ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Optimization of production conditions of extra cellular β-glucosidase in submerged fermentation of waterhyacinth using response surface methodology by *Rhizopus oryzae* MTCC 9642

Moumita Karmakar and Rina Rani Ray*

Microbiology Laboratory, Department of Zoology, Molecular Biology & Genetics,Presidency University, 86/1, College Street, Kolkata; 700 073, India.

ABSTRACT

In the present study, the effect of different production conditions on the yield of β-glucosidase by *Rhizopus oryzae* MTCC 9642 from waterhyacinth was investigated systematically using response surface methodology. A Central composite experimental design was applied to optimize its β-glucosidase enzyme production in liquid-state fermentation. The impact on enzyme production of three variables, namely substrate concentration, pH and temperature, was investigated by using water hyacinth based liquid medium. Highest activity of 153.033 U/ml was achieved at a substrate concentration of 1.25 % (w/v), pH 7.41 and temperature 26.04 ^{0}C. There was a direct correlation between the levels of enzymatic activities and the substrate concentration of wat er hyacinth as carbon source. The Central Composite Design was very efficient, providing much information on experiment variable effects and overall experimental error in a minimum number of required runs, consequently, it was also possible to determine a culture medium to obtain crude extracellular extracts with high β-glucosidase (157.033 U/ml) yield. This enzyme has gained industrial interest mainly due to their applications in wine and fruit juice processing because it releases aromatic compounds from flavorless glucosidic precursors.

Keywords: *Rhizopus oryzae,* Central composite experimental design, β-glucosidase.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Cellulose is the most abundant renewable natural biological resource, and constitutes a major portion of agricultural and forest wastes [1]. Thus they are the most promising feedstock for the production of energy, food and chemicals and their utilization could allow self-sustainable processes and products. Bioconversion of these wastes can be accomplished by cellulase, a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds [2]. The cellulase system in fungi comprises of three hydrolytic enzymes, of which β-Glucosidase (β –D glucoside glucohydrolase, EC 3.2.1.21) hydrolyses cellobiose, the end product of cellulose digestion, to glucose and thus plays an important role in complete saccharfications of cellulose [3]. Many microorganisms produce cellulolytic enzymes, but β-glucosidase is usually present at only relatively low activities in comparison with cellulase activity [3].Although the bioconversion of the agro waste material into fuel has received considerable interest during recent years, the high cost of production of these enzymes has hindered their industrial application [4]. To overcome this hindrance, extensive search is to be made for organisms capable of secreting copious amount of cellulase enzymes utilizing agrowastes, followed by the optimization of the parameters regulating enzyme production.

Response surface methodology is more satisfactory and effective than other methods such as classical one-at-a-time or mathematical methods because it can study many variables simultaneously with a low number of observations, saving time and costs [5] It has been successfully utilized to optimize compositions of fermentation medium [6],[7],[8] and enzyme characterization [9].As high amount of extra cellular β-glucosidase is not available from microbial sources and it is found to be present mostly as an intra cellular enzyme, the β-glucosidase of extra cellular origin may be of immense importance from industrial point of view.The present paper describes the statistical optimization of the parameters regulating the production of β-glucosidase from the submerged fermentation of water hyacinth, an aquatic weed by *Rhizopus oryzae* applying response surface methodology (RSM).

MATERIAL AND METHODS

Microorganism

A culture of *Rhizopus oryzae* MTCC9642 [10] was isolated from soil of India, West Bengal. Seed culture grown at 28°C for 48 h was used for building up of inoculum in Petri plates containing 20 mL of NA medium. A disk of 0.5 cm in diameter was added aseptically to flask (100 mL) containing 10 mL of medium with various concentrations of carbon source(water hyacinth), and the minor trace elements (g/L) of $(NH_4)_2HPO_4$ 0.4; KCl 0.1;$MgSO_4.H_2O$ 0.1 and peptone 0.9 . The inoculated flasks were incubated in static condition at 28°C for 48 h. The strain was maintained on PDA slants (peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; MgSO4.H2O 0.1: Dextrose 0.5 and agar 2) at 4° and -20°C for short-term and long-term storage, respectively.

