A Review of Xylanase Production by the Fermentation of Xylan: Classification, Characterization and Applications

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1. Introduction

The enzymatic hydrolysis of xylan, which is the second most abundant natural polysaccharide, is one of the most important industrial applications of this polysaccharide [1, 2]. The primary chain of xylan is composed of β-xylopyranose residues, and its complete hydrolysis requires the action of several enzymes, including endo-1,4-β-D-xylanase (EC3.2.1.8), which is crucial for xylan depolymerization [2]. Due to the diversity in the chemical structures of xylans derived from the cell walls of wood, cereal or other plant materials, a large variety of xylanases with various hydrolytic activities, physicochemical properties and structures are known. Moreover, xylan derivatives are frequently used to induce the production of xylanases [3] by microorganisms [4], using either solid-state or submerged fermentation [5].

Xylanases and the microorganisms that produce them are currently used in the management of waste, to degrade xylan to renewable fuels and chemicals, in addition to their use in food, agro-fiber, and the paper and pulp industries, where the enzymes help to reduce their environmental impact [6]. Oligosaccharides produced by the action of xylanases are further used as functional food additives or alternative sweeteners with beneficial properties [7].

To meet the needs of industry, more attention has been focused on the enzyme stability under different processing conditions, such as pH, temperature and inhibitory irons, in addition to its ability to hydrolyze soluble or insoluble xylans. Although many wild-type xylanases contain certain desired characteristics, such as thermostability, pH stability or high activity, no individual xylanase is capable of meeting all of the requirements of the feed and food industries. Moreover, as industrial applications require cheaper enzymes, the elevation of expression levels and the efficient secretion of xylanases are crucial to ensure the viability of

the process; therefore, genetic engineering and recombinant DNA technology have an important role in the large-scale expression of xylanases in homologous or heterologous protein-expression hosts.

Considering the future prospects of xylanases in biotechnological applications, the goal of this review chapter is to present an overview of xylanase production via fermentation and to describe some of the characteristics of these enzymes and their primary substrate, xylan. Moreover, this review will discuss the fermentation processes as well as the genetic techniques applied to improve xylanase yields.

2. Xylan

The three main components that constitute lignocellulosic substrates are cellulose, hemicellulose and lignin [8]. Schulze [9] first introduced the term 'hemicellulose' to represent the fractions isolated or extracted from plant materials using a dilute alkali. Hemicelluloses are composed of complex mixtures of xylan, xyloglucan, glucomannan, galactoglucomannan, arabinogalactan or other heteropolymers [8].

The substrate of xylanase, xylan, is the second most-abundant polysaccharide in nature, accounting for approximately one-third of the renewable organic carbon on Earth [10], and it constitutes the major component of hemicellulose, a complex of polymeric carbohydrates, including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose) and arabinogalactan (heteropolymer of D-galactose and arabinose) [11]. Xylan is primarily present in the secondary cell wall and together with cellulose (1,4-β-glucan) and lignin (a complex polyphenolic compound) make up the major polymeric constituents of plant cell walls [12]. Within the cell wall structure, all three constituents interact via covalent and non-covalent linkages, with xylan being found at the interface between lignin and cellulose, where it is believed to be important for fiber cohesion and plant cell wall integrity [1].

2.1. Structure and distribution

A complex, highly branched heteropolysaccharide, xylan varies in structure between different plant species, and the homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl units can be substituted to varying degrees with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl or p-coumaroyl side-chain groups [12,13] (Figure 1).

Xylan is distributed in several types of tissues and cells and is present in a variety of plant species [12], being found in large quantities in hardwoods from angiosperms (15–30% of the cell wall content) and softwoods from gymnosperms (7–10%), as well as in annual plants (<30%) [14]. Wood xylan exists as O-acetyl-4-O-methylglucuronoxylan in hardwoods and as arabino-4-O-methylglucuronoxylan in softwoods, while xylans in grasses and annual plants

are typically arabinoxylans [12]. Linear unsubstituted xylan has also been reported in esparto grass [15], tobacco [16] and certain marine algae [17,18], with the latter containing xylopyranosyl residues linked by both 1,3-β and 1,4-β linkages [17,19].

Similar to other polysaccharides of plant origin, xylan has a large polydiversity and polymolecularity [20]. The degree of polymerization in xylans is also variable, with, for example, hardwood and softwood xylans generally consisting of 150-200 and 70-130 β-xylopyranose residues, respectively [12].
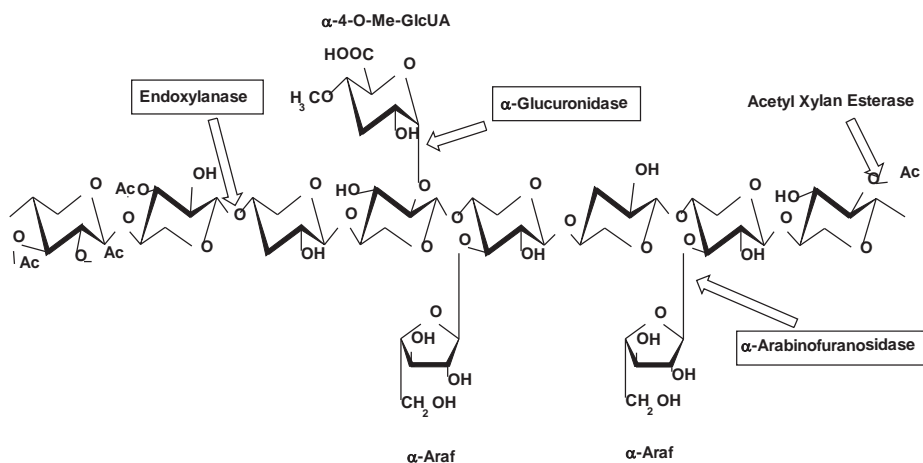

Figure 1. Structure of xylan and the xylanolytic enzymes involved in its degradation. Ac: Acetyl group; α-Araf: α-arabinofuranose; α-4-O-Me-GlcA: α-4-O-methylglucuronic acid. Source: Sunna and Antranikian [20].

