MOLECULAR & BIOCHEMICAL ASPECTS OF *Chainia* sp. (NCL 82.5.1)

A THESIS
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Dedicated to My Father.....

Late Shri Ratnakar Shamrao Mahajan

(October 10, 1917 → Dec 19, 1989)

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DECLARATION

This is to certify that the work incorporated in the thesis " Molecular and Biochemical Aspects of Chainia sp. (NCL 82.5.1)" submitted by Mrs. Kavita Rajiv Bandivadekar was carried out under my supervision . Such material as has been obtained from other sources has been duly acknowledged in the thesis.

V V Deshpande

Dr. (Mrs.) V V Deshpande

Research Guide

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Kavita Bandivadekar

ABBREVIATIONS

BSA	:	Bovine serum albumin
EDTA	:	5,5'-Dithiobis-(2-nitrobenzoic acid)
Gdn.HCl	:	Guanidine hydrochloride
IPTG	:	Isopropyl β-D-thiogalactopyranoside
NBS	:	N-bromosuccinimide
PAGE	:	Polyacrylamide gel electrophoresis
PEG	:	Polyethylene glycol
PFU	:	Plaque forming units
SDS	:	Sodium dodecyl sulphate
WRK	:	Woodward's Reagent K
X-gal	:	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

The current increased demand for better utilisation of replenishable sources and the pressure on the industries to operate within environmentally compatible limits has been a stimulus to the development of new concepts in Biotechnology. Agriculture and forestry waste contains polysaccharides, which could be converted to valuable fermentable sugars for the production of variety of chemicals like ethanol. Xylans or hemicelluloses are the heterologous polysaccharides and one of the important renewable resources found in the cell walls of plants. Xylan has a high potential for its degradation to useful end products. Xylanases produced by various organisms are useful in xylan degradation. *Chainia* (NCL 82.5.1) a sclerotial actinomycete species isolated in our laboratory produces many commercially important enzymes such as cellulase-free xylanase and substrate specific intra and extra cellular glucose/xylose isomerases. The ready accessibility of the enzyme to cellulose pulp due to its small size and the absence of cellulase are its advantageous features. In view of its industrial importance, it was planned to study the molecular and biochemical aspects of *Chainia* by studying structure-function relationship of the xylanase and molecular cloning of the gene responsible for its activity.

The present work describes –

- The structure-function relationship of xylanase II from *Chainia*.
  - Fluorometric analysis of tryptophan microenvironment.
  - Study of structural environment of essential cysteine residue.
  - Evidence for essential carboxylate residues.

Heterologous cloning and expression of xylanase II gene from *Chainia*

Enhancement of the pH and thermostability of the xylanase from *Chainia*.

SUMMARY OF WORK

Structure-function relationship of xylanase II from *Chainia*.

Study of microenvironment of tryptophan (trp) residue.

Trp is a useful built-in fluorescent probe. Its fluorescence is very sensitive to its microenvironment. Involvement of one out of three trp residues in the active site of the xylanase II from *Chainia* has been demonstrated previously, [Deshpande, Hinge and Rao (1990) Biochem. Biophys. Acta 1041, 172 - 177]. The work described here aims at : (i) deducing the structure-function relationship for the trp residue involved at the active site (a) by correlating the effect of N - bromosuccinimide (NBS) on the fluorescence and activity, and (b) by assessing the ability of xylan to protect against decrease in fluorescence versus activity of NBS-treated enzyme; and (ii) probing into the environment of the trp residues by studying the quenching of their fluorescence by various solute quenchers in presence and absence of guanidine hydrochloride (Gdn.HCl). Complete inactivation of NBS-treated enzyme occurs well before the loss of fluorescence. Full protection by xylan (0.5%), of the inactivation of enzyme by NBS compared with 30% protection for the decrease in fluorescence, confirms the participation of a single trp at substrate binding site of the xylanase. The xylanase exhibited a rather low fluorescence emission maximum at 310 nm. There was no shift in the emission maximum on treatment of the enzyme with Gdn.HCl (Upto 6.5 M), indicating the rigidity of the microenvironment around trp residues. The quenching studies with acrylamide suggested the occurrence of both collisional as well as static quenching processes. The enzyme retained full activity as well as the

characteristic emission maximum at 310 nm in the presence of acrylamide (100 mM), indicating that the quenching of fluorescence by acrylamide is a physical process. Acrylamide was more efficient as a quencher than CsCl and KBr. Treatment of the enzyme with Gdn.HCl resulted in an increase in the Stern-Volmer quenching constants (K_{sv}) of the solute quenchers. The analysis of K_{sv} and V values of KBr and CsCl suggests that the overall trp microenvironment in the xylanase II from *Chainia* is slightly electronegative.

Study of structural environment of an essential cysteine residue

N-(2,4-Dinitroanilino) maleimide reacts covalently with the thiol group of the xylanase II from *Chainia* leading to complete inactivation in a manner similar to N-ethylmaleimide, but provides a reporter group at the active site of the enzyme. Increasing amounts of xylan offered enhanced protection against inactivation of the xylanase by DAM. Xylan (5 mg) showed complete protection, providing evidence for the presence of cysteine at the substrate-binding site of the enzyme. Kinetics of the chemical modification of the xylanase by DAM indicated the involvement of 1 mol of cysteine residue per mol of enzyme, as reported earlier. The second order rate constant for the reaction of DAM with the enzyme was 3.61 X 10^3 $M^{-1}min^{-1}$. The purified xylanase was alkylated with DAM and digested with pepsin. The peptides were separated by gel filtration. The specific modified cysteinyl peptide was further purified by reverse phase HPLC. The active site peptide was located visually by its predominant yellow colour and characterised by a higher A_{340} / A_{210} ratio. The modified active site peptide has the sequence: Glu – Thr – Phe – Xaa – Asp. The sequence of the peptide was distinctly different from that of cysteinyl peptide derived from a xylanase from a thermotolerant *Streptomyces* species, but showed the presence of a conserved aspartic acid residue consistent with the catalytic regions of other glucanases.

The Evidence for essential carboxylate residue

The role of carboxyl group in the catalytic mechanism of the xylanase from *Chainia* has been demonstrated for the first time, based on the specific interaction of guanidine hydrochloride (Gdn.HCl) and Woodwards reagent K (WRK) with the carboxyl groups of the protein. The pH dependence of the kinetic parameters for hydrolysis of xylan implied the requirement of carboxyl groups for the activity of the enzyme. Low concentrations of Gdn.HCl inhibited the xylanase activity reversibly and competitively with a Ki of 0.63 M and the inhibition was unaccompanied by the structural changes in the protein. WRK completely inactivated the xylanase with the second order rate constant of 144 $M^{-1}min^{-1}$. Xylan afforded complete protection against inactivation by WRK, indicating the involvement of carboxylate group at the substrate binding region of the enzyme. Earlier observation of the presence of a single aspartate residue (Asp ^{21}) in the conserved catalytic region together with the present evidence for the essential carboxylate residue in the active site suggests that Asp^{21} is involved in the catalysis of xylan hydrolysis by *Chainia* xylanase.

Heterologous cloning and expression of the xylanase gene from *Chainia*

The genomic library of *Chainia* in λ gt10 was analysed for the expression of the xylanase gene using antixylanase antibodies. The recombinants exhibiting positive enzyme linked immunosorbent assay test (ELISA) showed clearance on RBB-xylan plate. The phage lysates showed xylanase activity in the range of 19 to 36 mU ml^{-1}, which was higher than that exhibited by the previously cloned xylanase gene from *Chainia*. The pooled DNA preparation of EcoR1 digests of the xylanase-positive λ gt10 recombinants showing higher activity was cloned in pUC8. One of the pUC8 recombinants (h_1) which exhibited

maximum activity (24 mU ml^{-1}) was shown to code for the low-molecular weight xylanase from *Chainia*. The xylanase produced by h_1 was inducible by isopropylthio-β-D-galactoside (IPTG) but not by xylan, suggesting that the DNA fragment containing the xylanase gene is in frame with, and its expression controlled by, the lacZ promoter.

🕮 Enhancement of pH and thermostability of the xylanase from *Chainia*

The ready accessibility of the xylanase II produced by *Chainia* to cellulose pulp due to its small size and the absence of cellulase are advantageous features. The enzyme is stable at 40°C for 1h and in a pH range of 5-9 at 4°C. Improved stability of the enzyme at higher temperature and pH are desirable. Effect of a variety of compounds was studied to enhance stability. Glycerol, sorbitol, mannitol (10%) or glycine (1M) had marginal effect on thermostability. Addition of Ca^{+2} or PEG (10mM) increased the half- life of the enzyme at 60°C. Cysteine (10mM) or Tween-80 (1%) showed 70% protection against thermal inactivation. Xylan (3%) offered complete protection against inactivation of the enzyme at 60°C and at pH 9.

LIST OF PUBLICATIONS :

1. K.R. **Bandivadekar** and V.V. Deshpande, (1994) "Enhanced Stability of Cellulase-free Xylanase from *Chainia*", **Biotech. Letters** Vol. 16 No. 2, Page 179-182.
2. **K.R. Bandivadekar** and V.V. Deshpande, (1996) "Heterologous Cloning of Xylanase Gene from *Chainia*" **Enzyme Microbial Technology** Vol. 18:439-443
3. **K.R. Bandivadekar** & V.V. Deshpande, (1996) "Structure-function relationships of Xylanases : Fluorometric analysis of tryptophan

environment", **Biochem. J** Vol. 315, Pages 583-587.

4. M Rao, S Khadilkar, **K.R. Bandivadekar** & V.V. Deshpande, (1996) "Structural Environment of an essential system residue of xylanase from *Chainia* sp. (NCL 82.5.1)", **Biochem. J** Vol. 316 pages 771-775 .
5. **Kavita Bandivadekar**, Suvarna Khadilkar, Mala Rao, Vasanti Deshpande (1997) "Evidence of the essential carboxylate residue in the active site of xylanase from *Chainia* (NCL 82-5-1)", abstract accepted for the **"AMI Annual Conference"** to be held at New Delhi, India, during Dec 97.

Chapter I

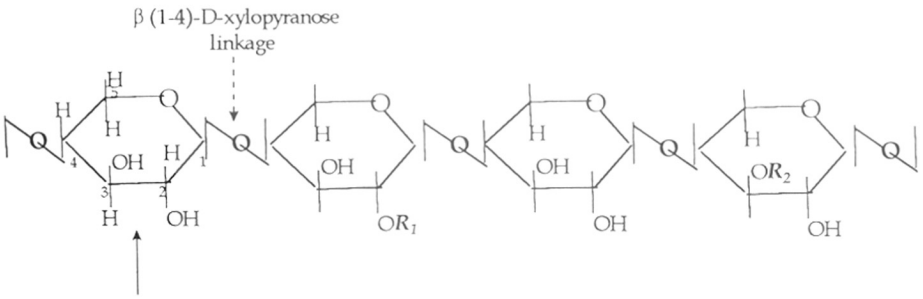
General Introduction

The current increased demand for better utilization of renewable resources and the pressure on industries to operate within environmentally compatible limits have been a stimulus to the development of new concepts in Biotechnology. Biotechnology avails of the nature's strategies to produce a desirable product , as the natural products are often biodegradable, harmless and do not cause damage to the environment. Increase in the knowledge about enzymes and their uses in various industries have expanded the horizons of biotechnology. The commercially important enzymes include glucose/xylose isomerases, α-amylase and glucoamylase, protease, lyases, xylanases and penicillin amidase. In contrast to commercial catalysts (metals), these biocatalysts (microbial enzymes) can be produced in virtually unlimited quantities and can be potentially inexpensive. Agriculture and forestry wastes contain polysaccharides that can be converted to fermentable sugars for the production of valuable chemicals like ethanol. Xylans or hemicelluloses are one of the prominent polysaccharides found in the cell walls of plants. Xylan accounts for approximately 50% of the biomass in the world. It is an important replenishable resource with a high potential for its degradation to useful end products. Xylanases are useful in xylan degradation and are produced by various organisms. As xylan- xylanase system plays an important role in a variety of applications, it demands more basic knowledge about the substrate and the enzymes involved in xylan degradation.

🕮 Xylans :

Hemicelluloses or xylans are noncellulosic polysaccharides that are found in plant tissues [1]. In the cell wall of terrestrial plants, xylan is the most common hemicellulosic polysaccharide, with β, 1 - 4 linked xylopyranosyl backbone and the side branches are formed of more than one type of substituents. The structural features of the plant cell wall xylan, its three-dimensional structure, interactions and properties are described in detail by Joseleau *et al* [2] and E. D. T. Atkins [3]. Based on the common substituents

found on the backbone, the xylans are divided into different categories namely linear homoxylan, arabinoxylan and glucuronoxylan [2]. However, in each category there is a microheterogeneity with respect to the degree and nature of branching. Moreover, complex side chains comprising of the oligosaccharide and non-oligosaccharide moieties have also been reported [2]. The basic backbone of xylan and possible substituents are shown in Fig. 1.



R_1 &/or R_2	:	Acetyl group
		α-L-Arabinofuranose
Or R1	:	4-O-Methyl-α-D-Glucuranopyranose
Or R2	:	β-D-Galactopyranose

Fig. 1 : Chemical structure of xylan

Although the chemical structure of xylan is known for a long time, a lot less is known about the primary structure of xylan due to the lack of information regarding the sequence and pattern of distribution of the side chains, along the xylan backbone [2]. The side chains determine the solubility, its physical structure, reactivity with other cellulosic components and, therefore, greatly influence the mode and extent of enzymatic cleavage. Due to the minor differences in chemical composition and side chain structure of different xylans, it becomes difficult to compare xylan-xylanase interactions from two different sources. One can use synthetic xylan substrate whose

chemical composition is known e.g. 4-methylumbellyferyl β-glycosides of xylobiose and xylotriose synthesized by Beilly *et al* [4]. But not all types of xylanases are detected by this substrate. Moreover, the interactions of xylanases in the natural environment would be different than on synthetic substrate. Availability of the three dimensional structural data on xylan in aqueous environment would be extremely important in understanding the xylan -xylanase interactions.

Xylans can be hydrolyzed either by acid or by enzyme yielding xylo-oligomers and the monomeric sugar D-xylose. Enzymatic hydrolysis yields the products under ambient condition with high degree of specificity while acid hydrolysis is nonspecific and yields an uncontrolled mixture of oligomeric products.

🕮 Xylanases

🗎 Occurrence of xylanase

Xylanases are ubiquitous in distribution and xylanase producers have been isolated from all ecological niches where plant material is deposited. They have been reported from prokaryotes as well as eukaryotes [1, 5 - 7]. The occurrence has been reported in marine and terrestrial bacteria, marine algae, fungi, rumen and ruminant bacteria, snails, crustaceans, seeds of terrestrial plants and a variety of invertebrate animals [8]. Xylanases have also been reported from *Bacillus* sp. found in Atlantic soils [9]. Microorganisms producing xylanases have been found in extremely diverse natural habitats and they utilize this activity to achieve a wide range of goals. In most of the microorganisms, which produce xylanases for utilization of xylan as a carbon source, they are secreted extracellularly, so that the high molecular size polymeric substrate; xylan can be assimilated easily. With the increasing applications of thermostable and alkalitolerant xylanases for biotechnological processes, recently, extremophiles have been investigated for xylanase

production. Many reports of xylanases from thermophilic [5, 10], alkalophilic [11, 12] and alkalothermophilic [13] microorganisms are available.

In case of plants, xylanases are involved in the growth, maturation and ripening of cereals and fruits [14]. Xylanases have also been implicated in pathogenesis or as a part of defense mechanism of plant cells [15]. Xylanases from plant pathogens disrupt the plant cell wall during invasion of the plant [16, 17]. Taiz and Honigman [18] have reported on the role of xylanases in degradation of arabinoxylan during germination of barley. Xylanases from *Trichoderma viridae* were able to induce biosynthesis of ethylene in Tobacco [19] and production of two of the three classes of pathogenesis-related proteins [20]. Xylanases are of different types.

Types of xylanases:

Xylan is a complex heterogeneous substrate having various chemical constituents. Therefore, complete hydrolysis of xylan requires participation of various xylanolytic enzymes such as endoxylanases and β-D-xylosidase. Xylanase attacks the polysaccharide backbone at β,1-4 linkages and β-xylosidase hydrolyses xylooligosaccharides to xylose. α-L-arabinofuranosidase and α-glucuronidase are involved in degradation of acetyl xylans . Acetylesterase and acetylxylan esterase deacetylate acetyl xylan and act synergistically with xylanase in the degradation of acetyl xylan. Generally, xylanases do not cleave linkages adjacent to the units carrying a side chain. Three different types of xylanases are involved in xylan degradation [1, 21, 22].

1. Endo-β-(1,4) - D xylanase: These enzymes act randomly on xylan to produce large amounts of xylooligosaccharides of various chain lengths . These are of two types: (a) non arabinose liberating endoxylanase and (b) arabinose liberating endoxylanase. The type (a) xylanases can be further divided into two categories depending on their inability to cleave particular branch points; viz. (i) the xylanases which cannot act on L- arabinosyl initiated

branch points at β, 1-4 linkages and produce xylooligosaccharides as small as xylobiose along with xylose and (ii) the enzymes which are unable to cleave at α, 1-2 and α, 1-3 branch points and produce xylooligosaccharides larger than xylobiose.

The type (b) xylanases are of two subtypes depending on the product of hydrolysis formed viz. (i) those which can attack at the branch points and produce arabinose along with xylobiose and xylose and (ii) xylanases producing arabinose and xylooligosaccharides of intermediate size.

2. Exo β 1-4 D xylanase: These enzymes are capable of cleaving the single xylose units from the reducing end of the xylan chain and are termed as exoxylanases.

3. β - xylosidases or xylobiases : Xylosidases hydrolyze disaccharides like xylobiose and higher xylooligosaccharides with decreasing specificity.

Fig. 2 shows mode of action of xylan degrading enzymes on xylan

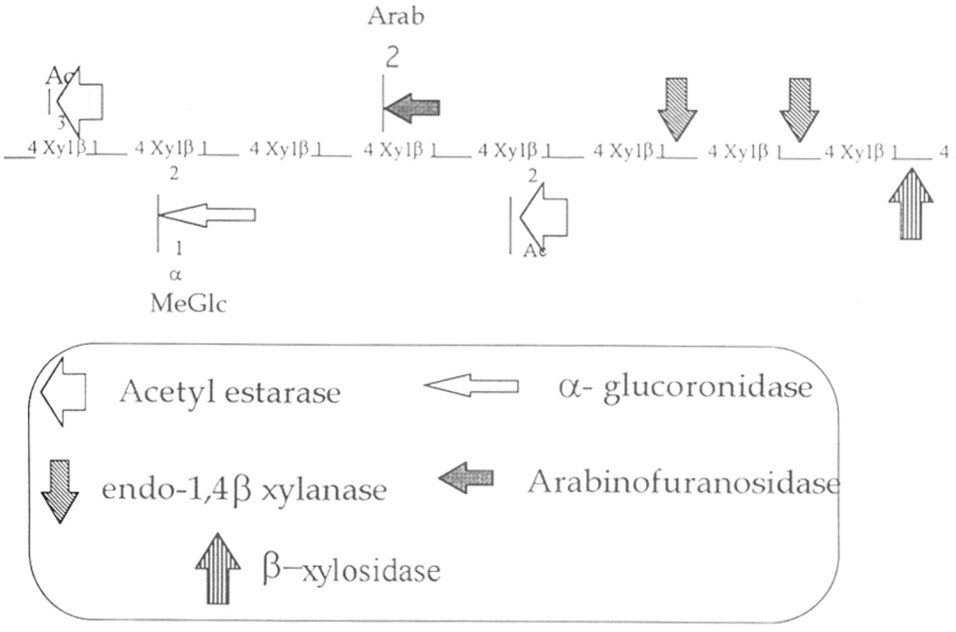


Fig. 2 : A representative structure of xylan and sites of action of various enzymes

(Xyl : xylose units, Arab : Arabinofuranose, Ac : Acetyl group, MeGlc : 4-O-Methyl glucoronic residue)

Xylanase families:

Xylanases are divided into two major families of glycosyl hydrolases on the basis of amino acid sequence identities in their putative catalytic domains and hydrophobic cluster analysis (HCA) [23, 24]. The HCA reveals similarities in secondary structures of proteins with very low sequence identities.. A numerical classification of glycosyl hydrolases by Bernard Henrissat in 1991 is based on amino acid sequence similarities with an intention to reveal the structural features of these enzymes rather than their sole substrate specificities [25]. The xylanases fall into family F and G which correspond to families 10 & 11 of Henrissat classification. The utility of this classification is its ability to predict the general three-dimensional structure and active site

topology of all the family members, provided this information is known for one or more representatives [26].

Till now about 77 family 10 (F) and 88 family 11(G) xylanases have been listed. Only one sequence of a bifunctional enzyme possessing both family F and family G catalytic regions was identified using sequence of either family 10 or family 11 enzymes [27]. Xylanases from both the families, that have been studied extensively with respect to their site directed mutagenesis, X-ray crystallography and 3D structure determination are listed in Table 1.

Table 1: X-ray crystallography and three dimensional structure of xylanases

(a) xylanases of G Family

Organism	Techniques used	Information derived	Ref.
Aspergillus niger	X-ray crystallization	Crystals are orthorhombic	[28]
	Molecular replacement	Refined crystal structure is given. It consists of single domain composed predominantly of β strands. Two β sheets are twisted around a deep , long cleft which is lined with many aromatic amino acid residues and is large enough to accommodate at least four xylose residues. The two conserved Glutamate residues ,Glu79 and Glu170 reach into this cleft from opposite sides.	[29]
Bacillus circulans	X-ray crystallography	Preliminary structure has been defined	[30]
	NMR studies Site directed mutagenasis	Glu78 functions as a nucleophile and Glu172 as a general acid catalyst during glycosylation ,and general base during deglycosylation deprotonating the attacking water.	[31]
		Positioning of acid/base catalyst is critical in functioning of the enzyme.	[32]

Organism	Techniques used	Information derived	Ref.
Bacillus pumilus IPO	X-ray crystallography	Preliminary x-ray studies and crystallization of xylanase showed monoclinic crystals	[33]
Thermophilic *Bacillus sp.*	X-ray crystallography	preliminary x-ray analysis has been done , orthorhombic crystals	[34]
Trichoderma reesei	X-ray crystallography	Two xylanases I and II have been crystallized , both are monoclinic.	[35]
	X-ray diffraction technique	The 3 D structure of xylanase II (optimum pH 5) have been defined .Single domain protein with 2 antiparallel β sheets and one α helix. Catalytic residues are Glutamates Glu86 & Glu177. Change in pH from 5 to 6.5 makes conformational changes in the protein, which are inhibitory to the activity of enzyme.	[36]
	X-ray diffraction technique and amino acid sequence homology studies	Two Glutamates in xyl I (Glu75 & Glu164) (Opt. pH 3-4) and xyl II (Glu86 & Glu177) (Opti. pH 5 - 5.5) are involved in activity. Inspection of the structures revealed that the width of the active site cleft and the number of subsites are different in xyl I and xyl II. In xyl I it is narrow with 3 subsites and in xyl II it is five. Variations in the surrounding residues are thought to explain the pH optimum differences observed in xyl I & xyl II.	[37]

(b) xylanases of F Family

Organism	Technique used	Information derived	Ref.
Cellulomonas fimi	X-ray crystallography	Preliminary x-ray analysis of the catalytic domain has been done.	[38]

Organism	Technique used	Information derived	Ref.
Pseudomonas fluorescens subsp. *Cellulosa*	X-ray crystallography	Catalytic domain has been expressed in *E.coli* and crystallized. Crystals are orthorhombic. Preliminary x-ray structure has been defined.	[39]
	Molecular replacement	Refined crystal structure & 3D structure. Tertiary structure is an eight fold β/α barrel, active site residues are a pair of Glutamates Glu127 and Glu246.	[40]
	Site directed mutagenesis	The preliminary role of the F subsite of Xyl A is to prevent small oligosaccharides from forming nonproductive enzyme-substrate complexes.	[41]
Streptomyces lividans	molecular isomorphous replacement x-ray crystallography	Primary structure is $(\alpha/\beta)_8$ barrel and the active site is located at the carbonyl end of the β barrel. The crystal structure supports the earlier assignment of Glu128 & Glu236 as the catalytic amino acids.	[42]
Thermoascus aurantiacus	X-ray crystallography	Preliminary x-ray analysis has been done , monoclinic crystals	[43]

Xylanases belonging to family 10 (F) exhibit high molecular weights (above 35KDa) and are characterized (i) by two domains viz. catalytic and cellulose binding domain, connected by a flexible linker region and (ii) by the presence of low pI values. Xylanases of family 11(G), on the other hand, exhibit low molecular weights i.e. below 25 KDa, high pI values, and do not display defined domain structure.