ISSN: 0975-8585

Enzyme extraction and enzyme assay:

The grown culture was filtered through filter paper (Whatman No1) and filtrate was used centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. To measure the activity of β-glucosidase, tubes containing the assay mixture (1ml) each containing an 0.5 ml of enzyme diluted with 0.1(M) phosphate buffer (pH-6) was incubated with 1 %(w/v) salicin for 15 minutes respectively at 33°C. The reducing sugar released in either case was measured by the dinitrosalicylic acid method [11] taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of β-glucosidase was defined as that amount of enzyme that liberated 1µ mole of glucose equivalent per ml per minute of reaction.

Statistical analysis:

An evaluation copy of the statistical software, Design-Expert version 7.1.6, from Stat-Ease, Inc., Minneapolis, USA was used for analysis of experimental data and to generate response surface plots. ANOVA was used to estimate the statistical parameters.

RESULTS AND DISCUSSION

Optimisation by response surface methodology

A Central Composite Design with three factors at five levels($-\alpha$, −1, 0, +1, $+\alpha$) (Table 1),

TABLE 1:Process variables and their levels

Independent variables	Symbol	Code levels			
		-1	+1	$-\alpha$	$+\alpha$
substrate conc(%)	A	0.5	1.25	0.244328	1.50567
pH	B	6	9	4.97731	10.0227
temp	C	18	40	10.5003	47.4997

20 experimental runs were performed in triplicate. Table 2 shows the three independent variables and their concentrations in actual values of the central composite design experiments (table 2). Upon completion of experiments, the average maximum enzymatic activity was taken as the dependent variable or response(Y). A second order polynomial equation was then fitted to the data by a multiple regression procedure. This resulted in an empirical model that related the responses measured to the independent variables of the experiment. For a three factor system the model equation was taken to be,

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{23}BC+\beta_{13}AC \quad \text{.....Eqn 1}$$

ISSN: 0975-8585

Where *Y*, predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients, β_{11}, β_{22}, β_{33}, squared coefficients; β_{12}, β_{23}, β_{13}, interaction coefficients and A denoted substrate concentration (% w/v), B was the pH and C was the temperature ($^{\circ}$C).

Table 2:Experimental design and results of the Central Composite model.

serial no.	substrate conc.	pH	temperature	enzyme unit (U/ml)
1	0.5	6	18	33.3
2	1.25	6	18	109.89
3	0.5	9	18	33.3
4	1.25	9	18	133.2
5	0.5	6	40	133.2
6	1.25	6	40	136.53
7	0.5	9	40	33.3
8	1.25	9	40	59.94
9	0.2	7.5	29	73.26
10	1.5	7.5	29	166.5
11	0.875	4.9	29	73.26
12	0.875	10.02	29	33.3
13	0.875	7.5	10.5	69.93
14	0.875	7.5	47.5	99.9
15	0.875	7.5	29	133.2
16	0.875	7.5	29	133.2
17	0.875	7.5	29	133.2
18	0.875	7.5	29	133.2
19	0.875	7.5	29	133.2
20	0.875	7.5	29	133.2

ISSN: 0975-8585

Factors affecting the β-glucosidase production:

On the basis of quadratic polynomial equation of response surface model (Eq. (1)), the present model and data analysis allowed not only to define optimum conditions for β-glucosidase production but also showed combined effect of independent variables such as substrate concentration(w/v), pH and temperature in terms of coded factors in Eq.(2).

β-glucosidase = +133.17+26.60 * A-16.14 * B+7.59 * C+5.83 * A * B-18.32 * A * C-24.98

* B * C-4.52 * A^2 -28.06 * B^2-16.88 * C^2 ……Eqn 2

Where response is the β-glucosidase activity,A is substrate concentration(w/v), B is the p^H ,C is the temperature in degree Centigrade.

