CHAPTER V

General Discussion
The genus *Fusarium* is composed of a highly diverse group of saprophytic and plant-pathogenic fungi. Asexual reproduction through microconidia and macroconidia is thought to predominate in the field, but many *Fusarium* anamorphs have *Gibberella* (*F. moniliforme*) or *Nectria* (*F. solani*) teleomorphs, that are elicited in the laboratory (Anderson et al., 1992). A strain of *Fusarium moniliforme* isolated from a mangrove ecosystem of the west coast of India produced microconidia and macroconidia on Czapec-Dox agar plates. Microconidia were attached to each other and formed un-branching chains. On the basis of this, the present isolate was identified as *F. moniliforme* and deposited in the National Collection of Industrial Microorganism as *Fusarium moniliforme* NCIM 1276.

Although this organism was isolated from an estuarine mangrove ecosystem and was highly salt and pH tolerant, it did not require salt for growth suggesting that it was adapted to the mangrove environment, and was not a true marine form. *F. moniliforme* has been reported to grow at acidic, neutral and alkaline pH (Thind and Madan, 1979). Thus the ability to grow in a wide range of pH indicates the adaptability of the organism to different environments including detritus-rich mangroves. The present isolate grows in the pH range between 2.0 to pH 11.0. It secretes polygalacturonase (EC 3.2.1.15) at acidic pH 5.0 and pectate lyase (EC 4.2.2.2) at pH 8.0.

Maximum polygalacturonase and pectate lyase was produced in submerged culture in the presence of 1% pectin and 0.2% glucose at pH 5 or pH 8 respectively. Increasing concentration of glucose above 1% repressed the production of polygalacturonase whereas pectate lyase production was not repressed. This suggested that polygalacturonase was an inducible enzyme and pectate lyase was produced constitutively. Considering that glucose-grown cells did not produce polygalacturonases it is possible that the end product triggered the induction of the enzyme. Collmer et al. (1982) suggested that degradation product of pectin triggered pectate lyase in *Erwinia chrysanthemi*.

There is a significant effect of pH on intracellular synthesis of both enzymes. ELISA based studies showed that although both proteins are produced by pectin grown cell at all pH, the quantity of intracellular synthesis varied from 5.2 µg to 6.2 µg at pH 2, 10, 11 and to a high value of 22.7 µg at pH 5 in the case of polygalacturonase, and 3.2 µg to 6.7 µg at pH 2, 10 and 11 and to a high value of 24.5 µg at pH 8 in case of pectate lyase. The
increase in intracellular synthesis was reflected in extracellular secretion. Immunogold labelling showed that at optimum pH the label is concentrated at the membrane in both cases. These data suggest that enzymes are excreted as fast as they are produced. At non-physiological pH there may be changes in the cell membrane which prevent transfer of mono or di-galacturonides into the cell acting as triggers to synthesis of enzymes with the consequence that enzyme production is affected. At non-physiological pH both proteins underwent changes in secondary structure resulting in loss of activity although antigenicity of the protein was maintained.

The amount of pectinase produced also depended on the type of fermentation (Pereira et al., 1993). In solid-state fermentation the present isolate produced three times more poligalacturonase activity as compared with submerged culture. Titration with antibodies showed that two forms of poligalacturonase and a single form of pectate lyase were produced by the isolate in solid-state fermentation. Therefore on complex substrates such as wheat bran the organism expressed more than one poligalacturonase gene. It is not known whether these two forms of poligalacturonase are products of a single gene or two different genes. Caprari et al. (1993) showed that four apparent forms of poligalacturonase produced by their strain of F. moniliforme were actually a single gene product with different glycosylation by post-translational modifications.

When inoculated with the present isolate healthy tomato (Lycopersicon esculentum) and cauliflower (Brassica oleracea botrytis) plants grown under field conditions showed yellowing and crinkling of leaves after 8 to 10 days. In the last stage of infection growth was arrested. At this stage root tips and hypocotyls showed decay and cortical tissue sloughed off. These symptoms are typical of vascular wilt.

