CHAPTER-XII
SUMMARY

The present study pertains to the plant cellwall degrading enzymes produced by two important plant pathogens viz. *R. niaricans* Ehrenberg causing soft-rot of papaya fruits and *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dowson, the incitant of leaf-spot of chillies. The role of pectolytic and cellulolytic enzymes in plant pathogenesis is associated with tissue maceration and comparatively recently with cell death (by physical action on membranes). The former enzymes, among the two, are more important which is explained to the easy repressibility of the cellulolytic enzymes. The production of these enzymes is indispensable for tissue maceration which is almost inseparable from pathogenesis. Nevertheless, the ability of these enzymes *per se* does not make the organism pathogenic.

The two organisms were isolated from their hosts and pathogenicity was proved as the first necessary step. The organisms were grown on different media with varying carbon sources and the culture filtrates were analyzed for the production of extracellular pectolytic and cellulolytic enzymes.
R. nigricans was grown on Czapek and Richard's media containing glucose, sucrose and pectin as the carbon source as well as on potato-pectin medium. The fungus failed to produce any pectolytic enzymes in glucose and sucrose containing media. However, it did produce these enzymes when pectin served as the carbon source. The nature of the enzyme synthesized differed for the various growth media. In Richard's pectin medium the fungus produced only hydrolytic enzymes while in Czapek-pectin medium it produced hydrolytic and also lyase enzymes simultaneously. In potato-pectin medium the two enzymes were produced in a temporal sequence; hydrocases first and lyases later. This experiment proved that R. nigricans produces the pectolytic enzymes inductively. This study for the first time reports lyase production by Rhizopus. The in vivo assay of pectolytic enzymes indicated only hydrolytic, chain-splitting enzymes and PME in the inoculated papaya fruits. There was no lytic activity.

The high PME activity in host prevents clear identification of the PG or PMG enzymes in the tissues.

X. campestris pv. vesicatoria also failed to synthesize pectolytic enzymes in media containing sugars. Thus, enzymes were produced inductively in presence of
pectin or NaPP as the carbon source. The bacterium produced only lyase enzymes in the Richard's-pectin medium in which the fungus produced only hydrolytic pectic enzymes. Thus, the same medium supported production of different enzymes by the fungus and the bacterium. The substrates were degraded at acidic as well as alkaline pH, though pectin was better degraded in alkaline range. An interesting observation was that in the above medium with NaPP as the carbon source the lyase enzyme that way produced was active only on pectin and not on NaPP which induced the enzymes. Thus, the enzyme activity was restricted to a limited pH of 10 and 11 only. These are important pointers to the complex pattern of regulation of the synthesis of these enzymes. Similar enzymes were produced in Czapek-pectin/NaPP media. Thus, while Rhizopus had a different pattern of enzyme production in the two media, the bacterium behaved similarly on the two media.

The fungus produced PME in Richard's-pectin medium, which was active at pH 6. The maximum activity occurred at 40°C upto 24 hrs, after which it declined. PME was assayed in healthy and infected fruits at various pH. In infected fruits PME activity was higher than in healthy
ones, at all the pH tested. The bacterium failed to produce PME in any of the media.

The study of effect of glucose along with pectin in the medium revealed that *R. nigricans* which synthesized only hydrolytic enzymes on Richard’s-pectin medium produced a new lyase enzyme also (PL) which degraded only pectin not NaPP. NaPP continued to be degradable by the hydrolase simultaneously synthesized as before. *X. campestris pv. vesicatoria* behaved as before. Thus, the presence of glucose did not alter the enzyme make up of the culture filtrates. At higher concentrations, however, the medium which showed peaks in TBA test suggesting lytic cleavage, failed to liquify the pectic substrate in viscosity test.

