MOLECULAR AND BIOCHEMICAL ASPECTS OF EXTREMOPHILIC ACTINOMYCETE

A THESIS SUBMITTED TO THE
UNIVERSITY OF POONA
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
(IN BIOCHEMISTRY)

BY

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NOV. 2001

DEDICATED TO MY DEAR PARENTS

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DECLARATION

It is certified that the work incorporated in the thesis "MOLECULAR AND BIOCHEMICAL ASPECTS OF EXTREMOPHILIC ACTINOMYCETE " submitted by Mr. Sudeep P. George was carried out by the candidate at the National Chemical Laboratory Pune, under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "MOLECULAR AND BIOCHEMICAL ASPECTS OF EXTREMOPHILIC ACTINOMYCETE" submitted for the degree of Doctor of Philosophy in Chemistry (Biochemistry), to the University of Pune has not been submitted by me for a degree to any other university or institution. This work was carried out at Division of Biochemical Sciences, National Chemical Laboratory, Pune, India.

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ACKNOWLEDGEMENT

I take this opportunity to express my heart-felt gratitude to my guide, Dr. (Mrs.) Mala Rao for her able guidance, unceasing encouragement, and sustained interest throughout the course of my work. I consider myself extremely fortunate to have her as my guide.

I express my thanks to Dr. (Mrs.) Vasanti Deshpande for her support and encouragement.

Thanks are due to Dr. Absar Ahmad for his help in microbiological work and valuable discussions. Thanks are also due to Dr.Murali Sastry providing me insight in the field of nanobiotechnology .

I thank all my seniors and ex-labmates Drs. Devyani, Urmila, Neeta,, Sneha, Mohini, Aparna and Kavita for their help and cooperation.

Thanks are also due to my labmates Anish, Prashant, Nilesh, Pankaj, Vinod, Swati, Kavita, Sharmili and Ajit, for maintaining a lively and cordial lab environment.

I was indeed fortunate to have a friend like CV with me throughout the course of my Ph.D. His help and support has enabled me to ease through many difficult situations.

Special thanks to my friends Rajesh, Jui, Maneesha, Anand, Ashavani, Arun, Manish, Lata and Ramchander for their constant help, encouragement and thoughtful gestures which made my stay at NCL more enjoyable.

I also express my sincere appreciation to our lab attendant Ramakant Lambate for his help in routine work. Thanks are also due to Mr. Kamthe, Karanjkar, Trehan, Indira, Usha and Satyali for their efficient and timely technical assistance.

I am thankful to Dr. Paul Ratnasamy, Director, NCL, and Dr. P. K. Ranjekar, Head, Biochemical Sciences Division for granting permission to submit this work in the form of thesis.

The fellowship received from the Council of Scientific and Industrial Research, India is duly acknowledged.

I am very fortunate to have understanding parents, who have given me the freedom to choose my own career and encouraged me in all my endevaours. It would have been difficult for me to complete my Ph. D. without the constant encouragement and support from Didi, Bhaiya, Benoy, Bhabhi, Sophie and all the near and dear ones in my family.

Nothing would have been possible without the blessings of our lord, for "The Lord is good; His mercy is everlasting and his truth endures for all generations " – Psalms 100:5

Sudeep P. George

ABBREVIATIONS

C(x,t)	:	Time and distance dependent protein concentration
D	:	Diffusion coefficient
DEP	:	Diethylpyrocarbonate
DNSA	:	Dinitrosalicylic acid
DTNB	:	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	:	Ethylene diamine tetra acetic acid
FTIR	:	Fourier transform infrared spectroscopy
IPTG	:	Isopropyl β-D-thiogalactopyranoside
L	:	Region of diffusion of protein.
LB	:	Luria Bertani
LB Amp	:	LB media containing 60 μg/ml Ampicillin
M(t)	:	Total mass uptake recorded as a function of time
m_0	:	Mass of protein
NEM	:	N-ethyl maleimide
OD	:	Optical density
ODA	:	Octadecylamine
OPTA	:	*o*-phthalaldehyde
PAGE	:	Polyacrylamide gel electrophoresis
PEG	:	Polyethylene glycol
PHMB	:	*p*-hydroxymercuribenzoic acid
PNPG	:	*p*-Nitrophenyl β-D-glucopyranoside
PNPX	:	*p*-Nitrophenyl β-D-xylopyranoside
QCM	:	Quartz crystal microgravimetry
SDS	:	Sodium dodecyl sulphate
TE	:	Tris EDTA
TEMED	:	N,N,N'N'- Tetramethyl ethylene diamine
TNBS	:	2,4,6-trinitrobenzenesulphonic acid

X-gal : 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

INTRODUCTION

Enzymes stable and active at high temperature and alkaline conditions are suitable for biotechnological applications. Extremophilic organisms are one of the natural sources for the identification of such enzymes. The complete enzymatic degradation of xylan to its monomer, xylose, is a complex process requiring the action of a battery of hydrolytic enzymes. Endo-(1→4)-β-xylanase, which attacks the polysaccharide backbone is one of the most crucial enzyme involved in xylan hydrolysis. Xylanases have possible application in waste treatment, fuel and chemical production, and in the development of eco friendly technologies for the manufacture and deinking of paper. Xylanases in conjunction with cellulases have been used for the bioconversion of agricultural biomass resources into useful products.

Investigations involving chemical modification of an enzyme can potentially yield insights into structure-function relationships. In comparison to the extensive studies on the biotechnological applications of xylanases, inadequate information is available concerning the structure-function aspects xylanases. For commercial realization and economic viability of xylanase production, it is necessary to hyperproduce the enzyme. Recombinant DNA techniques offer the means to enhance protein production.

The present study deals with the biochemical and molecular aspects of a xylanase isolated from an alkalothermophilic *Thermomonospora* sp.

The main features of the work done are:

1. Production of xylanase and cellulase from an extremophilic actinomycete
2. Purification and characterization of xylanase
3. Active site studies of xylanase I: Residues implicated and conformation of active site
   I) Evidence for the presence of an essential lysine residue
   II) Site and significance of a histidine residue
4. Encapsulation and interaction of xylanase in fatty lipid matrix

5. Construction of genomic library and identification of xylanase gene in *E. coli*

1. Production of xylanase and cellulase from an extremophilic actinomycete

A novel alkalothermophilic actinomycete having optimum growth at pH 9 and temperature 50 °C was isolated from self-heating compost from the Barabanki district of Uttar Pradesh, India. Based on its morphology, susceptibility of spores to heat and novobiocin, guanine-cytosine content of chromosomal DNA and cell wall composition, the organism was classified under *Thermomonospora*. The isolate produced 125 IU/ml xylanase and 23 IU/ml carboxymethyl cellulase (CMCase). The xylanase was active at broad range of pH (5-9) and temperature (40-90 °C). The optimum pH and temperature were 7 and 70 °C respectively. The enzyme was stable in the pH range 5-8 and was found to be thermostable with half-lives of 8 h and 4 h at 60 and 70 °C respectively, but only 9 min at 80 °C. The effect of a variety of compounds to enhance the stability of xylanase at 80 °C was studied. The CMCase was partially purified by fractional ammonium sulphate precipitation followed by cellulose affinity chromatography and Sephacryl S-200 gel filtration. The enzyme exhibited optimum activity at pH 5 and temperature 50 °C. The CMCase showed pH stability in the range 7-10. The enzyme retained 100 % activity at 50 °C for 72 h and had half-lives of 7 h and 3 h at 60 °C and 70 °C, respectively. The CMCase was stable in the presence of commercial detergents such as Ariel, Henko, and Surf Excel, indicating its potential as an additive to laundry detergent.

2. Purification and characterization of xylanase

A highly thermostable xylanase (Xyl I) produced by *Thermomonospora* sp. was purified to homogeneity by sequential fractional ammonium sulphate precipitation (35-55 %) followed by DEAE Sephadex ion exchange and Sephacryl S-200 gel filtration chromatography. The molecular weight of the enzyme was estimated to be 38,000 by SDS-PAGE. The pH and temperature optima for the enzyme were 7.5 and 80 °C respectively. The enzyme was extremely thermostable, retaining complete activity at 50 °C for up to 72 h. While at 80, 90 and 100 °C it had half-lives of 86 min, 30 min and 15 min respectively. Xyl I was stable in an expansive pH range of 5 to 10 with more than 75 % residual activity. The isoelectric point of

the enzyme was 4.1. The amino acid composition of Xyl I was determined and it was found that there was an almost equal composition of hydrophobic and hydrophilic amino acids.

3. Active site studies of xylanase I: Residues implicated, conformation and polarity of active site.

i) Evidence for the presence of an essential lysine residue .

Studies involving *o*-phthalaldehyde (OPTA) as the chemical initiator for fluorescent chemoaffinity labeling and trinitrobenzenesulphonic acid (TNBS) as chemical modifier have revealed the presence of a single lysine residue in the active site of Xyl I. The high pK value for the basic limb of the pH profile reflects the ionization of a lysine residue. The kinetics of inactivation of Xyl I with OPTA revealed that complete inactivation occurred due to the binding of one molecule of OPTA to the active site of Xyl I. The formation of a single fluorescent isoindole derivative corroborated these findings. The kinetics of inactivation of Xyl I with TNBS revealed that complete inactivation occurred due to the binding of one molecule of TNBS to the active site of Xyl I. The higher K_m values and similar k_{cat} values of the TNBS modified enzyme in comparison to native enzyme and the substrate protection against OPTA and TNBS, suggested the presence of the lysine residue in the substrate-binding site.

ii) Site and significance of a histidine residue

A fluorescent chemoaffinity label *o*-phthalaldehyde (OPTA) was used to ascertain the conformational flexibility and polarity at the active site of xylanase I (Xyl I). OPTA has been known to form a fluorescent isoindole derivative by cross-linking the proximal thiol and amino groups of cysteine and lysine. The involvement of cysteine in the formation of Xyl I-isoindole derivative has been negated by fluorometric and chemical modification studies on Xyl I, with group specific reagents and by amino acid analysis. The kinetic analysis of diethylpyrocarbonate modified Xyl I established the presence of an essential histidine at or near the catalytic site of Xyl I. Modification of histidine and lysine residues by diethylpyrocarbonate and 2,4,6-trinitrobenzenesulfonic acid, respectively, abolished the ability of the enzyme to form an isoindole derivative with OPTA, indicating that histidine and lysine participate in the formation of the isoindole complex. A mechanism for reaction of OPTA with histidine and lysine residues present in the protein structure has been proposed. Experimental evidence presented here suggests for the first time that the active site of Xyl I is

conformationally more flexible and it is more easily perturbed in presence of denaturants than the molecule as a whole. The changes in the fluorescence emission maxima of a model compound (isoindole adduct) in solvents of different polarity were compared with the fluorescence behaviour of Xyl I-isoindole derivative to conclude that the active site is located in a microenvironment of low polarity.

4. Encapsulation and interaction of xylanase in fatty lipid matrix.

Encapsulation of substrate protected xylanase I (Xyl I) from *Thermomonospora* sp. in thermally evaporated fatty amine films by a simple beaker-based immersion technique under enzyme-friendly conditions has been described. The approach is based on the diffusion of the enzyme from aqueous solution, driven primarily by attractive electrostatic interactions between charged groups on the enzyme surface and ionized lipid molecules in the film. The kinetics of Xyl I diffusion into the amine films was followed using Quartz Crystal Microgravimetry (QCM) measurements while the secondary and tertiary structure of the enzyme in the lipid matrix was studied using Fourier Transform Infrared (FTIR) and fluorescence spectroscopy measurements. The encapsulated Xyl I system was reusable and it was found that the lipid matrix enhances the optimum temperature conditions to a higher value. The advantages of this approach over other methods currently used for encapsulation of biomolecules are briefly discussed.

5. Construction of genomic library and identification of xylanase gene in *E. coli*

A genomic library of an alkalothermophilic *Thermomonospora* sp. was constructed in *E. coli* using standard approach of shotgun cloning. The genomic DNA from alkalothermophilic *Thermomonospora* sp. was isolated and digested with *Eco*RI. The 2-10 kb genomic DNA fragments were ligated with *Eco*RI digested and dephosphorylated pBluescript vector. The ligation mixture was used to transform competent *E. coli* XL1 cells. Based on blue\white selection, colonies containing recombinant plasmids were selected. The genomic library was screened using ^{32}P labeled xylanase probe from *Thermomonospora fusca* and xylan congo red plate clearance assay and the clone containing the xylanase gene was identified. The xylanase gene containing plasmid pATA93 was digested with different restriction enzymes.

A part of this work has been published as:

RESEARCH PAPERS

1. George, S.P ., Ahmad, A. & Rao, M.B. 2001. Studies on carboxymethyl cellulase produced by an alkalothermophilic actinomycete. *Bioresource Technol.* 77, 171-175.

2. George, S.P. , Ahmad, A. & Rao, M.B. 2001. A novel thermostable xylanase from *Thermomonospora* sp.: Influence of additives on thermostability. *Bioresource Technol.* 78, 221-224.

3. George, S.P. , Ahmad, A. & Rao, M.B. 2001. Involvement of a lysine residue in the active site of a thermostable xylanase from *Thermomonospora* sp. *Biochem. Biophys. Res. Commun.* 282, 48-54.

4. George, S.P. & Rao, M.B. 2001. Conformation and polarity of active site of xylanase I from *Thermomonospora* sp. as deduced by fluorescent chemoaffinity labeling: Site and significance of a histidine residue. *Eur. J. Biochem.* 268, 2881-2888.

5. George, S.P. , Gole, A.M., Sastry, M. & Rao, M.B. Interaction of xylanase I with fatty lipid matrix: fabrication, characterization and enzymatic activity of the enzyme-fatty lipid composite films. Communicated to *Journal of Physical Chemistry B.*

6. George, S.P ., Nath, D. & Rao, M.B. Thermophilic xylanases: molecular and biotechnological perspectives. Review in book *Advances in Microbiology* (in press).

7. George, S.P. and Rao, M.B. A novel bifunctional xylanase I from *Thermomonospora* sp has a single active site for the hydrolysis of xylan and p-nitrophenyl xylopyranoside. (manuscript in preparation)

ABSTRACTS

1. George, S.P ., Ahmad, A. & Rao, M.B. Studies on carboxymethyl cellulase produced by an alkalothermophilic actinomycete. Abstract published in 67th annual meeting of society of biological chemists (India) at New Delhi, Dec 19-21, 1998.

2. George, S.P ., Ahmad, A. & Rao, M.B. Thermostable xylanase from an alkalothermophilic actinomycete. Abstract published in American chemical Society (ACS), CELL and CARB divisions symposium held in New Orleans from Aug 21-26, 1999.

3. George, S.P ., Anish T.R. & Rao, M.B. Cloning and expression of cellulase gene from an extremophilic *Thermomonospora* sp. in *E. coli*. Abstract published in "Thermophiles", International symposium to be held at the department of Microbiology at Delhi University, South campus, New Delhi, India, from December 3-7, 2001.

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CHAPTER I

GENERAL INTRODUCTION

" The world is not running out of oil - at least not yet. What our society does face, and soon, is the end of the abundant and cheap oil on which all industrial n ations depend."

Colin J. Campbell and Jean H. Laherrére.
Scientific American, March 1998.

The analysis of the discovery and production of oil fields around the world, suggest that within the next decade the supply of conventional oil will be unable to keep up with the demand. The peak production of oil will occur between 2005 and 2010 after which there will be a decline in oil production and an increase in oil prices. There is an urgent need to analyze alternate sources of energy. Biomass is an alternative natural source of chemical feedstock with a replacement cycle short enough to meet the demand in the world fuel market. Xylan is a major constituent of hemicellulose and is the second most abundant renewable resource (Whistler & Richards, 1970). Eventually, if not in the near future, hemicellulose in combination with cellulose will supply most of the global demand for raw materials. Thus, it is not unrealistic to foresee that coal and crude oil are most likely to be substituted by biomass in another 50 years (Goheen, 1981).

In recent years, microbial capabilities have been exploited to use this plant biomass to the maximum. The use of microbial enzymes for the hydrolysis of lignocellulose is advantageous because of the high specificity of enzyme reactions, the mildness of the reaction conditions and the absence of substrate loss due to chemical modifications (Wong *et al*., 1988). These bioconversion processes are particularly attractive for the utilization of agricultural wastes. Cellulose is the major plant carbohydrate and its enzymatic hydrolysis by cellulase has been extensively investigated. The second most abundant polysaccharide is xylan, a major component of hemicellulose. Xylan together with cellulose accounts for more than 50 % of all plant biomass (Uffen, 1997). Hydrolysis products from lignocellulose may be subsequently converted into liquid fuel, single cell protein and chemicals using selective fermentative organisms (Biely, 1985). Based on total global plant biomass, the energy content present in both xylan and cellulose is almost equivalent to 640 billion tons of oil (Coughlan, 1993).

Due to the heterogeneity of xylan, the complete enzymatic degradation to its monomer is a complex process requiring the action of a battery of hydrolytic enzymes. They include Endo-(1→4)-β-xylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase and phenolic acid esterases. Endo-(1→4)-β-xylanase, which attacks the polysaccharide backbone is one of the most crucial enzyme involved in xylan hydrolysis.

Figure 1: The xylanolytic enzymes involved in the degradation of xylan
Ac: Acetyl group; α-Araf: α-arabinofuranose; α-4-*O*-Me-GlcA: α-4-*O*-methylglucuronic acid

Xylanases stable and active at high temperature and alkaline conditions are suitable for biotechnological applications. Extremophilic organisms are one of the natural sources for the identification of such enzymes (Nicolson *et al.*, 1988).

Microbial diversity

The microbial species that produce xylanases and other glycosidases have been found in extremely diverse natural habitats. Under mesophilic growth conditions, xylanolytic activity has been reported in a wide variety of different genera and species of bacteria, fungi, and yeasts (Gilkes *et al*., 1991). Microbial strains capable of growth at high temperatures at which most microorganisms fail to grow are grouped together as thermophiles. A host of microorganisms inhabit extreme environmental conditions where they thrive and grow at temperatures above 50 °C, at pH values 9 or greater (Uffen, 1997) and/or in high ionic strength aqueous systems containing salt approaching saturating conditions (Wong *et al*., 1988). *Bacillus stearothermophilus* (Gilead & Shoham, 1995), *Bacillus* sp. SPS-0 (Bataillon *et al*., 2000), *Bacillus* sp. XE (Debeire-Gosselin *et al*., 1992), *Clostridium stercorarium* (Bérenger *et al*., 1985), *Clostridium thermolacticum* TC 21 (Debeire-Gosselin *et al*., 1992), *Streptomyces* T_7 (Keskar *et al*., 1989), *Streptomyces thermoviolaceus* OPC520 (Tsujibo *et al*., 1992) and *Thermomonospora curvata* (Stutzenberger & Bodine, 1992) are some of the aerobic, thermophilic microorganisms shown to actively degrade plant polymers, including xylan. Hyperthermophilic eubacteria have been isolated that grow at temperatures above 80 °C. These microbes include *Thermotoga maritima* (Winterhalter & Liebl, 1995), *Thermotoga* sp. (Saul *et al*., 1995), *Caldocellum saccharolyticum* (Lüthi *et al*., 1990), *Dictyoglomus* sp. (Mathrani, & Ahring, 1991), *Pyrolobus fumarii* (Blöchl *et al*., 1997) and *Rhodothermus marinus* (Dahlberg *et al*., 1993). Anaerobic spore forming thermophilic cells include *Clostridium thermocellum, Clostridium thermohydrosullfuricum and Clostridium thermosaccharolyticum* (Biely, 1985). Additional anaerobic cells that grow and thrive at higher temperature with xylan include *Thermoanaerobacterium thanolicus, Thermoanaerobacterium acetigenum* (Nielsen *et al*., 1993), *Thermoanaerobium brockii, Thermoanaerobacterium* sp. strain JW/SL-Y S485 (Shao *et al*., 1995), and *Thermobacteriodes* species (Biely, 1985). Xylanolytic enzymes in these hyperthermophilic cells operate around neutral pH. There are few reports of *Bacillus* sp. that degrade xylan at high temperature and alkaline pH. Okazaki *et al*. (1984) were the first to report an alkalothermophilic *Bacillus* sp., capable of growth at pH 9-10 and 50 °C. *Bacillus* sp. (Dey *et al*., 1992) and *Bacillus* sp. TAR-1 (Takahashi *et al*., 2000) which are alkalothermophiles have also been reported to produce xylanolytic enzymes.

Scope of thermostable enzym es

Thermozymes are thermostable enzymes that function optimally between 60 and 125 °C. They have attracted increasing attention in recent years owing to their biotechnological potential. They are already used in molecular biology (Taq polymerase), detergent industry (proteases) and starch processing (α-amylase) and are excellent catalytic candidates for numerous additional applications that require high temperature stability. The fact that extremozymes have significant financial impact for the companies that exploit them is evident from the example of Taq polymerase, which has sales of $ 80 million per annum (Persidis, 1998). In general, parameters like temperature, pH and chemical as well as enzymatic stability are important for the industrial applicability of enzymes. The use of thermophilic enzymes reduces stability problems and in so doing, alleviates some of the expense of production and replacement in a reactor. The stability of enzymes from thermophiles should lead to higher recoveries at ambient temperatures than is possible for mesophiles. The low activity of extremely thermophilic enzymes at ambient temperatures eases handling and storage problems (Coolbear *et al*., 1992). Besides higher thermostability other expected advantages of thermophilic enzymes are increased chemoresistance, a longer useful shelf life and less contamination problems (Sonnleitner & Fiechter, 1983). Commercial application of xylanases demands identification of highly stable enzymes active under routine handling conditions. Most of the biobleaching of paper is carried out at high temperatures, therefore it is advisable to use enzymes that are active and stable at high temperatures. Xylanases are industrially important enzymes and many reviews have been published covering the various aspects of these enzymes (Biely, 1985; Debeire-Gosselin *et al*., 1992; Gilbert & Hazlewood, 1993; Kulkarni *et al.*, 1999). However, in recent times the biotechnological application of thermostable xylanases has stimulated research on its biochemical and molecular aspects.

Structure of xylans

In plant cells, xylan is one of the three major structural polysaccharides and is localized in the cell wall matrix. The relative distribution of lignocellulosic components in the cell walls is dependent on the plant species and on the stage of growth and development. Xylans are

typically polydispersed heteropolysaccharides with a homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl residues. The xylopyranosyl backbone is substituted at positions C-2, C-3 and C-5 to varying degrees depending upon the plant and the stage of development of the plant when the polymer was obtained (Joseleau *et al*., 1992). In monocots, at the C-2 positions 1→3 linked α-D-glucoronic acid or 4-O-methyl-α-D-glucoronic acid might occur, while at C-3 of xylopyranose, one frequently finds 1→3 linked α-L arabinofuranose. In some xylans, particularly in hardwoods, xylopyranose residues may be O-acetylated at the C-2 or (more commonly) the C-3 positions. Additionally, small amount of phenolic components, such as ferulic and *p*-coumaric acids (associated with lignin) may be esterified to xylan via their carboxyl groups to C-5 of arabinose branches (Kato & Nevins, 1985).

Xylanase production

Under natural conditions, xylanolytic enzymes appear to be inducible by the products of their own action and are subject to catabolite repression by carbon sources such as glucose or xylose. Xylan, being a high molecular mass polymer, cannot enter the cell. The induction of the enzymes are stimulated by low molecular fragments of xylan namely xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers, which are produced in the medium by small amount of constitutively produced enzyme. Xylan has been shown to be the best inducer of xylanase production in many cases (Simpson, 1956; Mishra *et al*., 1984; Kelly *et al*., 1989; Nakamura *et al*., 1992). However, a few organisms show constitutive production of the enzyme (Lyr, 1972; Debeire-Gosselin *et al*., 1990). Cellulose has also been observed to act as an inducer in a few cases (Bérenger *et al*., 1985; Deshpande *et al*., 1986; Morosoli *et al*., 1986; Stutzenberger & Bodine, 1992). Induction can also be achieved by various synthetic alkyl, aryl β-D-xylosides (Nakanishi & Yasui, 1980) and methyl β-D-xyloside (Nakanishi *et al*., 1992; Marui *et al*., 1985). These compounds enable the production of xylanolytic enzymes in the absence of xylan and xylooligosaccharides. Cheaper hemicellulosic substrates like corn cob, wheat bran, rice bran, rice straw, corn stalk and bagasse have been found to be most suitable for the production of xylanase in certain microbes. Wheat bran was found to be the best substrate for xylanase production by alkalothermophilic *Bacillus* sp. (Dey *et al*., 1992), alkalophilic *Streptomyces* VP5

(Vyas *et al*., 1990), *Streptomyces* T-7 (Keskar *et al*., 1992) and *Penicillium funiculosum* (Mishra *et al*., 1985). Highest levels of xylanase were formed when *Trichoderma brachiatum* was grown on wood pulp (Royer & Nakas, 1989). Xylanase production has been studied under both submerged and solid state fermentation. Haltrich *et al*. (1996) reported maximum xylanase activity (27,000 IU g^{-1}) produced by solid state fermentation from the fungus *Schizophyllum commune*. An increase in xylanase production under solid state fermentation (SSF) has also been reported from a bacterial strain *Bacillus licheniformis* A99 (Archana & Satyanarayana, 1997). Submerged fermentation is advantageous as it is well characterized, and homogeneous conditions are maintained throughout and it is easier to scale up. However, submerged fermentation being an energy intensive process, SSF is gaining more importance. SSF results in higher productivity, simplicity, low energy requirement, better recovery of product, lesser waste water output, the process being cheaper and there is no catabolite repression (Archana & Satyanarayana, 1997). However, its use is limited by the fact that not all organisms are able to grow in SSF, and the process cannot be well characterized. Fungi produce higher levels of xylanase than bacteria or yeasts. However, fungal xylanases are generally associated with cellulases (Steiner *et al*., 1987). Among fungi, the maximum activity reported is 3350 IU ml^{-1} from *Trichoderma reesei* (Haapala *et al*., 1994).

Properties of thermophilic xylanases

Recent reviews have described characterization of xylanases from microbial systems (Kulkarni *et al*., 1999; Sunna & Antranikian, 1997; Warren, 1996). The properties of some of the purified xylanases from extremophiles are described in Table 1. Xylanases from *Thermoanaerobacterium sp*. (Shao *et al*., 1995) and *Thermotoga thermarum* (Sunna *et al*., 1996) showed higher molecular weights of 350 and 266 kDa respectively. Few bacterial and fungal xylanases show maximal activities at temperatures 60-80 °C (Khasin *et al*., 1993; McCarthy *et al*., 1985). The purified endoxylanases from various species belonging to the genus *Thermotoga* are optimally active at temperatures between 80 and 105 °C (Simpson *et al*. 1991; Sunna *et al*., 1996; Winterhalter & Liebl, 1995). Xylanase from *Dictyoglomus* sp. exhibited a half- life of 80 min at 90 °C (Mathrani & Ahring, 1991). *Clostridium stercorarium* xylanase exhibited a temperature optimum of 70 °C and a half-life of 90 min at 80 °C. The thermophilic fungi

include *Thermoascus aurantiacus* (Khandke *et al*., 1989), which produces a thermostable xylanase reported to be stable at 70 °C for 24 h, *Paecilomyces variota* (Krishnamurthy *et al*., 1989) and *Talaromyces byssochlamydoides* (Yoshioka *et al*., 1981) with temperature optimum of 65-75 °C at pH 5-6.5.

Table 1: The properties of xylanases purified from thermophiles

Source		Growth Temp [°C]	Growth pH	Optimum pH	Optimum Temp [°C]	pI	MW [kD]	K_m [mg/ml]	References
Thermophilic bacteria									
Bacillus sp. TAR-1		50	10.5	Broad	70	>9.3	23	-	Takahashi *et al*., 2000
Bacillus sp.	W1 I	50	9-10	6	65	8.5	21.5	4.5	Okazaki *et al*.,
	W1 II			7-9	70	3.6	49.5	3.6	1984
	W2 I			6	65	8.3	22.5	8.3	
	W2 II			7-9.5	70	3.7	50	3.7	

Bacillus acidocaldarius	65	3.5-4	4	80	-	56	1.68	Uchino & Fakuda, 1983
Bacillus stearothermophilus T6	60	7-7.3	6.5	75	9	43	1.63	Khasin *et al*., 1993
Bacillus sp. SPS-0	60	8	6-7	75	-	99	0.7	Bataillon *et al*., 2000
Bacillus amyloliquefaciens	45	7	6.8-7	80	10.1	19.6	-	Breccia *et al*., 1998
Bacillus sp. XE	55	7	6	75	7.8	22	0.60	Debeire-Gosselin *et al*., 1992
Bacillus stearothermophilus 21	55	7	7	60	4.8	39.5	3.80	Nanmori *et al*., 1990
Clostridium stercorarium A	65	7	5.5-7	75	4.5	44	3.2	Berenger *et al*., 1985
B			5.5-7	75	4.4	72	2.9	
C			5.5-7	75	4.3	62	3.7	
Clostridium stercorarium HX-1	60	6-7	6.5	75	4.5	53	1.4	Sakka et *al*., 1991
Clostridium thermolacticum A	65	6-7	6.5	80	4.4	39	0.40	Debeire-Gosselin *et*

TC 21	B			6.5	80	4.5	55	0.53	*al*., 1992
	C			6.5	80	4.6	65	0.48	
Streptomyces T_7		50	7	4.5-5.5	60	7.8	21.8	10.0	Keskar *et al*.,1989
Streptomyces sp. B-12-2	1a	45		6	55	7.5	26.4	5.80	Elegir *et al*., 1994
	1b	50		6	60	8.3	23.8	3.40	
	2			7	60	5.4	36.2	3	
	3			7	60	5	36.2	1.20	
	4			6	60	4.8			
Streptomyces	I	50	7	7	70	4.2	54	-	Tsujibo *et al*., 1992
thermoviolaceus OPC520	II			7	60	8	33	-	
Thermoanaerobacterium sp.		60	6	6.2	80	4.3	350	3	Shao,*et al*., 1995
JW/SL-YS485									
Thermonospora curvata	1	55	6-7	7.8	75	4.2	36	2.50	Stutzenberger &
				7.2	75	7.1	19	1.40	Bodine 1992
				6.8	75	8.4	15	2.00	
Thermotoga sp. Fj SS3 B1		80	6.8-7	5.3	105	-	31	0.07	Ruttersmith *et al*.,

									1992
Thermotoga maritima	Xyn A	80	7	6.2	92	-	120	1.10	Winterhalter &
MSB8	Xyn B			5.4	105	-	40	0.29	Liebl, 1995
Thermotoga thermarum		77	6	6	80	-	266	0.36	Sunna *et al*., 1996
				7	90-100	-	35	0.24	
Thermophilic fungi									
Talaromyces	X-a	50	6.2	5.5	75	4.3	76	N.D.	Yoshioka *et al*.,
byssochlamydoides YH-50	X-b-I			4.5	70	3.8	54	N.D.	1981
	X-b-II			5	70	4	45	N.D.	
Talaromyces emersonii	II	45	4.5	4.2	78	5.3	78.4	N.D.	Tuohy &
CBS 814.70	III			3.5	67	4.2	58.2	N.D.	Coughlan, 1992
Thermoascus aurantiacus	C436	45	6	5.1	80	7.1	32	1.70	Tan *et al*., 1987

Multiplicity of xylanases

Bacterial and fungal cells produce a multiplicity of enzymes that belong to the same functional class and which sometimes exhibit broad plant polymer specificity (Wong *et al*., 1988; Gilkes *et al.,* 1991). These enzymes can be grouped into families based on conserved amino acid sequences in the catalytic domains and by hydrophobic cluster analysis. Thus, all high molecular weight xylanases belong to the family F/10, whereas low molecular weight xylanases belong to the family G/11. (Gilkes *et al*., 1991; Henrissat, 1992). Enzyme multiplicity has been studied in the fungus, *Trichoderma reesei* and *Trichoderma harzianum* (Wong *et al*, 1988). Results suggested that *Trichoderma reesei* produced four xylanases, each with different molecular weight and pI values. Törönnen *et al*. (1992) cloned two *Trichoderma reesei* genes, *xyn1* and *xyn2* that appeared to encode separate products, XYL1 and XYL2 exhibiting similar molecular weights 19 and 21 kDa respectively but had pI values of 5.2 and 9.0. Similarly, *Trichoderma harzianum* produced three distinct xylanases (Wong *et al*., 1986). In analogous studies, Shareck e*t al*. (1991) reported that *Streptomyces lividans*, produces three xylanases encoded by three different genes, *xlnA* belongs to the family F, while *xlnB* and *xlnC* are members of the family 11. Thomson (1993) suggested various mechanisms that could account for the multiplicity of function and specificity of the xylan degrading enzymes. Electrophoretically distinct xylanases could arise from posttranslational modification of a gene product such as differential glycosylation or proteolysis. The detection of minor xylanases may also be an artifact of the growth and /or purification conditions or these enzymes may have functions, which are not required in large amounts, e.g. hydrolysis of linkages not found frequently (Wong & Saddler, 1992). Multiple xylanases can also be produced from different alleles of the same gene (Wong *et al*., 1988) or may be a result of independent genes (Hazlewood & Gilbert, 1993).

