

Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology and Application

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Abstract

Xylanases are hydrolases depolymerising the plant cell wall component-xylan, the second most abundant polysaccharide. The molecular structure and hydrolytic pattern of xylanases have been reported extensively and mechanism of hydrolysis has also been proposed. There are several models for the gene regulation of which the present revealing could add to the wealth of knowledge. Future work on the application of these enzymes in paper and pulp, food industry, in environmental science i.e. bio-fuelling, effluent treatment and agro-waste treatment, etc. require a complete understanding of the functional and genetic significance of the xylanases. However, the thrust area has been identified as the paper and pulp industry. The major problem in the field of paper bleaching is the removal of lignin and its derivatives, which are linked to cellulose and xylan. Xylanases are more suitable in paper and pulp industry than lignin degrading systems.

KEY WORDS

Xylanase, Cellulase, *Bacillus*, Paper and pulp industries, Carbohydrate Binding Modules, Gene regulation

I. Introduction

Xylan, the second most abundant polysaccharide and a major component in plant cell wall consists of β -1,4-linked xylopyranosyl residues. The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated.¹⁻² Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%)- a group of carbohydrates in which xylan forms the major class- and lignin (20-30%). Xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronosyl and α -arabinofuranosyl residues linked to the backbone of β -1, 4, -linked xylopyranose units and has binding properties mediated by covalent and non-covalent interactions with lignin, cellulose and other polymers. Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucuronic acid residues.¹ The depolymerisation action of endo-xylanase results in the conversion of the polymeric substance into xylooligosaccharides and xylose. Xylanases are fast becoming a major group of industrial enzymes finding significant application in paper and pulp industry. Xylanases are of great importance to pulp and paper industries as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent.³ Viikari *et al.*⁴ were the first to demonstrate that xylanases are applicable for delignification in bleaching process. The applicability of xylanases increases day by day as Rayon, cellophane and several chemicals like cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethyl cellulose, methyl and ethyl

cellulose) are all produced from the dissolving pulp i.e. the pure form of cotton fibre freed from all other carbohydrates.

The importance of xylanases is not bound to the paper and pulp industry and there are other industries with equal importance of applicability. Potential applications of xylanases also include bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in consistency of beer and the digestibility of animal feed stock.⁵ Application of xylanase in the saccharification of xylan in agrowastes and agrofoods intensifies the need of exploiting the potential role of them in biotechnology. In all these cases xylan hydrolysis forms a chief factor. Thus a compendium of international xylanase research conducted during the past four decades is necessary for the analysis of future exploitation of xylanase technology. Most of the studies on xylanases were focused on only one single aspect of xylanase technology. The objective of this review is to discuss the properties and molecular biology of xylanases, genetics of microorganisms producing xylanases and applications.

Xylan, one of the major components of hemicelluloses found in plant cell wall is the second most abundant polysaccharide next to cellulose.⁶ The term hemicelluloses refer to plant cell wall polysaccharides that occur in close association with cellulose and glucans. In fact, the plant cell wall is a composite material in which cellulose, xylan and lignin are closely linked. Xylan, having a linear backbone of β -1, 4-linked xyloses is present in all terrestrial plants and accounts for 30% of the cell wall material of annual plants, 15-30% of hard woods and 7-10% of soft woods. Xylan is a heteropolysaccharide having O-acetyl, arabinosyl and 4-O-methyl-D-glucuronic acid substituents.¹

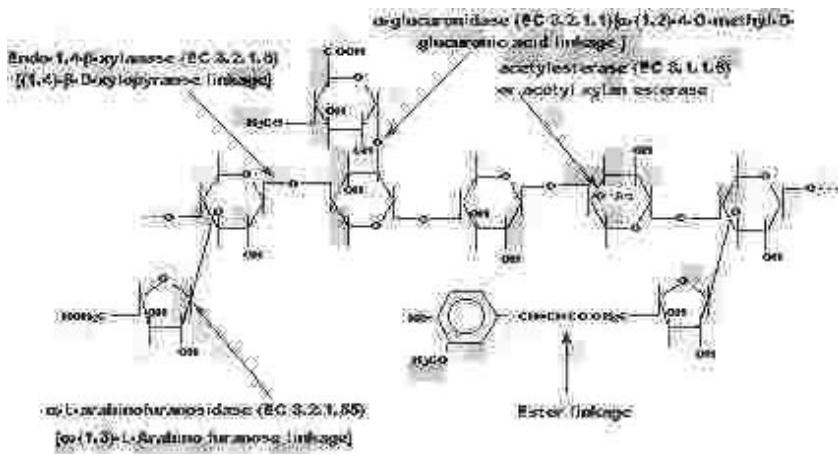


Fig. 1. Structure of arabinoxylan from grasses. The substituents are: Arabinose, 4-O-methyl-D-glucuronic acid, O-Ac (Acetyl group) and there is also ester linkage to phenolic acid group.¹

Similar to most of the other polysaccharides of plant origin xylan displays a large polydiversity and polymolecularity. It is present in a variety of plant species distributed in several types of tissues and cells. However, all terrestrial plant xylans are characterised by a β -1, 4-linked D-xylopyranosyl main chain

carrying a variable number of neutral or uronic monosaccharide subunits or short oligosaccharide chains. In the case of soft wood plants, xylan is mainly arabino-4-O-methyl glucuronoxylan which in addition to 4-O-methyl glucuronic acid is also substituted by α -arabinofuranoside units linked by α -1, 3-linkage to the xylan backbone and the ratio of arabinose side groups to xylose residue is 1:8. Rarely, acetyl groups are attached to the softwood xylan. The reducing ends of the xylan chains are reported to be linked to rhamnose and galacturonic acid in order to make alkali resistant end groups of xylan chain. Arabinoxylan is usually found in *Poaceae* (Fig. 1). Similar to other biopolymers xylan is also capable of forming intrachain hydrogen bonding, which supports a two fold extended ribbon like structure.⁷ The β -(1-4) D-xylan chain is reported to be more flexible than the two fold helix of β -(1-4) cellulose as there is only one hydrogen bond between adjacent xylosyl residues in contrast with two hydrogen bonds between adjacent glycosyl residues of cellulose. The absence of primary alcohol functional group external to the pyranoside ring as in cellulose and mannan has a dramatic effect on the intra and inter chain hydrogen bonding interactions. Intra-chain hydrogen bonding is occurring in unsubstituted xylan through the O-3 position which results in the helical twist to the structure. Nevertheless, the acetylation, and substitution disrupt and complicate this structure.⁸ An arabinose to xylose ratio of 0.6 is usually found in wheat water-soluble xylans.⁹

The most abundant hemicellulose in hard wood is *O*-acetyl-(4- *O*-methylglucurono) xylan. The backbone of this hard wood xylan consists of β -(1-4)-D-xylopyranose residues, with, on average, one α -(1-2)-linked 4- *O*-methyl glucuronic acid substituent per 10-20 such residues. Approximately 60-70% of the xylose units are esterified with acetic acid at the hydroxyl group of carbon 2 and/or 3 and on an average every tenth xylose unit carries an α -1,2-linked uronic acid side groups.^{1,9-11}

There are reports regarding covalent lignin carbohydrate bonds by means of ester or ether linkages to hemicelluloses but the covalent attachment to cellulose is less certain. In most primary plant cell walls, xyloglucans form the interface between the cellulose microfibrils and the wall matrix, but in some monocots (eg. Maize) this position is occupied by glucurono arabinoxylans. Finally the hemicelluloses are further associated with pectins and proteins in primary plant cell walls and with lignin in secondary walls, exact composition of which varies between organism and with cell differentiation.^{1,8,12}

II. Xylanolytic enzymes

The complex structure of xylan needs different enzymes for its complete hydrolysis. Endo-1, 4- β -xylanases (1,4- β -D-xylanxylanohydrolase, E.C.3.2.1.8) depolymerise xylan by the random hydrolysis of xylan backbone and 1,4- β -D-xylosidases (1,4, β -D-xylan xylohydrolase E.C.3.2.1.37) split off small oligosaccharides. The side groups present in xylan are liberated by α -L-arabinofuranosidase, α -D-glucuronidase, galactosidase and acetyl xylan esterase (Fig. 1).

Table1. A comparison of cellulase-poor / cellulase-free xylanase producing microorganisms.