Effect of several other sugars (fructose, sorbose, xylose, galactose, raffinose, cellobiose and lactose) on enzyme induction by pectin was examined. In *Rhizomus* clear-cut uniform behaviour was noted for xylose and sorbose while the former was stimulatory, sorbose was inhibitory for both FG and PMG enzymes. Except fructose all other sugars inhibited the FG enzyme. PMG production was stimulated by fructose, xylose and galactose. Sorbose also proved most inhibitory to PAL and PL production by *Xanthomomas*. No other sugar had such clear-cut drastic
effect as sorbose. Similar distinct effect was shown by galactose which proved stimulatory to PAL at higher concentrations and to PL at all concentrations. The trisaccharide raffinose and the disaccharides lactose and cellubiose proved inhibitory to PAL at all concentrations. None of the sugars affected the nature (hydrolytic or trans-eliminative) of the enzyme which has been reported for *Verticillium albo-atrum*, *V. dahliae* and *Helminthosporium sacchari* earlier from our laboratory.

Seven amino acids (phenylalanine, alanine, arginine, asparagine, glycine, glutamic acid and tryptophan) were examined for their impact on enzyme production in presence of pectin in the medium. Phenylalanine supported better growth of the fungus but rest of the amino acids showed inhibition (5-62%) as compared to the control. The amino acids behaved differently at the three concentrations, making any generalization difficult. But most significant differences can be made out. This is that phenylalanine proved stimulatory to pectolytic enzyme production for both the organisms (PG & FHG of *R. nigricans* and PAL and PL of *X. vesicatoria*). This is in contrast to the inhibition reported for pectolytic enzymes of *V. albo-atrum*, *V. dahliae*.
and \textit{H. sacchari}. In the present study there was no new enzyme synthesis which was earlier observed in phenylalanine media for the species of \textit{Verticillium} and \textit{Helminthosporium} in our laboratory.

The effect of metal ions is decisive on pectolytic enzyme production. This is best exemplified by the essentiality of calcium for lyase production by some bacteria and fungi. This aspect was examined critically for the reason that the previous works have also shown that calcium is dispensable and thus its essentiality is not universal. Moreover, occasionally, it is inhibitory to PAL. All the seven test metal ions proved inhibitory to PG of \textit{R. nigricans}. PME was stimulated by Na, Zn, Ca and Mg. While PG inhibition by metallic ions is well known, the stimulation of PME noted by us is not reported by earlier workers. The PAL and PL production by \textit{Xanthomonas} was also invariably inhibited. PAL inhibition occurred in the order \textit{Hg} > \textit{Mg} > \textit{Cu} > \textit{Co} > \textit{Zn} > \textit{Na} > \textit{Ca} while PL was inhibited by the ions in the following decreasing order of inhibition \textit{Hg} > \textit{Cu} > \textit{Mg} > \textit{Ca} > \textit{Zn} > \textit{Co}. Thus, the pectolytic enzyme production by both organisms was inhibited by the test ions. The effect of calcium on enzyme activity (by adding Ca in the reaction mixture) examined at seven
concentration $5 \times 10^{-5} \text{M}$ to $10^{-2} \text{M}$ and the enzyme activity was assayed at three pH (5, 6, 7). None of the different concentrations of calcium added to the reaction mixture proved stimulatory to FG and PMG activity of *R. nigricans*. Rather, reduced viscosity loss and low relative activity was noted at all concentrations. However, PMG was stimulated at one concentration ($5 \times 10^{-5} \text{M}$), in terms of % viscosity loss of pectin as well as the relative activity. The PAL and PL of *X. vesicatoria* were stimulated at lower concentrations of calcium but higher concentrations were distinctly inhibitory. In some instances the enzyme was inhibited or stimulated at the same concentration depending on the pH of the reaction mixture.

The effect of several phenolic compounds (catechol, cotechin, orcinol, phloroglucinol, resorcinol, salicylic acid and cinnamic acid) when included in medium, as well as in the reaction mixture, was examined. The phenols were used at the concentration of 50, 100 and 200 µg per ml of the medium. None of the phenols when added to the medium proved toxic to the fungus and, but for cinnamic acid (at 200 µg/ml conc which prevented FG and PMG synthesis as evidenced by TBA and viscosity test) all the phenols supported enzyme synthesis by the fungus. But the enzyme activities
were invariably retarded (20-30%). When added to the reaction mixture, the phenols didn't show noteworthy inhibition of PG; instead some of the phenols stimulated the enzyme activity. Surprisingly the phenols were more inhibitory at lower concentrations. PMG on the other hand showed retarded activity in presence of the phenols at all concentrations. The PAL and PL production by Xanthomonas was also not affected though the viscosity reducing property was invariably reduced which was proportional to the phenol concentrations. PL was inhibited many folds more than PAL. The inhibitory effect of inclusion of phenols in the reaction mixture was more pronounced on PL than on PAL. Most of the phenols inhibited the PL activity by 80-90% and the activity ceased after 10 min.