Fusarium sp. are well-known plant-pathogens and they cause vascular wilt in host plants. Zucker and Hankin (1970), Perley and Page (1971) and De Lorenzo et al. (1987) have reported that phytopathogenic organisms are known to produce one or more pectic enzymes. The present isolate apparently belongs to the group of Fusaria which are plant pathogens. These data confirm the hypothesis that this species has adapted to the environment from which it was isolated, and is not in itself a new form of the species.
Transverse sections of infected hypocotyls of both plants showed that polygalacturonase and pectate lyase are localized at the epidermal as well as vascular region of the section. It suggests that after infection of the plants, the present isolate secretes polygalacturonase and pectate lyase at these two regions of the plant tissue.

The present isolate produced larger amount of polygalacturonase in tomato tissues than cauliflower whereas pectate lyase production was enhanced in cauliflower plant, suggesting that acidic environment of the tomato cell sap influenced the secretion of polygalacturonase in tomato tissue rather than pectate lyase. The alkaline environment of cauliflower cell sap increased pectate lyase secretion as compared to polygalacturonase. These results duplicate behavior of the organism in submerged culture.

Pectate lyase from *F. moniliforme* NCIM 1276 was characterized by Rao *et al.* (1996A). In the present work a single endo-polygalacturonase (poly [1,4 -α-D galacturonic acid] glycanohydrolase EC 3.2.1.15) produced by the same isolate at pH 5 in submerged medium was purified. The enzyme had a molecular mass of 38 kDa and pI of 8.1. This enzyme showed higher affinity towards polygalacturonic acid than reported so far. The present enzyme showed two pKₐ values of 5.7 and 4.3 suggesting that histidine and carboxylate residues are important for the activity of enzyme. Chemical modification studies on endo-polygalacturonase showed the involvement of a histidine, carboxylate and arginine residue in catalysis and a tryptophan residue in substrate binding. Rexova-Benko (1970 and 1990) and Cooke *et al.* (1976) have shown earlier that histidine and carboxylate residues are essential for activity of endo-polygalacturonase produced by *A. niger*.

A single tryptophan residue was involved in the binding of substrate. Fluorescence data showed that tryptophan was in an electropositive microenvironment. Tryptophan has been shown to be involved in substrate binding in other polysaccharidases such as 1,4 β-glucanase (Ozaki and Ito, 1991), exo-(1,3)-β-D-glucanase (Ohio et al.;1989) and xylanase (Keskar *et al.*, 1989).

An arginine residue was involved in the active site of polygalacturonase which may be involved in catalysis or extending binding of the substrate. This is a first report about the involvement of an arginine residue at or near to the active site of the enzyme.
N-terminal sequence of 20 amino acids did not match with the N-terminal sequence of any reported polygalacturonases in the SWISS-PROT database. Studying the polygalacturonases produced by *Aspergillus* species Stratilova *et al.* (1993) reported that the amino acid sequences of the enzymes showed 72 to 75 % difference among the proteins and 61 to 71 % difference was observed between polygalacturonases from *Erwinia, Lycopersicon esculentum* (tomato) and *Prunus persica* (peach). Thus the fact that the present *Fusarium* protein did not show N-terminal sequence homology with reported proteins is not surprising.

Although the present isolate produced two pectinases at different pH *Fusarium* sp. are well known toxin producer. In order to produce food-grade enzymes from these organisms, overexpressing recombinants are required to be produced. Both polygalacturonase and pectate lyase are good candidates for this future technology development. One important aspect about this isolate is that it tolerates a high salinity. At 0.2 M salt 93% of polygalacturonase activity is produced compared to growth on distilled water medium (Chapter II and Page: 42) and pectate lyase production is similarly unaffected under these conditions.

This appears to be a first report of a *Fusarium* adapted to the marine ecosystem. In fact, in the Indian coastal context, the genus has been reported (D’souza and Araujo, 1979) only once before from the Mumbai region from where the organism was isolated, and has not been included in species lists from Karnataka state or the Sunderban mangroves of the Ganga-Brahmaputra delta (Chinnaraj, 1994). Compared to other coastal mangroves the Mumbai mangroves are highly degraded ecosystems. It is possible that regular cutting for firewood and other interference by man has encouraged *Fusarium* to spread into the mangrove from near-by agricultural fields.