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Do cultural conditions induce differential protein expression: Profiling of extracellular proteome of *Aspergillus terreus* CM20

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ABSTRACT

The present study reports the diversity in extracellular proteins expressed by the filamentous fungus, *Aspergillus terreus* CM20 with respect to differential hydrolytic enzyme production profiles in submerged fermentation (SmF) and solid-state fermentation (SSF) conditions, and analysis of the extracellular proteome. The SSF method was superior in terms of increase in enzyme activities resulting in 1.5–3 fold enhancement as compared to SmF, which was explained by the difference in growth pattern of the fungus under the two culture conditions. As revealed by zymography, multiple isoforms of *endo*-β-glucanase, β-glucosidase and xylanase were expressed in SSF, but not in SmF. Extracellular proteome profiling of *A. terreus* CM20 under SSF condition using liquid chromatography coupled tandem mass spectrometry (LC–MS/MS) identified 63 proteins. Functional classification revealed the hydrolytic system to be composed of glycoside hydrolases (56%), proteases (16%), oxidases and dehydrogenases (6%), decarboxylases (3%), esterases (3%) and other proteins (16%). Twenty families of glycoside hydrolases (GH) (1, 3, 5, 7, 10, 11, 12, 15, 16, 28, 30, 32, 35, 43, 54, 62, 67, 72, 74 and 125), and one family each of auxiliary activities (AA7) and carbohydrate esterase (CE1) were detected, unveiling the vast diversity of synergistically acting biomass-cleaving enzymes expressed by the fungus. Saccharification of alkali-pretreated paddy straw with *A. terreus* CM20 proteins released high amounts of glucose (439.63 ± 1.50 mg/gds), xylose (121.04 ± 1.25 mg/gds) and arabinose (56.13 ± 0.56 mg/gds), thereby confirming the potential of the enzyme cocktail in bringing about considerable conversion of lignocellulosic polysaccharides to sugar monomers.

1. Introduction

Lignocellulosic biomass is currently the most promising feedstock source for realizing sustainable energy demands of agrarian economies. The current availability of biomass in India is estimated at about 640 MT per year, of which 234 MT is available as surplus for energy generation (Hiloidhari et al., 2014). Lignocellulose, a composite of ~40–50% cellulose, ~25–35% hemicellulose and ~15–20% lignin represents a feasible and sustainable resource for renewable fuel (Holtzapple, 1993; Saritha et al., 2012). For the coherent conversion of the lignin-carbohydrate complex (LCC) to fermentable sugars, efficient methods of biomass pretreatment and biomass-hydrolysing machinery are necessitated (Jørgensen et al., 2007).

The most effective method of biomass hydrolysis involves the concerted action of cellulases, hemicellulases, and other glycoside hydrolases (Harris et al., 2010). The prominent of these are the cellulases, comprising of *exo*-β-glucanases and *endo*-β-glucanases (cleaving β-1,4-glycosidic bonds from chain ends and internally within chains, respectively), and β-glucosidases (cleaving the final β-1,4 linkage of cellobiose or small polysaccharides) (Davies et al., 1995). Hemicellulases are more complex, consisting of an array of xylanases, mannases, arabinases, and their corresponding glycosidases. Their synergistic action brings about hydrolysis of ester bonds and glycosidic bonds, thereby removing the side chains (Campos et al., 2014; Sweeney and Xu, 2012). To achieve complete conversion of the lignocellulose polysaccharides to sugar monomers, enzyme cocktails which are combinations

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of various glycoside hydrolases that act in synergy are indispensable.

Filamentous fungi are among the microorganisms which degrade lignocellulosic biomass through extracellularly produced glycoside hydrolases. Prominence has been given to the production of enzymes by fungi because of their ability to express ample amounts of hydrolases that are secreted into the medium, thus enabling effortless extraction and purification (de Souza et al., 2015; Mohanram et al., 2013; Polizeli et al., 2005). The production of cellulase and other lignocellulolytic enzymes has been widely studied in submerged culture processes in the laboratory, ranging from shake flask to 15,000 l fermentations (Sukumaran et al., 2005; Xia and Cen, 1999). The ease of handling and greater control of environmental factors of this technology requires special mention (Mrudula and Murugammal, 2011). Solid-state fermentation (SSF) is an alternative process for bioproducts production. The cultivation conditions in SSF are suitable for the growth of fungi which are able to grow at low water activities (Haltrich et al., 1996; Kumar et al., 2007). Commercial level production utilizes lignocellulosic biomass, such as cereal straw, spent hulls of pulses, bagasse, other agricultural residues, and paper industry wastes, as the carbon source (de-Limaa et al., 2005; Reczey et al., 1996; Szijarto et al., 2004). The use of nutrient-rich lignocellulosic materials is advantageous because the use of cheaper substrates can lower the cost of enzyme production (Sadhu and Maiti, 2013).

Recently, the hydrolytic efficiency of *Aspergillus terreus* has been utilized to hydrolyse highly ordered cellulose with high saccharification efficiencies (Narra et al., 2012; Nazir et al., 2012). However, identification and selection of promising targets for biorefinery applications require more insights into the hydrolytic machinery of microorganisms. The culture conditions influence the induction of specific enzyme isoforms leading to considerable variations in secretomes among different organisms and even within the same species (Girard et al., 2013; Hashemi et al., 2013; Li et al., 2013; Nazir et al., 2010). Thus, in the present study, a comprehension on the extracellular proteome of *A. terreus* and the differential expression of proteins under SmF and SSF conditions by the fungus was taken up.