Cloning and expression

For commercial realization and economic viability of xylanase production, it is necessary to identify organisms, which can hyperproduce the enzymes. Recombinant DNA techniques offer the means to enhance protein production. Genes for more than 70 xylanases have been cloned and sequenced (Schlacher *et al*., 1996). However, comparatively fewer genes of thermostable xylanases have been cloned.

a) *Heterologous cloning* : Thermostable xylanase genes have been isolated from an assortment of genera and expressed in *E. coli*. The xylanase gene cloned from *Bacillus stearothermophilus* T-6 was of interest because the enzyme was optimally active at pH 9 and 65 °C (Gat *et al*., 1994). Cloning and expression of a xylanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1 in *E. coli* has been reported (Gibbs *et al*., 1995). The recombinant enzyme was found to have an optimum temperature of 85 °C. Walsh *et al*. (1998) cloned the thermophilic xylanase from the extreme thermophile *Thermotoga* in the yeast *Kluyveromyces lactis*. The xylanase enzyme was correctly processed and secreted by *Kluyveromyces lactis* transformants into the culture broth. The xylanase cloned from *Thermotoga maritima* showed an optimum temperature of 90 °C at pH 5.5 and was stable upto 100 °C (Chen *et al*., 1997). The recombinant xylanase from *Thermotoga neapolitana* was found to be stable at 90 °C for 4 hours and a half-life of 2 hours at 100 °C, and it had an optimum temperature of 102 °C at pH 5.5 (Velikodvorskaya *et al*., 1997). Hayashi *et al*. (1997) were the first to report the cloning of the xylanase component of the cellulosome from *Clostridium thermocellum*. Xyn C comprises of a single catalytic domain of family 10 in addition to the thermostabilizing domain and a dockerin domain. The optimum temperature of the enzyme was 80 °C and the enzyme was stable at 70 °C for 10 min in the absence of substrate. The bacterial genes encoding xylanases have been seen in close proximity in the genome to other genes encoding cellulase related functions. In *Bacillus stearothermophilus* 21, the β-xylosidase gene (*xylA*) is located upstream of the xylanase gene (*xynA*) and the two genes are separated from each other by only two nucleotides. Nonetheless, the deletion analysis of the two genes indicates that they are independently transcribed (Baba *et al*., 1994). Similar linkage between xylanase and β-xylosidase genes has been shown in the case of *Bacillus pumilus* and *Caldocellum saccharolyticum*. Helbers *et al*. (1995) have shown the high level expression of the thermostable xylanase from *Clostridium thermocellum* into transgenic tobacco plants. The xylanase gene was introduced into the tobacco plant by an integration system with *Agarobacterium tumefaciens.*

b) *Homologous cloning* : A number of heterologous proteins cannot be efficiently expressed in *E. coli*, due to the relatively abundant occurrence of rare codons in the cloned gene, the need

for post synthetic modifications, toxicity of the coded protein to the host cells and susceptibility of the foreign protein to protease coded by *E. coli*. It is well known that higher expression levels are obtained in homologous host system. The homologous expression of the xylanase gene from an alkaliphilic, thermophilic *Bacillus* sp. has been demonstrated in a xyn^- mutant *Bacillus subtilis* A8 (Shendye & Rao, 1993), and *Bacillus subtilis* MI 111 (Shendye *et al*., 1994). Enhanced production of the xylanases has also been reported by chromosomal gene integration in an alkalophilic, thermophilic *Bacillus* sp. (Shendye & Rao, 1993a).

c) *Overexpression in E. coli* : In order to make the cloning of a gene a commercial success it is important to overexpress the protein. Moreover, crystallographic studies which require copious amounts of pure protein, the overexpression of the cloned gene is the most feasible option to attain the goal. Luthi *et al*. (1992) have cloned and overexpressed a thermostable xylanase from *Caldocellum saccharolyticum* in *E. coli*. High-level expression and single step purification of a thermostable xylanase from *Bacillus stearothermophilus* T-6 has been reported (Lapidot *et al*., 1996). The xylanase gene was cloned into T-7 polymerase expression vectors and the enzyme was found to constitute over 70 % of the cell protein. Xylanase A from extremely thermophilic eubacterial strain Rt8BB.4 was overexpressed in *E. coli*. The xylanase activity from domain 2 was associated with a 36 kDa protein, which was stable at 70 °C for at least 12 h at pH 7 (Dwivedi *et al*., 1996).

Sequence homology of xylanases

The sequences of many xylanases from family 10 and 11 are available in sequence databases. In general, sequences from the same family were closely related and exhibited more homology. This criterion has been used to assign particular sequences to particular families. The amino acid sequence of Xyn II (family 11) from *Trichoderma reesei* showed significant homology to alkaline low molecular mass xylanases from the same family, but also resembled more bacterial xylanase in several aspects (Törönnen *et al.*, 1992) *Schizophyllum commune* xylanase (family 11) was found to be the most similar to *Trichoderma* xylanases and distantly related to *Bacillus* xylanases (Oku *et al*., 1993). Xylanase B from the fungus *Penicillum purpurogenum* showed significant similarity with 38 other fungal and bacterial xylanases belonging to family 11. Xylanase B was more closely related to other fungal endoxylanases than to bacterial enzymes with 73 % homology to xylanase A from *Aspergillus awamori* (Diaz

et al., 1997). Xylanase A from *Bacillus stearothermophilus* 21 was 45-50 % identical to xylanases from other thermophilic organisms such as *Caldocellum saccharolyticum* and *Clostridium thermocellum* (Baba *et al.*, 1994a). Irwin *et al*. (1994) reported that the catalytic domain of xylanase (Tfx A) from *Thermomonospora fusca* showed 40-72 % identity with other xylanases. The xylanases from thermophilic organisms such as xylanase A from *Caldocellum saccharolyticum*, xylanase B from *Clostridium stercorarium* and xylanase T-6 from *Bacillus stearothermophilus* T-6 showed high homology to xylanase A from alkalophilic *Bacillus* C-125. *Bacillus stearothermophilus* xylanaseT-6 showed comparatively less homology to other xylanases from thermophilic organisms, such as *Clostridium saccharolyticum and Thermoanaerobacterium saccharolyticum* B6A-RI. The modular pattern found in the sequence of Xyn Z from *Clostridium thermocellum* was similar to the structural organization of several cellulases in which similar domains are shuffled at different locations within the sequences (Grepinet *et al.*, 1988). While, Xyn Y from *Clostridium thermocellum*, in common with xylanases from several thermophilic bacteria, contained a family 10 catalytic domain.

Thermostabilizing domain

The presence of repeated domains is common in bacterial xylanases, however, little is known about their function. The thermostability of a xylanase from *Thermoanaerobacterium saccharolyticum* has been correlated to the N-terminal domains of the enzyme (Lee *et al*., 1993). *Clostridium thermocellum* also contained a homologue of this domain that appeared to confer stability. There is a 28 % sequence homology between the thermostablilizing domains of xylanase Y from *Clostridium thermocellum* and xylanase A from *Thermoanaerobacterium saccharolyticum* (Fontes *et al*., 1995). The number and position of thermostablilizing domain are variable in respective enzymes; for example, a thermostabilizing domain occurs in the N terminal of *Clostridium thermocellum* Xyn C, whereas it occurs in the middle of *Clostridium thermocellum* Xyn Y and two domains occur in the N terminus of *Thermoanaerobacterium saccharolyticum* Xyn A (Hayashi *et al*., 1997). A similar thermostabilizing domain has been reported from a thermostable xylanase produced by a mesophilic *Bacillus* sp. strain NG-27 (Gupta *et al*., 2000). Thermostabilizing domains are found mainly in the thermophilic xylanase of family 10. Non-catalytic domains conferring thermostability have not yet been

detected in family 11 xylanases. One of the reasons may be that these enzymes are inherently more thermolabile, unlike family 10 xylanases in which folding is such that it has been easy for them to evolve into a thermophilic enzyme. Although removal of these domains reduced the thermostability and optimal temperature of *Clostridium thermocellum* Xyn Y and *Thermoanaerobacterium saccharolyticum* Xyn A, the interaction between catalytic domains and thermostabilizing domains responsible for thermostabilization remains to be studied.

Mechanism of action of xylanases

Based on amino acid sequence identities in their catalytic domains, β-glycanases have been grouped into 9 different families (A to I). The grouping is confirmed by hydrophobic cluster analysis, which reveals similarities in secondary structures. Most of the xylanases belong to family F and G (Gilkes *et al.*, 1991). The conservation of function may be reflected in the conservation of sequences, and therefore xylanases of individual families share common catalytic mechanism with other glycosidases. Glycosyl hydrolases are classified as retaining or inverting enzymes depending on the stereochemistry of the released product. Retaining enzymes liberate products with the same anomeric configuration as the substrate by a double-displacement mechanism involving a glycosyl enzyme intermediate (Figure 2).

Inverting enzymes form products with inverted configuration mediated by a single displacement mechanism. The stereochemistry of the reaction products released as shown by NMR studies for both family 10 and 11 indicated that catalysis occurs through double displacement mechanism with the retention of the anomeric configuration in both the families (Gebler *et al.*, 1992; Biely *et al.*, 1994). The hydrolysis reaction catalysed by xylanases as well as cellulases proceeds through an acid base mechanism involving two residues. The first residue acts as a general catalyst and protonates the oxygen of the osidic bond, whereas the second residue acts as a nucleophile which, in case of retaining enzymes reacts with the oxocarbonium intermediate or promotes the formation of an $-OH$ ion from a water molecule, as observed for inverting enzymes.

a

b

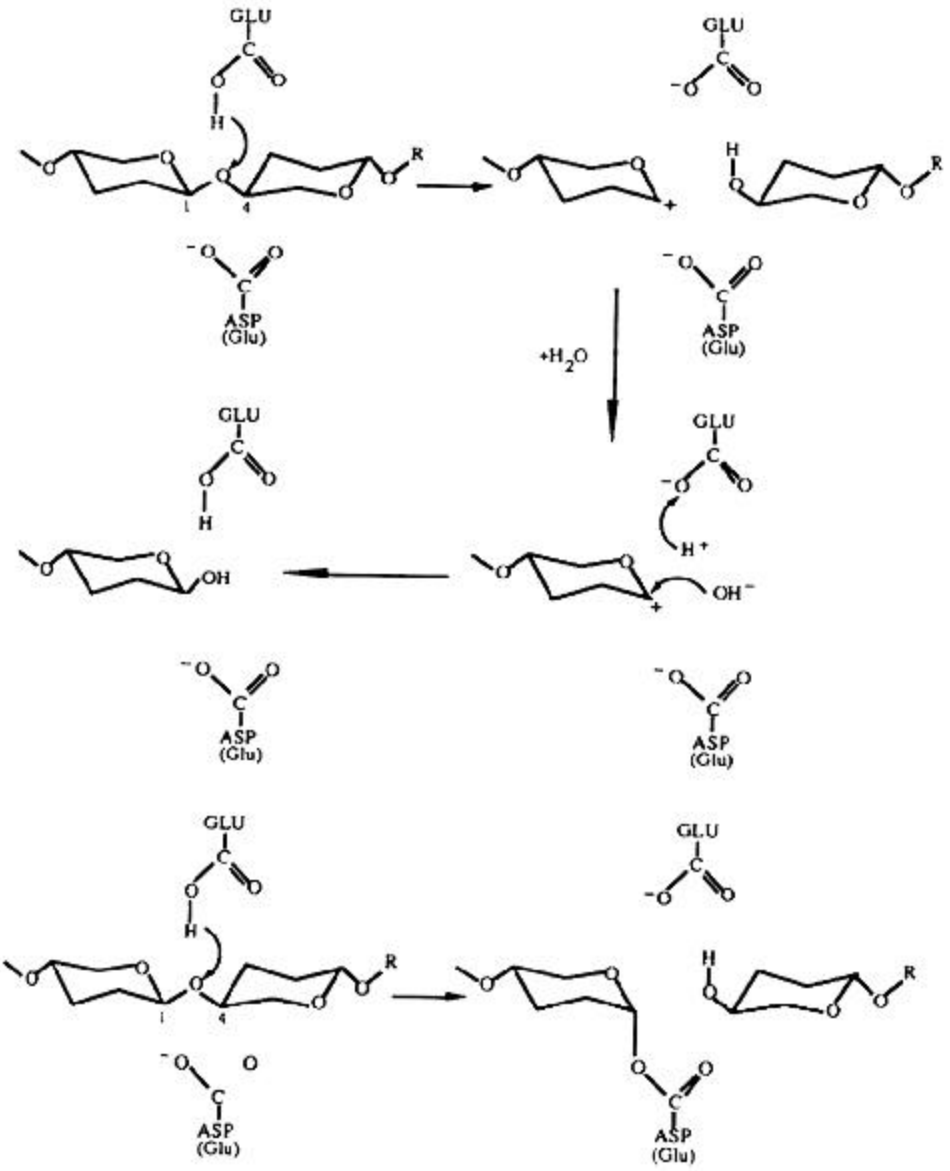


Figure 2: Reaction mechanism of the xylanases. Double displacement reaction (a) involving stabilization of an oxocarbonium ion by electrostatic interaction with the carboxylate of an Asp (or Glu) at the active site or (b) involving formation of a covalent intermediate by nucleophilic attack of the Asp (or Glu) on the incipient sugar

Reaction with retention of configuration involves a two-step mechanism in which proton transfer occurs to and from an oxygen molecule in an equatorial position at the anomeric center (Sinnot, 1990). This reaction mechanism is similar to that of lysozyme (Kelly *et al.*, 1989). Reactions leading to inversion of configuration proceeds through a single

substitution, as observed in the case of β-amylase (Thoma *et al*, 1971). It is, therefore, likely that a single amino acid residue (acid/base catalyst) is responsible in both proton transfer steps. Xylanases, mainly exhibit double displacement mechanism involving a glycosyl-enzyme intermediate, which is formed and hydrolysed via oxocarbonium ion like transition.

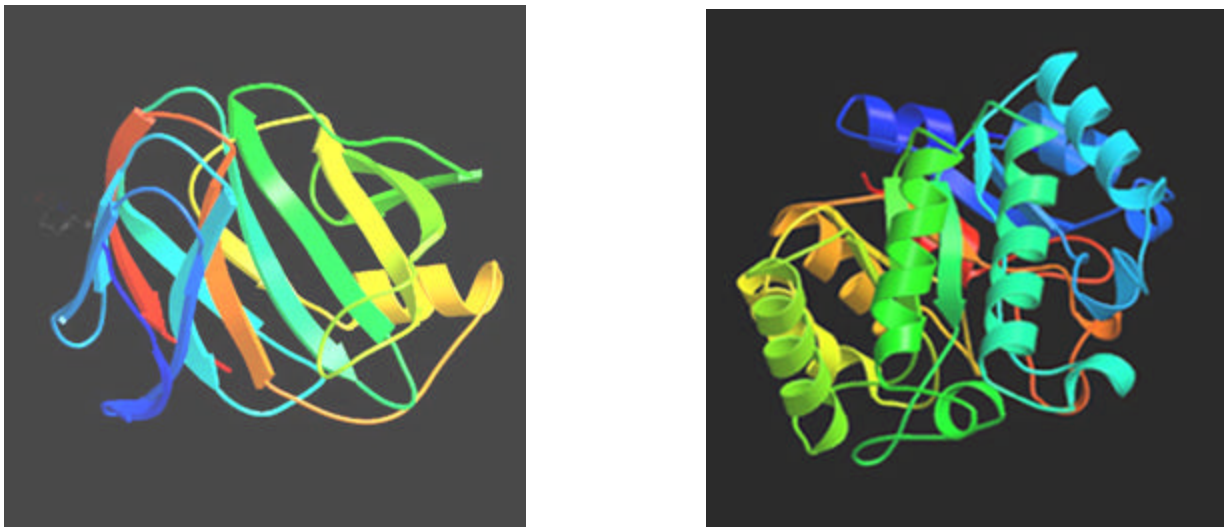
Site directed mutagenesis

Initially enzyme active site residues were determined by selective chemical modification. Rapid developments in molecular biology techniques have made it possible for the individual amino acid to be substituted by site directed mutagenesis (SDM). The conserved glutamate residues (Glu^{87} and Glu^{184}) have been shown to be present in the active site of xylanase A from *Schizophyllum commune* (Oku *et al*., 1993). The three dimensional structures of two bacterial xylanases from *Bacillus pumilus* and *Bacillus circulans* and a fungal xylanse from *Trichoderma harzianum* have also revealed the presence of two completely conserved glutamic acid residues corresponding to Glu^{87} and Glu^{184} of xylanase A from *Schizophyllum commune*. The highly conserved amino acid residues located at specific positions in xylanases are important in structure function analysis and hence are targeted for site directed mutagenesis. SDM can also be used to improve the properties of the enzyme by replacing the key residues in the active site of the enzyme. The xylanase A gene from *Streptomyces lividans* was modified by SDM, selecting for mutations that improve the catalytic activity and thermostability of the enzyme (Moreau *et al*., 1994). The amino acids at positions 155, 156 and 173 were replaced either by amino acids having same polarity but a different side chain, or by amino acids having the same side chain with a different polarity.All mutants synthesized a 43 kDa protein that reacted in immunoblots with specific anti-xylanase A antibodies. The F155Y, R156K, R156E and N173D mutant xylanases showed significantly higher activities than the wild type enzyme. An improvement in stability was obtained by replacing the arginine situated at position 156 of xylanase A by a glutamic acid residue (R156E). The mutation resulted in a modified enzyme with a temperature optimum that was 5 °C higher than that of wild type. In fact, the same substitution occurs naturally in a xylanase produced by *Bacillus* sp. C-125 and in a xylanase produced by *Caldocellum saccharolyticum* (Shareck *et al*., 1991). Both xylanases have an optimum temperature of 70 °C, which is the

same as for R156E. It was observed that the mutation N173D resulted in an enzyme that had enhanced stability at 60 °C. The substitution of Asn for Asp significantly increases the mean hydrophobicity. An increase in hydrophobicity in a region of a protein should reduce the number of water molecules having access to this locus or create a more compact protein as a result of new secondary structure interactions. This phenomenon could eventually increase protein stability as shown in the case of N173D xylanase. In *Bacillus circulans* mutant xylanase proteins, disulphide bridges conferred thermo protection as observed by the 15 °C increase in thermostability (Wakarchuk *et al*., 1994).

Crystal structure

There are very few thermostable xylanases whose crystal structure have been determined. The crystal structure of the thermostable xylanase from *Thermomyces lanuginosus*, as determined by single-crystal X-ray diffraction shows that it is a compact, globular protein with two heavily twisted β-sheets. Its structure is similar to other family 11 xylanases. One sheet forms the "outer" surface of the enzyme and consists of five antiparallel strands while the other β-sheet consists of nine antiparallel strands. The most prominent feature of the structure is a long cleft spanning the whole molecule and containing the active site. The reasons for thermostablity of *Thermomyces lanuginosus* xylanase were analyzed by comparing its crystal structure with known structures of mesophilic family 11 xylanases. The thermostability of this xylanase is due to the existence of a disulphide bridge, which connects the C-terminus of the β-strand B9 (residue 110) with the N-terminus of the α-helix (residue 154). The presence of electrostatic interactions between ion pairs has also been implicated in the stabilization of the protein structure (Gruber *et al*., 1998).



a b

Figure 3: High resolution crystal structures of thermostable xylanases. a) Crystal structure of family 11/G xylanase from *Thermomyces lanuginosus* (Resoultion: 1.55 Å). b) Crystal structure of family 10/F xylanase from *Thermoascus aurantiacus* (Resolution: 1.8 Å).

Thermoascus aurantiacus xylanase, belonging to family 10 of glycoside hydrolases is a single polypeptide chain that assumes $(\alpha/\beta)_8$ TIM barrel fold. The presence of a disulphide bridge between C terminus of helix α7 and the N terminus of the β8 and the presence of more than ten salt bridges might be contributing to the thermostability and activity of this protein at elevated temperatures. Salt bridges between oppositely charged groups are an important aspect to protein structure stability and, when buried, contribute 3-5 kcal/mol in its stability. The presence of six short helices termed as “thermo helix” also contribute to the stability of the protein (Auerbach *et al*., 1998). The presence of a proline residue in the middle of a α-helix may also contribute to the better packing in the protein and the formation of a more stable structure (Natesh *et al*., 1999).

Xyn B from *Dictyoglomus thermophilum* Rt46B.1 is folded into a single domain based on two β-sheets that pack against each other. Xyn B is the most thermostable xylanase yet to be structurally characterized having a temperature optimum of 75 °C. Several factors have been implicated for the thermostability of Xyn B. Firstly the solvent-accessible surface that is polar, is significantly higher than other xylanases. Xyn B has 83 % polar surface when compared to the 78 % polar surface area for *Bacillus circulans* xylanase. An increased

proportion of polar surface correlates strongly with increased thermostability (Vogt *et al*., 1997). Xyn B contains an extended B3-A5 connecting loop, which results in enhanced hydrogen bonding and results in higher thermostability.

The crystal structure of PVX, a thermostable, family 11 xylanase isolated from the thermophilic fungus *Paecilomyces varioti* Bainier has been determined by molecular replacement techniques using polyalanine coordinates of the *Thermomyces lanuginosus* xylanase. The structure has been determined at a high resolution (1.59 Å) and the final refined model has 194 amino acid residues and 128 water molecules. The structure belongs to an all β fold, with two curved β-sheets, forming the cylindrical active site cleft, and a lone α-helix, as present in other family 11 xylanases. The structure and the sequence of PVX were compared with the other available native structures of mesophilic and thermophilic xylanases belonging to family 11. The analysis provides the basis for the rationalization that the "hinge" region is made more compact in thermophiles by the addition of a disulphide bridge between Cys110 and Cys154 and a N-H…O hydrogen bond between Trp159 near the extremity of the lone α-helix and Trp138 on β-strand B8. Other factors which contribute to the thermostability of the enzyme are the associations involving buried water molecules, additional ion pairs and C-H…O and C-H…π type interactions dispersed in select areas (Kumar *et al*., 2000).

Biotechnological potentials of xylanases

The potential biotechnological application of xylan and xylanases has been of tremendous importance to researchers. The applications of xylanolytic enzymes can be categorized into two: the first one is their use in combination with cellulolytic enzymes and the second in absence of cellulases (Biely, 1985). A number of specific examples of how microbial cellulases and xylanases might be exploited in agricultural and industrial processes has been cited below.

1. Addition of cellulases and xylanases to pig and poultry cereal based diets elicits a significant improvement in nutrient utilization (Bedford & Classen, 1992). Poultry are incapable of efficiently digesting cereals, the major ingredient in their diets. The addition of xylanases to the feed results in an improved nutritive value of wheat based diets for broilers. Xylanases with

low pH optimum and broad pH stability would be most suitable for application in animal feed, where activity and stability at low pH is crucial.

2. The use of xylanases has also been proposed in clarification of juices and wines (Biely, 1985), maceration of vegetable matters (Beck & Scoot, 1974), liquefaction of coffee mucilage for making liquid coffee, extraction of flavors and pigments, plant oils and starch (McCleary, 1986).

3. Enzymic saccharification of agricultural and industrial wastes to provide sugar syrups for human consumption or for the production of fine chemicals through industrial fermentations. Xylose and xylooligosaccharides have possible applications in the food industry as thickeners or as fat substitutes and as an anti-freeze food additive. The hydrolysis products of xylan can be converted to solvents and artificial low calorie sweeteners (Wong & Saddler, 1992).

4. Xylanases are of great importance in the paper and pulp industry in the brightening process as they replace toxic chemicals such as elemental chlorine and chlorine-dioxide for developing environment friendly processes.

5. Enzymatic digestion of industrial wastes as an alternative to landfill deposition.

6. In the pharmaceutical industry, xylan is used as an agent for direct tableting and in combination with other components; for sustain release tablet construction.

7. Xylanases can be used in the production of dissolving pulps (purified cellulose) for making viscose rayons, cellulose esters and cellulose ethers (Paice & Jurasek, 1984) and to remove undesired hemicellulose content (Hinki *et al*., 1985; Viikari *et al*., 1993).

8. Improving the ensilage process by the introduction of cellulolytic capacity into silage bacteria and pretreatment of ensiled herbage with cellulase and hemicellulases (Wong & Saddler, 1992).

9. The use of xylanases in bakery has been suggested (Maat *et al*., 1992) and they were found to increase the specific volume, textural properties and shelf life of the bread.

Future prospects

The long-term application of xylanase for the conversion of renewable biomass into liquid fuels is not yet economically feasible. In order to make the application of xylanases realistic the improvement of enzyme yields is of prime importance. The complete bioconversion of xylans to the sugar monomers has so far not been achieved; this is the main

hurdle in the success of bioconversion process. The enzymatic cleavage of xylan to smaller oligosaccharides is itself a reversible reaction, hence a special process for the enzymatic cleavage of xylan needs to be designed. Such as the two-phase reactor in which the product can be continuously separated from the reactants. Cleaner biobleaching technology for the paper and pulp industry is currently concentrated in the developed countries, whereas renewable energy generation from agricultural waste has more relevance for the developing nations. In order to bring these ideas to reality further research has to be carried out. It is necessary to identify potent extremophilic xylanase producers, which are capable of producing enzymes active at extreme pH, and temperature. The crystal structures and sequence homology of few thermostable enzymes have been determined. Further studies on the gene sequence of thermostable enzymes will help to determine the basis of thermostability.

Present work

The present study deals with the biochemical and molecular aspects of cellulose and xylan hydrolyzing enzymes from an alkalothermophilic *Thermomonospora* sp. The thesis includes the following six chapters.

Chapter I: General introduction

Chapter II: Production of xylanase and cellulase from an extremophilic actinomycete

Chapter III: Purification and characterization of xylanase

Chapter IV: Active site studies of xylanase I: Residues implicated and conformation of active site

Section I: Evidence for the presence of an essential lysine residue

Section II: Site and significance of a histidine residue

Chapter V: Encapsulation and interaction of xylanase in fatty lipid matrix

Chapter VI: Construction of genomic library and identification of xylanase gene in *E. coli*

CHAPTER II

Production of XYLANASE AND CELLULASE from an extremophilic actinomycete

INTRODUCTION

Many microorganisms make their home in forbidding environments such as hot springs, glaciers and deep-sea vents. These microbes are called extremophiles because they thrive under conditions that, from the human vantage are extreme. Amazingly, these organisms do not merely tolerate but they do best in these punishing habitats and, in many cases, require one or more extremes in order to reproduce. The search for extremophiles has intensified recently, as scientists have recognized that the "survival kits" possessed by extremophiles can potentially serve in an array of applications. Of particular interest are the enzymes that help extremophiles to function in brutal circumstances.

One of the ways to identify enzymes, which are thermally stable and active at alkaline pH, is to exploit natural sources such as extremophilic organisms. They are known to produce enzymes having higher thermostability than those derived from their mesophilic counterparts (Nicolson *et al*., 1988). Xylanases and cellulases stable and active at high temperature and alkaline conditions are suitable for biotechnological applications. There are a number of reports on thermophiles producing cellulases (Stutzenberger *et al.*, 1972; Wilson *et al.*, 1988). A few alkalothermophilic *Bacillus* capable of producing enzymes of industrial importance are also known (Dey *et al.*, 1992; Okazaki *et al.*, 1984), but there is a lack of information relating to alkalothermophilc actinomycetes producing cellulase or xylanase. Cellulases have been investigated mainly with respect to their industrial use for the bioconversion of agricultural biomass resources into useful products. Recently cellulases have been successfully used as additives for laundry detergents. The current proportion of total enzyme production destined for the laundry detergent market exceeds 30 % (Horikoshi, 1996). Due to the increase in environmental pressure on the textile and paper industry, cellulases are expected to play a crucial role in developing eco-friendly technologies in denim processing and deinking of paper.

The present chapter describes the isolation and characterization of an alkalothermophilic actinomycete capable of producing xylanase and cellulase. The production and properties of the xylanase and cellulase have also been described.

MATERIALS AND METHODS

Materials

3,5-dinitrosalicylic acid (DNSA) and different substrates such as xylan (oat spelt), carboxymethyl cellulose (CMC), guar gum, *p*-nitrophenyl derivatives of β-D-xyloside, β-D-glucoside and β-D-galactoside were obtained from Sigma Chemical Co., USA. Sephacryl S-200 matrix was obtained from Pharmacia, Sweden. Peptone, Yeast extract, tryptone, pharmamedia and casamino acids were obtained from Difco Laboratories, USA. Corn cob, wheat bran, coconut stalk and saw dust were purchased locally. All other chemicals used were of analytical grade.

Methods

Isolation of the strain

The sample for the isolation of microorganism was obtained from self-heating compost from Barabanki district of Uttar Pradesh, India. The self-heating compost was suspended in sterile saline (0.85 % NaCl). In order to enrich the sample with xylanase producing organism, which are capable of growing at high pH and temperature, the sample was inoculated into a media containing wheat bran (1 %) as the only carbon source and grown at different conditions of high temperature (50-80 °C) and pH (8-11). The enriched cultures were then plated onto alkaline wheat bran (1 %) yeast extract (0.5 %) plates. The plates were grown at 50 °C for 24 to 48 h. The isolated cultures were streaked on alkaline nutrient agar plates containing 1 % xylan and were checked for clearance due to xylanase production by congo red method (Wood & Teather, 1988). The cultures producing clearance zones on nutrient agar xylan plates were inoculated in 50 ml alkaline wheat bran, yeast extract broth in 250 ml flask and incubated at 50 °C for 24-48 h at 200 rpm. Xylanase production was checked after different time intervals.

Identification of the microorganism

a) *Morphology of mycelia* : The aerial and substrate mycelia of the isolate were studied by growing the microorganism as a slide culture. Further details about the attachment of the

spores to the mycelia were ascertained by studying mycelium adhering to cover slips placed at an angle of 45° in growing culture plates (Cross, 1989). The cover slips were transferred to a slide and observed under Leitz compound microscope (10 X-100 X).

b) *Guanine-cytosine content of the DNA* : Chromosomal DNA was purified by the modified method of Hopwood *et al*. (1985) (as described in Chapter VI), and the guanine-cytosine content of the DNA was calculated from the thermal denaturation temperature (Marmur & Doty, 1962). DNA sample substantially free of protein RNA and phenol at a concentration of 20 μg/ml was taken in a stoppered cuvette. The cuvette was placed in a Beckman DU spectrophotometer attached to a variable temperature water bath. The temperature was increased at increments of 1 °C/10 min. The absorbance at 260 nm was recorded at each temperature. The increase in temperature results in denaturation of DNA causing a concomitant increase in the absorbance at 260 nm (hyperchromicity). The temperature corresponding to half the final increase in relative absorbance is designated as T_m.

The GC content was determined by the equation:

$GC = (T_m - 69.3)\ 2.44$

Where GC is the guanine-cytosine content of DNA and T_m is the melting temperature of DNA.

c) *Susceptibility of spores to heat and novobiocin* : The growth of heat-treated spores (90 °C, 10 min) or spores in the presence of 50 μg/ml novobiocin were observed.

d) *Cell wall composition* : Presence of the meso or L isomer of diaminopimelic acid (DAP) in the cell wall of the culture was detected as described by Staneck *et al*. (1974). Dried cells (10 mg) were hydrolyzed for 18 h with 1 ml of 6 N HCl in a sealed pyrex tube held at 100 °C in a sand bath. After cooling, the tubes were opened and the contents were filtered through paper. The solid material on the filter was washed with 4-5 drops of distilled water. The liquid hydrolysate was dried three consecutive times to remove most of the HCl. The residue was dissolved in 0.3 ml of distilled water. The sample was spotted on Whatman no. 1 paper along with reference standards for meso and L forms of diaminopimelic acid. Descending chromatography was carried out overnight by irrigation with methanol: water: 10 N HCl: pyridine in the ratio 80:17.5:2.5:10. Amino acids were detected by spraying the paper with acetonic ninhydrin (0.1 % w/v) followed by heating for 2 min at 100 °C.

Storage and Maintenance of the culture

The organism identified as *Thermomonospora* sp. was maintained on Luria Bertani (LB) wheat bran (pH 9) plates. The culture was grown at 50 °C for 72 h and the plates were preserved at 4 °C. For long term storage of the culture 15 % glycerol suspension of the spore suspension was kept frozen at - 70 °C. Another set of the culture was dispersed in sterile soil and stored at room temperature.

Xylanase Production

Xylanase production was studied in 500 ml Erlenmeyer flasks containing 100 ml of Reses medium. The composition of the medium was similar to that used by Mishra *et al*. (1984) except for the amount of yeast extract (1 %), Tween 80 (0.1 %) as surfactant and cellulose paper powder-cpp (4 %) as carbon source. Sterile 10 % sodium carbonate was used to adjust the pH of the medium to 9. A 10 % inoculum was added from an inoculum flask grown for 48 h at 50 °C. The microorganism was grown at 50 °C for 96 h on a rotary shaker maintained at 200 rev min^{-1}. At the end of 96 h the fermentation broth was centrifuged at 10,000 rpm for 10 min. The supernatant was used as the source of enzyme. Xylanase production was studied in the presence of different carbon, nitrogen sources and surfactants. All experiments were performed in triplicate.

