Research Article

The Optimization of Biosurfactant Production by *Bacillus amyloliquefaciens* subsp. *plantarum*

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Abstract

This research aimed to isolate biosurfactant-producing bacteria from fermented foods produced in Meuang district, Mahasarakham province based on oil-dispersion ability. Only one bacterial isolate from total 111 isolates, BS-VSA, that generates the largest clear zone on LBGS agar plate was selected as a biosurfactant-producing bacterium for optimization study. Taxonomic studies of its morphological, biochemical and the 16S rDNA sequence analysis indicated that BS-VSA isolate is closely related to *Bacillus amyloliquefaciens* subsp. *plantarum*. Optimization of culturing condition for biosurfactant production was carried out using Response Surface Methodology (RSM) with Box-Behnken design. Four of independent variables were studied here including carbon content, nitrogen content, pH, and %inoculum. It was found that the coefficient of determination (R^2) of the model was 0.6917, which indicated that the model was suitable for representing the relationships among the selected variables and advocated the significance of the model. Under the optimal condition, 10 g/L of glucose, 4.99 g/L of peptone, pH 9.0, and 10% of inoculum, oil displacement of biosurfactant was 2.13 cm^2. These results revealed that the BS-VSA isolate could be applied as the novel biosurfactant-producing bacterium.

Keywords: Biosurfactant, Oil displacement and Response Surface Methodology

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การปรับปรุงประสิทธิภาพการผลิตสารลดแรงตึงผิวชีวภาพโดย *Bacillus amyloliquefaciens* subsp. *plantarum*

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บทคัดย่อ

งานวิจัยครั้งนี้มีจุดมุ่งหมายเพื่อคัดแยกแบคทีเรียที่มีประสิทธิภาพในการผลิตสารลดแรงตึงผิวชีวภาพจากตัวอย่างอาหารหมักดอง โดยเก็บตัวอย่างในเขตอำเภอเมือง จังหวัดมหาสารคาม จากการวัดประสิทธิภาพค่าการกระจายตัวของน้ำมัน จากทั้งหมด 111 ไอโซเลท ไอโซเลท BS-VSA มีประสิทธิภาพค่าการกระจายตัวของน้ำมันสูง ซึ่งเมื่อศึกษาลักษณะทางสัณฐานวิทยา การทดสอบทางชีวเคมี และลำดับนิวคลีโอไทด์บริเวณ 16S rDNA พบว่า ใกล้เคียงกับ *Bacillus amyloliquefaciens* subsp. *plantarum* จากนั้นได้ศึกษาสภาวะที่เหมาะสมต่อการผลิตสารลดแรงตึงผิวชีวภาพ โดยใช้เทคนิคพื้นผิวตอบสนองที่ออกแบบการทดลองแบบบ๊อกซ์-เบห์นเคน เพื่อศึกษาอิทธิพลของ 4 ปัจจัย ได้แก่ ปริมาณคาร์บอน ปริมาณไนโตรเจน ความเป็นกรดด่าง และปริมาณหัวเชื้อเริ่มต้น ผลการทดลองพบว่า ค่าสัมประสิทธิ์ของการตัดสินใจเท่ากับ 0.6917 (R^2=0.6917) แสดงว่ารูปแบบการทดลองมีความเหมาะสมต่อความสัมพันธ์ของตัวแปรต่างๆ และจากสภาวะที่เหมาะสม ได้แก่ กลูโคส 10 ก./ลิตร เปปโตน 4.99 ก./ลิตร ความเป็นกรดด่างเริ่มต้นเท่ากับ 9 และปริมาณหัวเชื้อเริ่มต้น 10% สามารถผลิตสารลดแรงตึงผิวชีวภาพที่มีค่าการกระจายตัวของน้ำมันเท่ากับ 2.13 ซม^2 จากผลการทดลองแสดงให้เห็นว่า ไอโซเลท BS-VSA เป็นแบคทีเรียสายพันธุ์ใหม่ที่มีความสามารถในการผลิตสารลดแรงตึงผิวชีวภาพได้

คำสำคัญ : สารลดแรงตึงผิวชีวภาพ, ค่าการกระจายตัวของน้ำมัน และ วิธีการพื้นผิวตอบสนอง

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Introduction

Biosurfactants are amphipathic biomolecules with hydrophilic and hydrophobic portions that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces (Desai and Banat, 1997). These molecules are produced by various types of microorganisms including bacteria, fungi, and yeast. When biosurfactant molecules are on air-water or oil-water interfaces, they arrange themselves in micelles form by exposing their polar (hydrophilic) part in water phase and non-polar (hydrophobic) part in air or oil phase. The critical level of biosurfactant concentration that triggers the formation of supramolecular structures like micelles, bilayers, and vesicles is called as critical micelle concentration (CMC).

