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*Full Length Research Paper*

# Response surface analyses of rhamnolipid production by *Pseudomonas aeruginosa* strain with two response values

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**Response surface methodology (RSM) and Box-Behnken experiment design (BBD) were employed for optimization of biosurfactant shaking flask fermentation conditions of a Pseudomonas aeruginosa strain. Using Design Expert 8.0.5b software, multiple binomial mathematical models were established to study the influence of carbon sources (waste vegetable oil, WVO), nitrogen source (NaNO3) of and potassium source (KCl) levels in fermentation broth and their interactions on fermentation yield of biosurfactant. Significance tests showed that the model with the oil spreading circle (OSC) as response value was significant while that with rhamnolipid production by colorimetric method was narrowly significant (P=0.0531, R2=0.8218). Ultimately the optimal parameters were reached as WVO 5.04%, NaNO3 2.70 g/L, KCl 1.37 g/L with two index as response values. Under optimized conditions, the expected rhamnolipid yield increased dramatically. OSC measurement with diluted broth acting as the response value, rather as surface tension measurement or colorimetric method showed advantages such as easy and simple to conduct, the requirement for volume of broth, low cost and more precise to construct the optimal model.**

**Key words:** Pseudomonas aeruginosa, response surface methodology, rhamnolipid, aerobic fermentation.

## INTRODUCTION

Biosurfactants are amphipathic compounds produced by microorganisms with surface activities to reduce liquid surface tension (Gautam and Tyagi, 2006; Marchant and Banat, 2012). Biosurfactants have many advantages over synthetic surfactants, such as high surface activity, high biodegradability, lower critical micelle concentration (CMC), effectiveness at extreme environmental condi-tions and ecological acceptability (Gautam and Tyagi, 2006). Bio-surfactants have been applied in many fields, such as microbial enhanced oil recovery, bioremediation, cosme-tics, pharmaceuticals, detergents, food and other industries (Banat et al., 2000; Gautam and Tyagi, 2006).

The major classes of biosurfactants are known as glycol- and phospholipids, lipopeptides, lipoproteins, poly-meric and particulate surfactants (Desai and Banat, 1997). Glycolipids are among the most widely bacterially produced biosurfactants (Gautam and Tyagi, 2006), especially the rhamnolipid produced by Pseudomonas aeruginosa, with the highest potential to be the next generation of biosurfactants for the market (Müller et al., 2012). Even in recent years, a large number of journal papers on isolation and characterization of this species producing rhamnolipid are still being published (Hazra et al., 2011; Müller et al., 2011; Saikia et al., 2011; Zhang et al., 2012a). The reason why P. aeruginosa is often employed as biosurfactant-producing strain lies in its wide spread occurrence, rapid growth, the ease of isola-tion and screening (Zhang et al., 2012a).

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**Table 1.** Factors and levels of response surface analysis.

| Factor | Level | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| A: Carbon source (WVO, %) | 3 | 5 | 7 |
| B: Nitrogen source($NaNO_3$, g/L) | 1.5 | 2.5 | 3.5 |
| C: Potassium( KCl, g/L) | 1 | 1.5 | 2 |

To produce biosurfactant to compete with chemical surfactant, genetic improvement, using low cost raw-materials (Makkar and Cameotra, 2002), for instance, waste vegetable oil (WVO) (Raza et al., 2006; Zhang et al., 2012a, b), and fermentation conditions optimization are indispensible. Response surface methodology (RSM) has been employed as an efficient means for fermentation engineering, especially for optimization of fermentation media (Najafi et al., 2010; Wang et al., 2013). In this study, research interest was focused on fermentation medium composition optimization of a P. aeruginosa strain using RSM.

The factors affecting shaking fermentation yield include inoculum age, shaking speed (dissolved oxygen amount), incubation temperature and medium composition (including pH value) etc, and the medium composition is considered the most important one. Based on the previous studies (unpublished data), the objectives of this work, using P. aeruginosa strain Z41 as tested strain for shaking fermentation, were to evaluate the effects of the composition of the medium components including carbon source (WVO), nitrogen source (NaNO3) and potassium source (KCl) and to search for the optimal medium composition to produce rhamnolipids in shaking flasks to lay a solid base for further fermentation in fermentors or possible industrial production in the future. The oil spreading circle method was employed as well as rhamnolipid production per liter measured colorimetrically, to compare the two response values and to seek a very simple and low cost response value for RSM optimization of biosurfactant fermentation.

