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Author: Guihong Lan Qiang Fan Yongqiang Liu Yu Liu Yucheng Liu Xiaobo Yin Mina Luo

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Effects of the addition of waste cooking oil on heavy crude oil biodegradation and microbial enhanced oil recovery using *Pseudomonas* sp. SWP-4

Guihong Lan[a,b], Qiang Fan[a,b], Yongqiang Liu[c], Yu Liu[a], Yucheng Liu[a], Xiaobo Yin[b], Mina Luo[a]

[a] Key Laboratory of Oil&Gas Applied Chemistry of Sichuan Province, College of Chemistry and Chemical Engineering, Southwest Petroleum University, Chengdu 610500, PR China

[b] Key Laboratory of Development and Application of Rural Renewable Energy, Ministry of Agriculture,Chengdu 610041, PR China

[c] Faculty of Engineering and the Environment, University of Southampton, Southampton SO17 1BJ, United Kingdom

Correspondence author at: No. 8 Xindu Avenue, Xindu District, Chengdu 610500, PR China.

Tel.: +86 02883037306; fax: +86 02883037306.

E-mail address: guihonglan416@sina.com (G. Lan).

Highlights

► The addition of waste cooking oil stimulated the microbial activities. ► The addition of waste cooking oil shortened the crude oil degradation period. ► The addition of waste cooking oil enhanced the crude oil biodegradation. ► The addition of waste cooking oil helped the viscosity reduction of crude oil. ► The addition of waste cooking oil promoted the oil recovery efficiency.

Graphical abstract

Abstract

The present work aims to investigate the effects of the addition of waste cooking oil (WCO) on heavy crude oil biodegradation and microbial enhanced oil recovery (MEOR) using *Pseudomonas* sp. SWP-4. Growth kinetics show *Pseudomonas* sp.

SWP-4 had a maximum dry cell weight of 1.73 g/L and cell-surface hydrophobicity of 62.4% against n-hexadecane when degraded the crude oil with the addition of WCO. The maximum rhamnolipid concentration was 6.87 g/L, and the culture broth exhibited a higher emulsification efficiency of 58.3% on n-hexadecane and reduced the surface tension of broth to 22.7 mN/m. Meanwhile, *Pseudomonas* sp. SWP-4 reduced the viscosity of crude oil from 26,300 mPa·s to 550 mPa·s (40℃) and successfully degraded most of the n-alkanes. Furthermore, the fluidity of oil had been well improved after degradation. It can be concluded that not only could WCO stimulate the bacterial growth, but also it could enhance the crude oil degradation. Core displacement experiment demonstrates the efficiency of water flooding was just 5.8%, but the microbial flooding produced by *Pseudomonas* sp. SWP-4 with the addition of WCO effectively improved the oil recovery further with an additional oil recovery efficiency of 24.4%.

Key words: Waste cooking oil; Biodegradation; Microbial enhanced oil recovery; Growth kinetics; Viscosity; Bioremediation.

Abbreviations: EOR, enhanced oil recovery; MEOR, microbial enhanced oil recovery; WCO, waste cooking oil; CSH, cell-surface hydrophobicity; GC-MS, gas chromatography-mass spectrometer; LB, Luria-Bertani; MSM, mineral salts medium; OD_{600}, optical density at 600 nm; DCW, dry cell weight; E_{24} , emulsification index; Pr, pristane; Ph, phytane.

1. Introduction

With the continuous increase of worldwide energy demand, crude oil as a kind of main fossil fuel still plays an important role in the current industrial society. In China, the heavy crude oil reserve, such as in the Mao-8 zone of Zhongyuan oilfield in Inner Mongolia alone, reaches as much as 1.3×10^7 tons. However, the oil reserve is not equivalent to the oil recovery, which faces a big technical challenge due to the high viscosity and poor fluidity of oil. In terms of current volatile market for fossil fuel and large demand for petroleum, there is strong economic incentive to develop an alternative way to exploit the heavy crude oil resources. It is well-known that enhanced oil recovery (EOR) processes are conventionally applied to recover the residual heavy oil from reservoirs. However, after the primary and secondary technique, around two-thirds of crude oil remains trapped in reservoirs [1]. Thus,

developing the third stage of oil recovery such as chemical, physical and microbial processes to further recover oil has been a great concern. Nevertheless, chemical processes by using solvents, polymers and surfactants, and physical processes such as steam flooding or hot water flooding are expensive. Meanwhile, non-biodegradable residues were usually released to the environment with the chemical or physical processes [2]. By contrast, microbial enhanced oil recovery (MEOR) is a cost-effective and environment-friendly way to enhance the crude oil recovery.

