MOLECULAR ASPECTS OF XYLANASE FROM AN ALKALOTHERMOPHILIC *BACILLUS* SP. NCIM 59

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DECLARATION

This is to certify that the work incorporated in the thesis entitled **Molecular aspects of xylanase from an alkalothermophilic *Bacillus* (NCIM 59)"** submitted by Neeta Kulkarni - (Mrs. Rama K. Bhadekar) was carried out under my supervision at Biochemical Sciences Division, National Chemical Laboratory, Pune. Material obtained from other sources has been duly acknowledged in the thesis.

Mala Rao

Dr. Mala Rao

(Research Guide)

DEDICATED TO

MY PARENTS

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ABSTRACT

INTRODUCTION

The recent interest in biotechnology, coupled with the discovery of new, novel thermophiles and alkalophiles has prompted studies on the utilization of extremophiles and their enzymes for biotechnological purposes. Enzymes from extremophilic organisms are stable and active at high temperature and pH which are the prerequisites for their industrial application. Many advantages for the use of thermophiles in the biotechnology processes such as reduced contamination risk, faster reaction rates have been proposed. However , to date knowledge of alkalophilic life is almost entirely confined to certain members of the genus *Bacillus* . Many extracellular enzymes produced by alkalophilic bacilli, e.g. proteases, amylases, cellulases, pectinases , xylanases etc. have the potential application in commercial processes that operate under alkaline conditions since they exhibit optimal activity and stability at alkaline pH. Xylanases have recently attracted special attention as a means of converting biomass into energy and food resources or chemicals and also due to their potential application in pulp and paper industry. In view of the above applications, the xylanases from alkalophilic thermophilic *Bacillus* are potentially useful, and hence they were studied in greater detail.

Analysis of gene sequence is the first step for application oriented protein engineering studies. Several xylanases and their genes have been characterized from thermophilic bacteria. Also a few reports are available on xylanase gene sequences from alkalophilic bacteria. The alkalophilic thermophilic *Bacillus* (NCIM 59) (AT *Bacillus*) is isolated in our laboratory and secretes two cellulase free xylanases into the medium. The present studies deal with characterization of the recombinant xylanases I and II in *E. coli*. The isolation of the gene coding for XynII and its sequence analysis have also been described.

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The main features of the work done are:

1. Characterization of the recombinant xylanases I and II in *Escherichia coli* from AT *Bacillus*.
2. Expression of *xynII* in *E. coli.*
3. Extractive cultivation of recombinant *Escherishia coli using* aqueous two phase system.
4. Nucleotide sequencing of xylanase gene (*xynII*)
5. Application of xylanase from AT *Bacillus* in biobleaching of bagasse pulp.

SUMMARY OF THE WORK

1. Characterization of the recombinant xylanases from AT *Bacillus.*

The 4.5 kb gene fragment coding for xylanases from AT *Bacillus* has been cloned and expressed in *Escherichia coli* (Shendye and Rao, 1993, FEMS Microbiol. Lett., 108, 297-302). The extracellular xylanases produced by recombinant *E. coli* (pATBX 4.5) were purified to homogeneity. The Mr of the enzymes were estimated to be 35,000 (xylanase I) and 14,500 (xylanase II) by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified xylanases had similar temperature (60°C) and pH (6) optima and the isoelectric points were 4 and 8 respectively. The enzymes retained 100% activity at 50°C for 24 h however at 60^0C they showed half life of 2 h. The apparent Km values of xylanase I and II were 5.8 and 8.3 mg/ml and Vmax values were 0.010 and 0.520 μmol/min/mg respectively. The recombinant xylanases showed reduced ability to bind xylan and had lower specific activity and Km values than those of the xylanases from the parent *Bacillus* reported previously. Both the enzymes yielded different hydrolysis pattern when incubated with oat spelt xylan. The hydrolysis pattern of the recombinant xylanases was found to be distinctly different from that of the original xylanases which may be due to differential binding of the cloned enzymes to the substrate.

2. Expression of *xynII* in *E. coli*

The 4.5 kb gene fragment coding for two xylanases from AT *Bacillus* was subcloned. The recombinant *E. coli* harbouring 1.8 kb subfragment showed xylanase activity and immunoreactivity with the polyclonal antibodies raised against purified xylanase II from AT *Bacillus*. The immunoprecipitation of the extracellular extract of the recombinant pATBX 1.8 showed the presence of single protein band of Mr 14,300. The scanning electron microscopy of recombinant *E. coli* (pATBX 1.8) showed the presence of inclusion bodies. The treatment of *E. coli* (pATBX 1.8) cells with guanidine hydrochloride (Gdn/HCl) and Triton X-100 resulted in two fold increase in xylanase activity. However higher concentrations of Gdn/HCl and urea (1 to 8 M) caused irreversible denaturation of the enzyme.

3. Extractive cultivation of recombinant *E. coli* using aqueous two phase system

The recombinant *E. coli* (pATBX 1.8) secreting extracellular xylanase was used as a model system to study the application of aqueous two phase system for the extractive cultivation. An increase in the polymer concentrations from 6 to 20% in the polyethylene glycol - phosphate aqueous two phase system, resulted in an increase in the phase volume ratio with a concomitant decrease in the partition coefficient (K) and recovery of xylanase in the top phase. However varying phosphate concentrations from 8 to 16% decreased both the phase volume ratio and partition coefficient of xylanase. The polyethylene glycol (6%) and phosphate (12%) system was found to be optimum for extractive cultivation of *E. coli* where extracellular xylanase was selectively partitioned to the top phase giving a purification ratio of above 1.0 . The process was extended to semicontinuous operating mode at the optimal condition, wherein the top phase containing xylanase was recovered and the surviving cells were recycled together with the new top phase. The maximum recovery of xylanase was obtained after 12 h in the top phase with a two-fold increase in the specific activity as compared to the one obtained in the reference fermentation. The present chapter describes the use of two phase system for the extractive cultivation of recombinant *E. coli* (pATBX 1.8) with the purpose to obtain a simple and inexpensive separation procedure and to achieve the maximal extraction of xylanase to one phase.

4. Nucleotide sequence analysis of xylanase gene (*xynII*)

The nested deletions were carried out in recombinant plasmid (pATBX 1.8) using exonuclease III. A 1.0 kilobase gene fragment was found to code for a functional xylanase (XynII) in *E. coli*. The complete nucleotide sequence of the insert including the structural gene and the 5' and 3' flanking sequences has been determined (GenBank accession no. U83602). An open reading frame starting from ATG initiator codon comprising of 402 nucleotides gave a preprotein of 133 amino acids of calculated molecular weight 14.09 kDa. The molecular mass was in close agreement with that of the glycosylated mature protein from the parent *Bacillus* (15.8 kDa). The stop codon was followed by hairpin loop structures indicating the presence of transcription termination signals. The occurrence of three potential N-glycosylation sites in *xynII* gene was an unique feature especially of bacterial origin. The secondary structure analysis of XynII predicted that the polypeptide was primarily formed of β-sheets. XynII appeared to be a member of family G/11 of xylanases based on its molecular weight and basic pI (8.0). However, sequence homology revealed a striking similarity to family 10 xylanases. This article appears to be the first report linking the xylanases from neutrophilic and alkaliphilic organisms through the conserved motif (-SQTN-) that starts at an amino acid residue position 39 in XynII from alkaliphiic thermophilic *Bacillus.* The conserved triad (-VVX- where X is N or D) identified only in the xylanases from alkaliphilic organisms may play an important role in determining the functional conformation and activity at alkaline pH. Our results implicate the concept of convergent evolution for XynII and provide basis for research in evolutionary relationship among the xylanases from alkaliphilic and neutrophilic organisms.

5. Application of xylanase from AT *Bacillus* in biobleaching of bagasse pulp

The use of hemicellulolytic enzymes has recently attracted considerable interest as substitutes for chlorine chemicals in pulp bleaching due to the environmental concerns. The cellulase free xylanase from AT *Bacillus* was evaluated for prebleaching of the bagasse pulp. The U V absorption spectrum of the compounds released by enzyme treatment and after alkali extraction showed a characteristic peak at 280 nm indicating the presence of lignin in the released coloring matter. The

material released after enzyme treatment also absorbed strongly at 237, 280 and 465 nm. The biotreatment resulted in 2 units decrease in kappa number without altering the strength properties of the pulp. Subsequently the peroxide bleaching of the enzyme treated samples resulted in decrease in kappa number by 10 units and increase in the brightness by 2.5%. The viscosities of xylanase treated samples were found to be unaltered.

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CHAPTER I

GENERAL INTRODUCTION

Xylan is the major constituent of hemicellulose and is the second most abundant renewable resource with a high potential for degradation to useful end products. Hence the development of inexpensive technologies based on hemicellulose is called for. Eventually, if not in the near future, xylan, in combination with cellulose, will supply most of the global demand for raw materials. It is not unrealistic to foresee that coal and crude oil are likely to be substituted by biomass in another fifty years (Goheen, 1981). Microbial xylanases (1,4-β- D xylan xylanohydrolase EC 3.2.1.8) are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, and negligible substrate loss and side-product generation. Most of the bacteria and fungi secrete extracellular xylanases which act on the hemicellulosic material to liberate xylose as a directly assimilable end product allowing the organisms to grow heterotrophically on xylan. Ruminal microorganisms are known to be potent xylanase producers, possibly due to high dietary hemicellulose content of the feed of ruminant animals. The use of hemicellulolytic enzymes as a substitute for chlorine chemicals in pulp bleaching has recently attracted considerable interest because of environmental concerns. The limited hydrolysis of hemicellulose in pulps by hemicellulases, mainly xylanases, increases the extractibility of lignin from the kraft pulp in the subsequent bleaching sequences, reducing the chloro-organic discharges.

STRUCTURE OF XYLAN

The β-1,4-xylans are heteropolysaccharides with a homopolymeric backbone chain of 1,4-linked β-D-xylopyranose units. The backbone consists of O-acetyl, α -L-arabinofuranosyl, α -1,2-linked glucuronic or 4-O-methylglucuronic acid substituents (Aspinall, 1959). However, unsubstituted linear xylans have been isolated from guar seed husk, esparto grass and tobacco stalks (Eda et al, 1976). Wood xylans exist as O-acetyl-4-O-methylglucuronoxylans in hardwoods or as arabino-4-O-methylglucuronoxylans in softwoods. The degree of polymerization of hardwood xylans (150-200) is higher than that of softwoods (70-130) (Timell, 1965). The cereal xylans are made up of D-glucuronic acid and/or its 4-O-methyl ether and arabinose (Brillouet and Joseleau, 1987). Endospermic arabinoxylans of annual plants, also called pentosans, are more soluble in water and dilute alkali than xylans of lignocellulosic materials because of their branched structures (Ferreira-Filho, 1994). Based on the common substituents found on the backbone, xylans are categorised as linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan. However in each category

there exists a microheterogenity with respect to the degree and nature of branching.

INDUCTION

Despite the increase in knowledge of microbial xylanolytic systems in the past few years further studies on induction and secretion of xylanases are necessary to develop efficient xylanase producers for possible commercial applications. Xylanase production by various bacteria and fungi has been shown to be inducible. But rare examples of constitutive xylanase expression have also been reported (Srivastava and Srivastava, 1993). The substrate derivatives and the enzymatic end products may often play a key positive role in the induction of xylanases; they can also act as the end-product inhibitors, possibly at much higher concentrations. Xylan, being a high molecular weight polymer, cannot penetrate the cell wall. The low molecular weight fragments of xylan play a key role in the regulation of xylanase biosynthesis. These fragments include xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers. These molecules are liberated from xylan by the action of small amounts of constitutively produced enzyme. Sugarcane bagasse is found to be the best inducer of xylanase and β-xylosidase in *Cellulomonas flavigena* (Perez-Avalos et al, 1996). A synergistic effect on the synthesis of both the enzymes was observed when cellulose and hemicellulose were used together as the carbon source.

The xylanase from *Aspergillus* sp. (2MI), induced by pine xylan, exhibited higher stability than that induced by other xylans (Raquel et al, 1997). In the presence of xylose higher enzyme yields were obtained from *Bacillus pumilus* (Paul and Varma, 1990), *S. lividans* 66 (Kluepfel et al, 1990) and *Aureobasidium pullulans* (Leathers et al, 1984). However, in *Cryptococcus albidus* (Biely, 1985) xylose repressed xylanase production while in thermophilic actinomycetes (McCarthy et al, 1985) it had no influence on the regulation of xylanase expression. Xylobiose was found to be a specific inducer of xylanase in *Trichoderma reesei* (Hrmova et al, 1986) and *Aspergillus terreus* (Hrmova et al, 1991). Xylanolytic systems of yeasts and fungi were also induced by positional isomers of xylobiose. In *Cryptococcus albidus* the positional isomers of xylobiose behave differentially from xylobiose (Biely and Petrakova,1984) in that the response of the cells to them is slower, however, the enzyme yields are higher in the presence of isomeric xylobioses indicating that they are not direct inducers. D-xylan fragments, as well as

methyl β-D-xylopyranoside, induce xylanase in *Streptomyces* sp. (Marui et al, 1985) and in the yeasts of the genus *Cryptococcus* and *Trichosporon* (Yasui et al, 1984, Hrmova et al, 1984).

Regulation at molecular level

Xylanase biosynthesis and the phenomenon of enzyme induction at the molecular level are comparatively less investigated. Esteben et al (1992) have reported that xylanase was undetected in glucose grown cultures of *Bacillus circulans* WL-12 but xylose, mannose and cellobiose supported xylanase production. The xylanase and xylosidase in *Butyrivibrio fibrisolvens* GS 113 are shown to be under coordinate control, induced by xylan and repressed by glucose (Utt et al, 1991). An analysis of DNA fragments containing β xylanase genes from *B. pumilus* (Moriyama et al, 1987a) indicated that the xylanase and xylosidase genes are closely associated and are linked in a 14.4 kbp DNA fragment. However, they did not appear to be controlled by the same operon.

Catabolite repression

Catabolite repression by glucose is a common phenomenon observed in xylanase biosynthesis. Relatively few reports on the relation of cAMP to xylanase induction are available. In the case of yeast *C. albidus,* when xylan or methyl β -xylopyranoside were used as inducers, cyclic AMP caused two fold increase in xylanase production (Morosoli et al, 1989). However, cyclic AMP had no effect on the repression caused by D-xylose. It has been suggested that a 15-bp nucleotide sequence, upstream from the β -xylanase gene, may be a part of the cyclic AMP regulatory sequence. In *Aspergillus tubigensis,* a 158 bp region, 5' upstream from the xylanase gene, was shown to be involved in xylan specific induction (De Graff et al, 1994). The authors have also reported that catabolite repression of xylanase gene appeared to be controlled at two levels, directly by repression of gene transcription and indirectly by repression of transcriptional activator. The same pattern of regulation was observed in *A. niger* and *A. nidulans*. Mach et al (1996) have studied the carbon catabolite repression of xylanase gene expression. and have analysed the molecular basis for the absence of xylanase I formation by filamentous fungus *T. reesei*. They have concluded on the basis of northern blot analysis that the repression of basal xyn 1 transcription was mediated by the carbon catabolite repressor protein Cre 1. Cre 1 *in vivo* binds to two of four consensus sites (5'-SYG-GRG-3') in the xyn 1 promoter, which occurred in the form of

an inverted repeat .

Deletion and functional analysis of the xylanase genes from *A. niger* and *A. tubigensis* (De Graff et al, 1992) led to the discovery of a triplicated sequence that appears to control the enzyme induction. Gat et al (1994) have demonstrated that, in the case of *B. stearothermphilus* T-6, induction of xylanase synthesis by xylose was controlled at the transcriptional level, indicating the presence of a repressor protein mediating the regulation. Enzyme synthesis was also found to be repressed when easily metabolizable carbon sources were present in the growth medium, suggesting that the synthesis of the enzyme is controlled by transition state regulators and catabolite repression.

XYLANASES FROM ALKALOPHILIC MICROORGANISMS

Commercial applications of the xylanases demand identification of highly stable enzymes active under routine handling conditions. In general, parameters such as temperature, pH, and chemical and enzymatic stability are important for the industrial applicability of any enzyme. One of the ways to identify the industrially suitable xylanase preparations is to look for the enzymes from extremophilic microorganisms. The use of biocatalysts has been constrained due to their labile nature under extreme temperature and pH conditions. The study of extremophiles and their enzymes can extend the present understanding of protein chemistry in addition to expanding the potential applications of biocatalysts.

Studies of alkaliphiles have led to the discovery of a variety of enzymes which exhibit some unique properties. Alkaline xylanases have gained importance due to their application for the development of ecofriendly technologies used in paper and pulp industries. The enzymes are able to hydrolyze xylan which is soluble in alkaline solutions (Horikoshi, 1996). The first report on xylanase from alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa. The purified enzyme of *Bacillus* sp. C-59-2 exhibited a broad pH optimum ranging from 6.0 to 8.0. Many of the xylanases produced by alkaliphilic microorganisms such as *Bacillus sp.*(Okazaki et al, 1984) and *Aeromonas sp.* 212 (Ohkoshi et al 1985) with optimum growth at pH 10.0 showed remarkable stability at pH 9-10 but were not highly active above pH 8.0. The enzymes from *Bacillus sp.* TAR-1 (Nakamura et al, 1994), C-125 (Honda et al, 1985a) and alkaliphilic *Bacillus* sp (NCL-86-6-10) (Balakrishnan et al, 1992) were optimally active at pH 9-10.

Recently an alkali-tolerant xylanase from *Aspergillus fischeri* (Chandra and Chandra, 1996) was reported to exhibit remarkable (pH 9.0) stability. The xylanase from *Cephalosporium* was the only one reported from an alkalophilic fungus having activity at broad pH range of 6.5-9.0 (Bansod et al, 1993).

THERMOPHILIC XYLANASES

The xylanases from thermophilic bacteria such as *Thermonospora fusca* (McCarthy et al, 1985), thermophilic *Bacillus sp.* (Gruninger and Fiechter, 1986) and *Bacillus stearothermophilus* (Khasin et al, 1993) show an optimum temperature in the range of 65 to 80^0 C. The thermostable xylanase produced by a thermotolerant *Aspergillus* strain at 37^0 C showed maximum activity at 80^0 C (Mendicuti et al, 1997). Xylanase A from the thermophilic anaerobe *Clostridium stercorarium* has a temperature optimum of 70^0 C and a half life of 90 min at 80^0 C, whereas the xylanase from *Thermotoga sp.* has been shown to have temperature optimum of 105^0 C at pH 5.5 with a half life of 90 minutes at 95^0 C (Simpson et al, 1991). Xylanase from *Dictyoglomus sp.* exhibited a half life of 80 min at 90^0 C (Mathrani and Ahring, 1991). Among the thermophilic fungi, xylanase from *Thermoascus aurantiacus* has been reported to be stable at 70^0 C for 24 h with a half life of 54 min. at 80^0 C (Yu et al, 1987). The other thermophilic fungi producing thermostable xylanases include *Paecilomyces variota* (Krishnamurthy and Vithayathil, 1989) and *T. byssochlamydoides* (Yoshika et al, 1981) which show an optimum temperature of 65 to 75^0 C at pH 5 to 6.5. Recently endoxylanases from thermophilic actinomycete *Microtetraspora flexuosa* SIIX were reported to have optimum temperature of 80^0 C, at pH 6.0 (Berens et al, 1996). Despite the prevalence of xylan degrading enzymes in actinomycetes comparatively little information is available about xylanases from other groups of thermophilic actinomycetes such as *Microbispora, Saccharomonospora,* etc. Table I.1 describes a few of the extremophilic organisms producing xylanase.

Table I.1

Xylanases from extremophilic organisms

Source	Xylanase	Optimum conditions				Reference
		Growth		Activity		
		pH	Temp. (°C)	pH	Temp. (°C)	
Thermophilic bacteria						
Bacillus acidocaldarius		3.5-4.0	65	4.0	80	Uchino and Fukuda ,1983
Bacillus licheniformis A 99		7.0	60	7.0	50	Archana and Satyanarayana , 1997
Bacillus stearothermophilus T-6	T 6	7.0-7.3	60	9.0	65	Khasin et al ,1993
Bacillus stearothermophilus No.21	A	7.0	55	7.0	60	Nanmori et al ,1990
Bacillus thermoalkalophilus		9.0	60	6.0-7.0	60 , 70	Rajaram and Varma ,1990
Clostridium acetobutylicum ATCC 824	A	6.0	37	5.0	50	Lee et al , 1987
	B		37	5.5-6.0	60	
Clostridium stercorarium HX-1	D	6.0-7.0	60	6.5	75	Sakka et al ,1991
Clostridium stercorarium F-9	A	6.0-7.0	65	6.5	75	Berenger et al ,1985
Clostridium thermolacticum(TC21)		6.0-7.0	65	6.5	80	Gosselin et al ,1992

Table I.1 contd.

Dictyoglomus thermophilum Strain B1		7.0	68	7.0	80	Mathrani and Ahring , 1992
Microtetraspora flexuosa S II X		6.0	80	9.0	52	Berens et al ,1996
Thermophilic Bacillus Strain XE		7.0	55	6.0	75	Gosselin et al ,1992
Thermophilic Bacillus sp		7.0	65	6.5-7.0	78	Gruninger and Fiechter ,1986
Thermophilic bacteria ITI 283, ITI 236		7.5	65	8.0	80	Perttula et al ,1993
Thermoanaerobacterium sp. JW/SL -YS485		6.0	60	6.2	80	Shao et al ,1995
Thermotoga sp. (Fjss3-B.1)	A	6.8-7.0	80	5.3	105-110	Simpson et al ,1991
Thermotoga maritima (MSB 8)	A	7.0	80	6.2	92	Winterhalter and Liebl ,1995
	B		80	5.4	105	
	2			7.2	75	
	3			6.8	75	
Thermomonospora chromogena MT814		8.0	50	5.0-8.0	75	McCarthy et al ,1985
Thermomonospora fusca BD 21		8.0	50	6.0-8.0	65	Bachmann and McCarthy , 1991
Thermomonospora fusca YX		8.0	50	6.0-8.0	70	Irwin et al ,1994

Table I.1 contd.

Streptomyces thermoviolaceus OPC-520	I	7.0	50	7.0	70	Minami et al ,1992
	II		50	7.0	60	
Thermophillic fungi						
Gloephyllum trabeum			-	4.0	80	Ritschkoff et al ,1994
Talaromyces byssochlamydoides YH- 50		6.2	50	5.0	70	Yoshioka et al , 1981
Thermoascus aurantiacus		6.0	45	5.0	75	Yu et al , 1987
Thermomyces lanuginosus DSM5826		6.5	50	6.5	50	Gomes et al ,1993
Talaromyces emersonii CBS 814.7	II	4.5	45	4.2	78	Tuohy and Coughlan ,1992
	III	4.5	45	3.5	67	
Alkalophilic bacteria						
Alkalophilic Bacillus 41M-1		10.3	37	9.0	50	Nakamura et al ,1994
Bacillus sp. TAR-1		10.5	50	9.0	70	Nakamura et al ,1994
Bacillus sp. C-59-2		8.0	37	5.5-9.0	60	Horikoshi and Atsukawa ,1973
Bacillus sp. C-125		10.5	-	6.0-10.0	70	Honda et al ,1985c
Bacillus sp. NCIM 59		10.0	50	6.0	50-60	Dey et al , 1992
Aeromonas sp. 212		10.0	37	7.0-8.0	50	Kudo et al , 1985
Bacillus sp. NG-27		9.0-10.0	27	7.0,8.4	70	Gupta et al ,1992

Table I.1 contd.

Bacillus sp.		9.0-10.0	45-50			Okazaki et al ,1984
W1	I			6.0	65	
	II			7.0-9.0	70	
W2	I			6.0	65	
	II			7.0-9.5	70	
W3				6.0	65	
W4				6.0-7.0	70	
Bacillus NCL -87-6-10		9.5	28	8.0	60	Balakrishnan et al ,1992

CLONING AND EXPRESSION OF XYLANASE GENE(S)

The bacterial genes encoding xylan-degrading enzymes have been found to be adjacent on the genome or in close proximity to other genes encoding cellulase - related functions. In *P. fluroscens subsp. cellulosa*, xylanase and arabinofuranosidase genes were transcribed in the same direction and were separated by only 148 bp (Kellett et al, 1990), while the second xylanase gene mapped to within 125 bp of the endoglucanase gene.

Heterologous cloning

The xylanase genes have been isolated from different microbial genera and expressed in *E. coli*. Cloning and expression of a xylanase gene from the extreme thermophile *Dictyglomus thermophylum* Rt46B.1 in *E. coli* has been reported (Gibbs et al, 1995); the recombinant enzyme was found to have an optimum temperature of 85^0 C. The xylanase cloned from *Thermotoga maritima* showed an optimum temperature of 90^0 C at pH 5.5 and was stable upto 100^0 C (Chen et al, 1997). The recombinant xylanase from *Thermotoga neapolitana* was found to be stable at 90^0 C for 4h with a half life of 2 h at 100^0 C. (Veoikodvorskaya et al, 1997). The latter had an optimum temperature of 102^0 C at pH 5.5. Studies on the cloned xylanase from *Clostridium thermocellum* F1 revealed that it was optimally active at 80^0 C and was stable upto 70^0 C at neutral pH and over the pH range of 4 to 11 at 25^0 C (Hayashi et al, 1997). Keen et al (1996) have

reported the cloning of a xylanase gene from corn strains of *Erwinia chrysanthemi*. Sequence analysis revealed that the leader peptide of the protein was unusual and long. The protein was found to be distinct from xylanases belonging to glycohydrolase families 10 and 11 and appeared to be intermediate between families 5 and 30. The XYN 2 gene from *T. reesei* QM6a (la Grange et al, 1996) was expressed in yeast *Sacchromyces cerevisiae* under the control of alcohol dehydrogenase (ADH 2) and phosphoglycerate kinase (PGK 1) gene promoters and terminators, respectively. The recombinant strains produced 1200 and 160 nkat of xylanase activity per ml, respectively, under the control of ADH 2 and PGK 1 promoters. The recombinant xylanase had an optimum temperature of 60^0 C at pH 6 and retained more than 90% of its activity after 60 minutes at 50^0 C. Recently Graessle et al (1997) reported a system for regulated heterologous gene expression in the filamentous fungus *Penicillium chrysogenum*. The heterologous expression system was developed by placing the encoding sequences under the control of the repressible acid phosphatase gene promoter of *P. chrysogenum*.

As against the wealth of information available on the xylanase gene cloning of bacterial systems, very few reports are to be found on fungal systems. Only recently some detailed reports on the cloning and expression of xylanases from the fungi *Aspergillus kawachii* (Ito et al, 1992), *Trichoderma reesei* (Torronen et al, 1992), *Neocallimastix patriciarum* (Lee et al, 1993) and *Orpinomyces* PC-2 (Li et al, 1996a) have appeared. The expression of fungal xylanase in *E. coli* and rumen bacterium *Butyrivibrio fibrisolvens* OB 156 using putative xylanase promoter from *B. fibrisolvens* strain 49 has been reported (Xue et al, 1997).

Overexpression in *E. coli*

A thermostable xylanase from *C. saccharolyticum* has also been cloned and overexpressed in *E. coli* . The enzyme expressed by the recombinant had a thermal half life of 2-3 minutes at 80^0 C (Luthi et al, 1990). Although this enzyme cannot fit into the ideal criteria for industrial application, the isolated gene itself could prove a starting material for mutagenesis to further improve the properties of the engineered enzyme. Recently Koo et al (1996) have reported overexpression of xylanase from *Clostridium thermocellum* in *E. coli.* The recombinant enzyme had an optimum temperature of 60^0 C at pH 5.4. High level expression in *E. coli* has also been obtained with the modified domain II construct of xylanase c DNA from an anaerobic

fungus *Neocallimastix patriciarum*. The modified domain II xylanase produced in *E. coli* had a specific activity of 1229 U/mg protein. The high level expression was largely attributed to the presence of a favourable N-terminal coding sequence (Xue et al, 1995). Xue et al (1996) have reported temperature-regulated expression of recombinant xylanase from *N. patriciarum* in *E. coli* strain containing natural *lac* I gene under the control of *tac* promoter. The specific activities of recombinant xylanase and cellulase were 4.5 times higher at 42^0 C than those obtained at 23^0 C. Xylanase A from the extremely thermophilic eubacterial strain Rt8B.4 was overexpressed in *E. coli*. The xylanase activity from domain 2 was associated with a 36-kDa protein, which was stable at 70^0 C for at least 12 h at pH 7.0 (Dwivedi et al, 1996) .

Expression in plant system

In a recent report Helbers et al (1995) have shown the high level expression of the thermostable xylanase from *Clostridium thermocellum* (xylanase Z- truncated protein) into the transgenic tobacco plants. The xylanase gene was introduced into the tobacco plant by an integration system with *Agrobacterium tumefaciens*. The protein molecules were correctly targeted into the intracellular space with the help of the signal peptide from the proteinase II. Although the active enzyme was synthesized inside the tobacco cells, it did not harm the host cells due to either the high temperature optimum of the enzyme or the relative scarcity of the substrate molecules in the plant cell wall. The response of the transgenic plant to pathogenic stimuli has been shown to be mediated through the xylanase and other related enzymes secreted by the pathogen and hence the transgenic tobacco plants producing high levels of the xylanase from *C. thermocellum* assumes a special significance. Xylanase B (Xyn B) from *C. stercorarium* is also expressed in tobacco suspension cells (Nicotiana tabacum L.cv BY2 cell) under the control of CaMV 35S promoter and noparin synthetase terminator (Ohmiya et al, 1997). The amount of expressed Xyn B protein was also around 4-5% of total proteins in the soluble extracts of tobacco suspension cells.

