BIOCHEMICAL ECOLOGY OF *FUSARIUM MONILIFORME* ISOLATED FROM AN ESTUARINE ENVIRONMENT

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DEDICATED TO

MY PARENTS

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DECLARATION

Certified that the work incorporated in the thesis entitled **" Biochemical Ecology of *Fusarium moniliforme* Isolated from an Estuarine Environment**" submitted by **Suryakant K. Niture** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. Aditi Pant
Research Guide

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Suryakant Niture

LIST OF ABBREVIATIONS

CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
DEP	Diethylpyrocarbonate
DTNB	2,2-Dithiobisnitrobenzoic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylene diamine tetraacetic acid
GADL	Gluconic acid D-lactone
HEPES	2-(2-Hydroxyethyl) Piperazine-N-(4-Butanesulfonic acid).
HNBB	2- Hydroxy nitro benzyl bromide
HRP	Horse radish peroxidase
MES	2-(N-Morpholinolethanesulfonic acid)
NAI	N-acetylimidazole
NBS	N-Bromosuccinimide
NCIM	National Collection of Industrial Microorganisms
NEM	N-ethylmaleimide
NTEE	3-nitro-L-tyrosine ethylester
PGA	Polygalacturonic acid
PBS	Phosphate Buffer Saline
PHMB	p-Hydroxymercurybenzoate
PG	Phenyl glyoxal
PMSF	Phenyl methyl sulfonyl fluoride
*p*NPG	*p*-nitro phenyl glyoxal.
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
Texas red	Sulforhodamine
TCA	Trichloroacetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
WRK	Woodward's Reagent K

Summary

Fungi are known to produce a wide variety of enzymes, antibiotics, and growth regulators. They have the ability to degrade complex biopolymers such as polysaccharides, hydrocarbons and proteins. Pectic substances are structural polysaccharides occurring in the middle lamella and primary cell wall of the higher plants. This pectic material and hemicellulose are responsible for the integrity and coherence of plant tissues. Pectin is a complex polysaccharide, heterogeneous with respect to molecular weight and the method of isolation.

Pectin degrading enzymes are produced by many plants, plant-pathogens as well as saprophytic microorganisms such as bacteria and fungi. Among the bacteria *Erwinia sp.* and among the fungi *Aspergillus sp.* are known for pectinase production. There is a growing demand for pectinases in the food industry and their major use is in clarification of fruit juices. In order to investigate whether marine fungi produce unusual pectin-degrading enzymes which may be useful in industry, several pectin-degrading flora were isolated from detritus-rich coastal environments and intertidal mangrove communities of Maharashtra, Goa and Karnataka.

The present work refers to study of two different pectinases produced at two different pH by *Fusarium moniliforme* isolated from one of the environmental samples. At acidic pH 5 this isolate produced polygalacturonase (PG) (EC 3.2.1.15) and at alkaline pH pectate lyase (PL) (4.2.2.2).

The aim of the present work was 1) to optimize the production polygalacturonase by the organism and if possible, to study application of the enzyme in industry and 2) to understand the biochemical ecology of the isolate.

The work presented in this thesis deals with-

1) Optimization of culture conditions for polygalacturonase production in liquid culture as well as solid state fermentation and quantification of the pH dependent production of polygalacturonase and pectate lyase using ELISA.
2) The role of polygalacturonase and pectate lyase in plant-pathogenesis by the isolate.
3) Purification and characterization of polygalacturonase produced by the isolate.

Chapter 1:General introduction

The biochemical and ecological diversity of the fungi, pectinolytic enzymes from fungi as well as other sources such bacteria and plants, and their role in plant pathogenesis have been discussed in this chapter. Industrial applications of these enzymes are also reviewed. The chapter presents a framework for the studies conducted in this thesis.

Chapter 2: pH dependent induction of polygalacturonase and pectate lyase produced by *Fusarium moniliforme* NCIM 1276.

Several samples were collected from different estuarine environments. These samples were screened for pectinase production. One fungal isolate was found which produced polygalacturonase at acidic pH and pectale lyase at alkaline pH. This isolate was identified as *Fusarium moniliforme* on the basis of morphological characteristics and deposited in the National Collection of Industrial Microorganism (NCIM) as *Fusarium moniliforme* NCIM 1276. This organism is salt tolerant to 0.4 M NaCl and both enzymes retain full activity at 0.4 M NaCl.

The isolate produced polygalacturonase maximally (0.28 U/ml) at pH 5 in a liquid medium containing 1 % pectin, 0.2 % glucose, 0.5 % $NH_4(SO)_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4, 0.1% $CaCl_2$. The same organism produced pectate lyase maximally (8.2 U/ml) at pH 8 in the same liquid medium. Polygalacturonase production was induced in presence of 1% pectin but production of this enzyme was repressed when glucose (1%) was used as sole carbon source in the liquid medium at pH 5. On the other hand pectate lyase production was not affected in presence of (1%) glucose as sole carbon source in the liquid medium at pH 8. The thesis that polygalacturonase is an inducible enzyme whereas pectate lyase is produced constituvely by the organism is examined in the light of these data.

When the isolate was inoculated on a solid state medium containing wheat bran and orange pulp as carbon source, the production of polygalacturonase was three times more as compared with liquid culture medium. The optimal production was 80 U/gram of mixed substrate as compared to 28 U/ gram pectin in liquid culture. This organism produced single form of polygalacturonase and pectate lyase in liquid culture however two forms of

polygalacturonase and single form of pectate lyase were produced in solid state wheat bran medium.

Using Sandwich ELISA polygalacturonase and pectate lyase protein biomass were measured. These assays show that, this isolate produced extracellular and intracellular polygalacturonase and pectate lyase from pH 2 to11 but the maximum secretion of the enzymes occurred at pH 5 and at pH 8 respectively. Immunocytolocalization studies shows that both enzymes localized near to the cell membrane and vacuole regions of the spore and mycelium.

Chapter 3 : Crop plant pathogenecity of *Fusarium moniliforme* NCIM 1276.

Healthy tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*) plants inoculated with *Fusarium moniliforme* NCIM 1276 showed yellowing and crinkling of leaves after 8 to 10 days. Therefore this estuarine isolate is a pathogen on atleast these two crops.

Transverse sections of tomato and cauliflower hypocotyls examined under the light microscope showed that the fungus penetrates in about 4 days through the epidermal layer to the cortical tissue. Fluorescence labeled antibodies also showed that fluorescence of infected hypocotyls of cauliflower and tomato plants increase by 5 to 10 fold as compared to control plants

At physiological pH both enzymes are produced by this pathogen although the ratios between the enzymes varies between tomato and cauliflower systems. Tomato has an acidic cell sap of 6.6 whereas cauliflower has a cell sap pH of 7.6. In the roots of both plants the ratio between PG:PL production in tomato is 3.6 as compared to 1 in cauliflower clearly indicating that pectate lyase production is enhanced in the latter species.

The hypocotyl regions of both plants showed lower activity of enzymes. However in acidic environment such as in *Lycopersicon esculentum* (Tomato*)* there is greater production of the hydrolytic polygalacturonase (EC 3.2.1.15) as compared to the β-eliminative cleaver pectate lyase (EC 4.2.2.2) and conversely, in the neutral to alkaline cell sap host environment of *Brassica oleracea botrytis* (Cauliflower) pectate lyase production is enhanced.

Fusarium (Deuteromycotina, Moniliales) is a widely occurring plant-pathogen. The species is also known to exhibit a saprophytic mode of nutrition. The data presented here suggest that *Fusarium moniliforme* NCIM 1276 has adapted to estuarine conditions but has retained its virulence against crop plants through the production of pectin-degrading enzymes, which would also be useful in saprophytic modes of nutrition.

Chapter 4 : Purification and characterization of endo-polygalacturonase produced by *Fusarium moniliforme* NCIM 1276.

A single form of polygalacturonase, which is produced by the isolate in liquid culture, was purified to homogeneity by ion exchange chromatography using CM-Sephadex at pH 5 and Sephadex G-100 gel filtration column chromatography, with a yield of 28 %.

Biochemical characterization of the enzyme shows that, polygalcturonase had a M_r of 38 kDa and a carbohydrate content of 4 %. It has an alkaline pI of 8.1. The K_m is 0.12 mg ml^{-1}, V_{max} is 111.1 µM min^{-1} mg^{-1} and the k_{cat} is 4200 min^{-1}. It has a pH optimum of 4.8 and optimum temperature is 45°C. The enzyme activity was competitively inhibited by gluconic acid D-lactone (GADL) with k_i of 28 mM. The effect of metal ions on polygalacturonase activity shows that enzyme was inhibited by Zn^{++}, Hg^{++} and Fe^{+++} at concentration of 5 mM. Amino acid composition shows that, this protein contains 17% of acidic amino acids, and low quantity of sulfur containing amino acids. N- terminal amino acid sequence of the polygalacturonase is E-S-T-Q-L-N-P-I-P-S-T-V-I-H-G-A-T-G-Y-H- . This 20 amino acid sequence did not match with the N-terminal sequence of any reported polygalacturonase in the SWISS-PROT data base. The pK_a values of polygalacturonase are pK^1_a of 5.7 and pK^2_a of 4.3. Time dependant hydrolysis of polygalacturonic acid by the enzyme yielded di-and mono-galacturonic acid. Therefore this enzyme is an endo-polygalacturonase belonging to EC 3.2.1.15.

Active site characterization of endo-polygalacturonase was carried out by using amino acid groups specific chemical modifiers. The enzyme activity was inhibited by WRK, EDC, DEP, NBS, HNBB, Phenyl glyoxal, 2,3 Butanedione and pNPG, suggesting that, carboxylate, histidine, tryptophan and arginine residues are important for the activity

of enzyme. Kinetic inhibition studies and titration of enzyme with specific chemical modifier shows that a single residue of carboxylate, histidine, tryptophan and arginine is present at or near to the active site. Kinetic and fluorescence data show that tryptophan is present in the active site in an electropositive microenvironment and involved in binding. An arginine residue present at or near the active site may be involved in catalysis or extended binding of the substrate. A carboxylate and a histidine residue are involved in catalysis.

Chapter 5 : General Discussion

In this chapter the salient features of the thesis have been discussed with reference to the published literature and with reference to the stated objectives of the thesis, particularly industrial application.

CHAPTER

I

GENERAL INTRODUCTION

The plant cell wall consists of cellulose, hemicellulose, lignin and pectic substances. Pectic substances are structural polysaccharides occurring in the middle lamella and primary cell wall (Fig. 1.1). Pectin and hemicellulose are responsible for the integrity and coherence of plant tissues. The texture of vegetables and fruits are influenced by the amount of pectin present (Voragen, 1986). Most fruits have protopectin but during maturing and ripening it is converted into pectin and this conversion is economically important (Versteeg, 1978 and Rombouts and Pilnik, 1980). Degradation of pectic material is an important biological process in plants which includes elongation of cells, growth of the plant, ripening of fruits and abscission of leaves. Microbial pectin degradation is important for the decomposition of the plant material, digestion of plant food and the retting process (Voragen, 1989).

Plant pectin, a branched polysaccharide, consists of a backbone of α 1,4 glycosidically linked galacturonic residues with α 1,2 linked rhamnose at branch points. The L- rhamnopyranose residues of the sugars are attached to the C-1 and C-2 atoms in the main galacturonan chain. Depending upon the source of pectin galacturonate residues may be acetylated at C-2 and C-3 with neutral sugars such as galactose, arabinose and xylose. Enzymatic degradation shows that pectic substances consist of 'smooth' regions of homogalacturonan and highly branched 'hairy' regions which are rich in rhamnose and side chains of arabinose, galactose, and xylose (de Vries *et al.*, 1986).

The American Chemical Society has classified pectic substances into four main types (Kertesz, 1951).

1) Protopectin: A water insoluble parent pectic substance which occurs in plants, and from which pectic substances are produced.

2) Pectinic acid: Composed of polygalacturonic acid containing a small proportion of methyl ester groups. Pectinic acid is capable of forming a gel with acid and sugar.

3) Pectic acid: Consists of polygalacturonic acid and is essentially free of methyl ester groups.

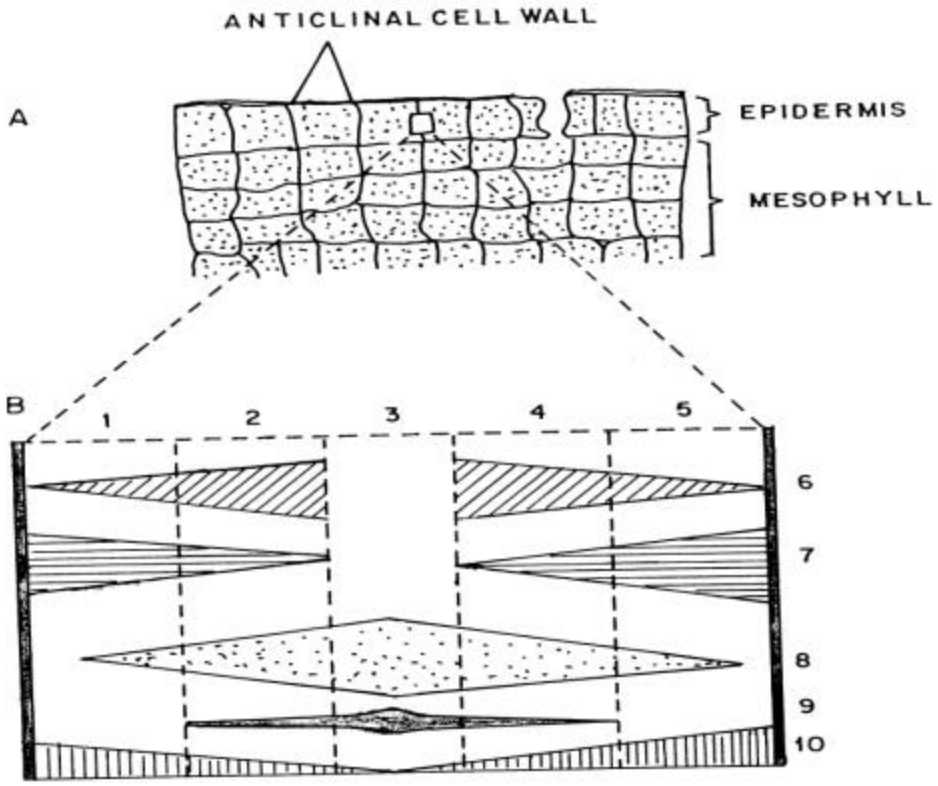
4) Pectin: A water soluble substance with a varying methyl ester content and degree of neutralization, so it forms a gel under acidic conditions.

Figure 1.1

A) Schematic representation of the epidermis and underlying mesophyll

B) Schematic representation of the cell wall structure at intracellular level

1: Secondary wall. 2: Primary wall. 3: Middle lamella. 4: Primary wall. 5: Secondary wall 6: Hemicellolose. 7: Cellulose. 8: Pectic substance. 9: Lignin. 10: Structural complexity. (Reproduced from: Bateman and Bashman, 1976)



Pectins are polymers where monomers of D-galacturonic acid are linked by α 1,4 glycosidic bonds to form polygalacturonic acid, and where the carboxylate groups of polygalacturonic acid are methoxylated. Methoxylation occurs in various degrees and is termed as degree of esterification. When esterification is more than 50 % the pectin is termed as high-methoxyl pectin whereas degree of esterification less than 50 % results in low-methoxyl pectins. When all the carboxyl groups in polygalacturonic acid are esterified the methoxyl content is 16.32 % and the degree of esterification is 100 %.

The molecular weight of different pectins varies from 10 kDa to 1,000 kDa depending upon the source and method of isolation. Pectic substances are highly viscous in aqueous solution and the viscosity of the material is dependent on the molecular weight, degree of esterification, ionic strength, pH, temperature, and concentration. Acid hydrolysis of pectic polysaccharides depends upon the temperature: at low temperature hydrolysis of ester bond takes place while higher temperatures are responsible for the depolymerization of the polysaccharide (Doesburg, 1965).

Degradation of pectin is an important activity in plant growth, saprophytic turn-over of carbon and pathogen attacks, and several key enzymes are involved in this process.

Pectinases are divided into two types: 1) Esterases and 2) Depolymerases. Pectin esterases catalyse the demethoxylation of pectin to form polygalacturonic acid. The depolymerases are further divided in two types 1) Hydrolases and 2) Lyases on the basis of their mode of action and type of substrate preferred. The classification of pectinases is based on mode of action (Table 1.1).

Table 1.1

Classification of pectinases

A) Esterase	Pectinesterase (EC 3.1.1.11), de-esterifies pectin to pectic acid by removal of methoxyl residues.
B) Depolymerase	
1) Acting on pectin	
a)	Polymethylgalacturonase (PMG)
(1)	Endo-PMG (EC 3.2.1.4), hydrolyses pectin in a random fashion.
(2)	Exo-PMG: hydrolyses pectin in a sequential fashion.
b)	Polymethylgalacturonate lyase (PMGL)
(1)	Endo-PMGL (EC 4.2.2.10), causes random cleavage in pectin by a transelimination process.
(2)	Exo-PMGL causes sequential cleavage in pectin by a transelimination process.
2) Acting on polygalacturonic acid	
a)	Polygalacturonase (PG)
(1)	Endo- PG (EC 3.2.1.15), hydrolyses polygalacturonic acid in a random fashion
(2)	Exo-PG (EC 3.2.1.67), hydrolysis of successive bond, releasing D-galacturonate.
(3)	Exo-PG (EC 3.2.1.82), hydrolysis of polygalacturonic acid from nonreducing end, releasing digalacturonate.
b)	Polygalacturonate lyase (pectate lyase, PGL)
(1)	Endo-PGL (EC 4.2.2.2), causes random cleavage in polygalacturonic acid by a transelimination process.
(2)	Exo-PGL (EC 4.2.2.9), causes sequential cleavage in polgalacturonic acid by a transelimination process.

In the chemical sense, hydrolytic cleavage is an acid-catalyzed reaction at the glycosidic bond of neutral polysaccharides and is estimated to be a slow reaction (Bochkov and Zaikov 1978). The mechanistic model for hydrolyses, where the acid is the proton donor, involves three steps 1) protonation of the glycosidic oxygen, to give the conjugate acid, 2) a unimolecular heterolysis of the conjugate acid, with the formation of a carbonium-oxonium ion and 3) addition of water to the carbonium-oxonium ion with the formation of a reducing end group and a proton (Sinnott, 1990). The same mechanism has been suggested for enzymatic hydrolysis of polysaccharides where either carbonyl or imidazole of the enzyme acts as a proton-donor. The mechanism of action of exo-β-(1-3)-D-glucanase (Jeffcoat *et al*., 1987), similar in action to β-glucosidase (Umerzurike, 1981) and α-L-aribinofuronidase (Selwood and Sinnott 1990) have been shown to be general acid catalysis.

Active site characterization of glycosidases identified those residues essential for polarization of the glysidic oxygen in the general acid hydrolysis mechanism. Matsuura *et al.*(1984) identified glutamic and an aspartic acid residue in the structure of Taka-amylase which are catalytically important. Henrissat (1989) also identified aspartic and glutamic acid residues as being involved in catalysis, during the amino acid sequence analysis of 21 different β-glucanases. A glutamic acid residue was identified in the sequence alignment of 28 different cellulase sequences, to be essential for the catalysis. Asn-Glu-Pro sequence is highly conserved in the endo-glucanases and it was suggested by the same author that this peptide may be involved in pyranose binding. Jeffcoat *et al*. (1987) implicated a histidine residue in an exo-β-(1-3)-D-glucanase.

In the pectin-degrading group of enzymes the hydrolases polygalacturonase (PG) and polymethylgalacturonase (PGM) cleave polygalacturonic acid or its methoxylated form in a manner apparently similar to that of other glycosidases acting on neutral polysaccharides. Henrissat (1991, 1993) suggested a classification of glycosyl hydrolases on the basis of their amino acid sequences. He classified 301 hydrolases corresponding to 39 EC entries into 35 families. Enzymes of one family are likely to have similar folding characteristics and active-site morphology. Henrissat (1989), by comparison of amino acid sequences of 21 β-glucanases, identified six families on primary structural homology.

Family 28 is polyspecific. Endo and exo- polygalacturonases belong in this family with 3.2.1.15 and 3.2.1.84 Enzyme Commission numbers respectively.

Endo-polygalacturonases (EC 3.1.2.15) act on polygalacturonic acid yielding saturated galacturonide as a end-product (Fig 1.2). Physiochemical properties of polygalacturonases show that, the pH optimum is between pH 3 to 5.5 (Kester and Visser, 1990; Waksman *et al*., 1991; Rao *et al*., 1996; Devi and AppuRao, 1996 and Gainvors *et al.*, 2000) with some exceptions like *Corticum rolsii* polygalacturonase which shows maximum activity at pH 2.5 (Kaji & Okada, 1969). The pH optimum of polygalacturonase also depends on the size of the substrate. The optimum pH of *Geotrichum candidum* shifted from 4.5 to 5.0 on polygalacturonic acid substrate to 3.5 on the trigalacturonate substrate (Brash and Eyal, 1970).

Kumari and Sirsi (1971), Urbanek and Zelewska-Sobczak (1975), Sakai and Takaoka (1985) and Devi and AppuRao (1996) have reported that the polygalacturonases studied by them showed temperature optima between 40 to 50°C, although unusually temperature stable enzymes have been reported (Mill & Tuttobello, 1961) and (Kumar and Palanivelu, 1999).

Polygalacturonases have molecular mass between 25 kDa to 80 kDa (Baldwin *et al*., 1989; Polizeli *et al*., 1991 and Kester and Visser, 1990). The polygalacturonase of *Neurospora sitophila* had a molecular mass of only 13 kDa (Fogarty and Dixon, as quoted by Fogarty and Kelly, 1983). Most organisms produce multiple forms of the enzyme (Kester and Visser; 1990; Devi and AppuRao, 1996 and Schwan *et al*., 1997) suggesting either that several genes are involved, or that post-translational modification results in different physical properties of the enzymes (Caprari *et al*., 1993).

Most polygalacturonases reported so far have acidic or neutral pI (Polizeli *et al*., 1991; Clasen and Green, 1996; Takasawa *et al.*, 1997). Polygalacturonase of *A. niger* produces five forms of polygalacturonases and all of these show acidic pI between 3.2 to 4.9 (Kester and Visser, 1990). Some polygalacturonases are basic molecules, for example polygalacturonase of *A. ustus* had a pI of 8.1 (Rao *et al*., 1996) and polygalacturonase of *Sclerotinia borealis* had a pI of 7.5 (Takasawa *et al*., 1997). Multiple forms produced by the same organism may also exhibit either acidic or basic pI (Cervone *et al*., 1977).

Polygalacturonase from different sources show different affinity towards the substrate (polygalacturonic acid). Most fungal polygalacturonases have K_m values between 1.5 mg/ml to 0.19 mg/ml (Wang and Keen, 1970, Manachini *et al*., 1987, Devi and AppuRao, 1996 and Rao *et al*., 1996). K_m value of polygalacturonase produced by *Fusarium oxysporum* has 0.54 mg/ml (Strand *et al*., 1976). On the other hand plant polygalacturonases show low affinities towards polygalacturonic acid for example tomato polygalacturonase has a K_m value of 2.7 mg/ml (Takehana *et al*., 1977). Properties of some fungal polygalacturonases are summarized in Table 1.2.

Wang *et al*. (1994) have reported two mechanisms of action for glycosidases; in one set of enzymes, direct displacement leads to net inversion of anomeric configuration. In the other set, anomeric configuration is retained via double displacement mechanism involving a glycosyl-enzyme intermediate. The mechanisms are quite distinct although same similarities are maintained such as both involve oxocarbonium ion-like transition states and both involve a pair of carboxylic acids. The carboxylic acids have different roles in the two cases. In the inverting enzyme one carboxyl acts as an acid catalyst and the other as a base catalyst, whereas in the retaining enzyme one functions as an acid/base catalyst and the other as a nucleophile.

Active site characterization of several of these hydrolytic enzymes has shown that histidine (Ohio *et al*., 1989; Helene *et al.*, 1991 and Gite *et al*., 1992) and carboxylate (Chipman and Sharon, 1969; Morosoli *et al*., 1986; Bray, 1990 and Deepal and Balasubramanian, 1986) are important residues for activity of these enzymes. Initially Cooke *et al*. (1976) reported the involvement of histidine residue in the active site of a polygalacturonase produced by *A. niger* and an early study by Rexova-Benkova and Mrackova (1978) on pH-dependant variation in K_m and V_{max} of polygalacturonase of *A. niger,* showed that there were two inflection points at pH 5.72 and 3.06 suggesting that histidine and carboxylate residues were important for the activity of the enzyme.

Figure 1.2

Products of pectinases

1: Unsaturated methoxylated galacturonide. **2:** Saturated methoxylated galacturonide. **3:** Unsaturated galacturonic acid. **4:** Saturated galacturonic acid. **(**Reproduced from: Alkorta *et al*., 1998)

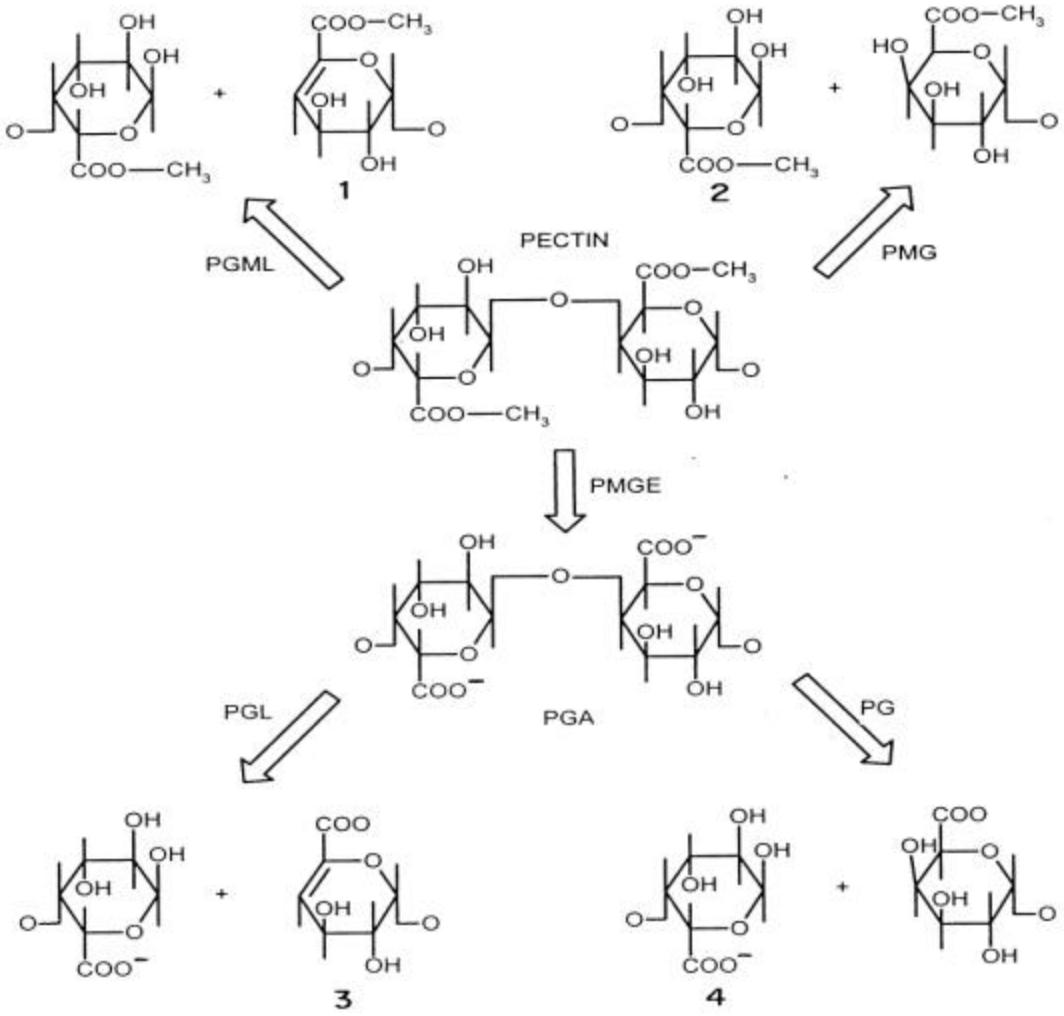


Table 1.2

Properties of some fungal polygalacturonases

Organism	**Forms**	**pH optimum**	**Mol. mass (kDa)**	**pI**	**Reference**
Aspergillus japonicus	I	4.5	35.5	--	Ishii & Yokotsuka (1972)
Aspergillus japonicus	I	4.7	25	--	Baldwin (1989)
	II	4.0	36.5	--	
Aspergillus nidulans	I	--	40	4.2	Dean and Timberlake(1989)
Aspergillus niger	I	4.1	35	--	Cooke *et al.* (1976)
	II	3.8	85	--	
Aspergillus niger	I	4.9	85	3.2-3.5	Kester & Visser (1990)
	II	4.8	38	4.6-4.9	
	IIIA	4.3	57	3.3	
	IIIB	4.5	57	3.3	
	IV	4.8	59	3.7	
Cryococcus albidus	I	5-6	45	6.8	Tanabe and Kamishima(1988)
Fusarium oxysporum	I	5.0	37	7.0	Strand *et al.* (1976)
	II	5.0	37	7.0	

Table: 1.2 Continued

Organism	forms	pH optimum	Mol. mass (kDa)	pI	Reference
Neurospora crassa	I	4.6	37	-	Polizeli *et al*. (1991)
Rhizoctonia fragariae	I	5.0	36	6.8	Cervone *et al*. (1977)
	II	5.0	36	7.1	
Rhizoctonia stolonifer	I	5.0	57	--	Manachini *et al*. (1987)
Kluyveromyces marxianus	I	5.0	45	5.9	Schwan *et al*. (1997)
	II	5.0	42	5.6	
	III	5.0	39	5.3	
	IV	5.0	36	--	
Corticium rolfsii	I	2.5			
Sclerotinia borealis	I	4.5	40	7.5	Takasawa *et al*. (1997)
Saccharomyces cerevisiae	I	3 - 4.5	42	--	Gainvors *et al*. (2000)
Postia placenta	I	3.2 - 3.9	34	3.2 - 3.4	Claasen & Green (1996)
Aspergillus ustus	I	5.0	36	8.2	Rao *et al*. (1996)
Thermomyces lanuginosus	I	5.5	59	--	Kumar and Palanivelu (1999)
Aspergillus carbonarius	I	4.0	61	--	Devi & AppuRao (1996)
	II	4.1	42	--	
	III	4.3	47	--	

Using chemical modification with diethylpyrocarbonate, Rexova-Benkova (1970) and Rao *et al*. (1996) have reported that the activity of polygalacturonases of *A. niger* and *A. ustus* were inhibited at pH 6 suggesting that a histidine residue was involved in activity. More recently Caprari *et al*. (1996) showed by site directed mutagenesis that a His^{234} was at the active site of an endo-polygalacturonase produced by *F. moniliforme.*

Rexova-Benkova (1990) showed that polygalacturonase of *A. niger* was completely inhibited by carboxylate modifing reagent EDC at pH 4.5. Recently, van Santen *et al*. (1999) showed that in *A. niger* endo-polygalacturonase II two carboxylate residues (Asp^{180} and Asp^{202}) at an appropriate distance of 5.7 A° in the active site are hydrogen bonded to a water molecule which donates a proton in the catalytic process (Wang *et al*., 1994). Armand *et al*. (2000) determined the active site topology of endo-polygalacturonase II produced by a different strain of *A. niger*, and suggested that the aspartic residues, Asp^{180}, Asp^{201} and Asp^{202} are conserved.

The indole ring of a tryptophan residue has affinity towards carbohydrates, and may be involved in substrate binding. The intrinsic tryprophan fluorescence of polygalacturonase was quenched with increasing concentration of substrate (polygalactutronic acid), and the reaction kinetics of polygalacturonase of *Colletotrichum lindemuthianum* (Waksman *et al*., 1992) suggested that involvement of the tryptophan was in substrate binding. Polygalacturonase of *A. ustus* was inhibited by tryptophan specific reagent HNBB suggesting that a tryptophan residue was present at or near to the active site (Rao *et al*., 1996). Tryptophan has been reported to be present in the active site of various other saccharidases like endo-1,4-β-glucanase (Ozaki and Ito, 1991), isomalto-dextranase (Okada *et al*., 1988) and xylanase (Kesker *et al*., 1989).

Urbanek and Zelewska-Sobczak (1975) have implicated cysteine and tyrosine residues in the active site of the enzyme produced by *Botrytis cinerea.* Stratilova *et al*. (1996) have reported a tyrosine residue in the active site of polygalacturonase produced by *A. niger*. Thus several amino acid residues have been implicated in enzyme active site of polygalacturonase produced by different microbes, although work on the enzymes produced by *Aspergillus* and *Fusarium* have suggested that histidine, carboxylate and tryptophan residues are involved in the catalytic process.

The crystal structures of glycosyl hydrolases suggest a common motif. The crystal structure of porcine pancreatic α-amylase (Buisson *et al*., 1987) showed three domains: the larger, N-terminal domain consists of parallel α-β barrel structure, the C- terminal domain forms a globular unit, where the chain folds into an eight-stranded anti-parallel β-barrel and the third domain is composed of anti-parallel β-sheets. The occurrence of enzymes from different sources such as α-amylase, β- galactosidase, xylanase, pyruvate kinese, aldolase, xylose isomerase, with different functions, but with similar super secondary structure indicate that enzymes can converge to a common peptide fold essential for catalytic function.

The first structure of polygalacturonase was solved by Pickersgill *et al*. (1998). The molecule comprises of 10 turn right handed parallel β-helix domain with two loop regions which forms a tunnel like substrate binding cleft (Fig 1.3A). Amino acid sequences which are conserved in the active site of polyagalacturonase are between these two loops. Alignment of 36 polygalacturonase sequences reveals four conserved regions Asn-Thr-Asp, Gly-Asp-Asp, Gly-His-Gly, and Arg-Ile-Lys. These conserved residues are located on β-strands 5,6,7 and 8 of polygalacturonase. Asn-Thr-Asp forms strand 5 and Arg-Ile-Lys forms strands 8. Gly-Asp-Asp forms the turn before β-strand 6, and Gly-His-Gly forms the turn before β-strand 7. The substrate binding cleft is formed by two long loops that precede strand 2 and 3 and three loops that follow strands 7,8 and 9. Pickersgill *et al*. (1998) suggests that Ser^{257} present on the seventh β-strand of polygalacturonase may be important in maintaining the geometry of the substrate binding cleft. The crystal structure suggests that Asp^{202} and Asp^{223} are directly involved in catalytic activity. Two water molecules, one of them bound to these carboxylates may be the nucleophiles in the reaction.

van-Santen *et al*. (1999) solved the crystal structure of polygalacturonase II from *A. niger*. The crystal structure represents enzyme folded into a right handed parallel β-helix with 10 complete turns. The β-helix is composed of four parallel β-sheets and has one very small α-helix near the N-terminus which occupies the enzymes hydrophobic core. Loop regions form a cleft on the exterior to the β-helix. Asp^{180}, Asp^{201}, Asp^{202}, His^{223}, Arg^{256}

and Lys^{258} are located in this cleft. Site-directed mutagenesis shows that these residues are involved either in substrate binding or in the catalytic mechanism.

More recently, Federici *et al*. (2001) solved the structure of polygalacturonases produced by the phytopathogenic fungus *Fusarium moniliforme*. The crystal structure showed that the right-handed parallel β -helix, consisting of 10 coils, each made up three to four β-strands (Fig 1.3 B). The active site of this protein showed that Lys^{269} and Arg^{267} are located inside the active site cleft, and His^{188} at the edge of the active site.

Thus the structures of polygalacturonases of *Erwinia*, *Aspergillus* and *Fusarium* show that all the proteins exhibit a "cleft" where catalytic residues are located. The molecular mass of *Erwinia, Aspergillus* and *Fusarium* polygalacturonases are 40 kDa, 35 kDa and 38 kDa respectively. The active site of *Erwinia* enzyme show that arginine, lysine and aspartate residues present. Polygalacturonase II of *Aspergillus niger* show Asp^{180}, Asp^{201}, Asp^{202}, His^{223}, Arg^{256} and Lys^{258} residues are located in the active site of enzyme. The active site cleft of *Fusarium* polygalacturonase has of Lys^{269}, Arg^{267} and His^{188} residues. Thus although all these enzymes having different molecular masses between 35 to 40 kDa, aspartate, arginine, lysine and histidine residues are conserved in the active site. All these enzymes showed 4.5%, 1.9%, and 2.4% of α helix component and 39.9%, 41%, and 43% of β sheet component suggesting that they have similar structural components, although they are produced by different organisms, in fact in the *A. niger* polygalacturonase II and the *E. carotovora* enzymes, 265 Cα atoms out of 335 are superimposible with an r.m.s.d. of 1.8 A° (Pickersgill *et al*., 1998, van Santen *et al*., 1999 and Federici *et al*., 2001).

The *Fusarium* polygalacturonase reported by Federici *et al*. (2001) shows 43.5% sequence identity to the *A. niger* II polygalacturonase. The amino acid sequence of *E. corotovora* polygalacturonase shows only 19 % sequence homology to the *A. niger* II polygalacturonase. Stratilova *et al*. (1993) compared amino acid sequence homology between polygalacturonase from *Aspergillus niger* I, II, *A. tubigensis, Lycopersicon esculentum* (tomato) and *Prunus persica* (peach) and showed that homology is seldom greater than 40 %. Henrissat, (1991) has shown that amino acid sequences of polygalacturonases do not share significant homology with other glycoside hydrolases either. Thus although homology is low, structure is conserved.