Over the last few decades, MEOR technology has been demonstrated successfully in laboratory or field conditions, especially for the low temperature oilfield reservoirs [3] . MEOR makes use of microbial activities and metabolic byproducts to reduce the viscosity and increase the fluidity of crude oil [4]. On the one hand, microorganisms can reduce the oil viscosity by degrading the long-chain saturated hydrocarbons and some of the other heavy oil fractions [5]; On the other hand, microorganisms can produce non-toxic products such as biosurfactants to increase the oil sweep efficiency through changing the reservoirs' physicochemical characteristics [6].

Among various microorganisms, only bacteria are considered as the promising candidates for MEOR due to their higher tolerance to extreme reservoir properties such as high salinity, pH, temperature, pressure, and nutrient availability [3]. The biosurfactant-producing microorganisms such as genus *Pseudomonas* [7], genus *Bacillus* [8], genus *Acinetobacter* [6] *et al.* are commonly employed in MEOR process. Nevertheless, most of the studies just evaluated the potential application of strains for light crude oil recovery, and the heavy crude oil was rarely discussed. So applying biotechnology to recover the heavy crude oil might be urgently needed in the future. Thereinto, genus *Pseudomonas* is known as a high-efficiency crude oil degrader [5, 9]. Meanwhile, genus *Pseudomonas* is able to produce rhamnolipid, which may be useful in the MEOR process [10]. In our previous study [11], we have isolated a strain *Pseudomonas* sp. SWP-4 that can consume waste cooking oil (WCO) to produce rhamnolipid. The produced rhamnolipid had low critical micelle concentration (27 mg/L) and high surface activities (reduced the surface tension of water from 71.8 mN/m to 24.1 mN/m and the interfacial tension against n-hexadecane from 29.4 mN/m to 0.9 mN/m). Meanwhile, the produced rhamnolipid showed excellent stability under high salinity (⩽80,000 ppm), wide pH (4–10) and high temperature (⩽100℃) conditions. All these excellent characteristics suggest that the strain *Pseudomonas* sp. SWP-4 has a great potential to be applied in MEOR process.

Even though many studies have applied genus *Pseudomonas* for crude oil degradation or MEOR, the degradation period is quite long and the oil recovery efficiency is not so satisfactory, which is mainly because of the poor growth of

bacteria in crude oil. For instance, Xia *et al.* [7] have demonstrated that adding bacterial biosurfactant solution of the employed bacteria could efficiently enhance the oil recovery. However, the biosurfactant purification is generally costly and complex. Analogously, Al-Hadhrami *et al.* [12] have reported that the addition of organic carbon sources such as cane sugar molasses was helpful to stimulate the bacterial growth when degraded the crude oil, but the degradation efficiency was also not prominent. Therefore, our aim is to explore an alternative but cheap substrate to promote the crude oil degradation as well as the oil recovery efficiency. Thereinto, WCO is a kind of less developed renewable energy but can stimulate the bacterial growth, thus we are going to employ it in present work. So far, there is no any report about the effects of the addition of WCO on heavy crude oil biodegradation or MEOR process.

2. Materials and methods

2.1 Chemicals and reagents

Tryptone and yeast extract were purchased from OXOID Company (Hampshire, England). Dichloromethane (guaranteed reagent grade) and the other chemicals (analytical reagent grade) were purchased from Kelong chemical regent factory (Chengdu, China), and deionized water (>18.25 MΩ cm) was used to make solutions. WCO (major compositions were palmitic acid, oleic acid and linoleic acid) was provided by Biogas Institute of Ministry of Agriculture (Chengdu, China). Heavy crude oil and wax samples were taken at 390−550 m depth in Ji-2-Ping-8 reservoir, Mao-8 zone, Zhongyuan oilfield (Inner Mongolia, China). The formation water used in core displacement experiment was also obtained from the Ji-2-Ping-8 reservoir. All the samples were stored in plastic buckets at 4℃ before use.

