Optimization of Xanthan Production with *Xanthomonas campestris* by Response Surface Methodology

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ABSTRACT

Xanthan gum, an important food additive, is used as a thickening agent and stabilizer in many food industrial applications. A 3-factor-3-level central composite rotatable design of response surface methodology was applied to model the fermentation parameters affecting xanthan production in a stirred tank bioreactor using *Xanthomonas campestris* TISTR 1100. The optimized production of xanthan gum was predicted and the interactive effects between fermentation parameters (yeast extract, fermentation time, and temperature) were investigated. Biomass concentrations, rheological property of fermentation broth and fermentation kinetics were evaluated and reported. Response surface analysis (RSM) showed that the data were adequately fitted to second-order polynomial model via quadratic regression relationship. The final mathematical model after eliminating the insignificant terms and refining the xanthan production was a quadratic model, xanthan yield = - 2.17 + 0.051 * fermentation+0.075 * temperature + 0.007 * fermentation * temperature - 0.002* fermentation^2 with R^2 = 0.965. RSM is an effective and useful method for optimizing the medium components and investigating the interactive effects, and can provide valuable information for xanthan scale-up fermentation using *Xanthomonas campestris* TISTR 1100.

Keywords: *Xanthomonas campestris* TISTR 1100, xanthan gum, response surface methodology, central composite design

Introduction

Xanthan gum has been used as an excellent additive substance for food applications because it has many advantages such as emulsion stabilization, temperature stability, compatibility with food ingredients, and its pseudoplastic rheological properties. Xanthan gum is therefore considered as the most well known heteropolysaccharide with a primary structure consisting of repeated pentasaccharide units formed by two glucose units, two mannose units, and one glucuronic acid unit, in the molar ratio 2.8 : 2.0 : 2.0 as a chemical structure shown in Figure 1. The growth of the microorganism and xanthan production are influenced by many factors such as the type of bioreactor used, the mode of operation (batch or continuous), the medium composition, and the culture conditions (temperature, pH, dissolved oxygen concentration) [1-3]. *Xanthomonas campestris* TISTR 1100 is one of extracellular product producing bacteria which is a plant pathogen in genus of the Pseudomonaceae family. Morphological study found that *Xanthomonas* cells occur as single straight rods, 0.4±0.7 mm wide and 0.7±1.8 mm long. The cells are motile, gram-negative, and they have a single

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polar flagellum (1.7±3 mm long) [1]. The microorganism is chemoorganotrophic and an obligate aerobe with a strictly respiratory type of metabolism that requires oxygen as the terminal electron acceptor. The bacterium cannot denitrify, and it is catalase-positive and oxidase negative. The colonies are usually yellow, smooth, and viscid [4]. Previously, the complete genome sequence of the *X. campestris* pv. *campestris* strain B100 was established. This investigation found that it consisted of a chromosome of 5,079,003 bp, with 4471 protein-coding genes and 62 RNA genes [5]. They also found from the comparative genomics showed that the genes required for the synthesis of xanthan and xanthan precursors were highly conserved among three sequenced *X. campestris* pv. *campestris* genomes, but differed noticeably when compared to the remaining four *Xanthomonas* genomes available [5]. This understanding lead us to further knowledge of the xanthenes synthesis. The xanthan synthesis is encoded by the *gum BCDEFGHIJKLM* genes, which are located in a single gene cluster of 12 kb that is mainly expressed as an operon from a promoter upstream of the first gene, *gumB* [6,7]. Xanthan synthesis is located at the cell membrane, where defined pentasaccharide repeat units of glucose–glucose–mannose–glucuronate–mannose as shown in Figure 1 are built from nucleotide sugars at a polyprenol lipid carrier [8]. *Xanthomonas* sp. is able to oxidize glucose and the Entner Doudoroff pathway is predominantly used for glucose catabolism [5].