The mode of action of polygalacturonases (EC 3.2.1.15) may be either a single-chain multiple attack mechanism in which end products appear rapidly or by a multi- chain attack where the monomers, dimers, and trimers accumulate only after further hydrolyses of higher oligogalacturonates. For example *Colletotrichum lindemuthianum* enzyme (English *et al*., 1972) produced only di or trigalacturonic acids whereas the *Kluyveromyces fragilis* enzyme (Phaff, 1966) hydrolyses pectate through a series of higher oligogalacturonides subsequently further hydrolysed to mono and digalacturonic acids. Activity on oligogalacturonates also decreases with decreasing chain length while some typical endo-polygalacturonases fail to hydrolyse digalacturonic acid and trigalacturonic acid (Rexova-Benkova & Markovic, 1976). Nasuno and Starr (1966) have reported that polygalacturonase of *E. carotovora* does not act on digalacturonic acid but hydrolyse trigalacturonic acid completely to form monogalacturonic acid. The initial rate of hydrolysis of substrate by polygalacturonase of *E. carotovora* depends upon the size of the substrate. It hydrolyses polygalacturonic acid, hexa-, penta-, tetra-, tri-galacturonic acid at the relative rates of 100, 15.8, 12.8, 2.2 and 1.7 respectively.

Endopolygalacturonases also show considerable differences in action patterns on oligogalacturonates. These differences depend on the nature of active site of the enzyme but more specifically by the size of the substrate binding and position of catalytic group (Rexova-Benkova 1973). Benen *et al*. (1999) have studied the kinetic characterization of endo-polygalacturonases I, II, C produced by a recombinant strain of *A. niger*. All these enzymes act on polygalacturonic acid by a random cleavage pattern. The authors used oligogalacturonides for the determination of sub-site of the enzymes. The sub-site maps show that the minimum number of galacturonic acids required for hydrolysis is seven for all three enzymes.

Polysaccharide lyases depolymerize acidic carbohydrates through β-eliminitive cleavage by abstraction of a proton (Fig 1.2). The abstraction of a proton from the α-carbon atom is assisted by the electron-withdrawing carboxyl group of the polysaccharide. Abstraction of the proton either enzymatically or by chemical base such as NaOH results in a direct eliminative cleavage forming an α-β unsaturated uronic acid residue on the non reducing and a hemiacetal on the reducing-end of the glycoside linkage (Albersheim *et al*., 1960). Lyases such as pectate lyase (PL) and polymethylgalacturonate lyase (PMGL)

catalyze a typical β-elimination (Albersheim *et al*., 1960), common to acting on acidic polysaccharides (Linnhardt *et al*., 1986) and belong in the Enzyme Commission number 4.2.2.2.

An important feature of the pectate lyase is the requirement of calcium, strontium or barium ion for their activity (Nagel & Wilson, 1970). It has been suggested that metal ion form salt bridge in tetraoligogalacturonides which are the true substrate of pectate lyase (Atallah and Nagel, 1977). Some of the characteristics of pectate lyases have been listed in Table 1.3. These enzymes acts on the polygalacturonic acid at alkaline pH and have pH optima in the range between 8 to 10 pH. The molecular mass of pectate lyase ranges from 12 kDa to 74 kDa. Although lyases show optimum activity at the alkaline pH range, the pI of the molecule varies from pH 4.6 to 10.3 (Miyazaki, 1991 and Sato and Kaji, 1980).

Figure 1.3

A)Three-dimensional structure of polygalacturonase from *Erwinia carotovora.*

Active site residues are shown in different colors. Blue: Lys^{282}, Purple: Asp^{202}, Asp^{205}, Asp^{223} & and Asp^{224} and Red: His^{251}. (Reproduced from: Pickersgill *et al.*, 1998)

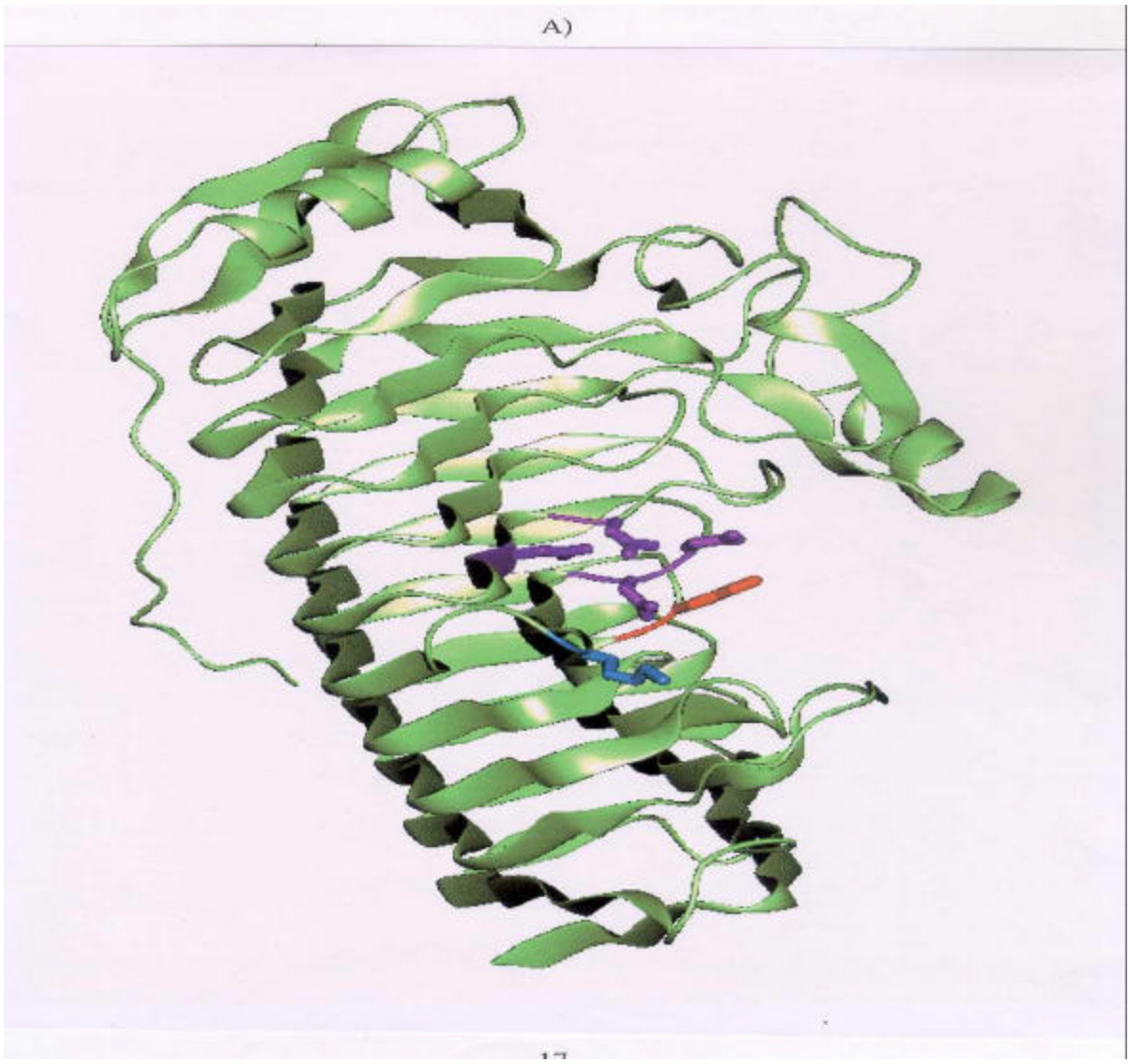
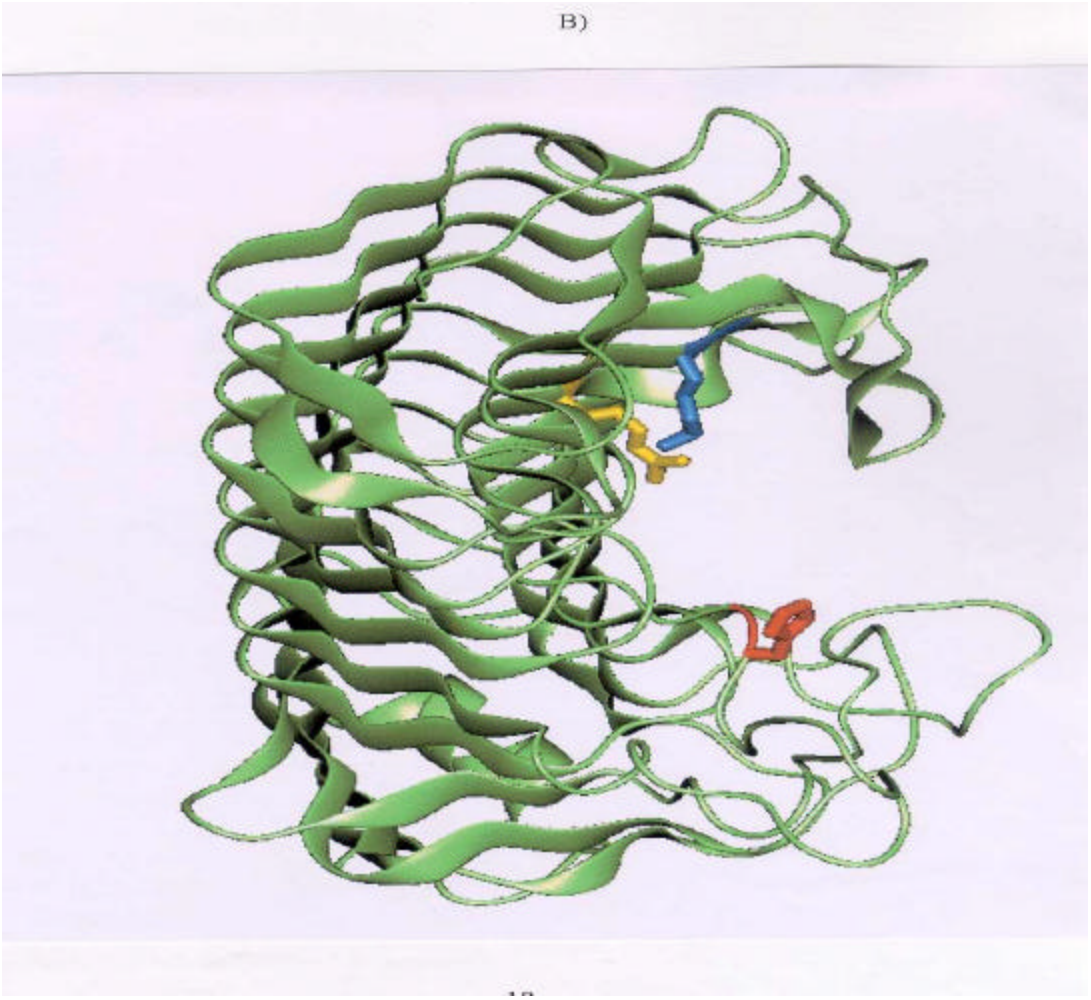


Figure 1.3

B) Three-dimensional structure of polygalacturonase from *Fusarium moniliforme.* Active site residues are shown in different colors. Blue: Lys^{269}, Yellow: Arg^{267} and Red: His^{188} (Reproduced from: Fiderici *et al.*,2001.)



Though pectate lyases have been purified and their preliminary characterization reported, there are a very limited studies on the active site characterization of these enzymes. The active site characterization of pectate lyase of *Fusarium moniliforme* NCIM 1276 showed that lysine and tryptophan residues are present at or near to the active site and Ca^{++} ions are essential of enzyme activity (Rao *et al*., 1996A). The authors showed that tryptophan fluorescence was quenched by the addition of substrate suggesting that tryptophan involved in the substrate binding.

Yodder *et al*. (1993) solved the crystal structure of the pectate lyase produced by *Erwinia chrysanthemi* and showed that the enzyme folds into a unique tertiary helix motif of parallel β- strands. Because of their functional similarities, these authors have suggested that all extracellular pectate lyases will have similar structure, differing only in the size and shape of the protruding loops. The authors have identified a common site on the protein, where acidic residues, Asp^{131}, Glu^{166} and Asp^{170} are conserved which are probably responsible for calcium binding. Pickersgill *et al*. (1994) solved the structure of pectate lyase produced by *Bacillus subtillus* in presence of calcium. These authors have shown that, the metal was attached to the acidic residues of glutamate and aspartate.

Table 1.3

Properties of pectate lyase from some microbial source

Source	Forms	Mol. mass kDa	pH optima	pI	Reference
Bacillus circulans	I	70	10.0	--	Joyce & Fogarty (1975)
	II	18	9.5	--	
Bacillus subtilus		33	8.5	9.85	Chesson & Codner (1978)
Bacillus stearothermophilus		24	9.0	--	Karbassi & Vaughn (1980)
Bacillus macerans		35	9.0	10.3	Miyazaki (1991)
Erwinia caratovara	I	41	--	9.1	Lei *et al*. (1985)
	II	44	--	9.4	
Erwinia aroideae		37	9.1	--	Kamimiya (1977)
Pseudomonas fuorescence		42.3	9.4	10.4	Rombouts *et al*. (1978)
Streptomyces nitrosporium		41	10.0	4.6	Sato & Kaji (1980)
Fusarium oxysporum	I	25	--	--	Artes & Tena (1990)
	II	37	--	--	
Fusarium solani		26	9.4	8.3	Crawford & Kolattukudy (1987)
Fusarium moniliforme		12	9.5	9.1	Rao *et al*. (1996A)
Klebsiella oxytoca	I	71	9.0	5.9	Pitkkanen *et al*. (1992)
	II	71	8.5	5.3	
Yersinia enterocolitica		55	8.8	5.8	Bagley (1979)

Polygalacturonases are produced by numerous fungi and bacteria and also by higher plants (Kester and Visser, 1990; Riou *et al*., 1992; Zheng *et al*., 1992; Kumar and Palanivalu, 1999; Wubben *et al*., 1999 and Lei *et al*., 1985). The plant enzymes are produced prior to budding or branch formation to break down existing cell wall.

Fungal and bacterial enzymes are produced during the course of saprophytic growth. Cooper *et al*. (1978) have reported that, pectic enzymes are the first polysaccharidases to be induced when fungi are cultured on isolated cell-wall.

When a pathogen attacks host plants, pectic enzymes act on plant cell wall pectins resulting in cell lysis and plant tissue maceration (Martinez *et al*., 1991). Karr and Albersheim (1970) showed that polysaccharide-degrading enzymes are unable to directly attack plant cell wall without prior action by wall modifying pectic enzymes. Several fungi exhibit both saprophytic and pathogenic modes of nutrition. *C. lindemuthianum* (English *et al*., 1972), *S. sclerotiorum* (Riou *et al*., 1991), *F. oxysporum f.sp.melonis* (Martinez *et al*., 1991), *F. solani f.sp pisi* (Crawford and Kolattukudy, 1987), *C. rolfsii* (Kaji and Okada, 1969), *A. niger* (Cervone *et al*., 1987), *A. nidulans* (Dean and Timberlake, 1989), *Monilinia ssp* (Fielding, 1981) and *P. frequentans* (Kawano *et al*., 1999) produce cell wall degrading enzymes and these species and *F. moniliforme* (De Lorenzo *et al*., 1987) are well known plant pathogens, which can also exhibit saprophytic modes of nutrition.

Polygalacturonases are inducible enzymes (Nyiri, 1968; Aguilar and Huitron, 1987 and Kawano *et al*., 1999). Inducers are galacturonic acid, its polymer (pectin and polypectate) and structural relatives (mucic acid, tartonic acid and dulcitol) (Keen and Horton, 1966 and Maldonado and Strasser de Saad, 1998). Polygalacturonase production may be repressed by the addition of glucose (Maldonado *et al*., 1989). Glucose is also known to repress the production of pectate lyase by a clinical strain of *Yersinia entercolitica* and a phyto-pathogenic strain of *Erwinia* (Chatterjee *et al*., 1979).

Polygalacturonases are used in fruit juice clarification. Whitaker (1984) reported that the juice yield and clarity increased in the presence of polygalacturonase, and rapid drop in viscosity facilitated filtration. Polygalacturonases are also used in extraction of juice by degradation of pectin resulting in higher yields. This enzyme is also used for the liquefaction of tissue. Thus polygalacturonases are important industrial enzymes besides their uses in nutrition and growth by different organisms. Industrial production of these

enzymes is carried out using mostly *Aspergillus niger*. Some species of *Aspergillus* are pathogenic to crop plants.

The production of polygalacturonase in submerged culture depends on carbon and nitrogen sources in the growth medium, temperature, pH and inoculum size. Polygalacturonase production has been optimized using submerged culture by Aguilar and Huitron (1987), Leuchtenberger and Meyer (1992) and Friedrich *et al*.,(1989). However, industrial production of polygalacturonase is generally carried out by semi-solid fermentation because this fermentation gives higher activity of the enzyme than submerged fermentation (Pereira *et al*., 1993). Agricultural by-products such as wheat bran (Rao, 1996), rice bran, sugar-cane bagasse (Periera *et al*., 1993) with beet peel and citrus peel (Siessere and Said, 1989) and orange peel as additional pectin sources have been used in this process. Acuna-Arguelles (1995) and Maldonado and de Saad (1998) have reported that polygalacturonase production by *A. niger* was six times higher in solid state fermentation than in submerged fermentation.

The occurrence of mangrove fungi in association with mangrove plants was first reported by Cribb & Cribb (1960). Hyde (1988) isolated 91 species of marine fungi recorded from mangroves all over the world. Chinaraj (1994) collected 94 species of higher marine fungi from mangrove forests of India. He identified 72 species of ascomycotina, 2 species of basidiomycotina and 20 species of deuteromycotina. From the deuteromycotina *Cirrenalia* genus was dominant in the coastal Indian mangrove followed by *Cladosporium, Cystopora, Phoma, Sporidesmium* (Chinaraj, 1994). D'souza and Araujo, (1979) isolated numerous fungi of deuteromycotina from the Mumbai region and have reported the occurrence of *Fusarium* from the coastal region.

During the screening for pectinase producer, Rao (1996) isolated a strain of fungus from decaying mangrove plants from Mumbai region. On the basis of morphological features the strain was identified as *F. moniliforme* and deposited in the National Collection of Industrial Microorganism at NCL as *F. moniliforme* NCIM 1276. Although in general *Fusarium* is a plant pathogen, some species cause symptomless infection in *Zea mays* (Bacon and Hinton, 1996 and Yetes *et al*., 1997). The present organism was isolated from decaying mangrove wood and it was desirable to determine its mode of nutrition.

The organism belongs to the order Moniliales and family Moniliaceae. It produces white colonies with septate mycelia. The spore bearing hyphae, are conidiophores. Conidiospores are thick walled and with tapering ends. This fungus produces two types of conidia, microconidia and macroconidia with separate conidiophore. Macroconidia are bigger in size as compared to the microiconidia, and are attached to each other at their ends and to form a chain.

This isolate secretes polygalacturonase (EC 3.2.1.15) at pH 5 and pectate lyase (EC 4.2.2.2) at pH 8 when supplied with pectin as a carbon source in submerged culture.

The objective of this thesis was to determine the mode of nutrition of this *Fusarium*. Since it produces two pectinases each having an acidic or alkaline pH optimum, the production of these two enzymes was optimized and further, the pathogenecity of the organism was tested on different host plants. The polygalacturonase was purified and its active site characterized.

Chapter 2 presents the data referring to the optimum culture conditions for polygalacturonase production in submerged and solid state fermentation and pH dependant quantification of polygalacturonase and pectate lyase using ELISA.

Chapter 3 presents data on polygalacturonase and pectate lyase production in different host plants.

Chapter 4 presents the characterization of active site of the purified polygalacturonase.

Chapter 5 presents a general discussion.

CHAPTER II

pH dependent induction of polygalacturonase and pectate lyase from highly pH tolerant *Fusarium moniliforme* NCIM 1276.

Summary

Several samples were collected from different estuarine environments. These samples were screened for pectinase production. One fungal isolate was found which produced polygalacturonase at acidic pH and pectale lyase at alkaline pH. This isolate was identified as *Fusarium moniliforme* on the basis of morphological characteristics and deposited in the National Collection of Industrial Microorganism (NCIM) as *Fusarium moniliforme* NCIM 1276. This organism is salt tolerant to 0.4 M NaCl and both enzymes retain full activity at 0.4 M NaCl.

The isolate produced polygalacturonase maximally (0.28 U/ml) at pH 5 in a submerged culture containing 1 % pectin, 0.2 % glucose, 0.5 % $NH_4(SO)_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4, 0.1% $CaCl_2$. The same organism produced pectate lyase maximally (8.1 U/ml) at pH 8 in the same submerged culture. Polygalacturonase production was induced in presence of 1% pectin but production of this enzyme was repressed when glucose (1%) was used as sole carbon source in the submerged culture at pH 5. On the other hand pectate lyase production was not affected in presence of (1%) glucose as sole carbon source in the submerged culture at pH 8. The thesis that polygalacturonase is an inducible enzyme whereas pectate lyase is produced constituvely by the organism is examined in the light of these data.

When the isolate was inoculated on a solid state medium containing wheat bran and orange pulp as carbon source, the production of polygalacturonase was three times more as compared with submerged culture medium. The optimal production was 80 U/gram of mixed substrate as compared to 28 U/ gram pectin in submerged culture. This organism produced single form of polygalacturonase and pectate lyase in submerged culture however two forms of polygalacturonase and single form of pectate lyase were produced in solid state wheat bran medium.

Using sandwich ELISA polygalacturonase and pectate lyase protein biomass were measured. These assays show that, this isolate produced extracellular and intracellular polygalacturonase and pectate lyase from pH 2 to11 but the maximum secretion of the enzymes occurred at pH 5 and at pH 8 respectively. Immunocytolocalization studies shows that both enzymes localized near to the cell membrane and vacuole regions of the spore and mycelium.

Introduction

Bench-top production of polygalacturonase and pectate lyase in submerged fermentation depends on critical control of media components and pH, temperature, aeration and agitation (Maldonado and Strasser de Saad, 1998) but may give lower productivity than solid-state fermentation although final conditions vary from organism to organism. In general solid state fermentation uses cheaper medium constituents (Acuna-Arguelles *et al*., 1995 and Maldonado and Strasser de Saad, 1998) and does not involve the critical control required for submerged fermentation (Pereira *et al*., 1993). The production of polygalacturonase by solid state fermentation has been done by growing fungi on agricultural by-products such as wheat bran (Rao, 1996), rice bran, sugar-cane bagasse (Periera *et al*., 1993) with beet peel and citrus peel (Siessere and Said, 1989) and orange peel as additional pectin sources.

The following chapter deals with the optimization of the growth conditions of *Fusarium* for maximum production of extracellular polygalacturonase in both submerged and solid state fermentation at the bench-top level. Antibodies raised against purified polygalacturonase and pectate lyase were used to determine the quantity of protein released in the cultures. The quantification of polygalacturonase and pectate lyase was done using sandwich ELISA. The antibodies were used to determine qualitative and quantitative differences in enzymes produced in submerged culture at different pHs. Immunolocalization of both enzymes was carried out using Transmission Electron Microscopy.

Materials

Pectin (Citrus fruit), polygalacturonic acid, Freund's complete and incomplete adjuvant, horseradish peroxidase, anti-rabbits IgG peroxidase conjugate and para-formaldehyde were purchased from Sigma Chemical Company USA. Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden. Tetramethyl benzidine/ hydrogen peroxide (TMB/H_2O_2), 5-bromo-4-chloro-3indolyl phosphate/nitro blue tetrazolium (BCIP) and anti-rabbits IgG gold conjugate were purchased from Banglore Genei Pvt Ltd India. Divinyl sulfone (DVS) was purchased from Aldrich Chemical Company USA. ELISA plates were purchased from Greiner Labortechnik Pvt Ltd Chandighad, India. Araldite A & B, DMP-40, gulteraldehyde, sodium cacodylate, uranyl acetate were purchased from Pellaco chemicals UK. Wheat bran and orange pulp was obtained from the local market. All chemicals and reagents except wheat bran and orange pulp used were of analytical grade.

Microorganism

Samples were collected from decaying wood of mangrove plants. Aliquots were put into enrichment media containing 1% pectin as a carbon source. The isolates were grown on agar medium containing 1% pectin and 0.2% K_2HPO_4 in sea-water, resulting colonies were isolated using standard microbiological techniques. The highest pectinolytic enzyme producer was chosen for the further studies. It was sub cultured on Czapek-Dox agar modified with1% pectin. The organism was identified on the basis morphological characters and deposited in the National Collection of Industrial Microorganisms at NCL, as *Fusarium moniliforme* NCIM 1276.

Methods

Enzyme assays

Polygalacturonase

Polygalacturonase was assayed by measuring the reducing sugars released from 0.3% polygalacturonic acid, by the method of Nelson (1944) and Somogyi (1945). The reaction mixture was made up in 0.1M sodium acetate buffer pH 5.0 with 0.7% NaCl and 0.25%

Na-EDTA. One unit of enzyme activity is defined as the amount of enzyme which released 1 µmol of galacturonic acid per minute at 40 °C at pH 5.0 (Collmer *et al*., 1988).

Pectate lyase

Pectate lyase activity was measured as the increase in absorbance at 232 nm of 0.24% polygalacturonic acid in 60 mM Tris/HCl buffer, pH 8.5 with 0.6 mM $CaCl_2$ at it's optimal temperature of 40°C. One unit of lyase activity was defined as the amount of enzyme which produced 1 µmol of unsaturated galacturonide (ε = 4600 $M^{-1}cm^{-1}$) per minute (Collmer *et al*., 1988).

Xylanases

The total reaction mixture of 1 ml contained 0.5 ml of suitable diluted enzyme in 50 mM sodium phosphate buffer, pH 6.0 and 0.5 ml of 1 % xylan (w/v) solution. The reaction mixture was incubated at 50°C for 30 min and then the reaction was terminated by the addition of 1 ml 3,5-dinitrosalicylic acid (DNSA). The reducing sugars liberated were determined by measuring the absorbance at 540 nm as described by Miller (1959). One unit (U) of xylanase activity is defined as the amount of enzyme required to liberate 1 µmole of xylose per min under standard assay conditions.

Amylase

Amylase activity was determined according to Bernfeld (1955). The reaction mixture contained 0.5 ml of 1% (w/v) soluble starch in 0.05 M acetate buffer pH 5.0 and 0.5 ml of suitably diluted enzyme. The reaction mixture was incubated at 50°C for 15 min and reducing sugars liberated were determined as glucose equivalents by the 3,5-dinitrosalicylic acid (DNSA) method (Miller, 1959). One unit (U) of the enzyme activity is defined as the amount of enzyme required to liberate 1 µmole of reducing sugars/min under standard assay conditions.

Cellulase

The reducing sugars liberated from the hydrolysis of 1% CMC (carboxymethyl-cellulose) as substrate were quantified using 3,5-dinitrosalicylic acids (DNSA). One unit of

celluloytic activity was defined as the amount of enzyme that released 1µmole of reducing sugar (glucose equivalent) per minute form 0.1 % CMC at 50 mM sodium acetate buffer pH 6 (Millar, 1959).

Protein assay

Protein concentration was determined in accordance with Lowry *et al*. (1951) with BSA standard.

Biomass estimation

Fungal biomass was harvested by centrifugation, washed with distilled water and dried at 40°C, till constant dry weight.

Optimization

Culture conditions

The inoculum was prepared in 100 ml conical flask using a medium containing 1% pectin, 0.2% glucose, 0.2 % $NH_4(SO)_4$ and pH 6. The flask was incubated for 48 h at 30°C and about 200 rpm. Exponentially growing cells were pelleted out, washed with sterile distilled water and resuspended in 100 ml of sterile distilled water. 1 ml of this suspension was inoculated in 50 ml of the culture medium in the all experiments.

The organism was grown in 250 ml conical flasks with 50 ml medium. The flasks were incubated for 5 days at 30°C and 200 rpm on rotary shaker. After centrifugation, biomass in terms of dry weight and the polygalacturonase activity in the cell free supernatant was determined under standard assay conditions.

All experiments were done in duplicate. In all the following experiments culture flasks were incubated at 30°C for 5 days except where otherwise stated.

Effect of salinity

Various concentrations of NaCl were used in the optimized growth medium (1% pectin, 0.2 % glucose, 0.5% $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2 % KH_2PO_4, 0.1% $CaCl_2$ at pH 5). After five days of growth, biomass was determined in terms of dry weight and mycelial-free broth was assayed for polygalacturonase activity.

Effect of carbon source

The medium containing 0.5% $(NH4)_2SO_4$, 0.2 % K_2HPO_4, 0.2 % KH_2PO_4, 0.1% $CaCl_2$ at pH 5. Pectin, starch, glucose, cellulose, xylan and wheat bran were used at a concentration of 1% w/v. Polygalacturonase activity was determined using standard assay conditions.

In the following series of experiments the basal medium was as above except where otherwise indicated.

Effect of pectin

Concentrations of pectin were varied in the basal medium. 0.2 % glucose was added at all pectin concentration.

Effect of glucose

10^6/ml of spore suspension of *Fusarium moniliforme* was inoculated in to the basal medium containing 1% pectin with different concentration of glucose. Samples were harvested after 96 hrs incubation. The cell biomass was determined by measuring dry weight. Enzyme activity was determined under standard assay conditions and protein biomass was determined by sandwich ELISA.

Effect of different nitrogen salts

Nitrogen salts used were: $(NH_4)_2SO_4$, KNO_3, $NaNO_3$, NH_4NO_3 and 0.5% peptone at 0.175 M nitrogen. The concentrations of the best nitrogen sources were then varied to determine maximum production of enzyme.

Effect of pH

Induction of different polysaccharidases of Fusarium moniliforme at pH 5 and 8.0

F. moniliforme spore suspention (10^6/ml) was inoculated in to 50 ml basal medium containing 37.9 mM $(NH4)_2SO_4$, 11.5 mM K_2HPO_4, 14.7 mM KH_2PO_4 and 6.8 mM $CaCl_2$: 2 H_2O with 1% sugars such as pectin, polygalacturonic acid, galacturonic acid, starch, glucose, cellulose, xylan and wheat bran. The flasks were incubated at 30°C and 200 rpm

on a shaker for 96 hrs. The residual activities of the corresponding enzymes were measured under standard assay conditions.

Effect of initial pH on polygalacturonase production

Initial pH of the optimized medium was adjusted from 2 to 11 with NaOH and HCl. After 5 days incubation at 30 °C, dry weight of mycelium and activity of polygalacturonase were determined.

Quantification of extracellular and intracellular PG and PL at different pHs

F. moniliforme spore suspension (10^6/ml) was inoculated into 50 ml basal medium containing 1% pectin and 0.2 % glucose as a carbon source. The initial pH of medium was adjusted from pH 2 to pH 11 using 1N HCl and 1N NaOH. The flasks were incubated at 30°C for 96 hrs. Extracellular PG and PL proteins biomass were determined using sandwich ELISA.

To determine intracellular protein concentrations whole cell biomass was sonicated for 5 minutes. The cell debris were removed by centrifugation. The supernatant cell extract was diluted up to 30 ml with distilled water. Intracellular concentration of polygalacturonase and pectate lyase were determined using sandwich ELISA.

CD spectra

2.56 µM purified pectate lyase and 1.82 µM polygalacturonase were incubated with 100 mM of various buffers from pH 2 to 11 for 24 hrs at 30 °C. CD spectra were recorded on a Jasco-710 spectropolarimeter from 190 nm to 250 nm using a 1 cm path length at 25 °C. CD analysis was carried out according to the method of Sreerama and Woody (1993). The data has been analyzed using the program SILCON.

Time course of polygalacturonase and pectate lyase production

Cultures were grown in respective optimized media upto 5 days. Polygalacturonase and pectate lyase enzyme activities were determined under respective standard assay conditions. Protein biomass of polygalacturonase and pectate lyase was quantified by

ELISA. Dry weight of fungal biomass was determined as above. The time course was run at 24 h intervals on duplicate flasks.

The effect of temperature

The culture was grown in the optimized pectin medium for 5 days at different temperatures raining from 10°C to 50°C. The fungal biomass in terms of dry weight and polygalacturonase activity were determined under standard assay conditions.

Effect of agitation

The organism was grown in the optimized medium for 5 days at 30°C at different agitation rates of 0, 50, 100, 150, and 200 rpm. The biomass of the fungus was determined as above and polygalacturonase activity in the cell free supernatant was determined under standard assay conditions.

Solid- state fermentation

A spore suspension was prepared from a 5 days-old Czapek-Dox agar slant. One ml of a spore suspension (10^6spore/ml) was used to inoculate the wheat bran medium in a 1 liter conical flask.

The basal medium used consisted of 0.5 gram $(NH_4)SO_4$ and 0.4 gram K_2HPO_4 in 80 ml of distilled water. The pH 5.0 and pH 8.0 of the medium was adjusted with 0.1 N NaOH and 0.1 N HCl, then 40 gram of wheat bran was added in the basal medium. Orange pulp was varied from 0 to 2.5 gram in the basal medium.

The contents of each flask were mixed thoroughly with sterile needle for uniform distributions of fungal spores in the medium. The culture flasks were incubated at 30°C for 8 days. Polygalacturonase and pectate lyase were extracted from the medium by the addition of 200 ml of 0.1 M sodium acetate buffer pH 5 and 0.1 M Tris/HCl buffer pH 8.0 respectively on a shaker for 2 hrs and medium was filtered through muslin cloth to remove cells and unused medium. After centrifugation, activity of the crude enzyme was determined using standard assay conditions. The activity was expressed as unit of polygalacturonase per gram of wheat bran.

Effect of moisture content

The moisture content of the wheat bran medium was varied from a ratio of 1:1, 1:2, 1:3 and 1:4 of the wheat bran. 40 gram wheat bran, with 2 gram of orange pulp, 0.5 gram $(NH_4)SO_4$ and 0.4 gram K_2HPO_4 were mixed with 40, 80, 120 and 160 ml distilled water.

Activity staining

Broth containing 0.1 mg of total protein as determined by Lowry *et al*. (1951) was used from both submerged and solid state fermentation was electrophoresed using 12% acidic native PAGE. The resultant gel was blotted onto polygalacturonic acid gel at pH 4.5 solidified by adding 1.5% agar. The activity band (s) were visualized by treatment with 3% cetrimide after 45 min incubation at 40°C.

Determination of k_i

0.13 µM polygalacturonase and 0.23 µM of pectate lyase was incubated with 1.35 mg and 2.7 mg polygalacturonic acid with different concentrations of respective antibodies and k_i was determined.

Titration of polygalacturonase and pectate lyase obtained from submerged and solid state medium with their antibodies.

Broth containing 0.1 mg of total protein as determined by Lowry *et al*. (1951) was used from both submerged and solid state fermentation and incubated with 1 to 8 µM of the respective antibodies. The antigen- antibody mixture was incubated at 4 °C for 8 hrs. The residual activities of the both enzymes were determined under standard assay conditions.

Purification of polygalacturonase and pectate lyase

Endo-polygalacturonase purification is described in chapter IV. Pectate lyase purification was carried out as described by Rao (1996).

Antibody preparation

Polyclonal antibodies against purified polygalacturonase and pectate lyase were raised in New Zealand white rabbits by injecting the purified enzymes (0.2 mg) with Freund's complete adjuvant subcutaneously at multiple site (Dunbar and Schwoebel, 1990). The first booster dose was given after 21 days and the second, 30 days after the first injection. The rabbits were bled after 20 days and the titer of the antibody was checked by Ouchterlony double diffusion test (Ouchterlony, 1953). When the titer of antibody reached 1:32 of pectate lyase and 1:16 of polygalacturonase the blood was collected by cardiac puncture, allowed to clot and the serum was separated from plasma by centrifugation. Then the serum was incubated at 50°C for 1 hour for inactivation the complement and stored at -20°C until required.

Partial purification of the antibodies

Partial purification of the antibodies were carried out according to Dunbar and Schwoebel (1990). Ammonium sulfate solution (77.7% w/v) was slowly added to the serum and the precipitate was collected by centrifugation, the precipitate was dissolved in 10 ml distill water and dialyzed extensively against 20 mM phosphate buffer pH 7.2. The dialysate was then loaded on DEAE cellulose column (1.5 × 30 cm) previously equilibrated at pH 7.2 with 20 mM phosphate buffer. The column was washed extensively with phosphate buffer till the fraction showed no detectable absorbance at 280 nm. Subsequently the column was washed with the same buffer with 50 mM NaCl. Fractions of 10 ml were collected at the flow rate of 20 ml/hour. Eluted fractions having A280 greater than 0.1 were pooled dialyzed extensively and concentrated using Amicon ultrafiltration unit fitted with a 10 K membrane. The concentrated solution of antibodies was stored at -20°C until required.

Preparation of immunoaffinity matrix

Immunoaffinity matrix was prepared according to the method of Sairam and Porath (1974), 5 ml Sepharose 4B was taken in a beaker and washed with 0.3 M carbonate buffer pH 10. Then the matrix was activated by addition of 0.5 ml of divinyl sulfone (DVS) and

the mixture was kept on a shaker at 50 rpm for 2 hours. The excess DVS was removed by centrifugation and the matrix was equilibrated with 0.3 M carbonate buffer pH 10. Purified polygalacturonase and pectate lyase (5 mg) were added to the matrix and the mixture was kept on the shaker for overnight. Unbound protein was removed by centrifugation, then the mixture was treated with 1 M glycine for 2 hours and the matrix was washed extensively with phosphate buffer pH 7.2 to remove excess glycine.

Purification of protein specific antibodies

Protein specific antibodies were purified according to the method described by of Sairam and Porath (1974). The immunoaffinity matrix from the above step was filled into an 0.5 cm × 2 cm column and the matrix was equilibrated with 10 mm Tris/HCl buffer pH 7.5. The column was re-equilibrated with freshly prepared 10 ml of 0.1 M triethyl amine pH 11.5 and washed 10 mM Tris/HCl buffer pH 7.5 followed by treatment 10 ml of 0.1 M glycine pH 2.5. The column was then washed with Tris/HCl buffer pH 7.5

Partially purified antiserum (5 mg) was passed through the column and after washing with Tris buffer at pH 7.5, acid sensitive antibodies were eluted by passing 10 ml of 0.1 M glycine solution pH 2.5 through the column. Base sensitive antibodies were eluted by passing 0.1 M trietyl amine solution at pH 11.5. Both fractions of antibodies were combined and dialysed against phosphate buffer pH 7.2. Protein specific antibodies were stored at - 20°C until required.

