Development of *Myrothecium verrucaria* as a mycopesticide for the control of plant pathogenic fungi and insects

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By

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Table of contents

List of tables

List of figures

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Certificate

Certified that the work incorporated in the thesis entitled "**Development of *Myrothecium verrucaria* as a mycopesticide for the control of plant pathogenic fungi and insects.**" submitted by Mrs. Medha Prashant Kulkarni was carried out under my supervision. Such material as has been obtained from other sources has been acknowledged in the thesis.

(M. V. Deshpande)
Research Guide

List of abbreviations

EDTA	Ethylene diamine tetra acetic acid
GlcNAc	*N*-Acetylglucosamine
4-MU-(GlcNAc)	4-methylumbelliferyl-β-D-*N*-acetyl –glucosaminide
4-MU(GlcNAc)$_3$	4-methylumbelliferyl-β-D-*N*-*N*'-*N*"triacetyl chitotrioside
µg	Microgram
µl	Microliter
mM	Milimole
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
nM	Nanomole
TCA	Trichloro acetic acid
w/v	Weight by volume

Abstract

India is one of the world's largest countries dependent on agriculture. The 1012.4 million population use agricultural commodities from 124.07 million hectares cropped area. Since early 1980's, improved irrigation, introduction of chemical fertilizers and use of high yielding crop varieties have led to a phenomenal growth in food grain production in the country (Wahab, 2004). However, on the other hand intensive and extensive cultivation of high yielding crop varieties, new cropping sequences, especially mono-cropping along with indiscriminate use of pesticides resulted in many problems of insect pests and diseases caused by fungi, bacteria, viruses and others.

The plant pathogenic fungi inflict considerable losses in harvestable yield; reduce aesthetic value and storage life of agricultural crops. Yield losses due to fungal diseases causing wilts, rots, cankers, rusts, smuts and mildews vary depending on the crop and region. While 200 major insect pests are of economic importance. Crop losses due to *Helicoverpa armigera*, a dreadful pest of cotton, pigeon pea, chickpea, peanut, sorghum, pearl millet, tomato, etc. have been estimated to be Rs. 10 million per year in India.

Biological control of plant fungal pathogens and pests involves the use of microbial antagonists such as bacteria, viruses, fungi and/or their products like enzymes, enzyme inhibitors and antibiotics. The viral and bacterial preparations are effective after ingestion while the fungal agents act by contact. The biocontrol of root infecting fungi was successfully achieved using the mycoparasitic fungi like *Trichoderma harzianum* and *Gliocladium virens* (Goldman and Goldman, 1998). The mycoparasitic fungi, secrete hydrolytic enzymes (mycolytic enzymes, ME) to degrade the main chemical constituents of the fungal cell wall, i.e., chitin, glucans

and proteins. Similarly, chitinase, protease and lipase, main components of a cuticle degrading enzyme complex (CDE) facilitated the penetration of entomopathogenic fungi like *Metarhizium anisopliae* and *Beauveria bassiana* into the insect body (Krieger de Moraes *et al*, 2003; St. Leger *et al*, 1986). Thus, the major interest in the present studies was to understand whether a fungus secreting high amounts of CDE/ME can be used to develop a biocontrol formulation with dual action, against fugal pathogens and insect pests. *M. verrucaria*, a saprophytic fungus known to produce high amounts of CDE/ME activities was selected for the present studies. Attempts were made to develop mycopesticide comprising of living propagules or CDE/ME components of *M. verrucaria* to control fungal pathogens and insect pests.

An extensive literature survey on development of mycopesticide for the control of plant pathogenic fungi and insect pests has been illustrated in the **Introduction (Chapter 1)**. Emphasis has been given to fungal pathogens and insect pests in agriculture, current biocontrol strategies, market status, production, formulation and application of fungal biocontrol agents, use of different fungal propagules and their enzymic and non-enzymic components in biocontrol, Integrated Pest Management and possible future strategies.

The **Materials and methods (Chapter 2)** utilized in the course of these investigations include details of growth and maintenance of *M. verrucaria* and plant pathogenic fungi such as *S. rolfsii* and *F. oxysporum*, shake flask and solid state fermentation experiments for the production of conidia and yeast like cells and hydrolytic enzymes and their formulations prepared for field trials using different agricultural wastes as carriers have been described. Assays for enzymes like chitinase, chitosanase, chitin deacetyalse, β-1,3-glucanase, alkaline protease, lipase, NAD- and NADP- dependent glutamate dehydrogenases, nitrate reductase and nitrilase have also been included. A novel spectrophotometric method

developed for chitinase assay was also included. The biochemical techniques include ultrafiltration and polyacrylamide gel electrophoresis. Molecular techniques including isolation of genomic DNA, PCR, DNA sequencing and analysis have also been given. The plate assays, pot experiments and field experiments have been given in this chapter.

Morphological, cultural, biochemical and molecular characterisation of *M. verrucaria* (Chapter 3) was carried out. Morphological and cultural characteristics of *M. verrucaria* were studied to develop a medium supporting good vegetative growth and sporulation of *M. verrucaria*. The maximum growth and sporulation were observed on potato dextrose agar (PDA), bean pod extract agar, carrot agar and maltose containing medium within 7 d incubation at 28°C.

For the first time it was observed that on a synthetic medium containing sucrose and ammonium sulphate, pH 4.0, *M. verrucaria* grew in an unicellular yeast like cells. On agar medium, cream coloured, mucoid colonies with grainy appearance were seen. Sudan black B staining to detect storage lipids revealed that the unicellular form of *M. verrucaria* resembled the actively metabolizing yeast cells rather than the resting chlamydospores. In a medium containing sodium nitrate, at pH 6.0, yeast like cells changed to hyphal form. The morphological transition in *M. verrucaria* was freely reversible. The conidia of *M. verrucaria* also displayed the dimorphic response during germination in presence of morphological triggers. Under the conditions favouring yeast form, conidia germinated into yeast cell while under hypha favouring conditions germ tube formation was observed.

A number of biochemical events precede to the morphological transition in fungi. Various biochemical correlates of the fungal morphogenesis were extensively reviewed by Deshpande (1998a). One of the significant biochemical

correlate, the chitinase complex comprising of endochitinase and *N*-acetylglucosaminidase played a significant role in several aspects of life cycle of fungi, such as hyphal tip growth, spore germination, cell separation etc. (Patil *et al*, 2000). The chitnolytic activities were estimated *in situ* using 4-methylumbelliferyl labeled fluorogenic substrates with permeabilised yeast-like cells/hyphae of *M. verrucaria*. During Y-H transition, the endochitinase activity increased 5.5 fold within first 12h of transition. Increase in endochitinase activity observed during the Y-H transition was attributed to its role in weakening the cell wall (Ghormade *et al*, 2000). *N*-Acetylglucosaminidase activity was also increased steadily throughout the Y-H transition. The reverse trend was noted during H-Y transition.

It has been reported that NAD- and NADP-dependent glutamate dehydrogenases have biochemical correlation with the morphological outcome (Amin *et al*, 2004; Ghormade *et al*, 2005a, b). In case of *M. verrucaria* the similar observation has been noted. For instance the relative proportion of NAD- and NADP-GDHs measured as the NADP-/NAD-GDH ratio was higher in the yeast form (7.7 ± 0.4) produced in the medium containing ammonium sulphate than in the hyphal cells (3.8 ± 0.2) produced in the medium containing sodium nitrate. The relationship between nitrate reductase involved in nitrate assimilation and NAD(P)-GDHs has been studied.

The sequence analysis of ribosomal DNA has been widely utilized for molecular systematics and identification of microorganisms (Mitchell *et al*, 1995; Sugita *et al*, 2002). The analysis of ITS (internal transcribed spacer) regions has been carried out mainly to identify or differentiate closely related species of pathogenic yeast (Chen *et al*, 2000; Gupta *et al*, 2000; Lott *et al*, 1998). To identify the genomic marker which can differentiate the *M. verrucaria* strain exhibiting

higher production of CDE/ME complex and hypha-yeast transition from other isolates of *M. verrucaria,* 18s rRNA and ITS1 sequence analysis was carried out. The genomic DNA was isolated from *M. verrucaria* mycelium using the DNA extraction protocol of Wendland *et al* (1996). Nuclear 18s rRNA gene and ITS 1 sequences were amplified using primers NS1 and ITS1, respectively. The sequence data was analyzed using NCBI server (http//:www.ncbi.nlm.nih.gov/BLAST). The 18s rRNA and ITS1 sequences were found highly homologous across the species. The analysis of genes or regions that have greater divergence may prove helpful in developing a genomic marker for *M. verrucaria* strain. The Genbank accession number for the 18s rRNA and ITS1 sequences reported in the thesis are: Myr 18s:AY129004 and Myr ITS1: Ay129005.

The characterisation studies were followed by studies on **Mycopesticide production (Chapter 4)**. Most of the fungal biocontrol formulations used conidia, while structures like blastospores, zygospores, chlamydospores, yeast cells and mycelia were also used (Deshpande, 1999). The aerial conidia of *M. verrucaria* were successfully used for the seed dressing, to control the infection of *Derechslera teres*, in barley (Mostafa *et al*, 1993). In present investigations it was seen that sorghum and pearl millet grains supported the highest production of conidia (1.3-1.6 x 10^9 conidia/g of substrate) as compared to rice, beaten rice and wheat (10^5 – 10^6 conidia/g of substrate). Wheat bran, rice bran and prawn shell waste did not support sporulation. Other biochemical and biophysical parameters were optimized for the production of *M. verrucaria* conidia **(Chapter 4A)**.

The extracellular CDE/ME complex was produced by growing *M. verrucaria*, in chitin containing medium using chitin (0.5% w/v) as a sole source of carbon. *M. verrucaria* produced high levels of chitinase (1.8 ± 0.2 U/ml), chitosanase (1.6± 0.2 U/ml), lipase (1.5 ± 0.2 U/ml), β-1,3 glucanase (0.23 ± 0.003 U/ml), mannanase

(0.019± 0.004 U/ml) and alkaline protease (0.013 ± 0.004 U/ml), under shaking conditions (200 rpm) at 28ºC for 7 d. Attempts were also made to produce CDE/ME complex in 15 l capacity stirred tank fermenter. Increase in the rate of aeration as well as agitation resulted in increase in foaming. The chitin particles entrapped in foam accumulate above the liquid surface making the substrate inaccessible to the growing mycelium of *M. verrucaria*. The fermentation was carried out at aeration rate of 0.5 vvm and agitation rate of 300 rpm, at 28°C for 5 d. The proper mixing of chitin particles in culture broth was observed under these conditions. High chitinase (2680 U/10L/day) activity was produced along with β-1,3-glucanase (260 U/10L/day), protease (23 U/10L/day) and lipase (1890 U/10L/day in stirred tank fermenter **(Chapter 4B)**.

Chapter 5 (Biocontrol efficacy of living propagules and enzymic components of *M. verrucaria* against fungal and insect pests) describes the studies on formulation, mode of application of different preparations and their efficacy to control pre-emergence rot of peanut caused by *S. rolfsii* under green house conditions. While biocontrol efficacy of different preparations against insect pests, *H. armigera*, *S. litura*, *P. xylostella* and *C. lanigera* was tested under laboratory and/or field conditions.

The conidia obtained by solid-state fermentation were harvested using 0.01% Tween 80. The conidial suspension ($1x10^{10}$ conidia/ml) was used to develop biocontrol formulation containing organic carriers like rice bran, wheat bran, prawn shell waste and conventionally used carriers like charcoal powder or precipitated silica powder. The preparations were stored in sealed plastic bags, at room temperature. *M. verrucaria* conidia in organic carriers wheat and rice bran with and without chitin supplement (2 g chitin per 100 g wheat or rice bran formulation) retained >80% viability when stored at 4ºC and 54-62% viability

after 18 months storage at room temperature. When prawn shell waste, charcoal powder and precipitated silica were used as carriers, the TVC after 18 months were 10, 22, 18 %, respectively). The rice bran formulation of conidia with and without chitin supplement was used for soil application to control the *S. rolfsii* infection in peanut.

M. verrucaria yeast-like cells were immobilised in alginate beads with and without 0.5% chitin. The air dried beads were then stored in air tight sterile plastic vials. *M. verrucaria* yeast like cells were stable in alginate beads with or without chitin (>80% TVC after 3 months storage) at room temperature. However, high chitinase (0.9 U/ml) activity was produced when chitin containing *M. verrucaria* beads were used as inoculum for chitinase production as compared to the beads without chitin.

Attempts were made to concentrate CDE /ME preparation, using polysulphone hollow fiber membrane of 10 kDa molecular weight cut-off. This was further concentrated using spray drying or freeze drying. The maximum recovery of chitinase activity (71.3%) was noted after concentration of enzyme preparation by freeze drying. While concentration by spray drying was found unsuitable as resulted in 67.2% loss in the recovery of chitinase activity.

The soil application with rice bran + chitin formulation of conidia and yeast-like cells immobilized in alginate with chitin was found to protect the seeds from the infection with *S. rolfsii*. The disease control was attributed to the slow release of mycolytic enzymes by vegetative cells of *M. verrucaria* in soil.

The pot experiment was carried out to study the effect of mycolytic enzyme preparation on the control of *S. rolfsii* infection in peanut. The CDE/ME preparation (chitinase 5-20 U/pot) was used for soil irrigation at a weekly interval. The treatment

with 10 and 20 U chitinase/pot, up to 3 weeks resulted in 40 and 70% increase in seed germination, respectively. Chitinase retained >80% activity in soil after 24 h.

The bioassays were carried out to check the effect of CDE complex on the insects, *H.armigera*, *S. litura*, and *P. xylostella* with appropriate controls. *P. xylostella* larvae were found to be most susceptible to enzyme preparation containing 2.0 U/ml chitinase (37% mortality within 72 h), while the same concentration of CDE preparation resulted in abnormal morphologies in *S. litura* during larval-pupal ecdysis. The 90, 80 and 73.33 % mortality of *P. xylostella*, *S. litura* and *H. armigera*, respectively was seen within 72 h when treated with CDE preparation containing 6.0 U/ml chitinase activity.

The effect of CDE preparation on the control of sugarcane woolly aphid, *Ceratovacuna lanigera* was studied under laboratory conditions. *C. lanigera* cuticle contains chitin and lipoproteins which was covered with woolly mass comprised of wax and lipids. The wooly mass was hydrolysed in the presence of CDE preparation containing 2 U/ml lipase. The mortality of *C. lanigera* nymphs and adults increased from 64 to 88% when the concentration of CDE preparation was increased from 1.0 to 6.0 U/ml chitinase. ME components of the enzyme preparation also affected the germination of fungal pathogen, *Capnodium* sp. causing secondary infection to sugarcane leaves.

The insect pathogenic fungi are being extensively used to control insect pests (Nahar *et al*, 2004). The major concern is of the possible change of the ecological balance of soil flora due to these exotic strains (Cook *et al*, 1996). The effect of enzyme preparation in combination with *M. anisopliae*, *B. bassiana* or *N. reliyi* conidia to control *H. armigera* in pulses was studied. *M. verrucaria* CDE preparation (chitinase 2 U/ml) in combination with the entomopathogenic fungi, reduced the

dose of entomopathogenic fungi by 100 times to achieve >70% mortality. Furthermore, the enzyme complex did not affect the viability of conidia.

The efficacy of CDE preparation of *M. verrucaria* to control *H. armigera* infestation in chickpea was studied in field in a randomized block design. The % efficacy of enzyme preparation 500 l/ha ($3x10^6$ U chitinase) was 60.94, the reduction in pod damage over untreated control was 56.49% and the increase in yield was 37.60%. The field trial results showed that the efficacy of CDE treatment for the control of *H. armigera* was at par with that of conventionally used chemical (endosulfan) or biological control agents (HaNPV).

Many plants produce cyanogenic glycosides, which may have role in defense against phytopathogens. These cyanoglycosides were oxidized by oxygenases to cyanohydrins, which were further converted to an aldehyde and HCN by oxynitrilases (hydroxynitrile lyases). This type of enzymatic system was found in many plants and insects but almost unknown in microorganisms. However, *M. verrucaria* is known to utilize nitrile compounds with the help of nitrilases (Maier-Greiner *et al*, 1991). In extracts of yeast/hyphal cells and conidia of *M. verrucaria* as well as in CDE/ME preparation, mandelonitrile lyase (oxynitrilase) activity required to produce HCN was not detected. This indicated its non-toxic nature, in general too.

A rapid and sensitive method was developed for the quantitative estimation of chitinase using the colloidal chitin prepared from Ostazin Brilliant Red labeled chitin (**Appendix 1**). The method was found suitable for the screening of chitinolytic organisms.

Thus present studies revealed that *M. verrucaria*, organism itself and extracellular hydrolytic enzymes can be effectively used to control fungal plant pathogens as well as insect pests (**Chapter 6 Conclusion**). The use of enzyme mixture in combination with an insect pathogenic fungus will be useful in an

integrated approach to control insect pests. Of course, the increased shelf-life and cost effective production of the enzyme mixture are the major concerns.

The **Reference** list is given in the **Chapter 7**.

Chapter 1

Introduction

1.1 Introduction

India is one of the world's largest agrarian countries. Total cultivable land area is 124.07 mha, major part of it falling under tropical climate. A variety of cereals, oil seeds, pulses, vegetables and horticultural crops are cultivated in India (Reddy and Zehr, 2004). Since the Green Revolution of 1980s, improved irrigation, introduction of chemical fertilizers and use of high yielding crop varieties have led to >30% increase in food grain production in the country (http://edugreen.teri.res.in/explore/bio/green.htm). This boost in agricultural produce and revenue made farmer happy because of his improved monetary condition giving favourable signals to the national economy. This seemingly favourable situation unfortunately did not last long. The fertile land slowly lost its texture, quality and fertility resulting in low levels of agricultural production. Many factors were responsible for this such as an intensive cultivation of high yielding crop varieties, change in cropping pattern, mono-cropping, extensive use of chemical fertilizers to name a few. Over and above excessive and indiscriminate use of chemical pesticides by farmers with lack of proper training and education made the situation worst. This resulted in many problems of insect pests and diseases caused by fungi, bacteria, viruses and others (Gupta, 2004; Wahab, 2004).

1.1.1 Pests and pathogens in agriculture

The fungal diseases such as wilt, rots, cankers, rusts, smuts and mildews cause considerable losses in yield, reduce the aesthetic value and storage life of agricultural commodities. 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 (Cheeke, 1995; Diekman and Green, 1992; Nelson *et al*, 1993). There are 200 major varieties of pests of economic importance belonging to insect orders Coleoptera, Diptera, Hemiptera, Lepidoptera,

Orthoptera, Thysanoptera and Acarina. Crop losses due to *Helicoverpa armigera*, a dreadful pest of cotton, pigeon pea, chickpea, peanut, sorghum, pearl millet, tomato, etc. have been estimated to be over Rs. 10 billion per year in India. Number of fungal pathogens and insect pests of cash crops are depicted in Table 1.1.

Table 1.1 Major fungal pathogens and insect pests of cash crops

Crop	Fungal pathogen	Loss in yield (%)	Insect pest	Loss in yield (%)
Wheat	*Puccinia recondiata*, *Helminthosporium sativum*	15-17	*Mythemna separata*	20-42
Rice	*Magnaporthe grisea*, *Rhizoctonia solani*	20-21	*Dicladispa armigera*	24 – 45
Pulses: Chickpea, Pigeon pea	*Fusarium udum*	20-24	*Helicoverpa armigera*	28-30
Cotton	*Phytophthora* sp.	5-7%	*Helicoverpa armigera*, *Bemisia tabaci*	18-31
Mustard	*Alternaria brasicae*	28-30	*Plutella xylostella*	20-35
Peanut	*Sclerotium rolfsii*	35-50	*Spodoptera litura*	60-71
Sugarcane	*Colletotricum falcatum*	14-20	*Ceratovacuna lanigera*	26-30
Vegetables: Cabbage, Cauliflower, Chilli	*Phytophthora capsici*	15-50	*Plutella xylostella*, *Spodoptera litura*	20-52

***** Compiled from: Alexander and Viswanathan, 1996; 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.

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.

1.2 Control of fungal pathogens and insect pests

1.2.1 Chemical pest control

In modern agriculture, the use of chemical fungicides and insecticides has a short history. However, they received a major position in routine agricultural practices due to their rapid action and low cost. Average use of herbicides in the world is about 45% followed by insecticides 36%, fungicides 17% and other chemicals 2%. While insecticides are used to a large extent, about 80% in India followed by fungicides 10%, herbicides 7% and other chemicals 3% (Wahab, 2004). Among the total pesticides used in the country, more than 60% of the pesticides, worth of nearly USD 630 million are consumed annually in the agriculture sector (Reddy and Zehr, 2004).

1.2.1.1 Fungicides

The discovery of Bordeaux mixture by P.M.A. Millardet (1878) is considered to be the first important landmark in the history of chemical disease control. This mixture was found effective in controlling the downy mildew of grapes caused by a fungus *Plasmopara viticola*. Other first generation fungicides are inorganic chemicals like copper and tin derivatives. The second generation of fungicides is of organic chemicals. These include Organotins, Quinines, Thiocarbamates (dialkyldithiocarbamates and monoalkyldithiocarbamates), Captan and related compounds, Chlorothalonil and Pentachloronitrobenzene (De Waard *et al*, 1993). These compounds are surface protectants like inorganic fungicides and are effective as a preventive measure. The development of resistance in target fungi led to the discovery of new fungicide molecules. Third generation fungicides are also organic but penetrated the plant tissue

and controlled established infections. These systemic fungicides were Carboxamides, 2-Aminopyrimidines, Benzimidazoles or Benzimidazole generating compounds, Phenylamides, Fosetyl-Al, Azoles and related compounds and Morpholines. Triazole fungicides, Etaconazole, Flusilazole, Myclobutanil, Penconazole, Triademefon and Triademenol were effectively used for the control of powdery mildew of grapes caused by *Uncinula necator* (Pearson *et al*, 1994). The designing of fourth generation of fungicides was governed by features *viz*. biodegradability in natural situations and selectivity in mode of action to reduce non-target effect (Leroux, 1996, 2003; Gullino *et al*, 2000). Howard and Valent (1996) reported that fungicides like Capropamid did not inhibit vegetative hyphal growth of *Magnaporthe grisea* but specifically interfere with melanin deposition in appresoria, an essential step for efficient pathogenecity of rice blast fungus. Quinoxyfen, a potent protectant fungicide against powdery mildew, prevents appressoria formation and host penetration. Sterol biosynthesis inhibitors (SBIs) that are active against ergosterol biosynthesis, a component of fungal cell membrane are being successfully used as fungicides (Beffa, 2004). Strobilurin group of fungicides is excellent inhibitors of fungal spore germination. However, the same mode of action shared by them led to the development of cross-resistance to other Strobilurin products. Consequently, the use of Strobilurins in the disease control programmes has to be managed to reduce the risk of resistance by other pathogens.

1.2.1.2 Insecticides

The earliest insecticide in the world was reported in France in 1763 with nicotine as an active ingredient. The mixture of lime and tobacco was used to control aphids (Zang *et al*, 2001). During 1800-1850, Pyrethrum a natural insecticide obtained from dried and crushed chrysanthemum flowers; having neurotoxic effects on insects was used in some areas of middle Asia. Pesticide research experienced very fast

development since the 1940s. At that time a number of new classes of pesticides with different structures were produced. Examples are Organochlorines, Organophosphates and Carbamates. DDT (dichloro diphenyl trichloroethane), BHC (benzene hexa-chloride), Strobane, Chlordane, Aldrin and Heptachlor are some of the examples of Organochlorine insecticides. Pesticide use in India began in 1948 when DDT was imported for the malaria control and BHC for locust control (Gupta, 2004). DDT was then used on more than three hundred different agricultural commodities as a general insecticide. Amongst the inorganic pesticides, DDT enjoyed a product life of 50 years all over the world before it got banned as a result of its harmful effects (Prasad, 2004).

Organophosphates like Diazinon, Chlorpyriphos, Endrin, Parathion and Terbufos were developed in early 1950s while Carbamates like Carbaryl, Propoxur, etc. were developed in late 1950s to early 1960s. The primary action of Organophosphates as well as Carbamates involves the inhibition of cholinesterase, an important enzyme in the nervous system. While Organophosphates, Kitazin-P, Parathion, Sulfenimide and Captan selectively prevent chitin formation in insects by inhibiting chitin synthase activity (Deshpande, 1998b). Carbaryl, the largest spectrum insecticide with the capacity of controlling 565 species of pests in 141 crops became the most popular insecticide in 1950s and today is still being widely used (Zang *et al*, 2001). Insecticides like Diazinon, Ethoprop, Trichlorfon, Bendiocarb, Halofenozide and Carbaryl are used as curative treatment for white grubs (muextension.missouri.edu/xplor). Fadare and Amusa (2003) reported the use of Carbaryl, Endosulfan and Monocrotophos for the control of cotton bollworm complex (*H. armigera* Hübner, *Earias insulana* Boisd, *E. biplaga* Wlk. and *Sylepta derogata* F.) in Southwestern Nigeria. For the control of recent outbreaks of *Ceratovacuna lanigera* Zehnter in sugarcane in Maharashtra and Karnataka, chemical insecticides such as Endosulfan, Phosalone, Monocrotophos,

Dimethoate and Metasystox sprays and Acephate and Methyl parathion dust were recommended (Joshi and Viraktmath, 2004).

Synthetic Pyrethroids, a new class of insecticides with neurotoxic effects on insects was introduced in late 1970s (Tikar *et al*, 2004). Examples of synthetic Pyrethroids are Allethrin, Syfluthrin, Cypermethrin, Deltamethrin and Fenvalerate. The Synthetic Pyrethroids contribute 25% of the total pesticides marketed in India (Verma and Singh, 2000). They are used mainly for the control of Organophosphate resistant populations of *P. xylostella* in crucifers and *H. armigera* in cotton and pulses.

To reduce the non-target effects of chemical insecticides a new class of pesticides was discovered, Insect Growth Regulators (IGRs). These were synthetically produced hormones of some insects. IGRs disrupt a number of normal processes in growth and development of insects; however, they did not kill the targeted pest. Juvenoids like Hydroprene and Fenoxycarb hamper the development of sexual organs of insects making them unable to reproduce. Buprofezin, a chitin synthesis inhibiting growth regulator along with Pyriproxyfen, a juvenile hormone analog have been used effectively to control white flies, *Bemisia tabaci* and *B. argentifolii* on cotton and other crops (Dennehy and Williams, 1997; Horowitz and Ishaaya, 1992). Diflubenzuron alone and in combination with Alphacypermethrin and Profenophos reduced the bollworm infestation in cotton significantly (Srinivasan and Uthamsamy, 2001). IGRs provide an ecofriendly alternative for the control of pests of stored grains like Rice moth, *Corcyra cephalonica* Stainton. Diflubenzuron reduced pupation, adult emergence and reproductive potentiality (fecundity, egg viability and longevity of adult) of *C. cephalonica* (Sharma and Bhargava, 2004).

1.2.1.3 Disadvantages of chemical pesticides

The excessive and indiscriminate use of chemical fungicides and insecticides led to several short term and long term adverse effects. The major problems faced by the agronomists were the large-scale destruction of natural enemies of pests and the development of resistance in target insect pests or fungal pathogens (Pawar, 2004). 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). In addition to these adverse effects, workers were exposed to occupational hazards in industrial settings and operational hazards during distribution and use in fields (Nigam *et al*, 1993). Pesticides also account for small but significant fraction of acute human and animal poisonings.

According to All India co-ordinated Research Project on Pesticide Residues under the Indian Council of Agricultural Research, 51% of food commodities were found contaminated with pesticide residues and out of these 20% had pesticide residues above maximum residue level (MRL) values as compared to 21% contamination with only 2% above MRL on worldwide basis. 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 (Dethe, 2001; Wahab, 2004). 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.

1.2.2 Biological control agents

Use of biopesticides for crop protection is one of the key elements for the sustainable agriculture. The biopesticides are classified mainly in two groups as biochemicals and microbials. The molecules, structurally and/or functionally related to the molecules of pests with a non-toxic mode of action are used as biochemical pesticides, e.g. pheromones, naturally occurring chemicals that insects use to find mates. Use of chemically synthesized pheromones disrupts insect mating by creating confusion during the search for mates, or by attracting insects to traps (Schmidt-Tiedelmann *et al*, 1999). While 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 a disease or preventing establishment of pest microorganisms through competition or other modes of action (Chavan *et al*, 2006). The living propagules of microorganisms and enzymes, enzyme inhibitors or antibiotics having antifungal or insecticidal properties produced by microorganisms are used singly or in combination.

The characteristics of biocontrol agents, in general which make them potential alternative to chemicals are: a narrow host range and highly specific mode of action, preventive in nature if timely applied, field persistence is generally non-toxic, compatible nature with other components of Integrated Pest Management (IPM) and use in organic farming situations where synthetic fungicides are restricted (Harman, 2000; Tsror *et al*, 2001) and generally safe to humans and plants.

The further discussion will highlight the status of bacteria, viruses and fungi as microbial pesticides with major emphasis on fungi and fungal products.

1.2.2.1 Viruses as biological control agents

The presence of one or more specific segments of viruses like double-stranded RNA (dsRNA) were found to cause diseases in plant pathogenic fungi characterized by reductions in their pathogenecity, ability to form sexual and asexual spores, spore viability and growth (Buck, 1998). It was also reported that dsRNA viruses are ubiquitous in fungi transmitted to compatible strains via cell fusion (McCabe *et al*, 1999). The use of fungal viruses to control fungal plant pathogens is relatively new strategy for biological control of plant diseases and was discovered in Europe after the European chestnut (*Castanea sativa*) was destroyed. The hypo-viruses of the chestnut-blight fungus, *Cryphonectria parasitica* were studied for their potential as biological control agents of fungi. Howitt *et al* (1995) worked on viruses of two common plant pathogens, *Botrytis cinerea* (Grey mold) and *Monoliniana fructicola* (brown rot) with the aim of identifying viruses that might be used as biological controls of pathogenic fungi. However, these reports though indicate the possibility of the use of viruses for the biological control of fungal pathogens, notable data is not available.

The viral nature of 'Jaundice disease' of silkworm, *Bombyx mori* was established in 1947, and soon it became clear that viruses were widespread in nature among economically important insect pests and so could potentially be used in pest management in agricultural practices. Since then more than 1600 different viruses infecting insects and mites have been reported. A special group of viruses, called as baculoviruses (nuclear polyhedrosis virus, NPV), to which about 100 insect species were susceptible, account for more than 10 percent of all insect pathogenic viruses (Deshpande, 1998b). Most NPVs were isolated from Lepidoptera (88%), Hymenoptera (6%) and Diptera (5%). These viruses develop in the nuclei of fat body, haemolymph, hypodermis and tracheal matrix. The oral ingestion of polyhedra transmits the disease. The infections may also be transmitted from an infected female via the egg to progeny.

Few commercial preparations available are: VFN80^{TM} to control Alfalfa looper (*Autographica californica*); Gemstar LC, Biotrol, Elcar against *Helicoverpa zea* in U.S.A.; in Europe for the control of *Trichoplusia*, *Helicoverpa*, *Diparopsis*, *Phthorimacea operculella* (potato tuber moth) a viral preparation is also available under the trade name Mamestrin^{TM} (from Database of Microbial Biopesticides (DMB), 2002). NPV studies in India are concentrated on control of *H. armigera* (on chickpea, pigeonpea, sunflower, cotton and tomato), *Spodoptera litura* (on cotton, tobacco, banana and castor), *Amsacta albistriga* (on peanut) and *Mythimna separata* (on sorghum) (Pokharkar, 2004). Loganathan *et al* (2000) reported the effective use of *H. armigera* NPV (HaNPV) for the control of *H. armigera* on chickpea. The formulations of NPV of *H. armigera* and NPV of *S. litura* have already been registered under the insecticide act, 1968, in India.

Although viruses are being used in fields for the pest control, they are specific to one genus ensuring relatively small market. Furthermore, their widespread use is hindered by several problems like rapid deactivation by environmental factors, requirement of living host for the production, their effectiveness only when ingested by the host and longer time from ingestion of lethal dose until death under field conditions (3-15 days) and the development of resistance to NPV in some insect pests like *P. xylostella*.

1.2.2.2 Bacteria as biocontrol agents

The rhizosphere resident antagonistic bacteria were used effectively to control soil borne fungal pathogens of many crop plants. Bacterial genera like *Bacillus*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* were amongst the most effective antagonists of fungi and their antifungal activity was attributed to the production of cell wall degrading enzymes, especially chitinase (Basha and Ulganathan, 2002; Guo *et al*,

2004; Nagrajkumar *et al*, 2004; Zhang and Yuen, 2000). While actinomycetes like *Streptomyces flavescens* and certain other bacteria like *Pseudomonas* sp. and *Bradyrhizobium* sp. were reported to produce antifungal antibiotics (Deshwal *et al*, 2003; Gupta *et al*, 2001; Woo *et al*, 2002). The secondary metabolites like Fe-chelating siderophores and cyanide were found associated with the fungal suppression by fluorescent pseudomonads (Nielsen *et al*, 1998). The genetically modified bacterial strains were also used as biopesticides in USA on a large scale. For example, *Agrobacerium radiobacter* k1026 was used to treat fruit and vegetable plants to prevent crown gall tumor formation.

Bacillus thuringiensis (Bt) has been the leading bacterial pesticide against lepidopteran insect pests since 1959 and it accounts for over 90% of total share of today's bioinsecticide market (Baum *et al*, 1999; Milner, 1994). The sporulating cells of *B. thuringiensis* contain inescticidal crystal protein that was found toxic to lepidipteran, dipteran as well as coleopteran insect species like *H. armigera*, *S. litoralis*, *P. xylostella,* Colorado Potato Beetle, Elm Bark Beetle, etc. Since 1990s the growth of Bt market was promoted by the introduction of new Bt strains and formulations with broader insect host ranges and increased insecticidal potencies of the toxins. Bt products in forestry effectively replaced the chemical pesticides. In Europe and North America Bt is used against defoliator larval moths while in Israel, it is used against *Thaumatopoea pityocampa* in pine forests. However, three major constraints affect field effectiveness of Bt products, the toxic protein being oral insecticide has to be consumed by the larva, environmental conditions reduce the efficacy of product and development of resistance in insect pests.

1.2.2.3 Fungi as biocontrol agents

Reports dating back to the 1930s first showed that fungal pathogens of plants could be infected or parasitised by other fungi (mycoparasites) (Howell, 2003). Weindling (1932) suggested the potential use of mycoparasitic fungi as biocontrol agents of plant pathogenic fungi. Since then number of mycoparasitic strains were isolated from their natural habitats and studied for their biocontrol efficacy against several plant pathogenic fungi in phylosphere or rhizosphere. Two types of mycoparasitic interactions, biotrophic and necrotrophic, were observed. Biotrophic mycoparasites like *Sporidesmium sclerotivorum* require a persistent contact with or occupation of living cells of the host *Sclerotinia minor* (Whips and Lumsden, 2000). *Sporidesmium sclerotivorum* hasutoria penetrate the host hyphae and trigger autolysis. Whereas necrotrophic mycoparasites kill the host cells often in advance of contact and penetration. Majority of the necrotrophic mycoparasites were developed as biocontrol agents due to the ease in production being saprophytic in nature, less specialized in their mode of action and broad host range. *Trichoderma* species (necrotrophic mycoparasites) were developed into several commercial products (Harman, 2000) and used for the control of numerous soil borne diseases, such as those caused by *Pythium ultimum* Trow. (Naseby *et al*, 2000), *Sclerotinia sclerotiorum* (Lib.) de Bary (Inbar *et al*, 1996) and *Fusarium oxysporum* Schlechtend (Sivan and Chet, 1993). While Elad (2000) reported the use of *Trichoderma harzianum* for the control of foliar pathogens like *B. cinerea*, *Pseuperonospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca fusca* in cucumber under commercial green house conditions. *P. ultimum* and *Rhizoctonia solani* causing damping-off disease of cotton and peanut wilt, respectively were parasitized by *Gliocladium virens* (Whipps and Lumsden, 2000). Other organisms, *Ampelomyces quisquqlis* was shown to be parasitic on powdery mildew hyphae and conidia (Falk *et al*, 1995; Sundheim and Krekling, 1982), whereas *Coniothyrum*

minitans and *Taloromyces flavus* (Kslocher) Stolk and Sasmon were parasitic on sclerotia of *Sclerotinia* species (Gerlagh *et al*, 1999; Huang *et al*, 2000; Madi *et al*, 1997), and *Pythium oligandrum* was parasitic on other *Pythium* spp., *F. oxysporum*, *Verticillium albo-atrum* Kleb. and *R. solani* (Benhamou *et al*, 1999). Urediniospores of the bean rust *Uromyces appendiculatus* were parasitized by *Cladosporium tenuissimum* (Assante *et al*, 2004). While *M. verrucaria* is known to parasitize hyphae of *Cochliobolus sativus* (Campbell, 1956).

