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Production of rhamnolipids with different proportions of mono-rhamnolipids using crude glycerol and a comparison of their application potential for oil recovery from oily sludge

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The use of efficient green cleaning agents, such as biosurfactants, is important in oil sludge treatment. Enhanced oil recovery from oily sludge by different rhamnolipids was comparatively evaluated. Using crude glycerol, the wild-type strain *Pseudomonas aeruginosa* SG and the recombinant strains *P. aeruginosa* PrhlAB and *P. stutzeri* Rhl produced 1.98 g L^{-1}, 2.87 g L^{-1} and 0.87 g L^{-1} of rhamnolipids, respectively. The three bacterial strains produced different rhamnolipid mixtures under the same conditions. The proportions of mono-rhamnolipids in the three rhamnolipid products were 55.92%, 94.92% and 100%, respectively. These rhamnolipid products also possessed different bioactivities. Emulsifying activity became higher as the proportion of mono-rhamnolipids increased. The three rhamnolipid products were stable at temperatures lower than 121 °C, pH values from 5–11 and NaCl concentrations from 0–15%. All three rhamnolipid products could recover oil from oily sludge, but oil recovery efficiency was positively related to the proportion of mono-rhamnolipids. Mono-rhamnolipids produced by the recombinant strain Rhl exhibited the best oil recovery efficiency (53.81%). The results reveal that mono-rhamnolipids are the most promising for oil recovery from oily sludge.

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Introduction

A large amount of oily sludge (solid waste containing oil) is produced in the process of oil production, transportation, refining and treatment of oily sewage.[1,2] Oily sludge has become a major source of soil oil pollution.[1] Oily sludge has been included in the national hazardous waste list in China. Oily sludge is the primary pollutant from the oil industry.[3] Although oily sludge is a solid waste product, it is also a high oil content resource.[4,5] How to recycle crude oil from sludge in an environmentally friendly and economically efficient way is significant to the treatment of oily sludge.

Surfactant-enhanced sludge cleaning is the focus of sludge treatment technology.[6,7] This mainly refers to the use of surfactant for desorption and emulsification of crude oil in oily sludge.[6] Then, separation of oil and sludge is completed under the action of an external force, such as centrifugal force. Surfactants are widely used and the consumption of surfactants is increasing. However, the residual chemical surfactants are not easily biodegraded.[8,9] So they may cause secondary pollution.[9] The consequent damage to the ecosystem has been paid more and more attention.

Compared with chemical surfactants, biosurfactants have good environmental compatibility, high surface activity and easy biodegradability.[10,11] The emulsifying activity of biosurfactant and the formation of micelles can disperse and solubilize petroleum hydrocarbons.[12] In oily sludge washing treatment, biosurfactants with high emulsification activity play a stronger role in solubilization of crude oil. The chemical structure of biosurfactants is diverse, which makes it possible to obtain biosurfactants with different activities.[13–15] Rhamnolipids are one of the most popular biosurfactants. Rhamnolipids are a series of homologues composed of rhamnoses and fatty acids.[16,17] Different rhamnolipid-producing bacterial strains afford rhamnolipid products with different structures.[18] The different structures of rhamnolipids may give quite different physico-chemical properties, such as emulsifying activity.[14,19]

In this study, three different rhamnolipid producers (*Pseudomonas aeruginosa* SG, *P. aeruginosa* PrhlAB, *P. stutzeri* Rhl) were used for rhamnolipid production. Crude glycerol was used as the low-cost substrate. Three different rhamnolipid products

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were extracted. The structural compositions and surface-active properties of the three rhamnolipids products were characterized. The rhamnolipids' stability against environmental stresses was evaluated at different temperatures, pH values and salinities. Further, the application potential of the three kinds of rhamnolipid products for oily sludge washing was also comparatively investigated.

