Statistical Optimization of Xanthan Gum Production and Influence of Airflow Rates in Lab-scale Fermentor

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Abstract

The present study was undertaken to investigate and optimize the possibility of xanthan gum production by *Xanthomonas campestris* PTCC1473 in 500ml shake flasks on the second grade date palm. Using an experimental response surface methodology (RSM) coupled with a central composite design (CCD), three major independent variables (nitrogen source, phosphor source and agitation rate) were evaluated for their individual and interactive effects on biomass and xanthan gum production in submerged fermentation. The optimum conditions selected for gum production were 3.15 g.l^{-1} for nitrogen source, 5.03 g.l^{-1} for phosphor source, and 394.8 rpm for agitation rate. Reconfirmation test was conducted, and the experimental value obtained for xanthan production under optimum conditions was about 6.72±0.26 g.l^{-1}, which was close to 6.51 g.l^{-1} as predicted by the model. A higher yield of biomass production was obtained at 13.74 g.l^{-1} for nitrogen source, 4.66 g.l^{-1} for phosphor source, and 387.42 rpm for agitation rate. In the next stage, scale-up from the shake flasks to the 1-L batch fermentors was carried. By using the optimum conditions for xanthan gum, the biomass and xanthan gum concentrations after 72h in three levels of air flow rate (0.5, 1 and 1.5 vvm) were obtained as 3.98, 5.31 and 6.04 g.l^{-1}, and 11.32, 15.16 and 16.84 g.l^{-1}, respectively. Overall, the second grade date palm seemed to exhibit promising properties that can open new pathways for the production of efficient and cost-effective xanthan gum.

Article Info

1. Introduction

Date fruit is one of the most widely cultivated crops in the south west of Asia. According to FAO official estimates, 75% of the world's palm date production belongs to Egypt, Iran, Soudi Arabia, Pakistan and Iraq [1]. During the last 50 years, Iranian palm date production has reached an average of 800.000 ton/year. Palm date harvesting is often accompanied by substantial fruit losses that occur during the picking, storage and conditioning processes [2]. Because of their inadequate texture, the lost dates, commonly named ''date by-products'', are not edible and are often discarded. They are composed of low grade and second grade dates. Currently, very little use is made of these by-products, and they are, most of the time, used for limited purposes such as animal feed [3, 4].

Xanthan is a polysaccharide derived from *Xanthomonas* spp., a plant-pathogenic bacterium genus, which has viscous properties [5]. Xanthan gum was discovered in the late 1950s by a US scientist, and is the first biopolymer produced industrially. In 1969, the Food and Drug Authority (FDA) authorized the use of xanthan gum in food products, marking the introduction of the first industrially produced biopolymer to the food industry. Since then, the demand for the xanthan gum produced from *Xanthomonas campestris* has progressively increased, at an annual rate of 5–10%.

It is widely used in foods, toiletries, cosmetics, as water-based paints, pharmaceutical

and petroleum industries because of its unique rheological properties. The xanthan gum applications have diversified its commercial value, and it has now turned into one of the widely used ingredients in food products [5-7].

Commercial production of xanthan gum uses glucose as the substrate, and generally batch fermentation instead of continuous fermentation as the batch processes have been proven to work successfully [6].

In fact, the cost of the fermentation medium has always been a major concern in the commercial production of xanthan. For this reason, recent research in the field has particularly focused on the search for cheaper natural alternatives for the currently used substrates, namely glucose or sucrose, so as to control the cost of the production process and the final product.

In this study, the authors postulate that the second grade date palm, which is abundantly available in nature as a waste of palm date harvesting and storing, can be used as a cheap substrate for xanthan gum production. Accordingly, the present work was undertaken to explore and further optimize xanthan gum production by *Xanthomonas campestris* in batch experiments on the second grade date palm using the response surface methodology (RSM). It evaluated the effect of three main independent variables, namely nitrogen source (yeast extract), phosphor source (K_2HPO_4) and agitation rate individually and in combination on optimum xanthan and biomass production. Furthermore, the effects of agitation levels on xanthan gum production in batch fermentor in comparison with shaking flasks were investigated.

