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Glycoside hydrolase production by *Aspergillus terreus* CM20 using mixture design approach for enhanced enzymatic saccharification of alkali pretreated paddy straw

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A successful lignocellulosic ethanol production process needs to address the technological impediments such as cost-competitiveness and sustainability of the process. Effective biomass utilization requires a repertoire of enzymes including various accessory enzymes. Developing an enzyme preparation with defined hydrolytic activities can circumvent the need for supplementing cellulases with accessory enzymes for enhanced hydrolysis. With this objective, mixture design approach was used in the present study to enhance glycoside hydrolase production of a fungal isolate, *Aspergillus terreus* CM20, by determining the proportion of different lignocellulosic components as enzyme inducers in the culture medium. A mixture of paddy straw and wheat straw (1.42:1.58) resulted in improved cellulolytic activities. The precipitated crude enzyme showed higher CMCase (365.03 18 IU $g^{-}1$), FPase (161.48 IU g^{-1}), avicelase (15.46 IU g^{-1}), β-glucosidase (920.92 IU g^{-1}) and xylanase (9627.79 IU g^{-1}) activities. The potential of the crude enzyme for saccharification of alkali pretreated paddy straw was also tested. Under optimum conditions, saccharification released 25.0 g L^{-1} of fermentable sugars. This indicates the superiority of the crude enzyme produced with respect to its hydrolytic enzyme components.

Keywords: Avicelase, Biomass, CMCase, β-glucosidase, Lignocellulose, Solid state fermentation, Xylanase

Lignocellulose, a common waste material from agriculture, is the most abundant and renewable biopolymer on earth. A promising strategy for efficient utilization of this renewable resource is microbial hydrolysis of the lignocellulosic biomass and fermentation of the resultant reducing sugars for production of desired metabolites or biofuel[1]. However, production of enzyme complex which can act synergistically to unlock and saccharify polysaccharides from the lignocellulose complex to fermentable sugars not only incurs major costs but also presents a great challenge[2].

Glycoside hydrolases (GH) comprise a pool of enzymes that include cellulases, hemicellulases, and other accessory enzymes used by microorganisms for biomass degradation[3,4]. Endoglucanases hydrolyze glycosidic bonds internally in cellulose chains at random and cellobiohydrolases act on chain ends, the synergistic action thus producing cello-oligosaccharides and cellobiose, which are then cleaved by β-glucosidase to glucose. The more complicated hemicellulose, with non-xylose substituents on xylan backbone, requires the action of several substituent-hydrolysing enzymes in addition to endoxylanase and β-xylosidase activities[5]. Almost all industrial cellulases are produced from filamentous fungi of the genera, *Trichoderma* and *Aspergillus*. However, the poor yield of β-glucosidase by *T. reesei* strains and the lower endoglucanase levels in *Aspergillus* sp. often warrant supplementation with commercial enzyme preparations for complete saccharification of lignocellulosic biomass[6]. Developing a hydrolytic enzyme preparation with defined cellulolytic and hemicellulolytic activities can circumvent this need for supplementing cellulases with accessory enzymes for enhanced hydrolysis.

Lignocellulosic materials such as agricultural residues are being used as substrates for production of glycoside hydrolases by filamentous fungi[7-9]. However, the complex nature of biomass coupled with the intricate regulation of fungal cellulolytic enzyme systems result in variable physiological responses of fungi[10]. Hence, designing an appropriate

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production medium is a crucial step in the fermentation process development. This involves screening of the critical medium components influencing production of the desired product and finding the optimum concentration of each component for maximum product formation[11]. Several statistical approaches used till date deal with single substrate source or randomly selected proportions of the mixture components. The synergetic effects of mixed substrates can be investigated by the mixture design method, which studies interactions among proportions of different components of a blend that have significant influence on the result, measured in one or more responses[12,1].

In the present study, we used 'mixture design' to evaluate the influence of various lignocellulosic components on glycoside hydrolases production by a fungal isolate, *Aspergillus terreus* CM20, under solid state fermentation. The concentrated crude enzyme was used for saccharification of alkali pre-treated paddy straw under optimum loadings of enzyme and substrate to obtain maximum sugar yields.