Specificity of the antibodies

The specificity of the polygalacturonase and pectate lyase antibodies were checked by both Ouchterlony double diffusion test (Ouchterlony, 1953) and Western dot blot. Ouchterlony double diffusion test was done using 1% (w/v) agarose gel in phosphate buffer saline (PBS) pH 7.2 at 4°C for 24 to 48 hrs (Fig 2.1 A and B).

The Western dot blot was developed according Towbin and Gordon (1984). 2 µg of purified polygalcturonase and pectate lyase were spotted on nitrocellulose paper. The nitrocellulose paper was air dry and was blocked with 1% BSA solution for 1 h at 37 °C. The Western dot blot was then washed two to three times with PBS/T at pH 7.2. The blot was incubated with 1:300 diluted first antibody solution for overnight at 4°C washed with

PBS/T two to three times and incubated with 1:5000 diluted anti-rabbit IgG secondary antibody labeled with alkaline phosphatase conjugate for 3 h at 37 °C. The Western dot blot was visualized by the addition of BCIP solution (Fig 2.1 C).

Figure 2.1

Ouchterlony double diffusion to check antigenic cross-reactivity of anti-polygalacturonase and anti-pectate lyase with each other and *Aspergillus niger* polygalacturonase

A) **1)** Center well anti-polygalacturonase antibody (1 mg) **2)** Wells 1 and 4 purified polygalacturonase **3)** Wells 2 and 5 *Aspegillus niger* polygalacturonase **4)** Wells 3 and 6 purified pectate lyase of *F. moniliforme* NCIM 1276.

B) **1)** Center well anti-pectate lyase antibody (1 mg) **2)** Wells 1 and 4 purified pectate lyase **3)** Wells 2 and 5 polygalacturonase of *Aspegillus niger* **4)** Well 3 and 6 polygalacturonase of *F. moniliforme* NCIM 1276.

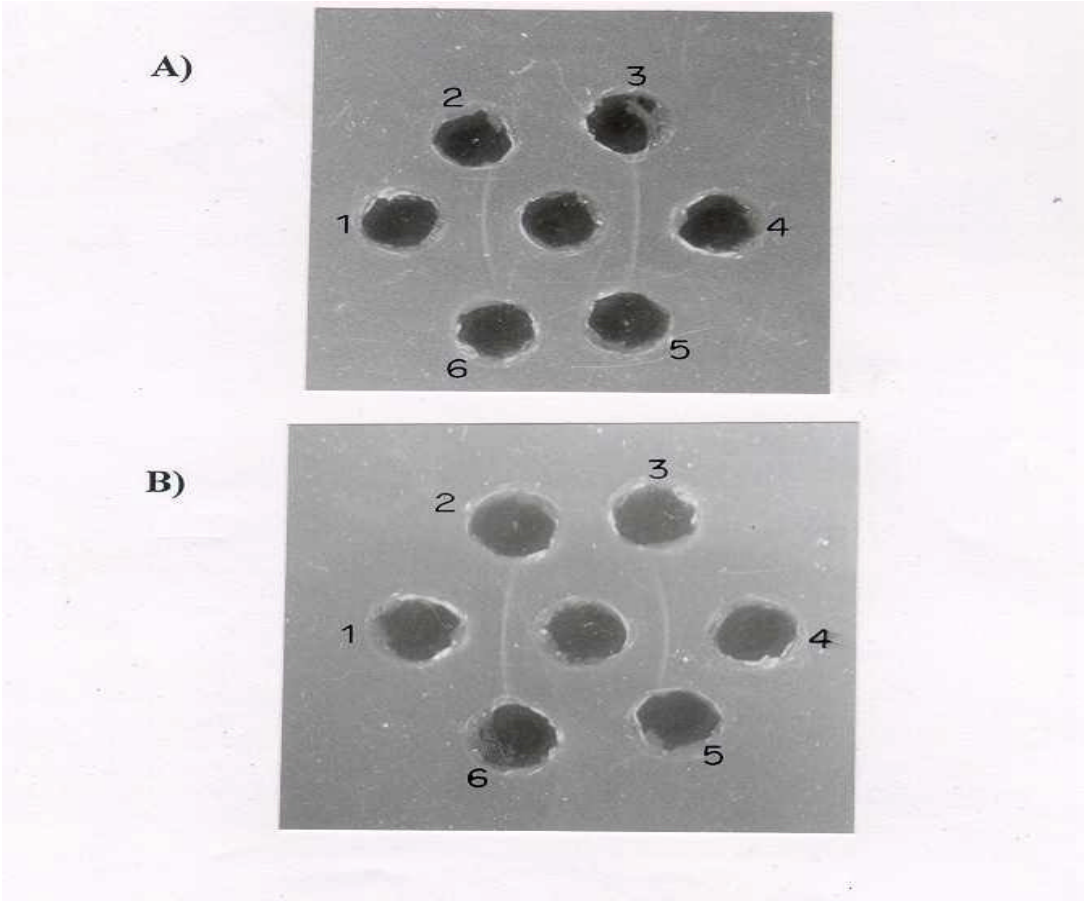
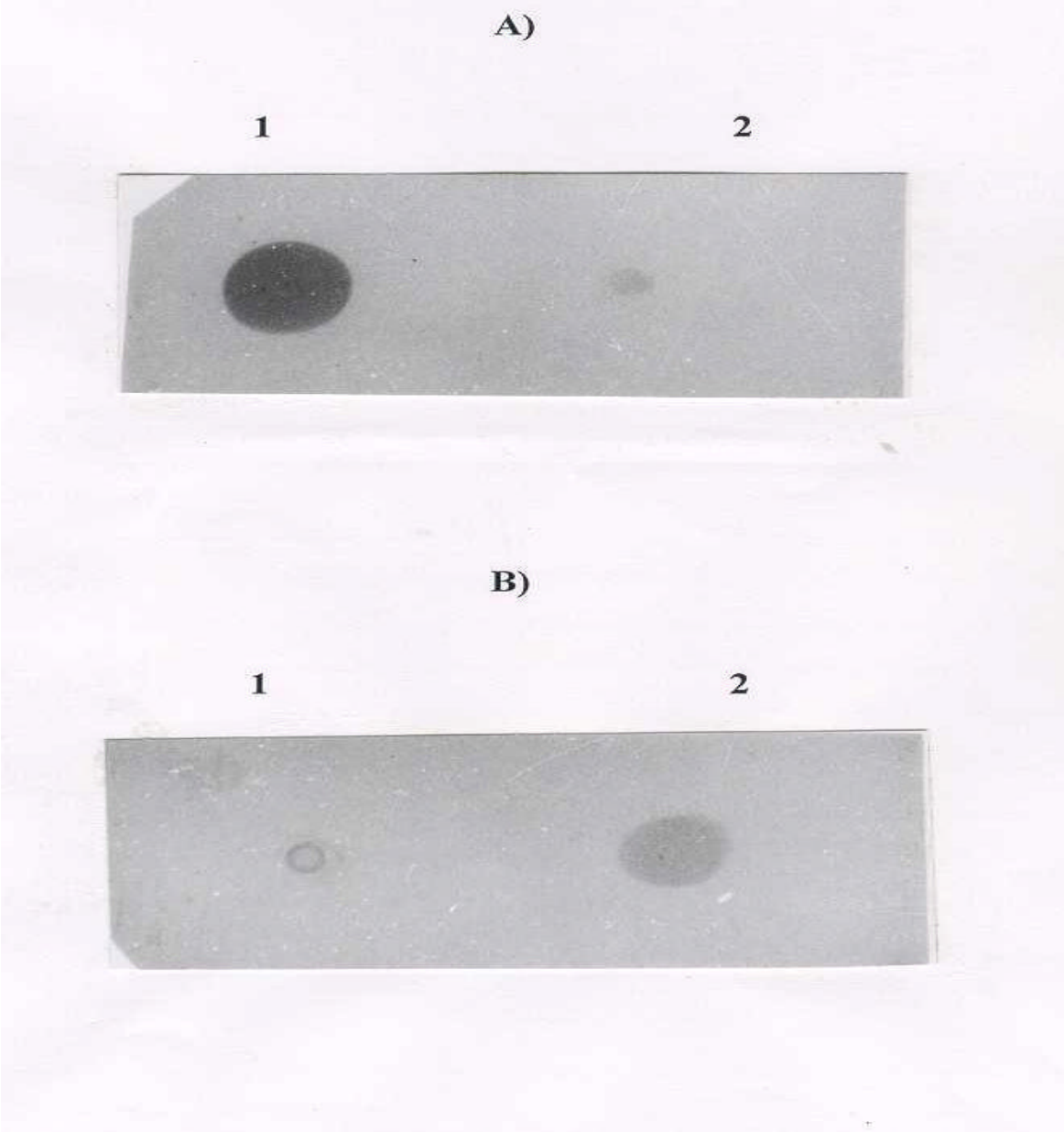


Figure 2.1

(C) Western dot blot

A) Polygalacturonase antibody used: Spot 1: polygalacturonase, Spot 2: pectate lyase.

B) Pectate lyase antibody used: Spot 1: polygalacturonase, Spot 2: pectate lyase.



Labeling of antibodies with horse rabbit peroxidase (HRP)

1.5 mg/ml of protein specific antibodies of polygalacturonase and pectate lyase were taken in beaker and 5 mg of HRP was added. The mixture was dialyzed against phosphate buffer pH 7.0 overnight. After dialysis 0.2% of glultaraldehyde solution was added and the mixture was kept on the shaker for 2 hrs and again dialyzed overnight at 4°C. The dialysate was then transferred to 50 mM Tris/HCl buffer containing 1 mM $MgCl_2$. The antibody conjugate was dialyzed against phosphate buffer pH 7.2, and stored at 4°C until required.

Sandwich ELISA

Sandwich ELISA was carried out according to the method of Perlmann & Perlmann (1994). 1:300 diluted primary antibody was coated to the ELISA plate the plate was incubated to 4°C for overnight. After blocking the wells with 0.05% gelatin, the plate was extensively washed with PBS/T and various concentrations of antigen were added in different wells. The plate was incubated at 37°C for 3 hrs and washed with PBS/T. Then 1:100 diluted primary antibody HRP conjugate was added again and the plate was incubated for 1 h. at 37 °C. Subsequently the plate was washed with PBS/T and 1:20 diluted TMB was added. The reaction was stopped with addition of 1N H_2SO_4. Resultant color was measured at 450 nm using automatic MR 700 Microplate ELISA reader and standard graph was plotted of absorbance against concentration of antigen (Fig 2.2 A and B).

In all further experiments antigens were quantified using this standard graph.

Immunocytolocalization of polygalacturonase and pectate lyase

Fixation and embedding

Mycelia and spores during logarithmic growth were taken, grown in presence of 1% pectin as a sole carbon source or 1% glucose as sole carbon source at pH 5 and at pH 8. The spores and mycelia were centrifuged and washed with 0.1 M sodium cacodylate buffer pH 7.0. The samples were fixed in 2% glutaraldehyde for 8 hrs and 1 % paraformaldehyde for 2 hrs at 25°C made in 0.1 M sodium cacodylate buffer (pH 7.0). Excess glutaraldehyde and paraformaldehyde were removed by washing twice with 0.1 M Na-cacodylate buffer.

Dehydration was performed in series of alcohol grade (20-95%) at room temperature. Infiltration was done with alcohol and Araldite A (1:1) for 1 h at 60 °C, followed by only Araldite A at 60 °C for 1 hr and then at room temperture. Cells were embedded in freshly prepared Araldite B (Araldite A 23 ml and 0.4 ml DMP-40). Blocks were polymerized at 60 °C for 48 to 72 hrs.

The blocks formed after polymerization were carefully trimmed to expose the underlying cells in the form of pyramid like shape to get serial sections. 600-700 A° thick sections were cut with glass knife on a LKB Bromma 2088 ultratome V. Sections were then picked up on Nickel grids.

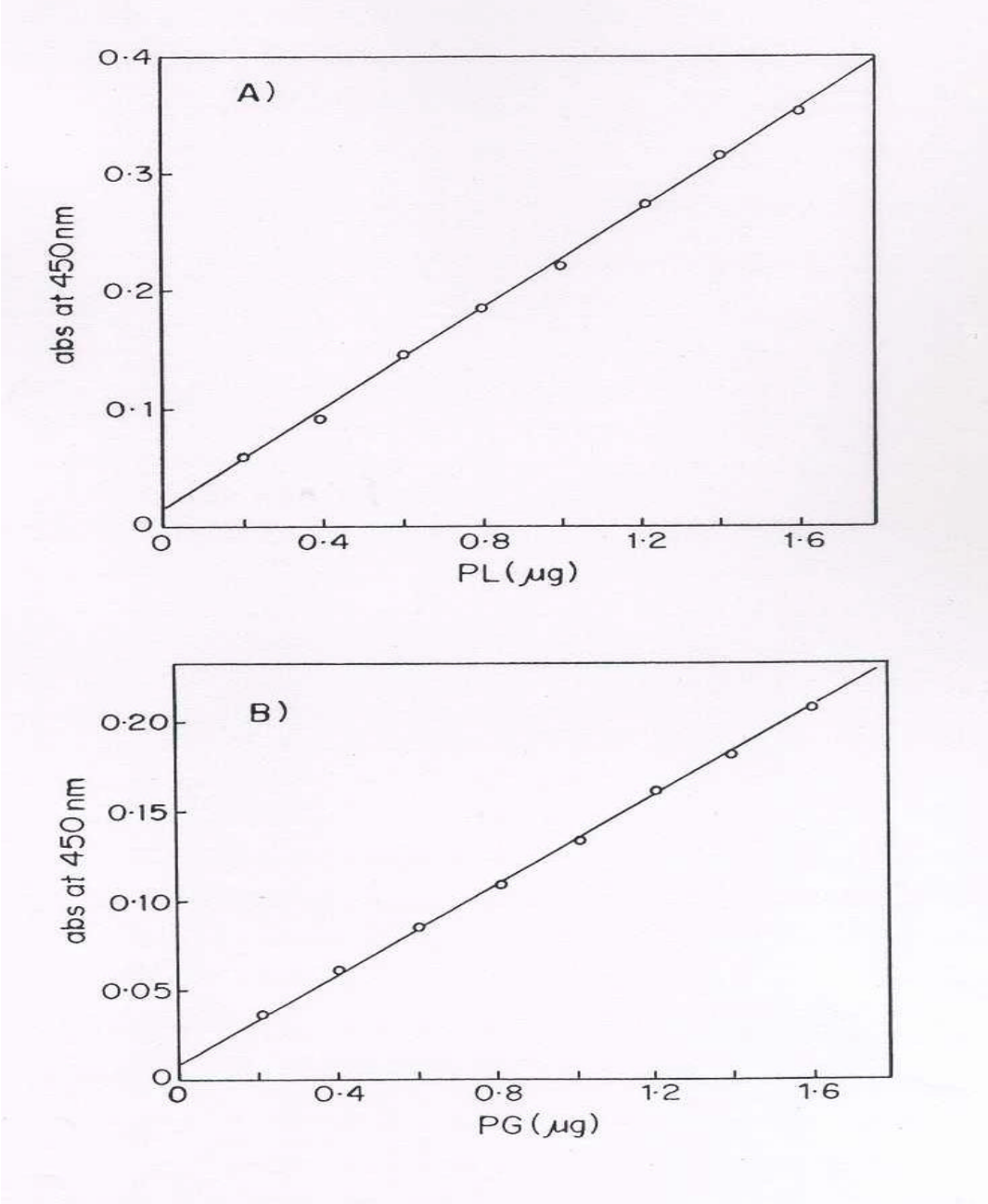
Immunostaining

Ultra thin sections were blocked with 1% BSA solution for 3 hrs at 25 °C. Sections were washed with 0.1 M sodium cacodylate buffer pH 7.0 and incubated with 1:300 diluted primary antibodies of polygalacturonase and pectate lyase at 4 °C overnight. Subsequently sections were washed with 0.1 M sodium cacodylate buffer pH 7.0 and incubated with 1:100 diluted anti-rabbit IgG gold-conjugate for 3 hrs at 25 °C. The sections were washed three times with 0.1 M sodium cacodylate buffer pH 7.0. The ultra thin sections were stained for 10 min in uranyl acetate in the dark and then washed thoroughly with deionised water followed by lead citrate 10 min and viewed with Ziess EM 109 Transmission Electron Microscope.

Figure 2.2

Standard graphs of polygalacturonase (PG) and pectate lyase (PL) of sandwich ELISA

A) Pectate lyase. B) Polygalacturonase.



Results and discussion

Samples were collected from different estuarine environments including mangroves of Maharashtra, Goa and Karnataka. Each was enriched in medium containing pectin. A fungal strain gave the largest clearing zone on pectin agar plate and this isolate was chosen for further studies. On Czapek-Dox agar the isolate produced fluffy white mycelia with both macroconidia and microconidia. The macroconidia were fusiform and tapering towards either end. The microconidia attached end to end and formed un-branched chains. On the basis of morphological characteristics the isolate was identified as *Fusarium moniliforme*. It has been deposited in the National Collection of Industrial Microorganisms (NCIM) as *Fusarium moniliforme* NCIM 1276 (Rao, 1996).

This *Fusarium* produces a pectate lyase at alkaline pH (Rao, 1996) and a polygalacturonase at acidic pH. Pectate lyase production is 8.1 U/ml when 1% pectin and 0.2 % glucose are supplied in the liquid medium at pH 8 and 200 rpm for 96 hrs. The present chapter reports studies on polygalacturonase production by the isolate.

Since this organism was isolated from an estuarine mangrove ecosystem, the first studies related to the effect of salt on enzyme production and whether the enzyme was salt tolerant. As shown in Table 2.1 increasing salt concentration did not affect the production of enzyme uptil a molarity of 0.4 M NaCl, thereafter both biomass and enzyme production were adversely affected. It is noted that although mangrove environments are influenced by tidal cycles they very rarely have water as saline as 0.4 M NaCl which is the salinity of the open ocean. In fact the salinity varies between 0.01 M in upstream areas where calcium and silicates are important metal ions, to 0.2 to 0.25 M salinity in downstream regions influenced by tidal cycles. Therefore the limit of salt tolerance required by estuarine species is fairly narrow. The occurrence of this organism in the estuarine mangrove suggests that it is not adversely affected by prevalent salinity.

This is a first report of a " salt-tolerant" *Fusarium*. Although it has long been recognized that this group of organisms are tolerant to pH variation in the environment. A mangrove environment, necessarily salty, is an unusual ecosystem to find a *Fusarium*.

Table 2.1

Effect of NaCl on growth and polygalacturonase production.

NaCl * (M)	Biomass (mg)	Polygalacturonase (U)
0.0	301	14.2
0.2	295	13.0
0.4	289	10.0
0.6	191	6.5
0.8	171	4.1
1.0	108	2.3

* The medium (50 ml) contained 1% pectin, 0.2 % glucose, 0.5 % $NH_4(SO)_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5.

Optimization

The basal medium consisted of 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5 with added nitrogen, pectin and or glucose. Adding different concentrations of pectin with 0.2% glucose showed that maximum production of enzyme occurred when 1% pectin was supplemented with 0.2% glucose (Table 2.2 and Table 2.3)

Polygalacturonase production was maximum at an aeration rate of 200 rpm. Ammonium sulfate was the best nitrogen source (Table 2.4) and maximum secretion of the enzyme occurred when 0.175 M nitrogen was available (Table 2.5). Higher concentration of nitrogen resulted in decreased biomass growth and also enzyme production suggesting a toxic effect. The organism grew best at 30°C and secreted a maximum of 0.28 U/ml polygalacturonase (Table 2.6).

Most mesophilic fungi produce enzyme at 30°C and the present species is mesophilic. Bailey (1990) showed that one strain of *Aspergillus niger* produced polygalacturonase most efficiently at 18 °C and Kumar and Palanivelu (1999) reported that the thermophilic fungus *Thermomyces lanugiosus* produced polygalacturonase at 50 °C.

The optimum conditions for production of polygalacturonase in submerged culture were 1% pectin, 0.2 % glucose, 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5 at 200 rpm and 30 °C. Under these conditions the fungus produced 0.28 U/ml of the enzyme. Rao (1996) showed and present author confirmed that pectate lyase production peaked in submerged culture when 1% pectin, 0.2 % glucose, 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 8 at 200 rpm and 30 °C. Under these conditions the fungus produced 8.1 U/ml pectate lyase.

Table 2.2

Effect of pectin on polygalacturonase production.

Pectin * (%)	**Biomass (mg)**	**Polygalacturonase (U)**	**Glucose**
0.0	50	ND	0.2 % of glucose was added in all cases.
0.5	165	6.5	
1.0	298	14.2	
1.5	300	13.6	
2.0	303	12.8	
2.5	311	12.6	

* The medium contained (50 ml) 0.2 % glucose, 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5.

Table 2.3

Effect of glucose on polygalacturonase production

Glucose * (%)	Biomass (mg)	Polygalacturonase (U)	Pectin
0.0	157	11.5	1% pectin was added In all cases
0.1	205	12.7	
0.2	290	14.2	
0.4	305	5.0	
0.6	315	4.5	
0.8	317	1.0	
1.0	320	ND	

* The medium contained 1% pectin, 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5. ND - Not Detectable

Table 2.4

Effect of different nitrogen source on polygalacturonase production.

Nitrogen source (0.5 %) *	Biomass (mg)	Polygalacturonase (U)
Ammonium sulfate	305	13.8
Ammonium nitrate	304	13.4
Potassium nitrate	190	9.3
Sodium nitrate	189	9.2
Peptone	110	6.0
Urea	105	5.1

* The medium contained 1% pectin, 0.2 % glucose, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5.

Table 2.5

Effect of Ammonium sulfate on polygalacturonase production

Ammonium sulfate (M) *	Biomass (mg)	Polygalacturonase (U)
0.125	288	11.3
0.15	302	12.4
0.175	306	13.9
0.20	260	11.1
0.225	233	9.3

* The medium contained 1% pectin, 0.2 % glucose, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5.

Table 2.6

Effect of temperature on polygalacturonase production.

Temperature (° C) *	Biomass (mg)	Polygalacturonase (U)
10	190	6.3
20	260	11.8
30	308	14.0
40	145	6.8
50	90	ND

* The medium contained 1% pectin, 0.2 % glucose, 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5. ND - Not Detectable.

Effect of different carbon sources

The isolate produced polygalacturonase irrespective of whether the medium contained pectin, cellulose, starch, xylan or wheat bran each at a concentration of 1% as a sole carbon source (Table 2.7). On glucose the isolate produced no polygalacturonase.

Table 2.7

Effect of different carbon sources on production of polygalacturonase

Carbon source * (1%)	**Polygalacturonase (U)**	**Glucose**
Glucose	No activity	
Pectin	11.5	No glucose
Cellulose	0.6	Supplied in
Starch	4.25	theses cases.
Xylan	3.25	
Wheat bran	2.04	

* The medium contained 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at pH 5.

Effect of glucose

The effect of glucose on polygalacturonase and pectate lyase production was different. As seen in Table 2.7 when only glucose was supplied in the medium there was no measurable polygalacturonase activity. Furthermore, as seen in Table 2.8, an increase in glucose from 0.1% to 0.2% glucose at pH 5 resulted in increase in both activity and µg of production of the enzyme but at higher glucose concentrations both activity and µg production fell until they were undetectable at 1% glucose. On the other hand, pectate lyase activity was measurable irrespective of glucose concentration up to 1% glucose (Table 2.8) and the production of this enzyme is higher at alkaline pH than at acidic pH.

When no glucose was supplied and pectin was the sole carbon source, the production of polygalacturonase at pH 5 was 11.5 Units (Table 2.7) These values increased

with increase in glucose concentrations upto 0.2% (Table 2.3, Page 44 and Table 2.8) to an optimum value of 14 Units. When more than 0.2 % glucose was supplied in the combined medium the biomass increased but polygalacturonase production decreased. Therefore, concentrations higher than 0.2 % of glucose repressed polygalacturonase production though an initial 0.2 % of glucose was required for induction of the enzyme. It has been shown by Aguillar and Huitron (1987) that in *Aspergillus* sp. extracellular polygalacturonase production is the result of a balance between availability of substrate and catabolite repression and a similar case obtains here.

Catabolite repression has been shown to limit enzyme production in a number of cases. De Lorenzo et al (1987) have shown this to occur in experiments with their strain of *Fusarium moniliforme*, and Keen and Horton (1966) Shinmyo *et al*. (1978) and Maldonado *et al*. (1989) showed that glucose catabolite repression may act at the level of either transcription or translation. When glucose was supplied at a concentration of 1% to the present strain as sole carbon source, immunolabeling and TEM showed that the enzyme was not produced (Fig 2.3 A). In presence of pectin at 1%, however enzyme was seen to be localized at the cell membrane (Fig 2.3 B). On the other hand, pectate lyase (Fig 2.4) is produced irrespective of carbon source.

Figure 2.3

Localization of polygalacturonase

A) Glucose grown spore cell of *Fusarium* B) Pectin grown spore cell of *Fusarium*

Gold particles localized near to the membrane (Arrow).

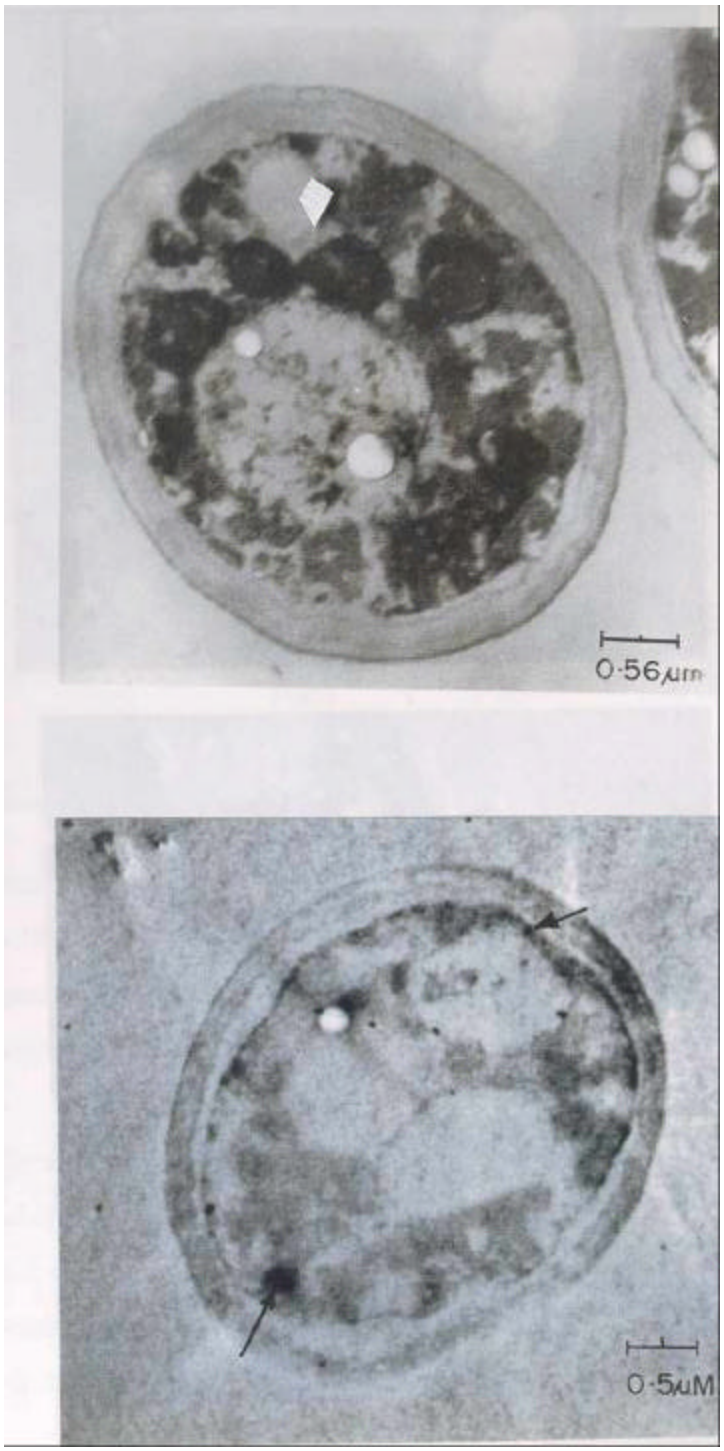


Figure 2.4

Localization of pectate lyase

A) Pectin grown mycelium cell B) Glucose grown mycelium cell of *Fusarium*

Gold particles localized near to the membrane and vacuole (Arrow).

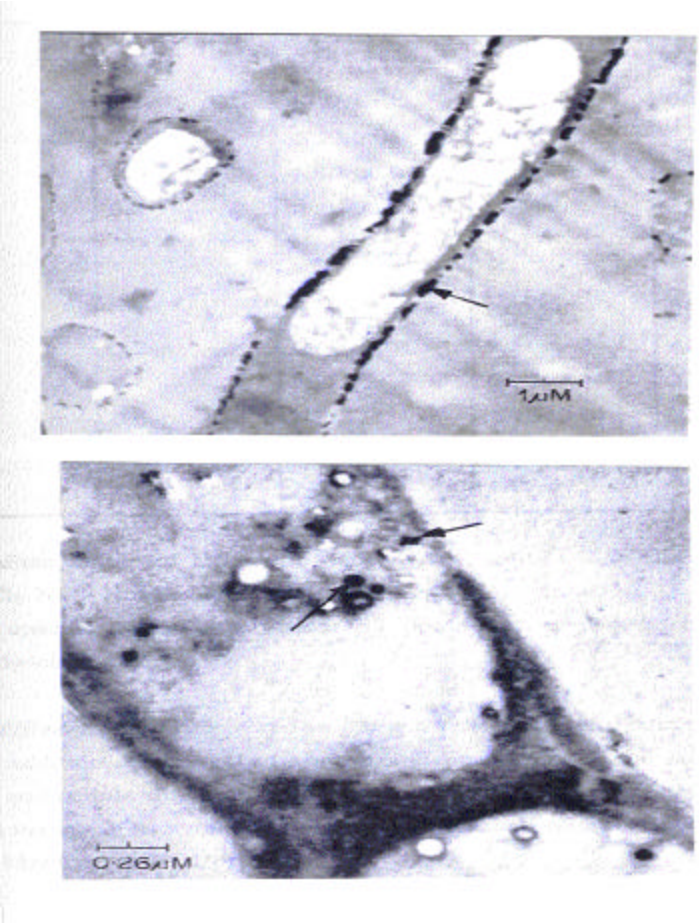


Table 2.8

Effect of glucose on polygalacturonase and pectate lyase production

Biomass (mg) *	**Glucose (%)**	**PG**		**PL**	
		(U)	**(mg)**	**(U)**	**(mg)**
At pH 5					
205±3.2	0.1	12.7±0.1	286±4.3	55.3±0.02	313±3.2
290±7.6	0.2	13.9±0.05	386±3.2	66.5±0.01	515±6.3
305±5.5	0.4	5.2±0.01	160±7.8	22.4±0.03	340±5.6
315±8	0.6	4.5.1±0.01	76.5±8.9	10.3±0.02	289±2.3
317±10	0.8	1.0±0.012	14±4.5	3.67±0.01	110±3.4
320±9.2	1.0	ND	ND	ND	86±7.3
At pH 8					
254±3.4	0.1	0.08±0.01	8±0.56	200±10	1100±12
306±5.4	0.2	0.02±0.01	7.5±0.42	420±8	1358±9.4
315±6.2	0.4	ND	6.25±0.4	310±5.8	1041±11.5
318±1.2	0.6	ND	1.45±0.01	220±5.4	638±9.6
323±2.3	0.8	ND	ND	103±2.1	408±8.3
338±4.3	1.0	ND	ND	83±08	250±7.8

* The medium contained 1% pectin, 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$. ND - Not Detectable.

Antibody specific polygalacturonase and pectate lyase protein biomass was determined using sandwich ELISA.

Effect of different sugar substrates on polygalacturonase and pectate lyase production.

In addition to polygalacturonase and pectate lyase the isolate produced amylase, xylanase, and cellulase (Table 2.9) It is well known that both pathogens and saprophytes produce a variety of enzymes for degrading cell wall components (Karr and Albersheim, 1970 and Martinez *et al.,* 1991).

Table 2.9

Induction of different polysaccharidases of *F. moniliforme* at pH 5 and at pH 8.

Substrate *	**PG** (U)	**PL** (U)	**Amylase** (U)	**Xylanase** (U)	**CMCase** (U)
At pH 5					
Galacturonic acid	5.2	3.1	ND	ND	ND
Polygalacturonic acid	10.3	4.2	1.23	ND	ND
Pectin	11.5	6	0.65	0.25	0.51
Cellulose	0.6	ND	0.25	3.12	1.55
Starch	4.25	ND	19.2	ND	ND
Xylan	3.25	ND	ND	22.1	0.61
Glucose	ND	16.15	ND	ND	ND
Wheat bran	2.04	7.7	16.0	28.7	2.5
At pH 8					
Galacturonic acid	1.23	16.5	ND	ND	0.52
Polygalacturonic acid	ND	110	0.32	1.2	2.1
Pectin	2.5	415	1	ND	ND
Cellulose	ND	50	ND	1.9	0.75
Starch	1.45	15	23	ND	ND
Xylan	0.95	30	ND	55	ND
Glucose	ND	2.3	ND	ND	ND
Wheat bran	ND	87	18.4	60	9

* 1 % substrate as sole carbon source was added in the basal submerged culture medium, after 96 hours incubation, activity of the corresponding enzyme was measured under standard assay conditions.

Effect of pH

The organism grew at all pHs tested (Fig 2.5). Even at pHs generally regarded as non-physiological such as pH 2 and pH 11 there was a measurable amount of biomass after 96 hrs of growth. This biomass appeared to be produced by using supplied glucose in the medium rather than by enzymatic degradation of pectin.

Polygalacturonase activity peaks at pH 5 and decreases thereafter. Amylase, xylanase and cellulase are also produced at both acidic and alkaline pH (Table 2.9), although it is not known whether identical proteins are produced at both pH.

The pH dependant variation in the production of both polygalacturonase and pectate lyase is shown in Table 2.10 and Figure 2.5. In general the production of pectate lyase was higher at all pHs than polygalacturonase (Table 2.10) 7.7 µg/ ml polygalacturonase was produced at pH 5 and pectate lyase peaked at pH 8 with 27. 2 µg/ ml. At pH 2 production of polygalacturonase was only 1.6% and at pH 11 it was 0.15 % as compared to that at pH 5. Pectate lyase production at pH 2 and 11 was slightly higher at 3.2 % and 2.2 % respectively as compared to production at pH 8.

Intracellular production of both the proteins (Fig 2.6) increased 20 fold from pH 2 to 4. Polygalacturonase reached an intracellular plateau at 17 µg/ml between pH 4 and 7, and pectate lyase between pH 5 to 8. Maximum mycelial biomass production was fairly constant between these pHs (Fig 2.5). Immunogold labelling (Fig 2.3 and 2.4) showed that at optimum pH for production of both enzymes the label is concentrated at the periphery of the cell, along the membrane. These data suggest that enzymes are excreted as fast as they are produced.

A second point suggested by these data was that environmental pH influences the production of the enzymes. Whether the change is at the level of the cell membrane (Takeuchi *et al*., 1985 and Sanders *et al*., 1989) by preventing signal transduction or whether the change was at the level of transcription or translation (Wubben *et al*., 2000 and Nir *et al*., 2000) is not known in the present case, but the rapid increase in intracellular production when external pH changes from pH 2 to pH 4 (Fig 2.6) indicates that mRNA synthesis may be enhanced. Extracellular accumulation of enzyme (Table 2.10) also starts at pH 2 and increases 30 fold at pH 4. A hypothesis derived from these observations is that enzymatic cleavage of extracellular pectin results in mono-, di-, or trigalacturonides which,

on transfer across the cell membrane, trigger intracellular synthesis of protein. Such triggering has been shown to occur in studies with *Erwinia chrysanthemi* by Collmer *et al*. (1982).

The quantity of extracellular secretion was not necessarily related to enzyme activity particularly at non-physiological pH of 2, 10, 11. Although measurable amount of protein was secreted by the present isolate as determined by ELISA (Table 2.10), CD spectra (Table 2.11) showed that both proteins undergo conformational changes at non-physiological pHs resulting in inactive proteins. As shown in Table 2.11 the percentage of α helix changed from about 14% at pH 2 to 16% at pH 5 which was the optimum pH of the enzyme. The percentage of β-sheet decreased from about 53% to 26.9% between pH 2 to 5. In comparison with these data, the *Erwinia carotovora* polygalacturonase had 4.5% α helix and 39.9% β-sheet (Pickersgill *et al*., 1998), *Aspergillus niger* II had 1.9% α helix and 41% β-sheet (van Santen *et al*., 1999) and *A. aculeatus* protein had 2.36% α helix and 43% β-sheet (Cho *et al*., 2001). A polyganacturonase purified from the broth of a species of *Fusarium moniliforme* (Federici *et al*., 2001) had an α helix component of 2.36% and β-sheet component of 43%. Although these values remain to be confirmed by diffraction, the data from the present protein indicate that the β-sheet component was less than in other reported proteins. It is interesting to note that activity increased when β-sheet component decreased as pH approached the optimum in the present protein. At alkaline pH the percentage of β-sheet again increased suggesting increased hydrogen bonding which resulted in decreased activity of the enzyme. Although these biophysical data are preliminary, they clearly show the relationship between pH and protein conformation and explain the inactivity of extracellular proteins at non-physiological pH (Fig 2.5) in comparison with the ELISA based determination of protein concentration (Table 2.10).