Microorganism	Xylanase IU/ml	Cellulase (IU/ml)		Reference
		FPase	CMCase	
FUNGI				
<i>Aspergillus awamori</i> VTT-D-75028	12.00	0.10	3.20	13
<i>Aspergillus niger</i> KKS	138	3.90	1.20	14
<i>Aspergillus niger</i> sp.	76.60	-	-	15
<i>Fusarium oxysporum</i> VTT-D-80134	3.70	0.10	0.70	13
<i>Thermomyces lanuginosus</i> strain	3576	-	-	16
<i>Phanerochate chrysosporium</i>	15-20	-	1.80-2.40	17
<i>Piromyces</i> sp. strain E 2	7.96	0.01	0.77	18
<i>Schizophyllum commune</i>	1244	65.30	5.00	19
<i>Talaromyces emersonii</i> CBS 814.70	56	26.70	2.41	20
<i>Thermomyces lanuginosus</i> ^a	650-780	0.01	0.01	21
<i>Trichoderma reesei</i> RUT C-30 ATCC 56765 ^a	400	-	6.00	22
<i>Trichoderma reesei</i> ^b	960	0.70	9.60	23
<i>Trichoderma viride</i>	188.10	0.55	-	24
BACTERIA				
<i>Bacillus</i> SSP-34	506	0.40	0.20	25
<i>Bacillus circulans</i>	400	0.05	1.38	26
<i>Bacillus stearothermophilus</i> StrainT6 ^a	2.33	-	0.02	3 27 28
<i>Bacillus</i> sp.	120	-	0.05	29
<i>Bacillus</i> sp.	11.50	-	1.2±0.13	30
<i>Bacillus</i> sp. strain NCL 87-6-10	93	-	-	31
<i>Bacillus circulans</i> AB 16	19.28	-	-	32
<i>Bacillus stearothermophilus</i> SP	0.35-0.6	-	-	33
<i>Clostridium absonum</i> CFR – 702	~ 258	0	0	34
<i>Rhodothermus marinus</i> ^a	1.8-4.03	0.05	0.025	35, 36
<i>Streptomyces cuspidosporus</i>	22-35	-	0.29	37
<i>Streptomyces roseiscleroticus</i> NRRL-B-11019 ^{a,c}	16.20	-	0.21	38
<i>Streptomyces</i> sp.	3.50	0	0	39
<i>Streptomyces</i> sp. QG-11-3	96	-	-	40
<i>Thermoactinomyces thalophilus</i> sub group C	42	0	0	41

a. Microorganisms reported to be producing `virtually` cellulase-free xylanases

b. Cellulase assay was performed using hydroxyethyl cellulose.

c. Cellulase assay carried out using 1 % acid swollen cellulose prepared from Solca floc SW 40 wood pulp cellulose

d. In Some cases either FPase or CMCase is not detected or absent.

Endo-xylanases are reported to be produced mainly by microorganisms (Table1); many of the bacteria and fungi are reported to be producing xylanases.^{5,7} However, there are reports regarding xylanase origin from plants i.e. endo-xylanase production in Japanese pear fruit during the over-ripening period and later Cleemput *et al.*⁴² purified one endo-xylanase with a molecular weight of 55 kDa from the flour of European wheat (*Triticum aestivum*). Some members of higher animals, including fresh water mollusc are able to produce xylanases.⁴³ There are lots of reports on microbial xylanases starting from 1960: Nevertheless, these reports have given prime importance to plant pathology related studies.^{25,44} Only during 1980's the great impact of xylanases has been tested in the area of biobleaching.⁴

Exo-1,4- β -D-xylosidase (EC 3.2.1.37) catalyses the hydrolysis of 1,4- β -D-xylo-oligosaccharides by removing successive D-xylose residues from the non-reducing end. The endoxylanases reported to release xylose during hydrolysis of xylan do not have any activity against xylobiose, which could be easily hydrolysed by β -xylosidases. There are reports regarding *Bacillus* sp.⁴⁵ and different fungi⁴⁶ producing intracellular β -xylosidases.

α -Arabinofuranosidases (EC 3.2.1.55) hydrolyse the terminal, non-reducing α -L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans. A number of microorganisms including fungi, actinomycetes and other bacteria have been reported to produce α -arabinosidases. The extreme thermophile *Rhodothermus marinus* is reported to produce α -L-arabinofuranosidase with a maximum yield of 110 nkat/ml (6.6 IU/ml).⁴⁷ Two different polypeptides with α -arabinofuranosidase activity from *Bacillus polymyxa* were characterised at the gene level for the production of α -arabinofuranosidases.⁴⁸

α -D-glucuronidases (EC 3.2.1.1) are required for the hydrolysis of the α -1, 2-glycosidic linkages between xylose and D-glucuronic acid or its 4-O-methyl ether linkage (Figs. 1). The hydrolysis of the far stable α -(1,2)-linkage is the bottleneck in the enzymatic hydrolysis of xylan and the reported α -glucuronidases have different substrate requirements. Similar to lignin carbohydrate linkage, 4-O-methyl-glucuronic acid linkage forms a barrier in wood degradation. There are number of microorganisms reported to be producing α -glucuronidases.⁴⁹

The complete hydrolysis of natural glucuronoxylans requires esterases to remove the bound acetic and phenolic acids (Fig. 1). Esterases break the bonds of xylose to acetic acid [acetyl xylan esterase (EC 3.1.1.6)], arabinose side chain residues to ferulic acid (feruloyl esterase) and arabinose side chain residue to p-coumaric acid (p-coumaroyl esterase). Cleavage of acetyl, feruloyl and p-coumaroyl groups from the xylan are helpful in the removal of lignin. They may contribute to lignin solubilisation by cleaving the ester linkages between lignin and hemicelluloses. If used along with xylanases and other xylan degrading enzymes in biobleaching of pulps the esterases could partially disrupt and loosen the cell wall structure.¹

III. Xylanase producing microorganisms

Several microorganisms including fungi and bacteria have been reported to be readily hydrolysing xylans by synthesising 1,4- β -D endoxylanases (E.C. 3.2.18) and β -xylosidases (EC.3.2.1.37). According to many of the early reports pathogenicity of xylanase producers to plants was a unifying character and it was thought that β -xylanases together with cellulose degrading enzymes play a role during primary invasion of the host tissues.⁵⁰ There are reports regarding the induction of the biosynthesis of ethylene⁵¹ and two classes of pathogenesis-related proteins in tobacco plants by the microbial xylanases.⁵² Thus these points reveal that certain xylanases can elicit defence mechanisms in plants. These actions may be mediated by specific signal oligosaccharides, collectively known as oligosaccharins or it may be due to the functioning of enzymes themselves or their fragments as the elicitors.⁵³⁻⁵⁴ Most of the fungal plant pathogens produce plant cell wall polysaccharide degrading enzymes.^{25,44} These enzymes result in the softening of the region of penetration by partial degradation of cell wall structures. Xylanases have been reported in *Bacillus*,

Streptomyces and other bacterial genera that do not have any role related to plant pathogenicity.⁵⁰ Since the introduction of xylanases in paper and pulp and food industries^{4,6} there have been many reports on xylanases from both bacterial and fungal microflora⁷.

A . Bacterial Xylanases

Bacteria just like in the case of many industrial enzymes fascinated the researchers for alkaline thermostable xylanase producing trait. Noteworthy members producing high levels of xylanase activity at alkaline pH and high temperature are *Bacillus* spp. *Bacillus* SSP-34 produced higher levels of cellulase poor xylanase activity under optimum nitrogen condition.⁵⁵ This bacterium also produced minimal level of protease activity at the selected nitrogen source of yeast extract and peptone combination. *Bacillus* SSP-34 produced a xylanase activity of 506 IU/ml in the optimised medium.²⁵ Earlier Ratto *et al.*²⁶ reported xylanase with an activity of 400 IU/ml from *Bacillus circulans*. It had optimum activity at pH 7 and 40% of activity was retained at pH 9.2. However, the culture supernatant also showed low levels of cellulolytic activities with 1.38 IU/ml of endoglucanase (CMCase EC 3.2.1.4) and 0.05 U/ml of cellobiohydrolases. *Bacillus stearothermophilus* strain T6, reported to be producing cellulase free xylanases was actually having slight cellulolytic activity of 0.021 IU/ml.^{3,27,28} *Streptomyces cuspidosporus* produced 40-49 U/ml in xylan medium and was associated with cellulases (CMCase, 0.29 U/ml).³⁷ *Bacillus* sp. strain NCL 87-6-10 produced 93 U/ml of xylanase in the zeolite induced medium which was more effective than Tween 80 medium.³¹ Another *Bacillus* sp. *Bacillus circulans* AB 16 produced 19.28 U/ml of xylanase when grown on rice straw medium.³² *Streptomyces* sp. QG-11-3 was found to be producing both xylanase (96 U/ml) and polygalacturonase (46 U/ml).⁴⁰ *Rhodothermus marinus* was found to be producing thermostable xylanases of approximately 1.8-4.03 IU/ml but there was also detectable amounts of thermostable cellulolytic activities.^{35, 36} Most of the other bacteria which degrade hemicellulosic materials are reported to be potent cellulase producers and include *Streptomyces roseiscleroticus* NRRL-B-11019 (xylanase 16.2 IU/ml and cellulase 0.21 IU/ml).³⁸ The strict thermophilic anaerobe *Caldocellum saccharolyticum* possesses xylanases with optimum activities at pH values 5.5-6.0 and at temperature 70°C.⁵⁶ Mathrani and Ahring⁵⁷ reported xylanases from *Dictyoglomus* sp. having optimum activities at pH 5.5 and 90° C, however merits the significant pH stability at pH values 5.5-9.0. Detailed description of all other organisms producing cellulases along with xylanases are given in Table 1.

