CHAPTER V

SUMMARY OF FINDINGS
The aim of the study was to find out the effect of meta-dinitrobenzene and pentachlorophenol on the growth and enzyme activity of Chaetomium globosum and Curvularia lunata and to investigate the mechanism of action of these fungicides.

2. Maximum growth and sporulation of both the fungi was obtained in modified Omeliansky's medium (pH 6.2-6.4), using carboxymethyl cellulose as substrate. In case of Chaetomium globosum, 28°C favoured the growth and sporulation, while Curvularia lunata showed peak growth at 30°C-33°C when grown on rotary shaker (200 cycles per min) for a period of 4-5 days. The cultures, when allowed to grow in stationary phase, took a longer time to attain maximum growth and sporulation. In both the organisms, 3.0 mL of spores suspension per 100 mL has been found sufficient to obtain maximum growth.

3. It appears that biotin, calcium pentothenate, riboflavin and thiamine are not required for the growth of the two organisms. However, in their presence, the growth as well
as the enzymes of both the organisms are activated. Maximum growth and enzyme activity is obtained with biotin, followed by calcium pantothenate.

4. An inverse correlation has been found to exist between the growth and enzyme production by the two organisms. The hyphal growth (73.0 mg) of *Curvularia lunata* was more profuse than that of *Chaetomium globosum* (52.0 mg), whereas, the enzymes elaborated by *Chaetomium globosum* were more active than those of *Curvularia lunata*.

5. The growth and sporulation of both the fungi was inhibited by meta-dinitrobenzene and pentachlorophenol.

6.0 x 10^{-6} M and 3.0 x 10^{-6} M solutions of meta-dinitrobenzene have been found to inhibit the growth and sporulation of *Chaetomium globosum* and *Curvularia lunata* respectively, whereas a solution of pentachlorophenol at 1.5 x 10^{-5} M and 9.0 x 10^{-6} M was required to completely check the growth and sporulation of *Chaetomium globosum* and *Curvularia lunata* respectively. In both the fungi, a higher concentration of pentachlorophenol was needed to inhibit the biological activity. The growth and sporulation of *Curvularia lunata* was inhibited at a lower concentration of the fungicides than was required for *Chaetomium globosum*. Meta-Dinitrobenzene has been found to be a stronger inhibitor than pentachlorophenol.

6. It has been found that both the fungicides were fungistatic at lower concentration and fungicidal at higher
concentration. Thus, the fungistatic/fungicidal activity depends on the different rates of reaction between the fungus and the toxicant and therefore seems to be apparently affected by the different concentrations of fungicides.

7. The effect of vapours of meta-dinitrobenzene and pentachlorophenol on the growth of two fungi has been studied. It has been found that the vapours of meta-dinitrobenzene completely inhibited as well as killed the superficial growth of *Chaetomium globosum* and *Curvularia lunata*, while, vapours of pentachlorophenol did not inhibit the superficial growth of these organisms, thus indicating that pentachlorophenol is not an effective fungicide in vapour phase.

8. When the cultures, grown in Petri dishes, containing nutrient agar, were exposed to fungicidal vapours, complete degeneration of superficial growth of *Chaetomium globosum* was obtained with 0.50 g/10.0 L of meta-dinitrobenzene, while 0.75 g/10.0 L of the same fungicide brought about only 80% degeneration of superficial growth of *Curvularia lunata*, within a period of 4 days. The undegenerated superficial growth (20%) of *Curvularia lunata* has been found to be non-viable, as it showed no sign of growth on being reinoculated on fresh potato-dextrose-agar medium. Whereas, the growth of the fungi, below the surface of the medium in vapour exposed Petri dishes, remained viable as, when reinoculated on fresh agar medium, normal germination was observed. Thus, it has been found that
the vapours of meta-dinitrobenzene could not penetrate the medium and kill the growth therein, while, the superficial growth of both the organisms was degenerated and completely killed by the vapours of this fungicide.

The effective concentration of fungicide depended on the volume of the container in which the experiment was carried out. However, there appeared to be no linear correlation between the volume of the container and concentration of fungicide.

9. Extracellular and intracellular enzymes, elaborated by these micro-organisms have been isolated to study the effect of meta-dinitrobenzene and pentachlorophenol on the activity of these enzymes.

Modified Omeliansky's medium has been found suitable for the production of enzymes by both the organisms. Maximum elaboration of intracellular enzymes have been recorded on the 3rd day of incubation, while, maximum extracellular enzymes have been obtained on the 4th day of incubation. In case of Chaetomium globosum, the extracellular enzymes have been found to be more active in hydrolysing as well as depolymerising the carboxymethyl cellulose than the intracellular enzymes. The extracellular and intracellular enzymes of Curvularia lunata hydrolysed the carboxymethyl cellulose nearly to the same extent. However, the extracellular enzymes, elaborated by Curvularia lunata, have been found weak in depolymerising the substrate as compared to the intracellular enzymes.
10. Maximum cellulase activity of both the fungi has been obtained when the enzymes were incubated with 1.0% (w/v) solution of carboxymethyl cellulose for a period of 60 min. at 35°-37°C and pH 5.0 to 5.2. Enzymes of both the organisms were found to denature and lose activity, as the temperature was raised to 65°-75°C.

