Xylanases–from Microbial Origin to Industrial Application

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Authors' contributions

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Review Article	

ABSTRACT

Xylanases are in the focus of research due to their potential to replace many current polluting chemical technologies by biochemical conversion. The field of application for xylanases is vast; it comprises industrial applications like wood pulp bio-bleaching, papermaking and bioethanol production. In addition, these enzymes can be applied as additives in food and beverage industry, and animal nutrition. However, considering the potential applications for these enzymes, the market share of xylanases is still low. Thus, the search for promising xylanases which tolerate relevant processing conditions and therefore can be used in industrial settings is an ongoing task. This review provides an overview of the enzymes reported from 2012 to mid 2014. Further, legal restrictions for the use of (genetically modified) organisms and enzymes are considered. This review provides an integrated perspective on the potential of specific xylanases for industrial applications.

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ABBREVIATIONS

AIA- advance informed agreement procedure; CBM- carbohydrate-binding module; CIBio- internal biosafety commission; CNBS- national biosecurity council; CTNBio- national biosecurity technical commission; ECF- elemental chlorine free; ECHA- european chemicals agency; EFSA- european food safety authority; EPA- US environmental protection agency; FDA- food and drug administration; FFDCA- federal food, drug and cosmetic act; GMO- genetic modified organism; GRAS- generally recognised as safe; MoA- ministry of agriculture; TCF- totally chlorine free; USDA- US department of agriculture; TSCA- toxic substances control act; XOS- xylo-oligosaccharides.

1. INTRODUCTION

Organisms use different strategies to obtain energy and basic building blocks for metabolism. Primary producers like plants utilise the energy from sunlight or inorganic substrates to produce organic molecules. Consumers instead feed on primary producers and other consumers. Finally, decomposers use remains of organisms and break it down to small organic molecules, *e.g.* sugars, amino acids and fatty acids. In contrast to dead animals the remains of plants are only poorly accessible due to their lignocellulosic cell wall. But, such plant material represents an enormous resource of organic matter and every year a new load is added to the environment from naturally and farmed sources. It comprises of cellulose, lignin and hemicelluloses, whereby from the latter the xylans are the most common ones. However, only a limited number of fungi and bacteria are able to degrade the plant cell wall into its monomers. These microorganisms are equipped with a set of hydrolytic enzymes to cleave and ultimately utilise the nutrients captured within these macromolecules. In contrast to the microorganisms, human used for a long time another way of exerting the energy bound in biomass, *i.e.*, direct burning. In many developing and underdeveloped countries burning wood still represents the most common energy source. However, at present times the usage of basic building blocks like sugars as basic chemicals for industry becomes more and more important. They have been used as starting material for the chemical and recently also biotechnological synthesis of various products, *e.g.* biofuel, bioplastics, and of high value fine chemicals. With evolving technology and commercial scale manufacturing processes, living standards have been increased over the last decades. However, many industrial production processes have a negative impact on the environment, due to the release of harmful and hazardous chemicals. Thus, to prevent persistent damages to nature, such as depletion of natural resources and environmental pollution, technologies have to become environment friendly.

A key to further development for the current polluting chemical technologies by biochemical conversion are enzymes, which are natural biocatalysts that perform under less extreme conditions than most chemical processes. Enzymes can play a vital role in chemical transformations since they offer advantages as compared to chemical (total) synthesis and degradation. They are very efficient, stereoselective, and mostly highly specific. Concerning plant biomass degradation xylanases (glycosidases which hydrolyse xylan) are of special interest, and these enzymes are largely used in various processes, *e.g.* bakery, distillery, diet of livestock, plant product processing, seed germination, and paper and pulp industry [1,2]. The molecular structure of xylanases and their mechanism of action are known [3]. Research on these enzymes had already been started in the early 50s as Sorensen [4] recorded the specificity and the products of xylanases activity from the fungus *Chaetomium globosum*. In the 80s it was claimed that the use of endoxylanases decreased the chemicals needed for bleaching Kraft pulp. Since then the production of industrial grade xylanases which are suitable for pulp industry – wherein they are used for pre-bleaching and deinking – represents the most important application for these enzymes. The global market of industrial enzymes in general is rapidly increasing. From one million dollars in 1990 it rose to 4.5 billion US$ in 2012. Current forecasts anticipate the global market to reach 7.1 billion by 2018. Because of their recognized potential as industrial biocatalysts, new xylanases are described in literature on a nearly weekly basis. This review provides an overview of the recently described enzymes by summarizing and comparing the reports of the last years (2012 – mid 2014).

2. XYLAN: AN OVERVIEW

The plant cell wall is made of three major polymeric components: (i) cellulose (polysaccharide fibres of 1,4-β-linked glucose units), (ii) lignin (complex polymeric structure of phenols) and (iii) hemicelluloses (polysaccharide fibres composed of glucose, mannans and xylan units).

After cellulose, xylan is the second most abundant polysaccharide in nature and accounts for one third of total renewable organic carbon on earth [5]. More than 10000 xylose units are polymerised by 1,4-β-linkages to form xylan strands (Fig. 1). Depending on the plant source, the xylans show structural variations. Different side chains can be attached to the linear backbone and based on their composition, xylans are grouped into four major families: (i) Arabinoxylans (AX) (side chains of single terminal units of α-$_L$-arabinofuranosyl subunits) (ii) Glucoronoxylans (GX) (α-$_D$-glucuronic acid or its 4-O-methyl ether derivative), (iii) Glucuroarabinoxylans (GAX) (α-$_D$-glucuronic acid and α-$_L$-arabinose) and (iv) galactoglucuronoarabinoxylans (GGAX) (characterised by the presence of β-$_D$-galactopyranosyl residues on complex oligosaccharide side chains).