Several review articles discussed the role of fungi in the control of various fungal pathogens (Bailey and Lumsden, 1998; Harman, 2006; Harman, 2000; Harman and Bjorkman, 1998; Handelsman and Stabb, 1996; Wilson, 1997). Besides mycoparasitic interaction, fungi also antagonize plant pathogenic fungi through the production of toxins and enzymes that inhibit the growth or reduce the competitive ability of other organisms (Harman, 2000; Harman *et al*, 1993; Howell *et al*, 1993). Cook (1988) reported that the control of phytopathogenic fungi was also achieved through competition for space and resources with highly competitive fungal biocontrol agents quickly colonizing plant surfaces, creating an effective 'living barrier' to subsequent pathogen invasion. While some fungal biocontrol agents act by mobilizing nutrients in the soil, a process that makes compounds in soil more available to plant uptake, resulting in increased general health and disease resistance (Harman, 2000). Fungi were also known to increase disease resistance in a way very similar to the phenomena of induced and systemic acquired resistance (Handelsman and Stabb, 1996; Harman, 2000). However, most of the biocontrol preparations were developed using mycoparasitic fungal strains. The list of these biocontrol agents is given in Table 1.2. The traits like higher competitiveness, production of antibiotics/toxins/cell wall degrading enzymes, mobilization of phosphates through siderophores and induction of systemic resistance are studied as additional factors that contribute to the biocontrol

Table 1.2 Fungi as biocontrol agents against plant pathogenic fungi and insects

Fungus	Host	Mode of action	Product trade name
Ampelomyces quisqualis	Powdery mildew	Hyperparasitism	AQ-10 (Ecogen, USA)
Trichoderma harzianum	*Sclerotium rolfsii*, *Phytophthora* sp., *Pythium* sp., *Sclerotinia* sp., *Rhizoctonia* sp., *Botrytis* sp.	Hyperparasitism, Competition, Antibiosis, Induction of systemic resistance	Root Pro (Efal Agri, Israel, Trichodex (Makhteshim Chemical Works, Israel), Som Phytopharma India Ltd., Ecotech Agro (India)
Coniothyrum minitans	*Sclerotinia* sp.	Hyperparasitism, Competition	Contans WG, Intercept WG (Prophyta Biologisher, Germany)
Gliocladium Virens GL-21	*Fusarium* sp., *Pythium* sp., *Rhizoctonia* sp., *Sclerotinia* sp., *Sclerotium rolfsii*	Antibiosis, Hyperparasitism, Competition	Soil gard (Certis, USA)
Pythium oligandrum	Root rot	Hyperparasitism	Polyversum (Biopreparaty, Czech Republic)
Pseudozyma flocculosa	Powdery mildew	Antibiosis	Sporodex Plant products, Canada
Metarhizium anisopliae	Vine weevil, Scarab larvae on pasture, Spittle bugs, Cockroaches, Termites	Entomopathogenesis	BIO 1020(Taensa, USA), Biogreen (Bio-care Technology, Australia), Bio-Path (Ecoscience, USA), Ecotech Agro (India)
Beauveria Bassiana	Coffee Berry borer, corn borer, grasshoppers, locusts, whitefly, aphids, thrips, boll worms, Colorado beetle	Entomopathogenesis	Conidia (AgrEvo, Germany), Ostrinil (NPP, France), Mycontrol GH and Mycontrol WP (Mycotech, USA), Mythri Crop Care (India), Veena Biotech (India)
Verticillium Lecanii	Whitefly, thrips, aphids	Entomopathogenesis	Mycotal and Vertalec (Koppert, Holland), Ecotech Agro (India)
Paecilomyces fumosoroseus	Whitefly	Entomopathogenesis	PFR-97 (ECO-tek, USA), PreFeRal (Agrisense, UK)
Hirsutella thompsonii	Coconut mite (*Aceria guerreronis*)	Entomopathogenesis	Mycohit (PDBC, India)

* Compiled form: Stephens (2002), Strasser *et al* (2000), www.indianindustry.com

efficacy. Fusaclean and Biofox C are known biocontrol preparations of non-pathogenic strains of *F. oxysporum* with induction of systemic induced resistance as the mechanism involved in biocontrol (Ramakrishnan *et al*, 2001).

Entomopathogenic fungi are key regulatory factors of insect populations in nature and are attracting attention as biocontrol agents for insect pests. There are 750 known species of entomopathogenic fungi spanning some 85 genera, the majority belonging to zygomycetes (Entomophthorales), Ascomycetes and Deuteromycetes (Carrutheres and Onsager, 1993). Fungi infect a broader range of insects than do bacteria and viruses. For instance, lepidopterans (moths and butterflies), homopterans (aphids and scale insects), hymenopterans (bees and wasps), coleopterans (beetles) and dipterans (flies and mosquitoes) were reported to be infected by fungi. The entomopathogenic fungi are the only practical microbial control for insects that feed by sucking plant juice and for many acridid and coleopteran pests which have no viral or bacterial pathogens (Hajek and St. Leger, 1994; St. Leger *et al*, 1996).

Beauveria bassiana (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Verticillium lecanii* (Zimmerman) Viegas and *Paecilomyces* sp. were reported to have broad host ranges that encompass most of the insect groups (McCoy, 1990). These fungi are thus favoured candidates in the area of pest control (Butt *et al*, 2000). *M. anisopliae* has been most extensively used in Brazil for the control of spittlebugs on sugarcane. *V. lecanii* is a pathogen that has demonstrated good 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* (Wetwood) and of *Thrips tabaci* Lindeman on cucumber. *B. bassiana* was identified in many insect species in temperate and tropical regions and used for pest control in Eastern Europe and China. A *B. bassiana* based mycoinsecticide was shown to be highly effective against coleopterans.

Epizootics caused by *Nomuraea rileyi* and *B. bassiana* were observed on *H. armigera* and *S. litura* in Indian fields since last 15 years as a result the insect population is highly susceptible to these fungi (Uma Devi *et al*, 2003). Different strains of *B. bassiana* were reported in 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 information on some commercially available entomopathogenic fungi is given in Table 1.2.

The viral and bacterial control agents infect insects via their digestive tract while fungi make entry into the host through the cuticle, the outermost protective covering in insects. Moreover, the fungal biomass can be produced easily and delivered in a variety of formulations that act as direct contact sprays, foliage sprays, bait or granules. Further advantages are: their mammalian safety and minimal impacts on non-target insects due to relative host specificity of different isolates of the same species, no toxic residues, environmentally safe and no known resistance in insect community. Another important aspect in fungus-fungus and fungus-insect interactions is the possibility of identifying a strain that has capability to show dual pathogenesis possibly mediated through specific hydrolytic enzymes. For instance, *T. harzianum*, a mycoparasitic species parasitized the elm bark beetle, *Scolytus* (Jassim *et al*, 1990). *V. Lecanii*, a pathogen of aphids was also reported to parasitize rust fungi (Whipps and Lumsden, 2000). However, research inputs are necessary to make them effective on a wide range of temperature and humidity and to have longer shelf life.

1.3 Host-pathogen interaction

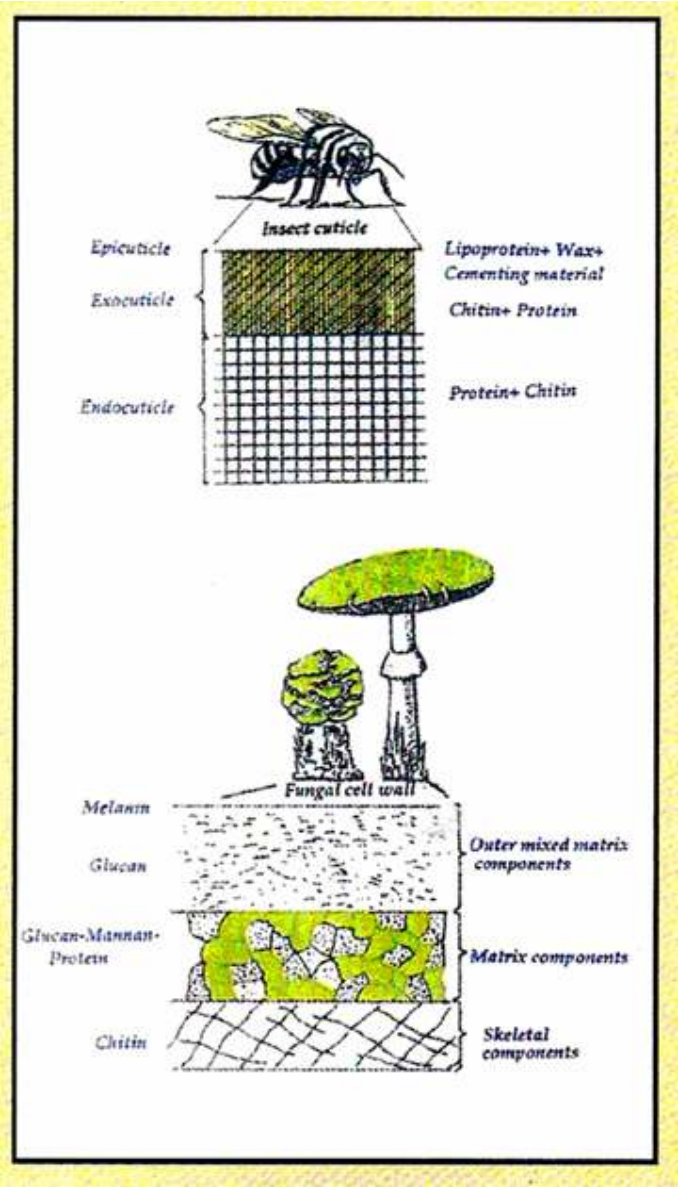
1.3.1 Structure of fungal cell wall and insect cuticle

The cell wall and cuticle are the outermost protective covers in fungi and insects, respectively. The fungal cell wall is not an inert protective shell but rather a

dynamic organelle required for the viability of the cell. Its complex structure serves many functions, such as osmotic protection to the protoplast, transport of molecules, growth and spore formation (de Nobel *et al*, 2000). In insects, the cuticle provides body armour, protecting the insect against predators, parasites and infecting microorganisms (Reynolds, 1987). Cuticle also stores energy during locomotion. Most importantly the cuticle protects insects from desiccation. Thus, any major disruption in organization or metabolism of cell wall and cuticle will be deleterious to both of them.

The fungal cell wall is composed primarily of polysaccharides like chitin, chitosan, 1,3-β- and 1,6-β-glucans and mannan (Fig. 1.1). In some fungi proteins are also the significant components of the cell wall and frequently associated with the polysaccharide components (Gooday, 1995). Lipids and melanins are the minor cell wall components. The shape- determining, more fibrillar polysaccharides, chitin, chitosan and chitin-glucan complexes make up the inner layer of the wall. These are also known as the structural or skeletal components. They provide a considerable mechanical strength and resistance to chemicals. The structural components are embedded in more gel-like matrix polymers such as glucans, glycoproteins and mannoproteins that make up the outer layer of the wall. The matrix components contribute to the flexibility of fungal cell wall. Other constituents such as lipids and pigments like melanins, though quantitatively minor, contribute a lot to the protection. The insect cuticle is composed of wax, lipids, protein and chitin (Fig. 1.1). The main component being chitin, its share is 25-50 % of dry weight of insect cuticle. Insect cuticle is a composite material consisting of a thin lipid-protein rich epicuticle covering the bulky procuticle. The epicuticle is a thin, dark pigmented multilayered membrane made up of cuticulin (lipoprotein), wax and cementing material of uncertain composition. Phenolics are an additional class of cuticular chemicals, which confer chemical and mechanical stability. The procuticle is composed of the exo- and endo-

Fig. 1.1 Structure of fungal cell wall and insect cuticle



cuticle comprising of chitin and protein, wherein the exo-cuticle is generally melanised (Anderson, 2002). The cells involved in the synthesis of cuticle are present below cuticle. Since the cuticle is incapable of growth, it must be shed from time to time as the insect grows, and a new and larger cuticle is laid down in its place. This is called as moulting or ecdysis. Synthesis and breakdown of chitin are active processes in insects and are immediately linked to the survival.

The fungal cell wall and insect cuticle share chitin as a common structural component (Fig. 1.1). Furthermore, chitin is absent in vascular plants and mammalsthus provide an attractive target for the control of fungal pathogens and insect pests. The fungal pathogens of fungi and insects act by contact and the chitinolytic enzymes were reported to play an important role in the penetration of host.

1.3.2 Fungus-fungus interaction

The interactions between plant pathogenic fungus and mycoparasitic one require close physical proximity of each other. Most of the observations of hyphal-hyphal interaction were made from petri dishes or detached plant tissues under controlled conditions. The hyphal interactions were extensively studied using light, fluorescence, scanning and transmission electron microscopy (Elad *et al*, 1983; Li *et al*, 2002). Recently, the gfp-tagged mutants of *Trichoderma atroviride* were used to study the *in situ* interaction of *T. atroviride* with the plant pathogens *R. solani* and *P. ultimum* in cocultures and directly on cucumber seeds, on roots and in sterile soil (Lu *et al*, 2004). Mycoparasitic fungi were reported to locate the host mycelium or spores, using chemical signals originated from the host fungus. These fungi attach themselves to the host hyphae by coiling, hooks or appresorium-like bodies (Deshpande, 1999). This was followed by penetration of cell wall and dissolution of cytoplasm as indicated by increased cellular vacuolization in host (Calvet *et al*, 1989). The penetration of *T.*

harzianum Rifai into the cell wall of other fungi was attributed to the production of enzymes, which are involved in its degradation (Zeilinger *et al*, 1999). Then antibiotics were released that permeate the perforated hyphae and prevent re-synthesis of the host cell wall (Lorito *et al*, 1996). After penetration of the cell wall, *T. harzianum* was found to cause dissolution of cytoplasm and its hyphae grew within the empty host hyphae (Inbar *et al*, 1996).

1.3.3 Fungus-insect interaction

Basic studies on the *in vivo* development of insect mycopathogens like *B. bassiana*, *M. anisopliae, N. rileyi* and *V. lecanii* helped in understanding the disease cycle in insects. Unlike the insect pathogenic bacteria and viruses, the fungi act by contact. The infective propagules are usually hydrophobic conidia as in case of *B. bassiana*, *M. anisopliae* and *N. rileyi* or sticky hydrophilic conidia seen in *V. lecanii* and *H. thompsonii* (Sosa-Gomez *et al*, 1997). The adhesion of conidia to insect cuticle may be a passive (adsorption) or active host-specific phenomena. Penetration of the insect cuticle is facilitated by the enzymatic degradation of insect cuticle involving proteases, chitinases and lipases as well as mechanical pressure. The fungus grew profusely in 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 also contribute to the demise of the insect. The fungus colonizes the dead cadaver of the insect and then produces conidia for dispersal. The sporulation is favoured in presence of high relative humidity, which enhance the possibility of horizontal or vertical transmission of the disease within the insect population.

1.3.4 Killing components

1.3.4.1 Mycolytic and cuticle degrading enzyme complexes

The fungal cells swell and burst when confronted with the cell wall degrading enzymes. Thus production of mycolytic enzymes is the most common strategy used by various organisms to combat fungal pathogens (de Nobel *et al*, 2000). The main components of mycolytic enzyme complex (ME) are chitinases, glucanases and proteases (Deshpande, 1999). The sequence of appearance of these extracellular enzymes was found to be corresponding to the layering of chemical constituents of the fungal cell wall, i.e., chitin, glucans and proteins (Goldman and Goldaman, 1998). When *T. harzianum* was grown on a mycelium of *R. solani*, an alkaline proteinase was produced first, followed by glucanases and chitinases. Haran *et al* (1996) reported the differential expression of a series of chitin-degrading enzymes of *T. harzianum* during mycoparasitism of *R. solani* and *S. rolfsii*, which varied with time and fungal host. *T. atroviride* endochitinase (ECH42) was implicated in the mycoparasitism of *R. solani* based on improved disease control by multiple–copy transformants (Carsolio *et al*, 1999). Expression of *ech42* from *T. atroviride* occurred before mycoparasitic contact following molecular exchange with the host *R. solani* (Kullnig *et al*, 2000) but in contrast, *chit33* was expressed during, but not before overgrowth of *R. solani* by *T. harzianum* (Dana *et al*, 2001). Also three to seven β-1,3-glucanases were produced by isolates of *T. harzianum* exposed to fungal cell walls, autoclaved mycelia or laminarin, and some enzymes were linked to mycoparasitism (Ramot *et al*, 2000; DE la Cruz *et al*, 1995). Exo-α-1,3- glucanase produced by *T. harzianum* also showed lytic and antifungal activity against plant pathogenic fungi like *A. niger*, *B. cinerea*, and *P. aurantiogriseum* (Ait-Lahsen *et al*, 2001). Involvement of chitinase (SECHI44) in mycoparasitic interaction between *Stachybotrys elegans* and *R. solani* was demonstrated by Morissette *et al* (2003). While secretion of an exo-β-1,3-glucanase by *Ampelomyces*

quisqualis was found to play an important role during the hyperparasitism of powdery mildew (Rotem *et al*, 1999). Cellulolytic enzymes of *Pythium oligandrum* were reported to play an important role in disrupting the integrity of the cellulose rich cell wall of phytopathogens, *Phytophthora and Pythium* species (Picard *et al*, 2000). While de Azevedo *et al* (2000) characterized an amylase produced by a *T. harzianum* isolate with antagonistic activity against *Crinipellis perniciosa*, the causal agent of witches's broom of cocoa. The importance of hydrolytic enzyme production in the biocontrol activity of yeasts vary with the organism (Saligkarias *et al*, 2002; Urquhart and Punja, 2002).

In vitro experiments showed that entomopathogenic fungi in submerged fermentation produce extracellularly cuticle degrading enzymes mainly chitinases, proteases and lipases when locust cuticle was used as a sole source of carbon (St. Leger *et al*, 1986). In case of sequence of appearance of extracellular enzymes in *Metarhizium* and *Beauveria* cultures a lot of studies were reported. Proteolytic enzymes such as esterase, endopeptidase, aminopeptidase and carboxypeptidase were produced within first 24 h of growth. *N*- acetylglucosaminidase appeared next and endochitinase, which hydrolyze chitin polymer randomly, was produced in significant quantities after 4 days. Lipases were detectable after 5 days (Charnley, 1989). Though chitin forms main structural mesh, the sequence of appearance of components of CDE was in accordance with cuticular structure. It was proposed that a cocktail of proteases act on peptides cross-linking the chitin fibrils providing available substrate for the fungal chitinases. St. Leger *et al* (1996) assigned major role to proteases in cuticle degradation by *M. anisopliae* as over expression of the subtilisin-like protease gene, *Pr1A*, was shown to significantly enhance the virulence of *M. anisopliae*. The virulent isolates of *N. rileyi* exhibited substantially higher levels of chitinase than avirulent strains (El-Sayed *et al*, 1989). Under *in vitro* conditions, *M. anisopliae* ME1 produced multiple chitinase

isozymes including both endochitinases (CHI1, CHI2 and CHI3) and an exochitinase (St. Leger *et al*, 1993). While, Jackson *et al* (1985) reported that chitinase-deficient mutants of *V. lecanii* were able to penetrate and infect aphids. Furthermore, recombinant *M. anisopliae* strains engineered to overproduce chitinase did not possess altered virulence to *Manduca sexta* larvae (Screen *et al*, 2001). Recently, Fang *et al* (2005) confirmed the involvement of Bbchit1 chitinase during penetration of cuticle by *B. bassiana*. The increased Bbchit1 production in transformants resulted in lower LC_{50} indicating that there was a dosage effect of Bbchit1 on infection efficiency. Nahar *et al* (2004) for the first time demonstrated an alternate mechanism of cuticle degradation with the involvement of constitutively produced chitin deacetyalse and chitosanase, the process of cuticle degradation in fungi that show low or delayed chitinase production.

The composition of CDE and ME is not drastically different. The dual pathogenecity of some fungi towards insects and fungi was attributed to the hydrolytic enzymes, which facilitate the entry of the pathogen in the respective hosts.

1.3.4.2 Antibiotics and toxins

The production of inhibitory metabolites is characteristic of many effective fungal and yeast biocontrol agents (Punja and Utkhede, 2003). Species of *Trichoderma* and *Gliocladium* are known to produce several secondary metabolites with antibiotic properties including polyketides, terpenoids and polypeptides (Vey *et al*, 2001). Gliotoxin and gliovirin are well-described antibiotics produced by *G. virens* (Howell, 2003). Gliovirin is reported to kill *pythium ultimum* by causing coagulation of its protoplasm. *Gliocladium* sp. also produced different enzyme inhibitors such as argifin, a potent chitinase inhibitor. Interestingly, argifin retarded the moulting process in cockroach larvae that again was attributed to the inhibition of insect chitinase activity (Nitoda *et al*, 2003). Recently, Soytong *et al* (2005) reported that chaetoglobosin-C and

trichotoxin significantly inhibited the spore production of pathogenic strains of *Colletotrichum gloeosporioides* and *C. acutatum* causing anthracnose disease of grapes. Claydon *et al* (1987) identified the antifungal, volatile alkyl pyrones produced by *T. harzianum*, which when added to a peat soil mixture reduced the incidence of damping off disease in lettuce caused by *R. solani*. *Trichoderma hamatum* was reported to produce trichoviridin, acrylic acid and three isonitriles among which isonitrin A possessed the broad spectrum antifungal activity (Ramakrishnan *et al*, 2001). A novel inhibitory substance 3-2(-hydroxypropyl), 4-(2,4-hexadienyl), 2(5H)-furanone, produced by *T. harzianum* was found to suppress growth of *F. oxysporum* and may be involved in the biocontrol of fusarial wilt. A novel trichothecene, harzianum A produced by *T. harzianum* is one of the killing components for the biocontrol of plant pathogenic fungi. While tricholin, a ribosome-inactivating protein produced by *T. Viride* was effective against *R. solani*. Nevertheless antifungal antibiotic production did 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 was not 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.

The entomopathogenic fungi, *B. bassiana*, *B. brongniartii*, *M. anisopliae*, *P. fumosoroseus*, *Tolypocladium* and *V. lecanii* produce different toxins such as bassianin, beauvericin, cytochalasin C, destruxins, dipecolonic acid, enfrapeptins, hirsutellin A, B, oosporin, swainsinone, etc. (Amiri-Besheli *et al*, 2000; Vey *et al*, 2001). Different insect species exhibited different levels of sensitivity towards these toxins. For example, lepidopteran insects were found highly susceptible to destruxins produced by *M. anisopliae*. Efrapeptins of *Tolypocladium* showed insecticidal and miticidal effects

against potato beetle, mites, budworm and diamondback moth and others. Oosporin produced by *B. brongniartii* affected enzyme malfunctioning in cockchafer larvae by redox reactions. The hyphomycete, *H. thompsonii* produced an extracellular insecticidal protein, hirsutellin A that was reported to be effective against citrus rust mite, mosquito larvae and others (Vey *et al*, 2001). *Fusarium proliferatum* and *Fusarium larvarum* were reported to produce a toxic metabolite, fumonisin 1 active against aphid *Schizaphis graminum* (Ganassi *et al*, 2001).

1.4 Development of mycopesticide

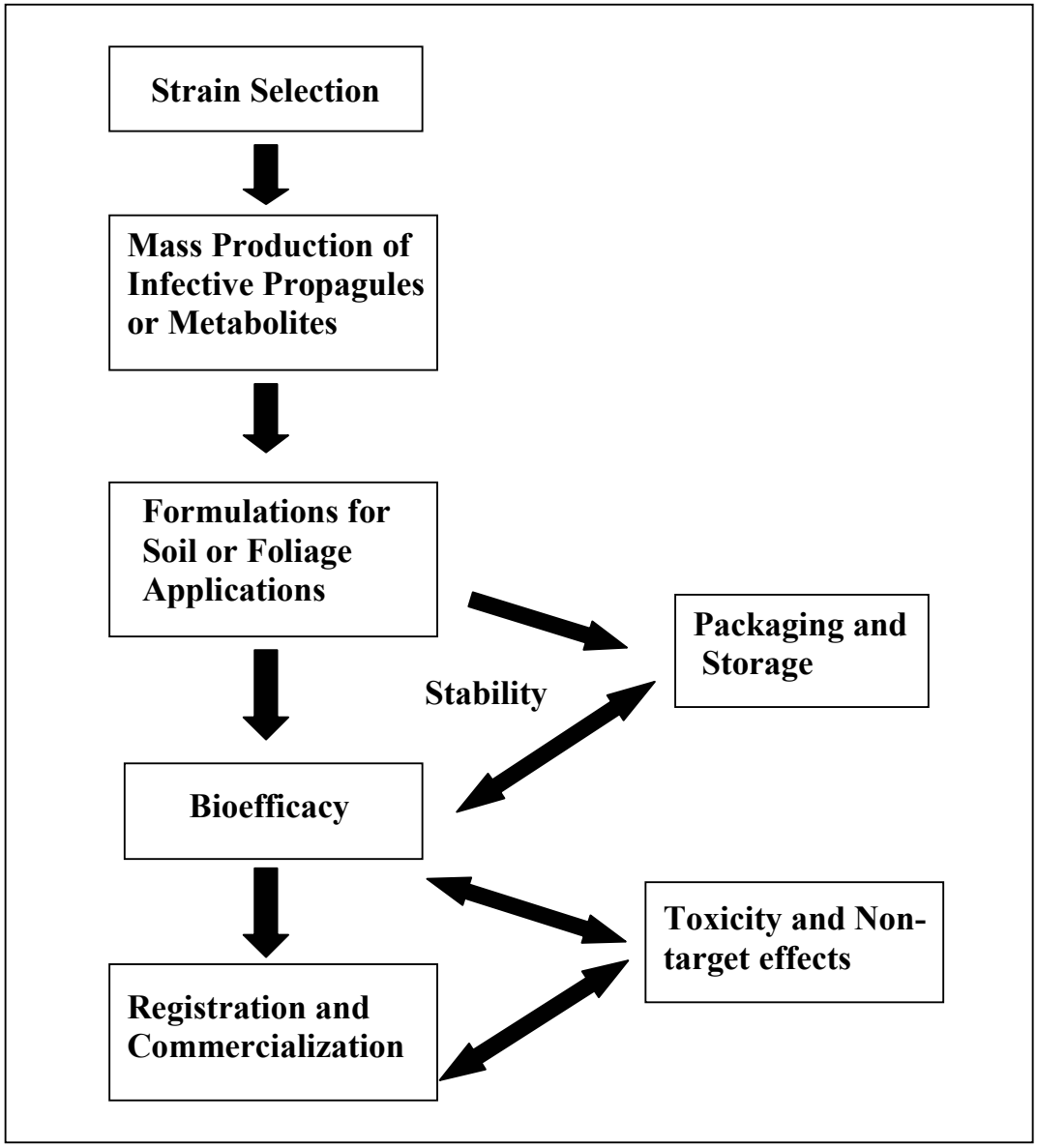
The steps involved in the development of mycopesticide are illustrated in Fig. 1.2.

1.4.1 Strain

1.4.1.1 Isolation

The soil dilution method is commonly used for the isolation of both mycoparasitic and entomopathogenic fungi (Goettel and Inglis, 1996). The baiting technique called the pre-colonized plate method in which field inoculum was placed on to the host fungus was used for the isolation of epiphytic mycoparasites (Krauss and Soberanis, 2001; Krauss *et al*, 1998). Zimmerman (1986) suggested the use of *Galleria* larvae as a bait for the isolation of insect pathogens. In *Galleria* bait method, the larvae are allowed to move through the soil in a small vial, which is moved upside down everyday. After 14 days, one can find dead or even mycosed larvae from which fungus can be isolated and further identified. Alternatively, mycosed larvae from fields may be collected to isolate highly virulent entomopathogens causing epidemics in particular insect host (Deshpande and Tuor, 2002). Screening and isolation of fungi producing extracellularly chitinase is usually done on media containing chitin, colloidal chitin or

Fig. 1.2 Approach and strategy for the development of mycopesticide



pretreated crab or shrimp shell waste as a sole source of carbon (Vyas and Deshpande, 1989; Wang and Huang, 2001). Recently, Ramirez *et al* (2004) developed a simple and sensitive method based on the use of colloidal chitin stained with Remazol Brilliant Blue R as a carbon source in a liquid medium for the selection or comparison of chitinolytic microorganisms.

1.4.1.2 Habitat association

Many past efforts to select biocontrol organisms were empirical. The random selection of organisms from soil and plant surfaces to chance encounters on culture media showing zones of inhibition was followed initially. Second approach was to examine healthy plants and their rhizosphere in otherwise heavily infested agricultural sites. Some agricultural fields were found to be disease suppressive, these were also used to isolate biocontrol organisms such as mycoparasite species. In the Rhone valley in France, *Fusarium* suppressive soils specific against wilt causing *Fusarium* were found. The saprophytic isolates of *Fusarium oxysporum* and *F. solani* repressed activity of pathogenic strains of *F. oxysporum* in melons. Another example is a soil suppressive to *Pythium ultimum* in California, where prior colonization of crop debris by *Pythium oligandrum* prevented damping off disease of sugar beet caused by *P. ultimum* (Deshpande, 1999).

Bidochka *et al* (2001) reported that the isolation of the entomopathogenic fungi was affected by the locality from where the soil was taken and also by the method of isolation selected. It was reported that *N. rileyi* isolates from different geographical locations showed different levels of virulence against two insect hosts, *H. armigera* and *S. litura* (Vimala Devi *et al*, 2003). In general the soil samples collected from the rhizosphere of crops where chemical insecticides are not routinely sprayed one can expect more number of isolates that had high infectivity against insect pest. For

instance, *Metarhizium* isolates from fields of tomato, okra and other vegetables heavily sprayed with chemicals were less virulent to *H. armigera* than the isolates from custard apple field rarely sprayed with chemicals (Nahar *et al*, 2003). This can also be correlated with the natural insect flora of the host plant, not necessarily pest that affects the virulence of entomopathogenic fungi.

The chitinolytic fungi like *Myrothecium*, *Trichoderma*, *Beauveria*, *Penicillium* and *Aspergillus* were isolated from rhizospheral soil samples, chitinous wastes and marine environments (Felse and Panda, 2000; Vyas and Deshpande, 1989).

1.4.1.3 Criteria for strain selection

The ability of the strains to produce differential levels of various cuticle degrading and mycolytic enzymes play an important role in strain selection (Deshpande, 1999; Goldman and Goldman, 1998; Krieger de Moraes *et al*, 2003; Nahar *et al*, 2004; Nahar *et al*, 2003; Patil *et al*, 2000; St. Leger *et al*, 1986). Interestingly, the spore characteristics such as the size, viability, production, speed of germination and relative hyphal growths are important for strain selection too (Liu *et al*, 2003). It was reported that in case of *P. fumosoroseus* the spore size and virulence towards *Plutella xylostella* were correlated with each other (Altre *et al*, 1999). While Sugimoto *et al* (2003) reported relatedness among genetic properties, conidial morphology and virulence of *V. lecanii* towards different insect hosts. Thus based upon the insect bioassay and level of cuticle degrading enzyme activities, selection of the best possible fungal strain along with the backup strains would be easier.

The environmental conditions like temperature, pH and water activity affect the growth and in turn efficacy of biocontrol agents (Antal *et al*, 2000; Fargues *et al*, 1997; Jackson *et al*, 1991). Thus the ability of biocontrol agents to withstand fluctuations in the environmental conditions requires consideration in strain selection. Certain other

criteria suggested by Jenkins *et al* (1998) that also governed the success of the biocontrol preparation were field performance studies, genetic stability, productivity, and stability of conidia in storage, stability in formulation, field persistence, mammalian safety, low environmental impact and capacity to recycle in the environment.

1.4.1.4 Strain improvement

Although naturally occurring antagonists provide a major source of new isolates, genetic improvement plays an important role in the development of biocontrol agents. In entomopathogenic as well as mycoparasitic fungi, mutagenesis was employed to understand the involvement of different genes in virulence and killing process as well as for the strain improvement (Valadares-Inglis *et al*, 1997; Delgado-Jarana *et al*, 1996). Protoplast fusion is the valuable tool for strain improvement that can overcome the incompatibility barrier for genetic recombination. Protoplast fusion of diauxotrophic mutants of *B. bassiana*, biocontrol agent against European corn borer and toxinogenic *Beauveria sulfurescens* (non-pathogenic to insects, but could produce an entomotoxic glycoprotein) was used to produce hybrids (Viaud *et al*, 1998). Molecular cloning to achieve overproduction, change the induction pattern or change in the localization (periplasmic/extracellular) of chitinases was reported in many systems (Shaikh and Deshpande, 1993). Many studies report the transformation of different *Trichoderma* strains with chitinase genes under the control of constitutive promoters (Deane *et al*, 1999; Limon *et al*, 1996; Zaldivar *et al*, 2001). The overexpression of genes encoding chitinase (*ech42*, *chit33*) (Baek *et al*, 1999; Limon *et al*, 1999), endoglucanase (*egl1*) (Migheli *et al*, 1998) and proteinase (*prb1*) (Flores *et al*, 1997), in transformed *Trichoderma* species improved the antagonistic potential of the agent against pathogens such as *Rhizoctonia* and *Pythium*, both *in vitro* and *in vivo*. Similarly, overexpression of subtilisin-like protease gene, *Pr1A* in *M. anisopliae* (St. Leger *et al*, 1996) and chitinase

gene, *Bbchit1* in *B. bassiana* (Fang *et al*, 2005) was linked with the increased virulence of these entomopathogenic fungi.

1.4.1.5 Preservation of fungi

The fungal spores are normally stored at -20°C or -80°C using skimmed milk, water or mineral oil. *P. fumosoroseus* spores remained viable when stored with water or mineral oil and preserved at -80°C. Alternately, fungal isolates can be stored for long period as dry alginate pellets (biomass plus sterile wheat bran and aqueous sodium alginate) at -20°C. The fungal conidia can be stored in carriers such as mineral oils, wheat bran, talc powder, silica, etc. to retain the viability and virulence of the conidia, which is an important requisite for the performance of the biocontrol preparation. Also, to retain the virulence, regular serial passage of fungus through the insect hosts for entomopathogenesis and for mycoparasitic ones growth in dual culture are essential (Chavan *et al*, 2006).

1.4.2 Production of fungal biocontrol agents

1.4.2.1 Living propagules

The fungal kingdom is a diverse assemblage of organisms with a great variety of structural types (Deshpande, 1999). The hypha and unicellular yeast are vegetative growth forms, while asexual spores such as conidia and sporangiospores are produced for dispersal. The lower fungi sexually produce resistant oospores or zygospores that are commonly used in the biocontrol formulations. Other resistant structures such as blastospores and chlamydospores are also used as infective propagules.

Production of fungi to use as biocontrol agents aims at the maximum possible number of living propagules that can infect pathogenic fungi and insects. Generally, fungal propagules in biopesticides must be alive at the time of their use and the

expected minimum shelf life is 18 months at 20°C (Deshpande, 1999). Thus for a stable biopesticide formulation, conidia are the favoured morphological structures as they retain viability and infectivity for long periods. Different fungal genera and even different isolates within the same fungal species, vary greatly in their requirements for nutrition, pH, water activity of the substrate (moisture content), temperature optima for mycelial growth and conidiation, light, aeration and incubation period. All these parameters must be thoroughly investigated and optimized to ensure that mass production process is not only efficient but also produces conidia of high quality and reliability (Chavan *et al*, 2006; Jenkins *et al*, 1998).

Both liquid and solid fermentation systems were used to obtain a desirable quantity of biomass, and former was the preferred approach. Submerged fermentation has obvious advantages of controlled processing, and both organism itself and the extracellular enzymic and non-enzymic products exhibiting biocontrol activity can be obtained easily. Using a wide range of controls for pH, agitation rate, aeration rate, temperature etc. the timing for harvesting of conidia can be precisely envisaged (Deshpande, 1999). Moreover, manipulation of cultural and nutritional parameters can be exploited to favour production of submerged conidia of *M. anisopliae* and *B. bassiana* (Kassa *et al*, 2004; Thomas *et al*, 1987). The phenomenon of microcycle conidiation was exploited in the production of conidia of *B. bassiana* (Bosch and Yantorno, 1999). Hegedus *et al* (1990) suggested a medium containing *N*-acetylglucosamine for the production of conidia of *B. bassiana* under submerged conditions. While Latorre *et al* (1997) used modified Richard's medium for the production of conidia of *T. harzianum* in stirred tank fermenter. Different mycoparasitic *T. harzianum* strains also produced chlamydospres in submerged fermentation, whereas conidia were formed on solid substrates. Chlamydospores of *G. virens* were produced in a stirred tank fermenter and the different fermentation stages were used to make the

effective biocontrol formulation (Eyal *et al*, 1997). Fargues *et al* (2002) described the conditions for mass production of blastospore like propagules of *M. flavoviride*. *Paecilomyces fumosoroseus*, a biocontrol agent against sweet potato whitefly, was also grown in the form of blastospores that were resistant to drying (Jackson *et al*, 1997). *Entomphthora virulenta* pathogen of aphids produced zygospores under submerged conditions in a medium containing dextrose and yeast extract (Latge *et al*, 1977). Shah *et al* (1998) reported the conditions for production of mycelia of *Erynia neoaphidis*, a pathogen of aphids. However, entrapment of mycelia in the alginate beads, for conidia production, was used to apply *E. neoaphidis* for the biocontrol of aphids (Shah *et al*, 1998).

Ferron (1981) reported the production of conidia of *B. bassiana* in unstirred shallow liquid culture (surface fermentation) where conidia produced at liquid-gas interface were harvested by peeling of the surface layer of the fungus. McQuilken *et al* (1990) reported the use of surface fermentation for the bulk production of oospores of *Pythium oligandrum*. A high yield of oospores was obtained on cane molasses within 2-3 weeks. High sterol contents of cane molasses supported the production of oospores.

The solid-state fermentation has also been successfully exploited for the production of aerial conidia of various biocontrol fungi (Jones *et al*, 2004; Kang *et al*, 2005; Krauss *et al*, 2002; Nava *et al*, 2006; Thangvelu *et al*, 2004). The advantages of solid-state fermentation are easy to carry out, raw material is cheap and most importantly conidia produced as living propagules tend to be more tolerant to desiccation and more stable as dry preparation compared with conidia produced in submerged fermentation (Deshpande, 1999). Furthermore, as large amounts of water is not added to the biological material during SSF, fermenter volumes remain small, necessary manipulations become less expensive and the cost of water removal at the end of fermentation is minimized. These advantages can overweigh the disadvantages

of SSF, which are lower product yields, slowness of fermentation, and the difficulty of controlling the process precisely (Pandey, 2003; Tengerdy, 1985).