Based on the common substituents found on the backbone, xylans are categorized as linear homoxylan, arabinoxylan, glucuronoxylan or glucuronoarabinoxylan. Homoxylans consisting exclusively of xylosyl residues are not widespread in nature; they have been isolated from limited sources, such as esparto grass, tobacco stalks and guar seed husks [20]. However, based on the nature of its substituents, a broad distinction may therefore be made among xylans, in which the complexity increases from linear to highly substituted xylans. Four main families of xylans can be considered [21]:

i.   Arabinoxylans, having only side chains of single terminal units of α-L-arabinofuranosyl substituents. In the particular case of cereals, arabinoxylans vary in the degree of arabinosyl substitution, with either 2-O- and 3-O-mono-substituted or double (2-O-, 3-O-) substituted xylosyl residues.

ii.   Glucuronoxylans, in which α-D-glucuronic acid or its 4-O-methyl ether derivative represents the only substituent.

iii.   Glucuronoarabinoxylan, in which α-D-glucuronic (and 4-O-methyl-α-D-glucuronic) acid and α-L-arabinose are both present.

iv.   Galactoglucuronoarabinoxylans, which are characterized by the presence of terminal β-D-galactopyranosyl residues on complex oligosaccharide side chains of xylans and are typically found in perennial plants.

In each category there exists microheterogeneity with respect to the degree and nature of branching. The side chains determine the solubility, physical conformation and reactivity of the xylan molecule with the other hemicellulosic components and hence greatly influence the mode and extent of enzymatic cleavage [12]. Endospermic arabinoxylans of annual plants, also called pentosans, are more soluble in water and alkaline solutions than xylans of lignocellulosic materials because of their branched structures [22].

2.2. Enzymatic hydrolysis of xylan

Due to the heterogeneity and complex chemical nature of plant xylan, its complete breakdown requires the action of a complex of several hydrolytic enzymes with diverse specificities and modes of action. Thus, it is not surprising for xylan-degrading cells to produce an arsenal of polymer-degrading proteins [1]. The xylanolytic enzyme system that carries out the xylan hydrolysis is normally composed of a repertoire of hydrolytic enzymes, including endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase   (α-glucosiduronase,   E.C.3.2.1.139),   α-arabinofuranosidase   (α-L-arabinofuranosidase, E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72) [23]. All of these enzymes act cooperatively to convert xylan into its constituent sugars [24]. Among all xylanases, endoxylanases are the most important due to their direct involvement in cleaving the glycosidic bonds and in liberating short xylooligosaccharides [8].

Xylan, being a high molecular mass polymer, cannot penetrate the cell wall. The low molecular mass fragments of xylan play a key role in the regulation of xylanase biosynthesis. These fragments include xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers. These molecules are liberated from xylan through the action of small amounts of constitutively produced enzymes [12]. Xylanase catalyzes the random hydrolysis of xylan to xylooligosaccharides, while β-xylosidase releases xylose residues from the nonreducing ends of xylooligosaccharides. However, a complete degradation requires the synergistic action of acetyl esterase to remove the acetyl substituents from the β-1,4-linked D-xylose backbone of xylan [25,26].

3. Xylanases

3.1. Classification and mode of action

Xylanases, as glycoside hydrolase members, are able to catalyze the hydrolysis of the glycosidic linkage (β-1,4) of xylosides, leading to the formation of a sugar hemiacetal and the corresponding free aglycone (nonsugar compound remaining after replacement of the glycoside by a hydrogen atom [27]). Xylanases have been classified in at least three ways: based on the molecular weight and isoelectric point (pI) [28], the crystal structure [29] and kinetic properties,

or the substrate specificity and product profile. As the first classification is not sufficient to describe all xylanases, several exceptions have been identified [10] because not all xylanases have a high molecular mass (above 30 kDa) and low pI or a low molecular mass (less than 30 kDa) and high pI [6]. Therefore, a more complete system, based on the primary structure and comparison of the catalytic domains, was introduced [10,30], analyzing both the structural and mechanistic features [10].

Updated information on the characteristics and classification of enzymes may be found in the Carbohydrate-Active Enzyme (CAZy) database. This is a knowledge-based resource specializing on enzymes that build and breakdown complex carbohydrates and glycoconjugates. This database contains information from sequence annotations found in publicly available sources (such as the National Center for Biotechnology Information, NCBI), family classifications and known functional information [31]. According to the CAZy database (http://www.cazy.org), xylanases (EC3.2.1.8) are related to glycoside hydrolase (GH) families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62. However, the sequences classified in families 16, 51 and 62 appear to be bifunctional enzymes containing two catalytic domains, unlike families 5, 7, 8, 10, 11 and 43, which have a truly distinct catalytic domain with endo-1,4-β-xylanase activity [10]. Using the same analysis, families 9, 12, 26, 30 and 44 may have residual or secondary xylanase activity.

