LIST OF PUBLICATIONS


Molecular characterization of multiple xylanase producing thermophilic/thermotolerant fungi isolated from composting materials

M. Sharma, B.S. Chadha, M. Kaur, S.K. Ghatora and H.S. Saini

Department of Microbiology, Guru Nanak Dev University, Amritsar, Punjab, India

Keywords
alkaline active xylanases, ITS and 26S rDNA sequences, molecular characterization, RFLP of 18S rDNA, thermophilic fungi.

Abstract

Aims: Molecular characterization of commercially important group of xylanase producing thermophilic/thermotolerant fungi.

Methods and Results: DNA from 16 thermophilic/thermotolerant fungal isolates was amplified by PCR using three sets of primers: (i) internal transcribed spacer sequence (ITS1-5.8S-ITS5), (ii) D1/D2 hyper variable region of 26S rDNA and (iii) 18S rDNA region. The amplified products of internal transcribed spacers (ITS) and D1/D2 region were sequenced and analysed using CLUSTALX, whereas, amplified 18S rDNA region was subjected to RFLP analysis based on restriction digestion with Rsal, Mbol and Hinfl.

Conclusions: The sequence based analyses of ITS1-5.8S-ITS5 as compared with D1/D2 region of 26-28S rDNA was found to be a better tool for phylogenetic resolution of thermophilic/thermotolerant fungi. The ITS1-5.8S-ITS5 sequence-based dendrogram indicates an early divergence of the alkaline active xylanase producing thermophilic fungal strains.

Significance and Impact of the Study: This study was the first report on phylogenetic characterization of thermophilic/thermotolerant fungi.

Introduction

Thermophilic fungi are unique group of moulds that include 30 odd species with an ability to thrive at temperatures between 45°C and 60°C (Maheshwari et al. 2000). These fungi are often associated with habitats such as retting guayule, mushroom compost, plant degrading materials, etc. The distribution, colonization and succession of thermophilic fungal population in compost is closely related with their ability to produce a variety of cell wall degrading enzymes (Sharma 1989) including cellulases, hemicellulases and other important hydrolases, i.e. amylases, lipases, acid proteases, etc. Xylanase, an important constituent of hemicellulases, which hydrolyses the xylan backbone of hemicellulosic fraction of plant cell wall, is considered biotechnologically important. The xylanases have a variety of industrial applications, i.e. clarification of juice and wine, starch separation and production of functional food ingredients, improving the quality of bakery products and animal feed biotechnology (Saha 2003). Xylanases with transglycosylation activities are also considered for tailor designing the drugs and preparation of neoglycoproteins (Eneyaskaya et al. 2003; Badhan et al. 2007). However, the biggest market for xylanases exists in bleaching of paper pulp where addition of thermostable alkaline active xylanases can replace up to 20–30% of chlorine and can reduce up to 50% organic halogens in the paper mill effluents (Bajpai et al. 1994). Some of the thermophilic fungi, Humicola insolens, Melanocarpus albomyces, Thermomyces lanuginosus have been reported to produce alkaline active thermostable xylanases (Dusterhoff et al. 1997; Saraswat and Bisaria 1997; Sonia et al. 2005). However, geographically diverse strains differ in their ability to produce xylanases (Chadha et al. 1999; Singh et al. 2000). Therefore, isolation, screening and cataloguing of diverse as well as novel isolates is an area of continued research interest.

Owing to their immense potential, novel xylanase producing indigenous thermophilic fungal strains must be
isolated and identified. Classical taxonomy based on colony morphology, hyphal structure and spore arrangement places the thermophilic fungi in one of the following classes, i.e. zygomycete, ascomycete and hyphomycete (Mouchacca 1997). However, morphological differences within strains grown on different media types and cultural conditions are usually observed. Moreover, the practice of interchangeably using names of asexual (anamorph) and sexual (teleomorph) stages of same fungus also results in confusion in identifying these fungi (Maheshwari & Mouchacca 1997) and a critical review on their physiology (Maheshwari et al. 2000) has highlighted these points. Therefore, it is timely relevant that the strains must be classified at molecular level.

The molecular analysis based on DNA sequences, recognized as the most reliable methods to reveal genetic relationships between the strains, could therefore, be unambiguously used to identify and evaluate the isolates at any taxonomic rank (Bruns et al. 1991). Various molecular approaches that can be used in the studies of evolutionary relationships and identification of filamentous fungi are ribosomal RNA (rRNA) sequence comparison, amplified rDNA restriction analyses (ARDRA) using restriction fragment length polymorphism (RFLP) and DNA-DNA complementarities (Taylor et al. 2000). Ribosomal DNA (rDNA) is one of the most conserved regions in the genome yet interspersed with variable regions that can be useful in deciphering phylogenetic divergence. This study for the first time reports the molecular characterization for taxonomic resolution of thermophilic (including thermotolerant) fungi, based on the alignment of amplified rDNA sequences of internal transcribed spacers (ITS) and hyper variable partial sequence of 26–28S region as well as RFLP-based analysis of 18S rDNA sequences.

**Materials and methods**

**Isolation of fungi**

Composting soil samples were collected from the sub-surface layers of soil (around 5–10 cm deep) from different areas of Amritsar region (Punjab, India) during different months of the year. The samples were suitably diluted and plated on YpSS (yeast potato soluble starch agar) medium of following composition (% w/v): Yeast extract, 0.4; K2HPO4, 0.1; MgSO4.7H2O, 0.05; soluble starch, 1.5; agar, 2.0; containing ampicillin (200 µg ml⁻¹) as antibacterial agent and incubated at 45/50°C for up to 7 days. The isolates were purified by repeated sub-culturing and maintained on YpSS agar slopes at room temperature. These cultures were identified and deposited at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India) as mentioned in Table 1. Some of these isolates were also studied for their xylanase producing capability and characterized for activity under alkaline pH (Ghatora et al. 2006).

**Extraction of DNA**

Fungal genomic DNA was extracted from finely powdered lyophilized mycelium (40 mg) and suspended in 550 µl of extraction buffer (50 mmol l⁻¹ Tris HCl, pH 8.0; 700 mmol l⁻¹ NaCl; 10 mmol l⁻¹ EDTA, 1% (v/v) β-mercaptoethanol and 1% (w/v) SDS) and 300 µl of equilibrated phenol. The contents were homogenized and incubated for 15 min at 65°C. The DNA in the aqueous phase was purified with repeated extractions using equal volumes of saturated phenol, chloroform, iso-amyl alcohol (PCI) mixture (25 : 24 : 1). The resultant DNA was precipitated with nine parts of ice-cold isopropyl alcohol and one part of sodium acetate (3 mol l⁻¹; pH 8.0) and kept at −20°C for 2 h, followed by centrifugation for 15 min at 8000 g. The resultant DNA pellet was rinsed with 70% (v/v) ethanol, air dried, suspended in 50 µl of sterilized double distilled water and stored at 4°C.

**PCR amplification**

DNA coding for ITS (ITS I and II) and the intervening 5-8S coding rDNA region was amplified using universal primers, ITS1 (5'-TCCGTAGGTGAAACTGCGG-3') and ITS4 (5'-TCCTCCGGCTTATGGATATGC-3'). The 18S rDNA region was amplified using primers NS1 (5'-GTAGTAGGTGAACCTGCGG-3') and NS8 (5'-TGGCAGGTTCACCTACGGA-3'). Whereas, the primer pair LR0R (5'-ACCCGCTGAACTTAAAGCC-3') and LR3 (5'-CGGTGTTTCCAA GACGCG-3') was used for amplification of hyper variable D1/D2 region of large subunit rDNA (26–28S) as described by White et al. (1999). The PCR amplification was carried out in 0.2-ml PCR tubes using an Eppendorf Mastercycler (Hamburg, Germany). PCR reaction mixture (50 µl) contained 25 µl of PCR mix (Genei, Bangalore, India), 2.5 µl of DMSO, 1 pmol l⁻¹ of each primer and 100 ng of DNA template. Thermal cycling conditions were as follows: initial denaturation (4 min at 95°C), followed by 30 cycles of denaturation (94°C for 50 s), annealing (51°C for 1 min), primer extension (72°C for 1 min), followed by final extension step for 10 min at 72°C. Amplification products were electrophoretically resolved on 1.4% (w/v) agarose gel containing ethidium bromide, using 1× TAE buffer at 70 V.

Restriction digestion of amplified DNA and analysis

Amplified 18S rDNA (0.5 µg) was digested with 2 units each of Rsal, (Biozymes, Espoo, Finland), HinfI and Mbol.
### Table 1: Characterization of thermophilic and thermotolerant isolates used in the study

<table>
<thead>
<tr>
<th>Culture</th>
<th>Morphological and microscopic characteristics</th>
<th>NCBI accession ITS-5-18S-ITSII</th>
<th>NCBI accession partial 26-285</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chaetomium thermophilum</em> MTCC 4891*</td>
<td>Characterized to have brownish black colonies on YpSS agar. Microscopic examination revealed wooly perithecum with branched terminal hairs.</td>
<td>EF550983</td>
<td>EF257371</td>
</tr>
<tr>
<td><em>Thermoascus aurantiacus</em> MTCC 4890*</td>
<td>Typically golden orange coloured colonies and reverse of the plate were brownish. The culture borne long branched conidiophores bearing irregular digitate philodes</td>
<td>EF550981</td>
<td>EU257370</td>
</tr>
<tr>
<td><em>Rhizomucor pusillus</em> MTCC 4895*</td>
<td>The culture showed deep neutral grey colonies with abundant dark sporangia. The apical portions of sporangiophore were sympodially branched and contained group of sporangia</td>
<td>EF550974</td>
<td>EU257379</td>
</tr>
<tr>
<td><em>Malbranchea flava</em> MTCC 4889*</td>
<td>The culture showed colonies with light yellow colour with pinkish tinge and dark brown pigmentation on the reverse of the plate. However, the hyphae showed typical coiled septate conidiophores on the terminal portions</td>
<td>EF550978</td>
<td>EU257368</td>
</tr>
<tr>
<td><em>Humicola insolens</em> MTCC 4617*</td>
<td>The culture showed dark brown to sooty black colonies on YpSS agar plates. It showed intercalary spores with lateral branches having chains/singly located aleurophores</td>
<td>EF550969</td>
<td>EU257375</td>
</tr>
<tr>
<td><em>Humicola fuscoatra</em> MTCC 6329*</td>
<td>Same as <em>H. insolens</em>, however spore shape differed</td>
<td>EF550968</td>
<td>EU257376</td>
</tr>
<tr>
<td><em>Mylceliphthora</em> sp. MTCC 6661*</td>
<td>The culture showed pale yellow colony, smooth pear shaped and ampulliform spores borne either by stalks or sessile</td>
<td>EF550985</td>
<td>EU257369</td>
</tr>
<tr>
<td><em>Melanocarpus albomyces</em> MTCC 3922*</td>
<td>The culture showed white cottony mycelia that grew profusely and touched the lid of plate within 2-3 days of incubation. The asporogenous hyphae coalesced afterwards and straw yellow exudates were observed</td>
<td>EF550980</td>
<td>EU257374</td>
</tr>
<tr>
<td><em>Mucor indicus</em> MTCC 6333*</td>
<td>Colony showed buff to antimony yellow colour with buff yellow to apricot yellow reverse, sporangiophores repeatedly branched sympodially with long branches</td>
<td>EF550975</td>
<td>EU257373</td>
</tr>
<tr>
<td><em>Thermomyces lanuginosus</em> D2W3*</td>
<td>The culture showed greenish grey to purple brown colour and typical wine red pigmentation at the reverse of agar plates. The mycelium was branched and septate with short aleurophores bearing terminal thick walled aleurospores</td>
<td>EF550981</td>
<td>EU257372</td>
</tr>
<tr>
<td><em>Corynascus sepedonium</em> MTCC 6490†</td>
<td>Colonies initially white, sometimes becoming pale brown, fluffy to felt, fairly dense throughout reverse cream-coloured. Conidia borne singly on short, narrow protrusions or 1-2 may be borne on globose, frequently ampulliform swellings</td>
<td>EF550982</td>
<td>EU257377</td>
</tr>
<tr>
<td><em>Acrophialophora rainiana</em> MTCC 6662†</td>
<td>Colonies growing rapidly, greyish-brown, reverse almost black. Hyphae pale brown, conidiophores arising singly, terminally and laterally on the hyphae, straight or slightly flexuosely, bearing ampulliform globular spores</td>
<td>EF550976</td>
<td>EU257378</td>
</tr>
<tr>
<td><em>Emericella nidulans</em> var. <em>nidulans</em> MTCC 6339†</td>
<td>The colonies were dark green with orange to yellow in areas of cleistothecial production. Reverse was purplish to olive. Hyphae are septate and hyaline. Conidial heads are columnar. Conidiophores are brown, with biseriate fruiting structures</td>
<td>EF550971</td>
<td>EU257370</td>
</tr>
</tbody>
</table>
Table 1

<table>
<thead>
<tr>
<th>Culture</th>
<th>Morphological and microscopic characteristics</th>
<th>NCBI accession</th>
<th>NCBI accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Emericella nidulans</em> var. <em>lata</em> MTCC 6327*†*</td>
<td>Same as above</td>
<td>EF550972</td>
<td>EU257370</td>
</tr>
<tr>
<td><em>Aspergillus caesitosus</em> MTCC 6326*†*</td>
<td>Mycelium extremely tough, tearing with difficulty, producing numerous dark green, hemispherical to loosely columnar heads in central colony areas, reverse colorless at first, becoming dark reddish purple in age, particularly dark dull yellow–green</td>
<td>EF550973</td>
<td>EU257370</td>
</tr>
<tr>
<td><em>Penicillium janthinellum</em> MTCC 6564*†*</td>
<td>Colonies showed tough, closely interwoven felt of fine hyphae, with delicately floccose surface irregularly wrinkled in central portions and radially furrowed in marginal colony areas at first white turning to greenish pale grey</td>
<td>EF550979</td>
<td>EU257370</td>
</tr>
</tbody>
</table>

*Thermophilic fungi; †Thermotolerant fungi.*

(Genei, Bangalore, India) as per manufacturer’s instructions. The digested products were resolved on 1.8% (w/v) agarose gels at 70 V and their molecular weights were determined using 100 bp/1 kbp ladder (Genei, Bangalore, India). Gels were photographed using Gene Snap (Gene Genius, Cambridge, UK) and analysed by Gene Tool software to calculate the molecular weight and amount of DNA followed by and matching of the DNA bands. The RFLP data were archived from the Gene Tool and further analysed using Gene Directory software, using restriction pattern of *Rhizomucor pusillus* amplified product as a reference track. A combinatorial cluster analyses of *Mbol, Rsal* and *Hinf I* digested products was performed by UPGMA (Unweighted Pair Group Method with Arithmetical Average) with Dice coefficient at 5% level to derive the dendrogram (Gene Genius, UK).

### Internal transcribed spacer sequence analysis

The purified amplified ITS region and D1/D2 hypervariable region of 26S–28S rDNA were sequenced by SPA services (Genei, Bangalore, India). The ITS sequences of different fungi were aligned, to each other as well as the sequences retrieved from NCBI databases, using multiple sequence alignment software (ClustalX). Dendrograms were generated using neighbour joining (NJ) plot and the boot strapping was carried using 100 replications. The ITS and D1/D2 variable region of 26S rDNA sequences were deposited with NCBI (Table 1).

### Xylanase production

The cultures were grown in 250-ml Erlenmeyer flasks that contained 50 ml of production medium of the following composition (% w/v): corn cobs 2-0; Oat spelt xylan 0-1, yeast extract 1-0, KH₂PO₄ 0-3, CaCl₂ 0-05, MgSO₄ 0-05 and 1% (v/v) of trace element solution containing (% w/v): (NH₄)₂SO₄ 0-2, KCl 0-5, CaCl₂ 0-1, MgSO₄ 0-5, ZnSO₄ 0-01 and CuSO₄ 0-005. The pH of the medium was adjusted to 6-0 prior to sterilization. The flasks were inoculated with two agar discs (6 mm in diameter) of 7–10 days old culture from YpSS agar plates and incubated under shaking conditions (120 rev min⁻¹) at 45/50°C up to 6 days (experiments were performed in triplicates). The crude enzymes were filtered and centrifuged (11 000 g) for 10 min. Xylanase activity was determined in the culture filtrates using birch wood xylan as substrate. The assay mixture containing 500 µl of 1% (w/v) birch wood xylan (×0502; Sigma, St Louis, MO) prepared in 50 mmol l⁻¹ sodium citrate buffer pH 6-5 and 500 µl suitably diluted enzyme was incubated at 50°C for 5 min. The reaction was stopped by addition of 3 ml dinitrosalicylic acid reagent and the contents were boiled for 15 min. The developed colour was read at 540 nm using Novaspec II spectrophotometer (Pharmacia). The amount of reducing sugars liberated was quantified using xylose standard. One unit of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose equivalents per minute.

### Isoelectric focusing gel electrophoresis and activity staining

The culture filtrates were concentrated using Amicon ultrafiltration cell fitted with PM-10 membrane (Millipore). These samples (protein-100 µg) were fractionated by isoelectric focusing (IEF) using 5% acrylamide gel containing 2-4% broad pH range (3-5–10-0) amphotile carrier ampholyte (Amersham Biosciences) in Mini-Protean II system (Bio-Rad). Ethanolamine (0-4% v/v) and sulphuric acid (0-2% v/v) were used as cathodic and anodic electrolyte solutions, respectively (Bhat and Wood 1989). IEF was
Molecular characterization of thermophilic fungi

M. Sharma et al.

carried out for 1 h each at constant voltage of 100 and 200 V followed by 500 V for 30 min. After fractionating the proteins on IEF, the gel in each lane was sliced (1-25-mm thickness). Each slice was incubated in 500 µl sodium citrate buffer (50 mmol l⁻¹, pH 6.0) for 72 h at 4°C. To identify alkaline active xylanase isoforms, the enzyme assay was performed against birch wood xylan prepared in Tris-HCl buffer (50 mmol l⁻¹, pH 9.0). Xylanase activity in the IEF gels was detected with 1% agarose replica containing covalently dyed RBB (Remazol Brilliant Blue)-Xylan prepared from oat spelt xylan (Sigma). Upon completion of electrophoresis, the gels were incubated in sodium acetate buffer (50 mmol l⁻¹, pH 6.0) for 30 min and then overlaid on RBB-Xylan containing gel for 30-60 min at 50°C. To avoid band diffusion, these gels were dried at 60°C.

Results

Sixteen different thermophilic/thermotolerant fungal strains, isolated from composting materials, were included in this study. The strains were characterized and identified on the basis of morphological features (Table 1) and further analysed at molecular level.

