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Statistical experimental design optimization of rhamsan gum production by *Sphingomonas* sp. CGMCC 6833

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Rhamsan gum is a type of water-soluble exopolysaccharide produced by species of *Sphingomonas* bacteria. The optimal fermentation medium for rhamsan gum production by *Sphingomonas* sp. CGMCC 6833 was explored definition. Single-factor experiments indicate that glucose, soybean meal, K_2HPO_4 and $MnSO_4$ compose the optimal medium along with and initial pH 7.5. To discover ideal cultural conditions for rhamsan gum production in a shake flask culture, response surface methodology was employed, from which the following optimal ratio was derived: 5.38 g/L soybean meal, 5.71 g/L K_2HPO_4 and 0.32 g/L $MnSO_4$. Under ideal fermentation rhamsan gum yield reached 19.58 g/L ± 1.23 g/L, 42.09% higher than that of the initial medium (13.78 g/L ± 1.38 g/L). Optimizing the fermentation medium results in enhanced rhamsan gum production.

Keywords: *Sphingomonas* sp. CGMCC 6833, rhamsan gum, medium optimization, central composite experimental design

Introduction

Sphingans are a class of water-soluble exopolysaccharides (EPS) produced by *Sphingomonas* species, including gellan gum, welan gum, rhamsan gum, S-657, and S-88 (Banik *et al.*, 2000). The common backbone structure of sphingan gums is a linear tetrasaccharide polymer of D-glucose (Glcp), D-glucuronic acid (GlcpA), and L-rhamnose (Rhap) in the ratio of 2:1:1. All sphingans share high viscosity and high thermal stability, thus seeing wide application as food thickeners, stabilizers and suspension agents (Morrison *et al.*, 1999; Bajaj and Singhal, 2007). Sphingans are commonly used in the pharmaceutical (Coviello *et al.*, 1998), petroleum (Dennis *et al.*, 1991; Vercaemer *et al.*, 1997), and concrete industries (Allen *et al.*, 1991; Plank, 2004), among others.

As a sphingan, rhamsan gum has the class common backbone structure. Rhamsan gum's specific side chains are composed of β-D-glucose, α-D-glucose substituted at O-6 by α-D-glucosyl-(1→6)-β-D-glucosyl disaccharide side-chains (Banik *et al.*, 2000). Rhamsan gum is non-gel forming, but it produces a thermostable, highly-viscous solution even at temperatures higher than 100°C. Compared with other sphingans, rhamsan gum tolerates high concentrations of phosphates and sodium chloride, enabling its broad application in the food industry (Kang and Pettitt, 1993). In 1996, rhamsan gum received approval as a food additive by the Japanese Ministry of Health and Welfare (Hagiwara *et al.*, 2010). As a versatile, anionic water-soluble ESP, rhamsan gum can also be used safely in plastic surgery (Paula *et al.*, 2007). Rhamsan gum is now produced commercially by CP Kelco Company.

Culture medium composition and conditions are essentials in high-efficiency fermentation (Jafarzade *et al.*, 2013). Carbon and nitrogen sources, inorganic salts and pH all influence cell growth rate, and subsequent accumulation of bacterial metabolic products. In the production of EPS, the medium is based on a high ratio of carbon substrate and limiting nutrients in submerged aerobic fermentation. Concentration of metallic ions in the medium alters synthesis, causing marked diversity in chemical composition and properties of produced EPS (Banik *et al.*, 2000). Studies on gellan gum show optimized experimental design produces significantly higher gum yields than previously reported methods (Bajaj *et al.*, 2006; Arockiasamy and Banik, 2008).

Current research on rhamsan gum depends primarily on the *Sphingomonas* sp. ATCC 31961 (Hagiwara *et al.*, 2010) whose rhamsan gum yield has been tested at 1.28% (Jerry *et al.*, 1983). Reports on rhamsan gum fermentation efficiency are very few, making further study necessary for increased yield. In this research, we apply single-parameter optimization and response surface methodology to investigate the effects of medium components. Higher rhamsan gum yield was achieved when the optimized medium was used.

Materials and Methods

Microorganism and medium

Sphingomonas sp. RH-1 (CGMCC 6833), a rhamsan gum production strain, was originally isolated from a soil sample collected from the Laoshan National Forest Park of Nanjing (Nanjing, China) (Xu *et al.*, 2012). The seed medium contained 20 g/L glucose, 1 g/L yeast extract, 3 g/L peptone, 2 g/L K_2HPO_4 and 0.1 g/L of $MgSO_4$ at pH 7.2–7.4. The initial fermentation medium contained 40 g/L glucose, 5 g/L yeast extract, 3 g/L K_2HPO_4, 0.4 g/L $MgSO_4$, and was pH-adjusted to 7.0–7.2.

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Culture methods

Sphingomonas sp. CGMCC 6833 was first inoculated into 100 ml of fresh seed medium in 500 ml flasks and aerobically incubated for 16 h with shaking at 200 rpm and 30°C. Seed culture (5%, v/v) was then inoculated into 100 ml of fermentation medium in 500 ml flasks and then incubated with shaking at 200 rpm and 30°C. In the single-factor experiments and response surface methodology experiments, *Sphingomonas* sp. CGMCC 6833 was cultured for 72 h. In the comparative experiments of the initial medium and optimised medium, *Sphingomonas* sp. CGMCC 6833 was cultured for 80 h.

The batch fermentation was conducted in batch fermentation in a 7.5 L, stirred bioreactor (Rushton impeller: BioFlo110, New Brunswick Scientific) with the following features: bioreactor internal diameter, 17.8 cm; height, 32.1 cm; and working volume, 4.5 L. The seed culture was inoculated as 5% (v/v). All cultivations were carried out at 30°C. The aeration rate and agitation speed were controlled at 1.0 L/L/min and 600 rpm in the batch fermentation.

Analytical methods

Biomass was determined for at least three 10 ml cell suspensions harvested by centrifugation, washed with distilled water, and dried at 60°C for 24 h to a constant weight. Using a biosensor equipped with a glucose oxidase electrode, the glucose concentration was analyzed (SBA-40C, Shandong Academy of Sciences, China) (Xu *et al*., 2005). Acidity/alkalinity was observed using a precision pH meter (Shanghai Leici Instrument Co., Ltd.). The rhamsan gum concentration was measured following these procedures:

First, pre-determined volume of fermentation broth was heated in a water bath at 80°C for 15 min. After cooling, the cells were removed by centrifugation at 12,000 × *g* for 30 min. Double volumes of anhydrous ethanol were added with stirring until a flocculent precipitate appeared. The solution was refrigerated at 4°C for 12 h. After centrifugation, the supernatant was removed. The process was then repeated using anhydrous ethanol. The precipitate was dried in an oven at 60°C to obtain a constant weight.

Single-factor experiments

Single-factor experiments were carried out in 500 ml flasks containing 100 ml medium at 200 r/min and 30°C. The carbon source (glucose, sucrose, fructose, maltose, lactose, soluble starch, glycerol), nitrogen source [yeast powder, soybean meal, yeast extract, beef extract, peptone, corn steep liquor, urea, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $(NH_4)_2HPO_4$], phosphate (K_2HPO_4, KH_2PO_4), metal ions ($MgSO_4$, $CuSO_4$, $ZnSO_4$, $MnSO_4$, $CoSO_4$, $FeSO_4$), glucose concentration (20 g/L, 30 g/L,40 g/L,50 g/L, 60 g/L) and pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.2, 7.5, 8.0) in the initial culture were varied in each experiment. Statistical analysis was generated by SPSS software (Version 21.0, IBM) and p levels at 0.05 were considered as significant.

Central composite design (CCD) and response surface methodology

After determining the significant factors, CCD and response surface methodology (RSM) were used to study the effects of three variables (soybean meal, K_2HPO_4, and $MnSO_4$) on rhamsan gum production in flasks. The three independent factors were studied at five different levels (-1.68, -1, 0, +1, and +1.68). These levels were selected on the basis of our preliminary experimental work that indicated that an optimum would be reached within these ranges. The variables were coded according to regression equation (1) as follows:

$$X_i = \frac{x_i - x_0}{\Delta x_i},\ i = 1,2,...k \quad (1)$$

Here, X_i is the coded independent factor, x_i is the real independent factor, x_0 is the value of x_i at the centre point and Δx_i is the step change value.

The design matrix used to evaluate the significance of the model was generated by Statistica software (Version 8.0, Statsoft Inc.). Sixteen experiments were performed at least three times each, and the results were presented as mean values of the three or more trials (Table 1). The design is represented by a second-order polynomial regression model as follows:

$$Y = a_o + \Sigma a_i X_i + \Sigma a_{ii} X_i^2 + \Sigma a_{ij} X_i X_j,\ i = 1,2,...k \quad (2)$$

In this case, Y is the predicted response, a_0 is the intercept, a_i is the linear coefficient, a_{ii} is the squared coefficient and a_{ij} is the interaction coefficient. X_i and X_j represent coded independent variables. The equation was used to generate response surface graphs for the maximum production of rhamsan gum, comparing experimental against predicted results to confirm the model's validity. Variables showing agreement between experimental and predicted results at confidence level (greater than 90%) were considered to have significant influence on rhamsan production.

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

Run	Variables			Rhamsan gum (g/L)	
	Soybean Meal, X_1	K_2HPO_4, X_2	$MnSO_4$, X_3	Observed[a]	Predicted
1	-1(4.00)	-1(3.00)	-1(0.20)	13.61±0.50	13.84
2	-1(4.00)	-1(3.00)	1(0.40)	14.71±0.51	14.62
3	-1(4.00)	1(7.00)	-1(0.20)	13.61±0.49	13.75
4	-1(4.00)	1(7.00)	1(0.40)	15.01±0.47	15.13
5	1(6.00)	-1(3.00)	-1(0.20)	15.08±0.53	14.71
6	1(6.00)	-1(3.00)	1(0.40)	15.82±0.50	15.43
7	1(6.00)	1(7.00)	-1(0.20)	17.13±0.45	16.96
8	1(6.00)	1(7.00)	1(0.40)	18.76±0.48	18.28
9	-1.68(3.22)	0(5.00)	0(0.30)	13.61±0.43	13.25
10	1.68(6.68)	0(5.00)	0(0.30)	15.97±0.52	16.63
11	0(5.00)	-1.68(1.64)	0(0.30)	14.35±0.45	14.55
12	0(5.00)	1.68(8.36)	0(0.30)	16.76±0.56	16.87
13	0(5.00)	0(5.00)	-1.68(0.13)	15.24±0.53	15.19
14	0(5.00)	0(5.00)	1.68(0.47)	16.60±0.57	16.96
15	0(5.00)	0(5.00)	0(0.30)	19.68±0.54	19.46
16	0(5.00)	0(5.00)	0(0.30)	19.31±0.58	19.46

[a] mean ± standard deviation (n = 3)

Results and Discussion

Effects of carbon sources

Carbon source is an important factor in the production of EPS (Freitas *et al.*, 2009) not only because it is a major constituent for the building of cellular materials, but also as an energy source for polysaccharide synthesis. In this research, glucose, sucrose, fructose, maltose, lactose, soluble starch and glycerol as carbon sources were investigated (Fig. 1). Among these carbon sources, glucose had the most positive effect on rhamsan gum production. Meanwhile, biomass yields highest with sucrose as the carbon source. The soluble starch and glycerol runs resulted in little biomass and rhamsan gum, indicating the glycometabolism of *Sphingomonas* sp. CGMCC 6833 could not make use of these substrates. The ANOVA and other statistical analyses reveal that the type of carbon sources significantly altered experimental yields of rhamsan gum and biomass. Some EPS-producing bacteria metabolize sucrose optimally as a carbon source (Yoon *et al.*, 2012). Furthermore, some grow optimally on soluble starch as the carbon source, starkly different from the case in *Sphingomonas* sp. CGMCC 6833 (Bajaj *et al.*, 2006). For the next trial, glucose was selected as the carbon source. None of the carbon sources was exhausted at the end of the fermentations. This phenomenon has also been observed in the fermentation of other members of the gellan gum family (Bajaj *et al.*, 2006).With glucose as carbon source (40 g/L), unused glucose in the medium persisted at 13.5 g/L after 72 h.