Estimation of xylanase activity

Xylanase activity was determined by incubating 1 ml of assay mixture containing 0.5 ml of 1 % xylan and 0.5 ml of suitably diluted enzyme in 50 mM phosphate buffer (pH 7) for 30 min at 50 °C. Enzyme and reagent blanks were also simultaneously incubated with the test samples. The reducing sugar formed was estimated by dinitrosalicylic acid method (Miller, 1959). One international unit (IU) of enzyme activity for xylanase was defined as the amount of enzyme releasing 1 μmol reducing sugar from xylan per minute using xylose as standard. The substrate xylan (1 %) was prepared by suspending two grams of xylan powder in 100 ml of distilled water and stirring the mixture for 16 h at 28 °C. The insoluble fraction was separated by centrifugation at 10,000 rpm for 10 min at 4 °C and the soluble fraction was used for the estimation of xylanase activity.

Estimation of CMCase activity

CMCase activity was determined by incubating 1 ml of assay mixture containing 0.5 ml of 1 % CMC and 0.5 ml of suitably diluted enzyme in 50 mM acetate buffer (pH 5) for 30 min at 50 °C. Enzyme and reagent blanks were also simultaneously incubated with the test samples. The reducing sugar formed was estimated by dinitrosalicylic acid method (Miller, 1959). One international unit (IU) of enzyme activity for CMCase was defined as the amount of enzyme releasing 1 μmol reducing sugar from CMC per minute using glucose as standard.

Determination of protein concentration

Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as standard.

Activity towards different subst rates

The supernatant was checked for its activity against different substrates. Ten milligrams of substrates such as chitin, guar gum, filter paper, gum arabic, lichenan, laminarin and gum locust bean were incubated with a suitably diluted culture filtrate in a total volume of 1 ml in 0.05 M sodium phosphate buffer, pH 7, at 50 °C for 30 min. The reducing sugars released were estimated by dinitrosalicylic acid method. The activity of the supernatant towards PNPX, PNPG and PNP-β-Gal was determined by incubating 1 ml of 0.3 % substrate with 0.1 ml of suitably diluted enzyme in 0.05 M phosphate buffer, pH 7, for 10 min at 40 °C. The liberated *p*-nitrophenol was measured at 400 nm. The activity was calculated according to the method of Mishra *et al*. (1984). The glucose and xylose isomerase activity were measured by incubating suitably diluted enzyme with in 50 mM phosphate buffer, pH 7 along with 100 mM glucose or 5 mM xylose in presence of 5 mM $MgSO_4.7H_2O$ and 1 mM $CoCl_2.4H_2O$ at 60 °C for 20 min. Fructose or xylulose formed was measured by the cysteine carbazol method (Dische & Borenfreund, 1951).

Effect of pH and temperature on the activity and stability of the enzyme

Estimation of xylanase and CMCase activity at different pH (4-10) and temperature (40-100 °C) values were carried out under standard assay conditions to determine optimum

pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 5 IU of enzyme for one hour, at 50 °C in buffers of desired pH and then estimating the residual activity under standard conditions. The temperature stability was determined by incubating 5 IU of enzyme at various temperatures. Aliquots were removed at different time intervals and their residual activity was estimated under standard assay conditions.

Thermostability of xylanase in the presence of different additives

The effect of various compounds on the thermostability of xylanase was determined by incubating the enzyme at 80 °C in the presence of various additives. At the end of incubation period the enzyme was cooled for 5 min and the residual activity was determined. Suitable controls were also taken. The time at which 50 % initial activity was reached corresponded to the half-life of the enzyme. The results obtained were expressed in the form of protective effect, which is defined as the ratio of xylanase half-life in the presence and absence of additive.

Partial purification of CMCase

After 120 h of aerobic cultivation under the specified growth conditions, cells were harvested by centrifugation at 10,000 rpm for 10 min. Unless otherwise stated all purification steps were done at 4 °C. The CMCase was precipitated from the supernatant by using fractional ammonium sulphate (30-55 %). The precipitate was dissolved in 50 mM sodium phosphate buffer pH 7. The enzyme solution was dialyzed against the same buffer for 24 h at 4 °C with periodical change of buffer. The resulting enzyme was loaded onto a cellulose affinity column and the bound CMCase was eluted by using glass-distilled water. The fractions having maximum CMCase activity were pooled, concentrated and loaded on a Sephacryl S-200 gel filtration column. The column was equilibrated and eluted with 50 mM sodium phosphate buffer pH 7 at the rate of 10 ml/h.

Stability of CMCase in c ommercial detergents

Stability of CMCase in the presence of the commercial detergents such as Ariel, Surf Excel and Henko were investigated by incubating the enzyme in the presence of the detergent (7 mg/ml) at 40 °C. Aliquots of enzymes were removed at intervals of 10 min for 1 h and the residual activity of the enzyme was determined under standard assay conditions.

RESULTS AND DISCUSSION

Isolation of xylanase producing alkalothermophilic organisms

The microorganisms were isolated from self-heating compost from the Barabanki district of Uttar pradesh, India. The isolates capable of growth at high temperature and pH were further screened for their ability to produce xylanase. Only one isolate was showing large clearance on LB xylan plate and was chosen for further studies (Figure 1).

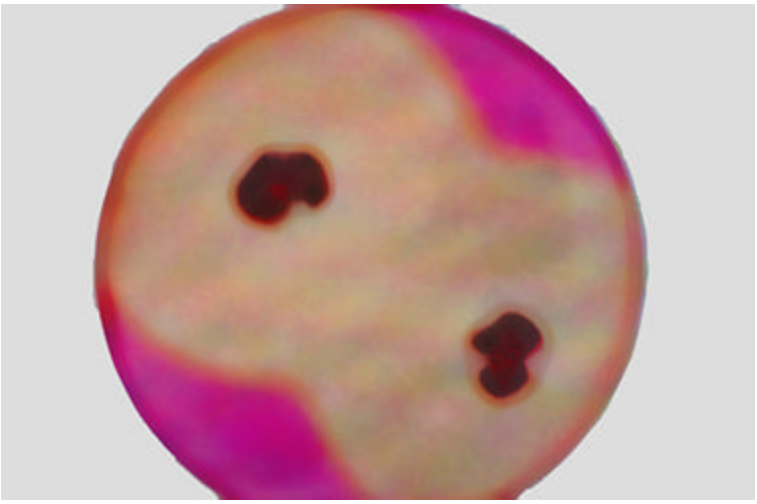


Figure 1: Clearance zone due to xylanase production: Microorganism was grown on xylan (0.5 %) containing LB agar plate and was incubated at 50 °C for 16 h.

Characterization of the isolate

The pH range for growth of the isolate was alkaline (8-9). No growth occurred below pH 8 and maximum growth occurred at pH 9. The optimum temperature for growth was 50 °C, however it failed to grow at 28 and 37 °C. The isolate was an aerobic, sporulating actinomycete which formed white colonies and produced both aerial and substrate mycelia. Single spores were attached to mycelia by sporophores (Figure 2). Based on these morphological characters the isolate could be classified under either *Thermoactinomycete* or *Thermomonospora*. Cell wall analysis indicated that it contained the meso form of DAP, which appeared as olive green spots fading to yellow on the paper chromatogram. The presence of 67 % guanine-cytosine in chromosomal DNA is a trait of the genus *Thermomonospora*. There was no growth from heat-treated spores (90 °C, 10 min) or in the presence of 50 μg/ml

novobiocin. These characteristics confirmed that the isolate is an alkalothermophilic actinomycete belonging to the genus *Thermomonospora*.

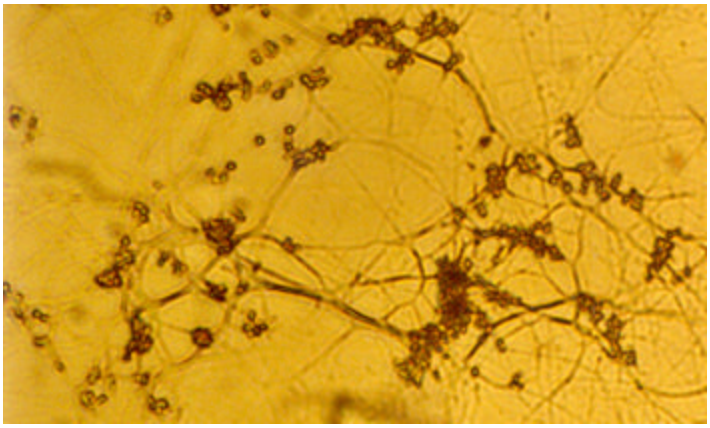


Figure 2: Microscopic photograph showing sporulation pattern of *Thermomonospora* sp.

Media optimization for xylanase production

An assortment of substrates, mainly agricultural residues were evaluated for xylanase production. Cellulose paper powder (cpp) at a concentration of 4 % was found optimal for maximum xylanase production (110 IU/ml). The production of comparable amounts of xylanase using corncob (105 IU/ml) is significant, as it is a cheap agricultural waste. Carbon sources such as wheat bran (30 IU/ml), xylan (22 IU/ml) and rice straw (18 IU/ml) produced moderate amounts of xylanase while coconut stalk and saw dust produced negligible amount of xylanase (Table 1).

Table 1: Effect of different carbon sources on xylanase production

	Carbon source (4 %)	Activity (IU/ml)
1.	Coconut stalk	2
2.	Corn cob	105
3.	Saw dust	3
4.	Cellulose paper powder (2 %)	80
	(3 %)	106
	(4 %)	110
	(5 %)	56
5.	Xylan	22
6.	Wheat bran	30
7.	Rice Straw	18

Using 4 % cpp as carbon source, different organic and inorganic nitrogen sources were evaluated for xylanase production. Yeast extract (115 IU/ml) was found to be the most suitable organic nitrogen source for the production of xylanase. Comparable results were obtained on using peptone and soyameal. When inorganic nitrogen sources were used, then a maximum xylanase production of 86 IU/ml was obtained with ammonium sulphate (Table 2). An increase in the concentration of inorganic nitrogen did not result in enhanced xylanase production. This signifies the use of organic nitrogen source for obtaining high yields of xylanase. Various surfactants at a concentration of 0.1 % were added separately to the medium containing 4 % cpp and 1 % yeast extract. Addition of Tween 80 resulted in a 10 % increase in the production of xylanase. No growth was observed in the presence of Triton X 100. The highest xylanase activity of 125 IU/ml was obtained by growing *Thermomonospora* sp. in Reses media containing 4 % cpp, 1 % yeast extract and 0.1 % Tween 80 for 96 h (Figure 3). The organism also produced 23 IU/ml cellulase, 1 IU/ml mannanase and 0.1 IU/ml β-xylosidase.

Table 2: Xylanase production in the presence of different nitrogen sources.

	Organic nitrogen source (1 %)	Activity (IU/ml)
1.	Tryptone	98
2.	Peptone	105
3.	Casaminoacid	94
4.	Yeast extract	115
5.	Soyameal	103
6.	Pharmamedia	85
	Inorganic ni trogen Source (70 mg/100 ml)	
7.	Ammonium chloride	78
8.	Sodium Nitrate	68
9.	Ammonium Sulphate	86
10.	Di Ammonium hydrogen phosphate	80

Xylanase production was carried out in modified Reses medium using cellulose paper powder (4 %) as the carbon source.

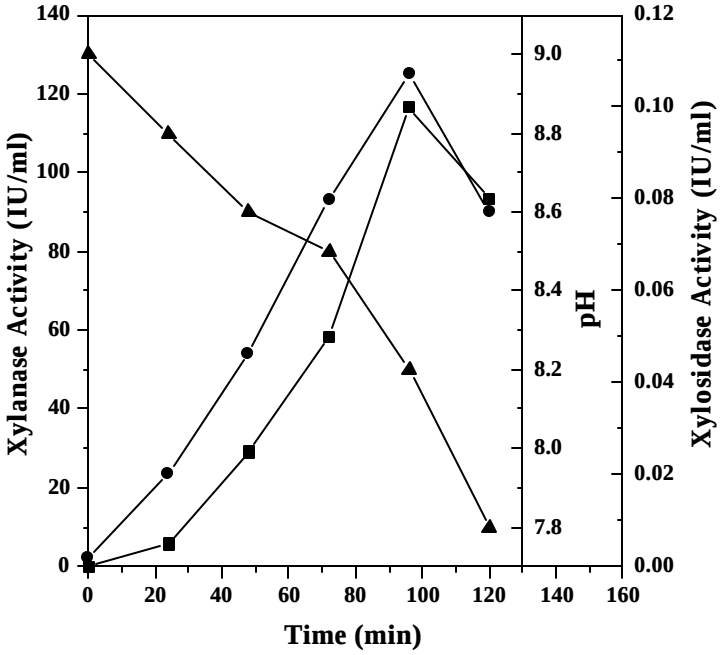


Figure 3: The time course of xylanase and **b**-xylosidase production by *Thermomonospora* sp. The media contained 4 % cpp as carbon source. Culture filtrate xylanase activity (●), xylosidase activity (■) and pH (▲).

The time course of xylanase production was studied in the same media. After a 10 % inoculum was added to the culture medium containing the inducer, xylanase production started without a lag and reached a maximum of 125 IU/ml at 96 h. A study of β xylosidase production was also included as the high xylanase activity could be due to concomitant β-xylosidase production. It was observed by Mishra *et al.* (1985) that the addition of exogenous β-xylosidase increased the ability of *Penicillium funiculosum* to hydrolyze xylan.

pH activity and stab ility of xylanase

The activity of xylanase was determined in buffers of different pH (4-10). The enzyme was active in a broad pH range (5-9) with maximum activity at pH 7. It yielded 68 and 44 % of its maximum activity at pH 8 and 9, respectively. Xylanase was remarkably stable in the pH range (5-8), with up to 80 % activity. It had 68 and 51 % residual activity at pH 9 and 10, respectively (Figure 4).

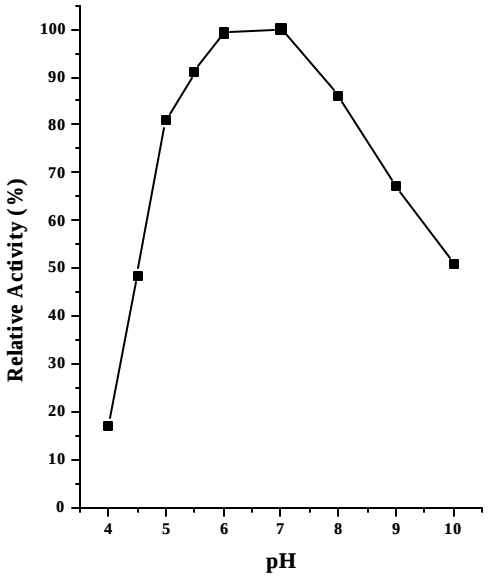


Figure 4: The pH optimum (▲) and pH stability (■) of xylanase action. The pH stability of the enzyme was measured by incubating 5 IU of enzyme for one hour, at 50 °C in buffer of desired pH. The following buffer systems were used: 0.05 M acetate buffer (pH 4-5), 0.05 M phosphate buffer (pH 6-7), 0.05 M TrisCl buffer (pH 8), 0.05 M carbonate-bicarbonate buffer (pH 9-11).

Temperature activity profile and thermal stability of xylanase

The xylanase had an optimum temperature of 70 °C (Figure 5a). It showed up to 80 % relative activity in the temperature range 50- 80 °C. However, at 90 and 100 °C there was a steep decline in activity with 24 and 12 % relative activity, respectively. The xylanase was exceptionally stable at 50 °C, it showed 100 % activity up to 72 h and had half lives of 8 h and 4 h at 60 and 70 °C, respectively (Figure 5b). At 80 °C the enzyme had half-life of only 9 min.

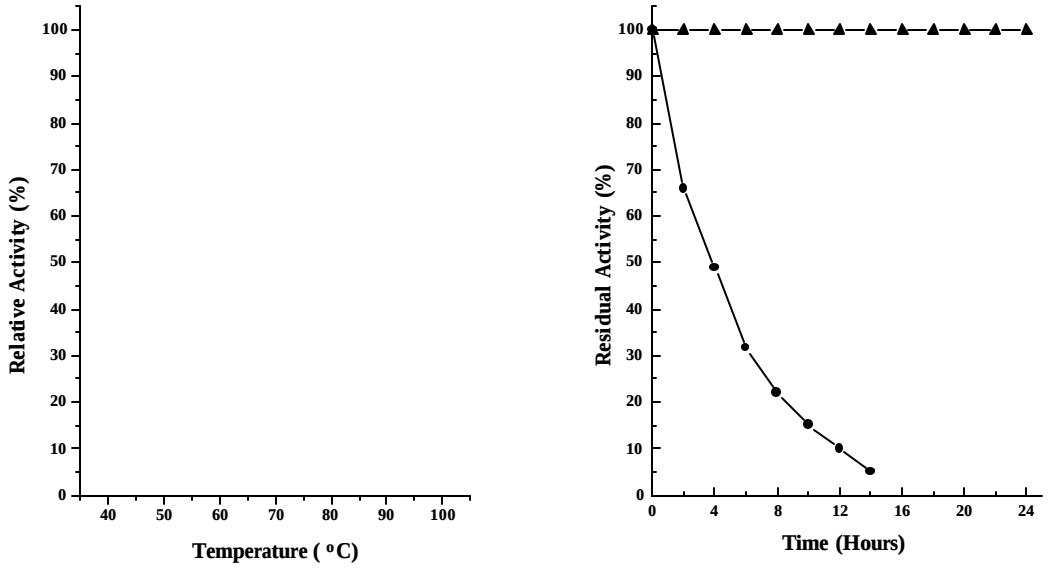


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Figure 5: Temperature optimum and stability of xylanase . a) The optimum temperature of xylanase in 0.05 M phosphate buffer, pH 7. The activity of the enzyme was determined in the range (40-100 °C). b) Thermal stability of xylanase: 5 IU of xylanase was incubated in 0.05 M phosphate buffer (pH 7) at 50 °C (▲), 60 °C (■) and 70 °C (●) for different intervals and residual activity was determined.

Effect of additives on the thermostability of xylanase

In order to improve the thermostability of xylanase at higher temperature, different additives were added to the enzyme solution. Naturally occurring osmolytes like amino acids, polyols and methylamines are known to confer stability to proteins against thermal inactivation. The xylanase from *Thermomonospora* sp. was highly thermostable at 60 and 70 °C, however at 80 °C it had a half-life of 9 min. The addition of alanine (0.5 M), cysteine (10 mM), β-mercaptoethanol and Tween 80 could not prevent the loss in activity at 80 °C. Similarly, various salts like $CoCl_2$, $CaCl_2$, KCl and NaCl at a concentration of 10 mM did not have any effect on the stabilization of xylanase. Nath & Rao (1995) have revealed the ability of substrate (xylan) to protect the enzyme from thermoinactivation at 60 °C but in case of xylanase from *Thermomonospora* sp., xylan (3 %) had a marginal effect on thermostabilization at 80 °C. Additives like trehalose, gelatin and trehalose/gelatin mixture also did not have a substantial effect on the thermostability of xylanase. Glycine at a concentration of 1M

provided a protective effect of 2.7. The effect of different polyols containing two to six carbon atoms [Ethylene glycol (C2), glycerol (C3), Sorbitol and Mannitol (C4)] on xylanase deactivation was studied at 80 °C. Figure 6a shows that all the polyhydroxylic additives had a positive effect on xylanase stability. The protective effect of polyols was proportional to the molecular size of the additives, which can be correlated to the number of hydroxyl groups contained per polyol molecule. An increase in the concentration of sorbitol brought about an increase in its protective effect. Sorbitol provided maximum protection at a concentration of 4 M (Figure 6b).

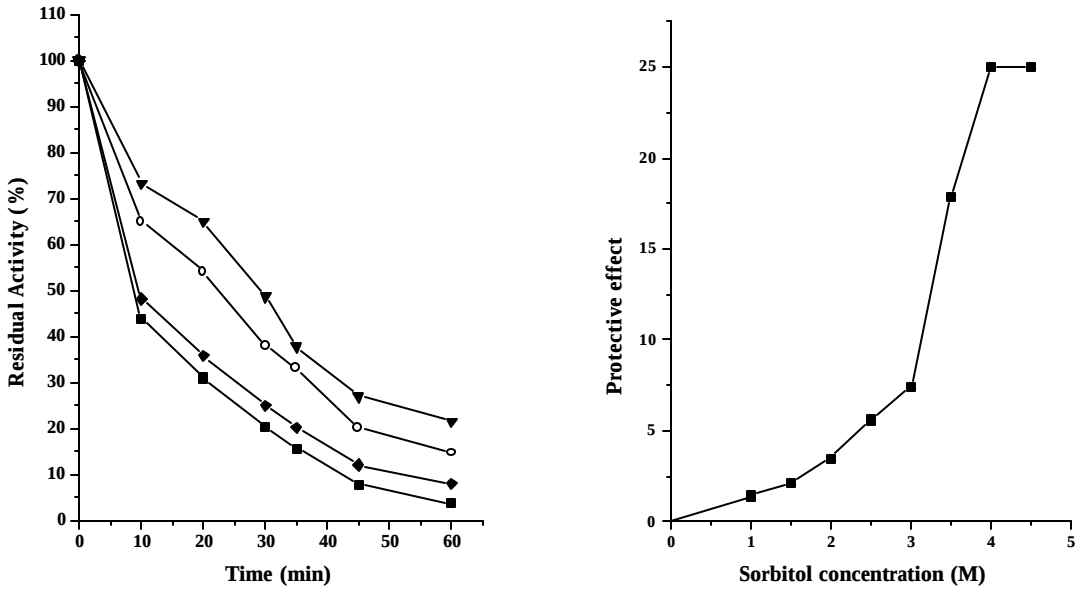

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Figure 6: a) Thermal stability of (■) xylanase at 80 °C in the presence of (◆) ethylene glycol, (▲) glycerol, (▼) mannitol, (●) sorbitol and ( ) glycine: 5 IU of xylanase was incubated in 0.05 M phosphate buffer (pH 7) in presence of additive. At the end of incubation period the enzyme was cooled for 5 min and the residual activity was determined. b) Effect of different concentrations of sorbitol on the thermostability of xylanase at 80 °C. The effect of sorbitol on the thermostability of xylanase was measured in terms of protective effect, which can be defined as the ratio of xylanase half-life in the presence and absence of additive.

Polyols have the ability to maintain the solvophobic interactions that play the key role in supporting the native conformation of the protein. They also have the capability to form hydrogen bonds, which plays an important role in protein stabilization. Polyols and glycine

have the ability to increase the hydration of proteins. Bull & Bresse (1978) have shown that any cosolvent having a tendency to maintain or increase the hydration of proteins would have a protective effect, while cosolvents with dehydrating effect on proteins would be considered as denaturing agents. The stabilizing effect of additives is not an absolute effect valid for all enzymes, but it depends on the nature of the enzyme, on its hydrophilic and hydrophobic character and on the degree of interaction with the additive. The use of thermostable biocatalysts in biotechnology has many advantages at high temperature, such as the increase in reaction rate, shift of thermodynamic equilibrium, high operational stability, increased solubility of reagents and decrease of viscosity of reaction medium.

CMCase production

The time course of CMCase and protein production in the extracellular culture filtrate by *Thermomonospora* sp. is shown in Figure 7. The microorganism was grown in a medium containing different carbon sources at 50 °C for 120 h. The maximum CMCase production of 23 IU/ml was obtained at 120 h, when cellulose paper powder (cpp) at a concentration of 4 % was used as the carbon source.

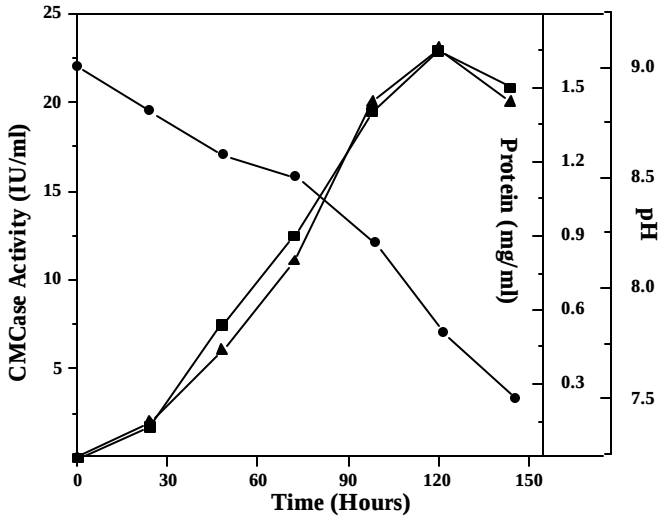

Figure 7: The time course of CMCase production by *Thermomonospora sp.* in a media containing 4 % cpp as carbon source. The extracellular culture filtrate was estimated for: Enzyme activity (■), pH of media (●) and protein (▲).

Wheat bran and Corncob were also suitable carbon sources for CMCase production (8.5-12.5 IU/ml). Very low activity was obtained when xylan, sawdust and coconut stalk were used as carbon sources.

Partial purification of CMCase

Maximum CMCase activity was obtained in the ammonium sulphate saturation range of 30-55 %, hence proteins precipitated in this range were used for purification. CMCase was further purified by sequential cellulose affinity and gel filtration chromatography. After elution from cellulose affinity column, the fractions having maximum specific activity were loaded on Sephacryl S-200 gel filtration column. Two CMCase peaks were observed and they were designated as CMCase I (fractions 44-51) and CMCase II (fractions 53-65). 3 ml fractions were collected and the fractions having highest specific activity were pooled and concentrated using a 10,000 molecular weight cutoff membrane. CMCase I and CMCase II were purified with 18.7 and 17.1 fold purification respectively. CMCase I was used for further studies as it showed higher specific activity. Table 3 summarizes the results for the partial purification of the CMCase.

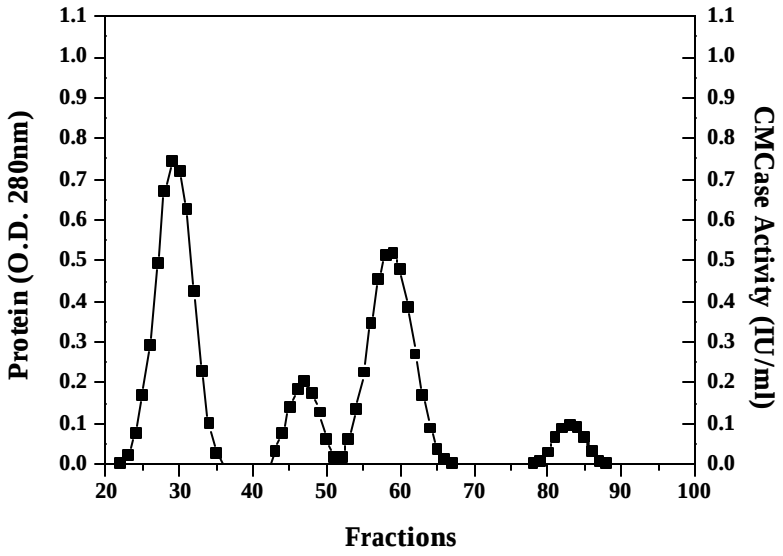

Figure 8: Fractionation of CMCase on Sephacryl S -200 column. CMCase was obtained after ammonium sulphate precipitation (30-55 %) and cellulose affinity column: CMCase activity (●), Absorbance at 280 nm ( ).

Table 3: Purification of CMCase from alkalothermophilic actinomycete.

Steps	Activity (IU/ml)	Protein (mg)	Sp activity (IU/mg)	Fold purification
Broth	23	1.65	13.9	1.0
$(NH_4)_2SO_4$ Precipitation	280.8	15.1	18.5	1.3
Cellulose affinity	350.1	6.2	56.3	4.0
Gel filtration				
CMCase I	285.92	1.1	259.93	18.7
CMCase II	427.84	1.8	237.69	17.1

Properties of CMCase

The CMCase had an optimum pH of 5. Significantly, high activity was found at pH 4 (86 %) but there was a steep decline in activity at pH 7 with only 31 % activity (Figure 9a). The CMCase showed maximum activity at 50 °C, on either side of this temperature there was a decline in activity, only 50 % residual activity was observed at 40 and 60 °C (Figure 9b).

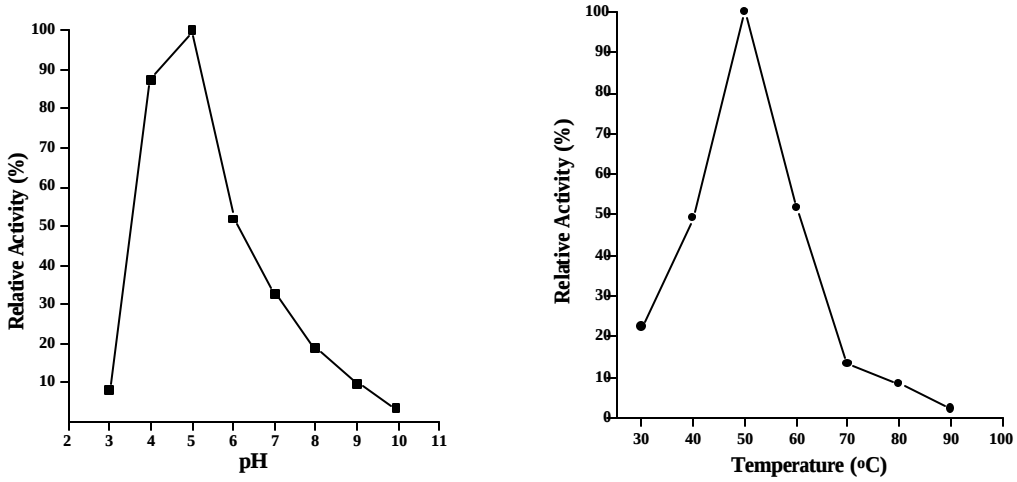

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Figure 9: (a) The optimum pH and (b) temperature of CMCase. The following buffer systems were used: 0.05 M acetate buffer (pH 3-5), 0.05 M phosphate buffer (pH 6-7), 0.05 M TrisCl buffer (pH 8), 0.05 M carbonate-bicarbonate buffer (pH 9-10).

The CMCase had a broad range of pH stability. It showed up to 85 % residual activity in the pH range 4-10. It was most stable at pH 7 (Figure 10a). The CMCase was highly stable at 50 °C, it showed 100 % activity up to 72 h and had half lives of 7 h and 3 h at 60 and 70 °C, respectively (Figure 10b).

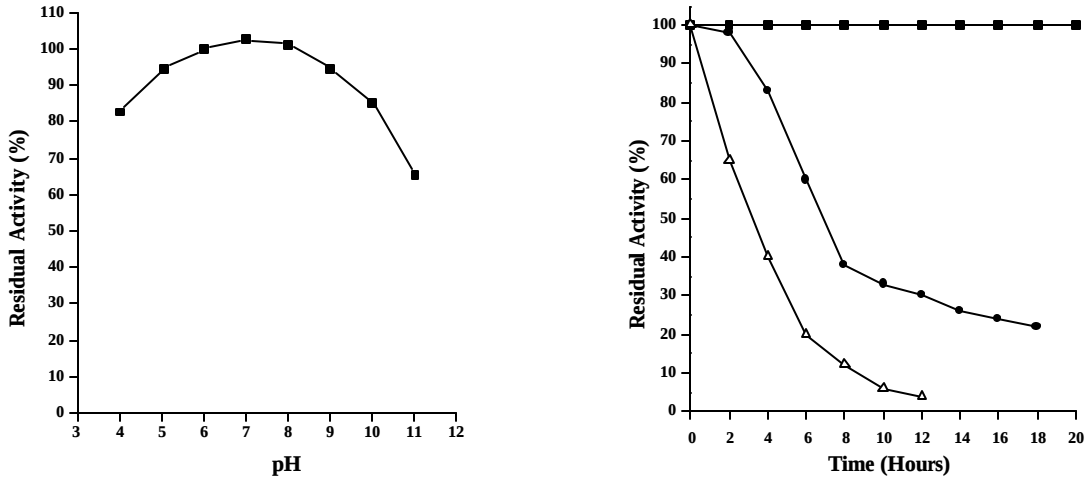

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Figure 10: a) pH stability of CMCase: The pH stability of the enzyme was measured by incubating 5 IU of CMCase for one hour, at 50 °C, in buffer of desired pH b) Thermal stability of CMCase: 5 IU of CMCase was incubated in 0.05 M phosphate buffer (pH 7) at 50 °C (■), 60 °C (●) and 70 °C (△) for different time intervals and residual activity was determined.

Stability of CMCase in the presence of detergents

CMCase was found to be stable in the presence of commercial detergents .The enzyme showed maximum stability in the presence of Ariel having residual activity of 85% after incubation at 40 °C for 1 hour. CMCase had 72 % and 66 % residual activities in the presence of Surf Excel and Henko, respectively under similar conditions (Figure 11).