2.2 Microorganism and cultivation

Pseudomonas sp. SWP-4 was stored in glycerol freezer stock at −40℃ in a freezer. The preculture was incubated at in Luria-Bertani (LB) medium: 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl. Crude oil degradation experiments were conducted in Mineral salts medium (MSM), which contained the following components: 4 g/L $NaNO_3$, 5 g/L NaCl, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_20$, 0.2 g/L $FeSO_4 \cdot 7H_20$, 0.2 g/L $CaCl_2$.

2.3 Identification of *Pseudomonas* sp. SWP-4 in hydrocarbonoclastic capacity

Pseudomonas sp. SWP-4, an excellent WCO degrader as demonstrated before [11], was tested for its potential application in crude oil degradation. In this part, CSH of *Pseudomonas* sp. SWP-4 was investigated against some typical hydrocarbons. In addition, the abilities of *Pseudomonas* sp. SWP-4 to grow on varieties of hydrocarbon substrates were evaluated.

2.3.1 CSH test

Pseudomonas sp. SWP-4 was firstly cultured in LB medium at 30℃, 150 rpm on a rocking incubator (QYC-200, Shanghai, China) for 24 h. Then, bacterial cells were harvested by centrifugation (3,000 rpm, 10 min). The collected cells were washed three times and re-suspended in PUM buffer, pH 7.2: 22.2 g K_2HPO_4, 7.26 g KH_2PO_4,

1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and deionized water to 1000 mL. Afterwards, the washed bacterial suspension was adjusted with PUM buffer to an optical density at 600 nm (OD_{600}) of 0.6±0.02 by an ultraviolet spetrophotometer (V-1800, Kyoto, Japan). Finally, 4 mL bacterial suspension and different volumes (0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mL) of hydrocarbons (n-hexadecane, n-dodecane, n-hexane, methybenzene) were added in each test tube. After pre-incubation at room temperature for 15 min, the mixtures were mixed by vortex for 2 min. To ensure the organic phase rose completely, the mixtures were left to stand for 15min. After that, the aqueous phase was transferred to a cuvette by an aseptic pipette and the OD_{600} values were measured [13, 14]. All the experiments were carried out in triplicate, and CSH percentage was calculated as Eq. (1):

$$CSH\% = \frac{OD_{600}{}^{0} - OD_{600}{}^{1}}{OD_{600}{}^{0}} \quad (1)$$

where $OD_{600}{}^{0}$ is the initial OD_{600} value, and $OD_{600}{}^{1}$ is measured after CSH test.

2.3.2 Growth on specific hydrocarbon substrates

The specific hydrocarbon substrates included n-hexane, n-dodecane, n-hexadecane, paraffin wax, methybenzene, dimethylbenzene, naphthalene. Theses experiments were conducted in test tubes supplemented with 4 mL MSM, and the addition of each hydrocarbon substrate was 5 g/L (solid hydrocarbons) or 0.5% (v/v, liquid hydrocarbons), respectively. Inoculum was 12 h-old LB-grown cultures and the inoculum ratio was 2% (v/v). Incubation was carried out at 30℃, 150 rpm for 72 h. In this part, growth was evaluated by measuring the dry cell weight (DCW) coupled with the surface view of the disappearance of the oil slick and the turbidity of the culture broth. DCW was determined by centrifuging (3000 rpm, 10 min) the culture broth to collect the cells, and then the cells were washed twice with distilled water and dried by heating at 60℃ until constant weight was obtained [7].