Effects of nitrogen sources

The choice of suitable nitrogen source also influences sphingans yield. Optimization of nitrogen manifests differently among various *Sphingomonas* species. In some studies, an inorganic nitrogen source [i.e., $(NH_4)_2SO_4$] has been reported as the best nitrogen source for the production of welan gum from *Alcaligenes* sp. CGMCC 2428 (Li *et al.*, 2011). Contrastingly, yeast extract, an organic nitrogen source, has also been reported as the best source for the production of gellan gum from *Sphingomonas paucimobilis* ATCC 31461 (Bajaj *et al.*, 2006). Therefore, the effects of nitrogen sources, including organic nitrogen source (yeast powder, soybean meal, yeast extract, beef extract, peptone, corn steep liquor) and inorganic nitrogen sources [urea, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $(NH_4)_2HPO_4$] were studied (Fig. 2). Here, results indicate that organic nitrogen sources have higher biomass and higher yield of rhamsan gum. Among organic nitrogen sources, the highest rhamsan gum production rate was achieved in medium supplemented with soybean meal. The yield of rhamsan gum and biomass found significant variance with the type of nitrogen sources as given by the corresponding F values

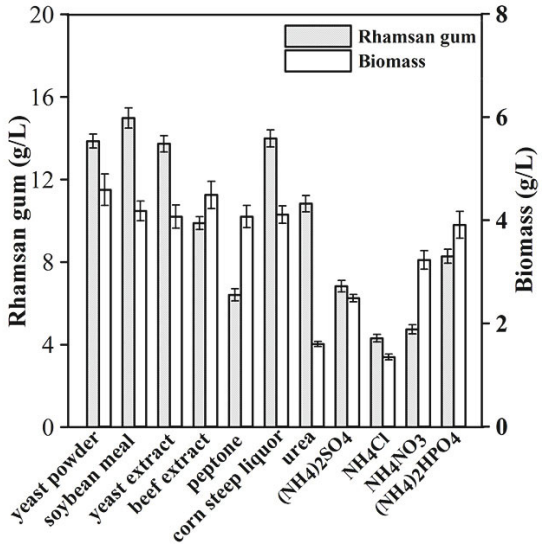

Fig. 2. **Effects of nitrogen sources (5 g/L) on rhamsan gum fermentation.**

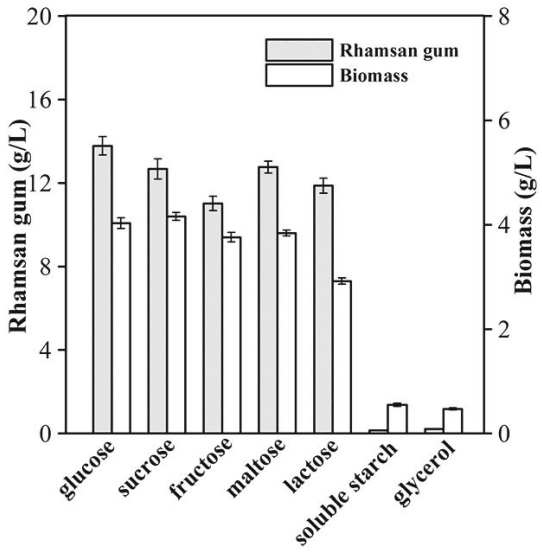

Fig. 1. **Effects of carbon sources (40 g/L) on rhamsan gum fermentation.**

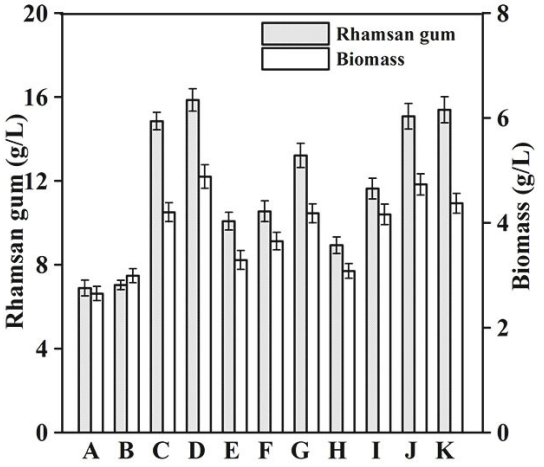

Fig. 3. **Effects of phosphate sources on rhamsan gum fermentation.** A, blank; B, 1 g/L K_2HPO_4; C, 3 g/L K_2HPO_4; D, 5 g/L K_2HPO_4; E, 1 g/L KH_2PO_4, F, 3 g/L KH_2PO_4; G, 5 g/L KH_2PO_4; H, 0.5 g/L K_2HPO_4 + 0.5g/L KH_2PO_4; I, 1.5 g/L K_2HPO_4 + 1.5 g/LKH_2PO_4; J, 2.5 g/L K_2HPO_4 + 2.5 g/L KH_2PO_4; K, 5 g/L K_2HPO_4 + 5 g/L KH_2PO_4.