Phylogenetic analysis based on RFLP of 18S rDNA region

Full length 18S rDNA region of the fungal isolates was amplified using NS1–NS8 primer pair. The PCR product ranged between 1.6 and 1.7 Kb in size. The amplified product from different fungal isolates was digested with Mbol, HinfI and Rsal to study the restriction pattern and perform ARDRA. The individual RFLP patterns of 18S rDNA sequence of different isolates are shown in Figs 1–3. The digested products resolved on 1-8% (w/v) agarose gel showed 1–4 visible bands of varying in sizes. The combined restriction pattern of the amplified 18S sequences of rDNA obtained using Mbol, HinfI and Rsal was used for cluster analyses by UPGMA. The combined cluster analyses resulted in a dendrogram (Fig. 4) with higher taxonomic resolution than that achieved by restriction pattern based on individual restriction enzyme (data not shown). The dendrogram revealed three distinct clades with members of mucorales, i.e. R. pusillus and Mucor indicus forming an out group. Other 14 fungal strains were resolved in two distinct clades. Where, H. insolens, H. fuscoatra, A. nainania showing high similarity were clustered together, whereas, T. lanuginosus showed an earlier divergence within this group. The other clade was characterized with Malbranchea flavia showing an early divergence, while, Myeliophththora sp. and Corynascus sepedonium showing high similarity coefficient were clubbed together.

Taxonomic relatedness based gene sequence of amplified ITS region and D1/D2 hyper variable region of 26S rDNA sequences

Using ITS1 and ITS4 primer pair, the rDNA containing the ITS1, ITS2 and the intervening 5·8S rRNA gene region was amplified from all the fungal isolates. The PCR product of amplified ITS region was approximately 530–580 bp except in M. indicus (630 bp). The dendrogram based on ITS1–ITS4 sequence analysis (Fig. 5) showed that thermostolerant fungi belonging to Aspergilli


© 2008 The Authors

Molecular characterization of thermophilic fungi


and Penicillii were clustered separately. However, some of the strains of genus Penicillium were paraphyletic and clubbed with Talaromyces flavus. Thermoascus aurantiacus, a true thermophile showed distinct and earlier genetic divergence as compared with thermotolerant Thermoascus crustatus and Byssochlamys verrucosa isolates. T. lanuginosus also a thermophilic deuteromycete fungus showed much earlier phylogenetic divergence as compared with other members of this clade. Similarly the thermophiles Melanocarpus albomyces and M. flavus also showed earlier phylogenetic divergence in their respective clades and were also characterized by longer branch lengths. H. insolens, H. fuscoatra, and Scytalidium thermophilum showed no molecular differences and were supported by high bootstrap value of 100. C. sepedonium, A. nainiana and Myceliophthora sp., were clustered together indicating close genetic relatedness. Their close relatedness was also supported by morphological features of bearing sessile conidia with lateral ampulliform stalks. The overall topography of dendrograms constructed on the basis of nucleotide sequences of ITS and D1/D2 hypervariable region of 26S rDNA were similar, except few exceptions, with R. pusillus and M. indicus forming an out group (Figs 5 and 6).

Diversity of xylanases

The fungal isolates included in this study produced diverse and multiple xylanases as observed in the zymogram developed against IEF gels (Fig. 7). These xylanases were characterized to be highly basic to acidic pI of 9.7–3.4. Screening of the fungal isolates for identifying hyper xylanase producers showed that thermophilic fungal isolates, T. lanuginosus, M. albomyces, C. thermophilum and Malbranchea sp. produced 1690, 2642, 1622 and 1417 units ml⁻¹ of xylanase, respectively. The observed activity levels were appreciably higher as compared with thermotolerant isolates (Table 2). Interestingly, few or all of the xylanases isoforms produced by these four isolates were characterized to be alkaline active (data not shown). These isolates showed distinct and early phylogenetic origin (Figs 4 and 5).
Molecular characterization of thermophilic fungi

Figure 5 ITS sequence-based phylogenetic tree of thermophilic fungi. A consensus NJ dendrogram with bootstrap values was based on multiple sequence alignment using ClustalX program.

Discussion

Thermophilic fungi are usually cultured on YpSS agar and are identified on the basis of morphological characteristics, i.e. colony coloration, pigmentation on the reverse side of plate, colony morphology, spore structure and arrangement, etc. (Cooney and Emerson 1964; Mouchacca 1997). This study reports the molecular characterization of xylanase producing thermophilic/thermotolerant fungi isolated from composting soils. Three alleles namely, 18S rDNA, ITS region and D1/D2 hyper variable region were considered for phylogenetic studies. Earlier studies using combination of two alleles, i.e. either 18S rRNA and ITS (Morakotkarn et al. 2007) or ITS and 26S rRNA (Voigt et al. 1999; Chen et al. 2001) have been used to delineate bamboo associated fungi, medically important yeast and zygomycetes, respectively. Upon comparing the dendro- grams of ITS region gene and D1/D2 region of 26S rDNA, similar topologies were observed (Figs 5 and 6). However, ITS gene tree was more robust as more nodes within ITS tree received higher measure of support from bootstrapping. Moreover, the branch lengths showed deeper nesting and better resolution in ITS tree as compared with 26S rDNA region. Similar observations have been made previously (Voigt et al. 1999; Chen et al. 2001). Furthermore, ARDRA-based analysis of the fungi was found to be rapid and reliable method of delineating thermophilic fungi. The RFLP pattern of 18S rDNA and ITS region have been used previously for molecular identification of sapstain fungi (Kim et al. 1999), air-borne fungi (Wu et al. 2003) as well as fungi associated with vascular discoloration of soybean (Harrington et al. 2000). However, in comparison with DNA sequences, no databases for RFLP pattern exist so these strains can only be compared amongst the isolates being included in study using well characterized reference strains.

© 2008 The Authors
The isolates included in this study showed differences in their abilities to produce xylanases in the production medium. Interestingly, thermophilic strains produced higher levels of xylanases when compared with thermotolerant strains. The fungi included in this study produced diverse and multiple xylanase isoforms with distinct catalytic as well as physiological properties (Badhan et al. 2004; Ghatora et al. 2006) that may act in tandem or sequentially to achieve superior hydrolysis of heterogeneous xylan containing substrates present in the compo-
Table 2 Production of xylanase by thermotolerant and thermophilic fungi

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Xylanase activity (units ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermophilic fungi</td>
<td></td>
</tr>
<tr>
<td>Chaetomium thermophilum</td>
<td>162.2 ± 8.3</td>
</tr>
<tr>
<td>Myceliophthora sp.</td>
<td>92.6 ± 6.2</td>
</tr>
<tr>
<td>Humicola fuscaatra</td>
<td>12.9 ± 1.2</td>
</tr>
<tr>
<td>Humicola insolens</td>
<td>17.2 ± 2.5</td>
</tr>
<tr>
<td>Malbranchea flav</td>
<td>141.7 ± 9</td>
</tr>
<tr>
<td>Melanocarpus albomyces</td>
<td>264.2 ± 11</td>
</tr>
<tr>
<td>Thermoascus aurantiacus</td>
<td>183.3 ± 2.5</td>
</tr>
<tr>
<td>Thermomyces lanuginosus</td>
<td>1650 ± 23</td>
</tr>
<tr>
<td>Thermotolerant fungi</td>
<td></td>
</tr>
<tr>
<td>Penicillium janthinellum</td>
<td>265 ± 1.5</td>
</tr>
<tr>
<td>Acrophialophora naniana</td>
<td>9.7 ± 1.0</td>
</tr>
<tr>
<td>Aspergillus caespitosus</td>
<td>257 ± 2.1</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>380 ± 4.2</td>
</tr>
<tr>
<td>Emericella nidulans var. lata</td>
<td>312 ± 2.3</td>
</tr>
<tr>
<td>Corynascus sepedonium</td>
<td>84 ± 4.2</td>
</tr>
</tbody>
</table>

Culture conditions: 250-ml Erlenmeyer flasks contained 50 ml of production medium; incubation temperature: 45/50°C; shaking at 120 rev min⁻¹; incubation time: 6 days.

sting materials (Wong et al. 1988). However, marked variation in the capability of geographically distinct strains to produce xylanases exists. Some of the strains of *T. lanuginosus* have been found to be prolific producers of xylanase, while others producing low levels of xylanase have also been reported previously (Chadha et al. 1999, Singh et al. 2000; Sonia et al. 2005). At phylogenetic level, this can be explained by observed lower bootstrap values at the nodes between different *T. lanuginosus* strains (Fig. 5) which indicates possible presence of cryptic sexual cycle or existence of horizontal flow of genetic information between strains (Hampf et al. 2001). The RAPD-based analysis has also pointed to the genetic variation between xylanase producing strains of *T. lanuginosus* (Singh et al. 2000). The RFLP and sequence-based data in this study also confirmed relative position of *H. insolens* and *H. fuscaatra* to be the variants of *S. thermophilum* (Mouchacca 1997). The genetic analysis based on ITS sequences (Boonlue et al. 2003) also suggested that these isolates cannot be resolved into distinct species.

The molecular characterization can also be a useful tool to phylogenetically relate the fungi on the basis of their characteristic morphological features (Fliegerova et al. 2004) as well as physiological and functional aspects. For example, the isolates producing alkaline active xylanases showed distinct and an early divergence within their respective clades. Similarly, two zygomycete fungi (*M. indicus* and *R. pusillius*), that expectedly formed out group during present study, and classified as mucorales 2 (Moncalvo 2005) are considered to have an early phylogenetic origin as compared with ascomycetes, were found to be good sources of phytases (Chadha et al. 2004; Gulati et al. 2007).

Acknowledgement

The financial support in the form of research project (BT/PR-5348/PID/04/178/2004) from Department of Biotechnology, Ministry of Science & Technology, Government of India is duly acknowledged.

References


Purification and characterization of two thermostable xylanases from *Malbranchea flava* active under alkaline conditions

Manju Sharma, Bhupinder Singh Chadha*, Harvinder Singh Saini

Department of Microbiology, Guru Nanak Dev University, Amritsar, Punjab, India

**Article Info**

- **Article history:**
  - Received 15 January 2010
  - Accepted 11 June 2010
  - Available online 13 July 2010

- **Keywords:**
  - Xylanases
  - Malbranchea flava
  - Peptide mass fingerprinting
  - Glycosyl hydrolases family 11
  - Glycosyl hydrolases family 10

---

**Abstract**

Two xylanases, MFXI and MFXII, from the thermophilic fungus *Malbranchea flava* MTCC 4889 with molecular masses of 25.2 and 30 kDa and pIs of 4.5 and 3.7, respectively were purified to homogeneity. The xylanases were optimally active at pH 9.0 and at 60 °C, exhibited a half-life of 4 h at 60 °C, and showed distinct mode of action and product profiles when applied to birchwood, oat spelt, and larchwood xylan, and to wheat and rye arabinoxylan. The xylanases were most active on larchwood xylan with *Km* values of 1.25 and 3.7 mg/ml. *Km/Kcat* values suggested that the xylanases preferentially hydrolyzed rye arabinoxylan. LC-MS/MS (liquid chromatography/mass spectrometry) analysis of tryptic digests of MFXI and MFXII revealed similarity with known fungal xylanases and suggests that they belonged to the GH 11 and 10 glycosyl hydrolase super families, respectively. These xylanases can potentially be used in enzyme-assisted bleaching of the pulp derived from agro-residues, as well as production of xylooligosaccharides for pre-biotic functional food applications.

© 2010 Elsevier Ltd. All rights reserved.

---

**1. Introduction**

Xylan is the second most abundant biopolymer in the plant cell wall and the major hemicellulosic polysaccharide. It is composed of a homopolymeric β-1,4-D-xlyopyranosyl backbone substituted to varying degrees with glucopyranosyl, 4-O-methyl-D-glucurono-pyranosyl, 3-O-arabinofuranosyl, acetyl, feruloyl and o-coumaroyl side chain residues (Saha. 2003). The complete hydrolysis of xylan requires the action of main and side chain-cleaving enzymes including endo-β-1,4-xylanase (EC 3.2.1.8), β-D-xylanidase (EC 3.2.1.37), -arabinofuranosidase (EC 3.2.1.20), acetyl xylan esterase (EC 3.1.1.72) and ferulic or p-coumaric acid esterase (EC 3.2.1.73) (Shallom and Shoham, 2003; Collins et al., 2005). Xylanases have potential uses in the food, feed, biofuels and pulp and paper industries (Dutta et al., 2007), and currently, the most promising application is in the prebleaching of pulps, mainly because of a desire to replace chlorine as a bleaching agent. This application requires that the xylanases are active and stable at high temperatures under alkaline conditions (Beg et al., 2001). Furthermore, paper mills in India are using rice and wheat straw as substrate for pulping, and since these agro-residues are rich in arabinoxylans, xylanases capable of showing high activity against arabinoxylans are desirable. In addition, xylanases can be used in hydrolysis of arabinoxylans for producing xylooligosaccharides (XOS) that are predominantly used as FOSHU (Foods for specified health use) in Japan and are being produced commercially from agro-residues in China and Japan (Vazquez et al., 2000). Many microorganisms have been investigated for their ability to produce endoxylanases that can degrade β-1,4-xylan in a random fashion and yield a series of linear and branched oligosaccharide fragments (Bhat and Hazelwood, 2001). Xylanases from thermophilic fungi such as *Thermomyces lanuginosus*, *Xylophilus thermophilus*, and *Thermosascus aurantiacus* have been studied for their potential industrial applications (Maheshwari et al., 2000; Singh et al., 2000). These xylanases are predominantly found in glycosyl hydrolase families GH 10 and GH 11 (Birley et al., 1997), but also in families 5, 8, 43 (Collins et al., 2005). Many microorganisms produce multiple endoxylanases. For example, 6, 10, and 12 types of xylanases are produced by *Chrysosporium lucknowense* (Ustinov et al., 2008), *Paenibacillus curdlanolyticus* B-6 (Pason et al., 2006) and a thermostolerant strain of *Myceliophthora* sp. (Badhan et al., 2007), respectively. The thermophilic fungus *Malbranchea flava* has previously been reported to produce xylanases that boost bleaching of pulps (Sonia et al., 2006). In the present study, two xylanases from this fungus were purified, characterized, and their ability to hydrolyze a variety of xylan types was determined.

**2. Methods**

**2.1. Cultivation and xylanase production**

*M. flava* MTCC 4889 (Microbial Type Culture Collection, Chandigarh, India) was grown on YpSs agar medium (Cooney and
Emerson, 1964) at 45 °C and stored at 4 °C. For xylanase production, solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks containing 5 g of rice straw and 17.5 ml of basal medium of the following composition (% w/v): yeast extract, 4.17; MgSO4·7H2O, 0.048; CaCl2·2H2O, 0.010; KH2PO4, 0.240. Prior to sterilization, the pH of the medium was adjusted to 7.0 with 1 N NaOH. The production medium was inoculated with 3 ml of a mycelial suspension from a 48-h-old culture grown under shaking conditions (150 rpm; 45 °C) in medium containing (% w/v): fructose, 1.5; yeast extract, 0.4; KH2PO4, 0.2; MgSO4·7H2O, 0.1; pH 7. SSF was allowed to proceed for 6 days under a water-saturated atmosphere at 45 °C.

2.2. Purification of xylanases from *M. flavus*

Fifty millilitre of sodium citrate buffer (0.05 M, pH 6.5) was added to the SSF culture and stirred gently at 45 °C for 1 h. The slurry was filtered through muslin cloth and centrifuged at 11,000g for 10 min at 45 °C. The filtrate was concentrated by ultrafiltration through a 10 kDa molecular weight cut-off membrane (Pall, USA) in a stirred cell (Millipore) and loaded on to a DEAE-Sepharose (Fast Flow) column (24 cm x 2.6 cm; Pharmacia), equilibrated with 20 mM Tris–Cl buffer (pH 6.5). The column was eluted first with two bed volumes of this buffer followed by a linear gradient of 0–1 M NaCl in 20 mM Tris–Cl buffer (pH 6.5) at a flow rate of 1 ml min–1. One xylanase peak was obtained during the isocratic and five during the NaCl gradient elution. The fractions corresponding to peak I were pooled, concentrated, desalted and equilibrated with 1.0 M (NH4)2SO4 in 50 mM acetate buffer (pH 5.0). The sample was applied to a Phenyl Sepharose 6 FF (Amersham Biosciences) hydrophobic interaction column (20 cm x 1.6 cm). The column was eluted with a linear gradient of (NH4)2SO4 from 1 to 0 M in 50 mM acetate buffer (pH 5.0) at a flow rate of 1 ml min–1. The fractions were pooled and the enzyme, designated MFX I, was characterized. The bound enzyme fractions (peak V) of DEAE-Sepharose column obtained during elution with ~0.45 M NaCl were pooled, concentrated, equilibrated with 1.0 M (NH4)2SO4 in 50 mM acetate buffer (pH 5.0) and resolved on Phenyl Sepharose 6 FF column as described above. The active fractions corresponding to xylanase (MFX II) were pooled, concentrated and applied onto polybuffer exchanger (PBE-94, Amersham Biosciences) column equilibrated with 20 mM acetate buffer (pH 5.0). The column was eluted with a linear gradient of NaCl from 0 to 1.0 M in 20 mM acetate buffer (pH 5.0) at a flow rate of 0.2 ml min–1. The fractions corresponding to pure MFX II were pooled and characterized.

2.3. Protein and xylanase assays

The protein content in the chromatographic fractions was monitored at 280 nm (UV mini Shimadzu-1240, Japan) and in the crude extract it was estimated with the method of Lowry et al. (1951) using bovine serum albumin as a standard. Xylanase activity was estimated as reported by Chadha et al. (2004). The liberated sugars were analyzed by the dinitrosalicylic acid (DNS) method and quantified using xylose standard curve. One unit (U) of xylanase activity was defined as the amount of enzyme that liberated 1.0 µmol of xylose equivalents from xylan per minute. The specific activity was defined as U per mg of protein.

2.4. Biochemical characterization of xylanases

2.4.1. SDS–PAGE and iso-electric focusing (IEF)

The homogeneity and molecular mass of MFX I and MFX II were determined by SDS–PAGE (Laemmli, 1970) in a 12% resolving gel using the Mini-PROTEAN II system (BIORAD). Iso-electric focusing (IEF) was performed according to the instructions provided by Novex (Invitrogen, Life Sciences, USA) using a 5% acrylamide gel containing 2.4% narrow range pH range (3–5) ampholine carrier servalyte (SERVA, Germany). The cathode buffer contained 0.35% (w/v) arginine and 0.29% (w/v) lysine, whereas 10 mM phosphoric acid was used as anode buffer. IEF was carried out for 1 h each at constant 100 and 200 V followed by 500 V for 30 min.

2.4.2. Protein identification

Protein bands fractionated on SDS–PAGE were excised and peptide mass spectrometry analysis was performed by 2D Nano LC/MS (Agilent 1100 series) at the TCGA (The Centre for Genomic Application, New Delhi). Mass spectrometry data were compared with data in the NCBI and Swiss Prot databases using the Mascot search algorithm (Vafaldi et al., 2010).

2.4.3. Zymogram analysis

Zymogram analysis was performed with a modification of the method of Taylor et al. (2006). After SDS–PAGE, the gel was incubated in 80 ml of refolding buffer [20 mM piperazine-N,N-bis (2-ethanesulfonic acid), pH 6.8, 2.5% Triton X-100, 2 mM dithiothreitol (DTT), and 2.5 mM CaCl2] for 1 h at room temperature and held overnight in fresh refolding buffer at 4 °C. The gel was thoroughly washed with distilled water and placed on 0.5% Remazol Brilliant Blue (RBB)-xylan (Sigma) containing gels for 4 h at 50 °C.