Homologous cloning

Several heterologous proteins cannot be efficiently expressed in *E. coli* due to any one of several reasons, such as the relatively abundant occurrence of rare codons in the cloned gene, the need for specific post-synthetic modification(s), structural complexity of the protein, toxicity of the coded protein to the host cells, and susceptibility of the foreign protein to proteases coded by *E. coli*. An *Aspergillus*

nidulans multicopy tranformant for the gene xylanase B coding for minor xylanase has been constructed recently (Espinar et al, 1996). The transformant was reported to secrete 114 U of xylanase/ mg protein.

The xylanase gene of *S. lividans* # 1326 was cloned by functional complementation of the xyn⁻ mutant of *S. lividans* using multicopy plasmid PIJ 702; a maximum enzyme production of 380 U/ml was reported (Mondou et al, 1986). Thus the cloning of a xylanase gene into a homologous system not only allowed excellent secretion but also yielded 60 times higher enzyme activity than that of the wild type. Iwasaki et al (1986) have also reported the molecular cloning of a xylanase gene from another strain of *Streptomyces* ; the recombinant showed maximum activity of 2830 U/ml of culture broth. The effects of signal peptide alterations and replacement on export of xylanase have been reported in *Streptomyces* (Page et al, 1996).

Cloning suitable for direct application

The cloning and expression of xylanases in non xylanolytic organisms has been largely restricted to microorganisms such as *Saccharomyces*, *Kluveromyces, Lactobacillus* and *Bacillus subtilis.* These organisms have been well characterized as far as their industrial applicability is concerned. The xylanase produced by the recombinant *L. plantarum* was able to release the fermentable carbohydrates from the ensiled crops and thereby improve the silage quality . *Saccharomyces* has been used to express the xylanases from *Aspergillus* and *Cryptococcus*. Recently cloning and expression of xylanase genes from *Meripilus giganteus, Myceloipthora thermophilum* and *Thielvavia terrestris* in *Aspergillus oryzae* have been reported (Sandal et al, 1996, 1997a, b), illustrating the examples of heterologous cloning of xylanase genes. The application of recombinant xylanase in the food and paper industries has also been described. Walsh and Bergquist have reported the expression of xylanase (Xyn A) from an extremely thermophilic anaerobe *Dictyoglomus thermophilum* Rt 46 B.1 in the yeast *Kluyveromyces lactis* (Walsh and Bergquist, 1997). The gene was fused in frame with the secretion signal of the *K. lactis* killer toxin in episomal expression vectors. Xyn A was secreted predominantly as an unglycosylated protein comprising of 90% of the total extracellular proteins. Also xylanase from thermophilic bacterium *Caldicellulosiruptor saccharolyticus* was secreted to a level of 10μg/ml in the same yeast. The reports on the expression of the cloned xylanase genes in heterologous host systems with relevant application potential are summarized in Table I.2

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Table I.2 : Cloning of xylanase genes in to hosts suitable for direct application

PARENT STRAIN	HOST	VECTOR	REFERENCE
Aspergillus Kawachii	*S. cerevisiae*	pVT 100	Ito et al ,1992
A. pullulans	*S. cerevisiae*	pYES 2	Li and Ljungdahl , 1996b
Clostridium acetobutylicum	*L. plantarum*	pWP 37	Scheirlinck et al ,1990
C. thermocellum	*L. plantarum*	pWP 37	Scheirlinck et al ,1990
Cryptococcus albidus	*S. cerevisiae*	pVT 100	Morosoli et al ,1992
	Pitchia stipitis	pJHS	
C. saccharolyticum	*S. cerevisiae*	pFLAGU 2	Donald et al ,1994

PROTEIN ENGINEERING

Protein engineering is one of the principal means of examining the active site of an enzyme to identify the roles of specific residues in catalysis; site- directed mutagenesis provides the technology required to redesign the protein. The identification of active site residues by chemical modification, X-ray crystallographic data and site-directed mutagenesis has provided the basic information regarding the structure- function correlation of the xylanases. These studies have formed the basis for the protein engineering of xylanases for specific manipulation of the gene for desired enzymatic properties.

Amino Acid modification

The participation of tryptophan in the active site of xylanases from *Chainia* (Deshpande et al, 1990) and *Streptomyces* (Keskar et al, 1989) has been reported. The fluorometric analysis of the xylanases from *Chainia* (Bandivadekar and Deshpande, 1996) and alkaliphilic thermophilic *Bacillus* (Nath and Rao, 1998) revealed that the tryptophan microenvironment was electronegative. Chemical modification of xylanases from the fungus *Schizophyllum commune* (Bray and Clarke, 1990) and an alkaliphilic thermophilic *Bacillus* sp.(Chauthaiwale and Rao, 1994) indicated the involvement of carboxyl groups in the catalysis. Evidence for the specific interaction of guanidine hydrochloride with the essential carboxyl group of xylanase from an alkaliphilic thermophilic *Bacillus sp.* has also been presented (Chauthaiwale and Rao, 1993). The

presence of cysteine in the active site of a few bacterial xylanases has been reported (Deshpande et al, 1990, Keskar et al, 1989). The characterization and sequencing of the cysteine containing active site peptide of the xylanase from *Streptomycs* T7 (Keskar et al, 1992) and *Chainia* have been reported. The peptides showed the presence of a conserved aspartic acid residue consistent with the catalytic regions of other glucanases.

X-ray crystallography studies

The three-dimensional structures of low molecular weight xylanases (family 11, Mr 20 kDa) from *B. pumilus* (Katsube et al, 1990), *B. circulans* , *T. harzianum* (Campbell et al, 1993), Thermophillic *Bacillus sp.* (Pickersgill et al, 1993) and *B. stearothermophilus* T-6 (Anna et al, 1997) have been reported. These studies have helped to determine the overall structure of xylanases, in possible identification of specific residues involved in substrate binding and catalysis. Crystallization and diffraction analysis of xylanases have been carried out at 1.5 - 3.0 Å. In *B. pumilus* IPO (Moriyama et al, 1987b). The enzyme molecule is of ellipsoidal shape (40 x 35 x 35 Å) with a well-defined cleft down one side of the molecule. However, the crystals of two major xylanases from the fungus *T. reesei* (Torronen et al, 1993) are reported to be monoclinic and those of *T. harzianum* to be orthorhombic (Rose et al, 1987). The crystallography studies of xylanase I from *A. niger* indicated a characteristic fold which is unique for family 11 xylanases (Krengel and Dijkstra, 1996). The enzymes of family 11 are single domain proteins composed of three antiparallel β sheets and one α -helix, with the active site lying between the second and third sheet. However, the three-dimensional structure of xylanase II from *T. reesei* (Torronen et al, 1994) has revealed that the β sheet structure is twisted, forming a large cleft on one side of the molecule. In the case of *T. harzianum* the xylanase contains two extra strands at the beginning of sheets I and II and a few insertions and deletions. The observed crystal structure of xylanase from *B. circulans* is in close agreement with the NMR-derived secondary structure of the protein (Plesniak et al, 1996a). In addition to conserved residues in the active site cleft of xylanases from *B. circulans* (Wakarchuk et al, 1994b) there are a number of other residues conserved on either side of the cleft. The three-dimensional structures of the xylanases from *T. harzianum* and *B. circulans* were found to be very similar. Many of the conserved amino acids of xylanases are believed to be structurally important for confirming the correct folding and packing. The putative

catalytic residues Glu 86 and Glu 177 of Xyn II from *T. reesei* are conserved. Also, a clear cluster of conserved residues consisting of Gln 136, Tyr 77 and Tyr 88 is observed around Glu 86. The hydrogen bond between Try 171 and Tyr 77 exists in other xylanases although the tyrosine residue is substituted by histidine in a few cases. However, the residues around Glu 177 are much less conserved. In addition to these amino acids, three residues, Pro 98, Asn 124 and Thr 133, are conserved although their role is not clear. It is speculated that they may take part in substrate binding. The flat Ser/Thr/ face of β-sheet A is also conserved in family 11 whose functional role may, to a certain extent be similar to that of cellulose-binding domains present in many cellulases (Torronen et al, 1994).

The three-dimensional structures of the catalytic domain of a few family 10 enzymes have been solved. These are xylanase/exoglucanse (Cex) from *C. fimi* , xylanase A from *P. fluorescens* , xylanase Z from *C. thermocellum* and xylanase from *S. lividans* . They all have an eight-fold α/β -barrel structure in which conserved glutamates function as catalytic nucleophile and acid/base catalytic residues. The presence of active site residues, Glu 127 on strand 4 and Glu 246 on strand 7 have been demonstrated in xylanase A from *P. fluroscens* subsp *cellulosa*. The three bulge-type distortions occurring on β strands 3,4, and 7 seem to be functionally significant as they serve to orient important active site residues. Recent crystal structure analysis revealed the presence of the Ca-binding site (located in loop 7) in xylanase A . This is the only xylanase reported to contain a Ca binding site. The authors have suggested that the occupation of Ca-binding loop with its ligand protected the enzyme from thermal inactivation, thermal unfolding and proteolysis (Spurway et al, 1997)

SITE DIRECTED MUTAGENESIS (SDM)

Previously the identification of enzyme active site residues relied on the chemical modification of proteins. The essential reactive groups were identified by selective chemical modification. Rapid developments in molecular biology techniques have made it possible for the individual amino acids to be substituted by site-specific mutagenesis. The knowledge derived from chemical modification and crystallographic studies of the active site facilitates the understanding of the structure-function relationship of the protein. Protein engineering is also applied to alter the substrate specificity, pH optima and to increase the thermal stability of the enzymes.

SDM and Structure-function analysis

The highly conserved amino acid residues located at specific positions in xylanases are important in structure-function analysis and hence are targeted for SDM. Based on the sequence similarity of xylanase from *Bacillus pumilus* with other xylanases of known origin and the knowledge of its three-dimensional structure, the authors have proposed the residues Glu 93 and Glu 182 to be the most suitable candidates for the essential catalytic activity of xylanase (Ko et al, 1992). Mutation of these residues resulted in decrease in specific activity, suggesting that they are essential catalytical residues. No change in protein conformation was observed, as confirmed by C.D. spectra of mutant enzyme. The conserved glutamate residues (Glu 87 and Glu 184) have also been shown to be present in the active site of xylanase A from *S. commune* (Oku et al, 1993). Recently mutagenesis and analysis of 3D structure of xylanase A from *S. lividans* revealed Asn 127 to be the important residue in maintaining the ionization states of two catalytic residues and in the stabilization of catalytic intermediates (Roberge et al, 1997a). The three-dimensional structures of two bacterial xylanases from *B. pumilus* and *B. circulans* and a fungal xylanase from *T. harzianum* have also revealed the presence of two completely conserved glutamic acid residues corresponding to Glu-87 and Glu-184 of *S. commune* xylanase A.

SDM for altered desirable properties

Site-directed mutagenesis of the cloned gene offers interesting research opportunities to change the properties of a protein suitable for its application. In the case of xylanase from *S. lividans* 1326 (Moreau et al, 1994) the thermostability is increased by replacing arginine 156 by glutamic acid. The modified enzyme has shown a temperature optimum- 5^0 C higher than that of the wild type. The half life of Arg156Glu was 6 min longer than that of the wild type enzyme, suggesting that although the engineered xylanase had a higher optimal temperature, the stability was not significantly affected. Previous reports suggested that the same substitution occurs naturally in the xylanases produced by *Bacillus sp.* C-125 and *C. saccharolyticum* (Luthi et al, 1992). Both the xylanases have an optimum temperature of 70^0C, which is the same as for the modified enzyme (Arg156Glu). The studies on cellulases from *T. reesei* TD beta-6 and *Thielavia terrestris* NRRL 8126 (Durand et al, 1984) have revealed that if two favorable mutations are combined, such as Arg 156 Glu and Asn 173 Asp, the resulting enzyme

is twice as stable as compared to the wild type, with half life of 220 minutes, at 60^{0}C. The introduction of disulfide crosslinks into proteins, to protect them from unfolding , requires the creation of cysteine residues that form disulfide bonds spontaneously in solution and do not obstruct functional domains. In *B. circulans* mutant xylanase proteins (Wakarchuk et al, 1994a), disulfide bridges conferred thermoprotection as observed by 15^0 C increase in thermostability.

Site-directed mutagenesis of a xylanase gene from *C. saccharolyticum* has yielded 6 mutant xylanases with altered temperature stability and temperature optimum (Luthi et al, 1992), whereas, no change was observed in the pH optimum. The stabilization of xylanases by random mutagenesis of the cloned gene fragment from *B. pumilus* IPO has been described (Arase et al, 1993). Four mutants, each showing a single amino acid substitution, have been selected on the basis of activity at 60^{0}C. Based on the computer graphic simulation it was confirmed that these substitutions do not change the wild type conformation. Kinetic analysis has revealed that the mutants are stabilized by a decrease in activation entropy, except for Asn 104 which was stabilized by an increase in activation enthalpy.

MECHANISM OF ACTION OF THE XYLANASES

It has been frequently suggested that the catalytic mechanism of glycosidases resembles that of lysozyme. The hydrolysis reaction catalyzed 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. The second residue acts as a nucleophile which, in case of retaining enzymes, interacts with the oxocarbonium intermediate or promotes the formation of an OH- ion from a water molecule, as observed for inverting enzymes. Reaction with retention of configuration involves a two-step mechanism in which proton transfer occurs to and from an oxygen atom in an equatorial position at the anomeric centre (Sinnot, 1990) (Fig.I. 1). This reaction mechanism is similar to that of lysozyme (Kelly et al, 1979). Reactions leading to inversion of configuration proceed 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 hydrolyzed via oxocarbonium ion like transition state.

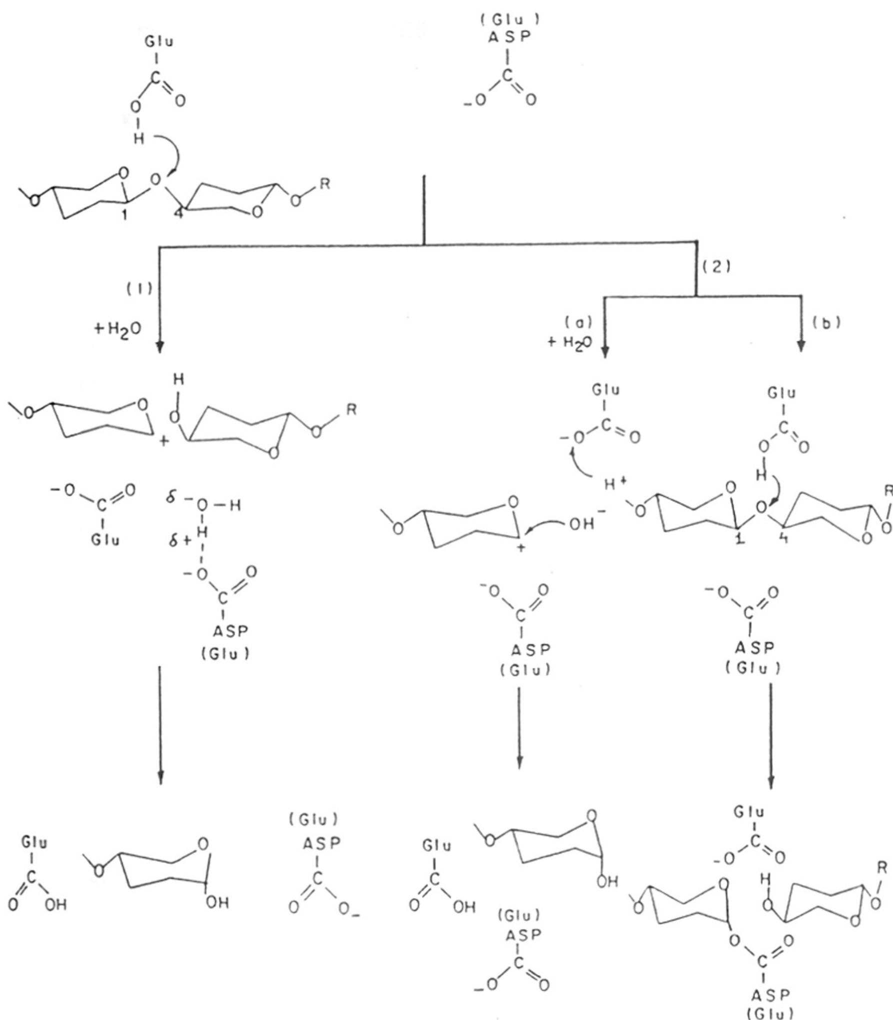

Fig. I.1 Reaction mechanism.

(1) Single displacement reaction. Involvement of a general acid (Glu), a general base (Asp or Glu) and attack by a nucleophilic water molecule is shown.

(2) 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

(b) involving formation of a covalent intermediate by nucleophilic attack of the Asp (or Glu) on C-1 of the incipient sugar (Based on ref. Sinnot, 1990).

DOMAIN ORGANIZATION OF XYLANASES

Hydrophobic cluster analysis (HCA) is a sensitive method for the comparison of amino acid sequences to derive structural, functional and evolutionary information. The HCA detects homologies between similar three-dimensional structures of proteins having low sequence identity. On the basis of amino acid sequence homology and hydrophobic cluster analysis catalytic domains of cellulases and xylanases were first classified into six families (A to F) (Henrissat et al, 1989). In a subsequent study, the families were upgraded to eleven (Gilkes et al, 1991) and in a third update to 45, including 482 glycosyl hydrolase amino acid sequences (Henrrissat and Bairoch, 1993). Presently Henrissat and Bairoch have classified all the available sequences of glycosyl hydrolases into 58 families (Henrissat and Bairoch, 1996). Based on the hydrophobic cluster analysis the xylanases are subdivided into two families, F and G, which are also shared by other glycanases such as endoglucanase, exoglucanase, and cellobiohydrolase (Henrrissat and Bairoch, 1993) . The families F and G, which are analogous to glycohydrolase families 10 and 11, comprise high and low molecular weight xylanases, respectively. Generally no significant homology was found between the xylanases from the two families, including the region around the catalytic residues, and they have an altogether different pattern of protein folding. At the molecular level the xylanase protein comprises functional or non-functional domains and linker regions.

Catalytic Domain

In general xylanases consist of a single catalytic domain. However, analysis of truncated forms of xylanase 3 from *Neocallimastix frontalis* has indicated that the full length protein contained two catalytic domains displaying similar substrate specificity (Durand et al, 1996). In the case of *Ruminococcus flavefaciens* two catalytic domains have been reported. Catalytic activity measurements and differential scanning calorimetry of exoglucanase/xylanase Cex from *C. fimi* suggested that binding and catalytic domains of the protein fold independently (Nikolova et al, 1997). The amino acid sequence of the endoxylanase from *Cryptococcus albidus* has some homology with the catalytic region of the eggwhite lysozyme (Morosoli et al, 1986). Based on the sequence homology it has been interpreted that the glycosidic bond cleavage in xylan is similar to that of lysozyme. However it has been shown that the catalytic domain of this enzyme is homologus to that of cellobiohydrolase from *C. fimi* and xylanase B from *B.*

subtilis C 125 (Coughlan, 1992). A xylanase from *C. saccharolyticum* (Luthi et al, 1990) is found to be homologous with cellobiohydrolase domain of the *Caldocellum* bifunctional exocellulase-endocellulase (*celB*), catalytic domain of cellobiohydrolase from *C. fimi* (O' Neill et al, 1988), xylanases from *B. subtilis* C 125 , *C. thermocellum* (Grepinet et al, 1988), and *Cryptococcus albidus* (Boucher et al, 1988). These homology studies suggest that these enzymes may have evolved by shuffling the two catalytic domains with several substrate binding domains. The full length xylanase C from *Fibrobacter succinogenes*, expressed in *E. coli* is reported to be less active than the two truncated xylanase C proteins, each possessing intact catalytic domain. It may be that the tertiary structure of enzyme is such that the catalytic sites are partially buried, as a consequence of improper folding in *E. coli.* Comparison of protein sequence by HCA showed a clear similarity in the secondary structure elements for the two catalytic domains of Xyn C and the *B. pumilus* xylanase, indicating a common ancestry as well as related three-dimensional organization. Two tryptophan residues in each domain are found to be completely conserved (Zhu et al, 1994).

Cellulose binding Domain (CBD)

The CBD of the mixed function glucanase-xylanase Cex from *C. fimi* contains 5 tryptophans, 2 of which are located within the $\alpha\beta$ -barrel structure and 3 are exposed on the surface. NMR analysis and chemical modification studies confirmed that the exposed aromatic residues play a direct role in binding to cellulose; their interaction with cellulose appeared to be reversible (Bray et al, 1996). The cellulose binding domains have also been detected in various xylanases such as xylanase A, B, and C from *Pseudomonas fluorescens,* xylanases from *Cellulomonas fimi,* and *Clostridium thermocellum* (Gilkes et al, 1991). These domains seem to play two possible roles, i.e., they either open the structure of the plant cell wall making it more accessible to enzymic hydrolysis or provide a general mechanism by which a consortium of hydrolases accumulate on the surface of the plant cell wall, resulting in the synergistic action between the enzymes. Black et al (1995) have studied cellulose and xylan binding domains from xylanase D of *C. fimi.* The deletion of the cellulose binding domain abolished the cellulose binding capacity of the enzyme without affecting the xylan binding properties. However, the Km values of the truncated xylanase for the insoluble xylan were higher, indicating that the internal cellulose binding homologue in xylanase D constitutes a discrete xylan binding domain which influences the affinity of

the enzyme for the insoluble substrate, but does not directly affect the xylanase activity. The existence of a truly functional internal CBD of the type found in *C. fimi* enzymes has not yet been demonstrated unambiguously (Tomme et al, 1994). Recently, domain organization and stability of xylanase A from *Thermotoga maritima* and its C terminal CBD were studied by expressing the two separate proteins in *E. coli*. CBD was expressed as a glutathione s-transferase fusion protein. Denaturation / renaturation studies have shown that the domain folds independently (Doris et al, 1997). As observed for all *Thermotoga* proteins investigated so far (Jaenicke et al, 1996), Xyn A exhibits extremely high intrinsic stability in the case of CBD. The apparent Tm value of both proteins exceeds 100^0 C. CBD was found to retain residual secondary structure even at a temperature beyond Tm. CBDs encoded by xylanase1 from *Rhodothermus marinus* are shown to be repeated in tandem at N-terminus exhibiting similarity with CBD family IV (Eva et al, 1997). It appears to be the first example of xylanase gene encoding a CBD family IV in combination with catalytic domain of glycosyl hydrolase family 10. The molecular architecture of four new xylanases, from the aerobic soil bacteria, *P. fluorescens subsp. cellulosa* and *C. mixtus* has been studied (Sadler et al, 1995). Each of the enzymes is modular and contains a novel cellulose binding domain that is separate from the catalytic domain. Characterization of xylanase A gene from *Thermoanaerobacterium thermosulfurigenes* EM 1 revealed the presence of two CBDs and a triplicated sequence at its C-terminus. The latter was found to be identical to S-layer like domains of previously characterized pullulanase from the same organism (Matuschek et al, 1996). In xylanase producing *Streptomyces* strains the proteolytic activity for the removal of CBD seems to be conserved (Arribas et al, 1997) since a similar xylanase pattern was obtained for all of them. It is suggested that autoprocessing of protein and protease activity are the two reasons that cause the loss of CBD that might help in the selective hydrolysis of xylan.

Thermostabilizing Domain

Repeated domains unrelated to the catalytic domain are relatively common in bacterial xylanases and glucanases; however, in most cases, little is known about their functions. Recent work on the thermostable xylanase from *Thermoanaerobacterium saccharolyticum* (Lee et al, 1993) has correlated the intrinsic thermostability to the N-terminal domains of the enzymes. Evidence has also been presented that XYL Y from *Clostridium thermocellum* (Fontes et al, 1995) contained a homologue of this domain

that also appeared to confer thermostability. The thermostabilizing domains have been closely associated with the xylanase catalytic domain. The 180 residue domain of xylanase Y from *C. thermocellum* has been shown to have 28% sequence identity with the thermostabilizing domain of xylanase A (residues 200-353) from *T. saccharolyticum*. Recently sequence analysis of xylanase C gene from *C. thermocellum* F1 revealed the presence of 165 amino acid region which was found to be homologous to the thermostabilizing domain (Hayashi et al, 1997). We compared the sequence of Xyn A from *T. saccharolyticum* with the other reported xylanases from thermophilic organisms. The analysis revealed that the xylanases from *B. stearothermophilus,* thermophilic bacterium RT8.B4, *T. maritima, Anaerocellum thermophilum* and *Caldicellulosiruptor sacchrolyticus* have a region similar to the thermostabilizing domain of Xyn A from *T. saccharolyticum*. Multiple sequence alignment of these xylanases with Xyn A from T. sacchrolyticum indicated conserved Gly and Tyr residues in the region corresponding to the thermostabilizing domain of Xyn A from *T. sacchrolyticum.* The identity of xylanases from other thermophilic organisms with the thermostabilizing domain ranged from 5 to 32%. Xylanase C from *C. thermocellum* showed maximum similarity of 32%, while xylanase from *B. stearothermophilus* showed very little identity.. It may be possible that these domains confer thermostability to the corresponding xylanases (Figure I.2). However, more experimental evidence is necessary to validate the concept that thermostability is conferred by a specific domain. Examination of the conserved residues in the thermostabilizing domain reveals the absence of cysteine. Predominance of bulkier aliphatic residues is observed, which may increase thermostability by increasing the compactness of the folded molecule (Mozhaev and Martinek, 1984). The occurrence of a large number of glycine residues supports the proposition that they may be located in the loop regions of thermophilic proteins (Ahern et al, 1987). However, comparatively few asparagine and glutamine residues are observed which can cause thermoinactivation, as they are more susceptible to denaturation at elevated temperatures .