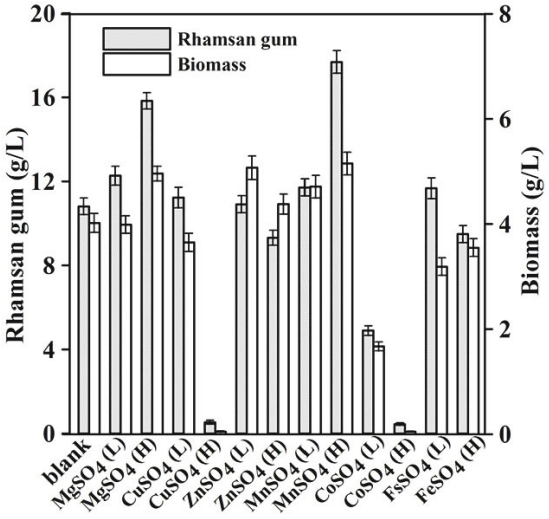

Fig. 4. **Effects of metal ions on rhamsan gum fermentation. L, 0.05 g/L; H, 0.5 g/L.**

of ANOVA. Consequently, soybean meal was chosen as the optimal nitrogen source.

Effects of phosphate sources

Bacteria contain phosphorus in compound form. As a buffer system, phosphates can adjust the medium's pH. Phosphates promote the basic metabolism of bacterial cells and affect biosynthesis. Ten total varied concentrations of two phosphate sources (K_2HPO_4 and KH_2PO_4) were observed (Fig. 3). In this study, sample A (non-phosphate sources) had the lowest levels of biomass and rhamsan gum production. Except for sample K, both biomass and rhamsan gum concentration increased when the phosphate concentration increased, possibly because phosphate has a relationship with some specific enzymes (phosphoglucose isomerase, dTDP-glucose pyrophosphorylase) which catalyze polysaccharide synthesis (Banik

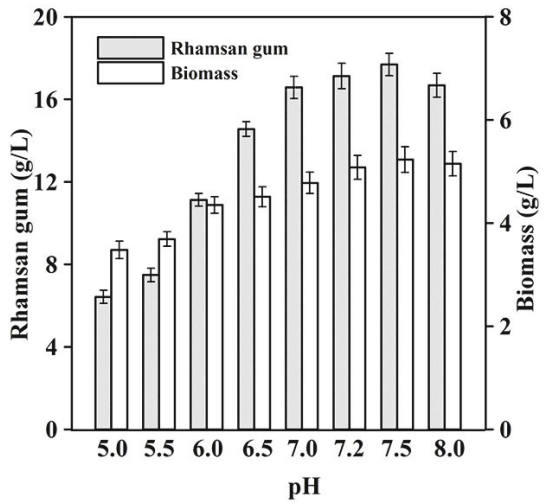

Fig. 5. **Effects of initial pH on rhamsan gum fermentation.**

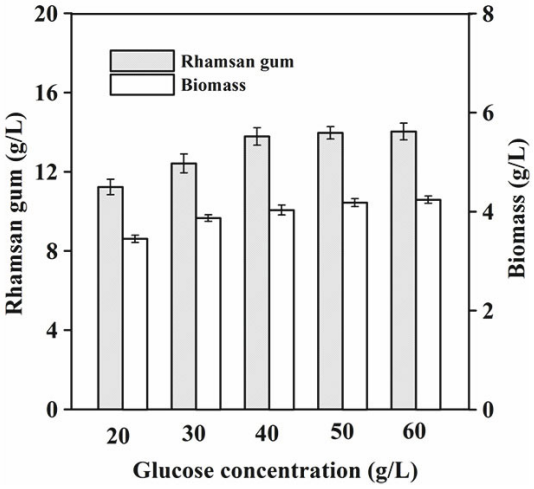

Fig. 6. **Effects of glucose concentration.**

et al., 2000; Li *et al*., 2010). Compared with KH_2PO_4, K_2HPO_4 yielded greater biomass and rhamsan gum production. The results of ANOVA and other statistical analyses reveal significant difference in the yield of rhamsan gum and biomass for varied phosphate sources. Potassium is the major intracellular cation in bacteria. Bacteria accumulate K^+ through a number of different transport systems that vary in kinetics, energy coupling, and regulation (Epstein, 2003). Therefore, a maximal concentration of K_2HPO_4 exists, which warrants further study.

