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Statistical Optimization of β-1,3 Glucan Production from the Novel Strain *Bacillus cereus* LVK13 (KC 898956)

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β-1,3 glucan, an Exopolysaccharide (EPS) that has enormous industrial applications can be produced commercially through microbial fermentations. The huge market demand necessitates the industries to search for new sources or to implement new strategies to maximize the yield of glucan. Hence, the present study focused on optimization of components of the culture medium and process variables to increase â-1,3 glucan production from *Bacillus cereus* LVK13 (KC 898956) using statistical tools. The essential nutrients that influence â-glucan production was screened using Plackett-Burman Design (PBD). Further, the three-screened components out of the eleven were analyzed with central composite design (CCD). The optimum concentrations of carbon, nitrogen and manganese sources were found to be 80, 1.5 and 0.03 g/l. Second order polynomial regression model accurately showed interpretation of experimental data with an R^2 value of 0.9563, Adjusted (Adj) R^2, Predicted (Pred) R^2 and F values of 0.9474, 0.9329 and 182.69 respectively.

Keywords: â-glucan, Sucrose, *Bacillus cereus* LVK13, Optimization, RSM.

Exopolysaccharides (EPSs) are either soluble or insoluble polymers obtained from various sources such as bacteria, yeast, fungi, or cereal plants. Many of these utilized in bioindustries are of microbial origin. Among this, â-Glucan, a homopolysaccharide of glucose bonded via â-(1,3 or 1,4 or 1,6)-D-glycosidic linkage is widely used in food and pharmaceutical industries due to its high anticancer and immunomodulatory effects[1].

EPSs can be produced by bacteria under all conditions, but the yield and chemical nature of EPS are strain dependent and affected by the nutritional and environmental conditions. The EPSs can be mass produced by fermentation of microorganisms on suitable media that aids increased yield of the product with stable costs independent of seasonal variations[2,3]. The structure, composition and viscosity of EPS depend on a number of factors such as the composition of culture medium, type of strain, and fermentation conditions[4].

Jung *et al* (2007) suggests the need for intensive research on bacterial glucan since the industries are using only the glucan from mushrooms and yeast lysate even they are low in purity and yield due to the availability of diminutive information about bacterial glucans[5].

It is a prerequisite to design an appropriate production medium in an efficient fermentation process to achieve higher cell density and yield of the desired metabolic product or enzyme[6]. Plackett-Burman design is used as the

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preliminary step to screen key factors among the process variables[7], which is generally followed by the steepest ascent (descent) method and central composite design[8]. Statistical analysis offers tools for optimizing medium components and perhaps response surface methodology (RSM) is the most widely used statistical methods for optimizing medium components. RSM can be used to find out most favourable conditions, ranges of controllable variables, and polynomial equations generation and also to assess relationships between controllable variables and observed results[9,10].

In the current research, the fermentation media for the production of â-1,3 glucan by the novel bacterial strain *Bacillus cereus* LVK13 (KC 898956)[11] was optimized by Plackett-Burman design and Response surface methodology.

MATERIALS AND METHODS

Microorganism and maintenance

Bacillus cereus LVK13 isolated from the agricultural field soil of Sathyamangalam, Tamil Nadu, India with NCBI gene repository accession number KC 898956 was used throughout this study. The isolate was maintained in nutrient agar slants containing the following ingredients (g/l); Peptone - 5, yeast extract - 1.5, beef extract - 1.5, NaCl - 5, agar - 15 at 4°C for further studies.

Production conditions

Seed culture was prepared by inoculating loopful of isolate on to seed medium containing (g/l): peptone - 5, yeast extract - 5 incubated at 30°C for 20 hours on a rotary shaker at 180 rpm. Approximately 10% (v/v) of seed culture was transferred to production medium containing (g/l); Sucrose - 100, KH_2PO_4 - 1.74, $CaCl_2.2H_2O$ - 0.015, K_2HPO_4 - 0.49, $MnCl_2.4H_2O$ - 0.01, $Na_2SO_4.10H_2O$ - 3.7, Citrate - 0.21, $MgCl_2.6H_2O$ - 0.25, NH_4Cl - 1.5, $FeCl_3.6H_2O$ - 0.024. 10% sucrose as carbon source was added to the media in order to induce glucan production. The initial pH of the medium before sterilization was adjusted to 6.5. Glucan was produced by shake flask culture at 30°C on a rotary shaker at 180 rpm and the product was analyzed periodically up to 96 hours[11].

