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Optimization of biodemulsifier production from *Alcaligenes* sp. S-XJ-1 and its application in breaking crude oil emulsion

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ABSTRACT

A biodemulsifier-producing strain of *Alcaligenes* sp. S-XJ-1, isolated from petroleum-contaminated soil of the Karamay Oilfield, exhibited excellent demulsifying ability. The application of this biodemulsifier significantly improved the quality of separated water compared with the chemical demulsifier, polyether, which clearly indicates that it has potential applications in the crude oil extraction industry. To optimize its biosynthesis, the impacts of carbon sources, nitrogen sources and pH were studied in detail. Paraffin, a hydrophobic carbon source, favored the synthesis of this cell wall associated biodemulsifier. The nitrogen source ammonium citrate stimulated the production and demulsifying performance of the biodemulsifier. An alkaline environment (pH 9.5) of the initial culture medium favored the strain's growth and improved its demulsifying ability. The results showed paraffin, ammonium citrate and pH had significant effects on the production of the biodemulsifier. These three variables were further investigated using a response surface methodology based on a central composite design to optimize the biodemulsifier yield. The optimal yield conditions were found at a paraffin concentration of 4.01%, an ammonium citrate concentration of 8.08 g/L and a pH of 9.35. Under optimal conditions, the biodemulsifier yield from *Alcaligenes* sp. S-XJ-1 was increased to 3.42 g/L.

1. Introduction

Every year, millions of tons of crude oil emulsions are generated by petroleum exploration [1]. Before being transported and refined, crude oil emulsions should be demulsified to reduce water content and recover crude oil because water and waterborne impurities present in the emulsion are corrosive to pipelines and containers [2]. Moreover, excess water in the emulsion also increases transportation costs, as it increases the total volume to be transported. As a consequence of demulsification, a large amount of separated water is produced, which is either recharged into the stratum or discharged to the ambient environment [3]. Currently, chemical demulsifiers are widely used to break crude oil emulsions. However, information on the effects of the demulsifier on the quality of separated water from the demulsification process is scarcely seen in the literature.

Since the early 1980s, biodemulsifiers have attracted increasing attention in research [4,5]. As a type of biosurfactants, biodemulsifiers are known for their high efficiencies in breaking water–oil emulsions. Compared with conventional chemical demulsifiers, biodemulsifiers are characterized by the following traits: low toxicity, environmental compatibility and high demulsifying efficiency under extreme conditions. However, studies on biodemulsifiers lag far behind research on other biosurfactants and remain in a preliminary stage. Most studies focus only on the screening of biodemulsifier-producing bacteria and evaluation of demulsification performance. Moreover, model kerosene–water emulsions are generally used in these studies, while crude oil emulsions are seldom seen. So it is still doubtful whether similar performance will be observed in the demulsification of crude oil emulsions.

In addition, cost-effective mass production of biodemulsifiers is a crucial issue in enabling its application. Biosurfactants (including biodemulsifiers) are synthesized by microorganisms under specific cultivation conditions [6,7]. The carbon and nitrogen sources are crucial components of culture media that have profound impacts on the production of biosurfactants [8]. Some recent studies on biosurfactant production found that biosurfactants produced from hydrophobic carbon sources were superior to those produced from hydrophilic carbon sources in terms of surface activity and productivity [9]. On the other hand, others found that when hydrophilic carbon sources were used, the extraction of biosurfactants could be simplified and therefore the production cost would be substantially reduced [10]. With respect to nitrogen sources, ammonium nitrate was proved to be the optimal nitrogen source in the production of lipopeptide [11] and rhamnolipid biosurfactant [12]. However, some studies showed that biosurfactants produced from

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nitrate nitrogen sources exhibited higher surface activities than those using ammonium nitrogen sources [11,13]. Thus it remains unknown how the production of biodemulsifiers will be affected by changes in carbon or nitrogen sources. Moreover, the optimization of cultivation conditions to increase biodemulsifier yield has not been studied for biodemulsifier-producing bacteria, other than for a few biosurfactant-producing bacteria.

The objective of this work was to optimize the cultivation conditions with respect to biodemulsifier yield for its application in the destabilization of crude oil emulsion. Demulsifying performance and the quality of separated water produced by a chemical demulsifier and the biodemulsifier were compared. To optimize production of the biodemulsifier, the influences of carbon sources, nitrogen sources and pH were studied in detail. Finally, the optimum conditions for biodemulsifier yield were identified using a central composite design (CCD) of response surface methodology (RSM).

2. Methods and materials

2.1. Microorganism and growth conditions

The tested strain of *Alcaligenes* sp. S-XJ-1 was isolated from petroleum-contaminated soil [14] and was kept at −4 °C on agar slant plate. It was inoculated in 100 mL of nutrient broth medium for enrichment, which consisted of 5.0 g/L beef extract, 10.0 g/L peptone and 5.0 g/L NaCl (pH 7.0). After the enrichment for 72 h, 10 mL of the fermented broth was transferred into 100 mL of modified mineral salts medium (MMSM) containing 4% (v/v) paraffin as a carbon source for another 7-day cultivation. The fermented broth was then tested for surface tension (ST), microbial adhesion to the hydrocarbon (MATH) and demulsifying ability. The composition of MMSM was (L^{-1}): NH_4NO_3 4.0 g, K_2HPO_4 4.0 g, KH_2PO_4 6.0 g, $MgSO_4{\cdot}7H_2O$ 0.2 g and trace mineral solution 1 mL. Trace mineral contained (L^{-1}) $CaCl_2{\cdot}2H_2O$ 1.0 g, $FeSO_4{\cdot}7H_2O$ 1.0 g and EDTA 1.4 g.