Different substrates like sorghum, wheat, barley, bajara, beaten rice and rice can be used to grow the fungal strains for conidia production (Vimala Devi, 1994). Agricultural waste like rice bran and rice bran-husk mixtures were used for mass production of *M. anisopliae* (Dorta *et al*, 1990). Hua and Feng (2003) reported the broomcorn millets as excellent substrate for production of granular cultures of *E. neoaphidis* that were capable of discharging primary conidia to infect *Myzus persicae*. Zheng and Shetty (1998) suggested the use of cranberry pomace, primary by-product of cranberry juice processing industry and fish protein hydrolysate, by-product of fishery for the production of species of *Trichoderma* having biocontrol potential. While another strain of *T. harzianum* grown on sorghum grains was mixed with organic materials like neemcake, coirpith, farmyard manure and decomposed coffee pulp before soil application (Saju *et al*, 2002). Recently, Thangvelu *et al* (2004) reported the mass production of *T. harzianum* on dried banana leaves dipped in jaggery solution prior to inoculation with the mycelial disks of biocontrol agent for the management of fusarial wilt of banana. The conidia of *Epicoccum nigrum*, biocontrol agent of the brown rot fungus *Monilinia laxa*, were produced on peat/vermiculite mixed with lentil meal (Larena *et al*, 2004).

1. 4.2.2 Fungal metabolites

Fungal metabolites may be used as biocontrol preparations. These preparations may have following advantages over living organism based preparations, wider spectrum, quicker action and environmentally safe. Furthermore, unlike chemicals they do not accumulate in fruits/vegetables (Shternshis, 2005).

Chitin hydrolyzing enzymes are the most promising candidates for use in pest and pathogen management. Chitinase alone and in combination with *B. thuringiensis* was reported to control larvae of spruce budworm (Smirnnoff, 1974; Smirnnoff, 1973). Mendosa *et al* (1996) used purified endochitinase of *M. verrucaria* for the control of mosquito larvae. While Govindsamy *et al* (1998) reported use of purified preparation of *M. verrucaria* chitinase to control groundnut rust, *Puccinia arachidis*. Recently, chitinase was used under field conditions for the control of raspberry midge blight in Siberia (Shternshis *et al*, 2002a). The synergy between the cell wall hydrolyzing enzymes and certain antibiotics was also reported. Several microbial chitinases, with β-1,3-glucanases, propane-2ol and polyoxyethelene lauryl ether were sprayed on rice field to control rice blight caused by *Pyricularia oryzae* (Tanaka *et al*, 1970). While 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).

Several attempts were reported on the laboratory- and/or large-scale production of fungal enzymes and antibiotics. However, reports on commercially available fungal metabolites, as biological control agents are sparse. Chitinolytic enzymes play an important role in the control of plant pathogenic fungi and insects containing chitin in their protective covers, cell wall and cuticle, respectively. Traditionally bacterial strains were used for the production of chitinolytic enzymes. Most of the chitinolytic bacteria like *Serratia marcescens*, *Bacillus pabuli*, *Pseudomonas stulzeri* and others express maximum chitinase during the third day of fermentation while fungi and actinomycetes express maximum chitinase around sixth day of fermentation under submerged fermentation conditions (Felse and Panda, 2000). However, bacterial chitinases are

primarily exo- instead of endo-cleaving enzymes, the former being substantially less effective than the latter in degrading chitin (Kramer and Muthukrishnan, 1997). Furthermore, fungal chitinases are active over a wide range of temperature and pH (Felse and Panda, 2000). Thus, recently the interest in fungal cultures extracellularly producing chitinases is increasing.

The details of production of chitinases by various fungal isolates were compiled in Table 1.3. The biocontrol fungus, *T. harzianum* is currently of major interest for large-scale production of chitinases. Other biocontrol fungi like *M. verrucaria* (Vyas and Deshpande, 1989), *V. lecanii* (Liu *et al*, 2003; Matsumoto *et al*, 2004) and *B. bassiana* (Suresh and Chandrasekaran, 1999; Zhang *et al*, 2004) were also reported as potent producers of chitinolytic enzymes. Saprophytic fungi like *Penicillium* sp. (Fenice *et al*, 1998; Parameswaran *et al*, 2005) and *Aspergillus* sp. (Rattanakit *et al*, 2002) were also used for the production of chitinolytic enzymes. Solid-state cultivation and metabolite production by fungi being the cost effective method many attempts were made to produce chitinolytic enzymes using solid substrates (Binod *et al*, 2005; Sandhya *et al*, 2005; Matsumoto *et al*, 2004). Chitin flakes, colloidal chitin or shrimp shell waste supplemented with agricultural by products like wheat bran or sugar cane bagasse were used as solid substrates for the production of chitinases. Supplementation of additional nitrogen source, nature and size of inoculum and biophysical parameters like moisture content, pH and temperature play an important role in improving the chitinase production by solid-state fermentation.

Many shake flask studies on the optimization of nutritional and biophysical parameters for the production of fungal chitinases under submerged conditions were also reported (Donzelli *et al*, 2005; El-Katatny *et al*, 2003; Felse and Panda, 1999; Sandhya *et al*, 2004). In most of the cases, addition of carbon source other than chitin reduced chitinase production but supported mycelial growth. While addition of urea

Table 1.3 Production of fungal chitinases

Organism	Type of fermentation	Substrate used	Reference
Myrothecium verrucaria	Submerged fermentation (Shake flask)	Crab shell chitin	Vyas and Deshpande, 1989
Fusarium chlamydosporium	Submerged fermentation (Shake flask)	Colloidal chitin and sucrose	Mathivannan *et al*, 1997
Penicillium janthinellum	Submerged fermentation (Shake flask and stirred tank fermenter)	Colloidal chitin	Fenice *et al*, 1998
Trichoderma harzianum	Submerged fermentation (Shake flask and stirred tank fermenter)	Crab shell chitin	Felse and Panda, 1999; Felse and Panda, 2000
Trichoderma viride	Shake flask studies	Colloidal shrimp shell chitin	Shindia *et al*, 2001
Aspergillus sp.	Solid-state fermentation	Shrimp shellfish waste	Rattanakit *et al*, 2002
Verticillium lecanii	Submerged fermentation (Shake flask and Stirred tank fermenter, air-lift fermenter)	Shrimp powder	Liu *et al*, 2003
Trichoderma harzianum	Submerged fermentation (Shake flask)	Colloidal chitin	Sandhya *et al*, 2004
Trichoderma harzianum	Solid-state fermentation	Wheat bran supplemented with colloidal chitin	Nampoothiri *et al*, 2004
Verticillium lecanii	Solid state fermentation, Submerged fermentation (Shake flask)	Sugarcane pith bagasse supplemented with shrimp waste silage, shrimp waste silage	Matsumoto *et al*, 2004
Beauveria bassiana	Solid-state fermentation	Wheat bran supplemented with silkworm chrysalis	Zhang *et al*, 2004
Penicillium aculeatum	Solid-state fermentation	Wheat bran medium supplemented with chitin flakes	Binod *et al*, 2005
Trichoderma atroviride	Submerged fermentation (shake flask)	Crab shell chitin	Donzelli *et al*, 2005
Trichoderma harzianum	Solid-state fermentation	Wheat bran supplemented with colloidal chitin	Sandhya *et al*, 2005

increased chitinase production by various fungal isolates. The optimization of aeration and agitation rates, pH and temperature plays a major role in enhancing chitinase production under submerged conditions. The attempts were also made to understand the effect of different process parameters such as agitation and aeration rates on fungal chitinase production in stirred tank fermenters.

Several studies were also carried out to optimize conditions for the production of fungal metabolites with antifungal or insecticidal properties. However, in many cases the industrial production of antifungal metabolites was limited by the low fermentation yields owing to the growth inhibition caused by very low product concentrations (≤ 100 mg/l). To mitigate this problem Sarhy-Bagnon *et al* (2000) suggested the use of solid-state culture for the large-scale production of 6-pentyl-α-pyrone, an antifungal compound produced by *T. harzianum*. While Serrano-Carreon *et al* (2002) demonstrated the use of two-phase liquid system to overcome the toxicity problem and increase the production of 6-pentyl-α-pyrone. In this system 6-pentyl-α-pyrone was extracted with n-hexadecane during cultivation. Gliotoxin, an epithiodiketopiperazine toxin produced by *T. viride* was found essential for curtailing the growth and multiplication of phytopathogens. Anitha and Murugesan (2005) reported the production of gliotoxin by *T. viride* on natural substrates like tapioca powder, sugarcane bagasse, rice bran, etc. The strategic manipulation of cultivation conditions resulted in enhanced production of gliotoxin on sugarcane bagasse as substrate. The destruxins A and B from *M. anisopliae* were found to be toxic substances to several pest insects. Liu *et al* (2000) reported the optimization of medium composition for destruxin production using response surface methodology. Maltose and peptone were the best carbon and nitrogen sources for production of destruxins by *M. anisopliae*. While Wang *et al* (2004) studied effect of different carbon-nitrogen ratios as well as different insect homogenates on the production of destruxins A and B. Recently, Feng *et al* (2004)

reported the optimization of cultivation conditions for the production of destruxin B by *M. anisopliae* in stirred tank fermenter and air-lift fermenter.

1.4.3 Formulation

Formulation is required to present the product in a usable form and to optimize the efficacy, stability, safety and ease of application of the product. The living propagules of fungi or their metabolite(s) active against fungal pathogens/insect pests may be used as biocontrol agents. The living propagules of fungi are required to get activated or multiplied after the application in order to control the target pest or pathogen. The formulation thus provides conditions that retain viability, virulence during preparation, storage and application and favour survival and establishment of biocontrol agent in the environment even after application. While in case of the fungal metabolite(s), formulation should retain metabolite in active form under the conditions used for storage and those in the field.

1.4.3.1 Types of formulations

Most of the commercially available fungal biocontrol preparations contain living propagules of fungi rather than their metabolites. These formulations were developed using dormant structures of fungi like conidia, chlamydospores or metabolically active forms like mycelia and yeast cells. Formulations containing dormant microbes tend to have longer shelf life, are easier to package and are tolerant to temperature fluctuations and to chemicals such as pesticides and sticker materials which may be required for successful application. On the otherhand, formulations containing metabolically active microbes are less tolerant to temperature extremes and chemicals, have shorter shelf life and have specific packaging requirements for gas and moisture exchange. In most cases,

fungi that are difficult to sporulate in culture are formulated in metabolically active state (Shah *et al*, 1998).

Fungal biocontrol agents are usually supplied as powders, granules or concentrated liquids. The intended use of the biocontrol agent (soil or foliar application) governs the type of formulation. The fungal condia produced by solid-state fermentation providing the carrier for the inoculum may be mixed with suitable diluent and used for soil application. The development of an aqueous formulation of hyphomycetous fungal conidia is hindered by their hydrophobic nature. In such cases addition of wetting agents like organosilicone was found effective (Wraight *et al*, 2001). The oils, vegetable and petroleum-derived, were generally found compatible with lipophilic conidia. It was suggested that the oils help conidia to stick to the insect cuticle and enable the fungus to kill insects even under low humidity conditions (Inyang *et al*, 2000; Vimala Devi and Prasad, 1996). Nahar *et al* (2003) reported the use of oil-diesel formulation of *M. anisopliae* aerial conidia for the control of *H. armigera* in pulses. While Kassa *et al*, (2004) demonstrated the use of oil-diesel formulations of aerial conidia or submerged spores of *M. anisopliae* var. *acridum* for the control of locusts and grasshoppers. The addition of diesel in formulation reduced the viscosity of oil thus facilitating easy spraying of the formulation. With respect to the biosafety, oil formulations eliminate the dust hazards. However, possible phytotoxicity of petroleum derived oil and a diesel spray is a major concern.

Torres *et al* (2003) developed a liquid formulation containing yeast cells of *Candida sake* for the control of *Penicillium expansum*, causing post harvest losses in apples. It was suggested that addition of 10% lactose as a protectant can improve viability, retain biocontrol efficacy and also render use of formulation economically feasible.

Dry powder and dry granules were most successfully produced with conidia. Concentrates of fungal propagules obtained after drying were usually formulated with an inert extender such as fine clay particles, peat, charcoal, clay, vermiculite, etc. Organic carriers like rice bran, wheat bran, and bagasse were also used. Wheat bran formulation provided a long-term shelf life to biocontrol agents, *Gliocladium virens* and *Trichoderma* isolates (Jackson *et al*, 1991). The biocontrol agents like *Trichoderma, Verticillium, Beauveria* and *Metarhizium* were also produced as wettable or water dispersible powders (Singh and Zaidi, 2005). The fungal propagules were mixed with diluents (e.g. charcoal, attapulgite) and surface-active agents (e.g. carboxymethyl cellulose, gelatin) to ensure suspending and wetting properties and stickers (e.g. xanthan gum).

The granular formulations were prepared using carriers such as alginate (Zohar-Perez *et al*, 2003) and cornstarch (Pereira *et al*, 1991). Alginate formulations of conidia, mycelia or ascospores of several biocontrol agents, such as *Trichoderma* sp., *Talaromyces* sp., *Gliocladium* sp. and others resulted in rapid increase in fungal biomass in the soil, enabling successful biological control of several plant diseases (Fravel *et al*, 1995; Lewis and Papavizas, 1987; Shaban and El-Komy, 2001). El-Katatny *et al* (2003) reported the use of chitin as adjuvant to enhance production of chitinase, an enzyme involved in biocontrol. Diatomaceous earth formulation containing conidia of *B. bassiana* was found more effective in controlling coffee berry borer as compared to the talcum powder formulation or sodium alginate pellet formulation (Niranjana, 2004).

The reports on biocontrol formulations containing fungal metabolites are sparse. Poole *et al* (1998) reported the use of different formulations of 6 pentyl-α-pyrone (neat, dispersed in 0.01% polysorbate 80 or dissolved in acetone or cold pressed oil) for the control of post harvest rots of Kiwifruits caused by *B. cinerea*. The formulations with

acetone and polysorbate 80 were found more effective as they could leave surface films of 6- pentyl-α-pyrone on the wound surfaces, which could contact surface lodged pathogen directly.

1.4.3.2 Storage and shelf life

This 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. The carrier that retains the maximum viability and virulence of conidia for a minimum of 6 months at 28°C, ideally 18 months at 20°C, is suitable for the further commercialization (Deshpande, 2005). A talc-based preparation of *T. virens* conidia retained 82% viability at 5°C in refrigerator after 6 months, while at room temperature (25-30°C) same level of viability was observed only up to 3 months (Singh and Zaidi, 2005). Prasad and Rangeshwaran (2000) identified kaolin and talc as better carriers for *T. harzianum* conidia as compared to bentonite. The talc-based formulation retained more than 10^6 viable propagules per g up to 3 months. Chlamydospores based formulations of *T. harzianum* and *T. virens* exhibited longer shelf life than conidia based formulations at room temperature. Formulation having mycelial fragments alone did not have longer shelf life (Singh and Zaidi, 2005).

1.4.4 Application

The application of mycopesticides (living propagules or metabolites of fungi) must be easy, effective, timely, to the appropriate site of action and compatible with the available agricultural equipments. In general, mycopesticides are applied as seed treatment, seedling root treatment, soil treatment and as foliar application.

Inoculation of seeds, corms, bulbs, tuber, etc. with the living propagules of antagonists help in preventing seed/corm decay and seedling blight. The diseases caused by seed-borne pathogens, particularly externally seed-borne, of even diseases affecting above ground plant parts can be controlled. While Selvakumar *et al* (2000) reported suppression of internally seed borne diseases like the loose smut of wheat by seed treatment with *Trichoderma*, probably due to effect of its metabolites. The seed dressing with *Trichoderma* conidia mixed in rice kanji or jaggary is also a common practice (Chavan *et al*, 2006). The soil application along with fertilizers is also an acceptable practice for the control of root pathogens (Spadaro and Lodivica, 2005; Soytong and Ratanacherdachai, 2005). In recent years, foliar sprays with oil based liquid formulations of biocontrol agents like *Trichoderma* and/or *Pseudomonas* were found to be quite effective against diseases affecting foliage or blossoms (Singh and Zaidi, 2005).

The soil dwelling insect pests can be controlled using soil application of entomopathogenic fungi. Biocane, the rice granule formulation of *M. anisopliae* was applied 20 cm below the soil surface during 'fill in' a few months after the sugarcane sets are planted (Milner, 2004). While peeled or squeezed barley kernels colonized with mycelium of *B. brongniartii* were applied with a drill machine to a soil depth 5-10 cm at a rate of 30-50 kg/ha for the control of European cockchafer (*Melolontha melolontha*) in grasslands and orchards (Keller, 2004).

For the control of locusts and grasshoppers, aqueous formulations of entomopathogenic fungi were primarily applied using conventional hydraulic sprayers like knapsack sprayers and CO_2 pressurized sprayers (Inglis *et al*, 2000). While the oil formulations were applied using very low (VLV; 5-20 l/ha) and ultra low (ULV; < 5 l/ha) volume sprayers. The oil-diesel formulations of *M. anisopliae*, *B. bassiana* and *N. rileyi* were applied using ultra low volume sprayers with controlled droplet application

for the control of *H. armigera* a dreadful pest on pulses (Nahar *et al*, 2004; Nahar *et al*, 2003). While Pu *et al* (2005) found the use of air-blast sprayer suitable for the spraying of the aqueous formulations of *B. bassiana* for the control of false eye leaf hopper, *Empoasca vitis* in tea canopy.

The mycopesticide preparations containing fungal metabolites, especially cell wall degrading enzymes were applied singly or in combination with other biocontrol agents for the control of insect pests or fungal pathogens under green house or field conditions. Foliar application of microbial chitinases was found effective for the control of raspberry midge blight in Siberia (Shternshis *et al*, 2002a). While Patil *et al* (2001) used mycolytic enzyme preparation of *M. verrucaria* containing chitinase, β-1, 3-glucanase and protease for soil irrigation to control *S. rolfsii* a root pathogen of peanut. Shternshis *et al* (2002b) applied the test composition containing *Mamestra brassicae* NPV (MbNPV) ($1x10^7$ polyhedra / ml), Bt *galleriae* ($2.5x10^7$ 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*, *Plutella xylostella* and *Pieris brassicae*. Poole *et al* (1998) reported the topical application of an antibiotic, 6-pentyl-α-pyrone dissolved in acetone on Kiwifruits to control post harvest rot caused by *B. cinerea*.

1.4.5 Risk assessment

Mycopesticides 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, mycopesticides may also pose risks to the non-target organisms. The potential non-target effects of fungal biocontrol agents include competitive displacement, allergenicity, toxicity and pathogenecity (Brimmer and Boland, 2003; Cook *et al*, 1996;

Simberloff and Stiling, 1996a; Simberloff and Stiling, 1996b). The potential of a biocontrol agent to infect organisms other than the target pests is of most concern from a risk management perspective (Cook *et al*, 1996).

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, 2003; 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 often being conducted under unnatural conditions. Strasser *et al* (2000) and Butt and Copping (2000) emphasized on the need to develop the tools and methodologies that will identify and detect fate of toxic fungal metabolites in the environment.

1.4.6 Registration and commercialization

The term mycopesticide is normally used for all fungal metabolites and living propagules of fungi that can be formulated for use as pesticide for the control of pests. In India there is registration committee constituted under the Insecticides act 1968. Every microbial pesticide, which is used, distributed, sold or offered for sale 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 final step in development of mycopesticide is the commercialization of the product. Market for fungal agents is considered to be 10-15% of the total biopesticide market in India. Around 125-150 tons of different fungal formulations worth Rs. 2-2.5 crores are being marketed in India per year (Chavan *et al*, 2006). The biocontrol preparations containing *T. harzianum, T. viride*, *Verticillium* sp., *Metarhizium* sp. and *B.*

bassiana are available in Indian market (www.indianindustry.com). Several economic and geo-political factors also influence the market potential for mycopesticides. The major concern is of course the acceptability by the farming community (Lacey *et al*, 2001).

The market development of mycopesticides faces several technical hurdles. Butt and Copping (2000) have extensively reviewed the key technical issues that need to be dealt with in the development of efficacious mycopesticides. One of the major criticisms of fungal biocontrol agents is that they act slowly and therefore, give limited protection to crops. The application of fungal metabolites may provide a quicker and ecofriendly alternative to control pathogens/pests on crop plants (Shternshis, 2005). The fungal metabolites may be used in combination with other biocontrol agents to reduce the time of kill of pest/pathogen. To mitigate the problems regarding the cost of production, relatively short shelf life of many products and specificity of fungal biocontrol agents a food industry based model with a more decentralized production strategy to encourage small-scale commercial enterprise has been suggested Chavan *et al* (2006).

1.5 Present investigations and thesis organization

In this Ph.D. work, an attempt has been made to develop a mycopesticide for the control of plant pathogenic fungi and insects using a saprophytic fungus, *Myrothecium verrucaria* that secretes appreciable amounts of mycolytic and cuticle degrading enzymes.

1.5.1 *Myrothecium verrucaria*

The fungal pathogens and insect pests share a common structural component, chitin in their outermost protective covers, cell wall and cuticle, respectively.

Furthermore, chitin is absent in vascular plants and mammals thus making it an attractive target for the control of both fungal pathogens and insect pests. In this thesis, *Myrothecium verrucaria*, a deuteromycetous saprophytic fungus has been used to develop a mycopesticide for the control of plant pathogenic fungi and insect pests. There are several qualities that make *M. verrucaria* a suitable system for the studies on the development of a mycopesticide using chitin as a target. *M. verrucaria* is known to secrete high amounts of chitinase along with other mycolytic (ME) and cuticle degrading (CDE) enzymes like β-1,3-glucanase, protease and lipase when grown in a medium containing chitin as a sole source of carbon (Vyas and Deshpande, 1989). It was reported that the mycolytic enzyme complex of *M. verrucaria* effectively degraded mycelial biomass of root pathogens like *F. oxysporum* and *S. rolfsii.* Recently, Patil *et al* (2001) demonstrated the control of pre-emergence rot of peanut caused by *S. rolfsii* in pot experiments using mycolytic enzymes of *M. verrucaria* for daily soil irrigation. *M. verrucaria* purified endochitinase was also found to be effective in killing the first and fourth instar larvae of a mosquito, *Aedes aegypti*, a vector of yellow fever and dengue, within 48 h (Mendosa *et al*, 1996). Thus in view of these results, the living propagules of *M. verrucaria* that can release CDE/ME complex or CDE/ME complex itself provide a suitable system to develop a mycopesticide against plant pathogenic fungi and insects.

1.5.2 Objectives of this study

The objectives of the present studies are as follows:

1. Morphological, cultural, biochemical and molecular characterization of *M. verrucaria*.

2. Production of living propagules of *M. verrucaria*, using solid state fermentation for the production of conidia, and submerged fermentation for the production of unicellular yeast-like cells.
3. Optimization for the production of CDE/ME of *M. verrucaria*, in shake flask and in 15 l fermenter.
4. Use of living propagules and enzymic components against plant pathogenic fungi and insects.
5. Formulation and toxicological studies.

1.5.3 Thesis organization

The experiments presented in this Ph.D. dissertation are organized into three main parts. The first part is morphological, cultural, biochemical and molecular characterization of *M. verrucaria* (Chapter 3). The morphological and cultural characteristics of *M. verrucaria* were studied to understand the triggers for H-Y reversible dimorphic transition. The differential expressions of chitinolytic enzymes and NAD-/NADP-dependent glutamate dehydrogenases and nitrate reductase were studied as biochemical correlates of H-Y dimorphic transition in *M. verrucaria*. The 18s rRNA and ITS1 sequence analysis was carried out for the molecular typing of the *M. verrucaria* strain exhibiting higher production of CDE/ME complex and hypha-yeast transition.

The second part of this research effort includes production of mycopesticide (living propagules of *M. verrucaria* and mycolytic and cuticle degrading enzyme complex of *M. verrucaria*) (Chapter 4). The production of *M. verrucaria* conidia under solid-state fermentation conditions while that of yeast-like cells under submerged fermentation conditions is discussed. The work on optimization of the conditions for the

production of mycolytic/cuticle degrading enzyme complex in a shake flask and in a stirred tank fermenter (15 l capacity) has also been described.

The third and final part of this dissertation concerns the formulation and application of living propagules and mycolytic/cuticle degrading enzyme complex of *M. verrucaria* for the control of plant pathogenic fungi, *S. rolfsii* and *Capnodium* sp. and insect pests, *H. armigera, Spodoptera litura* and *Plutella xylostella* (Chapter 5).

For the screening of chitinolytic organisms a rapid and sensitive method based on the use of Ostazin Red labeled colloidal chitin as a substrate for the quantitative estimation of chitinase was developed (Appendix 1).

Chapter 2

Materials and methods

2.1 Materials

The materials and chemicals used in the studies were purchased from suppliers as shown in Table 2.1.

Table 2.1 Source of chemicals and materials

Chemicals, Materials	Source
N-Acetylglucosamine (GlcNAc), Bovine serum albumin, Chitin, Chitosan, Ethylene glycol chitosan, Glucosamine, Gum arabic, α-Ketoglutarate, Laminarin, Mandelonitrile, Mandelonitrile lyase, 4-Methylumbeliferone, 4-Methylumbelliferyl-β-D-*N*-acetyl glucosaminide, 4-Methylumbelliferyl-β-N, N', N"- triacetyl chitotrioside, Tyrosine	Sigma Chemical Co., St. Louis, MO, USA
Dithiothreitol, N-(1-Naphthyl)ethylenediamine dihydrochloride, Nicotinamide adenine dinucleotide (NAD) and reduced form (NADH), Nicotinamide adenine dinucleotide phosphate (NADP) and reduced form (NADPH), Nitroblue tetrazolium, Phenazine methosulphate, Sulfanilamide	Sisco Research Laboratoies Ltd., India
Ammonium chloride, di Ammonium hydrogen orthophosphate, Ammonium nitrate, Ammonium sulphate, Calcium chloride, Cobaltous chloride, p-dimethyl aminobenzaldehyde, Ferrous sulphate, Glucose, Magnesium sulphate, Manganous sulphate, Mannitol, Potassium chloride, di-Potassium hydrogen orthophosphate, Potassium dihydrogen orthophosphate, Potassium nitrate, Sodium chloride, Sodium nitrate, Sodium nitrite, Sucrose, Urea, Zinc sulphate	s.d.fine-CHEM Ltd., India
Beef extract, Peptone, Sabouraud malt extract, Soyabean meal, Starch, Yeast extract	Hi-media, India
Oxgall	Loba Chemie, India
Ultra filtration membrane	Millipore Molsheim, France

* All other chemicals used were of analytical grade.

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	Purpose
Myrothecium verrucaria	Organism of present investigations on morphogenesis and biocontrol enzyme production
Sclerotium rolfsii, Fusarium oxysporum, Capnodium sp.	Plant pathogens
Benjaminiella poitrasii	Dimorphic fungus
Metarhizium anisopliae, *Beauveria bassiana*, *Nomurea rileyi*	Entomopathogenic fungi
Bacillus subtilis	Antagonist

2.2.1 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	Potato, 200; glucose, 20; agar, 20; pH, 5.5-6.0.
Sabouraud malt yeast extract peptone medium (SMYP)	Sabouraud malt extract, 3.0; yeast extract, 3.0; peptone, 5.0; glucose, 10.0; agar, 20.0; pH, 5.5-6.0.
Yeast extract peptone glucose medium (YPG)	Yeast extract, 3.0; peptone, 5.0; glucose, 10.0; agar, 20.0; 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/l): $FeSO_4$, 5.0; $MnSO_4$, 1.56; $ZnSO_4$, 3.34; $CoCl_2.2H_2O$, 2.0; pH, 6.0.
Basal 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; chitin, 5.0; trace metal solution, 1ml of (mg/l): $FeSO_4$, 5.0; $MnSO_4$, 1.56; $ZnSO_4$, 3.34; $CoCl_2.2H_2O$, 2.0; pH, 6.0.

Name	Constituents (g/l)
Medium for yeast form growth	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.
Medium for hyphal form growth	Sucrose, 20.0; $NaNO_3$, 6.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CaCl_2$, 0.5; pH, 6.0.
Czapek Dox medium	Sucrose, 40.0; $NaNO_3$, 2.0; $MgSO_4$, 0.5; $FeSO_4$, 0.01; K_2HPO_4, 1.0; agar, 20.0; pH, 5.0-5.5.
Minimal medium	Glucose, 5.0; $NaNO_3$, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 1.0; agar, 20.0; pH, 5.5-6.0.
Maltose medium	$NaNO_3$, 2.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; maltose, 4.0; agar, 20.0; pH, 5.5-6.0.
Carrot agar	Carrot, 200; agar, 20; pH, 5.5-6.0.
Bean pod extract agar	French beans, 50; agar, 20.0; pH, 5.5-6.0.
Soil agar	Soil, 20; agar, 20; pH, 6.5-7.0.
Nutrient agar	Beef extract, 3.0; NaCl, 5.0; peptone, 5.0; agar, 20.0; pH, 7.0.

2.2.2 Maintenance

M. verrucaria (MTCC 9151), *S. rolfsii* NCIM 1084, *F. oxysporum*, *M. anisopliae*, *B. bassiana* and *Capnodium* sp. were maintained on PDA slants. *N. rileyi* was maintained on SMYP agar. *B. poitrasii*, was maintained on YPG (containing Difco yeast extract, and peptone). *B. subtilis* was maintained on NA. The slants were incubated at 28°C and routinely subcultured after 15 d. The stock cultures were maintained at 4°C until used.

2.2.3 Growth of *M. verrucaria* on different media

Morphological and cultural characteristics of *M. verrucaria* were studied to identify a medium supporting good vegetative growth and sporulation of *M. verrucaria*.

The agar blocks (2 mm^2) with mycelial growth of *M. verrucaria* were inoculated in the center of the plates containing various complex solid media (potato dextrose agar, bean pod extract agar, carrot agar, soil agar, yeast extract peptone glucose medium and chitin medium) and synthetic solid media (Czapek Dox medium, Maltose medium and media for Y-form and H-form growth of *M. verrucaria*). The plates were incubated at 28°C for 7 d. The plates were then observed for colony characteristics and sporulation.

2.3 Hypha-yeast transition in *M. verrucaria*

2.3.1 Morphological triggers

M. verrucaria hyphal cells (48 h old) grown in a medium for H-form growth, pH 6.0, were used as inoculum for H-Y transition studies. While yeast inoculum was developed in a medium for Y-form growth, pH 4.0 (Table 2.3). To identify the morphological triggers for H-Y and reverse transition in *M. verrucaria*, effect of carbon and nitrogen sources and medium pH on the morphological outcome was studied. The more details of the media and incubation conditions used for this study are discussed appropriately under results (Chapter 3). Aliquots from liquid cultures were examined microscopically and % of yeast-like and hyphal cells per microscopic field were recorded.

2.3.2 Spore germination under yeast- and hypha-favouring conditions

M. verrucaria spore germination was studied in specific form supporting media. The spore germination was monitored on solid as well as in liquid media under shaking conditions (200 rpm) at 28°C. The liquid cultures were incubated on a rotary shaker (200 rpm). The growth was observed microscopically after every 3 h.

2.4 Aerial conidia production using solid-state fermentation

The attempts were made to produce *M. verrucaria* conidia using various solid substrates like grains (rice, beaten rice, sorghum, pearl millet, wheat), fishery waste (prawn shell waste) and agricultural waste (wheat bran, rice bran). The solid-state fermentation was carried out in a 500 ml capacity Erlenmeyer flask. *M. verrucaria* hyphal cells (48 h old) grown in YPG broth were used as an inocululm. After 14 d incubation, 0.01% Tween 80 (100 ml) was added to the solid substrate (50 g) and mixed thoroughly by keeping on a rotary shaker (200 rpm) for 30 min. Total number of conidia in the supernatant was quantified using Neubaur's chamber. While the total viable count was measured by serial dilution method. The soluble protein produced by *M. verrucaria* was measured as the indicator of its growth on various solid substrates according to Zheng and Shetty (1998).

2.5 Cuticle degrading/mycolytic enzyme complex of *M. verrucaria*

2.5.1 Shake flask studies

For the production of CDE/ME complex comprising of chitinase, β-1,3-glucanase, protease and lipase, *M. verrucaria* was grown in a medium containing either chitin (0.5 %) or autoclaved mycelium of *S. rolfsii* (5.0% wet wt) under shaking conditions (200 rpm), at 28°C, for 7 d as described earlier (Vyas and Deshpande, 1989).

2.5.2 Fermenter studies

Batch fermentations were carried out for CDE/ME complex production in 15 l capacity locally made stirred tank fermenter with the probes to monitor temperature, dissolved oxygen and pH. Attempts were made to optimize agitation and aeration conditions for the production of CDE/ME complex.

2.5.3 Concentration of cuticle degrading/mycolytic enzyme complex

The cell free broth of *M. verrucaria* grown in a chitin medium was used as the source of CDE/ME complex. Attempts were made to concentrate cell free broth by ultrafiltration using hollow fiber membrane of 10 kDa molecular weight cut off. For further concentration, two methods were tried, spray drying using Buchi 190 Mini Spray Dryer and freeze drying using DW6 Freeze dryer (Heto Lab Equipment, Denmark). The powder of CDE/ME complex obtained was stored at -20°C until used.

2.6 Plate assays to study antagonistic interaction

2.6.1 Dual culture plate assay

To study the interaction between *M. verrucaria* and plant pathogenic root infecting fungus, *S. rolfsii*, dual culture plate assay was carried out. The agar block from an actively growing colony of *S. rolfsii* was inoculated on minimal medium or a chitin agar plates. Simultaneously, agar block from *M. verrucaria* colony was inoculated at a distance of 3 cm. The plates were incubated at 28°C for 4 d. The plates where boundaries of two colonies meet were observed under light microscope, to observe hypha-hypha interaction. While % inhibition of mycelial growth of a pathogen was calculated when zone of inhibition was observed. The inhibition of the mycelial growth of fungal pathogen was calculated according to following formula,

$$\% \text{ Inhibition of mycelial growth} = \frac{R1 - R2}{R1} \times 100$$

R1 = Radius of the pathogen colony away from the antagonist's colony.
R2 = Radius of the pathogen colony towards the antagonist's colony.

2.6.2 Viability of sclerotia

The method adopted by Prasad *et al* (1999) was followed to assess the antagonistic potential of CDE/ME complex. Sclerotia of *S. rolfsii* were placed on the filter papers soaked in different solutions viz, filter sterilized (using the Milipore membrane of 0.45 μ pore size) CDE/ME preparations containing 0.1-1.0 U/ml chitinase and Carzim, 0.4 and 0.8 % w/v (chemical control). Sterile distilled water served as a control. Each treatment was replicated thrice. These plates were incubated at 28°C for 4 d. The number of viable sclerotia was estimated on the basis of their ability to give rise mycelial growth. The results were expressed as a percentage reduction in viability of sclerotia over control.

2.6.3 Effect of cuticle degrading/mycolytic enzyme preparation on germination of conidia

The spores of following fungi, *M. anisopliae*, *B. bassiana* and *N. rileyi* were mixed with filter sterilized CDE/ME preparation containing 2 U/ml chitinase. These suspensions were spread on PDA plates immediately or after 2 h incubation at room temperature. Germination of *N. rileyi* conidia was observed on SMYP plates. The plates were incubated at 28°C and observed under light microscope at an interval of 6 h. 10 microscopic fields per plate were observed. The reduction in % germination of spores was calculated by comparing the control plates spreaded with spore suspensions prepared in 0.01% Tween 80. The effect of CDE/ME preparation (chitinase 1-6 U/ml) on the germination of spores of plant pathogenic fungus, *Capnodium* sp. was also studied.

2.6.4 Peanut germination trial

Peanut seeds were surface sterilized using 0.5% v/v sodium hypochlorite and then washed with sterile distilled water. These seeds were then soaked in *M. verrucaria* conidial suspension ($1x10^8$ conidia/ml) at room temperature for 30 min. Seeds were then placed on a moist filter paper in a sterile petri plate. Seeds in test plates were coated with suspension of *S. rolfsii* mycelium (250 µl). Control seeds were soaked in sterile distilled water. The plates were incubated at room temperature up to 7 d. Whenever required filter papers were moistened with sterile distilled water. The percentage of seed germination and the length of a radicle were recorded.

2.7 Sudan black B staining of yeast-like cells

The yeast-like and hyphal cells of *M. verrucaria* were stained with Sudan black B using method of Burdon (1946). The slides of various samples were heat fixed and then flooded with Sudan black B solution (3.0 g/l in 70% ethanol). After 5 min, slide was washed with xylene and counterstained with safranine (5.0 g/l aqueous solution). The samples were observed using light microscope (Leica, Labor Lux). Yeast and hyphal cells of *B. poitrasii*, a known dimorphic fungus and chlamydospores and hyphal cells of *F. oxysporum* were used for comparison.

2.8 Enzyme assays

2.8.1 Chitinase assay

The chitinase activity in the cell free extracts was estimated as described earlier using colloidal chitin as the substrate (Vyas and Deshpande, 1989). Colloidal chitin was prepared from crab shell chitin (Sigma) and Ostazin Brilliant Red labeled chitin (a kind gift from Andy Holtman, Cook College, NJ, U.S.A). Chitin 10 g, was suspended in chilled o-phosphoric acid (300 ml, 88% w/v) and left at 4-8°C with stirring for 2 h. The

coloured chitin was stirred overnight. The acid swollen chitin was repeatedly washed with chilled distilled water, followed with a 1% w/v $NaHCO_3$ and was further dialyzed against cold distilled water. After homogenization in a Waring blender (1 min), acetate buffer (50 mM, pH 5.0) was added to the suspension to make the final substrate concentration, 7.0 mg/ml.

The reaction mixture for chitinase assay contained: 1 ml of acid swollen chitin, 1 ml of acetate buffer (50 mM, pH 5.0) and 1 ml enzyme. The reaction mixture was incubated at 50°C for 1 h. The assay mixture with heat-inactivated enzyme (5 min in boiling water-bath) was used as a control. The dye labeled GlcNAc released from the acid swollen Red-chitin was estimated at 530 nm. Simultaneously, the GlcNAc released was estimated colorimetrically with p-dimethyl amino benzaldehyde (DMAB) according to Reissig *et al* (1955). The unit of enzyme activity was defined as the activity that produced 1 μmole of GlcNAc per min.