2. Materials and methods

2.1. Isolation of microorganism and inoculum preparation

The fungal isolate CM20 was isolated as part of a bioprospecting survey from the Andaman coastal soils (Andaman and Nicobar Islands, India) by cultivation on Reese's mineral medium (Reese and Mandels, 1963) with acid swollen cellulose (1%) as sole carbon source. The culture was maintained on Potato Dextrose Agar (PDA) slants and was periodically sub-cultured.

2.2. Substrate for fermentation experiments

Dried, chopped straw of the rice variety Pusa Sugandh 5, collected from the farms of ICAR-Indian Agricultural Research Institute, New Delhi, was used as substrate for fermentation experiments. The cellulose, hemicellulose and lignin contents in the paddy straw were determined to be 39.3%, 22% and 16%, respectively, by the Updegraff (1969), the TAPPI (1996) and the NREL LAP-003 (Templeton and Ehrman, 1995) methods.

2.3. Comparative profiling of enzymes and other growth parameters under SmF and SSF conditions

2.3.1. Production of enzymes under SSF and SmF

For solid state fermentation, three grams of chopped substrate was taken in 250 ml Erlenmeyer flasks, to which 15 ml of Reese's mineral medium was added. For submerged fermentation, 30 ml Reese's mineral medium dispensed in Erlenmeyer flasks with 1% (w/v) of the substrate was used. For inoculation of both SSF and SmF cultures, 1 ml of spore suspension (1×10^7 spores/ml) of the fungal strain was used. The SSF cultures were incubated for seven days at 30 °C and the contents of the flasks were mixed manually on alternate days. The SmF cultures were incubated with agitation at 150 rpm in incubator shaker (Kuhner make) at 30 °C for seven days. After the incubation period, the contents of the flasks were extracted and were used as sources of extracellular enzymes.

2.3.2. Quantitative cellulolytic and xylanolytic enzyme assays

Filter paper lyase (FPase; total cellulase) and carboxy methyl cellulase (*endo*-β-1,4-glucanase; CMCase) activities were assayed as described by Ghose (1987). Avicelase (*exo*-β-glucanase) activity was assayed by the method of Zhang et al. (2009) and xylanase activity, by the method described by Ghose and Bisaria (1987). One unit of FPase/CMCase/Avicelase corresponded to 1 μM of glucose formed per minute during hydrolysis and one xylanase unit was expressed as 1 μM of xylose formed per minute during hydrolysis. β-glucosidase assay was performed using *p*-nitrophenyl-β-D-glucopyranoside as substrate (Wood and Bhat, 1988) and the activity was calculated in terms of μM of *p*-nitrophenol produced per ml of culture filtrate per minute. Proteins in the filtrates were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.The detailed protocols used in enzymatic assays have been provided as supplementary information (Supplementary text).

2.3.3. Scanning electron microscopy (SEM) analysis

The morphology and growth pattern of the fungus under SSF and SmF conditions were examined with SEM analysis. The samples were fixed in 2.5% glutaraldehyde overnight and were washed with 0.1 M phosphoric acid. The washed samples were then dehydrated with acetone by gradually increasing the concentration up to 100%. Finally, after palladium coating, SEM studies were done with Zeiss EVOMA10 scanning electron microscope at 20 KV/EHT and 10 Pa between 500× to 5000×.

2.3.4. Molecular weight determination by SDS-PAGE and activity staining by zymography

In order to determine the molecular weights of the various enzyme fractions produced in SSF and SmF, SDS-PAGE of the enzyme extract was carried out according to the method of Laemmli (1970). The crude enzyme extracts were initially concentrated by precipitation with ethanol in a ratio of 1:5 followed by resuspension of the precipitate in 0.05 M citrate buffer (pH 4.8).

For SDS-PAGE, the protein (10 μg) was loaded to 12% (w/v) SDS-PAGE gel and was run at 100 V for approximately 3 h. After electrophoresis, the gel was stained overnight in a solution of 0.1% (w/v) Coomassie Brilliant Blue R-250 in 30% (v/v) methanol and 10% (v/v) glacial acetic acid. After destaining, the gel was analyzed for bands along with a molecular weight marker. For the detection of cellulase and xylanase by zymography, 10 μg of *A. terreus* protein was boiled with SDS sample buffer (without β-mercaptoethanol) and loaded on zymogram gel made of 12% (w/v) PAGE gel with 1% CMC or birchwood xylan. Following electrophoresis, the zymogram

gel was soaked for 1 h in 2.5% (v/v) Triton X 100 for the renaturation of protein and washed thoroughly in distilled water prior to incubation at 50 °C for 30 min with 0.05 M citrate buffer (pH 4.8). The gel was washed with distilled water and stained with 0.1% Congo red for 10 min and washed with 5% NaCl until clear bands became visible. For β-glucosidase zymography, the sample was unboiled and the same amount of protein was loaded onto the gel. After renaturation and washing, the gel was incubated with 0.05 M citrate buffer (pH 4.8) containing 0.1% (w/v) esculin and 0.03% (w/v) ammonium iron (III) citrate for 10 min at 55 °C. The activity staining was visualized under UV light using Gel Doc EZ Imager (BioRad, Hercules, CA).

2.4. Concentration of extracellular proteome produced under SSF condition by organic solvent precipitation

Different organic solvents were tested for their ability to precipitate the enzymes in crude extract from SSF with maximum activity retention. Chilled solvents (acetone/acetonitrile/butanol/dimethyl sulfoxide (DMSO)/ethanol/propan-2-ol/methanol) were added to crude enzyme extracts in a ratio of 1:5 and were kept overnight at 4 °C. The protein precipitates were collected by centrifugation at 8832*g* for 10 min. The pellets were air-dried to remove residual solvent and were dissolved in 0.05 M citrate buffer (pH 4.8). The activity recovery % and fold-concentration of different enzymes were calculated.