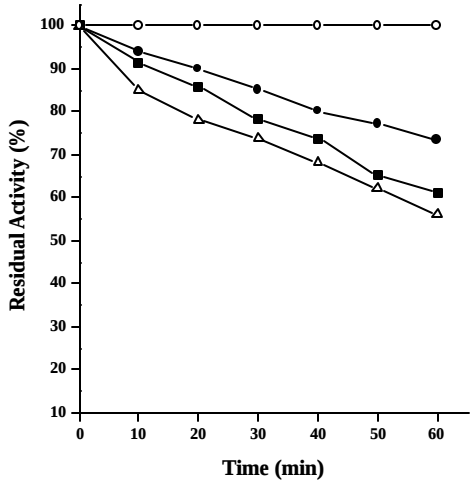


Figure 11: Stability of cellulase in various commercial detergents : CMCase was incubated at 40 °C in presence of various detergents and samples were removed after every ten minutes for 1 hour and residual CMCase activity was determined: Surf Excel (■), Ariel (●), Henko (△) and control (○).

A plethora of mesophilic microorganisms of bacterial and fungal origin capable of producing cellulases and xylanases are reported (Gilkes *et al.*, 1991). These enzymes display activity over a pH range from 4 to 6. However, enzymes with an alkaline pH optimum for activity (pH 10 or higher) are few. They are mainly alkalophilic bacteria from *Bacillus sp.* (Horikoshi *et al.*, 1984; Fukumori *et al.*, 1985) producing extracellular enzymes. A semi alkaline cellulase from an alkalophilic *Streptomyces* strain KSM-9 (Nakai *et al.*, 1987) has been reported. Shikata *et al.* (1990) have also isolated a series of alkalophilic *Bacillus* strains producing alkaline cellulases with pH optima in the range 8.5 to 9.5 having industrial application as laundry detergent additives. The main aim of isolation of an alkalothermophilic organism was in order to increase the possibility of production of alkalistable and thermostable enzymes by the organism. Although our organism was capable of growing at alkaline conditions, the cellulase produced by it had an optimum pH of 5 and the xylanase showed activity in a broad pH range of 5-9. The xylanase and CMCase were found to be highly stable at alkaline pH and were thermostable. The isolate was capable of producing xylanase and CMCase in the presence carbon sources such as wheat bran and corncob. These are agricultural wastes and they would be economical carbon sources during commercial

production of enzymes. The CMCase was also stable in the presence of commercial detergents such as Ariel, Henko and Surf Excel. In spite of the fact that the CMCase had an acidic optimum pH it could still be used in commercial detergents, as there are reports of detergents containing cellulase compositions enriched in acidic endoglucanases type components. These detergents impart good softness to cotton fabrics while retaining fabric colour during laundry (Clarkson *et al.*, 1992). Acidic cellulases also have application in the nonionic surfactant assisted acidic deinking of ONP (old news print) and OMG (old magazine). The use of acidic cellulases during deinking is advantageous as it improves the pulp freeness and repulping efficiency (Xia *et al.*, 1996).

SUMMARY

A novel alkalothermophilic actinomycete having optimum growth at pH 9 and temperature 50 °C was isolated from self-heating compost from the Barabanki district of Uttar Pradesh, India. Based on its morphology, susceptibility of spores to heat and novobiocin, guanine-cytosine content of chromosomal DNA and cell wall composition, the organism was classified under *Thermomonospora*. The isolate produced 125 IU/ml of xylanase when grown in shake flasks at pH 9 and 50 °C for 96 h. The xylanase was active at broad range of pH (5-9) and temperature (40-90 °C). The optimum pH and temperature were 7 and 70 °C respectively. The enzyme was stable in the pH range 5-8 and was found to be thermostable with half-lives of 8 h and 4 h at 60 and 70 °C respectively, but only 9 min at 80 °C. The effect of a variety of compounds to enhance the stability of xylanase at 80 °C was studied. Addition of sorbitol, mannitol and glycerol increased the thermostability of xylanase in proportion to the number of hydroxyl groups per polyol molecule. Glycine also offered protection against thermoinactivation. Xylan, trehalose, gelatin and trehalose/gelatin mixture had marginal effect on the thermostability of xylanase at 80 °C. The culture filtrate also contained cellulase (23 IU/ml), mannanase (1 IU/ml) and β-xylosidase (0.1 IU/ml) activities. The cellulase (CMCase) was partially purified by fractional ammonium sulphate precipitation followed by cellulose affinity chromatography and Sephacryl S-200 gel filtration. The enzyme exhibited optimum activity at pH 5 and temperature 50 °C. The CMCase showed pH stability in the range 7-10. The enzyme retained 100 % activity at 50 °C for 72 h and had half-lives of 7 h and 3 h at 60 °C and 70 °C, respectively. The CMCase was stable in the presence of commercial detergents such as Ariel, Henko and Surf Excel, indicating its potential as an additive to laundry detergents.

CHAPTER III

PURIFICATION AND CHARACTERIZATION OF XYLANASE

INTRODUCTION

Thermally stable enzymes are used at high temperatures for efficient hydrolysis of substrates and can be effectively recycled. Moreover, thermostability of an enzyme is also associated with a higher resistance to most chemical denaturants (Oshima, 1978). Xylanases stable and active at high temperature and alkaline conditions are suitable for biotechnological applications. There are very few reports of xylanases that are active and stable at both alkaline pH and elevated temperatures. The catalytic mechanism of an enzyme can be elucidated from a homogenously purified enzyme. Studies on structure function relationship and crystal structure of enzymes can be pursued only with purified enzymes. There are only two reports for the purification of xylanase from *Thermomonospora* sp. Bachmann *et al.* (1991) have purified a 23 kDa endoxylanase exhibiting three isomeric forms by fast protein liquid chromatography from *Thermomonospora fusca.* While Stutzenberger *et al.* (1992) have purified three xylanases designated as X_1, X_2 and X_3 by isoelectric focusing and size exclusion chromatography from *Thermomonospora curvata*.

The present chapter describes the purification and biochemical properties of a thermostable and alkalistable xylanase from *Thermomonospora* sp.

MATERIALS AND METHODS

Materials

Xylan (oat spelt), 3,5-dinitrosalicylic acid (DNSA), DEAE Sephadex A-50, protein molecular weight markers for SDS-PAGE and gel filtration were obtained from Sigma Chemical Co., USA. Sephacryl S-200 matrix and ampholytes were obtained from Pharmacia, Sweden. Proteins were concentrated in an ultrafiltration cell with UM-10 membranes from Amicon corp., Lexington, USA. All other chemicals used were of analytical grade.

Methods

Production of xylanase

Thermomonospora sp. was grown at 50 °C for 96 h on a rotary shaker maintained at 200 rev min^{-1}. The cultivation medium used for xylanase production was modified Reses media, which contained yeast extract (1 %), Tween 80 (0.1 %) as surfactant and cellulose paper powder (4 %) as the carbon source (Mishra *et al*., 1984). At the end of 96 h the fermentation broth was centrifuged at 10,000 rpm for 10 min. The supernatant was used as the source of enzyme.

Purification of Xylanase

All purification steps were carried out at 4 °C unless otherwise stated.

Ammonium sulphate precipitation: Protein precipitation by salting out technique using ammonium sulphate ($NH_4(SO_4)_2$) was carried out with constant gentle stirring. The amount of solid ammonium sulphate to be added was calculated according to the formula of Jagannathan *et al.* (1956).

$$X = \frac{50\,(S_2 - S_1)}{1 - 0.28\,(S_2)}$$

Where X = Amount of ammonium sulphate to be added for 100 ml.

S_1 = Initial concentration of ammonium sulphate.

S_2 = Required saturation of ammonium sulphate.

The culture filtrate (200 ml) was subjected to fractional ammonium sulphate precipitation (35 to 55 %). The culture filtrate was initially saturated to 35 % by the slow addition of 38.8 g of ammonium sulphate with gentle stirring. The solution was allowed to stand overnight at –20 °C. The precipitated solution was centrifuged at 10,000 rpm for 10 min at 4 °C and the pellet was discarded while the supernatant was subjected to 55 % saturation by the addition of 23.6 g of ammonium sulphate. The precipitate was recovered by centrifuging it at 10,000 rpm for 10 min at 4 °C. The pellet was dissolved in minimum amount of 0.05 M sodium phosphate buffer, pH 7 and dialyzed against 100 volumes of the same buffer with several changes for 24 h.

Ion exchange chromatography: The dialyzed fraction was applied to DEAE-Sephadex A-50 column (6×20 cm), previously equilibrated with 0.05 M sodium phosphate buffer at pH 7. The sample was loaded at a rate of 12 ml/h. The column was initially washed with 0.05 M sodium phosphate buffer, pH 7 at a rate of 16 ml/h to remove the unadhered and loosely adhered proteins. It was observed that xylanase does not elute out until a salt concentration of 0.35 M, therefore the column was first washed with two column volumes of 0.3 M sodium chloride in 0.05 M sodium phosphate buffer, pH 7. The elution of the adhered proteins was carried out by a linear gradient of sodium chloride (0.3 to 0.5 M) in 0.05 M sodium phosphate buffer, pH 7. Fractions of 8 ml each were collected and the fractions having maximum specific activity were pooled and concentrated by ultrafiltration through Amicon UM-10 membrane.

Gel filtration chromatography: The concentrated sample was loaded onto a Sephacryl S-200 column (2.5×100 cm), which was equilibrated with 0.05 M, pH 7 sodium phosphate buffer. Elution was carried out by using the same eluant at a flow rate of 10 ml/hr, and 2 ml fractions were collected. The fractions having maximum specific activity were pooled and concentrated by ultrafiltration through Amicon UM-10 membrane. The purity of the enzyme was checked by SDS-PAGE followed by staining with Coomassie brilliant blue R-250.

Effect of pH on activi ty and stability

Optimum pH for the activity Xyl I was determined by assaying the enzyme at different pH i.e. 50 mM sodium acetate (pH 4, 5), sodium phosphate (pH 6, 7), TrisCl (pH 8, 9) and

carbonate bicarbonate buffer (pH 10). The pH stability was determined by incubating the enzyme at 50 °C in different buffers in the range of pH 4-10 and the residual activities were measured under standard assay conditions.

Effect of temperature on activity and stability

In order to determine the optimum temperature of the enzyme, the activity was determined in the temperature range 40-100 °C at pH 7. The thermal stability of the enzyme was measured by incubating the enzyme in 50 mM sodium phosphate buffer, pH 7 at a given temperature. Aliquots were removed at different time intervals and the residual activity was determined. The temperature stability of the enzyme was determined in the range 40-100 °C.

Determination of molecular weight

a) Gel filtration: The molecular weight of xylanase was determined by gel filtration on Sephacryl S-200 column, according to the method of Andrews (1965). The void volume of the column (V_o) was determined with Blue Dextran 2000 ($2x10^6$ Da). Marker proteins and xylanase (2 mg/ml) were loaded separately on the column and eluted with the same buffer. Molecular weights of the markers used were bovine serum albumin (66 kDa), Ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). Fractions of 2 ml each were collected at a flow rate of 10 ml/h. The elution volume (V_e) of each protein was determined by measuring the absorbance at 280 nm. The molecular weight of the protein was determined by plotting the graph of V_e/V_o vs. log of molecular weight.

b) SDS-PAGE: SDS treatment neutralizes the native charge differences between the proteins and all proteins migrate as anions. SDS in combination with reducing agent (β-mercaptoethanol) and heat dissociates the proteins into their individual polypeptide units. Denatured polypeptides bind to SDS and become negatively charged. The amount of SDS bound is proportional to the molecular weight of the polypeptide. SDS-PAGE was carried out according to the method of Laemmli (1970). The relative electrophoretic mobility (R_f /R) i.e. the ratio of the distance of protein migration to the distance of dye migration were plotted against the log of molecular weight of standard proteins and the molecular weight of the protein was determined by regression analysis.

Isoelectric focussing

The isoelectric focussing of the enzyme was carried out in a vertical column according to the method of Sathivel *et al*. (1995). A glycerol gradient was formed within the vertical column by uniform mixing of high density solution (Glycerol 3 ml, Water 2 ml, Ampholyte-100 µl and 1 IU of pure enzyme) and low density solution (Water 5ml, 100 µl and 1 IU of pure enzyme) in a gradient mixer. Ampholine carrier ampholytes of pH range 2-12 were used. The lower end of the column is dipped in 0.1 N H_3PO_4 and the upper end is overlayed with 0.1 N NaOH. The positive electrode is dipped in the H_3PO_4 solution while the negative electrode is dipped in 0.1 N NaOH. A constant voltage of 400 V is applied for 8 h at 4 °C. After isoelectric focussing the protein, 200 µl each samples were collected in a microtitre plate and the fractions were assayed for xylanase activity and the pH was measured using a surface electrode.

Determination of Michaelis -Menten constant

The effect of substrate concentration on xylanase activity was determined by incubating the enzyme with different amounts of xylan (1-15 mg) under the assay conditions. The value of K_m and V_{max} was determined from Lineweaver Burk plot.

Determination of xylan degradation products

Xylanase I (1 IU) was incubated with xylan (5 mg) in 50 mM sodium phosphate buffer, pH 7 at 50 °C for 24 h in a volume of 0.1 ml. Aliquots were removed at different time intervals and were analyzed for the sugar products formed, by paper chromatography in a solvent system containing butanol, acetic acid and water in the ratio 3:1:1. The paper chromatograms were sprayed as described by Trevelyan *et al.* (1950).

Secondary structure of Xyl I

The CD spectra of purified Xyl I were recorded in a Jasco-J715 spectropolarimeter at ambient temperature using a cell of 1 mm path length. Replicate scans were obtained at 0.1 nm resolution, 0.1 nm bandwidth and a scan speed of 50 nm/min. Spectra were average of 6 scans with the baseline subtracted spanning from 250 nm to 195 nm in 0.1 nm increments. The

CD spectrum of the native Xyl was analyzed using the algorithm in K2d program (Andrade *et al*., 1993; Merelo *et al*., 1994), in order to determine its secondary structure.

Amino acid composition of Xyl I

Amino acid analysis of Xyl I was carried out on Pharmacia LKB alpha plus amino acid analyzer. Samples were hydrolyzed by standard acid hydrolysis conditions using 6 N HCl at 110 °C for 22 h. Excess acid was removed by evaporation in vacuum at room temperature. Cysteine residues were protected by pyridylethylation with 4-vinyl pyridine. Tryptophan was determined by the spectrophotometric method of Spande & Witkop (1967).

RESULTS AND DISCUSSION

Purification of xylanase I (Xyl I)

The culture filtrate was precipitated by fractional (35-55 %) ammonium sulphate saturation. Proteins precipitated within this range had maximum xylanase activity and was used for purification. Xyl I was further purified by sequential chromatography on DEAE Sephadex A-50 ion exchange column and Sephacryl S-200 gel filtration column. The enzyme was eluted from DEAE Sephadex column at a NaCl concentration of 0.35 M (Figure 1). The fractions (32-38) having maximum specific activity were concentrated and loaded onto Sephacryl S-200, gel filtration column. Analysis of fractions for xylanase activity and absorbance at 280 nm showed that maximum specific activity was obtained in fractions 42-50

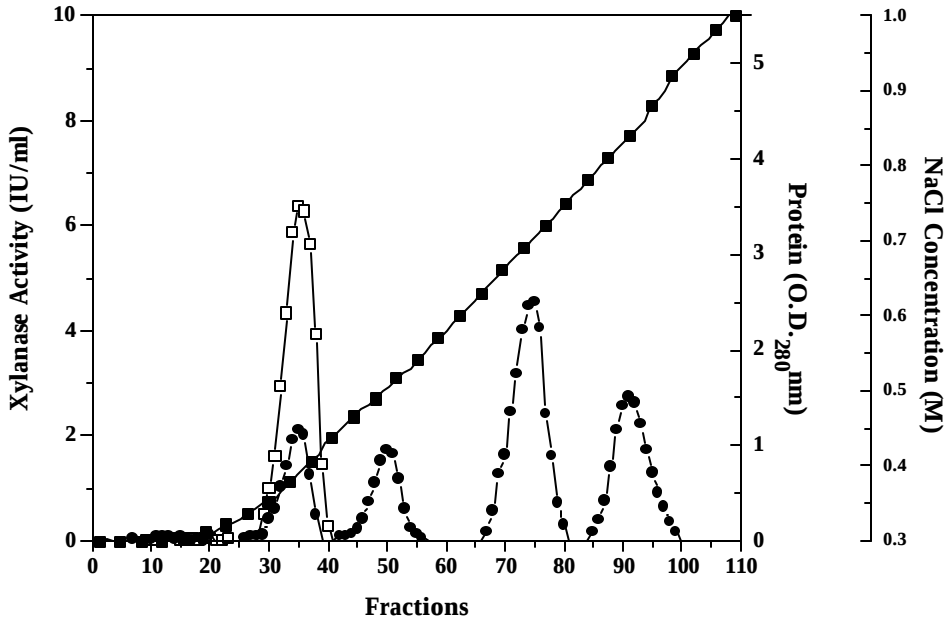


Figure 1: Anion exchange chromatography using DEAE Sephadex. The 35-55 % ammonum sulphate precipitate was loaded onto DEAE Sephadex column and the xylanase (□) along with other adhered proteins (●) were eluted by using a linear gradient of 0.3-1 M NaCl (■).

(Figure 2). These fractions were pooled and the enzyme thus obtained was found to be electrophoretically homogeneous by SDS-PAGE (Figure 3). Xyl I was purified thirteen fold with a specific activity of 455 IU/mg (Table 1).

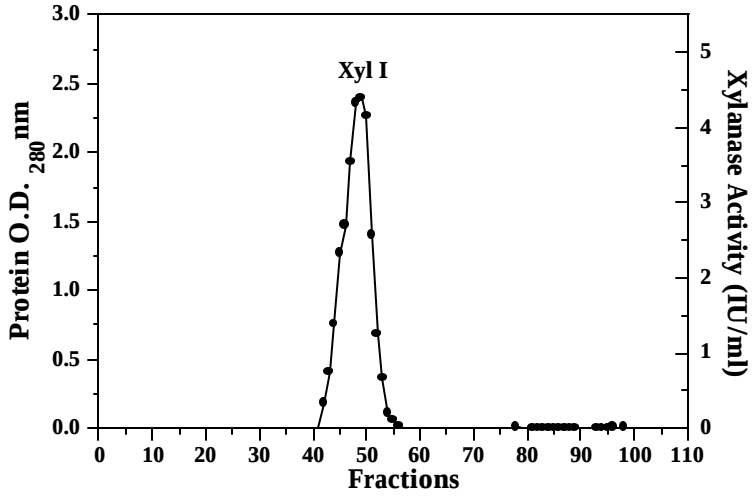


Figure 2: Fractionation of xylanase obtained after DEAE Sephadex on Sephacryl -S-200 column. The fractions were checked for xylanase activity (●) and absorbance at 280 nm (■).

Table 1: Purification of Xyl I

Steps	Volume (ml)	Activity (IU/ml)	Protein (mg/ml)	Specific Activity (IU/mg)	Fold Purification
Culture filtrate	200	125	3.6	34.2	1
$NH_4(SO_4)_2$ precipitation	40	1166.6	10.3	112.8	3.3
DEAE Sephadex	18	267.2	1.1	232.5	6.8
Sephacryl S-200	10	118.5	0.26	455	13.3

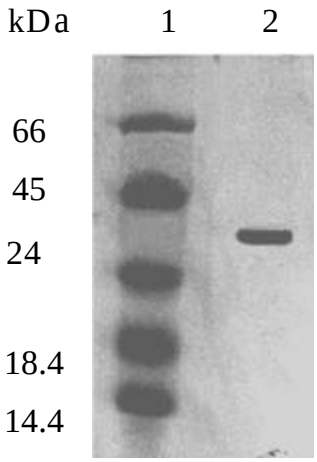


Figure 3: SDS -PAGE of purified Xyl I from *Thermomonospora* sp. in 10 % slab gel. Lane 1 contained standard marker proteins: bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), β-lactaglobulin (18.4 kDa) and lysozyme (14.4 kDa). Lane 2 contained purified Xyl I (2 μg).

Physicochemical properties of Xylanase I

Molecular weight

The molecular weight of the purified Xyl I as determined by gel filtration on Biogel P-100 (37.4 kDa) and SDS-PAGE (38 kDa) were found comparable, indicating the absence of subunits in the protein (Figure 4).

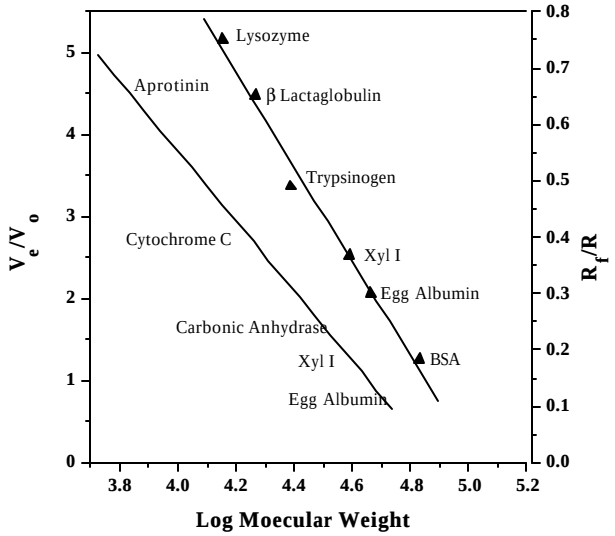


Figure 4: Determination of molecular weight of Xyl I. The molecular weight of Xyl I was determined by gel filtration (●) and SDS-PAGE (▲).

Determination of isoelectric point

Maximum xylanase activity was observed in a fraction having pH 4, indicating that the isoelectric point of Xyl I is 4.1 (Figure 5). An acidic pI indicates that Xyl I is an anionic (negatively charged) protein. Based on its molecular weight and pI, Xyl I can be classified as a family 10 xylanase.

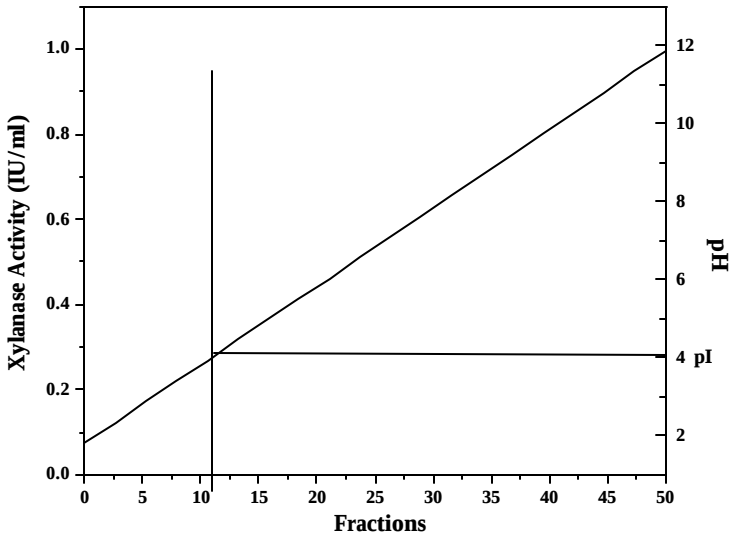


Figure 5: Isoelectric point of Xyl I. The fraction at which (▲) xylanase activity is focused by applying a voltage of 400 V across the (●) pH gradient is the isoelectric point of Xyl I.

Effect of pH on activity and stability

Xyl I was active in a wide range of pH from 5 to 10, with optimum activity at pH 7.5. It was stable in an expansive range of 5 to 10 with more than 75 % residual activity (Figure 6).

Effect of temperature on activity and stability

The temperature optima of the purified Xyl I was 80 °C and it showed up to 75 % relative activity at 90 °C (Figure 6). The enzyme was thermostable, retaining complete activity at 50 °C for up to 72 h. Xyl I had a half-life of 24 h and 12 h at 60 and 70 °C, respectively (Figure 7a), whereas at 80, 90 and 100 °C it had half-lives of 86 min, 30 min and 15 min

respectively (Figure 7b). Xyl I was more thermostable when compared to most of the other reported thermostable xylanases from wild strains. It was more stable than the xylanase isolated

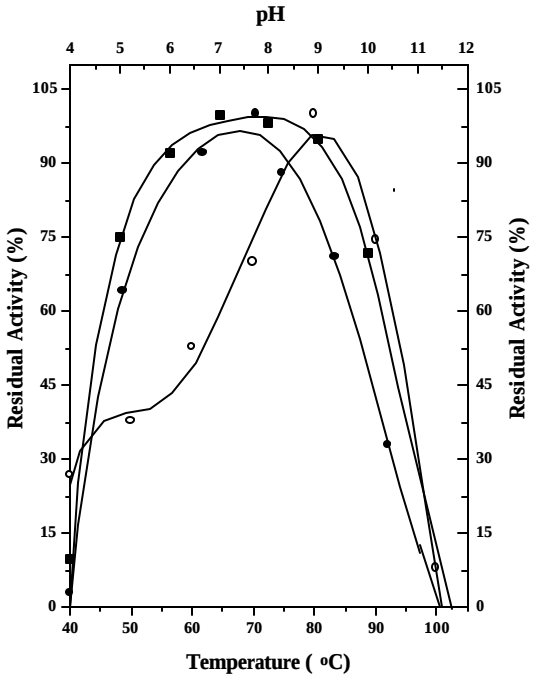
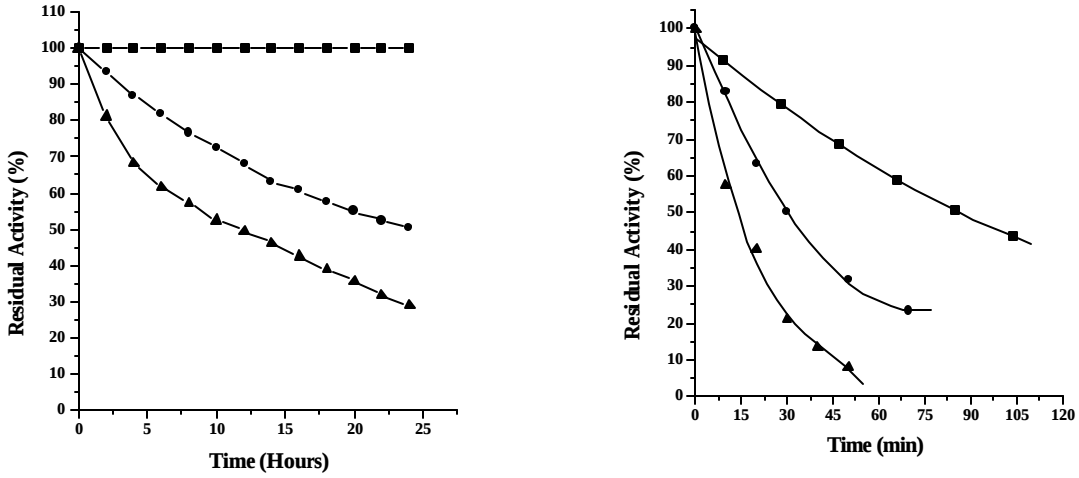


Figure 6: The pH optimum (●) and pH stability (■) of Xyl I. The pH stability of the enzyme was measured by incubating 5 IU of enzyme for one hour, at 50 °C in buffer of desired pH. The following buffer systems were used: 0.05 M acetate buffer (pH 4-5), 0.05 M phosphate buffer (pH 6-7), 0.05 M TrisCl buffer (pH 8), 0.05 M carbonate-bicarbonate buffer (pH 9-10). The optimum temperature (○) of Xyl I in 0.05 M phosphate buffer, pH 7. The activity of the enzyme was determined in the range (40-100 °C).

from *Thermotoga* sp. strain FjSS-B.1, which had a half-life of 8 min at 100 °C (Simpson *et al.*, 1991). However, a recombinant xylanase whose gene has been isolated from *Thermotoga neopalitina* and expressed in *E. coli* had a half-life of 30 min at 100 °C (Velikodvorskaya *et al*., 1997). The xylanase isolated from *Thermus thermophilus* was also highly thermostable with 61 % residual activity after incubation at 90 °C for 48 h. However, this xylanase was cell bound and had to be solubilized into the medium by sonication (Lyon *et al*., 2000).



a b

Figure 7: Thermal stability of Xyl I: 5 IU of xylanase was incubated in 0.05 M phosphate buffer (pH 7) at a) 50 °C (■), 60 °C (●), 70 °C (▲) and b) 80 °C (■), 90 °C (●) and 100 °C (▲) for different intervals and the residual activity was determined.

Secondary structure of Xyl I

The K2d analysis of the CD spectrum of Xyl I showed that the secondary structure of Xyl I is comparable with those of previously studied family 10 xylanases (Figure 8, Table 2). Based on crystallographic studies, Derewenda *et al*. (1994) have shown that the family 10 xylanase isolated from *Streptomyces lividans* has a TIM barrel structure, containing eight fold α/β barrels. The similarity in the contents of α and β structures in Xyl I with other family 10 xylanases suggests that it could have a tertiary structure similar to the eight fold α/β barrel structure of family 10 xylanases. Family 11 xylanases exist as a single domain that contains two mostly antiparallel β-sheets, which are packed against each other (Ohmiya *et al*., 1997).

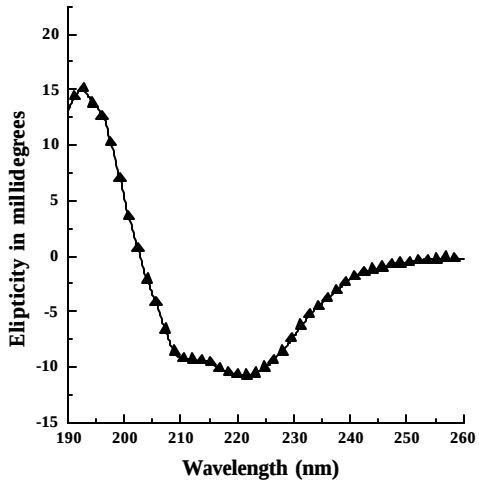


Figure 8: CD spectra of native Xyl I. Far-UV CD spectra were recorded for native Xyl I from 260-190 nm at 25 °C. The spectrum represents the average of six scans.

Table 2: Comparison of the secondary structure of Xyl I with other known family 10 xylanases.

Microorganism	**a**-Helix (%)	**b**-Sheet (%)	Random Coil (%)	Molecular weight (Da)
Penicillium simplicissimum	39.74	13.9	46.36	32,408
Pseudomonas fluorescens	32.85	14.7	52.45	38,411
Thermoascus aurantiacus	40.4	13.91	45.69	32,626
Thermomonospora sp. (Xyl I)	38	10	52	38,000

Amino acid composition of Xyl I

Amino acid analysis of Xyl I indicated the absence of cysteine residues in Xyl I (Table 3). The amino acid composition of Xyl I was comparable to the amino acid composition of *Thermomonospora alba* xylanase, the only family 10 *Thermomonospora* xylanase whose sequence is known (Blanco *et al.*, 1997). Xyl I had a higher composition of lysine, while

Thermomonospora alba xylanase contained three cysteine residues. Xyl I had equivalent number of hydrophobic and hydrophilic residues.

Table 3: Amino acid composition of Xylanase I

Amino acid	Number of residues in xylanase from	
	Thermomonospora sp. (Xyl I)	*Thermomonospora alba*
Aspartic acid	45	57
Glutamic acid	30	50
Alanine	35	44
Valine	23	30
Leucine	22	34
Isoleucine	18	27
Proline	15	24
Phenylalanine	10	13
Metheonine	4	7
Glycine	24	53
Serine	23	36
Threonine	20	26
Tyrosine	15	18
Arginine	10	26
Histidine	5	11
Lysine	14	8
Cysteine	-	3
Tryptophan	7**	14

** Determined by NBS method

Kinetic parameters of Xyl I

Xylanase was reacted with different concentrations of soluble xylan and the values for Michaelis-Menten constant (K_m) and V_{max} were calculated to be 3 mg/ml and 0.26 μmole.min^{-1}.mg^{-1}, respectively (pH 7, 50 °C).

Determination of end products

The end products of xylan hydrolysis were analyzed by paper chromatography. Xyl I produced xylose and xylobiose as the major products along with xylotriose, xylotetraose and higher oligosaccharides. Traces of xylose were produced within 90 min of hydrolysis. There were no traces of arabinose (Figure 9). The end products of xylan hydrolysis suggests that Xyl I is a non debranching endoxylanase.

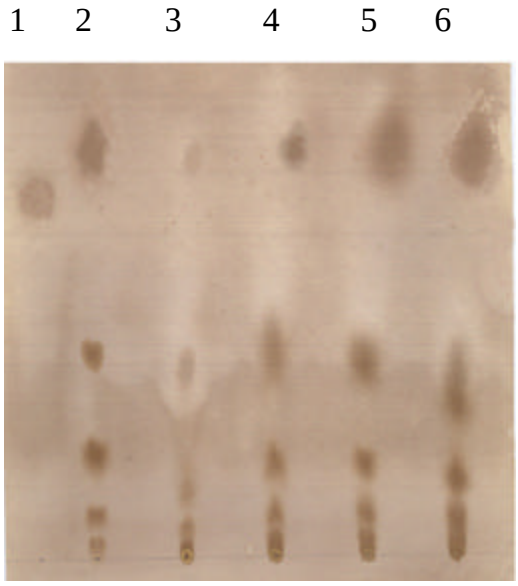


Figure 9: Paper chromatogram of xylan hydrolysis. Products from 5 mg xylan obtained by using 1IU of Xyl I at pH 7, 50 °C. Lane 1: Arabinose standard; Lane 2: Standard mixture of 10 μg each of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentose (X_5). Lane 3-6: 90 min, 5 h, 10 h and 27 h samples of hydrolysis products of Xyl I.

