African Journal of Biotechnology Vol. 9 (7), pp. 1024-1030, 15 February, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Optimizing the production of welan gum by *Alcaligenes facalis* NX-3 using statistical experiment design

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Accepted 26 March, 2009

Welan gum is an exopolysaccharide produced by *Alcaligenes facalis* NX-3. The composition of the medium for the production of welan gum was optimized with a stepwise strategy. Effects of various carbon and nitrogen sources were investigated. Plackett–Burman design was applied to elucidate the key ingredients in the media and the results indicated that the corn starch, cottonseed cake flour have significant effects on welan gum production. Central composite design was employed to search for the optimal concentration of the two key components and the experimental results were fitted to a second-order polynomial model at a 98% level of significance. By solving the regression equation and also by analyzing the response surface contour plots, the relative high welan gum production (22.85 g l^{-1}) was obtained at the optimal medium combinations and fermentation conditions as corn starch 43.6 g l^{-1}, cottonseed cake flour 4.1 g l^{-1}, 16-h-old inoculum of 3% (v v^{-1}) sizes, initial pH 7.0 and temperature 30°C. These predicted values were also verified by validation experiments.

Key words: *Alcaligenes facalis* NX-3, Medium optimization, Plackett–Burman design, statistical experiment design, welan gum.

INTRODUCTION

Welan gum is an exopolysaccharide produced by bacterium with many attractive properties. It is composed of tetrasaccharide repeating units of D-glucose, L-rhamnose and D-glucuronic acid with L-rhamnopyranosyl or L-mannosyl as the side chains (Kumar et al., 1996). Due to its excellent rheological properties, welan gum is used in many applications, mainly for concrete additives and enhanced oil recovery (E.O.R.) (Sébastien et al., 1999; Lachemi et al., 2004).

Although it has been several years since welan gum was first discovered, the process for the production has not been thoroughly studied. Many researchers have focused their efforts on the structure and characteristics of welan gum as well as the development of its applications (Sébastien et al., 1999; Lachemi et al., 2004; Eun et al., 1991; Ranieri et al., 1989). In contrast, there were few reports concerning culture medium and process parameters optimization. In biotechnology based industrial processes, the formulation of cultivation medium is of critical importance because their composition affects product concentration, yield and productivity. It is also important to reduce the costs of the medium as this may affect the overall process economics.

Statistical experiment design and data analysis provides an efficient approach to optimize the culture medium compared to the conventional method. The response surface methodology (RSM) is a powerful technique for testing multiple process variables. This methodology brings about the effect of interaction of various parameters, generally resulting in higher production yields and fewer experimental trials (Dean et al., 1999). One such method is the Plackett–Burman (P-B) design (Plackett et al., 1946), which has been frequently used for screening the key factors prior to optimization using RSM in recent years (Chakravarti et al., 2002; Liu et al., 2003; Tanyildizi et al., 2005). It is currently used for optimization studies in several biotechnological and industrial processes (Adinarayana et al., 2003; Almeidae et al., 2003; Rodrigues et al., 2003; Elibol, 2004).

However, it has not yet been reported as a means to study and optimize process parameters for welan gum

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Table 1. Variables and their levels for P–B experimental design.

Constituent	Symbol	Code levels	
		-1	+1
Corn starch (g l^{-1})	X_1	30	50
CCF (g l^{-1})	X_2	3	5
K_2HPO_4 (g l^{-1})	X_3	2	4
$MgSO_4$ (g l^{-1})	X_4	0.1	0.3

fermentation. The objective of this work was to determine the optimum culture medium for maximizing welan gum productivity. A stepwise optimization was performed including: (1) employment of a one-at-a-time method to screen the optimal carbon and nitrogen source, (2) elucidation of the medium component that affect welan gum production significantly using P–B design, (3) central composite design (CCD) was applied to optimize these significant ingredients.

MATERIALS AND METHODS

Bacterial strain

Alcaligenes facalis NX-3, a welan gum producing bacterium was isolated from soil in our laboratory. It was deposited in China General Microbiological Culture Collection Center with the accession number of CGMCC No. 2428 (Xu et al., 2006). It was maintained on agar slants containing (g l^{-1}): glucose 10; peptone 10; beef extract 3; NaCl 5 and agar 20 (pH 7.4-7.6). The slants were incubated at 30°C for 24 h and the fully grown slants were stored at 4°C.