Figure 2.5

Effect of initial pH on polygalacturonase and pectate lyase production.

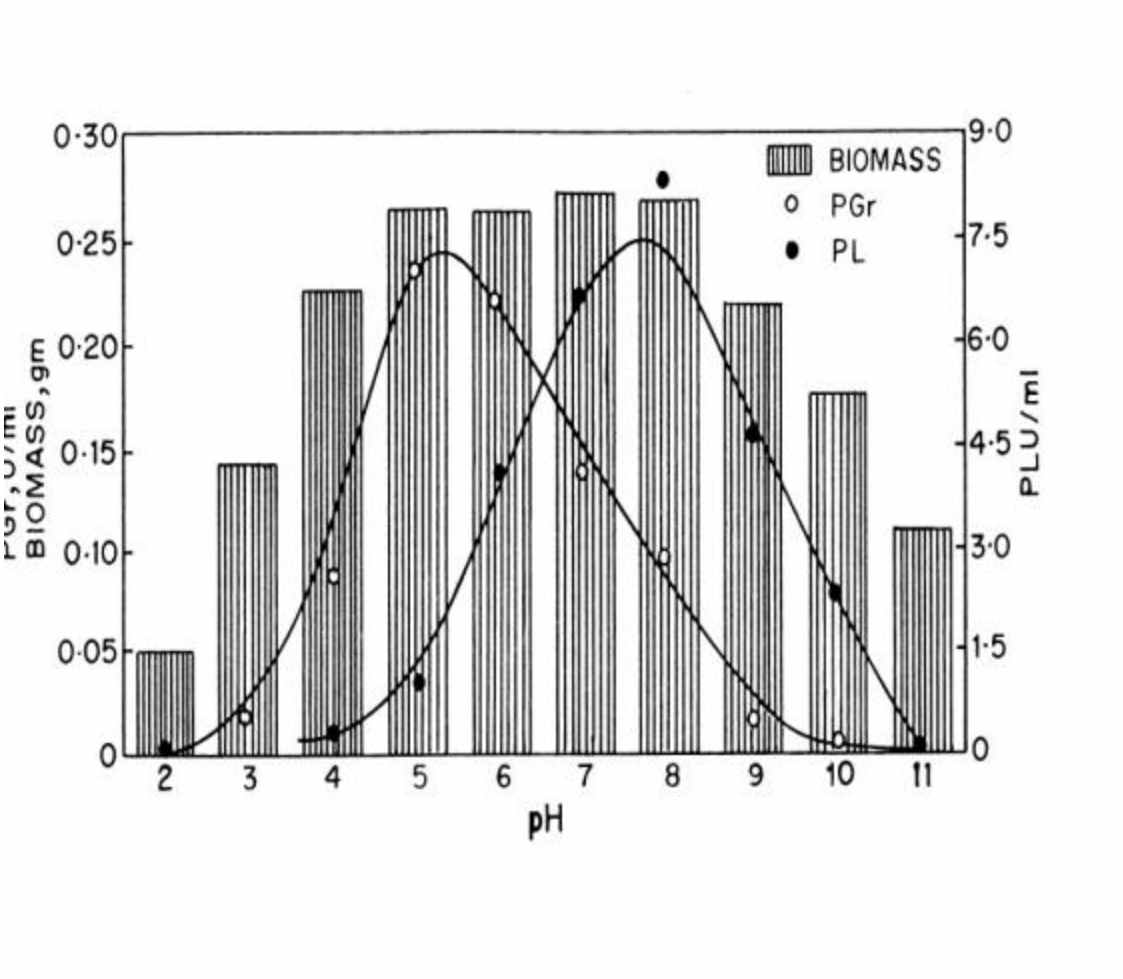


Table 2.10

Effect of initial pH on extracellular production of polygalacturonase and pectate lyase

pH	Biomass (mg)	PG (mg)	PL (mg)
2	0.35	6.25	43
3	88.5	32.25	102
4	211.5	175	378
5	295.5	385	510
6	315.3	277	630
7	299	27.5	1175
8	303	13.5	1360
9	191.5	10.6	1110
10	89	10	325
11	35	0.6	30

The isolate was grown in presence of 1 % pectin and 2 % glucose, 0.5 % $(NH_4)_2SO_4$, 0.2 % K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2$ at different pH.

Antibody specific polygalacturonase and pectate lyase protein biomass was determined using sandwich ELISA.

Figure 2.6

Effect of initial pH on intracellular production of polygalacturonase and pectate lyase

Intracellular antibody specific polygalacturonase and pectate lyase protein biomass was determined using sandwich ELISA.

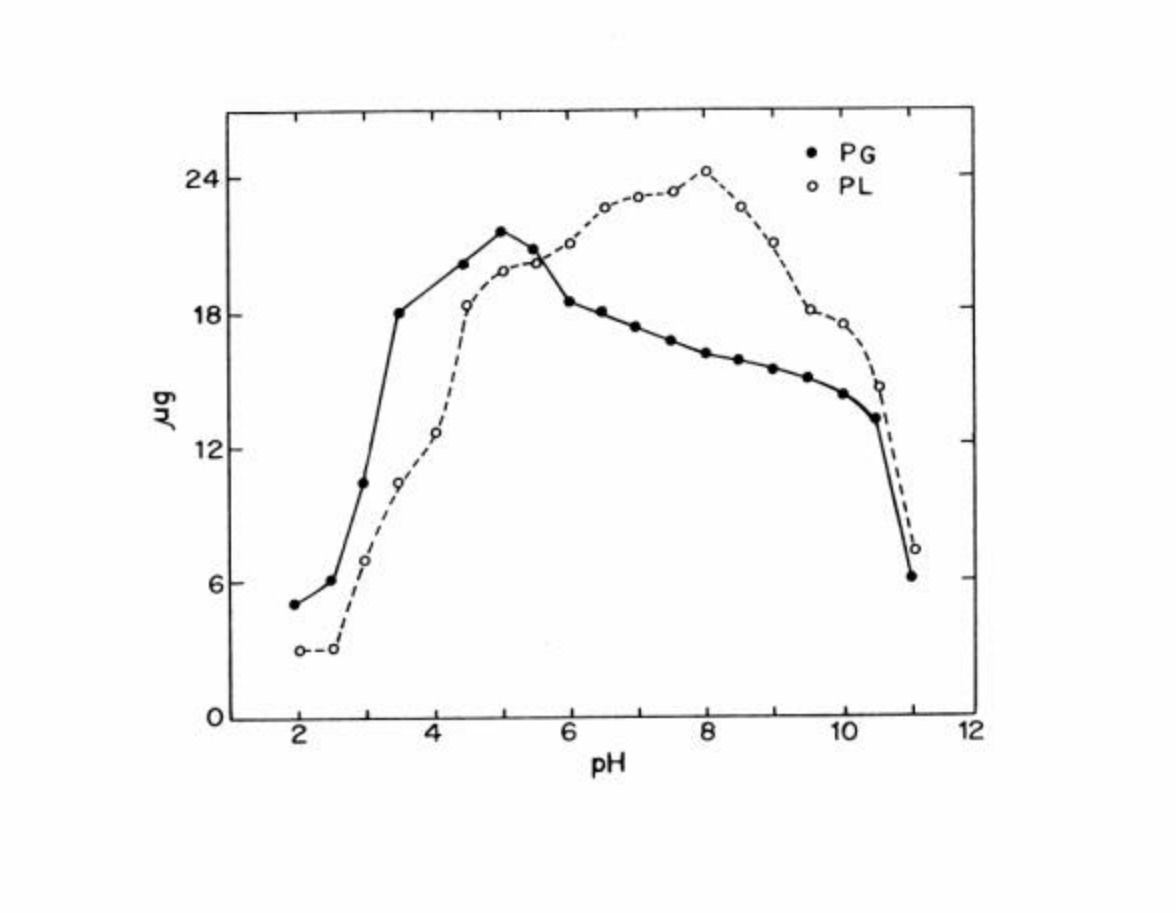


Table 2.11

CD analysis of polygalacturonase and pectate lyase

CD analysis was carried out according to the method of Sreerama and Woody (1993).

pH	a	b	**T**	**O**	S**F**
Polygalacturonase					
2	0.138	0.530	0.155	0.172	0.995
3	0.140	0.526	0.158	0.166	0.990
4	0.153	0.386	0.222	0.292	1.053
5	0.162	0.272	0.269	0.300	1.003
6	0.139	0.320	0.266	0.278	0.993
7	0.149	0.306	0.277	0.274	1.001
8	0.157	0.282	0.283	0.279	1.001
9	0.124	0.310	0.277	0.292	1.003
10	0.229	0.508	0.110	0.176	1.023
11	0.274	0.486	0.116	0.168	1.044
Pectate lyase					
2	1.002	0.028	0.004	0.011	1.044
3	0.999	0.007	0.002	0.002	1.009
4	1.006	0.024	0.005	0.005	1.040
5	1.001	0.028	0.004	0.006	1.041
6	1.000	0.016	0.002	0.002	1.020
7	1.008	0.016	0.001	0.002	1.022
8	0.259	0.388	0.123	0.231	1.001
9	0.361	0.342	0.077	0.187	0.967
10	0.762	0.150	0.039	0.088	1.038
11	1.002	0.013	0.01	0.001	1.017

Time course of polygalacturonase and pectate lyase production:

A time course showed that germ tube formation began 8 hours after spores were inoculated into fresh medium (Fig 2.7). After 8 hrs of inoculation 60 % of the spores had germinated. Septa formation and mycelium development started at 12 hrs, but enzyme activity and concentration were measurable in the broth at the 10^{th} hour (Table 2.12) after inoculation. Barash (1968) studied endo-polygalacturonase secretion during germination of spores of *Geotrichum candidium* and showed that the release of enzyme occurred even before germ-tube formation. The present isolate did not secrete polygalacturonase during the early stages of spore germination. The maximum increase in polygalacturonase occurred between 24 hrs to 48 hrs (Table 2.13). Protein concentration increased from 62 µg to 205 µg and activity double. The rate of enzyme production is higher at acidic pH than alkaline pH (Table 2.13) but in both cases reaches a plateau after 72 hrs. Pectate lyase production shows similar production (Table 2.13).

Solid state fermentation

Solid state media containing wheat bran and orange pulp as carbon sources increased the production of polygalacturonase three times compared with submerged cultures. The optimal production was 80 U/gram of mixed substrate as compared to 28 U/ gram pectin in submerged culture. Acuna-Arguelles *et al*. (1995) and Maldonado and Strasser de Saad (1998) have reported that polygalacturonase production by *A. niger* was six times higher in solid state fermentation than in submerged fermentation.

The effect of different concentrations of orange pulp in the medium showed that 2 gram of orange pulp with 40 gram wheat bran were necessary for maximum (80 U/gram) secretion of enzyme by the isolate (Table 2.14). Only 8 Units of enzyme was produced in presence of wheat bran alone because wheat bran contains only 0.1 to 0.15 mg pectin per gram of wheat bran and orange pulp is a better source of this material. Periera *et al*. (1993) added pectin in the solid state medium of *A. niger* and Siessere and Said (1989) used citrus peel in the fermentation by *Talaomyces flavus*, *Tubercularia vulgaris* and *penicillium* for production of pectic enzymes. In the present case addition of 2 gram of orange pulp increased polygalacturonase production almost 10 fold (Table 2.14)

Table 2.12

Time course of polygalacturonase production at pH 5.

Time hours	Polygalacturonase (U)	(mg)
2	ND	ND
4	ND	ND
6	ND	ND
8	ND	ND
10	1.1±0.01	6.1±0.1
12	1.8 ±0.01	18.56±0.14
14	1.92±0.1	27.23±0.16
16	2.34±0.08	30.43±0.1
18	3.6±0.03	40.23±0.12
20	4.8±0.01	58.34±0.14

Antibody specific polygalacturonase was determined using sandwich ELISA.

The effect of initial moisture content on enzyme production is that 40% moisture content was necessary for the maximum production of polygalacturonase (Table 2.15). Lonsane *et al*. (1985) and Babu and Satyanarayana (1995) pointed out that low moisture levels lead to sub optimal growth due to lower degree of substrate swelling and high surface tension. On the other hand high moisture levels decrease porosity, change wheat bran particle structure and promote development of stickiness thereby lowering oxygen tension which results in enhanced formation of aerial mycelium and consequent low enzyme production.

Figure 2.7

Germination of microconidia of *Fusarium moniliforme* NCIM 1276

A) 0 hrs B) 2 hrs C) 4 hrs D) 6 hrs E) 8 hrs F) 10 hrs G) 12 hrs after inoculation.

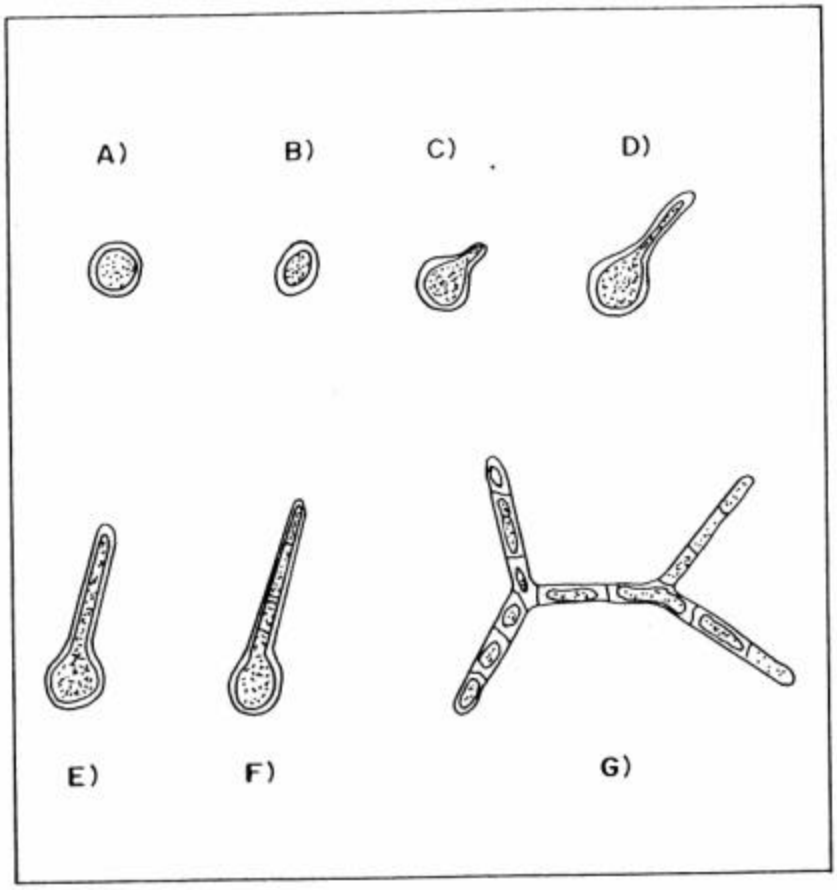


Table 2.13

Time course of polygalacturonase and pectate lyase production at pH 5 and at pH 8.

Time (hours)	Biomass (mg)	PG		PL	
		(U)	(mg)	(U)	(mg)
At pH 5					
0	--	--	--	--	--
24	23 ±5.2	5.3±0.1	62.5±1.6	11.3± 1.0	40±2.3
48	205±2.3	10.2±0.2	205±3.2	26.5±1.32	101±2.1
72	265±7.1	12.13±0.13	375±2.3	43.±0.086	401±1.3
96	302±4.5	13.93±0.2	381±7.2	63.2± 0.5	510±1.6
120	303±5.3	14.1±.03	382.5±2.3	67.23±3.2	515±1.7
At pH 8					
0	--		--		--
24	81.5±3.3	0.07±0.001	2.5±0.3	87.23±3.2	160±1.3
48	109.5±2.8	0.09±0.002	4.8±0.2	250.4±2.2	390±1.4
72	304.5±3.3	0.08±0.001	6.15±0.23	390.4±3.1	1261±8.4
96	314±1.9	0.07±0.003	12.5±0.13	410.6±5.7	1365±10.7
120	317±2.5	0.08±0.001	13.1±0.13	418.3±6.3	1366±9.5

Antibody specific polygalacturonase and pectate lyase protein biomass was determined using sandwich ELISA.

Table 2.14

Effect of orange pulp on polygalacturonase production in solid-state fermentation.

Media *	**Yield (U/ gram)**
Wheat bran- 40 gram	8
Wheat bran-40 gram + 0.5 gram orange pulp	23
Wheat bran-40 gram + 1 gram orange pulp	56
Wheat bran-40 gram +1.5 gram orange pulp	76
Wheat bran-40 gram + 2 gram orange pulp	80
Wheat bran-40 gram + 2.5 gram orange pulp	81

* The medium contained 0.5 gram $(NH_4)SO_4$ and 0.4 gram K_2HPO_4 in 80 ml of distilled water pH 5.0.

Table 2.15

Effect of moisture content on polygalacturonase production in solid medium.

Media *	**Water (ml)**	**Yield (U/gram)**
Wheat bran 40 g + orange pulp 2 g	40	55
Wheat bran 40 g + orange pulp 2 g	80	80
Wheat bran 40 g + orange pulp 2 g	120	52
Wheat bran 40 g + orange pulp 2 g	160	39

∗The medium contained 0.5 gram $(NH_4)SO_4$ and 0.4 gram K_2HPO_4 and at pH 5.0.

The present strain produced a single form of polygalacturonase in submerged culture. Solid state fermentation results in the production of two forms of polygalacturonase (Fig 2.8). It has been shown by Caprari *et al*. (1993) that four apparent forms of polygalacturonase produced by *F. moniliforme* were actually a single gene product with different glycosylation by post-translational modifications. In the present case antibodies raised against polygalacturonase cross-reacted only with itself. The antibodies did not cross-react with *A. niger* polygalacturonase, or with any other extracellular or intracellular protein released or produced by the present isolate when grown in submerged culture. These antibodies also did not cross react with pectate lyase from the same isolate (Fig 2.1 A, B and C).

Titration of polygalacturonase and pectate lyase with antibodies.

Polygalacturonase antibodies inhibited the enzyme competitively (Fig 2.9 A and Table 2.16) with a inhibition constant k_i of 2.82 μM. Similarly pectate lyase antibodies inhibited pectate lyase competitively (Fig 2.9 B and Table 2.17) with a k_i of 1.23 μM. Titration of these two antibodies in independent experiments with the culture broths from solid state fermentation showed that polygalacturonase activity was inhibited to 55 % (Fig 2.10 A) whereas pectate lyase was totally inhibited (Fig 2.10 B). These data show that solid state fermentation result in two polygalacturonases, probably products of different genes, but the pectate lyase is a single gene product in both types of fermentation.

Figure 2.8

Activity band(s) of polygalacturonase from submerged and solid state fermentation

A) Form submerged fermentation

B) From solid state fermentation

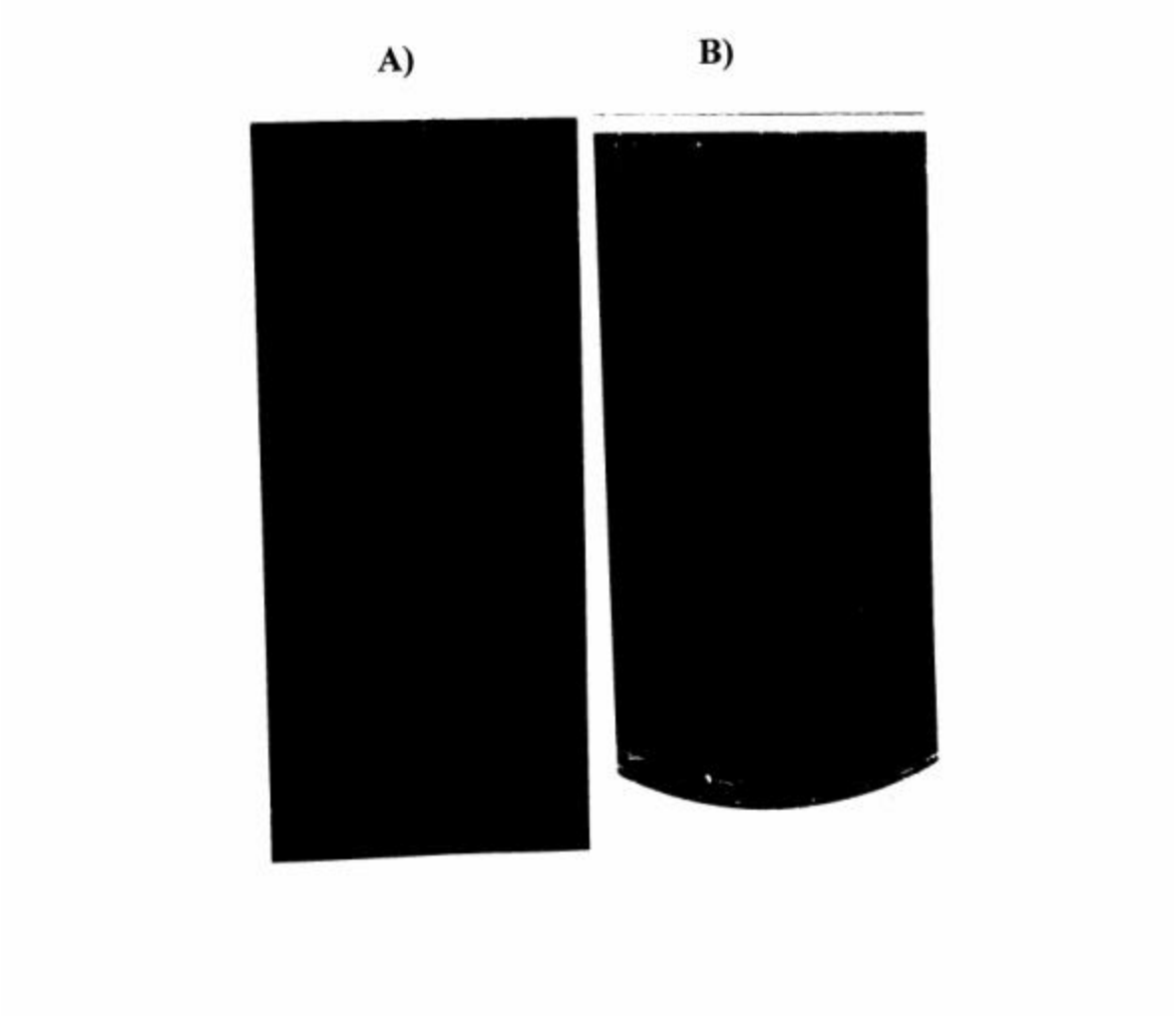


Figure 2.9

Competitive inhibition of polygalacturonase and pectate lyase with their antibodies

A) Polygalacturonase

B) Pectate lyase

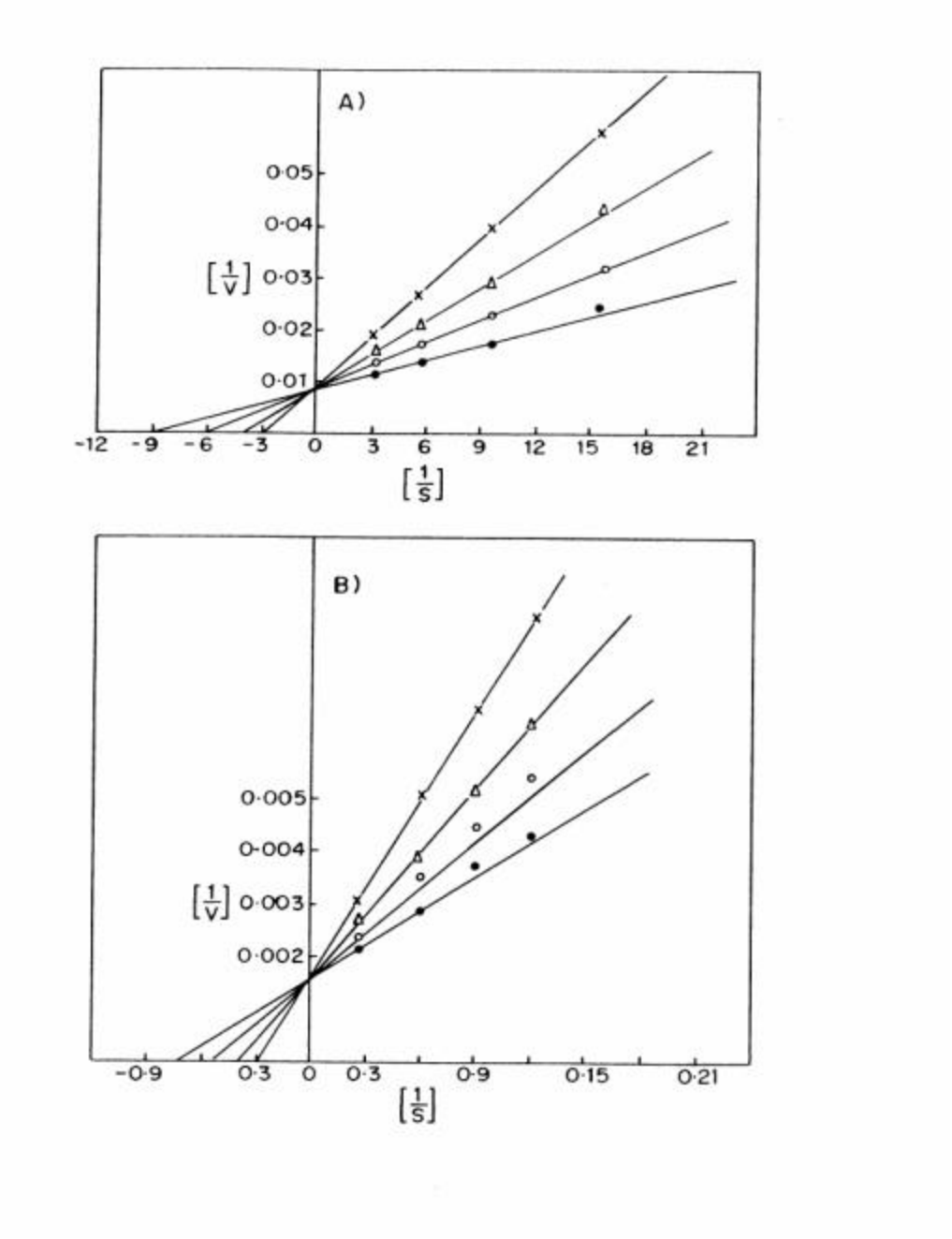


Table 2.16

***K*m and *k*cat values of partially inhibited polygalacturonase by antibodies**

% Activity	K_m (mg/ml)	k_{cat} $(min)^{-1}$
100	0.11	4,200
68	0.166	4,190
31	0.25	4,100
16	0.33	4,200

Table 2.17

***K*m and *k*cat values of partially inhibited pectate lyase by antibodies**

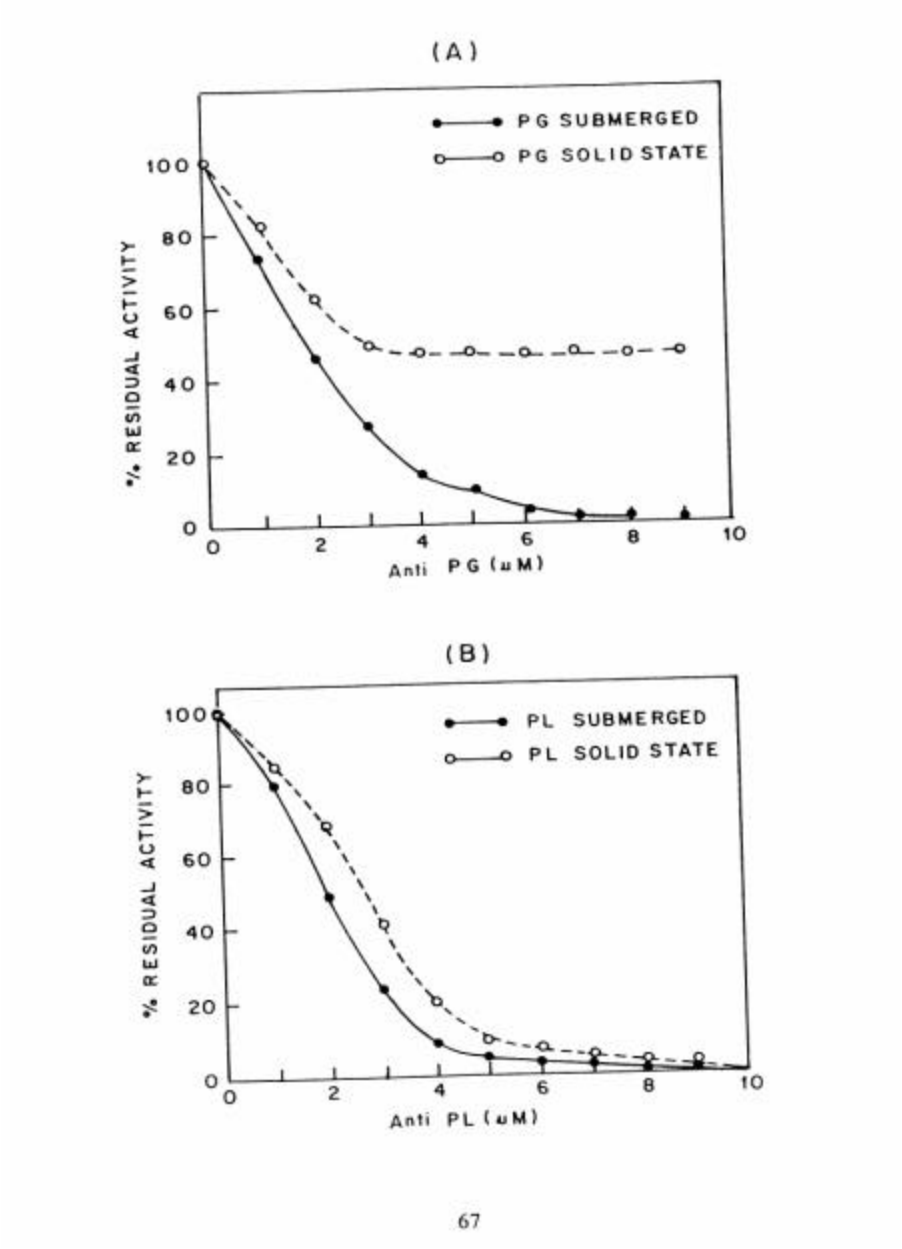
% Activity	K_m (mg/ml)	k_{cat} $(min)^{-1}$
100	1.2	7,891
70	1.72	7,890
50	2.5	7,895
30	3.33	7,890

Figure 2.10

Titration of polygalacturonase and pectate lyase with antibodies.

A) Polygalacturonase

B) Pectate lyase



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In conclusion the present isolate from an estuarine mangrove environment secretes polygalacturonase at acidic pH 5.0 and pectate lyase at alkaline pH 8.0. Maximum production of both enzymes occurred when the isolate was grown in submerged culture supplied with 1 % pectin and 0.2 % glucose. The production of polygalacturonase was totally repressed in 1% glucose. Pectate lyase production is not repressed suggesting that, polygalacturonase is an inducible enzyme and pectate lyase is produced constituvely. ELISA showed that both proteins were produced at all pH between 2 to 11 but at non-physiological pH the protein biomass was low compared to that as optimum pH. Spectral data showed that both proteins undergo conformational changes at non- physiological pHs resulting in inactive proteins. In solid state fermentation the present isolate produced 3 fold more polygalacturonase activity as compared with submerged fermentation. A single form of polygalacturonase is produced in submerged culture and two forms of polygalacturonase in solid state fermentation. Anti-polygalacturonase and anti-pectate lyase antibodies inhibited both enzymes activity competitively.

CHAPTER III

Crop plant pathogenecity of *Fusarium moniliforme* NCIM 1276.

Summary

Healthy tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*) plants inoculated with *Fusarium moniliforme* NCIM 1276 showed yellowing and crinkling of leaves after 8 to 10 days. Therefore this estuarine isolate is a pathogen on atleast these two crops.

Transverse sections of tomato and cauliflower hypocotyls examined under the light microscope showed that the fungus penetrates in about 4 days through the epidermal layer to the cortical tissue. Fluorescence labeled antibodies also showed that fluorescence of infected hypocotyls of cauliflower and tomato plants increase by 5 to 10 fold as compared to control plants

At physiological pH both enzymes are produced by this pathogen although the ratios between the enzymes varies between tomato and cauliflower systems. Tomato has an acidic cell sap of 6.6 whereas cauliflower has a cell sap pH of 7.6. In the roots of both plants the ratio between PG:PL production in tomato is 3.6 as compared to 1 in cauliflower clearly indicating that pectate lyase production is enhanced in the latter species. Therefore in acidic environment such as in *Lycopersicon esculentum* (Tomato*)* there is greater production of the hydrolytic polygalacturonase (EC 3.2.1.15) as compared to the β-eliminative cleaver pectate lyase (EC 4.2.2.2) and conversely, in the neutral to alkaline cell sap host environment of *Brassica oleracea botrytis* (Cauliflower) pectate lyase production is enhanced.

The hypocotyl regions of both plants showed lower activity of enzymes.

Fusarium (Deuteromycotina, Moniliales) is a widely occurring plant-pathogen. The species is also known to exhibit a saprophytic mode of nutrition. The data presented here suggest that *Fusarium moniliforme* NCIM 1276 has adapted to estuarine conditions but has retained its virulence against crop plants through the production of pectin-degrading enzymes.

Introduction

The role of pectinases in pathogenesis has been well established. Early studies (Karr and Albersheim, 1970) established what the authors referred to as Cell Wall Modifying Enzymes which were shown to be closely related to a polygalacturonase contained in one fraction: R-10. More recently polygalacturonase has been shown to determine virulence by *Erwinia caratovora* (Lei *et al*., 1985) and cause necrosis in *Vigna unguiculata* by *Aspergillus niger* (Cervone *et al*., 1987). A pectate lyase has been implicated in pathogenicity of *Fusarium solani* var *pisi* (Crawford and Kolattukudy, 1987) and pectinolytic enzymes are produced by the plant pathogen *Sclerotina sclerotium* (Riou *et al*., 1992). Polygalacturonase has been reported to be a virulence factor in *Agarobacterium tumefaciens* Biovar 3 (Rodriguez-Palenzuela *et al*., 1991) and pectin-degrading enzymes have been isolated from the culture of *Sclerotina borealis* (Takasawa *et al*., 1997). Although cutinases have also been reported to be produced during penetration by *Fusarium solani* f pisi into its host *Pisum sativum* (Shaykh *et al*., 1977) and it has been shown that specific inhibition of cutinase prevents infection (Maiti and Kolattukudy, 1979), lack of pectic enzymes for example in species like *Verticillium* makes strains of this species non pathogen (Leal and Villanueva, 1962). Thus, pectinases are important in pathogenecity of organisms.

Studies on *Erwinia* have shown that organisms have more than one set of genes responsible for the production of important enzymes like polygalacturonase (Reid and Collmer, 1986 and Kelemu and Collmer, 1993). It was therefore desirable to study whether the enzymes produced by *Fusarium* in submerged culture are antigenically similar to the enzymes produced in the host tissue.

The present strain of *Fusarium moniliforme* has been isolated from an unusual environment. Mangroves are tidal estuaries with diurnally changing conditions of salinity and pH. It was therefore desirable to determine whether the isolate was pathogenic to crop plants, and whether it produces polygalacturonase or pectate lyase when invading host tissue depending on cell sap pH of the host tissue.

The objective of the work reported in this chapter were to determine whether the isolate is a crop pathogen, whether the same genes are expressed in submerged culture and on the host tissue, and whether there is a difference in the ratios between the two enzymes

polygalacturonase (EC 3.2.1.15) and pectate lyase (EC 4.2.2.2) when the organism is grown on plants with different cell sap pH. For this study we used tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*).

Materials

Pectin (Citrus fruit), polygalacturonic acid, horseradish peroxidase, anti-rabbits IgG (whole molecule), sulforhodamine (Texas Red) were purchased from Sigma Chemical Company USA. Tetramethyl benzidine/ hydrogen peroxide (TMB/H_2O_2) was purchased from Bangalore Genei Pvt. Ltd. ELISA plates were purchased from Greiner Labortechnik Pvt Ltd Chandighgarh, India. Tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*) seeds were purchased from local seed store. All chemicals and reagents used were of analytical grade.

Methods

Microorganism and culture conditions

Growth, maintenance and production of polygalacturonase and pectale lyase from *Fusarium moniliforme* NCIM 1276 were described in Chapter 2.

Enzyme assay

Polygalacturonase and pectate lyase activity was determined using standard assay conditions as described in Chapter 2.

Protein assay

Protein concentration was determined in accordance with Lowry *et al*. (1951) with BSA standard

Labeling of anti rabbit IgG (whole molecule) with Texas Red

Labeling of anti rabbit antibodies with Texas Red was carried out according to Goldman (1968). 2 mg/ml of antibodies were incubated in carbonate buffer (0.5 M) pH 9.0 and mixed with 10 % solution of Texas Red. The reaction mixture was then passed through Sephadex G -20 (1.5 ×20 cm) column which was already equilibrated in phosphate buffer pH 7.2. Labeled antibody fractions were pooled and concentrated using ultrafiltration. Labeled antibodies were stored at 4ºC until required.

Pathogenicity tests

Pathogenecity of *F. moniliforme* was tested on healthy 30 days old tomato and cauliflower plants grown under field conditions. *Fusarium* culture grown in pectin medium for two days was harvested and used as an inoculum. 50 ml of inoculum was applied to exposed root around the base of each plant after making slight injury to the roots with the help of sterile needle. Control plants were inoculated with sterile distilled water. A moist, sterile cotton pad was placed over the site of inoculation to reduce desiccation. The inoculation was repeated after 5 days. Inoculated and control plants were observed periodically for the appearance of disease symptoms.