2.4.4. Temperature and pH optima

The activity of the purified xylanases was determined in sodium citrate buffer 0.05 M pH 6.5 with birchwood xylan as substrate at temperatures between 30 and 90 °C. The optimal pH was determined by measuring the activity against birchwood xylan (1% w/v) between pH 2 and 10 with 0.1 M HCl–KCl (pH 2), sodium citrate (pH 3–6), sodium phosphate (pH 7 and 8), and Glycine–NaOH (pH 9 and 10) buffers at 50 °C.

2.4.5. Thermal and pH stability of xylanases

The purified xylanases (MFX I and MFX II) were incubated at 50, 60 and 70 °C or at pH 5, 7 and 9 in 0.05 M sodium citrate, sodium phosphate and Glycine–NaOH buffers, respectively, for 0–4 h, and subsequently assayed for residual xylanase activity.

2.4.6. Effect of metal ions and other compounds

The purified xylanases were incubated in 5 mM solution of MgCl2, NaCl, KCl, MnCl2, CuCl2, ZnCl2, FeCl3, CaCl2, EDTA, DTT, β-mercaptoethanol, NBS and SDS for 30 min at room temperature in sodium citrate buffer (0.05 M, pH 6.5). The xylanase activity was measured using birchwood xylan as substrate.

2.4.7. Avicel adsorbable xylanase

The purified xylanases were characterized for their ability to adsorb to avicel as described by Badhan et al. (2007).

2.4.8. Substrate specificity and determination of kinetic parameters

The substrate specificity was determined by replacing birchwood xylan in the assay mixture with oat spelt xylan (OSX), debranched arabinan (DAX), larchwood xylan (LWX), wheat arabinoxylan (WAX), rye arabinoxylan (RAX), barley methoxy cellulose (CMC), 4-O-methyl glucuronoxylan (MGX), lichenin, laminarin, and xyloglucan (XG) at 1% (w/v). Activities of MFX I and MFX II were determined towards 3 mM p-nitrophenyl derivatives, pNP-α-L-arabinofuranoside, pNP-α-L-arabinopyranoside, pNP-β-D-celllobioside, pNP-α-L-mannopyranoside, pNP-β-o-galactopyranoside, pNP-β-o-galactopyranoside, pNP-β-o-glucopyranoside, pNP-β-o-xylopyranoside and pNP-β-o-xylopyranoside prepared in sodium acetate (0.05 M, pH 5) (Chadha et al., 2010).
et al., 2004). One unit (U) of activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per minute at 50 °C. The Michaelis–Menten kinetic parameters (Km and Vmax) and kcat were determined against BWX, OSX, LWX and RAX as substrates using Lineweaver–Burke plots.

2.5. Hydrolysis studies

2.5.1. Thin Layer (TLC) and high-pressure liquid chromatography (HPLC)

Nine-hundred microlitre of 1% (w/v) xylan (BWX, OSX, LWX, RAX, WAX and MGX) in sodium citrate buffer (0.05 M, pH 6.5) were incubated with 3 U of MF XI or MFX II at 50 °C for 72 h. Samples were withdrawn at intervals of 24 h, freeze dried and re-dissolved in methanol. TLC (0.25-mm layers of silica gel F-254 plates, Merck, India) was performed using a mixture of ethyl acetate:acetic acid:water (3:2:1, v/v) as solvent system. The hydrolysis products were detected by spraying the TLC plates with diphenylamine reagent followed by heating at 100 °C for a few minutes. A mixture of α-xylose (Sigma), xylobiose, xylotriose, xylotetraose and xylopentoase (Megazyme, Ireland) was used as a standard. HPLC was carried out with the DIONEX system (USA) equipped with a P680 pump, a thermostatted column compartment (TCC) and a differential refractive index detector (RI-101, SHODEX). The PL HI-PLEX NA column (Varioan 300 × 7.7 mm) was maintained at 60 °C with water as a mobile phase at a flow rate of 0.2 ml min⁻¹. Sugars in the hydrolysates were identified using xylose, xylobiose, xylotriose, xylotetraose and xylopentoase as standards.

3. Results and discussion

3.1. Xylanases from M. flava

M. flava MTCC 4889 produced high levels of xylanase (~15,000 units/g dry weight substrate) after 6 days of cultivation on rice straw at 45 °C (Fig. 1). This activity was higher than that of other thermophilic/thermotolerant fungal strains, except for the xylanases of M. flava of other thermophilic/thermotolerant fungal strains, except for Talaromyces thermophila and T. lanuginosus (Sonia et al., 2005; Li et al., 2006). Two M. flava xylanases, MF XI and MFX II, were purified to homogeneity (Table 1). MF XI showed a specific activity of 1798 U/mg protein while that of MFX II was 965 U/mg protein (Table 1). The molecular mass and iso-electric points of MF XI were 25.2 kDa and 4.5, respectively, while the corresponding values (Table 1). The molecular mass and iso-electric points of MFX I were 21,287 Da and 4.69, respectively (Fig. 4). Similar stability has been observed under alkaline conditions for xylanases from T. thermophila (Maalej et al., 2009), Myceliophthora sp., and Talaromyces thermophila were similar or somewhat higher (70–80 °C), but the pH optima for these xylanases were lower (pH 6–8) (Singh et al., 2000; Chada et al., 2004; Maalej et al., 2009). MF XI and MFX II were relatively stable at 60 °C and pH 9.0, since they retained 46% and 50% of their activity after 4 h of incubation, respectively (Fig. 4). Similar stability has been observed under alkaline conditions for xylanases from T. thermophila (Maalej et al., 2009), Myceliophthora sp. (Chada et al., 2004) and Bacillus sp. (Gessesse, 1998), but the stability of the M. flava xylanases appears to be better than that of Aspergillus caesius xylanases (half-life of 27 and 90 min at 55 °C and pH 8.0, respectively) which have been reported as potentially useful for Kraft pulp bleaching (Sandrin et al., 2005). Since bleaching of pulp with xylanases in paper mills is usually carried out at 55 °C for 2 h at pH 8.5 in the pulp digester (Bernier et al., 1994) the xylanases from M. flava would be expected to be suited for these conditions.

3.2. Identification of purified xylanases

Purified protein bands were excised from the polyacrylamide gel for protein identification by mass spectrometry (LC/MS). The MF XI and MFX II were identified with significant protein scores (p < 0.05) from Mascot searches of peptide mass fingerprints (Vafiadi et al., 2010).

The nominal mass of the MF XI and its calculated pi value were found to be 21,287 Da and 4.69, respectively. These values were close to those determined experimentally. In contrast, the nominal mass of MFX II and its calculated pi values were found to be 38,255 Da and 5.14, respectively, and thus different from the experimentally determined values. In case of MF XI, three peptides obtained by trypsin digestion matched the peptide sequence of an endoxylanase belonging to GH family 11 from T. lanuginosus, while in case of MF X II, only one peptide matched the sequence of xylanase belonging to GH family 10 from Pencillium chrysogenum (Table 4). The respective match scores for MF XI and II were 114 and 58. The results indicate that MFX I and MFX II from M. flava belong to GH 11 and GH 10 super family glycosyl hydrolases, respectively.

3.3. Temperature, pH optima and stability

The purified xylanases (MF XI and MFX II) were optimally active at 70 °C and at pH 9.0 (Fig. 3). The temperature optima for xylanases from the thermophilic fungi T. lanuginosus, Myceliophthora sp., and Talaromyces thermophila were similar or somewhat higher (70–80 °C), but the pH optima for these xylanases were lower (pH 6–8) (Singh et al., 2000; Chada et al., 2004; Maalej et al., 2009). MF XI and MFX II were relatively stable at 60 °C and pH 9.0, since they retained 46% and 50% of their activity after 4 h of incubation, respectively (Fig. 4). Similar stability has been observed under alkaline conditions for xylanases from T. thermophila (Maalej et al., 2009), Myceliophthora sp. (Chada et al., 2004) and Bacillus sp. (Gessesse, 1998), but the stability of the M. flava xylanases appears to be better than that of Aspergillus caesius xylanases (half-life of 27 and 90 min at 55 °C and pH 8.0, respectively) which have been reported as potentially useful for Kraft pulp bleaching (Sandrin et al., 2005). Since bleaching of pulp with xylanases in paper mills is usually carried out at 55 °C for 2 h at pH 8.5 in the pulp digester (Bernier et al., 1994) the xylanases from M. flava would be expected to be suited for these conditions.

3.4. Effect of metal ions and other compounds

The activity of MF XI was affected positively by EDTA but not Ca²⁺. Reductions in activity were observed in the presence of Mn²⁺, Mg²⁺, and to a lesser extent of Fe³⁺, DTT, β-mercaptoethanol and SDS (Fig. 5). In contrast, the activity of MFX II was promoted in the presence of β-mercaptoethanol, EDTA, DTT and SDS, while inhibition was observed in by Fe³⁺ (60%), Cu²⁺ (26%) and Zn²⁺ (68%). The increased activity of xylanases in presence of EDTA indicates lack of metal ions in their active sites. The inhibition of xylanase (MFX II) in the presence of Zn²⁺ may be due to oxidation of a thiol group that affect the native structure of enzyme thus destabilizing the conformational folding of the enzyme (Ohimya et al., 1995). The presence of a potentially reactive thiol group in MFX II, and the conformational folding of the enzyme (Ohimya et al., 1995). The presence of a potentially reactive thiol group in MFX II, and the conformational folding of the enzyme (Ohimya et al., 1995). The presence of a potentially reactive thiol group in MFX II, and the conformational folding of the enzyme (Ohimya et al., 1995). The presence of a potentially reactive thiol group in MFX II, and the conformational folding of the enzyme (Ohimya et al., 1995).
Table 1

Summary of purification of endoxylanases from *M. flava* MTCC 4889.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (Units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (µmol min⁻¹ mg protein⁻¹)</th>
<th>Purification (Fold)</th>
<th>Yield (%) recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Sephose</td>
<td>67535</td>
<td>800</td>
<td>84.418</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Peak I (Phenyl Sepharose)</td>
<td>6180</td>
<td>6.8</td>
<td>908.82</td>
<td>10.76</td>
<td>9.150</td>
</tr>
<tr>
<td>Peak V (Phenyl Sepharose)</td>
<td>2880</td>
<td>1.6</td>
<td>1800</td>
<td>19.14</td>
<td>9.150</td>
</tr>
<tr>
<td>PRE-94</td>
<td>2622</td>
<td>16.00</td>
<td>163.93</td>
<td>134</td>
<td>338</td>
</tr>
<tr>
<td>(MFX I)</td>
<td>250</td>
<td>0.260</td>
<td>961.53</td>
<td>2130</td>
<td>6.615</td>
</tr>
<tr>
<td>(MFX II)</td>
<td>250</td>
<td>0.260</td>
<td>961.53</td>
<td>2130</td>
<td>6.615</td>
</tr>
</tbody>
</table>

Fig. 2. (A) SDS-PAGE of purified xylanases from *M. flava*. Lane M, standard protein marker in the order of increasing molecular mass (PMWM, Genes). Lane 1, purified MFX I. Lane 2, purified MFX II. (B) Zymogram to detect xylanase activity in purified MFX I (Lane 1) and MFX II (Lane 2). (C) Analytical iso-electric focusing for determining the pI of purified MFX I and MFX II from *M. flava*. MFX I (Lane 1), MFX II (Lane 2), pI standard (Lane 3).

3.5. Adsorption of xylanase to avicel

MFX I, but not MFX II, activity decreased upon incubation with avicel, suggesting the presence of a cellulose binding domain (CBD) in MFX I, but not MFX II. The presence of CBD in family 11 xylanases, that are known to be low molecular weight proteins, is rare; however, XYN B (GH 11) from *Penicillium funiculosum* has been reported to be modular, whereas, XYN C (GH 11) from the same strain typically lacked a modular structure (Alcocer et al., 2002). Previously, we have shown the presence of both modular and non-modular xylanases in a thermotolerant *Myceliothorpa* sp. producing 10 functionally diverse xylanase isoforms (Badhan et al., 2007).

3.6. Substrate specificity

3.6.1. Activity against polysaccharides and pNP substrates

MFX I showed appreciably higher activity towards RAX, WAX, and OSX, in that order, as compared to BWX whereas MFX II showed comparable activity against substituted xylans (OSX, WAX and RAX) and unsubstituted BWX (Table 2). Both xylanases showed lower activity against LWX (substituted with mannose and glucose) as well as 4-O-methyl-o-glucuronoxylan (substituted with 4-O-methyl-o-glucuronic acid) when compared to BWX. While, MFX II showed release of pNP from various aryl
substituted substrates, MFX I did not recognize any of the pNP substituted substrates (Table 2). The observed activity of MFX II was much higher against pNP cellolobioside (pNPC) than pNPX, a property characteristic of xylanases belonging to family 10 (van Tilbeurgh and Clayssens, 1985; Collins et al., 2005). Based on the broad substrate specificity of MFX II and the higher specificity of the MFX I towards substituted xylans, the enzymes can be classified as belonging to the GH 10 and GH 11 families, respectively (Biely et al., 1997). Interestingly MFX I also recognized debranched arabinan (β-1,4-o-arabinose polymer) as substrate, though it failed to recognize pNP-α-L-arabinofuranosidase indicating a multifunctional endo-type action of this xylanase. The kinetics study demonstrated that both xylanases showed higher catalytic activity (\( V_{\text{max}} \) and \( k_{\text{cat}} \)) against rye arabinoxylan when compared to birchwood xylan. Furthermore, the \( k_{\text{cat}}/K_{M} \) ratio suggested that MFX I degraded birchwood, larchwood, and oat spelt xylan and rye arabinoxylan more efficiently than MFX II. These data differ from those of

---

**Fig. 4.** Stability of MFX I (A–C) and MFX II (D–F) at different temperatures (50–70 °C) and at pH 5.0, 7.0, 9.0 as a function of time.

**Fig. 5.** Effect of metal ions and chemicals on the activity of MFX I and MFX II from *M. flavo*.
a recent finding that family 10 xylanases were catalytically more efficient than the family 11 xylanases of *C. lucknowense* (Ustinov et al., 2008). The xylanases (MFX I and II) differed in their affinity towards polysaccharides as evident from $K_m$ values. The observed lower $K_m$ values of both the xylanases towards LWX indicate their high affinity towards this substrate (Table 3).

### 3.7 Mode of action of purified MFX I and MFX II against different xylan types

The comparative mode of action of purified MFX I and MFX II on different xylan types were studied by analyzing the hydrolysis products by TLC (Fig. 6) as well as HPLC (Fig. 7). Hydrolysis of birch wood xylan (BWX), a glucuronoxylan mainly composed of 4-O-methylglucuronic acid in the ratio of 12:100 (Jacobs et al., 2001) with purified MFX I resulted in xylobiose ($X_2$) as the major product followed by xylotriose ($X_3$) as the other main product. In addition, larger xylotriosyl oligomers $X_5$ as well as iso-$X_5$ putatively identified as aldopentaenic acid were detected. In contrast, hydrolysis of BWX with MFX II, resulted in $X_2$ as major product followed by xylotetraose ($X_4$), in addition an isomeric product between $X_4$ and $X_5$, putatively identified as aldotetraenic acid was observed. The absence of $X_3$ and $X_5$ was evident in the hydrolysis products of MFX II. The observed product profile is consistent with the characteristic products formed during hydrolysis of methylglucuronoxylan by xylanases belonging to family GH 11 and 10, respectively (Biely et al., 1997; Kolenova et al., 2006). Similar hydrolysis products were observed during hydrolysis of BWX with xylanase from an alkalophilic *Cephalosporium* sp. (Kang et al., 1996), however, Lv et al. (2008) observed that an alkaline active xylanase purified from

**Table 2**

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>(Relative activity) MFX I</th>
<th>MFX II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birchwood xylan</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oat spelt xylan</td>
<td>132</td>
<td>106</td>
</tr>
<tr>
<td>Rye arabinoxylan</td>
<td>165</td>
<td>96</td>
</tr>
<tr>
<td>Wheat arabinoxylan</td>
<td>147</td>
<td>110</td>
</tr>
<tr>
<td>Larchwood xylan</td>
<td>85</td>
<td>87</td>
</tr>
<tr>
<td>Methyl glucuronoxylan</td>
<td>83</td>
<td>71</td>
</tr>
<tr>
<td>Xyloglucan</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Debranched arabinan</td>
<td>30</td>
<td>3.6</td>
</tr>
<tr>
<td>Laminarin</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Lichenan</td>
<td>13</td>
<td>0.0</td>
</tr>
<tr>
<td>Carboxy methyl cellulose</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pNP-β-D-xlyopyranoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-β-D-cellobioside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-β-D-galactopyranoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-α-D-galactopyranoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-β-D-glucopyranoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-α-D-mannopyranoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-α-L-arabinofuranoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-α-D-arabinopyranoside</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pNP-acetate</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* and ++, intensity of detectable released colour of pNP. - , no release of pNP detected.
Table 3

<table>
<thead>
<tr>
<th>Xylan</th>
<th>Xylanase</th>
<th>$K_m$ (mg ml$^{-1}$)</th>
<th>$V_{max}$ (µmol min$^{-1}$ mg protein$^{-1}$)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (ml min$^{-1}$ mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birchwood</td>
<td>MFX I</td>
<td>6.6</td>
<td>3333</td>
<td>42,012</td>
<td>6365.5</td>
</tr>
<tr>
<td>Xylan</td>
<td>MFX I</td>
<td>5.0</td>
<td>1923</td>
<td>21,449</td>
<td>4289.7</td>
</tr>
<tr>
<td>Rye arabino-</td>
<td>MFX I</td>
<td>6.0</td>
<td>5000</td>
<td>63,025</td>
<td>10,504</td>
</tr>
<tr>
<td>Xylan</td>
<td>MFX II</td>
<td>10.0</td>
<td>3846</td>
<td>42,897</td>
<td>4289.7</td>
</tr>
<tr>
<td>Larchwood</td>
<td>MFX I</td>
<td>1.25</td>
<td>1666</td>
<td>20,999</td>
<td>10,799</td>
</tr>
<tr>
<td>Xylan</td>
<td>MFX II</td>
<td>3.7</td>
<td>1923</td>
<td>21,449</td>
<td>5797</td>
</tr>
<tr>
<td>Oat spelt</td>
<td>MFX I</td>
<td>10.0</td>
<td>5000</td>
<td>63,025</td>
<td>6302.5</td>
</tr>
<tr>
<td>Xylan</td>
<td>MFX II</td>
<td>6.6</td>
<td>2564</td>
<td>28,587</td>
<td>4331.4</td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th>Name</th>
<th>Pred MW/pl</th>
<th>Expt MW/pl</th>
<th>Peptides matched</th>
<th>Closest relative</th>
<th>Match score</th>
<th>% Sequence coverage</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFX I</td>
<td>21287/4.69</td>
<td>25210/4.5</td>
<td>3</td>
<td>Thermomyces lanuginosus</td>
<td>114</td>
<td>17</td>
<td>AGLNNGDHY YQIVATEYG DGYAR</td>
</tr>
<tr>
<td>MFX II</td>
<td>35255/5.14</td>
<td>30000/3.7</td>
<td>1</td>
<td>Penicillium chrysogenum</td>
<td>58</td>
<td>6</td>
<td>ITVADVG</td>
</tr>
</tbody>
</table>

Fig. 7. HPLC chromatogram showing profile of hydrolysis products obtained by the action of MFX I against different substrates. (A) standard, (B) OSX, (C) BWX, (D) WAX.