2.5. Analysis of extracellular proteome of A. terreus CM20 produced under SSF condition

2.5.1. Protein digestion

To 0.5 mg of protein, 25 μl of 100 mM ammonium bicarbonate (ABC) solution was added and the proteins were denatured with trifluoroethanol (25 μl) and 200 mM dithiothreitol (2.5 μl). The mixture was vortexed and heated to 60 °C for 30 min. Then, 10 μl of 200 mM iodoacetamide (IAM) solution was added for alkylation and allowed to stand at room temperature for 1 h in the dark. About 300 μl of water and 100 μl of 100 mM ABC solution were added to raise the pH to 8.0. Sequencing grade modified porcine trypsin (Promega, Madison, WI, US) in ABC was added in the ratio of 1:50 and incubated overnight at 37 °C. Formic acid (2 μl) was added to lower the pH in order to stop the trypsin activity. The peptides were dried using speedvac and stored at −80 °C until MALDI-MS analysis.

2.5.2. LC–MS/MS analysis

The peptides were reconstituted in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA), and injected into the chromolith Caprod RP-18e (150-0.1 mm) column. The extracted peptides were spotted with α-cyano-4-hydroxycinnamic acid (CHCA) matrix in a 1:1 ratio. MALDI-MS data was acquired automatically over a mass range of 800–3500 Da in the reflector ion mode on an AB (Applied Biosystems) 4800 MALDI-TOF/TOF Analyzer with 4000 Series Explorer v3.5 software, using a fixed laser intensity for 1500 shots/spectrum, with a uniformly random spot search pattern. The potential difference between acceleration voltage and floating collision cell defines the collision energy, which was 1 keV in all experiments. Re-optimized instrument settings were employed to achieve optimal sensitivity. Air was used as the collision gas such that nominally single collision conditions were achieved. In each MS spectrum, the 10 most abundant MS peaks were selected for MS/MS using an acquisition method that excluded ions with S/N less than 50, and which filtered out identical peaks detected in adjacent spots, selecting only the strongest precursor. The precursor ions with the weakest S/N were acquired first in order to achieve the maximum signal intensity for low-abundance peptides. A 1 kV MS/MS operating mode was used, the relative precursor mass window was set at 250 (full width half mass), with metastable suppression enabled. MS/MS acquisition of selected precursors was set to a maximum of 1250 shots with 50 shots per subspectrum using fixed laser intensity. The stop-condition criteria were set to a minimum of 100 S/N on more than seven peaks within the spectrum after the minimum 1000 shots. The data were searched using the online MASCOT search engine (Matrix Science, Boston, MA, USA). A maximum of one missed cleavage, peptide tolerance 100 ppm and MS/MS tolerance 0.4 Da were used in the MASCOT search engine. Searches were conducted against the fungal database available in NCBI.

2.6. Enzymatic saccharification of alkali-pretreated paddy straw

Paddy straw was pretreated with alkali as described by Pandiyan et al. (2014). Saccharification of the substrate (4% w/v) was carried out in plastic bottles with the acetone-precipitated *A. terreus* CM20 proteins (56 FPU/gds) at 50 °C and 150 rpm in a temperature controlled shaker water bath for 48 h. Aliquots were taken out periodically from the reaction mixture and the amount of reducing sugars released was quantified by high performance liquid chromatography (HPLC, Waters Corporation, Milford, MA, USA) equipped with Waters 515 pump and Waters 2414 refractive index detector. The Aminex HPX-87H column was operated with 5 mM H_2SO4 as mobile phase at a flow rate of 0.5 ml/min and the oven temperature was kept at 40 °C.

All experiments were carried out in triplicate. Statistical analyses, such as critical difference and mean standard error, were performed using SPSS 16.0 software package.

3. Results and discussion

3.1. Identification of the microorganism

The fungal isolate CM20 which was able to grow on cellulose as sole carbon source produced white mycelium on potato dextrose agar with numerous brownish spores. The isolate was identified by Indian Type Culture Collection (ITCC), New Delhi on the basis of their morphological characteristics followed by sequencing of the ITS region and BLAST search. The isolate was identified as *Aspergillus terreus* (NCBI GenBank accession number KM401596).

3.2. Comparative profiling of enzymes and other growth parameters under SmF and SSF conditions

3.2.1. Enzyme production under SmF and SSF

The fungus was found to secrete higher amount of proteins under the solid-state fermentation condition (0.25 mg/ml) as against the submerged fermentation (0.05 mg/ml). Also the lignocellulose-induced profiles of cellulase and xylanase production under the two fermentation conditions were found to be significantly different. The levels of extracellular enzymes produced in SSF were significantly higher than those produced in SmF. Under SSF, *A. terreus* CM20 produced 3 times the CMCase and FPase, 2 times the xylanase, and 1.5 times the pNPGase produced under submerged fermentation (Table 1). Moreover, significant cellobiohydrolase activity (Avicelase) could be detected in solid state fermentation.

Presently, cellulases are produced from fungi, bacteria and actinomycetes, using either submerged fermentation (SmF) or solid state fermentation (SSF) on several carbon sources (Cunha et al., 2012; El-Hadi et al., 2014; Gamarra et al., 2010; Oberoi et al., 2010).

Table 1
Cellulolytic and xylanolytic enzymes (IU/ml) produced by *A. terreus* CM20 under submerged (SmF) and solid state (SSF) fermentation conditions.

