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Bench scale production of xanthan from date extract by *Xanthomonas campestris* in submerged fermentation using central composite design

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In this research, xanthan production from date extract was done by using bacterium *Xanthomonas campestris* PTCC1473 in submerged fermentation (SmF). The impact of different initial concentrations of carbon (date extract) and nitrogen (NH_4NO_3) sources on cell growth and xanthan production was evaluated. Inoculum (72 h) from the YMB medium was added to the substrate with defined chemical components (nitrogen, sugar, moisture, ash and pH) and incubated. Central composite design (CCD) was used to evaluate the effects of carbon and nitrogen sources on production yield. The results indicate a decrease of cell growth in carbon source concentration up to 50 g/l. The highest cell growth and xanthan production were achieved at 40 g/l concentration of carbon source. The nitrogen source concentration did not cause a significant effect on cell growth; but the highest concentration of xanthan was produced in 0.2 g/l of nitrogen source. The ratio of carbon to nitrogen content had a significant impact on xanthan production. In the optimum condition, maximum concentration of produced xanthan yield and productivity was seen at 11.2 g/l and 8.19 g/kg.day, respectively.

Key words: Xanthan, submerged fermentation, date extract, *Xanthomonas campestris*, central composite design.

INTRODUCTION

Xanthan is a biopolymer which microbial production can be scaled up by conducting batch aerobic fermentation of *Xanthomonas campestris* from glucose substrate. However, the increase in both price and demand of this product indicates the necessity of application of an economic glucose substrate (Garcia-Ochoa et al., 2000; Katzbaur, 1998). Iran is ranked second in the world for date production, supplying the world with 20% of the total dates, a large amount of which is wasted (Katzbaur,

1998). Hence, this rich source of sugar can be utilized as a domestic and available industrial medium for cell growth and xanthan production. Most research on xanthan microbial production is focused on screening different types of microorganisms which are produced and studying the impact of culture medium variables such as carbon, nitrogen and phosphor sources on the production yield (Garcia-Ochoa et al., 1998; Esgalhado et al., 1995; Casas et al., 2000; Khosravi-Darani et al., 2009). In submerged culture, production of enough xanthan makes the medium highly viscous, which results in problems of agitation. Solid state is applied on different kinds of substrates example, apple and malt pomace, citrus waste, olive extraction wastage, sugar beet, whey,

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cassava bagasse, potato and coffee beans waste. Most of the previously mentioned agro-industrial substrates need acidic, basic and/or enzymatic pretreatments before the fermentation process, except for citrus, apple and malt wastage (Rodriguez-Couto et al., 2006). *X. campestris* requires macro elements (example, carbon, nitrogen and phosphor) and also microelements (example potassium, ferrous and calcium) to produce xanthan. Optimization of type and concentration of chemical components of a medium affects the yield and cost of production (Garcia-Ochoa et al., 1998; Esgalhado et al., 1995; Casas et al., 2000; Khosravi-Darani et al., 2009). Variables which have impact on the yield of xanthan production as well as range finding have been studied (Khosravi-Darani et al., 2009). Process variables include type and concentration of carbon (date extract and sugar beet molass in the range of 2 to 5%), nitrogen and phosphor sources, agitation, temperature (28 to 30°C), fermentation time and pH as well as inoculum age (24 to 25 h) and size (5 to 10%). Among the earlier mentioned factors, temperature, concentration and type of carbon, nitrogen (inorganic source example ammonium nitrate and diammonium phosphate) and phosphor (KH_2PO_4 and K_2HPO_4) sources play the most operative role in cell growth and production using Placket-Burman design (Khosravi-Darani et al., 2009). Baghi-Nejad et al., (2011) conducted research and focused on using date waste as an industrial culture medium to produce xanthan from *X. campestris*. They studied the effect of phosphor (K_2HPO_4), nitrogen (NH_4NO_3) and carbon (date extract) sources using CCD. The maximum concentration of produced biopolymer was reported as 13.2 g/l (Rodriguez-Couto et al., 2006). Khosravi-Darani et al. (2009) also compared the yield of xanthan production using date extract and dried wastage of date extraction, in submerged and solid state fermentations. The results showed that the xanthan production yield (the weight ratio of produced xanthan to consumed sugar) and productivity (g/g. day) are increased in submerged fermentation (22.4%, 7.46) as compared to solid state fermentation (13.3, 4.43). The productivity of the process increased from 7.46 to 8.19 g/kg per day. Obtained results indicated that the kind of carbon and nitrogen sources significantly affects the productivity, and also, applying a two-stage incubation temperature increases the yield (Baghi-Nejad et al., 2011).

