Statistical Optimization of Fermentation Parameters for Cellulase Production Utilizing Banana Peel

Modhuleena Mandal[1] and Uma Ghosh[1*]

[1]*Department of Food Technology and Biochemical Engineering, Jadavpur University, Kolkata -700032, India.*

Authors' contributions

This work was carried out in collaboration between both authors. Authors MM and UG designed the study. Author MM performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors UG and MM managed the analyses of the study. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: To optimize the physical parameters of solid state fermentation (SSF) by implementing statistical tool for production of cellulase, one of the potential biocatalyst in industries.
Study Design: Fermentation was carried out by the employment of response surface methodology (RSM) based on the Box-Behnken design (BBD) available in software Design-Expert (Version 7) Stat-Ease, Inc.
Place and Duration of Study: Department of Food Technology and Biochemical Engineering, Jadavpur University, Kolkata, India, between June 2014 and December 2014.
Methodology: The optimization of environmental factors for production of cellulase (FPase) was carried out by response surface methodology (RSM) based on the Box-Behnken design (BBD). The design included a total of 29 experimental trials that comprised time and temperature of fermentation, amount of substrate and hydration ratio as model factors for three levels.
Results: The mutual interaction between the independent variables under optimized conditions yielded FPase at the level of 8.05 U/ gds and total dissolved protein at the level of 1.4 mg/ml which

_____

Corresponding author: E-mail: ughoshftbe@yahoo.co.in;

were close to the predicted values from the model (7.95 U/gds and 1.39 mg/ml respectively for FPase and protein). The ideal parameters for cellulase production by *Aspergillus* sp. were 25% of hydration, 33ºC, 3.1 g of substrate and 7 days of fermentation.

Conclusion: A good correspondence between the predicted values from the model and the experimental values were seen. Consequently, we can use this methodology to adequately describe the interaction effect between the important independent variables influencing the fermentation process and the responses.

1. INTRODUCTION

The cellulases are classified under the glycoside hydrolase (GH) families [1] that catalyzes the hydrolysis of 1, 4 β-D glycosidic linkages in cellulose and are chiefly produced by fungi and bacteria and is reported to have a broad range of industrial and commercial applications [2-4]. Fungi are significant organisms for degradation of plant material in nature, they accomplish this degradation by means of secreted enzymes that are usually stable under environmental conditions. Fungal cellulases are deployed in biorefineries for conversion of biomass to fermentable sugars. The enzyme has wide range of potential applications in food biotechnology as well. Cellulases plays an imperative part of macerating enzyme complex which improves the quality and performance of food products without additional capital investment [5]. Fungal cellulases are produced either in submerged fermentation or in solid state fermentation. Submerged fermentation is amenable to high levels of process control and scrutinizing, so the method can be complex, on the other hand solid state fermentation (SSF) can be economic, simple and less energy intensive. In SSF, the chances of contamination is also low as the hyphal growth of the filamentous fungi can access the cellulose within the substrate in the absence of free water, this property of fungi gives them an edge over bacteria which can also serve as cellulase producer. Multifactorial system or one-factor at a time is the traditional technique for the optimization of process parameters, this type of method is time intense and does not represent the interactive effects between components [6]. In the present study, an attempt was made to identify the optimal conditions for cellulase production under SSF by employing response surface methodology (RSM) which is an influential mathematical approach. The optimization is achieved by analyzing the relationships among a number of parameters that influence the process as a whole. Besides that, the article focuses on meaningful utilization of horticultural waste, banana peel that can be biotechnologically converted to value added products which can reduce environmental pollution to a great extent. As banana peel is reported to be rich in nutrients which supports growth of cellulase producer [7] it can be extensively used as substrate for SSF.

2. MATERIALS AND METHODS

2.1 Substrate and Chemicals

Whatman No1 filter paper with particle retention 11 µm was obtained from Sigma Aldrich. 3, 5-Dinitrosalicylic acid and Folin-Ciocalteu were procured from Merck. Ripe banana peels were collected from local sources for solid state fermentation.

