Volume 60(1):39-48, 2016
Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Statistical optimization of cellulase biosynthesis by isolated cellulolytic fungi utilizing horticultural waste

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ABSTRACT Cellulase production by isolated cellulolytic fungi was carried out by utilizing horticultural waste, banana peel under solid state fermentation (SSF). The isolated fungus was previously tested for its cellulose hydrolyzing ability by observing the clear zone pattern around the colonies on CMC amended agar plates followed by secondary screening on wheat bran. The isolate named SS3 (5) that exhibited maximum enzyme activity at the level of 19.9 U/gds and 3.7 U/gds for CMCase and FPase respectively was chosen for further morphological and genotypic identification. The isolate was found to be similar to *Aspergillus niger* isolates, based on 18S rRNA sequencing and BLAST analysis. Statistical optimization of cellulase biosynthesis by the addition of nutritional supplements to a banana peel based medium was studied by response surface methodology (RSM) involving Box-Behnken Design (BBD), which resulted in an improved cellulase yield. The cellulase production was found to be increased by 2.01-fold and 1.4-fold for CMCase and FPase, respectively, under statistically optimized nutritional conditions (i.e. medium supplemented with 0.06% $MgSO_4$, 0.4% Triton X-100 and 0.52% $NaNO_3$) compared to the control (*i.e.* medium without supplements).
Acta Biol Szeged 60(1):39-48 (2016)

KEY WORDS

Aspergillus sp.
CMCase
FPase
response surface methodology
solid-state fermentation

Introduction

Cellulase refers to an assortment of enzymes, which synergistically acts to hydrolyze cellulose, the major structural carbohydrate of the plant cell wall and most copious biopolymer on the Earth (Guo et al. 2008). Several efforts have been made in biotechnology industry to come up with new enzyme-based biotechnological techniques for conversion of cellulosic biomass to value-added products. Cellulase is a multienzyme system composed of several enzymes, which include endo-1,4-β-D-glucanase [EC 3.2.1.4], exo-1,4-β-D-glucanase [EC 3.2.1.91] and β-glucosidase [EC 3.2.1.21] (Bhat and Bhat 1997). The endoglucanase randomly attacks β-1,4 bonds in cellulose producing glucan chains of different lengths, whereas exoglucanase acts on the ends of the cellulose chain and releases β-cellobiose as the end product, which is eventually converted to glucose by β-glucosidase. Cellulases are produced by a large number of microorganisms and are reported to be either intracellular or extracellular (Rathnan et al. 2012). Several fungi are reported to degrade cellulose, but a limited number of them can produce considerable quantities of free enzyme, which can proficiently hydrolyze crystalline cellulose. Therefore, it becomes imperative to obtain novel fungal strains producing extracellular cellulases with higher specific activities and greater efficiency. Considering the importance of microbial cellulases, in various industries including paper, textile, biofuel production, food, feed, brewing and agriculture industries (Kuhad et al. 2011), screening of naturally occurring cellulase synthesizing microorganisms may be the finest way to obtain novel microbial strains (Narasimha et al. 2006).

Production of cellulase enzymes has been extensively studied in submerged fermentation, but the high expenditure of enzyme production has constrained its application in scale-up processes. Solid state fermentation is a promising technique to produce cellulases, and the use of cellulosic solid wastes as substrate rather than expensive pure cellulose makes the technique more economically feasible (Das and Ghosh 2009). Furthermore, optimization of fermentation parameters has become indispensable to increase the yield of the enzyme. Response surface methodology is applicable to optimize some bioprocessing parameters (Vaidya et al. 2003; Pan et al. 2008) and it is also able to study the combined effect of the fermentation parameters and the responses. The present study reports on (i) the isolation of fungal isolates for efficient and cost effective production of cellulases, (ii) optimization of solid substrate for fermentation and (iii) employment of response surface methodology to optimize the nutritional components of medium under SSF for cellulase production.

Submitted April 19, 2016; Accepted Sept 13, 2016
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Materials and Methods

Substrate and chemicals

Carboxymethylcellulose (CMC) was procured from LOBA chemicals. Medium components and Congo Red were obtained from Hi-Media Pvt. (India). Whatman No. 1 filter paper of retention 11 µm and glucosamine were procured from Sigma-Aldrich. 3,5-Dinitrosalicylic acid, Folin-Ciocalteu and nutritional supplements were obtained from Merck. Wheat bran, agricultural and horticultural wastes were collected from local sources for solid state fermentation.

Pre-treatment of substrate

The different agro and horticultural wastes were dried at 80 °C for 5 h and were ground to a fine powder by passing it through a sieve of size 1.1 mm.

