Optimization Studies for Enhancing Cellulase Production by *Penicillium janthinellum* Mutant EU2D-21 Using Response Surface Methodology

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Extracellular fungal cellulases are key enzymes for the degradation of lignocellulosic biomass. Greater production of these enzymes could reduce the cost of biofuels production. In this study, the basal medium for cellulase production by *a Penicillium janthinellum* mutant (EU2D-21) in submerged fermentation conditions was optimized using response surface methodology (RSM). Initial studies using a Plackett-Burman design (PBD) showed that $(NH_4)_2SO_4$ and urea are significant factors for improving β-glucosidase and FPase production. A central composite design (CCD) was applied to obtain the maximum response, which resulted in the optimal production of β-glucosidase (5.79 IU/mL) and FPase (5.76 IU/mL). These values were 1.87 and 1.67 times higher than the corresponding values obtained under un-optimized conditions.

Keywords: P. janthinellum EU2D-21; Cellulase production; Central composite design (CCD); Plackett-Burman design (PBD)

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INTRODUCTION

Biofuels are good substitutes for fossil fuels; they are considered to be carbon neutral because any CO_2 produced during fuel combustion is consumed by subsequent biomass re-growth (Zaldivar *et al*. 2001). Biofuels are produced from lignocellulosic plant biomass, which is a mixture of carbohydrate polymers (cellulose, hemicellulose, and pectin, to varying degrees) and the non-carbohydrate polymer lignin (Asenjo *et al*. 1991; Knauf and Moniruzzaman 2004; Lin and Tanaka 2006). Degradation of biomass may be attributed to the synergistic and complementary action of many enzymes, including cellulase, hemicellulase, and other accessory enzymes (Henrissat *et al*. 1985; Ding *et al*. 2012). Cellulases are the key enzymes required for the degradation of lignocellulosic polysaccharides into simple monomeric sugars, which can be converted through microbial fermentation processes to biofuels or other value-added products. The development of large-scale biomass (inedible parts of plants, grasses, and forest waste) conversion processes would alleviate shortages of food (including animal feeds) and solve waste disposal problems (Singhvi and Gokhale 2013).

Cellulose is degraded by the synergistic action of three types of enzymes in cellulase (glycoside hydrolases): exo-1,4-β-D-glucanase (EC 3.2.1.91), endo-1,4-β-D-glucanase (EC 3.2.1.4), and β-glucosidase (EC 3.2.1.21). Among fungi, *Trichoderma* and *Aspergillus* have been extensively studied, particularly due to their ability to secrete cellulose-degrading enzymes. The hyper-production of β-glucosidase and β-xylosidase by

Aspergillus niger NCIM 1207 isolated in a laboratory has been reported (Khisti *et al*. 2011). Cellulase production by *Penicillium decumbens* (Wang *et al.* 2013a) and its application in cellulose hydrolysis (Wang *et al.* 2013b) has been reported. However, the search for efficient and better sources of cellulase continues, which may result in a lower cost of enzymes. Unfortunately, the conversion of cellulose to glucose is not yet commercially feasible, and efforts are needed to produce cellulases that can be used to hydrolyze biomass at an affordable cost.

Penicillium janthinellum NCIM 1171 has been identified as a cellulase producer using bagasse as carbon source (Adsul *et al*. 2004), and its application in hydrolysis has been studied (Adsul *et al*. 2005). Mutants of *P. janthinellum* NCIM 1171 that are capable of producing enhanced levels of cellulases have been isolated (Adsul *et al*. 2007). One of the mutants, *P. janthinellum* EU2D-21, produced high levels of endoglucanase and β-glucosidase in solid-state fermentation. This enzyme preparation is also known to be stable in ionic liquids (Adsul *et al.* 2009). In addition, the superior performance of cellulase preparations produced by *Penicillium* species over *Trichoderma* enzymes has been well documented (Gusakov 2011).

Studies that optimize the bioprocess are essential for the development of economically feasible bio-based products. Cellulase production is influenced by medium components, especially carbon and nitrogen sources, and physical variables such as pH, temperature, inoculum density, and incubation time. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions. It has been successfully used in the optimization of bioprocesses (Wejse *et al*. 2003; Hao *et al*. 2006). RSM provides mathematical models that show the dependence of enzyme production on controlling/significant factors and helps to determine their optimum levels for maximum production. In this study, we initially employed a Plackett-Burman design (PBD) of experiments for identifying significant medium components involved in cellulase production. This was followed by a central composite design (CCD) to find optimal values of the significant factors identified by PBD for enhancing enzyme production using *Penicillium janthinellum* EU2D-21. The overall strategy led to a significant enhancement in enzyme production that should prove to be very useful.