2.8.2 *In situ* estimation of endochitinase and *N*-acetylglucosaminidase

The chitinolytic activities in the vegetative cells were estimated according to Ghormade *et al* (2000). The endochitinase and *N*-acetylglucosaminidase assays were performed using fluorogenic substrates, 4-methylumbelliferyl-β-D-*N*-*N*'-*N*"triacetyl chitotrioside [4-MU-$(GlcNAc)_3$] and 4-methylumbelliferyl-β-D-*N*-acetyl-glucosaminide (4-MU-GlcNAc), respectively prepared in 50% v/v ethanol. The reaction mixture contained 5 mg (wet wt) of whole cells suspended in 300 μl of sodium acetate buffer (50 mM, pH 5.0) and 20 μl of 700 μM fluorogenic substrate. After incubation at 37°C for 30 min the reaction was terminated by the addition of 2.2 ml 0.2 M Na_2CO_3. After centrifugation at 2000 g for 10 min at 4°C, the supernatant was collected and the fluorescence was measured in a Perkin-Elmer Fluorescence spectrophotometer, using

excitation at 360 nm and emission at 445 nm. One unit of enzyme activity was defined as the activity that produced 1 nmole of 4-methylumbelliferone per mg wet wt of whole cells per min.

2.8.3 Chitosanase assay

The chitosanase activity was estimated using acid swollen chitosan as the substrate. The assay mixture contained 1 ml acid swollen chitosan (7 mg/ml), 1 ml acetate buffer (50 mM, pH 5.0) and 1ml enzyme that was incubated at 50°C for 1 h. The glucosamine liberated was estimated using the modified Morgan-Elson method described by Good and Bessman (1964). One international unit of enzyme activity was defined as the activity that produced 1 μmole of glucosamine per min.

2.8.4 Protease assay

Protease activity was measured using Hammerstein casein as a substrate (Vyas and Deshpande, 1989). The reaction mixture, 2 ml, contained an aliquot of a suitably diluted enzyme solution, 10 mg Hammerstein casein and sodium carbonate buffer (0.2 mM, pH 9.7). Enzyme reaction was carried out at 35°C for 20 min and terminated by the addition of 3 ml trichloroacetic acid (TCA) (2.6 ml 5.0% TCA + 0.4 ml 3.3 N HCl). The absorbance of the TCA soluble fraction was measured at 280 nm. One international unit was defined as enzyme activity that produced 1 μmole of tyrosine per min.

2.8.5 Lipase assay

Lipase activity was determined as described by Pignede *et al*, (2000). The substrate emulsion was prepared with olive oil, 50 ml and gum arabic, 50 ml (10% w/v). The reaction mixture contained 1 ml enzyme, 5 ml substrate emulsion and 2 ml phosphate buffer (50 mM, pH 6.8) and was incubated for 1 h at 37°C with shaking. 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 acid released with 50 mM NaOH. One international unit was defined as enzyme activity that produced 1 μmole of fatty acid per min.

2.8.6 β-1,3-Glucanase assay

The extracellular, β-1,3-glucanase was estimated using laminarin as a substrate (Vyas and Deshpande, 1989). An aliquot of 0.5 ml enzyme solution was mixed with 0.5 ml of 1% laminarin prepared in the acetate buffer (50 mM, pH 5.0) and incubated at 50°C 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.

2.8.7 Mannanase assay

The mannanase activity was estimated using yeast mannan as a substrate (Mendosa *et al*, 1996). The 0.5 ml of enzyme sample was mixed with 0.5 ml yeast mannan (1%) prepared in a potassium phosphate buffer (200 mM, pH 5.8) and was incubated at 50°C for 1 h. Reducing sugars were estimated as mannose equivalents as described by Somogyi (1952). One international unit was defined as the enzyme activity that liberated 1 μmole of mannose per min.

2.8.8 Amylase assay

The amylase activity was measured using soluble starch as a substrate as described by Bernfeld (1951).

2.8.9 Cellulase assay

The cellulase activity was estimated using carboxymethyl cellulose as a substrate as described earlier by Sadana *et al* (1979).

2.8.10 NAD(P)-glutamate dehydrogenase assay

To measure the NAD(P)-GDH activities in hyphal and yeast-like cells of *M. verrucaria*, cell extracts were prepared by the method of Ferguson and Sims (1971). The hyphal-/yeast-like cells were collected on Whatmann filter paper No. 1, washed with chilled distilled water followed by potassium phosphate buffer (5 mM, pH 7.2) containing 0.25 mM EDTA and 50 mM K_2SO_4 and homogenized in a Braun's homogeniser for six cycles of 15 sec each for yeast cells and 4 cycles for hyphal cells. The homogenate was then centrifuged at 12500 g for 15 min and the supernatant was used for the determination of NAD(P)-GDH activity.

NAD(P)-GDH was measured by the method of Khale *et al* (1992). The reductive amination of α-ketoglutarate was measured by monitoring the decrease in the A_{340} of NAD(P)H. The reaction mixture (1 ml) contained 50-100 µl of crude extract, 200 mM NH_4Cl and 30 mM α-ketoglutarate in a potassium phosphate buffer (100 mM, pH 8.0). The reaction was initiated by the addition of 0.125 mM NADH or NADPH to the sample cuvette. A control in which α-ketoglutarate was omitted from the reaction mixture was run for each assay. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µmole of NAD(P)H per min.

2.8.11 Nitrate reductase assay

The nitrate reductase activity in the extracts of hyphal and yeast-like cells of *M. verrucaria* was measured using the method described by Redinbaugh and Campbell (1985). The reaction mixture (2 ml) contained: 1.8 ml of potassium phosphate buffer

(25 mM, pH 7.3) with 10 mM potassium nitrate and 0.05 mM EDTA, 0.1 ml 2.0 mM NADH and 0.1 ml enzyme sample. The reaction mixture was incubated at 30°C for 2 min. The reaction was then stopped by adding 58 mM sulfanilamide solution prepared in 3 M hydrochloric acid. The 0.77 mM *N*-(1-Naphthyl) ethylenediamine dihydrochloride solution (1 ml) was then added for the colour development. The tubes were then incubated at 25°C for 10 min. The absorbance was read at 540 nm. One unit of nitrate reductase activity was defined as the amount of enzyme that reduces 1 μmole of nitrate to nitrite per min in NADH system at pH 7.3 at 30°C.

2.8.12 Mandelonitrile lyase assay

Mandelonitrile lyase assay was performed by monitoring the decomposition of (R, S)-mandelonitrile (Kuroki and Conn, 1989). The amount of benzaldehyde produced was measured by recording the absorbance at 249.6 nm. Standard reaction mixtures contained Mes.KOH buffer (100 nM, pH 5.5), 395 μM (R, S)-mandelonitrile and make up the volume to 1 ml by adding suitably diluted enzyme. Reaction was initiated by adding enzyme and the increase in absorbance was then followed. Since, (R, S)-mandelonitrile also decomposes slowly at this pH in the absence of enzyme, the non enzymic rate was determined for each assay and subtracted from the total rate to obtain the true enzymic rate. One unit of mandelonitrile lyase activity was defined as the amount of enzyme that catalyses the production of 1 μmole of benzaldehyde per min.

2.9 Estimation of reducing sugars

The glucose equivalents were estimated by Somogyi (1952), GlcNAc and glucosamine were measured by Morgan-Elson method (Reissig *et al*, 1955) and modified Morgan-Elson method (Good and Bessman, 1964), respectively.

2.10 Protein estimation

Protein was estimated according to Lowry *et al* (1951) using bovine serum albumin as a standard. Protein staining after native polyacrylamide gel electrophoresis was done using silver nitrate (Blum *et al*, 1987).

2.11 Thin layer chromatography

Thin layer chromatography was used for the detection of GlcNAc produced during hydrolysis of colloidal chitin (Sigma) and colloidal Red chitin. The samples were kept in a boiling water bath for 5 min to precipitate out soluble proteins and were used after centrifugation. The solvent system used was n-butanol: pyridine: water (6:4:3 v/v/v). GlcNAc was visualised by spraying with a solution of 3% w/v p-anisidine in butanol.

2.12 Native polacrylamide gel electrophoresis and activity staining of NAD- and NADP- glutamate dehydrogenases

To detect whether different NAD- and NADP-dependent GDHs are expressed in *M. verrucaria* H and Y form cells, the activity staining was done according to the procedure of Laycock *et al* (1964). The intracellular proteins from 72 h old H and Y form cells of *M. verrucaria* were separated on a non-denaturing 7.5% (w/v) polyacrylamide gels (pH, 8.3) (Davis 1964). The staining mixture contained 100 mM L-glutamate, 0.06 mM phenazine methosulphate, 0.22 mM nitroblue tetrazolium and 0.026 mM NAD(P), potassium phosphate buffer (100 mM, pH 8.0). The gels were incubated in staining solution until blue coloured bands appeared. In order to eliminate possibility of an artifact caused due to alcohol dehydrogenase the staining was carried out in the absence of glutamate (substrate) (Kapoor and Grover, 1970). The gels were stored thereafter in a methanol: acetic acid: water (40:10:50 v/v/v) mixture.

2.13 18s rRNA gene sequence analysis

The genomic DNA was isolated from 72 h old mycelium of M. verrucaria grown in a synthetic medium containing sucrose and sodium nitrate, pH 6.0. DNA extraction protocol suggested by Wendland *et al* (1996) was used for DNA isolation. Nuclear 18s rRNA gene and ITS 1 sequences were amplified using primers NS1 and ITS1, respectively according to White *et al* (1990). DNA (50 ng) was used for PCR. For 50 µl of reaction, 2 mM dNTPs 5 µl, 10X buffer 5 µl and 0.5 U of Taq polymerase, were used. The reaction volume was made to 50 µl using sterile double distilled water. Following conditions were used for PCR, initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec, followed by 10 min extension at 72°C using MJ Research DNA Engine PTC-200. PCR product (5 µl) was mixed with 1U shrimp alkaline phosphatase (Amersham) and 1U exonuclease I and incubated at 37°C for 15 min and heat denaturated at 75°C for 15 min. This purified sample was used for sequencing using primers NS1 (for 18s rRNA gene) and ITS1 (for ITS1 region). Sequencing was done using Big Dye Terminator Kit (ABI-Perkin-Elmer, USA). The sequencing reactions were run on ABI-310 automated sequencer. The sequence data was analyzed using NCBI server (http//:www.ncbi.nlm.nih.gov/BLAST).

2.14 Immobilization of yeast-like cells in alginate beads

M. verrucaria yeast-like cells (72 h old) were separated from the culture broth using centrifugation. The yeast biomass (1 g wet wt) was mixed in sterile sodium alginate (2% w/v) solution or sodium alginate solution containing chitin powder (0.5% w/v). The mixture was then dropped through pipette having 2 mm orifice, into a sterile calcium chloride (1% w/v) solution. The beads thus produced

were harvested from the solution, rinsed in a sterile distilled water and spread in a single layer in trays lined with a filter paper. These beads were air dried in a laminar airflow for 1 h. The beads were stored in a sterile, airtight plastic vials, at room temperature or at 4°C. The viability of *M. verrucaria* in beads was quantified before beads formation, same day when beads were produced (0 d) and on every subsequent 7 d after beads formation. The beads were disintegrated in 0.01% Tween 80, which was taken as a suspension of yeast cells. The population of *M. verrucaria* was enumerated by a dilution plate method using PDA.

2.15 Formulation of conidia with different carriers

M. verrucaria conidia produced on sorghum grains were harvested using 0.01% v/v Tween 80. The conidial suspension was mixed with organic carriers like rice bran, wheat bran, prawn shell waste and conventionally used carriers like charcoal powder or precipitated silica powder. The rice bran and wheat bran supplemented with 2% chitin were also used as carriers. These conidial preparations contain $1x10^9$ conidia/g carrier. The preparations were stored in sealed plastic bags, at room temperature or at 4°C. The viability of conidia with different carriers was assessed at an interval of 15 d. The conidial preparations (10 g) were mixed with 100 ml of 0.01% Tween 80. The viability of *M. verrucaria* conidia in the suspension was quantified by a serial dilution method using PDA.

2.16 Biocontrol of fungal pathogens of plants (Pot experiments)

The root pathogen, *S. rolfsii* was grown in a YPG broth (100 ml), under shaking conditions, 200 rpm for 72 h, at 28°C. The soil:sand (2:1) mixture (6 kg) was sterilized by autoclaving and infested with the mycelial mass of *S. rolfsii*. Peanut seeds (2 Nos.) were sown per pot containing 200 g soil. Following soil treatments

(5 g/pot) were used to detect their efficacy to control *S. rolfsii* infection in peanut: rice bran formulations of *M. verrucaria* conidia with and without chitin and immobilized yeast-like cells of *M. verrucaria* with and without chitin. The results were compared with conventionally used chemical fungicide treatment, Carzim (0.8 % w/v). Untreated controls in sterile and *S. rolfsii* infested soil were also kept for comparison. The plants were irrigated with sterile distilled water. In another experiment, seeds were sown in *S. rolfsii* infested soil and were irrigated with CDE/ME preparation (chitinase, 5-20 U/pot) at an interval of 7 d for 2 weeks. Plants were allowed to grow up to 3 weeks. Percentage seed germination was recorded and plant vigour was assessed in terms of shoot and root length. To measure the chitinase activity secreted by different morphologies of *M. verrucaria* in soil, or the residual chitinase activity after soil irrigation with CDE/ME preparation, 100 g soil per pot was thoroughly mixed in 100 ml acetate buffer (50 mM, pH 5.0). The supernatant was used to measure the acid swollen chitin degrading activity in soil.

2.17 Insect bioassay

The initial culture of *Helicoverpa armigera* was established by collecting larval and pupa stages from the chickpea fields. The rearing of the larvae was done individually in plastic vials on vegetable diet (Okra) disinfected for 10 min with 0.5% sodium hypochlorite as described by Ignoffo *et al*, (1975). The larvae of *Spodoptera litura* were collected from castor plants and were reared on castor leaves in a plastic tub. *Plutella xylostella* larvae were collected from infested cauliflowers before use in bioassay. The sugarcane leaves infested with nymphs and adults of *Ceratovacuna lanigera* were collected from sugarcane fields and used for the bioassay. The temperature and relative humidity in the insect rearing room was maintained at 25 ± 2 °C and 65 ± 5%, respectively.

For bioassay, conidial suspensions ($1x10^5$ and $1x10^7$ conidia/ml) of fungal isolates, *M. verrucaria*, *M. anisopliae*, *B. bassiana* and *N. rileyi* were prepared in sterile 0.01% Tween 80 solution. CDE/ME preparation of *M. verrucaria* containing chitinase 2-6 U/ml was used singly or in combination with the conidial suspensions of fungal isolates. Susceptibility of larvae of *H. armigera*, *S. litura* and *P. xylostella* was evaluated by dipping the larvae in conidial suspensions and/or CDE/ME preparation for 5 sec. Three replicates of 20 larvae were used in each experiment. As control 3 sets of 20 larvae were treated with sterile distilled water and 3 sets of larvae were treated with heat inactivated CDE/ME preparation. After treatment, each larva was kept in a separate plastic vial containing moist Whatman No. 1 paper and allowed to feed on diet, okra piece, castor leaf and cauliflower piece for larvae of *H. armigera*, *S. litura* and *P. xylostella*, respectively. The vials were kept at 25 ± 2 °C and 65 ± 5% RH. The mortality was recorded upto 14 days. The dead larvae receiving treatment of conidial suspensions were placed in a sterile petriplate containing moist cotton swab to allow mycelial growth over the cadaver for 3 days. Mortality was corrected by Abott's (1925) formula.

The sugarcane leaves infested with *C. lanigera* were cut into pieces (3 cm^2) and placed on moist filter paper in sterile petriplate. The effect of *M. verrucaria* conidial suspension and CDE/ME complex on nymphs and adults of *C. lanigera* was assessed by spray method. These suspensions (2 ml/piece of sugarcane leaf) were sprayed using hand compression spray. The plates were incubated at 25 ± 2 °C and 65 ± 5% RH. The mortality was recorded upto 14 days. The dead numphs/adults receiving treatment of conidial suspensions were placed in a sterile petriplate containing moist cotton swab to allow mycelial growth over the cadaver for 3 days. Mortality was corrected by Abott's (1925) formula. The wooly mass was separated from the adult aphids and was incubated with CDE/ME complex (2 U/ml lipase) of

M. verrucaria for 5 h. These samples were observed under light microscope to assess its hydrolysis.

2.18 Field trial

Field trial was conducted at College of Agriculture, Pune-5 during *Rabi*, 2001-2002, to evaluate the efficacy of CDE/ME preparation of *M. verrucaria* to control *H. armigera* infestation in chickpea. The chickpea plot size was 5 x 5 m^2. The normal agronomy practices except plant protection measures were carried out. The CDE/ ME preparation 500 l/ha ($3x10^6$ U chitinase), endosulfan (0.2% v/v) 500 l/ha, *Ha*NPV, 250 l/ha and *M. anisopliae* (10^7 conidia/ml) 500 l/ha were sprayed with hand compression knapsack sprayer. The treatments were given in randomized block design with four replications. Five plants per plot were selected randomly and tagged for recording observations. Treatments were initiated when infestation was 3-4 larvae per plant. Counts for live larvae were recorded a day before treatment and third, fifth, eighth, eleventh, and fifteenth day after treatment. Field studies were evaluated on the basis of percent efficacy (Henderson and Tilton, 1955), percent pod damage and percent yield, which were determined according to Hassani (2000).

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Chapter 3

Morphological, cultural, biochemical and molecular characterization of *M. verrucaria*

3.1 Introduction

Myrothecium verrucaria is a common saprophytic fungus with a cosmopolitan distribution. It has been found on various plant material, cellulosic waste and soil (Domsch *et al*, 1980). *M. verrucaria* is known for the extracellular production of the hydrolytic enzymes like chitinase, β-1,3 glucanase, lipase and protease involved in degradation of fungal cell wall and insect cuticle, when grown in a medium containing chitin as a sole source of carbon (Vyas and Deshpande, 1989). It was reported that the mycolytic enzyme complex (ME) effectively degraded mycelial biomass of root pathogens like *Fusarium oxysporum* and *Sclerotium rolfsii.* Daily soil irrigation with mycolytic enzyme preparation of *M. verrucaria* was found to control the *S. rolfsii* infection in peanut (Patil *et al* 2001). *M. verrucaria* purified endochitinase was also found to be effective in killing the first and fourth instar larvae of a mosquito, *Aedes aegypti*, a vector of yellow fever and dengue, within 48h (Mendosa *et al*, 1996). Thus, *M. verrucaria* was selected to develop a mycopesticide formulation, which will be effective against both the fungal pathogens of plants and insect pests.

M. verrucaria isolate MTCC 9151, known to produce appreciable amounts of cuticle degrading and mycolytic enzymes, was used in the present studies. The morphological and cultural characteristics of this isolate have been described in the present chapter. The formation of unicellular yeast-like cells in *M. verrucaria* was observed for the first time. The attempts were made to identify the triggers for hypha to yeast (H-Y) and reversible transitions and to optimize conditions for the production of yeast-like cells under submerged fermentation conditions.

In our laboratory extensive work has been carried out to find biochemical correlation between NAD- and NADP-dependent glutamate dehydrogenases (NAD- and NADP-GDH) and yeast-hypha (Y-H) transition in *B. poitrasii*. The significance

of the relative proportion of both NAD- and NADP-GDH, measured as NADP-/NAD- GDH ratio in the yeast-hypha transition was reported by Khale *et al* (1992). Furthermore, Khale-Kumar and Deshpande (1993) described the involvement of cAMP in the regulation of NADP-/NAD-GDH ratio and in dimorphic transition of *B. poitrasii*. Amin *et al* (2004) reported the presence of one NAD-GDH in both yeast and mycelial forms of *B. poitrasii* and two active NADP-GDHs one expressed in hyphal form and the other one in yeast form. Recently, Ghormade *et al* (2005a) reported the presence of active yeast form specific NADP-GDH and high NADP-/ NAD-GDH ratio in the sporangiospores and zygospores which result in a dimorphic commitment of the organism (*B. poitrasii*) during the entire life-cycle rather than restricting it to a particular phase. While Ghormade *et al* (2005b) demonstrated the involvement of polyamines, which are known to regulate cell differentiation in the regulation of NADP-GDH activities in *B. poitrasii*. Ghormade *et al* (2000) also emphasized the significance of chitinolytic activities in a mixed membrane fractions of yeast/hyphal cells of *B. poitrasii* in the morphological transition. Thus, in present studies attempts were made to study differential expressions of ammonia assimilating enzymes (NAD- and NADP-dependent glutamate dehydrogenases) and chitinolytic enzymes (endochitinase and *N*-acetylglucosaminidase) in hyphal and yeast-like cells of *M. verrucaria*.

Lastly, the18s rRNA gene sequence analysis was carried out to identify the strain specific marker for *M. verrucaria* MTCC 9151.

3.2 Results and discussion

3.2.1 Morphological and cultural characteristics of *M. verrucaria*

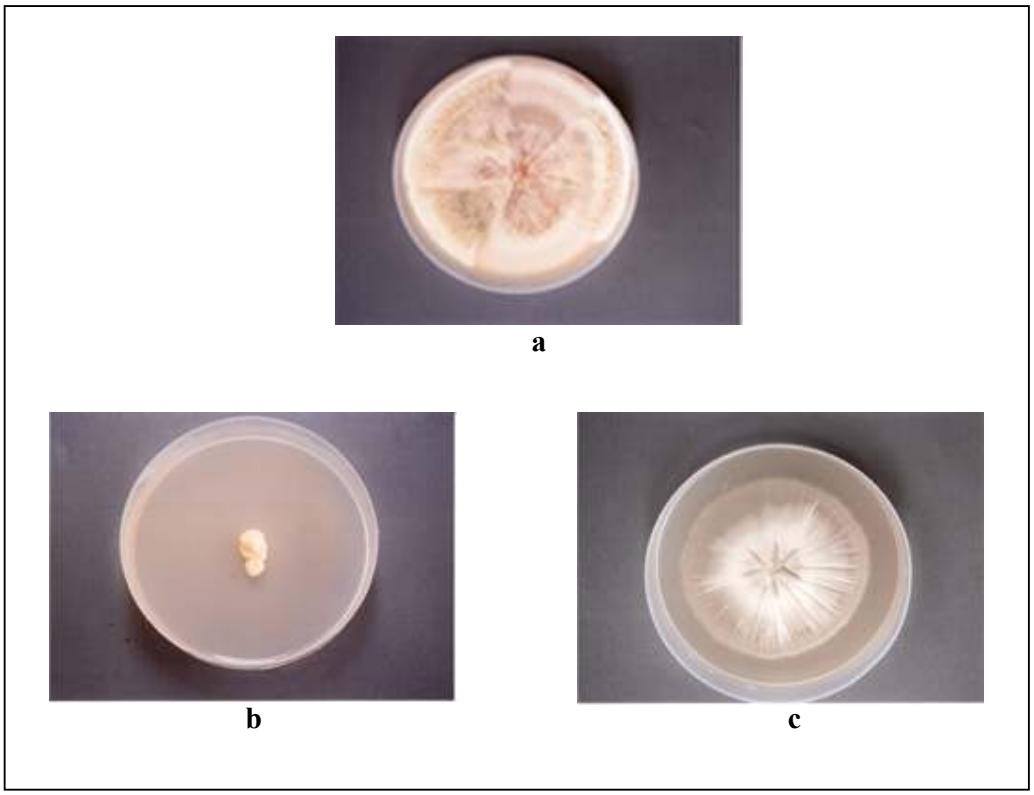
M. verrucaria has been classified under Class: Deuteromycetes, Order: Moniliales, Family: Moniliaceae (Barnet and Hunter, 1987). *M. verrucaria* was

reported to produce white mycelium bearing diffuse or coalescent greenish sporodochia. The conidiophores were repeatedly branched bearing conidia terminally. The conidia were subhyaline to coloured, one celled, ovoid to elongate. Morphological and cultural characteristics of *M. verrucaria* MTCC 9151 were studied to identify a medium supporting good vegetative growth and sporulation. Agar blocks (2 mm^2) with mycelial growth of *M. verrucaria* were inoculated in the center of the plates containing following media: potato dextrose agar, bean pod extract agar, carrot agar, soil agar, YPG, Czapek Dox agar, Maltose agar, Chitin medium, medium for yeast form growth and medium for hyphal form growth. The plates were incubated at 28°C for 7 d.

All the media used in present study except medium for Y form growth, supported mycelial growth of *M. verrucaria*. The luxuriant mycelial growth as well as maximum sporulation of *M. verrucaria* was observed on potato dextrose agar (PDA), bean pod extract agar, carrot agar and maltose containing medium. Dense aerial mycelium and sporulation in sectors was observed on PDA (Fig. 3.1) and carrot agar while that on bean pod extract agar and maltose containing medium was in concentric rings. Good sporulation of *M. verrucaria* on maltose containing media and also on pea, bean and flax seed was reported by Domsch *et al* (1980). Very less mycelial growth and sporulation of *M. verrucaria* was observed on Czapek Dox agar and soil agar. While *M. verrucaria* sporulation was not observed even after 14 d incubation on chitin medium and YPG. The potato dextrose agar slants were used for the routine maintenance of *M. verrucaria*.

On a sucrose containing medium with ammonium sulphate as a nitrogen source, pH 4.0 (medium for yeast form growth) unicellular yeast-like growth of *M. verrucaria* was observed for the first time. Cream coloured, mucoid colony with grainy

Fig. 3.1 Cultural characteristics of *M. verrucaria* on various media



* *M. verrucaria* growth on a. PDA, b. medium for Y-form growth and c. medium for H-form growth at 28˚C after 7 d incubation.

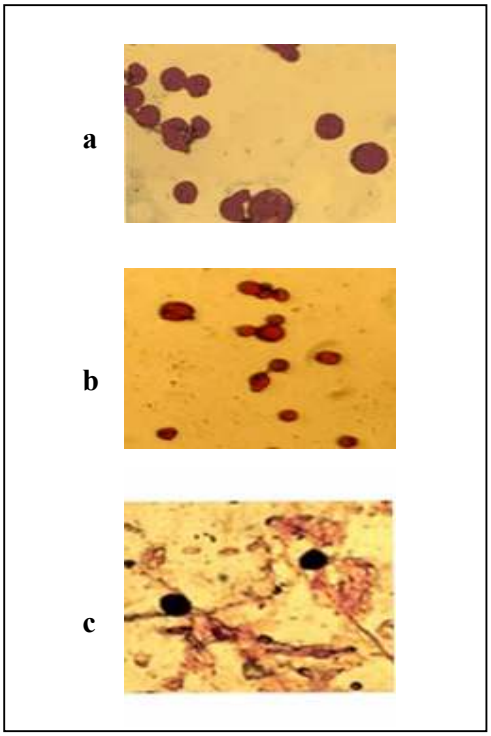
appearance was seen on solid medium for Y-form growth (Fig. 3.1). Earlier, unicellular growth of *M. verrucaria*, in the presence of cell wall growth inhibitors produced by *Vollutellospora* sp. was reported (Thirumalachar, 1956). The medium with sucrose and sodium nitrate as a nitrogen source, pH 6.0 (medium for hyphal form growth) supported filamentous growth of *M. verrucaria*. The mycelial growth in concentric rings was observed on this medium. As compared to the growth on PDA, less aerial mycelium and sporulation was observed on medium for hyphal form growth after incubation at 28˚C for 7 d.

3.2.2 Morphological transition in *M. verrucaria*

3.2.2.1 Nature of unicellular growth

The hyphal cells (48 h old) of *M. verrucaria* when inoculated in a medium for Y-form growth, exhibited morphological transition to a unicellular growth. These cells were produced by interseptal swelling of hyphae leading to a question whether these were vegetatively growing yeast-like cells or chlamydospore-like resting structures. Cole *et al* (1991) faced a similar problem in identifying the nature of round cells of *Candida albicans* produced in gastric mucosa of immuno-compromised mice. *C. albicans* produced oval yeast-like cells under *in vivo* as well as *in vitro* conditions while round chlamydospores under *in vitro* conditions. To identify the nature of round cells in gastric mucosa of mice, they were stained with sudan black B. The chlamydospores and round cells showed darkly stained cytoplasm containing abundant lipid droplets while actively metabolizing yeast cells of *C. albicans* which do not contain storage lipids, were stained with counter stain, eosin. Hyphal and yeast form cells of *M. verrucaria* remained unstained with sudan black B which can be attributed to the absence of storage lipids. Fig. 3.2 reveals the yeast-like cells of *M. verrucaria* and the yeast cells of

Fig. 3.2 Sudan black B staining of yeast cells and chlamydospores



* a. yeast-like cells of *M. verrucaria*, b. yeast cells of *B. poitrasii*, c. chlamydospores of *F. oxysporum*

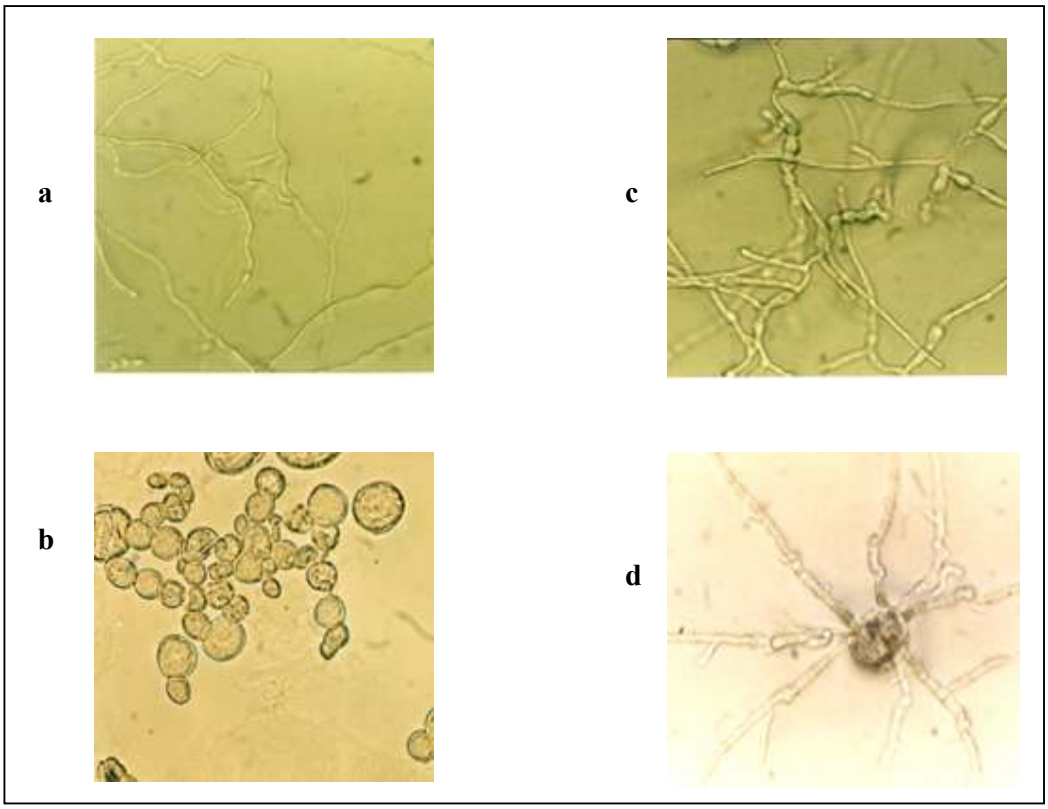
B. poitrasii, a dimorphic fungus used for comparison stained with counter stain eosin while the chlamydospores of *F. oxysporum* containing storage lipids were stained black with sudan black B. The present studies thus revealed that the unicellular form of *M. verrucaria* resemble the actively metabolizing yeast cells rather than the resting chlamydospores.

3.2.2.2 H-Y reversible transition

The hypha-yeast (H-Y) transition in *M. verrucaria* was studied in a medium for Y-form growth, pH 4.0. The flasks were incubated under shaking conditions (200 rpm) at 28°C for 72 h. The different stages of H-Y transition are depicted in Fig. 3.3. For the first 24 h, the hyphal cells appeared to be swollen (Fig. 3.3b) and eventually these changed into unicellular yeast-like cells, which further proliferated by budding (Fig. 3.3c). The yeast-like cells of *M. verrucaria* were round with a diameter of 23 ± 3 μ. The H-Y transition with the development of intermediate transitional forms appearing as chains of yeast like cells was observed in *Histoplasma capsulatum* and *Paracoccidiodes brasiliensis* (Maresca and Kobayashi, 1989; San-Blas and San-Blas, 1985).

The morphological transition in *M. verrucaria* was found to be freely reversible. As seen in Fig. 3.3d the yeast-like cells produced germ tubes within 6-8 h when inoculated in a medium for H-form growth, pH 6.0. Hyphal cells became predominant within 24 h of incubation under shaking conditions (200 rpm) at 28°C. The Y-H transition was a rapid process as compared to H-Y transition in *M. verrucaria*.

Fig. 3.3 H-Y reversible transition in *M. verrucaria*



* a. hyphal cells (0 h), b. interseptal swellings in hyphal cells (24 h), c. budding yeast-like cells (72 h), d. germ tube forming yeast-like cells (8 h)

3.2.2.3 Morphological triggers

The reversible morphological transition between the two vegetative growth forms (filamentous hyphal form and unicellular yeast form) in response to the environmental perturbations is exhibited by many fungi. Some of the important morphological triggers are carbon source, nitrogen source, pH of the medium, temperature, serum, etc. (Ruiz-Herrera and Sentandreu, 2002). The effect of different carbon sources on the formation of yeast-like cells of *M. verrucaria* was studied in the medium containing ammonium sulphate, pH 4.0. All the mono- and di-saccharides used in the present investigations supported the formation of yeast-like cells (Table 3.1). Sucrose (2%) was found to be the most suitable carbon source for the production of yeast-like cells within 72 h of incubation.

Interestingly, the production of yeast-like cells of *M. verrucaria* in the presence of mono- and di-saccharides as carbon source was found to be associated with decrease in pH from 4.0 to 3.2 ± 0.3 (Table 3.1). While in the presence of chitin, H-Y transition in *M. verrucaria* was retarded with concomitant rise in pH from 4.0 to 5.9.

Table 3.1 Effect of carbon source on H-Y transition in *M. verrucaria*

Carbon source	Concentration (% w/v)	Morphology (72 h)	pH (72 h)
N-acetyl Glucosamine	0.5	Y	3.2
Glucose	2.0	Y	3.0
Sucrose	1.0	H>Y	3.5
Sucrose	2.0	Y	2.9
Sucrose	3.0	Y	2.9
Sucrose	5.0	Y	2.8
Lactose	2.0	Y	3.1
Maltose	2.0	Y	3.2
Chitin	0.5	H	5.9

*Y: yeast-like cells, H: hyphae
H>Y: more than 50% of the cells are in hyphal form
ND: not detected

It has been reported that hexoses at high concentration favour Y form growth in *B. poitrasii* (Khale *et al*, 1990) while galactose was found to trigger unicellular growth in *Penicillium marneffei* (Wong *et al*, 2001). In case of *C. albicans* (Sullivan *et al*, 1984) and *B. poitrasii* (Ghormade *et al*, 2000) *N*-acetylglucosamine (GlcNAc) favoured hyphal form. However, in the case of *M. verrucaria* GlcNAc supported yeast form growth. It can be attributed to the final pH 3.2 after 72 h. Only a few studies highlight the importance of change in pH when using particular carbon source on morphological outcome. Barathova *et al* (1977) reported that as compared with changes of pH occurring on certain nitrogen sources together with H-Y transition in *Paecilomyces viridis*, changes of pH of media with various carbon sources like glucose, sucrose, maltose, lactose, etc. did not influence the morphology. In case of *Yarrowia lipolytica*, growth in hyphal form was observed in the presence of glucose as a carbon source. However, the use of citrate or phosphate buffer to avoid acidification of this medium was suggested to play an important role in deciding the morphological outcome (Ruiz-Herrera and Sentandreu, 2002).

The effect of nitrogen source on the H-Y transition in *M. verrucaria* was studied in a medium for Y form growth, pH 4.0, supplied with different inorganic and organic nitrogen sources added to it on equal nitrogen basis (nitrogen 0.98 g/l). In *M. verrucaria*, Y form growth was induced in the presence of ammonium salts e.g.-ammonium sulphate, ammonium hydrogen phosphate or ammonium chloride as a nitrogen source, associated with the decrease in pH from 4.0 to 3.1 ± 0.3 (Table 3.2). While in the presence of sodium nitrate or potassium nitrate, hyphal form was predominant and rise in pH (up to 4.9-5.0) was observed within 72 h of incubation. These results also emphasized the importance of acidification of the medium as a result of uptake of ammonium ions as nitrogen source in triggering the H-Y transition in *M. verrucaria*. Barathova *et al* (1977) reported similar observations for *P. viridis*. Nitrogen

source was also found an important morphological trigger in *Ustilago maydis* (Ruiz-Herrera *et al*, 1995). However, in *U. maydis*, medium acidification (pH 3.0 to 2.54) observed in the presence of ammonium sulphate or ammonium nitrate as a nitrogen source favoured hyphal form while rise in medium pH from pH 3.0 to 6.0 was found associated with the uptake of potassium nitrate as a nitrogen source and triggered yeast form growth. The acidification of the medium in the presence of ammonium salts as

Table 3.2 Effect of nitrogen source on H-Y transition in *M. verrucaria*

Nitrogen source	Morphology (72 h)	pH (72 h)
Ammonium sulphate	Y	2.9
Ammonium chloride	Y	3.2
Ammonium hydrogen phosphate	Y	3.4
Ammonium nitrate	Y>H	4.3
Sodium nitrate	H	4.9
Potassium nitrate	H	5.0
Urea	H	6.0
Peptone	H>Y	4.0

*Y: yeast-like cells, H: hyphae, Y>H: more than 50% of the cells are in yeast form
H>Y: more than 50% of the cells are in hyphal form

a nitrogen source was attributed to the uptake of ammonium ions (NH_4^+) mediated by H^+-antiport transport systems at expenses of ATP (Dubios and Grenson, 1979; Ruiz-Herrera *et al*, 1995). While rise in pH of the medium in the presence of nitrate as nitrogen source was ascribed to the uptake of nitrate ions (NO_3^-) by the proton symport (Ruiz-Herrera *et al*, 1995).