B. Fungal xylanases and associated problems.

There has been increased usage of xylanase preparations having an optimum pH ≤ 5.5 produced invariably from fungi (⁵⁸Subramaniyan and Prema, 2000). The optimum pH for xylan hydrolysis is around 5 for most of the fungal xylanases although they are normally stable at pH 3 - 8 (Table 2). Most of the fungi produce xylanases, which tolerate temperatures below 50°C. In general, with rare exceptions, fungi reported to be producing xylanases have an initial cultivation pH lower than 7. Nevertheless it is different in the case of bacteria (Table 1).

The pH optima of bacterial xylanases are in general slightly higher than the pH optima of fungal xylanases.²⁷ In most of the industrial applications, especially paper and pulp industries, the low pH required

Table 2 Characterisation of xylanases from different microorganisms

Microorganisms	Mol. Wt. (KDa)	Purification fold	Yield (%)	Optimum pH and Temperature		Stabilities at ^d		pI	Km (mg/ml)	Vmax (μmol / min / mg)	Reference
				pH	Temperature	PH (hrs)	Temp. (hrs)				
FUNGI											
<i>Acrophialophora nainiana</i>	22	0.98	1.6	7.0	55	-	60 (1)	-	16 - 40.91	-	59
<i>Aspergillus awamori</i>	39	-	-	5.5-6	55	-	-	5.7-6.7	1.0	10000	60
	23	-	-	5.0	50	-	-	3.7	0.33	3333	60
	26	-	-	4.0	45-50	-	-	3.3-3.5	0.09	455	60
<i>Aspergillus nidulans</i>	34	24	7.5	6	56	4.0-6.7	56	3.4	0.97	1091	61
<i>Aspergillus sojae</i>	32.7	8.2	9	5.0	60	5-8 (24)	50 (10 minutes)	3.5	-	-	62
	35.5	4.6	5	5.5	50	5-8 (24)	35 (10 minutes)	3.75	-	-	62
<i>Aureobasidium pullulans Y-2311-1</i>	25	5.8	10.3	4.8	54	4.5	50	9.4	7.6	2650	63
<i>Aureobasidium pullulans ATCC 42023</i>	21	38	6.3	3-4.5	35	-	-	-	2.93	866	64
<i>Cephalosporium sp. strain RYM-202</i>	35	17.3	9.9	7.5-8.0	50	-	-	6.3	5.26	118.4	65)
	24	22.9	15.0	7.5-8.0	50	-	-	4.4	4.16	145.2	66
<i>Erwinia chrysanthemi</i>	42	19.9	3.12	5.5	55	4-7	35	8.8	-	-	66
<i>Humicola insolens</i>	6.0	-	-	6-6.65	55-60	-	-	9.0	-	-	67
	21	-	-	6-6.5	55-60	-	-	7.7	-	-	67
<i>Penicillium purpurogenum</i>	33	15.8	5.7	7.0	60	6.0-7.5 (24)	40 (3)	8.6	-	-	68
	23	5	4.3	3.5	50	4.5-5.5 (24)	40 (3)	5.9	-	-	68
<i>Trichoderma longibrachiatum</i>	37.7	55.8	5.1	5-6	45	5	-	-	10.14	4025	69
<i>Trichoderma viride</i>	22	16	12.5	5	53	-	-	9.3	4.5	160	70
<i>Trichoderma harzianum</i>	20	7.5	-	5.0	50	-	40	-	0.58	0.106	71

Table 2 Contind.

Microorganisms	Mol. Wt. (KDa)	Purification fold	Yield (%)	Optimum pH and Temperature		Stabilities at ^d		pI	Km (mg/ml)	Vmax (μmol / min / mg)	Reference
				pH	Temperature	pH (hrs)	Temp. (hrs)				
BACTERIA											
<i>Aeromonas caviae</i> ME1	20	-	-	7	50	3.0-4.0	6.5-8	7.1	9.4	4330	72
<i>Bacillus amyloliquefaciens</i>	18.5-19.6	7.3	53.9	6.8-7.0	80	9	50	10.1	-	-	73
<i>Bacillus circulans</i> WL-12	85	-	-	5.5-7	-	-	-	4.5	8	-	50
<i>Bacillus sp.</i> Strain SPS-0	99	36	25	6.0	75	-	70 (4)		0.7	145	
<i>Bacillus sp.</i> W1 (JCM2888)	21.5	124.6	25	6	65	4.5-10	-	8.5	4.5	-	74
	49.5	9.6	2.6	7.9	70	4.5-70	-	3.7	0.95	-	74
<i>Bacillus sp.</i> strain 41-1(36)	36	3.6	15.3	9	50	-	-	5.3	3.3	1100	75
<i>Bacillus sp.</i> strain TAR-1	40	-	-	6	75	-	-	4.1	-	-	76
<i>Bacillus sp.</i> strain K-1	23	-	-	5.5	60	12	50	-	-	-	77
<i>Bacillus stearothermophilus</i> T-6	43	38.9	46	6.5	75	-	70 (14.5 ^{1/2})	9	1.63	288	27
<i>Streptomyces</i> T-7	20.643	41.3	6.7	4.5-5.5	60	5.0 (144)	37 (264)	7.8	10	7600	78
<i>Streptomyces sp.</i> No 3137	50	48	33	5.5-6.5	60-65	5.5-6.5	55	7.1	9.1 ^a	-	79
	25	2.85	2	5.0-6.0	60-65	5.0-6.0	55	10.06	-	-	..
	25	3.6	8	5.0-6.0	60-65	5.0-6.0	55	10.26	11.2 ^a	-	..
<i>Thermotoga maritima</i>		217	54	6.5	85		95 (12 ^{1/2})				
<i>Thermotoga thermarum</i>	266 ^b	22.5	16.2	6	80	-	-	-	0.36	1.18	80
	35 ^c	1.9	1.5	7	90-100	-	-	-	0.24	19.5	..

a. Km estimated on xylotetrose. b. Dimer of 105 kDa and 150 kDa. c. Monomer

d. The stability in hours was given in bracket. Numbers preceding ^{1/2} represents the half-life time.

for the optimal growth and activity of xylanase necessitates additional steps in the subsequent stages which make fungal xylanases less suitable. Although high xylanase activities were reported for several fungi, the presence of considerable amount of cellulase activities and lower pH optima make the enzyme less suitable for pulp and paper industries. Gomes *et al.*²⁴ reported xylanase activity (188.1 U/ml-optimum pH 5.2) and FPase activity (0.55 U/ml-optimum pH 4.5) from *Trichoderma viride*. Similar to *T. viride*, *T. reesei* was also known to produce higher xylanase activity - approximately 960 IU/ml - and cellulase activity - 9.6 IU/ml.²³ Like *Trichoderma* spp., *Schizophillum commune* is also one of the high xylanase producers with a xylanase activity of 1244 U/ml, CMCase activity of 65.3 U/ml and FPase activity of 5.0 U/ml.¹⁹ Among white rot fungi, a potent plant cell wall degrading fungus - *Phanerochaete chrysosporium* produced a xylanase activity of 15-20 U/ml in the culture medium, but it also produced high amounts of cellulase activity measuring about 12% of maximum xylanase activity.¹⁷ Singh *et al.*,¹⁶ reported a xylanase activity of 59,600 nkat/ml (approximately 3576 U/ml) from *Thermomyces lanuginosus* strain. *Aspergillus niger* sp. showed only 76.60 U/ml of xylanase activity after 5.5 days of fermentation.¹⁵ Reports on fungal xylanases with negligible cellulolytic activity are very rare like the *Thermomyces lanuginosus* xylanase with a trace cellulase activity of 0.01 U/ml.²¹ All other fungal strains were showing considerable levels of cellulase activities (Table 1). Another major problem associated with fungi is the reduced xylanase yield in fermenter studies. Agitation is normally used to maintain the medium homogeneity, but the shearing forces in fermenter can disrupt the fragile fungal biomass leading to the reported low productivity.⁵⁸ Higher rate of agitation speed leading to hyphal disruption may decrease xylanase activities.

