Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(6):870-878

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Statistical medium optimization for the production of β-galactosidase from *Aspergillus terreus* KUBCF1306 using Response Surface Methodology

B. Vidya[1], M. Palaniswamy[3], S. Sowmya[1], P. Anusooriya[1], D. Malarvizhi[1], S. Priyanga[1], K. Devaki[1], P. Chella Perumal[2], Pratibha Prabhakaran[2] and V. K. Gopalakrishnan[1,2*]

[1]*Department of Biochemistry, Karpagam University, Coimbatore*
[2]*Department of Bioinformatics, Karpagam University, Coimbatore*
[3]*Department of Microbiology, Karpagam University, Coimbatore*

ABSTRACT

β-galactosidase is a vital enzyme with diverse applications in food and pharmaceutical industries. Response surface methodology was used to study the effects of fermentation parameters on β-galactosidase enzyme production. The effect of pH, temperature, carbon source, nitrogen source, inoculum size and incubation time on the production of β-galactosidase from Aspergillus terreus KUBCF1306 were studied by employing statistical methods. Objective of the present work is to improve the yield of β-galactosidase activity by using statistical methods like Placket & Burman designs (PBD) and Response surface methodology (RSM). Screening of variables to find their relative effect on β-galactosidase production was done using Plackett-Burman design. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the number of individual experiments. Out of the six factors screened, pH, carbon source and temperature were found to influence the enzyme production significantly. Results of the statistical analysis showed that the lack of fit, of the model was good in all cases. The response surface methodology was found to be useful in optimizing and determining the interactions among process variables in β-galactosidase enzyme production.

Key words: β-galactosidase, Response surface methodology, Plackett-Burman design, *Aspergillus terreus*, Central composite design.

INTRODUCTION

Enzymes are proteins with specific catalytic functions that are produced by all living cells. β-galactosidase is a vital enzyme with diverse applications in molecular biology and industries [1]. Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in food industry to improve sweetness, solubility, flavor and digestibility of dairy products. It is realised by enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), it is also called as lactase. Free preparations of β galactosidases have been exploited in various applications such as industrial, biotechnological, medical, analytical and in different other applications. β-galactosidase occurs widely in nature and is produced by a number of microorganisms [2], the major industrial enzymes are obtained from *Aspergillus* sp. and *Kluyveromyces* sp. β galactosidase from *Kluyveromyces lactis* is one of the most widely used enzymes [3,4,5,6].

Optimization of biochemical coupling can be done by employing either univariate or multivariate strategies. Univariate procedure may fail since the effect of one variable may be

dependent on the level of others involved in the optimization. Multivariate optimization schemes involve experimental designs for which the levels of all the variables are changed simultaneously. Response surface methodology (RSM) has become very popular in recent years, with wide range of applications in biochemical

process optimization [1]. The conventional approach of optimizing one-factor-at-a-time method and it involves changing one factor at a time and maintaining the rest factors at a fixed level. This method is extremely time saving and is unable to detect the effect of interaction of various factors [7]. To overcome this difficulty, response surface methodology (RSM), which is a collection of statistical techniques applicable to experimental design, model building, evaluating the effects of factors and screening optimum conditions of factors for desirable responses.

Furthermore, this method allows the development of mathematical models that permit assessment of the relevance, statistical significance of the factors being deliberate as well as evaluates the interaction effects between the factors [8]. In addition, response surface methodology (RSM) is an competent strategic experimental tool by which the optimal conditions of a multivariable system may be determined. The aim is to obtain mathematical models showing the dependence of the enzyme activity on independent variables. The mathematical dependences are used for the prediction of the optimum values of the independent variables, ensuring optimal yield [9, 10].

Plackett–Burman design [11] is one such method that has been frequently used for screening multiple factors at a time. This experimental design is particularly useful for initial screening as it is used for the estimation of only the main effects. The significant factors obtained from the screening experiments could be further optimized by employing response surface methodology that enables the study of interaction effects among different variables.

The principle objective of this study was to optimize the production of extracellular β-galactosidase from *Aspergillus terreus* KUBCF1306 using statistical methods to achieve maximum yield.

EXPERIMENTAL SECTION

Organism and inoculum preparation

Fungal strains were isolated from garden soil obtained from Coimbatore, Tamil Nadu by dilution plate method. The strains were morphologically identified by Agharkar Research Institute, Pune. Fungal strains were maintained on PDA at 30ºC for subsequent use.

Submerged fermentation

The basal medium for maintenance of the culture was composed of the following (in g/L): Lactose - 20.0, $NaNO_3$ - 2.0, K_2HPO_4 - 1.0, KCl - 0.5, $MgSO_4$. $7H_2O$ - 0.5, $FeSO_4$. $5H_2O$ - 0.01, with pH adjusted to 5.0 (Citrate phosphate buffer, 100m mol L^{-1}).