## MATERIALS AND METHODS

### Strains

Z41, Pseudomonas aeruginosa, isolated from crude oil contaminated soil samples in the oil field (Zhang et al., 2012b).

### Chemicals and samples

KH2PO4 (purity ⩾99.5%) was purchased from Shaihai Linfeng Chemical Reagent Co., Ltd (China), NaNO3, NaCl, KCl, K2HPO4.H2O, Na2HPO4.12H2O MgSO4.7H2O, CaCl2.2H2O and peptone (total N 12.5-15.0%), were purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. Diesel (0#) was purchased from a local gas station in Yancheng, China. Beef extract (total N ⩾13%) was purchased from Beijing Aoxing Biotechnology Co., Ltd (China), yeast extract (total N 10.0%-12.5%) was purchased from Oxid Co., Ltd (England), agar (bacterial grade, gel strength ⩾1300 g/cm2) was purchased from Hirono Co., Ltd. (Japan).

WVO, originating from salad oil, was collected from restaurants in Yancheng Teachers University, Yancheng City, China.

### Media

Nutrient broth (NB) or seed medium was composed of, in g/L deionized water: peptone 10, beef extract 5, and NaCl 5. For the preparation of nutrient agar plates or slants, 15.0 g/L agar (strength 1300) were added (Zhang et al., 2012b).

Biosurfactant fermentation broth were all prepared as referenced (Zhang et al., 2012b) and modified as required for optimization.

### Methods

#### Response surface tests

Experimental Box-Behnken design (BBD) was employed as shown in Table 1. Fermentation conditions, including inoculum age, shaking speed (dissolved oxygen amount), incubation temperature and medium pH value, the optimal kinds of carbon source, nitrogen and potassium, and the design of the levels of each tested factor in RSM were determined by previously implemented single-factor and orthogonal tests (unpublished).

In total, 17 runs of experiments were carried out (Table.2) Each treatment had a control without inoculum. Two response values, OSC and rhamnolipid production were employed. After 3 days' incubation, the two response values were measured accordingly.

#### Verification test

A new run of shaking flask fermentation was carried out using the optimal parameters of RSM experiment. The OSC and ST values of broth and rhamnolipid production values were measured and compared with expected values.

#### Biosurfactant production measurement

The protocols of incubation, fermentation and evaluation of biosurfactant production using the oil spreading method, ST method and colorimetric method (at 483nm) were the same as in our previous report (Zhang et al., 2012b).

#### Statistics

All the data in the RSM test were analyzed by Design Expert (version 8.0.5bb) software to find the most appropriate fermentation medium composition for RSM modeling of rhamnolipid producing medium composition.

All experiments were conducted in triplicate. Results were evaluated for statistical significance using ANOVA.

## RESULTS

### Standard curve of colorimetric method to determine rhamnolipid production

As shown in Figure 1, a new standard curve was plotted by measuring the optical density at 483 nm, different from

**Table 2.** Experimental Box-Behnken design (BBD) runs in Design Expert (8.0.5b version).

| Run number | A | B | C |
|---|---|---|---|
| 1 | 0 | -1 | 1 |
| 2 | 0 | 1 | 1 |
| 3 | -1 | 0 | -1 |
| 4 | 1 | 1 | 0 |
| 5 | 0 | 0 | 0 |
| 6 | -1 | -1 | 0 |
| 7 | 0 | 0 | 0 |
| 8 | 1 | 0 | -1 |
| 9 | 1 | 0 | 1 |
| 10 | 1 | -1 | 0 |
| 11 | 0 | 0 | 0 |
| 12 | -1 | 1 | 0 |
| 13 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 |
| 15 | 0 | 1 | -1 |
| 16 | -1 | 0 | 1 |
| 17 | 0 | -1 | -1 |

