Optimization Using 3³ Full-Factorial Design for Crude Biosurfactant Activity from *Bacillus pumilus* IJ-1 in Submerged Fermentation

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Received: August 5, 2019 / Revised: October 22, 2019 / Accepted: November 29, 2019

This study aimed to optimize the culture conditions to improve the crude biosurfactant activity of *Bacillus pumilus* IJ-1, using a 3³ full-factorial design of response surface methodology (RSM). It was found that submerged fermentation of *B. pumilus* improved the activity of the crude biosurfactant. The factors selected for optimization were NaCl concentration, temperature, and tryptone concentration. Response surface analysis revealed that the fitted quadratic model was statistically significant and produced an adequate *R*² value (0.9898) and a low probability value (<0.0001). The optimum level for each factor was found to be 0.567% (w/v) NaCl, 21.851°C and 0.765% (w/v) tryptone, respectively. Crude biosurfactant activity was found to be most affected by tryptone concentration; then temperature, and finally NaCl concentration. Our results may potentially facilitate large-scale biosurfactant production from *B. pumilus* IJ-1.

Keywords: 3³ full-factorial design, *Bacillus pumilus* IJ-1, biosurfactant, optimization, response surface methodology, surface tension reduction ratio

Introduction

Biosurfactants are extracellular surface-active amphiphilic biomolecules derived mainly from microorganisms [1]. These biomolecules can be classified into different groups on the basis of the species of biosurfactant-producing microorganisms and the characteristic of their chemical structure [2]. Biosurfactants effectively decrease surface tension or interfacial tension in aqueous solutions or hydrocarbon mixtures [2] and serve as highly efficient emulsifiers [3, 4]. The numerous advantages of biosurfactants over chemically synthesized surfactants include lessened toxicity, enhanced biodegradability, better environmental compatibility, producing capacity from renewable and cheaper sub-

strates, greater foaming properties, better stability at extreme conditions of temperatures, pH and salinity, and ecological acceptability [1, 3, 5]. For these reasons, biosurfactants have potential applications to the food processing, cosmetics, pharmaceuticals and oil recovery industries. Moreover, biosurfactants are used in the bioremediation for oil spills and removal of residual oil in storage tanks [3, 5, 6].

However, the low yields and high costs associated with biosurfactant production severely restrict their large-scale use [7]. One of the approaches to compensate for the aforementioned limitations involves the determination of optimal production medium and culture conditions to maximize biosurfactant productivity [5]. In this regard, the control of biosurfactant production is strongly influenced by temperature and medium pH, salinity, and composition [8].

Statistical approaches have been applied to optimize cultural conditions in biotechnological processes.

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Response surface methodology (RSM) is the most extensively used method for culture medium optimization. The RSM is more effective than the classical 'one-factor-at-a-time' method because it helps assess the given factors simultaneously with fewer observations, thus saving time, labor, and cost [9, 10]. This is a statistics-based technique which helps to design appropriate experiments, building mathematical models, evaluating the effect of factors and establishing optimal conditions [11, 12]. The RSM has been successfully employed in various areas of biotechnology to optimize bacterial growth media and culture conditions [13–17].

Despite its availability, there are comparatively fewer reports showing its utilized instances in optimization of biosurfactant production [18–20]. Statistical experimental designing is essential for optimizing culture conditions. The factorial design is one of the best tools to analyze the effect of relevant factors and their potential interactions. Multiple regression analysis formulated equations that adequately indicate the influence of interdependent factors on selected responses. Experiment through the factorial design and the RSM are commonly conducted in optimization of culture conditions [21, 22].

We previously stated the consequence of medium composition and culture conditions on crude biosurfactant activity from *Bacillus pumilus* IJ-1, using an established method [23]. According to a literature survey, this paper is another report to elucidate optimization of culture conditions for crude biosurfactant activity from *B. pumilus*, using RSM at a flask level.

Materials and Methods

Microorganism

In this study, the bacterial strain *B. pumilus* IJ-1 was used for crude biosurfactant activity (Fig. 1). The strain was previously isolated from contaminated crude oil collected in Taean, Korea and characterized in our laboratory [24]. It has been entitled by the Culture Center of Microorganisms (KCCM), Korea (Accession no. KCCM11319P).

Media

The bacterial cells were cultured on Luria-Bertani (LB) agar containing (w/v) 1.0% NaCl (Promega, USA),

Fig. 1. Phase-contrast microscopic observation of *Bacillus pumilus* IJ-1 (Magnification: $\times 1,000$).