The microbial community produced X$_1$, X$_2$, and X$_3$ as the hydrolysis products from BWX, while xylanase from alkaliphilic Bacillus firmis yielded X$_1$, X$_2$, and X$_3$ as the products (Tseng et al., 2002). The observed differences in the hydrolysis products obtained from BWX by the xylanases of GH 11 may be due to differences in the composition of BWX used by these workers as MALDI mass spectrometry has revealed that 4-O-methylglucuronic acid (4-O-MeGlcA) ratio in BWX may vary from 5:100 to 12:100 and furthermore, the distribution of the 4-O-methyl glucuronic acid residues is irregular along the xylan backbone chain (Jacobs et al., 2001). The hydrolysis of LWX with MFX I resulted in similar products to those identified with BWX as substrate. The hydrolysis of LWX with MFX II, however, showed the presence of X$_1$, X$_2$, iso-X$_3$, and iso-X$_4$ as substituted products as well as X$_5$. Upon hydrolysis of LWX with xylanase (GH 10) from finger millet, the presence of X$_2$, X$_3$, and iso-X$_4$ (arabinosyl substituted xylotriose) were observed (Chithra and Muralikrishna, 2008). Khandke et al. (1989) identified X$_1$, X$_2$, and aldotetrauranonic acid as the hydrolysis products of LWX with xylanase (GH 10) from T. aurantiacus. The differences in the products observed may be due to differences in the extraction procedures followed for separating larch wood xylan. LWX is reportedly composed of arabinogalacturonoxylan with an ara/4-O-MeGlcA ratio of 10:12:100 (Jacobs et al., 2001), whereas, according to Li et al. (2000), it is composed of xylogalactomannan. Jacobs et al. (2001) have shown that LWX contains arabinogalacturonoxylan as well xylogalactoglucomannan. When LWX is treated with potassium hydroxide, galactoglucomannan precipitates and the clear filtrate containing arabinogalacturonoxylan is separated eventually using ethanol/acetic acid. The
hydrolysis of OSX with MFX I resulted in X₃ > X₄ > X₅ > X₆ as the main products. In addition, minor levels of iso-X₃ and iso-X₅ were evident (Fig. 5). X₆ was not produced during hydrolysis of OSX with MFX II. The production of shorter xylooligosaccharides during hydrolysis of OSX by GH 10 than by GH 11 enzymes from Trichoderma sp. has previously also been observed (Chen et al., 2009). Hydrolysis of RAX with MFX I produced X₂ and X₆ as the predominant products with lower amounts of X₁ and X₅ whereas, MFX II yielded X₁ and substituted X₂, X₃ and X₄ xylooligosaccharides (XOS). Similarly, hydrolysis of WAX, which is highly substituted with arabinose, with MFX I yielded unsubstituted products (X₁~X₅), whereas, MFX II resulted in X₃ as major unsubstituted product along with iso-X₃ and iso-X₄, iso-X₅ as the major substituted products. The presence of unsubstituted X₁~X₅ and substituted arabinoxylooligosaccharides (AXOS) such as iso-X₃ and iso-X₅ during hydrolysis of WAX with endoxylanase I (GH 11) and the presence of mainly substituted XOS upon hydrolysis with endoxylanase III (GH 10) from A. awamori have also been shown previously (Kormelink et al., 1993). The XOS reportedly possess important pre-biotic properties that support the growth of Bifidobacterium sp., while, arabinoxylooligosaccharides (AXOS) with higher average DP (around 5) suppressed the formation of short chain fatty acids (SCFA) which has desirable effects on the human gut bacteria (Craeyveld et al., 2008). Similarly aldopentauronic acid (4-O-MeGlcA-X₄) formed during hydrolysis of methylglucuronoxylan by xylanases (GH 11) has been shown to possess anti proliferative properties (Tramice et al., 2009).

4. Conclusion

M. flavus is good source of alkaline active thermostable xylanases with high activity on substituted arabinoxylans and potential for use in enzyme-assisted bleaching of the pulp derived from agro-residues. The wide range of substituted and unsubstituted xylo-oligosaccharides obtained from different xylan types with these xylanases could potentially find applications as pre-biotics.

Acknowledgement

The financial support to B.S.C. by Department of Biotechnology, Ministry of Science and Technology carrying out this project (No. BT/PR-5348/PID/04/178/2004), is duly acknowledged.

References


Evaluation of Glycosyl Hydrolases in the Secretome of *Aspergillus fumigatus* and Saccharification of Alkali-Treated Rice Straw

Manju Sharma • Rohit Soni • Asiya Nazir • Harinder Singh Oberoi • Bhupinder Singh Chadha

Received: 13 June 2010 / Accepted: 9 August 2010 / Published online: 21 August 2010 © Springer Science+Business Media, LLC 2010

**Abstract** A thermotolerant *Aspergillus fumigatus* strain isolated from composting pile of mixed industrial waste was found to produce a spectrum of cellulase and hemicellulases when cultured on rice straw solidified substrate. The two-dimensional electrophoresis (2DE) resolved the secretome into 57 distinct protein spots. The zymograms developed against 2DE gels identified the presence of three β-glucosidases and five CBH1/EG1 isoforms in the secretome. The peptide mass fingerprinting of 17 protein spots by liquid chromatography mass spectrometry characterized the secretome into different glycosyl hydrolase families. The enzyme cocktail produced by *A. fumigatus* was capable of efficient hydrolysis of alkali pretreated rice straw (at 7% and 10% w/v) resulting in 95% and 91% saccharification, respectively.

**Keywords** Secretome • Peptide mass fingerprinting • Glycosyl hydrolases • Activity detection of CBH1/EGI and β-glucosidases in 2DE gels • Saccharification

M. Sharma • R. Soni • A. Nazir • B. S. Chadha (✉)
Department of Microbiology, Guru Nanak Dev University, Amritsar 143005, Punjab, India
e-mail: chadhabs@yahoo.com

M. Sharma
e-mail: manju_scorpian05@yahoo.co.in

R. Soni
e-mail: rohitsoni15@yahoo.co.in

A. Nazir
e-mail: asiya_nazir@yahoo.com

H. S. Oberoi
Central Institute of Post Harvesting Technology, Ludhiana 141004, Punjab, India
e-mail: hari_manu@yahoo.com
Introduction

Lignocellulosics in the form of agro-residues and forestry biomass constitutes potentially enormous source of feedstock for bioconversion into biofuel, feed, and specialty chemicals [1]. Lignocellulosics comprises of cellulose, hemicellulose, and lignin that are present as intertwined complex fibril macromolecular structure. The structural heterogeneity in terms of proportion of cellulose, hemicellulose, and lignin in different plant species as well as the spatial distribution of the constituent molecules is perhaps one of the major hindrances in developing universal enzyme-based bioconversion technologies for their optimal utilization [2, 3]. Nature is abound with a rich diversity of bacteria, fungi, and actinomycetes that cohabit in the ecological niches and produce a vast diversity of glycosyl hydrolases (cellulases and hemicellulases), lignin-degrading enzymes, and other supporting proteins in order to degrade complex lignocellulosic structure into monomeric/simpler sugar moieties [4]. One of the key enzyme of the plant cell wall degrading enzymes is cellulase, which is a complex of endoglucanases (1,4-β-D-glucan-4-glucanohydrolases, EC 3.2.1.4) that randomly cuts the internal bonds within cellulose polymer, exoglucanases (1,4-β-D-glucan cellobiohydrolases or cellobiohydrolases, EC 3.2.1.91) that processively remove cellobiose units from reducing and non-reducing ends of the cellulose polymer and β-glucosidases (cellobiase or β-D-glucoside glycohydrolase, EC 3.2.1.21) that catalyzes the conversion of cellobiose into glucose moieties [5]. Due to the heterogeneity and complex chemical nature of hemicellulose (xylan), its hydrolysis into simpler constituents (monomers, dimers, or oligomers) requires the action of a wide spectrum of enzymes with diverse catalytic specificity and modes of action. Therefore, it is not surprising that microorganisms produce an arsenal of hemicellulolytic enzymes [6]. Most important of these enzymes is endoxylanase (EC 3.2.1.8) that cleaves β-1,4 linked xylose backbone while β-xylosidase (EC 3.2.1.37) hydrolyses xyl-o-oligomers. In addition, a variety of debranching enzymes, i.e., α-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), acetyl xylan esterase (EC 3.1.1.72), α-galactosidases (EC 3.2.1.22), and β-mannosidases (EC 3.2.1.25) are required for efficient utilization of hemicellulosic fraction [7].

From biotechnological standpoint few of the fungi (Trichoderma reesei, Aspergillus niger, Acremonium cellulolyticus, and Penicillium decumbens) have been isolated and developed for producing high levels of the cellulases [8-10]. The cellulase production capabilities of an organism is usually determined for endoglucanase (CMCase), cellobiohydrolase, and β-glucosidase activities against chemically modified substrates [11], but these assays do not indicate the presence of specific GH families to which a particular component of the enzyme activity can be ascribed. Sometimes, even the enzyme extracts exhibiting higher activities against the substrates used for assay fail to perform efficient hydrolysis of the natural lignocellulosic substrates because of either lack of key component enzyme in the enzyme mix or due to the steric hindrances caused by the structural complexities of substrate in its native/pretreated form [2]. So, it becomes important to evaluate and characterize the strains for variety of glycosyl hydrolases for achieving efficient hydrolysis of specific kind of cellulosic feedstock substrate.

Over 200 known glycosyl hydrolases have been identified and classified in different families based on to their amino acid sequence similarity [12] and are clustered in carbohydrate active enzyme databases [13]. Each of the cellulolytic microbial strain produces a specific set of signature GHs depending primarily upon the genetic capacity as well as culture conditions employed for their production [14]. With the advent of proteomic-based technologies (MALDI TOF and liquid chromatography tandem mass spectrometry (LC MS/MS)), it has now become much easier to know the distribution and
prevalence of characteristically different GH and other plant cell wall degrading enzymes in different strains [15], which can immensely improve our understanding about the hydrolytic potential of enzyme mix vis-a-vis cellulotic substrate [16]. During the search for efficient cellulase-producing strains, we isolated a thermotolerant *Aspergillus fumigatus* fresenius strain that was found to produce cellulases optimally on solidified rice straw medium. The cellulases from the strain showed very good potential for deinking of the composite paper waste as well saccharification of Solka Floc and steam pretreated bagasse [17, 18]. This study reports the secretome analysis following two-dimensional electrophoresis (2DE) and LC MS/MS approaches to identify glycosyl hydrolases and other proteins produced by *A. fumigatus* under optimized culture conditions on solidified rice straw medium. The zymogram technique was employed to identify the β-glucosidases and cellobiohydrolases (CBH/EGI) in the 2DE gels, while diverse xylanases, endoglucanases, and acetyl esterases were identified using 1D (isoelectric focusing (IEF)) gels. Furthermore, the potential of the secreted proteins (cellulase/hemicellulase) for efficient hydrolysis of alkali-treated rice straw into fermentable sugars was also evaluated.

**Materials and Methods**

**Culture**

A thermotolerant fungal strain isolated from degrading paper/polythene composite industrial waste was identified as *A. fumigatus* fresenius (AMA) on the basis of morphological and molecular characterization [17]. The fungus was grown and maintained on yeast potato soluble starch of following composition (% ; w/v), starch 1.5, yeast extract 0.4, KH$_2$PO$_4$ 0.2, K$_2$HPO$_4$ 0.23, MgSO$_4$.7H$_2$O 0.05, citric acid 0.057, and agar 2.0. The pH of the medium was adjusted to 7.0. The fungus was cultured at 40 °C for 7 days and stored at 4 °C.

**Enzyme Production**

Solid state fermentation was carried out in Erlenmeyer flasks (250 ml) that contained ground rice straw as a carbon source (5 g) and basal medium BM (15 ml) of the following composition (% ; w/v) (NH$_4$)$_2$SO$_4$, 1.1, beef extract 0.25, KH$_2$PO$_4$, 0.4, Tween-80, 0.25. Prior to sterilization, the initial pH and moisture content of the medium were adjusted to 7.0 and 75%, respectively. The culture medium then was inoculated with a mycelial suspension (2 ml) of 24-h-old culture grown on glucose pre-cultured medium (% w/v; glucose, 1.5; yeast extract, 0.4; K$_2$HPO$_4$, 0.2; MgSO$_4$.7H$_2$O, 0.1; pH 7.0) and incubated in a water-saturated atmosphere at 45 °C for 5 days in an incubator. Thereafter, the enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks and kept at 45 °C for 1 h under mild shaking. The resultant slurry was filtered through muslin cloth and centrifuged at 8,800×*g* for 10 min, and the extracts were used for enzyme assay.

**Enzyme Assays**

The EG, xylanase, and polygalactouronase activities were determined using CM-cellulose (1% w/v), birch wood xylan (1% w/v), and polygalactouronic acid (0.24% w/v), prepared in sodium citrate buffer (50 mM, pH 6.0), as respective substrates. The reaction mixture (1 ml) containing equal amounts of suitably diluted enzyme and substrate was incubated at 50 °C.
for 10, 5, and 30 min, respectively. The reaction was stopped by addition of dinitrosalicylic acid followed by boiling [19]. The developed color was read at 540 nm using Novaspec II spectrophotometer (Pharmacia), and the amounts of released glucose, xylose, and galactouronic acid were quantified using respective standards. The avicel absorbable activity (AAEG) was assayed as described by Ref. [20]. The reaction mixture containing 0.5 ml of sodium acetate buffer (25 mM, pH 5.0), 0.5 ml of culture supernatant, and 100 mg of avicel was kept at 4 °C for 1 h. After centrifugation, the residual EG activity in the supernatant was measured as described above. AAEG was measured indirectly by subtracting avicel non-adsoibable EG activity from total EG activity. Total cellulase activity (Fpase) was measured by using Whatman No. 1 filter paper strip (1×6 cm) as substrate [21]. The β-glucosidase, cellobiohydrolase (CBHI/EGI), β-xilosidase, α-arabinofuranosidase, and β-mannosidase activities were assayed by micro-titer plate based method [22] using p-nitro phenyl-β-D-glucopyranoside, p-nitro phenyl-β-D-lactoside, p-nitro phenyl-β-D-xiloside, p-nitro phenyl-β-D-arabinofuranoside, and p-nitro phenyl-β-D-mannoside (pNPMa), as respective substrate. The reaction mixture contained 50 μl of sodium acetate buffer (50 mM, pH 5.0) and suitably diluted enzyme (25 μl), and the reaction was initiated by adding 25 μl of respective substrates (3 mM). The micro-titer plate was incubated at 50 °C for 30 min, and the reaction was terminated thereafter by adding 100 μl of NaOH-glycine buffer (0.4 M, pH 10.8). The developed yellow color was read at 405 nm using an ELISA Reader (MULTISKAN; Lab system). For assay of acetyl esterase and feruloyl esterase activities, the titer plate-based method [23] was employed. For estimation of CBHI fraction in CBHI/EGI, the assay was performed in the presence of 5 mM celllobiose so as to inhibit CBH I activity [24]. One unit of activity was expressed as the amount of enzyme required to release 1 μmol of pNP per minute under assay conditions. The resultant enzyme activities were expressed as unit per gram dry weight substrate.

Two-Dimensional Electrophoresis

The enzyme extract was desalted and concentrated using ultrafiltration Amicon cell fitted with PM-10 membrane (10-kDa cut off). Protein (150 μg) samples were loaded by passive in-gel rehydration at 20 °C in 125 μl rehydration buffer (8 M urea, 2% CHAPS, Destreak reagent, 1% IPG buffer pH 3–5.6 and 0.005% bromophenol blue in Milli Q grade sterilized water). The IPG strips (7 cm) were rehydrated for 16 h at room temperature in rehydration buffer. The IEF was performed using Etta IGPHor 3 system (GE, Healthcare Biosciences) in a stepwise manner using voltage hour program that increased linearly in the following steps: 100 V, 2 h; 300 V, 2 h; 1,000 V, 2 h; 5,000 V, 3 h (gradient); 5,000 V, 6 h (step), with a total of 51,000 V h. Prior to SDS-PAGE, the IPG strips were incubated for 15 min in 6 ml of 0.05 M Tris–Cl (pH 8.8), 8 M urea, 30% (v/v) glycerol, 2% (v/v) SDS, 60 mM dithiothreitol (DTT), and traces of bromophenol blue followed by incubation for 15 min in the same buffer except that DTT was replaced with 50 mM iodoacetamide. The equilibrated IPG strips were transferred onto 12% polyacrylamide gels without stacking gel and overlaid with 0.5% low melting agarose. The second dimension was run at constant of 25 mA. The electrophoresis was carried out using a Hoefer mini VE system (GE, Healthcare Biosciences), and the gels were stained using silver nitrate.

Zymograms for Detection of Enzyme Activities in 2DE and IEF Gels

For in-gel activity assay of β-glucosidase and CBHI/EGI, the resultant 2DE gels were incubated in 80 ml of refolding buffer (20 mM PIPES [piperazine-N,N-bis (2-
ethanesulfonic acid) buffer [pH 6.8], 2.5% Triton X-100, 2 mM DTT, and 2.5 mM CaCl$_2$) for 1 h at room temperature and then held overnight in fresh refolding buffer at 4 °C. The gels then were thoroughly washed with Milli Q grade sterilized water (18.2 MΩ) followed by incubation with 10 mM 4-methylumbelliferyl β-d-glucoside and 4-methylumbelliferyl β-d-lactopyranoside (MUL) prepared in sodium acetate buffer (50 mM; pH 5.0) at 50 °C for 45 min. Regions of enzymatic activity were visualized on an UV trans-illuminator. Similarly, β-glucosidase, CBH I/EG I, and acetyl esterase activity bands were visualized for protein samples resolved by IEF. IEF was performed according to the instructions provided by Novex, using 5% acrylamide gel containing 2.4% broad pH range (3–10.0) ampholine carrier ampholyte in a Mini-protein II system (Biorad). Ethanolamine (0.4% v/v) and sulphuric acid (0.2%, v/v) were used as cathodic and anodic electrolyte solutions, respectively [21]. Isoelectric focusing was carried out for 1 h each at constant voltage of 100 and 200 followed with 500 V for 30 min. After electrophoresis, the gels were incubated for 15 min in 0.05 M sodium acetate buffer (pH 5.0) and overlaid on polyacrylamide gel containing CMC and xylan (1%; w/v) for 2 h at 50 °C. The overlay gel was removed and stained with 0.2% Congo Red. Bands corresponding to EG and xylanase appeared as clear zone against a dark background after destaining with 1 M NaCl followed by treatment with 10% (v/v) acetic acid solution. For detection of acetyl esterases, the gels were incubated with pNP-acetate (3 mM) using methodology as described above for β-glucosidase and CBH/EGI.

Protein Identification

The purified protein bands fractionated by 2DE were excised and sent to TCGA (The Centre for Genomic Application, New Delhi) for peptide mass spectrometry analysis by LC/MS (Agilent 1100 series 2D NanoLC MS). Mass spectrometry data were compared with data in the NCBI and Swiss Prot databases using the Mascot search algorithm.

