Appl Biochem Biotechnol (2007) 142:60–70
DOI 10.1007/s12010-007-0019-2

Improved Cellulase Production by *Trichoderma reesei* RUT C30 under SSF Through Process Optimization

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Received: 23 June 2006 / Revised: 28 August 2006 / Accepted: 29 August 2006 /
Published online: 30 May 2007

Abstract The major constraint in the enzymatic saccharification of biomass for ethanol production is the cost of cellulase enzymes. Production cost of cellulases may be brought down by multifaceted approaches which includes the use of cheap lignocellulosic substrates for fermentation production of the enzyme, and the use of cost efficient fermentation strategies like solid state fermentation (SSF). The current study investigated the production of cellulase by *Trichoderma reesei* RUT C30 on wheat bran under SSF. Process parameters important in cellulase production were identified by a Plackett and Burman design and the parameters with significant effects on enzyme production were optimized for maximal yield using a central composite rotary design (CCD). Higher initial moisture content of the medium had a negative effect on production whereas incubation temperature influenced cellulase production positively in the tested range. Optimization of the levels of incubation temperature and initial moisture content of the medium resulted in a 6.2 fold increase in production from 0.605 to 3.8 U/gds of cellulase. The optimal combination of moisture and temperature was found to be 37.56% and 30 °C, respectively, for maximal cellulase production by the fungus on wheat bran.

Keywords Cellulase · *Trichoderma reesei* · Solid state fermentation · Wheat bran · Plackett and Burman · Central composite design

Introduction

Large-scale use of ethanol in fuel applications are looked upon as a renewable alternative to using fossil fuels. The use of lignocellulosic feedstock offers good perspectives for application of ethanol in transportation fuels [1], and the bioethanol produced from biomass feedstock is identified as a cost-effective option for CO_2 emission reduction [2]. A variety

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of metabolites can be synthesized working on the concept of "Biorefinary" [3] where glucose serves as base for the microbial synthesis of metabolites and this glucose is in turn derived by hydrolysis of the cellulose in biomass.

Enzymatic hydrolysis of cellulosic biomass is considered as the most efficient and least polluting methods for generating glucose from lignocellulosics, constrained only by the production cost of cellulases [4]. The cost of cellulase enzyme accounts for a significant share of the cost of saccharification [5] and large reductions in cellulase cost are envisaged for developing an economical process for bioethanol production from lignocellulosic biomass. One of the major parameters influencing the cost of cellulase production is the type of substrate used in fermentation production of the enzyme [6, 7]. Typically, cellulose of varying purity levels is used as substrates in cellulase production under submerged fermentation (SmF), which adds up to the cost of enzyme production. However, solid state fermentation technology relies on use of cheaper substrates for production of the cellulases [7–10], making it more cost effective. Also, the technology is promising due to the high product concentration, low cost of dewatering and the lower input of infrastructure and skill [11–13]. Higher yields of cellulase are obtained in solid state fermentation (SSF) compared to SmF [14] and production cost is reduced considerably [15]. With the appropriate technology, improved bioreactor design and operation controls SSF is envisaged to become a competitive method for the production of cellulases [16].

In this the present study, the filamentous fungus *Trichoderma reesei* RUT C30 was used for cellulase production using wheat bran as substrate under SSF. The medium components and environmental variables affecting cellulase production were optimized in two stages using statistical design of experiments (DOE). The parameters with significant effects on cellulase production were identified using a fractional factorial design and their levels were optimized using a central composite response surface method to improve cellulase yield.

Materials and Methods

Microorganism and Preparation of Inoculum

The fungus *T. reesei* RUT C30, a kind gift from Prof. George Scakacs, Technical University of Budapest, Hungary, was used in the present study. Culture was maintained on potato dextrose agar slants and was incubated at 30 °C. The fully sporulated slants obtained after 5 days were used immediately or stored at 4 °C in refrigerator. For preparing the spore inoculum, sterile saline was added to slants and the spores were dislodged into it by gentle pipetting under aseptic conditions. The suspension was recovered by aspiration and transferred to sterile 15-ml tubes. The suspension was appropriately diluted with sterile saline to obtain the required spore count. One milliliter of this spore suspension was used to inoculate the substrate.