Unless otherwise mentioned, liquid cultures (100 mL) were incubated in flasks (250 mL) at 35 °C on a rotary shaker (DKY-II, Shanghai Duke Automation Equipment Company, China) at 140 rpm for 7 days.

2.2. Comparison of water produced by the demulsification processes

A series of bench tests were carried out to simulate the production and treatment of separated water from the demulsification process in oilfields. In a bench-scale demulsification test of crude oil emulsion, the biodemulsifier (produced by *Alcaligenes* sp. S-XJ-1) and the chemical demulsifier (polyether) were compared. The polyether was obtained from Karamay Oilfield, which was purchased from Karamay Xinkeao Chemical Co. Ltd. (China). The mixture of demulsifier and emulsion was vigorously stirred at 900 rpm for 4 min to achieve complete mixing status and then allowed to separate under stagnant conditions for 150 min in a 35 °C water bath. The separated water was then siphoned out and treated in bench tests of coagulation, sedimentation and filtration. The optimal dosage of coagulants was evaluated by a series of jar tests. In the coagulation test, 250 mg/L of polyaluminium chloride was added, followed by fast stirring at 200 rpm for 1 min. Three milligrams per liter of polyacrylamide was then added, followed by slow agitation at 80 rpm for 5 min and then by 30 min of static settling. The supernatant was then filtered through filter paper to stimulate the large-scale filtration process.

All parameters were analysed according to standard methods (APHA). To measure soluble COD, each sample was filtered through 0.45 μm filter paper, prior to open reflux digestion and titration. Suspended solids and oil content were measured using the gravimetric method.

2.3. Effect of carbon source and nitrogen source

To study the effects of carbon sources on the production of the biodemulsifier, *Alcaligenes* sp. S-XJ-1 was cultured on MMSM supplemented with 4% (w/v) of various hydrophilic carbon sources (including lactose, sucrose, starch, sodium acetate and sodium citrate) or 4% (v/v) of various hydrophobic carbon sources (including peanut oil, sunflower oil, rapeseed oil, palm butter, sesame oil, crude coconut oil and soy bean oil). In the study of the effects of nitrogen sources, 4% (v/v) liquid paraffin was used as the sole carbon source, while ammonium nitrate in MMSM was replaced by ammonium chloride, ammonium sulfate, ammonium molybdate, ammonium citrate, potassium nitrate, sodium nitrate or urea, separately, at the same nitrogen concentration (0.14%, w/v). Yeast powder was added to the medium at the same concentration as ammonium nitrate (0.4%, w/v).

The modified culture media were all adjusted to pH 7 before the cultivation.

2.4. Effect of temperature and pH

The cultivation was carried out in a temperature-controlled rotary shaker (DKY-II, Shanghai Duke Automation Equipment Company, China) to study the effects of cultivation temperature. The cultivation temperatures were set at 20, 30, 35, 40 and 50 °C. To study the effect of pH, the initial pH values of the culture media were adjusted to 5, 6, 7, 8 and 9.5 with 6 mol/L of HCl and 2 mol/L NaOH.

2.5. Measurement of surface tension (ST)

The surface tension of fermented broth was measured with a Du Nouy ring tensiometer (DT-102, Zibo Huakun Electrical Equipment Limited Company, China) according to the procedure described by Bodour and Miller-Maier [15]. Each reported observation was expressed in terms of mean ± standard deviation of triplicate measurements. The surface tensions of sterilized medium and distilled water were 70.1 ± 0.2 mN/m and 72.0 ± 0.2 mN/m, respectively.

2.6. Measurement of microbial adhesion to the hydrocarbon (MATH)

The microbial adhesion to the hydrocarbon (MATH) was used to denote the cell surface hydrophobicity of the microbial species. It was measured according to the protocol suggested by Thavasi et al. [16]. On the 7th day of cultivation, cells harvested by centrifugation were rinsed with phosphorus buffer (pH 7.0) twice and then diluted to an initial optical density measured at 580 nm (initial OD_{580}) of around 0.8–1.0. Four milliliter of this cell suspension was then vortex-mixed with 4 mL of kerosene at 1800 rpm for 3 min. The mixture was left undisturbed for 20 min and then the final OD_{580} of the aqueous phase was measured. MATH was calculated as follows:

$$\text{MATH} = \left[\frac{1 - \text{OD}_{580(\text{final})}}{\text{OD}_{580(\text{initial})}}\right] \times 100\%$$

This value varies from 0 to 1. A higher value indicates higher cell surface hydrophobicity.

2.7. Determination of biodemulsifier yield

Fermented broth was centrifuged for 10 min at 10,000 rpm. The obtained cell pellets were thoroughly cleansed with distilled water

Table 1
Experimental ranges and levels of the independent variables for central composite design used in the medium optimization of biodemulsifier produced by *Alcaligenes* sp. S-XJ-1.

Variables	Code	Range and levels				
		−1.682	−1	0	+1	+1.682
Paraffin (%)	X_1	0.64	2.00	4.00	6.00	7.36
Ammonium citrate concentration (g/L)	X_2	4.64	6.00	8.00	10.00	11.36
pH	X_3	6.48	7.50	9.00	10.50	11.50

and then dried at 105 °C for 24 h. The dried powder product was weighed and calculated to identify the biodemulsifier yield.