Growth medium and fermentation conditions

A loop of *A. facalis* NX-3 cells was inoculated into 30 ml preculture medium in 250 ml flasks and incubated for 16 h at 30°C with shaking at 200 r min^{-1}. The preculture medium contained (g l^{-1}): glucose 20, yeast extract 1, peptone 4, K_2HPO_4 2, $MgSO_4$ 0.1 (pH7.2-7.4).

All batch fermentations were carried out in Erlenmeyer flasks (500 ml) with 100 ml of fermentation medium. The composition of media varied according to the experimental design described in this work. The initial pH of media was adjusted to 7.2-7.4 and the media were sterilized at 121°C for 15 min. Cultivations were conducted by 16-h-old inoculum of 3% (v v^{-1}) sizes, at 30°C in a rotatory shaker at 200 r min^{-1} for 66 h according to the previous study .

Determination of cell growth

This was done by dry cell weight estimation. Broth was diluted appropriately by distilled water to lower the viscosity and centrifuged at 10000×g for 30 min to separate the cell mass. The biomass was washed twice with distilled water, then dried to a constant weight in a hot-air oven at 60°C for 6 h and weighted.

Determination of welan gum concentration

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 10000×g for 10 min, and dried in a hot-air oven (60°C, 4 h).

Determination of broth viscosity

The viscosity of the fermentation broth was measured by a rotational viscometer (Model NDJ-1, Shanghai Heng-Ping Scientific Instrument Co. Ltd., China) with disk spindle 4 at 30 r min^{-1} at 25°C.

Screening the optimal nitrogen and carbon sources

Six different carbon sources at concentration of 40 g l^{-1} (glucose, sucrose, maltose, lactose, glycerol and corn starch), seven sources of nitrogen (peptone, yeast extract, defatted soybean powder(DSP), defatted peanut powder(DPP), cottonseed cake flour(CCF), ammonium nitrate and ammonium sulfate) at concentrations of 4 g l^{-1}, were pre-tested. For this investigation, cultivations were carried out with media containing K_2HPO_4 2 g l^{-1}; $MgSO_4$ 0.1 g l^{-1}.

P-B design

In order to determine which nutrients had a significant effect on welan gum production, P-B design was used. Based on the P-B design, each factor was examined at two levels: -1 for a low level and +1 for a high level. Table 1 illustrated the factors under investigation as well as levels of each factor used in the experimental design. The mean of the welan gum production obtained was taken as the response. Variables with $p<0.05$ were considered to influence welan gum production significantly.

Optimization of key ingredients with central composite design (CCD)

The optimization of key components concentration to maximize the welan gum production was determined with CCD (Liu et al., 2003; Liu et al., 2007). The variables were coded according to the following regression equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}$$

where x_i is the coded variable of a nutrient factor, X_i is the natural variable of the nutrient factor. X_0 is the value of the natural variable at the center point, and ΔX_i is the step change value. The variables and levels were shown in Table 2.

Table 3 shows 12 trials of the two variables, each at five levels. The design was made up of a central composite design with four replications of the central points (all factors at level 0) and the four star points. In each case, welan gum production was determined. All experiments were performed in triplicates for the mean calculation. The value of welan gum production was taken as the response. The experimental results were fitted with a second-order polynomial function as follows:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where Y is the response variable, β_0 the constant, β_i the coefficient for the linear effect, β_{ii} the coefficient for the quadratic effect, β_{ij} the coefficient for the interaction effect, and x_i and x_j the coded level of variable X_i and X_j. The above quadratic equation was used to plot surfaces for the variables.

Time course of welan gum production

To confirm the above predictions, further experiments were performed comparing the optimized medium in this work and a non-

Table 2. Variables and their levels for CCD.

Constituent	Symbol	Code levels				
		-1.41	**-1**	**0**	**+1**	**1.41**
Corn starch (g l^{-1})	X_1	25.85	30	40	50	54.14
CCF (g l^{-1})	X_2	2.58	3	4	5	5.41

Table 3. Experimental design and results of CCD.

Trial no.	Corn starch		CCF		Yield of welan gum（g l^{-1}）			
	X_1(g l^{-1})	Code	X_2(g l^{-1})	Code	Experimental value			Predict value
					Set1	Set2	Average	
1	30.00	-1	3.00	-1	15.91	17.4	16.66	16.74
2	30.00	-1	5.00	1	18.71	18.09	18.40	18.10
3	50.00	1	3.00	-1	18.76	18.93	18.85	18.76
4	50.00	1	5.00	1	20.77	20.54	20.66	20.17
5	25.85	-1.41	4.00	0	16.89	17.18	17.04	17.11
6	54.14	1.41	4.00	0	19.63	19.74	20.60	20.01
7	40.00	0	2.58	-1.41	16.87	18.01	17.44	17.36
8	40.00	0	5.41	1.41	18.87	18.81	18.84	19.31
9	40.00	0	4.00	0	21.05	20.83	21.94	20.98
10	40.00	0	4.00	0	20.97	21.11	22.04	20.98
11	40.00	0	4.00	0	20.96	21.13	22.85	20.97
12	40.00	0	4.00	0	21.07	20.87	21.97	20.98

optimized medium used for the production of welan gum by *A. facalis* NX-3 before, which contained (g l^{-1}): sucrose 40; peptone 1; yeast extract 5; K_2HPO_4 4; $MgSO_4$ 0.1. 7.5 l bioreactor was filled with 4l of production medium and sterilized at 121°C for 15 min. It was inoculated with 3% (v v^{-1}) of 16-h-old culture. The bioreactor was operated at 30°C, and agitation was shifted from 200 to 600 r min^{-1} for a constant aeration of 1.5 v v^{-1} min^{-1}

Data analysis

All statistical experimental designs and results analyses were carried out using Statistica 6.0 software (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities p(t) were determined by Student's t-test; The variance explained by the model is given by the multiple determination coefficient R^2.

RESULTS AND DISCUSSION

Effects of carbon sources on welan gum production

The growth of cells, the yield of welan gum, and the viscosity of the fermentation broth were investigated using six different carbon sources. As shown in Figure 1(A), the maximum biomass (5.8 g l^{-1}), the broth viscosity (11000 cp), and the synthesis of welan gum (19.1 g l^{-1}) were observed when corn starch served as the carbon source, followed by sucrose and glucose. Utilization of lactose by *A. facalis* NX-3 was poor, and neither the production of welan gum nor the viscosity of the fermentation broth was satisfactory.

Effects of nitrogen sources on welan gum production

The effects of nitrogen sources on the welan gum production by *A. facalis* NX-3 was investigated using corn starch (40 g l^{-1}) as carbon source. The results were shown in Figure 1(B), the highest yield (18.6 g l^{-1}) was obtained with CCF, while yeast extract produced the highest biomass (6.08 g l^{-1}) with welan gum production of 16.1 g l^{-1}. When $(NH_4)_2SO_4$ and $(NH_4)_3PO_4$ were used, welan gum production was low, that is to say inorganic nitrogen sources had poor effect on growth and welan gum production. CCF was thus chosen as the nitrogen sources for further experiments.

P–B design

The most important nutrient factors were screened by applying the P-B design as described in Table 1. The experimental design and the results were illustrated in Table 4. The welan gum production varied greatly from 12.03 to 19.23 g l^{-1} with different combinations of the components in the media. The highest values of welan