Enzyme Production and Assay

Preparation of Substrate for SSF

Wheat bran was used as substrate in the present study. It was dried at 60 °C overnight in a hot air oven to remove moisture. Five grams of the substrate was weighed into 250-ml Erlenmeyer flasks and was moistened with mineral salt medium to attain appropriate initial moisture content. The basal mineral salts solution used for the experiment had following

composition: KH_2PO_4—0.5%, NH_4NO_3—0.5%, $MgSO_4 \cdot 7H_2O$—0.1%, peptone—0.1%, NaCl—0.1%, and $CaCl_2$—0.05% (trace elements: $FeSO_4 \cdot 7H_2O$—0.005%, $MnSO_4 \cdot 7H_2O$—0.001%, $ZnSO_4 \cdot 7H_2O$—0.001%, and $CoCl_2$—0.0002%). The pH of the salt solution was adjusted with 1 N HCl or 1 N NaOH wherever required. It was then sterilized by autoclaving at 121.5 °C for 15 min at 15 lbs pressure.

Enzyme Production

Erlenmeyer flasks containing 5 g of substrate moistened with the mineral salts medium were inoculated with 1 ml of spore suspension containing the desired number of spores. The contents were mixed thoroughly and were incubated under controlled conditions of temperature and humidity. Incubation was continued for the duration indicated in the experimental designs and at the end of incubation period enzyme was recovered by extraction with 0.1 N citrate buffer (pH 4.8). The extract was centrifuged to remove debris at 6,000 rpm for 10 min at 4 °C and was used as the crude enzyme sample.

Enzyme Assay

Filter paper assay was used to estimate total cellulase activity in the crude enzyme preparation as given below. A rolled Whatman No 1 filter paper strip of dimension 1.0× 6 cm (50 mg) was placed into each assay tube. The filter paper strip was saturated with 0.5 ml of Na–citrate buffer (0.05 M, pH 4.8) and was equilibrated for 10 min at 50 °C in a water bath. Half milliliter of an appropriately diluted (in Na–citrate buffer—0.05 M, pH 4.8) enzyme was added to the tube and incubated at 50 °C for 60 min. Appropriate controls were also run along with the test. At the end of the incubation period, each tube was removed from the water bath and the reaction was stopped by addition of 3 ml of DNS reagent. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The reaction mixture was diluted appropriately and was measured against a reagent blank at 540 nm in a UV–VIS spectrophotometer. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of cellulase activity was defined as the amount of enzyme required for liberating 1 mg of reducing sugar per milliliter per minute and was expressed as U/gds (units per gram dry substrate).

Optimization of Parameters for Improving Cellulase Production

Optimization of parameters for cellulase production was performed in two stages. Initially, 11 variables were screened using a fractional factorial design to identify the parameters, which significantly influenced enzyme production and in the second stage the levels of these parameters were optimized using a response surface design.

Screening of Parameters Affecting Cellulase Production by Fractional Factorial Design

A Plackett and Burman [17] design was employed to determine the effect of individual parameters affecting cellulase production by the fungus under SSF. The composition of mineral salt solution used for wetting the substrate and the important physical parameters affecting enzyme production were screened in a design with 11 variables at two levels in a total of 12 experimental runs (Table 1). The parameters tested were: initial moisture content

Table 1 Plackett and Burman design matrix for the screening of variables influencing cellulase production.

Std order	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Cellulase activity (U/gds)	
												Observed	Predicted
1	1	−1	1	−1	−1	−1	1	1	1	−1	1	0.26	0.93
2	1	1	−1	1	−1	−1	−1	1	1	1	−1	1.03	0.57
3	−1	1	1	−1	1	−1	−1	−1	1	1	1	0.59	0.93
4	1	−1	1	1	−1	1	−1	−1	−1	1	1	0.17	0.57
5	1	1	−1	1	1	−1	1	−1	−1	−1	1	1.85	1.66
6	1	1	1	−1	1	1	−1	1	−1	−1	−1	0.58	0.37
7	−1	1	1	1	−1	1	1	−1	1	−1	−1	2.97	2.76
8	−1	−1	1	1	1	−1	1	1	−1	1	−1	2.27	2.23
9	−1	−1	−1	1	1	1	−1	1	1	−1	1	1.70	2.20
10	1	−1	−1	−1	1	1	1	−1	1	1	−1	0.60	0.39
11	−1	1	−1	−1	−1	1	1	1	−1	1	1	1.52	1.49
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	2.04	1.47

of the medium, particle size of substrate, initial pH of the medium, incubation temperature, inoculum concentration, age of the inoculum, concentration of NH_4NO_3 in the medium, concentration of peptone, concentration of inducer (cellobiose), concentration of Tween 80, and the incubation time. The variables were tested at two levels: a higher level designated as +1 and a lower level designated as −1. The actual and coded values tested for each parameter are given in Table 2. Experimental runs were performed according to the design and the response (enzyme activity) was recorded. A factorial model was fitted for the main effects using Design Expert software (Statease Corp, USA). The effects of individual parameters on cellulase production was calculated by the following equation (Eq. 1)

$$\varepsilon = \left(\sum \mu_+ - \sum \mu_-\right)\Big/ n \tag{1}$$

Where ε is the effect of parameter under study and "μ_+" and "μ_-" are responses (cellulase activities) of trials at which the parameter was at its higher and lower levels respectively and "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of