This research aims to study the curvature of response surfaces related to variables of carbon and nitrogen sources in a defined data range using response surface method. The central composite design is used to evaluate the main effect and interaction of initial concentration of date extract and ammonium nitrate on cell growth and xanthan gum production in submerged fermentation. According to the obtained results in previous research, the data range of variables is defined with the aim of optimization. The strategy of changing temperature during the cell growth (28°C) and stationary phase (32°C) was applied.

MATERIALS AND METHODS

Inoculum and medium preparation

X. campestris bacterium was purchased from Iranian Research Organization of Science and Technology (IROST), and was cultivated in GYC medium (containing glucose monohydrate, calcium carbonate and yeast extract in 20, 17, 20, 10 g/l, respectively). The inoculum was prepared in YMA and YMB (without agar) and inoculation control was applied as described in previous research (Khosravi-Darani et al., 2009). Date extract was obtained from Dombaz Company (Hormozgan, Iran) and factors such as ash, nitrogen content, pH and sugar were analyzed according to the methods reported in the previous study (Khosravi-Darani et al., 2009). The detailed composition of date extract is as follows: dry substance 77.89; pH 4.77; ash 0.07% w/w; acidity (as citric acid) 0.05; conductivity 4 μS/cm; fructose 37.4%; dextrose 34.1%; sucrose 0.08%.

Fermentation condition

Experiments were conducted using 500-ml Erlenmeyer flasks containing 200 ml of production medium. Date extract and ammonium nitrate (NH_4NO_3) were used as the carbon and nitrogen sources, respectively. Other components of fermentation medium were (g/l) H_3BO_3 2.1, $MgCl_2$ 0.507, Na_2SO_4 4.6, H_3BO_3 0.006, ZnO 0.006, $Fe_2Cl_3.6H_2O$ 0.020, $CaCO_3$ 0.020, $FeSO_4$ 0.008 and HCl 0.13 ml. Fermentation was applied in an incubator at 28°C for 72 h with a shaking rate of 200 rpm. This experiment was repeated three times to reduce the experimental errors and to ensure that data collection was done correctly.

Biomass determination

Biomass and xanthan were determined gravimetrically. Production medium was diluted 4 times and cells were collected by centrifugation for 40 min at 21000 *xg*. Then, biomass was resuspended in isopropanol (IPA) twice to wash out gum residues. Cells were dried in an oven for 24 h at 80°C and weighed.

Gum determination

For separation of xanthan gum, IPA was added to supernatant of culture medium (at 300%, v/v) containing 1 g/l NaCl and centrifuged for 20 min at 21000 *xg*. Precipitated gum was dried in an oven for 48 h at 80°C and weighed. Viscosity was measured by a viscometer (Brookfield, USA) with spindle No. 2 and shaking rate of 100 rpm.

Statistical analysis

Statistical analysis was performed using SPSS-11 software. After confirmation of data normality, results were statistically analyzed and reported as average ± standard deviation with a confidence limit of 95%. The CCD design of the experiment was conducted by Mini Tab-14 software.

RESULTS

Central composite design

Trials were conducted according to experimental design

Table 1. CCD for xanthan production from date syrup in submerged fermentation.

Sequence	Carbon Source code	Nitrogen source code	Carbon source (g/l)	Nitrogen source (g/l)	Xanthan concentration (g/l)	Biomass concentration (g/l)
1	0	-1	50	0.2	8.40±0.39	4.18±0.15
2	-1	-1	40	0.2	11.20±0.44	4.31±0.19
3	1	-1	60	0.2	4.80±0.48	3.80±0.18
4	1	1	60	0.3	4.04±0.27	3.16±0.16
5	-1	0	40	0.25	11.06±0.37	4.30±0.13
6	0	0	50	0.25	8.01±0.44	3.89±0.17
7	0	-1	50	0.2	8.60±0.43	4.30±0.22
8	-1	0	40	0.25	11.03±0.51	4.24±0.21
9	0	0	50	0.25	8.30±0.38	4.10±0.16
10	1	0	60	0.25	4.87±0.38	3.43±0.15
11	1	1	60	0.3	4.26±0.33	3.10±0.18
12	0	1	50	0.3	8.00±0.52	3.80±0.22
13	-1	1	40	0.3	10.50±0.65	4.20±0.23
14	1	0	60	0.25	5.15±0.17	3.50±0.14
15	-1	-1	40	0.2	11.30±0.64	4.30±0.13
16	0	1	50	0.3	7.80±0.39	3.93±0.11
17	1	-1	60	0.2	5..40±0.38	3.80±0.10
18	-1	1	40	0.3	10.90±0.66	4.20±0.25

