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produced by microorganisms which have colonized environments extreme from an anthropocentric point of view. The thermophiles, alkaliphiles and acidophiles have been the most extensively investigated of the extremophilic xylanases comparing to the cold-adapted xylanases.

1.2.3.1.1 Thermophiles
A number of thermophilic and hyper-thermophilic xylanase producing microorganisms have been isolated from a variety of sources, including hot pools, self-heating decaying organic debris, terrestrial and marine solfataric fields and thermal springs (Singh et al 2003; Harris et al 1997; Vieille & Zeikus 2001; Cannio et al 2004; Sunna & Bergquist 2003; Sunna et al 1997). The majority of the thermophilic xylanases produced have been found to be from families 10 and 11 so far and not even one was from any of the other glycoside hydrolase families. The gene for the thermostable xylanase with half life of 8 minutes at 100 °C from the extreme thermophilic archaeon *Thermococcus zilligii* (Uhl & Daniel 1999) was fascinatingly obstinate to cloning with family 10 and 11 consensus primers (Sunna & Bergquist 2003), signifying that this enzyme belongs to one of the other glycoside hydrolase families like 5, 7, 8 or 43 or indeed to another as yet unknown xylanase family.

Family 10 xylanases have been secluded from a variety of thermophilic and hyperthermophilic organisms including *Bacillus stearothermophilus* (Khasin et al 1993), *Caldicellulosiruptor* sp. (Luthi et al 1990), *C. thermocellum* (Leggio et al 1999), *Rhodothermus marinus* (Abou-Hachem et al 2003), *Thermotoga* sp. (Winterhalter et al 1995; Zverlov et al 1996) and *Thermoascus aurantiacus* (Leggio et al 1999). A family 10 xylanase, XynA from *Thermotoga* sp. strain FjSS3-B1 is in fact, one of the most thermostable xylanases with an perceptible optimum temperature for activity of 105 °C and a half life of 90 minutes at 95 °C (Simpson et al 1991). While less frequent, family 11 thermophilic xylanases have also been isolated from *Bacillus* strain D3.


a. those with a molecular weight of less than 30 kDa and basic pI and 
b. those with a molecular weight of greater than 30 kDa and acidic pI.

Conversely, several exceptions to this pattern have been found (Sunna & Antranikian 1997; Matte & Forsberg 1992) and around 30% of currently known xylanases, especially fungal xylanases, cannot be classified by this system.

A more complete classification system was launched later (Henri ssat et al 1989) that allocated not only xylanases, but glycosidases in general (EC 3.2.1.x), and that has now turned out to be the benchmark way for classifying these enzymes. This system is anchored in primary structure similarities of the catalytic domains only and clusters enzymes in families of related sequences (Henrissat & Coutinho 2001). The initial classification grouped cellulases and xylanases into 6 families from A to F (Henrissat et al 1989) that was restructured to 77 families in 1999 as from 1 to 77 (Henrissat & Coutinho 2001) and continues to grow as new glycosidase sequences are identified. At present, 133 glycoside hydrolase families are present (CAZYPEDIA 2013), with approximately 33% of these being polyspecific enzymes with diverse substrate specificities.

This classification system imitates both structural and mechanistic characteristics as the enzyme structure and molecular mechanism are linked to its primary structure. Enzymes within a particular family encompass analogous three-dimensional structures (Henrissat & Coutinho 2001) and analogous molecular mechanism (Gebler et al 1992) and they might have analogous specificity of action on small, soluble, synthetic substrates (Claeyssens & Henrissat 1992). Besides, contrary evolution has resulted in some families having allied three-dimensional structures and hence the grouping of families into higher hierarchical levels known as clans has been brought in (Bourne & Henrissat 2001). At the moment, 14 different clans namely GH-A to GH-N have been added.


52. Coughlan, MP, Touhy, MG, Filho, Puls, J, Claeyssens, M, Vrsanska, M & Hughes, MM. 1993. Enzymological aspects of microbial hemicellulases
Fungal and bacterial endo-xylanases are almost exclusively single subunit proteins with molecular weight (MW) values ranging from 8.5 to 85 kDa and isoelectric point (pI) values between 4.0 and 10.3 and most of them are glycosylated (Coughlan et al 1993; Polizeli et al 2005). An interesting physicochemical property of fungal and bacterial endo-xylanases is the apparent strong relationship between their MW and pI, with some exceptions, endo-xylanases fall in two main classes: those with MW of less than 30 kDa are usually basic proteins, and those with MW values in excess of 30 kDa are usually acidic. Most characterised endo-xylanases are optimally active at temperatures ranging between 45 °C and 75 °C, only a small number show maximal activity at temperatures above 80 °C. The latter include endo-xylanases from thermophilic bacteria of the genus *Thermotoga* (Winterhalter & Liebl 1995).