2. Materials and Methods

2.1 Samples and substrate preparation

The date samples used in the current study were second grade dates purchased from the Jihad-e-Agriculture of Bushehr region (South of Iran).

After being sorted (in -18°C to prevent fermenting), the dates were pitted and the fleshes were mixed by adding some water up to the final concentration (50 and 70 g.l⁻¹). The date juice was prepared by filtering the mixture by using Whatman filter papers (No. 1). Then it was autoclaved at 121°C (15 psi) for 20min.

2.2 Microorganism, growth conditions and inoculum preparation

A strain of *Xanthomonas campestris* PTCC 1473 was used for the present study. The microorganism was purchased from Iranian

Research Organization for Science and Technology (IROST). The preparation of the inoculum was performed by the transfer of the microorganism from the stock culture into the GYC (Glucose Yeast Calcium Carbonate) Agar and its subsequent incubation for 48 h at 28°C. Subculturing was carried out once in every two weeks, and the culture was stored at 4°C.

The strain was adapted to high date juice concentrations as described below. For the first adaptation passages, *Xanthomonas campestris* was cultured for 24h in a rotatory shaking incubator (250 rpm) at 28°C in the LB medium (in g.l⁻¹: yeast extract 5, tryptone 10 and NaCl 10) containing 25g glucose, whereas for the next four passages, it was cultured under the same conditions in 50 g.l⁻¹ date juice. Two last passages were carried out in 70 g.l⁻¹ date juice. In all passages, the LB medium was added. The xanthan gum production cultures (in shake flasks and fermentor) were subsequently inoculated with a 5% (v/v) inoculum from the last culture. At each passage, optical density (OD) was measured at 600nm by the spectrophotometer (Scinco UV 2100, South Korea) to estimate the biomass quantity, and the best result at the above 7 passages was obtained (OD=6.8).

2.3 Fermentation

The experiments were carried out in 500 ml shake flasks with 100ml of medium containing date juice with 70 g.l⁻¹ concentration, in 250 rpm, 30°C for 2 days. The cultures were grown in duplicates, and sterile additives (LB medium) were added under aseptic conditions after autoclaving. In all flasks, trypton (10 g.l⁻¹) and sodium chloride (10 g.l⁻¹) were added.

2.4 Xanthan gum estimation

Xanthan gum was assayed according to a previous report [8] with the difference that the potassium chloride solution was supplemented with EDTA to achieve a final EDTA concentration of 4mM [9].

2.5 Biomass estimation

The precipitation obtained from the previous stage was dried in oven at 105°C for 24 h and weighed [8].

2.6 Fermentation in batch fermentor

Fermentation in batch fermentor was carried out with the aim of studying the effect of aeration on xanthan gum production. Since the goal of the study was optimization of xanthan gum production, the optimum conditions for xanthan gum (nitrogen source, phosphor source and agitation rate) obtained from the last stage

were chosen to use in the fermentor. NaCl and trypton were added in the same value.

For this purpose, F1-S-3L bioreactor (Majer Science, Taiwan) was used as a scale-lab fermentor. The fermentation medium without the nitrogen source was sterilized while in the fermentation vessel. The nitrogen source was autoclaved separately, and then introduced into the vessel aseptically.

The composition of the culture medium was the same used for the flask cultures. The air flow rate was adjusted at 0.5, 1 and 1.5vvm (air volume/medium volume/minute). Runs were terminated after 72 h of culture. The pH of the culture medium was 7.0 after sterilization, and it was automatically maintained at 7±0.1 by adding 1.5N NaOH and 1.0N HCl. The temperature was maintained constant at 30ºC for 72h. During the process, the concentration of the biomass and xanthan gum was measured in the culture medium in three samples of 50ml each, every 24h. To prevent foaming, sufficient volume of anti-foam agent (silicone) was added automatically.