It was observed that xylanases from thermophilic organisms exhibited more homology in their catalytic domain. The conserved residues in the catalytic domains could suggest the close evolutionary relationship between the thermophilic bacteria that might have arisen through the lateral transfer of a single ancestral gene between them. The thermophilic xylanase A of *Thermomonospora fusca* is the one reported to date,

belonging to family 11 (Irwin et al, 1994). However, 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 enzymes in which folding is such that it has been relatively easy for the enzyme to evolve into thermophilic enzyme (Fontes et al, 1995). The data infer that domains which confer *increased* thermostability may be a common phenomenon among family 10 xylanases from thermophilic organisms. Such domains are located only in xylanases and have not been observed in a number of thermophilic endoglucanases characterized to date.

```
1-167 MYVESPD-PTLEYYIDDVVVTTQNPI-QVGNVIANETFENGNTSGWIGTG-SSVVKAVYG
2-  6 LLTLLTVFALLTVGICGSFLPLPKAS---AAALIYDDFET-GLNGWGPRG-PETVELTTE
3- 74 IENPDPNPQKPNILIPFAKEEIQREFH---VATDTWKAGDLTFTIYSPVKAVPNPETADE
4-167 LIVNMPTSKSDSFYIDLFTIKDLENA----YVLKQENFENKNTGGFLPEDKNCKITLAKD
5-176 LIIVASKNTNFNFYLDKVQVLAPKES-GP-KVIYETSFEN-GVGDWQPRG-DVNIEASSE
6-169 LKVYVPTSTTN-FYVDLFTLKVADKS----HLIKFENFEDKSIAGFISQDKKCKLSISKE
7-168 LIVNMPTSKSDSFYIDLLVIKDLENA----YVLKQENFENKNTGGFLPEDKNCKIALTKD
8-  8 AKITALVVLLGVFFVLPSNISQLYAD----YEVVHDTFEV-NFDGWCNLGVDTYLTAVEN

                          .
1-    VAHSGDYSLLTTGRTA--NWNGPSYDLTGKIVPG-QQYNVDFWVKFVNGNDT-EQIKATV
2-    EAYSGRYSLKVSGRTS--TWNGPMVDKTDVLTLG-ETYKLGVYVKFVGDSYSNEQRFSLQ
3-    EELKLALVPAVIVEMTIDNTNGTRARRAFFGFEGTDPYTSMRRIDDTCPQLRGVGQGRIL
4-    RAYSSAYSLKVQPSQK-TKNGKILFPIKGLLQKG-GTYDFSLLVYQDSSKP--VNFSAGI
5-    VAHSGKSSLFISNRQK--GWQGAQINLKGILKTG-KTYAFEAWVYQNSGQDQ-TIIMTMQ
6-    KAYQGTYSIKVQQTVK-RQNTTVILPVKGTFEKG-KSYSISFYMHQSILKS--LNFAVGI
7-    KAYSSTYSLKVPAILKRARMERFLFPIKGLLQKG-RTYDFSVLVYQNSSKP--VSFLAGI
8-    EGNNGTRGMMVINRSS--ASDGAYSEKGFYLDGG-VEYKYSVFVKHNGTGT-ETFKLSVS

                                       *   *     .
1-    KATS-DKDNYIQVNDFANVNKGEWTEIKGSFTLPV-ADYSGISIYVESQNPTLE------
2-    LQYNDGAGDVYQNIKTATVYKGTWTLLEGQLTVPS--HAKDVKIYVETEFKNSPSPQDLM
3-    SIVSKDEGVRSALHFSMEDILTAQLEENWTFGLGK---VGALIVDVPAGEKKT-------
4-    KLND-GKSTKEIVLAKQNVAPKKWTQLFATLDLDTRFSAKDVSFFVKPAAAIS-------
5-    RKYSSDASTQYEWIKSATVPSGQWVQLSGTYTIPAGVTVEDLTLYFESQNPTLE------
6-    RFLESGKNMREIVLGRVTIPKNKWTEVFASYTPSLDSKIKDFVIFIRPLSDISY------
7-    KIND-GKSTKEIVLTKQSVTPKKWAELFATLDLDTKFSAKDVSFFVKPAVAIP-------
8-    YLDSETEEENKEVIATKDVVAGEWTEISAKYKAPK--TAVNITLSITTDSTVD-------

1-333 -FYIDDFSVIG---EISNNQITIQNDIPDLYSVFKDYFPIGVAVDPSRLNDADP---HAQ
2-177 DFYIDDFTATP------ANLPEIEKDIPSLKDVFAGYFKVGGAA-TVAELAPKP---AKE
3-242 --YQFAVCFYR----G-GYVTAGMDASYFYTRFFQNIEEVGLYALEQAEVLKEQSFRSNK
4-332 -YYLDLYSISD----ENWGQPVPDYNLPSLCEKYKNYFKIGVAV-PYRALTNPV---DVE
5-344 -FYVDDVKIVDTTSAEIKIEMEPEKEIPALKEVLKDYFKVGVAL-PSKVFLNPK---DIE
6-335 -YYIDNFTISD----DGWYSAVPDLDLPSLREKYKNYFKIGVAV-PYRALTNPV---DVA
7-334 -YYIDLYSISD----ENWGQPIPDYNLPSLCEKYKNYFKIGVAV-PYRALTNPV---DVE
8-171 -FIFDDVTITR---KGMAEANTVYAANAVLKDMYANYFRVGSVL-NSGTVNNSS---IKA
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Fig. I.2

Fig. I.2 Homology of the thermostabilizing domain of xylanase Y from *Clostridium thermocellum* with other xylanases. Accession numbers are in parentheses .1) *T. saccharolyticum* B6A-RI (A48490) xylanase 2) *C. thermocellum* F1 xylanase C (D84188) 3) *B. stearothermophilus* 21 xylanase (D28122) 4) Thermophilic bacterium RT8.B4 (S12745) 5) *T. maritima* xylanase A (Z462664) 6) *Anaerocellum thermophilum* xylanase A (Z69782) 7) *Calidicellulosiruptor saccharolyticus* xylanase I (AF005382) 8) *C. thermocellum* YS xylanase Y (X83269). The conserved amino acid residues are indicated by ' * ' . Homology to the xylanase from *T. saccharolyticum* B6A-RI is shown by bold letters. Dots indicate chemically similar amino acids.

MOLECULAR EVOLUTION

The HCA analysis of amino acid sequences of xylanases and cellulases has revealed that isoenzyme forms of these enzymes, observed in several organisms, are a consequence of large multigene families and are not solely the result of processing of a single gene product. Cellulase and xylanase genes from different families are often present in a single organism suggesting that microorganisms have acquired multiple plant cell wall hydrolase genes not exclusively through gene duplication but via extensive horizontal gene transfer (Gilbert and Hazlewood, 1993). The occurrence of both fungal and bacterial enzymes in the families 5,6,10 and 12 and the presence of prokaryotic and plant enzymes in family 9 led to the proposition that lateral transfer of cellulase and xylanase genes has also occurred.

Many xylanases and cellulases are known to possess a modular structure comprising a catalytic domain linked to a non-catalytic cellulose binding domain (CBD). It is necessary for xylanases and other hemicellulases, to act cooperatively on plant cell walls which consist of cellulose, hemicellulose and lignin. Hence the binding of hemicellulases to cellulose via CBDs should be advantageous in the hydrolysis of hemicellulose in plant cell walls. This may be the reason for the evolution of xylanases containing CBDs. Although xylan-binding domains have also been reported in xylanases, such domains need to be specifically evolved for each xylan due to the heterogeneous nature of the substrate. The integration of CBD may be the efficient way to save the gene capacity exclusively for xylan.

Spurway et al (1997) have reported the presence of Ca binding site (located in loop 7) in xylanase A from *P. fluoresces subsp cellulosa*. The literature survey indicated that only 5 of the 28 family 10 xylanases contain extended loop 7. Phylogenetic analysis has revealed a relationship between xylanases containing loop 7 and a common ancestral sequence containing DNA insertion in region encoding loop 7. Since similar sequences occur in taxonomically diverse groups of organisms a considerable horizontal gene transfer between family 10 xylanases seems to have occurred.

We have compiled the amino acid sequences of xylanases from the PIR databank (Protein International Resource, Release 46.0) . The sequences were aligned using Clustal V multiple sequence alignment (Higgins and Sharp, 1988). A minimum mutation distance matrix was then constructed from the pairwise comparisons of 54 sequences. The distance matrix was then used to obtain an evolutionary tree using

TAXAN (Release 2.0, Information Resources Group, University of Maryland, USA). The evolutionary tree (Fig. I.3) implies that the xylanases from fungal and bacterial origin appear to be related with each other forming seven different groups. Although xylanases produced by the same organism share maximum homology as observed in the case of *T. aurantiacus, T. viride* and *S. commune,* xylanases from two different fungi, i.e., *N. frontalis* and *F. floriforme* are also identical. Fungal xylanases from *T. viride, T. aurantiacus, A. niger* with bacterial xylanases from *C. stercorarium* and *R. flavefaciens* form one group. Thermophilic xylanases from *C. acetobutylicum* and *C.thermocellum* seem to be related. However, xylanases produced by two strains of *P. fluorescens* were found to be distantly related to each other.

Thus the xylanases that make up a family/ group appear to have diverged from a common evolutionary ancestor. Such enzymes are apt to retain similar secondary and tertiary structure and have the same amino acid residues at 20-50% of the corresponding positions in their primary sequences. The folding of the polypeptide chain is essentially the same in family 11 enzymes with substantial variations occurring only in external loops, e.g., xylanases from *T. ressei* and *T. harzianum.* These enzymes are classical illustrations of diverging evolution from a common ancestor. Recently xylanase from *Streptomyces vividosporus* T7A has been found to be different from the reported xylanases from family 10 or 11. It does not fall into any of the two families. The large size of this protein may be explained by gene duplication of the original ancestral gene, a common occurrence in *Streptomyces* (Magnuson and Crawford, 1997).

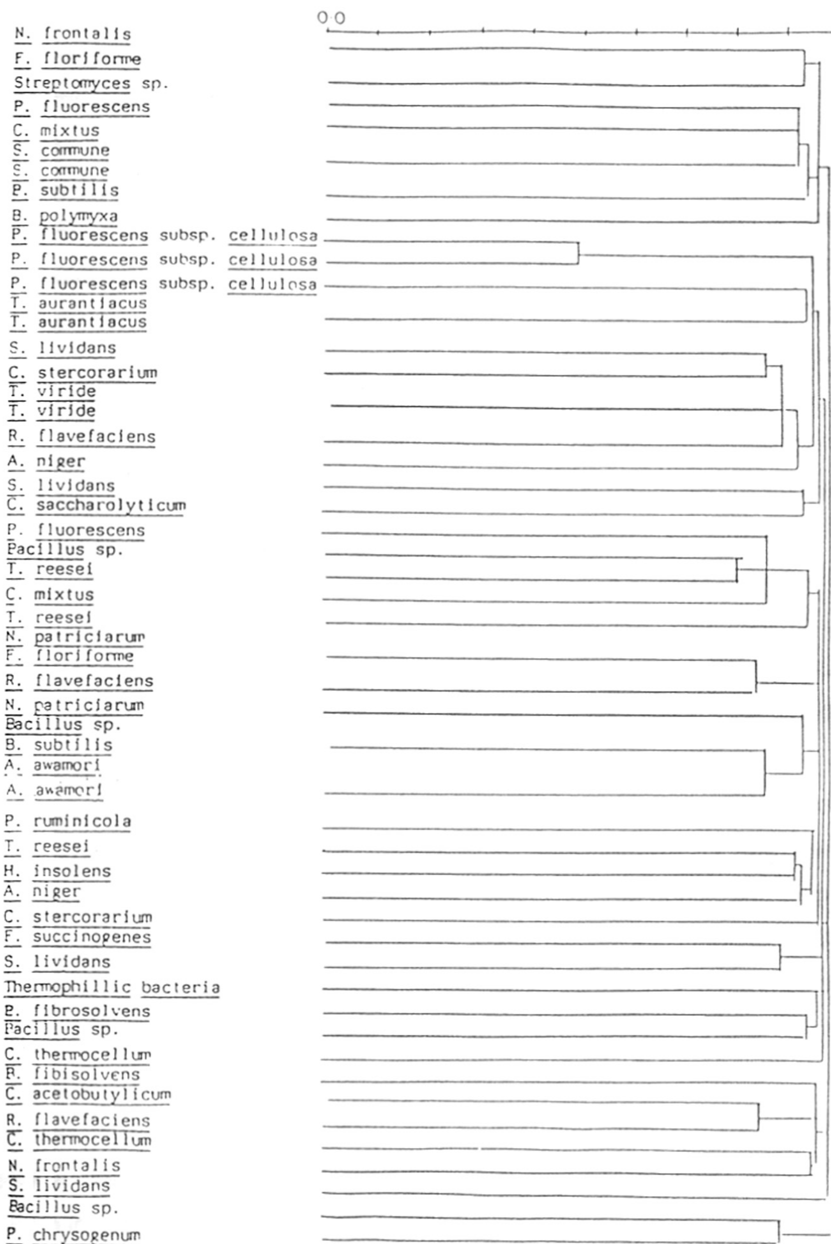


Fig. I.3 Dendrogram showing possible evolutionary relationships among xylanases. The amino acid sequences of xylanases compiled from PIR (Release 46.0) were aligned using Clustal V multiple sequence alignment. Minimum mutation distance matrix then constructed was used to obtain the dendrogram using TAXAN (Release 2.0, Information Resources Group, University of Maryland, USA).

BIOTECHNOLOGICAL POTENTIALS OF XYLANASES

During the last decade, the potential biotechnological applications of xylan and xylanases have been of particular interest to researchers. At present, the major end-products of xylan, which are of considerable importance, are furfural and xylitol. Furfural production is derived mainly from agricultural residues whereas xylitol is obtained from wood residues. Xylanases also play a key role in the maceration of vegetable matter, (Beck and Scoot, 1974) protoplstation of plant cells , clarification of juices and wine (Biely, 1985), liquefaction of coffee mucilage for making liquid coffee , recovery of oil from subterranean mines, extraction of flavors and pigments , plant oils and starch (McCleary, 1986) and to improve the efficiency of agricultural silage production (Wong and Saddler, 1992a). The purified xylanase from *Trichoderma viridae* was found to induce the biosynthesis of ethylene and two other pathogen-related proteins in tobacco, suggesting that xylanases can also play a role in induction of plant defense mechanism (Bailey et al, 1995). The xylanases from the germinating plant seed primarily convert the reserve food to the assimilable end- product. It is also proposed that they play a role in cell elongation, they seem to be involved in fruit softening , and are believed to have yet undiscovered important physiological functions.

The xylanases find application in the bakery and the fodder industries due to the presence of substantial amounts of residual hemicellulose in the raw material. In bakeries the xylanases act on the gluten fraction of the dough and help in the even redistribution of the water content of the bread (Wong and Saddler, 1992b), thereby significantly improving the desirable texture, loaf volume and shelf life of the bread. The use of xylanases along with other hemicellulases increases the nutritive value of the animal feed. Cellulase free xylanases have potential applications in biobleaching of the kraft pulp. Applications of xylanases in pulp and paper industry are described in detail in chapter V. These biotechnological potentials of xylanases have prompted the search for suitable enzymes and technologies for large-scale economic production.

The alkalophilic thermophilic (AT) *Bacillus* is an isolate from our laboratory which produces cellulase free xylanases at 50^0C and pH 10.0. The purification and characterization of the two xylanases have been described earlier (Dey et al, 1992).. The gene fragment coding for xylanases has been cloned and expressed in *E. coli* (Shendye and Rao, 1993).

PRESENT WORK

The present investigations deal with characterization of xylanases from the recombinant *E. coli.* The restriction analysis and the isolation of the gene fragment coding for Xyn II have been carried out. The nucleotide sequencing of *xynII* and comparison with other xylanases have been documented. A part of the thesis deals with application of xylanases from AT *Bacillus* in biobleaching of bagasse pulp.

The thesis includes:

Chapter I: General introduction

Chapter II: The extracellular xylanases produced by recombinant *E. coli* (pATBX 4.5) were purified and characterized. The properties of recombinant xylanases were compared with those from the parent *Bacillus.*

Chapter III: The gene fragment of 4.5 kb coding for xylanases from AT *Bacillus* was subcloned. The expression of XynII in recombinant *E. coli* harbouring 1.8 kb subfragment was studied.

Chapter IV: The use of aqueous two phase system for the extractive cultivation of recombinant *E. coli* (pATBX 1.8) was studied for production and separation of xylanase.

Chapter V: The sequencing of the gene coding for low molecular weight xylanase (Xyn II) from AT *Bacillus* and comparison with other xylanases were carried out.

Chapter VI: The application of xylanases from AT *Bacillus* in biobleaching of bagasse pulp was studied.

CHAPTER II

CHARACTERIZATION OF RECOMBINANT XYLANASES FROM AT *BACILLUS*

SUMMARY

The 4.5 kb gene fragment coding for xylanases from AT *Bacillus* has been cloned and expressed in *Escherichia coli* (Shendye and Rao, 1993). The extracellular xylanases produced by recombinant *E. coli* (pATBX 4.5) were purified to homogeneity. The Mr of the enzymes were estimated to be 35,000 (xylanase I) and 14,500 (xylanase II) by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified xylanases had similar temperature (60°C) and pH (6) optima and the isoelectric points were 4 and 8 respectively. The enzymes retained 100% activity at 50°C for 24 h however at 60°C they showed half life of 2 h. The apparent Km values of xylanase I and II were 5.8 and 8.3 mg/ml and Vmax values were 0.010 and 0.520 μmol/min/mg respectively. The recombinant xylanases showed reduced ability to bind xylan and had lower specific activity and Km values than those of the xylanases from the parent *Bacillus* reported previously. Both the enzymes yielded different hydrolysis pattern when incubated with oat spelt xylan. The hydrolysis pattern of the recombinant xylanases was found to be distinctly different from that of the original xylanases which may be due to differential binding of the cloned enzymes to the substrate.

INTRODUCTION

Xylanase genes from different microbial genera have been cloned and expressed in *E. coli.* The expression in *E. coli* is generally found to be lower than the parent organism and the recombinant proteins were found to be confined to the cytoplasmic or the periplasmic fractions. The intracellular accumulation of the recombinant xylanases and the absence of post-translational modifications such as glycosylation in *E. coli* have been reported to be the key reasons for the low levels of xylanase activity. The extracellular activity has been reported in recombinant *E. coli* for the xylanases from alkalophilic *Aeromonas* (Kudo et al, 1985), alkalophilic *Bacillus* (Honda et al, 1985b), AT *Bacillus sp.* (Shendye and Rao, 1993) and *Cellulomonas sp.* (Bhalerao et al, 1990). The recombinant xylanase from *Bacillus stearothermophilus* T-6 was was optimally active at pH 9.0 and 65^0 C (Gat et al, 1994). The recombinant proteins secreted by *E. coli* are nonglycosylated (Jenkins and Curling, 1994). Enhanced xylanase production has been achieved in case of alkaline *Aeromonas* sp. (Kudo et al, 1985), *Bacteroides ruminicola* (Whitehead and Hespell, 1989) and *Fibrobacter succinogenes* 135 (Hu et al, 1991) by cloning in *E. coli.*

Overexpression of *B. subtilis* and *B. circulans* xylanases in *E. coli* has also been described. A gene encoding mature *B. circulans* xylanase has been designed to imitate the frequency of degenerate codons used in *E. coli.* Synthetic *B. circulans* gene is then converted to *B. subtilis* xylanase gene via single codon substitution (Thr 147 Ser). The plasmids containing both synthetic genes were further modified for direct expression in *E. coli.* Under the control of *lac* promoter, recombinant xylanase has been produced at levels as high as 300 mg/L in solution form in cytoplasm. Characterization of the products indicated that the purified recombinant protein was correctly processed and enzymatically active (Sung et al, 1993). *B. subtilis* xylanase gene, fused to the 5' end of *C. fimi* cenA, has been overexpressed in *E. coli* (Tomme et al, 1994). Fusion protein exhibited strong affinity for both microcrystalline cellulose and regenerated cellulose. Recently Lapidot et al (1996) have reported overexpression and single-step purification of a thermostable xylanase from *B. stearothermophilus* T-6. The xylanase gene was cloned into T-7 polymerase expression vectors. The enzyme was found to constitute over 70% of the cell protein and was efficiently purified from the host proteins by a single heating step. Over 2g soluble and active enzyme per l culture were achieved.

The gene fragment coding for xylanases from AT *Bacillus* has been cloned and expressed in *Escherichia coli* (Shendye and Rao, 1993) from this laboratory. The present chapter deals with the purification and characterization of the extracellular xylanases produced by *E.coli* (pATBX 4.5) and the comparison of nonglycosylated recombinant xylanases with those from the parent *Bacillus*.

MATERIALS AND METHODS

Purification

E. coli (pATBX 4.5) was grown at 37°C with shaking (200 rpm) for 18 h in LB broth containing ampicillin (100µg/ml) (Shendye and Rao, 1993). Culture broth (100 ml) was centrifuged and the supernatent was precipitated with ammonium sulfate (90% saturation) followed by dialysis against 50 mM potassium phosphate buffer, pH 7.0. The ammonium sulfate precipitate was dissolved in 1.0 ml of 25 mM potassium phosphate buffer, pH 7.0 and was subjected to gel filtration on a Bio-Gel P-100 column (packed volume 1x95 cm) equilibrated with 25 mM potassium phosphate buffer pH 7.0. The column was eluted with the same buffer. The fractions were collected at a rate of 2.0 ml per 10 min., scanned for their absorbance at 280 nm and assayed for xylanase activity. The active fractions were pooled, concentrated by lyophilisation and dialyzed against 50 mM potassium phosphate buffer, pH 7.0. All steps were carried out at 4° C unless otherwise mentioned.

Estimation of xylanae activity

Two grams of xylan powder was suspended in distilled water and stirred for 16 h and 28°C. The insoluble fraction was removed by centrifugation and the soluble fraction was used as the substrate for estimation of xylanase activity. Xylanase activity was estimated by mixing the suitably diluted enzyme with 0.5 ml xylan solution (1%) in a final volume of 1 ml and incubating at 50°C for 30 min. The reducing sugars released were determined by the dinitrosalicylic method (Miller, 1959) using D- xylose as standard. One unit of xylanase activity was defined as the amount of enzyme that produced 1 µmol of xylose equivalent per minute from xylan under the assay conditions. The protein concentration was measured according to Bradford (1976).

Determination of molecular weight

The molecular weights were determined by gel filtration on Bio-Gel P-100 column. The markers used were aprotinin (6,500), cytochrome c (12,400), carbonic anhydrase (29,000) and albumin (66,000). SDS-PAGE was performed as described by Laemmli (1970).

Isoelectric focusing

Analytical isoelectric focusing in polyacrylamide gel was carried out according to Vesterberg (1972). The ampholyte range used was pH 3-10.

Amino acid analysis

The recombinant xylanase II was hydrolyzed with 6 M HCl for 22 h, at 110°C, and analyzed on a Pharmacia LKB Alpha Plus Amino Acid Analyzer. Protection for cysteine, methionine and tyrosine was carried out using appropriate protecting agents (Ozols, 1990).

Determination of Km and Vmax

Suitably diluted xylanases were incubated with different substrate concentrations (2-10 mg of xylan) under standard assay conditions. Km and Vmax were determined from Lineweaver-Burk plots.

Determination of xylan degradation products

Xylanase (1 U) was incubated with xylan (5 mg) in 0.05 M phosphate buffer, pH 7.0 at 50° C for 16 h in a volume of 0.2 ml. Hydrolysates were analyzed for the sugars by paper chromatography in a solvent system containing butanol-acetic acid-water (3:1:1 v/v) and detected as described by Trevelyan et al (1950).

Adsorption on xylan

Xylan (10 mg) was washed with 50 mM potassium phosphate buffer, pH 7.0 and was incubated with 1 U of xylanase at 4°C for 60 min. After centrifugation the supernatent was checked for xylanase activity.

Media composition:

Luria Bertani (LB) medium:

Tryptone	1.0%
Yeast extract	0.5%
NaCl	0.5%
pH	7.2-7.4
Agar (for solid media)	2%

RESULTS

The extracellular culture filtrate of *E. coli* (pATBX 4.5) was precipitated with ammonium sulfate (90% saturation) and subjected to column chromatography on Bio-gel P-100 (Fig. I.1). Two xylanase peaks were observed and designated as xylanase I (fractions 20-35) and xylanase II (fractions 70-90). The purification steps are summarized in Table I.1. The purified enzymes were found to be homogeneous and showed single bands on SDS-PAGE.

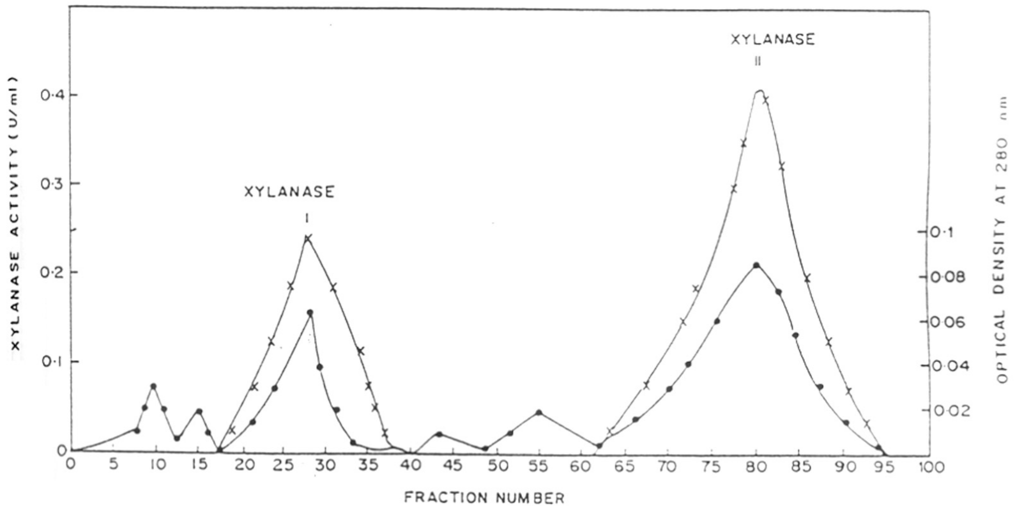


Fig. II.1 Fractionation on Bio-Gel P-100 of the ammonium sulfate precipitated protein from the culture filtrate of *E.coli* (pATBX 4.5). Fractions of 2.0 ml were collected. X xylanase activity; ● , absorbance at 280 nm.

Table II.1: Purification of xylanases from *E. coli* (pATBX 4.5)

Steps	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification
Culture supernatent	100.00	2.00	1.50	1.33	1.00
Ammonium sulphate precipitate	5.00	31.00	2.48	12.50	9.39
Bio-Gel P-100					
Xylanase I	1.00	17.90	0.81	22.00	16.54
Xylanase II	1.00	98.40	1.29	76.00	57.14

Molecular weight

The molecular weights of xylanase I and II as determined by Bio-gel P-100 column were 32,400 and 8,900 respectively. By SDS-PAGE the corresponding molecular weights were 35,000 and 14,500 respectively (Fig. II.2).

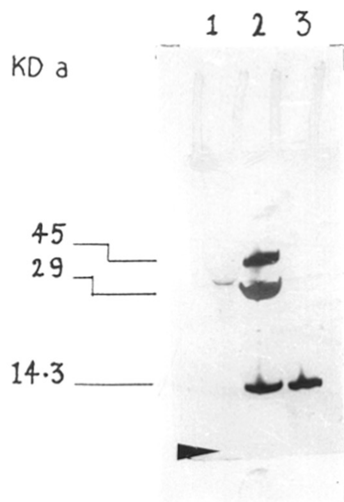


Fig. II. 2. SDS-PAGE of purified recombinant xylanases. Lane I, xylanase 1 (15 μg); lane 2, protein standards (from the top): ovalbumin (45,000), carbonic anhydrase (29,000), lysozyme (14,300); lane 3, xylanase II (30 μg). Arrow indicates the dye front.

Effect of pH on activity and stability

The pH was adjusted with 0.05 M potassium phosphate buffer (pH 5-7) or NaOH-glysine buffer (pH 8-10).Xylanase I and II at 50°C showed pH optima of 6 but showed 60-70% activity at pH 8. Xylanase I was stable at pH 6-8 whereas xylanase II was stable at a lower pH range (pH 6-7). At pH 8 xylanase II showed 20% loss in activity. At pH 9 and 10 xylanase I showed 50 and 60% loss in activity whereas xylanase II showed 30 and 40% loss in activity respectively (Fig II.3).

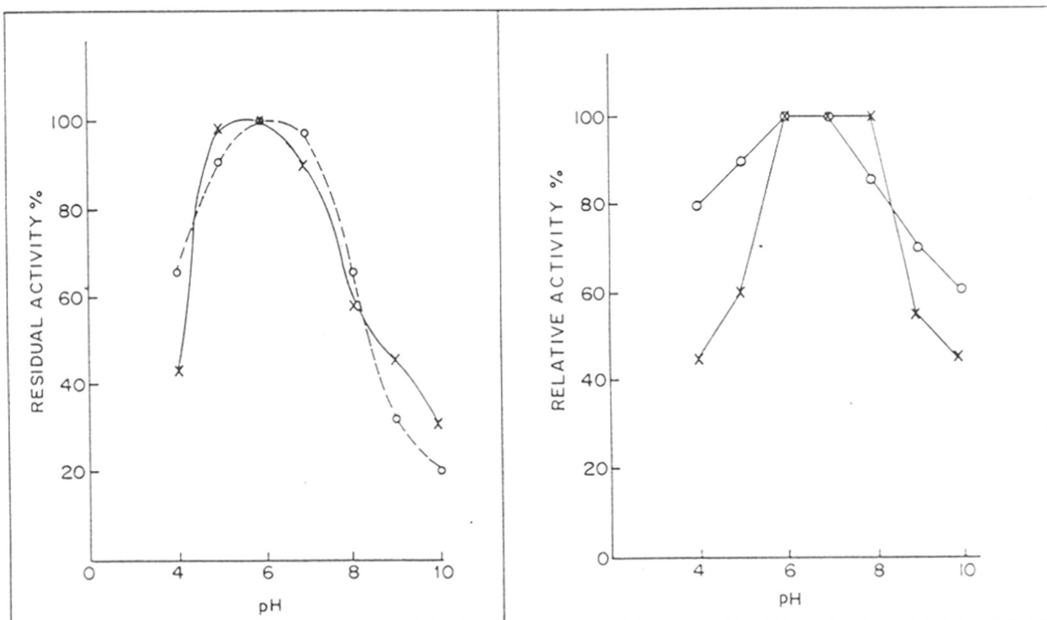


Fig. II.3. Effect of pH on stability of xylanases. Potassium phosphate buffer (50 mM) was used for pH values between 5 and 7, and 50 mM glycine-NaOH buffer was used for pH 8.0-10.0. The activity of 5 U at 50°C and pH 6.0 was taken as 100%. x, xylanase I; o, xylanase II

Effect of temperature on activity and stability

Xylanase I and II showed temperature optima at 60° C . Both the enzymes were stable at 50°C for 24 h. However at 60°C they showed half life of 2 h (Fig.II.4) whereas the AT *Bacillus* xylanases I and II showed 50% loss in activity after 48 h and 4 h respectively under identical exprimental conditions (Dey et al, 1992).

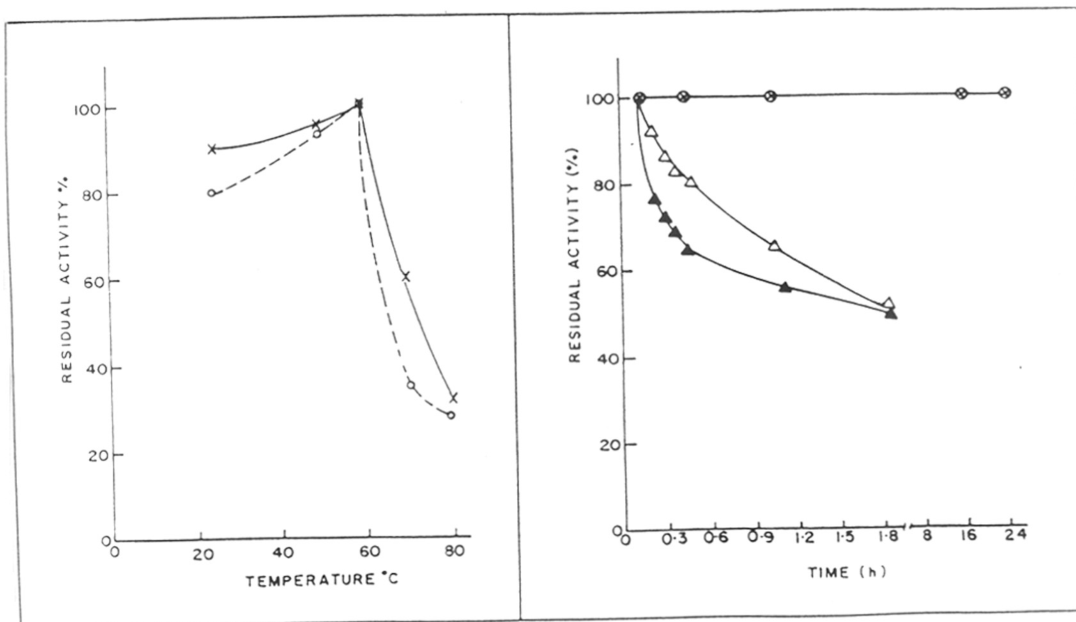


Fig. II.4. Thermal stability of xylanases. The enzymes were incubated at 50 and 60°C in 0.05 M phosphate buffer, pH 7.0. At different time intervals the aliquats were withdrawn and residual activities were measured under standard conditions.

Xylanase I : x , at 50°C ; Δ , at 60°C .

Xylanase II : o , at 50°C ; ▲ , at 60°C

Other properties of cloned xylanases

The isoelectric points of xylanase I and II were found to be 4 and 8 respectively. With xylan as a substrate the Km values were 5.8 and 8.3 mg/ml and Vmax values were 0.010 and 0.520 μmol/min/mg for xylanase I and II respectively. Amino acid composition of xylanase II (Table II.2) revealed that the protein contains high amounts of serine, glutamic acid, glycine and lysine. Methionine could not be detected and is reported to be present in low amounts in xylanases (Pace and Jurasek, 1979).

Table II.2 Amino acid composition of xylanase II

Amino acid	(mol/mol)
Aspartic acid	2.91
Threonine	2.09
Serine	11.60
Glutamic acid	11.44
Proline	1.79
Glycine	9.30
Alanine	4.79
Cysteine	ND*
Valine	1.94
Methionine	ND*
Isoleucine	1.60
Leucine	2.12
Tyrosine	1.16
Phenylalanine	1.14
Histidine	2.35
Lysine	8.65
Arginine	1.11
Tryptophan	ND

NOTE : ND, not determined. ND*, not detected

End product determination of xylan hydrolysis

Xylanase I degrades xylan preferentially to xylobiose, triose and higher xylooligosaccharides. The absence of xylotetraose and the presence of traces of xylose was also observed. Xylanase II appeared to cut the substrate into longer xylooligosaccharides with significant absence of xylobiose and xylose in the hydrolysate (Fig. II.5)

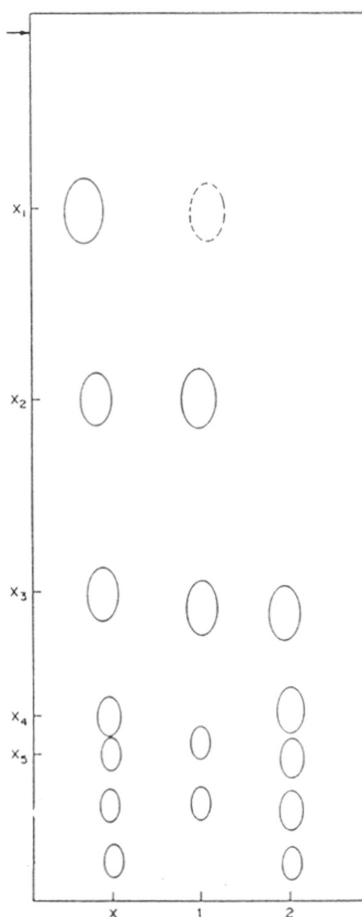


Fig. II.5 Schematic representation of paper chromatogram of hydrolysis. Products from 5 mg xylan obtained using 1U of xylanases at pH 7.0. Lane X: Standard mixture of 10 μg each of xylose (x1), xylobiose (x2), xylotriose (x3), xylotetraose (x4), xylopentose (x5) and higher xylooligosaccharides. Lanes 1 and 2: 16 h samples of hydrolysis products of xylanase I and II respectively.

Adsorption on xylan

Table II.3 shows the adsorption pattern of the xylanases from the recombinant and the parent *Bacillus*. The culture filtrates of the *Bacillus* and *E. coli* (pATBX 4.5) showed 81 and 61% adsorption on xylan respectively. The purified xylanase I from the recombinant and the *Bacillus* differed considerably in the ability to bind xylan whereas xylanase II showed 70 and 86% adsorption on xylan respectively.

Table II.3: Adsorption of xylanases

	% Adsorption	
	AT *Bacillus*	*E. coli* (pATBX 4.5)
Xylanase I	40	0
Xylanase II	86	70
Culture filtrate	81	61

Xylanase (1 U) was incubated with xylan (10 mg) for 60 min. at 4°C and the supernatent was checked for xylanase activity.

DISCUSSION

Cloning and expression of xylanase genes in *E. coli* from a plethora of organisms has been reported, the extreme thermophiles include *Dictyoglomus thermophylum* (Gibbs et al, 1995), *Thermotoga maritima* (Chen et al, 1997), and *T. neapolitana* (Veoikodvorskaya et al, 1997). Extensive cloning work has been undertaken on *Clostridium* sp. to track down the enzymatic specificities of the individual proteins that form the complex cellulosome and xylanasome structures (Thomson, 1993). However very few xylanases from recombinant *E. coli* have been purified and characterized such as those from *B. subtilis* (Bernier et al, 1983) *Trichoderma reesei* and *T. viride* (Sung et al, 1995).

In the present chapter we have described the purification of xylanases from *E. coli* (pATBX 4.5) and charactrized them. Reports of the genes coding for the xylanolytic enzymes cloned and expressed in *E. coli* have shown that most of the enzymatic properties of the recombinant xylanases are similar to those produced by the original organisms (Sipat et al, 1987, Zappe et al, 1987). In case of alkaliphilic *Bacillus* sp. strain C-125 the recombinant xylanase was slightly unstable at low pHs compared with the original enzyme (Honda et al, 1985b). However our data suggest that the properties of recombinant xylanases differ markedly from the original xylanases which may be due to the difference in the post-translational modification such as glycosyl substitution of the proteins. Xylanase I from the recombinant *E. coli* and the parent *Bacillus* showed the same molecular weight. However the recombinant xylanase II had a lower molecular weight as compared to that of the native xylanase II (Mr 15,800). The lower molecular weight of the xylanase II observed by gel filtration compared to SDS-PAGE indicates retardation of the protein due to interaction with the gel matrix. The cloned xylanases were found to be less stable at higher temperature (60°C) as compared to xylanase I and II from the AT *Bacillus* which showed the half life of 48 h and 4 h respectively at 60°C (Dey et al, 1992).The difference in the stability may be due to glycosylation which has been implicated in the stabilization of glycanases against environmental extremes (Merivuori et al, 1985). In case of cellulases from *Cellulomonas fimi* it has been shown that glycosylation does not significantly affects their stabilities towards heat and pH but it prevents protyolytic cleavage between functional domains (Langsford et al, 1987). The Vmax values of the xylanases from *E. coli* (pATBX 4.5) and the parent *Bacillus* (0.0172 and 0.742 μmol/min/mg for xylanase I and II respectively) (Dey et al, 1992) are almost identical. However the higher Km values of

cloned xylanases compared to that of AT *Bacillus* xylanases (1.58 and 3.5 mg/ml respectively) show significantly lower substrate affinity. In case of *Trichoderma reesei* cellulases glycosyl substitution has been implicated in adsorption of cellulases to insouble substrates (Chanzy et al, 1984), however the binding is not affected in cellulases from *C. fimi* (Langsford, 1987). The difference in the adsorption pattern obtained on the oat spelt xylan between AT *Bacillus* and recombinant xylanases indicates probably the glycosyl moities may be mediating the substrate interaction. Results of the xylan hydrolysis suggest that the xylanases from the parent *Bacillus* and *E. coli* (pATBX 4.5) have different modes of action, which may be due to the differential binding of the recombinant xylanases to the substrate. The absence of xylotetraose in the hydrolysate of xylanase I and absence of xylobiose and xylose in the hydrolysate of xylanase II may be due to lack of possible transglycosylation reaction of the cloned xylanases. The absence of methionine in the amino acid composition of the recombinant xylanase II compared to its presence in low amounts in the xylanase II from the parent *Bacillus* may be due to the differential processing of the enzymes in the *Bacillus* and *E. coli.* The observed differences in the properties of the recombinant xylanases could be due to nonglycosylation, and/or changes in the amino acid profile.

CHAPTER III

EXPRESSION OF *XYNII* IN *E. COLI*

SUMMARY

The 4.5 kb gene fragment coding for two xylanases from AT *Bacillus* was subcloned. The recombinant *E. coli* harbouring 1.8 kb subfragment showed xylanase activity and immunoreactivity with the polyclonal antibodies raised against purified xylanase II from AT *Bacillus*. The immunoprecipitation of the extracellular extract of the recombinant pATBX 1.8 showed the presence of single protein band of Mr 14,300. The scanning electron microscopy of recombinant *E. coli* (pATBX 1.8) showed the presence of inclusion bodies. The treatment of *E. coli* (pATBX 1.8) cells with guanidine hydrochloride (Gdn/HCl) and Triton X-100 resulted in two fold increase in xylanase activity. However higher concentrations of Gdn/HCl (upto 6 M) and urea (1 to 8 M) caused irreversible denaturation of the enzyme.

INTRODUCTION

Escherichia coli is the most commonly employed bacterial host for heterologous gene expression since a large body of information is available on its genetic and physiological characteristics. However cytoplasmic expression in *E. coli* is not always achievable without many undesirable effects. For example, foreign proteins, especially the smaller polypeptides, are often very rapidly degraded to truncated variants by intracellular proteases (Goff and Goldberg, 1987). In addition many recombinant polypeptides are unable to fold properly within the cell and associate to form large protein aggregates called inclusion bodies (IBs). Aggregates resulting from the expression of recombinant proteins have also been observed in both pro and eukaryotic cells such as *B.subtilis, Erwinia carotovora* and *Saccharomyces cerevisiae* (Bowden and Georgiou, 1990). *E.coli* as a gram-negative bacterium contains both an outer and a cytoplasmic membrane. As a consequence, a periplasmic space is created into which proteins can be secreted if they contain an N-terminal extension called a signal sequence. Many reports are available describing the presence of IBs for the enzymes that are either intracellular or secreted in periplasmic space (Marston, F. A. O., 1986) and majority of them are about β lactamase (Chalmers et al, 1990), human growth harmone (Szoka et al, 1986) and interleukin-2 (Devos et al, 1983). Despite the widespread occurrence of IBs, the mechanism responsible for their formation is not well understood. The size of IBs is probably determined by the polypeptide sequence and cellular factors. Solubilization of the aggregated proteins usually requires strong denaturating conditions.

The relation between intracellular conditions, such as ionic strength or interactions with cellular components and protein aggregation is not clear. Many of the processes that are thought to play a role in intracellular protein aggregation, such as the rate of translation and the interactions of partially folded polypeptides with chaperonins, exhibit strong temperature dependence (Chalmers et al, 1990). The choice of signal sequence for secretion affects the aggregation of mature protein. The recovery of biologically active products from the aggregated state is typically accomplished by unfolding with chaotropic agents or acids, followed by dilution or dialysis into optimized refolding buffers. To date, no definitive correlation has been established between the amino acid sequence of the protein and its propensity to aggregate *in vivo*. Molecular chaperones are known to interact with folding intermediates and facilitate their proper folding.

This chapter describes restriction analysis and subcloning of 4.5 kb gene fragment coding for xylanases. The immunological data and the molecular weight determination indicated that the recombinant *E. coli* harbouring 1.8 kb subfragment codes for xylanase II.

Vector pGEM-7Zf(+) and *E. coli* host strain (DH5α) were purchased from Promega. Ampicillin and other chemicals used were purchased from Sigma. *E. coli* (pATBX 4.5) cells were grown at 37^0 C in 250 ml Erlenmeyer flasks containing 50 ml LB with 100μg/ml ampicillin with shaking at 200 rpm.

Restriction digestion

Plasmid DNA (pATBX 4.5) was extracted and purified using Qiagen tubes (Diagen Gmb H, FRG) as suggested by the manufacturer. The insert DNA of 4.5 kb was purified using low melting agarose gel. Restriction digestion, ligation, agarose gel electrophoresis and other DNA manipulations were carried out using standard protocols (Sambrook et al, 1989).

Xylan-congo red plate clearance assay

The recombinants; *E. coli* (pATBX 1.8), were patched on LB plates containing xylan (0.5%) and ampicillin 100μg/ml. After 16 h incubation at 37^0C, 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 2mg/ml lysozyme and after the agarose was set, plates were incubated inverted at 37^0C for 30 min. to ensure the complete lysis of the recombinant colonies. The plates were then treated with 1M NaCl to visualize the possible clearance due to intracellular expression. The plates were further incubated at 50^0C for 3-4 h in order to enhance the xylan clearance.

Preparation of protein extracts from the recombinants

The culture broth of recombinant *E. coli* (pATBX 1.8) was centrifuged and the supernatant was referred to as extracellular extract. The periplasmic protein extract was prepared by lysozyme/ EDTA (ethylene diamine tetra-acetic acid) treatment as described by DePamphilis and Adler (1971). Cells were treated with 2 ml of 0.25% lysozyme in 0.1 M Tris.HCl, pH 7.8, 0.2 M NaCl and 0.1 M EDTA in 0.1 M Tris.HCl pH 7.8 and the supernatant was considered as periplasmic extract. The intracellular extracts were prepared by sonication of the cells using Virsonic sonicator at the maximum current setting. The cell suspension was chilled and while it was held on ice, three shocks of 30 sec each were applied. The care was taken to keep the suspension below 10^0C. The cell

lysate was centrifuged at 12,000 rpm for 30 min and the resulting clear solution was designated as intracellular extract.

Immunoprecipitation

The crude extract of the *E. coli* (pATBX 1.8) was immunoprecipitated using polyclonal antixylanase II antibodies. The protein extracts (50-500 μg) were mixed with the antibodies (5μl) in PBS (phosphate buffered saline). The mixture was incubated at 37^{0}C for 2 h and then centrifuged at 12,000 rpm for 15 min. The pellets were washed thrice with 200μl PBS buffer and then dissolved in 0.1% SDS solution by heating at 50^{0}C for 30 min. The resulting protein solutions were used for denaturating polyacrylamide gel electrophoresis.

Electron microscopy

The samples prepared for this were studied by the scanning electron microscope (SEM) Leica Stereoscan 440 Model. The *E. coli* (pATBX 1.8) cells were fixed o . n. in glutraldehyde fixation buffer (2% glutraldehyde in phosphate buffer). Then the cells were dried by repeated washing with increasing concentrations of alcohol (10 to 100%). The samples were then mounted on the standard specimen mounting stubs by using conducting silver paste. The samples were coated with a thin layer of gold in Polaron coating unit E5000 to prevent the charging of the specimen. For comparative study the electron beam parameters were kept constant while analyzing all the samples. The micrographs of the samples with 10KV EHT and 25pA beam current were recorded by 35mm camera attached to the high resolution recording unit.

Chemical permeabilization

Cells were recovered from fermentation broth by centrifugation at 4^0 C and washed once in cold treatment buffer (0.05 M phosphate, pH 7.0). Cells resuspended in treatment buffer were then added to flasks containing similarly cold solutions of Gdn.Hcl and/or Triton X-100, to yield final solutions with the indicated concentrations of treatment chemicals. The control consisted of periplasmic fraction obtained by lysozyme-EDTA treatment of cells. The flasks were left gently shaking at 4^0 C (o. n.). Samples were withdrawn, centrifuged, decanted and saved at 4^0 C for later assay.

Buffer composition:

PBS-Phosphate buffered saline

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Distilled water	800ml

pH adjusted to 7.4 with HCl.

H_2O was added to make the volume to 1 lit.

RESULTS AND DISCUSSION

DNA manipulations

The plasmid DNA of pATBX 4.5 was isolated and digested with Eco RI and Hind III. The insert DNA of 4.5 kb was purified by low melting agarose gel and digested with Pvu II. The gel electrophoresis of the insert cut with Pvu II showed the presence of two sub fragments of the sizes 2.7 kb and 1.8 kb (Fig. III.1).

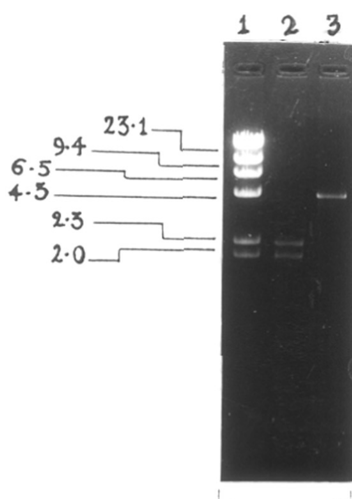


Fig. III.1 Restriction digestion of the 4.5 kb DNA insert.

Lane 1: λ Hind III marker, lane 2: 4.5 kb DNA digested with Pvu II, lane 3: 4.5 kb DNA insert,

Subcloning

The subfragments 1.8 and 2.7 kb were purified from low melting agarose gels and then mixed with suitably digested pGEM-7Zf (+) in 3:1 ratio. The recombinant *E. coli* harbouring 1.8 kb subfragment coding for xylanase is designated as pATBX 1.8 (Fig. III.3). Fig. III.2a shows vector map of pGEM-7Zf(+) and fig. III.2b shows structure of pATBX 1.8.

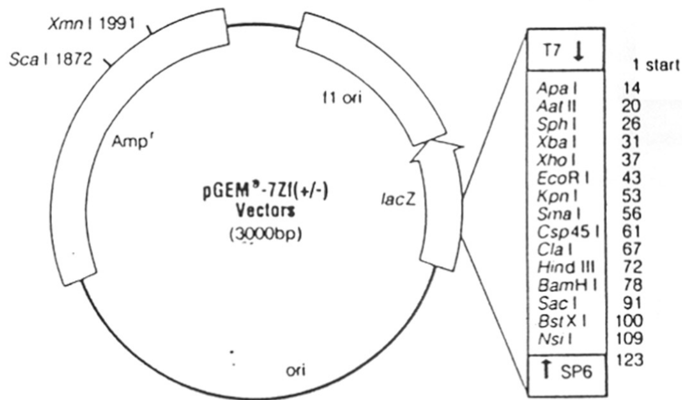
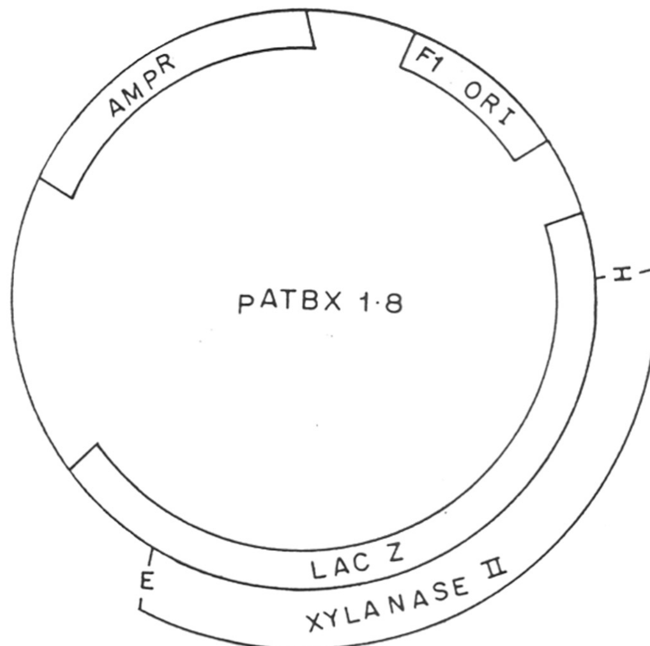


Fig. III.2 a) Vector map of pGEM-7Zf(+)



b) Map of plasmid pATBX 1.8

The *xynII* gene is contained in a 1.8 kb Eco RI-Hind III insert in the plasmid pGEM-7Zf (+). Boxes show the positions of the replication origin of F1 phage, and the genes coding for XynII, β-lactamase (Amp) and lac Z.

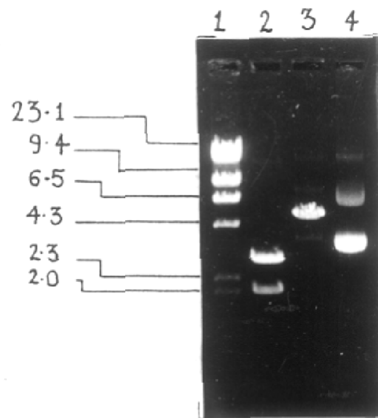


Fig. III.3: Restriction digestion of the recombinant plasmid pATBX 1.8. lane 1: lambda Hind III marker DNA. lane 2: Eco RI-Hind III digestion of the recombinant plasmid pATBX 1.8, lane 3: pATBX 1.8 linearised with Eco RI, lane 4: undigested plasmid pATBX 1.8

Expression of xylanase gene

