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Xylanases: An Overview

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Authors' contributions

This work was carried out in collaboration between all authors. Author MS prepared the outlines and wrote the first draft of the manuscript. Author AK thoroughly checked the manuscript, added new references, wrote few sections related to molecular biology and managed the literature. Both the authors read and approved the final manuscript.

Review Article

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ABSTRACT

Endo-1, 4-β-xylanase (Endo-β-1, 4-xylan, xylanohydrolase; EC. 3.2.1.8, commonly called xylanase) is an industrially important enzyme which degrades xylan randomly and produces xylooligosaccharides, xylobiose and xylose. It is mainly present in microbes and plants but not in animals. Xylanases from fungal and bacterial sources have been extensively studied and produced commercially. Its potential use in paper industries has been discussed which is directly related to reduction in environmental pollution. It has role in bio-bleaching paper pulp and increasing pulp brightness. Besides, it can be exploited for ethanol production and as an additive in animal feedstock to improve its nutritional value. Endo-1, 4-β-xylanase can also be exploited in baking and fruit juice industries. Here, we reviewed its distribution, structural aspects and industrial/ biotechnological applications. Besides, we also discussed studies related to cloning of the gene encoding endo-1, 4-β-xylanase with the objectives of overproducing the enzyme and altering its properties to suit commercial applications.

Keywords: Endo-1; 4-β-xylanase; birchwood xylan; animal feedstocks; xylooligosaccharides; paper industries; baking.

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1. INTRODUCTION

Enzymes are the catalytic cornerstone of metabolism and as such they are the focus of intense research worldwide, not only in the biological community, but also with process designers/engineers, chemical engineers and researchers working in other scientific fields.

Microbial enzymes are a fast growing field in biotechnology. The global market of industrial enzymes was closed to a billion dollars in 1990 and crossed the $2.0 billion mark in 2005 [1]. The market has been estimated at $3.3 billion in 2010 and is expected to reach $4.4 billion by 2015 (http://www.bccreseach.com/report/enzymes-industrial-applications-bio03of.html). The production cost and the yields of enzymes are considered the major problems in commercial exploitation.

Microbial enzymes have tremendous potential for different applications. Over the years due to their remarkable features, enzymes have occupied the central stage of all the biochemical and industrial processes. Enzymes can carry out their myriads of biochemical reactions under ambient conditions, which make their use eco-friendly and often the best alternative to polluting chemical technologies. Currently, the chemical additives are being replaced by the enzymes in numerous food applications. The use of enzymes has become a need of the time, because they promote effects similar to those of chemical additives with the advantage of being considered as safe natural additives [2]. Enzymatic treatment provides the same level of output as it is achieved through conventional methods that use harsh chemicals. Therefore, usage of enzymes at various industrial levels has also gained momentum.

Organic wastes from renewable forest and agricultural residues comprise cellulose, hemicelluloses and lignin in an average ratio of 4:3:3 [3]. The exact percentage of these three components varies from source to source [4].

In view of rising prices of crude oil due to increasing fuel demands, the need for alternative sources of bio-energy is expected to increase sharply in the coming years. Among potential alternative bio-energy resources, lignocelluloses have been identified as the prime source of bio-fuels and other value-added products. The primary components of lignocellulosic biomass, cellulose and hemicelluloses are the most abundant organic compounds on earth and have the potential to be a renewable source for energy and chemicals. The estimated global annual production of biomass is 1 x 10 tons, sequestering 2 x 10 J of energy [5]. Large quantities of lignocellulosic wastes are generated through forestry, agricultural practices and industrial processes, particularly from agro-allied industries [6] which generally accumulate in the environment and thereby cause pollution problem [7]. There are number of reports on the uses of these renewable sources [8-10]. Since last decade, there has been increasing research interest in the value of bio-sourced materials recovered from residual biomass and agro-wastes [11]. The agro-wastes being the enriched source of lingocellulosic residues can be effectively used as the raw material for the commercial production of various value added materials by bringing about a significant reduction in the cost of production [12].

It is apparent that plant biomass provides an extensive renewable resource; however, its exploitation has been limited due to the cost of hydrolysis of plant structural polysaccharides into their component sugars. Efforts are being done to exploit microbial cellulases and xylanases for conversion of plant structural polysaccharides into their monosaccharide sugars. Hemicellulose is the second abundant renewable biomass in nature, and xylan is the major hemicellulose component and approximately amounts to 20-35% of plant cell wall dry

weight [13]. The structural polysaccharide, hemicelluloses (xylan being the major component of hemicelluloses) together account for greater than 50% of plant biomass and consequently are the most abundant terrestrial organic molecules. The value of plant biomass as a renewable and exhaustible resource is immediately apparent if one considers that its estimated total energy content is equivalent to 640 billion tonnes of oil [14]. Plant structural polysaccharides provide the major source of nutrients for ruminant livestock, and have the potential through microbial fermentations to provide renewable substrates for the chemical, pharmaceutical and feed industries.

Hemicelluloses are the second most abundant fraction available in nature. These are the storage polymers in seeds [15] and they form the structural component in cell walls of woody plants [16]. The monomers of various hemicelluloses are useful in the production of different antibiotics, alcohols, animal feed, chemicals and fuels [17,18]. The future requirement of mankind for chemicals and animal feed might be fulfilled by using hemi-cellulosic agricultural and industrial wastes [19,20].

