

ORIGINAL ARTICLE

Application of Response Surface Methodology for Xanthan Gum and Biomass Production Using *Xanthomonas campestris*

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KEYWORDS

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ABSTRACT: Xanthan gum is an extracellular polysaccharide produced by various *Xanthomonas* species such as *X. campestris*. The objective of present study was to investigate the influence of different carbon and nitrogen sources on xanthan gum production by *X. campestris*. Using an experimental Response Surface Methodology (RSM) complemented with a Central Composite Design (CCD), the impact of peptone, lactose, glucose and ammonium nitrate in medium were estimated for their individual and interactive effects on biomass and xanthan gum production. The optimal concentrations of peptone, lactose, glucose and ammonium nitrate for xanthan gum yield and biomass production was determined as 9.25 g/l, 53.37 mmol, 29.31 mmol and 4.58 g/l for xanthan gum yield and 6.77 g/l, 52.65 mmol, 38.12 mmol and 3.54 g/l for biomass production. Under the optimum experimental conditions, the xanthan gum yield reached to its maximum value (8.42 g/l). The results provide the support data for xanthan gum production on a large scale.

INTRODUCTION

In recent years, due to health-related problems of chemically synthesized additives and changes in consumer's preferences for natural and socially more acceptable additives, use of natural additives have led to an extensive research on developing healthy food.

The primary structure of xanthan gum as a hetero polysaccharide is made of repeated pentasaccharide units consisting of two glucose and two mannose, and one glucuronic acid unit (molar ratio of 2.8:2.0:2.0). Xanthan gum's toxicity and safety for food products

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and pharmaceutical applications have been largely studied by many scientists. It is a non-sensitizing material and does not cause skin or eye irritation. The use of xanthan gum as a food additive without any particular quantitative restrictions has been approved by FDA (the United States Food and Drug Administration) [1]. In 1980, it was added to the list of food emulsifier/stabilizer (as item E-415) by EEC (the European Economic Community). Xanthan gum is a bio-material being produced by microorganism and due to its excellent properties and has a wide range of applications in food formulations, pharmaceutical industries, petroleum industry, cosmetics and personal, care products and agriculture [2]. For different important reasons as temperature and emulsion stabilization, compatibility with other food products and finally, its pseudo-plastic rheological properties, xanthan gum has been widely used in various food products [3].

X. campestris is used for producing xanthan gum. Cells of *Xanthomonas* are aerobic, Gram-negative and straight rods (usually 0.4-0.7 wide * 0.7-1.8 μm long) with a single polar flagellum. Their colonies usually occur in yellowish color, smooth and butyrous or sticky [4]. It can be cultured at different temperatures ranging between 25 and 35 °C in neutral pH [1].

Experimental design consists of a small set of experiments, in which the levels of all contributing variables are changed simultaneously in a systematic manner. This approach offers several advantages over conventional experimental methods based on changing levels of one variable at a time, while keeping the other variables constant. The changing levels of one variable at a time approach provides no information about what happens when factors are varied simultaneously (ignores interactions), provides less information about the variability of the response, provides no mapping of experimental space and therefore does not lead to real optimum. In contrast, the experimental design is composed of mutually connected experiments that are linked in a logical manner, thus, it provides more precise information about the studied system, because the joint influence of all factors is assessed. By subse-

quent analysis of data, the optimal conditions, the factors that most influence the results and the presence of interactions can be determined [5, 6].

Among different types of experimental design, the RSM has become the standard approach for much of the experimentation carried out for optimization purposes, both in laboratory and industry. RSM is mostly concerned with approximating a complex unknown function with a polynomial, usually either a first-order model or a second-order model. Therefore, designs for matching the models are of great importance which results in the estimation of interaction and even quadratic effects. Thus, they give an idea of the shape of the understudy response surface, accordingly called response surface designs. [7]. A response surface design include some significant characteristics as follows: minimum residuals or errors of prediction, minimum number of treatment combinations, desirable information distribution across the experimental domain, good paucity of fit detection and good graphical analysis in the simple data patterns [8].