Materials and methods

Strains

In this study, three different rhamnolipid producers (*Pseudomonas aeruginosa* SG, *P. aeruginosa* PrhlAB, *P. stutzeri* Rhl) were used for rhamnolipid production. Strain SG is the wild-type strain, which was isolated from production water of Xinjiang oil reservoir, China.[20] Strain PrhlAB is a recombinant strain derived from strain SG by increasing the copy numbers of the *rhlAB* genes.[21] Strain Rhl is also a recombinant strain, constructed by heterologous expression of *rhlABRI* genes in strain *P. stutzeri* DQ1.[22]

Medium and culture conditions

Luria–Bertani (LB) medium was used to prepare seed culture. The three strains were cultured at 37 °C and 200 rpm for 16 h. The medium used for rhamnolipid production contained 60 g L^{-1} crude glycerol, 3.4 g L^{-1} KH_2PO_4, 4.0 g L^{-1} $K_2HPO_4 \cdot 3H_2O$, 0.80 g L^{-1} $MgSO_4 \cdot 7H_2O$, 3.5 g L^{-1} $NaNO_3$, 0.50 g L^{-1} KCl, 0.05 g L^{-1} $CaCl_2$, 0.50 g L^{-1} NaCl. The pH of the medium was adjusted to 6.8 using 1 M NaOH. Crude glycerol was purchased from an agency company in Jinan, China. This crude glycerol contained 90% glycerol, some water, methanol, and esters. The other chemicals were analytical grade, and were purchased from Sinopharm Chemical Reagent Co., Ltd, China. The culture conditions for rhamnolipid production were 37 °C, 200 rpm for 5 days. The inoculum amount of seed culture was 3% (v/v). In cultivation experiments, 250 mL Erlenmeyer flasks containing 120 mL medium were used.

Rhamnolipid extraction

Cultures of the three strains were centrifuged at 10 000 rpm for 10 min. Cell-free culture was collected and heated at 80 °C for 30 min. The soluble protein in cell-free culture degenerated and precipitated. Treated cell-free culture was centrifuged at 10 000 rpm for 10 min again. The supernatant was collected. The pH of the supernatant was adjusted to 1.5 using 6 M HCl. The supernatant was placed at 4 °C for 16 h. Then the sample was centrifuged at 10 000 rpm for 10 min. The precipitate was collected and then dissolved in 0.1 M $NaHCO_3$. The rhamnolipid product in $NaHCO_3$ solution was extracted using chloroform/methanol (v/v, 2 : 1). The extraction solution was dried by vacuum rotary evaporation (65 °C, 50 rpm). The obtained yellow solid substance was the rhamnolipid product.

Quantitative analysis by oil-spreading method

Rhamnolipid concentrations in the three bacterial cultures were quantified by the oil-spreading method.[23] The three rhamnolipid products were respectively dissolved in distilled water. Rhamnolipids-water solutions were prepared with different concentrations (100, 200, 300, 400, 500, 600, 700 and 800 mg L^{-1}). The oil-spreading circle diameters of the rhamnolipid solutions were measured as previously described.[23] Standard curves of oil-spreading circle diameters and rhamnolipid concentrations were prepared. Linear correlations were established between the oil-spreading circle diameters and rhamnolipid concentrations. The oil-spreading circle diameters of the bacterial cultures were measured. The rhamnolipid concentrations in the three bacteria cultures were calculated using the related standard curves.

Qualitative analysis by TLC and FTIR

Thin-layer chromatography (TLC) analysis was performed according to previous studies.[20,22] The three rhamnolipid products were respectively dissolved in methanol to a concentration of 200 mg L^{-1}. Then, 10 μL of sample was spotted on silica gel G plates (Qingdao Marine Chemical Factory, Qingdao, China). Chloroform/methanol/distilled water (90 : 25 : 2, v/v/v) was used as developing solvent. The silica gel G plates were finally visualized by sulfuric acid–phenol reagent (concentrated H_2SO_4, 80% phenol solution) at 95 °C for 10 min.

Fourier transform infrared spectroscopy (FTIR) analysis was also used to identify the functional groups of the rhamnolipids.[20,22] A NICOLET380 FTIR spectrometer (Thermo Electron Corporation, USA) was used. The resolution was 0.5 cm^{-1}. FTIR spectra were collected at wave numbers between 400 cm^{-1} and 4000 cm^{-1}. Solid rhamnolipid product (10 mg) was mixed with spectral purity KBr (100 mg). Then a translucent disc was made at 25 Mpa for 30 s.