Fermentation condition	CMCase	FPase	Avicelase	pNPGase	Xylanase
Submerged (SmF)	4.4 ± 0.21	0.4 ± 0.01	0.02 ± 0.001	12.8 ± 0.68	41.5 ± 1.5
Solid-state (SSF)	12.9 ± 0.75	3.4 ± 0.15	0.95 ± 0.05	14.09 ± 0.89	97.01 ± 1.8

When *Aspergillus niger* was used for cellulase production in SmF and SSF using coir waste as substrate, almost 14.6 fold higher productivity of cellulase was obtained in SSF (Mrudula and Murugammal, 2011). Higher enzyme yields and volumetric productivities associated with SSF have been reported by various researchers (Gamarra et al., 2010; Mazotto et al., 2013; Renovato et al., 2011).

3.2.2. *Growth pattern of* A. terreus *CM20 in submerged fermentation and solid state fermentation conditions*

To gain more insights, the growth pattern of the fungal strain under the two culture conditions was studied by SEM analysis. Morphologically, the fungus was identical to other reported strains of *A. terreus* (JX155853; ITCCI-8575.11) (Louis et al., 2013, 2014). However, under SmF, *A. terreus* CM20 displayed a dispersed filamentous growth (Supplementary Fig. 1a), while extensive attachment of mycelium onto the substrate, resembling the natural growth condition of fungus, was seen in SSF. This interaction between the microorganism and the substrate established in SSF is stated to be responsible for the high level of enzyme production in SSF (Cunha et al., 2012; Singhania et al., 2010). The excretion of hydrolytic enzymes at the hyphal tip, without large dilution like in the case of SmF, also makes their action very efficient under SSF (Raimbault, 1998). It has been previously reported that in *Trichoderma reesei*, sporulation coincides with the upregulation of a broad array of carbohydrate-active enzymes so as to prepare the spores for rapid germination in a cellulose-rich medium (Metz et al., 2011). This effect was observed under the SSF culture conditions where *A. terreus* CM20 displayed heavy sporulation and secreted high concentrations of hydrolytic enzymes (Supplementary Fig. 1b).

3.2.3. *Quantitation of proteins expressed under SmF and SSF conditions*

Differential expression of enzymes in SSF and SmF was further investigated in terms of the hydrolytic enzyme production profiles. SDS-PAGE and zymogram analysis were done using ethanol-precipitated enzyme extract. *A. terreus* CM20 secreted more proteins under solid state fermentation conditions than under submerged fermentation conditions and the protein bands ranged from 17–165 kDa, as evident from the SDS-PAGE gels (Fig. 1a). Oda et al. (2006) examined the production of extracellular proteins by *Aspergillus oryzae* under both SSF and SmF culture conditions. He found that during 32 h and 40 h of growth in SSF culture, *A. oryzae* secreted 53.7 and 77.3 mg protein per g dry mycelium, respectively, while in SmF culture the values were only 13.2 and 11.9 mg protein, respectively.

Zymograms for each of the assayed enzyme activities are given in Fig. 1(b)–(d). The images reveal clear differences in the enzyme activity profiles expressed under the two bioprocess conditions. CMCase displayed two major isoforms of 26 kDa and 33 kDa in the SSF culture while only the 33 kDa band was evident in the SmF culture (Fig. 1b). Zymogram analysis of xylanase activity performed with birch wood xylan showed significant difference between the SmF- and SSF-expressed activities. Five bands of approximately 20, 28, 30, 34 and 42 kDa were observed in SSF samples, whereas only a single band of 34 kDa was observed in the SmF sample (Fig. 1c). The β-glucosidase bands were revealed only when the samples were not boiled and two prominent bands of ~85, ~82 kDa were observed in activity based zymography of both SmF and SSF samples. Additionally, the SSF-produced enzyme showed an extra band of ~50 kDa. Peterson et al. (2011) had reported that β-glucosidase activity was eliminated by boiling the samples and the zymogram produced from unboiled samples revealed very high β-glucosidase activity.

The results were in perfect correlation with the manifold increase in extracellular enzymes activities obtained in SSF as compared to SmF. Fairly similar results were observed during xylanase production by the fungus, *Simplicillium obclavatum* under solid state and submerged fermentation conditions (Roy et al., 2013). While the fungus produced multiple xylanases and endoglucanases in SSF, only a single xylanase isoform was detected in SmF. Multiple isoforms of enzymes secreted in SSF, but not in SmF, has been attributed to the low water activity of the solid medium that can induce specific gene expression, thereby affecting the metabolite production (Ferreira et al., 2010; Ishida et al., 1998). Expression of certain genes specifically in the solid state fermentation conditions has been demonstrated in *Aspergillus oryzae* by subtractive cloning of cDNA (Akao et al., 2002) as well as by comparative proteome and Northern analyses of expressed proteins (Oda et al., 2006). Therefore, as opined by Li et al. (2013), SSF is the most promising method for the production of hydrolytic enzymes and other bioactive compounds. Most importantly, about 10-fold reduction in production cost has been observed when SSF was employed for cellulase production (Tengerdy, 1996). As all the parameters examined were found to be better under SSF conditions, further analysis was done using the SSF extracellular proteome.

3.3. *Organic solvent precipitation of crude enzyme extract*

Seven different organic solvents were checked for precipitation of *A. terreus* CM20 crude enzyme extract with maximum activity retention, the details of which are given in Table 2. Among the solvents, dimethyl sulfoxide (DMSO) failed to cause significant precipitation of the proteins. About 83.0–84.1% of the proteins could be recovered by using ethanol or acetone as the precipitant. All the enzymes assayed also retained their maximum activities with either acetone or ethanol, and the activity retentions obtained with the two solvents were statistically at par. β-glucosidase retained around 88.4–88.9% activity and xylanase retained 83.0–84.1% activity. Also, 42.3–46.1% CMCase activity and 41.0–59.7% FPase activity could be retained. Avicelase, but showed lesser activity retention of 16.2–17.5%. Propan-2-ol and methanol were able to cause recovery of 70.0–76.1% of total proteins, with significant enzyme activities. Acetonitrile and butanol, though caused considerable protein precipitation, did not maintain high enzyme activities. Several studies have shown acetone and ethanol to be the solvents better suited for precipitation and partial purification of enzymes (Illanes, 2008; Tiwari et al., 2014; Trentini et al., 2015).