of Table 1. Different rows of the table were done randomly to eliminate the interferential errors caused by time. The concentration of xanthan and cell mass were determined and given in last columns of Table 1. According to results given in Table 1, a quadratic equation with all terms was proposed to represent a suitable model for xanthan production, with concentration of carbon (C) and nitrogen (N) sources (Equation 1):

$$Y = \beta_0 + \beta_1 C + \beta_2 N + \beta_3 C^2 + \beta_4 N^2 + \beta_5 CN \qquad (1)$$

Where, β_i is the equation coefficient and its presence and amount can be calculated by linearization. Calculations were done using MINITAB-14 software and variables with P more than 0.05 were eliminated. Calculations showed that an interaction effect (between nitrogen and carbon source concentrations, with P = 0.942) and also the square effect of nitrogen concentration (P = 0.863) can be neglected. After elimination of ineffective factors and recalculations of Equation 1, the P value of all remaining terms was replaced to obtain Equation 2. The amount of produced xanthan can be achieved by putting different concentrations of sugar (carbon) and nitrogen sources in Equation 2 as model:

$$Y = -15.2075 + 0.777833C + N - 0.00876667C^2 \qquad (2)$$

Model R-sq had a correlation coefficient of 99.8% which indicates that the model had a good accuracy and conformity with experimental data. Furthermore, the low P value related to lack-of-fit parameter (0.137) also indicates the suitability of the model.

Variables' main effects and their interactions on cell growth and xanthan production

Figures 1 and 2 show the main effects of each variable on cell growth and xanthan production. As shown in the results increasing the nitrogen concentration from 0.2 to 0.3 (g/l) caused increased cell growth but decreased xanthan production. Generally, the nitrogen factor is vital for cell growth, but not for xanthan production. In fact, nitrogen is not a structural participant in xanthan polysaccharide. Its presence is necessary only for cell growth and enzyme production of catabolic and anabolic pathways. Maximum xanthan is produced with the least amount of nitrogen (0.2 g/l). An increase in carbon concentration from 40 to 60 (g/l) caused a decrease in cell growth and xanthan production. No more cell generation and xanthan production can be observed in carbon concentrations up to 50 g/l. Maximum xanthan production was obtained in 40 g/l concentration and it decreased in higher concentrations. High levels of sugar source in the medium could cause that decrease. On the other hand, it could be attributed to various compounds in date extract as a complex medium, which can act as inhibitor factors of cell growth. These compounds can
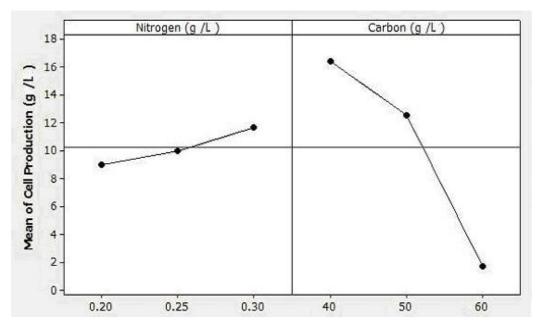
Figure 1. The impact of initial concentration of carbon and nitrogen sources on the cell growth.
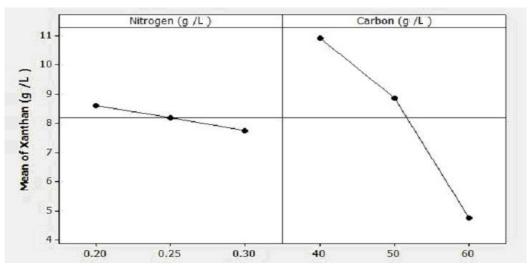

Figure 2. The impact of initial concentration of carbon and nitrogen sources on xanthan production.

affect gum production and cell growth in a direct or indirect way. Most growth inhibitor compounds found in date extract are acid, phenol and quinine compounds (Leela et al., 2008).