Enzymatic Hydrolysis

The enzyme produced by *A. fumigatus* was used for saccharification of alkali-treated rice straw (ground rice straw particle size 5–7 mm was treated with 1% NaOH at solid to liquid ratio of 1:15 at 15 psi for 20 min). The saccharification was carried out at substrate concentration of 7% and 10% (w/v) with enzyme loading rate of 10.27 FPU/g dry weight rice straw (pH 6.0 at 50 °C) for up to 96 h. The released sugars in the hydrolysates were analyzed using a HPLC system (DIONEX, USA) equipped with a P680 pump, a Thermostatted Column Compartment, and a differential refractive index detector (RI-101, SHODEX). The PL HI-PLEX NA column (Varian 300 × 7.7 mm) was maintained at 60 °C with water as a mobile phase at a flow rate of 0.2 ml min$^{-1}$. Sugars in the hydrolysates were identified using glucose, xylose, cellobiose, xylobiose, and cellotriose (Sigma-Aldrich) as standards.

Results and Discussion

Production of Glycosyl Hydrolases by *A. fumigatus* Fresenius

AMA isolated from composting industrial waste was found to produce cellulases capable of efficient deinking and enzymatic hydrolysis of Solka Floc and bagasse [17, 18]. The strain
on solidified rice straw culture medium produced a variety of glycosyl hydrolases (Table 1). The culture produced higher levels of cellulases (EG, CBHI, βG, and FPase) as well as xylanase activities when compared with the specific activities achieved by industrially important strains of *T. reesei* RUT C-30 [25] as well as *Acremonium cellulolyticus* [26]. The β-glucosidase of *A. fumigatus* was resistant to glucose inhibition as no loss of activity was observed in the presence of 300 mM glucose (data not shown). The β-glucosidases resistant to glucose are useful for carrying out saccharification of lignocellulosics at high substrate loading rates for obtaining higher concentration of sugars in the hydrolysates [27]. The culture also exhibited appreciable growth on PDA supplemented with 0.5% (w/v) of 2-deoxy glucose, a toxic analog of glucose, usually employed to isolate catabolite repression resistant strains/mutants [28]. *A. fumigatus* AMA thus exhibited industrially important traits for developing bioconversion technologies.

Two-Dimensional Electrophoretic Profiling

The crude enzyme extract, prepared as described in “Materials and methods”, was resolved by 2DE (Fig. 1). Fifty-seven proteins spots were detected in pI range of 3.0–5.6. The zymograms for detection of β-glucosidase and cellulase (CBHI/EGI) were developed by activity staining using MUG and MUL as substrates, respectively. The activity staining of the gel for β-glucosidases (Fig. 2a) showed the presence of activity in three distinct regions, with a major activity band (β-G I) appearing at pI ~3.2 and molecular weight of ~85 kDa. In addition, five β-glucosidase activity spots were detected; of these, four isoforms of apparently same molecular weight, but of different pI (β-G II), were found as a train of spots and an additional active β-glucosidase spot (β-G III) with pI ~5.6 was observed. The resolution of crude extract on single dimension pI gel indicated the presence of four β-glucosidases (Fig. 2b). Kim and coworkers [5] in a previous study detected two active β-glucosidases (β-G I and β-G II) in *A. fumigatus* proteome. However, there were evident differences in the intensity of β-G I band/spot observed by these workers and those observed in the present study. Kim and coworkers found that the intensity of the β-G I in 2DE zymogram diminished with increasing denaturant urea (7 M) concentration, whereas we observed intense active spot of β-G I even in the presence of 8 M urea. In addition to β-G I and β-G II, we could also localize β-G III isoform (Fig. 2a). The observed higher intensity of the β-G I activity spot and localization of an additional isoform may be attributed to the fact that 150 µg of protein was loaded in the present protocol as compared with 100 µg protein loaded in previous study [5]. Secondly, we did not subject the sample to deglycosylation, which is known to decrease the stability of glycoproteins [29]. For localizing CBHI/EGI (Fig. 3a), zymogram was developed using 4-methylumbelliferyl β-D-lactopyranoside (MUL). All five activity spots for CBHI/EGI activity were detected. This is the first report on localizing CBHI/EGI active spots in 2DE gels. Since MUL is not a specific substrate for assay of CBH I activity, therefore, it could not be ascertained whether the observed spots were of EGI or CBH I [25]. However, by simultaneously developing two zymograms against the proteins resolved on IEF gels (pI 3–10) for CBHI/EGI and endoglucanases using MUL and CMC as respective substrates [18, 30], we found that only one of the three bands, i.e., CBHI/EGI b (Fig. 2b) corresponds to CBHI while other two were apparently EGI (Fig. 4c).

Seventeen distinct protein spots (Fig. 1) in the pI range of 3.5–5.6 were picked for identification using LC MS/MS approach. The secretome (Table 2) showed the presence of different glycosyl hydrolases, including cellulases, hemicellulases, polygalactouronases, chitinase, as well as low molecular weight Asp-hemolysin and dipeptidyl peptidase. The
Table 1  Comparison of the glycosyl hydrolase activities (cellulase/hemicellulases) in the culture extract of *A. fumigatus* grown on solidified rice straw medium

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EG</th>
<th>AAEG</th>
<th>EGI</th>
<th>CBHI</th>
<th>β-G</th>
<th>FPase</th>
<th>Xylanase</th>
<th>β-Xyl</th>
<th>α-Ara</th>
<th>Acetyl esterase</th>
<th>Feruloyl esterase</th>
<th>β-Mann</th>
<th>PG</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fumigatus</em> (units/g substrate)</td>
<td>240</td>
<td>41.4</td>
<td>12.4</td>
<td>23.9</td>
<td>245</td>
<td>10.2</td>
<td>3,400</td>
<td>8.8</td>
<td>1.51</td>
<td></td>
<td></td>
<td></td>
<td>68</td>
</tr>
<tr>
<td><em>A. fumigatus</em> (units/mg protein)</td>
<td>18.4</td>
<td>3.1</td>
<td>0.95</td>
<td>1.8</td>
<td>18.8</td>
<td>0.78</td>
<td>261.5</td>
<td>0.67</td>
<td>0.12</td>
<td>3.6</td>
<td></td>
<td>1.3</td>
<td>5.2</td>
</tr>
<tr>
<td><em>T. reesel</em> [25] (units/mg protein)</td>
<td>8.94</td>
<td>ND</td>
<td>0.25</td>
<td>1.1</td>
<td>0.48</td>
<td>0.58</td>
<td>119.1</td>
<td>0.75</td>
<td>1.1</td>
<td>0.33</td>
<td></td>
<td>ND</td>
<td>4.5</td>
</tr>
<tr>
<td><em>A. cellulolyticus</em> [26] (units/mg protein)</td>
<td>4.52</td>
<td>ND</td>
<td>ND</td>
<td>0.26</td>
<td>1.2</td>
<td>0.66</td>
<td>12.4</td>
<td>0.017</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>1.1</td>
</tr>
</tbody>
</table>

β-G β-glucosidase, β-Xyl β-xylosidase, α-Ara α-arabinofuranosidase, β-Mann β-mannosidase, PG polygalacturonase activities, ND not determined

a The culture was grown on Solka Floc (SF)

b The mentioned activities in Ref. [25] have been changed from nkat/mg protein to units/mg protein for comparison
c Activity given as mannanase
d Activity as avicelase
secretome produced by lignocellulolytic strains *T. reesei*, *Phanerocheate chrysosporium*, and *Aspergillus oryzae* have been characterized previously [4, 14, 31, 32]. The proteome analysis of *A. fumigatus* has also been previously documented [33]; this, however, is the first report on the glycosyl hydrolases produced in the secretome of *A. fumigatus*.

Not surprising that all, 17 deduced peptide sequences showed a high degree of similarity ($E=0.0$) to the non-redundant protein sequences annotated from *A. fumigatus* Af 293 genome sequence [34, 35] taken from NCBI databases. However, the homology search with peptide sequences using Swiss Prot database showed only few matches with *A. fumigatus* protein sequences implying lack of reported work on protein sequences of cell wall degrading enzymes of this fungus. On the basis of homology search (Swiss Prot), 2DE resolved proteins spot 2 and spot 9 shown in Fig. 1 were identified as two distinct β-D-glucan cellobiohydrolases (CBH I) belonging to GH7 family that showed a high degree of similarity ($E=0.0$) to the protein sequences of *A. fumigatus* (accession nos. Q4WM08) and *Aspergillus aculeatus* (O59843.1) GH7, respectively. The CBH I corresponding to spot 9 have been purified to homogeneity and characterized (being reported elsewhere). CBH I are
the cellobiohydrolases that processively remove cellobiose units from the reducing end of the cellulose chain are the major component of cellulase in industrially important strains of *T. reesei* [36]. The protein spot (I) in Fig. 1 was identified as β-D-glucan glucanohydrolase (β-G) that belongs to super family GH-3 and showed homology to matched peptide sequence of β-glucosidase of *A. aculeatus* (Swiss Prot accession nos. P48825.1). Previously, Kim and coworkers [5] also arrived at the same conclusion during the proteome analysis of β-glucosidase from *A. fumigatus*. However, they estimated the molecular weight of the β-G I to be 240 kDa whereas we found it to be of 85 kDa, which was also confirmed through SDS-PAGE of the purified β-glucosidase (being reported elsewhere). Rudick and Elbein [37] had also previously shown that purified β-G I from *A. fumigatus* is a multi-subunit protein of 340 kDa. The deduced molecular weight of β-G I (85 kDa) in the present study closely fit into the description of being a tetramer. The protein spot 8 in the secretome (Fig. 1) was identified as endoglucanase (GH12) with close similarity to EG from *A. aculeatus* (Swiss Prot accession nos. P22669) (Table 2). The endoglucanase of GH family 12 is possibly the major EG isoform in *A. fumigatus*, which is known to lack CBM and can recognize a wide variety of substrates such as barley β-glucan, CMC and xyloglucan, laminarin, etc. [38, 39]. The culture extract also

**Fig. 3** a Detection of CBHI/EGI activities after 2DE of secreted proteins of *A. fumigatus* grown on rice straw. b Detection of CBHI/EGI activities in the crude extract of *A. fumigatus* after one-dimensional IEF (pH 3–10)
Table 2 Identification of the protein spots (shown in Fig. 1) in *A. fumigatus* secretome by LC/MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mascot score</th>
<th>Identified protein</th>
<th>Query matches</th>
<th>Matched peptides</th>
<th>Closest relative (Swiss Prot databases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>60</td>
<td>1,4-β-1-Glucan celllobiohydrolase GH7</td>
<td>1</td>
<td>TFYGPGMTVDTK</td>
<td><em>A. fumigatus</em> 293* XP751044.1</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>Arabinanase putative GH-43</td>
<td>1</td>
<td>AVEDYQFGWNQLK</td>
<td><em>A. fumigatus</em> 293* XP731479.1</td>
</tr>
<tr>
<td>4</td>
<td>130</td>
<td>N-Acetyl hexaminidase GH 20</td>
<td>4</td>
<td>APSSLQFVNVK, EGSDT IQTAK ILEQLDAMSLSK TYNDLSQYWVDHAVPIFR</td>
<td><em>A. fumigatus</em> XP 747307</td>
</tr>
<tr>
<td>5</td>
<td>243</td>
<td>β-Xylosidase GH-3</td>
<td>6</td>
<td>LAVCDTSLVTR LGYFDPAEDOPYR AAGEGIVLLK TLLLWATQAQGYDVK QADVVVFYAGIDNTIEAEGR ALGPYNTAALVSR</td>
<td><em>A. fumigatus</em> XP Q4WF6.1</td>
</tr>
<tr>
<td>6</td>
<td>134</td>
<td>β-Mannosidase GH-47</td>
<td>3</td>
<td>GPAADLVEDR, LSDLTGQEQYAK, ADLIDFGLK</td>
<td><em>A. fumigatus</em> XP 752825.1</td>
</tr>
<tr>
<td>8</td>
<td>118</td>
<td>Cellulase putative GH-12</td>
<td>5</td>
<td>RVSQWTSASYN, SYANSQVSLTK</td>
<td><em>A. fumigatus</em> Q4WBR5.1</td>
</tr>
<tr>
<td>9</td>
<td>88</td>
<td>1,4-β-1-glucan celllobiohydrolase GH7</td>
<td>2</td>
<td>LYLGPDKNYVMML, AQNPTTHVFSNIR</td>
<td><em>A. fumigatus</em> XP 731479.1</td>
</tr>
<tr>
<td>A</td>
<td>206</td>
<td>Asp hemolysin (aegerolysin superfamily)</td>
<td>4</td>
<td>TAPPGGSVNNSCGRS, DASSGTTGGF, DLYDGNNTNDFDVERTK, YGGAIGTVDVEVR</td>
<td><em>A. fumigatus</em> Q00050.2</td>
</tr>
<tr>
<td>B</td>
<td>161</td>
<td>Arabinofuranosidase GH-43</td>
<td>6</td>
<td>WENDWPSV, STSATGGFVDK, QEAAFUFER, GFVLYHYADTR, AVEDYQFGWNQLK, AVEDYQFGWNQLK</td>
<td><em>A. fumigatus</em> XP 731479.1</td>
</tr>
<tr>
<td>D</td>
<td>183</td>
<td>Arabinofuranosidase GH-62</td>
<td>3</td>
<td>K.DISPAGWASPKN, R.SQVQDQMTISPC, L.R.LALLTQTNASC</td>
<td><em>A. fumigatus</em> XP 749229.1</td>
</tr>
<tr>
<td>E</td>
<td>102</td>
<td>Polygalacturonase putative GH-28</td>
<td>2</td>
<td>NVPSVAQC, GASGATLPD</td>
<td><em>A. fumigatus</em> XP753090.1</td>
</tr>
<tr>
<td>F</td>
<td>109</td>
<td>Arabinofuranosidase GH-62</td>
<td>3</td>
<td>LALLTQTNASC, LALLTQTNASC, SQVDQMTISPC</td>
<td><em>A. fumigatus</em> XP 749229.1</td>
</tr>
</tbody>
</table>

*a* Unpublished results.
Table 2 (continued)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mascot score</th>
<th>Identified protein</th>
<th>Query matches</th>
<th>Matched peptides</th>
<th>Closest relative (Swiss Prot databases)</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>65</td>
<td>Polygalacturonase</td>
<td>2</td>
<td>GASGATLPDGAR,</td>
<td><em>P.olsonii</em> Q94833.1</td>
<td>3e-127</td>
</tr>
<tr>
<td></td>
<td></td>
<td>putative GH-28</td>
<td></td>
<td>GVTSQITLSSIR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>101</td>
<td>Cellobiose dehydrogenase</td>
<td>2</td>
<td>VVLSAGTFGSAR,</td>
<td><em>A.fumigatus</em> 293* XP756097.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDH cytochrome</td>
<td></td>
<td>SGIGPSQLEIVK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>superfamily</td>
<td></td>
<td></td>
<td><em>P. chrysogenum</em> Q01738.1</td>
<td>5e-124</td>
</tr>
<tr>
<td>I</td>
<td>56</td>
<td>1,4-β-α-glucan</td>
<td>2</td>
<td>DTISSNIDGR,</td>
<td><em>A. fumigatus</em> 293* XP748896*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>glucanhydrolase</td>
<td></td>
<td>AGVASVMCSYNK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GH3</td>
<td></td>
<td></td>
<td><em>A. aculeatus</em> P48825.1</td>
<td>2e-177</td>
</tr>
<tr>
<td>K</td>
<td>143</td>
<td>Arabinosidase putative</td>
<td>4</td>
<td>VDDNTFVR,</td>
<td><em>A. fumigatus</em> 293* XP749202</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GH 43</td>
<td></td>
<td>AIFIWESR,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VSGPVVEYSR,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DVSIPEGVGR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>220</td>
<td>Dipeptidyl peptidase</td>
<td>5</td>
<td>NLVSPVK,</td>
<td><em>A. fumigatus</em> 293* AAB67282</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(esterase lipase</td>
<td></td>
<td>SEAPDPSGK,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>superfamily)</td>
<td></td>
<td>TLIVGESDLGR,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IASANIEIPELK,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GDSSSPVFSPNGDK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Non-redundant protein sequences annotated from *A. fumigatus* Af 293 genome sequence

contained cellobiose dehydrogenase (spot H; Fig. 1) that showed homology to *Penicillium chrysogenum* (Swiss Prot accession Q01738.1). This enzyme belongs to CDH cytochrome super family, which oxidizes cellobiose residues by two electrons and can reduce a variety of electron acceptors [15].

A variety of hemicellulases were also identified in the secretome. The culture was found to produce five distinct xylanases (highly acidic to moderate acidic pI) on IEF zymogram (Fig. 4a). *A. fumigatus* have been previously reported to produce xylanases that belongs to family GH 10 and GH 11 [40, 41]. The protein spot K (Fig. 1) identified as arabinofuranosidase GH 43 with high mascot score of 143 and 4 peptide matches with the non-redundant protein sequences annotated from *A. fumigatus* genome (accession nos. XP 749202) did not show any significant protein match in the Swiss Prot database, indicating that this protein from *A. fumigatus* strain AMA or its homolog is yet to be reported (Table 2), whereas spot B (Fig. 1) also identified as arabinofuranosidase GH 43 showed similarities to *A. niger* arabinofuranosidase (Swiss Prot accession no. P 42256.1) with E=2e-96. Two more arabinofuranosidases corresponding to spots D and F (Fig. 1) were classified as members of GH 62 showing close match to arabinofuranosidase of *Streptomyces coelicolor* (Swiss Prot accession nos. Q54161.1). The arabinofuranosidases of GH 43 and GH 62 differ in their mode of action, where GH 43 is known to release O-3 linked arabinofuranosyl residues from double-substituted xylose; in contrast, GH 62 releases O-2- or O-3-linked arabinofuranosyl from mono-substituted xylose [42]. A β-xyllosidase (spot 3; Fig. 1) characterized as GH-3 (Table 1) matched completely to the *A. fumigatus* Swiss Prot protein sequence (Q4WF16.1). β-Xylosidases (EC 3.2.1.37) are exo-type glycosidases that hydrolyze short xylo-oligomers into single xylose units. The spatial similarity between D-xylopyranose and L-arabinofuranose leads to bifunctional xylosidase, arabinosidase enzymes, found mainly in families 3, 43, and 54 [43]. The
culture extract was found to contain five acetyl esterases isoforms as detected in zymogram developed against IEF gel (Fig. 4b). The acetyl xylan esterases are essentially required for removal of acetic acid moieties that esterifies the xylose units at the O-2 or O-3 position. The presence of α-mannosidase (GH 47) in the secretome (protein spot 6;
Fig. 1), which is known to be involved in the maturation of Asn-linked oligo-saccharides during N-glycosylation of the proteins [44], was confirmed by high mascot score (143) and homology (E=0.0) to closely related α-mannosidase from *A. fumigatus* (Swiss Prot accession nos. Q4WRZ5.1) (Table 2). The protein spots E and G (Fig. 1) were identified as polygalacturonase (GH 28) and spot 4 as N-acetyl hexaminidase (GH 20) in the secretome also indicated the ability of *A. fumigatus* strain to utilize pectin [45] and chitin [46] in addition to cellulose. N-Acetyl hexaminidase (GH 20) catalyzes the specific exohydrolisis of chitooligosaccharides from the non-reducing end generating monomers of N-acetylglucosamine [46]. The secretome also showed the presence of dipeptidyl peptidase (spot L; Fig. 1) that has also been reported in the secretome of *A. oryzae* during solid-state fermentation on rutin [14]. The presence of Asp-hemolysin (spot A; Fig. 1), a determinant of pathogenesis in *A. fumigatus*, was confirmed by PMF. This protein belongs to the aegrolysin family that has been observed in various molds, including edible mushrooms *Pleurotus ostreatus*, where it is specifically expressed during the formation of primordia and fruiting bodies of mushrooms [47].