2.7 *Experimental methodology*

The next step in the study involved determining the optimum levels of the significant variables for xanthan production. In fact, the more factors are involved in a desired response, the more sophisticated the optimization process becomes. In such cases, RSM is often reported to offer effective tools for the optimization of the response [10, 11]. Accordingly, RSM was selected for the present study to maximize the xanthan production. The main and interaction effects of the phosphor source (2-6 g.l^{-1}), nitrogen source (0-15 g.l^{-1}) and agitation (200-400 rpm) on xanthan production (Y_1) and biomass production (Y_2) as response variables were studied.

In order to determine the significant experimental variables and develop a response surface for medium optimization, the major factors mentioned above were further investigated by CCD. The experimental range for each factor was selected on the basis of the results obtained from 18 preliminary experiments carried out by using CCD. A second order polynomial model was fitted for the production of xanthan gum and biomass (Y), giving an equation of the following form:

$$Y = a_0 + a_1 A + a_2 B + a_3 C + a_{12} AB + a_{13} AC + a_{23} BC + a_{11} A^2 + a_{22} B^2 + a_{33} C^2 \quad (1)$$

Where, Y is the measured response, a_0 is the intercept term, a_1, a_2 and a_3 are linear coefficients, a_{12}, a_{13} and a_{23} are the interaction coefficients, a_{11}, a_{22} and a_{33} are the quadratic coefficients, and A, B and C are the coded independent variables. The obtained response values were used to estimate the model coefficients by the least square method using the Design Expert software (version 7.0.0).

For the sake of reliability and accuracy of findings, mathematical models need to be validated before actual exploitation. In the case of the composite design employed in the current study, the validation of the model was carried out by subjecting the data to the analysis of variance (ANOVA). The method can be described as follows [12]:

The total sum of squares SST (with 35 degrees of freedom) is divided into the sum of squares SSX due to regression (with 5 degrees of freedom) and the residual sum of squares SSR (with 30 degrees of freedom):

$$SST = SSX + SSR$$

The residual sum of squares (SSR) can be partitioned into two parts: the first part is due to pure experimental error (SSE) and is computed as the sum of squares deviations in the center point calculated with 21 degrees of freedom, and the second part (SSL) corresponds to the lack of fit, which is used to assess the significant of the model. SSL is determined with 9 degrees of freedom.

The fitted model is considered adequate if the variance due to the lack-of-fit is not significantly different (F-test at the level 95%) from the pure error variance.

3. Results and Discussion

Table 1 presents the level of independent variables in coded and encoded forms according to the experimental design and the responses: xanthan (Y_1) and biomass production (Y_2) for all experiments. The experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

3.1 *Analysis of experimental data*

The final equations for xanthan (Y_1) and biomass production (Y_2) derived from the application of the method (after eliminating non-significant terms by the forward method) are given below:

$$Y_1 = -1.35488 + 0.17168 \, A + 0.018045 \, B + 0.40062 \, C - 9.6483 \, E - 4 \, BC - 0.018786 \, C^2 \quad (2)$$

Y_2= -1.90068 + 0.11429 A + 0.021225 B - 0.06591 C + 0.014075 AC + 0.000543 BC - 0.000034 C^2 (3)

Where, A is phosphor source, B is agitation rate, and C is nitrogen source. It can be noted that Eqs. (2) and (3) have mathematical rather than physiological meanings particularly because they can encompass the values of the three factors (phosphor source, nitrogen source and agitation rate) and estimate the xanthan and biomass yields.

3.2 Xanthan production (Response Y_1)

Thirty six experiments were carried out according to the conditions indicated in Table 1. Xanthan yield was determined and reported. The

magnitudes of linear coefficients presented in Table 2 indicate that the nitrogen source had a more positive effect on xanthan production than the phosphor source. P-values <0.05 were obtained for the nitrogen and phosphor sources and agitation rate. The relationship between the response and the experimental variables could be graphically illustrated by response surface plots.

Figure 1 shows the effect of interaction of incubation nitrogen and agitation rate on the rate of xanthan production. The nitrogen source was observed to have a significant effect on xanthan production (Figure 1). In fact, the xanthan production was noted to increase proportionally with the increase in the nitrogen source values up to 3.95 g.l^{-1} just at low agitation rates.