In the present chapter, we have described the purification of a highly thermostable xylanase produced by *Thermomonospora* sp. This is significant because moderately thermophilic organisms such as *Thermomonospora* sp. have dual advantage over hyperthermophilic organisms. It is capable of growing at 50 °C, thereby preventing contamination during upstream processing. However, in comparison to hyperthermophilic organism (80 °C) it grows at lower temperature and therefore has lower consumption of energy for its growth and enzyme production. Moreover, the thermostablity of Xyl I is comparable to that of xylanases produced by hyperthermophilic organisms like *Thermotoga* sp.

SUMMARY

A highly thermostable xylanase (Xyl I) produced by *Thermomonospora* sp. was purified to homogeneity by fractional ammonium sulphate precipitation (35-55 %) followed by ion exchange (DEAE Sephadex) and gel filtration chromatography (Sephacryl S-200). Xyl I was purified with thirteen-fold purification and specific activity of 455 IU/mg. It was classified as a family 10 xylanase based on its molecular weight (38,000 Da) and isoelectric point (4.1). The optimal temperature for the activity of Xyl l was 80 °C. Xyl I was highly thermostable with half-lives of 86 min, 30 min and 15 min at 80, 90 and 100 °C, respectively. Xyl I was stable in an expansive pH range of 5 to 10 with more than 75 % residual activity.

The Michaelis-Menten constant and V_{max} values were estimated to be 3 mg/ml and 0.26 μmole.min^{-1} .mg^{-1}, respectively (pH 7, 50 °C). Xyl I is a non-debranching endoxylanase. K2d analysis showed that the secondary structure of Xyl I was made up of 38 % α-helix and 10 % β-sheet. Amino acid analysis of Xyl I indicated the absence of cysteine residues in Xyl I. The amino acid composition of Xyl I from *Thermomonospora* sp. was comparable to the amino acid composition of *Thermomonospora alba* xylanase.

CHAPTER IV

Active site studies of xylanase I: Residues implicated and conformation of active site

INTRODUCTION

In an attempt to understand the catalytic mechanism of an enzyme, it is essential to study the structural elements and the three-dimensional conformation of the active site. Chemical modification of reactive amino acid side chains in the active site helps to identify residues that are important for catalysis. The utility of chemical modification is greatly enhanced by its use in conjunction with site directed mutagenesis, which mutually supplement each other. Though extensive studies have been carried out on the industrial applications of xylanases, there are comparatively fewer reports on the molecular enzymology of this class of enzymes. The role of carboxyl groups in the catalytic action of xylanase from an alkalothermophilic *Bacillus* sp. has been delineated through kinetic and chemical modification studies using Woodward's reagent K (Chauthaiwale & Rao, 1994). Bray & Clarke (1990) have also reported the involvement of carboxyl groups in the catalytic mechanism of xylanase from *Schizophyllum commune*. Site directed mutagenesis of the Glu-93 and Glu-182 residues of *Bacillus pumilus* xylanase resulted in a drastic decrease in its specific activity, indicating the role of the glutamate residues in catalysis (Ko *et al*., 1992). The participation of cysteine residues in the active site of xylanase appears to be restricted only to actinomycete xylanases. Keskar *et al*. (1989) have shown the presence of a cysteine residue in the active site of a xylanase from a thermotolerant *Streptomyces* T_7, by group specific modification with *p*-hydroxymercuribenzoate (PHMB). The role of the cysteine and tryptophan residue in substrate binding of a xylanase from *Chainia* sp has been deduced based on its reaction with N-(2,4-Dinitroanilino)maleimide (DAM) and N-bromosuccinimide (NEM), respectively (Rao *et al*., 1996). Chemical modification studies of xylanase from *Schizophyllum commune* by tetranitromethane implicated the predominant role of tyrosine in substrate binding (Bray & Clarke, 1995b). An arginine residue is also shown to be involved in the binding of a xylose residue in the active site of xylanase from *Bacillus circulans* (Wakarchuk *et al*., 1994b). There have been reports of the presence of histidine in the active site of family 10 xylanases from *Cellulomonas fimi*, *Pseudomonas fluorescence* and *Penicillium simplicissimum* based on the crystal structure of the enzymes (White *et al*., 1994; Harris *et al*., 1996; Schmidt *et al*., 1998). Roberge *et al*. (1997) have shown by site directed mutagenesis the importance of histidine residues in the active site of a family 10 xylanase from *Streptomyces lividans*. Recently,

Charnock *et al*. (1997) have suggested the possible role of a lysine residue in positioning the substrate in the active site of a xylanase from *Pseudomonas fluorescence*.

Structure-function relationships are one of the central issues in the investigation of biological macromolecules. The conformational integrity of an enzyme is essential for its activity. In the present chapter, we have determined the residues involved in catalysis and substrate binding. We have also attempted to correlate the loss in catalytic activity to the conformational changes occurring in Xyl I. The conformational changes in the active site were probed by utilizing the fluorescent properties of *o*-phthalaldehyde (OPTA). OPTA is a fluorescent chemoaffinity label (FCAL), which forms a fluorescent isoindole derivative by cross-linking the thiol and amine groups of cysteine and lysine, respectively (Simons & Johnson, 1978a). Data presented here suggests that the active site of Xyl I has low polarity and it is conformationally more fragile than the rest of the molecule.

MATERIALS AND METHODS

Materials

2,4,6-trinitrobenzenesulfonic acid (TNBS), diethyl pyrocarbonate (DEPC), *p*-hydroxymercuribenzoic acid (PHMB), N-ethyl maleimide (NEM) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. USA. All other chemicals used in the present work were of analytical grade. An isoindole derivative of *o*-phthalaldehyde (isoindole adduct) was synthesized as described by Rönnberg *et al*. (1984). The product showed absorption spectra similar to the one described by the above authors.

Methods

Steady state kinetic studies

The rate of hydrolysis of xylan was determined at different substrate concentrations (2 to 15 mg). The kinetic parameters Michaelis-Menten constant (K_m) and turnover number (k_{cat}), were obtained from Lineweaver-Burk plots. The k_{cat} values for Xyl I hydrolysis were determined from the average of at least three measurements at each substrate concentration and pH. The $k_{cat}.K_m^{-1}$ were calculated from the slope of the Lineweaver-Burk plots.

Reaction of Xyl I with chemical modifiers

Xyl I was incubated with the respective chemical modifiers under the specified conditions. Aliquots were removed periodically for the measurement of residual enzyme activity. Substrate protection studies were performed by preincubating the enzyme with the substrate xylan (5 mg) for 10 min prior to the addition of the modifier. Control tubes having only enzyme or only inhibitor or inhibitor and substrate were incubated under identical conditions. The inactivation data was then analyzed as described by Levy *et al*. (1963).

The apparent first order rate constant of inactivation depends on the concentration of the modifier and can be expressed as.

$$K_{app}=K(M)^n$$

Where K_{app} is the apparent first order rate constant, K is the second order rate constant, M is the concentration of the modifier and n is the number equal to the average order of the reaction with respect to the concentration of the modifier. K_{app} can be calculated as a slope

from a semilogarithmic plot of residual activity as a function of time. The second order rate constant for inactivation was determined from the slope of the plot of pseudo-first order rate constant against inhibitor concentration. The order of reaction (*n*) was estimated by determining K_{app} at different concentrations of the modifier.

$\log K_{app} = \log K + n\log(M)$

A plot of log K_{app} against log *M* gives a straight line with the slope equal to *n*, where *n* is the number of molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme-inhibitor complex.

Modification of Xyl I by OPTA

Fresh OPTA solution was prepared in methanol for each experiment. The modification was carried out by incubating 2 μM of Xyl I in 50 mM sodium phosphate, pH 7 with varying concentrations of OPTA, at 25±1 °C. Methanol had no effect on the activity of the enzyme and was always less than 2 % (v/v). At different time intervals, an aliquot was withdrawn from the reaction mixture and the residual activity was measured on termination of the reaction by adding 5 μl of 10 mM cysteine. The formation of Xyl I-isoindole derivative was followed spectrofluorometrically by monitoring the increase in fluorescence at 410 nm with the excitation wavelength fixed at 338 nm. The modified enzyme was passed through a Biogel P-2 column to separate the isoindole derivative from the unreacted *o*-phthalaldehyde and cysteine in the reaction mixture. The isoindole content of the modified enzyme was calculated spectrophotometrically at 338 nm using a molar absorption coefficient of 7.66 $mM^{-1}cm^{-1}$ (Simons & Johnson, 1978a).

Reaction with TNBS

Xyl I (5 μM) was incubated with varying concentrations of 2,4,6-trinitrobenzenesulfonic acid (4 to 10 mM) in the presence of 0.25 ml 4 % sodium bicarbonate at 37 °C in a reaction volume of 0.5 ml. Aliquots were withdrawn at suitable time intervals and the reaction was terminated by adjusting the pH to 4.5. The degree of inactivation in presence of 1 % xylan was also determined. Control tubes with only enzyme, only inhibitor and inhibitor/substrate were incubated under identical conditions.

Modification with DEP

Xyl I (5 μM) in 2 ml of 0.05 M sodium phosphate buffer at pH 7 was incubated with varying concentrations of DEP (2-10 mM) at 25±1 °C. Freshly prepared DEP in absolute ethanol was used. Samples were removed periodically at different time intervals and the reaction was arrested by the addition of 50 μl of 10 mM imidazole buffer, pH 7.5. The residual activity of the diluted enzyme derivative was determined under standard assay conditions and expressed as a percentage of the control. The DEP concentration was determined spectrophotometrically at 230 nm. The amount of N-carbethoxyimidazole formed was calculated by using a molar absorption coefficient of 3000 $M^{-1}cm^{-1}$ (Melchior & Fahrney, 1970). Decarbethoxylation of the DEP modified enzyme was carried out according to Miles (1977). The DEP modified enzyme samples were incubated with 50 mM hydroxylamine, pH 7, at 25 ±1 °C for 4 h and the enzyme activity was determined under standard assay conditions.

Reaction of Xyl I with PHMB, NEM and DTNB

Xyl I (2 μM) was incubated with high concentrations of PHMB (10-50 mM) in 50 mM phosphate buffer, pH 7 at 25±1 °C. Samples were removed at different time intervals and assayed for residual xylanase activity. Control tubes having only enzyme or only inhibitor were incubated under identical conditions. Similar experiments were performed in the presence of NEM. Xyl I was titrated with DTNB according to the method of Ellman (1959), to assess the number of free thiol groups present. A 1 ml sample of Xyl I (10 μM) in 8 M urea/50 mM Tris buffer, pH 8, was added to a quartz cuvette with 2 mM DTNB. The change in absorbance at 412 nm was continuously monitored at 25 °C for 60 min, and the number of cysteine residues was estimated spectrophotometrically at 412 nm by using a molar absorption coefficient of 1.36×10^{4} $M^{-1}cm^{-1}$ for the release of 5-mercapto-2-nitrobenzoic acid.

Fluorescence studies

Fluorescence measurements were performed on a Perkin Elmer spectrofluorometer LS 5B at 25±1 °C using an excitation and emission slit width of 5 nm. Each fluorescence

measurement was made using 2 ml samples of Xyl I (4 μM) in 50 mM sodium phosphate buffer, pH 7. Fluorescence emission spectra were recorded with an excitation wavelength of 338 nm. The fluorescence of the buffer and reagents were also measured at an appropriate wavelength and were used to correct the observed fluorescence. Corrections were also made to compensate for the dilution upon addition of modifiers.

CD measurements

CD spectra were recorded in a Jasco-J715 spectropolarimeter at ambient temperature using a cell of 1 mm path length. Replicate scans were obtained at 0.1 nm resolution, 0.1 nm bandwidth and a scan speed of 50 nm/min. Spectra were average of 6 scans with the baseline subtracted spanning from 250 nm to 195 nm in 0.1 nm increments. The CD spectra of the native, DEP (10 mM) and OPTA (0.5 mM) modified Xyl I (5 μM) were recorded in 50 mM sodium phosphate buffer (pH 7).

Determining the po larity of microenvironment at active site

The polarity of the active site in Xyl I was determined by studying the microenvironment of the isoindole derivative in Xyl I. The isoindole adduct (15 μM), an isoindole derivative of OPTA, was dissolved in solvents of varying polarity. The sample was excited at 338 nm and fluorescence emission for each was determined. Polar solvents cause bathochromic shifts in fluorescence of the isoindole adduct. A linear relationship was found between the molar transition energies (E_T) and emission maximum (λ_{em}) of the adduct, dissolved in different solvents. The correlation between E_T and emission maximum is given by the equation $E_T = 2.985\lambda_{em} - 1087.28$ (Dimroth *et al*., 1963).

RESULTS AND DISCUSSION

I) EVIDENCE FOR THE PRE SENCE OF AN ESSENTIAL LYSINE RESIDUE

pH dependence of the enzyme activity

The ionizable groups essential for the function of Xyl I activity were determined through the effects of pH on k_{cat} and $k_{cat}.K_m^{-1}$ values. The plots of k_{cat} and $k_{cat}.K_m^{-1}$ against pH are shown in Figure 1. The dependence of initial velocity upon substrate concentration was hyperbolic at each pH investigated and all Lineweaver-Burk plots were linear. The plots indicate the dependence of Xyl I activity on the ionization of at least two groups. The log k_{cat} versus pH plot gave pK values of 5 and 10.1 for the enzyme substrate complex. Values of 5.7 and 8.1 were obtained from the log $k_{cat}.K_m^{-1}$ versus pH plot for the free enzyme. The values obtained from the acidic limb of the curves are consistent with the participation of a carboxylate in the Xyl I catalysis which is in agreement with the proposed mechanism of action. However, the basic limb of the rate profile may reflect the ionization of a lysine residue since lysine groups in proteins have pK values usually in the range of 9–10 (Raetz & Auld, 1972). Slopes obtained from the log $k_{cat}.K_m^{-1}$ versus pH and log k_{cat} versus pH plots were +0.41, -0.28 and +0.69, -0.89 respectively. The slopes of the acidic and basic limb of the curves are less than unity, indicating that the simple model of the pH dependence of enzyme action does not describe adequately the Xyl I enzyme system. The divergence from unit slopes may arise from electrostatic perturbation of ionization constants, multiple intermediates or conformational effects. This may indicate the interaction of two ionizable groups on the enzyme suggesting a complex mechanistic pathway for the Xyl I system (Wakim *et al*., 1969). The nature of the essential residues for the function of Xyl I activity was resolved through specific chemical modification of the enzyme.

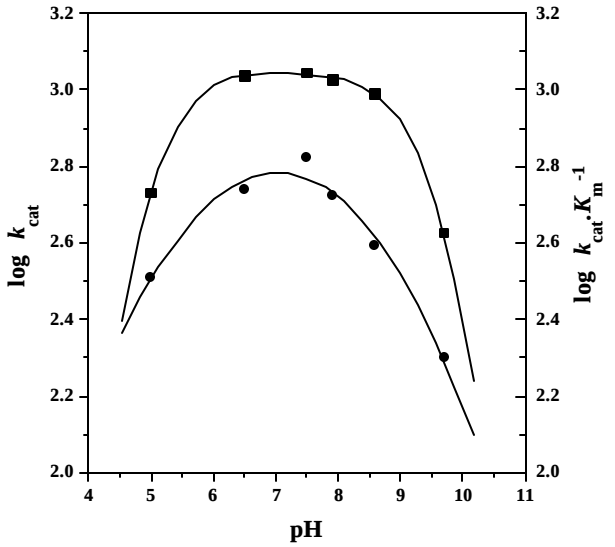


Figure 1: Dependence of kinetic parameters on pH. Xyl I (1 μM) was reacted with different concentrations of substrate (2-15 mg, of soluble xylan) in 50 mM buffers of pH ranging from 5-10. K_m and k_{cat} values were derived from Lineweaver-Burk plots. The effect of pH on log k_{cat} (■) and log $k_{cat}.K_m^{-1}$ (●).

In an attempt to decipher the residues involved in the active site, Xyl I was subjected to modification with different group specific reagents. Since the basic end of the rate profile showed the possible ionization of a lysine residue, Xyl I was subjected to chemical modification with lysine specific reagents like OPTA and TNBS.

Inactivation of Xyl I by OPTA

The incubation of Xyl I with increasing concentration of OPTA (0.05-0.5 mM) resulted in a time dependent decrease in enzyme activity. The natural logarithm of percent activity remaining was plotted against time to obtain linear pseudo first order plots (Figure 2). When the pseudo first order rate constants obtained at each concentration were replotted against OPTA concentration, a linear relationship was observed. Analysis of the order of inactivation with respect to OPTA concentration by method of Levy *et al*. (1963), yielded a slope of 0.84, indicating that one molecule of OPTA binds to one molecule of enzyme at the active site (Figure 1, inset). OPTA has been used as a fluorescent probe and a modifier of lysine and cysteine residues (Simons & Johnson, 1978b; Palcazewski *et al*., 1983).

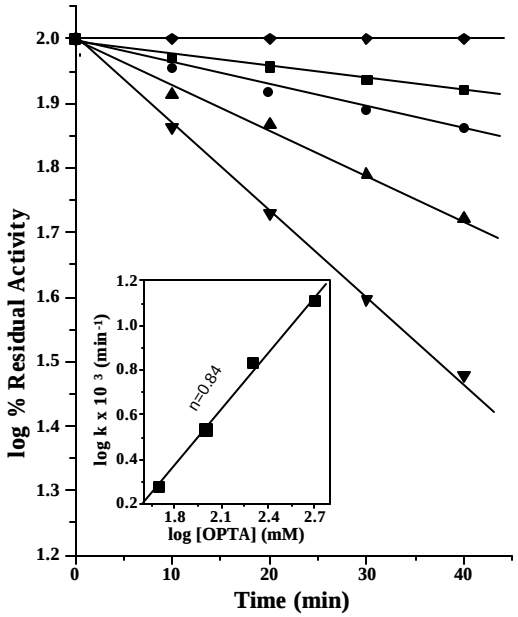


Figure 2: Kinetics of inactivation of Xyl I by *o*-phthalaldehyde. Pseudo first order plots for the inactivation of Xyl I by OPTA. Xyl I (2 μM) was incubated with 0.0 mM (◆), 0.05 mM (■), 0.1 mM (●), 0.2 mM (▲) and 0.5 mM (▼) OPTA at 25 °C. Aliquots were removed at indicated time intervals and the reaction was terminated by 10 mM cysteine. Inset: Double logarithmic plot of pseudo first order rate constants as a function of OPTA concentration.

Inactivation due to OPTA occurs due to the simultaneous modification of lysine and cysteine residues at or near the active site of the catalytic domain. OPTA reacts with lysine and cysteine residues to form a fluorescent isoindole derivative. The inactivation of Xyl I by OPTA resulted in concomitant increase in fluorescence at 415 nm (excitation wavelength, 338 nm), which is characteristic for the formation of an isoindole derivative (Figure 3). Therefore indicating that the SH and NH_2 groups of Xyl I are involved in the reaction with OPTA are situated at the active site of Xyl I. Upon complete inactivation, an average of one isoindole derivative per molecule of enzyme was found, based on the increased absorbance at 337 nm. As shown in figure 2, the increase in the amount of isoindole derivative formed correlated well with the decrease in enzyme activity, suggesting that OPTA causes inactivation of Xyl I by the formation of a single isoindole derivative.

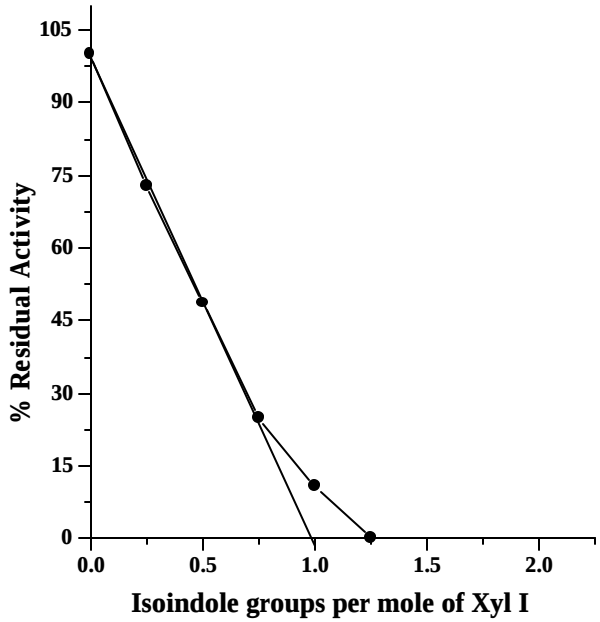


Figure 3: Stoichiometry of *o*-phthalaldehyde inactivation. Plot of residual activity against number of isoindole derivatives formed as deduced by spectroscopic studies

SDS-PAGE of OPTA modified Xyl I showed no higher molecular weight bands other than the one corresponding to Xyl I. This indicates that the reaction between Xyl I and OPTA was intramolecular and not intermolecular in nature i.e. one OPTA molecule reacts with the cysteine and lysine residue of the same Xyl I molecule and not between two different Xyl I molecules. The CD spectra of native and OPTA modified Xyl I were identical, indicating that modification by OPTA does not result in a gross change in the enzyme structure (Figure 4). The similarity of the CD profile of OPTA modified and native Xyl I implies that the loss in activity due to OPTA modification is only due to the modification of the active site residues and not due to the change in gross conformation caused due to binding of excessive reagent molecules to the protein.

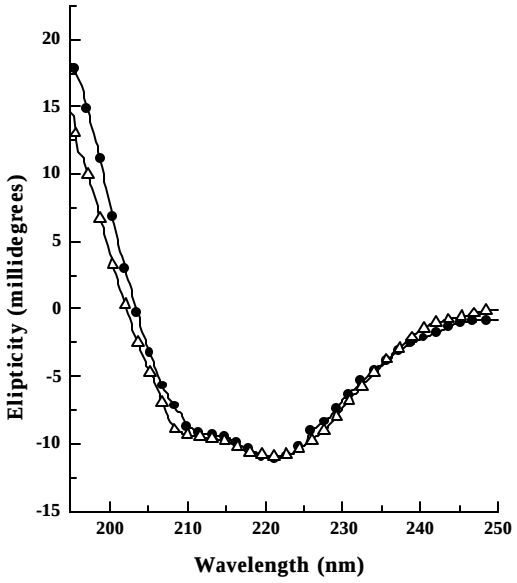


Figure 4: CD spectra of native and OPTA modified Xyl I. Far-UV CD spectra were recorded for native (●) and OPTA modified (△) Xyl I from 250-195 nm at 25 °C. Each spectrum represents the average of six scans.

Modification of Lysine residue by TNBS

To further confirm the involvement of lysine residue in the active site of Xyl I, it was modified with TNBS, which is a group specific reagent for lysine. Incubation of Xyl I (5 μM) with different concentrations of TNBS resulted in a time and concentration dependent loss of enzyme activity as shown in Figure 5. The reaction followed pseudo first order kinetics. The pseudo first order rate constants (*K*) were linearly related to the concentrations of the reagent, suggesting that no reversible complex was formed during the inactivation process. Furthermore a reaction order of 1.05 with respect to the modifier was determined from the slope of the double logarithmic plots (Figure 5, inset), indicating that 1 mol of TNBS inactivated 1 mol of enzyme.

The CD measurements revealed no effect of the modifier on the α-helix and β-sheet content of Xyl I. Hence the TNBS induced inactivation of Xyl I is a result of direct chemical modification of an essential lysine residue and cannot be attributed to the disruption of the enzyme structure (Figure 6).

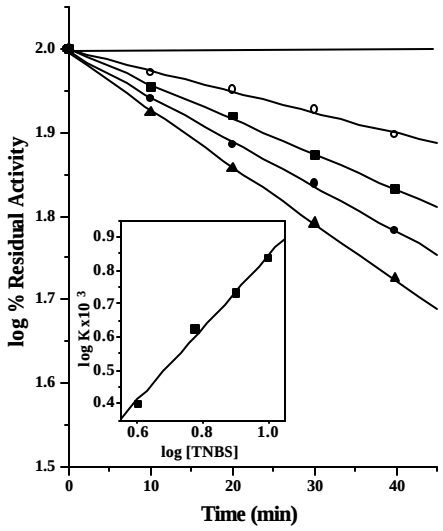


Figure 5: Kinetics of inactivation of Xyl I by TNBS. Pseudo first order plots for the inactivation of Xyl I by TNBS. Xyl I (2 μM) was incubated with 4 mM (○), 6 mM (■), 8 mM (●), 10 mM (▲) TNBS and control (▽) at 25 °C. Aliquots were removed at indicated time intervals and the reaction terminated by adjusting the pH to 4.5. Inset: Double logarithmic plots of pseudo first order rate constants as a function of TNBS concentration.

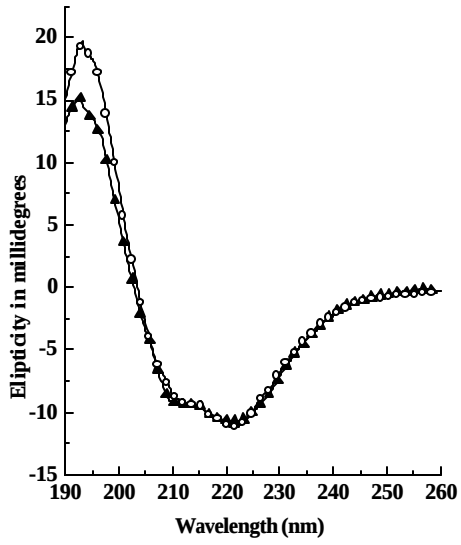


Figure 6: CD spectra of native and TNBS modified Xyl I. Far-UV CD spectra were recorded for native (○) and TNBS modified ( ) Xyl I from 260-190 nm at 25 °C. Each spectrum represents the average of six scans.

The kinetic parameters for TNBS modified and native enzyme were analyzed to determine whether the modified lysine residue is present in the catalytic or the substrate-binding site. No change was observed in the k_{cat} values of native and modified enzyme, while the K_m values were 3 mg/ml and 7 mg/ml respectively (Table 1).

Table 1: Kinetic parameters of native and TNBS modified xylanase I

Xylanase I	Residual activity (%)	K_m (mg/ml)	k_{cat} (min^{-1})
Native	100	3	13400
TNBS modified	60	4.2	13500
	52	6.1	13250
	35	7	13300

TNBS modified Xyl I was prepared by incubating 5 μM of Xyl I with 5 mM TNBS for different time intervals in a total a volume of 1 ml at 25±1 °C. The reaction was terminated by adjusting the pH to 4.5. The K_m and k_{cat} values were determined from Lineweaver-Burk plots. The substrate concentrations used for the determination of K_m and k_{cat} were in the range of 1-15 mg.

Table 2: Substrate protection of Xylanase I a gainst inhibition by OPTA and TNBS
Enzyme samples (5 μM each) were incubated with different concentrations of xylan for 10 min before addition of inhibitors.

Reaction	Residual activity (%)
None (Control)	100
OPTA (1 mM)	0
Xylan(1 mg) + OPTA	65
Xylan (2 mg) + OPTA	80
Xylan (5 mg) + OPTA	98
TNBS (10 mM)	0
Xylan (1 mg) + TNBS	70
Xylan (2 mg) + TNBS	85
Xylan (5 mg) + TNBS	100

The comparable k_{cat} values suggested that lysine was not present in the catalytic site, while the significant increase in the K_m indicated the presence of lysine at the substrate-binding site. The modification of the lysine residue present in the substrate binding site results in a decrease in the efficiency of binding of the substrate to the substrate binding site and results in an increase of K_m. The complete protection of Xyl I against inactivation by TNBS and OPTA, by the substrate, confirms the presence of lysine at the substrate-binding site (Table 2).

The three dimensional structure of family 10 xylanases have revealed several highly conserved residues that are on the surface of the active site. To the best of our knowledge there is only a single report of the presence of lysine in the active site of a xylanase from *Pseudomonas fluoresens*. Charnock *et al*. (1997) have suggested that Lys-47 plays an important role in positioning the substrate into the active site of Xylanase A from *Pseudomonas fluoresens*. The present section demonstrates the presence of an essential lysine residue in the active site of Xyl I by fluorescent chemoaffinity labeling and group specific modification.

II) SITE AND SIGNIFICANCE OF A HISTIDINE RESIDUE

Formation of fluorescent isoindole derivative

Fluorescence excitation and emission spectroscopic data showed that an isoindole derivative was formed following the reaction between Xyl I and OPTA (Figure 7). The fluorescent isoindole derivative is formed by cross-linking the proximal cysteine and lysine groups of the protein with OPTA. During the isoindole formation, these residues must be oriented such that the ε-NH_2 group of lysine and SH group of cysteine are about 3 Å apart (Puri *et al.*, 1985).

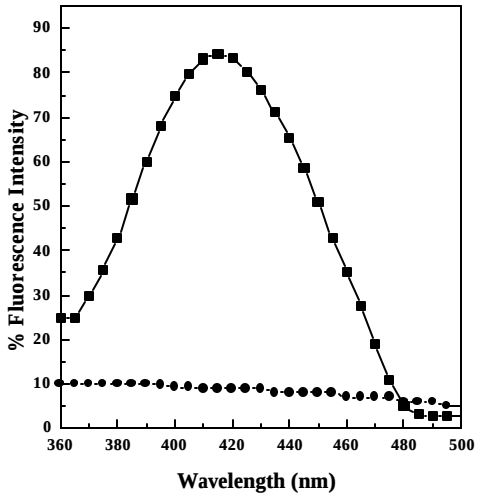


Figure 7: Isoindole fluorescence of Xyl I modified by OPTA. Xyl I sample (5 μM) was incubated with 1 mM of OPTA for 10 minutes at 25 °C. The isoindole fluorescence of OPTA modified Xyl I (■) and Xyl I (●) was monitored at λ_{ex} 338 and λ_{em} 415.

Non involvement of cysteine residues in isoindole formation

The inactivation of Xyl I by OPTA suggested the possible involvement of a cysteine residue in the active site and in the formation of an isoindole derivative. The participation of cysteine groups in the mechanism of catalysis was investigated using cysteine specific reagents such as PHMB and NEM. It was observed that Xyl I was not inactivated in the presence of high concentrations of PHMB and NEM (50 mM) and retained complete activity. Titration of Xyl I with 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 8 mM urea at pH 8 over an

extended time period did not result in the release of 5-mercapto-2-nitrobenzoic acid, suggesting that no free thiol groups exist in the Xyl I. Amino acid analysis of Xyl I indicated the absence of cysteine residues in Xyl I (Chapter III).

Role of histidine residue in active site

Reaction of Xyl I with DEP resulted in progressive loss in activity. The inactivation was dependent on the concentration of DEP. The pseudo first order rate constants for different concentrations of DEP (2-10 mM) were calculated and the slope of the plots of log K against log [DEP] produced a value of 0.96 (Figure 8).

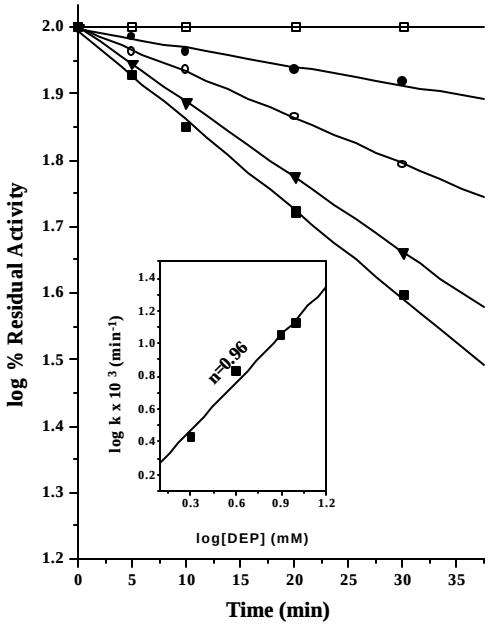


Figure 8: Time course of inactivation of Xyl I by DEP. Each incubation mixture contained 5 μM Xyl I in 0.05 M sodium phosphate buffer, pH 7 and 0 mM (□), 2 mM (●), 4 mM (○), 8 mM (▼) and 10 mM (■) DEP. Inset: Double logarithmic plot of pseudo first order rate constants as a function of log DEP concentrations.

These results showed that the loss in activity was due to the modification of a single histidine residue. DEP is specific for histidine at neutral pH, but it also reacts to a lesser extent with tyrosine, cysteine and lysine (Miles, 1977). The modification of tyrosine residues by DEP was excluded, as there was no decrease in the absorbance of the modified protein at 278 nm.

Moreover, there was no loss in activity on treating the purified enzyme with N-acetyl imidazole, which is a tyrosine specific reagent. The modification of cysteine could be ruled out as Xyl I does not contain any cysteine residues. The recovery of total activity in the presence of hydroxylamine further proved the involvement of DEP in histidine modification and negated the modification of lysine by DEP. Carbethoxylation of the enzyme at pH 7 for 30 min resulted in 60 % loss of its initial activityThe CD measurements revealed no effect on the α-helix and β-sheet content of Xyl I. Hence the DEP induced inactivation of Xyl I is a result of direct chemical modification of an essential histidine residue and cannot be attributed to the disruption of the enzyme structure (Figure 9).

