MOLECULAR CLONING AND EXPRESSION OF XYLANASES FROM ALKALOPHILIC THERMOPHILIC BACILLUS SP. (NCIM 59)

A THESIS
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Shendye A.P.

Abhay Shendye

DECLARATION

Certified that the work incorporated in the thesis "MOLECULAR CLONING AND EXPRESSION OF XYLANASES FROM ALKALOPHILIC THERMOPHILIC BACILLUS SP. (NCIM 59)" submitted by Mr. Abhay P. Shendye was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Mala Rao

Mala Rao
Research Guide

ABSTRACT OF THE THESIS

INTRODUCTION

Recent studies on the extremophilic microorganisms have demonstrated that the boundaries and borderlines in our thinking about the conditions which allow life on earth have to be enlarged. A further deepening of biological research on extremophilic organisms is of particular importance for our understanding of the early evolution of prokaryotes, the relation between structure and function of the cell components and enzymes, the evolution of survival strategies, mechanisms of energy transduction and conservation, while its significance for biotechnology is thought to be enormous (Prins *et al* 1990). Many advantages for the use of thermophiles in the biotechnology processes such as reduced contamination risk, faster reaction rates, reduced fermenter cooling cost etc. have been proposed. Amylases, proteases, Taq DNA polymerase and restriction endonucleases such as Bst II, Bcl I etc. are some of the examples of the commercially important enzymes from thermophiles (Edward and Jones 1983).

The alkalophilic microorganisms have already made large impact in the application of biotechnology for the manufacture of mass market consumer products. While proteases, lipases and cellulases from the alkalophiles have already been commercially applied, few enzymes such as amylase, glucanase, β lactamase, pectinase, pullulanase and xylanase are being investigated, and there is a lot of scope for

future development (Grant *et al* 1990).

An alkalophilic thermophilic *Bacillus* has been isolated in our laboratory which produces cellulase free xylanase activity (Hinge *et al* 1989, Dey *et al* 1992). Xylanases (1,4 - β - D xylan xylanohydrolase EC 3.2.1.8) catalyze the hydrolysis of xylan to xylooligosaccharides and xylose which are useful as feedstock for food and fuel. The use of xylanases in conjunction with cellulases for the complete conversion of the cellulosic biomass to sugars is widely studied (Biely 1985), and it can greatly improve the overall economics of the processing of the lignocellulosic biomass (Flinckinger *et al* 1980). Large quantities of xylan are released as effluent in the paper and pulp industry and their bioconversion may be of economic significance. Recently the use of cellulase free xylanases for the selective hydrolysis of hemicellulose component in paper and pulp is receiving consideration (Paice and Jurasek 1984). Thermally stable xylanases active at alkaline pH may be particularly useful for the treatment of alkaline pulps.

In the view of the above application, the xylanases from alkalophilic thermophilic *Bacillus* are potentially useful, and hence they were studied in greater detail. The present studies deal with the molecular cloning of the xylanase gene fragment from alkalophilic thermophilic *Bacillus* and the expression of the recombinant xylanases in *E. coli* and *Bacillus*. *E. coli* harboring a recombinant plasmid pATB 507I and capable of growth at 50^{o} C was obtained from the

genomic library of alkalophilic thermophilic *Bacillus*. The recombinant was analyzed further for the thermal adaptation which was found to be a transformable property.

The main features of the work done are:

1. Construction of genomic library of alkalophilic thermophilic *Bacillus* and identification of the xylanase gene fragment.
2. Analysis of gene expression in *E. coli*.
3. Homologous expression in *Bacillus subtilis*.
4. Gene integration and enhanced xylanase expression .
5. Genetic transfer of thermophilic trait from alkalophilic thermophilic *Bacillus* to *E. coli*.

SUMMARY OF THE WORK

1. CONSTRUCTION AND SCREENING OF GENOMIC LIBRARY IN *E. COLI*.

A genomic DNA library of alkalophilic thermophilic *Bacillus* was constructed in *E. coli* using standard approach of shot gun cloning. The high molecular weight genomic DNA was isolated from alkalophilic thermophilic *Bacillus* and conditions for Hind III digestion were standardized. The size fractionation was carried out using sucrose density ultracentrifugation. The DNA fragments in the range of 6 - 12 kb were used for ligation with Hind III digested and dephosphorylated pUC 8, an expression vector. Based on α complementation of the β galactosidase gene, 4,500 colonies were identified as recombinants which represented the alkalophilic thermophilic

Bacillus genome with more than 99 % confidence.

The genomic library was screened using xylan congo red plate clearance assay and antibodies raised against the purified xylanase II from alkalophilic thermophilic *Bacillus*. Six out of 4,500 recombinants from the genomic library showed clearance zone on the xylan congo red plate. These clones also showed a positive ELISA reaction with the antixylanase II antibodies. The plasmid DNA was isolated from the six putative positive clones and digested with Hind III. Identical restriction pattern was obtained with each recombinant showing three inserts of sizes 6.5, 3.2 and 1.7 kb.

2. SUBCLONING AND ANALYSIS OF GENE EXPRESSION IN *E. COLI*

The Southern hybridization of Hind III digested pATB X 4.5 with labeled genomic DNA of alkalophilic thermophilic *Bacillus* confirmed the origin of the insert to be from alkalophilic thermophilic *Bacillus*. The Hind III digest of pATB X 11.5 was cloned in pUC 8 and the recombinants were screened for xylan plate clearance. The xylanase gene was localized in the 6.5 kb Hind III fragment. The insert had unique site for Eco RI and Pvu II and had no cleavage site for Bgl II, Cla I, Pst I and Sma I. The 4.5 kb Eco RI Hind III subfragment was found to code for the xylanase activity.

The recombinant pATB X 4.5 secreted out xylanases [2 U/ml] in the culture filtrate. The recombinant coded for two xylanases which were identified as xylanase I and II using

double diffusion immunoreactivity with the antixylanase II antibodies. The molecular weights of the two xylanases were 35 and 14.5 KD as detected by SDS PAGE of the immunoprecipitated extract. The xylanase activity was constitutive and addition of xylan or IPTG or reorientation of the insert did not alter the xylanase activity, indicating that the xylanase expression was under its own promoter.

3. HOMOLOGOUS EXPRESSION IN *B. SUBTILIS*

The overexpression of a cloned gene is possible in a homologous host system because of the efficient transcription, translation and secretion. Hence the 6.5 kb xylanase gene fragment from alkalophilic thermophilic *Bacillus* was cloned in a shuttle vector pLP 1202 and the expression of the xylanases was studied in the xylanase negative (*B. subtilis* A8) and positive (*B. subtilis* MI 111) hosts. The recombinant xylanases were secreted out by both the strains. The expression of the xylanases was found to be constitutive. The expression of xylanases in *B. subtilis* A8 and *B. subtilis* MI 11 was 140 and 310 mU/mg protein respectively.

4. GENE INTEGRATION AND ENHANCED XYLANASE EXPRESSION

The level of expression of the xylanases in *B. subtilis* strains was higher than that in *E. coli* but lower than that in alkalophilic thermophilic *Bacillus*. The low levels of expression may be attributed to the weak recognition of the alkalophilic thermophilic *Bacillus* signals which is an unusu-

al extremophilic species. Hence chromosomal integration and xylanase gene amplification was carried out using alkalophilic thermophilic *Bacillus*. An electroporation mediated transformation of alkalophilic thermophilic *Bacillus* was carried out with the recombinant plasmid pATB X6.5. The integrants were characterized by larger zone of xylan clearance than the parent culture and hybridization with pUC8. The Hind III digested genomic DNA of alkalophilic thermophilic *Bacillus* when probed with 6.5 kb Hind III fragment harboring the xylanase gene, showed a single hybridization band at 6.5 kb. However DNA digests of the integrant strain CII 11 showed two positive signals at 5.1 and 3.2 kb whereas strain CIII 6 showed three positive signals at 4.8, 3.2 and 2.9 kb. These results indicated the occurrence of homologous recombination in 6.5 kb xylanase gene region of the genomic DNA and suggested a non Campbell mode of recombination. The integrants were checked for xylanase production up to ten subcultures and consistently showed two fold higher xylanase activity than the parent strain. The xylanase productivity (U/ml/h) of the parent and the integrant strains was maximum at 20 and 16 h respectively. The maximum xylanase productivity of 7.4 U/ml/h was obtained for strain CIII 6.

5. GENETIC TRANSFER OF THERMOPHILIC TRAIT FROM ALKALOPHILIC, THERMOPHILIC *BACILLUS* TO *E. COLI*

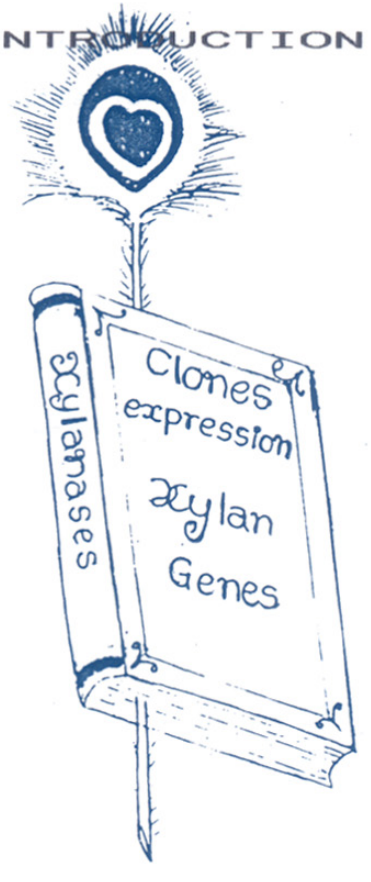
The genomic DNA library of alkalophilic thermophilic *Bacillus* was screened for a possible recombinant capable of growth at 50° C. Out of 10,000 recombinants one was found to grow at 50 ° C. The recombinant plasmid pATB 507 I showed 20 bands on agarose gel electrophorasis, suggesting that the plasmid was unstable. The *E. coli* recombinant pATB 507 I after curing showed resistance to ampicillin and retained the thermal adaptation property. The southern blot analysis of the total DNA from the recombinant with the labeled genomic DNA from alkalophilic thermophilic *Bacillus* showed hybridization of *E. coli* genomic DNA with the probe, indicating that the plasmid pATB 507 I had undergone recombination(s) with the *E. coli* genome. When the plasmid DNA was transformed in *E. coli* JM 109, one in 10^4 transformants was capable of growth at 50° after an overnight incubation at 37 ° C. These transformants were also associated with multiple plasmid bands, indicating the possible prerequisite of recombination(s) for growth at high temperature. The recombinant pATB 507I had no rec A activity as assayed using mytomycin C.

A part of this work has been published as:

1. Hinge, J., **Shendye, A.**, Srinivasan, M.C. and Rao, M. (1989) Indian Patent Application No. 796-DEL-89.
2. Dey, D., Hinge, J., **Shendye, A.**, and Rao, M. (1992) Can. J. Microbiol. 38, 436-442.
3. **Shendye, A.** and Rao, M. (1993) FEMS Microbiol. Letts. 108, 297-302.
4. **Shendye, A.** and Rao, M. (1993) Enzyme Microb. Technol. 15, 343-347.
5. **Shendye, A.** and Rao, M. (1993) Biochem. Biophys. Res. Comm. 195, 776 - 784.
6. **Shendye, A.**, Gaikaiwari, R. and Rao, M. (1994) W. J. Microb. Biotechnol. (In press).

CHAPTER I

GENERAL INTRODUCTION



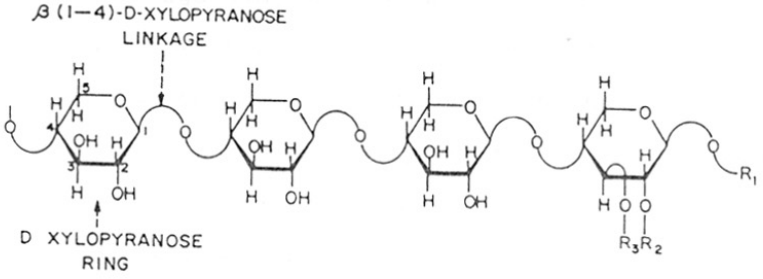
Xylans are heterogeneous polysaccharides found in the plant cell wall and have a backbone of β- 1,4 - linked xylopyranose residues. Xylan is the second most abundant renewable resource with high potential for its degradation to useful end products. Microbial xylanases (1,4-β- D xylan xylanohydrolase EC 3.2.1.8) are the most preferred catalyst for xylan hydrolysis due to their high specificity, the mild reaction conditions, and negligible substrate loss and side product generation (Thomson 1993). The thorough understanding of the xylanolytic enzymes is therefore necessary for their optimum utilization.

Xylanases are produced by a variety of organisms which include bacteria, algae, fungi, protozoa, gastropodes, arthropodes, and higher plants (Dekker and Richards 1976, Taize and Honigman 1976). Most of the bacteria and fungi show extracellular xylanase secretion. The secreted xylanases act on the hemicellulosic material to liberate xylose as a directly assimilable end product and thus allow 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 ruminant animals. Xylanases play a key role in invasiveness of the plant pathogens by hydrolysis of the xylan in the plant cell wall (Anderson 1978, Dean and Anderson 1991, Dean *et al* 1991, Doux-Gayat *et al* 1978). Purified xylanase from *Trichoderma viridae* was found to induce the biosynthesis of ethylene and two other pathogen related proteins in tobacco (Taize and

Honigman 1976), suggesting that the xylanases could also play a role in induction of plant defense mechanism. The xylanases from the germinating plant seeds primarily convert the reserve food into the assimilable end product (Slade *et al* 1989), but their role is also proposed in the cell elongation. Xylanases from the plants seem to be involved in fruit softening (Lobavitch and Greve 1983) and beleived to play - yet undiscovered - important physiological roles.

Structure of xylan

The β-1,4-xylans are amorphous substances that exist in the highly branched heteropolymeric state. Unlike cellulose, crystalline regions are absent in xylan and the side branches are formed of more than one type of substituents. Based on the common substituents found on the backbone, the xylans are divided in to different categories namely linear homoxylan, arabinoxylan, glucuranoxylan, and glucuranoarabinoxylan. However in each category there is microheterogenity with respect to the degree and nature of branching. Moreover complex side chains comprising of the oligosaccharide and nonoligosaccharide moieties have also been reported (Joseleu *et al* 1992). The basic backbone and the possible substituent groups are shown in the figure 1.



R_1:

p Coumaric acid

α-D-Glucuronic acid

α-D-Arabinofuranose

R_2:

Acetyl group

α-D-Glucuranopyranose [Cα Lignin]

4-O-Methyl-α-D-Glucuranopyranose [Cα Lignin]

α-L-Arabinfuranose [Cα and Cβ Lignin, Ferulic acid, Acetyl group, p Coumaric acid]

Cα Lignin

R_3:

Acetyl group

α-L-Arabinofuranose [Cα and Cβ Lignin, Ferulic acid, acetyl group, p Coumaric acid]

β-D-Galactopyranosyl (1—5) α-L-Arabinofuranose

β-D-Xylopyranosyl (1—2) α-L-Arabinofuranose

α-L-Arabinofuranosyl(1—2,1—3,1-2,3 Arabinofuranose)n

β-D-Galactopyranosyl (1—4) D-Xylopyranosyl (—2)-α-L-Arabinofuranose

FIG 1.1: CHEMICAL STRUCTURE OF XYLAN

Although the chemical structure of the xylan is known for a long time; most of the description is regarding the alkali soluble major fraction, with no or very little information on the minor structural elements. A lot less is known about the true primary structure of xylan due to lack of information regarding the sequence and pattern of distribution of the side chains, along the xylan backbone (Joseleau et al 1992). The side chains determine the solubility, physical conformation, and reactivity of the xylan molecule with the other hemicellulosic components and hence greatly influence the mode and extent of enzymatic cleavage. The three dimensional structure of the xylan molecules has been recently described by Atkins (1992). The xylan backbone shows a "three fold left - handed conformation under crystallized conditions" and the geometry of the glycosidic linkage is not affected by the side chains (FIG. 2). However availability of the three dimensional structure data on xylan in aqueous environment would be extremely important in understanding the xylan and xylanase interactions.

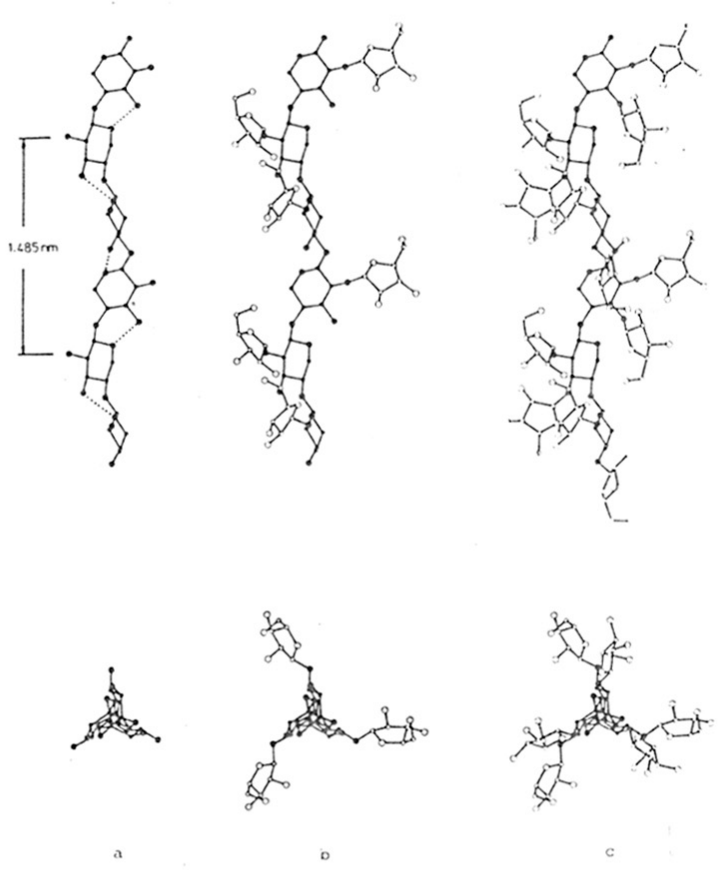


FIG 1.2:THREE DIMENSIONAL STRUCTURE OF XYLAN

Projections perpendicular (top) and parallel (bottom) views of (a) xylan backbone, (b)backbone with one L-arabinose side group, (c) backbone with two L-arabinose side groups. Hydrogen bonds are shown dotted.

Xylanolytic enzymes

Xylan being a heterogeneuos substrate having various chemical substituents, the complete hydrolysis of xylan requires the action of several hydrolytic enzymes. Xylanases attack the polysaccharide backbone at β- 1,4-linkages and β-xylosidase hydrolyses xyloligosaccharides to D- xylose. α -L-Arabinofuranosidase and α -glucuronidase are involved in degradation of acetyl xylan. Acetylesterase or acetyl xylan esterase deacetylate acetyl xylan and act synergistically with xylanase in the degradation of acetyl xylan. Generally xylanases do not cleave linkages adjacent to the units carrying a side chain. Three different types of xylanases are involved in xylan degradation (Reilly 1981, Woodward 1984, Dekker 1985).

1. Endo-β-(1,4)-D-xylanase: These enzymes act randomly on xylan to produce large amounts of xylooligosaccharides of various chain lengths. They are classified as follows:

(a) **Non-arabinose liberating endoxylanases**: These xylanases cannot act on L-arabinosyl initiated branch points at β -(1,4) linkages.

(b) **Arabinose liberating endoxylanase**: These xylanases can attack at the branch points and produce arabinose.

The arabinose liberating and arabinose non liberating xylanases are further classified on the basis of their ability to degrade the xylan to xylose and xylobiose along with the other smaller oligosaccharides.

2. Exo-β-1,4-D-xylanase: The enzymes capable of cleaving the single xylose units from the non-reducing end of the xylan chain are the exoxylanases.

3. β-Xylosidase or xylobiase: Xylosidases hydrolyse disaccharides like xylobiose and higher xylooligosaccharides with decreasing specific affinity.

Xylanase production

Xylanolytic enzyme systems of the bacteria and fungi have been shown to be inducible. But rare examples of constitutive xylanase synthesis have also been reported (Shoham Y. 1994, Srivastava and Srivistava 1993). Almost all the xylanase producers show low levels of constitutive xylanase activity, which has a role to play in the induction mechanism. Xylan - being a high molecular size polymeric substance - can not penetrate the cell membrane, and the low molecular weight derivatives of xylan play key role in the regulation of the xylanase expression. These low molecular derivatives 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. Cellulose has also been shown to act as an inducer of the xylanases in few cases (Biely *et al* 1991, Thomson 1993, Wong *et al* 1988), but whether the cellulose or the contaminating xylan fraction has the inducing effect is unclear at present (Thomson 1993, Biely *et al* 1992). Xylan has been found to be the best inducer of xylanase biosynthesis in *Aspergillus sydowii*, *Neurospo-*

ra crassa , *Paecilomyces varioti*, and *Pichia stiptis* (Ghosh and Nanda 1991, Kelly *et al* 1989, Mishra *et al* 1984, Ozcan *et al* 1984). However, cheaper hemicellulosic substrates like corn cob, wheat bran, rice bran, rice straw, corn stalk and bagasse have also been found to be better inducers in case of certain microorganisms such as *Streptomyces* T_7 , alkalophilic *Streptomyces* VP5, *Penicillium funiculosum*, *Thielaviopsis basicola* and *Phanerochaete chrysosporium* (Dobozi *et al* 1992, Ghosh and Dep 1988, Keskar 1992, Mishra *et al* 1985, Vyas *et al* 1990). The xylanase induction is a complex phenomenon and multiple chemical substances are known to induce the xylanase expression to different degrees. The response to an individual inducer is species and gene specific. The occurance of two xylanases in the single culture filtrate - one inducible and the other constitutive, have been reported (Debeire *et al* 1990). An inducer chemical showing maximum xylanase expression in one species may be actually the inhibitor of xylanase expression in the other species (Hrmova *et al* 1986, 1989, 1991). Since the substrate derivatives - that happen to be the enzymatic end products - often play key positive role in the induction of xylanases, the same chemical substances also act as end product inhibitors of the enzyme synthesis - possibly at much higher concentration. The heterodisaccharides and their positional isomers are synthesized by more than one enzymes which include xylanase, xylosidase, xylobiase, and transglycosidase. The low molecular weight subatances that have been identified as the xylanase inducers need transferase enzymes for their translocation into the cyto-

plasm. Hence presence and the level of all these or the required enzymes in the culture filtrate also affects the xylanase synthesis. The possible factors representing the regulation of xylanase biosynthesis have been diagramatically represented in the figure 1.3, which is largely based on the figure by Thomson (1993).

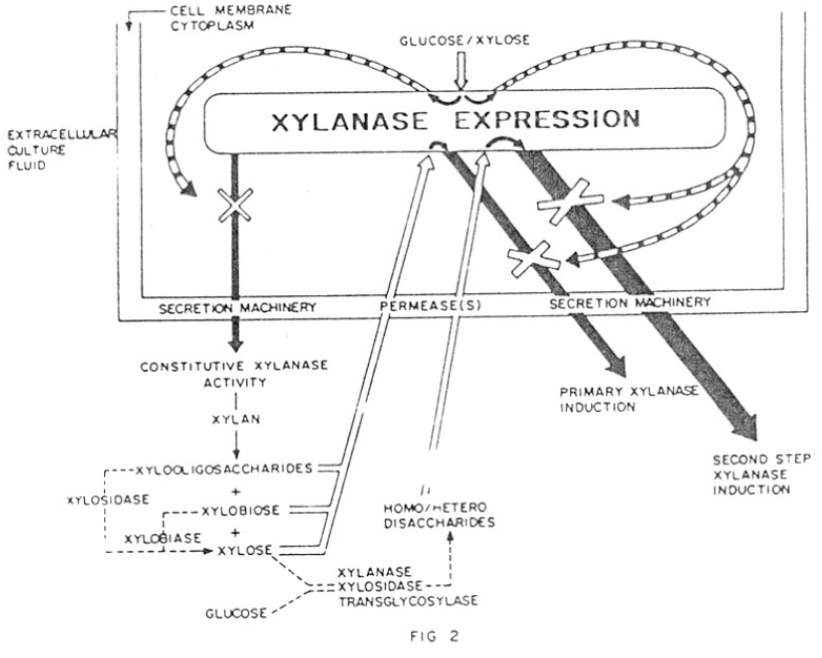


FIG 1.3: REGUL ATION OF XYLANASE SYNTHESIS

The level of xylanase expression is regulated by various additional factors when the xylanase fermentation is carried out on the complex heterogeneus substrates. These include xylan accesibility; rate, amount, and chemical nature of the released xylooligosaccharides; and quantity of xylose released - which acts as the carbon source as well as inhibitor of xylanase synthesis in most of the cases - and have a combined effect on the level of xylanase expression. Generally, the slow release of the inducer molecules and the possibility of conversion of the inducer to its non metabolizable derivative by the culture filtrate are believed to boost the level of xylanase expression.

Multiplicity of xylanases

Most of the microorganisms producing the xylanases show multiple protein bands associated with the xylanase activity. The multiplicity of the xylanases arises due to several reasons such as: presence of distinct xylanase genes, differential post translational modification, proteolysis and broad specificity of the other glycanases towards the xylan (Wong *et al* 1988). The multiple xylanases have been shown to posses differential microspecificity towards the heterogeneus substrate - the xylan - in various aspects of substrate specificity such as branching status of the substrate, affinity towards the insoluble and / or soluble substrate, and ability to cleave various oligosaccharides.

The multiple enzyme components of the xylanase enzyme

systems are known to exhibit cooperativity in the aspects of xylan degradation such as reducing the degree of polymerization of xylan, solubilization of xylan, generation of small oligosaccharides, and xylose production.

Biochemical characteristics of xylanases

The xylanases from different sources display extreme variability with respect to their biochemical and physicochemical properties. Microbial xylanases are single subunit proteins with *Mr* and pI values in the range of 8.5 - 145 kD and 4.0 - 10.4 respectively. The pH optima of xylanases are generally in the range of 4.0 - 7.0 (Coughlan 1992), but a few exceptions have been reported (Akiba and Horikoshi 1981, Wood *et al* 1981, Nakamura *et al* 1994). The Dixon plot of the xylanase activity Vs pH is by an large a bell shaped curve which indicates that two reactive groups with ionization optima values falling on the two sides of the peak are involved in the catalysis. Most of the xylanases have the temperature optima in the range of 40 - 50 ^{o}C. Thermostable xylanases with enzymatic activity in the temperature span of 70 - 90 ^{o}C have been reported (Dahlberg *et al* 1992, Mathrani and Ahring 1992, Nakamura *et al* 1994), and are believed to have better application potential (Jurasek and Paice 1986). Multiple forms of xylanases are usually found in the culture filtrates of various microorganisms. Xylanases also vary in their catalytic activities; subsite maps and mode of action; affinity for soluble and insoluble substrate; and the presence and organization of the functional domains. These

aspects have been extensively reviewed (Bastawde 1992, Biely 1985, Gilkes *et al* 1991, Henrissat 1992, Wong *et al* 1988). However, significant amount of data on substrate specificity and domain structure of xylanases has been added in the recent years.

Substrate specificity:

In the early work, the xylanases have been described as arabinose liberating (debranching) and non liberating enzymes that are further classified depending on their ability to generate small oligosaccharides (X3, X4) along with xylose and xlyobiose (Reilly 1981, Woodward 1984). The xylosidase-, xylobiase- and exoxylanase- like activity associated with the xylanases has also been reported. Low levels of activities towards the non-xylose oligosaccharides have been described as the nonspecific reactivity of the corresponding xylanases (Lee *et al* 1987, Toda *et al* 1975). The recent studies have deepened our understanding regarding the much diverse results on substrate specificity. The xylanases vary in their sugar and linkage specificity. The purified xylanases have been reported to cleave number of polysaccharides such as CMC and avicel (Tan *et al* 1987), starch (Yoshiko *et al* 1981), and p- nitrophenyl - ß- D glucoside (Shei *et al* 1985). They exhibit variable action pattern with respect to main and side chain cleavage and the substituents may be a requirement or hinderance to the activity of an individual xylanase. These properties lead to broad / additional sub-

strate specificities in different xylanases. The broad substrate specificity of the xylanases has been shown - in some cases - as a result of multiple functional domains present in the same enzyme. The xylanase specificity can be better characterized with the help of subsite mapping which also gives an insight in to the mechanistic behaviour of the enzyme. These studies have been carried out using the xylooligosaccharides of defined lengths that are radiolabeled at the reducing end. The predictions about the subsites are made on the basis of frequencies for cleavage of the particular bonds.The subsite mapping studies on the xylanase from *Aspergillus niger* have shown that the substrate binding site consists of eight subsites and the catalytic site is at the center. Hence the xylobiose molecule can not form a productive complex with the enzyme (Meagher *et al* 1988). The acidic xylanase from *A. niger* is consisting of seven subsites (Vrsanska *et al* 1982). The xylanase from *Ceratocystis paradoxa*, which preferentially cleaved highly substituted xylan, had four substrate binding subsites with the catalytic site at the center (Dekker and Richards 1975 a,b). The xylanases from *Cryptocs albidus* was found to have four substrate binding subsites (Beily *et al* 1981).

Domain structure:

Two distinct classes of xylanases have been revealed by hydrophobic cluster analysis (Glikes *et al* 1991). The individual xylanases from the two classes have similar molecular weights, domain structures and catalytic sequences.

However, the position of the catalytic domain with respect to the N terminal end has not been considered. In addition to catalytic and substrate binding domains, cellulose binding, cellulosome forming and other homologous domains with unknown function have been described.

TABLE 1.1 : GROUPS OF XYLANASES ON THE BASIS OF THE DOMAINS

GROUP	ENZYME	ORGANISM	REFERENCE
Group I	Xyn A	*Aspergillus kawachii*	Ito *et al* 1992
	Xyn A	Alkalophilic *Bacillus* sp. C-125	Hamamoto *et al* 1987
	Xyn A	*Butyrivibrio fibrisolvens* 49	Mannarelliet *al* 1990
	Xyn B	*B. fibrisolvens* H17C	Lin and Thomson 1991
	Xyn A	*Coldocellum saccharolyticum*	Luthi *et al* 1990
	Xyn Z	*Clostridium thermocellum*	Grenipet *et al* 1988
	Xyn	*Cryptococcus albidus*	Boucher *et al* 1988
	Xyn	*Penicillium chrysogenum*	Williams and Withers 1992
	XyL A	*Psuedomonas fluorescens* subsp. *cellulosa*	Hall *et al* 1989
	XyL B	*Pseudomonas fluorescens* subsp. *cellulosa*	Kellet *et al* 1990
	XyL A	*Ruminococcus flavefaciens* 17	Zhang and Flint 1992
	Xln A	*Streptomyces lividans*	Shareck *et al* 1991
	STX I	*Streptomyces thermooviolaceus* OPC-520	Tsujibo *et al* 1992
	Xyn	*Thermoascus aurantiacus*	Srinivasa *et al* 1990

GROUP	ENZYME	ORGANISM	REFERENCE
Group II	Xyn C	*A. kawachii*	Ito *et al* 1992
	Xyn	*Aureobasidium*	Leathers 1988
	Xyn	*Bacillus circulans*	Yang *et al* 1988b
	Xyn A	*Bacillus pumilus*	Fukusaki *et al* 1984
	Xyn	*Bacillus subtilis*	Paice *et al* 1986
	Xyn II	*Chainia*	Bastawde *et al* 1991
	Xyn B	*Clostridium acetobutylicum*	Zappe *et al* 1990
	XyL A	*R. flavefaciens* 17	Zhang and Flint 1992
	Xyn	*Schizophyllum commune*	Paice *et al* 1978
	Xyn B	*S. lividans*	Shareck *et al* 1991
	Xln C	*S. lividans*	Shareck *et al* 1991
	STX II	*S. thermoviolaceus* OPC 520	Tsujibo *et al* 1992
	Xyn	*Trichoderma reesei*	Torronen *et al* 1992
	Xyn	*Trichoderma harzianum*	Yaguchi *et al* 1992a
	Xyn	*Trichoderma viridae*	Yaguchi *et al* 1992b

Mechanism of action of xylanases

The involvement of various amino acid such as cysteine and/or cystine (Beguin 1990, Biswas*et al* 1990, Deshpande *et al* 1990), tryptophan (Deshpande *et al* 1990, Keskar *et al* 1989, Yasui *et al* 1988), histidine (Keskar *et al* 1989), tyrosine (Bray and Clarke 1990), and carboxyl group (Bray and Clarke 1990, Chauthaiwale and Rao 1993, Ko *et al* 1992) in the essential residues for catalysis has been demonstrated. But involvement of cysteine/cystine is doubtful in most of the cases (Coughlan 1992). The xylanases and lysozyme are believed to have similar mechanism of substrate cleavage (Koshland 1953). Both the enzymes catalyze hydrolysis of the substrate by cleavage of equatorial pyranosidic linkage. The recent results suggest two distinct catalytic mechanisms for xylanases on the basis of final molecular configuration of the product (Sinnott 1992). Some of the xylanases operate via a double displacement mechanism - like lysozyme - in which anomeric conformation is retained (Hardy and Poteete 1991), while the others catalyze single displacement reaction in which the conformation is reverted (Sinnott 1990).

Biotechnological potentials of xylan and xylanases

In recent years, the potential biotechnological applications of xylan and xylanases have been of particular interest. Purified xylan is an excellent source of additive for direct tableting, which has potentials for delayed release

tablet construction (Gamerith and Strutzenberger 1992). The role of dietary xylan as a regulator of human immune system has been demonstrated (Maidz et al 1992). The hydrolysis products of xylan - xylose and xylooligosaccharides - have direct and indirect utility. Xylose is presently being used in the food and pharmaceutical industry, and could also serve as a raw material in synthesis of speciality products. The xylooligosaccharides have potential applications as food thickener, which will substitute fats (Zeikus *et al* 1991); and as an antifreeze food additive (McCleary 1986). Some of the xylooligosaccharides have been claimed to act as hypertension / hypotension modulant , hepatotrophic agent, and anti HIV agent. The xylan hydrolysis products can be subsequently converted into liquid fuel, single cell proteins, and solvents (Wong and Saddler 1992), artificial low calorie sweetener (Magee and Kosaric 1985), claimed anticarcinogenic agent and activating factor for *Bacillus bifidus*.

Applications of xylanases are generally viewed from the perspective of bioconversion or selective degradation of hemicelluloses. Combinations of xylanases, other hemicellulases and cellulases in varying concentrations are selected for different applications. Cellulase free xylanases are desirable for their application in the paper and pulp industry. Xylanases play a key role in maceration of vegetable matter (Beck and Scott 1974, Lahaye and Vigouroux 1992); protoplastation of the plant cells (Butenko and Kochko 1979); clarification of juices and wine (Biely 1985, 1989); liqui-

faction of coffee mucilage for the manufacture of liquid coffee (Wiseman 1975); recovery of oil from subterrenian mines; extraction of flavors and pigments (Johonson *et al* 1978), plant oils and starch (McCleary 1986); and in improvement of production efficiency of agricultural silage (Wong and Saddler 1992). Applications of xylanases in paper and pulp, animal feed, and bakery have been worked out in greater details. Xylanases help in the pulp bleaching process by solubilization of chromophore, swelling of fiber matrix to disrupt the adsorptive interactions, solubilization of the redeposited xylan, and cleavage of lignin carbohydrate linkage (Wong *et al* 1988). They play important role in debarking, prebleaching of kraft pulp, deinking of recycled fibers, and in purification of cellulose for preparation of dissolving pulp (Paice and Jurasek 1984, Gamerith and Strutzenberger 1992). The xylanases assume special importance in the paper and pulp industry as they replace the toxic chemicals such as elemental chlorine and chlorine dioxide. Several review articles have summarized the potentials of xylanases in paper and pulp industry (Eriksson 1990, Pommier 1991, Reid 1991, Trotter 1990, Viikari *et al* 1991). Some of the recent articles describe the processes which may be useful in developing total chlorine free bleaching method with the help of microbial xylanases (Yan *et al* 1993 , Pommier *et al* 1990). The dietary hemicelluloses have little nutritional significance for the non - ruminant organisms as they lack the appropriate digestive enzymes. These undigested fibers increase the viscosity of the food in the gut, which interferes with

penetration of digestive enzymes, absorption of the digested food and may support pathogenic conditions especially in broiler chicks (Annison and Choct 1991, Fry *et al* 1958 , Schutte 1990, White *et al* 1983,). The use of xylanases along with other hemicellulases corrects the problems and also increases the nutritive value of the feed. In bakery the xylanases act on the gluten fraction of the dough and help in even redistribution of the water content of the bread (Roza *et al* 1992), thereby significantly improving the desirable texture, loaf volume and the shelf life of the bread. These biotechnological potentials of the xylanases have prompted the search for suitable enzymes and technologies for large scale economic production of highly active and stable enzyme preparations.

Molecular cloning of xylanases

The xylanase genes have been cloned from different microbial genera and expressed in *E. coli* (table 1.2). The xylanase expression in *E. coli* is generally found to be lower than the parent organism. The xylanases expressed in *E. coli* are generally 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 low levels of xylanase gene expression. However, low level of extracellular activity has been reported in *E. coli* for the xylanases from alkalophilic *Aeromonas*,

alkalophilic *Bacillus* and *Cellulomonas* sp. (Bhalerao *et al* 1990, Honda *et al* 1985, Kudo *et al* 1985). Although the analysis of the xylanase gene expression is presented in the table, the relative vertical comparison of the activity values is not desired; because of the high standard deviation values (108 %) associated with the xylanase assay using different substrate preparations and / or minor modifications of the assay method.