Optimization of â-glucan production

Plackett-Burman design

The relative importance of various components of the medium that influences the production of glucan was evaluated using Plackett-Burman design based on the first-order polynomial model:

$$Y = \beta 0 + \sum \beta iXi$$

Where Y is the response, âo is the model intercept, âi is the linear coefficient and Xi is the level of the independent variable. In the present work, 11 assigned variables were screened with 14 treatment combinations. Each independent variable was tested at high (+1) level and low (-1) level (Table 1) using Minitab 17 statistical software package. From regression analysis, the variables which are significant at the 95% level ($p<0.05$) were considered to have a greater impact on glucan production.

Response surface methodology

CCD was used for determining the optimum concentration of the significant factors that are screened using Plackett-Burman design. The second-order polynomial model used to correlate the relationship between the glucan yield and medium components was

$$Y = \beta_o + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 \Sigma\Sigma\beta_{ij} X_i X_j$$

Where Y is the predicted response, âo, âi, âii, âij are the model constant, linear coefficient, quadratic coefficient and interaction coefficient respectively. Xi and Xj are the coded independent variables or factors.

The experimental design protocol for RSM was developed using Minitab 17 statistical software package. The analysis of variance (ANOVA) table was generated and the effect and regression coefficients of individual linear and quadratic interactions were determined. The importance of all the terms in the polynomial was predicted statistically by computing the F value at a probability (p) of 0.05. Regression coefficient was used to make statistical calculations to create response surface curves from the regression models.

In order to test the model accuracy, R^2, adjusted R^2 ($Radj^2$) and predicted R^2 ($Rpred^2$) were assessed. Kolmogorov-Smirnov normality test was performed for normality assumption and the outliers were checked by studentized residual values. Using Minitab response optimizer, the second-order polynomial equation was maximized

under a global solution of desirability equal to one to obtain the optimal levels of the independent variables. The accuracy of the values was verified by comparing the predicted values obtained with the mathematical model and the measured values obtained after the experiments under the same conditions.

RESULTS AND DISCUSSION

Assessment of factors affecting glucan production

The first optimization step was a 14 run Plackett-Burman design to find out the significant factors affecting glucan production by *Bacillus cereus* LVK 13 (KC 898956). A wide variation in glucan content from 0.52 - 3.95 g/l was obtained with three replicates (Table 2). This variation reflected the significance of factors.

Table 1. Range of variables used in the Plackett - Burman design and Response Surface Methodology

S.No	Variables	Code	Levels (g/l)		
			-1	0	+1
1	Sucrose	X1	4	8	12
2	Glucose	X2	4	8	12
3	Galactose	X3	4	8	12
4	NH_4Cl	X4	0.5	1.5	2.0
5	KNO_3	X5	0.5	1.5	2.0
6	$NaNO_3$	X6	0.5	1.5	2.0
7	KH_2PO_4	X7	0.5	1.75	3.0
8	K_2HPO_4	X8	0.1	0.5	1.0
9	$MnCl_2$	X9	0.01	0.03	0.05
10	$MgCl_2$	X10	0.1	0.25	0.4
11	$FeCl_3$	X11	0.02	0.06	0.1

The analysis of regression coefficients and the t value of the three medium components (Table 3) demonstrated that sucrose (X1), ammonium chloride (X4) and manganese chloride (X9) had significant effects on glucan production. KNO_3 (X5) and Glucose (X2), $NaNO_3$ (X6) and $FeCl_3$ (X11) were found to be insignificant with positive coefficients. Neglecting the variables that were insignificant, the first-order model equation for glucan production is:

$$Y = 0.37 + 0.1127\ X1 + 0.0127\ X2 - 0.0594\ X3 + 0.0261\ X4 + 0.0183\ X5 + 0.0061\ X6 + 0.648\ X7 - 1.14\ X8 + 13.0\ X9 + 8.40\ X10 - 0.65\ X11$$

Pareto Chart (Fig. 1) states the effects of different variables on the response. Based on this, X1, X4 and X9 were selected for further optimization that had the most significant effects on â-1,3 glucan production.

Optimization of culture conditions by RSM

The three components, X1, X4 and X9 determined by Plackett Burman Design was further optimized to maximize the production of glucan using response surface methodology. The run order was provided by Minitab 17 statistical package. The corresponding measured and predicted values are analyzed for the variance. Table 4 shows that the quantity of other variables