2.2 Fungal Strain

Aspergillus sp. isolated from soil obtained from saw mill was maintained at 4ºC on PDA (Potato Dextrose Agar).

2.3 Pre-treatment of Substrate

Ripe banana peels (Yellow variety) were dried at 70ºC for 5 h and were ground to fine powder and was passed through sieve of size 1.1 mm.

2.4 Solid State Fermentation

SSF was carried out in 100 ml Erlenmeyer flasks containing banana peel as substrate moistened with distilled water (w/v). The flasks were sterilized by autoclaving at 15 psi for 15 min, and cooled to room temperature and inoculated with 5 ml of spore suspension (10^9 spores/ml). The contents of the flasks were mixed well with sterilized glass rod to distribute the inoculum throughout the substrate and incubated at desired temperature, according to the runs provided by the software Design Expert version 7. The fermentation factors and levels studied are given in Table 1.

2.5 Extraction of Crude Enzyme

After fermentation, the crude enzyme was extracted from the solid medium by mixing the medium with (1:10 w/v) distilled water and agitated on a rotary shaker at 140 rpm at 30°C for 1 h. Dampened cheese cloth was used to filter the extract and extracts were then centrifuged at 8000 rpm for 5 min at 4°C [8]. The clear supernatant was used as a source of crude extracellular enzyme [9].

2.6 Assay of Cellulase

The cellulase (FPase) activity was determined by the method of Mandels and Weber [10]. The reducing sugar liberated was estimated spectrophotometrically at 540 nm after addition of DNS. One Filter paper unit (FPU) is defined as amount of enzyme in the filtrate releasing 1 µmol of reducing sugar from filter paper/ml/min [11]. The enzyme activities were expressed as U/gds (i.e. Unit per gram dry substrate). Dry weight of the samples were determined by drying them in a hot air oven at 60°C to a constant weight.

2.7 Protein Estimation

Culture filtrates were used for extracellular protein estimation according to the method of Lowry [12]. Bovine serum albumin (BSA) was used as standard.

2.8 Statistical Design for Optimization of Cellulase Production (Box-Behnken Design)

RSM is a collection of statistical techniques for designing an experiment, evaluating the effects of factors and searching for optimal conditions for desirable responses [13]. For optimization of process parameters, *Aspergillus* sp. isolated from soil sample was used in this study. The statistical tool was implemented to optimize the most important variables of fermentation to maximize cellulase production following Box-Behnken design. Four vital parameters viz. time (A), temperature (B), substrate amount (C) and hydration ratio (D) were chosen as the independent variables and FPase activity (Y_1), expressed in U/gds and extracellular protein content (Y_2), expressed as mg/ml were the dependent response variables. Three different levels were studied for each independent variable including hydration at 25, 50 and 75%,

substrate amount 3, 5, 7 g, incubation temperatures at 23, 30 and 37°C, and incubation time for 3, 5 and 7 days as shown in Table 1. Total 29 experiments were conducted for four independent variables. All the experiments were done in triplicate and average FPase activity and protein obtained were taken as dependent variables. The following second-order polynomial equation was adopted to study the effects of independent variables on the responses (Eq. 1).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{44}D^2 + \beta_{12}AB + \beta_{13}AC + \beta_{14}AD + \beta_{23}BC + \beta_{24}BD + \beta_{34}CD \quad (1)$$

Where *Y* is the response (FPase activity and extracellular protein) β_0 is the constant term; β_1, β_2 and β_3 are the coefficient of linear terms; β_{11}, β_{22} and β_{33} and so on are the coefficient of quadratic terms; and β_{12}, β_{13} and β_{23} are the coefficient of cross product terms, respectively. Design expert statistical software (version 7) was used for RSM analysis of experimental data. ANOVA was used to establish the significance of the model parameters.

