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Two-Stage Statistical Medium Optimization for Augmented Cellulase Production via Solid-State Fermentation by Newly Isolated *Aspergillus niger* HN-1 and Application of Crude Cellulase Consortium in Hydrolysis of Rice Straw

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ABSTRACT: Cellulolytic enzyme production by newly isolated *Aspergillus niger* HN-1 was statistically optimized using Plackett−Burman and central composite design (CCD). Optimum concentrations of 2, 0.40, 0.01, and 0.60 g L^{-1} for KH_2PO_4, urea, trace elements solution, and $CaCl_2{\cdot}2H_2O$, respectively, were suggested by Design-Expert software. The two-stage optimization process led to a 3- and 2-fold increases in the filter paper cellulase (FP) and β-glucosidase activities, respectively. FP, β-glucosidase, endoglucanase, exopolygalaturonase, cellobiohydrolase, xylanase, α-L-arabinofuranosidase, β-xylosidase, and xylan esterase activities of 36.7 ± 1.54 FPU gds^{-1}, 252.3 ± 7.4 IU gds^{-1}, 416.3 ± 22.8 IU gds^{-1}, 111.2 ± 5.4 IU gds^{-1}, 8.9 ± 0.50 IU gds^{-1}, 2593.5 ± 78.9 IU gds^{-1}, 79.4 ± 4.3 IU gds^{-1}, 180.8 ± 9.3 IU gds^{-1}, and 288.7 ± 11.8 IU gds^{-1}, respectively, were obtained through solid-state fermentation during the validation studies. Hydrolysis of alkali-treated rice straw with crude cellulases resulted in about 84% glucan to glucose, 89% xylan to xylose, and 91% arabinan to arabinose conversions, indicating potential for biomass hydrolysis by the crude cellulase consortium obtained in this study.

KEYWORDS: *Aspergillus niger HN-1, crude cellulase consortium, Plackett−Burman, hydrolysis, rice straw, response surface methodology*

INTRODUCTION

Lignocellulosic waste residues obtained from energy crops, wood, and agricultural residues represent the most abundant global source of renewable biomass.[1] Cellulase enzymes provide a key opportunity for achieving benefits of biomass utilization through the bioconversion of the abundant cellulosic wastes into the simplest carbohydrate monomers. In future, cellulases are likely to play an important role in the bioconversion of renewable cellulosic biomass to commodity chemicals.[2] In recent years, some important agro-industrial residues and byproducts, such as sugar cane bagasse, Kinnow waste, wheat bran, rice straw, leaves, and corn stover, have been used as substrates for the production of cellulases through solid-state fermentation.[3,4]

The contribution of enzyme cost to ethanol production from lignocellulosic biomass is still quite significant and is higher than the estimates available in the literature.[5] Reduction in the production cost and improvement in cellulase yield could be achieved through the use of appropriate and low-cost carbon and nitrogen sources in the formulation of fermentation medium. Because of nonavailability of proper infrastructure or an established commercial use of rice straw, huge quantities of rice straw generated every year are burned in fields, leading to biomass loss and environmental pollution in many developing countries, including India.[6] Thus, the use of rice straw for cellulase production seems to be a good alternative for its efficient management. Most commercial cellulases are produced by filamentous fungal strains of *Trichoderma reesei* and *Aspergillus niger*.

Cellulolytic enzymes comprise endoglucanase (EG; 1,4-β-D-glucan-4-glucano-hydrolases; EC 3.2.1.74), cellobiohydrolase (CBH; EC 3.2.1.91 and EC 3.2.1.74), and β-glucosidase (BGL; EC 3.2.1.21). Endoglucanase cleaves glycosidic bonds randomly within the interior of cellulose polymer chain. Cellobiohydrolases act progressively on the reducing or nonreducing ends of cellulose chains, releasing either cellobiose or glucose as major products. The β-glucosidase hydrolyzes soluble cellodextrins and cellobiose to glucose.[7] Cellobiohydrolases are important components of the multienzyme cellulase complexes, and the potent cellulolytic fungi generally produce two different CBHs: CBHI and CBHII.

The type of strain, culture conditions, the nature of the substrate, and availability of nutrients are some of the important factors that affect enzyme yields. Substrates such as cellulose, glucose, yeast extract, peptone, urea, KH_2PO_4, $(NH_4)_2SO_4$,

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$MgSO_4$, $FeSO_4$, $MnSO_4$, $CoCl_2$, and $CaCl_2$ have been reported for cellulase production.[8,9] As large numbers of basal ingredients are screened for each new isolate and optimized in fermentation processes, experiments based on multifactorial design are difficult and laborious. Therefore, statistics-based experimental methods have been designed for medium optimization.

The Plackett−Burman factorial designs allow screening of main factors from a large number of process variables. Such designs are useful in preliminary studies, wherein the major objective is to select variables that can be fixed or eliminated for further optimization. Response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal condition of multivariable systems can be determined. Therefore, the objectives of the present study were to screen the medium ingredients using Plackett−Burman experimental design; obtain optimum level of screened variables by employing central composite design (CCD); validate the design using optimized concentrations of significant components in static tray SSF process using a newly isolated *A. niger* HN-1 strain; and evaluate the potential of crude cellulase consortium produced by *A. niger* HN-1 in hydrolysis of alkali-treated rice straw.

MATERIALS AND METHODS

Materials. Rice straw of variety PR-127 was procured from the research fields of Punjab Agricultural University (PAU), Ludhiana, Punjab, India. Rice straw was dried, cut into small pieces with a chaff cutter, and ground into smaller particles of about 2 mm each. Wheat bran was procured from the local flour mill. The dehydrated media, medium ingredients, and analytical grade chemicals were procured from Hi-Media Laboratories Pvt Ltd. (Mumbai, India) and Fisher Scientific (Mumbai, India), respectively. The substrates used for enzyme assays and the sugar standards used for HPLC determinations were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards for oligosachharides, such as xylobiose, xylotriose, and cellotriose, were procured from Megazyme, International (Ireland).

