# Chapter 7
# Microbial Xylanases: Sources, Types, and Their Applications

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**Abstract** Biomass conversion to an utilizable energy sources such as monomer sugars using enzymatic hydrolysis has been emerged as the current technology which promises the future energy. In nature, bioconversion process of biomass is mediated by a group of biofunctional hydrolytic enzymes. These enzymes generally work in cooperative synergetic action to facilitate enhanced effective degradation of biomass. Xylanase is one of the crucial hydrolytic enzymes involved in hydrolysis of xylan, the hemicellulose which constitutes 15–30 % of the plant biomass. This chapter discusses in detail about the enzymatic hydrolysis of xylan by the xylolytic enzyme endo-1,4-β-xylanase, its occurrence in nature and mode of action, structure and classifications, current methods for its production, purification, and characterization. In addition, the major and recent industrial applications of this enzyme were highlighted as well.

## 7.1 Introduction

Nowadays, the governments promote extensive researches for the development of an alternative transportation fuel from a renewable energy sources, realizing the upcoming major energy crisis due to the depletion of petroleum-derived fuels. As an initiative, the department of energy in US has started producing biofuels with a set target of 60 billion gallon per year by 2030. Europe also has the similar targets by that time to replace 25 % of petroleum-based liquid transportation fuel by biofuels (Himmel et al. 2007). However, it is a challenging target since the biofuel production using substrates such as sugar cane and corn has limited capacity to supply such a huge volumes. Apart from this, in many countries people protest against the use of food crops for biofuel production and many debates that it leads to a major food crisis. As a remedy, researchers found out that the lignocellulose

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biomass which consists of 75 % polysaccharide sugars can be used as a significant feedstock for biofuel production (Lynd et al. 1991). If the attraction is more, lignocellulose biomasses are renewable energy source and exist abundant in nature and it can be derived predominantly from agricultural wastes (Gomez et al. 2008). Lignocelluloses are heteropolysaccharides that give structural rigidity to all kinds of plant. They account for approximately more than 50 % of the total biomass in the nature, synthesized (estimated 10–50 $\times$ $10^{12}$ tons $year^{-1}$) by plants through photosynthesis (Claassen et al. 1999). Lignocelluloses are composed of three major polymeric constituents such as cellulose (an unbranched linier polymers of long β-1-4 glucan between 2000 and 27,000 units), hemicellulose (a heterologous polysaccharides which are complex in nature with sugar monomeric subunits such as D-xylose, D-mannose, D-galactose, and L-arabinose) and lignin (complex phenolic structure act as linkage between cellulose and hemicellulose.

The name hemicellulose was first introduced by Schulze (1891). These heteropolysaccharides are abundant in plant cell wall and have complex structures when compared to cellulose due to the enormous number of sugar monomeric side chains (500–3000). It creates a cross-linked network between the cellulose microfibrils and lignin and plays a major role in structural integrity of plant cell walls. It also regulates the rate of expansion of primary cell wall and acts as a shield over cellulose to protect it from enzymatic hydrolysis. Hemicellulose is the second most abundant renewable biomass that is available after cellulose within the lignocellulose matrix representing about 20–35 % of total biomass (Saha 2003). Table 7.1 summarizes the approximate composition of various biomass materials. Classification of hemicellulose was made based on the types of sugar monomer units present on them. As mentioned above, hemicelluloses are composed of xylan, mannan, galactan, and arabinan polymers. Among these xylan is the most frequently occurring polymer composed mainly of β-D-xylopyranosyl residues which linked by β-1,4-glycosidic bonds (Beg et al. 2001).

In hardwoods plants, hemicellulose primarily consists of xylans and glucomannans whereas in softwood plants, it is composed of arabinoglucuronoxylans

**Table 7.1** Approximate composition (as a percentage) of various biomass materials

| Biomass | Cellulose (%) | Hemicellulose (%) | Lignin (%) | References |
|---|---|---|---|---|
| Bermuda grass | 47.8 | 13.3 | 19.4 | Li et al. (2010) |
| Reed canary straw | 42.6 | 29.7 | 7.6 | Bridgeman et al. (2008) |
| Corn cobs | 35–39 | 38–42 | 4.5–6.6 | Okeke and Obi (1994) |
| Rice straw | 41 | 21.5 | 9.9 | Lee (1997) |
| Bagasse | 38.1 | 26.9 | 18.4 | Lee (1997) |
| Coffee pulp | 24.0 | 8.9 | 19.4 | Dijkerman et al. (1997) |
| Coconut fiber | 17.7 | 2.2 | 34 | Dijkerman et al. (1997) |
| Rice hulls | 24–29 | 12–14 | 11–13 | Okeke and Obi (1994) |
| Corn stove | 39 | 19.1 | 15.1 | Lee (1997) |
| Wheat straw | 36.6 | 24.8 | 14.5 | Lee (1997) |
| Sawdust | 45.0 | 15.1 | 25.3 | Dijkerman et al. (1997) |

**Table 7.2** The hemicellulolytic enzymes and their substrates (Shallom and Shoham 2003)

| Enzymes | Substrates |
|---|---|
| Endo-β-1,4-xylanase | β-1,4-Xylan |
| Exo-β-1,4-xylosidase | β-1,4-Xylooligomers |
| α-L-Arabinofuranosidase | α-Arabinofuranosyl, (1–3) xylooligomers, α-1,5-arabinan |
| Endo-α-1,5-arabinanase | α-1,5-Arabinan |
| α-Glucuronidase | 4-*O*-methyl-a-glucuronic acid, (1–2) xylooligomers |
| Endo-β-1,4-mannanase | β-1,4-Mannan |
| Exo-β-1,4-mannosidase | β-1,4-Mannooligomers, mannobiose |
| β-Galactosidase | α-1,6-Galactopyranose, mannooligomers |
| β-Glucosidase | β-1,4-Glucopyranose, mannopyranose |
| Endo-galactanase | β-1,4-Galactan |

(xylans), xyloglucans, glucomannans, and arabinogalactans. Xylans are the major form of hemicellulose made of D-xylopyranosyl units which are linked by β-1,4-glycosidic bonds. β-mannan-based polymers are the second major form of cellulose which are made of β-1,4-linked mannose and/or glucose residues. Galactoglucomannans which consist of α-1,6-linked galactose side chains are also grouped under these polymers. They are found abundant in hardwood hemicellulose, which comprised varying ratios of D-mannopyranose units α-(1–6)-substituted galactopyranose side chains with *O*-acetyl side chains and β-1-4-linked-D-glucopyranose units. Arabinan and arabinogalactans are also considered as hemicelluloses which are generally composed of α-1,5-linked L-arabinofuranosyl units. Table 7.2 summarizes the major enzymes involved in the breakdown of cross-linked hemicelluloses. Those groups of enzymes which are involved in hydrolysis of xylan are generally known as xylanases. The current book focuses on one of the crucial xylolytic enzymes known as endo-1-4-β-xylanase.

## 7.2 Enzymatic Hydrolysis of Xylan

In most terrestrial plant species, secondary cell wall thickening takes place by the deposition of xylan and they are found in-between cellulosic fiber sheets and lignin with complex binding relationship through hydrogen bonding. Xylan thus forms a think wall over cellulose and enables the protection from degradation by different cellulose degrading enzymes. Xylan constitutes 15–30 % of the plant biomass in hardwood and 7–12 % of the plant biomass in softwood. Since they are the major hemicellulose group in most of the plant species, they have been considered as one among the renewable source of energy in the form of biomass. Structure of xylan is complex in nature as they are composed of β-1,4 linked-D-xylopyranose units with α-L-arabinofuranose and 4-*O*-methyl-α-D-glucuronopyranosyl acid side chains. Figure 7.1 shows the major enzymes involved and their site of action during the enzymatic hydrolysis is xylan (Polizeli et al. 2005).

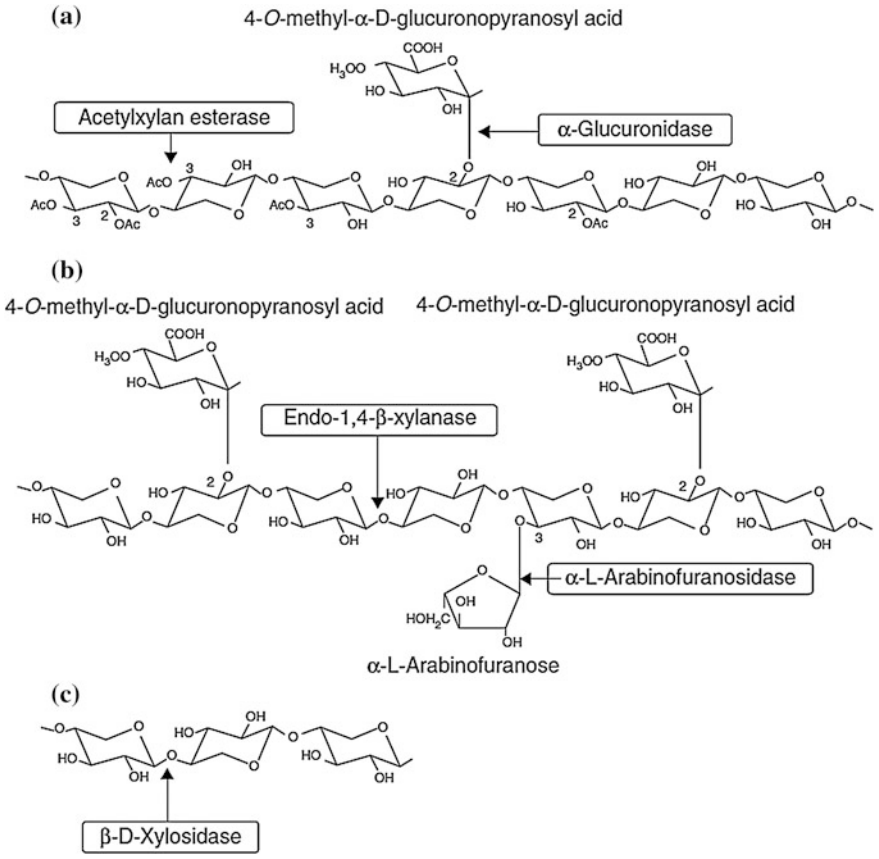


**Fig. 7.1** Enzymatic hydrolysis of xylan (Polizeli et al. 2005)

Hardwood xylan (e.g., birchwood xylan) consists of more than 70 β-1,4 linked-D-xylopyranose units linked by β-1,4 glycosidic bonds and 4-*O*-methyl-α-D-glucuronopyranosyl acid side chains which are found attached at second carbon of every tenth xylopyranose units. Acetylation rate is higher in these groups and it is quite frequent at second and third carbon atom on xylopyranose ring. In softwood xylan, acetylation rate is zero but instead of an acetyl group they possess α-L-arabinofuranose linked by α-1-3 glycosidic bond at the third carbon atom on xylopyranose ring. Based on the side chains of the xylan backbone, they are classified into different groups such as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan. These four groups are heterogenic in terms of their degree and nature of branching. In many plants, xylans are found partially acetylated, which protect them from complete degradation by xylolytic enzymes. This could be the reason behind the fact that the complete degradation of xylan is accomplished only by synergetic interaction of acetyl xylan esterase and endo-xylanase enzymes.

Due to the heterogeneity and complex chemical structure of polymer, the complete breakdown of xylan requires the synergetic action of several hydrolytic enzymes which are specific in their mode of actions. Generally, xylans are not attacked randomly, but the bonds selected for hydrolysis depend on several biochemical factors that determine the nature of the substrate molecules such as the chain length, the degree of branching, and the presence of substituents.

The major xylolytic enzymes involved in xylan hydrolysis are as follows:

- Endo-1,4-β-xylanase—cleaves long xylan backbone into short xylooligosaccharides.
- β-xylosidases—also attacks the β-1,4-glycosidic linkages to liberate xylose from short oligosaccharides.
- α-L-arabinofuranosidases—remove L-arabinofuranose side chains.
- α-D-glucuronidases—hydrolyze the methyl glucuronate residues.
- Acetyl xylan esterases—hydrolyze acetate groups on xylan backbone.
- Ferulic acid esterases—hydrolyze the aromatic acids groups linked on arabinofuranoside residues.

Endo-1,4-β-xylanase: (EC 3.2.1.8) cleaves the glycosidic bonds between the D-xylopyranose units on the xylan backbone and as a result, short xylooligosaccharides are formed. β-D-xylosidases: (EC 3.2.1.37) liberate xylose from short xylooligosaccharides which are formed during xylan hydrolysis by endo-1,4-β-xylanase. These enzymes are classified based on their relative affinity for the xylooligosaccharides into distinct entities such as xylobiases and exo-1,4-β-xylanases. Even though they are two different enzymes, they have been recognized as β-D-xylosidases in general, and releases β-D-xylopyranosyl residues from small xylooligosaccharides and xylobiose. Their affinity for the xylooligosaccharides is inversely proportional to its complexity. Xylobiose was found to be the best substrate for these enzyme groups. These groups of enzymes play important role in xylan hydrolysis when there is endo-1,4-β-xylanase inhibition due to the accumulation of short oligomers of β-D-xylopyranosyl units, by hydrolyzing these products and remove the cause of inhibition thereby enhancing the xylan hydrolysis (Andrade et al. 2004). The molecular weights of these enzyme groups are relatively high, between 60 and 260 kDa and their pH optima is between 4.0 and 5.0. Generally, these enzymes are thermophilic in nature and their optimum temperature varies from 40° to 80°. However, in most cases they exhibit highest activity around 60 °C (Rizzatti et al. 2001). α-L-arabinases: These enzymes remove L-arabinose side chains which are substituted at second and third carbon on β-D-xylopyranosyl ring of arabino xylan. They have been classified as exo-α-L-arabinofuranosidase (EC 3.2.1.55) and endo-1,5-α-L-arabinase (EC 3.2.1.99), based on their distinct mode of action. The first group, which is most common, degrades branched arabinans and *p*-nitrophenyl-α-L-arabinofuranosidase, whereas the second group hydrolyzes only the linear arabinans (de Vries et al. 2000). The α-D-glucuronidases (EC 3.2.1.131) can hydrolyze the α-1,2 linkages between glucuronic acid and xylose residues found in glucuronoxylan. They are classified under glycoside hydrolase family 67 and rarely found in nature. During biodegradation of glucuronoxylan, these

enzymes are able to release glucuronic acid. However, the enzyme activity is only limited on short xylooligomers due to the partial hindrance by acetyl group present in xylan backbone (Puls et al. 1991). Acetylxylan esterase: (EC 3.1.1.6) it acts by removing the *O*-acetyl groups from second and third carbon on β-D-xylopyranosyl ring of acetyl xylan. This enzyme plays important role in xylan hydrolysis by removing *O*-acetyl side chains, since *O*-acetyl groups in acetyl xylan interfere the activity of endo-1,4-β-xylanase and β-D-xylosidases on xylan backbone. Xylan extraction from hardwoods is usually mediated by alkali treatment, resulting in acetyls hydrolysis, and probably this could be the reason of the late discovery of this enzyme (Shao and Wiegel 1995; Blum et al. 1999). Ferulic acid esterase: Ferulic acid esterases (EC 3.1.1.-) act by hydrolyzing the bond between arabinose side chain and ferulic acid group attached (Crepin et al. 2004). The cooperative functioning among the above-mentioned xylolytic enzymes enhance the complete biodegradation of xylan. It was also observed that acetylxylan esterase activity on xylan results in deacetylation of xylan backbone and these deacetylated xylans are more easily hydrolyzed by endo-xylanase. Similarly, there was a significant increase in hydrolysis efficiency when arabinoxylan was pretreated by α-arabinofuranosidase. This was due to the removal of arabinan side chains which act as hindrance to endo-xylanase activity (de Vries et al. 1999).

## 7.3 Occurrence of Endo-1,4-β-xylanase

Endo-1,4-β-xylanase are also known as endo-(1→4)-β-xylan 4-xylanohydrolase, endo-1,4-xylanase, xylanase, β-1,4-xylanase, endo-1,4-xylanase, endo-β-1,4-xylanase, 1,4-β-xylan xylanohydrolase, β-xylanase, β-1,4-xylan xylanohydrolase, endo-1,4-β-xylanase, β-D-xylanase, 4-β-D-xylan xylanohydrolase, endo-(1→4)-beta-xylan 4-xylanohydrolase, beta-1,4-xylanase, endo-beta-1,4-xylanase, endo-1,4-beta-D-xylanase, 1,4-beta-xylan xylanohydrolase, beta-xylanase, beta-1,4-xylan xylanohydrolase, endo-1,4-beta-xylanase, beta-D-xylanase, and 4-beta-D-xylan xylanohydrolase. In nature, the endo-1,4-β-xylanase enzymes are widely distributed among eukaryotes and prokaryotes. The occurence of these enzymes was also reported in higher eukaryotes such as plants, protozoa, small insects, and several marine species. A Japanese pear fruit was reported producing endo-xylanase during its over-ripening period. Endo-xylanase of molecular weight 55 kDa was also isolated from wheat flour (Cleemput et al. 1997). Apart from these sources, members of higher animals such as insects and fresh water mollusc were also reported as xylanase producers (Yamura et al. 1997). However, bacteria, fungi, and actinomycetes were found to be the largest source of xylanase enzyme.