The recombinant colonies were screened for the expression of the xylanase gene using the xylan plate clearance. The colonies were spotted on the plate at a regular distance. The *E. coli* DH5α harbouring pGEM-7Zf (+) was included as a negative control (Fig. III.4). The host strain *E. coli* DH5 α (pGEM-7zf) had no detectable intra or extracellular xylanase activity. The recombinant *E. coli* pATBX 1.8 showed maximum xylanase activity of 60mU/ml. Extracellular xylanase activity accounted for more than 60% of the xylanase production. The remaining xylanase activity was obtained in the periplasmic fraction. However intracellular extract of *E. coli* (pATBX 1.8) had no xylanase activity (Table III.1).

Table III.1

Localization of xylanase activity of the recombinant pATBX 1.8

	Xylanase (mU/µg)	% Activity
Extracellular extract	0.21	66
Periplasmic fraction*	0.01-0.06	20-40
Intracellular extract	-	nil

* 20% activity (specific activity 0.01mU/µg) was obtained in periplasmic fraction by lysozyme-EDTA method while chemical permeabilization of cells yielded 40% xylanase activity (specific activity 0.06 mU/µg).

Antixylanase analysis and molecular weight determination

The extracellular extract of recombinant pATBX 1.8 showed single precipitin line which completely fused with that of purified xylanase II from AT *Bacillus* (Fig. III.5). The immunoprecipitation of the extracellular extract of recombinant pATBX 1.8 showed the presence of single protein band of Mr 14,300 (Fig. III.6) along with two high molecular weight protein bands corresponding to heavy and light chains of Abs. *E. coli* (pGEM-7zf) and intracellular extract of the recombinant *E. coli* (pATBX 1.8) did not show precipitation with antixylanase antibodies and no protein bands were detected on SDS-PAGE. These results suggested that the recombinant pATBX 1.8 secreted xylanase and gene fragment of 1.8 kb coded only for low molecular weight xylanase from AT *Bacillus*.

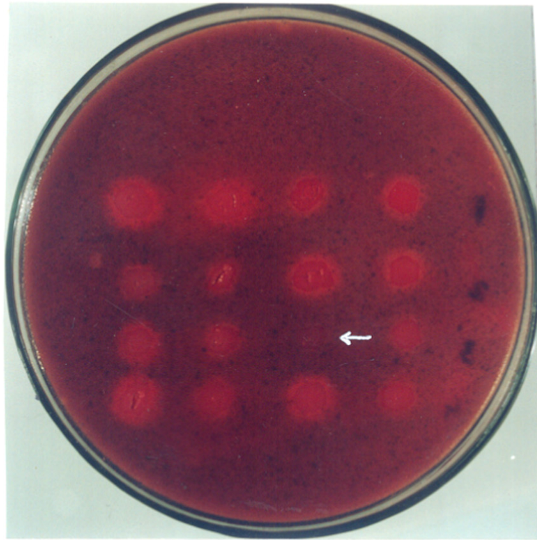


Fig. III.4 Xylan-congo red plate clearance shown by the recombinant *E. coli* (pATBX 1.8). The arrow points to the *E. coli* harbouring vector DNA (negative control). The clearance zone was observed after incubation at 37^{0}C for 18 h followed by 2 h incubation at 50^{0}C.

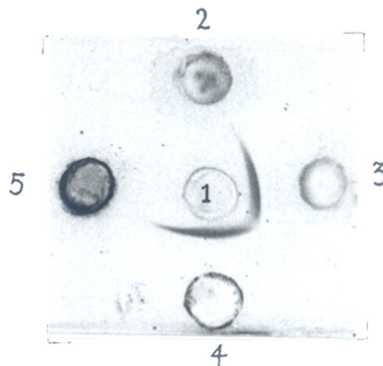


Fig. III.5: Ouchterlony double diffusion test.

1. rabbit antiserum against purified XynII, 2. extracellular extract of *E. coli* (pGEM-7Zf (+)), 3. extracellular extract of *E. coli* (pATBX 1.8) , 4. purified xylanase II from AT *Bacillus*, 5. Intracellular extract of *E. coli* (pATBX 1.8)

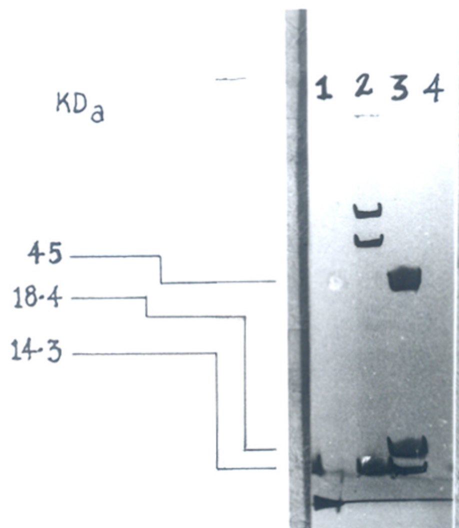
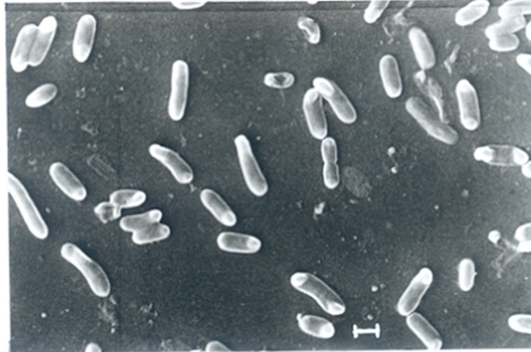


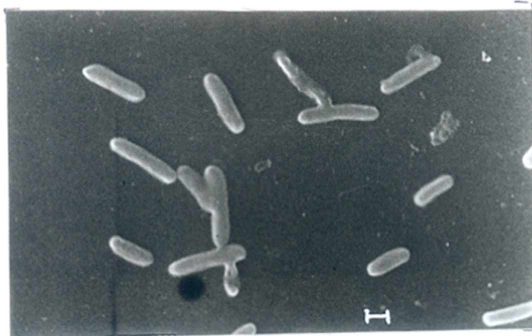
Fig. III.6: SDS-PAGE of crude culture broth immunoprecipitated with antixylanase II antibodies. lane 1: *E.coli* DH5α (pGEM-7Zf) crude broth. lane 2: Extracellular extract of *E. coli* (pATBX 1.8), lane 3: Molecular weight marker, lane 4: Intracellular extract of *E. coli* (pATBX 1.8). Arrow indicates the dye front.

Scanning electron microscopy of E. coli (pATBX 1.8)

The recombinant *E. coli* (pATBX 1.8) cells were examined by scanning electron microscope (SEM). The inclusions were observed in recombinant *E. coli* cells. They were irregular with a semi-spherical appearance. These inclusions were not observed in the nonplasmid-containing strain of *E. coli* or in *E. coli* containing parental plasmid vector pGEM-7Zf (+) (Fig. III.7). The morphology of the cells producing recombinant xylanase was distinct from that of the nonplasmid-containig cells in that the edges of the former were filled with a moderately electron opaque material.



a



b

Fig. III.7 Scanning electron micrograph of

a) *E. coli* (pATBX 1.8) cells fixed in late log phase of growth. Electron dense area is a periplasmic IB. The bar represents 1 μ (x5000).

b) Cells of *E. coli* strain containing vector DNA. IBs are not observed in such cells (x5000)

The size of IBs is probably determined by the polypeptide sequence and cellular factors. It is suggested that the formation of protein aggregates depends on the protein synthesis rate and amino acid sequence of the leader peptide; which directs transport across the cytoplasmic membrane. Aggregation can be prevented by growing the cells in the presence of non-metabolizable saccharides or at low temperatures. At present there is relatively little information regarding folding and assembly of secreted proteins in prokaryotes. In the cytoplasm the precursor form of secreted polypeptides must be maintained in a conformation competent with export. At least two elements of secretory process serve to modulate the tertiary structure of the precursor. 1) the presence of the signal sequence which in addition to its other roles, also serves to retard folding and 2) interactions with chaperonins such as Gro EL, Gro ES, trigger factor and Sec B (Thomas and Baneyx, 1996). The effect of signal sequence on the folding pathway of the mature protein may be explained by 2 different mechanisms viz. 1) depending on the leader peptide, the precursor may be exported through separate pathways, involving different components of the secretory system 2) signal sequence influences folding of the mature protein, which in turn, leads to aggregation (Bowden and Georgiou, 1990).

There is strong, although not conclusive, evidence to suggest that protein aggregation is a highly specific process mediated by specific amino acid interactions and is also dependent on the growth conditions (Valax and Georgiou, 1993). β-lactamase can form IBs in either the periplasmic space or the cytoplasm in *E. coli* depending on whether it is expressed with or without signal sequence. The increased rate of protein synthesis in case of β-lactamase most likely leads to accumulation of a putative folding intermediate which is prone to intramolecular association. Although, the conformation of the aggregated protein appears to be similar, the IBs exhibit marked differences in morphology, in their resistance to protease digestion and in solubilization by denaturants (Bowden et al, 1991). Indirect evidence suggests that the conformation of different proteins in the IBs can vary from a native-like state to completely misfolded molecules.

Analysis of aggregated proteins

The strain studied was *E. coli* (pATBX 1.8), producing XynII from AT *Bacillus*. The treatment of *E. coli* (pATBX 1.8) cells with Gdn (upto 1 M) resulted in two fold increase in xylanase activity. A significant % of overall protein was extracted with increasing concentrations of Gdn combined with 0.5% TritonX-100 (Fig. III.8). However further increase in Gdn concentration (upto 6M) and treatment with urea (1-8M) yielded no xylanase activity.

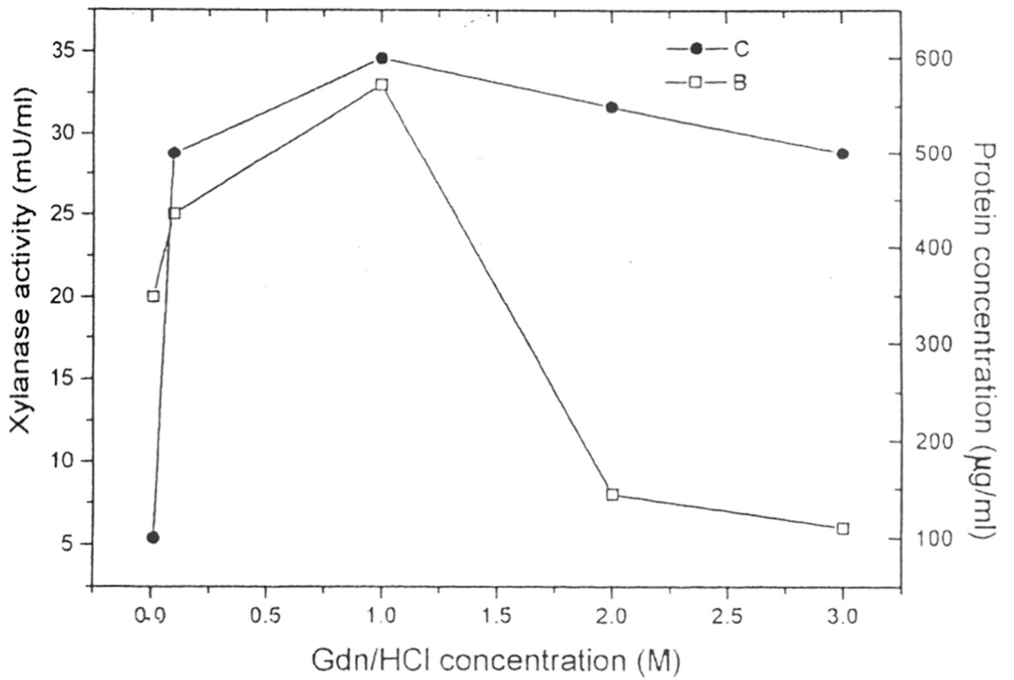


Fig. III.8 Overall protein release (●) and xylanase activity (□) from *E. coli* (pATBX 1.8) by solubilization of IBs as a function of Gdn concentration at constant 0.5% TritonX-100.

Gdn is a chaotropic agent capable of solubilizing some normally hydrophobic compounds, perhaps due to its interactions with the hydrogen bonding of water. Triton X-100 is a nonionic detergent known for its ability to solubilize phospholipid bilayer membranes. Gdn may disrupt the outer membrane exposing the inner membrane to attack by Triton X-100 (Naglak and Wang, 1990). We observed that chemical permeabilization of *E. coli* (pATBX 1.8) cells with Gdn and TritonX-100 yielded twofold increase in xylanase activity as compared to that obtained from lysozyme-EDTA treated cells. This may be due to solubilization of periplasmic IBs. This observation also indicates that recombinant xylanase exist in the aggregated form (IBs) which is also evident by SEM. It can be tentatively concluded that the interactions leading to the association of the polypeptide chains in the periplasmic space are weaker and are more readily disrupted by denaturants. It is found that the yield of enzymatically active β-lactamase upon Gdn/HCl solubilization and renaturation of IBs is generally low, whereas under the same experimental conditions the purified protein can be refolded quantitatively. This may be due to the presence of nonpolypeptide components, which interfere with protein refolding from IBs.

A potential solution to preventing the entrapment of secreted proteins in the membrane fraction is the use of periplasmic-leaky mutants as hosts. These mutants, which may be induced by ultraviolet irradiation or nitrosoguanidine treatment, release periplasmic enzymes directly into the extracellular milieu.

CHAPTER IV

EXTRACTIVE CULTIVATION OF RECOMBINANT *E. COLI* USING AQUEOUS TWO PHASE SYSTEM

SUMMARY

The recombinant *E. coli* (pATBX 1.8) secreting extracellular xylanase was used as a model system to study the application of aqueous two phase system for the extractive cultivation. An increase in the polymer concentrations from 6 to 20% in the polyethylene glycol - phosphate aqueous two phase system, resulted in an increase in the phase volume ratio with a concomitant decrease in the partition coefficient (K) and recovery of xylanase in the top phase. However varying phosphate concentrations from 8 to 16% decreased both the phase volume ratio and partition coefficient of xylanase. The polyethylene glycol (6%) and phosphate (12%) system was found to be optimum for extractive cultivation of *E. coli* where extracellular xylanase was selectively partitioned to the top phase giving a purification ratio of above 1.0 . The process was extended to semicontinuous operating mode at the optimal condition, wherein the top phase containing xylanase was recovered and the surviving cells were recycled together with the new top phase. The maximum recovery of xylanase was obtained after 12 h in the top phase with a two-fold increase in the specific activity as compared to the one obtained in the reference fermentation. The present chapter describes the use of two phase system for the extractive cultivation of recombinant *E. coli* (pATBX 1.8) with the purpose to obtain a simple and inexpensive separation procedure and to achieve the maximal extraction of xylanase to one phase.

INTRODUCTION

Recent developments in biotechnology have opened up new avenues toward the production of many biomolecules of importance for research, pharmaceutical/clinical, and industrial usage. The separation of compounds in conventional chemical processes such as centrifugation, electrophoresis or column chromatography, scale up problems are considerable, making them uneconomical unless the product is of high value. Therefore, there is a need for alternative approaches to the problem. Aqueous two phase extraction (ATPE) is one such method and is recognized as a potential and powerful primary purification step in the overall enzyme/protein recovery train. The selective partitioning of product in one phase yields the product in a concentrated form, thus reducing volume to be handled in the following purification steps. Further it offers high yields and selectivity, low process time and energy, easy scale up and continuous operation. Aqueous two phase system (ATPS) constitute mild environmental conditions for biological material due to the high water content and the low surface tension between the phases. An important feature of ATPE is that partitioning of enzymes/proteins, in general, does not depend on their concentration and the volume of the system over a wide range. Scaling up of the process does not change the conditions considerably. The use of polymer aqueous two phase systems for the partition of various macromolecules has been thoroughly investigated by Albertson (1971). ATPS are mainly of two types, polymer-polymer and polymer-salt. Much research has been devoted to extractive bioconversion such as the production of biosurfactant (Drouin and Cooper, 1992) and intracellular enzymes (Tanaka et al, 1993) from *Bacillus subtilis.* However little attention has been given to extractive cultivation in PEG/ salt media, since the high salt concentration for this system are thought to inhibit the growth of normal cells such as *B. subtilis* and *E. coli* (Stanier et al, 1979).

This chapter describes the use of polymer ATPS for extractive cultivation of recombinant *E. coli* (pATBX 1.8) for the production and separation of xylanase.

MATERIALS AND METHODS

E. coli (pATBX 1.8) has been described in chapter III. It was grown on LB plates or in Luria Bertani broth at 37^0C for 18 h with shaking (200 rpm) in the presence of ampicillin (100 μg/ml).

Poly ethylene glycol (PEG 6000) and ampicillin were obtained from Sigma Chemical Company. The salts and other chemicals used were of analytical grade.

Cultivation

Escherichia coli (pATBX 1.8) was grown at 37^0C with shaking for 18 h in Luria broth (LB) containing ampicillin (100μg/ml). Xylanase activity was estimated as described in chapter II. One miliunit (mU) of xylanase activity was defined as the amount of enzyme that produced 1nmol of xylose equivalent per minute from xylan under the assay conditions. The protein concentration was measured according to Bradford (1976). The cell concentration was determined by measuring the optical density of the culture broth at 660 nm.

Two-phase systems

PEG 6000/ phosphate aqueous two phase medium was utilized in the cultivation of *E. coli* (pATBX 1.8) cells. The phosphate salt KH_2PO_4-Na_2HPO_4 in a weight ratio of 3:7 were used as the phase forming component. PEG 6000 was taken as % w/v basis. In all partition experiments, the top and bottom phases were separated using a separating funnel and the volumes of the two phases were determined. The enzyme activity and protein concentration of both the phases were estimated. On the basis of these data the partition coefficient (K), and yield coefficient (Y_b) of the enzyme, as well as the phase ratio (R), were determined as described by Huddleston and Lyddiatt (1991).

$K = C_t / C_b$

where C_t and C_b are concentrations of the enzyme in the top and bottom phases respectively.

$Y_b = 100/1 + [(1/R)x(1/K)]$

and

$R = V_t/V_b$

where V_t and V_b are the volumes of the top and the bottom phases respectively.

Purification ratio is defined as the ratio of the % recovery of enzyme and total proteins in the top phase.

Semicontinuous operation

The recombinant *E. coli* cells were grown in LB (100 ml) containing PEG and phosphate. After every 4 h the upper phase was carefully recovered and stored. A new top phase was added and the bottom phase counting surviving *E. coli* cells was reused.The system was mixed, the phases separated and the top phase removed as before. This procedure was repeated four times. The concentration of xylanase and total protein in both the phases was determined after each extraction.

RESULTS AND DISCUSSION

Growth behaviour of E. coli (pATBX 1.8)

The growth rate of *E. coli* (pATBX 1.8) was initially lower in the PEG 6% phosphate (12%) system as compared to that in LB (Fig. IV.1) However it was observed to be higher during the log phase. Replacing NaCl with Na_2SO_4 (1 to 5%) had no effect on growth pattern. It might be due to the time needed for the organisms to adapt to polymer rich media and also PEG is known to contain some inhibitory compounds which may exert toxic influence on cells. Prolongation of lag phase has been earlier observed for cells in aqueous two phase systems (Larsson et al, 1988).

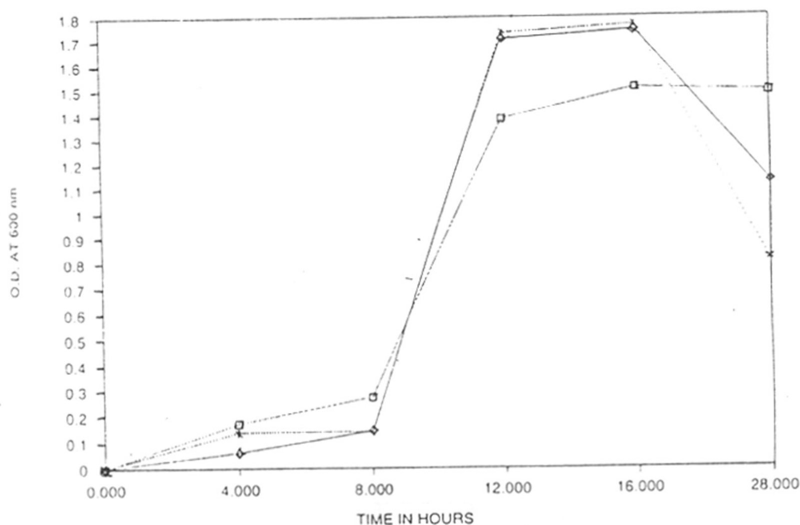


Fig. IV.1 Growth curve of *E. coli* (pATBX 1.8). Cells were grown in LB (-□-), LB containing 6% PEG and 12% phosphate (-x-), LB containing 6% PEG and 12% phosphate where 1% NaCl was replaced by 1% Na_2SO_4 (-◇-).

Biphasic systems

E. coli (pATBX 1.8) was grown in LB containing 10% phosphate, while PEG concentration was varied from 6 to 20% (Fig. IV.2a). Maximum purification ratio of 1.18 was obtained at 6% PEG concentration at which volume of the top phase was minimum (5ml). In another experiment *E. coli* cells were grown in LB at 6% PEG concentration, while salt concentration was varied from 8 to 18% since at 6% salt concentration phases could not separate. The specific activity of xylanase was maximum in top phase in the phase system consisting of 6% PEG and 12% salt. Maximum purification ratio of 1.21 was obtained at 12% salt concentration (Fig. IV.2b). Increase in salt concentration resulted in decrease in the volume of the top phase.

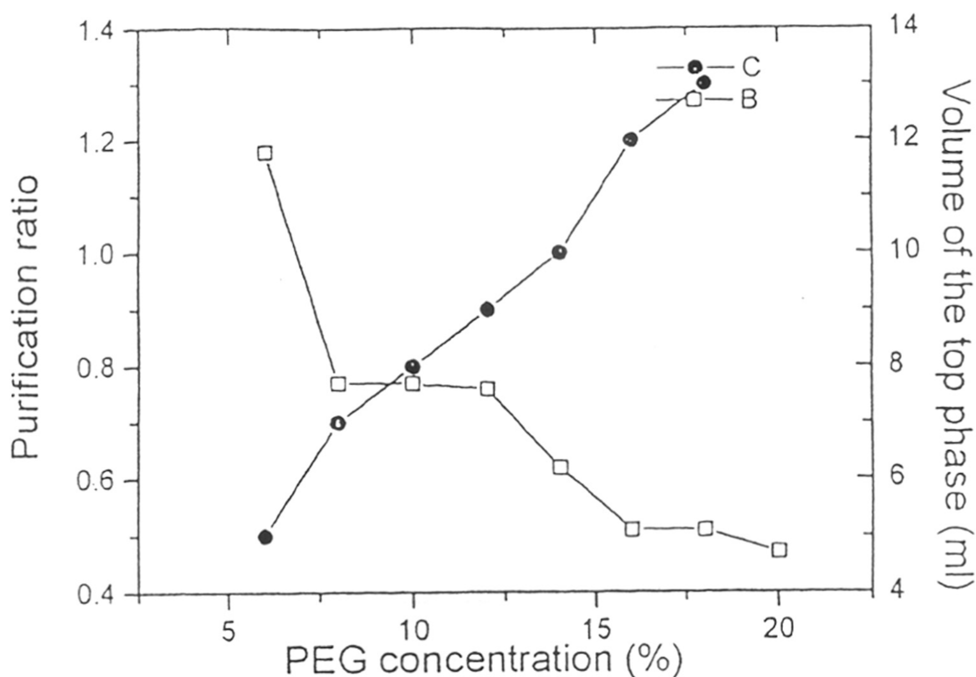


Fig.IV.2: (a) Effect of PEG concentration on purification ratio of xylanase (-□- B) and volume of the top phase (-●- C). *E. coli* (pATBX 1.8) cells were cultivated in a biphasic system consisting of 10% phosphate salt, while PEG concentration was varied from 6 to 20%.

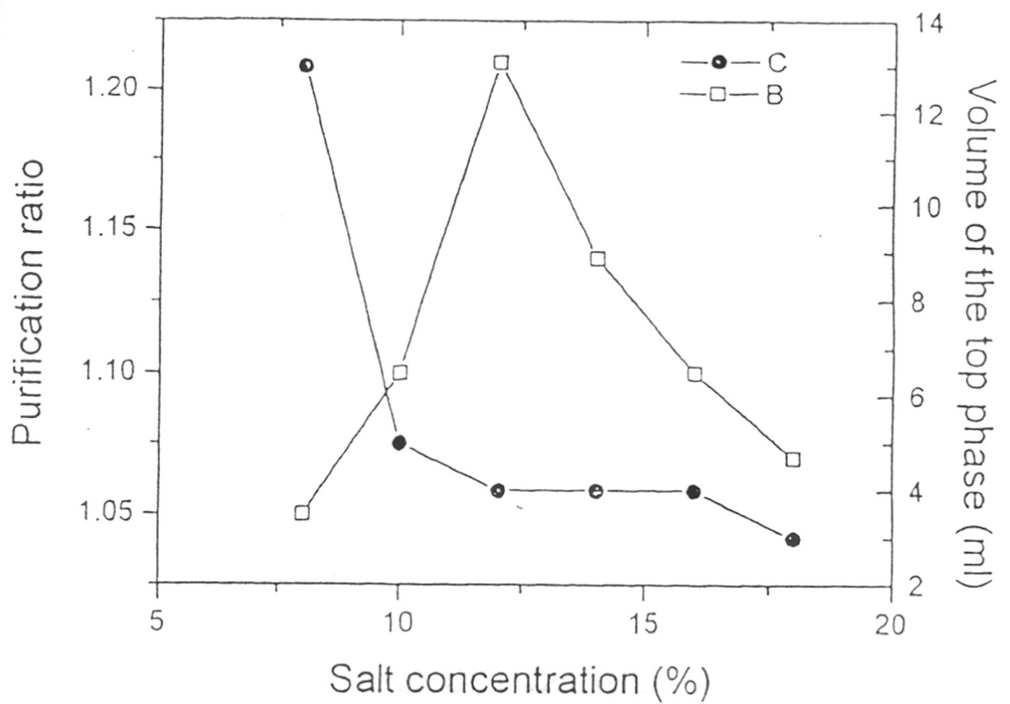


Fig. IV.2 (b) Effect of salt concentration on purification ratio of xylanase (-□- B) and volume of the top phase (-●- C). *E. coli* (pATBX 1.8) cells were cultivated in a biphasic system consisting of 6% PEG, while salt concentration was varied from 8 to 18% .

Phase volume ratio was found to increase with increasing polymer concentration while partition coefficient of xylanase decreased when concentration of PEG was increased from 6 to 20%. At PEG concentration of 6% the partition coefficient was 1.4 (Fig. IV.3a). As against this both, the phase volume ratio and partition coefficient of xylanase decreased with increasing salt concentration (Fig. IV.3b).

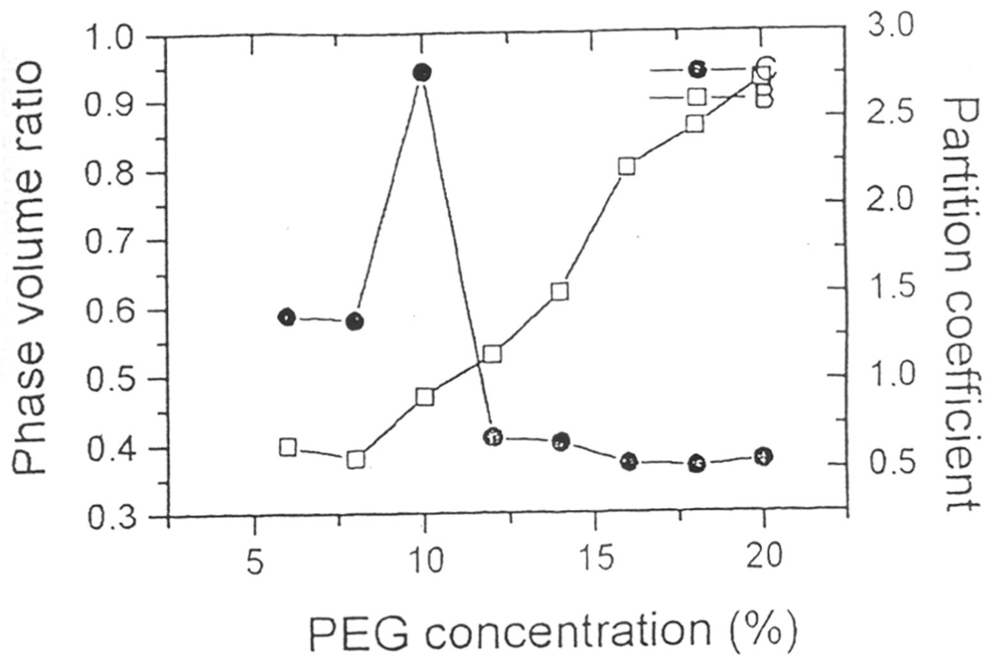


Fig.IV.3: (a) Effect of PEG concentration on phase volume ratio (-□- B) and partition coefficient (-●- C) of xylanase.

Cultivation coditions of *E. coli* (pATBX 1.8) were as described for fig. IV.2 (a).

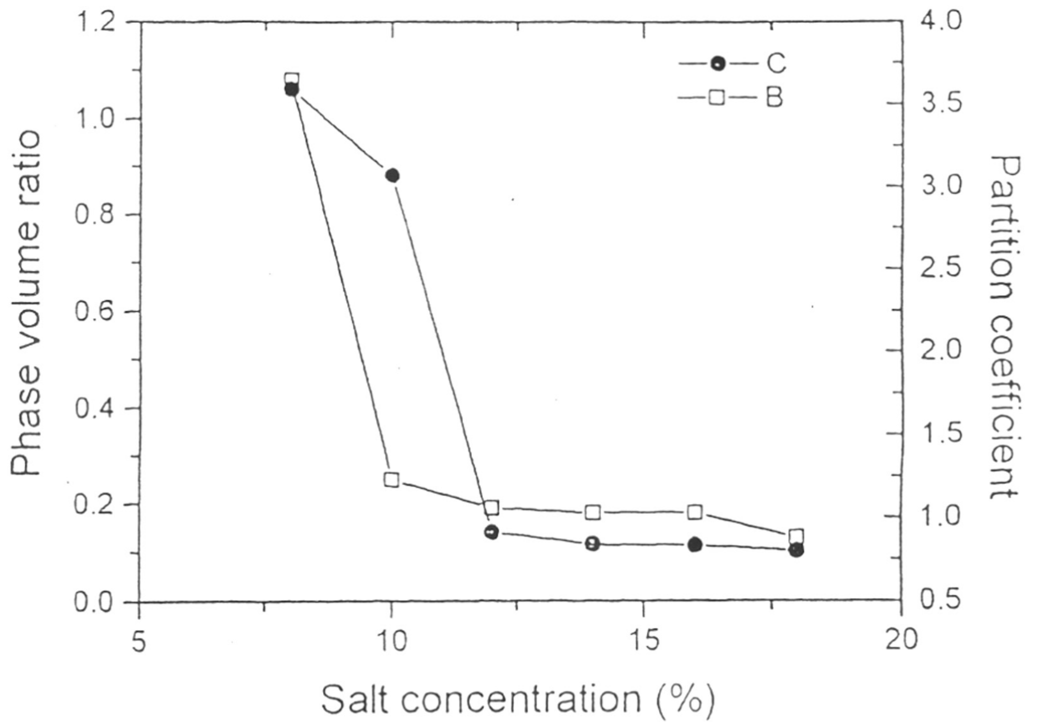
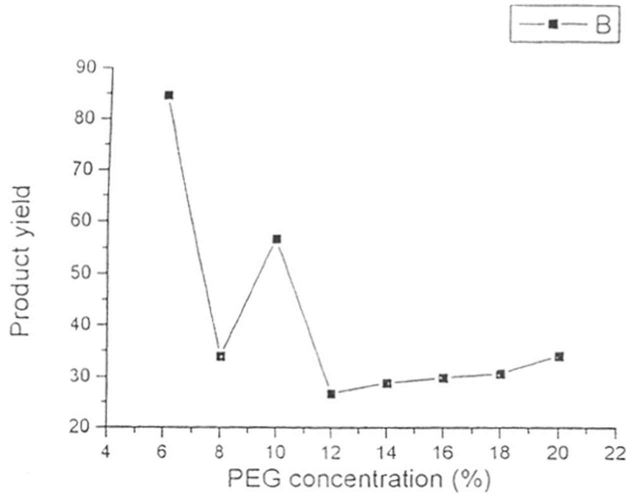


Fig. IV.3 (b) Effect of salt concentration on phase volume ratio (-□- B) and partition coefficient (-●- C) of xylanase.

Cultivation conditions of *E. coli* (pATBX 1.8) were as described for fig.IV.2 (b).

The product yield was found to be maximum at 6% PEG and 10% phosphate concentration (Fig. IV.4a) Increase in salt concentration caused decrease in the product yield (Fig. IV.4b).



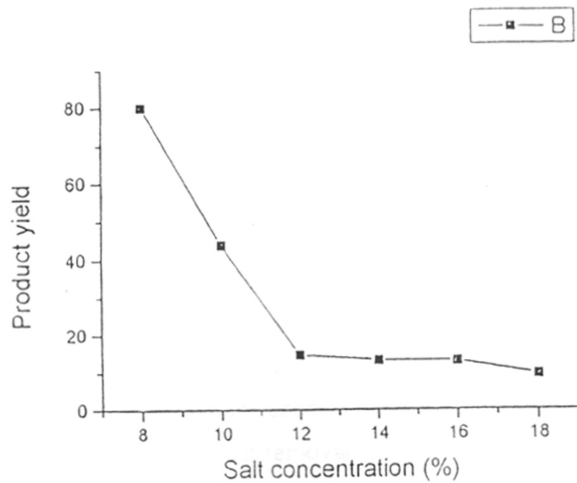


Fig.IV.4: (a) Effect of PEG concentration on product yield (-■- B).

(b) Effect of salt concentration (b) on product yield (-■- B).

Cultivation conditions of *E. coli* (pATBX 1.8) were as described for fig.IV.2(a) and (b) respectively.

Kuboi et al (1995) have reported extractive cultivation of *E. coli* in ATPS to produce intracellular β-galactosidase. The PEG/ phosphate system was optimized with added Na_2SO_4 for extractive cultivation and enzyme was partitioned to the top phase while total soluble proteins and cells partitioned to the bottom phases observed in the case of *E. coli* (pATBX 1.8). ATPS has also been used earlier by Veide et al (1983) for large scale isolation of β-galactosidase from a suspension of disintegrated *E. coli* cells wherein 75% of the enzyme was recovered in the lighter PEG phase. Purification ratio was found to increase with decreasing phase volume ratio. However we observed that both , the purification ratio and phase volume ratio decreased with increasing polymer concentration. Partitioning of α-amylase to the dextran rich bottom phase has been reported by Park and Wang (1991) wherein cells also preferentially partitioned to the dextran phase in both, batch and continuous fermentations.

The mechanism for the protein behavior in a PEG-salt two phase system has been suggested by Busby and Ingham (1980) to be that the two phase system provides an opportunity for proteins to lower their chemical potential by escaping into the bottom salt phase when the PEG concentration in the top phase is increased. In a PEG solution of the corresponding concentration, they would be precipitated. One possible explanation, why xylanase remained in the PEG phase could be the hydrophobic interactions. The distribution of xylanase among the two phases appeared to be dependent on the composition of phase system and chemical nature of the enzyme. Thus this fast and efficient isolation step in an ATPS, using inexpensive chemicals and conventional techniques, provides a suitable basis for further purification.

Semicontinuous operation

E.coli (pATBX 1.8) was inoculated in LB containing 6% PEG and 12% phosphate . After every 4h the top phase was recovered and analyzed for xylanase activity and protein concentration (Table IV.1). The equal volume of top phase was replaced and fermentation was continued till 16 h. Maximum specific activity in the top phase was obtained at the end of 12 h. Integration of product removal with the fermentation might have reduced the end product inhibition and thus improved the productivity. The repeated use of cells seemed to improve their catalytic efficiency.

Table IV.1

Semicontinuous extraction in phase system

Time (h)	Recovery in top phase (%)	Specific activity Top Phase (U/mg)	Specific activity Reference (U/mg)
4	57	13	10
8	70	19	15
12	100	44	21
16	73	30	25
18	50	9	8

Culturing of bacterial cells in two phase system has been carried out earlier e.g. *A. simplex* (Kaul and Mattiasson, 1986). The toxin was produced by *Cl. tetani* cultured in a phase system containing 2% dextran and 12% PEG (Mr 4000). The total yield of toxin was much higher when produced in this manner as compared to the production in conventional medium (Mattiasson, 1983). Similarly large scale isolation of β galactosidase from *E. coli* cells using PEG 6000 and K-PO_4 is reported (Veide et al, 1983).

The effect of the phase forming polymers on the growth rate of recombinant *E. coli* and xylanase extraction in an ATPS was examined. Aqueous polymer two phase systems have been used for the separation of a variety of biological macromolecules, cell organelles, cells and viruses. Also, polymer seemed to have stabilizing effect on cell organelles. The use of ATPS for large scale isolation of enzymes has been developed by Kula et al (1981). Examples of extractive bioconversions in such systems include ethanol production from glucose and starch, acetonebutanol from glucose, hydrolysis of casein proteins etc. (Kwon et al, 1996).

Cellulolytic enzyme production in ATPS composed of PEG 8000 5% and dextran T500 7% with *T. reesei* Rut C30 (Persson et al, 1991) has been reported. With *B. subtilis* (Andersson et al, 1985) and *Asp. phoenicis* (Persson et al, 1989) an enhanced enzyme production was observed. Recently partitioning of xylanase produced by *Penicillium janthinellum* in an ATPS using PEG and phosphate was reported by Costa et al (1998). The extractive cultivation of recombinant *E. coli* cells to produce , release and separate heat shock proteins using PEG/ dextran ATPS was developed by Umakoshi et al (1996).

The partition coefficients of HSPs were found to improve to greater values when phosphate salts were added at a concentration of more than 0.1 M. However, so far there is no report on the use of ATPS for extractive cultivation of recombinant *E. coli* producing xylanase. Thus ATPS offer an interesting alternative to conventional cultures. An environment containing high concentration of polymer per se may stabilize biological structures- a fact that may be better exploited in the future.

CONCLUSION

The extractive cultivation process of recombinant *E. coli* cells using ATPS for the production and separation of extracellular xylanase has been developed. In two phase, batch fermentation, a lower maximum cell density, longer lag phase and generally higher xylanase activity were observed in the presence of PEG. The rate of xylanase synthesis decreased at high concentrations of PEG. The salt-rich bottom phase's affinity for cells was exploited in the design of semicontinuous cell recycle system in which the bottom phase was reused and the virtually cell-free top phase is withdrawn for further product purification. This could have application in affinity partitioning for the purification of xylanase e.g. by attaching a specific affinity ligand to the high molecular weight PEG in the two-phase system, the loss of enzyme to the bottom phase could be minimised and the recovery of xylanase improved.

CHAPTER V

NUCLEOTIDE SEQUENCE ANALYSIS OF XYLANASE GENE (*XYNII*)

SUMMARY

The1.8 kb gene fragment from AT *Bacillus* was found to code for a functional xylanase (XynII) in *E. coli.* The complete sequencing of 1.8 kb gene fragment was carried out using the deletents obtained by exonuclease III. The nucleotide sequence of the insert including the structural gene and the 5' and 3' flanking sequences has been determined (GenBank accession no. U83602) .An open reading frame starting from ATG initiator codon comprising of 402 nucleotides gave a preprotein of 133 amino acids of calculated molecular weight 14.09 kDa. The molecular mass was in close agreement with that of the glycosylated mature protein from the parent *Bacillus* (15.8 kDa). The stop codon was followed by hairpin loop structures indicating the presence of transcription termination signals. The occurrence of three potential N-glycosylation sites in *xynII* gene was an unique feature especially of bacterial origin. The secondary structure analysis of XynII predicted that the polypeptide was primarily formed of β-sheets. XynII appeared to be a member of family G/11 of xylanases based on its molecular weight and basic pI (8.0). However, sequence homology revealed a striking similarity to family 10 xylanases. The xylanase from AT *Bacillus* appears to link the xylanases from neutrophilic and alkaliphilic organisms through the conserved motif (-SQTN-) that starts at an amino acid residue position 39 in XynII. The conserved triad (-VVX- where X is N or D) identified only in the xylanases from alkaliphilic organisms may play an important role in determining the functional conformation and activity at alkaline pH. Our results implicate the concept of convergent evolution for XynII and provide basis for research in evolutionary relationship among the xylanases from alkaliphilic and neutrophilic organisms.

INTRODUCTION

Xylanases from extremophilic organisms have gained more importance due to their stability at high temperature and pH which are the prereqisites for commercial applications. Analysis of gene sequence leads to a better comprehension of the transcription regulatory mechanism and is the first step for application oriented protein engineering studies. A plethora of nucleotide squences of xylanase genes from various thermophilic organisms such as *Caldocellum saccharolyticum* (Grepinet et al., 1988) , *Bacillus stearothrmophilus T-6* (Gat et al., 1994), *Clostridium stearcorarium* (Baba et al., 1994), *Thermomonospora fusca* (Irwin et al., 1994), *Dictyoglomus thermophilum* (Gibbs et al., 1995) and *Streptomyces thermoviolaceus* (Tsujibo et al., 1997) are available. However a few xylanase sequences from alkaliphilic bacteria such as alkaliphilic *Bacillus* strain C - 125 (Hamamoto et al., 1987) and *Bacillus* sp. N 137 (Tabernero et al., 1995) are reported. So far there is no report on xylanase gene sequence from an alkaliphilic thermophilic organism .

A number of reports on sequence homology of xylanases are documented. In general, sequences from the same family (10 or 11) were closely related and exhibited more homology. This criterion has been used to assign particular sequences to particular families. Xylanase A from *B. stearothermophilus* 21 was 45-50% identical to xylanases from other thermophilic organisms such as *C. sacchrolyticum* and *C. thermocellum* (Baba et al, 1994). Xylanase C (acid xylanase) from *A. kawachii* showed 40-50% homology with xylanases from *B. pumilus, B. circulans* and *C. acetobutylicum.* However, Xyn C from A. kawachii showed no significant homology with xylanase A from the same organism and glucanases from other filamentous fungi (Ito et al, 1992). The xylanases from thermophilic organisms such as, xylanase A from *C. sacchrolyticum* (Luthi et al, 1990), xylanase B from *C. stercorarium* (Fukumura et al, 1995) and xylanase T-6 from *B. stearothermophilus* T-6 (Gat et al, 1994) showed high homology to xylanase A from alkaliphilic *Bacillus* C-125. *B. steararothermophilus* xylanase T-6 showed comparatively less homology to other xylanases from thermophilic organisms, such as *Clostidium sacchrolyticum, T. sacchrolyticum* B6A-RI. Domain A of xylanase C from *F. succinogenes* showed more homology with fungal ruminal enzyme, whereas domain B exhibited similarities with the bacterial enzymes. It was suggested that these organisms were found in environmental niches totally different from the ruminal organisms, suggesting that their catalytic domains might have had a common origin but they had diverged a long time ago. On the other hand, the enzymes from the three ruminal organisms (*R. flavefaciens, N. patriciarum*

and *F. succinogenes*) shared similar multiple catalytic domain structure, which might stem from environmental selection (Paradis et al, 1993). The structures of xylanases from the two strains of the *P. ruminicola* represent a departure from the recognised evolutionary route for xylanases and other glycoside hydrolases, which is thought to have involved reassortment of integral domain blocks associated with catalytic, substrate binding and other functions.

The modular pattern found in sequence of XynZ from *C. 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). Xyn Y from *C. thermocellum*, in common with xylanases from several thermophilic bacteria, contained a family 10 catalytic domain. This observation could reflect the close evolutionary relationship between thermophilic bacteria. Thus the evolution of xylanase activity might have risen through the lateral transfer of a single ancestral gene between thermophilic bacteria.

This chapter describes the primary structure of xylanase II from the AT *Bacillus* and its comparison with other xylanases. The evolutionary relationship among xylanases from neutrophilic and alkaliphilic organisms is discussed.

MATERIALS AND METHODS

The cultivation of AT *Bacillus* and *E. coli* (pATBX 1.8) are described earlier.

DNA Preparations

Plasmid DNA was purified using Quagen columns (Diagen, GmbH, FRG) following the protocol recommended by the manufacturer. Transformation of *E. coli*, agarose gel electrophoresis and the general use of nucleic acid modifying enzymes were as described by Sambrook et al (1989).

Genration of unidirectional deletions with Erase-a-Base system

A series of nested deletions of pATBX 1.8 was constructed using the exonuclease III / S1 nuclease kit supplied by Promega (Promega Corp. Madison, WI). 10 μg of recombinant DNA (pATBX 1.8) was digested to completion with Sac I (generates exonuclease III resistant 3' overhang) and HindIII (generates 5'overhang). The digested DNA was purified using phenol/chloroform. The DNA pellet was dried under vacuum and then resuspended in 60μl of Exo III 1X buffer. Meanwhile, 7.5 μl of S1 nuclease mix was added to each of 24 small tubes and left on ice. The DNA tube was warmed to the digestion temperature in water bath. 300-500 units of ExoIII were added mixing as rapidly as possible (Digestion proceeds at about 450 bases/minute at 37^{0}C). 2.5 μl of samples were removed at 30 second intervals into the S1 tubes on ice, pipetting up and down briefly to mix. After all the samples have been taken, the tubes were moved to room temperature for 30 minutes. Then 1μl of S1 stop buffer was added and tubes were heated at 70^{0}C for 10 minutes to inactivate the S1 nuclease. The samples from each time point were transferd to 37^{0}C and 1μl of Klenow mix was added to each sample. Samples were incubated for 3 minutes and then 1μl of the dNTP mix was added. Further the samples were incubated for 5 minutes at 37^{0}C. The samples were then moved to room temperature and 40μl of ligase mix was added to each sample. The samples were mixed well and incubated at room temperature for 1 h and then used for transformation of competent *E. coli* (DH5α) cells.The plasmid DNAs were isolated from the transformants obtained and the sizes of linearised plasmids were analysed by agarose gel electrophoresis. The plasmids with decreasing insert sizes were used for sequencing.

Sequencing using T7 DNA polymerase (Sequenase version 2.0)

The nested deletions were sequenced using Sequenase 2.0^{TM} dye-deoxy terminator sequencing kit supplied by USB. Labeling was done with ^{35}S-dATP (Amersham). The double-stranded DNA templates (3-5μg) were denatured by alkaline-denaturation method (Lim and Pene, 1988). The DNA pellet was redissolved in 7μl of distilled water. 2μl of

sequenase reaction buffer and 1μl of primer were added. Annealing was done by warming the tubes to 37°C for 15-30 minutes. The tubes were centrifuged briefly and left on ice. While cooling, the tubes were labeled, filled and capped with 2.5 μl of each termination mixture (G, A, T, C) and the tubes were prewarmed in 37°C bath. To the ice-cold annealed DNA mixture (10μl), DTT, 0.1 M (1μl), diluted labeling mix (2μl), [^{35}S]dATP (0.5μl), diluted sequenase polymerase (2μl) were added. After mixing the tubes were incubated at room temperature for 2-5 minutes. 3.5μl of labeling reaction was trasfered to each termination tube (G, A, T, C), mixed and incubation of the termination was continued at 37°C for 5 minutes. The reactions were stopped by adding 4 μl of stop solution. Samples were heated to 75°C, 2 minutes immediately before loading onto sequencing gel. 2-3 μl were loaded in each lane.

Sequencing using Vent DNA polymerase

To determine the sequence in the region of nucleotide bp 300 to 400 the primer was synthesized as follows:

5' CTG CTT CTC GCC GAA ACG TTT GGT 3'

The sequencing was carried out using Circumvent DNA sequencing kit from New England Biolabs (UK) where the synthesized oligo was used. Reaction was performed as follows:

DNA	3μl
Primer	1.5 μl
10X buffer	1.5 μl
30 X Triton X-100	1 μl
Water	5 μl
^{35}S	2 μl
Vent DNA polymerase	1 μl

3.5 μl of this mixture was added to each of the four termination tubes labelled as A, G, C and T. One drop of mineral oil was added and polymerase chain reaction (PCR) was run as follows:

Denaturation at 75°C for 20 minutes.

Annealing at 55°C for 20 minutes.

Plolymerization at 72°C for 20 minutes.

After running PCR, 4 μl of stop solution was added to each tube and the samples were then used for sequencing.

Denaturating gel electrophoresis

For all sequencing reactions 6% gels were used.

Gel recipe (for 100 ml gel solution):

Acrylamide/Bis-acrylamide	5.7g/0.3 g
Urea 7-8.3 M	42-50 g
H_2O	~45 ml

After dissolving, the volume of the gel solution was adjusted to 100 ml with H_2O, filtered and degassed. At the time of pouring 1 ml of 10% ammoniun persulphate and 25 μl of TEMED (N, N, N', N' tetramethyl-ethylenediamine) were added. Gels were prepared 2-20 hours prior to use and pre-run for 15-60 minutes. Gels were soaked after running in 5% acetic acid and 15% methanol to remove the urea.The gels were then dried at 80^{0}C. For ^{35}S gels, exposure was done with direct contact between the dried gel and the emulsion side of the film. In general, overnight to 36 hour exposures were sufficient when using fast film (Hyperfilm-MP).

Buffers and solutions

Exo III 10X buffer:	**Klenow buffer:**
660 mM Tris-Hcl, pH 8.0	20mM Tris-HCl, pH 8.0
6.6 mM $MgCl_2$	100mM $MgCl_2$
S1 7.4X buffer:	**S1 nuclease mix** (for 25 time points)
0.3M potassium acetate, pH 4.6	172μl deionized water
2.5M NaCl	27μl S1 7.4X buffer
100mM $ZnSO_4$	60u S1 nuclease
50% glycerol	S1 stop buffer
dNTP mix:	0.3M Tris base
0.125mM each of dATP, dCTP, dGTP and dTTP	0.05M EDTA
Klenow mix:	**Ligase mix:**
30μl klenow buffer	790μl deionized water
3-5u klenow DNA polymerase	100μl ligase 10X buffer
Ligase 10X buffer:	100μl 50% PEG
500mM Tris-HCl, pH 7.6	10μl 100mM DTT
100mM $MgCl_2$	5u T_4 DNA ligase
10mM ATP	

Sequenase buffer (5X)	**Labeling mix (dGTP) (5X)**
200mM Tris-HCl, pH 7.5	7.5μM dGTP
100mM $MgCl_2$	7.5μM dCTP
250mM NaCl	7.5μM dTTP

ddG Termination mix	**ddA Termination mix**	**ddT Termination mix**	**ddC Termination mix**
80μM dGTP	80μM dGTP	80μM dGTP	80μM dGTP
80μM dATP	80μM dATP	80μM dATP	80μM dATP
80μM dCTP	80μM dCTP	80μM dCTP	80μM dCTP
80μM dTTP	80μM dTTP	80μM dTTP	80μM dTTP
8μM ddGTP	8μM ddATP	8μM ddTTP	8μM ddCTP
50mM NaCl	50mM NaCl	50mM NaCl	50mM NaCl

Stop solution

95% formamide

20mM EDTA

0.05% Bromophenol Blue

0.05% Xylene Cyanol FF

Enzyme dilution buffer

10mM Tris.Hcl, pH 7.5

5mM DTT

0.5mg/ml BSA

Sequenase Version 2.0 T7 DNA Polymerase

13units/ml in 20mM KPO_4, pH 7.4

1mM DTT

0.1mM EDTA

50% glycerol

RESULTS AND DISCUSSION

Deletion analysis

The nested deletions were carried out in recombinant plasmid (pATBX 1.8) from 5' end by using Erase-a-Base technique (fig. V.1) and altogether 200 deletents were obtained. Plasmid DNAs were isolated from the deletents and analysed by agarose gel electrophoresis (fig. V.2).

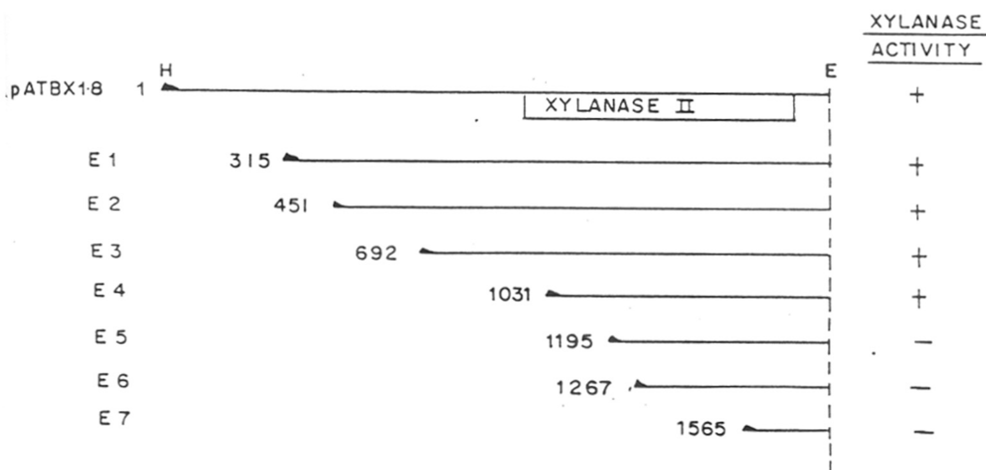


Fig. V.1 Construction of deletion derivatives

Clone pATBX 1.8 was used to construct various deletion derivatives. Numbers at the arrow tails indicate the deletion endpoints. Xylanase activity is indicated by +and -. Abbreviations of restriction enzymes; H, Hind III; E, Eco RI.

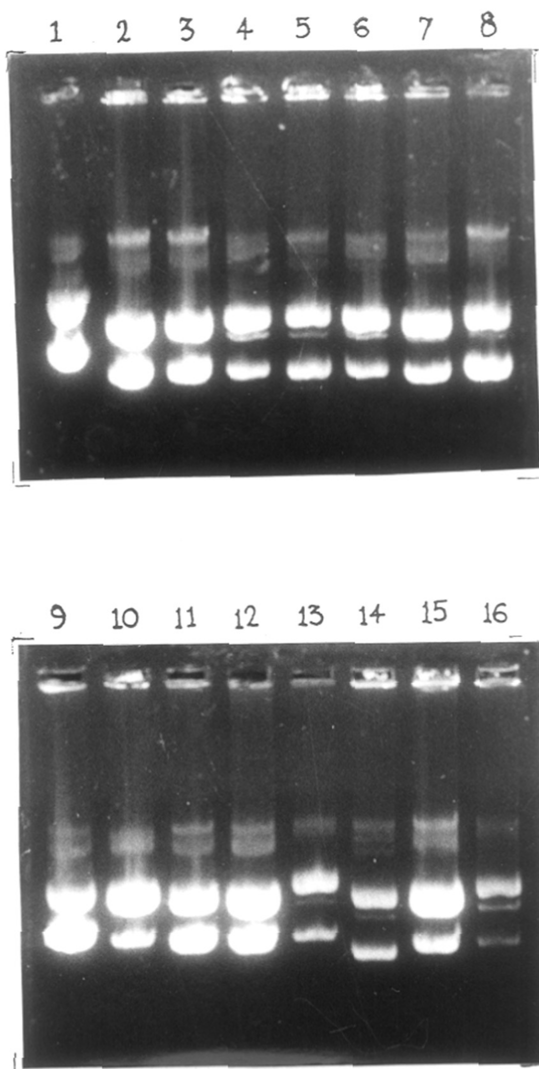


Fig. V.2 Representative figure of the plasmids isolated from the deletents . Lanes 1 and 9- plasmid pATBX 1.8; lanes 2 to 8 and 10 to 16- plasmid DNAs isolated from the deletent clones.

Plasmid DNAs isolated from the deletents were linearized with Eco RI and it was observed that many of them were carrying almost same size of the insert. Out of 200 clones, E1, E2, E3, E4, E5, E6 and E7 (fig. V.3) were observed to harbour the inserts with decreasing sizes . They were used to obtain the overlapping sequences along with the original clone pATBX 1.8.

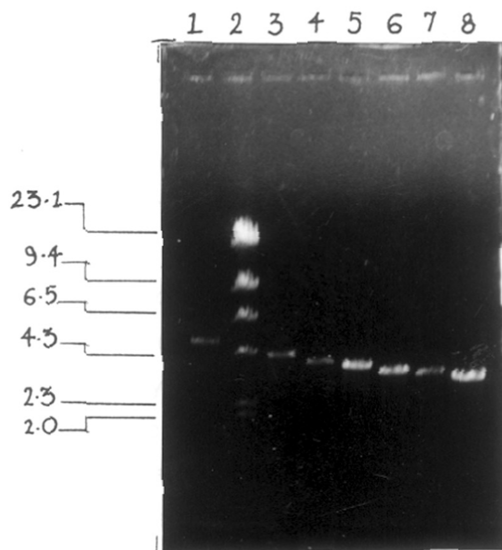


Fig. V.3 The plasmids of the deletent clones *linearized with ECo RI . lane 1, plasmid* pATBX linearized with ECoRI, lane 2, λ HindIII marker DNA, , lane 3 to 8 plasmids of the deletent clones linearized with ECo RI.

Nucleoide sequence of xynII

Full sequencing of 1.8 kb gene fragment was carried out, using the chain termination method (Sanger et al, 1977). Altogether more than 2000 bp were read on both strands (Fig V.4) . The analysis of sequence revealed an open reading frame (ORF) located about 1 kb downstream from the 5' end of the gene fragment. This ORF contained 402 nucleotides, representing 133 amino acids, of calculated molecular weight 14.09 kD. The molecular mass was in close agreement with that of the glycosylated mature protein from the parent *Bacillus* (15.8 kDa). The ORF showed the presence of 17 amino acid putative leader sequence (Fig. V.5). The signal sequence of 17 residues represented a typical gram positive signal peptide and conformed to -3-1 rule proposed by von Heijne (1988). Proline residue at position -4 from the cleavage site might allow a turn in the polypeptide which presumably exposes the cleavage site to signal peptidase. The apparent homology between the signal sequences of xylanases from the AT *Bacillus* and C-125 (4/17 are identical) probably arose as a consequence of these regions' role in protein export. The N-terminal part exhibited all the features of a signal peptide except for a negatively charged residue (Asp) in the n region. The presence of one or more negatively charged residues in the n region of signal peptides of xylanase from *S. lividans* (Shareck et al., 1991); rat liver cytochrome p-450 and rat epoxide hydrolase (von Heijne, 1986) is reported. From a thermodynamic point of view it is expected that , negative charges should have a tendency to move spontaneously from the cytosolic to the extracytosolic side of the prokaryotic inner membrane. The activation energy for transmembrane movement is less for negatively charged molecules than for positively charged ones. It was also suggested that negative net charge in *E. coli* lipoprotein signal sequence n region impairs the synthesis of protein. The presence of a net negatively charged NH_2 terminus caused initial cytoplasmic accumulation of prolipoprotein which was post-translationally translocated across the cytoplasmic membrane at a rate which was dependent on the positive charges present in this region (Vlasuk et al., 1983).