2.8. Evaluation of demulsification performance

A crude oil emulsion was obtained from the Karamay Oilfield and stored at 4 °C. During long-time storage, oil and water were separated. Fresh crude oil emulsion was prepared by mixing the separated oil and water at 1:1 ratio (v/v) with a digital mechanical mixer (S312-40, Shanghai Jing Sheng Scientific Instrument Limited Company, China) at 900 rpm for 15 min. The emulsion was identified as W/O type by the dilution method. The prepared emulsion was very stable, showing no evidence of water separation within 24 h at 35 °C. In a demulsification test, 2 mL of fermented broth or cell suspension was added to a 20 mL graduated test tube containing 18 mL of the crude oil emulsion. The test tube was manually inverted 200 times to achieve complete mixing. The tubes were then left undisturbed in the water bath at 35 °C. The change in volume of the water phase was recorded at certain intervals. Demulsifying performance was evaluated by calculating the demulsification ratio as shown below.

$$\text{Demulsification ratio} = \frac{\text{water volume (on the bottom)}}{\text{water volume in original emulsion} + \text{added culture volume}} \times 100\%$$

Demulsifying speed was assessed by emulsion half-life ($t_{1/2}$), which was the reaction time when 50% of the demulsification ratio was achieved [17]. The demulsification ratio of the blanks (by dosing 2 mL of sterilized medium) was 0% within 24 h.

Fermented broth was dosed as biodemulsifier in demulsification tests except when comparing two demulsifiers or evaluating the pH effect. To compare the demulsifying ability of two demulsifiers, cell pellet biodemulsifier suspension and polyether solution were made in the same concentration. Cell pellet suspension was prepared by suspending dried cell pellet in distilled water to achieve certain concentration. Before dosing, the samples were ultrasonically vibrated for 1 min to make the dried cell pellet fully dispersed in the solution. When studying the pH effect, a cell pellet suspension was prepared by diluting wet cells harvested by centrifuge into distilled water immediately before the test.

2.9. Experimental design and optimization of the biodemulsifier yield

Optimum conditions for biodemulsifier yield by *Alcaligenes* sp. S-XJ-1 were determined by CCD and RSM. This method is suitable for fitting a quadratic surface and helps to optimize the effective parameters with a minimum number of experiments. The ranges and the levels of the variables investigated are given in Table 1. The variables studied were paraffin (X_1), ammonium citrate concentration (X_2) and initial pH (X_3), based on the results obtained for the effects of cultivation conditions on biodemulsifier yield. The number of experimental runs in the CCD for the three variables consisted of 8 factorial points, 6 axial points and 6 replicates at the centre points, indicating a total of 20 experiments were required. The independent variables were coded to the (−1, 1) interval, in which the low and high levels were coded as −1 and +1, respectively. The axial points were located at (±1.682, 0, 0), (0, ±1.682, 0), (0, 0, ±1.682), where 1.682 was the distance of the axial point from the centre and makes the design rotatable. The three factors, five levels CCD experimental design and the results obtained are presented in Table 2. For the evaluation of experimental data, the

Table 2
Experimental design matrix and experimental responses in the medium optimization of biodemulsifier produced by *Alcaligenes* sp. S-XJ-1.

Run	Coded values			Real values			Biodemulsifier yield (g/L)
	X_1	X_2	X_3	Paraffin (%)	Ammonium citrate (g/L)	pH	
1	1	1	1	6.00	10.00	10.5	1.96
2	1	1	−1	6.00	10.00	7.5	0.39
3	1	−1	1	6.00	6.00	10.5	2.34
4	1	−1	−1	6.00	6.00	7.5	0.28
5	−1	1	−1	2.00	10.00	7.5	1.23
6	−1	1	1	2.00	10.00	10.5	2.24
7	−1	−1	1	2.00	6.00	10.5	2.01
8	−1	−1	−1	2.00	6.00	7.5	1.05
9	1.682	0	0	7.36	8.00	9.0	1.51
10	−1.682	0	0	0.64	8.00	9.0	0.97
11	0	1.682	0	4.00	11.36	9.0	1.63
12	0	−1.682	0	4.00	4.64	9.0	1.11
13	0	0	1.682	4.00	8.00	11.5	0.57
14	0	0	−1.682	4.00	8.00	6.5	0.09
15	0	0	0	4.00	8.00	9.0	3.63
16	0	0	0	4.00	8.00	9.0	3.59
17	0	0	0	4.00	8.00	9.0	3.45
18	0	0	0	4.00	8.00	9.0	3.54
19	0	0	0	4.00	8.00	9.0	3.77
20	0	0	0	4.00	8.00	9.0	3.40

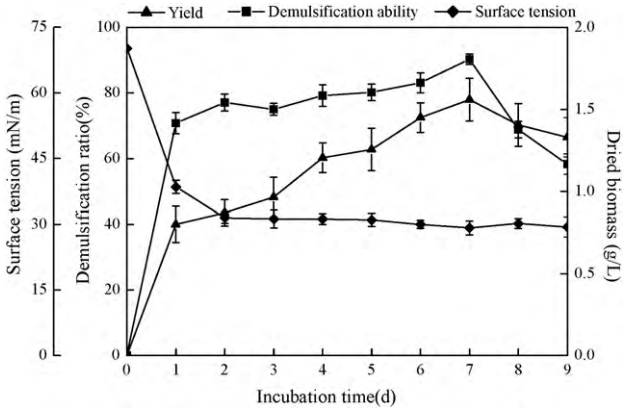

Fig. 1. Production of biodemulsifier by *Alcaligenes* sp. S-XJ-1 in batch cultivation for 7 days with 4% paraffin as the carbon source.