Ammonium nitrate was included in the present study as a N source to find out which form of nitrogen (the ammonium ion or nitrate ion) was preferentially taken up and did this uptake of nitrogen affect morphological outcome. As seen for the ammonium sulphate addition as a nitrogen source, ammonium nitrate favoured H-Y transition up to 72 h. This could be attributed to the decrease in pH. On further

incubation hyphal form was predominant with concomitant increase in pH. The initial decrease of pH could be due to the utilization of ammonium ions while further growth was predominantly due to the utilization of NO_3^- nitrogen. In other words, the trigger for morphological change could be correlated with the pH change.

The results clearly indicate that ammonium ions were preferentially taken up as a nitrogen source in the presence of nitrate ions. The suppression of nitrate assimilation in the presence of ammonia is common to many fungi and is believed to have an adaptive significance in natural habitats. Ammonia, even in a very low concentration, was reported to completely suppress nitrate assimilation by *Scopularis brevicaulis* when both the sources of nitrogen were present together (Mortan and Macmillan, 1954). A rapid decay of nitrate uptake and nitrate reductase activity of *Penicillium chrysogenum* was observed in the presence of ammonium ions (Goldsmith *et al*, 1976). The other nitrogen sources have also been shown to have influence on the morphology of the fungus. Gagiano *et al* (2002) reported the differentiation of yeast cells of *S. cerevisiae* in pseudohyphal invasive growth in response to the limitation of a nitrogen source in particular ammonia, and the availability of proline. Similarly, nitrogen deprivation has been suggested to stimulate filamentation in *C. albicans* (Brown and Gow, 1999). In the case of *M. verrucaria* it would be interesting to study whether filament formation in presence of nitrate as a nitrogen source is the response of the fungus to the presence of poor nitrogen source or it is the response towards the increase in pH of the medium associated with nitrate uptake.

The organic nitrogen sources like peptone were reported to favour Y form growth in *B. poitrasii* as compared to an inorganic nitrogen sources such as ammonium hydrogen phosphate (Khale *et al*, 1990). While organic sources of nitrogen, amino acids and peptone were strong inducers of hyphal growth in *Y. lipolytica* (Szabo and Stofanikova, 2002). In case of *M. verrucaria* the morphological outcome in the

presence of organic nitrogen sources (urea and peptone) was found to be correlated with the change in pH associated with the uptake of nitrogen source (Table 3.2). In the presence of urea, H-Y transition was completely retarded where rise in pH from 4.0 to 6.0 was observed during incubation. While in the presence of peptone change in pH was not observed, and transition towards Y form was favoured resulting in swollen hyphae (< 50%) after 72 h of incubation. Leija *et al* (1986) observed a correlation between the final pH of the medium and morphology of *M. rouxii* when different amino acids were used as the nitrogen source.

Changes in the pH of the medium were shown to induce a strong morphogenetic response in many dimorphic fungi such as *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962), *Mycotypha africana* (Hall and Kolankaya, 1974), *C. albicans* (Odds, 1985; Soll, 1985), *Aureobasidium pullulans* (Andrews *et al*, 1993) *Y. lipolytica* (Szabo, 1999) as well as in *B. poitrasii* (Ghormade and Deshpande, 2000). All these fungi grew in a yeast form in an acidic environment, whereas they exhibited transition to hyphal form upon increase of pH to near neutral. The effect of pH was opposite in the case of *U. maydis* (Martinez-Espinoza *et al*, 2004; Ruiz-Herrera *et al*, 1995). *U. maydis* grew in yeast like form at pH near neutral while hyphal form was induced at pH 3.0-4.0. In case of *Y. lipolytica* and *S. cerevisiae* the extracellular pH was suggested to govern the morphological outcome by affecting the rates and efficiency of transport of certain nitrogen sources across the membrane by changing the affinity of sensor components as well as activities of membrane transporters (Szabo and Stofanikova, 2002; Horak, 1997). While Dubios and Grenson (1979) reported that extracellular pH affected the uptake of ammonium salts by *S. cerevisiae*. At pH (< 7.5), ammonium ion (NH_4^+) formation was favoured and in *S. cerevisiae* ammonium uptake was mediated by an H^+-antiport transport systems at expenses of ATP resulting in medium acidification. While,

at higher pH the ammonia (NH_3) formed accumulated in growing cells essentially by diffusion.

The effect of initial pH of the medium on the morphology of *M. verrucaria* was studied in the presence of ammonium sulphate or sodium nitrate as nitrogen source. The H-Y transition was favoured in the presence of ammonium sulphate as a nitrogen source at initial pH values in the range of 3.0-7.0. The H-Y transition was found to be associated with the decrease in pH to 3.1 ± 0.3. While, H-Y transition was retarded at initial pH values 8.0-10.0 where decrease in pH was not sufficient to induce the unicellular growth (Table 3.3). From these results it can be suggested that in *M. verrucaria* the uptake of ammonium ions was regulated by the external pH similar to that observed in *S. cerevisiae* (Dubios and Grenson, 1979) and medium acidification was found to play an important role in inducing unicellular yeast-like growth at pH values 3.0-7.0.

Table 3.3 Effect of initial pH of the medium on morphology of *M. verrucaria*

Initial pH	Ammonium sulphate		Sodium nitrate	
	pH (72 h)	Morphology (72 h)	pH (72 h)	Morphology (72 h)
3.0	2.8	Y	3.7	Y>H
4.0	2.9	Y	4.9	H
5.0	2.9	Y>H	5.7	H
6.0	3.1	Y< H	6.2	H
7.0	3.4	Y< H	7.2	H
8.0	7.5	H	7.6	H
9.0	7.8	H	8.4	H
10.0	8.2	H	9.2	H

*Y: yeast like cells, H: hyphal cells, Y< H: more than 50% of the cells were in hyphal form, Y>H: more than 50% of the cells were in yeast form

The transition to Y form at pH values 3.0-4.0 was rapid than that at pH 5.0-7.0 (Table 3.3). In the presence of sodium nitrate, yeast form cells were produced only at pH 3.0 where rise in pH was not sufficient to retard the transition to Y form. These results also emphasized the importance of the pH in deciding the morphological outcome for *M. verrucaria*.

To confirm the role of pH in triggering the dimorphic transition in *M. verrucaria*, the pH of the medium was maintained at an initial value 4.0 or 6.0 by the addition of 0.1 N HCl or NaOH after every 24 h incubation during transition or by preparing the media in a citrate buffer (100 mM, pH 4.0 or 6.0). The experiment was carried out using three nitrogen sources, ammonium sulphate, ammonium nitrate and sodium nitrate. The flasks in which pH was not maintained to initial value served as a control. Results of this experiment are compiled in Table 3.4. The morphological

Table 3.4 Effect of continuous maintenance of initial pH on H-Y transition in *M. verrucaria*

Nitrogen source	Initial pH 4.0		Initial pH 6.0	
	Morphology (72 h)	pH (72 h)	Morphology (72 h)	pH (72 h)
Ammonium sulphate[c1]	Y	2.9	Y	3.1
Ammonium sulphate[a]	Y	4.0	H	6.0
Ammonium sulphate[b]	Y	4.0	H	6.0
Ammonium nitrate[c2]	Y>H	4.3	Y<H	4.7
Ammonium nitrate[a]	Y	4.0	H	6.0
Ammonium nitrate[b]	Y	4.0	H	6.0
Sodium nitrate[c3]	H	4.9	H	6.2
Sodium nitrate[a]	Y	4.0	H	6.0
Sodium nitrate[b]	Y	4.0	H	6.0

*Y: yeast-like cells, H: hyphal cells, Y>H: more than 50% of the cells in yeast form, Y<H: more than 50% of the cells in hyphal form.

c1: ammonium sulphate (0.46%), pH of the medium was not controlled.

c2: ammonium nitrate (0.28%), pH of the medium was not controlled.

c3: sodium nitrate (0.6%), pH of the medium was not controlled.

a: Initial pH of the medium was maintained by the addition of 0.1N HCl or NaOH.

b: Initial pH of the medium was maintained using citrate buffer (100 mM, pH 4.0 or 6.0).

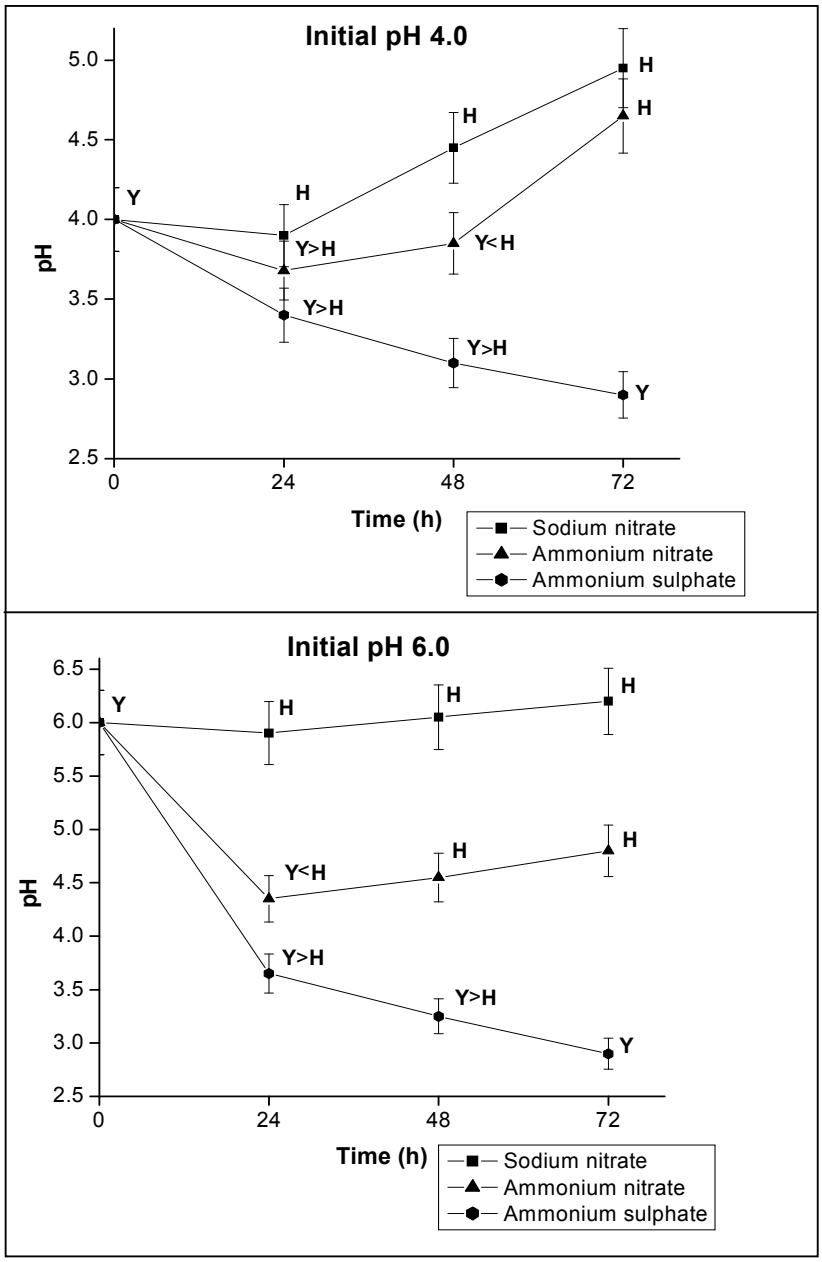
outcome correlated well with the pH of the medium rather than a nitrogen source. When pH of the medium was maintained at 4.0, in the presence of all the nitrogen sources, yeast form was favoured while at pH 6.0 hyphal form was predominant (Table 3.4).

These results confirm the major role of pH (4.0) in triggering the unicellular growth in *M. verrucaria*. Ruiz-Herrera and Setandreu (2002) reported the use of media containing phosphate and citrate buffer to study morphological transition in *Y. lipolytica*. In contrast to the observations on *M. verrucaria*, in *Y. lipolytica* the morphological outcome was governed by nitrogen source (ammonium sulphate or glutamine) in glucose containing medium with phosphate buffer (50 mM, pH 7.0). Under these conditions Y-H transition was favoured in presence of the ammonium sulphate while it was completely retarded in the presence of glutamine as a nitrogen source.

3.2.2.4 Y-H transition in *M. verrucaria*

The Y-H transition in *M. verrucaria* was studied in the presence of different nitrogen sources such as ammonium sulphate, ammonium nitrate and sodium nitrate, at initial pH 4.0 or 6.0. The flasks were incubated under shaking conditions (200 rpm) at 28°C. Morphological transition in *M. verrucaria* was freely reversible. The rapid Y-H transition was observed at pH 6.0 in presence of the sodium nitrate as a nitrogen source (Fig. 3.4). Most of the yeast cells produced germ tubes within 6-8 h and filamentous growth was observed within 24 h. While at pH 4.0, in sodium nitrate containing medium though Y-H transition was favoured it was slow (48 h). In the presence of ammonium sulphate at pH 6.0 as well as at pH 4.0, Y-H transition was completely retarded due to the acidification of medium as a result of uptake of ammonium ions. In the presence of ammonium nitrate, initial uptake of ammonium ions resulted in medium acidification and Y form growth was predominant up to 48 h

Fig. 3.4 Effect of nitrogen source and pH on Y-H transition in *M. verrucaria*



* The pH profiles and vegetative morphologies of *M. verrucaria* in the presence of different nitrogen sources at initial pH 4.0 and 6.0.

at pH 4.0. Later uptake of nitrate resulted in rise in pH, and Y-H transition was favoured. While at pH 6.0, though medium acidification was observed, pH did not decrease to 4.0. The Y-H transition at pH 6.0 was rapid as compared to that at pH 4.0.

3.2.2.5 Spore germination under H and Y form triggering conditions

M. verrucaria spore germination was monitored on a solid medium for Y-form growth, pH 4.0 and medium for H-form growth, pH 6.0. The spores of *M. verrucaria* displayed a dimorphic response during germination (Fig. 3.5). On medium for Y form growth, *M. verrucaria* spores produced yeast cells within 12 h which multiply by budding and gave rise to cream coloured, mucoid colonies within 24-48 h. The spores gave rise to small germ tubes within 3-4 h when grown on a medium for H-form growth, at pH 6.0. Within 24-48 h, white, cottony, spreading mycelial colonies of *M. verrucaria* were observed. Although the presence of the dimorphic response was not emphasized, the sporangiospores of *Mucor rouxii* germinated by budding and in hyphal form in response to anaerobiosis and change in glucose concentration (Bartnicki-Garcia and Nickerson, 1962). The conidia of *H. capsulatum*, *P. brasiliensis*, in response to a change in temperature, which was again a dimorphism trigger for them, also exhibited yeast and hyphal type germination (Anderson, 1978). Ghormade and Deshpande (2000) suggested that the dimorphism is the intrinsic ability of the fungus *B. poitrasii* as besides the vegetative forms, asexual as well as sexual spores recognized the morphological triggers, glucose, pH and temperature. From the present investigations it is evident that in case of *M. verrucaria*, yeast cells may be produced by the transition of the vegetative filamentous form or by the direct germination of asexual spores into yeast cells under yeast favouring conditions. The details are given in the Chapter 4.

3.2.3 Biochemical characterization of *M. verrucaria*

3.2.3.1 Endochitinase and *N*-acetylglucosaminidase

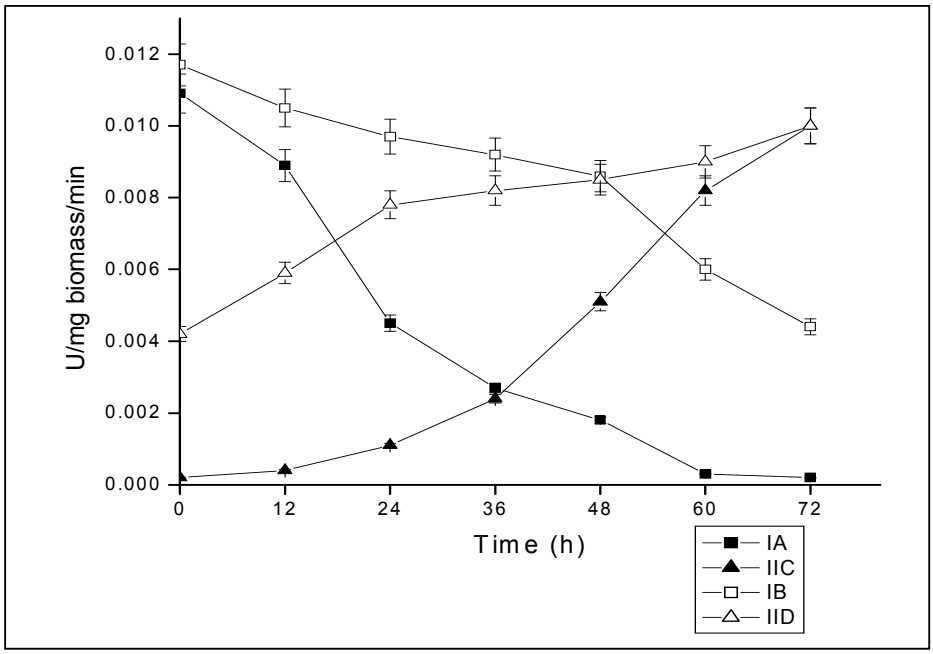
Chitin, a 1,4 β-D-*N*-acetylglucosamine linked polymer is the main structural component of most fungal cell walls (Kopecek and Raclavsky, 1999; Zindarsic *et al*, 1999). The chitinase complex comprising endochitinase and *N*-acetylglucosaminidase

Fig. 3.5 *M. verrucaria* spore germination on H and Y form favouring media

* a: spore gave rise to germ tube on medium for H-form growth
  b: spore germinated into yeast-like cell on medium for Y-form growth

activities has been implicated in several aspects of life cycle of fungi such as hyphal tip growth, spore germination, cell separation and autolysis (Kuranda and Robbins, 1991; Lima *et al*, 1999; Patil *et al*, 2000; Rast *et al*, 1991; Sahai and Manocha, 1993). In addition, reports available in the literature suggested that membrane bound chitinases significantly contributed in the morphogenetic event in fungi (Adams *et al*, 1993; Ghormade *et al*, 2000; Rast *et al*, 1991). Chitinolytic enzymes act by weakening the fungal cell wall for the germ tube formation and also by supplying *N*-acetylglucosamine for the synthesis of new cell wall at the growing tip. Therefore, chitnolytic activities were estimated *in situ* using 4-methylumbelliferyl labeled fluorogenic substrates with permeabilised yeast and hyphal cells of *M. verrucaria* grown in medium containing sucrose and ammonium sulphate, pH 4.0 and that containing sucrose, sodium nitrate, pH 6.0, respectively. *N*-Acetylglucosaminidase activity increased steadily throughout

Fig.3.6 Intracellular chitinolytic activities of *M. verrucaria* during dimorphic transition



* IA, Endochitinase activity during H-Y transition; IB, *N*-acetylglucosaminidase activity during H-Y transition; IIC, Endochitinase activity during Y-H transition; II D, *N*-acetylglucosaminidase activity during Y-H transition

the Y-H transition (Fig. 3.6). The activity in 12 h (56% germ tube formation) was 1.2 fold higher than at time zero. The endochitinase activity also increased 5.5 fold within first 12 h of transition. During H-Y transition (Fig. 3.6), the trend of the enzyme activities was reverse to the trend observed for Y-H transition. The high quantities of chitinolytic enzymes in hyphal cells appeared to decrease. The decrease in chitinolytic activities could be attributed to either proteolysis of the enzymes that were not required at a given point of time or the inhibition by specific inhibitors, if any (Deshpande, 1992).

3.2.3.2 NAD- and NADP-dependent glutamate dehydrogenases and nitrate reductase

It has been reported earlier that the yeast form growth in *B. poitrasii* (Khale *et al*, 1992) and *M. rouxii* (Peters and Sypherd, 1979) was favoured by a complex nitrogen source. The extensive work has been carried out in our laboratory to understand the role and regulation of glutamate dehydrogenase enzymes involved in NH_4^+ assimilation during morphological transition in *B. poitrasii* (Amin *et al*, 2004; Ghormade *et al*, 2005a,b; Khale-Kumar and Deshpande, 1993). In case of *M. verrucaria*, the yeast-like cells were produced in a medium containing ammonium salts as a nitrogen source while nitrate was used as a nitrogen source to obtain the hyphal form. Furthermore, when ammonium nitrate was used as a nitrogen source, H-Y transition was favoured during first 48 h of incubation while later Y-H transition was triggered suggesting the prior uptake of ammonium as a nitrogen source followed by the uptake of nitrate as a nitrogen source. These observations suggest the differential expression of the enzymes involved in nitrogen metabolism. The expression of NAD- and NADP-dependent glutamate dehydrogenases and nitrate reductase enzymes in yeast and hyphal forms of *M. verrucaria* was studied during present investigations.

M. verrucaria hyphal cells were inoculated in glucose containing medium with different nitrogen sources, at pH 4.0. The flasks were incubated under shaking conditions (200 rpm) at 28°C. Different morphological forms produced were harvested after 48-96 h. The NADP- and NAD-GDH and nitrate reductase activities in cell extracts were then estimated. The quantitative relationship between the NAD-GDH and NADP-GDH was expressed as NADP-/NAD-GDH ratio as described earlier in case of *B. poitrasii* (Khale *et al*, 1992). The results are given in Table 3.5.

Table 3.5 Differential expression of NADP- and NAD-glutamate dehydrogenases and nitrate reductase during H-Y reversible transition in *M. verrucaria*

Nitrogen Source	Time (h)	Morphology	pH	NADP-/ NAD-GDH ratio	Nitrate reductase (U/mg)
Ammonium sulphate	72	Y	2.9	7.7 ± 0.4	ND
Sodium nitrate	72	H	5.9	3.8 ± 0.2	14.2 ± 0.7
Ammonium nitrate	48	Y	3.4	4.9 ± 0.1	2.3 ± 0.2
Ammonium nitrate	72	H>Y	3.8	3.7 ± 0.1	36.8 ± 1.8
Ammonium nitrate	96	H	4.9	3.8 ± 0.2	39.0 ± 1.9

* Y: yeast-like cells, H: hyphal cells, H>Y: more than 50% of the cells in hyphal form

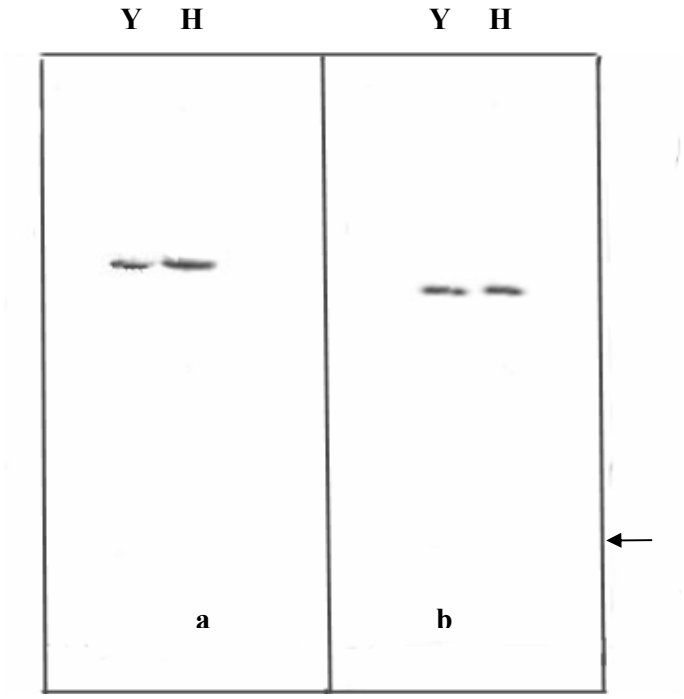
The hyphal cells retained their morphology in sodium nitrate containing medium. Specific activities of NADP-GDH (0.095 ± 0.002 U/mg), NAD-GDH (0.025 ± 0.002 U/mg) and nitrate reductase (14.2 ± 0.7 U/mg) were detected in 72 h old hyphal cells. In ammonium sulphate containing medium hyphal cells reverted to yeast-like cells within 72 h. The Y form cells of *M. verrucaria* (72 h old) exhibited 2-fold higher specific activity of NADP-GDH (0.177 ± 0.002 U/mg) as compared to that in hyphal cells while NAD-GDH levels (0.023 ± 0.001 U/mg protein) did not change significantly. A higher NADP-/NAD-GDH ratio (7.7 ± 0.4) was found

associated with the yeast form cells of *M. verrucaria* than the hyphal cells (3.8 ± 0.02). Earlier association between higher GDH ratio and yeast form as compared to that of hyphal form was reported in case of *B. poitrasii* (Khale *et al*, 1992). The nitrate reductase activity was not detected in yeast-like cells of *M. verrucaria*.

In ammonium nitrate containing medium, initially H-Y transition was favoured with a decrease in pH (4.0-3.4) and within 48 h swollen hyphae were observed. Low nitrate reductase activity (2.3 ± 0.2 U/mg) was detected in these cells. Also, GDH ratio (4.9 ± 0.1) higher than that observed in hyphal cells (3.8 ± 0.02) was observed in these cells. On further incubation, pH started increasing and swollen hyphae reverted to hyphal form. The increase (15-fold) in the specific activity of nitrate reductase (39.0 ± 1.9 U/mg) and decrease in the GDH ratio (4.9 ± 0.1 to 3.8 ± 0.2) was observed. From these results it may be suggested that when ammonium nitrate was used as a nitrogen source, *M. verrucaria* selectively utilized nitrogen in ammonium form, which resulted in a medium acidification and in turn favoured H-Y transition. After depletion of ammonium ions, *M. verrucaria* started utilizing nitrate ions as a nitrogen source. The pH of the medium increased due to nitrate uptake and transition to hyphal form was favoured. Goldsmith (1973) reported that ammonium at very low concentrations caused a rapid decay of nitrate uptake and nitrate reductase activity in *P. chrysogenum*. The nitrate reductase activity was induced when NH_4^+-grown *P. chrysogenum* was incubated with inorganic nitrate in synthetic medium in the absence of NH_4^+. In these cells ammonium transport system was maximally derepressed.

Recently, Amin *et al* (2004) reported the presence of one NAD-GDH in both yeast- and mycelium- form cells and two active NADP-GDHs, one expressed in mycelium form and other one in yeast form of *B. poitrasii*. The extracts of hyphal and

Fig. 3.7 Activity staining of NADP- and NAD- dependent glutamate dehydrogenases in *M. verrucaria*



* **a**: NADP-GDH in Y and H forms of *M. verrucaria*, **b**: NAD-GDH in Y and H forms of *M. verrucaria*, ⟶ indicates the dye front.

yeast-like cells of *M. verrucaria* were subjected to polyacrylamide gel electrophoresis. The activity staining revealed that *M. verrucaria* has two glutamate dehydrogenases, one requiring NADP- and the other one requiring NAD- as a coenzyme in both the morphological forms (Fig. 3.7).

3.2.4 Molecular characterization of *M. verrucaria*

The sequence analysis of ribosomal DNA has been widely utilized for molecular systematics and identification of microorganisms (Mitchell *et al*, 1995; Sugita *et al*, 2002). The analysis of ITS (internal transcribed spacer) regions has been carried out mainly to identify or differentiate closely related species of pathogenic yeast (Chen *et al*, 2000; Gupta *et al*, 2000; Lott *et al*, 1998). To identify the genomic marker which can differentiate the *M. verrucaria* strain exhibiting higher production of CDE/ME complex

and hypha-yeast transition from other isolates of *M. verrucaria,* 18s rRNA and ITS1 sequence analysis was carried out. The genomic DNA was isolated from *M. verrucaria* mycelium using the DNA extraction protocol of Wendland *et al* (1996). Nuclear 18s rRNA gene and ITS 1 were amplified using primers NS1 and ITS1, respectively. The sequence data was analyzed using NCBI server (http//:www.ncbi.nlm.nih.gov/ BLAST). The Genbank accession number for the 18s rRNA and ITS1 sequences reported in the thesis are: Myr 18s:AY129004 and Myr ITS1: Ay129005. These 18srRNA gene and ITS1 sequences were given in Fig. 3.8. The 18s rRNA and ITS1 sequences were found highly homologous within the species. The analysis of genes or regions with greater divergence may prove helpful in identifying a genomic marker for *M. verrucaria* MTCC 9151.

Fig. 3.8 Myr 18s: AY129004, the sequence of *Myrothecium verrucaria* 18s rRNA gene and Myr ITS1:AY129005, the sequence of ITS1 region

Myr 18s: AY129004

1 tatacagcga aactgcgaat ggctcattat ataagttatc gtttatttga tagtaccttа
61 ctacttggat aaccgtggta attctagagc taatacatgc taaaaatccc gacttcggaa
121 gggatgtatt tattagatta aaaaccaatg ccctctgggc tctctggtga tttatgataa
181 cttctcgaat cgcatggcct tgtgccggcg atggttcatt caaatttctt ccctatcaac
241 tttcgatgtt tgggtagtgg ccaaacatgg ttgcaacggg taacggaggg ttagggctcg
301 accccggaga aggagcctga gaaacggcta ctacatccaa ggaaggcagc aggcgcgcaa
361 attacccaat cccgacacgg ggaggtagtg acaataaata ctgatacagg gctcttttgg
421 gtcttgtaat tggaatgagt acaatttaaa tcccttaacg aggaacaatt ggagggcaag
481 tctggtgcca gcagccgcgg taattccagc tccaatagcg tatattaaag ttgttgtggt
541 taaaaagctc gtagatgaac cttgggcctg gctggccggt ccgcctcacc gcgtgtactg
601 gtccggccgg gcctttccct tt

Myr ITS1: AY129005

1 gaggtcatta ccgagtttac aaactcccaa acccttttgtg aaccttacca tattgttgct
61 tcggcgggac cgccccggcg ccttcgggcc cggaaccagg cgcccgccgg aggccccaaa
121 ctcttatgtc tttagtggtt ttctcctctg agtgacacat aaacaaataa ataaaaactt
181 tcaacaacgg atctcttggt tctggcatcg atgaaaaacg cagca

The H-Y transition in *M. verrucaria* was reported for the first time in the present chapter. The external pH, which governs the mode of uptake of nitrogen source, was found to be the important morphological trigger. The results of 18s rRNA gene and ITS1 sequence analysis suggest that further analysis of genes or regions with greater divergence may prove helpful in identifying a genomic marker for *M. verrucaria* MTCC 9151.

Chapter 4

Mycopesticide production

4 A Production of living propagules, conidia and yeast-like cells of *M. verrucaria*

4A.1 Introduction

The production of fungi to use as biocontrol agents aims at the maximum possible number of living propagules that can infect pathogenic fungi and insects and/or release metabolites such as mycolytic/cuticle degrading enzymes, antibiotics or toxins to kill fungal and insect pests. Generally, fungal propagules in biocontrol formulations must be alive at the time of their use and expected minimum shelf life for a commercially feasible product is 18 months at 20°C (Deshpande, 1999). The fungal kingdom is a diverse assemblage of organisms with a great variety of structural types. Several different stages of fungal life cycle, vegetative growth forms such as mycelia and unicellular yeasts, asexual spores such as conidia and sporangiospores and sexually produced oospores and zygospores were used to develop biocontrol formulations of fungi (Chavan *et al*, 2006; Deshpande, 1999). However, aerial conidia are propgules of choice for the development of mycopesticides. The aerial conidium is the infectious and dissemination unit in case of many mycoparasitic and entomopathogenic fungi yet in a dormant stage, safe and easy for storage and application as biocontrol formulations (Horaczek and Viernstein 2004).

The solid-state fermentation (SSF) was found advantageous for the large-scale production of conidia. It was easy to carry out, raw material is cheap, and most importantly, conidia produced as living propagules tend to be more tolerant to desiccation and more stable as a dry preparation as compared with conidia produced in submerged fermentation (Deshpnade, 1999; Jenkins and Goettel, 1997). SSF was successfully used for the conidia production in different biocontrol fungi such as *M. anisopliae* (Deshpande and Tuor, 2002; Nahar *et al*, 2003), *B. bassiana* (Dalla Santa *et al*, 2004), *N. rileyi* (Vimala Devi *et al*, 2000) and *C. minitans* (Jones *et al*, 2004).

Different nutritional factors such as the choice of solid substrate and requirement of additional carbon or nitrogen source or salt solutions etc. and environmental factors like initial substrate pH, moisture content of the substrate and incubation temperature were found to affect the spore germination, vegetative growth and spore production in fungi. Optimization of these factors was reported to improve the production of spores under SSF conditions, thus making the production of biocontrol agent economically viable.

M. verrucaria is known to secrete high amounts of mycolytic enzymes when grown in chitin containing medium. Patil *et al* (2001) reported the use of these mycolytic enzymes for the control *S. rolfsii* infection on peanut. However, in view of the stability of enzymes under different field conditions it would certainly be advantageous to use organism itself for the seed dressing or soil application and for further slow release of enzymes. Thus in present investigations attempts were made to produce conidia of *M. verrucaria* using SSF while yeast-like cells were produced under submerged conditions as an alternative propagule.

4A.2 Results and discussion

4A.2.1 Production of conidia using solid-state fermentation

4A.2.1.1 Selection of the solid substrate

Conidia production of *M. verrucaria* by SSF using agro-industrial residues and cereal grains was optimized with respect to substrate and cultivation conditions in Erlenmeyer flasks. The selection of the substrate was found to be an important feature of any SSF as the production of conidia significantly differed among the fungal isolates on different agricultural products used as substrates (Vimala Devi *et al*, 1994; Zhang and Watson, 1997). Different cereal grains (sorghum, pearl millet, rice and wheat), wheat and rice bran and marine waste (prawn shell waste) were screened to select the

Table 4.1 Production of *M. verrucaria* conidia on various solid substrates

Substrate	Conidia / g substrate
Sorghum	1.6×10^9
Pearl millet	1.3×10^9
Rice	1.2×10^6
Beaten rice	1.4×10^5
Wheat	2.3×10^5
Wheat bran	-
Rice bran	-
Prawn shell waste	-

* The flasks were incubated at 28°C for 14 d.

substrate suitable for *M. verrucaria* conidia production (Table 4.1). All the substrates supported good mycelial growth of *M. verrucaraia*. While sporulation was observed only on sorghum, pearl millet, rice, beaten rice and wheat grains. On sorghum and pearl millet maximum sporulation ($1.3\text{-}1.6 \times 10^9$ conidia/g substrate) of *M. verrucaria* was seen. A high yield of conidia of *N. rileyi* (F) Samson (2.8×10^9 conidia/g substrate) was obtained on crushed sorghum (Vimala Devi *et al*, 2000). Deshpande *et al* (2001) reported that sorghum supported sporulation in case of *Metarhizium* and *Beauveria* sp. and barley was suitable for *Nomuraea* sp. While, rice supported maximum production of conidia in all the three strains of *M. anisopliae*, *B. bassiana* and *N. rileyi* (Nahar *et al*, 2003). The wheat bran was reported to support conidia production in *B. bassiana* CS-1 (Kang *et al*, 2005). While in case of *M. verrucaria*, on wheat bran as well as rice bran sporulation was not seen upto 14 d. Similarly, prawn shell waste was also found to support only mycelial growth of *M. verrucaria* (Table 4.1).

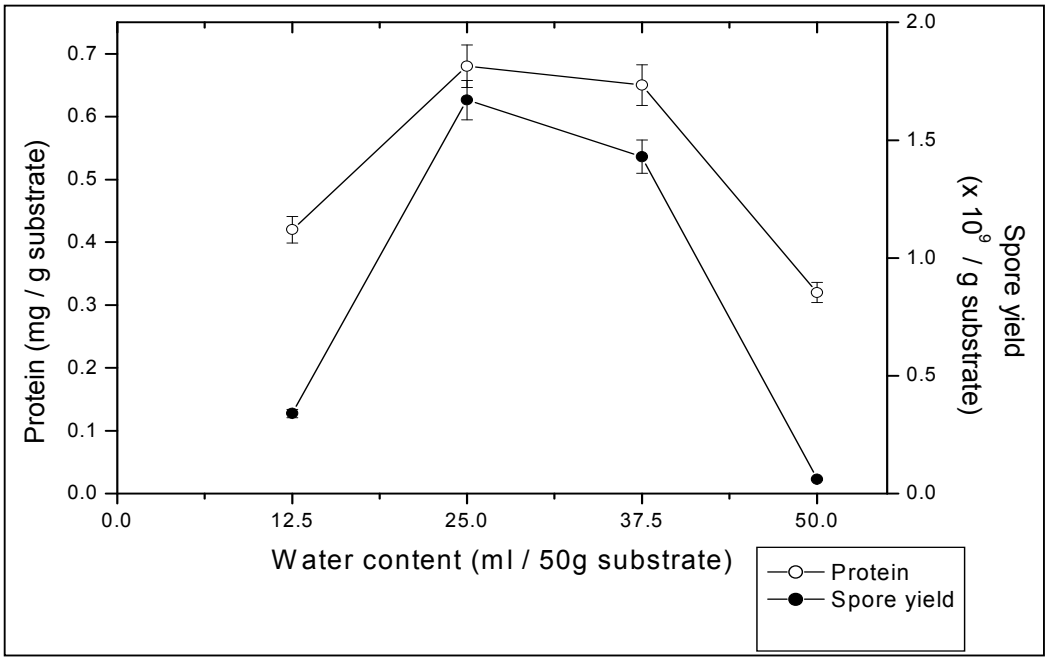
On sorghum grains *M. verrucaria* sporulation started on the 7th day and maximum sporulation was observed after 14 d of incubation. Further increase in incubation period up to 20 d did not result in the increase in spore yield. Besides this on

further incubation, the solid medium became soggy and affected the spore recovery from the substrate. In further experiments, sorghum was used as a solid substrate for the production of conidia of *M. verrucaria*.