Table 1. Central composite design of the variables and the corresponding experimental yields for xanthan gum and biomass in shake flasks

Run	Phosphor source (g/l)	Agitation rate (rpm)	Nitrogen source (g/l)	Xanthan production Y_1 (g/l)	Biomass production Y_2 (g/l)
1	6.00(+1)	400.00(+1)	15.00(+1)	2.235	4.727
2	4.00(0)	300.00(0)	7.50(0)	4.256	2.912
3	2.00(-1)	400.00(+1)	0.00(-1)	6.084	3.314
4	4.00(0)	300.00(0)	7.50(0)	4.077	2.784
5	6.00(+1)	400.00(+1)	0.00(-1)	6.618	1.596
6	2.00(-1)	200.00(-1)	15.00(+1)	1.556	1.769
7	4.00(0)	200.00(-1)	7.50(0)	2.744	1.96
8	2.00(-1)	400.00(+1)	15.00(+1)	2.023	3.986
9	6.00(+1)	300.00(0)	7.50(0)	5.107	3.648
10	4.00(0)	300.00(0)	0.00(-1)	3.72	1.202
11	4.00(0)	300.00(0)	7.50(0)	3.898	2.657
12	4.00(0)	300.00(0)	15.00(+1)	1.839	4.346
13	2.00(-1)	200.00(-1)	0.00(-1)	2.476	1.111
14	4.00(0)	300.00(0)	7.50(0)	4.666	2.404
15	4.00(0)	400.00(+1)	7.50(0)	5.58	3.04
16	6.00(+1)	200.00(-1)	0.00	3.366	1.445
17	2.00(-1)	300.00(0)	7.50(0)	3.041	2.172
18	6.00(+1)	200.00(-1)	15.00(+1)	1.683	3.333
19	2.00(-1)	200.00(-1)	0.00(-1)	2.719	1.651
20	6.00(+1)	200.00(-1)	0.00(-1)	3.983	1.985
21	2.00(-1)	400.00(+1)	0.00(-1)	6.936	1.854
22	6.00(+1)	400.00(+1)	0.00(-1)	7.157	2.136
23	2.00(-1)	200.00(-1)	15.00(+1)	1.628	2.309
24	6.00(+1)	200.00(-1)	15.00(+1)	1.989	3.873
25	2.00(-1)	400.00(+1)	15.00(+1)	2.552	4.526
26	6.00(+1)	400.00(+1)	15.00(+1)	2.719	5.267
27	2.00(-1)	300.00(0)	7.50(0)	3.6	2.712
28	6.00(+1)	300.00(0)	7.50(0)	4.625	4.188
29	4.00(0)	200.00(-1)	7.50(0)	3.197	2.5
30	4.00(0)	400.00(+1)	7.50(0)	5.055	3.58
31	4.00(0)	300.00(0)	0.00(-1)	4.395	1.742
32	4.00(0)	300.00(0)	15.00(+1)	3.636	4.886
33	4.00(0)	300.00(0)	7.50(0)	5.071	3.197
34	4.00(0)	300.00(0)	7.50(0)	5.427	2.944
35	4.00(0)	300.00(0)	7.50(0)	5.806	3.452
36	4.00(0)	300.00(0)	7.50(0)	6.209	3.324

This data can be explained that during the microbial fermentation, the nitrogen source is just needed to prepare growth conditions and produce enzymes used in the synthesis of biological xanthan production. Among bacterial exopolysaccharides are synthesized, xanthan production is greater in the media containing higher ratios of carbon to nitrogen (C/N) [13]. Owing to the fact that, in this study, carbon

concentration in medium was almost instant in higher concentration of nitrogen, the ratio C/N decreased, and consequently, the rate xanthan production decreased.

Moreover, higher yields of xanthan production could be obtained with increasing the agitation level from 200 to 400 rpm (Figure 1). Agitation is then an important factor in the batch fermentation of *Xanthomonas campestris*. The

beneficial effects of increased agitation have been attributed by some investigators to a thinning slime layer, enhancing this way the transfer of nutrients and oxygen for xanthan formation [14]. This could explain the different values of xanthan production at various speeds applied in this work. These results are in agreement with other previously reported findings in the literature [15, 16], showing that low concentration of nitrogen source has a positive effect on xanthan production, which also increases upon increasing of the agitation level.