The catalytic domain of Family 10(G) xylanases consists of a cylindrical 8 fold α/β barrel resembling a salad bowl, with the catalytic site at the narrower end, near the carboxyl terminus of β barrel [41]. There are usually five to eight xylopyranose binding subsites. The xylanases tend to form oligosaccharides of low degree of polymerization. The overall structure

resembles a tadpole [44] with a caralvic (amino terminal) 'head' and a cellulose binding domain (carboxyl terminal) 'tail'. Family 11(G) xylanases comprise of β pleated sheets formed into a two layered trough that surrounds the catalytic site and a single α helix [45]. It has been compared with the palm and fingers [36]. On the basis of crystal structure results and mutational studies a lysozyme type mechanism has been proposed for family G xylanase [46].

Production and multiplicity of xylanases:

Pure xylan is the most preferred carbon source for the production of xylanase [47 - 51]. However, use of pure xylan is not economically feasible for commercial production. Therefore, agricultural residues rich in hemicellulose such as wheat bran, rice bran, rice stalk, corn cobs, bagasse, corn stalk and canola meat etc. have been used as substrates for microbial xylanase production [52 - 56].

Majority of the xylanases are produced extracellularly although a few reports of intracellular xylanase from rumen bacteria are also available [57, 58]. Almost all xylanolytic systems of bacteria and fungi have been shown to be inducible. A few examples of constitutive xylanases have been reported [47, 59 - 62]. Derivatives formed by enzymatic cleavage of substrate play a positive role in inducing xylanase production.

A single organism can produce many biochemically different xylanases. Genetic multiplicity has been observed in at least three ways ; (I) Regardless of the origin, (anaerobic or aerobic, bacterial or fungal), genomes of many organisms maintain several non-polycistronic functional copies of loci that encode proteins with overlapping functions [63 - 66]. In few cases , especially in eukaryotes , detection of redundant xylanase polypeptides is the result of differential post translational processing [67]. (II) In several cases a single gene product encodes multiple catalytic domains. (III) In many microorganisms, multiple copies of redundant or related loci are polycistronic e. g. a xylanase fragment found in *Caldocellum saccharolyticum* contains five open reading frames encoding a xylanase , a β - xylosidase, an acetyl esterase

and two ORF's of unknown function [68]. Wong *et al*,1988 [67] have investigated the origins and functions of multiplicity in xylanolytic enzymes. Multiplicity may be due to the complex xylan structure. It can also be due to the presence of distinct xylanase genes, differential post translational modification, proteolysis and broad specificity of the other glycanases towards xylan. The multiple enzyme components of the xylanase enzyme systems are known to exhibit co-operativity of xylan degradation such as reducing the degree of polymerization of xylan, solubalization of xylan, generation of small oligosaccharides and xylose production. The observed pattern of genetic multiplicity may point to a two step evolutionary process, where initially primitive proteins with low substrate recognition specificity (recognize both cellulose and xylan) are duplicated and evolved independently [69].

Usually one organism produces two types of xylanases, a low molecular weight and a high molecular weight, with high and low pI values respectively. The low molecular weight enzyme plays a major role in initiating xylan degradation. The low molecular weight derivative of xylan i.e. xylose, xylobiose, xylooligosaccharides, heterosaccharides of xylose etc. also play an important role in regulation of xylanase expression. In some cases, cellulose is also known to act as an inducer of xylanase production [7, 67, 70].

Xylanosome concept:

Evidences for the discrete multifunctional multienzyme complexes called "xylanosomes" found on the surface of some xylanolytic microorganisms exist in literature. Xylanosome is structurally analogous to cellulosome or proteasome. Xylanosome existing as a multisubunit protein aggregate has been reported in *Butyrivibrio fibrisolvens* H17c [71]. Similarly *Candida papyrosolvens* C7 also possesses a multicomplex cellulase-xylanase system. Presence of xylanosome in the anaerobic bacterium *Thermoaenarobacterium saccharolyticum* B6A - RI was reported by Zeikus *et al* [72].

Synergism of xylanolytic enzymes :

In xylan hydrolysis, co-operativity or synergism has been observed between enzymes acting on the β1—4,D-xylan backbone (endo-xylanase) and side chain cleaving enzymes (α-L- arabinofuranosidase, acetylxylan esterase and α-glucuronisidase). The synergistic action between acetylxylan esterase and endoxylanase results in efficient degradation of acetylated xylan. The release of acetic acid by acetylxylan esterase increases the accessibility of the polysaccharide backbone for endoxylanase attack. The endoxylanase creates shorter acetylated polymers, which are the preferred substrates for esterase activity [5, 73]. Synergism also occurs between various endoxylanases. *F. Succinogenes* S85 secretes two types of endoxylanases. Endoxylanase 1 possesses a debranching activity, liberating arabinose from xylan. Release of arabinose precedes the hydrolysis of xylan backbone into xylooligosaccharides, which allows endoxylanase 2 to act on the xylan backbone [74].

Properties of Xylanases :

Xylanases from different sources display extremely variable characteristics with respect to their biochemical and physico-chemical properties. There seems to be a strong relationship between their molecular weights and isoelectric values. Wong *et al*, in 1988, [67] suggested that endoxylanases could be grouped into those that are basic proteins with molecular weight below 30,000 and those that are acidic with molecular weight above 30,000. There are many exceptions to these general patterns, [65, 75]. The pH optima of xylanases are generally in the range of pH 4.0 - 7.0, but few exceptions have been reported [76, 77]. The Dixon plot of xylanase activity *Vs* pH is by and large a bell shaped curve, which indicates that two reactive groups with ionization optima values falling on the two side of the peak are involved in the catalysis. Many of the characterized xylanases are optimally active at temperature ranges between 45° to 75° C. The purified endoxylanase

from various species, belonging to *Thermotoga* are optimally active at temperatures between 80°C - 105°C [78 - 81].

The biochemical characters of xylanases from different organisms have been listed in various recent reviews [5, 6, 23, 67, 79, 82, 83]. The smallest endoxylanase 6000 Kda was isolated from *Chainia* sp. and its 43 amino acid residues were sequenced [84]. The primary sequences of over 70 xylanases have now been determined [40] and a few have been solved by x- ray crystallography (Table 1) .

Most of the xylanases are glycoprotein in nature. Glycosylation has no apparent effect on catalytic activity but it does afford protection against proteolytic cleavage of the interdomain linker when the enzyme is adsorbed to cellulose or xylan [85]. *Chainia* xylanase is also a glycoprotein [86].

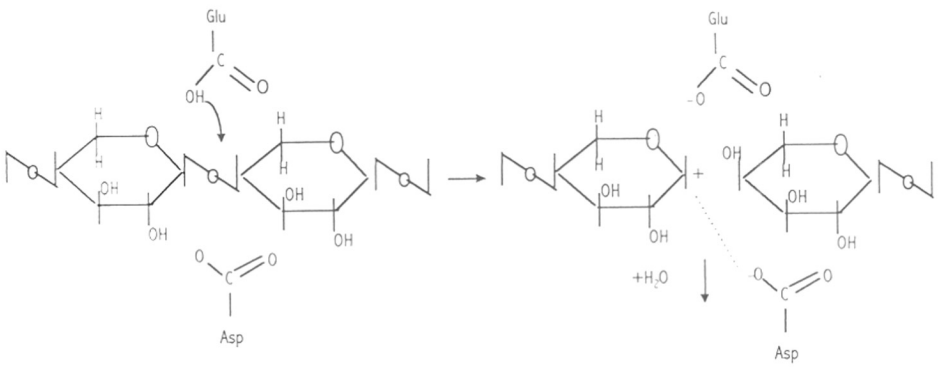
Substrate specificity and mode of action:

Xylanases vary in their sugar and linkage specificity. Information about substrate specificity can be utilized for investigating active site structure and mode of action of xylan-degrading enzymes. Xylanases are hydrolytic enzymes requiring acid/base catalyst to carry out the reaction, however, all bonds are not hydrolysed from the complex substrate. Evidences suggest that there are a number of subsites present at the active site which position the monomer residues of xylan properly to carry out the particular bond breakage. These subsites are responsible for the difference in specificity of different xylanases. It has also been suggested that the enzyme-substrate specificity is the result of the non-covalent interactions between the substrate and subsites of the enzyme (usually located around the catalytic center), endo-cleaving enzymes are likely to be more specific than exo-cleaving enzymes. Further, the stringency of the substrate-enzyme interaction is not uniform throughout xylanase families, and in many cases these enzymes also recognise and cleave a second substrate but with much lower specificity (e. G. CMC) [87]. The sites attacked along with xylan chain and the frequency of bond cleavage is also governed by the structure of the heteroxylans (i. e. In the degree and

frequency of side-chain substituents) [88]. The early product of degradation are xylooligosaccharides, some of mixed constitution i.e. usually containing arabinose and /or glucuronic acid or its 4-O-methyl derivative. The ultimate size of the other end product is determined by the specificity of the enzyme. The purified xylanases have been reported to cleave a number of polysaccharides such as CMC and avicel [89] , starch [90] and *p* – nitrophenyl β - D – Glucoside [91]. They exhibit variable action pattern with respect to main and side chain cleavage and the substituents may be a requirement or hindrance to the activity. The analysis of product obtained after hydrolysis gives information about the mode of action of xylanases. The involvement of various amino acids at the active site (as described in section on active site studies) also provides information about the probable mode of action.

All glycanases and glycosidases examined to date catalyze stereoselective hydrolysis i. e. the configuration about the anomeric center is either inverted (single displacement reaction) or retained (double displacement reaction) upon hydrolysis of glycosidic linkage [26].

The actual mode of action of a typical xylanase can be shown as follows (Fig. 3) . The requirement of two carboxylic amino acids Glu/ Glu or Asp/Glu is necessary for the bond cleavage.



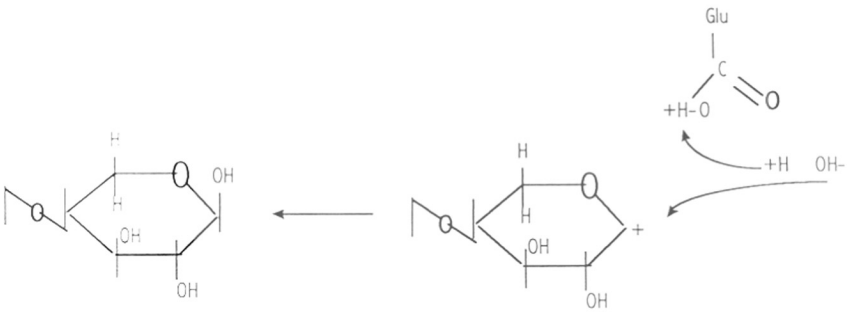


Fig. 3 : hydrolysis of xylan by xylanase

(A typical xylanase is shown possessing Glu and Asp as the essential active site residues for the cleavage of the β-1,4 linkage)

🕮 Structure-function relationship of xylanases:

It is necessary to improve many properties of enzymes like solubility, catalytic activity and selectivity for their industrial application. Therefore, a detailed study on the primary, secondary and tertiary structure of the xylanases, and correlation of the structural specificities to a particular function is required. In this respect, the studies are concentrated on the active site of the enzyme which is most important for determining the catalytic activity of the enzyme.

🖹 Active site studies:

Chemical modification technique is used for probing the active site structure of the enzyme. The studies on the effect of various amino acid group-specific reagents on the xylanase have indicated the presence of essential catalytic residues. Some of the commercially important xylanases have been shown to possess Trp, Cys, His, Tyr and carboxy amino acids at their active sites. Xylanases are the typical hydrolytic enzymes which need acid-base catalysis for carrying out the reaction. In case of many xylanases Glu and Asp are shown to act as the acid-base catalyst and also the nucleophile to carry out the reaction.

The enzymes from family F consist of 8 fold α/β barrels containing deep active site grooves consistent with their endo mode of action. Some also have subsites for xylose binding [40]. All acidic pI xylanases of family G (11) have conserved Asp residue, whereas basic xylanases have conserved Asn residue. In case of endo 1 4, β-xylanase from *Trichoderma reesei* at the optimum pH 5.0, Tyr88 is shown to interact with Trp77, whereas at pH 6.8, Tyr88 bonds with Glu177, thereby disrupting the catalytic activity [35]. Carboxy amino acids play an important role in catalytic mechanism of xylanase while Trp, Tyr, Cys are structurally important and form the cluster of other conserved residues which are capable of fine tuning the properties of catalytic Glu/Asp residues by hydrogen bond network. Other conserved residues are important in order to confirm the correct folding and packing of the enzyme protein. Xylanase being carbohydrate binding and hydrolysing protein, Trp, Tyr or Cys residues participate in the binding of the carbohydrate residue by packing against a flat face of the carbohydrate ring [92]. Table 2 summarizes the information on the xylanases studied for their active site structure.

Table 2 : Active site studies on xylanases

Organism	Essential active site residue	References
Alkalothermophilic *Bacillus* NCIM 59	Trp, carboxyl amino acids	[93], [94]
Bacillus strain 41M – 1	Trp/Tyr, carboxyl amino acids	[95]
Chainia	Trp, Cyst, carboxyl amino acids	[96], [97], [98]
Clostridium stercorarium	Cyst	[99]
Fusarium Avinaceum	Trp	[100]
Schizophyllum commune	Tyr, carboxyl amino acids	[101]
Thermotolerant *Streptomyces* sp.	Trp, Cyst	[102], [103]
Streptomyces sp. 3137	Trp	[104]
Trichoderma koningii ATCC 26113	Trp, Cyst	[105]

Site directed mutagenesis

Besides the potential role of site directed mutagenesis in protein engineering for altering the properties of the enzyme; it is also useful for confirming the role of the active site residues of the enzyme in catalysis. Identification of active site residues by chemical modification has been reported for several xylanases (Table 2), but very few have been confirmed by site directed mutagenesis studies . The xylanase from *Bacillus pumilus* IPO has been stabilized by random mutagenesis of cloned gene fragment [106]. Appropriate mutations of xylanase A were constructed and biochemical properties of the mutated enzymes were evaluated for investigating role of conserved active site residues on xylanase A (family F) of *Pseudomonas fluorescence* subsp. *Cellulosa* [40]. The results suggested that the additional (sixth xylose binding) subsite F of xylanase A prevents small oligosaccharides from forming nonproductive enzyme-substrate complex. The site directed mutagenesis studies also provided insights into the importance of several residues located in the F, E and C subsites of xylanase. The data showed that highly conserved residues do not necessarily play important roles in different xylanases from the same family. Carboxy amino acids play an important role in actual hydrolysis of xylan . Xylanases act by double displacement mechanism involving a covalent α - glycosyl enzyme intermediate. The formation and hydrolysis of this covalent intermediate occurs via oxocarbenium ion similar to a transition state , with the assistance of two key active site residues. In case of *Bacillus circulans* xylanase, the critical distance (~5.5A°) between two catalytic carboxyl amino acid residues has been altered by mutagenesis of putative acid/base catalyst viz. Glu172. An increase in the separation (Glu172Asp) resulted in decreased catalytic activity towards xylan and the decrease in separation (Glu172Cys) caused 25 fold reduction in xylan hydrolysis. Complete removal of carboxyl group had a more dramatic effect. Glu172Cys and Glu172Gln mutants exhibited no measurable activity on xylan or phenyl xylobioside, a substrate which requires acid catalysis. The results

emphasize the functional importance of the carboxyl group found at this position [33]. Cysteine may be involved in hydrogen bonding with the substrate. Cysteines, even when at some distance from the active site may be critical to the proper folding of the molecule. Their modification may distort the 3D configuration of the active site with resultant loss of activity [107]. Tryptophan may participate by forming hydrogen bonds with the substrate as has been shown in case of lysozyme [108, 109]. Histidine is involved in stabilizing the structure for overall reaction mechanism. Plesniak *et al,* [110] have shown that *Bacillus circulans* xylanase contains 2 His residues, one solvent exposed (His156) and the other (His149) is buried within its hydrophobic core. With the help of NMR spectroscopy, it is shown that His149 is involved in a network of hydrogen bonds with an internal water and Ser130, as well as in a potential weak aromatic interaction with Tyr105. The structural importance of this buried His residue was confirmed by the destabilizing effect of substituting Phe or Gln at position 149.

🕮 Biotechnological exploitation:

Xylanases have considerable potential in many biotechnological industries. The enzyme assumes special importance in the paper and pulp industries as it has a potential to replace the toxic chemicals such as elemental chlorine and chlorine dioxide that are employed currently in paper and pulp industry [111]. Cellulase-free xylanases are desirable for their application in biobleaching during pulp manufacture. Xylanases help in pulp bleaching process by solubilization of the chromophore, swelling of fiber matrix to disrupt the absorptive interactions, solubilization of the redeposited xylan and cleavage of lignin carbohydrate linkage [67]. Xylanases are also used for the production of fuel and chemical feed stock [107], liquefaction of coffee mucilage [1], extraction of pigments and flavors [112] , protoplast formation [113] and the production of modified hemicelluloses for industrial use [114]. The enzymes play an important role in debarking and prebleaching of kraft

pulp and de-inking of recycled fibers. Xylanases have been used along with other enzymes like cellulases and xylosidases in bioconversion of wastes such as those from the forest and agricultural industries, and in the clarification of vegetable and fruit juices. Xylanases are useful in bakery for increasing the quality and volume of dough. They have been used in pig diets for improving the feed digestibility and feed conversion ratios. In these diets, multienzyme mixtures containing xylanase and cellulase are used which depolymerise arabinoxylan and mixed linkage beta glucan, thus reducing their viscosity building properties. This results in more rapid and more efficient absorption of nutrients from the gut. Recently, xylanases have also been used in pet diets to decrease the amount and frequency of faeces [115].

Chemical pulps are made from wood, in a pulp and paper mill to generate a variety of paper products. Mostly, the lignin present in the wood pulp turns the pulp into dark brown color, which is necessary to be removed from the pulp for production of the better quality of paper. Chemical bleaching process involves elemental chlorine and peroxide. Byproducts of chemical bleaching are chlorinated organics, and other hazardous chemicals. To overcome this problem bleaching technologies devoid of chlorine have been evolved using oxygen, greater amount of chlorine dioxide, hydrogen peroxide, ozone and biological catalysts such as enzymes. Enzymes, especially xylanases, secreted by various microorganisms are useful as replacement for conventional bleaching agents. They selectively hydrolyze the hemicellulose component of wood pulp. Extraction of the wood using caustic soda (alkaline extraction) removes the xylan fragments along with associated lignin from the craft pulp [116]. Most advantageous bleaching operations are performed at high temperatures and alkaline pH. Therefore, cellulase-free xylanases that are able to function under these conditions are desirable. Most of the microorganisms produce xylanases for utilizing xylan as a carbon source for growth and survival. They also produce other enzymes such as cellulases that synergistically degrade the plant material. Very few organisms produce

xylanases that are cellulase-free. *Chainia* produces extracellular and cellulase-free xylanase [117]. Improvement in its stability to higher temperatures and alkaline conditions would be beneficial for its use in paper and pulp industry.

🕮 Molecular cloning of xylanases:

Molecular cloning of genes from a novel organism to a well studied organism is a very well established technique. It is employed for most of the commercially and genetically important proteins and other cell products. In xylanolytic systems, the main objectives for the recombinant DNA technology are (i) construction of xylanase producer strain free of cellulase activity, (ii) improvement of productivity and fermentation characteristics of xylan degrading organism, so that xylan can be utilized and converted totally into desired chemicals like ethanol., (iii) understanding of xylanase gene expression and the secretion at the molecular level and (iv) amplification of the expression of genes already present , for increased yield of xylanase . Chromosomal gene integration have been tried in case of alkalothermophilic *Bacillus* sp.[118] which yielded two fold increase in production of enzyme. Cloning of xylanase gene from various microorganisms into *E.coli*, *Streptomyces*, *Bacillus* and yeast has been reported (Table 3 & 4). A variety of vectors such as pBR322, λ derived vectors, expression vectors of pUC series have been used for cloning of xylanase into *E. coli* and other organisms.

📄 Heterologous cloning of xylanase :

Initially, most of the heterologous gene cloning was carried out in *E. coli* . Well-studied organisms like *E. coli* and availability of a large variety of cloning vectors offered great help in constructing genomic libraries in this organism. However, the xylanase expression in *E. coli* is generally found to be lower than the parent organism and it is usually confined to the cytoplasmic or periplasmic fractions. The intracellular accumulation of the recombinant xylanases and the absence of post translational modifications such as glycosylation in *E. coli* are the main reasons for low levels of xylanase

expression. However, cloning in *E. coli* has two main advantages viz. easy access to sequencing and for manipulation of the genes.

Heterologous cloning of the xylanase genes in the non-xylanolytic hosts other than *E. coli* has also been reported. The hosts such as *Saccharomyces*, or *Lactobacillus* offer fermentative advantages in utilizing the products of hemicellulose degradation material as the fermentable carbon source. Reports of heterologous cloning of xylanase genes are listed in Table 3 and Table 4.