Materials and Methods

Isolation and characterization of the microorganism

The fungus was isolated from the coastal soils of Little Andaman island (10° 30′N: 92° 37′E) (Andaman and Nicobar Islands), India on minimal medium with cellulose as sole carbon source. The culture was maintained on Potato Dextrose Agar slants and was periodically sub-cultured. For molecular identification, total DNA was isolated and the rDNA gene cluster, consisting of ITS-1, the 5.8S rDNA and ITS-2, was amplified with primers homologous to conserved sequences within the small subunit (SSU) rRNA gene. The ITS primers used were ITS-1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS-4 (5' TCCTCCGCTTATTGATATGC 3') to amplify fragment of approximately 650 bp. The amplified products were outsourced to Xcelris Labs Ltd, Ahmedabad, India for DNA sequencing. The partial sequences of the isolate were compared with sequences available by the BLAST search in the National Center for Biotechnology Information (NCBI) database (http://www.nvbi.nlm.nih.gov) to identify the nearest taxa and were submitted to NCBI GenBank.

Mixture design for production of glycoside hydrolases using lignocellulosic substrates

The lignocellulosic substrates used in the study were paddy straw (variety Pusa Sugandh 5) with 39.3% cellulose, 22.2% hemicellulose and 16% lignin, wheat straw (variety HD 2967) with 34.6% cellulose, 26.8% hemicellulose and 17.2% lignin and corn cob (hybrid variety HQPM-1) with 37.4% cellulose, 32.2% hemicellulose and 18.4% lignin. The effects of these substrates on the glycoside hydrolase production by *A. terreus* CM20 were established using mixture design which was set up as a simplex centroid design for three components at a fixed total concentration of 3 g.

The design comprised ten experiments, including three centre point experiments. The design table for these experiments is presented in Table 1. The design and analysis was performed with Minitab (Release 16) statistical software (Minitab Inc., USA). The different combinations of the chopped substrates (1 cm) were taken in 100 mL Erlenmeyer flasks and were sterilized by autoclaving at 121°C for 15 min. Inoculation was done using 5 mycelial discs (6 mm diameter) of the 7 days old fungal growth on PDA plates and 15 mL of sterilized Reese's mineral medium[15] was added to each flask. The flasks were incubated at 30°C and the contents of the flasks were mixed manually on alternate days. After 7 days of incubation, the crude extracellular enzyme was collected by extraction with 0.05 M citrate buffer (pH 4.8) followed by filtration and centrifugation at 10000 rpm. The enzyme was stored at 4°C for further use.

Quantitative 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[16] and avicelase (exo-β-glucanase) activity by the method of Zhang *et al.*[17]. Xylanase activity was assayed by the method described by Ghose and Bisaria[18]. β-glucosidase assay was performed using *p*-nitrophenyl-β-D-gluco pyranoside as substrate[19]. Proteins in the filtrates were determined by the Bradford method[20] using bovine serum albumin (BSA) as standard.

Large scale production of hydrolytic enzymes and their concentration

A. terreus CM20 was grown on the optimized substrate mixture (60 g) in 1 L Erlenmeyer flasks along with 300 mL of sterilized Reese's mineral medium. The incubation conditions were as discussed earlier. The extracted crude enzyme was concentrated by precipitation with acetone, following the method of Tiwari *et al.*[21].

SDS-PAGE and zymography

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. The gel was then stained overnight using Coomassie Brilliant Blue R-250 and, after destaining, the gel was analyzed for bands along with a molecular weight marker[20]. The cellulose, xylanase and β-glucosidase activities were detected by zymography, following the procedure of Tiwari *et al*.[21].

