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RESEARCH ARTICLE

Optimization of Xanthan Gum Production Using Cheese Whey and Response Surface Methodology

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Abstract Cheese whey lactose was used as a carbon source for xanthan gum production with *Xanthomonas campestris* and *Xanthomonas pelargonii.* Proteins were precipitated and removed from whey prior to fermentation. Box-Behnken response surface methodology was used for optimization of the carbon, magnesium, and phosphate source concentrations in the culture medium to maximize xanthan gum production. After 48 h of fermentation using *X. campestris*, the highest xanthan concentration (16.4 g/L) was achieved at 65.2 g/L of cheese whey (39.1 g/L of lactose), 14.8 g/L of phosphate ($K H_2PO_4$), and 1.1 g/L of magnesium ($MgSO_4 \cdot 7H_2O$). The corresponding optimum cheese whey, phosphate, and magnesium concentrations in cultures of *X. pelargonii* were 80.0, 6.7, and 0.8 g/L, respectively, which resulted in a xanthan production of 12.8 g/L. The xanthan gum yield (g of xanthan/g of lactose) was 0.42 for *X. campestris* and 0.27 for *X. pelargonii*.

Keywords: xanthan, whey, *Xanthomonas,* optimization

Introduction

Xanthan gum is a water soluble heteropolysaccharide composed of glucose, mannose, and glucronic acid units that is produced industrially using the Gram negative bacterium *Xanthomonas campestris* mainly using glucose and sucrose rich substrates (1,2). Several specific physical properties, including a pseudo plastic behavior and a high viscosity, have expanded applications in different industries, including food products (bakery, prepared foods, and beverages) and drug formulations in tablets and suspensions, and opened new areas of application in ceramic glazes, papermaking, oil drilling, and agricultural chemicals (2-5). It is reported that 60% of the xanthan gum that is used is consumed in non-food industries and, to provide more beneficial applications, several strategies have been used to reduce production costs (6). For instance, the yield and recovery of xanthan production have been maximized by setting the important affecting factors of culture conditions, bioreactor type, and aeration and agitation rates at appropriate levels (7-11). The substrate cost (mainly a carbon source) substantially contributes to the overall production cost of xanthan gum. Therefore, using less expensive and more abundant substrates can enhance the economy of the fermentative xanthan production process. Thus, the low cost substrates sugar cane molasses (12), sugar beet molasses (13,14), and agricultural wastes (1,8,15-19) have been used instead of pure glucose for industrial production of xanthan.

Cheese whey is a by-product of dairy industry processes that is characterized by a high organic content and poses a severe threat to the environment with inappropriate disposal. The Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) values of cheese whey range between 27-60 and 50-102 kg/m^3, respectively (20). The organic load of cheese whey mainly results from a high lactose content (39-60 kg/m^3). Proteins and fats also contribute to the organic content of cheese whey (20-21). Although cheese whey can be reprocessed and reused based on valorization technologies, such as spray drying for whey

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powder production and ultrafiltration for recovery of proteins and lactose, these processes are energy intensive and costly. Therefore, cheese whey is usually considered as a major problem in the dairy industry (22-23). Annually, approximately 145 million tons of cheese whey are produced by the dairy industry (22). Biological conversion of cheese whey lactose into more valuable ethanol, methane, bioplastic, and single cell protein products is a possible remedy to reduce the BOD value up to 4× (21).

In this study, cheese whey as a cheap and abundant substrate was used to produce xanthan with the bacterial 2 species *X. campestris* and *X. pelargonii.* Since whey proteins can be recovered and used for different purposes, such as infant food formulation, a pretreatment (precipitation) stage was performed to separate proteins from cheese whey. In addition, Box-Behnken methodology was used to determine optimum levels of the concentrations of lactose, phosphate, and magnesium in a culture medium to maximize the yield of xanthan production.

Materials and Methods

Cheese whey Cheese whey was provided by Pegah Dairy Industries (Isfahan, Iran) in a powder form. The composition of cheese whey used in this work is shown in Table 1.

Cheese whey pretreatment An amount of 200 g of cheese whey powder was dissolved in 1,000 mL of water. This solution was boiled for 10 min in a beaker, cooled to 60°C and centrifuged (3-30 k; Sartorius, Goettingen, Germany) at 10,000×*g* for 10 min. The pH of the supernatant was adjusted to 4.0-4.5 using 0.1 N H_2SO_4 (Merck, Darmstadt, Germany), then was boiled for 10 min and the pH was adjusted again using 1 N NaOH (Merck) to 7.0. The precipitate was then removed by filteration through Grade 1 qualitative filter papers (GE Healthcare, Pittsburgh, PA, USA) (24).

