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

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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 CBHI/EGI 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 CBHI/EGI and β-glucosidases in 2DE gels · Saccharification

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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 glucohydrolase, 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 xylo-oligomers. In addition, a variety of debranching enzymes, i.e., α-arabinofuranosidase (EC 3.2.1.55), α-glucouronidase (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 celluloyticus*, 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 cellulosic 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 1 D (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_2PO_4 0.2, K_2HPO_4 0.23, $MgSO_4.7H_2O$ 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*) $(NH_4)_2SO_4$, 1.1, beef extract 0.25, KH_2PO_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_2HPO_4, 0.2; $MgSO_4.7H_2O$, 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-adsorbable 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-xylosidase, 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-xyloside, *p*-nitro phenyl-β-D-arabinofuranoside, and *p*-nitro phenyl-β-D-mannoside (pNPMan), 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 cellobiose 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 Ettan 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% (*w*/*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 p*I* 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 p*I*~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 p*I* (β-G II), were found as a train of spots and an additional active β-glucosidase spot (β-G III) with p*I*~5.6 was observed. The resolution of crude extract on single dimension p*I* 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 (p*I* 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 p*I* 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

Enzyme	EG	AAEG	EGI	CBHI	β-G	FPase	Xylanase	β-Xyl	α-Ara	Acetyl esterase	Feruloyl esterase	β-Mann	PG
A. fumigatus (units/g substrate)	240	41.4	12.4	23.9	245	10.2	3,400	8.8	1.51	47.5	16.8	0.6	68
A. fumigatus (units/mg protein)	18.4	3.1	0.95	1.8	18.8	0.78	261.5	0.67	0.12	3.6	1.3	0.04	5.2
T. reesei[a] [25][b] (units/mg protein)	8.94	ND	0.25	1.1	0.48	0.58	119.1	0.75	1.1	0.33	ND	4.5[c]	ND
A. cellulolyticus[a] [26] (units/mg protein)	4.52	ND	ND	0.26[d]	1.2	0.66	12.4	0.017	ND	ND	ND	1.10	ND

β-G β-glucosidase, *β-Xyl* β-xylosidase, *α-Ara* α-arabinofuranosidase, *β-Mann* β-mannosidase, *PG* polygalactouronase 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

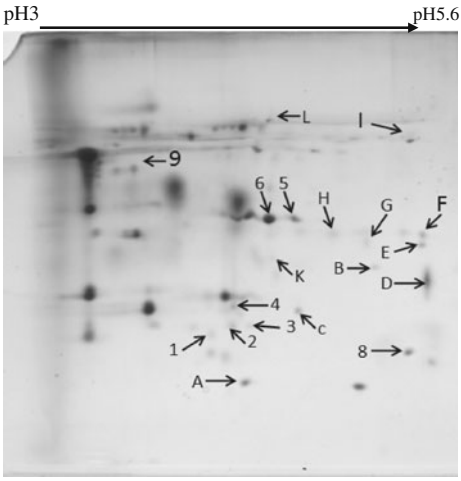

Fig. 1 2DE secretome pattern of *A. fumigatus* grown on rice straw

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). CBHI are

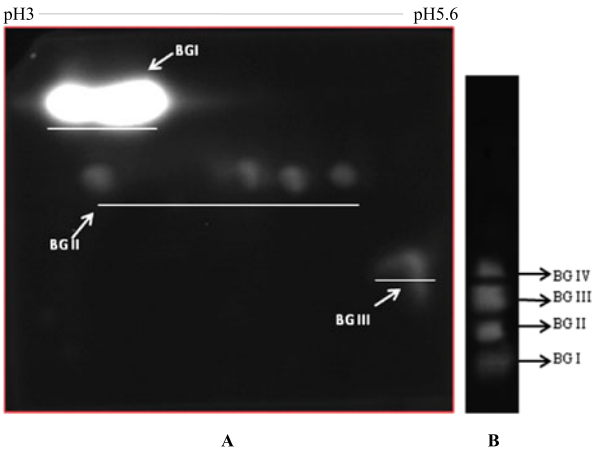

Fig. 2 **a** Detection of β-glucosidase activities after 2DE of secreted proteins of *A. fumigatus* grown on rice straw. **b** Detection of β-glucosidase activities in the crude extract of *A. fumigatus* after one-dimensional IEF (p*I* 3–10)

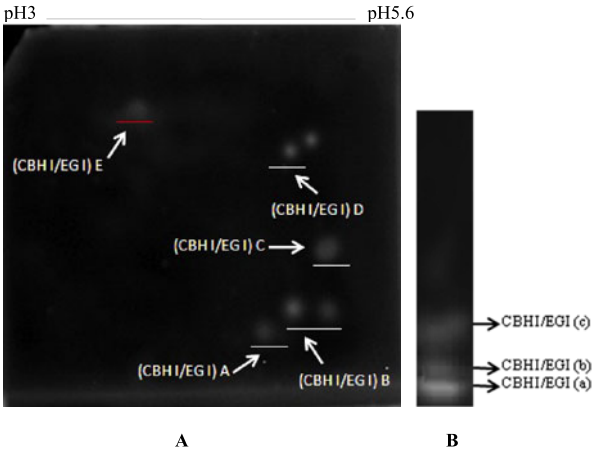

Fig. 3 **a** Detection of CBHI/EGI activities after 2DE of secreted proteins of *A. fumigatus* grown on rice straw. **b** Detection of CBHI/EGI activities in the crude extract of *A. fumigatus* after one-dimensional IEF (p*I* 3–10)