There have been several reports on microbial endo-1,4-β-xylanase since 1960. Nevertheless, most of these xylanase enzymes remained unnoticed. During 1980s first studies on xylanase-mediated bio-bleaching in pulp industry was reported and only since then the scientific world realized the great impact of xylanase enzymes in industrial applications. As mentioned above, a number of microorganisms including

bacteria and fungi have the capacity for hydrolyzing xylans by synthesizing a variety of xylolytic enzymes. Early reports reveal that many of these microbial species are plant pathogens as they play an important role in degradation and invasion of plant tissues. It also shows that xylanases can elicit defense mechanisms in plants by a collective functioning with other cellulolytic enzyme groups.

### 7.3.1 Fungal Endo-1,4-β-xylanase

Many of the fungal species that are pathogenic to plants produce plant cell wall polysaccharide degrading enzymes. Endo-1,4-β-xylanase is one among the major group of such enzymes and they result in partial degradation of cell wall structures to the region of penetration (Subramaniyan 2000). A hypothetical model of fungal invasion on plant tissue is shown in Fig. 7.2.

A number of fungal species have been used to produce xylanase since they are the major producer of xylanases in nature. Table 7.3 shows the major fungal species used to produce xylanases. *Phanerochaete chrysosporium*, a potent plant pathogen, reported to produce xylanase activity of 15–20 U $mL^{-1}$ along with considerable amount of cellulose activity (Copa-Patino et al. 1993). Other study also showed that the thermophilic fungus *Thermomyces lanuginosus* has the capacity of high xylanase production up to 3576 U $mL^{-1}$ (Singh et al. 2000). Many fungal species have been reported as xylanase producers; however, species with high xylanase activity and negligible cellulase activity are found to be very rare in nature. It was also observed that the pH optima of many of these fungal xylanases are between pH 5 and 6 even though they are stable at pH between 3 and 8. On the other hand, bacterial xylanases showed slightly higher pH optima that makes them suitable for many of the industrial applications. Thermal tolerance of the majority of fugal xylanase reported so far has been found below 50 °C and in most cases this

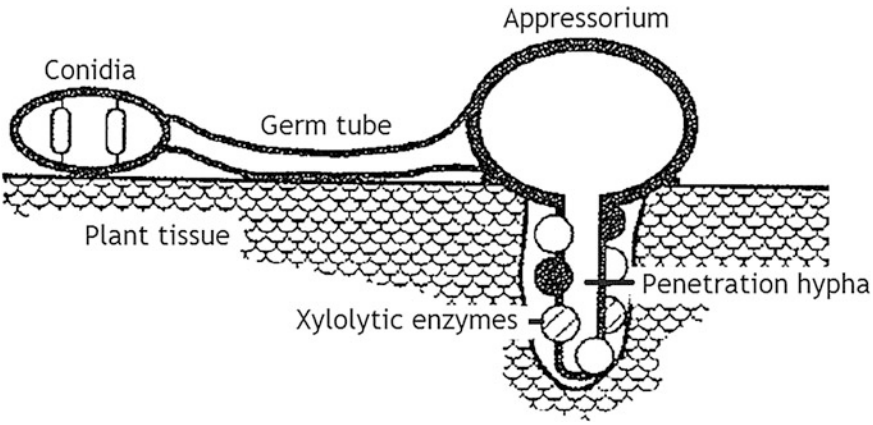


**Fig. 7.2** Fungal invasion on plant tissue by xylolytic degradation (Prade 1996)

**Table 7.3** Major fungal xylanase and its characteristics

| Source | Molecular weight (kDa) | Optimum conditions | | References |
|---|---|---|---|---|
| | | Temperature (°C) | pH | |
| *Aspergillus awamori* | 39, 23, 26 | 45–55 | 4.0–5.5 | Kormelink et al. (1993) |
| *A. fumigatus* | 19, 8.5 | 55 | 5.5 | Silva et al. (1999) |
| *A. kawachii* | 35, 26, 29 | 60, 55, 50 | 5.5, 4.5 | Ito et al. (1992) |
| *A. nidulans* | | 55 | 8 | Taneja et al. (2002) |
| *A. oryzae* | 35 | 60 | 5 | Kitamoto et al. (1999) |
| *A. nidulans* | 22, 34 | 62, 56 | 5.5, 6.0 | Fernández-Espinar et al. (1994) |
| *A. sojae* | 32.7, 35.5 | 60, 50 | 5.0, 5.5 | Kimura et al. (1998) |
| *A. sydowii* | 33 | 50 | 4 | Ghosh and Nanda (1994) |

particular property of fungal xylanase makes them less favorable for application in harsh industrial conditions. Reduced xylanase yield in fermenter studies is another major problem associated with fungi xylanase. This was due to poor oxygen transfer, shear force during fermentation, and the enzyme production process in fungi is highly regulated by growth morphology (El Enshasy et al. 1999, 2006; El Enshasy 2007). Generally, the fungal species has filamentous growth pattern, the fungal growth in fermenters is restricted due to the shear stress, and eventually this results in poor xylanase production (Dean et al. 1991).

### 7.3.2 Bacterial Endo-1,4-β-xylanase

Since 1980s, several bacterial strains which are capable of producing alkaline and highly thermophilic xylanase enzymes have been reported. Among these wide spectrum of bacteria, *Bacillus*s species are the most predominant producers of endo-1,4-β-xylanase enzyme with negligible amount of cellulose activity under optimized growth conditions. Other author showed a high yield of xylanase up to 506 IU $mL^{-1}$ by *Bacillus* SSP-34 (Subramaniyan and Prema 2002). In general, bacterial endo-1,4-β-xylanase is suitable for industrial application since they have a wide pH optima and improved thermostability when compared to fungal xylanases. Ratto et al. (1992) reported *Bacillus circulans* strain with xylanase activity of 400 IU $mL^{-1}$. They observed the optimal enzyme activity was at pH 7 and retained 40 % of activity at high pH up to 9.2. Many other researchers reported cellulose-free xylanase enzyme from *Bacillus stearothermophilus* strain T6 (Khasin et al. 1993; Lundgren et al. 1994). A relatively high xylanase activity was reported in *Bacillus* sp. strain NCL when it was grown in zeolite-induced medium (Balakrishnan et al. 2000). Thermophile bacteria such as *Rhodothermus marinus* produced approximately 1.8–4.03 IU $mL^{-1}$ of thermostable xylanase along with

**Table 7.4** Major bacterial xylanase and its characteristics

| Source | Molecular weight (kDa) | Optimum conditions | |
|---|---|---|---|
| | | Temperature (°C) | pH |
| *Aeromonas caviae* ME1 | 20 | 50 | 7 |
| *Bacillus amyloliquefaciens* | 18.5–19.6 | 80 | 9 |
| *B. circulans* WL-12 | 85 | – | – |
| *Bacillus* sp. *strain* SPS-0 | 99 | 75 | – |
| *Bacillus* sp. W1 (JCM2888) | 21.5, 49.5 | 65, 70 | 4.5–10, 4.5–7 |
| *Bacillus* sp. *strain* 41-1(36) | 36 | 50 | 9 |
| *Bacillus* sp. *strain* TAR-1 | 40 | 75 | 6 |
| *Bacillus stearothermophilus* T-6 | 43 | 75 | 6.5 |
| *Streptomyces* T-7 | 20.6 | 60 | 4.5–5.5 |
| *Streptomyces* sp. No 3137 | 50, 25, 25 | 60–65 | 6-May |
| *Thermotoga maritima* | – | 85 | 6.5 |
| *T. thermarum* | – | 80–100 | 7 |

Modified from Subramanya and Prema 2000

detectable amount of cellulose activity. Other strain such as *Bacillus circulans* showed high yield of xylanase (400 IU $mL^{-1}$) which has pH optima of 7 but with a high stability at alkaline pH (Beg et al. 2000). The concept of TCF (total chlorine free) bleaching of pulp was developed during this period and it was achieved with xylanase from *Bacillus stearothermophilus* strain T6 which has optimum activity at pH 6.5 (Kohli et al. 2001). A detailed description of major bacterial xylanase and their characteristics are given in Table 7.4

## 7.4 Biosynthesis and Regulation of Microbial Endo-1,4-β-xylanase

Biosynthesis of xylanase enzyme by microorganisms and the phenomenon of xylanase induction are less investigated at molecular level due to the difficulty in setting up a cell-free system under experimental conditions. However, Srivastava and Srivastava (1993) introduced a hypothetical model of xylanase biosynthesis and regulation to describe similar conditions. Xylans are comparatively complex in structure with high molecular weight and thus it is usually hard to be utilized directly by microbes. Xylans are converted into smaller molecules such as xylose, xylobiose, xylotriose, or xylooligosaccharides by the constitutively produced small amount of endo-1,4-β-xylanase enzymes. These simpler molecules can be easily utilized by microbial cell as the carbon source (Zhao et al. 1997). These low molecular weight molecules can act as inducer for further xylanase expression and this has been one of the possible explanations for direct induction of xylanase gene.

Xylanases are usually secreted in the media which contains pure xylan or xylan residues in cultures of different species such as *Aspergillus awamori*, *Trametestrogii*,

and *Streptomyces* sp. QG-11-3 (Beg et al. 2001). However, several exceptional cases have also been reported such as in *Cellulomonas flavigena*; xylan acts as a poor inducer of xylanase gene (Avalos et al. 1996). In some rare cases, for example, in yeast strain *Trichosporon cutaneum*, xylanase biosynthesis is induced by certain positional isomers. In many bacterial species, the xylanase induction is also possible with various sugars such as D-xylose, D-maltose, D-glucose, and D-arabinose. On the other hand, in many fungal species, the natural lignocelluloses such as corn cobs, rice straw, sugarcane bagasse, and wheat bran were found to be capable of triggering the xylanase induction (Gupta et al. 2000). Other study conducted by Kumar and Satyanarayana (2011) reported on the wheat bran-mediated xylanase induction in *Bacillus halodurans* TSEV1 strain. However, the hypothetical mechanism of direct induction is questionable when the transportation across the cell wall can be blocked by larger molecules. Based on the study of Gomes et al. (1994), there has been a universally supported concept based on intracellular β-xylosidases. They explains that the larger xylooligomers formed during xylan hydrolysis are directly transported into the cell matrix and it was further degraded into xylose residues by β-xylosidase which are present in the cytoplasm.

Hydrolysis of xylooligomers by hydrolytic transporter proteins during their transportation through cell membrane is another possible explanation for above phenomenon. There are also rare cases in which the high xylose concentration in the media inhibits the xylanase gene expression (Strauss et al. 1995). Exclusion of inducer transport across the cell membrane is another possible reason for poor gene expression. In similar cases such as in *E. coli* it has been observed that the presence of glucose in the media prevents the lactose transportation through the cell membrane which is inducer for *lac* operon (Borralho et al. 2002). It was also observed that the xylanase biosynthesis is mediated by complex metabolic pathway in which the inducer level and the level of repressor molecule to that particular inducer vary with the organism and their growth medium. For example, the xylanase production by *Streptomycetes* sp. was increased when cellulose substrates are used in the growth media, whereas in *Cellulomonas favigena*, sugarcane bagasse was found to be the best inducer of xylanase enzyme (Alejandro et al. 2007). Addition of xylose in fermentation media increased xylanase production to a significant amount in *Bacillus* sp. (Gupta and Kar 2009). However, other studies showed that the presence of readily utilizable sugars such as glucose, xylose, and galactose can suppress xylanase biosynthesis in other strains such as in *Streptomyces* sp. (Bajaj and Singh 2010) and in *T. reesei* (Mach-Aigner et al. 2010).

## 7.5 Classification and Structures of Xylanase

Xylanases are considered as one of the major hydrolyzing enzyme groups as they mediate the xylan degradation to simpler utilizable units. Activity of these enzymes depends on the substrate specificity as well as substrate complexity. Wong et al. (1988) classified xylanases into two major groups based on the end product of

hydrolysis reaction. They were debranching enzymes which liberate arabinose and non-debranching enzymes which do not liberate arabinose residues from α,1,3,L-arabinofuranosyl. A few years later, another classification system was introduced based on the physiological properties of xylanase enzymes such as isoelectric point (PI) and molecular weight (MW). Therefore, xylanases were classified into two major groups: end xylanase enzymes with molecular weight lower than 30 kDa and basic pH and those with molecular weight higher than 30 kDa and acidic pH. However, few years later, this classification system was found incorrect since it matches for only a few xylanase enzymes.

Henrissat and Bairoch (1993) proposed a broad classification system based on the similarities in amino acid sequence and catalytic module. The glycoside hydrolases are further classified into different families. This classification system has got wide acceptance since it gives information regarding the structural properties, the catalytic mechanisms of the enzyme, and their evolutionary relationship within the group. Xylanases were grouped under glycoside hydrolases (GH) 5, 8, 10, and 11 families. However, the enzymes with multi-domain that exhibit detectable amount of xylanase activity are grouped into GH7, 16, 43, and 62 families (Cantarel et al. 2009). Table 7.5 comprises major xylolytic enzymes, their classification into CE, GH families, and their current crystallographic structure. Some of these GH families are further classified into super families that represent a more distant common evolutionary ancestor. Major GH family xylanase and their general structures are shown in Fig. 7.3 (Table 7.6).

**Table 7.5** Xylolytic enzymes, their classification into CE, GH families, and the their current crystallographic structure status

| Enzyme | Enzyme family | | Structure status |
|---|---|---|---|
| | EC | GH | |
| Endo-β-1,4-xylanase | 3.2.1.8 | 5, 8, 10, 11 and 43 | GH5-Cryst. (1BQC)<br>GH8-Cryst. (1IS9)<br>GH10-1FH7, 1CLX, 1XYZ<br>GH11-1H4G, 1BCX, 1IGO<br>GH43-(1GYD) |
| Exo-β-1,4-xylosidase/β-xylosidases | 3.2.1.37 | 3, 39, 43, 52 and 54 | GH3-Cryst. (1EX1)<br>GH39-Cryst.<br>GH43-(1GYD) |
| α-L-Arabinofuranosidase | 3.2.1.55 | 3, 43 and 51 | GH3-(1EX1)<br>GH43-(1GYD)<br>GH51-Cryst |
| Endo-α-1,5-arabinanase | 3.2.1.99 | 43 | 1GYD |
| α-Glucuronidase | 3.2.1.139 | 67 | 1K9D, 1GQI |
| Acetyl xylan esterase | 3.1.1.72 | 1 | (1JJF) |
| | | 5 | 1QOZ, 1G66 |
| Ferulic acid esterases | 3.1.1.73 | 1 | 1JJF, 1GKK |