3.4. *Extracellular proteome analysis of* A. terreus *CM20*

The soluble secreted proteins which have major roles in a cell's central biological processes constitute the extracellular proteome

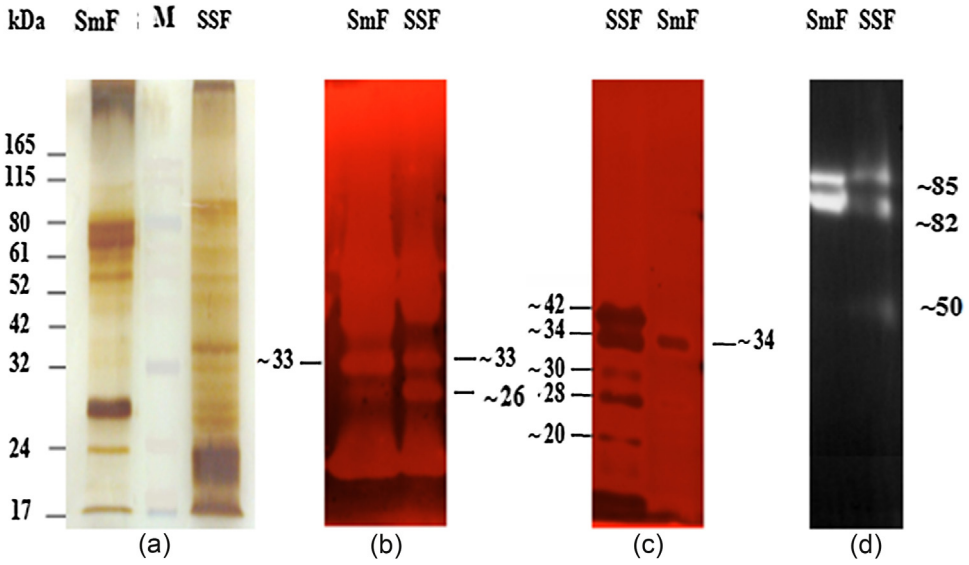

Fig. 1. SDS-PAGE gels and zymograms of enzyme extracts of *A. terreus* CM20 expressed under submerged fermentation (SmF) and solid state fermentation (SSF)—(a) SDS-PAGE gels (M-Pink Plus prestained protein marker), (b) CMCase zymogram, (c) xylanase zymogram, (d) β-glucosidase zymogram.

Table 2
Organic solvent precipitation of crude enzyme extract of *A. terreus* CM20.

Organic solvent	Activity recovery (%)					Protein recovery (%)
	CMCase	FPase	Avicelase	pNPGase	Xylanase	
Crude enzyme	100.0	100.0	100.0	100.0	100.0	100.0
Methanol	22.5	7.7	13.8	44.9	50.9	70.0
Acetonitrile	5.0	10.6	7.2	9.0	17.6	22.4
Acetone	42.3	59.7	17.5	88.4	61.8	84.1
Propan-2-ol	36.2	27.8	14.2	81.0	55.7	76.1
Butanol	19.2	8.2	1.9	20.4	26.5	48.1
Ethanol	46.1	41.0	16.2	89.9	62.7	83.0
DMSO	–	–	–	–	–	–

of the cell (Braaksma et al., 2010). The analysis of proteome using mass-spectrometry presents a chance to understand the various enzymes secreted by the fungus as well as to study their complex interactions in bringing about biomass degradation (Singh et al., 2014; Tiwari et al., 2014). So, to better understand the identities and functions of the enzymesexpressed by *A. terreus* CM20, analysis of the extracellular proteome was carried out. The proteome was digested with trypsin and the resultant peptides were analyzed by liquid chromatography coupled tandem mass spectrometry (LC–MS/MS).

A total of 63 different proteins in the extracellular proteome were detected and identified through database searches (Mascot,

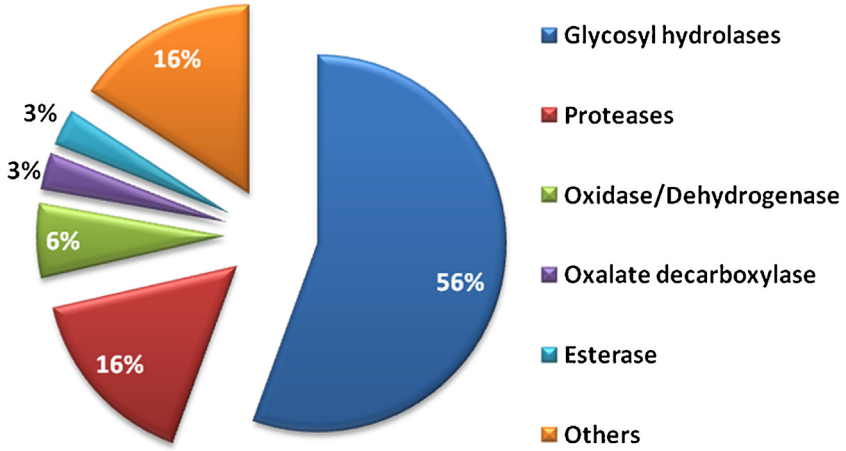

Fig. 2. Functional classification of proteins detected in the *A. terreus* CM20 secretome.

Table 3
Hydrolytic enzymes detected in the extracellular proteome of *A. terreus* CM20.