β-Galactosidase assay

β-Galactosidase activity was determined by using o-nitrophenyl- β –d galactopyranoside (oNPG) as substrate. 50µl of crude enzyme sample were added to 950µl of 2.5mM oNPG solution in 100mM citrate-phosphate buffer. Following 10 min incubation in a water bath at 60ºC, 1ml of 10% (w/v) sodium carbonate solution was added and the absorbance of the final mixture was measured at 410 nm in order to determine the amount of released o-nitro-phenol (oNP). One unit of β-galactosidase activity was defined as the amount of the enzyme required to liberate 1 µmole of oNP per minute under assay conditions [12].

Experimental Design

One variable at a time method

Optimization of β-galactosidase production was done by using different physical parameters one at a time approach keeping the other as constant. The different physical parameters are pH, temperature, carbon sources, nitrogen sources, incubation time, inoculum size. The first optimization step was carried out using ‘one variable at a time’ experimental approach to identify the significant factors affecting β-galactosidase production by *Aspergillus terreus* KUBCF1306 and it was investigated by using different agro-industrial wastes as a substrate for submerged fermentation.

Plackett-Burman design

For the selection of various variables, “Design Expert 9.0.3.1” (Stat-Ease Inc, Minneapolis, USA) was used to generate and analyze the experimental design of Plackett-Burman. The optimum concentrations of the variables were obtained by the graphical and numerical analysis using the Design Expert program, based on the criterion of desirability. Plackett and Burman design was firstly applied to screen the significance of sixteen components based on improved enzyme production. Finally, Box-Behnken design and response surface methodology were further adopted to derive a statistical model for optimizing the medium components for β galactosidase production.

Box–Behnken design

A Box–Behnken design was employed to investigate the optimization of the most significant variables affecting the enzyme production. Box–Behnken design [13] followed for the production of β-galactosidase by *Aspergillus terreus* KUBCF1306. This method is useful for optimizing a small number of variables at a few levels.

Central Composite Design

The central composite design (CCD) is the most frequently and extensively used RSM design. Central composite design is a well established widely used statistical technique for determining the key factors from a large number of medium components by a small number of experiments [14]. Two–level factorial part (the core) of the design consists of all possible combinations of the plus or minus ("– 1" or "+ 1") levels of the factors. Center points are usually repeated to get an estimate of experimental error. Thus the central composite design requires five coded levels of each factor: "– 1" or "+ 1" (factorial points), "– α" or "+ α" (axial points), and the all zero level (center point). Central Composite Designs are intended to estimate the coefficients of a quadratic model.

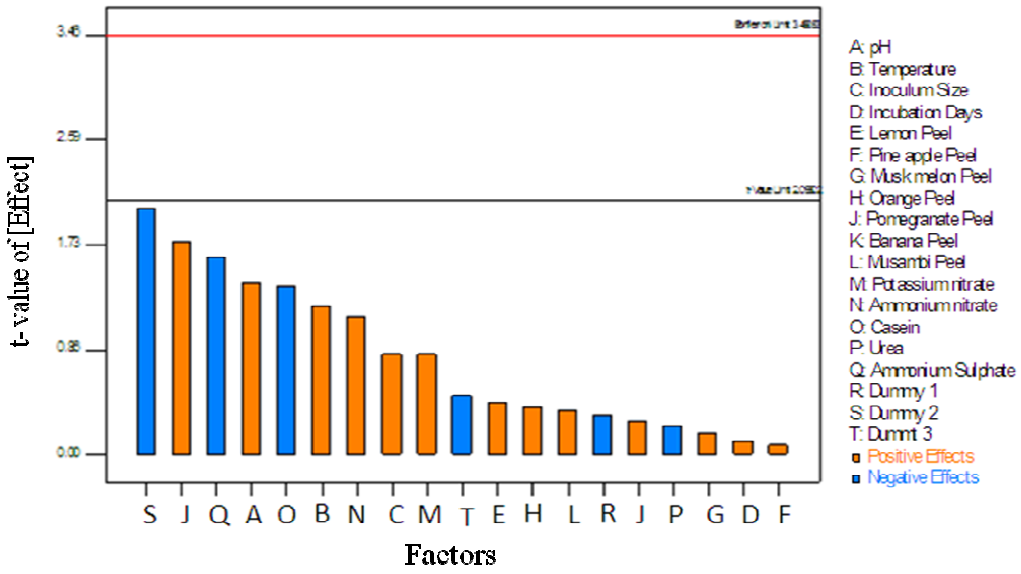
Validation of the model

The statistical model was validated with respect to all significant variables within the design space. A random set of six experimental combinations under the optimized conditions was used for validation of the statistical model.