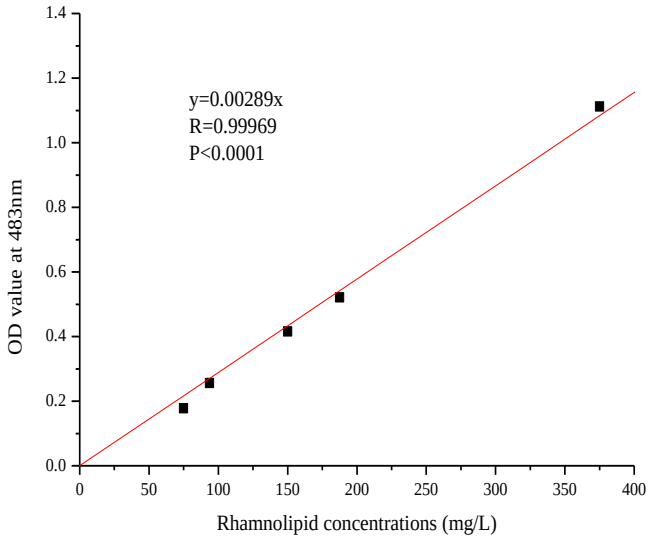


**Figure 1.** Standard curve of OD value at 483nm.

our previous report (Zhang et al., 2012b), of a series of dilutions of standard rhamnolipid solutions. The regression equation was y = 0.00289x, R2 = 0.99969, P<0.0001.

## The total data of RSM test

Figure 2 shows the results of the RSM test. It could be concluded that the changing trend of rhamnolipid concentration was largely the same as the OSC, but the former's experimental errors, represented by error bars in Figure 2, were bigger than those of OSC.

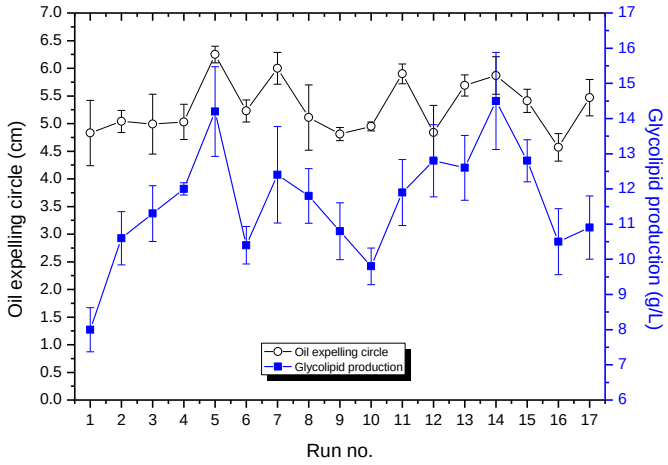


**Figure 2.** Contrast of data of oil spreading circle diameters and rhamnolipid production (vertical bars indicate the SD of means of triplicates).

## Setup of response surface model

Design-Expert software suggested a quadratic equation for OSC and rhamnolipid production as follows (both in terms of coded factors):

Y = 5.98 + 0.034 * A - 0.020 * B - 0.22 * C + 0.12 * A * B + 0.030 * A * C + 0.067 * B * C - 0.64* A2 - 0.33 * B2 - 0.47 * C2

Z =13.12-0.075A + 1.14 * B - 0.86 * C-0.050 * A * B – 0.050 * A * C + 0.17 * B * C - 0.67* A2 - 1.20 * B2 -1.35 * C2

Where, Y means OSC (cm) and Z means rhamnolipid production (g/L).

ANOVA results of the quadratic model in Table 3 revealed that the model equation derived by RSM in Design-Expert software based on OCED as the response value could adequately be used to describe the bio-surfactant production under a wide range of operating conditions ("Prob>F"=0.0004). For the model, there was no lack of fit and the quadratic R2 was 0.9602. The model equation derived based on rhamnolipid production (by colorimetric method) was also suitable to describe the biosurfactant production ("Prob>F"=0.0531>0.05, not so ideal yet). There was obvious lack of fit and the quadratic R2 was only 0.8218.

