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Review

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Thermostable xylanases from thermophilic fungi and bacteria: current perspective

B.S. Chadha[a*], Baljit Kaur[a], Neha Basotra[a], Adrian Tsang[b]., Ashok Pandey[c]

a: Department of Microbiology, Guru Nanak Dev University, Amritsar-143 005 (India)

b: Center for Structural and Functional Genomics, Concordia University, Canada

c: Centre for Innovation and Translational Research, CSIR-Indian Institute of Toxicology Research, Lucknow-226 001- India

Bhupinder Singh Chadha, Ph.D (Corresponding author)

Department of Microbiology,

Guru Nanak Dev University, Amritsar, Punjab,

India.

E-mail: chadhabs@yahoo.com

Tel: +91-183-2258802 Ext 3317

Fax: +91-183-2258820

Baljit Kaur. Ph.D.

Department of Microbiology,

Guru Nanak Dev University, Amritsar, Punjab,

India.

E-mail: baljit_sohal@rediffmail.com

Neha Basotra. M.Sc.

Department of Microbiology,

Guru Nanak Dev University, Amritsar, Punjab,

India.

E-mail: nehabasotra506@gmail.com

Ashok Pandey. Ph.D.

Centre for Innovation and Translational Research, CSIR-Indian Institute of Toxicology Research.

Lucknow-226 001- India

ashok.pandey1@iitr.res.in

Adrian Tsang, Ph.D.

Center for Structural and Functional Genomics, Concordia University

Sherbrooke Street West, Montreal, Quebec H4B 1R6, Canada

adrian.tsang@concordia.ca

Abstract

Thermostable xylanases from thermophilic fungi and bacteria have a wide commercial acceptability in feed, food, paper and pulp and bioconversion of lignocellulosics with an estimated annual market of USD 500 Million. The genome wide analysis of thermophilic fungi clearly shows the presence of elaborate genetic information coding for multiple xylanases primarily coding for GH10, GH11 in addition to GH7 and GH30 xylanases. The transcriptomics and proteome profiling has given insight into the differential expression of these xylanases in some of the thermophilic fungi. Bioprospecting has resulted in identification of novel thermophilic xylanases that have been endorsed by the industrial houses for heterologous over- expression and formulations. The future use of xylanases is expected to increase exponentially for their role in biorefineries. The discovery of new and improvement of existing xylanases using molecular tools such as directed evolution is expected to be the mainstay to meet increasing demand of thermostable xylanases.

Keywords: Thermophilic fungi and bacteria, Thermostable xylanases, Glycoside hydrolases, Genomics and metagenomics, production

1. Introduction

Xylanase represents a key component of hemicellulases that catalyzes the breakdown of β 1-4 linkage present in the xylan backbone of hemicelluloses for subsequent conversion to xylose moieties. Xylanases have found beneficial role in feed and food by liberating essential nutrients through hydrolysis/cleavage of non-degradable hemicellulose fibers (Leisola et al., 2002). Commercially, xylanase as an important ingredient has attained a good market proportion in paper and pulp industry (Kumar et al., 2016). Besides, some of the potential applications of microbial xylanase includes, as a food additive ingredient for poultry, in baked products, coffee extractions, agriculture silage, as well as functional foods, etc. In baking industry, xylanases are being employed for enhancing the quality of dough such as stability, flexibility and extensibility by acting on soluble as well as non-soluble pentosans in flour (Butt et al., 2008; Ahmad et al., 2014). The xylanase market is expected to be robust during the forecast period due to increase requirement in animal feed industry. The estimated market share of this enzyme is between 200-300 million dollars and slated to reach 500 million dollars by 2023. In animal feed, nutritional additives are a prime need for poultry industry as they are involved in improving digestibility of broiler chickens by lowering the viscosity level in their intestines thus leading to the improvement in the weight gain and feed conversion efficiency (Collins et al., 2005; Nagar et al.,2012). In comparison to other operational costs, the expenses of animal feed in production of poultry and livestock are very high. Thus, to improve the feed digestivity of livestock and cut down the expenses with profit gain, enzymes are the best and safe option to be employed in feed industry. In addition to animal feed industry, the paper and pulp industry

also aids in the growth of global xylanase market. The eco-friendly use of xylanase enzyme over the harsh chemical usage further fuels the global xylanase market. On the basis of grade, global xylanase market is segmented into: feed grade and food grade whereas on the basis of their application, the global xylanase market is dissected according to their use in different sectors such as feed and livestock, bleaching of pulp, as an additive (in poultry), bakery, and bioconversion of agro-wastes. The global xylanase market is geographically divided in to five key regions including North America, Latin America, Europe, Asia-Pacific and Middle East Africa. Among these, Asia Pacific, especially China, Indonesia holds maximum share of xylanase market due to major poultry production, followed by Asia Pacific is North America, Europe, Latin America and MEA. In Europe, the presence of poultry companies such as PHW-Gruppe Lohmann & CO. AGxylanase, Plukon Food Group, LDC Group momentous the growth of xylanase market.

Xylanases are produced by diverse variety of micro-organisms including extremophilic fungi, bacteria, yeast etc. which are classified in several glycoside hydrolase (GH) families (5, 7, 8, 10, 11, 26, 30 and 43). Xylanases from these sources have been characterized and possess different characteristics such as pH and temperatures to retain their functionality. The industrial application of xylanase demands that the enzymes must be able to withstand the harsh conditions such as acid/alkaline environment and elevated temperature, where alkaline xylanase is beneficial for pulp and kraft bleaching while the preferred xylanases in poultry feed must be active under acidic to neutral pH, the prevalent conditions in the gut. Therefore, bioprospecting for thermostable xylanases keeping in mind the pH and temperature optima has been considered as two major criteria for their application besides being resistant to proteases,

metal ion, etc. This review keeping in view the immense potential of xylanases from thermophilic fungi and bacteria primarily focuses on the research and development that has taken place in last 5-8 years and explore the methodologies being used for discovery of novel xylanases.