Isolation of fungi with relevant enzyme producing capability

In order to isolate cellulase producing fungi, three soil samples were collected from areas containing decaying leaves, garden soil and an area near a saw mill. These soil samples were named as SS1, SS2 and SS3, respectively. The soil samples were subjected to a serial dilution up to 10^{-10} in sterilized distilled water by taking 1 g of soil from each original sample. Fungal colonies were isolated by pour plate method (10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} dilutions were plated) (Khan and Singh 2011) on Czapek-Dox agar medium with the following composition (per liter): 3 g glucose, 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4$, 20 g agar; pH 5.0 (Sri Lakshmi and Narasimha 2012). Inoculated plates were incubated at 30 °C for 7 days. After incubation, different fungal isolates were chosen based on the variation in their size, shape and color. The fungal strains were named according to the corresponding soil sample and a number was given to the isolate.

Primary screening by plate staining

Different fungal colonies selected from pour plate technique, were screened for their cellulase producing abilities by following the standard procedure (Teather and Wood 1982). Modified Czapek-Dox medium was used, which contained 1% carboxymethylcellulose (CMC) as sole carbon source. The pH of the medium was 5.0. The fungal isolates were added as spore suspensions made with sterile water in cups made by Cork borer on the solidified medium. The plates were incubated at 30 °C for 72 h followed by incubation at 50 °C for 18 h, which is reported to be the optimum temperature for most fungal cellulase activity (Sri Lakshmi and Narasimha 2012). After incubation, 10 ml of 1% aqueous solution of Congo Red was added to the plates and were shaken for 15 min. The Congo Red staining solution was then discarded and 1 N NaOH was used for destaining the plates. Finally, the fungal spore inoculated plates and the control plate where distilled water was added instead of spore suspension were analyzed by noticing the formation of yellow zones around the inoculated wells (Sri Lakshmi and Narasimha 2012).

Secondary screening by solid state fermentation (SSF)

Isolates, which gave encouraging results in primary screening, were tested for their ability to produce CMCase and FPase on wheat bran which was used as a substrate for solid state fermentation. Three grams of wheat bran were moistened with distilled water (1:1 w/v) in a 100-ml Erlenmeyer flask and autoclaved. The flasks were then inoculated with 3 ml spore suspension of the screened organism (10^9 spores/ml) and were incubated at 30 °C for 7 days.

Extraction of crude enzyme

After fermentation, the crude enzyme was extracted from each solid moldy medium by mixing them homogeneously with distilled water (1:10 w/v) (Mrudula and Murugammal 2011) followed by agitation at 120 rpm on a rotary shaker at 30 °C for 1 h. Filtration of the extracts was done by dampened cheese cloth. The pooled extracts were centrifuged at 8000 rpm for 5 min at 4 °C (Khan and Singh 2011). The supernatant obtained were used as the crude extracellular enzyme preparation.

Enzyme assay with crude enzyme

Standard endoglucanase (CMCase) activity was measured using an assay based on Ghose's methodology (Ghose 1987). The filter paper hydrolyzing (FPase) activity was determined by the method of Mandels and Weber (Mandels and Weber 1969). The reducing sugar liberated for both the cases were estimated spectrophotometrically at 540 nm after addition of dinitrosalicylic acid reagent using a HITACHI U-2000 spectrophotometer. One filter paper unit (FPU) was defined as an amount of the enzyme in the filtrate, which releases 1 µmol of reducing sugar from filter paper/ml/min (Pradeep and Narasimha 2011). One unit of CMCase was the amount of the enzyme releasing 1 µmol of reducing sugar from carboxymethylcellulose/ml/min (Pradeep and Narasimha 2011). The enzyme activities were expressed as U/gds (Unit per gram dry substrate). The dry weight of the samples was determined by drying them in a hot air oven at 80 °C to a constant weight.

Protein estimation in crude enzyme

The culture filtrates were used for estimation of total dissolved protein, according to the method of Lowry (Lowry et al. 1951). Bovine serum albumin (BSA) was used as the protein standard.

Biomass determination

Fungal biomass estimation was carried out by determining the N-acetyl glucosamine released by the acid hydrolysis of the chitin, one of the constituents of the fungal cell wall (Ramachandran et al. 2005). Glucosamine was used as the standard. The results were expressed as mg glucosamine per gram dry substrate.