Even though there are differences in the growth conditions including pH, agitation and aeration, and optimum conditions for xylanase activity^{17,19, 21,23,24,26,38,58,82,83} there is considerable overlapping in the molecular biology and biochemistry of prokaryotic and fungal xylanases.⁸⁴

IV. Classification of xylanases

Wong *et al.*⁵ classified microbial xylanases into two groups on the basis of their physicochemical properties such as molecular mass and isoelectric point, rather than on their different catalytic properties. While one group consists of high molecular mass enzymes with low pI values the other of low molecular mass enzymes with high pI values, but exceptions are there. The above observation was later found to be in tune with the classification of glycanases on the basis of hydrophobic cluster analysis and sequence similarities.⁸⁵

The high molecular weight endoxylanases with low pI values belong to glycanase family 10 formerly known as family 'F' while the low molecular mass endoxylanases with high pI values are classified as glycanase family 11 (formerly family G).⁸⁶ Recently there has been the addition of 123 proteins in Family 11 out of which 113 are xylanases/ORFs for xylanases, 1 unnamed protein and 9 sequences from US patent collection. But, 150 members are present in family 10 of which 112 are having xylanase activities. (<http://afmb.cnrs-mrs.fr/~cazy/index.html>). Biely *et al.*⁸⁷ after extensive study on the differences in catalytic properties among the xylanase families concluded that endoxylanases of family 10 in contrast to the members of family 11 are capable of attacking the glycosidic linkages next to

the branch points and towards the non-reducing end.⁸⁸ While endoxylanases of family 10 require two unsubstituted xylopyranosyl residues between the branches, endoxylanases of family 11 require three unsubstituted consecutive xylopyranosyl residues. According to them endoxylanases of family 10 possess several catalytic activities, which are compatible with β -xylosidases. The endoxylanases of family 10 liberate terminal xylopyranosyl residues attached to a substituted xylopyranosyl residue, but they also exhibit aryl- β -D-xylosidase activity. After conducting an extensive factor analysis study Sapag *et al.*⁸⁵ applied a new method without referring to previous sequence analysis for classifying Family 11 xylanases, which could be subdivided in to six main groups. Groups I, II and III contain mainly fungal enzymes. The enzymes in groups I and II are generally 20 kDa enzymes from *Ascomyceta* and *Basidiomyceta*. The group I enzymes have basic pI values while those of group II exhibit acidic pI. Enzymes of group III are mainly produced by anaerobic fungi. Meanwhile, the bacterial xylanases are divided in to three groups (A, B and C). Group A contains mainly enzymes produced by members of the *Actinomycetaceae* and the *Bacillaceae* families, strictly aerobic gram-positive ones. Groups B and C are more closely related and contain mainly enzymes from anaerobic gram-positive bacteria, which usually live in the rumen. Xylanases from aerobic gram-negative bacteria are found in subgroup Ic as they closely resemble the fungal enzymes of group I. Unlike previous classifications they also reported a fourth group of fungal xylanases consisting of only two enzymes.⁸⁵

V. Multiple forms of xylanases

Streptomyces sp. B-12-2 produces five endoxylanases when grown on oat spelt xylan.⁸⁹ The culture filtrate of *Aspergillus niger* was composed of 15, and *Trichoderma viride* of 13 xylanases.⁸⁷ The most outstanding case regarding multiple forms of xylanases was production of more than 30 different protein bands separated by analytical electrofocusing from *Phanerochaete chrysosporium* grown in Avicel.⁹⁰ There are several reports regarding fungi and bacteria producing multiple forms of xylanases.^{5,91} The filamentous fungus *Trichoderma viride* and its derivative *T. reesii* produce three cellulase free β -1, 4-endoxylanases.⁶ Due to the complex structure of heteroxylans all of the xylosidic linkages in the substrates are not equally accessible to xylan degrading enzymes. Because of the above hydrolysis of xylan requires the action of multiple xylanases with overlapping but different specificities.⁵

The fact that protein modification (e.g. post translational cleavage) leads to the genesis of multi-enzymes has been confirmed by various reports.^{92,93} Leathers^{92,94} identified one xylanase, APXI with a molecular weight of 20 kDa and later another xylanase APX II (25 kDa) was purified by Li *et al.*⁶³ from the same organism *Aureobasidium*. However, according to Liang *et al.* APXI and APXII are encoded by the gene xyn A. This suggestion was based on their almost identical N-terminal amino acid sequences, immunological characteristics and regulatory relationships and the presence of a single copy of the gene and the transcript.⁹³ Purified APX I and APX II from *Aureobasidium pullulans* differ in their molecular weights. Post-translational modifications such as glycosylation, proteolysis or both could contribute to this phenomenon.^{63,92} Therefore several factors could be responsible for the multiplicity of xylanases. These include differential mRNA processing, post-secretional modification by proteolytic digestion, and post-

translational modification such as glycosylation and autoaggregation.⁶ Multiple xylanases can also be the product from different alleles of the same gene.⁵ However, some of the multiple xylanases are the result of independent genes.⁴⁹

VI. Purification and properties of xylanases

Column chromatographic techniques, mainly ion exchange and size exclusion are the generally utilised schemes for xylanase purification, but there are also reports of purification with hydrophobic interaction column chromatography.⁹⁵ There are several reports regarding the purification of xylanases to electrophoretic homogeneity, however, the yield and purification fold varies in different cases (Table 2). In all the cases the culture supernatants are initially concentrated using precipitation or ultrafiltration techniques. A moderately thermostable xylanase was purified from *Bacillus* sp. Strain SPS-0 using ionexchange, gel and affinity chromatographies.⁹⁶ Thermostable xylanases from thermophilic organisms like *Dictyoglomus* and *Thermotoga* spp which grow at a temperature higher than 80°C could be easily purified by the inclusion of one additional step of heating.⁸⁰ Use of cellulose materials as the matrix in column chromatography is impaired by the fact that certain xylanases are having cellulose binding domains, which will interact with the normal elution process.⁷⁷ Takahashi *et al.*,⁹⁷ purified a low molecular weight xylanase (23 kDa) from *Bacillus* sp. strain TAR-1 using CM Toyopearl 650 M column. This xylanase with optimum activity at 70°C had broad pH profile. Kimura *et al.*⁹⁸ purified *Penicillium* sp. 40 xylanase with molecular weight 25 kDa which was induced by xylan and repressed by glucose.

VII. Structure of Xylanases

The three-dimensional structure of family 10 and 11 endoxylanases has been determined for several enzymes, from both bacteria and fungi. The endoxylanase 1BCX from *Bacillus Circulans* is having the features of Family 11.^{8, 12} The catalytic domain folds into two β sheets (A and B) constituted mostly by antiparallel β strands and one short α helix and resembles a partly closed right hand.⁸⁵ The differences in catalytic activities of endoxylanases of family 10 and 11 can be attributed to the differences in their tertiary structure. The family 11 endoxylanases are smaller and are well packed molecules with molecular organisation mainly of β -pleated sheets.^{99,100} The catalytic groups present in the cleft accommodate a chain of five to seven xylopyranosyl residues. There is a strong correlation in that the residue hydrogen bonded to the general acid/base catalyst at position 100 is Asparagine in the so-called 'alkaline' xylanases, where as it is aspartic acid in those with more acidic pH optimum. Thermostability is an important property due to their proposed biotechnological applications. Thermophilic nature and thermostability may be explained by a variety of factors and structural parameters. Of these, the importance of S---S bridges and aromatic sticky patches can be analysed by sequence alignment. However, Sapag *et al.*⁸⁵ showed that S---S bridges are unlikely to be of importance in the thermophily of family 11 xylanases. The overall structure of the catalytic domain of family 10 xylanase is an eight -folded barrel.¹⁰¹ The substrate binds to the shallow groove on the bottom of the 'bowl'. The (α / β) barrel appears to be the structure of two other endoxylanases of family 10. The substrate binding sites of the family 10 endoxylanases are apparently not such deep cleft as the substrate binding sites of family 11 endoxylanases. This fact together with a possible

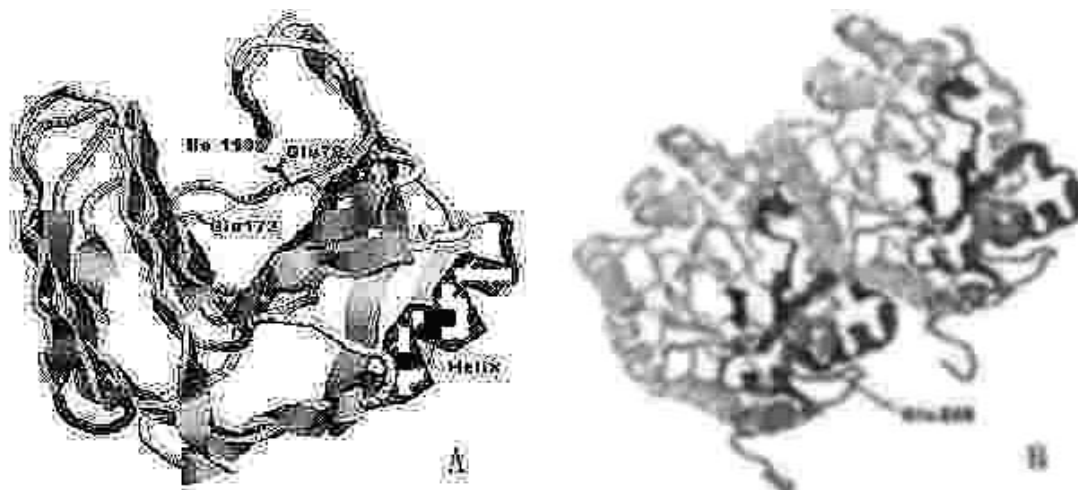


Fig. 2. The three-dimensional structures of A. Family 11 xylanase (PDB Code - 1BCX) from *Bacillus circulans*. β -pleated structure is present more than 50% while extreme right side structure denotes α -helical structure. Glu 172 and Glu 78 are at the catalytic site. B. Family 10 Xylanase (PDB Code - 1IE0X) from *Streptomyces Lividans*. Glu 236 forms covalent link with the substrate. (Courtesy, Protein Data Bank – PDB) <http://www.rcsb.org/pdb/index.html>

greater conformational flexibility of the larger enzymes than of the smaller ones may account for a lower substrate specificity of family 10 endoxylanases.^{8,12}

VIII. Catalytic sites

The structure of *Bacillus* 1,4- β -xylanases as mentioned earlier, have a cleft, which according to Torronen *et al.*⁹⁹ can be the active site. There are two members from the family 11 xylanases, (XYNII from *Trichoderma harzianum* and 1XNB from *Bacillus circulans*) which clearly show this kind of catalytic sites.^{99,102} The *Bacillus circulans* xylanase has two proximal carboxylates, Glu 172 and Glu 78, which act as an acid catalyst and nucleophile respectively.⁹⁹ The abnormally high pKa of Glu 172, the character that enabled Glu172 to act as acid catalyst is resulting from the electrostatic interactions with neighbouring groups like the Arg 112¹⁰², (Fig. 2A). Endo-1,4- β xylanase of the F10 xylanases is having a cylindrical [α]/ [β] barrel resembling a salad bowl with the catalytic site at the narrower end, near the C-terminus of the barrel⁸⁶ and there are five xylopyranose binding sites (Fig. 2B). The high molecular weight F10 xylanases tend to form low DP oligosaccharides. Xylanase cex from *Cellulomonas fimi* has a catalytic (N-terminus) region and a cellulose-binding domain (C-terminus), the former resembling the head and the latter the tail of a tadpole structure (¹⁰³White *et al.*, 1994).