4A.2.1.2 Effect of water content

Moisture content of solid substrate is an important factor during microbial growth in SSF as it causes swelling of the substrate and facilitates utilization of nutrients by the organism. Thus optimum moisture content for fungal growth and conidiation needs to be identified for each substrate/isolate combination (Jenkins *et al*, 1998). To provide the optimal moisture content for conidia production by *M. verrucaria*, sorghum grains were soaked in distilled water (12.5-50 ml/50 g sorghum grains) overnight, autoclaved and then used for conidia production. The soluble protein produced by *M. verrucaria* in the sorghum medium was measured as the indicator of growth (Fig. 4.1). Increase in mycelial growth (0.42 –0.68 mg protein/g substrate) as

Fig. 4.1 Effect of initial water content on *M. verrucaria* conidia production under solid state fermentation conditions

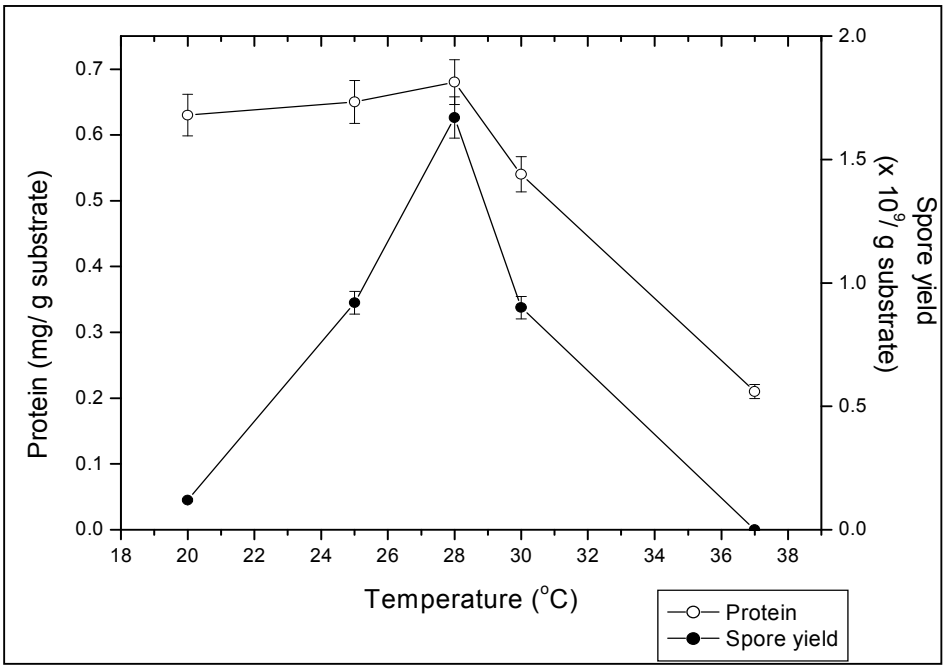


well as spore yield (0.34-1.67 x 10^9 conidia/g substrate) was observed with increase in water addition up to 25 ml/50 g substrate (Fig. 4.1). Further addition of water resulted in decrease in mycelial growth and spore yield. A higher moisture level was suggested to affect mycelial growth of *Trichoderma* sp., *Penicillium* sp. and *Rhizopus oligosporous* on cranberry processing waste by decreasing porosity or intracellular spaces, lower oxygen diffusion and gas exchange while a low moisture level resulted in decreased substrate swelling and decreased mycelial growth and sporulation (Zheng and Shetty, 1998). Addition of 25 ml water per 50 g of sorghum was found optimum to provide enough moisture for the maximum growth and sporulation of *M. verrucaria*.

4A.2.1.3 Effect of incubation temperature

The SSF flasks were incubated at different incubation temperatures for 14 d to study the effect of temperature on the production of *M. verrucaria* conidia. The optimum incubation temperature for *M. verrucaria* conidia production was found to be

Fig. 4.2 Effect of incubation temperature on *M. verrucaria* conidia production under solid state fermentation conditions



28°C (spore yield $1.6x10^9$ conidia/g of substrate) though good mycelial growth (0.59-0.68 mg protein/g substrate) was observed at 20-30°C (Fig. 4.2). At incubation temperatures below as well as above 28 °C, conidia production decreased drastically. Sparse mycelial growth with no sporulation was observed when SSF flasks were incubated at 37°C. Incubation temperature was reported to be a critical factor governing the sporulation of number of biocontrol fungi grown on solid substrates. Maximum sporulation of *Penicillium commune* was observed over incubation temperature range 28-30°C and sporulation decreased drastically with further increase in temperature (Nava *et al*, 2006). While *Pandora neoaphidis* conidia production was carried out at 20°C. The optimum temperature for *B. bassiana* conidia production was reported to be 25-26°C (Dalla Santa *et al*, 2004; Ye *et al*, 2006).

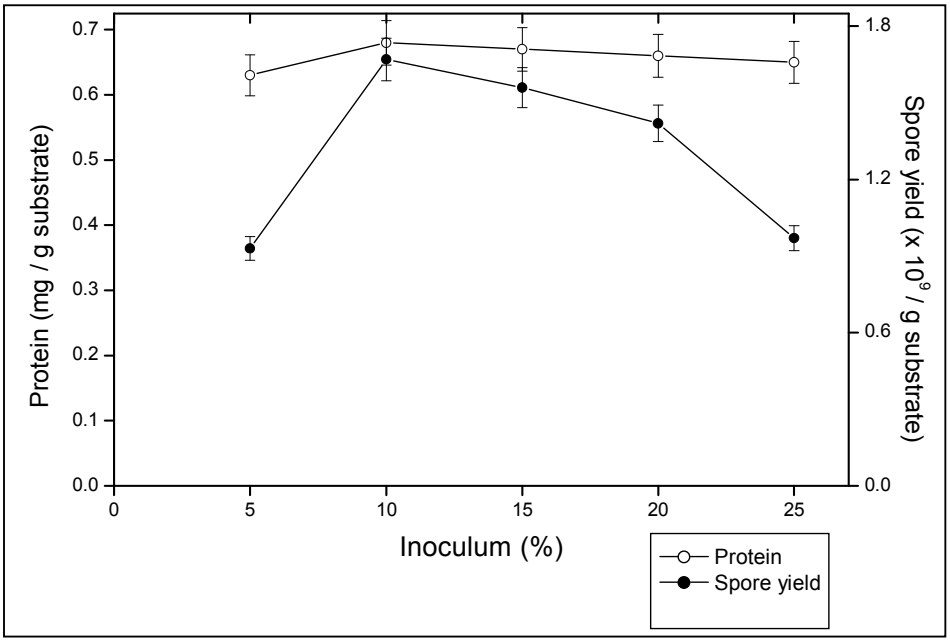
4A.2.1.4 Effect of an inoculum

The use of fungal mycelium produced in liquid culture as inoculum was suggested to provide following benefits during conidia production under SSF conditions:

- The competitiveness of the fungus is enhanced, reducing the risk of colonization of the substrate by contaminating microorganisms.
- Colonization and conidiation are more rapid thus reducing incubation time.
- It ensures an even coverage of the solid substrate resulting in homogenous growth throughout the substrate (Jenkins *et al*, 1998).

Thus in the present study *M. verrucaria* mycelium grown in YPG broth (72 h) was used as an inoculum for the production of conidia. Fig. 4.3 reveals the effect of inoculum concentration (5-25%) on mycelial growth and sporulation of *M. verrucaria* on sorghum. Increase in the inoculum size (5-10% v/w) resulted in increase in spore

Fig. 4.3 Effect of inoculum concentration on growth and sporulation of *M. verrucaria* on sorghum grains



yield (0.7-1.67x10^9 conidia/g substrate). With further increase in the inoculum size the mycelial growth was favoured while decrease in sporulation (≈10 fold) was observed. Use of 10% v/w inoculum was found optimum for the production of conidia of *M.verrucaria* on sorghum. Nahar *et al* (2003) reported the use of biomass grown in YPG broth (10%) as inoculum for the production of conidia of *M. anisopliae*, *B. bassiana* and *N. rileyi* on rice grains.

Commercial application of biocontrol agent depends on the production of large quantities of conidia. Most of the potential biocontrol fungi were reported to produce conidia in the range of 10^9 to10^{10} conidia/g substrate. Nahar *et al* (2003) reported the production of conidia of *M. anisopliae*, *B. bassiana* and *N. rileyi* in the range 10^9-10^{10} conidia/g rice within 14 d incubation. While another isolate of *N. rileyi* was reported to yield 2.8x10^9 conidia/g crushed sorghum within 10 days (Vimala Devi *et al*, 2000). During present studies, with sorghum as a solid substrate, optimum conditions observed for *M. verrucaria* conidia production were: initial water content (25 ml/50 g substrate),

incubation temperature (28°C) and 72 h old YPG grown mycelium as an inoculum (10% v/w). The highest conidia production ($2x10^9$ conidia/g substrate) was observed after 14 d incubation under the optimized fermentation conditions.

4A.2.2 Production of yeast-like cells of *M. verrucaria*

Liquid fermentations were also successfully used for the large-scale production of fungal propagules. The submerged fermentation systems have obvious advantages of controlled processing. Using a wide range of controls for pH, agitator speed, aeration rate, temperature, etc. the timing for harvesting of e.g. blastospores can be precisely envisaged to obtain suitable biomass for storage (Deshpande, 1999; Gibbs *et al*, 2000). Submerged fermentation conditions were reported to be extensively used for the large-scale production of fungal propagules like blastospores, chlamydospores, mycelia and yeast cells (Belanger *et al*, 1994; Fargues *et al*, 2002; Hebbar *et al*, 1997; Issaly *et al*, 2005; Jadubansa *et al*, 1994) of various potential biocontrol fungi.

Under submerged fermentation conditions hyphal as well as yeast-like cells of *M. verrucaria* can be produced within 72 h as compared to the production of condia within 14 d under SSF conditions. However, isolated yeast-like cells could be uniformly mixed with the carrier as compared to the hyphal cells produced as large pellets (4-5 mm diameter). Thus, attempts were made to optimize conditions for the production of yeast-like cells of *M. verrucaria* as an alternative living propagule for the slow release of enzymes.

4A.2.2.1 Effect of nature of inoculum

The low pH (4.0) was found to be the morphological trigger for the formation of yeast-like cells of *M. verrucaria*. Sucrose (2%) and ammonium sulphate (0.46%) were

found to be the most suitable carbon and nitrogen sources, respectively for the production of yeast-like cells. The effect of nature of inoculum on the production of yeast biomass was studied. Different morphologies of *M. verrucaria*, hyphal and yeast-like cells (48 h old) and conidia (7 d old slant) were used as inoculum for production of yeast-like cells. Table 4.2 reveals that these inocula yielded yeast biomass in the range of 4.46-4.6 g/l wet wt within 72 h. However, when conidia or yeast-like cells were used as inoculum, % of isolated yeast-like cells (≥ 50%) was higher as compared to that

Table 4.2 Effect of nature of inoculum on the production of *M. verrucaria* yeast-like cells

Inoculum/100ml medium	Biomass (g/l)
Conidia ($1x10^{7}$)	4.5 ± 0.3
Yeast like cells (5% wet wt)	4.6 ± 0.4
Hyphal cells (5% wet wt)	4.46± 0.3

observed in presence of hyphal cells as inoculum (28% isolated yeast-like cells). While remaining yeast-like cells formed small aggregates or chains. The isolated yeast-like cells can be uniformly mixed with the carrier to develop biocontrol formulation. Therefore, the yeast inoculum providing maximum biomass and isolated cells was selected for the mass production of yeast-like cells.

Under the optimized conditions for production of yeast-like cells: sucrose (2%), ammonium sulphate (0.46%), initial pH 4.0 and 48 h old yeast-like cells (5% wet wt) as inoculum, maximum yeast biomass (4.6 ± 0.4 g/l wet wt) was obtained under shaking conditions (200 rpm) at 28°C after 72 h.

4A.2.2.2 Production of mycolytic enzymes using yeast-like cells

The secretion of mycolytic enzymes plays an important role in a biocontrol of fungal pathogens. Thus it was necessary to study production of mycolytic enzymes

using yeast-like cells of *M. verrucaria* as an inoculum before developing the biocontrol formulation. For comparison, hyphal cells and conidia were used as an inoculum. The production of mycolytic enzymes was studied in a medium containing chitin or autoclaved mycelium of a plant pathogen, *S. rolfsii* as a sole source of carbon. The hyphal cells remained unchanged during the extracellular production of mycolytic enzymes. The spores germinated and gave rise to hyphal form. The yeast-like cells undergo reversion to the hyphal form within 24 h in the above-mentioned media (initial pH 6.0) prior to the production of mycolytic enzymes and there after continued to grow in hyphal form as the pH of the medium increased (6.0-7.2) during the incubation (Table 4.3).

Table 4.3 Production of mycolytic enzymes using different morphologies of *M. verrucaria* as inoculum

Inoculum	Morphology (7d)	Final pH (7d)	Chitinase (U/ml)	β-1,3 Glucanase (U/ml)	Protease (U/ml)
Chitin 0.5% (Carbon source)					
Conidia suspension ($1x10^7$conidia/ml)	H	7.7	1.8 ± 0.2	0.23 ± 0.03	0.013± 0.002
Hyphal cells (5% wet wt)	H	7.4	1.62 ± 0.2	0.19 ± 0.02	0.012 ± 0.002
Yeast cells (5% wet wt)	H	7.2	1.41± 0.01	0.18 ± 0.03	0.01 ± 0.002
S. rolfsii mycelium (5.0% wet wt) (Carbon source)					
Conidia suspension ($1x10^7$ conidia/ml)	H	7.5	0.061± 0.002	0.73 ± 0.12	0.012 ± 0.002
Hyphal cells (5% wet wt)	H	7.3	0.047 ± 0.003	0.74 ± 0.11	0.011 ± 0.001
Yeast cells (5% wet wt)	H	7.1	0.025± 0.003	0.49 ± 0.13	0.010± 0.001

*H: Hyphal cells

The maximum production of mycolytic enzymes was observed in the presence of *M. verrucaria* conidia used as an inoculum. Hyphal and yeast-like cells when used as an inoculum, marginally lower (8-17% and 22-24%, respectively) production of mycolytic enzymes was observed. Irrespective of the inoculum used high chitinase activity was induced in chitin containing medium as compared to *S. rolfsii* autoclaved mycelium containing medium. Whereas a high β-1,3-glucanase activity was expressed in medium containing *S. rolfsii* autoclaved mycelium. The production of protease did not change significantly with different inocula or with different media. From these results it can be suggested that the yeast-like cells of *M. verrucaria* which can be mass produced easily within 72 h and which exhibited transition to hyphal form secreting mycolytic enzymes within 24 h may be used as alternative living propagule for slow release of mycolytic enzymes for the control of plant pathogenic fungi.

4 B Production of cuticle degrading and mycolytic enzyme complex of *M. verrucaria*

4B.1 Introduction

In recent years, the use of chitinolytic enzymes in applicative fields such as biological pest control, the degradation of chitin rich wastes and the production of chitin hydrolysates for pharmaceutical or chemical purposes and for food and feed industry has become of increasing interest (Donzelli *et al*, 2003; Horsch *et al*, 1997; Patil *et al*, 2000). Traditionally selected strains of *Serratia marcescens* were used for the production of chitinolytic enzymes (Green *et al*, 2005; Mejia-Saules *et al*, 2006). 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), *Penecillium* sp. (Fenice *et al*, 1998; Parameswaran *et al*, 2005), *V. lecanii* (Liu *et al*, 2003; Matsumoto *et al*, 2004) and *B. bassiana* (Suresh and Chandrasekaran, 1999; Zhang *et al*, 2004) 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; El-Katatny *et al*, 2003; 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, 2000; Liu *et al*, 2003). The objective of the present study was to optimize the conditions for the production of chitinase of *M. verrucaria* at shake flask as well as at 15 l capacity fermenter level. The production of other enzyme components in CDE/ME complex, β-1,3-glucanase, protease and lipase was also studied under the conditions optimized for chitinase production.

4B.2 Results and discussion

4B.2.1 Shake flask studies

4B.2.1.1 Effect of different chitin sources

Chitinases were reported to be the inducible enzymes and various studies report the use of chitin in different forms such as crab shell chitin (Felse and Panda, 1999; Felse and Panda, 2000; Vyas and Deshpande, 1989), low molecular weight chitin (Onte *et al*, 2003), colloidal chitin (Fenice *et al*, 1998), shrimp shell waste (Liu *et al*, 2003), shrimp waste sillage (Matsumoto *et al*, 2004), etc. as source of carbon for the production of fungal chitinases under submerged fermentation conditions. *M. verrucaria* was grown in a medium containing chitin from different sources, crab shell chitin (Sigma and Hi-Media) and chitin containing fishery waste, prawns shell waste (0.5% w/v). The chitinase activity produced by *M. verrucaria* with Sigma chitin was higher (chitinase 1.68 ± 0.2 U/ml) as compared to other commercial chitin (Hi media) (chitinase 1.29 ± 0.2 U/ml). This could be due to the purity and particle size of chitin.

4B.2.1.2 Effect of corn steep liquor and molasses

Vyas and Deshpande (1989) reported increased production of chitinase by *M. verrucaria* in the presence of yeast extract and peptone. In the present studies attempts were made to identify cheaper raw materials that can replace yeast extract and peptone. The maximum chitinase activity (1.68 ± 0.2 U/ml) was produced in the presence of yeast extract and peptone (Table 4.4). While chitinase activities lower than that produced in the basal medium were seen in the presence of corn steep liquor (CSL) or molasses. Thus in further experiments basal medium supplemented with yeast extract and peptone was used for the production of CDE/ME complex.

Table 4.4 Effect of addition of corn steep liquor and molasses in medium on chitinase production by *M. verrucaria*

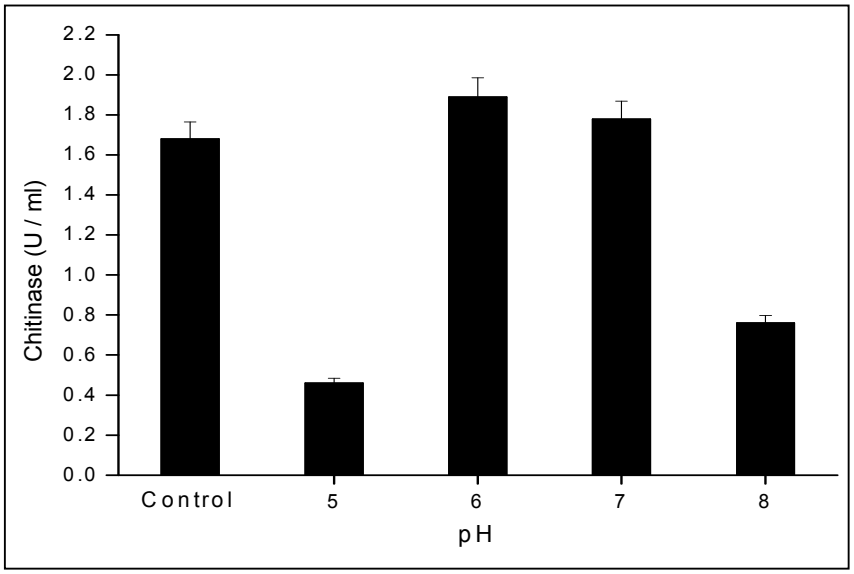
Medium constituents	Chitinase (U/ml)
Basal medium	1.42 ± 0.1
Basal medium + yeast extract 0.5% + peptone 0.5%	1.68 ± 0.2
Basal medium + 0.5% CSL	0.61 ± 0.07
Basal medium + 1.0% CSL	0.51 ± 0.05
Basal medium + 2.0% CSL	0.16 ± 0.01
Basal medium + 0.5% molasses	0.88 ± 0.09
Basal medium + 1.0% molasses	1.00 ± 0.1
Basal medium + 2.0% molasses	1.21± 0.1
Basal medium + 3.0% molasses	1.12 ± 0.1

* Basal medium with chitin as a sole carbon source as described under materials and methods

4B.2.1.3 Effect of controlled pH

Vyas and Deshpande (1989) reported that initial pH of the medium was important for the chitinase production by *M. verrucaria*. At pH 6.0, the chitinase activity was consistently high. However, increase in pH of the medium (pH 6.0-7.6) was observed during the course of chitinase production. Thus, in present investigations effect of controlled pH on chitinase production by *M. verrucaria* was studied. The initial pH was adjusted in a range of 5.0-8.0. Constant pH was maintained at the initial value by addition of sterile 1 N HCl after every 24 h of incubation. Maintaining the pH at 6.0, optimum pH for chitinase production resulted in 16% increase in chitinase production as compared to that in the control flask where pH of the medium was not adjusted to initial value (Fig. 4.4). The initial pH of the medium significantly affects the production of different extracellular enzymes by fungi. *T. viride* F-9 was reported to produce maximal chitinase activity in pH range 7.0-8.0 (Rogalski *et al*, 1997) while

Fig. 4.4 Effect of controlled pH on chitinase production by *M. verrucaria*



* Control: pH of the medium was allowed to increase during the course of fermentation from the initial of 6.0.

optimum pH of 4.9 was reported for chitinase production using *T. harzianum* (Felse and Panda, 1999). Maximal chitinase production by *Penicillium janthinellum* was observed at an initial medium pH of 4.0 (Fenice *et al*, 1998). However, in contrast to our results, when *P. janthinellum* was grown at the constant pH 4.0, the chitinase production decreased.

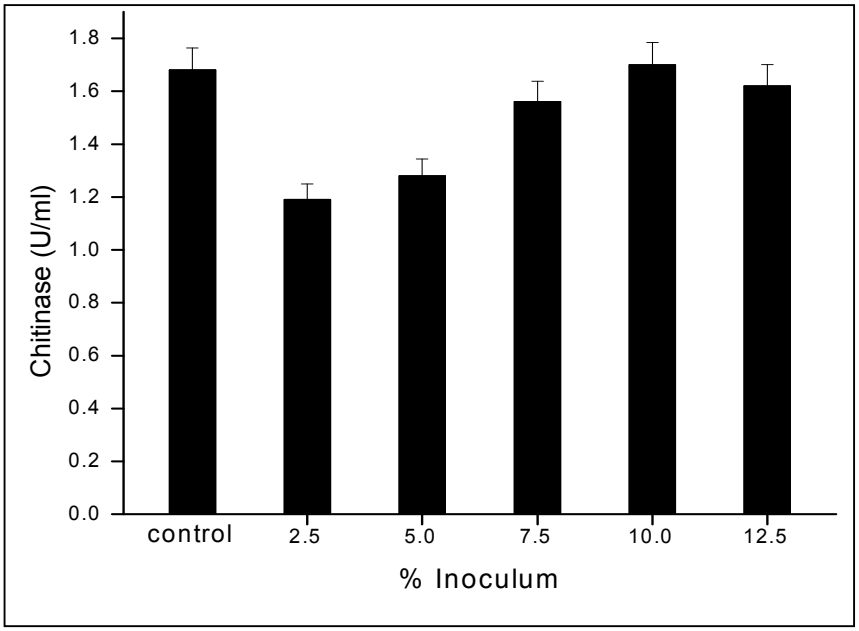
4B.2.1.4 Effect of an inoculum

The production of fungal chitinases was initiated using either spore or mycelial inocula. The use of mycelia of *V. lecanii* grown in chitin containing medium as inoculum was reported to significantly improve *N*-acetylhexosaminidase yields as compared to spore 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). Furthermore, inoculum concentration was also found as crucial parameter governing the chitinase production.

Lower inoculum concentration was not sufficient to effect maximal transformation of biological materials while larger inocula resulted in the decreased chitinase production by *T. harzianum* due to the shortage of nutrients available for the larger biomass and faster growth of culture (Sandhya *et al*, 2004). In the present investigations, chitinase production using *M. verrucaria* spore inoculum was compared with those using different concentrations of mycelial inocula. In case of *M. verrucaria*, after 48 h of initial lag period steady increase in chitinase production was observed. Thus, in the present study, 4 d old mycelium grown in chitin containing medium was used as an inoculum.

The nature as well as concentration of inoculum was observed to significantly affect the chitinase production by *M. verrucaria*. The mycelial inoculum (10%) led to production of the chitinase comparable to that obtained with spore inoculum (Fig. 4.5). High chitinase activity (1.68 ± 0.2 U/ml) was produced using these inocula. While in

Fig. 4.5 Effect of mycelial inoculum density on chitinase production by *M. verrucaria*



* Control: *M. verrucaria* spore suspension (10^7spore/ml) was used as inoculum.

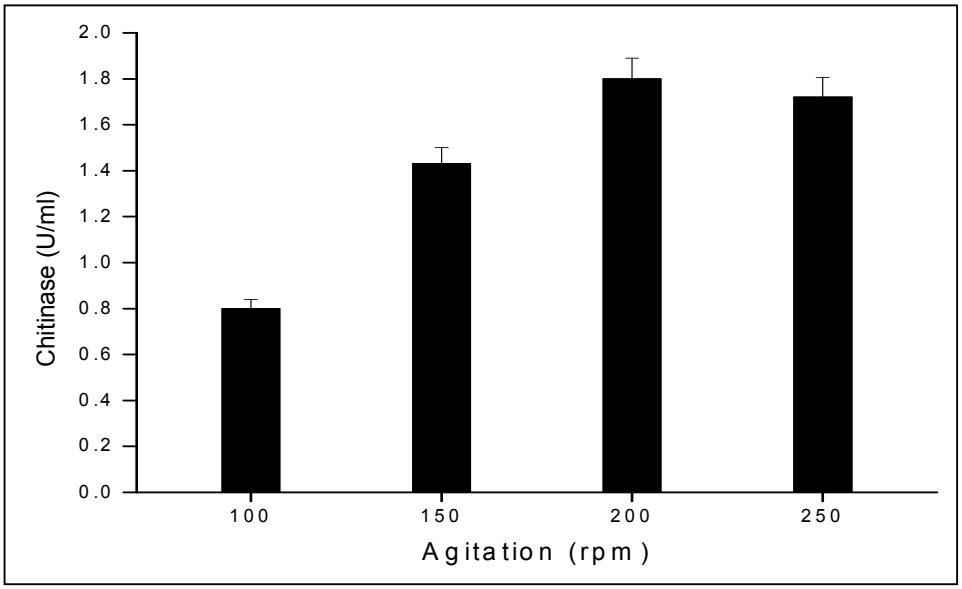
the presence of 2.5-7.5% mycelial inoculum, lower chitinase production (1.18 ± 0.18 to1.54± 0.2 U/ml chitinase) was observed. Slight reduction in chitinase production (1.58 ± 0.15 U/ml chitinase) was also observed when 12.5% inoculum was used.

Interestingly, it was observed that when spore inoculum was used, mycelium developed on chitin particles and small pellets (size ≤1 mm) of *M. verrucaria* were produced. In contrast, bigger pellets (size 2-3 mm) were produced when mycelial inoculum was used. The pellet size was reported to affect the production of various fungal metabolites (Feng *et al*, 2004; Gibbs *et al*, 2000; Zindarsic, 2001). The establishment of nutrient gradients through larger pellets causing transportational limitations was suggested to reduce the metabolite production. The increase in chitinase production using mycelial inoculum as compared to spore inoculum as observed in case of *V. lecanii* (Matsumoto *et al*, 2004) and *T. harzianum* (Sandhya *et al*, 2004), was not observed in case of *M. verrucaria*. This may be attributed to the production of larger pellets in the presence of mycelial inoculum as compared to those in the presence of spore inoculum.

4B.2.1.5 Effect of agitation

Increase in chitinase production (0.8 ± 0.05 to 1.8 ± 0.2 U/ml chitinase) by *M. verrucaria* was observed with increase in agitation rate from 100-200 rpm (Fig. 4.6). The highest chitinase activity (1.8 ± 0.2 U/ml) was produced at agitation rate 200 rpm. Further increase in agitation rate resulted in decrease in chitinase production. Lower chitinase production by *V. lecanii* at higher agitation rate was ascribed to the shearing effect on the cells by Liu *et al* (2003).

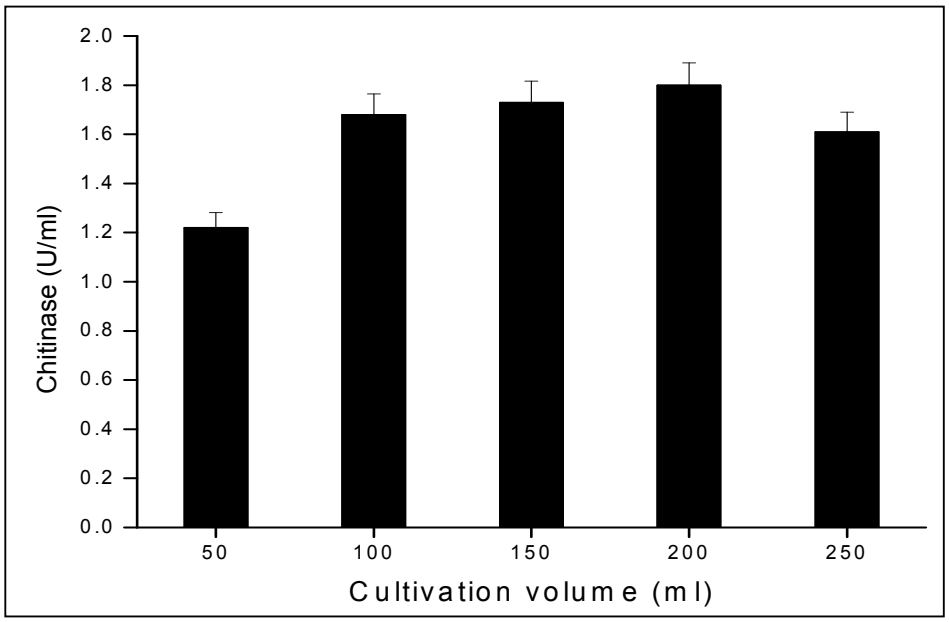
Fig. 4.6 Effect of agitation on chitinase production by *M. verrucaria*



4B.2.1.6 Effect of cultivation volume

The effect of different cultivation volumes of chitin medium (50-250 ml) in the 500 ml Erlenmeyer flask on chitinase production by *M. verrucaria* was studied. Vyas and Deshpande (1989) reported maximum chtinase production (1.8 ± 0.2 U/ml) *M. verrucaria*, with cultivation volume 100 ml. However, in the present studies maximum chitinase activity (1.8 ± 0.2 U/ml) was produced in flasks containing 200 ml cultivation volume (Fig. 4.7). Chitinase production increased from 1.2 ± 0.1 to 1.8 ± 0.2 U/ml with increase in cultivation volume from 50 to 200 ml. While with further increase in cultivation volume decrease in chitinase activity was observed possibly due to less dissolved oxygen. Liu *et al* (2003) studied the effect of cultivation medium volume on chitinase production by *V. lecanii*. The highest chitinase activity in this case was also obtained when 200 ml cultivation medium volume in 500 ml capacity Erlenmeyer flask was used. The highest production of chitinase was suggested due to the compromise between the mass transfer and/or shear stress at this volume (Liu *et al*, 2003).

Fig. 4.7 Effect of cultivation volume on chitinase production by *M. verrucaria*

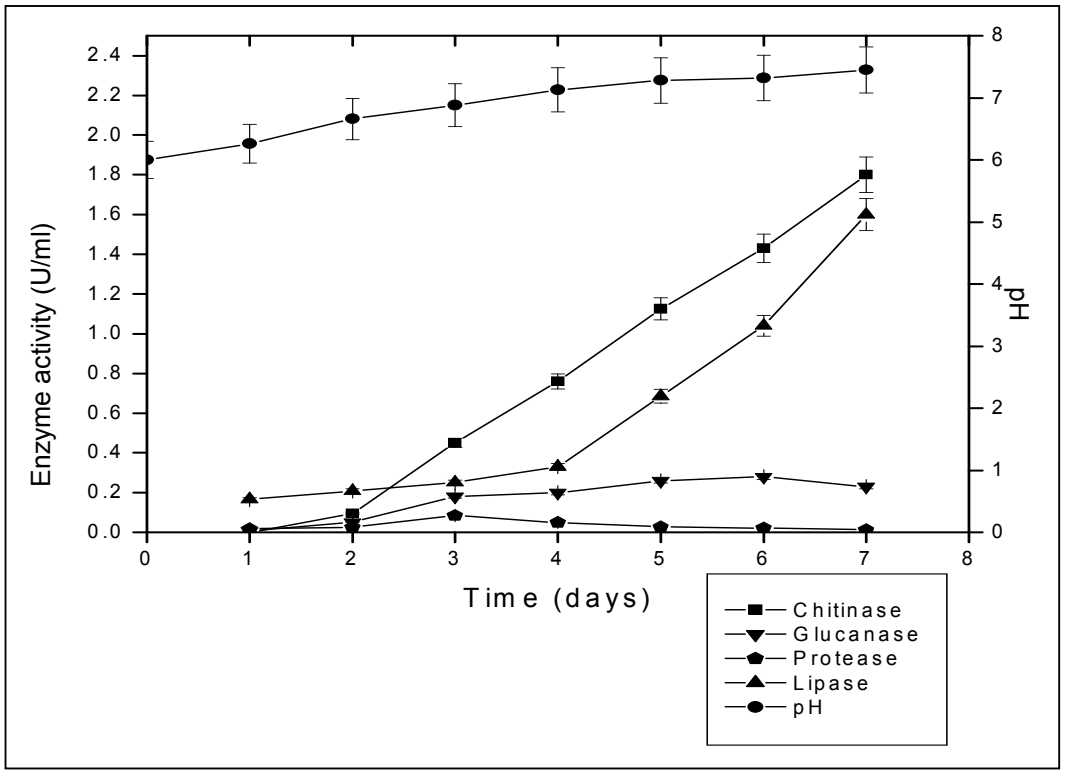


Taking into account above experiments, the highest chitinase production by *M. verrucaria* could be achieved in an optimized culture medium by using culture volume at 200 ml with agitation rate at 200 rpm, at 28°C within 7 days.

4B.2.1.7 Time course for production of cuticle degrading / mycolytic enzyme complex in shake flask

The conditions optimized for chitinase production by *M. verrucaria* in shake flask: chitin (Sigma) as carbon source, supplementation of yeast extract and peptone in production medium, constant medium pH 6.0, mycelial inoculum (10% v/v) grown in chitin medium, agitation 200 rpm and cultivation volume 200 ml in 500 ml capacity Erlenmeyer flask. Under these conditions production of other enzymes in CDE/ME complex, β-1,3-glucanase, protease and lipase was also monitored. Fig. 4.8 reveals the production profile of the CDE/ME complex of *M. verrucaria* under optimized shake flask conditions. Steady increase in chitinase activity was observed up to 7 d after the initial lag period of 2 d. The highest chitinase activity (1.8 ± 0.2 U/ml) was obtained on

Fig. 4.8 The production profile of cuticle degrading/mycolytic enzyme complex by *M. verrucaria* under shake flask conditions



7 d of incubation. Similarly, the highest lipase activity (1.6 ± 0.2 U/ml) was produced on 7 d of incubation. β-1,3-Glucanase activity increased from 0.0074 ± 0.0006 U/ml to 0.28 ± 0.004 U/ml within 6 d. On further incubation up to 7 d, 18% reduction in β-1,3 glucanase activity (0.23 ± 0.003 U/ml) was observed. The maximum alkaline protease activity (0.084 ± 0.0008 U/ml) was obtained on 3 d. With further increase in incubation period, protease production decreased slowly, and on 7th day 0.013 ± 0.004 U/ml protease activity was observed. The chitosanase and mannanase activities were also measured in the 7th day culture filtrate. Chitosanase (1.6 ± 0.2 U/ml) and mannanase (0.019 ± 0.004 U/ml) activities were also detected on 7th d of incubation. The 7th day culture filtrate was used as CDE/ME preparation for the control of plant pathogenic fungi and insects.

4B.2.2 Fermenter studies

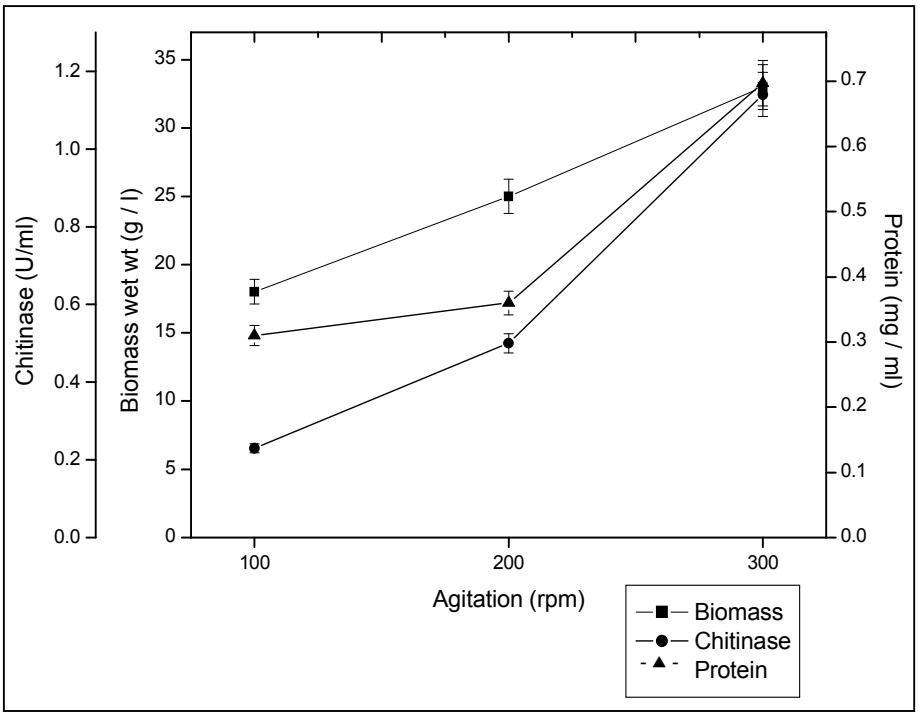
Batch fermentations were carried out for CDE/ME complex production in 15 l capacity locally made stirred tank fermenter with the probes to monitor temperature, dissolved oxygen and pH. Attempts were made to optimize agitation and aeration conditions for the production of CDE/ME complex.