Figures 3 and 4 illustrate the interaction of variables on cell growth and xanthan production. In the range of 0.2 to 0.3 g/l, any change in nitrogen concentration does not cause any significant changes in both dependent variables, that is, growth and xanthan production. But, the role of the carbon source concentration was more significant on both responses. The ratio of carbon to nitrogen (C/N) was one of the effective parameters in this

process. It can be observed (Figure 4) that the most xanthan production was obtained at a low level of carbon concentration (40 g/l). Also it increased when nitrogen concentration was low (increase in C/N ratio). Cell growth reached the maximum level in 40 g/l of carbon source, and the reduction of nitrogen content (increase in C/N ratio) caused decreased cell growth.

Optimum variables concentration

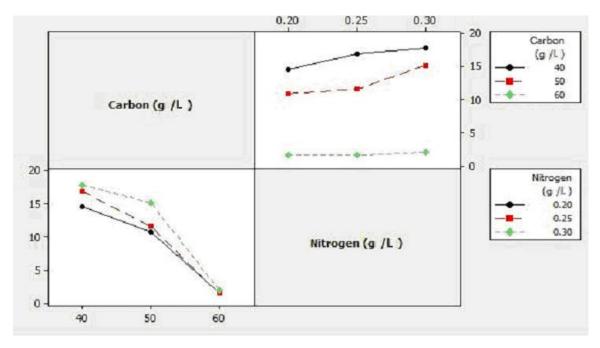Figures 5 and 6 show changes in cell concentration and

Figure 3. The interaction of initial concentration of carbon and nitrogen sources on the cell growth.
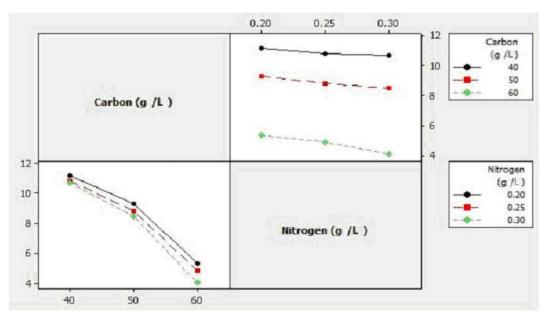
Figure 4. The interaction of initial concentration of carbon and nitrogen sources on xanthan production.

xanthan production based on changes in process variables. Figure 6 shows that in 40 to 45 g/l concentration of carbon source and 0.2 to 0.3 g/l of nitrogen source, cell growth is at its maximum. This result is in agreement with the previous reports (Leela et al.,2008; Yoo and Harcum, 1999; Souw and Demain, 1980; Funahashi et al., 1987; Lo and Yang, 1996; Esgalhado et al., 1995; Casas et al., 2000). The results show that the variation in nitrogen source does not have

a significant effect on cell growth. In the range of 40 to 43 g/l of carbon source and 0.2 to 0.22 g/l of nitrogen source, maximum xanthan production is obtained. The slope of the plots showed that nitrogen concentration has a small impact on xanthan production, while carbon concentration has a significant impact. However, since the amount of optimum concentration of carbon and nitrogen sources are at the edge of the data range, it is necessary to investigate the effect of smaller amounts of
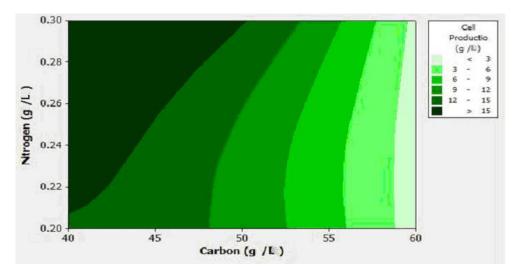
Figure 5. The plot of variation of cell concentration based on variation of carbon and nitrogen.
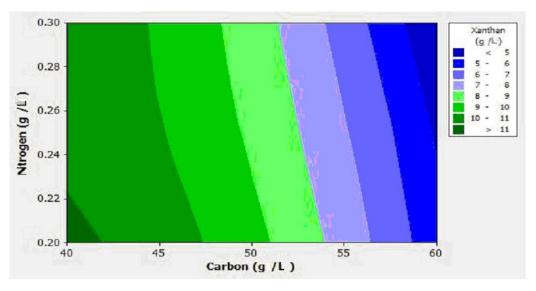

Figure 6. The plot of variation of xanthan concentration based on variation of carbon and nitrogen.

these sources.