Table 3 : Cloning of xylanase gene in *E. coli* :

Parent strain	Vector	Reference
Aeromonas caviae ME -1	λ Dash II	[119]
Aspergillus kawachii	λg 11/ pUC 118	[120]
Aureobasidium pullulans Y-2311-1	λ ZAP II / pBluescript	[121]
Bacillus polymyxa	pBR 322	[122]
Bacillus pumilus IPO	pBR 322	[123]
Alkalophilic thermophilic *Bacillus* sp. NCIM 59	pUC 8	[124]
Bacillus stereothermophilus T - 6	λ EMBL 3	[125]
Bacillus stereothermophilus	pUC 8	[126]
Bacillus subtilis CD4	pUC 8	[127]
Bacteroides ovatus V 975	pUC 18	[128]
Butyrivibrio fibrosolvens # 49	pUC 19	[129]
Caldocellum saccharolyticum	λ 1059	[130]
Cellulomonas fimi	λ ZAP II	[131]
Cellulomonas sp. NCIM 2353	pUC 18	[132]
Chainia sp. NCL 82.5.1	λgt 10 /pUC 8	[133] , [134]
Clostridium stercorarium F 9	pBluescript II KS	[135]
Dictyoglomus thermophilus	λ ZAP II	[136]
Fibrobacter succinigenes # 135	λgt WES λB	[137]

Parent strain	Vector	Reference
Neocallimastix patriciarum	λ ZAP II	[138]
Penicillium chrysogenum	λ EMBL 3	[139]
Penicillium purpurageneum	λ Ziplox. Arms	[140]
Pseudomonas fluorescence subsp. *Cellulosa*	λ 47.1	[141]
Thermotoga maritima DSM 3109 (MSB 8)	pUC 19	[142]

Table 4 : Cloning of xylanase genes in hosts other than *E. coli* :

Parent strain	Host	Vector	Ref.
Aspergillus kawachii	*Saccharomyces cereviciae*	pG3	[143]
Alkalophilic thermophilic *Bacillus* sp. NCIM 59	*Bacillus subtilis A 8* *Bacillus subtilis M III*	pLP1202	[144], [145]
Clostridium acetobutyrilum	*Lactobacillus plantarum*	pWp 37	[146]
Clostridium stercorarium	Tobacco cells	pXY B	[147]
Clostridium thermocellum	*Bacillus subtilis*	pUB 110	[148]
Clostridium thermocellum	*LactoBacillus plantarum*	pWp 37	[146]
Streptomyces halstelli JM 8	*S. lividans JI 66* *S. parvulus JI 2283*	pIJ 702, pJM 9,	[149]
Streptomyces lividans 1326	*S. lividans*	pIJ 702	[150]
Streptomyces parvulus IMET 41380 and 8852 and *Streptomyces vinaceus* NCIB 8852	*lividans TK 24 &* *S. coelicolor A3(2)*	pIJ 702	[151]
Streptomyces **sp. No. 36a**	*S. lividans* TK 21 *S. kasugansis* 63	pIJ702 pSK 2	[152]

Parent strain	Host	Vector	Ref.
Streptomyces thermoviolaceus OPC-520	*S. griseus* PSR 2	pIJ 702	[153]
Thermomonospora alba ULJB 1	*Streptomyces lividans*	pIJ 699	[154]
Thermomonospora fusca	*S. lividans*	pIJ 702	[155]

Homologous cloning of xylanase genes:

The cloning of genes in the homologous hosts offers many advantages. Similarity in codon usage permits efficient transcription, translation and secretion of proteins. In case of *Streptomyces* the promoter elements may not be recognized by the *E. coli* σ factor, in turn, leading to difficulties in the expression of the corresponding gene. Hence the construction of genomic library and the screening of the xylanase gene has been carried out from *Streptomyces* sp. # 36a and *S. lividans* # 1326 using suitable homologous host systems. In case of *Bacillus pumilus* IPO and alkalothermophilic *Bacillus* the extracellular secretion of the xylanase was achieved using the homologous host *B. subtilis*. The level of expression of the xylanase is relatively higher in homologous host system than that in heterologous like *E. coli* .

🕮 Present work :

Chainia is a sclerotial Actinomycete isolated at National Chemical Laboratory, Pune, from the soil of Haldighat, Rajasthan [117] (Fig. 4). It is a Gram positive and non acid fast, saprophytic, aerobic, mesophilic organism. It has high G+ C content which is typical of *Streptomyces* spp.and susceptibility to polyvalent *Streptomyces* phages [156]. It has L L diaminopropionic acid and glycine, but no characteristic sugars in the peptidoglycan of cell walls. It produces many commercially important enzymes such as extracellular, cellulase-free xylanases [117], substrate-specific intra-and extra-cellular glucose/xylose isomerases [157] and protease [unpublished results]. It secretes

8-10 IU/ml of xylanase on media containing agricultural residues like cereal brans, while a higher activity of 26 IU/ml is secreted with medium containing pure xylan. The major component of xylanase is a low molecular weight protein with optimum pH in the range of 5 - 7 and temperature in the range of 55 - 60°C. The enzyme has been purified by gel filtration and ion exchange chromatography [86], and involvement of tryptophan and cysteine residues has been demonstrated at the active site [93]. Present work reports studies on the structure-function relationship of the active site residues of the xylanase from Chainia sp. (NCL 82.5.1) and cloning of the gene responsible for its expression.

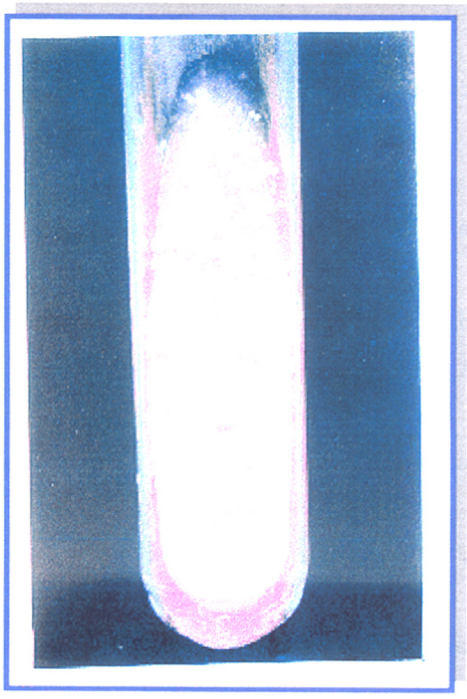


Fig. 4 : Growth of Chainia spp. (NCL 82.5.1) when grown on MGYP slant at 28°C for 5 days.

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Chapter II

Structure-function Relationship Of Xylanase II From Chainia

🕮Summary of Chapter II

Part A : Study of microenvironment of tryptophan (trp) residue.

Trp is a useful built-in fluorescent probe. Its fluorescence is very sensitive to its microenvironment. Involvement of one out of three trp residues in the active site of the xylanase II from *Chainia* has been demonstrated previously, [Deshpande, Hinge and Rao (1990) Biochem. Biophys. Acta 1041, 172 - 177]. The work described here aims at : (i) deducing the structure-function relationship for the trp residue involved at the active site (a) by correlating the effect of N - bromosuccinimide (NBS) on the fluorescence and activity, and (b) by assessing the ability of xylan to protect against decrease in fluorescence versus activity of NBS-treated enzyme; and (ii) probing into the environment of the trp residues by studying the quenching of their fluorescence by various solute quenchers in presence and absence of guanidine hydrochloride (Gdn.HCl). Complete inactivation of NBS-treated enzyme occurs well before the loss of fluorescence. Full protection by xylan (0.5%), of the inactivation of enzyme by NBS compared with 30% protection for the decrease in fluorescence, confirms the participation of a single trp at substrate binding site of the xylanase. The xylanase exhibited a rather low fluorescence emission maximum at 310 nm. There was no shift in the emission maximum on treatment of the enzyme with Gdn.HCl (Upto 6.5 M), indicating the rigidity of the microenvironment around trp residues. The quenching studies with acrylamide suggested the occurrence of both collisional as well as static quenching processes. The enzyme retained full activity as well as the characteristic emission maximum at 310 nm in the presence of acrylamide (100 mM), indicating that the quenching of fluorescence by acrylamide is a physical process. Acrylamide was more efficient as a quencher than CsCl and KBr. Treatment of the enzyme with Gdn.HCl resulted in an increase in the Stern-Volmer quenching constants (K_{sv}) of the solute quenchers. The analysis of K_{sv}

and V values of KBr and CsCl suggests that the overall trp microenvironment in the xylanase II from *Chainia* is slightly electronegative.

Part B : Structural environment of essential cysteine residue

N-(2,4-Dinitroanilino) maleimide reacts covalently with the thiol group of the xyl II from Chainia leading to complete inactivation in a manner similar to N-ethylmaleimide, but provides a reporter group at the active site of the enzyme. Increasing amounts of xylan offered enhanced protection against inactivation of the xylanase by DAM. Xylan (5 mg) showed complete protection, providing evidence for the presence of cysteine at the substrate-binding site of the enzyme. Kinetics of the chemical modification of the xylanase by DAM indicated the involvement of 1 mol of cysteine residue per mol of enzyme, as reported earlier. The second order rate constant for the reaction of DAM with the enzyme was 3.61×10^3 $M^{-1}min^{-1}$. The purified xylanase was alkylated with DAM and digested with pepsin. The peptides were separated by gel filtration. The specific modified cysteinyl peptide was further purified by reverse phase HPLC. The active site peptide was located visually by its predominant yellow colour and characterized by a higher A_{340} / A_{210} ratio. The modified active site peptide has the sequence: Glu - Thr - Phe - Xaa - Asp. The sequence of the peptide was distinctly different from that of cysteinyl peptide derived from a xylanase from a thermotolerant Streptomyces species, but showed the presence of a conserved aspartic acid residue consistent with the catalytic regions of other glycanases.

Part C : The Evidence for essential carboxylate residue

The role of carboxyl group in the catalytic mechanism of the xylanase from *Chainia* has been demonstrated for the first time, based on the specific interaction of guanidine hydrochloride (Gdn.HCl) and Woodwards reagent K (WRK) with the carboxyl groups of the protein. The pH dependence of the

kinetic parameters for hydrolysis of xylan implied the requirement of carboxyl groups for the activity of the enzyme. Low concentrations of Gdn.HCl inhibited the xylanase activity reversibly and competitively with a Ki of 0.63 M and the inhibition was unaccompanied by the structural changes in the protein. WRK completely inactivated the xylanase with the second order rate constant of 144 $M^{-1}min^{-1}$. Xylan afforded complete protection against inactivation by WRK, indicating the involvement of carboxylate group at the substrate binding region of the enzyme. Earlier observation of the presence of a single aspartate residue (Asp ^{21}) in the conserved catalytic region together with the present evidence for the essential carboxylate residue in the active site suggests that Asp^{21} is involved in the catalysis of xylan hydrolysis by *Chainia* xylanase.

🕮General Introduction :

Application of enzymes on an industrial scale, requires consideration of their properties such as solubility, catalytic activity, and selectivity of the enzyme. It is known that enzymes have evolved to work in a natural environment defined by the characteristic of the organism that synthesized them. Therefore, various methods have been developed, for making the enzyme work *in vitro* at desired conditions. The identification of the amino acids responsible for the formation of enzyme-substrate complex and determination of their three dimensional structure is a prerequisite for manipulating the protein for desirable properties. Chemical modification of amino acids using group specific reagents is used to study the role of amino acids present at the active site [1,2]. Another indirect approach employed for tentative assignment of catalytic role to amino acids was the study of effect of pH on enzymatic activity. X-ray crystallography is the most powerful technique currently available for visualizing an accurate picture of three dimensional structure of an enzyme and its active site [3,4]. Classical chemical probes have also been refined to investigate the active site and the reaction pathway. Development of affinity analogues and active site directed photoaffinity or fluorescent labels have contributed significantly in the development of enzyme research and more specifically in understanding the architecture and function of the active site.

Study of the environment prevailing at the active site and critical role of constituent amino acids, is very important in structure-function analysis and protein engineering.

Active site studies of xylanases:

The studies on the catalytic domains which include the active site have been carried out in detail for many xylanases with the help of chemical modification [5,6,7], site directed mutagenesis and x-ray crystallography [8,9,10]. Involvement of cysteine [11, 12], tryptophan [13], histidine [14, 15],

tyrosine [7] and carboxyl amino acids [16, 17, 18] at the active site has been demonstrated. The presence of carboxyl group containing amino acids is absolute in case of all xylanases as they are directly shown to be involved in catalysis [18]. Other essential amino acids are responsible for generating the particular structural arrangement of the active site cleft and for defining the actual distances between the catalytic residues. These are also involved in stabilizing the enzyme-substrate complex for carrying out the hydrolysis reaction [18].

This chapter deals with the active site studies of the xyl II from Chainia. The chapter has been divided into three sections. Section A describes the fluorometric analysis of the tryptophan microenvironment in xyl II. The structural environment of an essential cysteine residue in xyl II has been deduced in section B while evidence for the existence of an essential carboxyl group has been presented in section C.

Part A

Study Of Microenvironment Of Tryptophan Residue

🕮 Introduction:

Majority of proteins are globular. The folding of a polypeptide chain to form a relatively compact protein inevitably results in the burial of certain amino acid residues from the external aqueous environment. Other residues either by choice or by chance lie on the surface exposed to the polar solvent . For studying the structure of proteins in solution, the mapping of the residues that are exposed or buried is important. Since most proteins contain relatively small number of tryptophan residues , this amino acid is of great use in such topographical studies. To probe the exposure of indole ring , solvent perturbation [19, 20] and chemical modification techniques [21] are often employed. However, there are many limitations and experimental difficulties with these methods. A very promising spectroscopic technique involves the measurement of the degree of quenching of the fluorescence of tryptophanyl residue by the addition of various low molecular weight agents termed as quenchers. These solutes decrease the fluorescence intensity of the residues via a physical contact with the excited indole ring. Hence the ease with which the fluorescence is quenched depends upon the "exposure" of the Trp residues to the quencher .

📄 Basis of protein fluorescence:

The three aromatic amino acids present in a protein are tryptophan, tyrosine and phenyl alanine. These are responsible for the emission of fluorescence after absorption of UV light. The absorption characteristics have been reviewed by Wetlaufer [22]. Absorption of tyrosine is pH dependent. The emission bands of phenyl alanine , tyrosine and tryptophan are at 282 , 303 and 248 nm respectively [23]. The fluorescence characteristics of the aromatic groups in proteins are not identical to those of neutral solutions of the free amino acids. Furthermore, these fluorescence characteristics are changed as the conformations of proteins are modified [23] . In a protein containing all the three aromatic amino acids, the observed emission is due mainly to tryptophan

residue. Proteins containing both tyrosine and tryptophan do not show an emission band due to tyrosine [23]. The fluorescence spectrum of Trp in proteins usually has a maximum at shorter wavelengths than Trp in water. Changes in protein conformation can lead to changes in emission maximum or quantum yield [24, 25, 26]. Studies indicate that once the relationship between fluorescence emission maximum and / or quantum yield and conformation has been established for a particular protein the parameters may be used as a criterion for determining its conformational change [23]. Fluorescence measurements appear to be more sensitive to molecular environment than many other physical methods, and reveal even small structural transitions of proteins . Although it is useful to have techniques available that will report small or large conformational changes in proteins, fluorescence studies of the above type yield more detailed information about changes around the emitting fluorophores.

Tryptophan is a natural built-in probe for quenching studies. Therefore, the effect of reagents which have interaction with the Trp residues present at the active site of the protein provides valuable information of the microenvironment around the essential Trp residue.

Involvement of tryptophan residue in the active site of the low molecular weight xylanase from *Chainia* has been demonstrated earlier [5].

The work described in this chapter aims at (i) deducing the structure-function relationship for the tryptophan residue involved at the active site a) by correlating the effect of N-bromosuccinimide (NBS) on the fluorescence and activity, and b) by assessing the ability of xylan to protect against decrease in fluorescence versus activity of NBS treated enzyme and (ii) probing into the environment of tryptophan residue by studying the quenching of their fluorescence by various solute quenchers in the presence and absence of guanidium hydrochloride (Gdn.HCl).

Reaction with N-bromosuccinimide:

NBS is a strong oxidizing agent which oxidizes the indole residue to oxyindole derivative [1] (Fig. 1).

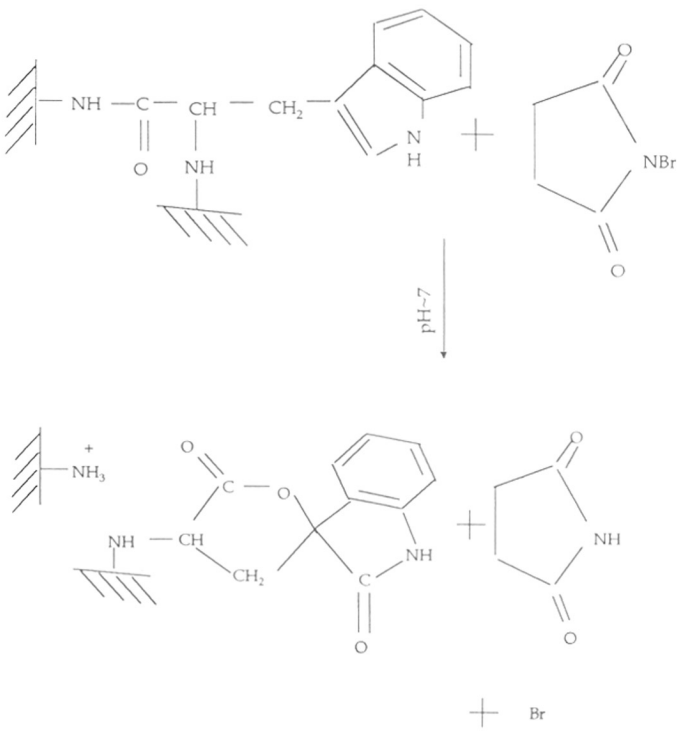

Fig. 1 Reaction of N-bromosuccinimide with Trp residue

This oxidation reaction can be used to probe the accessibility and the reactivity of Trp residues in proteins. The indole chromophore of Trp absorbs strongly at 280 nm which on oxidation with NBS is converted to oxyindole, a chromophore showing weak absorbance at this wavelength. It is also reactive with sulfhydryl residues.

Reaction with Guanidium hydrochloride:

Guanidium hydrochloride (Gdn.HCl) is a known denaturing agent, used to investigate the change in protein conformation. Gdn.HCl, due to its

denaturing action either 'exposes' or 'buries' the Trp residues present in the protein.

Effect of solute quenchers:

Solute quenchers are the chemicals which quench or decrease the native fluorescence of the Trp fluorophore. There are three types of quenchers which are usually used for quenching studies viz. positively charged , negatively charged and neutral. These by collisional or static quenching processes quench the fluorescence of Trp residues. The negatively charged quenchers like iodide (KI) or bromide (KBr), and the positively charged quenchers like CsCl, because of their chemical properties are limited to quench the surface tryptophanyl residues. Also the ability to quench is dependent upon location of neighboring charged groups. The neutral quenchers like acrylamide or molecular oxygen are very efficient quenchers. These quenchers can penetrate the protein matrix without any chemical hindrance and hence can reach the fluorophore buried inside the protein structure [27]. Therefore, quenching studies can be used to reveal details about the microenvironment of the Trp residues.

Materials and Methods:

Materials

NBS, acrylamide, CsCl, KBr, xylan (oat spelt), 1-anilinonaphthalene -8-sulphonate (ANS) and guanidium hydrochloride (Gdn.HCl) were purchased from Sigma chemical Co., St. Louis, MO, USA. beef extract , peptone, yeast extract were also from Sigma Co., Sweden. All other chemicals were of analytical grade.

Purification of enzyme:

The enzyme was purified according to the procedure described by Bastawade [28] with slight modifications. All steps were carried out at 4°C unless otherwise stated . The culture filtrate (500 ml) was obtained by centrifugation of the fermentation broth at 8000xg for 20 min. The supernatant

(7,890 U, 9,850 mg) was concentrated by the addition of three volumes of chilled ethanol. The precipitate was recovered by centrifugation at 10,000xg for 25 min, dried under vacuum and was dissolved in 20 ml of 10 mM phosphate buffer, pH 7.0. The undissolved solid particles were removed by centrifugation and the clear supernatant (7,570 U, 3,788 mg) was treated with DEAE-cellulose equilibrated with 10 mM phosphate buffer ,pH 7.0. The slurry was filtered and the filtrate containing enzyme was collected. The cake was washed with 10 ml of buffer to recover the enzyme. The pooled filtrate (40 ml, 2,705 U, 344 mg) was concentrated under vacuum, and used for further purification by gel filtration using Sephadex G-50 column. 1 ml portion of this enzyme preparation was applied to the column (1.5 x 100 cm) equilibrated with 10 mM acetate buffer, pH 5.8. The fractions were collected at the rate of 2 ml/min and estimated for xylanase activity. The active fractions of low molecular weight xylanase were pooled (902 U, 22 mg) and further concentrated by drying in a vacuum decicator. This enzyme preparation was used for further studies.

Enzyme activity and protein assay:

Two g of xylan was suspended in 100ml of 50mM sodium acetate buffer (pH 5.7) and stirred for 12 to 16 h. The insoluble fraction was removed by centrifugation and the soluble fraction was used for xylanase assay. The xylanase was assayed by incubating 0.5 ml of appropriately diluted enzyme with 0.5 ml of xylan (1%) at 50°C for 30 min [29] . The reducing sugar was estimated by the 3,5-dinitrosalicylic acid (DNSA) method using D-xylose as a standard [30]. One unit of xylanase was defined as the amount of enzyme that produces 1 μmol of xylose min^{-1} from xylan. Protein was determined by the method of Lowry et al [31] and spectrophotometrically [32].

Fluorescence measurements:

Fluorescence measurements were performed with an Aminco SPF-500 automatic recording spectrofluorometer with an excitation and emission

bandwidth of 4 mm in a quartz cuvette. An excitation wavelength of 280 nm was used. For every fresh experiment, blanks for cuvette, enzyme, reagents were determined. Fluorescence spectra were recorded. For quenching studies, fluorescence of the native enzyme was treated as 100 % and decrease in fluorescence was calculated accordingly.

Chemical modification of xylanase with NBS:

The effect NBS on the activity and fluorescence of xyl II was determined after incubation of the purified enzyme (0.5 U) with different aliquots of NBS at 25°C for 15 min in acetate buffer (50 mM). Decrease in fluorescence and residual activity were determined and the graphs were plotted against molar ratio of NBS : xylanase.

Substrate protection studies of xylanase :

The enzyme (0.5U) was incubated with different amounts of xylan (0.01 -0.5 %) for 15 min before the addition of NBS (3.3 X 10^{-5} M). The effect on Trp fluorescence was determined.

Treatment of enzyme with Gdn.HCl

Enzyme (6.5×10^{-7}M) was incubated with different concentrations of Gdn.HCl ranging from 0.05M to 6M at 25°C for 1h in 0.5ml. Emission spectra of the enzyme in presence of Gdn.HCl were recorded in 0.5ml volume in 50mM acetate buffer (pH 5.8). 0.5ml xylan (1%) was added to determine the residual activity.

Quenching of fluorescence

Relative fluorescence intensity of protein (1.31×10^{-5}M) in 50mM acetate buffer was recorded in the absence and presence of varying concentrations of acrylamide, CsCl and KBr. The quenching data was analyzed using Stern-Volmer equation [27, 33].

$F_o / F = 1 + K_{sv}[Q]$

where, F_o is the fluorescence of the protein in the absence of quenchers and F is

the observed fluorescence at the concentration [Q] of the quencher. K_{sv} is the collisional quenching constant which was determined from the slope of the Stern-Volmar plot at lower concentrations of quencher.

Conformational changes in the protein and the accessibility of fluorophore were monitored by analyzing the quenching data using modified Stern-Volmer equation :

$$F_o / \Delta F = \{1 / f_a K_{sv}[Q]\} + 1 / f_a$$

where, f_a is the fractional degree of fluorescence at concentration [Q] and ΔF is the difference in the native fluorescence and that after quenching.