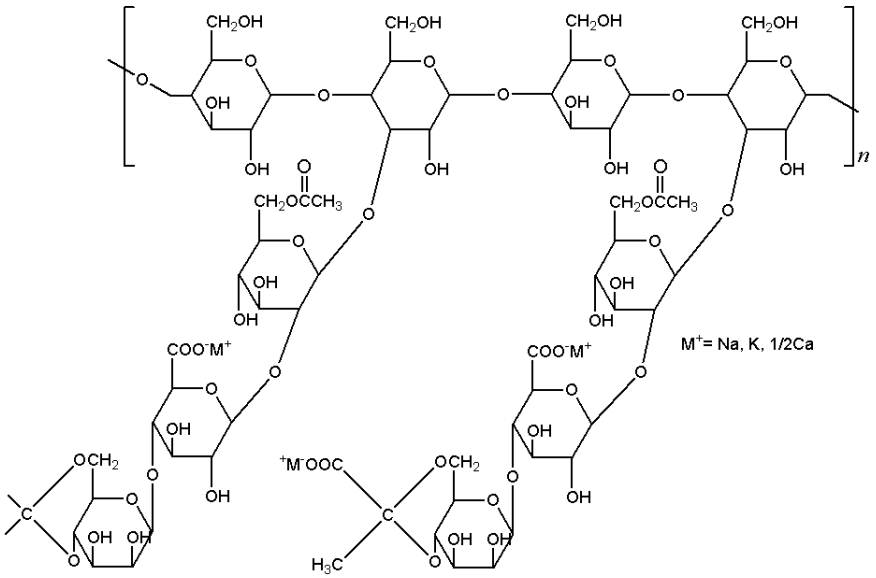

Figure 1 Chemical structure of xanthan gum [1]

The important concern for xanthan gum production is the process which depends on the chance of minimizing the operating cost and time and maintaining the production in high performance. With production of this product by *X. campestris* in a nutrient

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medium, the cells productivity could be improved by minimizing the possible occurrence of growth limiting nutrients in the medium [3]. Optimum pH at pH7 and the temperature ranging between 25 and 35°C were usually used as culture conditions [4]. Not only the growth kinetics and alternative substrates as coconut juice, molasses and modified media but also physical parameters for xanthan gum production from *X. campestris* TISTR 1100 have been reported and explored [9-11]. Recently, the attempt to implement by imitating the production process by simulation of the xanthan gum production in continuous fermentation systems and optimization of parameters effect on xanthan production is still explored [12,13]. The objective of this work is to optimize the xanthan production by *X. campestris* TISTR 1100 in batch fermentations using response surface methodology. The individual and interactive effects of three independent variables of nitrogen source, yeast extract (0.10-0.50%), fermentation time (0-90 h) and temperature (25–35°C) on xanthan gum and biomass production were studied, using a face-centered composite design of experiments.

Materials and methods

1. Microorganism and inoculum preparation

Xanthomonas campestris TISTR 1100 was used as biological catalyst throughout this experiment. Inoculum preparation was transferred from the stock solution to YM agar slants and incubated for 2 days at 28°C. Following this period, colonies were transferred into the broth media, which contained the following in g/L: yeast extract (1, 3, and 5); malt extract 3; peptone 5; glucose 10. Inoculum media was sterilized in 100 ml Erlenmeyer flasks at 121°C for 20 min. Following sterilization, the medium pH was 7. Cultures were then incubated at 28°C in an orbital shaking incubator with agitation of 250 rpm for 24 h until they reached an optimal density of 0.8 at 600 nm. These 10% (v/v) seed cultures were used as starter for initiate the fermentation in the stirred tank bioreactor.

2. Fermentations

Batch fermentations were carried out in a 3.7 litre benchtop fermenter (Type KLF 2000, Bioengineering AG, Wald Switzerland). The working volume was 2.7 L for both with various concentration of juice medium. Experiments were conducted at 25, 30, 35°C with aeration rate 150 L/h resulting in 15 ppm of the dissolved oxygen at 300 agitation rate in the fermentation broth under three supplement nitrogen sources, yeast extract at 0.10, 0.30 and 0.5 w/v. The broth cultures were harvested at the fermentation time of 0, 45 and 90 h. All runs were in duplicate and averaged values are presented in this work.