Structural composition analysis by HPLC-MS

The three rhamnolipid products were respectively dissolved in 10% acetonitrile–water to a concentration of 500 mg L^{-1}. High-pressure liquid chromatography-mass spectrometry (HPLC-MS) analysis was carried out according to previous studies.[24,25] A liquid chromatography-mass spectrometer (Waters, Milford Massachusetts, USA) equipped with a reversed-phase C18 column (ø 2 mm × 150 mm × 0.5 μm) was used. The injection sample volume was 20 μL. The mobile phase was acetonitrile-water with gradient from 10% to 60%. Mass spectrum scanning mass number ranged from 50 m/z to 800 m/z. Rhamnolipid congeners were identified based on m/z.[24] Their relative proportions were calculated using the area normalization method.

Bioactivity and stability analysis

Three rhamnolipid products were respectively dissolved in distilled water to a concentration of 200 mg L^{-1}. The rhamnolipid surface activity, emulsifying activity and stability against environmental stresses were evaluated. Surface tension was measured at 30 °C using a surface tension meter BZY-1 (Shanghai Hengping Instrument and Meter Factory, Shanghai, China). The emulsifying activity was measured as described previously.[20] Crude oil sampled from the Xinjiang oil field was

used. The emulsion index (EI_{24}) (%) is defined as the height of the oil layer (mm) divided by the total height of the mixture (mm) and multiplied by 100.[20] Stability of rhamnolipids was evaluated under diverse environmental conditions. Rhamnolipid solutions were treated at different temperatures (50 °C, 80 °C, 100 °C and 121 °C), pH values (2, 4, 6, 8,10, 11 and 12) and NaCl concentrations (0%, 3%, 6%, 9%, 12%, 15%, 18%, 21% and 25%) for 30 min. The surface tension and EI_{24} were measured to evaluate the rhamnolipids' stability against environmental stresses.

The critical micelle concentration (CMC) of the rhamnolipid products was also measured. Rhamnolipid solutions with concentrations ranging from 0 to 120 mg L^{-1} were prepared. The surface tension of the solutions was measured. Curves of surface tension against rhamnolipid concentration were prepared. The concentration at the inflection point of the surface tension curve is the CMC of rhamnolipids.

Oily sludge washing experiments

The three rhamnolipid products were investigated for their capacity to remove oil from oily sludge. Experiments and analyses were performed as previously described.[26] The oily sludge was sampled from an onshore oilfield in the northwest of China. It contained 13.66% total petroleum hydrocarbons (TPH). Using the extracted rhamnolipids and water, three kinds of rhamnolipid solutions (200 mg L^{-1}) were prepared. In Erlenmeyer flasks, 10 g oily sludge was mixed with 100 mL rhamnolipid solution. Distilled water was used as negative control. The Erlenmeyer flasks were shaken at 180 rpm and 60 °C for 24 h to wash the oily sludge. Then, samples were centrifuged at 5000*g* for 10 min to separate the oil, water and oil sludge. The washed oil sludge samples were collected. Using tetrachloromethane, TPH in the oily sludge samples were extracted. The extraction liquid was collected and naturally dried at room temperature (28 °C) in 90 mm plates. The weight of TPH was calculated, named as A. The weight of washed-out oil was the initial TPH amount in 10 g oily sludge (1.366 g) minus A. The washing efficiency (%) was defined as the weight of washed-out oil divided by the initial TPH amount (1.366 g) multiplied by 100.

Results and discussion

Rhamnolipid production by the three strains using crude glycerol

As shown in Fig. 1, all three strains can efficiently produce rhamnolipids using crude glycerol, decreasing the surface tension of the culture from 64 mN m^{-1} to 26 mN m^{-1}. Using crude glycerol, strain SG produced 1.98 g L^{-1} of rhamnolipids (Fig. 1A), strain PrhlAB produced 2.87 g L^{-1} of rhamnolipids (Fig. 1B), and strain Rhl produced 0.87 g L^{-1} of rhamnolipids (Fig. 1C). The results demonstrated that crude glycerol can be used as a good carbon source by different rhamnolipid-producing strains.