Determination of the lactose content in pretreated cheese whey The concentration of lactose in the supernatant was determined using an HPLC apparatus (Knauer, Geretsried, Germany) with a Nucleosil® 100-5 NH_2 RP, 250×4.6 mm column (Macherey-Naggel, Düren, Germany) and an RI detector. Briefly, 20 μL of supernatant was injected into the column. Acetonitrile: water at a ratio of 80:20 (v/v) and flow rate of 1 mL/min at room temperature was used for elution. Standard solutions of lactose were prepared by dissolving different amounts of standard lactose (Merck) in water and analyzed with HPLC. The amount of lactose in the whey was equal to 60% (w/w) of the initial whey powder. The treated cheese whey (low protein) was autoclaved (110N; Iran Tolid, Tehran, Iran) at 121°C for 20 min prior to fermentation.

Table 1. Composition of cheese whey powder

Parameter	Mean value
Fat % (w/w)	1.25±0.55
Protein % (w/w)	6.75±0.25
Total solids % (w/w)	95.75±0.75
Acidity (Dornic degree)	11±0.5
Lactose % (w/w)	61±1

Table 2. Range of factors for fermentation

+1	0	-1	Level % (w/v)
8 (4.8)	5 (3)	2 (1.2)	Cheese whey (lactose)
1.5	1.0	0.5	KH_2PO_4
0.18	0.12	0.06	$MgSO_4 \cdot 7H_2O$

Microorganisms and inoculum preparation *X. campestris* PTCC1473 and *X. pelargonii* PTCC1474 were purchased from the Persian Type Culture Collection (PTCC, Tehran, Iran). The microorganisms were cultivated on GYC plates containing (g/L) glucose (20), calcium carbonate (20), yeast extract (10), and agar (20) (Merck) for 48 h at pH 7 and 28°C (25). Four loops of cells were transferred from YPC plates to flasks containing 100 mL of YPD medium with (g/L) glucose (20), peptone (20), and yeast extract (10) (Merck). Flasks were incubated (B28; Binder, Tuttlingen, Germany) at 28°C and 200 rpm for 24 h and grown cells were used as an inoculum for production of xanthan gum. The inoculum (5% v/v) was added to cotton plugged Erlenmeyer flasks containing 50 mL of an autoclaved (110N; Iran Tolid) solution of 2 g/L of $(NH_4)NO_3$, 2 g/L of citric acid, 0.006 g/L of H_3BO_3, 0.006 g/L of $ZnCl_2$, 0.0024 g/L of $FeCl_3$, and 0.02 g/L of $CaCl_2$. Flasks were supplemented with cheese whey (lactose), potassium dihydrogen phosphate (KH_2PO_4), and magnesium sulfate ($MgSO_4 \cdot 7H_2O$) at concentration levels shown in Table 2. The pH values of the solutions were adjusted by addition of 1 N NaOH to 7.0 before autoclaving. Cheese whey (lactose) was separately autoclaved (110N; Iran Tolid) and added to the culture medium (7,26). Cultivation (B 28; Binder) was performed at 28°C and 250 rpm for 48 h.

Recovery and purification of xanthan gum Forty-eight hours after incubation, 1.5 mL samples were taken from every culture and centrifuged (3-30 k; Sartorius) for 30 min at 15,000 rpm to remove bacterial cells. The cell-free supernatant was mixed with a 3 mL solution of 0.1% calcium chloride in isopropanol, resulting in xanthan precipitation. Precipitated xanthan was collected by centrifugation (3-30 k; Sartorius) (45 min, 50,000×*g*) and oven-dried (3493; Behdad, Tehran, Iran) at 50°C for 48 h (2,25).

FT-IR Spectra of xanthan gum FT-IR spectra of dried xanthan gum powders were recorded (FT/IR-6300; JASCO Analytical Instruments, Tokyo, Japan) for comparison of functional groups of the synthesized xanthan gum with a standard pure xanthan sample (Sigma-Aldrich, St. Louis, MO, USA). The dry sample powder was mixed with KBr (spectroscopic grade) (Sigma-Aldrich) and pressed into pellets under reduced pressure using an evacuable pellet press (161-1900; PIKE Technologies, Madison, WI, USA) for 2 min. FTIR spectra were obtained by scanning (FT/IR-6300; JASCO Analytical Instruments) between 4,000 and 400 1/cm (8).