RESULTS AND DISCUSSION

The Pareto chart displays the magnitude of each factor estimate and it is a convenient way to view the results of Plackett-Burman experimental design. The main effect was calculated as the difference between the average of measurements made at the high level setting (+) and the average of measurements observed at the low level setting (-) of each factor. Figure 1 shows the Pareto chart for the effect of selected nineteen factors on β-galactosidase production.

Figure 1: Pareto chart showing the effect of the selected nineteen factors on β-galactosidase production



Plackett-Burman experimental design is based on the first order model with no interaction among the factors. The first step involved the screening of variables and the second step involved the optimization of significant variables. Plackett–Burman design, a widely used fractional factorial method was adopted for the screening of cultural and nutritional parameters influencing β-galactosidase from *Aspergillus terreus* KUBCF1306 in submerged fermentation. All the experiments were carried out in triplicates according to a design matrix (Table 1), which was based on the number of variables to be investigated. Each row of the matrix represented a trial and each column represented an independent factor whose levels were varied. The total number of trials to be carried out was n+1, where n was the number of variables under study.

In this study, for selection of the most significant medium composition affecting the value of desirability, a total of 19 variables, including sixteen factors namely, pH (X1), temperature (X2), inoculums size (X3) incubation days (X4), lemon peel (X5), orange peel (X6), pomegranate peel (X7), muskmelon peel (X8), banana peel (X9), musambi

peel (X10), pine apple peel (X11) potassium nitrate (X12), ammonium nitrate (X13), casein (X14), urea (X15), ammonium sulphate(X16) and three dummy or unassigned variables (X17, X18, X19) were tested and identified by the Plackett-Burman design experiment. The principal effects of each variable on the value of desirability were represented at each variable and was evaluated at two levels, a high (+) and a low (-) level. The experimental design with the variables, symbol code, units and experimental level of the variables are shown in Table 1. The range and levels of the variables investigated are listed in Table 1.

Table 1: Plackett Burman design for screening variables at different level for the production of β-galactosidase

Code	Variables	Units	Levels	
			Low	High
A	pH	-	4	7
B	Temperature	˚C	30	70
C	Inoculum size	%	1	5
D	Incubation period	Days	1	7
E	Lemon peel	%	1	5
F	Orange peel	%	1	5
G	Pomegranate peel	%	1	5
H	Muskmelon peel	%	1	5
J	Banana peel	%	1	5
K	Musambi peel	%	1	5
L	Pineapple peel	%	1	5
M	Potassium nitrate	%	1	5
N	Ammonium nitrate	%	1	5
O	Casein	%	1	5
P	Urea	%	1	5
Q	Ammonium sulphate	%	1	5
R	Dummy 1	-	-1	1
S	Dummy 2	-	-1	1
T	Dummy 3	-	-1	1

R, S, T is three dummy variables

RSM mainly exploits two designs of experiments for process optimization viz., Central Composite design and Box-Behnken design. Ferreira et al. (2007) made a comparative study between these two designs of experiment and concluded that Box-Behnken design is more efficient than Central Composite design [8]. Empirical models and statistical analysis are extremely important to elucidate basic mechanisms in complex situations, thus providing better process control and understanding. In most RSM problems, the relationship between the response and independent variables is unknown. Placket and Burman experiments and their levels were further optimized for enhanced β-galactosidase production by employing a Box – Behnken design and central composite design [11, 13]. According to this program, above mentioned sixteen factors were chosen and three dummy variables were used to evaluate experimental error. The statistical significance of the regression coefficients was determined by the model equation was determined by Fischer's test and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2 [15].

Finally, the physical factors pH, temperature, inoculum size, incubation days, pomegranate peel and ammonium nitrate for each run, the experimental responses along with the predicted response obtained from the regression equation for the 54 combinations are shown in Table 2.

This design contains only a subset of all possible factor-setting combinations and generates information about the main effects of the design variables with the smallest possible number of experiments. The random error variability and test for the statistical significance of the parameter estimates can be determined using the design. The regression coefficient, P value and confidence level were determined and the variables with confidence level greater than 90% were considered to be more significant for β-galactosidase production.

Analysis of variance (ANOVA) was performed to establish the adequacy and significance of predicted quadratic model as given in Table 3.