The secretome characterization (Table 2) hence gave very useful information about the presence of a combination of a variety of glycosyl hydrolases (GH-3, GH-7, GH-12, GH-20, GH-28, GH-43, GH-47, and GH-62) in the crude extract of *A. fumigatus*, which distinctively differs from the glycosyl hydrolases present in the secretome of *Saccharophagus degardans* [48], which was reported to be a versatile cell wall degrading bacteria from marine ecosystem, as well as those present in commercially important cellulase-producing *T. reesei* strains [4, 31].

Hydrolysis of Alkali-Treated Rice Straw

The hydrolysis of alkali-treated rice straw with the crude enzyme extract at 7% and 10% (w/v) substrate concentration resulted in 95% and 91% saccharification in 96 h (Fig. 5). The HPLC profile of the hydrolysis products obtained by saccharification of 1 g alkali pretreated substrate showed the presence of glucose (550.6 mg), xylose (123.1 mg), xylobiose (60.05 mg), cellobiose (6.83 mg), and celotriose (6.00 mg) in the resultant hydrolysate (Fig. 6), implying that this enzyme mix, though balanced, is deficient in β-xylosidase activity. The observed rate of hydrolysis observed in this study is comparatively higher that those reported for cellulase from *Trametes hirsuta* [49]. Furthermore, the amount of enzyme used (as Fpase/g substrate) in our experiment was ~3.5-folds lower. Further work on developing the strain with higher specific activities so as to cut down the enzyme loading is in progress.

Acknowledgement The financial support from NAIP (ICAR) for carrying out this research project (NAIP/ Comp-4/C-30030) “Novel biotechnological processes for production of high value products from rice straw and bagasse” is duly acknowledged.

References


BIOFUELS
ALTERNATIVE FEEDSTOCKS AND
CONVERSION PROCESSES

A. PANDEY
C. LARROCHE
S.C RICKE
C.-G. DUSSAP
E. GNANSOUNOU
CHAPTER 9

Production of Hemicellulolytic Enzymes for Hydrolysis of Lignocellulosic Biomass

Sharma Manju, Bhupinder Singh Chadha*
Department of Microbiology, Guru Nanak Dev University, Amritsar-143 005, India
*Corresponding author: E-mail: chadhabs@yahoo.com

1 INTRODUCTION

Lignocellulosics in the form of agroresidues and forestry biomass constitute a potentially enormous source of feedstock for bioconversion into biofuel, feed, and specialty chemicals (Kamm and Kamm, 2004; Ohara, 2003). Lignocellulosics are comprised of cellulose, hemicellulose, and lignin that are present as intertwined complex fibril macromolecular structure. The structural heterogeneity in terms of proportion of cellulose, hemicellulose, and lignin in different plant species, as well as the spatial distribution of the constituent molecules, is perhaps one of the major hindrances in developing universal enzyme-based bioconversion technologies for their optimal utilization (Sharma et al., 2010a,b). In this chapter, we focus on the technologies available for the utilization of hemicellulosic fraction.

2 STRUCTURE OF HEMICELLULOSE

The term hemicellulose refers to a group of homo- and heteropolymers consisting of xylopyranose, mannopyranose, glucopyranose, and galactopyranose main chains with a number of substituents resulting in structurally complex polymer (Girio et al., 2010; Zheng et al., 2009). The hemicelluloses derived from different plant sources also show significant differences in their composition and structure. Few of the recent reviews give a detailed account of the hemicellulose structure (Girio et al., 2010; Scheller and Urvskov, 2010). β-1,4-xylans, the major components of hemicellulose, are the second most abundant polymer
in nature, accounting for one-third of the renewable biomass available on earth and constitutes around 20-30% of the dry weight of tropical hardwood and annual plants (Dhiman et al., 2008). The homopolymeric backbone of β-1,4-linked d-xylopyranose units is substituted to varying degrees with 4-O-methylglucuronopyranosyl, α-L arabinofuranosyl, α-d-glucuronyl residues, acetyl, feruloyl, and/or p-coumaroyl side chain units (Sun et al., 2005). Xylan exists as O-acetyl-4-O-methylglucuronoxylan in hardwoods and as arabino-4-O-methylglucuronoxylan in softwoods, while xylans in grasses and annual plants are typically arabinoxylans consisting of β-1,4-linked backbone of d-xylopyranosyl residues to which α-L-arabinofuranosyl (Araf) residues are linked at C-3 and C-2 (Izydorczyk and Dexter, 2008). Arabinoxylan agroresidues such as straws have been identified in wheat, rye, barley, oat, rice, sorghum, corn fiber, rye grass, etc. (Polizeli et al., 2005). Arabinoxylans from rice, sorghum, finger millet, and maize bran are more complex than those from barley arabinoxylans. The former contain, in addition to arabinose residues, small amounts of xylopyranose, galactopyranose, and α-d-glucuronic acid or 4-O-methyl-α-d-glucuronic residues. One of the unique features of arabinoxylans is the presence of hydroxycinnamic acids, ferulic and p-coumaric, esterified to O-5 of Araf linked to O-3 of the xylose residues (Medina et al., 2010) where ferulate esters can dimerize via phenoxy radicals into dehydrodi-ferulate esters, which are responsible for covalent crosslinking between arabinoxylan chains and arabinoxylans and other cell wall constituents (Lazaridou et al., 2007). In addition, acetyl groups may be esterified at C-2 or C-3 of the xylose residues. The relative amount and the sequence of distribution of these structural elements vary depending on the source of arabinoxylans. The majority of arabinofuranosyl residues in arabinoxylans are present as monomeric substituents; however, a small proportion of oligomeric side-chains, consisting of two or more Araf residues, are linked via 1 → 2, 1 → 3, and 1 → 5 bonds (Wong, 2006).

In case of hardwood xylan, approximately seven out of 10 xylosyl residues carry α-O-methylglucuronyl residue at O-2. They are associated with the lignin via ester, ether, and glycosidic bonds in plant cell walls (Sun et al., 2005). A small percentage of hardwood is also composed of glucomannans which consist of β-(1-4) linked glucose and mannose units forming chains that are slightly branched. The ratio of mannose:glucose is about 1.5:1 or 2:1 in most hardwoods (Sande et al., 2009). At the C-2 position, D-galacturonic acid is linked with an L-rhamnose, whereas the L-rhamnose is connected to the xylose chain at its C-3 position (Vries and Visser, 2001). The differences in acetylation as well as the presence of O-2 substituted 4-O-methyl-α-d-glucuronic acid units in xylans, in addition to terminal methyl glucuronic acid units linked to the xylan backbone, have also been documented (Pinto et al., 2005).

Arabinoglucuronoxylan is a minor component of softwood hemicelluloses. The backbone of softwood xylan is made up of β-L, 4-xylose units, with branches at C-2 and C-3 position. For about every 10 units of xylose, there are two 4-O-methyl-α-d-glucuronic acid groups substituted at the C-2 position and one α-L-arabinose unit at C-3 position (Izydorczyk and Dexter, 2008). L-arabinose and 4-O-methyl-α-d-glucuronic acid groups help maintain the xylose backbone, which is otherwise degraded during base-catalyzed reaction (Peng et al., 2010). In softwoods, hemicelluloses are mainly in the form of galactoglucomannan that forms the backbone of linear or slightly branched chain of β-(1-4) linked d-mannopyranose and d-glucopyranose units. Galactoglucomannan can be roughly divided into two types: one with a low galactose content, sometimes referred to simply as glucomannan, and the other with a high galactose content. The ratios of galactose to glucose to mannose are 0.1-0.2:1:3-4 and
ENDOXYLANASES

1:1:3 in the two types respectively. The hydroxyl groups at positions C-2 and C-3 in the backbone units are partly substituted by O-acetyl groups, on average one group per 3-4 hexose units (Girio et al., 2010); the degree of substitution varies with the source. In addition, arabinogalactan, xyloglucan, glucomannan, and other glucans are present (Albertsson et al., 2010; Laine, 2005; Scheller and Ulvskov, 2010).

Arabinogalactan is mainly known as a component of the heartwood of larches. The backbone consists of β-1-3-linked D-galactopyranose units and is highly branched at C-6 with side chains composed of β-1-6-linked D-galactose units, D-galactose, and L-arabinose units or single L-arabinose units and single D-glucuronic acid units. Other hemicelluloses include xyloglucan that are present mainly as a polysaccharide in the primary cell wall of higher plants and similar to structure of cellulose with β-(1 → 4)-linked D-glucosyl backbone containing α-D-Xylose-(1 → 6)-glucose substitutions. The xylosyl residues can be substituted at O-2 with β-Galactose, α-L-arabinose, or α-L-Fucose (Lopez et al., 2010). As well as β-glucans, there are linear homopolymers of D-glucopyranosyl (Glcp) residues linked mostly via two or three consecutive β-(1 → 4) linkages that are separated by a single β-(1 → 3) linkages (Lazaridou et al., 2007; Scheller and Ulvskov, 2010).

3 HEMICELLULASES

Due to the heterogeneity and complex chemical nature of hemicellulose, its hydrolysis into simpler constituents (monomers, dimers, or oligomers) requires the action of a wide spectrum of enzymes with diverse catalytic specificity and modes of action. Therefore, it is not surprising that microorganisms produce an arsenal of hemicellulolytic enzymes. Most important of these enzymes is endoxylanase (EC 3.2.1.8) that cleaves β-1,4-linked xylose backbone, while β-xylanase (EC 3.2.1.37) cleaves xylose monomers from the nonreducing end of xylooligosaccharides and xylobiose. In addition, a variety of debranching enzymes, that is, α-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72), α-galactosidases (EC 3.2.1.22), and β-mannosidases (EC 3.2.1.25), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), and p-coumaric acid esterases (EC 3.1.12) are required for efficient utilization (Figure 1) of hemicellulosic fraction (Shallom and Shoham, 2003).

4 ENDOXYLANASES

Xylanases-producing microorganisms have been isolated from diverse ecological niches like Southern Caucasus and Amazon forests, Antarctica, hot springs, composting soils, guts of earthworm, to name a few. Various bacterial and fungal cultures have been isolated and documented in several reviews (Maheshwari et al., 2000; Subramaniyan and Prema, 2002; Sunna and Antranikian, 1997; Vries and Visser, 2001). However, being an area of continued research, each passing year adds to the existing information about xylanases from different sources. A number of new species of microbes from diverse environments and ecological niches are being studied for the production of xylanolytic enzymes. Several new strains of thermophilic fungi, Myceliophthora sp., Chrysosporium lucknowense, Malbranchea flavia, Talaromyces thermophila
(Chadha et al., 2004; Maalej et al., 2009; Sharma et al., 2008; Ustinov et al., 2008) have been isolated from composting piles where the temperature rises to ~70 °C reported to produce xylanolytic enzymes. Owing to their higher thermostability and other technical traits, xylanases from thermophilic strains of bacteria and fungi are important from biotechnological viewpoint. Some of the other novel xylanase-producing microorganisms reported in the recent past include basidiomycete Cerioposis subvermispora (Magalhaes and Milagres, 2009); facultative anaerobe Anoxybacillus pushchinoensis A8 (Kacagan et al., 2008), Alicyclobacillus sp. A4 (Bai et al., 2010) as well as actinomycete strains of Streptomyces thermonitrificans, S. thermocarboxydus (Cheng et al., 2009; Kim et al., 2010). The isolation of genes from metagenomic library encoding for xylanases has also been reported in recent years. The environmental DNA library prepared from insect gut, manure waste, soil, and dairy cow rumen has yielded clones containing gene coding for xylanases (Brennan et al., 2004; Kim et al., 2008; Zhao et al., 2010). Novel xylanase showing 59% identity to endo-β-1-4-xylanase from Cellulomonas pachnodae was isolated from the soil metagenome (Kim et al., 2008). Clones harboring novel xylanases with two catalytic domains of family 43 and two CBD of family IV have been characterized (Zhao et al., 2010). The characterization of the crystal structure of CelM2, a bifunctional glucanase xylanase protein from the metagenome library, has revealed the metal effect and substrate-binding moiety (Nam et al., 2009).

Xylanases have been classified in families 5, 7, 8, 10, 11, and 43 on the basis of their amino acid sequences, structural folds, and mechanisms for catalysis (Collins et al., 2005; Cantarel et al., 2009). GH 10 and 11 xylanases represent the best studied xylanase families, and they differ in the number of subsites they possess, with GH 10 having four or five subsites and GH 11 having at least seven subsites (Dodd and Cann, 2009). While endoxylanases belonging to family 10 are characterized by high molecular weight (usually >30 kDa) and acidic pI, the members of family 11 have low molecular weight and basic pI, though exceptions do occur in some cases (Lagaert et al., 2009; Wong et al., 1988). The process of classifying xylanases in

![FIGURE 1 Enzymatic breakdown of arabinoxylan. Source: www.google.com.](image-url)
different families is supported by hydrophobic cluster analysis that predicts distinct protein-folding patterns as well as nucleotide sequences in these xylanases (Arora et al., 2009; Sapag et al., 2002). Compared to GH10 and GH11 endoxylanases, only a limited amount of data is available on the catalytic properties of xylanases from GH families 7, 8, and 43. The recent studies have reported the characterization of novel xylanases from *Trichoderma reesei* and *Erwinia chrysanthemi* belonging to glycoside hydrolase family 5 with exoacting mechanisms (Larson et al., 2003), and an endoacting xylanase from *Pseudalteromonas haloplanktis* in family 8 (Collins et al., 2002). The GH5 enzyme from *E. chrysanthemi* is specialized for hydrolysis of 4-O-methyl-D-glucuronoxylan or its acetylated counterparts and does not attack other types of xylans, linear β-1,4-xylooligosaccharides, or esterified aldoarionic acids (Vrsanska et al., 2007). However, a new bacterial xylanase belonging to GH 5 was found to be active on neutral, nonsubstituted xylooligosaccharides, showing a clear difference from other GH 5 xylanases characterized to date that show a requirement for methyl-glucuronic acid side chain for catalysis (Gallardo et al., 2010). The crystal structure of family 8 xylanase from an Antarctic bacterium, *P. haloplanktis*, showed that it appeared to have less salt bridges and increased number of hydrophobic residues that were exposed to the surroundings revealing their adaptation toward cold environment (Van Petegam et al., 2003). Pollet et al. (2010) evaluated the substrate preference and hydrolysis product profiles of different GH 8 xylanases in order to investigate their activities and substrate specificities. The findings of this study showed that GH 8 xylanases have narrow substrate specificities and the subtle amino acid changes in the glycon as well as the aglycon subsites probably form the basis of the observed differences between GH 8 xylanases. The GH 7 enzyme from *Trichoderma reesei* is considered as a nonspecific endo β-1,4-glucanase (Kleywegt et al., 1997), and the GH 43 enzyme from *Paenibacillus polymyxa* displays both xylanase and α-L-arabinofuranosidase activities (Gosalbes et al., 1991). Diverse physicochemical and functional characteristics, as well as folds and mechanisms of action of all the xylanase of different families, have been well discussed in an excellent review by Collins et al. (2005).

Catalytically, xylanases from families 10 and 11 can be differentiated on the basis of lower and higher substrate specificities, respectively. The lower substrate specificity of family 10 xylanases was demonstrated by their ability to catalyze the hydrolysis of cellulase substrate, pNP-cellobioside at a gluconic linkage, while the members of family 11 xylanase failed to recognize this as substrate (Biely et al., 1997; Collins et al., 2005). The substrate specificity of xylanases is reflected by the structural features of their active site. Each xylose is accommodated in a subsite (−) and (+), depending on whether it binds the glycone or aglycone regions of the substrate, respectively. Kinetic and structural investigations of GH11 xylanases indicate that their active sites potentially have up to three (−) subsites and three (+) subsites (Janis et al., 2005). In contrast, GH7 and GH10 xylanases have four to five subsites (Collins et al., 2005). Another feature that distinguishes GH10 and GH11 xylanases is the nature of the reaction products released from decorated xylans. GH11 xylanases produce substituted xylooligosaccharides both at the aglycone and glycone subsites (Maslen et al., 2007). The family 10 and 11 xylanases also differ in their action on 4-O-methyl-D-glucurono-D-xylan and rhodymenan, a β-1,3-β-1,4-xylan (Biely et al., 1997). A recent study assessed the activity of several GH10 and GH11 proteins with purified xylooligosaccharides substituted with MeGA and revealed that GH10 enzymes cleave xylan chains when MeGA is linked to xylose at the +1 subsite, whereas GH11 enzymes cleave when
MeGA is appended at the +2 subsite (Kolenova et al., 2006). Direct evidence for these results was reported in a recent study on the mass spectra of the products of hydrolysis for GH10 and 11 with arabinoxylan substrates (Maslen et al., 2007; Vardakou et al., 2008). These results suggest that EXs of family 10 are able to hydrolyze xylose linkages closer to side-chain residues and thus help to explain why these enzymes release shorter products than EXs of family 11 when incubated with arabinoglucuronoxylan substrates (Biely et al., 1997). This difference in substrate specificity for xylanases has important implications in the deconstruction of xylan (Dodd and Cann, 2009).