1. Xylanase coding genes in thermophilic fungi

It is well recognized that thermophilic fungi produce multiple xylanases (Hinz et al., 2009). The current advances in genome sequencing, annotation and analysis of thermophilic fungi (www. fungal genomics.ca) shows the presence of multiple genes coding for xylanases (Table 1). The majority of the xylanases from thermophilic fungi harbor either GH10 or GH11 xylanase except for *Myceliophthora sepedonium, Pseudocercosporella, Thermomyces stellatus, Scytalidium thermophilum* that also code for functionally distinct GH 30 xylanases (Table 1). GH30 xylanases specifically active against methyl-glucouronoxylans have been studied in thermophilic *Clostridium thermocellum* (st John et al., 2016) but have not been functionally purified from thermophilic fungi. Of the different thermophilic fungi genome sequenced, *Myceliophthora sepedonium,* codes for maximum xylanase genes (seventeen) including eight that belongs to GH10 and six categorized to GH11. The genome of other strains *Pseudocerco sporella* and *Myceliophthora thermophila* also coded for 14 and 13 xylanase genes, respectively. Whereas, *Thermomyces lanuginosus*, considered as one of the most prolific xylanase producer and *Thermoascus aurantiacus* coded for single GH11 and GH10 xylanase genes (Singh et al., 2003; Winger et al., 2014).Thermophilic bacterial genome, however, do not contain such elaborate diversity and multiplicity of xylanases possibly owing to smaller

genome size. Most of thermophilic bacterial genome has genetic information for GH 10/GH 11 xylanases (Chakdar et al., 2016).

2. Mode of action of thermophilic xylanases

The mode of action of the xylanases has been elucidated in excellent reviews previously (Collins et al., 2005; Dodd and Cann, 2009). Based on recent understanding on the mode of action and structure of the thermophilic xylanases belonging to different families (Table 2), the GH10 family xylanase from *Thermoascus aurantiacus* revealed the presence of $(\alpha/\beta)_8$ Tim barrel fold comprising of eight major parallel β-strands and eight major α helices. In addition to this, six short helices are present along the polypeptide chains. Side view of the molecule resembles a "Salad bowl" as the upper face of the molecule on the β-barrel side possess a greater radius due to the extended loops of β-α loops. The bottom face has a narrow radius with α-β turns (Pollet et al., 2010).The catalytic site of enzyme comprises of two glutamate residues where one acts as an acid/base catalyst and the other behaves as a nucleophile. The catalytic domain comprising of 250 to 450 amino acids are highly conserved in xylanases but the affinity differences between these sub-sites significantly affect their modes of action, substrate preference and product preference (Bar et al., 2004). In order to decipher the mechanism underlying their heat stability, the crystal structure of many thermostable xylanases have been resolved. The stability at high temperature is thought to result from intermolecular and intra-molecular interactions (like hydrogen and disulfide bonds), interactions with carbohydrate binding units and stabilized C- and N-terminal ends. Through these interactions, the N-terminal region increases the conformational stability and prevents the destabilization of proteins at higher temperatures (Cheng et al., 2014). Since the terminal regions of the proteins are generally more flexible

than other parts of the structure, an increased terminal flexibility may enhance the process of denaturation and promote irreversible aggregation. Thus, the formation of a disulfide bond increases the surface pKa and hydrogen bonds for stabilizing the N-terminal random structure, which are the key determinants for the catalytic activity under conditions of high temperature and pH (Boonyapakron et al., 2017).

The structural elucidation of GH11 xylanase exhibits the presence of catalytic domain that displays a β-jelly-roll architecture comprising of two anti-parallel β sheets and one single major α-helix forming fingers and palm. The presence of loops in between the structure depicts thumb and cord. The structure is compact and closely packed and mimics partially closed right hand. However, a unique structure for a GH11 xylanase (XynCDBFV) has been reported in ruminant fungus *Neocallimastix patriciarum*. It harbors an extended N-terminal region comprising 11 amino acids that adheres to several β strands and spanned the convex side of the palm β-sheet. The N-terminal region is attached to the catalytic core by hydrogen bonds with stacking forces along a disulfide bond between Cys-4 and Cys-172. Through these interactions, the N-terminal region clumps the catalytic core into a more restrained structure, which confers to its stabilization at elevated temperatures (Cheng et al., 2014).The active site of thermophilic GH11 xylanase is a deep (~9 Å), narrow (~4 Å) and long (~25 Å) cleft with the catalytic dyad located in the middle, which supports the endo mechanism of GH11 xylanases. The active site residues are two molecules of glutamic acid acting as a nucleophile and an acid/base catalyst that actively participates in a double-displacement catalytic mechanism and the existence of a covalent glycosyl-enzyme intermediate complex. Xylanases from the GH11 family have low molecular weight and high pI values (Alvarez-cervantes et al., 2016).