3. Analytical methods

3.1 Determination of viscosity and biomass concentration

Broth samples were taken at regular intervals. The apparent viscosity was measured in the fermentation broth. For viscosity determination a Brookfield viscometer DV-I (Massachusetts, USA) with spindle number 27 at 100 rpm was used. Biomass was calculated by dry cell-weight estimation. Aliquots of 5 mL

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was added 1% KCl to reduce the viscosity and then the cell were collected after centrifugation at 12,000 rpm for 30 min at 4°C. The supernatant was collected for determination of residue sugar and xanthan content. The biomass residue was then washed with 1 mL of conc. HCl and distilled water to remove traces of xanthan before passed through the 0.2 μm cellulose nitrate membrane. Finally, cell were dried to a constant weight at 60°C for 48 h and weighed.

3.2 Determination of xanthan gum concentration

The collected exopolysaccharide was recovered from the cell-free supernatant by precipitation with two volumes of 95% ethyl alcohol. The solution was then centrifuged at 14,000 x g at 4°C for 30 min. The supernatant was saved for total sugar determination. The precipitates were further rinsed with 95% ethyl alcohol and passed through 0.45 μm cellulose nitrate membrane. The residues were dried in an oven at 60°C for 48 h and weighed. Dry weight determination was then calculated as described in an earlier publication [14].

4. Response surface methodology and central composite design

In order to evaluate the effect of factors on the response surface in the region of investigation, a three-factor-five-level CCD was performed. Based on the best results of the one-at-a-time method, the ranges and levels of three variables; yeast extract (*A*), fermentation time (B) and temperature (*C*) are listed in Table 1. The statistical software package Design-Expert 8.0.6 was used to analyze the experimental design. The total number of experiments with three factors was 20 (2k+2k+6, when k=3, where *k* is the number of factors). The design matrix with three

Variables (yeast extract, fermentation time and temperature) × three levels (−1, 0, +1) are presented in Table 1. All the variables were taken at the coded values. In order to control the error bar, 20 runs were performed in a random order in which there were six replications at the center points to evaluate the pure error. Process performance was evaluated by analyzing the concentration of xanthan gum production. In optimization, the response can be related to chosen factors by linear or quadratic models. A quadratic model, which also includes a linear model, is given as follows:

$$Y=\beta_0+\beta_{1A}+\beta_{2B}+\beta_{3C}+\beta_{12AB}+\beta_{13AC}+\beta_{23BC}+\beta_{11A2+}\beta_{22B2}+\beta_{33C2}\text{.....Equation 1}$$

Where Y is the predicted response whereas response 1 for viscosity, response 2 for biomass concentration and response 3 for xanthan gum concentration; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{12}, β_{13}, β_{23}, interaction coefficients; β_{11}, β_{22}, β_{33}, squared coefficients. Data were processed for Eq. (1) using the Design-Expert 8.0.6 program including analysis of variance (ANOVA) to obtain the interactive effects between the process variables and the response. The quality of fit of the polynomial model was expressed by the coefficient of determination R^2, and its statistical significance was reevaluated by the F-test in the same program.

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Table 1 Experimental design for three fermentation factors for three responses for xanthan gum production

Run	Factor 1 A:Yeast Extract (%)	Factor 2 B:Fermentation (h)	Factor 3 C:Temperature (°C)	Response 1 Viscosity (cP)	Response 2 Biomass (g/L)	Response 3 Xanthan (g/L)
1	0.5	0	35	7.50	0.04	0.05
2	0.3	45	30	247.50	1.5	8.93
3	0.3	45	30	247.50	1.5	8.93
4	0.1	45	30	327.50	1.46	9.13
5	0.5	90	35	535	1.92	12.88
6	0.3	45	30	247.50	1.5	8.93
7	0.5	90	25	155	2.39	6.9
8	0.1	0	25	7.5	0.03	0.09
9	0.3	90	30	455.50	1.88	11.52
10	0.5	45	30	272.50	1.85	7.55
11	0.1	90	35	647.50	1.82	14.71
12	0.3	0	30	7.5	0.03	0.09
13	0.3	45	25	127.50	1.83	5.31
14	0.1	90	25	192.50	1.9	7.57
15	0.3	45	35	385	1.58	12.3
16	0.3	45	30	247.50	1.5	8.93
17	0.3	45	30	247.50	1.5	8.93
18	0.5	0	25	7.50	0.06	0.04
19	0.3	45	30	247.50	1.5	8.93
20	0.1	0	35	7.50	0.04	0.09