*Source* Shallom and Shoham 2003

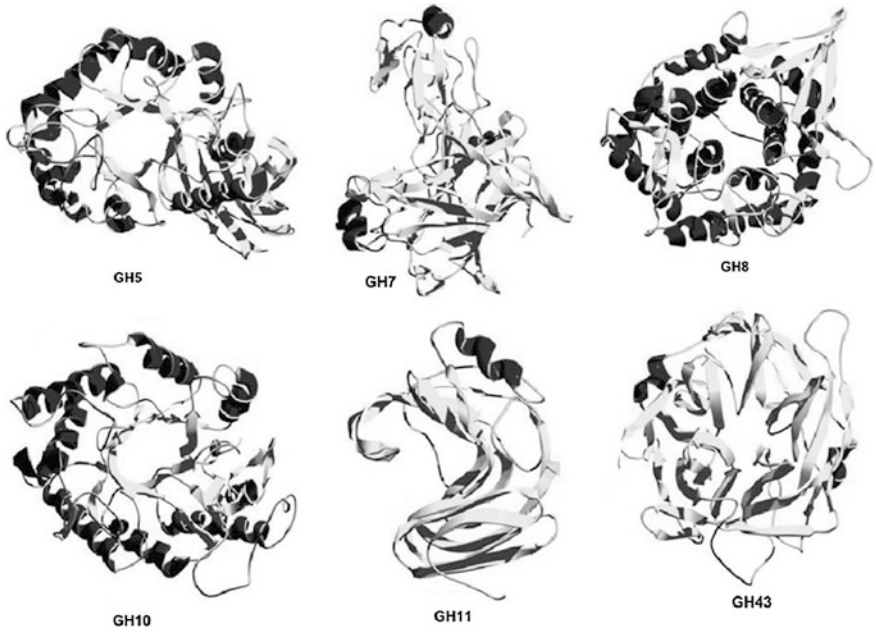


**Fig. 7.3** Structures of xylanase major GH family xylanase (Collins et al. 2005a, b)

**Table 7.6** CBMs of known structure linked to xylanases (Berrin and Juge 2008)

| CBMs family | Source of xylanase | GH family | PDB code |
|---|---|---|---|
| CBM-2 | *Cellulomonas fimi* | 10A, 11A | 1EXG, 2XBD, 1HEH |
| CBM-4 | *Rhodothermus marinus* | 10A | 1K45 |
| CBM-6 | *Clostridium thermocellum* | 11A | 1UXX, 1NAE, 1UY4 |
| CBM-9 | *Thermotoga maritima* | 10A | 1I8A |
| CBM-10 | *Cellvibrio japonicus* | 10A | 1QLD |
| CBM-13 | *Streptomyces olivaceovidris* | 10A | 1XYF |
| CBM-15 | *Cellvibrio japonicus* | 10C | 1GNY |
| CBM-22 | *Clostridium thermocellum* | 10B | 1DYO |
| CBM-36 | *Paenibacillus polymyxa* | 43A | 1UX7 |

### *7.5.1 GH10 Xylanases*

Most of the glycoside hydrolases which are classified under GH10 are endo-β-1,4-xylanases. Apart from these enzyme, G10 group also consists of a small number of endo-β-1,3-xylanases (EC 3.2.1.32), which cleave β-1,3-glycosidic linkages randomly in β-1,3-xylans backbone. A typical GH10 xylanases has a wide spectrum of substrate specificity as they are able to hydrolyze various forms of xylans in nature. These enzymes are capable to attack not only the linear forms of xylan but also the highly branched heteroxylans and xylosomes. Kolenova et al. (2006)

analyzed the hydrolysis product from glucuronoxylan and confirmed that GH10 xylanases can attack xylan at its non-reducing end if there are two or more unbranched xylose residues which are present on backbone. It was also found that GH10 xylanases can even hydrolyse the highly branched arabinoxylan into small fragments. Plant xylanases which have been identified so far are grouped under GH10 family. In recent years many studies were carried out to characterize these plant xylanases and it was observed that these enzymes have limited substrate specificity (Chithra and Muralikrishna 2008). The study of Van Campenhout et al. (2007) was focused on the characterization of GH10 xylanase isolated from barley and they reported that the enzyme can release small xylose units from various substrates. GH10 xylanases, in addition to their xylolytic activity, are also active against glucose-derived substrates like aryl-cello-oligosaccharides (Charnock et al. 1997; Andrews et al. 2000).

Three-dimensional structure of xylan was first described by Atkins (1992). Subsequently, more studies were carried out using NMR spectrometry and X-ray crystallography techniques to obtain more carbohydrate structure details. It was found that under crystallized condition, the xylan backbone exhibits a threefold left-handed confirmation and the geometry showed that there is no effect on β-1,4 glycosidic bonds by the side chains of those attached. Electron diffraction pattern study confirmed the structure and the hexagonal morphology. The hydrogen at the fifth carbon atom on xylose ring has binding properties either intra- or interchain. The study also suggested that the structural confirmation of D-xylose ring indicated the di-equatorial binding property of both 1–4 and 1–3 glycosidic linkages.

The X-ray crystallographic image of endo-β-1,4-xylanases enzyme isolated from *Thermoascus aurantiacus* has been used to explain the general structure of GH family 10 xylanase (Fig. 7.4). It was found that the 32.5 kDa long polypeptide chain with a β/α-fold TIM barrel structure consists of eight major β strands which are arranged side by side and parallel, forming a cylinder in the center followed by eight major α helixes. In addition, around six short helixes are also found attached with the polypeptide chain. The catalytic domains of family 10 xylanases are found in cylindrical shape and the overall side view resembles a 'Salad bowl' (Fig. 7.5). In this configuration, the catalytic sites are seen relatively closer to the carboxyl terminus end of xylan backbone. Since the molecular weight of these xylanase is higher, they always exhibit low degree of oligosaccharide polymerization. Both the disulfide bond and salt bridges present on the xylan structure improve its thermostability. In the overall structure, the top phase of the molecule which is β-barrel side has larger than the bottom face, the α-β turns. This was due to the elaborate architecture of β–α loops (Natesh et al. 1999).

### *7.5.2 GH11 Xylanases*

GH11 xylanase unlike the other xylanase families, they only consist of endo-β-1,4-xylanases that are exclusive enzymes cleaves β-1,4-xylosidic bonds between xylose monomers. These enzymes are often sub-classified based on their

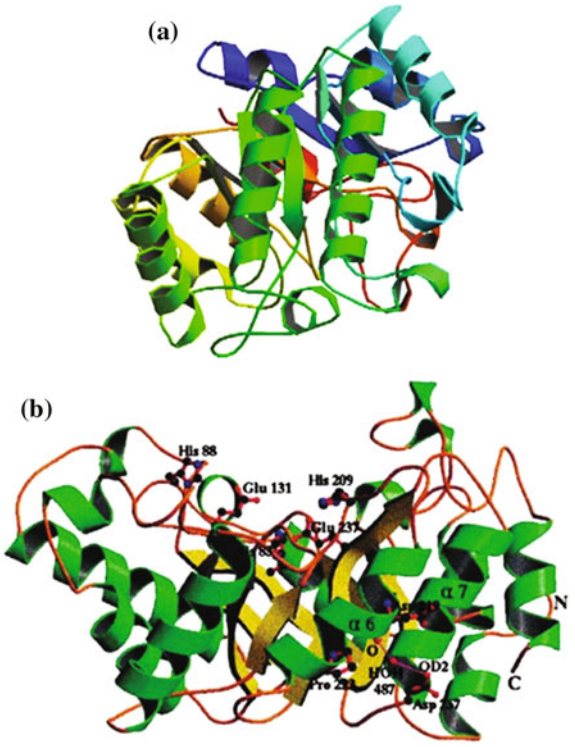


**Fig. 7.4** **a** A front view showing the (α/β) 8 TIM barrel fold, **b** salad bowl view exposing the substrate binding cleft of endo-1,4-β-xylanase from *T. aurantiacus* (Natesh et al. 1999)

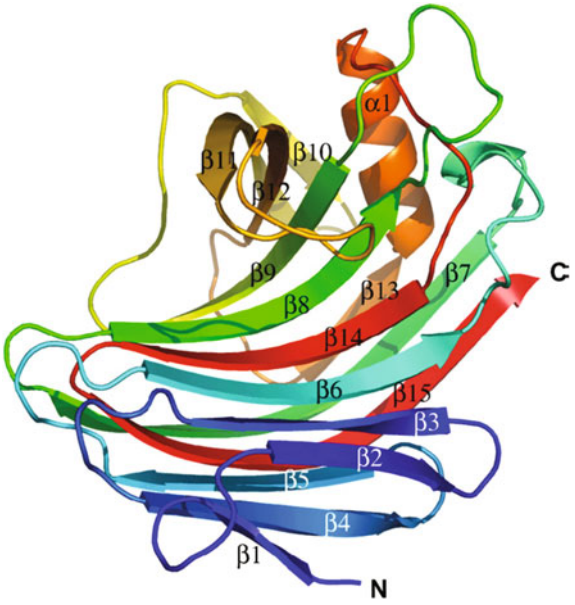


**Fig. 7.5** Three-dimensional structure of NpXyn11A. The protein schematic is color ramped from the N terminus (*blue*) to the C terminus (*red*) (Vardakou et al. 2005)

PI values into acidic and alkaline xylanases (Joshi et al. 1997). Many studies demonstrated the correlation between the pH optima and the amino acid residues which are present adjacent to the catalytic site of these enzymes. In many cases, it was observed that the acidic xylanase (pH < 5) consists of asparagine, whereas in alkaline xylanase has arginine (Krengel and Dijkstra 1996; Fushinobu et al. 1998). This was also confirmed by other study which reported that arginine introduction to bacterial xylanase shifts the pH optimum to acidic range (Pokhrel et al. 2013). Heteroxylan, xylobiose, and xylotriose are the major subunits formed during GH11 xylanase-mediated xylan hydrolysis. Further hydrolytic activities on these subunits were found negligible; however, hydrolysis products such as xylotetrose, xylopentose, and xylohexose are further hydrolyzed by GH11 xylanases (Cervera-Tison et al. 2009). These enzymes cleave the xylose backbone at unsubstituted regions which are quite away from the branched xylose present at non-reducing end. It was also found that GH11 xylanases require minimum of three unsubstituted consecutive xylose residues for the primary binding and initiation of hydrolysis (Biely et al. 1997; Katapodis and Christakopoulos 2008).

Ko et al. (1992) reported the first structural description of a GH11 xylanase that was isolated from *Bacillus pumilus*. But his analysis was not very precise and he failed to deposit the 3D structure in protein database. To date, more than 100 GH11 xylanase 3D structures from different fungal and microbial species have been solved and made available in CAZy database. Xylanases isolated from a thermophilic fungi *Thermomyces lanuginosus* are widely used to explain the general structure of GH11 xylanases. The structure shows a globular protein composed of two β-sheets (β-a, β-b) with a molecular weight of 25 kDa. The outer β-sheet (β-a) is composed of five antiparallel β strands with polar and uncharged amino acids such as threonine and serine. The inner β-b sheet is made up of nine antiparallel β strands of which the outer sides are developed to catalytic sites and the inner sides are found attached with β-a forming a hydrophilic core (Torronen et al. 1994). In general, most of the GH11 xylanases are made of only a single catalytic domain and found in β-jelly roll structure. It also resembles the shape of partially closed right hand in which two β-sheets represent the 'fingers,' the loops between β-strands of β-b such as B7 and B8 represent the 'thumb,' and the twisted inner sides of β-b and α-helix represent the 'palm' of the hand (Gruber et al. 1998).

While comparing these two major GH families, the GH11 family consists only of 'true xylanases' displaying substrate specificity toward D-xylose containing xylan substrates. Their catalytic versatility is lower when compared to GH10 xylanases and the products from its action such as xylobiose and xylotriose usually required further hydrolysis by other xylanase enzymes. The GH10 xylanases can mediate hydrolysis until the release of xylose as end product. Another interesting feature of GH10 xylanases is their ability to tolerate glucose-derived to a certain extend in addition to their xylanolytic activity that exhibits in addition to their xylanolytic activity (Collins et al. 2005a). It is generally accepted that GH10 can cleave decorated regions on AX backbone and its activity is less hampered by α-L-arabinofuranosyl and acetyl or 4-*O*-methyl-D-glucuronate side chains present on xylan

backbone, whereas GH11 xylanases are very specific in their action as they cleave only at the unsubstituted regions (Biely et al. 1997). This property is also reflected in the shape of their active binding sites, where GH10 xylanase has a shallow groove active site which has less affinity toward the unsubstituted regions and GH11 xylanases possess a cleft-shaped active site which has higher affinity toward unsubstituted consecutive xylose.

### *7.5.3 GH8 and GH5 Xylanases*

In addition to the above-mentioned GH families, GH8 family xylanase is another most studied xylanase which consists of both endo- and exo-xylanases enzymes. Exo-xylanases are those enzymes which reduce xylooligosaccharides (XOS) and release xylose subunits. A very few number of endo-xylanase have been identified so far that belong to GH8 family such as xylanases isolated from *Pseudoalteromonas haloplanktis* (Collins et al. 2005a); GH8 exo-oligoxylanases was also isolated from *Bifidobacterium adolescentis* (Lagaert et al. 2007) and from *Bacillus halodurans* (Honda and Kitaoka 2004). In general, the diversity in substrate specificity of these enzymes is large in nature and rXyn8 isolated from *P. haloplanktis* is the best example which shows highest activity on mixed linkage (β-1,3- and β-1,4-bonds) homoxylan (Pollet et al. 2010). Studies on three-dimensional structure of GH8 xylanase isolated from P. *haloplanktis* xylanase show that it is a single-domain molecule with six (α/α)-fold barrel which is found similar to GH48 enzymes (De Vos et al. 2006). Glycoside hydrolases 5 (GH5) is another largest enzyme family which consists of more than 1800 entries in the CAZy database. Under this family, several enzymes with apparent activity on xylan have been reported. Endo-xylanases from *Aeromonas punctuate* (Suzuki et al. 1997) and *Meloidogyne incognita* (Mitreva-Dautova et al. 2006) are the major reported GH5 xylanases so far. However, certain enzymes in this group such as xylanase from *H. jecorina* also exhibit exo-activity. Larson et al. (2003) was the first one who reported the structure of GH5 xylanase (XynA) isolated from *E. chrysanthemi.* In recent years, many other X-ray analysis of GH5 xylanase has been reported. St John et al. (2009) reported X-ray crystallographic analysis of XynC from *B. subtilis*, which shows a multidomain protein (consist of a catalytic domain along with a C-terminal CBM) with (α/β) 8-fold barrel.

## 7.6 Catalytic Mechanisms of Endo-1,4-β-xylanase

Hydrolysis of glycosidic bonds is carried out mainly by two different catalytic residues that are present in the active site of the enzyme. Hydrolysis takes place at the anomeric center of the substrate molecules either by inversion or retention of the configuration and this has been depended on factors such as the structure and

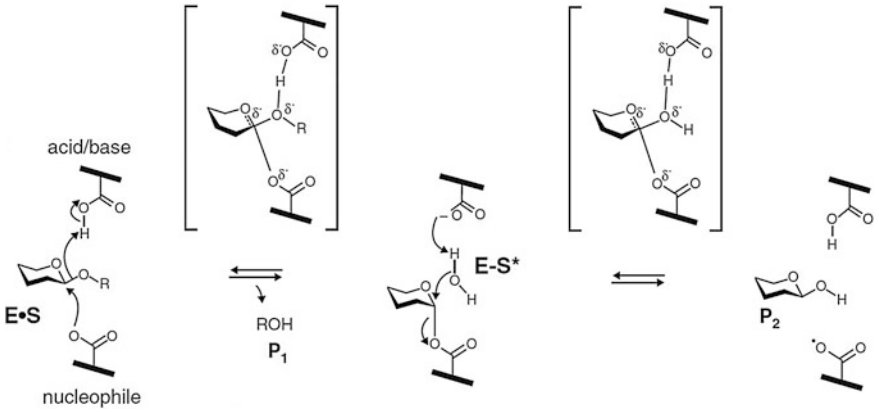


**Fig. 7.6** A hypothetical model of catalytic mechanism of GH xylanase. The double-displacement reaction mechanism for retaining glycosidases (Koshland mechanism). Modified from Paës et al. (2012)

position of catalytic residues on the enzymes (Collins et al. 2005b). Many of the endo-1,4-β-xylanase exhibit a double-displacement mechanism in their operation on specific substrate molecules (Fig. 7.6). In most cases, the first acid/base catalytic residue initiate the hydrolysis by protonating the xylopyranosyl linkages between the xylan monomers and this process is termed as glycosylation. The second catalytic residue which acts as a nucleophile attacks the same linkage and results in the formation of enzyme–glycosyl intermediate while passing through an oxocarbenium-ion-like transition state. In consecutive steps which are also termed as deglycosylation, the first catalytic residue exhibits basic properties by activating the incoming water molecule and abstracting a proton from it. The activated water molecule readily attacks the anomeric carbon of the enzyme–glycosyl complex formed in the previous step and release the product with an α-configuration at the anomeric carbon (Sidhu et al. 1999; Chiku et al. 2008).

