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Optimization of exopolysaccharide welan gum production by *Alcaligenes* sp. CGMCC2428 with Tween-40 using response surface methodology

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ABSTRACT

The effect of additives on welan gum production produced by fermentation with *Alcaligenes* sp. CGMCC2428 was studied. Tween-40 was the best additive for improving welan gum production and welan gum displayed better rheological properties than that obtained by control fermentation without additives. Response surface methodology was employed to optimize the culture conditions for welan gum production in the shake flask culture, including Tween-40 concentration, pH and culture temperature. The optimal conditions were determined as follows: Tween-40 concentration 0.94 g/l, pH 6.9 and temperature 29.6 °C. The corresponding experimental concentration of welan gum was 23.62 ± 0.60 g/l, which was agreed closely with the predicted value (23.48 g/l). Validation experiments were also carried out to prove the adequacy and the accuracy of the model obtained. The welan gum fermentation in a 7.5 l bioreactor reached 24.90 ± 0.68 g/l.

1. Introduction

Welan gum is an anionic exopolysaccharide produced by fermentation using *Alcaligenes* species (O'Neill, Selvendran, Morris, & Eagles, 1986). It is composed of tetrasaccharide repeating units of D-glucose, D-glucuronic acid and L-rhamnose at a ratio of 2:1:1 with L-rhamnopyranosyl or L-mannopyranosyl side chains (Chandrasekaran, Radha, & Lee, 1994). At least 85% of the repeat units also have an acetyl substituent at O(2) of the 3-linked glucose. Due to its high and stable viscosity in aqueous solutions over a broad range of temperatures (up to 150 °C) and pH (2–12), welan gum has great commercial potential in the food industry, as a concrete additive and for enhancing oil recovery (EOR) (Kang, Veeder, & Cottrell, 1983). Welan gum is especially valuable in EOR because of its excellent stability at high temperatures.

Welan gum fermentation has been improved by breeding high producing strains (Guo, Qiao, Li, Li, & Xu, 2007) and by optimizing the culture conditions (Li, Xu, & Shi, 2004). However, welan gum production using *Alcaligenes* species was relatively lower than xanthan gum production from *Xanthomonas campestris*. For example, the conversion rate of welan gum from 50 g/l glucose was 30–40% using *Alcaligenes*, whereas xanthan gum production from 50 g/l glucose was 70–90% efficient in fermentations with *Xanthomonas campestris* (Hsu & Lo, 2003). Thus, it is necessary to greatly improve the fermentative production of welan gum to achieve similar efficiency.

Previous studies have shown that additives like surfactants and polar organic solvents (Tween-40, Tween-80, Triton X-100, dimethyl sulfoxide DMSO or glycerol) can enhance the production of many biomolecules produced by fermentation, including cellulase (Stutzenberger, 1987), β-amylase, pullulanase (Reddy, Reddy, & Seenayya, 1999), glutathione (Wei, Li, Du, & Chen, 2003), γ-glutamic acid (Wu et al., 2008), gellan gum (Arockiasamy & Banik, 2008) and xanthan gum (Galindo & Salcedo, 1996). These additives may enhance production by increasing cell membrane permeability, which is a natural barrier to the transport of extracellular substrates and the secretion of products (Wu et al., 2008). Presently, there are no reports documenting the effects of these additives on the production of welan gum by fermentation. Considering that welan gum has the same linear polymer structure as gellan gum, we speculated that these same additives might also influence the permeability of *Alcaligenes* sp. CGMCC2428 cell membranes and improve the biosynthesis or recovery of welan gum.

A response surface methodology for the optimization of product synthesis by fermentation was applied successfully for the

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optimization of medium constituents and other critical reaction parameters (Gan & Latiff, 2011; Sun, Liu, & Kennedy, 2010). Response surface methodology overcomes the limitations of single-parameter optimization, which is both time-consuming and cannot assess the complex interactions among the various physicochemical parameters (Ye & Jiang, 2011). In this work, the effect of several additives on exopolysaccharide welan gum production in shake flask culture was investigated and an optimized production protocol designed by defining the best additive, additive concentration, pH and temperature. This is the first report on the enhanced production of welan gum using additives.