3. ENZYMATIC HYDROLYSIS OF XYLAN

Due to abovementioned structural and chemical variability in xylans, an array of specific hydrolytic enzymes is needed to break down these polymers to monomers, *i.e.* xylose residues. Complete xylan degradation is performed by a system of xylanolytic enzymes which catalyse several reactions: (i) Glycoside hydrolases, *i.e.* endo-1,4-β-xylanases and 1,4-β-xylosidase, which hydrolyse glycosidic bonds, (ii) Carbohydrate esterases, which hydrolyse ester bonds, and glucuronoyl esterases, and (iii) Polysaccharide hydrolases, *i.e.* α-$_L$-arabinofuranosidase, α-galactosidase and α-4-O-methyl-$_D$-glucuronidase, which are responsible to cleave off glycosidic bonds mainly in the chain substituent or side chains (Fig. 1). The cleavage of 1,4-β-glycosidic bonds in the xylan backbone by endoxylanases results in xylo-oligosaccharides (XOS) ranging from 2-10 monomers. Xylosidases cleave xylose residues from non-reducing termini of these short chain XOS. The other enzymes prepare the xylans for an effective hydrolysis by endoxylanases. Thus, carbohydrate esterases remove acetyl, feruloyl, and glucuronoyl groups. Acetylxylan esterases remove acetyl esters from O-2 or O-3 sites of backbone glycosyle units, whereas ferulic acid esterases deacetylate feruloyl groups from α-$_L$-arabinoxylans. *p*-coumaric acid esterases hydrolyse ester linkages between arabinose side chain residues and *p*-coumaric acid. Further, glucuronoyl esterases demethylate O-6-methyl glucuronoyl in glucuronoarabinoxylans, and α-$_L$-arabinofuranosidases remove α-$_L$-arabinofuranosyl residues from non-reducing ends. α-galactosidases remove glycosidic bonds between galactose substituents and galactomannan. In hardwood xylan α-4-O-methyl-$_D$-glucuronidases hydrolyse 1,2-α glycosidic bonds between $_D$-glucuronic acids (ether 4-O-methyl-$_D$-glucuronic acid) and xylose residues of XOS.

4. INDUSTRIAL APPLICATIONS OF XYLANASES

In 1819, Henry Braconnot had already reported the hydrolysis of cellulose into simple sugars by sulphuric acid [6]. Since then this procedure has been used for commercial scale hydrolysis of lignocellulosic biomass. However, acidic hydrolysis needs very high energy input as the reaction takes place at high temperature, and due to the low pH at which the hydrolysis takes place, the complete equipment needs to be acid resistant. These energy and cost consuming requirements reduce the cost effectiveness of the process. An alternative approach for the hydrolysis of cellulose and hemicelluloses is the use of suitable enzymes like xylanases. Dependent on the downstream following application xylanases are used to degrade the carbon-rich macromolecular fibres to different degrees. In biofuel production the xylan strands are degraded completely into fermentable sugars which subsequently can be used for fermentation and distillation of ethanol. For other applications longer xylan units (XOS) are the preferred compounds. A further field of application is the supplementation of products with xylanases to support downstream following biochemical reactions.
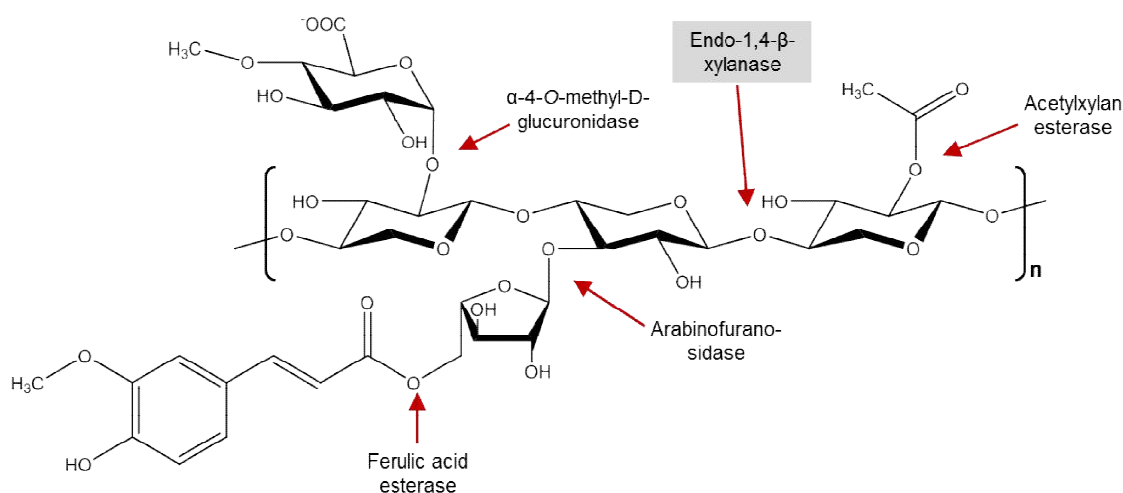
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Fig. 1. Section of a xylan structure and the sites of its attack by xylanolytic enzymes
The backbone (in brackets) of the substrate is composed of 1,4-β-linked xylose residues. The endo-1,4-β-xylanase, cleaving within the backbone, is highlighted in grey.

In addition, to produce xylanases for industrial applications, the easy access to the basic materials needed for their production is a clear advantage. For instance agricultural "waste", including crop harvest residue, is rich in lignocellulosic plant matter and can serve as raw material for xylanase production.

4.1 Bioethanol Production

Using plant biomass as starting material for the production of ethanol is a complex process which involves several conversions. First, the lignocellulosic biomass has to be delignified; yielding xylan and cellulose, which then can be hydrolysed to XOS and cellobiose, respectively. These oligosaccharides can subsequently be converted into simple sugars, *i.e.* xylose and glucose. The latter represent the compounds of interest for bioethanol production. Thus, here bifunctional xylanases, *i.e.* endo-1,4-β-xylanase with additional cellulase activity, can reduce the need for various enzymes [7]. This may help to make the saccharification process of lignocellulosic biomass more efficient and cheaper. A pretreatment of straw, using xylanases and cellulases can effectively improve downstream saccharification and fermentation, important for bioethanol production. Even though the biofuel technology continues to advance, the demand is stalling since the yields have to be increased and the costs decreased to compete with conventional fuels.

4.2 Animal Feedstock

Animal feed, including cereals like wheat, triticale and soy-based diet, are rich in lignocelluosic biomass. However, the viscous properties of lignocelluloses hinder their digestion. By enzyme supplementation to feedstock, nutrients entrapped in the macromolecules are released and thereby the digestion enzymes have better access to their substrate. In that way the nutritional value of the diet increases. It was shown that enzymatic treatment of forages can improve the quality of silage, since it results in an improved rate of plant cell wall digestion in ruminants [8]. Addition of exogenous fibrolytic enzymes to the feedstock showed positive effects on animal growth by increasing the amount of fibres digested [9]. The *in trans* supplemented enzymes support the pre-digestion by breaking up the lignocellulosic biomass. This enables a more efficient use of the energy conserved in the sugar molecules. An additional effect of this pre-digestion is that the resulting sugars represent also nutritionally suitable compounds for the desired ruminal microflora, which in turn further assists digestion [10].