The members of family F11 have catalytic domains formed from β -pleated sheets that form a two layered trough surrounding the catalytic site.⁸ The trough has been likened to the palm and fingers while the loop resembles the thumb of the right hand. The loop protrudes into the trough and terminates in an isoleusin.^{8, 12}

Xylanases possess three to five subsites for binding the xylopyranose rings in the vicinity of the catalytic site. Meagher *et al.*¹⁰⁴ observed five pyranose binding sites in *Trichoderma reesei* Xyn2, while three were found in Xyn I. The subsites for binding xylopyranose residues are defined by the presence of tyrosine as opposed to tryptophan.^{100,105} Tryptophan, essential for substrate binding in most glycosides, is not reported to have a role in xylanase action. Xylanase from *Pseudomonas fluorescens* binds to the substrate - a xylopentose at sites ⁻¹ to ⁺⁴.¹⁰¹

IX. Carbohydrate binding modules (CBMs)

Most of the plant cell wall hydrolysing enzymes typically comprises a catalytic module and one or more carbohydrate binding modules (CBMs) that bind to a plant cell wall polysaccharide (Hachem *et al.* 2000¹⁰⁶). The justifiable function of these substrate-binding domains is to allow unerring alignment of the soluble enzyme with the insoluble polysaccharide, thereby increasing enzyme concentration at the point of attack. However, they are not essential for hydrolysis of the substrate.¹⁰⁷ Binding of CBMs to insoluble substrates was significantly enhanced by the presence of Na⁺ and Ca²⁺ ions. However, these binding modules were not having any contribution with synergistic effects on xylan hydrolysis. The CBMs are classified into different families based mostly on a comparison of primary structure with previously characterised sequences. Many of the modules in this classification system are not functionally characterised and their precise roles in hemicellulose hydrolysis are not yet fully understood. Of the different families of CBMs (more than ten) family 4 include thermostable *Rodothermus marinus* xylanase CBM with affinity for both insoluble xylan and amorphous cellulose.¹⁰⁶ CBMs attach the enzyme to the plant cell wall and by bringing the enzyme into close and prolonged association with its recalcitrant substrate increase the rate of catalysis. CBMs have also been reported to display additional functions such as substrate disruption and sequestering and feeding of single polysaccharide chains into active site of the catalytic modules.¹⁰⁸ CBMs have been grouped into 23 different families, many of which are further divided into subfamilies.



.Fig. 3. Two classes of carbohydrate binding modules of xylanases bound to respective ligands. **A.** Cellulose binding domain (CBD) of hydrolase C_{ex}.¹⁰⁹ **B.** Structure of xylanase XBD1 xylan binding domain bound to a xylohexaose. Unlike CBD, the binding face is not planar, but instead forms a 'twisted' site with the TRP residues in an almost perpendicular arrangement. These aromatics are naturally oriented to form stacking interactions with two sugar rings in the xylan helix (Courtney, Simpson PJ, http://www.shef.ac.uk/uni/projects/nmr/PJS/xbd_x6.pdb). RasMol and Adobe Photoshop are used to generate the 3D structure.

Substrate binding domains are more common in F10 than in F11 xylanases. Although the overall fold of most CBMs is conserved, consisting of sandwiched β sheets, the topology of the actual substrate binding sites varies between families. Trp54 and Trp 72 play a central role in binding cellulose while Trp 17 might be less important for cellulose binding, but could participate in the binding of longer β -1, 4-glucans in cellulose.¹⁰⁹ CBDs usually have a planar binding face which is thought to complement the flat binding surface presented by the crystalline cellulosic substrate (Fig 3. A). In families 1, 2a, 3a, 5 and 10 the CBM is interacting preferentially with crystalline substrate. This cellulose binding domain (CBD) is a flat surface that contains a planar strip of highly conserved aromatic residues. The flat surface presumably enables the proteins to interact with the multiple planar chains found in crystalline cellulose. However these binding sites in members of family 4 and 22 have a shallow cleft like appearance that can accommodate only a single cellulose or xylan chain probably via a combination of stacking interactions and hydrogen bonding. Cellulose-binding domains (CBD) are found in several xylanases. (¹¹⁰Black *et al.*, 1997). The reason for the presence of CBD on plant cell wall hydrolases is possibly due to the performance of cellulose as a general receptor of plant cell wall hydrolases.¹¹⁰ It is the only non-variable structural polysaccharide in the cell wall of all plant species, although there are some marginal changes in the degree of crystallinity of cellulose. Tfx A binds both to cellulose and xylan. Recently there are increasing number of reports on xylan binding domains (XBDs) in family 11 (family G) xylanases. Xylan binding domain has been reported in endo-xylanase of *Bacillus* sp. Strain K-1.⁷⁷ The family F/10 xylanase from *Streptomyces olivaceoviridis* E-86⁸⁶ is having a XBD. The STX I and STX II xylanases from *Streptomyces violaceus* OPC-520 are having xylan binding domains.⁹¹ Recently the xylan-binding domain (XBD) was solved by NMR. The overall structure of the proteins is very similar to that of the CBDs of family 2a. The surface tryptophan of XBD are arranged in a perpendicular rather than planar orientation with respect to one another (Fig 3. B). This enables the XBDs to interact with the 3- fold helix of a xylan chain, rather than the planar chain found in cellulose Unlike CBD, the binding face is not planar, but instead forms a 'twisted' site with the TRP residues in an almost perpendicular arrangement. These tryptophan residues are naturally oriented to form stacking interactions with two sugar rings in the xylan helix. Binding is mediated *via* several co-planar, solvent-exposed aromatic rings which form stacking interactions with the sugars in the polysaccharide and also through hydrogen bonding.¹⁰⁸

X. Mode of Action of Xylanases

Several models have been proposed to explain the mechanism of xylanase action. Xylanase activity leads to the hydrolysis of xylan. Generally hydrolysis may result either in the retention or inversion of the anomeric centre of the reducing sugar monomer of the carbohydrate. This suggests the involvement of one or two chemical transition states. Glycosyl transfer usually results in nucleophilic substitution at the saturated carbon of the anomeric centre and take place with either retention or inversion of the anomeric configuration. Most of the polysaccharide hydrolyzing enzymes like cellulases and xylanases are known to

hydrolyse their substrates with the retention of the C1 anomeric configuration. There is the involvement of double displacement mechanism for the anomeric retention of product.¹¹¹ The double displacement mechanism involves the following features;

- (i) an acid catalyst which protonates the substrate
- (ii) a carboxyl group of the enzyme positioned on
- (iii) a covalent glycosyl enzyme intermediate with this carboxylate in which the anomeric configuration of the sugar is opposite to that of the substrate.
- (iv) this covalent intermediate is reached from both directions through transition states involving oxo carbonium ions.
- (v) various non-covalent interactions providing most of the rate enhancement¹¹¹ (Fig. 3).