A response surface methodology was successfully applied for the optimization of medium constituents and other critical reaction parameters by fermentation [9, 10]. Response surface methodology overcomes the limitations of single parameter optimization, which is both time-consuming and cannot assess the complex interactions among the various physicochemical parameters [11].

In the present study, optimization of xanthan gum production by *X. campestris* in batch experiments was attempted using response surface methodology where the simultaneous effect of the four independent variables (peptone, lactose, glucose and $(\text{NH}_4)_2\text{NO}_3$) were investigated for optimal xanthan and biomass production. Optimization of xanthan gum production which leads to increase in xanthan gum production efficiency and finally reduction the cost of final product is inevitable

MATERIALS AND METHODS

Microorganism and inoculum preparation

X. campestris ATCC 33913, a wild-type strain, was used throughout this study. A synthetic medium (yeast malt) containing: 3.0 g/L of yeast extract and malt extracts; 5.0 g/L of peptone and 10.0 g/L of glucose at pH=7 was used as the inoculum medium. The preparation of the inoculum was performed by the transfer of the microorganism from the stock solution to the yeast malt agar plates (YM agar) and its subsequent incubation for 48 h at 30 °C. A single colony of cells from the (YM) plates was then transferred to a 100 mL conical flask containing 25 mL of the sterile YM medium and incubated for 24 h at 30 °C and 180 rpm. This was ultimately used as the inoculum medium. Fermentation was carried out in 250 mL Erlenmeyer flasks, each of which contained 50 mL of the sterile production medium. The medium was inoculated for 24 h with 5% (v/v) of the *X. campestris* culture.

Analytical Methods

Determination of dry cell weight (DCW)

The cells were collected after centrifugation for 30 min at 12,000 rpm while the supernatant was discarded. The biomass was subsequently washed twice with alcohol to remove traces of xanthan before being subjected to another centrifugation for 10 min at 9000 rpm. The cells were then dried in a hot air oven for 3 h at 105 °C and finally weighed.

Xanthan gum production and concentration

Xanthan production was made through aerobic fermentation in batch in an orbital shaker set at 30 °C and 180 rpm for 72 h. The fermented broth was centrifuged for 30 min at 12,000 rpm to remove bacterial cells.

The cell-free supernatant (10 mL) obtained through the procedures described above was then added to three volumes of ice cold ethyl alcohol, and the mix-

ture was kept at 4 °C for 12 h to precipitate xanthan gum. Afterwards, the precipitate was recovered by centrifugation for 30 min at 4 °C and 10,000 rpm. The xanthan gum separated by centrifugation was then washed with ethyl alcohol and dried in a hot air oven for 24 h at 50 °C. The production of the biopolymers of this strain was evaluated by measuring the weight of the dry product per liter of fermented broth and the average was expressed in g/L.

Experimental design and data analysis

Central composite design and response surface methodology

The effects of four variables (peptone, lactose, glucose and ammonium nitrate) on xanthan gum production in flasks were studied using central composite design [12] and response surface methodology [13–15]. The independent variables were investigated at five different levels (–2, –1, 0, +1 and +2), and then coded according to the following equation (regression) (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3, \dots, k \quad (\text{eq. 1})$$

Where,

x_i and X_i are respectively the coded and real independent variables, X_0 is the value of X_i at center point and ΔX_i the value of step change [16]. A total of thirty experiments were conducted in flasks with each factor at five different levels (Table 1). The regression coefficients were calculated and the obtained experimental data were compatible with a second-order polynomial model. The model equation is given by:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j, \quad i = 1, 2, 3, \dots, k \quad (\text{eq.2})$$

Where,

Y is the response variable, β_0 the constant, β_i , β_{ii} and β_{ij} are respectively coefficients for the linear, quadrat-

ic and interaction effects, and x_i and x_j are the coded independent factors. The quadratic equation (eq. 2) was employed to plan the related surfaces for the variables [17, 3].