Various microorganisms are actively involved in the degradation of hemicelluloses. The presence of microorganisms that degrade hemicelluloses, particularly xylan, a major constituent of hemicelluloses, was reported over 100 years ago by Hoppe-Seyler in 1889 [21] who described a gas production process using wood xylan suspension and river mud microbes. Microbial enzymes act cooperatively to convert xylan to its constituent simple sugars. These enzymes include β-1,4-endoxylanases (xylanases; EC 3.2.1.8), which cleave internal glycosidic bonds within the xylan backbone; arabinofuranosidase (EC 3.2.1.55) which hydrolyzes arabinose side-chains; α-glucuronidase which removes glucuronic acid side-chains from the xylosyl units; xylan esterase (EC 3.1.1.6) which releases acetate group; and finally β-xylosidase (EC 3.2.1.37), which hydrolyzes xylobiose to xylose [22]. It is apparent that there is a considerable degree of synergy between these enzymes [23]. For example, many xylanases do not cleave glycosidic bonds between xylosyl units if substituted. Therefore, side chains must be cleaved before the xylan backbone can be completely hydrolyzed [24]. Conversely, several accessory enzymes will only remove side chains from xylo-oligosaccharides. These enzymes therefore require xylanases to partially hydrolyze the plant structural polysaccharides, before side-chains can be cleaved [23].

The enzymes which break down hemicelluloses are referred as hemicellulases. These have been defined and classified according to the substrates on which these act. These are collectively grouped as glycan hydrolases.

β-1, 4-Xylans are heterogeneous polysaccharides found in the cell walls of all land plants and in almost all plant parts [25]. The hydrolysis of their characteristic backbone, consisting of β-1, 4-linked D-xylosyl residues, involves β-1, 4-xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidases (1,4-β-D- xylan xylohydrolase; EC 3.2.1.37). In general terms, endo-1, 4-β-xylanases attack on the internal xylosidic linkagesin the backbone and β-xylosidases release xylosyl residues by end wise attack on xylo-oligosaccharides. Although many endo-1, 4-β-xylanases are known to release xylose during the hydrolysis of xylan or xylo-oligosaccharides, xylobiase activity has only been reported in β-xylosidases. These enzymes are the major components of xylanolytic systems produced by the bio-degradative microorganisms such as fungi and bacteria [26-29], whose activities are important for the maintenance of carbon flow in the carbon cycle and biomass turnover in nature.

Plant cell walls, the major reservoir of fixed carbon in nature, have three major polymeric constituents: cellulose (insoluble fibers of β-1,4-glucan), hemicelluloses (non-cellulosic polysaccharides including glucans, mannans, and xylans), and lignin (a complex polyphenolic structure). After the deposition of a primary wall during elongation, many plant cells (particularly in vascular tissues) undergo secondary wall thickening during differentiation [30]. The β-1,4-xylans are mainly found in secondary cell walls, the major component of mature cell walls in woody tissue [31]. These also represent the major hemicelluloses in the primary cell walls of monocots. Xylans generally constitute a minor component of the primary cell walls in dicots [32,33]. The importance of xylans as a carbon reservoir is well illustrated in birch wood, of which 35% of the dry weight is xylan [31]. In general, xylan is the major hemicellulose in wood from angiosperms but is less abundant in wood from gymnosperms. It accounts for approximately 15 to 30 and 7 to 12% of the total dry weight, respectively [25].

Xylanase is not just an interesting protein from an industrial and agricultural viewpoint, but is also of intrinsic scientific interest. Xylanases are widely distributed. These occur in both prokaryotes and eukaryotes [27]. Amongst the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases [26]. Here, we reviewed xylanase distribution, structural aspects and industrial/ biotechnological applications.

1.1 Xylan: An Overview

Schulze in 1891 [34] introduced the name hemicellulose for the fractions isolated or extracted from plant materials with dilute alkali. The principal monomers present in most of the hemicelluloses are D-xylose, D-mannose, D-galactose and L-arabinose. Hemicelluloses include xylan, mannan, galactan and arabinan as the main heteropolymers. Xylan contains D-xylose as its monomeric unit and traces of L-arabinose. The galactan contains D-galactosyl units whereas mannan is made up of D-mannosyl units. The arabinan is composed of L-arabinose.

Xylan is an important structural component of plant biomass left in agricultural wastes. It is the most abundant of all of the hemicellulosic materials in the world comprising over 30% of renewable organic carbon on this planet [35;36]. Xylan is a polysaccharide made up of D-xylosyl units connected by β-1,4-glycosidic bonds. It is often branched with predominantly acetyl, arabinofuranosyl and glucuronic side chains attached to the xylose backbone [37,38].

Xylan is the most abundant non-cellulosic polysaccharide present in both hardwoods and annual plants and accounts for 20-35% of the total dry weight in tropical plant biomass [39-41]. In temperate softwoods, xylans are less abundant and may comprise about 8% of the total dry weight [42]. Xylan is found mainly in the secondary cell wall and is considered to be forming an interface between lignin and other polysaccharides. It is likely that xylan molecules are covalently linked with lignin's phenolic residues and also interact with polysaccharides, such as pectin and glucan. In simplest form, xylan is a linear homo-polymer that contains D-xylose monomer linked through β-1, 4-glycosyl bonds [43,44].

Xylanase secretion often is associated with low or high amount of cellulases. Using xylanase for pulp treatment is preferable to using cellulase free xylanase since cellulase may adversely affect the quality of the paper pulp [45-49]. The most practical approach is the screening for naturally occurring microbial strains capable of secreting cellulose-free xylanases under optimized fermentation conditions. In order to use xylanase prominently in bleaching process, it should be stable at high temperature and alkaline pH [50,51].

Xylanase is produced by diverse genera and species of bacteria, actinomycetes and fungi. While several bacterial species secrete high levels of extra-cellular xylanase, filamentous fungi secrete high amounts of extra-cellular proteins where xylanase secretion often accompanies cellulolytic enzymes [52].

Microbial endo-1,4-β-xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss, and side product generation. However, cost of the enzymatic hydrolysis of a biomass is one of the main factors limiting the economic feasibility of this process. Therefore, the production of xylanase must be improved by finding more potent fungal or bacterial strains or by inducing mutant strains that can secrete greater amounts of the enzyme [27].

Xylanase immobilisation involves the covalent attachment or entrapment of an enzyme to a carrier [53]. It potentially offers a way to reduce the cost of large-scale production. The immobilised enzymes can easily be recovered and reused, therefore, cost of the enzyme required may be reduced [53,54]. The ability to reuse enzymes, along with longer half-life of immobilised enzymes could allow the development of a process with continuous production [53,55]. Besides, immobilisation of the whole microorganism can also be considered to continuously produce endo-1,4-β-xylanase since this enzyme is mostly secreted as an extra-cellular form.