TABLE 1.2: CLONING AND EXPRESSION OF XYLANASE GENES IN *E. COLI*

SOURCE ORGANISM	VECTOR	DESIGNATION		MW		EXPRESSION OF CLONED XYLANASE			REFERENCE
		PROTEIN	VECTOR	P	R	I:E:P	MAX. EXPRESSION U/ml	U/mg	
Aeromonas sp.212 (ATCC 31085)	pBR 322	XylL	pAX 1	145	135	60:40:0	1.63	-	Kudo *et al* 1985
Aspergillus kawachi	\gt 11	XylA	-	35	32.7	-	-	-	Rozaet *al* 1992
	pUC 118	XylC							
Bacillus C125	pBR 322	XylA	pCX 311	43	43	3.5:82:14	0.57		Honda *et al* 1985
Bacillus sp. YA14	-	XylS	-	-	-	-	-	-	Bernier *et al* 1985
B. circulans NRC 9024/USDA 729	pUC 19	-	pBCX549	59 22	59 22	>90:?:?	0.04	-	Yang *et al* 1989
B. polymyxa NRC 2288/NRRL 8505	pBR 322	-	pBPX277	48	51	?:<10:major	0.037	-	Yang *et al* 1988
	pUC 13	-	-	-	22	?:35%:?	-	-	
B. polymyxa NCIB 8158/ATCC 842	pBR 322	-	pX1	-	-	intra.	10.0	0.1	Sandhu 1984

TABLE 1.2 (CONTINUED)

SOURCE ORGANISM	VECTOR	DESIGNATION		MW		EXPRESSION OF CLONED XYLANASE			REFERENCE
		PROTEIN	VECTOR	P	R	I:E:P	MAX. EXPRESSION		
							U/ml	U/mg	
B. pumilus IPO	pBR 322	XN/ XylA	pOXN 29	-	-	93:0:7	-	0.002	Panbangred *et al* 1983
B. subtilis PAP115	pBR 325	-	pRH 271	-	22	intra	0.5	-	Bernier *et* 1983
B. subtilis CD4	pUC 8	-	pBX 3	-	-	-	2.8	-	Srivastava 1993
Bacteroides ovatus V 975	pUC 18	-	-	-	-	intra	-	12.9	Whitehead 1990
B. ruminicola #23	pUC 18	-	pRX 1	-	-	intra	-	1.1	Whitehead 1988
Butyrivibrio fibrosolvens # 49	pUC 19	XylA	pML 110	45	46.6	93.2:5:2.8	-	0.01	Mannarelli 1990
Caldocellum saccharolyticum	\1059 pBR 322	XylA	pNZ1076	-	42	-	-	-	Clarke *et a* 1991
Cellulomonas sp. NCIM 2353	pUC 18	-	pCX 11	-	45	extra	-	0.056	Bhalerao *et* 1990

TABLE 1.2 (CONTINUED)

SOURCE ORGANISM	VECTOR	DESIGNATION		MW		EXPRESSION OF CLONED XYLANASE			REFERENCE
		PROTEIN	VECTOR	P	R	I:E:P	MAX. EXPRESSION		
							U/ml	U/mg	
C. bioaztea	pHC 79	-	pXA 1	-	-	intra	-	-	Clarke *et al* 1991
DSM 20112	/pUC 19								
Chainia sp.	\gt 10	-	pVX 8	6	-	intra	0.003	0.018	Chauthaiwale 1992
NCL 82-5-1	pUC 8								
Clostridium sp. F1	pBR 322		-	-	-	intra	-	-	
C. acetobutylicum	pEcoR251	-	pHZ 300	28	28	intra	[64]	4.0	
# P 262									
C. stercorarium	pHC 79	XylX	-	-	70,62,42		-	3.1	Sakka *et al*
NCIB 11745		CelX	-	80/	80,120	intra	-	1.1	
		CElW	-	120	42		-	1.2	

TABLE 1.2 (CONTINUED)

SOURCE ORGANISM	VECTOR	DESIGNATION		MW		EXPRESSION OF CLONED XYLANASE				REFERENCE
		PROTEIN	VECTOR	P	R	I:E:P	MAX. EXPRESSION U/ml	U/mg		
C. thermocellum NCIB 10682	pUC 8	XylZ	-	90	41,39	intra	-	1.5		Mac Kenzie *et al* 1989
C. thermocellum ATCC 27405	pUC 19	-	pCTX736-1	-	-	-				Mac Kenzie *et al* 1989
			pCTX736-2	-	25	>90% intra	-	-		
Cryptococcus albidus	pUC 13	-	-	40	44	-	-	-		Morosoli *et a* 1988
F. succinigenes # 135	\WES\B pBR 322	-	pFSX02	-	-	20:20:60	0.57	28.5	4.3	Hu *et al* 1991

TABLE 1.2 (CONTINUED)

SOURCE ORGANISM	VECTOR	DESIGNATION		MW		EXPRESSION OF CLONED XYLANASE				REFERENCE
		PROTEIN	VECTOR	P	R	I:E:P	MAX. EXPRESSION U/ml	U/mg		
Neocallimastix patriciarum	\ZAP II	XylB	-	93	73,42	intra	-	193.75	-	Lee *et al* 1993
Pseudomonas	\47.1	-	pJHH1	-	-	2:16:82	-	0.059	-	Gilbert *et al* 1988
fluorescens			pJHH4	-	-	0:9:91	-	0.061	-	
subsp. *cellulosa*			pJHH5	-	-	1:12:87	-	0.039	-	
Ruminococcus albus	pBR 322	-	pRAL1	-	56	intra	-	0.013	-	
# SY3			pRAL2	-			-	<0.001	-	Romaniec *et al* 1989
R. flavefaciens	\EMBL3	-	L9	-	-	-	1.6	-		Flint *et al*
# 17		XylB	X10	-	20-30	-	0.9	-		

TABLE 1.2 (CONTINUED)

SOURCE ORGANISM	VECTOR	DESIGNATION		MW		EXPRESSION OF CLONED XYLANASE				REFERENCE
		PROTEIN	VECTOR	P	R	I:E:P	MAX. EXPRESSION U/ml	U/mg		
R. flavefaciens		-	X7	-	-	-				
# 17		XylA	X4	-	30&>	-	1 :	-		
Streptomyces flacogriseus	pUC 8	-	pSX4	18	18	intra	7.85	-		
Thermomonospora fusca YX	\gtWES\B	-	-	-	-	52:27:0	0.79	-	0.025	Ghangas *et al* 1989
Trichoderma reesei	pGEM5Z(+)	XylI	-	19	19	-	-	-		Torronen *et al*
C 30		Xyl II	-	21	20.7	-	-	-		1992

VECTOR DESIGNATION CORRESPONDS TO THE RECOMBINANT CHIMERA

P AND R CORRESPOND TO PARENT AND RECOMBINANT PROTEIN

I:E:P IS THE RATIO OF INTRACELLULAR, EXTRACELLULAR AND PERIPLASMIC XYLANASE ACTIVITY

Cloning of xylanase gene in heterologous host systems other than *E. coli*

The cloning and expression of the xylanases in the non xylanolytic organisms such as *Saccharomyces*, and *Lactobacillus* have been shown to offer fermentative advantages to the host in utilizing the hemicellulosic materials as the fermentable carbon source. Expression of the xylanases in non homologous yet related host has been shown to better than that in *E. coli*. These studies are summerized in table 1.3.

TABLE 1.3: CLONING OF XYLANASE GENES IN HETEROLOGOUS HOSTS OTHER THAN E. COLI

PARENT STRAIN	HOST	VECTOR	REFERENCE
Aspergillus kawachii	*S. cerevisiae*	pVT 100	Ito *et al* (1992)
Clostridium acetobutylicum	*L. plantarum*	pWP 37	Scheirlinck (1990)
C. thermocellum	*L. plantarum*	pWP 37	Scheirlinck (1990)
	B. subtilis	pUB 110	Pack 1993
Cryptococcus albidus	*S. cerevisiae*	pVT 100	Morosoli *et al* 1992
	P. stipitis	pJHS	

Homologous expression of the xylanase genes

The cloning of genes in the homologous host system offer expression advantages because of the efficient transcription, translation and secretion of the protein. The gene regulation studies carried out on xylanases in the homologous host systems are of particular significance since they provide the enzymes such as transglycosylases and xylosidases that are necessary for the xylanase induction (Thomson 1993). In case of *Streptomyces* the promoter elements may not be recognised by the *E. coli* σ factor, in turn leading to difficulties in expression of the corresponding gene. Hence the construction of genomic library and the screening for the xylanase gene has been carried out from *Streptomyces* sp.# 36a and *S. lividans* strain #1326 using the suitable homologous host system. In case of *B. pumilus* IPO, the extracellular secretion of the xylanase was achieved using the homologous host *B. subtilis*. The level of expression of the xylanases is relatively higher in homologous host system than that in *E. coli*. The reports of the homologous cloning and expression of the xylanases are summerized in the table 1.4.

TABLE 1.4: HOMOLOGOUS EXPRESSION OF XYLANASE GENES

PARENT STRAIN	HOST	VECTOR	REFERENCE
Aspergillus niger var *kawachii*	*A. niger*	pAW 14S	Roza *et al* (1992)
Bacillus pumilus IPO	*B. subtilis* MI 111	pUB 110	Panbangred 1985
Streptomyces #36a	*S. lividans*	pIJ 702	Mondou *et al* 1986
	S. kasugansis	pSK 2	
S. llividans	*S. lividans*	pIJ 702	Iwasaki *et al* 1986
Streptomyces EC3	*S. lividans*	pIJ 702	Servais *et al* 1992
	S. paravulus	pIJ 702	

Protein engineering of xylanases

Biotechnological applications of the xylanases demand for the identification of highly stable enzyme preparations that show the optimum activity at extremes of pH and temperature range. Hence the protein engineering studies on xylanases have gained importance. The X - ray crystallogrphic studies are useful to elucidate the structure function correlation of the enzyme which is one of the basic prerequisite for the specific manipulation of the enzymatic properties. The preliminary X - ray crystal data has been obtained for xylanases from *Bacillus* sp. (Inaoka and Soda 1956), *B. subtilis* (Wakarchuk *et al* 1992), *B. pumilus* (Katsube *et al* 1989), *Gliocladium virens* (Takahashi and Katsumi 1979), *Thermoascus aurantiacus* (Khandke *et al* 1989), *Trichoderma harzianum* (Rose *et al* 1987), and *T. viride* (Hashimoto *et al* 1971). However electron density map and further analysis is available only for the *B. pumilus* xylanase. Identification of the active site residues by chemical modification has been reported for several xylanases, but very few have been confirmed by site directed mutagenesis studies. These include Glu 79 and Glu 172 residues of the xylanase from *B. subtilis* (Wakarchuk *et al* 1992), Glu 78 of xylanase from *B. circulans* (Yang *et al* 1988), Glu 93 of *B. pumilus* (Katsube *et al* 1989).

The stabilization of the xylanases by random mutagenesis of the cloned gene fragment from *Bacillus pumilus* IPO has been described (Arase A. *et al* 1993). Four mutants - each showing a single amino acid substitution - were selected on

the basis of activity at 60 ^{o}C. The changes in the specific activities of the mutant enzymes were observed. The mutant N104 (Gly 38 to Ser) showed five fold decrease in the specific activity where as N 102 mutant (Gly 38 to Asp) showed 80 % increase. In the studies on the site specific mutagenesis of xylanase A gene from *Streptomyces lividans* # 1326, single amino acid substitutions - Arg 156 to Glu, Arg 156 to Lys and Asn 173 to Asp - have resulted into the formation of three mutant enzymes which show increased specific activity as well as thermal half life at 60 ^{o}C. The double mutant - 156 Glu and 173 Asp - however showed increased half life but decreased specific activity. One mutant - Phe 155 to Tyr - had increased specific activity and decreased thermal half life (Moreau A. *et al* 1994).

One of the ways of identifying the biotechnologically suitable xylanase preparations is to look for the extremophilic microorganisms as their enzymes are expected to be stable and active under the conditions of their optimum growth. Recent studies on the extremophilic microorganisms have demonstrated that the boundaries and borderlines in our thinking about the conditions which allow life on earth have to be enlarged. A further deepening of biological research on extremophilic organisms is of particular importance for our understanding of the early evolution of prokaryotes, the relation between structure and function of the cell components and enzymes, the evolution of survival strategies,

mechanisms of energy transduction and conservation, while its significance for biotechnology is thought to be enormous (Prins *et al* 1990). Many advantages for the use of thermophiles in the biotechnology processes such as reduced contamination risk, faster reaction rates, reduced fermenter cooling cost, etc. have been proposed. Amylases, proteases, Taq DNA polymerase and restriction endonucleases such as Bst II, Bcl I, etc. are some of the examples of the commercially important enzymes from thermophiles (Edward and Jones 1983). The alkalophilic microorganisms have already made large impact in the application of biotechnology for the manufacture of mass market consumer products. While proteases, lipases and cellulases from the alkalophiles have already been commercially applied, few enzymes such as amylase, glucanase, β lactamase, pectinase, pullulanase and xylanase are being investigated, and there is a lot of scope for future development (Grant *et al* 1990). Recently xylanases from *Dictioglomus* sp. have been described which show thermal half life of 1.3 h at 90 ^{o}C and 25 % of its optimum activity at pH 9.5(Mathrani and Ahring 1992). The crude xylanase preparation from *Bacillus* sp. # TAR 1 was found to be optimally active at 75 ^{o}C and pH 7.0 or at 70 ^{o} and pH 9.0. These xylanases from the extremophilic microorganisms may prove to be useful in the future biotechnological exploitations.

The alkalophilic thermophilic *Bacillus* NCIM 59 is an isolate from our laboratory which produces cellulase free xylanases at 50 ^{o}C and pH 10. The xylanase fermentation,

purification and characterization of the two xylanases has been described earlier (Hinge *et al* 1989, Dey *et al* 1992). Involvement of tryptophan and carboxylic group in the active site of the purified xylanase I (Mr 35,000) has also been reported (Deshpande *et al* 1990, Chauthaiwale and Rao 1993).

The present investigations deal with the cloning and expression of the xylanases from the alkalophilic thermophilic *Bacillus* NCIM 59 in to *E. coli* and *B. subtilis*. Analysis was also carried out on the *E. coli* recombinant capable of growth at 50 ^{o}C. The thesis includes:

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: CONSTRUCTION AND SCREENING OF GENOMIC LIBRARY IN *E. COLI*.

A genomic DNA library of alkalophilic thermophilic *Bacillus* was constructed in *E. coli* using standard approach of shot gun cloning. The high molecular weight genomic DNA was isolated from alkalophilic thermophilic *Bacillus* and conditions for Hind III digestion were standardized. The size fractionation was carried out using sucrose density ultracentrifugation. The DNA fragments in the range of 6 - 12 kb were used for ligation with Hind III digested and dephosphorylated pUC 8, an expression vector. Based on α complementation of the β galactosidase gene, 4,500 colonies were identified as recombinants which represented 99% of the alkalophilic thermophilic *Bacillus*.

The genomic library was screened using xylan congo red

plate clearance assay and antibodies raised against the purified xylanase II from alkalophilic thermophilic *Bacillus*. Six out of 4,500 recombinants from the genomic library showed clearance zone on the xylan congo red plate. These clones also showed a positive ELISA reaction with the antixylanase II antibodies. The plasmid DNA was isolated from the six putative positive clones and digested with Hind III. Identical restriction pattern was obtained with each recombinant showing three inserts of sizes 6.5, 3.2 and 1.7 kb.

CHAPTER 3: ANALYSIS OF GENE EXPRESSION IN *E. COLI*

The Southern hybridization of Hind III digested pATB X 4.5 with labeled genomic DNA of alkalophilic thermophilic *Bacillus* confirmed the origin of the insert. The Hind III digest of pATB X 11.5 was cloned in pUC 8 and the recombinants were screened for xylan plate clearance. The xylanase gene was localized in the 6.5 kb Hind III fragment. The insert had unique site for Eco RI and Pvu II and had no cleavage site for Bgl II, Cla I, Pst I and Sma I. The 4.5 kb Eco RI Hind III subfragment was found to code for the xylanase activity. Further subcloning resulted in identification of 1.8 kb Eco RI Pvu II fragment which coded for the xylanase activity.

The recombinant pATB X 4.5 secreted out xylanases [2 U/ml] in the culture filtrate. The recombinant coded for two xylanases which were identified as xylanase I and II using

double diffusion immunoreactivity with the antixylanase II antibodies. The molecular weights of the two xylanases were 35 and 14.5 KD as detected by SDS PAGE of the immunoprecipitated extract. The xylanase activity was constitutive and addition of xylan or IPTG or reorientation of the insert did not alter the xylanase activity, indicating that the xylanase expression was under its own promoter.

CHAPTER 4: HOMOLOGOUS EXPRESSION IN *B. SUBTILIS*

The over expression of a cloned gene is possible in a homologous host system because of the efficient transcription, translation and secretion. Hence the 6.5 kb xylanase gene fragment from alkalophilic thermophilic *Bacillus* was cloned in a shuttle vector pLP 1202 and the expression of the xylanases was studied in the xylanase negative (*B. subtilis* A8) and positive (*B. subtilis* MI 111) hosts. The recombinant xylanases were secreted out by both the strains. The expression of the xylanases was found to be constitutive. The expression of xylanases in *B. subtilis* A8 and *B. subtilis* MI 11 was 140 and 310 mU/mg protein respectively.

CHAPTER 5: GENE INTEGRATION AND ENHANCED XYLANASE EXPRESSION

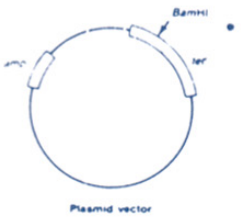
The level of expression of the xylanases in *B. subtilis* strains was higher than that in *E. coli* but lower than that in alkalophilic thermophilic *Bacillus*. The low levels of expression may be attributed to the weak recognition of the

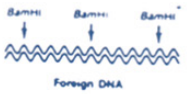
alkalophilic thermophilic *Bacillus* signals which is an unusual extremophilic species. Hence chromosomal integration and xylanase gene amplification was carried out using alkalophilic thermophilic *Bacillus*. An electroporation mediated transformation of alkalophilic thermophilic *Bacillus* was carried out with the recombinant plasmid pATB X6.5. The integrants were characterized by larger zone of xylan clearance than the parent culture and hybridization with pUC8. The Hind III digested genomic DNA of alkalophilic thermophilic *Bacillus* when probed with 6.5 kb Hind III fragment harboring the xylanase gene, showed a single hybridization band at 6.5 kb. However DNA digests of the integrant strain CII 11 showed two positive signals at 5.1 and 3.2 kb whereas strain CIII 6 showed three positive signals at 4.8, 3.2 and 2.9 kb. These results indicated the occurrence of homologous recombination in 6.5 kb xylanase gene region of the genomic DNA and suggested a non Campbell mode of recombination. The integrants were checked for xylanase production up to ten subcultures and consistently showed two fold higher xylanase activity than the parent strain. The xylanase productivity (U/ml/h) of the parent and the integrant strains was maximum at 20 and 16 h respectively. The maximum xylanase productivity of 7.4 U/ml/h was obtained for strain CIII 6.

CHAPTER 6 : GENETIC TRANSFER OF THERMOPHILIC TRAIT FROM ALKALOPHILIC THERMOPHILIC *BACILLUS* TO *E. COLI*

The genomic DNA library of alkalophilic thermophilic *Bacillus* was screened for a possible recombinant capable of growth at 50° C. Out of 10,000 recombinants one was found to grow at 50 ° C. The recombinant plasmid pATB 507 I showed 20 bands on agarose gel electrophoresis, suggesting that the plasmid was unstable. The *E. coli* recombinant pATB 507 I after curing showed resistance to ampicillin and retained the thermal adaptation property. The southern blot analysis of the total DNA from the recombinant with the labeled genomic DNA from alkalophilic thermophilic *Bacillus* showed hybridization of *E. coli* genomic DNA with the probe, indicating that the plasmid pATB 507 I had undergone recombination(s) with the *E. coli* genome. When the plasmid DNA was transformed in *E. coli* JM 109, one in 10^4 transformants was capable of growth at 50° after an overnight incubation at 37 ° C. These transformants were also associated with multiple plasmid bands, indicating the possible prerequisite of recombination(s) for growth at high temperature. The recombinant pATB 507I had no rec A activity as assayed using the antibiotics mytomycin C.

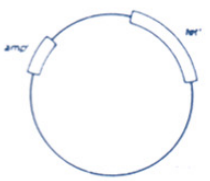
CHAPTER II



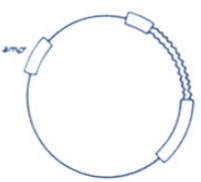


CONSTRUCTION AND SCREENING OF GENOMIC LIBRARY IN E. COLI

Ligate with bacteriophage T4 DNA ligase



Reconstitution of the original *Bam*HI site without insertion of a segment of foreign DNA restores the *tet*^{r} gene. Bacteria transformed with this non-recombinant plasmid can grow in the presence of ampicillin or tetracycline.



Insertion of a segment of foreign DNA inactivates the *tet*^{r} gene. Bacteria transformed with this recombinant plasmid can grow in the presence of ampicillin but not tetracycline.

ELISA

Y Y
wash

Add enzyme-labelled antigen only

Detection of Antigen

Summary

The genomic library of alkalophilic thermophilic *Bacillus* was constructed in *E. coli* using vector plasmid pUC 8. Standard shot gun cloning approach was used. The genomic DNA fragments size fractionated in the range of 6 - 12 kb were taken for the genomic library construction. The construction of the genomic library has resulted in to the generation of 4,500 recombinants with 8.5 kb average insert size. The library represents the genome of alkalophilic thermophilic *Bacillus* with more than 99.9 % confidance. Six xylanase positive recombinants were identified on the basis of xylan congo red plate clearance and the enzyme linked immunosorbant assay reaction with the antixylanase II antibodies. Preliminary characterization of the xylanase positive recombinants has shown that all the six recombinants have identical insert size.

Introduction

The recent studies on the molecular characterization of the bacterial xylanases have suggested that these enzymes could be useful model systems for the gene expression, secretion, and protein engineering studies (Yang *et al* 1988). Cloning of the xylanase gene(s) in *E. coli* has been reported from various sources and has already been described in the Chapter 1.

The xylanase gene cloning is undertaken for one of the several reasons.

1. The cloning of xylanase genes has been carried out to understand the gene structure, and to mutate the xylanase gene in order to obtain altered and desirable enzymatic properties.
2. To obtain the cellulase free xylanase preparation (Wong *et al* 1988) which is desired for the application in paper and pulp industry.
3. To isolate the genes and to identify the individual enzyme specificities of enzyme complexes eg. the enzyme specificities of xylanosomes and cellulosomes have been analysed in case of *Clostridium* sp. (MacKenzie *et al* 1989).
3. The xylanases have also been cloned with the aim of overproduction, but the success has been limited (Wong *et al* 1988).
4. The multiplicity of xylanases which may arise due to

various reasons such as multiple genes, proteolysis, or differential glycosylation has been analysed with the help of gene cloning approach (Wang *et al* 1992, Hazzlewood and Gilbert 1992).

5. The cloned xylanases have been transferred to and expressed in different heterologus hosts, are known to impart significant fermentative advantage to the host. Isolation of the desired gene by its cloning in *E. coli* could be the first step in such studies.

6. The composition of the crude enzyme preparation could be altered to suit the applications of hemicellulase and/or cellulase preparations, by knocking off the expression of the corresponding gene(s) with the help of the cloned gene fragments .

In recent years the interest in the xylanases has been increasing due to their potential applications especially in the paper and pulp industry. In this respect the xylanases from alkalophilic and/or thermophilic organisms could assume greater importance as their enzymes are expected to be more stable and/or active at high pH and temperature. The studies on cloning of the xylanase gene (s) from alkalophilic thermophilic *Bacillus* NCIM 59 were therefore undertaken with the aim of isolation and characterization of the xylanase gene fragment(s). The studies on expression and regulation of the cloned xylanases could also be of an academic interest for the better understanding of the gene regulation in extremophilic microorganism.

The present chapter describes the construction of

genomic library of the alkalophilic thermophilic *Bacillus* NCIM 59, using pUC 8 vector. The genomic library was screened using xylan congo red plate clearance and Enzyme linked immunosorbant assay, leading to identification of the xylanase positive recombinants.

Materials and Methods

Chemicals

Agarose, Ampicillin, IPTG, PEG 8000, pronase, RNase I, SDS, X-gal, xylan (oatspelt), were purchased from Sigma Chemical Co., USA. Bacto agar, tryptone, and yeast extract were from Difco Laboratories, USA. All the restriction endonucleases, T4 DNA ligase and calf intestinal phosphatase, and molecular weight marker [lambda DNA digested with Hind III] were obtained from Amersham (UK), Boehringer Mannheim (Germany), New England Biolabs (USA) or Pharmacia LKB (Sweden). The random primer labeling kit was from Boehringer Mannheim (Germany). The transfer membranes Hybond-N and nitrocellulose were from Amersham (UK) and Advanced Microdevices Ambala, India, respectively. The Qiagen columns (from Diagen GmbH, FRG) were used for DNA purification. The radiolabeled α P ^{32} dCTP and α P ^{32} dATP were supplied by Bhabha Atomic Research Centre, Bombay. Konica (Japan) X-ray films, commercial developer preparation (IPC 163: Kodak) and Sodium thiosulphate were used for autoradiography.

All other chemicals used were of analytical grade (AR) obtained from SRL, Qualigenes or E. Merck.

Host and vector system

Plasmid vector pUC 8 was used for construction of the genomic library of alkalophilic thermophilic *Bacillus* NCIM 59, and reorientation studies respectively. The *Escherichia*

coli strain JM 105 and JM 109 were used for construction of genomic library and propagation of the recombinant plasmid(s) from the selected recombinants. The genotypes of the strains (Yanish - Perron *et al* 1985) are given below:

E. coli JM 105 : F' *traD36 lacI*^{q} **(lacZ)M15 proA*$^{+}$*B*$^{+}$*/thi rpsL* (Str^{r}) *endA sbcB15 sbcC? hsdR4*($r_k^- r_k^+$) **(lac-proAB)*

E. coli JM 109 : F' *traD36 lacI*^{q} **(lacZ)M15 proA*$^{+}$*B*$^{+}$*/e14*$^{-}$ (McrA^{-})

**(lac-proAB) thi gyrA96* (Nal^{r}) *endA1 hsdR17* (r_k^- m_k^+) *relA1 recA1 supE44.*

Choice of the cloning vector

The pUC 8 (Yanisch- Perron *et al* 1985) plasmid was chosen as a vector in the present studies for its multiple cloning sites and the high copy number (300) which could facilitate cloning of DNA fragments generated with various restriction endonuclease specificities and the copy number effect could be useful to yield sizable expression. The restriction map of the plasmid is shown in figure 2.1. The plasmid has an ampicillin resistance gene as a selectable marker, which has been derived from pBR 322 plasmid. The vector has a polylinker - consisting of nine different cloning sites - that is placed between the lac O/P and the lac Z' gene fragment. The lac Z' gene fragment codes for N terminal peptide of the ß - galacosidase which undergoes intracellelic

α complementation with the mutant galactisidase protein (M 15 mutation) coded by the genomic DNA of *E. coli* JM 105 and gives rise to a functional β - galactosidase. The galactosidase cleaves the chromogenic substrate to its indole derivative which imparts blue colour to the colonies. When an insert DNA fragment is cloned in the multiple cloning site the lac Z' gene fragment is disrupted and the resulting colonies are white in colour. Thus the recombinants could be phenotypically selected as the ampicillin resistant white colonies.

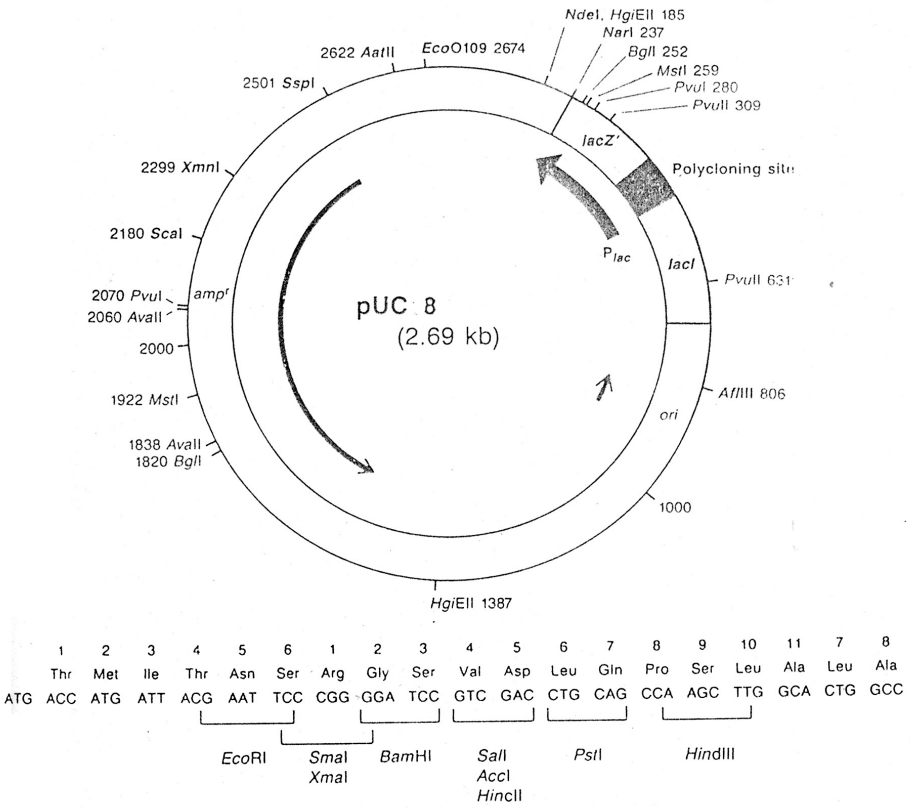


FIG. 2.1: RESTRICTION MAP OF PLASMID pUC 8

Culture maintainance

The alkalophilic thermophilic *Bacillus* and *E. coli* stocks were stored as 15% glycerol suspensions of the 16 h grown liquid cultures at -70°C. All the recombinants were grown in media containing ampicillin (100 µg/ml) for 16 h, washed twice with the medium without antibiotics and then preserved in 15 % glycerol suspension at - 70 °C. Short term maintainance of the cultures was at 4 °C. The alkalophilic thermophilic *Bacillus* and *E. coli* strains were maintained respectively on wheat bran yeast extract slants and M9 minimal medium (Yanisch - Perron *et al* 1985) supplemented with thiamine. The recombinants were maintained on LB plates containing ampicillin (100 µg/ml). All the media and buffers were autoclaved at 15 pounds per square inch for 20 min. Heat labile components like antibiotics, IPTG, X-gal were filter sterilized through autoclaved millipore (0.45 um) membranes and added freshly to cooled media (45°C). Ampicillin (sodium salt) solution was prepared at a concentration of 100 mg/ml in sterile autoclaved double distilled water and 100 µg/ml final concentration was used in the medium. 10 µl of 100 mM IPTG and 40 µl 2% X-gal (in dimethyl formamide) was used per 25 ml of LB agar.

MEDIA COMPOSITION (G %)

1. Luria Bertani (LB) medium

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

2. Wheat bran - yeast extract medium

Wheat bran (washed)	: 5
Yeast Extract	: 0.5
Agar	: 2
(for solid media)	
Na_2CO_3	: 1
(separately streilized)	

3. M9 Minimal medium

$Na_2HPO_4 \cdot 7H_2O$	: 1.28
KH_2PO_4	:0.3
NaCl	:0.05
NH_4Cl	: 0.1

BUFFER COMPOSITION

1. TE

Tris-Cl (pH 8.0) : 10 mM

EDTA : 1 mM

2. SET

Sucrose : 8 g%

in TE

3. TAE

Tris-acetate : 40 mM

EDTA (pH 8.0) : 1 mM

4. 20 X SSC

NaCl : 3 M

Trisodium citrate: 0.3 M

5. GET

Glucose : 50 mM

EDTA : 10 mM

Tris-Cl (pH 8.0) : 25 mM

6. PBS

NaCl : 150 mM

Phosphate buffer : 10 mM

(pH 7.5)

7. ST

Sucrose : 8 g %

Triton X 100 : 0.1 g %

in TE

8. TNE

NaCl : 100 mM

in TE

Isolation of *Bacillus* DNA

Alkalophilic thermophilic *Bacillus* was grown in 50 ml of LB containing 1% Na_2CO_3 at 50°C for 16 h with shaking at 200 rpm. DNA was isolated according to modified method of Chater *et al.* Cells were separated by centrifugation at 5000 rpm for 5 min and washed twice with TE buffer and then suspended in 5 ml SET buffer containing 2 mg/ml lysozyme. After an incubation for 30 min at 37°C, 1.2 ml of 0.5 M EDTA and 130 µl of pronase solution (200 µg/ml) were added and allowed to stand for 5 min at room temperature. SDS solution (10%) was then added to make the final concentration 0.1 %, mixed gently and incubated at 37°C for 45 min. The clear suspension was then mixed with equal volume of equilibrated phenol, followed by addition of equal volume of chloroform and isoamyl alcohol (24:1) and thorough mixing. After the centrifugation at 15,000 rpm for 20 min, the aqueous layer was treated again with mixture (1:1) of phenol chloroform followed by two washes of chloroform. The aqueous layer was then precipitated with two volumes of ethanol. Sodium chloride 0.1 M was used to obtain quantitative recovery. The spool of high molecular weight DNA formed was removed and washed with 70% ethanol and dissolved in TE buffer. The DNA solution was treated with DNase free RNase (50 µg/ml final concentration) at 37°C for 30 min. The DNA (2 µl) from the preparation was loaded on 0.8% agarose gel to confirm the RNA removal. The DNA preparation was again subjected to phenol - chloroform treatment and ethanol precipitation as mentioned

above. The resulting solution of DNA was subjected to spectrophotometric quantitation and purity analysis.

Quantitation of DNA

Absorption spectra of suitably diluted DNA solution was recorded on a Shimadzu double beam spectrophotometer (model UV 210-A) in the range of 220-320 nm . The preparations with following absorbance ratios were considered as suitable for further work .

$A_{280} : A_{260} < 0.45$ to 0.55 $A_{230} : A_{260} < 0.45$

$A_{300} : A_{260} < 0.05$

The DNA was quantitated by using equation:

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

Plasmid isolation

The plasmid isolation was performed using alkaline lysis method or boiling method depending on the scale of plasmid preparation.

Minipreparations

The plasmid DNA was isolated by the boiling method as described by Maniatis *et al*(1982). The culture (5 ml) grown in LB broth with ampicillin (100 µg/ml) for 16 h at 37°C on shaker (200 rpm) was centrifuged in an Eppendorf tube at 13,000 rpm for 1 min. The supernatant was discarded and the pellet was suspended in 0.25 vol of ST buffer. Freshly pre-

pared lysozyme solution (10 mg/ml in 10 mM Tris) 0.02 V was added and the tube was held in boiling water bath for 1 min. The lysate was centrifuged immediately at 13,000 rpm for 10 min and the pellet was removed with a sterile toothpick. The supernatant was treated with phenol-chloroform and the precipitation was carried out with 0.6 V of isopropanol at room temperature for 30 min. After centrifugation the pellet was dissolved in 50 µl TE buffer and further treated with DNase free RNase for 30 min at 37°C.

Large scale plasmid preparation

The large scale plasmid preparation was carried out by alkaline lysis method (Maniatis *et al* 1982). The culture (500 ml) grown for 16 h with antibiotics was centrifuged in GSA rotor (Sorvall) at 6,000 rpm for 10 min and the resulting pellet was washed twice with TE. The pellet was finally suspended in GET (20 ml) and lysozyme solution was added in the final concentration of 0.1 mg/ml. The suspension was mixed gently but thoroughly, and allowed to stand on ice for 15 min. Freshly prepared alkali and SDS were added to this suspension in the final concentrations of NaOH 0.1 M and SDS 0.1 %. The suspension was mixed thoroughly and allowed to stand at room temperature for 20 min. The resulting clear lysate was mixed with 5M potassium acetate (pH 4.6) to make the acetate final concentration of 150 mM. The suspension was mixed and centrifuged at 12,000 rpm for 20 min. The clear supernatant was processed for phenol - chloroform and RNase treatment as described in plasmid minipreparation. The plas-

mid was precipitated with 2.5 volumes of ethanol and 100 mM NaCl was added to achieve quantitative recovery.

Transformation of *E. coli* with plasmid DNA

Incorporation of plasmid molecules in suitable *E. coli* host was performed by transformation of the competent cells prepared either by the treatment with $CaCl_2$ (Maniatis *et al* 1982) or using a modified tretment with TSS buffer (Chang *et al* 1989) containaing PEG 6000 (0.2 %), DMSO (0.01 %), and $MgCl_2$ (10 mM).

E. coli host was inoculated into 5 ml LB broth and grown at 37°C for 16 h at 200 rpm. 100 µl of 16 h grown culture was inoculated in 25 ml LB broth and incubated at 37°C with vigorous shaking till A_{600} of the culture was 0.5. The culture was chilled on ice for 10 min and centrifuged at 5000 rpm for 10 min at 4°C. The pellet was suspended in 12.5 ml 100 mM chilled $CaCl_2$ and 10 mM Tris-HCl pH 8.0 mixture and kept on ice for 30 min. Cells were separated by centrifugation and resuspended in 1 ml of $CaCl_2$ Tris- HCl or TSS buffer solution and divided into aliquotes of 250 µl. Upto 40 ng of supercoiled plasmid DNA was added per aliquot and incubated on ice for 30 min, followed by the heat stock at 42°C for 2 min. The tubes were immediately chilled on ice and then incubated at 37°C for 60 min without shaking, after adding 1 ml of LB broth. Different aliquotes were spread on LB agar plates containing ampicillin. Competent cells with or without transformation with vector were spread on antibiotic

containing LB agar and competent cells inoculated on LB agar without antibiotic were referred to as control plates. Plates were incubated at 37°C for 16 h. The transformation efficiency was expressed as the number of transformants per microgram of plasmid DNA.

Agarose gel electrophoresis

The agarose gels (0.8 %) were used for monitoring restriction digestions of genomic and plasmid DNA. The gel electrophoresis was carried out in 1X TAE buffer at a constant current of 50 mA till the tracking dye (bromophenol blue) reached the other end of the gel. After electrophoresis the gels were stained in dark with ethidium bromide (50 µg/ml) visualized on a long wavelengh (302 nm) UV-transilluminator (UV products, San Gabriels, California, USA) and photographed with a 35 mm SLR camera (Cannon A1 with microphotoghraphy and zoom lens system) using a red filter.

Restriction digestion

The primary tools used by the molecular biologist in manipulating the DNA are restriction enzymes and other modifying enzymes. The restriction enzymes bind specifically to and cleave double stranded DNA at specific sites known as the recognition sequence. The conditions can be manipulated to obtain partial and complete digestion of the DNA.

Complete digestion

Plasmid DNA was digested with 3 - 5 U of restriction endonuclease per µg of DNA in reaction volume of 20 µl at 37°C for appropriate time. Commercially supplied buffers or LS, HS, and MS buffers described by Maniatis *et al* were used. The restriction enzymes were inactivaed by heating for appropriate time and temperature as per the manufacturers suggestions.