EXPERIMENTAL

Chemicals

Cellulose powder, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), and 3,5-dinitro-salysilic acid were obtained from Sigma-Aldrich Co., St. Louis, USA. Yeast extract and peptone were obtained from Hi-Media Laboratories Pvt. Limited, Mumbai, India. All other chemicals were of analytical grade and were obtained locally.

Microbial Strains and Enzyme Production

P. janthinellum EU2D-21 was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The strain was maintained on potato dextrose agar (PDA) and sub-cultured once every 3 months. The fermentation basal medium consisted of (g/L) KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, yeast extract 0.1 g, bacto peptone 0.25 g, $MgSO_4.7H_2O$ 0.3 g,

$FeSO_4.7H_2O$ 0.005 g, $MnSO_4.7H_2O$ 0.0016 g, $ZnSO_4.7H_2O$ 0.0014 g, $CoCl_2$ 0.002 g, and polysorbate 80 (Tween® 80) 0.1%. The pH of the fermentation medium was adjusted to 5.2. Submerged fermentation (SmF) was carried out in 250-mL Erlenmeyer flasks with 70 mL of fermentation medium containing 1% (w/v) cellulose powder and 2.5% (w/v) wheat bran (Adsul *et al*. 2007). Shake flask experiments were carried out in 250-mL Erlenmeyer flasks with 70 mL of fermentation medium containing the appropriate amount of substrate. The flasks were inoculated with spores (approximately 10^7) from 15-day-old culture grown on PDA slants and incubated at 30 °C with shaking at 180 rpm. The samples were removed at various time intervals and centrifuged at 3000 rpm for 10 min. The supernatant was analyzed for extracellular enzyme activities. Filter paper cellulase (FPase) and β-glucosidase activities were determined as previously reported (Adsul *et al.* 2007). FPase was assayed by incubating the suitably diluted enzyme (0.1 mL) with 1.9 mL of citrate buffer (50 mM, pH 4.5) containing Whatman No. 1 filter paper (50 mg, 2 to 6 cm). The reaction mixture was incubated at 50 °C for 60 min. The β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) activity was estimated using *p*NPG as substrate. The total assay mixture (1 mL), consisting of 0.9 mL of pNPG (1 mg/mL) and 0.1 mL of suitably diluted enzyme, was incubated at 50 °C for 30 min. The liberated *p*-nitrophenol was measured at 410 nm after developing the color with 2 mL of sodium carbonate (2%). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose, *p*-nitrophenol produced from the appropriate substrates / min / mL of crude filtrate under the assay conditions.

Optimization Using Response Surface Methodology

Plackett-Burman design (PBD) of experiments

The PBD is a very useful and widely employed statistical technique for identifying the major independent variables or factors that have a significant effect on a particular response (Plackett and Burman 1946). According to PBD, for *n* factors to be assessed, the total number of experiments to be carried out is $n + 1$, where $n + 1$ is usually a multiple of four. In the present study, a careful selection of ten factors (Table 1)

Table 1. Screening of Variables for Cellulase Production by Plackett-Burman Design

Factor Code	Factor Name	Unit	High (+1)	Low (-1)
A	KH_2PO_4	g/L	3.000	1.000
B	$CaCl_2$	g/L	0.450	0.150
C	Urea	g/L	0.450	0.150
D	$(NH_4)_2SO_4$	g/L	2.100	0.700
E	Yeast extract	g/L	0.150	0.050
F	Peptone	g/L	0.450	0.150
G	$MgSO_4$	g/L	0.450	0.150
H	Salts*	g/L	0.015[#]	0.005[$]
J	Polysorbate 80	%	0.150	0.050
K	Incubation time	Days	6	4

* Contains $FeSO_4$, $MnSo_4$, $ZnSO_4$, $CoCl_2$,
[#] respective amount 0.0075 g/L, 0.0024 g/L, 0.0021 g/L, 0.0030 g/L
[$] respective amount 0.0025 g/L, 0.008 g/L, 0.0007 g/L, 0.0010 g/L

for carrying out screening studies using the PBD was made, with the eleventh factor kept as a dummy. This approach has known advantages for optimization by the introduction of a degree of freedom for maneuvering through the design space.