3. RESULTS AND DISCUSSION

3.1 Optimization of Physical Parameters for Cellulase Production by Box Behnken Design

Response surface methodology has been successfully applied in the optimization of fermentation medium components and fermentation processes. It allows the calculation of maximum enzyme production based on sets of experiments in which all the factors are varied within selected range and is also used to study interactive effects of a variety of process parameters [14]. RSM could infer and analyse the combined influence of the parameters affecting production efficiency, and furthermore predict the optimal enzyme producing parameters [15]. The software Design Expert (version 7) used for RSM applied second order model to optimize the responses. In order to check the prospective of the isolated *Aspergillus* sp. to synthesize cellulase for biotechnological applications, and to optimize the environmental parameters for fermentation, determination of cumulative effect of multiple parameters regulating the rate of enzyme production have become warranted. RSM is more satisfactory than classical one-factor-at-a-time (OFAT) models because it can study several variables concurrently with low number of observations,

causing reduction in time and costs [16]. The data were analyzed by multiple regression analysis and the regression coefficients for equations were determined. The suitability and adequacy of the model were judged by the coefficient of determination (R^2). The R^2 which can also be called multiple correlation coefficient can be defined as the ratio of the explained variation to the total variation and was determined by degree of fit. The closer the R^2 value to unity, better the correlation between the observed and predicted values [13].

Equation in Terms of Coded Factors:

$$FPase\ (Y1) = +4.06 - 0,033 \times A + 0.20 \times B - 0.075 \times C - 1.96 \times D + 0.10 \times AB - 0.10 \times AC - 0.32 \times AD - 0.84 \times BC + 0.45 \times BD + 0.21 \times CD - 0.57 \times A^2 - 0.18 \times B^2 + 0.040 \times C^2 + 1.21 \times D^2 \tag{2}$$

Equation in Terms of Coded Factors:

$$Protein(Y2) = +0.76 + 0.0910 \times A - 0.14 \times B - 0.039 \times C + 0.062 \times D - 0.70 \times AB - 0.21 \times AC - 0.075 \times AD - 0.098 \times BC - 0.13 \times BD + 0.34 \times CD - 0.11 \times A^2 - 0.14 \times B^2 + 0.065 \times C^2 + 0.19 \times D^2 \tag{3}$$

Here (Y_1) and (Y_2) are the predicted responses (cellulase activity and extracellular protein content respectively). A, B, C, D are coded factors viz. incubation time, temperature, substrate amount and hydration ratio respectively. Tables 2 and 3 shows the results which were tested by the Fisher's statistical test for the analysis of variance (ANOVA) using Design Expert software. The coefficients of determination, R^2, from the Eq. 2 and 3 were found to be 0.99 and 0.99 for the regressed models predicting the FPase activity and protein content, respectively. The values suggests a good fit for the model. The significance of each coefficient was determined by using the F-test and p-value (Tables 2 and 3). The F value 1310.82 for response 1 i.e FPase implies that the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. For response 2 (protein content) the F-value was found to be 3724.91 which implied that the model is significant (p<0.05).There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The lack of fit measured the failure of the model to represent data in the experimental domain at points which were not included in the regression. The value of "lack of fit F value" were 2.03 and 1.96 for cellulase and protein respectively and it revealed that the quadratic model is statistically significant for the responses. The contour plots obtained from the model shows the variation with respect to simultaneous change in two variables. For maximum yield of cellulase, selection of appropriate fermentation parameters is desirable and contour plots based on well fitted model provides these options [17]. Less prominent interactions are generally shown by circular nature of the contour plots whereas prominent interactions are shown by elliptical nature of contour plots. For the interaction terms to be significant atleast one parent term needs to be significant. For FPase the parent terms B, C, D and the interaction terms AB, AC, AD, BC, BD, CD were found to be significant. 3D plots given in (Figs. 1a-f) shows the interactive effect of independent variables selected. Highest cellulase production was achieved at moisture level around 25% (w/v), with 3.1 g of solid substrate and maintaining temperature around 33℃. On the other hand for protein AB, AC, AD, BC, BD, CD interaction terms were found to be significant (3D plots given Figs. 2a-f). The twisted nature of the 3D plots determines that there is significant interaction between the chosen independent variables and the responses.