Results and Discussion

1. Analysis of experimental data

The statistical analysis of the data showed that the level of significance was 95%. The analysis was done using coded values. When the regression model is determined with coded values of the variables, the size of each coefficient gives a direct measurement of the importance of each effect. The coefficients of multiple determinations (R^2) give the percentage variation in the response explained by our regression model. As shown in Table 2, where the model coefficients are listed together and associated probabilities, in case of viscosity, biomass and xanthan production we can explore 97.43, 98.58 and 96.94% of the variation, respectively. A p value <0.05 suggests that less than 5% chance will be true (i.e. reject the null hypothesis). In case of xanthan and biomass production had only two fermentation parameters affected to the yields which are fermentation time and temperature

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(p <0.0001) while no effect of yeast extract was detected (Table 2). The coefficients of those parameters for xanthan production are significant as well (p <0.0001). The analysis of variance is shown in Table 3. In addition the second order model should be appropriate for our study. Our results further indicated that the models chosen can be satisfied to explain the effects of yeast extract (A), temperature (B), time (C) on viscosity, biomass and xanthan production. The models fitting the above response variables are in uncoded (actual) values and the final equations derived from the application of the method are given below and the ANOVA tables associated with the fitted model were shown in Table 3-5

Table 2 Estimated coefficients of multiple determinations (R^2) for viscosity (A), biomass (B) and xanthan (C) production using coded values

Term	Coefficient	SE coefficient	p value
(A) Viscosity [S= / R^2=0.9824 / R^2(adj)=0.9743]			
Yeast extract	-20.5	9.16	0.0434
Fermentation	194.8	9.16	<0.0001
Temperature	109.25	9.16	<0.0001
Yeast extract x fermentation	-18.75	10.24	0.0901
Fermentation x temperature	104.38	10.24	<0.0001
Fermentation x fermentation	-57.45	12.95	0.0007
(B) Biomass production [S= /R^2=0.9925 / R^2(adj)=0.9858]			
Yeast extract	0.1	0.029	0.0061
Fermentation	0.97	0.029	<0.0001
Temperature	-0.081	0.029	0.0196
Yeast extract x fermentation	0.07	0.033	0.0574
Yeast extract x temperature	-0.052	0.033	0.1386
Fermentation x temperature	-0.068	0.033	0.0654
Yeast extract x yeast extract	0.05	0.056	0.3941
Fermentation x fermentation	-0.65	0.056	<0.0001
Temperature x temperature	0.1	0.056	0.1038
(C) Xanthan production [S= / R^2=0.9758 / R^2(adj)=0.9694]			
Fermentation	5.32	0.26	<0.0001
Temperature	2.01	0.26	<0.0001
Fermentation x temperature	1.64	0.29	<0.0001
Fermentation x fermentation	-3.39	0.36	<0.0001

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Table 3 Analysis of variance for viscosity using coded values

Summary

Source	Sequential p-value	Adjusted R-Squared	Predicted R-Squared	
Linear	< 0.0001	0.7753	0.5767	
2FI	0.0002	0.9371	0.7174	
Quadratic	0.0059	0.9753	0.9053	Suggested
Cubic	0.3372	0.9787	-7.2576	Aliased

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	1067451	1	1067451.013			
Linear vs Mean	503029	3	167676.175	22.857	< 0.0001	
2FI vs Linear	90669	3	30222.917	14.711	0.0002	
Quadratic vs 2FI	18630	3	6209.877	7.688	0.0059	Suggested
Cubic vs Quadratic	3908	4	976.962	1.406	0.3372	Aliased
Residual	4169	6	694.914			
Total	1687855	20	84392.763			