Isolation of Fungi. The fungal strain used in this study was isolated from partially rotten wood samples collected from PAU, Ludhiana, India. One gram of sample was aseptically transferred to the 250 mL Erlenmeyer flask containing 50 mL of sterile distilled water. The flask was vortexed vigorously for 15 min on a vortex shaker. Fungi were isolated by serial dilution method using sterilized Rose Bengal Chloramphenicol (RBC) agar medium plates. The plates were incubated at 30 °C for 4−6 days and were observed regularly for colony development. Different characteristic colonies, based on their morphological features, were picked, and pure fungal isolates were obtained by successive subculturing of these colonies on the fresh potato dextrose agar (PDA) medium plates. Stock cultures were preserved at 4 °C on PDA slants in a refrigerator for further identification and characterization.

Screening of Cellulolytic Fungi. Inoculum from the culture tubes of each of the isolates was plated on CMC agar medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.02% peptone, 0.2% CMC, and 1.7% agar). The plates were incubated at 30 °C for 48 h and were flooded with Gram's iodine (2.0 g KI and 1.0 g of iodine in 300 mL of distilled water) for 5 min. The production of extracellular cellulase by the organisms was indicated by a zone of clearance around the colony.[10] Fungal isolates showing clear zone around the colony were further tested for cellulase production by filter paper cellulase (FP) and β-glucosidase assay. The isolate producing relatively higher cellulase concentration in less time was selected for enzyme production studies. The identity of the selected isolate was confirmed through molecular characterization. Molecular characterization of the selected isolate was done with genomic DNA extracted using a fungal genomic DNA isolation kit (Chromous Biotech Pvt Ltd., Bangalore, India). The large subunit (LSU) of the 28s rRNA gene was amplified with the LROR (5′-ACCCGCTGAACTTAAGC-3′) and LR 7 (5′-TACTACCACCAAGATCT-3′) primers. Details about the polymerase chain reaction (PCR) protocol and data analysis of the sequences are described in our previously published paper.[11]

Enzyme Production and Extraction. The most efficient selected fungal strain was cultured on the PDA plates and was used as inoculum source for enzyme production studies. After sporulation, each plate was washed with 25 mL of sterile water, and the spores were collected in sterilized bottles. The spore count was determined using a hemocytometer, and the inoculum concentration was adjusted to a spore concentration of 1×10^8 spores mL^{-1}. Solid-state fermentation was employed for cellulase production using rice straw and wheat bran in the ratio 4:1.[3,4]

All of the enzyme production experiments were conducted in 250 mL Erlenmeyer flasks with each flask containing 10 g of substrate. Moisture content of 70% was made up with the Mandel Weber (MW) medium.[12] The flasks were autoclave-sterilized for 15 min, cooled, and inoculated with 1 mL of fungal spore suspension. Flasks were incubated at 30 °C for 96 h, and enzyme extraction from the flasks was done by addition of a suitable volume of citrate buffer (0.1 M, pH 4.8) followed by vortexing for 30 min, filtration, and centrifugation at 5000*g* at 4 °C for 10 min. Supernatant was collected and analyzed for filter paper cellulase (FP) and β-glucosidase production.

Enzyme Assays. Enzyme assay for EG, exopolygalacturonase (exo-PG), and xylanase was done using CMC (1%, w/v), pectin (1%, w/v), and birch wood xylan (1%, w/v), respectively. The substrates were prepared in sodium citrate buffer (50 mM, pH 5.5). The reaction mixture containing equal amounts of appropriately diluted enzyme (0.5 mL) and substrate (0.5 mL) was incubated at 50 °C. The reaction time for EG was 30 min, whereas that for exo-PG and xylanase was 10 min. Glucose and xylose were determined from the standard curves of glucose and xylose, respectively, prepared using the dinitrosalicylic acid. Galacturonic acid (GA) released by the action of exo-PG was determined using the modified dinitrosalicylic acid method.[13] One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of the molecule (glucose, GA, and xylose) per milliliter per minute under standard assay conditions and expressed as international units per gram dried substrate (IU gds^{-1}). Filter paper cellulase activity was measured using Whatman no.1 filter paper (1 cm × 6 cm) strip as substrate.[14] Cellobiohydrolase I is the major component of the cellulase system required for degradation of crystalline cellulose and constitutes about 60% of the proteins secreted by *Trichoderma reesei*.[15] Endoglucanase I (EGI) and CBHI were assayed using *p*-nitrophenyl-β-D-lactopyranoside (pNPL) as a substrate. The activity obtained using pNPL was that of EGI, CBHI, and β-glucosidase (BGL). The CBHI activity was therefore obtained by the subtraction method described previously.[16] The β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, and xylan esterase activities were determined using 4-nitrophenyl β-D-glucopyranoside, 4-nitrophenyl-β-D-xylanopyranoside, 4-nitrophenyl-α-L-arabinofuranoside, and 4-nitrophenyl-acetate as substrate, respectively.[17,18] Appropriately diluted enzyme (25 μL) was mixed with 50 μL of sodium acetate buffer (50 mM, pH 5.0) and 25 μL of the respective substrate. The reaction mixture was incubated at 50 °C for 30 min, and the reaction was terminated by the addition of 100 μL of NaOH−glycine buffer (pH 10.8, 0.4 M), and the *p*-nitrophenol released was analyzed at 405 nm with ELISA reader (Multiskan Ascent, Thermo Electron Corp., USA). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per mililiter per minute under standard assay conditions and expressed as international units per gram dried substrate (IU gds^{-1}). Total protein content was determined according to the protein−dye binding method.[19]

Plackett−Burman Design. Plackett−Burman design involved 12 experimental runs with 11 independent variables (including 3 dummy variables). The selected independent variables were urea, KH_2PO_4, trace element solution composed of $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CoCl_2.2H_2O$; $CaCl_2 \cdot 2H_2O$, yeast extract, $FeSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, and K_2HPO_4. The concentration of trace elements such as Mn, Zn, and Co in the MW medium ranges from 0.002 to 0.016 g L^{-1}, indicating their requirement in very low concentrations. On the other

hand, the concentration of elements such as Ca in MW medium is 0.3 g L^{-1}. Therefore, it was decided to evaluate the effect of the incorporation of a mixture of Mn, Zn, and Co in the growth medium on cellulase production by *A. niger* HN-1. For each variable, high (+) and low (−) levels were selected. Response variables selected were FP activity (FPU gds^{-1}) and β-glucosidase activity (IU gds^{-1}). Filter paper cellulase activity is a relative measure of the overall cellulose-hydrolyzing capacity of microbial cellulase preparations.[14]

The main effect of each variable was determined with the equation

$$\mathrm{Exi} = (\Sigma \mathrm{Mi}^{+} - \Sigma \mathrm{Mi}^{-})/N$$

where Exi is the variable main effect, Mi^+ and Mi^- are the response percentage in trials, in which the independent variable (xi) is present in high and low concentrations, respectively, and N is the half-number of trials. The significance of each variable was determined via Student's t test. Table 1 shows the variables and their corresponding levels used in the experimental design. The values of two levels were fixed on the basis of our previous preliminary experimental studies and also on the basis of the concentrations of such nutrients in the MW medium. Design-Expert version 7.1 (Stat-Ease Inc., Minneapolis, MN, USA) was used for designing experiments statistically as well as for the analysis of the experimental data. This model does not describe interactions among the variables and is used to evaluate and select the important factors that influence the response.