1.0% tryptone (Acumedia, USA), 0.5% yeast extract (BioShop, USA) and 1.5% agar (Junsei, Japan), pH 7.0. For inoculum preparation, a mineral salt medium (A-medium) was used involving the following components (w/v): 0.3% KNO_3 , 0.22% Na_2HPO_4 , 0.06% MgSO_4 , 0.014% KH_2PO_4 , 0.004% CaCl_2 , 0.002% FeSO_4 , 0.001% NaCl and 0.1 ml of a trace element solution, pH 7.0. The trace element solution consisted of (g/l): 2.32 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.78 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 g ethylenediaminetetraacetic acid, 0.56 g H_3BO_3 , 0.42 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.39 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ [25]. Culture media used for crude biosurfactant activity experiments consisted of tryptone and NaCl in accordance with that formulated by Park and Kim [23]. By using 1 N NaOH solution, the initial pH of the media was adjusted to 9.0.

Preparation of crude biosurfactant

For seed culture, a bacterial colony was transferred to 5 ml of A-medium. Seed culture was conditioned at 35°C, and 14–16 h of shaking incubation (200 rpm, $\text{OD}_{600} = 0.8\text{--}0.9$). Then, 1 ml of the bacterial culture suspensions was inoculated in 1-L Erlenmeyer flasks containing 100 ml of crude biosurfactant production medium. Cultivations containing tryptone and NaCl at various concentrations were performed at temperatures ranging from 15 to 25°C (intervals of 5°C) with agitation (200 rpm) for 72 h. Each experiment was carried out in triplicate. Culture broth was centrifuged at $9,300 \times g$ and 4°C for 15 min to eliminate bacterial cells. Subsequently, the remaining cell-free broth was filtered through a 0.22- μm pore-size membrane (Minisart, Sartorius Stedium Biotech GmbH, Germany). The filtered sample served as the source of the crude biosurfactant and was used for surface tension determination.

Surface tension determination

Reducing the surface tension of the bacterial growth medium, the effects of biosurfactants can be indirectly measured by determining surface tension [26, 27]. The surface tension was determined on the crude biosurfactants with a Surface Tension Analyser DST-60 digital surface tensiometer (Surface Electro Optics, Korea), using the Du Nouy ring method at 20°C [28]. To validate these measurements, calibration used distilled water (ST = 71.5 dyne/cm $\pm$ 0.5) before obtaining sample measurements. Each nutrient source required an uninoculated control flask to measure the initial surface tension. To enhance accuracy, each measurement resulted from three separate experiments, and the surface tension of the sample was expressed into the mean value in each measurement.

Validation of the experimental design of 3³ full-factorial design

The critical optimal conditions via single-factor-at-a-time experiments were previously reported [23]. NaCl, tryptone, and culture temperature were determined as major factors affecting significant crude biosurfactant activity. Based on previous studies, appropriate models were devised to analyze factor interactions. For its validity, the parameter range was selected in accordance with the previous report [23]. Experiments were based on 3³ full-factorial design: NaCl (0, 0.5, 1.0%, w/v), tryptone (0, 0.5, 1.0%, w/v), and temperature (15, 20, 25°C) by *B. pumilus* IJ-1 inoculated with 1% (w/v) seed culture at pH 9.0 in shaking incubator (200 rpm) for 72 h.

The specified codes and the range of the parameters were enlisted in Table 1. Each factor of the design was assessed at 3 different levels from low (-1), to medium (0), and to high (+1). In developing the regression equation, the test factor was encoded as follows:

$$X_i = \frac{x_i - x_i^0}{\Delta x_i / 2}$$

where the i -th test factor X_i is the dimensionless coded value, x_i is the real one, x_i^0 is the real one at the center point, and Δx_i is the range, so that X_i is $-1 \leq X_i \leq 1$.