Thus, a total of 12 experiments in the PBD were planned. Each independent variable was investigated at two levels, a high level (+1) and a low level (-1). The experimental design was constructed using Design-Expert Software (DES) (V7.1.6, Stat-Ease, Minneapolis, MN, USA). Based on an analysis of the PBD results, two of the chosen ten factors were identified to be significant in the range of levels employed with respect to the chosen factors. All experimental runs were carried out in triplicate, and the average β-glucosidase and FPase activities were taken as the corresponding response values.

Central composite design (CCD) of experiments

Central composite design (CCD) was used to study the optimization of two significant factors obtained by PBD, *viz.*, $(NH_4)_2SO_4$ and urea. The respective minimum and maximum ranges were chosen and investigated (Table 3). CCD is a 2^k factorial design in which the total number of experimental design trials is $N = 2^k + 2k + n_0 = 13$, where $k = 2$ is the number of factors, and $n_0 = 5$ is the chosen number of replicates at center points. The CCD design matrix is presented in Table 3. To determine the relationship between factors and responses, the experimentally evaluated response data was statistically analyzed using ANOVA and regressed with a general quadratic model. Thirteen experimental runs were carried out at 30 °C, and the β-glucosidase and FPase enzyme activities were monitored after 8 days of incubation.

RESULTS AND DISCUSSION

Screening of Variables for β-glucosidase and FPase Production by PBD

For screening purposes, the chosen ten factors were evaluated using the PBD of experiments. Interestingly, the results suggested with a high level of confidence that only two variables, namely $(NH_4)_2SO_4$ and urea, were significant in the production of both β-glucosidase and FPase (Table 2).

In the case of β-glucosidase, a best fit linear response model (as analysed with DES) in terms of the actual factors $(NH_4)_2SO_4$ and urea, denoted by A and B in g/L, respectively, were obtained as:

$$[\beta\text{-glucosidase}] = +1.87925 - 1.06369 * A + 2.72722 * B \quad (1)$$

This model satisfied all of the statistical tests by ANOVA. Thus, a model F-value of 9.96 implied that the model is significant and suggested that there is only a 0.52% chance that a model F-value this large could occur due to noise. The values of Prob > F were less than 0.05 for both urea and $(NH_4)_2SO_4$. The ANOVA statistics, calculated using DES, also reports other statistical measures for model acceptability, *viz.,* a) coefficient of determination R^2, representing the amount of model variation around the mean, b) Adj-R^2, the amount of variation around the mean and adjusted for the number of terms in the model, and c) Pred- R^2, the amount of variation in new data explained by the model. All these model acceptability measures are seen to be satisfied in the present study, with the

coefficients of determination R^2 = 0.69 with Adj-R^2 = 0.61 and the Pred-R^2 = 0.45 lying within 0.16 agreement of each other.

Similarly, in the case of FPase, a best fit response model in terms of actual factors was obtained as:

$$[\text{FPase}] = 3.06583 - 0.36310 * A + 2.93889 * B \qquad (2)$$

The above model was also found to satisfy all of the statistical tests. Thus, a model F-value of 16.76 implied that the model is significant and suggested that there is only a 0.09% chance that a model F-value this large could occur due to noise. The values of Prob > F were less than 0.05 for both $(NH_4)_2SO_4$ and urea. The coefficient R^2 is 0.79 with the Pred-R^2 of 0.62 in agreement with Adj-R^2 of 0.7. It should also be pointed out that the signs of the coefficients in Equations 1 and 2 for both β-glucosidase and FPase show that $(NH_4)_2SO_4$ has a negative effect, while urea has a positive effect, in the ranges of the levels employed. For the reasons stated above, $(NH_4)_2SO_4$ and urea were selected as factors for further optimization studies with CCD to further improve the maximum response values.

Table 2. PBD Design Matrix and Evaluated β-glucosidase and FPase Activities

Run	A	B	C	D	E	F	G	H	J	K	β-glucosidase IU/mL	FPase IU/mL
1	-1	1	-1	1	1	-1	1	1	1	-1	0.004	2.51
2	1	1	-1	1	1	1	-1	-1	-1	1	0.216	3.16
3	-1	-1	-1	1	-1	1	1	-1	1	1	0.042	2.68
4	1	1	-1	-1	-1	1	-1	1	1	-1	1.737	3.31
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.013	2.82
6	-1	1	1	-1	1	1	1	-1	-1	-1	1.024	4.05
7	1	-1	-1	-1	1	-1	1	1	-1	1	1.783	3.49
8	-1	1	1	1	-1	-1	-1	1	-1	1	1.203	3.73
9	1	-1	-1	1	-1	1	1	1	-1	-1	1.152	3.82
10	1	-1	1	1	1	-1	-1	-1	1	-1	0.165	3.19
11	**-1**	**-1**	**1**	**-1**	**1**	**1**	**-1**	**1**	**1**	**1**	**3.090**	**4.43**
12	1	1	1	-1	-1	-1	1	-1	1	1	3.070	4.06