The rhamnolipid concentrations in bacterial culture were determined by the oil-spreading method.[23] The linear correlations for quantification of rhamnolipids produced by strain SG, PrhlAB and Rhl were as follows: $y = 0.0624x + 5.4286$, $R^2 = 0.9908$; $y = 0.0609x + 5.9643$, $R^2 = 0.9879$; $y = 0.0602x + 6.7857$, $R^2 = 0.9906$. In the linear correlations, y (mm) is the diameter of the oil-spreading circle, and x (mg L^{-1}) is rhamnolipid concentrations in the bacterial culture. Values of x are between 100 mg L^{-1} and 800 mg L^{-1}. The linear correlations for quantification of three rhamnolipids were different, which indicated different bioactivity of the three rhamnolipid

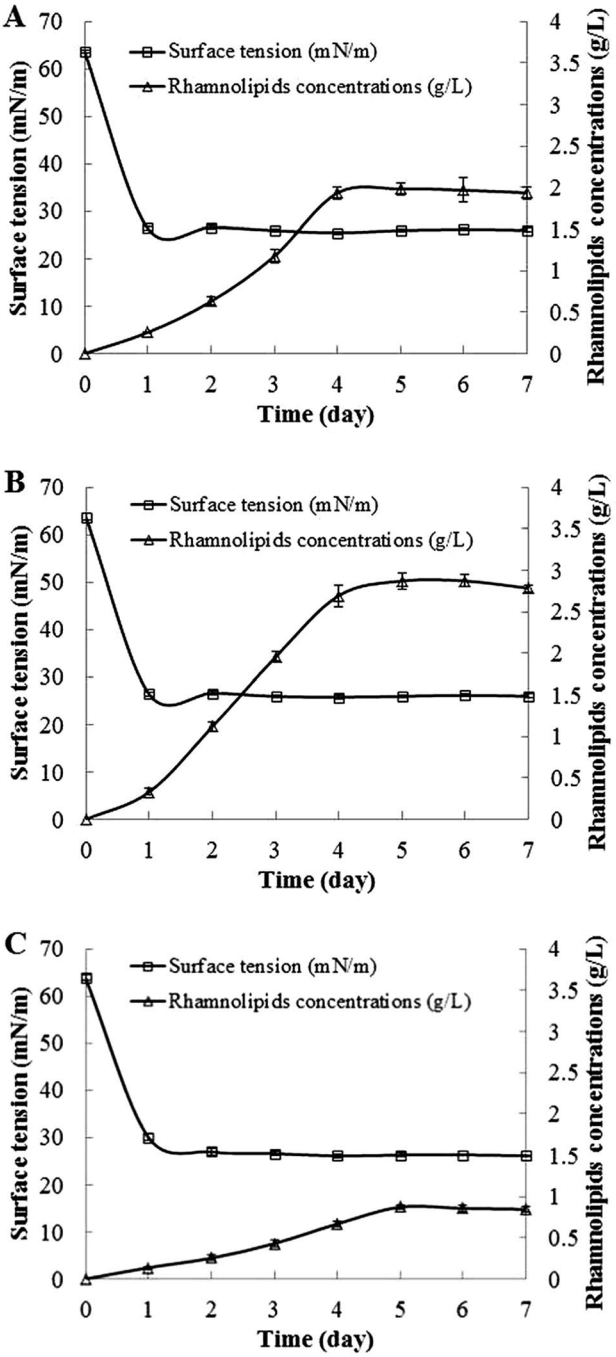

Fig. 1 Rhamnolipid production by strains SG (A), PrhlAB (B) and Rhl (C) grown on crude glycerol.

compositions, such as different emulsifying activity.[23] So the structural compositions of the three rhamnolipids might be different. Rhamnolipids are a series of congeners composed of rhamnose and fatty acid.[14,16] Previous studies reported that different strains produced rhamnolipids with different compositions.[14,24,27] The industrial waste (crude glycerol) would be a low-cost substrate for producing different rhamnolipids products.

TLC and FTIR analysis of the three rhamnolipid products

All three biosurfactant samples formed yellow spots on TLC silica plates when stained with sulfuric acid–phenol reagent. TLC results showed that all three rhamnolipids contained reducing carbohydrates. The FTIR spectra of the three rhamnolipid products are shown in Fig. 2. The absorption bands around 2928 cm^{-1}, 2857 cm^{-1} and 1457 cm^{-1} were caused by the stretching vibrations of C–H in aliphatic groups. The absorption band at 1731 cm^{-1} was caused by ester groups. These absorption bands were characteristic of rhamnolipids. These FTIR spectra are similar to the spectra of previously reported rhamnolipids.[20,28] The TLC and FTIR analysis results confirmed that the three strains produced rhamnolipids using crude glycerol.