Enzymatic saccharification of pretreated lignocellulosic substrate

The concentrated crude enzyme extract of *A. terreus* CM20 was used for saccharification of alkali pretreated paddy straw. Dried and chopped paddy straw of the variety Pusa Sugandh 5 was used as substrate in the experiment. Alkali pretreatment was done by treating the paddy straw with 1% NaOH as described by Pandiyan *et al*.[22]. The treated samples were washed with distilled water until neutral pH and stored at 4°C. These samples were characterized for cellulose content according to the method of Updegraff[23] and hemicelluloses and lignin, according to TAPPI method[24]. The saccharification experiments were carried out in 50 mL screw capped plastic bottles in 0.05 M citrate buffer (pH 4.8) at 50°C and 150 rpm in a constant temperature shaker water bath. The effect of substrate concentration (1–10% w/v) and enzyme loading (10-90 FPU gds^{-1}) on saccharification of alkali pre-treated paddy straw was studied. Aliquots were taken out periodically from the reaction mixture and the amount of reducing sugars released was quantified by high performance liquid chromatography (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_2SO_4 as mobile phase at a flow rate of 0.5 mL min^{-1} and the oven temperature was kept at 40°C.

Results and Discussion

Characterization of the fungal isolate

The fungal isolate CM20 was able to grow on cellulose as sole carbon source and produced white mycelium on potato dextrose agar and produced numerous brownish spores. The isolate was identified as *Aspergllus terreus* (NCBI GenBank accession number KM401596) through sequencing of the ITS region followed by BLAST search.

Effect of lignocellulosic substrates on glycoside hydrolase production

The 3 lignocellulosic substrates, paddy straw (PS), wheat straw (WS) and corn cob (CC) were used for glycoside hydrolase production by *A. terreus*, under solid state fermentation, in different combinations as given in Table 1. The CMCase, FPase, β-glucosidase and xylanase values ranged from 8.76-103.29 IU g^{-1}, 10.46-27.30 IU g^{-1}, 34.24-72.40 IU g^{-1} and 83.98-269.16 IU g^{-1}, respectively (Table 1). The results were analyzed using the analysis of variance (ANOVA) to identify the combination of input variables that jointly optimize the responses. Regression coefficients for the responses are shown in the following equations.

$$\text{CMCase (IU g}^{-1}) = 24.69\times PS + 23.96\times WS + 3.95\times CC \quad \ldots (1)$$

$$\text{FPase (IU g}^{-1}) = 8.80\times PS + 7.78\times WS + 3.40\times CC \quad \ldots (2)$$

$$\beta\text{-glucosidase (IU g}^{-1}) = 24.21\times PS + 13.80\times WS + 11.43\times CC - 3.71\times PS\times CC \quad \ldots (3)$$

$$\text{Xylanase (IU g}^{-1}) = 80.31\times PS + 63.81\times WS + 84.42\times CC + 20.71\times PS\times WS \quad \ldots (4)$$

Table 1—Various hydrolytic enzyme activities produced by *Aspergillus terreus* CM20 using paddy straw, wheat straw and corn cob as culture medium components utilizing the mixture design approach

Run number	Paddy straw (g)	Wheat straw (g)	Corn cob (g)	CMCase (IU g^{-1})	FPase (IU g^{-1})	Avicelase (IU g^{-1})	β-glucosidase (IU g^{-1})	Xylanase (IU g^{-1})
1	3.0	0.0	0.0	71.55	26.29	10.04	72.40	239.16
2	0.0	3.0	0.0	73.41	23.64	21.13	41.43	198.06
3	0.0	0.0	3.0	8.76	10.46	19.15	34.24	249.13
4	1.5	1.5	0.0	103.29	27.30	23.44	61.21	269.33
5	1.5	0.0	1.5	50.68	17.41	22.49	46.43	201.08
6	0.0	1.5	1.5	56.23	14.84	30.17	34.63	198.62
7	1.0	1.0	1.0	50.59	19.55	23.27	44.03	219.90
8	2.0	0.5	0.5	71.47	21.45	10.58	57.42	237.31
9	0.5	2.0	0.5	59.10	18.21	33.44	42.23	192.63
10	0.5	0.5	2.0	36.10	10.67	31.75	35.23	226.76

Positive coefficients for all the three components (PS, WS and CC) showed a statistically significant positive influence on the enzyme activity titers, with individual *P* values <0.05 (data not shown). Though the values for avicelase were found to range from 10.04-33.44 IU g^{-1} (Table 1), the components were not found to exert a significant effect on the enzyme activity (*P* >0.05). In a study on bioconversion of paddy straw by *Streptomyces viridiochromogenes* under solid-state fermentation conditions, it was shown that avicelase activity was highly increased by the addition of NH_4Cl as N-source to the fermentation medium[25].

