Comparison of Methods to Quantify Rhamnolipid and Optimization of Oil Spreading Method

A rapid and reliable method to quantify rhamnolipid is indispensable to study and evaluate rhamnolipid-producing bacteria. Five methods were attempted to quantify rhamnolipid in bacterial culture. The oil spreading method better predicted the rhamnolipid concentration and is simpler than the other methods. The potential influencing factors (temperature, pH, salinity, metals, bacterial cells, carbon sources) were investigated. The common substance in fermentation broth has little effect on oil spreading circle. An optimized protocol of the oil spreading method to quantify rhamnolipid was proposed. A positive linear correlation ($R^2 = 0.9908$) was established between the diameter of the transformed oil spreading circle and the concentration of rhamnolipid ($100–800$ mg L$^{-1}$). Results of a large number of samples suggested that the oil spreading method is easy, rapid and reliable to analyze quantitatively rhamnolipids.

**Key words:** Rhamnolipid, quantification, oil spreading method, optimization


**Stichwörter:** Rhamnolipid, Quantifizierung, Ölspreitungsmethode, Optimierung

1 Introduction

Rhamnolipid is one kind of biosurfactants produced by microorganisms, consisting of two parts, a hydrophilic moiety (one or two rhamnoses) and a hydrophobic moiety ($\beta$-hydroxy fatty acid with different carbon chain lengths) [1–3]. Among the biosurfactants, rhamnolipid has been extensively studied because of its remarkable surface activity and emulsifying properties [4, 5]. Rhamnolipid has broad potential applications in various fields, including microbial enhanced oil recovery (MEOR) [6–8] and environmental remediation [9–11].

In the screening process of rhamnolipid-producing bacteria, a quantitative analysis of rhamnolipid is indispensable. Moreover, the development of a rapid and reliable method to quantify rhamnolipid is essential to study and evaluate rhamnolipid-producing bacteria. However, there is no uniform method for a quantification of rhamnolipid in bacteria culture. At present, the quantitative methods for rhamnolipid include the anthrone–sulfuric acid method [12, 13], critical micelle concentration (CMC)-surface tension method [14], the orcinol assay [15, 16], the organic solvent extraction [17] and the oil spreading method [25, 26]. At present, few studies were focused on comparing these methods to quantify rhamnolipid in bacteria culture.

In the present work, five methods to quantify rhamnolipids, the anthrone–sulfuric acid method, the CMC-surface tension method, the orcinol assay, the organic solvent extraction and the voil spreading method, were compared for their ease of operation and their reliability comparing to the real rhamnolipid amount. The oil spreading does not require specialized equipment or chemicals, more importantly, it excludes the bias which results from the interference of medium. It was found to be more rapid and reliable than other methods. Furthermore, influencing factors on the diameter of oil spreading circle was investigated. The oil spreading method was optimized, and a protocol was proposed. The results of this study will contribute to providing with a convenient and reliable method to analyze quantitatively rhamnolipids in a bacteria culture.

2 Materials and Methods

2.1 Chemicals and media

All the chemicals used for the experiments are of analytical grade, such as anthrone, orcinol, sulfuric acid, chloroform, methanol, NaCl, MgCl$_2$, ZnCl$_2$, FeCl$_3$, CuCl$_2$, MnCl$_2$, CaCl$_2$, AlCl$_3$, glucose and glycerol. The used chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd, China. Both distilled water and tap water were used. The crude oil was sampled from Xinjiang oilfield with density of 0.886 g cm$^{-3}$ and viscosity of 5.6 mPa·s. Edible soybean oil and olive oil were used. All cultures were incubated in Glycerol-Nitrate (GN) medium [18]. Using chloroform/methanol (v/v, 2:1) [19], the crude rhamnolipid product was extracted from culture of *Pseudomonas aeruginosa* SG (GenBank accession number KJ995745) [20].
2.2 Comparison of methods to quantify rhamnolipid

To compare the five methods to quantify rhamnolipid, the culture supernatant of \textit{P. aeruginosa} SG and the solution of 625 mg L\(^{-1}\) rhamnolipid (125 mg L\(^{-1}\) \textit{P. aeruginosa} SG rhamnolipid product was dissolved into 200 mL GN medium) were prepared. In this work, all experiments were conducted three times, and the average data were used.

For the anthrone-sulfuric acid method [12, 13], 1 mL of rhamnolipid sample was placed in an ice bath. 4 mL of a solution containing 0.2% anthrone (in 95% \(H_2SO_4\)) were added. After heating for 30 min at 100°C, the samples were cooled down to room temperature. The value of OD\(_{550}\) (Optical density value of the tested solution at light of 550 nm) was measured. The rhamnolipid concentration was calculated from standard curves prepared with L-rhamnose (0–300 mg L\(^{-1}\)) and expressed as rhamnose equivalent.