2. Materials and methods

2.1. Microorganism and medium

Alcaligenes sp. CGMCC2428 was used in this study. It was deposited in the China General Microbiological Culture Collection Center. The seed medium contained 20 g/l glucose, 1 g/l yeast extract, 3 g/l peptone, 2 g/l $K_2HPO_4{\cdot}3H_2O$, 0.1 g/l $MgSO_4$ (pH 7.2–7.4). The fermentation medium was comprised of 50 g/l glucose, 8 g/l $(NH_4)_2SO_4$, 3 g/l $K_2HPO_4{\cdot}3H_2O$ and 0.4 g/l $MgSO_4$. The initial pH was adjusted to 7.0.

2.2. Cultivation conditions

Shake flask studies were performed in 80 ml of fermentation medium in 500 ml shake flasks. The flasks were incubated under different conditions that were varied based on the experimental design.

Fermentor studies were carried out in a 7.5 l bioreactor (Rushton-style impeller, d. 6 cm; bioreactor i.d. 17.8 cm, height 32.1 cm, BioFlo 110, New Brunswick Scientific, USA) with a working volume of 4.5 l. *Alcaligenes* sp. CGMCC2428 was first inoculated into 135 ml of fresh seed medium in 1 l flasks and aerobically incubated for 12–14 h at 30 °C and 200 rpm. Seed culture (3%, v/v) was then inoculated into the fermentation medium. The pH of the medium was controlled by the automatic addition of 3 M NaOH. The bioreactor was agitated at 600 rpm and aerated at a fixed rate of 1.0 volume of air per volume of liquid per minute (vvm). The additives were added to the medium at a measured concentration at the beginning of the fermentation. Controlled fermentations without additives were also carried out to serve as controls.

2.3. Analytical methods

Dry cell weight (DCW) was determined during fermentation from at least three 10 ml cell sample suspensions that were then harvested by centrifugation, and then the precipitate was washed with distilled water and dried at 105 °C. The supernatant was collected and the welan gum was precipitated by using two volumes of ice-cold alcohol. The precipitate was recovered by centrifugation at 10,000 × g for 10 min, and dried in a hot-air oven (60 °C, 4 h). The welan gum concentration was determined (Li et al., 2004). The glucose concentration was analyzed by a biosensor equipped with a glucose oxidase electrode (SBA-40C, Shandong Academy of Sciences, China). The pH and DO were monitored with Ingold pH probe and polarographic electrodes on the bioreactor. The weight-average molecular weight (MW) and viscosity of welan gum (1% in water, 25 °C) were measured as previously described (Li et al., 2011).

2.4. Experimental design and data analysis

2.4.1. Central composite design and response surface methodology

The effects of three variables (additive concentration, pH and temperature) on welan gum production in flasks were studied using central composite design and response surface methodology. These three independent factors were studied at five different levels (−1.68, −1, 0, +1 and +1.68) selected on the basis of our preliminary experimental work that indicated an optimum could be reached within these ranges. The variables were coded according to the regression equation (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \quad i = 1, 2, \ldots, k \tag{1}$$

In Eq. (1), x_i is the coded independent factor, X_i is the real independent factor, X_0 is the value of X_i at the center point and ΔX_i is the step change value.

Sixteen experiments were carried out with each at five levels (Table 1) in flasks. Experimental data were fitted to a second-order polynomial model and regression coefficients were obtained. The model equation for the analysis is given by:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j, \quad i = 1, 2, \ldots, k \tag{2}$$

where Y is the response variable, β_0 is the constant, β_i is the coefficient for the linear effect, β_{ii} is the coefficient for the quadratic effect, β_{ij} is the coefficient for the interaction effect, and x_i and x_j

Table 1
Experimental design and results of the central composite design.

Run	Tween-40 (g/l)	pH	Temperature (°C)	Welan gum (g/l)	
	X_1	X_2	X_3	Observed[a]	Predicted
1	0.50	6.50	28.00	22.41 ± 0.65	22.39
2	0.50	6.50	32.00	21.61 ± 0.60	21.57
3	0.50	7.50	28.00	22.54 ± 0.70	22.24
4	0.50	7.50	32.00	21.60 ± 0.55	21.56
5	1.50	6.50	28.00	22.32 ± 0.67	22.19
6	1.50	6.50	32.00	22.05 ± 0.58	22.19
7	1.50	7.50	28.00	21.15 ± 0.55	21.03
8	1.50	7.50	32.00	21.30 ± 0.40	21.16
9	0.16	7.00	30.00	21.84 ± 0.62	22.00
10	1.84	7.00	30.00	21.43 ± 0.67	21.50
11	1.00	6.20	30.00	22.35 ± 0.71	22.30
12	1.00	7.80	30.00	21.04 ± 0.48	21.32
13	1.00	7.00	26.70	22.12 ± 0.59	22.38
14	1.00	7.00	33.40	21.84 ± 0.54	21.81
15	1.00	7.00	30.00	23.46 ± 0.68	23.42
16	1.00	7.00	30.00	23.41 ± 0.64	23.42

[a] Mean ± standard deviation ($n = 3$).