4B.2.2.1 Effect of agitation

Agitation rate is an important parameter as it plays a significant role in determining the productivity by providing mixing and 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, 2000; Liu *et al*, 2003). Every fermentation has its own optimum agitator speed. This agitator speed will depend upon the resistance of the organism to shear, its morphological state, the nutrient composition, pH and many other conditions (Felse and Panda, 2000).

To study the effect of agitation on chitinase production, a series of experiments were conducted at different agitator speed. The agitator speed was varied between 100 and 400 rpm. In these experiments, except the change in agitator speed, all the other conditions were maintained as follows: initial chitin concentration 5.0 g/l; initial pH 6.0 (uncontrolled) and mycelial inoculum (10% v/v); working volume, 10 l; aeration rate, 1.0 vvm. A consistent increase in biomass (18 ± 0.9 to 33 ± 1.5 g/l), extracellular protein secretion (0.31 ± 0.006 to 0.69 ± 0.005 mg/ml) and chitinase production (0.23 ± 0.003 to 1.14 ± 0.009 U/ml) was observed with increase in agitator speed from 100 to 300 rpm (Fig. 4.9). The highest chitinase activity (1.14 U/ml) was produced by *M. verrucaria* within 5 days of incubation with the agitator speed of 300 rpm. Further increase in agitator speed (400 rpm) resulted in increased foaming. The chitin particles

Fig. 4.9 Variation in biomass, extracellular protein and chitinase production with agitation rate



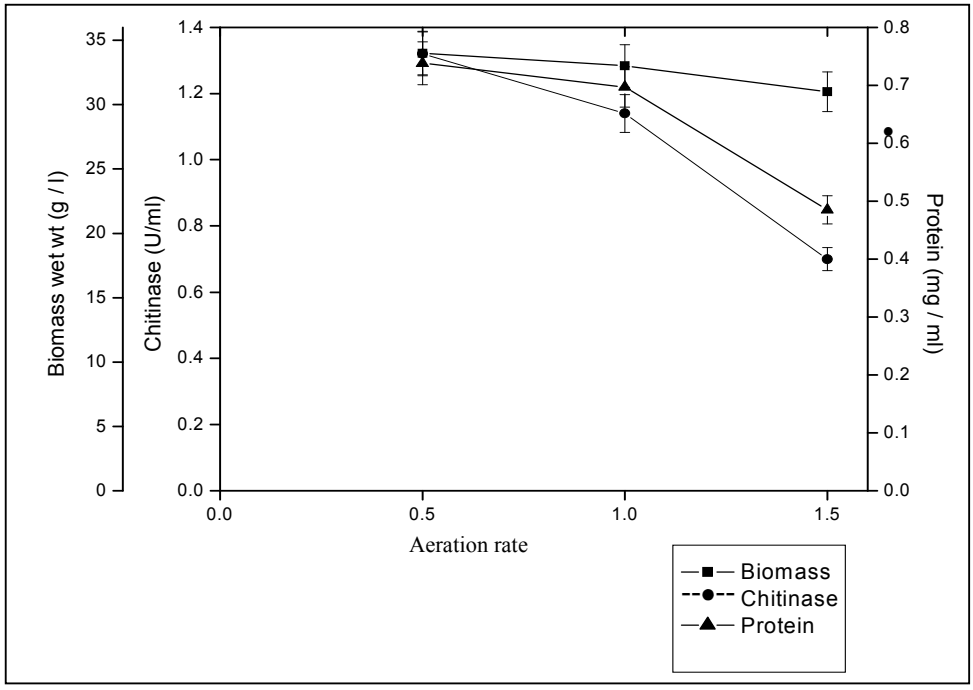
entrapped in foam accumulated above the liquid surface making the substrate inaccessible to the growing mycelium of *M. verrucaria*. Felse and Panda (2000) reported the maximal chitinase production by *T. harzianum* at agitator speed of 224 rpm. While the optimum agitator speed for the chitinase production by *P. janthinellum* was observed to be 500 rpm (Fenice *et al*, 1998). The highest chitinase production by *V. lecanii* F091 was observed with the agitator speed of 150 rpm. All these studies indicate that the production of chitinase is facilitated with certain critical agitator speed specific for a particular fungal 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*, 1998) and incomplete mixing and/or mass transfer resistance (Felse and Panda, 2000). 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*, 1998; Liu *et al*, 2003). From the present studies 300 rpm was found to be optimal agitator speed for the production of chitinase by *M. verrucaria*.

4B.2.2.2 Effect of aeration

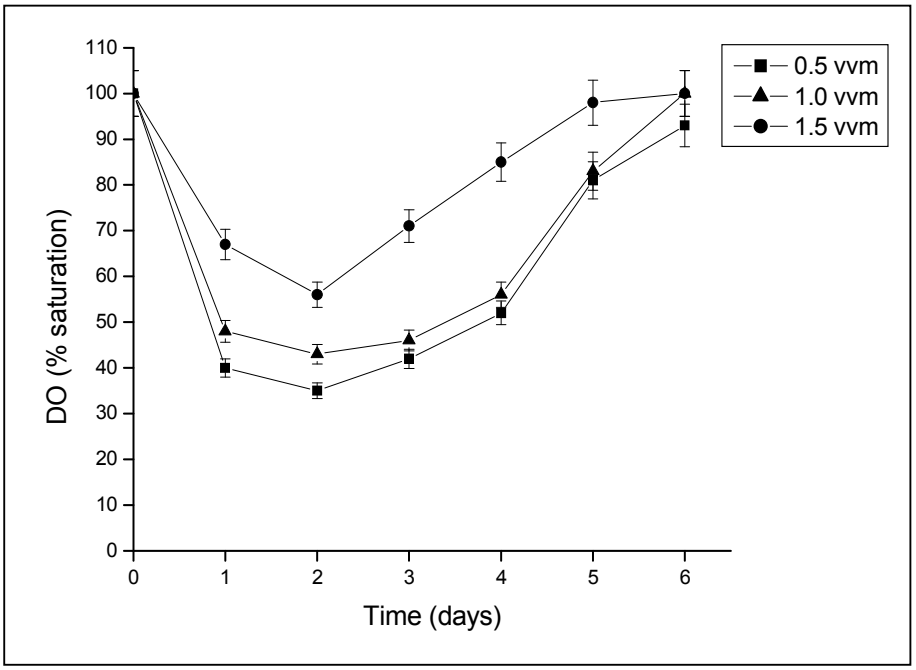
With optimum agitation rate 300 rpm, the effect of aeration was investigated by comparing the chitinase production at three aeration rates, namely 0.5, 1.0 and 1.5 vvm. The time course for chitinase activity and dissolved oxygen (DO) during cultivation of *M. verrucaria* in chitin medium were depicted in Fig. 4.10 and Fig. 4.11, respectively. Decrease in biomass (34 ± 2.3 to 31± 1.6 g/l), extracellular protein (0.73 ± 0.04 to 0.48 ± 0.02 mg/ml) and chitinase (1.32 ± 0.1 to 0.7 ± 0.08 U/ml) production by *M. verrucaria* was observed with increase in aeration rates. Chitinase activities on 5th day of incubation with aeration rates of 0.5, 1.0 and 1.5 vvm were 1.32, 1.14 and 0.7 U/ml, respectively (Fig. 4.10). With increase in aeration rate significant decrease in biomass was not observed, but the decrease in chitinase production was very rapid.

Fig. 4.10 Time course of chitinase production using *M. verrucaria* under different aeration rates



The DO concentration profiles were slightly different under different aeration rates (Fig. 4.11). At low aeration rate of 0.5 and 1.0 vvm, DO levels were between 40-50% saturation for most of the incubation period. In contrast, DO levels were above

Fig. 4.11 Time course of DO level under different aeration rates during chitinase production using *M. verrucaria*

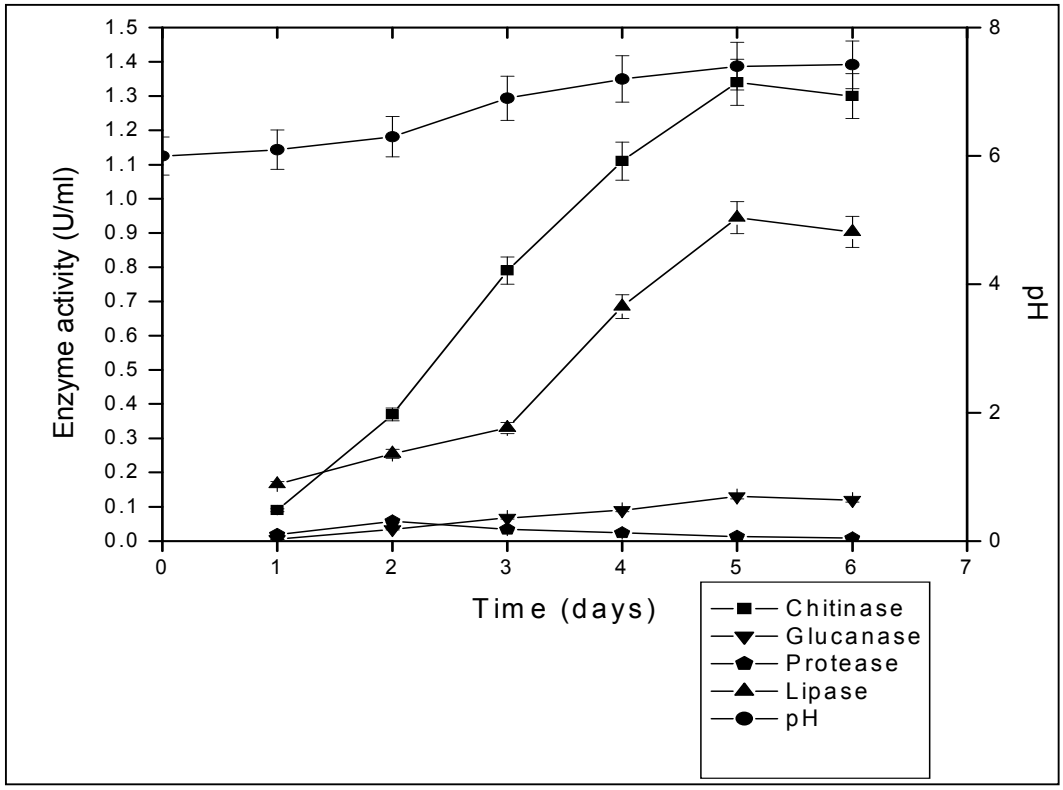


55% saturation at higher aeration rate, 1.5 vvm. From these results it may be suggested that the chitinase production was affected by higher DO saturation levels (≥ 55%) inculture broth. The aeration rate, 0.5 vvm was selected for the chitinase production. Fenice *et al* (1998) studied the effect of aeration rate 0.5-1.5 vvm on the chitinase production by *P. janthinellum*. The maximum chitinase production was observed with 1.5 vvm aeration rate.

4B.2.2.3 Time course for production of cuticle degrading/mycolytic enzyme complex by *M. verrucaria* in the fermenter

The time course for the production of cuticle degrading / mycolytic enzymes was studied under the conditions optimized for chitinase production (Fig. 4.12). The fermentation was carried out with aeration 0.5 vvm and agitation 300 rpm, at 28°C for 6

Fig. 4.12 Time course for cuticle degrading/mycolytic enzyme complex production in stirred tank fermenter

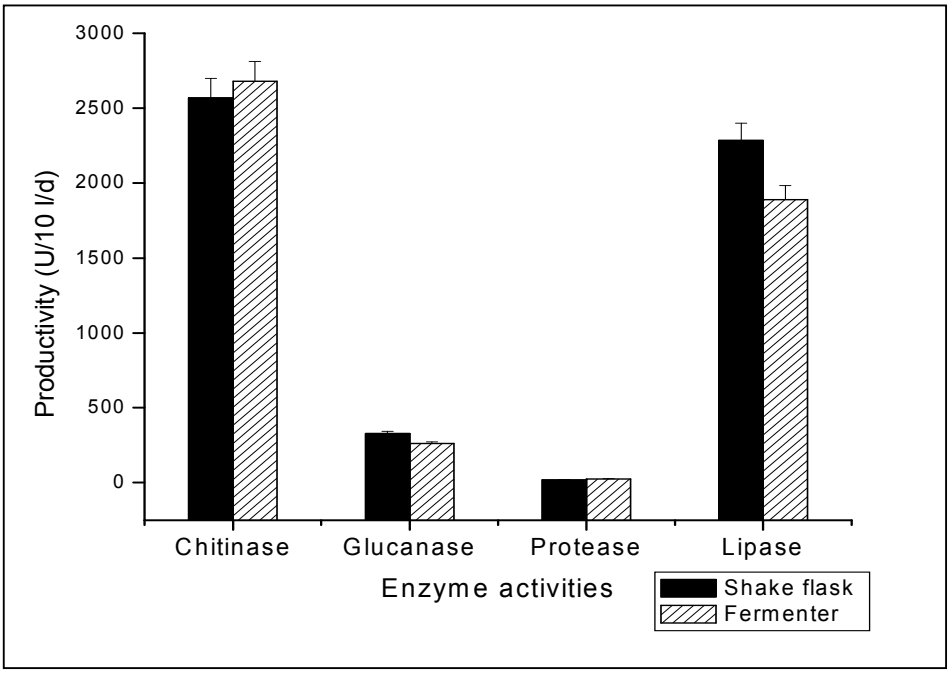


d. In the fermenter, chitinase, 1.32 ± 0.06 U/ml; β-1,3-glucanase, 0.13 ± 0.005 U/ml, protease 0.011 ± 0.001 U/ml and lipase 0.945 ± 0.005 U/ml were produced within 5 d. Further increase in incubation period did not result in increase in production of enzymes. Thus, fermentation was terminated after 5 d of incubation. Fig. 4.13 reveals the productivities of different cuticle degrading and mycolytic enzymes under shake flask and fermenter (15 l) levels. Though the enzyme activity levels obtained in

fermenter were lower (25-43%) than the shake flask, the productivities of these enzymes were found to be comparable. Marginal increases (6-14%) in chitinase and protease productivities were observed at fermenter level as compared to that at the shake flask level. While decreases (17-20%) in β-1,3-glucanase and lipase productivities were observed at fermenter level.

Thus, in the present studies agitation (300 rpm) and aeration (0.5 vvm) rates were optimized for the production of CDE/ME complex by *M. verrucaria* in stirred tank fermenter. It was observed that *M. verrucaria* produced larger pellets (diameter ≥ 3 mm) when grown under the optimized aeration agitation conditions in stirred tank

Fig. 4.13 Productivity of different cuticle degrading and mycolytic enzymes under shake flask and fermenter (15 l) levels.



fermenter. Further investigations on the effect of pellet size or morphology (pelleted or dispersed) of *M. verrucaria* on enzyme production and appropriate changes in culture conditions may help in increasing enzyme productivity. Furthermore, controlled pH (6.0) in shake flask studies resulted in 16% increase in chitinase production. Further

studies on controlled pH with optimized agitation and aeration will possibly be useful to obtain maximum CDE/ME activities to make the production commercially viable.

Chapter 5

Biocontrol efficacy of living propagules and enzymic components of *M. verrucaria* against fungal and insect pests

5.1 Introduction

Interest in the control of seed borne or soil borne plant pathogenic fungi has greatly increased in recent years as these fungi reduce the seed dormancy, percentage seed germination and adversely affect the seedling vigour resulting in significant yield losses. Moreover, a seed borne inoculum plays a vital role in the disease cycle leading to epidemics. *S. rolfsii* is the devastating soil-borne fungal pathogen infecting over 500 species of plants. The wide host range, prolific growth and ability to produce persistent sclerotia contribute to the large economic losses associated with *S. rolfsii* in agricultural crops like peanut, cowpea, pigeon pea, corn and horticultural plants like jasmine, lilium, chrysanthemum, etc. (Patibanda *et al*, 2002; Uma Maheshwari *et al*, 2002). Several fungicides have been reported to inhibit the mycelial growth of *S. rolfsii* (Patil and Rane, 1982; Singh and Dwivedi, 1989). However, none of the chemical fungicides tested were fungicidal to sclerotia of the pathogen (Singh and Dwivedi, 1989).

Other than fungal pathogens the agricultural pests known so far include 2,000 species of insects. Among the insect pests *H. armigera*, a lepidopteron insect is reported to cause more than 50% losses in yield of the important crops such as cotton, pulses, vegetables and sunflower, in India (Wahab, 2004). The development of resistance in *H. armigera* to chemical insecticides has aggravated the problem. *S. litura*, another lepidopteron pest on cotton, rocked the fertile cotton districts in Andhra Pradesh in year 1998. It destroyed 370,000 hectares of crop, which led to suicides by 44 farmers frightened by the complete harvest loss in cotton fields (News: Pestilence drives Andhra farmers to suicide, January 19, 1998). While in year 2000, outbreak of *S. litura* caused severe losses in soybean in Rajasthan. The diamondback moth (DBM), *P. xylostella* is the major pest in crucifers. Its outbreak was reported in cauliflower and cabbage crops around Delhi in 1999. This pest has

an extraordinary propensity to develop resistance to every synthetic insecticide used to control it. The escalating cost of managing this pest on commercially grown crucifers reveals the inadequacy of existing management efforts. Sugarcane woolly aphid, *C. lanigera* was reported in outbreak proportions from western and southern India in the year 2002 (Joshi and Viraktamath, 2004). The woolly aphid infestation on sugarcane not only severely affected the yield but also posed health hazards to farmers and cattle.

In view of the devastating nature of fungal and insect pests the development of a common agent to control both could be advantageous. The outermost protective covers that are, cell wall in fungal pathogens and cuticle in insect pests share common structural component, chitin. Thus, it provides an opportunity to develop a common agent targeting the protective covers for the control of both fungal pathogens and insect pests. *M. verrucaria* produces extracellularly the hydrolytic enzymes like chitinase, β-1,3 glucanase, lipase and protease involved in degradation of fungal cell wall and insect cuticle, when grown in a medium containing chitin as a sole source of carbon (Vyas and Deshpande, 1989). The aim of the present study was to demonstrate the effective use of living propagules (conidia / yeast-like cells) of *M. verrucaria* and/or the hydrolytic enzyme complex to control both plant pathogenic fungi and insects.

5.2 Results and discussion

5.2.1 Formulation studies

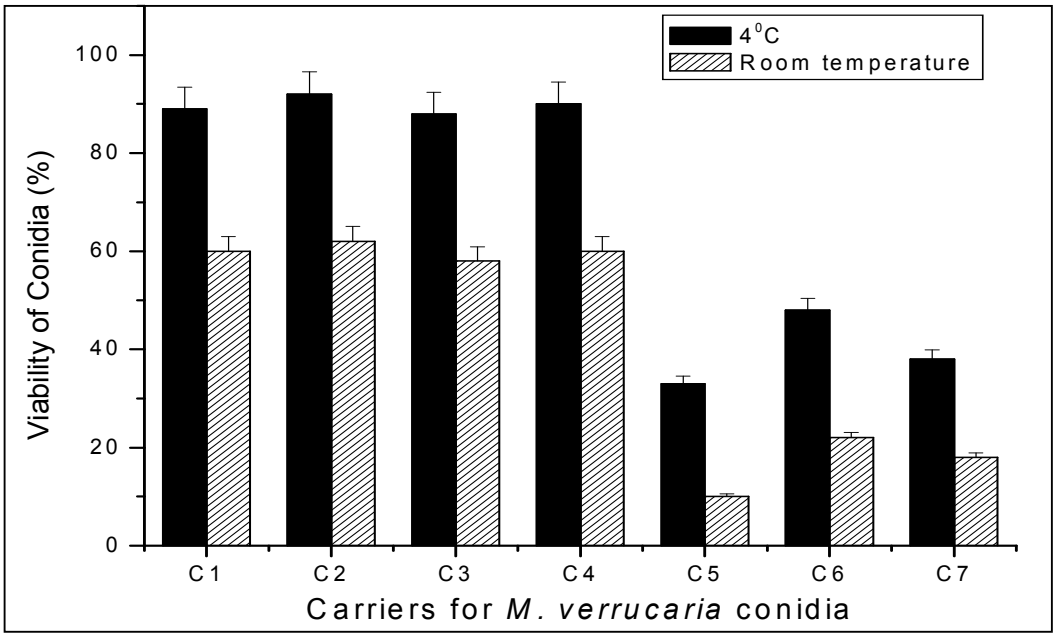
5.2.1.1 Conidial formulation

The microbial agents are usually formulated with different carriers or agents that facilitate packaging, extend shelf life and render the preparation easy to handle (Paau, 1988). Recently, Batta (2004) reported the use of dust carriers like charcoal

powder, oven-ash and chalk powder to prolong the shelf-life of *M. anisopliae* conidia used to control rice weevil in stored grains. Organic carriers like rice bran, wheat bran, baggase were also used as carriers for different biocontrol fungi (Chavan *et al*, 2006). Similar formulations can also be used to control plant pathogenic fungi.

M. verrucaria conidia produced by solid-state fermentation on sorghum grains were harvested after 14 d of incubation using 0.01% Tween 80. The conidial suspension (1 ml containing $1x10^{10}$ conidia) was mixed with sterile 100 g organic carriers like rice bran, wheat bran, prawn shell waste and conventionally used carriers like charcoal powder or precipitated silica powder. The preparations were stored in sealed plastic bags at room temperature or at 4°C until used. From Fig. 5.1 it can be seen that the viability of conidia in different carriers was more at 4°C than

Fig. 5.1 Effect of temperature on viability of *M. verrucaria* conidia formulated with different carriers



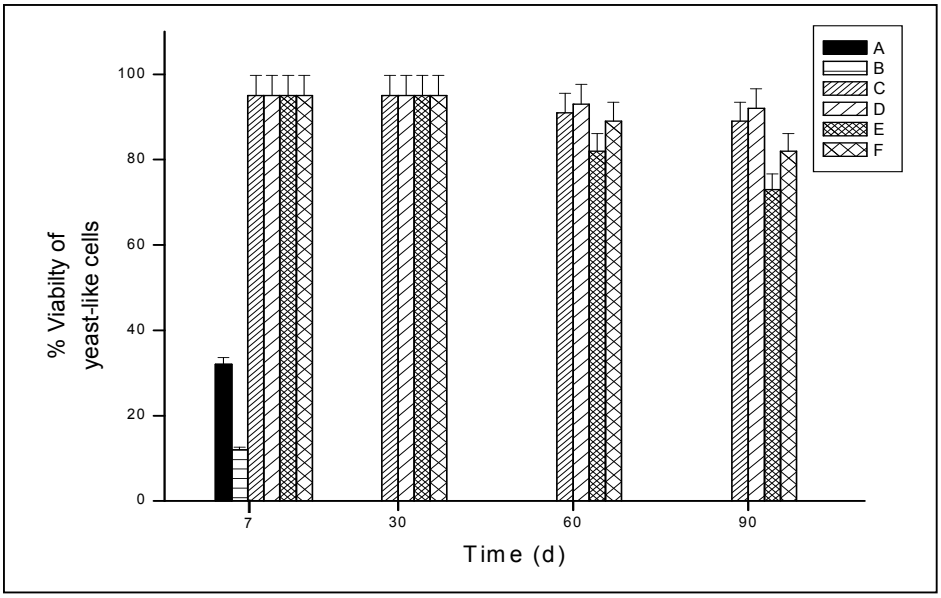
* *M. verrucaria* conidia were mixed with different carriers namely, C1: Rice bran, C2: Rice bran + chitin, C3: Wheat bran, C4: Wheat bran + chitin, C5: Prawn shell waste, C6: Charcoal powder and C7: Precipitated silica powder. These preparations were stored at 4°C and room temperature for 18 months.

at room temperature when tested after 18 months, a desirable period for the potential commercial preparation. *M. verrucaria* conidia in organic carriers wheat bran and rice bran with and without chitin supplement (2 g chitin in 100 g wheat bran or rice bran) retained >80% viability when stored at 4°C and 54-62% viability after 18 months storage at room temperature (Fig. 5.1). When prawn shell waste, charcoal powder and precipitated silica were used, as carriers the TVC after 18 months were 10, 22, 18 %, respectively. The, rice and wheat bran with and without chitin supplement (C1-C4) were found to be the best carriers for *M. verrucaria* conidia. Though, the chitin supplement did not significantly improve the shelf life of conidia. It was found to be useful in the production of chitinolytic enzymes by *M. verrucaria* after application of conidia to soil. The conidia mixed in rice bran with and without chitin supplement were used for the soil application to control pre-emergence rot of peanut caused by *S. rolfsii*.

5.2.1.2 Yeast-like cells formulation

The vegetative yeast-like cells provided an alternative living propagule of *M. verrucaria* to develop biocontrol preparation. The yeast-like cells were immobilized in alginate beads with or without 0.5% w/v chitin under aseptic conditions. The beads initially 5-6 mm in diameter, shrunk to 3-4 mm in diameter after air-drying in laminar airflow for 1 h. From yeast-like cells (1 g wet wt, 4.2×10^{11} cfu/g wet wt), ≈20 g of alginate beads were produced. TVC of yeast-like cells was $(1.9 \pm 0.3) \times 10^{9}$ cfu/g of alginate beads. To study the effect of temperature on viability of free as well as immobilized yeast-like cells, they were stored in sterile, airtight plastic vials and incubated at 4°C and room temperature. Fig. 5.2 reveals that the viability of free yeast cells decreased rapidly when stored at room temperature as well as at 4°C (12

Fig. 5.2 Effect of temperature on the viability of yeast-like cells of *M. verrucaria* entrapped in alginate beads with or without chitin



*A: Yeast-like cells stored at 4°C; B: Yeast-like cells stored at room temperature; C: Yeast-like cells immobilized without chitin, stored at 4°C; D: Yeast-like cells immobilized with chitin, stored at 4°C; E: Yeast-like cells immobilized without chitin, stored at room temperature; F: Yeast-like cells immobilized with chitin, stored at room temperature

and 33 % TVC after 7d, repectively). While up to 60 days the viability of yeast-like cells was not significantly affected by storage temperature. With further increase in incubation period viability of yeast-like cells (>73% TVC after 3 months storage) stored at room temperature decreased rapidly. The immobilized yeast-like cells retained >90% viability at refrigeration temperature (4°C) after 3 months storage. Chitin supplementation did not significantly improve the viability of yeast-like cells during storage at different temperatures.

The chitinase production by yeast-like cells immobilized with or without chitin was compared with that of free yeast-like cells of *M. verrucaria*. The chitinase production using immobilized yeast-like cells (1% w/v) as an inoculum was higher (0.9 U/ml) with added chitin than the immobilized yeast-like cells without chitin (0.6 U/ml) and free yeast-like cells (0.55 U/ml) under identical incubation conditions

(28°C, under shaking conditions 200 rpm, for 7 d). El-Katatny *et al* (2003) reported the enhanced chitinase production by conidia of *Trichoderma* species entrapped in alginate matrix along with chitin as compared to those entrapped without chitin. The immobilized yeast-like cells of *M. verrucaria* with and without chitin were used for soil application to control *S. rolfsii* infection in peanut.

5.2.1.3 Cuticle degrading/mycolytic enzyme preparation

M. verrucaria was grown in chitin containing medium under shaking conditions (200 rpm) at 28ºC for 7 d. The cell free broth containing chitinase, β-1,3-glucanase, protease and lipase was used as CDE/ME preparation. The pH of the culture filatrate was adjusted to 5.0 using citric acid (0.2 M). For the use of enzyme preparation for control of fungal pathogens or insect pests, the major constraint was its volume, which was affecting storage and transportation. Attempts were made to concentrate enzyme preparation, using polysulphone hollow fiber membrane of 10 kDa molecular weight cut-off. This was further concentrated using spray drying or freeze drying.

Table 5.1 Concentration of cuticle degrading/mycolytic enzyme preparation using different methods

Concentration method	Quantity	Chitinase (U)	Recovery (%)
Culture filtrate	10,000 ml	18,000	-
Ultra filtration	500 ml	14,760	82
Freeze drying	32 g	12,841	71.3
Spray drying	25 g	5,904	32.8

* Ultrafiltration concentrate was further concentrated using either freeze drying or spray drying.

Table 5.1 reveals the % recovery of chitinase activity during different enzyme concentration methods. Using ultra filtration, 10 l enzyme preparation was concentrated to 500 ml. This ultra filtration concentrate was further concentrated to powder form (32 g) by freeze-drying using DW6 Freeze dryer (Heto Lab Equipment, Denmark). The maximum recovery of chitinase activity (71.3%) was noted after concentration of enzyme preparation by freeze drying. The ultrafiltration concentrate when further concentrated to powder form (25 g) by spray drying using Buchi 190 Mini Spray Dryer at 60°C resulted in 67.2% loss in the recovery of chitinase activity. Thus, the combination of ultrafiltration and freeze-drying methods was found suitable to concentrate the enzyme preparation. The powder obtained after freeze drying can be stored in airtight plastic bottles at –20°C up to 3 months without significant loss in chitinase activity. For the control of plant pathogenic fungi and insect pests, enzyme powder was diluted using distilled water to get chitinase activity (1-6 U/ml) and applied to soil or on insects.

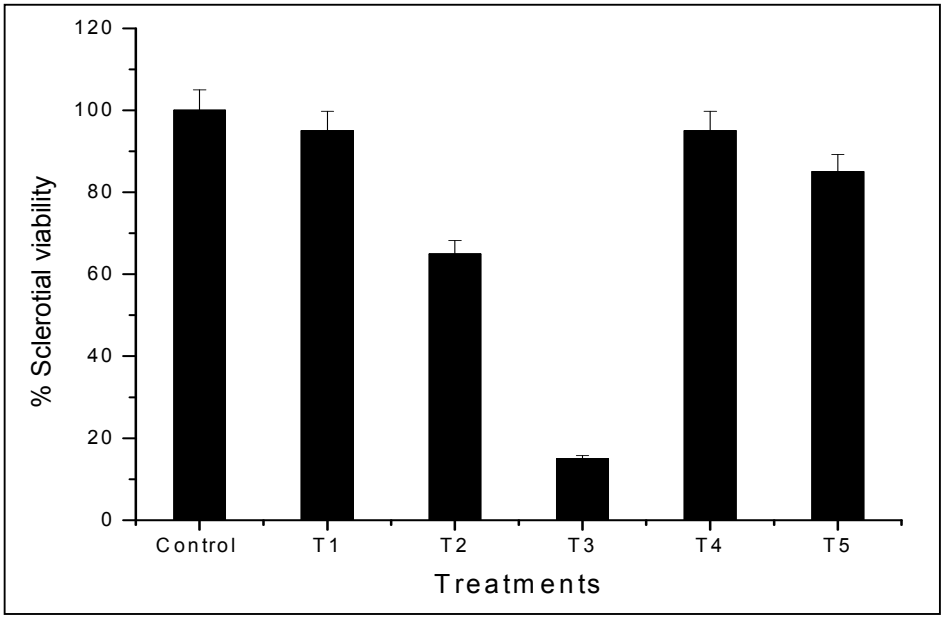
5.2.2 Control of *Sclerotium rolfsii* infection in peanut

5.2.2.1 Plate assays

To study the fungus-fungus interaction between *M. verrucaria* and plant pathogenic root infecting fungus, *S. rolfsii*, dual culture plate assays were carried out using minimal medium and chitin medium. On chitin medium, 54% inhibition of the mycelial growth of *S. rolfsii* was observed after 4 d of incubation at 28°C. While on minimal medium containing glucose, growth of *S. rolfsii* mycelium was not significantly affected. This can be attributed to the production of mycolytic enzymes by *M. verrucaria* on chitin medium. Unlike mycoparasitic fungi, microscopic observation of the dual culture plate assays did not reveal any hyphal interaction between *M. verrucaria* and *S. rolfsii*.

Elad *et al* (1983) and Prasad *et al* (1999) reported that culture filtrates of *Trichoderma* species containing chitinase and β-1,3-glucanase affected the viability of sclerotia of *S. rolfsii*.The effect of mycolytic enzyme complex of *M. verrucaria* on the viability of sclerotia, the resting structures of *S. rolfsii* was studied using a method of Prasad *et al* (1999). The effect of various treatments on sclerotial viability was assessed by placing sclerotia on sterile Whatman No. 1 filter paper impregnated with mycolytic enzume preparation or chemical fungicide, carzim and that impregnated with sterile distilled water served as control. The sclerotial viability was affected significantly with 1.0 U/ml chtinase activity (Fig. 5.3). The marginal decrease (5-15%) in sclerotial viability was observed in the presence of a chemical fungicide Carzim (0.4-0.8 % w/v, concentration recommended for control of *S.*

Fig. 5.3 Effect of mycolytic enzyme complex of *M. verrucaria* on the viability of sclerotia under plate assay conditions



* Treatments: Control. Distilled water, T1. Chitinase (0.1 U/ml), T2. Chitinase (0.5 U/ml), T3. Chitinase (1.0 U/ml), T4. Carzim (0.4% w/v), T6. Carzim (0.8% w/v).

rolfsii in field). Thus mycolytic enzyme treatment was found more effective in reducing the sclerotial viability under laboratory assay conditions.

Germination trial was carried out to assess the potential of *M. verrucaria* to control *S. rolfsii* infection on peanut. The peanut seeds coated with *S. rolfsii* suspension and those soaked in sterile water served as controls. The test seeds were soaked in conidial suspension of *M. verrucaria* and then coated with *S. rolfsii* mycelial suspension. The treated seeds were placed on moist filter paper in sterile Petri plates and incubated at room temperature upto 7 d. The germination of peanut seeds was affected (80% reduction) in the presence of *S. rolfsii* treatment. The coating of seeds with *M. verrucaria* conidia was found to protect the seeds from the infection with *S. rolfsii*. In this set 60% seed germination was observed. The length of the radicle was measured on 5th day after germination to assess the plant growth promoting effect of the treatment. Average length of the radicles in control (sterile water) and *M. verrucaria* conidial treatment receiving sets was 3.5 ± 0.3 cm, suggesting the absence of any growth promoting effect of *M. verrucaria.* However, germination trial results confirm the potential of *M. verrucaria* to control *S. rolfsii* infection in peanut.

5.2.2.2 Pot experiments

Pot experiments were carried out to assess the efficacy of different preparations containing living propagules (conidia and yeast-like cells) or mycolytic enzymes of *M. verrucaria* to control pre-emergence rot of peanut caused by *S. rolfsii*. In the first pot experiment efficacy of the rice bran-conidia preparation with and without chitin and immobilized yeast-like cells with and without chitin was compared with the conventionally used chemical fungicide, Carzim. Table 5.2 depicts the results of this experiment. In the infested soil only 30% seeds germinated. Furthermore, the effect was

Table 5.2 Control of pre-emergence rot in peanut using various formulations containing *M. verrucaria* conidia or yeast-like cells

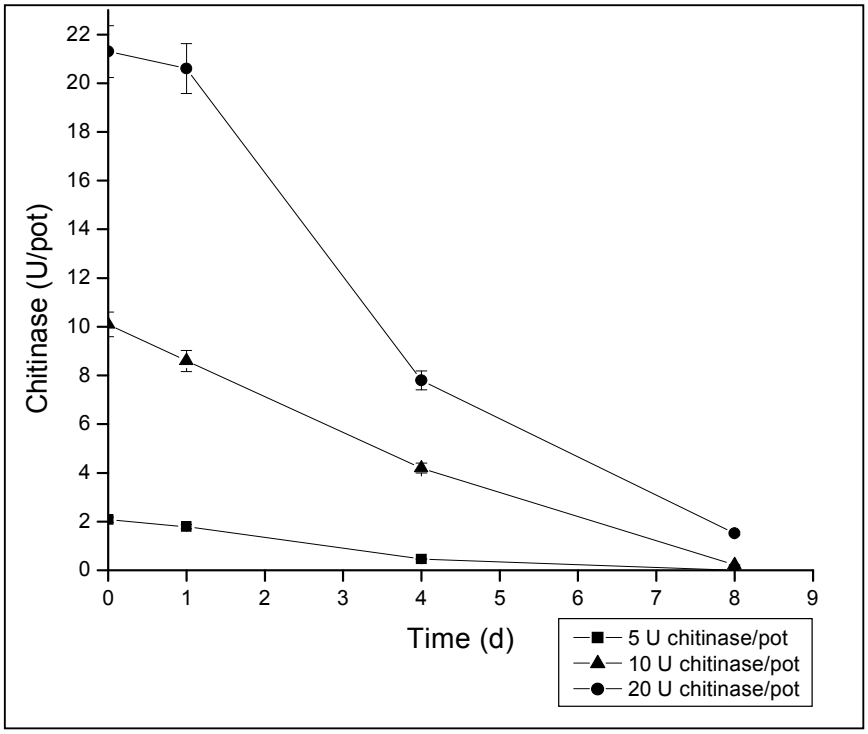
Formulations	Seed germination (%)	Average shoot length (cm)	Average root length (cm)
Control (Sterile soil)	100	15.0	16.0
Control (Infested soil)	30	4.3	4.2
Rice bran formulation	50	14.4	14.3
Rice bran + chitin formulation	90	14.6	15.0
Yeast-like cells	40	12.9	13.5
Immobilized yeast-like cells	45	13.5	14.2
Immobilized yeast-like cells + chitin	60	15.0	15.2
Carzim	70	12.7	13.2

pronounced on the germinated seeds with respect to the shoot and root length (Table 5.2). The germination of seeds was found to be improved in the presence of different treatments. The conidia mixed with rice bran and chitin were found to be the most effective for the control of pre-emergence rot of peanut caused by *S. rolfsii*. Interestingly, for number of treatments though the seed germination was not improved drastically, the germinated seeds were healthy and plant vigour was good as compared to the plants grown in infested soil. The chitinolytic activity (0.008 to 0.012 U/g soil) was detected in soil receiving the treatment of *M. verrucaria* condia or yeast-like cells as against the untreated controls. dal Soglio *et al* (1998) reported the use of *T. harzianum* Th008 for the control of *R. solani* causing root rot. The disease control was correlated with the production of endochitinase by *T. harzianum* Th008 in the rhizosphere of soybean.