Xylanases have been primarily classified as GH 10 and 11 based on the hydrophobic cluster analysis of the catalytic domains and similarities in the amino acid sequences [8]. Although members of these two families have been thoroughly studied, the catalytic properties of the members of the remaining families (5, 7, 8 and 43) are recent and remain very limited [32].

Members of GH families 5, 7, 8, 10, 11 and 43 differ in their physicochemical properties, structure, mode of action and substrate specificities [10]. Several models have been proposed to explain the mechanism of xylanase action. Xylanase activity leads to the hydrolysis of xylan. Generally, this hydrolysis may result either in the retention (GH families 5, 7, 10 and 11) or the inversion (GH families 8 and 43) of the anomeric center of the reducing sugar monomer of the carbohydrate [33,34].

Families 5, 7, 10 and 11 contain enzymes that catalyze the hydrolysis with the retention of the anomeric configuration, with two glutamate residues being implicated in the catalytic mechanism. This indicates a double-displacement mechanism, in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolyzed, and two carboxylic acid residues, suitably located in the active site, are involved in the formation of the intermediate; one acts as a general acid catalyst by protonating the substrate, while the second performs a nucleophilic attack, which results in the departure of the leaving group and the formation of the α-glycosyl enzyme intermediate (β to α inversion). In the second step, the first carboxylate group instead functions as a general base, abstracting a proton from a nucleophilic water molecule, which attacks the anomeric carbon. This leads to a second substitution, in which the anomeric carbon again passes via a transition state to give rise to a product with the β configuration (α to β inversion) [10,34].

In contrast to the mechanism mentioned above, the enzymes in families 8 and 43 generally act via an inversion of the anomeric center, and glutamate and aspartate may be the

catalytic residues. Inverting enzymes work via a single displacement reaction, in which one carboxylate provides for a general acid-catalyzed leaving group departure. The second function of these enzymes, acting as general base, activates a nucleophilic water molecule to attack the anomeric carbon, thereby cleaving the glycosidic bond and leading to an inversion of the configuration at the anomeric carbon. Generally, the distance between the two residues allows for the accommodation of the water molecule between the anomeric carbon and the general base [10,34].

3.1.1. GH families 10 and 11

Xylanase from the GH10 family (or family G) have a low molecular mass with a pI between 8–9.5, while those from the GH11 family (or family F) have a high molecular mass and lower pI values [35,36].

Glycoside hydrolase family 10 is composed of endo-1,4-β-xylanases and endo-1,3-β-xylanases (EC 3.2.1.32) [34]. Members of this family are also capable of hydrolyzing the aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond. Furthermore, these enzymes are highly active on short xylooligosaccharides, thereby indicating small substrate-binding sites. Crystal structure analyses, kinetic analyses of the activity on xylooligosaccharides of various sizes and end product analyses have indicated that family 10 xylanases typically have four to five substrate-binding sites [37]. Members of this family also typically have a high molecular mass, a low pI and display an $(\alpha/\beta)8$-barrel fold [10,34,38].

Compared to other xylanases, GH11 members display several interesting properties, such as high substrate selectivity and high catalytic efficiency, a small size, and a variety of optimum pH and temperature values, making them suitable in various conditions and in many applications [39]. Family 11 is composed only of xylanases (EC3.2.1.8), leading to their consideration as "true xylanases," as they are exclusively active on D-xylose-containing substrates. GH11 enzymes are generally characterized by a high pI, a low molecular weight, a double-displacement catalytic mechanism, two glutamates that act as the catalytic residues and a β-jelly roll fold structure. Additionally, the products of their action can be further hydrolyzed by the family 10 enzymes [37]. Similar to family 10 xylanases, these enzymes can hydrolyze the aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond, but they are inactive on aryl cellobiosides. Furthermore, in contrast to the family 10 xylanases, but similar to the family 8 cold-adapted xylanases, these enzymes are most active on long-chain xylooligosaccharides, and it has been found that they have larger substrate-binding clefts, containing at least seven subsites [10].

Xylanases belonging to GH10 exhibit greater catalytic versatility and lower substrate specificity than those belonging to GH11 [37,40]. According to Davies *et al*. [41], the binding sites for xylose residues in xylanases are termed subsites, with bond cleavage occurring between the sugar residues at the -1 (non-reducing) and the +1 (reducing) ends of the polysaccharide substrate. As observed in assays using arabinoxylan as the substrate, GH10 products have arabinose residues substituted on xylose at the +1 subsite, whereas GH 11 products have arabinose residues substituted at the +2 subsite [42]. These results suggest that GH 10 enzymes are able to hydrolyze xylose linkages closer to the side-

chain residues [43]. Therefore, xylanases from family 11 preferentially cleave the unsub-stituted regions of the arabinoxylan backbone, whereas GH10 enzymes cleave the decorated regions, being less hampered by the presence of substituents along the xylan backbone [37]. The xylan side-chain decorations are recognized by xylanases, and the de-gree of substitution in xylan will influence the hydrolytic products; this difference in substrate specificity has important implications in the deconstruction of xylan [43].