STATISTICAL ANALYSIS

Experimental design and subsequent regression analysis of the experimental data were performed using the Design Expert 7 [18, 19]. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) [20, 21]. The polynomial model equation's quality was statistically justified with respect to the determination coefficient (R^2) and its statistical significance was computed using an F-test approach.

Experimental validation of the optimized conditions

In order to validate the above optimization model, optimal conditions were tested in triplicate and compared with the predicted results from optimized conditions with student t test.

RESULTS AND DISCUSSION

According to the results of our preliminary experiments, the suitable concentrations of peptone, lactose, glucose and ammonium nitrate in medium for biomass and xanthan gum production were determined for further CCD experiments. Five levels of each variable were set by software of Design Expert, which are presented in Table 1. In the next step, 30 trials of CCD were carried out to optimize the production of biomass and xanthan gum production. The results of CCD experiments were summarized in Table 2.

Table 1. Coded levels of different variables in the central composite design

Variables	Coded levels					
	Unit	-2	-1	0	1	2
A: Peptone	g/L	0	3.75	7.5	11.25	15
B: Lactose	mmol	0	20.81	41.63	62.44	83.25
C: Glucose	mmol	0	13.88	27.75	41.63	55.50
D:(NH₄)₂NO₃	g/L	0	2.5	5	7.5	10

The biomass production and xanthan gum yield displayed a considerable variation from 0.72 to 2.22 and 6.47 to 8.47 g/L, respectively. Based on the results of CCD experiments, a second-order polynomial regression model between xanthan gum yield and the tested independent variables was derived by software of Design Expert as shown in equation 3:

$$Y = +3.787 + 0.154A + 0.038B + 0.124C + 0.464D + 0.0021 \times AB - 0.0032 AC + 0.016AD - 0.0021BD - 0.0024CD - 0.013A^2 - 0.0004 \times B^2 - 0.0014 \times C^2 - 0.046D^2 \quad (\text{eq. 3})$$

In order to determine whether the quadratic regression model was significant or not, the ANOVA ana

lyses were conducted, which are summarized in Table 3 and Table 4 for xanthan and biomass production, respectively. The ANOVA of the quadratic regression model demonstrated that the model was highly significant, evident from the Fisher's F-test with a very high model F-value (24.12 and 30.76 for xanthan gum and biomass production, respectively) but a very low p-value ($P < 0.0001$). The goodness of the model was examined by the determination coefficients (R^2) and the Pearson correlation coefficients (R).

Table 2. Experimental design (conditions and responses) for xanthan and biomass production

Run no.	Peptone (g/L)	Lactose (mmol)	Glucose (mmol)	(NH ₄) ₂ NO ₃ (g/L)	Xanthan gum (g/L)		Biomass (g/L)	
					Actual value	Predicted value	Actual value	Predicted value
1	3.75	20.81	41.63	2.50	6.99	7.14	0.72	0.90
2	3.75	20.81	13.88	2.50	7.01	7.09	1.29	1.43
3	7.50	41.63	27.75	0.00	7.11	7.28	1.45	1.24
4	7.50	0.00	27.75	5.00	7.72	7.91	2.16	2.21
5	3.75	62.44	13.88	7.50	7.77	7.74	1.85	1.86
6	3.75	62.44	13.88	2.50	7.10	7.00	1.48	1.38
7	7.50	41.63	27.75	5.00	7.90	7.87	2.00	2.20
8	3.75	20.81	13.88	7.50	8.03	7.82	2.20	2.15
9	7.50	41.63	27.75	5.00	7.06	6.88	0.92	0.91
10	11.25	62.44	41.63	2.50	7.63	7.43	1.52	1.44
11	11.25	20.81	41.63	2.50	6.47	6.56	0.83	0.88
12	7.50	41.63	27.75	5.00	7.94	7.80	1.92	1.85
13	11.25	62.44	13.88	2.50	7.33	7.47	1.59	1.49
14	7.50	41.63	27.75	5.00	7.35	7.34	0.85	1.00
15	11.25	20.81	13.88	7.50	7.29	7.15	1.65	1.45
16	3.75	20.81	41.63	7.50	7.75	7.71	1.46	1.41
17	11.25	20.81	13.88	2.50	7.45	7.33	1.16	1.19
18	7.50	41.63	0.00	5.00	7.65	7.83	1.68	1.68
19	7.50	41.63	27.75	10.00	7.25	7.29	1.48	1.38
20	0.00	41.63	27.75	5.00	7.77	7.79	1.99	2.12
21	11.25	20.81	41.63	7.50	7.11	7.00	0.95	0.92
22	11.25	62.44	13.88	7.50	7.33	7.50	1.38	1.44
23	7.50	83.25	27.75	5.00	7.51	7.36	2.08	1.96
24	7.50	41.63	27.75	5.00	6.77	6.98	1.07	1.22
25	3.75	62.44	41.63	2.50	8.26	8.34	2.22	2.11
26	11.25	62.44	41.63	7.50	8.36	8.34	2.18	2.11
27	7.50	41.63	55.50	5.00	8.20	8.34	2.03	2.11
28	3.75	62.44	41.63	7.50	8.33	8.34	2.13	2.11
29	15.00	41.63	27.75	5.00	8.41	8.34	1.98	2.11
30	7.50	41.63	27.75	5.00	8.48	8.34	2.10	2.11