Table 2
Production of welan gum by *Alcaligenes* sp. CGMCC2428 with additives in medium. The culture was performed in 500 ml shake flasks containing 80 ml of the fermentation medium and the flasks were incubated in a rotary shaker at 30 °C and 200 rpm for 72 h. The addition concentrations of Tween-40, DMSO and glycerol were 0.5 g/l, 1.25 g/l and 1.25 g/l, respectively.

Sample	DCW (g/l)[a]	Welan gum (g/l)[a]	MW (×10^5)[a]	Viscosity (Pa s)[a]
Control	5.71 ± 0.18	19.05 ± 0.72	9.02 ± 0.09	2.55 ± 0.05
Tween-40	6.38 ± 0.21	22.50 ± 0.65	9.31 ± 0.08	3.12 ± 0.07
DMSO	5.99 ± 0.22	20.51 ± 0.61	9.06 ± 0.10	2.68 ± 0.06
Glycerol	6.13 ± 0.24	21.15 ± 0.70	9.15 ± 0.11	2.81 ± 0.06

[a] Mean ± standard deviation (n = 3).

are the coded independent factors. The quadratic equation (2) was used to plot surfaces for the variables.

2.4.2. Statistical analysis

Statistica version 8.0 (StatSoft, Inc., Tulsa, USA) was used for the experimental design and regression analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2 and its statistical significance was determined by an F-test.

2.4.3. Experimental validation of the optimized condition

In order to validate the above optimization model, three tests were carried out using the optimized condition to confirm the result from the analysis of the response surface.

3. Results and discussion

3.1. Improved welan gum production with additives

Of the 11 additives investigated (cetyltriethylammonium bromide, sodium dodecyl sulfate, Triton X-100, Span-20, Span-40, Span-80, Tween-20, Tween-40, Tween-80, DMSO and glycerol), Tween-40, DMSO and glycerol were found to be the most effective at improving welan gum production in *Alcaligenes* sp. CGMCC2428 fermentation. The optimum concentrations were 0.5 g/l Tween-40, 1.25 g/l DMSO and 1.25 g/l glycerol. Among these three additives, fermentation with Tween-40 yielded the highest welan gum concentration at 22.50 ± 0.65 g/l, followed by DMSO (20.51 ± 0.61 g/l) and glycerol (21.15 ± 0.70 g/l) compared with control fermentation without additives (19.05 ± 0.72 g/l) (Table 2). It was also found that all these three additives promoted cell growth.

The exopolysaccharide obtained from the fermentation with additives showed better rheological properties than that produced by the control fermentation. The viscosity of welan gum obtained from the fermentation with Tween-40, DMSO or glycerol was higher (3.12 ± 0.07 Pa s, 2.68 ± 0.06 Pa s and 2.81 ± 0.06 Pa s, respectively) than that obtained by control fermentation (Table 2). The welan gum MW was also investigated. The MW reached a maximum value of (9.31 ± 0.08) × 10^5 Da in the presence of Tween-40, but was only (9.02 ± 0.09) × 10^5 Da in the control fermentation. For welan gum, the higher the MW, the higher is the viscosity. This might be responsible for the superior rheological property of welan gum obtained from fermentation with additives.

All these three additives could increase the permeability of bacterial cell membranes, thereby enhancing the substrate transport into the cells and the release of welan gum polymer back into the media (Arockiasamy & Banik, 2008). The DCW, glucose consumption and DO concentration profiles during welan production with or without 0.5 g/l Tween-40 in a 7.5 l bioreactor are compared in Fig. 1. Compared with the control fermentation without Tween-40, the DO concentration exhibited an increasing trend and the glucose consumption rate was higher during fermentation with Tween-40. Moreover, the residual glucose concentration at the end of the fermentation time was lower (6.00 ± 0.24 g/l) in the presence of Tween-40 than at the end of control fermentation (12.00 ± 0.48 g/l). This suggests that the additives enhanced the mass and oxygen transfers during fermentation, leading to the increased utilization of nutrients for welan gum production.