Additionally pathogenecity of the *Fusarium moniliforme* was tested on healthy tomato and cauliflower plants grown in tissue culture. 30 days old seedlings were raised in sterile Whites basal tissue culture medium. 1 ml of (10^6/ml) spore suspension of *Fusarium* was injected to the hypocotyls and roots region of the plants without disturbing the plants themselves using sterile syringe. Samples of infected plant part were collected after 8-10 days. The media composition of Whites medium was as published by Whites (1943).

Isolation and identification of the pathogen from field grown infected tomato and cauliflower plants

Infected plants and their roots were collected from the field, washed thoroughly in running tap water and examined under the microscope.

The samples were cut to 2 cm small size, washed with sterile distilled water, surface sterilized using 0.01 % mercuric chloride solution for 1 min, washed again with sterile distilled water and blotted on filter paper. Then the roots and hypocotyls were placed on Czapec-Dox agar plate modified with 1% pectin. The plates were incubated at 30°C for 4 days and colony emerging around the infected hypocotyls and roots were purified by single spore isolation method. The pathogen was identified using morphological characteristics as *Fusarium moniliforme* NCIM 1276 and it was maintained on Czapec-Dox agar at 10 °C.

Isolation polygalacturonase and pectate lyase from infected tissue of tomato and cauliflower plants grown in Whites basal medium

Cauliflower and tomato were grown under sterile conditions in Whites basal medium for one month at 25ºC under constant light intensity of 2.5 W m^{-2}. Then 1 ml spore suspension *Fusarium moniliforme* (10^6 spores) was injected using sterile syringe around the roots and hypocotyls region of the plants. Care was taken that the plants were not damaged. The infected and control plants were incubated for 4 to 8 days at 25ºC under constant light intensity 2.5 W m^{-2}. The infected plants roots and hypocotyls were collected, washed with sterile distilled water, and 1 gram of infected tissues of roots and hypocotyls were taken for the isolation of polygalacturonase and pectate lyase enzymes. The infected tissues were frozen in liquid nitrogen and ground in a mortar and pestle. The powder of the frozen tissue was dissolved in 0.25 M NaCl, and after centrifugation, the supernatant solution was used for the determination of polygalacturonas and pectate lyase activity. The antibody specific protein in the supernatant was measured using sandwich ELISA as described in chapter 2. Control plants were grown under the same conditions without infection by *Fusarium moniliforme* spores.

Localization of the pathogen in host tissue

Transverse sections of hypocotyls of infected and control tomato and cauliflower plants were stained with cotton blue. The sections were washed with distilled water two to three times and observed under the light microscope.

Localization of polygalacturonase and pectate lyase in Whites medium grown tomato and cauliflower plants

Transverse sections of the hypocotyls of tomato and cauliflower infected and control plants were equilibrated with PBS/T at pH 7.0. The sections were blocked with 1% BSA for 1 h. Then sections were washed three times with PBS. Thereafter they were incubated with 1:300 diluted polygalacturonase and pectate lyase antibodies overnight at 4ºC. Then sections were again washed with two to three times with PBS and incubated with 1:100 diluted second antibody labeled with Texas Red at 37ºC for 3 hrs. The sections were

washed repeatedly with PBS and observed under Leitz Laborlux S fluorescent microscope using N_2 filter (excitation wavelength between 540-600 nm).

100 µm thin sections of infected and control hypocotyls were cut using Leica ultramicrotome Model RM 2155. Both sections were blocked with 1% BSA and after washing with PBS/T, the sections were incubated with 1:300 diluted primary antibody for overnight at 4ºC. The sections were washed with two to three times with PBS and incubated with 1:100 diluted second antibody labeled with Texas Red at 37ºC for 3 hrs. After washing with PBS/T, a single section each of infected and control hypocotyls was crushed in 2 ml 10 mM phosphate buffer at pH 7.0. The cell debris was removed by centrifugation. The supernatant solution was excited at 596 nm and fluorescence intensity of the Texas Red (I_0) was measured using a Perkin Elmer Spectrofluorimeter LS 5B at 25ºC.

Results and discussion

When healthy tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*) plants grown under field conditions were inoculated with *Fusarium moniliforme* NCIM 1276 they showed yellowing and crinkling of leaves after 8 to 10 days. In the last stage of infection growth was arrested (Fig 3.1, Fig 3.2). At this stage root tips and hypocotyls showed decay and cortical tissue sloughed off. These are symptoms typical of vascular wilt. Infected material incubated on Czapec-Dox agar medium containing 1% pectin at 30°C, showed fungal colonies morphologically identified as *Fusarium moniliforme* NCIM 1276.

Bacon and Hinton (1996) and Yetes *et al*. (1997) have shown that some species of *Fusarium* cause endophytic symptomless infection in *Zea mays* but the present isolate apparently belongs to the main 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 it self a new species.

Pectic enzymes are the first polysaccharidases produced by fungal pathogen in their attack on plants (Collmer and Keen, 1986). Bateman (1966) has reported the production of hydrolytic and trans-eliminative enzymes at different pH by *Rhizoctonia solani*. Young cultures of *Rhizoctonia* grown at acidic pH contained primarily polygalacturonase whereas older, alkaline cultures contains mostly pectate lyase. In the case of the present organism varying the pH of the medium in which the organism was cultured (Chapter 2) resulted in a similar shift in the type of enzyme produced showing that virulent strains express a variety of enzymes to suit the local pH conditions.

Figure 3.1

Pathogenicity testing of *Fusarium moniliforme* NCIM 1276 on field grown tomato plant

A) 8 days after infection B) Control tomato plant.

Figure 3.2

Pathogenicity testing of *Fusarium moniliforme* NCIM 1276 in Whites basal medium grown tomato and cauliflower plants

A) Tomato plant 1) Control 2) 4 days after infection 3) 8 days after infection

B) Cauliflower plant 1) Control 2) 4 days after infection 3) 8 days after infection.

Transverse section of infected *in vitro* tomato and cauliflower hypocotyls showed that the fungus penetrates in about 4 days through the epidermal layer to the cortical tissue (Fig 3.3). Sporulation occurs around the 6^{th} day after infection. The hypocotyls were sectioned 4 days after inoculation with fungal spores and the section challenged with Texas Red labeled second antibodies. Fluorescence intensity in the vascular bundles of both infected tomato and cauliflower plants clearly shows that both enzymes were produced in both host tissues (Fig 3.4, Fig 3.5). The fluorescence intensity differed by 5 to 10 fold between control and infected plants (Fig.3.6). Crawford and Kolattukudy (1987) and De Lorenzo *et al*. (1987) have shown that *F. solani* f.sp.pisi and *F. moniliforme* use pectate lyase and polygalacturonase for penetration into host tissue.

During optimizations studies (Chapter 2) it has been shown that only one polygalacturonase and only one pectate lyase is produced in liquid culture by the organism at acidic and alkaline pH respectively. Although it is not known whether different enzymes may be produced if these two genes are knocked out, for example in mutated forms of the organism, the present data shows that the polygalacturonase produced in culture and on host are antigenically similar (as is also the case for the pectate lyase) and therefore likely to be produces of the same gene whether the organism is grown on submerged culture or in host.

Figure 3.3

Transverse section of tomato hypocotyl.

A) Control transverse section B) Infected transverse section of tomato hypocotyl.

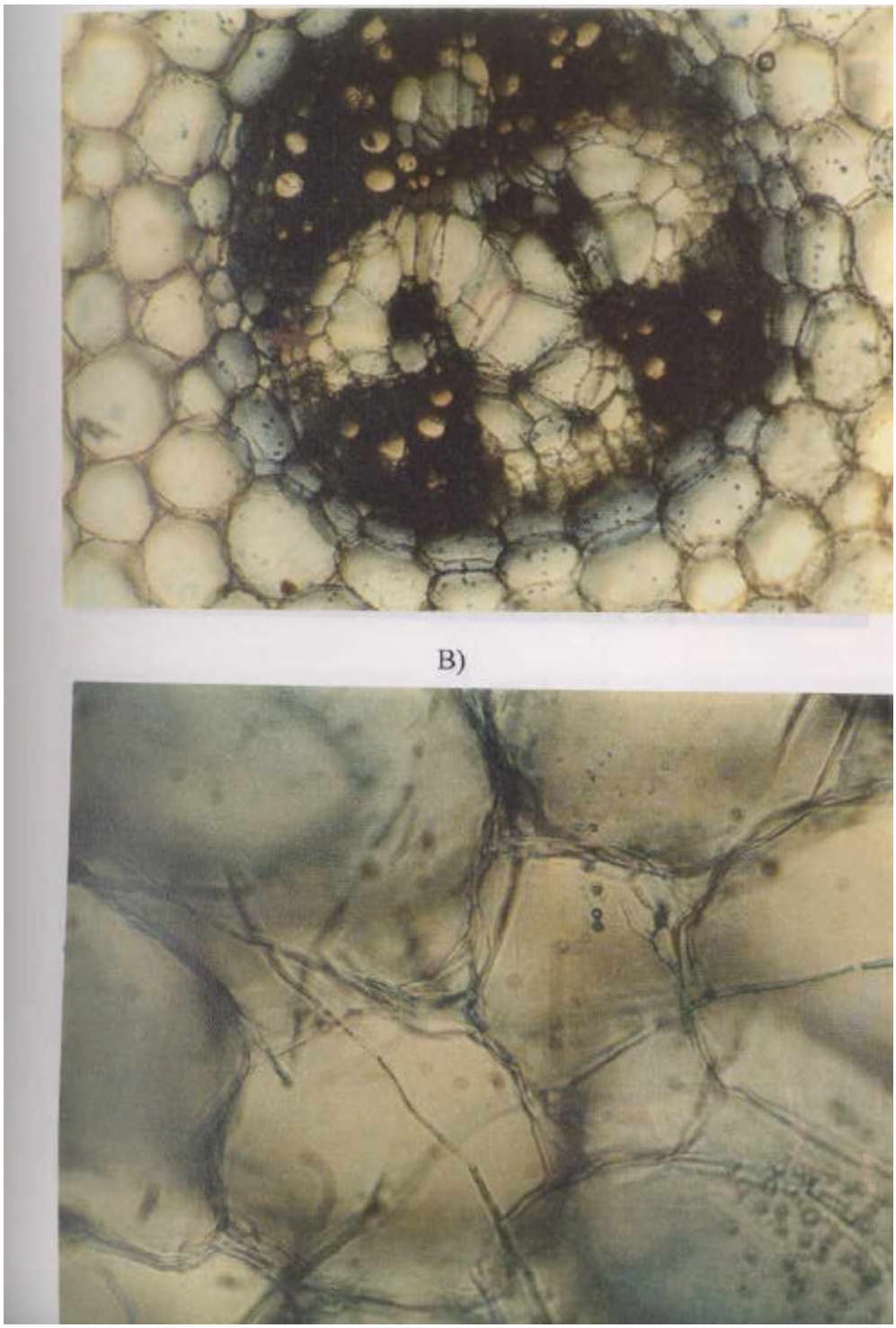


Figure 3.4

Transverse section of tomato hypocotyl incubated with Texas Red labeled second antibodies. (Staining procedure was described in Methods Section)

A) Control B) Infected

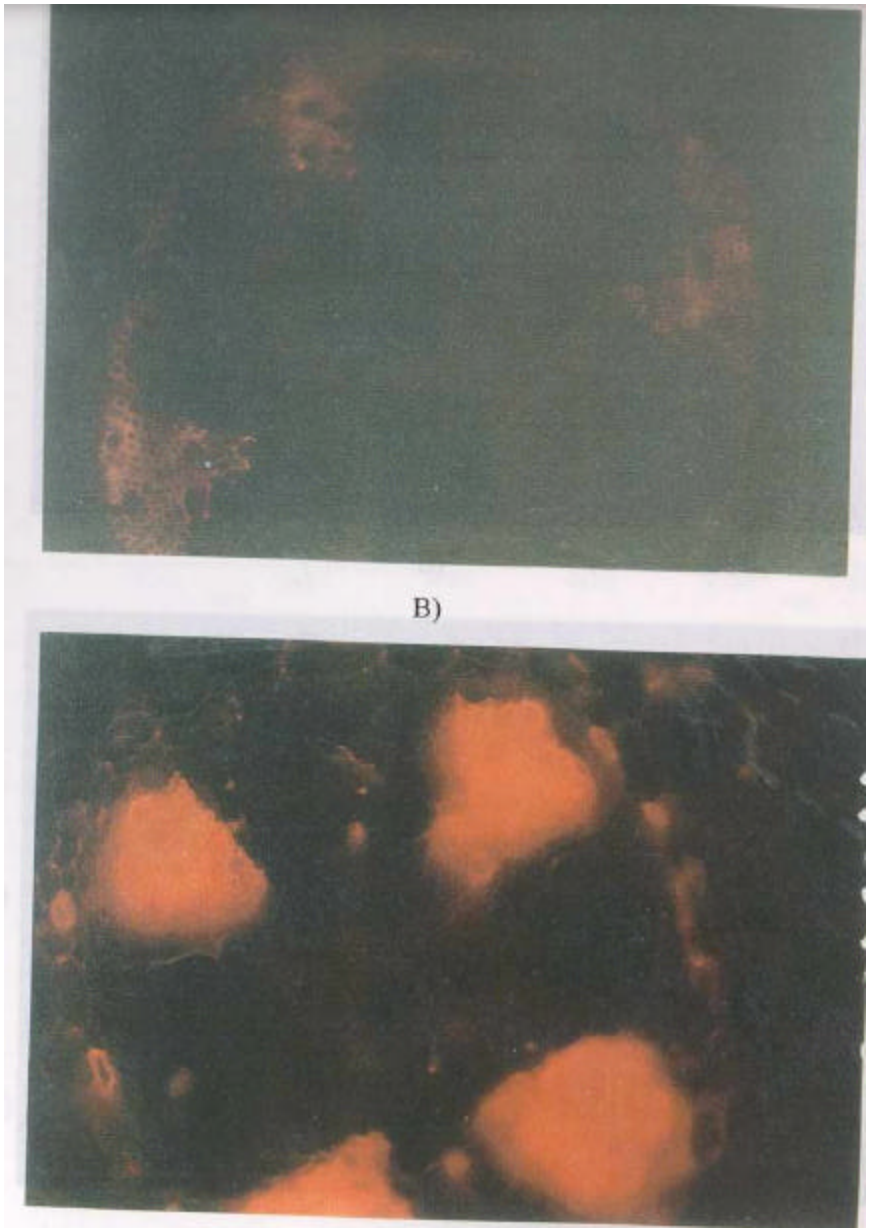
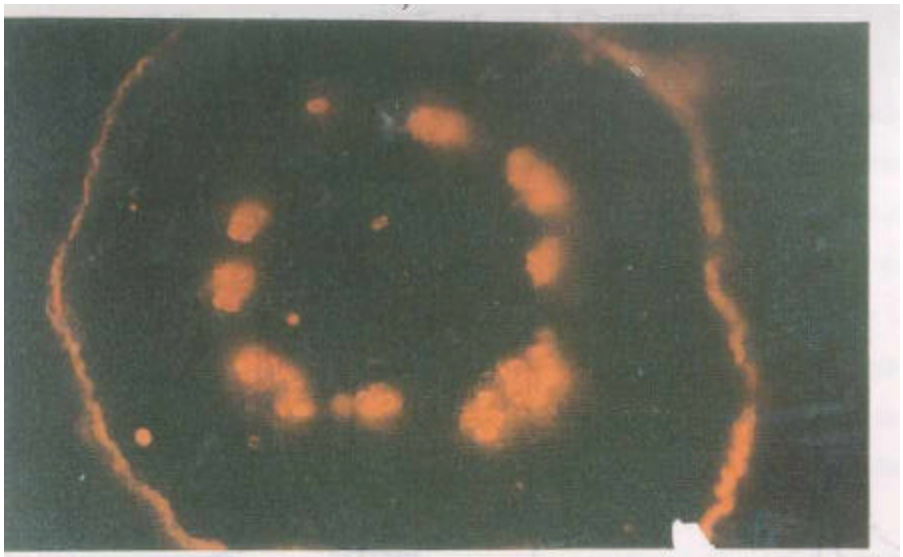


Figure 3.5

Transverse section of cauliflower hypocotyls incubated with Texas Red labeled second antibodies. (Staining procedure was described in Methods Section)

A) Control B) Infected

A)



B)

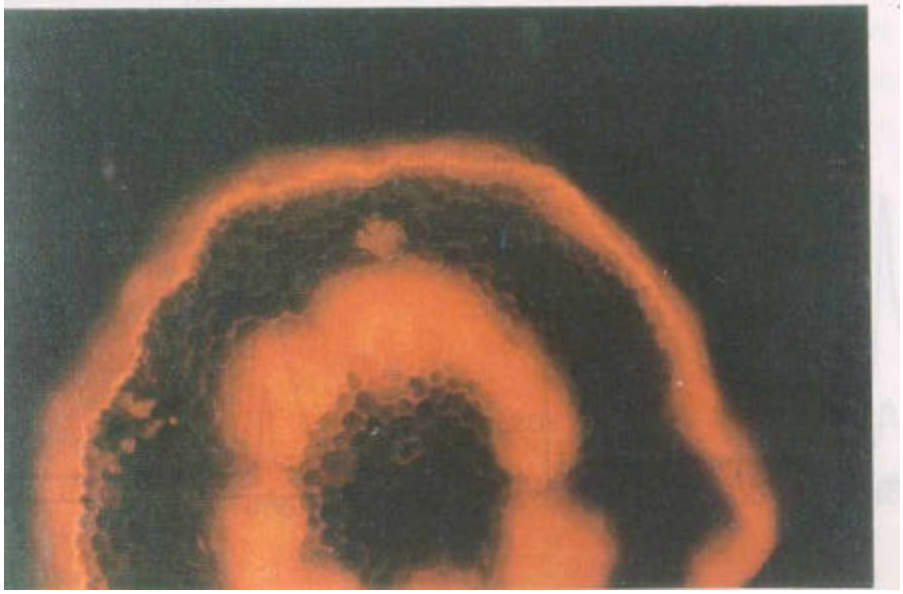


Figure 3.6

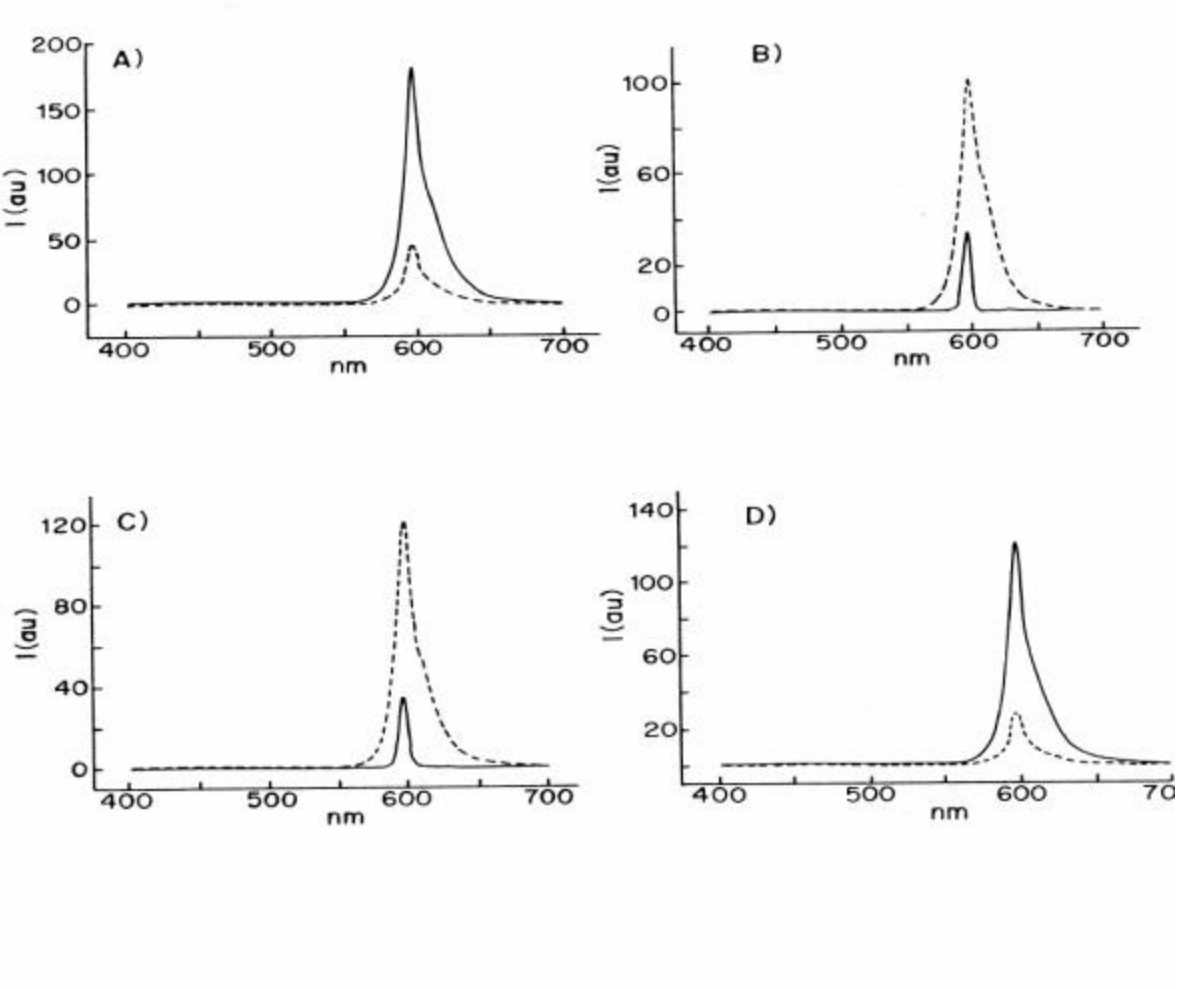
Fluorescence intensity spectrum of tomato and cauliflower control and infected hypocotyls transverse section

PG I st antibodies used A) Tomato hypocotyls: Test (—), Control (------).

B) Cauliflower hypocotyls : Test (------) , Control (—).

PL Ist antibodies used C) Tomato hypocotyls: Test (-------), Control (—).

D) Cauliflower hypocotyls : Test (——) , Control (------)



As seen in Table 3.1 extracts from the control plants showed very low amount of polygalacturonase and pectate lyase activity. Furthermore these enzymes did not cross-

react with antibodies raised against *Fusarium* proteins indicating that the plants themselves produce antigenically different polygalacturonases and pectate lyases. In fact *Fusarium* polygalacturonase and pectate lyase antibodies do not cross react with the polygalacturonase or pectate lyase produced by either *Aspergillus ustus* or *A. niger*. On infection of tomato and cauliflower plants with *Fusarium* polygalacturonase and pectate lyase activity increase and the proteins produced cross reacted with the purified antibodies.

Tomato which has an acidic cell sap of 6.6 showed an 77 fold increase in polygalacturonase activity between infected roots and control. In comparison pectate lyase activity increased only 30 fold from 0.04 units to 1.2 units per gram of infected tissue (Table 3.1). Infected cauliflower roots which have a cell sap pH of 7.5 shows a 125 fold increase in polygalacturonase production and a 167 fold increase in pectate lyase over control plant. The ratio between PG:PL production in tomato is 3.6 as compared to 0.93 in cauliflower (calculated from Table 3.1) clearly indicating that pectate lyase production is enhanced in the roots of the latter species.

In general the hypocotyls showed lower activity of enzymes both in control and after infection (Table 3.1). However the increase in polygalacturonase activity in infected tomato was over 130 fold as compared to the control whereas pectate lyase activity increased only 3 fold. In cauliflower on the other hand the increase in polygalacturonase in infected hypocotyls was only 19 fold as compared to a 40 fold increase in pectate lyase.

Table 3.1

Distribution polygalacturonase and pectate lyase in control and infected tomato and cauliflower plants

Item	PG		PL	
	(U)	(mg)	(U)	(mg)
Control tomato hypocotyls	0.0262	ND	0.021	ND
Control tomato roots	0.056	ND	0.041	ND
Infected tomato hypocotyls	2.6	35	0.061	13.6
Infected tomato roots	4.32	90	1.2	17
Control cauliflower hypocotyls	0.051	ND	0.012	ND
Control cauliflower roots	0.021	ND	0.014	ND
Infected cauliflower hypocotyls	0.992	18	1.483	25
Infected cauliflower roots	2.63	45	2.74	78

ND - Not Detectable.

Protein biomass was determined using sandwich ELISA as described in chapter 2.

In conclusion *Fusarium moniliforme* NCIM 1276 produces only two enzymes, possibly representing two gene products, whether in submerged culture or in host tissue. In host tissue there is a difference in the ratio of the two enzymes produced which appears to depend on the internal cell sap pH. In acidic environments there is greater production of the hydrolytic polygalacturonase (EC 3.2.1.15) over the β-eliminative cleaver pectate lyase (EC 4.2.2.2) and conversely in a neutral to alkaline cell sap host environment, pectate lyase is produced in larger quantity. These results duplicate the behavior of the organism in submerged culture.

CHAPTER IV

Purification and characterization of endo-polygalacturonase produced by *Fusarium moniliforme* NCIM 1276.

Summary

A single form of polygalacturonase, which is produced by the isolate in the submerged culture, was purified to homogeneity by ion exchange chromatography using CM- Sephadex at pH 5 and Sephadex G-100 gel filtration column chromatography, with a yield of 28 %.

Biochemical characterization of the enzyme shows that, polygalcturonase had a M_r of 38 kDa and a carbohydrate content of 4 %. It has an alkaline pI of 8.1. The K_m is 0.12 mg ml^{-1}, V_{max} is 111.1 µM min^{-1} mg^{-1} and the k_{cat} is 4200 min^{-1}. It has a pH optimum of 4.8 and optimum temperature is 45°C. The enzyme activity was competitively inhibited by gluconic acid D-lactone (GADL) with k_i of 28 mM. The effect of metal ions on polygalacturonase activity shows that enzyme was inhibited by Zn^{++}, Hg^{++} and Fe^{+++} at concentration of 5 mM. Amino acid composition shows that, this protein contains 17% of acidic amino acids, and low quantity of sulfur containing amino acids. N- terminal amino acid sequence of the polygalacturonase is E-S-T-Q-L-N-P-I-P-S-T-V-I-H-G-A-T-G-Y-H-. This 20 amino acid sequence did not match with the N-terminal sequence of any reported polygalacturonase in the SWISS-PROT data base. The pK_a values of polygalacturonase are pK^1_a of 5.7 and pK^2_a of 4.3. Time dependant hydrolysis of polygalacturonic acid by the enzyme yielded di-and mono-galacturonic acid. Therefore this enzyme is an endo-polygalacturonase belonging to EC 3.2.1.15.

Active site characterization of endo-polygalacturonase was carried out by using amino acid groups specific chemical modifiers. The enzyme activity was inhibited by WRK, EDC, DEP, NBS, HNBB, Phenyl glyoxal, 2,3 Butanedione and pNPG, suggesting that, carboxylate, histidine, tryptophan and arginine residues are important for the activity of enzyme. Kinetic inhibition studies and titration of enzyme with specific chemical modifier shows that a single residue of carboxylate, histidine, tryptophan and arginine is present at or near to the active site. Kinetic and fluorescence data show that tryptophan is present in the active site in an electropositive microenvironment and involved in binding. An arginine residue present at or near the active site may be involved in catalysis or extended binding of the substrate. A carboxylate and a histidine residue are involved in catalysis.

Introduction

Endo-polygalacturonases (poly [1,4-α-D-galacturonide] glycanohydrolase) EC 3.2.1.15 are produced by a large variety of organisms such as bacteria, fungi and plants (Kester and Visser, 1990; Rodriguez *et al*., 1991; Riou *et al*., 1992; Zheng *et al*., 1992; Kumar and Palanivalu, 1999 and Wubben *et al*., 1999). Polygalacturonases have been purified and characterized from a number of fungi such as *Aspergillus niger* (Cooke *et al*., 1976), *Postia placenta* (Clausen and Green, 1996), *Rhizopus stolonifer* (Manachini *et al*., 1987), *Kluyveromyces marxianus* (Barnby *et al*., 1990), *Fusarium oxysporum* (Strand *et al*., 1976) and *Fusarium moniliforme* (De Lorenzo *et al*., 1987).

These enzymes showed extensive variation in their physical and chemical properties in relation to pH optima, temperature optima, molecular mass, pI, kinetics constants and active site residues.

Most polygalacturonase have optimum pH between 4.0 to 6.0 and optimum temperatures between 30 to 45ºC (Urbanek and Zelewska-Sobczak, 1975; Sakai and Takaoka, 1985 and Baldwin and Pressey, 1989). Molecular mass of the enzymes varies from 25 kDa to 85 kDa (Baldwin and Pressey, 1989 and Kester and Visser, 1990).

Fungi produces numerous acidic as well as basic polygalacturonases. Isoelectric point of reported polygalacturonases ranging from 3.2 to 8.1 (Tobis *et al*., 1993 and Rao *et al*., 1996).

Binding affinities towards substrate values (K_m) of the reported polygalacturonase varies from 0.67 mg/ml for the *Aspergillus carbonarius* enzyme I (Devi and AppuRao, 1996) to 0.19 mg/ml for the *Rhizopus stolonifer* enzyme (Manachini *et al*., 1987).

Studies show involvement of histidine, carboxylate, tyrosine, cysteine and tryptophane residues at or near the active site of different polygalacturonases (Rexova-Benkova and Slezarik, 1970; Rexova-Benkova, 1990 and Urbanek and Zelewska-Sobczak, 1975).

The histidine residue was shown to be important for enzyme activity of polygalacturonase of *A.niger* (Rexova-Benkova and Slezarik, 1970). Rao *et al*. (1996) have suggested that, a histidine residue was present at or near to the active site of *A. ustus* enzyme. That the histidine residue was involved in the catalysis has also been determined by site directed mutagenesis (Caprari *et al*., 1996 and Armand *et al*., 2000). Rexova-

Benkova and Mrachova (1978) used pK_a to show that carboxylate and histidine residues are involved in the catalytic process of a polygalacturonase produced by *A. niger*. The presence of the carboxylate residue in the active site of this polygalacturonase was also showen by chemical modification (Rexova-Benkova, 1990).

Devi and AppuRao (1998) have reported that tryptophan residue was responsible for activity of polygalacturonase produced by *A. carbanarius*. Waksman *et al*. (1992) and Rao *et al*. (1996) have shown that tryptophan residue was involved in the binding site of the polygalacturonase of *C. lindemuthianum* and *A. ustus* respectively using fluorescence quenching studies.

Urbanek and Zelewska-Sobczak (1975) implicated cysteine and tyrosine residues in the active site of the enzyme produced by *Botrytis cinerea* and Stratilova *et al*. (1996) have reported a different strain of *A. niger* produced a polygalacturonase which has tyrosine in the active site. Thus several amino acid residues have been implicated in active site of polygalacturonase from different soucres.

The present study involves the purification and active site characterization of endo-polygalacturonase produced by *Fusarium moniliforme* NCIM 1276. The data presented are based on chemical modification.

Materials

Pectin (sodium polypectate), polygalacturonic acid, galacturonic acid, CM-Sephadex, Sephadex G-100, SDS-PAGE and gel filtration molecular weight markers, bovine serum albumin, phenyl glyoxal (PG), N-ethylmaleimide (NEM), 2,2-dithiobisnitrobenzoic acid (DTNB), phenylmethylsulfonylfluoride (PMSF), p-hydroxymercurybenzoate (PHMB), diethylpyrocarbonate (DEP), 3-nitro-L-tyrosine ethylester (NTEE), 1-ethyl-3-(3-dimethylaminoproply) carbodiimide (EDC), 2- hydroxy - 5- nitrobenzylbromide (HNBB), 2,4,6-trinitrobenzenesulfonic acid (TNBS), N-bromosuccinimide (NBS), N-acetylimidazole (NAI), 2,3 butanedione, para-nitroacetophenone, succinic anhydride, Woodward's reagent K (WRK), trichloroacetic acid (TCA), HEPES, and MES were purchased from Sigma Chemical Company USA. Analytical grade chemicals and reagents were used.

Methods

Preparation of reagents

The stock of HNBB was prepared in 1,4 dioxane. The stock of WRK was prepared in 1 mM HCl. The stocks of DEP and phenyl glyoxal were prepared in ethanol. The stock of succinic anhydride was prepared in dioxane. pNPG was prepared in methanol

Para-nitrophenylglyoxal (pNPG) was synthesized according to the procedure of Steinbach and Becker (1954). A solution of selenium dioxide (3.9 g) in 2.4 ml of distilled water and 15 ml of glacial acetic acid was refluxed at 120°C for 1 h with 5 g of p-nitroacetophenone. The reaction mixture was cooled and selenium was removed by filtration. The crystals of pNPG were purified by repeated recrystallization from glyoxal. The purity of pNPG was checked by melting point (98°C) and IR - spectrum.

Organism and culture conditions

Growth, maintenance and production of polygalacturonase from *Fusarium moniliforme* NCIM 1276 are described in the chapter 2.

Enzyme assay

Polygalacturonase activity was determined using standard assay conditions as described in Chapter 2.

Protein assay

Protein concentration was determined in accordance with Lowry *et al*. (1951) with BSA standard.

Purification of polygalacturonase from Fusarium moniliforme NCIM 1276

The cell-free broth was concentrated by ultrafiltration through a 5K cellulose acetate Amicon® membrane and dialyzed in 50 mM acetate buffer pH 5. Dialyzed material was loaded onto a 30 cm x 2.5 cm CM- Sephadex C-50 column equilibrated with 50 mM acetate buffer pH 5. Enzyme was eluted at a flow rate of 12 ml hr^{-1} with a linear gradient of 0.01 to 0.5 M NaCl in the same buffer. The active fractions were pooled, dialyzed and baded again onto a 30 cm x 1 cm Sephadex C-50 column equilibrated with 50 mM acetate buffer at pH 5 and eluted with a flow rate of 9 ml hr^{-1} using a gradient of 0.01 to 0.5 M NaCl in acetate buffer at pH 5. The active protein eluted between 0.35 to 0.38 M NaCl on both columns. The active fractions from the second eluate were pooled, concentrated on a Savant Speedvac Model SC110A and loaded onto a 120 cm x 1 cm Sephadex G-100 column equilibrated with 0.1M acetate buffer with 0.1M NaCl. The active fractions were pooled, concentrated and dialyzed extensively against acetate buffer, and stored at -10°C until required.

Preliminary characterization of endo-polygalacturonase

Electrophoresis of polygalacturonase

Homogeneity of purified polygalacturonase was determined by 12% acidic native PAGE at pH 4.5 in acetate / KOH buffer, the protein band was visualized by silver staining.

Activity staining of polygalacturonase was done by blotting 12% acidic native PAGE onto solidified polygalacturonic acid gel containing 1.5% agar at pH 4.5, the activity band was visualized by 3% cetrimide after 45 min incubation at 40°C.

A tube gel was prepared using 7.5% acrylamide and ampholines in the range of pH 3 to 10 for determination of isozymes.

Determination of molecular mass of polygalacturonase

By Gel filtration

A Sephadex G-100 (100 × 1 cm) column was equilibrated with 50 mM acetate buffer pH 5 with 0.1M NaCl and calibrated with 1 mg/ml of standard proteins: 45 kDa; egg-albumin, 36 kDa; glyceraldehyde 3-phosphate dehydrogenase, 29 kDa; carbonic anhydrase, and 24 kDa; trypsinogen. 1 mg/ml polygalacturonase was co-chromatographed with the above proteins and elution volume (V_e) of the polygalacturonase was calculated.

By SDS-PAGE

The molecular weight of polygalacturonase was determined by using 12 % SDS-PAGE (Laemmli, 1970). 20 µg of purified polygalacturonase was co-chromatographed with standard SDS proteins markers: 66 kDa; bovine serum albumin, 45 kDa; ovalbumin, 29 kDa carbonic anhydrase, 20.1 kDa; trypsin inhibitor and 14.2 kDa; ∝ lactalbumin. The proteins bands were visualized with Coomassie Blue R-250, and R_f values were calculated for standard proteins and polygalacturonase.

pH optima and stability of polygalacturonase

The optimum pH for polygalacturonase activity was determined by incubating 0.3% polygalacturonic acid with purified enzyme from pH 2.0 to pH 11.0. The pH stability also determined by incubating enzyme at pH ranging from 2.0 to 11.0 for 2 hours. The residual activity of the enzyme was determined under standard assay conditions.

Temperature optima and stability of polygalacturonase

The optimum temperature for polygalacturonase activity was determined by incubating enzyme with substrate at different temperatures from 5°C to 70°C. Stability of enzyme at different temperatures was determined by incubating 5 µg/ml polygalacturonase at each temperature for 2 hours. Residual activity of the enzyme was measured under standard assay conditions.

Determination of pI

Isoelectrofocussing was performed in the modified straight-tube method of Chinnathambi *et al*. (1994).

Determination of kinetic constants

The kinetic constants K_m, V_{max} and k_{cat} of enzyme were calculated by fitting the data on activity at different substrate concentration to a linear regression on Lineweaver-Burk double-reciprocal plots using ORIGIN 4.1 (Microcal).

pK_a of endo-polygalacturonase was determined by assaying the enzyme activity at different substrate concentrations (PGA) and pH between 3 to 8. The pH dependent K_m and V_{max} values were determined from Lineweaver-Burk plots.

Determination of K_i

At saturating concentrations of polygalacturonic acid, inhibition of the enzyme by Gluconic Acid D-lactone (GADL) was tested at various concentrations of the inhibitor. The kinetic constants K_m, k_i and k_{cat} were determined under standard assay conditions using 30 to 300 µg PGA and 5 to 40 mM GADL. The constants were calculated by fitting the data to a linear regression on Lineweaver-Burk plots using Origin 4.1 (Microcal).