```
   1 AAGCTTTAAT GCGGTAGTTT ATCACAGGTA AATTGCTAAC GCAGATCAGG
  51 CACCGTGTAT GAAATCTAAC AATGCGCTCA TCGTCATCCT CGGCACCGTC
 101 ACCCTGGATG CTGTAGGCAT AGGCTTGGTT ATGCCGGTAC CTGCCGGGCC
 151 TCTTGCGGGA TATCGTCCAT TCCGACAGCA TCGACCAGGT CACTATTGGC
 201 GATGCTGCTA GCGTCTATAT GCGTTTGATG CAATTTCTAT GCGTCACCCG
 251 ATTCTCGGAG CACTGTCCGA CCGATTTGGC CGCCGCCCAG TCCTGCTCGC
 301 ATTCGCTACT TGGAGCCACT ATCGACTACG CGATCATGGC GACCACACCC
 351 GTCCTGTGGA TCCTCTACGC CGGACGCATC GTGGCCGGCA TCACCGGCGC
 401 CACAGGTGCG GTTGCTGGCG CTATATCGCC GACATCACCG ATGGGGAAGA
 451 TCGGGCTCGC CACTTCGGGC TCATGAGCGC TTGATTTCGG CGTGGGTATG
 501 GTGGCAGGCC CGTGGCGGGG ACTGTTAGGC GCATCTCCTT GCATGCACCA
 551 TTCCTTCGCG CGGTGCTTCA ACGGCCTCAA CCTACTACTG GGCTGCTTCC
 601 TAATGCAGGA GTCGCGAGAG CCTTCAACCC AGTCAGCTCC TTCCGGTGGC
 651 GCGGGGCATG ACTATCGTCG CCGCACTTAT GACTGTCTTC TTTATCATGC
 701 AACTCGTAGG ACAGGTGCCG GCAGCGCTCT GGGTCATTTT CGGCGAGGAC
 751 TACTTTGCTG GAGCGCGACG ATGATCGGCC TGTCAGCTTG CGGTATTCGG
 801 AATCCTTGCA ACGCCCTCGC TCAAGCCTTC GTCACTGGTC CCGCCAACCA
 851 AACGTTTCGG CGAGAAGCAG GCCATTATCG CCGGACTGGC GCCGACGCGC
 901 TGGGCTACGT CTTGCTGGCG TTCGCGACGC AGGCTGGATG GCCTTCCCCA
 951 TTATGATTCT TCTCGCTTCC GGCGCATCGG GATGCGCCGC GTTGCAGGCC
1001 ATGCTGTCCA GGCAGGTAGA TGACGACCAT CAGGGACAGC TTCAAGGATC
1051 GCTCGCGGCT ACTTACCAGC CTAACTTCGA TCATGGACCA GCTGATCGTC
1101 ACGGCGATTT ATGCCGCCTC GGCAGCACAT GGAACGGGTT GGCATGGATT
1151 GTAGGCGGCC GACCTATACC TTGTCTCGCC TCCCCGCGTT GCTCGCGGTG
1201 CATGGTAGCC GGGCCACCTC GAACCTGAAT GGAAGCCGGC TAGGCACCTC
1251 GCTAACGGAT TCACTCCAAG AATTGGAGCC AATTCTTGCA GTGAGAACTG
1301 TGAATAGCAG TCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTTCCGC
1351 ATCTCCAGCT CGCCGCACGT AGGCGCATCT CGGGCAGCGT TGGGTCCTGG
1401 CACGGGTGCG AATGAAGTCG TTGATCTGTC AGTTGAGGAC CGGCTAGGCT
1451 GGCGGGTTGC CTTACTGGTT AGCAGAATAA TCACCATACG CGAGCCGGAA
1501 CGTAAGCACT CTGCTGCAAA CGTCTGCGAC CTGAGCAACA ACTGAATGGT
1551 CTTCGGTTTC GTAAGTCTGG AAACCGGGGA AGTCAGCGCG CCCTGCACCA
1601 TCCATGTTCC GGATCCTGCA TCGCGGATGC TGGCTTGGCC TACCCTGTGG
1651 AACACCCTAC CCTTCGATAA CGAAGGCGCT GGCATTGACC CTTAGGTGAT
1701 TTCTCTGGTC CCGCCGCATC CATACCGACC GTTTGTTTCA CCCTCACAAC
1751 GTTCCAGTAC CGGGCATGTT CATCATCAGT AGCCCGTATC GTGAGCATCC
1801 CTCCTCTCGT TTCATCGGAT CATTACCCCC ATGAACAGAA ATCCCCCTTA
1851 CCACCGGAGG CATCAGTGAC CAAACAGGAA AAACCGCCCT TACATGGCCG
1901 CTTTCAGAGG CCAGACATAA CGCTCGAGGA AACCTCAACC GAGGTGGACC
1951 GCGATGACCG GCGCCTCGGA TCCGTTCCCG CCACGCTGAT GAGCTTTCCG
2001 CGGGGGTCCG AATTCCTCGA GTCTAGAGGA GCTGCGACGT CGGGCCCATT
2051 CGCCCTATAG TAGTCGTATT ACATTCACTG GCCGTCGGTT TTCAACGTCG
2101 TGACTGGGAA ACCCTGCTTA CCACTTATCG CTTGCGCCAT CCCCTTTCGC
2151 CAGCTGGCCT AATGCAGAGG CCCGCACCGA TCGCCTCCAC GTGCCCGTGA
2201 ATGGCAATGG ACG
```

Fig.V.4 Nucleotide sequence of 1.8 kb gene fragment .