For more than fifty years the addition of enzymes has been known to improve animal performance, but their use as additives in animal feed was limited because it was assumed that enzymes will not survive proteolysis in the rumen. However, β-glucanases were introduced in

poultry diet during the early 1980s in Scandinavia, and subsequently xylanases followed. Nowadays the 2014 world feed enzyme market is worth above 1 billion US$ and continues to increase by >5% annually. Positive effects of xylanases on animal growth were reported for fishery, piggery and poultry [11-13], as well as for cattle. Supplementation of fibrolytic enzymes like xylanases and cellulases significantly increased the average daily milk yield in Murrah buffaloes [8]. Similar results were obtained for goats [14]. The timing of the enzyme addition seems to be flexible, since an improved milk yield was also obtained by spraying forages with xylanases and cellulases from *Myceliopthora thermophilia* in appropriate doses just prior feeding [15].

However, it has to be noted that animal's response to enzyme supplementation may vary with its mode of implementation, *e.g.* whether the supplementation is applied to dry/fresh forage or silage or infused directly into the rumen, applied onto the complete diet or added to a component of the diet [16].

4.3 Production of Xylo-Oligosaccharides as Food Additive

Endoxylanase catalysed xylan hydrolysis results in XOS. The latter showed pre-biotic effects, and can be used in functional foods. They enhance the growth of potentially health promoting bifidobacteria in colon and thereby restrict the growth and proliferation of other harmful bacteria [17]. Therefore, XOS are used in beverages like soy milk, tea and coffee, dairy products, desserts like pastries, cakes, biscuits, puddings, jellies and jams, and special preparations for elderly people and children, or as active component of symbiotic preparations [17,18]. Further, XOS are reported to inhibit the growth of sarcoma-180 and other tumours, probably by indirectly stimulating the non-specific immunological host defence [19]. In addition to the immune-stimulating effects, anti-inflammatory activity was reported too [17]. These benefits make XOS interesting for the pharmaceutical industry.

4.4 Baking Industry

The use of enzyme preparations in bread making to produce fluffier bread has been reported long ago [20]. Xylans play an important role in bread making due to their water absorption capability and interaction with gluten. Utilization of xylanases improves bread qualities, *e.g.* good volume rise, smooth texture and appearance, and dry, balanced dough with prolonged shelf time. In 2012, xylanases, produced by a *Bacillus licheniformes* strain, were made commercially available by Novozyme as an additive for baking industry under the brand name "Panzea". Beside that one, different microbial sources for xylanases, which putatively can be applied in baking industry, were reported. The activity variation of these xylanases is given in the percentage of final loaf volume rise. Additional quantitative biochemical data are missing. Xylanase from *Aspergillus foetidus* was reported to increase the loaf volume by 53% [21]. Xylanases isolated from *Thermomyces maritime* and *Thermomyces lanuginosus* resulted in 60.3% rise [22]. An *Aspergillus oryzae* variant showed improved bread quality when compared to the xylanases produced by *Humicolain solens* and *Trichoderma reesei*.

4.5 Paper and Pulp Industry

Xylanases, as well as other enzymes, *e.g.* amylases, lipases and esterases, are applied in the paper and pulp industry. However, for their industrial application the xylanases has to be cellulose free, since the latter is the desired product of the paper industry. During the Kraft process, *i.e.*, the chemical conversion of wood into wood pulp (lignocellulosic fibrous material), the lignin-carbohydrate complex is hydrolysed. Thus, endo-1,4-β-xylanase can be used in this process to increase the extraction of lignin. In that way it becomes more accessible for bleaching. By this approach the consumption of chlorine chemicals for bleaching is reduced. In addition it was reported that, beside the positive effect on the environment, enzymatically treated pulp is brighter and has improved fibre quality. In 1979 it was observed that the incubation of unbleached kraft pulp with the fungus *Phanerochaete chrysosporium* resulted in delignification and in reduction of bleaching chemicals [23]. If this effect was due to xylanases alone or to the mix of enzymes produced by this strain was not further investigated. Later on, xylanase was isolated from the fungus *Schizophylum commune* by fractional precipitation and shown to have a significant hydrolytic activity against the hemicellulose content of the pulp [24]. Through the increasing portion of recycled paper used, xylanases gained further importance. This is due to the fact that such secondary (recycled) fibres

require deinking to ensure a quality that could compete with that of virgin fibres.

4.6 Xylitol Production

Xylitol is a five carbon polyol with sweetness comparable to that of sucrose and is used in food and pharmaceutical industries. Several features foster its application in food industry, *e.g.* it is tolerable for diabetics, does not cause acid formation, has low viscosity, and has a cooling effect when dissolved in a solution [25]. Xylose can be reduced to xylitol in the presence of NADPH-dependant xylose reductase and thereby represent the substrate for xylitol production [26].

4.7 Fruit Juice and Beer Finishing

Xylanases together with many other hydrolytic enzymes like pectinases, amylases, tannases and carboxymethylcellulases are used in the brewing industry to reduce the turbidity of liquids and to clarify juices. Endo-1,4-β-xylanases help in juice extraction and filtration from vegetables and fruits by reducing the viscosity of the juices [27]. Further, they improve the yield of fermentable sugar from barley, which is useful in beer industry. Additionally, brewing liquid filterability is improved by the reduction of its viscosity [10]. Production of coffee, plant oils and starch represent further examples of application, since their extraction is facilitated by xylanases, which cleave the compact xylan layer of the seeds [28].

4.8 Degumming

In combination with a mild chemical pre-treatment, pectinases and xylanases play a vital role in the degumming and better separation of bast fibres. Enzymatic hydrolysis of bamboo materials make them suitable for textile processing [29]. Xylanases from *Bacillus subtilis* B10 were successfully used for degumming of ramie bast fibres [30]. Further, xylanases and pectinases are also used in the debarking of wood [28].

4.9 Seed Germination and Fruit Ripening

Germinating seeds naturally produce xylanases late in the germination process, which might help to access required nutrients for the better growth of the new plant. Xylanases are also reported to take active part in softening of fruits *e.g.* papaya. As the fruit starts to soften during ripening, endoxylanases play an important role by modifying the polysaccharides in the cell wall matrix [31]. Thus, xylanases might in the future play a role in the commercial scale ripening process of fruits. Many (exotic) fruits are usually harvested and transported in an unripe state and have to finish the ripening process in the country of destination.