Experimental methodology Box-Behnken response surface methodology (RSM) (27) was used to optimize lactose, phosphate, and magnesium concentrations for xanthan production. The array of designed experiments is shown in Table 3. Both individual and interactive effects of the cheese whey (lactose) concentrations of 20 (12), 50 (30), and 80 (48) g/L, the phosphate concentration (5, 10, and 15 g/L), and the magnesium concentration (0.6, 1.2, and 1.8 g/L) on polysaccharide production using *X. campestris* (Y1) and *X. pelargonii* (Y2) as the response variables were studied. The experimental range of each factor was selected on the basis of results obtained from preliminary experiments (data not shown). Factor levels (in coded values) were −1, 0, +1, where 0 corresponded to the central point. Designed experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors.

A second order polynomial model was developed for xanthan gum production (Y) using a least squares method. The resultant equation was in the form of:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{12}X_1X_2 +b_{13}X_1X_3+b_{23}X_2X_3 \quad (1)$$

where X_1, X_2, and X_3 represent coded levels of the independent variables, b_0, b_i, b_{ij} (i, j=1, 2, 3) represent coefficient estimates where b_0 is the interception, b_i is the linear term, b_{ij} is the quadric term, and b_3 is the interaction term (26). Results were analyzed using the Design Expert® 8 package and an analysis of variance (ANOVA) method with RSM. The statistically significant factors were distinguished by (p value <0.05).

Results and Discussion

Xanthan gum production Xanthan gum is industrially produced using mainly glucose or sucrose as the sole carbon source. However, commercially available xanthan gum is relatively expensive and this has hindered wider applications. Using cheaper and more abundant carbon sources, such as cheese whey, for xanthan manufacturing can reduce the production cost and, therefore, enhance the economy of the process (1). In addition to lactose, cheese whey is a rich source of valuable proteins with different applications (21). Most proteins were precipitated in a pre-treatment step and the remaining lactose was used as the sole carbon source.

Xanthan production values for *X. campestris* and *X. pelargonii* after 48 h are shown in Table 3. The lowest xanthan gum production values of 5.34 g/L for *X. campestris* and 7.96 g/L for *X. pelargonii* were obtained in run 1. The highest xanthan gum concentrations were 16.65 g/L (run 4) for *X. campestris* and 12.28 g/L (run 8) for *X. pelargonii*. Generally, *X. campestris* exhibited a higher xanthan gum production yield than *X. pelargonii* under different conditions.

Modeling and statistical analysis Levels of the independent variables in coded and natural forms according to the experimental design and the responses (xanthan gum concentrations, Y_1, Y_2) are shown in Table 3. The adequacy of the model and the assumptions made for statistical analysis were controlled using residuals plots. Normal probability plots of the residuals for *X. campestris* and *X. pelargonii* are shown in Fig. 1A and B. There were no serious outliers in the plots, which validated the assumption of a normal distribution for the error. Plots of residuals vs. fitted values with no apparent structure are shown in Fig. 2A and B, which verified the assumption of constant variance for analysis using both *X. campestris* and *X. pelargonii*.

Analysis of experimental data Derived models explained the effects of cheese whey (lactose X_1), KH_2PO_4 (X_2), and $MgSO_4 \cdot 7H_2O$ (X_3) on xanthan production. The final equation for xanthan production using *X. campestris* (Y_1) was:

$$Y_1=14.72+3.57X_1+1.54X_2+1.08X_3-2.94X_1^2-0.67X_2^2 -0.98X_1^2+0.39X_1X_2+0.6X_1X_3-1.33X_2X_3 \quad (2)$$

and for xanthan produced by *X. pelargonii* (Y_2) was:

$$Y_2=10.26+1.28X_1+0.69X_2-0.60X_3-1.29X_1X_2 -0.047X_1X_3+0.08X_2X_3 \quad (3)$$

The equations presented above have more mathematical than physiological meanings, particularly because the equations encompass the values of the 3 factors (carbon source, phosphate source, and magnesium source) and estimate xanthan gum production for 2 *Xanthomonas* strains.

