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Modeling and Optimization of Synthetic Production Medium for the Production of Xanthan Gum using Response Surface Methodology

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Response surface methodology (RSM) was used to evaluate the effects of variables, namely the concentration of glucose, citric acid, KH_2PO_4 and NH_4Cl, in the synthetic production medium for xanthan production. Central composite design was used in the design of experiments. The experimental results showed that the optimum concentration of the components were (in g/l): glucose, 34.20; citric acid, 2.11; KH_2PO_4, 4.01 and NH_4Cl, 2.13. The maximum xanthan production was 12.5 g/L. This method was efficient; only 24 experiments were necessary to assess these conditions, and model adequacy was very satisfactory as the coefficient of determination was 0.9856.

Keywords: optimization, central composite design, response surface methodology, synthetic production medium, xanthan production.

Xanthan gum, an extracellular polysaccharide produced by bacterium *Xanthomonas campestris*[1] is an important industrial biopolymer because of its unique properties e.g. aqueous solution of xanthan gum has comparatively high viscosity and stability over a wide range of temperature and pH[2,3]. It has wide applications in food, cosmetic and pharmaceutical industries as a suspending, stabilizing and thickening agent[4].

Xanthomonas bacteria need a carbon source, a nitrogen source, and other nutrients including potassium, phosphorus, magnesium, iron and calcium salts to produce xanthan gum. Previous authors have studied the influence of different nutrients, carbon and nitrogen sources on the xanthan production by limiting nutrient technique. Davidson[5] showed that polysaccharide production was improved using carbon and phosphorus sources as limiting nutrients in the production medium. Souw and Demain[6] found glucose and sucrose as the best carbon sources and glutamate as the best nitrogen source at a level of 15 mmol, with an inhibitory effect on growth at higher concentrations. Addition of low amount of some organic acids, such as citric or succinic, in the production medium improve xanthan production. *Jana et al.*[7] also reported that the addition of small quantity of citric acid in the production medium enhances the pyruvic content of xanthan gum, resulting in higher viscosity of its aqueous solutions. Further Souw *et al.*[8] reported that citrate at concentration 4.7 - 9.4 mmol stimulated xanthan formation in pH-uncontrolled fermentation in several media, when pH was controlled the beneficial effect was

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eliminated. Tait *et al.*[9] studied xanthan production using batch, and continuous culture, and found that the growth and the production were increased when nitrogen and carbon concentration decreased. De Vuyst[10] found that bacterial growth and xanthan production were increased by low and high C/N ratio respectively and therefore suggested a two-step fermentation process[11]. Other authors also reported similar findings with variation in the concentrations of carbon and nitrogen sources and in fermentation conditions[12-15]. Garcia-Ochoa[16] showed that nitrogen, phosphorous and magnesium influenced growth whereas nitrogen, phosphorus, and sulfur influenced the production of xanthan. During nutritional studies several authors have reported the application of both synthetic and complex production medium[17-19].

The aim of this work is to develop an empirical model and the optimization of synthetic production medium components for the production of xanthan gum by means of Response Surface Methodology (RSM), a powerful statistical tool able to deal with this type of problem. RSM has been successfully applied in related fields for optimization of the process raw materials and conditions for the production of final product[20-24]. The medium components selected for optimization (variables) were glucose, citric acid, KH_2PO_4 and NH_4Cl (these have been predicted to play a significant role in enhancing the production of xanthan gum) and the other components (trace nutrients: Na_2SO_4, $MgCl_2.5H_2O$, $FeCl_3.6H_2O$, $ZnCl_2$, $CaCl_2.2H_2O$, H_3BO_3, Na_2CO_3) were kept at constant concentration in the medium. The individual and interaction effects of the components on the xanthan production were also examined with the help of contour plots. This will helpful for further study on xanthan gum production, like enhancement and cost reduction of xanthan gum production, by providing these nutrients from natural or cheaper substrates.

MATERIAL AND METHODS

Microorganism

Xanthomonas campestris NCIM 2956 was used in the present study. It was maintained on YMPGA medium at 4°C and subcultured every 15 days.