The predicted versus experimental plot for the two response values showed that the actual values were distributed near to the straight line (Figure 3), which indicated that actual values were very close to the predicted ones, especially OSC. Thus, both were the appropriate models to predict the biosurfactant production using factors mentioned above when the other conditions were fixed.

## Interactions of factors tested

C, N and K sources are three most important nutrition

**Table 3.** The analysis of variance (ANOVA) for the response surface quadratic model.

| Response value | Source | Sum of square | Degree of freedom | Mean square | F-value | Prob>F |
|---|---|---|---|---|---|---|
| OSCD | Model | 3.91 | 9 | 0.43 | 18.75 | 0.0004 |
| | Residual | 0.17 | 7 | 0.023 | | |
| | Lack of fit | 0.062 | 3 | 0.023 | 0.83 | 0.5421 |
| | Pure error | 0. 100 | 4 | 0.025 | | |
| | $R^2$ | | 0.9602 | | | |
| Rhamnolipid production determined by colorimetric method | Model | 33.73 | 9 | 3.75 | 3.59 | 0.0531 |
| | Residual | 7.32 | 7 | 1.05 | | |
| | Lack of fit | 1.97 | 3 | 0.66 | 0.49 | 0.7076 |
| | Pure error | 5.35 | 4 | 1.34 | | |
| | $R^2$ | | 0.8218 | | | |

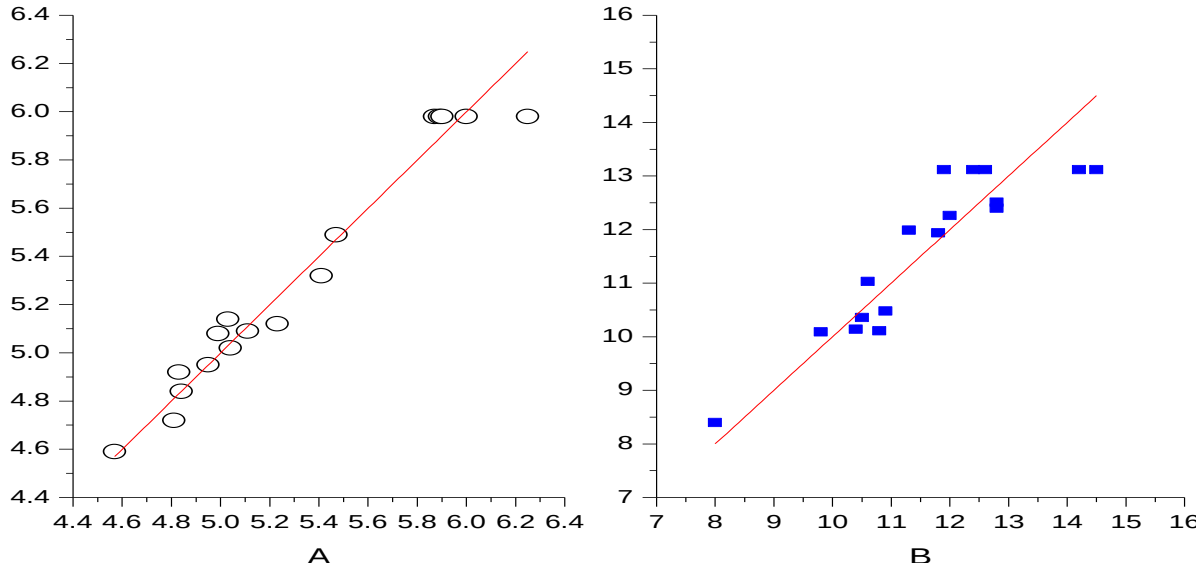


**Figure 3.** Experimental values vs. predicted values (A: OSCD; B: Rhamnolipid production determined by colorimetric method).

elements in fermentation media, so these should be the three factors considered most important. It can be seen from Figure 4 that the levels of each factor and interactions of each two factors influenced biosurfactant production represented by OSC and colorimetric values. In every figure, the biosurfactant production increased with rising of each factor's level until reaching the peak (the stable points, or maximum values) then went down again. The fluctuating trends changed in similar way whether the OSC value or colorimetric value was employed as response value, although the peak differed a little. It can be concluded that the 3D curves of OSC based model were steeper (with more dense contour lines) than that of colorimetric value, indicating that the interactions of factors were stronger in OSC based model.