1.2.2 Multiplicity and multiple domains of Xylanases

In addition to the production of a variety of xylanolytic enzymes, many microorganisms generate multiple xylanases (Gilbert & Hazlewood 1993; Gilbert et al 1988; Yang et al 1989). These may have diverse physicochemical properties, structures, specific activities and yields, as well as overlying but dissimilar specificities, thereby increasing the efficiency and extent of hydrolysis, but also the enzyme complexity and diversity. Typical examples of microorganisms that produce xylanase isoenzymes include *Aspergillus niger* that produces fifteen extracellular xylanases and *Trichoderma viride* that secretes thirteen (Biely et al 1985). This multiplicity might be the consequence of genetic redundancy (Wong et al 1988), but differential post-translational processing has also some impact (Biely 1985). The isoenzyme genes might be polycistronic or non-polycistronic multiple copies within the genome and in a few cases several xylanases are expressed as a distinct gene product. The xylanase, β-xylosidase and acetyl esterase genes of *Caldocellum saccharolyticum* now known as *Caldocellulosiruptor saccharolyticus* are polycistronic (Luthi et al 1990), while with emphasis on fungal systems. *In:*, MP COUGHLAN & , GP HAZLEWOOD, (eds). *Hemicellulose and Hemicellulases.*, London: Portal Press, pp.53-84.


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1.1.1 Xylan degrading enzymes

Due to the heterogeneity of xylan, its hydrolysis requires the action of a complex enzyme system. The interaction of a number of main chain and side chain cleaving enzyme activities is then necessary. The main chain enzymes involved are endo-\(\beta\)-1,4-xylanases, \(\beta\)-1,4-xylosidases and a more recently discovered enzymes named exoxylanases (Gasparic et al 1995). Depending on both the source and the procedure used in its extraction and purification, the required side chain cleaving activities would include \(\alpha\)-L-arabino-furanosidases and \(\alpha\)-D-glucuronidases as well as the esterases liberating acetyl, coumaroyl and feruloyl substituents. Among the different functions suggested for xylanases are biodegradation in order to provide a source of metabolizable energy, degradation of cell wall components in concert with other polysaccharide degrading enzymes, degradation of xylan during germination of barley and digestion of dietary vegetation.

1.2 XYLANASE

Xylanases are glycosidases, also called O-glycoside hydrolases, (EC 3.2.1.x) that catalyze the endohydrolysis of 1,4-\(\beta\)-D-xylosidic linkages in xylan. They are a prevalent collection of enzymes concerned with the xylose production. Xylose was a prime carbon source for cell metabolism and is produced by a plethora of organisms including bacteria, algae, anthropods, fungi, gastropods, and protozoa in plant cell infection (Prade 1995). They were initially termed pentosanases (Whistler & Masek 1955) and were documented by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 when they were allocated the enzyme code EC 3.2.1.8. Their authorized name is endo-1,4-\(\beta\)-xylanase, but regularly used synonymous terms include \(\beta\)-1,4-xylanases.


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acetylated and holds L-arabino furanose, 4-O-methyl-β-D-glucuronic acid and ß-D-xylopyranose in a ratio of 13:20:100 (Puls & Schuseil 1993). The 4-O-methyl glucuronose residues are connected to C-2 and the L-arabino furanose residues bonded to the C-3 of the relevant xylopyranose backbone units by α-1,3-glycosidic bonds (Figure 1.1) (Polizeli et al 2005). The arabinoyl components come about almost 12% of the xylosyl residues.

Figure 1.1 Xylan degrading enzyme action sites in hardwood (A) and in softwood (B).

The ester linked components like acetyl, p-coumaroyl and feruloyl residues are lost from substrates by solubilization in alkali. Some or all of the feruloyl substituents in native lignocellulolytic material are implicated in covalent cross linking with other polysaccharides. But other natural functions of


plants. It is second only to cellulose in plants. Hemicellulose can be removed from the original or the delignified tissue by extraction with aqueous alkali or, less frequently, with water. Unlike cellulose, it is chemically complex, containing a number of polymeric and hetero-glycan components as mentioned above. In woody plants, hemicellulose is built up from relatively few sugar residues, the most common of which are D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methylglucuronic acid, D-galacturonic acid and glucuronic acid. The variety of sugar residues found in hemicellulose from grasses and cereals is small; however there is a great variety of different branch types according to the source (Puls & Schuseil 1993).

