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Application of RSM to Optimize Medium for Exo-Polysaccharide Production by *Bacillus* sp

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The combined effect of various media components for exo polysaccharide production by *Bacillus* spp was studied using RSM. The extracted polysaccharide showed good anti tumor activity and an attempt was made to increase the production of EPS using RSM .The temperature (30°C±1°C) and pH value (7.0± 0.1) were kept constant throughout the experimental time. A conventional method was used to fix the best yielding substrate and Box Behnken Design was employed to design the composition of the media components. The optimal value for the critical components was obtained as follows. Sucrose -30 gm, Yeast extract -2 gm ,Calcium chloride - 4.3gm,Casamino acid -10ml/l with the predicted value of the model 10.02gm/l ±0.001. A quadratic polynomial analysis was applied for predicting the various responses of media components. Under an experiment the yield of exo polymer was 10gm/l ± 0.001. The determination coefficient (R^2) was 0.9813 that ensures adequate credibility of the model.

Key words: Optimization , Box-Behnken design, Exopolysaccharide, Response surface methodology.

Media are traditionally optimized by one-at-a time strategy i.e. varying one factor while keeping all others constant. Though the conventional method is simple and easy to apply without the need for statistical analysis, designing more number of experiments is uneconomical. Using statistical methods to design an optimal medium is economical and often accurate with fewer residues.

RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of various factors and conclude with an optimal condition that gives best results. RSM has been successfully used in optimization of many Bioprocesses.

Interest in exo polysaccharide producing bacteria has increased because of its importance in determining the rheological properties of dairy products and many non dairy applications[1,2]. Nitrogen limiting conditions increase the production of EPS. The effect of various stimulating agents on the production of EPS was studied[3]. In recent years there has been a continuous search for new water soluble polysaccharides, particularly those produced by microorganisms. These polymers have been attracting interest due to their great application potential in food, cosmetic, pharmaceuticals and oil industries. The reach of optimized fermentation conditions particularly associated to physical and chemical parameters is of primary and great importance for the development of any process due to their impact upon its economics and practicability[4,5]. Production of Exo polysaccharide by a soil bacterium influenced by pH and temperature was determined by conventional method insitu[6]. Optimization of

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media components (glucose, peptone & salt solution) have been predicted to play a significant role in enhancing the production of alkaline protease[1,7]. Box Behnken is a response surface methodology used to examine the relationship between one or more response variables and a set of quantitative experimental parameters. In this work the design is used to determine the interaction of 4 variables on the yield of Exo polysaccharides. The critical values of the independent variable were estimated using a statistical package of Design Expert – 7. The study aims to optimize media component to increase polysaccharide production for *Bacillus* sp. isolated from a kitchen source and proved as anti-cancer agent.

MATERIALS AND METHODS

Microbiology

A new strain of *Bacillus* sp. isolated from a kitchen source was found to produce water soluble polysaccharides that are active anti-oxidant and anti-tumor agents. It was grown at 37°C for 24 hours and maintained on nutrient agar slants at 4°C and sub cultured at 4 weeks intervals.

Preparation of Inoculum: An 18 hour 50 ml stock culture was prepared for inoculation by inoculating 50ml nutrient broth in 250ml flask. The composition of the medium includes 1% glucose and 1ml casamino acid in excess. The flask was kept on a rotary shaker at 180rpm at 37°C.

Optimization by RSM

The method involves number of empirical techniques to evaluate the correlation of experimental factors and predict the critical concentration of dependent and independent variables. However a prior knowledge and understanding of the process and process variables facilitate more realistic model. Based on prior experiments sucrose, yeast extract, calcium chloride and casamino acid were found to be major variables in polymer production when temperature and pH were kept constant.

Thus, these variables were selected to find the optimized conditions for higher polymer production, using CCD & RSM .The range and the levels of experimental variables investigated in this study are presented in Table 1.