Partial digestion of *Bacillus* DNA

The genomic DNA of alkalophilic thermophilic *Bacillus* (20 µg) was mixed with the restriction buffer 10 µl and the final volume was made up to 100 µl with sterile glass distilled water. The solution was mixed thoroughly and the aliquotes of 10 µl each were dispensed in to eight eppendorf vials leaving behind 20 µl solution in the first tube to which 20 units of Hind III was added. The contents were mixed gently and serial transfers of 10 µl solutions were carried out leading to enzyme concentrations in the range of 5 - 0.005 U / µg of DNA. All the tubes were incubated at 37°C for 6 h. The reaction was terminated by heating the tubes at 75 ^{o}C for 10 min and the pattern of digestion was monitored by gel electrophoresis.

Size fractionation of Hind III digested *Bacillus* DNA

The size fractionation was carried out by sucrose density gradient centrifugation. Sucrose gradient was prepared by carefully pouring 6 ml of 10% sucrose in TNE buffer on the top of 6 ml of 40% sucrose in TNE buffer. The tube was sealed and rotated slowly to the horizontal position and kept at room temperature for 4 h. The tube was then gently turned back to the vertical position and 200 µg of partial digest of the genomic DNA of alkalophilic thermophilic *Bacillus* DNA was loaded on the top. Tubes were then centrifuged at 35,000 rpm for 16 h at 20°C in Beckman SW 41 swing out rotor. Fractions of 500 µl each were collected after centrifugation and 10 µl samples from the selected fractions were loaded on 0.8 % agarose gel. The fractions in the desirable size range (6 - 12 Kb) were pooled and precipitated with 0.6 volumes of isopropanol.

Dephosphorylation

The Hind III digested plasmid DNA was dephosphorylated with calf intenstinal alkaline phosphatase (CIP). DNA was dissolved in phosphatase buffer (1 mM $ZnCl_2$, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3) and was incubated with 1 U of enzyme for 1 h at 37°C. At the end of incubation period CIP was removed by incubating at 65°C for 30 min followed by phenol-chloroform treatment and precipitation with ethanol.

Ligation

The predigested and dephosphorylated vector DNA as well as partially digested size fractionated donor DNA were purified and precipitated as mentioned above. The DNA precipitates were dissolved in sterile glass distilled water and the solutions were mixed in appropriate proportions. The mixture was warmed to 45°C for 5 min to melt the cohesive termini that could have reannealed, and allowed to cool slowly. Mixture was chilled to 0°C. To this solution following components were added : bacteriophage T_4 DNA ligase (0.1 U), 5 mM ATP (1 µl), bacteriophage T_4 DNA ligase buffer (200 mM Tris-HCl, 50 mM $MgCl_2$, 50 mM dithothreitol, 500 ug/ml bovine serum albumin) 10 X solution (1 µl), and sterile distilled water to make up the volume to 10 µl; however the order of addition was exactly opposite. The mixture was incubated at 10°C for 16 h, and an additional aliquot of ligase and ATP was added. The reaction was continued for 4 - 6 h at 16 ^{o}C.

Colony blotting

The LB agar plates containing desirable colonies - grown in presence of the antibiotics for 6 h - were covered with hybond N circle of the appropriate diameter. The colonies were allowed to grow for another 10 - 12 h, during which, the colonies adhered to the membrane and the subsequent lifting was easy. Before lifting the membrane, three asymmetric holes were made on the paper through the plate. Membranes were

gently lifted and kept on the Whatman No. 3 filter presoaked in GET containing lysozyme (1 mg/ml) and incubated at room temperature for 20 min. The membranes were then washed with the alkali (NaOH 0.1 M) - SDS (0.1 %) solution and twice with the neutralizing solution (1.0 M Tris-HCl, pH 7.5, 1.5 N NaCl). The blots were then briefly washed with 2 X SSC and air dried. The dry blots were exposed to U. V. light for 10 min with DNA side facing the U. V. source.

Labelling of DNA

Probes were labeled with α P^{32} dCTP or α P^{32} dATP (specific activity 3000 Ci/M mole) by random priming method of Fienberg and Vogelstein (1983, 1984) which is based on the hybridization of a mixture of random hexanucleotides to the DNA to be labeled, which acts as a primer for synthesis of second strand of DNA in which ^{32}P dCTP is incorporated. Using this method specific activity > 10^8 CPM/µg DNA could be achieved. 50 ng of the probe DNA in a suitable volume of TE buffer was denatured by boiling for 10 min and was immediately chilled on ice. 2 µl reaction buffer with primer, 1 µl each of dATP, dTTP, dGTP and 5 ul of alpha ^{32}P dCTP and 2 µl Klenow fragment (4 U) were added to denatured probe DNA; to a final volume of 20 µl. The mixture was incubated at 37°C for 1 h. The reaction was stopped by adding EDTA (final concentration, 10 mM).

Removal of unincorporated label

To prevent background of non-specific hybridization it is necessary to separate the labeled probe from unincorporated labeled nucleotides. This was carried out by selective precipitation. DNA labeling reaction mixture was precipitated with 4 M ammonium acetate pH 4.5 and ethanol at -70°C for 1 h. Before centrifugation the tube was transferred to 37°C water bath. The incubation at 37°C redissolves nucleotide which has precipitated along with the probe. After centrifugation the pellet was first washed with 0.67 M ammonium acetate with 67% ethanol, at room temperature with gentle shaking. The pellet was then washed with 90% ethanol and dried. Finally the DNA was dissolved in TE buffer and used as probe.

The radioactivity of the purified labeled probes was counted in Rack-beta liquid scintillation counter.

Hybridization and washing:

Before hybridization with labeled probe, it is essential to block the sites on the membrane that bind single or double stranded DNA non-specifically giving high background to the autoradiograms. Prehybridization with nonspecific DNA such as calf thymus DNA serves this purpose.

Prehybridization

Southern blots or colony blots were prehybridized for 3-4 h at appropriate temperature in heat sealed plastic bag

containing prehybridization mixture [5 x SSC, 0.1% SDS, 5 X Denhardts' solution (containing 1 % w/v each of BSA, Ficoll, and polylyvinylpyrolidone) and 100 µg/ml calf thymus DNA]. The amount of prehybridization solution added was 200 µl/cm^2 of the blot.

Hybridization

After prehybridization, the solution was removed and replaced by hybridization solution (same as prehybridization mixture) containing the labeled probe. The purified probe was denatured by boiling for 10 min which was chilled immediately, added along with the hybridization solution to the blots. Hybridization was carried out overnight with gentle shaking.

Washing of filters and autoradiography

After hybridization, the filters were washed for removal of the unbound or non-hybridizing probes using solutions of different stringencies depending on the probe used for hybridization. The filters were washed twice with 2 X SSC and 0.1% SDS mixture each for 15 min at 63°C. This was then followed by two washes respectively with 1 X SSC, 0.1% SDS and 0.1 X SSC, 0.1% SDS each for 10 min at 63 ^{o}C. The filters were dried, wrapped in saran wrap and exposed to X-ray film for desired time at -70°C using vinyl X-ray cassettes with built in intensifying screens.

Analysis of the genomic library

After the transformation of the ligation mixture the cells were plated out on LB containing ampicillin, X-gal and IPTG. This allowed to select for the plasmid bearing cells and the recombinants were identified as white colonies. The representative recombinants were used for colony blotting and hybridization with labeled genomic DNA from alkalophilic thermophilic *Bacillus*. The plasmid DNA was isolated from few recombinants, digested with Hind III and the insert size was estimated.

Screening of the genomic library:

The genomic library was screened for the xylanase positive recombinant with the help of the polyclonal antibodies raised against the purified xylanase II from alkalophilic thermophilic *Bacillus*. The purification of the xylanase, antibody preparation and ELISA are described below.

Polyclonal antibody preparation

The xylanase II from alkalophilic thermophilic *Bacillus* was purified as described by Dey *et al* (1992). The extracellular culture filtrate (1,000 ml) was precipitated with 3 volumes of chilled ethanol. After 4 h of incubation at 4 ^{o}C, the precipitate was harvested by centrifugation, dried under vaccum and redissolved in 20 ml volume of 50 mM phosphate buffer (pH 7.0). The xylanases were separated on Bio-

Gel P 10 column and the fractions containing xylanase II were pooled, desalted and lyophilised. The enzyme preparation was analysed by SDS - PAGE and was found to be homogeneous preparation with a single protein band at Mr 15,800. The purified protein preparation was used for raising the antiserum in New Zealand white rabbit. The rabbit was immunized with the purified xylanase II (0.5 mg) mixed with complete Freund's adjuvant. Three booster injections (0.5 mg each) were given after 6 weeks intervals. One week after the end of the three booster doses, the titre of the antiserum was determined by double diffusion analysis. The antiserum was serially double diluted up to 1:32 dilution and was used for testing its reactivity with ammonium sulfate precipitated crude xylanase preparation of alkalophilic thermophilic *Bacillus*. Agarose (1%) in PBS buffer was used as the diffusion medium and the distance between the wells was 1 cm. The serum was collected when the titre reached 1:16.

Enzyme linked immunosorbant assay (ELISA)

The recombinants were grown and transferred to sterile nitrocellulose membrane as described in the colony blotting. After the lysozyme treatment the membranes were blocked with 2 % goat serum which was previously treated at 65 ^{o}C for 30 min to inactivate the interferon. The treatment was given in PBS buffer with gentle shaking. The membranes were then washed thrice with PBS buffer (10 min each) and then allowed to react with antibodies raised against low molecular weight xylanase from alkalophilic thermophilic *Bacillus*. The anti-

bodies were diluted (1:3,000 - 1:5,000) in PBS buffer containing 2 % goat serum and the reaction was carried out with gentle shaking at 37 ^{o}C for 1 h. The membranes were washed thrice with PBS (10 min each) and challenged with 1:5000 dilution of anti-rabbit IgG- peroxidase conjugate (raised in goat) After three washes with PBS, the bound peroxidase was detected

Xylan - congo red plate clearance assay:

The recombinants were patched on LB plates containing xylan (0.5 %) and ampicillin 100 µg/ml. After 16 h incubation at 37^{o} C, the plates were layered with 1 % congo red solution and allowed to stand for 20 min. The excess congo red solution was carefully decanted without disturbing the colonies. The plates were checked for the possible clearance produced by the extracellular synthesis of xylanase(s). The plates were then overlaid with 0.4 % agarose containing 2 mg /ml lysozyme; and after the agarose was set, plates were incubated inverted at 37 ^{o}C for 30 min to ensure the complete lysis of the recombinant colonies. The plates were then treated with 1 M NaCl to visualize the possible clearance due to intracellular xylanase expression. The plates were further incubated at 50 ^{o}C for three to four hours in order to enhance the xylan clearance.

Results and Discussion

Construction of genomic library

The standard shot gun cloning strategy was used for construction of the genomic library of alkalophilic thermophilic *Bacillus* NCIM 59 in *E. coli*. The high molecular weight genomic DNA was purified from the alkalophilic thermophilic *Bacillus* NCIM 59 (FIG 2.2A).

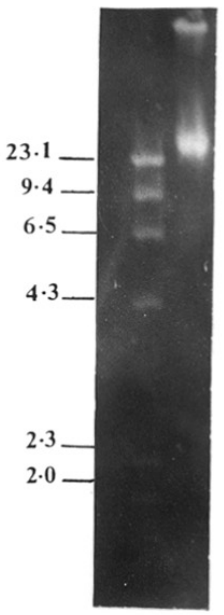


FIG. 2.2A: Agarose gel electrophoresis of DNA isolated from alkalophilic thermophilic *Bacillus*

lane 1: Lambda Hind III marker

lane 2: high molecular weight DNA

The spectrophotometric analysis of the DNA showed tthe following absorbance ratios:

A_{280} : A_{260} = 0.50 A_{230} : A_{260} = 0.40

A_{300} : A_{260} = 0.05

The DNA preparation (FIG 2.2B)was used for further work.

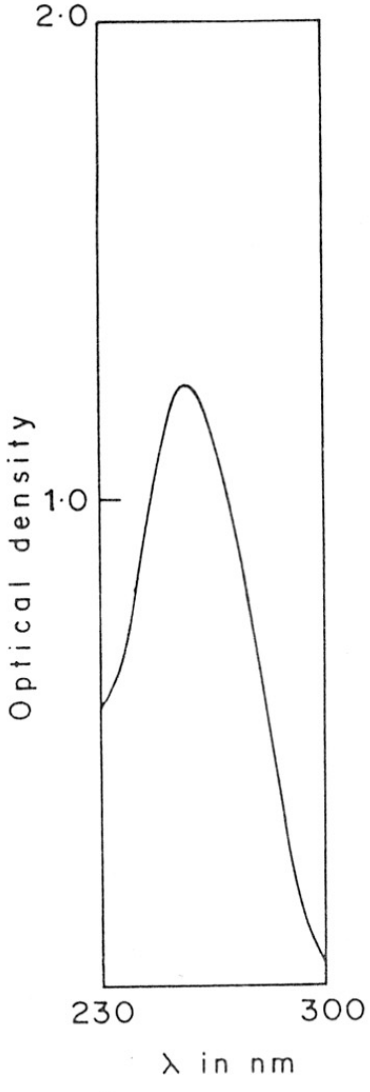


FIG 2.2B: UV absorption spectrum of genomic DNA of alkalophilic thermophilic *Bacillus*

The DNA was digested partially with Hind III and size fractionation was carried out using sucrose density gradient (FIG. 2.3) and DNA fragments in the range of 6 - 12 kb in size were used for construction of the genomic library.

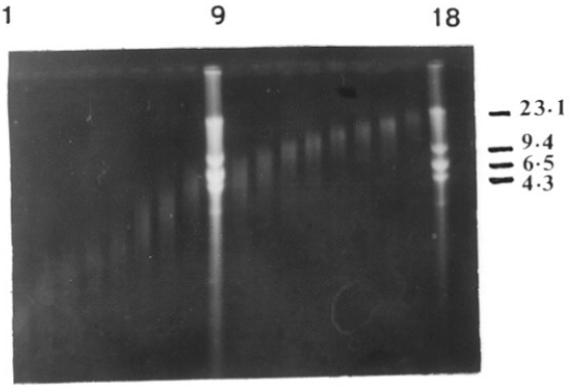


FIG 2.3: Size fractionation of Hind III digested *Bacillus* DNA
lane 9 and 18: Lambda Hind III marker
lane 1 - 8 and 10 - 17 fractions of digested DNA

The pUC 8 DNA was linearised with Hind III and was treated with alkaline phosphatase. The digested genomic DNA (1 µg) and dephosphorylated pUC 8 (200 ng) were mixed and subjected to phenol - chloroform treatment. The ligation and transformation using $CaCl_2$ or TSS treated competent cells of *E. coli* was carried out as described in materials - methods.

As a result of twenty independent transformation reactions using 10 ng DNA with respect to the vector, 5,500 transformants were obtained and the transformation efficiency was 2.75×10^4 transformants/µg of DNA. Out of the 5,500 transformants, 4,500 colonies were white; indicating that the ratio of recombinants to the non-recombinants was 85:15 (FIG. 2.4). This ratio was obtained with the vector to insert ratio of 1:5 on µg basis which is approximately corresponding to molar ratio of 2:3.

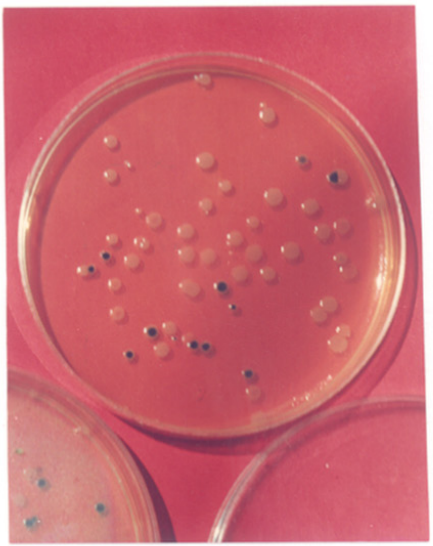


FIG 2.4: Identification of the recombinants (white colonies) on LB plates containing X - gal and IPTG

Analysis of the genomic library:

The randomly selected recombinants were patched on a separate LB plate with pUC 8 transformant as a control, and subjected to colony blot analysis. The labeled genomic DNA from alkalophilic thermophilic *Bacillus* was used as the hybridisation probe. All the colonies showed positive signals of equal intensity, whereas the pUC transformant showed no hybridization (FIG. 2.5). The results suggested that all white colonies had insert DNA from the alkalophilic thermophilic *Bacillus*.

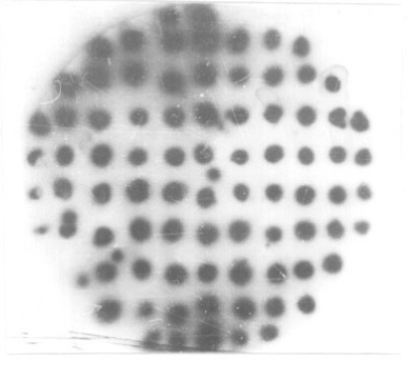


FIG 2.5: Colony hybridisation of the recombinants with labaled genomic DNA from alkalophilic thermophilic *Bacillus*

The plasmid DNA from ten representative recombinants was isolated and digested with Hind III. The gel electrophoresis of the digested plasmids showed the presence of insert DNA in the range of 6 - 11 kb (FIG. 2.6), which is in agreement with the DNA size fractionation range.

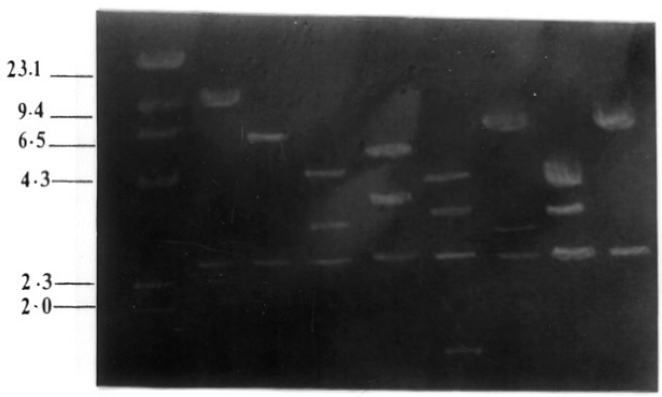


FIG. 2.6: Digestion of the representative recombinant plasmids with Hind III Lane 1 is lambda Hind III marker.

The genome size of *Bacillus subtilis* has been reported to be 5 x 10^3 kb (Itaya M. and Tanaka T. 1991). Assuming similar genome size for alkalophilic thermophilic *Bacillus* the following formula for calculating the representativeness of the genomic library was applied.

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

where N = necessary no. of recombinants

p = desired probability of obtaining a single copy recombinant

f = average fractional proportion of the genome in a single recombinant

Since the possibility of insertion of DNA into plasmid decreases with increasing size, the average insert size of 8.5 kb was taken for the calculations which is based on the actually detected range of insert (6 - 11 kb). The number of recombinants required to represent a single copy gene with 99.9 % confidance was found to be 3,500; which is less than the number of recombinants obtained (4,500). Hence the library was considered to be a representative library and used for screening of the recombinants harbouring the xylanase gene fragment(s).

Screening of the genomic library for the xylanase positive recombinant(s):

A genomic library can be screened for the desirable gene either at gene level by hybridization with a suitable probe, or with the help of the protein expressed by the desirable gene. The protein can be detected as an antigen with the help of specific antibodies against the protein or its epitope, or with the help of enzymatic activity of the protein. Since xylanases from bacterial sources - especially from *Bacillus* sp. are not expected to have an expression barrier in *E. coli*, the genomic library of alkalophilic

thermophilic *Bacillus* was screen using ELISA reaction and xylan congo red plate clearance assay.

Xylan plate clearance assay:

The recombinant colonies were screened for the expression of the xylanase gene using the xylan plate clearance. Fifty colonies were spoted on per plate at a regular distance, and keeping a minimum distance of 0.5 cm from the edges of the plate. This ensured elimination of false positive reactions, which are commonly observed because of overcrowding. The *E. coli* JM 105 harbouring pUC 8 was included as a negative control in each plate. The presence of clearance was confirmed by repeating the plate clearance. Six recombinants showing weak clearance consistantly in three independent colony clearance assays were picked up as xylanase positive recombinants.

Characterization of the anti - xylanase II antiserum:

The preimmune serum from the rabbit, collected before initiation of the immunisation with xylanase II had no reactivity with the crude xylanase preparation from the alkalophilic thermophilic *Bacillus*. The antiserum collected one week after the last booser dose was checked against the crude culture filtrate of alkalophilic thermophilic *Bacillus*, for

the antibody titre by double diffusion analysis. The titre was found to be 32 (FIG 2.7). However two arcs were obtained corresponding to each well; indicating that in addition to xylanase II, one more protein was reacting with the antiserum. The antiserum was also used to test its reactivity with the two purified xylanases of alkalophilic thermophilic *Bacillus*. The double diffusion analysis showed that the antibodies react with the xylanase I (Mr 35,000) and xylanae II (Mr 15,800) of alkalophilic thermophilic *Bacillus* and the two arcs were corresponding to the two xylanases (FIG. 2.8).

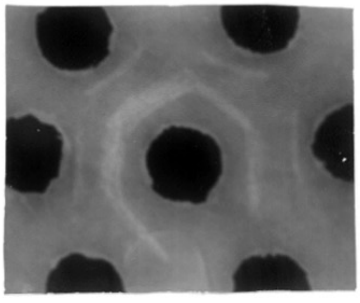


FIG. 2.7: Determination of antibody titre

Well 1 - 5 contain the following antibody dilutions 1. 1:2, 2. 1:4, 3. 1:8, 4. 1:16 5. 1:32. Well 6 contains preimmune serum. Central well contains crude xylanase extract from alkalophilic thermophilic *Bacillus*.

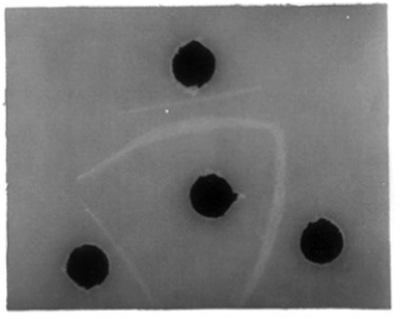


FIG 2.8: Double diffusion test between the xylanase I and II Central well contains antibodies against xylanase II. Well 1: xylanase II, Well 2: alcohol precipitated culture filtrate, Well 3: xylanase I.

The antixylanase II antibodies were checked for the ELISA reaction with *E. coli* JM 105 harbouring pUC 8, and were found to be non reactive at 1:3,000 or higher dilutions. The antibodies however reacted with the colonies of alkalophilic thermophilic *Bacillus* and crude enzyme preparation at dilutions as high as 1:5,000. The antibodies were found to be precipitating and neutralizing type.

Screening of the genomic library by the enzyme linked immunosorbant assay (ELISA):

The genomic library was also screened by the ELISA reaction of the recombinant colonies blotted on the nitrocellulose paper. The dilution of the antibodies was 1: 5,000. When the total genomic library was screened with ELISA, six recombinants - distintly positive in the reaction were obtained (FIG. 2.9). These recombinants perfectly coinsided with those identified by the xylan plate clearance assay. No additional recombinants were detected in the ELISA reaction, which is supposed to be a highly sensitive method.

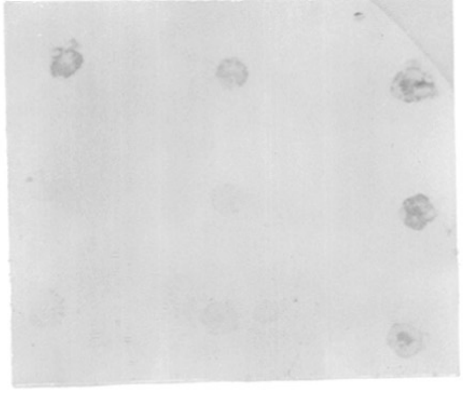


FIG. 2.9: The xylanase positive recombinants showing positive ELISA reaction

Preliminary characterization of the xylanase positive recombinants:

The xylanase positive recombinants were subjected to plasmid isolation, digestion with Hind III and agarose gel (0.8 %) electrophoresis. The digestion pattern of all the six recombinants showed four idebtical bands per well, indicating that the insert had two internal Hind III sites. One of the recombinants was analysed further and the sizes of the three subfragments were found to be 6.5, 3.2 and 1.7 kb (FIG 2.10); and the total insert size was predicted to be 11.4 kb.

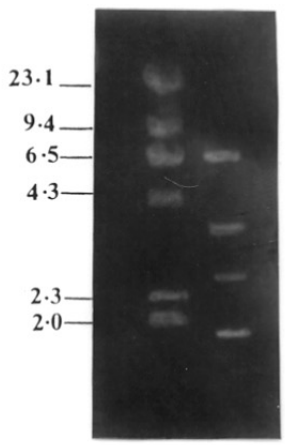


FIG. 2.10: Digestion of the recombinant plasmid pATB X 11.4

lane 1: lambda Hind III marker DNA

lane 2: Hind III digestion of the recombinant plasmid pATB X 11.4

The construction of the genomic library has resulted in to the generation of 4,500 recombinants with 8.5 kb average insert size. The library represents the genome of alkalophilic thermophilic *Bacillus* with more than 99.9 % confidance. Six xylanase positive recombinants were identified on the basis of xylan congo red plate clearance and the enzyme linked immunosorbant assay reaction with the antixylanase II antibodies. Preliminary characterization of the xylanase positive recombinants has shown that all the six recombinants have identical insert size. Further studies on subcloning and expression are described in chapter 3.

Summary

The recombinant plasmid pATB X 235 was analysed for the linkage between the three subfragments. The 6.5 kb Hind III fragment was located in between the 1.7 and 3.2 kb fragments. The xylanases were also localised to the 6.5 kb fragment. Further restriction digestion and subcloning studies resulted in to identification of 4.5 kb Eco RI - Hind III fragment that coded for the xylanases. Equivqlent amount of xylanase activity was detected from IPTG induced or non induced recombinants irrespective of the orientation of the 4.5 kb fragment with respect to the lac O/P, indicating that the xylanase gene expression was under the control of its own promoter. 95 % of the xylanase activity (2 U/ml) was found in the extracellular culture filtrate. The hydrolysis of xylan by the recombinant xylanases yielded mainly xylobiose with traces of xylose, xylotriose and higher oilgosaccharides.

Introduction

Examination of xylanase multiplicity in *Bacillus* sp. suggests that these bacteria produce two distinct xylanases differing in molecular mass and isoelectric points (Wong and Saddler 1991). However, molecular cloning of both the xylanases has been achieved only in case of *Bacillus circulans* (Yang et al 1989). In case of *Bacillus* C 125 (Honda *et al* 1985), *B. polymyxa* (Yang *et al* 1988), *B. pumilus* IPO (Panbangred *et al* 1983), *B. subtilis* PAP 115 (Bernier *et al* 1983), and CD4 (Srivastava and Srivastava 1993), only one out of the two xylanases has been expressed in *E. coli*.

The alkalophilic thermophilic *Bacillus* - like the other *Bacillus* sp. - produces two distinct xylanases. The screening of the genomic library has resulted in to identification of a 11.4 kb fragment coding for the xylanase activity. Therefore it is essential to determine the number of xylanases coded by the insert. Also, the restriction mapping and subcloning studies would pinpoint the position of the xylanase gene(s). Hence the present chapter describes subcloning, restriction mapping, and expression studies on the xylanase gene fragment from alkalophilic thermophilic *Bacillus* in *E. coli* (Shendye and Rao 1993 a).

Materials and Methods:

Chemicals

Low melt agarose, Dinitrosacylic acid, and protein molecular weight markers for PAGE were obtained from Sigma chemicals (USA). All other reagents were of AR grade and were procured from SRL, Qualigens or E. Merck.

Low melt agarose gel electrophoresis and DNA purification

The electrophoresis was performed as described in the previous chapter using low melting agarose (1 % w/v) and the electrophoresis was performed at 10 ^{o}C. After the electrophoresis and staining with ethidium bromide (0.1 mg %) the gel was visualized with minimum UV exposure, and the desired DNA bands were cut from the gel using a sharp and clean plastic blade.

The agarose gel blocks containing the desired DNA bands were transferred to eppendorf tubes and then incubated at 50 ^{o}C to melt the agarose. Phenol - chloroform treatment was given to the molten agarose and the DNA was precipitated with ethanol. After redissolving, the DNA was checked by agarose gel electrophoresis. The DNA was purified further using Qigen column and then used for ligation with the desired vector.

Southern blotting

The transfer of DNA from agarose gels to hybond N (Amersham) membranes was carried out according to Maniatis *et*

al (1982). After electrophoresis the gel was soaked in 0.25 N HCl at room temperature for 10 min for depurination of DNA. The DNA was then denatured by soaking the gel twice in 1.5 M NaCl and 0.5 M NaOH for 30 min at room temperature with gentle shaking. The gel was neutralized by soaking it in 1 M Tris-HCl, pH 7.4 and 1.5 M NaCl, 30 min, (twice) with constant shaking. The capillary blotting of DNA onto the membrane was carried out for 16 h in 20 X SSC, after which the membrane was rinsed in 5 X SSC, air dried and exposed to U.V. irradiation for 10 min with DNA side facing the UV source.

Subcloning and reorientation

The insert DNA samples digested with a single restriction endonuclease i.e. Hind III were mixed with the Hind III digested and dephosphorylated pUC 8 in 5:1 ratio. The double digested insert fragments were purified from low melting agarose gels as previously described , and then mixed with suitably digested vector DNA in 3:1 ratio. The Eco RI - Hind III fragments were cloned in pUC vectors by completely replacing the multiple cloning site. The Eco RI - Pvu II and the Hind III - Pvu II fragments were cloned in pUC vectors using Eco RI or Hind III along with Sma I. The restriction endonuclease Sma I is a blunt end generating endonuclease and the resulting end could religate with the Pvu II cut end without generation of any restriction endonuclease specificity. Further purification, ligation and transformation were carried out as described in chapter 2. In order to obtain the

insert DNA in both the orientations with respect to the lac O/P, the purified DNA fragments of the insert were simultaneously ligated with both pUC 8 and pUC 9 vectors in which the multiple cloning site is present in the opposite orientations with respect to lac O/P.

Preparation of protein extracts from the recombinants

The culture broth was centrifuged and the supernatant was referred to as extracellular extract. The periplasmic extract was prepared by the osmotic shock (Anraku Y. and Heppel L. 1967) with the phosphate buffer (pH 7.0) saline containing Mg^{2+} (Final concentration 100 mM). 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 ^{o}C. The cell lysate was centrifuged at 12,000 rpm for 30 min and the resulting clear solution was designated as intracellular extract. The protein was estimated by the Lowry's method (Lowry O. H. *et al* 1951).

Estimation of xylanase activity

Two grams of xylan powder was suspended in distilled water and stirred for 16 h and 28^{o}C. The insoluble fraction was removed by centrifugation and the soluble fraction was was used as the substrate for estimation of the xylanase activity.

The enzyme was assayed by mixing a suitably diluted aliquot of the enzyme with 0.5 ml xylan solution in phosphate buffer (pH 7.0) and the final volume was 1.0 ml. The reaction was incubated at 50 ^{o}C for 30 min and the reducing sugars formed were determined using the dinitro salicylic acid reagent (Miller 1959). One unit of the xylanase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars per min using xylose as a standard. The xylanase activity was also estimated by the comparatively sensitive method using 4- hydroxy benzoate reagent which can detect 10 - 50 µg of xylose. Properly diluted enzyme was mixed with 200 µl of 1% xylan in a final volume of 1.0 ml with potassium phosphate buffer (final concentration 50 mM), pH 7.0. The reaction was carried out at 50°C for 30 min and 5 ml of freshly prepared reagent was added. The tubes were kept in boiling water bath for 10 min and the absorbance was noted at 420 nm. Standard curve for xylose in the range of 10 to 50 µg was used to calculate the enzyme activity (Dubois *et al* 1956).

Immunoprecipitation of the xylanases

The xylanases from the crude extract of alkalophilic thermophilic *Bacillus* as well as the recombinant extracts were immunoprecipitated using the polyclonal antixylanase II antibodies. The protein extracts (50 - 500 µg) were mixed with the 32 titre antibodies (5 µl) in PBS buffer. The mixtures were incubated at 37 ^{o}C for 2h and then centrifuged at

12,000 prm for 15 min. The pellets were washed thrice with 200 µl PBS buffer and then dissolved in 0.1 % SDS solution by heating at 50 ^{o}C for 30 min. The resulting protein solutions were used for denaturing PAGE analysis.

Polyacryl amide gel electrophoresis (PAGE)

The polyacryl amide gel electrophoresis was carried out as described by Laemli (1970). The polyacryl amide gel (12 %), tris glycine buffer (pH 8.9) and the denaturing conditions (SDS 0.1 %) were used. The gels were run at constant current of 20 mA, till the tracking dye (Bromophenol blue) reached the other end of the gel. The gels were stained for 2 - 4 h with Brilliant blue R 250 solution (0.1 %) and the solvent was methanol, acetic aid, and water mixture in 4:1:5 proportion. The gels were destained with the same solvent till the distinct contrast developed between the protein bands and the clear gel background.

Xylan hydrolysis and end product determination

The xylanase preparations (0.1 U each) from the recombinant and the parent organisms were mixed with xylan (5 mg) in 200 µl final volume of phosphate buffer (final concentration 50 mM). The reaction mixture was incubated at 50 ^{o}C for 24 h in a tightly closed eppendorf tube. After the hydrolysis the reaction mixture was analysed by paper chromatography. Whatman filter paper (# 2) was used as the stationary solid phase and the solvent phase was a mixture of butanol, acetic

acid and water in 3:1:1 ratio. The chromatograms were developed using silver nitrate reagent (Trevelyan *et al* 1950).

Results and discussion

Analysis of *E. coli* (pATBX 11.4)

The intracellular, periplasmic and extracellular extracts of the recombinant were subjected to xylanase assay. The activity was found to be largely localized to the extracellular extract, and approximately 30 % was found in the intracellular extract. No activity was detected in the periplasmic fraction. The concentrated extra and intra cellular extracts were subjected to double diffusion analysis using antixylanase II antibodies. The recombinant extracts produced two precipitin lines that coincided with those produced by the crude culture filtrate of alkalophilic thermophilic *Bacillus*, indicating that the insert coded for both the xylanases produced by the parent *Bacillus*.

The analysis of the linkage of the three subfragments of 6.5, 3.2 and 1.7 kb from the pATB X 11.4 plasmid was carried out with the help of southern blot analysis of partial and complete Hind III digests of the recombinant plasmid. The labeled genomic DNA from alkalophilic thermophilic *Bacillus* was used as a probe. In the partial digest, the hybridization signals were obtained at 8.2 and 9.7 kb which correspond to 6.5 + 1.7 and 6.5 + 3.2 kb fragments. No band appeared at 4.9 kb (a possible addition product of 3.2 and 1.7 kb fragments), indicating that the 3.2 and 1.7 kb fragments are not directly linked to each other. No additional low molecular weight bands were obtained. The 2.6 kb band

corresponding to the linearised pUC 8 did not hybridise with the labeled genomic DNA of alkalophilic thermophilic *Bacillus*. These results also confirmed that the insert DNA had originated from the alkalophilic thermophilic *Bacillus* genomic DNA (FIG 3.1).

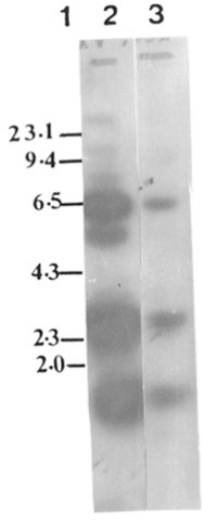


FIG. 3.1: Southern blot analysis of the digested plasmid pATB X 11.4 with labeled genomic DNA of AT *Bacillus*.

lane 1: Molecular weight marker, Lambda DNA digested with Hind III, 2: and 3: Partial and complete Hind III digest of pATB X 11.4 hybridised with $\alpha^{32}P$ labeled genomic DNA from AT *Bacillus*

Restriction analysis and subcloning of the xylanase gene fragment

The Hind III digest was further subcloned in pUC 8 and the recombinants were screened for xylan plate clearance. The xylanase activity was localised in the 6.5 kb Hind III fragment. The plasmid harbouring the 6.5 kb xylanase gene insert was designated as pATB X 6.5. The 6.5 kb DNA fragment was subjected to further restriction analysis and the insert was found to have unique sites for Eco RI and Pvu II. However no cleavage was obtained with the restriction endonucleases Bgl II, Cla I, Pst I and Sma I. The Eco RI -Hind III double digested pATBX 6.5 yielded two subfragments of 2 and 4.5 kb which were subcloned in pUC 8. The recombinant with 4.5 kb insert (pATBX 4.5) showed clearance zone on LB - Xylan plate and positive colony ELISA reaction (FIG. 3.2). The xylanase activity of the subclones was monitored along with the restriction analysis. The results are summarized in figure 3.3.

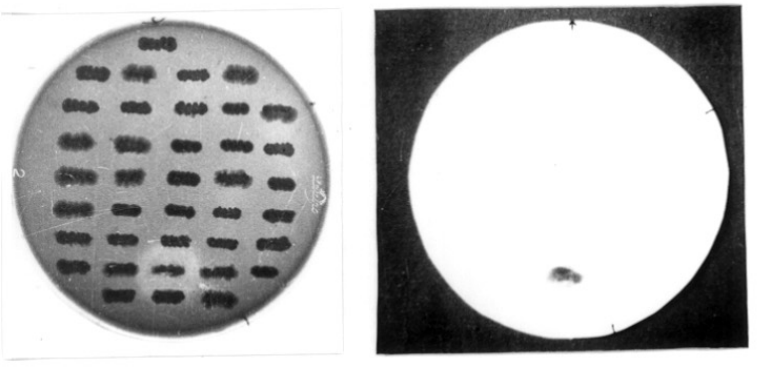


FIG. 3.2 A: Xylan - congo red plate clearance shown by the recombinant pATB X 4.5. The arrow points to the *E. coli* (pATB X 4.5) recombinant surrounded by a clearance zone on LB plate containing 1 % xylan. The clearance was observed after incubation at 37 ^{o} C for 18 h followed by 2 h incubation at 50 ^{o} C. The other colonies are recombinants from the genomic library as negative control. B: The recombinant pATB X 4.5 showing positive colony ELISA reaction.