Xanthan production using *X. campestris* and *X. pelargonii* (responses Y_1 and Y_2) A total of 15 experiments were carried out under conditions listed in

Table 3. A Box-Behnken array with responses for xanthan gum production after 48 h of fermentation for 2 *Xanthomonas* strains

Run Order	Cheese whey:lactose X_1 (% w/v)	KH_2PO_4 X_2 (% w/v)	$MgSO_4 \times 7H_2O$ X_3 (% w/v)	Xanthan gum (g/L)	
				X. campestris (Y_1)	*X. pelargonii* (Y_2)
1	5: 3 (0)	0.5 (-1)	0.18 (1)	13.07	8.53
2	5: 3 (0)	1.5 (1)	0.18 (1)	14.72	10.31
3	5:3 (0)	1.5 (1)	0.06 (-1)	15.71	11.56
4	8: 4.8 (1)	1.5 (1)	0.12 (0)	16.65	10.55
5	5: 3 (0)	1 (0)	0.12 (0)	15.16	10.6
6	8: 4.8 (1)	1 (0)	0.18 (1)	15.65	11.18
7	5: 3 (0)	1 (0)	0.12 (0)	15.01	10.46
8	8: 4.8 (1)	1 (0)	0.06 (-1)	11.79	12.28
9	5: 3 (0)	0.5 (-1)	0.06 (-1)	8.76	10.10
10	2: 1.2 (-1)	1.5 (1)	0.12 (0)	7.45	11.09
11	2: 1.2 (-1)	1 (0)	0.06 (-1)	7.14	9.11
12	5: 3 (0)	1 (0)	0.12 (0)	13.98	10.6
13	8: 4.8 (1)	0.5 (-1)	0.12 (0)	14.00	11.97
14	2: 1.2 (-1)	1 (0)	0.18 (1)	8.60	8.2
15	2: 1.2 (-1)	0.5 (-1)	0.12 (0)	6.34	7.36

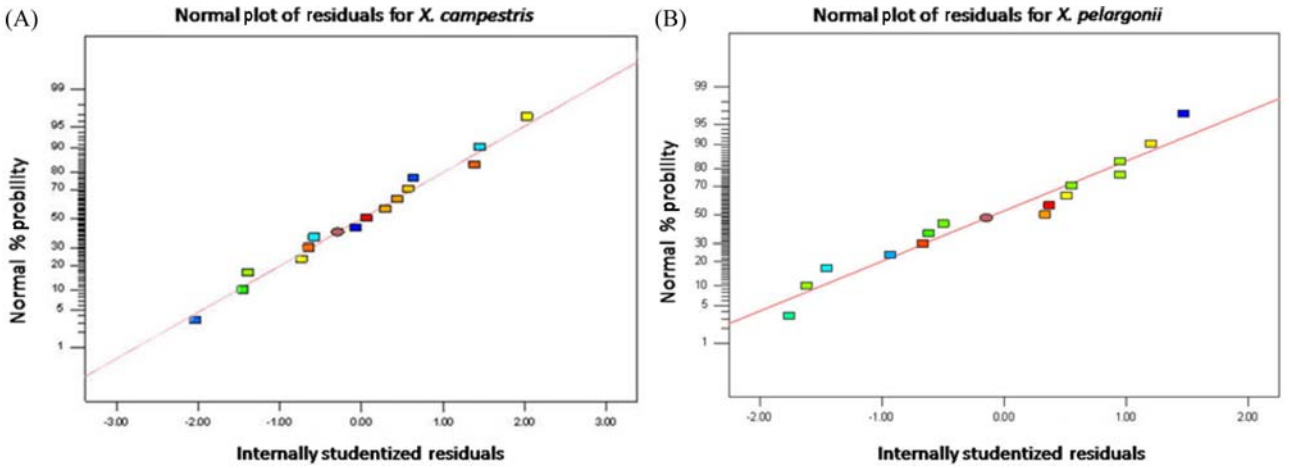

Fig. 1. A normal probability plot of residuals for production of xanthan gum using. (A) *X. campestris*; (B) *X. pelargonii*