2.4 Physicochemical properties analysis of the employed oil and formation water

Before the following experiments being carried out, the physicochemical properties of the employed heavy crude oil were analyzed. Water content in crude oil was measured as described by Hammer *et al*. [15]. Wax, resins, and asphaltenes contents in crude oil were determined according to literature [5]. Solidifying point was determined by a kryoscope (SYP 1022-I, Shanghai, China) and viscosity was determined at 30 ℃ by a rotational viscometer (DNJ-5S, Shanghai, China). Meanwhile, the viscosity-temperature curve of the employed heavy crude oil was plotted. Density measurement was conducted at 30℃ using the hydrometer method. The salinity of the formation water and its chemical compositions were analyzed by an atomic absorption spectrophotometer (AA-7020, Beijing, China) and an ion chromatograph (833, Metrohm, Switzerland).

2.5 Effects of the addition of WCO on heavy crude oil biodegradation

To evaluate the effects of the addition of WCO on crude oil degradation, these assays were performed in 250 mL Erlenmeyer flasks containing 50 mL MSM and 0.2 ±0.01 g crude oil with 0.2 mL WCO at 30℃, 150 rpm. Meanwhile, the same assays without addition of WCO were conducted as control. In this part, each medium was

autoclaved at 121℃ for 20 min and then inoculated with a inoculum ratio of 4% (v/v). Inoculum was prepared by cultivating *Pseudomonas* sp. SWP-4 for 12 h in LB medium.

2.5.1 Time course of crude oil degradation

At specific time intervals (every day for 7 days), the amount of biomass in the culture broth was monitored by measuring the DCW as described above. CSH measurement was carried out to investigate the cell activity of *Pseudomonas* sp. SWP-4 when grew on the crude oil, and the tested hydrocarbon was n-hexadecane with the additive volume of 2.5 mL. Surface tension of each cell-oil-free broth was measured by a digital tensiometer (DT-102, Zibo, China) using the ring method [16]. Emulsification index (E_{24}) measurement was determined by adding 3 mL n-hexadecane to the same volume of cell-oil-free broth in the test tube, and then the mixture was mixed by vortex for 3 min and left to stand at 25℃ for 24 h prior to measurement. E_{24} was expressed as the percentage of the height of the emulsified layer divided by the total height of the liquid column [17]. Meanwhile, quantification of rhamnolipid production was determined by anthrone-sulfuric acid colorimetric method, and the rhamnose value was calculated from the standard curves prepared with L-rhamnose. The concentration of rhamnolipid was determined by multiplying rhamnose value by a coefficient of 3.4, obtained from the correlation of pure rhamnolipid/rhamnose [18-20].

2.5.2 Viscosity reduction of crude oil

To measure the viscosity reduction of crude oil after being degraded by *Pseudomonas* sp. SWP-4, each culture broth was demulsified at 40℃ in water bath for 2 h at specific time intervals (every day for 7 days). Then, the upper residual oil was carefully collected and carried out to measure the viscosity by the rotational viscometer at 40℃. All these measurements were carried out in triplicate.

2.5.3 Analysis of crude oil degradation by GC-MS

The residual crude oil was extracted with an equal amount of dichloromethane at the end of cultivation (7 days). Then, the mixture was vigorously oscillated for 3 min and the lower organic phase was carefully collected. Afterwards, the organic phase was condensed at 40℃ until the dichloromethane was completely evaporated. The dried crude oil sediment was dissolved in 20 mL dichloromethane and dehydrated with 2 g anhydrous sodium sulfate [5]. 0.5 μL oil sample was injected in an Agilent GC-MS (7890A/5975C, USA) with a split ratio of 5:1. GC-MS analysis was carried out by the following temperature programming: initial temperature was 60℃, then raised at a rate of 10℃/min to 300℃. The percentages of n-alkanes degradation were calculated using normalization method.

2.6 Core displacement experiment

Core displacement experiment was employed to investigate the potential application of *Pseudomonas* sp. SWP-4 in MEOR process. The schematic view of this experiment was shown in Fig. 1. The test was performed at 30℃ in a constant temperature incubator, which simulated Ji-2-Ping-8 reservoir's temperature. Two man-made cores (named 1# and 2#) with a diameter of 2.5 cm and a length of 30 cm

were filled with 40–60 mesh quartz sands. Drive fluid was the deionized water and the tested fluid used the formation water. The cores were flooded with the formation water at a constant flow velocity of 5 mL/min. The porosity Φ_t was calculated as Eq. (2) using the phase difference method, and permeability K was illustrated in Eq. (3) [21].

$$\phi_t = \frac{V_P}{V_T} \times 100\% \quad (2)$$

where V_P is the pore volume and V_T is the core volume.

$$K = \frac{Q\mu L}{A\Delta P} \times 10^{-1} \quad (3)$$

where Q is the flow of core fluid (mL/s), μ is viscosity of formation water (mPa • s), L is the core length (cm), A is the core sectional area (cm^2) , $\triangle P$ is differential pressure of the core on both ends (MPa).