Patil *et al* (2000) reported that daily soil irrigation with mycolytic enzymes of *M. verrucaria* was found effective in the control of *S. rolfsii* infection in peanut. Hence

in the present studies mycolytic enzyme preparation was used for soil application at an interval of 1 week. The stability of the mycolytic enzymes in soil and their efficacy in controlling *S. rolfsii* infection in peanut was studied. The ME preparation (15 ml) containing 5-20 U chitinase was used per pot at a weekly interval. Residual chitinase activity in soil was estimated on 1, 4 and 7 days in first week and at the end of 2nd and 3rd week (Fig. 5.4). Under the pot experiment conditions (soil pH, 6.5 and

Fig. 5.4 Residual chitinase activity in soil after weekly soil application with ME preparation for the control of pre-emergence rot in peanut



temperature, 25-32°C), chitinase activity in soil decreased rapidly within 7 d. The residual chitinase activities of 0.22 ± 0.02 and 1.52 ± 0.2 U/pot, were observed in the pots receiving treatment of 10 and 20 U chitinase/pot, respectively.

The seed germination (20-60%) in *S. rolfsii* infested soil was observed with increase in chitinase concentration (5-20 U/pot) applied per pot (Table 5.3). The application of ME preparation containing 20 U chitinase resulted in 60% increase in peanut seed germination which was marginally higher than the chemical control,

Table 5.3 Control of pre-emergence rot in peanut using mycolytic enzyme preparation of *M. verrucaria*

Treatments	Seed germination (%)	Shoot length (cm)	Root length (cm)
Control (Sterile soil)	100	15.2	15.8
Control (Infested soil)	20	4.0	3.8
Chitinase 5.0 U/pot	40	13.7	14.2
Chitinase 10.0 U/pot	60	15.0	16.0
Chitinase 20.0 U/pot	80	14.9	15.6
Carzim	70	12.7	13.2

* NA: not applicable

Carzim (50% increase in seed germination). Assessment of plant vigour in terms of root and shoot lengths revealed that plants receiving ME preparation treatment were healthier than carzim treated plants. The root and shoot lengths of ME treated plants were comparable to those in plants in sterile soil.

5.2.3 Control of insect pests

5.2.3.1 Insect bioassays

M. verrucaria enzyme preparation contains all the components (chitinase, protease and lipase) required for insect cuticle degradation. Thus in present studies CDE preparation was tested against following insect pests, *H. armigera*, *S. litura*, *P. xylostella* by larval dip method. The >70% mortality was observed in all, with chitinase 6 U/ml (Table 5.4). The enzyme mixture was most effective against *P. xylostella* (37% mortality with chitinase 2 U/ml) within 72 h. While the same concentration of CDE preparation resulted in abnormal morphologies in *S. litura* during larva-pupa ecdysis

Table 5.4 Effect of *M. verrucaria* cuticle degrading enzyme preparation on 3rd instar larvae of *H. armigera, S. litura* and *P. xylostella*

Chitinase (U/ml)	Mortality (%), (72 h)		
	H. armigera	*S. litura*	*P. xylostella*
1.0	0.00	0.00	16.65
2.0	13.32	20.00	37.00
4.0	50.00	53.33	70.00
6.0	73.33	80.00	90.00
Control[1]	3.33	0.00	3.33
Control[2]	0.00	3.33	0.00

*Control[1]: Heat inactivated CDE preparation, Control[2]: Distilled water

(Fig. 5.5). The enzyme treated larvae of insect pests exhibited change in the colour of their cuticles within 48-72 h. Brown-black patches were observed on their cuticles. From these results it may be suggested that with increase in the concentration of CDE preparation dissolution of cuticle components increased resulting in death of the insect larvae.

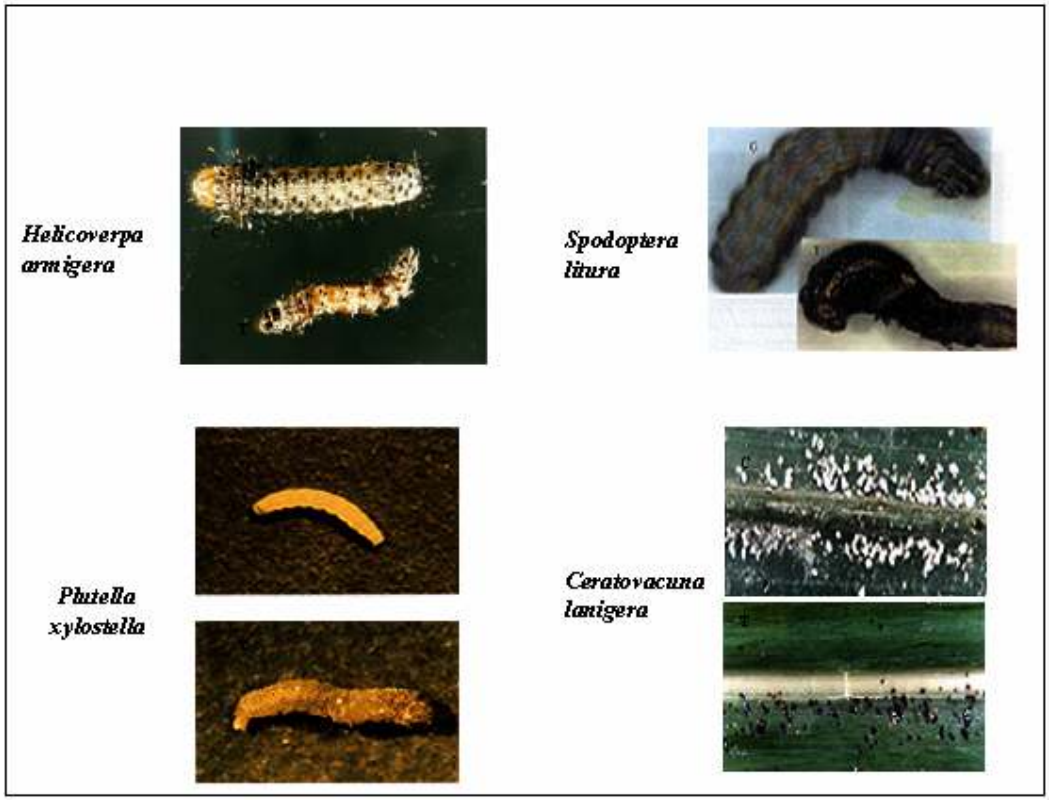
Effect of CDE preparation on sugarcane woolly aphid, *C. lanigera*, a sucking pest was studied by spray method. The mortality of woolly aphids increased from 64 to 88% when the concentration of CDE preparation was increased from 2-6 U/ml chitinase (Table 5.5). In enzyme treated adults woolly mass was hydrolyzed and aphids turned

Table 5.5 Effect of cuticle degrading enzyme preparation on *C. lanigera*

Chitinase (U/ml)	% Mortality (72 h)
1.0	64.0
2.0	70.0
4.0	78.0
6.0	88.0
Control[1]	13.0
Control[2]	9.0

*Control[1]: Heat inactivated CDE preparation, Control[2]: Distilled water

Fig.5.5 Effect of cuticle degrading enzyme preparation on insect pests



black (Fig. 5.5). Lipase activity in CDE preparation may play an important role in hydrolyzing woolly mass comprised of wax and lipids. Complete hydrolysis of wooly mass was observed within 5 h when incubated with CDE preparation of *M. verrucaria* (lipase 2.0 U/ml) at room temperature.

Woolly aphid excretes honeydew like substances, which favour the growth of *Capnodium* sp. (sooty mold) on sugarcane leaves. The fungal growth was reported to affect the photosynthesis and cause reduction in sugarcane yield and quality. The effect of *M. verrucaria* enzyme preparation on germination of *Capnodium* sp. was studied. With increase in chitinase concentration (2-6 U/ml) the *Capnodium* spore germination decreased (39-92%). Similarly, the mycolytic enzymes (chitinases, β-1,3-glucanases and proteases) of *Trichoderma* sp. were reported to degrade cell walls and inhibit spore germination or germ tube elongation in various phytopathogenic fungi such as *S. rolfsii*, *R. solanii* and *B. cinerea* (Markovich and Kononova, 2003). The present investigations revealed that the cuticle degrading / mycolytic enzyme preparation of *M. verrucaria* plays a dual role. It has the potential to control both the fungal pathogen *Capnodium* sp. as well as insect pest *C. lanigera* on sugarcane.

The cuticle degrading enzyme complex comprising of chitinase, protease and lipase was reported to facilitate the penetration of entomopathogenic fungi like *M. anisopliae*, *B. bassiana* into the insect body (Krieger de Moraes *et al*, 2003; St. Leger *et al*, 1986). In view of the effectiveness of the CDE preparation of *M. verrucaria* in disrupting the insect cuticle its combination with insect pathogenic fungi like *M. anisopliae*, *B. bassiana* and *N. reliyi* was tested against *H. armigera* larvae under laboratory conditions. The conidia of entomopathogenic fungi were mixed with the CDE preparation (2 U/ml chitinase). This preparation was used immediately or 2 h after mixing in the bioassay. CDE preparation did not affect the viability of conidia significantly even when conidia were incubated with CDE preparation for 2 h at

Table 5.6 Use of entomopathogenic fungi and cuticle degrading enzyme preparation of *M. verrucaria* singly or in combination against 3rd instar *H. armigera* larvae

Treatment	Spore germination (%)	Mortality (%)	LT_{50} (d)
B. bassiana (10^7 spores/ml)	85.1	87.5	5.0
B. bassiana (10^5 spores/ml)	84.9	50.0	6.0
B. bassiana (10^5 spores/ml) + Chitinase	80.5	85.0	2.7
B. bassiana (10^5 spores/ml) + Chitinase*	78.9	80.0	2.5
M. anisopliae (10^7 spores/ml)	81.2	95.0	3.8
M. anisopliae (10^5 spores/ml)	83.2	53.5	5.2
M. anisopliae (10^5 spores /ml) + chitinase	80.7	95.0	2.3
M. anisopliae(10^5 spores/ml) + chitinase*	78.6	90.0	2.2
N. rileyi (10^7 spores/ml)	75.3	70.0	5.1
N. rileyi (10^5 spores/ml)	76.7	47.5	6.2
N. rileyi (10^5 spores/ml) + chitinase	75.3	87.5	2.5
N. rileyi (10^5 spores/ml) + Chitinase*	73.3	80.0	2.6
Chitinase	NA	20.0	-
Distilled water	NA	3.3	-

* *B. bassiana* / *M. anisopliae* /*N. rileyi*+ chitinase*: The fungal spores were incubated with chitinase (2 U/ml) prior to the use in bioassay
NA: Not applicable

room temperature (Table 5.6). With the 10^7 spores/ml of *B. bassiana* 87.5% mortality was seen within 14 d, while it was 95% with *M. anisopliae* and 70% with *N. rileyi*. The treatment with CDE preparation measured as chitinase (2 U/ml) showed only 20% mortality after 14 d. The % mortalities with 10^5 conidia/ml were

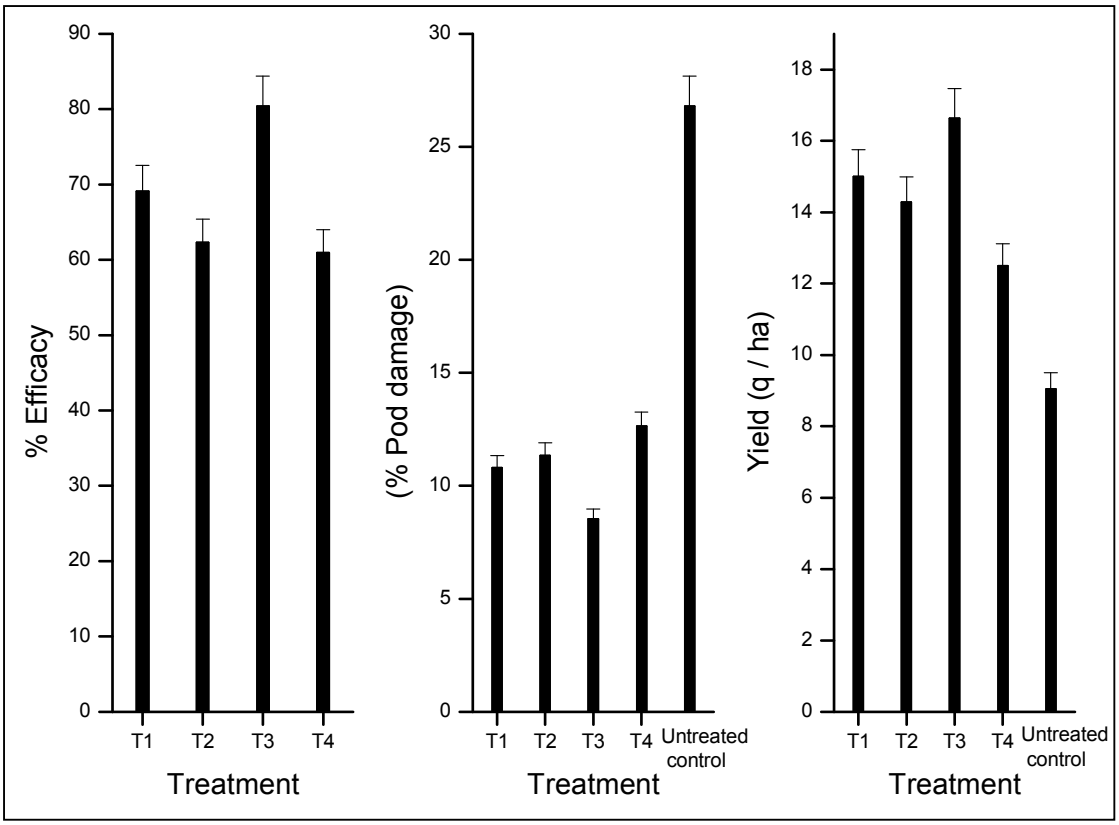
50, 53.5 and 47.5% with *B. bassiana*, *M. anisopliae* and *N. rileyi* respectively, within 14 d. However, the combination of CDE preparation with conidia of *B. bassiana*, *M. anisopliae* and *N. rileyi* (10^5 /ml) increased the % mortality. Recently, Shternshis (2005) reported that % mortality of *P. sticticalis* doubled when *B. thuringiensis* subsp. *sotto* was applied in combination with bacterial chitinase preparation. The insecticidal activity of two granulosis viruses (GV of *P. sticticalis* and *Cydia pomonella*) and NPV of *M. brassicae* 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. NPV as well as *B. thuringiensis* act on the insect after ingestion. The addition of exogenous chitinase favoured their penetration through chitinous peritrophic membrane in the gut resulting in improvement in biocontrol efficacy. The combination of chitinase preparation and entomopathogenic fungi such as *B. bassiana* and *V. lecanii* was tried with the expectation of increase in biocontrol efficacy through improved penetration of the fungus through the insect cuticle. However, a contradictory data was obtained due to inhibition of fungal growth in the presence of chitinase (Shternshis, 2005). The present studies revealed that germination of spores of entomopathogenic fungi namely, *M. anisolpliae*, *B. bassiana* and *N. rileyi* was not affected in the presence of cuticle degrading enzyme complex (measured as chitinase, 2.0 U/ml) of *M. verrucaria* and thus they can be effectively used in combination for the control of *H. armigera*.

5.2.3.2 Field trial

The CDE preparation of *M. verrucaria* was used to control *H. armigera* infestation in chickpea under field conditions. The results of the field trial were assessed using three different parameters viz., percent efficacy, percent pod damage

and yield (q/ha) (Fig. 5.6). Percent efficacy of various treatments after two sprays was found to be in the range of 60.1 to 80.38% against *H. armigera.* The treatment with *M. anisopliae* was found to be the most effective for the control of *H. armigera*.

Fig. 5.6 Efficacy, pod damage and yield of different treatments against *H. armigera* infestation on chickpea under field conditions



* T1: endosulfan, T2: HaNPV, T3: *M. anisopliae*, T4: *M. verrucaria* CDE preparation

The percent efficacy of CDE preparation was marginally less (60.1) than the endosulfan and NPV. As compared to the untreated control plot (26.8% pod damage) all the treatments showed % pod damage in the range of 8.54-12.64. Application of CDE preparation resulted in 53% reduction in %pod damage as compared to untreated control. The average yield in untreated control plots was 9.05 q/ha while it increased up to 13.87 q/ha in CDE treated plots. The chickpea yield was in the range of 14.28 – 16.64 q/ha when treated with endosulfan, NPV or *M.*

anisopliae. The field trial results showed that the efficacy of CDE treatment for the control of *H. armigera* was at par with that of edosulfan and NPV.

Thus present studies revealed that living propagules (conidia/yeast-like cells) of *M. verrucaria* formulated with different carriers along with chitin may be effectively used for the control of pre-emergence rot in peanut caused by *S. rolfsii*. While the CDE/ME preparation may be used effectively for the control of insect pests like *H. armigera*, *S. litura*, *P. xylostella* and *C. lanigera* singly or in combination with entomopathogenic fungi like *B. bassiana*, *M. anisopliae* and *N. rileyi*. Furthermore, soil application of ME preparation significantly reduced the incidence of pre-emergence rot in peanut.

5.2.4 Toxicity studies

Finally, the toxicity of the *M. verrucaria* organism and its enzyme preparation was tested in the laboratory by measuring oxynitrilase activity. Many plants produce cyanogenic glycosides, which may have role in defense against phytopathogens. These cyanoglycosides were oxidized by oxygenases to cyanohydrins, which were further converted to an aldehyde and HCN by oxynitrilases (hydroxynitrile lyases). *M. verrucaria* is known to utilize nitrile compounds with the help of nitrilases (Maier-Greiner *et al*, 1991). In extracts of yeast/hyphal cells and conidia of *M. verrucaria* as well as in CDE/ME preparation, oxynitrilase (mandelonitrile lyase) activity was not detected. This indicated its non-toxic nature, in general too.

Appendix 1

Development of a new method for estimation of chitinase

A 1 Introduction

In the literature different methods were reported to estimate the chitinase activity. The most sensitive methods used radiolabeled (^{3}H-chitin) and fluorogenic (4-methyl umbeliferyl labeled) substrates (Patil *et al*, 2000). However, these methods require specialized instruments and expensive reagents to measure chitinolytic activity. The use of less expensive soluble- and insoluble- chitin substrates linked with different dyes (Ostazin Brilliant Red labeled chitin, Remazol Brilliant Blue labeled chitin) in solid or liquid media as carbon source was reported to allow detection of chitinolytic activity (Bauer *et al*, 1998; Ramirez *et al*, 2004). The use of these dye-labeled chitin substrates for quantitative estimation of chitinases was not reported. Routinely, quantitative estimation of total chitinolytic activity is carried out using the acid swollen chitin as a substrate. However, the assay is time consuming as it involves the incubation with colloidal chitin and a colorimetric estimation of *N*-acetylglucosamine (GlcNAc) released using reducing sugar estimation by Somogyi method or Reissig method (Reissig *et al*, 1955; Somogyi, 1952). The present work describes a new method employing the colloidal chitin prepared from Ostazin Brilliant Red labeled chitin for the quantitative estimation of total chitinolytic activity.

A 2 Results and discussion

A 2.1 Substrate preparation

The o-phosphoric acid treatment was used to prepare swollen substrate from chitin (Sigma) and from Ostazin Brilliant Red labeled chitin. The acid swollen chitin (Sigma) was white and fluffy mass. To obtain similar consistency of swollen coloured chitin, overnight stirring with o-phosphoric acid was needed. The dye labeled GlcNAc was not released under the conditions used for the preparation of colloidal chitin.

The non-enzymatic parameters of the enzyme assay such as, acetate buffer (50 mM, pH 5.0), incubation temperature and time (50 °C, 1 h) and termination of reaction by keeping tubes in boiling water bath for 5 min did not release dye labeled GlcNAc and/or only dye from the substrate. The dye labeled chitin was found to be stable at 50 °C up to 3 h at pH ranging from 4.0 to 7.0.The dye binding with the chitinous substrate could either be through weak interactions such as hydrogen bonds and hydrophobic interactions or covalent linkages (Ramirez *et al*, 2004). As there was no significant release of dye from colloidal Red chitin at alkaline pH (8.0) it can be suggested that weak hydrogen bonds were not involved in dye binding.

A 2.2 Effect of substrate concentration

The effect of different substrate concentrations (3-15 mg/ml) on the release of dye labeled GlcNAc measured at 530 nm and GlcNAc (labeled as well as unlabeled) measured using DMAB was studied with 0.3 U/ml of chitinase activity measured on acid swollen substrate (Sigma). As shown in Table A 1, with 7.0 mg/ml concentration of the substrate the absorbance at 530 nm was 0.066 and the GlcNAc was 5.0 ± 0.3

Table A 1 Effect of the substrate concentrations on the chitinase activity

Substrate concentration	Colloidal Red-chitin	
	Absorbance	GlcNAc
(mg/ml)	(530 nm)	(μM/ml/h)
3.0	0.033	2.5±0.4
5.0	0.048	3.7±0.3
7.0	0.066	5.0±0.3
10.0	0.069	5.25±0.4
15.0	0.072	5.4±0.4

***** *M. verrucaria* chitinase (0.3 U/ml, estimated using colloidal chitin-Sigma) was used in the experiment.

μM/ml within 1 h. There was no proportionate increase with increase in substrate concentration. Therefore, in subsequent experiments 7.0 mg/ml substrate was used.

A 2.3 Effect of incubation time

The effect of incubation time (15-120 min) on the release of dye labeled GlcNAc measured at 530 nm and GlcNAc (labeled as well as unlabeled) measured using DMAB was studied. As shown in Table A 2, the absorbance at 530 nm increased from 0.048 to 0.074 when the incubation time was increased from 15-120 min. There

Table A 2 Effect of incubation time on dye and *N*-acetylglucosamine released from colloidal Red-chitin

Incubation time (min)	Colloidal Red-chitin	
	Absorbance (530 nm)	GlcNAc (μM/ml/h)
15	0.048	3.62±0.3
30	0.051	3.82±0.4
60	0.066	5.0±0.3
90	0.070	5.32±0.3
120	0.074	5.59±0.4

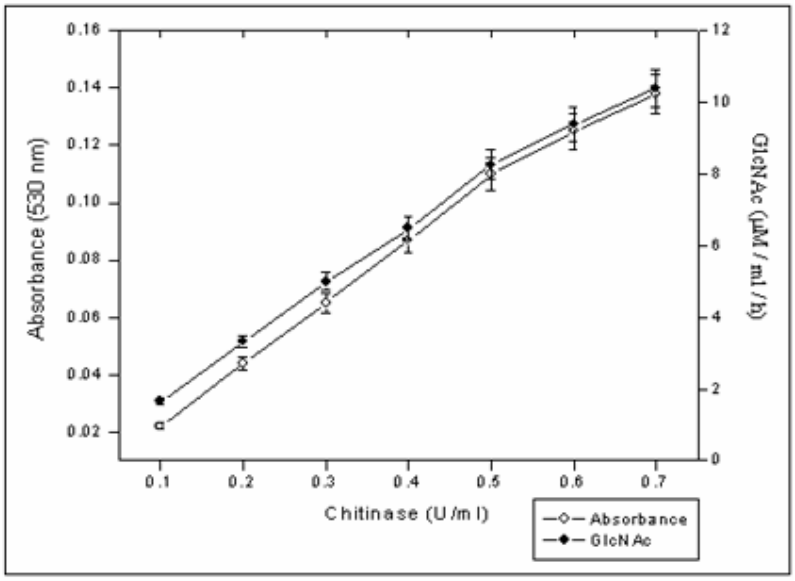
***** *M. verrucaria* chitinase (0.3 U/ml, estimated using colloidal chitin-Sigma) was used in the experiment.

was no appreciable increase in the activity, if the reaction mixture was incubated up to 120 min. Therefore in subsequent experiments 60 min incubation period was used.

A 2.4 Effect of enzyme concentration

The acid swollen Red-chitin was hydrolysed using *M. verrucaria* chitinase (0.1-0.7 U/ml). The absorbance was measured at 530 nm and the GlcNAc was estimated

Fig. A 1 Correlation between absorbance at 530 nm with the *N*-acetylglucosamine produced during chitinase assay

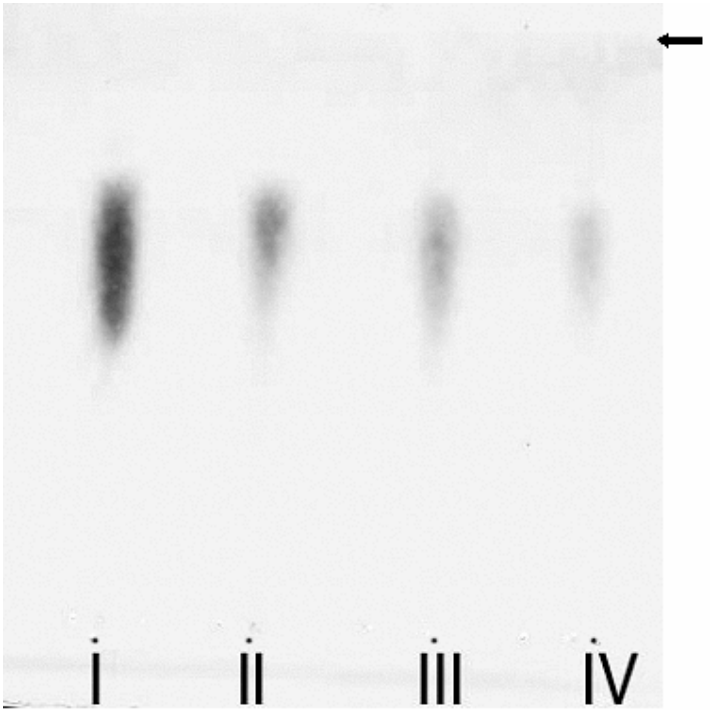


using Reissig *et al* method (1955). Both of methods showed correlation between released GlcNAc (dye labeled and unlabeled) and the increase in enzyme concentration ($P < 0.01$) (Fig. A 1). With the increase in enzyme concentration (0.1-0.5 U/ml) absorbance at 530 nm increased from 0.022 to 0.11 and concentration of released GlcNAc increased from 1.67 to 8.36 µM/ml within 1 h. With further increase in enzyme concentration proportional release in dye as well as GlcNAc was not observed. This may be attributed to the non-uniformity in the labeling of the substarte. Lower GlcNAc release from colloidal Red-chitin as compared to colloidal chitin (Sigma) may be attributed to the nature of the substrate and /or less accessibility to the enzyme as compared to the colloidal chitin (Sigma).

A 2.5 Thin layer chromatography

The Rf value for GlcNAc and for mixed spot of GlcNAc from colloidal chitin (Sigma) and colloidal Red chitin was 0.66. The Rf value for GlcNAc released from colloidal chitin (Sigma) in the reaction mixture was marginally higher 0.68. While

Fig. A 2 Thin layer chromatogram showing *N*-acetylglucosamine released from labeled and unlabeled chitin substrates



* I: standard GlcNAc, II: GlcNAc released from colloidal chitin (Sigma), III: mixture of GlcNAc released from colloidal -chitin (Sigma) and -Red chitin, IV: GlcNAc released from colloidal Red chitin. →, indicates solvent front.

lower Rf value (0.63) was observed for GlcNAc released from colloidal Red chitin (Fig. A 2). These results were found significant ($P< 0.05$). The difference in the Rf values of GlcNAc and colored GlcNAc also confirm the non-uniformity in the labeling (Fig. A 2). The thin layer chromatography was not sensitive to detect chitooligosaccharides (Rajamohanan *et al,* 1996).

A 2.6 Definition of unit

The absorbance 0.05 at 530 nm was considered as 1U, which was equivalent to 3.8 μM/ml GlcNAc produced in 1 h.

A 2.7 Validation and specificity of the assay

Beauveria bassiana and *Bacillus subtilis* were grown in chitin medium under shaking conditions (200 rpm) at 28ºC for 4 d. For validation, chitinase activity in cell free broths of *B. bassiana* and *B. subtilis* and of commercially available amylase and cellulase preparations were estimated using colloidal chitin (Sigma) and colloidal Red-chitin (Table A 3). There was no release of coloured GlcNAc due to amylase and cellulase activities under the experimental conditions.

Table A 3 Effect of different enzymes on colloidal Red-chitin

Enzymes	Chitinase[1] (U/ml)	Chitinase[2] (U/ml)
Chitinase (*M. verrucaria*)	1.8±0.2	7.8±0.85
Chitinase (*Beauveria bassiana*)	0.008±0.001	0.034±0.004
Chitinase (*Bacillus subtilis*)	0.006±0.001	0.026±0.004
Cellulase	ND	ND
Amylase	ND	ND

*Chitinase[1]: Activity was estimated using colloidal chitin (Sigma).
*Chitinase[2]: Activity was estimated using colloidal Red-chitin.
*ND: Not detected.

The assay can be adapted to the 96 well plate method, which can be used to measure chitinolytic activity in large number of samples. The test variability did not increase significantly by the use of reduced volumes (SD ≤ 5%). To avoid the interference due to insoluble coloured substrate, in reading the intensity of dye released, filtration based assay using multiscreen solubility plate may be developed.

In conclusion, the use of colloidal chitin prepared from Ostazin Brilliant Red

labeled chitin allowed the development of a new specific and sensitive method for the quantitative estimation of chitinases. The method is suitable for the scrrening of chitinolytic organisms.

Chapter 6

Conclusion

A variety of cereals, oil seeds, pulses, vegetables and horticultural crops are cultivated in India. The food grain production in the country has substantially increased since the Green Revolution of 1980s. However, in recent years agricultural production suffered 15-50% losses in yields due to soil borne fungal pathogens such as *S. rolfsii*, *F. oxysporum*, *R. solanii* and insect pests like *H. armigera*, *S. litura*, *P. xylostella*, *C. lanigera* and others. The chemical fungicides and insecticides currently being used for the control of fungi and insect pests face the problems of resistance development. Moreover their use is not ecofriendly, they cause destruction of natural enemies and affect non-target population through bio-magnification in the environment. Thus, for the sustainable agriculture, there is a need to use novel biological approaches for the control of fungal pathogens and insect pests.

Chitin is the unique structural polymer, which is present in the protective coverings of both fungi and insects while absent in vascular plants and mammals. The glucans and proteins are other structural components in fungal cell wall while insect cuticle contains proteins and lipids. Mycoparasitic fungi like *Trichoderma* and *Gliocladium* are known to degrade fungal cell wall using mycolytic enzyme complex, ME comprising of chitinase, glucanase and protease. While entomopathogenic fungi secrete cuticle degrading enzyme complex, CDE (chitinase, protease and lipase) for the penetration into insect host. The, major interest in the present studies was to understand whether a fungus secreting high amounts of CDE/ME can be used to develop a biocontrol formulation with dual action, against fugal pathogens and insect pests.

M. verrucaria, a saprophytic fungus known to produce high amounts of CDE/ME activities was selected for the present studies. Attempts were made to develop mycopesticide comprising of living propagules and/or CDE/ME components of *M. verrucaria* to control fungal pathogens and insect pests.

During present studies, unicellular growth of *M. verrucaria* was observed for the first time in a synthetic medium containing sucrose and ammonium sulphate (Chapter 3). The medium pH was found to be the important morphological trigger favouring H-Y reversible transition. The conditions were optimized for the production of yeast-like cells that can be used for slow release of mycolytic enzymes for the control of plant pathogenic fungi. Under the optimized conditions (sucrose, 2%; ammonium sulphate, 0.46%; initial pH, 4.0) for the production of yeast-like cells: with conidia or yeast (5%) as inoculum, maximum yeast biomass (4.6 ± 0.4 g/l wet wt) was obtained under shaking conditions (200 rpm) at 28°C within 72 h (Chapter 4A).

Attempts were also made to optimize conditions for the production of *M. verrucaria* conidia using solid state fermentation. Under the optimized conditions, sorghum grains as solid substrate, initial moisture content adjusted using 25 ml distilled water per 50 g substrate, 10% mycelial inoculum and incubation temperature 28°C, highest yield of conidia ($2x10^9$ conidia/g of substrate) was obtained (Chapter 4A).

The production of cuticle degrading/mycolytic enzymes of *M. verrucaria* was studied in shake flask as well as in 15 l capacity stirred tank fermenter. Under shake flask conditions chitinase production was significantly enhanced with chitin as a sole source of carbon. The production of chitinase in stirred tank fermenter was significantly affected by agitation and aeration. The agitation rate 300 rpm and aeration rate 0.5 vvm were found to be optimum for the chitinase production. Under optimized conditions, chitinase (1.32 U/ml), β-1,3 glucanase (0.13 U/ml), protease (0.011U/ml) and lipase (0.945 U/ml) were produced in a fermenter within 5 d. The enzyme productivities at fermenter level were comparable to those in shake flask conditions. However, to improve the chitinase production in the fermenter, the studies on effect of controlled pH and effect of morphology (pelleted/dispersed) may prove helpful (Chapter 4 B).

The conidia and yeast-like cells were formulated with different carriers for use in soil application to control soil-borne fungal pathogens (Chapter 5). *M. verrucaria* conidia retained >80% and >50% viability after 6 and 18 months storage at room temperature respectively when stored with rice bran alone or supplemented with chitin as carrier. The yeast-like cells were immobilized in alginate matrix with and without chitin. The storage at room temperature significantly affected the viability of immobilized yeast-like cells. The yeast-like cells retained >80% viability after 2 months storage at room temperature. The supplementation of chitin with carriers help in inducing chitinase production after soil application, however it did not significantly enhance the shelf-life of conidia with rice bran or immobilized yeast-like cells. For the use of CDE/ME enzyme preparation to control fungal pathogens or insect pests, the major constraint was its volume, which was affecting storage and transportation. Using combination of ultrafiltration and freeze drying, 10 l enzyme preparation was concentrated to powder form (32 g) which retained >70% of the initial chitinase activity.

The bio-efficacy of different formulations were carried out to control rot of peanut caused by *S. rolfsii*, under green house conditions. Rice-bran +chitin formulation of conidia and yeast-like cells immobilized with chitin significantly reduce the incidence of pre-emergence rot in peanut. The biocontrol efficacy of these propagules was correlated with the slow release of chitinolytic enzymes in soil. Significant increase in % peanut seed germination in *S. rolfsii* infested soil was observed when mycolytic enzyme preparation (10-20 U chtinase / pot) was applied to soil at weekly interval.

CDE preparation was found effective against the insect pests *H. armigera*, *S. litura*, *P. xylostella* and *C. lanigera* under bioassay conditions. The insect mortality was observed within 72 h. The bioassay results suggest that CDE preparation, (Chitinase 2 U/ml) in combination with entomopathogenic fungi may be used to increase %

mortality, decrease in the killing time due to hydrolysis of cuticle to facilitate penetration process and the decrease in the load of conidia sprayed in the field. The efficacy of the CDE preparation (chitinase 6 U/ml) to control *H. armigera* in chickpea under field conditions was at par with the coventional chemical (Endosulfan) and biological (HaNPV) treatments. Lastly, the toxicity studies revealed that application of living propagules or CDE/ME preparation may be considered safe as in these preparations oxynitrlase (mandelonitrile lyase) activity (producing HCN from cyanoglycosides) was not detected.

For the screening of chitinolytic organisms a rapid and sensitive method based on the use of Ostazin red labeled colloidal chitin as substrate for the quantitative estimation of chitinase was developed (Appendix 1).

In conclusion, *M. verrucaria*, organism itself and extracellular hydrolytic enzymes can be effectively used to control plant pathogenic fungi as well as insect pests. The use of enzyme mixture in combination with an insect pathogenic fungus will be useful in an integrated approach to control insect pests. *M. verrucaria* is being considered as a scheduled organism by Central Insecticide Board. This will indeed facilitate the use of organism itself and/or extracellular hydrolytic enzymes for the control of plant pathogenic fungi and insects. Of course, the increased shelf-life and cost effective production of the enzyme mixture are the major concerns.

Chapter 7

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List of Research Papers and Reviews / Patents / Presentations

Research Papers and reviews

F. Shirazi, M. Kulkarni and M.V.Deshpande (2006) A rapid and sensitive method for screening of chitinase inhibitors using Ostazin Brilliant Red labeled chitin as a substrate for chitinase assay. In: Letters in Applied Microbiology (In Press)

S. Chavan, M. Kulkarni and M.V. Deshpande (2006) Status of Microbial Pesticides in India In: Annual Review of Plant Pathology, Vol. 4, (ed. S.M. Reddy and H.N. Gour) (In press).

S. Chavan, M. Kulye, M. Kulkarni, and M.V. Deshpande (2006) *Myrothecium verrucaria*: A potential biocontrol agent for the control of insect pest. In : Proceedings of the National Symposium of Biomanagement of Insect pests, Annamalainagar, (In Press).

Patents

M.V. Deshpande, S. Chavan and M. Kulkarni.
A composition for controlling wooly aphid infestation and a method of treatment thereof. Indian patent has been filed in 2004, File No. NF 414/2004.

M.V. Deshpande, M. Kulkarni, M. Chitnis and R. Patil.
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Presentations

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