Strain identification

The morphological identification of fungal isolate SS3 (5) was carried out using staining technique with lactophenol cotton blue. To confirm the morphological identity of the selected strain, 18S rRNA sequencing was applied. Isolation of genomic DNA, amplification of DNA by PCR and 18S rRNA sequencing were done in GCC Biotech (India). Isolation of fungal genomic DNA was carried out followed by its amplification in 20 µl of PCR reaction mixture containing 10 ng of genomic DNA template, 2.5 mM $MgCl_2$, 0.25 mM dNTP Mix, forward, reverse primers and 0.75 U Taq DNA polymerase. Amplification was carried out in a thermal cycler (Veriti, Applied Biosystems) with a primary denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. Termination of thermal cycles was done after a final extension for 5 min at 72 °C. Fungal specific primers for 18S rRNA gene amplification were the following: 18S-F1-GCAAGTCTGGTGCCAGCAGCC, 18S-R1-CTTCCGTCAATTCCTTTAAG, 18S-F2-AACTTAAAGGAATTGACGGAAG, 18S-R2-GCATCACAGACCTGTTATTGCCTC. PCR products were used in sequencing reactions with the same primer using a Big Dye Terminator Cycle Sequencing Reaction Kit version 3.0 (Applied Biosystems). Sequencing was performed on ABI 3500 sequencer (Applied Biosystems). Acquired sequences were assembled to get the full-length sequence of the gene by DNA Dragon. The BLAST (Singh et al. 2006) facility of the NCBI GenBank database was used to compare the resulting sequence with known fungal sequences.

Substrate optimization

Different agro and horticultural wastes were tested for their competence to act as a substrate for production of extracellular cellulases by SSF. Fermentation was carried out with banana peel, pea peel, potato peel, rice straw, ribbed gourd peel, pumpkin peel, mango peel, amaranth, litchi peel, sugarcane bagasse, newspaper and cabbage at 30 °C for 7 days under stationary condition, and enzyme assay was done in each case. Fermentation using wheat bran was used as the control. The best solid substrate achieved by this step was fixed for subsequent experiments.

Table 1. The coded level of variables chosen for the experiments.

Variable	Code	Range and level		
		-1	0	+1
Nitrogen source ($NaNO_3$) %	A	0.4	0.5	0.6
Metal salt ($MgSO_4$) %	B	0.04	0.05	0.06
Surfactant (Triton X-100) %	C	0.2	0.3	0.4

Optimization of nutritional parameters for cellulase production employing response surface methodology (RSM)

RSM using Box-Behnken Design (BBD) was applied for optimization of cellulase production, which comprised of full factorial search by detecting simultaneous and systematic variation of important components of the fermentation process. As banana peel, (ripen yellow variety) gave maximum cellulase production among the substrates tested, it was selected for further optimization studies employing response surface methodology. Three important parameters, namely the nitrogen source $NaNO_3$ (A), the metal salt $MgSO_4$ (B) and the surfactant Triton X-100 (C), were selected as the independent variables, and cellulase activity (U/gds), protein content (mg/ml) and biomass (mg/gds) were the dependent response variables. Each of these independent variables was studied at three different levels per BBD with a total of 17 experimental runs. Cellulase activities (U/gds), protein content (mg/ml) and biomass (mg/gds) corresponding to the combined effects of three variables were studied in their specified ranges as shown in Table 1. Temperature, amount of substrate, hydration ratio and time was maintained as 30 °C, 5 g, 25% and 5 days, respectively, throughout all experiments. CMCase and FPase activity obtained under these optimized physical conditions were 60 U/gds and 8 U/gds, respectively. This served as control as no additional nutritional supplement was added at this stage. All the flasks were analyzed for cellulase activity, biomass and extracellular protein content as planned in BBD. This methodology allows the modeling of a second order equation that describes the process of fermentation (Chapla et al. 2010). Cellulase production data were analyzed and response surface model was fitted with multiple regressions.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 \sum \sum_{i<j=1}^{n} \beta_{ij} X_I X_J.$$

Where Y is the measure of responses; β_0, β_i, β_j and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j are the coded value of the i^{th} and j^{th} independent variables. The variables X_iX_j represents the first order interaction between X_i and X_j for (i<j) (Hoa and Hung 2013). The nitrogen source ($NaNO_3$), metal salt ($MgSO_4$) and surfactant (Triton X-100), which produced maximum cellulase activity by one-factor-at-a-time (OFAT) methodology, were considered for statistical optimization after screening different compounds (data unpublished).

Comparative analysis of cellulase production by isolated Aspergillus sp. and Aspergillus niger NCIM612

SSF was carried out under optimized conditions with the isolated *Aspergillus sp.* and *Aspergillus niger* NCIM612, and cellulase production from the respective strains was determined.