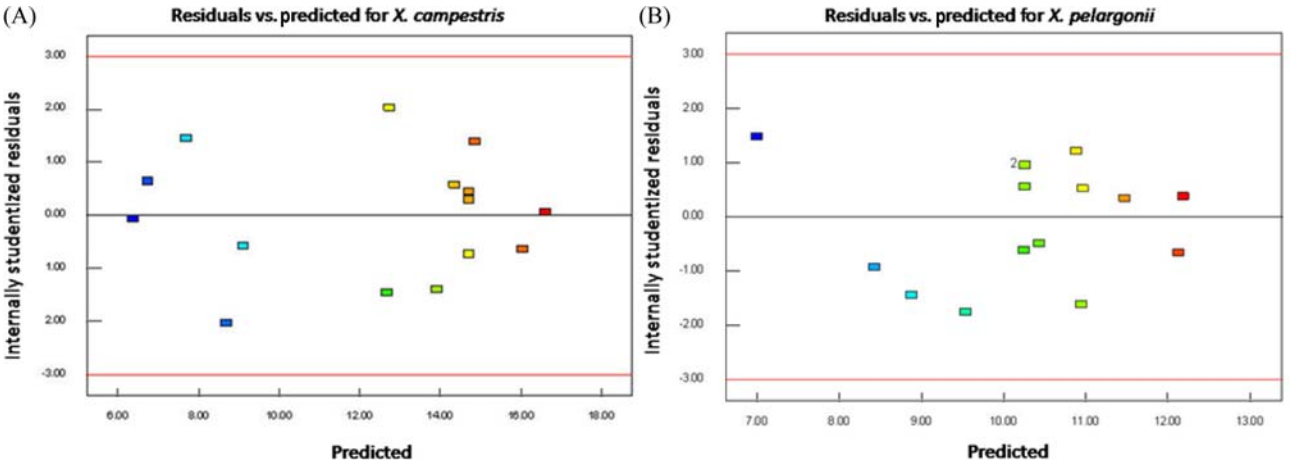

Fig. 2. A plot of residuals vs. production of xanthan gum using. (A) *X. campestris*; (B) *X. pelargonii*

Table 4A. Matrix for a Box-Behnken design with responses of xanthan production for *X. campestris*

Source (*X. campestris*)	DF[1)]	SS	MS	*F*	*p*-value
(R^2, R^2 (adj))=(95.82%, 88.31%)					
Regression	9	174.01	19.33	12.75	0.006
Cheese whey (A)	1	101.96	101.96	67.24	0.000
KH_2PO_4 (B)	1	19.10	19.10	12.59	0.016
$MgSO_4$ (C)	1	9.33	9.33	6.15	0.055
AB	1	0.59	0.59	0.39	0.559
AC	1	1.44	1.44	0.95	0.375
BC	1	7.02	7.02	4.63	0.084

Table 4B. Matrix of a Box-Behnken design with responses of xanthan production for *X. pelargonii*

Source (*X. pelargonii*)	DF[1)]	SS	MS	*F*	*p*-value
(R^2, R^2 (adj))=(96.03%, 93.06%)					
Regression	9	26.49	4.41	32.27	0.000
Cheese whey (A)	1	13.06	13.06	95.43	0.000
KH_2PO_4 (B)	1	3.85	3.85	28.14	0.001
$MgSO_4$ (C)	1	2.92	2.92	21.32	0.002
AB	1	6.63	6.63	48.47	0.000
AC	1	0.01	0.01	0.06	0.804
BC	1	0.03	0.03	0.19	0.677

[1)]DF, degrees of freedom; SS, sum of squares; MS, mean of squares; *F*, *F*-value

Table 3. The xanthan yield was determined and analyzed using the Design Expert software package. The values of coefficients shown in Table 4 indicate that all 3 sources had strong positive effects on xanthan gum production, but the carbon source was determined to be the most important factor.

The carbon source concentration as the driving force for sugar transport to cells is an important factor in microbial xanthan fermentation. Determination of the optimum concentration of a carbon source has been the subject of several studies (5,25,28-29). The highest xanthan titers were obtained at sugar concentrations of 30-40 g/L (5,25, 28-29), and glucose concentrations above 50 g/L exhibited inhibitory effects on xanthan production (5). Consistent with these results, the highest amounts of xanthan gum production in this study were obtained at 65.21 and 78.96 g/L of cheese whey (39.12 and 47.37 g/L lactose) for *X. campestris* and *X. pelargonii*, respectively.