Biosurfactants are categorized to 6 groups based on their chemical compositions including; i) glycolipid; ii) lipopeptides and lipoproteins; iii) fatty acids and natural lipids; iv) phospholipids; v) polymeric biosurfactants; and vi) particulate surfactants. Based on different chemical compositions, these biosurfactants have different properties in physical, chemical, and biological aspects, which lead to broad industrial applications, such as, in production of agricultural products, construction, food, alcoholic beverage, leather, paper, and medicine (Desai and Banat, 1997). One of the main demands of biosurfactant is the chemical synthesis process in petrochemical industry. However, chemically-synthesized surfactant is toxic, and its accumulated residues cause the environmental problem in the long-term (Mann and Boddy, 2000; Mann and Bidwell, 2001).

Nowadays, the awareness of environmental quality worldwide and the enforcement of law regulation, especially in environment-related case lead to the increase in interest of biosurfactants production to substitute the use of chemically-synthesized surfactants (Banat *et al.*, 2000). The reasons that biosurfactants gain more attention because they are biodegradable, lower toxic, stable at extreme temperatures, pH and salinity, and they can be produced from renewable sources (Mercade *et al.*, 1993; Plaza *et al.*, 2006).

Although biosurfactants have wide range of applications, such as emulsifier, separation, solvent, anti-fouling, anti-viscous, foam stabilizer, they still cannot replace the use of chemical surfactant because of their relatively-high production cost. Investigations of microorganisms that are efficient producer of biosurfactants and production process optimization is now become an interesting area for research and development sectors (Kosaric *et al.*, 1984). The patterns of natural biosurfactant production are possible: (a) growth-associated production, (b) production under growth limiting conditions, (c) production by resting/non-growing cells, and (d) production associated with the precursor addition. In the event of growth-associated production, there is a parallel relationship between the substrate utilization, growth and production (Rodrigues *et al.*, 2006). Cell growth and biosurfactant accumulation are influenced by culture compositions, such as carbon source (water-soluble and water-insoluble carbon), nitrogen source (organic and inorganic nitrogen) and growth factor. This study aimed to isolate biosurfactant-producing bacteria from fermented foods to apply them in food industries. Additionally, to improve biosurfactant capability, the media formulation and culturing condition were optimized by statistical design tools. Response Surface Methodology (RSM) with Box-Behnken design that composed of four factors at three levels of variations was used and oil displacement value was measured as the response.

Materials and methods

1. Isolation of biosurfactant-producing bacteria from fermented foods

Twenty-five grams of fermented foods (pickled fish, pickled vegetable, pickled garlic,

pickled bamboo shoots etc.) were mixed and serially diluted in PBS buffer, then samples were plated in modified LBGS agar media (containing Bacto-tryptone 10 g/L, yeast extract 5 g/L, glucose 10 g/L, NaCl 5 g/L, agar 15 g/L, pH 7.5 (Roongsawang *et al.*, 2002)). After incubation at 35±1°C for 48 h, each bacterial isolate, which grew on plates, was inoculated in LBGS broth media. After incubation at the same condition, each bacterial isolate was kept as stock culture separately in -20°C.

2. Screening and selection of biosurfactant-producing bacteria

Each bacterial isolate was reinoculated from stock to fresh modified LBGS agar media that covered the top surface with Murban light crude-oil (Roongsawang *et al*., 2002). After incubation at 35±1°C for 24-48 h, the diameters of clear zones (oil-displaced halos) developed on the plates were measured as described in previous study (Morikawa *et al.,* 1993). In this study, all bacterial isolates were tested and only one isolate that showed the largest clear zone was selected as a biosurfactant-producing bacteria for culturing condition optimization experiment.

3. Identification of bacterial isolates by sequencing of 16S rDNA gene

To identify the selected bacterial isolate, the characteristic, morphology and biochemical properties were tested based on Bergey's manual Systematic Bacteriology. Additionally, a genus and species of the selected isolate was identified based on the similarity of 16S rDNA gene sequences. The genomic DNA of the selected bacterium was extracted by using Genomic DNA mini Kit (Geneaid Biotech Ltd., Taiwan). The genomic DNA was used as a template in a PCR reaction to amplify a fragment of the 16S rDNA gene by using thermal cycler machine (Major Science, USA). The PCR mixture was prepared by using the following two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3', positions 9-27 on 16S rDNA) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA).