The static quenching constant V was determined by using the following equation :

$$F_o / F = \{ 1 + K_{sv} [Q] \} e^{V[Q]}$$

Quenching of fluorescence in presence of 3M Gdn.HCl was also carried out. The differences in quenching constants and fluorophore accessibility were extrapolated from the Stern-Volmer plots.

Binding of 1-8 ANS to Xyl II:

Enzyme fluorescence was recorded in presence and absence of 1-8 ANS (40 μM) , in the emission range of 400 nm to 660 nm with an excitation wavelength of 355nm.. Fluorescence spectra were also recorded after denaturing the protein by Gdn.HCl (3 M).

Results:

Chemical modification with NBS:

Xyl II from *Chainia* shows an emission maximum at 310nm when excited at 280nm (Fig 2A). Oxidation of Trp in xyl II by NBS resulted in decrease in fluorescence at 310nm (Fig 2D). About 80% of the total fluorescence is abolished at an NBS : xyl II molar ratio of 50 whereas loss in enzymatic activity occurred at a molar ratio of 10 (Fig 3). Increasing amount of xylan offered increased protection for the loss in fluorescence that occurred upon treatment of enzyme with NBS (Fig 2C,B). Xylan (0.5%) gave 100%

protection against inactivation by NBS concomitant to 30% protection for quenching of fluorescence by NBS (Fig 2 B).

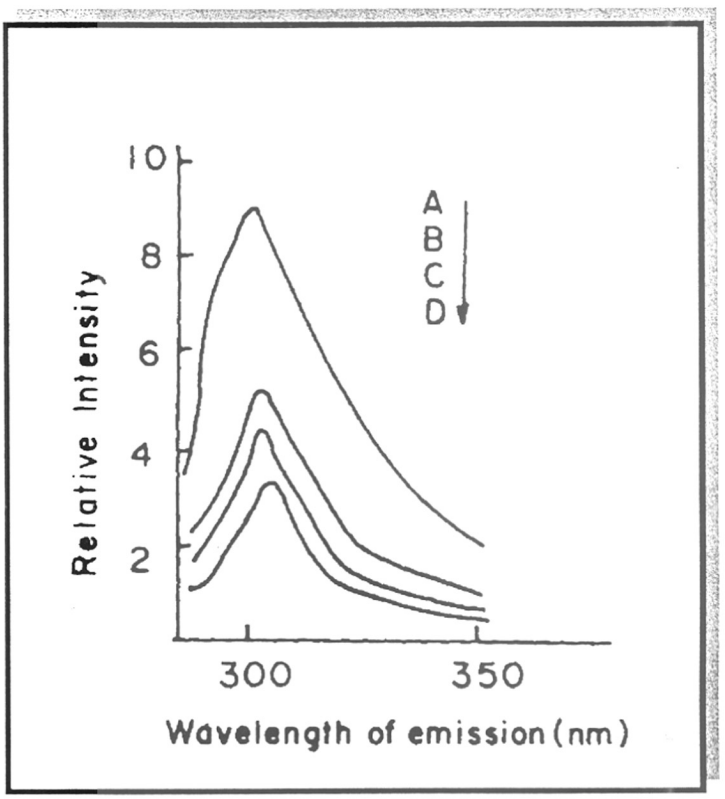

Fig. 2 : Fluorescence of native and NBS-treated xyl II

Native enzyme (A) was treated with NBS (3.3×10^{-5}) (D) in presence of 0.016% (C) and 0.5% (B) xylan

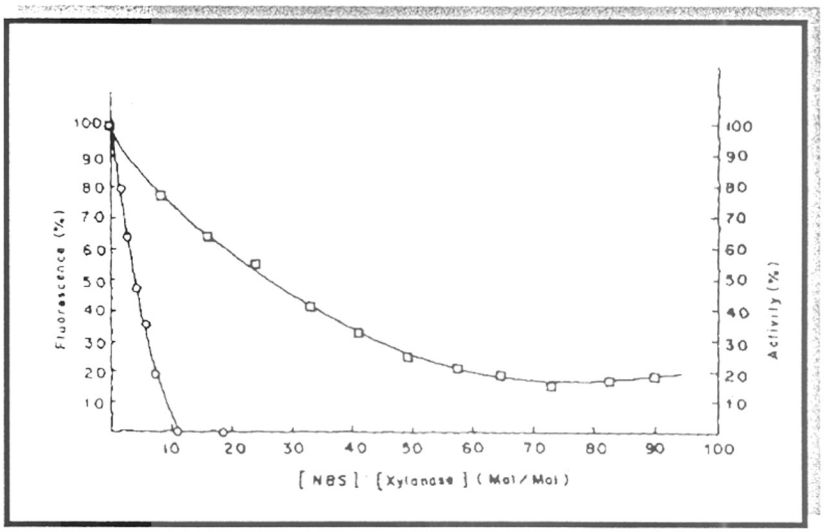

Fig. 3 : Effect of NBS on activity and intrinsic fluorescence of xyl II from *Chainia*

Residual activity (O) and fluorescence (□) after treatment with NBS

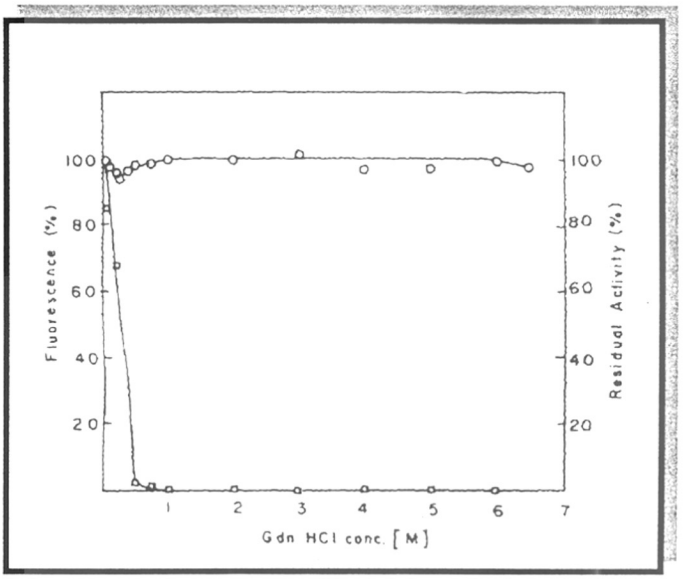

Fig. 4 : Effect of treatment with Gdn.HCl on fluorescence and activity of xyl II

Fluorescence (O), residual activity (□)

Effect of Gdn.HCl on fluorescence and activity of Xyl II:

Fig. 4 shows the effect of Gdn.HCl on the fluorescence and activity of Xyl II. There is no decrease in the tryptophanyl fluorescence of xyl II up to the Gdn.HCl concentration of 6.5 M, whereas there is a rapid decrease in the activity of Gdn.HCl treated enzyme. Complete inactivation of enzyme occurs at 750mM Gdn.HCl.

Quenching of fluorescence :

Treatment of xyl II with acrlamide, CsCl and KBr resulted in quenching of fluorescence (Fig 5). 25% quenching was obtained with 20mM acrylamide, 150mM CsCl and 500mM KBr indicating that acrylamide was the most efficient quencher. The enzyme retained full activity in presence of acrylamide (100mM) and the observed quenching in fluorescence by acrylamide was not due to the different K_{sv} and f_{eff} values for low and high concentrations of the quenchers (Fig. 5B-c, C-c). The K_{sv} and f_{eff} values as extrapolated from the graph are shown in Table 1. In presence of 3M Gdn.HCl, there is an increase in the efficiency of quenching in case of acrylamide (Fig. 5A-a), a slight decrease in efficiency in case of CsCl (Fig. 5B-a) whereas no change in the efficiency of quenching by KBr (Fig. 5C-a). However, there is an overall increase (approximately 3 to 5 fold) in the collisional quenching constant (K_{sv}) in the presence of Gdn.HCl. There was no change in the fluorescence of ANS , before and after treatment with Xyl II.

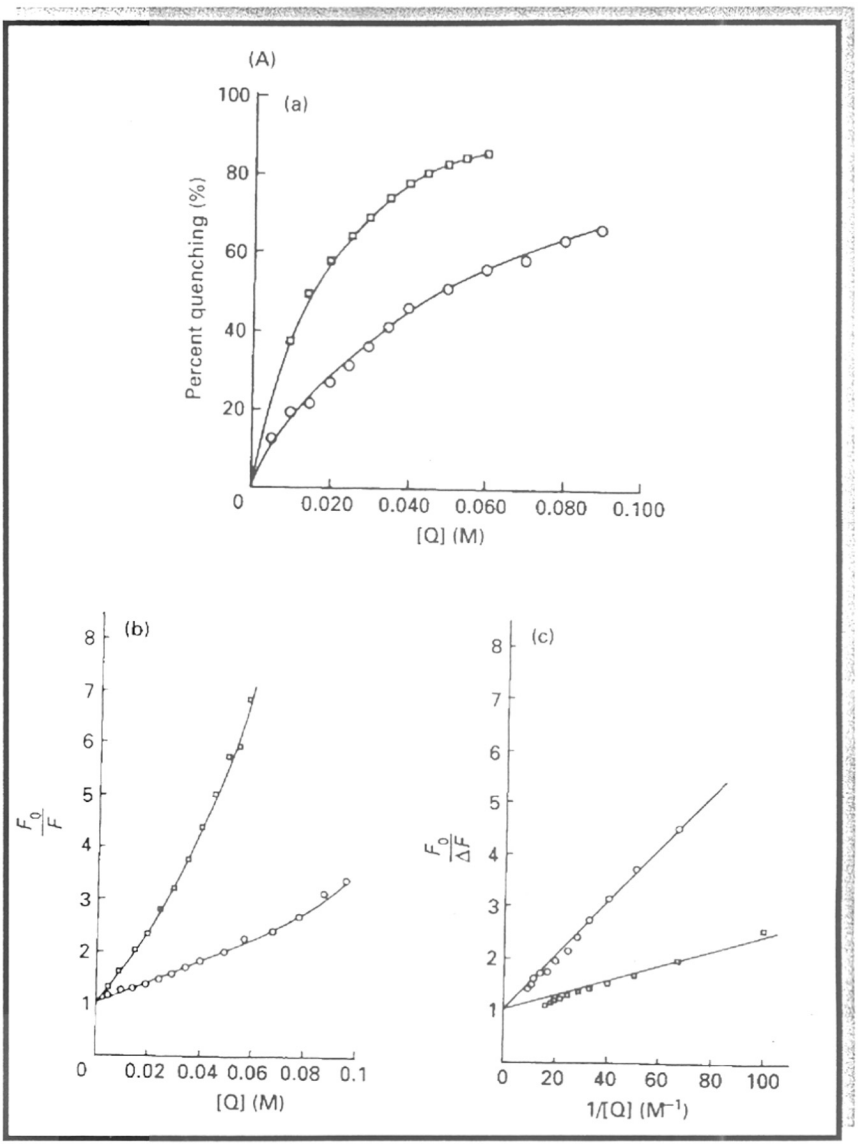

Fig. 5 : (A) Effect of Acrylamide on fluorescence of enzyme (○) in presence of Gdn.HCl (□)

(a) Effect of addition of acrylamide on enzyme fluorescence, (b) Stern-Volmer plot, (c) modified Stern-Volmer plot

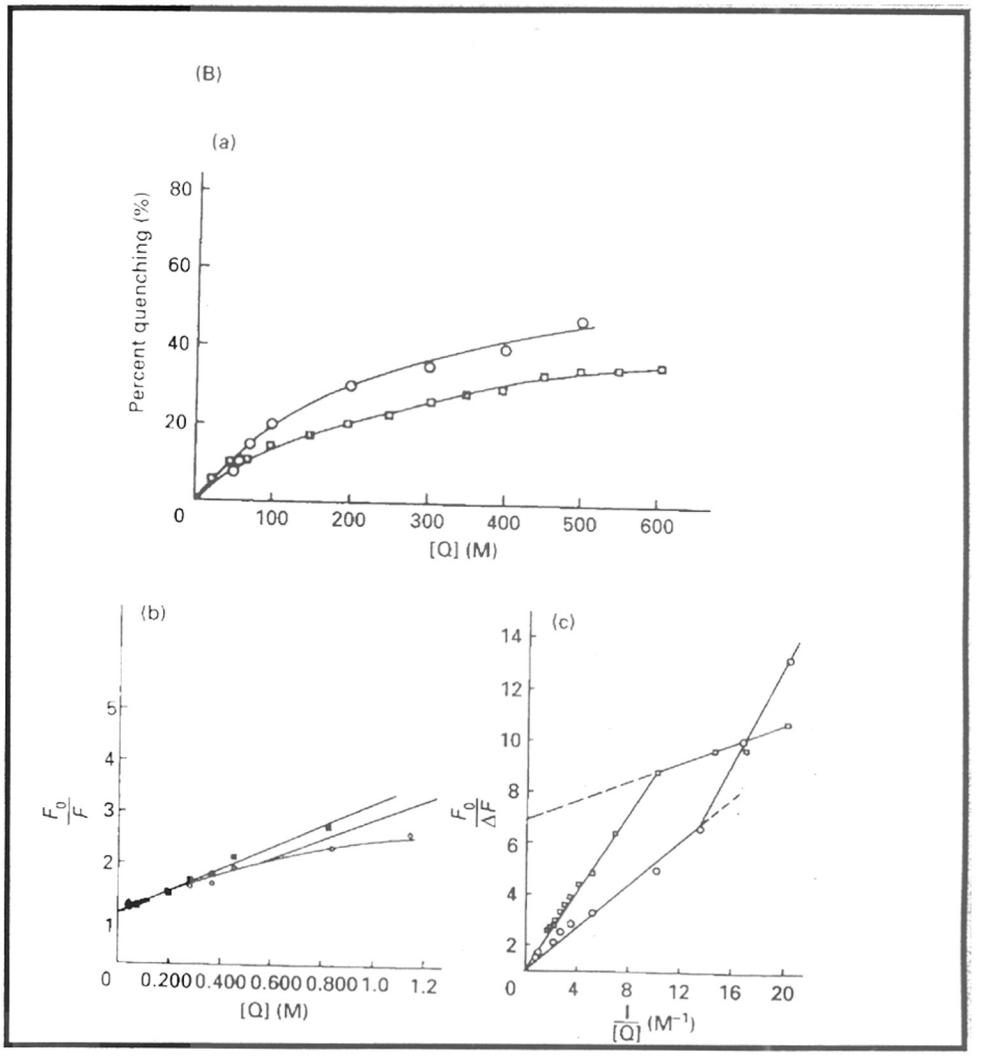

Fig. 5 : (B) Effect of CsCl on fluorescence of enzyme (○) in presence of Gdn.HCl (□)

(a) Effect of addition of CsCl on enzyme fluorescence, (b) Stern-Volmer plot, (c) modified Stern-Volmer plot.

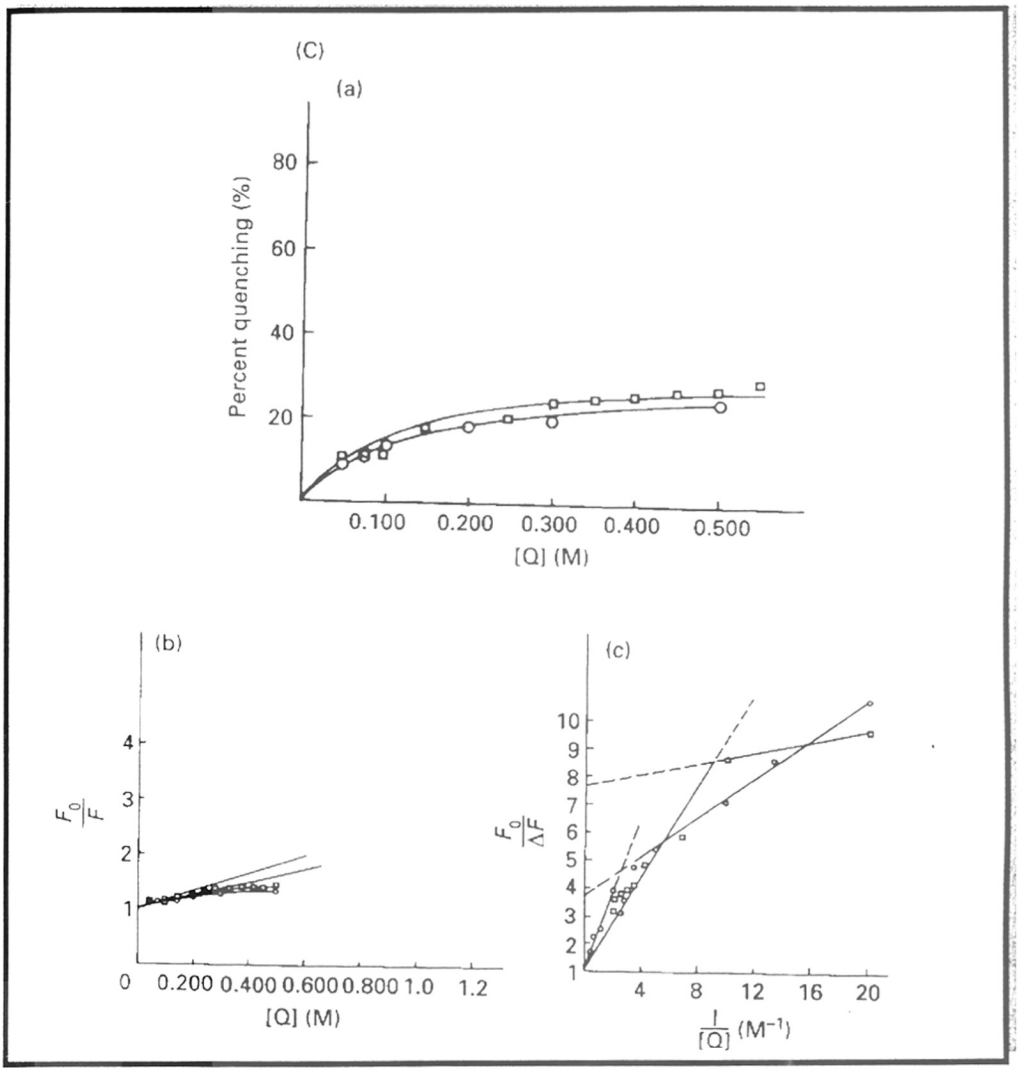

Fig. 5 : (C) Effect of KBr on fluorescence of enzyme (○) in presence of Gdn.HCl (□)

(b) Effect of addition of KBr on enzyme fluorescence, (b) Stern-Volmer plot, (c) modified Stern-Volmer plot.

Table 1: Quenching parameters

Quencher concentration	Gdn.HCl (3 M)	K_{sv}	V	f_{eff}
Acrylamide (5-100 mM)	-	19.23	0.785	1
Acrylamide (5-100 mM)	+	70	2.5	1
CsCl (50-75 mM)	-	1	1.27	ND
CsCl (50-100 mM)	+	5.6	3.23	0.15
CsCl (100 mM-1.5 M)	-	2.5	-0.34	1
CsCl (100 mM-1.5 M)	+	1.23	-0.15	1
KBr (50-385 mM)	-	2.85	-1	0.27
KBr (50-111 mM)	+	9.33	-5.44	0.13
KBr (385 mM-1.5 M)	-	0.75	0.33	1
KBr (111 mM-1.5 M)	+	1.14	-0.17	1

🕮 Discussion:

The low molecular weight xylanase from *Chainia* contains three Trp groups based on its amino acid composition [28] . Chemical modification of xylanase by tryptophan-specific reagents such as NBS and hydroxy nitro-benzyl bromide and the detailed kinetic analysis of the modified enzyme according to Levy et al [34] revealed the participation of one Trp group in the active site of the enzyme [5]. In the present work , we have investigated the structure-function relationship of the xylanase by correlating the loss in fluorescence and activity of the NBS-treated enzyme. Treatment of enzyme with NBS leads to loss in both activity and fluorescence. However, it was observed that complete inactivation precedes the loss in fluorescence (Fig 2)

corroborating our earlier results that not all tryptophans are essential for activity. This has been further substantiated by the substrate protection studies. Xylan gives full protection to the enzyme against inactivation by NBS but is able to offer only 30% protection for the loss in fluorescence, probably due to the binding of xylan to the enzyme through Trp residue present at the active site rendering its indol group inaccessible for oxidation by NBS (Fig 1 B). These studies provide full support to our earlier observation that a single Trp is essential for the activity of xylanase from *Chainia* and it is involved in the substrate-binding region of the enzyme. It has been shown in glycanases that Trp is essential for binding of the enzyme to substrate for carrying out hydrolysis [35]. The residual fluorescence (20%) observed at higher molar ratio (66 : 1) of NBS : Xylanase indicates that a part of the fluorophore is inaccessible to modification by NBS and is situated in a relatively rigid environment.

The Trp residues in Xyl II from *Chainia* exhibit unique spectroscopic features. The enzyme shows a fluorescence maximum at 310nm upon excitation at 280nm. This fluorescence maximum is the most blue shifted signal observed so far in glycanases [35, 36]. Other examples of such high energy emission are those of RNAase T [37], azurin [38] and paralbumin [39]. Buried Trp residues in proteins usually show emission maxima in the range of 325 to 335nm [40]. Emission maximum of L-Trp in water is 340nm. The Trp emission maximum in a protein is highly influenced by its conformation and the amino acids present in the vicinity of the Trp residues [23]. Interaction between the ground or excited states of aromatic groups with side chains of neighboring amino acids in the protein are known to occur [41]. In case of xyl II, it has been shown that amino acids containing carboxyl groups [42] and cysteine [43] are present at the active site along with tryptophan. These amino acids may be responsible for emission of high energy photons in the microenvironment of the Trp resulting in a lower emission maximum of 310nm.

Gdn.HCl is known to react reversibly with the carboxyl groups of the

amino acids [44]. At low concentrations of Gdn.HCl, Trp residues are shielded by the interaction of Gdn.HCl with carboxyl amino acids, present at the active site . This may be responsible for the initial decrease in fluorescence. As the Gdn.HCl concentration is increased the conformational changes occurring in the protein due to its unfolding, may result in the regain of Trp fluorescence.