## 7.7 Factors Affecting Endo-1,4-β-xylanase Functionality

### *7.7.1 GH Family Origin*

Among the above-mentioned families, endo-xylanases GH10 and GH11 are the most studied. Endo-xylanase enzymes with relatively higher molecular weight are grouped into family F/10 and they are found with cellulose-binding domain and a catalytic domain that are usually connected by linker peptide. Enzyme belonging to this family also found to have a (β/α)-fold TIM barrel structure (Biely et al. 1997), whereas the family G/11 xylanase is generally of low molecular weight and is further

classified into two major groups based on their PI value. Similarly, β-xylosidases, another major xylolytic enzyme is grouped under GH classification system into various families such as 3, 39, 43, 52, and 54. Studies showed that β-xylosidases from the families 3, 39, 52, and 54 use a retaining mechanism to hydrolyze xylooligomers, while those from family 43 mediate the hydrolysis by inverting the anomeric configuration. Advanced bioinformatics tools such as Basic Local Alignment Search (BLAST) and pair-wise alignments of the protein sequences have been used to identify the xylanase enzymes and compared with closely related enzymes within the families. The sequences of family 10 xylanase are generally used to identify mutually exclusive enzymes using BLAST search. Further confirmations are also made using X-ray crystallographic studies. A continuous update to all these GH family classifications is provided by the carbohydrate-active enzyme database (CAZy) since there is a direct relationship between the protein folding and the sequence. This system is universally accepted as it can provide structural features of enzymes that help to predict their sole substrate specificity; it helps to identify and reveal the evolutionary relationships between the enzyme groups, and also act as a convenient tool to derive mechanistic information.

### *7.7.2 Carbohydrate-Binding Modules (CBMs)*

The X-ray crystallographic studies on the xylanase structure reveal that most of them have a modular structure composed of a catalytic site and one or more carbohydrate-binding modules (CBMs) that are interconnected by flexible linkages (Kulkarni et al. 1999; Subramaniyan and Prema 2002; Collins et al. 2005b). A CBM is defined as a specially arranged amino acid sequence with high affinity to carbohydrate molecules within a carbohydrate-active hydrolytic enzyme. These unique sites are designed to bind with specific polysaccharides on plant tissue and mediate the structural damage by enzymatic hydrolysis (Bolam et al. 2001; Boraston et al. 2002). Similar to the catalytic modules of glycoside hydrolases, CBMs are classified into 64 families according to their amino acid sequence similarity and this classification is available in CAZy database (Tomme et al. 1994). A study of Boraston and his group reported a detailed overview about the structure and functions of CBMs (Boraston et al. 2000). However, a very few members of GH11 xylanases carry CMB. Among these only a few of them have been experimentally demonstrated, but others are hypothetically explained based on their sequential similarities with well-studied ones. It have been also reported that certain CBMs from Xyl-11 are specific for cellulose and this characteristic feature helps the xylanase to localize the substrates indirectly even when they are in close association with cellulose. Major CBMs of known structure which are linked to xylanases are presented in Table 7.7. Xylanases isolated from fungi such as *Penicillium funiculosum* XynB (Brutus et al. 2004) and *Neocallimastix patriciarum* XynS20 (Liu et al. 2008) are found to have CMB1 modules. It was also reported that the

**Table 7.7** Sensitivity of GH11 xylanases to proteinaceous inhibitors (XIP-1, TAXI-1 and TLXI) (Gebruers et al. 2008)

| Xylanase source | XIP-1 | TAXI-1 | TLXI |
|---|---|---|---|
| Fungi | | | |
| *Penicillium purpurogenum* | − | + | − |
| *Fusarium graminearum* | + | − | − |
| *P. funiculosum* | + | + | + |
| *P. funiculosum* | + | + | + |
| *Trichoderma longibrachiatum* | + | + | − |
| *T. viride* | + | + | + |
| Bacteria | | | |
| *Bacillus agaradhaerens* | − | − | − |
| *B. subtilis* | − | + | − |
| *Fibrobacter succinogenes* | − | − | − |

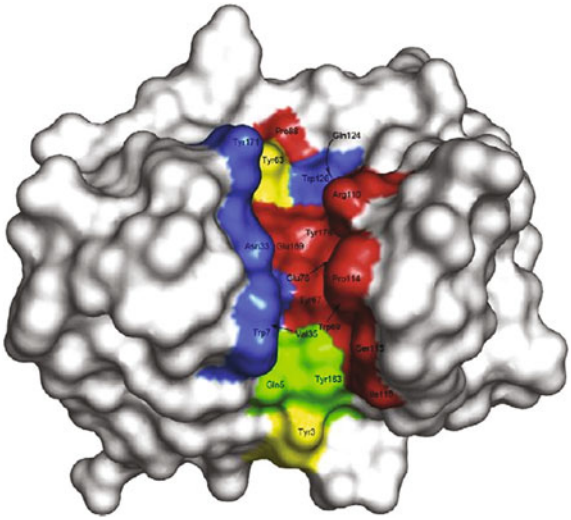

**Fig. 7.7** Spatial conservation of residues paving the catalytic cleft of Xyl-11 (Paës et al. 2012)

occurrence of a C-terminal CBM1 in Xyl-11 isolated from *Podospora anserina* found to have significantly improved ability to reduce the sugar during the hydrolysis of wheat straw (Couturier et al. 2010). Figure 7.7 shows the three-dimensional structure of NpXyn11A which belongs to GH11. Some GH11 xylanases, apart from the CBMs, also consist of dockerin domains that are key elements which help in the formation of cellulose-binding multi-enzyme complex known as cellulosomes. These enzyme complexes are originally observed in *Clostridium thermocellum* (Lamed et al. 1983). In addition to cellulolytic enzymes, cellulosomes are also found with hemicellulase such as xylosidases, xylanases, and arabinofuranosidases. Other research showed also the similar results in XynC isolated from *Fibrobacter succinogenes* S85 (Marrone et al. 2000).

### 7.7.3 Xylanase Enzyme Inhibitors

Almost all the plant species have developed a self-defense mechanism as response to the pathogenic microbial attack by hydrolases enzymes. Xylanase inhibition by the proteinaceous inhibitors is one among those defense response widely seen in both soft- and hardwood plants. A numerous studies have been carried out to understand this defense mechanism since 1990s and it was found that *Triticum aestivum* xylanase inhibitor (TAXI) and xylanase inhibitor protein (XIP) are the two major groups of proteins responsible for Xyl-11 in specific (Debyser et al. 1999; Rouau et al. 2006). Apart from the above two groups, the third group of inhibitor known as thaumatin-like xylanase inhibitor (TLXI) also been isolated from wheat. It was also found that none of these xylanase inhibitors are effective against plant origin xylanase enzymes (Dornez et al. 2010a, b). Occurrence of these inhibitors seems to be a significant hindrance in xylanase-mediated industrial process such as bread making and brewing as they inhibit effective hydrolysis of substrates (Sorensen and Sibbesen 2006). Therefore, it has been an important topic of research to understand the mode of action of these inhibitors. Sensitivity of GH11 xylanases to proteinaceous inhibitors (XIP-1, TAXI-1, and TLXI) are as given in Table 7.7 (Fig. 7.8).

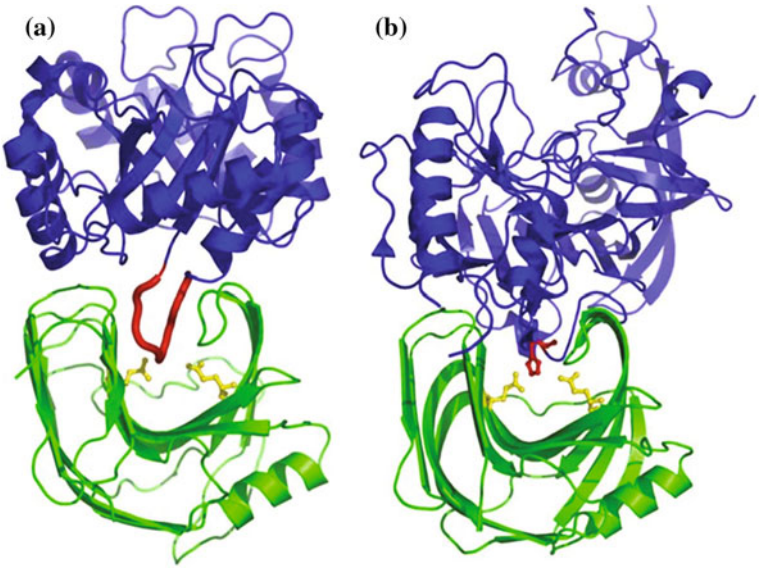


**Fig. 7.8** **a** Structure of the inhibition complex between *P. funiculosum* Xyl-11 (in *green*) and XIP-I inhibitor (in *blue*). XIP-I inhibiting loop is in *red*, catalytic residues are in *yellow*. **b** Structure of the inhibition complex between *B. subtilis* Xyl 11 (in *green*) and TAXI-IA inhibitor (in *blue*). His374 residue is in *red*, catalytic residues are in *yellow* (Paës et al. 2012)

#### 7.7.3.1 *Triticum Aestivum* Endo-xylanase Inhibitor (TAXI)

These are proteins with pI values of $\geq 8.0$, which occur in two different molecular forms known as A and B (Gebruers et al. 2008). Form A is seen as full-length proteins with molecular mass near to 40 kDa, whereas the form B consists of two polypeptide chains (a longer chain and a shorter chain of size 28–30 and 10–12 kDa, respectively) that bound together by disulfide bonds (Croes et al. 2009). These inhibitors are seen largely in wheat grains during the early stages of grain development and specifically inhibit GH11 xylanase disrespect to their origin (Tables 7.8, 7.9). Further analysis of originally identified TAXI inhibitor proteins revealed that it is a mixture of two different proteins of different pI values. They are named as TAXI-I and TAXI-II and have different inhibition activities on GH11 endo-xylanase isolated from *A. niger* (Gebruers et al. 2004). Other research carried out by Raedschelders et al. (2004) identified and isolated the genes coding for these two inhibitor proteins from ray and barley grains and based on this study Fierens et al. (2004) developed a *Pichia pastoris*-based expression system for the large-scale production. By 2005, two more new TAXI class named TAXI-III and TAXI-IV were also identified (Igawa et al. 2005). TAXI-type inhibitors are found to be very specific in their inhibitory characteristics. The available research data shows that they inhibit only GH11 endo-xylanases of bacterial and fungal origin. However, the subclass TAXI-II was turned to be an exception, as they are unable to inhibit GH11 endo-xylanases such as XynBc1 and ExlA isolated from *B. cinerea* and *A. niger*, respectively (Brutus et al. 2005).

**Table 7.8** Occurrence of TAXI-type inhibitors in different cereals (Raedschelders et al. 2004)

| Cereal | Inhibitor proteins | | |
|---|---|---|---|
| | TAXI-I | TAXI-II | Inhibitor level (ppm) |
| Wheat | + | + | ca. 38 |
| Durum wheat | + | + | ca. 11 |
| Rye | + | − | ca. 21–37 |
| Barley | + | − | ca. 4–5 |
| Maize, rice, oats, buckwheat | − | − | − |

**Table 7.9** Occurrence of XIP-type inhibitors in different cereals (Raedschelders et al. 2004)

| Cereal | Inhibitor proteins | |
|---|---|---|
| | XIP-I | Inhibitor level (ppm) |
| Wheat | + | ca. 80 |
| Durum wheat | + | ca. 14 |
| Rye | + | ca. 95 |
| Barley | + | ca. 3 |
| Maize | + | ca. 2 |
| Rice, oats, buckwheat | − | − |
| Sorghum | − | − |

#### 7.7.3.2 Endo-xylanase Inhibiting Proteins (XIP)

Basically, these are glycosylated single-chained proteins with molecular weight of 30 kDa and its pI value falls between 8.0 and 9.0. The crystallography study shows that it has similar structural features of GH18 with typical (α/β) 8-fold TIM barrel shape (Payan et al. 2003). However, they are found to be passive on GH18 enzymes such as chitinases due to the high degree of mismatch at its enzyme biding site. In general, these inhibitors consist of two independent enzyme-binding sites that mediate binding with almost all GH10 and GH11 endo-xylanases that are fungal origin. However, GH11 endo-xylanase from *Neocallimastix patriciarum* (Payan et al. 2004) and GH10 endo-xylanase from *Aspergillus aculeatus* (Flatman et al. 2002) are found to be insensitive to XIP. Similar to TAXI-type inhibitors, XIP-type inhibitors are also categorized into different polymorphic families based on the recent proteomics studies. Followed by identification of Xip-I (the most common inhibitor in the XIP) class, Durand et al. (2005) identified Xip-II class in *T. turgidum* ssp. In addition to this, Xip-III type inhibitor encoding gene was discovered in *T. aestivum* and was observed that these genes are transcribed very rarely under stressed leaves. Later on, several other XIP-type genes were also identified known as Xip-R genes, OsXip that are of plant origin (Takahashi-Ando et al. 2007). Biely et al. (2008) confirmed the existence of XIP-type inhibitors in maize leaves and roots during the first week after germination.

#### 7.7.3.3 Thaumatin-like Xylanase Inhibitor (TLXI)

Thaumatin-like endo-xylanase inhibitors (TLXI) consist of a single amino acid chain with molecular weight of 18 kDa. This type of inhibitor was first discovered in wheat by Fierens et al. (2007). These proteins have high pI and usually exhibit glycosylation at varying extents that found to have a very high stability (no denaturation was observed at 100 °C at extreme pH ranges even after 120 min). These inhibitors are grouped into class 5 pathogenesis-related proteins as they show very high sequential similarity with certain antifungal proteins (Trudel et al. 1998). Even though there are no crystallographic studies that have been reported on structure of TLXI inhibitor, the structure has been predicted and shows the existence of five disulfide bridges and three β-sheets (Fierens et al. 2009). The inhibitory mechanism of these proteins was found to be specific against Xyl-11. The xylanase inhibitors were recently reviewed by Dornez et al. (2010a, b).

## 7.8 Recombinant Endo-1,4-β-xylanase

The advances in molecular biology and genetic engineering opened up several new applications of recombinant DNA technology. The new opportunity for the construction of GM microbial strains with selected enzyme machinery was one of the

greatest breakthroughs. On the basis of this, more efficient endo-1,4-β-xylanase-producing microorganisms were constructed to improve the biodegradation of hemicellulosic residues and thus find many industrial applications. In this section, we will highlight in detail the recent advance in recombinant xylanase production. Moreover, studies on xylanase gene regulation and biosynthesis at molecular level and various tools applied to enhance the novel characteristic of xylanase enzymes will be discussed.

Several xylanases have been isolated and identified from various sources. However, very limited numbers of these enzymes are found to be suitable for industrial applications due to their poor performance in harsh process conditions. Poor thermostability is the biggest issue associated with most of the xylanases. Enzymes with higher thermostability can enhance the efficiency of industrial process with an increased residual time. Therefore, there has been a huge demand for thermostable xylanases in pulp and paper industry as they are applied during pulp bleaching process which usually carried out at temperatures more than 90 °C. Most of the xylanases we have today are not able to withstand this high temperature. However, highly thermophilic xylanases have been reported from thermophilic bacteria such as *G. thermoleovorans* and *Thermotoga* sp. (Sharma et al. 2007). Alkali stability is also another major problem associated with industrial applications. It was observed that most of the highly thermophilic xylanases are less stable under alkaline conditions which limit their potential use in industries. Most of the xylanases reported so far have optimal activity at either acidic or neutral pH (Wang et al. 2010). Exceptional cases are also reported, such as xylanases from *Bacillus halodurans* which exhibit high alkali tolerance (Mamo et al. 2006), but these enzymes are found to have poor thermostability. Cellulose-free xylanase is another desirable characteristics required in pulp bleaching industries to mediate efficient hydrolysis without disturbing the cellulosic content of the pulp. Studies also shows that low molecular weight xylanases are more efficient in pulp bleaching as they can easily diffuse into pulp fibers. In practical and economical point of view, growing these extremophilic microorganisms in large scale is not feasible. Moreover, it is difficult to have a single enzyme with all desired characteristics. Conventional enzyme stabilization techniques such as helix capping and salt bridging (Puchkaev et al. 2003) have been practiced to improve the enzyme characteristics. However, these methods are found to be ineffective when the enzyme is required in large quantities. To overcome these problems and enhance the large-scale production for the industrial application, recombinant xylanases were introduced using gene cloning and expression techniques. rDNA technology has been widely used nowadays to make alteration or modifications in existing wild-type xylanase to improve their specific characteristics such as thermostability and alkaline stability. A number of xylanase coding genes from different microbial species have been isolated and expressed successfully in suitable expression hosts. The expression level of xylanase gene in host cell usually depends on several factors such as cloning strategies used, host cell, biosynthesis mechanism, gene regulation, fermentation media, and many bioprocessing parameters. Bacterial and fungal species are generally used to express xylanases in large scale. The cloning vector selection

is usually carried out based on the expression host and a wide variety vectors have been introduced in last few decades. Even though the expression rate is higher in recombinant strain, the enzyme activity is usually less than in the native producer strain. Several reasons have been identified for this activity loss and the intercellular accumulation of the enzyme due to the lack of post-translational modification is suggested to be the key reason (Schlacher et al. 1996).