The optimal conditions for xanthan production that were selected using the Design Expert 7.0.0 software package were: 3.15 $g.l^{-1}$ of nitrogen source, 5.03 $g.l^{-1}$ of phosphor source, and 394.8 rpm. Under these conditions, the expected value of the xanthan yield was 6.51 $g.l^{-1}$. A supplementary experiment was carried out under the selected optimal conditions, and led to an experimental xanthan yield value of 6.72±0.26 $g.l^{-1}$, which was very close to the one expected by the model (6.51 $g.l^{-1}$).

3.3 *Biomass production (Response Y_2)*

The findings in Table 2 indicate that, contrary to the case of xanthan production, phosphor source has a more positive linear effect on biomass production than nitrogen source and that nitrogen source exerts a negative linear influence. P-values <0.05 were obtained for nitrogen and phosphor sources and agitation rate.

The plots for biomass production are illustrated in Figure 2 and Figure 3. As shown in Figure 2, the maximum biomass production (5.26 $g.l^{-1}$) can be obtained at high nitrogen sources, and at phosphor values increasing from 4 to 6 $g.l^{-1}$.

Nitrogen is one of the most important key factors in the growth of Xanthomonas cells. Since the nitrogen source is rare in palm date; consequently, it has a significant effect on biomass production. In high concentrations of nitrogen (more than 3.75 $g.l^{-1}$), decreasing in xanthan production was observed, whereas the biomass production increased with the addition of nitrogen. This later effect can clearly be explained by the fact that nitrogen usually regulates growth [17]. In fact, similar results were achieved in a previous study [18]. Results showed that high nitrogen concentration caused an increase in biomass production. Also higher mass production did not lead to higher xanthan gum production [8, 19]. With respect to Figure 3, it can be noted that increased biomass production can be obtained at high nitrogen sources and at agitation rate increasing from 336-400 rpm. In the low level of nitrogen, increasing in the agitation rate has insignificant effect on biomass production. Since, in the low level of nitrogen, the growth of microorganism cells is reduced, as a result, they do not need to the oxygen obtained from agitating.

Table 2. Analysis of variance (ANOVA) for the quadratic models of xanthan and biomass production

| Term	Coefficient	F-value	P-value
A:(Y_1) Xanthan production (R^2 = 0.8512)			
Constant	-1.35488		
Phosphor source	0.17168	4.518	0.0432
Agitation rate	0.018045	44.776	0.0001<
Nitrogen source	0.40062	62.761	0.0001<
Agitation rate× Nitrogen source	-0.00096	16.054	0.0005
Nitrogen source × Nitrogen source	-.018786	8.059	0.0087
B:(Y_2) Biomass production (R^2 = 0.8933)			
Constant	-1.90068		
Phosphor source	0.11429	23.62	0.0432
Agitation rate	0.21225	31.10	0.0001>
Nitrogen source	-0.065910	161.38	0.0001>
Phosphor source× Nitrogen source	0.014075	4.36	0.0458
Agitation rate × Nitrogen source	0.000543	16.20	0.0004
Agitation rate × Agitation rate	0.000034	6.19	0.0189