Endoxylanases, belonging to GH family30 (GH30), are classified into 8 different subfamilies of which subfamily 7 (GH30-7) is derived from fungi and subfamily 8 (GH30-8) belongs to bacterial strains and are single -domain enzymes that act on xylans that are decorated with 4-O-methylglucuronic acid (MeGlcA) moieties to yield glucouronic acid (GlcA) containing xylooligosaccharides or aldouronates (Valenzuela et al., 2012; Padhila et al., 2014). However, unlike well characterized GH30 glucurono-xylanases from *Bacillus subtilis* and *Erwinia chrysanthemi* (st. John et al., 2017), xyn30D reported from the secretome of *Paenibacillus barcinonensis* is a modular enzyme comprising of carbohydrate binding module belonging to family CBM35 (Sainz-polo et al., 2014). Moreover it has been reported that recent GH30-8 xylanase from thermophilic anaerobic bacterium *Clostridium thermocellum* (CtXyn30A) show preference for GlcA containing substrates as well as possess a significantly high activity on wheat arabinoxylan (WAX) and several other biomass derived polysaccharides that do not contain GlcA (Verma *et al.,* 2016). The three dimensional structure of endo 1-4 xylanase (CtXyn30A) showed $(\beta/\alpha)_8$ TIM domain which is specific characteristic of clan A. This domain comprises of Val11–Pro295 residues, with a core of eight β strands and is surrounded by eight α helices. Whereas, the strongly attached side β-domain contains His3 to Gln10 and Gly296 to Val386 residues. The structure analysis has revealed that Glu 136 and Glu 225 are absolutely conserved within the subfamily and are the key constituents of the catalytic residues. The distance between these carboxyl residues is less than 5Å thus support retention mechanism for hydrolysis. CtXyn30A is active over a broad range of pH and displays an optimum temperature of 70^{o}C. The thermostability of the enzyme may be attributed to the presence

of higher number of salt bridges and strong hydrogen bonding between the side chain residues (Freire et al., 2016).

GH family 8 alongwith endo-1,4-β-xylanases also contain cellulases, chitosanases, lichenases . The cold-adapted xylanase act on xylan to get converted to primarily xylotriose and xylotetraose and was found to be most active on long chain xylo-oligosaccharides. Modeling of the three-dimensional (3D) structure of Rex8A from *Paenibacillus barcinonensis* BP-23 shows an $(\alpha/\alpha)_6$ barrel fold topology where the loops connecting the α-helices contour the active site. Moreover Rex8A enzyme has been reported for the first time that acts on branched xylo-oligosaccharides (Valenzuela et al., 2016). It has been proposed that like GH11 family xylanases, GH 8 xylanases contain the catalytic site in the middle alongwith the large substrate binding cleft comprising of at least six xylose binding residues. But they differ from GH10 and GH11 by their inverting single displacement reaction mechanism (Pollet et al., 2010). According to CAZy database, GH 43 family includes an array of enzymes such as β-xylosidase, α-L-arabinofuranosidase, arabinase, xylanase and galactan 1,3-β-galactosidase (Lombard et al., 2013). The structural elucidation of GH 43 family shows that proteins of this family contain five bladed β-propeller domains and possess inverting mechanism where three residues are involved in catalysis (Till et al., 2014).

3. Characteristics, properties and applications of xylanases from thermophilic fungi

3.1 *Myceliophthora strains*

M. thermophila, a thermophilic fungus (optimum growth temperature of 45˚C) previously identified as *Chrysosporium lucknowense* has been found to be an efficient source of

hemicellulolytic enzymes including xylanases (Hinzet al., 2009). The genome of *C. lucknowense* strain was annotated to contain 11 xylanases genes for GH10 and seven for GH11 (Hinz et al., 2009). Whereas, *Myceliophthora thermophila* genome was later found to contain 12 xylanase genes (Karnaouriet al., 2014). A thermophilic strain identified as *Myceliophthora* sp (IMI, 38079) by IMI Kew, UK and as *C. lucknowense* (MTCC) by Microbial Type Culture Collection, Chandigarh, India, had also been reported previously to produce functionally distinct multiple xylanases (Badhanet al., 2004). Ten different functionally diverse xylanases from *Myceliophthora* sp.were resolved electrophoretically using PAGE/IEF and were found to show characteristically different activity against unsubstituted xylans, arabinoxylans and methyl-glucouroxylan (Badhan et al., 2004). Ustinov and co-workers (2008) purified six xylanases from *C. lucknowense* (3 each of GH10 and GH11 xylanases) and found that GH11 in comparison to GH10 xylanases displayed high substrate specificity when subjected to catalysis against different xylans (Table 3). Structurally two of the GH10 xylanases (Xyl 10 B and Xyl 10 C) were found to be distinct with modular configuration and were tethered by CBM1 at N and C terminal, respectively. Further they found these xylanases to be catalytically versatile and exhibited high thermostability. *Myceliophothora thermophila* is also known to produce low molecular weight xylanase (14 kDa) that is active under extreme alkaline condition (Boonrunget al., 2016). *Myceliophthora* is now known to contain four species namely *M. theromophila, M. heterothallica, M. Hinulea and M. fergusis* (van de Brink et al., 2013), where *M. thermophila* is better producer of xylanases when compared to *M. heterothallica*. The transcriptome profiling of *M. thermophilia* showed that the expression of GH 1 xylanases was up-regulated when cultured in presence of monocot cereals, whereas, the

expression of GH10 showed no selective up-regulation in presence of either monocots/dicots as substrate and its level remained unchanged (Kolbusz et al., 2014). *M. thermophila* JCP 1-4 strain from Brazil (Pereiera et al., 2015) was found to produce 931.1 (units/g) of xylanase on sugarcane bagasse, whereas, *Myceliophthora s*p., isolated from composting soils of India was reported to produce 2366 (units/g) of xylanase when cultured on rice straw employing solid state fermentation (Badhan et al., 2007). Recently two GH11 xylanase genes MYCTH_49824 and MYCTH_56237 from *M. thermophila* have been cloned and expressed in *Pichia pastoris*. The multiple template alignment for modelling further revealed their catalytic domains (Basit et al., 2018).