1.2 Xylanase Distribution

Over 100 years ago the role of enzymes in the breakdown of xylan was observed by Hoppe-Seyler [56]. Xylanases, the xylan hydrolyzing enzymes, are ubiquitous and diverse by nature [36]. A number of different sources, including bacteria [57-59], fungi [57,60], actinomycetes [61], and yeast [62,63] have been reported to produce xylanase.

In the last decade, a number of hyperthermophilic archaea have been isolated that are able to grow around the boiling point of water [64]. Various bacterial and fungal species viz. *Thermomonospora* sp, *Bacillus*, sp, *Melanocarpus albomyces*, *Chaetomium thermophilum*, *Nonomuraea flexuosa*, *Streptomyces* sp., *Dictyoglomus* sp., *Thermotogales* sp., *Thermoactinomyces thalophilus, Thermoascus aurantiacus, Fusarium proliferatum, Clostridium abusonum, Arthrobacter* etc. are reported to produce alkalo-thermophilic xylanases [42,65-75].

Bacteria just like many industrial enzymes fascinated the researchers for alkaline thermostable endo-1, 4-β-xylanase producing trait. *Bacillus* SSP-34 produced higher levels of endo-1, 4-β-xylanase activity under optimum nitrogen condition. *Bacillus* SSP-34 produced a endo-1, 4-β-xylanase with activity of 506 IU/ml in the optimized medium [76]. Earlier, Ratto et al. [77] reported endo-1,4-β-xylanase with an activity of 400 IU/ml from *Bacillus circulans*. It had optimum activity at pH 7 and 40% of the activity was retained at pH 9.2. However, the culture supernatant also showed low levels of celluloytic 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 has been reported to produce cellulase free endo-1,4-β-xylanase. It actually had slight cellulolytic activity of 0.021 IU/ml. Mathrani and Ahring [78] reported endo-1,4-β-xylanases from *Dictyoglomus* sp. having optimum activities at pH 5.5 and 90ºC. It merits the significant pH stability at a range of pH values 5.5 to 9.0.

Most of the fungi produce endo-1, 4-β-xylanases which tolerate temperatures up to 50ºC. In general, with rare exceptions, fungi are reported to produce endo-1,4-β-xylanases and have an initial cultivation pH lesser than pH 7.0.

The pH optima of bacterial endo-1,4-β-xylanases are in general slightly higher than the pH optima of fungal endo-1,4-β-xylanases. In most of the industrial applications, especially paper and pulp industries, the low pH required for the optimal growth and activity of endo-1, 4-β-xylanase necessitates additional steps in the subsequent stages which make fungal endo-1,4-β-xylanases less suitable.

Even though there are differences in the growth conditions including pH, agitation, aeration, and optimum conditions for endo-1, 4-β-xylanase activity, there is considerable overlapping in the molecular biology and biochemistry of prokaryotic and fungal xylanases [77,79-87].

1.3 Endo-1,4-β-xylanase Structural Aspects

The three-dimentional structures of GH 10 and 11 endo-1,4-β-xylanases have been determined for several enzymes both from bacteria and fungi. The endo-1,4-β-xylanase IBCX from *Bacillus circulans* has been found to have the features of GH 11. The catalytic domain folds into two β sheets (A and B) constituted mostly by antiparallel β strands and one short α helix and resembles with a partly closed right hand. The differences in catalytic activities of endo-1, 4-β-xylanases of GH 10 and 11 can be attributed to the differences in their tertiary structure. The GH 11 endo-1,4-β-xylanases are smaller and are well packed molecules with molecular organization mainly of β-pleated sheets. 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 is asparagine at position 100 in the so-called 'alkaline' endo-1,4-β-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 themo-stability may be explained by a variety of factors and structural parameters. Of these, the importance of disulfide bridges and aromatic sticky patches can be analyzed by sequence alignment. However, Sapag et al. [88] showed that disulfide bridges are unlikely to be of importance in the thermophilic behavior of GH 11 endo-1,4-β-xylanases. The overall structure of the catalytic domain of GH 10 endo-1,4-β-xylanase is an eight-folded barrel. 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 endo-1,4-β-xylanases of GH 10. The substrate binding sites of the GH 10 endo-1,4-β-xylanases are apparently not such deep cleft as the substrate binding sites of GH 11 endo-1,4-β-xylanases. The fact together with a possible greater conformational flexibility of the larger enzymes than of the smaller ones may account for a lower substrate specificity of GH 10 endo-1,4-β-xylanases.

2. BIOCHEMICAL PROPERTIES OF ENDO-1, 4-B-XYLANASES (XYLANASE)

2.1 Bioseparation

It has been observed that conventional techniques for separation and purification of biomaterials have disadvantages viz. insufficient selectivity, high energy consumption and denaturation especially with the use of organic solvents. Most of the disadvantages are overcome by conducting bioseparation in aqueous two phase systems. Aqueous two-phase

systems are formed when two mutually incompatible polymers or polymer and salt are mixed above certain concentration [89]. Gupta et al. [90] purified xylanase from *Trichoderma viride* by precipitation with an ionic polymer Eudragit S 100. The purified enzyme was tested free from contaminant proteins and recovery was 89%. They showed that binding of the enzyme to the polymer was predominantly by electrostatic interaction. Bronnenmeier et al. [91] purified cellulose binding enzymes including xylanase from *Clostridium stercorarium* using cellulose-affinity chromatography. Santos et al. [92] purified xylanase to near homogeneity using packed and expanded beds and by making use of cationic adsorbent, Streamline SP, in the presence of *Bacillus pumilus* cell mass. Breccia et al. [93] isolated extracellular xylanase from the whole broth of *Bacillus amyloliquefaciens* by adsorption on a cation exchanger, Amberlite IRC-50 in fluidized bed with low degree of expansion. They eluted the adsorped enzyme from Amberlite IRC-50 by increase in pH. The enzyme was sufficiently pure with 82% yield. Higher yield with sufficient purity with bioseparation is of much advantage for industrial applications.