The value of the determination coefficient adj-R² (0.9120 and 0.8921 for xanthan gum yield and biomass production, respectively) demonstrated that the total variation of 91.20% and 89.21% for xanthan gum yield and biomass production was attributed to the tested independent variables and only about 8.40%

and 10.79% for xanthan gum yield and biomass production of the total variation could not be explained by the model.

As presented in Table 2, the amount of residual value which calculated from differences between the experimental and predicted xanthan gum yield and biomass

production for the 30 trials of CCD were very small, nearly close to zero.

Table 3. Estimated coefficients of multiple determinations (R^2) for xanthan gum production using coded values

Source	Sum of squares	DF	Mean square	F- value	Prob > F
Model	7.57	13	0.58	24.12	< 0.0001
A-Peptone	0.38	1	0.38	15.63	0.0011
B-Lactose	0.38	1	0.38	15.65	0.0011
C-Glucose	0.38	1	0.38	15.86	0.0011
D-(NH₄)₂NO₃	0.22	1	0.22	9.04	0.0084
AB	0.47	1	0.47	19.28	0.0005
AC	0.47	1	0.47	19.31	0.0005
AD	0.37	1	0.37	15.28	0.0013
BD	0.20	1	0.20	8.47	0.0102
CD	0.11	1	0.11	4.71	0.0454
A²	0.98	1	0.98	40.61	< 0.0001
B²	1.09	1	1.09	45.02	< 0.0001
C²	2.02	1	2.02	83.79	< 0.0001
D²	2.33	1	2.33	96.58	< 0.0001
Residual	0.39	16	0.02		
Lack of Fit	0.34	11	0.03	3.18	0.1058
Pure Error	0.05	5	0.01		
Cor Total	7.96	29			

R^2 : 0.9515; Adj R^2 : 0.9120; Pred R^2 : 0.7782

The model's failure was measured by the lack-of-fit factor in order to represent the data at points not included in the regression. The F-value for lack-of-fit were 3.18 and 2.54 while the corresponding P -value were 0.105 and 0.156 (>0.05), which implied the lack-of-fit was not significant relative to the pure error due to noise. Insignificant lack-of-fit confirmed the validity of the model (Table 3 and 4).

The coefficients of the quadratic polynomial model, along with their corresponding p -values, are calculated and presented in Table 3 and 4 for xanthan

gum yield and biomass production, respectively. The P -value served as a device for controlling the significance of each coefficient. It was also used as an indication of the interaction strength between each independent coefficient.

The P -value had a reverse relationship with the significance of the obtained corresponding coefficient. According to Table 3 and 4, it can be inferred that all regression coefficients of the quadratic polynomial model with low P -values were highly significant.