5 β-D-XYLOSIDASES

β-D-xylosidases (EC 3.2.1.37) are exotype glycosidases that hydrolyze short xylooligomers into single xylose units. An important role ascribed to β-xylosidases comes into play after the xylan has suffered a number of sequential hydrolyses by xylanase. This reaction leads to the accumulation of short oligomers of β-D-xylopyranosyl, which may inhibit the endoxylanase. β-xylosidase then hydrolyzes these products, removing the cause of inhibition, and increasing the efficiency of xylan hydrolysis (Zanoelo et al., 2004). Purified β-xylosidases usually do not hydrolyze xylan; their best substrate is xylobiose and their affinity for xylooligosaccharides is inversely proportionate to its degree of polymerization (Polizeli et al., 2005). β-xylosidases from filamentous fungi are usually liberated into the growth medium, that is, they are extracellular proteins. Although xylose is the end product inhibitor of β-xylosidases, it can act as inducer of xylanolytic gene expression. High yields of β-xylosidase on xylose were observed with T. reesei (Kristufek et al., 1995) and A. versicolor (Andrade et al., 2004). Recently, an extracellular xylose-tolerant β-xylosidase from Paecilomyces thermophila J18 was purified to homogeneity from the cell-free culture supernatant (Yan et al., 2008). However, cell-associated β-xylosidases have been reported from the cell extract of Penicillium sp., Sclerotium sp. grown on oat spelt xylan (OSX; Knob and Carmona, 2009). β-xylosidases from fungi are often monomeric glycoproteins, but some have been reported to possess two or three subunits (Polizeli et al., 2005; Xiong et al., 2007). They are grouped into five different families (GH3, GH39, GH43, GH52, and GH54) and their reaction mechanisms either result in inversion (GH43) or retention (GH3, GH39, GH52, and GH54) of stereoechemical configuration at the anomeric carbon. The best characterized β-xylosidases are from GH3 and GH43 (Dodd and Cann, 2009). The crystal structures for two biochemically characterized GH43 β-xylosidases from Selenomonas ruminantium and Geobacillus stearothermophilus have revealed the presence of two domains, an N-terminal five-bladed β-propeller domain and a C-terminal α/β-sandwich domain (Brunzelle et al., 2008). These enzymes possess two subsites for sugar binding and it is anticipated that only two xylose units will bind to the active site, thus extending the rest of the xylose units out into the solution. This prediction is further corroborated by biochemical analyses of GH43 β-xylosidases that reveal a decrease in catalytic efficiency ($k_{cat}/K_M$) when active on xylooligosaccharides longer than X2, thus suggesting that these enzymes possess only two xylose-binding sites (Wagschal et al., 2009). Although most members of the GH43 family have bacterial origin, few filamentous fungi, namely, Aspergillus oryzae, Penicillium herquei, and Cochliobolus carbonum possess DNA sequences that encode putative
family 43 β-xylosidases (Ito et al., 2003; Suzuki et al., 2010). Bravman et al. (2001a,b) reported the overexpression, purification, and biochemical characterization of a GH39 family β-xylosidase from *Bacillus steaothermophilus* T-6, and provided firm support for the assignment of Glu 160 as the acid-base catalyst of family 39 GHs. GH39 β-xylosidases have also been reported from *B. halodurans* (Muzard et al., 2009). GH3 represents a large group of glycosidic enzymes and possesses several distinct enzymatic activities including β-glucosidases, β-xylosidase, arabinofuranosidase, and N-acetyl-β-D-glucosaminidase activities (Faure, 2002). The spatial similarity between D-xylopyranose and L-arabinofuranose leads to bifunctional xylosidase/arabinosidase enzymes, found mainly in families 3, 43, and 54 (Ma et al., 2000). A bifunctional cell associated β-xylosidase belonging to GH3 family was purified from the cell extract of dimorphic fungus *Aureobasidium pullulans* strain ATCC 20254, grown on OSX (Ohta et al., 2010). The enzyme also showed some α-L-arabinofuranosidase activity (a novel mutant with AtBXL1 which encodes putative bifunctional β-D-xylosidase/α-L-arabinofuranosidase) has been identified in *Arabidopsis* mucilage secretory cells (Arsovski et al., 2009). The extensive structural and biophysical characterization of a family 52 β-xylosidase from *Geobacillus steaothermophilus* describes it as highly hydrated dimer protein whose active site was formed by the two promoters, and it probably involved aromatic residues (Contreras et al., 2008).

### 6 α-ARABINOFRANOSIDASES

α-Arabinofuranosidases (AFase) are accessory enzymes that hydrolyze the terminal, nonreducing α-L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans and act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of hemicelluloses and pectins (Saha, 2000). Arabinan-degrading enzymes have been classified on the basis of their mode of action, that is, endoacting or exoacting. The arabinan-degrading enzymes that act in an endofashion are called endo-1,5-α-L-arabinanases (EC 3.2.1.99) and those that act in an exofashion are called α-L-arabinofuranosidases (EC 3.2.1.15). Exoacting α-L-arabinofuranosidases (EC 3.2.1.55) are active against 1-nitrophenyl-α-L-arabinofuranoside and on branched arabinans, whereas endo-1,5-α-L-arabinofuranosidases (EC 3.2.1.99) are active only toward linear arabinans, and are not able to hydrolyze p-nitrophenyl-α-L-arabinofuranoside or arabic gum (Polizeli et al., 2005). Most of the arabinan-degrading enzymes reported in the literature are of the exoacting type. However, there are some reports of α-L-arabinofuranosidases capable of hydrolyzing both 1,3- and 1,5-α-L-arabinofuranosyl linkages in arabinoxylan (Corral and Ortega, 2006; Ichinose et al., 2008). Moreover, in some cases, α-L-AFases possessing β-xylosidase activity or xylanases with α-L-arabinofuranosidase activity also have been described (Arsovski et al., 2009). These enzymes expedite the hydrolysis of the glycosidic bonds by more than 1017 fold, making them one of the most efficient catalysts known. Arabinofuranosidases exist as monomers, but dimeric, tetrameric, and octameric forms have also been found (Panagiotou et al., 2003). They are classified into five GHs families, that is, GH3, GH43, GH51, GH54, and GH62 (Allgaier et al., 2010) and can hydrolyze glycosidic linkages at net inversion (GH43) or retention (GH51, 54) of stereochemical configuration at the anomeric carbon (Dodd and Cann, 2009; Carapito et al., 2009). AFs belonging to GH51 and 62 family release O-2 and O-3 linked arabinofuranosyl units from monosubstituted xylose.
A family 51 α-L-arabinofuranosidase from *Penicillium purpurogenum* was purified to homogeneity and characterized; the monomer with a molecular weight of 70 kDa exhibited low activity toward short arabinooligosaccharides and differed in some properties from other enzymes of this family (Fritz et al., 2008). Arabinofuranosidases of GH43 family result in the release of O-2 and O-3 substituted arabinose from monosubstituted xylose and display a variety of different substrate specificities. They are known to release O-3 linked arabinofuranosyl residues from double-substituted xylose (Hinz et al., 2009). A novel GH43 α-L-arabinofuranosidase from *Humicola insolens* that was cloned and expressed in *A. oryzae* was found to selectively hydrolyze arabinofuranosyl residues of doubly substituted xylopyranosyl residues in arabinoxylan. The synergistic action of two α-L-arabinofuranosidases from *H. insolens* belonging to GH51 along with the earlier-mentioned GH43 enzyme resulted in the removal of single sitting \((1 \rightarrow 2)\)-α-L-arabinofuranosyl units released after the GH43 enzyme had catalyzed the removal of \((1 \rightarrow 3)\)-α-L-arabinofuranosyl residues on doubly substituted xylopyranosyls in wheat arabinoxylan (Sorensen et al., 2006). Recently, crystal structures have been reported for GH43 arabinofuranosidase from *S. ruminantium* (Brunzelle et al., 2008) and *Bacillus subtilis* (Vandermarliere et al., 2009) which have the same N-terminal five bladed β-propeller fold common to GH43 enzymes but differ in the C-terminal domain. Due to this difference, these enzymes exhibit distinct substrate preferences with the *S. ruminantium* enzyme (SXA) having high activity on pNP-β-D-xylopyranoside followed by pNP-α-L-arabinofuranoside and xylooligosaccharides (Jordan et al., 2007), whereas the *B. subtilis* showed highest activity on pNP-α-L-arabinofuranoside and water extractable arabinoxylans (Bourgois et al., 2007).

### 7 ACETYLXYLAN ESTERASES

Acetylxyylan esterases (3.1.1.72) are enzymes that are able to hydrolyze the ester linkage between acetyl and xylose residues in xylans. This deacetylation makes the xylopyranosyl units of the main xylan chain more accessible to degradation by endo-β-1,4-xylanases (EC 3.2.1.8). Acetylxyylan esterases play an important role in the hydrolysis of xylan, as the acetyl side-groups can interfere with the approach of enzymes that cleave the backbone by steric hindrance, and their elimination thus facilitates the action of endoxylanases (Javier et al., 2007). The enzyme action on polysaccharide substrates creates new sites on the xylan main chain, suitable for productive binding with depolymerizing endoxylanases. The degradation of acetylxyylan with endoxylanases proceeds faster and to a higher degree in the presence of acetylxyylan esterases. They also deacetylate the partially acetylated xylooligosaccharides which makes the oligosaccharides fully susceptible to the action of β-xylosidases (Hinz et al., 2009). Two purified acetylxyylan esterases from *C. lucknowense* were found to release all acetyl groups from acetylated xylan oligosaccharides except one, which was found to be located at the nonreducing end of the oligosaccharide suggesting that the esterases are able to cleave all ester linkages at the reducing end (Hinz et al., 2009). The production of acetylxyylan esterases by various fungi and bacteria has been reported, but it has been important to distinguish between nonspecific acetyl esterase activity and acetylxyylan esterases by using appropriate substrates (Li et al., 2008). They suggested that most of the esterases are serine type which attack on low molecular mass substrates such as 4-nitrophenoxy acetate.
or 4-methylumbelliferyl acetate and employ a Ser-His-Asp (Glu) catalytic triad for catalysis. This mechanism involves the initial phase of acylation of the nucleophiles of serine residue followed by deacylation with water acting as a nucleophile (Taylor et al., 2006). However, the carbohydrate esterases of family 4, which also contain chitin deacetylases, do not operate on the earlier-mentioned aryl acetates and also do not possess Ser-His-Asp catalytic triad (Taylor et al., 2006). Cleavage of acetyl groups from the xylan is helpful in the removal of lignin. They may contribute to lignin solubilization by cleaving the linkages between lignin and hemicelluloses (Subramaniyan and Prema, 2002).

Feruloyl esterases (EC 3.1.1.73) are enzymes which hydrolyze the ester bond between the arabinose substitutions and ferulic acid. This later ester bond is involved in crosslinking xylan to lignin. Due to the ability of these residues to crosslink xylan and pectin polysaccharides to each other and to lignin, they are important for the structural integrity of the plant cell wall. Although some prokaryotic feruloyl esterases have been purified, the majority of these enzymes have been studied from eukaryotic systems. Feruloyl esterases can be divided into small monomeric enzymes, large dimeric enzymes, and monomeric enzymes based on molecular mass. On the basis of substrate specificity toward synthetic substrates and their capability to liberate diferuloyl bridges, these esterases can be divided into 4 groups, namely, A-D (Crepin et al., 2004). Benoit et al. (2008) introduced another classification of the ferulic acid esterases based on amino acid sequence homology and their activity toward methyl ferulate, methyl sinapate, and methyl caffeate. Most of the feruloyl esterases are extracellular and are active against xylan and xylan-derived oligosaccharides, from which they are able to release ferulic acid. Ferulic/coumaric acid esterases belong to the carbohydrate esterase (CE) family 1, whereas acetylxylan esterase activity has been described for members of CE 1-7, 12 and the recently discovered family 16 (Li et al., 2008).

8 α-D-GLUCURONIDASES

α-D-Glucuronidases (EC 3.2.1.131) are the enzymes that hydrolyze the α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan. However, the substrate specificity varies with the microbial source, and some glucuronidases are able to hydrolyze the intact polymer (Wet and Prior, 2004). Acetyl groups close to the glucuronosyl substituents are known to partially hinder the α-glucuronidase activity. To date, all of the α-D-glucuronidases are classified as family 67 glycosidases, which catalyze the hydrolysis via the inverting mechanism (Shallom et al., 2004).

9 MANNANASES

Endo-1,4-β-D-mannanase (EC 3.2.1.78) catalyzes the random cleavage of β-D-1,4-mannopyranosyl linkages within the main chain of galactomannan, glucomannan, galactoglucomannan, and mannnan. They liberate short-chain β-1,4-manno-oligomers, which can be further hydrolyzed to mannose by β-mannosidases (EC 3.2.1.25; Li et al., 2006). A variety of different organisms, including bacteria, fungi, higher plants, and animals, are known to produce mannanases (Chen et al., 2008; Li et al., 2006). Multiple extracellular mannanases have been
reported among many fungi like *Trichoderma reesei*, *T. harzianum*, and *Aspergillus* sp. (Fattah et al., 2009). The interest in β-mannanase has recently increased, partly because of their potential pertinence in the food and paper and pulp industries (Dhawan and Kaur, 2007). Endo-1,4-β-D-mannanases are classified in GH families 5 and 26, whereas β-mannosidases are described in GH families 1, 2, and 5 (Cuong et al., 2009; Songsiriritthigul et al., 2010).

10 METHODS FOR ASSAY OF HEMICELLULOLYTIC ACTIVITY

Birchwood xylan (BWX) which is least substituted and contains 94% of carbohydrate as xylose (more than 90% is in the form of soluble xylan) is an ideal substrate for standardizing the activity of endoxylanase. Xylanase assay is usually done using BWX which is mainly present as methyl-glucouronoxylan as substrate and contains 90% xylan. The most widely used assay method that has been standardized after carrying out interlaboratory studies was suggested by Bailey et al., (1992). They found that given the nature of substrate and variation in batch to batch up to 17% standard deviation can be tolerated. Today, this method is most widely used as indicated by over 650 citations of the method. The use of arabinoxylans (wheat arabinoxylan; WAX/Rye arabinoxylan; RAX) shows high activity when compared to oatspelt xylan (OSX) and BWX. So, even though there are pitfalls in these methods, the hydrolysis of BWX using DNS method stands out as the most widely used method. Other methods involving the use of RBB (Remazol Brilliant Blue) dyed methyl glucuronoxylan which initiates the release of RBB have also been advocated; however, the high cost of this substrate is one of the limiting factors in its wide use. Megazyme, an Irish company, has also introduced azo-dyed xylan as substrate for xylanase activity. It has been observed that many authors bring about changes in the protocol which may lead to inaccurate assays and sometimes workers have erroneously reported the results where μg of xylose released instead of μmol of xylose released has been shown as enzyme units (Lakshmi et al., 2009). In this way, the xylanase activity is overestimated by 100 times. There are few reports where 4-nitrophenyl and 4-methylumbelliferyl glycosidases of xylobiose and xylotriose have been used as substrates for assay of endoxylanase activity (Ziser and Withers, 1994) which is considered to provide stable and linear hydrolysis over the period of assay when compared to xylan which show decrease in hydrolysis with time as the number of positions susceptible to hydrolysis decrease steadily. The use of fluorogenic substrates 6,8-difluoro-4-methylumbelliferyl β-p-xylobioside for ultrasensitive continuous assay of xylanase has also been suggested. This HPLC-based method provides speed and sensitivity for measuring xylanase activity, as well as screening xylanase inhibitors in a high-throughput format (Ge et al., 2007). In yet another high-throughput screening (HTS) approach, multiplexed glycochip enzymatic assays based on a nanostructure initiator mass spectrometry (NIMS) have been developed by Northen and Coworkers at JBEI (Joint Bio Energy Institute, CA). In this NIMzyme assay, the enzyme substrates are immobilized on mass spectrometry surface using fluorous phase interactions (DOE Report, 2009).

The arabinofuranosidase activity is usually measured using pNp α-L-arabinofuranoside as substrate. However, cereal xylans are mono- and disubstituted with (1→2) and (1→3) linked α-L-arabinofuranosyl (α-L-Araf) residues. In addition, ferulic and p-coumaric acids are ester linked to arabinoxylans at O-5 of α-L-Araf units (Mastihubova and Biely, 2010; Pastel et al., 2009). In order to know the substrate specificity of α-L-arabinofuranosidase, the substrates
β-D-xylp (1-2), α-L-araf (1-3), α-L-araf (1-3) mono, α-L-araf (1-3) di, and α-L-araf (1-2) di are employed, whereas pNP β-D xylopyranoside is substrate of choice for assay of β-xylosidase activity. Acetylxylan esterase activity is measured using pNP acetate or α- or β-naphthyl acetate or methylumbelliferyl acetate which though are nonspecific substrates for acetylxylan esterase activity but have been widely used in screening and identification of active fractions during purification (Blum et al., 1999). However, the most objective method uses hardwood acetylxylans where the amount of released acetic acid is determined either by HPLC- or enzyme-based assay (Megazyme) and few other commercial kits. Recent reports suggest using pNP ferulate as substrate for assay of feruloyl esterase activity; however, synthetic esters of cinnamic acid can be used as substrate where release of ferulic acid can be monitored using HPLC or determined spectrophotometrically at 340 nm (Ghatora et al., 2006; Mastihuba et al., 2002). Recent reports suggest the 4-nitrophenyl 5-O-transferuloyl α-L-arabinofuranoside and 4-nitrophenyl-2-O-transferuloyl α-L-arabinofuranoside as suitable substrates for determination as well as difference of FAE activity. α-glucuronidase catalyzes the liberation of Me Glca and GlcA from aldouronic acid on which MeGlca or GlcA residues are linked to single xylopyranosyl residue or a non-reducing terminal xylopyranosyl residue of xylooligosaccharide. Therefore, glucuronoxylans can be used as substrate for assay of α-glucuronidase activity only in the presence of xylan depolymerizing enzyme (Puls and Schuseil, 1993). The most common substrate for α-glucuronidase activity is the aldouronic acids obtained by acidic/enzymatic hydrolysis of glucuronoxylan. An indirect method quantifies 4 nitrophenyl 2-O-(4-O-methyl-α-D-glucuronopyrimosyl) β-D-xylopyranose as substrate. Liberation of MeGlca from compounds yields an equivocal amount of pNP β-D-xylopyranoside which is hydrolyzed by β-xylosidase (Biely et al., 2000).

11 DOMAIN ORGANIZATION OF HEMICELLULASES

Most of the plant cell wall hydrolyzing enzymes typically comprise a catalytic module and one or more carbohydrate-binding modules (CBMs) that bind to a plant cell wall polysaccharide (Hachem et al., 2000). The primary function of CBMs is to increase the catalytic efficiency of the enzymes against soluble and/or insoluble substrates, and they do so by allowing inert alignment of the soluble enzyme with the insoluble polysaccharide. CBMs are also known to display some additional functions such as substrate disruption and sequestering and feeding of single polysaccharide chains into the active site of the catalytic modules (Subramaniyan and Prema, 2002). CBMs are located either at the N- or C-terminal, or both, and are classified into 61 different families in the CAZY database by sequence similarity and biochemical function (Coutinho and Henrissat, 1999). A wide variation exists in binding specificity within these types, for example, CBMs belonging to families 1, 2a, 3, 5, and 10 bind mainly to crystalline cellulose, whereas members of families 2b, 4, 6, 13, and 22 prefer xylan (Charnock et al., 2000). Three-dimensional structures of members of several CBM families have been elucidated and are now available from crystallographic as well as nuclear magnetic resonance (NMR) spectroscopic studies (Fujimoto et al., 2000).

Xylanases generally are known to have three types of domains, catalytic, noncatalytic (cellulose-binding domains), and thermostabilizing domains. Family 11 xylanases are found to contain a smaller catalytic domain than that of family 10 xylanases, and thus show lesser
catalytic versatility (Biely et al., 1997; Sapag et al., 2002). Although xylanases contain a single catalytic domain, certain enzymes from Neoclimaxis patriciarum, Fibrobacter succinogenes, and N. frontalis (Durand et al., 1996; Gilbert et al., 1992; Paradis et al., 1993) were found to contain two family 11 catalytic domains each. Structural analysis of both family 10 and 11 catalytic domains using X-ray crystallography revealed that family 10 has an eightfold β/α-barrel-shaped structure (Harris et al., 1996), while catalytic domains of family 11 xylanases fold into two β-sheets constituted mostly by antiparallel β-strands and one short α-helix (Gruber et al., 1998). Several studies have reported CBMs to potentiate the catalytic activity of cellulases against crystalline substrates, and xylanases against cellulose/xylan complexes. However, these domains do not potentiate the activity of GHs against soluble substrates (Ali et al., 2001). A family 2b CBM was found to increase the catalytic activity of a thermostable single domain family 10 xylanase (XynB) from Thermotoga maritima when fused at the C-terminus (Kittura et al., 2003). Similarly, Mangala et al. (2003) reported that the addition of a family 6 CBM to B. halodurans xylanase enhances its activity toward insoluble xylan. Araki et al. (2004) elucidated the essential role of the family-22 CBMs for β-1,3-1,4-glucanase activity of Clostridium stercorarium Xyn10B. Binding of CBMs to insoluble substrates was significantly enhanced by the presence of Na⁺ and Ca²⁺ ions. Talabani et al. (2004) reported the structure determination of the xylan-binding CBM 36 domain of the Paenibacillus polymyxa xylanase 43A. The structural analysis revealed the molecular basis for its unique Ca²⁺-dependent binding of xylooligosaccharides through coordination of the O₂ and O₃ hydroxyls, thus displaying its great potential for mapping the "glyco-architecture" of plant cells. In a recent study, the usefulness of synthetic xylan-binding modules as specific probes in analysis of hemicelluloses (xylan) in wood and fiber materials was demonstrated (Filonova et al., 2007). CBMs have also been used as affinity tags for purification of xylanases from Myceliophthora sp. (Badhan et al., 2007).