DISCUSSION

In this research, xanthan was produced by the method of submerged fermentation, using *X. campestris* PTCC 1473. The initial concentrations of carbon (date extract) and nitrogen sources (NH_4NO_3) were optimized for cell growth and xanthan production. The results show that maximum cell growth and xanthan production were obtained at 40 g/l of carbon source. Changing the nitrogen source concentration in the selected data range had no significant impact on cell growth. The maximum amount of xanthan production was obtained in 0.2 g/l of nitrogen source. Maximum concentration of xanthan

production was 11.2 g/l which is 10% higher than what was obtained in a similar process organized at a constant temperature of 28°C. Ben Salah et al. (2010) investigated and optimized the possibility of xanthan gum production by *X. campestris* NRRL B-1459 in batch experiments on date palm juice. The optimal conditions were 84.68 g/l for carbon source, 2.7 g/l for nitrogen source and 30.1°C for temperature. In fact, usage of date extract as a carbon source in this study for xanthan production reduces the requirement of high level of a separated nitrogen source concentration. Lower consumption of nitrogen source may lead to decreasing medium cost in large scale, and can promote commercialization of the xanthan production from date syrup. Results of this research showed that the carbon concentration to nitrogen ratio (C : N) has a significant impact on the xanthan production process. Its

optimum level varies during the xanthan generation phases. In fact, as much as the C/N ratio decreased, more cells were generated, and as much as it increased, more xanthan were produced (Leela et al., 2008; Yoo and Harcum, 1999; Souw et al., 1980; Funahashi et al., 1987; Lo and Yang, 1996). In fact, a small ratio of C/N in the cell growth phase (first 24 h of the process) causes an increase in cell generation which is due to protein and biomass production. However, since the structure of xanthan does not include the nitrogen atom, abundance of nitrogen source does not affect the production of this gum. Most metabolic cycles tend to produce biomass, while in xanthan production phase (the next 48 h of the process), the increase in the C/N ratio acts as a signal to produce xanthan hetero polysaccharide. The limited nitrogen source in the production phase (as much as it does not prevent cell growth) increases the yield; while in the growth phase, it could act as a cell growth inhibitor. Other researchers also stated that nitrogen is one of important nutrients for growth of *Xanthomonas* sp., which can be used either in organic or inorganic forms in the medium (Cadmus and Knutson, 1983). The impact of nitrogen source on xanthan production is in contrast with those of carbon source. By increasing the concentration of a nitrogen source with a constant concentration of carbon source, specific growth rate and cell productivity will increase, but rate and yield of xanthan production will decrease (Letisse et al., 2001; Cadmus and Knutson, 1983). Cadmus et al. (1983) showed that nitrogen content of the culture medium highly affects the pyruvate content, and the organic nitrogen source can act as an inhibitor factor of xanthan production. According to this report, inorganic nitrogen is not a suitable source because of its high cost, low availability, low quality of produced xanthan (which contains pyruvic and citric acid), its dark color, insoluble substances content and its expensive purification cost. However, produced xanthan should be free of insoluble and colored substances in industrial usage, and it must also contain approximately 3.3% pyruvate (Letisse et al., 2001). Letisse et al. (2001) and Ochoa et al. (2004) reported that ammonium nitrate and diammonium phosphate cause maximum yield of xanthan production among inorganic sources. High viscosity which is due to xanthan production caused difficulties in the fermentor agitation. This is why *Xanthomonas* sp. is an aerobic bacterium and a decrease in the soluble oxygen content of the system not only reduces the agitation feasibility but also cause high energy consumption during the agitation procedure. Thus, kinetic control and modeling would be impossible (Rottava et al., 2009). Although, this defi-ciency is not problematic in lab scale (due to the specific shape of the mixer and the good performance of the sparger), it can cause serious problems at an industrial scale. For further studies in this field, investigation of xanthan production in lower level of carbon and nitrogen sources is suggested. Also, bioreactor scale of up to 20 L as well as different feeding strategies needs to be studied. Furthermore, studying the rheological properties of xanthan and optimizing the fermentation conditions (for food application purposes) could be an innovative and informative research. For instance, applying different temperatures, dissolved oxygen and compounds of culture medium are suggested to change the viscosity.

This research was conducted to evaluate the effects of initial concentrations of carbon (date extract) and nitrogen sources (NH_4NO_3) on cell growth and xanthan production by *X. campestris* PTCC 1473. The results show that maximum cell growth and xanthan production were obtained at 40 g/l of carbon source. Reversely, increase of nitrogen source concentration has negative and slight effect on xanthan production in the range of 0.2 to 0.3 g/l. In fact, use of date extract as a carbon source in medium of xanthan production, reduces the requirement of high level of a separated nitrogen source concentration. Lower consumption of nitrogen source may lead to decreasing medium cost in large scale, and can promote commercialization of the xanthan production from date syrup.

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