The emission maxima of native and denatured Xyl II were alike suggesting that the Trp residues in the enzyme are present in a highly rigid environment. To determine the polarity and accessibility of these fluorophores, quenching of fluorescence with neutral (acrylamide), negatively charged (CsCl) and positively charged (KBr) solutes was investigated. Solute quenching of protein fluorescence is a technique that can yield information regarding the exposure of protein-bound intrinsic or extrinsic fluorophore. It is a dynamic method since the presence of solutes alters the fluorescence properties of the protein. In presence of acrylamide, xyl II shows an upward curving Stern-Volmer plot, indicating the occurrence of both collisional and static quenching processes and the presence of more than one fluorophore in the protein. While analyzing heterogeneously emitting systems like Xyl II, it is necessary to consider the collisional quenching constant k_a(the association constant for the formation of one : one complex between the quencher and the fluorophore) for each individual fluorophore. If K_a is small, the association is weak and vice versa. However, it is difficult to determine K_a for each individual fluorophore. K_{sv} represents the resultant effective collisional quenching constant for all the fluorophores and can be determined from the Stern-Volmer plots. The K_{sv} value for acrylamide quenching (19.23) is much higher than those for CsCl (1) and KBr (2.85) at low concentrations of the quencher, indicating that acrylamide is an efficient quencher. One of the most attractive features of acrylamide as a quencher has been the fact that it does not interact significantly with proteins [27]. Retention of full enzyme activity and emission peak at 310 nm in presence of acrylamide indicate that quenching of fluorescence by acrylamide is a physical process. V represents

the active volume element surrounding the excited fluorophore which can be determined from K_{sv} values. Increase in K_{sv} and V values in case of acrylamide and CsCl, in presence of Gdn.HCl indicates increased accessibility for the fluorophores upon unfolding (Table 1). However, the decrease in V value in case of KBr shows its inability to reach the fluorophore even after unfolding of the protein. The bimodal modified Stern-Volmer plots in case of CsCl and KBr suggest that quenching of fluorescence by these two solutes is highly affected by their concentrations in the reaction mixture giving two different linear plots for low and high concentration ranges. Almost linear Stern-Volmer plots, in case of KBr indicate very little static quenching, which is also evident from the negative V values. As KBr is a negatively charged quencher, this situation will arise if the microenvironment of Trp is also electronegative thereby not allowing the quencher to come into the active volume element of the fluorophore. Higher efficiency of quenching by CsCl as compared to KBr also points to the presence of electronegative environment around Trp residues in Xyl II.

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Part B

Structural Environment Of An Essential Cysteine Residue

🕮 Introduction:

Various kinds of approaches have been used to identify the active site peptides of proteins. Use of radioactive labels such as ^{32}P labeled di-isopropylfluorophosphate was employed by Cohen and Warringa for determining active site peptide of choline esterase [1]. Second powerful tool for determining structure-function relationship is affinity labeling. These reagents are structurally similar to the substrate. Affinity labeling takes the advantage of the normal enzyme-substrate interactions to ensure that the large local concentration of reagent exists at the active site. It depends upon the affinity label to form a stable linkage with the enzyme [2, 3, 4]. Fluorescence labeling procedures have also been developed to bind certain fluorescent dyes to proteins as a means to increase fluorescence of proteins in which the natural fluorescence is absent [4] . Because of the known reactivity of the alkylating agents with cysteine, they have wide applications in the protein chemistry. The wide use of 1-fluoro-2,4-dinitrobenzene (FDNB) for labeling amino groups with the yellow DNP residue and for characterising the N-terminal amino acids in polypeptide chains indicated the value of coloured reagents in protein chemistry [5]. N-ethyl maleimide (NEM) is a potent modifier for cysteine residues. NEM reacts rapidly with thiols forming a stable thioether link and can very well be used for modification of active site thiol groups of proteins [6, 7]. However, NEM produces a colourless derivative. A number of other substituted maleimides have proven useful, since they introduce a chromogenic substituents into the protein. N-(2,4- dinitroaniline) - maleimide (DAM) [8] is one such coloured derivative. This reagent allows modification of sulfhydryl groups in the protein and ready detection of the appropriate peptide, after proteolytic degradation and separation [9].

Reports regarding presence of cysteine at the active site of xylanase are available [10 - 13]. It has been shown that tryptophan and cysteine residues are present at the active site of *Chainia* xylanase [14 - 16]. This chapter deals with the isolation and identification of amino acids involved in the cysteinyl active

site peptide using a chromophoric reagent.

Materials and Methods

Materials:

DAM (Fig. 1) was synthesized in our laboratory according to Clark-Walker and Robinson [8]. Xylan (Oat spelt), pepsin and 3,5-dinitrosalicylic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex G-25 and G-50 were obtained from Pharmacia (Uppsala, Sweden). Pepsin and standard amino acid mixture were purchased from Sigma Chemical Co., USA All other chemicals used were of analytical grade.

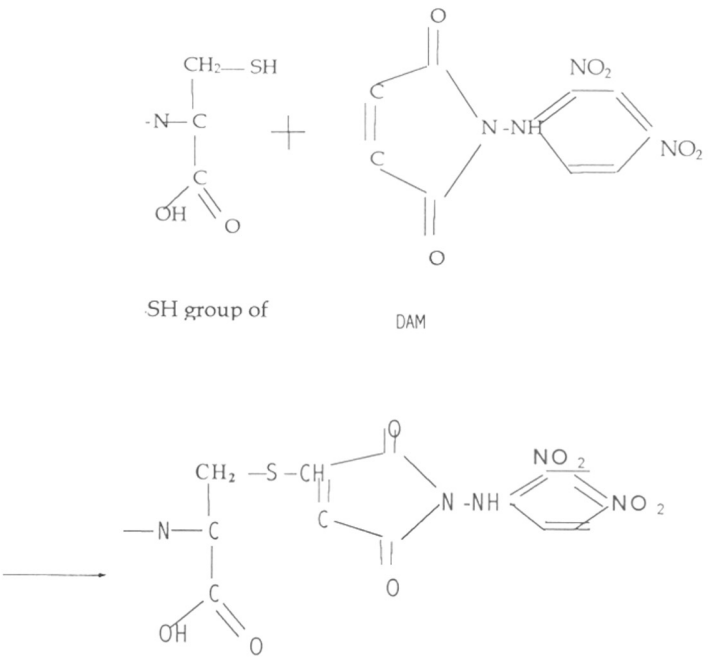

Fig. 1 Reaction of DAM with the thiol group of cysteine

The product formed is unable to create a phenylthiohydantion

Purification of enzyme and assay:

Purification of Chainia xylanase and its assay were carried out as described earlier in Chapter II, Part A.

Kinetics of inactivation of xylanase by DAM :

The purified xylanase (5μg) was incubated with different concentrations of DAM in 50 mM sodium acetate buffer pH 5.8 in a total volume of 2.5 ml at 25°C Aliquots of 0.5 ml were removed periodically and the residual activity was determined by the addition of 0.5 ml of xylan (1%) followed by incubation at 50°C for 30 min. The pseudo-first-order rate constant (K) was determined from the slope of the plot of logarithm of the residual activity against time of reaction. The second order rate constant was calculated from the slope of the plot of pseudo-first-order rate constant against concentration of the inhibitor [17]. Controls containing enzyme or DAM alone were simultaneously run. DAM was dissolved in methylcellosolve. The amount of methylcellosolve carried in the reaction mixture had no effect on the enzyme activity.

Substrate protection of xyl II against inactivation by DAM:

The xylanase (5 μg) was incubated with different amounts of xylan (1 - 5 mg) for 10 min at 4°C. 10 μl of DAM (500 μM) was added and the reaction mixture was incubated at 25°C for 15 min in a total volume of 250 μl. The xylanase activity was estimated by adding xylan to a final concentration of 5 mg per reaction mixture. Parallel controls for enzyme activity for various amounts of xylan in the absence of DAM were run.

Modification of xyl II with DAM :

The enzyme (20 mg) was treated with 5 ml of DAM (50 mM) for 3 h at 25°C in a total volume of 10 ml in 50 mM sodium acetate buffer, pH 5.0. The modified enzyme was precipitated with 3 volumes of ethanol at O°C. The precipitated protein was recovered by centrifugation at 10,000 g for 30 min and was dried under vacuum. The activity and the absorption spectra of the native and modified enzyme were monitored.

Proteolysis of DAM-treated xyl II

The yellow precipitate of DAM-treated enzyme was dissolved in 2.5 ml

of 10 mM HCl and digested with 400 µg of pepsin at a protein to proteinase ratio of 10:1 (w/w) at 37°C for 24 h at pH 2.0.

Gel filtration on Sephadex G-25

The peptic digest of DAM-treated xylanase was subjected to gel filtration on a Sephadex G-25 column (25 x 25 cm) in water. Fractions (2.5 ml each) were collected at the rate of 30 ml h^{-1} and analyzed for absorption at 210 and 340 nm. Fractions showing maximum absorption at 210 nm and 340 nm were pooled and processed separately.

Purification of cysteinyl peptide by HPLC

The pooled preparation of yellow fractions from Sephadex G-25 column was further purified by RP-HPLC on a vydac 218TP510 semi-preparative column (10 x 250 mm; 5 µm, 3000 A) with Beckman system consisting of two model 112 pumps with a model 420 gradient controller. The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) and water in 0.1% TFA. The eluted peaks were detected with a model 165 variable wavelength detector and displayed on a Hewlett-Packard model 3390 A integrator.

Sequencing of cysteine-containing peptide

Sequence analysis of the HPLC-purified peptide was carried out by automated gas-phase Edman sequencing with an Applied Biosystems model 477A sequencer.

Results:

Purification of enzyme :

The low molecular weight xylanase (xyl II) from *Chainia* was purified by ethanol precipitation and DEAE-cellulose chromatography followed by gel filtration on Sephadex G-50. The homogeneity of the enzyme was confirmed by SDS-PAGE [18]. A total purification of 9.2 fold over the crude culture filtrate was obtained and the specific activity of the purified enzyme was 41 U/mg with the final enzyme yield of 11%. The molecular weight of xylanase

from *Chainia* was determined to be 5000-6000 by gel filtration and SDS-gel electrophoresis [19, 20].

Chemical modification of xylanase by DAM

Reaction of the low molecular weight xylanase from *Chainia* with *p*-hydroxy-mercuric-benzoate and subsequent kinetic analysis implied the presence and participation of 1 mol of cysteine in the active site of the enzyme [14]. In the present work, the functional cysteine group was labeled by reaction with DAM, a chromophoric thiol-specific derivative of NEM. The treatment of xylanase with DAM resulted in progressive inhibition of enzyme activity with increasing concentration of the reagent (Fig.2).

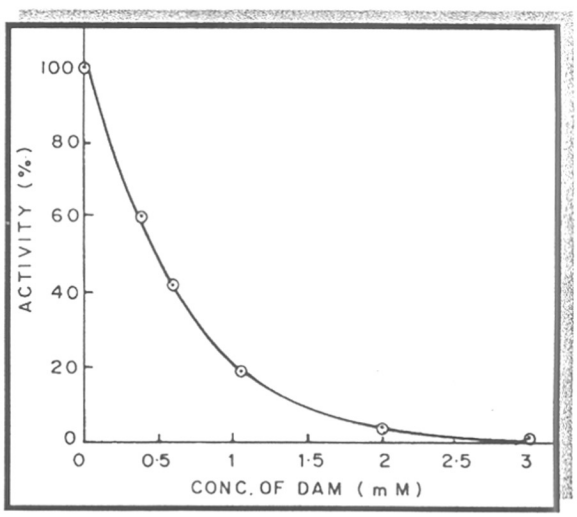

Fig. 2 : **Effect of treatment of DAM on the activity xyl II from *Chainia***

A double logarithmic plot of the observed pseudo-first-order rate constant against reagent concentration (Fig. 3) yielded a reaction order of 0.95 (Fig 3, inset). The second order rate constant for the inactivation of the xylanase by DAM (Fig. 4) was calculated to be 3.61 X 10^3 M^{-1} min^{-1}. The DAM-treated enzyme showed complete inhibition of the enzyme activity with a concomitant increase in absorbance at 340 nm (Fig. 5) characteristic of DAM-cysteine and corresponded to an incorporation of 0.9 mol of DAM mol^{-1} of enzyme.

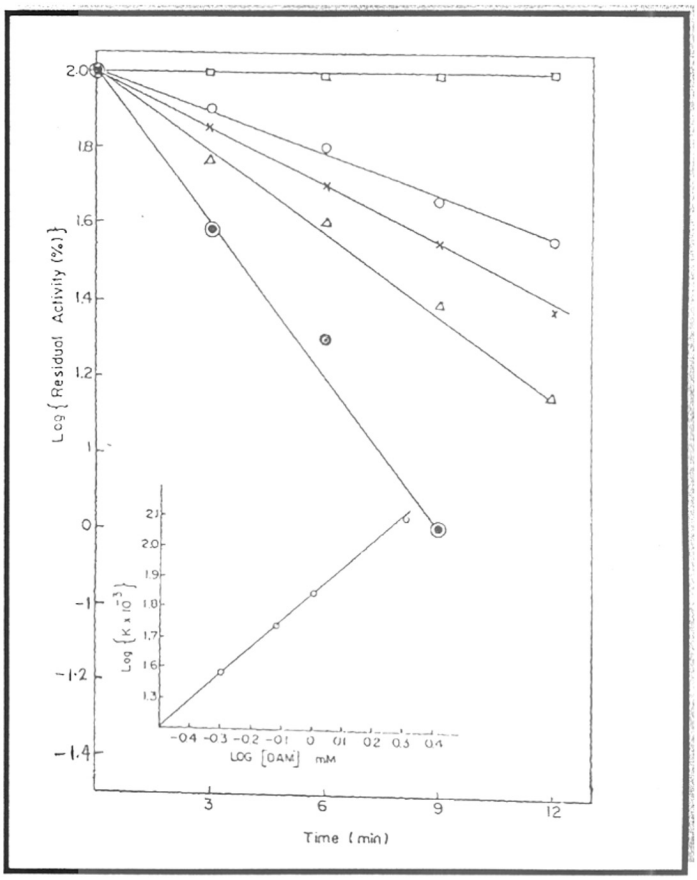

Fig. 3: Kinetics of inactivation of xylanase by DAM.

Enzyme (5 μg) was preincubated at 25°C with DAM (0.5to 3mM) (□, mM;.○, 0.5 mM; ▽, 1 mM; △, 2 mM; ⊙, 3 mM) The inset shows the apparent order of reaction with respect to reagent concentration. The logarithms of observed pseudo-first order rate constants (k) calculated from Fig 2 were plotted against the logarithm of the inhibitor concentration.

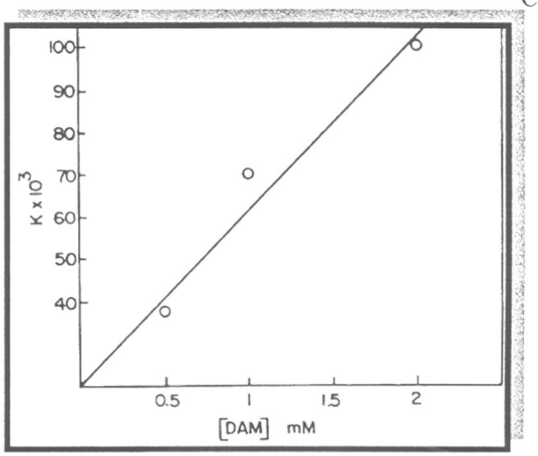

Fig. 4 : The second order rate constant for inactivation of xyl II by DAM

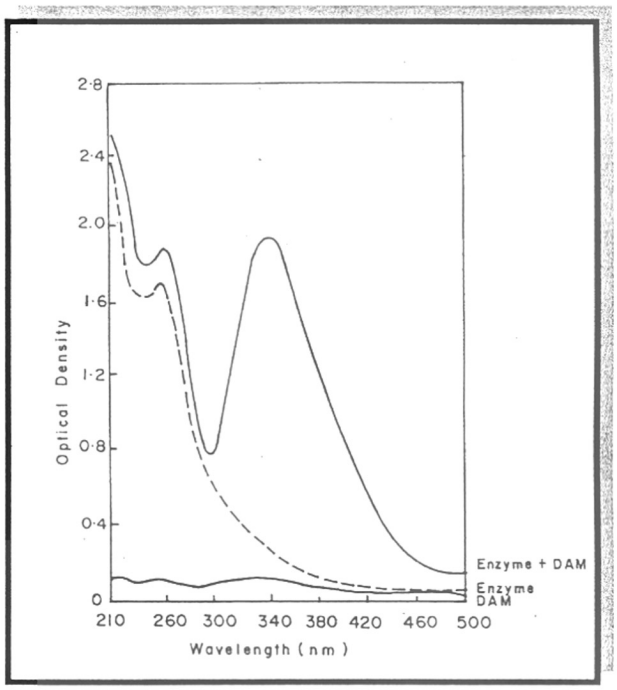

Fig.5 : Absorption spectrum of the native and modified enzyme

The enzyme (20 mg) was incubated with 5 ml of DAM (50 mM) in a total volume of 10 ml in 50 mM sodium acetate buffer, pH 5.0 for 3 h at 25°C and precipitated with ethanol. 'Optical Density' ≡ absorbance

Evidence for the presence of cysteine in the active site :

Fig. 6 depicts the results of protection of xylanase by varying amounts of xylan against inactivation by DAM. A progressive protection of inactivation by DAM was observed with increasing amounts of xylan indicating that xylan and DAM compete for the same binding site viz. the cysteine residue in the active site. Xylan (5 mg) offered complete protection against inactivation of the enzyme by DAM. These results confirm that the cysteine is present at or near the substrate-binding region of the enzyme and the thiol modified by DAM is in the active site.

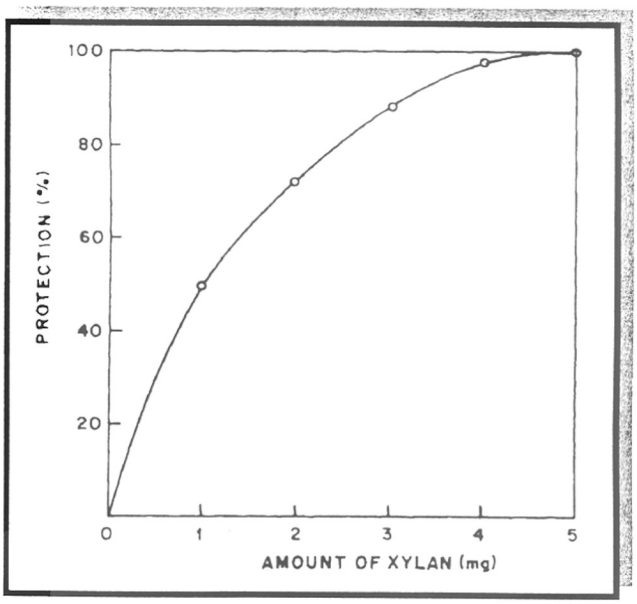

Fig. 6 : **Protection of xylanase by substrate against inactivation by DAM**

The xylanase (5 μg) was incubated with varying amounts of xylan for 10 min at 4°C prior to incubation with 10 μl of DAM (500 μM) at 25 °C for 15 min, in a total volume of 250 μl. The xylanase activity was assayed by making xylan to the final concentration of 5 mg per reaction mixture.

Isolation and purification of the cysteine-containing peptide

In order to locate the reactive cysteine-containing peptide, the DAM-treated enzyme was hydrolyzed with pepsin. The mixture of peptides was subjected to gel filtration on a Sephadex G-25 column. (Fig. 7).

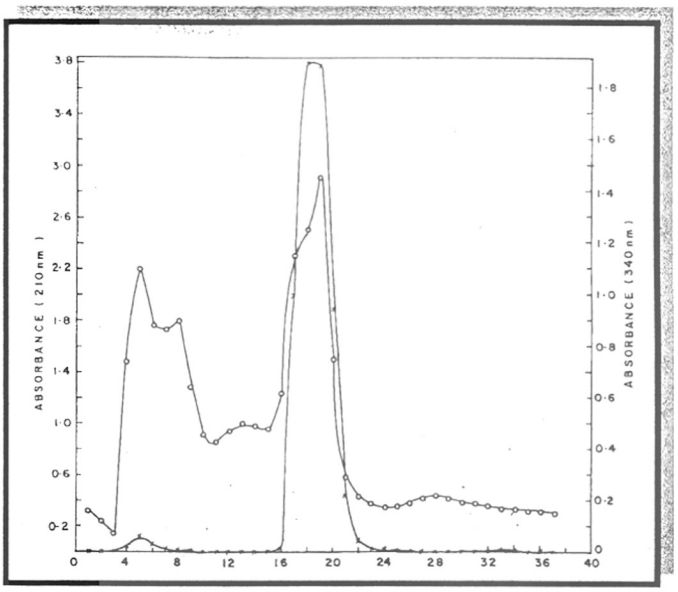

Fig. 7: Gel filtration on Sephadex G-25 of peptic peptides of modified xylanase.

DAM treated enzyme (4 mg) was digested with pepsin (400 μg) at 37°C for 24 h at pH 2 and the digest was subjected to gel filtration on sephadex G-25. Fractions (2.5 ml) were collected and monitored for absorption at 210 nm (O) and 340 nm (X).

The fractions showing distinct yellow color and higher A_{340}/A_{210} ratio were pooled and lyophilized. Further purification of the yellow peptide was achieved by its separation on HPLC (Fig. 8). The peptide containing the DAM-cysteine (peak A) was separated from the unreacted DAM (Peak B).

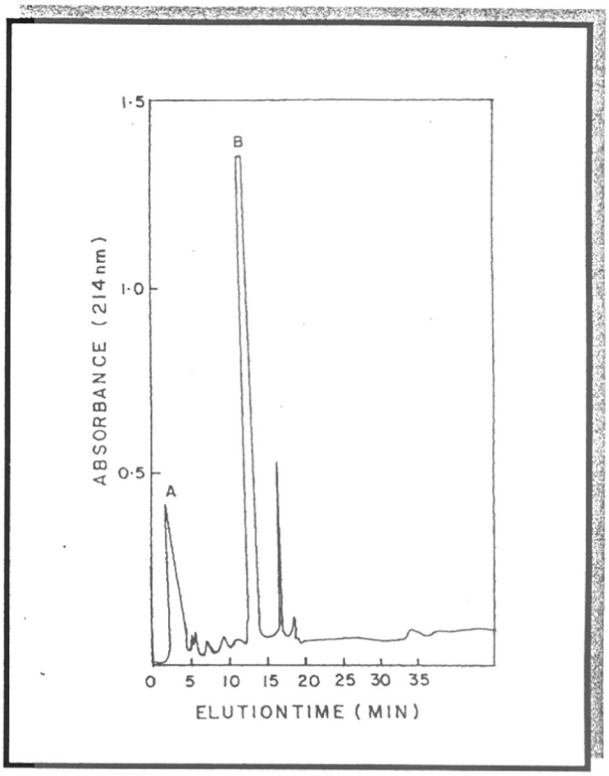

Fig. 8: Purification of the cysteinyl peptide by RP-HPLC

The pooled yellow fraction from sephadex G-25 column was subjected to RP-HPLC according to the conditions described in Materials and Methods.

Sequencing of the cysteine-containing peptide

Analysis of the cysteinyl peptide (Fig 8, Peak A) yielded the sequence Glu-Thr-Phe-Xaa-Asp. The Xaa represents a modified cysteine residue which is unable to form a phenylthiohydantoin derivative.