3.2 Rationale of the Model

Repeat fermentation was carried out according to the optimal conditions for cellulase and protein production by RSM analysis to test the accuracy of the model. The optimal conditions were found as hydration ratio of 25.37%, substrate amount 3.1 g, temperature 33.3ºC, and incubation time of 6.77 days under SSF (Table 4), but these conditions are difficult to exercise in reality, and some deviations were expected. Therefore, optimal conditions were targeted as hydration ratio of 25%, substrate amount 3.1 g, temperature 33ºC and incubation time of 7days under SSF. As a result FPase and protein produced by *Aspergillus* sp. under targeted optimal conditions were 8.05 U/gds which was found to be higher than Hoa et al. [13] on the other hand protein content was found to be 1.4 mg/ml. The obtained data were not significantly different from predicted cellulase activities and protein content (7.95 (U/gds) and 1.39 (mg/ml), respectively meaning that the realistic fermentation condition above is acceptable and can be applied to obtain the highest cellulase activitiy under the same condition.

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

Variable	Code	Unit	Range and level		
			-1	0	+1
Time	A	Days	3	5	7
Temperature	B	Degrees	23	30	37
Substrate amount	C	Grams	3	5	7
Hydration ratio	D	Percentage	25	50	75

Table 2. ANOVA for response surface quadratic model (FPase)

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P –value Prob>F	
Model	66.25	14	4.73	1310.82	<0.0001	Significant
A-time	0.01	1	0.01	3.69	0.0752	
B-temperature	0.50	1	0.50	138.56	<0.0001	
C-substrate amt	0.068	1	0.068	18.70	0.0007	
D-hydration ratio	46.22	1	46.22	12802.04	<0.0001	
AB	0.04	1	0.04	11.08	0.0050	
AC	0.04	1	0.04	11.08	0.0050	
AD	0.42	1	0.42	117.03	<0.0001	
BC	2.81	1	2.81	777.16	<0.0001	
BD	0.81	1	0.81	224.37	<0.0001	
CD	0.18	1	0.18	50.03	<0.0001	
A^2	2.12	1	2.12	587.18	<0.0001	
B^2	0.21	1	0.21	56.87	<0.0001	
C^2	1.06	1	1.06	292.29	<0.0001	
D^2	9.49	1	9.49	2628.81	<0.0001	
Residual	0.05	14	3.610E-003			
Lack of fit	0.01	10	1.854E-003			
Pure error	0.03	4	8.000E-003	0.23	0.9723	Not significant
Cor total	66.30	28	66.30			

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

Table 3. ANOVA for response surface quadratic model (Extracellular protein)

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P –value Prob>F	
Model	1.76	14	0.13	3724.91	<0.0001	Significant
A-time	0.09	1	0.09	2938.76	<0.0001	
B-temperature	0.25	1	0.25	7317.60	<0.0001	
C-substrate amt	0.18	1	0.18	546.40	<0.0001	
D-hydration ratio	0.04	1	0.04	1354.49	<0.0001	
AB	0.02	1	0.02	581.77	<0.0001	
AC	0.17	1	0.17	5111.9	<0.0001	
AD	0.02	1	0.02	667.84	<0.0001	
BC	0.03	1	0.03	1128.66	<0.0001	
BD	0.07	1	0.07	2084.42	<0.0001	
CD	0.47	1	0.47	13927.5	<0.0001	
A^2	0.07	1	0.07	2214.61	<0.0001	
B^2	0.13	1	0.13	3827.72	<0.0001	
C^2	0.02	1	0.02	807.20	<0.0001	
D^2	0.23	1	0.23	6687.43	<0.0001	
Residual	4.717E-004	14	3.369E-005			
Lack of fit	3.917E-004	10	3.917E-005	1.96	0.2703	Not significant
Pure error	8.000E-005	4	2.000E-005			
Cor total	1.76	28				

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

(a)