In terms of CMCase and FPase activities, interactions between components (PS × WS, PS × CC and WS × CC) were not significant, with *P* >0.05. However, with respect to xylanase, the term PS × WS had a positive effect on the activity (Eq. 4). The most significant effect on the enzyme activities, as suggested by the results, was exerted by paddy straw and wheat straw, followed by corn cob (Eq. 1-3). But in terms of xylanase, corn cob had the maximum effect, followed by paddy straw and wheat straw. This may be due to the comparatively high content of hemicelluloses in corn cob, as stated earlier. However, the combination of paddy straw and wheat straw had a significant positive effect on xylanase production. This may be attributed to the comparatively higher content of lignin in corn cob which interferes with the accessibility of polysaccharides to hydrolytic enzymes. The culture medium with a combination of 1.5 g paddy straw and 1.5 g wheat straw (run 4) gave the highest values for xylanase, CMCase and FPase while, medium with 3 g paddy straw (run 1) gave the highest value for β-glucosidase (Table 1). Sherief *et al.*[26] found that *Aspergillus fumigates* produced maximum xylanase and endoglucanase on a 1:1 mixture of paddy straw and wheat bran while, higher β-glucosidase and exoglucanase were detected when the content of rice straw in the mix was increased.

Contour plots were created using this model to obtain information concerning the interaction of substrate components on production of these enzymes. The results showed that although all the 3 components had significant effects in maximizing the enzyme production, the PS and WS components maximized the production of 4 enzymes (CMCase, FPase, β-glucosidase and xylanase) (Fig. 1a,b,d,e). Avicelase production was found to be better in a mix of all the 3 substrates (Fig. 1c), as also indicated by the Table 1 (run 7). This may be attributed to the chemical nature and nutrient availability of the different substrates used. The predicted optimum levels of the tested variables obtained were 1.42 g paddy straw and 1.58 g wheat straw with a composite desirability of 0.87. Verification of the predicted values was conducted by using optimal levels of the tested variables in the medium for glycoside hydrolase production and this returned the following values: CMCase: 83.41 IU g^{-1}; FPase 20.18 IU g^{-1}; avicelase 5.85 IU g^{-1}; β-glucosidase 84.5 IU g^{-1} and xylanase 257.3 IU g^{-1}. These values were superior to those reported in the literature[27,28].

Delabona *et al.*[10] used experimental 'mixture design' as a tool for production of glycosyl hydrolases by *Trichoderma harzianum* P49P11 strain under submerged fermentation and found that a mixed culture medium could significantly maximize GH biosynthesis rate. The substrates did not exert a significant effect on avicelase production may be because of the inherent differences in the substrates, their preparation and experimental conditions, as suggested by Maurer[29]. A study by Rocky-Salimi and Hamidi-Esfahani[30] showed that avicelase activity of *Trichoderma reesei* QM9414 was highly sensitive to particle size and that maximum avicelase activity was obtained when particle size was decreased. Several studies of glycoside hydrolase production by using different substrates and microorganisms have been found with different yields[7-9,27]. This is because various natural substrates are able to induce secreted