```
   1 GTT GCA GGC CAT GCT GTC CAG GCA GGT AGA TGA CGA CCA TCA GGG ACA   48
                                                    -35
  49 GCT TCA AGG ATC GCT CGC GGC TAC TTA CCA GCC TAA CTT CGA TCA TGG   96
                                   -10
  97 ACC AGC TGA TCG TCA CGG CGA TTT ATG CCG CCT CGG CAG CAC ATG GAA  144

 145 CGG GTT GGC ATG GAT TGT AGG CGG CCG ACC TAT ACC TTG TCT CGC CTC  192
   1   SD            Met Asp Cys Arg Arg Pro Thr Tyr Thr Leu Ser Arg Leu   13

 193 CCC GCG TTG CTC GCG GTG CAT GGT AGC CGG GCC ACC TCG AAC CTG AAT  240
  14 Pro Ala Leu Leu Ala Val His Gly Ser Arg Ala Thr Ser Asn Leu Asn   29

 241 GGA AGC CGG CTA GGC ACC TCG CTA ACG GAT TCA CTC CAA GAA TTG GAG  288
  30 Gly Ser Arg Leu Gly Thr Ser Leu Thr Asp Ser Leu Gln Glu Leu Glu   45

 289 CCA ATT CTT GCA GTG AGA ACT GTG AAT AGC AGT CAA ACC AAC CCT TGG  336
  46 Pro Ile Leu Ala Val Arg Thr Val Asn Ser Ser Gln Thr Asn Pro Trp   61

 337 CAG AAC ATA TCC ATC GCG TTC CGC ATC TCC AGC TCG CCG CAC GTA GGC  384
  62 Gln Asn Ile Ser Ile Ala Phe Arg Ile Ser Ser Ser Pro His Val Gly   77

 385 GCA TCT CGG GCA GCG TTG GGT CCT GGC ACG GGT GCG AAT GAA GTC GTT  432
  78 Ala Ser Arg Ala Ala Leu Gly Pro Gly Thr Gly Ala Asn Glu Val Val   93

 433 GAT CTG TCA GTT GAG GAC CGG CTA GGC TGG CGG GTT GCC TTA CTG GTT  480
  94 Asp Leu Ser Val Glu Asp Arg Leu Gly Trp Arg Val Ala Leu Leu Val  109

 481 AGC AGA ATA ATC ACC ATA CGC GAG CCG GAA CGT AAG CAC TCT GCT GCA  528
 110 Ser Arg Ile Ile Thr Ile Arg Glu Pro Glu Arg Lys His Ser Ala Ala  125

 529 AAC GTC TGC GAC CTG AGC AAC AAC TGA ATG GTC TTC GGT TTC GTA AGT  576
 126 Asn Val Cys Asp Leu Ser Asn Asn ***                             134