3.2 *Thermomyces lanuginosus*

Thermomyces lanuginosus with optimum growth temperature of 50˚C is considered as one of the most prolific producer of alkaline active virtually cellulase free thermostable xylanases that exhibited 3500 (units/ml) and 48,000 (units/g substrate) of activity under shake flask and solid substrate fermentation, respectively (Sonia et al*.,* 2005; Kumar et al*.,* 2009; Winger et al., 2014). However, *T. lanuginosus* strains isolated from different geographical regions vary in their xylanase production capacity. A *T. lanuginosus* strain VAPS isolated from decaying wood sample produced as low as (61 units/ml) that was improved to 132.5 **(**units/ml) using combination of optimization approaches such as Genetic Algorithm-Response Surface Methodology (GA-RSM), Artificial Neural Network (ANN), Genetic Algorithm-Artificial Neural Network (GAANN) (Kumar et al., 2016). Heterologous production of cloned *T. lanuginosus* gene using *E. coli* and *Pichia pastoris* as host has also been reported (Mchunu et al., 2009). Upon comparison, it was found that expression of xylanase by alkaline stable variant NC38 in *P. pastoris* (261. 7±

0.61Uml) was 545-folds higher than observed in *E. coli* (Mchunu et al., 2009). Engineering of an N-terminal disulfide bridge resulted in improved thermal performance of recombinant *T. lanuginosus* GH11 xylanase as evidenced by apparent upward temperature optima shift upwards at pH 6.5 by about 10°C to 75°C (Wang et al*.,* 2012). *T. lanuginosus* produces low molecular weight (25.0 kDa) and highly thermostable xylanase that is active between pH 5.0 and 9.0 and has been classified as GH 11 family member. The secretome based analysis of *T. lanuginosus* grown on corn-cob based medium showed high proportion of protein as xylanase (22-30%) (Winger et al. 2014) and may be the reason for its purification using simple two-step process involving weak anion exchanger and gel filtration (Bennet et al., 1998). Recent studies have shown that the xylanase purification was also possible by partitioning using PEG1000/NaCit based two phase aqueous system. The separation based on partitioning is best because of high Kp value of 17.7 ± 0.3 thus making it applicable for industrial purification of xylanase (Loureiro et al., 2017). Xylanase from *T. lanuginosus* is commercially produced as recombinant enzyme using *Fusarium venentatum* as host organism and is marketed as Novozyme 899 as processing aid in baking industry to improve dough stability and crumb structure. Similarly, Pentopan Mono BG, a recombinant xylanase from *T. Lanuginosus* expressed in *A. oryzae,* is known to improve bread loaf volume by 41% (Butt et al., 2008). Ronozyme WX CT, an endoxylanase derived from *T. Lanuginosus* employing submerged fermentation by Novozymes A/S (Denmark), and marketed by DSM Nutritional Products (Switzerland) is authorized in the EU (Commission Regulations (EC) No 1332/2004 and No 2036/2005) as feed enzyme. The xylanase product was found to positively influence the feed conversion efficiency in non-ruminants (Nielsen et al., 2008). The treatment of Kraft pulp during milling with *T.*

lanuginosus xylanase was found to partially hydrolyze xylan associated with cellulose fibres thus resulting in subtle changes in the pulp structure and enhanced susceptibility of the pulp to refining (refining energy was significantly reduced) and improved the static strength properties of paper (Buzala et al., 2016).

3.3 *Malbranchea* strains as source of xylanase

Malbranchea strains produce multiple forms of thermostable alkaline active xylanases (Sharma et al., 2010). The culture produces high levels of xylanases (27193 units/g substrate) at 45˚C on solidified culture medium containing rice straw as carbon source (Mahajan et al., 2014). Employing transcriptomics profiling it was observed that the expression of *M. cinnamomea* GH11 xylanase gene was up-regulated by 301.8 times when cultured on xylan and 22.79 times on wheat bran as compared to glucose (Huttner et al., 2017).The xylanases MEX-I(GH11) and MEX-II(GH10) from a thermophilic isolate *Malbranchea flava* were purified and characterized and found to be optimally active at pH 9.0 and 70^{O}C (Sharma et al., 2010). The secretome profiling of the *M. Cinnamomea* indicated it to be a source of thermostable metal dependent glycosyl hydrolases (Mahajan et al., 2016) including xylanases that are involved in boosting the hydrolytic potential of commercial cellulase Cellic Ctec 2 during saccharification of rice straw, carrot grass and corn stover (Sharma et al.,. 2016). The cloning of 50 kDa thermostable GH10 xylanase MpXyn10A and its expression in *Aspergillus nidulans* was carried out and the expressed enzyme was optimally active at pH 5.8 and 80°C and found to be 16% glycosylated and thermostable, preserving 85% activity after 24 h at 65°C. Circular dichroism showed high alpha-helical content consistent with the canonical GH10 family $(\beta/\alpha)_8$ barrel fold observed in molecular modeling. The xylanase resulted in effective hydrolysis of native and

pretreated sugarcane bagasse (Rebeiro et al., 2014). In a recent study, two xylanase genes (GH10 and GH11) designated as XYN10A_MALCI and XYN11A_MALCI from *Malbranchea cinnamomea*, respectively, were cloned and expressed in *P. pastoris* X33. The resultant clones of GH11 and GH10 produced 573.3 and 24.3 (units/ml) of xylanase, respectively, when fed with methanol under shake flask cultures (Basotra et al., 2018) were purified and the recombinant rXYN11A MALCI was found to be stable at 70°C and catalytically active against a variety of substituted (arabinoxylans) as well as unsubstituted xylans. The recombinant xylanases were found to act synergistically with commercial cellulases and resulted in 1.54and 1.58 folds improved hydrolysis of acid and alkali treated rice straw (Basotra et al., 2018).