Table 4. Estimated coefficients of multiple determinations (R^2) for biomass production using coded values

Source	Sum of squares	DF	Mean square	F- value	Prob > F
Model	6.27	12	0.52	30.76	< 0.0001
A-Peptone	0.43	1	0.43	25.12	0.0001
B-Lactose	0.73	1	0.73	42.86	< 0.0001
C-Glucose	0.34	1	0.34	19.78	0.0004
D-(NH₄)₂NO₃	0.94	1	0.94	55.57	< 0.0001
AB	0.26	1	0.26	15.31	0.0011

Table 4. Continued

AC	0.89	1	0.89	52.57	<0.0001
BD	0.20	1	0.20	11.66	0.0033
CD	0.21	1	0.21	12.46	0.0026
A ²	0.80	1	0.80	47.24	<0.0001
B ²	0.23	1	0.23	13.75	0.0017
C ²	1.51	1	1.51	89.01	<0.0001
D ²	0.50	1	0.50	29.34	<0.0001
Residual	0.29	17	0.02		
Lack of Fit	0.25	12	0.02	2.54	0.1564
Pure Error	0.04	5	0.01		

R²: 0.9384; Adj R²: 0.8921; Pred R²: 0.7430

Response Surface and Contour Plots Analyses

The graphical representations of the quadratic polynomial regression equation are the three-dimensional (3D) response surface and two-dimensional (2D) contour plots. Visualization of the relationship between each variable's responses and the experimental steps as well as the interaction of any two tested variables from the circular or elliptical nature of contour is made possible through them. A

circular contour plot is suggestive of the negligibility of the interaction of corresponding variables. Moreover, and elliptical nature of the contour is indicative of the significance of the interaction of corresponding variables. In the present study, the 3D response surfaces and 2D contour plots are presented in Figure 1 for xanthan gum and Figure 2 for biomass production, which were generated by employing the software of Design-Expert.