The phosphate concentration is also an important factor for cell growth and xanthan production. Phosphate is needed both for cell growth and as a buffering agent to prevent pH fluctuations (4,13). However, xanthan production is inhibited at high phosphate concentrations (28-29). Phosphate can also affect the composition of xanthan gum, and low pyruvate containing xanthan gum has been reported at low phosphate concentrations (30). Maximum xanthan titers in this study were obtained at phosphate concentrations of 14.78 and 6.69 g/L for *X. campestris* and *X. pelargonii*, respectively. The interaction between phosphate and carbon sources was significant ($p<0.05$) for gum production using *X. pelargonii,* but had no significant effect ($p>0.05$) on gum production using *X. campestris.*

Magnesium, a cofactor for many enzymes, is present in the cell membrane and the cell wall, and plays a role in activation of sugar uptake (29,31). Therefore, xanthan production can be influenced by the magnesium source concentration. However, in the range examined herein, the magnesium concentration significantly ($p<0.05$) affected xanthan production only for *X. campestris*.

Silva *et al*. (4) used a central composite design (CCD) method for optimization of carbon, phosphate, and magnesium concentrations for xanthan gum production with a mixture of whey and sucrose as a carbon source. The optimum levels of carbon, magnesium, and phosphate sources were reported to be 80, 20, and 1 g/L, respectively. However, only the phosphate concentration was identified as a significant factor for xanthan production. Savvides *et al.* (32) studied xanthan gum production using *X. campestris* on non-hydrolyzed whey and partially hydrolyzed whey containing a mixture of lactose, glucose, and galactose. The highest xanthan titer in a batch culture was 12.46 g/L in a culture medium containing 43 g/L of a carbon source, which is equivalent to a yield of 29%. The yields of xanthan gum from carbon sources obtained in this work were 42 and 27% for *X. campestris* and *X. pelargonii*, respectively.

The coefficient of multiple determination (R^2) indicates the percentage of variation in a response explained by a