Recent studies report the characterization of a cellulose-binding domain from Clostridium cellulovorans endoglucanase-xylanase D and demonstrated that this domain can serve as a bifunctional fusion tag for solubilization of fusion partner as well as a domain for the immobilization, enrichment, and purification of molecules or cells on regenerated amorphous cellulose (Xu and Foong, 2008). The crystal structure of the family 31 CBM of β-1,3-xylanase from Alcaligenes sp. strain XY-234 (AlcCBM31) which shows affinity only with β-1,3-xylan was reported for the first time. The structure is based on typical immunoglobulin fold quite similar to CBM structures of families 34 and 9, which also adopt structures based on immunoglobulin folds (Hashimotoa et al., 2005).

CBDs have also been reported in other plant cell wall hydrolases such as mannanase (Stalbrand et al., 1995), acetylxylan esterase (Ferreira et al., 1993), and arabinofuranosidases (Black et al., 1996). Recently, a family 54 α-L arabinofuranosidase was reported to possess a CBM belonging to family 42 which specifically binds the arabinofuranose side chain of hemicellulose (Miyanaga et al., 2006).

### 12 Multiplicity of Hemicellulases

The production of a multienzyme system of xylanases, in which each enzyme has a special function, is one strategy for microorganisms to achieve effective hydrolysis of xylan. Most of the fungi-degrading lignocelluloses produce functionally diverse hemicellulases with many
isoforms (Badhan et al., 2004; Wong et al., 1988). Perhaps the structural complexity of lignocelluloses has resulted in the need for these multiple forms. Various mechanisms have been suggested to account for the multiplicity of function and specificity of the xylan-degrading enzymes. Electrophoretically distinct xylanases could arise from post-translational modification (Martin et al., 2007) of a gene product such as differential glycosylation or proteolysis. The detection of minor xylanases may also be an artifact of the growth and/or purification conditions or these enzymes may have functions, which are not required in large amounts, for example, hydrolysis of linkages not found frequently (Wong and Saddler, 1992). Multiple endoxylanases can also be expressed by distinct alleles of one gene, or even by completely separate genes (Chavez et al., 2002; Lagaert et al., 2009). Heterogeneity of xylan substrates may be one of the reasons for the production of multiple forms of xylanases, and some of these isoforms may be substrate specific or may show wide specificity, while it may be a secondary activity for others (Wong et al., 1988). Many microorganisms are able to produce multiple endoxylanases in order to acclimatize to various plant structural polysaccharides. For example 2, 6, 10, and 12 types of xylanases are produced by Bacillus firmus and M. flavus (Sharma et al., 2010a,b; Tseng et al., 2002), C. lucknowense (Ustinov et al., 2008), Paenibacillus curdlanolyticus B-6 (Pason et al., 2006), and a thermotolerant strain of Myceliophthora sp. (Badhan et al., 2007), respectively. Sharma et al. (2008) reported the molecular characterization of 16 different thermophilic/thermotolerant fungi isolated from composting materials capable of producing multiple xylanases (Figure 2a). Recently, two-dimensional electrophoresis approaches were employed to study the expression of multiple xylanases from S. termostrificans NTU-88 (Cheng et al., 2009). Presence of inducers or inhibitors in the medium also affects the production of enzymes, as expression of some of the genes may get induced or repressed by the presence of these agents. Expression of four Cochliobolus carbonum endo-1,4-β-xylanase genes (XYL1, XYL2, XYL3, and XYL4) and one exo-1,4-β-xylosidase gene (XYPI) was observed in the culture medium containing xylan; however, addition of glucose resulted in repression of all the four endoxylanases. The comparative analyses of the expression pattern of two genes from P. purpurogenum, xynA and xynB responsible for the production of endoxylanases XynA and XynB of families 10 and 11, respectively, were carried out under several induction and repression conditions. It was observed that the endoxylanase gene xynB was efficiently expressed with all the inducers (birch wood xylan, OSX, xylose, and xylitol), whereas xynA gene was expressed only in presence of OSX (Chavez et al., 2002). However, in case of production of multiple xylanases from thermophilic fungus Myceliophthora sp., it was observed that in addition to the type of carbon source, culture conditions also play an important role in multiplicity of xylanases, where rice straw induced expression of 3 and 5 xylanase isoforms under shake flask and solid-state fermentation (SSF), respectively (Badhan et al., 2004). Expression of multiple xylanases can also be induced by the positional isomers formed as a result of transglycosylation activity of enzymes produced at constitutive level (Saraswat and Bisaria, 1997). Multiple forms of enzymes may also result from horizontal gene transfer in the microorganisms living in similar ecological niche, and thereafter, evolving separately adapting to particular environmental conditions (Cpeljnik et al., 2004). The study of the functional importance of three xylanases from the saprophytic fungus T. harzianum showed a high degree of complementation of these xylanases in the hydrolysis of aspen xylan. Furthermore, the functional diversity of 10 xylanases from thermophilic fungus Myceliophthora sp. was analyzed using different types of xylan substrates, and it was concluded that xylanases are not redundant enzymes since each contributes
significantly and uniquely to the hydrolysis of the xylan. In spite of the fact that the multiform enzymes catalyze same reaction, they may differ in kinetic properties, regulatory characteristics, and/or stabilities (Naessens and Vandamme, 2003). Therefore, in order to elucidate the functional variations, the catalytic potential of each isoxylanase should be assayed against different substituted and unsubstituted xylan types (Ghatora et al., 2006; Wong et al., 1988).

Multiplicity has also been observed in β-xylosidases, α-L-arabinofuranosidases (AFs; Figure 2b and c), and acetylxylan esterases and feruloyl esterases (Ghatora et al., 2006; Vries and Visser, 2001). Two β-xylosidases liberated from the cell surface of P. herquei were purified and identified as GH43 enzymes (Ito et al., 2003). Three different forms of α-L-arabinofuranosidases from P. purpureogenum were separated by isoelectrofocusing and detected using the zymogram technique, out of which one arabinofuranosidase has been
purified and identified as GH54 enzyme. Sinitzyna et al. (2003) isolated two arabinofuranosidases (AF-60 & AF-70) from among the major components of the xylanase system of *Penicillium canescens*. *B. subtilis* produces two α-L-arabinofuranosidases capable of releasing arabinosyl oligomers and L-arabinose from plant cell walls, both belonging to family 51 GHs but differing significantly in their substrate specificities (Inacio et al., 2008). Recently Hinz et al. (2009) reported the selective production, purification, and characterization of four arabinofuranosidases, two acetylxylan and ferulic acid esterases, and α-glucuronidase from the filamentous fungus *C. lucknowense*, thus demonstrating high potential of this fungus as a producer of hemicellulolytic enzymes. Thermophilic fungi including *H. insolens*, *Chaetomium thermophilum*, and *Melanocarpus* sp. were identified as prolific producers and expressed multiple esterases that were putatively classified as xylan acetyl esterase and feruloyl esterases on the basis of distinct preferential substrate specificities toward p-nitrophenyl acetate and p-nitrophenyl ferulate, respectively (Ghatora et al., 2006).

### 13 FUNCTIONAL GENOMIC APPROACH FOR STUDYING HEMICELLULASES

Functional genomics for system analysis of bacteria- and fungi-producing GHs have been important in profiling the expression of cellulases and hemicellulases predicting the functional strategy these fungi employ for degradation of plant cell wall. The analysis of the transcriptome and secretome datasets has been evaluated to identify the gene/proteins that are overexpressed in *Neurospora crassa* (Tian et al., 2009), *Postia placenta*, and *Phanerochaete chrysosporium* (Martinez et al., 2009; Wymelenberg et al., 2010). Viewed together with transcript profiles, *P. chrysosporium* employs an array of extracellular GHs to simultaneously attack cellulose and hemicelluloses. In contrast, under these same conditions, *P. placenta* secretes an array of hemicellulases but few potential cellulases (Wymelenberg et al., 2010). The studies reporting comparative secretomes of the fungal strains grown under submerged and SSF of *A. oryzae* (Oda et al., 2006), between two hypersecretory strains of *T. reesei* (Gimbert et al., 2008) or those grown in presence of different carbon sources, have also highlighted differential expression profiles and have also led to the identification of unreported putative arabinofuranosidases (Gimbert et al., 2008). Comparative studies have also highlighted differences in the relative abundance of proteases, cellulase/hemicellulase in the extracts of *T. reesei* Rut C-30, and commercial enzyme preparation Spezyme CP from the same organism (Nagendran et al., 2009). Quantitative iTRAQ secretome analysis of *A. niger* has revealed the presence of novel hydrolytic enzymes (Adav et al., 2010). The secretome of *A. fumigatus* has revealed the presence of variety of GHs that was found to be efficient in carrying out the saccharification of alkali-treated rice straw (Sharma et al., 2010a,b).

### 14 ENZYME PRODUCTION

A wide spectrum of cell wall-degrading enzymes including cellulases and hemicellulases (GHs) are produced by different fungi and bacteria. However, these microorganisms differ appreciably in their capability to produce these enzymes in terms of their activities as well as the spectrum of different GHs. Each microorganism differs in its genetic capacity
(Hinz et al., 2009) and consequently secretes a specific combination of GHs. However, most of the commercially important sources of hemicellulases are limited to fungi. Recent studies showed that the difference in the activity profiles of commercial cellulolytic/hemicellulolytic strains, for example, *T. reesei*, *A. niger* (Sorensen et al., 2005), and *C. lucknowense* (Emalfarb et al., 2003) could be related to their genetic capacity. Where *T. reesei* is known to be a good source of celllobiohydrolases and endoglucanases, *A. niger* is known to be a good source of β-glucosidase/xylosidase, whereas the *C. lucknowense* genetic system was found to be most elaborate for the expression of hemicellulases (Hinz et al., 2009).

Most of the studies on the production of hemicellulases are primarily focused on xylanases which are specifically required in the paper and pulp industry, for generating xylooligosaccharides (Pastel et al., 2009; Puchart and Biely, 2008; Sharma et al., 2010a,b). Most of the other applications however require the complete spectrum of hemicellulolytic enzymes, especially in the bioconversion process for converting hemicellulose fraction to monomeric sugars for further fermentation into biofuels and specialty chemicals (Ohara, 2003). Because of the differences in the structural composition of hemicellulose, defining the right balance of enzyme mixture is not easy. Alternative bioreactors such as the air-lift or bubble-column, which have a lower shear stress, seem to produce better results. For example, studies on xylanase and cellulase production by *A. niger* in various bioreactors showed that in general, better yield and productivity were obtained in a bubble-column and an air-loop air-lift than in the stirred-tank reactor. However, the relatively high cost of enzyme production has hindered the industrial application of the enzymatic process. Recent trends show that SSF which involves the growth of fungi on wet solids in the absence of free water is an attractive proposition because of the economic and engineering advantages (Rodrigues et al., 2007). The low moisture content results in low-energy consumption and prevents bacterial contamination and the problems caused by low gas distribution during submerged cultures, which make the SSF system good (Leite et al., 2007; Pandey et al., 2000). The hyphal mode of fungal growth and their good tolerance to low water activity and high osmotic pressure conditions make it efficient and competitive in natural microflora for bioconversion of solid substrates (Raimbault, 1998).

Some of the prolific producers of xylanase, *T. reesei* and *T. lanuginosus*, are known to produce >3000 U/mL under shake flask/submerged culture (Haapala et al., 1994; Singh et al., 2000), while *T. lanuginosus* has been reported to produce 48,000 (U/g substrate) under SSF (Sonia et al., 2005). There are several references in literature that suggest that fungi produce appreciably higher levels of xylanases when cultured under SSF in comparison to SmF. For example, *P. brasiliense*, *A. niger*, *Melanocarpus albomyces* produced almost 5-10 times higher activities under SSF as compared to SmF (Jorgensen et al., 2005; Narang et al., 2001; Thygeson et al., 2003). Similar observations have also been made on the production of arabinofuranosidase from *Arthrobacter* sp., where 0.1 (U/ml) was produced under shake flask conditions compared to 35 (U/g substrate) under SSF (Khandeparker et al., 2008). It has been estimated that SSF is 100 times more economical for cellulase production as compared to SmF (Antoine et al., 2010).

In order to produce a complete spectrum of hemicellulases, the nature and composition of the carbon sources used for induction of enzyme production plays a crucial role. Various carbon sources such as rice straw, wheat straw, wheat bran, corn cobs, bagasse, banana peels, etc. have been used for production hemicellulases (Sonia et al., 2005; Thygeson et al., 2003) The growth of cultures on different carbon sources has been shown to be associated with differential expression of functionally distinct xylanases (Badhan et al., 2007). It has been
observed that not all the components of hemicellulases are produced in presence of one type of carbon source as *T. lanuginosus* produced maximal levels of xylanase and β-xylosidase in presence of corn cobs, whereas OSX was found to induce maximal levels of arabinofuranosidase, acetylxylan esterase, feruloyl esterase, and β-mannosidase which clearly suggests it is judicious to go for production of optimal level rather than maximal levels of production. Most of the work on optimization has been focused on endoxylanases, and there is dearth of work done where debranching has been considered during optimization. In a recent report, optimization of xylanases and debranching enzymes by thermophilic fungal strain *M. flava* grown on sorghum straw was optimized employing response surface methodology. Under optimal conditions, *M. flava* produced 16390, 9.49, 3.40, 69.8, and 2.25 (units/g substrate) of xylanase, β-xylosidase, arabinofuranosidase, acetyl esterase, and feruloyl esterase, respectively (Sharma and Chadha, 2010). In addition to carbon source, type of nitrogen sources, C:N ratio, initial medium pH, incubation temperature, inoculum level, inoculum age, initial moisture levels, etc. also play an important role in production of hemicellulases (Jatinder et al., 2006a; Sonia et al., 2005). The process optimization can be done by classical method that involves modification of one independent variable at a time, while all others are fixed at a certain level. The optimized conditions for production of hemicellulases have been reported for *Rhodothermus marinus* (Gomes et al., 2000), *Penicillium brasiliannum* (Jorgensen et al., 2005), *Thermomyces lanuginosus* (Sonia et al., 2005), *Melanocarpus* sp. MTCC3922 (Jatinder et al., 2006a). Statistical approaches like response surface methodology, central composite design, multiple linear regression, back propagation neural network, and lazy learning algorithm have also been used for optimization of hemicellulases (Guerfali et al., 2010; Jatinder et al., 2006b, Meshram et al., 2008).

### 15 APPLICATIONS OF HEMICELLULASES

The xylanolytic enzymes used in the paper and pulp industry mainly for biobleaching and pectinolytic enzymes have been used for debarking; in addition to bleaching capability, xylanases have been found useful in other applications also, that is, clarification of juice and wine, starch separation and production of functional food ingredients, improving the quality of bakery products, in animal feed biotechnology, in debarking, deinking of recycled fibers, and in preparation of dissolving pulp (Beg et al., 2001; Polizeli et al., 2005; Techapun et al., 2003). The use of hemicellulases along with glucanases, cellulases, proteases, amylases, phytase, galactosidasıes, and lipases has become a common practice in the field of animal feed biotechnology. These enzymes bring about the breakdown of plant cell wall complex present in the ingredients of feed and reduce the viscosity of raw material. If xylanase is added to feed containing maize and sorghum, both of which are low-viscosity foods, it may improve the digestion of nutrients in the initial part of the digestive tract, resulting in a better use of energy (Polizeli et al., 2005). Addition of xylanases to rye-based diet of broiler chickens has been shown to increase weight of chicks (Bedford and Classen, 1992); moreover, the use of xylanases in combination with phytases has resulted in increase in egg and albumen weight from white and brown egg-laying hens (Silversides et al., 2006). Some of the family 11 xylanases produced by rumen bacteria of genera *Pseudobutyrivibrio* and *Butyrivibrio* show the cleavage of OSX into tetra or higher oligomers; these xyloooligosaccharides could be
helpful in promoting the proliferation of beneficial microflora (Craeyveld et al., 2008). Therefore, xylanases produced by these strains could be used as a feed additive for animals, and such strains can be used as probiotic for animals (Cpeljnik et al., 2004). Xylanase also play an important role in improving the quality of bread, breaking down hemicellulose in wheat flour, helping in the redistribution of water, and leaving the dough softer and easier to knead, resulting in increase in bread volumes and improved resistance to fermentation (Shah et al., 2006). Synergistic action of xylanases and related hemicellulases can be employed for generation of biofuel such as ethanol and xylitol from lignocellulosic biomass. Xylitol used as sweetener in food has odontological applications such as teeth hardening, and is used in chewing gum and toothpaste formulation (Beg et al., 2001). Xylanases with transglycosylation activities can also be used for designing the drugs and preparation of neoglycoproteins (Eneyaskaya et al., 2003). The use of xylanases in production of alkyl glycosides by hydrolysis of polysaccharides is a challenging opportunity. Xylanase purified from a strain of A. pullulans has been used for direct transglycosylation of xylan with 1-octanol and 2-ethylhexanol into octyl-β-D-xylobioside and 2-ethylhexyl-β-D-xylobioside, respectively (Matsumura et al., 1999). Xylan-debranching enzymes such as acetylxylan esterase and feruloyl esterases may enhance the process of solubilization of lignin-carbohydrate complex by removing substitutions and linkages between polymers during pulping (de Graaff et al., 2000). Acetylxylan esterase can be used in deinking of paper by aiding in the removal of substituents groups which hinder main-chain-degrading enzymes. Recently, esterases especially feruloyl esterases have been reported as being used for the bioconversion of lignocellulosic wastes, synthesis of esters in organic solvents, and isolation of phenolic acids as precursors of value-added chemicals (Garcia-Conesa et al., 2005). α-L-Arabinofuranosidases have been employed for aromatizing musks, wines and fruit juices, for delignification of paper pulp, for digestibility enhancement of animal feedstock, and for fractionation of sugar beet pulp into pectin, cellulose, and arabinose (Saha, 2000). A potential utilization of pectinases is in the treatment of softwoods, which has been shown to improve the efficiency of preservative treatment by rendering the wood more permeable for chemical preservatives (Gregorio et al., 2002).

References


REFERENCES


REFERENCES


9. PRODUCTION OF HEMICELLULOLYTIC ENZYMES FOR HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS