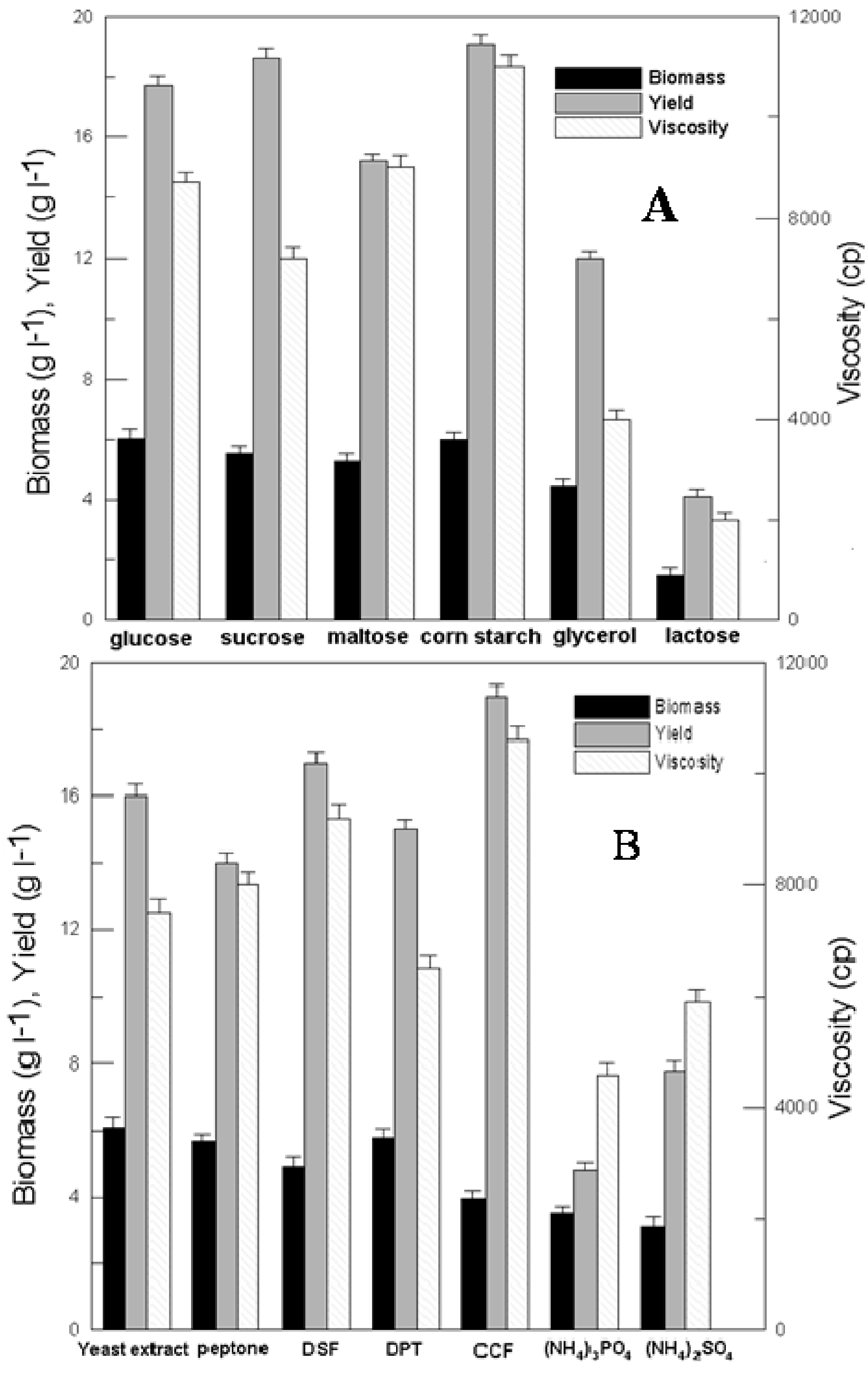

Figure 1. Effects of different carbon (A) and nitrogen (B) sources on welan gum production.

gum yield was obtained when the maximal level of corn starch and CCF, the minimal level of inorganic salts were used. Table 5 shows the statistical analysis of the studied variables on welan gum production. As can be seen from Table 5, welan gum production was greatly affected by corn starch and CCF ($P<0.05$). The other components in the media showed no significant influence welan gum production. The effect estimate of corn starch and CCF was positive, which suggested that the increase of their concentration in the medium will result in the promotion of welan gum production, while the effect estimates of K_2HPO_4 and $MgSO_4$ were negative, which meant low level of their concentration would benefit welan gum synthesis. The two nutrient components, corn starch and

Table 4. Experimental design and results of P-B design.

Trial	Corn starch	CCF	K_2HPO_4	$MgSO_4$	Welan gum (g l^{-1})
1	-1	-1	-1	-1	12.83
2	1	-1	-1	1	15.98
3	-1	1	-1	1	15.69
4	1	1	-1	-1	19.23
5	-1	-1	1	1	12.03
6	1	-1	1	-1	14.35
7	-1	1	1	-1	17.37
8	1	1	1	1	18.21

Table 5. Statistical analysis for welan gum production from the result of P-B design.

Factors	Medium components	Effect	Std.Err.	*t*-value	*P*-value
Mean/Interc.		15.7112	0.3476	45.2006	0.0000
X_1	Corn starch	2.4625	0.6952	3.5423	0.0383
X_2	CCF	3.8275	0.6952	5.5058	0.0118
X_3	K_2HPO_4	-0.4425	0.6952	-0.6365	0.5697
X_4	$MgSO_4$	-0.4675	0.6952	-0.6725	0.5494

Table 6. Analysis of variance (ANOVA) for the selected quadratic model and the parameter estimates.

Factor	Effect	Std.Err.	*t*-value	*F*- value	*P*-value
Mean/Interc	22.1990	0.2042	108.7174		
X_1	2.3692	0.2887	8.2056	67.3316	0.000177
X_1^2	-3.2989	0.3228	-10.2204	104.4574	0.000051
X_2	1.3623	0.2887	4.7188	22.2674	0.003262
X_2^2	-3.9709	0.3226	-12.3094	151.5224	0.000018
X_1 X_2	0.0350	0.4084	0.0857	0.0073	0.9345

CCF will be further optimized with CCD.

Optimization of the key nutrient component in the media with CCD

Based on the results of P-B design, K_2HPO_4 and $MgSO_4$ were found to be non-significant. Therefore, for the next step of optimization, the concentrations of K_2HPO_4 and $MgSO_4$ were set to the low level. Table 3 represents the experimental design with the results obtained and predicted by model for welan gum synthesis. The yield varied from 16.66 to 22.85 g l^{-1} according to the different levels of the components in the medium.

The statistical program was used for the regression analysis of the experimental data. A full second-order polynomial model was used to fit the dependent variable using the following equation:

$$Y = -43.1425+1.4310X_1-0.0165X_1^2+16.4947X_2-1.9854X_2^2+0.0017X_1X_2 \quad (1)$$

Where, Y is predicted response of welan gum, and X_1 and X_2 are the coded values of corn starch and CCF, respectively. For welan gum production, regression analysis shows that the model is very reliable, with R^2 (multiple correlation coefficient) value of 0.9807, which means that the model could explain 98% variation in the response. Statistical testing of the model was done for analysis of variance (ANOVA) and the results were shown in Table 6. Table 6 showed that all the two linear and quadratic terms had significant effect ($P < 0.05$) on welan gum production, which indicated that corn starch and CCF can act as limiting factors and even small increases in their concentrations will significantly increase or decrease welan gum production, respectively, while the interaction of these two factors showed no significant effect on welan gum synthesis.

The coefficient estimates of Eq. (1), along with the corresponding *P*-values are presented in Table 7. The

Table 7. Regression analysis of a full second-order polynomial model for CCD.

Factor	Regression Coeff.	Std.Err.	*t*-value	*P*-value
Mean/Interc	-43.1425	5.1012	-8.4574	0.000149
X_1	1.4310	0.1534	9.3258	0.000086
X_1^2	-0.0165	0.0016	-10.2204	0.000051
X_2	16.4947	1.5330	10.7594	0.000038
X_2^2	-1.9854	0.1613	-12.3094	0.000018
$X_1 X_2$	0.0017	0.0204	0.0857	0.9345
R-sqr	**0.9807**			

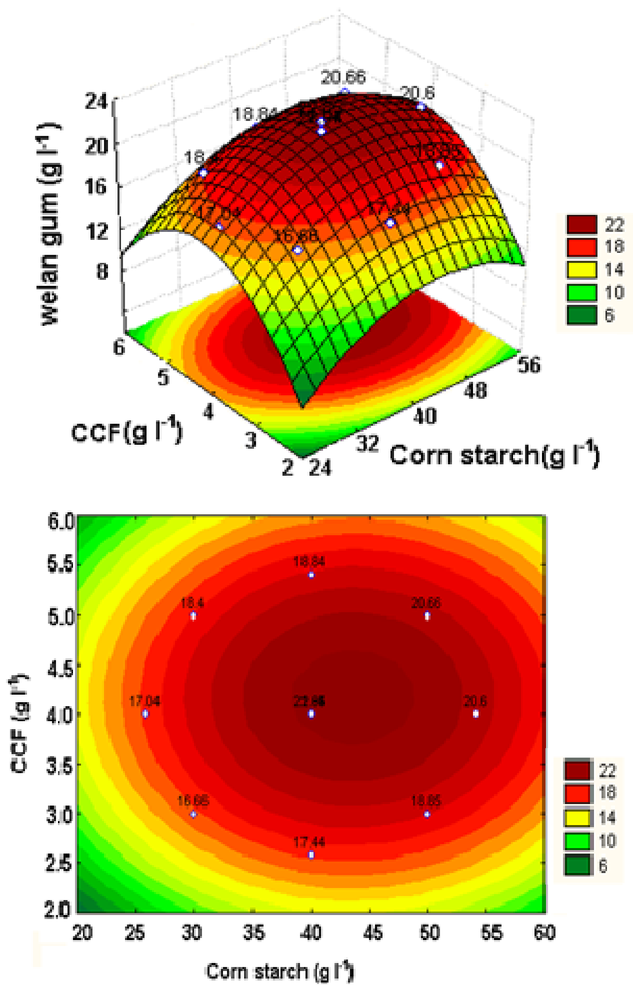

Figure 2. 3D response surface and 2D contour plots showing interaction between corn starch concentration and CCF concentration on the production of welan gum.