response variable was fitted by a second-order model in the form of a quadratic polynomial equation given below:

$$Y = b_0 + \sum_{i=1}^{n} b_i \chi_i + \sum_{i=1}^{n} b_{ii} \chi_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} \chi_i \chi_j \quad (1)$$

where Y is the predicted response, b_0 is the constant coefficient, b_i, b_{ii} and b_{ij} are the linear, interaction and quadratic coefficients, respectively. χ_i and χ_j are the coded values of the variables. The analysis of variance (ANOVA) and response surfaces were performed using Statistical Analysis System software (SAS, version V8.0, SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Production of biodumulsifier by Alcaligenes sp. S-XJ-1

Strain of *Alcaligenes* sp. S-XJ-1 was cultivated in the MMSM containing 4% liquid paraffin as the carbon source and the changes of surface tension and demulsifying ability of the fermentation broth were recorded each day. As shown in Fig. 1, the surface tension quickly dropped to 31.5 ± 1.3 mN/m on the 2nd day and stayed constant thereafter, which suggests that the biodemulsifier produced by *Alcaligenes* sp. S-XJ-1 had a high surface activity and its critical micelle concentration was achieved within 2 days' cultivation. The maximal biodemulsifier yield (1.56 ± 0.13 g/L) and demulsification ratio (90.3 ± 1.6%) were achieved on day 7 and both started to decline from day 8. This indicates that cell growth probably entered a decline phase when the endogenous metabolism became dominant due to the limitation of substrate concentration. As a result, the length of the fermentation period was set at 7 days in subsequent biodemulsifier production runs.

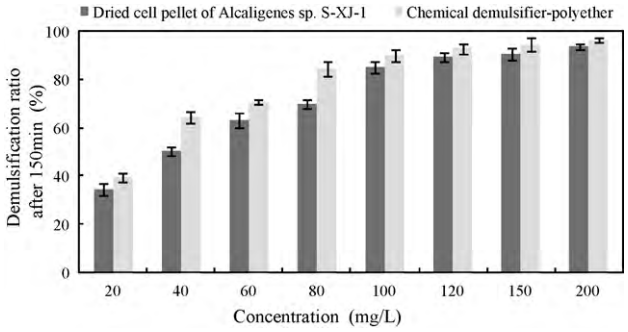

Fig. 2. Comparison of biodemulsifier produced by *Alcaligenes* sp. S-XJ-1 with chemical demulsifier with respect to their demulsifying ability.

After being harvested by centrifugation and dried at 105 °C, the powder-dried biodemulsifier retained 90% of its demulsifying ability [14], which indicated that the biodemulsifier produced by *Alcaligenes* sp. S-XJ-1 (using paraffin as a carbon source) was cell wall associated and possessed excellent heat-resistance. This feature would greatly facilitate its transportation to and storage at the site of application.

3.2. Comparison between biodemulsifier and chemical demulsifier

The dried cell pellet biodemulsifier was compared with the chemical demulsifier for the demulsification of a fresh crude oil emulsion. The demulsification ratio at 150 min was used to evaluate the performance. It is shown in Fig. 2 that the demulsification ratio gradually increased with an increasing dosage of the demulsifiers. However, the chemical demulsifier performed better than the biodemulsifier at the same concentration. The demulsifying difference became marginal when the dosage exceeded 100 mg/L.

To evaluate the quality of the separated water produced by the demulsification process, demulsification tests were carried out with 120 mg/L of biodemulsifier and 100 mg/L of chemical demulsifier, at which a similar demulsification ratio (90%) was achieved. As shown in Fig. 3, the separated water from the biological demulsification showed a much lower soluble COD concentration (163.0 ± 6.2 mg/L) but a slightly higher suspended solid (SS) concentration. This may be explained by the different characteristics of these two demulsifiers. In the form of dried powder, the biodemulsifier was insoluble in water. It remained suspended at the oil/water interface at the end of the demulsification process, which accounted for the higher SS of the separated water. In contrast, the chemical demulsifier, polyether, was miscible in water and therefore contributed to soluble COD of the separated water. The oil concentration in the separated water from biological demulsification was slightly lower than that from chemical demulsification, as

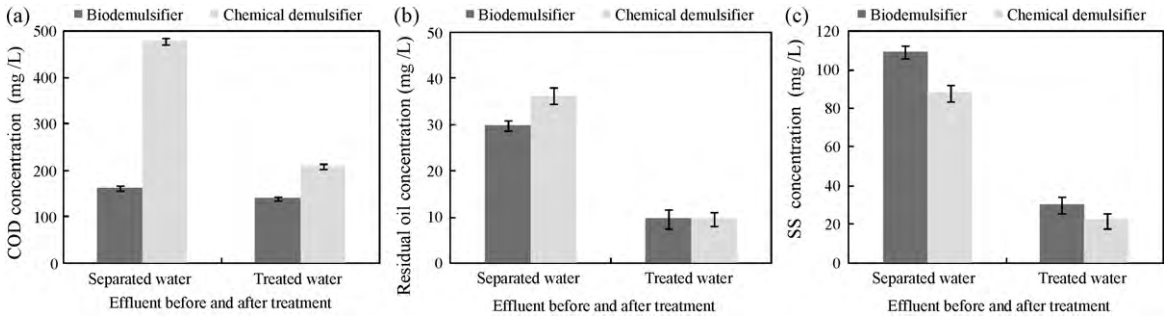

Fig. 3. Comparison of biodemulsifier produced by *Alcaligenes* sp. S-XJ-1with chemical demulsifier with respect to (a) COD, (b) residual oil concentration, and (c) SS of the separated water.