3.1.2. GH families 5, 7, 8 and 43

GH family 5 (or family A) is the largest glycoside hydrolase family, and only seven amino acid residues, including the nucleophile and the general acid/base residue, are strictly conserved among all members [10]. Structural alignment among the members of family 5 and 10 showed that these enzymes are as structurally different within family 5 as they are to the family 10 enzymes, therefore both families are classified into clan GH-A. The concept of clan or superfamily demonstrates a broader relationship between GH families, suggesting a more distant common evolutionary ancestor [44,45]. Further-more, the activity of these enzymes is affected by substituents on the xylan main chain, and it is unable to cleave linkages adjacent to substituted residues. Hydrolysis studies have shown that the shortest substituted fragments formed from glucuronoxylan and arabinoxylan are substituted xylotrioses, with the substitution being found on the inter-nal xylose residue. Therefore, the products produced by family 5 are shorter than those produced by family 7 [10].

GH family 8 (or family D) is composed of cellulases (EC 3.2.1.4), and also contains chitosanases (EC 3.2.1.132), lichenases (EC 3.2.1.73) and endo-1,4-β-xylanases (EC 3.2.1.8). This family of cold-adapted xylanases was found to hydrolyze xylan to xylotriose and xylotetraose and was most active on long-chain xylooligsaccharides. Similar to family 11 xylanases, a large substrate binding cleft containing at least six xylose-binding residues, with the catalytic site in the middle, was proposed [6]. However, unlike family 10 and 11 xylanases, enzymes from family 8 were found to catalyze hydrolysis with the inversion of the anomeric configuration and, under the conditions used, were found to be inactive on aryl β-glycosides of xylose, xylobiose and xylotriose [10,31].

GH families 7 and 43 contain only a few enzymes exhibiting xylanase activity that have been identified and studied. Family 7 has characteristics in common with both family 10 and 11 xylanases. Similar to the former family, those in family 7 have a high molecular weight and low pI, as well as a small substrate-binding site, containing approximately four subsites, with the catalytic site in the middle [10]. The members of family 43 have not been as thoroughly studied, and the structure of only one member has been determined, indicating that members of this family may display a five-blade β-propeller fold. Furthermore, a glutamate and aspartate in the center of a long V-shaped surface groove formed across the face of the propeller have been suggested as the catalytic residues. Family 43 is grouped with family 62 in clan GH-F, and, as also demonstrated in the family 8 enzymes, its members are believed to catalyze hydrolysis via a single displacement mechanism [10,31].

3.2. Properties and applications

The heterogeneity and complexity of xylan have resulted in a diverse range of xylanases, which differ in their physicochemical properties, structure, mode of action and substrate specificities [10]. As the xylosidic linkages in lignocellulose are neither equivalent nor equally accessible, the production of an enzymatic system with specialized functions is a strategy to achieve superior xylan hydrolysis [28]. Together with the heterogeneous nature of xylan, the multiplicity of xylanases in microorganisms may be caused by a redundancy in gene expression. Generally, a single xylanase gene encodes multiple xylanases, and xylanase multiplicity may arise from posttranslational modifications, such as differential glycosylation, proteolysis or both [23].

The potential applications of xylanases also include the bioconversion of lignocellulosic material and agro-wastes into fermentative products, the clarification of juices, the improvement of the consistency of beer and the digestibility of animal feedstocks [28]. One of the most important biotechnological applications of xylanase is its use in pulp bleaching [46]. Xylanases may also be applicable to the production of rayon, cellophane and several chemicals such as cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethyl cellulose and methyl and ethyl cellulose), which are all produced by dissolving pulp and purifying fibers from other carbohydrates [33].

3.2.1. The paper and pulp industries

During the past several years, the use of enzymes in paper and pulp bleaching has caught the attention of researchers and industries all over the world. Xylanase enzymes have proven to be a cost-effective means for mills to take advantage of a variety of bleaching benefits [47]. Xylanases and other side-cleaving enzymes have been used in pulp bleaching primarily to reduce lignin and increase the brightness of the pulp [20,46]. The importance of xylanase in the pulp and paper industries is related to the hydrolysis of xylan, which facilitates the release of lignin from paper pulp and, consequently, reduces the usage of chlorine as the bleaching agent [33].

Bleaching is the process of lignin removal from chemical pulps to produce bright or completely white finished pulp [1]. Thus, the bleaching of pulp using enzymes or ligninolytic microorganisms is called biobleaching [48]. This process is necessary due to the presence of residual lignin and its derivatives in the pulping process, which causes the resultant pulp to gain a characteristic brown color. The intensity of this pulp color is related to the amount and chemical state of the remaining lignin [33].

The bleaching of pulp involves the destruction, alteration or solubilization of the lignin, colored organic matter and other undesirable residues on the fibers [33]. Bleaching of kraft pulp usually requires large amounts of chlorine-based chemicals and sodium hydrosulfite, which cause several effluent-based problems in the pulp and paper industries. The use of these chemicals generates chlorinated organic substances, some of which are toxic, mutagenic, persistent, and highly resistant to biodegradation, in addition to causing numerous harmful disturbances in biological systems and forming one of the major sources of environmental pollution [1,33,49].