### 7.8.1 Cloning and Expression of Fungal Endo-1,4-β-xylanase Gene

It has been more than three decades since filamentous fungi are used in the production of xylanase enzyme. Fungal cells are the widely used biofactories in large-scale production of xylanases and have been considered as more potent xylanase producers as they are capable of secreting much higher amounts of xylanolytic enzymes in fermentation media than bacteria or yeast (Bergquist et al. 2002; Fang et al. 2008). Large numbers of fungal strains are widely used for xylanases production such as many species from *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, and *Disporotrichum.* However, in many of these fungi, the difficulty in isolating pure form of xylanase was the major problem since they generally exhibit a low amount of cellulase activity. Recombinant DNA technology has been widely applied to overcome this drawback and production of xylanase expression systems which are free of cellulase activity which improved its acceptability in industrial applications. Apart from the bacterial expression hosts, filamentous fungi are also attractive hosts for xylanase expression. It is because of their ability to secrete the proteins into the fermentation media in large quantities. *Aspergillus* and *Trichoderma* species are the widely used expression hosts in homolog expression system. Kitamoto et al. (1999) successfully overexpressed XynF1 gene in *A. oryzae* under a strong TEF1 promoter in glucose-based submerged fermentation. XynF1, XynF3, and XynG2 are another important xylanase genes expressed efficiently in *A. oryzae* under stronger promoter called P-No 8142 in similar fermentation conditions. *Aspergillus niger* is another attractive host for recombinant xylanases production due to its ability to produce and secrete protein in high capacity up to 30 g $L^{-1}$ in the fermentation media (Hessing et al. 1994). Xylanase coding gene from *T. reesei* was successfully expressed in *A. niger* cD15 stain under G6P-dehydrogenase promoter and glaA terminator which is isolated from *Aspergillus awamori* (Rose and van Zyl 2002). This recombinant xylanase retained its 75 % activity even after 3 h of incubation at 50 °C. The optimal condition of the enzyme activity was observed at temperature between 50 and 60 °C and pH 5–6. Levasseur et al. (2005) also reported on the potential use of *Aspergillus niger* as host to express xynB under promoter sequence isolated from *Aspergillus nidulans*. They showed that, *A. nidulans* can be used as efficient host for xylanase expression and production. Kimura et al. (1998) expressed xynG1 in *A. nidulans* under its own promoter showed that the xylanase expression was induced in the presence of

**Table 7.10** Major fungal xylanase expression system

| Source of xylanase gene | Expression host | Vector | MWt (kDa) | References |
|---|---|---|---|---|
| Heterologous expression host | | | | |
| *Aspergillus niger* | *P. pastoris* GS115 | pHIL-D2 | 30 | Berrin et al. (2000) |
| *A. usamii* E001 | *E. coli* BL21 | pET-28a (+) | 20 | Zhou et al. (2008a) |
| *Thermobifida fusca* NTU22 | *P. pastoris* KM71H | pPICZα A | 36 | Cheng et al. (2005) |
| *Cochliobolus sativus* | *E. coli* SOLR | Lambda ZAP | 25 | Emami and Hack (2001) |
| *A. niger* BCC14405 | *P. pastoris* KM71 | pPICZαA | 21 | Ruanglek et al. (2007) |
| *Aureobasidium pullulans* | *E. coli* | pYES2 | – | Ohta et al. (2001) |
| *Penicillium* sp. 40 | *E. coli* DH5α | pUC119 | 20.7 | Kimura et al. (2000) |
| *Trichoderma reesei* Rut C-30 | *E. coli* BL21 (DE3) | pET-28a | 24 | Jun et al. (2008) |
| *T. reesei* PC-3-7 | *E. coli* JM109 | pT7Blue-T | 33.1 | Ogasawara et al. (2006) |
| Homologs expression host | | | | |
| *A. oryzae* KBN 616 | *Aspergillus oryzae* KBN616 | pNAN8142 | 32 | Kimura et al. (2000) |
| *A. niger* BRFM281 | *Aspergillus niger* D15#26 | pAN52.3 | 23 | Levasseur et al. (2005) |
| *Acrophialophora nainiana* | *Trichoderma reesei* Rut C-30 | pHEN11 exp | 19 | Salles et al. (2007) |
| *Orpinomyces* sp. PC-2 | *Hypocrea jecorina* | pT3C | 28 | Li et al. (2007) |

glucose in the media. Filamentous fungi enable extracellular secretion of heterologous protein which facilitates the downstream processes and ease the purification and characterization of the produced enzyme. Moreover, most genes from fungi have introns and filamentous fungi are able to recognize them and express with minimal miscoding. In addition, fungal proteins are glycosylated and the protein expression in another filamentous fungi results in similar glycosylation pattern. For expressing highly hemophilic xylanase, many researches have been selected filamentous fungi because of above-mentioned advantages when compared to other expression hosts like bacteria. Table 7.10 shows major fungal xylanase expression system.

Yeast-based heterologous gene expression is considered as an excellent alternative system when based on their ease scaling up compared to fungi. The presence of eukaryotic post-translational modification mechanism is one of the main reasons behind the wide application of yeast expression systems in industry (Cheng et al. 2005). *S. cerevisiae* is one of the important hosts for xylanase production by cloning of xylanase cDNA obtained by RT-PCR through crossing over. The presence of intron sequence (lake of proper splicing of introns) that hinders the xylanase gene expression is the major disadvantage of these expression systems (Moreau et al.

1992). It has been also observed that a significant increase in xylanase expression in yeast transformed with the xynA gene without intron (26.2 U $mL^{-1}$) when compared to the expression from the gene with intron (16.7 U $mL^{-1}$) (Li and Ljungdahl 1996). It is also worthy to note that Chavez et al. (2004) made the first successful attempt to clone and express the xynA gene which is isolated from *P. purpurogenum* in *S. cerevisiae*. Isolated xynA gene that consists of eight introns regions was spliced correctly and was integrated on yeast chromosome. The Xln A gene was then expressed under transcriptional control (XlnR and CreA) where xylose or xylan acts as inducer and glucose acts as repressor (van Peij et al. 1997). The methanotropic yeast *Pichia pastoris* is another widely used xylanase expression host which has many advantages over *S. cerevisiae* such as improved secretion efficiency, ease to attain high cell density culture in inexpensive media, and relative ease in scaled up fermentation process (Cereghino and Cregg 2000). Moreover, the presence of strong and regulatory alcohol oxidase (*AOX I* and *AOX 11*) promoters which are involved in methanol metabolism promotes the overexpression of xylanase gene upon the addition of methanol in the fermentation media (Tsai and Huang 2008). Table 7.11 summarizes some examples of successful cloning of fungal xylanase in yeast.

### 7.8.2 Cloning and Expression of Bacterial Endo-1,4-β-xylanase Gene

La Grange et al. (2000) successfully co-expressed xylanase genes (xln B from *Bacillus pumilus* along with xyn2 isolated from *T. reesei*) in yeast *S. cerevisiae*. *E coli*-based expression systems are widely used to express the xylanase. Endo-1,4-β-xylanase coding genes isolated from *A. usamii* were cloned in *E. coli* BL-21 with the help of PET-28a plasmid vector and expressed successfully and a maximum xylanase activity of 49.4 U $mg^{-1}$ was reported. The gene expression was induced by IPTG and the protein was expressed with 6-X histidine tags that facilitate the purification of expressed protein (Zhou et al. 2008a). Similarly, for the xylanase gene from *B. circulans* Teri-42 when cloned in *E. coli* DH5-alpha using

**Table 7.11** Cloning of different fungal xylanase genes in yeast

| Xylanase source | Expression host | Vector | MWt (kDa) | References |
|---|---|---|---|---|
| *Aspergillus niger* | *P. pastoris* GS115 | pPIC9K | 20 | Liu et al. (2006) |
| *A. niger* | *P. pastoris* GS115 | pHIL-D2 | 30 | Berrin et al. (2000) |
| *A. niger* BCC14405 | *P. pastoris* KM71 | pPICZαA | 21 | Ruanglek et al. (2007) |
| *A. sulphureus* | *P. pastoris* X33 | pGAPZαA | – | Cao et al. (2007) |
| *Aureobasidium pullulans* | *P. pastoris* GS115 | pPIC3.5 | 39 | Tanaka et al. (2004) |
| *A. pullulans* | *S. cerevisiae* INVSc1 | pYES2 | – | Ohta et al. (2001) |
| *Thermobifida fusca* NTU22 | *P. pastoris* KM71H | pPICZα A | 36 | Cheng et al. (2005) |
| *T. fusca* | *P. pastoris* GS115 | pPIC9K | 31 | Sun et al. (2007) |

pUC19 plasmid vector, the xylanase expression was found to be reduced. However, when the same gene is expressed in *B. subtilis* using plasmid pBA7, 14-fold increase in expression was observed. Therefore, *Bacillus* became more attractive hosts compared to *E. coli* and thus used widely in heterogeneous protein production. *Bacillus* sp. used in industrial fermentation are non-pathogenic in nature and in bacillus-based expression, the proteins are usually secreted out of the cell. Moreover, they are gram-positive bacteria and therefore, they do not produce endotoxins (lipopolysaccharides). These features could be of great advantage by reducing some purification steps in terms of breaking the cells and extraction from large number of intracellular proteins.

### 7.8.3 Overexpression in Escherichia coli

For the past several decades, *E. coli* has been recognized as the ideal platform for expression of recombinant proteins. Factors such as rapid growth on inexpensive media, non-pathogenicity, easy to make gene modification without effecting the cell growth, easy methods required for isolation and purification of expressed proteins made *E. coli* the most successful expression host for industrial use. In spite of these advantages, due to the lake of specific post-translational modification such as glycosylation and disulfide bond formation, many heterologous proteins were not successfully expressed in *E. coli*. Xylanase was one of those which require N-glycosylation during post-translation stage, whereas *E. coli* only can perform O-glycosylation. However, many exceptional cases such as successful expression of a glycosylated β-xylosidase gene isolated from thermophilic fungi *P. thermophile* have been reported (Teng et al. 2011). It was also observed that the endo-1,4-β-xylanase expression levels are significantly higher in gram-positive hosts such as *Lactobacillus* species and *B. subtilis* compared to *E. coli* as they can perform N-glycosylation.

In general, the recombinant xylanases expressed in *E. coli* are accumulated in the cytoplasm or in periplasmic place in the absence of a proper secretory channel on cell wall (Schlacher et al. 1996). However, in many cases, xylanase activity has also been detected outside the cell (Karlsson et al. 1997; Ebanks et al. 2000). Xylanase expression rate in *E. coli* mainly depends on the efficiency of transcription of xylanase gene which is cloned under promoter sequence. Even though there are several cloning vectors and host strains have been introduced, eukaryotic gene expression in *E. coli* is unable to carryout due to the absence of a functional promoter sequence. Basaran et al. (2001) isolated β-xylanase gene from *Pichia stipites* and successfully cloned and expressed in *E. coli*. The recombinant enzyme activity was low (4 U $mg^{-1}$) when compared to the activity original enzyme activity (30 U $mg^{-1}$) in wild stain. Although a variety of cloning vectors have been used, pET expression vector systems were reported to be the most efficient for protein expression in *E. coli*. Xylanase genes isolated from different sources are cloned under the control of a strong T7 promoter which is isolated from bacteriophage genome. This promoter

**Table 7.12** Major cloning vectors used in *E.coli*-based expression system

| Source of xylanase gene | Cloning vector | References |
|---|---|---|
| Bacteria | | |
| *AeromoA75:C93nas* sp. *212* | pBR 322 | Kudo et al. (1985) |
| *Bacillus circulans* | pUC 19 | Yang et al. (1988) |
| *B. polymyxa* | pUC 13, pBR 322 | Yang et al. (1988) |
| *B. ruminicola* | pUC 18 | Whitehead and Hespell (1989) |
| *Butyrivibrio fibrosolvens* | pUC 19 | Mannarelli et al. (1990) |
| *Caldocellum saccharolyticum* | λ1059, pBR 322 | Luthi et al. (1990) |
| *Cellulomonas* sp. | pUC 18 | Bhalerao et al. (1990) |
| *Chainia* sp. | pUC 8, λgt 10 | Chauthaiwale and Deshpande (1992) |
| *Clostridium stercorarium* F9 | pBR 322 | Sakka et al. (1991) |
| *C. thermocellum* | pUC 8 | Hu et al. (1991) |
| *Neocallimastix patriciarum* | λZAP II | Lee et al. (1993) |
| Fungi | | |
| *Aspergillus oryzae* KBN 616 | pNAN-d | Kimura et al. (2000) |
| *A. usamii* E001 | pET-28a(+) | Zhou et al. (2008b) |
| *Aureobasidium pullulans* Y-2311-1 | pCRII, λ ZapII | Li and Ljungdahl (1996) |
| *Cochliobolus sativus* | λ ZAP | Emami and Hack (2001) |
| *Helminthosporium turcicum* H-2 | pBluescript SK(+) | Degefu et al. (2004) |
| *Neocallimastix patriciarum* | pBTac2 | Xue et al. (1992) |
| *Penicillium* sp. 40 | pUC119 | Kimura et al. (2000) |
| *Trichoderma reesei* Rut C-30 | pET-28a | Jun et al. (2008) |
| *T. reesei* PC-3-7 | pT7Blue-T | Ogasawara et al. (2006) |
| *T. reesei* C30 | pGEM5Z(+) | Torronen et al. (1994) |

remains silent until T7 RNA polymerase is produced by the host cell genome. Table 7.12 shows the major cloning vectors used in *E. coli*-based expression system.

Ogasawara et al. (2006) amplified xynIII gene from *T. reesei* genomic DNA using a RT-PCR and was cloned and expressed in *E. coli* JM109 using pAG9-3 plasmid vector. When the xylanase gene expression was induced by IPTG, they could produce 26 mU $mL^{-1}$ of xylanase activity in cellular extract, which was very low. However, the XynIII activity increased by about 500-fold (13.2 U $mL^{-1}$) compared to the activity in soluble supernatant, when the xylanase inclusion bodies were refolded in 8 mM urea solution. Zhou et al. (2008a) reported the maximum xylanase activity up to 49.4 U $mg^{-1}$ when they cloned and expressed xynII cDNA isolated from *A. usamii* in *E. coli* BL21-Codon plus (DE3) RIL strain using pET-28a (+) expression vector. The xylanase protein expressed is attached to 6X-His-tag which ease protein purification by affinity chromatography. Jun et al. (2008) also used the same pET vector to clone and express a β-xylanase gene, xyn2, isolated from *T. reesei* in *E. coli* and reported the enhanced xylanase activity of 650 U $mg^{-1}$. In some cases, the heterologous cloning and expression results in the

change in original characteristics of xylanase gene. For example, a non-glycosylated XynA gene when expressed in *E. coli* exhibited significant loss in its substrate specificity when compared to the glycosylated xylanase from the wild strain *P. stipites*. The stability of the enzyme also decreased due to the change in optimum temperature rage. However, improved thermostability and pH stability have been reported when Xyn2 from *T. reesei* was expressed in *E. coli*. Table 7.13 shows the properties of major endo-β-1,4-xylanase enzyme expressed in *E. coli.*

**Table 7.13** Properties of major endo-β-1,4-xylanase enzyme expressed in *E. coli*

| Source of xylanase gene | MWt (kDa) | Optimum pH | Optimum temperature (°C) | References |
|---|---|---|---|---|
| *Geobacillus thermoleovorans* | 45 | 8.5 | 80 | Verma and Satyanarayana (2012) |
| *Paenibacillus macerans* IIPSP3 | 205 | 4.5 | 60 | Dheeran et al. (2012) |
| *Thermoanaerobacterium saccharolyticum* NTOU1 | 50 | 6.5 | 63 | Hung et al. (2011) |
| *Clostridium* sp. TCW1 | 53, 60 | 6 | 75 | Lo et al. (2010) |
| *Actinomadura* sp. S14 | 21, 30 and 40 | 6 | 80 | Sriyapai et al. (2011) |
| *Alicyclobacillus* sp. A4 | 42.5 | 7 | 55 | Bai et al. (2010) |
| *Anoxybacillus* sp. E2 | 38.8 | 7.8 | 65 | Wang et al. (2010) |
| *Nesterenkonia xinjiangensis* CCTCC AA001025 | 22 | 7 | 55 | Kui et al. (2010) |
| *Bacillus* sp. JB 99 | 20 | 8 | 70 | Shrinivas et al. (2010) |
| *Paenibacillus* sp. DG-22 | 20 | 6 | 60 | Lee et al. (2007) |
| *Bacillus licheniformis* 77-2 | 40 | 8 | 75 | Damiano et al. (2003) |
| *Enterobacter* sp. MTCC-5112 | 43 | 9 | 100 | Khandeparkar and Bhosle (2006) |
| *Geobacillus* sp. MT-1 | 36 | 7 | 70 | Wu et al. (2006) |
| *Bacillus* sp. | 44 | 6.5, 8.5 and 10.5 | 50 | Sapre et al. (2005) |
| *Bacillus thermantarcticus* | 45 | 5, 6 | 80 | Lama et al. (2004) |
| *Bacillus thermoleovorans* | | | | |
| K-3d | 40, 69 | 7 | 70, 80 | Sunna et al. (1997) |
| *Bacillus flavothermus* LB3A | 80–130 | 7 | 70 | Sunna et al. (1997) |
| *Thermotoga* sp. FjSS3-B.1 | 31 | 5.5 | 80 | Simpson et al. (1991) |