2.2 Molecular Weight

Fontes et al. [94] showed the molecular weight of xylanase from *Cellvibrio mixtus* to be 43413 Da based on the coding sequence of *Xyl*C gene. However, Gallardo et al. [95] reported the molecular weight of xylanase from *Bacillus sp.* BP-7 to be 24 kDa based on the coding sequence of *Xyn*A gene. Lee et al. [96] also reported the molecular weight 23 kDa for xylanase from *Bacillus licheniformis*. Lee et al. [97] reported the molecular weight 20 kDa for xylanase from *Paenibacillus* sp. DG-22. Xylanase isolated from *Trichoderma* sp. has been reported to have molecular weight of 21 kDa [75]. Nishimoto et al. [98] reported molecular weight of two xylanases from *Bacillus* sp. strain AR-009 coded by *Xyl*A and *Xyl*B genes, to be 23 kDa and 48 kDa, respectively. Sharma et al. [99] reported the molecular weight nearly 43 kDa for xylanase isolated from *Enterobacter* sp. Khandeparkar and Bhosle [42] reported the molecular weight nearly 20 kDa for xylanase isolated from *Arthrobacter* sp. Ratanakhanokchai et al. [100] reported the molecular weight nearly 23 kDa for xylanase isolated from *Bacillus* sp. strain K-1. However, Ananda Muniswaran et al. [101] reported the molecular mass of xylanase to be 36 kDa isolated from *Bacillus* sp. strain 41M. Xylanases have been grouped into GH 10 having high molecular weight (more than 30 kDa), and family 11 having low molecular weight (less than 30 kDa) [102,103].

2.3 Amino Acid Composition and Sequencing

The complete amino acid analyses of endo-1, 4-β-xylanases from bacterial as well as fungal origin have been reported. Amino acid composition of *Streptomyces* sp. 3137 [104]; *Bacillus subtilis* [40], *B. pumilus* [105]. *Aspergillus Niger* [106], *Sporotrichum thermophile* [107], *Schizophyllum commune* [108] and *Chainia* sp.[109] have been reported. The smallest endo-1,4-β-xylanase with a molecular weight of 5500 to 6000 Da was isolated from *Chainia* sp. and its 43 amino acid residues were sequenced [110]. Paice et al. [105] determined the complete amino acid sequence of xylanases from *B. subtilis* as well as *B. pumilus*. The *B. subtilis* endoxylanse has 185 amino acid residues in its chain whereas *B. pumilus* xylanase has been found to have 201 amino acid residues in its chain. The partial amino acid sequences were reported for two endoxylanses from fungal sources. The first 72 amino acid residues of xylanase from *Cryptococcus albidus* from the N-terminal end have been sequenced [111], whereas the first 27 residues from the N-terminal end of xylanase from *Schizophyllum commune* are sequenced [108]. Amino acid sequencing of number of

xylanases is available in CAZy (www.cazy.org) and ENZYME (enzyme.expasy.org;www.brenda-enzyme.org) databases.

2.4 Crystallization and X-Ray Crystalographic Studies of Xylanases

Crystallization and X-ray Crystallographic studies of xylanases were first carried out with the endo-1,4-β-xylanase from *Bacillus* sp. [112]. More recently X-ray crystallographic studies have been reported for the endo-1,4-β-xylanases from *Bacillus pumilus*-IPO and *Trichoderma harzianum* [113,114]. The crystals of endo-1, 4-β-xylanase from *Bacillus pumilus*-IPO are reported to be monocline, and space group was P2, with a = 4.08 nm, b= 6.68nm, c =3.47 nm and β = 103.0º, whereas the crystals of *Trichoderma harzianum* endo-1,4-β-xylanase unit cells are reported to be orthorhombic, and space group was P 2,2 with a = 4.42 nm, b = 9.41 nm and c = 5.16 nm. The molecular weights as determined by X-ray analysis have been found to be 20000 Da for *T. harzianum* and 22500 Da for *Bacillus pumilus*. The crystal formation of another endo-1,4-β-xylanase has been reported from *Cheatomium thermophile* [115].

2.5 Carbohydrate Binding Module

The structures of endo-xylanases of glycoside hydrolase families 10 and 11 are studied. Family F/10 endo-xylanases are composed of a cellulose binding domain and a catalytic domain connected by a linker peptide with a (β/α) (8) fold TIM barrel. Family G/11 endo-xylanases have a β-jelly roll structure and are considered to be able to pass through the pores of hemicellulose network owing to their smaller molecular sizes [116].Carbohydrate binding modules have much potential for applications concerning the use of plant material as food and feed as well as for the utilization and basic analysis of fibres out of these materials (www.immun.lth.se/research). Gullfot et al. [117] studied the xyloglucan specific carbohydrate binding module (CBM) in xylanase by the use of X-ray crystallography. Von Schantz et al. [118] studied structural basis for carbohydrate binding specificity taking into consideration two genetically engineered CBMs. Cicortas Gunnarsson et al. [119] isolated variants with substantially improved specificity against xylan. Kiyohara et al. [120] showed specificity in CBMs. They showed that β-1, 3-xylanase from Vibrio sp. specifically binds 1,3-β-xylan and 1,4-β-xylan does not bind at all. Okazaki et al. [121] also showed that CBM in β-1,3-xylanase from *Alcaligenes* sp. specifically binds 1,3- β-xylan and not the 1,4-β-xylan. However, Pavon-Orozco et al. [122] showed that CBM in *Cellulomonas flavigena* xylanase significantly enhances the synergy with a cellulase. They also showed that CBM binds to xylans and to a lesser extent to cellulose. Bolam et al. [123] reported the presence of two CBMs in *Cellulomonas fimi* xylanase 11A, where one CBM binds xylan and another CBM binds cellulose. They also determined three-dimensional structure of CBM2b-2 by NMR spectroscopy, a module specific for xylan binding.The module has a twisted β-sandwich architecture and the two surface exposed tryptophans, Trp570 and Trp602, which are in perpendicular orientation with each other, are shown to be essential for ligand binding. They also showed that upon changing Arg573 to glycine altered the polysaccharide binding specificity of the module from xylan to cellulose. Abou-Hachem et al. [124] produced two N-terminally repeated CBMs (CBM4-1 and CBM4-2) encoded by xyn10A gene from *Rhodothermus marinus* in *Escherichia coli*. Binding studies showed binding of these CBMs to insoluble xylan and to phosphoric acid swollen cellulose and binding is enhanced significantly in the presence of Na+ and Ca^{+} ions. The studies indicate that CBM plays an important role in the catalysis of xylanase by specifically binding xylan.