The design was comprised of 29 experimental trials including 27 for factorial design, and 2 for replication of the central points (Table 2). The reduction ratio of surface tension (%) was considered response values (Y). All experiments were conducted in triplicate on different days and the mean value from the three experiments has been presented. As the initial value differed in run types, its difference from final value was calculated [5]. In Table 2, reduction ratio (%) is calculated as:

Reduction ratio of surface tension =

$$(\text{initial tension} - \text{final tension}) / \text{initial tension} \times 100$$

Determination of optimal conditions by RSM

RSM was required to ascertain the interactive effects of three factors at three levels. The consequent data was then subjected to multiple regression analysis on the basis of the empirical model. After completing the experiments, the relationship between the independent factors and their responses was fitted to the following predictive second-order polynomial equation:

$$Y_i = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i < j}^k \beta_{ij} X_i X_j + \sum_{i=1}^k \beta_{ii} X_i^2 \quad (1)$$

where Y_i is the predicted response, subscripts i and j take values from 1 to k of the number of factors, β_0 is a constant, β_i 's are the linear coefficients, β_{ii} 's are the quadratic ones, β_{ij} 's are the cross-product ones, and X_i and X_j are the coded dimensionless values of these factors in Table 1.

The optimal values of the experimental conditions

Table 1. Range of the parameters used for modeling and the specified codes for each parameter.

Independent factors	Symbols		Coded values and the corresponding values of parameters		
	Uncoded	Coded	-1	0	1
NaCl (w/v)	x_1	X_1	0	0.5	1
Temperature (°C)	x_2	X_2	15	20	25
Tryptone (w/v)	x_3	X_3	0	0.5	1

Table 2. Experimental 3³ full-factorial design and the response of reduction ratios of the surface tension.

Trial number	Factor 1: NaCl (w/v)	Factor 2: Temperature (°C)	Factor 3: Tryptone (w/v)	Response	
				Reduction ratio of surface tension (%)	
				Experimental value	Predicted value
1	0	15	0	1.370	0.595
2	0	15	0.5	46.543	45.136
3	0	15	1	42.983	45.283
4	0	20	0	1.107	3.973
5	0	20	0.5	49.113	49.197
6	0	20	1	50.550	50.027
7	0	25	0	4.077	1.979
8	0	25	0.5	47.093	47.887
9	0	25	1	50.640	49.400
10	0.5	15	0	5.433	2.144
11	0.5	15	0.5	45.707	46.897
12	0.5	15	1	48.653	47.256
13	0.5	20	0	2.683	5.743
14	0.5	20	0.5	51.667	51.180
15	0.5	20	0.5	51.430	51.180
16	0.5	20	0.5	51.427	51.180
17	0.5	20	1	52.630	52.222
18	0.5	25	0	2.977	3.971
19	0.5	25	0.5	50.740	50.091
20	0.5	25	1	50.333	51.817
21	1	15	0	1.207	0.256
22	1	15	0.5	40.140	45.222
23	1	15	1	46.547	45.793
24	1	20	0	2.483	4.077
25	1	20	0.5	53.533	49.726
26	1	20	1	52.860	50.981
27	1	25	0	3.927	2.526
28	1	25	0.5	49.160	48.859
29	1	25	1	48.380	50.797

resulted from solving the regression equation and analyzing the response-surface contour plots. The responses to each run were subjected to multiple regression analysis with the help of SAS 9.2 software (SAS Institute, USA) to obtain the coefficients of the polynomial equation. The model was statistically validated via the analysis of variance (ANOVA) provided in the same software. The equation was then expressed in the form of a three-dimensional response surface and their respective contour plots, using the software. These plots were used to elucidate the interaction among different factors and to

predict optimal conditions.

Results and Discussion

Optimization of crude biosurfactant activity using 3³ full-factorial design and RSM

We have studied the relationship between response (surface tension reduction ratio) and three independent factors (NaCl, temperature, and tryptone), using a 3³ full-factorial design and RSM. The design and results of the 3³ full-factorial design experiments are presented in

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model.

Source	Degree of freedom (DF)	Sum of squares	Mean square	F-value	Prob > F
Model	9	40135	4459.444	832.37	<0.0001
Linear	3	29306	9768.667	1823.35	<0.0001
Quadratic	3	10809	3603.000	672.49	<0.0001
Crossproduct	3	20	6.667	1.26	0.2951
Total error	77	413	5.357		
Lack of fit	17	319	18.765	12.04	<0.0001
Pure error	60	94	1.559		
Corrected total	86	40547			
R^2	0.9898				

Table 2. For the data, we fitted the second-order polynomial equation (1) and obtained ANOVA table and the parameter estimates by SAS 9.2.

The results of ANOVA in the quadratic model illustrated in Table 3; the equation could adequately be employed to describe crude biosurfactant activity under various operating conditions. It came up with an F -value of 832.37 with an extremely low p -value (<0.0001). These results implied that it was highly significant for the response variables assessed herein (Table 3). The R^2 was 0.9898, and the coefficient of variation (CV) was relatively low (6.68%), indicating a good precision and reliability [29]. These estimators revealed that the model was acceptable by the statistical point-of-view to predict the response in the considered factor range.

The t -values and the corresponding p -values were calculated from the Student's t distribution, along with the parameter estimates (Table 4). The following quadratic equation was formulated for the uncoded data:

$$\begin{aligned} \text{Reduction ratio (\%)} = & -41.762 + 5.204x_1 + 4.435x_2 \\ & + 129.376x_3 + 0.089x_1x_2 + 0.850x_1x_3 + 0.273x_2x_3 \\ & - 6.873x_1^2 - 0.107x_2^2 - 88.789x_3^2 \end{aligned} \quad (2)$$

where reduction ratio (%) is about the surface tension (dyne/cm), and x_1 , x_2 and x_3 are uncoded values corresponding to the NaCl concentration (% w/v), temperature ($^{\circ}\text{C}$), and tryptone concentration (% w/v), respectively. For the coded data this equation was obtained:

$$\begin{aligned} \text{Reduction ratio (\%)} = & 51.180 + 0.264X_1 + 1.597X_2 \\ & + 23.240X_3 + 0.222X_1X_2 + 0.213X_1X_3 + 0.683X_2X_3 \\ & - 1.718X_1^2 - 2.685X_2^2 - 22.197X_3^2 \end{aligned} \quad (3)$$

The p -value was for assessing the significance of each of the coefficients, subsequently necessary to understand the pattern of the mutual interactions among factors [30]. The smaller p , the more significant the corresponding coefficient.

Table 4. Least-squares fit and parameter estimates.

Parameter	DF	Estimate	Standard error	t -value	Pr > t	Parameter estimate from coded data
Intercept	1	-41.762	8.347	-5.00	<.0001	51.180
x_1	1	5.204	3.849	1.35	0.1804	0.264
x_2	1	4.435	0.839	5.29	<.0001	1.597
x_3	1	129.376	3.849	33.61	<.0001	23.240
x_1*x_2	1	0.089	0.154	0.57	0.5672	0.222
x_1*x_3	1	0.850	1.543	0.55	0.5833	0.213
x_2*x_3	1	0.273	0.154	1.77	0.0805	0.683
x_1*x_1	1	-6.873	2.073	-3.32	0.0014	-1.718
x_2*x_2	1	-0.107	0.021	-5.18	<.0001	-2.685
x_3*x_3	1	-88.789	2.073	-42.83	<.0001	-22.197

Table 5. Sum of squares partitioned by the factors.

Factor	DF	Sum of Squares	Mean Square	F-value	Pr > F
x_1 (NaCl)	4	66.058075	16.514519	3.08	0.0207
x_2 (Temperature)	4	300.119598	75.029899	14.00	<.0001
x_3 (Tryptone)	4	39010	9752.598149	1820.37	<.0001

The sum of squares was partitioned to assess the effect of individual factors (Table 5). Each factor was subjected to a joint test on all the parameters. For example, the test for x_1 examines the null hypothesis that x_1 and x_1x_i 's ($i = 1, 2, 3$) are all zero. Crude biosurfactant activity was most prominently affected by tryptone (p -value < 0.0001), followed by temperature (p -value < 0.0001), and NaCl (p -value = 0.0207).

The actual and predicted plot indicated that experimental values were distributed proximal to the straight line (Fig. 2), indicating that actual values were highly similar to the predicted ones ($R^2 = 0.9898$). Thus, equation (2) was a suitable model for predicting the crude biosurfactant activity efficiency under the aforementioned experimental conditions.

The three-dimensional response surfaces and their respective contour plots are the graphical representations of equation (2) (Fig. 3). Each plot shows the effect of two independent factors varies within the given range, while the other factor is fixed at its center point level. This visualization allowed for the associations among the levels of each factor and the response and interactive types between factors.

Fig. 3(A) illustrates the effects of NaCl concentration and temperature on crude biosurfactant activity at a

Fig. 2. Experimental vs. predicted reduction ratio of surface tension.

definite tryptone concentration of 0.5% (w/v). Temperature had a slightly greater effect on crude biosurfactant activity than NaCl concentration. The maximum surface tension reduction ratio was achieved at 0.55% (w/v) NaCl at 21.5°C.