## 7.9 Improvement of Endo-1,4-β-xylanase Characteristics

In general, enzymes are classified based on their conserved regions on them. Xylanases are specific in their identity due to the random variations in amino acid sequence and it was found that some very specific stretches of amino acids defines the structural and functional behavior of enzyme. Existing xylanases can be improved in terms of their activities and other novel characteristics such as substrate specificity, thermostability, and pH stability using protein engineering approaches. Critical amino acid exchanges are usually practiced in order to enhance the characteristics of existing xylanase enzymes. Therefore, before amino acid sequence modification a detailed studies on the native xylanase amino acid sequences are required to fully understand the structural and functional behaviors such as the presence of catalytic domains, specificity of substrate-binding sites, occurrence of thermo-stabilizing domains, and the presence of covalent/non-covalent interactions. The significance of a particular acid or a set of amino acids for novel enzyme characteristics can be identified easily using specific amino acid inhibitors such as *N*-bromosuccinimide (tryptophan), phenylmethylsulfonyl fluoride (serine), and iodo acetate (cysteine). It was also found that cysteine plays critical role in the formation of covalent glycosyl enzyme intermediate during most bacterial xylanase activity. Many of the family 10 xylanases possess three different amino acids such as Phe4, Trp6, and Tyr 343. They were identified as crucial in protein folding and enzyme stability even under extreme conditions. Torronen and Rouvinen (1997) studied the significance of asparagines and aspartic acid on the pH optima of family 11 xylanases. The studies conducted on xylanase isolated from *B. circulans* revealed that the presence of asparagine at 52nd position of amino acid sequence increased the thermal fluctuations at unstable regions. However, when it is substituted with tyrosine, the overall thermostability of the enzyme exhibited significant increased (Joo et al. 2011). It was also reported that the catalytic activity of *B. circulans* xylanase increased when the Asn at position 35 is mutated to Asp (Li and Wang 2011). Other study showed also a significant increase in thermostability (10–15 °C) as a result of point mutation (aspartic acid to asparagine) at position 56 (Yin et al. 2010). However, in many other cases, the point mutation was reported to have negative effects such as loss in substrate-binding affinity, alkaline stability, and thermostability. Simpson et al. (1999) reported the complete loss of carbohydrate-binding module affinity toward the xylan when the arginine was mutated to glycine. In case of *B. circulans* xylanase, change in pH optima was reported when R73V and Q167M were mutated (Yang et al. 2008). Table 7.14 comprises significant achievements made in xylanase protein engineering to increase enzyme stability.

**Table 7.14** Significant achievements made in xylanase protein engineering (Verma and Satyanarayana 2012)

| Xylanase source | Approaches/alterations | Significant inferences | References |
|---|---|---|---|
| *Bacillus* sp. NG-27 | Deletion of Phe4 and Trp6 | Complete loss in activity, while Trp6 and Tyr343 affected folding | Bhardwaj et al. (2010) |
| *B. circulans* | Amino acid modification (N52Y) | Slight increase in half-life and Tm as compared to wild type | Joo et al. (2011) |
| *B. circulans* | Asn35Asp | Improvement in catalytic activity | Li and Wang (2011) |
| *N. patriciarum* | D57N | Enhancement in thermostability by 10–15 °C | You et al. (2010) |
| *G. stearothermophilus* | Substitution with N-terminal of *T. fusca* | Significant improvement in thermostability | Sun et al. (2007) |
| *T. reesei* | Improvement of C-terminal packaging | Half-life of the mutant xylanase was increased by 63 min | Turunen et al. (2001) |
| *T. lanuginosus* DSM5726 | Introduction of disulfide bond between Cys100 and Cys154 | Improvement in thermostability | Gruber et al. (1998) |
| *B. stearothermophilus* No. 236 | Introduction of between Ser and Cys) 100 and (Asn to Cys) 150 | Enhancement in thermostability by 5 °C | Jeong et al. (2007) |
| *A. niger* BCC144505 | Substitution of arginine in place of Ser/Thr | Enhancement in the thermostability | Sriprang et al. (2006) |
| *Thermobifida fusca* | Error prone PCR to obtain a mutant having T21A, G25P, V87P, I91T, and G217L | Improved stability at alkaline pH, 4.5-fold increase in Km and 12-fold enhancement in Kcat/Km | Wang and Xia (2008) |
| *Thermomyces lanuginosus* | Error prone PCR A54T | Alkali-stable mutant | Stephens et al. (2009) |

## 7.10 Industrial Production of Endo-1,4-β-xylanase

Although many microbial species such as bacteria, fungi, and actinomycetes have been reported as xylanase producers, a few number of strains are acceptable by the industrial society. To meet the current and forecasting market demands, xylanase production need to be increased by many folds through efficient production

**Table 7.15** Major commercial xylanases produced by SmF techniques (Polizeli et al. 2005)

| Commercial name | Distributors | Microorganism | Application |
|---|---|---|---|
| Allzym PT | Alltech | *Aspergillus niger* | Animal feed improvement |
| Bio-Feed Plus | Novo Nordisk | *Humicola insolens* | Animal feed |
| EcopulpX-200 | Primalco | *Trichoderma reesei* | Cellulose pulp bleaching |
| Ecosane | Biotec | *Trichoderma reesei* | Animal feed |
| Grindazym GP, GV | Danisco ingredients | *A. niger* | Bird and pig feed |
| Irgazyme 40 | Nalco-Genencor, Ciba, Geigy | *T. longibrachiatum* | Paper industry and animal feed |
| Solvay pentonase | Solvay Enzymes | *T. reesei* | Starch and bread making |
| Xylanase | Seikagaku | *Trichoderma* sp. | Carbohydrate structural studies |
| Xylanase GS35 | Iogen | *T. reesei* | Pulp bleaching |

strategies. Therefore, optimizations of production and purification processes are the key to obtain maximum xylanase yield either by solid-state fermentation (SSF) or submerged fermentation (SmF) (Narang and Satyanarayana 2001). Nowadays, the most of commercial xylanases production processes are carried out by SmF as shown in Table 7.15. Cultivation media plays important role in the production of xylanases in industrial scale. A suitable and economically viable media formulation which supports the maximum xylanase production is usually required for large-scale production. Therefore, abundantly available natural agriculture residues are widely used for xylanase production. Agricultural wastes such as corn cob, con stover, and wheat straw were successfully used as suitable substrates for fungal xylanases production (Damiano et al. 2003; Gupta et al. 2000).

### *7.10.1 Physical and Nutritional Parameters Affecting Endo-1,4-β-xylanase Production*

In SSF, biomass and xylanase productions depend on several factors such as the type of microorganism, inoculums size, temperature, oxygen diffusion, the period of cultivation, the substrate used and its pretreatment, size, water activity, relative humidity, pH, and many other factors. On the other hand, for SmF, the temperature, pH, agitation, and dissolved $O_2$ are the most critical physical parameters affecting xylanase production in SmF. Most of the xylanase-producing microorganisms reported so far are either mesophilic or thermophilic (Coughlan and Hazlewood

1993). It was observed that these microbes show highest xylanase activity and optimum growth at temperature between 40 and 70 °C. However, those extremophilic such as *Thermotoga* sp., *Dictyoglomus*, and *Geobacillus* exhibit even higher optimum temperature range of up to 80 °C (Ko et al. 2011; Sharma et al. 2007; Qureshy et al. 2002). Xylanases of fungal origin usually are found less thermophilic in nature. Even though some exceptional cases have been reported, the optimum temperature for most fungal xylanases is below 55 °C. This has been one of the major drawbacks of fungal xylanase in industrial application. On the other hand, bacterial xylanases exhibit higher thermostability. The pH of the fermentation media is another major factor effecting the xylanase production as well as the enzyme stability. Fungal xylanases usually are highly alkaliphilic as they can be active at wide ranges of pH, usually between 5.0 and 10.0, whereas the bacterial xylanases have quite narrow pH spectrum (Dhillon et al. 2000). Aeration and agitation rates during fermentation are the other crucial factors in case of xylanase production process. Studies show that xylanase production by *Bacillus* sp. is enhanced at an agitation rate of 200–250 rpm and 30 % and above dissolved $O_2$ saturation (Anuradha et al. 2007; Sanghi et al. 2009). In cretin fungal species which exhibit mycelia growth pattern, higher agitation rate results in poor growth as well as less xylanase production due to the high sheer force. Studies made by Archana and Satyanarayana (1998) and Sharma et al. (2007) deal with the optimization of physical parameters effecting xylanase production.

In addition, carbon and nitrogen sources and their concentration in the fermentation media are found to be not only effecting the xylanase production but also the localization of enzymes. The fungal xylanase producers are able to utilize wide verity lignocellulosic-derived substrates such as corn cob, corn straw, wheat bran, rice straw, and sugarcane bagasse. For production in SSF system, these substrates are used as they are cheaper and easily available raw materials (Chauhan et al. 2007). However, in bacterial cells in SmF, simpler and readily utilizable nutrients are used. Organic nitrogen sources such as tryptone, peptone, yeast extract, and soybean meal are widely used as they enhance the xylanase production as well as biomass. Kapoor et al. (2008) observed a significant increase in xylanase production which was observed when the *Bacillus* sp. SSP-34 was provided with yeast extract in combination with peptone. In similar study, xylanase production by *Bacillus sam*-3 was increased up to 25-folds higher when the inorganic nitrogen source was replaced by soybean meal. Use of corn steep powder also significantly enhanced xylanase production by *T. reesei* (Lappalainen et al. 2000). Apart from these major nutrients, the incorporation of micronutrients such as trace elements, vitamins, and amino acids improved the xylanase production to great extend in cases of thermo-anaerobe-mediated xylanase fermentation. In cases of recombinant strains, the xylanase expression greatly depends on the gene inducers present in the fermentation media. IPTG is the most widely used chemical inducer for '*lac*'-based expression system (Mamo et al. 2007). However, in large-scale production, IPTG is not applicable due to its high cost.

In cases of recombinant xylanase, the expression of xylanase genes usually depends on several aspects such as biosynthesis mechanism, gene regulation, vector

construction, expression host used, expression media, and other growth parameters during the fermentation. For commercial applications, xylanases have to be ideally produced quickly and in large quantities. The conventional production strategies have been followed are found to be less effective to meet the growing need in the present xylanase market. Therefore, many researches are going on for developing recombinant strains for xylanase production. However, a very limited number of these recombinant strains are studied further for the scaling up of xylanase production in pilot scale. Hence, more investigations on this area are required and it is very significant to have more efficient and economically viable process to reduce the cost of xylanase production.

### 7.10.2 Optimization of Endo-1,4-β-xylanase Production

Media screening and medium composition optimization are usually the preliminary steps involved in bioprocess optimization which are always carried out in shake flask level to select the most suitable medium formulation. Both physical and chemical parameters effecting the cell growth as well as xylanases production are monitored and optimized at this stage with a number of experiments. Basar et al. (2010) observed that the recombinant xylanase production in *E. coli* is a growth-associated process. They observed a rapid growth of *E. coli*, reaching the stationary phase after 16 h and the maximal xylanase production up to 324.72 U $mL^{-1}$ was obtained in defined medium. They also reported the effects of different concentrations of glucose in growth media on cell growth and xylanase production. Farliahati et al. (2009) reported the xylanase expression in recombinant *E. coli* DH5α in different media compositions (defined, semi-defined, and complex). Optimization study of pH and agitation ranges also carried out in shake flask cultivation mode. From these studies, they observed the highest xylanase production (up to 2122 U $mL^{-1}$) in defined media at initial pH 7 and agitation speed of 200 rpm. A suitable cultivation strategy for the production of two truncated thermostable recombinant xylanases (XynlAN and XynlANC), isolated from *Rhodothermus marinas*, was developed by Karlsson et al. (1998). They found that the fed-batch cultivations of *E. coli* strain BL21 (DE3) with a controlled exponential glucose feed led to high specific production of the recombinant proteins and addition of complex nutrients such as Tryptone Soya Broth (TSB) to the media exhibits positive impact on cell growth rate and cell productivity. Single addition of either lactose or isopropyl-thio-go-galactoside (IPTG) was used for induction. In lactose-induced cells, the production of recombinant xylanase was delayed for approximately 30 min compared to those induced with IPTG, but the specific product levels were comparable at 3 h after induction. Huang et al. (2006) cloned a xylanase gene from *B. subtilis* into *E. coli* and found that the xylanase distributions in extracellular, intracellular, and periplasmic fractions were in ratio 22.4, 28.0, and 49.6 %, respectively, with an optimal enzyme production at pH 6.0 and 50 °C. The

superiority of defined medium for xylanase production using recombinant bacteria was also reported by Farliahati et al. (2009).

Catabolic repression of the xylanases biosynthesis is found to be a common phenomenon usually in the presence of glucose in culture medium. When glucose is abundant in the grown medium, the repression of catabolic enzyme synthesis takes place due to the gene regulation. When the microbe can easily absorb the readily available glucose in the medium, they will not utilize for other complex nutrients by spending energy to metabolize complex nutrients. Xylanase production reported to be reduced due to the catabolic repression that makes the xylanase coding gene in inactive mode. In another study by Basar et al. (2010) they enhanced xylanase production using recombinant *E. coli* by optimizing the media composition and other growth factors. Two basal mediums (defined media and complex media) have been used and found out that the defined media gives the highest growth as well as the xylanase production under experimental conditions. At 20 % enhanced dissolved oxygen tension (DOT) in lab scale bioreactor, they increased xylanase production up to 1784 $mL^{-1}$.

### *7.10.3 Application of Response Surface Methodology (RSM) in Optimization of Xylanase Production*

The conventional media optimization method based on step-by-step process varying one variable at a time (OFAT) while keeping the other variables constant is a time-consuming process and less reliable in many cases. To overcome this problem, statistical approach using experimental designs was applied. Response surface methodology (RSM) is the most reliable statistical application that has been used for optimization of fermentation media. Optimizations of physiological and nutritional growth factors are found to be very important procedures to reduce the production cost of xylanases in industrial scale. Many researches have been carried out based on RSM which used to understand and evaluate the significant interaction between the physiological and nutritional factors effecting the cell growth and product formation. A prior knowledge about these growth factors and their interactions is necessary for the designing of a more realistic model. Hence, many of the RSM studies are found to be based on the results obtained from OFAT method. Selection of these growth parameters has been done by checking their significance level using a 2FFD Pareto chart before proposing an experimental design. Most significant factors were then used as variables in central composite design (CCD) (Farliahati et al. 2010). The effect of various growth parameters in production of xylanase enzyme was studied by Mullai et al. (2010). In his study, statistical experimental designs (CCD) were constructed with the help of statistical application MINITAB 14. Plackett–Burman (PB) design for seven different variables, $NH_4NO_3$, $KH_2PO_4$, $CaCl_2$, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}7H_2O$, and NaCl at two levels +1 and −1 (higher and lower level, respectively) and the

statistical significance were studied by determining the *F*-value. The optimum levels of selected variables from the Plackett–Burman design were investigated using central composite design (CCD). The response was measured in terms of xylanase activity. Counter plots and response surface 3D graphical representations generated could show the interactions of different variables under experimental conditions. Garai and Kumar (2012) optimized the xylanase production by *A. candidus* in SSF. They studied the effect of various nitrogen sources in the production media and they found that xylanase production was enhanced when the ammonium nitrate was used as sole nitrogen source. In the same study, applying RSM-based BoxBehnken design, the researcher also optimized the physical parameters such as time of incubation, temperature, and moisture content which effects xylanase activity. The crude xylanase recovered at the end of solid-state fermentation was used for the scarification of ammonia-treated lignocellulose materials to study the substrate-binding assay. Among the various substrates used, corn comb was found releasing the maximum reduced sugar (438.47 mg $g^{-1}$) when it was treated with crude xylanase enzyme. In RSM-based optimization, xylanase production was optimized by Box–Behnken design. Initially, Planket–Berman design was employed to identify the key ingredients and process conditions which exhibit the most significant effect on the xylanase expression. In the later stage, using Box–Behnken design, the optimal level of the identified key ingredients in production media as well as the optimum range of process parameters such as cultivation time, pH, temperature, and agitation are identified. Using this approach 6.83-fold increase in xylanase production was obtained under optimized conditions when compared to that of initial un-optimized conditions.