The cores were firstly saturated with 5% (w/w) crude oil, and then aged at 30℃ for 24 h. Afterwards, the cores were flooded with formation water at 5 mL/min until no oil was produced, and the efficiency of water flooding was expressed as the percentage of the oil mass of water flooding divided by the mass of original saturated oil. After that, microbial flooding (prepared by cultivating *Pseudomonas* sp. SWP-4 for 24 h in 50 mL MSM with 2% (v/v) WCO) was injected in the cores with a flow rate of 5 mL/min, and then the cores were incubated for 24 h. At last, the cores were flooded again with the microbial flooding until no oil was recovered, thus the additional oil recovery efficiency was obtained.

Fig. 1. Schematic view of the core displacement experiment.

3. Results and discussion

3.1 Identification of *Pseudomonas* sp. SWP-4 in hydrocarbonoclastic capacity

3.1.1 CSH test

As the Fig. 2 shows, *Pseudomonas* sp. SWP-4 exhibited the highest CSH against n-hexadecane than the other tested hydrocarbons. When the volume ratio of bacterial suspension to n-hexadecane was 4:2.5, CSH percent reached the maximum value of 68.6%. Similarly, *Pseudomonas* sp. SWP-4 gave the maximum CSH percentages of 66.4%, 52.6% and 49.8% against n-dodecane, methybenzene and n-hexane. However, it could be clearly seen that the inhibitory effect increased with the rise of hydrocarbon volume, which was mainly because that excess hydrocarbon had a certain toxicity to bacteria. The CSH capacity affects the bacterial absorption and degradation of hydrophobic organic, and it also affects the bacterial adsorption to a variety of biological and non-biological surface and interface. Kumari *et al.* [22] demonstrated that the high oil degradation efficiency was attributed to the bacterial

high CSH capacity. Abbasnezhad *et al*. [14] also demonstrated that CSH was an important parameter in biodegradation of hydrocarbons to enhance uptake and metabolism of compounds with very low aqueous solubility. Thus, *Pseudomonas* sp. SWP-4 presented a great potential to be applied in biodegradation of crude oil owing to its good CSH capacity.

Fig. 2. CSH capacity of *Pseudomonas* sp. SWP-4 against specific hydrocarbons. The error bars represents standard deviation values of three independent experiments (n = 3).

3.1.2 Growth on specific hydrocarbon substrates

The abilities of *Pseudomonas* sp. SWP-4 to grow on specific hydrocarbon substrates were investigated as shown in Table 1. *Pseudomonas* sp. SWP-4 degraded all of the tested hydrocarbon substrates, but it was more inclined to grow on n-alkanes compared with polyaromatic hydrocarbons. *Pseudomonas* sp. SWP-4 showed luxuriant growth on n-hexadecane and n-dodecane and good growth on n-hexane, giving average DCW of 0.98 g/L, 0.83 g/L, and 0.65 g/L after 72-hour cultivation, respectively. Meanwhile, *Pseudomonas* sp. SWP-4 grew fairly on paraffin wax, methybenzene, dimethylbenzene and naphthalene. Obayori *et al.* [23] also investigated the substrates susceptibility of *Pseudomonas* strains on various components of crude oil, but their isolates performed worse susceptibility compared with the present *Pseudomonas* sp. SWP-4. Therefore, due to its good viability on various hydrocarbon substrates, *Pseudomonas* sp. SWP-4 exhibits high potential for crude oil degradation.

Table 1
Abilities of *Pseudomonas* sp. SWP-4 to grow on specific hydrocarbon substrates, cultivated at 30℃, 150 rpm for 3 days[a].