### Determination of optimized parameters

It can be seen from Figure 3A and B that the rhamnolipid production represented by OSC and colorimetric value reached the peak at about 5% of carbon source. In Figure 3A and C, the peak was at more than 2.5 g/L of nitrogen source and in Figure 3B and C the peak was lower than 1.40 g/L. After optimization process by software, the results of the optimized parameters are shown in Table 4.

### Verification test

The optimal levels of three factors were used again for a new run of fermentation, as shown in Table 5. In conclusion, after optimization biosurfactant production by Z41 strain increased from 28.2% and 43.0% in OSC and colorimetric values, separately.

## DISCUSSION

The factors affecting the biosurfactant production in labo-

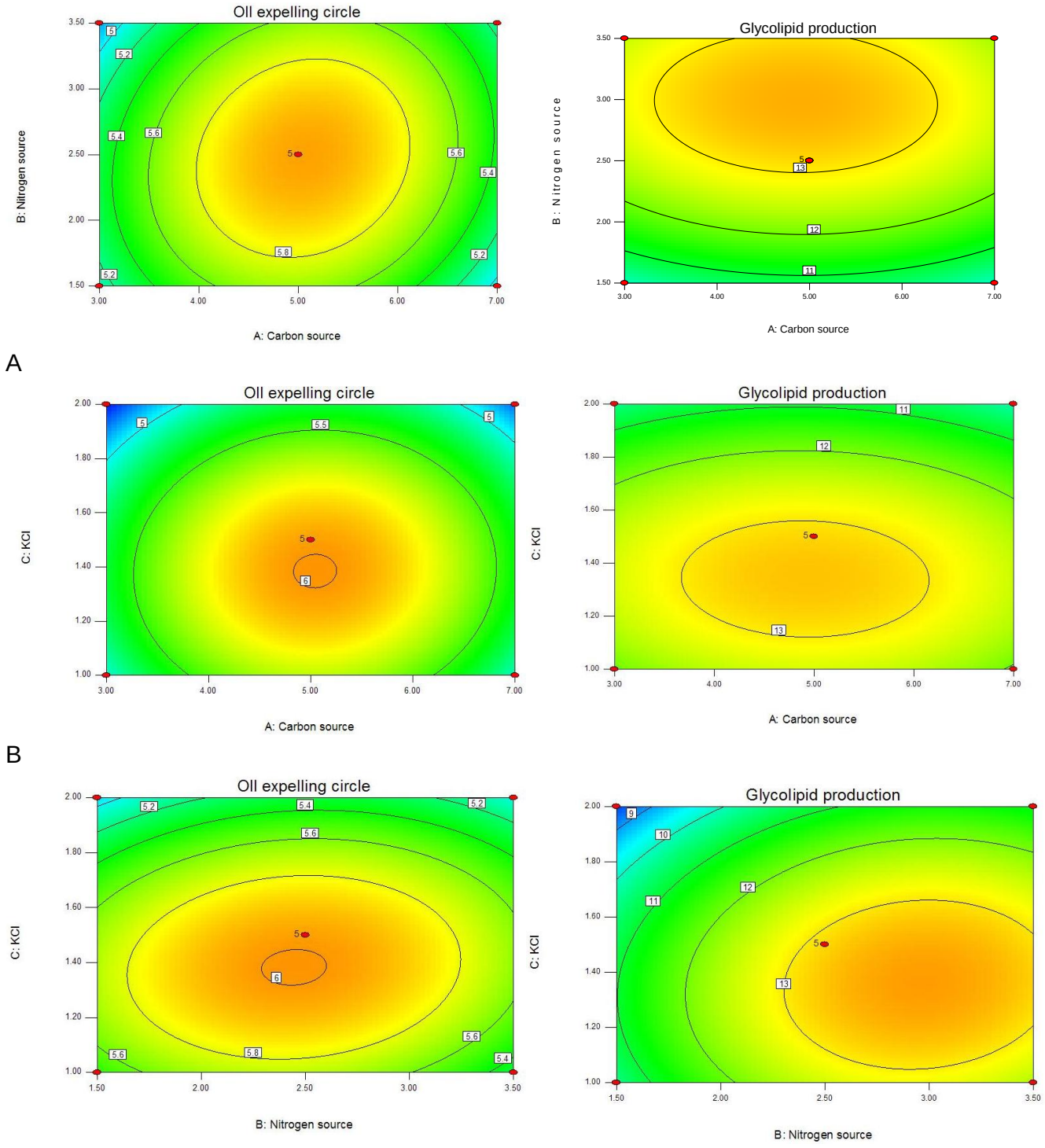


**Figure 4.** The contours of OSCD (left) and rhamnolipid production determined by colorimetric method (right) (contours were generated using the data shown in Figure 2. Inputs were the 17 experimental runs carried out under the conditions established by the BBD design. To avoid redundancy, the 3D surface plots were not shown. A: C & N, B: C & K, C: N & K).

ratory scale have been extensively studied in recent years (Abalos et al., 2002), but there is no universal opinion as to the response value selection. Usually ST (Najafi et al., 2010), biomass concentration (Rodrigues et al., 2006), or combination of biomass and biosurfactant concentration (Abalos et al., 2002) are used as response values. How-

**Table 4.** Optimized parameters for two models.

| Response value | Carbon source (%) | Nitrogen source (g/L) | KCl (g/L) | Expected OSCD (cm) | Expected rhamnolipid production (colorimetric method, g/L) |
|---|---|---|---|---|---|
| OSCD | 5.04 | 2.70 | 1.37 | 5.99 | 13.42 |
| Rhamnolipid production | 5.04 | 2.70 | 1.37 | 5.99 | 13.42 |

**Table 5.** Results of verification test.

| Parameter | OSCD (cm) | Surface tension (mN/m) | Rhamnolipid production (colorimetric method, g/L) |
|---|---|---|---|
| Initial composition | 5.07±0.25 | 37.54±1.10 | 9.16±0.91 |
| Optimized composition | 6.05±0.42 | 34.47±2.76 | 13.10±1.29 |
| Increase by (%) | 28.2 | | 43.0 |

ever, a very simple and low cost method, the oil spreading circle method has been ignored. This study used OSC as well as biosurfactant production as response values.