Results and Discussion

Isolation and primary screening of microorganisms

A total of fifteen fungal isolates were obtained on the basis of plate staining technique (Table 2). All the isolates exhibited clear zones of varying diameter around the colonies after staining with Congo Red solution followed by destaining the plates with 1 N NaOH (Sri Lakshmi and Narasimha 2012). The strains displaying zone of hydrolysis greater than 1 mm in diameter were selected for further studies of screening. Five fungal isolates namely SS2 (4), SS3 (5), SS3 (2), SS1 (5) and SS1 (6) were found to have a better hydrolyzing capability, of which SS3 (5) showed the maximum zone of hydrolysis having a value of 2.2 mm in diameter. As plate, screening technique has poor association between enzyme activity and colony to clear zone ratio it cannot be considered quantitative (Maki et al. 2009). Secondary screening was performed with the above mentioned strains for quantitative analysis.

Secondary screening

SSF was carried out in 100 ml Erlenmeyer flasks using wheat bran as substrate at 30 °C under stationary condition for the fungal isolates that showed the best result during primary screening. Isolate SS3 (5) showed maximum production for both CMCase and FPase (Fig. 1) at the levels of 19.9 U/gds and 3.7 U/gds, respectively.

Table 2. Fungal isolates having cellulolytic property.

Fungal isolates	Colony color	Colony diameter (mm)	Zone of hydrolysis (mm)
SS3 (5)	Black	4.4±0.1	2.2±0.1
SS3 (2)	Light brown	3.0±0.1	2±0.1
SS2 (6)	Brown	3.0±0.05	1.7±0.07
SS1 (5)	Brown	4.2±0.1	1.6±0.1
SS1 (6)	Black	4.4±0.1	1.6±0.05
SS2 (4)	Black	4.8±0.07	0.8±0.05
SS1 (2)	Black	3.0±0.05	0.8±0.1
SS3 (3)	Black	3.2±0.1	0.6±0.1
SS1 (3)	Black	3.1±0.1	0.6±0.1
SS1 (4)	Brown	3.0±0.1	0.4±0.1
SS2 (3)	Black	4.0±0.05	0.4±0.15
SS2 (7)	Black	2.8±0.05	0.3±0.1
SS3 (1)	Brown	3.1±0.1	0.3±0.1
SS3 (4)	Dark brown	2.5±0.1	0.2±0.15
SS2 (9)	Black	2.7±0.1	0.1±0.05

*Each value is a mean of three replicates ± standard deviation.

Strain identification

The microscopic characteristics of the strain as observed under the light microscope showed that the morphology was similar to *Aspergillus sp.* (Devi and Kumar 2012). Radial colonial head and conidia were observed, which appeared black to brownish black under 40× objective.

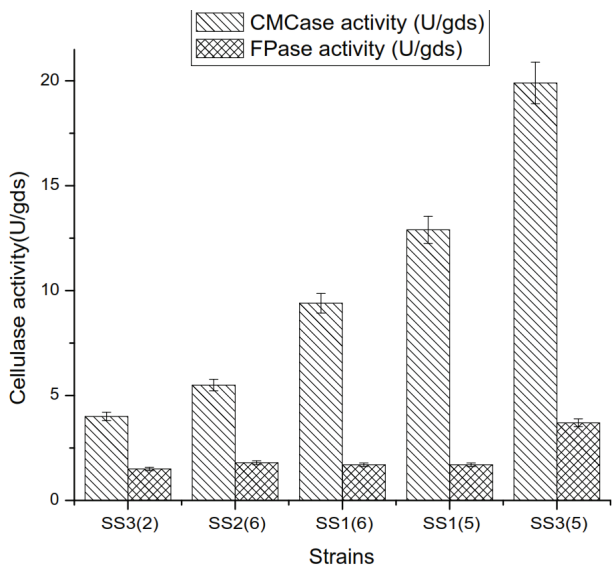

Figure 1. Quantitative screening of the fungal isolates by SSF. Assay was performed at 30 °C for 7 days. Error bars with standard error are shown.

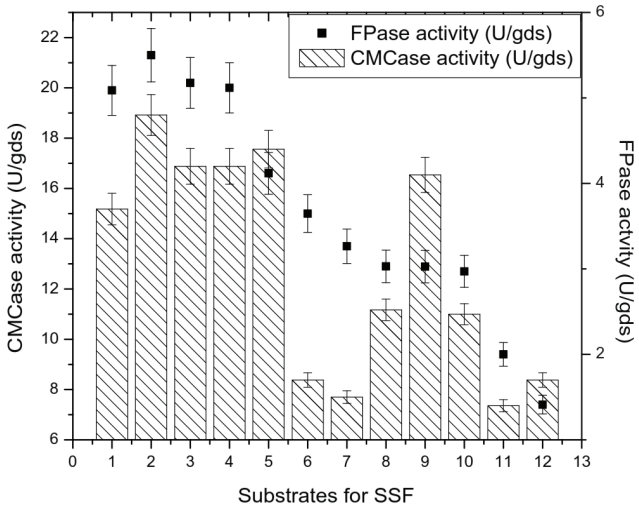

Figure 2. Optimization of solid substrates for production of CMCase and FPase by SSF. Error bars with standard error are shown. Substrates were the followings: 1. wheat bran, 2. banana peel, 3. potato peel, 4. newspaper, 5. amaranth, 6. ribbed gourd, 7. mango peel, 8. pea peel, 9. rice straw, 10. cabbage, 11. sugarcane bagasse, 12. litchi peel.