11. The products of cellulosic degradation in the absence of fungicides have been identified as cellobiose and glucose by paper chromatography. However, in the presence of fungicides no reducing sugars could be identified, indicating that these fungicides inhibit the hydrolysis of carboxymethyl cellulose.

12. The activity of extracellular enzymes has been found to be inhibited by meta-dinitrobenzene and pentachlorophenol. Within a period of 60 min, 85% inhibition of enzyme activity has been achieved by meta-dinitrobenzene, at a concentration of 1.5 x 10^{-3} M, in case of Chaetomium globosum, while, in Curvularia lunata 87% inhibition has been obtained with the same concentration of fungicide. Pentachlorophenol, at the concentration of 1.5 x 10^{-3} M, inhibited the enzymes, elaborated by Chaetomium globosum and Curvularia lunata, to the extent of 80% and 84% respectively within a period of 60 min. meta-Dinitrobenzene has been found to be more effective than pentachlorophenol in inhibiting the enzyme activity, as has been found for growth and sporulation.
13. The extracellular enzymes, elaborated by Chaetomium globosum, have been purified by dialysis, ammonium sulphate salt precipitation, ion exchange chromatography, using DEAE Sephadex A-50 and finally by gel filtration, using Sephadex G-100 and G-200. 118 fold purification was achieved by combining these methods.

14. The kinetics of purified enzyme preparation have been studied. The Km value for the hydrolysis of carboxymethyl cellulose by Cx enzymes was 2.85 mg/mL. Maximum carboxymethyl cellulase (Cx) activity was obtained at pH 5.0 and the enzyme was found stable up to 40°C. The thermostability decreased by 50% when incubated at 50°C for 24 hr. At 70°C, the enzyme was found to be completely inactive.

15. The purified enzymes have been found to be inhibited by meta-dinitrobenzene and pentachlorophenol against cellulosic degradation to the extent of 100% and 85% respectively at a concentration of 1.5 x 10^-3 M within a period of 30 min. Thus, the purified enzymes were more susceptible to fungicidal action than crude enzymes.

16. The toxicity of pentachlorophenol, at 1.5 x 10^-2 M concentration, on fall in viscosity of carboxymethyl cellulose, has been found more pronounced than meta-dinitrobenzene at the same concentration.

17. It has been found that the effect of varying concentrations of two fungicides did not obey simple mass action
theory. Plots of relative enzyme activity against fungicide concentration gave sigmoid shaped curves rather than rectangular hyperbolas, suggesting that more than one fungicide molecule per enzyme molecule participated in the formation of inactive enzyme-fungicide complex.

18. In order to establish structure-fungicidal activity relationship of dinitrobenzenes, the effect of o-, m- and p-dinitrobenzenes on the activity of carboxymethyl cellulase \((C_x)\) has been investigated. It has been found that the nitro group at m- position in the nitrobenzene nucleus potentiates the fungicidal efficacy than at o- or p-positions.

19. To find out the mechanism of action of the two fungicides, the effect of adding different activators in the growth medium and in the enzyme preparation, containing toxic concentration of the fungicides has been studied.

Out of the seven activators tested, only sodium thioglycollate and tryptophan have been found to reverse the inhibited growth of the two fungi. However, these two activators behave differently. When the inhibited spores of the two fungi were reactivated with sodium thioglycollate, elongated germ-tubes were obtained. Whereas, with tryptophan only short and branched germ-tubes were produced. When these cultures were grown in stationary phase, mycelial mat formation was obtained with sodium thioglycollate, as is evident by the formation of long hyphae. However, no mycelial mat was formed in presence
of tryptophan, probably due to the short and branched hyphae.

The growth of *Curvularia lunata* was stimulated in presence of sodium thioglycollate, as the cell mass of the organism increased. This increase in cell mass may be attributed to a denser packing of hyphae and thickening of cell membrane. However, with tryptophan, the cell mass of both the fungi remained low, as compared to the percentage of spores germination.

20. It is indicated from the reactivation of inhibited growth that the fungicides bind at the -SH and -NH₂ groups of protein moiety, inhibiting the biosynthesis of amino acids having these groups. These binding sites are considered to be located in the cell, as the cell multiplication ceases to take place in presence of the fungicides and gradually the mycelia degenerate.

21. The inhibited carboxymethyl cellulase activity was reversed appreciably by sodium thioglycollate and tryptophan followed by glycerol and cysteine. Various concentrations of these activators were tried and it has been found that 1.5 x 10⁻² M solution of sodium thioglycollate and tryptophan reactivated the inhibited enzymes at pH 5.0 and temperature 37°C at which the enzymes were most stable and active. That sodium thioglycollate acted as a stronger reactivator than cysteine, may be due to the fact that at pH 5.0, cysteine is only slightly dissociated. At higher temperature, reversal
of the inhibition action was not achieved, probably due to
denaturation of enzymes.

22. The reactivation studies with inhibited enzymes also
indicate that m-dinitrobenzene binds at the -SH as well as
-NH₂ groups nearly to the same extent. However, pentachloro-
phenol mainly blocks the -SH groups and to some extent inhibits
the -NH₂ dependent systems.

23. It is, therefore, assumed that the toxic action of
both the fungicides on the growth and enzyme activity of the
organisms may be due to excessive withdrawal of electrons at
the active sites, thus oxidising the active groups of protein
moiety.