The central value zero chosen for experiment were glucose – 2gm/100ml, yeast extract – 0.3gm/100ml, salt – 0.45gm/100ml and 1ml of Casamino acid .For statistical calculations the variables X_i were coded to x_i according to eq. 1.

$$X_i = x_i (x_i - x_i^2) \Delta x \qquad ...1$$

After considering several experimental designs, a four variable experimental design proposed by Box Behnken was used to optimize the critical composition required for high yield of Exo polysaccharide Table 2. A quadratic model was used to estimate the response of dependent variable .Where Y is predicted response and A, B, C, D are independent variables, b_0 is constant and b_1 ,b_2 ,b_3 ,b_4 are coefficients.

$$Y = b_o + b_1 A + b_2 B + b_3 C + b_4 D \qquad ... 2$$

The production was optimized by using Box Behnken design when EPS production is related to independent variables by a response eq. 3

$$Y = R_0 + \Sigma R_i X_i + \Sigma 2 R_{ii} X_i 2 + \Sigma\Sigma R_{ij} X_i X_j + E \qquad ...3$$
$$Y = f(x_{1,} x_{2,} x_3, x_4 \ldots\ldots\ldots\ldots x_n)$$

The true relation between Y and X may be complicated; in most cases it is not known. A quadratic polynomial can be used to represent the function in the range interest (Annadurai & Sheeja) where $X_{1,}$ $X_{2,}$ are independent variables which affect the response Y. R_o, R_i, R_{ii}, R_{ij} (i=1-k and j=1-k) are known parameters, E is the random error.

Statistical testing of the model was done by partial sum squares – Type III ANOVA and the results are tabulated Table 4. The calculation of regression analysis gives the value of the determination coefficient (R^2 = 0.9813) which indicates that only 1.87% of the total variations are not explained by the model and the P value Prob F less than 0.0500 indicate model terms are significant. Thus in this model sugar, yeast, calcium chloride and casamino acid. Sugar + casamino acid, Yeast + casamino acid are significant terms.

RESULTS AND DISCUSSION

The statistical analysis of the design shows a high precision of the quadratic model that reflects the high degree of fitting between the predicted and the experimental data. The data obtained by varying sugar concentration, yeast extract, calcium chloride and casamino acid is tabulated in table-3.

This great similarity between the predicted and the observed results reflects the accuracy and the applicability of the Box Behnken model in the optimization process.

As the isolated EPS is reported to good anti-oxidant and anti-tumor property at its low concentration an attempt is made to increase its yield. Neeraj *et al.,* showed that the EPS and LPS production by *Sinorhizobium* freedi can be induced by Tn 5 mutants infecting vigna radiate. Besides the linear effect of the substrate/exopolysaccharide factor, Yp/s the response surface method also gives an insight about the parameters quadratic and combined effect[8,9]. Thus in this experiment the yield increases as the yeast concentration is limiting (Fig. 1). Whereas, the sugar and casamino acid shows a linear effect in increasing the yield of polysaccharide (Fig. 2,3).

All the calculations involved as well as the drawing of all three dimensional surface have been obtained using the Design-Expert software Version-7.

Table 1. Range and the levels of experimental variables

Code Unit	Sugar	Yeast Extract	Salt	C.A.A.
-1	1	0.2	0.40	0.5
0	2	0.3	0.45	1.0
+1	3	0.4	0.50	1.5