Table 2: Box – Behnken design matrix experimental and predicted values of β-galactosidase production by *A.terreus* KUBCF1306

Run	A: pH	B: Temperature (˚C)	C: Inoculum size (%)	D: Incubation period (days)	E: Pomegranate peel (%)	F: Ammonium nitrate (%)	β-galactosidase activity (U/ml)	
							Observed Value	Predicted Value
1	7	30	3	1	3	3	63.04	61.25
2	5.5	30	3	4	1	5	45.26	48.17
3	5.5	50	1	1	3	1	87.57	83.26
4	7	50	1	4	3	5	115.04	115.75
5	4	30	3	1	3	3	106.67	106.23
6	4	70	3	1	3	3	124.41	120.48
7	4	50	5	4	3	1	86.69	88.94
8	7	50	1	4	3	1	67.13	65.34
9	5.5	30	5	4	5	3	134.43	132.47
10	7	30	3	7	3	3	145.39	145.62
11	7	50	5	4	3	5	76.47	76.42
12	5.5	50	5	7	3	5	142.31	141.87
13	7	50	5	4	3	1	139.61	139.65
14	7	70	3	1	3	3	158.13	158.42
15	5.5	70	5	4	1	3	169.63	165.30
16	5.5	50	3	4	3	3	174.48	170.45
17	5.5	70	5	4	5	3	159.20	156.37
18	7	50	3	7	5	3	126.42	127.81
19	5.5	30	1	4	1	3	148.00	146.27
20	7	50	3	1	1	3	123.46	120.59
21	5.5	70	3	4	1	5	168.06	169.8
22	5.5	70	3	4	5	5	135.71	133.65
23	5.5	50	1	7	3	1	138.61	140.20
24	4	50	3	7	5	3	124.32	124.54
25	5.5	70	3	4	1	1	157.52	154.36
26	4	50	3	1	1	3	92.18	93.47
27	5.5	50	1	1	3	5	67.13	66.54
28	5.5	50	5	1	3	1	66.15	63.48
29	5.5	30	3	4	1	1	41.26	43.21
30	5.5	50	5	1	3	5	158.91	152.47
31	4	50	3	7	1	3	141.26	145.37
32	5.5	30	1	4	5	3	120.44	121.45
33	5.5	50	3	4	3	3	140.31	142.36
34	4	50	3	1	5	3	180.27	184.34
35	5.5	50	3	4	3	3	175.61	175.64
36	4	50	1	4	3	1	73.62	73.41
37	5.5	70	3	4	5	1	61.43	60.17
38	4	70	3	7	3	3	86.35	82.64
39	5.5	50	1	7	3	5	46.17	41.35
40	5.5	30	3	4	5	5	64.68	64.76
41	5.5	70	1	4	1	3	146.36	142.84
42	7	50	3	1	5	3	168.47	165.42
43	5.5	30	5	4	1	3	170.85	172.16
44	5.5	30	3	4	5	1	46.74	43.52
45	5.5	70	1	4	5	3	59.63	57.34
46	4	50	1	4	3	5	68.44	67.25
47	5.5	50	5	7	3	1	91.67	93.42
48	5.5	50	3	4	3	3	113.12	110.23
49	5.5	50	3	4	3	3	158.07	157.63
50	5.5	50	3	4	3	3	124.14	124.04
51	4	50	5	4	3	5	173.16	170.59
52	7	70	3	7	3	3	168.34	164.22
53	7	50	3	7	1	3	167.61	171.34
54	4	30	3	7	3	3	175.36	176.81

As shown in Table 3, The F-value of the model was 1.98 for β-galactosidase activity yield, it implied that the model was very significant, and there was only a 0.01% chance that a "Model F-Value" could occur due to noise. Moreover, the *P*-values (<0.001) of the model and the lack of fit (0.4299) also suggested that the obtained experimental data was a good fit with the model. The value of determination coefficient R^2= 0.9856 for β-galactosidase yield, ensured a satisfactory adjustment of the quadratic model to the experimental data, and also indicated a high correlation between the predict values and the practical values. Normally, a regression model having an R^2 value higher than 0.9 is considered and a model with an R^2 value between 0.7 and 0.9 is considered as having a high correlation [16, 17]. The quality of fit of the model was checked by coefficient of determination (R^2),

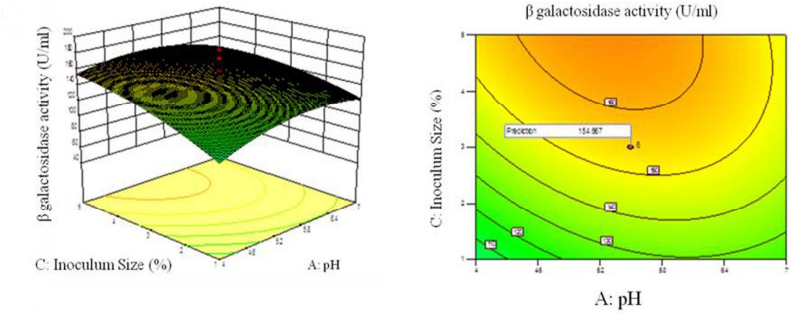
the value of 0.9260 indicates that 92.6% of the variability in the response could be explained by the model. The P-value serves as a tool for checking the significance of each of the coefficients.