The PCR products were sent for sequencing. The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) program. The identification of phylogenetic neighbors was initially carried out by the BLASTN program against the database containing type strains with validly published prokaryotic names.

4. Experimental design and fermentation for culturing-condition optimization

Optimization of culturing condition for biosurfactant production (*Y*) was carried out using Response Surface Methodology (RSM) with Box-Behnken design. Four of independent variables were studied here including carbon content (X_1), nitrogen content (X_2), pH (X_3), and %inoculum (X_4). For each variable, three levels (max = +1, mid = 0, min = -1) was selected for the optimization, with a total of 29 runs. The range and levels of independent variables and code values were represented in Table 1. Experimental data were analyzed using the Design-Expert software (version 7.0.0, STAT-EASE Inc., USA), to fit the second-order polynomial regression model as shown in Equation (1):

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \quad \text{..... (1)}$$

Where *Y* is the response variable (oil displacement), β_0 is the constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the two factor interaction coefficient. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination (R^2).

To culture the selected bacterium as designed based on RSM, the bacterial isolate was inoculated in 50 ml of broth media (containing $(NH_4)_2SO_4$ 7.0 g/L, $Na_2HPO_4.12H_2O$ 3.8 g/L, $MgSO_4.7H_2O$ 0.7 g/L, (Joshi *et al.,* 2008)) with vary carbon

content, nitrogen content, pH and %inoculum and incubated at room temperature with stirring using rotary shaker at 150 rpm for 72 h. The culture broth was centrifuged at 10,000 rpm for 10 min to get cell free broth and measured the oil displacement according to Morikawa *et al.,* 1993.

Results and Discussion

1. Screening and identification of biosurfactant-producing bacteria

Biosurfactant-producing bacteria were screened from fermented food samples based on oil-dispersion ability. Only one bacterial isolate, BS-VSA, from total 111 isolates that generates the largest clear zone on LBGS agar plate was selected for further experiment for culturing condition optimization.

The morphological observation further demonstrated that BS-VSA is a rod shaped Gram-positive bacterium. To identify the BS-VSA, a fragment of 16S rDNA was generated by PCR reaction and sequenced as described in the method. The sequencing result has 99.75% homology to 16S rDNA fragment of *Bacillus amyloliquefaciens* subsp. *plantarum* (Accession number CP000560).

2. Optimization of biosurfactant production using Response Surface Methodology

After identification of BS-VSA isolate, this selected bacterium was aimed to be subjected in optimization experiment to enhance the biosurfactant production using RSM with Box-Behnken design. Here, carbon content of 10-50 g/L (X_1), nitrogen content of 0.1-5 g/L (X_2), pH of 4-9 (X_3), and %inoculum of 1-10% (X_4) were tested to optimize the biosurfactant production (Table 1). The experiment trials were designed for 29 runs with three different coded levels (high (+1), medium (0), low (-1)) based on Box-Behnken design as shown in Table 2. A response factor, oil displacement, of all samples obtained from different culturing conditions was measured.

The statistic software package, Design-Expert software version 7.0.0 (STAT-EASE Inc., USA), was used for regression analysis of experimental data and to plot response surface. One-way analysis of variance (ANOVA) was used to estimate the statistical parameters. The responses of Box-Behnken design were well fitted with the second-order polynomial equation as shown in Equation (2).

$$Y = -1.76101 + 0.052891X_1 + 0.089155X_2 + 0.38562X_3 + 0.034358X_4 - (9.93083\times10^{-3})\, X_1X_3 \quad \text{..... (2)}$$

In this equation, Y is the oil displacement, and X_1, X_2, X_3 and X_4 are the coded value of the test variables as carbon content, nitrogen content, pH, and %inoculum, respectively. The statistical significance of the model was evaluated by the *F*-test for ANOVA (Table 3). ANOVA of the model (2FI vs. Linear) suggested that the model is significant with the value of "Prob > *F*" was 0.0002, which indicated that the computed model was statistically significant with a confidence interval of 99.95% (P-value < 0.05). The model *F*-value (7.81) implied that the model was significant and there was only a 0.02% chance that a "Model *F*-value" could occur because of noise. The "Lack of Fit" test also confirmed the statistical significance of the model because the "Prob>*F*" value was 0.5437 (insignificant Lack of Fit, P-value > 0.05).