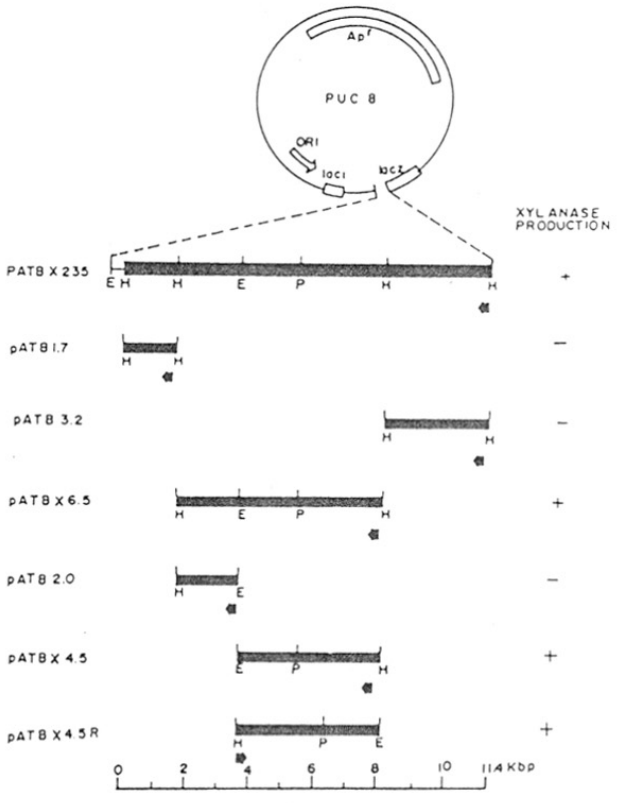


FIG 3.3: Restriction enzyme map of the genomic DNA fragments of AT *Bacillus* cloned in pUC 8 that code for xylanase activity. The solid bars represent AT *Bacillus* genomic DNA. The arrow indicates functional orientation of the lac promoter of pUC 8. The plasmid pATB X 4.5R was constructed by subcloning of the 4.5 kb fragment in pUC 9. Abbreviations for the restriction sites are as follows: E - Eco RI, H - Hind III, P - Pvu II. DNA size is estimated in kilobases. + and - indicate the presence and absence of xylanase activity estimated by DNSA method (see materials and methods).

Reorientation of the 4.5 kb insert

The 4.5 kb EcoRI - Hind III fragment of the plasmid pATBX 4.5 was cloned into pUC 9 where the orientation of the insert with respect to the lac O/P was reversed (pATBX 4.5 R). Equivalent amount of xylanase activity was produced by transformants pATBX 4.5 and pATBX 4.5 R (TABLE 3.1), indicating that the expression was under the xylanase gene promoter.

Xylanase production by the recombinant *E. coli* JM 105

The host strain *E. coli* JM 105 (pUC 8) had no intra or extracellular xylanase activity. In *E. coli* recombinants expression of the xylanases improved by subcloning (Table 1). The recombinant pATBX 4.5 showed maximum xylanase activity of 2 U/ml which was higher as compared to most of the cloned xylanase gene products from various *Bacillus* sp. (Bernier *et al* 1983, Honda *et al* 1985, Panbangred *et al* 1983, Yang *et al* 1988, 1989). The xylanase activity was extracellular accounting for 95 % of the xylanase production. The periplasmic fraction did not show any xylanase activity. The enzyme activity was observed even in the absence of xylan and addition of IPTG had no effect. The hydrolysis of xylan with the extracellular culture filtrate of pATBX 4.5 yielded xylobiose as the major product along with xylose and traces of xylotriose and xylotetrose (FIG. 3.4).

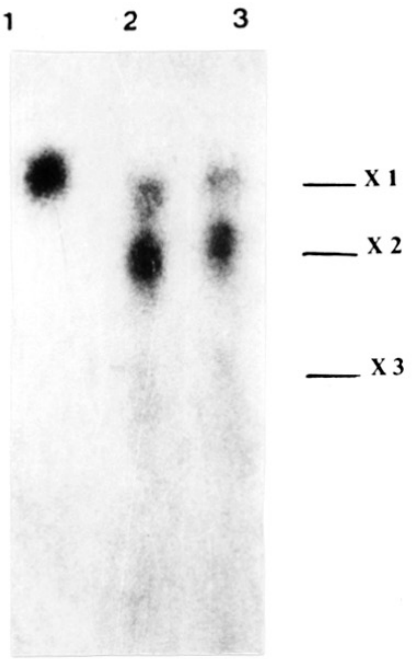


FIG. 3.4: Paper chromatogram of xylan hydrolysis products. Lane 1: Xylose standard 10 µg and 2: and 3: 24 h sample of hydrolysis products of extracellular culture filtrates of alkalophilic thermophilic *Bacillus* and *E. coli* recombinant pATB X 4.5 respectively. X1, X2 and X3 denote respectively xylose, xylobiose and xylotriose.

TABLE 3.1: Induction and localization of xylanase activity of the recombinant

RECOMBINANT	INDUCER	XYLANASE ACTIVITY (U/mg protein)		% ACTIVITY	
		I*	E*	I	E
pATBX6.5	Nil	0.004	0.044	6	94
	IPTG(20 µg/ml)	0.005	0.038	5	95
	Xylan(0.5%)	0.005	0.042	7	93
pATBX4.5	Nil	0.015	1.333	3	97
	IPTG (20 µg/ml)	0.018	1.358	2	98
	Xylan (0.5%)	0.020	1.500	4	96
pATBX4.5R	Xylan (0.5%)	0.016	1.300	4	96

* I and E denote intracellular and extracellular protein fractions respectively

Antixylanase analysis and molecular weight determination

The intra and extracellular extract of recombinant pATBX 4.5 showed two precipitin lines which completely fused with those of AT *Bacillus* culture filtrate (FIG. 3.5). These results suggest that the recombinant harboring the 4.5 kb insert coded for the two xylanases. Immunoprecipitation of crude culture filtrate of AT *Bacillus* followed by SDS PAGE showed the presence of only two bands corresponding to xylanase I and II. *E. coli* (pUC 8) did not show precipitation

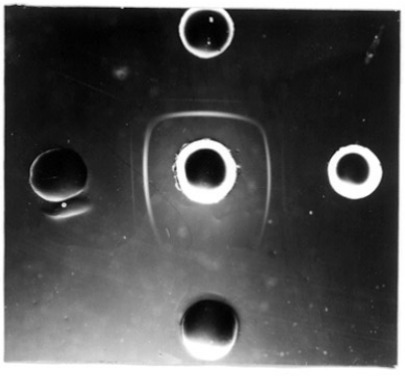


FIG. 3.5: Ouchterlony double diffusion test. Well 1: rabbit antiserum against purified xylanase II, 2: culture supernatent of AT *Bacillus*., 3: extracellular extract of *E. coli* (pATB X 4.5). 4: extracellular extract of *E. coli* (pUC 8). 5: intracellular extract of *E. coli* (pATB X 4.5).

with antixylanase antibodies and no protein bands were detected on SDS PAGE. The immunoprecipitated xylanases from the intra and extracellular extract of the recombinant showed protein bands at Mr 14,500 and 35,000 (FIG. 3.6) which were approximately of the same size as the xylanases of AT *Bacillus* (Mr 15,800 and 35,000). The difference in the molecular weight of the xylanase II may be due to proteolytic modification or absence of glycosylation. Presence of xylanases in the intracellular extracts of the recombinant without significant enzyme activity suggests possibility of protein aggregation or improper folding.

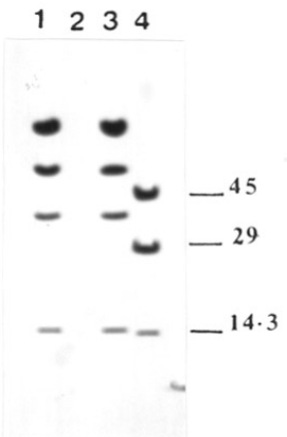
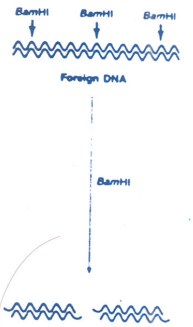


FIG. 3.6: SDS - PAGE of cell extracts immunoprecipitated with antixylanaseII antibodies. Lane 1: Intracellular extract of pATB X 4.5. , 2: *E. coli* JM105 (pUC 8) intracellular extract., 3: Extracellular extract of pATB X 4.5., 4: Molecular weight marker. Arrow indicates the dye front.

The available restriction mapping and sequencing data on the location of xylanase gene on the genomic DNA of various *Bacillus* sp. indicates the presence of a flanking region of 5 to 6 kb suggesting the possible magnitude of distance between the two xylanase genes. In contrast both the xylanases of AT *Bacillus* are coded by a single 4.5 kb Eco RI - Hind III fragment. It is possible that the two xylanase are coded by closely linked or overlapping genes. If the two xylanases are assumed to be coded by a single gene, then the proteolytic processing of a high molecular polypeptide has to be considered. Then in double diffusion analysis, the antibodies raised against the low molecular weight xylanase would have also reacted with unprocessed protein giving rise to arcs that merge with spur formation (Ouchterlony 1949). However our results show the presence of two distinct arcs corresponding to the two xylanases (Fig. 3.5) suggesting that they are not the products of proteolytic processing. In addition, no high molecular weight unprocessed form is detected in the immunoprecipitated extracts from the recombinant. The two xylanases seem to be the proteins of two distinct origins sharing common immunologically reactive domains. The expression of the recombinant xylanases in *E. coli* has largely been intracellular (see table 1.2). Extracellular secretion of the recombinant xylanases has so far been reported only in case of *Bacillus* C 125 xylanase A gene (Honda *et al* 1987). In case of *Cellulomonas* sp. (NCIM 2353), the recombinant xylanases are detected in the extracellular

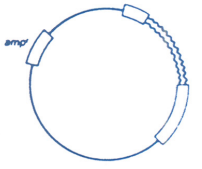
culture filtrate of *E. coli*; but no data has been presented regarding the localization studies (Bhalerao *et al* 1992). The extracellular activity of this recombinant may be because of the leakage of a small fraction of the total activity produced, which is a common observation in case of many recombinant xylanases (see table 1.2). The extracellular expression of the xylanases by the recombinant *E. coli* harboring the 4.5 kb DNA fragment from alkalophilic thermophilic *Bacillus* could prove to be a biotechnologically important feature.

CHAPTER IV

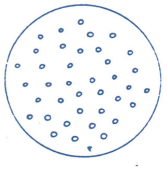


HOMOLOGOUS EXPRESSION IN

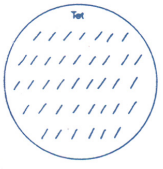
BACILLUS SUBTILIS

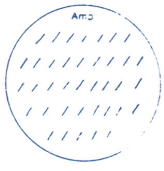


Insertion of a segment of foreign DNA inactivates the *tet* gene. Bacteria transformed with this recombinant plasmid can grow in the presence of ampicillin but not tetracycline



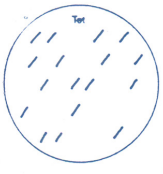
Colonies of transformed bacteria growing in the presence of ampicillin

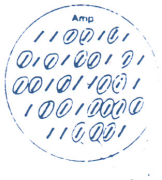




Individual colonies transferred with a sterile toothpick to media containing tetracycline (left) or ampicillin (right)

Plates incubated overnight at 37°C





Amp⁺ Tet⁺ colonies recovered for further analysis

SUMMARY

The 11.4 kb Hind III fragment of genomic DNA of alkalophilic - thermophilic *Bacillus* , coding for two xylanases, has been cloned in *Escherichia coli* recombinant plasmid pATB X235. The 6.5 kb Hind III subfragment was cloned in a *Bacillus* plasmid pLP1202 at Hind III site ; inactivating the tetracycline resistance gene. *Bacillus subtilis* A8 - a xylanase negative mutant or MI 111 - a xylanase proficient host - were transformed with the ligation mixture using electroporation. The recombinants were chloramphenicol resistant and tetracycline sensitive. The *B. subtilis* A8 transformants were characterised by the clearance on LB plates having xylan and congo red. The expression of the recombinant xylanases in *B. subtilis* MI 111 was confirmed by immunological cross reactivity with the antibodies raised against purified xylanase II (Mr 15,800) from alkalophilic thermophilic *Bacillus*. The xylanase activity obtained in *B. subtilis* MI 111 and A8 was five and fifteen fold higher respectively as compared to *E. coli* harboring pATB X235. Nintyfive percent of the enzyme activity was extracellular. The xylanases produced by the A8 recombinant showed molecular weights of 35 and 14.5 KDa. The hydrolysis of xylan by the A8 recombinant xylanases yielded mainly xylobiose. Xylose was also detected along with traces of xylotriose and xylotet-

rose. The hydfrolysis pattern was similar to that of alkalophilic thermophilic *Bacillus*. However, the paper chromatographic analysis of the xylan hydrolysis products of MI 111, suggested that the xylanases from alkalophilic thermophilic *Bacillus* and host *B. subtilis* MI 111 had different modes of action. The hydrolysis products were identical, except for the xylopentaose which was detected only in case of alkalophilic thermophilic *Bacillus*. The host *B. subtilis* MI 111 produced low levels of xylanase activity which was enhanced by incorporation of xylan or wheat bran in the growth medium. However, no significant enhancement in the expression of recombinant xylanases was observed, indicating that the host plays a key role in the regulation of xylanase gene expression.

Introduction

Isolation of the xylanase gene fragment from alkalophilic thermophilic *Bacillus* represents an essential step in the engineering of a more efficient microorganism. The low levels of expression of alkalophilic thermophilic *Bacillus* xylanases in *E. coli* is consistent with the previous reports on cloning and expression of xylanases in *E. coli*. The high growth rate and the availability of multicopy plasmid(s) are the main advantages of using *E. coli* as a cloning host, that are reflected in to the level of expression of the foreign gene(s). In general few problems are frequently associated with the use of *E. coli* as a host for production of foreign proteins (Sibakov and Palva 1987). The high level of expression in *E. coli* leads to aggregate formation within the cells. These aggregates are improperly folded and insoluble protein complexes. The renaturation of these protein complexes to the active protein form is very difficult and seldom yields more than 5 % of the total protein present in the complex form.

Several heterologus proteins can not be efficiently produced in *E. coli* due to:

a) the relatively abundant occurrence of rare codons in the cloned gene (Sharp and Li 1986).

b) the need for specific postsynthetic modification and / or the structural complexity of the protein

c) toxicity of the accumulated foreign protein to the *E. coli* host.

d) succeptibility of the foreign protein to *E. coli* protease(s).

Cloning and expression of the cloned gene(s) in the homologous host system is of significant advantage as the translation and secretion efficiency is expected to be high. Also, in the homologous system, accurate postsynthetic modification may be possible; and the problems such as toxicity, codon usage differences, and succeptibility to the host protease(s) could be minimized. Hence the expression of the cloned xylanase gene fragment from alkalophilic thermophilic *Bacillus* in *B. subtilis* is of particular interest. Since most of the *Bacillus* sp. produce xylanolytic enzymes, the ease of detection of the cloned xylanases needs to be considered. The use of xylanase negative host would be the first choice. However, xylanase proficient host could also be used if the differentiation between the host and the recombinant xylanases is possible.

A generally accepted view on the regulation of xylanase synthesis is that the low constitutive levels of xylanases produce small soluble oligosaccharides which are able to enter the cells and induce the synthesis of the corresponding xylanases (Hrmova *et al.*, 1991). Recent studies on the regulatory elements indicated the key role of few heterodisaccharides such as 4-O-ß-D- xylopyranosyl-L arabinose (Hrmova *et al.*, 1986; Biely and Petrakova, 1984) and 1,2 - ß - linked disaccharides of glucose and xylose (Hrmova *et al.*,

1989, 1991) in induction of xylanases. The role of transglycosylating enzymes in the synthesis of the positional isomers of these heterodisaccharides was suggested in case of *Aspergillus terreus* and *Trichoderma reesei* (Hrmova *et al.*, 1991). In the model explaining induction of xylanases Thomson (1993) has described two levels of induction, in which xylooligosaccharides act as primary inducers and the heterodisaccharides further induce the xylanase synthesis in a more effective way.

Hence, the low level of expression of the recombinant xylanases in the non xylanolytic hosts may be justified as these organisms need not posses the proper inducers such as heterodisaccharides and/or inducer synthesizing machinery. Also the xylanase negative mutation might have accompanied with the overexpression of the xylanase represser.

On the basis of the above information the studies on the induction and expression of the xylanases from alkalophilic thermophilic *Bacillus* were planned. The present chapter describes cloning of the 6.5 kb xylanase gene fragment from the alkalophilic thermophilic *Bacillus* in a *E. coli* - *Bacillus* shuttle plasmid vector pLP 1202. The expression of the xylanases was studied using two *Bacillus subtilis* hosts: a xylanase negative mutant designated as A8 strain and a xylanase proficient strain designated as MI 111.

Materials and Methods

Bacteria and culture media

E. coli JM 105, *B. subtilis* A8 and *B. subtilis* M 111 were used through out the studies. *B. subtilis* A8 (BGSC Strain # 1A 651) and *B. subtilis* MI 111 (BGSC strain # 1A 253) were obtained from *Bacillus* Genetic Stock Center. The *E. coli* strain has been described in the chapter 2. Both the *Bacillus* strains have been derived from *B. subtilis* strain # 168 and carry auxotrophic selectable markers. The genotypes of these hosts are given below.

B. subtilis A8 : lys-3 xynA8.

B. subtilis MI 111 : SP10(S) arg(GH)15 hsrM leuA8 m(-)168.

The *Bacillus* and *E. coli* strains were routinely grown in LB at 37 ^{o}C with shaking for 18 h, and the respective recombinant strains were grown in presence of chloramphenicol (7.5 µg/ml) and ampicillin (30 µg/ml).

The plasmid pLP 1202 is derived from two plasmids :

pUB 110 : A 4.548 kb plasmid from *Staphylococcus* which can replicate in *Bacillus* and codes for chloramphenicol resistance in both *E. coli* (15 µg/ml) and *B. subtilis* (7.5 µg/ml).

pBR 322 : The well documented *E. coli* plasmid (size :4.361 kb) coding for ampicillin resistance in *E. coli* (100 µg/ml) and tetracycline resistance in both *E. coli* (15 µg/ml)

and *B. subtilis* (2.5 µg/ml).

The shuttle plasmid has been constructed (see FIG. 4.1) by ligating the pBR 322 plasmid linearized with Pvu II, with the fragment of pUB 110 that contains replicon for *B. subtilis* and chloramphenicol acyl transferase gene (Ostroff G. R. and Pene J. J. 1984).

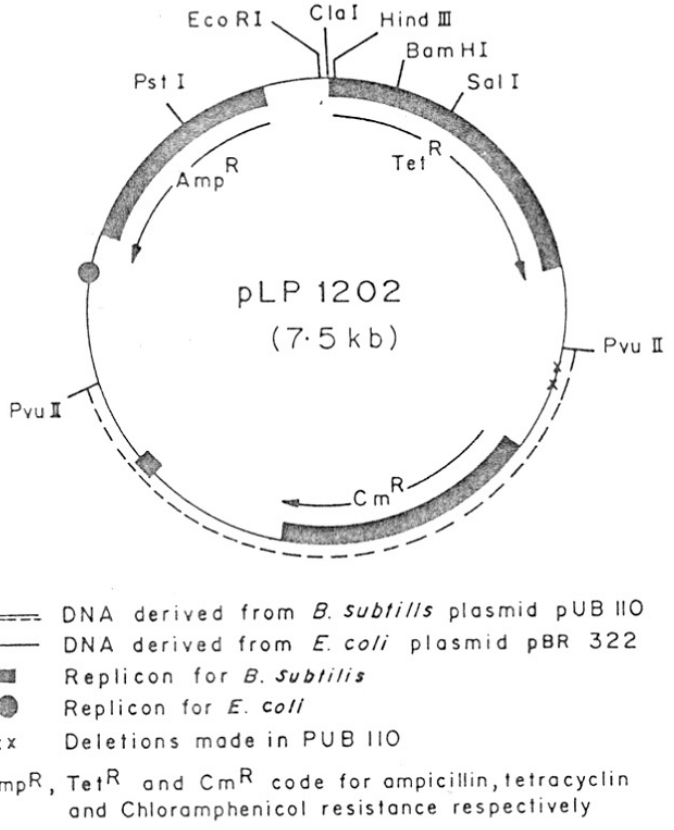


FIG. 4.1: Restriction map of the plasmid pLP 1202

DNA Manipulations.

Plasmid DNA was extracted from *E. coli* and *Bacillus* as described in the first chapter. In case of *Bacillus*, however, the lysozyme treatment, was given for 30 min at room temperature in TE containing sucrose (8 % w/v). Restriction endonuclease digestion, ligation, gel electrophoresis, and transformation in *E. coli* was performed as already described. The recombinant plasmid pATB X235 was digested with Hind III and 6.5 kb fragment was purified from an agarose gel using Qiagen tubes. The purified 6.5 kb fragment was ligated to pLP 1202 that was linearized with Hind III followed by alkaline phosphatase treatment.

Transformation in *B. subtilis*

The *B. subtilis* cells grown for 16 h in 10 ml LB with shaking (200 rpm), were used to inoculate fresh LB (1,000 ml). The cells were allowed to grow for 6 - 8 h and the final OD_{600} was 0.8 - 1.0. The cells were washed twice with equal volume of sterile distilled water prechilled to 4 ^{o}C under aseptic conditions. Two more washes were given respectively with 0.5 and 0.2 V of prechilled sterile distilled water. The cells were finally resuspended with 3 ml of sterile glycerol (10 %) solution, and preserved in 200 µl alliquotes at - 70 ^{o}C. The *B. subtilis* competent cells suspended in 10 % glycerol, were transformed with the ligation mixture (50 ng with respect to the vector) using the BTX T 100 electroporator. A single pulse of 659 V and 4.68 m sec was applied.

Screening for the xylanase positive recombinant

The transformants were plated initially on LB agar supplemented with chloramphenicol (7.5 µg/ml) and then screened for tetracycline sensitive phenotype. The *B. subtilis* A8 transformants were selcted using a xylan / congo red plate clearance assay.

The *B. subtilis* MI 111 recombinants showing chloramphenicol resistant and tetracycline sensitive phenotype were grown in liquid medium for crude xylanase protein preparation. The crude extract was subjected to double diffusion analysis against the antibodies raised against the purified xylanase II from alkalophilic thermophilic *Bacillus*. The colonies corresponding to the positive double diffusion test [arc(s) formation] were considered as the confirmed xylanase positive recombinants.

Preparation of cell extracts and enzyme assay

The culture broth (50 ml) was centrifuged and the supernatent, referred to as extracellular extract, was concentrated by ammonium sulfate precipitation or directly taken for xylanase assay. The cells were washed and sonicated or treated with lysozyme in 3 ml final volume. The supernatent after centrifugation, referred to as intracellular extract, was assayed for xylanase. Protein estimation was carried out by the method of Lowry *et al.* (1951). The xylanase activity was determined as described earlier.

The other methods such as molecular weight determination, immunological characterization, and determination of xylan degradation products were exactly followed as described in the first chapter.

RESULTS

Cloning of Xylanase Gene fragment in pLP 1202

Plasmid transformation in *B. subtilis* can be accomplished either with competent cells or by protoplasts in the presence of polyethylene glycol (Bennet and Grinsted 1984). The cells could be either in the naturally competent stage or the competence may be induced chemically (Fiedler and Wirth 1988). Both the transformation methods suffer the disadvantage of low transformation efficiency and the regeneration time of the protoplasts is also too long (Taylor and Burke 1990). The electroporation is however, a rapid simple and effective method of introducing plasmid DNA in to bacterial cells and specific methods for electroporation mediated transformation of *Bacillus* have recently been described (Brigidi *et al* 1990, Bone and Ellar 1989, Brian and Trevors 1989, Taylor and Burke 1990).

Electroporation

The electroporation mediated transfer of DNA in to plant, animal, fungal, protozoan, yeast and bacterial cells gas already been described (Dower *et al* 1988). The bacteria from different genera such as *Bacillus, Bordetella, Campylobacter sp., Clostridium, Corynebacterium sp., Cyanobacteria sp., Enterococcus, Erwinia, Escherichia, Haemophilus, Klebsiella, Listeria, Lactobacillus, Lactococcus, Leuconostoc, Mycobacterium, Myxococcus, Pediococcus, Propionibacterium,*

Pseudomonas, *Rhizobium*, *Salmonella*, *Staphylococcus*, *Streptococcus*, and *Vibrio* have been succefully transformed using electroporation (Chassy *et al* 1988). Amongst bacilli, electroporation has been demonstrated in *B. anthracis*, *B. cereus*, *B. sphericus*, *B. subtilis*, and *B. thuringensis* using the intact cells (Brigidi *et al* 1990, Bone and Ellar 1989, Brian and Trevors 1989, Chassy *et al* 1988, Taylor and Burke 1990).

The electroporation technique involves the application of a brief, high intensity electric pulse to the cells, which reversibly permeabilizes the cell membrane due to the formation of transient ultrafine pores (Knight 1981, Dower *et al* 1988). The field strength of the electroporation pulse is defined as " *actual applied voltage in kV per cm interelectrode distance*", and is known to be the major factor affecting the transformation efficiency. The application of a single pulse of the field strength in the range of 12 - 16 [kV/cm] and with the pulse length of the order of 5 msec has been shown to be optimum for various bacterial species (Dower *et al* 1988, Marcus *et al* 1990, McIntyre and Harlander 1989, Oultram *et al* 1988); however low field strength electroporation is generally suitable for obtaining 10^3 - 10^4 transformants / µg of intact plasmid DNA (Delorme 1989, Phillips-Jones 1990, Powell *et al* 1988).

The schematic representation of the cloning of the 6.5 kb xylanase gene fragment in pLP 1202 is shown in figure 4.2. The results of the transformation of the ligation mixture in *B. subtilis* A 8 and MI 111 are summarized in table 4.1. All

the chloramphenicol resistant and tetracycline sensitive colonies of A8 transformants were surrounded by the clear hallow on the xylan / congo red plates. The plasmid isolated from the representative colonies showed 6.5 kb insert on the subsequent Hind III digestion (FIG 4.3).

TABLE 4.1: Transformation of the ligation mixture in *B. subtilis*

DNA in µg (vector)	Strain	Number of transform.	Number of recombi.	Transformation efficiency
0.05	A 8	200	120	4×10^{3}
	MI 111	265	150	5.3×10^{3}

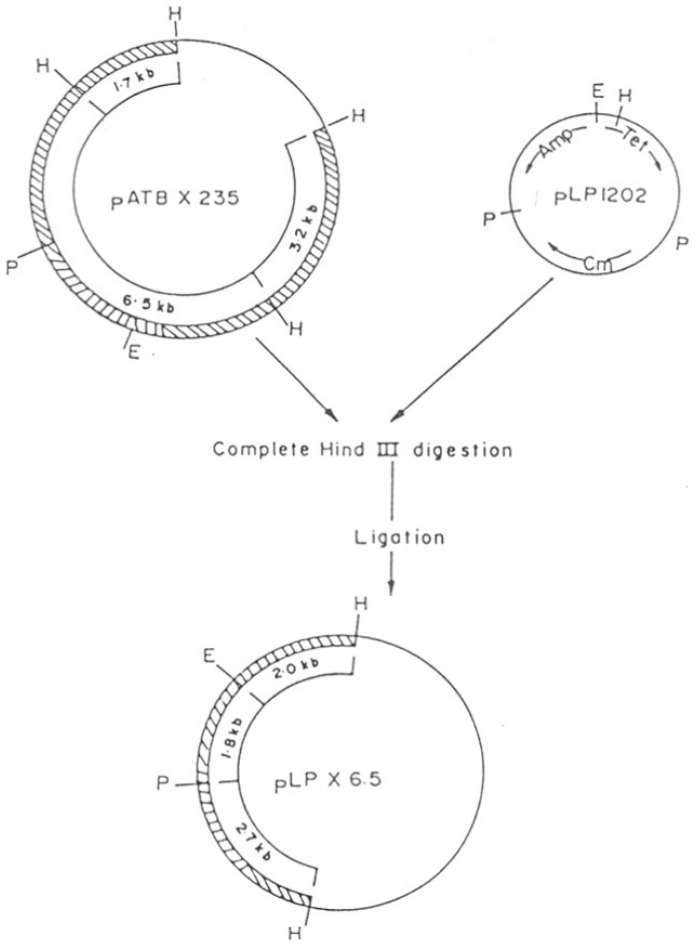


FIG. 4.2 : Construction of Recombinant Plasmid. The 6.5 kb A T *Bacillus* chromosomal fragment (marked area) from plasmid pATB x 235 was ligated to pLP 1202 linearized with Hind III followed by alkaline phosphatase treatment. The xylanase positive recombinant pLP X 6.5 was tetracycline sensitive and chloramphenicol resistant. The restriction sites are shown in the figure. E - Eco RI, H - Hind III, P - Pvu II. Amp, Tet and Cm are ampicillin, tetracycline and chloramphenicol resistance genes respectively.

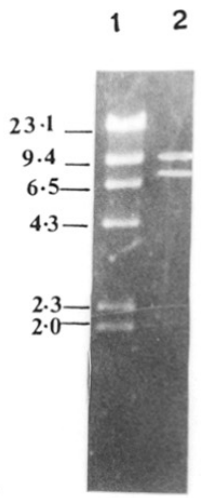


FIG. 4.3 : Digestion of the recombinant plasmid DNA with Hind III

lane 1: Lambda Hind III digest molecular size marker

lane 2: Digested pLP X 6.5 showing vector and insert.

Confirmation of the xylanase positive recombinants in *B. subtilis* MI 111

The protein extract of the host *B. subtilis* MI 111 did not show any precipitin line in double diffusion analysis with the antixylanase II antibodies indicating that the xylanase(s) of the host are immunologically distinct from the xylanases of alkalophilic thermophilic *Bacillus*. The protein extract of the transformant harboring pATBX 6.5 also showed two precipitin lines which were identical to those of the protein extract of alkalophilic thermophilic *Bacillus* (Fig. 4.4). These results confirmed the expression of recombinant xylanases in *B. subtilis* MI 111.

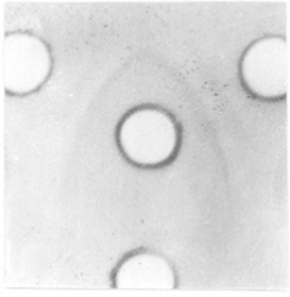


FIG. 4.4 Ouchterlony double diffusion test
Well 1 contains rabbit antiserum against purified xylanase II. Well 2, 3 and 4 contain extracellular protein extract of alkalophilic thermophilic *Bacillus*, *B. subtilis* MI 111 (pLP X 6.5) and *B. subtilis* MI 111 host respectively.

Xylanase activity of the *B. subtilis* recombinants

The *Bacillus subtilis* A 8 recombinants produced five fold higher activity as compared to the *E. coli* recombinants. Around nintyfive percent of the total activity was confined to the extracellular broth. The level of expression of the recombinant xylanases in *B. subtilis* MI 111 was three fold higher than that of *B. subtilis* A8. The xylanase activity of the recombinants is summarized in the Table 4.2. The xylanase activity of the *B. subtilis* A 8 recombinant was higher (1.5 times) when grown on xylan (TABLE 4.2), whereas the *B. subtilis* MI 111 recombinant did not show any significant increase (TABLE 4.3). The xylanase activity of the host *B. subtilis* MI 111 was induced three fold with xylan and four fold with wheat bran inducers. But this apparent increase in the xylanase activity of the transformant in the presence of inducer may be due to the induction of the host xylanase activity, indicating that the recombinant xylanases are not induced in *B. subtilis* MI 111.

TABLE 4.2: Induction and localization of xylanase activity of the recombinant *B. subtilis* strains

B. subtilis strain	INDUCER	XYLANASE ACTIVITY (mU/mg protein)		% ACTIVITY	
		I	E	I	E
A8	Nil	2	100	4	96
	Xylan (0.5%)	4	140	6	94
MI 111	Nil	5	300	3	97
	Xylan (0.5%)	7	310	5	95

The *B. subtilis* cells were grown at 37°C for 18 h in L broth containing chloramphenicol (7.5 µg/ml).

I: intracellular extract, E: extracellular extract.

Table 4.3: Production of recombinant xylanases by *B. subtilis* MI111

Inducer	Extracellular xylanase activity (mU/mg biomass)	
	B. subtilis MI 111	*B. subtilis* MI 111 (pLPX6.5)
Nil	67	441 (374)
Xylan (0.5%)	186	563 (377)
Wheat bran (5%)	278	548 (270)

The fermentations were carried out at 37°C for 18 h using LB as basal medium. Inducers were added as specified in the Table. The transformants were grown in the media containing chloramphenicol (7.5 µg/ml). Values in the parentheses correspond to the xylanase activities of the recombinant derived by subtracting the xylanase activity of the host from that of the transformant grown under identical conditions.

Determination of Molecular Weight of Recombinant Xylanases in *B. subtilis* A 8.

B. subtilis A8 did not show precipitation with antixylanase antibodies and no protein bands were detected on SDS PAGE. Immunoprecipitated xylanase from the extracellular extract of the recombinant showed two protein bands at Mr 14,500 and 35,000 which were approximately of the same size as the xylanases of AT *Bacillus.* (FIG 4.5).

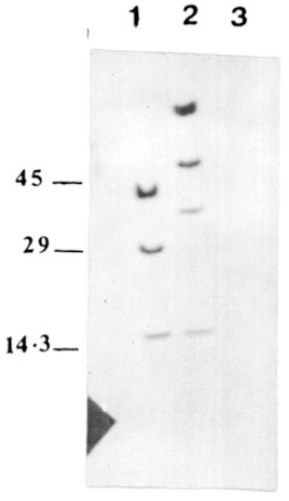


FIG. 4.5 : SDS PAGE of the cell extracts immunoprecipitated with the antixylanase II antibodies.

lane 1: Protein molecular weight marker

lane 2: Extracellular extract of recombinant *B. subtilis* A8 (pLP X 6.5)

lane 3: Extracellular extract of the host *B. subtilis* A8

Arrow indicates the dye front.

Xylan Hydrolysis

The hydrolysis of xylan with the extracellular culture filtrates of AT *Bacillus* and *B. subtilis* A8 producing recombinant xylanases yielded xylobiose as the major product. Xylose and traces of xylotriose and xylotetrose were also produced. The hydrolysis patterns were overall similar . However, the paper chromatographic analysis of the xylan hydrolysis products of alkalophilic thermophilic *Bacillus* and the *B. subtilis* MI 111 transformant showed showed distinct differences. Both the hydrolysis products showed the presence of xylose, xylobiose and xylotetraose as the major end products along with xylotriose, xylohexaose and higher oligosaccharides. However xylopentaose was detected only in the hydrolysis products using the xylanases from alkalophilic thermophilic *Bacillus*. The extracellular extract of the host *B. subtilis* MI 111 produced xylobiose and xylotetraose as the major products with traces of xylose and higher oligosaccharides. The presence of xylotriose and xylopentaose was not detected. (FIG. 4.6) These results suggested that the xylanases from alkalophilic thermophilic *Bacillus* and host *B. subtilis* MI 111 had different modes of action. The absence of a specific xylooligosaccharide such as xylopentaose in the hydrolysis products of the transformant could be due to either lack of the possible transglycosylation reaction of the cloned xylanases, or absence of other transglycosylating

enzymes or efficient degradation of the oligosaccharide by the host xylanases.

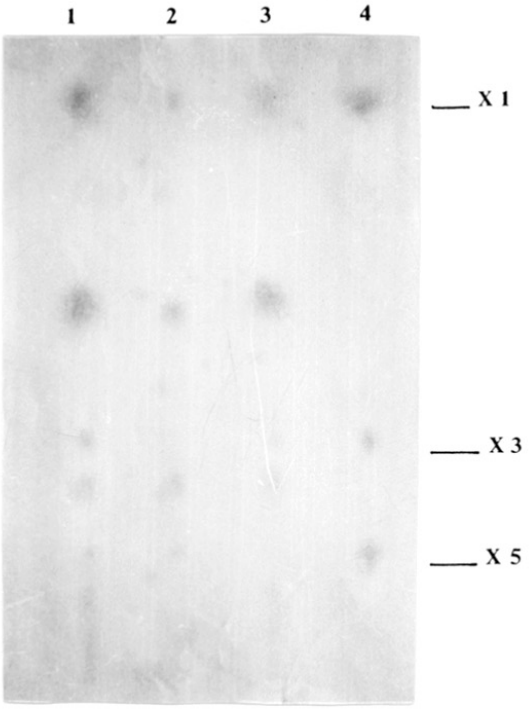


FIG. 4.6: **Paper chromatogram of xylan hydrolysis.** Products from 5 mg of xylan obtained using 1 U of xylanase at 50 ^{o} C, pH 7.0 for 24 h. Lane 1-3 are hydrolysis products of the extracellular culture filtrates of alkalophilic thermophilic *Bacillus*, host *B. subtilis* MI 111, and the transformant harboring pLPX 6.5 respectively. Lane 4 is standard mixture of xylose (X1), xylotriose (X3) and xylopentaose (X5). X2, X4 and X6 denote xylobiose, xylotetraose and xylohexaose respectively.

Panbangred *et al* (1985) have reported expression of xylanase gene from *B. pumilus* in *B. subtilis* M III. The recombinant showed 2 - 3 times higher activity than the parent *B. pumilus*. The level of expression of the recombinant xylanases of alkalophilic thermophilic *Bacillus* in the two *B. subtilis* hosts was comparable with the constitutive level of xylanase expression in alkalophilic thermophilic *Bacillus* (Dey *et al.*, 1992). The low levels of expression of xylanases from alkalophilic thermophilic *Bacillus* in *B. subtilis* could be due to nonrecognition of the signals from the extremophilic source. The copy number of pLP 1202 in *B. subtilis* is much lower than that of pUc 8 in *E. coli*. This may be attributed to the narrow increase in the enzyme activity in *B. subtilis* as compared with *E. coli*. However, the regulatory studies on the genes expressed in heterologus hosts are not necessarily a true reflection of the situation in the parent organism (Thomson, 1993).