The morphological identification of *Aspergillus sp*. was further authenticated with molecular study. Fungal 18S rRNA gene was sequenced from both directions and the obtained sequences were assembled to get the full-length sequence of the gene by DNA Dragon. BLAST query in the NCBI GenBank database indicated that the isolate SS3 (5) is similar to *A. niger*.

Substrate optimization

In SSF, the selection of an appropriate solid substrate for the fermentation is a critical factor and involves screening of a number of agro and horticultural materials for enzyme production and microbial growth. The biosynthesis of enzymes depends significantly on culture conditions and carbohydrates generally encourage the production of cellulolytic enzymes. Best cellulase production has been reported on cellulosic materials (Juhász et al. 2005). In the current study, 12 different substrates, such as, newspaper, potato peel, amaranth, banana peel, rice straw, sugarcane bagasse, pea peel, cabbage (outer leaves), litchi peel, pumpkin peel, mango peel and ribbed gourd peel were tested for cellulase production by the isolated strain. Wheat bran was used as the control. Among the substrates that were assessed banana peel produced maximum cellulases at the levels of 21.3 U/gds and 4.8 U/gds for CMCase and FPase (Fig. 2) followed by potato peel, which may suggest that the peel provided all the necessary nutrients required for the growth of the isolated microorganism (Nagarajaiah and Prakash 2011). Application of peel for fermentation can not only reduce the production costs, but also may help to reduce the pollution related problems as the peels, that are accumulated can be meaningfully utilized for the production of value-added products. In comparison to *Trichoderma viride* GIM 3.0010 (Yan et al. 2011), enhanced yield of CMCase was obtained with the isolated *Aspergillus sp.* using banana peel as a substrate.

Statistical optimization of medium components using Box-Behnken design

RSM has been proven to be a powerful statistical tool for optimization of fermentation parameters for SSF. This method has been successfully applied in the optimization of fermentation medium components and physical parameters for the fermentation process (Katapodis et al. 2007). The design

Table 3. ANOVA statistics for CMCase (response 1).

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P–value Prob>F	
Model	244.88	9	27.21	111.68	<0.0001	Significant
A-Nitrogen	35.7	1	35.7	146.53	<0.0001	
B-Metal salt	56.71	1	56.71	232.73	<0.0001	
C-Surfactant	1.45	1	1.45	5.93	0.0451	
AB	3.06	1	3.06	12.57	0.0094	
AC	10.24	1	10.24	42.03	0.0003	
BC	16.81	1	16.81	68.99	<0.0001	
A^2	77.04	1	77.04	316.20	<0.0001	
B^2	16.47	1	16.47	67.58	<0.0001	
C^2	30.64	1	30.64	125.75	<0.0001	
Residual	1.71	7	0.24			
Lack of fit	1.3	3	0.43	4.24	0.0983	Not significant
Pure error	0.41	4	0.10			
Cor total	246.59	16				

*P ≤ 0.0001 indicates highly significant values, P ≤ 0.05 indicates significant values, P > 0.05 indicates values that are not significant.

Table 4. ANOVA statistics for FPase (response 2).

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P–value Prob>F	
Model	3.87	9	0.43	52.24	<0.0001	Significant
A-Nitrogen	0.38	1	0.38	46.48	0.0002	
B-Metal salt	1.09	1	1.09	132.07	<0.0001	
C-Surfactant	0.25	1	0.25	30.60	0.0009	
AB	0.01	1	0.01	1.9	0.2108	
AC	0.25	1	0.25	30.35	0.0009	
BC	0.15	1	0.15	18.47	0.0036	
A^2	0.73	1	0.73	89.21	<0.0001	
B^2	1.08	1	1.08	131.54	<0,0001	
C^2	3.853E-003	1	3.853E-003	0.47	0.516	
Residual	0.05	7	8.236E-003			
Lack of fit	0.04	3	0.01	4.83	0.0812	Not significant
Pure error	0.01	4	3.120E-003			
Cor total	3.93	16				

*P ≤ 0.0001 indicates highly significant values, P ≤ 0.05 indicates significant values, P > 0.05 indicates values that are not significant.

Table 5. ANOVA statistics for Biomass (response 3).