Structural compositions of the three rhamnolipids products

The liquid chromatogram results of the three rhamnolipids products are shown in Fig. 3. Because crude extracts of rhamnolipids products were used, some impurity peaks occurred in the chromatograms. According to Déziel's analysis methods,[24] rhamnolipids produced by strain SG (Fig. 3A), PrhlAB (Fig. 3B) and Rhl (Fig. 3C) contained 8, 5 and 5 rhamnolipid congeners, respectively. As shown in Table 1, the rhamnolipid product of strain SG contained three mono-rhamnolipid and five di-rhamnolipid congeners; the rhamnolipid product of strain PrhlAB contained four mono-rhamnolipid and one di-rhamnolipid congeners; the five rhamnolipid congeners produced by strain Rhl were all mono-rhamnolipids. HPLC results confirmed that the three bacterial strains produced rhamnolipids using crude glycerol. Moreover, the structural compositions of three rhamnolipid products were quite different. The proportions of mono-rhamnolipids in the rhamnolipid products of strains SG, PrhlAB and Rhl were 55.92%, 94.92% and 100%, respectively.

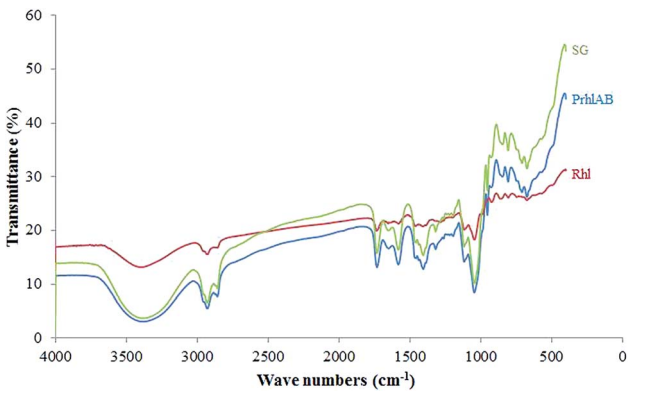

Fig. 2 Fourier transform infrared (FTIR) spectroscopy analysis of the three rhamnolipid products from strains SG, PrhlAB and Rhl.

Emulsifying activity of the three rhamnolipid products

As shown in Fig. 4, all three rhamnolipid solutions (200 mg L^{-1}) emulsified crude oil with EI_{24} >60%. Rhamnolipids produced by recombinant strain Rhl exhibited best emulsifying activity for crude oil, with EI_{24} = 83.3%. Only *rhlAB* genes were introduced into a non-rhamnolipid-producing strain DQ1 to construct recombinant strain Rhl.[22] Rhamnolipids produced by recombinant strain Rhl were all mono-rhamnolipids. Mono-rhamnolipids contain only one rhamnose. Di-rhamnolipids contain two rhamnoses. Mono-rhamnolipids are less hydrophilic relatively lipophilic than di-rhamnolipids.[25] So mono-rhamnolipids have better emulsifying activity, whereas di-rhamnolipids have better surface activity.[25] In this study, the emulsifying activity for crude oil is positively related to the proportion of mono-rhamnolipids in the rhamnolipid products. Good emulsifying activity of biosurfactant is promising for bioremediation of hydrophobic pollutants.[29,30]

Surface activity of the three rhamnolipid products

As shown in Fig. 5, the surface tension first decreased with the increase of rhamnolipid concentrations. Then the surface tension remained constant with the increase of rhamnolipid concentrations. Rhamnolipids produced by wild-type strain SG decreased the water surface tension to 27.2 mN m^{-1}. The critical micelle concentration (CMC) was 60 mg L^{-1} (Fig. 5A). Rhamnolipids produced by recombinant strain PrhlAB and strain Rhl also decreased the water surface tension to lower than 30.0 mN m^{-1}. The CMCs of rhamnolipid mixtures produced by strain PrhlAB and strain Rhl were 80 mg L^{-1} (Fig. 5B) and 90 mg L^{-1} (Fig. 5C). Other studies have reported that the CMCs of rhamnolipids are in the range 40–150 mg L^{-1}.[14,31] Sodium dodecyl sulfate (SDS) is a commonly used synthetic surfactant. The CMC of SDS is 2100 mg L^{-1}.[32,33] Compared with synthetic surfactants, the three rhamnolipid products are highly excellent.