Table 3
The effect of carbon source on the properties and demulsifying ability of biodemulsifier produced by *Alcaligenes* sp. S-XJ-1.

Carbon source	Surface tension (mN/m)[a]	Biodemulsifier yield (g/L)	MATH (%)	Demulsification ratio after 150 min (%)[b]	$t_{1/2}$ (min)
Starch	64.7 ± 1.3	3.06 ± 0.44	18.0 ± 2.2	20.8 ± 1.0	>150
Sodium acetate	62.3 ± 0.4	1.51 ± 0.22	17.5 ± 1.5	20.8 ± 1.3	>150
Sodium citrate	59.2 ± 0.3	1.78 ± 0.22	20.1 ± 2.6	20.0 ± 0.9	>150
Sodium succinate	61.7 ± 0.9	2.96 ± 0.30	18.2 ± 2.3	19.2 ± 1.5	>150
Sucrose	67.4 ± 0.4	3.20 ± 0.31	14.5 ± 0.8	18.3 ± 1.6	>150
Lactose	68.0 ± 0.5	1.83 ± 0.52	12.3 ± 1.1	12.5 ± 1.1	>150
Peanut oil	38.1 ± 0.2	2.08 ± 0.22	75.7 ± 1.3	91.6 ± 1.6	45
Sunflower oil	42.0 ± 0.1	0.66 ± 0.17	72.3 ± 2.6	68.8 ± 1.4	30
Rapeseed oil	36.1 ± 0.2	1.87 ± 0.20	69.8 ± 0.9	58.3 ± 2.1	120
Palm butter	39.2 ± 0.6	3.01 ± 0.32	69.3 ± 1.8	45.8 ± 0.9	>150
Sesame oil	40.0 ± 0.6	0.48 ± 0.15	68.8 ± 1.5	45.8 ± 2.5	>150
Crude coconut oil	38.2 ± 0.1	0.43 ± 0.16	62.5 ± 0.4	20.8 ± 0.8	>150
Soy bean oil	38.0 ± 0.4	1.45 ± 0.26	62.1 ± 2.1	16.7 ± 0.5	>150
Paraffin	30.1 ± 0.1	1.45 ± 0.12	65.5 ± 1.4	90.2 ± 2.3	60

[a] The surface tension of sterilized culture medium and distilled water was 70.1 ± 0.2 mN/m and 72.0 ± 0.2 mN/m, respectively.
[b] The demulsification ratio of blanks (sterilized medium with various carbon sources) was 0% within 24 h.

shown in Fig. 3b, indicating that the biodemulsifier can be as efficient as the chemical demulsifier with respect to the oil recovery ratio.

To evaluate the quality of separated water after treatment, the separated water was treated by coagulation, sedimentation and filtration, which have been widely used in oilfields in China for the removal of COD, SS and oil. Fig. 3a shows that the COD concentration of the treated water from chemical demulsification was still 210.2 ± 5.8 mg/L, much higher than that of the treated water from biological demulsification (140.0 ± 4.9 mg/L). As shown in Figs. 3b and c, residual oil and SS could be efficiently removed from both separated waters during the treatment process, which indicates that the application of biodemulsifier had no negative effects on the effluent compared to the chemical demulsifier.

Treatment of the wastewater produced in the oil extraction industry has been a critical issue in recent years [2]. Traditional treatment methods applied in oilfield produced water are just combinations of several treatment units, such as coagulation, sedimentation and filtration. This physical–chemical technology can only eliminate the SS and oil from the wastewater but has limited efficiency on removal of dissolved organic pollutants. Recently, many approaches have been attempted to remove the organic compounds from separated water, including the hydrolysis acidification/bio-contact oxidation system (HA/BCO) [18], photoelectrocatalytic decontamination [19] and Al_2O_3-PVDF nanocomposite tubular ultrafiltration membranes [20]. Despite their effectiveness, all these approaches incur substantial treatment costs. Use of the biodemulsifier would eliminate the secondary pollution risk to the environment produced by demulsification. In this study, the concentration of organic pollutants in the separated water produced by biodemulsifier was lower than that produced by chemical demulsifier. Thus, it can be concluded that using a biodemulsifier would reduce the difficulty and the costs of separated water treatment.

3.3. The effect of carbon source on the production of biodemulsifier

On the 7th day of cultivation, the fermented broth produced by different carbon sources were tested for surface tension, biodemulsifier yield, cell surface hydrophobicity and demulsifying ability. It is shown in Table 3 that hydrophilic carbon sources resulted in lower surface activities (with surface tensions higher than 60 mN/m) and lower demulsification ratios than hydrophobic carbon sources, even though they yielded higher biodemulsifier concentrations. On the other hand, the emulsion half-life ($t_{1/2}$) for the biodemulsifier produced from all hydrophilic carbon sources was longer than 150 min, suggesting that hydrophilic carbon sources were not efficient in the production of biodemulsifier. Apart from soybean oil and coconut oil, biodemulsifiers produced from hydrophobic carbon sources all exhibited demulsifying ability. The biodemulsifier produced from sunflower oil was most efficient, giving an emulsion half-life ($t_{1/2}$) of just 30 min. Peanut oil showed the highest demulsification ratio (91.6 ± 1.6%) within 150 min. When hydrophilic carbon sources were used, microbial adhesion to hydrocarbon (MATH) was lower than 20%. In contrast, it was over 62% when the hydrophobic carbon sources were used. MATH of cells in peanut oil fermentation broth reached 75.7 ± 1.3%,

Table 4
The effect of nitrogen source on the properties and demulsifying ability of biodemulsifier produced by *Alcaligenes* sp. S-XJ-1.

Nitrogen source	Surface tension (mN/m)[a]	Biodemulsifier yield (g/L)	Demulsification ratio after 150 min (%)[b]	$t_{1/2}$ (min)
Ammonium citrate	30.2 ± 1.5	1.82 ± 0.39	93.3 ± 2.6	10
Yeast powder	30.2 ± 0.3	6.23 ± 0.39	86.7 ± 1.3	45
Urea	34.1 ± 1.2	4.45 ± 0.32	62.5 ± 3.1	45
Potassium nitrate	35.3 ± 1.5	1.37 ± 0.17	60.4 ± 2.4	60
Ammonium sulfate	31.5 ± 1.2	1.19 ± 0.25	54.2 ± 3.2	90
Ammonium chloride	36.2 ± 1.1	1.25 ± 0.23	54.2 ± 2.1	60
Sodium nitrate	36.0 ± 0.4	1.24 ± 0.21	54.2 ± 1.8	90
Ammonium molybdate	55.4 ± 0.5	0.72 ± 0.21	12.5 ± 1.9	>150
Ammonium nitrate	30.2 ± 0.1	1.51 ± 0.13	91.3 ± 3.7	60

[a] The surface tension of sterilized culture medium and distilled water was 70.1 ± 0.2 mN/m and 72.0 ± 0.2 mN/m, respectively.
[b] The demulsification ratios of blanks (sterilized medium with various nitrogen sources) was 0% within 24 h.

Table 5
The effect of temperature and pH on the properties and demulsifying ability of biodemulsifier produced by *Alcaligenes* sp. S-XJ-1.

Cultivation conditions	Surface tension (mN/m)[a]	Biodemulsifier yield (g/L)	Demulsification ratio after 150 min (%)[b]	$t_{1/2}$ (min)
T = 20 °C, pH 7	31.3 ± 0.2	1.15 ± 0.17	20.8 ± 1.2	>150
T = 30 °C, pH 7	29.1 ± 0.4	1.27 ± 0.12	70.8 ± 2.4	60–90
T = 35 °C, pH 7	30.1 ± 1.1	1.39 ± 0.20	90.3 ± 1.5	60
T = 40 °C, pH 7	29.4 ± 0.3	1.30 ± 0.15	72.9 ± 3.2	15–30
T = 50 °C, pH 7	41.9 ± 0.2	0.44 ± 0.05	37.5 ± 2.3	>150
T = 35 °C, pH 5	34.9 ± 0.5	0.29 ± 0.09	56.2 ± 1.2	90
T = 35 °C, pH 6	32.7 ± 0.1	0.68 ± 0.11	66.7 ± 2.1	45
T = 35 °C, pH 7	30.1 ± 1.1	1.39 ± 0.20	90.3 ± 1.5	60
T = 35 °C, pH 8	32.8 ± 0.2	1.99 ± 0.22	95.8 ± 3.4	5–10
T = 35 °C, pH 9.5	31.6 ± 0.4	2.84 ± 0.15	97.9 ± 2.4	0–5

[a] The surface tensions of sterilized culture medium and distilled water were 70.1 ± 0.2 mN/m and 72.0 ± 0.2 mN/m, respectively.
[b] In demulsification tests, the demulsification ratio of blank (with the dosing of 2 mL of sterilized MMSM culture medium) was 0% within 24 h. The demulsification ratios of blanks with sterilized medium cultured at various initial pH levels were <10% within 24 h.

which was highest of all hydrophobic carbon sources. As shown in Table 3, the demulsification ratios appear to be positively correlated with cell surface hydrophobicity when various hydrophobic carbon sources were used.

In reported studies on biodemulsifier production, both hydrophilic and hydrophobic carbon sources can be found. However, they seem to produce different types of biodemulsifiers. For example, hydrophilic carbon sources are seldom reported for the production of biodemulsifiers, except for an extracellular biodemulsifier, acetoin [21]. On the contrary, cell wall associated demulsifiers are mostly produced from hydrocarbons such as crude oils [22], tetradecane [8], cetane [4,5] and kerosene [23], indicating that hydrophobic carbon sources would induce the production of cell wall associated biodemulsifiers. One possible explanation is that the presence of amphiphilic substances (such as a biodemulsifier) on the cell wall could facilitate utilization of hydrophobic carbon sources and thus enable the bacteria to survive in a hydrocarbon-polluted environment.

The presence of cell wall associated biodemulsifier also resulted in higher cell surface hydrophobicity. This feature facilitated the dispersion of biodemulsifiers into the oil phase and adsorption onto an oil/water interface. Due to the high surface activity, the biodemulsifier eventually displaced the emulsifier adsorbed on the oil–water interface and resulted in demulsification. A similar phenomenon was also observed in other studies. For example, Ma et al. [24] reported that a hydrophobic substance produced on the cell surface of *Rhodococcus* sp. PR-1 could break a W/O emulsion.

Among all tested hydrophobic carbon sources, sunflower oil, rapeseed oil and peanut oil were identified as suitable candidates for biodemulsifier synthesis. As edible oils, the abundant source of waste frying oils produced from food processing can be used as carbon sources in the synthesis of biodemulsifiers to lower the production cost.

3.4. The effect of nitrogen source on the production of biodemulsifier

The effects of various nitrogen sources were investigated and the results are shown in Table 4. It can be clearly observed that the highest demulsification ratio (93.3%) and shortest emulsion half-life ($t_{1/2}$) (10 min) were achieved simultaneously when ammonium citrate was used as the nitrogen source. Compared to ammonium nitrate, ammonium citrate increased the biodemulsifier yield by only 20% (much lower than urea and yeast extract), while it dramatically increased both the demulsification ratio and the speed. However, it was interesting to see the poor demulsification performance when citrate was used as the sole carbon source together with ammonium nitrate as the nitrogen source (as shown in Table 3). The only difference in the compositions of the culture media used in these cases was the presence of paraffin. This implies that citrate would only stimulate the demulsifying activity and production of biodemulsifier in the presence of hydrophobic carbon sources (such as paraffin). As an intermediate product of the tricarboxylic acid (TCA) cycle, citric acid would be able to strengthen the metabolic process and somehow to facilitate the production of biodemulsifier.

Comparing ammonium nitrogen with nitrate nitrogen, no significant advantages were observed with respect to demulsifying ability or biodemulsifier yield. This was different from what was reported in the literature on other biosurfactants. For example, Abouseoud et al. found that sodium nitrate and ammonium nitrate were more efficient in enhancing the production of the biosurfactant rhamnolipid by *Pseudomonas fluorescens* than ammonium chloride [12]. Previous studies have shown that biosurfactants [25] and biodemulsifiers [26] were composed of a few homologues. The change of carbon sources or nitrogen sources would probably change the composition and dominant biosurfactant homologue

Table 6
Estimated regression coefficients and corresponding standard deviation, t_{exp} and significance level for biodemulsifier yield of *Alcaligenes* sp. S-XJ-1.

Coefficient	Value	Standard deviation	t_{exp}[a]	Pr > |t|
X_1	0.89	0.75	1.18	0.2639
X_2	2.90	0.84	3.46	0.0061
X_3	8.29	1.28	6.46	<0.0001
X_1^2	−0.17	0.04	−4.82	0.0007
X_2^2	−0.16	0.04	−4.49	0.0012
X_3^2	−0.45	0.06	−7.06	<0.0001
X_1X_2	−0.024	0.05	−0.51	0.6227
X_1X_3	0.074	0.06	1.16	0.2745
X_2X_3	−0.022	0.06	−0.35	0.7354

[a] t_{exp} was obtained from the t-test, which indicates the significance of the regression coefficients.

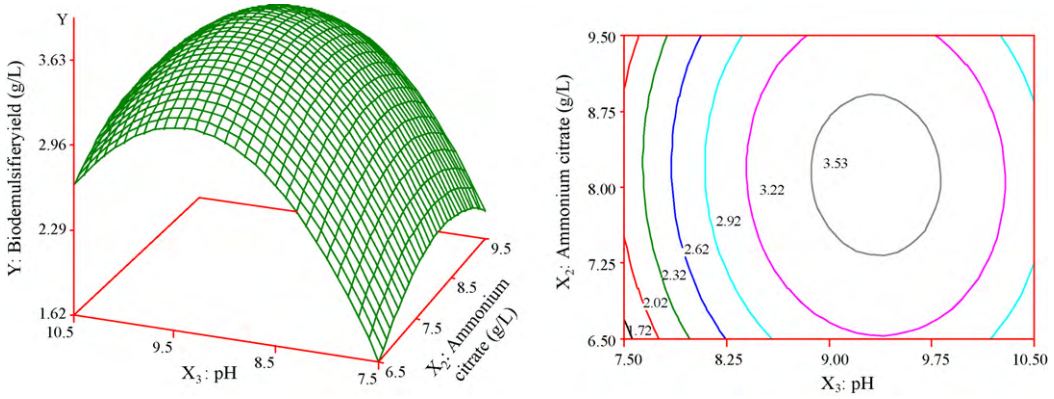

Fig. 4. Response surface plot and the contour plot for biodemulsifier yield of *Alcaligenes* sp. S-XJ-1: effects of ammonium citrate and pH.

species and consequently influence overall surface activity. In this study, the evaluation indicators, demulsification ratio and biodemulsifier yield, may not be able to differentiate subtle distinctions of biodemulsifier homologues produced from various nitrogen sources.

Both urea and yeast extract greatly increased the biodemulsifier yield during the same cultivation time. However, their corresponding demulsification ratios were still lower than the control (ammonium nitrate). To improve the efficiency of demulsification and biodemulsifier yield, ammonium citrate is suggested as the optimal nitrogen source.

3.5. Effect of pH and temperature on the production of biodemulsifier

As a mesophilic strain, *Alcaligenes* sp. S-XJ-1 was cultivated at a temperature range between 20 and 50 °C to determine its optimal growth temperature. It is shown in Table 5 that the demulsifying ability of the biodemulsifier decreased at both high temperature (>40 °C) and low temperature (<35 °C). In conclusion, the optimal temperature for the production of biodemulsifier was around 35 °C, which was set as a constant in the subsequent optimization experiments.