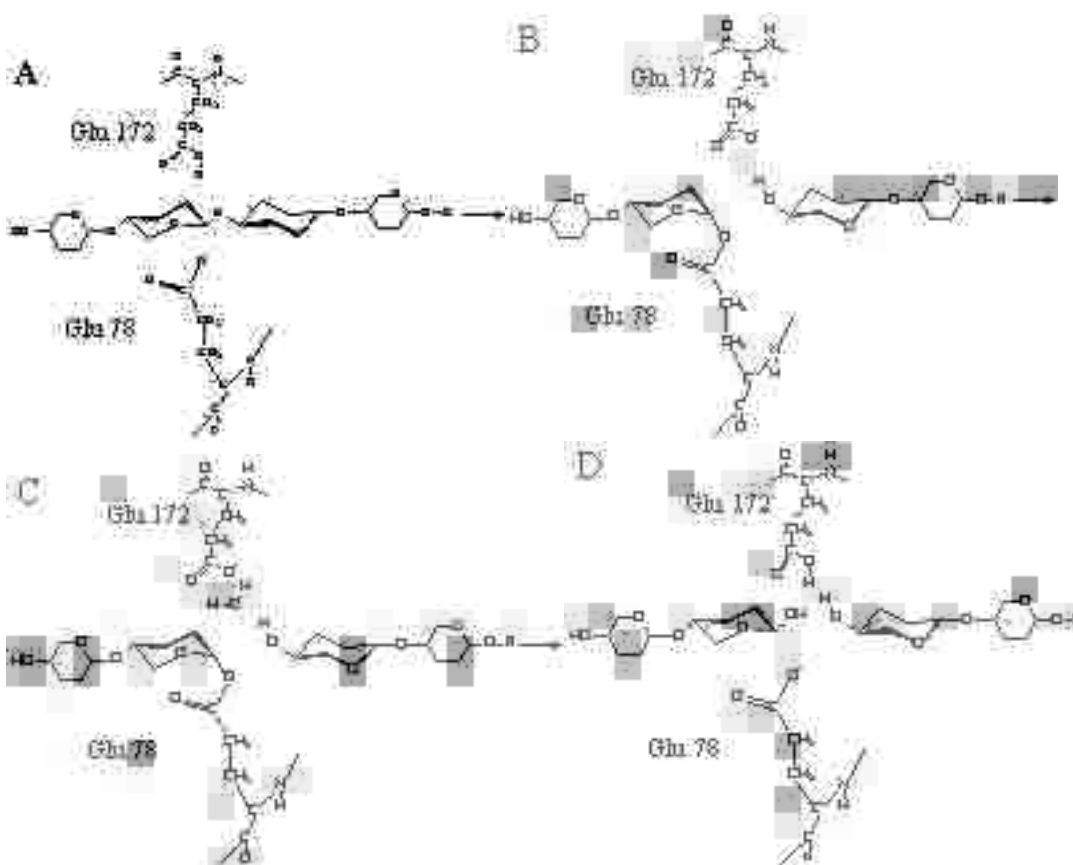


Fig. 3. Reaction mechanism by *Bacillus circulans* xylanase (1XNB). A) The helical xylan structure is positioned in the trough formed between Tyr 65 and Tyr 69. Glu 172 is the acid/base catalyst and Glu 78 is the nucleophile. B) The glycone is bound to Glu 78. This intermediate is retained during transglycosylation reactions. C) Water displaces the nucleophile. D) Dissociation and diffusion of the glycone (xylobiose) allow movement of the enzyme to a new position on the substrate. Xylanases of family 11 exhibit a random endo-mechanism rather than progressive cleavage. This is because the aglycone is released in step B and the glycone in D.^{8,12}

Based on the crystallographic study of xylopentose binding to *Pseudomonas fluorescens* Xylanase A Leggio *et al.*¹⁰¹ proposed a most suitable enzyme mechanism which combine the classical concepts listed above and facts derived from their study. According to them (1.) xylan is recognised and bound by xylanase as a left-handed three fold helix (2.) the xylosyl residue at subsite -1 is distorted and pulled down toward the catalytic residues, and the glycosidic bond is strained and broken to form the enzyme-substrate covalent intermediate (3.) the intermediate is attacked by an activated water molecule, following the classic retaining glycosyl hydrolase mechanism and the product is released.¹⁰¹

There are several reports regarding the hydrolytic pattern of xylanases from *Bacillus* spp. and most of them are mainly releasing xylobiose, xylotriose and xylotetraose while formation of xylose occurred only during prolonged incubation. Xylanases A and B from *Trichoderma reesei* and C and D from *Trichoderma harzianum* under different combinations showed synergistic interactions on different xylan substrates. Xylanase combinations were more effective than single xylanase for hydrolysing pine holocellulose.¹¹² Xylanase II of *Bacillus circulans* WL-12 (pI 9.1)⁵⁰ hydrolysed xylan principally to xylobiose, xylotriose and xylotriose. This enzyme was shown to be requiring a minimum of four xylopyranoside residues to form the productive complex, thus xylotriose out of other substrates tried was the most preferred substrate to saturate all binding sites of the enzyme. But the Xylanase I from the same source degraded xylan rapidly to xylotriose and prolonged incubation resulted in xylose, xylobiose and xylotriose as the main end products.

XI. Xylanase Gene Regulation

In most of the reports regarding xylanases there is the occurrence of constitutive enzyme production.^{113,114} Xylanase attacks xylan, comparatively a large heteropolysaccharide, which is prevented from entering the cell matrix by the cell membrane. The products of xylan hydrolysis are small molecular weight xylose, xylobiose, xylotriose and other oligosaccharides.^{113,114} These molecules easily enter the microbial cells and sustain the growth by acting as energy and carbon source. The products of hydrolysis can stimulate xylanase production by different methods. Xylose being a small pentose molecule can enter the bacterial and fungal cells easily and induce xylanase production.^{6,114} However, the larger molecules pose problem in transportation, which questions the direct induction role of these macromolecules on enzyme synthesis.¹¹⁴ There are two plausible explanations for the inductive role of larger molecules based on the reports of Wang *et al.*¹¹³ and Gomes *et al.*¹¹⁵(Fig.5). One of the explanations is that the xylo-oligomers formed by the action of xylanase on xylan are directly transported into the cell matrix where they are degraded by the intracellular β -xylosidase which releases the xylose residues in an exo-fashion from the xylo-oligomers. The above concept is supported by the universal occurrence of intracellular β -xylosidases^{45,116} in microorganisms. The other possibility is that the oligomers are hydrolysed to monomers during their transportation through the cell membrane into cell matrix by the action of hydrolytic transporter having exo β -1,4- bond cleaving proteins like the β -xylosidases. The above idea stemmed from the reports on β -xylosidases with transferase activity.¹¹⁷ In both the ways the resulting xylose molecules as mentioned earlier results in the enhanced production of xylanase. However, there are rare cases where the

xylose molecules repress the xylanase production (*Bacillus thermoalkalophilus*¹¹⁸) where the inducer may be yet another derivative from the xylan hydrolysates. If glucose, the most effective carbon source, is present in the growth medium there is repression of synthesis of catabolic enzymes which may be occurring at the transcriptional level or by mere inducer exclusion of the respective inducers of these enzymes. The first one i.e. the catabolic repression at the transcriptional level has been clearly explained by Saier and Fagan¹¹⁹ (1992).

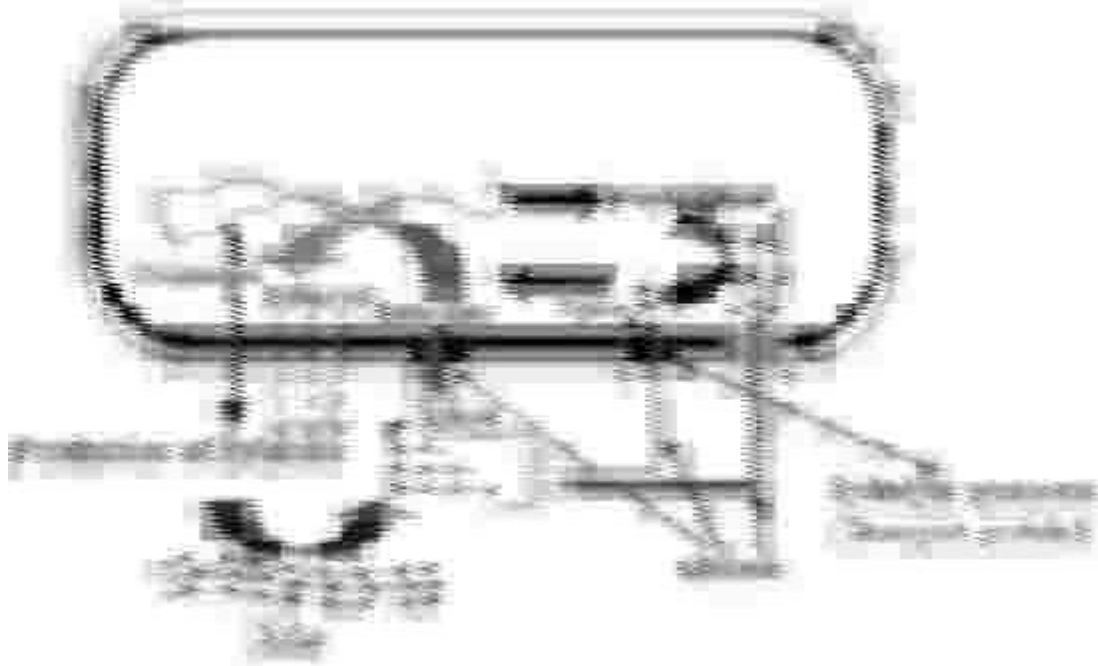


Fig. 4 Hypothetical model for xylanase gene regulation in bacteria based on the reports of Wang *et al.*¹¹³, Zhao *et al.*¹¹⁴ and Gomes *et al.*¹¹⁵ 1. Xylose monomers can be easily transported through the cell membrane which induces the enhanced xylanase synthesis. 2. The action of constitutively produced xylanases results in xylooligosaccharides eg. xylotriiose¹¹⁴, the transportation of which in to the cell later cause the enhanced synthesis. 3. The hydrolytic permeator can result in the transportation-coupled hydrolysis of xylooligomers from the constitutive xylanase action. All the cases could be affected by the presence of glucose.

