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Evaluation of metal ions on the production and flocculating activity of polysaccharide-based bioflocculant from *Paenibacillus mucilaginosus*

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ABSTRACT

The effect of metal ions on the production and flocculating activity of polysaccharide-based bioflocculants (PSBs) by *Paenibacillus mucilaginosus* GIM1.16 were studied in liquid culture. In single-factor experiments, supplementing with Mg^{2+}, Ca^{2+} or Fe^{3+} to the culture medium could increase the PSBs production by 197%, 44%, 109% and increase the flocculating activity by 19%, 9%, 15% compared to that in basal medium, which showed more important and positive effects than Zn^{2+} and Cu^{2+}. Response surface methodology was then employed to further optimize the concentrations of these metal ions, and the maximum PSBs production (2.236 g/L) was achieved with 1150 μM $MgSO_4$, 290.1 μM $CaCl_2$ and 19.1 μM $FeCl_3$. The results of batch fermentation for PSBs under the optimal conditions further confirmed that adding a small amount of metal ions to the medium not only could shorten the fermentation period for about 12 h, but also increase the conversion rate of carbon source and the flocculating activity of PSBs by 199% and 26.3%, respectively. The work in this paper is significant for improving the production and feasibility of PSBs.

KEYWORDS

Bioflocculants;
Flocculating activity;
Metal ion;
Paenibacillus mucilaginosus;
Response surface methodology.

INTRODUCTION

Recently, bioflocculants have attracted a great deal of attention in many industrial fields such as wastewater treatment, downstream processing, fermentation, and food industries, due to their innocuity and biodegradability over traditional flocculants[1-4]. The main components of bioflocculants are biomacromolecules produced by microorganisms during their growth, such as polysaccharides and proteins[5]. The polysaccharide-based bioflocculants (PSBs) have been the focus of research and development due to their unique structures, excellent physical-chemical properties, and excellent selectivities. In fact, PSBs have been used in treating many kinds of pollutants, including dyeing pigment, heavy metal ions, and other suspended pollutants[6-8]. However, high production cost, low yield and low flocculating activity are the major bottlenecks for

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the large-scale application of PSBs[9,10]. The common method for improving the production and flocculating activity of PSB was optimization of carbon and nitrogen sources[7,10,11]. In fact, the carbon and nitrogen sources were known to be relatively expensive ingredients for industrial production. Thus, there is a necessity to search for a method for economical and high production of PSBs with high flocculating activities.

In the past decades, metal ions have drawn much attention in the metabolite biosynthesis due to their important roles on the cell growth and microbial metabolism[12]. Several previous reports have shown that the metabolite biosynthesis of microorganisms could be significantly increased by adding small amount of metal ions to the culture medium. It has been reported that the fusaricidins production by *Bacillus subtilis* could be increased by 40% after adding 1.3 mM Mg^{2+} to the culture medium[12]. For *Rhizopus nigricans*, 0.0001% Mn^{2+} showed significant enhanced effects in citrate lyase and glucoamylase activities, resulting in the increase in the production of microbial lipids by 30%[13]. However, to our knowledge, most of the current studies only focused on the effects of single metal ion on the metabolite biosynthesis. In fact, there might be synergies between metal ions. Therefore, it is predicted that certain metal ions complex might be more efficient in promoting PSBs production.

Paenibacillus mucilaginosus is one kind of soil bacteria that has been widely studied in the aspect of silicate mineral weathering and agriculture[14,15]. In recent years, *P. mucilaginosus* and its metabolites (mainly PSBs) played potential roles in wastewater treatment and heavy ions absorption[16,17]. However, the studies of *Paenibacillus mucilaginosus* in PSBs production are rare. In the present study, single-factor experiments were conducted to screen the optimal concentration of individual metal ion for the PSBs production, and then response surface methodology (RSM) was used to optimize the concentrations of these metal ions complex on PSBs production by *P. mucilaginosus* GIM1.16 and a quadratic predictive model was developed. In addition, batch fermentation was conducted to further evaluate the effects of the metal ions complex on the production and flocculating activities of PSBs in liquid culture.

MATERIALS AND METHODS

Microorganism and culture conditions

P. mucilaginosus strain GIM1.16 (isolated from soil sample) was provided by the Microbial Culture Collection Center of Guangdong Institute of Microbiology, Guangzhou, China. The strain was cultured in nitrogen-free slant (10 g sucrose, 1 g Na_2HPO_4, 0.2 g $MgSO_4$, 0.1 g $CaCO_3$, 5 mg $FeCl_3$, 20 g agar in 1 L distilled water, pH 7.5) at 30°C for 3 d, and then stored at 4°C. Seed cultures were prepared from nitrogen-free medium (10 g sucrose, 1 g Na_2HPO_4, 0.2 g $MgSO_4$, 0.1 g $CaCO_3$, 5 mg $FeCl_3$ in 1 L distilled water, pH 7.5) at 30°C with stirring at 200 rpm.