P-values are used as a tool to check the significance of each coefficient, which also indicates the interaction strength between each independent variable (Liu et al., 2003). The smaller the *P*-values mean the bigger the significance of the corresponding coefficient (Muralidhar et al., 2001). It can be seen from Table 7 that all regression coefficients are significant except the interaction of corn starch and CCF.

The 3D response surface and the 2D contour plots are the graphical representations of the regression equation. Both plots are presented in Figure 2. The main goal of response surface is hunt efficiently for the optimum values of the variables such that the response is maximized. By analyzing the plots, the optimal values of the fermentation conditions for obtaining approximately welan gum production lie in the following ranges of the tested variables: corn starch concentration from 38 to 48 g l^{-1} and CCF concentration from 3.6 to 4.6 g l^{-1}. The model predicted that the maximum yield of 22.47 g l^{-1} was obtained at the optimal point $X_1 = 43.6$ g l^{-1}, $X_2 = 4.1$ g l^{-1}. The final optimized medium composition contains (g l^{-1}): corn starch 43.6; CCF 4.1; K_2HPO_4 2; $MgSO_4$ 0.1. In order to verify the predicted results, an experiment was performed with the optimized levels of nutrients in shake-flask. As a result, 22.85 g l^{-1} welan gum was obtained, which was suggesting that experimental and predicted values of welan gum production were in good agreement, validating the model.

Time course of welan gum production

Figure 3 shows the time course of biomass and welan gum production by the cultivation in two different media: (1) optimized in this work; (2) non-optimized media used before. Although biomass was a little higher in the previous medium (4.47 g l^{-1}) compared with our optimized medium (4.0 g l^{-1}), welan gum production reached 22.85 g l^{-1}, which was higher than the previous medium (18.5 g l^{-1}). And an increase of approximately 18% of welan gum production, further supporting the usefulness of the P-B design and CCD as optimization techniques to improve the production of welan gum.

Conclusion

In this study, the statistical methodology, combination of P-B design with CCD, is demonstrated to be effective and reliable in selecting the statistically significant factors and finding the optimal concentration of those factors in welan gum production medium.

Corn starch and CCF were identified as most influenc-

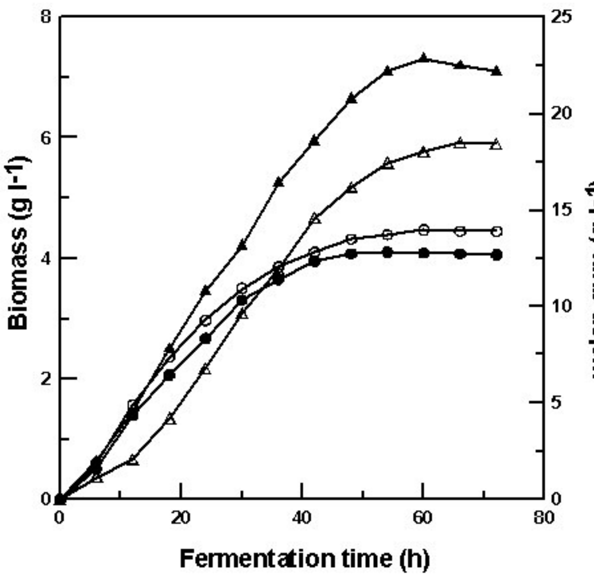

Figure 3. Comparison of time course of welan gum production by *A. facalis* NX-3. The symbols were used: biomass in the optimized media (●), welan gum yield in the optimized media(▲), biomass in the previous media(○), welan gum yield in the previous media (Δ).

ing components for enhancing welan gum production by using P-B design, and then their optimal concentrations were obtained by using response surface methodology. It was shown that the model was adequate to predict the optimization of welan gum production of *A. facalis* NX-3. From the present study, it is evident that P-B design and CCD is demonstrated to be effective and reliable in selecting the statistically significant factors and finding the optimal concentration of those factors in welan gum production. The optimization of the medium resulted not only in a 23% higher welan gum production than media previously used but also in a reduction of constituents costs, as corn starch and CCF was used in the medium. This is particularly important for scaling-up the cultivation process. The information obtained is considered fundamental and useful for the development of *A. facalis* NX-3 cultivation process for efficient production of welan gum on a large scale.

ACKNOWLEDGEMENTS

The authors would like to thank for the supports obtained from National Fundamental Research 973 Program of China (No. 2007CB714304), National High Technology Research and Development Program of China (No. 2006AA03Z453), and Nature Science Foundation of China (N0. 20674308).

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