The cut-off criteria of statistically significance with "Prob > *F*" with < 0.1 was applied to each "model term", and, in this case, X_1, X_2, X_3, X_4, and X_1X_3 are significant model parameters to the oil displacement of biosurfactant production in the BS-VSA. The coefficient of determination (R^2) of the model was 0.6917, which indicated that the model was suitable for representing the relationships among the selected variables and advocated the significance of the model. Based on the computed model (Equation (2)), the maximal oil displacement of biosurfactant (2.13334 cm^2)

was obtained when 10 g/L of glucose, 4.99 g/L of peptone, pH 9.0, and 10% of inoculum were used.

Response surface plots of the RSM as a function of two variables at a time are helpful in understanding both the main and the interaction effects of these variables (Fig. 1). Based on the ANOVA analysis of model term, X_1, X_2, X_3, X_4, and X_1X_3 are statistically significant. When separately considering the effect of each independent variable, it is interesting that X_1 has negative effects on oil displacement of biosurfactant, however X_2, X_3, and X_4 have positive effects. The effects of interactions between two variables, X_1X_3 (glucose and pH), on oil displacement of biosurfactant are complicated (Fig. 1). At lower carbon content, oil displacement of biosurfactant increased when pH increased, while, at higher carbon content, the oil displacement is decreased when pH increased.

Growth conditions of microorganism and environmental factors such as pH, temperature, and oxygen availability have impact on biosurfactant production through their effects on cellular metabolism. For example, Kim *et al*. (1997), they found that the surface tension reducing activity of *Bacillus subtillis* C9 was stable to pH over the range of pH of 5.0-9.5. Based on the measurement of surface tension, Gumaa *et al*. (2010) observed that the optimal pH for biosurfactant production of *Serratia marcescens* was at pH 7.0. Also Mahdy *et al.* (2012) optimized the production of biosurfactant from three *Candida* strains and found that each strain have different optimal pH. These evidences suggested that each type of microorganisms have specific condition to maximize biosurfactant production. The result of RSM here suggests that finding the optimal points of these parameters for culturing BS-VSA is necessary to maximize production of biosurfactants.

Conclusion

Biosurfactants are interesting natural products for biotechnological and industrial applications. They have several advantages over their synthetic compounds, such as lower toxicity, higher biodegradability, better environmental compatibility and the ability to be synthesized from renewable feedstock. Here, a bacterium, BS-VSA isolate that is closely related to *Bacillus amyloliquefaciens* subsp. *plantarum,* was selected to be a potential candidate for biosurfactant producer. Based on statistical analysis in RSM experiments, carbon content, nitrogen content, pH, and %inoculum are factors that significantly affect on the oil dispersion. The optimum levels of biosurfactant were produced by the BS-VSA isolate when grown in culture broth medium containing 10 g/L of glucose, 4.99 g/L of peptone with a C/N ratio of 2:1 at pH 9.0 and 10% inoculum, incubated at room temperature and shaken at 150 rpm for 72 hr. Additionally, further experiments should be conducted to identify the impact degree of each variable factors on the biosurfactant production. Thus, the BS-VSA isolate was proved to be a potential source of biosurfactant that could be used for the industrial processes especially in food industries because it is originally isolated from fermented food.

Acknowledgements

The authors wish to thank the Program in Biology, Faculty of Science and Technology Rajabhat Maha Sarakham University for providing necessary laboratory infrastructure to carry out the work.

Table 1 Coded factors and actual value of independent variables

Factor	Range of variable		
	Low (-1)	Mid (0)	High (1)
Carbon content (glucose g/L) (X_1)	10.00	30.00	50.00
Nitrogen content (peptone g/L) (X_2)	0.10	2.55	5.00
pH (X_3)	4.00	6.50	9.00
Inoculum (%) (X_4)	1.00	5.50	10.00

Table 2 Experimental design to test the effect of independent variables on protein production (Y)