3.4 *Thermoascus aurantiacus* as source of xylanase

T.aurantiacus (with optimal growth temperature at 50˚C), a well known source of GH10 xylanase, has been reported to produce xylanase both under solid state fermentation where wheat bran supported 6543 (units/g substrate) (Jainet al., 2015) and shake flask culture (130 units/ml) using corn cob as substrate (Olivirea et al., 2010). *T.aurantiacus* has also been found to express xylanase constitutively using xylose as inducer where, Schuerg et al. (2017) showed that feeding xylose continuously in fed batch mode resulted in production of 80 (units/ml) of xylanase. The crystal structure of *T.aurantiacus* GH10 xylanases had been shown to complex with xylobiose substitute with arabinofuranosyl feraloyl ester side chain and the enzyme. The enzyme showed fourfold higher substrate specificity for xylotriose which is linked to arabinose at non reducing xylan moiety compared to xylose alone (Vardakou et al., 2005).*Thermoascus aurantiacus* M-2, producing novel acidophilic and thermostable xylanase 39.07 (U/mL) was purified. The purified xylanase (31.0 kDa) was

characterized to be active at 75^{o}C and pH 5.0 and maintained stability at 80^{o}C (Ping et al., 2018).

3.5 *Scytalidium thermophilium* and other thermophilic fungal strains as source of xylanases

Xylanases produced from *Humicola insolens* (syn. *S. thermophilum* and now *Mycothermus thermophilus*) being GRAS organism has been approved by FDA and is used commercially for application in food (brewery, bread, starch extraction from wheat) and feed industry (European commission report, 2016). The xylanases from *H. Insolens* are capable of improving the starch extraction from wheat and is sold commercially as Shearzyme by Novozymes. The optimum growth temperature of *H. insolens* is 45˚C (Basotra et al., 2016). Four GH10 xylanases from *H. insolens* have been purified and characterized to be optimally active between 70-80°C and at pH 6.0-7.0 (Du et al., 2013). Later Shi et al., (2015) cloned and expressed multi-modular GH11 xylanases form *H. insolens*Y1that was found to be alkaline tolerant.

Besides the strains cited above, different thermophilic fungus like *Thielavia terrestaris* (Berka et al., 2011; Garcia-Haunte et al., 2017), *Corynascus thermophilus* (van de Brink et al., 2013), *Rhizomucor pusillus*(Huttner et al., 2018) have also been reported as source of xylanase. *T.terrestris* was found to produce hyper-thermophilic active xylanase (Tt Xyn A) with molecular weight of 82 kDa and was found to be optimally active at pH 5.5 and at 85 ^{o}C. The xylanase exhibited half life of 23.1 days at 65^{o}C. The thermostability was found to be associated with gain in secondary structures at high temperature (Garcia-Haunte et al., 2017).*C. thermophilus* previously known as *Myceliophthora fergusii,* reported to produce

low levels of xylanases (van de Brink et al., 2013), was taken up for expression of GH11 xylanase for heterologous over expression in *P. pastoris*. To obtain perfect expression, the 870 bp gene sequence was codon optimized and synthesized. The recombinant Xyn11A was found to be optimally active at pH 7.4 and 70^{o}C. The enzyme was resistant to metal ions and protease such as pepsin which makes it suitable for biotechnological applications (Yang & Zhang, 2017). *Paecilomyces thermophile*, a thermotolerant fungus that had been reported to produce high levels of thermostable xylanases was cloned and expressed in *P.pastoris*. The recombinant xylanase produced 8.1 (g/L) of protein and 2940 (U/ml) of activity in 5L fermenter fed sequentially with methanol. The recombinant enzyme was active at 80^{o}C for 30 min (Fanet al., 2012). A highly catalytically efficient thermophilic xylanase belonging to family GH10 from *Achaetomium* Sp X2-8 was stable at 75^{o}C and at broad range of pH 4.0 to 10.0 with Kcat/ Km of 3710/m/s/mg (Zhao et al., 2013). The xylanase was comparable to Ultraflo, commercially produced from *H. insolens* by Novozyme and was used for filtration of brewing mash and was found to perform much better in combination with β-glucanase when compared to Ultraflo. Surprisingly, the genome of thermophilic fungus *Rhizomucor pusillus* which do not harbor any gene for xylanase (Huttner et al., 2018) was reported to produce 6 (Units/ml) of xylanase when cultured on beechwood xylan (Huttner et al., 2018). *R. pusillus,*isolate from maize silage, has also previously been shown to produce 824 (U/g) of xylanase which was found to be stable at 75^{o}C (Robeldo et al., 2016). The observed xylanase activity in the secretome can be ascribed to the broad specificity of some of other glycosyl hydrolases like GH5 and even auxiliary activity proteins in the secretome against the xylan as substrate (Fromhaggen et al., 2016).*Talaromyces emersonii* now renamed as *Rasmasonia emersonii* is an important

source of xylanases that is commercially produced by Novozymes DSM and is in the list of food enzymes approved for use in mashing process in breweries as well as biorefineries. A thermotolerant strain *T. Leycettanus,* reported as a novel source of the thermostable GH10 xylanase, was cloned and expressed in *P. pastoris.* The purified recombinant TlXyn10A was acidic and hyper-thermophilic and retained stability over the pH range of 2.0–6.0 and at 90^{o}C (Wang et al., 2017). Later Wang and co-workers (2017) prepared three mutants of GH10 xylanases by swapping gene of a xylanase of another fungal strain *Bispora* sp., MEY-1 reported to be source of thermostable xylanases and showed site directed mutation of E229I, F232E and G145D resulted in weakened substrate specificity and improved catalytic efficiency. *Melanocarpus albomyces*, a thermophilic and non-sporulating fungus that is considered as proficient strain for production of alkaline active xylanase with application in paper and pulp industry was subjected to strain development. The mutant *M. albomyces* IITD3A produced 415 (IU/mL) xylanase on alkaline lignocellulosic extract in a 14 L bioreactor with volumetric productivity of 11,530 (IU/L/h),which was 8-fold higher than that of the wild-type strain (Biswas et al., 2010, Gupta et al., 2013). The cyclic maintenance of pH of fermentation medium between 7.8 and 8.2 increased the productivity to 16, 670 (IU/L/h), it was found that change in the fungal morphology to a pellet form resulted in very high xylanase productivity of 22,000 IU/L/h (Biswas et al., 2010).