As hemicellulose is easier to depolymerize than lignin, biobleaching of pulp appears to be more effective with the use of xylanases than with lignin-degrading enzymes. This is due to the fact of the removal of even a small portion of the hemicellulose could be sufficient to open up the polymer, which facilitates removal of the residual lignin by mild oxidants [33,50].

The use of xylanase in bleaching pulp requires the use of enzymes with special characteristics. A key requirement is to be cellulose-free, to avoid damaging the pulp fibers [3], as cellulose is the primary product in the paper industry [33]. Other desirable characteristics are stability at high temperatures [51] and an alkaline optimal pH [48].

Madlala *et al*. [52] used different preparations of commercial Xylanase P and crude xylanase from *Thermomyces lanuginosus* to evaluate the bleaching process of paper pulp. It was demonstrated that the use of enzymes could increase the pulp brightness (over 5 brightness points over the control) and reduce the amount of bleaching chemicals used (up to 30% for chlorine dioxide). Chipeta *et al*. [53] evaluated crude xylanase preparations from *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 and found that at a charge of 10 U per gram of pulp it was possible to reduce the usage of chlorine dioxide up to 30% without compromising the pulp brightness.

3.2.2. Bioconversion of lignocellulose in biofuels

Currently, second-generation biofuels are the primary products of the bioconversion of lignocellulosic materials. According to Taherzadech and Karimi [54], ethanol is the most important renewable fuel in terms of volume and market value, and following the fossil fuel crisis, it has been identified as an alternative fuel [48]. Despite the primarily first-generation production of ethanol, from sugar and starch, the second-generation production of ethanol has only begun to be tested in pilot plants [55]. And, unlike first-generation biofuels, second-generation biofuels do not compete with food production and can provide environmental, economic, and strategic benefits for the production of fuels [56].

Xylanase, together with other hydrolytic enzymes, can be used for the generation of biological fuels, such as ethanol, from lignocellulosic biomass [1,57]. However, enzymatic hydrolysis is still a major cost factor in the conversion of lignocellulosic raw materials to ethanol [56]. In bioethanol fuel production, the first step is the delignification of lignocellulose, to liberate cellulose and hemicellulose from their complex with lignin. The second step is a depolymerization of the carbohydrate polymers to produce free sugars, followed by the fermentation of mixed pentose and hexose sugars to produce ethanol [1,58]. Simultaneous saccharification and fermentation is an alternative process, in which both hydrolytic enzymes and fermentative microorganism are present in the reaction [48,59].

3.2.3. The pharmaceutical, food and feed industries

Xylanase, together with pectinase, carboxymethylcellulase and amylase, can be used for the clarification of juices because the turbidity observed is due to both pectic materials and other materials suspended in a stable colloidal system [60]. Xylanase may also improve the extraction of coffee, plant oils, and starch [25]. The xylose resulting from xylan depolymerization may

also be converted to xylitol, a valuable sweetener that has applications in both the pharmaceutical and food industries [61-63].

In the bakery industry, xylanase may improve the quality of bread, by increasing the bread's specific volume. In combination with amylases, this characteristic was enhanced, as observed upon the introduction of *Aspergillus niger* var. *awamori* [64]. According to Collins *et al.* [65], psychrophilic enzymes may be suitable for use in the baking industry as they are generally optimally active at the temperatures most frequently used for dough preparation (at or below 35 °C). These enzymes could also be used as more efficient baking additives than the currently used commercial mesophilic enzymes, which are optimally active at higher temperatures.

Xylanase may also improve the nutritional properties of agricultural silage and grain feed. The use of this enzyme in poultry diets showed that the decrease in weight gain and feed conversion efficiency in rye-fed broiler chicks has been associated with intestinal viscosity [66]. The incorporation of xylanase from *Trichoderma longibrachiatum* into the rye-based diet of broiler chickens reduced intestinal viscosity, thus, improving both the weight gain of the chicks and their feed conversion efficiency [67].

Xylanases can also be used in cereals as a pretreatment for arabinoxylan-containing substrates, as arabinoxylans are partly water soluble and result in a highly viscous aqueous solution. This high viscosity of cereal grain water extract may lead to brewing problems, by decreasing the rate of filtration or haze formation in beer. Additionally, it is unfavorable in the cereal grains used in animal feeding [68,69].

The enzymatic hydrolysis of xylan may also result in oligomers known as xylooligosaccharides (XOs), which may be used in pharmaceutical, agriculture and feed products. XOs have prebiotic effects, as they are neither hydrolyzed nor absorbed in the upper gastrointestinal tract, and they affect the host by selectively stimulating the growth or activity of one or a number of bacteria in the colon, thus improving health [70-72]. Among their key physiological advantages are the reduction of cholesterol, maintenance of gastrointestinal health, and improvement of the biological availability of calcium. They also inhibit starch retrogradation, improving the nutritional and sensory properties of food [73]. For the production of XOs, the enzyme complex must have low exoxylanase or β-xylosidase activity, to prevent the production of high amounts of xylose, which has inhibitory effects on XO production [74,75].