 577 CTG GAA ACC GGG GAA GTC AGC GCG CCC TGC ACC ATC CAT GTT CCG GAT  624

 625 CCT GCA TCG CGG ATG CTG GCT TGG CCT ACC CTG TGG AAC ACC CTA CCC  672

 673 TTC GAT AAC GAA GGC GCT GGC ATT GAC CCT TAG GTG ATT TCT CTG GTC  720

 721 CCG CCG CAT CCA TAC CGA CCG TTT GTT TCA CCC TCA CAA CGT TCC AGT  768

 769 ACC GGG CAT GTT CAT CAT CAG TAG CCC GTA TCG TGA GCA TCC CTC CTC  816

 817 TCG TTT CAT CGG ATC ATT ACC CCC ATG AAC AGA AAT CCC CCT TAC CAC  864

 865 CGG AGG CAT CAG TGA CCA AAC AGG AAA AAC CGC CCT TAC ATG GCC GCT  912

 913 TTC AGA GGC CAG ACA TAA CGC TCG AGG AAA CCT CAA CCG AGG TGG ACC  960

 961 GCG ATG ACC GGC GCC TCG GAT CCG TTC CCG CCA CGC TGA TGA GCT TTC 1008

1009 CGC GGG GGT CC                                                   1019
```

Fig. V.5

Fig. V.5 Nucleotide and amino acid sequence of the xylanase II gene.

Numbering of the amino acids starts at the initiation codon (ATG). The possible promoter (-10 and -35), Shine-Dalgarno sequence (----SD---) and trancription termination sequences (-----) are indicated. The possible signal peptide cleavage site is indicated by arrowhead. Three potential N-glycosylation sites are indicated with upward arrows. The posiible transcription termination sequences at positions 570 and 828 are indicated.

The completed DNA sequence for XynII was found to contain a translation start site ATG for the secreted protein at nucleotide position 154 (Fig. V.5). At position -5 from the translation start site there exists a putative Shine-Dalgarno (SD) sequence which allows base pairing between mRNA and the 3' end of bacterial 16s r RNA . It showed good homology to SD sequence of *B. subtilis* (Fig. V.6a) and that of xylanases from other *Bacilli* (Fig. V.6b) except for the presence of cytosine. The putative promoter sequences TTCGATCA for -35 region and ATTTAT for -10 region with 23 bp spacing between them were located upstream of ATG start codon from position 86 to 122. These sequences resemble the consensus promoter sequences for σ^{43} factor found in *B. subtilis* (Helmann and Chamberlin, 1988). At nucleotide positions 570 and 828 downstream from the stop codon there are two palindromoic sequences which can be formed into a stable hairpin structure (ΔG=-9.60 and -7.20 kcal/mol respectively) and which might act as transcription terminators.

E. coli	CTAGTGGA GGAAT
B. subtilis	A**GAA**A**GG**A GGTGATC
AT *Bacillus* sp.	G**GAA**C**GG**G

(a)

Bacillus sp.	RBS sequence	Accession Number
B. pumilus (*xyn* A)	AA**AA**A**GG**AGAGAA	X00660
B. stearothermophilus (T6 xylanase)	**G**AGGG**GG**AA	Z29080
Bacillus sp. YA-14 (*xyn* S)	**GGA**GG	X59058
Bacillus sp. (*xyn* Y)	**GGA**GG	X59059
AT *Bacillus* sp. (*xyn* II)	**GGAA**C**GG**G	U83602

(b)

Fig. V.6 Comparison of ribosome binding site sequences.

(a) Homology of SD sequence of xylanase II from the AT *Bacillus* with that of *E. coli* (Gold and Stormo, 1987) is shown by underlines and homology with SD sequence of *B. subtilis* (Moran et al,1982) is indicated by bold letters.

(b) Comparison with SD sequences of xylanases from bacilli.

Glycoprotein nature

Three potential N-glycosylation sites (Asn-Xaa-Ser/Thr) (Gavel and von Heijne, 1990) are present in this mature xylanase protein at amino acid positions 13, 38, and 47. Biochemical analysis of XynII has revealed that it is a glycoprotein (Dey et al., 1992). The presence of carbohydrate molecules may be the reason for high molecular weight of xylanase II produced by the AT *Bacillus* (15.8 kD) as compared to that from recombinant *E. coli* (pATBX 1.8). The occurrence of carbohydrate moiety is a common phenomenon for many eukaryotic xylanases (Funaguma et al., 1991) . However few xylanases from prokaryotic sources such as *Clostridium stearcorarium* (Biely, 1991) , *Streptomyces sp.* (Marui et al, 1985) and the AT *Bacillus* (Dey et al., 1992) have also been found to be glycoproteins. Many extracellular proteins contain at least a small amount of covalently bound carbohydrate, the function of which is not always known. The carbohydrate moiety is never known to be involved in the active site, but may help to stabilize the molecules and thus increases their biological life.

Secondary structure prediction

An analysis of the secondary structure of XynII by the method of Chou and Fasman (1978) predicted that the polypeptide chain primarily formed β sheets. The conserved amino acids are often in positions of β-turns, linking two β-sheets suggesting that the 3-dimensional structure of xylanaseII has been extremely conserved during evolution. The conserved glycin residues seem to be distributed throughout the primary sequence which might be essential for the proper folding of the enzyme.

Codon usage

The codon usage for *xynII* from the AT *Bacillus* is described in table V.1. The occurrence of CAA for Gln and AGC for Ser in the ORF appeared to be different than those for *E. coli* and *B. subtilis* genes and might be characteristic of the extremophilic species. No obvious preference was observed for G or C in the third position. The codon usage, overall, appeared to be unbiased as reported for other genes in *B. subtilis* (Ogasawara, 1985). The coding region contained 22% A, 21% T, 29% C, 28% G. Codon usage for Ile, Pro, Asp, Glu and Arg resembled that of alkaliphilic *Bacillus* 137 (Tabernero et al., 1995) and *B. stearothermophilus* T6 (Gat et al., 1994). Positively charged His in *Bacillus* xylanases showed bias for CAT, however in AT *Bacillus* xylanase CAT was used only once as compared to CAC which was used twice. The codon usage for signal sequence of *xynII* revealed that bias observed in the mature protein is not retained in the signal peptide except for Asp and Cys. The preference for rare codons for two amino acid residues

(CGG for Arg and ATA for Ile) may be affecting the level of expression in *E. coli* (pATBX 1.8). Rare codons in *E. coli* are those used very infrequently as a consequence of very low cognate tRNA abundance (Sharp and Li, 1986).

Table V.1

Codon usage of the xylanase gene from AT Bacillus			
Codon	Amino acid	Count	Percent
TTT	Phe	0	0
TTC	Phe	1	0.7
TTA	Leu	1	0.7
TTG	Leu	4	3
TCT	Ser	3	2.2
TCC	Ser	2	1.5
TCA	Ser	2	1.5
TCG	Ser	3	2.2
TAT	Tyr	1	0.7
TAC	Tyr	0	0
TGT	Cys	1	0.7
TGC	Cys	1	0.7
TGG	Trp	2	1.5
CTT	Leu	1	0.7
CTC	Leu	3	2.2
CTA	Leu	3	2.2
CTG	Leu	4	3
CCT	Pro	2	1.5
CCC	Pro	1	0.7
CCA	Pro	1	0.7
CCG	Pro	3	2.2
CAT	His	1	0.7
CAC	His	2	1.5
CAA	Gln	2	1.5
CAG	Gln	1	0.7
CGT	Arg	1	0.7
CGC	Arg	3	2.2
CGA	Arg	0	0
CGG	Arg	6	4.5
ATT	Ile	1	0.7
ATC	Ile	3	2.2
ATA	Ile	3	2.2
ATG	Met	1	0.7
ACT	Thr	1	0.7
ACC	Thr	6	4.5
ACA	Thr	0	0
ACG	Thr	2	1.5
AAT	Asn	3	2.2
AAC	Asn	6	4.5

Table 1 continued			
Codon	Amino acid	Count	Percent
AAA	Lys	0	0
AAG	Lys	1	0.7
AGT	Ser	1	0.7
AGC	Ser	6	4.5
AGA	Arg	2	1.5
AGG	Arg	1	0.7
GTT	Val	4	3
GTC	Val	2	1.5
GTA	Val	1	0.7
GTG	Val	3	2.2
GCT	Ala	1	0.7
GCC	Ala	2	1.5
GCA	Ala	4	3
GCG	Ala	5	3.7
GAT	Asp	3	2.2
GAC	Asp	2	1.5
GAA	Glu	3	2.2
GAG	Glu	3	2.2
GGT	Gly	3	2.2
GGC	Gly	4	3
GGA	Gly	1	0.7
GGG	Gly	0	0

Comparison with other xylanases

On the basis of the low molecular weight and basic pI (8.0) XynII appears to be a member of family 11 of xylanases according to the classification by Henrissat and Bairoch (1993). The low apparent mass (14.3 kD) and high pI value (8.0) of Xyn II are similar to those of xylanases from *Robillarda sp* Y-20 (17.6 kD, pI=9.7) (Koyama et al., 1990), *Trichoderma viride* (22kD, pI=9.3) (Ujiie et al, 1991), and *Streptomyces roseiscleroticus* (22.6 kD, pI=9.5) (Grabski and Jeffries, 1991). The first two amino acids in the predicted N-terminus of Xyn II i.e. Ala-Val are identical to that of xylanase from the parent *Bacillus*.

The comparison of *xynII* sequence with those of the other reported xylanases revealed 50-60% homology at nucleotide level. However at amino acid level, no striking similarity was obtained. Multiple alignments of XynII with family 11 xylanases from bacilli (fig. V.7) showed 17% identity whereas comparison with xylanases from other organisms revealed no significant homology (Fig. V.8).

Trp44 in XynII was found to be conserved (Fig. V.7 and V.8). The conserved Trp residues in XynC from *F. succinogenes* (Zhu et al., 1994) are believed to bind to the polysaccharides by hydrophobic interactions, with the stacking of aromatic residues along the face of the sugar rings. Trp residues have also been suggested to play important roles in the maintaince of the tertiary structure, effective substrate binding and keeping pKa of catalytic carboxyl residues abnormally high in case of chicken lysozyme (Inoue et al., 1992).

```
1_B.circul   MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
2_B.subtil   MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
3_Xyn_II     MDCRRPTYTLSRLPALLAVHGSRATSNLNGSRLGTSLTDS---LQELEPILAVRTVNSSQ
             *   . .. .    **...    **.    .      **    .. .       .** *.

1_B.circul   TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
2_B.subtil   TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
3_Xyn_II     TN-----P-WQNISIAFRISSSP-------------------------HVGASRAALGPG-
             *.       * . *    *                             *  *  .  *

1_B.circul   TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFTNHVNA
2_B.subtil   TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA
3_Xyn_II     ------TGANEVVDLSVEDRLGWRVA----LLVSRIITIREPERKHSAANVCDLSNN---
                      .   *.    * .           ..  ..*.  *   .      .*.

1_B.circul   WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTV-W
2_B.subtil   WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW-
3_Xyn_II     ----------------------------------
```

Fig. V.7 Comparison of primary structure of Xyn II with that of the family 11 xylanases from the bacilli. * and . below the lines indicate conserved and chemically similar amino acid residues respctively. Underlined residues indicate the motif (-SQTN-) in the AT *Bacillus* xylanase. Xylanases are (accession numbers in parentheses) : 1-*B. circulans* xylanase (S01734); 2-*B. subtilis* xylanase (S39157) ; 3- AT *Bacillus* Xyn II (U 83602).

```
1  ITTNQTG-YDGMYYSFWTDGGGSVSMTLNGGGSYSTQWT--NCGNFVAGKGWGNGG-RRT
2  GTPSSTGTDGGYYYSWWTDGAGDATYQNNGGGSYTLTWSG-NNGNLVGGKGWNPGAASRS
3  --------AGINYVQNYNGNLADFTYDESAG-TFSMYWEDGVSSDFVVGLGWTTGS-SNA
4  --------HNGFYYSWWTDNGAQATYTNNAGGSYSITWS--GNGNLVGGKGWNPGS-ARN
5  --GPKHLAARQDYNQNYKTG-GNIQYNPTSN-GYSVTFS--GAQDFVLGKGWKQGT-TRT
6  GTPSSQGTHNGCFYSWWTDGGAQATYTNGAGGSYSVNWK--TGGNLVGGKGWNPGA-ART
7  VTPNAEGWHNGYFYSWWSDGGGQVQYTNLEGSRYQVRWR--NTGNFVGGKGWNPGT-GRT
8  -LIERAGPGGINYVQNYNGNLGQFTYNENAG-TYSMYWNNGVNGDFVVGLGWSTGA-ARS
9  NTPNGEGTHNGCFWSWWSDGGARATYTNGAGGSYSVSWG--SGGNLVGGKGWNPGT-ART
10 STPSSTGTSGGYYYSFWTDGGGDVTYTNGDGGSYTVEWT--KVGNFVGGKGWNPGS-SQT
11 TIQPGTGYNNGYFYSYWNDGHGGVTYTNGPGGQFSVNWS--NSGNFVGGKGWQPGTKNKV
12 -----------NYVQNYNGNVANFEYSQYDG-TFSVNWNG--NTDFVCGLGWTVGT-GRT
13 ----------INYVQNYNGNLGAFSYNEGAG-TFSMYWQQGVSNDFVVGLGRSTGS-SNP
14 ITSSETGTNNGYYYSFWTNGGGDVEYTNGNGGQYSVKWT--NCDNFVAGKGWNPGS-AKT
15 STPSSEGYHNGYFYSWWTDGGGSAQYTMGEGSRYSVTWR--NTGNFVGGKGWNPGT-GRV
16 ----------------MDCRRPTYTLSRLPA-LLAVHGSR-ATSNLNGSRLGTSLT-DSL

1  VRYSGYFNPSGNG-YGCLYGWTSN-PLVEYYIVDNWGSYRPTG------EYRGTVYSDGG
2  ISYSGTYQPNGNS-YLSVYGWTRS-SLIEYYIVESYGSYDPSSA----ASHKGSVTCNGA
3  ISYSAEYSASGSSSYLAVYGWVNY-PQAEYYIVEDYGDYNPCSS----ATSLGTVYSDGS
4  VTYSANYRPNGNS-YLSVYGWTRN-PLVEYYVVENFGTYDPSSQ----ASRKGTINVDGA
5  VKYTGSTQAQAGTVLVALYGWTRGSKLVEYYIQDFTSGGSGSAQ----GQKMGQVTCDGS
6  ITYSGTYSPSGNS-YLAVYGWTRN-PLIEYYVVENFGTYDPSSQ----ATVKGSVTADGS
7  INYGGYFNPQGNG-YLAVYGWTRN-PLVEYYVIESYGTYNPGSQ----AQYKGTFYTDGD
8  ITYSSNYQASGGS-YLSVYGWINS-PQAEYYIVESYGSYNPCGAGQSGVTQLGTVCSDGA
9  ITYSGTYNYNGNS-YLAVYGWTRN-PLVEYYVVENFGTYDPSSQ----SQNKGTVTSDGS
10 ISYSGSFIPSGNG-YLSVYGWTQN-PLIEYYIVESYGDYNPGTA----GTHQGTLESDGS
11 INFSGSYNPNGNS-YLSVYGWSRN-PLIEYYIVENFGTYNPSTG----ATKLGEVTSDGS
12 ITYSGSYNPGYSGSYQAIYGWTGQGSLSEYYVIDNYGGYNPCTGSG--VTQLGSLYSDGS
13 ITYSASYSASGGS-YLAVYGWVNS-PQAEYYVVEAYGNYNPCSSGS--ATNLGTVSSDGG
14 VTYSGEWESNSNS-YVSLYGWTQN-PLVEYYIVDKYGDYDPSTG----ATELGTVESDGG
15 INYGGAFNPQGNG-YLAVYGWTRN-PLVEYYVIESYGTYNPSSG----AQVKGSFQTDGG
16 QELEPILAVRTVN-SSQTNPWQNI--SIAFRISSSP--HVGASR-----AALGPGTGANE
                        *                               *

1  TYDIYKTTRYNAPSVEGTRTFDQYWSVRQSKVIGS----GTITTGNHFDAWARAGMNLGQ
2  TYDILSTWRYNAPSIDGTQTFEQFWSVRNPKKAPGGSISGTVDVQCHFDAWKGLGMNLGS
3  TYQVCTDTRTNEPSITGTSTFTQYFSVRESTRT-----SGTVTVANHFNFWAQHGFGNS-
4  TYQVAQSTRTNQPSIDGTRTFYQYWS----------------------------------
5  VYDIWQHTQVNQPSIVGTTTFVQYISNRVSKRS----TGGTITTKCHFDAWAKLGMNLGN
6  SYKIAQTQRTNQPSIDGTQTFQQYWSVRQNKRS-----SGSVNMKTHFDAWAAKGMKLG-
7  QYDIFVSTRYNQPSIDGTRTFQQYWSIRKNKRV-----GGSVNMQNHFNAWQQHGMPLG-
8  TYTVYTDTRTNQPSITGTSTFKQYWSVRQTKRT-----SGTVTTGNHFAYWAKYGFGNS-
9  SYKIAQSTRTNQPSIDGTRTFQQYWSVRQNKRS-----SGSVNMKTHFDAWASKGMNLG-
10 TYDIYTATRENAPSIEGTATFTQFWSVRQSKRT-----SGSVTTQNHFDAWSQLGMTLG-
11 VYDIYRTQRVNQPSIIGTATFYQYWSVRRNHRS-----SGSVNTANHFNAWAQQGLTLG-
12 SYQVCTHTQYNQPSIVGTTTFPQYFSVRQNKRS-----SGSVNMQNHFNYWAQHGFPNR-
13 TYQVCTDTRVNQPSITGTSTFTQFFSVRQGSRT-----SGTVTIANHFNFWANDGFGNS-
14 TYKIYKTTRENAPSIEGTSTFNQYWSVRQSGRVG-----GTITAQNHFDAWANVGLQLG-
15 TYNVAVSTRYNQPSIDGTRTFQQYWSVRQQKRV-----GGSVNMQNHFNAWSRYGLNLG-
16 VVDLSVEDRLGWR---VALLVSRIITIREPERK-----HSAANVCDLSNN----------
```

Fig. V.8 Sequence alignment of Xyn II with the xylanases belonging to family 11 produced by organisms other than bacilli. * and . below the lines indicate conserved and chemically similar amino acid residues respctively. Xylanases are (accession numbers in parentheses) : 1- *Streotomyces* sp EC3 xylanase (X81045), 2- *Schizophyllum commune* Xyn A (id 140875), 3- *Aspergillus kawachii* Xyn C (S45138) 4- *Magnaporthe grisea* xylanase (L81127), 5- *Cochliobolus carbonum* xylanase (U58916), 6- *Ascochyta pisi* xylanase (Z68891), 7- *Humicola insolens* xylanase (X76047), 8- *Auriobasidium pullulans* Xyn A (U10298), 9- *Cochliobolus carbonum* XYL 1 (Q06562), 10- *Emericella nidulans* xylanase (P55333), 11- *Hypocrea jecorina* xylanase 2 (P36217), 12- *Cryptococcus* sp S-2 xylanase (JC 4909), 13- *Penicillium purporogenum* Xyl B (Z50050), 14- *Aspergillus oryzae* Xyn G1 (AB003085), 15- *Cochliobolus sativus* Xyl 2 (AJ 004802), 16- AT *Bacillus* Xyn II (U 83602).

Asp22 and Asp77 of XynII were found to be analogous to Asp11 and Asp106 of *B. circulans* mature xylanase respectively. The mutational analysis of *B. circulans* xylanase revealed that acidic side chain of Asp11 was not essential for catalysis suggesting that Asp106 might play an important role in the catalytic mechanism. The Glu residues from XynII were not found to be congruous to Glu78 and Glu172 of *B. circulans* xylanase which were reported to be absolutely conserved and were known to participate in catalytic mechanism. Arg83 of XynII was found to correspond to Arg112 of *B. circulans* xylanase. Mutational and crystallograhic analysis of the catalytic residues of *B. circulans* xylanase have revealed that Arg112 plays an important role in the active site of the enzyme (Wakarchuk et al., 1994b). Interestingly XynII did not show close resemblance with thermophilic xylanases belonging to family 10 and 11 (fig. V.9).

```
1 -------------------------------------------------MKLKKKMLTLL
2 ------------------------------------------------------------
3 MLRRKVIFTVLATLVMTSLTIVDNTAFAATNLNTTESTFSKEVLSTQKTYSAFNTQAAPK
4 ----------------------------------AAQSVDQLIEKVYFGVATDQNRLTTG
5 ------------------------------------------------------------

1 LTASMSFGLFGATSSAATDYWQYWTDGGGMVNAVNGPGGNYSVTWQN-TGNFVVGKG---
2 XTTPNSEGWHDG------YYYSWWSDGGAQATYTNLEGGTYEISWGD-GGNLVGGKG---
3 TITSNEIGVNGG------YDYELWKDYGNTSMTLK-NGGAFSCQWSN-IGNALFRKGKKF
4 KNAAIIQADFGQVTP---ENRMKWDATEPSQGNFNFAGADYLVNWAQQNGKLIYGHTLVW
5 ------------------------MDCRRPTYTLSRLPALLAVHGSR-ATSNLNGSR---

1 ------WTVGSPNRVINYNAGIWEPSGNGY-LTLYGWTR-------NALIEYYVV----D
2 ------WNPGLNARAIHFEG-VYQPNGNSY-LAVYGWTR-------NPLVEYYIV----E
3 NDTQ--TYKQLGNISVNYDC-NYQPYGNSY-LCVYGWTS-------SPLVEYYIV----D
4 WSQLPPWVVSITDKMKNYIGKAFAWDVVNEAFNITGRLEVAAASRTDPNAKLYINDYNLD
5 ------LGTSLTDSLQELEP-ILAVRTVNS-SQTNPWQ--------NISIAFRIS----S

1 SWGTYRPTG--NYKGTVNSDG-GTYDIYTTMRYNAPSIDGTQTFQQFWS-----VRQSKR
2 NFGTYDPSSGATDLGTVECDG-SIYRLGKTTRVNAPSIDGTQTFDQYWS-----VRQDKR
3 SWGSWRPPGG-TSKGTITVDG-GIYDIYETTRINQPSIQGNTTFKQYWS-----VRRTKR
4 SARYPKTQAIVNRVKQWCAAGVPIIGIGNQTARAAITVWGVADPDRWWLREVIGICWGGV
5 SPHVGASRA---ALGPGTGAN-EVVDLSVEDRLG---WRVALLVSRIIT-----IREPER

1 PTGSNVSITFSNHVNAWR-SKGMNLGSSWAYQVLATEGYQSS-------G----RSNVTV
2 TSG---TVQTGCHFDAWA-RAGLNVNGDHYYQIVATEGYFSS-------G----YARITV
3 TSG---TISVSKHFAAWE-SKGMPLG-KMHETAFNIEGYQSS-------G----KADVNS
4 PNQFDASAGTPEAFLNVIGEDYIPIAFKNTLTNVMKNSIRASTTPLLFDGNFNPKPAYNA
5 KHS---AANVCDLSNN--------------------------------------------

1 -------W
2 ADVG----
3 MSINIGK-
4 IVQGLQ--
5 --------
```

Fig. V.9 Sequence comparison of Xyn II with he xylanases belonging to family 11 produced by themophilic organisms. Xylanases are (accession numbers in parentheses) : 1- *B. stearothermophilus* Xyn A (U15985) , 2- *Thermomyces lanuginosus* xylanase (1942242), 3- *Clostridium acetobutylicum* Xyn B (S12745), 4-*Thermoascus aurantiacus* (A40147), 5.- AT *Bacillus* Xyn II (U 83602).

The comparison of amino acid sequence of XynII with family 10 xylanases from alkaliphilic organisms revealed significant homology (fig. V.10) rather than with family 11 xylanases (fig. V.11). Glu100, Asn115, His3 and Ser 5 were found to be conserved. Glu100 may function as nucleophile acid-base catalyst. Asn127 in xylanaseA from *S. lividans* (Roberge et al., 1997a) has shown to be conserved and is reported to play a key role in maintaining the ionization states of the two catalytic residues. The structural analysis of xylanaseA has revealed that 2 His residues are part of important hydrogen bond network in the vicinity of two catalytic residues (Roberge et al, 1997b). Plesniak et al (1996b), based on NMR assignments, have suggested that His149 present in *B. circulans* xylanase may be buried in within its hydrophobic core and involved in a network of hydrogen bonds with an internal water and Ser130, as well as in a potential weak aromatic-aromatic interaction with Tyr105. The histidine, serine and tyrosine residues are absolutely conserved in all known family 11 xylanases. It has been suggested that since the side chains of the conserved residues are located on the "backside" of the enzyme, relative to the active site cleft, they are more likely to fulfill important roles in establishing the structural stability of xylanase, instead of having a direct catalytic function. In the absence of conserved Tyr residue in AT *Bacillus* xylanase, Trp86 probably, may play a similar role.

```
1  MITLFTKPFVAGLAISLLVGRGLGNVAAAQGGPPQSGVFGENHKRNDQPFAWQVASLSER
2  MITLFRKPFVAGLAISLLVGGGIGNVAAAQGGPPKSGVFGENEKRNDQPFAWQVASLSER
3  ------------------------------------------------------------
4  ----------------------------------------MNQQLN-------IPNLYEI

1  YQEQFDIGAPVEPYQLEGRQAQILKHHYNSLVAENAMKPVSLQPREGEWNWEGADKIVEF
2  YQEQFDIGAAVEPYQLEGRQAQILKHHYNSLVAENAMKPESLQPREGEWNWEGADKIVEF
3  ------MDCRRPTYTLSR-LPALLAVHG-SRATSNLNGS-----RLG---TSLTDSLQEL
4  YKDFFSIGAAVNSKTLES-EKELLKKHYNSLTAENEMKFELLQPEQGNFNFTQADKLVAF
         . .      *      .*  *  *  . *           *      .* .

1  ARKHNMELRFHTLVWHSQVPEWFFIDENGNRMVDETDPEKRKANKQLLLERMENHIKTVV
2  ARKHNMELRFHTLVWHSQVPEWFFIDEDGNRMVDETDPDKREANKQLLLERMENHIKTVV
3  --EPILAVR---TVNSSQTNPWQNIS---------------IAFRISSSP----HVGASR
4  ANEHNMKLRGHTLVWHNQTTGWLFQNSDG-----------TQVNRETLLQRMEAHISTVL
        . .*    *   *   *                      .         *. .

1  ERYKDDVTSWDVVNEVIDDGGG--LRESEWYQITGTDYIKVAFETARKYGGEEAKLYIND
2  ERYKDDVTSWDVVNEVIDDGGG--LRESEWYQITGTDYIKVAFETARKYGGEEAKLYIND
3  AALGPGTGANEVVDLSVEDRLG-------WRVALLVSRIITIREPERKHSAANVCDLSNN
4  GRYKGQFYSWDVVNEAISDDDSEYLRKSKWLDIIGEDFIAKAFEFAHQADPN-ASLFYND
           . .**   . *          *        *    *  ..          *

1  YNTEVPSKRDDLYNLVKDLLEQGVPIDGVGHQSHIQIGWPSIEDTRASFEKFTSLGLDNQ
2  YNTEVPSKRDDLYNLVKDLLEQGVPIDGVGHQSHIQIGWPSIEDTRASFEKFTSLGLDNQ
3  ------------------------------------------------------------
4  YNESHPNKRERIYRLVKSLLDKDVPIHGVGLQAHWNVHDPSLDDIRAAIERYASLGIQLQ

1  VTELDMSLYGWPPTGAYTSYDDIPEELFQAQADRYDQLFELYEELSATISSVTFWGIADN
2  VTELDMSLYGWPPTGAYTSYDDIPAELLQAQADRYDQLFELYEELAADISSVTFWGIADN
3  ------------------------------------------------------------
4  ITEMDVSMFSWDNR--RADLTQPTEKMLNLQEERYDQFFQLFREYKDVISNVTFWGANDS

1  HTWLDDRAREYNNGVGVDAPFVFDHNYRVKPAYWRII-D
2  HTWLDGRAREYNNGVGIDAPFVFDHNYRVKPAYWRIID-
3  ---------------------------------------
4  YTWLNDFPVRGRK----NWPFIFDEKGDPQRVFLENR--
```

Fig. V.10 Comparison of Xyn II with that of the family 10 xylanases produced by alkaliphilic organisms. * and . below the lines indicate conserved and chemically similar amino acid residues respctively. Underlined residues indicate the motif (-SQTN-) in the AT *Bacillus* xylanase. Xylanases are (accession numbers in parentheses) : 1- Alkalitolerant xylanase (A45296), 2-*Bacillus* sp C-125 Xyn A (D00087), 3- AT *Bacillus* Xyn II (U 83602) , 4- *Bacillus* sp (N 137) xylanase (I40356).