Solid-state fermentation (SSF) is also widely used for large-scale production of xylanase enzymes. It is basically designed to mimic the natural habitat of microbes that are less likely to grow in submerged fermentation (SMF) conditions. In SSF, the solid bed is used as nutrient source as well as the mechanical support for cell growth. *A. niger*, *T. reesei*, and *T. koningii* are the major fungal species used to produce xylanase under SSF conditions (Dhiman et al. 2008; Garg et al. 1998). However, xylanase production by bacteria is commonly carried out in submerged fermentation conditions where the physical parameters such as temperature, pH, and dissolved $O_2$ level involved are controlled to the optimum levels with more ease, and therefore an enhanced production can be achieved. Esteban et al. (1982) reported that the large-scale production of xylanase in SmF is the growth-associated process. However, several exceptional cases were also been reported such as xylanase production using *Bacillus* sp. (Dey et al. 1992). Later study by Bocchini et al. (2002) reported the production of highly thermostable cellulase-free xylanase in SMF using *B. circulans.* Similar study was also carried out by Sharma et al. (2007) who used *Geobacillus thermoleovorans* AP7 to produce highly thermo-alkali-stable xylanase. The switch to xylanase-producing recombinant *E. coli* is seen as an economic strategy toward higher productivity and easier downstream purification. Sandhu and Kennedy (1984) reported a large-scale production of xylanase by transforming *E. coli* with recombinant plasmids carrying a 1,4-β,D-xylanase gene from *B. polymyxa.* In another study, xylanase gene isolated

from *A. usmani* was cloned in pET-28a expression vector and when it is expressed in *E. coli* BL21, the maximum xylanase activity was found to be 49.4 U $mg^{-1}$. The gene expression was induced by IPTG and the xylanase was found expressed with 6-X histidine tag that facilitates an easy purification of expressed protein (Zhou et al. 2008b). A very successful cultivation strategy for the production of two truncated thermostable recombinant xylanases (XynIAN and XynIANC), isolated from *Rhodothermus marinas*, was developed by Karlsson et al. (1999). They found that the fed-batch cultivations of *E. coli* strain BL21 (DE3) with a controlled exponential glucose feed led to high specific production of the recombinant proteins with additional complex medium. Highest xylanase production in defined medium was achieved by Farliahati et al. (2009). In this study, xylanase production reached up to 2122.5 U $mL^{-1}$ at pH 7.4 and when $(NH_4)_2HPO_4$ was used as the main nitrogen source. They also reported that the xylanase production was a growth-associated process as the enzyme secretion was greatly dependent on biomass concentration for strain *E. coli* DH5α.

Biswas et al. (2010) used mutant IITD3A of *Melanocarpus albomyces* for the production of high titer cellulose-free xylanase in 14-L stirred tank bioreactor where an optimized (RSM based) production media and process parameters are used. The authors reported the xylanase activity reached up to 415 U $mL^{-1}$ after 24 h by pH recycling strategy (between 7.8 and 8.2) during the production phase. They also reported that the xylanase production is significantly influenced by aeration and agitation. A 5.2-fold increase (overall volumetric productivity of 22,000 U $L^{-1}$ $h^{-1}$) in activity was observed when the cells exhibited pellet form at an agitation speed of 600 RPM.

Kumar et al. (2009) optimized the xylanase production in lab scale stirred tank bioreactor using mycelial fungal strain *T. lanuginosus* MC 134. Fermentation media contains corn cob as a sole source of carbon which was inoculated with *T. lanuginosus* MC 134 and observed the production in terms of xylanase activity over a period of 9 days at various process parameters. A highest xylanase activity, 2009 U $mL^{-1}$, was obtained after 6 days of fermentation. However, a significant decrease (40 %) in productivity was observed in bioreactors when compared to shake flask culture. Similarly, xylanase production by several microbial strains using corn cob was reported and from these studies it was clear that the production largely depends on rheological factors in bioreactors and the type of extraction methods used. Ruanglek et al. (2007) successfully cloned and expressed XylB (564 bp) encoding endo-1,4-β-xylanase obtained from *A. niger* BCC14405 in *Pichia pastoris* under AOX1 promoter. A maximum of 7352 U $mg^{-1}$ of xylanase-specific activity was obtained in 2 L lab scale bioreactor with BMGY/BSM medium under exponential feeding strategy. During the batch phase which was aimed to achieve HCDC, the temperature and pH were maintained at 30 °C and 5.0, respectively. Agitation was maintained in the range of 600–1000 rpm. Fed-batch phase was started after 16–20 h by feeding 67 % (v/v) glycerol plus 1 % (v/v) trace mineral solution in exponential manner. Table 7.16 shows examples for xylanase production in lab and pilot-scale submerged fermentation by different fungal strains.

**Table 7.16** A comparison of xylanase production in lab and pilot-scale submerged fermentation using fungal strains

| Name of the organism | Substrate used | Mode of operation | Xylanase activity (IU $L^{-1}$) | Productivity (IU $L^{-1}$ $h^{-1}$) | References |
|---|---|---|---|---|---|
| *Aspergillus niger* | Xylan (from corncob) | Batch (20 L) | 290,000 | 3820 | Yuan et al. (2005) |
| *Aspergillus oryzae* | Spent sulfite liquor | Batch (15 L) | 199,000 | 13,100 | Chipeta et al. (2008) |
| *Thermomyces lanuginosus DSM 10635* | Oat husk hydrolysate | Batch (2 L) | 210,000 | 4380 | Xiong et al. (2004) |
| *Thermomyces lanuginosus MC 134* | Coarse corncob | Batch (5 L) | 2,010,000 | 13,950 | Kumar et al. (2009) |
| *Trichoderma reesei* | Oat husk hydrolysate | Fed-batch (5 L) | 1,350,000 | 14,060 | Xiong et al. (2005) |
| *Melanocarpus albomyces* | Soluble wheat straw extract | Batch with pH cycling (14 L) | 550,000 | 22,000 | Biswas et al. (2010) |

### 7.10.4 pH Optima and Stability of Endo-1,4-β-xylanase Enzymes

In general, endo-1,4-β-xylanases are active at wide pH ranges and their pH optima depend on the producer organism and the physiochemical properties of the substrate molecules. Fungal xylanases are usually acidophilic in nature. However, the optimal pH for bacterial xylanases is usually above 5.5. Endo-1,4-β-xylanase from *A. kawachii* reported as the most acidophilic xylanase which has a very low pH optimum of 2. On the other hand, xylanase from *Bacillus* sp. 41 M1 reported as the most alkalophilic xylanase which has pH optimum 9.0 (Nakamura et al. 1993). In certain cases, different pH optimums were observed in different xylanases secreted by same organism. For example, XynI and XynII produced by *H. jecorina* were reported to have pH optimums 3–4 and 5–5.5, respectively. This showed that the pI values of the enzymes are correlated with the amino acid residues at various positions on xylanase. However, among Xyl-11 family, those are acidophilic in nature possess an Asp residues at position 33 even though there are very rare exceptions such as *A. kawachii* IFO 4308 xylanase which has pH optimum close to 5.0 (Zhu et al. 1994). However, many alkalophilic xylanases such as Xyn11 from *A. versicolor* (Carmona et al. 1998), XynC from *Uccinogenes* sp. (Zhu et al. 1994) also displayed an Asp at the same position. Similarly, studies on *P. griseofulvum* Xyl-11 reported that it displays a Ser residue at position 44 and mutating this

residue resulted significant changes in pH optimum of the enzyme (Andre-Leroux et al. 2008).

There have been many different explanations for the factors determining the optimal pH value of xylanases. However, it believes that certain amino acid residues of xylanase structure play key role in the determination of the optimal pH value of the enzyme. In case of acidic pH optima, the Asp/Glu residues near the catalytic cleft become protonated and it decreases the electrostatic repulsion between the substrate and enzyme, whereas in alkaline xylanases, instead of acidic residues more alkaline residues such as arginine are seen. de Lemos Esteves et al. (2005) experimented various mutations in *Streptomyces* sp. S38, and observed a pH optimum shift from 6.0 to 7.5, which enhanced the xylanase enzyme activity several folds higher than the enzyme from the wild type. The pI values of catalytic domain present on xylanase play a key role in its pH activity profile. Many recent studies reveals that, among GH11 xylanase, the residues those contribute positive charges and hydrogen bonds to the catalytic domain help in increasing or decreasing the pI values depending upon the electrostatic interaction between the substrate molecules (Joshi et al. 1997). Apart from these residues, factors such as occurrence of salt bridges are also reported to have significant effects on pH optima (Hakulinen et al. 1998).

### *7.10.5 Endo-1,4-β-xylanase of Extremophilic Origin*

Considerable progress has been made in the isolation of extremophilic microorganisms and their successful cultivation in the laboratory. Commercial applications of the xylanases demand highly thermostable enzyme which can work effectively under elevated temperature. Many advantages such as reduced contamination risk and faster reaction rates have been proposed for the use of thermophiles in biotechnology processes. In general, parameters such as temperature, pH, and chemical and enzymatic stability are important for the industrial applicability of any enzyme. Recently, a simple model, based on the thermodynamic and kinetic parameters for inactivation of the enzyme, has been presented for predicting (1) thermostabilities in the presence of substrate and (2) residual activities of enzymes in harsh processing environments. The study of extremophiles and their enzymes can extend the present understanding of protein chemistry in addition to expanding the potential applications of biocatalysts. Studies of alkaliphiles have led to the discovery of a variety of enzymes which exhibit some unique properties. Biological detergents contain enzymes such as alkaline proteases and/or alkaline cellulases from alkaliphiles. Commercial production became economical only after the discovery of alkaliphilic *Bacillus*-producing CGT with enhanced pH stability. Alkaline xylanases have gained importance due to their application for the development of eco-friendly technologies used in paper and pulp industries. The enzymes are able to hydrolyze xylan which is soluble in alkaline solutions.

#### 7.10.5.1 Alkaliphilic and Acidophilic Endo-1,4-β-xylanase

The pH tolerance of microbial endo-1,4-β-xylanase enzymes depends on the natural growing environment of the microbial species. Endo-1,4-β-xylanase-producing alkalophilic microbes grow optimally at higher pH above 9.0 and those produce acidophilic xylanases grow between pH 1.0–5.0 (Kimura et al. 2000). These organisms are usually isolated from various natural environments such as soil, decomposing organic matters, paper pulp industry wastes, and many other sources. *Bacillus* sp. C-59-2 was the first reported xylanase-producing alkaliphilic microorganism (Nakamura et al. 1993). Since then, there have been a number of reports on xylanases production using alkaliphilic and acidophilic microbes. However, the studies indicated that the intracellular xylanase produced by many of these microbes may not be adapted to extreme environmental growth conditions of their hosts. A large number of alkaliphilic microbes found to produce xylanase at optimal pH close to neutral although their activity can be retained in alkaline conditions. Many exceptional cases are also reported such as XylB from *Bacillus* sp. which has pH optima of 9.0–10 (Gessesse and Mamo 1998). Many acidophilic xylanases of fungal and bacterial origin have also been reported in last two decades. *Cryptococcus* sp. S-2 and *Penicillium* sp. 40 (Kimura et al. 2000) are the most studied extremely acidophilic (pH optima 2–3) xylanase producers.

#### 7.10.5.2 Psychrophilic Endo-1,4-β-xylanase

Even though the xylanase-producing microorganisms are abundant in nature, only a very less number of endo-1,4-β-xylanase-producing psychrophilies have been identified so far. Gram-negative bacteria such as *Pseudoalteromonas haloplanktis* TAH3a (Collins et al. 2005a) and *Flavobacterium frigidarium* (Humphry et al. 2001); gram-positive *Clostridium* sp. PXYL1 (Akila and Chandra 2002); fungal species such as *Alternaria alternate*, *Penicillium* sp., and *Phoma* sp.; and yeast sp *Cryptococcus adeliae* (Petrescu et al. 2000) are the major psychrophilic organisms reported to be endo-1,4-β-xylanase producers. Almost all these microbial species are isolated from Antarctic regions. The best studied two psychrophilic xylanases, which are from *Pseudoalteromonas haloplanktis* and *Cryptococcus adeliae*, were classified under GH 8 and GH10, respectively. The (α/α) 6 barrel structure of these structures is described by Van Petegem et al. (2003). The limited studies revealed that these enzyme groups have very poor stability due to the reduced number of salt bridges present on it but higher catalytic activity even at very low temperature. Georlette et al. (2004) also observed that psychrophilic xylanases are characterized by up to 10-folds higher activity even at mild cool temperature of 5 °C when compared to mesophilic xylanase activity. Moreover, the psychrophilic xylanase displayed higher catalytic and retained 60 % of maximum activity at very low temperature. However, the psychrophilic xylanases are totally unstable at high temperature (temperature 55 °C) and exhibited 12 times shorter half-life compared to mesophilic xylanases (Collins et al. 2005b).

#### 7.10.5.3 Thermophilic Endo-1,4-β-xylanase

A large number of thermophilic and hyper-thermophilic (optimal growth temperature 50–80 °C and more than 80 °C, respectively) microbes which are able to produce xylanases have been isolated from various sources such as thermal hot springs, deep marine fields, self-heating organic debris, etc. Almost all of these enzymes were grouped under families 10 and 11. Extremophile microbial species such as *Caldicellulosiruptor* sp. (Luthi et al. 1990), *Rhodothermus marinus* (Abou Hachem et al. 2000), and *Bacillus stearothermophilus* (Khasin et al. 1993) are the major producers of thermophilic family 10 xylanases, whereas family 11 thermophilic xylanases are less common and have been isolated from extremophiles such as *Bacillus* strain D3 (Connerton et al. 1999), *Chaetomium thermophilum* growing medicinal mushrooms in Egypt as a new strategy for cancer treatment, *dictyoglomus thermophilum* (McCarthy et al. 2000), *paecilomyces variotii* (Kumar et al. 2000), and *thermomyces lanuginosus* (Schlacher et al. 1996).

Optimum activity range of these thermophilic xylanases was found between 65 and 80 °C, whereas the xylanases from *Thermotoga* sp. exhibit optimal activity at 105 °C and pH 5.5. X-ray crystallographic structure and amino acid sequential analysis of many of these thermophilic xylanase indicated very close similarities with mesophilic xylanase and probable reason for the higher thermostability could be the minor changes in amino acid alignment. Many mutagenic studies also have indicated that minor modifications in mesophilic aminoacid array enhanced the thermostability to many folds (Winterhalter and Liebl 1995).

#### 7.10.5.4 Features Responsible for Endo-1,4-β-xylanase Thermostability

Thermostability comparison made between the enzymes are usually misleading since in most cases, the enzymes compared do not share the same experimental conditions such as incubation time, concentration of protein, nature, and concentration of the substrate. Many researchers believe that the high thermostability of xylanases is due to the presence of a network of certain charged amino acid residues near to the catalytic sites. These residues are usually found coupled with the disulfide bridges between β-strand and the α-helix. However, many recombinant thermostable xylanases are expressed in *E. coli* and are found to have thermostability ranging from 70 to 80 °C. When analyzing their protein structure, comparatively longer N-terminal ends are observed which may account for its higher thermostability (Morris et al. 1996). Many other analyzes also propose that the thermostability is due to the presence of highly flexible α-helix and C-terminal regions where the protein unfolding is generally initiated (Kumar et al. 2000). However, several other factors have also been identified that explain the xylanase thermostability such as follows:

- Presence of disulfide bridges which may strengthen the whole protein framework would give more stability to the enzyme.
- Presence of ion and aromatic pairs and their interactions may favor to more stable framework.
- Strategic position of water molecules on the framework, since the readily available water bindings are required for the catalytic activity even at higher temperature.
- Complex folding nature of enzyme. The higher the folding complexity, the higher the stability.
- Occurrence of oligomerization and the presence of certain aromatic residues result in the hydrophobic patches which allows stronger monomeric interactions enhancing the enzyme stability.