For the CMC-surface tension method, the samples were serially diluted. Then the surface tension of each dilution was measured by the BZY-1 automatic surface tension meter (Shanghai equitable Instruments Factory, china). The Dcmc values were estimated from the dilution at which a sharp rise in the surface tension value occurred. The CMC value of the rhamnolipid product is 80 mg L\(^{-1}\) [20]. The rhamnolipid concentration (mg L\(^{-1}\)) of sample was the Dcmc value multiplied by 80 (the CMC value).

For the orcinol assay [15, 16], 0.5 mL of a sample was extracted three times using 1 mL of ether. The upper organic phases were collected and evaporated; afterwards, 0.5 mL of distilled water were added. Approximately 4.5 mL of 0.19% orcinol solution (in 53% \(H_2SO_4\)) was then added to 0.5 mL of the diluted samples. After heating for 30 min at 80°C, the samples cooled down to room temperature and the OD\(_{421}\) values were measured. The rhamnolipid concentrations were calculated from the standard curve prepared with L-rhamnose (0–50 mg L\(^{-1}\)).

For the organic solvent extraction method [17], the pH of the samples was adjusted to 2.0 with 1 mol L\(^{-1}\) HCl and kept at 4°C overnight to reduce the rhamnolipid solubility. Two volumes of chloroform/methanol (v/v, 2:1) were added to the culture supernatant and shaken for 30 min. The lower organic phases were collected and evaporated to dry using a rotary evaporator (60 rpm, 45°C). The rhamnolipid residue was thin and yellowish. The rhamnolipid concentration (mg L\(^{-1}\)) of sample was the mass of the thin yellowish product divided by the volume of sample.

For the boil spreading method, 30 ± 1 mL of distilled water was added in a petri dish (90 mm in diameter). Then (20 ± 1) mL of crude oil was added to the surface of water. A thin oil membrane was formed. (10 ± 1) mL of sample was then gently dropped onto the center of the oil membrane. A clearly oil spreading circle was formed. The diameter of oil spreading circle was measured as the amount of rhamnolipid. The diameters of oil spreading circles showed linear relations with the rhamnolipid amount in bacteria culture samples.

2.3 Influencing factors of oil spreading method

A 500 mg L\(^{-1}\) rhamnolipid sample was prepared as previously described (50 mg rhamnolipid product was dissolved into 100 mL GN medium). Four factors (water types, water amount, oil types, and oil amount) which may affect the oil spreading method were investigated. (1) Effect of water types: 30 mL of tap water or distilled water were added to a petri dish (90 mm diameter) followed by addition of 20 mL of crude oil to the surface of the water. Then 10 mL of 500 mg L\(^{-1}\) rhamnolipid sample were added to the surface of oil. The diameter of the clear zone on the oil surface was measured. (2) Effect of the water amount: According to the method described in section (1), the effect of different amounts (20, 25, 30, 35 mL) of distilled water on the diameter of the clear zone on the oil surface was studied. (3) Effect of oil types: 20 mL of n-hexadecane, liquid paraffin or crude oil were added to the surface of water to evaluate the effect of oil type on the diameter of the clear zone on the oil surface. (4) Effect of oil amount: According to the method described in section (1), the effect of different amounts (10, 15, 20, 25, 30, 35 mL) of crude oil on oil spreading method was studied. The diameter of clearly formed oil spreading circle was measured.

2.4 Effect of physico-chemical factors on oil spreading activity of the rhamnolipid

In order to evaluate the effect of physico-chemical factors on the oil spreading activity, 500 mg L\(^{-1}\) rhamnolipid samples were used. Effect of different temperatures (i.e., 4, 30, 60, 80 and 100°C), pH (i.e., 1, 2, 4, 6, 8, 10 and 12) and different salinity (i.e., NaCl concentration of 0%, 5%, 10%, 15% and 20%) on oil spreading activity of rhamnolipid samples were studied. To investigate the effect of the metallic ion on oil spreading activity, several metallic ions (e.g., Mg\(^{2+}\), Zn\(^{2+}\), Fe\(^{3+}\), Cu\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\), Al\(^{3+}\)) with concentration of 5 g L\(^{-1}\) were added into rhamnolipid samples, respectively. The diameter of a clearly formed oil spreading circle was measured.