Effect of metal ions on polygalacturonase activity

10 µg / ml of purified enzyme was dialyzed against deionized water for 8 hours. The enzyme was then incubated with 5 mM of different metal chlorides for 30 minutes. Residual activity was measured using 0.3% polygalacturonic acid (PGA) in 0.1M acetate buffer at pH 5.

Determination of hydrolysis product of polygalacturonic acid

1 % polygalacturonic acid in acetate buffer pH 5 with 1 unit of polygalacturonase (5.6 µg) was incubated at 40 °C. After each 15 minutes interval, 0.1 ml of the reaction mixture was removed and boiled for 5 min. Hydrolyzed material was loaded onto Whatman paper No.1. The solvent system was pyridine-ethyl acetate-acetic acid- water (5:5:5:5). The chromatogram was visualized by spraying with pthalate anisidine reagent.

Determination of amino acid composition

Amino acid composition was done on a pharmacia LKB alpha plus amino acid analyser by hydrolysing the purified enzyme in 6 N HCl at 110°C for 24 hrs, cysteine methionine and tyrosine were protected. Tryptophan residues were estimated by titration with N-bromosuccinimide by the method of Spande and Witkop (1967). Free cysteine and disulphide were estimated by the method of Habeeb (1972) and Cavallini *et al*. (1966).

Elecroblotting and N-terminal sequence

Electroblotting of the enzyme, on the PVDF membrane was carried out according to Le Gendre *et al*. (1993). The purified enzyme fraction was separated on the 12 % w/v SDS-PAGE at pH 8.8. After electrophoresis, the gel and PVDF membrane were sandwiched between Whatman papers and placed in the blotting cassette. The tank was filled with 10 mM CAP buffer pH 11.0 containing 10 % methanol and the protein was transferred under a constant current of 250 mA for 45 minutes. The PVDF membrane was then washed with milli Q water and stained with Coomassie Blue R-250. The N-terminal amino acid sequence of the first 20 residues of the enzyme were determined by Edman degradation on an automated Shimadzu Model PSQ-1 protein sequencer.

Active site characterization

Histidine residues

Reaction with diethylpyrocarbonate (DEP)

0.13 µM of polygalacturonase was incubated in 50 mM sodium phosphate buffer at pH 6.0 at 25ºC with 5 mM DEP. The reaction mixture was dialysed and residual activity of the enzyme was determined under standard assay conditions.

. Reactivation of the inhibited enzyme was carried out by incubating the inhibited enzyme with 2 M hydroxylamine and 2 mM EDTA for 60 min at room temperature. The mixture was dialyzed against 0.1 M acetate buffer at pH 4.5. The activity of dialyzed enzyme was determined under standard assay conditions.

Kinetics of inhibition the of DEP modified enzyme were determined by incubating 0.13 µM polygalacturonase with 20 to 80 µM of DEP. Aliquots were removed at 5 min

intervals. Residual activity of the modified enzyme was determined by using standard assay conditions.

Quantification of modified histidine residues ware carried out by the stepwise addition of an aliquots of (5 µM) DEP to 1.97 µM in 1ml of polygalacturonase. After each addition, an aliquot of the mixture was removed and the residual activity and absorbance at 240 nm was measured. The number of histidine residues modified was calculated on the basis of molar absorbance coefficient of 3,200 $M^{-1}cm^{-1}$ for carbethoxyhistidine at 240 nm (Ovadi *et al*., 1967).

0.131 µM of polygalacturonase was incubated with 40 µM DEP. After dialysis excess reagent was removed. The K_m and k_{cat} values of partially modified enzyme with residual activity 39%, 50% and 58% were determined

Substrate protection studies were carried out by the incubating 2 mg/ml polygalacturonic acid with 0.131 µM enzyme before addition of DEP.

. Conformational changes of endo-polygalacturonase after the modification with DEP were checked with circular dichromism measurement. The CD spectra of native and DEP modified enzyme samples 2.56 µM in 1 ml in 10 mM sodium phosphate buffer at pH 6 were recorded on a JASCO-710 Spectropolarimeter from 190-250 nm using a 1 cm path length at 25 °C.

Carboxylate residues

Reaction with Woodwords Reagent K (WRK)

0.131 µM of purified polygalacturonase was incubated with 100 mM of Woodwords reagent K in 50 mM phosphate buffer at pH 6 at 25ºC. The reaction mixture was dialyzed and residual activity of the enzyme was determined under standard assay conditions.

Reaction with 1-ethyl-(3-dimethylaminopropyl) carbodiimide (EDC)

0.131 µM of polygalacturonase was incubated in 50 mM MES/HEPES buffer 75:25 v/v at pH 6 with 100 mM EDC. The reaction mixture was dialyzed and residual activity of the enzyme was determined under standard assay conditions.

131 μM of polygalacturonase was incubated in 50 mM MES/HEPES buffer 75:25 v/v at pH 6 with 10 to 50 mM of EDC. Aliquots were removed at 10 min intervals and added 0.1 ml of 100 mM of acetate buffer at pH 4.5 to arrest the reaction. The residual activity of the modified enzyme was determined under standard assay conditions.

0.131 μM of polygalacturonase was incubated in 50 mM MES/HEPES buffer 75:25 v/v at pH 6 with 20 mM EDC. After dialysis excess reagent was removed. The K_m and k_{cat} values of partially modified enzyme with residual activity 45%, 55% and 70 % were determined.

Reaction with EDC/ Nitrotyrosine ethylester (NTEE)

5.26 μM /ml of enzyme was incubated in 50 mM MES/HEPES buffer 75: 25 w/v at pH 6 with 100 mM of EDC and 30 mM of NTEE at 25°C for 45 min. Then the reaction was stopped by the addition of 10 % TCA and the precipitate of the enzyme was collected by centrifugation. The precipitate was washed two to three times in chilled acetone and dried. Then the precipitate was dissolved in 0.1 M NaOH in 1 ml. The number of nitrotyrosyl groups incorporated was determined spectrophotometrically at 430 nm using a molar absorption coefficient of 4,600 $M^{-1}cm^{-1}$ (Pho *et al*., 1977).

Substrate protection studies were carried out by the incubating 0.131 μM enzyme with 3 mg/ml polygalacturonic acid before addition of EDC.

CD spectra of native and EDC modified enzyme samples (2.56 μM/ml) in 10 mM of MES / HEPES buffer pH 6 were recorded on a Jasco-710 spectropolarimeter from 190 nm to 250 nm using a 1 cm path length at 25°C.

Arginine residues

Reaction with phenyl glyoxal

0.13 μM of purified polygalacturonase was incubated in 50 mM of sodium phosphate buffer at pH 7.8 with 5 mM phenyl glyoxal at 25ºC. After incubation for 30 min the reaction mixture was dialyzed against same buffer and residual activity was measured.

Kinetics of inhibition of the polygalacturonase with phenyl glyoxal were carried out by incubating 0.13 μM of purified enzyme with 20 to 50 μM of phenyl glyoxal at 30°C.

Aliquots from the reaction were removed at 3 min intervals and residual activity of the modified enzyme was determined under standard assay conditions.

0.131 µM of polygalacturonase was incubated with 20 µM phenyl glyoxal. After dialysis excess reagent was removed. The K_m and k_{cat} values of the partially phenyl glyoxal modified enzyme with residual activity 55%, 70% and 80% were determined.

Reactivation of the phenyl glyoxal modified enzyme was carried out by dialyzing the reaction mixture against 10 mM Tris / HCl buffer pH 7.8.

Reaction with 2,3 butanedione

0.13 µM of polygalacturonase was incubated in 20 mM HEPES and 1 mM borate buffer at pH 7.8, with 10 mM 2,3 butanedione. The reaction mixture was dialyzed to remove the excess reagent and residual activity of the enzyme was determined under standard assay conditions.

The enzyme was incubated with 1 mM to 9 mM of 2,3 butanedione at 30ºC. Aliquots were removed after 15 min intervals and residual activity of the enzyme was determined under standard assay conditions.

K_m and k_{cat} values of partially modified enzyme with 2,3 butanedione residual activity 15%, 35%, and 50% were determined.

Substrate protection of the polygalacturonase against phenyl glyoxal and 2,3 butanedione was carried out by incubating the enzyme with (2 to 3 mg/ml) of polygalacturonic acid before addition of reagents.

Reaction with para-nitrophenyl glyoxal (pNPG)

5.26 µM in 1 ml of enzyme was incubated in 50 mM sodium phosphate buffer pH 7.8 with 5 mM pNPG at 30°C for 30 min. Aliquots were removed at 5 min intervals and residual activity of the enzyme was determined under standard assay conditions.

Quantification of modified arginine residues was carried out by the addition of different concentration of pNPG to 5.26 µM in 1 ml of enzyme. Reaction mixture was dialyzed against 100 mM sodium pyrophosphate buffer at pH 9.0 containing 150 mM sodium ascorbate. After dialysis color of the reaction was measured at 475 nm. The

number of arginine residues modified was calculated using molar absorption 3,300 $M^{-1}cm^{-1}$ (Yamasaki *et al*., 1981).

Conformational changes of the enzyme after arginine modification were determined by CD. The CD spectra of native and phenyl glyoxal modified enzyme were recorded on a Jasco-710 spectropolarimeter from 190 nm to 250 nm using a 1 cm path length at 25°C.

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Tryptophan residues

Reaction with 2 hydroxy-5-nitrobenzylbromide (HNBB)

0.13 µM in 1 ml polygalacturonase in 10 mM phosphate buffer pH 6 was incubated with 10 mM of freshly prepared HNBB and allow to react for 30 min. After dialyses, residual activity of the enzyme was measured under standard assay conditions.

Kinetics of the inhibition of polygalacturonase by HNBB were carried out by incubating the enzyme 3 to 15 mM with HNBB. At 10 min intervals aliquots from the reaction mixture were removed and residual activity of the modified enzyme was determined under standard assay conditions.

The K_m and k_{cat} values of partially inhibited enzyme residual activity 30%, 55% and 75% were determined under standard assay conditions.

Reaction with N-Bromosuccinimide (NBS)

0.131 µM/ml Polygalacturonase in 40 mM phosphate buffer pH 6.5 was incubated with 1 mM of NBS and allow to react for 30 min. After dialysis excess reagent was removed. The residual activity of the enzyme was measured under standard assay conditions.

Kinetics of inhibition of polygalacturonase by NBS were also carried out by incubating 0.13 µM / ml the enzyme with 2 to 10 µM of NBS. Aliquots from the reaction mixture were removed at 5 min intervals and residual activity of the enzyme was determined under standard assay conditions.

K_m and k_{cat} values of the partially inhibited residual activity 25%, 50% and 75% enzyme were determined.

Quantification of tryptophan residues was carried out by titrating 1 ml of enzyme solution (1.97 µM) with freshly prepared NBS. The NBS mediated reaction was followed

by measuring the change in absorbance at 280 nm. The reagent was added till the protein NBS ratio reached 1:10. After each addition of reagent aliquot, 5 µl of reaction mixture aliquot was removed and residual activity of the enzyme was determined under standard assay conditions. The number of tryptophan residues modified was determined spectrophotometrically using a molar absorbance coefficient of 5,500 $M^{1}cm^{-1}$ (Spande and Witkop, 1967) .

Substrate protection of the polygalacturonase was carried out by addition 2 mg/ml of polygalacturonate before addition of HNBB and NBS.

Conformational changes of the enzyme after tryptophan modification were determined by CD spectra of native and NBS modified enzyme. CD spectra were recorded on a Jasco-710 spectropolarimeter from 190 to 250 nm using a 1 cm path length at 25 °C.

Tyrosine residues

Reaction with N-acetylimadazole (NAI)

0.13 µM of enzyme was incubated in 40 mM sodium borate buffer pH 7.5 with 10 mM N-acetylimidazole for 30 min at 25ºC. The reaction mixture was dialyzed and residual activity of the modified enzyme was measured under standard assay conditions. The number of tyrosine residues modified was calculated by the titration of 5.26 µM in 1 ml of enzyme with stepwise addition of NAI. After each addition, the residual activity of the modified enzyme was determined under standard assay conditions. The number of tyrosine residues modified was calculated by using molar absorption coefficient 1,160 $M^{-1}cm^{-1}$ (Riordan and Vallee, 1972).

Cysteine residues

Reaction with N- ethylmaleimide (NEM)

0.13 µM of polygalacturonase in 50 mM sodium phosphate buffer pH 6 was incubated with 5 mM of NEM at 30°C for 30 min. The reaction mixture was dialyzed and residual activity of the enzyme was determined under standard assay conditions.

Reaction with p-hydroxymercurybenzoate (PHMB)

0.13 µM of enzyme was incubated with 10 mM of PHMB in sodium phosphate buffer pH at 6.0. The reaction mixture was dialyzed and residual activity of the enzyme was determined under standard assay conditions.

Reaction with 2,2'- dithiobisnitrobenzoic acid (DTNB)

10.5 µM in ml of enzyme was incubated with 1 mM of DTMB at 30°C for 45 min in sodium phosphate buffer pH 7.8. Aliquots were removed at 10 min intervals and residual activity of the enzyme was determined. The number of free cysteines modified was calculated using a molar absorption coefficient of 13,600 $M^{1}cm^{-1}$ at 412 nm (Habeeb, 1972).

Determination of total sulfhydryl groups was carried out by incubating 0.4 mg of protein in 0.5 ml of phosphate buffer pH 7.2 with 8 M urea as a reducing agent with 0.1 ml of 0.1 M Na-EDTA. After that, 2.5 % of 1ml freshly prepared sodium borohydrate was slowly added to the protein solution. The reaction tubes were well shaken and incubated at 38°C for 30 min. After incubation, 0.5 ml of 1 M K_2HPO_4 with 0.2 N HCl was added in the reaction tubes with 2 ml of acetone for the complete distruction of borohydrate. The tubes were bubbled with nitrogen gas for 5 min. and added 0.5 ml of 10 mM of DTMB. The reaction was allowed to proceed for 15 min at 30ºC. Then nitrogen gas was bubbled in the tube through the mixture for 2 min. Absorbance was determined at 412 nm. Using extinction coefficient of 12,000 $M^{-1}cm^{-1}$ (Cavallini *et al*., 1966) total number of sulfhydryl groups modified was calculated.

Lysine residues

Reaction with succinic anhydride

0.13 µM of purified polygalacturonase was incubated in sodium phosphate buffer pH 7.0 with 10 mM of succinic anhydride prepared in dioxane. After incubation for 30 min at 25°C, the excess reagent was removed by dialysis and residual activity of the enzyme was determined under standard assay conditions.

Reaction with 2, 4, 6, trinitobenzenesulfonic acid (TNBS)

0.131 µM in1 ml enzyme was incubated in potassium phosphate buffer pH 7.5 with 5 mM TNBS in the dark at 30°C for 1 hour. The reaction mixture was dialyzed against acetate buffer pH 5 and residual activity of the enzyme was determined under standard assay conditions. The number of amino groups modified was determined spectrophotometrically using a molar absorption coefficient of 9,950 M^{-1} cm^{-1} at 335 nm for trinitrophenylated lysine (Habeeb, 1966).

Serine residues

Reaction with phenylmethylsulfonyl fluroride (PMSF)

0.13 µM of polygalacturonase was incubated in 50 mM sodium phosphate buffer pH 7.0 with 5 mM of PMSF at 30°C for 30 min. The reaction mixture was dialyzed against same buffer and residual activity of the enzyme was determined under standard assay conditions.

Fluorescence measurements

Fluorescence spectra of endo-polygalacturonase were obtained using a Perkin Elmer Spectrofluorimeter LS 5B at 25°C, using an excitation and emission slit width of 5 nm. The fluorescence spectra of native enzyme at a concentration of 2.6 µM in 2 ml and severally modified polygalacturonases were obtained by using 280 nm excitation wavelength. The spectrum range was 300 to 400 nm. Fluorescence quenching of native and histidine modified, carboxylate modified, arginine modified and tryptophan modified modified enzyme was calculated using substrate (PGA), and GADL as a competitive inhibitor.

Tryptophan fluorescence was excited at 295 nm and spectrum range was 300 to 390 nm. Tryptophan fluorescence quenching of native and tryptophan modified enzyme also calculated by addition of PGA and GADL.

The microenvironment of the tryptophan residue was determined in a series of experiments. The enzyme 5.26 µM in 2 ml of 10 mM acetate buffer at pH 5 was excited at 295 nm and the emission was recorded at 336 nm. The enzyme was titrated with 8M acrylamide, 5M KI and 5M CaCl. The fluorescence quenching data was analysed by the

Stern-Volmer and modified Stern-Volmer equations (Stern and Volmer, 1919) using Microcal Origin 4.1.

The equations used were:

$$F_0/\Delta F = 1 + K_a [Q]$$

$$F_0/\Delta F = [1/(f_a K_a)\ 1/(Q) + 1/ f_a]$$

where F_0 is the fluorescence intensity without quencher, ΔF is the difference in fluorescence intensity with and without quencher, f_a is effective fractional accessible tryptophan fluorescence, K_a is effective quencheing or Stern-Volmer constant and $[Q]$ is the quencher concentration.

Stern-Volmer constants of the native polygalacturonase, in presence of substrate 4 mg and 50 mM gluconic acid D-lactone which is a competitive inhibitor of enzyme were determined.

Results and discussion

Purification of polygalacturonase from Fusarium moniliforme NCIM 1276

The endo-polygalacturonase was purified 125-fold with 28.8% yield from the culture broth of *Fusarium moniliforme* NCIM 1276. The purified enzyme has a specific activity 178 U / mg. (Table 4.1). Sephadex G-100 gel filtration resulted in a single homogenous peak (Fig 4.1).

Table 4.1

Purification of endo-polygalacturonase from *Fusarium moniliforme* NCIM 1276.

Purification step	**Vol. (ml)**	**Total activity (U)**	**Total protein (mg)**	**Specific activity (U/ml)**	**% Yield**	**Fold purification**
Broth	1000	260	180	1.44	--	--
Ultrafiltration	40	220	100	2.2	84	1.5
CM-Sephadex I	50	107	2.8	38.2	41.15	26.5
CM-Sephadex II	30	90	0.92	97.8	34.61	67.77
Sephadex G-100	10	75	0.42	178.57	28.84	124.9

Homogeneity of polygalacturonase

The protein thus purified gave a single activity band on native PAGE at pH 4.5 (Fig. 4.2 A &B). Isoelectric focussing shows a single protein band at pI 8.1 (Fig 4.3). Therefore a single form of polygalacturonase is produced by this isolate in submerged culture.

Several authors have reported different forms of polygalacturonase produced by *Fusarium moniliforme* (De Lorenzo *et al*., 1987), *F. oxysporum f sp lycopersici* (Strand *et al*., 1976) and *F. oxysporum f. sp. melonis* (Martinze *et al*., 1991) in submerged culture. De Lorenzo *et al*. (1987) purified four polygalacturonases produced by *F. moniliforme.* All

forms showed a single pI of 6.7. Later Caprari *et al*. (1993) showed that the only difference in the protein relates to glycosylation and suggested that they were all products of a single gene. *Sclerotinia sclerotiorum* produces numerous acidic isoforms of polygalacturonase between pI 4.7 to 5.0 (Waksman *et al*., 1991). Kester and Visser (1990) Purified five forms of endo-polygalacturonase from *A. niger*, all have been shown to have acidic pI between 3.2 to 5.9. *Botrytis cinera* releases five forms of polygalacturonase four with acidic pI and one with alkaline pI (Tobis *et al*., 1993). In contrast *Aspergillus ustus* Rao *et al*. (1996) and the present *Fusarium* both produce one endo-polygalacturonase each and both molecules have alkaline pI.

Figure 4.1

Elution profile of endo-polygalacturonase from Sephadex G-100

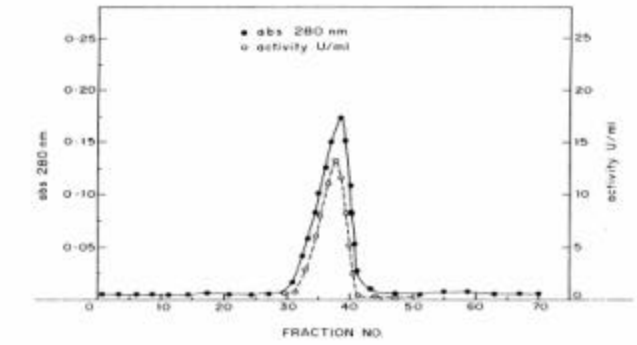

Figure 4.2

(A) Native acidic PAGE of purified Polygalacturonase

10 µg of polygalacturonase was loaded onto 12% w/v, polyacrylamide slab gel, pH 4.5 (acetate / KOH). The protein band was visualized using silver nitrate.

(B) Activity staining of polygalacturonase

12% native acidic polyacrylamide slab gel was blotted on agarose gel containing 0.3% polygalacturonic acid at pH 5 & incubated at 40°C for 45 min. After incubation the activity band was visualized by the addition of 3 % cetrimide.

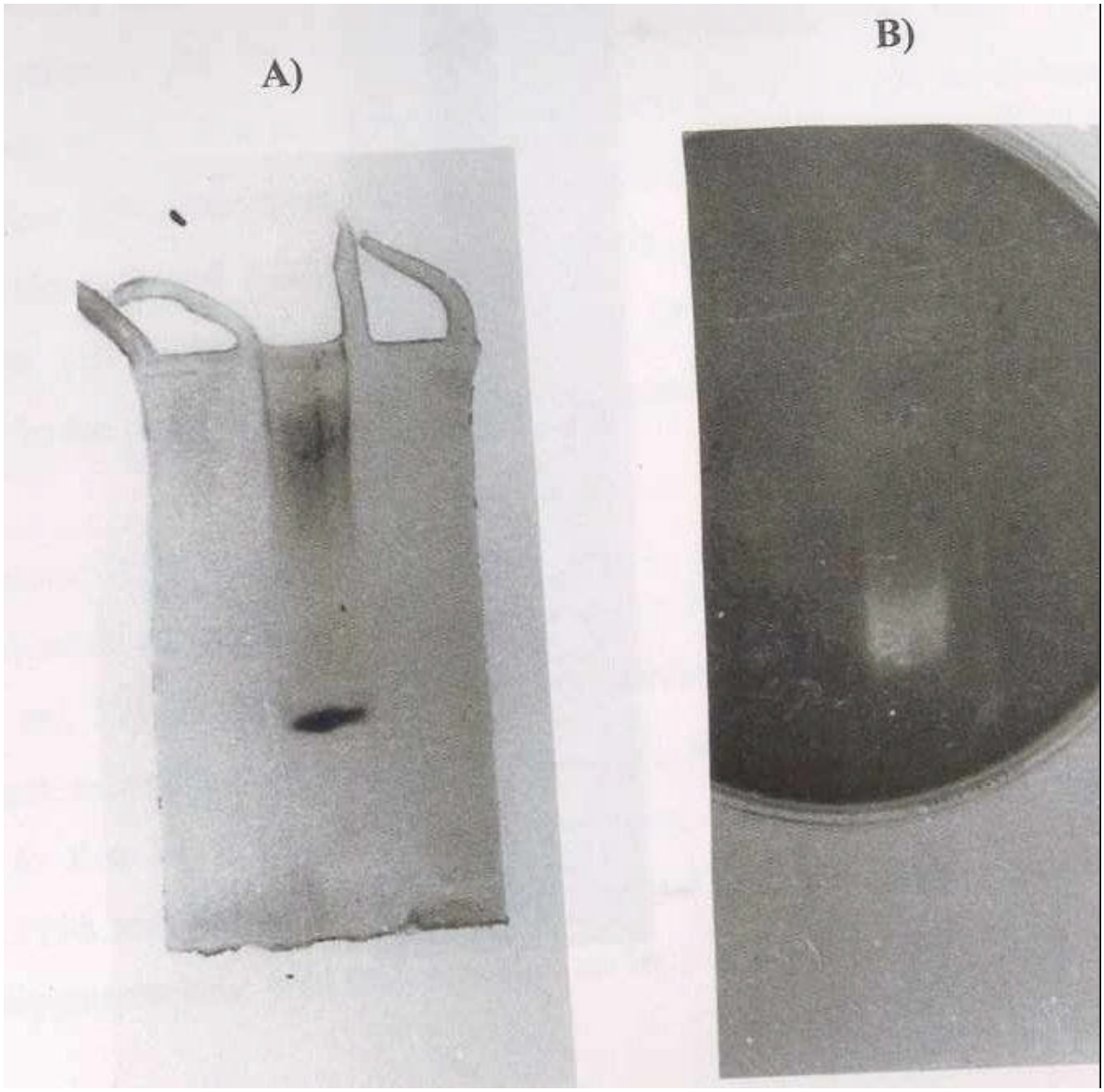

Figure 4.3

Isoelectric focussing of endo-polygalacturonase

Purified polygalacturonase 50 µg was loaded onto 7.5% (w/v) polyacrylamide tube gel using ampholines in the range of pH 3 to 10 pH. The band was visualized by Coomassie Blue R-250.

Preliminary characterization of polygalacturonase

Molecular mass determination

The molecular mass of purified enzyme was determined by gel filtration and SDS-PAGE (Fig 4.4 & 4.5). In both cases the molecular mass was 38 kDa. The match indicated that the protein is monomeric. Molecular masses of polygalacturonases produced from different sources show that, molecular masses of the enzyme range from 32 kDa to 85 kDa (Strand *et al*., 1976; De Loranzo *et al*., 1987; Baldwin and Pressey, 1989 and Kaster and Visser, 1990). The *Fusarium* polygalacturonase is with in this range.

pH and temperature optima

The optimum pH for activity of polygalacturonase was 4.5. The optimum temperature was 45°C. The enzyme was stable from pH 4 to 8 pH for 2 hours (Fig.4.6). The enzyme lost 50% activity at 50°C when incubated for 2 hours (Fig.4.7). Kumari and Sirsi (1971), Urbanek and Zelewska-Sobczak (1975), Sakai and Takaoka (1985) and Devi and AppuRao (1996) have reported that most of the polygalacturonase showed the optimum pH in the range of 4 to 5.5 pH and temperature optimum between 40 to 50°C.

Kinetic constants of polygalacturonase

The kinetic constants K_m, V_{max} and k_{cat} of polygalacturonase were determined to be 0.110 mg / ml, 111.11 µmol of product/mg/ minute and 4.2×10^3 / minute respectively. The apparent second-order rate constant K_{cat}/K_m was 3.5×10^4. K_m value is low as compared to that of reported polygalacturonases (Manachini *et al*., 1987; Devi and AppuRao, 1996 and Rao *et al*., 1996) so it appears that this enzyme has a higher affinity towards polygalacturonic acid than reported so far.

K_i of polygalacturonase

At substrate saturation the kinetics of gluconic acid D-lactone inhibition showed that this sugar is a competitive inhibitor to polygalacturonic acid with a $k_{i,}$ of 28 mM (Fig. 4.8). The double reciprocal plots indicated no change in V_{max} though there was a change in K_m with k_{cat}/K_m values of the order of 3.5×10^4, 2.5×10^4, 1.9×10^4, 1.2×10^4 for the

native, 33% inhibited, 48% inhibited, and 63% inhibited enzyme respectively, suggesting that the gluconic acid D- lactone acts at the site of binding rather that of catalysis (Fig 4.9).

pK_a *of polygalacturonase*

The pH dependent kinetic parameters were determined using different substrate concentrations. The plot of log V_{max}/K_m verses pH resulted in a curve with two inflection points at pK^1_a of 5.7 and pK^2_a of 4.3 (Fig 4.10). It was concluded that the imidazole moiety of histidine and at least one carboxylic group of either aspartate or glutamate are involved in catalysis.

Similar results were reported by Rexova-Benkova and Mrackova (1978) who determined the active groups of polygalacturonase of *A. niger* on the basis of pK_a values suggested that, histidine and carboxylate residues were important for the activity.

N- terminal sequence of endo- polygalacturonase:

N- terminal was determined by the Edman degradation procedure. Endo-polygalacturonase showed E-S-T-Q-L-N-P-I-P-S-T-V-I-H-G-A-T-G-Y-H- amino acid sequence. This 20 amino acid sequence did not match with the N-terminal sequence of any reported polygalacturonase in the SWISS-PROT data base.

Figure 4.4

Determination of molecular mass of polygalacturonase by gel filtration

Sephadex G-100 column (100 × 1 cm) was equilibrated with 50 mm acetate buffer pH 5.0 containing 0.1 M NaCl and calibrated with (a) egg-albumin, (45 kDa), (b) glyceraldehyde-3-phosphate dehydrogenase (36 kDa), (c) carbonic anhydrase (29 kDa) (d) trypsinogen (24 kDa). V_o - void volume and V_e - elution volume.

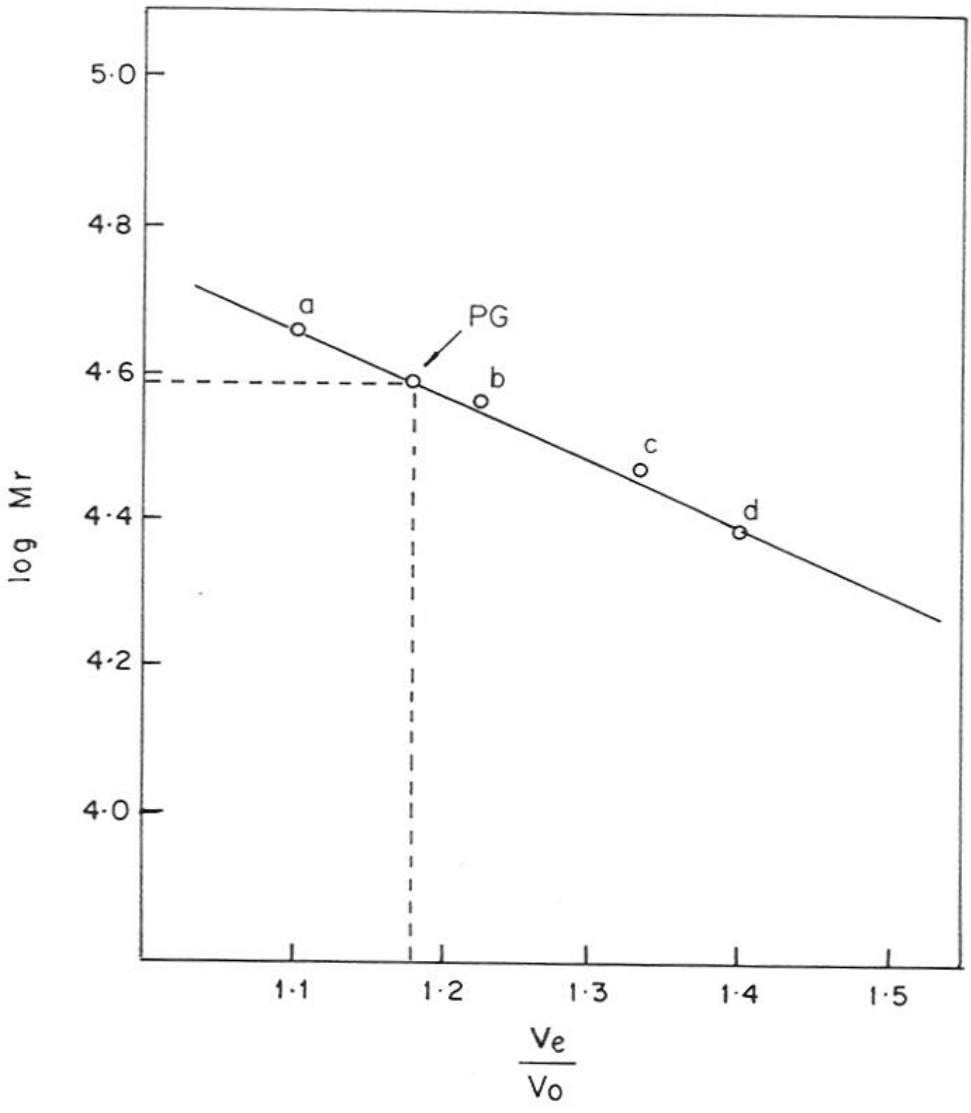

Figure 4.5

SDS-PAGE of purified endo-polygalacturonase

12% SDS-PAGE with molecular weight markers (top to bottom) a) Bovine serum albumin, (66 kDa) b) Ovalbumin, (45 kDa) c) Carbonic anhydrase, (29 kDa) d) Trypsin inhibitor, (20.1 kDa) and e) α - Lactalbumin, (14.2 kDa).

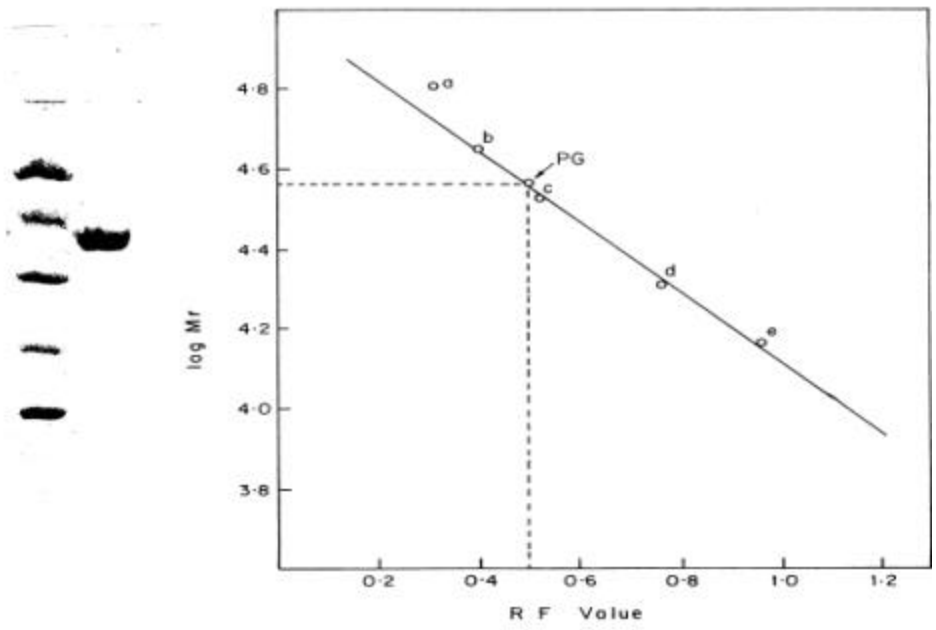

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Figure 4.6

pH optima and stability of polygalacturonase

Polygalacturonase was assayed at different pH values (2.0-11.0) in universal buffers at 40ºC as described in methods. For stability the enzyme solution was incubated at different pH for 2 hrs.

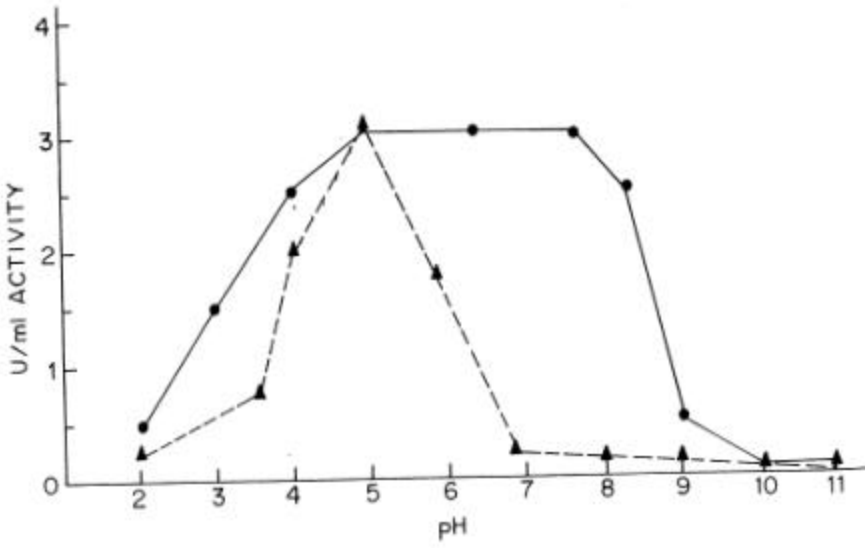


Figure 4.7

Temperature optima and stability of polygalacturonase

Polygalacturonase was assayed at various temperatures (30-70ºC) at pH 5 as described in methods. For stability the enzyme was incubated at different temperatures for 2 hrs.

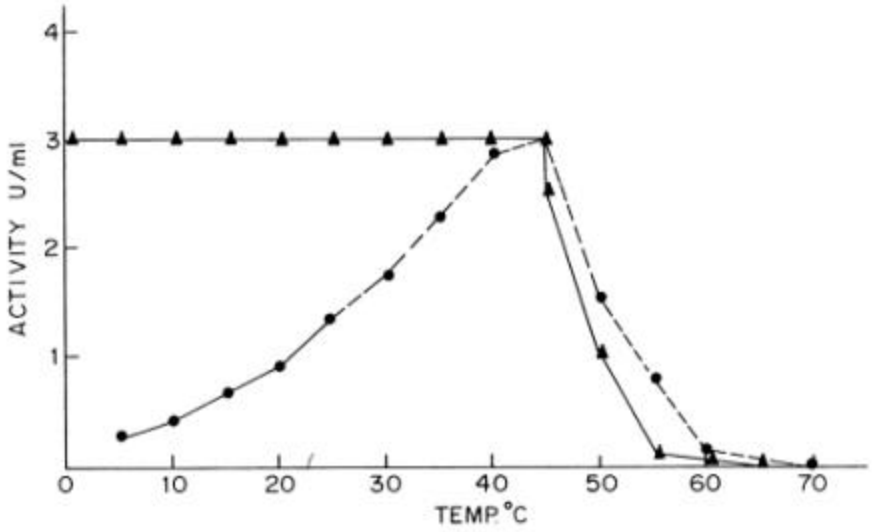


Figure 4.8

Determination of k_i

0.13 µM polygalacturonase was incubated with 1.35 mg (o---o) and 2.7 mg (•----•) PGA with different concentrations of GADL and k_i was determined.