Table 2. Box Benhken Design

Sucrose	Yeast Extract gm/100ml	Calcium Chloride gm/100ml	Casamino acid gm/100ml	EPS Yield ml/100ml
2.00	.3	.4	1.5	.65
2.00	.3	.4	.5	.55
2.00	.2	.5	1	.54
2.00	.4	.5	1	.5
3.00	.3	.45	1.5	.95
2.00	.3	.50	1.5	.5
2.00	.2	.45	1.5	.76
2.00	.3	.45	1	.7
1.00	.3	.45	.5	.45
2.00	.3	.45	1	.62
2.00	.3	.50	.5	.45
2.00	.2	.40	1	.68
1.00	.3	.45	1.5	.4
3.00	.4	.45	1	.89
1.00	.3	.50	1	.4
3.00	.3	.40	1	.88
1.00	.4	.45	1	.45
3.00	.2	.45	1	1
3.00	.3	.50	1	.83
1.00	.2	.45	1	.48
2.00	.4	.45	.5	.6
2.00	.4	.40	1	.56
1.00	.3	.40	1	.4
2.00	.3	.45	1	.68
3.00	.3	.45	.5	.8
2.00	.3	.45	1	.67
2.00	.4	.45	1.5	.64
2.00	.3	.45	1	.6
2.00	.2	.45	.5	.55

Table 3. Observed and Predicted value

Test No.	Observed value	Predicted value	Residual value
1.	.48	.48	_3.750
2.	1.00	.99	.001
3.	.45	.46	7.083
4.	.89	.88	7.917
5.	.55	.53	0.023
6.	.45	.47	_0.015
7.	.65	.63	0.020
8.	.50	.52	_0.019
9.	.45	.44	.013
10.	.80	.80	_2.5
11.	.40	.42	_0.016
12.	.95	.98	_0.031
13.	.68	.68	4.167
14.	.56	.57	_9.167
15.	.54	.55	_9.167
16.	.50	.52	_0.022
17.	.40	.42	_0.019
18.	.88	.90	_0.019
19.	.38	.35	0.033
20.	.83	.80	0.033
21.	.55	.57	_0.017
22.	.60	.60	_4.167
23.	.76	.75	.015
24.	.61	.58	.031
25.	.67	.65	.016
26.	.68	.65	0.026
27.	.70	.65	0.046
28.	.60	.65	0.054
29	.62	.65	0.034

The model allowed the evaluation of the effects of linear, quadratic and combined effects of the independent variables. The p-values were used as a tool to check the significance of the interaction effects among the variables. A p-value lower than 0.01 indicates that the model is considered to be statistically significant .Smaller value of p indicates the corresponding coefficient term[10].

The statistical significance of the ratio between the mean square variation, due to regression and the mean square residual error was tested using analysis of variation. (Table 4). The ANOVA table also shows a term for residual error which measures the amount of variation in the response data left unexplained by the model. The type of the model chosen to explain the relationship between the factors and the response is quadratic polynomial model which shows the model to be highly significant and adequate to represent the actual relationship between the response and the input variables with very small p-values (0.0001).

The process for EPS production is carried out under constant temperature and pH. According to[11,12] for a rise in biomass and polymer from aerobic microorganisms a vigorous aeration is required and thus the work approach towards optimizing the aeration and agitation[13].

Attempted to investigate the effect of various stimulating agents on EPS synthesis in a

Table 4. ANOVA

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F value	P value
A	.65	1	.057	52.43	<.0001
B	0.013	1	.65	594.40	.0036
C	0.023	1	.013	12.22	.0005
D	0.018	1	.023	20.65	.0011
AB1.600E-003	1	1.600E-003	.018	16.87	-
AC2 250E-004	1	2.250E-004	1.47	0.2460	-
AD0.010	1	0.010	0.21	.6567	-
BC1 .006E-003	1	1.600E-003	9.16	.0091	-
BD0.010	1	0.010	1.47	.2460	-
CD6.250E-004	1	6.250E-.004	9.16	.0091	-
$A^2$0.012	1	0.012	.57	.0054	-
B^2.602E-004	1	2.602E-004	10.78	.6329	-
$C^2$0.043	1	0.043	0.24	<0.001	-
$D^2$9.081E-003	1	9.081E-003	39.16	.0120	-
Pure error	7.120E-003	4	8.32	_	-
Correlation total	.82	28	_	_	-