Table 2. Plackett-Burman design for 11 variables

Run Order	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Y (â-1,3 glucan yield)
1	1	1	-1	1	1	-1	1	-1	-1	-1	1	3.11
2	-1	-1	1	1	1	-1	1	1	-1	1	0	1.9
3	-1	1	1	1	-1	1	1	-1	1	-1	0	2.12
4	-1	1	1	-1	1	-1	0	-1	1	1	1	1.56
5	0	0	0	0	0	0	-1	0	0	0	-1	2.79
6	-1	-1	-1	1	1	1	0	1	1	-1	1	1.29
7	1	1	-1	1	-1	-1	0	1	1	1	0	2.65
8	-1	1	-1	-1	-1	1	1	1	-1	1	1	1.85
9	1	1	1	-1	1	1	0	1	-1	-1	0	0.96
10	1	-1	-1	-1	1	1	1	-1	1	1	0	3.95
11	1	-1	1	1	-1	1	0	-1	-1	1	1	2.05
12	1	-1	1	-1	-1	-1	1	1	1	-1	1	1.93
13	-1	-1	-1	-1	-1	-1	0	-1	-1	-1	0	0.52
14	0	0	0	0	0	0	-1	0	0	0	-1	2.86

were same as those in the standard media. Data were analyzed using Minitab 17 and the mathematical expression relating the glucan yield with variables is shown below:

$$Y = 0.015 + 0.4127\ X1 - 0.0552\ X4 - 29.4\ X9 - 0.01102\ X1*X1 + 0.00282\ X4*X4 + 484\ X9*X9 + 0.00004\ X1*X4 + 1.036\ X1*X9 + 0.342\ X4*X9$$

To test the fit of the model equation, the regression based determination R^2 coefficient was evaluated. The R^2 value provides a measure of how much variability in the observed response values can be interpreted by the experimental factors and their interactions. The R^2 value is always between 0 and 1[12,13]. The model conferred a high determination coefficient (R^2=0.9563) explaining 95.63% of the variability in the glucan production. The adjusted determination coefficient ($Radj^2$) and predicted determination coefficient ($Rpred^2$) were 0.9474 and 0.9329 respectively.

The $Radj^2$ corrects the R^2 value for the sample size and for the number of terms in the model. Normality test was performed for judging the model adequacy which showed a p value of >0.010. Hence, confirming the normality assumption. The studentized residual values were calculated for checking the outliers. All the values are within the range of -2 and +2, thereby affirming the model (Fig. 2). According to Anderson *et al* (2005) studentized residual values greater than -3.5 and +3.5 are regarded as outliers[14].

Table 3. Statistical analysis of a Plackett - Burman design showing coefficient values and t and P values for each variable for glucan production

Term	Coefficient	t	P
Constant	0.01429	139.33	0.005
Sucrose	0.01429	31.55	0.020
Glucose	0.01429	3.56	0.174
Galactose	0.01429	-16.62	0.038
NH_4Cl	0.01429	13.71	0.046
KNO_3	0.01429	9.62	0.066
$NaNO_3$	0.01429	3.21	0.192
KH_2PO_4	0.01429	34.00	0.019
K_2HPO_4	0.01429	-15.92	0.040
$MnCl_2$	0.01429	18.14	0.035
$MgCl_2$	0.01429	23.50	0.027
$FeCl_3$	0.01429	-1.81	0.322

Table 4. CCD experimental design matrix of three variables in real units and amount of â-1,3 glucan production

Run Order	X1	X4	X9	Yield (g/l) Predicted	Expected
1	0	0	0	2.39	2.38
2	1	1	-1	3.53	3.54
3	0	0	0	2.38	2.38
4	1	1	-1	3.54	3.54
5	-1	-1	1	1.39	1.36
6	-1	1	-1	1.29	1.29
7	1	1	1	4.29	4.29
8	1	-1	1	3.95	3.27
9	1	-1	1	3.93	3.27
10	-1	1	-1	1.29	1.29
11	1	-1	-1	3.24	3.26
12	1	-1	1	3.94	2.45
13	0	0	0	2.37	2.38
14	1	1	1	4.31	4.29
15	-1	-1	-1	1.17	1.15
16	0	0	0	2.35	2.38
17	0	0	0	2.33	2.38
18	-1	-1	-1	1.15	1.15
19	-1	-1	-1	1.12	1.15
20	1	-1	-1	3.27	3.26
21	0	0	0	2.36	2.38
22	0	0	0	2.35	2.38
23	0	0	0	2.39	2.38
24	0	0	0	2.36	2.38
25	0	0	0	2.39	2.38
26	0	0	0	2.37	2.38
27	-1	-1	1	1.36	1.36
28	1	-1	-1	3.26	3.26
29	4	-1	1	1.33	1.36
30	1	1	1	4.28	4.29
31	-1	1	1	1.84	1.85
32	-1	1	-1	1.29	1.29
33	-1	1	1	1.85	1.85
34	1	1	-1	3.56	3.56
35	0	0	0	2.38	2.38
36	-1	1	1	1.83	1.85
37	0	0	0	2.37	2.38
38	0	1	0	2.65	2.64
39	0	0	-1	2.23	2.21
40	1	0	0	2.63	2.63
41	0	0	0	2.37	2.38
42	0	0	1	2.47	2.45
43	0	0	0	2.38	2.38
44	0	0	-1	2.23	2.21
45	0	0	1	2.45	2.47
46	0	0	-1	2.2	2.21
47	-1	0	0	1.31	1.32
48	1	0	0	2.64	2.63
49	1	0	0	2.63	2.63
50	0	0	0	2.38	2.38
51	-1	0	0	1.32	1.32
52	0	-1	0	1.97	1.32
53	0	1	0	2.64	2.64
54	0	-1	0	1.96	1.97
55	-1	0	0	1.32	1.32
56	0	-1	0	1.96	1.97
57	0	0	0	2.37	2.38
58	0	1	0	2.63	2.64
59	0	0	0	2.35	2.38
60	0	0	1	2.44	2.47