Table 2 Actual levels of variables tested with the factorial design and their effects on cellulase production.

Code	Parameter name	Low level (−1)	High Level (+1)	Effect estimate	$P>F$
X_1	Moisture (%)	40	60	−1.10	0.006
X_2	Particle size (μM)	300–500	500–1,000	0.25	–[a]
X_3	pH	4	7	−0.32	–[a]
X_4	Temp of Incubation (°C)	27	32	0.73	0.036
X_5	Inoculum size (spores/ml)	10^5	10^8	−0.07	–[a]
X_6	Inoculum age (days)	5	7	−0.08	–[a]
X_7	NH_4NO_3 (g/L)	2.5	7.5	0.56	0.0885
X_8	Peptone (g/L)	1	3	−0.14	–[a]
X_9	Cellobiose (g/L)	0.001	0.01	−0.21	–[a]
X_{10}	Tween 80 (g/L)	0.1	0.5	−0.54	0.0999
X_{11}	Incubation time (h)	96	144	−0.57	–[a]

[a] Terms not included in the model.

Table 3 Actual and coded levels of variables tested with the central composite design.

Code	Parameter name	Levels of parameters				
		$-\alpha$ (−1.414)	−1	0	+1	$+\alpha$ (+1.414)
X_1	Moisture (%)	33.96	35	37.5	40	41.04
X_2	Temp of Incubation (°C)	28.2	29	31	33	33.8

α = Axial/star point (s) are levels larger than the chosen range of parameters.

individual parameters on cellulase production. The most significant parameters affecting cellulase production were identified.

Optimization of Significant Parameters

The significant parameters identified by the Plackett and Burman design were optimized using a response surface methodology (RSM). Specifically, a central composite rotary design (CCD) [18] was used for this study where the effect of the significant variables was studied at five different levels (Table 3). The design matrix with 14 experimental runs in two blocks, where the midpoint is replicated six times is shown in Table 4. The screened variables: initial moisture content of the medium and the incubation temperature were coded as X_1 and X_2, respectively.

The behavior of the system was modeled by a second order polynomial equation. The model equation used for the analysis is given below (Eq. 2)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \Sigma \sum_{i<j} \beta_{ij} X_i X_j + \varepsilon \tag{2}$$

Table 4 Central composite design matrix for optimization of parameters identified by the fractional factorial design.

Std order	Block	Moisture (%)		Temperature (°C)		Cellulase activity (U/gds)	
		Actual	Coded	Actual	Coded	Observed	Predicted
1	1	35	−1	29	−1	3.12	3.19
2	1	40	1	29	−1	3.1	3.24
3	1	35	−1	33	1	2.9	2.75
4	1	40	1	33	1	2.8	2.72
5	1	37.5	0	31	0	3.6	3.53
6	1	37.5	0	31	0	3.5	3.53
7	1	37.5	0	31	0	3.48	3.53
8	2	33.96	−1.41	31	0	2.9	2.95
9	2	41.04	1.41	31	0	3	2.96
10	2	37.5	0	28.17	−1.41	3.26	3.11
11	2	37.5	0	33.83	1.41	2.27	2.43
12	2	37.5	0	31	0	3.78	3.42
13	2	37.5	0	31	0	3.26	3.42
14	2	37.5	0	31	0	3.24	3.42

Where, Y is the predicted response; β_0 is the offset term; β_i is the linear effect; β_{ii} is the squared effect, β_{ij} is the interaction effect, X_i and X_j are coded terms for independent variables under study and ε is the error factor.

For two variable systems, the model equation is given below (Eq. 3)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (3)$$

Regression analysis and estimation of the coefficients were performed using Design Expert. Pareto chart of the effect estimates, the three dimensional response surfaces, and contour plots were generated using Statistica software (Statsoft Inc., USA) or Design Expert. The ideal levels and combinations of parameters were identified by optimization functions in the software and experiments were run at these levels for validation of the model.