2.6 Xylanase Inhibitors

During plant pest infection, plant cells produce a group of proteins named as pathogenesis related proteins. These proteins range from cell wall/ membrane degrading enzymes to protease inhibitors and proteins related to oxidative metabolism [125]. Some of them act as attack molecules to damage the pathogen, while others act as defence molecules to protect plant cells from the molecular attack of pathogens. Xylanase inhibitor proteins are potential defence molecules which could act to prevent plant cell wall degradation by fungal hydrolytic enzymes. Vasconcelos et al. [126] isolated a new chitinase like xylanase inhibitor protein from coffee (*Coffea arabica*) that at 1.5 µg/µl concentration inhibited the germination of spores of Soybean Asian rust (*Phakopsora pachyrhizi*) by 45%. This xylanase inhibitor protein also inhibited xylanases from *Acrophialophora nainiana* by approximately 60% when present at 12:1 (w/w) enzyme: inhibitor ration. Earlier, Durand et al. [127] reported a chitinase like xylanase inhibitor protein isolated from rice which showed specificities towards fungal GH11 xylanases but did not exhibit any chitinolytic activity. Biely et al. [128] reported the presence of a xylanase inhibitor protein in both leaves and roots extracts of germinated maize. They reported unusual thermostability of these inhibitors. They reported a half life of more than 10 hours for these inhibitors when tested at pH 4.5 and at 100ºC. These inhibitors inhibited microbial xylanases but did not inhibit a plant endoxylanase. Therefore, they predicted the possible role of these inhibitors in maize defense against phytopathogens. Tokunaga and Esaka [129] reported that xylanase inhibitor protein expression is enhanced by various stresses viz. citrate, sodium chloride treatment, wounding, external ascorbic acid addition. The inhibitor in roots was induced by methyl jasmonate treatment in the root. However, it did not induce in the shoot by wounding and stress related phytohormones. On the basis of their study, they suggested that xylanase inhibitor protein involves in defense mechanism in rice. Overall, it may be concluded that xylanase inhibitor protein plays a defense role against phytopathogens.

2.7 Biotechnological Applications

The xylanase has been used for a number of commercial applications. In 1979, an U.S. patent for a method of xylanase production was issued. It was a mixture of enzymes used as an animal feed additive for dairy cattle [75]. Some important applications for xylanases are as follows:

2.7.1 Bioethanol production

The process of ethanol production from lignocellulosic biomass includes de-lignification of plant biomass and hydrolysis of cellulose and hemicellulose to monosaccharides [43]. The hydrolysis process can be performed by treatment with acids at high temperatures or by enzyme action. The acidic hydrolysis requires a significant energy consumption and acid resisting equipment which makes the process more expensive. However, enzymatic hydrolysis does not have these disadvantages. Because of the complex composition of lignocellulosic biomass, the synergistic action of several enzymes viz. endoglucanases, (EC 3.2.1.4), β-glucosidases (EC 3.2.1.21), endo-1, 4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) is required for complete hydrolysis [130]. In some cases, endo-1, 4-β-xylanase has been reported to be a bifunctional enzyme having endo-1,4-β-xylanase as well as cellulase activity. Bi-functionality of endo-1,4-β-xylanase could result in more efficient and cheaper saccharification process of the agricultural residues, municipal and industrial wastes used for bio-ethanol production as it can degrade both cellulose and xylan residues.

Saccharification of the cellulose and hemicellulose in biomass results in sugar-rich liquid streams useful for the production of a variety of value-added products including ethanol, furfural, and various functional biopolymers [131]. An increased possibility of fermentation of both hexoses and pentoses sugars present in lignocelluloses into methanol has also been reported [132].

2.7.2 Animal feedstocks

It has been found that addition of Endo-1,4-β-xylanase in animal feed stimulates animal growth rates by improving digestibility and improving the quality of animal litter [133,134]. The endosperm cell walls of cereal grains are enriched in polysaccharides that are usually in the form of arabinoxylans mixed with linked β-glucans, celluloses, mannans, and galactans [135]. Of these, arabinoxylans and β-glucans constitute major parts. For example, wheat, triticale, and rye are enriched in arabinoxylans [136], whereas oats and barley contain more β-glucans [137,138]. Generally, the viscous properties of the polysaccharides make them difficult to digest by domestic animals. Therefore, inclusion of xylanase in wheat- or rye-based diets [139,140] or β-glucanase in barley-based diets [141] is an important measure to enhance the availability of the polysaccharides. Endo-1,4-β-xylanase and 1,3-1,4-β-glucanase have been widely applied for breakdown of the internal β-1,4-linkages of 1,4- β-D-xylan backbone [36] and specific cleavage of 1,4- β-D-glucosidic bonds adjacent to β-1,3-linkages in the mixed-linked- β-glucans, respectively [142]. Therefore, endo-1,4-β-xylanase thins out the gut contents and allows increased nutrient absorption and increased diffusion of the pancreatic enzymes. Endo-1,4-β-xylanase also converts hemicellulose to sugars and thereafter, nutrients trapped within the cell walls are released and chickens get sufficient energy from lesser feed. The barn is cleaner due to more thoroughly digested feed and the chicken waste is drier and lesser sticky. In addition, chicken eggs are cleaner due to dry laying area [75]. Mathlouthi et al. [143] showed that feeding a rye based diet to chickens reduces villus capacity for nutrient absorption and bile acid capacity for fat solubilization and emulsification.