The hydrophilic moiety of di-rhamnolipids contains two rhamnoses. Di-rhamnolipids exhibit are more hydrophilic than mono-rhamnolipids. Previous studies also reported that di-rhamnolipids had stronger surface activity than mono-rhamnolipids.[25,34] Rhamnolipids produced by strain SG contained the most abundant di-rhamnolipids. So the rhamnolipids produced by strain SG exhibited the best surface activity, such as lowest CMC and surface tension. Oher studies have also reported that mono-rhamnolipids, containing only one rhamnose (hydrophilic moiety), are less soluble and adsorb to surfaces more strongly, so they have higher CMC for hydrocarbon solubilization than di-rhamnolipids.[35] The rhamnolipids produced by strain Rhl were all mono-rhamnolipids. So this product showed highest CMC and surface tension value.

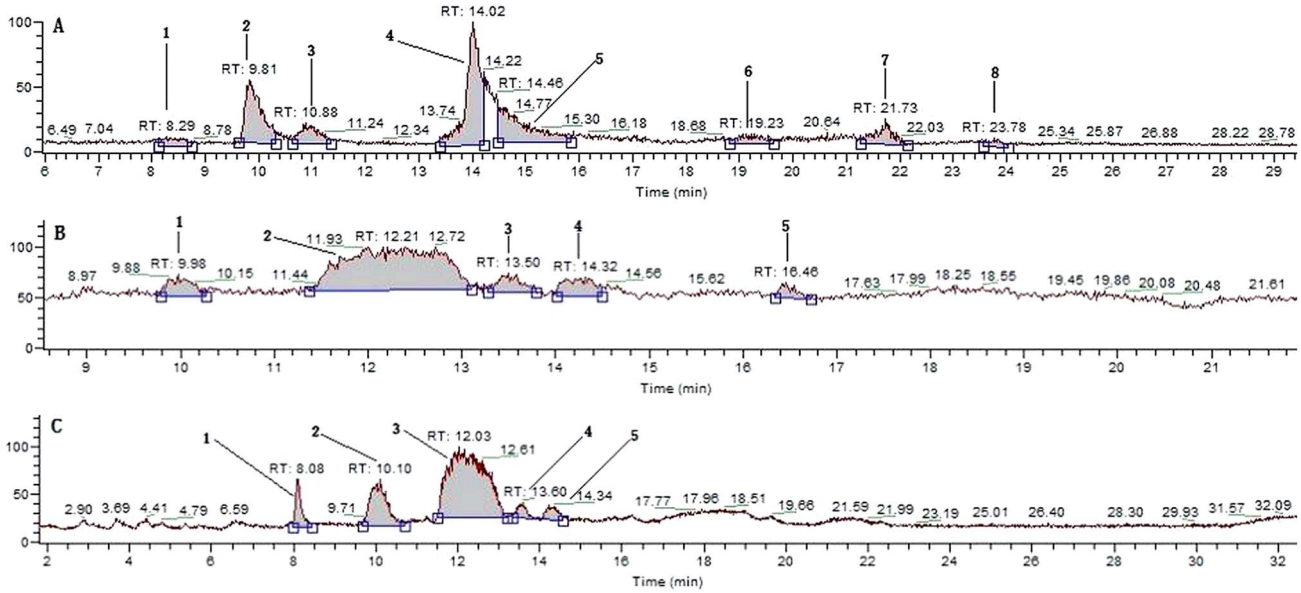

Fig. 3 Liquid chromatogram results for the three rhamnolipid products: (A) rhamnolipids produced by strain SG, (B) rhamnolipids produced by strain PrhlAB, (C) rhamnolipids produced by strain Rhl.