The statistical significance of Eq. (2) was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 3. It is evident that the model was highly significant, as suggested by the model *F* value and a low probability value (*P*model > *F*). The Model F-value of 194.64 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. The "Pred R-Squared" of 0.9569 is in reasonable agreement with the "Adj R-Squared" of 0.9892. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable[12]. Your ratio of 43.083 indicates an adequate signal. This model can be used to navigate the design space. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared. Usually, the higher the value of CV, the lower the reliability of experiment is. Here, a lower value of CV (4.57) indicated a better precision and reliability of the experiments [13]. The precision of a model can be checked by the determination coefficient (*R*2) and correlation coefficient (*R*). The determination coefficient (*R*2) implies that the sample variation of 99.4% for β-glucosidase production was attributed to the independent variables, and only about 0.6% of the total variation cannot be explained by the model. Normally, a regression model having an *R*2 value higher than 0.9 is considered to have a very high correlation [14]. The closer the value of *R* (correlation coefficient) to 1, the better the correlation between the experimental and predicted values. Here, the value of *R* (0.989) for Eq.(2) indicates a close agreement between the experimental results and the theoretical values predicted by the model equation (Fig. 1). The residuals from the least squares fit play an important role in judging model adequacy [15]. By constructing a normal probability plot of the residuals, a check was made for the normality assumption, as given in Fig. 2. The normality assumption was satisfied as the residual plot approximated along a straight line. The plot (Fig 2) is satisfactory, so we conclude that the empirical model is adequate to describe the β-glucosidase activity by response surface. Three dimensional response surface plots graphically represent regression equations and are generally used to demonstrate relationships between the response and experimental levels of each variable (Fig 3,4,5).

ISSN: 0975-8585

Table 3: Analysis of variance table for β-glucosidase production in SmF(submerged fermentation)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	36193.26	9	4021.473	194.6393	< 0.0001	Significant
A-substrate conc	9662.94	1	9662.94	467.6863	< 0.0001	
B-pH	3556.405	1	3556.405	172.13	< 0.0001	
C-temp	787.1676	1	787.1676	38.09892	0.0001	
AB	271.6781	1	271.6781	13.14922	0.0046	
AC	2683.514	1	2683.514	129.8821	< 0.0001	
BC	4990.005	1	4990.005	241.5163	< 0.0001	
A^2	293.8255	1	293.8255	14.22116	0.0037	
B^2	11348.56	1	11348.56	549.2706	< 0.0001	
C^2	4104.99	1	4104.99	198.6815	< 0.0001	
Residual	206.6115	10	20.66115			
Lack of Fit	206.6115	5	41.32231			
Pure Error	0	5	0			
Cor Total	36399.87	19				

R^2-0.994, Adj $R^2$0.989, Pred R^2-0.956, C.V. %-4.57.