Lack of fit tests

Source	Sum of Squares	df	Mean Square
Linear	117375.713	11	10670.519
2FI	26706.963	8	3338.370
Quadratic	8077.332	5	1615.466
Cubic	4169.482	1	4169.482
Pure Error	0	5	0

Model summary statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	85.650	0.811	0.775	0.577	262640.239	
2FI	45.325	0.957	0.937	0.717	175345.503	
Quadratic	28.421	0.987	0.975	0.905	58721.776	Suggested
Cubic	26.361	0.993	0.979	7.258	5123073.344	Aliased

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Table 4 Analysis of variance for biomass production using coded values

Summary

Source	Sequential p-value	Adjusted R-Squared	Predicted R-Squared	
Linear	< 0.0001	0.8117	0.7276	
2FI	0.8616	0.7808	0.1854	
Quadratic	< 0.0001	0.9858	0.9314	Suggested
Cubic	0.2169	0.9898	-2.9540	Aliased

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	33.359	1	33.359			
Linear vs Mean	9.596	3	3.199	28.310	< 0.0001	
2FI vs Linear	0.098	3	0.033	0.248	0.8616	
Quadratic vs 2FI	1.625	3	0.542	63.637	< 0.0001	Suggested
Cubic vs Quadratic	0.048	4	0.012	1.979	0.2169	Aliased
Residual	0.037	6	0.006			
Total	44.763	20	2.238			

Lack of fit tests

Source	Sum of Squares	df	Mean Square
Linear	1.808	11	0.164
2FI	1.710	8	0.214
Quadratic	0.085	5	0.017
Cubic	0.037	1	0.037
Pure Error	0.000	5	0.000

Model summary statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.3361	0.8415	0.8117	0.7276	3.1068	
2FI	0.3627	0.8500	0.7808	0.1854	9.2897	
Quadratic	0.0923	0.9925	0.9858	0.9314	0.7821	Suggested
Cubic	0.0782	0.9968	0.9898	2.9540	45.0913	Aliased

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Table 5 Analysis of variance for xanthan production using coded values

Summary

Source	Sequential p-value	Adjusted R-Squared	Predicted R-Squared	
Linear	< 0.0001	0.7489	0.6026	
2FI	0.2625	0.7701	0.0124	
Quadratic	< 0.0001	0.9704	0.8729	Suggested
Cubic	< 0.0001	0.9999	0.9474	Aliased

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	1005.504	1	1005.504			
Linear vs Mean	325.457	3	108.486	19.886	< 0.0001	
2FI vs Linear	22.375	3	7.458	1.494	0.2625	
Quadratic vs 2FI	58.490	3	19.497	30.364	< 0.0001	Suggested
Cubic vs Quadratic	6.403	4	1.601	543.467	< 0.0001	Aliased
Residual	0.018	6	0.003			
Total	1418.247	20	70.912			

Lack of fit tests

Source	Sum of Squares	df	Mean Square
Linear	87.286	11	7.935
2FI	64.911	8	8.114
Quadratic	6.421	5	1.284
Cubic	0.018	1	0.018
Pure Error	0	5	0.000

Model summary statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	2.3357	0.7885	0.7489	0.6026	164.0424	
2FI	2.2345	0.8427	0.7701	0.0124	407.6325	
Quadratic	0.8013	0.9844	0.9704	0.8729	52.4514	Suggested
Cubic	0.0543	1.0000	0.9999	0.9474	21.7154	Aliased