Inoculum preparation

Inoculum was prepared by transferring cells from freshly prepared slant to test tube containing 2.5 ml growth medium and afterwards scaling it up to the required volume in Erlenmeyer flasks using 10 % inoculum. The composition of the growth medium was (in g/l): glucose, 10; peptone, 5; yeast extract, 3; malt extract, 3; with pH 7.0. The flasks and test tubes were kept on an orbital shaker at 220 rpm and at 28°C. After 24 h incubation, the whole broth was taken as inoculum for next stage.

Fermentation experiments

The production medium contained (in g/l) : Na_2SO_4, 0.114; $MgCl_2.5\ H_2O$, 0.163; $FeCl_3.6\ H_2O$, 0.0014; $ZnCl_2$, 0.0061; $CaCl_2.2\ H_2O$, 0.012; H_3BO_3, 0.006; Na_2CO_3,0.500; and variables (glucose, citric acid, KH_2PO_4 and NH_4Cl).

The range and levels of the variables are given in Table 1. Concentrations of these variables were varied in production medium according to the experimental design shown in Table 2. pH was adjusted to 7.0 with 1 N NaOH solution before sterilization. The solution containing carbon source (i.e. glucose) was sterilized separately and added to the medium at the time of inoculation.

Table 1. Experimental range and levels of the variables

Variables	Levels and Range				
	-2	-1	0	1	2
A: glucose (g/l)	18	24	30	36	42
B: citric acid (g/l)	0	1	2	3	4
C: KH_2PO_4 (g/l)	2	3	4	5	6
D: NH_4Cl (g/l)	1	1.5	2	2.5	3

The fermentation experiments were carried out for 48 h in orbital shaker (Künher, Switzerland) at 30°C and 220 rpm using 250-ml Erlenmeyer flasks containing 50 ml of specific medium with 10 % inoculum.

Analytical method

Xanthan was measured as culture viscosity with a Brookfield Viscometer (LVT) at room temperature using spindle no. 4 and 60 rpm. Calibration was done by the solutions of precipitated xanthan. Xanthan was precipitated from broth sample with isopropyl alcohol at an

alcohol/broth ratio of 3:1. The precipitate was washed and dried.

Experimental design

Response surface methodology is a collection of mathematical and statistical technique that is useful for the modeling and analysis of problem in which a response of interest is influenced by several variables and the objective is to optimize the response.

In order to describe the nature of the response surface in optimum region, a central composite design (CCD) with five coded levels was performed. For the four variables, this design was made up of a fractional 2^{4-1} factorial design with its eight cube points, augmented with eight replications of the center points (all variables at level '0') and the eight star points i.e. points having, for one factor, an axial distance to the center of $\pm \alpha$, whereas the other factors are at level '0'. The axial distance α was chosen to be 2. The complete CCD is given in Table 2.

For statistical calculations, the variable Xi was coded as x_i according to the following equation:

$$xi = \frac{X_i - X_o}{\delta X} \qquad ...(i)$$

Where,

X_i is the coded value of the i^{th} variable.
X_i is the real value of the i^{th} variable.
X_o is the real value of the i^{th} variable at the centre point.
δX is the step change value.

The central values (level '0') of the variables chosen for experimental design were (in g/l): glucose, 30.0; citric acid , 2.0; KH_2PO_4, 4.0; NH_4Cl, 2.0 ; and other coded values are

Table 2. Central composite design consisting of 24 experiments for the study of four experimental variables in coded units.

Experiment No.	A: glucose	B: citric acid	C: KH_2PO_4	D: NH_4Cl	Coefficien t assessed by
1	-1	-1	-1	-1	Fractional 2^{4-1}
2	1	-1	-1	1	factorial design
3	-1	1	-1	1	
4	1	1	-1	-1	
5	-1	-1	1	-1	
6	1	-1	1	1	
7	-1	1	1	1	
8	1	1	1	-1	
9	2	0	0	0	Star points
10	-2	0	0	0	(8 points)
11	0	2	0	0	
12	0	-2	0	0	
13	0	0	2	0	
14	0	0	-2	0	
15	0	0	0	2	
16	0	0	0	-2	
17	0	0	0	0	Central points
18	0	0	0	0	(8 points)
19	0	0	0	0	
20	0	0	0	0	
21	0	0	0	0	
22	0	0	0	0	
23	0	0	0	0	
24	0	0	0	0	

given in Table 1.