Table 1. Independent Variables and Their Concentrations Used in the Plackett−Burman Experimental Design

		exptl value	
variable (g L^{-1})	symbol	low (−1)	high (+1)
KH_2PO_4	A	1	3
K_2HPO_4	B	1	3
dummy 1	C	0	0
$CaCl_2{\cdot}2H_2O$	D	0.20	0.40
$(NH_4)_2SO_4$	E	1	2
dummy 2	F	0	0
$FeSO_4{\cdot}7H_2O$	G	0.002	0.01
urea	H	0.10	0.30
dummy 3	J	0	0
yeast extract	K	0.10	0.30
trace element solution[a]	L	0.002	0.01

[a]Trace element solution was composed of $MnSO_4$, $ZnSO_4$, and $CoCl_2$ in the ratio 1:0.875:1.25.

Optimization Using Central Composite Design. The next step in formulation of the medium was to determine the optimum levels of significant variables for enhancing cellulase production. For this purpose, response surface methodology (RSM) using central composite design (CCD) was employed. CCD consisting of 27 experimental runs, with 8 axial points ($\alpha \pm 2$) and 3 replications at the central point (0), was employed to optimize the concentration of significant factors. The design space consisted of four variables, which are KH_2PO_4, urea, trace element solution, and $CaCl_2{\cdot}2H_2O$, as they showed significant positive effect on cellulase production during analysis of Plackett−Burman statistical design. Response variables were FP activity and β-glucosidase activity. The range selected for the medium components was 2−4 g L^{-1} for KH_2PO_4 (X_1), 0.15−0.40 g L^{-1} for urea (X_2), 0.01−0.03 g L^{-1} for trace element solution (X_3), and 0.20−0.60 g L^{-1} for $CaCl_2{\cdot}2H_2O$ (X_4). The relationship between independent variables and the response was calculated by the second-order polynomial, and the behavior of the system was explained by the quadratic equation

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_4 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2$$

where Y is the predicted response; β_0 is the intercept term; β_1, β_2, β_3, and β_4 are the linear effects; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are the interaction terms; β_{11}, β_{22}, β_{33}, and β_{44} are the square terms; and X_1, X_2, X_3, and X_4 are the independent variables. Results of the RSM experiment were analyzed using Design-Expert 7.1 evaluation software. Analysis of variance (ANOVA) was used to estimate the statistical difference among the parameters.

Validation Studies. Validation experiments were conducted using solid-state tray fermentation in stainless steel trays of 36 cm length × 25 cm width × 2.5 cm height.[4] Two hundred twenty-two grams of substrate was impregnated with 444 mL of mineral solution containing optimized concentrations of the selected medium components in conical flasks. The initial pH of the fermentation process was adjusted to 5.0, and the contents were thoroughly mixed and autoclave-sterilized for 15 min. The flasks were inoculated with 40 mL of inoculum having a spore concentration of 1×10^8 spores mL^{-1}. The material after inoculation was spread in trays to achieve a depth of about 1.5 cm. All of these operations were carried out under sterile air draft in the laminar air flow. The trays were covered with muslin cloth and incubated in the fumigated environmental chamber, maintained at 30 °C and 95% humidity for 96 h. All of the experiments were conducted in triplicate, and the mean and SD values were determined with MS Excel software.

Analytical Methods. Rice straw was extracted with ethanol and subjected to proximate compositional analysis.[6] Ash and moisture contents were determined using previously described procedures.[20] Acid-insoluble lignin (AIL) was analyzed according to the laboratory analytical procedure (LAP) of the National Renewable Energy Laboratory (NREL). Acid-soluble lignin (ASL) was determined using the previously described procedure.[6] All of the experiments were carried out in triplicate, and the mean and standard deviation (SD) values were calculated using the MS Excel program. Glucose, xylose, arabinose, galactose, cellobiose, cellotriose, xylobiose, and xylotriose were analyzed with HPLC (Dionex Corp., Sunnyvale, CA, USA) using a Shodex SP-0810 column (300 × 7.8 mm) fitted with an SP-G guard column (Waters, Milford, MA, USA).[21] Degassed HPLC grade water was used as a mobile phase at a flow rate of 1.0 mL min^{-1}. The column oven and refractive index (RI) detector were maintained at 80 and 50 °C, respectively. Samples were diluted, centrifuged, and filtered through 0.45 μm RC membranes (Phenomenex Corp., Santa Clara, CA, USA). Peaks were detected by the RI detector and quantified on the basis of the area and retention time of the respective sugar or oligosaccharide standards.