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P–value Prob>F	
Model	144.27	9	16.30	7.10	0.0085	Significant
A-Nitrogen	11.76	1	11.76	5.21	0.0564	
B-Metal salt	63.85	1	63.85	28.29	0.0011	
C-Surfactant	0.03	1	0.03	0.01	0.9096	
AB	20.7	1	20.7	9.17	0.0192	
AC	3.61	1	3.61	1.6	0.2465	
BC	2.72	1	2.72	1.21	0.3084	
A^2	12.27	1	12.27	5.44	0.0525	
B^2	15.49	1	15.49	6.86	0.0344	
C^2	15.31	1	15.31	6.78	0.0352	
Residual	15.80	7	2.26			
Lack of fit	3.3	3	1.12	0.36	0.7859	Not significant
Pure error	12.44	4	3.11			
Cor total	160.07	16				

*P ≤ 0.0001 indicates highly significant values, P ≤ 0.05 indicates significant values, P > 0.05 indicates values that are not significant.

allowed us to predict maximum cellulase production based on few sets of experiments, in which all the selected factors were varied within a specific range. The implemented design could also study the interactive effects of various process parameters (Chapla et al. 2010). ANOVA (analysis of variance) was employed to determine the significant effects of variables on cellulase, protein and biomass production. Experimental results suggested that the variables selected for the fermentation process had a strong effect on cellulase production. On the basis of these experimental values, statistical testing was carried out using the Fisher's 'F'-test. ANOVA for cellulase production showed that fitted second order polynomial RSM is highly significant with F test, the values, of which were found to be 111.68, 52.24, 7.10 and 10.59 for CMCase, FPase, biomass and protein, respectively, as shown in Table 3-6. The p-values from ANOVA table suggested that the combination of independent variables had a significant effect on the four responses (CMCase, FPase, biomass and protein content). The coefficient of determination R^2 for CMCase, FPase, biomass and protein respectively were 0.99, 0.98, 0.90 and 0.93, which indicated that there is a good agreement between the predicted and experimental values. The contour plots described by the regression model were prepared by using Design-Expert Stat-Ease (version 7) software. Nitrogen ($NaNO_3$) concentration of the optimized fermentation medium was found to be 0.52%, which is close to the central value selected for the independent variable. For cellulase production, 0.4% surfactant (Triton X-100) and 0.06% metal salt ($MgSO_4$) were considered ideal, which were different from the central level of values selected. An increase or de-

Table 6. ANOVA statistics for Protein (response 4).

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P–value Prob>F	
Model	1.63	9	1.63	10.59	0.0026	Significant
A-Nitrogen	3.613E-003	1	3.613E-003	0.21	0.6642	
B-Metal salt	0.15	1	0.15	8.75	0.0211	
C-Surfactant	1.23	1	1.23	70.05	<0.0001	
AB	0.11	1	0.11	6.00	0.0400	
AC	0.03	1	0.03	1.84	0.2169	
BC	2.500E-003	1	2.500E-003	0.14	0.7174	
A^2	4.516E-003	1	4.516E-003	0.26	0.6280	
B^2	0.11	1	0.11	6.34	0.0399	
C^2	0.02	1	0.02	1.18	0.3131	
Residual	0.12	7	0.01			
Lack of fit	0.01	3	4.825E-003	0.18	0.9064	Not significant
Pure error	0.11	4	0.02			
Cor total	1.80	16				

*P ≤ 0.0001 indicates highly significant values, P ≤ 0.05 indicates significant values, P > 0.05 indicates values that are not significant.