3.3. Xylanase assays

The xylanase activity is often assayed based on measurement of reducing sugar released during the course of hydrolysis of xylan, by DNS or Nelson-Somogyi methods. Due to absent of standardization, Bailey *et al.* [76] compared the measurement of xylanase activity by twenty different laboratories. According to the author, the major source of variation between apparent xylanase activities was probably the substrate chosen, although small differences in protocols were also significant. After standardization of substrate

and method, the interlaboratory standard variation of the results decreased from 108% to 17% from the mean. Others researchers use the 4-o-methylglucuronoxylan covalently dyed with Remazol Brilliant Blue (RBB xylan) as substrate, and the xylanase is assayed based on the release of the dyed fragments [77]. There are also available some commercial methods for xylanase assays, as the fluorescence-based method EnzChek® Ultra Xylanase Assay Kit (Invitrogen, Carlsbad, CA) or the Xylazyme tablet (Megazyme, Bray, Ireland), which employs azurine-crosslinked arabinoxylan (AZCLArabinoxylan) as substrate and its hydrolysis by xylanase produces water soluble dyed fragments.

3.4. Producing microorganisms

Microorganisms, in particular, have been regarded as a good source of useful enzymes because they multiply at extremely high rates and synthesize biologically active products that can be controlled by humans. In recent years, there has been a phenomenal increase in the use of enzymes as industrial catalysts. These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons: they exhibit high catalytic activity and a high degree of substrate specificity, they can be produced in large amounts, they are highly biodegradable, they pose no threat to the environment and they are economically viable [4].

In this context, microbial xylanases are the preferred catalysts for xylan hydrolysis, due to their high specificity, mild reaction conditions, negligible substrate loss and side product generation. Xylanases derived from microorganisms have many potential applications in the food, feed, and paper pulp industries [10,12,78]. Complete xylanolytic enzyme systems, which including all of these activities, have been found to be widespread among fungi [20,24], actinomycetes [79] and bacteria [12], and some of the most important xylanolytic enzyme producers include *Aspergillus*, *Trichoderma*, *Streptomyces*, *Phanerochaetes*, *Chytridiomycetes*, *Ruminococcus*, *Fibrobacteres*, *Clostridia* and *Bacillus* [12,78,80,81]. The ecological niches of these microorganisms are diverse and widespread and typically include environments where plant materials accumulate and deteriorate, as well as in the rumen of ruminants [78,82,83].

Although there have been many reports on microbial xylanases since the 1960s, the prime focus has been on plant pathology related studies [84]. Only during the 1980's did the use of xylanases for biobleaching begin to be tested [85]. Since 1982, several microorganisms, including fungi and bacteria, have been reported to readily hydrolyze xylans by synthesizing 1,4-β-D endoxylanases (E.C. 3.2.18) and β-xylosidases (EC.3.2.1.37) [86]. Table 1 presents a list of some of the xylanase-producing microorganisms and their activities.

The production of xylanases must be improved by finding more potent fungal or bacterial strains or by inducing mutant strains to excrete greater amounts of the enzymes. Moreover, the level of microbial enzyme production is influenced by a variety of nutritional and physiological factors, such as the supply of carbon and nitrogen, physical circumstances and chemical conditions [98].

Microorganisms	Xylanases	Cultivation conditions	Media	Reference
Penicillium canescens	18,895 IU/g	pH 7.0; 30 °C	Soya oil cake and casein peptone	[87]
Streptomyces sp. P12–137	27.8 IU/mL	pH 7.2; 28 °C	Wheat bran and KNO_3	[88]
Thermomyces lanuginosus SD-21	8,237 IU/g	pH 6.0; 40 °C	Corn cob and wheat bran and $(NH_4)_2SO_4$	[89]
Penicillium fellutanum	39.7 IU/mL	30 °C	Oat spelt xylan, urea, peptone and yeast extract	[90]
Penicillium clerotiorum	7.5 IU/mL	pH 6.5; 30 °C	Wheat bran	[91]
Acremonium furcatum	33.1 IU/mL	30 °C	Oat spelt xylan, urea, peptone and yeast extract	[90]
Aspergillus niger PPI	16.0 IU/mL	pH 5.0; 28 °C	Oat and urea	[92]
Neocallimastix sp. strain L2	1.13 IU/mL	50 °C	Avicel (PH 105) from Serva (Heidelberg, Germany)	[93]
Cochliobolus sativus Cs6	1,469.4 IU/g	pH 4.5; 30 °C	Wheat straw and $NaNO_3$	[94]
Bacillus circulans D1	8.4 IU/mL	pH 9.0; 45 °C	Bagasse hydrolysates	[95]
Streptomycessp. strain Ib 24D	1,447.0 IU/mL	pH 7.5; 28 °C	Tomato pomace	[96]
Paecilomyces themophila J18	18,580.0 IU/g	pH 6.9; 50 °C	Wheat straw and yeast extract	[97]

Table 1. Review of xylanases-producing microorganisms.

3.4.1. Fungi

Filamentous fungi are particularly interesting producers of xylanases and other xylan-degrading enzymes because they excrete the enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria. In addition to xylanases, fungi produce several auxiliary enzymes required for the degradation of substituted xylan [2].

The fungal genera *Trichoderma*, *Aspergillus*, *Fusarium*, and *Pichia* are considered great producers of xylanases [99]. White-rot fungi have also been shown to produce extracellular xylanases that act on a wide range of hemicellulosic materials, are useful as food sources [100] and produce metabolites of interest to the pharmaceutical, cosmetic, and food industries [78]. White-rot basidiomycetes normally secrete large amounts of these enzymes to degrade lignocellulosic materials. For example, *Phanerochaete chrysosporium* produces high levels of α-glucuronidase [101], and *Coriolus versicolor* produces a complex xylanolytic combination of enzymes [102]. Xylanase is also produced by *Cuninghamella subvermispora* when growing on plant cell-wall polysaccharides or on wood chips [103].