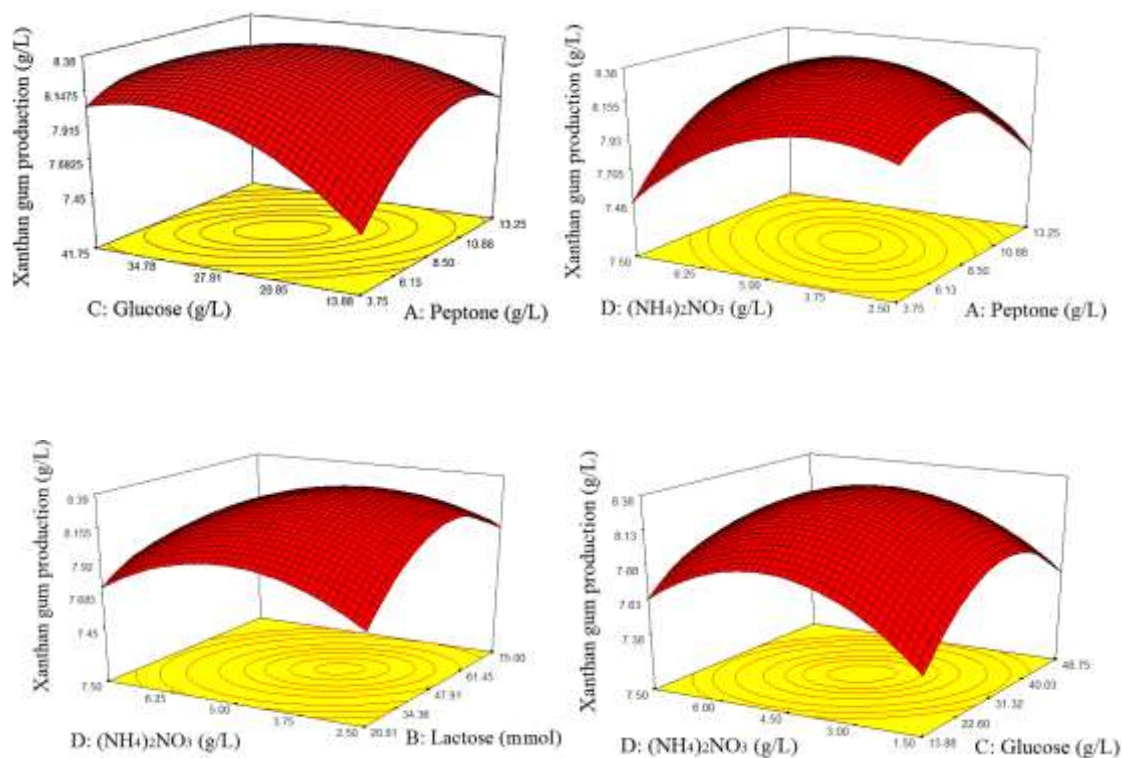


Figure 1. The 3D-response surface and 2D-contour plots of xanthan gum yield (g/L) versus the tested variables (g/L)

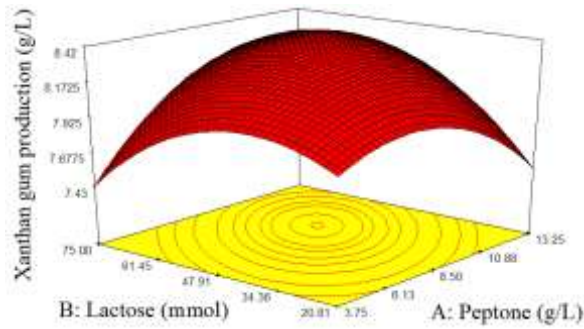


Figure 1. Continued

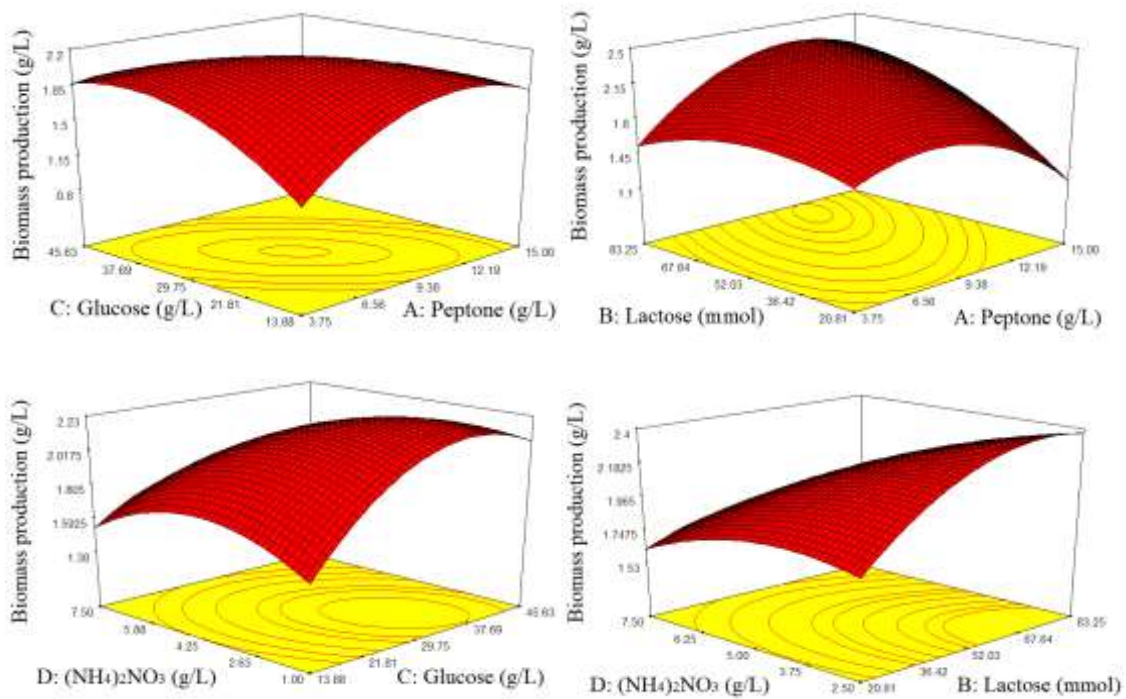


Figure 2. The 3D-response surface and 2D-contour plots of biomass production (g/L) versus the tested variables (g/L)

Studying the 3D response surfaces and their corresponding 2D contour plots led to the easy investigation of two variables' interaction and the efficient location of their optimum ranges until the response reached its maximum level. The confined surface in the contour diagram's smallest ellipse indicates the expected maximum response.