Fig. 3(B) shows the effect of the interaction between NaCl and tryptone concentrations on crude biosurfactant activity at 20°C. Tryptone concentration had a more significant effect than that of NaCl. The maximal production was achieved at 0.55% (w/v) NaCl and 0.76% (w/v) tryptone.

The combined effects of temperature and tryptone concentration are illustrated in Fig. 3(C) with NaCl concentration fixed at 0.5% (w/v). The strain contributed to the maximal production with 0.76% (w/v) tryptone supplementation at 21.8°C. Crude biosurfactant activity was shown to be less affected by NaCl concentration than by the other factors. Higher concentrations of tryptone and medium temperature increased the surface tension reduction ratio. As observed in Fig. 3, tryptone concentration had a definite effect on crude biosurfactant activity. These results verified the predicted model.

Table 6 includes experimental values along with predicted values in both factors of culture conditions and reduction ratio of surface tension. A comparison of these values pointed to some reasonable concurrence. In the former factor, predicted values were $x_1 = 0.567$, $x_2 = 21.851$, and $x_3 = 0.765$. The corresponding value in the latter was 57.649%. The concurrence between the two values supported the model.

The important features of the current statistical approach to optimize the biosurfactant production using *Bacillus* sp. in shake-flask fermentation is shown in Table 7 [31–33]. To our knowledge, very few studies have reported the optimization of biosurfactant production from *Bacillus pumilus*. Recently, medium components for biosurfactant production by *B. pumilus* 2IR were investigated [32]. The central composite design (CCD) performed by Fooladi *et al.* [32] revealed that glu-

Fig. 3. Two and three dimensional contour plots for the reduction ratio of surface tension. RSM plots were induced the data in Table 2. Inputs were 29 experimental runs under the conditions by the 3^3 full-factorial design. (A) On function of NaCl concentration and temperature. (B) On function of NaCl and tryptone concentrations. (C) On function of temperature and tryptone concentration.

cose, crude oil, KNO_3 , and $(\text{NH}_4)_2\text{SO}_4$ most significantly influenced biosurfactant production by *B. pumilus* 2IR. The present results indicate that crude biosurfactant activity by *B. pumilus* IJ-1 depended on tryptone concentration, temperature, and NaCl concentration.

Statistical approaches facilitate the formulation of bio-

surfactant production media and may be crucial to enhance productivity [34]. This study attempted to optimize culture conditions for crude biosurfactant activity by *B. pumilus* IJ-1. Using a combination of a 3^3 full-factorial design and RSM, we determined the optimal operating conditions for large-scale biosurfactant production

Table 6. Predicted values vs. experimental values for maximal crude biosurfactant activity.

Factor	Culture conditions		Reduction ratio of surface tension (%)	
	Experimental value	Predicted value	Experimental value	Predicted value
x_1 (w/v, %)	1	0.567		
x_2 (°C)	20	21.851	53.88%	57.649%
x_3 (w/v, %)	0.5	0.765		

Table 7. Comparison of the current studies on *Bacillus* sp. shake-flask fermentation regarding optimization of biosurfactant production.

Strain	Isolation source	Optimization method	Optimal conditions	Surface tension (Reduction ratio of surface tension)	Reference
<i>B. pumilus</i> IJ-1	Contaminated crude oil collected in Taejeon, Korea	3^3 full-factorial design	0.567% (w/v) NaCl 0.765% (w/v) tryptone 21.851 °C	26.06 mN/m (57.649%)	This work
<i>B. subtilis</i> JK-1	Traditional Korean soybean-fermented food, Chungkookjang	Central composite rotatable design	1.550% (w/v) soluble starch 0.477% (v/v) skim milk 0.096% (w/v) KNO ₃ 37.145 °C	27.28 mN/m (45.353%)	[31]
<i>B. pumilus</i> 2IR	Oil-contaminated soil samples from Iranian oil field	Central composite design	3.031% (w/v) glucose 0.8% (v/v) crude oil 0.288% (w/v) KNO ₃ 0.24% (w/v) (NH ₄) ₂ SO ₄	30.52 mN/m (-)	[32]
<i>B. subtilis</i> MG495086	Crude oil sample from Assam oil reservoir field	Central composite design	3.8% (v/v) light-paraffin oil 62.4 °C pH 7.7	29.85 mN/m (-)	[33]

by *B. pumilus* IJ-1. Therefore, further research is required to optimize the biosurfactant yield in specialized bioreactors.

Acknowledgments

This work was supported by a grant from Research year of Inje University in 2016.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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