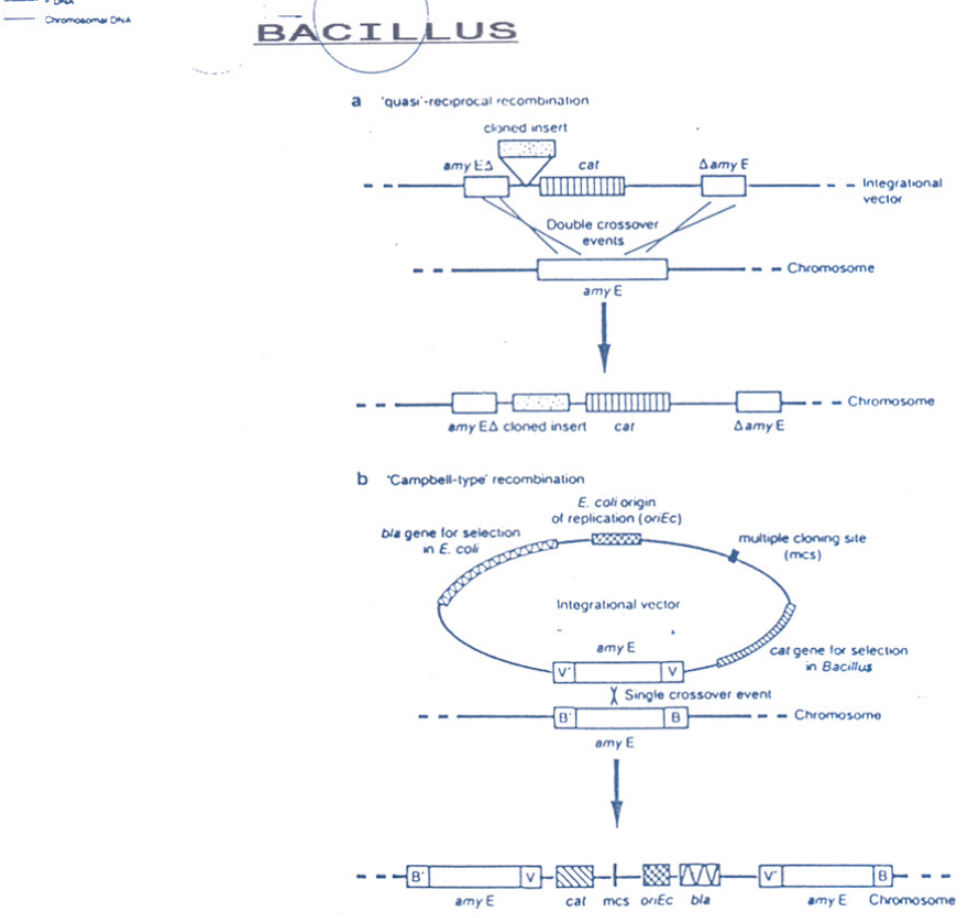
The constitutive expression of the xylanases in the recombinant *B. subtilis* strains may be caused by absence or nonrecognition of the regulatory elements. In the xylanase fermentation generally insoluble substrates are used as inducers. The constitutive expression of xylanases is advantageous to eliminate the insoluble inducers from large scale fermentations.

The present studies establish the inter-species differences in the induction profiles of the cloned xylanases. They provide a foundation for detailed studies on the role of the enzyme machinery of the host such as the transglycosylat-

ing enzymes. These enzymes may be important in the synthesis of proper inducer molecules such as heterodisaccharides and their positional isomers, and thus may play a critical role in the induction of the cloned xylanase genes.

CHAPTER V

GENE INTEGRATION AND ENHANCED XYLANASE EXPRESSION IN ALKALOPHILIC THERMOPHILIC BACILLUS



SUMMARY

Chromosomal integration and xylanase gene amplification was demonstrated for the first time in an alkalophilic thermophilic *Bacillus* sp. (NCIM 59). An electroporation mediated transformation of alkalophilic thermophilic *Bacillus* was carried out with the recombinant plasmid pATB X6.5 harboring the xylanase gene from the same organism. The integrants were characterized by larger zone of xylan clearance than the parent culture and hybridization with pUC8. Repeated transformation strategy was used for further amplification of the xylanase gene. The Hind III digested genomic DNA of alkalophilic thermophilic *Bacillus* when probed with 6.5 kb Hind III fragment harboring the xylanase gene, showed a single hybridization band at 6.5 kb. However DNA digests of the integrant strain CII 11 showed two positive signals at 5.1 and 3.2 kb whereas strain CIII 6 showed three positive signals at 4.8, 3.2 and 2.9 kb. These results indicated the occurrence of homologous recombination in 6.5 kb xylanase gene region of the genomic DNA and suggested a non campbell mode of recombination. The integrants were checked for xylanase production up to ten subcultures and consistently showed two fold higher xylanase activity (128 U/ml) than the parent strain. The xylanase productivity (U/ml/h) of the parent and the integrant strains was maximum at 20 and 16 h respectively.

INTRODUCTION

The biotechnological applications of xylanases have stimulated the search for stable and highly active enzyme preparations which can be obtained in bulk quantities. For the commercial realization and economic viability of enzyme production, it is necessary to identify organisms which can hyperproduce the xylanases. The xylanase genes show low levels of expression in the heterologous hosts. Hence there is a need for trying alternate approaches to enhance the expression of the cloned xylanases. From the results on the homologous gene expression of the xylanases from the alkalophilic thermophilic *Bacillus*, it may be concluded that the low level of expression may be because of the factors mediated by the host. Thus the studies on the expression of the two xylanases using the alkalophilic thermophilic *Bacillus* as a host are of importance. The plasmid systems available for the transformation of *Bacillus* sp. such as pLP 1202, pUB 110, pMK3 and others that use the antibiotics selective pressures are not suitable for their use with alkalophilic thermophilic *Bacillus* as the antibiotics under consideration i.e. tetracycline, chloramphenicol, neomycin etc are unstable at alkaline pH and 50 ^{o}C; and the alkalophilic thermophilic *Bacillus* shows growth in presence of high concentrations of these antibiotics. The auxotrophic mutants of the alkalophilic thermophilic *Bacillus* are not available; and the studies on the high temperature mutants of *Bacillus* (Droffner and Yamamoto) suggest that the auxotrophic mutations may be

very difficult to detect as the extremophilic strains require highly nutritious media for their growth. Hence plasmid vectors using the prototrophic markres were not used for alkalophilic thermophilic *Bacillus*.

The strategy for cloning and overexpression of the xylanases in alkalophilic thermophilic *Bacillus* was therefore developed using the innovative genetic technique of plasmid integration. The cloned gene, borne up on a plasmid, can integrate with the *Bacillus* genome via Campbell - type insertion at its homologous site (Kooistra *et al* 1988). Plasmid that are unable to replicate in *Bacillus* can exist only if they integrate in to its chromosome. The Campbell type single cross over results in to duplication of the homologous DNA, and the double cross over results in to genetic exchange without DNA amlification (FIG 5.1). The non campbell mode of recombination has also been reported in case of *B. subtilis* (Albertini and Galizzi 1985, Davidoff and Dubnau 1973, Yasbin *et al* 1975), where integration of a single stranded fragment of the plasmid with the genomic DNA possibly at gaps (Duncan *et al* 1978, Emori *et al* 1988). However, alkalophilic thermophilic *Bacillus* is an extremophilic isolate of local origin, and the techniques for its genetic manipulations - especially using this organism as a host - are not yet standardized.), generating the restriction fragments of varied lengths.Special integrational vectors have been developed for *B. subtilis* that also carry antibiotics resistance markers (Kallio *et al* 1987, Kooistra *et al* 1988, Yuki 1975). The antibiotics may be used for the selection of the integrants and an in-

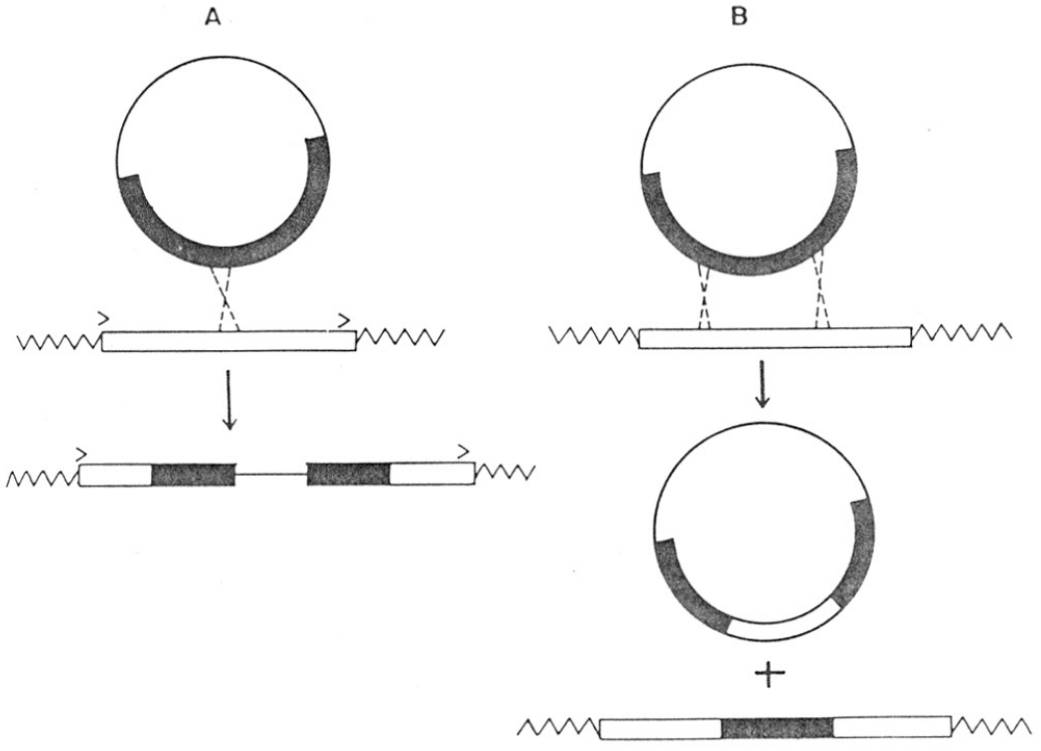


FIG. 5.1: Campbell type mode of recombination: A) Single cross over and B) Double cross over.

non-homologous plasmid DNA

homologous insert DNA

homologous region of the genomic DNA

rest of the non-homologous genomic DNA

crease in the concentration may be used to select for the in vivo amplified strains which carry multiple copies for the antibiotics resistance gene. In most of the cases the desired gene is also amplified along with the antibiotic resistance gene, giving rise to linear copy number rise of the desired gene with increasing antibiotic concentration (in a limited range). Since the antibiotics selective pressure can not be used with alkalophilic thermophilic *Bacillus*, the special integration vectors may not be of much significance.

The present chapter describes enhanced xylanase production using a recent and promising approach of chromosomal gene integration. The pUC recombinant plasmid pATB X 6.5 was therefore chosen for the integration studies. The gene amplification was performed using the alternative procedure of repeated transformations (Kallio *et al* 1987).Although the process of gene integration is well known in *B. subtilis* (Duncan *et al* 1978, Haldenwang *et al* 1980, Jaanniere *et al* 1985, Saunders *et al* 1984,) its application in genetic engineering has been so far reported only for the stabilization of expression of the β- lactamase gene of *Staphylococcus aureus* in *B. subtilis* (Saunders *et al* 1984) and for the overexpression of *B. amyloliquefaciens* α- amylase gene in *B. subtilis* (Kallio *et al* 1987). The use of the technique has not been reported in the biotechnology of an extremophile, such as alkalophilic thermophilic *Bacillus*. Our results represent the first report on gene integration mediated enhanced xylanase production.

MATERIALS AND METHODS

Bacteria, growth and fermentation media:

Alkalophilic thermophilic *Bacillus* was routinely grown on LB medium. Screening of the transformants was carried out on L broth supplemented with xylan (oat spelt, Sigma) 5 g/l, congo red (Sigma) 0.1 g/l, solidified using agar 20 g/l. Fermentation studies were carried out either in xylan yeast extract medium or in wheat bran yeast extract medium containing either xylan 5 g/l or washed wheat bran 50 g/l along with yeast extract 2.5 g/l and NaCl 5 g/l. The media were made alkaline (pH 10.5) after autoclaving by addition of separately sterilized sodium carbonate 1% (w/v) final concentration. The cultures were routinely grown at 50°C with shaking (200 rpm) and the samples were removed at designated time intervals. The fermentations was carried out for 48 h and the inoculum was 10 % (v/v) 16 h grown culture using the medium same as the fermentation medium.

Transformation and screening of the integrants:

The recombinant plasmid pATBX 6.5 was used to transform the AT *Bacillus* cells prepared according to Dower (see chapter 4). The AT *Bacillus* cells suspended in 10% glycerol were transformed with the help of a single pulse set at 650 V and

5 msec using BTX T100 electroporator. The transformants were screened using xylan-congo red plate clearance assay and colony hybridization with labeled pUC8 vector. The selected strain was subjected to second transformation cycle. Southern blot analysis of the selected strains was carried out using labeled 6.5 kb Hind III fragment of pATBX 6.5. The DNA labeling was performed using multiprime labeling kit (Amersham) and [α ^{32}P] dATP (3000 Ci/m mol). Hybridization and washing were at highest stringency conditions (63°C) and final washing was with 17 mM sodium chloride-sodium citrate buffer (pH 7.0) having 0.1% (w/v) sodium dodecyl sulfate (SDS).

Maintenance and cultivation of integrants:

The overnight grown cells of the integrants were suspended in glycerol (15% v/v) and stored as frozen glycerol stocks at -70°C. They were also grown on wheat bran yeast extract slants and maintained in sporulated form at 4°C. The sporulated AT *Bacillus* and integrants were routinely subcultured to fresh slant before every fermentation experiment and 16 h growth from these slants was used to inoculate fermentation media containing wheat bran, glucose, xylose or lactose as sole source of carbon. The xylan medium used for xylanase production was inoculated with the bacteria grown on xylan yeast extract slants.

Enzyme and protein determination:

The xylanase activity of the extracellular culture supernatent was determined in 50 mM sodium phosphate buffer (pH 7.0) by incubating the enzyme with 1% soluble xylan at 50°C for 30 min. The reducing sugars formed were estimated by dinitrosalicylic acid reagent.

One unit of xylanase activity was defined as the amount of enzyme releasing one µmol of reducing sugar per min using xylose as standard. Protein estimation was carried out by the method of Bradford.

RESULTS AND DISCUSSION

Integration of the xylanase gene fragment in to the chromosome of alkalophilic thermophilic *Bacillus*:

The recombinant plasmid pATBX 6.5 containing the xylanase gene fragment from AT *Bacillus* was used in the present studies.This was done in order to keep excess homologous regions for the genetic recombinations. The pATBX 6.5 is a pUC8 derived *E. coli* vector, which cannot replicate in *Bacillus* sp., and does not have any other homologous region except the insert which may aid in its recombination with the genomic DNA of AT *Bacillus*. As a result of the transformation of AT *Bacillus* with pATBX 6.5, 1285 colonies developed on to the screening medium. These colonies merely reflect the number of cells that survived after the electroporation shock. No information regarding the number of transformants could be obtained due to the lack of selective pressure at this stage. However from the standard data on the electroporation mediated bacterial transformation (Dower *et al* 1988), around 80 % of the surviving colonies were expected to have received the plasmid; which might have been integrated or degraded - due to its foreign nature and absence of replicon - by the enzymes of alkalophilic thermophilic *Bacillus*. When the representative colonies from the transformants were screened for hybridization with the labeled pUC 8 DNA, fif-

teen showed positive hybridization test (Fig. 5.2A). It could be also possible that some of the integrants had been derived by recombination of the DNA fragment devoid of vector and hence did not have pUC integrated with the alkalophilic thermophilic *Bacillus* genome. Thus we might have missed few of the recombinants due to limitations in the screening method. In xylan congo red plate clearance assay 14 out of these 15 strains showed distinctly larger zones of xylan clearance whereas one strain (CII 14) showed smaller clearance zone as compared to AT *Bacillus* (Fig. 5.2B). One of the integrants CII 11, was selected for further amplification of xylanase gene copies. Gene multiplication was achieved by carrying out successive transformation of strain CII 11 with pATBX 6.5. These transformants could not be screened with pUC 8 as the CII 11 strain had already shown positive hybridization with pUC 8. The putative positive integrants were identified on the basis of xylan clearance and strain CIII6 was selected for further studies.

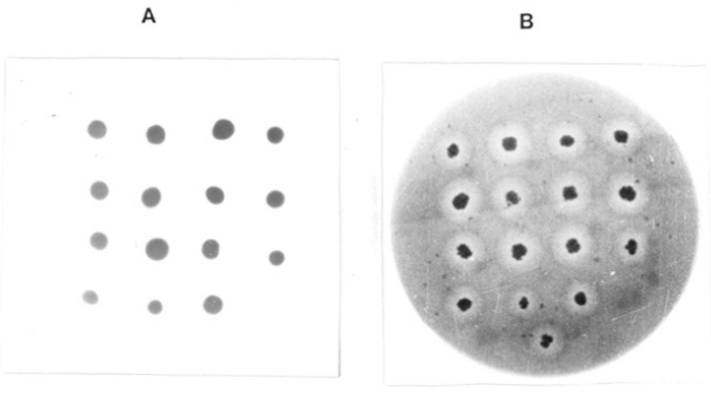


FIG. 5.2: (A) Integrants showing positive hybridization with labeled pUC 8.
Arrow indicates position of AT *Bacillus* colony as a negative control

(B) AT *Bacillus* and the integrants showing xylan plate clearance.

Characterization of the integrants:

The Hind III digests of genomic DNA from AT *Bacillus* and the two integrant strains CII 11 and CIII 6 were probed with labeled 6.5 kb xylanase gene fragment (fig. 5.3). A single band of hybridization was observed at 6.5 kb in the DNA digest of the AT *Bacillus*. However the restriction digest of integrant CII 11 showed two positive signals at 5.1 and 3.2 kb whereas CIII 6 showed three signals at 4.8, 3.2 and 2.9 kb. These results indicated addition of 1.8 and 4.4 kb DNA by gene integration in CII 11 and CIII 6 strains respectively. The strains CII 11 and CIII 6 also showed addition of 1 and 2 Hind III sites respectively, indicating that the mode of recombination was different than the Campbell type. The southern blot analysis revealed hybridization bands of varied sizes, and the mode of recombination is predicted to be of non campbell type. The mechanism of integration by the non - Campbell type recombination is illustrated in figure 5.4, This mode of recombination results in generation of tandem repeats which are known to be more prone to deletions, and a deletion in the essential region for enzyme expression may result in decrease or loss of enzyme activity. The strain CII 14 showed lower xylanase production than AT *Bacillus* (table 5.1), which may be due to deletion in the essential region of the xylanase gene.

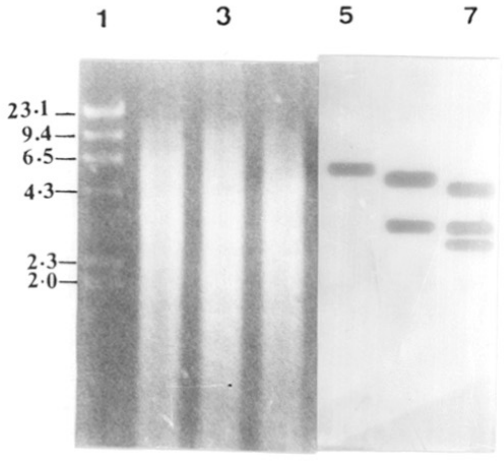


FIG. 5.3: Southern blot analysis

Hybridization of the genomic DNA samples of AT *Bacillus* and the integrants CII 11 and CIII 6 digested with Hind III and probed with 6.5 kb Hind III fragment coding for xylanases of AT *Bacillus*.

Lane 1: Lambda Hind III digest DNA molecular size marker.

Lane 2 - 4: Genomic DNA digests of AT *Bacillus* and the integrants CII 11 and CIII 6 respectively.

Lane 5 - 7: Positive hybridization signals of AT *Bacillus* and the integrants CII 11 and CIII 6 respectively.

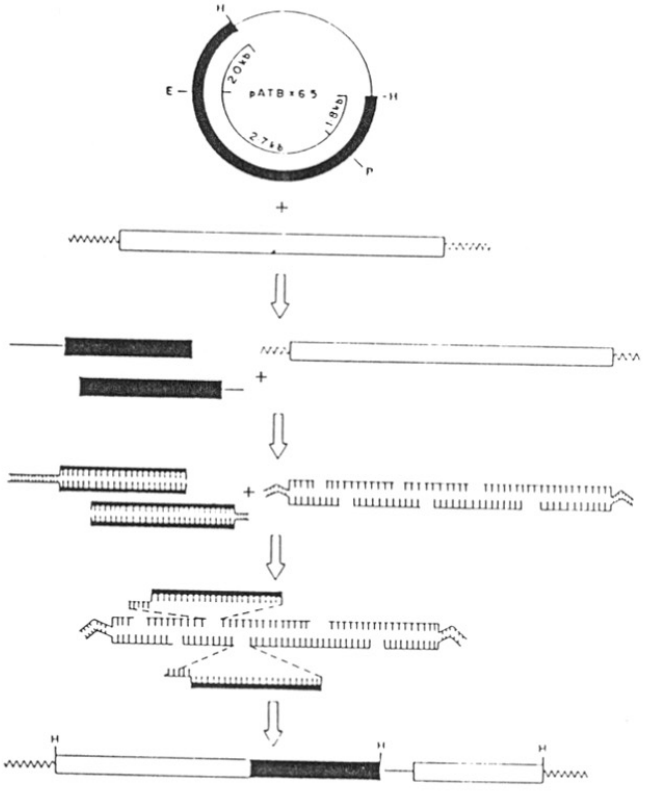


FIG. 5.4: Integration of the recombinant plasmid pATB X 6.5 with xylanase gene region of AT *Bacillus* genomic DNA.

Proposed mode of recombination is according to Yasbin *et al.* (1975).

——— pUC DNA.

▬▬ xylanase gene fragment from pATB X 6.5.

▭▭ AT *Bacillus* genomic xylanase fragment.

∧∧∧ AT *Bacillus* genomic DNA.

ıılıı ıı nicked genomic DNA of AT *Bacillus*.

ıılıılıılıı single stranded fragment containing a part of insert plus vector.

Xylanase production:

Alkalophilic thermophilic *Bacillus* showed low levels of xylanase activity (1.3 - 2.6 U/ml) in media containing 1% (w/v) glucose, lactose or xylose. Both the integrant strains showed elevated levels of xylanase activities (2.8 - 3.2 U/ml) which leveled off after 14 h fermentation. These results suggest that there is marked enhancement in the constitutive xylanase production by the integrants at least in the initial phase of fermentation.

In case of alkalophilic thermophilic *Bacillus* xylan is a poor inducer (Dey *et al* 1992) producing 6.2 /ml of xylanase activity. The integrant strains CII 11 and CIII 6 showed xylanase production of 10.5 and 12.0 U/ml respectively in 28 h of fermentation in a xylan containing medium. The maximum xylanase productivity (U/ml/h) also coincided with the maximum production (Fig. 5 A). AT *Bacillus* and the integrants CII 11 and CIII 6 showed maximum xylanase activity of 66, 96 and 128 U/ml respectively in a medium containing wheat bran in 20 h of fermentation period. The integrants showed maximum productivities at 16 h of fermentation time (Fig. 5 B). The integrants showed stable growth and xylanase production up to 10 slant to slant subcultures. The protein and the specific activity data (Table 1) of the extracellular xylanases of AT *Bacillus* and the integrants indicated that there are no significant differences in the sp. activities when

xylan medium was used. On wheat bran medium highest sp. activities were obtained at 16 h, which decreased later on due to increase in the protein synthesis and decrease in the xylanase productivity.

TABLE 5.1: Xylanase activity of AT *Bacillus* and the integrant strains.

Fermentation medium	Time (h)	Strain	Extra-cellular xylanase yield (U/ml)	Extra cellular protein yield (mg/ml)	Specific activity (U/mg) protein
Xylan-Yeast extract	28	AT *Bacillus*	6.1	0.290	21.03
		CII 11	10.4	0.375	27.73
		CIII 6	11.9	0.465	25.50
	48	AT *Bacillus*	6.6	0.760	8.86
		CII 11	8.8	1.000	8.80
		CIII 6	11.2	1.080	10.37
Wheat bran Yeast extract	16	AT *Bacillus*	46.0	0.325	141.55
		CII 11	83.0	0.480	172.91
		CIII 6	111.0	0.610	193.44
		CII 14	20.0	0.307	65.12
	20	AT *Bacillus*	66.0	0.380	173.68
		CII 11	95.0	0.540	175.92
		CIII 6	128.0	0.750	170.66
		CII 14	30.0	0.410	73.17
	48	AT *Bacillus*	51.5	1.333	38.63
		CII 11	77.5	1.550	50.00
		CIII 6	98.0	1.833	53.46
		CII 14	26.5	1.250	21.35

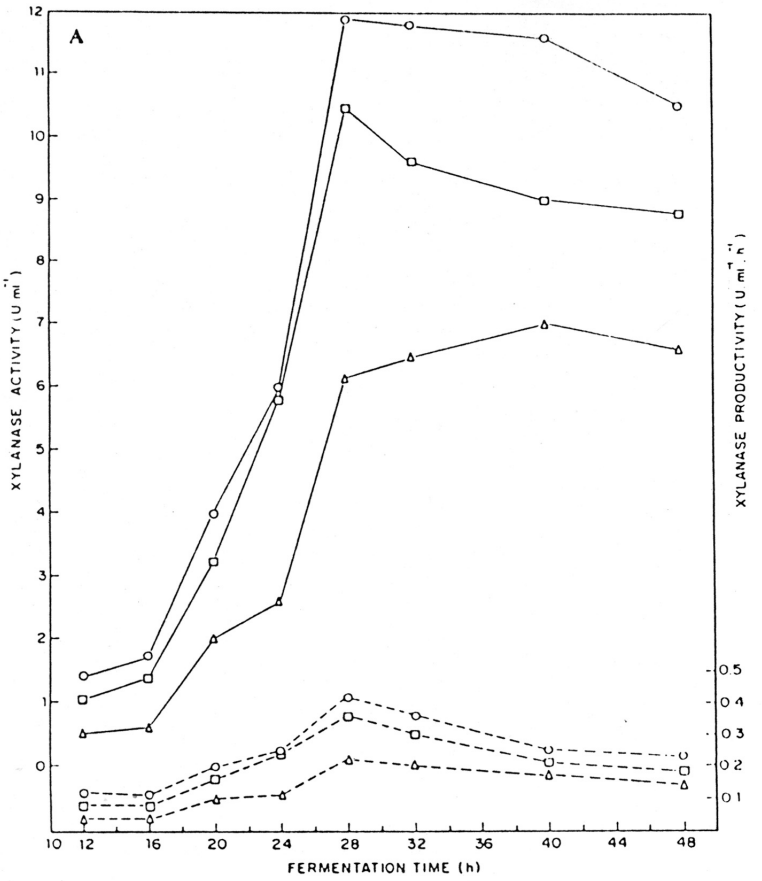


FIG. 5.5: Xylanase production of AT *Bacillus* and the integrants CII 11 and CIII 6

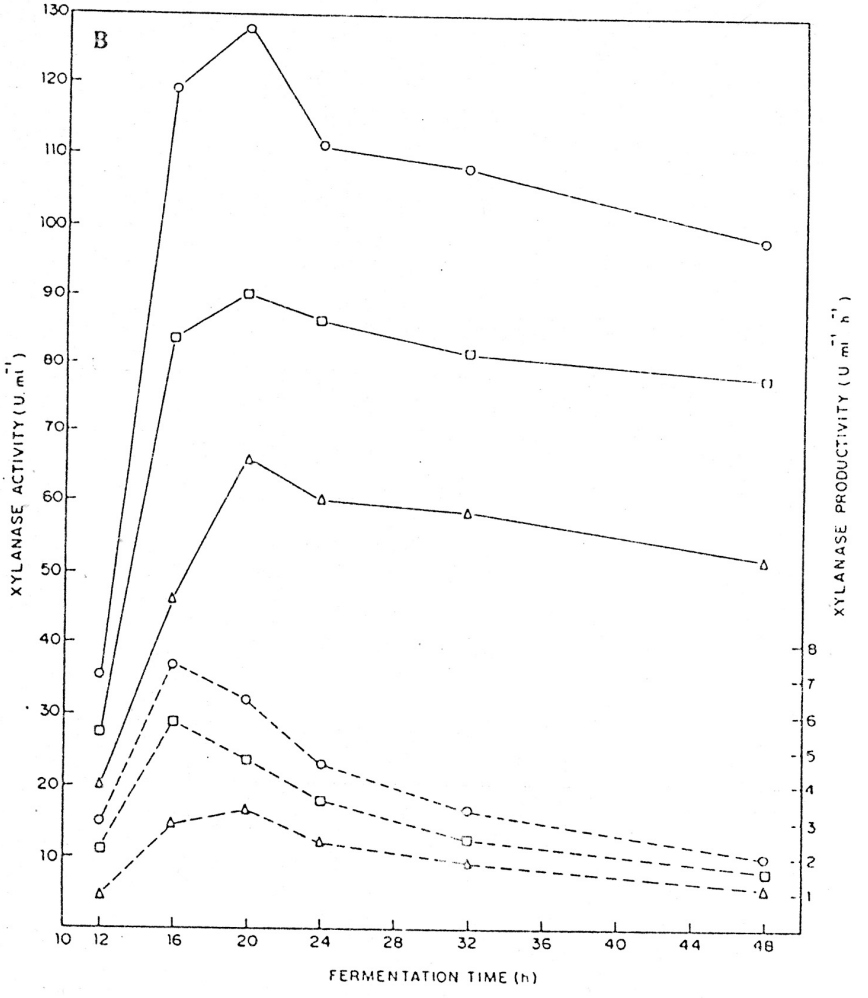
(A) using the media containing xylan and

(B) wheat bran as a sole carbon source

———— xylanase production (U/ml).

— — — — xylanase productivity (U/ml/h).

Δ AT *Bacillus*, □ strain CII 11 and ○ strain CIII 6


B
XYLANASE ACTIVITY (U.ml⁻¹)
XYLANASE PRODUCTIVITY (U ml⁻¹ h⁻¹)
FERMENTATION TIME (h)
130
120
110
100
90
80
70
60
50
40
30
20
10
8
7
6
5
4
3
2
1
10 12 16 20 24 28 32 36 40 44 48

The present studies demonstrated a simple method of gene amplification using pUC8 cloning vector. Gene integration may be advantageous in protein hyperproduction because of gene dosage effect (Kullio *et al* 1987, Albertini and Galizzi 1985)), efficient expression and stabilization of the protein product (Janniere *et al* 1985), and higher stability of the integrants as compared to plasmid bearing strains (Janniere *et al* 1985, Kullio *et al* 1987). The alkalophilic thermophilic *Bacillus* integrants seemed to show high stability and enhanced xylanases production which correlated with the gene dosage. Xylanase hyperproduction has been shown in case of *B. circulans* (Yang *et al* 1989) and *B. pumilus* (Panbangred *et al* 1985), but the productivity values reported are 0.29 and 0.11 U/ml/h respectively. Enhanced xylanase production has been achieved in alkaline *Aeromonas* (Kudo *et al* 1985), *Bacteroides ruminicola* (Whitehead *et al* 1989), *Caldocellum saccharolyticum* (Luthi *et al* 1990), *Fibrobacter succinogenes* 135 (Hu *et al* 1991) and *Streptomyces lividans* (Mondou *et al* 1986) using recombinant DNA techniques (TABLE 5.2). Although these recombinants showed 1.2-80 fold higher yields of xylanases as compared to the parent strains, the actual productivity values were in the low range of 0.1 -0.3 U/ml/h. The recombinant *Streptomyces* sp. showed maximum production of 2830 U/ml/28 which corresponds to 101.4 U/ml/h productivity. The CIII6 strain of AT *Bacillus* derived by gene integration showed stable growth and production of 7.4 U/ml/h xylanase activity. This may be biotechnologically important for large

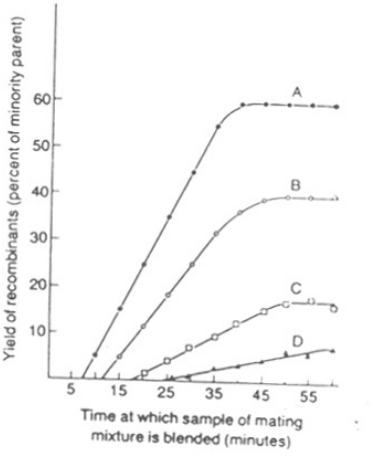
scale xylanase production and can be used for further gene amplification by the same method.

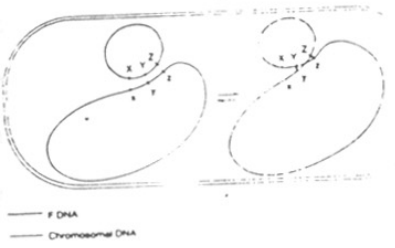
TABLE 5.2: ENMANCED EXPRESSION OF XYLANASES BY GENE CLONING

PARENT STRAIN	CLONING HOST	XYLANASE ACTIVITY	
		X PARENT	U/ ml/h
Aeromonas sp.	*E. coli*	80	0.1
Bacteroide ruminicola	*E. coli*	1.2	1.1×10^{-3}
Caldocellum saccharolyticum	*E. coli*	55	0.22
Bacillus circulans	*E. coli*	-	0.29
Bacillus pumilus	*B. subtilis*	3	0.11
Fibrobacter succinogenes (Strain # 135)	*E. coli*	5	0.09
Streptomyces Strain# 36a	*Streptomyces*	70	101.4
Streptomyces lividans	*S. lividans* TK 64	60	5.2

CHAPTER VI

GENETIC TRANSFER OF THERMOPHILIC TRAIT FROM ALKALOPHILIC BACILLUS TO E. COLI





Summary

The genomic library of alkalophilic thermophilic *Bacillus* constructed in *E. coli* was screened for a possible recombinant capable of growth at high temperature. A single recombinant [pATB 507 I] out of 10,000 colonies showed growth at 50^{o} C. The recombinant produced large (5 mm), opaque and mucilaginous colonies on LB. The IMViC biochemical tests suggested that the strain was *E. coli*, and scanning electron microscopy revealed increased cell length. The plasmid DNA from the thermophilic recombinant showed multiple bands. The cured strain showed ampicillin resistance (10 µg/ml) and presence of insert DNA in the genome, as revealed by southern blot analysis. The thermophilic trait was transformable with 10^{-4} frequency of expression amongst the pATB 507I transformants. The expression was invariably associated with multiple band pattern and hybridization of *E. coli* genomic DNA with labeled genomic DNA from alkalophilic thermophilic *Bacillus*, and suggested that the genetic recombination(s) of the insert DNA was an essential prerequisite for expression of thermophilic trait. The conjugative plasmid (RP4) mediated mobilization of thermophilic trait resulted in thermophilic conjugants after 2 h incubation. Southern blot analysis revealed that a single locus [5.6 kb Bam HI fragment] was involved in the transfer of thermophilic trait.

Introduction

Temperature is one of the most important environmental factor controlling the activities and evolution of the organisms. Not all temperatures are equally suitable for the growth and reproduction of living organisms, and it is therefore important to consider the optimum temperatures of the organisms (Brock 1967). In recent years the upper and lower temperature limits of growth of pure cultures of microorganisms have been extended at least to 110 ^{o}C and -14 ^{o}C respectively (Wiegel 1990). But there are no organisms which grow at both the extremities of the temperature limits and hence, the microorganisms are grouped according to their growth temperatures in to psychrophiles, mesophiles and thermophiles. The thermophiles have been further subgrouped depending on the growth temperature in to following classes (Wiegel 1990):

thermotolerants : opt.< 45^{o}C max > 50^{o}C

temperature tolerant thermophiles : < 25^{o}C - > 50^{o}C

temperature tolerant extreme thermophiles :< 45 - > 70^{o}C

extreme thermophiles :> 45 - > 70^{o}C

barothermotolerants :optimum temperature < 100 ^{o}C, but growing also above 100 ^{o}C.

barothermophiles : strictly > 100 ^{o}C.

There are some microorganisms that usually do not grow at higher temperatures, but may be made to grow at elevated temperatures by simple adaptations or mutations. Tsien *et al* (1980) have defined such organisms as cryptic thermophiles. The cryptic thermophiles are characterized as follows:

1) The organisms mainly posses thermostable enzymes, lipids, membrane components and means to stabilize their DNA at elevated temperatures.
2) Organisms that have the information to synthesize the thermostable isoenzymes and/or alter their lipids in to the thermostable form, but apparently are not using the information.

Both the classes of the cryptic thermophiles can not grow at elevated temperature as they lack one or two key components. The possibility of a greater number of strains belonging to the genera *Bacillus* and *Clostridium* being the cryptic thermophiles has been suggested (Wiegel 1990, Achenbach-Richter 1987).

Many advantages of the use of the thermophiles in the biotechnological processes have been proposed (Ayala 1986, Sonnleitner and Fiechter 1983, Wiegel and Ljungdahl 1986). They include :

1) relatively higher growth rates and faster reaction times.

2) reduced risk of contamination

3) reduced cooling costs of operating large fermentations

4) reduction in the energy requirement for agitation because of the lower viscosity at elevated temperature.

5) increase in the ionization and solubility leading to higher mass transfer coefficients

6) pathogen free operations

7) ease of product recovery - especially of those recovered by distillation or volatilization

In spite of the biotechnological potentials of the thermophiles, the studies on the molecular mechanisms of thermophily have largely confined to analysis of upper temperature of growth and its extension using simple mutations. There are very few reports dealing with the identification and / or transfer of the genes governing thermophily possibly because the ways in which the various organisms have adapted to respond to changes in the temperature are numerous (Edward 1989), eventhough the underlying principles are the same. Genes responsible for the growth at or around the upper temperature have been identified in *E. coli* (Dean and Richard 1991, Neidhardt *et al* 1983). Intrageneric transfer of thermophilic trait has been studied in *Bacillus* using the genomic DNA transformation approach. Involvement of thermal adaptation genes possibly located at two distinct loci in the transformable thermophilic trait has been proposed for *Bacillus* sp.(Droffner and Yamamoto 1985, Lindsay and Creaser 1875, McDonald and Matney 1963). But there is no abundant evi-

dence for the occurrence of the thermal adaptor genes and thermophily is presently regarded as an intrinsic property of the corresponding microorganism (Wiegel 1990).