The correlation plot was made between the measured values of glucan content and the predicted values determined by the model (Fig. 3). The R^2 value was found to be 94.2% and the Pearson correlation of predicted yield and expected yield was 0.958. For each variable, model coefficients were predicted by regression analysis (Table 5). The significance of each coefficient was determined by t and P values and the larger t and the smaller P value indicate the high significance of the corresponding coefficient[15,16]. A value of p <0.05 implies that the model is significant. The results revealed that sucrose, ammonium chloride and manganese chloride have a significant effect on glucan production. Positive coefficients of sucrose, ammonium chloride and manganese chloride indicated a linear effect of the increase in glucan production.

The graphical depiction provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between test variables to deduce the optimum conditions. One such response surface representing glucan production in the present study was a function of the concentration of sucrose and ammonium chloride

Table 5. Regression analysis of CCD for glucan production

Term	Coefficient	t	P
Constant	2.2970	58.39	0.000
X1	1.0723	30.63	0.000
X4	0.1947	5.56	0.000
X9	0.483	6.96	0.000
X1* X1	-0.325	-2.61	0.012
X4* X4	0.1587	2.35	0.023
X9* X9	0.1937	2.87	0.006
X1* X4	0.0013	0.03	0.975
X1* X9	0.0829	2.12	0.039
X4* X9	0.0512	1.31	0.197