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

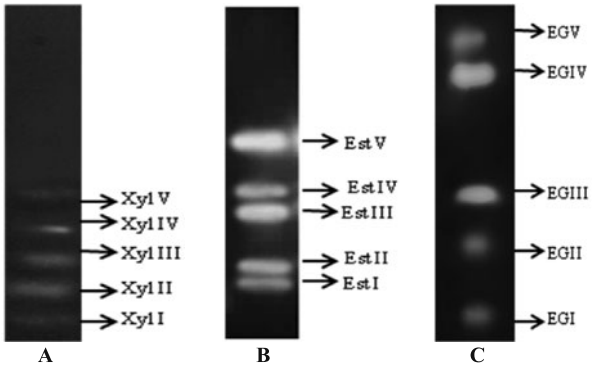

Fig. 4 Zymograms for detection of **a** xylanase, **b** esterase, and **c** endoglucanase isoforms secreted by *A. fumigatus*. The proteins were resolved by one-dimensional IEF (p*I* 3–10)

Table 2 Identification of the protein spots (shown in Fig. 1) in *A. fumigatus* secretome by LC/MS

Sample	Mascot score	Identified protein	Query matches	Matched peptides	Closest relative (Swiss Prot databases)	*E*
2	60	1,4-β-D-Glucan cellobiohydrolase GH7	1	TFYGPGMTVDTK	*A. fumigatus* 293[a] XP751044.1	0
					A. fumigatus Q4WM08	0
3	50	Arabinanase putative GH-43	1	AVEDYQFGWNQLK	*A. fumigatus* 293[a] XP 731479.1	0
					A. niger P42256.1	$2e^{-96}$
4	130	*N*-Acetyl hexaminidase GH 20	4	APSSLQFVNVK, EGSDTIQITAK ILEQLDAMSLSK TYNDLSQYWVDHAVPIFR	*A. fumigatus*[a] XP 747307	0
					C. albicans P43077	$8e^{-153}$
5	243	β-Xylosidase GH-3	6	LAVCDTSLDVTTR LGYFDPAEDQPYR AAGEGIVLLK TLLWAATQAGYDVK QADVVVYAGGIDNTIEAEGR ALGPYNTAALVSR	*A. fumigatus* 293[a] Xp748529.1	0
					A. fumigatus Q4WFI6.1	$2e^{-94}$
6	134	β-Mannosidase GH-47	3	GPAADLVEDR, LSDLTGDQEYAK, ADLIDFGLK	*A. fumigatus*[a] XP 752825.1	0
					A. fumigatus Q4WBR5.1	0
8	118	Cellulase putative GH-12	5	RVSQWTASVN, SYANSQVSLTK	*A. aculeatus* P22669	$2\ e^{-89}$
9	88	1,4-β-D-glucan cellobiohydrolase GH7	2	LYLGPDKNYVMLK, AQNPTTHVVFSNIR	*A. fumigatus* XP[a] B747897.1	0
					P. chrysogenum P13860	1e−105
A	206	Asp hemolysin (aegerolysin superfamily)	4	TAPPGGSVNVNSCGRS, DASSGTTGGF, DLYDGNTNDFDVGERTK, YGGAIGTVDVEVGR	*A. fumigatus* 293[a] XP748379.1	$1e^{-75}$
					A. fumigatus Q00050.2	$4e^{-77}$
B	161	Arabinofuranosidase GH-43	6	WENDWPSV, STSATGGFVDK, QEAAFMFER, GFVLYYHYADTR, AVEDYQFGWNQLK, AVEDYQFGWNQLK	*A. fumigatus* 293[a] XP 731479.1	0
					A. niger P42256.1	$2e^{-96}$
D	183	Arabinofuranosidase GH-62	3	K.DISNPAGWSAPK.N, R.SQVDQTMTISPCK.L, R.LALLTQTNSAC	*A. fumigatus* 293[a] XP749229.1	0
					Streptomyces coelicolor Q54161.1	$2e^{-57}$
E	102	Polygalactouronase putative GH-28	2	NVPSVAQC, GASGATLNPDGAR	*A. fumigatus* 293[a] XP753090.1	0
					P. olsonii Q94833.1	$3e^{-127}$
F	109	Arabinofuranosidase GH-62	3	LALLTQTNSAC, LALLTQTNSAC, SQVDQTMTISPCK	*A. fumigatus* 293[a] XP749229.1	0
					Streptomyces coelicolor Q54161.1	$3e^{-57}$

Table 2 (continued)