In the present chapter we report for the first time an intergeneric transfer of thermophilic trait from an alkalophilic thermophilic *Bacillus* to *E. coli* using gene cloning approach. A thermophilic recombinant showed growth at 50 ^{o}C produced distinctly different colonies than those of host *E. coli*. Biochemical, genetic and electron microscopic analysis of the thermophilic recombinant confirmed that the strain belongs to genus *Escherichia*. Involvement of a single genetic locus in the transformable thermophilic trait was confirmed by Southern blot analysis. The recombination(s) of the insert DNA from *Bacillus* with *E. coli* genome was demonstrated, which is an essential prerequisite for expression of the thermophilic trait.

Materials and Methods

Bacteria and Culture Media:

Alkalophilic thermophilic *Bacillus* (NCIM 59) and *E. coli* JM strains JM 105, JM 109, and HB 101 were used through out the present studies. The genotype of the *E. coli* HB 101 strain used (Boyer and Roulland-Dussoix 1969) is given below:

E. coli HB 101 : F^- **(gpt-proA)62 leu supE44 ara14 galK2 lacY1*

**(mcrC-mrr) rpsL20*(Str^r) *xyl-5 mtl-1 recA13*

The antibiotics were procured from the Sigma chemicals USA and were used as specified. The biochemical tests for indole and acetoin production, lactose fermentation, and utilization of citrate as a sole source of carbon were performed as described in Bergey's manual (1984).

Electron Microscopy:

Cells grown in LB were mixed with equal volume of gluteraldehyde (6% v/v) and $CaCl_2$ was added in 0.01 % (w/v) final concentration. The suspension was gently mixed and allowed to stand on ice for 1 h. The cells were centrifuged and the pellet was washed thrice with 100 mM phosphate buffer (pH 7.0). The cells were finally suspended in the buffer and spread on aluminium foil. The foil was air dried and the sample was subjected to scanning electron microscopy.

DNA Manipulations:

The genomic library was constructed in pUC 13 using 2 - 6 kb size fractionated Bam HI digest of the genomic DNA of alkalophilic thermophilic *Bacillus*. The details of the protocols for the genomic library construction were exactly the same as described in the previous chapter (chapter 2).

Plasmid Curing Transformation and Conjugation:

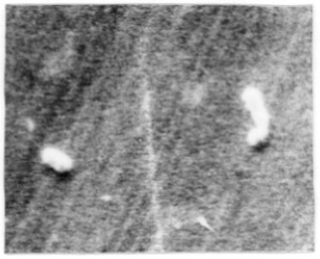
Plasmid transformation was carried out using competent cells prepared by $CaCl_2$ method. Cured strains were obtained from the cultures grown without the selective pressure. Conjugation was performed by broth mating (Yanish - Perron *et al* 1985). The strains for conjugation were grown for 12 - 16 h. The cells were mixed and centrifuged at low speed (1,000 rpm) for 5 min. The resulting pellet was recovered by removing the supernatent carefully. The conjugation was allowed to take place in the pellet for time intervals varying from 5 min to 2 h. The conjugation was arrested by mixing the pellet with fresh LB medium and vortexing. The cells from the suspension were then plated out on the appropriate media to select for the conjugants.

Results

Isolation and Characterization of the Thermophilic Recombinant:

Out of 10,000 recombinants plated out on LB containing ampicillin (100 µg/ml), one showed growth at 50^{o}C. The colony was mucoid and opaque and the diameter after 16 h growth was 5 mm. The cells were Gram negative coccobacilli which produced indole, but did not produce acetoin or utilize citrate as a sole carbon source. Lactose was not fermented. The scanning electron microscopy of the thermophilic recombinant revealed that the recombinant cell size was larger than that of the host *E. coli* JM 105 (FIG 6.1). The plasmid isolation from the thermophilic recombinant yielded a unique multiple band pattern. The thermophilic recombinant designated as pATB 507 I was used for the further studies.

A



B

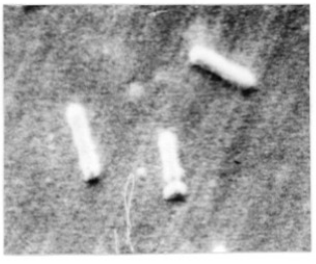


FIG 6.1: Scanning electron micrograph (a) *E. coli* JM 105 and (b) the thermophilic recombinant. The 16 h grown cells were fixed using gluteraldehyde and spread on aluminium foil. The dry cell layer was subjected to SEM at 10 kV, 15,000 X. The bar (⌊⌋) represents 1 µm.

Genetic analysis of the recombinant pATB 507 I:

The plasmid isolated from the thermophilic recombinant (pATB 507 I) was used to transform *E. coli* JM 105 and 109. Both the strains accepted and stably maintained the plasmid. The recombinant was grown in absence of ampicillin for 16 h and the cells were plated on LB with or without ampicillin to select spontaneously cured cells. One hundred and thirty cured colonies were obtained from three experiments, but all of them were resistant to 10µg/ml ampicillin whereas the host *E. coli* JM 105 was sensitive to 5 µg/ml antibiotics. The cured cells of the thermophilic recombinant showed growth at 50 ^{o}C. The cured cells were made competent and used to check transformability of pUC 8 and pBR 322. The plasmids were transformed with 10^5 cfu/µg efficiency and were stably maintained.

Transformation of the thermophilic trait in *E. coli*:

The *E. coli* JM 105 and 109 cells transformed with pATB 507 I plasmid were plated out on LB plates containing ampicillin (100 µg/ml) and incubated in two sets respectively at 37 and 50 ^{o}C. The transformants were scored at 37 ^{o}C but no colony developed at 50 ^{o}C. The transformants were allowed to grow at 37 ^{o}C for 16 h and the resulting colonies were then replica plated and incubated at 50 ^{o}C. The transformants from both JM105 and 109 strains showed 10^{-4} frequency of expres-

sion of the thermophilic trait. When the plasmid DNA from pATB 507 I transformants expressing thermophilic trait was analyzed by agarose gel electrophoresis, multiple band pattern was observed. The transformants unable to express thermophilic trait did not show multiple band pattern.

Mobilization of thermophilic trait:

The spontaneously cured *E. coli* JM 109 (Amp^R) and HB 101 (Str^R) were used as donor and recipient strains respectively. The mobilization was mediated through RP4 (Kan^R), which is a conjugative plasmid of *E. coli* (Achenbach - Richter *et al* 1987). The conjugants were selected on LB plates containing ampicillin (100 µg/ml), kanamycin (15 µg/ml) and streptomycin (15 µg/ml). The conjugants obtained after 2 h of incubation expressed thermophilic trait (growth at 50 ^oC) and preincubation at 37 ^oC was not necessary.

Analysis of the recombination:

In order to analyze the possible mode of recombination of plasmid pATB 507 I, *E. coli* JM 109 cells (pATB 507 I) were transformed with RP 4. The total plasmid DNA was isolated after three successive subcultures and overnight incubations under selective pressure. The RP 4 plasmid was recovered from the agarose gel and used for southern hybridization with labeled pATB 507 I. The isolated RP 4 plasmid failed to hybrids with labeled pATB 507 I in the subsequent southern blot analysis.

Southern blot analysis:

Southern blot analysis of the total DNA from thermophilic recombinant, probed with labeled genomic DNA from alkalophilic thermophilic *Bacillus* showed positive signals for plasmid as well as genomic DNA. The transformants of the plasmid pATB 507 I which did not express thermophilic trait, showed positive signals corresponding to the plasmid bands. No signal was obtained for the genomic DNA of these transformants and that of the host *E. coli* JM 105 (FIG 6.2).

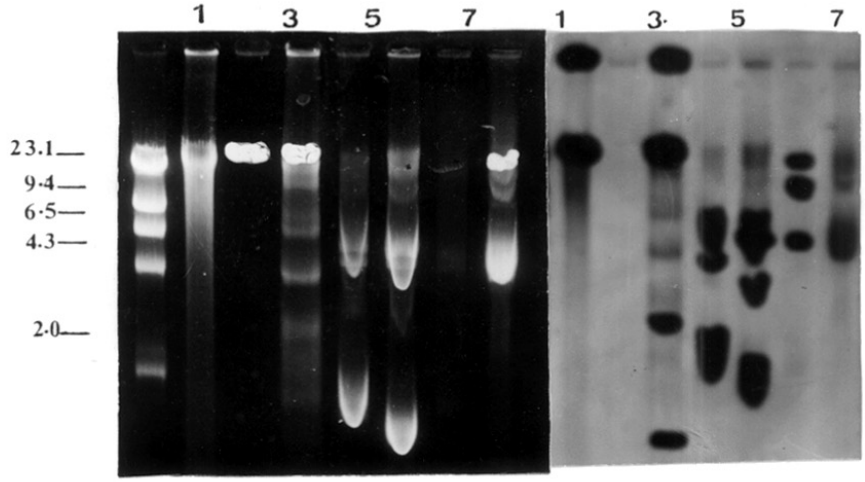


FIG 6.2: Southern blot analysis of the thermophilic recombinant. The genomic DNA from alkalophilic thermophilic *Bacillus* was labeled using α P 32 dATP (3,000 Ci/ mM) with the random primer labeling kit (Boehringer). The hybridization and washing was carried out at 60 ^{o} C. The final washing was at highest stringency conditions (0.1 X SSC with 0.1 % SDS). (a) Gel electrophoresis of 1: Lambda DNA Hind III digest, 2: genomic DNA from cured thermophilic recombinant, 3: *E. coli* JM 105 genomic DNA, 4: Plasmid bearing thermophilic transformant, 5-8: pATB 507I transformants not expressing the thermophilic trait.

(b) Southern blot autoradiogram showing 1: genomic DNA from cured thermophilic recombinant, 2: *E. coli* JM 105 genomic DNA, 3: Plasmid bearing thermophilic transformant, 5-8: pATB 507I transformants not expressing the thermophilic trait.

Bam HI digested genomic DNA of alkalophilic thermophilic *Bacillus* was probed with labeled pATB 507 I, and a single hybridization signal was obtained at 5.6 kb (data not shown).

DISCUSSION:

A distinct DNA fragment from alkalophilic thermophilic *Bacillus* conferred thermophilic trait of growth at 50 ^{o} C to *E. coli*. Biochemical, genetic and electron microscopic evidence clearly demonstrated that the strain was an *E. coli*. This represented a unique example of interspecific transfer of thermophilic trait involving a single defined genetic locus. The mucoid colony character and the altered cell size of the thermophilic recombinant may be because of altered expression of lon (Maurizi 1985) or defective cell wall synthesis (Yanish - Perron *et al* 1985).

Multiple band pattern of the plasmid observed in the strain that expressed thermophilic trait, along with the ampicillin resistance (10 µg/ml) of the cured strain and hybridization of the genomic DNA of thermophilic *E. coli* with that of alkalophilic thermophilic *Bacillus*; clearly demonstrated involvement of genetic recombination(s) and rearrangements. The fact that these characteristics were uniquely associated with the cells expressing the thermophilic trait indicate the recombination event(s) could be an essential prerequisite for the expression of the thermophilic trait. This observation was consistent with the previous work on homologous expression of the cloned htp^{R} gene from *E. coli* where recA mediated homologous recombination between the cloned gene and the chromosomal DNA resulted in the expression of the htp^{R} coded character i.e. growth at 42 ^{o}C (Neidhardt *et al* 1983).

The frequency of obtaining the expression of the thermophilic trait amongst the pATB 507 I transformants was identical in *E. coli* JM 105 and 109, irrespective of the differences in their genotype especially with respect to the recombination genes such as mcrA, recA, sbc B15, and gyr 96 (Yanish-Perron *et al* 1985). The results indicated that the genetic recombinations of the thermophilic trait marker was independent of the recombination pathways involving the above mentioned genes. Since the marker transfer between the plasmid pATB 507 I and RP 4 was not detected, the possibility of involvement of transposon could be ruled out. The plasmid replication is known to take place via theta (FIG: 6.3a) or sigma (FIG: 6.3b) mode of replication, which may not directly explain the genetic recombinations observed in the present studies. Also it is possible that the replication machinary is non functional at high temperature. We suggest that the recombination(s) could be taking place via recombination mediated plasmid replication (Viret *et al* 1991), which is diagramatically represented in figure 6.3 (c) along with the ordinary theta (4a) and sigma (4b) modes.

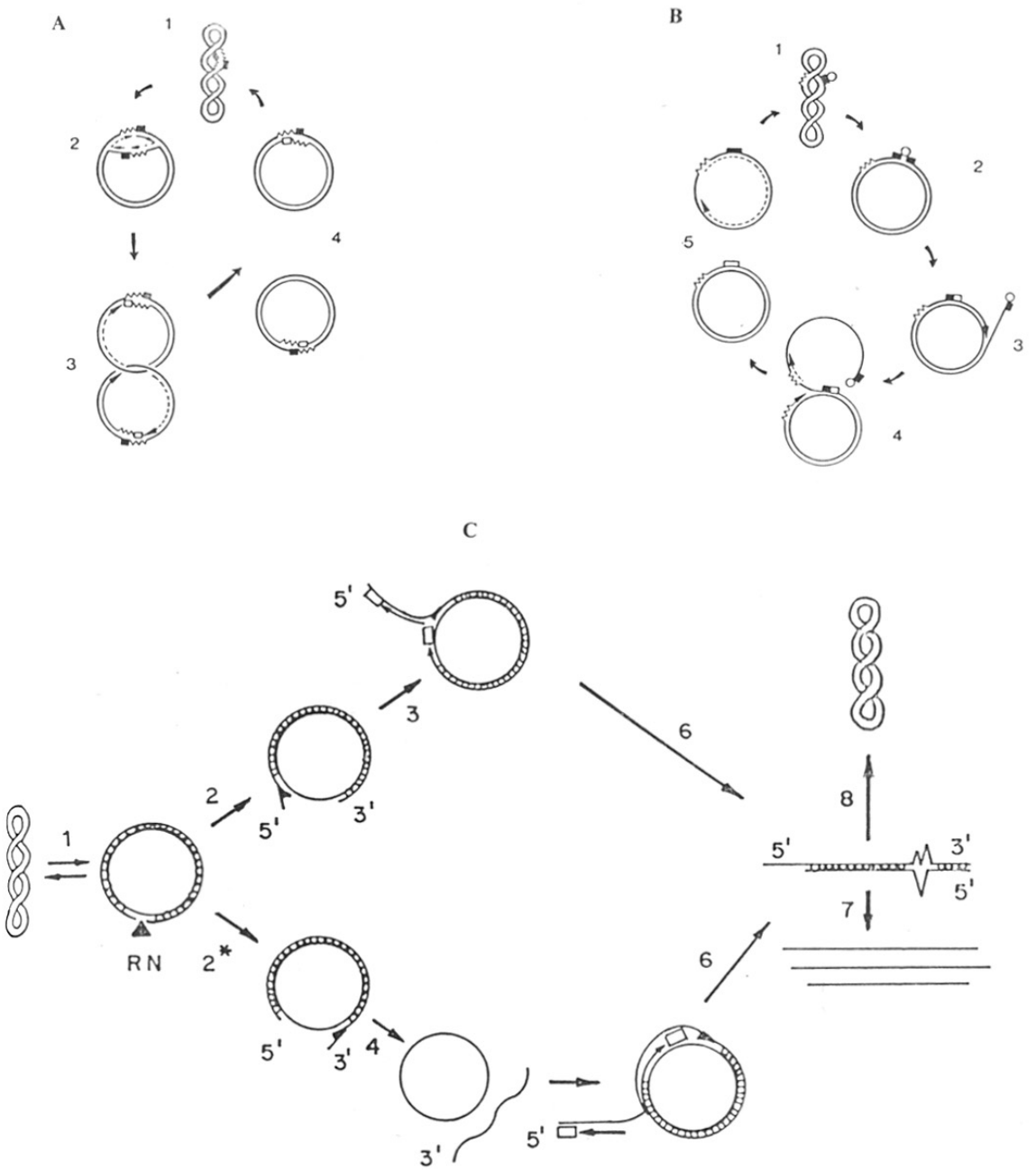


FIG 5.4: Mode of replication of the plasmid DNA:

a) theta mode b) sigma mode

c) recombination dependent concatemeric plasmid replication.

The replication via a new heat inducible recombination pathway independent of Rec A has been reported by Delaney (1990) for thermotolerant *E. coli*. The thermophilic recombinants could also be replicaing by a similar mode. In order to grow at high temperature, a mesophile must posses thermostable cell membrane and protein synthesis machinery, as well as enzymes that are active at high temperature (Brock 1967). Since these properties are coded by distinct genetic loci, it is generally believed that several genes need to be transferred to convert a mesophile in to a thermophile (McDonald and Matney 1967, Lindsey and Creaser 1975, Wiegel 1990). Our results seem to be contradictory to this belief. But, in ac cordance to the theory of thermophilic origin of present day organisms (Achenbach-Richter *et al* 1987, Brock 1967), we believe that some of the genetic information needed for growth at high temperature is available in the genome of the present day organisms. Earlier reports on *E. coli* pointing out existence of machinery for stabilization of the cell components at 50 ^{o} C (Delaney 1990) and on the archaebacterial ATPase related protein which may have role in high temperature adaptation (Phipps *et al* 1991), support our belief. The cloned fragment could be a high temperature regulon from AT *Bacillus* which is functional in *E. coli*. Since the frequency of expression of thermophilic trait amongst the pATB 507I transformants is low, alternative possibility of modification of one or more genetic loci with the help of specific recombinations of the insert needs to be considered. In

the earlier work on *Bacillus* involving transformation of genomic DNA as a tool to transfer thermophilic trait (Drofner and Yamamoto 1985), thermophilic transformants were obtained with a lower marker transfer frequency (10^{-7}) as compared to that for single marker transfer (10^{-3}) and the conclusion was drawn to effect the presence of two unlinked loci. As we note, the transformation of genetic material that codes for thermophilic trait and the phenotypic expression of thermophily are separated by event of genetic recombination(s) which occurs at a frequency of 10^{-4}. The low marker transfer frequency can thus be explained on the basis of genetic recombination(s). The role of recombinations has also been demonstrated in the phenotypic expression of htp^{r} temperature regulon of *E. coli*. Hence we propose that the requirement of genetic recombination(s) for expression of thermophilic trait may be a rational phenomenon. Further studies on the role and mechanism of recombinations in the expression of the thermophilic trait could be of particular importance for our understanding of the early evolution of prokaryotes, relation between structure and function of the cell components and enzymes, and the evolution of strategies for survival at the extremes of environmental conditions. The studies may lead to the identification of genetic routs for developing thermophilic strains, which is of enormous biotechnological significance.

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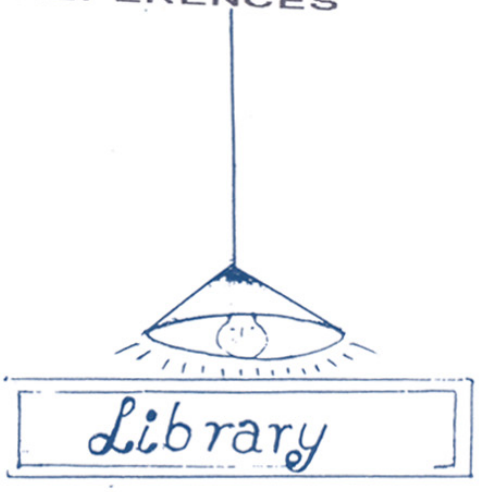

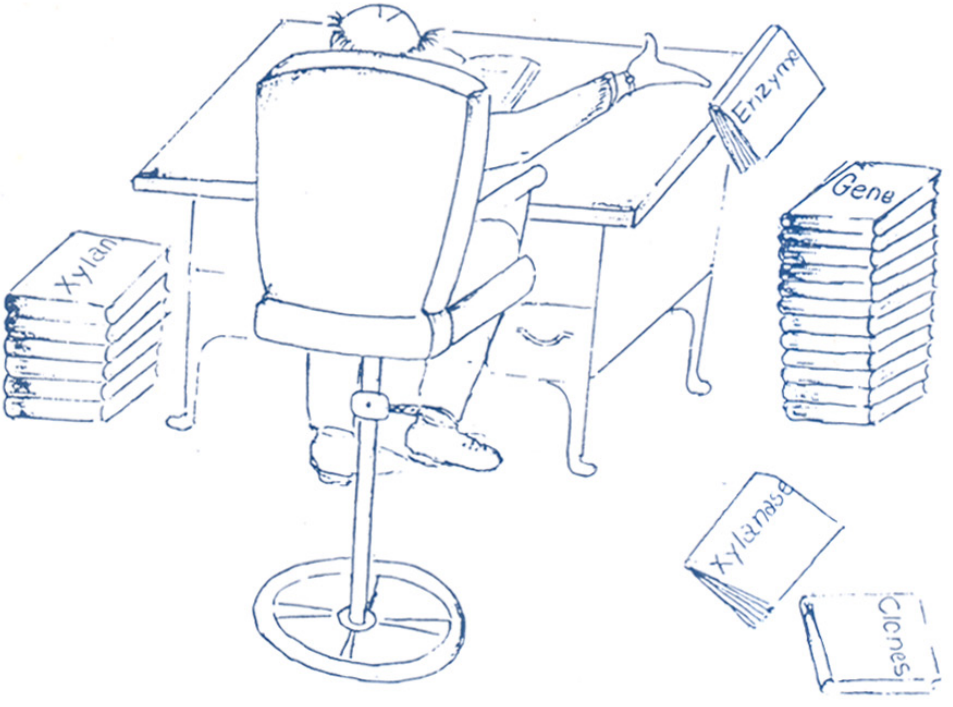

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REPRINTS OF THE PUBLISHED PAPERS

Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp.[1]

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DEY, D., HINGE, J., SHENDYE, A., and RAO, M. 1992. Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp. Can. J. Microbiol. 38: 436–442.

An alkalophilic thermophilic *Bacillus* sp. (NCIM 59) isolated from soil produced two types of cellulase-free xylanase at pH 10 and 50°C. The two enzymes (xylanase I and II) were purified to homogeneity by ethanol precipitation followed by Bio-Gel P-10 gel filtration and preparative polyacrylamide gel electrophoresis. The molecular weights of xylanase I and II were estimated to be 35 000 and 15 800, respectively, by sodium dodecyl sulfate gel electrophoresis. The enzymes exhibited immunological cross-reactivity and were glycoproteins. They had similar temperature (50–60°C) and pH (6) optima. Both xylanases were stable at 50°C at pH 7 for 4 days. However, xylanase I was comparatively more stable than xylanase II at 60°C. The isoelectric points of xylanase I and II were 4 and 8, respectively. The apparent K_m values, using xylan as substrate, were 1.58 and 3.5 mg/mL, and V_{max} values were 0.0172 and 0.742 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. Both xylanases were inhibited by *N*-bromosuccinimide, suggesting the involvement of tryptophan in the active site. The hydrolysis patterns demonstrated that the xylanases were endoenzymes. Xylanase I and II yielded mainly xylobiose, xylotriose, and higher xylooligosaccharides, with traces of xylose from xylan.

Key words: cellulase-free xylanase, alkalophilic thermophilic *Bacillus* sp., enzyme purification, characterization.

DEY, D., HINGE, J., SHENDYE, A., et RAO, M. 1992. Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp. Can. J. Microbiol. 38 : 436–442.

Une souche alcalophile thermophile de *Bacillus* sp. (NCIM 59) isolée du sol produit à pH 10 et à 50°C deux types de xylanase exempte de cellulase. Les deux enzymes (xylanase I et II) ont été purifiées jusqu'à homogénéité par précipitation à l'éthanol suivie d'une filtration sur du Bio-Gel P-10 et d'une électrophorèse préparative sur gel de polyacrylamide. L'électrophorèse sur gel de dodécyl sulfate de sodium a permis d'établir que les poids moléculaires des xylanases I et II étaient de 35 000 et 15 800, respectivement. Ces enzymes sont des glycoprotéines et présentent une réactivité immunologique croisée. Elles ont toutes deux la même température optimale (50–60°C) et le même pH optimal (6). Ces deux xylanases demeurent stables pendant 4 jours à 50°C et pH 7. À 60°C par contre, la xylanase I est comparativement plus stable que la xylanase II. Les pI des xylanases I et II sont de 4 et 8, respectivement. Lorsque la xylane est utilisé comme substrat, les valeurs de K_m sont de 1,58 et 3,5 mg/mL respectivement et celles de la V_{max} de 0,0172 et 0,742 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Ces deux xylanases sont inhibées par le *N*-bromosuccinimide suggérant ainsi la présence de tryptophane au site actif. Les profils d'hydrolyse confirment que ces xylanases sont des endoenzymes. À partir du xylane, les xylanases I et II produisent surtout du xylobiose, du xylotriose, des xylooliogosaccharides supérieurs et des traces de xylose.

Mots clés : xylanase exempt de cellulase, *Bacillus* sp. alcalophile thermophile, purification enzymatique, caractérisation enzymatique.

[Traduit par la rédaction]

Introduction

Xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) catalyze the hydrolysis of xylan to xylooligosaccharides and xylose, which can be useful feedstock for food and fuel. Xylan is the major component of hemicellulose, which includes, in general, the noncellulosic polysaccharides of plant cell walls (Timell 1967). The use of xylanases in conjunction with cellulases for the complete conversion of cellulosic biomass to sugars is widely studied (Biely 1985), and it can greatly improve the overall economics of the processing of the lignocellulosic biomass (Flickinger *et al.* 1980). Large quantities of xylan are released as effluents in the paper and pulp industries, and their bioconversion may be of economic significance. Recently, the use of cellulase-free xylanases for the selective hydrolysis of the hemicellulose components in paper and pulp has received consideration (Jurasek and Paice 1986).

Xylanases occur widely in bacteria, actinomycetes, and fungi (Wong *et al.* 1988). Cultivation of microorganisms under conditions of high temperature and pH might be considered advantageous in preventing contamination. Thermally stable xylanases can be used at high temperatures for the efficient hydrolysis of xylan and may also be effectively recycled. Many reports of xylanases from thermophilic or alkalophilic microorganisms are available (Grüninger and Fiechter 1986; Bérenger *et al.* 1985; Honda *et al.* 1985), but very few are from alkalophilic and thermophilic organisms (Okazaki *et al.* 1984). Cellulase-free xylanases active at alkaline pH may be particularly useful for the treatment of alkaline pulps. The present paper reports the isolation of an alkalophilic thermophilic *Bacillus* sp. that produces cellulase-free xylanase (Hinge *et al.* 1989). The purification and properties of the enzymes are also discussed.

Materials and methods

Larch wood xylan, polyacrylamide gel electrophoresis (PAGE) reagents, *N*-bromosuccinimide, carboxymethylcellulose (sodium salt), and lichenan were obtained from Sigma Chemical Co.,

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TABLE 1. Effect of carbon sources on production of xylanase at pH 10 and 48-50°C

Carbon source	Concn. in medium (%)	Xylanase activity (U/mL)
D-Xylose	1	0.44
D-Glucose	1	1.30
Xylan	1	3.00
Wheat bran	5	50.00

St. Louis, Mo., U.S.A. Laminarin was from Koch-Light, England, Bio-Gel chromatographic materials were from Bio-Rad (U.S.A.), ampholytes were from Pharmacia (Sweden), and yeast extract and peptone were from Difco Laboratories. The xylooligosaccharide standards were kindly provided by Professor Peter Reilly, Iowa State University. All other reagents used were of analytical grade.

Sceening and isolation of alkalophilic thermophilic Bacillus

The soil sample from a hot-water spring (Vajreshwari District, Thane), Bombay, India, was suspended in sterile water and was spread on alkaline agar plates (yeast extract, 10 g; agar, 10 g; wheat bran, 50 g; Na_2CO_3, 35 g; water, 1 L; final pH 10). Sodium carbonate was sterilized separately prior to addition to the medium. The plates were incubated at 50°C for 48 h. About 2000 colonies developed, and they were transferred onto alkaline xylan plates (xylan, 10 g; yeast extract, 10 g; Na_2CO_3, 10 g; agar, 15 g; water, 1 L) and incubated at 50°C. One strain, designated as alkalophilic thermophilic *Bacillus*, showed clearance on the xylan plate and was thus identified as a potent producer of xylanase.

Bacillus sp. was grown on liquid Luria-Bertani medium in the pH range 7-11, adjusted with sodium carbonate-bicarbonate buffer. Growth at different temperatures and pH was monitored by absorbance measurements at 600 nm.

Characterization and identification of bacteria

Morphological properties and taxonomic characteristics of the bacteria were studied according to methods in *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1974) and those described for the genus *Bacillus* by Gordon *et al.* (1973).

Determination of base composition

Chromosomal DNA was purified according to the method of Chater *et al.* (1982). The DNA composition (G + C content) was calculated from the thermal denaturation temperature as described by Mamur and Doty (1962).

Enzyme assays

Xylanase was assayed by mixing the enzyme with 0.5 mL of 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 dinitrosalicyclic method (Miller 1959), using D-xylose as standard. The xylan (2 g) substrate was prepared by suspending in 100 mL of 50 mM pH 7.0 sodium phosphate buffer, followed by stirring for 16 h at 30°C. The insoluble fraction was removed by centrifugation and the soluble fraction was used for xylanase assay. One unit (U) 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 Lowry *et al.* (1951) or to Bradford (1976); using bovine serum albumin as standard. Total carbohydrate was measured by the phenol-H_2SO_4 method, as described by Dubois *et al.* (1956), using glucose as standard.

Activity towards different substrates

Ten milligrams of substrate carboxymethylcellulose (sodium salt), filter paper (25 mg, Whatman No. 1), lichenan, gum arabic, or laminarin was incubated with a suitably diluted enzyme in a total volume of 1 mL in 50 mM phosphate buffer, pH 7, at 50°C for 30 min. The red ng sugars formed were estimated by the dinitrosalicyclic acid method. Activity towards 4-nitrophenyl-β-D xyloside (PNPX) or glucoside (PNPG) was determined by incubating 1 mL of 0.3% substrate with 0.1 mL of suitably diluted enzyme in 50 mM phosphate buffer, pH 7, for 30 min at 40°C. The liberated *p*-nitrophenol was measured at 400 nm (Mishra *et al.* 1984).

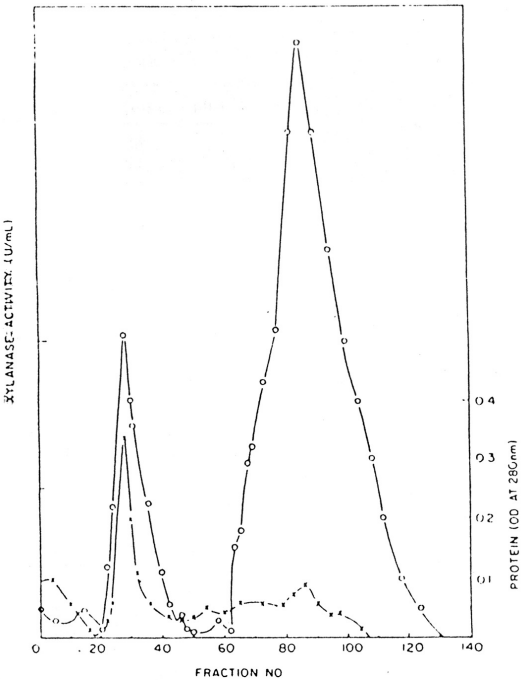

FIG. 1. Fractionation on Bio-gel P-10 of the alcohol-precipitated protein from the culture filtrate of an alkalophilic thermophilic *Bacillus* isolate. Fractions of 2.5 mL were collected. ○, xylanase activity; ×, absorbance at 280 nm.

Effect of N-*bromosuccinimide on xylanase activity*

Xylanase (0.1 mg) in 0.05 M sodium acetate buffer (pH 6) was incubated with *N*-bromosuccinimide (1 mM) for 10 min. The residual activity was estimated as described earlier.

Enzyme production

Enzyme was produced in 250-mL flasks with 50 mL medium containing washed wheat bran (5%) and yeast extract (1%). The inoculum (10%) was grown in the same medium (but with only 1% wheat bran) for 18 h. The pH of the medium was initially adjusted to 10 with 1% Na_2CO_3, and the culture was grown for 48 h at 48-50°C.

Purification of the enzyme

All steps were carried out at 4°C unless otherwise specified. Polyacrylamide gel electrophoresis was performed as described by Ornstein (1964).

Molecular weight determination

The polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) for purified xylanases was performed in slab gels according to the method of Laemmli (1970), using 10% gels. Molecular weight markers used were lysozyme (14 300), carbonic anhydrase (18 500), ovalbumin (45 000), and bovine serum albumin (66 000). The molecular weight of the purified enzymes was also determined by

TABLE 2. Purification of xylanases

Steps	Volume (mL)	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Fold purification
Culture supernatant	100	30	2.0	15	1.0
Alcohol precipitate	5.0	275	3.0	91	6.0
Bio-Gel P-10					
Xylanase I	1.0	30	0.6	50	3.0
Xylanase II	1.0	160	0.6	267	18.0
PAGE					
Xylanase I	0.4	9.0	0.1	120	8.0
DEAE-cellulose					
Xylanase II	2.0	30	0.1	300	20.0

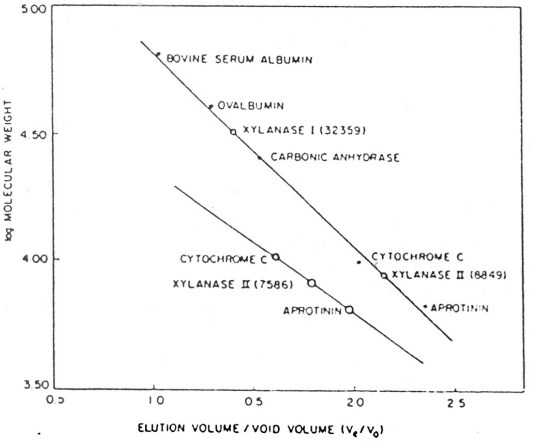

FIG. 2. Determination of molecular weights by Bio-Gel filtration. *, xylanase I and II on Bio-Gel P-100; ○, xylanase II on Bio-Gel P-10, with standard markers.

gel filtration on Bio-Gel P-100 and P-10 columns. The markers used were aprotinin (6 500), cytochrome *c* (12 400), carbonic anhydrase (29 000), and albumin (66 000). The molecular weights of the xylanases were calculated by regression analysis.

Preparation of antiserum

Antiserum against xylanase II was obtained from rabbits immunized with the purified xylanase II (0.5 mg) mixed with the complete Freund's adjuvant. Three booster injections (0.5 mg each) were given in the same manner after 6 weeks, and the serum was collected 1 week after the last injection. The immunodiffusion test (Oüchterlony 1949) was carried out in 0.05 M phosphate buffer, pH 7.0, containing 1.0% agarose.

Isoelectric focussing

Analytical isoelectric focussing in polyacrylamide gel was carried out according to Vesterberg (1972). The ampholyte range used was pH 3.5–10.

Determination of K_m *and* V_{max}

Suitably diluted xylanases I and II were incubated with different amounts of xylan (2–10 mg) under the assay conditions described. K_m and V_{max} were determined from Lineweaver–Burk plots.

Hydrolysis of xylan

The hydrolysis was carried out in stoppered tubes with 1 U of xylanase and 25 mg of xylan in 0.05 M phosphate buffer, pH 7, in a reaction mixture volume of 0.5 mL at 50°C. The aliquots were removed at different time intervals, and the reducing sugars were assayed by the dinitrosalicylic assay method, using xylose as standard. Percentage hydrolysis was calculated as shown below, and it was multiplied by a factor of 0.9 to account for the addition of water molecule during hydrolysis.

$$\% \text{ hydrolysis} = \frac{\text{reducing power as xylose}}{\text{weight of xylan}} \times 100 \times 0.9$$

Determination of xylan degradation products

Xylanase I or II (1 U) was incubated with xylan (5 mg) in 0.05 M phosphate buffer, pH 7.0, at 50°C for 24 h in a volume of 0.1 mL. The aliquots at different time intervals were analysed for the sugar products formed by paper chromatography in a solvent system containing butanol – acetic acid – water (3:1:1). The paper chromatograms were sprayed as described by Trevelyan *et al.* (1950).

Results and discussion

Characteristics of the Bacillus *isolate*

The isolated strain was an aerobic, gram-positive, motile, spore-forming, rod-shaped bacterium (6 × 0.5 μm). On alkaline nutrient agar at 50°C the colonies were butyrous, glistening, and pale cream coloured. The strain showed negative reactions for production of indol, hydrogen sulfide, ammonia, and urease but positive reaction for hydrolysis of starch, for the Voges–Proskuaer test, for production of catalase, for hydrolysis of casein, and for reduction of nitrate. The organism produced oxidase, and ammonia was utilized as a nitrogen source. Acid but not gas was produced aerobically or anerobically from D-glucose, D-mannose, D-fructose, and sucrose. Based on these characteristics the organism was identified as belonging to the genus *Bacillus*, according to Buchanan and Gibbons (1974). The guanine-cytosine content of DNA was 46.7%. The isolate was similar to *Bacillus pumilus*, although the growth pH and temperature were higher. The range of pH for growth of the isolate was alkaline, from 8.0 to 11.0. No growth occurred at and below pH 7.0, and maximum growth occurred at pH 9.0–10.0. The temperature range for growth was 27–55°C. The isolate therefore appeared to be an alkalophilic thermophilic *Bacillus*.

Enzyme production

The *Bacillus* isolate was grown in a simple wheat-bran yeast-extract medium at pH 10.0 at 50°C for 48 h. Wheat bran was a good source for xylanase production (50–60 U/mL). When xylan was used in the medium, the maximum xylanase activity was 3.0 U/mL. The extracellular culture broth showed no activity towards carboxymethyl-

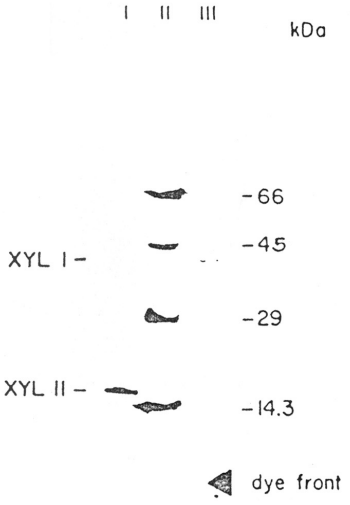

FIG. 3. SDS-PAGE of purified xylanases (25 μg) in 10% slab gel. Lane I, xylanase II; lane II, protein standards (from the top): bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), lysozyme (14 300); lane III, xylanase I. XYL, xylanase.

cellulose and filter paper. Xylose and glucose were poor inducers (Table 1).