The effect of ten fungicides (basamid, benlate, brassicol, cuprasol, frumin, hexasan, manozeb, thiram, zinex and ziram) on growth of R. nigricans was examined. Except hexasan which supported better growth (25% more over control) rest of the fungicides proved inhibitory in the following order: brassicol > cuprasol > manozeb > ziram > thiram > frumin > zinex > benlate > basamid. The PG was not detectable spectrophotometrically in the culture
filtrates of media containing benlate, brassicol, hexasan, thiram and ziram. Nevertheless, the enzyme activity could be seen in the viscosity test. In some cases even more depolymerase activity was noted. The PMG activity, however, was detectable in the TBA test for all fungicide amended media except the one containing brassicol (which also showed 59% reduced activity in viscosity test). The viscosity test showed greater depolymerization by all enzyme samples except the one derived from thiram-containing medium. Xanthomonas campestris pv. vesicatoria also metabolized all the fungicides and produced the lyase enzymes detectable in TBA test. Except manozeb, which proved little stimulatory, all other fungicides showed retarding effect in the viscosity experiment. PL activity was more inhibited than PAL.

The cellulase production by R. nigricans in presence of carboxymethylcellulose (CMC, cellulose powder and filter paper pulp was examined in Richard's and Czapek media upto 27 days of growth. In both media, containing cellulose powder, there was no activity. Similarly the Czapek medium containing filter paper pulp also did not support cellulase production. CMC and filter paper
containing media showed endoglucanase and \( \beta \)-glucosidase activity but exoglucanase was missing in terms of cotton activity. Endoglucanase activity was checked in media containing sugars to compare their endoglucanase-inducing property as compared to CMC. This was done by viscometric assay. Disaccharides, in the order cellobiose > lactose sucrose, supported better endoglucanase production than CMC. Glucose medium showed least endoglucanase activity. The endoglucanase from CMC medium was best active at pH 5 and at the temperature of 55°C (It was active upto 80°C, though).

*Xanthomonas* also failed to elaborate exoglucanase in Richard's-CMC medium though feeble activity was detectable in the Czapek-CMC medium. Endoglucanase and \( \beta \)-glucosidase were detectable in the CMC-containing media. The endoglucanase was more active in the alkaline pH (8), though little differences in pH preference was noted for enzyme samples of different intervals derived from the two media. The endoglucanase activity was more in the Czapek-CMC medium and the enzyme was best active at pH 7. The endoglucanase in 72 h culture filtrate of Czapek-CMC medium was best active at 60°C and activity was detectable at 80°C also.
The sugars, glucose, cellobiose and lactose were provided at 5 different concentrations (0.01, 0.05, 0.1, 0.5 and 1.0%) and the endoglucanase activity was assayed viscometrically. Glucose proved inhibitory to the enzyme production for both the organisms at all concentrations. Cellobiose and lactose, especially latter proved stimulatory but the inhibition by glucose was more marked than the stimulation by the disaccharides.

The effect of five growth hormones (Indole acetic acid, Gibberellic acid, Naphthoxy acetic acid, Maleic hydrazide and 2,4-Dichlorophenoxy acetic acid) at one (0.05%) concentration was examined on endoglucanase production. Thus, it can be concluded that all the hormones except Gibberellic acid inhibited the endoglucanase production. Gibberellic acid showed marked stimulation on endoglucanase production by both the organisms, but it was more pronounced in R. nigricans where the enzyme activity in this medium maintained its higher activity till the end of the test period.