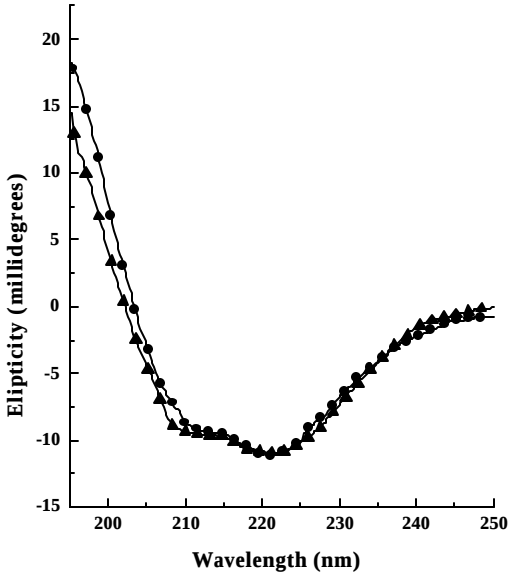


Figure 9: CD spectra of native and DEPC modified Xyl I. Far-UV CD spectra were recorded for native (●) and DEPC modified ( ) Xyl I from 260-190 nm at 25 °C. Each spectrum represents the average of six scans.

The kinetic parameters Michaelis-Menten constant (K_m) and turnover number (k_{cat}), were obtained from Lineweaver-Burk plots for native and DEP modified Xyl I. There was no change in the K_m values of modified and native enzyme, while the k_{cat} values were 4200 min^{-1} and 13400 min^{-1} respectively (Table 3).

Table 3: Kinetic parameters of native and DEP modified Xylanase I
DEP modified Xyl I was prepared by incubating 5 μM of Xyl I with 5 mM DEP for different time intervals (35, 45 and 60 minutes), in a total volume of 1 ml at 25±1 °C. The reaction was terminated by the addition of 50 μl of 10 mM imidazole buffer. The K_m and k_{cat} values were determined from Lineweaver-Burk plots. The substrate concentrations used for the determination of K_m and k_{cat} were in the range of 1-10 mg/ml.

Xylanase I	Residual activity (%)	K_m (mg/ml)	k_{cat} (min^{-1})
Native	100	3.0	13400
DEP modified	62	3.2	6500
	45	3.1	5600
	33	3.0	4200

The similar K_m values and the significant decrease in the k_{cat} suggested that histidine was not present in the substrate binding site and was present at or near the catalytic site of Xyl I. Xylan did not provide any protection to Xyl I against inactivation by DEP, which may be because xylan binds only at the substrate binding site, therefore making the catalytic site susceptible to attack by DEP. The histidine residue in the active site may be involved in a network of hydrogen bonds, which are responsible for maintaining the ionization state of the two catalytic residues responsible for the hydrolysis of the β-1,4 glycosidic bond. The structural analysis of the active site of family 10 xylanases from *Cellulomonas fimi*, *Pseudomonas fluorescens* and *Penicillium simplicissimum* have shown the presence of a hydrogen bonding network involving histidine residues in the active site (White *et al*., 1994; Harris *et al*., 1996; Schmidt *et al*., 1998). White *et al*. (1994) have shown that the trio of residues Glu233-His205-Asp235 is highly conserved within family 10. Roberge *et al*. (1997) have undertaken the site directed mutagenesis for xylanase A derived from *Streptomyces lividans*. They have proved that the histidine residues His81 and His207 are very important in the hydrogen-bonding network of the active site. Mutation of these residues modifies the interaction necessary to maintain the ionization state of the two catalytic glutamic acids of xylanase A. The histidine present in the active site of Xyl I may play a role similar to that played by the conserved histidine residues in other family 10 xyalanaes.

Isoindole formation by OPTA with histidine and lysine

Inactivation of Xyl I by OPTA resulted in the formation of an isoindole derivative, which is characterized by excitation and emission maxima at 338 nm and 415 nm, respectively. OPTA is a bifunctional agent that forms isoindole derivatives due to its reaction with SH and NH_2 groups of lysine and cysteine residues (Palczewski *et al*., 1983). The involvement of a lysine residue in the formation of an isoindole ring was confirmed by the inability of TNBS modified Xyl I to form a Xyl I-isoindole derivative with OPTA. The prior modification of a lysine residue by TNBS makes lysine unavailable for further reaction with OPTA. Similar experiments were performed by modifying histidine residues in Xyl I by diethylpyrocarbonate. DEP modified Xyl I was also unable to form an isoindole derivative with OPTA (Figure 10). However, Xyl I pretreated with PHMB and NEM was unable to abolish the formation of the isoindole peak at 415 nm with OPTA (excitation wavelength, 338 nm). These results indicated the involvement of lysine and histidine residues in the formation of an isoindole derivative.

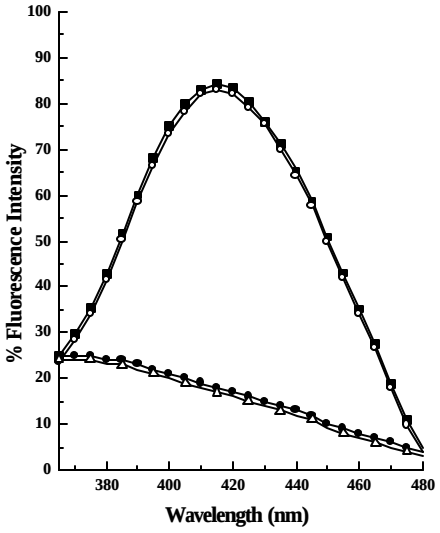
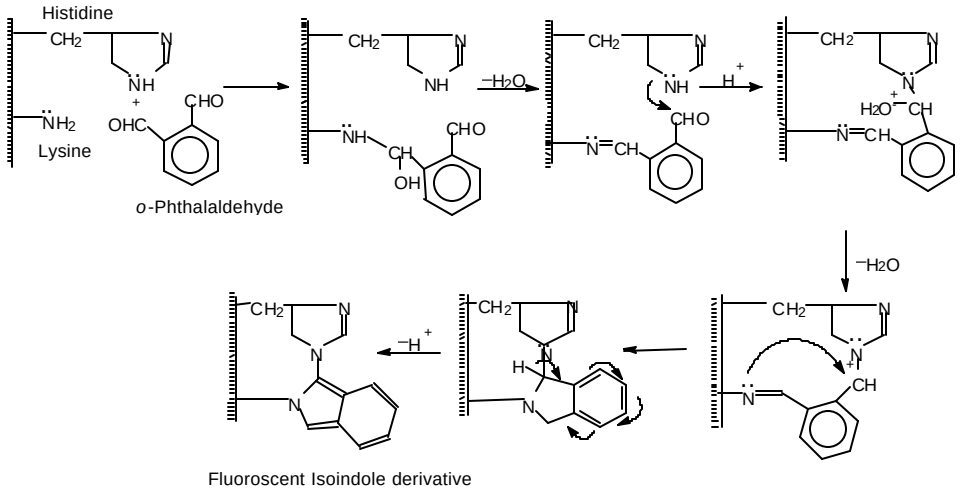


Figure 10: Effect of (△) histidine, (●) lysine and (○) cystei ne modifiers on the (■) isoindole fluorescence of Xyl I. Xyl I samples (2 μM) were incubated with 10 mM each of DEP, TNBS and PHMB for 20 minutes at 25 °C. The modified Xyl I samples were then treated with 0.5 mM OPTA for 10 min at 25 °C. The change in the isoindole fluorescence was monitored at λ_{ex} 338 and λ_{em} 415.

A mechanism for the formation of an isoindole derivative by OPTA with histidine and lysine residues in the active site of Xyl I has been proposed based on well established chemical reactions. Simons & Johnson have shown the mechanism for the reaction between OPTA, amine and thiol groups (Simons & Johnson, 1978a). Moreover Yoshimura *et al*. have elucidated the mechanism for isoindole formation between histidine and OPTA (Yoshimura *et al.*, 1990). The reaction mechanism proposed by us is based on these two reported reactions. Yoshimura *et al.* have shown the formation of a 1:2 adduct of histidine and OPTA, wherein a single histidine molecule reacts with two OPTA molecules resulting in the formation of a fluorescent adduct. In case of Xyl I kinetic analysis shows that only one molecule of OPTA binds to one Xyl I molecule (Figure 2). Moreover the scheme shown by Yoshimura is applicable for histidine in the free form, in case of proteins the primary amine of histidine reacts with the carboxyl group of adjacent amino acid to form a peptide bond and is not available for reacting with OPTA. Therefore, the only possible reactive group in histidine is the secondary amine in the imidazole ring. OPTA contains two aldehyde groups, one of these aldehyde groups reacts with the primary amine in lysine resulting in the release of a water molecule. The residual aldehyde group reacts with the secondary amine in the imidazole ring of histidine and results in the release of another water molecule and forms a fluorescent isoindole derivative (Scheme 1).



SCHEME 1

Scheme 1: Proposed mechanism of the fluorescence reaction of OPTA with histidine and lysine residues present in Xyl I.

Effect of urea on active si te conformation

Low concentrations of urea were used as a perturbant of the active site in order to study its effect on the activity, gross conformation of the enzyme and conformation of the active site. There have been divergent views on the flexibility of the active site. In the case of creatine kinase (Yao *et al*., 1984) and ribonuclease A (Liu & Tsou, 1987), it has been shown that inactivation of the enzyme precedes its gross conformational change, thereby suggesting that the active site is more fragile than the molecule as a whole. Some authors have reported a concurrent process for the inactivation and unfolding of enzyme molecules during denaturation (Nieto & Ayala, 1977; Busby *et al*. 1981), while in the case of xylose reductase the active site is relatively less fragile (Rawat & Rao, 1997b).

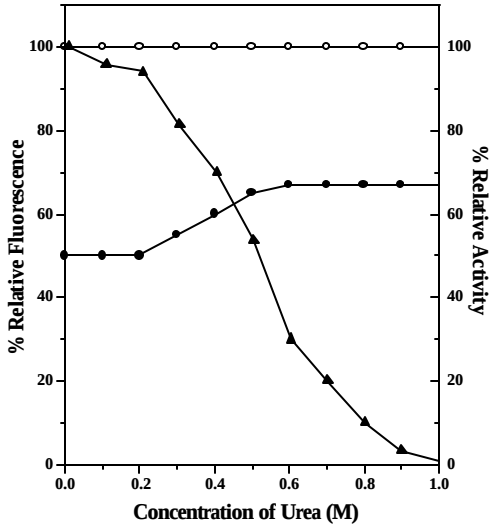


Figure 11: Effect of increasing concentration of urea on (●) isoindole fluorescence, (▲) activity and (○) tryptophanyl fluorescence of xylanase I. OPTA labeled Xyl I was prepared by incubating 2 μM of Xyl I with 0.5 mM OPTA for 10 minutes at 25 °C in 0.05 M sodium phosphate buffer at pH 7. The labeled Xyl I was incubated in varying concentrations of urea. The changes in isoindole fluorescence (λ_{ex} 338 nm, λ_{em} 415 nm) were monitored. Similar experiments were carried out with unmodified Xyl I. The residual activity and tryptophanyl fluorescence (λ_{ex} 295 nm, λ_{em} 336 nm) of Xyl I were also measured in different concentrations of urea.

The gross conformation of Xyl I was studied by the change in the intensity or shift of tryptophanyl fluorescence, while the active site conformation was monitored by the change in the intensity of isoindole fluorescence of the OPTA labeled active site. When Xyl I was

subjected to low concentrations of urea it showed a simultaneous decrease in activity and increase in isoindole fluorescence at the active site (Figure 11). This indicates that the loss in activity of xylanase at low concentrations of urea is due to conformational changes at the active site. There was no change or shift in the tryptophanyl fluorescence, thereby showing that the gross conformation of the enzyme is intact. These results suggest that the active site of Xyl I is conformationally more flexible and it is more easily perturbed in presence of denaturants than the molecule as a whole.

Polarity of isoindole binding site

The polarity of the active site can be deduced by studying the microenvironment of the isoindole ring bound to the active site of xylanase. The nature of the solvent greatly influences the λ_{em} of an isoindole derivative (Simons & Johnson, 1978b). The polarity of the solvent causes characteristic shifts of the charge transfer band in absorbance spectra (Palczewski *et al*., 1983; Kosower, 1958). We have used the E_T polarity scale which reflects the dependence (blue shift) of π-π^* transition energies of the N-phenolpyridinum betaines on the solvent.

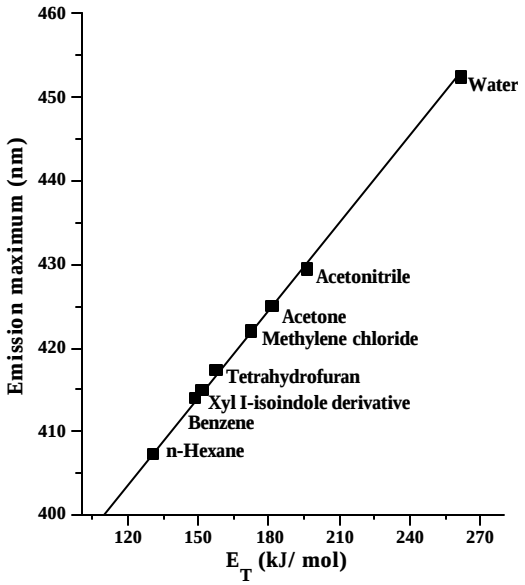


Figure 12: Correlation between fluorescence maximum of isoindole adduct and betaine iodide transition energies (E_T) in solvents of varying polarity. Excitation wavelength was fixed at 338 nm.

The variation in the fluorescence emission maximum of a model compound (isoindole adduct) in solvents of different polarity was studied (Figure 12). The fluorescence emission maxima of Xyl I-isoindole derivative was compared with the E_T values of the isoindole adduct in different solvents. The fluorescence emission maximum of Xyl I isoindole derivative was at 415 nm. An E_T value of 151.4 kJ/mol was obtained from the equation $E_T=2.985\lambda_{em}-1087.28$. This corresponds to the E_T of the isoindole adduct in a mixture of benzene and tetrahydrofuran, indicating that the microenvironment at the active site of Xyl I has low polarity.

SUMMARY

I) EVIDENCE FOR THE PRESENCE O F AN ESSENTIAL LYSINE RESIDUE .

Studies involving OPTA as the chemical initiator for fluorescent chemoaffinity labeling and TNBS as chemical modifier have revealed the presence of a single lysine residue in the active site of Xyl I. The high pK value for the basic limb of the pH profile reflects the ionization of a lysine residue. The kinetics of inactivation of Xyl I with OPTA revealed that complete inactivation occurred due to the binding of one molecule of OPTA to the active site of Xyl I. The formation of a single fluorescent isoindole derivative corroborated these findings. The kinetics of inactivation of Xyl I with TNBS revealed that complete inactivation occurred due to the binding of one molecule of TNBS to the active site of Xyl I and confirmed the presence of a single lysine residue in the active site of Xyl I. The higher K_m values and similar k_{cat} values of the TNBS modified enzyme in comparison to native enzyme and the substrate protection against OPTA and TNBS, suggested the presence of the lysine residue in the substrate-binding site.

II) SITE AND SIGNIFICANCE OF A HISTIDINE RESIDUE

A fluorescent chemoaffinity label *o*-phthalaldehyde (OPTA) was used to establish the presence of histidine in the active site of a family 10 xylanase isolated from *Thermomonospora* sp. OPTA has been known to form a fluorescent isoindole derivative by cross-linking the proximal thiol and amino groups of cysteine and lysine. The involvement of cysteine in the formation of Xyl I-isoindole derivative has been negated by fluorometric and chemical modification studies on Xyl I, with group specific reagents and by amino acid analysis. The kinetic analysis of diethylpyrocarbonate modified Xyl I established the presence of an essential histidine at or near the catalytic site of Xyl I. Modification of histidine and lysine residues by diethylpyrocarbonate and 2,4,6-trinitrobenzenesulfonic acid, respectively, abolished the ability of the enzyme to form an isoindole derivative with OPTA, indicating that histidine and lysine participate in the formation of the isoindole complex. A mechanism for reaction of OPTA with histidine and lysine residues present in the protein structure has been proposed. OPTA was also used to ascertain the conformational flexibility and polarity at the active site of

xylanase I (Xyl I). Experimental evidence presented here suggests for the first time that the active site of Xyl I is conformationally more flexible and it is more easily perturbed in presence of denaturants than the molecule as a whole. The changes in the fluorescence emission maxima of a model compound (isoindole adduct) in solvents of different polarity were compared with the fluorescence behaviour of Xyl I-isoindole derivative to conclude that the active site is located in a microenvironment of low polarity.

Chapter v

Encapsulation and interaction of Xylanase in fatty lipid matrix

INTRODUCTION

Proteins interact with membranes in different ways. They can adsorb at the lipid head group region, partially penetrate the hydrophobic core or fully span the lipid bilayer. The outcome of the interaction depends on the structure and chemical composition of both the protein and lipid membrane (May & Ben-Shaul, 1999). Protein-lipid interactions are probably the single most crucial factor that determines the action of protein molecules in biological membranes (Mouritsen & Bloom, 1987). It is important to expand this concept by elucidating the nature of protein-lipid interaction. On the other hand, for technological and biomedical applications, studies on protein-lipid interactions have played a significant role in developing protocols for drug delivery and immunosensing systems (Rui *et al*., 1998; Wink *et al*., 1998; Puu, 2001). For such applications, it is required that the proteins/enzymes be encapsulated into suitable supports. Furthermore, immobilized biocatalysts are advantageous for commercial applications due to convenience in handling, ease of separation from the product and reuse, low product costs, possible increase in thermal and pH stability (Tischer & Wedekind, 2000). Encapsulation also protects the enzymes against degradation, aggregation and deamidation while rendering the enzymes accessible to substrates and cofactors for biosensing and enzymatic applications (Avnir & Braun, 1996; Shabat *et al*., 1997). Till date, proteins/enzymes have been immobilized in/onto various matrixes such as phospholipid bilayers (Himachi *et al*., 1994), sol-gel matrix (Ellerby *et al*., 1992), cross-linked crystals (Lalonde *et al*., 1995), SAMs (Fang & Knobler, 1996), Langmuir-Blodgett films (Nicolini *et al*., 1993), polymer matrixes (Zhu *et al*., 2000), zirconium phosphates (Kumar & McLendon, 1997), hydrophobic controlled pore glasses (Bosley & Clayon, 1994), DNA-directed immobilization (Niemeyer *et al*., 1999), films of fatty lipids (Gole *et al*., 2000a) as well as onto 3-D supports such as organic (Caruso & Mohwald, 1999) and inorganic (Keating *et al*., 1998) colloidal particles.

We have studied the interactions of a biotechnologically important enzyme, xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) with the lipid octadecylamine (ODA, $C_{18}H_{37}NH_2$, cationic lipid). The industrial importance of xylanase comes from its applications in clarification of juices and wines, conversion of renewable biomass into liquid fuels and in

development of environmentally sound bio or pre-bleaching processes in paper and pulp industries. The objective of this work on one hand adds to our on-going efforts to understand protein-lipid interactions. Secondly, the study helps in developing robust enzyme-lipid composite materials for eventual enzymatic and biotechnological applications. The relevance of our findings throw light upon the mechanism of protein-lipid interaction and opens a new avenue in nano-biotechnology.

MATERIALS AND METHODS

Materials

Octadecylamine (ODA; cationic lipid) was obtained from Aldrich Chemicals. All buffer salts were from standard commercial sources and of highest quality available.

Methods

Deposition of ODA thin films

250 Å thick ODA films were deposited on gold-coated AT cut quartz crystals (for QCM measurements) and Si (111) substrates (for FTIR, and enzymatic activity measurements) by thermal evaporation in an Edwards E308 chamber. The deposition was done at a pressure of 1 x 10^{-7} Torr and the film deposition rate and thickness was monitored in-situ using an Edwards thickness monitor.

Substrate protection

A 5 μM solution of Xyl I at pH 7 was mixed with xylan (1 %) in different proportions and the mixture was preincubated at 4 °C for 5 minutes. The ODA films were incubated for 30 min in these solutions. The optimum Xyl I : xylan ratio was calculated by determining which ratio results in maximum xylanase activity in the biocomposite films.

Encapsulation studies

Solutions of Xyl I, substrate protected Xyl I and xylan solutions were prepared, in sodium phosphate buffer (0.05 M, pH 7). The diffusion of enzyme/substrate into the thermally evaporated ODA films was monitored by immersion of the ODA covered gold-coated AT cut quartz crystals in the enzyme/substrate solutions for different time intervals. The frequency change of the crystals was measured ex-situ after thorough washing (in deionized water) and drying (in flowing nitrogen) of the crystals. The frequency counter used was an Edwards FTM5 instrument operating at a frequency stability and resolution of $\pm$ 1 Hz.

For the 6 MHz crystal used in this investigation, this translates into a mass resolution of 12 ng/cm^2. The frequency changes were converted to mass loading using the standard Sauerbrey formula (Sauerbrey, 1959).

Contact angle measurements.

Contact angle measurements of a sessile water drop (1 μl) on 250 Å thick ODA films deposited on Si (111) substrates before and after encapsulation of the substrate protected Xyl I, and films formed by drying a drop of the substrate protected Xyl I on ODA matrix as well as that on Si (111) substrate were carried out on a Rame Hart 100 Goniometer.

Studies of secondary structure of encapsulated substrate protected Xyl I

The enzyme–ODA biocomposite film was prepared by immersion of a 250 Å thick ODA film on Si (111) substrate in the pH 7 substrate protected Xyl I solution for 1h as explained previously. After thorough washing in deionised water and drying the biocomposite films in flowing N_2, FTIR measurements were performed on a Shimadzu FTIR-8201 PC instrument operated in the diffuse reflectance mode at a resolution of 4 cm^{-1}. To obtain good signal to noise ratios, 256 scans were taken of the biocomposite film in the range 400 to 4000 cm^{-1}. For comparison, FTIR spectra of an as-deposited 250 Å thick ODA film on Si (111) substrate was also recorded under similar conditions.

Fluorescence studies

Fluorescence measurements were performed on a Perkin Elmer spectrofluorometer LS 5B at 25 ± 1 °C using an excitation and emission slit width of 5 nm. Each fluorescence measurement was made using 2 ml samples of Xyl I (4 μM) in 50 mM sodium phosphate buffer, pH 7. Fluorescence emission spectra were recorded with an excitation wavelength of 338 nm. The fluorescence of the buffer and reagents were also measured at an appropriate wavelength and used to correct the observed fluorescence. Corrections were also made to compensate for the dilution upon addition of modifiers.

Enzymatic activity measurements

250 Å thick substrate protected Xyl I - ODA biocomposite films on Si (111) substrates (4 cm^2 substrate area), were formed by encapsulation of a mixture of different ratios of Xyl I : xylan (w/w) at pH 7 (0.05 M sodium phosphate buffer). Care was taken not to dry the films as this dehydrates the film and results in a loss in the activity. The enzymatic activity of the encapsulated Xyl l molecules in the lipid matrix was determined by reaction with an aqueous solution of xylan (5 mg/ml) in sodium phosphate buffer (0.05 M) at pH 7 and incubating the mixture at 50 °C for 10 min. The reducing sugar released was determined by the dinitrosalicylic acid method (Miller, 1959). Protein concentration was determined according to the method of Bradford (Bradford, 1976), using bovine serum albumin as standard. In order to check the reproducibility of the enzyme activity, 5 substrate protected Xyl I - ODA biocomposite films were reacted with xylan as described above. Suitable controls were taken to estimate the enzymatic activity of an as deposited 250 Å thick ODA film under similar conditions.

Temperature profile of enzymatic activity of the substrate protected Xyl I - ODA biocomposite films.

The enzymatic activity of the substrate protected Xyl I - ODA (1:4 ratio of Xyl I : xylan; w/w) biocomposite films (film dimensions = 4 cm^2) formed at pH 7 was tested in the temperature range 40-90 ^{o}C and compared with that of the enzyme in solution. These measurements were carried out for five substrate protected Xyl I - ODA biocomposite films at each one of the temperatures.

Reusability of the substrate protected Xyl I – ODA biocomposite films

The reusability of the encapsulated enzyme was checked for 5 cycles of reaction with xylan. For comparison, the enzymatic activity of Xyl I in solution at pH 7 was also estimated in the manner above.

RESULTS AND DISCUSSION

The motivation for encapsulating an enzyme in a lipid matrix was two-fold. From a fundamental point of view, this study can be seen as a step to understand protein-lipid interactions, an area of considerable current interest. Secondly, the utilization of enzymes in different areas of chemical, pharmaceutical and agricultural industries is now fairly well established and the technique proposed would have immediate application for such industrial applications in addition to the more thoroughly studied enzymatic applications.

QCM studies

Xyl I (5 μM), substrate protected Xyl I and xylan were incorporated into thermally evaporated 250 Å thick ODA films by simple immersion of the fatty amine films in the respective solutions held at pH 7 (0.05 M, sodium phosphate buffer). Figure 1 shows the QCM kinetics of mass uptake at pH 7 into 250 Å thick independent ODA covered gold coated AT-cut quartz crystals during immersion in the solutions of Xyl I (circles), substrate protected Xyl I (1:4 w/w Xyl I : xylan; squares) and xylan (triangles). The error bars are a 5 % standard deviation to the data obtained by five separate experiments in each case. The kinetics of enzyme incorporation in the ODA films studied by QCM measurements may be analyzed in terms of a one-dimensional (1-D) diffusion model as has been demonstrated for carboxylic acid derivatized colloidal particles of silver, gold and CdS in fatty amine films (Patil *et al*., 1997). There is considerable swelling of the films after enzyme incorporation and therefore the film thickness values used in the 1-D analysis are different from the as-deposited thickness of the films (250 Å). The thickness of the composite films after protein incorporation was measured by ellipsometry and are listed in Table 1 along with the values of the protein and xylan concentration at the film-enzyme solution interface (C_O molecules cm^{-3}) and the enzyme diffusivity (D, $Å^2$ min^{-1}) obtained from an analysis of the QCM data shown in Figure 1.

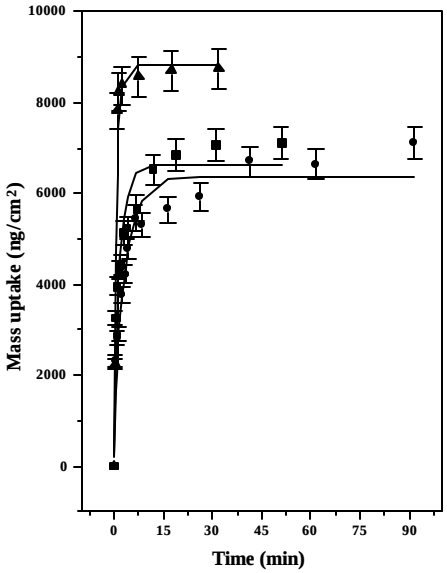


Figure 1: QCM kinetics of diffusion. QCM kinetics of incorporation of xylan (triangles), substrate protected xylanase, (xylanase : xylan, 1:4 ratio, w/w; squares) and xylanase (circles) in the ODA matrix with time during immersion of 250 Å thick ODA films in respective solutions at pH 7 (0.05 M sodium phosphate buffer. The solid lines are fits to the data obtained using a 1-D diffusion model. The error bars shown are 5% standard deviation to the data as measured by 5 separate experiments under similar conditions.

The solid lines in the figure are the fits based on the 1-D diffusion analysis of the QCM mass uptake data. It is also seen that equilibration of the enzyme density within the films occurs in all cases within 1 h of immersion in the enzyme solution. The enzyme immobilization time obtained by this procedure is considerably better than that reported for other techniques (Ellerby *et al*., 1992; Gill & Ballesteros, 1998; Kumar & McLendon, 1997). It is believed that electrostatic interactions are mainly responsible for the incorporation of the enzyme molecules into the lipid matrix. At pH 7, both the Xyl I (pI 4.1, anionic) and ODA (pK_b ~ 10.5, cationic) molecules are completely ionized leading to maximum attractive electrostatic interaction between the host and guest. Furthermore, xylan being a polysaccharide, does not contain ionizable groups, yet does show a significant amount of incorporation in the lipid matrix. This can be clearly seen from the QCM data (Figure 1, triangles) and the values for diffusion coefficient (D) and the concentration at the interface

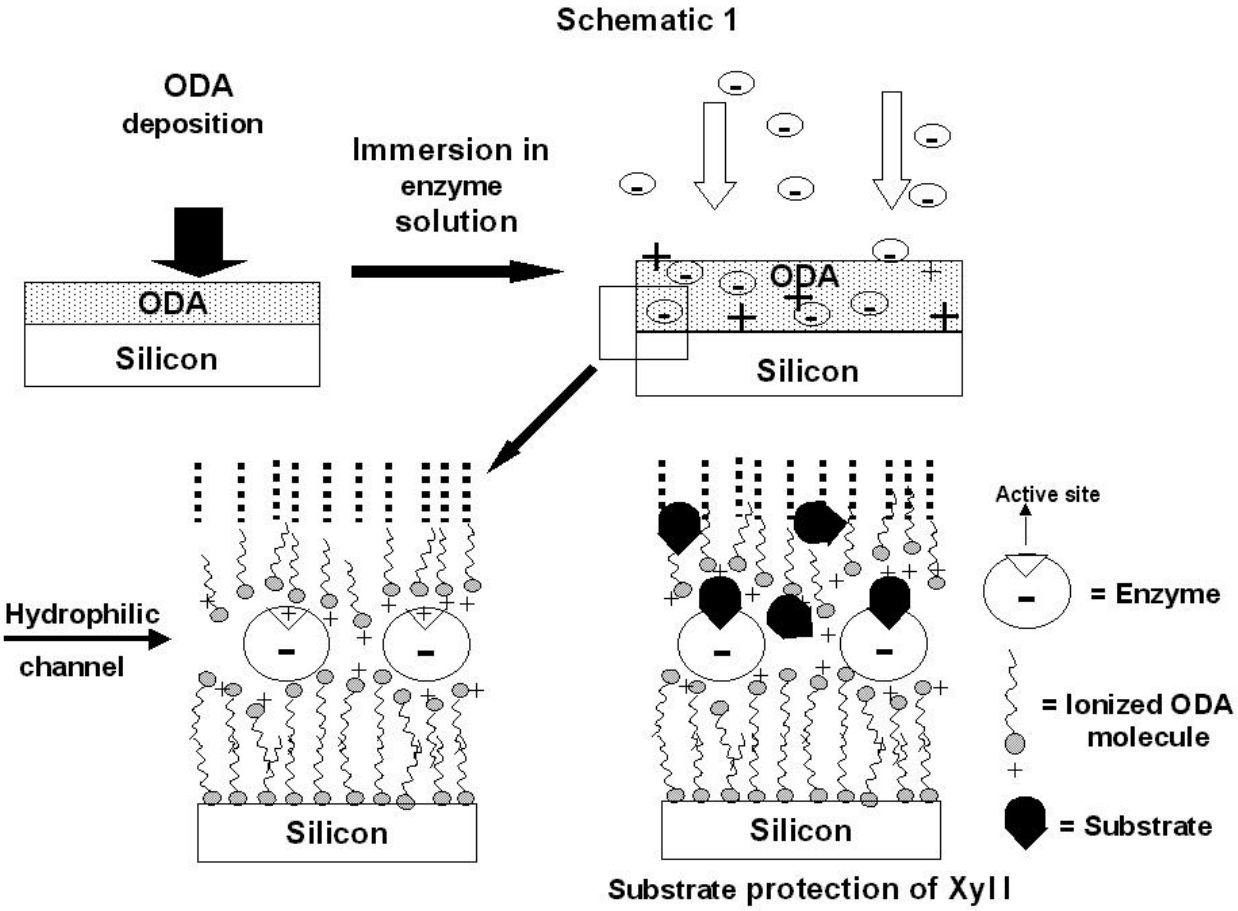
(C_O), as shown in Table. 1, which are greater than that for the Xyl I and substrate protected Xyl I.

Table 1: Parameters obtained from a 1-D diffusion analysis of QCM mass uptake measurements during incorporation of xylanase/xylan in fatty amine matrix at pH 7.