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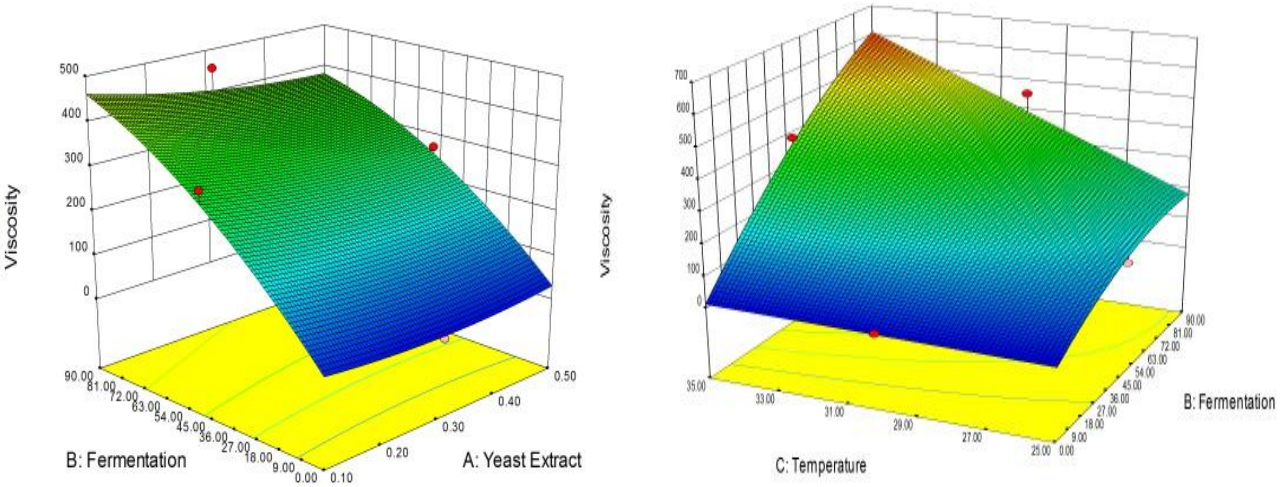
For viscosity; Viscosity = -19.13 - 8.75* Yeast Extract - 6.41* Fermentation + 0.96* Temperature - 2.08* Yeast Extract * Fermentation + 0.46* Fermentation * Temperature - 0.03* Fermentation^2

For xanthan gum; Xanthan = -2.17 + 0.05 * Fermentation + 0.07 * Temperature + 0.07 * Fermentation * Temperature - 0.01* Fermentation^2

For biomass; Biomass = 4.23 + 0.15 * Yeast Extract + 0.06 * Fermentation - 0.29 * Temperature + 0.08 * Yeast Extract * Fermentation - 0.003 * Fermentation * Temperature - 0.003 * Fermentation^2 + 0.04 * Temperature^2

2. The effect of fermentatin parameters on xanthan gum production

Under the consideration of the three factors, in all these cases, was held constant at the center point, for fermentation time 45 h, at temperature of 30°C and yeast extract 0.3% (w/v), respectively. As illustration in the graphs of Figure 1 show that, in general, viscosity has increased as the increasing yield of xanthan production is obtained with biomass concentration (Figure 1-3).



(a) (b)

Figure 1 The contour plots for (a) viscosity at varying fermentation time and yeast extract, (b) viscosity at varying temperature and fermentation time