2.7.3 Production of xylooligosaccharides in paper industries

A recent and exciting application of xylanases is the production of xylo-oligosaccharides (XOs) [144], these are newly developed functional oligosaccharides that feature many beneficial biomedical and health effects, such as the stimulation of human intestinal *Bifidobacteria* growth [37]. Currently, XOs are produced mainly by enzymatic hydrolysis due to the high specificity, the negligible substrate loss, and side product generation [144].

Pulp and paper industry is one of the major polluting industries in the developed as well as developing countries [145]. In recent past, environment protection groups have taken strict actions to restrict the release of toxic components to water bodies. Therefore, in order to cope-up with the increasing pressure to protect the environment and to develop the cleaner technologies, various trials have been carried out by employing numerous enzymes [146], mediators [147] and microorganisms [148] for pulp and paper industries.

For paper production, it is important to retain cellulose with the removal of lignin from the paper pulp. An old classical method is based on bleaching of the paper pulp by chlorine. Endo-1, 4-β-xylanase is capable of splitting hemicellulose chains responsible for the close adherence of lignin to the cellulose network. Therefore, endo-1, 4-β-xylanase treatment is more suitable than lignin-degrading systems. The use of xylanases for pre-bleaching kraft pulp is known since long time [75,149]. Application of xylanase results in a lower chlorine

dosage, a lower chemical cost, and lower chloro-organic concentrations in pulp and effluent [76,150,151].

Xylan degradation requires the interaction of several enzymatic activities, including endo-1,4-β-xylanase, xylosidase and arabinosidase. The end products of this degradation are xylose, arabinose, and methyl-glucuronic acid containing oligosaccharides. Xylooligosaccharides are sugar oligomers showing potential for practical applications in a variety of fields, including pharmaceuticals, feed formulations, agricultural purposes and food applications [152]. As additives for functional foods, XOs have pre-biotic action showing positive biological effects such as improvement in the intestinal function by increasing the number of healthy *Bifidobacteria* [153-155]. These xylooligosaccharides if used as dietary supplements may be beneficial to gastrointestinal health and may reduce the risk of colon cancer [156]. As food ingredients, XOs have an acceptable odor, and are non-carcinogenic [157,158] and have low-calorie value, allowing their utilization in anti-obesity diets [159,160]. In food processing, XOs show advantages over insulin in terms of resistance to both acids and heat, allowing their utilization in low pH juices and carbonated drinks [161].

2.7.4 Pulp fiber morphology

After comparing micrographs of soft wood sulphate pulp with that of the same pulp after xylanase pre-bleaching and alkali extraction, Pekarovicova et al. [162] found that there is no marked change in the shape of fiber after xylanase pre-bleaching. However, flattening of the fiber 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 fiber surfaces [163].

2.7.5 Need for cellulase free endo-1,4-β-xylanase

The public concerned 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 bio-bleaching. 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 endo-1, 4-β-xylanases with tenuous or no cellulase activity can be applied in paper industry because the loss of pulp viscosity is at the minimum level.

2.7.6 Biobleaching

Endo-1, 4-β-xylanases have also been applied to bio-bleaching processes, as their removal of the xylan layer enhances the bleaching effect of chemical reagents [164-166]. Therefore, numerous bio-bleaching studies using endo-1, 4-β-xylanases or laccases have been reported [167-169]. A few studies have assessed both enzymes in sequence or jointly [166,170-172].

Chlorination of pulp does not show any decolorizing effect, and in fact, the color of the pulp may increase with chlorination and it is the oxidative mechanism which aids pulp bleaching. At low pH, the main reaction of chlorine is chlorination rather than oxidation. Therefore, chlorine selectively chlorinates and degrades lignin compounds rather than the carbohydrates moieties (eg. hemicelluloses –xylan) in the unbleached pulp. Enzymatic hydrolysis of the re-precipitated and relocated xylans on the surface of the fibers apparently renders the structure of the fiber more permeable.

2.7.7 Kraft process

Removal of residual lignin from kraft pulp is physically and chemically restricted by hemicelluloses. Lignin has been reported to link with hemicelluloses and there are reports regarding the isolation of lignin carbohydrate complexes from the kraft pulp [173].

2.7.8 Bread fluffier

Xylanases are used as additives in the baking industry to increase the elasticity of the gluten network. Elasticity improves handling and stability of the dough. Thereafter, the enzyme gets denatured and inactivated during bread baking. Addition of xylanases to wheat flour for bread making changes arabinoxylans resulting in nearly 10% more voluminous loaf [75]. Arabinoxylans are highly branched xylans present in wheat and rye flour. Several xylanases from bacterial and fungal sources are used in baking industries [174]. Xylanase action also increases crumb softness after storage. It is important to note that due to differences in substrate specificities, action patterns, interactions with inhibitors and kinetics capabilities, all xylanases are not effective in baking [75]. On the comparison of the abilities of different xylanases isolated from *Aspergillus oryzae*, *Humicola insolens* and *Trichoderma reesei* to improve the quality of bread made from wheat flour, it was shown that the most effective is xylanase from *A. oryzae* [175].The specific volume of bread increased by 8–13%, crumb firmness decreased by 15–24% compared to bread without xylanase. However, maximum anti-staling effect was observed with xylanase from *T. reesei.* Jiang et al. [176] isolated a xylanase from thermophilic bacteria, *Thermomyces lanuginosus* CAU44 and showed its application in bread making.