4. Thermostable Xylanases from thermophilic bacteria

4.1 *Geobacillus* strains as source of thermostable xylanase

Geobacillus perhaps is the most widely reported thermophilic bacteria for its ability to produce thermostable xylanase. *Geobacillus* sp. WSUCEF1 (grows optimally at 60˚C)

isolated from soil (Bhalla et al., 2015) and strain DUSELR 13 isolated from deep gold mines of South Dakota (Bibra et al., 2018) have been reported for producing highly thermostable enzyme whereas xylanase from the former strain showed half life of 18 and 12 days at 60°C and 70°C, respectively, while DUSELR 13 produced 31 (U/ml) after optimization and exhibited t ½ of 13 days at 60°C and 70°C. The enzymes also showed better hydrolysis of beechwood xylan when compared to commercial xylanases Cellic HTEC 2 and Accelarase XY. A GH10 xylanase from *Geobacillus* sp WSUCFI cloned and expressed in *E. coli* exhibited 461 (units/mg) of specific activity and exhibited t ½ at 60°C after 60 h (Bhalla et al., 2014). *G. stearothermophilus* KIBGE-IB29, a soil isolate was found to produce xylanase optimally at 60°C and at pH 6.0 after 24 h of incubation period (Bibi et al.,2014).The GH10 xylanase from *G. stearothermophilus* and *Rhodothermus marinus* (Mathew et al., 2018) produced xylo-oligosaccharides (XOS) and arabino-xylooligosaccharides (AXOS) such as A3X, A2XX and A2+3XX. The strain produced a single 45kDa xylanase; however unlike *G. Stearothermophilus*, xylose was not an inducer of the resultant xylanase (Gerasimova & Kuisiene, 2012). The xylanase expression in *G. stearothermophilus* was found to be controlled by multiple regulatory mechanisms including XylR as the master negative regulator in addition to carbon catabolite repression. The genes for xylanase expression were positively modulated in presence of xylose as inducer which negates the effect of XylR, in addition quorum sensing played an important role as evidenced from 50 fold increase in expression of xylanase during exponential growth phase (Shulami et al., 2014). *G. galactosidasius* BS61 isolated from geothermal resource in Turkey producing (15 U/ml) xylanase was purified and applied for clarification of juices of orange and pomegranate as evident by reduced turbidity (Sari et al., 2018).

Geobacillus thermodenitrificans JK1 is another well studied strain for the production of xylanase that was shown to produce two xylanase (XynA1 and XynA2) isoforms that synergistically act with β-xylosidases and arabinofuranosidase from efficient hydrolysis of birchwood xylan (Huang et al., 2017). The xylanase gene GtxynA1 of *G. thermodenitrificans* was found to be associated with HUS (hemicellulose utilization) locus. This GH10 family protein was optimally active at 60^{o}C in pH range of 3.0 - 9.0 and its stability was shown to be improved by applying protein engineering where mutants of *G. thermodenitrificans* C5 with enhanced temperature by 11^{o}C substituting histidine, tryptophan and aspartic acid with glutamic acid and proline (H82E/ W185P/ D186E) was reported (Irfan et al., 2018).

4.2 *Caldicellulosiruptor* as source of thermostable xylanase

C.bescii. *C. lactoaceticus* and *C. owensensis* have been reported for the secretion of thermostable xylanases in recent past (An et al., 2014, Jia et al., 2014 and Liu et al., 2018). The GH10 xylanase from *C. bescii* was over-expressed in *E.coli* and found to exhibit high catalytic activity with half life of 7.7 h at 60^{o}C and the enzyme was optimally active at 70^{o}C at pH 7.0 with 85% activity at pH 4.0 and 12.0 (Anet al., 2014).The N-terminal domain of GH10 xylanase from *C.bescii* was found to be multidomain cbxyn10C/cel48B capable of degrading crystalline cellulose besides xylan and barley β-glucan (Xue et al., 2015), thus making it useful candidate for bioconversion. *C. lactoaceticus* is also known to express GH10 xylanase (47 kDa) that exhibited good stability at 80^{o}C at pH4.5. The enzyme was shown to liberate branched XOS with methyl-glucouronic acid sub-chains (Jia et al., 2014). Recently, *C. owensensis* has been shown to produce highly thermostable xylanase which is optimally active at 90^{o}C. The recombinant CoXynA was shown to