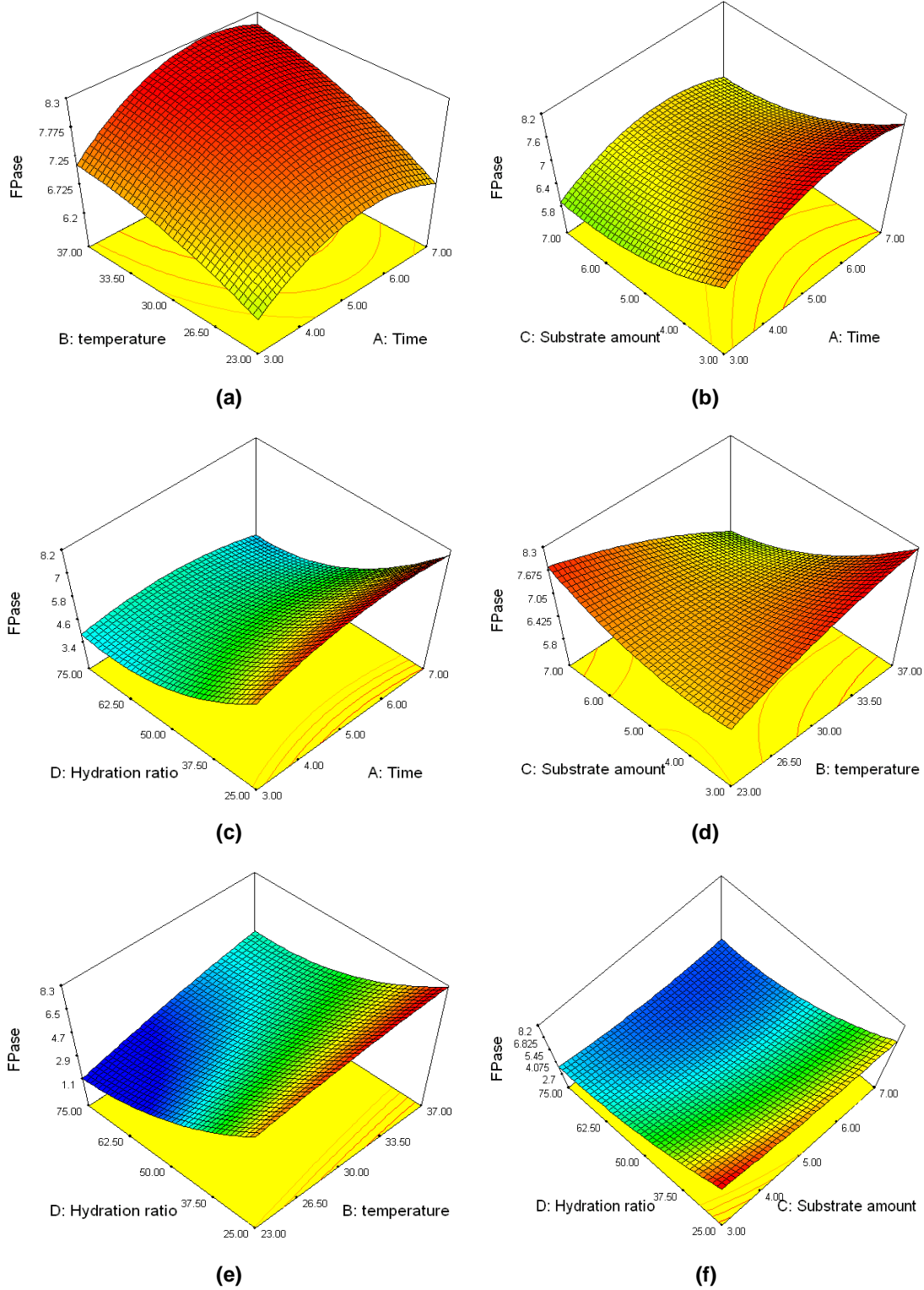**(b)**

(c)

(d)

(e)

(f)

Fig. 1. (a-f)-3D plots of significant interactions AB, AD, AC, BC, BD, CD for FPase production

(a)