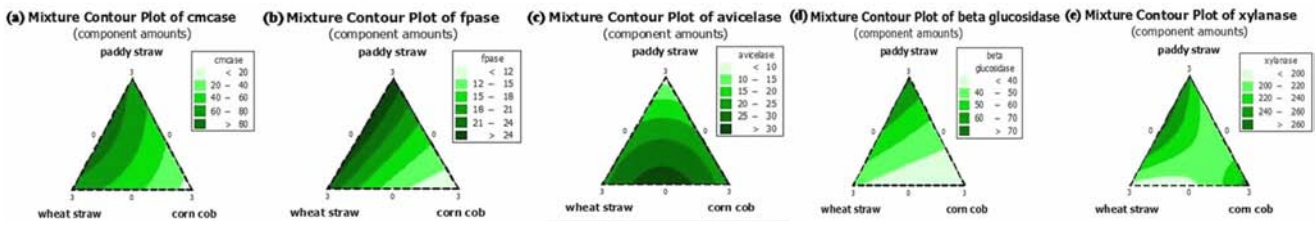

Fig. 1—Contour plots for the responses; (a) CMCase, (b) FPase, (c) avicelase, (d) β-glucosidase and (e) xylanase of *A. terreus* CM20 mixture design experiment

enzymes which are favorable to degrade particular combinations of polysaccharides and chemicals found in the carbon source[31]. The production of cellulases and hemicellulases has been shown to be inducible and is affected by the nature of the substrate used in fermentation[9]. Also, there are evidences for coordinated expression of cellulases and hemicellulases[10,32]. Improved cocktails with synergistic action of cellulases and hemicellulases are of need in the biofuel and bioproduct industries. The crude enzyme produced by *A. terreus* CM20 in the optimized medium showed a significant enhancement in the hydrolytic enzyme activities and thus, could be favourably used in bioconversion processes.

Large scale production of hydrolytic enzymes and their concentration

The crude enzyme produced on large scale fermentation of the optimized substrate mix by *A. terreus* CM20 were concentrated by precipitation with acetone and 4.2, 5.9, 1.8, 8.8 and 6.2 fold concentration of CMCase, FPase, avicelase, β-glucosidase and xylanase, respectively was achieved, with respective activity recoveries in the range of 17.5% to 88.4% (Table 2). Tiwari *et al.*[21] tried precipitation of hydrolytic enzymes with different organic solvents and found that acetone precipitation could maintain high activities of endoglucanase, FPase, β-glucosidase, cellobiohydrolase and xylanases. The concentrated crude enzyme was used for further studies.

Enzyme activity staining by zymography

In SDS-PAGE, the protein bands ranged from 20-80 kDa (data not shown). Zymogram of CMCase displayed four isoforms of 25-45 kDa and of xylanase showed 3 bands of approximately 27, 29 and 32 kDa (data not shown). Although most of the *Trichoderma* and *Aspergillus* produce low molecular weight xylanase (16-22 kDa), several *Aspergillus* xylanases possess molecular weights between 22-43 kDa[33]. The β-glucosidase bands were revealed only when the samples were not boiled and three prominent bands of ~42, ~100, ~165 kDa were observed in activity based zymography. Peterson *et al.*[34] had reported that β-glucosidase activity was eliminated by boiling and the zymogram produced from unboiled samples revealed high β-glucosidase activity. The molecular weight of zymogram bands could be partly the result of protein complexes or aggregates containing the protein with enzyme activity, particularly for samples that were not boiled[34]. The zymograms expressed effectively the presence of various hydrolytic enzymes in the crude extract of enzymes produced by *A. terreus* CM20 under solid state fermentation of the optimized substrate combination.

Enzymatic saccharification of lignocellulosic biomass using the crude enzyme preparation

The concentrated crude enzyme preparation was used for enzymatic saccharification of alkali pretreated paddy straw. Pretreatments showed significant increase in the carbohydrate content (46 % cellulose and 27.5% hemicelluloses) of the paddy straw as alkali pretreatment enhances lignin solubilization. Alkali pretreatment is the method of choice for materials that have relatively low lignin content like agricultural residues[35]. Also, washing after pretreatment removes enzyme inhibitors and improves the release of sugars from pretreated solids[36].