Table 3: Analysis of variance (ANOVA) for the model developed for β-galactosidase production

Source	Sum of Squares	Degree of Freedom	Mean squares	F- Value	P- Value prob > F	
Model	56310.96	27	2085.59	1.98	0.0433	significant
A-pH	273.37	1	273.37	0.26	0.6151	
B-Temperature	1568.17	1	1568.17	1.49	0.2339	
C-Inoculum size	8400.04	1	8400.04	7.96	0.0091	
D-Incubation period	126.04	1	126.04	0.12	0.7325	
E-Pomegranate peel	4240.04	1	4240.04	4.02	0.0556	
F-Ammonium nitrate	145.04	1	145.04	0.14	0.7139	
AB	6160.50	1	6160.50	5.83	0.0230	
AC	420.50	1	420.50	0.40	0.5335	
AD	85.56	1	85.56	0.081	0.7781	
AE	561.12	1	561.12	0.53	0.4725	
AF	612.50	1	612.50	0.58	0.4531	
BC	528.12	1	528.12	0.50	0.4857	
BD	2664.50	1	2664.50	2.52	0.1242	
BE	12.25	1	12.25	0.012	0.9150	
BF	1596.13	1	1596.13	1.51	0.2299	
CD	1012.50	1	1012.50	0.96	0.3365	
CE	1035.12	1	1035.12	0.98	0.3312	
CF	1225.00	1	1225.00	1.16	0.2913	
DE	4560.12	1	4560.12	4.32	0.0477	
DF	242.00	1	242.00	0.23	0.6361	
EF	2016.13	1	2016.13	1.91	0.1788	
A^2	2154.29	1	2154.29	2.04	0.1651	
B^2	1866.87	1	1866.87	1.77	0.1952	
C^2	738.29	1	738.29	0.70	0.4106	
D^2	76.22	1	76.22	0.072	0.7903	
E^2	72.38	1	72.38	0.069	0.7955	
F^2	12942.29	1	12942.29	12.26	0.0017	
Residual	27450.69	26	1055.80			
Lack of fit	23115.35	21	1100.73	1.27	0.4299	not significant
Pure error	4335.33	5	867.07			
Cor total	83761.65	53				

Furthermore, three dimensional response surface plots and corresponding 2D contour plots, (Figures 1 and 2) which graphically represent regression equations, were used to demonstrate relationships between the response and experimental levels of each variable. Figure 1 and 2, shows the response surface plots and their respective contour plots of β-galactosidase production. Each figure presents the effect of two factors while the other factor was held at zero level.

Figure 1: Three dimensional response surface plots showing effects of pH and inoculum size with corresponding contour plots showing predicted optimal response



As shown in Figures 1 and 2, there was significant interaction between each pair of variables. Each contour curve in a 2D plot represents an infinite number of combinations of two test variables with all the others at fixed levels. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram [18]. The shape of the contour plot, circular or elliptical, indicates whether the mutual interactions between the corresponding variables are significant or not [19]. If it is circular, the interactions between the variables are negligible and if it is elliptical the interaction between the variables are significant [20]. Figure 3 shows three dimensional response surface plots and corresponding counter plots that represent effect of incubation days and

ammonium nitrate, while the other factors are maintained at constant. Figure 4 shows the effect of pH and incubation days for the enzyme production.

Figure 2: Three dimensional response surface plots showing effects of pH and ammonium nitrate with corresponding contour plots showing predicted optimal response

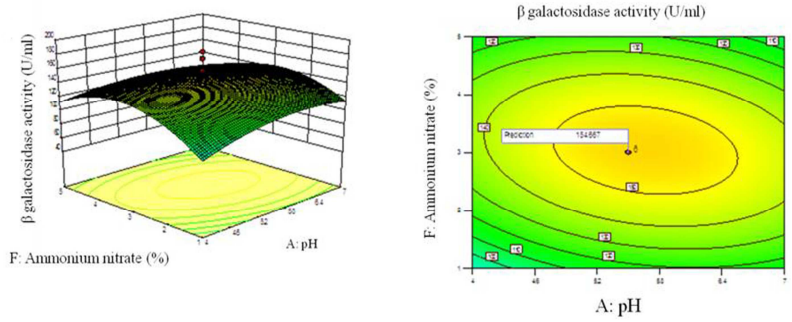


Figure 3: Three dimensional response surface plots showing effects of ammonium nitrate and incubation days with corresponding contour plots showing predicted optimal response