Run Order	Factor				Oil displacement (cm^2)
	glucose (g/L)	peptone (g/L)	pH	Inoculum (%)	
1	50	0.10	6.5	5.5	0.4798
2	30	2.55	6.5	5.5	0.5315
3	30	2.55	6.5	5.5	0.9633
4	10	5.00	6.5	5.5	1.0710
5	50	2.55	6.5	1.0	0.4037
6	30	5.00	9.0	5.5	1.0076
7	30	5.00	6.5	1.0	0.6542
8	50	2.55	9.0	5.5	0.4536
9	30	2.55	6.5	5.5	0.4896
10	10	2.55	9.0	5.5	2.4154
11	10	2.55	6.5	1.0	0.7936
12	30	2.55	6.5	5.5	1.0837
13	30	2.55	9.0	10.0	1.1084
14	30	2.55	4.0	1.0	0.5697
15	30	0.10	6.5	10.0	0.4817
16	50	2.55	4.0	5.5	0.4817
17	30	2.55	9.0	1.0	0.4332
18	30	0.10	4.0	5.5	0.5525
19	30	2.55	6.5	5.5	0.9633
20	10	2.55	6.5	10.0	1.2427
21	50	0.10	6.5	5.5	1.2569
22	50	2.55	6.5	10.0	0.6585
23	30	5.00	6.5	10.0	1.2537
24	10	0.10	6.5	5.5	0.5525
25	30	0.10	9.0	5.5	0.9031
26	30	2.55	4.0	10.0	0.6233
27	30	0.10	6.5	1.0	0.6585
28	30	5.00	4.0	5.5	1.0058
29	10	2.55	4.0	5.5	0.4573

Table 3 ANOVA analysis of the design to optimize biosurfactant production

Source	Sum of squares	df	Mean squares	F value	p-value Prob > F	
Model	3.08	5	0.62	7.81	0.0002	Significant
A-glucose	0.65	1	0.65	8.29	0.0085	
B-peptone	0.57	1	0.57	7.28	0.0129	
C-pH	0.58	1	0.58	7.33	0.0126	
D-Inoculum	0.29	1	0.29	3.64	0.0688	
AC	0.99	1	0.99	12.53	0.0017	
Residual	1.81	23	0.079			
Lack of fit	1.51	19	0.079	1.05	0.5437	Not significant
Pure Error	0.30	4	0.076			
Cor total	4.89	28				

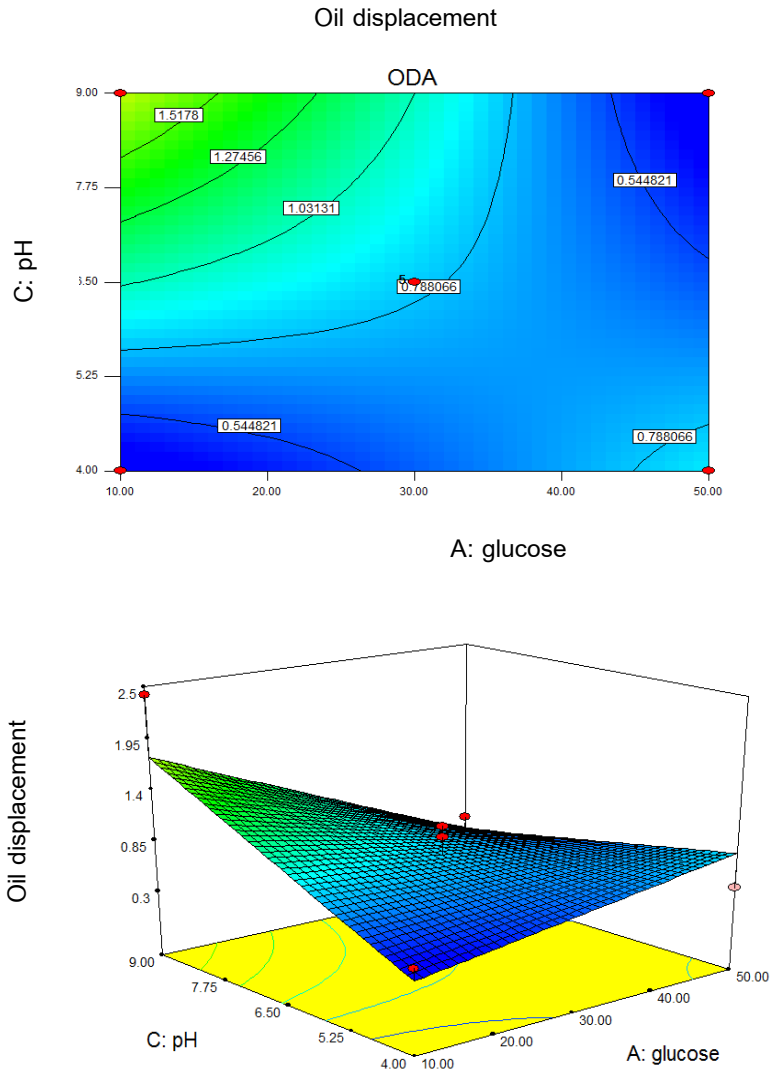

Fig. 1 Response surface plots showed the effect of interaction between the two independent variables (incubation time (X_1) and nitrogen content (X_3)) to the protein production.

Contour plot (a) and 3D surface plot (b).

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