Pretreatment and Enzymatic Hydrolysis of Alkali-Treated Rice Straw. Rice straw after size reduction was dried in a hot-air oven at 70 °C to a constant weight. Dried rice straw, ground to a particle size of about 1 mm, was treated with 1% (w/v) sodium hydroxide (NaOH) at a solid-to-liquid ratio of 10% (w/v). Pretreatment conditions were selected on the basis of the results obtained from our previous experiments.[21] The solid residue was collected by filtration and washed extensively with distilled water to a neutral pH. The residue was dried at 60 °C to a constant weight and used for hydrolysis. Enzymatic hydrolysis was conducted in 250 mL screw-capped polycarbonate flasks containing 10 g of dried alkali-treated rice straw and 4 mL of crude enzyme with enzyme loading at 15 FPU gds^{-1}. Tetracycline and cyclohexamide, each at a concentration of 50 μg mL^{-1}, were added to the flasks prior to hydrolysis. An appropriate amount of citrate buffer (50 mM, pH 5.0) was added to achieve a 10% solid-to-liquid ratio. The flasks were placed in the rotary incubator shaker at 120 rpm at 50 °C. Aliquots for sugar analysis were collected at 24 h intervals until 96 h. The experiment was conducted in triplicate, and data were analyzed using JMP software (SAS Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Isolation and Screening of Cellulolytic Fungal Strains. Among the nine fungal isolates that showed characteristic diversity for colony morphology, color of the spores, structure of spores, and their arrangements, only three isolates showed

clear characteristic zones on the CMC agar plates. On the basis of the microscopic characteristics of the three isolates, it could be confirmed that all three isolates belonged to the genus *Aspergillus*. One isolate produced more cellulase than the other two in relatively less time (data not shown) and was therefore selected for the optimization studies. Filter paper cellulase and β-glucosidase activity for this isolate were found to be 13.6 ± 0.60 and 125 ± 5.8, respectively, after 72 h of incubation. Sequencing and analysis of the LSU of the 28s rDNA region of the 898 bp amplicon of the selected isolate showed that the isolated fungal strain had the highest identity with *A. niger* (HM008328). Phylogenetic relationships were obtained through the alignment and cladistic analysis of homologous nucleotide sequences of known microorganisms (Figure 1). On

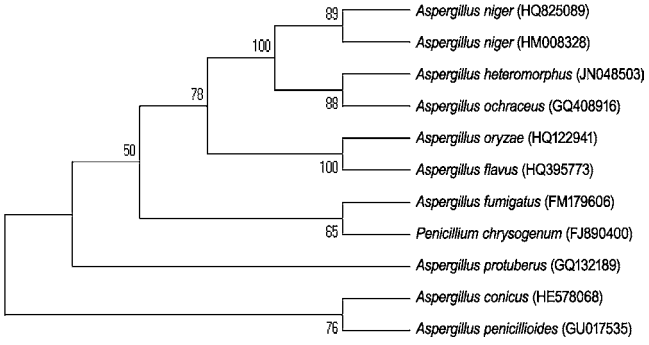

Figure 1. Phylogenetic dendrogram for *Aspergillus niger* HN-1 and related strains with their accession numbers based on the 28s rRNA gene sequence.

the basis of the morphology and comparison of the gene sequences, the isolated fungal strain was identified as a strain of *A. niger* and is henceforth referred to as *A. niger* HN-1. The 28s rRNA gene sequence for the newly isolated strain of *A. niger* HN-1 was submitted to GenBank with the accession no. HQ825089. *A. niger* HN-1 culture has been deposited with the Microbial type culture collection and gene bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India, with the accession no. MTCC 11097.

Selection of Significant Medium Components through Plackett–Burman Design Analysis. A large variation in FP (13.3–30.9 FPU gds^{-1}) and β-glucosidase (94.5–223.5 IU gds^{-1}) activities was observed, which reflects the importance of medium optimization to attain higher yields of the desired product (Table 2). Analysis of the main effect of eight medium components showed that urea, trace element solution, KH_2PO_4, and $CaCl_2{\cdot}2H_2O$ were the positive significant factors that had a significant effect in increasing FP and β-glucosidase activity. The magnitude of the main effect reflects the effect of each variable on the measured response and ranks the variables accordingly (Figure 2). The significant

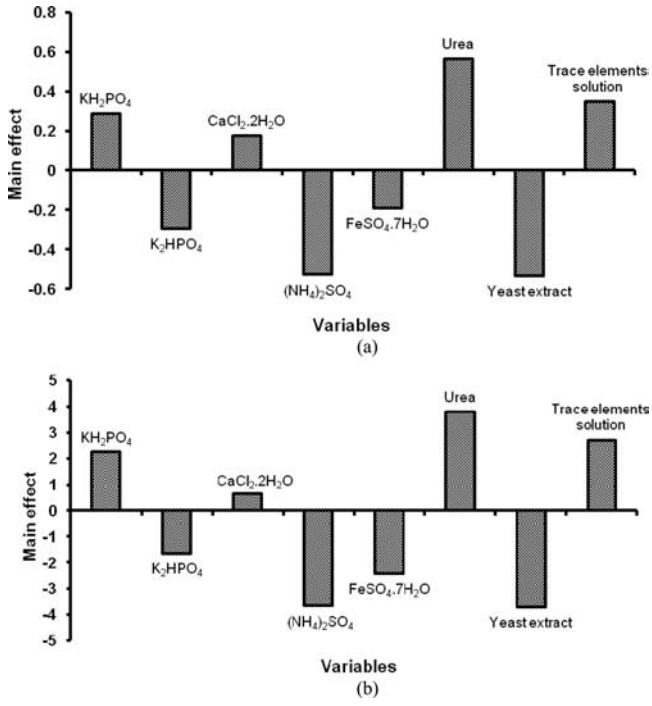

Figure 2. Plackett–Burman design to evaluate the effect of medium constituents on (a) filter paper cellulase and (b) β-glucosidase production.

effect of the medium components in enhancing cellulase production increased in the following order: $CaCl_2{\cdot}2H_2O$, KH_2PO_4, trace element solution, and urea (Figure 2a). On the other hand, yeast extract, $(NH_4)_2SO_4$, K_2HPO_4, and $FeSO_4{\cdot}7H_2O$ showed a negative effect on cellulase production (Figure 2b). The model equations for FP activity and β-glucosidase activity were expressed as