Sl No.	gi accession	Description	Score*	Matches	Sequence coverage (%)	Mass (kDa)	pI	Protein family	Substrate binding domain
Cellulases									
1	115397177	Reducing end-acting cellobiohydrolase	732	12(8)	18	57.26	4.77	GH7	CBM1
2	115436902	Periplasmic β-glucosidase	697	12(7)	24	82.71	5.34	GH3	–
3	115400906	Endo-β-1,4-glucanase	556	6(6)	32	25.88	5.86	GH12	–
4	115449373	Endo-β-1,4-glucanase	169	3(1)	7	69.00	5.98	GH5	–
5	915102397	β-glucosidase	128	2(1)	3	71.64	4.55	GH1	–
6	629663225	β-glucosidase	103	2(1)	3	69.61	4.68	GH1	–
7	179743727	Exo-cellobiohydrolase	89	3(1)	4	37.89	4.44	GH7	CBM1
8	333980940	Endoglucanase	78	4(0)	18	42.99	5.23	GH5	–
9	115388467	Fungal 1,3(4)-β-D-glucanase	76	1(1)	6	33.03	6.39	GH16	CBM1
10	950686	Reducing end-acting cellobiohydrolase	69	3(0)	1	55.69	4.95	GH7	–
11	156048578	Reducing end-acting cellobiohydrolase	58	2(0)	3	47.39	4.57	GH7	–
12	361131160	1,4-β-D-glucan cellobiohydrolase	54	2(1)	4	51.55	6.22	GH7	–
13	70983939	β-glucosidase	53	2(0)	3	65.31	5.09	GH1	–
14	70996706	Periplasmic β-glucosidase	53	1(1)	1	82.63	5.14	GH3	–
15	302880422	Periplasmic β-glucosidase	53	1(1)	1	87.53	5.91	GH3	CBM1
16	816331821	Reducing end-acting cellobiohydrolase	52	1(1)	1	56.74	4.57	GH7	–
Hemicellulases									
17	115401944	α-L-arabinofuranosidase	575	10(5)	23	52.17	6.05	GH54	–
18	115390723	Arabinan *endo*-1,5-α-L-arabinosidase	352	6(5)	24	35.37	6.11	GH43	–
19	115400988	Endo-β-1,4-xylanase	258	4(3)	19	24.74	6.05	GH11	CBM42
20	818166321	α-L-arabinofuranosidase	191	4(2)	8	51.39	6.04	GH54	CBM42
21	596540951	α-L-arabinofuranosidase	141	3(1)	5	51.96	6.14	GH54	CBM42
22	915705830	α-L-arabinofuranosidase	96	2(1)	3	51.88	5.46	GH54	–
23	45269104	α-L-arabinofuranosidase	94	1(1)	8	23.30	4.58	GH62	CBM1
24	682272144	α-L-arabinofuranosidase	94	1(1)	4	40.89	8.29	GH62	–
25	115402831	Endo-1,4-β-xylanase	78	2(1)	6	33.21	8.46	GH10	–
26	584145179	α-L-arabinofuranosidase	77	1(1)	5	34.98	8.38	GH62	–
Proteases									
27	115442704	Serine carboxypeptidase	326	5(4)	12	59.66	4.66	S10	–
28	115490965	Serine carboxypeptidase	314	5(4)	8	60.37	4.74	S10	–
29	115438046	Serine carboxypeptidase	256	5(3)	10	67.62	5.01	S10	–
30	169777249	Serine carboxypeptidase	210	3(3)	7	67.44	4.74	S10	–
31	115491521	Peptidase	138	2(2)	10	27.94	4.55	G1	–
32	303318879	Serine carboxypeptidase	105	2(1)	3	72.62	4.57	S10	–
33	212535660	Serine carboxypeptidase	71	1(1)	1	70.87	4.35	S10	–
34	285611	Aspergillopepsin-like aspartic protease	68	1(1)	2	42.27	4.85	Aspartyl protease family	

Table 3 (*Continued*)

Sl No.	gi accession	Description	Score*	Matches	Sequence coverage (%)	Mass (kDa)	pI	Protein family	Substrate binding domain
35	169779013	Serine carboxypeptidase	55	1(1)	1	59.62	4.75	S10	–
36	189188566	Serine carboxypeptidase	47	1(0)	2	60.86	4.71	S10	–
Others									
37	115388667	β-1,6-glucanase	243	4(3)	12	49.56	4.72	GH30	–
38	115395828	Glucoamylase	232	5(2)	9	67.75	5.16	GH15	CBM20
39	115449417	Invertase	188	3(2)	5	70.35	5.08	GH32	CBM66
40	115388721	Monoamine oxidase	179	4(2)	10	51.37	5.43	Flavin containing amineoxidore-ductase family	
41	115384178	Glucooligosaccharide oxidase	166	3(2)	6	61.02	6.17	AA7	–
42	115396640	Hydrophobic surface binding protein A	161	3(2)	16	26.35	5.10	HsbA	–
43	115386510	FAD/FMN-containing dehydrogenase	136	3(1)	6	61.57	5.71	AA7	–
44	115383814	Cerato-platanin	123	4(0)	8	50.96	4.60	Cerato-platanin family	
45	115384120	Cerato-platanin	111	3(0)	37	14.48	8.73	Cerato-platanin family	
46	115400505	Feruloyl esterase	102	2(1)	3	91.98	5.95	Tannase family	–
47	115492149	β-galactosidase	94	3(0)	3	105.25	6.13	GH35	–
48	115401674	Oxalate decarboxylase	90	2(1)	4	49.92	4.47	Bicupin family	–
49	115387173	Esterase	88	1(1)	5	35.52	7.49	CE1	CBM1
50	115401562	Polygalacturonase	77	1(1)	6	24.20	4.32	GH28	–
51	630015437	Oxalate decarboxylase	69	1(1)	1	58.64	4.69	Bicupin family	–
52	115396494	Sialidase	63	2(0)	2	88.87	5.06	GH74	CBM1
53	115396724	β-1,3-glucanosyltransferase	59	1(1)	2	56.68	4.69	GH72	CBM43
54	685411221	RNA recognition motif	55	1(1)	0	160.46	7.04	RRM	–
55	591486600	Transcriptional regulator	54	2(0)	5	46.38	4.53	–	–
56	443918739	Glucose-1-dehydrogenase	53	1(1)	1	97.25	9.71	SDR	–
57	156063954	Chromatin organization modifier domain	50	2(0)	0	141.05	6.95	–	–
58	1705640	Catalase	48	1(0)	1	80.41	5.44	–	–
59	918823217	Phox-like protein	48	2(0)	2	40.99	9.52	SNARE	–
60	291510286	Chromosome segregation protein	47	1(0)	3	35.08	6.44	SMC	–
61	630018265	α-glucuronidase	47	1(0)	2	58.03	4.89	GH67	–
62	115385609	α-mannosidase	96	1(1)	3	59.46	5.19	GH125	–
Hypothetical protein									
63	615400357	Hypothetical protein	47	1(0)	4	26.04	4.86	–	–