Results and Discussion

Optimization of Process Parameters for Enhancing Cellulase Production

Trichoderma reesei RUT C30 produced 0.605 U/gds of cellulase activity in the unoptimized basal medium. Screening of most important variables and their optimization was attempted to improve the yield of cellulase under SSF on wheat bran substrate.

Screening of Parameters Affecting Cellulase Production

Eleven variables were screened for determining the parameters with most significant effects on cellulase production. These parameters included environmental variables and medium

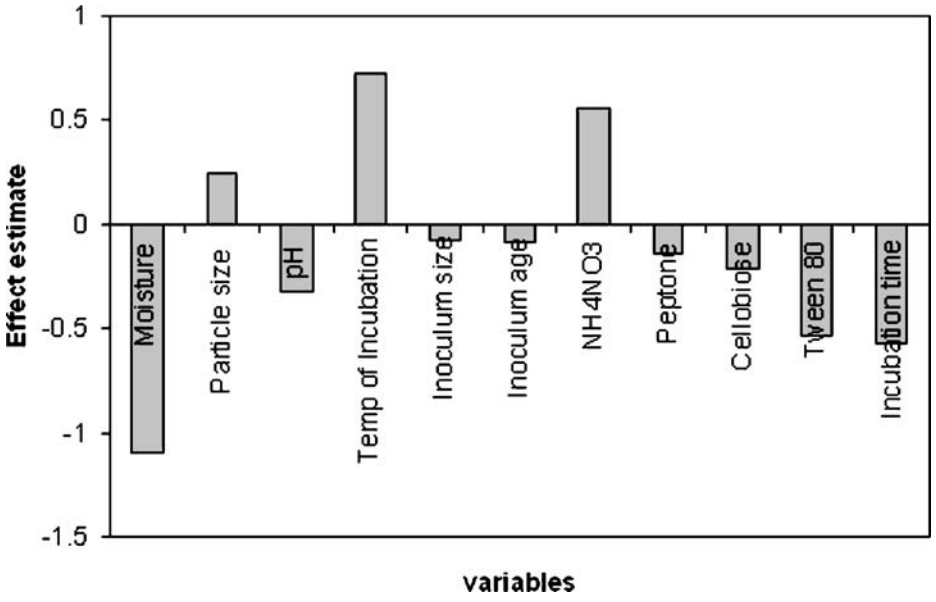

Fig. 1 Effect of independent variables on cellulase production by *T reesei* under SSF on wheat bran based on Plackett and Burman experiments

components as given in Table 2. Plackett and Burman experiments showed a wide range of difference in the cellulase yield from *T reesei*. The minimal and maximal activities obtained were 0.17 and 2.97 U/gds, respectively (Table 1). The main effects of parameters on cellulase production were calculated as ε values as given in the "Materials and Methods" section. The parameters with statistically significant effects were identified using the Fisher's test for ANOVA. Initial moisture content (X_1) and incubation temperature (X_7) had a confidence level higher than 95% ($P>F=0.05$ or lesser). The ε values (effect estimates of individual parameters on cellulase production) are represented in Fig. 1. It can be deduced that the cellulase production increased with increase in incubation temperature and with a decrease in initial moisture content of the medium. Based on these results, initial moisture content and incubation temperature were selected for further optimization to improve cellulase production.

Optimization of the Levels of Significant Parameters Identified by Plackett and Burman Experiments

The two variables which showed a confidence level above 95% in the screening experiment were selected and were optimized further using a central composite design. Based on the results of Plackett and Burman experiment, the levels of media components were set at their middle levels in the CCD with the following exceptions. The inducer cellobiose was eliminated from the medium because it had an insignificant and negative effect. Particle size had a positive effect and considering its role in proper aeration, the higher level was taken. The level of Tween 80 was fixed at its lower level and incubation was performed till 96 h because the effects of both were negative. Table 4 shows the central composite experiment design and the experimental and predicted responses obtained for cellulase production by *T reesei*. The data was analyzed by multiple regression analysis and a second order polynomial equation (Eq. 4) was derived to represent the cellulase production as a function of the independent variables tested.

$$Y = 3.48 + 0.0026X_1 - 0.24X_2 - 0.23(X_1)^2 - 0.32(X_2)^2 - 0.02X_1X_2 \quad (4)$$

Where Y = predicted response (cellulase yield), X_1 and X_2 are coded values of initial moisture content and incubation temperature, respectively.