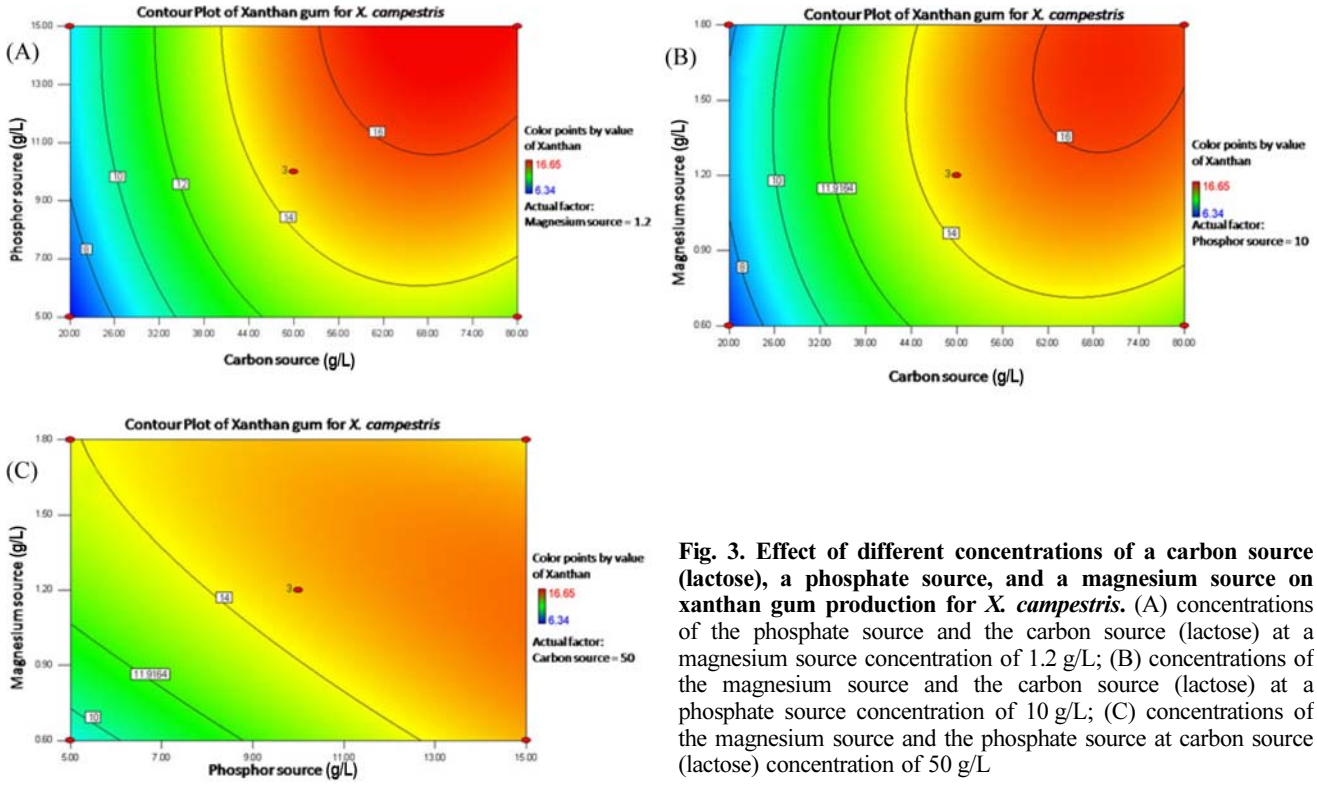

Fig. 3. Effect of different concentrations of a carbon source (lactose), a phosphate source, and a magnesium source on xanthan gum production for *X. campestris*. (A) concentrations of the phosphate source and the carbon source (lactose) at a magnesium source concentration of 1.2 g/L; (B) concentrations of the magnesium source and the carbon source (lactose) at a phosphate source concentration of 10 g/L; (C) concentrations of the magnesium source and the phosphate source at carbon source (lactose) concentration of 50 g/L

regression model (33,34). The fitted models were subjected to an analysis of variance and results showed that the regression models were statistically (p<0.05) significant for xanthan production. The R^2 values as a measure of a model goodness-of-fit (15,34), were 0.9582 and 0.9603 for *X. campestris* and *X. pelargonii*, respectively, indicating that only 4.2 and 4% of the total variation was not explained by the model. The adjusted R^2 value for the model was close to the ordinary R^2 value, at 0.8831 for *X. campestris* and 0.9306 for *X. pelargonii*.

Contour plots for xanthan production are shown in Fig. 3 for *X. campestris* and Fig. 4 for *X. pelargonii*. It should be noted that the third factor of the carbon source, phosphate source, and magnesium source in all cases was held constant at the center point (carbon, 50 g/L; lactose, 30 g/L; phosphate, 10 g/L; magnesium; 1.2 g/L). Furthermore, the graphs in Figs. 3 and 4 show that, in general, the carbon and phosphate sources both had a significant (p<0.05) effect on xanthan production for both *X. campestris* and *X. pelargonii.* An increased xanthan production yield was obtained with increasing carbon and phosphate source concentrations. The magnesium concentration had a significant (p<0.05) effect on xanthan gum production for *X. pelargonii,* while a decrease in the xanthan gum production yield for *X. pelargonii* was obtained with an increasing magnesium concentration.

Optimum range of xanthan production The 3 independent variables (carbon, phosphate, and magnesium source concentrations) all influenced xanthan gum production. The obtained models could be used to predict xanthan production over the full range of factor levels for both bacterial strains. Also, the optimum conditions for maximum xanthan production could be predicted using the models. The optimal conditions of xanthan yield for *X. campestris* and *X. pelargonii* were, respectively, cheese whey concentrations of 65.2 and 79.0 g/L (lactose; 39.12 and 47.37 g/L), phosphate source concentrations of 14.78 and 6.69 g/L, and magnesium source concentrations of 1.09 and 0.80 g/L. Under these conditions, the expected values for xanthan yield were 16.77 and 12.32 g/L for *X. campestris* and *X. pelargonii*, respectively. Supplementary experiments were carried out under selected optimal conditions that led to experimental xanthan concentrations of 16.40 and 12.78 g/L, close to predictions made using the model (16.77 and 12.32 g/L).

FT-IR Spectra of xanthan gum FTIR spectra of synthesized xanthan gum for identification of functional groups were obtained and compared with the spectrum of standard xanthan (Table 5). Xanthan samples showed the presence of hydroxyl, carbonyl, carboxyl, and acetal groups in the xanthan gum structure. Xanthan produced using both strains had slightly higher proportions of acetal groups (1065, 1089 1/cm for *X. campestris* and *X. pelargonii*, respectively) than the standard xanthan gum (1032 1/cm).