Fungal xylanases are generally associated with celluloses [104]. On cellulose these strains produce both cellulase and xylanase, which may be due to traces of hemicellulose present in the cellulosic substrates [105]; however, selective production of xylanase may be possible using only xylan as the carbon source. The mechanisms that govern the formation of extracellular

enzymes with regards to the carbon sources present in the medium are influenced by the availability of precursors for protein synthesis. Therefore, in some fungi, growing the cells on xylan uncontaminated by cellulose under a lower nitrogen/carbon ratio may be a possible strategy for producing xylanolytic systems free of cellulases [106]. 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 the fermenter can disrupt the fragile fungal biomass, leading to the reported low productivity. Higher rates of agitation may also lead to hyphal disruption, further decreasing the xylanase activity [50].

3.4.2. Bacteria

Xylanases have been reported in *Bacillus*, *Streptomyces* and other bacterial genera that do not have any role related to plant pathogenicity [86]. The extreme thermophile *Rhodothermus marinus* has been reported to produce α-L-arabinofuranosidase [107], and two different polypeptides with α-arabinofuranosidase activity from *Bacillus polymyxa* were characterized at the gene level for the production of α-arabinofuranosidases [108].

Bacteria, just like many other industrial enzymes, have fascinated researchers due to their alkaline-thermostable xylanase-producing trait [33]. The optimum pH of bacterial xylanases are, in general, slightly higher than the optimal pH of fungal xylanases [109], which is a suitable characteristic in most industrial applications, especially the paper and pulp industries. Noteworthy producers of high levels of xylanase activity at an alkaline pH and high temperature are *Bacillus* spp. [33]. When considering only temperature, a handful of xylanases that show optimum activity at higher temperatures have been reported from various microorganisms. These include *Geobacillus thermoleovorans*, *Streptomyces sp.* S27, *Bacillus firmus*, *Actinomadura sp.* strain Cpt20 and *Saccharopolyspora pathunthaniensis* S582, all of which produce xylanases that show activity between 65 and 90 °C [8]. One xylanase, reported from *Thermotoga sp.* [110], has been shown to exhibit a temperature optima between 100 and 105 °C.

3.5. Production of xylanases under SSF and SmF

Xylanases are produced by either solid-state or submerged fermentation [5]. Although most xylanase manufacturers produce these enzymes using submerged fermentation (SmF) techniques (nearly for 90% of the total xylanase sales worldwide) [2], the enzyme productivity via solid-state fermentation (SSF) is normally much higher than that of submerged fermentation [5]. The growing interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes, including xylanases from fungal origins, is primarily due to the economic and engineering advantages of this process [111].

The advantages of SSF processes over SmF include a low cultivation cost for the fermentation, lower risk of contamination [1], improved enzyme stability, mimicking the natural habit of the fungus, production of enzymes with higher specific activities, generation of a protein-enriched byproduct, and easier downstream processing of the enzymes produced [112]. SSF conditions are especially suitable for the growth of fungi, as these organisms are able to grow at relatively

low water activities, contrary to most bacteria and yeast, which will not proliferate under these culture conditions [113].

On the contrary, submerged fermentation allows better control of the conditions during fermentation [114]. The submerged fermentation of aerobic microorganisms is a well-known and widely used method for the production of cellulase and xylanase [115]. In general, SmF is the preferred method of production when the preparations require more purified enzymes, whereas synergistic effects from a battery of xylan-degrading enzymes can easily be found in preparations obtained by SSF using complex substrates, though the latter is commonly sought in applications aimed at improving animal feed [113].

The choice of the substrate is of great importance for the selection of the fermentation process and the successful production of xylanases. In this context, purified xylans can be excellent substrates because the low molecular weight compounds derived from them are the best xylanase inducers. The use of such substrates has led to increased yields of xylanase production and a selective induction of xylanases, with concomitantly low cellulase activity in a number of microorganisms. However, for large-scale processes other alternatives have to be considered due to the cost of such substrates. Some lignocellulolytic substrates such as barley husk, corn cobs, hay, wheat bran or straw have been compared in relation to pure substrates, and many have performed significantly better than isolated xylans (or celluloses) with respect to the yields of xylanase in large-scale production processes. Solid-state fermentation processes are practical for complex substrates, including agricultural, forestry and food processing residues and wastes, which are used as inducing carbon sources for the production of xylanases [113]. The use of abundantly available and cost-effective agricultural residues, such as wheat bran, corn cobs, rice bran, rice husks, and other similar substrates, to achieve higher xylanase yields via SSF allows the reduction of the overall manufacturing cost of biobleached paper. This has facilitated the use of this environmentally friendly technology in the paper industry [1].

3.6. Cloning and expression of xylanases

To meet specific industrial needs, an ideal xylanase should have specific properties, such as stability over a wide range of pH values and temperatures, high specific activity, and strong resistance to metal cations and chemicals [116]. Other specifications include cost-effectiveness, eco-friendliness, and ease of use [32]. Therefore, most of the reported xylanases do not possess all of the characteristics required by industry [8].