Table 2. Evaluation of Plackett–Burman Design To Study the Effect of Medium Components on Cellulase Production

	exptl value (g L^{-1})												
run order	A	B	C	D	E	F	G	H	J	K	L	FP activity (FPU gds^{-1})	β-glucosidase activity (IU gds^{-1})
1	1	1	0	0.4	1	0	0.01	0.1	0	0.3	0.01	19.9	124.9
2	3	3	0	0.2	1	0	0.01	0.1	0	0.3	0.002	15.1	102.1
3	1	3	0	0.4	1	0	0.002	0.3	0	0.3	0.01	25.3	178.5
4	1	3	0	0.4	2	0	0.01	0.3	0	0.1	0.002	20.8	132.5
5	3	3	0	0.4	2	0	0.002	0.1	0	0.3	0.002	13.3	94.5
6	3	1	0	0.4	2	0	0.002	0.1	0	0.1	0.01	25.2	176.7
7	1	1	0	0.2	2	0	0.002	0.3	0	0.3	0.002	15.7	108.7
8	3	1	0	0.2	2	0	0.01	0.3	0	0.3	0.01	23.4	158.4
9	3	1	0	0.4	1	0	0.01	0.3	0	0.1	0.002	29.4	190.4
10	3	3	0	0.2	1	0	0.002	0.3	0	0.1	0.01	30.9	223.5
11	1	3	0	0.2	2	0	0.01	0.1	0	0.1	0.01	14.4	97.5
12	1	1	0	0.2	1	0	0.002	0.1	0	0.1	0.002	23.9	168.8

$$FP = 25.3083 + 1.4417A - 1.4750B + 8.750D - 5.2833E - 235.4167G + 28.0833H - 26.5833K + 435.4167L$$

$$\beta\text{-glucosidase} = 182.7583 + 11.225A - 8.2750B + 32.0833D - 36.6500E - 3018.7500G + 189.583H - 185.2500K + 3385.4167L$$

Analysis of variance (ANOVA) was applied to test the significance and adequacy of the model. The model F value of 10.63 FPU gds^{-1} for FP activity and 9.69 IU gds^{-1} for β-glucosidase activity and the correlation coefficients (R^2) of 0.97 and 0.96 for FP and β-glucosidase activity, respectively, suggest the significance of the model. This indicates that 97 and 96% of the total variation around the average could be explained by regression for FP and β-glucosidase activity, respectively. Analysis using Plackett–Burman design showed that only four components had a positive significant effect on enzyme production by *A. niger* HN-1. Use of inorganic nitrogen sources, such as urea, resulted in higher enzyme production than the organic nitrogen sources. This may be attributed to the fact that complex substances such as amino acids and vitamins in organic nitrogen sources could trigger the biomass production, thereby making it unnecessary for the fungus to produce cellulase.[22] The advantages associated with using urea as a nitrogen source are its easy availability, low price, and buffering ability. Supplementation of fermentation medium with urea protected cellulase produced by *A. niger* from inactivation caused by a sudden drop in pH of the medium.[23] The results obtained in the present study showed a negative influence of $(NH_4)_2SO_4$ on cellulase production. The β-glucosidase activity was not detected when $(NH_4)_2SO_4$ was used as a nitrogen source.[24] As mentioned previously, addition of $CaCl_2$ showed a significant positive effect in enhancing the FP and β-glucosidase acitivity. Calcium may be responsible for some changes in the permeability of the cell wall that result in a more rapid excretion of the enzymes, which, in turn, improves cellulase synthesis.[25]

Optimization by Response Surface Methodology. The actual values of the test variables and experimental results are presented in Table 3. The values reported for the responses are the average of three replications for each run. Analysis of variance (ANOVA) was applied to test the significance and adequacy of the model for FP and β-glucosidase acitivities. The model F values of 10.40 FPU gds^{-1} and 6.70 IU gds^{-1} for FP and β-glucosidase acitivity, respectively, imply that the model was significant. The nonsignificant value for lack of fit of 0.0529 for FP activity and 0.3884 for β-glucosidase showed that the quadratic model was valid for this study. The R^2 values for FP and β-glucosidase activity were 0.92 and 0.88, respectively. The multiple regression analysis was used to fit the second-order polynomial, which resulted in the following equations for the response only in terms of significant variables obtained after optimization:

$$FP = 58.93 - 1.93X_1 - 15.8X_1X_2 + 513.8X_1X_3 + 78.0X_2X_4 - 918.75X_3X_4 - 1.28{X_1}^2 + 110.53{X_2}^2 \quad (1)$$

Table 3. Effect of Four Independent Variables on Cellulase Production by *Aspergillus niger* HN-1

run	KH_2PO_4 (X_1)	urea (X_2)	trace element solution (X_3)	$CaCl_2 \cdot 2H_2O$ (X_4)	FP activity (FPU gds^{-1})	β-glucosidase activity (IU gds^{-1})
1	3	0.275	0.02	0.4	20.1	154.6
2	3	0.525	0.02	0.4	25.9	148.8
3	3	0.275	0.04	0.4	22.7	151.8
4	4	0.15	0.01	0.6	16.2	158.9
5	3	0.275	0.02	0.4	21.3	173.6
6	4	0.4	0.01	0.2	11.2	139
7	3	0.275	0.02	0.8	19.1	145.5
8	1	0.275	0.02	0.4	17.5	187.4
9	4	0.4	0.03	0.6	25.8	145.9
10	4	0.4	0.03	0.2	23.3	157.5
11	5	0.275	0.02	0.4	11.4	153
12	3	0.275	0.02	0.4	21.2	159.9
13	2	0.4	0.03	0.6	23.1	156.7
14	4	0.15	0.01	0.2	17.5	158.7
15	2	0.15	0.03	0.2	18.1	149.2
16	4	0.15	0.03	0.6	18.5	154.4
17	4	0.4	0.01	0.6	19.5	161.5
18	2	0.15	0.03	0.6	10.1	154.6
19	2	0.15	0.01	0.2	31.5	182.4
20	2	0.15	0.01	0.6	29.5	233.7
21	2	0.4	0.01	0.6	35	224
22	2	0.4	0.01	0.2	32.4	220.3
23	3	0.025	0.02	0.4	27.1	139
24	4	0.15	0.03	0.2	29.9	195.7
25	3	0.275	0	0.4	24.5	205.9
26	3	0.275	0.02	0	21.3	161
27	2	0.4	0.03	0.2	28	150

$$\beta\text{-glucosidase} = 248.71 - 42.72X_1 - 6729.91X_3 + 1783.12X_1X_3 - 30.40X_3X_4 \quad (2)$$

The interactions of KH_2PO_4 with urea, KH_2PO_4 with trace element solution, urea with $CaCl_2 \cdot 2H_2O$, and trace element solution with $CaCl_2 \cdot 2H_2O$ had a significant effect on FP activity at the 95% confidence level. Coefficients of the model suggested that β-glucosidase activity was highly sensitive to KH_2PO_4 and trace element solution concentrations. The interactions of KH_2PO_4 with trace element solution and that of trace elements solution with $CaCl_2 \cdot 2H_2O$ had a significant effect on β-glucosidase activity at the 95% confidence level.