5. MICROBIAL SOURCES OF XYLANASES

Xylanases are present in various microorganisms, *e.g.* free-living soil bacteria and fungi, or endosymbionts of ruminants and termites. Initially the enzymes have been identified by screening isolated environmental samples, *e.g.* axenic cultures, or heterogenic solutions of microorganisms, phenotypically for xylanase degrading activity. By the increasing number of available sequence information this process was speed up in the last years. Databases could be screened *in silico* to identify putative xylanase-encoding genes. Using molecular biological methods, *i.e.* cloning, enabled the overexpression of the target gene. By heterologous expression approaches even metagenomic environmental DNA samples became available for activity-based screenings. In Table 1 the most promising xylanases – judged by the activity reported under the given conditions – from different origin are given, and the top ten are described in detail. (A more comprehensive list can be found in the annex: Supplementary Table S1 and S2.)

B1 The highest xylanase activity reported from a bacterial host was 36633 IU/mg xylanase, using birchwood and oat spelt xylan as substrate. This was achieved using a purified xylanase originating from *Bacillus subtilis* CXJZ [32]. The enzyme is a weakly acidic, cellulase free xylanase with a molecular weight of 23.3 kDa and pI 9.63. The strain was obtained by screening ramie stems collected from the fields and subsequently partially submerged in natural river water. A genomic library of *B. subtilis* CXJZ was constructed and the resulting *E. coli* clones were screened for xylanolytic activity. From positive clones xylanase genes were amplified using non-degenerated primers. The sequence is deposited in GenBank under the accession number EU233656. The activity optima determined were 60°C and pH 5.8. The enzyme

was stable up to 70°C and was easy to purify by ultracentrifugation and subsequent gel filtration chromatography. Further, it is reported that the strain is employed in the degumming lines of ramie textile mills in Hunan, China [32].

B2 A xylanase gene (*xyn11A*) coding a glycosyl hydrolase, a linker region and a carbohydrate-binding module (CBM) was cloned from the genomic library of alkalophilic *Bacillus* sp. SN5 [33]. The intact Xyn11A (366 amino acids) and Xyn11A-LC, the latter represents a truncated version without the CBM-linker, were expressed in *E. coli* BL21. Both purified proteins showed highest activity at 55°C. Optimal pH determined for Xyn11A was 7.5 and Xyn11A-LC showed a broad pH profile of 5.5-8.5 with >80% activity. Both enzymes were found to be alkali-tolerant and retained over 80% residual activity for 1 h after pre-incubation at pH 8.5-11.0 at 37°C. Xyn11A-LC was found to be more stable with lower affinity and catalytic activity using insoluble xylan as substrate but with higher specific activity for soluble beechwood xylan than Xyn11A. The presence of a CBM fused to Xyn11A implies that it might play an important role in degradation of insoluble xylan. Being alkali tolerant and cellulase free, Xyn11A-LC shows potential for applications in paper industry.

B3 An endoxylanase from *Bacillus brevis* ATCC 8246 showed activity in neutral to alkaline pH and stability at temperature of >55°C [34]. Enzyme production was carried out in liquid state cultures of *B. brevis* with wheat straw as main source of carbon. Using a combination of ammonium sulphate precipitation, gel filtration and ion exchange chromatography, the enzyme was purified to a 2.4 fold activity increase. The enzyme was stable between 45°C-90°C, and showed optimal activity at 55°C and pH 7.0. At 85°C the enzyme retained 50% of its activity. At 70°C its half-life was 2 h compared to 3 h at optimal temperature. High temperature tolerance of this *B. brevis* xylanase makes it a suitable candidate for Kraft pulp bio-bleaching.

B4 The bacterial strain *Paenibacillus macerans* IIPSP3A, producing a highly thermostable, halotolerant xylanase, was isolated from the gut of wood feeding termites using culture enrichment medium with beechwood xylan [35]. The purification of a xylanase with a molecular mass of 205 kDa and a pI of 5.38 by strong cation exchanger and gel filtration chromatography was reported [35]. Optimum pH

and temperature of the purified enzyme were 4.5 and 50°C, respectively. At 60°C the half-life was 6 h, and at 90°C the half-life was reported to be 2 h. High specific activity and stability at high temperature and pH make this enzyme suitable in bioethanol production and in paper and pulp industry as well.

B5 A thermostable bacterial xylanase was identified from *Bacillus halodurans* C-125 [36]. Xylanase C-125 was successfully cloned and expressed in *Pichia pastoris* and showed thermophilic and alkalophilic properties with an optimal activity at 70°C and pH 9.0. The enzyme was found to be cellulase-free. These properties make it compatible for applications in pulp and paper industry. Further, the enzyme enhanced brightness while preserving paper strength properties such as tensile index, tearing index, bursting index and post-colour number. C-125 XynA pre-treated wheat straw pulp allowed for a 10% decrease in chlorine consumption, maintaining brightness and kappa number at the same level.

B6 In a screening for strains producing thermostable xylanases, *Paenibacillus* sp. NF1 was isolated. Growing as a submerged culture, this strain showed high levels of extracellular xylanase activity [37]. The observed xylanase activity was 211.79 IU/mg. This value could be increased to 3081.05 IU/mg using oat spelt xylan as substrate after a 14.55-fold purification. The 37 kDa XynNF enzyme showed maximal activity at 60 °C and pH 6.0, respectively. Its optimal pH range is acidic, ranging from pH 4 to 7.

B7 A new xylanase Xyn11E was isolated and characterized from *Paenibacillus barcinonensis* BP-23 [38]. The complete sequence of the xylanase-encoding gene was obtained by gene walking on amplified DNA fragments. The xylanase was found to be dependent on *P. barcinonensis* BP-23 lipoprotein (LppX) for its expression in an active form using *E. coli* DH5α as heterologous host. Protein purification was carried out by immobilized metal affinity chromatography using a HisTrap HP column. The 20.7 kDa protein has a pI of 8.7. Optimal assay conditions were reported to be 50°C and pH 6.5. Enzyme activity on bleached pulps from different origin was also investigated. Xyn11E released reducing sugars from Elemental Chlorine Free (ECF) and Totally Chlorine Free (TCF) pulps from eucalyptus, sisal and flax, proving it to be suitable for enzymatic-assisted

production of high cellulose-content pulps from paper-grade pulps.