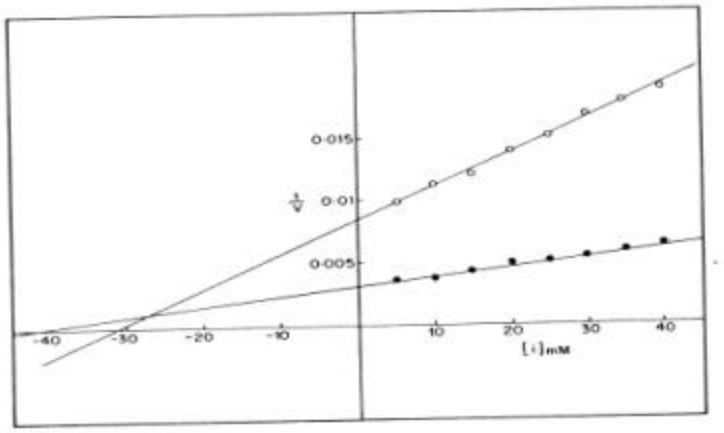


Figure 4.9

Competitive inhibition of polygalacturonase by GADL

0.13 µM polygalacturonase was incubated with 10 mM (•), 15 mM (Δ) and 20 mM (∇) GADL with native enzyme (□).

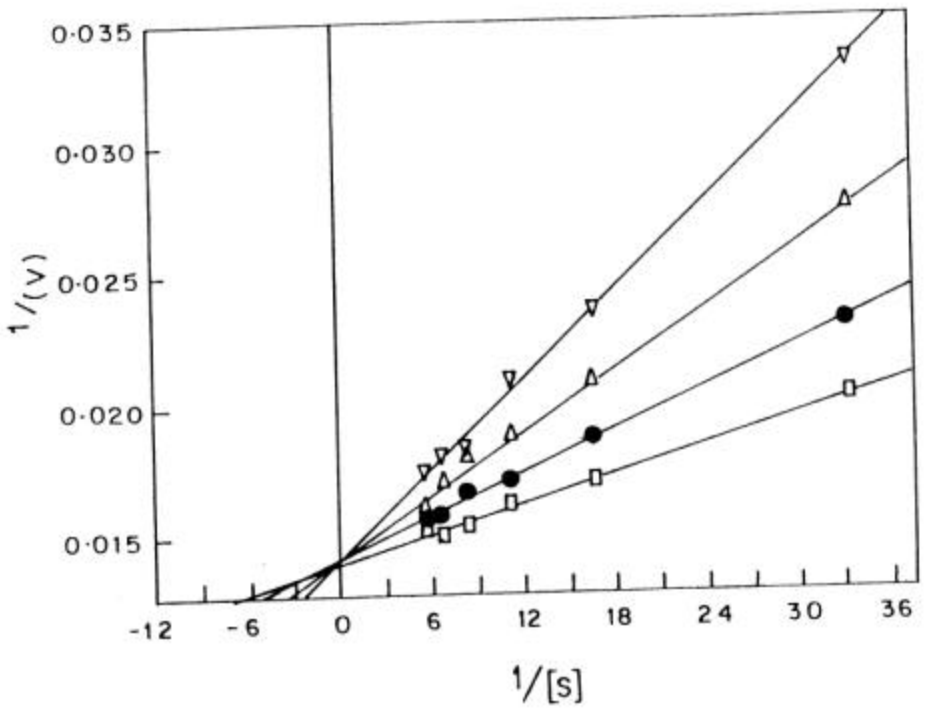
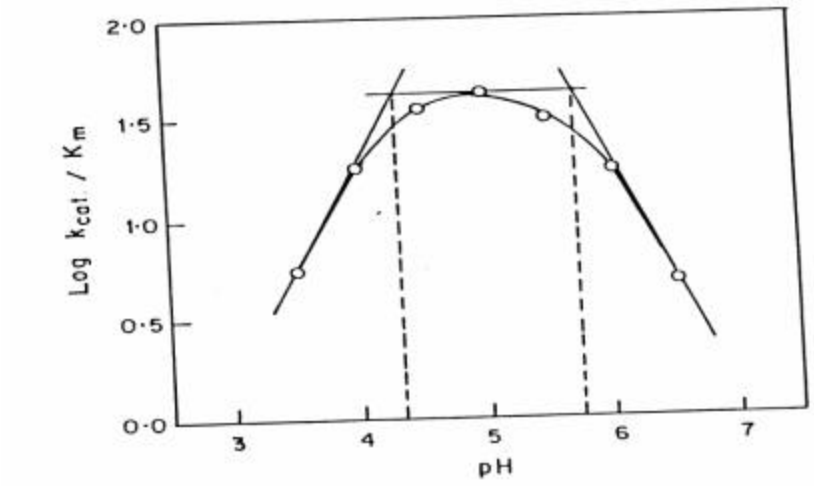


Figure 4.10

Determination of pK_a

pH-dependent K_m and V_{max} values were determined from Lineweaver-Burk plots by varying the substrate concentration at each pH value.



Sensitivity of polygalacturonase towards metal ions

The effect of metal ions on polygalacturonase is shown in (Table 4.2) *Fusarium* polygalacturonase does not require metal ions for activity. EDTA did not affect this enzyme. However, enzyme was inhibited by Zn^{++}, Hg^{++} and Fe^{+++} at concentration of 5 mM. Kumari and Sirsi (1971) showed that the polygalacturonase from *Ganoderma lucidum* was not inhibited by Fe^{++} and Fe^{+++} ions. Devi and AppuRao (1996) reported Hg^{++} ion inhibited polygalacturonase activity of *Aspergillus cabonarius*. Polygalacturonase of *Rhizopus stolonifer* was inhibited by Mn^{++} and Zn^{++} and activity was stimulated by Fe^{+++}, Mg^{++}, and Co^{++} (Manachini *et al*., 1987).

Table 4.2

Effect of metal ions on polygalacturonase activity

Metal ion	Concentration (mM)	Residual activity (%)
EDTA	5	100
Na^{+}	5	96
K^{+}	5	98
Ca^{++}	5	95
Al^{+++}	5	91
Ba^{++}	5	86
Mg^{++}	5	75
Mn^{++}	5	55
Zn^{++}	5	55
Hg^{++}	5	25
Fe^{+++}	5	5

Amino acid composition of polygalacturonase

As seen in (Table 4.3) the amino acid composition of the protein shows marginal differences with other reported polygalacturonases. This protein contains 17% of acidic amino acids, 2 cysteine residues which formed a single disulfide linkage. There were no

free cysteines in the protein molecule. Similar data have bee reported by Strand *et al*. (1976), Kester and Visser (1990), Devi and AppuRao (1996) and Rao *et al*. (1996).

Table 4.3

Amino acid composition of polygalacturonase produced by *Fusarium moniliforme* NCIM 1276 and *Aspergillus ustus* (Rao *et al*., 1996).

Amino acid	***F. moniliforme*** **Mol/ Mol**	***A.Ustus*** **Mol/Mol**
Asx	45	38
Thr	25	35
Ser	33	36
Glx	25	22
Gly	40	48
Ala	25	20
Val	19	23
Met	13	1
Ile	13	24
Leu	13	17
Tyr	14	13
Phe	8	13
His	8	7
Lys	23	29
Arg	6	8
Pro	15	-
•Trp	3	4
? Cys	2	2

• Tryptophan was calculated according to Spande and Witkop (1967)

? Cysteine was calculated according to Cavallini *et al*. (1966)

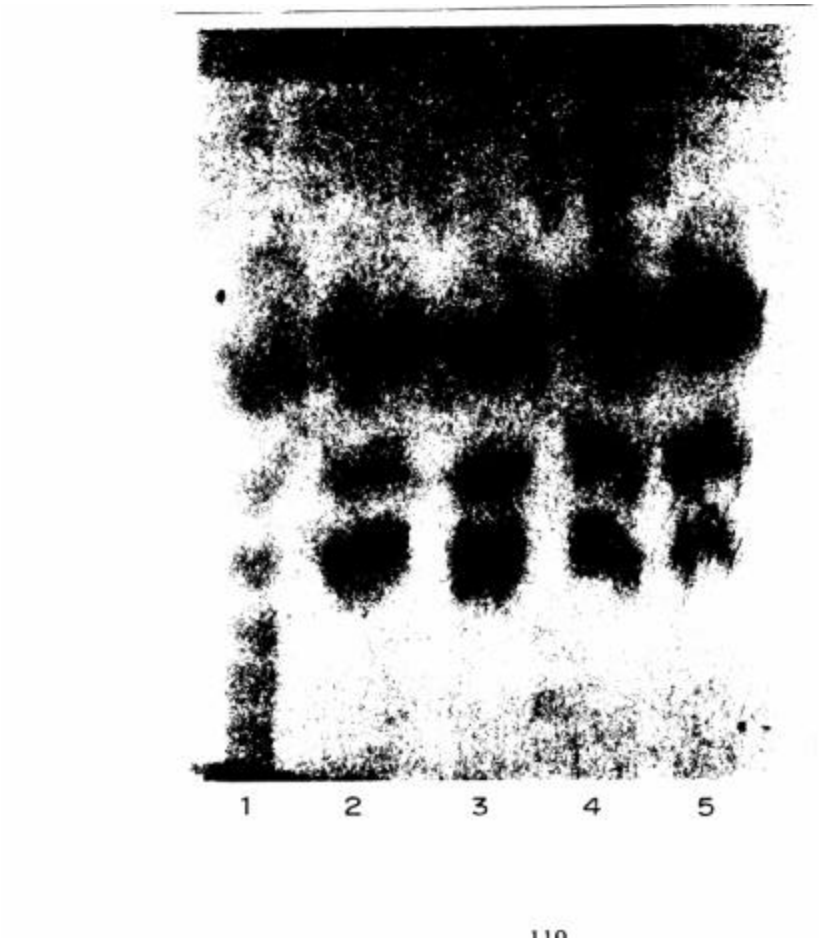
Mode of substrate hydrolysis by polygalacturonase

Time dependent hydrolysis of polygalacturonic acid by the enzyme yielded di-and monogalacturonic acid (Fig 4.11) after 45 minute. Therefore this enzyme is an endo-polygalacturonase belonging to EC 3.2.1.15. English *et al*. (1972) reported that, polygalacturonase of *Collectotrichum lindemuthianum* produced predominately trigalacturonic acid and digalacturonic acid as end product. Strand *et al*. (1976) showed that *F. oxysporum* f.sp. *lycopersici* enzyme produced monogalacturonic acid.

Figure 4.11

Time dependant hydrolysis products of polygalacturonic acid with polygalacturonase

Lane 1-4, samples after incubation for 15, 30, 45 and 60 min. Lane 5, mono-,di- and tri-galacturonic acid standards. The details are in the methods section.



Active site characterization

The pK_a determined from the k_{cat}/K_m vs pH plot shows two inflexion points at pK^1_a of 5.7 near that of histidine, and an acidic pK^2_a of 4.3, suggesting that a histidine and carboxylate residues are involved at or near the catalytic site of the enzyme (Fig. 4.10).

To substantiate these results chemical modification studies were carried out. The effect of different chemical modifiers on the activity of the polygalacturonase is shown in (Table 4.4). WRK, EDC, DEP, NBS, HNBB, Phenyl glyoxal, 2,3 Butanedione and pNPG inhibited enzyme activity suggesting that, histidine, carboxylate, tryptophan and arginine residues are important for the activity of the enzyme. NEM, NAI, PHMB, PMSF, TNBS, Succinic anhydride and DTNB did not inhibit enzyme activity indicating that, tyrosine, lysine, cysteine and serine residues do not have any role in enzyme activity.

Modification of Histidine residues

Carboxyethylation of the enzyme at a ratio of 1: 23 DEP resulted in 100% loss in activity. Lysine is known to react with DEP in alkaline conditions (Miles, 1977), therefore the loss of enzyme activity subsequent to modifications of lysine was determined using TNBS which is specific for ε-amino groups (Habeeb, 1966). 5 µM protein treated with TNBS at a ratio of 1: 200 showed on titration that of the total 23 residues of lysine in the protein 15.6 residues were modified without loss of activity confirming that a histidine residue is located at or near the active site. On treatment with hydroxylamine the enzyme regained 62% of its original activity confirming that a histidine residue is involved in the enzyme activity. Substrate protection studies showed that the enzyme retains 83% of its original activity in presence of 2 mg polygalacturonic acid (Table 4.5). The CD spectra of the native and DEP-modified enzyme were almost identical, showing that the modification of the histidine residue does not lead to any gross conformational change in the enzyme (Fig 4.12).

The kinetics of inhibition of the enzyme by DEP was done by plotting the log of residual activity against time at 5 min intervals at different concentrations of the inhibitor (Fig. 4.13). DEP mediated inactivation followed pseudo-first-order kinetics at each concentration of the inhibitor. The pseudo-first-order rate constants were calculated from the slope of the plots of log [percent residual activity] against log [DEP] (Levy *et al*.,

1963). The plot gave a slope of 1.05. Therefore inhibition kinetics follow a single exponential, suggesting that one residue of histidine is modified.

Time and concentration dependent titration with DEP gives similar results. 5 µM Aliquots of DEP were added to the enzyme solution. Increase in absorbance was measured at 240 nm and simultaneously decrease in residual activity was calculated. Using a molar absorption coefficient 3,200 $M^{-1}cm^{-1}$ for carbethoxyhistidine at 240 nm, it was determined that one histidine residue is essential for enzyme activity (Fig 4.14).

There was no change in the K_m value of the partially inactivated enzyme, however a significant decrease in the K_{cat} was observed as compared to the native enzyme (Table 4.6). This indicates that the histidine residue is involved in catalysis.

Rexova -Benkova (1970) and Cooke *et al*. (1976) have reported the importance of histidine in the catalysis of endo-polygalacturonase of *A. niger*. Rao *et al*. (1996) have also reported a critical role of histidine residue in the catalytic process of polygalacturonase *of A.ustus* using chemical modification. The authors were pointed out that a single proton was transferred from the histidine residue of the enzyme to the glycosidic oxygen. Caprari *et al*. (1996) showed that a histidine 234 residue was involved in catalytic process of endo-polygalacturonase of *F. moniliforme* by site directed mutagenesis.

Table 4.4

Effect of different chemical modifiers on the activity of endo-polygalacturonase

Chemical Reagent	**Concentration**	**Buffer**	**Residul activity (%)**
NEM	5 mM	Sodium phosphate, 50 mM, pH 7.5	100
NAI	10 mM	Sodium Borate, 20 mM, pH 7.5	100
PHMB	10 mM	Sodium phosphate, 50 mM, pH 6.0	100
PMSF	5 mM	Sodium phosphate, 50 mM, pH 7.5	100
TNBS	5 mM	Sodium phosphate, 50 mM, pH 7.8	97
Succinic anhydride	10 mM	Sodium phosphate, 50 mM, pH 7.8	100
DTMB	1 mM	Sodium phosphate, 50 mM, pH 7.8	100
Woodward's Reagent K	100 mM	Sodium phosphate, 50 mM, pH 6.5	90
EDC	100 mM	MES / HEPES, 50 :15 mM pH 6.0	20
DEP	5 mM	Sodium phosphate ,50 mM, pH 6.0	0
NBS	1 mM	Sodium acetate, 50 mM, pH 4.5	0
HNBB	10 mM	Sodium acetate, 50 mM, pH 4.8	0
Phenyl glyoxal	5 mM	Sodium phosphate, 50 mM, pH 7.8	0
2,3 Butanedione	10 mM	HEPES / Borate, 25: 1 mM, pH 7.8	10
pNPG	5 mM	Sodium phosphate, 50 mM, pH 7.8	0

Purified polygalacturonase (0.131 μM) was incubated with various chemical modifiers and reaction mixture was dialyzed against respective buffer. Residual activity of the enzyme was determined under standard assay conditions.

Table 4.5

Substrate protection of histidine residues

Incubation Mixture	**Residual activity (%)**
Control	100
Enzyme + DEP (20 μM)	10
Enzyme + 2 mg (PGA) + DEP	83

Table 4.6

***K*$_m$ and *k*$_{cat}$ values of partially DEP inhibited polygalacturonase.**

(%) Activity	**Residue Modified**	**Reagent used**	***K*$_m$ (mg / ml)**	***k*$_{cat}$ (min$^{-1)}$)**
100	--	--	0.110	4200
61	Histidine	DEP	0.105	3400
50	,,	,,	0.107	2200
42	,,	,,	0.102	1900

Figure 4.12

The CD spectra of native and DEP treated polygalacturonase

The CD spectra were recorded on a JASCO -710 spectropolarimeter from 190 - 250 nm using 1 cm path length at 25 °C at enzyme concentration of 2.56 μM, in 10 mM phosphate buffer pH 6, native enzyme (——) and DEP treated (------) enzyme.

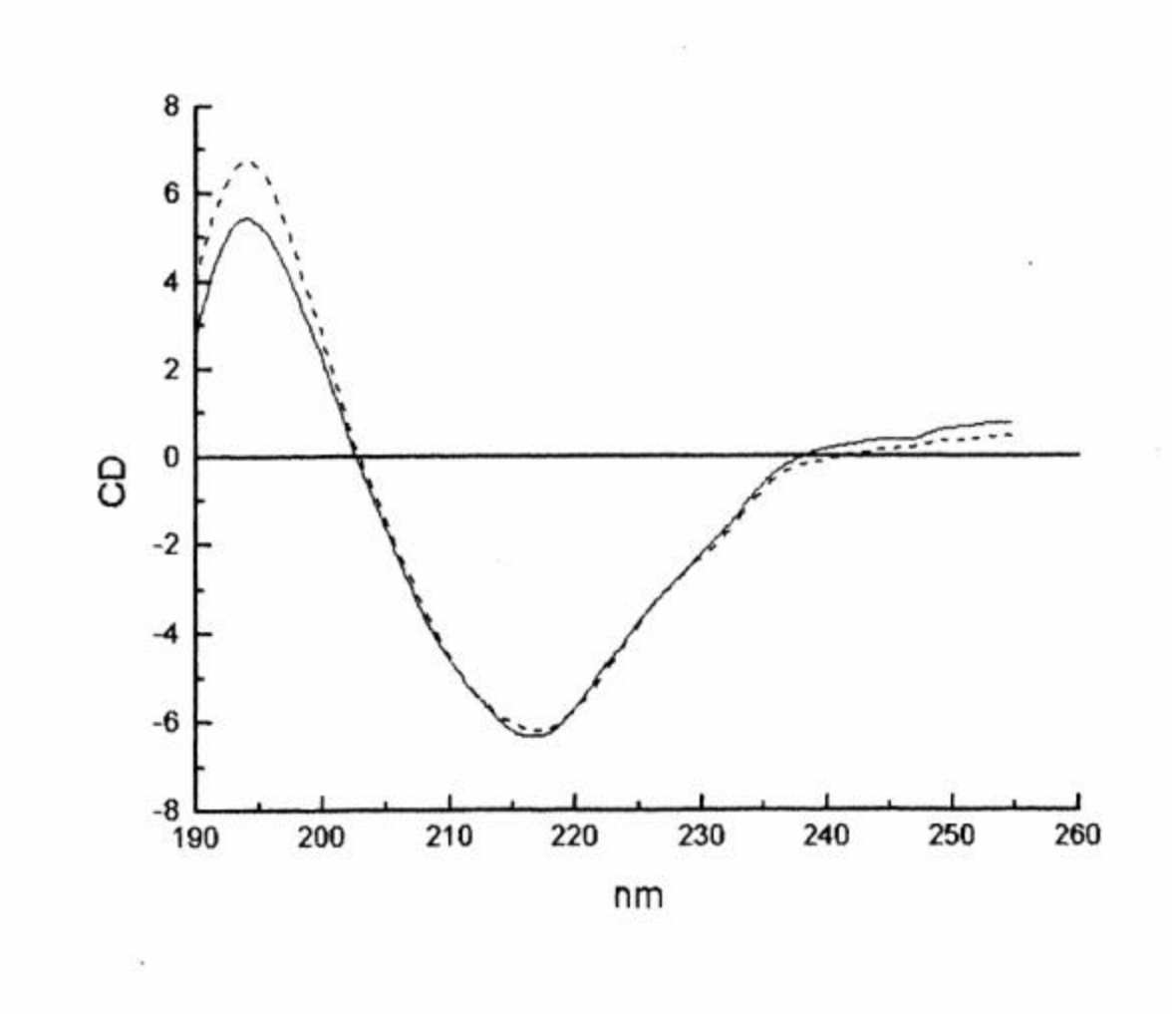


Figure 4.13

Kinetics of inhibition of endo-polygalacturonase by DEP

The pseudo-first order rate constants (K_{app}) at 0 µM (♦), 20 µM (∇), 40 µM (ð), 60 µM (Δ), 80 µM (•) and 100 µM (o) DEP were plotted against the inhibitor concentrations (inset plot).

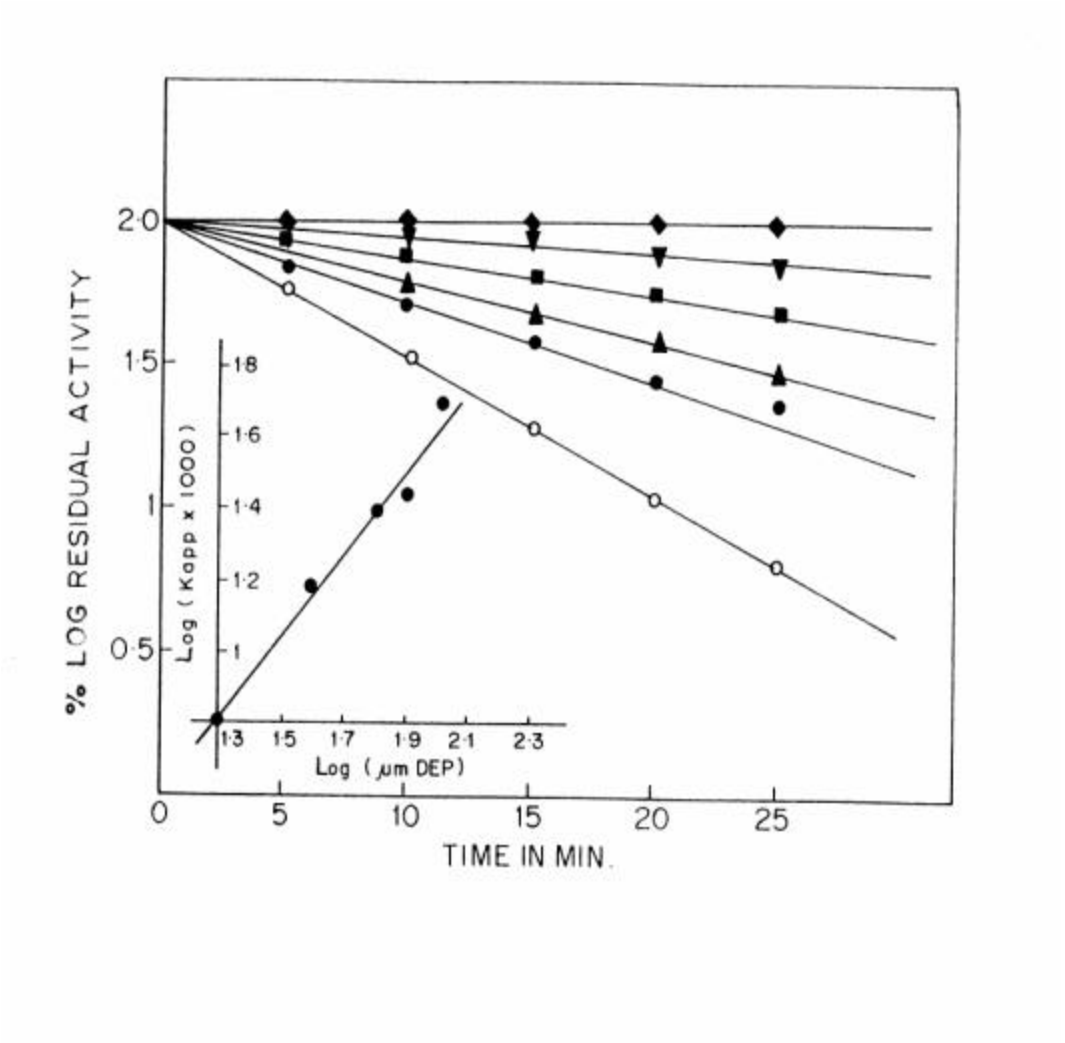
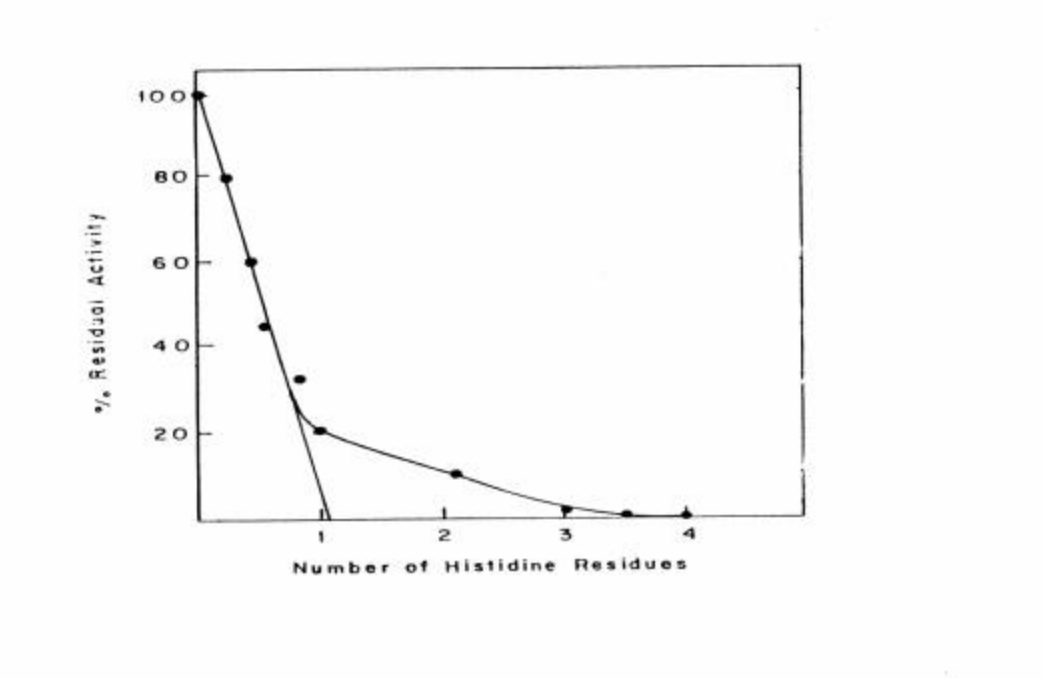


Figure 4.14

Titration of endopolygalacturonase (1.97 mM / ml) with DEP

Modification of histidine residues was carried out with stepwise addition of DEP as described in the methods section.



Modification of carboxylate groups

EDC-promoted amide formation resulted in loss of activity. Incubation with 100 mM EDC caused the enzyme to lose 80 % of its activity. Pho *et al.* (1977) reported that EDC reacted with specific carboxylate residues at an acidic pH of 5.5 to 6.0. EDC also reacts with cysteine tyrosine and lysine residues at alkaline pH. When 10.5 µM of enzyme was incubated with 1 mM of DTMB (Habeeb, 1972) at 30°C for 45 min at pH 7.8 there was no loss of activity indicating that there was no free cysteine in the enzyme. Furthermore neither NAI nor TNBS (Riordan and Vallee 1972 and Habeeb, 1966) showed any effect on polygalacturonase activity. This suggests that EDC modified carboxylate residues rather than cysteine, tyrosine or lysine residues.

Substrate protection showed that 56% original activity was retained in presence of 3 mg of polygalacturonic acid (Table 4.7). CD spectra in EDC modified enzyme were identical to the native enzyme (Fig 4.15) indicating that loss in enzyme activity was due to specific modification of carboxylate residues and not due to gross conformational changes in the enzyme molecule.

The kinetics of inhibition of the enzyme by EDC was done by plotting the log residual activity against time at 10 min intervals at different concentrations of the inhibitor. The log of residual activity plotted as the function of time at various EDC concentrations was linear to 10 % of the initial activity. EDC mediated inactivation followed pseudo-first-order kinetics at any fixed concentration of the reagent. The pseudo-first-order rate constants were calculated from the slope of [log percent residual activity] versus reaction time and the order was determined from the plots of log [K_{app}] against log [EDC]. The slope of the line was 0.92 indicating that the loss of activity occurred as a result of modification of a single carboxylate residue (Fig 4.16).

Similar results were obtained when EDC mediated inactivation was carried out in the presence of glycinemethylester. Modification of the enzyme with EDC in presence of NTEE resulted in the incorporation of 1.2 nitrotyrosyl residues per molecule of the enzyme suggesting that the inactivation of the enzyme was due to the modification of a single carboxylate residue (Fig 4.17).

Kinetic constants of partially inhibited enzyme were calculated. There was no change in the K_m value of the partially inactivated enzyme, however a significant decrease in the k_{cat} was observed as compared to the native enzyme (Table 4.8). This indicated that the involvement of the carboxylate residues in catalysis.

Participation of the carboxyl group in the active site of polygalacturonase *of A. niger* has been reported by Rexova-Benkova and Mrachova (1978) on the basis of kinetic data. Chemical modification of carboxyl group with EDC was used to show the involvement of carboxyl in the activity of polygalacturonase of *A. niger* (Rexova-Benkova, 1990). Wang *et al*. (1994) have suggested a model for the activity of the glycosyl-hydrolases where two carboxylate residue hold the activated water molecule that acts as proton donor. A number of reports are available on the involvement of the carboxylate residue in the active site of a variety of glycosyl hydrolases including lysozyme (Chipman and Sharon, 1969), cellulase (Morosoli *et al*. 1986), xylanases (Bray and Clarke, 1990) and α galactosidases (Deepal and Balasubramanium, 1986).

Table 4.7

Substrate protection of carboxylate residue

Incubation mixture	**Residual activity (%)**
Control	100
Enzyme + EDC (40 mM)	29
Enzyme + 3 mg (PGA) + EDC	56

Table 4.8

K_m and k_{cat} values of partially carboxylate inhibited polygalacturonase

% Activity	Residue modified	Reagent used	K_m (mg / ml)	k_{cat} (min^{-1})
100	--	--	0.110	4200
70	Carboxylate	EDC	0.105	3496
55	,,	,,	0.108	3205
45	,,	,,	0.110	2747

Figure 4.15

The CD spectra of native and EDC treated polygalacturonase

The CD spectra were recorded on a JASCO -710 spectropolarimeter from 190 - 250 nm using 1 cm path length at 25 °C at enzyme concentration 2.56 μM in 10 mM phosphate buffer pH 6, native enzyme (——) and EDC treated (------).

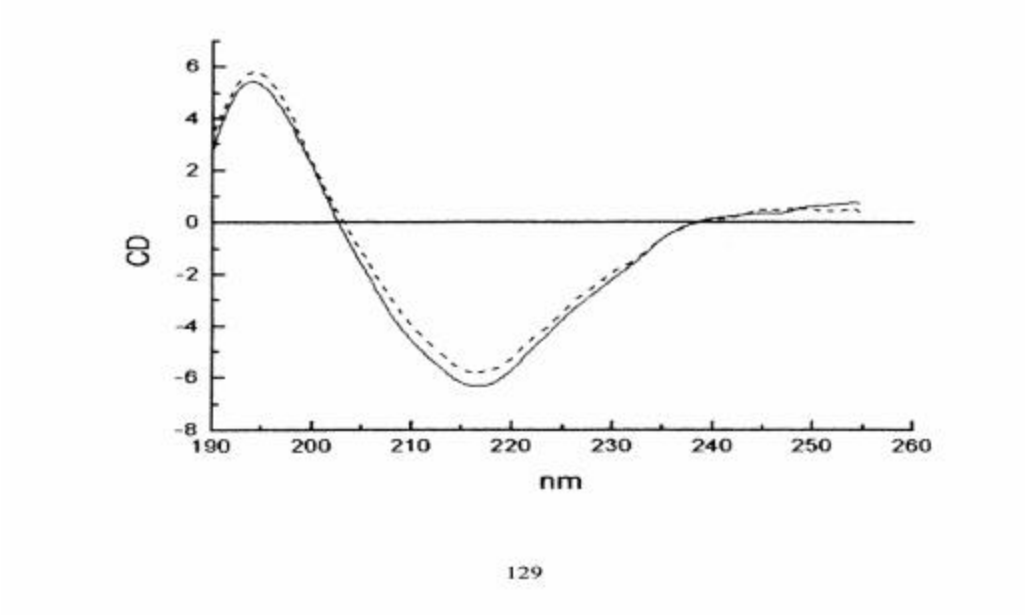


Figure 4.16

Kinetics of inhibition of endo-polygalacturonase by EDC

The pseudo- first order rate constants K_{app} at 0 mM (•), 10 mM (♦), 20 mM (Δ), 30 mM ( ), 40 mM (o), and 50 mM (▽) EDC were obtained against the inhibitor concentrations (inset plot).

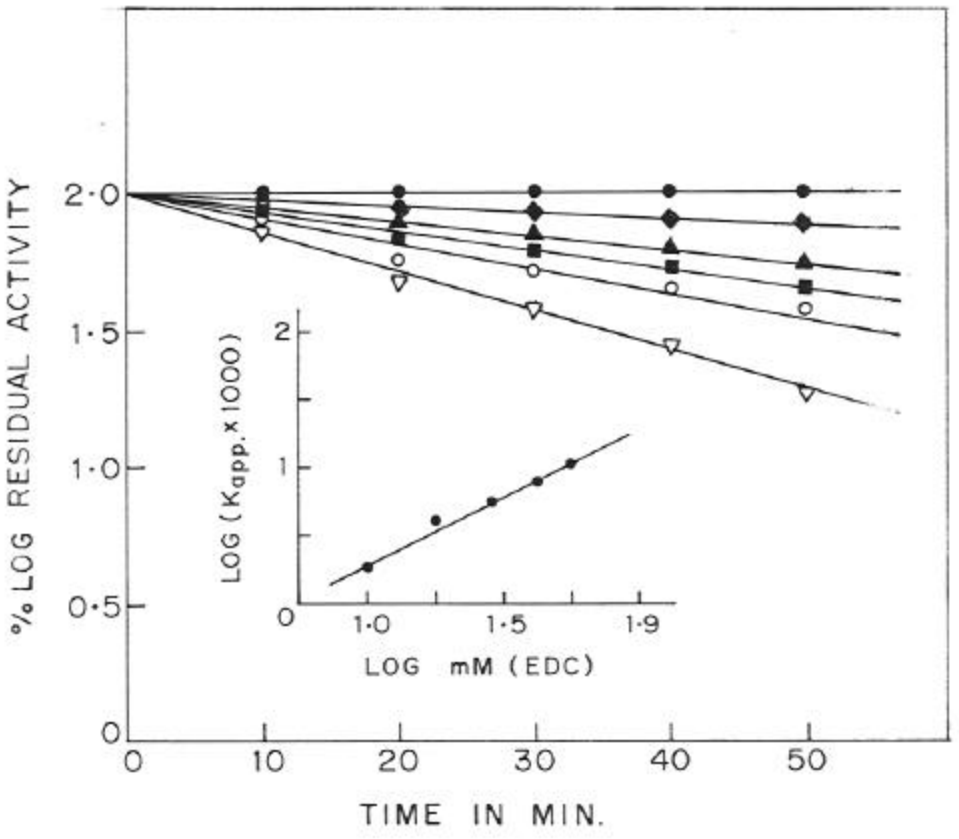
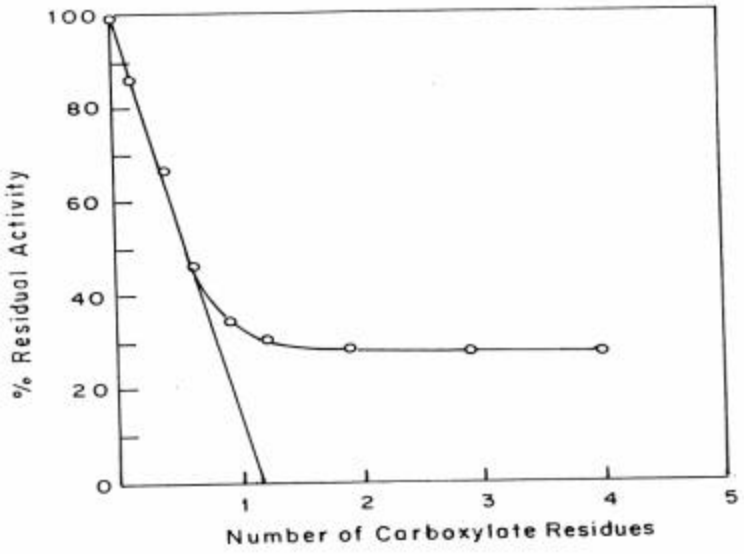


Figure 4.17

Titration of endo-polygalacturonase with EDC/ NTEE

Quantification of carboxylate residues modified was carried out by the addition of reagent as described in the methods section.



Modification of arginine residues

Takahashi (1968) reported that phenyl glyoxal reacts with guanido group of arginine at pH 7 to 8 and at 25ºC. Riordan (1979) has showed that 2,3 butanedione also reacts with the guanido group of arginine in the presence of borate ions. When 0.131 µM polygalacturonase was incubated with 5 mM phenyl glyoxal and 10 mM 2,3 butanedione the enzyme lost its 100% and 90 % of its original activity respectively. No loss of activity was observed in the control sample.