(b)
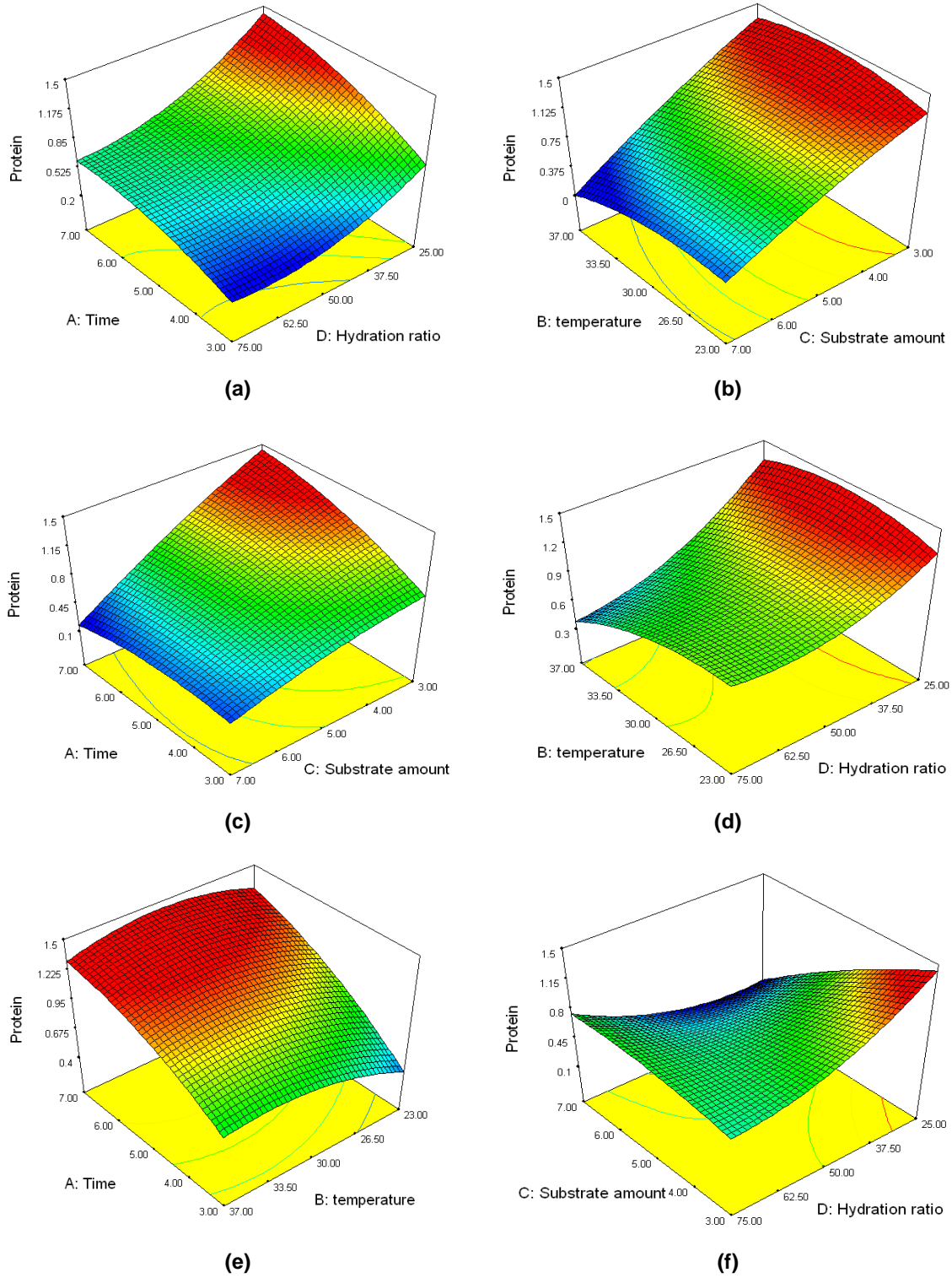
(c)

(d)

(e)

(f)

Fig. 2. (a-f) 3D plots of interaction terms AB, AC, AD, BC, BD, CD for extracellular protein estimated

Table 4. Rationale of RSM

Factor	Name	Level	Low level	High level	Std. dev.	Coding	
A	Time	6.77	3.00	7.00	0.000	Actual	
B	Temperature	33.37	23.00	37.00	0.000	Actual	
C	Substrate amount	3.10	3.00	7.00	0.000	Actual	
D	Hydration ratio	25.37	25.00	75.00	0.000	Actual	
Response	Prediction	SE mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
FPase	7.95867	0.064	7.82	8.10	0.088	7.77	8.15
Protein	1.39565	6.216E-003	1.38	1.41	8.505E-003	1.38	1.41