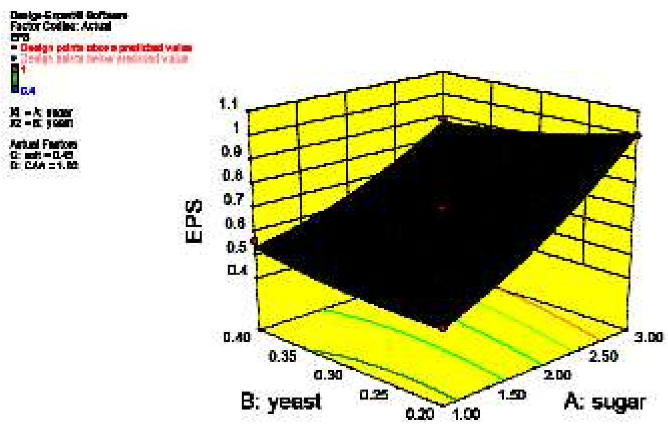

Fig. 1. EPS Yield based on sugar and yeast concentration

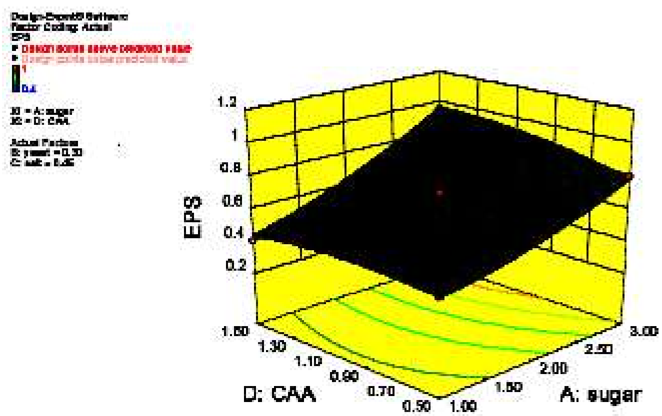

Fig. 2. EPS Yield based on varying concentrations of sugar and casaminoacid

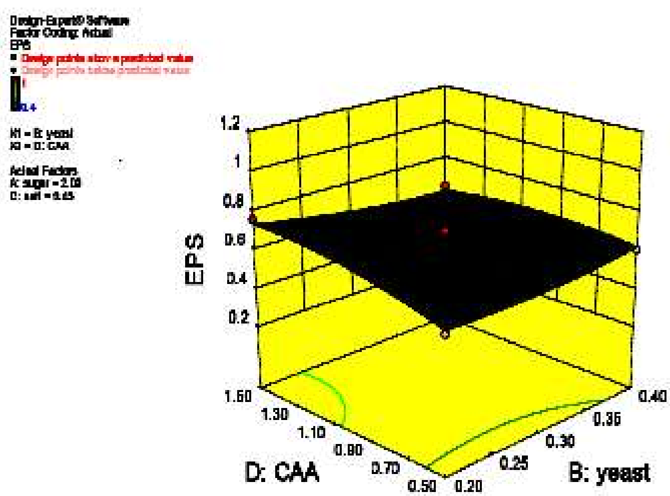

Fig. 3. EPS Yield based on casamino acid and yeast

batch culture by *Rhizobium legumosarum*, *Leuconostac mesenteroides* and reported that the highest yield was with corn oil yielding 8.61 g/g biomass.

Reported data indicate that the production of EPS is more during limiting of nitrogen as reported by[14,15]. Use of statistical methods for optimization process was proved to a cost effective process as reported by .Here in this process use of RSM has helped to locate the optimal level of media components for EPS with minimum time and effort.

CONCLUSION

Sucrose being the best carbon source for EPS synthesis by Gram positive bacteria in contrast to Gram negative where the glucose serve as best carbon source . The production of EPS is more during limiting of nitrogen. Furthermore the sugar concentration had a marked effect on the EPS production. Thus by increasing the sucrose concentration the yield of this exo polysaccharide increases. In the present study the yield of EPS has been increased 2-fold with minimum effort and time.

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