Phenyl glyoxal and butanedione mediated inactivation could be prevented to a significant extent, by incubating the enzyme with excess 3 mg of polygalacturonic acid (Table 4.9). When the phenyl glyoxal modified enzyme was dialysed against 10 mM Tris/HCl buffer pH 7.8, the enzyme gained its 58 % of original activity indicating that, the inactivation of enzyme occurred due to modification of arginine residues. Moreover the CD spectra of both native and phenyl glyoxal modified enzyme were similar (Fig. 4.18) indicating that the modification does not lead to gross change in the conformation of the enzyme.

Kinetics of inhibition by phenyl gloyxal and butanedione followed pseudo-first-order kinetics at any fixed concentration of the reagents. The pseudo-first-order constants were calculated from the slope of the plots of log percent residual activity against reaction time. The order of the reaction was determined from the plot of log [K_{app}] against log [phenyl glyoxal] and log [butanedione] concentrations (Levy *et al*., 1963). The plots gave slopes 1.2 and 0.65 respectively (Fig 4.19 & 4.20). These plots indicated that the loss of activity of polygalacturonase was due to the modification of a single arginine residue.

Colorimetric titration of enzyme with pNPG results in 95 % loss in activity. The inactivation of the enzyme activity depends on concentration of the reagent. The plot of residual activity against the number of arginine residues modified (Fig 4.21) revealed that the loss of activity resulted from the modification of a single arginine residue.

There was no change in the K_m of the partially phenyl glyoxal and butanedione inactivated enzyme, however there was a significant decrease in k_{cat} values as compared to the native enzyme (Table 4.10) which suggested that the involvement of arginine residue in the catalytic process.

This is the first report of the involvement of an arginine residue in catalysis by polygalacturonase reported so far. Pickersgill *et al*. (1998) reported that 36 polygalacturonases from different sources showed that arginine residue is one of the conserved residues in the active site cleft. van Santen *et al*. (1999) also showed that an arginine residue was present in the cleft of the active site although in neither case arginine was shown to be actively involved in the catalytic process.

Table 4.9

Substrate protection of arginine residue

Incubation Mixture	**Residual activity (%)**
Control	100
Enzyme + Phenyl glyoxal (25µM)	0
Enzyme+ 3 mg (PGA) + Phenyl glyoxal	65
Enzyme + 2,3 Butanedione (5mM)	26
Enzyme + 2 mg (PGA) + Butanedione	52

Table 4.10

K_m and k_{cat} values of partially arginine inhibited polygalacturonase

(%) Activity	**Residue Modified**	**Reagent Used**	**K_m (mg / ml)**	**k_{cat} (min^{-1})**
100	--	--	0.110	4200
45	Arginine	Phenyl glyoxal	0.109	2900
30	,,	"	0.110	2200
20	,,	"	0.100	1800
85	,,	2,3 Butanedione	0.106	4100
65	,,	"	0.105	3300
50	,,	"	0.108	2600

Figure 4.18

The CD spectra of native and phenyl glyoxal treated polygalacturonase

The CD spectra were recorded on a JASCO -710 spectropolarimeter from 190 - 250 nm using 1 cm path length at 25 °C at an enzyme concentration of 2.56 µM, in 10 mM phosphate buffer pH 6, native enzyme (——) and phenyl glyoxal treated (----) enzyme.

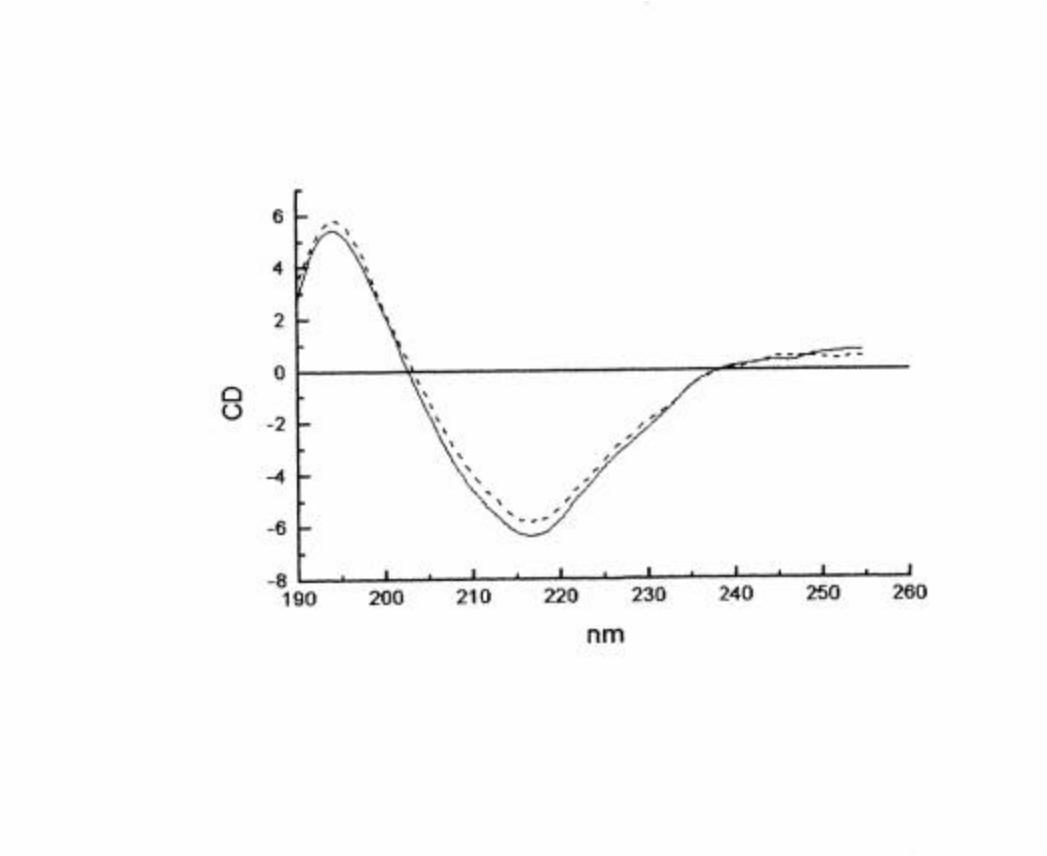


Figure 4.19

Kinetics of inhibition of endo-polygalacturonase by phenyl glyoxal

The pseudo-first order rate constants (K_{app}) at 0 μM (•), 20 μM (o), 30 μM (Δ), 40 μM ( ) and 50 μM (O) phenyl glyoxal were plotted against the inhibitor concentrations (inset plot).

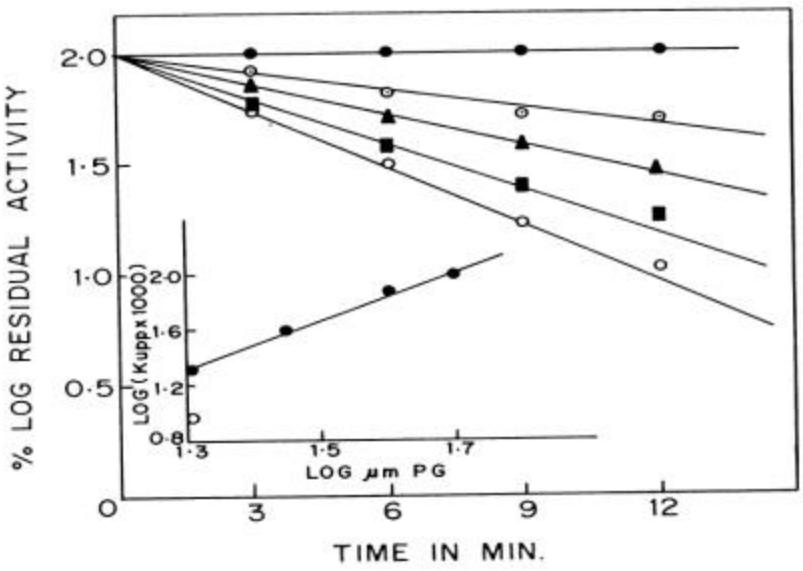


Figure 4.20

Kinetics of inhibition of endo-polygalacturonase by 2,3 -butanedione

The psudo-first order rate constants (K_{app}) at 0 mM (♦), 1mM (•), 3 mM (Δ), 6mM ( ) and 9 mM (o) 2,3 butanedione were plotted against the inhibitor concentrations (inset plot).

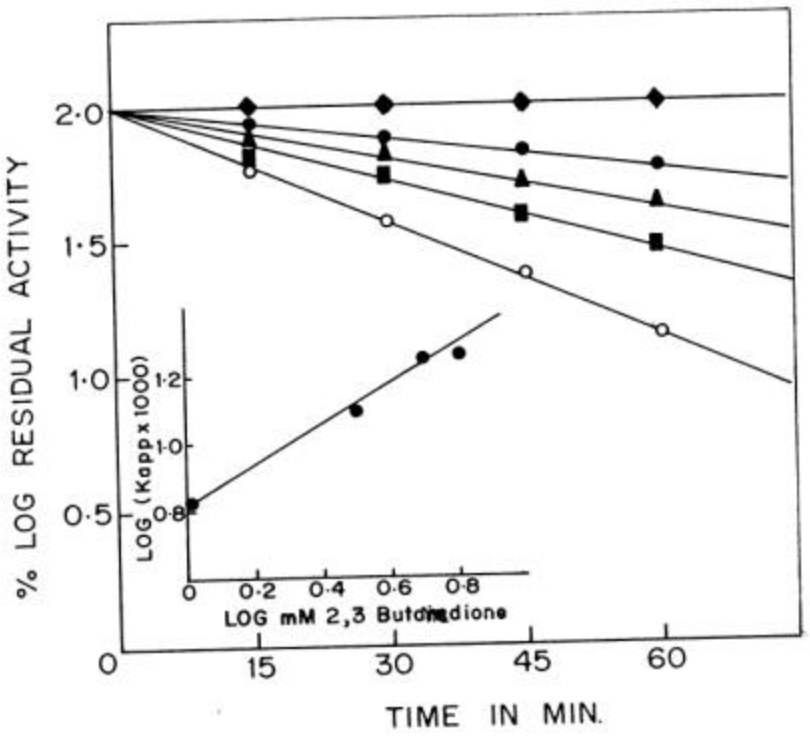
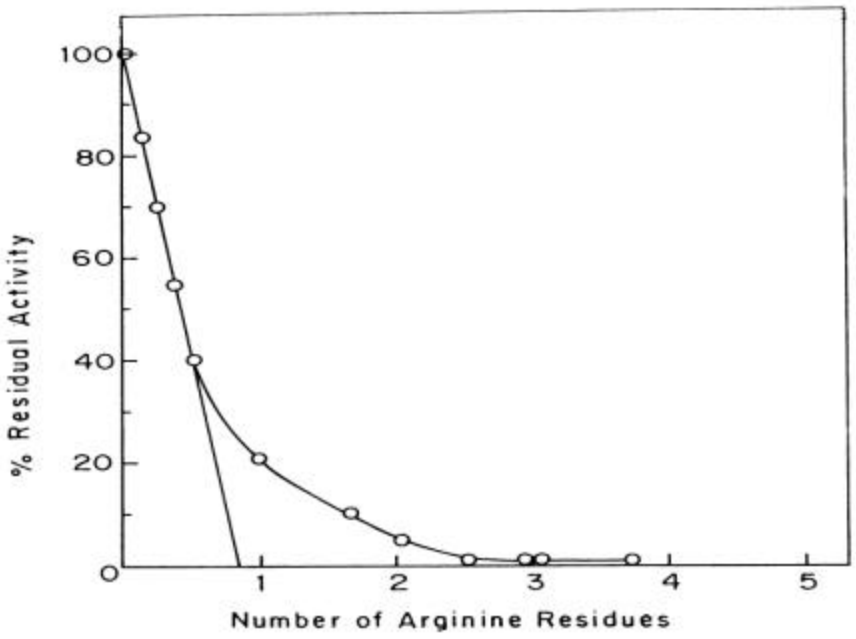


Figure 4.21

Titration of endopolygalacturonase (5.25 mM / ml) with pNPG

Quantification of arginine residues was carried out with addition of pNPG as described in methods section.



Modification of tryptophan residues

When the purified polygalacturonase was incubated with 10 mM HNBB in sodium acetate buffer pH 4.5 it lost 90 of its original activity (Horton and Koshland, 1965). HNBB also reacts with cysteine at alkaline pH. However when 10.5 μM of polygalacturonase was titrated with 1 mM DTNB (Habeeb, 1966) enzyme activity was not affected showing that HNBB reacts with tryptophan residues rather than cysteine under the conditions used.

Spande and Witkop (1967) have showed that NBS oxidizes tryptophan residues at acidic conditions however, NBS also reacts with tyrosine residues (Riordan and Vallee 1972). The protein contains 14 tyrosine residues. NAI modified 8.6 of these residues without loss of activity suggesting that NBS modified tryptophan residues rather than tyrosine in this case.

HNBB and NBS mediated inactivation could be prevented to a significant extent by incubating the enzyme with 2 mg of polygalacturonic acid (Table 4.11). Moreover the CD spectra of both native and NBS modified enzyme were similar (Fig 4.22) indicating that the modification does not lead to any gross change in the enzyme conformation.

Kinetic analysis of the inactivation of the enzyme by HNBB and NBS was done by plotting the log residual activity against time at different concentrations of the inhibitors. The pseudo-first-order rate constants were calculated from the slope of the plot of log percent residual activity versus reaction time, and order of the reaction was determined from the plot of [log (k_{app})] against log [HNBB] & log [NBS]. The plots gave the slopes 1.32 and 1.39 respectively, indicating that the inhibition kinetics followed a single exponential of tryptophan residue (Fig 4.23 & 4.24).

Similar results were obtained when polygalacturonase was titrated with NBS leading to an inhibition which was time and concentration dependant. The plot of percent residual activity against number of tryptophan residues modified (Fig 4.25) showed that the loss of activity resulted from the modification of a single tryptophan residue.

The kinetic constants of partially inhibited polygalacturonase by HNBB and NBS indicated that there was a significant change in the K_m values. The K_m values of partially inhibited enzyme increased with decreasing activity of the enzyme. This indicated that modified enzyme had a lower affinity towards substrate as compared to the native enzyme. The k_{cat} of partially tryptophan modified enzyme remained the same as compare to the

native enzyme, indicating that the tryptophan residues were involved in the binding of the substrate and not in the catalysis (Table 4.12).

Additional evidence for the role of tryptophan residue involved in binding was provided by spectrofluorimetric data (Table 4.13). This showed that when native polygalacturonase was titrated with substrate and GADL, the fluorescence quenching was 10.5% and 27.49 % respectively. Further when the one tryptophan was modified chemically the fluorescence quenching is only 1% and 1.8 % with PGA and GADL respectively, suggesting that a single tryptophan was present at or near to the active and involved in binding.

Waksman *et al*. (1992) studied the reaction kinetics of polygalacturonase from *C. lindemuthianum* by fluorescence quenching in the presence of substrate. The authors have reported that the involvement of tryptophan is in substrate binding. Devi and AppuRao (1998) and Liu *et al*. (1994) have also shown that a tryptophan residue was responsible for the activity of the enzyme. Tryptophan has been reported to be present in the active site of various saccharidases like endo-1,4-β-glucanase (Ozaki and Ito, 1991), isomalto-dextranase (Okada *et al*., 1988) and xylanase (Kesker *et al*., 1989). Tryptophan thus appears to have affinity towards carbohydrates, and could be involved in substrate binding in carbohydrate-degrading enzymes including polygalacturonase.

Table 4.11

Substrate protection of tryptophan residue

Incubation Mixture	**Residual Activity (%)**
Control	100
Enzyme + HNBB (5 mM)	15
Enzyme + 2 mg (PGA) + HNBB	85
Enzyme + NBS (20 μM)	10
Enzyme + 2 mg (PGA) + NBS	68

Table 4.12

K_m and k_{cat} values of partially tryptophan inhibited polygalacturonase

% activity	**Residue modified**	**Reagent used**	**K_m (mg/ml)**	**k_{cat} (min^{-1})**
100	--	--	0.110	4200
70	Tryptophan	HNBB	0.116	4180
45	,,	,,	0.224	4200
25	,,	,,	0.344	4200
75	,,	NBS	0.169	4200
50	,,	,,	0.200	4110
30	,,	,,	0.333	4200

Table 4.13

Flurescence quenching of native and single tryptophan modified polygalacturonase.

Purified polygalacturonase (5.26 μM, 2 ml) in10 mM sodium acetate buffer pH 4.5 was excited at 295 nm and fluorescence emission maxima was recorded at 336 nm.

Item	**Chemical modifier**	**Activity (%)**	λ_{max}	**Quencher**	**Q (%)**
Native enzyme	--	100	336	PGA	10.5
1 tryptophan modified	NBS	10	332.5	PGA	1.0
Native enzyme	--	100	336	GADL	27.49
1Tryptophan modified	NBS	10	332	GADL	1.8

Figure 4.22

The CD spectra of native and NBS treated polygalacturonase

The CD spectra were recorded on a JASCO-710 spectropolarimeter from 190 - 250 nm using 1 cm path length at 25 °C at an enzyme concentration of 2.56 µM, in 10 mM phosphate buffer pH 6, native enzyme (——) and NBS treated (------) enzyme.

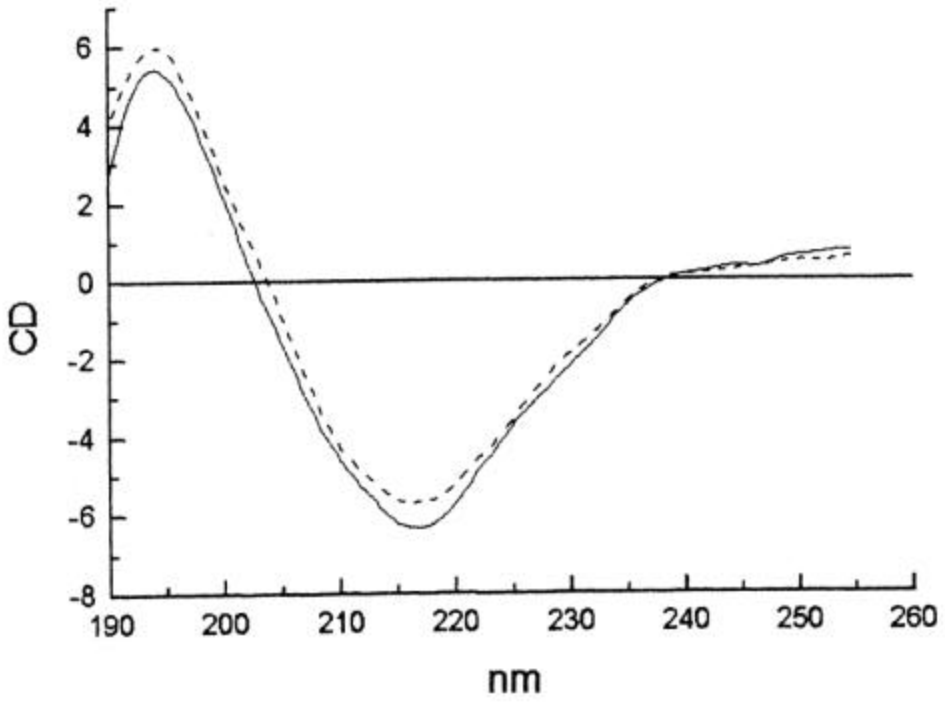


Figure 4.23

Kinetics of inhibition of polygalacturonase by HNBB

The pseudo - first order rate constants (K_{app}) at 0 mM (•), 3 mM (∇), 6 mM (Δ), 9 mM ( ), 12 mM (×) and 15 mM (o) HNBB were plotted against the inhibitor concentrations (inset plot).

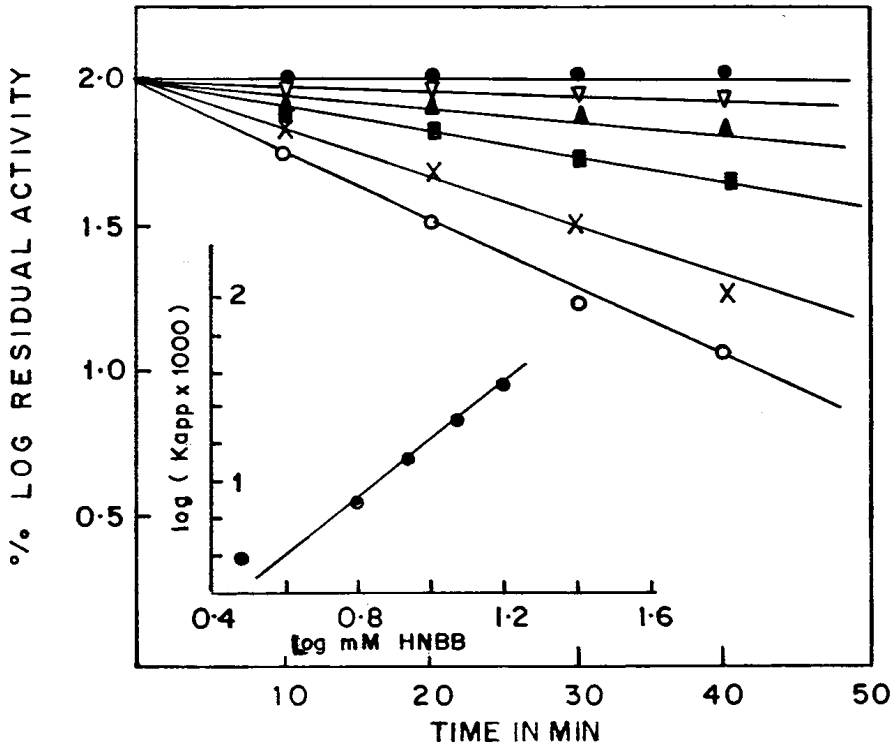


Figure 4.24

Kinetics of inhibition of endo-polygalacturonase by NBS

The pseudo -first order rate constants (K_{app}) at 0 μM (•), 2 μM (∇), 4 μM (Δ), 6 μM ( ), 8 μM (×), 10 μM (o) NBS were plotted against the inhibitor concentrations (inset plot).

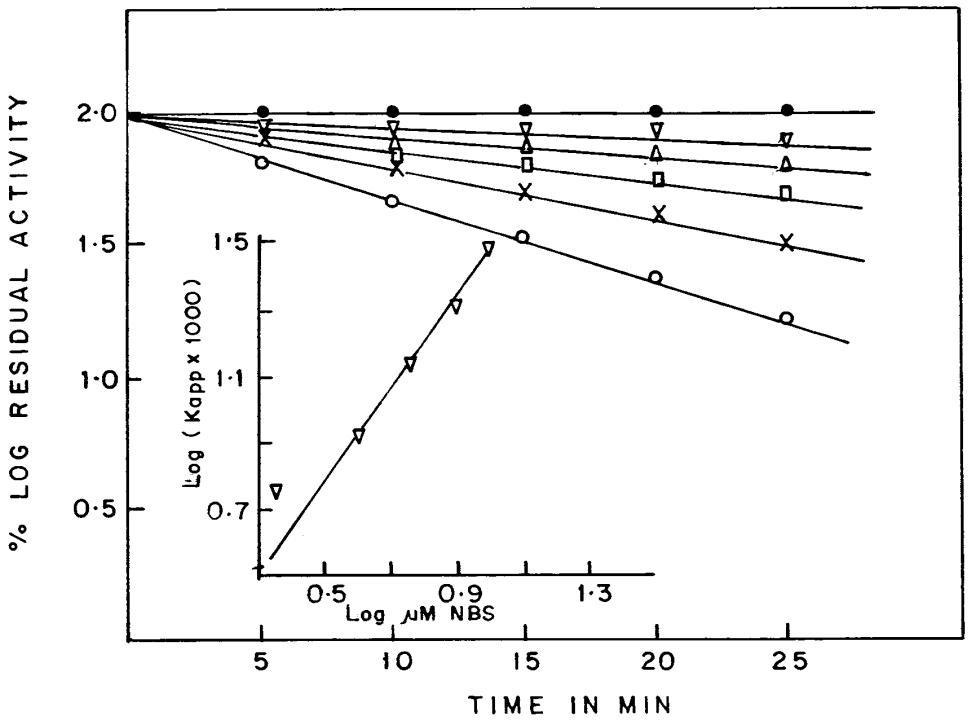
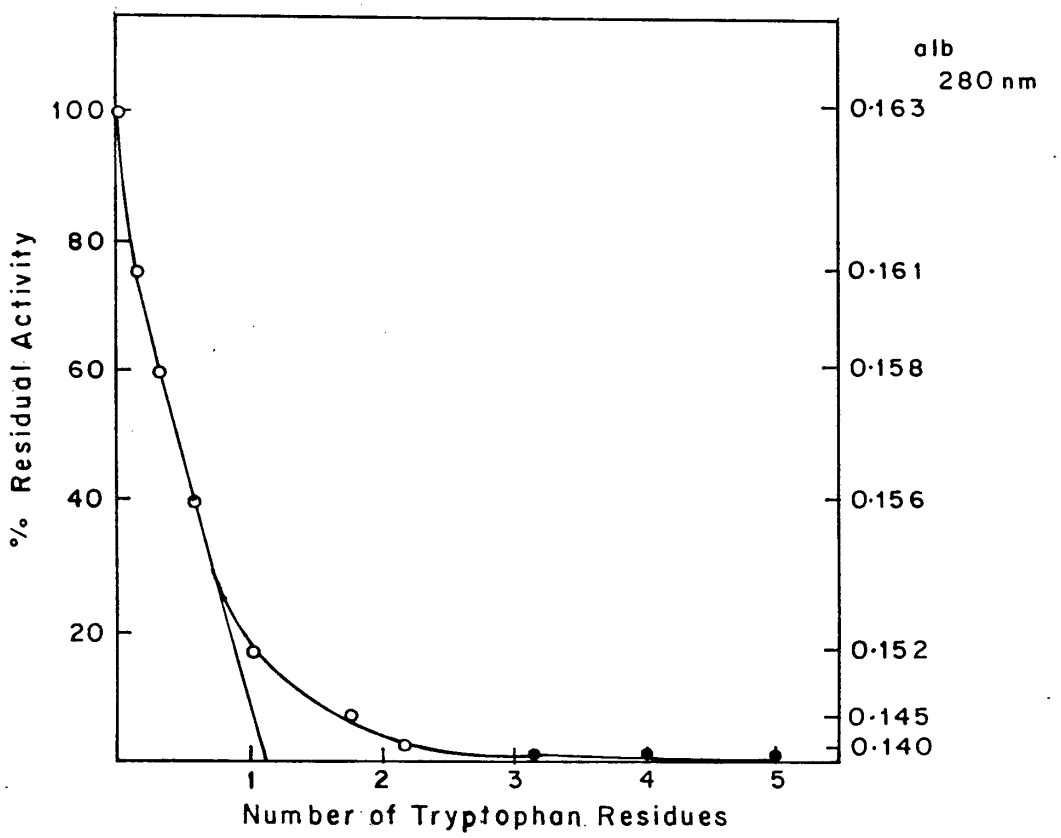


Figure 4.25

Titration of endopolygalacturonase (1.97 mM /ml) with NBS

Modification of tryptophan residue was carried out with stepwise addition of NBS as described in methods section.



Microenvironment of active site tryptophan residue

The Stern-Volmer plots from the fluorescence of the native enzyme with acrylamide, I^- and Cs^+ indicated collisional quenching (Fig 4.26 a, b, c). The F_0/F versus [Q] plots with I^- and Cs^+ both show a downward curve suggesting that the three tryptophans in this protein exist in different environments. Fitting the data presented in the modified Stern-Volmer plots (Fig 4.26 d, e, f) to the equation described in the Material and Methods section showed that the flourophore is 50% accessible to I and 14% to Cs^+ (Table 4.14). Moreover, the f_a (0.5) for iodide is lower and the K_a (5.7) is higher than with the acrylamide-quenched enzyme (f_a = 1.16 and K_a = 3.26) leading to the conclusion that at least one tryptophan is localized on the surface of the enzyme in an electropositive enviroment. Modification of one tryptophan residue with NBS (Fig 4.27) results in a drop in fluorescence and the λ_{max} of the modified protein is blue-shifted from 337 nm to 332 nm showing that the remanant fluorescence is from hydrophobic tryptophans. The difference spectrum shows a red shift from 337 nm to 347 nm suggesting that the residue modified by NBS is on the surface of the protein (Fig 4.27). Titrating the single-residue modified protein with either PGA or GADL (Table 4.13) decreased the percent quench in tryptophan fluorescence from 10.5 to 1 % with PGA and from 27.5 to 1.8 % with GADL as compared to the native enzyme, which further suggests that the surface tryprophan residue, which is in an electropositive environment, is involved in binding. Thus substrate binding by tryptophan is supported by both the kinetic and fluorescence data.

Table 4.14

Stern-Volmer constants of the native polygalacturonase in presence of substrate (PGA) and GADL

5. 26 μM (2 ml) of polygalacturonase was excited at 295 nm and emission was recorded at 336 nm (λmax). The K_a and f_a values were calculated.

Quencher	**Q (%)**	f_a	K_a	λ_{max}
Acrylamide				
Native	79	1.16	3.26	336.21
Enzyme-Substrate	65	0.93	2.89	336.41
Enzyme-GADL	74	0.97	3.8	336.23
KI				
Native	29.7	0.5	5.66	335.72
Enzyme -Substrate	3.4	0.05	16.8	335.52
Enzyme-GADL	30	0.46	14.3	335.21
CsCl				
Native	19.7	0.14	24.2	335.6
Enzyme -Substrate	2.5	--	--	335.17
Enzyme-GADL	19.4	0.32	5.0	335.72

Figure 4.26

Stern-Volmer and modified Stern-Volmer plots of fluorescence quenching of native polygalacturonase by acrylamide (8 M), KI (5 M), and CsCl (5 M)

The native protein 5.26 µM (2 ml) in 50 mM sodium acetate buffer pH 5 was excited at 295 nm and emission was recorded at 336 nm

A) Stern -Volmer plots

a) Acrylamide b) KI c) CsCl

B) Modified Stern - Volmer Plots

d) Acrylamide e) KI f) CsCl

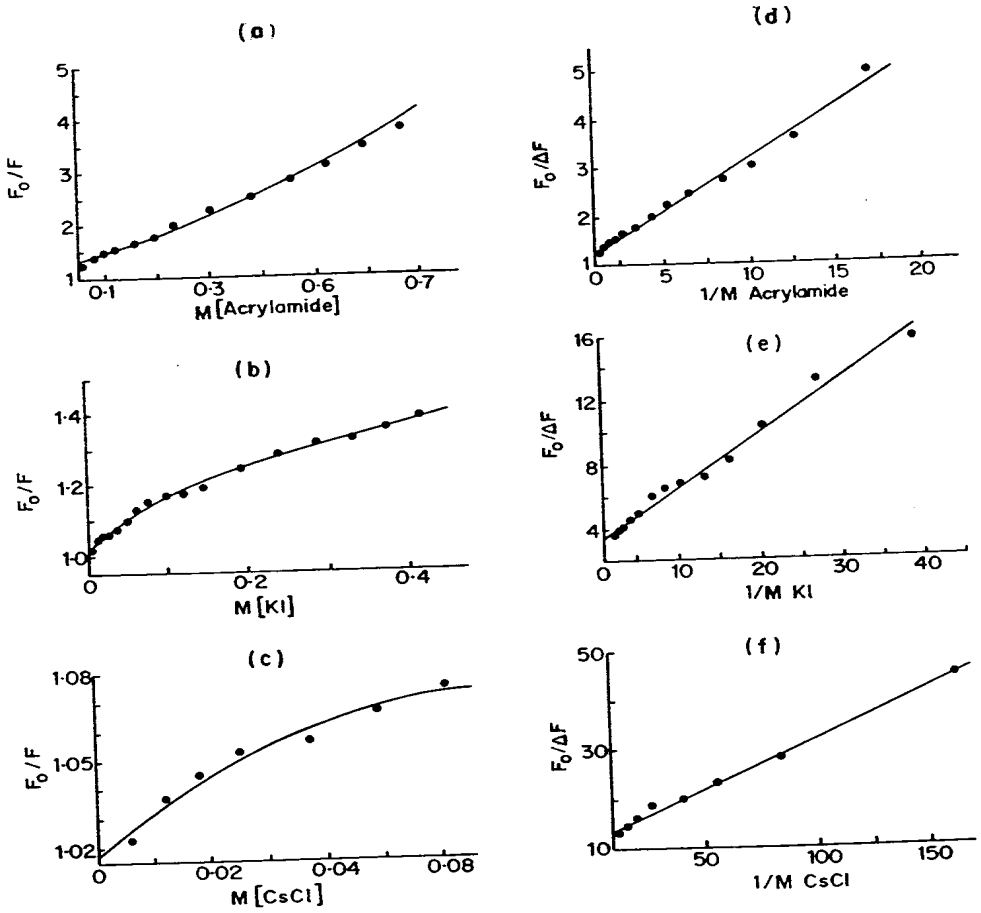
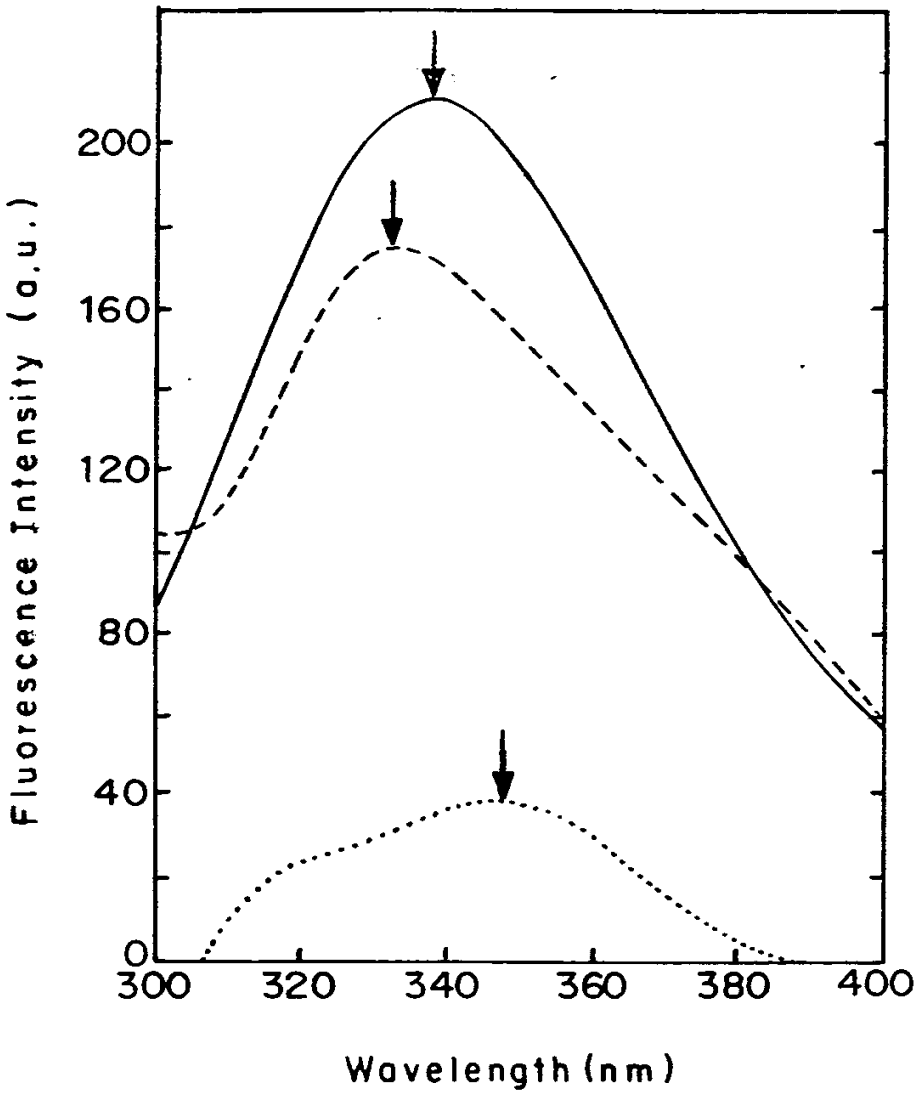


Figure 4.27

Difference fluorescence spectra with a single tryptophan residue modified with NBS.

Polygalacturonase (2.56 μM) was excited at 295 nm and λ_{max} was recorded at 336 nm, native spectra (——), NBS modified spectra (------) and difference spectra (·····).



In conclusion this organism produced a single extracellular endo-polygalacturonase in submerged culture which had an M_r of 38 kDa and a carbohydrate content of 4 %. It had an alkaline pI of 8.1. The K_m was 0.12 mg ml^{-1}, V_{max} was 111.1 µM min^{-1} mg^{-1} and the k_{cat} was 4200 min^{-1}. It had a pH optimum of 4.8. Kinetic and fluorescence data showed that a tryptophan residue was present in the active site in an electropositive microenvironment and was involved in binding. An arginine residue at or near the active site may be involved in catalysis or extended binding of the substrate. A carboxylate and a histidine residue were involved in catalysis.

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-branched 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 polygalacturonase activity as compared with submerged culture. Titration with antibodies showed that two forms of polygalacturonase 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 polygalacturonase gene. It is not known whether these two forms of polygalacturonase are products of a single gene or two different genes. Caprari *et al*. (1993) showed that four apparent forms of polygalacturonase 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 galacturonide] 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 pK_a 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-Benkova (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 data base. 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.

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1) Niture, S. K., Pant, A. & Ameeta R. Kumar (2001) Active site characterization of the single endo-polygalacturonase produced by *Fusarium moniliforme* NCIM 1276, *European Journal Biochemistry*. **268,** 832-840.

2) Niture, S. K. & Pant A. The role of pH in polygalacturonase and pectate lyase production by *Fusarium moniliforme* NCIM 1276 (manuscript under preparation).

3) **Niture S. K** & Pant A. (2001) Extracellular production of pectinases from *Fusarium moniliforme* NCIM 1276, **27th** meeting of mycological society of India & International symposium on frontiers of fungal diversity and diseases in south east Asia *Gorakhpur* (U.P.) India.