Interestingly, OSC measurement with diluted broth acting as the response value, rather as ST measurement or colorimetric method showed advantages, whether in the establishment of multiple binomial mathematical models or in the analyses of interactions of different factors, that was relevant to the actual process of measurement. The sulfuric acid-phenol method was much more complicated than the oil spreading circle method, leading to more random errors in experiments and instability of results. The longer time needed for the colorimetric measurement and the storing of samples in the refrigerator also added possible errors. Another reason was that the surface activity of the broth was not only caused by rhamnolipid. OSC measurement, compared to the colorimetric method, was easy and simple to conduct as it only required 10 µL original or diluted (10 times) broth and was low in cost. The use of OSC is strongly recommended by the authors for the optimization of biosurfactant-producing fermentation parameters. As for the ST as a response value used by other researchers (Najafi et al., 2010), in our case, was found hard to employ. It was found that its measurement of original broth without dilutes did not make notable difference, since the CMC was only about 128 mg/L (unpublished). To measure the ST proportional to the rhamnolipid concentration, the broth had to be diluted to below the CMC. It was really very complicated to calculate and operate, so the ST was not considered as a good response value for RSM in this study.

The rhamnolipid production was 13.10 g/L under RMS optimized conditions, a little lower than the yield optimized with a central composite design (CCD) algorithm in *P. aeruginosa* fermentation in some other reports (Abbasi et al., 2012). This might be caused by difference in strain capacity in fermentation yield and measuring methodology.

Although there are some differences in equations of the models, contours and response surface plots (not shown in this paper), the optimized levels of three factors from two models based on two response values were the same. This showed that although the RSM is a good tool for optimization of fermentation conditions, the success in the optimization experiment should be based on careful design of the kinds and the levels of the tested factors and accurate measurements of response values, especially in biological studies with more unexpected conditions affecting the experiments results.

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