2.7.9 Baking

Enzymes play a central role in baking technology. Xylanase has been reported to have use in bread making [43]. It has been described that enzymatic hydrolysis of non-starch polysaccharides leads to improvement of rheological properties of dough, bread specific volume, and crumb firmness [177]. Endo-1,4-β-xylanase (xylanase) randomly attacks arabino-xylan backbone to cause a decrease in the degree of polymerization, hence leaving a strong impact on arabino-xylan structure and function [178,179]. At the optimum doses, xylanase improves dough machinability, dough stability, oven spring, loaf volume, crumb structure, and shelf life [180,181]. As a consequence of the hydrolytic action of xylanase, free sugars such as pentoses could be released, which might be used by microbes for fermentation. The non-starch polysaccharides in the endosperm cell wall of wheat constitute up to 75% of the dry matter weight [182]. There are 3-4% (w/w) pentosans in normal wheat flour which are partially soluble [179]. Several endo-1, 4-β-xylanases from bacterial and fungal sources have been used in baking industries [174]. Endo-1, 4-β-xylanase action also increases crumb softness after storage. It is important to note that due to differences in substrate specificities, action patterns, interactions with inhibitors and kinetics capabilities, all endo-1, 4-β-xylanases are not effective in baking. On comparison of the abilities of different endo-1, 4-β-xylanases isolated from *Aspergillus oryzae*, *Humicola insolens* and *Trichoderma reesei* to improve the quality of bread made from wheat flour, it was shown that the most effective is endo-1,4-β-xylanase from *A. oryzae* [175].The specific volume of bread increased by 8–13%, crumb firmness decreased by 15–24% compared to bread without endo-1,4-β-xylanase. However, maximum anti-staling effect was observed with endo-1,4-β-xylanase from *T. reesei.* Jiang et al. [176] isolated a endo-1,4-β-xylanase from a thermophilic bacteria, *Thermomyces lanuginosus* CAU44 and showed its application in bread making. Laurikainen et al. [183] observed increase in softening of wheat dough from 90 BU in control

to 170 BU in endo-1,4-β-xylanase supplemented dough upon addition of *Tricoderma* culture filtrate enriched in endo-1,4-β-xylanase. Martinez-Anaya and Jimenez [177] reported that starch and non-starch hydrolyzing enzymes result in the release of free water and change of soluble fraction of dough. Redgwell et al. [184] reported consistent decrease in viscosity of wheat flour batter and demonstrated that the viscosity change is caused due to the action of endo-1, 4-β-xylanase on the crude enzyme preparation. Jiang et al. [176] also reported an improvement in specific volume of wheat bread using endo-1, 4-β-xylanase B from *Thermotoga maritima.* Pescado–Piedra et al. [185] studied the effect of the addition of glucose oxidase, peroxidase and endo-1, 4-β-xylanase on dough rheological parameters and bread quality. They reported that the addition of peroxidase and endo-1,4-β-xylanase increased water absorption, while the incorporation of glucose oxidase had no effect on it. Crumb characteristics were only improved by endo-1, 4-β-xylanase. High concentration of glucose oxidase or endo-1, 4-β-xylanase decreases stability [75].

2.7.10 Fruit juice and beer clarification

Endo-1,4-β-xylanase helps in increasing juice yield from fruits or vegetables and also in the maceration process. Besides, it reduces the viscosity of the fruit juice improving its filterability [133]. Endo-1, 4-β-xylanase also improves extraction of more fermentable sugar from barley and therefore useful for making beer, as well as processing the spent barley for animal feed. In addition, added endo-1, 4-β-xylanase reduces the viscosity of the brewing liquid, improving its filterability [75].

2.7.11 Improving silage

It is known that endo-1,4-β-xylanase and cellulase treatment of forages produces a better quality silage that improves the subsequent rate of plant cell wall digestion by ruminants. There is a considerable amount of sugar sequestered in the xylan of plant biomass. As a result of endo-1,4-β-xylanase treatment, there is increased nutritive sugar and that is useful for digestion in cow and other ruminants. It is also known that endo-1,4-β-xylanase also produces compounds which are the nutritive source for many ruminal microflora [75].

2.7.12 Bioconversion

For bioconversion process that converts lignocellulose to fermentative products, maximal utilization of the various polymeric sugars is desirable. Present knowledge clearly suggests that “complete” xylanolytic (and cellulolytic) systems are required to achieve maximum hydrolysis of complex substrates to yield monomeric residues.

In certain bioconversion processes, total hydrolysis of substrate may not be required or desirable at the hydrolysis phase. For example, fermentative organisms such as *Klebsiella pneumonae* can utilize disaccharides such as xylobiose [186]. Furthermore, hydrolysis limitations associated with product inhibition can be relieved by using a method such as sequential co-culture [186] or simultaneous saccharification and fermentation [187]. Finally, the release of acetyl, glucuronosyl, or phenolic residues, or all the three, during hydrolysis may interfere with fermentation.

2.7.13 Bioenergy

Chiranjeevi et al. [188] optimized the influential parameters for the production of holocellulases (mixture of cellulases and xylanases) from *Cladosporium cladosporioides*

under submerged cultivation system. They discussed that efficient holocellulases cocktail plays a significant role in commercialization of biorefinery, textile, detergent formulation and paper manufacturing industries. Song et al. [189] reported that wheat straw is an abundant coproduct of the agri-food industry and is considered to be a primary source of lignocellulosic biomass for second generation biorefining. They engineered GH11 xylanase by mutating at position 111 using a directed evolution strategy in order to develop better biomass degrading ability in it. However, they also pointed out that enzyme engineering alone can not overcome the limits imposed by the complex organization of the plant cell wall and the lignin barrier. Cavka et al. [190] investigated the possibility to utilize fiber sludge, waste fibers from pulp mills and lignocellulose based biorefineries for combined production of liquid biofuel and biocatalysts. They showed the potential of converting waste fiber sludge to liquid biofuel and enzymes as coproducts in lignocellulose based biorefineries. A most thermal and acid stable xylanase called as xtreme xylanase has been discovered in a catalogued bacteria, *Alicyclobacillus acidocaldarius* originally isolated from Nymph Creek in Yellowstone National Park's Norris Geyser basin. The enzyme is stable and active at temperatures ranging from hot tap water to nearly boiling, and in acidic conditions ranging from battery acid to acid rain. The enzyme is capable of efficiently converting hemicellulose and cellulose components of biomass into energy rich sugars and these sugars can be used to make fuels and high value chemicals (www.inl.gov/research/xtreme-xylanase/).