The second possibility of catabolite inhibition may be inducer exclusion occurring at the level of inducer transport across the cell membrane.^{120, 121} An example of inducer exclusion is the fact that glucose will prevent the uptake of lactose, the inducer for the *lac* operon of *E. coli*^{120, 121} The xylanase inducer proteins resulting in the transcriptional activation have recently been elucidated by Peij *et al.*¹²² The *xln R* gene of *Aspergillus niger* controls all the xylanolytic enzymes and other two endoglucanases suggesting the occurrence of common regulatory systems in microorganisms.¹²²

XII. Xylanase Gene cloning

La-Grange *et al.*⁴⁵ successfully cloned (coexpressed) *Bacillus pumilus* β -xylosidase (xynB) gene along with *Trichoderma reesei* β -xylanase-2 (xyn2) gene in the yeast *Saccharomyces cerevisiae*. Genomic DNA from *Bacillus circulans* Teri-42 was cloned in *Escherichia coli* DH5-alpha using plasmid pUC19, however, 14 fold increase in expression was observed in *Bacillus subtilis* clone harbouring recombinant plasmid pBA7.¹²³ Both genes under common promoter and terminator sequences resulted in 25% increase in the amount of reducing sugar released from Birchwood xylan. *Bacillus* sp. strain NG-27 xylanase (47 kDa) active at 70⁰ C and pH 8.4 was cloned in *Escherichia coli* using shot gun library method.¹²⁴ Xylanase gene from *Vibrio* sp. strain XY-214 was also manipulated by using the host *Escherichia coli*. The 1383 bp long gene was responsible for 51,323 Dalton protein.¹²⁵ Similarly *Paenibacillus* sp. xylanase was also cloned in *Escherichia coli*.¹²⁶ There are several reports regarding the genetic manipulation on xylanase producing microorganisms.¹²⁷⁻¹³³ During the early periods of xylanase research, the lack of hyper producing potent culture resulted the taming of xylanase genes from the already available cultures. Gene manipulation has the advantage of production of microbial strains with selected enzyme machinery. According to Biely⁶ the main objectives for gene cloning are: 1.) Construction of producers of xylanolytic systems free of cellulolytic enzymes and 2.) Improvement of fermentation characteristics of industrially important xylose fermenting organisms by introducing xylanase and xylosidase genes so that the direct fermentation of xylan would be possible. *Bacilli* have many features attractive for a microorganism to be used as a host for the production of heterologous proteins. Most *Bacilli* used in industry and research are non-toxic and have the generally recognised as safe (GRAS) status as they lack cellular components of metabolic products toxic to human being or animals.¹³⁴ This fact is obvious because the members of the genus *Bacillus* are gram-positive organisms and do not contain endotoxins (lipopolysaccharide), which are ubiquitous in all gram-negative bacteria including *Escherichia coli*. These endotoxins from gram-negative bacteria are difficult to remove from many proteins in the process of purification. Another important feature of all *Bacillus* spp. used in practical applications is their apathogenicity and the well-proved safety of appropriate industrial processes using them. The secretory production could be advantageous for industrial production. Purification of a secreted protein is simpler and more economical than that of a product produced intracellularly, the prevalent mode of production in most

Table 3. Gene cloning of some important bacterial xylanases

Organism	Host		Vector used	Reference
	Name	Remarks		
<i>Actinomadura</i> sp. strain FC7	<i>Escherichia coli</i> N4924 N/14 (periplasmic-leaky), xylanase- and cellulase-negative <i>Streptomyces lividans</i>	2 Classes of recombinants were isolated. Plasmid pJF1 and plasmid pJF6	plasmid pFD666	135
<i>Bacillus circulans</i> Teri-42	<i>Escherichia coli</i> DH5 α <i>Bacillus subtilis</i> - (expression)	Expression low in <i>E. coli</i> , the 1.7 kb insert of the clone <i>E. coli</i> (pAQA) ligated to plasmid pUB110 for transforming <i>Bacillus subtilis</i> .	plasmid pUC19	123
<i>Bacillus ltyicus</i>	<i>Escherichia coli</i> and <i>Bacillus subtilis</i>	Shuttle vector pHB 201 was used in <i>B. subtilis</i>	PUC 19 (<i>E. coli</i>) and pHB 201 (<i>B. subtilis</i>)	136
<i>Bacillus</i> sp. strain NG-27	<i>Escherichia coli</i>	Shotgun library	Plasmid pBR322 (At the <i>HindIII</i> site) - one clone out of a total of 5X10 ³ recombinants	124
<i>Bacillus subtilis</i>	<i>Bacillus subtilis</i>	Self cloning	-	137
<i>Clostridium thermocellum</i> ATCC 27405	<i>Bacillus subtilis</i>	A series of subclones & deletion derivatives of the chromosomal DNA are analysed.	plasmid pCX64. (chromosomal DNA fragment + plasmid pUC19)	130
<i>Clostridium thermocellum</i>	<i>Bacillus subtilis</i> DB104	Protease-deficient host	Plasmid pJX18 - gene under the control of a strong <i>Bacillus</i> promoter	138
<i>Streptomyces</i> sp.S38	<i>Streptomyces lividans</i> TK24 and <i>Streptomyces parvulus</i>	Enzyme production was 40-fold higher with original <i>Streptomyces</i> sp. S38	Plasmid pDML1000 and pDML1001	139
<i>Streptomyces</i> sp. Ec3	<i>Streptomyces lividans</i>	Host prepared by nitrosoguanidine mutagenesis of strain TK24	plasmid pIJ702 and plasmid pIJ699 (8 from 22,000 clones,	133
<i>Streptomyces thermoviolaceus</i> OPC-520	<i>Escherichia coli</i> JM109 and <i>Streptomyces griseus</i> PSR2	Three genes encoding 2 types of endo-xylanase (STX-I and STX-II) and an acetyl xylan-esterase (AXE)	plasmid pUC18, plasmid pUC19, phage M13mp18, phage M13mp19 and plasmid pIJ702	91
<i>Vibrio</i> sp. Strain XY-214	<i>Escherichia coli</i> DH5 α	<u>β-1,3-Xylanase</u> The <i>Bgl</i> III and <i>Xba</i> I fragments of about 4.4 kbp were used, The recombinant plasmid yielded from 1 of the 22 clones was termed pTXY1.	pBluescript II KS(2) - 22 of the 860 clones hybridized to the alkaline phosphatase-labeled probe	125

gene cloning systems. More over the overproduction of an intra-cellular protein often leads to its aggregation resulting in the denatured condition. Harbak and Thygesen¹³⁷ found that the xylanase expressed in a self-cloned strain of *Bacillus subtilis* does not have acute and subchronic oral toxicity even at higher doses. Thus there is an increased attention towards *Bacillus* expression system instead of other systems including *Escherichia coli* (Table 3). The early studies on cloning of xylanase gene include the works on *Bacillus* spp.⁶ In addition to permitting the introduction of novel genes, cloning techniques could enable amplification of the expression of genes already present. For instance, the production of xylanase in *Bacillus subtilis* was enhanced successfully using a plasmid vector carrying the *Bacillus pumilus* gene. The transformant produced approximately three times more extracellular xylanase than the donor strain. More over, the enzyme was produced constitutively, suggesting that regulatory elements of the donor organism were absent in the vector used for the transformation.¹²⁷ The xylanase genes xyn A and B of *Bacillus subtilis* were cloned in *Escherichia coli*.¹⁴⁰ An alkalophilic *Bacillus* sp. strain C125 produced two types of xylanases (N and A) whose molecular weights were 43 and 16 kDa respectively. The xyn A gene located on a 4.6 kbp DNA fragment was cloned in *E. coli*, and more than 80 % of the activity could be detected in the culture medium.¹²⁸ Sung *et al.*¹²⁹ successfully completed the over expression of *Bacillus subtilis* and *Bacillus circulans* genes in *E.coli* by constructing synthetic genes with multiple unique restriction sites. The synthetic genes encoded only the mature enzymes and the results showed 10-100 folds increase in activity over all previous experiments. According to them the repeated usage of degenerate codons in the *Bacillus* derived genes if present in *E.coli* may deplete the supply of specific tRNA thus limiting the expression.

Gat *et al.*¹³¹ using *E. coli* cloned the 1236 bp open reading frame of *Bacillus stearothermophilus* T-6 xylanase gene. They also found that the β -xylosidase gene was present 10 kb down stream of the xylanase gene, but it was not a part of the same operon. Despite the future role of *Bacillus* expression system there are few reports regarding the xylanase gene cloning using *Bacillus* sp. Jung and Pack¹³⁰ cloned *Clostridium thermocellum* xylanase gene in *Bacillus subtilis*. They constructed the vector pJX18 by inserting a Bam HI 1.6 kb DNA fragment of pCX18, which contained the xylanase structural gene. However, the glycosylation of the over expressed protein was not considered in this case which resulted in the proteolytic degradation leading to the formation of different bands of proteins with hydrolytic nature.¹³⁰ Cho *et al.*¹³⁸ tried to validate this aspect by using a protease-deficient *Bacillus subtilis* DB104 for cloning endoxylanase (I) from *Clostridium thermocellum*. The transformed cells successfully secreted xylanases into the culture broth and this technique is highly valuable considering the problems associated with intra-cellular production of proteins. There are reports regarding the cloning of xylanases from organisms other than *Bacillus* spp., like *Streptomyces thermoviolaceus* OPC-520,⁹¹ *Actinomadura* sp. strain FC7,¹³⁵ *Streptomyces lividans*,¹³² and *Streptomyces* sp. strain EC3¹³³ A detailed description of the major recombinant clones along with the vector characteristics and remarks were included in the Table 3.