Optimization of β-glucosidase and FPase Production by CCD

The generated experimental design with 13 runs for the chosen two factors, $(NH_4)_2SO_4$ and urea, as well as enzyme activities, is shown in Table 3. The low and high level values employed in the CCD for $(NH_4)_2SO_4$ were chosen to be 0.5 and 1.0, respectively. The range was shifted to the left when compared to PBD because of the

negative effect of $(NH_4)_2SO_4$ in the PBD models in Eqs. 1 and 2. On the other hand, because of the positive effect shown by urea in the PBD models (Eqs. 1 and 2), its low and high range were shifted to the right and chosen to be 0.3 and 0.6, respectively. The levels of the other factors except incubation time were kept at the median levels considered in the PBD. The incubation time was chosen to be 8 days because it was observed in a time course experiment that the highest enzyme production activity was obtained on the eighth day. Experiments based on the CCD design were carried out, and the observed responses were analyzed by ANOVA to obtain the best fit models for β-glucosidase and FPase production.

In the case of β-glucosidase, the best fit response model in terms of actual factors was obtained as a quadratic model with two factor interactions:

$$[\beta\text{-glucosidase}] = -15.94102 + 61.48088 * A - 5.48616 * B - 37.02848 * A * B - 49.97982 * A^2 + 64.98687 * B^2 + 67.47071 * A^2 * B - 86.27138 * A * B^2 \quad (3)$$

Table 3. CCD of Experiments with Evaluated β-glucosidase and FPase Activities

Run	$(NH_4)_2SO_4$ g/L	Urea g/L	β-glucosidase IU/mL	FPase IU/mL	Final pH
1	1.00	0.60	2.77	5.06	4.05
2	0.75	0.45	4.35	4.27	4.56
3	0.75	0.66	5.39	5.30	5.39
4	1.10	0.45	1.14	4.56	3.68
5	0.40	0.45	2.61	4.29	6.09
6	0.75	0.45	4.14	4.60	4.52
7	0.75	0.45	4.20	4.60	4.62
8	0.75	0.45	4.14	4.52	4.55
9	0.75	0.24	3.29	4.18	4.38
10	0.75	0.45	4.30	4.60	4.58
11	**0.50**	**0.60**	**5.79**	**5.76**	**6.23**
12	1.00	0.30	1.03	3.91	3.78
13	0.50	0.30	2.03	2.70	5.43

Thus, the obtained F-value of 160.34 implied that the above model is significant and suggested that there is only a 0.01% chance that a model F-value this large could occur due to noise. The values of Prob > F were less than 0.05 for both urea and $(NH_4)_2SO_4$. The coefficient of determination R^2 value is 0.99 with the Pred-R^2 of 0.80. This is in reasonable agreement with the Adj-R^2 of 0.99. These tests imply that the predicted responses using the model (Eq. 3) and those of the actual responses fit

exceedingly well (Fig. 1), and the model was used to predict the activities in the chosen ranges of the key factors.

In the case of FPase, the best fit response model in terms of actual factors was again obtained as a quadratic model involving two factor interactions:

$$[FPase] = -19.45992 + 55.91137 * A + 49.39108 * B - 117.77657 * A * B - 33.15697 * A + 2.43333 * B^2 + 70.02882 * A * B \quad (4)$$

Here, the obtained F-value of 33.39 implied that the above model is significant and suggested that there is only a 0.02% chance that a model F-value this large could occur due to noise. The values of Prob > F were less than 0.05 for both urea and $(NH_4)_2SO_4$ with the coefficient of determination R^2 value equal to 0.97. Here also the Pred-R^2=0.97 is in reasonable agreement with the Adj-R^2=0.56 and suggests that the model (Eq. 4) is significant (Fig. 2).