System	Lipid Matrix	Film Thickness (Å)	C_O molecules cm^{-3}	D $Å^2$ min^{-1}
Xylan	ODA	500	2.79×10^{11}	1.10×10^{5}
Xylanase	ODA	500	2.02×10^{11}	2.71×10^{4}
Xylanase+xylan (1:4 w/w)	ODA	500	2.10×10^{11}	5.05×10^{4}

This underlines the fact that secondary interactions such as hydrogen bonding, hydrophobic interactions etc. also contribute to the interaction between xylan and ODA film. In the case of Xyl I and substrate protected Xyl I, electrostatic interactions between the ionized amino acid groups on the enzyme surface and the lipid matrix are mainly responsible for the intercalation. The values for diffusion coefficient (D) and concentration at the interface (C_O) for the substrate protected Xyl I system are more than that for pure Xyl I system which may be due to the contribution of xylan in the diffusivities for substrate protected Xyl I system. The discrepancies in the diffusivity data reflected by enhanced diffusivities for xylan as compared to that of the Xyl I and substrate protected Xyl I system may be due to a very simple electrostatic model being used to describe the protein incorporation process. We would like to emphasize that this model is highly idealized and represents a first step in the analysis of the kinetics of the protein incorporation process. Further refinement of the analysis will be done as our understanding of the different protein-lipid interactions operative in these experiments advances. In studies on pepsin (Gole *et al.*, 2000a) and fungal protease (Gole *et al.*, 2000b) incorporation into fatty lipid films, electrostatic interactions significantly contributed to the incorporation process as compared to secondary interactions. But this study indicates that secondary interactions dominate the intercalation process as evidenced by enhanced xylan diffusion as compared to that of the enzyme into the lipid films. It is clear that a purely

electrostatic picture of the process is not correct and supports earlier studies of polyion-ionized lipid monolayer interactions where a Poisson-Boltzmann electrostatic model was clearly shown to be inadequate in explaining the complexation observed (Cuvillier & Rondelez, 1998). The situation on hand regarding protein-lipid interactions is expected to be far more complex. It is expected that the Xyl I molecules are immobilized within the hydrophilic regions of the ODA matrix as shown in Schematic 1. It should be possible for analytes in solution to communicate with the enzyme molecules via hydrophilic water channels during immersion of the biocomposite films in different solutions.



Schematic1: Diagram showing the various steps involved in enzyme -ODA b iocomposite film formation. A possible microscopic structure of the enzyme-lipid composite film and the mechanism of substrate protection of Xyl I by xylan is also shown.

Wetting properties

It is well known that proteins spontaneously adsorb at phase boundaries (Razumovsky & Damodaran, 1999), and therefore, surface binding of Xyl I to the ODA films as a mechanism for the enzyme uptake observed (Figure 1) must be ruled out. Contact angle measurements with a sessile water drop on a 250 Å thick ODA film deposited on Si (111) substrates before and after immersion in the substrate protected Xyl I solution at pH 7 for 1 h yielded 90° and 88° respectively. It is pertinent to mention here that the contact angles measured for the bare Si (111) surface and a substrate protected Xyl I film deposited on the Si (111) surface and on 250 Å thick ODA film by evaporation of a drop of the enzyme solution yielded values of 18°, 14° and 20° respectively. The high contact angles observed for the as-deposited ODA films do not reduce after immobilization of the enzyme, as would have been the case for surface adsorption of the enzyme, which would result in reduction of the contact angle.

Secondary structure of encapsulated enzyme

The amide linkages in the amino acid residues in polypeptides and proteins give rise to well-known signatures in the infrared region of the electromagnetic spectrum. The position of the amide I and II bands in the FTIR spectra of proteins is a sensitive indicator of conformational changes in the protein secondary structure (Kumar & McLendon, 1997; Dong *et al*., 1992) and may be used to study the Xyl I molecules in the ODA matrix. A number of vibrational bands can be seen in both the curves in Figure 2 for the as-deposited 250 Å thick ODA film (curve 1) and the 250 Å thick bioconjugate film (curve 2). The amide I band, which is assigned to the stretch mode of the carbonyl group coupled to the amide linkage, occurs at ca.1633 cm^{-1} (Figure 2, curve 2, feature a) for the substrate protected Xyl I - ODA biocomposite film and is in good agreement with the assignments obtained by Templeton *et al*. (1999) for monolayers of coenzyme A on gold clusters. Whereas a small feature at this wavenumber does occur in the as-deposited ODA film (Figure 2, curve 1), the intensity of this band increases in curve 2 clearly showing that it originates from the enzyme molecules in the biocomposite film. The position of this band is close to that reported for native proteins (Kumar & McLendon, 1997; Dong *et al*., 1992), and indicates that the

secondary structure of the protein in the ODA environment is relatively unperturbed. The N-H stretch vibration in amide linkages (the amide II band) is observed at 1548 cm^{-1} in the biocomposite film (curve 2, feature b), which is clearly missing in the as-deposited ODA film (curve 1) and compares well with the work by Templeton *et al*. (1999).

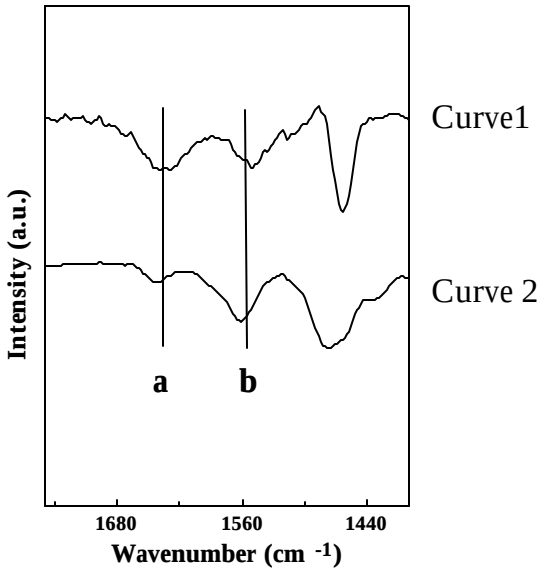


Figure 2: FTIR spectra of Xyl I in ODA film. FTIR spectra recorded from a 250 Å thick as-deposited ODA film (curve 1) and a 250 Å thick substrate protected Xyl I biocomposite film (curve 2; see text for details).

A feature at about 1560 cm^{-1} is observed in the as-prepared ODA film (curve 1) and is assigned to the N-H deformation vibration mode in the ODA matrix. The presence of well-defined amide bands as seen from Figure 2, indicates intactness of the secondary structure of the enzyme. The enzymatic activity of the encapsulated enzyme would largely depend on the tertiary structure of the enzyme remaining unperturbed within the lipid matrix.

Enzymatic activ ity and Substrate protection

Initial experiments indicated that Xyl I encapsulated in the ODA films failed to show any enzymatic activity. To study the possible role of ODA in inhibiting the enzymatic activity, a separate experiment was carried out in which Xyl I (2 μg) was incubated along with ODA (100 μM) and the residual activity was measured. It was found that ODA inhibits Xyl I. Studies on protection of Xyl I active site by precomplexing the enzyme along with its substrate (xylan) in different ratios, before immobilization into 250 Å thick ODA films was carried out at pH 7. Table 2 shows that complete protection of enzyme active site was accomplished at an Xyl I : xylan ratio of 1:4 (w/w), which yields optimum catalytic activity (Table 2).

Table 2: Substrate protection of Xyl I at pH 7 by addition of different concentrations of the substrate xylan prior to encapsulation in 250 Å thick ODA film

S. No	Xyl I : xylan ratio (w/w)	Relative Activity (%)
1	1:1	20
2	1:2	45
3	1:3	85
4	1:4	98
5	1:5	95

An increase in the Xyl I : xylan ratio brought about a decrease in the activity, probably due to the decrease in the diffusion of enzyme in comparison to the substrate. Hence all further experiments were carried out with substrate-protected Xyl I (1:4) biocomposite films. In earlier studies on pepsin (Gole *et al*., 2000a) and fungal protease (Gole *et al*., 2000b) enzyme molecules entrapped in thermally evaporated lipid films, It was observed that the encapsulated enzyme molecules showed significant enzymatic activity without substrate

protection. As discussed above, substrate protection is important in the case of Xyl I molecules entrapped in ODA films and a possible explanation is that the enzyme molecules are encapsulated in the hydrophillic regions of the lipid bilayers as illustrated in Schematic 1. The location of the negatively charged amino acid residues on the enzyme surface would determine the electrostatic coordination of the enzyme with the protonated amine groups in the film and hence, the orientation of the enzyme molecules within the ODA bilayers. It is conceivable that if these negatively charged sites are located close to the active site of the enzyme, then the orientation of the enzyme in the lipid bilayers would not favour easy access of substrate molecules from solution to the "blocked" active sites of the enzyme. Heller *et al*. (1998) have observed that complexing flexible polycations with lactate and glycolate oxidases before immobilization into silicate sol gels dramatically stabilizes the enzyme by reducing the electrostatic interaction of the silica and the arginine groups in the active sites, wherein now the polycations bind to the sol gel matrix. A similar protection mechanism might be possible in our case as well but in the absence of crystallographic information on the Xyl I system, we are unable to make a definite statement on this aspect. Experimentally, however, an enzyme : substrate ratio of 1:4 (w/w) was determined to be ideal in optimising the enzymatic activity of the encapsulated enzyme in the lipid matrix and is an important result of this investigation. Furthermore, it was found that the Xyl I–ODA biocomposite film loses enzymatic activity when dried in air. This might be due to the dehydration of the biocomposite material. A similar loss in activity albeit to a lesser extent was found in studies of fungal protease-fatty lipid biocomposite films (Gole *et al*., 2000b).

Effect of ODA on the isoindole fluorescence

In order to determine the residues involved in the interaction between ODA and Xyl I a fluorescent chemoaffinity label, *o*-phthalaldehyde (OPTA) was used. OPTA is a bifunctional agent that forms a fluorescent isoindole derivative due to its reaction with SH and NH_2 groups of lysine and cysteine residues, which gives a characteristic isoindole peak at 415 nm with the excitation wavelength fixed at 338 nm (Simons & Johnson, 1978; Palczewski *et al*., 1983). Xyl I (4 μM) was preincubated with ODA (10 mM) for 10 min and it was then reacted with

OPTA (0.5 mM). ODA modified Xyl I was unable to form an isoindole derivative with OPTA (Figure 3).

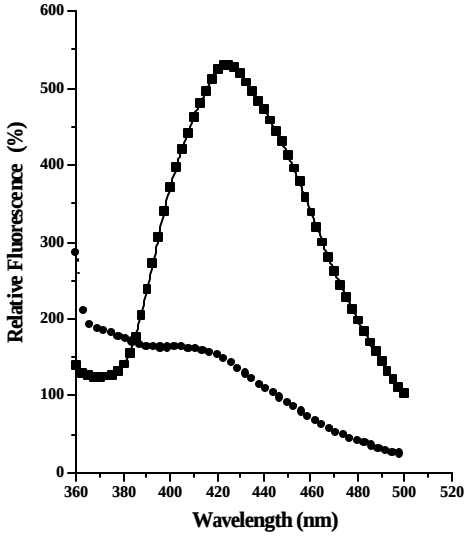


Figure 3: Effect of ODA on the isoindole fluorescence of OPTA treated Xyl I. Xyl samples (4 μM) were incubated with 10 mM ODA for 20 minutes at 25 °C. The modified Xyl I samples were then treated with 0.5 mM OPTA for 10 min at 25 °C. The change in the isoindole fluorescence was monitored at λ_{ex} 338 and λ_{em} 415.

Thus, prior treatment of Xyl I with ODA makes the residues in the active site unavailable for further reaction with OPTA. We have found that the active site of Xyl I contains a histidine and a lysine residue that are able to form a fluorescent isoindole derivative with OPTA (Chapter IV). It is likely that ODA interacts with either histidine or the lysine residue in the active site, thereby preventing additional reaction with OPTA. Earlier kinetic studies on Xyl I have shown that the histidine residue is located at the catalytic site in the active site and is involved in a network of hydrogen bonds, which are responsible for maintaining the ionization state of the two catalytic residues responsible for the hydrolysis of the β-1,4 glycosidic bond. The lysine residue is present in the substrate-binding site and it plays an important role in positioning the substrate into the active site (Chapter IV). Since the inactivation of Xyl I by ODA can be prevented by the protection of the active site with the substrate, it is possible that ODA is interacting with the lysine residue present in the substrate binding site. The interaction of lysine residue with the lipid matrix can be elucidated based on

the "snorkel" model (Segrest *et al*., 1992). In the snorkel model, it has been proposed that in the interfacial positively charged amino acid residues of the peptide, when associated with the lipid insert the charged moieties into the aqueous environment for solvation. Thus in the snorkel orientation, the entire uncharged van der Waals surface is buried within the hydrophobic interior of the lipid bilayer. The bulk of the van der Waals surface area of the positively charged lysine is hydrophobic. The lipid matrix interacts with this van der Waals surface area of the lysine residue making it inaccessible for reaction with the substrate, thereby leading to the inactivation of the enzyme. Preincubation of Xyl I with the substrate prevents the interaction between the lysine groups of the enzyme and the matrix thus avoiding inactivation of the enzyme. In earlier studies on pepsin (Gole *et al*., 2000a) and fungal protease (Gole *et al*., 2000b) enzyme molecules entrapped in thermally evaporated lipid films, we have observed that the encapsulated enzyme molecules showed significant enzymatic activity without substrate protection. Pepsin and fungal protease are aspartic proteases, which have a bilobal structure with the active site cleft, located between the lobes. The active site contains aspartic acid residue that is situated between the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr (Sielecki *et al*., 1991). Unlike Xyl I active site, these proteases does not contain any His or Lys residue in the active site for interaction with ODA. Moreover the microenvironment of the active site may also play a part in the interaction with ODA.

Reusability

The Xyl I-ODA biocomposite film was found to be re-usable for five cycles with progressive decrease of activity measurements as shown in Table 3. During the first two runs up to 83 % activity was retained, following which there was a rapid decrease in activity after every cycle. The diminishing activity as a function of number of cycles may be due to the replacement of the enzyme by the substrate which would also lead to blockage of the diffusion pathways of solution based xylan molecules and thus contribute further to a reduction in catalytic activity. The decrease in activity with every cycle could also be attributed to the increase in interaction between the enzyme and the lipid matrix leading to the inactivation of the enzyme.

Table 3: Reusability of Xyl I -ODA biocomposite films.

System	Activity of Xyl I (IU)[#]	Specific Activit y (IU/mg)	Residual Activity (%)
Xyl I in solution (Control)	1.35	0.45 ± 0.05	100
Substrate protected Xyl I-ODA biocomposite film	1.29	0.44 ± 0.05	100
Biocomposite film, run 1	1.28	0.44 ± 0.04	100
Biocomposite film, run 2	1.08	0.36 ± 0.06	83
Biocomposite film, run 3	0.72	0.24 ± 0.05	55
Biocomposite film, run 4	0.48	0.16 ± 0.05	35
Biocomposite film, run 5	0.15	0.05 ± 0.04	11

[#] One unit of xylanase activity was defined as the amount of enzyme that produced a change in absorbance of 0.3 at 540 nm and 50 °C.

Temperature profile of encapsulated enzyme

The temperature dependence of the enzymatic activity of Xyl I molecules in solution at pH 7 (Figure 4, squares) was compared with that of the substrate-protected Xyl I molecules encapsulated in 250 Å thick ODA films (4 cm^2 substrate area) at pH 7 (Figure 4, circles). The optimum temperature of operation of Xyl I in solution was 80 °C and it shifts to 85 °C in the encapsulated form. The reproducibility was checked for five similar films at each temperature value.

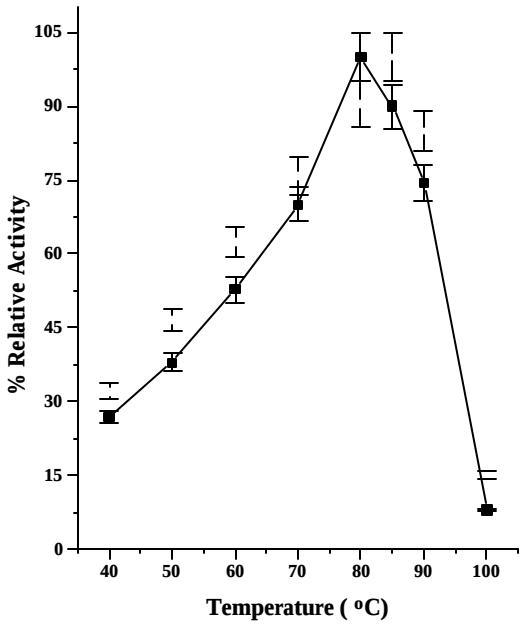


Figure 4: Optimum temperature of Xyl I in solution and in ODA film. Variation in the percentage enzymatic activity from substrate-protected Xyl I molecules in solution (circles) and substrate-protected Xyl I molecules entrapped in 250 Å thick ODA films grown at pH 7 (squares) as a function of temperature. The readings at each temperature are the average of 5 independent readings. Care was taken to encapsulate equal amount of enzyme in each film.

The enzyme in solution is susceptible to adverse environmental conditions. As mentioned earlier, one of the requisites for a matrix used for immobilization should be its ability to stabilize the protein and protect it from external harsh conditions. Furthermore, the entrapped protein should be accessible to analytes from solution such as substrates. It is well known that immobilized enzymes have restricted mobility that prevents structural changes and 'locking' the enzyme in some preferred orientation. The lipid matrix in our case not only protects the enzyme from external harsh conditions such as temperature and increases the optimum temperature of operation, but also possibly 'locks' the enzyme in some orientation preventing mobility and aggregation. The enhanced thermal stability of an immobilized enzyme has been reported and may be due to the hindered mobility (Weetal, 1969; Hernaiz & Crout, 2000).

In conclusion, we have demonstrated the formation of Xyl I - fatty amine biocomposite films by a simple beaker-based immersion process. The enzyme molecules are

entrapped in the lipid matrix by primarily electrostatic interactions. Secondary interactions such as hydrophobic and hydrogen bonding also play an important role in the encapsulation process. Substrate-protection of the Xyl I molecules with its substrate, xylan, was required to obtain optimum enzymatic activity of the entrapped enzyme molecules. The shift in the optimum temperature of operation of the enzyme in the lipid matrix shows that the matrix protects the enzyme from external harsh conditions. Access of the enzyme molecules to substrate molecules in solution is possibly provided via hydrophilic channels in the films (Schematic 1). The enzyme molecules in the lipid films are encapsulated without significant distortion to their native structure as evidenced by FTIR and enzymatic activity measurements. The elasticity of the bilayers may be primarily responsible for this and enables the matrix to adopt the contours of the enzyme guest molecule (Schematic 1). The reasonably fast time-scales for the synthesis of the enzyme-lipid biocomposites under mild encapsulation conditions (close to physiological pH conditions, encapsulation at 4 °C) is a major improvement over other techniques. The enzyme-loading factor can be easily controlled by depositing thicker lipid films with an additional degree of freedom provided by the chain length of the lipid amphiphiles. The technique demonstrated above shows promise for the encapsulation of other biomacromolecules such as DNA (Sastry *et al*., 2001).

SUMMARY

The encapsulation and interaction of xylanase I (Xyl I) from *Thermomonospora* sp. in thermally evaporated fatty amine films by a simple beaker-based immersion technique under enzyme-friendly conditions has been described. The approach is based on the diffusion of the enzyme from aqueous solution, driven primarily by attractive electrostatic interactions between charged groups on the enzyme surface and ionized lipid molecules in the film. The encapsulated Xyl I molecules initially failed to show catalytic activity possibly due to the interaction of residues in the enzyme active site to the matrix thereby causing its inactivation. It was found that encapsulating Xyl I into the lipid matrix along with its substrate (xylan) resulted in catalytic activity of the biocomposite film comparable to that of the free enzyme molecules in solution. The kinetics of Xyl I diffusion into the amine films was followed using quartz crystal microgravimetry (QCM) whereas Fourier transform infrared spectroscopy (FTIR) and bio-catalytic activity measurements confirmed the stable native conformation of the encapsulated enzyme. The interaction of the lipid matrix with the active site of Xyl I was ascertained by fluorescent chemoaffinity labelling with *o*-phthalaldehyde. The encapsulated substrate-protected Xyl I system was reusable and it was found that the lipid matrix stabilizes the enzyme and shifts the optimum temperature for catalytic activity from 80 °C to 85 °C. The present results describe a radically new approach for the entrapment of biocatalysts into thermally evaporated lipid films and in understanding towards protein-lipid interaction.

CHAPTER vI

Construction of genomic library and identification of xylanase gene in e. coli

INTRODUCTION

The economic feasibility of utilizing xylanase for biotechnological applications depends on its hyperproduction. Molecular cloning and enhancement of expression levels of xylanase gene is one of the approaches to achieve it. Protein engineering can also bring about enhancement of catalytic properties and stability of xylanases resulting in their suitability for commercial applications. The identification of xylanase gene represents the first step towards the construction of an efficient microorganism. To the best of our knowledge there are only two reports of cloning of xylanase gene from *Thermomonospora* sp. Ghangas *et al.* (1989) have cloned a xylanase gene from *Thermomonospora fusca* into bacteriophage Lambda and have expressed it in *E. coli* and *Streptomyces lividans*. Two different endo-β-1,4-xylanases from *Thermomonospora alba* ULJB1 have been cloned by Blanco *et al.* (1997). *xylA*, has been sequenced, subcloned and overexpressed in *Streptomyces lividans.*

The present chapter describes the construction of a partial genomic library of alkalothermophilic *Thermomonospora* sp., using pBluescript as vector in *E. coli*. The presence of the xylanase gene was detected by southern hybridization with xylanase probe from *Thermomonospora fusca.* The recombinant *E. coli* containing pATA93 showed intracellular xylanase activity.

MATERIALS AND METHODS

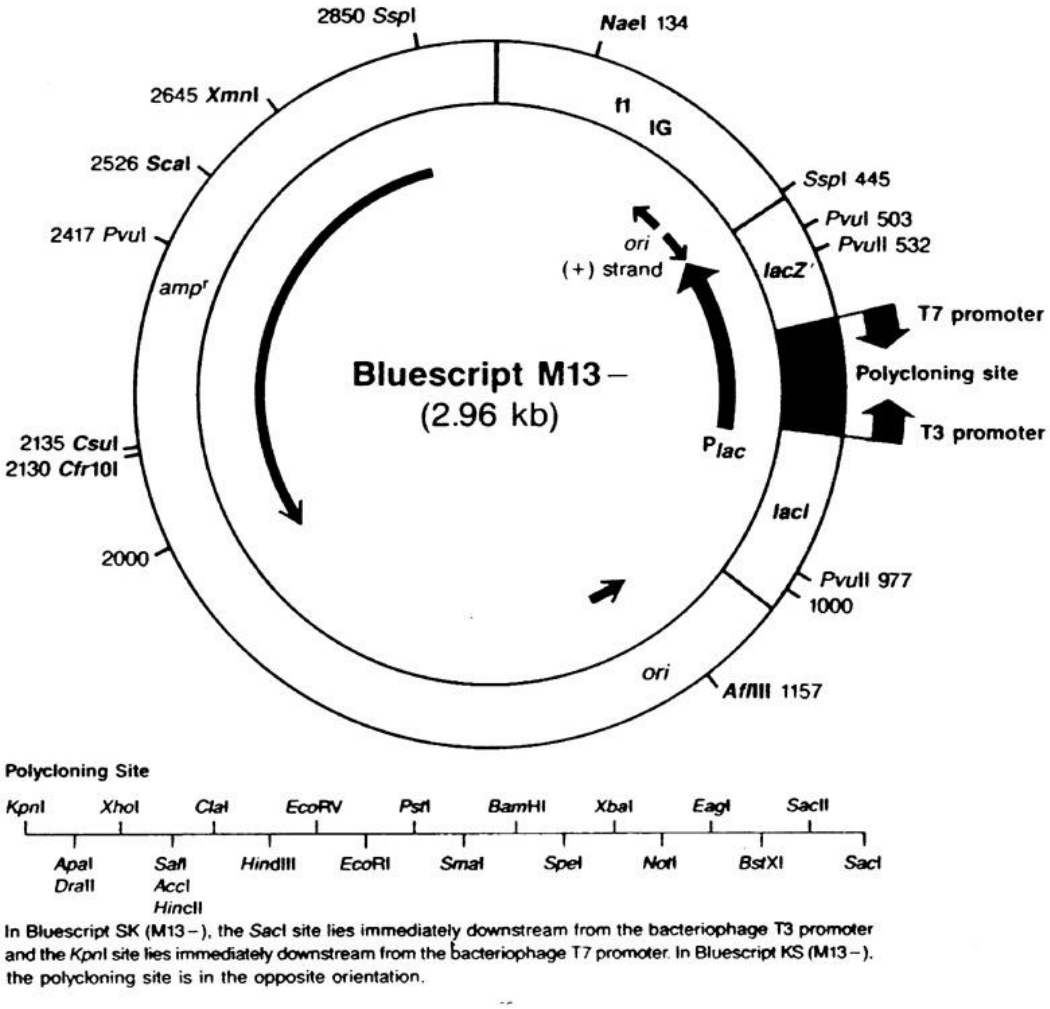
Materials

Chemicals : Agarose, ampicillin, SDS, X-gal, IPTG, RNAase, lysozyme were purchased from Sigma Chemical Co., USA. Lambda *Hind*III digest, calf intestinal phosphatase (CIP), T_4 DNA ligase, all restriction endonucleases were obtained from Boehringer Mannheim (Germany), random primer labeling kit was purchased from Bangalore Genei, Hybond N transfer membrane was obtained from Amersham (UK). Radiolabelled α ^{32}P dATP were supplied by Bhabha Atomic Research Center, Mumbai. Kodak X-ray films were used for autoradiography.

E. coli harbouring plasmid pYY01 containing xylanase gene from *Thermomonospora fusca* was a gift from Prof. David Wilson and Prof. Diana Irwin from Cornell University, New York. All other chemicals used were of analytical grade obtained from various local sources.

Host system : The *E. coli* strain XL1-Blue was used for the construction of the genomic library and propagation of the recombinant plasmid from the selected recombinants.
E. coli XL1-Blue genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacI*^{q}*ZDM15* Tn*10* (Tet^r)]^c

Vector : Plasmid vector pBluescript (pBS) was used for the construction of the genomic library of alkalothermophilic *Thermomonospora* sp. pBS plasmid was chosen as a vector in the present study for its multiple cloning sites and high copy number. pBS contains ampicillin resistance gene as marker and blue/white selection for the screening of recombinants.



In Bluescript SK (M13–), the *Sac*I site lies immediately downstream from the bacteriophage T3 promoter and the *Kpn*I site lies immediately downstream from the bacteriophage T7 promoter. In Bluescript KS (M13–), the polycloning site is in the opposite orientation.

Figure 1: Restriction map of pBluescript

Culture maintenance

E. coli was stored as 15 % glycerol suspensions of the 16 h grown liquid culture at –70 °C. *Thermomonospora* sp. spores were also stored as a suspension in 15 % glycerol at –70 °C. All recombinants were grown in media containing ampicillin (60 μg/ml) for 16 h, and preserved as 15 % glycerol suspension at –70 °C. Short-term maintenance of the cultures was at 4 °C. The recombinants were maintained on LB plates containing ampicillin (60 μg/ml). All media and buffers were autoclaved at 15 pounds per square inch for 20 min. Heat labile components like antibiotics, IPTG, X-Gal were filter sterilized through autoclaved Millipore

(0.45 μm) membranes and added to cooled media (45 °C). Ampicillin solution was prepared as a stock of 100 mg/ml and was used at a final concentration of 60 μg/ml in the medium. 4 μl of 200 mg/ml IPTG and 40 μl of 20 mg/ml X-gal (in dimethyl formamide) were used per 25 ml of LB agar.

Media and Buffer composition

1. Luria Bertani (LB) medium (%)
   Tryptone :1.0
   Yeast Extract : 0.5
   NaCl : 0.5
   Agar : 2.0
   pH : 7.2-7.4

2. TE
   TrisCl (pH 8) : 10 mM
   EDTA (pH 8) : 1 mM

3. STE buffer
   8 % sucrose in TE

4. 20X SSC
   NaCl : 3 M
   Trisodium citrate : 0.3 M

5. TAE
   Tris-acetate : 40 mM
   EDTA (pH 8) : 1mM

6. 20X SSPE
   NaCl : 3.6 M
   Sodium phosphate : 0.2 M
   EDTA, pH 7.4 : 0.02 M

7. 100X Denhardt's
   2 % (w/v) Bovine serum Albumin
   2 % Ficoll
   2 % (w/v) PVP (polyvinylpyrrolidone)

Methods

Isolation of *Thermomonospora* sp. DNA

Thermomonospora sp. was grown in 100 ml of LB (pH 9) at 50 °C for 48 h with shaking at 200 rpm. DNA was isolated according to the method of Hopwood *et al*. (1985) with slight modifications. Mycelium (1 g) was washed in 5 ml TE buffer in a centrifuge tube. The suspension was centrifuged at 10,000 rpm and the pellet was resuspended in 5 ml TE. 10 mg lysozyme (2 mg/ml) was added and swirled to dissolve. It was incubated at 30 °C, triturating every 15 minutes until a drop of suspension on a microscopic slide was completely cleared with a drop of 10 % SDS (90 minutes). 1.2 ml of 0.5 M EDTA was added to the mixture followed by 0.7 ml 10 % SDS (i.e. to 1 %). The solution was swirled and incubated at 37 °C for 2 h. Proteins were extracted from the aqueous phase by adding 6 ml of a mixture of phenol: chloroform: IAA (25:24:1). The mixture was shaken for 5 min and then centrifuged at 10,000 rpm, 4 °C for 10 min. Aqueous phase was transferred to another tube and extraction with phenol: chloroform was repeated. The aqueous phase was weighed and 40 μg/g of RNAase was added and incubated at 37 °C for 1 h. DNA was precipitated by adding equal volume of isopropanol and was stored at room temperature for 1 h. DNA was spooled with a glass rod and transferred to a fresh tube. DNA was dissolved in 5 ml of TE buffer.

Quantitation of DNA

Absorption spectra of suitably diluted DNA solution was recorded on a Varian Cary WinUV spectrophotometer in the range 220-320 nm. The preparation with a A_{260}:A_{280} ratio > 1.8-2 were considered suitable for further work. The DNA was quantitated according to the following equation.

Absorbance of 1.0 at 260 nm= 50 μg/ml DNA (Maniatis *et al.*, 1982)

Isolation of plasmid DNA (Maxiprep)

A 30 ml culture of *E. coli* containing the plasmid pBS was grown to late log phase (OD_{600} = 0.6). LB media (500 ml) containing the appropriate antibiotic in a 2 L flask was

inoculated with 25 ml of late log phase culture. The culture was incubated at 37 °C for 2.5 h with vigorous shaking. In order to get high yields of plasmid (2-5 mg/500 ml culture) the culture was grown in the presence of chloramphenicol (170 μg/ml). The culture was incubated for a further 12-16 h at 37 °C with vigorous shaking. The bacterial cells were harvested from a 500 ml culture by centrifugation at 4,000 rpm for 15 min at 4 °C. The bacterial pellet was resuspended in 10 ml of solution I [50 mM glucose, 25 mM TrisCl (pH 8), 10 mM EDTA (pH 8)]. 1ml of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM TrisCl, pH 8) was added to the solution. This was followed by the addition of 20 ml of freshly prepared solution II [0.2 N NaOH, 1 % SDS]. The centrifuge bottle was closed and the contents were mixed thoroughly by gently inverting the bottle several times. The bottle was stored at room temperature for 5-10 minutes. 15 ml of ice-cold solution III [5 M potassium acetate (60 ml), glacial acetic acid (11.5 ml), H_2O (28.5 ml)] was added and the contents were mixed by shaking the bottle several times till there was no longer two distinguishable liquid phases. The bottles were stored on ice for 10 min followed by centrifugation of the bacterial lysate at 4,000 rpm for 15 min at 4 °C. The supernatant was filtered through four layers of cheesecloth. Isopropanol (0.6 volume) was added to the filtrate and mixed well. The mixture was stored at room temperature for 10 min. The plasmid was recovered by centrifuging at 5,000 rpm for 15 min at room temperature. The pellet of nucleic acid was dissolved in 3 ml of TE.

Purification of plasmid by polyethylene glycol

The plasmid solution was transferred to a 15 ml centrifuge tube and 3 ml of ice-cold solution of 5 M LiCl was added. The solution was mixed well and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was transferred to a fresh tube and an equal volume of isopropanol was added to it. The solution was mixed well and the precipitated nucleic acid was recovered by centrifugation at 10,000 rpm for 10 min at room temperature. The pellet was dissolved in 500 μl of TE (pH 8) containing RNAase (20 μg/ml). The solution was transferred to a microfuge tube and the solution was stored at room temperature for 30 min. 500 μl of 1.6 M NaCl containing 13 % (w/v) polyethylene glycol (PEG 8000) was added and mixed well. The plasmid DNA was recovered by centrifugation at 10,000 rpm for 5 min at 4

°C. The pellet was dissolved in 400 µl of TE (pH 8) and the solution was extracted with phenol: chloroform. The aqueous layer was removed and 100 µl of 10 M ammonium acetate was added to it. 2 volumes of ethanol were added and it was mixed well and the tube was stored for 10 min at room temperature. The plasmid DNA was recovered by centrifugation at 10,000 rpm for 5 min at 4 °C. The pellet was washed with 70 % ethanol and dried to remove the last traces of alcohol. The pellet was dissolved in 500 µl of TE.

Restriction digestion of genomic and plasmid DNA

The DNA solution (0.2-1 µg) was placed in a sterile microfuge tube and the volume was made up to 18 µl by adding sterile MilliQ water. 2 µl of digestion buffer was added and it was mixed by tapping the tube. 1-2 units of restriction enzyme was added and mixed by tapping the tube. The mixture was incubated overnight at 37 °C. The reaction was stopped by adding 0.5 M EDTA (pH 8) to a final concentration of 10 mM. Small aliquots were removed during the course of reaction and analyzed on minigel to monitor the progress of digestion.