* Individual ions scores >47 indicate identity or extensive homology ($p < 0.05$).

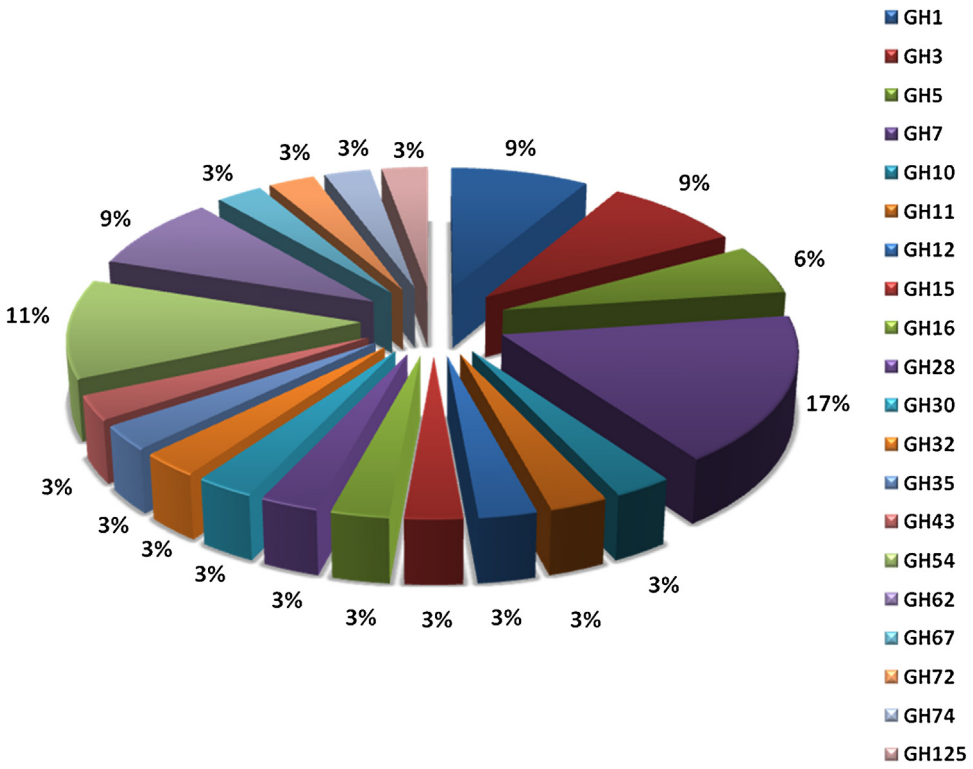

Fig. 3. Distribution of various glycoside hydrolase (GH) families in the extracellular secretome of *A. terreus* CM20.

NCBI and CAZy). These included cellulases, hemicellulases, proteases, and other enzymes and proteins involved in polysaccharide modification and cell maintenance, the details of which are given in Table 3. Manual interpretations of peptide MS/MS spectra were also checked for the proteins which are included in the table. The detected proteins had molecular weights ranging from 10–170 kDa and isoelelectric points (pI) from 4.0–10.0 (Supplementary Fig. 2). Nevertheless majority of the proteins were of low molecular weight (54% proteins of 40–70 kDa and 24% of <40 kDa) and almost 86% of the total proteins clustered within an acidic pI range of 4–6. Functional classification of the identified proteins, depicted in Fig. 2, showed a major proportion of glycoside hydrolases (56%), the superfamily of cellulases, hemicellulases and other accessory enzymes involved in polysaccharide hydrolysis. Other detected hydrolytic enzymes included proteases and lesser amounts of oxidases, dehydrogenases, esterases, decarboxylases and other minor proteins. The glycoside hydrolases (GH) were further categorized into GH families based on their sequence homology and they were found to belong to 20 different families. About 17% of the enzymes belonged to the family GH7 which houses *endo*-β-1,4-glucanase (EC 3.2.1.4) and reducing end-acting cellobiohydrolase (EC 3.2.1.176), and 11% belonged to GH54, encompassing α-L-arabinofuranosidase (EC 3.2.1.55) and β-xylosidase (EC 3.2.1.37) (Fig. 3). The GH family 62, also containing α-L-arabinofuranosidase (EC 3.2.1.55) constituted 9% of the glycoside hydrolases detected. Another 9% consisted of each of the families, GH1 and GH3, comprising of β-glucosidases (EC 3.2.1.21). Moreover, less common glycoside hydrolases belonging to the family GH125 were also detected. Besides, several polysaccharide-side chain hydrolysing enzymes and esterases were also detected, which belonged to the AA7 (Accessory Activities) and CE1 (Carbohydrate Esterase) families, respectively. The presence of esterases in the extracellular proteome is also noteworthy because they act in concert with xylanases to cause hemicellulose degradation, as suggested by Biely et al. (2014).