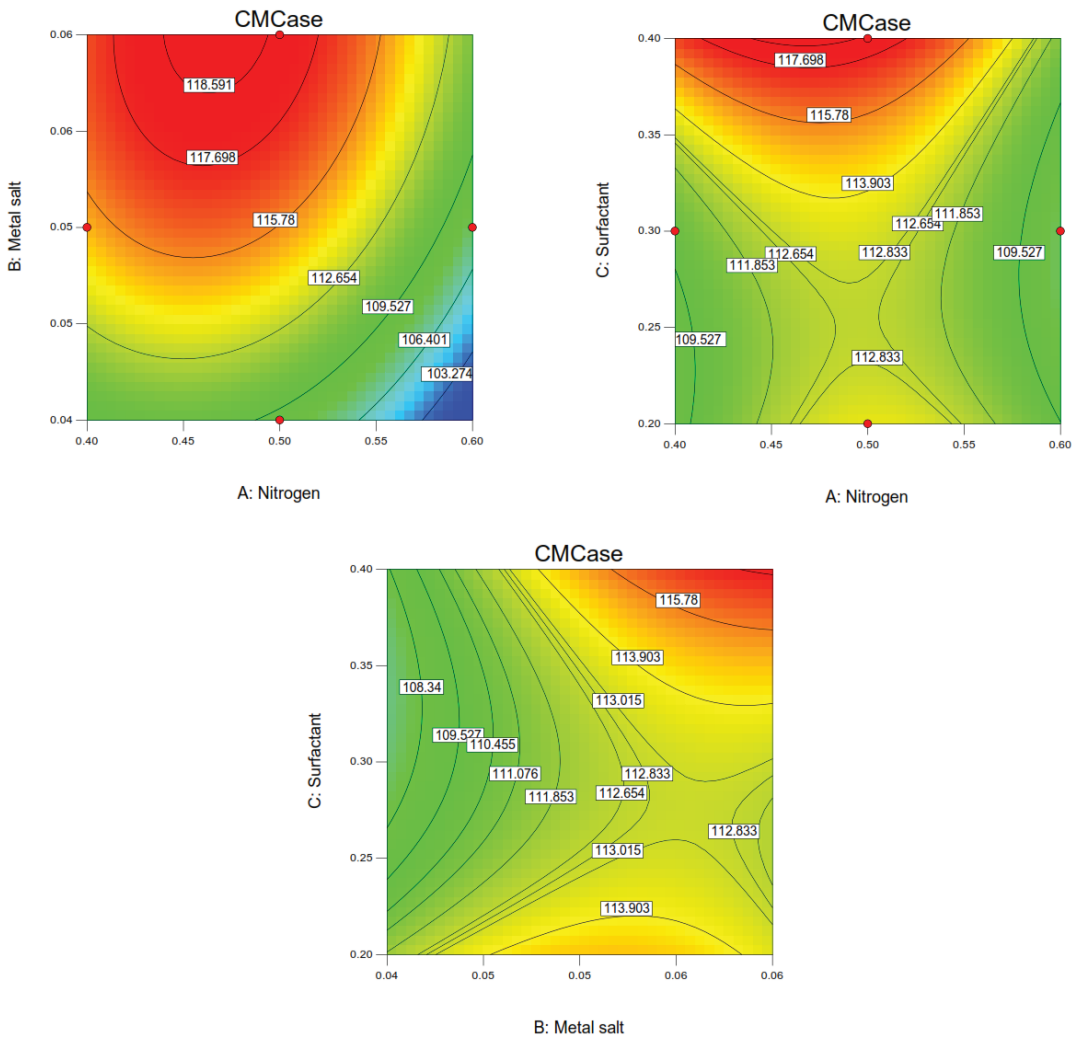

Figure 3. Contour plots showing significant interactions between independent variables on CMCase production.

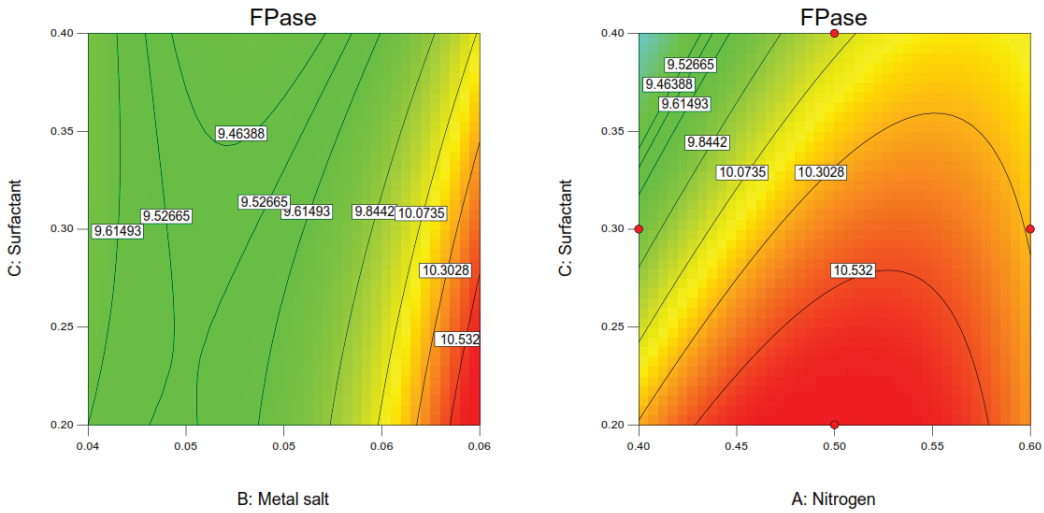

Figure 4. Contour plots showing significant interactions between independent variables on FPase production.