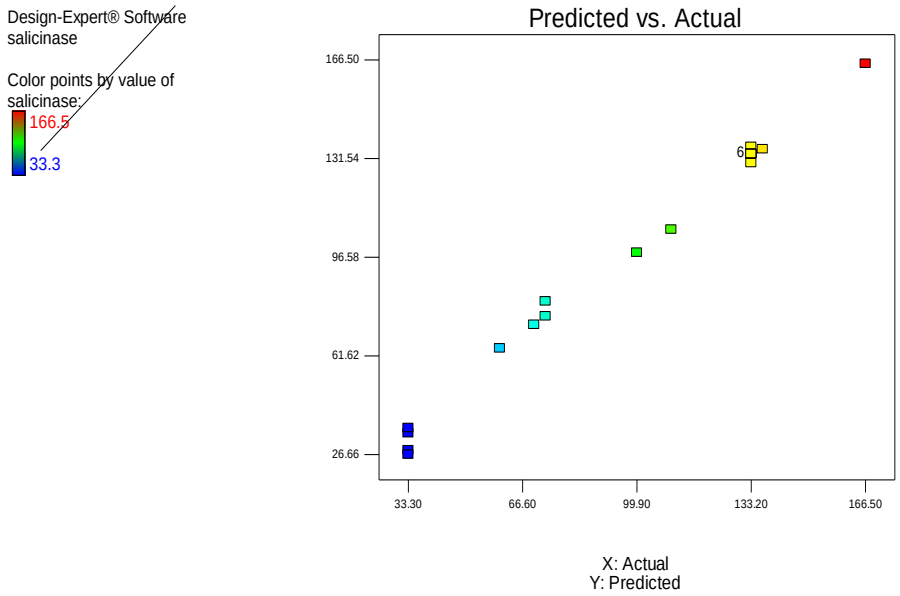

Fig.1 Actual vs. predicted values of the response

ISSN: 0975-8585

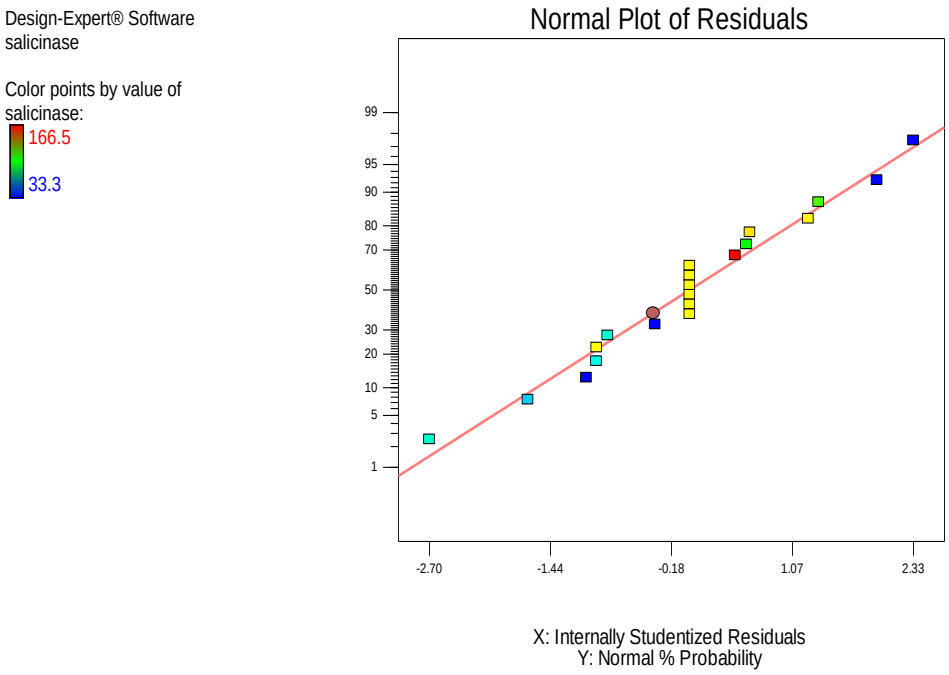

Fig. 2 Normal plot of residuals

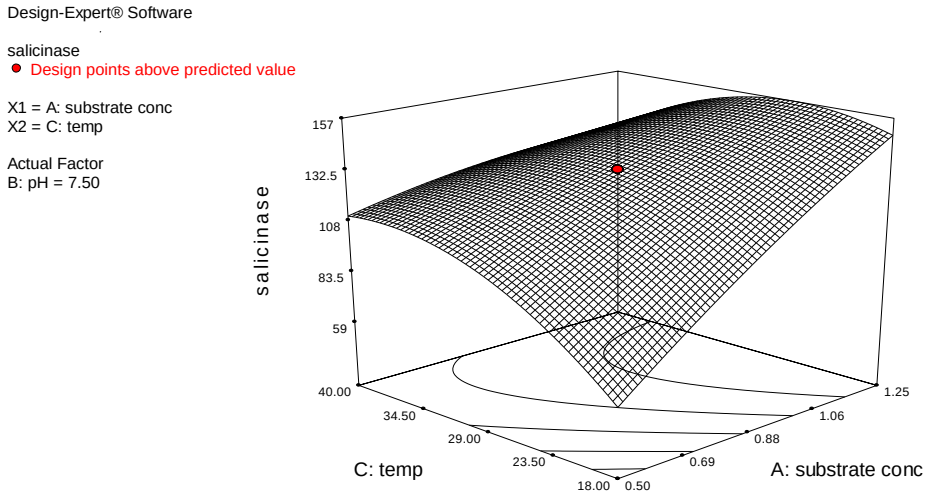

Fig. 3 Response surface plot showing the effect of pH and substrate concentration