Stability of the three rhamnolipid products

The three rhamnolipids retained their same properties after treating at 121 °C for 30 min, which indicated that the three rhamnolipids were thermostable. In the presence of 15% of NaCl, all three rhamnolipids solutions had surface tension lower than 30.0 mN m^{-1} and emulsified crude oil with EI_{24} higher than 60.0%. These results revealed that the three rhamnolipids were salt-tolerant. After treatment with different pH values in the range 4–10, all three rhamnolipids could decrease the surface tension to lower than 35.0 mN m^{-1} and emulsify crude oil with EI_{24} higher than 60.0%. The three rhamnolipids are stable at temperatures lower than 121 °C, pH values 4–10 and salinities lower than 15% NaCl. Therefore, all three rhamnolipids can potentially be used in complex and extreme environments, such as oil reservoirs and petroleum-contaminated soil.

Enhanced oil removal from oily sludge by the three rhamnolipid products

The initial total petroleum hydrocarbons (TPH) amount in oily sludge is 13.66%. After washing with rhamnolipids of strains SG, PrhlAB and Rhl, the TPH amounts in the washed oily sludge samples were 9.47%, 7.11% and 6.31%, respectively. High concentrations of petroleum hydrocarbons in soil are toxic to

Table 1 Structural composition of rhamnolipids produced by strains SG, PrhlAB and Rhl

Strain	Chromatographic peak number	Retention time (min)	Mass spectrum signal (m/z)	Rhamnolipid homologue	Relative abundance (%)
SG	1	8.29	447	Rha-C_8-C_8	4.24
	2	9.81	475	Rha-C_8-C_{10}	19.47
	3	10.88	621	Rha-Rha-C_8-C_{10}	7.77
	4	14.02	503	Rha-C_{10}-C_{10}	32.22
	5	14.46	649	Rha-Rha-C_{10}-C_{10}	21.25
	6	19.23	677	Rha-Rha-C_{10}-C_{12}	4.71
	7	21.73	675	Rha-Rha-C_{10}-$C_{12:1}$	7.85
	8	23.78	705	Rha-Rha-C_{12}-C_{12}	2.50
PrhlAB	1	9.98	475	Rha-C_8-C_{10}	9.50
	2	12.21	503	Rha-C_{10}-C_{10}	64.58
	3	13.50	529	Rha-C_{10}-$C_{12:1}$	11.02
	4	14.32	531	Rha-C_{10}-C_{12}	9.83
	5	16.46	649	Rha-Rha-C_{10}-C_{10}	5.08
Rhl	1	8.08	447	Rha-C_8-C_8	6.46
	2	10.10	475	Rha-C_8-C_{10}	15.84
	3	12.03	503	Rha-C_{10}-C_{10}	68.55
	4	13.60	529	Rha-C_{10}-$C_{12:1}$	4.56
	5	14.34	531	Rha-C_{10}-C_{12}	4.58

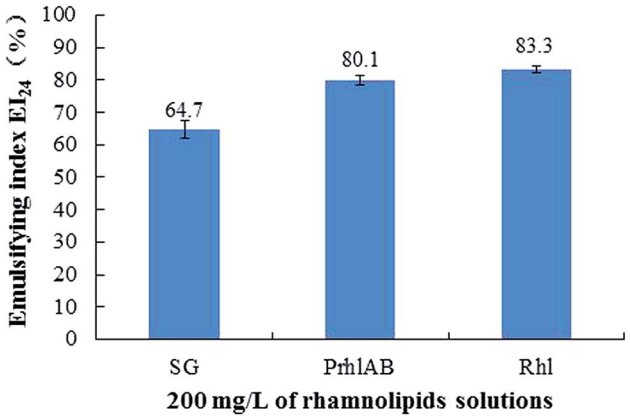

Fig. 4 Emulsifying activity of the three rhamnolipid products from strains SG, PrhlAB and Rhl.