2.7.14 Cloning of the gene

Endo-1,4-β-xylanase genes have been cloned from various species and homology has been determined. Information on several cloned endo-1,4-β-xylanase genes have been summarized in Table 1.

3. PROTEIN ENGINEERING

Since xylanase is of much industrial importance, efforts have been done to get more thermostable xylanases by using protein engineering approach. Mining new genes from nature, rational engineering of known genes and directed evolution of these genes are required to get tailor made xylanaaes for various industrial applications [116]. Wang et al. [206] introduced a disulfide bridge Q1C-Q24C in the N-terminal region of xylanase TLX isolated from *Thermomyces lanuginosus* GH11. The engineered enzyme has been shown to exhibit higher temaperature optimum. The resistance to thermal inactivation was also found to increase by about 10ºC. The N-terminal disulfide bridge increased both kinetic and thermodynamic stability almost equally. Hokanson et al. [207] reported that a diverse set of mutations including N71D, Y73G, T95G and Y96Q in*Trichoderma reesei* xylanase GH11 when combined into a single construct (Hjx-81), the enzyme was active even after heating at 100^{o}C for 20 minutes. You et al. [208] reported a mutant of a family 11 xylanase (xyn-CDBFV) from *Neocallimastix patriciarum* with a mutation, D57N in the active center and showed stabilization of this mutant by the substrate. Zhang et al. [209] engineered thermostable xylanase XT6 from Geobacillus stearothermophilus having 13 amino acids substitutions and showed that engineered enzyme had half life of inactivation 52 fold of that of the wild type. Therefore, the mutant enzyme is of potential interest for industrial applications. Roberge et al. [210] investigated various mutations in xylanase A from Streptomyces lividans. They showed that W266 is important for both substrate binding and ifficient catalysis by the enzyme.

Table 1. Cloning of endo-1, 4-β-xylanase genes from various species

Gene	Species	Sequence identity	Total no. of amino acid residues	Molecular weight	Reference
Endo-1,4-β-xylanase	*Verticillium dahliae*	72% sequence identity on comparison with endo-1,4-β-xylanase genes from *Cochlibolus carbonum* and *Cochlibolussativus*			191
Xyn A4	*Alicyclobacillus species* A4	53% identity on comparison with endo-1,4-β-xylanase gene from *Geobacillusstearothermophilus*	338		192
Xyl C	*Cellvibrio mixtus*				94
Xyl OC	*Bispora species* MEY-1	57.3% identity with endo-1,4-β-xylanase gene from *Aspergillus fumigatus* Af 293			193
Bna A	*Neocallimastixpatriciarum*				194
Xyn 11 A	*Lentinula edodes*		283		195
Xyn A	*Bacillus species* BP-7			24kDa	95
Xyn A	*Pacilomyces thermophila*				196
Xyl A	*Bacillus subtilis* AMX-4		213		197
Endo-1,4-β-xylanase	*Streptomyces* species TN 119	48.8% identity with endo-1,4-β-xylanase from *Cellulosimicrobium species* HY-12			198
Endo-1,4-β-xylanase	*Bacillus licheniformis*			23kDa	96
Xyn A	*Paenibacillus species* DG-22	76% identity with endo-1,4-β-xylanase from *Bacillus halodurans* C-125	210	20kDa	97

Xyn 5	*Paenibacillus species* W-61		1326		199
Xyn B527	*Steptomyces species* S-27	75.9% identity with endo-1,4-β-xylanase from *steptomyces species* Zxy19	230		200
Syn AS9		50.85% identity with endo-1,4-β-xylanase from *Steptomyces avermitilis*	465		201
Endo-1,4-β-xylanase	*Aspergillus terreus* BCC-129		302	32kDa	202
Endo-1,4-β-xylanase	*Eubacteria* Rt8b.4			36 KDa	203
Xyn C	*Clostridium stercorarium*		1031		204
Xyn 10A	*Paenibacillus curdlnolyticus* B-6		1276		205

4. CONCLUSION

Endo-1, 4-β-xylanase is an industrially important enzyme which degrades xylan randomly and produces xylooligosaccharides, xylobiose and xylose. It is mainly present in microbes and plants but not in animals. Endo-1,4-β-xylanase has been extensively studied from fungal and bacterial sources and it has been commercially produced. Its potential use in paper industries has been discussed which is directly related to reduction in environmental pollution. It has role in bio-bleaching paper pulp and increasing pulp brightness. Besides, it can be exploited for ethanol production and as an additive in animal feedstock to improve its nutritional value. Endo-1,4-β-xylanase can also be exploited in baking and fruit juice industries. Here, we reviewed its distribution, structural aspects, industrial/ biotechnological applications. Bioseparation technique is proved to be much advantageous for isolation and purification of xylanase in getting much higher yield with sufficient purity. In plants, xylanase inhibitor proteins have been found which have been shown to act as defense molecule against phytopathogens.Besides, we also discussed studies related to cloning of the gene encoding endo-1,4-β-xylanase with the objectives of overproducing the enzyme and altering its properties to suit commercial applications. Various studies related to protein engineering have been discussed. Protein engineering is a strong tool to get more thermostable xylanase having industrial applications. Besides, importance of carbohydrate binding module has been discussed. It has been reported by several workers that carbohydrate binding module has much specificity. Upon changing the specific amino acid into another amino acid, change in polysaccharide binding specificity has been reported. Therefore, there is much scope in protein engineering (tailoring) research of xylanase in order to exploit it more appropriately for industrial applications. Xylanase can also be exploited to revolutionize biorefineries.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

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