A linear increase in FP activity was seen when the concentration of urea was increased from 0.15 to 0.4 g L^{-1} (Figure 3a). The presence of KH_2PO_4 and trace element solution at concentrations of 4 and 0.05 g L^{-1}, respectively, led to an increase in FP activity (Figure 3b). An increase in the concentration of urea with an increase in $CaCl_2 \cdot 2H_2O$ concentration had a positive effect on FP activity (Figure 3c) but at 0.6 g L^{-1} $CaCl_2 \cdot 2H_2O$, an increase in concentration of trace element solution from 0.01 to 0.05 g L^{-1} had an adverse effect on FP activity (Figure 3d). It is evident from these results that addition of KH_2PO_4 and trace element solution at their lower level of selected range and of urea and $CaCl_2 \cdot 2H_2O$ at their higher level of selected range enhanced FP activity. Maximum β-glucosidase activity was obtained at 2 g L^{-1} KH_2PO_4 and 0.01 g L^{-1} trace element solution concentrations (Figure 4a). Increase in $CaCl_2 \cdot 2H_2O$ concentration from 0.2 to 0.6 g L^{-1} at a fixed trace element solution concentration of 0.01

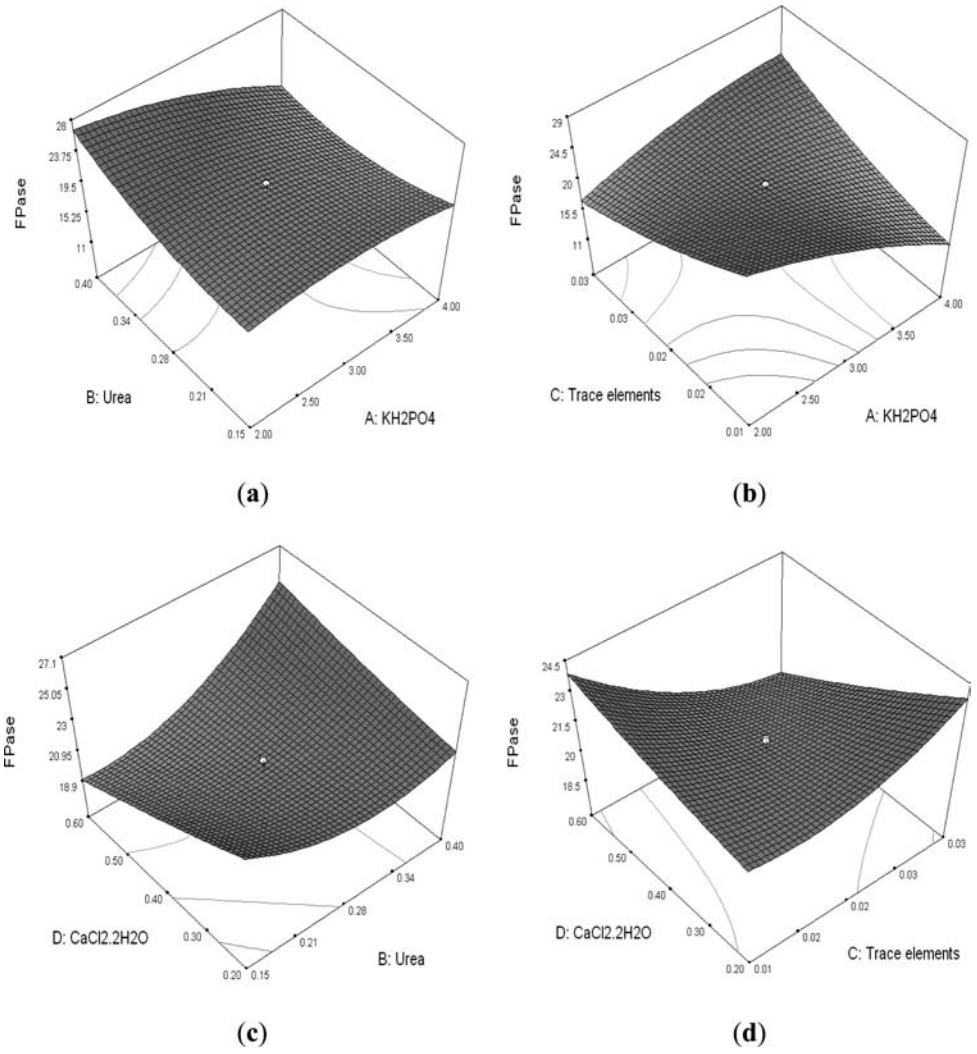

Figure 3. Response surface plots for filter paper cellulase activity showing (a) interaction of KH_2PO_4 with urea, (b) interaction of KH_2PO_4 with trace elements solution, (c) interaction of urea with $CaCl_2{\cdot}2H_2O$, and (d) interaction of $CaCl_2{\cdot}2H_2O$ with trace element solution.

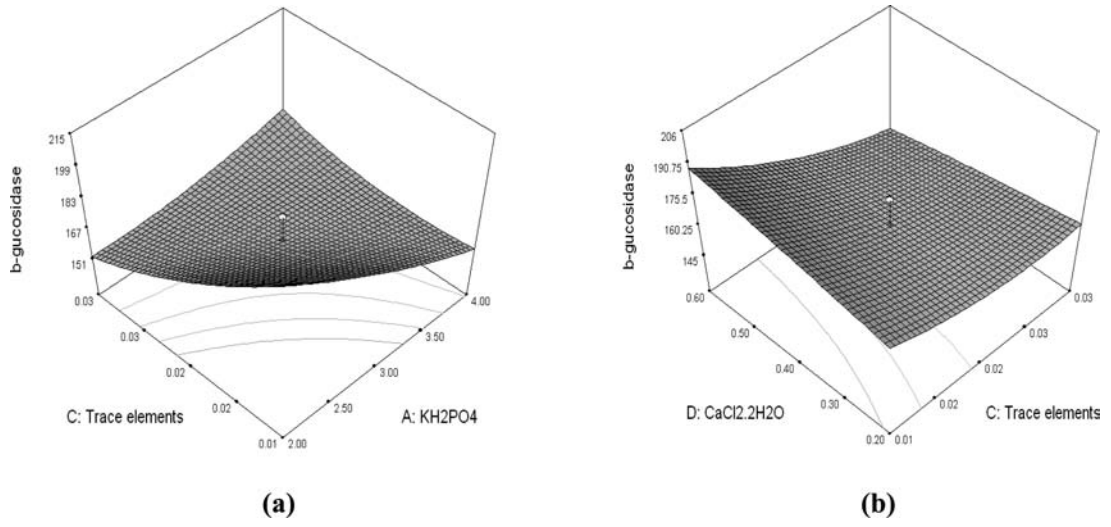

Figure 4. Response surface plots for β-glucosidase activity showing (a) interaction of (a) KH_2PO_4 or (b) $CaCl_2{\cdot}2H_2O$ with trace element solution.