Plots of response surface, shown in Figure 1, represent the different variables' effects on xanthan gum yield and their interactions when other variables were fixed at zero level. Xanthan gum yield showed an

increasing tendency with the increasing of the concentrations of different variables, and then decreased slightly.

According to the response surface plot, an increase in the medium's carbon concentration increases the production of xanthan gum. In addition, xanthan production is similarly but slightly influenced by the phosphorous concentration. However, increased nitrogen concentration causes a decrease in gum production [22]. In fact due to its lack of participation in polysaccharide structure, high concentration of nitrogen

source is not suitable for xanthan production. However, it is only necessary for cell growth and enzyme production for catabolic and anabolic pathways of bacterial cells [22].

A significant effect of carbon source was observed on xanthan production. In fact, the xanthan production yield was noted to increase proportionally with the increase of the carbon source values from 40 to 80 g/L. This data can be explained that during microbial fermentation, the carbon source not only acts as a major constituent for the building of cellular materials, but is also used in the synthesis of these associate growth polysaccharides [23].

A full elliptic contour in Figure 1 was observed, showing an important interaction between the tested variables for xanthan gum production. It was consistent with the analyses of coefficients of the regression equation (Table 3 and 4). Figure 2 represents the effects of different variables on biomass production and their interaction when other variables were fixed at zero level. When the concentrations of all of the tested variables in medium were increased from the lowest to the highest levels, biomass production was increased initially and then decreased. The elliptic contour in Figure 2 indicated the significant interaction between peptone and glucose for biomass production.

The biomass production's contour plots are shown here. It can be noted that increased biomass production yields can be obtained at high nitrogen sources and at temperature values lowered from 35 to 30 °C, with the maximum at 3.74 g/L. The temperature of incubation was considered as an important factor in biomass biosynthesis [23]. All commercial polysaccharide-producing microorganisms are mesophiles [24].

By analyzing the 3D response surface and 2D contour plots, the corresponding point to the maximum of xanthan gum yield should be located on the response surface's peak, which is illustrated in the contour diagram's smallest ellipse.

Optimization of the variables and verification of the model

By solving the inverse matrix of the regression polynomial equation (eq. 3) employing the software of Design-Expert, the optimum values of the tested parameters in uncoded units were obtained as follows: peptone 9.25 g/L, lactose 53.37 mmol, glucose 29.31 mmol and ammonium nitrate 4.58 for xanthan gum production and peptone 6.77 g/L, lactose 52.65 mmol, glucose 38.12 mmol and ammonium nitrate 3.54 for biomass production. Under the optimum conditions, the prediction of xanthan gum yield reached to the maximum (8.42 g/L). The data were experimentally rechecked through the deduced optimal conditions in order to validate the appropriation of the model equation for portending the value of optimum response. Under the determined conditions, a mean value of xanthan gum yield of 8.42 g/L ($n = 5$) was obtained from the actual experiments, slightly higher than the predicted maximum value (8.42 g/L). Nevertheless, there was no significant difference between the expected and experimental yield at the time of *t*-test which presents the model as satisfactory and adequate for reflecting the expected optimization.

CONCLUSIONS

Xanthan production by *X. campestris* PTCC-1473 was studied by using RSM. The optimal concentrations of peptone, lactose, glucose and ammonium nitrate for xanthan gum yield and biomass production were determined as 9.25 g/L, 53.37 mmol, 29.31 mmol and 4.58 g/L for xanthan gum yield and 6.77 g/L, 52.65 mmol, 38.12 mmol and 3.54 g/L for biomass production, respectively.

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