For predicting the optimal point, a second order polynomial function was fitted to the experimental results (given below):

$$Y=\beta_o+\sum_{i=1}^{k}\beta_i x_i+\sum_{i=1}^{k}\beta_{ii}x_i x_i+\sum_{i<j}^{k}\beta_{ij} x_i x_j \quad ...(ii)$$

where, Y is response variable.
β_o is the interception coefficient.
β_i is the linear effect of variables.
β_{ii} is the quadratic effect of variables .
β_{ij} is the interaction effect of variables ($i \neq j$).

Data analysis

The Design -Expert software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots.

RESULTS AND DISCUSSION

The final concentrations of xanthan obtained with 24 experiments in the chosen experimental design are given in Table 3.

The application of response surface methodology[25-29] yielded the following regression equation, which is an empirical relationship between the xanthan produced and variables:

$$Y = 12.33 + 0.35 * A + 0.25 * B + 0.094 * C + 0.28 * D - 0.24 * A^2 - 1.04 * B^2 - 1.05 * C^2 - 0.58 * D^2 - 0.11 * A * B - 0.11 * A * C + 0.013 * A * D + 0.088 * B * C + 0.21 * B * D - 0.012 * C * D \quad ...(iii)$$

where,

Y is the response, i.e. the xanthan produced in g/l and A, B, C, and D are the coded values of the variables glucose, citric acid, KH_2PO_4 and NH_4Cl respectively.

The results of second order response surface model fitting in the form of analysis of variance (ANOVA) are given in Table 4. The model F-value of 44.02 implies that the model is significant. The goodness of fit of the model was checked by the determination coefficient (R- squared). In this case the value of determination coefficient (R- squared = 0.9856) indicates that only 1.44 % of the total variations are not explained by the model. The value of the adjusted determination coefficient (Adj R- squared = 0.9632) is also very high to advocate for a high significance of the model. A relatively lower value of the coefficient of variation (CV =3.12%) indicates a better precision and reliability of the experiments carried out. All the above considerations indicate a good adequacy of the regression model.

Table 3. Final xanthan concentration of each experiment

Experiment No.	Xanthan conc.(g/l)	Experiment No.	Xanthan conc.(g/l)
1	8.8	13	8.7
2	10.1	14	7.4
3	9.9	15	10.5
4	9.6	16	9.4
5	8.6	17	12.2
6	9.4	18	12.3
7	10	19	12.4
8	9.3	20	12.3
9	12	21	12.3
10	10.6	22	12.4
11	8.6	23	12.3
12	7.6	24	12.4

The significant of each term of the model was determined by F-values and values of "Prob>F" (Table 4). The larger the magnitude of F-value and the smaller the value of "Prob>F ", the more significant is the corresponding coefficient[26,28]. This implies that the first order main effects of all variables are less significant as compared to their quadratic main effects, because of their respective high "Prob>F" values. This indicates that little variations in their concentration will alter final product concentration. Further the "Prob>F" values of terms B^2 and C^2 are very low as their respective F-values are very high showing that the little variation in concentration of variables (B: citric acid and C: KH_2PO_4) in the medium plays a important role in the final product formation.

Contour plot of the response surface as a function of two variables at a time, holding all other variables at fixed levels ('0', for instance), are more helpful in understanding both the individual and the interaction effects of these two variables. These

Table 4. ANOVA for Response Surface Quadratic Model Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	FValue	Prob > F	
Mode	64.46	14	4.6	44.02	< 0.0001	significant
A	0.98	1	0.98	9.37	0.0135	
B	0.5	1	0.5	4.78	0.0566	
C	0.14	1	0.14	1.34	0.2761	
D	0.6	1	0.6	5.78	0.0396	
A2	1.67	1	1.67	15.95	0.0031	
B2	30.9	1	30.9	295.41	< 0.0001	
C2	31.64	1	31.64	302.54	< 0.0001	
D2	9.56	1	9.56	91.4	< 0.0001	
AB	0.051	1	0.051	0.48	0.5042	
AC	0.1	1	0.1	0.97	0.3509	
AD	6.25E-04	1	6.25E-04	5.98E-03	0.9401	
BC	0.061	1	0.061	0.59	0.4637	
BD	0.18	1	0.18	1.73	0.2213	
CD	1.25E-03	1	1.25E-03	0.012	0.9153	
Residual	0.94	9	0.1			
Lack of Fit	0.91	2	0.45	90.62	< 0.0001	significant
Pure Error	0.035	7	5.00E-03			
Cor Total	65.4	23				
And,						
Std. Dev.	0.32	R-Squared		0.9856		
Mean	10.38	Adj R-Squared		0.9632		
C.V.	3.12	Pred R-Squared		0.4136		
PRESS	38.35	Adeq Precision		18.261		