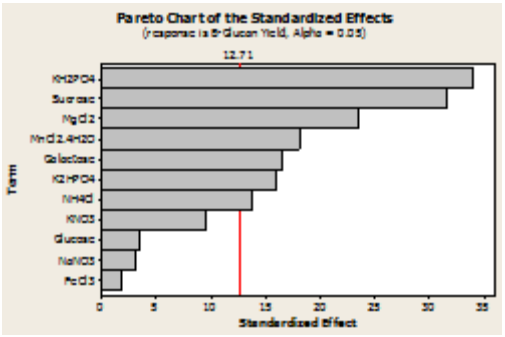

Fig. 1. Pareto chart showing the effect of media components on glucan production

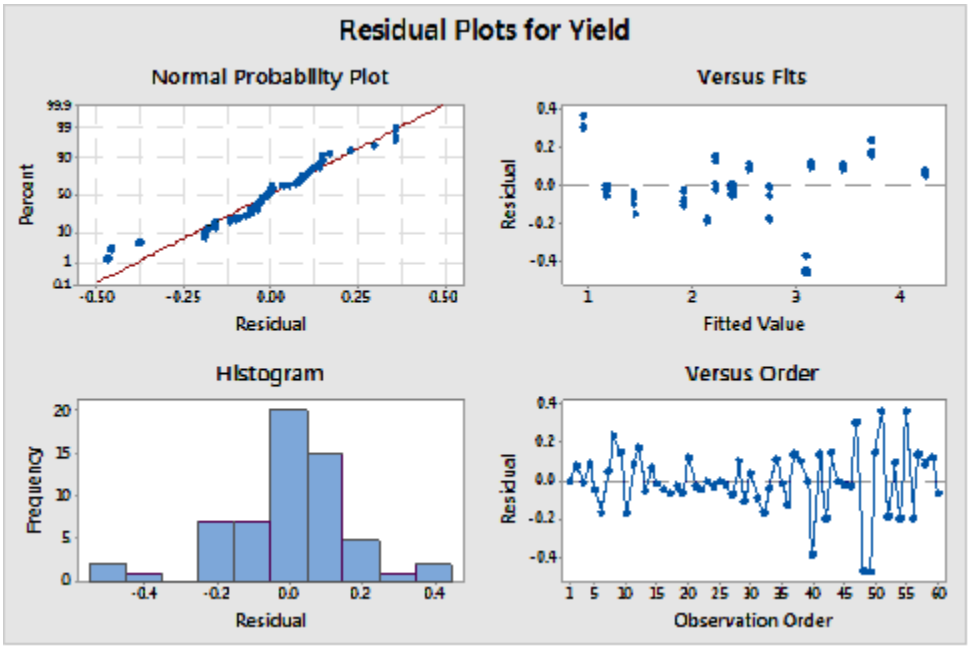

Fig. 2. Residual Plots for yield

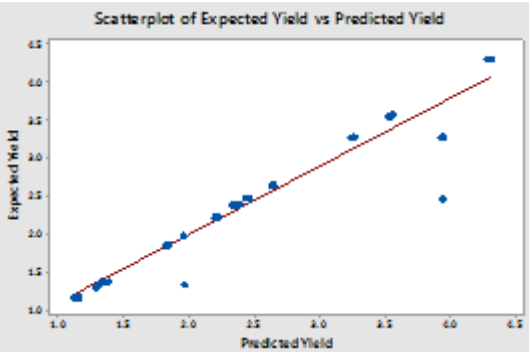

Fig. 3. Linear correlation plot between measured Vs. predicted glucan yield

with manganese chloride at an optimum level (Fig. 4). The steep slope shows that glucan production is sensitive to that factor.

The model predicted a maximum glucan yield of 4.31 g (Dry weight) per litre of the fermentation medium by solving the regression equation and analyzing the response surface plot using Minitab software. The optimum levels of the remarkable variables are sucrose - 80 g/l; ammonium chloride - 1.5 g/l and manganese chloride - 0.03 g/l. To validate the predicted model, three experiments were conducted using this optimum medium composition.

Glucan yield of 4.12 g/l was obtained at this medium composition, which agreed well with the predicted value 4.31 g/l. As a result, the developed model was considered accurate and reliable for the production of â-1,3 glucan from *Bacillus cereus* LVK13 (KC 898956).

CONCLUSION

The three components, sucrose, ammonium chloride and manganese chloride were determined through Plackett Burman Design to be the most significant variables forming essential nutrients for the growth and production of â-1,3 glucan. The optimized concentration of sucrose, ammonium chloride and manganese chloride are statistically analyzed by Response Surface Methodology using CCD and a significant mathematical model with a co-efficient determination of $R^2 = 0.95$. The interactive effects of the variables were determined to be significant. The optimum concentration of the process variables is: sucrose (80 g/l), ammonium chloride

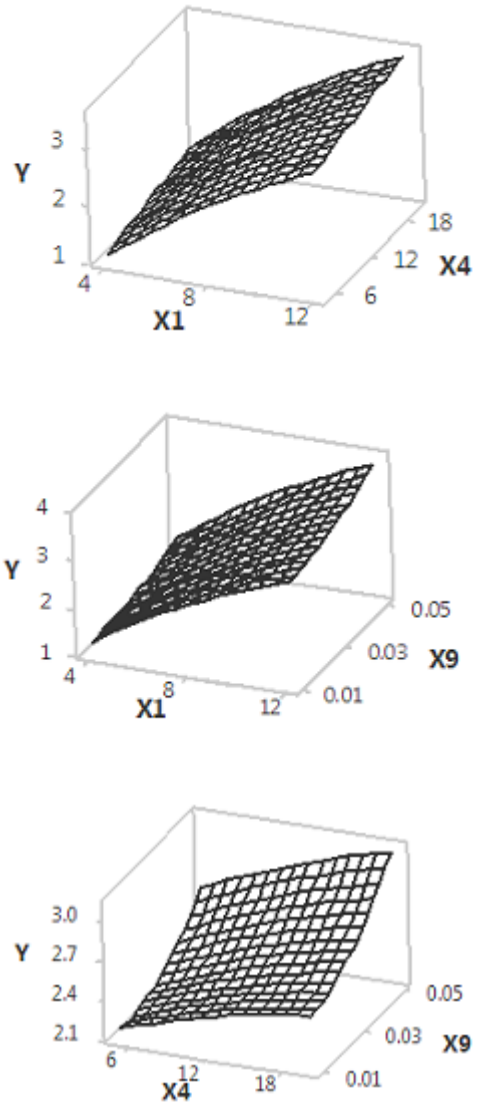

Fig. 4. Response surface curves of glucan production showing interaction between various factors

(1.5 g/l) and manganese chloride (0.03 g/l). Using the optimized components and concentrations, the glucan yield reaches 4.12 g/l. The results show a close concordance between the expected and obtained production level.

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