Purification of xylanases

The culture filtrate (100 mL) was precipitated with three volumes of chilled ethanol. The precipitate (15 mg) was recovered by centrifugation, dried under vacuum, and dissolved in 5 mL of 50 mM phosphate buffer, pH 7.0. One millilitre was then subjected to gel filtration on a Bio-Gel P-10 column (2.5 × 90 cm) equilibrated with 0.025 M acetate buffer, pH 6.0. The column was eluted with the same buffer and the fractions were collected, scanned for their absorbance at 280 nm, and assayed for xylanase activity (Fig. 1). Two xylanase peaks were observed, and they were designated as xylanase I (fractions 25–32) and II (fractions 78–88), respectively. The results of the purification of xylanases from the culture filtrates are summarized in Table 2.

Final purification of xylanase I

The pooled fractions containing xylanase I were concentrated by lyophilization and dialysed against 0.001 M phosphate buffer, pH 7.0. This fraction (0.6 mg) was then subjected to preparative polyacrylamide gel electrophoresis. Electrophoresis was carried out anodically at pH 8.9 in glass tubes (19 × 120 mm) at 4°C for 30 h with 20-mA current and 250 V. After the run, the gel was removed, and a vertical strip was cut and stained with Coomassie Brilliant Blue to reveal the protein pattern. The gel was cut into eight equal portions and eluted with 0.05 M phosphate buffer, pH 7, concentrated by lyophilization, and dialysed.

Final purification of xylanase II

Fractions 78–88 from the Bio-Gel P-10 column were pooled, lyophilized, and dialysed. Xylanase II (0.6 mg) was

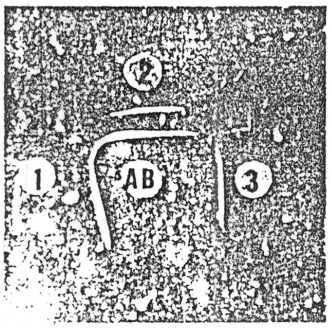

FIG. 4. Double diffusion test between the xylanases I and II. Well: AB, rabbit antiserum against xylanase II; (1) xylanase II (20 μg); (2) alcohol precipitated culture filtrate (100 μg); (3) xylanase I (20 μg).

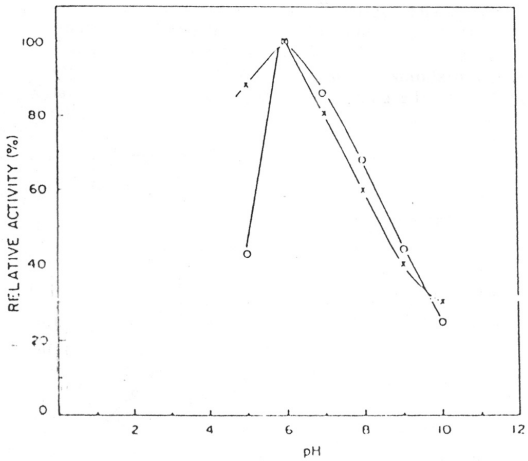

FIG. 5. Optimum pH of xylanases I and II at 50°C. Potassium phosphate buffer (50 mM) was used for pH values between 5 and 7, and 50 mM glycine-NaOH buffer was used for pH 8.0–10.0. The activity of 5 U at 50°C and pH 6.0 was taken as 100%. ×, xylanase I; ○, xylanase II.

mixed with DEAE-cellulose (50 mg) equilibrated with 0.01 M phosphate buffer, pH 7.0, in a batchwise manner. The unadsorbed supernatant fraction was lyophilized and dialyzed.

Molecular weights of xylanase I and II

The molecular weights of xylanase I and II as determined by gel filtration on a Bio-Gel P-100 column were 32 359 and 8 849, respectively (Fig. 2). The M_r for xylanase II determined on a P-10 column was 7 586. By SDS-PAGE the corresponding molecular weights were 35 000 and 15 800, respectively (Fig. 3).

Immunological properties

The Ouchterlony double diffusion tests for the xylanase I and II was carried out using polyclonal antibody raised against purified xylanase II. As shown in Fig. 4, xylanase I and II gave arches of precipitate. Both the enzymes did

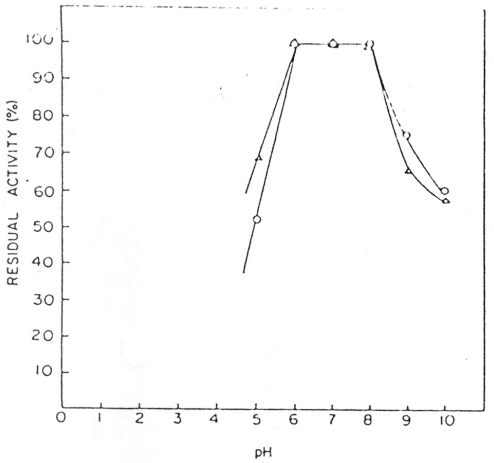

FIG. 6. Effect of pH on stability of xylanases. Enzyme solutions were incubated at different pHs at 50°C for 24 h, and the residual activities were measured by the standard assay procedure. The activity of 5 U at 50°C and pH 7.0 was taken as 100%. △, xylanase I; ○, xylanase II.

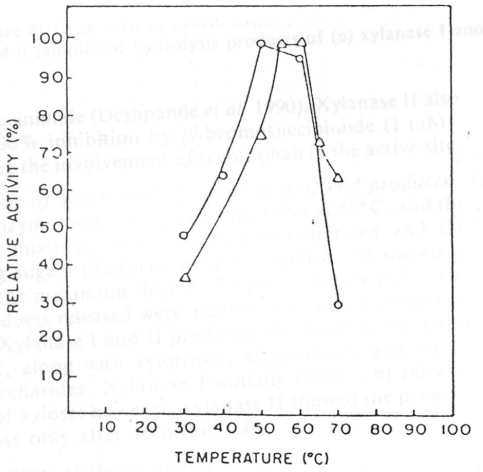

FIG. 7. Optimum temperature of xylanases in 0.05 M phosphate buffer, pH 7.0. △, xylanase I; ○, xylanase II.

not give precipitate with the serum from preimmunized rabbit.

Effect of pH on activity and stability

The pH was adjusted with 0.05 M potassium phosphate buffer (pH 5–7) or NaOH–glycine buffer (pH 8–10). Xylanase I and II at 50°C showed pH optima of 6, but had 60–70% activity at pH 8 (Fig. 5). The stability of the enzyme was determined by incubating 5 U at 50°C for 24 h at different pH, and the residual activities were measured. Both xylanases were stable at pH 6–8 (Fig. 6).

Effect of temperature on activity and stability

Xylanase I was most active at 55–60°C, whereas xylanase II had an optimum temperature of 50°C (Fig. 7). The

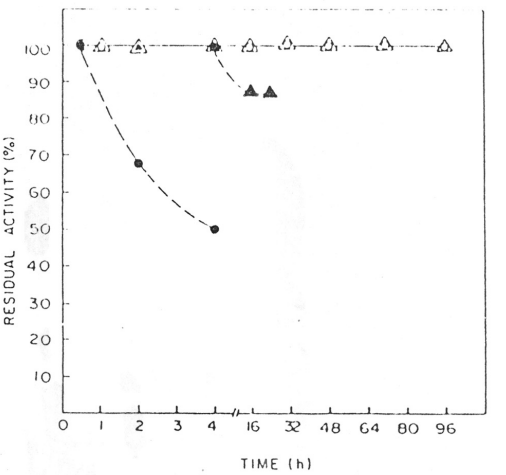

FIG. 8. Thermal stability of xylanases. The enzymes were incubated at 50 and 60°C in 0.05 M phosphate buffer, pH 7.0. At different time intervals the aliquots were withdrawn, and residual activities were measured under standard conditions. Xylanase I: △, at 50°C; ▲, at 60°C. Xylanase II. ○, at 50°C; ●, at 60°C.

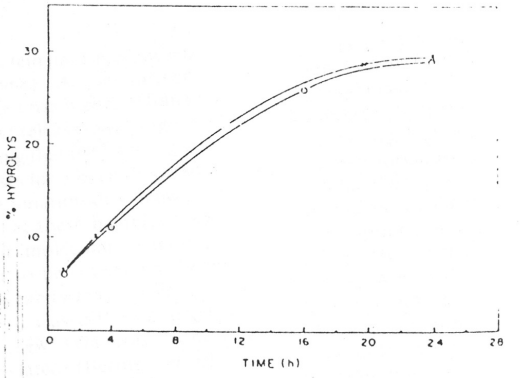

FIG. 9. Time course of hydrolysis of 25 mg xylan by 1 U xylanases at pH 7.0 and 50°C. ×, xylanase I; ○, xylanase II.

thermal stability of the enzymes was measured by incubating 5 U in 0.05 M phosphate buffer, pH 7, at 50 and 60°C for different time intervals. Both enzymes were stable at 50°C for 96 h (Fig. 8). At 60°C xylanase I did not show loss in activity for 4 h, whereas xylanase II showed only 50% activity.

Other properties of the xylanases

The enzymes did not hydrolyze *p*-nitrophenyl-β-D xylopyranoside or *p*-nitrophenyl glucopyranoside and had no effect on carboxymethylcellulose. The isoelectric points of xylanases I and II were found to be 4 and 8, respectively. The carbohydrate content of xylanase I and II was 15 and 30%, respectively. With xylan as substrate the K_m values were 1.58 and 3.5 mg/mL and V_{max} values were 0.0172 and 0.742 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for xylanase I and II, respectively. Xylanase I was inactivated completely by 1 mM N-

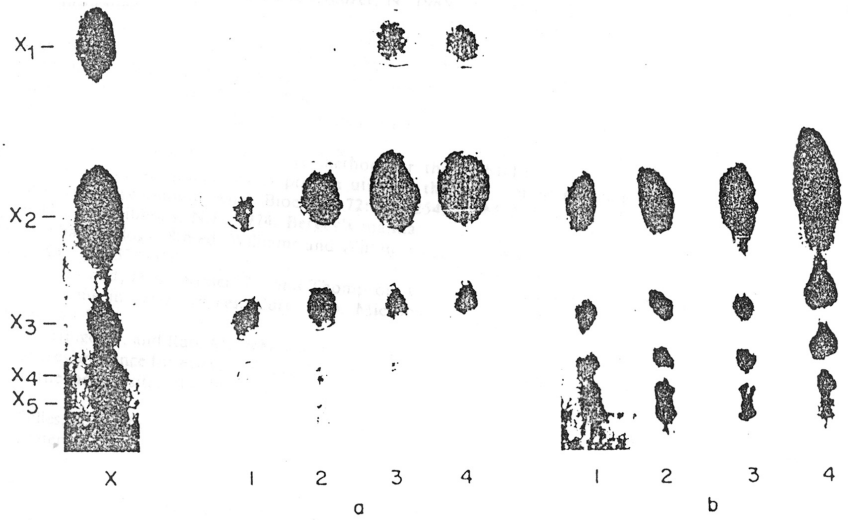

FIG. 10. Paper chromatogram of hydrolysis. Products from 5 mg xylan obtained using 1 U of xylanases at pH 7.0. Lane X, standard mixture of 10 μg each of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and xylopentose (X_5); Lanes 1–4, 1-, 4-, 16-, and 24-h samples of hydrolysis products of (*a*) xylanase I and (*b*) xylanase II, respectively.

bromosuccinimide (Deshpande *et al.* 1990). Xylanase II also showed 90% inhibition by *N*-bromosuccinimide (1 mM), suggesting the involvement of tryptophan in the active site.

Hydrolysis of xylan and determination of end products

The enzymes were incubated with xylan at 50°C, and the reaction mixtures were periodically withdrawn and the reducing sugars produced were determined. As shown in Fig. 9, the maximum degree of hydrolysis was 28%. The end products released were analysed by paper chromatography. Xylanase I and II produced xylobiose as the major product, along with xylotriose, xylotetraose, and higher oligosaccharides. Xylanase I initially (after 1 h) released traces of xylose; however, xylanase II showed the presence of xylose only after 16 h (Fig. 10).

Comparison of the xylanases

The *Bacillus* strain isolated from the soil samples showed growth characteristics similar to other alkalothermophilic bacteria. The optimum temperature and pH of the two xylanases did not show any striking difference. A large difference in pH optima has been observed with other alkaline xylanases (Honda *et al.* 1985; Okazaki *et al.* 1985). The xylanases from the present studies were more stable at 50°C as compared with the earlier reported alkaline xylanases; however, the pH stability cannot be compared because the previous tests were carried out at 4°C (Honda *et al.* 1985). The xylanases were immunologically cross-reactive and probably share similar antigenic epitopes. The two xylanases differed in their molecular weights, isoelectric points, K_m values, and degree of carbohydrate content. The difference in the molecular weights of the xylanases observed by the gel filtration and the SDS method may be due to the glycoprotein nature of the enzymes (Pitt-Rivers and Impiombato 1968). A comparison of K_m values suggests that xylanase I has higher affinity for xylan than xylanase II. The xylanase releases xylooligosaccharides of intermediate size, indicating that they are endoxylanases (Reilly 1981). Multiple xylanases have been reported in numerous microorganisms. An examination of xylanase multiplicity in *Bacillus* sp. suggests that these bacteria produce two xylanases, differing in their molecular weights and isoelectric points, which appear to be conserved (Wong *et al.* 1988). The xylanase heterogeneity may arrive from posttranslational modification, such as differential glycosylation or proteolysis, or both. Few xylanases from *Bacillus* sp. are apparently glycosylated (Bernier *et al.* 1983). However, data on xylanases from alkalophilic and (or) thermophilic *Bacillus* are not available. Many of the xylanases are apparently translated as precursors with peptide signal sequences (Fukusaki *et al.* 1984; Hamamoto *et al.* 1987; Paice *et al.* 1986). A pair of xylanases have been purified from many organisms, including *Bacillus* sp. (Honda *et al.* 1985; Okazaki *et al.* 1985), and two distinct genes have been identified in *Bacillus circulans* (Yang *et al.* 1989).

Acknowledgements

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Cloning and extracellular expression in *Escherichia coli* of xylanases from an alkaliphilic thermophilic *Bacillus* sp. NCIM 59 *

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Abstract: A genomic DNA library of an alkaliphilic thermophilic *Bacillus* was constructed in *Escherichia coli* with pUC 8 vector and was screened using a Congo red xylan plate clearance assay. Six xylanase positive transformants having identical inserts showed immunological reactivity towards polyclonal antibodies raised against purified xylanase (M_r 15800) from the *Bacillus*. A 4.5-kb *Hin*dIII–*Eco*RI subfragment was found to code for two xylanases of M_r 14500 and 35000. Equivalent amounts of xylanase activity were detected from IPTG induced and noninduced recombinants irrespective of the orientation of the 4.5-kb insert with respect to the lac promoter, indicating that xylanase gene expression was under the control of its own promoter. 95% of the xylanase activity (2 U/ml) was found in the extracellular culture filtrate. The hydrolysis of xylan by the recombinant xylanases yielded mainly xylobiose.

Key words: Xylanase gene; Cloning in *Escherichia coli*; Extracellular expression; Recombinant xylanase; Alkaliphilic *Bacillus*; Thermophilic *Bacillus*

Introduction

Xylanases (1,4-β-D xylan xylanohydrolase; EC 3.2.1.8) catalyze the hydrolysis of xylan to xylo-oligosaccharides and xylose, which are useful as feedstock for production of food and fuel. Cellulase-free xylanases active at alkaline pH also have potential application in the paper and pulp industry [1,2]. Examination of xylanase multiplicity in *Bacillus* sp. suggests that these bacteria produce two distinct xylanases differing in molecular mass and isoelectric points [3], but only one of the two xylanases from various *Bacillus* spp. [4–6] has been cloned and expressed in *E. coli*. However, in *B. polymyxa* [7] and alkaliphilic *Bacillus* C 125 [8] two distinct genes that code for two xylanases have been demonstrated. There are a few reports of xylanase production by alkaliphilic-thermophilic bacteria [9,10], but there have been no reports of cloning and gene expression.

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Recent studies on the molecular characterization of bacterial xylanases suggest that these enzymes will be useful model systems for gene expression, secretion and protein engineering studies [11]. Earlier we reported the purification and properties of xylanase I (M_r 35 000) and xylanase II (M_r 15 800) from the alkaliphilic, thermophilic *Bacillus* NCIM 59 (AT *Bacillus*) [10,12]. Tryptophan has been shown to be involved in the active site of the xylanases [13]. The present paper reports the cloning and extracellular expression in *E. coli* of xylanases from AT *Bacillus* NCIM 59.

Materials and Methods

Bacteria and culture media

AT *Bacillus* (NCIM 59) and *E. coli* JM 105 were routinely grown in L broth or on LB plates [14]. For *Bacillus*, the initial pH of the medium was adjusted to 10.5 with 1% (w/v) Na_2CO_3 and at the end of the growth period the pH was found to be the same. The growth temperature was 50°C. *E. coli* recombinants were grown at 37°C with shaking (200 rpm) for 18 h in the presence of ampicillin (100 μg/ml).

DNA manipulation

Chromosomal DNA was isolated as described previously [15]. Plasmid DNA was extracted and purified using Qiagen tubes (Diagen GmbH, FRG) as suggested by the manufacturer. Restriction endonuclease digestion, ligation, agarose gel electrophoresis, reorientation and other DNA manipulations were carried out using standard protocols [14].

Construction and screening of genomic library

Partial *Hin*dIII digest of AT *Bacillus* genomic DNA size fractionated in the range of 6–12 kb in size (1 μg) was ligated to pUC 8 (200 ng) linearised with *Hin*dIII followed by treatment with alkaline phosphatase [14]. The ligation mixture was used to transform $CaCl_2$-treated competent cells of *E. coli* JM 105 [14]. Screening for xylanase positive clone was carried out by the xylan-Congo red clearance plate assay [16]. Enzyme-linked immunosorbant assay (ELISA) of the xylanase-positive transformant colonies used polyclonal antibodies raised against purified xylanase II. Purification of the xylanase and preparation of the antibodies were as already described [10]. Southern blot analysis of the recombinant was carried out [14] under high stringency conditions. Hybridization and washing were done at 63°C. Final washing was with 17 mM sodium citrate-sodium chloride buffer (pH 7.0) having 0.1% w/v sodium dodecyl sulfate (SDS).

Analysis of the recombinant xylanases

The xylanase positive recombinant culture broth (100 ml) was centrifuged and the supernatant was concentrated by ammonium sulfate precipitation followed by dialysis to 3 ml. This concentrated culture filtrate was referred to as extracellular extract. Periplasmic and intracellular extracts were prepared by osmotic shock and sonication respectively [17]. Protein was estimated by the Lowry method [18]. The xylanase assay was performed as described [10]. The reducing sugars formed were estimated by using the dinitrosalysilic acid reagent [19], or 4-hydroxybenzoate reagent [20]. One unit of enzyme was defined as the amount releasing 1 μmol of reducing sugar per min using xylose as a standard. The immunoprecipitation [21] and SDS-PAGE [22] were carried out as described. The extracellular culture broth of *E. coli* pATBX 4.5 (0.1 U) was incubated with xylan (5 mg) for 24 h. The reaction mixture was analysed by paper chromatography using butanol : acetic acid : water (3 : 1 : 1) as the solvent system [23].

Results and Discussion

Cloning of xylanase genes

Six out of 4500 recombinants from the *Hin*dIII genomic library of AT *Bacillus* DNA showed a clear zone around the colony of xylan Congo red plates. These clones also showed a positive ELISA reaction with the antixylanase II antibodies. Plasmid DNA isolated from the six clones when digested with *Hin*dIII, gave an identical restriction pattern, with each insert having two internal *Hin*dIII sites and giving rise to three subfrag-

ments of sizes 6.5, 3.2 and 1.7 kb. These *Hin*dIII fragments were subcloned into pUC 8 and the recombinants were screened using the xylan plate clearance assay. The xylanase gene was localized to the 6.5-kb *Hin*dIII fragment. This restriction fragment had unique site for *Eco*RI and *Pvu*II and had no cleavage site for *Bgl*II, *Cla*I, *Pst*I or *Sma*I (Fig. 1). The *Eco*RI-*Hin*dIII double digested pATBX 6.5 yielded two subfragments of 2 and 4.5 kb which were subcloned into pUC8. The recombinant with the 4.5-kb *Eco*RI-*Hin*dIII fragment (pATBX 4.5) showed a clearance zone on LB-xylan plate and positive colony ELISA reaction (Fig. 2). Southern hybridisation of *Hin*dIII digested pATBX 4.5 with labelled genomic DNA of AT *Bacillus* confirmed the origin of the insert.

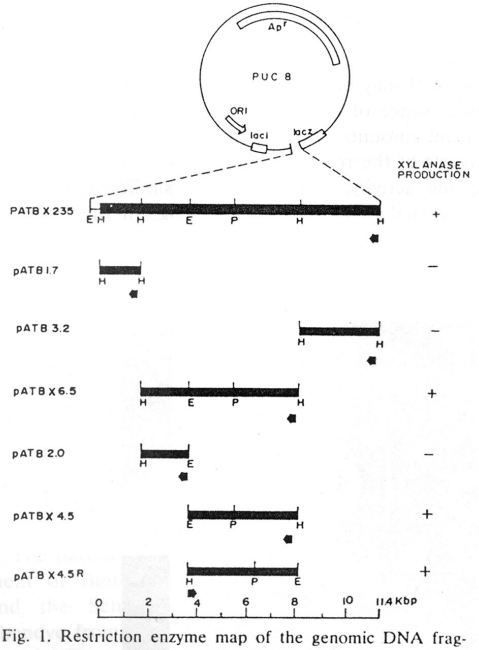

Fig. 1. Restriction enzyme map of the genomic DNA fragments of AT *Bacillus* cloned in pUC 8 that code for xylanase activity. The solid bars represent AT *Bacillus* genomic DNA. The arrow indicates functional orientation of the lac promoter of pUC 8. The plasmid pATBX 4.5 R was constructed by subcloning of the 4.5-kb fragment in pUC 9. Abbreviations for the restriction sites are as follows: E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II. DNA size is estimated in kilobases. + and – indicate the presence and absence of xylanase activity estimated by DNSA method (see Materials and Methods).

Reorientation of insert 4.5-kb

The 4.5-kb *Eco*RI-*Hin*dIII fragment from the plasmid pATBX 4.5 was cloned into pUC 9 where the orientation of the insert with respect to the lac O/P was reversed (pATBX 4.5 R). Equivalent amount of xylanase activity was produced by transformants pATBX 4.5 and pATBX 4.5 R (Table 1), indicating that enzyme expression was under the xylanase gene promoter.

Xylanase production by the recombinant E. coli JM 105

The host strain *E. coli* JM 105 (pUC 8) had no detectable intra- or extracellular xylanase activity. In *E. coli*, expression of the xylanase improved by subcloning (Table 1). The recombinant pATBX 4.5 showed maximum xylanase activity of 2 U/ml which was higher than most of the cloned xylanase gene products from various *Bacillus* sp. [4–6,24]. Extracellular xylanase activity accounted for 95% of the xylanase production. The periplasmic fraction did not show any xylanase activity. The enzyme activity was observed even in the absence of xylan and the addition of IPTG had no effect. The hydrolysis of xylan with the extracellular culture filtrate of pATBX 4.5 yielded xylobiose as the major product along with xylose and traces of xylotriose and xylotetrose.

Anti-xylanase analysis and molecular mass determination

In the double diffusion analysis, anti-xylanase II antibodies showed cross-reactivity with xylanase I of AT *Bacillus* [10]. The intra- and extracellular extract of the recombinant containing pATBX 4.5 showed two precipitin lines. The precipitin line corresponding to the xylanase II of the culture filtrate of the parent organism completely fused with the precipitin lines of the intracellular and extracellular extracts of the recombinant, whereas the arcs corresponding to the xylanase I were identical (Fig. 3).

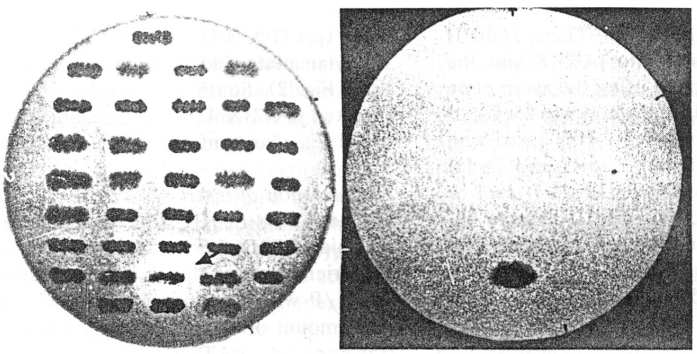

Fig. 2. (A) Xylan–Congo red plate clearance shown by the recombinant pATBX 4.5. The arrow points to the *E. coli* (pATBX 4.5) recombinant surrounded by a clearance zone on the LB plate containing 1% xylan. The clearance was observed after incubation at 37°C for 18 h followed by 2 h incubation at 50°C. The other colonies are recombinants from the genomic library as negative control. (B) The recombinant pATBX 4.5 showing positive colony ELISA reaction.

Immunoprecipitation of crude culture filtrate of AT *Bacillus* followed by SDS-PAGE showed the presence of only two bands corresponding to xylanases I and II. *E. coli* (pUC 8) did not show precipitation with antixylanase antibodies and no protein bands were detected on SDS-PAGE. The immunoprecipitated xylanases from the intra- and extracellular extract of the recombinant showed protein bands at M_r 14500 and 35000 (Fig. 4) which were approximately of the same size as the xylanases of AT *Bacillus* (M_r 15800 and 35000). The difference in the relative molecular mass of the xylanase II may be due to proteolytic modification or absence of glycosylation. The presence of significant amounts of xylanases in the intracellular extracts of the recombinant, without significant enzyme activity, suggests the possibility of protein aggregation or improper folding. In the

Table 1

Induction and localization of xylanase activity of the recombinant pATBX 235

Recombinant	Inducer	Xylanase (U/mg protein)		% activity	
		I	E	I	E
pATBX 6.5	Nil	0.004	0.044	6	94
	IPTG (20 μg/ml)	0.005	0.038	5	95
	xylan (0.5%)	0.005	0.042	7	93
pATBX 4.5	Nil	0.015	1.333	3	97
	IPTG (20 μg/ml)	0.018	1.358	2	98
	xylan (0.5%)	0.020	1.500	4	95
pATBX 4.5 R	xylan (0.5%)	0.016	1.300	4	96

I: denotes intracellular extract.
E: denotes concentrated extracellular extract.

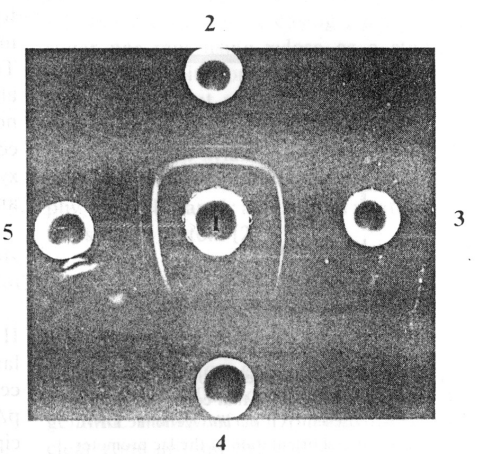

Fig. 3. Ouchterlony double diffusion test. Wells: 1, rabbit antiserum against purified xylanase II; 2, culture supernatant of AT *Bacillus*; 3, extracellular extract of *E. coli* (pATBX 4.5); 4; extracellular extract of *E. coli* (pUC 8); 5, intracellular extract of *E. coli* (pATBX 4.5). The arc towards well 1 corresponds to xylanase II.

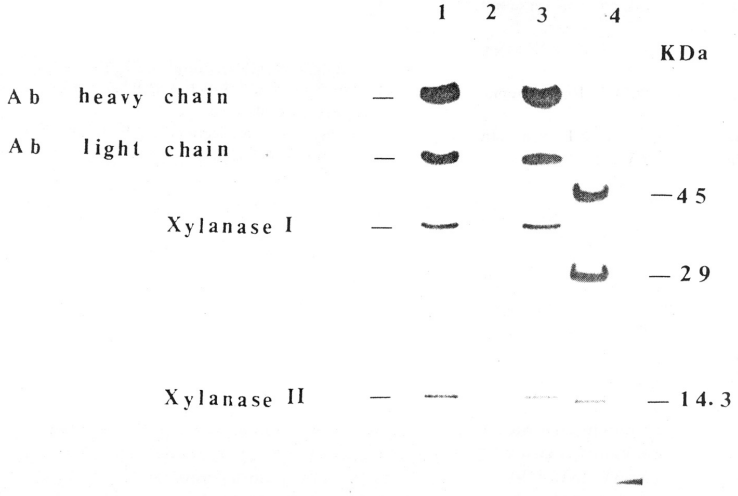

Fig. 4. SDS-PAGE of cell extracts immunoprecipitated with antixylanase II antibodies. Lanes: 1, intracellular extract of pATBX 4.5; 2, *E. coli* JM105 (pUC 8) intracellular extract; 3, extracellular extract of pATBX 4.5; 4, molecular mass marker. Arrow indicates the dye front.

present study, no high molecular mass unprocessed form was detected in the immunoprecipitated extracts from the recombinant. Hence the two xylanases seem to be the proteins of two distinct origins sharing common immunologically reactive domains.

Acknowledgements

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Molecular cloning and expression of xylanases from an alkalophilic thermophilic *Bacillus* (NCIM 59) in *Bacillus subtilis* A8

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The 6.5-kb Hind III fragment of alkalophilic-thermophilic Bacillus *genomic DNA, coding for two xylanases, was cloned from* Escherichia coli *recombinant plasmid pATB X235 in a* Bacillus *plasmid pLP1202 at the Hind III site, inactivating the tetracycline resistance gene.* Bacillus subtilis *A8 was transformed with the ligation mixture using electroporation. The recombinants were chloramphenicol-resistant, tetracycline-sensitive, and showed clearance on LB plates having xylan and immunological cross-reactivity with the antibodies raised against the purified xylanase (M_r 15,800) from alkalophilic-thermophilic* Bacillus. *The xylanase activity obtained in* B. subtilis *A8 was fivefold higher than in* E. coli *harboring pATB X235. Ninety-five percent of the enzyme activity was extracellular. The xylanases produced by the recombinant showed molecular weights of 35 and 14.5 kDa. The hydrolysis of xylan by the recombinant xylanases yielded mainly xylobiose. Xylose was also detected, along with traces of xylotriose and xylotetrose.*

Keywords: Xylanase cloning; alkalophilic-thermophilic *Bacillus;* homologous gene expression; xylanase-negative mutant; electroporation

Introduction

Xylan is a major component of the hemicellulose residue of plant biomass.[1] The use of xylanases in conjunction with cellulases for the complete conversion of the cellulosic biomass to sugars has been widely studied,[2] and it can greatly improve the overall economics of the processing of the lignocellulosic biomass.[3] Large quantities of xylan are released as effluent in the paper and pulp industry, and their bioconversion may be of economic significance. Recently the use of cellulase-free xylanases for the selective hydrolysis of the hemicellulose component in paper and pulp has been receiving consideration.[4] Thermally stable xylanases active at alkaline pH may be particularly useful for the treatment of alkaline pulps. Earlier we reported[5] the purification and properties of xylanases from the alkalophilic-thermophilic *Bacillus* NCIM 59 (AT *Bacillus*). A 6.5-kb Hind III fragment of AT *Bacillus* genomic DNA coding for two xylanases has been cloned and expressed in *E. coli* using pUC 8 vector.[6] One of the xylanases (M_r 14,500) was detected in the extracellular broth, accounting for 70% of the enzyme activity, whereas the other xylanase (M_r 35,000) was found to be cytoplasmic.

Isolation of the xylanase gene fragment from AT *Bacillus* represents an essential step in the engineering of a more efficient microorganism. The expression of several *Bacillus* xylanases cloned in *E. coli* is lower than in the parent organism.[7–9] The expression of the cloned gene can be modified by coupling the gene to a strong promoter, eliminating the operators sensitive to repression, and by increasing the efficiency of translation.[10] Since the translation efficiency is expected to be high in the homologous system, the present paper reports the expression of the cloned xylanase gene fragment in *B. subtilis* A8.

Materials and methods

Bacteria and culture media

E. coli JM 105 and xylanase-negative *B. subtilis* A8 were used throughout the studies. *B. subtilis* A8 was

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obtained from Bacillus Genetic Stock Center (strain No. 1A 651). *Bacillus* and *E. coli* were routinely grown in liquid medium (L broth) or on LB plates (2% w/v agar) containing (in grams per liter) NaCl 5, tryptone 10, and yeast extract 5 (Difco). For AT *Bacillus*, the pH of the medium was made alkaline (10.5) with 1% w/v Na_2CO_3, and the temperature of growth was 50°C. *E. coli* and *Bacillus* recombinants were grown at 37°C with shaking (200 rev min^{-1}) for 18 h with ampicillin (100 $\mu g\ ml^{-1}$) and chloramphenicol (7.5 $\mu g\ ml^{-1}$), respectively.

The plasmid pLP 1202 had already been described.[11] The pATB X235 is a recombinant plasmid having an 11.4-kb insert from AT *Bacillus*, which codes for two xylanases.[6]

DNA manipulations

Chromosomal DNA was isolated according to the method described by Chater *et al.*[12] Plasmid DNA was extracted and purified using Qiagen tubes (DIAGEN GmbH, FRG) as suggested by the manufacturer. The restriction endonuclease digestion and ligation was performed with the enzymes from New England Biolabs, MA, according to the methods suggested by the manufacturer, followed by electrophoresis on 0.8% (w/v) agarose gels.

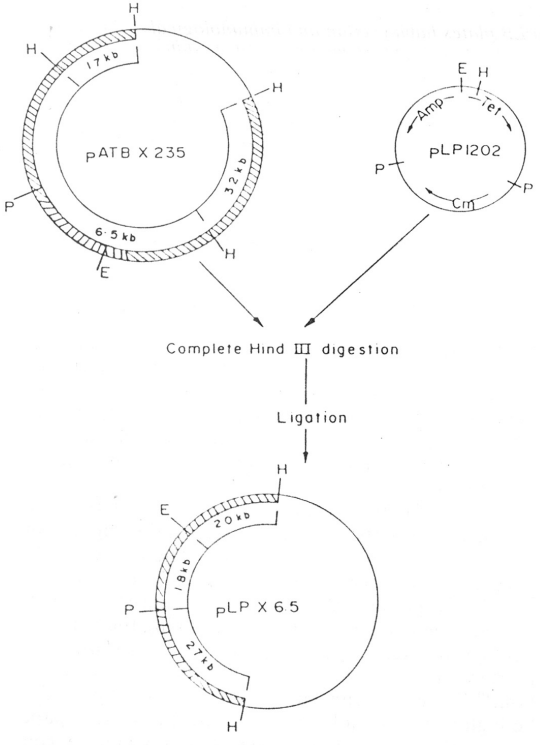

Figure 1 Construction of recombinant plasmid. The 6.5-kb AT *Bacillus* chromosomal fragment (marked area) from plasmid pATB × 235 was ligated to pLP 1202 linearized with Hind III followed by alkaline phosphatase treatment. The xylanase-positive recombinant pLP × 6.5 was tetracycline-sensitive and chloramphenicol-resistant. The restriction sites are shown in the figure. E, Eco RI; H, Hind III; P, Pvu II. Amp, Tet, and Cm are ampicillin, tetracycline, and chloramphenicol resistance genes, respectively

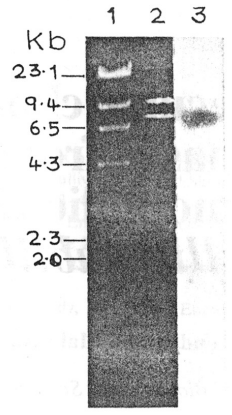

Figure 2 Southern hybridization of the digested plasmid with labeled genomic DNA of AT *Bacillus*. Lane 1: Lambda Hind III digest molecular size marker. Lane 2: Digested pLP × 6.5 showing vector and insert. Lane 3: 6.5-kb insert hybridized with the labeled genomic DNA of AT *Bacillus*

Subcloning and screening for recombinant B. subtilis

The recombinant plasmid pATB X235 was digested with Hind III, and a 6.5-kb fragment was purified from an agarose gel using Qiagen tubes. The purified 6.5-kb fragment was ligated to pLP 1202 linearized with Hind III followed by alkaline phosphatase treatment. The *B. subtilis* A8 competent cells were prepared[13] and resuspended in 10% glycerol. The cells were transformed with the ligation mixture using a BTX T 100 electroporator. A single pulse of 659 V and 4.68 msec was applied. The transformants were plated initially on LB agar supplemented with chloramphenicol (7.5 $\mu g\ ml^{-1}$) and then screened for tetracycline-sensitive and xylanase-positive phenotype using a xylan/congo red plate clearance assay.[14] Southern blot analysis of the recombinant was carried out[15] using labeled genomic DNA of AT *Bacillus*. The DNA was labeled using α P 32 dATP (3,000 Ci $mmol^{-1}$) by multiprime labeling (Amersham). Hybridization and washing were done at high stringency conditions (63°C). Final washing was with 17 mM sodium chloride-sodium citrate buffer (pH 7.0) having 0.1% sodium dodecyl sulfate (SDS).

Preparation of cell extracts and enzyme assay

The culture broth (50 ml) was centrifuged and the supernatant, referred to as extracellular extract, was con-

Table 1 Induction and localization of xylanase activity of the recombinants

Organism	Inducer	Xylanase activity (mU mg^{-1} protein)		% Activity	
		I	E	I	E
E. coli	Nil	4	39	25	75
(pATB X235)	Xylan (0.5%)	5	40	30	70
B. subtilis	Nil	2	100	4	96
(pLP X6.5)	Xylan (0.5%)	4	140	6	94

The *E. coli* or *B. subtilis* cells were grown at 37°C for 18 h in L broth containing ampicillin (100 μg ml^{-1}) or chloramphenicol (7.5 μg ml^{-1}), respectively. I, Intracellular extract; E, extracellular extract

centrated by ammonium sulfate precipitation or directly taken for xylanase assay. The cells were washed and sonicated or treated with lysozyme in 3 ml final volume. The supernatant after centrifugation, referred to as intracellular extract, was assayed for xylanase. Protein estimation was carried out by the method of Lowry *et al.*[16] The xylanase activity was measured in 50 mM phosphate buffer (pH 7.0) at 50°C, for 30 min using 1% (w/v) soluble Oat Spelt xylan (Sigma). The reducing sugars formed were estimated by dinitrosalisylic acid reagent[17] or by p-hydroxybenzoate reagent.[18] One unit was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute using xylose as standard.