g L^{-1} led to an increase in β-glucosidase activity (Figure 4b). Addition of $CaCl_2$ at a concentration of 0.60 g L^{-1} enhanced the FP and β-glucosidase activities. It could be inferred from this study that medium supplementation with KH_2PO_4 at a concentration of 2 g L^{-1} enhanced enzyme production. Addition of trace element solution at 0.01 g L^{-1} had a

Table 4. Comparison of Cellulase Production by Different *Aspergillus* Strains Using Cellulosic Substrates

microbial strain	substrate	FP activity (FPU gds $^{-1}$)	EG activity (IU gds^{-1})	β-glucosidase (IU gds^{-1})	xylanase (IU gds^{-1})	CBH (IU gds^{-1})	ref
Aspergillus MAM-F35	wheat straw	79	487			35	28
Aspergillus niger KK2 mutant	rice straw	19	129	100	5070		29
Aspergillus niger KKS	rice straw (submerged)	84		370	9100		30
Aspergillus ustus	rice straw/ wheat bran/ Toyoma mineral solution (7:3:40)	6	12	40	650		31
Aspergillus fumigates	rice straw/ wheat bran (7:3)	0.93	14.71	8.51	42.7	0.68	32
Aspergillus fumigatus Fresenius	rice straw	9.73	240.2	470	2800	15	33
Aspergillus niger HN-1	rice straw/ wheat bran (4:1)	36.7	416.3	252.3	2593.5	8.9	present study

significant effect on cellulase production. Metal cations, such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+}, were necessary for cellulase synthesis by *Trichoderma viride* QM6a.[26] It is felt that the effect of balance between different metal ion concentrations could be more significant than their individual effects.[25] Thus, it is clear that different constituents can work as inducers for cellulase production in one organism, whereas their presence retards enzyme production in others. Studies are in progress to appraise the effect of incorporation of individual elements used in the trace element solution along with a few more elements in the growth medium on cellulase production by *A. niger* HN-1.

Validation Using Optimized Parameters. On the basis of the numerical optimization results and model graph analysis, concentrations of 2, 0.40, 0.01, and 0.60 g L^{-1} for KH_2PO_4, urea, trace element solution, and $CaCl_2{\cdot}2H_2O$, respectively, were found to be optimum and, thus, the validation studies were conducted using the optimized concentrations of the selected constituents. According to the model eqs 1 and 2, the predicted responses for FP and β-glucosidase activity were 34.9 FPU gds^{-1} and 233.4 IU gds^{-1}, respectively, whereas the actual responses obtained after validation for FP and β-glucosidase activities were 36.7 ± 1.54 FPU gds^{-1} and 252.3 ± 7.4 IU gds^{-1}, respectively, indicating successful validation of the statistical model. Endoglucanase, xylanase, exo-PG, CBHI, α-L-arabinofuranosidase, β-xylosidase, and xylan esterase activities of 416.3 ± 22.8, 2593.5 ± 78.9, 111.2 ± 5.4, 8.9 ± 0.50, 79.4 ± 4.3, 180.8 ± 9.3, and 288.7 ± 11.8 IU gds^{-1}, respectively, were observed during the validation study. The total protein content in the crude extract was 0.7 ± 0.04 mg mL^{-1}. In comparison to the previously reported cellulase titers obtained using different *Aspergillus* strains, cellulase produced by *A. niger* HN-1 after two-stage optimization showed comparable or higher values for different cellulase components (Table 4). Validation studies using selected medium constituents in optimized concentrations showed 3- and 2-fold increases in FP activity and β-glucosidase activities, respectively, compared to the activity obtained using MW medium. In a previous study, we reported an increase in FP activity in a SSF process using Kinnow pulp and distilled water compared to the activity obtained using Kinnow pulp and MW medium.[3] As the isolate used for cellulase production in this study was not characterized for cellulase production using different nutrients, it can be inferred that supplementing the substrate with a medium composed of 2.0 g L^{-1} KH_2PO_4, 0.40 g L^{-1} urea, 0.01 g L^{-1} trace element solution, and 0.60 g L^{-1} $CaCl_2{\cdot}2H_2O$ enhanced cellulase production significantly.

Most of the celluloytic strains reported in the literature are deficient in production of one enzyme component or another (Table 4). Besides cellulase, *A. niger* HN-1 also produced auxiliary enzymes required for production of pentose sugars needed for efficient ethanol production from lignocellulosics. In addition to the cellulase components, auxiliary enzymes, such as glucuronidase, acetylesterase, α-arabinofuranosidase, feruloyl esterase, xylanase, β-xylosidase, galactomannanase, and glucomannanase, attack hemicellulose and release mainly xylose, mannose, and arabinose. Thus, production of different cellulase components and auxiliary enzymes by *A. niger* HN-1 makes it an ideal candidate for further evaluation. Most of the *Aspergillus* strains are known for efficient production of β-glucosidase, but generally lack the ability to produce CBH in significant amounts (Table 4). In comparison, the enzyme assays clearly indicate that *A. niger* HN-1 showed potential for production of a complete cellulolytic enzyme system in a single cocktail desired for effective hydrolysis of pretreated cellulosic biomass.