2.5 Effect of carbon sources and bacterial cells on oil spreading method

To evaluate the effect of residual carbon sources (glucose, glycerol, soybean oil, olive oil) on oil spreading activity, media with different carbon sources were used as solvent of the extracted rhamnolipid produced by \textit{P. aeruginosa} SG. 50 mg of the extracted rhamnolipid product were dissolved into 100 mL medium to prepare 500 mg L\(^{-1}\) rhamnolipid solutions, respectively. The other medium composition is identical to the composition of GN medium, except for the carbon source. Furthermore, the influence of bacterial cells to oil spreading activity was examined. Using glycerol as carbon source, aerobic cultures of \textit{P. aeruginosa} SG and \textit{P. aeruginosa} WJ-1 [21], and their cell-free culture supernatant (10,000 rpm, 10 min) were prepared. Aerobic cultures of \textit{P. aeruginosa} SG, and its cell-free culture supernatant were diluted 16-fold, and aerobic cultures of \textit{P. aeruginosa} WJ-1, and its cell-free culture supernatant were diluted 32-fold. The diameter of clearly formed oil spreading circle was measured.

2.6 Effect of the common substance in fermentation broth on oil spreading method

\textit{Escherichia coli} DH5\(\alpha\) could not produce rhamnolipid. The strain \textit{E. coli} DH5\(\alpha\) was fermented using GN medium. In order to investigate the effect of common substance (salts, proteins, lipids) in fermentation broth on oil spreading activity, 50 mg rhamnolipid product was dissolved into 100 mL of fermentation broth of \textit{E. coli} DH5\(\alpha\) to prepare a 500 mg L\(^{-1}\) rhamnolipid sample. The diameter of clearly formed oil spreading circle was measured.

3 Results and Discussion

3.1 Comparison of methods

Samples of strain SG cultures and the 625 mg L\(^{-1}\) rhamnolipid solution were used for comparison of methods for quan-
tification of rhamnolipid. The results are shown in Table 1. In this work, the statistical analysis of all experimental data was carried out by methods referred to previous studies [22, 23].

Compared to the real rhamnolipid concentration in samples, the anthrone-sulfuric acid method and the orcinol assay largely overestimated the rhamnolipid concentration. These two methods are based on the colorimetric determination of sugar moieties to quantify the rhamnolipid in bacteria cultures. In the bacteria cultures, other sugar metabolites might have strong interferences to these two methods. These two methods require a specialized equipment and sulfuric acid and overestimate the rhamnolipid concentration of samples. They are also relatively complex methods. For the organic solvent extraction method, a lot of organic solvent (chloroform and methanol) was used. This method is too slow to quantify the rhamnolipid in the culture. It is impossible to quantify the rhamnolipid in a large number of samples through organic solvent extraction method. The CMC-surface tension method requires the high-resolution surface tension meter. The samples need to be serially diluted in this method. Therefore, it is not suitable for a rapid determination of a large numbers of samples.

Among the five methods, the oil spreading method is more accurate and reliable. Compared to the other four methods, no specialized equipments or chemicals were used in this method. The oil spreading method is also less time-consuming and easier to perform and to standardize than the other methods, which makes it applicable to determine a large number of samples.

Rhamnolipid as a potent natural biosurfactant has a wide range of potential applications, including MEOR [4–8] and environmental remediation [9–11, 24]. Isolation of the high yield of rhamnolipid-producing strains is essential to MEOR and environment bioremediation. According to the literature and study reports, there is no unique method for quantification of rhamnolipid. Therefore, a rapid and reliable method is urgently needed for a quantification of rhamnolipid in bacteria cultures. In our study the oil spreading method was considered to be the best method to analyze quantitatively rhamnolipid due to its rapidness, reliability, and simplicity. Oil spreading method contributes to screen high yield of rhamnolipid-producing bacteria and to study their rhamnolipid production kinetics.

3.2 Influencing factors to oil spreading method

Water types (tap water and distilled water) have a significant effect on the oil spreading method. Tap water led to the uneven distribution of oil film on the water surface and the formation of irregular oil spreading area. However, clear oil spreading circles were formed on the oil film when using distilled water. The water amounts (25–35 mL) have no effect on the oil spreading method. Less than 25 mL of water could not cover the whole 90-mm petri dish. Oil types, like n-hexadecane and liquid paraffin are colorless, which is difficult to observe the oil spreading zone. Using crude oil can solve this problem. Less than 15 μL of crude oil can not form a whole oil membrane on the water surface. Moreover, more than 25 μL of crude oil formed a too thick oil film. The 500 mg L⁻¹ rhamnolipid sample forms an oil spreading circle of 20.0 mm in diameter on this thick oil film. However, the 500 mg L⁻¹ rhamnolipid sample theoretically forms an oil spreading circle of 36.7 mm in diameter. Therefore, using more than 25 μL of crude oil can underestimate the rhamnolipid concentration. Using 15 μL or 20 μL of crude oil, the 500 mg L⁻¹ rhamnolipid sample formed an oil spreading circle of 35.3 mm in diameter. The result demonstrated that water types, water amount, oil types, and oil amount all influenced the oil spreading method.