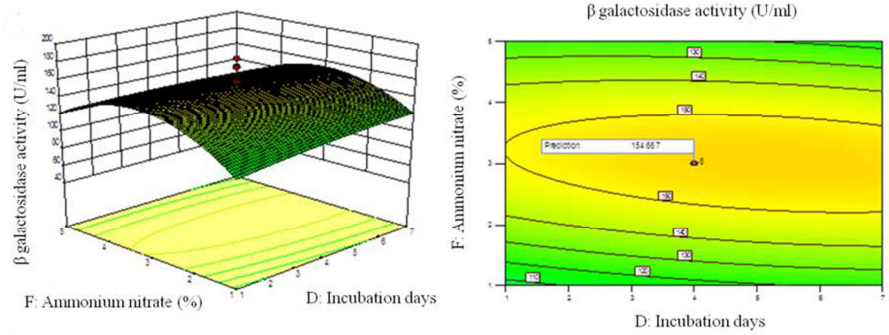


Figure 4: Three dimensional response surface plots showing effects of incubation days and pH with corresponding contour plots showing predicted optimal response

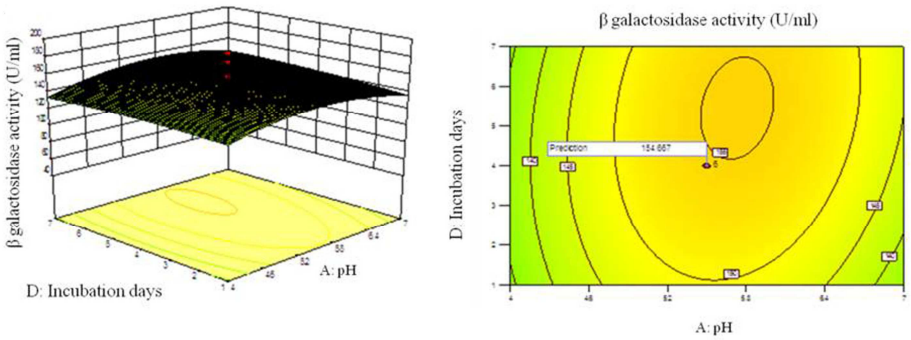


Figure 5: Three dimensional response surface plots showing effects of inoculum size and temperature with corresponding contour plots showing predicted optimal response

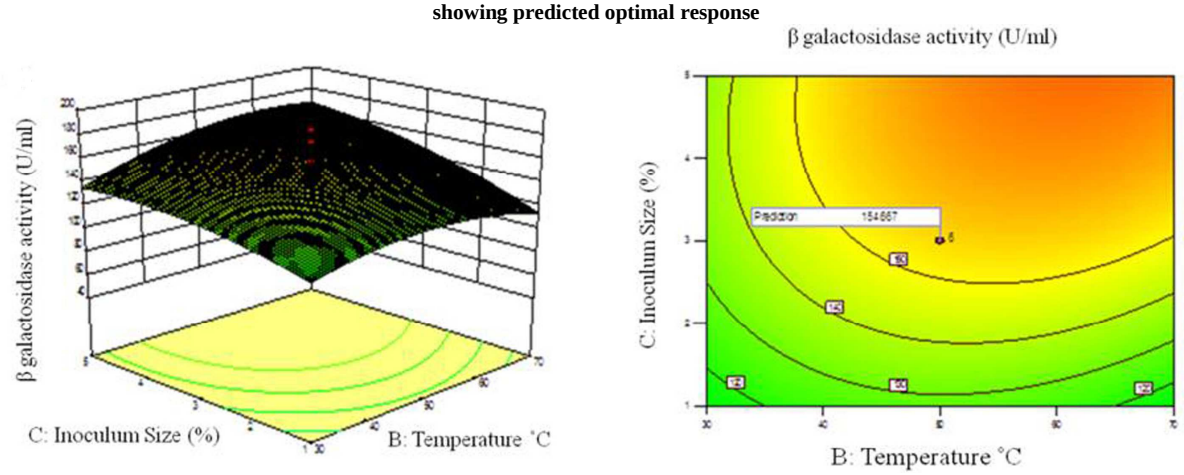


Figure 5 shows three dimensional response surface plots and corresponding counter plots which represent the effect of inoculums size and temperature. Figure 6 shows the effect of ammonium nitrate and temperature.