🕮 Discussion:

The xylanase from *Chainia* belongs to the G family of xylanases which encompasses the low molecular mass, basic xylanases, most of which have been isolated from *Bacillus* sp. [21]. Xylanases are functionally related to cellulases and lysozyme. Tryptophan and carboxylic acids have been shown to be involved in the catalytic mechanism of xylanases, cellulases and lysozyme. However, involvement of cysteine residues in the active site of xylanases appears to be restricted only to actinomycetes xylanases [13, 14]. The xylanase from *Cellulomonas fimi* has been shown to contain distinct cellulose and xylan-binding domains [22]. The protein sequences of the catalytic domains of cellulases and xylanases have been compared [23]. However, the sequences around the functional residues have not been delineated. As a step towards the understanding of structure-function relationship of xylanases, the primary objective of the present work was to decipher the structural environment of the functional cysteinyl residue in the xylanase from *Chainia* and compare it with that of xylanase from *Streptomyces*. NEM is a potent modifier for cysteine residues. However, it is of little value as a marker of such groups as it forms colourless derivatives. The strategy used in the present work was to use DAM, the chromophoric derivative of NEM, as a reporter for isolating the peptide containing the cysteine group. The inability of DAM to inactivate the enzyme in the presence of xylan provides evidence that the modified thiol is in the active site. The taxonomic status of *Chainia* is controversial. While chemical and genetic data indicate a close relationship between the genus *Chainia* and *Streptomyces*, the development of vegetative mycelium into sclerotic granules and the presence of 2,3-diaminopropenoic acid in the cell wall are the main distinguishing features of *Chainia*. Our results reveal that although both the cysteinyl peptides contain phenylalanine and the aspartic acid residues, the sequence of amino acids around the functional cysteine of xylanase from the thermotolerant *Streptomyces* T-7 [13] is distinctly different from that around the

reactive cysteine of xylanase from *Chainia*. Comparison of the partial amino acid sequence of the xylanase from *Chainia* with the catalytic regions of xylanases from alkalophilic *Bacillus* sp. [25] *Clostridium thermocellum* [26] and *Cryptococcus albidus* [27, 28] revealed a homologous region containing one aspartic and two glycine residues as the highly conserved residues. The active site cysteinyl peptide from *Chainia* shows the presence of conserved aspartic acid residue but lacks the universal glycine residues. Whether the essential tryptophan residue of the xylanase from *Chainia* lies in the neighborhood of the cysteine or is distributed distantly on the polypeptide chain and constitutes a substrate-binding pocket on folding of the protein would await collection of sequencing data for peptide containing the essential tryptophan residue. Nevertheless, the knowledge of amino acid sequence around the reactive cysteine will be useful for deciphering the structure-function relationship of the xylanase using site-specific mutagenesis.

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Part C

Evidence For Essential Carboxylate Residue

Introduction:

Xylanases are hydrolytic enzymes which are grouped under glycanases. The glycanases hydrolyze the polysaccharides through the acid/base catalysis by the carboxyl amino acids. Carboxyl groups have been implicated to play a catalytic role in the active site of cellulase [1] and lysozyme [2] which are functionally related to xylanases. Tryptophan and cysteine have been shown to participate in the active site of *Chainia* xylanase, [Part A & B]. This chapter presents evidence for the presence of essential carboxyl group at the substrate binding site of the *Chainia* xylanase based on the inhibition of the xylanase by Gdn.HCl and Woodward's reagent K (WRK) and establishes its close structural relationship with glycosidases.

Materials and Methods

Materials

WRK (N-ethyl-5-phenylisoxazolium-3'-sulphonate), p-hydroxyl-mercuric-benzoate (*p*HMB), Gdn.HCl, urea and other chemicals used were of analytical grade. Purification of enzyme and assay were carried out as described earlier in this chapter in Part A.

Reaction of xylanase with Gdn.HCl

The xylanase (10 µg) in 0.5 ml of 50 mM sodium acetate buffer, pH 5.7 was incubated with different concentrations of Gdn.HCl or SDS for 15 min. The residual activity was determined by addition of 0.5 ml of xylan (1%) under the standard assay conditions. The enzyme sample incubated in absence of denaturant served as a control. The kinetics of inhibition of the enzyme was studied by estimating the activity in the presence of xylan in the range of 0.1 to 1.8 mg/ml in presence and absence of 0.4 M Gdn.HCl. The PHMB modified xylanase was prepared by treatment of the enzyme with PHMB (1 mM) till there was no increase in absorbance at 250 nm. The reaction of WRK with PHMB- modified xylanase before & after preincubation with Gdn.HCl (0.4 M) for 15 min was monitored by increase in absorbance at 340 nm.

Fluorescence spectra of the enzyme (120 μg) in the presence and absence of Gdn.HCl were recorded at excitation wavelength of 295 nm using Aminco SPF 500 spectrofluorometer.

Modification of xylanase by reaction with WRK

The xylanase (10 μg) in 0.5 ml of 50 mM sodium phosphate buffer (pH 5.7) was incubated for 30 min with various concentrations of WRK (freshly prepared in l mM HCl). Aliquots were withdrawn at suitable intervals of time and the residual activity was determined under the standard assay conditions. Control tubes containing enzyme with HCl in the absence of WRK were also run. The corresponding amounts of HCl had no effect on the stability and activity of the enzyme during the incubation period. The degree of modification of enzyme was measured by monitoring the increase in absorbance at 340 nm assuming a molar absorption coefficient of WRK to be 7000 $M^{-1}cm^{-1}$ [3].

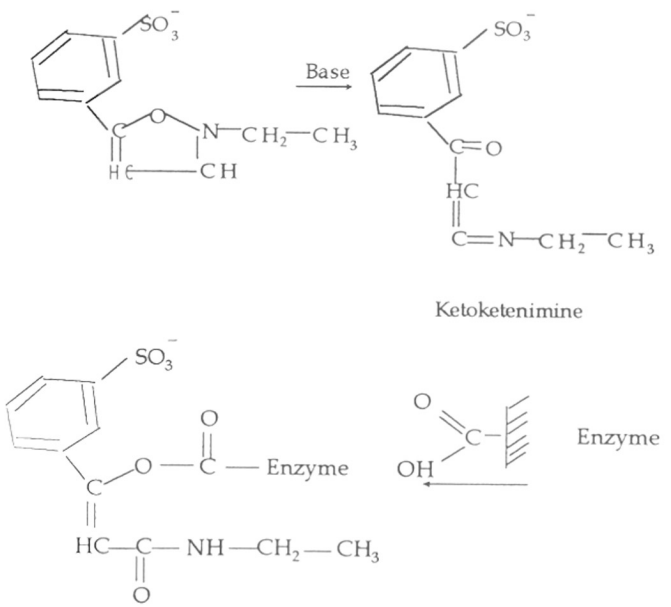

Fig. 1 Reaction of WRK with xylanase

Results and Discussion

Effect of pH on the kinetic functions

The pH dependence of kinetic parameters of the xylanase activity was studied in the pH range of 4 to 10.0. The plot of log Vmax/Km *Vs.* pH revealed the presence of critical ionizable groups with pKa values of 5.4 and 7.2 (Fig. 1). The lower pKa value (5.4) indicated the likely involvement of a carboxyl group in the mechanism of catalysis.

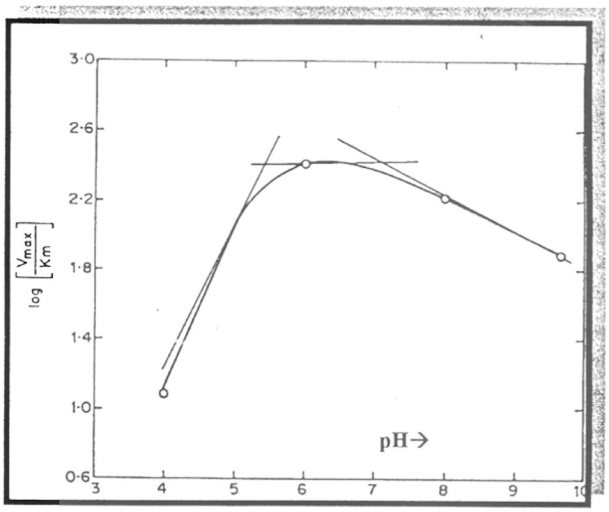

Fig.2 : Effect of pH on the kinetic parameters of xylanase activity
The Km and Vmax at different pH were determined from Lineweaver Burk plots.

Interaction of Gdn.HCl with xylanase

Specific interaction of Gdn.HCl with the carboxyl groups of proteins has been demonstrated [4]. The reaction is more specific than the conventional carboxyl specific reagents such as carbodiimide or trialkyl oxonium fluoroborate. Therefore, the participation of carboxyl groups in the mechanism of catalysis of *Chainia* xylanase was investigated using Gdn.HCl as a carboxyl-specific reagent. The xylanase was inhibited to 60% by 0.4 M Gdn.HCl (Fig. 3).The extent of inactivation of the enzyme was higher at lower (< 1 M) concentrations of Gdn.HCl. SDS (up to 4%) had no effect on the enzyme activity indicating

that the observed inactivation of the enzyme by Gdn.HCl was not due to its denaturation but was due to the specific interaction of the guanidino group with the carboxyl group of the enzyme essential for activity. The fluorescence emission spectrum of the xylanase on excitation at 295 nm was unaffected by treatment with 0.4 M Gdn.HCl. Thus the observed loss in activity of 0.4 M Gdn.HCl-treated enzyme cannot be attributed to the structural changes in the protein. NaCl, KCl or LiCl (0.4 M each) did not inhibit the xylanase ruling out the possibility that the inhibition of enzyme by Gdn.HCl is due to the salt effect and supporting the view point that it is due to the interaction of Gdn.HCl with the carboxyl groups of the enzyme. Inhibition of the xylanase by 0.4 M Gdn.HCl in the presence of different concentrations of xylan was purely competitive (Fig. 4). In the presence of Gdn.HCl, the Km of the enzyme increased to 13 from 5 mg/ml. The Ki was calculated to be 0.63M. Xylan (0.5 mg/ml) protected the enzyme significantly (60%) against the inhibition by Gdn.HCl revealing that the inhibition was reversible and was due to the interaction of Gdn.HCl with the carboxyl residues that are involved at or near the substrate binding site of the enzyme.

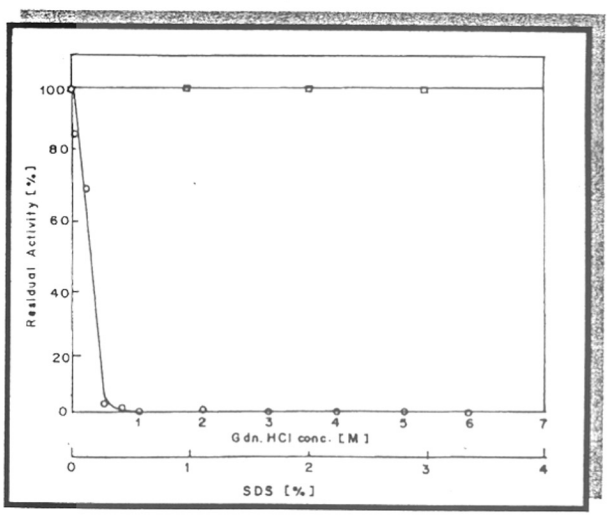

Fig. 3 : Effect of denaturants on the xylanase activity

Xylanase (.05 U) was incubated with different concentrations of Gdn.HCl (O-O), or SDS (□-□) for 15 min at 25°C in 0.05 M acetate buffer, pH 5.7 in a total volume of 0.5 ml.

Reaction of WRK with xylanase

The involvement of the carboxyl group in the active site of the enzyme was also verified using a carboxyl-specific chemical modifier, WRK. The xylanase was inactivated rapidly by 10 mM WRK. The semi-log plots of percentage residual activity as a function of time at various concentrations of WRK (Fig. 5) indicated that the inactivation process exhibits pseudo-first order kinetics with respect to time at any fixed concentration of the inhibitor. Based on the analysis described by Levy *et al* [5], the pseudo-first order rate constants were calculated from the slope of the plots of logarithm of the residual activity against the time of reaction. The order of reaction was estimated from the slopes of the plots of log of pseudo-first-order rate constant against log of inhibitor concentration (Fig. 5 inset) which indicated that the loss of enzyme activity by treatment with WRK resulted from reaction of only one carboxylate group per molecule of enzyme.

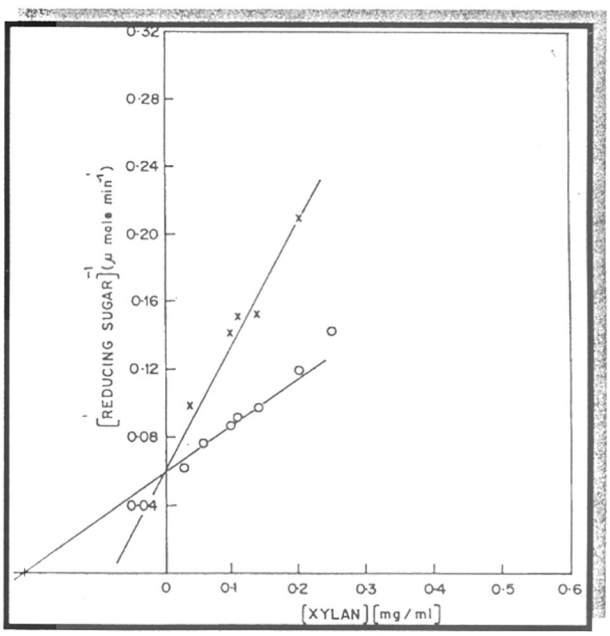

Fig. 4 : Kinetics of inhibition of xylanase by Gdn.HCl.
The xylanase activity was assayed at different concentrations of xylan in the absence (O- O) and presence of (X-X) Gdn.HCl.

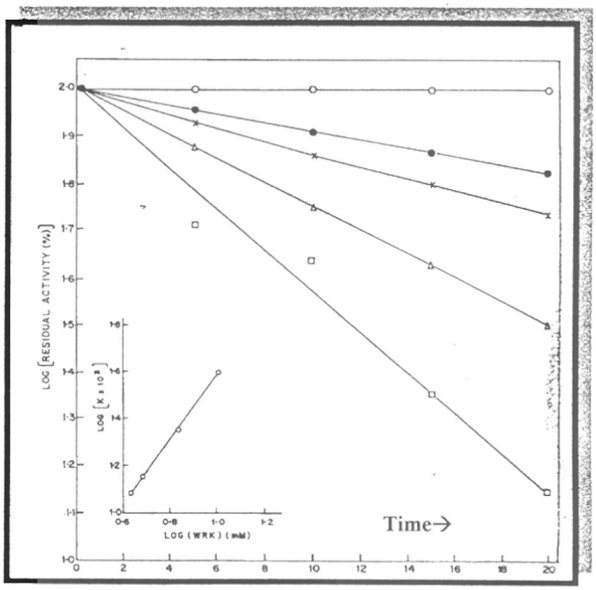

Fig. 5: Kinetics of inactivation of xylanase by WRK :

The xylanase (10 μg) was incubated with 4mM (●), 5mM (X), 7mM (Δ)or 10mM (□) of WRK in 0.05 M sodium phosphate buffer, pH 6.0 at 25°C. The inset shows the apparent order of reaction with respect to reagent concentration. The enzyme (60 μg) was incubated with 0.4 M Gdn.HCl in 50 mM acetate buffer, pH 5.7 or 10 mM WRK in 50 mM sodium phosphate buffer, pH 5.7 at 25°C for 10 min.

Treatment of enzyme with xylan (0.5 mg/ml) prior to its incubation with WRK (7 mM) rendered 100% protection against inactivation by WRK confirming the presence of essential carboxylate residue at the substrate-binding site. The modification of aspartate or glutamate residues in the protein by WRK results in a concomitant increase in absorbance of the protein at 340 nm and allows the quantification of carboxylate modification [3]. In order to rule out the possibility of Participation of -SH group in the overall reaction, the xylanase was treated with *p*HMB (1 mM) prior to its reaction with WRK. Spectral analysis of the WRK-treated enzyme at 340 nm revealed that complete inactivation of the enzyme was accompanied by the modification of three carboxylate residues (Table 1). Pretreatment of the xylanase with Gdn.HCl resulted in the failure of access of carboxyl groups to WRK confirming that

Gdn.HCl competes with WRK in interacting with carboxyl groups. Incubation of the enzyme with xylan prior to its reaction with WRK prevented modification of one carboxylate residue with retention of enzyme activity.

Table 1: Reaction of xylanase with WRK

Ser. No.	Sample	Absorbance at 340 nm
1.	Enzyme	0.010
2.	Enzyme + Gdn.HCl (0.4M)	0.020
3.	Enzyme + WRK (10 mM)	0.223
4.	Enzyme + Gdn.HCl (0.4M) + WRK (10mM)	0.020
5.	Enzyme + xylan (0.5mg/ml) + WRK (10mM)	0.150

The *Streptomyces* xylanases differ from *Bacillus* xylanases and from other functionally related hydrolytic enzymes in possessing cysteine [6, 7] addition to tryptophan as an essential amino acid residue for activity. Carboxyl groups have been shown to be integral part of the active site of xylanase from *Schizophyllum commune* [8] and alkalothermophilic *Bacillus* [9]. The objective of the present investigations was to ascertain whether the carboxyl groups are essential for the activity of the xylanase from *Chainia* which resembles *Streptomyces*. Comparison of the amino acid sequences of catalytic regions of endoxylanases from microbial sources revealed that the homology is restricted to residues 21 to 24 (D- GG) of low molecular weight xylanase from *Chainia* [10]. The presence of a single Asp residue in the conserved region[10] and the present evidence for the essential carboxylate residue prompt us to postulate that Asp^{21} is the carboxylate residue required for catalysis. Whether the three essential amino acid residues viz. tryptophan, cysteine and aspartate/glutamate in *Chainia* xylanase lie in close proximity or are distributed distantly in the polypeptide chain and come together to constitute

the substrate binding pocket upon folding of the enzyme would await collection of data on isolation and sequencing of peptides containing the specific amino acid residue. Nevertheless, the knowledge of the essential amino acid groups together with the identification of the *Chainia* xylanase gene on a 1.4 kb DNA fragment[11] and truncated 0.15 kb DNA fragment [12] will facilitate its manipulation by site directed mutagenesis to obtain an altered protein with desirable properties such as stability at high pH and temperature which are prerequisites for its application in biotechnology.

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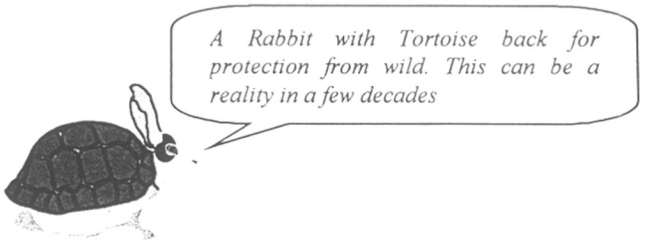
Chapter III

Heterologous Cloning Of Xylanase Gene From Chainia (NCL 82.5.1)



🕮Summary

The genomic library of *Chainia* in λgt10 was analyzed for the expression of the xylanase gene using antixylanase antibodies. The recombinants exhibiting positive enzyme linked immunosorbent assay test (ELISA) showed clearance on RBB-xylan plate. The phage lysates showed xylanase activity in the range of 19 to 36 mU ml^{-1}, which was higher than that exhibited by the previously cloned xylanase gene from *Chainia*. The pooled DNA preparation of EcoR1 digests of the xylanase-positive λ gt10 recombinants showing higher activity was cloned in pUC8. One of the pUC8 recombinants (h_1) which exhibited maximum activity (24 mU ml^{-1}) was shown to code for the low-molecular weight xylanase from *Chainia*. The xylanase produced by h_1 was inducible by isopropylthio-β-D-galactoside (IPTG) but not by xylan, suggesting that the DNA fragment containing the xylanase gene is in frame with, and its expression controlled by, the *lac* Z promoter.

🕮 Introduction:

Cloning of genes from one organism to the other well studied organism is a fairly known technique. For the last two decades, techniques in this field are improving and emerging to satisfy various needs encountered by the scientists. As the application of enzymes in various industries is increasing day by day, procedures have been developed to obtain enzymes with desired characters. With the help of these gene manipulation techniques, foreign DNA fragments can be introduced in the desired host, through a proper vector, maintained stably and expressed, overcoming the natural barrier of recombination. The gene of interest can be further sequenced and manipulated for site directed mutagenesis studies. With the help of these techniques many enzymes like proteases, amylases and restriction endonucleases are produced commercially from genetically tailored microorganisms.

There are various ways for cloning of genes, these include -

(i) Homologous cloning i.e. cloning of genes of one organism into same species

(ii) Heterologous cloning i.e. cloning from one species in a different species, and

(iii) Amplification of the copy number of the genes of interested enzymes in the same organism.

Various genes from different organisms have been cloned in a large variety of hosts. Many of these synthesize commercially important enzymes which are utilized in the industry. Genes from various species of *Streptomyces* have been cloned in *E. coli* [1, 2] and *Streptomyces* [3 - 6]. In case of *Streptomyces* genes, recognition of *Streptomyces* promoters in *E. coli* is the key step in gene expression. *Chainia* is a *Streptomyces* sp., producing high activity of cellulase-free xylanase . Cloning of its gene in *E. coli* would be of great interest for overproduction of the enzyme as well as for studying the regulatory aspects of the gene.

Materials and Methods :

Materials:

Agarose , ampicillin, SDS , PEG 8000, X-gal, IPTG, xylan (Oat spelt), DNAase, RNase and Protinase K were purchased from Sigma Chemical Co., (USA). Tryptone, yeast extract , malt extract , glucose, bactoagar were from Difco Laboratories (India). All the restriction endonucleases used were obtained from Amersham (UK) or Boeringer Mannheim (Germany). T_4 DNA ligase and calf intestinal phosphatase, molecular weight markers (λ DNA digested with Hind III and Eco RI or 1 kb ladder) were purchased from Boeringer Mannheim (Germany). The transfer membrane, nitrocellulose was from Advanced Microdevices Ambala, India. Elutip D (from Schleicher and Shuell, Germany) and Quaigen columns (from Diagen gumbo, Germany) were used for DNA purification . The vectors Eco RI cut and dephosphorylated λgt 10 arms along with packaging extracts in cloning kit from Amersham (UK), and pUC8 from Pharmacia, Sweden were used in cloning experiments.

Host and Vector System:

λgt10 vector was used for the construction of genomic library of *Chainia* and subcloning was carried out using plasmid vector pUC8. The details of the host and vector are as follows:

Host : Genotypes

E. coli	Genotype
NM 514	hsd R 514 ($rk^- mk^-$), Arg H, gal E, gal X, str A, lycB7, hfl+
JM 109	F′ tra D36 $lacI^q$ Δ (lac Z) M15 pro AB/rec A1 end A1gyr A96

Vectors : Properties

Name	Size (Kb)	Arm length	Cloning sites
λgt10	43.34	32.71 , 10.59	Eco RI
pUC8	2.67	----	EcoRI, BamHI, PstI, HindIII, Sal I

Maintenance of cultures and phage stocks:

The working stock of *E.coli* cultures (recombinants as well as cloning hosts) were stored on agar plates containing appropriate antibiotic , if any, at 4°C for short term while for the long term storage, 15% glycerol suspensions of liquid cultures were kept frozen at -70°C.

All the lysates of bacteriophage vectors , amplified library and recombinant clones were stored as 1% chloroform stocks at 4°C. Dimethylsulfoxide (DMSO) was added to the bacteriophage lysates to the final concentration of 7% v/v, mixed gently, and stored at -70°C for long term storage.