3.3 Effect of physico-chemical factors on oil spreading activity

Figure 1 shows the effect of physico-chemical factors on the oil spreading activity of 500 mg L⁻¹ rhamnolipid samples. A wide range of temperature (4–100 °C) has no difference on the diameter of oil spreading zone (Fig. 1a). Heating the rhamnolipid samples up to 100 °C caused no effect on the biosurfactant performance (the surface tension is still lower than 30 mN m⁻¹) and its oil spreading activity. In Fig. 1b, the oil spreading activity of rhamnolipid samples was affected by the pH. When the pH was too acidic or too alkaline, the oil spreading activity was limited (Fig. 1b). However, the oil spreading activity of rhamnolipid samples was relatively stable between pH 2 and pH 10 (Fig. 1b). Figure 1c shows the effect of salinity on the oil spreading activity of rhamnolipid samples. Negligible changes occurred in oil spreading activity with an increase in the NaCl concentration up to 15 %. However, at the highest level of NaCl concentration (20 %), the oil spreading activity was severely dropped to 72 %. Figure 1d demonstrated that the oil spreading activity decreased when metal ions were added into the rhamnolipid samples. However, negligible changes were occurred when Mg²⁺ and Al³⁺ were added into rhamnolipid samples.

Figure 2 illustrates the effect of hydrophilic carbon sources (glucose, glycerol) and hydrophobic carbon sources (soybean oil, olive oil) on the oil spreading activity. The 250 mg L⁻¹ rhamnolipid sample theoretically forms an oil spreading circle of 21.3 mm in diameter. Using hydrophilic carbon sources (glucose and glycerol), oil spreading circles of 20.3-mm in diameter were obtained. However, the diameters of formed oil spreading circles were more than 40 mm using the hydrophobic carbon sources (soybean oil and olive oil) (Figure 2). The results demonstrate that the hydrophilic carbon sources cause no effect on the oil spreading activity.

3.4 Effect of carbon sources and bacterial cells on oil spreading activity

Figure 2 illustrates the effect of hydrophilic carbon sources (glucose, glycerol) and hydrophobic carbon sources (soybean oil, olive oil) on the oil spreading activity. The 250 mg L⁻¹ rhamnolipid sample theoretically forms an oil spreading circle of 21.3 mm in diameter. Using hydrophilic carbon sources (glucose and glycerol), oil spreading circles of 20.3-mm in diameter were obtained. However, the diameters of formed oil spreading circles were more than 40 mm using the hydrophobic carbon sources (soybean oil and olive oil) (Figure 2). The results demonstrate that the hydrophilic carbon sources cause no effect on the oil spreading activity. While the hydrophobic carbon sources interfere and enhance the oil spreading activity due to the hydrophobic performance similar to crude oil. As shown in Fig. 3, bacterial
cells have no effect on oil spreading activity. Therefore, when using the oil spreading method to quantify rhamnolipid the bacterial cells need not to be removed by centrifugation of the cultures. However, with regard to samples containing hydrophobic organic substances (such as soybean oil, olive oil and crude oil), the hydrophobic organic substances need be removed before determination. The hydrophobic organic substances can be removed from cultures by filtration and centrifugation before determination.

3.5 Effect of the common substance in fermentation broth

Using the fermentation broth of *E. coli* DH5α as solvent, the 500 mg L⁻¹ rhamnolipid sample forms an oil spreading circle of 35.0 mm in diameter. The 500 mg L⁻¹ rhamnolipid sample forms theoretically an oil spreading circle of 36.7 mm in diameter. The result showed that the common substances (salts, proteins, lipids) in fermentation broth had little effect on oil spreading method.