Determination of PSBs production

To quantify PSBs production, the fermentation broth was centrifuged at 10, 000 g for 20 min at room temperature. The resulting supernatant was mixed with three volumes of cold ethanol, stirred vigorously and kept overnight at 4°C. The precipitate was collected by centrifugation and dissolved in distilled water. The PSBs concentrations were determined by the phenol-sulfuric acid method[18].

Flocculating activity analysis

One milliliter of the culture supernatant was collected after centrifugation at 10, 000 rpm for 20 min. Three volumes of ethanol were added to the supernatant with vigorous stirring at 4°C. The PSBs were obtained by collecting the precipitate after centrifugation at 10, 000 rpm for 10 min, and then re-dissolved in 1ml distilled water and dialyzed against distilled water. The flocculating activity analysis was conducted as follows: a 1 L beaker containing 5 g/L kaolin suspension was placed in a six league of electric mixer for agitation, first at 150 rpm for 4 min, then at 50 rpm for 10 min, with a final standing still for 10 min. The prepared PSBs were put into the kaolin suspension with 2 mM $CaCl_2$. The absorbance of the sample and the blank were measured at 550 nm with a spectrophotometer (APL-752N, Shanghai, China), respectively. The flocculating activity was determined by the following equation:

$$\text{Flocculating activity (\%)} = (OD_{blank} - OD_{sample})/OD_{blank} \times 100\% \quad (1)$$

Shaking flask culture of *P. mucilaginosus* GIM1.16

Single-factor experiments

Liquid culture was carried out in a 250-ml flask containing 100 ml basal medium (20 g sucrose, 0.5 g yeast extract, 2 g Na_2HPO_4 in 1 L distilled water, pH 7.5). Initial Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+} contents in basal medium, measured by atomic absorbance spectrometer (Z-2000, Hitachi, Japan), were 12, 28, 1.7, 1.1 and 0.9 μM, respectively. Single-factor experiments were conducted to determine the optimal concentration of individual metal ion for PSBs production by *P. mucilaginosus* GIM1.16. The cultures were supplemented with $MgSO_4$, $CaCl_2$, $FeCl_3$, $ZnSO_4$ and $CuSO_4$, for Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+}, respectively. The levels considered for Mg^{2+} were 800, 1200, 1600 μM, for Ca^{2+} were 250, 500, 1000 μM, for Fe^{3+} were 10, 20, 40 μM, for Zn^{2+} and Cu^{2+} were 5, 10, 20 μM, respectively. Three replicates and control cultures without supplemented metal ions were conducted for each experiment.

Fractional factorial design (FFD)

Five metal ions (Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+}) were chosen as variables for FFD. The parameters for these five variables were designated as X_1, X_2, X_3, X_4 and X_5, respectively. The concentrations of these five metal ions for maximum PSBs production determined by single-factor experiments were used as the highest level (+1 level) in FFD. A total 10 experiments including two center points (0 level) were carried out. The predicted response of FFD was based on the following first-order polynomial model:

$$Y = A_0 + \sum A_i X_i \tag{2}$$

where *Y* was the predicted response (PSBs production), A_0 was the model intercept, A_i was the linear coefficient, and X_i was the level of independent variable. The variables significant at 95% of confidence level (P<0.05) were considered to exert significant effects on PSBs production.

Path of steepest ascent experiment

Three important variables (Mg^{2+}, Ca^{2+} and Fe^{3+}) were screened by FFD. To determine the appropriate levels of these variables for the maximizing PSB production, path of steepest ascent experiment was conducted. The experiments were conducted till a maximum response was obtained. This point would be near the optimal point and could be used as the center point for central composite design (CCD).

Central composite design (CCD)

Central composite design, one of the most common experimental designs used in RSM, was employed to investigate the optimal concentration of metal ions (Mg^{2+}, Ca^{2+} and Fe^{3+}) for the maximum PSBs production by *P. mucilaginosus* GIM1.16. The CCD used was generated by "Design-Expert" software (Trial version 8.0.6; Stat-Ease Inc., Minneapolis, MN, USA). According to the results of FFD and steepest ascent experiment, the three most significant variables (Mg^{2+}, Ca^{2+} and Fe^{3+}) were studied at five different levels (-1.68, -1, 0, +1, +1.68) and a set of 20 experiments were conducted. The relationships and interrelationships of the variables were explained by the following second-degree polynomial equation:

$$Y = A_0 + \sum_{i=1}^{n} A_i X_i + \sum_{i<j}^{n} A_{ij} X_i X_j + \sum_{j=1}^{n} A_{jj} X_j^2 \tag{3}$$

where *Y* was predicted response, A_0 was a constant, A_i was the linear coefficients, A_{ij} was the second-order interaction, and A_{jj} was the quadratic coefficient. X_i and X_j were the level of independent variable. In this study, three variables were involved, therefore, n=3, Eq. (3) came to be the following second-order polynomial equation:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2 \tag{4}$$

where *Y* was the response, X_1, X_2 and X_3 were the input variables; A_0 was a constant; A_1, A_2 and A_3 were the linear coefficients; A_{12}, A_{13} and A_{23} were the cross product coefficients; A_{11}, A_{22} and A_{33} were the quadratic coefficients.