B8 The hyperthermophilic eubacterium *Thermotoga petrophila* RKU 1 was isolated from an oil reservoir in Japan and was found to produce an endo-1,4-β-xylanase [39]. The strain was cultivated anaerobically in MMI medium. The xylanase encoding gene was amplified from the genomic DNA and heterologously expressed in *E. coli* strain BL21 Codon plus. The extracellular enzyme with a molecular mass of 40 kDa was purified by heat treatment, followed by anion and cation exchange chromatography. The optimal temperature and pH for this enzyme are 95°C and 6.0, respectively. The recombinant enzyme exhibited high stability with a half-life of 54.5 min at 96°C. The extremely high thermostability makes it a suitable candidate for application in paper and pulp industry.

B9 Another thermostable xylanase XynG1-1 was identified from *Paenibacillus campinasensis* G1-1 [40]. The bacterial strain was isolated from cotton stalk stockpile samples and cultivated in complex medium for xylanase production. A xylanase gene was amplified from the genomic DNA, subsequently cloned and expressed in *E. coli* BL21 (DE3). The protein with a mass of 41.3 kDa was purified to homogeneity by hydrophobic interaction, gel filtration, and ion exchange chromatography. Optimal conditions for the cellulase-free XynG1-1 were determined to be 60°C and pH 7.5, respectively. Stability was preserved between 70°C-80°C and over a wide pH range of 5.0-9.0. The authors state that the enzyme should be greatly valuable in various industrial applications, especially for pulp bleaching pre-treatment.

B10 A putative endo-1,4-β-xylanase (XylB8) was identified by sequence analysis of cloned metagenomic DNA from termite gut [41]. An actinobacterial xylanase gene was sequenced, cloned and heterologously expressed in *E. coli*. The recombinant protein was purified by affinity chromatography and its specificity appeared to be limited to xylan. Maximal activity was determined at 55°C and pH 5.0. The enzyme was reported to have potential application in animal feed, textile and paper industries.

F1 A promising xylanase (XynR8) was cloned from un-purified rumen fungal cultures. Later on *Neocallimastix* sp. was suggested as the real origin [42]. Heterologous expression of XynR8 in

E. coli resulted in a highly active (66672 U/mg) and stable (pH 3-11) protein. The optimal temperature was 55°C. By inserting utations N41D and N58D, which according to the authors should stabilize two loops within the protein structure, the tolerance to pH 2 was increased by 24.6%. In addition, the activity was increased 1.4 fold for N58D (96689 U/mg) and 1.1 fold for N41D (79645 U/mg) compared to the wild type, putting these xylanases among the most active onestowards soluble oat spelt xylan. The activity of this enzyme at very low pH makes it an interesting candidate in biofuel production.

F2 The highest xylanase activity from crude extract was obtained by using a gene originating from *Aspergillus niger* IME-216, which was overexpressed in an industrial strain of *Saccharomyces cerevisiae* [43]. Using cDNA as template a fusion fragment consisting of phosphoglycerate kinase promoter, α-factor signal peptide, xylanase, and a terminator was constructed. This construct was integrated into the genome of *S. cerevisiae* YS2. The xylanase production of the transgenic yeast was 1.5 fold higher (74800 U/mL) than the value reached by the host strain under flask culture at 28°C for 72 h. The authors speculate about the use of recombinant *S. cerevisiae* YS2 strains in bioethanol production by simultaneous fermentation of glucose and xylose, even though the 1.5 fold increase in a transgenic host is only slightly above the wild type strain.

F3*Paecilomyces thermophila* xylanase (XynA) was functionally expressed in *Pichia pastoris* GS115 [44]. Optimal activity of the recombinant XynA was observed at 75°C and neutral pH with 30 min stability recorded at 80°C. The high activity (52940 U/mg) and the robust expression in yeast make PtxynA worthy of further investigations.

F4 The putative xylanase gene *xyl10* from *Aspergillus niger* was codon-optimised for *P. pastoris* and synthesized [45]. The final gene product was inserted in *P. pastoris* X33 under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter. Purification of the recombinant protein was carried out by size exclusion chromatography. Recombinant Xyl10 showed optimal activity at 60°C and pH 5.0. The enzyme maintained 90% of its original activity at 60°C for 30 min at pH 5.0, and around 74% after incubation at pH 3.0-13.0 for 2 h at 25°C. Recombinant Xyl10 exhibits great potential in

various industrial applications, due to its high activity (32000 U/mg).

F5 *Aspergillus usamii* E001 was used as donor of a xylanase gene encoding AuXyn11A [46]. The N-terminal amino acids were replaced by the corresponding ones from the hyper thermostable xylanase Syxyn11. The *auXyn11A* part was amplified from total RNA whereas the *syXyn11* part was synthesized. Both genes were transferred to *E.coli* JM109, serving as parent genes for the construction of a hybrid xylanase gene, *AExynM*. AuXyn11A and AEXynM were expressed in *P. pastoris* GS115. A combination of ammonium sulphate precipitation, ultrafiltration, and gel filtration was used for purification. Optimum temperature reached 75°C for the thermo-stabilised version, compared to 50°C for AuXyn11A. Further, three amino acids in the replaced N-terminus were tested and shown to contribute to high thermal stability.

F6 The fungus *Trichoderma reesei* is an established host for expression of homologous or heterologous genes. Xylanase II was homologously overexpressed in *T. reesei* QM9414 by putting the gene under the control of the promoters of *pdc* (encoding pyruvate decarboxylase) and *eno* (encoding enolase) [47]. By real-time reverse-transcription polymerase chain reaction these promoters were shown before to be extremely active under high glucose conditions. The recombinant strains produced 9266 U/ml (*pdc* promoter) and 8866 U/ml (*eno* promoter) of xylanase activities in the culture supernatant. Xylanase II accounted for >80% of the total protein secreted by the recombinant strains, indicating the high potential for rational promoter screening for optimising homologous and heterologous xylanase production.

F7 A 26 kDa endoxylanase was obtained from the wood-rotting fungus *Schizophyllum commune* [48]. The corresponding gene was cloned and expressed with a C-terminal His$_6$-tag in *P. pastoris* GS115. Following Ni-NTA affinity purification, optimal conditions for activity were determined to be 50°C and pH 5. Enzyme activity improved in the presence of cations like potassium, sodium, lithium, cadmium and cobalt whereas EDTA, mercury and ferric ions inhibited its activity. It was suggested that this enzyme might serve as a major component of a lignocellulosic degrading enzyme cocktail.