*Differences were significant at P<0.05.
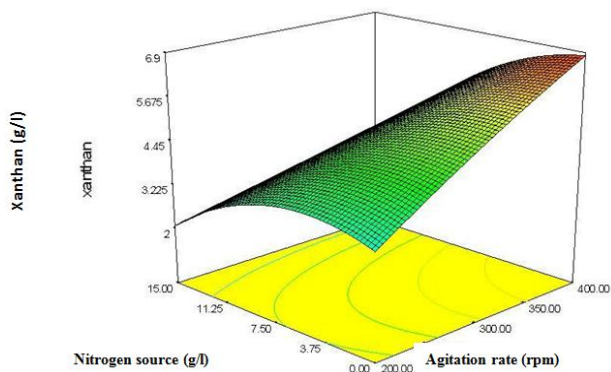
Figure 1. Surface of xanthan production as a response to nitrogen source and agitation rate changes in the shake flasks (phosphor source =5.03 g.l^{-1}).
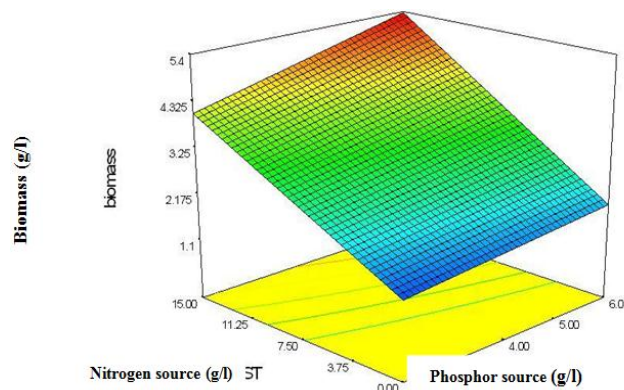

Figure 2. Surface of biomass production as a response to nitrogen and phosphor source changes on in the shake flasks (agitation rate =387.42 rpm).
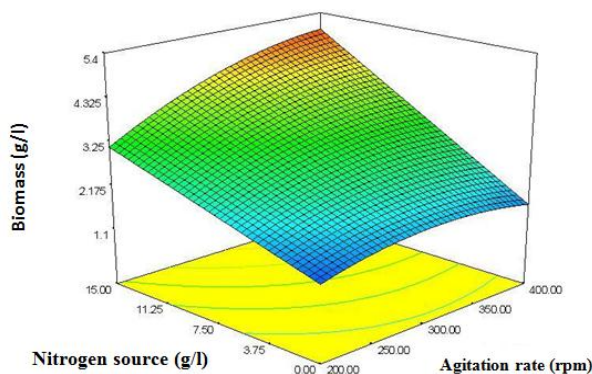

Figure 3. Surface of biomass production as a response to nitrogen source and agitation rate in the shake flasks (phosphor source =4.66 g.l^{-1}).

The optimal conditions selected for biomass production by the Design Expert software were 4.66 g/l for phosphor source, 13.74 g/l for nitrogen source and 387.42 rpm. The expected value of biomass was 4.87±0.06 g/l (predicted response 4.66 g/l), indicating the efficacy of the model for prediction of the amount of biomass production under different situations of the medium.

3.4 *Statistical analysis and validation of the model*

The value of determination coefficient (R^2), as a measure of the model's goodness-of-fit, was 0.8512 for xanthan production and 0.8933 for biomass production (Table 2). This indicates that only about 14.88% and 10.67% of the total variations were not explained by the two models, respectively. The lack-of-fit value can be indicative of a model's failure to represent data in the experimental domain at points, which are not included in the regression. In fact, the lack-of-fit values for the regression equations (2) and (3) were not significant (P>0.05), and thus demonstrating that the two model equations were

adequate for predicting xanthan and biomass production under any combination of the values of the variables involved (Table 2).

To model validation, the experiments for xanthan gum production were carried out in optimum conditions. The values of xanthan and biomass were 6.72±0.26 and 2.6±0.14 g.l^{-1}, respectively. These results are very close to the predicted values by using RSM.

3.5 *Fermentor*

All of the experiments were carried out in a stirred tank bioreactor, with 3L of working volume, equipped with pH, dissolved oxygen, temperature and foam props. After inoculation, the samples were collected at regular time intervals (24, 48 and 72h) thereafter. The effects of fermentation time and aeration rate on exopolysaccharide and biomass production are shown in Figure 4 and Figure 5, respectively.

An increasing in the air flow rate yields an increasing in biomass and xanthan gum production, as a consequence of a greater oxygen mass transfer rate that produces an increase in the dissolved oxygen [20].

Xanthan production is correlated to aeration rate. Similar results have been achieved in previous studies [14, 21], suggesting that higher aeration rate leads to higher rate of xanthan production.