XIII. Application of xylanases

Potential application of xylanases in biotechnology include biobleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuels.

A. Paper Industry

Chlorinated phenolic compounds as well as polychlorinated biphenyls, produced during conventional pulp bleaching being toxic and highly resistant to biodegradation, form one of the major sources of environmental pollution.

1. Kraft Process :

Removal of residual lignin from Kraft pulp is physically and chemically restricted by hemicelluloses. Lignin has been reported to link with hemicelluloses^{1,141} and there are reports regarding the isolation of lignin carbohydrate complexes from the kraft pulp.¹⁴²

The most common pulping process is the Kraft process or Sulphate process where cooking of wood chips is carried out in a solution of Na_2S / NaOH at about 170°C for two hours resulting in the degradation and solubilisation of lignin. The resulting pulp has a characteristic brown colour which is primarily due to the presence of residual lignin and lignin derivatives. The intensity of pulp colour is a function of the amount and chemical state of the remaining lignin. To obtain pulp of very high brightness and brightness stability, all the lignin must be removed from the pulp. For that, chemical pulping is more effective than mechanical pulping. However, there is the formation of residual lignin which has to be removed by bleaching process. The residual lignin in chemical pulp is dark in colour because it has been extensively oxidized and modified in the cooking process. This residual lignin is difficult to be removed due to its covalent binding to the hemicellulose and perhaps to cellulose fibres. The bleaching of the pulp can be regarded as a purification process involving the destruction, alteration or solubilization of the lignin, coloured organic matters and other undesirable residues on the fibres.¹⁴³

2. Biobleaching

Bleaching of chemical pulp to a higher brightness without complete removal of lignin has not been successful so far. Conventionally chlorine is used for bleaching. Chlorination of pulp does not show any decolourising effect, and in fact, the colour of the pulp may increase with chlorination and it is the oxidative mechanism which aids the pulp bleaching.¹⁴⁴ At low pH the main reaction of chlorine is chlorination rather than oxidation. Thus chlorine selectively chlorinates and degrades lignin compounds rather than the carbohydrates (e.g. hemicelluloses – xylan) moieties in the unbleached pulp. The dominant role of chlorine in bleaching is to convert the residual lignin in the pulp to water or alkali soluble products. The effluent that are produced during the bleaching process, especially those following the chlorination and the first extraction stages are the major contributors to waste water pollution from the pulp paper industry.⁵⁸ During the Kraft process part of the xylan is relocated on the fibre surfaces. Considerable amount of xylan is present in the fibres after pulping process. Enzymatic hydrolysis of the reprecipitated and relocated xylans on the surface of the fibres apparently renders the structure of the fibre more permeable. The

increased permeability allows the passage of lignin or lignin-carbohydrate molecules in higher amounts and of high molecular masses in the subsequent chemical reactions.

Ligninases and hemicellulases (xylanases) were tested for biobleaching. Use of hemicelluloses was first demonstrated by Viikari *et al.*⁴ which resulted in the reduction in chlorine consumption. Lundgren *et al.*²⁸ even tried a Mill trial on TCF (total chlorine free) technology for bleaching of pulp with xylanase from *Bacillus stearothermophilus* strain T6 which is having optimum activity at pH 6.5.²⁷⁻²⁸ Eventhough there are many reports on microbial xylanases only a limited number of them are having characteristics applicable in paper and pulp industry (Table 2).

Two types of phenomena are involved in the enzymatic pretreatment. The major effect is due to hydrolysis of reprecipitated and readsorbed xylan or xylan-lignin complexes that are separated during the cooking process. As a result of the enzymatic treatment, the pulp becomes more accessible to the oxidation by the bleaching chemicals. A minor effect is due to the enzymatic hydrolysis of the residual non-dissolved hemicellulose by endoxylanases. Residual lignin in unbleached pulp (Kraft pulp) is linked to hemicellulose and that cleavage of this linkage will allow the lignin to be released.²⁸

3. Why Xylanases?

Xylans do not form tightly packed structures hence are more accessible to hydrolytic enzymes. Consequently, the specific activity of xylanase is 2-3 times greater than the hydrolases of other polymers like crystalline cellulose.⁸⁴ In the pulping process, the resultant pulp has a characteristic brown colour owing to the presence of residual lignin and its derivatives. The intensity of pulp colour is a function of the amount and chemical state of the remaining lignin. In order to obtain white and bright pulp suitable for manufacturing good quality papers, it is necessary to bleach the pulp to remove the constituents such as lignin and its degradation products.²⁸ Biobleaching of pulp is reported to be more effective with xylanases than with lignin degrading enzymes. This is because the lignin is cross-linked mostly to the hemicellulose and the hemicellulose is more readily depolymerised than lignin.⁵⁸

Removal of even a small portion of the hemicellulose can be sufficient to open up the polymer and facilitate removal of the residual lignin by mild oxidants. The principal objective of the application of biotechnological methods is the achievement of selective hemicellulose removal without degrading cellulose. Degradation of cellulose is the major problem associated with conventional pulping process, which invariably affects the cellulose fibre, and thus the quality of paper^{3, 143}. Removal of xylan from the cell walls leads to a decrease in energy demand during bleaching.¹⁴⁵ Therefore enzymatic treatments of pulp using xylanases have better prospect in terms of both lower costs and improved fibre qualities.

4. Pulp fibre morphology

After comparing SEM micrographs of soft wood sulphate pulp with that of the same pulp after xylanase prebleaching and alkali extraction, Pekarovicova *et al.*¹⁴⁶ found that there is no marked change in the shape of fibre after xylanase prebleaching. However, flattening of the fibre arise after alkaline extraction, confirming that the lignin extraction from the cell wall results in its collapse. Another report on application of xylanases for bagasse sulfite pulp pre-treatment also confirmed the formation of 'peels' and

'cracks' of fibre surfaces.² Perhaps this can be explained as resulting from the digestion of the readsorbed linear xylan from the pulp fibre surface. Surface modification and the subsequent penetration of surface layers aid the easy removal of chromophoric compounds by mild oxidising agents.

5. Need for Cellulase free Xylanase

The public concern on the impact of pollutants from paper and pulp industries, which use chlorine as the bleaching agent act as strong driving force in developing biotechnology aided techniques for novel bleaching i.e. biobleaching.¹⁴⁵ As mentioned earlier, xylanases are more preferable to ligninases. However the occurrence of cellulase contamination in most of the reported fungi (Table 1) is posing a major threat in applying the xylanases in biobleaching. The cellulases easily result in the hydrolysis of cellulose, which should be the main recovered product in paper industry. However, the enzyme preparations from microorganisms producing higher levels of xylanases with tenuous or no cellulase activity can be applied in paper industry because the loss of pulp viscosity is at minimum level.

B. Other applications of xylanolytic enzymes

In cereals like barley arabinoxylans form the major non-starch polysaccharide. Arabinoxylans constitute 4-8% of barley kernal and they represent ~ 25 and 70 % of the cell wall polysaccharides of endosperm and aleurone layer respectively. The arabinoxylanases are partly water soluble and result in a highly viscous aqueous solution. This high viscosity of cereal grain water extract might be involved in brewing problems (decreased rate of filtration or haze formation in beer) and is a negative parameter for the use of cereal grains in animal feeding.⁹⁻¹⁰ A better solution for this problem could be derived from the application of xylanases for pre-treating the arabinoxylan containing substrates. The xylanolytic enzymes are also employed for clarifying juices and wines,⁵⁻⁶ for extracting coffee, plant oils and starches,^{5,147} for improving the nutritional properties of agricultural silage and grain feed.^{5,148} Xylanases are also having application in rye baking where the addition of xylanase makes the doughs soft and slack.^{9, 10,137} Xylanases are used as dough strengthners since they provide excellent tolerance to the dough towards variations in processing parameters and in flour quality. They also significantly increase volume of the baked bread.¹³⁷ Sugars like xylose, xylobiose and xylooligomers can be prepared by the enzymatic hydrolysis of xylan.⁵ Bioconversion of lignocelluloses to fermentable sugars has the possibility to become a small economic prospect. It is because massive accumulation of agricultural, forestry and municipal solid waste residues create large volume of low value feedstock.¹⁴⁹ If the feed stock is variable, a complete xylanolytic system would appear desirable to ensure maximal hydrolysis. Such an enzyme system would include xylanases, β -xylosidases, and the various debranching enzymes.

Production of environmentally friendly fuel is gaining great importance as the energy sources are shrinking. There are reports regarding the production of ethanol from the agrowastes by incorporating xylanase treatment.¹⁵⁰

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