Batch fermentation of the PSBs in fermentor

Seed cultures were prepared by the method mentioned in section 2.1, and the cultures were added to a 3 L jar fermentor (New Brunswick BF-115, Germany) containing 2 L of basal medium or metal ions complex-supplemented medium that gave maximum PSBs production in CCD, respectively. The cultures in the jar fermentors were cultivated at 30°C with stirring at 200 rpm and aeration rate of 1 vvm throughout the whole

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TABLE 1 : The effect of the metal ions (Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+}) with different concentrations on PSBs production and flocculating activity after *P. mucilaginosus* GIM1.16 cells were cultivated for 72h

Levels		PSB (g/L)	FA (%)	Levels		PSB (g/L)	FA (%)
Mg^{2+} (μM)				**Ca^{2+} (μM)**			
(I)	0	0.404±0.029	69.6±1.1	(I)	0	0.410±0.031	69.1±0.9
(II)	800	0.807±0.054	77.3±2.6	(II)	250	0.527±0.036	73.4±2.2
(III)	1200	1.198±0.069	82.7±2.5	(III)	500	0.592±0.020	75.2±2.8
(IV)	1600	0.656±0.033	76.1±1.8	(IV)	1000	0.526±0.031	73.6±2.1
Fe^{3+} (μM)				**Zn^{2+} (μM)**			
(I)	0	0.424±0.026	68.2±1.6	(I)	0	0.408±0.024	68.3±1.4
(II)	10	0.767±0.069	74.2±3.1	(II)	5	0.415±0.026	67.4±1.5
(III)	20	0.888±0.052	78.1±2.5	(III)	10	0.441±0.049	70.5±2.7
(IV)	40	0.836±0.040	75.8±1.7	(IV)	20	0.416±0.028	68.9±3.6
Cu^{2+} (μM)							
(I)	0	0.405±0.033	67.1±2.6				
(II)	5	0.421±0.039	70.9±2.4				
(III)	10	0.492±0.034	72.8±1.3				
(IV)	20	0.407±0.024	71.3±1.2				

***FA : Flocculating activity**

process. Dissolved oxygen and pH in cultivation were uncontrolled. At specific time intervals, fermentation broth was collected for analysis. The growth of the strain was determined by dry cell weight. The culture medium was centrifuged at 10, 000 rpm for 20 min. The pellet was collected and then dried to a constant weight at 105°C. The PSBs in the culture supernatant were precipitated with three volumes of ethanol at 4°C overnight. The precipitate was collected by centrifugation and used for PSB concentration analysis, while the supernatant was used for the residual sugar (sucrose, glucose and fructose) analysis according to the method by[19].

RESULTS AND DISCUSSION

Single-factor experiments

The effect of individual metal ion (Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+}) on PSB production by *P. mucilaginosus* GIM1.16 was shown in TABLE 1. The results revealed that the five metal ions shared similar patterns in affecting the production and flocculating activity of PSBs, that is, the production and flocculating activity of PSBs were first increased then decreased with the increase in the concentrations of Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+} in the culture, and the maximum PSBs production and flocculating activity were achieved at the metal ion concentration of level III (1200 μM Mg^{2+}, 500 μM Ca^{2+}, 20 μM Fe^{3+}, 10 μM Zn^{2+} and Cu^{2+}, respectively). Compared with the PSBs production in the basal medium, the metal ions Mg^{2+} (1200 μM), Ca^{2+} (500 μM), Fe^{3+} (20 μM), Cu^{2+} (10 μM) and Zn^{2+} (10 μM) could increase PSBs production by 197%, 44%, 109%, 21% and 8%, respectively, indicating that Mg^{2+}, Ca^{2+} and Fe^{3+} might exert more positive influences on the production of PSBs than Cu^{2+} and Zn^{2+}. Similar results were observed that Mg^{2+} and Ca^{2+} exerted positive and important effects on exopolysaccharide biosynthesis in *Pseudomonas aeruginosa*[20]. Fe^{3+} could significantly stimulate exopolysaccharide production in *Paenibacillus polymyxa* SQR-21, but Zn^{2+} failed[12]. In *Pseudomonas fluorescens* WR-1, Ca^{2+} showed more positive effect on exopolysaccharide production than Cu^{2+}[21]. Metal ions are known to influence the types and quantity of metabolite by affecting the enzyme activity involved metabolite biosynthesis, because metal ions were the cofactors for many enzymes involved in the growth and metabolism of microorganisms[12]. For example, Mg^{2+} is the activators for phosphoglucomutase which was the key enzyme involved in the biosynthesis of alginate and Lipopolysaccharide in *Pseudomonas aeruginosa*[22]. Fe^{3+} is the most impor-

TABLE 2 : Fractional factorial design arrangement and responses for PSBs production

Runs	$MgSO_4$ (μM) X_1	$CaCl_2$ (μM) X_2	$FeCl_3$ (μM) X_3	$ZnSO_4$ (μM) X_4	$CuSO_4$ (μM) X_5	PSB (g/L)	
						Actual	Predicted
1	600 (-1)	250 (-1)	10 (-1)	10 (1)	10 (1)	1.073	1.140
2	1200 (1)	250 (-1)	10 (-1)	5 (-1)	5 (-1)	1.436	1.444
3	600 (-1)	500 (1)	10 (-1)	5 (-1)	10 (1)	1.085	1.019
4	1200 (1)	500 (1)	10 (-1)	10 (1)	5 (-1)	1.247	1.240
5	600 (-1)	250 (-1)	20 (1)	10 (1)	5 (-1)	1.358	1.292
6	1200(1)	250 (-1)	20 (1)	5 (-1)	10 (1)	1.808	1.801
7	600 (-1)	500(1)	20 (1)	5 (-1)	5 (-1)	1.104	1.171
8	1200 (1)	500 (1)	20 (1)	10 (1)	10 (1)	1.589	1.597
9	900 (0)	375 (0)	15 (0)	7.5 (0)	7.5 (0)	1.297	1.323
10	900(0)	375 (0)	15 (0)	7.5 (0)	7.5 (0)	1.349	1.323

tant micronutrient utilized by bacteria and is being required as a cofactor for many enzymes and iron-containing proteins[12]. In our case, the PSBs production enhanced by Mg^{2+}, Fe^{3+} and Ca^{2+} might be due to the significant effects of these metal ions on the enzymes involved in the growth and metabolism of *P. mucilaginosus* GIM1.16.

In addition, the metal ions Mg^{2+} (1200 μM), Ca^{2+} (500 μM), Fe^{3+} (20 μM), Cu^{2+} (10 μM) and Zn^{2+} (10 μM) could also enhance the flocculating activities of PSBs by 19%, 9%, 15%, 8% and 3%, respectively, comparing with the basal medium counterparts. Moreover, results showed that for all the concentrations of the metal ions, there was a positive correlation between the PSBs production and the PSBs flocculating activities, that is, the higher in the PSBs production, the higher in the PSBs flocculating activities. It was assumed that the increase of PSBs flocculating activity might be caused by the increase in PSBs production. Similar phenomena were observed in some microorganisms, such as *Cellulomonas* sp., *Halomonas* sp. and *Bacillus* sp.[9,23,24].

Fractional factorial design

For the FFD experiment, Mg^{2+} (X_1), Ca^{2+} (X_2), Fe^{3+} (X_3), Zn^{2+} (X_4) and Cu^{2+} (X_5) were considered as the variables with three levels (-1, 0, +1). The concentration of each metal ion for maximum PSBs production determined by single-factor experiment (1200 μM Mg^{2+}, 500 μM Ca^{2+}, 20 μM Fe^{3+}, 10 μM Cu^{2+} and 10 μM Zn^{2+}) was used as the highest level (+1 level). As shown in TABLE 2, the PSBs production varied from 1.073 to 1.808 g/L in the media with different levels of metal ions complex, which indicated the necessity to optimize the metal ions concentrations for maximum PSBs production. According to the regression analysis, Mg^{2+}, Fe^{3+} and Cu^{2+} showed positive effects on PSBs production, while Ca^{2+} and Zn^{2+} played negative roles in PSB production (Eq. (5)). The analysis of the variables with confidence levels showed that Mg^{2+} (P=0.0018), Ca^{2+} (P=0.0304) and Fe^{3+} (P=0.0068) were considered as the most significant variables affecting PSBs production. The predicted response and test variables could be obtained by the following first-order polynomial equation:

$$Y = 1.33 + 0.18X_1 - 0.081X_2 + 0.13X_3 - 0.021X_4 + 0.051X_5 \quad (5)$$

where Y was the predicted response (PSBs production), X_1, X_2, X_3, X_4 and X_5 were coded values of Mg^{2+}, Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+}, respectively.

The analysis of variance (ANOVA) showed that F-value and the P-value were 19.31 and 0.0066, respectively, indicating that the model was highly significant. The determination coefficient R^2 of the model was 0.96, which indicated that 96% of the variability in the response could be explained by the model.

Path of steepest ascent experiment

Since the results of FFD were far from the actual optimum, the steepest ascent experiment was carried out to determine the optimum domain of PSBs production. The steepest ascent experiment started from the center point of FFD and moved along the path where the concentrations of Mg^{2+} and Fe^{3+} increased, while Ca^{2+} concentration decreased (TABLE 3). The results showed that the maximum PSBs production (2.151 g/

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L) was achieved in run 5, indicating that the maximum PSBs production would be near this point. Therefore, the metal ions complex of run 5 was chosen as the center point of CCD.

Central composite design (CCD)

Based on the results of steepest ascent experiment, CCD was carried out to determine the optimum concentrations of the metal ions (Mg^{2+}, Ca^{2+} and Fe^{3+}) for the maximum PSBs production. The value of responses (PSBs production) obtained under different conditions of combined application of metal ions were shown in TABLE 4. By employing multiple regression analysis to the experimental data of PSBs production, the predicted response and test variables could be obtained by the following second-order polynomial equation:

$$Y_{PSB} = 2.17 + 0.15X_1 + 0.058X_2 + 0.064X_3 + 0.035X_1X_2 - 0.036X_1X_3 - 0.060X_2X_3 - 0.089X_1^2 - 0.12X_2^2 - 0.049X_3^2 \quad \textbf{(6)}$$

The statistical significance of regression equation was checked by F-test, and the analysis of variance for response surface quadratic polynomial model was conducted by Design-Expert. The determination coefficient R^2 of the model was 0.95 for Eq. (6), which indicated there was high degree of correlation between the experimental and predicted values for PSBs production. The F-values (21.23) and the low P-values (<0.0001) demonstrated that the models were highly significant (TABLE 5). In addition, the P-value for lack of fit (0.5262) implied the lack of fit were not significant relative to the pure error (TABLE 5). Therefore, the model for PSBs production was found to be adequate for prediction within the range of variables employed. The

TABLE 3 : Experimental design and results of the steepest ascent path

Run	$MgSO_4$ (μM)	$CaCl_2$ (μM)	$FeCl_3$ (μM)	PSB production (g/L)
1	900	375	15	1.331
2	950	350	16	1.534
3	1000	325	17	1.703
4	1050	300	18	1.891
5	1100	275	19	2.151
6	1150	250	20	1.916
7	1200	200	21	1.742
8	1250	175	22	1.548
9	1300	150	23	1.387

TABLE 4 : Central composite design arrangement and responses for PSBs production

Run	$MgSO_4$ (μM)X_1	$CaCl_2$ (μM)X_2	$FeCl_3$(μM)X_3	PSB (g/L)	
				Actual	Predicted
1	1050(-1)	250(-1)	18.00(-1)	1.562	1.582
2	1150(1)	250(-1)	18.00(-1)	1.923	1.889
3	1050(-1)	300(1)	18.00(-1)	1.811	1.748
4	1150(1)	300(1)	18.00(-1)	2.168	2.193
5	1050(-1)	250(-1)	20.00(1)	1.914	1.901
6	1150(1)	250(-1)	20.00(1)	1.990	2.064
7	1050(-1)	300(1)	20.00(1)	1.782	1.827
8	1150(1)	300(1)	20.00(1)	2.138	2.129
9	1016(-1.68)	275(0)	19.00(0)	1.654	1.666
10	1184(1.68)	275(0)	19.00(0)	2.206	2.178
11	1100(0)	233(-1.68)	19.00(0)	1.763	1.740
12	1100 (0)	317(1.68)	19.00(0)	1.928	1.934
13	1100 (0)	275 (0)	17.32 (-1.68)	1.893	1.929
14	1100 (0)	275 (0)	20.68 (1.68)	2.195	2.143
15	1100 (0)	275 (0)	19.00(0)	2.237	2.174
16	1100 (0)	275 (0)	19.00(0)	2.072	2.174
17	1100 (0)	275 (0)	19.00(0)	2.145	2.174
18	1100 (0)	275 (0)	19.00(0)	2.142	2.174
19	1100 (0)	275 (0)	19.00(0)	2.220	2.174
20	1100 (0)	275 (0)	19.00(0)	2.223	2.174

coefficient estimates of Eq. (6), along with the corresponding P-value, were summarized in TABLE 5. The linear coefficients (X_1, X_2 and X_3), cross-product coefficient (X_2X_3) and quadratic term coefficients (X_1^2, X_2^2 and X_3^2) were significant, while the other term coefficients (X_1X_2, X_1X_3) were not significant.

The dimensional response surface curves were plotted to study the effects of the interaction between two test variables on the response (Figure 1), and provided the location of optimum experimental conditions[10]. According to the analysis of the response surface contour plots, the model showed that the optimum levels of the three variables were at 1150 μM $MgSO_4$, 290.1 μM $CaCl_2$ and 19.1 μM $FeCl_3$, and predicted that the maximum PSBs production was 2.25 g/L. Five additional experiments in the shaking flasks with optimum conditions were conducted to validate the adequacy of the predicted value, and the actual mean value of PSBs production was 2.236 g/L, which was in close agreement with the predicted value.

TABLE 5 : Analysis of variance for predictive equation for PSBs production by *P. mucilaginosus* GIM16

Source	Sum of squares	df	Mean square	F-value	Prob > F
Model	0.770	9	0.085	21.23	< 0.0001**
Residual	0.040	10	4.026E-003		
Lack of fit	0.020	5	3.901E-003	0.94	0.5262NS
Pure error	0.021	5	4.150E-003		
Cor total	0.810	19			
X_1	0.32	1	0.32	78.57	< 0.0001**
X_2	0.045	1	0.045	11.28	0.0073**
X_3	0.055	1	0.055	13.70	0.0041**
X_1X_2	9.522E-003	1	9.522E-003	2.37	0.1551NS
X_1X_3	0.010	1	0.010	2.54	0.1421NS
X_2X_3	0.029	1	0.029	7.09	0.0238*
X_1^2	0.11	1	0.11	28.40	0.0003**
X_2^2	0.20	1	0.20	50.65	< 0.0001**
X_3^2	0.034	1	0.034	8.51	0.0154*

*** < 0.05; ** < 0.01; NS: non-significant.**

Batch fermentation for PSBs production by *P. mucilaginosus* GIM1.16

To further evaluate the effects of metal ions complex on the production and flocculating activities of PSBs by *P. mucilaginosus* GIM1.16, batch fermentation for PSBs in 3 L jar fermentor containing basal medium or metal ions-supplemented medium with optimal metal ions concentrations in CCD was conducted, respectively (Figure 2). The profile of cell growth showed good correspondence to the production and flocculating activities of PSBs in the two media, indicating that the PSBs was formed during cell growth rather than during cell lysis[23]. These results were also in agreement with those of single-factor experiments. In addition, it was clear that the maximum dry cell weight (6.93 g/L), maximum PSBs production (2.513 g/L) and maximum flocculating activity (95.2%) in metal ions-supplemented medium, respectively, were obtained at 18 h, 21 h and 18 h, which were 9 h, 12 h and 12 h earlier than in basal medium. These results indicated the fermentation pe-

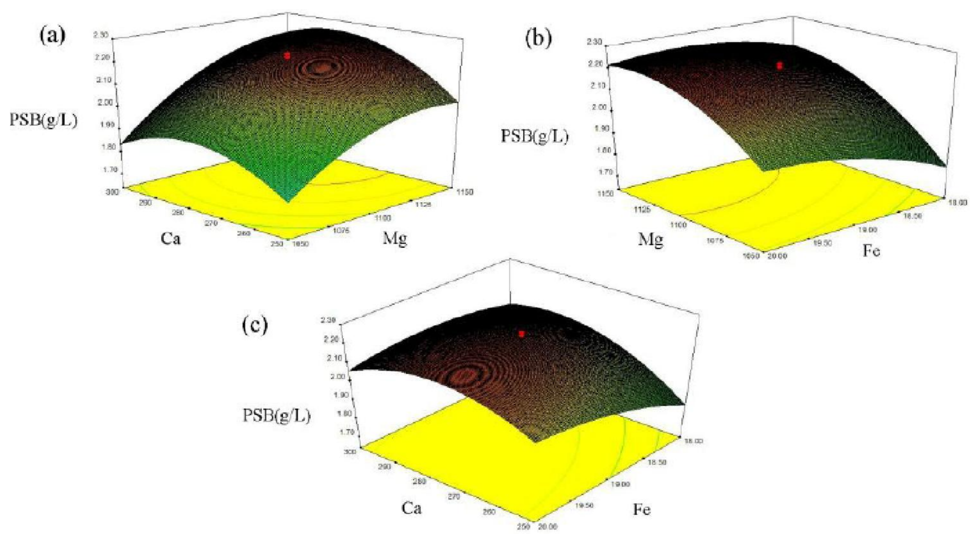

Figure 1 : Response surface 3D plots of the influence of (a) Mg^{2+} and Ca^{2+}, (b) Mg^{2+} and Fe^{3+}, (c) Ca^{2+} and Fe^{3+} and their interactive interactions on the PSBs production by *P. mucilaginosus* GIM1.16

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riod could be shorten when *P. mucilaginosus* GIM1.16 cells grew in metal ions-supplemented medium, comparing with that of basal medium. Metal ions are known to exert significant effects on maintaining cellular metabolism and enzyme activities[25], and the increase in certain enzyme activities could shorten the fermentation period[26]. In our case, the metal ions might promote the activities of several key enzymes involved in cell growth and PSBs biosynthesis to shorten the fermentation period.

The results (Figure 2) also showed that the maximum flocculating activity of PSBs in the metal ions-supplemented medium could be increased by 26.3%, comparing with that in basal medium. It appeared that the reason for the increase in flocculating activity of the PSBs from metal ions-supplemented medium was the enhanced production of PSBs.

From Figure 2b, the maximum PSB production in metal ions-supplemented medium was 2.513 g/L, which was about 6.11 times over than that of basal medium.

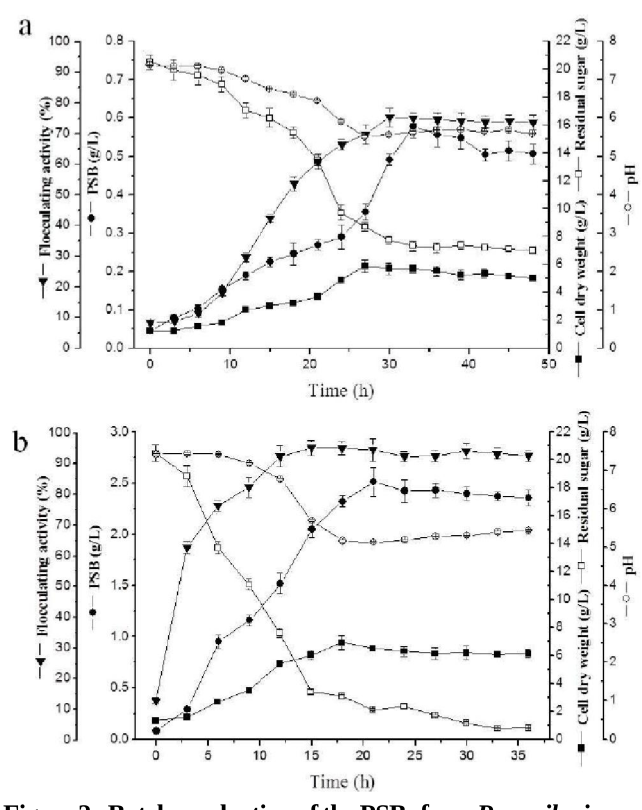

Figure 2 : Batch production of the PSBs from *P. mucilaginosus* GIM1.16 in the (a) basal medium and the (b) metal ions complex-supplemented medium. Flocculating activity (*filled triangle*), PSB (*filled circle*), Cell dry weight (*filled square*), pH (*open circle*), residual sugar (*open square*)

The residual sugar content in the metal ion-supplemented medium was only 0.83 g/L after 36 h cultivation, indicating a higher consumption efficiency of sucrose (96%) than that in basal medium (66%). It is worthwhile noting that adding a small amount of metal ions to the culture medium not only could enhance the PSBs production, but also significantly increase the conversion rate of carbon source to PSBs by 199% compared with that in basal medium. Liu et al.[10] optimized the exopolysaccharide production by *Paenibacillus polymyxa* EJS-3 through the optimization procedure of carbon and nitrogen sources, and the conversion rate of carbon source to exopolysaccharide was only increased by 23.5%. Li et al.[7] employ similar method to enhance the PSB production by *Paenibacillus elgii* B69, only 16% increase in the conversion rate of carbon source to PSB was obtained. In our case, adding small amount of metal ions complex to the culture medium could significantly enhance the production and flocculating activity of PSBs by *P. mucilaginosus* GIM1.16. Thus, as comparing with another common way to enhance PSBs production and activities by increasing carbon and nitrogen sources which are known to be expensive ingredients for industrial production, metal ions-complex is relatively cheaper but with better performance in improving PSBs production and activities, and this method might be a promising way for the industrial production of PSBs.

CONCLUSIONS

In the present study, the single-factor experiments showed that Mg^{2+}, Ca^{2+} and Fe^{3+} exerted more important and positive effects than Zn^{2+} and Cu^{2+} on the production and flocculating activity of PSBs, because these three metal ions (Mg^{2+}, Ca^{2+} and Fe^{3+}) could increase the PSBs production by 197%, 44%, 109% and increase the flocculating activity of PSBs by 19%, 9%, 15%, respectively. RSM was then used to further optimize the concentrations of the three important metal ions, and the maximum PSBs production (2.236 g/L) was achieved with 1150 μM $MgSO_4$, 290.1 μM $CaCl_2$ and 19.1 μM $FeCl_3$. The results of batch fermentation for PSBs production indicated that adding a small amount of metal ions to the medium not only could shorten the fermentation period for about 12 h, but also increase the conversion rate and the flocculating activity of PSBs by 199% and 26.3%, respectively. Such work may also be helpful to other microbial fermentation processes for enhanced PSBs production under laboratory and commercial conditions.

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REFERENCES

[1] H.M.Oh, S.J.Lee, H.M.Park, H.S.Kim, H.C.Kim, J.H.Yoon, G.S.Kwon, B.D.Yoon; Harvesting of Chlorella vulgaris using a bioflocculant from Paenibacillus sp.Biotechnol.Lett., **23**, 1229-1234 **(2001)**.

[2] P.Prasertsana, W.Dermlima, H.Doelleb, J.F.Kennedy; Screening, characterization and flocculating property of carbohydrate polymer from newly isolated Enterobacter cloacae WD7. Carbohydr.Polym., **66**, 289-297 **(2006)**.

[3] M.F.Elkady, S.Farag, S.Zaki, G.Abu-Elreesh, D.Abd-Ei-Haleem; Bacillus mojavensis strain 32A, a bioflocculant-producing bacterium isolated from an Egyptian salt production pond. Bioresour.Technol., **102**, 8143-8151 **(2011)**.

[4] F.Freitasa, V.D.Alvesb, C.A.V.Torresa, M.Cruza, I.Sousab, M.J.Meloa, A.M.Ramosa, M.A.M. Reisa; Fucose-containing exopolysaccharide produced by the isolated Enterobacter strain A47 DSM 23139. Carbohydr.Polym., **83**, 159-165 **(2011)**.

[5] L.F.Liu, W.Cheng; Characteristics and culture conditions of a bioflocculant produced by Penicillium sp. Biomed.Environ.Sci., **23**, 213-218 **(2010)**.

[6] S.B.Deng, R.B.Bai, X.M.Hu, Q.Luo; Characteristics of a bioflocculant produced by Bacillus mucilaginosus and its use in starch wastewater treatment. Appl. Microbiol. Biotechnol., **60**, 588-593 **(2003)**.

[7] O.Li, C.Lu, A.Liu, L.Zhu, P.M.Wang, C.D.Qian, X.H.Jiang, X.C.Wu; Optimization and characterization of polysaccharide-based bioflocculant produced by Paenibacillus elgii B69 and its application in wastewater treatment. Bioresour. Technol., **134**, 87-93 **(2013)**.