F8 The activity of an extracellular endoxylanase PoXyn2 from the filamentous fungus *Penicillium occitanis* Po16 was reported to be 2368 U/mg in an assay using oat spelt xylan as substrate. The cDNA was cloned in an expression vector that allowed to fuse a N-terminal His$_6$-tag to the enzyme. The construct was integrated in *P. pastoris* X-33 under the control of the constitutive GAP promoter. Recombinant protein was expressed extracellular and purified to its homogeneity by using immobilized metal affinity chromatography (Ni-NTA resin). By using *P. pastoris* X33 as heterologous host the activity towards oat spelt xylan was increased to 8550 U/mg [49]. The 30 kDa enzyme showed optimal activity at 50°C and pH 3. Ability to work under acidic pH optimum and wide pH stability allow to predict potential applications of PoXyn2 in feed and food industries.

F9 Two extracellular xylanases, designated Xyn-1 and Xyn-2, were isolated from *Rhizopus oryzae* NRRL 29086, a strain previously isolated from dew-retted flax [50]. The two proteins Xyn-1 (32 kDa) and Xyn-2 (22 kDa) were expressed in *E.coli* and characterised after purification to electrophoretic homogeneity. The activities determined were 605 U/mg and 7710 U/mg, respectively. Melting temperature of these enzymes was estimated to fall in the range of 49.5-53.7°C. Xyn-1 and Xyn-2 demonstrated release of xylose from flax shives-derived hemicelluloses as model feed-stock.

F10 A 20 kDa heat resistant xylanase was isolated from *Aspergillus niger* SCTCC 400264 (Li 2014) [51]. The corresponding *xynB* gene was transferred and expressed as His-tagged protein in *E. coli* as well as in *P. pastoris*, whereby enzyme production was four times more important in *P. pastoris*. Maximal enzyme activity was recorded to be 4757 U/mg under optimal conditions, *i.e.* 55°C and pH 5.0. The enzyme showed 86% residual activity after 10 min heat treatment at 80°C (74% after 30 min). The gene integration was stable, and after 50 generations the cells were tested positive for the *xynB* gene. This xylanase could be used in animal feed applications, since it has 82% residual activity at body temperature, and can preserve >50% relative activity under acidic pH. Further, the enzyme is thermostable, a feature necessary for high-temperature requirements of typical feed pelleting process.

Table 1. Xylanases of bacterial and fungal origin.[a]

Strain number[b]	Natural producer	Heterologous host	Enzyme purification	Xylanase activity [IU/mg]	Used substrate	Optimal conditions	Ref.
B1	*Bacillus subtilis*	*E.coli* DH5α	Purified	36633.4	Birchwood and oat spelt xylan	60°C pH 5.8	[32]
B2	*Bacillus* sp. SN5	*E. coli* BL21 (DE3)	Purified	4511.9	Beechwood xylan	55°C pH 7.5	[33]
B3	*Bacillus brevis* ATCC 8246	__	Purified	4380	Agro-waste *e.g.* Wheat straw	55°C pH 7.0	[34]
B4	*Paenibacillus macerans* IIPSP3	__	Purified	4170±23.5	Beechwood xylan	60°C pH 4.5	[35]
B5	*Bacillus halodurans* C-125	*Pichia pastoris*	Crude	3361 U/ml	Wheat Straw Pulp	70°C pH 9.0	[36]
B6	*Paenibacillus* sp. NF1	__	Purified	3081.05±4.12 3045.93±4.34 2533±5.66 2460±6.74	Oatspelt Xylan Birchwood Xylan Rice hull Xylan Bran Xylan	60°C pH 6.0	[37]
B7	*Paenibacillus barcinonensis* BP-23	*E. coli*	Purified	3,023	Beechwood xylan	50°C pH 6.5	[38]
B8	*Thermotoga petrophila* RKU 1	*E.coli* BL21	Purified	2600 1655	Birchwood xylan Beechwood xylan	96°C pH 6.0	[39]
B9	*Paenibacillus campinensis* G1-1	*E.coli* BL21 (DE3)	Purified	1865.5	Birchwood xylan	60°C pH 7.5	[40]
B10	*Actinobacterium*	*E.coli*	Purified	1837	Xylan	55°C pH 5.0	[41]
B11	Microbial consortium	*E.coli* DE3	Purified	1794.4±136.6 1029.5±55.2	Birchwood xylan Beechwood xylan	65°C-70°C pH 9.0-10	[52]
B12	*Bacillus pumilus* SSP-34	__	Purified	1723	Oat spelts xylan	50°C pH 6.0	[53]
B13	*Gracilibacillus* sp. TSCPVG	__	Purified	1667	Birchwood xylan	pH 7.5	[54]
B14	*Thermobacillus*	*E.coli* JM109 (DE3)	Purified	1170	Birchwood xylan	60°C	[55]

Strain number[b]	Natural producer	Heterologous host	Enzyme purification	Xylanase activity [IU/mg]	Used substrate	Optimal conditions	Ref.
	xylanilyticus					pH 6.2	
B15	Rumen microbiota of Hu sheep	*E.coli* BL21 (DE3)	Purified	1150	Birchwood xylan	50°C pH 5.0	[56]
B16	*Bacillus* sp. QH14	*E.coli* BL21 (DE3)	Purified	Wild type: 1148.56 *E. coli*: 700.47	n.g	60°C pH 9.2	[57]
B17	*Cellulosimicrobium* sp. MTCC 10645	__	Purified	960	Birchwood xylan	50°C pH 7.0	[58]
B18	*Streptomyces* sp. Strain S9	*Pichia pastoris*	Purified	950.8+23.12	Birchwood xylan	70°C pH6.5	[59]
B19	*Cellulosimicrobium* sp. Strain HY-13	__	Crude	788.3	Birchwood xylan	45°C pH 6.0	[60]
B20	*Bacillus amyloliquefaciens* CH51	*E.coli* BL21	Purified	701.1	Birchwood xylan	25°C pH 4.0	[61]
F1	*Neocallimastrix* sp.	*E.coli*	Purified	N58D: 96689 N41D: 79645 Wild type: 66672	soluble oat spelt xylan	55°C pH 2.0	[42]
F2	*Aspergillus niger* IME-216	*Saccharomyces cerevisiae* YS2	Crude	74800 U/ml	Xylan	28°C n.g.	[43]
F3	*Paecilomyces thermophile*	*Pichia pastoris* GS115	Purified	52940	Birchwood xylan	75°C pH 7.0	[44]
F4	*Aspergillus niger*	*Pichia pastoris* X33	Purified	32000	Beechwood xylan	60°C pH 5.0	[45]
F5	*Aspergillus usamii* E001	*Pichia pastoris* GS115	Purified	AuXyn11A: 9266 AeXynM: 8866	Corncob xylan	50 & 70C pH 4.6	[46]
F6	*Trichoderma reesei* QM9414	*E. coli* Top10F'	Crude	P_{pdc}: 9266 P_{eno}: 8866	Birchwood xylan	50°C pH 5.3	[47]
F7	*Schizophyllum commune*	*Pichia pastoris* GS115	Purified	9000	Beechwood xylan	50°C pH 5.0	[48]
F8	*Penicillium occitanis* Pol6	*Pichia pastoris* X-33	Purified	8549.85	Oat spelt xylan	50°C pH 3.0	[49]
F9	*Rizopus oryzae* strainNRRL 29086	*E. coli*	Purified	Xyn2: 7710 Xyn1: 605	Birchwood xylan	35°C pH 5.0	[50]
F10	*Aspergillus niger* SCTCC	*Pichia pastoris*	Crude	4757	Oat spelt xylan	80°C	[51]

