V. SUMMARY AND CONCLUSIONS
Chitinases are well known enzymes involved in growth, morphogenesis and interactions of chitin containing organisms like fungi, nematodes, arthropods etc. Besides the biocontrol potential of chitinase, the potential of the soluble hydrolysis products of chitin in various fields like medicine and agriculture has generated interest in obtaining some commercially suitable chitinase preparations. The success in using enzymes for different application depends on the supply of highly active preparation at a reasonable cost. The thermostable enzymes possess advantages of obtaining higher reaction rates, ability to use higher substrate concentrations, longer shelf life and lower contamination problems. This investigation, thus aimed at production of a thermostable chitinase from thermophilic organisms, particularly fungi.

In the present investigation, a potent chitinase producing thermophilic fungus *Myceliophthora thermophila* was selected amongst one hundred and thirty four thermophilic fungal isolates, isolated from various environmental samples collected from different geographical regions of India. A cost effective production process with high yields is required for any enzyme to be applicable on industrial scale. Thus an attempt was made to understand and improve the production aspects and a high chitinase producing submerged and solid state fermentation medium was formulated. An endeavour was also undertaken to purify and characterize the enzyme and study its applicability in chitin hydrolysis and biocontrol of fungal, nematode and insect pests. The significant findings of the present investigation are summarized below:

Of the twenty eight chitinase producing strains among 134 thermophilic fungal isolates, *Myceliophthora thermophila* was selected based on higher chitinase titres. The identity of the selected strain was confirmed by amplification and sequencing of the ITS region and its phylogenetic analysis. The chitinase production by this fungus was inducible in the presence of chitin, its hydrolysis products and lactose.

In submerged fermentation, *Myceliophthora thermophila* produced chitinase optimally after 6 days at 45°C at pH 6.0. Potassium nitrate, magnesium sulphate and incubation days were identified as important factors affecting chitinase
production by Plackett-Burman design, which were further optimized by RSM. Statistical optimization resulted in 7.1-fold improvement in chitinase titres. A sustainable chitinase titre was also attained in Erlenmeyer flasks of varied volumes and in laboratory fermentor. The fermentation time was reduced to half (72 h) in the fermentor as compared to that in Erlenmeyer flasks (144 h), with minor enhancement (7.4 fold) in enzyme yield.

*Myceliophthora thermophila* grew well on wheat bran and produced chitinase in the presence of an inducer in a growth associated manner. The optimal chitinase titres were obtained after 10 days of incubation at 45°C and pH 6.0. Optimization with Plackett-Burman design revealed the significance of wheat bran, glucose and initial moisture level among others in obtaining higher chitinase titres. Optimizing the values of these variables by response surface methodology helped in achieving 10.58 fold enhancement in chitinase production. A slight reduction in chitinase titres in shake flasks of higher volume and enamel coated metallic trays was observed. However, this decline could be circumvented with optimization of bed depth in trays and 11 fold enhancement over initial unoptimized medium was achieved. *M. thermophila* secreted other hydrolytic enzymes (cellulose, xylanase, lipase etc.) along with chitinase in SSF. Chitinase production by *M. thermophila* was 7.6-fold higher in SSF (192300 U kg⁻¹ DMR) than that in SmF (25382.4 UL⁻¹).

The crude culture filtrate was concentrated by lyophilization with 16.8 fold better recovery of chitinase in presence of lactose. The chitinase was purified to homogeneity from the concentrate by using affinity adsorption followed by hydrophobic interaction chromatography. The purified native chitinase is a monomer of 43 kD with a pl of 4. It exhibited an optimum temperature and pH of 55°C and 4, respectively with a t₁/₂ of 9 hours at 60°C and 3 hours at 70°C. The MALDI–ToF–MS/MS analysis of 11 trypsin digested peptides of the protein showed homology with a glycosyl hydrolase family 18 protein of *M. thermophila* ATCC 42464. One of the two signature sequences was also present in the identified peptides, confirming the identity of the purified protein as a chitinase from *M. thermophila*. 
The preferred substrate for chitinase was swollen chitin however crystalline chitin and chitosan were also hydrolyzed. The maximum hydrolysis rate ($V_{\text{max}}$) and apparent Michaelis-Menten constant ($K_m$) for colloidal chitin were 25.25 U/mg protein and 0.386 mg/ml, respectively. Chitinase activity was not affected by most of the organic solvents and detergents. The enzyme does not require any metal ion for activity. Chitinase activity is inhibited by Al$^{2+}$, Hg$^{2+}$ and Fe$^{2+}$ ions. Carboxyl groups and tryptophan residues are important for chitinase activity as the activity is inhibited by woodwards reagent K and N-bromosuccinimide. Chitinase activity is inhibited by PMSF, DTT and IAA to varied extent indicating the importance of sulphhydryl groups and serine residues for enzyme activity or stability. The enzyme is stable to storage under refrigeration and retained 82% of its activity after 7 months storage at 4°C.

Chitin hydrolysis with the chitinase yielded N-acetylglucosamine and chitobiose initially revealing the exo- mechanism of enzyme action. As the reaction progresses the chitobiose is hydrolyzed to NAG completely. This chitobiase activity was confirmed by assay with pNP-N-acetylglucosamine. Higher oligosaccharides were not detected in the hydrozylate even after prolonged incubation. Thus the chitinase is useful in generating both NAG and chitobiose.

Chitinase was effective against fungi, nematode and insects. The cell walls of spores/hyphae were disrupted in *Fusarium oxysporum*, *Curvularia* sp. and *Stachybotrys* sp. by chitinase treatment. The chitinase inhibited hatching of the eggs in the nematode, *Meloidogyne incognita* with the enzyme treated eggs showing clearance of egg cytoplasm. The chitinase induced mortality in two insect pests tested, 3rd instar larvae of the mosquito, *Aedes aegypti* and *Maconellicoccus hirsutus*.

The bifunctional thermoacidstable chitinase from *Myceliophthora thermophila* is particularly suitable for N-acetylglucosamine and chitobiose production at high temperatures with increased reaction rates. The non-requirement of metal ions for its activity and longer shelf-life makes it an enzyme of choice in industry. The enzyme possesses a broad spectrum biocontrol potential against fungi, nematode and insects. The chitosanase activity associated with the chitinase
makes it particularly suitable for use in biocontrol as chitin in nature is almost always associated with chitosan in organisms. A significant increase in enzyme production was achieved in submerged and solid state fermentations. The studies on chitinase production in trays and fermentor suggested the feasibility for large scale industrial production of the enzyme. An aspect that needs attention is the cloning and over expression of chitinase gene of *M. thermophila* in a suitable host for lowering the cost of production.