Sample	Mascot score	Identified protein	Query matches	Matched peptides	Closest relative (Swiss Prot databases)	*E*
G	65	Polygalactouronase putative GH-28	2	GASGATLNPDGAR, GVTYSGITLSSIR	*P. olsonii* Q94833.1	$3e^{-127}$
H	101	Cellobiose dehydrogenase CDH cytochrome superfamily	2	VVLSAGTFGSAR, SGIGPSDQLEIVK	*A. fumigatus* 293[a] XP756097.1	0
					P. chrysogenum Q01738.1	$5e^{-124}$
I	56	1,4-β-D-glucan glucanhydrolase GH3	2	DTISSNIGDR, AGVASVMCSYNK	*A. fumigatus* 293[a] XP748896[a]	0
					A. aculeatus P48825.1	$2e^{-177}$
K	143	Arabinosidase putative GH 43	4	VDDNTFVR, AIFIWESR, VSGPVVEVSR, DVSIVEGVGR	*A. fumigatus* 293[a] XP 749202	0
L	220	Dipeptidyl peptidase (esterase lipase superfamily)	5	NLVSPVK, SEAIPDPSGK, TLIVGSEDLGR, IASANEIDPELK, GDSSSPVFSPNGDK	*A. fumigatus* 293[a] AAB67282	0

[a] 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 p*I*) 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-xylosidase (spot 3; Fig. 1) characterized as GH-3 (Table 1) matched completely to the *A. fumigatus* Swiss Prot protein sequence (Q4WFI6.1). 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

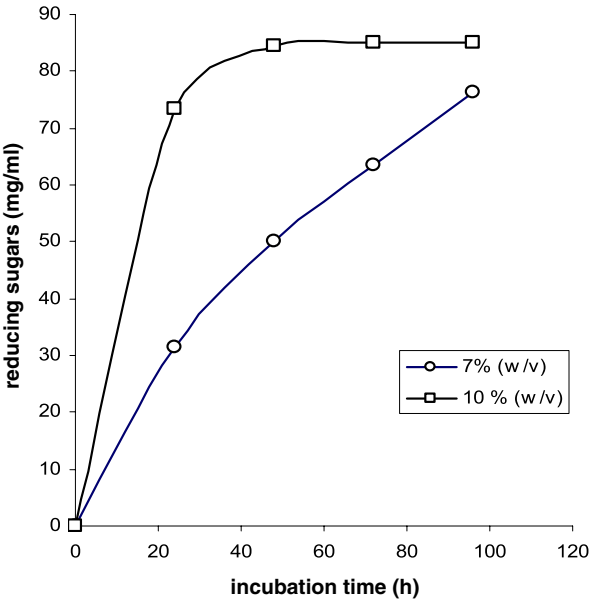

Fig. 5 The hydrolysis of alkali-treated rice straw (7% and 10%) using enzyme preparation of AMA

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;

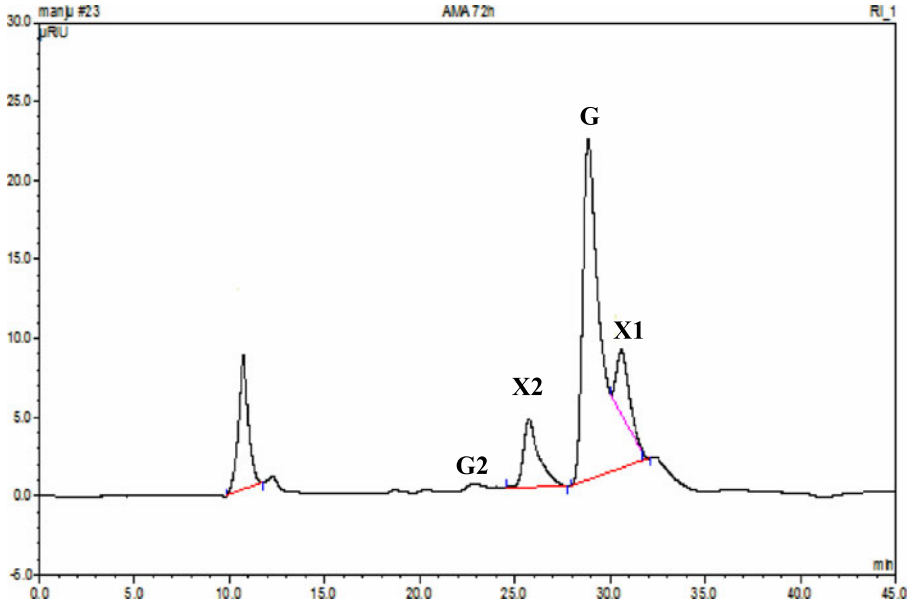

Fig. 6 HPLC chromatogram showing profile of hydrolysis products obtained by the action of crude extract of *A. fumigatus* on alkali pretreated rice straw. X_1 xylose, X_2 xylobiose, G glucose, G_2 cellobiose. The retention time (min) on the *x*-axis, and RI units on the *y*-axis

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 polygalactouronase (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 exohydrolysis 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 sectretome 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 cellotriose (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.

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