Fig. 1. Model-predicted (Eq. 3) *vs.* actual response of β-glucosidase activity for the 13 CCD experiments (IU/mL)

Fig. 2. Model-predicted (Eq. 4) *vs.* actual response values of FPase activity (IU/mL) for the thirteen CCD experiments

The maximum activity for β-glucosidase (5.79 IU/mL) and FPase (5.76 IU/mL) was attained in run 11 (Table 3); the maximum values were 1.87 and 1.67 times higher, respectively, than those initially reported for the basal medium.

The 3-D plots (Figs. 3 and 4) of activity *vs.* $(NH_4)_2SO_4$, and urea clearly show the significance of the quadratic and interaction terms in the model equations (3 and 4) by displaying the nonlinearity that arises due to the complex nature of the interactions that exist among the two variables. The 3-D surface curves of calculated responses for both β-glucosidase and FPase clearly show that the maximum activity was achieved at lower levels for $(NH_4)_2SO_4$ and higher levels for urea.

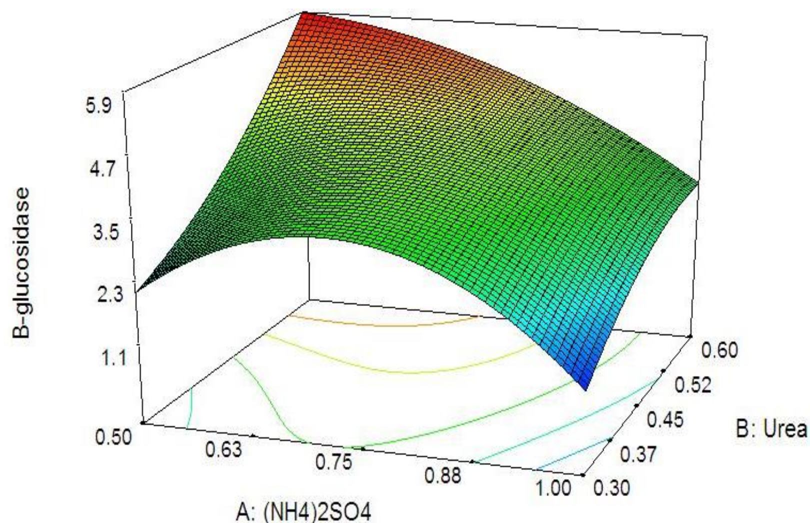

Fig. 3. CCD model (Eq. 3) analysis shows the negative effect of $(NH_4)_2SO_4$ and positive effect of urea on β-glucosidase production (IU/mL).

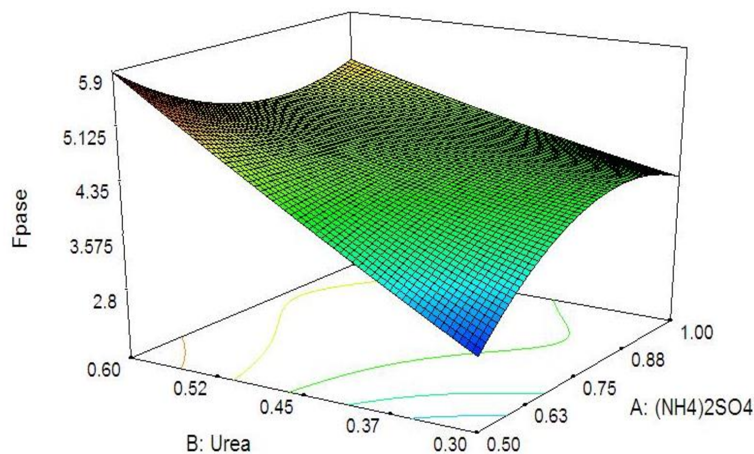

Fig. 4. CCD model (Eq. 4) analysis displays the negative effect of $(NH_4)_2SO_4$ and positive effect of urea on FPase production (IU/mL).

CONCLUSIONS

1. The cost of cellulase plays a crucial role in economics of cellulosic biofuels and this mainly arises due to the high enzyme loads required for hydrolysis of lignocellulosic materials. Cellulase cost can be reduced by optimizing the media conditions for its production; this study has shown the possible improvement by using the combined statistical strategy of PBD and CCD.
2. The results of the screening of 10 variables using PBD experiments suggested the significance of two variables, namely, $(NH_4)_2SO_4$ and urea which contributed to improvements in both β-glucosidase and FPase production. The further optimization studies with CCD using selected two factors resulted in further enhancement in enzyme activities.
3. Exploration of nutrient components in fermentation media by RSM studies bring out the observation that nitrogen sources such as $(NH_4)_2SO_4$ and urea are the main factors for enhancing the production of β-glucosidase and FPase.

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