on β-glucosidase production in LSF with other variable constant at middle level

ISSN: 0975-8585

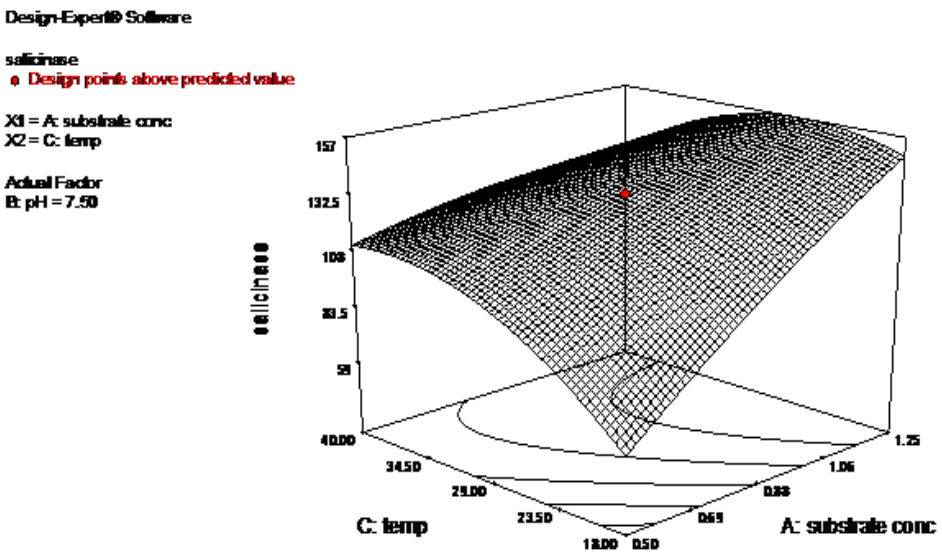

Fig 4 Response surface plot showing the effect of temperature and substrate concentration on β-glucosidase production in LSF with other variable constant at middle level