Table 5 Analysis of variance for the selected quadratic model.

Source	Sum of squares	Degrees of freedom	Mean square	F value	$P>F$
Model	1.5639	5	0.3128	7.26	0.0108
X_1	0.0001	1	0.0001	0.00	0.9719
X_2	0.4608	1	0.4608	10.70	0.0137
$(X_1)^2$	0.3999	1	0.3999	9.28	0.0187
$(X_2)^2$	0.7810	1	0.7810	18.13	0.0038
X_1X_2	0.0016	1	0.0016	0.04	0.8526
Residual	0.3015	7	0.0431		
Lack of fit	0.1058	3	0.0353	0.72	0.5898
Pure error	0.1957	4	0.0489		
Corrected total	1.9100	13			

Coefficient of variation (CV)=6.57%, coefficient of determination (R^2)=0.8384 correlation coefficient (R)= 0.9156, and adjusted R^2 =0.7229.

Testing of the model was performed by the Fisher's statistical test for the ANOVA using Design Expert software and the results are shown in Table 5. ANOVA of the quadratic regression model suggests that the model is significant with a computed F value of 7.26 and a $P>F$ lower than 0.05. The value of multiple correlation coefficient (R) was 0.9156. The closer the value of R to 1, the better is the correlation between the observed and predicted values and the R value obtained indicated a better correlation. A lower value for the coefficient of variation suggests higher reliability of the experiment and in this case the obtained CV value of 6.57% demonstrated a greater reliability of the trials. Table 5 also gives the P values of each of the parameters and their quadratic and interaction terms. The effect estimates of the terms are provided as a Pareto chart in Fig. 2 which clearly shows the significance of incubation temperature, and the quadratic terms of initial moisture content and incubation temperature on cellulase production. The significance of individual variables can be evaluated from their P values, the more significant terms having a lower P value. The values of $P>F$ less than 0.05 indicates that the model terms are significant and this case X_2, $(X_1)^2$, and $(X_2)^2$ were found to be significant model terms (Table 5 and Fig. 2). There was no significant interaction between the parameters.

Response surface curve was plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal cellulase yield. Subpanels A and B of Fig. 3 represent the response surface and the contour plot obtained for the effects of incubation temperature and initial moisture content on cellulase yield, respectively. The shapes of contour plots indicate the nature and extent of the interactions. Prominent interactions are shown by elliptical plots, whereas less prominent or negligible or less prominent interactions are shown by circular contour maps. It is clearly observed from the response surface and contour plot that there is no significant interaction between the tested variables. Regardless of the incubation temperature, the maximal cellulase yield was obtained at an initial moisture content between 37 and 38% and variations in initial moisture content did not affect the temperature optima between 30–31 °C, confirming the lack of interaction between these parameters. The optimal temperature and initial moisture content was within these ranges where the maximal activity of 3.52 U/gds was predicted by the model. Moisture content of the medium in SSF systems is an important parameter which affects the productivity [19]. The moisture levels in SSF processes, which vary between 30 and 85%, has a marked effect on growth kinetics [20]. The optimal moisture content for growth of microorganisms differ with respect to the substrate since the water holding capacity of substrates are different which have significant effects on water

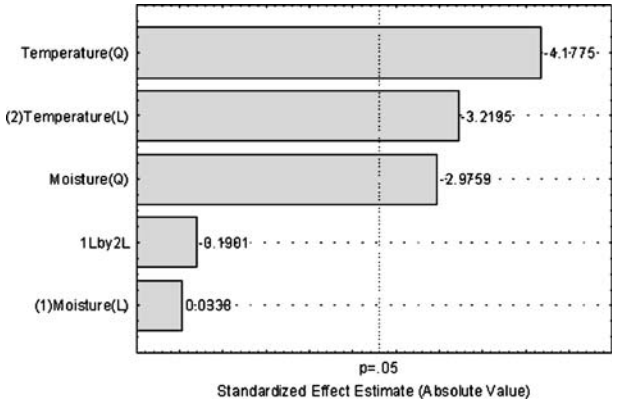

Fig. 2 Pareto chart for the standardized effects of process terms on cellulase production