Within the tested pH range, an acidic pH was unfavorable to the production and demulsifying performance of biodemulsifier (Table 5). As the pH of the culture medium increased to the neutral or alkali range, both the biodemulsifier yield and the demulsification ratio remarkably increased. At pH 9.5, both reached maximum levels, together with the fastest demulsification speed ($t_{1/2}$ < 5 min). This suggests that *Alcaligenes* sp. S-XJ-1 might be a facultative alkaliphilic bacterium, which probably had a specific alkaliphilic enzyme to facilitate its survival and production of biodemulsifier under alkali conditions. To date, only a few species have been identified as alkaliphilic bacteria, such as *Bacillus* sp., *Micrococcus* sp., *Pseudomonas* sp., *Achromobacter* sp. [27]. In this paper, a strain of *Alcaligenes* sp. was firstly reported as alkaliphilic bacteria.

To eliminate the effect of pH of the whole broth on demulsification, cell suspensions, rather than fermented broth, were applied in demulsification tests. The cell suspension obtained from pH 9.5 fermented broth still performed better than that obtained from the pH 7 culture. Moreover, the former showed a MATH value of 85%, much higher than the MATH of the latter (65.5%). Therefore, it was concluded that the alkali condition (pH 9.5) can increase the production of biodemulsifier or demulsification efficiency. It will be investigated in future work how the alkali condition affects the synthesis of biodemulsifiers.

3.6. Optimization of biodemulsifier production using response surface methodology

From the above discussion, it was found that paraffin, ammonium citrate and pH had significant effects on the production of biodemulsifier. A full factorial CCD was employed to determine the individual and interactive effects of three parameters on biodemulsifier yield. Table 6 shows the ANOVA of regression parameters of the predicted response surface quadratic model for biodemulsifier production. The larger the magnitude of the t_{exp}-values and the smaller Pr > |t| values, the more significant are the corresponding coefficients. As can be seen from the table, the linear effect of X_2 and X_3 and their quadratic effects were significant to the response.

The statistical significance of the quadratic model was evaluated as presented in Table 7. The result shows that this regression was statistically significant at an F-value of 10.17 and values of Prob > F (0.0006). The value of the correlation coefficient (R^2 = 0.9015) indicates that only 9.85% of the total variation could not be explained by the empirical model. Hence, the response surface model was considered to be significant. The final regression model in terms of real factors was expressed by the following second-order polynomial equation:

$$Y = -48.7 + 0.89X_1 + 2.90X_2 + 8.29X_3 - 0.17X_1^2 - 0.024X_1X_2 - 0.16X_2^2 + 0.074X_3X_1 - 0.022X_2X_3 - 0.45X_3^2 \quad (2)$$

Table 7
Analysis of variance (ANOVA) for the response surface quadratic model for biodemulsifier yield of *Alcaligenes* sp. S-XJ-1.

Source of data	Sum of squares	Degree of freedom (DF)	Mean square	F-Value	Prob. > F
Model	26.98	9	3.00	10.17	0.0006
Linear	3.18	3	1.06	3.60	0.0538
Quadratic	23.30	3	7.77	26.34	<0.0001
Interaction	0.51	3	0.17	0.57	0.6464
Residual	2.95	10	0.29	–	–
Lack of fit	2.86	5	0.57	32.71	0.0008
Pure error	0.09	5	0.02	–	–

R^2 = 0.9015.

Fig. 4 shows the three-dimensional response surfaces and two-dimensional contour plots of the quadratic model, showing the effects of the ammonium citrate concentration and pH on the biodemulsifier yield, with the paraffin kept constant at its zero level. As can be seen, the biodemulsifier yield increased with increasing pH or ammonium citrate concentration to its peak but then decreased with a further increase of the two variables. Hence, the influence of these two variables on biodemulsifier yield was significant. In addition, the biodemulsifier yield was shown to be very sensitive to changes of the initial pH, compared to ammonium citrate.

With the objective being to maximize the biodemulsifier yield, the optimum conditions were identified using the SAS statistical analysis software package. The maximum response value for biodemulsifier yield was estimated at 3.6 g/L with the optimum conditions found at a paraffin concentration of 4.01%, an ammonium citrate concentration of 8.08 g/L and a pH of 9.35. Under the above optimum conditions, the yield of biodemulsifier was experimentally obtained at 3.42 g/L. It was observed that the experimental value obtained was in good agreement with the value predicted from the model. When applied to the demulsification of a crude oil emulsion, 98% and 95% demulsification ratios were achieved by 10% (v/v) fermented broth and 120 mg/L powder-dried biodemulsifier, respectively.

The above discussion illustrates that biodemulsifier yield was increased and its demulsifying ability was enhanced by optimization using CCD of RSM. To the authors' knowledge, optimization of biodemulsifier yield has not previously been reported in the literature. Further studies will focus on the mechanism of the effects produced by ammonium citrate and the initial culture pH.

4. Conclusion

The biodemulsifier produced by *Alcaligenes* sp. S-XJ-1 was compared with a chemical demulsifier for demulsification tests of a crude oil emulsion. The biodemulsifier not only showed satisfactory demulsifying ability but also improved the quality of separated water, which is expected to reduce the environmental impact produced by separated water. Effects of various cultivation factors on the production of biodemulsifiers were investigated. The results showed that hydrophobic carbon sources were more favorable to the synthesis of this cell wall associated biodemulsifier than hydrophilic carbon sources. Ammonium citrate was the most suitable nitrogen source among those tested. When optimizing biodemulsifier yield, the optimal levels of paraffin, ammonium citrate and pH were identified as 4.01%, 8.08 g/L and 9.35, respectively. Under the above optimum conditions, the yield of biodemulsifier was experimentally increased from 1.45 to 3.42 g/L. The biodemulsifier produced by *Alcaligenes* sp. S-XJ-1 is expected to be promising for future industrial applications.

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