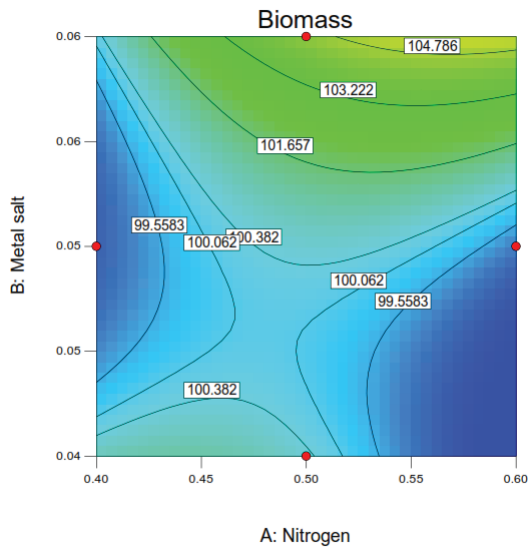

Figure 5. Contour plot showing significant interaction between independent variables on biomass production.

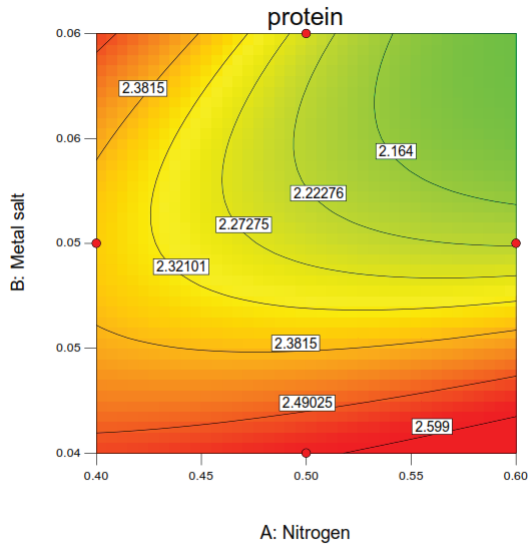

Figure 6. Contour plot showing significant interaction between independent variables on protein production.

crease from these values resulted in a reduction of cellulase yield. The contour plots of only the significant interactions between the two independent variables are given here for the responses (Fig. 3-6). The interaction terms AB, AC, BC were found to be significant for CMCase. The parent terms or the coded factors A, B, C and the interaction terms AC and BC were found to be significant for FPase. AB was found to be the only significant interaction term in case of response 3 and 4 (biomass and protein). The elliptical nature of the contour plots indicated a substantial interaction between independent and dependent variables. Besides that, the contour plots also showed the communicative change with respect to simultaneous alteration in two variables keeping the third variable at its central point. A suitable choice of medium component is necessary to obtain maximum enzyme yield and the contour plots obtained from the model provides these alternatives.

Authentication of the model

A fermentation was done by the isolated *Aspergillus sp.* under optimal conditions (*i.e.* 0.06% $MgSO_4$, 0.4% Triton X-100 and 0.52% $NaNO_3$) to test the optimized parameters given by

the model. The cellulase, biomass and protein production under optimized parameters were 120.8 U/gds for CMCase, 11.5 U/gds for FPase, 105.6 mg/gds for biomass and 2.25 mg/ml for protein. The values obtained under the above conditions were close to the predicted values (117.9 and 10.09 U/gds for CMCase and FPase, 104.8 mg/gds for biomass and 2.2 mg/ml for protein) given by the model. Hence, the model was fit and the fermentation conditions above, can be satisfactorily applied to obtain high cellulase activities. Compared to control (without $NaNO_3$, $MgSO_4$, Triton X-100) where CMCase and FPase activities were found to be 60 and 8 U/gds respectively, 2.01- and 1.4-fold increase was observed for CMCase and FPase under the optimized nutritional conditions.

Conclusion

The present study attempts to investigate the potential of an indigenously isolated fungal strain to produce cellulase on lignocellulosic solid wastes. SS3 (5) strain isolated from a soil sample collected from saw mill exhibited the highest CMCase and FPase production. The isolate was found to be similar to *Aspergillus niger*, based on 18S rRNA sequencing. By optimization of the solid substrate for SSF, an approximately 1.07- and 1.29-fold increase of the CMCase and FPase activity could be achieved. The closeness of the experimental and the predicted values of RSM suggests the precision of the optimization method. Under the optimized conditions, CMCase and FPase production were obtained up to 120.8 U/gds and 11.5 U/gds, respectively, which were 2.01 and 1.4 times higher than those of the control (without the addition of nutritional supplements). These attributes made banana peel a proficient substrate for SSF and the isolated *Aspergillus sp.* a potential candidate for cellulase production.

Acknowledgement

The authors gratefully acknowledge the financial assistance by University Grants commission, New Delhi.

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