Certain GH11 endo-1,4-β-xylanases display disulfide bridges at position between β-strand and α-helix. However, these enzymes do not exhibit higher thermostability, whereas endo-1,4-β-xylanase produced by *Dictyoglomus thermophilum* exhibits thermostability even though that do not have any disulfide bridges on their structure. Therefore, the presence of disulfide bridges does not necessarily account for the thermophilic characters of the enzymes (Table 7.17).

**Table 7.17** Techniques used for purification of endo-1,4-β-xylanase enzymes

| Source | Purification | Buffer solution | References |
|---|---|---|---|
| *Acrophialophora nainiana* | Gel filtration and ion exchange chromatographies | 50 mM acetate | Salles et al. (2000) |
| *Bacteroides xylanisolvens* XB1A | Metal affinity resin and batch/gravity-flow column | 50 mM phosphate | Mirande et al. (2010) |
| *Sulfolobus solfataricus* | Ultrafiltration and AKTA Fast Protein Liquid Chromatography | 50 mM Tris–HCl | Cannio et al. (2004) |
| *Streptomyces* sp. AMT-3 | Filtration and overnight dialysis | 50 mM sodium citrate | Nascimento et al. (2002) |
| *Glaciecola mesophila* KMM 241 | Precipitation with 60 % saturation of ammonium sulfate and His-Tag Ni-affinity column | 50 mM citrate | Guo et al. (2009) |
| *Bacillus* sp. YJ6 | Sephacryl S-100 h chromatography | 20 mM citrate (pH 3.0–6.0), sodium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.0–9.0), sodium carbonate (pH 8.0–11.0) | Yin et al. (2010) |
| *Paenibacillus barcinonensis* | Precipitation with 20 % ammonium sulfate and cation exchange chromatography | 50 mM phosphate | Valenzuela et al. (2010) |
| *Aspergillus versicolor* | DEAE–Sephadex and HPLC GF-510 gel filtration | McIlvaine (pH 4.0–8.0), Tris–HCl (pH 8.0–9.0), glycine–NaOH (9.0–9.5) | Carmona et al. (2005) |

#### 7.10.5.5 Improvement of Endo-1,4-β-xylanase Thermostability

In last two decades, thermostability of xylanase enzymes was continuously improved through the protein engineering approaches. In most of these studies, the researches considered to modify the mesophilic xylanase enzyme structure to design a new structural model similar to the thermophilic xylanase. Arase et al. (1993) used chemical random mutagenesis in XYNA from *B. pumilus* and this strategy was successful to increasing the enzymes' half-life from 4 to 12 min at 57 °C. In a similar study, the specific activity and optimum temperature of Xyl1 from *Streptomyces* sp. S38 was enhanced from 2100 to 2500 IU $mg^{-1}$ with the shift of the optimal temperature from 57 to 66 °C (Georis et al. 2000). Other study showed that the replacement of the N-terminal end of mesophilic xylanase xlnB from *Streptomyces lividans* with those from thermostable xylanases TfxA from *T. fusca* by random gene shuffling and observed a significant increase in thermostability (Shibuya et al. 2000). Introduction of two disulfide bridges, one between B9 strand and the α-helix and another between N and C-terminal ends, also enhanced the thermostability of Tx-xyl (Paës and O'Donohue 2006). The authors also reported that the occurrence of residues that met at N-terminal end brought negative impact on xylanase thermostability and temperature optimum which are reduced by 10-folds and 5 °C, respectively. From these engineering strategies applied in xylanases protein engineering, researchers concluded that:

- The α-helix on xylanase structure seems very sensitive to thermal unfolding—making these are more rigid and may enhance the thermostability of the enzyme. However, it may also diminish the catalytic activity (Muilu et al. 1998).
- N-terminal end plays key role in thermostabilization in GH11 xylanase—linking the N-terminal extremity to A2 strand or C-terminal extremity enhanced the thermostability of xylanase. Introduction of an additional complete β strand by mutagenesis also beneficial for improved thermostability.
- Presence of disulfide bridges does not necessarily enhance the thermophilic behavior of the enzymes. Therefore, addition of these bridges does not always bring positive impact on enzyme thermostability.

## 7.11 Industrial Applications of Endo-1,4-β-xylanase

Industrial production of xylanases has been grown significantly during last few decades based on their increased application. Xylan can be converted into many useful products such as xylitol and furfurol through enzymatic hydrolysis (Subramaniyan and Prema 2002). The hydrolysis could be also carried out through acid treatment process which is relatively easy, fast, and cheap. However, the application of this method in industries is limited due to the formation of toxic end

substances that may have cross reactions with product of interest (Beg et al. 2001). Therefore, enzymatic hydrolysis method is widely applied even though it is a slow process but more efficient and eco-friendly. During industrial application, the xylolytic enzymes are not commonly applied in pure form but in combination with other hydrolytic enzymes to achieve better results. The industrial applications of xylanases cover different areas and the list of applications increased by time and thus increases the market demand of xylanases accordingly.

### *7.11.1 Animal Feed Industry*

Xylanases are widely used in animal feed manufacturing. They are usually applied in combination of other hydrolytic enzymes such as pectinase, protease, cellulose, lipase, glucanase, alginase, and phytase. These enzymes are used to reduce the viscosity of raw materials used in animal feeds. The complex arabinoxylan that is present on the cell wall of most grains may reduce the effective digestion and absorption of nutrients in poultry. Studies on this area have reported that the xylanase-treated feed has better digestibility and nutrient absorption rate when compared to raw feeds (Bedford and Classen 1992). In animal feed industries, the feed conversion rate (FCR) is defined as the ratio of feed consumed to the body weight gained by the animal. A lower FCR means improved feed digestibility and nutrient absorption. Xylanase enzymes are widely incorporated in poultry feed to reduce the FCR. It have been reported that the addition of endo-xylanase is isolated from *Acidothermus cellulolyticus* in poultry feed and observed a significant decrease in FCR, indicating an improved digestibility of feed (Clarkson et al. 2001). Similar results were observed when endo-xylanase is isolated from *Neocallimastix patriciarum* added to canola seed after oil extraction and applied for cattle feeding. The digestibility and stability of nutritive value of silage for cattle are found to be enhanced when it was treated with xylanase along with probiotic lactobacillus. The xylose formed by the de-polymerization of hemicellulose was fermented by the probiotic lactobacillus and thus enhanced the feed nutritive value. It was also reported that xylanase supplementation in the animal diet improved the animal growth through the better nutrition absorbability and enhancement of immune response as well as the gut micro-flora (Gao et al. 2008). In other study, Vandeplas et al. (2010) reported that when *T. viride* xylanase is incorporated in poultry feed, it increased nutrient digestion by 60 % and resulted in a significant increase in chicken weight. Pretreatment of forage crops with xylanase leads to better digestion in cattle and these enzymes are often found in synergy with other cellulolytic enzymes. The assimilation of ruminant feed was also improved many folds by xylanases addition (Yang and Xie 2010).

### 7.11.2 Food and Beverages Industry

Food and beverages industries use also xylanases in wide range of applications. Xylanases are capable of hydrolyzing the hemicellulose in wheat that enhances the water absorption of dough at leavening level in bread making process. This could possibly enhance the fermentation rate and as a result the softness of the bread and volume was increased (Rouau 1993). Xylanase cocktails are often used to reduce the stickiness of the dough thus enhance the crumb (Goesaert et al. 2005; Butt et al. 2008). Other study showed also that under specific process condition, incorporation of xylanase in the dough increased the shelf life of bread and also improved the volume up to 24 % (Verjans et al. 2010). However, enzyme selection is critical in these conditions since the cereals used for bread making often exhibit the xylanase inhibition by TAXI, XIP, or TLXI (Gebruers et al. 2004). Pasta processing is another area where xylanases are widely used as solubilizing agent of durum wheat semolina without changing its rheological properties (Ingelbrecht et al. 2001; Brijs et al. 2004). It was also reported that xylanase enzymes can improve the separation of wheat flour into starch and gluten (Frederix et al. 2004). However, in certain cases, action of Xyl-11 does not favor the separation due to high viscosity caused by the un-extractable arabino xylanase hydrolysis. In such conditions, synergetic dosages of Xyl-10 and Xyl-11 are used as it can mediate-improved agglomeration of gluten protein even though the wheat quality is very poor (Van Der Borght et al. 2005). Table 7.18 shows some examples of commonly used xylanase preparations for the baking industry. Xylanases are widely used also in juice, beer, and wine making industries. The enzymatic breakdown of fruits and vegetables hemicellulose can increase the clarity and stability of juice and wine. Xylanases can be used along with several other enzymes such as pectinase and amylase to improve the quality of beverage products in terms of aroma, taste, clarity, and stability (Wong et al. 1988). It was reported that up to 27 % decrease in insoluble fibers was achieved in juice clarification by addition of xylanase enzyme isolated from *Sclerotinia sclerotiorum* (Olfa et al. 2007). In beer production, partial hydrolysis of arabinoxylan found in the barley cell wall gives a middy viscous appearance to the fermented beer. In such

**Table 7.18** Examples of commonly used xylanase preparations for the baking industry (Collins et al. 2005a, b)

| Supplier | Brand | Enzyme source |
|---|---|---|
| Beldem-Puratos | Bel'ase B210 | *Bacillus subtilis* |
| | Bel'ase F25 | *Aspergillus niger* |
| Genencor Intl. | Multifectw | *Trichoderma reesei* |
| Danisco | Grindamyle H | *Aspergillus niger* |
| DSM | Bakezymew HS, BXP | *Aspergillus niger* |
| Novozymes | Pentopan Mono | *Thermomyces lanuginosus* |
| AB enzymes | Veronw 191 | *Aspergillus niger* |
| | Veronw Special | *Bacillus subtilis* |

cases, various xylanase enzyme groups are used to increase the filtration performance of beer wort by hydrolysis of viscous AXs fractions. Xylanase inhibitors present in the malt are the major hinderance to xylanase action. The xylanase activity is retained with the help of other synergetic enzymes used in the process (Debyser et al. 1997).

### 7.11.3 Paper and Pulp Industries

Endo-1,4-β-xylanase enzymes play significant role in paper and pulp industry since they facilitate an eco-friendly bio-bleaching of wood pulp by reducing the use of chemical bleaching agents such as chlorine and chlorine dioxide. For the past few decades, kraft pulp bleaching industry is recognized as the biggest area of xylanase application. Several studies reported that the removal of lignin content can be achieved by xylanase enzymes far better than by other lignin-degrading enzymes (Lundgren et al. 1994). Use of chemical bleaching agents in conventional pulp bleaching methods cause serious environmental pollution issues and this drove the researcher's attention on xylanase enzymes (Subramaniyam and Prema 2002). Table 7.19 shows major endo-1,4-β-xylanase supplier in pulp bleaching industry. Application of endo-1,4-β-xylanase enzymes in pulp bleaching can reduce using chlorine as well as the total energy requirement for this process. Kulkarni et al. (1999) observed that xylanase pretreatment of kraft pulp lowers the usage of chemicals by 10–20 %. The concept of TCF (total chlorine free) bleaching technologies was also recently introduced to make the process more eco-friendly. However, cellulase-free endo-1,4-β-xylanase is required in this process since the cellulose enzyme may degrade the cellulose which is the main target of the industry

**Table 7.19** Major xylanase supplier in pulp bleaching industry (Beg et al. 2001)

| Supplier | Product trade name |
|---|---|
| Alko Rajamaki, Finland | Ecopulp |
| Sandoz, Charlotte, N.C. and Basle, Switzerland | Cartazyme |
| Clarient, UK | Cartazyme HS, HT and SR 10 |
| Genercor, Finland; Ciba Giegy, Switzerland | Irgazyme 40–4X/Albazyme 40–4X |
| Novo Nordisk, Denmark | Pulpzyme HA, HB and HC |
| Biocon India, Bangalore | Bleachzyme F |
| Rohn Enzyme 0Y; Primalco, Finland | Ecopulp X-100, X-200, and X-200/4 |
| Solvay Interox, USA | Optipulp L-8000 |
| Thomas Swan, UK | Ecozyme |
| Iogen, Canada | GS-35, HS70 |

(Bajpai and Bajpai 2001). Relatively lower molecular weight of xylanase enzymes helps them to penetrate more deep into the lignocelluloses substrates and facilitate better hydrolytic actions (Beaugrand et al. 2005; Beg et al. 2001). Higher thermostability and alkaline stability are other key features that make xylanase more favorable for bio-bleaching industry (Buchert et al. 1995). The mechanisms of pulp bleaching by xylanase have been studied and many researchers believe that xylanase hydrolysis takes place at lignin xylan interaction and thus results in a loosely packed cellulosic framework (Shatalov and Pereira 2007). However, it was recently reported the potential use of *Aspergillus oryzae* MDU-4-based fungal xylanase for effective deinking of newspaper pulp where a significant improvement in optical properties such as brightness up to 57.9 % ISO with decreased (211 ppm) residual ink concentration (Chutani and Sharma 2015).

### *7.11.4 Biofuel Production*

The next-generation fuel production is based on renewable sources such as lignocellulosic biomass. As first step, the complex lignocellulosic biomass needs to be breakdown to a mixture of relatively smaller units made of hexoses and pentoses or their monosaccharides that are further fermented to ethanol or other biofuels (Margeot et al. 2009). The process of converting the lignocellulosic feedstocks into biofuels usually involves the following steps:

- Enzyme hydrolysis: the critical step involved where the hydrolysis of lignocelluloses into various sugar monomers by the action of cocktail of hydrolytic enzymes.
- Fermentation: production of biofuel by the utilization of sugar monomers formed by various microbial species, usually carried out by different bacterial and yeast strains depends on the desired type of biofuel.
- Distillation: separation and purification of biofuel produced.

The controlled hydrolysis of lignocelluloses using enzymatic cocktail to produce the desirable products is the challenge involved in this process. Xylolytic enzymes, such as Endo-1,4-β-xylanase in particular, are very essential in biomass hydrolysis as they initiate progressive cell wall in the assembly and thus mediate the cell wall penetration by other cellulolytic enzymes (Beaugrand et al. 2005).

### *7.11.5 Other Applications*

Apart from the traditional applications in pulp beaching industry, xylanases have been used in several other areas as well. The broad spectrum of xylanase application includes the saccharification of xylan to xylose, plant cell protoplasting, coffee

mucilage liquefaction, vegetable softening, extraction of plant pigments and oils, etc. (Kulkarni et al. 1999). In laundry industries, endo-1,4-β-xylanase was effectively used to remove the plant-based stains. The stain-removing capacity of family 11 endo-xylanase was improved when it chemically linked with cellulose-binding domain of cellulases. In textile industries, xylanase enzymes can be used to process the plant fiber as they can decrease the lignin content and improve the color and quality of fibers (Csiszár et al. 2001; Battan et al. 2012). Until now, xylanase applications in pharmaceutical industries are very limited. However, it is used in some dietary supplements in combination with other enzymes (Polizeli et al. 2005). Commercial products such as chewing gum contain xylitol and it can be easily produced by hydrolysis of xylan using xylanase enzymes.

### *7.11.6 Future Perspectives*

The current list of highly diversified applications of xylanases clearly demonstrates the increased demand of this type of enzymes. In addition, the continuous improvement of the enzyme catalytic properties increases the potential uses of this enzyme by adding many new industrial applications. The improvement of catalytic properties of the xylanases by shifting the optimal operation conditions of the enzyme and by isolation of new potential xylanases from extremophilic microbes are further expressed in suitable mesophilic host to industrialize the process. In addition, with the increased knowledge about protein engineering, it become possible also to increase enzyme stability and kinetic properties using different approaches such as changing of amino acid sequence, creation of sulfide bridge within the molecule, and changing the enzyme 3D configuration. All these will help to increase the potential application of xylanases under extreme operation conditions.

However, some recent studies were skeptic about the future application of xylanases in biofuel sector based on the current dramatic drop of oil price which make biofuel non-competitive alternative to fossil fuel in economic point of view. However, xylan biorefinery is not limited to fuel market but the biohydrolysis products of xylan such as pentose oligomers and monomers are also used as intermediate in the production of many biochemicals. Moreover, the increased public awareness about the negative impacts of chemicals on the environment, ecosystem, and human and animal health increased the pressure on chemical industries to replace the current chemical-based catalytic processes to a biobased and eco-friendly processes especially in paper/pulp, leather, and detergent industries. In addition, there is significant increased demand for xylanases application in food and feed industries for the production of safe and high-quality product. These all together support our current estimation of the continuous increase of xylanases demand in many industrial applications.

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