4. CONCLUSION

Statistical design RSM was implemented in this study to optimize cellulase and protein production by SSF employing isolated *Aspergillus* sp. as a cellulase producer. By using the methodology the optimal range of hydration ratio, temperature, time of fermentation, substrate amount were easily achievable rather than a specific value, making it more flexible for process advancement. Comparison of predicted and experimental values revealed good correspondence between them, it also indicated that the empirical models derived from RSM can be used satisfactorily to describe the relation between the important factors involved in fermentation eventually leading to enhancement of cellulase production by isolated *Aspergillus* sp*.* Consequently we can also conclude that banana peel, which is a horticultural waste can be used competently for production of one of the most important industrial enzyme cellulase in a cost effective way.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Henrissat B, Davies G. Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol. 1997;7: 637-644.

2. Bhat MK. Cellulases and related enzymes in biotechnology. Biotechnol Adv. 2000;18: 355-383.

3. Adsul MG, Bastawde KB, Varma AJ, Gokhale DV. Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. Bioresour Technol. 2007;98:1467-1473.

4. Kaur J, Chadha BS, Kumar BA, Saini HS. Purification and characterization of two endoglucanases from *Melanocarpus* sp. MTCC 3922. BioresTechnol. 2007;98:74-81.

5. Busk PK, Lange L. Cellulolytic potential of thermophilic species from four fungal orders. AMB Express. 2013;3:47.

6. Sarkar N, Aikat K. Cellulase and Xylanase production from rice straw by a locally isolated fungus *Aspergillus fumigates* NITDGPKA3 under solid state fermentation – statistical optimization by response surface methodology. J. Technol. Innov. Renew. Energy. 2012;1:54-62.

7. Nagarajaiah SB, Prakash J. Chemical composition and antioxidant potential of peels from three varieties of banana. J. Food Ag-Ind. 2011;4(1):31-46.

8. Khan JA, Singh SK. Production of cellulase using cheap substrate by solid state fermentation. Int J Pl An and Env Sci. 2011;1(3):179-187.

9. Mandal M, Ghosh U. Production of Multi-enzyme system by isolated *Aspergillus* sp. under solid state fermentation and submerged fermentation. IJLTET. 2015; 5(1):186-192.

10. Mandels M, Weber J. The production of cellulases. In: Gould RF (ed) Cellulases and its application: Advances in chemistry Series: American Chemical Society; Washington; 1969.

11. Pradeep MR, Narasimha G. Utilization of pea seed husk as a substrate for cellulase production by mutant *Aspergillus niger*. Insight Biotechnol. 2011;1(2):17-22.

12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem. 1951; 193:265-275.

13. Hoa BT, Hung PV. Optimization of nutritional composition and fermentation conditions for cellulase and pectinase production by *Aspergillus oryzae* using response surface methodology. Int Food Res J. 2013;20(6):3269-3274.

14. Dave BR, Parmar P, Sudhir A, Panchal K, Subramanian RB. Optimization of process parameters for cellulase production by *Bacillus licheniformis* MTCC 429 using RSM and molecular characterization of cellulase gene. J Bioproces Biotech. 2015; 5(3):212.

15. Zhang W, Lin Y, Zhang Qi, Wang X, Wu D, Kong H. Optimization of simultaneous saccharification and fermentation of wheat straw for ethanol production. Fuel. 2013; 112:331-337.

16. Kumar S, Sharma N, Vyas G, Tandon D. Optimization of process parameters for cellulase free xylanase production by *Bacillus amyloliquefaciens* SH8 in submerged fermentation using response surface methodology. Biocatal Biotranfor. 2014;3(1):1-7.

17. Mohana S, Shah A, Divecha J, Madamwar D. Xylanase production by *Burkholderia* sp. DMAX strain under solid state fermentation using distillery spent wash. Bioresour Technol. 2008;99:7553-7564.

_____