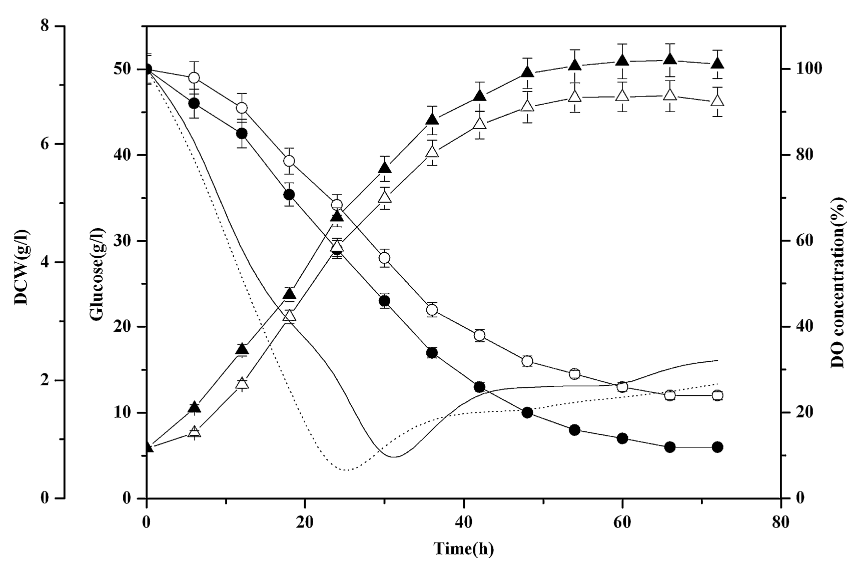

Fig. 1. DCW, glucose consumption and DO concentration during welan gum production by *Alcaligenes* sp. CGMCC2428. The fermentation was performed in a 7.5 l bioreactor containing 4.5 l of the fermentation medium at 30 °C and agitated at 600 rpm. The pH was maintained at 7.0 by the addition of sterilized 3 M NaOH. DCW-Tween-40 (▲), DCW-control (△), glucose-Tween-40 (●), glucose-control (○), and DO-Tween-40 (—), DO-control (. . .).

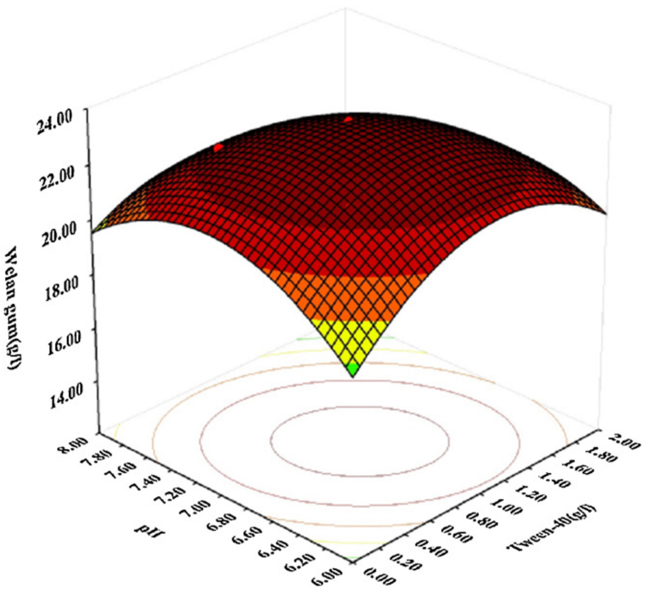

Fig. 2. Response surface curve for welan gum production by *Alcaligenes* sp. CGMCC2428 as a function of Tween-40 and pH, when temperature was maintained at 30.00 °C.