Molecular weight determination and immunological properties

SDS-PAGE was carried out as described[19] using 10% gels. The antixylanase antibodies were raised as already described.[5] The immunoprecipitation and ELISA were performed as described.[20]

Determination of xylan degradation products

Concentrated culture filtrates (0.1 U) were incubated with xylan (5 mg) in 50 μl 50 mM phosphate buffer (pH 7.0) for 24 h. The aliquots were analyzed for the sugar products formed by paper chromatography in the butanol-acetic acid-water (3 : 1 : 1) solvent system. The paper chromatograms were developed as described by Trevelyan *et al.*[21]

Results

Cloning of xylanase gene fragment in pLP 1202

The complete Hind III digestion of the recombinant plasmid pATB X235 showed presence of three subfragments of 6.5, 3.2, and 1.7 kb. Subcloning of these fragments in *E. coli* has shown that the 6.5-kb fragment codes for the two xylanases.[6] Cloning of the 6.5-kb fragment in pLP 1202 gave rise to 200 chloramphenicol-

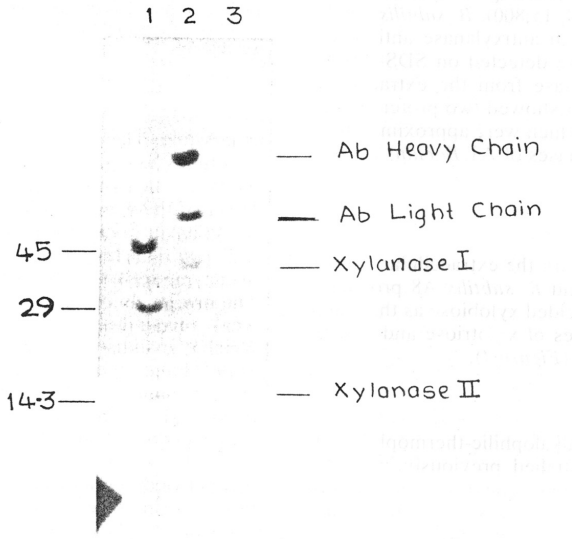

Figure 3 SDS-PAGE of the cell extracts immunoprecipitated with the antixylanase II antibodies. Lane 1: Protein molecular weight marker. Lane 2: Extracellular extract of recombinant *B. subtilis* A8 (pLP × 6.5). Lane 3: Extracellular extract of the host *B. subtilis* A8. Arrow indicates the dye front

resistant transformants of *B. subtilis*, which when replica-plated on LB plates containing xylan (0.5%) yielded 120 colonies having halos round them. All these colonies were tetracycline-sensitive, and showed positive test for colony and dot blot ELISA with the antixylanase antibodies raised against purified xylanase (M_r 15,800) from AT *Bacillus*. The plasmid isolated from one of the colonies on Hind III digestion showed the 6.5-kb insert. The insert had unique sites for Eco RI and Pvu II and no sites for Bgl II, Pst I, and Sma I (*Figure 1*). The southern hybridization with AT *Bacillus* genomic DNA confirmed the origin of the insert (*Figure 2*).

Xylanase activity of the B. subtilis *recombinants*

The *Bacillus subtilis* recombinants produced fivefold higher activity as compared to the *E. coli* recombinants.[6] Around 95% of the total activity was confined to the extracellular broth. The xylanase activity of the *B. subtilis* recombinant was higher when grown on xylan, whereas the *E. coli* recombinant did not show any significant difference. The xylanase activity of the recombinants is summarized in *Table 1*.

Determination of molecular weight of recombinant xylanases

Immunoprecipitation of crude culture filtrate of AT *Bacillus* followed by SDS-PAGE showed the presence of only two bands, corresponding to xylanase I (M_r 35,000) and xylanase II (M_r 15,800). *B. subtilis* A8 did not show precipitation with antixylanase antibodies, and no protein bands were detected on SDS-PAGE. Immunoprecipitated xylanase from the extracellular extract of the recombinant showed two protein bands at M_r 14,500 and 35,000, which were approximately of the same size as the xylanases of AT *Bacillus* (*Figure 3*).

Xylan hydrolysis

The hydrolysis of xylan with the extracellular culture filtrates of AT *Bacillus* and *B. subtilis* A8 producing recombinant xylanases yielded xylobiose as the major product. Xylose and traces of xylotriose and xylotetrose were also produced (*Figure 4*).

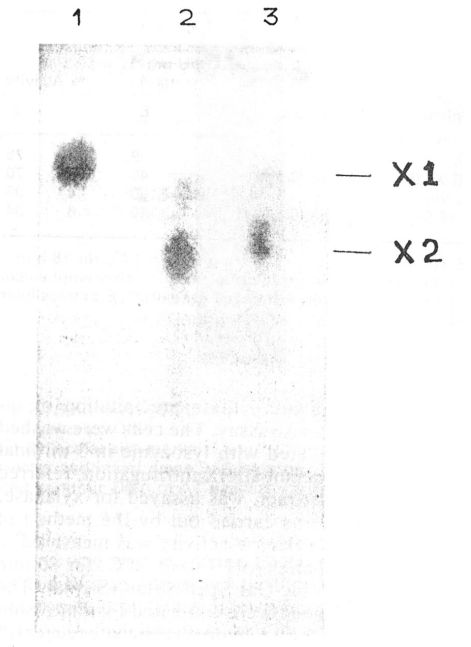

Figure 4 Paper chromatogram of xylan hydrolysis. Products from 5 mg of xylan obtained using 0.1 U xylanase at pH 7.0. Lane 1: Xylose standard (10 μg). Lanes 2 and 3 are 24-h samples of hydrolysis products of extracellular culture filtrates of AT *Bacillus* and the recombinant pLP × 6.5. X1 and X2 denote xylose and xylobiose

Discussion

Xylanase production by alkalophilic-thermophilic microorganisms has been studied previously.[5,22] However, molecular cloning and gene expression have not so far been reported. When the genes for extracellular enzymes of Gram-positive bacteria are cloned in *E. coli*, the gene products are generally transported to the membrane and/or periplasmic space.[7–10] However, the xylanases of AT *Bacillus* cloned in *E. coli* showed 70% extracellular enzyme production. In a homologous host system, due to efficient transcription, translation, and secretion, overexpression of the cloned gene product is possible. The recombinant *B. subtilis* A8 produced five times as much xylanase as the *E. coli* recombinants, but this value does not seem to reflect the direct effect of the gene copy number. The copy number of pLP 1202 in *Bacillus* is much lower than that of pUC 8 in *E. coli*. This may be attributed to the narrow increase in enzyme activity in *Bacillus* as compared with *E. coli*. The low levels of expression may be attributed to the weak recognition of the signals from AT *Bacillus*, which is an unusual extremophilic species. Panbangred *et al.*[10] have reported expression of the xylanase gene from *B. pumilus* in *B. subtilis* M 111. The host used in these studies secreted small amounts of xylanases, and the recombinant showed two to three times higher activity. However, the host *B. subtilis* A8 used in the present studies is a xylanase-negative mutant. This mutation might have been accompanied by the overexpression of xylanase gene repressor, possibly interfering with the expression of the cloned xylanase. The constitutive expression of the xylanases in the recombinants *E. coli* and *B. subtilis* A8 may be caused by the absence or nonrecognition of the regulatory elements. In xylanase fermentation, insoluble substrates are generally used as inducers. The constitutive expression of

xylanases is advantageous to eliminate the insoluble inducers from large-scale fermentations.

Acknowledgements

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Expression of the cloned xylanases from an alkalophilic, thermophilic *Bacillus* in *Bacillus subtilis*

A. Shendye, R. Gaikaiwari and M. Rao*

A recombinant plasmid construct, pLPX6.5, harbouring a 6.5 kb *Hind*III fragment of genomic DNA, from an alkalophilic, thermophilic *Bacillus* NCIM 59 and coding for xylanase activity, was electroporatically transformed into *Bacillus subtilis* MI 111. The expression of the recombinant xylanases was confirmed by cross-reactivity with antibodies raised against purified xylanase II (*M*, 15,800) from NCIM 59. However, as there were different xylan hydrolysis products from NCIM 59 and the host *B. subtilis*, the two xylanases appear to have different modes of action. Xylanase expression in the transformants was 6-fold higher than in the host. There was no significant enhancement in the expression of recombinant xylanases by adding xylan to the growth medium.

Key words: Alkalophilic thermophilic *Bacillus*, homologous expression, recombinant xylanases.

Studies on induction and secretion of xylanases (1,4-β-D xylan xylanohydrolase; EC 3.2.1.8) are necessary to develop efficient xylanase producers for their possible commercial development. Induction of xylanases requires the key participation of a few hetero-disaccharides (Biely & Petrakova 1984; Hrmova *et al.* 1986, 1989, 1991). The role of transglycosylating enzymes in the synthesis of the positional isomers of these hetero-disaccharides was indicated in *Aspergillus terreus* and *Trichoderma reesei* (Hrmova *et al.* 1991). This may explain the low level expression of recombinant xylanases in heterologous, non-xylanolytic hosts, as these organisms may not possess the proper inducers, such as the hetero-disaccarides, and/or the necessary inducer synthesising machinery. An understanding of the expression of the cloned xylanases in a xylanase proficient, homologous host system is therefore needed. The cloning of a 6.5 kb xylanase gene fragment of an alkalophilic, thermophilic *Bacillus* has been reported (Shendye & Rao 1993a) and the expression of xylanases in *Echerichia coli* and the xylanase-negative mutant. *Bacillus subtilis* A8 described (Shendye & Rao 1993b). The cloned xylanases can be expressed from their own promoter elements. The extracellular activity of the recombinant *B. subtilis* A8 was 5-fold higher than that of the recombinant *E. coli* but was lower than that of the alkalophilic, thermophilic *Bacillus* (Dey *et al* 1992). The present study was on the induction and expression of the cloned xylanases in a xylanase-proficient host, *Bacillus subtilis* MI111.

Materials and Methods

The recombinant construct pLPX 6.5 has been described earlier (Shendye & Rao 1993b). The host, *B. subtilis* MI 111, was obtained from the *Bacillus* Genetic Stock Center [BGSC no. 1A 253; SP 10(S) arg (GH) 15 hsr M leuA8 m(–) 1687] and grown in Luria broth (LB) containing (% w/v): Tryptone, 1; yeast extract, 0.5; and NaCl, 0.5. The cultures were incubated at 37°C for 16 h with shaking (200 rev/min). The cells were then centrifuged, washed extensively with sterile distilled water and competent cell suspension was prepared in 10% (v/v) sterile glycerol (Dower *et al.* 1988). The transformation was carried out using an electroporator. The cell suspension (150 µl) was mixed with 10 ng plasmid solution and a single pulse set at 650 V and 5 ms was applied using an inter-electrode gap of 1.9 mm. The transformants were selected on LB agar plates containing chloramphenicol (7.5 µg/ml). The plasmid isolation and analytical agarose (0.8% w/v) gel electrophoresis were performed according to Maniatis *et al.* (1982).

Antiserum against xylanase II (*M*, 15,800) from alkalophilic, thermophilic *Bacillus* (NCIM 59) was obtained from a New Zealand white rabbit. The rabbit had been immunized with the purified xylanase II (0.5 mg) mixed with complete Freund's adjuvant and then given three booster injections (0.5 mg each) at 6-week intervals. The serum was collected 1 week after the last injection. The anti-xylanase II antibodies were used to study the cross-reactivity of the *B. subtilis* MI 111 xylanase(s) and for the

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identification of the recombinant xylanases. Immunodiffusion tests were carried out in 0.05 M phosphate buffer, pH 7.0, as already described (Dey *et al.* 1992).

Fermentation studies were carried out using LB medium alone or LB medium supplemented with xylan (0.5% w/v) or wheat bran (5% w/v). The selective pressure was maintained by adding chloramphenicol (7.5 µg/ml) to the culture media. The fermentation broth was centrifuged and the supernatant was estimated for xylanase activity by incubating with 1% oat spelt xylan (Sigma) in 50 mM phosphate buffer (pH 7.0) at 50°C for 30 min. Reducing sugars formed were estimated by the dinitrosalicylic acid method. One unit was defined as the amount of enzyme releasing 1 µmol reducing sugar/min using xylose as a standard.

Extracellular xylanase extracts (1 U each) from alkalophilic, thermophilic *Bacillus* NCIM 59, *B. subtilis* MI 111 and the transformant harbouring pLPX 6.5 were each incubated with xylan (5 mg) in 0.05 M phosphate buffer (pH 7.0) at 50°C for 24 h in 0.1 ml. The sugar products formed were analysed by paper chromatography in a solvent system containing butanol/acetic acid/water (3:1:1 by vol.) The paper chromatograms were developed as described by Trevelyan *et al.* (1950).

Results and Discussion

The plasmid pLPX6.5 (Figure 1) was transformed in *B. subtilis* MI 111 and the transformants were selected for chloramphenicol resistance. Six selected transformants showed the presence of plasmid DNA, with an electrophoretic pattern identical to pLPX6.5 (date not shown). In double-diffusion analyses using the antibodies raised against purified xylanase II (M_r 15,800) from alkalophilic, thermophilic *Bacillus*, the protein extract of the transformant harbouring pATBX6.5 showed two precipitin lines which were identical to those of xylanase I and xylanase II from the protein extract of the alkalophilic, thermophilic *Bacillus*. The protein extract of the host, *B. subtilis* MI 111, did not show any precipitin line, indicating that the xylanase(s) of the host are immunologically distinct from the xylanases of the alkalophilic, thermophilic *Bacillus* (Figure 2). These results confirmed the expression of recombinant xylanases in *B. subtilis* MI 111.

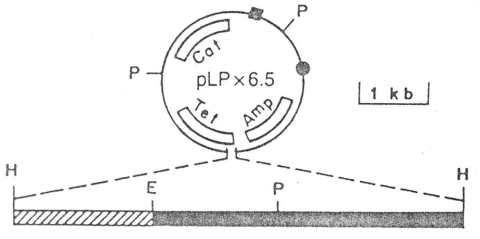

Figure 1. Recombinant plasmid pLPX6.5 harbouring the xylanase gene fragment from an alkalophilic, thermophilic *Bacillus*. A 6.5 kb *Hind*III fragment of the *Bacillus* genomic DNA was cloned in plasmid pLP 1202 (Ostroff & Pene 1984), inactivating the tetracycline resistance gene. Cat—Chloramphenicol resistance; amp—ampicillin resistance; ●—*E. coli* replicon; ■—*B. subtilis* replicon; ▩—region of the AT *Bacillus* genomic DNA coding for xylanase activity; ▨—flanking region of the AT *Bacillus* genome; E—EcoRI; H—*Hind*III; P—*Pvu*II. The 1 kb scale bar refers to the lower, expanded section.

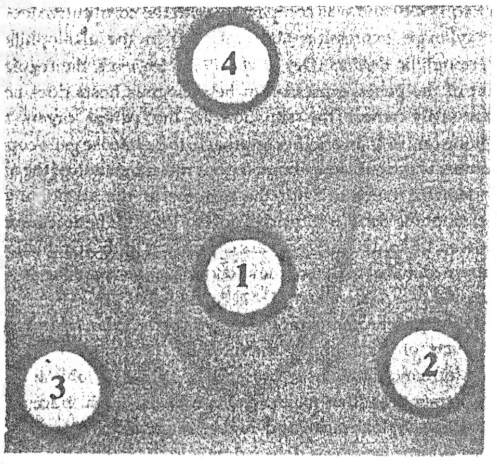

Figure 2. Ouchterlony double-diffusion test. Well 1 contained rabbit antiserum against purified xylanase II. Wells 2, 3 and 4 contained extracellular protein extracts of alkalophilic, thermophilic *Bacillus*, *B. subtilis* MI 111 (pLPX6.5) and the *B. subtilis* MI 111 host, respectively.

The transformant had six times the activity of the host in Luria broth. The xylanase activity of the host, *B. subtilis* MI 111, was enhanced 3-fold with xylan and 6-fold with wheat bran inducers (Table 1). The apparent increase in the xylanase activity of the transformant in the presence of inducer may be due to the induction of host xylanase activity, indicating that the recombinant xylanases are constitutively expressed in *B. subtilis* MI 111. The level of expression of the recombinant xylanases in *B. subtilis* MI 111 was 3-fold higher than that already reported in xylanase-negative *B. subtilis* A8 (Shendye

Table 1. Production of recombinant xylanases by *B. subtilis* MI 111.*

Inducer	Extracellular xylanase activity (mU/mg biomass)	
	***B. subtilis* MI 111**	***B. subtilis* MI 111 (pLPX6.5)**
None	67	441
Xylan (0.5%)	186	563
Wheat bran (5%)	278	548

* Fermentations were carried out at 37°C for 18 h using Luria broth as basal medium. Inducers were added as specified in the Table. The transformants were grown in the media containing chloramphenicol (7.5 µg/ml). Biomass was determined as the dry weight of the bacterial cell pellet.

& Rao 1993b) and was comparable with the constitutive level of xylanase expression (1300 mU/ml) in the alkalophilic, thermophilic *Bacillus* (Dey *et al.* 1992). However, the regulation of the genes expressed in heterologous hosts does not necessarily reflect the situation in the parent organism (Thomson 1993). In another approach, the xylanase gene copy number was doubled by homologous recombination of the 6.5 kb *Hind*III fragment with the genome of the alkalophilic, thermophilic *Bacillus* (Shendye & Rao 1993c). The integrants showed 1.5-fold higher xylanase expression, with an induction pattern identical to that of the alkalophilic, thermophilic *Bacillus*.

The paper chromatographic analysis of the xylan hydrolysis products of alkalophilic, thermophilic *Bacillus* and of the transformant showed the presence of xylose, xylobiose and xylotetraose as the major end products, with traces of xylotriose, xylohexaose and higher oligosaccharides. However, xylopentaose was detected only in the hydrolysis products of the xylanases from the alkalophilic, thermophilic *Bacillus*. The extracellular extract of the host, *B. subtilis* MI 111, produced xylobiose and xylotetraose as the major products, with traces of xylose and higher oligosaccharides. The presence of xylotriose and xylopentaose was not detected.

These results indicate that the xylanases from the alkalophilic, thermophilic *Bacillus* and the *B. subtilis* MI 111 host had different modes of action. The absence of a specific xylo-oligosaccharide such as xylopentaose in the hydrolysis products of the transformant could be due to the absence of the possible transglycosylation reaction of the cloned xylanases, or efficient conversion of the xylo-oligosaccharide by the host xylanase(s).

The present studies establish the inter-specific differences in the induction profiles of the cloned xylanases. They provide a foundation for detailed studies on the role of the enzyme machinery of the host, such as the transglycosylating enzymes. These enzymes have been shown to be important in the synthesis of proper inducer molecules such a heterodisaccharides and their positional isomers (Hrmova *et al* 1991), and thus may play a critical role in the induction of the cloned xylanase genes.

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CHROMOSOMAL GENE INTEGRATION AND ENHANCED XYLANASE PRODUCTION IN AN ALKALOPHILIC THERMOPHILIC *BACILLUS* SP. (NCIM 59)@

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Chromosomal integration and xylanase gene amplification were demonstrated for the first time in an alkalophilic thermophilic *Bacillus* sp. (NCIM 59). The integrants were characterized by larger zone of xylan clearance than the parent culture and hybridization with vector (pUC8) DNA. Repeated transformation strategy was used for further amplification of the xylanase gene. The results of Southern blot analysis indicated the occurance of homologous recombination in the 6.5 kb xylanase gene region of the genomic DNA and suggested a non campbell mode of recombination. The integrants were checked for xylanase production up to ten subcultures and consistently showed two fold higher xylanase activity than the parent strain with the maximum xylanase productivity (U/ml/h) at 16 h.

Xylanases [1,4-ß-D-xylan xylanohydrolase, E.C. 3.2.1.8] find potential uses in the complete conversion of plant biomass and biobleaching of paper pulp (1). The biotechnological applications of xylanases have stimulated the search for stable and highly active enzyme preparations which can be obtained in bulk quantities. For the commercial realization and economic viability of enzyme production, it is necessary to identify organisms which can hyperproduce the xylanases. Recombinant DNA techniques offer means to enhance protein production. However, the xylanase genes show low levels of expression in heterologous hosts probably due to absence of glycosylation machinery or intracellular localization and improper processing (1-3). In the large scale fermentation process, use of the recombinants is restricted due to the plasmid instability and constant requirement of antibiotic selective pressure.

Earlier we have reported isolation of an alkalophilic thermophilic *Bacillus* sp. (NCIM 59) [AT *Bacillus*] producing cellulase free xylanases (4,5). The gene fragment coding for the

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two xylanases has been cloned (6) and the extracellular expression in *E. coli* and *B. subtilis* has been studied (7). The maximum xylanase production obtained in the recombinants was lower than that of the parent AT *Bacillus*.

In this paper we present enhanced xylanase production using a recent and promising approach of chromosomal gene integration. Although the process of gene integration is known in *B. subtilis* (8-11) its application in genetic engineering of an extremophile, such as AT *Bacillus* is so far not studied. Our results represent the first report on gene integration mediated enhanced xylanase production.

MATERIALS AND METHODS

Bacteria, growth and fermentation media: The AT *Bacillus* used in this study has been described (4,5). For transformation AT *Bacillus* was routinely grown on L broth containing (in grams per liter) NaCl 5, tryptone (Difco) 10 and yeast extract (difco) 5. Screening of the transformants was carried out on L broth supplemented with xylan (oat spelt, Sigma) 5 g/l, congo red (Sigma) 0.1 g/l, solidified using agar 20 g/l. Fermentation studies were carried out either in xylan yeast extract medium or in wheat bran yeast extract medium containing either xylan 5 g/l or washed wheat bran (4,5) 50 g/l along with yeast extract 2.5 g/l and NaCl 5 g/l. The media were made alkaline (pH 10.5) after autoclaving by addition of separately sterilized sodium carbonate 1% (w/v) final concentration. The cultures were routinely grown at 50°C with shaking (200 rpm) and the samples were removed at designated time intervals. The fermentations were carried out as already described (4,5).

Transformation and screening of the integrants: The recombinant plasmid pATBX 6.5 (6) was used to transform the AT *Bacillus* cells prepared according to Dower (12). The AT *Bacillus* cells suspended in 10% glycerol were transformed with the help of a single pulse set at 650 V and 5 msec using BTX T100 electroporator. The transformants were screened using xylan-congo red plate clearance assay (13) and colony hybridization with labeled pUC8 vector (14). The selected strain was subjected to second transformation cycle. Southern blot analysis of the selected strains was carried out (14) using labeled 6.5 kb Hind III fragment of pATBX 6.5. The DNA labeling was performed (14) using multiprime labeling kit (Amersham) and [a ^{32}P]dATP (3000 Ci/m mol). Hybridization and washing were at highest stringency conditions (63°C) and final washing was with 17 mM sodium chloride-sodium citrate buffer (pH 7.0) having 0.1% (w/v) sodium dodecyl sulfate (SDS).

Maintainance and cultivation of integrants: The overnight grown cells of the integrants were suspended in glycerol (15% v/v) and stored as frozen glycerol stocks at -70°C. They were also grown on wheat bran yeast extract slants and maintained in sporulated form at 4°C. The sporulated AT *Bacillus* and integrants were routinely subcultured to fresh slant before every fermentation experiment and 16 h growth from these slants was used to inoculate fermentation media containing wheat bran, glucose, xylose or lactose as sole source of carbon. The xylan medium used for xylanase production was inoculated with the bacteria grown on xylan yeast extract slants.

Enzyme and protein determination: The xylanase activity of the extracellular culture supernatent was determined in 50 mM sodium phosphate buffer (pH 7.0) by incubating the enzyme with 1% soluble xylan at 50°C for 30 min. The reducing sugars formed were estimated by dinitrosalicyclic acid reagent (15).

One unit of xylanase activity was defined as the amount of enzyme releasing one umol of reducing sugar per min using xylose as standard. Protein estimation was carried out by the method of Bradford (16).

RESULTS AND DISCUSSION

Integration of the xylanase gene fragment in to the chromosome of alkalophilic thermophilic *Bacillus*: The recombinant plasmid pATBX 6.5 containing the xylanase gene fragment from AT *Bacillus* is shown in Fig. 1. The xylanase activity is coded by the 4.5 kb EcoRI-Hind III subfragment (6). pATBX 6.5 is a pUC8 derived *E. coli* vector, which cannot replicate in *Bacillus* sp., and the presence of homologous region aids in its recombination with the genomic DNA of AT *Bacillus*. As a result of the transformation of AT *Bacillus* with pATBX 6.5, 1285 colonies developed on to the screening medium out of which fifteen showed positive pUC hybridization test (Fig. 2A). In xylan congo red plate clearance assay 14 out of these 15 strains showed distinctly larger zones of xylan clearance whereas one strain (CII 14) showed smaller clearance zone as compared to AT *Bacillus* (Fig. 2B). One of the integrants CII 11, was selected for further amplification of xylanase gene copies. Gene multiplication was achieved by carrying out successive transformation of strain CII 11 with pATBX 6.5. These transformants could not be screened with pUC 8 as the CII 11 strain had already shown positive hybridization with pUC 8. The putative positive integrants were identified on the basis of xylan clearance and strain CIII6 was selected for further studies. Southern blot analysis was performed to confirm the gene multiplication in the strain CIII6.

Chromosomal gene amplification has been generally carried out with the help of specially designed integration vectors using increased levels of selective pressure (17 -21). The plasmid pATBX 6.5 used in the present studies lacked antibiotics marker selectable in *Bacillus* sp. Also high temperature (50°C) and pH (10.5) necessary for growth of AT *Bacillus* render inactivation of the antibiotics. Hence the integrants could not be amplified using selective pressures. An alternative method of repeated transformation was therefore used for multiplication of xylanase gene copies in AT *Bacillus*.

Characterization of the integrants: The Hind III digests of genomic DNA from AT *Bacillus* and the two integrant strains CII 11 and CIII 6 were probed with labeled 6.5 kb xylanase gene fragment (fig. 3). A single band of hybridization was observed at 6.5 kb in the DNA digest of the AT *Bacillus*. However the restriction

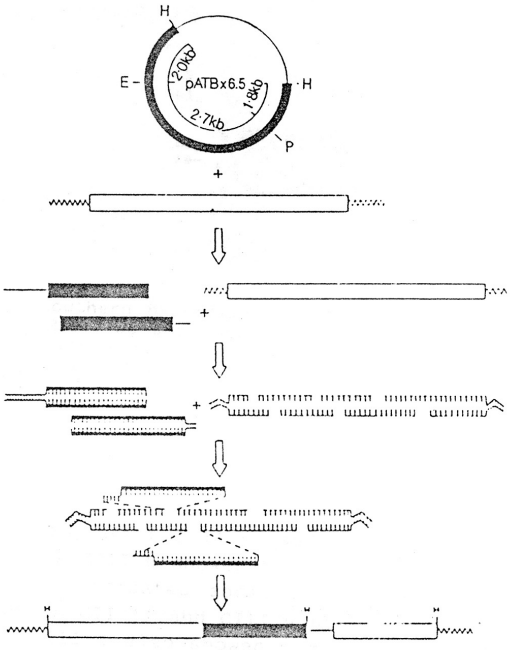

FIG. 1. Integration of the recombinant plasmid pATB X 6.5 with xylanase gene region of AT *Bacillus* genomic DNA. Proposed mode of recombination is according to Yasbin *et al.* (21).

- pUC DNA.
- xylanase gene fragment from pATB X 6.5.
- AT *Bacillus* genomic xylanase fragment.
- AT *Bacillus* genomic DNA.
- nicked genomic DNA of AT *Bacillus*.
- single stranded fragment containing a part of insert plus vector.

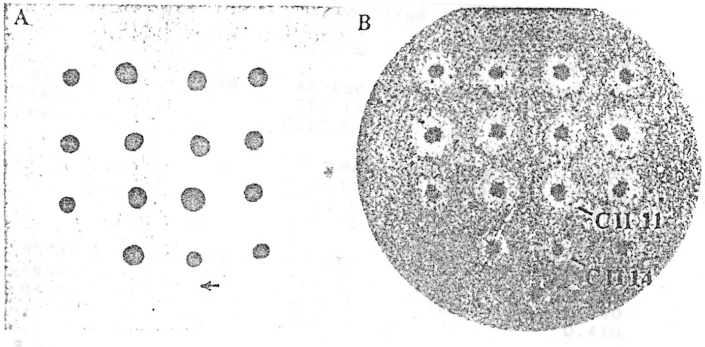

FIG. 2. (A) Integrants showing positive hybridisation with labeled pUC 8. Arrow indicates position of AT *Bacillus* colony as a negative control.
(B) AT *Bacillus* and the integrants showing xylan plate clearance.

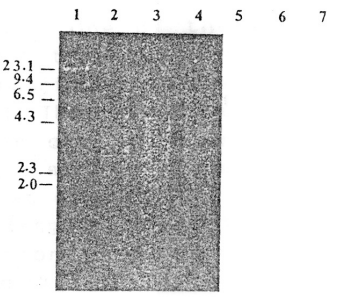

FIG. 3. Southern hybridisation of the genomic DNA samples of AT *Bacillus* and the integrants CII 11 and CIII 6 digested with Hind III and probed with 6.5 kb Hind III fragment coding for xylanases of AT *Bacillus*.
Lane 1: Lambda Hind III digest DNA molecular size marker.
Lanes 2 - 4: Genomic DNA digests of AT *Bacillus* and the integrants CII 11 and CIII 6, respectively.
Lanes 5 - 7: Positive hybridisation signals of AT *Bacillus* and the integrants CII 11 and CIII 6, respectively.

digest of integrant CII 11 showed two positive signals at 5.1 and 3.2 kb whereas CIII 6 showed three signals at 4.8, 3.2 and 2.9 kb. These results indicated addition of 1.8 and 4.4 kb DNA by gene integration in CII 11 and CIII 6 strains respectively. The strains CII 11 and CIII 6 also showed addition of 1 and 2 Hind III sites

Table 1: Xylanase activity of AT *Bacillus* and the integrant strains

Fermentation medium	Time (h)	Strain	Extra-cellular xylanase yield (U/ml)	Extra cellular protein yield (mg/ml)	Specific activity (U/mg) protein
	28	AT *Bacillus*	6.1	0.290	21.03
		CII 11	10.4	0.375	27.73
		CIII 6	11.9	0.465	25.50
Xylan-Yeast extract	48	AT *Bacillus*	6.6	0.760	8.86
		CII 11	8.8	1.000	8.80
		CIII 6	11.2	1.080	10.37
	16	AT *Bacillus*	46.0	0.325	141.55
		CII 11	83.0	0.480	172.91
		CIII 6	111.0	0.610	193.44
		CII 14	20.0	0.307	65.12
Wheat bran Yeast extract	20	AT *Bacillus*	66.0	0.380	173.68
		CII 11	95.0	0.540	175.92
		CIII 6	128.0	0.750	170.66
		CII 14	30.0	0.410	73.17
	48	AT *Bacillus*	51.5	1.333	38.63
		CII 11	77.5	1.550	50.00
		CIII 6	98.0	1.833	53.46
		CII 14	26.5	1.250	21.35

respectively (fig. 1). One of the possible modes of gene duplication is campbell type recombination resulting in addition of identical sized restriction fragment as a second copy of the desired gene (17, 20). The non campbell mode of recombination has also been reported in case of *B. subtilis* (19 - 21), where integration of a single stranded fragment of the plasmid with the genomic DNA possibly at gaps (8, 22), generating the restriction fragments of varied lengths. Since the southern blot analysis revealed hybridization bands of varied sizes, the mode of recombination is predicted to be of non campbell type (fig. 1). This mode of recombination results in generation of tandem repeats (8) which are known to undergo deletions (19), and a deletion in the essential region for enzyme expression may result in decrease or loss of enzyme activity. The strain CII 14 showed lower xylanase production than AT *Bacillus* (table 1), which may be due to deletion in the xylanase gene region.

Xylanase production: AT *Bacillus* showed low levels of xylanase activity (1.3 - 2.6 U/ml) in media containing 1% (w/v) glucose, lactose or xylose. Both the integrant strains showed elevated levels of xylanase activities (2.8 - 3.2 U/ml) which leveled off after 14 h fermentation. These results suggest that there is marked enhancement in the constitutive xylanase production by the integrants at least in the initial phase of fermentation.

In case of AT *Bacillus* xylan is a poor inducer (5) producing 6.2 /ml of xylanase activity. The integrant strains CII 11 and CIII 6 showed xylanase production of 10.5 and 12.0 U/ml respectively in 28 h of fermentation in a xylan containing medium. The maximum xylanase productivity (U/ml/h) also coincided with the maximum production (Fig. 4 A). AT *Bacillus* and the integrants CII 11 and CIII 6 showed maximum xylanase activity of 66, 96 and 128 U/ml respectively in a medium containing wheat bran in 20 h of fermentation period. The integrants showed maximum productivities at 16 h of fermentation time (Fig. 4 B). The integrants showed stable growth and xylanase production up to 10 slant to slant subcultures. The protein and the specific activity data (Table 1) of the extracellular xylanases of AT *Bacillus* and the integrants indicated that there are no significant differences in the sp. activities when xylan medium was used. On wheat bran medium highest sp. activities were obtained at 16 h, which decreased later on due to increase in the protein synthesis and decrease in xylanase productivity.

The present studies demonstrated a simple method of gene amplification using pUC8 cloning vector. Gene integration may be advantageous in protein hyperproduction because of gene dosage effect (17, 19), efficient expression and stabilization of the protein product (11), and higher stability of the integrants as compared to plasmid bearing strains (11, 17). The AT *Bacillus* integrants seemed to show high stability and enhanced xylanases production which correlated with the gene dosage. Xylanase

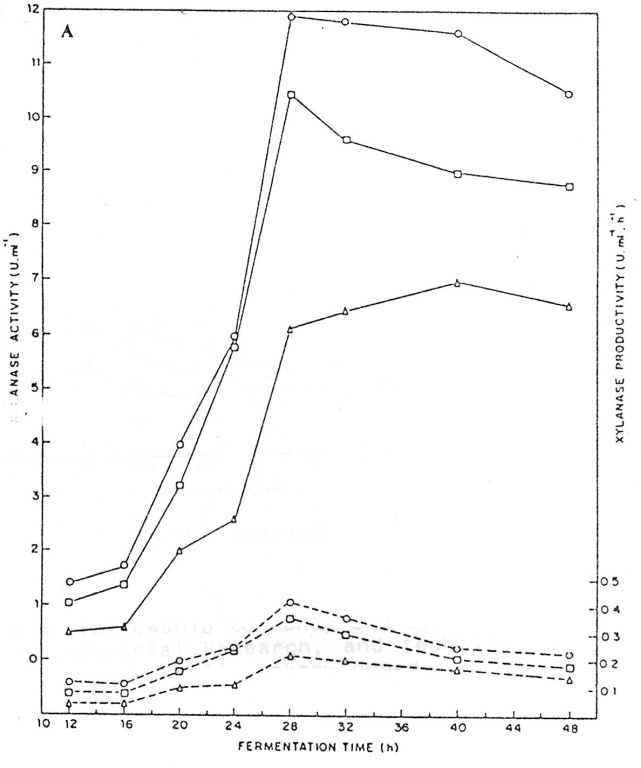

FIG. 4. Xylanase production of AT *Bacillus* and the integrants CII 11 and CIIII 6 on media containing (A) xylan and (B) wheat bran as a sole carbon source.
——— xylanase production (U/ml).
— — — xylanase productivity (U/ml/h).
Δ AT *Bacillus*, □ strain CII 11 and ○ strain CIII 6.

hyperproduction has been shown in case of *B. circulans* (23) and *B. pumilus* (24), but the productivity values reported are 0.29 and 0.11 U/ml/h respectively. Enhanced xylanase production has been achieved in alkaline *Aeromonas* (25), *Bacteroides ruminicola* (26), *Caldocellum saccharolyticum* (27), *Fibrobacter succinogenes* 135 (28) and *Streptomyces lividans* (2) using recombinant DNA techniques. Although these recombinants showed 1.2-80 fold higher yields of xylanases as compared to the parent strains, the actual productivity values were in the low range of 0.1 -0.3 U/ml/h. The recombinant *S. lividans* showed maximum production of 380 U/ml/72 which corresponds to 5.3 U/ml/h productivity. The CIII6 strain of AT *Bacillus* derived by gene integration showed stable growth and production of 7.4 U/ml/h xylanase activity. This may be biotechnologically important for large scale xylanase production and can be used for further gene amplification by the same method.

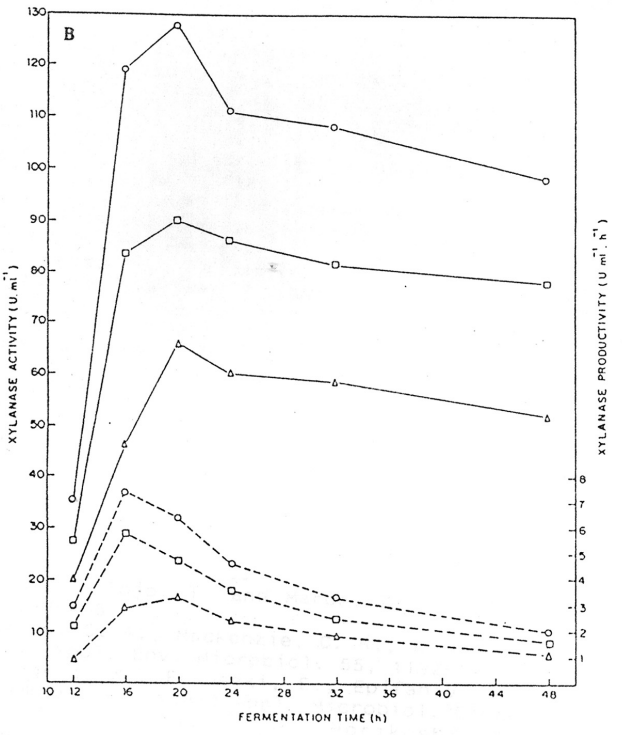

FIG. 4 - Continued

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