Figure 6: Three dimensional response surface plots showing effects of ammonium nitrate and temperature with corresponding contour plots showing predicted optimal response

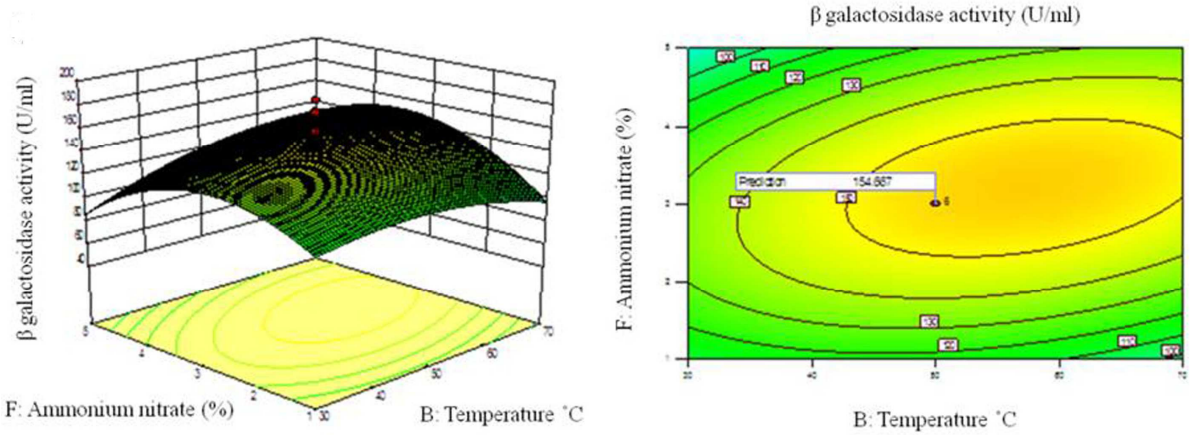


Figure 7 shows the three dimensional response surface plots and corresponding counter plots, represent effect of ammonium nitrate and inoculums size. Figure 8 shows the three dimensional response surface plots and corresponding counter plots shows the effects of pomegranate peel and temperature.

Figure 7: Three dimensional response surface plots showing effects of ammonium nitrate and inoculum size with corresponding contour plots showing predicted optimal response

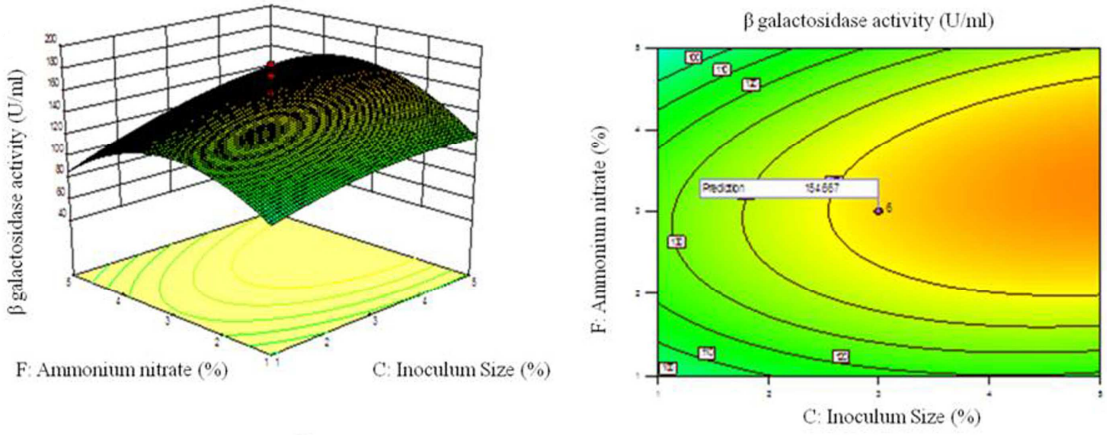
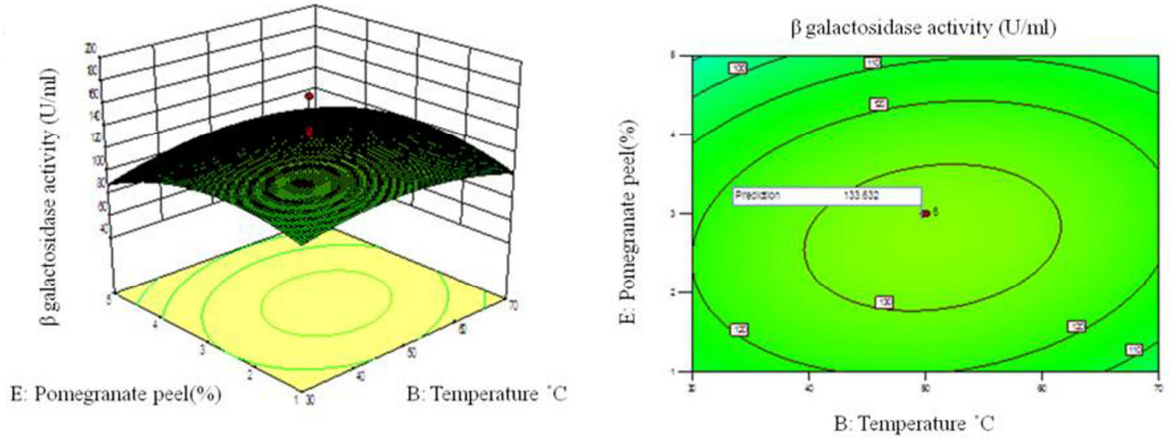