Note: This table is an output of Design-Expert software.
Terms used in table can be easily understood from the software's help.

plots can be easily obtained by calculating from the model, the values taken by one variable where the second varies (from -2 to +2, step 0.5 for instance) with constraint of a given Y value. The response values of different concentrations of the variables can also be predicted from the respective contour plots (Figs. 1-6). The maximum predicted response value is indicated by the surface confined in the smallest curve in the contour diagram.

The slope of each contour plot of each variable is almost independent of the concentration of the other. These contour plots show that the optimal concentrations of the variables are (aprox): glucose, 0.7; citric acid, 0.1; KH_2PO_4, 0.0 and NH_4Cl, 0.2 in coded unit and the corresponding natural values are (in g/l) 34.2, 2.1, 4.0 and 2.1 respectively. However the interactive effect of two variables on the xanthan production is estimated by the nature of plot; elliptical nature shows that variables have interactive effect and circular nature shows that there is no interactive effect of the variables.

From the plots, it can be concluded that variables (citric acid and KH_2PO_4) have a interaction with other variables (Figs. 1,2,5,6; having elliptical nature) but there is no interaction between them (Fig. 4; having circular nature) for the final product formation, showing that these variables plays a more sensitive role in the xanthan production. Fig. 3 shows that a minor influence of the nitrogen source on the glucose concentration.

It was also concluded from the ANOVA that citric acid and KH_2PO_4 are sensitive medium components and a little variation from their optimum concentrations will cause an inhibitory effect on xanthan production. Previous reports also