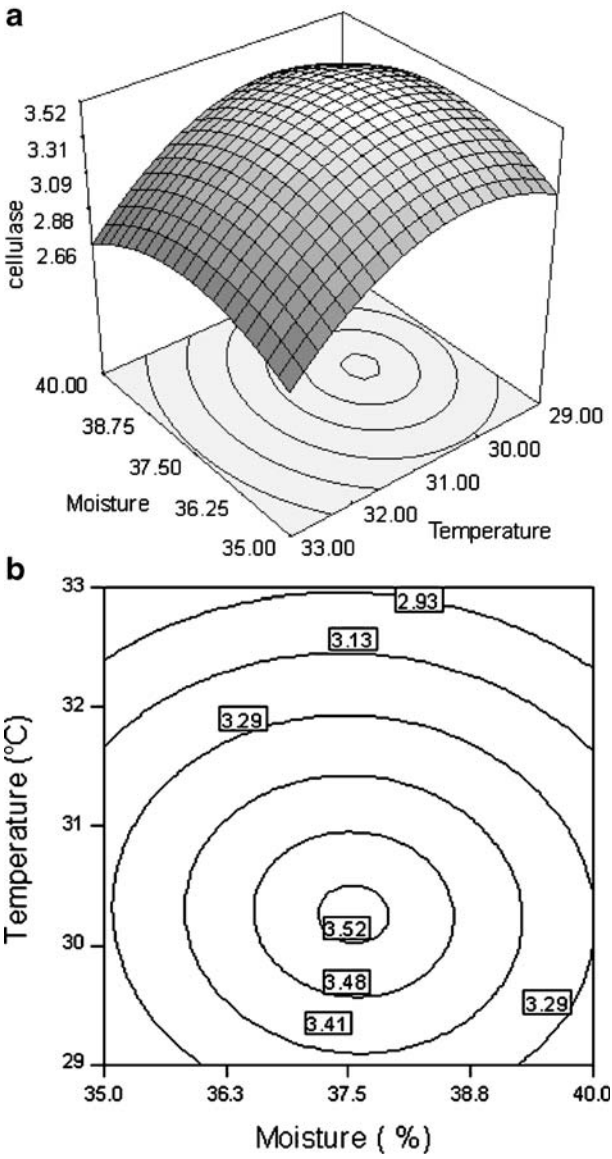

Fig. 3 Three-dimensional response surface (a) and the corresponding contour plot (b) for cellulase production in relation to initial moisture content and incubation temperature

activity—A_w [12]. Wheat bran has a higher water holding capacity compared to the lignocellulosic substrates like sugar cane bagasse and this would account for the lower value of initial moisture content for optimal yield of the enzyme. Effect of incubation temperature is rather an organism-dependent parameter. Though a large number experiments for cellulase production has used an incubation temperature of 28 °C [21, 22], the fungus has been cultivated largely at 30 °C for production of the enzyme [7, 13]. Lower temperatures also have been reported for cellulase production by the fungus [23]. In all these cases, the substrates were different and the effect of substrate cannot be ruled out as an interfering factor in controlling the optimal temperature of the fungus. However, this is

Table 6 Model validation experiments.

Trial No.	Moisture (%)	Temperature (°C)	Cellulase activity (U/gds)	
			Observed	Predicted
1	36	30	3.58	3.49
2	37	30	3.8	3.58
3	37.5	30	3.78	3.59

not proven and will require further studies to consolidate. In the current study, temperature was found to be the parameter with largest significant effect on cellulase production. The regression equation (Eq. 4) was solved using Design Expert and the optimal values for obtaining the highest yield was determined to be an initial moisture content of 37.56% and an incubation temperature of 30 °C. At this combination, the maximum predicted yield was 3.52 U/gds.

Validation of the Model

The adequacy of the model equation (Eq. 4) was validated by performing a total of three verification experiments within the experimental range as given in Table 6. The data of the validation runs were also statistically analyzed to find the correlation between observed and predicted values. The correlation coefficient (R) between experimental and predicted values was found to be 0.9703 indicating that the group of experimental values is in good agreement with those of the predicted demonstrating the accuracy of the model.

Conclusion

To date, though several reports are available which deals with SSF production of cellulases and its optimization, a majority of them deals with the classical approaches where one factor is tested at a time. The identification of important process variables by Plackett and Burman experiments and the optimization of their levels by the central composite design had helped to improve cellulase yield from 0.605 to 3.53 U/gds. Validation trials could yield a maximum of 3.8 U/gds and the overall increase in cellulase was 6.2-fold which is considerably good. The results indicate the application of statistical methods in improving cellulase production and demonstrate the potential of SSF technology in economical production of cellulase from *T. reesei*.

Acknowledgements The authors are thankful to the Council of Scientific and Industrial Research, Government of India, for the research grant on project CMM013 which funded this study.

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