Figure 8: Three dimensional response surface plots showing effects of pomegranate peel and temperature with corresponding contour plots showing predicted optimal response



CONCLUSION

The statistical design of experiments offers efficient methodology to identify the significant variables and to optimise factors using a minimum number of experiments for β-galactosidase production. A maximum β-

galactosidase activity of 4.62 U/ml was obtained by *Aspergillus terreus* KUBCF1306 using the optimised medium determined by the Plackett– Burman design. The response surface methodology based on Box–Behnken design and Central composite design enabled the determination of optimal conditions for obtaining greater β-galactosidase production. A significant improvement in the production of β-galactosidase was accomplished using agro industrial waste. The optimized medium established in this work might result in a significant reduction in the cost of medium constituents making the process economically viable.

Acknowledgements
We, the authors are thankful to our Chancellor, Chief Executive Officer, Vice Chancellor and Registrar of Karpagam University for providing facilities and encouragement.

Financial support
Financial assistance provided by INSPIRE, Ministry of Science and Technology, Department of Science & Technology, Government of India (IF120793).

REFERENCES

[1] D Kishore; M Talat; ON Srivastava; AM Kayastha, *PLoS One*, **2012**, 7(7), 1-13.
[2] S Sen; L Ray; P Chattopadhyay, *Appl. Biochem. Biotech*., **2012**, 167, 1938-1953.
[3] QZK Zhou; XD Chen, *Biochem. Eng. J*., **2001**a, 9, 33–40.
[4] E Jurado; F Camacho; G Luzon; JM Vicaria, *Enzy. Microb. Technol.*, **2002**, 31, 300-309.
[5] DH Lee; SG Kang; SG Suh; JK Byun, *Mol. Cells*, **2003**, 15, 68–74.
[6] R Klewicki, *Food. Chem*., **2007**, 100, 1196–1201.
[7] WG Cochran, GM Cox. Experimental Designs, 4th edition, Wiley Publishers, New York, **1992**.
[8] SLC Ferreira; RE Bruns; HS Ferreira; GD Matos; JM David; GC Brandao; EGP Silva; LA Portugal; PS Reis; AS Souza WNL Santos, *Anal. Chim. Acta.*, **2007**, 597, 179–186.
[9] J Li; C Ma; Y Ma; Y Li; W Zhou; P Xu, *Appl. Microbiol. Biotechnol*., **2007**, 74, 563–571.
[10] R Usha; KK Mala; CK Venil; M Palaniswamy, *Pol. J. Microbiol.*, **2011**, 60, 213-21.
[11] RL Plackett; JP Burman, *Biometrika*, **1946**, 34, 255–272.
[12] DG Hatzinikolaou; E Katsifas; D Mamma; AD Karagouni, *Biochem. Eng. J*. 2005, 24, 161–172.
[13] GEP Box; DW Behnken, *Technometrics*, **1960,** 2, 455–475.
[14] P Soni; M Singh; AL Kambel; UC Banerjee, *Bioresource. Technol*., **2007**, 98, 829-833.
[15] RA Fisher, *J. Min. Agri*., **1926**, 33, 503-513.
[16] JP Guilford, B Fruchter. Fundamental statistics in psychology and education. McGraw-Hill, New York, **1973**.
[17] PD Haaland. Statistical problem solving. In: Haaland P.D. (Ed.), Experimental Designs in Biotechnology. Marcel Dekker, Inc., New York and Basel, **1989,** 1-18.
[18] M Elibol, *Process Biochem.*, **2004**, 39, 1057-1062.
[19] R Manimekalai; T Swaminathan, *Bioproc. Eng*., **1999,** 21, 465-468.
[20] JZ Liu; LP Weng; QL Zhang; H Xu; LN Ji, *World. J. Microbiol. Biotechnol*., **2003**,19, 317-323.