3.6 Optimized oil spreading method

In the present study, the influencing factors of oil spreading method were investigated. An optimized oil spreading method was proposed: 15 µL of crude oil were added to the surface of 25 ml of distilled water in a petri dish (90 mm in diameter) to form a thin oil membrane. 10 µL of sample were then gently dropped onto the center of the oil membrane. The diameter of clearly formed oil displaced circle was measured. The rhamnolipid concentration was calculated from linear regression model prepared with rhamnolipid extract (100–800 mg L⁻¹). When hydrophobic carbon sources are used for fermentation, the hydrophobic organic

![Figure 1](image1.jpg) The effect of physico-chemical factors on oil spreading activity: temperature (a); pH (b); salinity (c); metal ions (d). The 500 mg L⁻¹ rhamnolipid sample theoretically forms an oil spreading circle of 35.0 mm in diameter. Error bars indicate the standard deviation of three independent measurements

![Figure 2](image2.jpg) Effect of carbon sources on oil spreading activity: Hydrophilic carbon sources (glucose, glycerol) and hydrophobic carbon sources (soybean oil, olive oil) were used. The 250 mg L⁻¹ rhamnolipid sample theoretically forms an oil spreading circle of 21.3 mm in diameter. Error bars indicate the standard deviation of three independent measurements

![Figure 3](image3.jpg) Effect of bacterial cells on oil spreading activity: Two strains *P. aeruginosa* SG and WJ-1 were used. Error bars indicate the standard deviation of three independent measurements

![Figure 4](image4.jpg)
substance need to be removed from bacteria cultures by filtration and centrifugation before determination.

3.7 Linear regression model of rhamnolipid quantification using oil spreading method

Rhamnolipid product of *P. aeruginosa* SG was extracted by chloroform/methanol (v/v, 2:1). Rhamnolipid solutions with concentration of 0–1000 mg L\(^{-1}\) were prepared. The diameter of oil spreading zone linearly increased with the concentration of rhamnolipid over a concentration range of 100–800 mg L\(^{-1}\) (Fig. 4). Youssef et al. reported the linear correlation between the diameters of the clear zone and the concentration of surfactin (50–400 mg L\(^{-1}\)) [25]. Rhamnolipid and surfactin may have a different surface activity. According to the data in Fig. 4, the following linear regression model was obtained by linear regression analysis:

\[
y = 0.0616x + 5.9524, \quad R^2 = 0.9908 \quad \text{(Eq. 1)}
\]

Where \(y\) is the diameter of oil spreading zone (mm); \(x\) is the rhamnolipid concentration (mg L\(^{-1}\)). The concentration of rhamnolipid less than the critical micelle concentration will not be quantified by the oil spreading method. Samples containing a high concentration of rhamnolipid need to be diluted to lower than 800 mg L\(^{-1}\).

The oil spreading method is based on the activity of surfactants. So the oil spreading method is not specific to rhamnolipids. However, the sort of biosurfactants in the fermentation broth is usually known before the analysis. The diameter of oil spreading circle formed by a surfactant-containing solution is directly proportional to the concentration of tested biosurfactants [25, 26]. Different compositions and molecular structures of rhamnolipid may have a different oil spreading activity. However, we can solve this problem by re-establishing the linear regression model between the diameters of formed oil spreading circle and the concentration of the tested rhamnolipid product. We attempted to determine lipopeptide biosurfactants produced by a *Bacillus amyloliquefaciens* strain using the oil spreading method. A very strong, positive, linear correlation between the diameters of clear zone and the concentrations of lipopeptide biosurfactants from 100 mg L\(^{-1}\) to 1000 mg L\(^{-1}\) \((R^2 = 0.9931)\) was found. Therefore, oil spreading method can also quantify other biosurfactants in culture.

The efficiently rhamnolipid producing bacteria are significant for microbial enhanced oil recovery or environmental remediation. To isolate and evaluate rhamnolipid-producing bacteria, a quantitative analysis of rhamnolipid is indispensable.

4 Conclusions

In the present study, a comparison of methods to quantify rhamnolipid was carried out. The oil spreading method was considered to be a better method with some advantages, such as easy operation, saving time, convenient and simple. Moreover, the oil spreading method is most valuable for the analysis of a large number of samples. No specialized equipments or chemicals were used in measurement procedure of oil spreading method. The potential influencing factors (temperature, pH, salinity, metals, bacterial cells, carbon sources) were investigated to oil spreading method. The common substance (such as salts, proteins, lipids) in fermentation broth has little effect on oil spreading method.
An optimized protocol to quantify rhamnolipid by oil spreading method was proposed.

References


7. Feng, Zhao et al.: Comparison of methods to quantify rhamnolipid and optimization of oil spreading method (mm) and the concentration of rhamnolipid (mg L$^{-1}$). Error bars indicate the standard deviation of three independent measurements. The solid line is the least square fit with an R$^2$ of 0.9098

Figure 4

An optimized protocol to quantify rhamnolipid by oil spreading method was proposed.

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