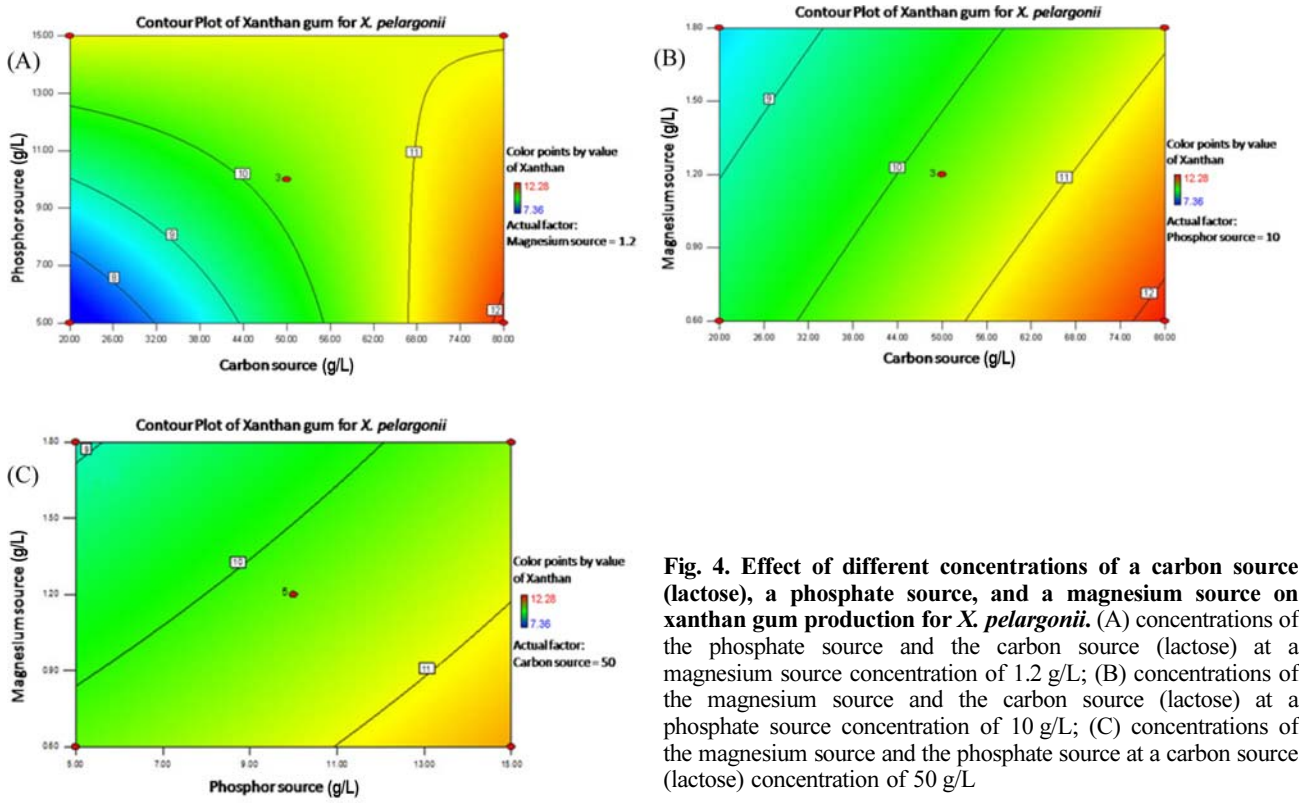

Fig. 4. Effect of different concentrations of a carbon source (lactose), a phosphate source, and a magnesium source on xanthan gum production for *X. pelargonii*. (A) concentrations of the phosphate source and the carbon source (lactose) at a magnesium source concentration of 1.2 g/L; (B) concentrations of the magnesium source and the carbon source (lactose) at a phosphate source concentration of 10 g/L; (C) concentrations of the magnesium source and the phosphate source at a carbon source (lactose) concentration of 50 g/L

Table 5. Spectra of functional groups

Functional group/ Wave number (1/cm)	Hydroxyl	Carbonyl	Carboxyl	Acetal
Standard xanthan gum	3,426	1,617	1,412	1,032
Xanthan gum synthesized with *X. campestris*	3,422	1,623	1,415	1,065
Xanthan gum synthesized with *X. pelargonii*	3,427	1,621	1,408	1,089

More acetal groups in xanthan gum results in a higher solubility of xantan which, in turn, can increase the range of industrial applications of xanthan (8).

Deproteinated cheese whey was used in this study as a cheap and abundant source of carbon for microbial xanthan gum production using *X. campestris* and *X. pelargonii*. Optimum concentrations of carbon, magnesium, and phosphate sources in a culture medium were determined using RSM. Lower xanthan concentrations were obtained for *X. pelargonii* than for *X. campestris*. Xanthan properties make *X. pelargonii* attractive for industrial applications.

Disclosure The authors declare no conflict of interest.

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