Native enzymes are not sufficient to meet the demand, due to low yields and incompatibility of the standard industrial fermentation processes [35]. Therefore, molecular approaches must be implemented to design xylanases with the required characteristics [8]. Heterologous expression is the main tool for the production of xylanases at the industrial level [35]. Protein engineering (alteration or modification of existing proteins) by recombinant DNA technology could be useful in improving the specific characteristics of existing xylanases [8]. Genetic engineering and recombinant DNA technology allow the large-scale expression of xylanases in homologous or heterologous protein-expression hosts. As industrial applications require cheaper enzymes, the elevation of expression levels and efficient secretion of xylanases are vital for ensuring the viability of the process [23].

An increasing number of publications have described numerous xylanases from several sources and the cloning, sequencing, mutagenesis and crystallographic analysis of these enzymes [12]. The available amino acid sequence data, X-ray crystallographic data, molecular dynamics and computational design of xylanases provide information that authenticates the relationship between the structure and function of xylanases. All of these methods aid in the design of xylanases that are required in industrial processes, such as improvement of the stability of xylanases at higher temperatures and alkaline pHs [8].

To attempt these processes for commercial purposes, genes encoding several xylanases have been cloned in homologous and heterologous hosts [12,48]. Recombinant xylanases have shown equivalent or better properties than the native enzymes, and the xylanase genes from anaerobic microorganisms have also been expressed successfully in hosts that can be employed in the fermentation industry [35].

3.6.1. Expression in bacteria

Escherichia coli is known for its ease of manipulation, inexpensive growth conditions, simple techniques required for transformation and accumulation of high levels of product in the cell cytoplasm; therefore, this organism has become the most widely used expression host [117]. Despite *Escherichia coli's* use as a good cloning host for recombinant proteins, it does not provide efficient and functional expression of many xylanases [23,24], and not all genes are easily expressed in *E. coli* [117]. This problem may be due to the repetitive appearance of rare codons and the requirement for specific translational modifications, such as disulfide-bond formation and glycosylation [23]. Therefore, this microorganism is useful for the detailed study of xylanase gene structure and for the improvement of the enzymes via protein engineering [35].

Lactobacillus species and *Bacillus subitilis* have been attractive hosts for the production of heterologous proteins, obtaining higher expression levels than *E. coli* [23,118]. *B. subtilis* and *Lactobacillus* are gram-positive and perform N-glycosilation [119]. Their primary interest in industry and research, is due to the fact that are non-toxic and are generally recognized as safe (GRAS) [23,118]. Members of the genus *Bacillus*, unlike *E. coli*, do not contain endotoxins (lipopolysaccharides), which are difficult to remove from many proteins during the purification process. The secretory production could also be advantageous in industrial production [33].

3.6.2. Expression in yeast

Heterologous protein expression in yeast systems is highly attractive because they provide additional benefits over bacterial expression systems. Among these benefits are the ability to perform eukaryotic post-translational modifications, the ability to grow to very high cell densities and the ability to secrete proteins into the fermentation media. Moreover, yeast are free of toxins and the majority have GRAS status [23].

Saccharomyces cerevisiae secretes high amounts of xylanases into the culture medium. Because it has already been established as an industrial microorganism, it can be used conveniently for

the industrial production of xylanases at low costs [35]. *Pichia pastoris* has also emerged as an excellent host for the commercial production of xylanases due to very high expression under its own promoters [35]. However, the success of this methylotrophic yeast, similar to *Hansenula polymorpha*, is reached with the promoters of alcohol oxidase, an enzyme involved in the methanol-utilization pathway [23]. Therefore, these promoters have limited use at the large scale due to the health and fire hazards of methanol [35].

3.6.3. Expression in filamentous fungi

Filamentous fungi are capable producers of xylanases, via both heterologous and homologous gene expression, and reach high expression yields with their own promoters [35]. Filamentous fungi have already undergone intricate strain improvement for high-level protein secretion and are feasible when using the native xylanase-expressing machinery for functional expression of foreign xylanases from remote sources. The xylanase gene from *P. griseofulvum* has been successfully expressed in *A. oryzae* [120].

4. Conclusion

Xylan, the major hemicellulose component, requires the synergistic action of several hemicellulase enzymes for its complete hydrolysis to monomer sugars. The principle enzyme in this processes is endo-1,4-β-xylanase, which cleaves the glycosidic bonds between xylosides, generating short xylooligosaccharides. The majority of the studied xylanases have been classified into the GH10 or GH11 families, whereas studies of the xylanases in families 5, 7, 8 and 43 are still emerging.

The conversion of xylan to useful products represents part of our efforts to strengthen the overall economics of the processing of lignocellulosic biomass and to develop new means of energy production from renewable resources. Among these products are xylanases, enzymes that have a wide range of important industrial applications. Therefore, in the future, new methods will be developed for easier and cheaper production of these enzymes to fulfill the demands of various industries. In this context, the use of lignocellulosic agricultural waste for the production of these enzymes by either submerged or solid-sate fermentation has been very attractive, in addition to molecular techniques that are being tested to improve the enzyme's characteristics and increase its expression rates. Moreover, as the native enzyme does not fulfill all of the process requirements, bioprospecting for new genes, rational engineering and directed evolution of known genes are powerful tools that can be used to improve these enzymes.

Acknowledgements

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