In all levels of aeration rate, xanthan production increased rapidly to a maximum at 48h and then decreased gradually. The decline in the xanthan formation rate might be associated with gum formation, as the increase in broth viscosity due to gum formation would significantly reduce the mass transfer rate for oxygen and other nutrients [20]. As shown in Figure 5. Biomass production markedly increases with increasing of the fermentation time to 24h. Then the production rate decreases.

Stirrer speed was regulated from 400 to 800 rpm. Due to increase in viscosity, the stirrer speed must not be maintained constant throughout the process, because oxygen mass transfer would be dramatically affected [20]. The results showed that at 48 to 72h, difference in the xanthan production rate in the levels of 1 to 1.5 vvm is lesser than 0.5 to 1 vvm.
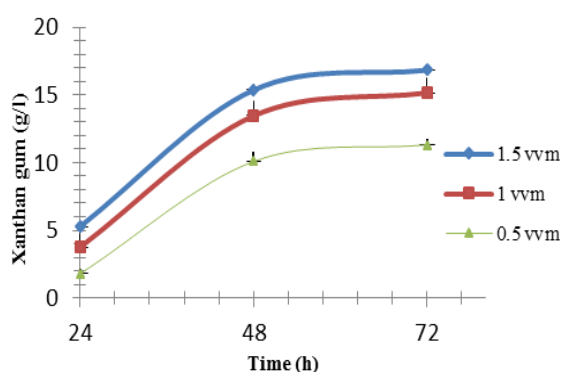

Figure 4. Effect of aeration rate on xanthan production in lab-scale fermentor.
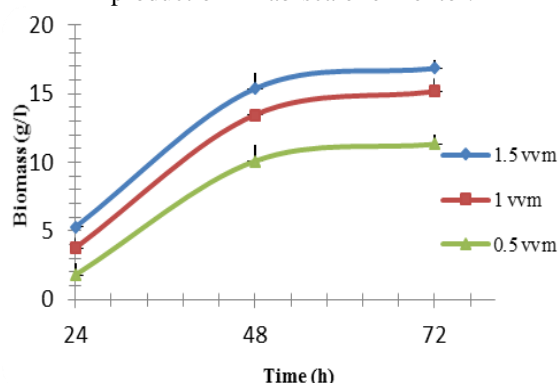

Figure 5. Effect of aeration rate on biomass production in lab-scale fermentor.

The mean of xanthan production by using optimal conditions in the shake flasks after 48h was 6.72±0.26, while it was 10.09, 13.46 and 15.37 g.l^{-1} in the batch fermentor after 48h fermentation (in 0.5, 1 and 1.5vvm), respectively.

4. Conclusion

This current work investigated the possibility of using second grade date juice as substrate for xanthan gum and biomass production by *Xanthomonas campestris* PTCC 1473. The influence of phosphor and nitrogen sources and agitation rate was determined using the response surface methodology (RSM). The selected optimal conditions for xanthan production (phosphor source: 5.03 g.l^{-1}, nitrogen source: 3.15 g.l^{-1} and agitation rate: 394.8 rpm) were checked and confirmed by a supplementary experiment. The experimental yield of xanthan was found to be in good agreement with the predicted one (6.72±0.26 versus 6.51 g.l^{-1}, respectively).

Since the goal of the study was optimization of xanthan gum production, the optimum conditions for xanthan gum production were used for the medium in the fermentor. Xanthan gum and biomass rate after 72 h in the batch fermentor in three levels of air flow rate (0.5, 1 and 1.5vvm) were obtained as 11.32, 15.16 and 16.84 g.l^{-1} (for xanthan) and 3.98, 5.31 and 6.04 g.l^{-1} (for biomass), respectively.

Overall, the findings of the present study indicate that the second grade date palm presented in the current study seems to have strong potential and promising properties that can open new pathways for the production of efficient and cost-effective xanthan gum. It can, therefore, be considered as a strong candidate for future industrial and commercial applications related to xanthan gum. To our knowledge, the present study is rare in the literature to explore and report on the direct exploitation of lost date, an abundant agro-industrial residue, for xanthan production. The results are, in fact, promising in that they suggest that xanthan gum production can be industrially extended and maximized through the use of this low cost substrate.

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