exhibit half life of 1 h at 80°C. The authors further solved crystal structure of the recombinant xylanase. The relative high thermostability of the enzyme was proposed to be due to increased overall protein rigidity resulting from reduced length and fluctuation of loop 7 (Liu et al., 2017). The hemicellulase from *C. owensensis* was capable of degrading hemicellulose of native corn stover and cob efficiently (Peng et al., 2015). *Herbivorax saccincola* A7 isolated through enrichment culture using untreated corn stover as carbon source was found to grow optimally at 55°C and at alkaline pH (9.0). The draft genome of the culture revealed the presence of GH11 xylanase and essential genes for xylose metabolism, xylose isomerase, xylose transporter etc. (Aikawa et al., 2018). A high xylanase producing *Caldicoprobacter algeriensis* isolated from hydrothermal hot spring of Guelma produced 250 (U/ml) of xylanase in a medium comprising birchwood and oat spelt xylan at 70°C in 24 h (Amel et al., 2016). Modular trifunctional xylanolytic Axy43A has also been reported from *Paenibacillus curdlanolyticus* strain B-6. This GH43 hydrolase with family 6 carbohydrate binding module exhibited endoxylanase, β-xylosidase and arabinoxylan hydrolyzing activities (Teeravivattanaki et al., 2016). Modular family 8 xylanase from marine bacteria *Glaciecola mesophila* was found to have new type of CBM with eight β-strands (Chen et al., 2018). Improved expression of *Thermoanaerobacterium aotearoense* SCUT27 GH10 xylanase harbouring CBM modules and three surface layer homology domains (Huang et al., 2015) expressed in *B.subtilis* was optimally active at 80°C and at pH 6.5. The hydrolysis of beechwood xylan resulted in xylobiose and methylglucourono-xylotriose as the main products.

4.3 *Thermopolyspora flexuosa* as source of industrial xylanase

A GH10 xylanase from *Thermopolyspora flexuosa* (*Nonomuraea flexuosa*) DSM43186 which is an actinomycete known to produce thermophilic xylanase exhibited temperature optima of 65-70°C against insoluble xylan and 75-80°C in presence of 3 % xylan was found to be stabilized in presence of ionic liquid [EMIM]OAc at inactivating temperatures (80-90 °C). The increased stability of TfXYN10A indicated to the binding of IL molecules that stabilized the protein structure. Furthermore, the xylanase exhibited low K_m (~1 mg/mL) demonstrated higher tolerance to ILs than xylanases with higher K_m (Anbarasan et al., 2017). The GH10 xylanase from *T. flexuosa*was was taken up for commercial production. The gene was cloned and expressed in *Trichoderma reesei* by AB enzymes. The enzyme produced in GRAS organism has been cleared by Canadian government for application in baking, brewery, potable alcohol and grain processing (GRAS Notice No. GRN000628). The recombinant xylanase produced by *T. Reesei* at industrial scale was subjected to ultrafiltration and spray drying and is marketed in powdered form that has an activity of 2,28,200 (units/g).

In addition, some of the novel thermophilic bacterial strains capable of producing xylanase including, *Caldoprocter, Herbinix*, *Herbivorax saccinola* from bovine manure as well as biogas digester beside *Rhodothermus marinus* from marine environment have also been reported (Amel et al., 2016; Mechelke et al., 2017; Aikawa et al., 2018; Mathew et al., 2018).

5. Metagenomic DNA as source of thermostable xylanases

Owing to the search for novel enzymes for industrial exploitation, metagenomic approaches can overcome the limitations of culture-dependent methods and can facilitate the discovery of enzymes from uncharacterized microorganisms. The screening of metagenomic libraries coding for xylanases has resulted in few novel ORF's coding for thermostable xylanases. The environmental DNA isolated from different niches such as insect gut, manure, waste water, hot environmental samples, chicken cecum, crater of Avachinsky volcano have been reported as sources of xylanases (Mientus et al., 2013). The xylanases from the chicken cecum metagenome was found to require high concentration of salt and organic solvents for their activity (Darkazali et al., 2017).Furthermore it was proposed that poultry feed is primarily composed of high ratio of non-starch polysaccharides that include xylans and arabinoxylans so the microorganisms capable of hydrolyzing these polysaccharides should be abundant in chicken intestine (Mead et al., 1989). A novel alkali-stable and thermostable GH11 endoxylanase encoding gene (Mxyl) was retrieved by functional screening of a compost soil metagenome. The recombinant xylanase (1077bp) exhibited activity at 80^{o}C and pH 9.0 (Verma et al., 2013). The thermostability of this enzyme was subsequently engineered through the enrichment of surface β-sheets with arginine residues by substituting serine/threonine by site directed mutagenesis (Verma and Satyanarayana, 2013) whereas xylanases from volcano metagenome was found to be maximally active at 95^{o}C and exhibited half life of 22 h at 85^{o}C (Mientus et al., 2013). Similarly, the expression of xylanase gene xyl7 derived from termite gut, in *E. coli* showed higher thermostability with 10^{o}C increase in optimal temperature and was active under broad pH range of 5.5-10.0 (Qian et al., 2015). Ming-Zhe

et al. (2015) cloned xylanase gene from metagenomic DNA of cow dung and expressed in *B. Megaterium*. The recombinant xylanase was found to be optimally active at pH7 and 75^{o}C.

Conclusion

Bioprospecting of new genes for novel xylanases from diverse bacteria and metagenomics can be exploited for future applications. Furthermore, through taking advantage of rapid development of genome editing and synthetic biological techniques in future, the enzyme producing microorganisms are compelling subjects to explore the growing importance of thermophilic xylanases in various industrial applications. The development of technology based on these xylanases is foreseen as they will be able to cater to the growing needs of the industry. Therefore this area of research with expected increase in market share by three folds in next ten years will be hotly pursued.