```
1     MSQKKLTLINLFSLFALTLPARISQAQIVTDNSIATRGGYDYEFWKDSGGSGTMILNHGG
2     -------MFKFKKNFLVGLSAALMSISLFSATASAASTDY-WQNWTDGGGIVNAVNGSGG
3     ------------------------------------------------------------
4     ---------------------------------------------MDCRRPTYTLSRLPA

1     TFSAQWNNVNNILFRKGKKFNETQTHQQVGNMSINYGANFQPNGNAYLCVYGWTVDPLVE
2     NYSVNWSNTGNFVVGKG-----WTTGSPFRTINYNAGV-WAPNGNGYLTLYGWTRSPLIE
3     ------------------------------------------------------------
4     LLAVHGSRATSNLNGSR-----------LGTSLTDSLQELEPILAVRTVNSSQT-NPWQN

1     YYIVDSWGNWRPPGATPKGTITVDGGTYDIYETLRVNQPSIKG--IATFKQYWSVRRSKR
2     YYVVDSWGTYRPTG-TYKGTVKSDGGTYDIYTTTRYNAPSIDGD-RTTFTQYWSVRQTKR
3     ----------------------------HLAKGSLFNXNIIAGQVPSNFSPYWNQVTPEN
4     ISIAFRISSSPHVG---ASRAALGPGTGANEVVDLSVEDRLGWRVALLVSRIITIREPER

1     TSG---TISVSNHFRAWENLGMNMG-KMYEVALTVEGYQSSGSANVYSNTLRINGNPLST
2     PTGSNATITFSNHVNAWKSHGMNLGSNWAYQVLATEGYQSSGSSNVTVW-----------
3     STK---NGNVQ-------------------------------------------------
4     KHS---AANVCDLSNN--------------------------------------------

1     ISNNESITLDKNN
2     -------------
3     -------------
4     -------------
```

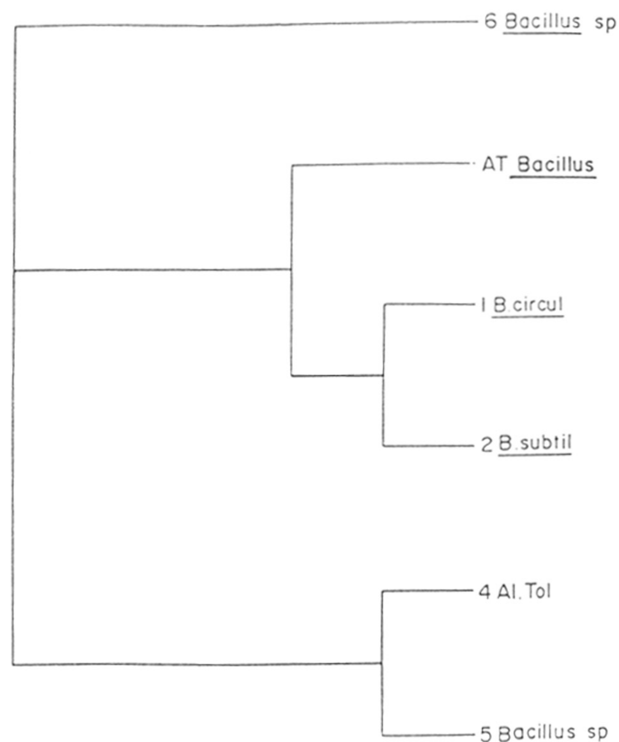
Fig. V.11 Multiple sequence alignment of Xyn II with the xylanases belonging to family 11 produced by alkaliphilic organisms. Xylanases are (accession numbers in parentheses) : 1- alkalitolerant xylanase (A45313), 2- *Bacillus* sp YA-14 Xyn S (S48126), 3- *Bacillus* sp BP-23 Xyn A (id 173920), 4- AT *Bacillus* Xyn II (U 83602).

A flotilla of xylanase sequences and their homology have been documented. There are a few high molecular weight xylanases which show similarity to family 11 xylanases. The catalytic domain of xylanase (STXII) from *Streptomyces thermoviolaceous* OPC-520 (Tsujibo et al., 1997) and the amino terminal domain of xylanaseD from *Ruminococcus flavefaciens* (Flint et al., 1993) showed significant similarity to xylanases belonging to family 11. Domains A and B of xylanaseC from ruminant organism *Fibrobacter succinogenes* S85 shared homology with family 11 xylanases from *B.circulans, B. subtilis and B. pumilus* (Paradis et al., 1993). XynII from the AT *Bacillus* represents the first example showing similarity to family 10 xylanases although it belongs to family 11.

We have attempted to elucidate the evolutionary relationship among xylanases produced by neutrophilic and alkaliphilic organisms by comparing the amino acid sequences. The dendrogram (fig. V.12) illustrates the relatedness between Xyn II and other xylanases produced by neutrophilic and alkaliphilc organisms. Both the groups appeared to be linked through the almost universal sequence (-SQTN-) that started at an amino acid residue position 39 of the AT *Bacillus* XynII (fig. 7 and 10). However the conserved triad -VVX- (where X is N or D) that was identified in xylanases produced only by alkaliphilic organisms, may play an important role in determining the functional conformation and activity at alkaline pH.

The structural studies of various xylanases and XynII from the little-investigated extremophilic *Bacillus* sp is interesting from an evolutionary point of view. Hydrophobic cluster analysis of xylanases (Henrissat and Barioch, 1993) has shown that they share a considerable sequence homology. Their similar crystal structures and folding patterns corroborate that they are derived from a common ancestral precursor through divergent evolution. Proteins with analogous functions generally have evolutionary relationships revealed by very similar amino acid sequences. Surprisingly, a comparison of XynII from AT *Bacillus* with other xylanases has proven that- despite many similar properties, the primary structure showed very limited homology (less than 20%) suggesting that XynII might have evolved independently. This would represent the case of a convergent evolution of xylanase at molecular level. Although convergent evolution is uncommon among xylanases, comparison of subtilisin and animal serine proteases has also shown that they have evolved independently since their primary and three dimensional structures are totally different, despite many similar properties (Smith et al., 1968). XynII exhibited similar identity with families 10 and 11 of xylanases suggesting that it probably

represents the first member of an additional family. Further work on three dimensional structure of XynII is in progress which will help in structural comparison with other reported xylanases. However more data on xylanases from alkaliphilic organisms is necessary to corroborate the validity of the hypothesis.



V.12 Dendrogram showing possible relationship of Xyn II with xylanases from alkaliphilic and neutophilic organisms, created by treeview package (Page, 1996). The differences between the sequences are proportional to the length along the horizontal axis. Abbreviations of the species are as follows: (Accession numbers of xylanase sequences are given in the legends to figures 7 and 10) 1.B. circul.-*B. circulans* , 2. B. subtil-*B. subtilis*, 3. Xyn II from the AT *Bacillus*, 4. Al. Tol. Alkali tolerant xylanase , 5. *Bacillus* sp. C-125, 6. *Bacillus* sp. (N137).

CHAPTER VI

APPLICATION OF XYLANASE FROM AT *BACILLUS* IN BIOBLEACHING OF BAGASSE PULP

SUMMARY

The use of hemicellulolytic enzymes has recently attracted considerable interest as substitutes for chlorine chemicals in pulp bleaching due to the environmental concerns. The cellulase free xylanase from AT *Bacillus* was evaluated for prebleaching of the bagasse pulp. The U V absorption spectrum of the compounds released by enzyme treatment and after alkali extraction showed a characteristic peak at 280 nm indicating the presence of lignin in the released coloring matter. The material released after enzyme treatment also absorbed strongly at 237, 280 and 465 nm. The biotreatment resulted in 2 units decrease in kappa number without altering the strength properties of the pulp. Subsequently the peroxide bleaching of the enzyme treated samples resulted in decrease in kappa number by 10 units and increase in the brightness by 2.5%. The viscosities of xylanase treated samples were found to be unaltered.

INTRODUCTION

Environmental regulations have put a restriction to the usage of chlorine in bleaching processes in paper and pulp industry, especially in Western European countries and in North America (Viikari et al., 1986). The hemicellulase-aided bleaching method represents a new type of technology, adopted by the pulp and paper industry. Hemicellulases are the first group of specific enzymes to be used in a large-scale application in this industrial area. The approach also represents a truly sustainable technology.

Wood is mainly composed of cellulose, hemicellulose and lignin. Cellulose is the most abundant of the components, generally representing 40-45% of the wood dry weight (Sjostrom, 1993). The two main hemicelluloses in wood are xylans and glucomannans. The softwood xylan has a backbone of arabino-4-O-methyl-glucuronoxylan, which is composed of D-xylopyranose units connected via β-1,4-glucosidic linkages. Hardwood xylan contains 4-O-methyglucuronic acid and acetyl side groups. Methylglucuronic acids are linked to the xylan backbone by β-1,2-glucosidic bonds, and the acetic acids are esterified at the carbon 2 and/or 3 hydroxyl group. Galactose is the main fraction of glucomannan in softwoods whereas it is present only in a small amount in hardwoods. The two main glycanases depolymerizing the hemicellulose backbone are endo-1,4-β-D-xylanase and endo-1,4-β-D mannanase. Enzymatic bleaching is reported to result from the cleavage of bonds between lignin and carbohydrate and the opening up of the pulp structure (Paice et al., 1988).Viikari et al. (1986) first showed that treating of pulps with hemicellulases can reduce subsequent chlorine bleaching requirements and subsequently other investigators (Clark et al., 1990; Manoir et al., 1991; Paice et al., 1988) have confirmed these studies. Xylanases play an important role in debarking, deinking of recycled fibers, and in the purification of cellulose for the preparation of dissolving pulp (Jager et al, 1992). Xylanases promote bleaching by the hydrolysis of relocated, reprecipitated xylan on the surface of the pulp fibers, allowing for better chemical penetration and thus improving lignin extractibility (Kantelinen et al, 1993). The reprecipitated xylan forms a barrier for the extraction of lignin, both, in hardwood and softwood pulps. Scanning electron microscopic studies of xylanase pretreated pulps revealed increase in the porosity of pulp fiber aiding in pulp accessibility to bleaching chemicals (Pekarovicova et al., 1992). However no traversal cracks were observed on the fibers that might decrease the fibre mechanical strength (Fig. VI.1.I and II).

Xylanases from different organisms have been evaluated for their interaction with various kinds of pulps. At the laboratory scale, xylanases from *Streptomyces roseiscleroticus* (Patel et al, 1993), actinomyces (Davis et al, 1992), *T. harzianum* (Senior et al, 1988), and *Humiola sp.* (Silva et al, 1994) have been used for enzymatic pulp treatment to test their bleach boosting abilities. Recently, the thermostable xylanase from *Thermotoga maritima* was compared with commercial pulpzyme HC and was found to be efficient in releasing lignin from kraft pulp (Chen et al, 1997).The cloned xylanases expressed in *B. cereus* (Tremblay et al, 1993) and *E. coli* (Paice et al, 1988) have also been reported to improve the delignification of unbleached kraft pulps, thus reducing the chlorine required to achieve a certain degree of brightness. The thermostable xylanase produced by thermophilic, anaerobic bacterium *Dictyoglomus sp.* (Ratto et al, 1994), has been evaluated for its suitability in pulp bleaching. Xylanase pretreatment at 80^0 C and pH 6-8 resulted in an increase in brightness by 2 ISO units in one stage peroxide delignification. Thermostable xylanase from *B. sterarothermophilus* T-6 bleached the pulp effectively at 65^0 C and pH 9.0, and has been implicated successfully on industrial scale mill trial (Lapidot et al, 1996). The first commercial xylanase preparation available for pulp bleaching was marketed by Novo Nordisk A/s, under the name "Pulpzyme HA", which was produced by a strain of *T. reesei.* Recently three commercially available xylanases, viz., Ecopulp (from Alko-ICI), Cartazyme-NS-10 (from Clariant) and Pulpzyme HC (from Novo Nordisk), were tested in the bleaching of Eucalyptus kraft pulps. The results indicated a significant decrease in consumption of ClO_2 and H_2O_2 (Vicuna et al, 1997). Recently total chlorine free (TCF) bleaching methods are being developed in which enzymes have been combined with O_2, O_3 and/or hydrogen peroxide (Farell et al, 1996). In TCF bleaching sequences, the addition of enzymes increases the final brightness value, which is a key parameter in marketing the chlorine-free pulps. In addition to this, savings in the TCF bleaching chemicals are important with respect to both costs and the strength properties of the pulp. The more efficient xylanase yielding strains and technologies will offer a low investment xylanase-aided bleaching that is both environmentally and economically advantageous.

From western countries many reports using xylanases from different sources for evaluating their interaction with various kinds of pulps are available (Senior et al., 1991; Viikari et al., 1994). However it is necessary to assess and study the processes under conditions specific to different countries with various kinds of pulps which are locally available. In Maharashtra, (India) where sugarcane is the abundantly grown crop,

bagasse is one of the major cheap raw materials available for paper making. Bagasse is the residue from the crushing of sugar cane and it contains about 65% fiber, 25% pith cells and 10% water solubles and foreign material. Bagasse fiber averages 1.5 to 2 mm in length and the fibers are relatively fine as compared with wood pulp. Bagasse contains a high proportion of hemicellulose and high-yield bagasse pulp produces dense, well-bonded sheets with low tearing strength.

The most significant feature of xylanases from AT *Bacillus* is their cellulase free nature which is one of the necessary prerequisites for use in the paper and pulp industry. The present chapter describes the application of the cellulase free xylanase active in the alkaline pH from AT *Bacillus* in enzymatic treatment of the bagasse pulp.

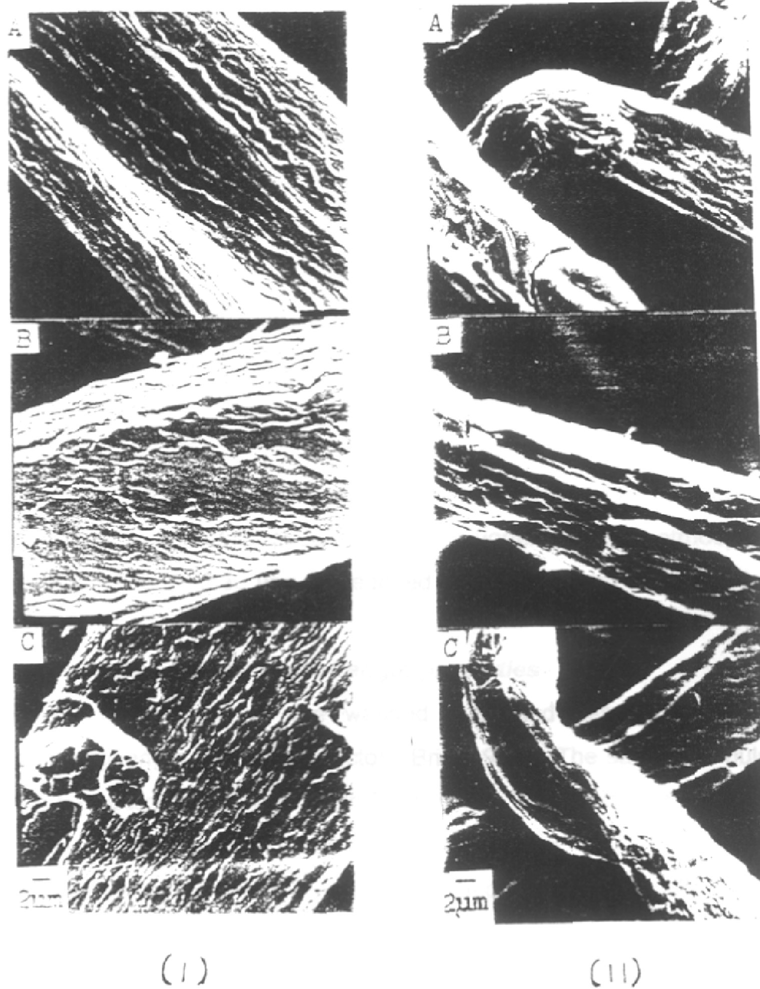


Fig. VI. 1 .I. Electron micrographs of mixed softwood sulhate pulp.

II. Electron micrographs of mixed hardwood sulphate pulp.

a) original pulp b) xylanase prebleached c) xylanase prebleached and alkaline extracted.

(Based on Pecarovicova et al, 1992)

MATERIALS AND METHODS

Production of enzyme

AT *Bacillus* was isolated from a hot water spring (Vajreshwari District, Thane, Bombay, India). It is an aerobic, gram positive bacterium growing at 50^0C, pH 10.0 (Dey et al., 1992). For enzyme production AT *Bacillus* was cultivated in shake flasks in a 50 ml medium containing wheat bran (5% w/v) and yeast extract (0.5% w/v) and Na_2CO_3 (1%). The fermentation was carried out at 50^0C for 48 h using 10% inoculum. The centrifuged medium was precipitated with 3 volumes of chilled ethanol as described by Dey et al. (1992). The precipitate obtained after centrifugation was dissolved in 10 ml of 50 mM phosphate buffer, pH 7.0 and was used as crude xylanase (500 U/ml) for further studies. Enzyme assay was carried out as described in chapter II.

Preparation of bagasse pulp: mechanochemical method

The bagasse was obtained after crushing and extraction of the sugar cane. The depithing was carried out by mechanical method (Casey, 1960). The depithed bagasse was then treated with caustic soda (pH 11) in a vortex pulper at atmospheric pressure for 1 h at 75^0C. The washed and unbleached pulp in this form was used in the following experiments.

Estimation of kappa number and strength properties

Handsheets were made of the washed pulp and used to determine kappa number,breaking length and burst factor (Britt, 1970). The sample of pulp (3-4 g) was exposed to the action of 0.1 N $KMnO_4$ (acidified) in a volume of 1000 ml at 25^0C for 10 min. The reaction was stopped by adding excess of KI solution and KMnO4 consumed was determined from the results of back-titrating the liberated iodine with standard sodium thiosulphate. The kappa number so obtained was the ml of 0.1 N $KMnO_4$ consumed per gram of pulp (Tappi method T 236). It is a measure of residual lignin in the pulp. Breaking length was determined using special crushers machined in such a manner that the length of specimen to be broken was effectively zero.

Determination of viscosity

Viscosity was determined by dissolving delignified pulp in cupriethylenediamine (CED) and measuring the viscosity of a 0.5% solution with an Ostwald viscometer (Tappi method T 230). Brightness of a paper sheet was determined at 681 nm with an Elrepho instrument.

Properties of crude xylanase from AT Bacillus

The activity of extracellular xylanase from AT *Bacillus* was estimated at 60, 70 and 80^{0}C and at pH 7, 8, 9 and 10. Relative residual activity was determined considering the activity obtained at 60^{0}C and pH 7 as 100%. Eventhough the enzyme showed maximum activity at 60^{0}C and pH 7, 52% activity was retained at 70^{0}C and pH 9. At 80^{0}C the enzyme exhibited 42 and 30% activity at pH 7 and 10 respectively (Fig. VI.2)

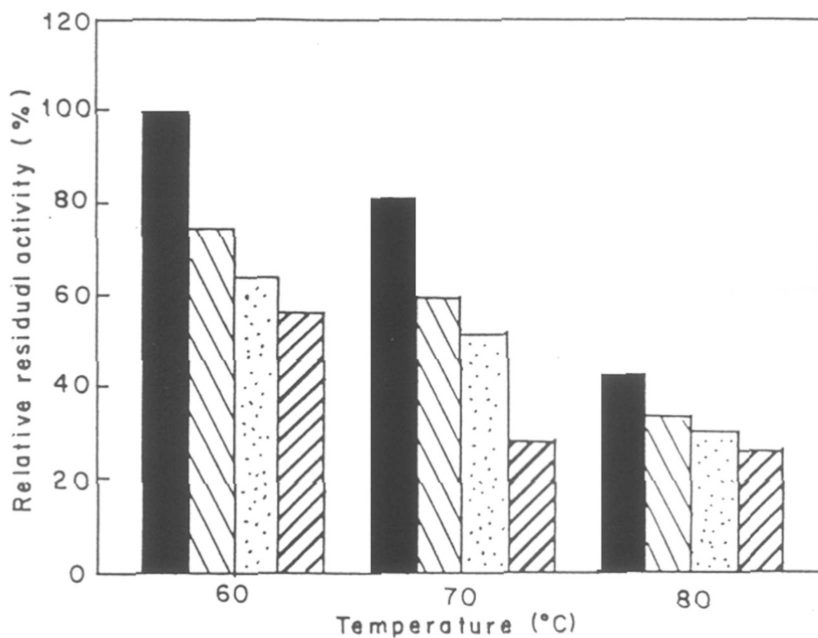


Fig. VI.2 Properties of crude xylanase from AT *Bacillus*. Activity at pH 7.0 and 60^{0}C was considered as 100%. Residual activity was estimated at temperatures 60, 70 , 80^{0}C and at pH 7 (■), 8 (▧), 9 (▭) and 10 (▨).

Colour removal from the bagasse pulp

Enzyme treatment (stage 1).

The bagasse pulp was treated with crude xylanase in 50 mM phosphate buffer (50 ml), pH 7.0. The enzyme dosage levels from AT *Bacillus* ranged from 0 to 40 U/ g oven dry pulp. The pulp samples were mixed with the suitably diluted enzyme and then incubated at 50^{0}C for 4 h. The control was treated in a similar manner but with water substituted for enzyme. Following incubation the pulp samples were washed and the absorbance of the filtrate was determined spectrophotometrically. It was observed that colour removal increased with enzyme dose upto 40 U/ g oven dry pulp as determined by the increase in absorbance at 237, 280 and 465 nm (Fig. VI.3).

Alkali extraction (stage 2).

The pulps were washed with water and preheated to 65^{0}C prior to the addition of 1.5 g NaOH per 150 g oven dry pulp. The pulps were extracted for 1 h at 65^{0}C, and the absorbance of the filtrate at 237, 280 and 465 nm was determined. The samples were subsequently washed with water until the pH of the washings were neutral. The absorbance at 237, and 465 nm was also found to increase when the alkali extract of the enzyme treated pulp was used (Fig. VI.3).

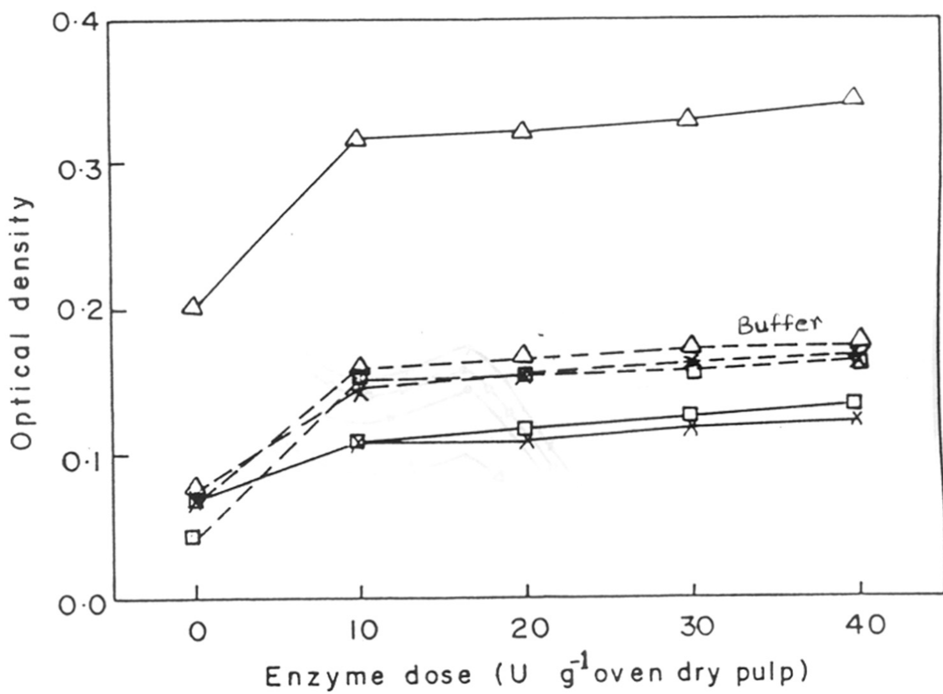


Fig.VI.3 Absorbance of the buffer (---) and alkali extracts (–) from enzyme treated pulp samples. Symbols represent optical density at 237 nm (□), at 280 nm (×), and at 465 nm (△) respectively.

The peak at 280 nm in the U V spectrum indicated the presence of lignin in the released coloring matter (Fig.VI.4).

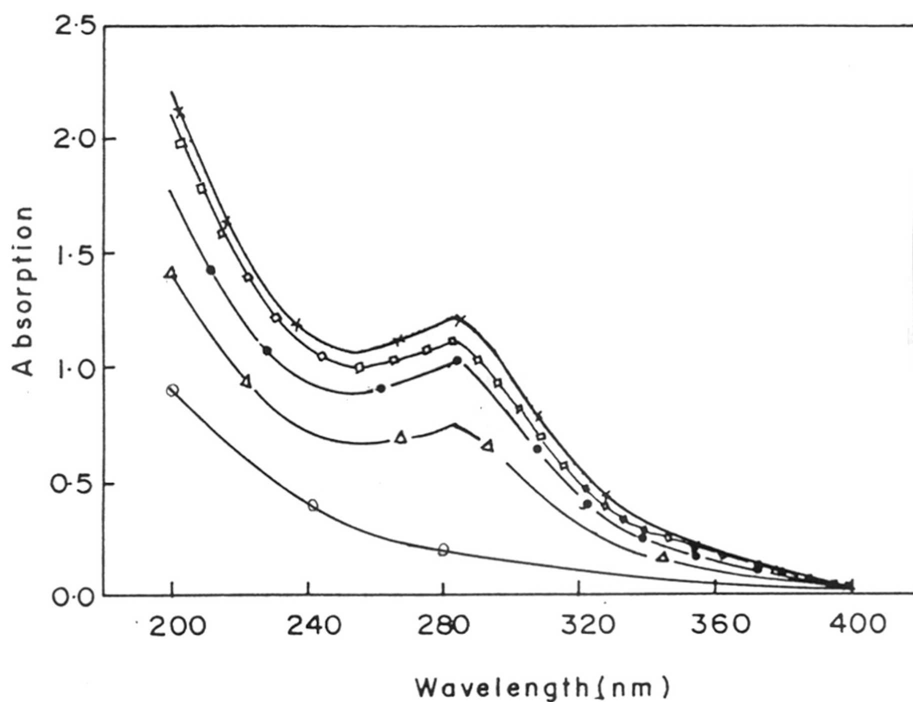


Fig. VI.4 U V spctrum of coloured compounds released during enzyme treatment at 0 (○), 10 (△), 20 (●), 30 (□) and 40 (X) enzyme units per g oven dry pulp respectively.

This result was consistent with kappa numbers obtained (Table VI.1). Maximum reduction was obtained at 10 U/g oven dry pulp. Further increase in the enzyme dosage did not seem to reduce the kappa number eventhough there was an increase in the absorbance at 280 nm supporting the observation by Ziobro (1990 a, b) that carbohydrate degradation products also attribute to the coloring matter.

Table VI.1

Effect of biotreatment on kappa number and strength properties:

Enzyme dose	Kappa number	Breaking length	Burst factor
(U/g oven dry pulp)		(meters)	
0	32.22	3826.77	20.76
10	30.20	3823.70	20.98
20	30.22	3810.75	20.50
30	30.30	3815.60	21.09
40	30.25	3840.10	20.10

The unbleached bagasse pulp was treated with different concentrations of crude xylanase from *Bacillus* and the samples were incubated for 4 h at 50^0 C. The handsheets made of these samples were used to measure kappa number and strength properties.

Estimation of strength properties

The strength properties of the handsheets made of the biotreated pulp were not found to be altered as compared to that of the reference pulp (Table VI.1) due to the cellulase free nature of the enzyme. It has been shown that for normal papers, the values obtained are linearly related to the fibre strength. The burst factor indicates the degree of bonding between the fibres of a sheet and is the ratio between normal and the zero span breaking lengths of the test sheets. It provides means for determining an index of average ultimate strength of individual fibres either in a pulp or in a commercial paper sample.

Peroxide delignification of the enzyme treated pulp

In another set of experiment the unbleached bagasse pulp was treated with crude xylanase (10 U/g oven dry pulp). Treatment was given as described previously. However the samples were removed after 4, 8 and 12 h. After washing the samples were used for

peroxide bleaching followed by two hypochlorite stages instead of conventional chlorine bleaching. Kappa number was found to reduce by 10 U after peroxide treatment compared to 3 U decrease in the control pulp (Table VI.2). However relative shrinkage in peroxide stage was more for biotreated samples as compared to that obtained in the hypochlorite stages. No change was observed in the viscosity of the enzyme treated samples. The sample removed after 4 h showed maximum increase in the ISO brightness (2.5%) suggesting that longer period of treatment was not needed which is another useful feature of the enzyme for use in the pulp bleaching.

Table VI.2

Effect of peroxide treatment on enzyme treated pulp:

	Reference pulp	Enzyme treated samples (Treatment time in h)		
		4	8	12
Kappa no. (unbleached pulp)	42.00	42.00	42.00	42.00
Kappa no. (after biotreatment)	42.00	41.00	41.20	41.20
Peroxide stage				
H_2O_2 added (%)	1.75	1.75	1.75	1.75
H_2O_2 consumed (%)	0.92	1.34	1.34	1.34
Kappa no.	39.00	32.00	33.70	33.00
Hypo-I				
Hypochlorite added (%)	5.50	5.50	5.50	5.50
Hypochlorite consumed (%)	5.42	5.42	5.42	5.42
Hypo-II				
Hypochlorite added (%)	2.50	2.50	2.50	2.50
Hypochlorite consumed (%)	2.31	2.31	2.31	2.31
Relative shrinkage (%)				
(peroxide stage)	2.00	6.70	5.60	8.00
Relative shrinkage (%)				
(hypo-I,II stages)	11.50	9.50	10.50	8.00
Viscosity cp (CED)	11.80	12.30	12.30	12.00
Brightness (% ISO)	70.00	72.50	72.00	72.00

The unbleached bagasse pulp was treated with 10 U of xylanase per g oven dry pulp for different time intervals at 50^0C. Biotreatment was followed by peroxide bleaching.

H_2O_2, pulp 12%, 70^0C, 3 h.

Hypo-I and Hypo-II, pulp 8%, 40^0C, 75 min.

% chemicals are based on oven dried pulp.

DISCUSSION

Xylanases from various fungi and bacteria have been reported to facilitate the bleaching of kraft pulp, thereby reducing chlorine demand by decreasing the kappa number. It has been suggested that xylanases mainly act on reprecipitated xylan on the surface of the microfibrils (Kantelinen et al., 1991) allowing for better chemical penetration and thus improve lignin extractability. Alkaline stable lignin-carbohydrate complexes present in the wood seem to be the major obstacles for solubilisation of the residual lignin. During conventional bleaching, these linkages are cleaved by acidic bleaching stages e.g. chlorine or chlorine dioxide. However the degradation products adversely contribute to the effluent. In contrast to this, hemicellulose degrading enzymes selectively hydrolyze polysaccharide chains attached to lignin, thereby decreasing the amount of chemicals required for pulp bleaching. It is observed that xylanase from AT *Bacillus* retains more than 50% activity at 70^{0}C and pH 9. Thus it seems to have potential for commercial application. Very few reports are available in which clear correlation between action pattern of the enzyme and nature of chromophore released is described (Patel et al., 1993). Absorbance at 465 nm correlates with release of color by the enzyme action (as determined by absorbance at 237 nm). Our results showing increase in absorbance at 280 nm with a peak in the U V spectrum emphasize the presence of lignin in the released coloring matter from the bagasse pulp (Nissen et al., 1994). However corresponding decrease was not observed in kappa number suggesting that increase in absorbance at 280 nm may be due to the release of lignocellulose compounds (Fry, 1988). Paice et al. (1992) have reported loss in the fibre strength of black spruce kraft pulp when bleached with commercial xylanase. However the strength properties of the paper made from the biotreated bagasse pulp in the present work remain unaltered, thus proving the suitability of the cellulase free xylanase from AT *Bacillus* in pulp bleaching. The major content of crude xylanase preparation from AT *Bacillus* has been reported to be of low molecular weight (15,800) basic (pI 8) xylanase (Dey et al., 1992). This has the added advantage in pulp bleaching due to better substrate accessibility and more beneficial electrostatic interactions between fibres and enzyme. Considerable reduction in kappa number (10 U) was obtained when the biotreated samples were subjected to peroxide treatment compared to 3 U in that of the reference pulp. Also relative shrinkage in hypochlorite stage was less for enzyme treated samples. The cellulose chain length is indicated in terms of viscosity and the

nonspecific endoglucanases were observed to reduce the viscosity of softwood kraft pulp indicating the degradation of cellulose chains (Viikari et al., 1992; Da Silva et al., 1994). However no decrease in the viscosities of the bagasse pulps treated with xylanase from AT *Bacillus* were observed even after long incubation period of 12 h. Our studies indicate that xylanase activated the pulp towards bleaching with hydrogen peroxide suggesting that the future applications will not be focused solely on chlorine and chlorine dioxide bleaching. Further work using different bleaching sequences that may yield paper of higher brightness and better quality is in progress.

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