Media, Buffers and solutions used:

Luria Broth (LB)		TNE Buffer		Congo-red xylan agar	
Tryptone :	1 %	Tris.Cl (pH 8.0) :	10 mM	Oat spelt xylan	1 %
Yeast Extract :	0.5%	NaCl :	100 mM	Congored	1 %
NaCl :	1 %	EDTA (pH 8.0) :	1 mM		
pH :	7.2 - 7.4				

20 X SSC		10 X PBS		PEG/NaCl g%	
NaCl :	3 M	NaCl :	1.5 M	PEG	20
Tri sodium citrate :	0.3 M	Sodium Phosphate (pH 7.5) :	1 M	NaCl	11.7

SM Buffer	g%	TAE Buffer		GET Buffer	
NaCl	0.58	Tris acetate	40 mM	Glucose	50 mM
MgSO4.7H2O	0.2	EDTA	1 mM	EDTA	10 mM
Tris base	0.605			Tris.Cl	25 mM
2% gelatin	0.001			(pH 8.0)	
pH	7.5				

For preparation of PEG/NaCl solution, the contents were dissolved in 75 ml of SM buffer and volume was adjusted to 100 ml.

All the media were prepared in single distilled water and were autoclaved at 15 psi (pounds per square inch) for 20 min. Agar (2%) was added (for solid media) to the medium before autoclaving and plates were poured after the mixture was cooled to 45°C. Agarose (0.7%) was included in the broth for the top agar. Heat labile components like antibiotics, IPTG, maltose were filter sterilized through autoclaved millipore (0.45 μm) membranes and added freshly to the cooled media (45°C). Ampicillin (sodium salt) solution was prepared at a concentration of 50 mg/ml in sterile autoclaved double distilled water and 50 μg/ml final concentration was added in the medium. X-gal (5 bromo-4chloro-3indolyl β D-galactoside) was dissolved in dimethyl formamide at 2% concentration. 10 μl of IPTG (Isopropyl thiogalactoside) (100 mM) and 40 μl of X-gal (2%) was used per 25 ml of LB agar plate.

Agarose gel electrophoresis:

Analytical gel electrophoresis

Agarose gel (0.7%) was used for monitoring digestions of genomic DNA while single and double digestions of plasmids were monitored on 1.2% agarose gels. The gel electrophoresis was carried out in 1 X TAE buffer at constant current of 50 mA for 3-4 h using bromophenol blue as a tracking dye. After electrophoresis the gels were stained in dark with ethidium bromide (100 ng/ml), visualized on a UV - transilluminator (302 nm)(UV products, San Gabriels, California USA) and photographed with a 35 mm SLR camera (Minolta X 700 with microphotography and zoom lens system) using a red filter.

LMT agarose gel electrophoresis :

Low melting temperature (LMT) agarose was used to elute specific fragments of DNA from the gel. LMT agarose was melted in TAE buffer by

heating at 70°C for 30 min and cooled to 37°C. After pouring the gel, it was allowed to solidify for 1 h at 15°C. After completion of electrophoresis, it was stained with ethidium bromide as above and visualized on UV transilluminator. The specific bands were cut with the help of a sharp scalpel and collected in eppendorf tubes. Approximately 5 volumes of TE buffer was added to the gel pieces and the mixture was heated at 65°C for 20 min so that the agarose is completely melted. Equal volume of phenol was added to the molten agarose and mixed thoroughly. It was centrifuged in an eppendorf centrifuge for 10 min and the supernatant was treated with phenol/chloroform until the interface was not visible. The aqueous phase was then washed twice with chloroform and DNA was precipitated with 2.5 volumes of ethanol. The tubes were kept overnight at -70°C and DNA was pelleted by centrifugation. The pellet was repeatedly washed with 70% ethanol, dried and purified on Elutip-D column as follows:

The DNA sample was dissolved in low salt buffer (200 mM NaCl; 20mM Tris.HCl pH 7.4; 1.0mM EDTA). The column was washed with 3ml of high salt buffer (1.0M NaCl; 20mM Tris.HCl pH7.4; 1.0mM EDTA) followed by priming of column by two washes of 5 ml each of low salt buffer. DNA sample was absorbed on to the primed column by forcing it slowly with the help of a syringe. The column was washed twice with 3 ml of low salt buffer and DNA was eluted using 0.4 ml of high salt buffer. The DNA was precipitated with two volumes of ethanol, centrifuged and the pellet was washed twice with70% ethanol to remove the salts. The purified DNA was dissolved in TE buffer.

Immunoscreening of the λgt10 clones :

Immunoscreening was carried out using antixylanase antibodies raised in rabbit against the electrophoretically homogenous preparation of the low molecular weight xylanase from *Chainia*. The anti-rabbit-IgG-peroxidase conjugate (Boehringher Mannheim , Germany) was used to locate the

xylanase-positive clones.

Preparation of plating cells

The single colony of the *E. coli* NM514 culture was inoculated in 2ml of LB broth and incubated O/N at 37°C with vigorous shaking. 100 µl of O/N culture was inoculated in 25 ml LB broth containing maltose (4 mg/ml) and incubated until the A_{660} reached to 0.5. The cells were pelleted by centrifugation at 5000 rpm for 15 min at 4°C. The pellet was resuspended in 12.5 ml of chilled $MgSO_4$ (10 mM) and stored at 4°C.

ELISA

The 20 putative xylanase-positive λgt10 clones from the genomic library of *Chainia* in E.coli [7] were patched on an LB plate overlaid with soft agar (0.7%) containing plating cells (500 µl in each plate), in triplicates, and incubated overnight at 37°C. After development of plaques, one of the plates was covered with sterile nitrocellulose membrane for 10 min. The membrane was then lifted and immersed in PBS buffer containing lysozyme (2mg/ml) and BSA (100 µg/ml) and kept overnight. It was then washed twice with PBS buffer (10 min each) and incubated with 1:100 diluted anti-low molecular weight xylanase antibodies in PBS containing 100 µg/ml BSA for 1 h with gentle shaking. It was washed twice with PBS (10 min each) and challenged with anti-rabbit IgG -peroxidase conjugate (1: 2000 diluted in PBS containing 100 µg/ml BSA) for 1 h. After washing thrice with PBS buffer the bound peroxidase was detected with 3,3 diaminobenzidine tetrahydrochloride (0.5%) in 0.5M sodium citrate buffer pH 5, containing H_2O_2 (0.03%).

Determination of xylanase activity

The ELIZA-positive clones were patched on Congo-red agar plates containing xylan (1%) [8] to verify the xylan degrading activity as monitored by the clearance of xylan on a plate.

The xylanase activity was estimated as described in Chapter II Part. For determining the activity of phage lysates, 1 ml of the lysate broth was removed in the eppendorf tubes before addition of chloroform. The broth was centrifuged at 10,000 rpm for 5 min and the supernatant was used for estimation of xylanase activity.

Extracellular activity of pUC8 recombinants was checked by taking 0.5 ml of centrifuged broth of the clone grown overnight in LB containing ampicillin (50 μg/ml). The cell pellet was extracted at 4°C for 12 - 14 h with 2 ml of 0.05 M phosphate buffer, pH 7.2 containing 0.5% triton X-100 and 200 mM guanidinium hydrochloride [9], for determining the intracellular activity. The supernatant was stored at -20°C and the pellet was extracted with 1 ml of 50 mM phosphate buffer by freeze thawing . Both the supernatant and the extracted pellet were estimated for the xylanase activity.

Isolation of bacteriophage λ DNA

The DNA from λgt10 clones was isolated using liquid lysis method [10] with slight modifications.

Transfection and lysis

500 μl of plating cells were inoculated with a single plaque or approximately 10^7 pfu and incubated for 20 min at 37°C.5 ml of LB broth and 5 μl of $CaCl_2$ (0.5M) were added and incubated at 37°C for 7 to 8 h with vigorous shaking. When the lysis was visualized, 50 μl chloroform was added and shaking was continued for 10 min. The lysate was centrifuged for 10 min at 5000 rpm to remove cell debris. The clear lysate was treated with DNase I and RNase (1 μg/ml each) for 30 min at 37°C. 5 ml of PEG/NaCl solution was added and the mixture was kept on ice for 1 h and centrifuged at 10,000 rpm for 20 min. The solution was drained out carefully to remove PEG. The phage pellet was resuspended in 500 μl of SM buffer and was briefly centrifuged to remove undissolved particles. It was then treated with 500 μl of chloroform to remove the traces of PEG.

Isolation of phage DNA

The 500 µl phage suspension in the previous step was treated with 5 µl of EDTA (0.5 M) and 50 µl SDS (10%) and incubated at 65°C for 20 min. It was then treated with phenol followed by phenol/chloroform and two washes of chloroform. The DNA was precipitated with equal volume of isopropanol, kept at room temperature for 30 min, centrifuged, dried and dissolved in 50 µl TE.

Digestions of DNA with restriction enzymes

For all digestions of vector DNA (pUC8 / λgt10) with restriction enzymes, minimum 10U of restriction enzyme per µg of DNA was used in a reaction volume of 20 µl at 37°C for appropriate time. Either the commercially supplied buffers or the buffers prepared according to Sambrook *et al,* were used. The enzyme digestions were stopped by the addition of EDTA to a final concentration of 10 mM.

The digested λgt10 DNA was visualized by the agarose gel electrophoresis as described earlier. The bands of inserts were eluted from LMT agarose gel, purified and all the inserts were pooled together for further ligation with pUC8.

For the double digestions of pUC8, DNA was first digested with the enzyme requiring low salt buffer, followed by incubation with high salt requiring enzyme. Digestions were allowed to proceed at 37°C. The reaction was terminated as described above.

Dephosphorylation

Linearized plasmid vector (pUC8 - EcoRI digest) was dephosphorylated with calf intestinal alkaline phosphatase (CIP). DNA (1 µg) was dissolved in 50 µl phosphatase buffer (50 mM Tris.HCl pH 7.5; 1 mM EDTA; 10 mM $MgSO_4$) and incubated with 1U of enzyme for 1 h at 37°C. The phosphatase was removed by the treatment with phenol/chloroform.

Ligation

Eco RI-digested dephosphorylated vector DNA and the pooled preparation of inserts from xylanase-positive λgt10 clones were mixed and warmed to 45°C for 5 min to melt the cohesive termini that might have reannealed . Mixture was chilled to 0°C. 1μl of 10 X bacteriophage T_4 DNA ligase buffer (200 mM Tris.HCl; 50 mM $MgCl_2$, 50 mM dithiothreitol; 500 μg/ml bovine serum albumin), 0.1 U of bacteriophage T_4 DNA ligase, 1 μl of 5 mM ATP were added to the mixture to the final volume of 10 μl adjusted with distilled water. The mixture was incubated at 16° C for 16 h for ligation.

Transformation of *E. coli* with recombinant plasmid DNA

E. coli JM 109 from the glycerol stock was streak-inoculated on M9 medium, to guarantee the presence of F'episome. A single colony from this plate was inoculated into 5 ml LB broth and kept at 37 °C O/N with shaking. 100 μl of overnight grown culture was inoculated in 25 ml LB broth and incubated at 37 °C with vigorous shaking till O. D. at A_{600} of the culture was 0.5. The culture was chilled on ice for 10 min and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant liquid was discarded and the pellet was resuspended in 12.5 ml 100 mM chilled $CaCl_2$/10mM Tris HCl pH 8 and kept on ice for 30 min. The cells were again pelleted by centrifugation as above and resuspended in 1 ml of $CaCl_2$ solution and divided into aliquots of 200 μl. Upto 40 ng of supercoiled plasmid DNA was added to each aliquot and incubated on ice for 30 min followed by the heat shock at 42 °C for 2 min. The tubes were transferred immediately to ice for 2 min and incubated at 37 °C for 60 min without shaking after adding 1 ml LB broth. Different aliquots were plated on LB plates containing ampicillin. The transformation efficiency was expressed as the number of transformants per μg of DNA.

Isolation of plasmid DNA

From the white recombinants colonies, the plasmid DNA was isolated according to Kieser with slight modifications [11].

Minipreparations

Two ml of LB with ampicillin (50µg/ml) was inoculated with the culture and grown overnight at 37°C on an orbital shaker. 1 ml of overnight grown culture was centrifuged in the eppendorf tube for 5 min. The supernatant was discarded and the cell pellet was resuspended in 100 µl GET buffer. 200 µl of SDS (1%) containing NaOH (0.2N) was added and mixed thoroughly. The clear suspension was kept for 5 min at room temperature and 150 µl of chilled potassium acetate (1M) was added. After vortexing briefly, the tubes were kept on ice for 10 min and centrifuged for 5 min. To the supernatant liquid, 450 µl of phenol/chloroform was added, centrifuged and the aqueous layer was treated once with chloroform. The nucleic acids were precipitated by adding 400 µl isopropanol and kept at room temperature for 30 min. After centrifugation, the pellet was dissolved in 50 µl TE, treated with RNase for 30 min at 37°C. 20 µl from this solution was restricted with appropriate enzyme and visualized on 1% agarose gel.

Large scale preparation

500 µl of overnight grown culture was inoculated into 100 ml of LB with ampicillin (50µg/ml) and incubated for approximately 16 h. The cells were pelleted by centrifugation, and resuspended in 10 ml GET buffer. The suspension was treated with 20 ml of 1% SDS/0.2N NaOH. The contents were mixed thoroughly and the tube was incubated at 55°C for 30 min. After centrifugation the supernatant liquid was treated with phenol/chloroform followed by a chloroform wash. 35 ml of isopropanol was added to the aqueous layer, mixed and incubated for 30 min. It was centrifuged at 10,000 rpm for 20 min. The pellet was washed with 70% ethanol, dried and dissolved in 500 µl TE. It was then treated with RNase for 30 min, washed with phenol/chloroform and chloroform and precipitated with 50 µl 3M sodium acetate and 1 ml of ethanol. After overnight incubation at -20°C, the DNA was pelleted by centrifugation, vacuum dried and dissolved in 200 µl TE.

The plasmid DNA isolated from recombinants was digested by EcoRI to excise the insert. It was then loaded on agarose gel to visualize the insert.

Amplification by polymerase chain reaction (PCR)

PCR was performed in an automated thermal cycling device (M. J. Research, USA). The reaction mixture contained 500 ng of template DNA with Tris-HCl, pH 8.3 (10mM), $MgCl_2$ (2mM), dNTPs (0.2mM), primers (forward and reverse, 0.2 µM each), and Taq DNA polymerase (2.5 U) (Promega) in a total volume of 50µl. To denature the template, the reaction mixture was heated at 98°C for 1 min followed by 40 cycles, each consisting of denaturation for 1 min at 95°C, annealing for 1 min at 60°C and extension for 2 min at 72°C accompanied by a final extension step of 5 minutes at 72°C. After termination of PCR, the DNA was precipitated with ethanol, and visualized on agarose (1%) gel electrophoresis.

Characterization of the recombinant xylanase

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in duplicate sets at pH 4.8 and 8.9 according to Reisfeld [12] and Maurer[13] respectively. One set of gels was cut into eight equal portions and the protein was extracted by homogenization followed by centrifugation. The supernatants were estimated for xylanase activity. The other set of gels was stained with Coomassie brilliant blue G-250.

Immunoprecipitation

Ouchterlony double diffusion was carried out in 1 X phosphate buffer saline (PBS) at 4°C for 48 h. Precipitin lines were visualized after drying the gel followed by staining with Coomassie brilliant blue R-250.

Induction of xylanase activity

The recombinant under study was inoculated in 10 ml LB containing 0.125% IPTG or 0.5% xylan for observing the probable effect of IPTG and xylan

on induction of xylanase activity, respectively. After O/N incubation the extracellular broths were estimated for enzyme activity.

Results and Discussion

Immunoscreening of the oligo-positive λgt10 recombinants

The screening of a genomic library of *Chainia* in *E. coli* with a mixed oligonucleotide probe synthesized corresponding to the sequence of six amino acids of the xylanase (Met-Tyr-Ser-Phe-Thr-Trp) followed by cloning and expression of putative xylanase fragments in pUC8 indicated that an indigenous promoter from *Chainia* was responsible for the xylanase activity [7].Therefore in an attempt to select for a higher xylanase yielding recombinant, the twenty xylanase positive λgt10 clones were analyzed for their expression using antixylanase antibodies. All the plaques showed positive ELISA test .

The lysates showed production of xylanase in the range of 19-36mU ml^{-1} (Table 1).

Table 1: Xylanase activity of λgt10 clones

Clone Number	Activity ($mUml^{-1}$)	Clone Number	Activity ($mUml^{-1}$)
1	25	11	22
2	19	12	25
3	36	13	21
4	26	14	24
5	18	15	24
6	25	16	26
7	21	17	25
8	19	18	30
9	21	19	20
10	19	20	21

The observed expression of the xylanase gene may be either due to the

promoter activity of the cI of bacteriophage λgt10 gene or due to presence of indigenous promoter of the xylanase gene from *Chainia* as observed earlier [7].

Subcloning of λgt10 inserts

The phage DNA from all the ELISA-positive clones was isolated by transfection of E. coli NM514. Restriction digestion of phage DNA with EcoRI showed the presence of inserts of varying sizes (0.5 to 4 Kb) (Fig.1).

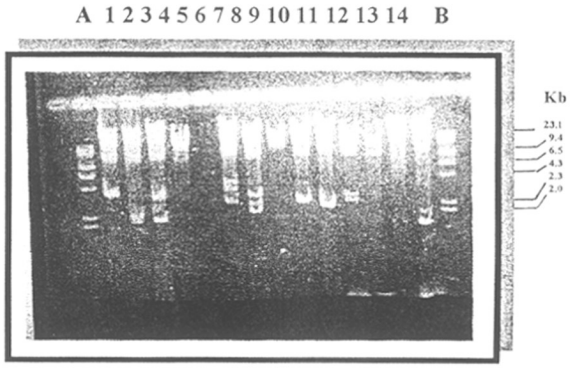


Fig. 1 : Restriction digestion of λgt10 clones with Eco RI
Lane A & B : λ Hind III molecular weight marker
Lane 1 to 14 : DNA of λgt10 clones digested with Eco RI

Further subcloning of the inserts from the xylanase-positive λgt10 recombinants in pUC8 was carried out for the possible amplification of the product. Total 15 white colonies of recombinants were obtained. Out of these three recombinants (h_1, h_2, h_3) showed measurable activity in the extracellular broth (Table 2) and exhibited visible clearance zones around their colonies on RBB-xylan plates (Fig.2)

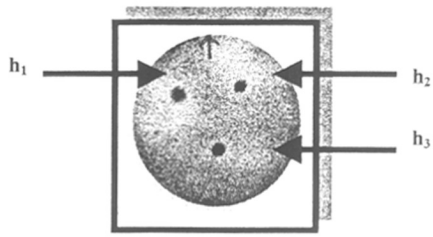


Fig. 2 : RBB-xylan clearance shown by pUC 8 recombinants :
h_1, h_2 and h_3 were patched on LB containing RBB-xylan (0.1 %), and incubated overnight at 37°C for 1h.

Table 2 : Xylanase activity of pUC8 recombinants

Clone	Activity (mU ml^{-1})
h_1	24
h_2	21
h_3	22

Out of these xylanase positive recombinants, h_1 (24 mU ml^{-1}) showed higher activity than that exhibited by the xylanase gene cloned earlier [7] and was therefore selected for further characterization .

Restriction digestion of recombinant plasmid DNA

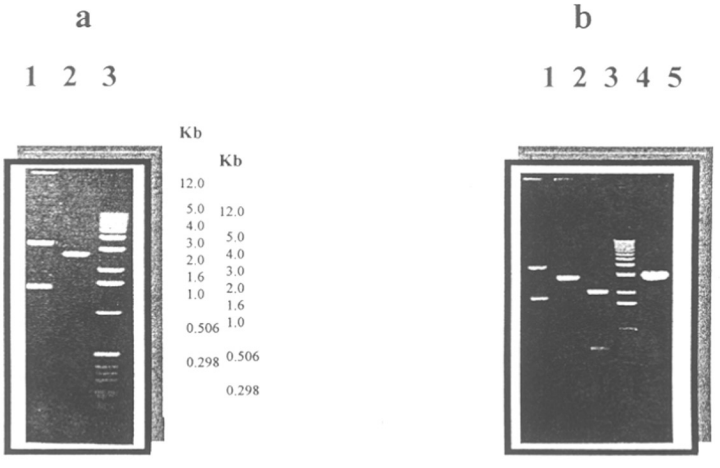


Fig. 3 : Restriction digestion of pRH1.

a) pRH1 (Lane 1) was digested with EcoRI (Lane 2), DNA Molecular Weight marker (0.075 - 12 kb) (Lane 3). b) pRH1 (Lane 1) was digested with Pst I (lane 2); Rsa I (Lane 3) and Hind III (lane 5); DNA molecular weight marker (Lane 4).

Digestion of the plasmid (pRH1) isolated from h_1 with Eco RI showed linearization of the plasmid with no visible insert (Fig. 3a), suggesting lack of regeneration of one of the two Eco RI sites flanking the cloned DNA. Digestion of the plasmid with Hind III or Pst I also resulted in linearization of the

plasmid (Fig.3b). Double digestions of the plasmid with Eco RI and Hind III or Bam HI also failed to excise the insert (data not shown) revealing that the Eco RI site distal to the polylinker is not regenerated contrary to that in pVX8 where the Eco RI site attached to the polylinker was lost [7].

Amplification of the fragment

The PCR method for in vitro amplification of specific DNA fragments opened up a number of fields in molecular biology to overcome the lacunas in the sensitive analytical methods. The PCR is based on the use of two oligonucleotides to prime DNA polymerase-catalyzed synthesis from opposite strands across a region flanked by the priming sites of the two oligonucleotides. By repeated cycles of DNA denaturation, annealing of oligonucleotide primers and primer extension an exponential increase in copy number of a discrete DNA fragment can be achieved [10].

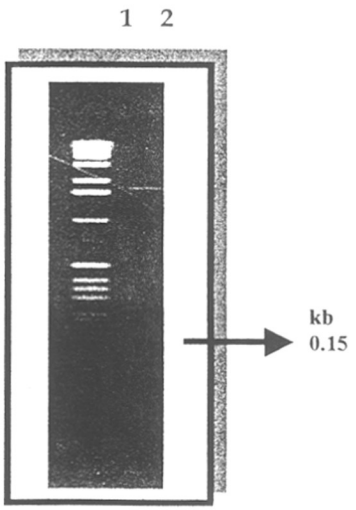


Fig.4:Amplification of pRH1 by PCR :

DNA molecular weight marker (Lane 1), pRH1 was amplified (Lane 2),

In order to investigate whether the cloned fragment of pRH1 is too small to be visualized on electrophoresis, amplification of the fragment using

PCR was carried out. After amplification a distinct band (approximately 0.15 Kb) was located on the gel (Fig.4) besides the band at 2.7Kb which is due to the unamplified band of the original circular plasmid.