Hydrolysis of Alkali-Treated Rice Straw Using Crude Cellulase Consortium. Glucan, xylan, arabinan, AIL, ASL, ash, and protein contents in rice straw were 36.2 ± 1.23, 24.8 ± 1.1, 3.28 ± 0.54, 12.12 ± 0.87, 1.97 ± 0.21, 14.13 ± 0.98, and 3.23 ± 0.45%, respectively. Either galactans, mannans, or rhamnans were not present in the rice straw used in this study or their concentration was too low to be determined by HPLC. Glucan concentration increased by about 50%, whereas xylan, lignin, and ash decreased by about 40, 49, and 51%, respectively, after pretreatment. The total biomass loss during alkali treatment with 1% NaOH was about 42%. Hydrolysis using the crude cellulase consortium resulted in about 84% glucan conversion to glucose, 89% xylan conversion to xylose, and 91% arabinan conversion to arabinose (Figure 5). The

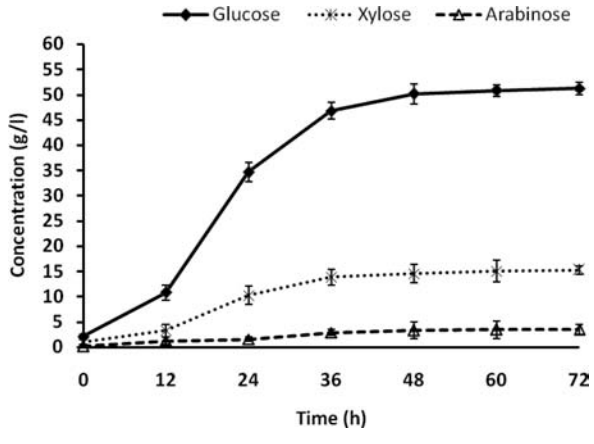

Figure 5. Hydrolysis of alkali-treated rice straw using crude cellulase obtained from *Aspergillus niger* HN-1.

Table 5. Summary of Some of the Published Results on Hydrolysis of Pretreated Rice Straw Using Crude Cellulase Enzyme

microbial strain	substrate loading (%, w v^{-1})	enzyme concn (FPU gds^{-1})	reducing sugar concn (mg gds^{-1})	hydrolysis (%)	hydrolysis time (h)	ref
Trametes hirsute	2.5	30	685	88.9	96	34
Penicillium sp. B1	5	19	316	57.9	48	35
Trichoderma reesei A1	5	19	374	70.5	48	35
Aspergillus fumigates	7	10.27		95	96	36
Aspergillus fumigates	10	10.27		91	96	36
Aspergillus niger HN-1	10	15	680	86	48	Present study

hydrolysate obtained after 48 h of hydrolysis was composed of about 680 mg reducing sugars gds^{-1} (501.7 mg of glucose, 145.6 mg of xylose, and 32.8 mg of arabinose), which corresponds to about 394 mg reducing sugars gds^{-1} of the untreated biomass comprising about 74% glucose, 21% xylose, and 5% arabinose. No cellobiose, cellotriose, xylobiose, or xylotriose was detected in the hydrolysate. Oligosaccharides such as xylobiose and xylotriose are formed because of the absence of β-xylosidase or the presence of this enzyme in low concentrations in the hydrolytic enzyme mixture. The absence of oligosaccharides in the hydrolysate suggests that auxiliary enzymes such as β-xylosidase were present in required concentrations in the crude cellulase complex. Although we did not characterize endoglucanase for its isoforms, the absence of cellotriose in the hydrolysate suggests that different isoforms of endoglucanase were present in the crude cellulase consortium. However, studies on the expression of isoforms of different cellulase components and the secretome analysis of the cellulase produced by *A. niger* HN-1 are needed to arrive at the role of different components in hydrolysis. Hydrolysis rate was maximum during 12−36 h of hydrolysis, after which a significant increase in sugar concentration was not seen (Figure 5). It is also felt that solubilization of cellulose and hemicellulose during hydrolysis leads to increased lignin and ash content, which in turn reduces cellulase efficiency. Hemicellulose and lignin restrict the access of cellulolytic enzymes by coating cellulose fibers, and in some lignocellulosics, pectin could also exert a similar effect.[27] The results obtained through the present study are significant in terms of substrate loading, hydrolysis time, and sugar yield (Table 5).

A hHigh solid-to-liquid ratio might lead to reduced sugar concentrations in the hydrolysate because of substrate inhibition and lower accessibility of cellulose to hydrolytic enzymes. However, for commercial success of ethanol production from lignocellulosic biomass, it is important to use high substrate loading to obtain a significant concentration of fermentable sugars. It is therefore important that future research is directed to produce enzyme consortia that are able to efficiently hydrolyze substrates at loading levels of 15% (w/v) or higher. Previous studies have reported hydrolysis of different lignocellulosic materials using crude enzymes (Table 5). However, in most of the previous studies, either low substrate concentration was used for hydrolysis or low sugar concentrations were obtained or longer hydrolysis time was required to achieve a desired sugar concentration. The comparison of sugar concentration, substrate loading, and hydrolysis time in overall terms showed that the results obtained in the present study for hydrolysis of alkali-treated rice straw using crude cellulase preparations are better than the previously reported results (Table 5). Higher hydrolytic efficiency for crude cellulase preparation shows promise for conducting experiments at a higher scale of operation. Our future research is aimed at molecular characterization of the cellulase produced by *A. niger* HN-1, process optimization for production of cellulases and hemicellulases by *A. niger* HN-1, and study of enzyme kinetic parameters and evaluation of the potential of partially purified cellulase consortium in hydrolysis of a variety of lignocellulosic biomass at higher substrate concentrations.

This study showed that the two-stage statistical medium optimization using a newly isolated strain of *A. niger* significantly enhanced the production of cellulolytic enzyme system. Hydrolysis of alkali-treated rice straw using the crude cellulase consortium led to the production of 680 mg reducing sugars g^{-1} in 48 h. Furthermore, the presence of different cellulase components in a single cellulolytic enzyme system (consortium) obviates the use of enzymes separately for hydrolysis of lignocellulosic biomass, thereby reducing the production cost. Experimental methods to enhance the enzyme production are imperative for success in the biofuels industry, which uses enzymatic and microbial fermentation platforms.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CCD, central composite design; RSM, response surface methodology; HPLC, high-performance liquid chromatography; FP, filter paper cellulase; EG, endoglucanase; CBH, cellobiohydrolase; Exo-PG, exopolygalacturonase; MW, Mandel Weber; SSF, solid state fermentation; CMC, carboxymethylcellulose; LSU, large subunit; PDA, potato dextrose agar

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