Effects of metal ions

Metal ion concentration is critical for microbial growth and metabolite production. Hence, effects of different metal ions on rhamsan gum production rate and biomass were analyzed (Fig. 4). The ANOVA of the data on the effect of metal ions on rhamsan gum production and biomass reveals that metal ion type is a statistically significant variable. Lower concentration of Cu^{2+} (0.05 g/L) shows little influence on the biomass and yield of rhamsan gum. However, the concentration of rhamsan gum decreased with increase in Cu^{2+} concentration (0.5 g/L). Co^{2+} inhibited the growth of bacteria at both higher and lower concentrations. Therefore, Co^{2+} and higher Cu^{2+} have pernicious effects on *Sphingomonas*

Table 2. **Analysis of the central composite design results of the rhamsan gum production medium**

Source	Regression coefficient	Standard error	t-value	p-value
Intercept	-39.609	6.331	-6.256	0.001
X_1	15.550	1.763	8.818	0.000
X_1^2	-1.596	0.162	-9.843	0.000
X_2	1.966	0.654	3.007	0.024
X_2^2	-0.331	0.041	-8.174	0.000
X_3	74.095	13.838	5.355	0.002
X_3^2	-119.601	16.211	-7.378	0.000
$X_1 X_2$	0.293	0.087	3.361	0.015
$X_1 X_3$	-0.163	1.744	-0.093	0.929
$X_2 X_3$	0.744	0.872	0.853	0.427

sp. CGMCC 6833. Zn^{2+} and Fe^{2+} had no remarkable influence in both higher and lower concentrations. Mg^{2+} and Mn^{2+} increased both the biomass and the yield of rhamsan gum in lower concentration (0.05 g/L) and higher concentration (0.5 g/L). Mn^{2+} was more effective than Mg^{2+}, possibly because Mn^{2+} is a co-factor for some enzymes and may thus significantly improve both biomass and rhamsan gum production. Utter and Werkman have shown that Mg^{2+} and Mn^{2+} activate an important enzyme that converts phosphoglycerate to physphopyruvate (Utter and Werkman, 1942). However, trace addition of toxic metals to the medium actually reduces the activity of the phosphoglycerate-converting enzyme or stops it completely. This raises the possibility that apparently toxic effects may be explained by the incorporation of Cu^{2+} and Co^{2+} in an enzyme usually employing Mg^{2+} or Mn^{2+} as an activator. During the fermentation process, the pH of the fermentation broth with Mn^{2+} decreased slowly, suggesting that it may be an important factor in achieving a higher yield of rhamsan gum. The importance of Mn^{2+} in rhamsan gum production is demonstrated here for the first time. Similar results have been found in the fermentation of gellan gum (Monot and Quinn, 1996). However, the function of Mn^{2+} with regard to sphingan production is still unclear and worthy of further study.

Effects of pH

Initial pH is critical in fermentation (Seo *et al.*, 2010). The effects of different initial pH values on rhamsan gum production were investigated in this research (Fig. 5). The results of ANOVA substantiate the significant differences in the yield of rhamsan gum and biomass at different pH. When the pH value increased from 5.0 to 7.5, a parallel increase in rhamsan gum production and biomass took place. However, the yield of rhamsan gum and biomass decreased when pH reached 8.0. At lower pH of 5.0–6.0, bacterial growth was inhibited, so less rhamsan gum was produced. Earlier reports state that maximum production of ESP gellan gum is obtained at pH 6.5–7.0 (Lobas *et al.*, 1992; Jin *et al.*, 2003). By the results, a sub-alkaline initial medium pH is better to produce rhamsan gum.

Effects of glucose concentration

Glucose concentration was investigated by experiment (Fig. 6) and found 40 g/L glucose was ideal for this medium. On one hand, when carbon source concentration was higher, rhamsan gum yield showed limited increase. On the other hand, previous research on sphingans shows that high carbon source to nitrogen source ratio is beneficial for production (Pollock, 2002). So glucose concentration was adjusted and soybean meal, K_2HPO_4, and $MnSO_4$ was selected as variable factors for response surface methodology.