Characterization of the recombinant xylanase

Extracellular production of low molecular weight xylanase

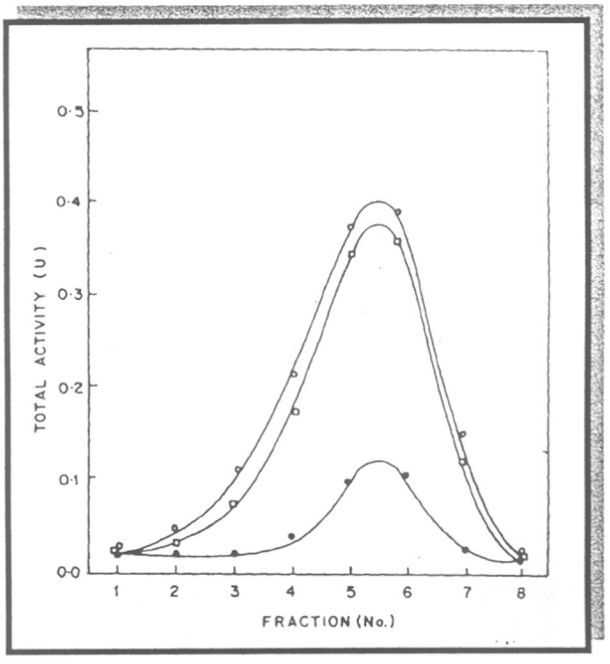


Fig.5 :Xylanase activity in the fractions after gel electrophoresis :

Electrophoresis was carried out at pH 4.8. The gels were cut, homogenised and xylanase activity in the supernatant was estimated. *Chainia* xylanase crude extract (O-O) Purified low molecular weight xylanase (□-□) Extracellular extract of pRH1 (●-●)

Chainia produces a low (Mr 6,000) and a high(Mr 21,000) molecular weight xylanase[14]. In order to investigate which of the two proteins is produced by the cloned gene, the purified low and high molecular weight xylanases, the crude extract of *Chainia* and the extracellular extract from the recombinant h_1 were subjected to gel electrophoresis at pH 4.8. Estimation of

the activity in the gel fractions showed that the xylanase activity of the recombinant corresponded with that of the purified low molecular weight xylanase (Fig. 5). The anodic run did not show the presence of xylanase activity in the recombinant suggesting that the cloned insert codes only for the low molecular weight xylanase.

E. coli has widely been used as a cloning host due to the wealth of genetic and physiological knowledge available about the organism. Moreover, the genes cloned in *E. coli* can be subjected to site-specific mutagenesis to obtain a mutant protein with altered desirable properties. However, E.coli normally does not release the recombinant proteins into the extracellular medium. The extracellular expression of *Chainia* xylanase in *E.coli*, reported here, is an interesting observation. It has been shown earlier that the induction of synthesis of the periplasmic enzymes from the strong promoters such as *lac* or *tac* results in the excretion of 90% of the synthesized proteins [15]. The extracellular expression of *Chainia* xylanase in *E. coli* JM 109 may also be attributed to the defective nature of its cell wall [10] .

Immunodiffusion

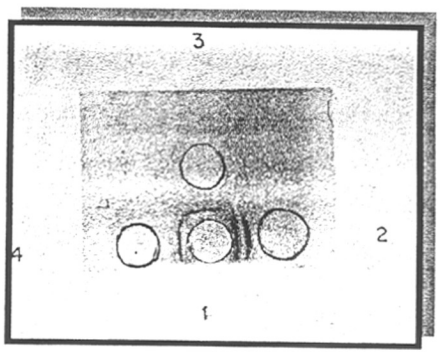


Fig. 6 : Immunodiffusion pattern of the recombinant xylanase :

Antixylanse antibodies (Well 1), *Chainia* xylanase, crude enzyme (Well 2), Purified low molecular weight *Chainia* xylanase (Well 3) and Extracellular extract of h_1 (Well 4)

The cloned gene produces a xylanase which cross reacts with antibodies raised against the low molecular weight xylanase and exhibits a precipitin arc which resembles with that produced by the low molecular weight xylanase (Fig 6),providing further support that the truncated *Chainia* DNA fragment contained in pRH1 codes for the low molecular weight xylanase.

Induction of xylanase activity of pRH1

The enzyme xylanase produced by clone h_1, was inducible by IPTG and not by xylan, suggesting that the cloned gene is in frame with the Lac Z promoter and the expression of xylanase gene is under the control of Lac Z promoter (Table 3).

Table 3 : Induction of xylanase activity of h_1

Clone	Inducer	Activity (mU/ml)
h_1	None	24
h_1	IPTG (0.125%)	43
h_1	Xylan (0.5%)	22
E. coli (pUC8)	IPTG (0.125%)	01.3

Earlier, it has been shown that an indigenous promoter from *Chainia* was responsible for the induction of xylanase activity in pVX8 [15] and that inspite of high guanine plus cytosine content of *Chainia* ,E.coli does recognize a few *Chainia* promoters [16]. Considering the small size of the insert in h_1, the observed lack of induction by xylan may be attributed to the absence of regulatory elements from *Chainia* in the truncated gene.

In conclusion, a higher-yielding (24 mU ml^{-1}) recombinant capable of producing an extracellular xylanase activity was isolated by immunoscreening of the genomic library of *Chainia* in λgt10. The cloned *Chainia* DNA fragment from pRH1 codes for the low molecular weight xylanase from *Chainia*. The cloned xylanase is inducible by IPTG but not by xylan suggestive of the absence of *Chainia* regulatory elements on pRH1. Our results represents first report on molecular cloning and expression of the xylanase gene from

Streptomyces in *E.coli.*

Comparison of *Chainia* recombinant xylanases

Comparison of the two xylanase-positive recombinants pVX8 & pRH1 (Table 4) reveals significant difference in xylanases expressed by them ,

pRH1[a]	pVX8[b]
Xylanase activity	
- Extracellular (43 mU/ml)	- Intracellular (5.4 mU/ml)
- inducible by IPTG	- not inducible by IPTG
- Preparative PAGE reveals activity of only low molecular weight xylanase	- Not done
Recombinant plasmid	
-EcoRI site distal to the polylinker is not regenerated	-EcoRI site attached to the polylinker is not regenerated
-Insert size ≈ 0.15 kb	- Insert size ≈ 1.2 kb
Immunodiffusion	
-Reveals presence of only one arc corresponding to low molecular weight xylanase	-Reveals presence of both arcs of low and high molecular weight xylanases

[a] - source present work [17]
[b] - source [7, 18]

The xylanase coded by pRH1 is extracellular, inducible by IPTG and corresponds with the protein band of low molecular weight xylanase of *Chainia* on PAGE as well as in immunodiffusion. The recombinant xylanase of pVX8, on the other hand, is intracellular, not inducible by IPTG and shows the presence of low and high molecular weight xylanases by immunodiffusion.

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Chapter IV

Enhancement Of pH & Themostability Of Xylanase From Chainia

🕮 Summary :

The ready accessibility of the xylanase II produced by *Chainia* to cellulose pulp due to its small size and the absence of cellulase are advantageous features. The enzyme is stable at 40°C for 1h and in a pH range of 5-9 at 4°C. Improved stability of the enzyme at higher temperature and pH are desirable. Effect of a variety of compounds was studied to enhance stability. Glycerol, sorbitol, mannitol (10%) or glycine (1M) had marginal effect on thermostability. Addition of Ca^{+2} or PEG (10mM) increased the half- life of the enzyme at 60°C. Cysteine (10mM) or Tween-80 (1%) showed 70% protection against thermal inactivation. Xylan (3%) offered complete protection against inactivation of the enzyme at 60°C and at pH 9.

🕮 Introduction :

In order to be suitable for technological applications, the enzymes should be stable under industrial operational conditions for several weeks or months. This necessitates the use of enzymes that are thermostable. However, not all enzymes which have high catalytic activity, selectivity and productivity, are stable at high temperature which is a prerequisite for their application in most of the existing enzymatic industrial processes. Therefore, it is mandatory to improve the protein stability at higher temperatures and pH. The ideal approach to stabilization of an enzyme would be to delineate the mechanism of its inactivation and to design the specific stabilizing procedure which would prevent the occurrence of inactivation. The exact mechanism of thermal inactivation of most of the commercially important enzymes is not known [1]. Therefore, various methods are developed to obtain stable enzymes; e. g.

(I) Isolation of the enzyme from extremophilic organism,

(II) Cloning of genes from extremophiles to non-extremophilic host (easy production of enzyme)

(III)Stabilization of unstable enzyme by modification, achieved by;

(a) immobilization,

(b) chemical modification

(c) association of proteins with non-protein compounds such as substrates, solvents and salts, or by

(d) protein engineering using (i) random mutagenesis or (ii) site specific mutagenesis [2].

There are many reports of enzyme stabilization by the above methods, e. g. the thermostability of D-xylose isomerase from *Thermoanaerobacterium thermosulfurigenes* was enhanced by site-directed substitutions of aromatic amino acids in the active site. This enhancement was explained as the consequence of the reduction of the area of water-accessible hydrophobic

surface [3]. The xylanase A from *Streptomyces lividans* was modified by site-directed mutagenesis. The mutant enzymes F155Y, R156E, R156K, and N173D were respectively 28, 10, 50 and 25% more active than the wild type enzyme [4].

In nature, proteins are stabilized by the interactions with the constituent amino acids by hydrogen bonding, electrostatic and hydrophobic interactions. In some proteins, cross-linking and presence of metal ions and cofactors play substantial role in stabilizing the structure. Vital protein conformation for biological activities is maintained by water-protein interactions through electrostatic force [5]. At the active site clefts, water molecules are likely to affect the interaction between substrates and enzymes, thereby influencing the mechanism of substrate binding and enzymatic activity. Enzymes, in their native form are not always stable at high temperature and pH, therefore, the need arises of making them suitable for the end use.

Increasing the stability of enzymes by associating proteins with non-protein compounds (additives) is easy to manipulate and economical. The different additives used are osmolytes, salts and the substrate or the substrate analogues. The osmolytes include the sugars, polyols, free amino acids and other polymers. The solutions of sugars and polyols have lower dielectric constants than pure water. Thus, electrostatic interactions are stronger in these solutions than in water. The subtle changes in the solution composition may be enough to alter the protein stability [Table 1].

Table 1 : Effect of additives on activity of various enzymes studied

ORGANISM (ENZYME)	EFFECT OF ADDITIVE	REFERENCE
Cucurbita facifolia (protease)	Glycerol, mannitol- increase in half life by 2-fold, PEG, casein	[6]
Aspergillus niger (glucose oxidase)	Polyhydric alcohols, PEG	[7]

ORGANISM (ENZYME)	EFFECT OF ADDITIVE	REFERENCE
Sclerotium rolfsii (pullulanase)	Xylitol, sorbitol - 100% retention of enzyme activity	[8]
Bacillus stearothermophilus (α-amylase)	Polyols, dimethyl formamide, and dimethyl sulfoxide - increased half-life by 2-fold	[9]

Reduced surface energy of polyol solutions has also been implicated in the stabilization of proteins by them [10]. The amino acids may influence the thermostability of enzymes in three different ways, viz. neutral, stabilizing and destabilizing effect [11]. The stabilizing effect of cysteine hydrochloride is attributed to the re-establishment of the free -SH groups on the enzyme [12].

In this chapter we have used different additives to improve the thermostability and pH stability of *Chainia* xylanase.

🕮 Materials and Methods:

🖹 Materials:

Malt extract, yeast extract, peptone and agar were from Difco laboratories, USA. Glycerol, Mannitol, Sorbitol and polyethylene glycol (PEG) (8000) were from Loba Chemie Ltd., India. Mercaptoethanol, SDS, Tween-80, cysteine hydrochloride, glycine and xylan (Oat spelt) were obtained from Sigma Chemical Co., USA.

🖹 Enzyme production and enzyme assay:

Production and estimation of the enzyme were carried out as described in previous chapter (Chapter II Part A).. The culture filtrate was obtained by centrifugation of the fermentation broth at 8000 X g for 20 min. The supernatant was concentrated by the addition of three volumes of chilled ethanol. The precipitate was recovered by centrifugation at 10,000xg for 25 min, dried under vacuum and was dissolved in 20 ml of 10 mM phosphate buffer, pH 7.0. The undissolved solid particles were removed by centrifugation and the clear supernatant of crude extract was used for further

studies, as in industry it is economical to use crude rather than the purified enzyme.

Thermal Stability of *Chainia* xylanase :

The thermal inactivation of xylanase was studied by incubating 5U of enzyme at different temperatures in 50 mM acetate buffer, pH 5.8. Effect of additive on thermal stability was determined by incubating the enzyme in presence of an additive at a desired temperature for a stipulated period of time. At the end of incubation the enzyme was cooled on ice for 15 min and the residual activity was determined. The enzyme incubated at 4°C in the absence of an additive was assumed to have 100 % activity.

pH stability:

Effect of pH an the stability of xylanase was studied by incubating the enzyme with buffers covering a pH range of 5.8 - 10.0.Different buffers used were : Sodium acetate buffer, pH 4 - 5.8; Sodium phosphate buffer, pH 6 - 7;

Results and Discussion:

The xylanase from *Chainia* was stable at 40°C and retained 70% of its activity after incubation for 90 min. The half life of the enzyme at 50°C was 90 min. However, at 60°C, there was a rapid inactivation of enzyme with a half-life period of only 15 min (Fig 1). The application of xylanase in pulp and paper industry demands that the enzyme is stable in the temperature range of 60 to 70°C which is the temperature of the incoming pulp for bleaching operations. Application of *Chainia* xylanase in conjunction with conventional bleaching of pulp was effective in improving the brightness of paper [13]. Therefore, we have made efforts to increase the thermostability of the enzyme by using various additives.

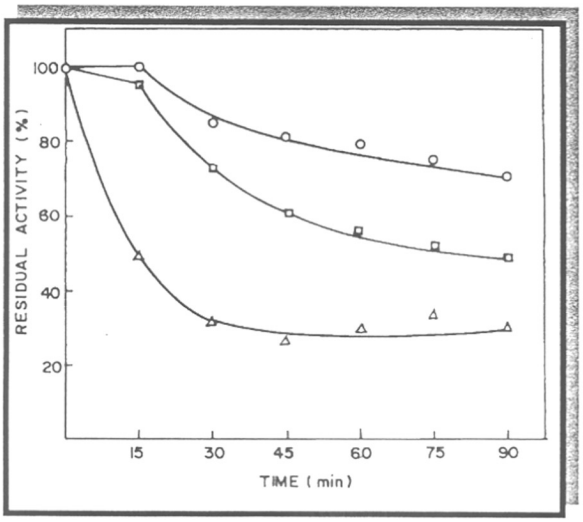

Fig. 1 : Stability of *Chainia* xylanase.

At 40°C (○), 50°C (□), 60°C (Δ)

Effect of additives on thermostability of xylanase:

Naturally occurring osmolytes such as amino acids, polyols and methylamines are known to confer stability on the proteins against thermal inactivation by promoting refolding and reactivation of the thermally unfolded proteins [14]. The xylanase from *Chainia* retained 70 % of its activity at 60°C in presence of cysteine hydrochloride (10 mM). However, addition of mercaptoethanol (10 mM) did not prevent the loss in activity at 60°C. The xylanase from *Chainia* has been shown to possess a cysteine residue at the active site of the enzyme [15, 16]. SDS (1 %) had no effect on the thermostability but Tween-80 (1%) was effective in enhancing the stability of the enzyme at 60°C. Water plays an important role in influencing the thermostability of enzymes. Polyhydric alcohols modify the structure of water and strengthen the hydrophobic interactions among non polar amino acids thereby making the enzyme resistant to thermal denaturation . Glycerol, sorbitol and mannitol (10%) had marginal effect (20 % protection) on the stability of the enzyme. On the other hand PEG 8000 increased the half-life of

the enzyme at 60°C from 15 to 60 min. Calcium chloride (10mM) was also effective in increasing the thermostability of the xylanase by doubling its half-life at 60°C (Fig 2) probably due to the binding of the Ca^{+2} to the proteolysis sites in the xylanase and preventing its proteolysis by the proteases present in the crude extract. Glycine (1 M) had no effect on the stability of the enzyme.

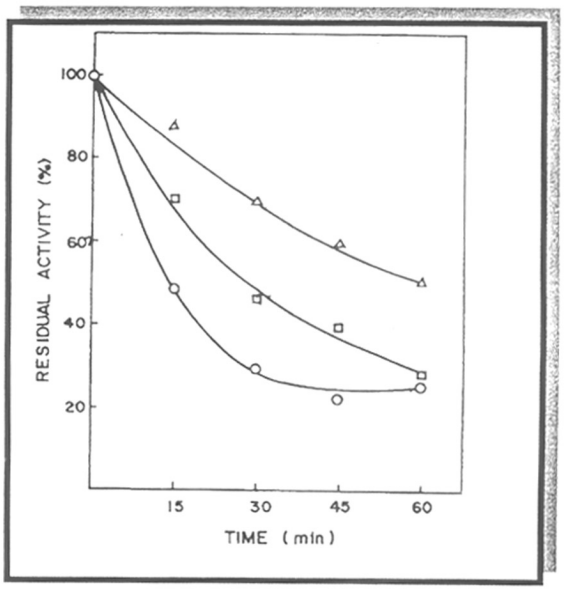

Fig. 2 : Effect of additives on Chainia xylanase.

Effect of PEG (Δ) and $CaCl_2$ (□) on Chainia xylanase (○) incubated at 60°C for 1 h.

Table 2 :Effect of additives on the stability of *Chainia* xylanase

ADDITIVE (final conc.)	RESIDUAL ACTIVITY(%)
PEG 8000 (1 mM)	70
(10 mM)	50
(25 mM)	30
(50 mM)	25
Proline (1 M)	25
Glycine (1M)	20

ADDITIVE (final conc.)	RESIDUAL ACTIVITY(%)
Cysteine hydrochloride (10mM)	70
β-mercaptoethanol (10&25 mM)	30
Tween-80 (1%)	70
SDS (1%)	24
$CaCl_2$ (10 & 50mM)	50
Glycerol (10%)	100
Urea (2M)	36
Xylan (3%) at 70°C	50

(PEG - Polyethyleneglycol; SDS - Sodium Dodesyl Sulphate)

Another approach to prevent thermal inactivation is by protecting the active site of the enzyme by its binding to the substrate or inhibitor. Addition of xylan to the xylanase from *Chainia* during incubation at 60°c was effective against thermal inactivation. Increasing concentrations of xylan exhibited increased protection against loss in activity (Fig 3) . In presence of 3 % xylan the enzyme was fully active at 60°C and retained 50 % of its activity at 70°C for 15 min.

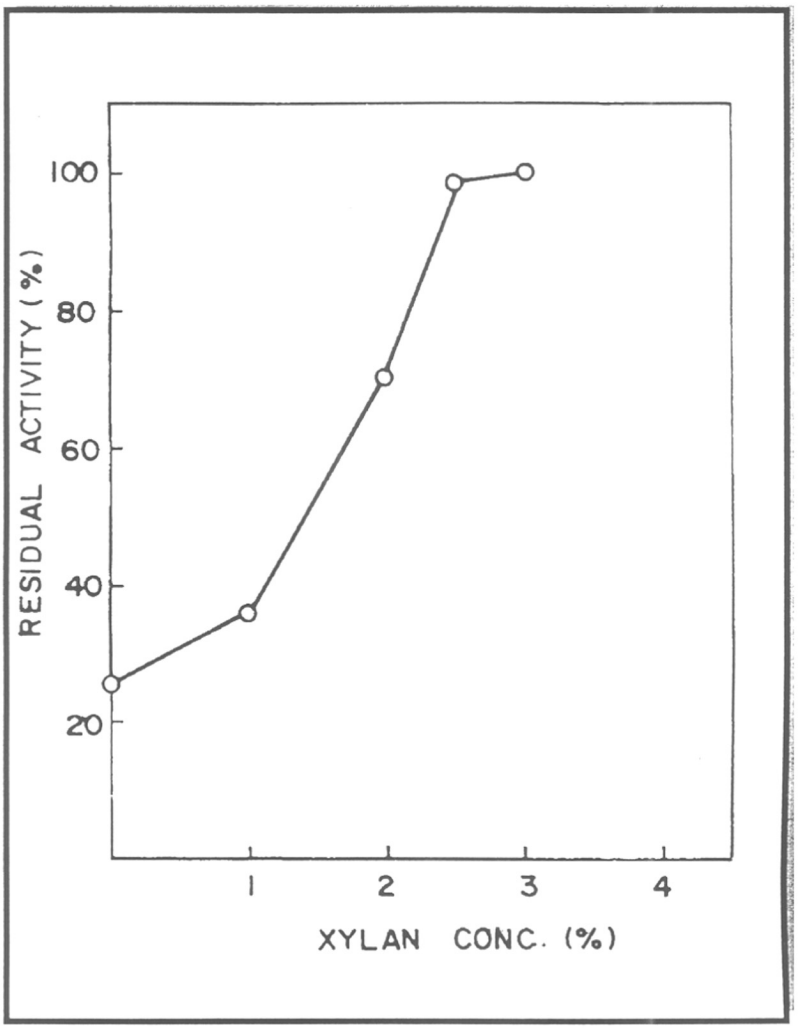

Fig. 3 : Effect of addition of substrate on thermostability of Chainia xylanase at 60°C for 1 h.

pH stability of xylanase :

Besides thermostability, stability at alkaline pH is a desirable trait for biotechnological application of xylanase . The xylanase from *Chainia* is active in a broad pH range of 6 to 9 at 4°C. However, at 60°C, it showed only 50 % of its original activity between pH 6 to

above pH 9. Addition of xylan (3 %) to the enzyme during incubation at 60°C was effective in conferring full stability between pH 6 to 9. The enzyme retained 50 % of its original activity at pH 10 (Fig 4).

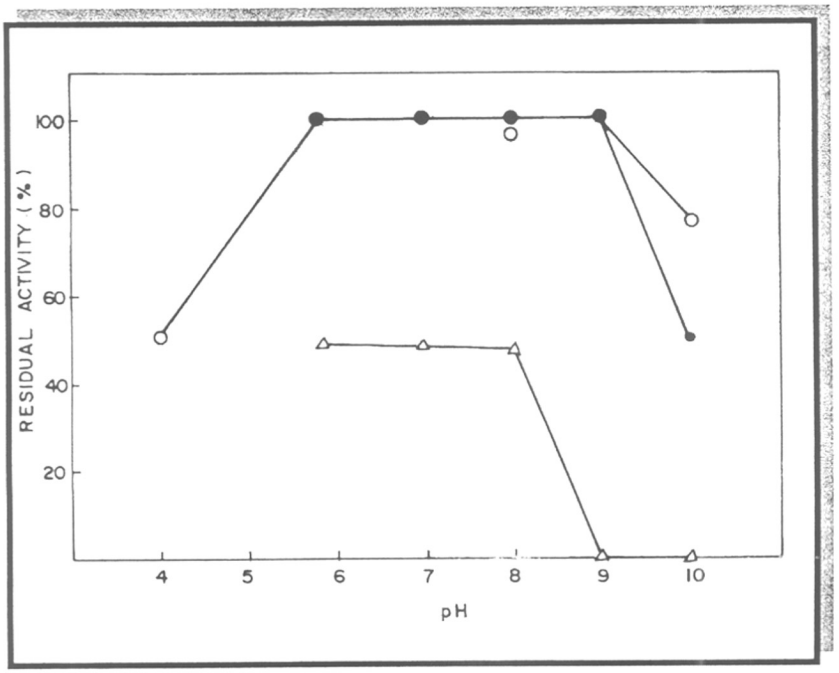

Fig. 4 : Stability of *Chainia* xylanase at different pH

At 4°C (O), 60°C (Δ) and at 60°C (•)

🕮 Conclusion:

Although the xylanase from *Chainia* does not possess stability at alkaline pH and at high temperature, its stability is improved by the addition of compounds such as cysteine hydrochloride, Ca^{+2} , Tween 80 or PEG. In presence of xylan, the enzyme was fully active for 1 h at 60°C and at pH 9 which are the prerequisites for its application in paper and pulp industry.

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