Seven of the hydrolytic enzymes, which belonged to GH3, GH7, GH16, GH62, GH 74 and CE1 families had the module CBM1, which according to Lombard et al. (2014) is a cellulose-binding domain found exclusively in fungi. Three enzymes, which belonged to GH11 and GH 54 families, were found to contain the CBM42 module, known to bind to the non-reducing end L-arabinofuranosyl residues, present in hemicelluloses like arabinoxylan, arabinogalactan and arabinan (Miyanaga et al., 2004). Other enzymes were found to possess CBM20, a starch-binding module (Lombard et al., 2014), CBM43, involved in β-1,3-glucan binding (Barral et al., 2005) and CBM66, which targets the terminal fructoside residue of fructans (Cuskin et al., 2012). The presence of these substrate-binding modules further points to the targeting and adhesive properties of the proteins which increase the effective concentration of catalytic modules on the surface of respective substrates (Shoseyov et al., 2006; Singh et al., 2014).

Besides cellulases and hemicellulases, several proteases, oxidases and oxalate decarboxylase were also detected. The proteases have a major role in assimilating nitrogen from the complex organic nitrogen source in the medium used for induction of the enzymes. There are also a few reports on the inclusion of proteases as auxiliary enzymes in novel enzyme mixtures that can be used directly on lignocellulosic biomass (Duck et al., 2004; Nadine et al., 2003). The role of oxalate decarboxylase is postulated to be in cell-wall degradation by complexing with calcium and weakening the cell-wall structure (Sato et al., 2007).

Hence, analysis of the extracellular proteome revealed the great diversity of synergistically acting glycoside hydrolases and other proteins expressed by *A. terreus* CM20, the harmonious action of which can cause appreciable biomass bioconversion.

3.5. Enzymatic saccharification of alkali-pretreated paddy straw using the crude enzyme preparation from A. terreus CM20

The synergistic action of the *A. terreus* CM20 enzyme cocktail was assessed by saccharification of alkali-pretreated paddy straw. Alkali pretreatment showed significant increase in the carbohydrate content (49% cellulose and 30% hemicelluloses) of the paddy straw as alkali pretreatment enhances lignin solubilisation. Alkali pretreatment is the method of choice for materials that have relatively low lignin content like agricultural residues (Saritha et al., 2012).

Saccharification of the substrate was carried out using acetone-precipitated enzyme extract because acetone had the advantage of being relatively inexpensive and also caused protein precipitation and enzyme activity recoveries identical to ethanol. After 48 h of saccharification, fermentable sugar yield of 616.8 mg/gds was obtained. Soto et al. (1994) and Xu et al. (2007) have reported the attainment of maximum saccharification of alkali treated sunflower hulls and ammonia pretreated soybean straw, respectively, with an enzyme concentration of 50 FPU/g. Although doubling of enzyme loading increases the sugar yield by more than 13% (Chang et al., 1996), it makes the process uneconomical. However, high enzyme loadings can compensate for the loss in cellulose conversion at high solids loadings, which is important for the bioethanol process to be economical (Humbird et al., 2010). The reducing sugar yield obtained in the present study was significantly higher than several reports in the literature. Enzymatic hydrolysis of alkali pretreated rice straw using cellulase from *T. harzianum* SNRS3 resulted in a reducing sugar yield of 600 mg/g substrate (Rahnama et al., 2014). Steam pretreated rice straw when saccharified with commercial cellulase (Sigma) and β-glucosidase (Sigma) supplemented with indigenously produced xylanase, released 553.33 mg/gds of reducing sugars (Choudhary et al., 2014). In a recent study, saccharification of phosphoric acid pretreated wheat straw with cellulase from *T. reesei* NCIM 1186 exhibited a maximum sugar release of 371.44 mg/gds (Kuila et al., 2015).

Moreover, HPLC analysis detected the presence of monosaccharides—glucose (439.63 ± 1.50 mg/gds), xylose (121.04 ± 1.25 mg/gds) and arabinose (56.13 ± 0.56), in the hyrolysates of alkali-pretreated paddy straw saccharified with crude enzyme from *A. terreus* CM20 (Supplementary Fig. 3). Sugar yields similar to the present study have been obtained previously on saccharification of alkali pretreated paddy straw with *A. terreus* (Narra et al., 2012). However, thin layer chromatography of hydrolysates detected mainly glucose with very low amounts of xylose. Mahajan et al. (2014) also carried out saccharification of alkali pretreated rice straw at 10% (w/v) substrate loading with glycoside hydrolases from the thermophilic fungus, *Malbranchea cinnamomea*. They obtained saccharification corresponding to 44.9 mg/ml of reducing sugars, and the hydrolysate was quantified to contain only glucose and xylose. The distinct profiles of monosaccharide sugars obtained on analysis of hydrolysates by HPLC clearly demonstrate the improved properties of *A. terreus* CM20 cellulase complex in terms of its enhanced glycoside hydrolase activities which act in synergy.

The study, therefore, signifies the cellulolytic potential of the native isolate, *A. terreus* CM20, which when tailor-made according to specific bioprocess requirements, can be utilized in the development of low-cost and substrate-specific cellulase preparation befitting the indigenous bioethanol industry. However, advanced proteomic platforms are required to explain the possible posttranscriptional regulations in the fungus in response to the environment resulting in differential protein expression profiles under different culture conditions.

Conflict of interest

The authors declare that no conflict of interest, financial or other, exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2016.06.006.

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