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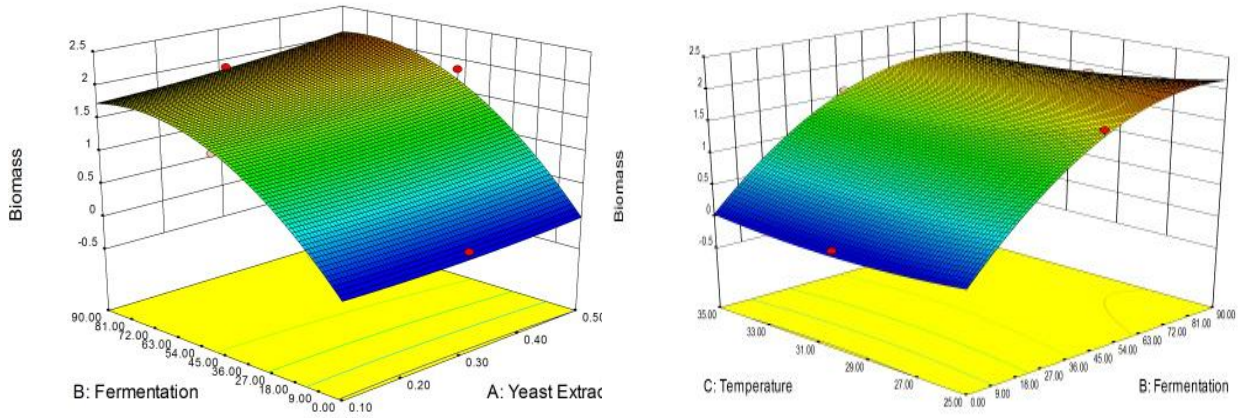
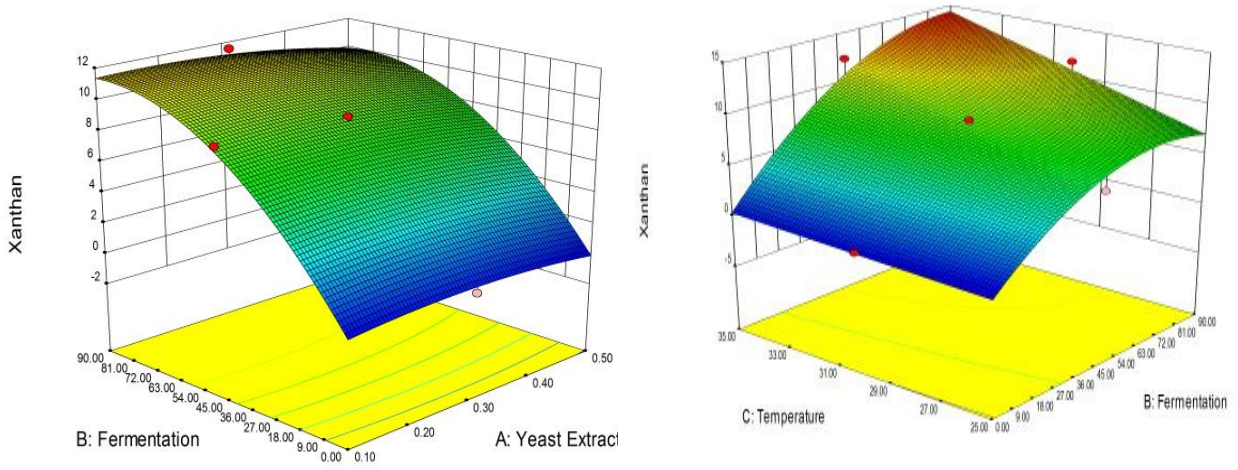


(a) (b)

Figure 2 The contour plots for (a) biomass at varying fermentation time and yeast extract, (b) biomass at varying temperature and fermentation time



(a) (b)

Figure 3 The contour plots for (a) xanthan at varying fermentation time and yeast extract, (b) xanthan at varying temperature and fermentation time

Results showed no contribution effect found of the increasing yeast extract as the nitrogen source on xanthan production. For temperature, the highest yields of xanthan production can be reached at 35°C (Figure 3b). Higher yields of xanthan production can be obtained by increasing the time of cultivation up to 90 h. Our results are in agreement with previous reports [15,16]. As higher values of xanthan gum at temperatures between 30 and 33°C were obtained [16,17], whereas our investigation found the same trend at a bit

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higher temperature at 35°C. However, the xanthan gum production is varied and depends on the medium. Moreover, higher yields of xanthan production can be succeeded when the time of cultivation increase. The maximum value of xanthan production (14.36 g/L) was obtained when the fermentation medium contained 0.10% yeast extract and the stirred tank bioreactor was operated at 35°C over 90 hours fermentation time.

Conclusions

Our investigation results show that fermentation of xanthan gum in a stirred tank bioreactor under the batch process the higher levels of xanthan gum (up to 14 g/L) and biomass can be reached at increasing temperature and the time of fermentation with the maximum value up to 90 h, adjusting the temperature to 35 °C. However, the longer fermentation time will make higher cost of operation. Therefore, an attempt to minimize time of cultivation will be one of the important factors to consider for xanthan gum production in the pilot and industrial scales while maintaining a relatively high xanthan yield 9.52 (g/L) which is approximately 33% reduction. Under this consideration gain a shorter fermentation by 2.5 times, an alternative fermentation can be operated at 37 h with the yeast extract 0.10% at 35°C.

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