[8] H.J.Zhao, H.T.Liu, J.G.Zhou; Characterization of a bioflocculant MBF-5 by Klebsiella pneumoniae and its application in Acanthamoeba cysts removal. Bioresour. Technol., **137**, 226-232 **(2013)**.

[9] J.He, Q.W.Zhen, N.Qiu, Z.D.Liu, B.J.Wang, Z.G.Shao, Z.N.Yu; Medium optimization for the production of a novel bioflocculant from Halomonas sp. V3a2 using response surface methodology. Bioresour. Technol., **100**, 5922-5927 **(2009)**.

[10] J.Liu, J.G.Luo, H.Ye, Y.Sun, Z.X.Lu, X.X.Zeng; Medium optimization and structural characterization of exopolysaccharides from endophytic bacterium Paenibacillus polymyxa EJS-3. Carbohydr. Polym., **79**, 206-213 **(2010)**.

[11] A.H.R.Aljuboori, A.Idris, N.Abdullah, R.Mohamad; Production and characterization of a bioflocculant produced by Aspergillus flavus. Bioresour.Technol., **127**, 489-493 **(2013)**.

[12] W.Raza, X.M.Yang, H.S.Wu, Q.W.Huang, Y.C.Xu, Q.R.Shen; Evaluation of metal ions (Zn^{2+}, Fe^{3+} and Mg^{2+}) effect on the production of fusaricidin-type antifungal compounds by Paenibacillus polymyxa SQR-21. Bioresour.Technol., **101**, 9264-9271 **(2010)**.

[13] V.Jambhulkar; Effect of various metal ions on glucoamylase and citrate lyase activities of Rhizopus nigricans in production of lipids. Asiatic J.Biotech.Res., **3**, 1134-1139 **(2012)**.

[14] X.F.Hu, J.S.Chen, J.F.Guo; Two phosphate- and potassium-solubilizing bacteria isolated from Tianmu mountain, Zhejiang, China. World J.Microb.Biot., **22**, 983-990 **(2006)**.

[15] B.B.Basak, D.R.Biswas; Influence of potassium solubilizing microorganism (Bacillus mucilaginosus) and waste mica on potassium uptake dynamics by sudan grass (Sorghum vulgare Pers.) grown under two alfisols. Plant Soil, **317**, 235-255 **(2009)**.

[16] B.Lian, Y.Chen, J.Zhao, H.H.Teng, L.J.Zhu, S.Yuan; Microbial flocculation by Bacillus mucilaginosus: Applications and mechanisms. Bioresour. Technol., **99**, 4825-4831 **(2008)**.

[17] B.B.Mo, B.Lian; Hg (II) adsorption by Bacillus mucilaginosus mechanism and equilibrium param-

FULL PAPER

eters. World J.Microb.Biot., **27**, 1063-1070 **(2010)**.

[18] M.Dubois, K.A.Gilles, J.K.Hamilton, P.A.Rebers, F.Smith; Colorimetric method for determination of sugars and related substances. Anal.Chem., **28**, 350-356 **(1956)**.

[19] A.Lazaridou, T.Roukas, C.G.Biliaderis, H.Vaikousi; Characterization of pullulan produced from beet molasses by Aureobasidium pullulans in a stirred tank reactor under varying agitation. Enzyme Microb.Tech., **31**, 122-132 **(2002)**.

[20] L.O.Martins, L.C.Brito, I.Sa-Correia; Roles of Mn^{2+}, Mg^{2+} and Ca^{2+} on alginate biosynthesis by Pseudomonas aeruginosa. Enzyme Microb. Tech., **12**, 794-799 **(1990)**.

[21] W.Raza, W.Yang, Y.Jun, F.Shakoor, Q.W.Huang, Q.R.Shen; Optimization and characterization of a polysaccharide produced by Pseudomonas fluorescens WR-1 and its antioxidant activity. Carbohydr. Polym., **90**, 921-929 **(2012)**.

[22] R.W.Ye, N.A.Zielinski, A.M.Chakrabarty; Purification and characterization of phosphomannomutase/phosphoglucomutase from Pseudomonas aeruginosa involved in biosynthesis of both alginate and lipopolysaccharide. J.Bacteriol., **176**, 4851-4857 **(1994)**.

[23] Y.Zheng, Z.L.Ye, X.L.Fang, Y.H.Li, W.M.Cai; Production and characteristics of a bioflocculant produced by Bacillus sp. F19. Bioresour.Technol., **99**, 7686-7691 **(2008)**.

[24] U.U.Nwodo, A.I.Okoh; Characterization and flocculation properties of biopolymeric flocculant (glycosaminoglycan) produced by Cellulomonas sp. Okoh.J.Appl.Microbiol., **114**, 1325-1337 **(2013)**.

[25] J.Isar, L.Agarwal, S.Saran, R.K.Saxena; Succinic acid production from Bacteroides fragilis: Process optimization and scale up in a bioreactor. Anaerobe, **12**, 231-237 **(2006)**.

[26] X.Y.Ge and W.G.Zhang; A shortcut to the production of high ethanol concentration from jerusalem artichoke tubers. Food Technol.Biotechnol., **43**, 241-246 **(2005)**.