CCD and response surface analysis

Response surface methodology (RSM) using CCD was employed to determine the optimal levels of the three selected factors (soybean meal, K_2HPO_4, and $MnSO_4$) that influence rhamsan gum production. Utilizing multiple regression analysis, the second-order polynomial equation model (Eq. 2) explains the rhamsan gum production level of *Sphingomonas* sp. CGMCC 6833.

$$Y = 15.550X_1 - 1.596X_1^2 + 1.966X_2 - 0.331X_2^2 + 74.095X_3 - 119.601X_3^2 + 0.293X_1X_2 - 0.163X_1X_3 + 0.744X_2X_3 - 39.609 \quad (3)$$

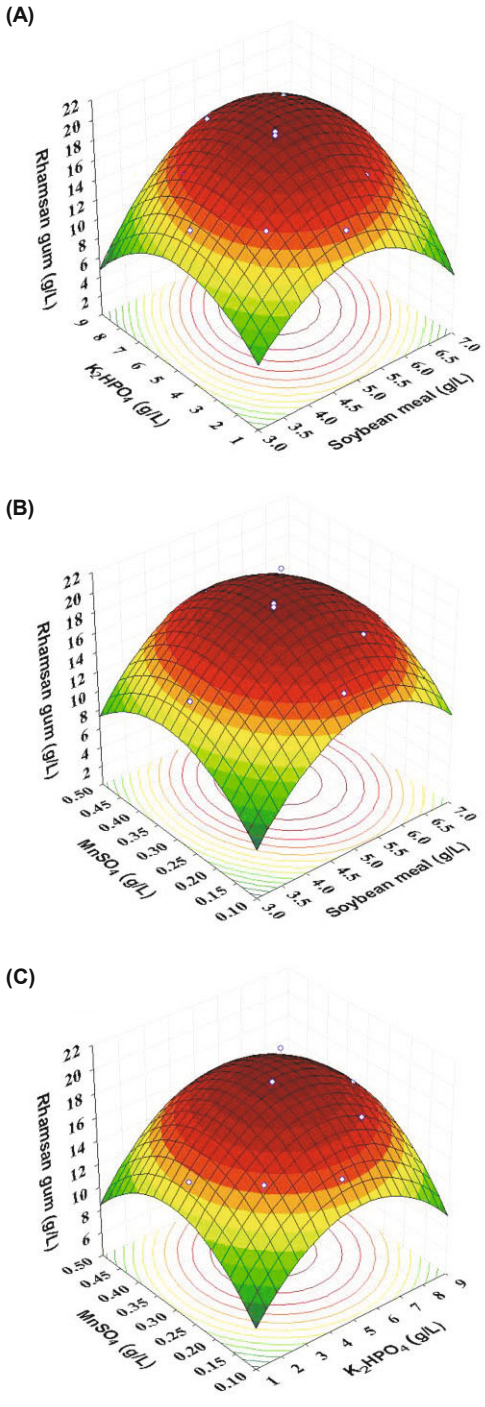

Fig. 7. Response surface curve for rhamsan gum production by *Sphingomonas* sp. CGMCC 6833. (A) A function of soybean meal and K_2HPO_4 when $MnSO_4$ was maintained at 0.3 g/L, (B) A function of soybean meal and $MnSO_4$ when K_2HPO_4 was maintained at 5.0 g/L, (C) A function of $MnSO_4$ and K_2HPO_4 when soybean meal was maintained at 5.0 g/L.