Strain number[b]	Natural producer	Heterologous host	Enzyme purification	Xylanase activity [IU/mg]	Used substrate	Optimal conditions	Ref.
	400264					pH 4.0-6.0	
F11	*Chaetomium globosum*	*E.coli*	Purified	4530+230	Rice straw	40°C pH 5.5	[62]
F12	*Armillaria gemina* SKU2114	__	Crude	1270 U/ml	Rice straw	n.g.	[63]
F13	*Armillaria gemina*	__	Purified	1270 U/ml	Birchwood xylan	n.g.	[64]
F14	*Penicillium oxalicum* B3-11	*Trichoderma reesei* Qm9414	Purified	1856+53.5	Beechwood xylan	50°C pH 5.0	[65]
F15	*Aspergillus usamii* E001	*Pichia pastoris* GS115	Purified	912.6 U/ml	Insoluble Corncob xylan	50°C pH 5.0	[66]
F16	*Aspergillus niger* DSM 1957	*Pichia pastoris* GS115	Purified	808.5	Birchwood xylan	50°C pH 7.0	[67]
F17	*Gloephyllum trabeum*	*Pichia pastoris* GS115	Purified	727.2+15.75	Beechwood Xylan	70°C pH 7.0	[68]

[a]*The best xylanases (activity >700 U/mg) reported in recent years are given;* [b]*bacterial hosts are numbered with B and fungal hosts with F; n.g.: not given; Ref.: reference*

6. LEGAL RESTRICTIONS FORTHE INDUSTRIAL USE OF XYLANASES

Additionally to scientific issues related to xylanase research, it has to be kept in mind that legal regulations influence research and application. While the application in industrial processes like pulp bleaching seems rather unproblematic, legal issues are obviously at stake for enzymes used in the food chain. For the application of xylanases in non-food related industrial processes, general product safety regulations and directives for genetic modified organisms (GMOs) safety which differ from country to country, have to be taken into account. When used in terms of food processing or feeding, additionally food safety regulations have to be considered.

Researchers and companies working with wild type strains have the advantage that for the final product as well as for the production process, no GMO regulations have to be taken into account. Independently from the fact that the whole crude extract or purified enzymes are used. However, wild type strains may have several disadvantages, *e.g.* slow growth rates, low yield, or special growth requirements, which hinder their application due to economic reasons. Since most of these wild type strains are fungi, special cultivation methods like solid state fermentation have to be used for large scale cultivation, an important disadvantage over classical liquid fermentation methods. Another disadvantage is that depending on the process, *e.g.* the bleaching of pulp, only xylanase activity is desired, while the wild type strains might also express other hydrolytic enzymes such as cellulases. Further, enzymatic degradation of lignocellulose demands the interplay between a whole set of different hydrolytic enzymes, that are usually not expressed in one organism providing all activities required. Therefore, heterologous expression of xylanase genes has advantages, *e.g.* vast yield enhancement by (heterologous) overexpression in host strains with optimised cultivation methods, and access to proteins encoded in the genome of so far unculturable microorganisms, solely detected in metagenomics approaches using environmental DNA samples.

The handling of GMOs and GMO products is essentially regulated by two international agreements as well as the respective national laws. In the following, a short overview of the international agreements and the statutory regulations in Brazil, China, EU and USA is given.

International Regulations The "Cartagena Protocol on Biosafety to the Convention on Biological Diversity" regulates the handling of living genetically modified organisms in terms of trade between states. This protocol is an addition to the "Convention on Biological Diversity" from 1993, it has been signed by 166 countries and became valid in 2003 [69,70]. The basic claim of the protocol is that the importing country's environment and the population's health have to be protected from the possible risks posed by the uncontrolled release of GMOs. Therefore, the importing countries have to get access to all scientific information about the GMO allowing to make an informed decision regarding whether an import is allowed or not. This principle is referred to as "Advance Informed Agreement Procedure" (AIA). Particularly excluded from the AIA are GMOs for pharmaceutical use and for food, feeding and respective processing purposes. Before using GMOs it shall be ensured that the organism underwent some monitoring processes that correlate with the respective generation time. In addition, prior to an intentional release of GMOs into the environment, risk assessments have to be performed by the particular party [71]. The "Nagoya – Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety" (Supplementary Protocol) is an addition to the original Cartagena Protocol. This protocol regulates the responsibilities in case of damages caused by GMOs. This includes the damage by authorised releases as well as unintentional or illegal trans-boundary movement [72].

Brazil is the second largest producer of genetically modified plants for livestock feeding, and one of the biggest biofuel producers worldwide. The Brazilian constitution defines the intact environment as public good, necessary for a healthy lifestyle and following the precautionary principle, the biosafety law (Law No. 11,105) [73] which sets safety regulations for the handling of GMOs and GMO products was enforced by the congress. It distinguishes between laboratory (development and testing) and commercial (production and processing) use of GMOs. According to this law, the "National Biosecurity Technical Commission" (CTNBio) has to set standards for the handling of GMOs, *e.g.* safety guidelines. This central authority reviews all biotechnological projects and decides whether to approve them or not case by case. The "National

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Biosecurity Council" (CNBS) has to approve, and if necessary to create administrative regulations for, the introduction of GMOs and GMO products considering social, economic, and national interest. Additionally, each institution dealing with the creation, production or research of GMOs has to set up an "Internal Biosafety Commission" (CIBio). Thus, the release of GMOs has to be approved by the CTNBio, as well as the CNBS if the release is for commercial purposes. Food and feed containing GMOs or GMO products have to be labelled according to the ministerial act No. 2.658 if the GMO content is higher than 1% [74]. Recombinant proteins are considered GMO products. Therefore, research and production of xylanases, if obtained from transgenic strains, have to be approved by the CTNBio in each case and have further to be reviewed by the CNBS if a product, containing recombinant xylanases, enters the market.