Those hetero-polysaccharides based on a backbone structure of ß-1,4-linked D-xylose residues are collectively referred to as the ß-1,4-D-xylans, and constitute the main polymeric component of the hemicellulose fraction of plant cell walls. Xylans from different sources in nature exhibit considerable variation in composition and structure. For instance, the ß-1,3-linked materials are only found in marine algae, those containing a mixture of ß-1,3 and ß-1,4 linkages are found in seaweeds like Rhodymenia palmata, while the ß-1,4-linked xylans are characteristic of hardwoods, softwoods and grasses (Sunna & Antranikian 1997).

The xylan in hardwood that comprises 10-35% of the total dry weight is acetyl-4-O-methyl glucuronoxylan with a degree of polymerisation (DP) between 150 and 200. Around 10% of the ß-D-xylopyranose backbone units is replaced with a 1,2-linked 4-O-methyl-ß-D-glucuronic acid residue at C-2, whereas 70% is acetylated at C-2 or C-3 or both (Figure 1.1). Birch xylan in reality has more than 1 mole of acetic acid for every two moles of xylose. The partial xylan solubility in water was due to the existence of these acetyl groups that are readily separated when xylan is subjected to alkali extraction.

Softwoods have 10 to 15% xylan as arabino-4-O-methylglucuronoxylan with a DP between 70 and 130. This material is not
CHAPTER 1
INTRODUCTION
This chapter emphasizes the significance and characterization of xylanase enzyme along with its fungal source, *Trichoderma reesei* and its cloning in *Escherichia coli*. A brief review of literature pertaining to the present work is also presented.

1.1 XYLAN

The major renewable source of polysaccharide in nature is the plant cell walls and they are made of three major polymeric components:

1. **cellulose** - an insoluble polymer composed of ß-D-glucopyranosyl residues linked by ß-1,4-glycosidic bonds;
2. **hemicellulose** - a sequence of heteropolysaccharides including arabinans, xylans, mannans and glucans;
3. **lignin** - a complex polyphenol familiarly interrelated with the hemicelluloses, making a matrix that environs the systematic cellulose microfibrils.

Contiguous cells are bound by this lignin that is present in high concentration in wood. Overall biomass include on average 40% cellulose, 33% hemicellulose and 23% lignin, by dry weight. They do not accumulate naturally, however they go through microbial degradation as part of the carbon cycle (Biely 1993).

Hemicellulose has been defined as that fraction of plant cell walls which remains after the removal of cellulose and pectin. It is associated with cellulose and lignin and accounts for up to 35% of the total dry weight of higher plants.


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ABSTRACT

The growing public concern regarding environmental impact of paper and pulp industry pollutants was the sturdy thrust behind the endeavours leading to novel bleaching practices. Phenol and chlorinated chemicals and polychlorinated biphenyls produced during conservative pulp bleaching methodology arise from residual lignin present in wood pulp. The majority of the chloroaromatic compounds released at the time of the pulp bleaching process is noxious and accumulates in the living and non-living components of the environment. The enzyme xylanase are becoming a chief group of industrial enzymes finding significant function in paper and pulp industry. It is used in paper and pulp industries as the xylan hydrolysis facilitates lignin release from paper pulp and reduces bleaching by chlorine. The significance of xylanase is not limited to the paper and pulp industry and there are other industries with equivalent applicability like clarification of fruit juices, bioconversion of lignocellulosic material and agro-wastes to fermented products, development in beer uniformity and the animal feed materials digestibility. The present investigation is focused on extracting xylanase from Trichoderma reesei, distinguishing it in SDS PAGE and its activity authentication by zymography, resolving its optimal pH and temperature conditions, and gene identification. The identified xyn2 gene was obtained from Genbank and synthetically raised and cloned. Synthetic nucleotide sequence had 840 bp long DNA fragment of xyn2 gene with open reading frames encoding polypeptides of 229 amino acid residue. The xyn2 gene were made by two exon, first one from 59 to 348 bp and second one from 411 to 810 bp and one intron sequence from 349 to 410 bp. Xylanase encoding gene of Trichoderma reesei was ligated into the pUC19 vector and numbered as GS57308 pUC19-xyn2 system. The ligation mixture containing xyn2-pUC19 DNA was then...