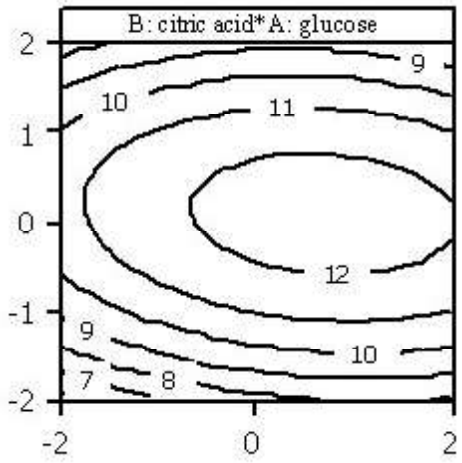

Fig. 1

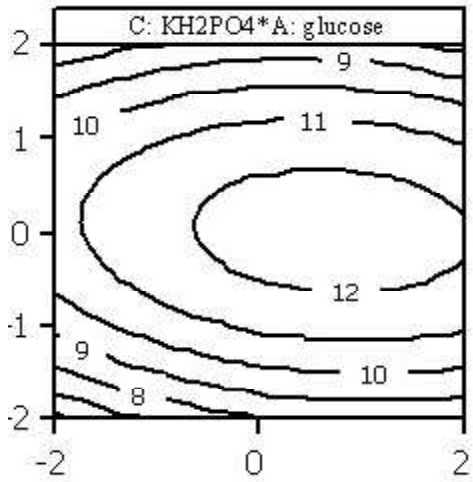

Fig. 2

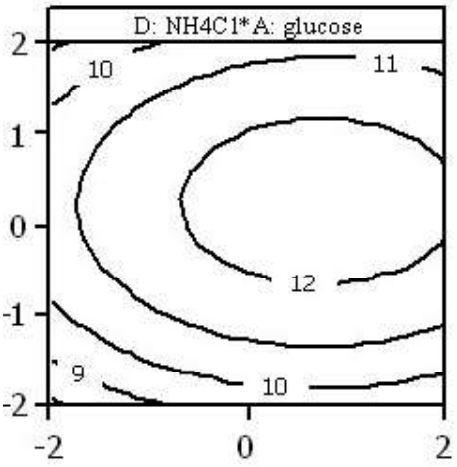

Fig. 3

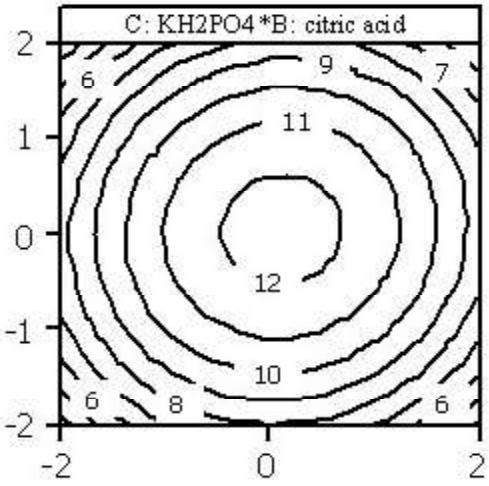

Fig. 4

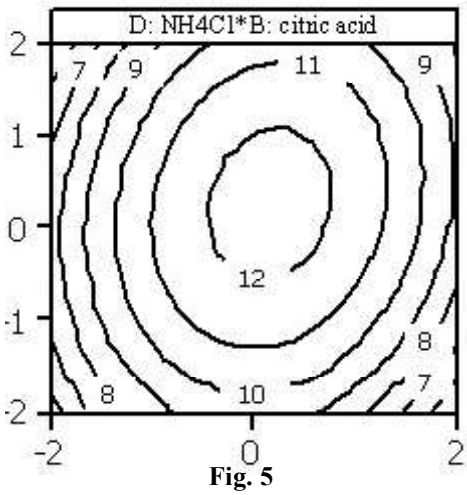

Fig. 5

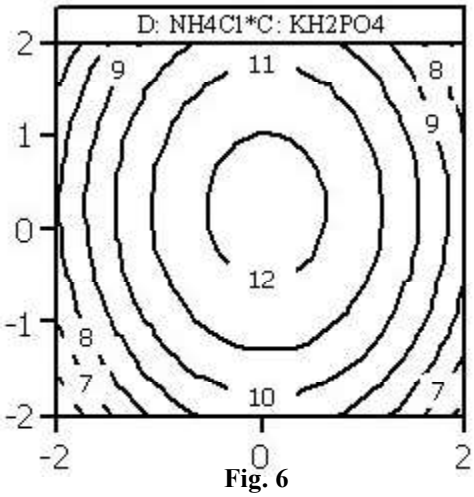

Fig. 6

Fig. 1-6. Contour plots; effect of two variables and their interaction on xanthan production. Other variables held at zero level.

support our findings indicating that improvement in xanthan production when these are used as limiting nutrients in the production medium[5-16].

The optimum values of the medium components were obtained by solving the regression equation (iii) and these are found to be (in coded units) as follows: glucose, 0.70; citric acid, 0.11; KH_2PO_4, 0.01 and NH_4Cl, 0.26. The coded values were substituted in equation (i) to obtain the natural values (in g/l); glucose, 34.20; citric acid, 2.11; KH_2PO_4, 4.01 and NH_4Cl, 2.13. The model predicts that the maximum xanthan concentration can be obtained using the above optimum concentrations of the variables is 12.4983 g/l. The verification of the results using the optimum concentrations was accomplished by carrying out shake flask experiment, which showed final concentration of xanthan of about 12.5 g/l, a close agreement with the model predictions.

CONCLUSION

Although the media optimization for xanthan gum production for a standard culture is very old story, but in best of our knowledge the use of response surface methodology with study of four major medium components has not been carried out in past time. In the present study, response surface methodology was proved to be the potent tool in optimizing medium composition and in the study of collective effect of the components for xanthan production by *Xanthomonas campestris* NCIM 2956. As mentioned earlier this study will further helpful us for the cost reduction of xanthan gum production by providing these nutrients from natural or cheaper substrates.

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