The fig. 2a shows the effect of enzyme loading (10-90 FPU gds^{-1}) on the saccharification of pretreated paddy straw. For a 48 h incubation period, increasing the enzyme loading from 10 to 70 FPU gds^{-1} resulted in an increase of carbohydrate concentrations from 101.0 to 295.5 mg gds^{-1}. Additional increases in enzyme loading proved to be ineffective, possibly due to steric hindrance on the cellulosic surface combined with decreased adsorption efficiencies[27]. At an enzyme loading of 70 FPU gds^{-1}, saccharification was carried out at

Table 2—Concentration of enzymes produced by *Aspergillus terreus* CM20 by single-step acetone precipitation

	Enzyme activity (IU ml^{-1})					Proteins (mg ml^{-1})	Specific activity (IU mg^{-1} proteins)				
	CMCase	FPase	Avicelase	pNPGase	Xylanase		CMCase	FPase	Avicelase	pNPGase	Xylanase
Crude extract	8.6	2.7	0.9	10.4	155.7	0.2	43.0	13.5	4.5	52.0	778.5
Extract after acetone precipitation	36.5	16.15	1.55	92.09	962.78	1.15	31.74	14.04	1.35	80.08	837.20
	% Recovery						Fold concentration				
	42.3	59.7	17.5	88.4	61.8		4.2	5.9	1.8	8.8	6.2

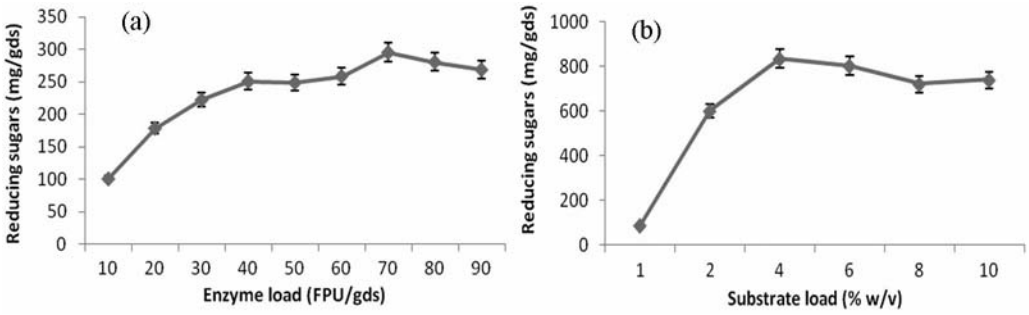

Fig. 2—Effect of (a) enzyme load; and (b) substrate load on enzymatic saccharification of alkali pretreated paddy straw

substrate concentrations ranging 1-10 % w/v (Fig. 2b). A substrate concentration of 4 % w/v yielded 25.0 g L^{-1} of fermentable sugars. The results obtained were higher than the values cited in the literature[27,37].

HPLC analysis detected the presence of glucose, xylose and arabinose in the hyrolysates of alkali pretreated paddy straw saccharified with crude enzyme from *A. terreus* CM20 (data not shown). The distinct profiles of sugars obtained, thus, clearly demonstrated the improved properties of the *A. terreus* CM20 crude enzyme, in terms of its enhanced glycoside hydrolase activities which acted in synergy, to bring about complete saccharification of lignocelluloses.

The optimization of the technology for conversion of biomass polysaccharides to fermentable sugars is of utmost importance in driving a cost-effective and sustainable bioproducts production process[2]. Optimized biomass combinations that increase enzyme titers may prove to be an effective method to decrease bioconversion processing costs. Thus, 'mixture design' can be employed to tailor enzyme mixtures of the future comprising accessory enzymes for essential lignocellulolytic activities to achieve improved overall performance yields.

Conclusion

Enzyme technology is generally considered the most sustainable technology in a green economy and the bioconversion process requires the synergistic action of enzyme complexes. The present study used the mixture design approach for enhanced glycoside hydrolases production by *A. terreus* CM20 under solid state fermentation. The results showed that the 3 lignocellulosic substrates (paddy straw, wheat straw and corn cob) tested, exerted a significant positive effect on the enzyme production and a mixture of paddy straw and wheat straw was found to induce better activity of glycosidases. Saccharification experiment with the concentrated crude enzyme resulted in higher release of sugars, both pentoses and hexoses, which could be further used for production of biofuels and other biobased chemicals. The study thus, demonstrates usefulness of the mixture design approach in developing tailor-made enzyme cocktails better suited to biorefineries.

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