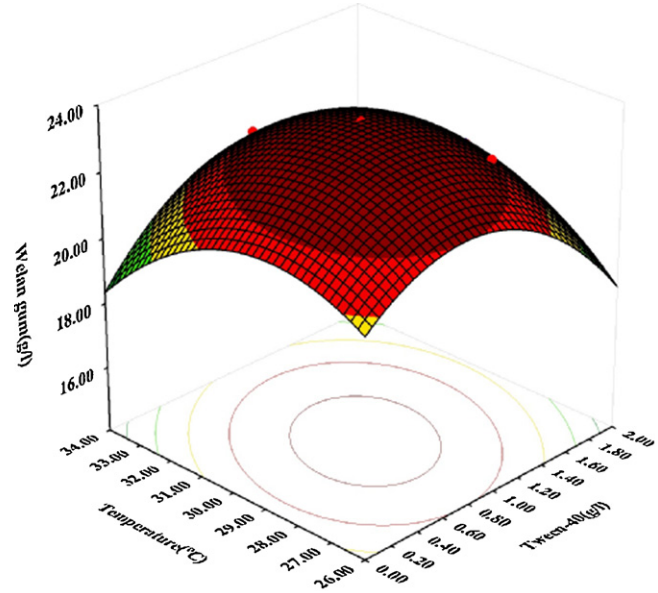

Fig. 3. Response surface curve for welan gum production by *Alcaligenes* sp. CGMCC2428 as a function of Tween-40 and temperature, when pH was maintained at 7.00.

3.2. Optimization of Tween-40 concentration, pH and temperature for welan gum production

Response surface methodology using central composite design was employed to determine the optimal levels of the three selected factors (Tween-40 concentration, pH and temperature) that influenced welan gum production. Utilizing the multiple regression analysis, we found that the second-order polynomial equation model (Eq. (2), Section 2.4.1) explained the welan gum production by *Alcaligenes* sp. with Tween-40.

$$Y = -182.058 + 5.480X_1 - 2.356X_2 + 31.179X_3 - 2.271X_1^2 + 6.472X_2^2 - 0.117X_3^2 - 1.020X_1X_2 + 0.202X_1X_3 + 0.035X_2X_3 \quad (3)$$

In Eq. (3), Y is the predicted welan gum production, X_1, X_2 and X_3 are coded values of Tween-40 concentration, pH and temperature, respectively.

The statistical significance of Eq. (3) was checked by F-test and the ANOVA for response surface quadratic model is summarized in Table 3. The model can be proved fit and to adequately account for the variation observed if the F-test for the model is significant at $P < 0.05$. The smaller P-value indicated that the corresponding variable was more significant. As shown in Table 3, except for X_2X_3, the P-values for others were much less than 0.05, indicating that all these variables were more significant for welan gum production than the interaction between pH and temperature. If the F-test for lack of fit is significant ($P < 0.05$), then a more complicated model is required to fit the data. We considered a regression model with an R^2-value higher than 0.9 as having a very high correlation (Li, Lu, Gu, & Mao, 2005). The P-value for lack of fit (0.1024) and $R^2 = 0.9551$ indicated that the experimental data obtained fit well into the model and could be used to explain the effects of Tween-40 concentration, pH and temperature on welan gum production.

Figs. 2–4 illustrate the three-dimension response surface curves of welan gum production for each pair of parameters by keeping the third factor constant at its zero level. These 3D plots and their respective contour plots provided a visual interpretation of the interaction between two factors and facilitated the identification of the optimum experimental conditions. According to the response surface analysis, the predicted maximum production of welan gum is 23.48 g/l when Tween-40 is 0.94 g/l, pH is 6.9 and temperature is 29.6 °C. This theoretical maximum is 23.25% higher than the yield from control fermentation without additives (19.05 ± 0.72 g/l).

3.3. Experimental validation of the optimized condition

In order to validate the adequacy of the model, verification experiments were carried out at the predicted optimal conditions. The mean concentration of the obtained welan gum from triplicate trials in shake flask was 23.62 ± 0.60 g/l, which was much near the predicted valve (23.48 g/l). Furthermore, the suitability of the model was investigated by batch fermentation of welan gum

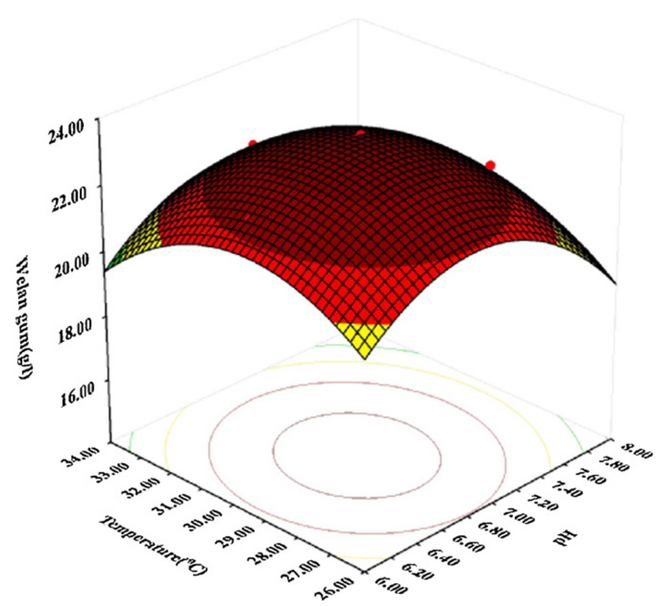

Fig. 4. Response surface curve for welan gum production by *Alcaligenes* sp. CGMCC2428 as a function of pH and temperature, when Tween-40 was maintained at 1.00 g/l.