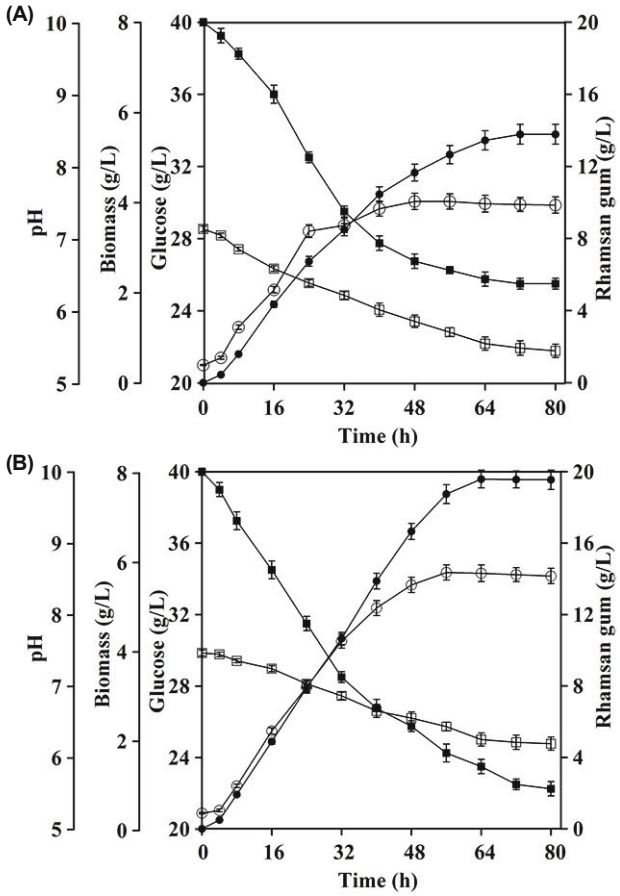

Fig. 8. Time course of rhamsan gum fermentations with the initial and optimised medium by *Sphingomonas* sp. CGMCC 6833 (A, Initial medium; B, Optimised medium). Notes: Glucose (■), Rhamsan gum (●), Biomass (○), pH (□).

In Equation 3, Y is the predicted rhamsan gum production, and X_1, X_2, and X_3 are the coded values of soybean meal, K_2HPO_4, and $MnSO_4$, respectively.

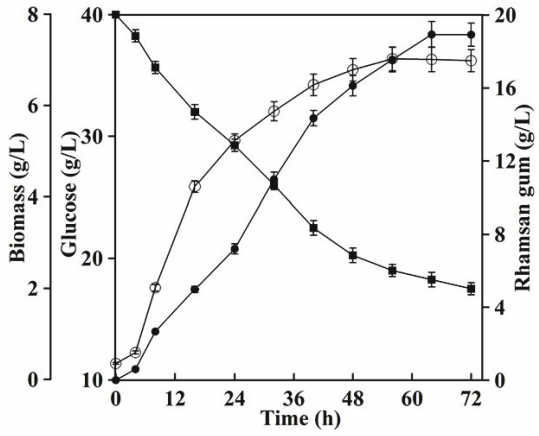

Fig. 9. Time course of rhamsan gum fermentations with optimised medium by *Sphingomonas* sp. CGMCC 6833 in 7.5 L bioreactor. Glucose (■), Rhamsan gum (●), Biomass (○), pH (□).

The statistical significance of Equation 3, as checked by t-value and p-value in terms of response surface quadratic model, is summarized in Table 2. A smaller p-value indicates that corresponding variables are more significant. As shown in Table 2, excepting X_1X_3 and X_2X_3, the p-values are much less than 0.05, indicating that all variables are more significant for rhamsan gum production yield than the interaction between soybean meal and $MnSO_4$, and that between K_2HPO_4 and $MnSO_4$. $R^2 = 0.975$ indicates that the obtained experimental data fit well into the model and illustrate the effects of soybean meal, K_2HPO_4 and $MnSO_4$ during rhamsan gum production.

With the third factor constant at zero, 3D response surface curves for rhamsan gum production at each pair of parameters are as illustrated in Fig. 7A–C. These 3D plots and their respective contour plots provide a visual interpretation of the interaction between two factors and facilitate the identification of optimal experimental conditions. According to the response surface analysis, the predicted maximum production of rhamsan gum is 19.84 g/L when soybean meal is 5.38 g/L, K_2HPO_4 is 5.71 g/L and $MnSO_4$ is 0.32 g/L.

To validate the adequacy of the optimized medium, verification experiments were executed at predicted optimal conditions (Fig. 8). In optimized medium, the mean concentration of obtained rhamsan gum from triplicate trials in a shake flask is 19.58 g/L ± 1.23 g/L, which is very near the predicted value (19.84 g/L). The productivity of rhamsan gum here is 3.44 g/g DCW (dry cell weight). Furthermore, the yield of rhamsan gum in optimized medium is 42.09% higher than the yield in the initial medium (13.78 g/L ± 1.38 g/L).

The batch fermentation of rhamsan gum was conducted to investigate the suitability of our model, using *Sphingomonas* sp. CGMCC 6833 in a 7.5 L bioreactor. As shown in Fig. 9, the highest concentration of rhamsan gum reaches 18.53 g/L ± 1.77 g/L, demonstrating our model's adequacy.

Acknowledgements

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