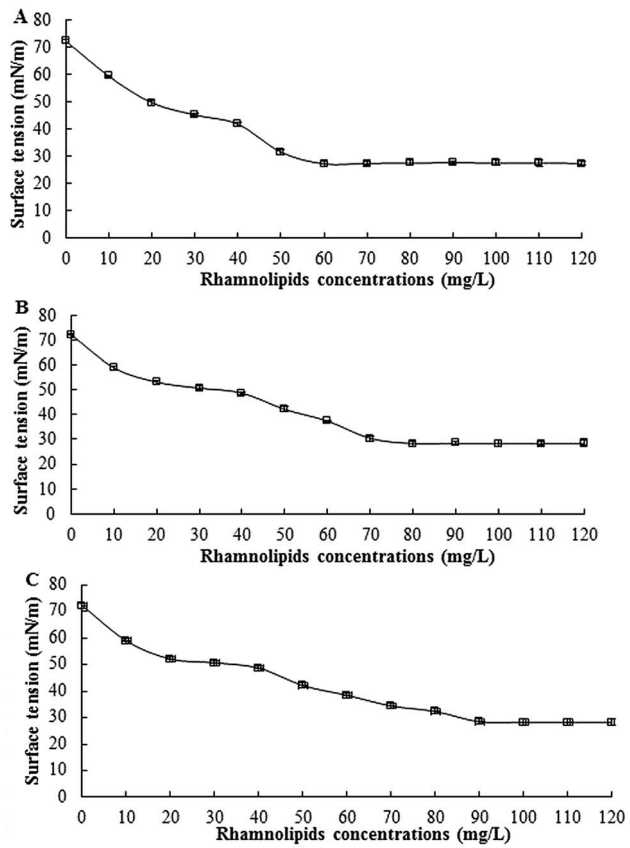

Fig. 5 Critical micelle concentration determination based on relationship graphs between surface tension and rhamnolipid concentrations: (A) rhamnolipids produced by strain SG, (B) rhamnolipids produced by strain PrhlAB, (C) rhamnolipids produced by strain Rhl.

microbes.[36,37] The rhamnolipid washing process significantly reduced the TPH amount in oily sludge, which was beneficial to microbial remediation of oily sludge. The washing efficiency of the three rhamnolipid products from strains SG, PrhlAB and Rhl were 30.67%, 47.95% and 53.81%, respectively. The washing efficiency of distilled water was 8.20%. Compared with distilled water, all three rhamnolipid solutions (200 mg L^{-1}) efficiently removed TPH from oily sludge. Previous studies reported that biosurfactants are excellent agents for oily sludge washing.[38–40] Another study also showed that biosurfactants (200 mg L^{-1}) produced by a *Bacillus subtilis* strain removed 62% of TPH from contaminated soil.[26] In the present study, mono-rhamnolipids produced by recombinant strain Rhl exhibited the best washing efficiency for oily sludge. Mono-rhamnolipids had better emulsifying activity. The results indicated that mono-rhamnolipids are excellent agents for oily sludge washing.

Perspectives

A previous study showed that glycerol, which has good solubility in water, can be easily absorbed and metabolized by microorganisms.[22] Moreover, crude glycerol is the main by-product of biodiesel and saponification processess.[41,42] In this study, crude glycerol was used as a cheap carbon source for rhamnolipid production by different strains. Therefore, crude glycerol is a promising and inexpensive carbon source for rhamnolipid production. Rhamnolipids are a mixture, consisting of one or two rhamnoses (hydrophilic moiety) and one or two β-hydroxy fatty acids with different lengths (hydrophobic moiety).[16] Different bacterial strains, culture media and cultivation conditions produce rhamnolipid mixtures with different types and proportions of congeners.[18,43] Their physico-chemical properties and applications depend on the structural compositions of the rhamnolipid mixtures.[14] In this study, the emulsifying activity for crude oil is positively related to the proportion of mono-rhamnolipids in the rhamnolipid products. Good emulsifying activity of biosurfactant is promising for bioremediation of hydrophobic pollutants.[29,30] In this study, mono-rhamnolipids exhibited the best washing efficiency for oily sludge. Therefore, future research should concentrate on ways to enhance mono-rhamnolipid production, such as biosynthesis pathway regulation and medium optimization.

Conclusions

Industrial waste (crude glycerol) can be used for rhamnolipid production using three different bacterial strains: *Pseudomonas aeruginosa* SG, PrhlAB and Rhl. The three strains produce different rhamnolipid mixtures. The proportions of mono-rhamnolipids in the rhamnolipid products from strains SG, PrhlAB and Rhl are 55.92%, 94.92% and 100%, respectively. The three rhamnolipid products are thermostable and salt-tolerant. The emulsifying activity for crude oil is positively related to the proportion of mono-rhamnolipids in the rhamnolipid products. The surface activity is positively related to the proportion of di-rhamnolipids. All three rhamnolipid products efficiently removed TPH from oily sludge. Mono-rhamnolipids exhibited the best washing efficiency for oily sludge.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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