Table 3
ANOVA for the second-order polynomial model.

Source	SS	DF	MS	F	P
X_1	0.30	1	0.30	241.29	0.0409
X_2	1.17	1	1.17	938.73	0.0208
X_3	0.40	1	0.40	318.26	0.0356
X_1^2	3.21	1	3.21	2571.07	0.0126
X_2^2	2.99	1	2.99	2389.22	0.0130
X_3^2	2.02	1	2.02	1616.44	0.0158
X_1X_2	0.52	1	0.52	416.16	0.0312
X_1X_3	0.33	1	0.33	262.44	0.0392
X_2X_3	0.01	1	0.01	7.84	0.2184
Residual error	0.34	6	0.057		
Lack of fit	0.34	5	0.068	54.61	0.1024
Pure error	0.0013	1	0.0013		
Total SS	7.63	15			

R^2 = 0.9551, $R^2(Adj)$ = 0.8878; SS, sum of squares; DF, degrees of freedom and MS, mean square.

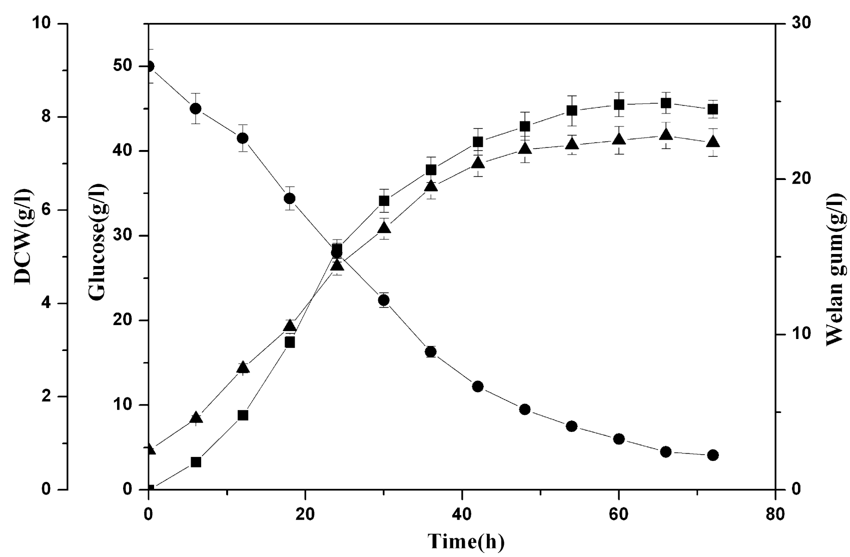

Fig. 5. Time course of batch fermentation in 7.5 l bioreactor for welan gum production by *Alcaligenes* sp. CGMCC2428 under the optimized condition with Tween-40. DCW (▲), glucose (●), and welan gum (■).

using *Alcaligenes* sp. CGMCC2428 in a 7.5 l bioreactor. The glucose consumption, DCW and the production of welan gum were monitored during the cultivation (Fig. 5). As shown in Fig. 5, the highest concentration of 24.90 ± 0.68 g/l welan gum was achieved with an initial concentration of 50 g/l glucose. The model was proved to be adequate.

4. Conclusions

This paper studied the effect of additives on exopolysaccharide welan gum production using *Alcaligenes* sp. CGMCC2428. Of the 11 additives tested, Tween-40 at 0.5 g/l proved to be the best for enhancing of welan gum yield, MW and viscosity. Through response surface methodology, the optimal levels for Tween-40 concentration (0.94 g/l), pH (6.9) and temperature (29.6 °C) were determined that together enhanced welan gum yield by 23.25% compared with the yield obtained without additives. Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and the results showed that the predicted value agreed with the experimental values well. The optimum conditions obtained in this experiment gives a basis for further study with large scale batch fermentation in a bioreactor for the production of welan gum from *Alcaligenes* sp. CGMCC2428.

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