Size fractionation of *Eco*RI digested *Thermomonospora* sp. DNA

The *Eco*RI digested genomic DNA of *Thermomonospora* sp. was separated on a 0.8 % agarose gel by using 1X TAE running buffer (40 mM Tris acetate, 1 mM EDTA). The DNA fragments corresponding to 2-10 kb were eluted from the gel by using Agarose gel DNA extraction kit from Boehringer Mannheim. The 2-10 kb DNA fragments were cut out from the agarose gel using a sharp scalpel, taking care to leave as much agarose gel as possible. The sample was transferred to a preweighed reaction tube. 300 µl of agarose solubilization buffer containing sodium perchlorate was added per 100 mg of agarose gel. Silica suspension (10 µl) was added to the sample. The mixture was incubated for10 min at 60 °C and vortexed every 2-3 min. The sample was centrifuged for 30 s and the supernatant was discarded. The matrix containing the DNA was resuspended with 500 µl of nucleic acid binding buffer on a vortex mixer. Centrifuge and discard supernatant. The pellet was washed with 500 µl of washing buffer, centrifuged and the supernatant discarded as before. The washing was repeated and the pellet was dried at room temperature for 15 min. 50 µl of TE buffer was added for the elution

of the DNA. After centrifugation at maximum speed for 30 s, the DNA containing solution was transferred to a new reaction tube. The elution cycle was repeated. The eluted genomic DNA was precipitated by adding 2 volumes of ethanol. The plasmid DNA was recovered by centrifugation at 10,000 rpm for 5 min at 4 °C. The pellet was resuspended in TE to get a final concentration of 100 μg/ml.

Dephosphorylation of linearized plasmid

The digested plasmid (10-20 μg) containing solution was extracted with phenol: chloroform and precipitated with two volumes of ethanol for 15 minutes at 0 °C. The DNA was recovered by centrifugation at 10,000 rpm for 10 min at 4 °C, and dissolved in TrisCl (pH 8.3). 10 μl of 10X Calf intestinal alkaline phosphatase (CIP) dephosphorylation buffer and 1 unit of CIP were added and incubated at 37 °C for 30 min. EDTA, pH 8 (5 mM) was added and it was heated at 65 °C for 1 h to terminate the reaction. The reaction mixture was cooled to room temperature and extracted with phenol: chloroform. To the aqueous layer 0.1 volume of 3 M sodium acetate (pH 7) was added and the DNA was precipitated by adding two volumes of ethanol. The DNA was recovered by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellet was washed with 70 % ethanol and the pellet was dissolved in TE (pH 7.6).

Ligation reaction between genomic DNA and pBS

*Eco*RI digested and dephosphorylated pBS and *Eco*RI digested genomic DNA in a molar ratio of 1:2 were mixed. The mixture was warmed at 45 °C for 5 min to melt the cohesive termini that could have reannealed, and allowed to cool slowly. The DNA was precipitated in the presence of 2.4 M ammonium acetate using two volumes of ethanol. The pellet was dried and suspended in 5 μl of DNA ligase buffer. 1 unit of ligase was added to the mixture and the mixture was incubated for 4 h at 16 °C.

Preparation of competent *E. coli*

Fresh competent cells were prepared by using calcium chloride. A single colony was picked from a freshly grown plate of *E. coli* and was inoculated into 100 ml LB broth in a 1 liter flask. The culture was incubated at 37 °C for approx 5 h with vigorous shaking (300 rpm). The cells were transferred asceptically to a sterile SS-34 tube and centrifuged at 4,000 rpm, for 10 min at 4 °C. The pellet was suspended in 10 ml of freshly prepared ice cold 0.1 M $CaCl_2$ and stored on ice for 30 min. The cells were centrifuged at 4,000 rpm, for 10 min at 4 °C and the supernatant was discarded. The cells were resuspended in 2 ml of ice cold $CaCl_2$ for each 50 ml original culture. The efficiency of transformation was increased 4-6 fold by storing the cells at 4 °C for 12-24 h. The competent cells (200 µl) were transferred to sterile microfuge tube by using chilled sterile tips.

Transformation of competent *E. coli* cells with ligation mixture

The ligation mixture (50 ng) was added to 200 µl of competent *E. coli* and the contents were mixed by gentle swirling. The tubes were stored on ice for 30 min. The tubes were transferred to a water bath that has been preheated to 42 °C and incubated for exactly 90 s. The reaction tubes were rapidly transferred to an ice bath and were allowed to chill for 1-2 min. 800 µl of LB was added to the cells and the tubes were incubated at 37 °C for 45 min to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid. 200 µl of the transformed competent cells were plated onto SOB agar medium containing 20 mM $MgSO_4$ and 60 µg/ml ampicillin. The plates were incubated at 37 °C for 12-16 h.

Identification of recombinant colonies

In order to differentiate between the colonies containing recombinant plasmids from the non-recombinant colonies, blue white selection of the colonies was performed. To the SOB plates containing ampicillin (60 µg/ml), 40 µl of a stock solution of X-gal (20 mg/ml in dimethylformamide) and 4 µl of a solution of isopropylthio-β-D-galactoside (IPTG) (200 mg/ml). The mixture of X-gal and IPTG were spread uniformly onto the surface of agar plate

by using a sterile glass spreader. The plates were incubated at 37 °C until all the mixture is absorbed into the agar. The transformants were plated onto the plates and incubated at 37 °C for 12-16 h. After the appearance of the colonies, the plates were placed at 4 °C for several hours to allow the blue colour to develop fully. This colour test helps to differentiate the recombinants constructed in plasmid vectors from the non recombinant plasmids.

Capillary transfer of DNA

DNA was transferred from the gel to nylon membrane by capillary transfer. After the gel was run, the unused areas of the gel were removed by a razor blade and the bottom right end of the gel was cut to orient the gel. The DNA was denatured by soaking the gel for 45 min in several volumes of denaturing solution (1.5 M NaCl, 0.5 N NaOH), with constant gentle agitation. The gel was rinsed briefly in deionized water and then neutralized by soaking for 30 min in several volumes of a solution of 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant agitation. The neutralization solution was changed and the soaking was continued for a further 15 min. A piece of 3MM Whatman paper was wrapped around a stack of glass plates and it was placed in a large tray. The tray was filled with transfer buffer (20X SSC) and the paper was saturated with it. The gel was removed from neutralization solution, inverted and placed on center of the support. A nylon filter about 1 mm larger than the gel in both dimensions was cut and wetted in both deionized water and transfer buffer for 5 min each. The membrane was placed and aligned on the gel. Two pieces of 3MM Whatman papers, cut exactly to the size of gel were wet with 2X SSC and placed over the filters. A stack of paper towels was cut and placed over the 3MM papers. The paper towels were weighed down by 500 g of weight. The transfer was allowed for 24 h. After the transfer was completed, the paper towels and 3MM paper were removed and the filter was dried on a dry 3MM paper. The filter was soaked in 6X SSC to remove pieces of agarose gel. The filter was then covered with 3MM paper and baked in an oven at 80 °C for 2 h to fix the DNA onto the membrane.

Colony blotting

A suitably cut nylon membrane was placed on an agar plate containing ampicillin (50 μg/ml). Recombinant colonies were transferred onto the nylon filter and then onto a master

plate. Small streaks 2-3 mm in length were arranged in a grid pattern. Each colony was streaked on identical position on both plates. Suitable control colonies were also streaked. The plates were incubated at 37 °C till they start growing rapidly and then the filters were transferred onto an agar plate containing 170 μg/ml chloramphenicol. The colonies were incubated at 37 °C for an additional 12 h for the amplification of the recombinant plasmids. The filters were marked at three asymmetric locations by stabbing through it with an 18 gauge needle. The master plate is stored at 4 °C. In order to liberate the DNA from the bacterial colonies and to bind it to the filters the following method based on the procedure of Grunstein & Hogness (1975) was used. Four pieces of 3MM Whatman paper were cut to appropriate size and shape and were placed in four different trays. Each 3MM paper was saturated with 10 % SDS, denaturing solution (0.5 N NaOH, 1.5 M NaCl), neutralizing solution (1.5 M NaCl, 0.5 M TrisCl [pH 7.4]) and 2X SSC. The excess liquid was poured out. The nylon filter was transferred to SDS impregnated 3MM paper and incubated for 3 min. The filter was then sequentially transferred to denaturing solution, neutralizing solution and 2X SSC saturated 3MM papers. They were incubated in each of the solutions for 5 min each. Dry the filters for 30 min at room temperature. The DNA was fixed onto the filters by baking at 80 °C for 2 h.

Purification of probe

The probe containing plasmid (pYY01, 4.88 kb) used in the present study is a recombinant containing xylanase gene isolated from *Thermomonospora fusca* (Ghangas *et al*., 1989). The plasmid pYY01 was restricted by *Sal*I and the fragments were separated on low metling agarose (0.8 %). The band representing the xylanase gene was cut from the gel using a scalpel and transferred to a clean tube. About five volumes of 20 mM TrisCl (pH 8), 1 mM EDTA (pH 8) is added to the agarose pieces. The tube was incubated at 65 °C for 5 min to melt the gel. The solution was cooled to room temperature and was extracted with phenol: chloroform. The aqueous solution was transferred to a sterile tube and 0.2 volume of 10 M ammonium acetate and 2 volumes of ethanol were added to the tube and stored at –20 °C for 1 h to precipitate the DNA. The DNA was recovered by centrifugation at 10,000 rpm for 10 min. The pellet was dissolved in TE and its purity was checked on gel.

Labeling of prob e

Labeling involves the invitro synthesis of DNA from denatured double stranded template DNA using random hexanucleotides as primers by klenow fragment and ^{32}P dATP (Fienberg & Vogelstein, 1983). In order to label the probe, 25 ng of probe was taken in a clean eppendorf tube and 9 µl of autoclaved MilliQ water was added. The contents were boiled for 5 min in a boiling water bath and chilled in ice for 5-10 min to denature to DNA. The sample was centrifuged at 8,000 rpm for 30 s. 2.5 µl of 10X labeling buffer (900 mM HEPES (pH 6.6) and 100 mM $MgCl_2$) was added to the sample. 1 µl of random primer (100 µg/ml) was added, followed by 2.5 µl of 20 mM DTT solution and 2 µl of dCTP, dGTP and dTTP mix (2.5 mM each). 3 µl of the radiolabel ^{32}P dATP (specific activity > 3,000 Ci/mmole; 10 µCi/µl) was also added to the mixture. Finally, 1 µl of 3 units/µl klenow fragment was added to the reaction mixture and mixed gently. The mixture was incubated at room temperature for at least 2 h.

Prehybridization

Before hybridization with labeled probe, it is essential to block the other areas on the blots that do not contain DNA of interest and are capable of binding single or double stranded DNA non-specifically. This nonspecific binding results in high background in the autoradiogram. Prehybridization with nonspecific DNA such as calf thymus DNA or Salmon sperm DNA serves this purpose. Southern blots or colony blots were prehybridized for 3-4 hours at 65 °C in a rotatory shaker containing prehybridization mixture.

Stock soluti on	Volume (ml)	Final concentration
20X SSPE	6.25	5X SSPE
100X Denhardt's	1.25	5X Denhardt's
10% (W/V) SDS	1.25	0.5 % SDS
H_2O	16.25	-

The prehybridization solution was prewarmed to 65 °C before the blots were placed in it. 2 ml of freshly boiled (denatured) calf thymus DNA (5 mg/ml) was added to the prehybridization mixture.

Hybridization

After hybridization, the solution was removed and replaced by hybridization solution (same as prehybridization mixture) containing labeled probe. The labeled probe was denatured by boiling for 5 min followed by chilling in ice for 5 min, before adding it to the hybridization mixture. The hybridization was undertaken at 65 °C, overnight with rotary shaking.

Washing of filters and autoradiography

After hybridization, the filters were washed for removal of the unbound or non-hybridized probes using solutions of different stringencies depending on the probe used for hybridization. The filters were washed twice at room temperature with a solution containing 2X SSC and 0.1 % SDS for 10 min. This was followed by a single hot wash at 65 °C with a solution containing 1X SSC and 0.1 % SDS for 5 min. The moist filters were wrapped with saran wrap and exposed to X-ray film for desired time at –70 °C, using vinyl X-ray cassettes with built in intensifying screens.

Identification of xylanase gene containing colonies

The recombinants were patched on LB plates containing xylan (1 %) and ampicillin (100 μg/ml). After 16 h incubation at 37 °C, the plates were layered with 1 % congo red solution and allowed to stand for 20 min. The excess congo red solution was carefully decanted without disturbing the colonies. The plates were checked for the possible clearance produced by the extracellular synthesis of xylanase. The plates were then overlaid with 0.4 % agarose containing 2 mg/ml lysozyme. After the plates were set, it was incubated at 37 °C to ensure the complete lysis of the recombinant colonies. They were then treated with 1 M NaCl to visualize the possible clearance due to intracellular xylanase expression. The plates were further incubated at 50 °C for six hours.

Preparation of intracellular extract

The intracellular xylanase activity was estimated from clones grown in 50 ml LB broth with ampicillin (60 μg/ml) at 37 °C for 16 h. Cells were separated by centrifugation at 4 °C, 10,000 rpm for 10 min. Pellet was washed twice with 50 mM phosphate buffer, pH 7 and resuspended in 2 ml of the same buffer. Cells were ruptured by sonication (Virsonic) for 90 s (30 s, three shocks) and cell debris was removed by centrifugation. Clear supernatant obtained (referred to as intracellular extract) and extracellular broth were used for checking the xylanase activity. The intracellular proteins were further concentrated by subjecting it to ammonium sulphate precipitation (90 % saturation). The precipitated proteins were resuspended in minimum volume of 0.05 M sodium phosphate buffer, pH 7.

RESULTS AND DISCUSSION

Isolation of *Thermomonospora* sp. genomic DNA

The quality of the genomic DNA was checked on 0.8 % agarose gel and was found to be high molecular weight, intact DNA (Figure 2). The spectrophotometric analysis of the DNA showed that the DNA had a A_{260}:A_{280} ratio of 2.0, which indicates the purity of the DNA preparation.

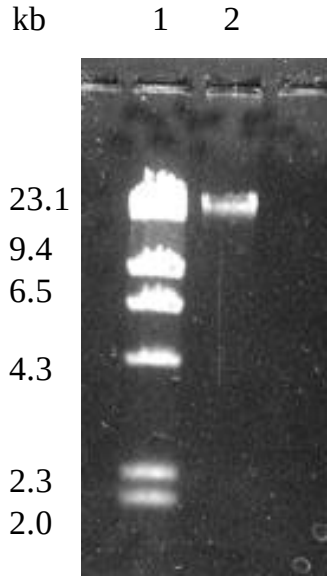


Figure 2: Genomic DNA isolated from alkalothermophilic *Thermomonospora* sp. Lane 1: Lambda *Hind*III marker, Lane 2: *Thermomonospora* sp. genomic DNA

Construction of the genomic library

The genomic DNA of *Thermomonospora* sp. was digested with *Eco*RI and separated on 0.8 % agarose gel. The fragments corresponding to 2-10 kb were eluted from the gel and were used for the construction of the genomic library. The vector pBS was linearised with *Eco*RI and was treated with alkaline phosphatase (Figure 3). The digested genomic DNA and dephosphorylated plasmid were mixed in a molar ratio of 2:1 and were ligated. The ligation mixture was transformed into competent *E. coli* XL1 cells. As a result of ten independent transformation reactions using 50 ng DNA for each transformation, 1300 transformants were obtained. Out of the 1300 transformants, 850 were white, indicating that the ratio of recombinants to non-recombinants was 2:1. The vector (pBS) contains the regulatory

sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lac*Z). These vectors are used in hosts (*E. coli* XL1) that code for the carboxy terminal of β-galactosidase. Neither the host encoded nor the plasmid encoded fragments are active by themselves but they can associate to form an active protein, which give a blue coloured product when it acts on the chromogenic substrate X-gal. The insertion of the foreign DNA into the plasmid at the multiple cloning site of pBS causes the formation of an amino terminal that is not capable of α-complementation and forms white colonies

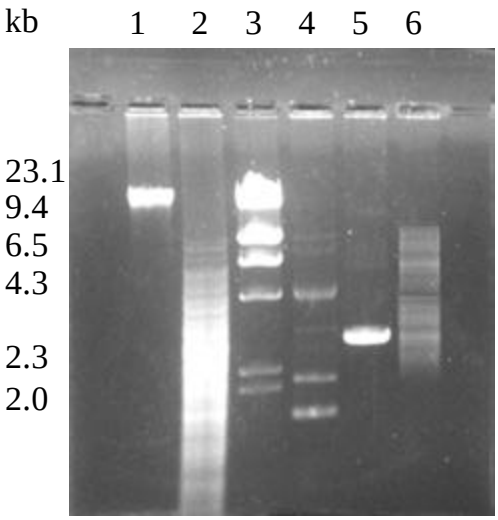


Figure 3: Agarose gel electrophoresis of *Eco*RI digested genomic DNA and the vector pBS. Well 1: *Thermomonospora* sp. genomic DNA, Well 2: *Eco*RI digested genomic DNA, Well 3: Lambda *Hind*III digest marker, Well 4: Unrestricted pBS, Well 5: *Eco*RI restricted pBS, Well 6: *Eco*RI digested and fractionated (2-10 kb) genomic DNA.

Analysis of genomic library

The genome size of *Thermomonospora fusca* is 3.656×10^3 kb, assuming similar genome size for *Thermomonospora* sp. the following formula for calculating the representativeness of the genome library was applied.

$$N=\ln (1-p)/\ln (1-1/n)$$

Where N=necessary number of recombinants

p=desired probability of obtaining a single colony recombinant (95 %)

n= size of the genome relative to a single cloned fragment $\frac{3.656 \times 10^3}{6} = 609$

$$N= \frac{\ln (1-0.95)}{\ln (1-1/609)} = 1823$$

Since the possibility of insertion of DNA into plasmid decreases with increasing size, the average insert size of 6 kb was taken for the calculations. The number of recombinants required to represent a single copy gene with 95 % confidence was found to be 1823, which is more than the number of recombinants obtained (850). The partial library was screened for recombinants harboring the xylanase gene fragment (Figure 4).

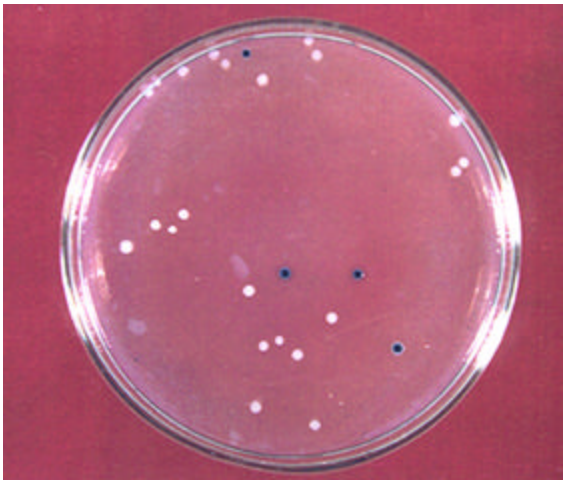


Figure 4: Identification of recombinants and non -recombinants. The recombinants appear as white colonies while the non-recombinants produce blue coloured colonies when grown on LB plate containing X-gal and IPTG.

The randomly selected recombinants were patched on a LB Amp plate with pBS transformants as a control. The ^{32}P labeled genomic DNA from *Thermomonospora* sp. was used as the hybridization probe. All the colonies showed positive signals of equal intensity, whereas the pBS transformant showed no hybridization. The results suggest that all the recombinant colonies had insert DNA from *Thermomonospora* sp. The plasmid DNA from eight representative recombinants was isolated and digested with *Eco*RI. The gel electrophoresis of the digested plasmids on 0.8 % agarose gel showed the presence of insert DNA in the range 2-10 kb (Figure 5), which is in agreement with the DNA size fractionation range.

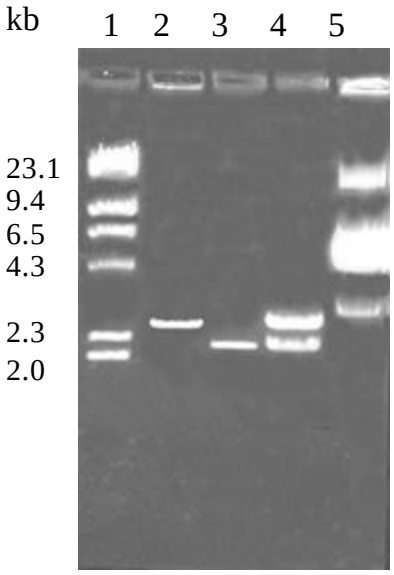


Figure 6: Purification of xylanase probe: Well 1: Lambda *Hind*III digest, Well 2: *Eco*RI restricted pUC18, Well 3: Probe fragment extracted from gel, Well 4: *Sal*I restricted pYY01, Well 5: pYY01.

Souther n hybridization of genomic digest with probe

To confirm whether the xylanase gene (2.19 kb) from *Thermomonospora fusca* would hybridize with the genomic DNA of *Thermomonospora* sp., the *Eco*RI and *Sal*I digested genomic DNA of *Thermomonospora* sp. were checked for hybridization with the probe. The xylanase gene hybridized with the *Eco*RI digested genomic DNA at a fragment of size 5.3 kb, while it hybridized with a 2 kb fragment in *Sal*I digested genomic DNA, confirming the presence of homology between their genomes (Figure 7). Therefore, the xylanase gene from *Thermomonospora fusca* was further used as a probe to screen the genomic library by colony hybridization.

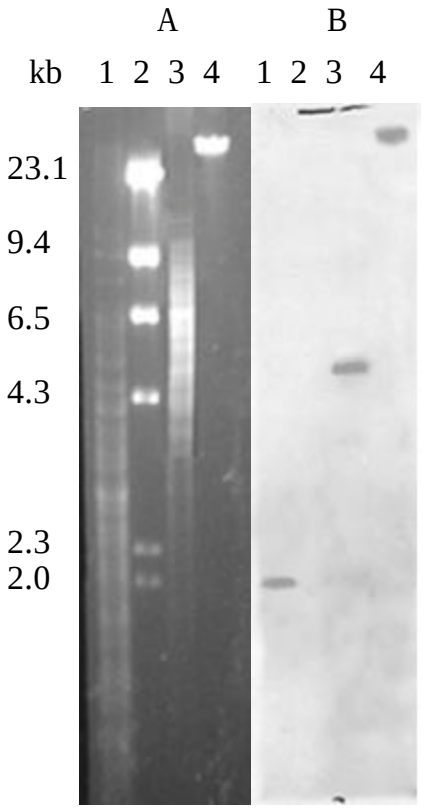


Figure 7: Southern blot analysis using 2.19 kb *Sal*I fragment from *Thermomonospora fusca* as probe. (A) Gel stained with ethidium bromide. Lane 1: *Sal*I digested genomic DNA of *Thermomonospora* sp., Lane 2: Lambda *Hind*III digest, Lane 3: *Eco*RI digested genomic DNA of *Thermomonospora* sp., Lane 4: Undigested genomic DNA. (B) Autoradiogram after 3 days exposure using xylanase probe from *Thermomonospora fusca*.

Analysis of Colony blots

The expression of a gene may not be under the control of β-galactosidase promoter or the fragment may not be in frame with the promoter or the orientation of the fragment may not be right. Under such conditions, the protein encoded by the gene will not be expressed. In such a case, the presence of a DNA fragment containing the desired gene can be effectively identified by using a labeled probe. The advantage of hybridization method is that it is independent of expression of the foreign DNA by the *E. coli* host. The colony blots representing the 450 clones were further challenged with labeled probe (pYY01 xylanase fragment) to screen for the presence of partial or complete xylanase gene. Washing the blots at

low stringency showed hybridization with ten colonies (Figure 8). These colonies were selected and further used to check for presence of both intracellular and extracellular xylanase.

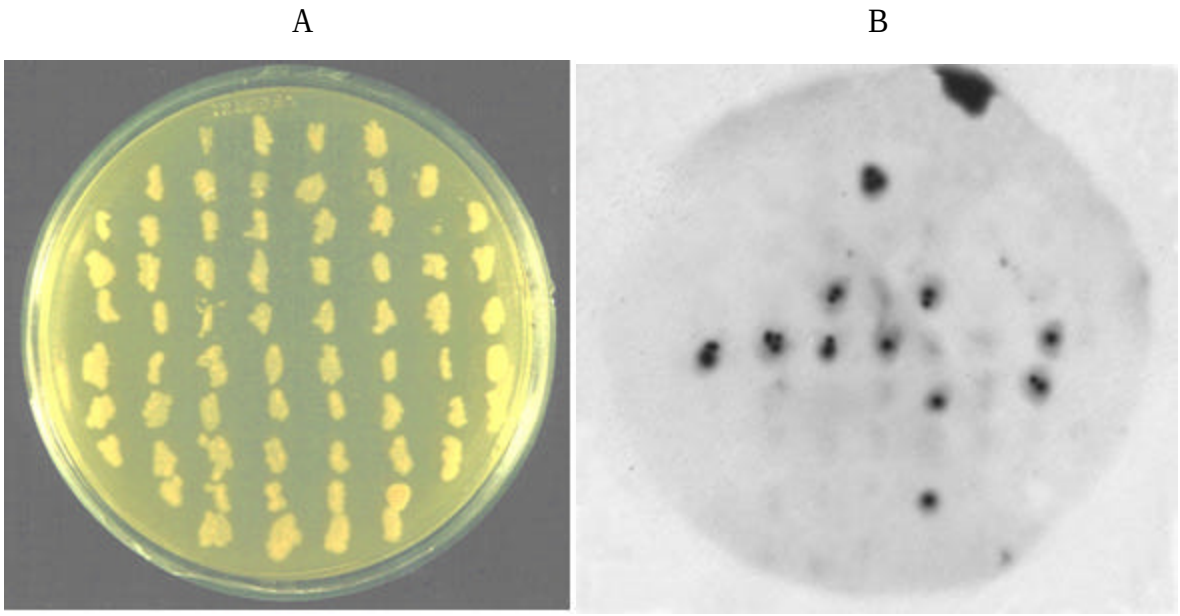


Figure 8: Colony hybridization of the recombinants with rad iolabeled xylanase probe. (A) Recombinants patched on LB amp plate. (B) Colony blots showing positive hybridization.

Identification of xylanase positive recombinants

A genomic library can be screened for the desirable gene either at the gene level by hybridization with a suitable probe, or with the help of the protein expressed by the desired gene. In case of xylanase the congo red plate assay can be used effectively to determine the presence of xylanase enzyme produced by a colony. Fifty colonies were spotted per plate at regular distance keeping a minimum distance of 0.5 cm from the edges of the plate. This ensured elimination of false positive reactions, which are commonly observed because of overcrowding. The *E. coli* XL1 harboring pBS plasmid was included as a negative control in each plate. Two recombinants showing clearance on xylan congo red plate consistently, in three independent colony clearance assays were picked up as xylanase positive recombinants (Figure 9). These recombinants also showed hybridization with the labeled probe. One of the *E. coli* recombinants harboring plasmid containing xylanase gene (pATA93) was used for further studies.

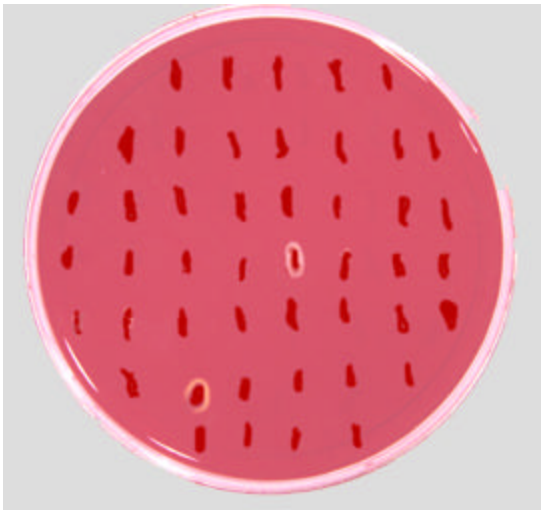


Figure 9: Xylan congo red plate clearance shown by two recombinants. The clearance was observed after incubation at 37 °C for 18 h followed by incubation at 50 °C for another 6 h. The other colonies are recombinants from the genomic library.

Restriction digestion of plasmid from xylanase positive clone

Plasmid pATA93 from the xylanase positive recombinant *E. coli* was isolated and digested with different restriction endonucleases. Digestion with *Eco*RI showed the presence of two bands. The 2.96 kb band corresponds to pBS vector, while the 5.3 kb fragment represents the insert (Figure 10). Restriction digestion of pATA93 with *Sal*I, *Pst*I and *Sma*I resulted in the formation of 4, 3 and 7 distinct fragments, respectively (Table 1). All the three restriction enzymes have a single restriction site in the multiple cloning site of pBS, therefore the vector will always be in a fragment which has a size equal to or greater than 2.96 kb. From Table 1, it can be concluded that the fragments 3.76, 5.6 and 3.56 produced by *Sal*I, *Pst*I and *Sma*I, respectively correspond to part xylanase fragment and the vector. *Sal*I, *Pst*I and *Sma*I have 4, 3 and 7 restriction sites, respectively in pATA93.

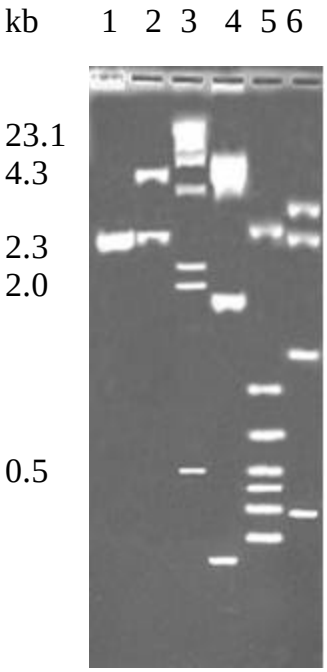


Figure 10: Restriction digestion of xylanase gene containing recombinant plamid pATA93 using different restriction enzymes. Well 1: *Eco*RI digested pBS, Well 2: *Eco*RI digested pATA93, Well 3: Lamda *Hind*III digest, Well 4: *Pst*I digested digested pATA93, Well 5: *Sma*I digested pATA93, Well 6: *Sal*I digested pATA93.

Table 1: Restriction fragments of pATA93 with different restriction enzymes

Fragment	Fragments obtained after restriction digestio n of plasmid pATA93 by			
	*Eco*RI	*Sal*I	*Pst*I	*Sma*I
1	2.96	3.768	5.6	3.56
2	5.3	2.886	1.859	1
3		1.272	0.792	0.8
4		0.434		0.565
5				0.500
6				0.451
7				0.393

Expression of xylanase gene

The xylanase positive clone containing the plasmid pATA93 was grown in LB broth for 16 h at 37 °C. Xylanase activity was not detected in the extracellular broth. Initial estimation of the intracellular cell extract did not show any xylanase activity, however ammonium sulphate precipitation (90 %) of the intracellular cell extract of the *E. coli* recombinant (pATA93) produced 10 mU/mg of intracellular xylanase.

Construction of genomic library and identification of the gene of interest are important prerequisites for gene cloning experiments. The present chapter has described the construction of the genomic library of *Thermomonospora* sp. in *E. coli* and the identification of a clone containing xylanase gene. Additional work like restriction mapping, subcloning, sequencing and overexpression of the xylanase gene can be undertaken. The present work forms the basis for further studies like site directed mutagenesis and gene shuffling, to improve the alkalistability or thermostability of enzymes. Hajime *et al*. (2000) have significantly increased the thermostability of *Streptomyces lividans* xylanase B by replacement of its N-terminal region with the corresponding region from *Thermomonospora fusca* xylanase A. They have also shown that random gene shuffling between a mesophilic enzyme and its thermophilic counterpart represents a facile approach for improving the thermostability of a mesophilic enzyme. Similar chimeric xylanases with improved thermostability have been constructed between family 10 xylanase FXYN from *Streptomyces olivaceoviridis* E-86 and XylA from *Thermomonospora alba* ULJB1 (Ahsan *et al*., 2001).

SUMMARY

The genomic library of *Thermomonospora* sp. was constructed in *E. coli* using standard approach of shotgun cloning. Genomic DNA fragments of size 2-10 kb were inserted at *Eco*RI site in pBS vector and transformed into *E. coli* XL1. Based on blue/white selection, 850 recombinant colonies were obtained with average insert size of 6 kb. The presence of xylanase gene was identified by hybridization of xylanase probe from *Thermomonospora fusca* with genomic DNA digest of *Thermomonospora* sp. Ten recombinant colonies containing xylanase gene were identified, on screening the genomic library with the xylanase probe. The genomic library was also screened using xylan congo red plate clearance assay and two clones containing the xylanase gene were confirmed. The xylanase gene containing plasmid was designated as pATA93. Preliminary characterization of pATA93 showed that it contained a 5.3 kb fragment with 4, 3 and 7 restriction sites for *Sal*I, *Pst*I and *Sma*I, respectively. The production of xylanase was intracellular (10 mU/mg), no extracellular xylanase was detected.

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