China´slegislation on GMOs mainly focuses on genetically engineered organisms for agricultural use, including plants, animals or microorganisms. GMOs underlie the "Regulations on Administration of Agricultural Genetically Modified Organisms Safety" [75]. According to the regulations, research and testing of GMOs has to take place in facilities that have the standard for the biosafety level required by the organism investigated. If this involves organisms requiring biosafety level 3 or 4, the Ministry of Agriculture (MoA) has to be informed before the start of the project. New products derived from GMOs need a so called GMO Safety Certificate, issued by the MoA after three stages of product testing. GMOs and GMO products for food and feeding purposes can be approved by the Ministry of Health after risk assessment [76]. The regulations on GM food also include a labelling system and a tracing system, which forces producers of GM food to keep tracking files of their products at least for two years and to label products containing GMOs and their products accordingly. Since no further legislation concerning GMOs has been enforced yet, it is not possible to make specific statements about the situation with xylanases in China. While the food, drug and agricultural sectors are well regulated, it is however not clear if recombinant enzymes are considered as GMO products or as chemical substances. Additionally, it seems that there are no regulations for non-food, non-drug or non-agricultural biotechnology applications other than basic environment protection laws.

EU authorities have legislative competences for all 28 members, especially for healthcare, consumer and environment protection [77]. The EU directives represent a minimum standard that has to be fulfilled for products to be produced and marketed within the EU. While each of the 28 countries can deny products that fulfil the EU standards, it is not possible to loosen the national regulations below the EU standards. To protect the environment and the population's health, these EU standards follow the precautionary principle of the Cartagena Protocol. Essentially three directives regulate the handling of GMOs and GMO products within the EU. The directives as such have to be implemented into national laws by EU members, determining which authorities are in charge of control. Directive 1907/2006 determines that all substances imported into or manufactured in the EU in amount over one ton have to be registered by the "European Chemicals Agency" (ECHA) [78]. The registration requires, among others detailed information about the manufacturer, the identity of the substance and safety guidelines. Particularly excluded are substances for food and feeding purposes. Directive 2009/41/EG regulates the handling of GMOs within closed systems [79]. These regulations apply to every production process that includes transgenic microorganisms and states that all appropriate measures have to be taken to avoid damage to the environment and/or the public health. This includes measures to ensure that no living organisms escape the closed system. Directive 2001/18/EC regulates the intentional release of GMOs into the environment [80]. This also applies to production methods where the remaining of the GMO within a closed system cannot be ensured. Before the release of a GMO into the environment, a case by case environmental impact assessment has to be performed. This includes practical experiments to ensure the safety of the organism in the respective environment and requires detailed knowledge about the host organism, the implemented gene, the used vector, the application and processing and potential health and environmental threats. Toxic products and potential allergens (each new protein) are also recognised as potential health treats. The directive 1829/2003 regulates the utilisation of GMOs and GMO derived products in food and feeding [81]. A GMO or GMO product that is utilised in food and feeding has to be approved by the "European Food Safety Authority" (EFSA). Additionally, it has to be ensured that all ingredients can be traced back to the producer

and that products containing GMOs or GMO products are labelled as such. Labelling is not necessary if the GMO content is unintended but inevitable due to the process conditions and lies below 0.9% of the product. This would apply to purified (whether GMO or non GMO derived) enzymes, which have to be approved by the EFSA as ingredients in food or feeding products. This is especially important, since natural producers, *e.g.* fungal strains, are known to produce toxins. The dilution or even the removal of toxins by technical processes is forbidden. To bypass the requirement to label the product as GMO product, it is also necessary to purify the xylanase at least below the previously mentioned threshold. This is of great interest, since the consumer acceptance of GMO products is very low in certain EU member states.

USA, as the largest producer of GMO products, has no particular federal laws regulating the use and release of genetically modified organisms or their products. Instead, the regulations relevant to the particular product are applied. Therefore, the products are categorised as agricultural, food and drug and as general chemical products. Depending on the product, it has to undergo the approval procedures of one or more of the institutions. These regulations focus on the safety of the end product rather than the production process. The authorities in charge are the US Department of Agriculture (USDA), the Food and Drug Administration (FDA) and the US Environmental Protection Agency (EPA) [82]. Within this scheme genetically modified microorganisms for non-food and non-healthcare application are generally considered as chemical substances by the Toxic Substances Control Act (TSCA) [83] and their safety has to be approved by the EPA. Products derived from GMOs for non-pharmaceutical, non-food, feeding or processing purposes also have to be approved by the EPA unless they are listed in the TSCA Chemical Substance Inventory [84]. Microorganisms and derived products for pharmaceutical use or use in food, feeding or processing, however, are under the responsibility of the Federal Food, Drug, and Cosmetic Act (FFDCA) or the Public Health Service Act (PHSA) and have to be approved by the FDA. Considering this, strains for recombinant production of xylanases at industrial scale within the USA have to be approved by the EPA while the production of xylanases using wild type strains does not need any approval. The product (endo-1,4-β-xylanase (CAS-Nr: 9025-57-4)) also does not have to be approved by the EPA, since

it is already listed on the TSCA Chemical Substance Inventory. Xylanases for application in food, feeding and processing are under the responsibility of the FFDCA, regardless of the production process. The FDA however approves (endo-1,4-β-)xylanases as food and feeding additives as well as for food processing as "generally recognised as safe" (GRAS) [85]. Nevertheless, novel formulations and mixed enzyme formulations have to be approved by the FDA.

7. CONCLUSION

Although the use of wild type strains is not as strictly regulated as the use of GMOs and GMO products, it seems that only the optimisation of microbial strains renders them, or their products, promising for industrial applications. Enzymes identified by screening various habitats show different qualities. The exhaustive analysis of existing data will promote the rational design of new enzymes with the desired properties. Industrial producer strains will form the basis for heterologous hosts. Progress in biotechnology will unleash the potential of xylanases in the next years.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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