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Table 1.Genome wide analysis of thermophilic fungi coding for diverse xylanases

Fungal strains	Number of genes	GH 7	GH 10	GH 11	GH 30
Chaetomium thermophilic	8		3	4	1
Myceliophothora sepedonium	17				
Myceliophothora fergussi	9		4	5	0
Malbranchea cinnamomea	4		3	1	
Rasamsonia byssochlamys	6	1	2	1	1
Pseudocercosporella	14		6	6	2
Rhizomucus pusilus	7				
Scytalidium thermophilium	10		7	3	1
Thermoascus aurantiacus	1		1		
Thermomyces lanuginosus	1			1	
Thermomyces stellatus	10		4	4	2
Thielavia australiensis	7		3	3	1
Thielavia terrrestaris	9		5	4	
Myceliophthora themophilum	13		5	8	

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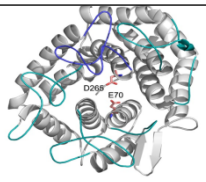
Table2.: Structure and folding topologies of xylanases of different GH families.

GH families	Organism	Folding topology	Structure	Mechanism	References
8	*Paenibacillus barcinonensis*	$(\alpha/\alpha)_6$		Inverting	Valenzuela et al.,2016

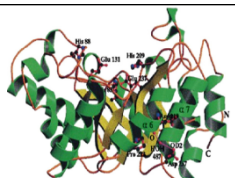
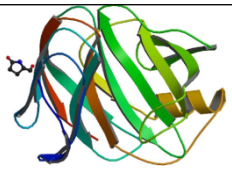
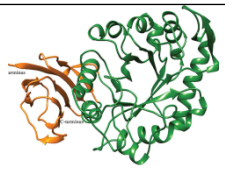
10	*Thermoascus auranticus*	$(\beta/\alpha)_8$Tim barrel		Retaining	Pollet et al., 2010
11	*Neocallimastix patriciarum*	β Jelly roll		Retaining	Chenget al., 2014
30	*Clostridium thermocellum*	$(\beta/\alpha)_8$Tim barrel		Retaining	Freire et al., 2016
43	*Butyrivibrio proteoclasticus*	β-propeller		Inverting	Tillet al., 2014

Table 3. Production and properties of purified xylanases from thermophilic and thermotolerant fungi

Fungal strain, (xylanase activity)	MW (kDa) /pI	Optimum pH/Temp	Km (mg/ml) /Vmax(U/mg/min)	GH Family	Reference
Humicola insolens (16.4 units/ml)	Xyn W (44.0)[w]/NR Xyn A (41.5)[a]/NR Xyn B (42.0)[a]/NR Xyn C (44.0)[a]/NR	6.0/70[o] C 6.0/80[o] C 7.0/70[o] C 6.0/70[o] C	2.8 / 311.8 1.6 / 974.4 1.1 / 306.8 2.1 / 287.7	GH10 GH10 GH10 GH10	Du et al.,2013
T. lanuginosus DSM 10635 (1180 units/ml)	(25.5)/N.R	6.5/70[o] C	3.85/ N.R	NR	Xiong et al.,2004 Le and Wang,

T. lanuginosus[a] (36 units/ml)	(44.0)[f]/N.R	6.0/65° C	N.R/ N.R	GH 11	2014
Thermoascus aurianticus (575.9 units/ml)	(33.0)/N.R	4.0-4.5/70- 75° C	N.R/ N.R	GH10	Kalogeris et al., 2001
Chaetomium thermophile (61.1 units/ml)	Xyl I (26.0)/N.R Xyl II (7.0)/N.R	5.4-6.0/ 70° C 4.8-6.4/ 60° C	0.55/58.8 0.1/5.26	NR	Ganju et al., 1989 Katapodis et al., 2007
Melanocarpus albomyces (415 units/ml)	Xyl I (38.0)/N.R Xyl II (24.0)/N.R	6.6/65° C 5.5/65° C	0.30/311 1.69/500	NR	Biswas et al., 2010
Myceliophthora sp. IMI 387099 (16.2 units/ml)	Xyl Ia (53.0)/5.2 Xyl IIa (53.0)/4.8	6.0/75° C 6.0/75° C	1.63/55.5 1.69/33.3	GH10 GH10	Chadha et al., 2004
Malbranchea flava MTCC 4889 (164 units/ml)	MFX I (25.2)/4.5 MFX II (30.0)/3.7	9.0/70° C 9.0/70° C	1.25/1666 3.7/1923	GH11 GH10	Sharma et al., 2010
Chrysosporiium lucknowenese (96.6 units/ml)	Xyn 10A (47.8)/4.7 Xyn 10a (31.0)/8.9 Xyn 10B (57.0)/4.4 Xyn 10b (46.0)/4.3 Xyn 10C(40.0)/4.8 Xyn 11A(24.0)/7.7 Xyn 11B (23.0)/8.4 Xyn 11C (22.0)/6.7	5.5-7.0/65-70° C 5.5-7.0/65-70° C 5.5-7.0/80-85° C 5.5-7.0/80-85° C 5.0/80°C 5.5-7.0/70° C 5.5-7.0/65-70°C 4.5/65°C	N.D/65[d] N.D/83[d] N.D/39[d] N.D/85[d] N.D/32[d] N.D/395[d] N.D/169[d] N.D/300[d]	GH10 GH10 GH10 GH10 GH10 GH11 GH11 GH11	Ustinov et al., 2008
Sporotrichum thermophile	StXyn I (24.0)/8.7 StXyn II (48.0)/8.0	5.0/60° C 5.0/60° C	NR	GH11 GH10	Vafiadi et al., 2010
Remersonia thermophila	rtXylI (42.0)/N.R	6.5/60° C	2.48 /5.79	GH 10	McPhillips et al., 2014

a: recombinant xylanases; f; fusion protein xylanase, w: wild; d: (units/mg)

Highlights

- Thermophilic fungal genomes coding for thermostable xylanase genes
- Thermophilic fungi as efficient producer of xylanases
- Novel thermophilic bacterial strains as good source of highly thermostable xylanases
- Metagenomic DNA from diverse ecological niches as source of thermostable xylanases