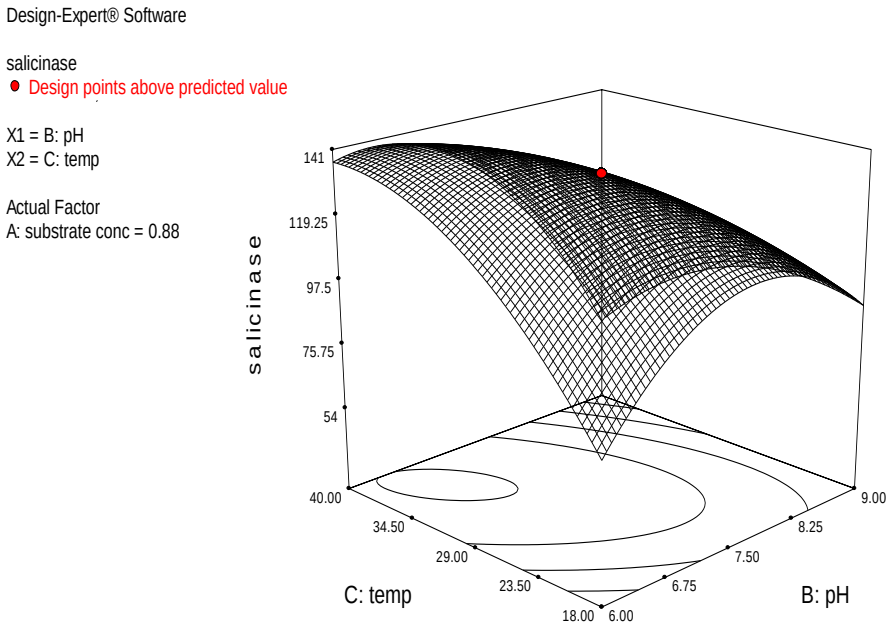

Fig. 5 Response surface plots showing the effect of temperature and pH on β-glucosidase production in LSF with other variable constant at middle level.

VALIDATION OF THE MODEL

ISSN: 0975-8585

The statistical optimal values of variables were obtained where the central composite design and the response at the different points yielded maximum β-glucosidase SmF. A repeat fermentation of water hyacinth for highest production of β-glucosidase, by *Rhizopus oryzae* MTCC 9642 under optimal conditions was carried out for verification of the optimization.The maximum β-glucosidase and endoglucanase production is 150 U/ml which is 4.46% less than the predicted value respectively. This discrepancy was probably due to the slight variation in experimental conditions. In unoptimized condition the production of β-glucosidase was shown in run number 1 of Table II which is 33.3 U/ml. After optimization the maximum production of β-glucosidase 150 U /ml which is 4.5 fold increase in the enzyme production.

CONCLUSION

Statistical optimization of cultivation conditions using the central composite design of response surface methodology appeared to be a valuable tool for the production of β-glucosidase in submerged fermentation(SmF) by *Rhizopus oryzae* MTCC 9642. The predicted and verifiable β-glucosidase activity under optimal conditions in static flasks experiments were 157.033 U/mL and 150 U/mL, respectively at substrate concentration of 1.25%, pH 7.41 and temperature of 26.04 $^{\circ}$C.

ACKNOWLEDGEMENT

The authors wish to thank Department of Science and technology, West Bengal, India for financial assistance.

REFERENCES

[1] Singh A, Singh N, Bishnoi NR. Int J Civil Env Eng 2009; 1(1):23-26.
[2] Chellapandi P, Jani HM. Braz J Microbiol 2008; 39: 122-127.
[3] Roy SK, Raha RK, Sadhukhan RK, Chakrabarty SL. World J Microbiol Biotechnol 1991; 7:613-618.
[4] Narasimha G, Sridevi A, Buddolla V, Subhosh CM, Rajsekhar RB. Afr J Biotechnol 2006; 5(5): 472-476.
[5] Deepak V, Kalishwaralal K, Ramkumarpandian S, Venkatesh Babu S, Senthilkumar SR , Sangiliyandi G. Biores Technol 2008; 99(17): 8170-8174.
[6] Cui F, Li Y, Xu Z, Xu H, Sun K, Tao W. Biores Technol 2006;97: pp 1209–1216.
[7] Zhang X, Li Y, Zhuge J. J Chem Technol Biotechnol 2006;81:1075–1078.
[8] Saval L, Pablos, Sanchez S. Biores Technol 1993;43(1):19-25
[9] Karmakar M, Ray RR. Res Rev Biosc 2010; 4(1):50-55
[10] Karmakar M, Ray RR. Asian J Biotechnol 2010; 2(1) : 27-36.
[11] Bernfeld P. Method Enzymol 1955; 1:149-150.
[12] Design expert 7.1.5 users guide manual.
[13] Box GEP, Hunter WG, Hunter JS. Statistics for experimenters. New York: Jhon Wiley and Sons. 1978; 291–334.

ISSN: 0975-8585

[14] Haaland PD. Separating signals from the noise. In: Experimental design in biotechnology. New York: Marcel Dekker. 1989; 61–83.
[15] Myers RH, Montgomery DC. Response surface methodology. USA:Wiley; 2002.