3.2 Physicochemical properties analysis of the employed oil and formation water

The physicochemical properties of the employed heavy crude oil were measured as shown in Table 2. The water content in crude oil was just 3.66% and the heavy oil components were relatively high. Wax, resins and asphaltene contents were 11%, 4.32% and 23.86%, respectively. The high contents of the heavy oil fractions resulted in high viscosity and density of crude oil. The viscosity and density were measured to be 220,000 mPa·s (30℃) and 0.9488g/cm^3 (30℃), respectively. The solidifying point of crude oil was dependent on the wax contents, and it was determined to be 11℃. These results help to explain the poor fluidity of the employed crude oil. The

viscosity-temperature curve of the employed heavy crude oil was shown in Fig. 3, from which we can see that the viscosity plummeted when temperature increased from 30℃ to 40℃. In view of the convenience and operability of the measurement, the subsequent viscosity reduction experiments were carried out at 40℃.

The total salinity of the formation water ranged between 0.6% and 1% (w/v) and its chemical composition was (g/L): Chloride, 5.58; Bicarbonate, 0.61; Sulfate, 0.78; Carbonate, 0.08; Calcium, 0.15; Magnesium, 0.12; Sodium/Kalium, 3.48. From these results we can see that the total salinity of the formation water is nearly equal to the normal saline (0.9%) and MSM (1.1%). Meanwhile, Ji-2-Ping-8 is a low temperature oilfield reservoir (25.4−30.7℃). Thus we can infer that both salinity of the formation water and reservoir temperature are quite suitable for microbial growth and MEOR process.

Table 2
Physicochemical properties of the employed heavy crude oil[a].

Fig. 3. Viscosity-temperature curve of the employed heavy crude oil. The error bars represents standard deviation values of three independent experiments (n = 3).

3.3 Effects of the addition of WCO on heavy crude oil biodegradation

3.3.1 Time course of crude oil degradation

Fig. 4a shows the typical time course profile of the crude oil degradation. From DCW curve, we can see that bacterial growth was in the stationary phase from day 4 to day 6, during which *Pseudomonas* sp. SWP-4 grew vigorously and exhibited a maximum DCW of 1.11 g/L. The increasing law of CSH was in line with the bacterial growth, and the highest CSH (against n-hexadecane) capacity of 58.3% was obtained during the stationary phase. Rhamnolipid, as a typical secondary metabolite, was largely produced in the stationary phase. However, rhamnolipid was also steadily accumulated even in the decline phase, giving the maximum concentration of 2.79 g/L on day 7. Meanwhile, the increase of E_{24} was in proportion to the accumulation of rhamnolipid, and the cell-oil-free broth showed the highest emulsification efficiency on n-hexadecane, 55.7%. Similarly, surface tension was also an important parameter to reflect the process of crude oil degradation. The collected cell-oil-free broth could decrease the surface tension of water from 72.4 mN/m to 25.4 mN/m.

From Fig. 4b, we can see that the addition of WCO gave positive effects on crude oil degradation. *Pseudomonas* sp. SWP-4 entered the stationary phase on day 3 and had a maximum amount of biomass of 1.73 g/L. Furthermore, the highest CSH

capacity (against n-hexadecane) was proven to be 62.4%. The maximum concentration of rhamnolipid was 6.87 g/L, which was almost two-and-a-half times compared with the result obtained from the same assays without the addition of WCO. In the meantime, surface activities of the collected cell-oil-free broth were also better, which exhibited a higher emulsification efficiency of 58.3% on n-hexadecane and reduced the surface tension of water to a lower value, 22.7 mN/m.

Zhang *et al.* [9] also proved that *Pseudomonas aeruginosa* had the capacity to produce biosurfactant as well as degrade petroleum hydrocarbons when grew on a hydrophobic phase. Kumari *et al.* [22] showed the oil degradation ability of genus *Pseudomonas* was greater than other bacterial species, which might link to several inherent factors such as higher biosurfactant production. In our previous study [11], we have demonstrated that *Pseudomonas* sp. SWP-4 was able to grow well on the MSM with WCO as the sole carbon source, and this waste oil had significant influences on bacterial growth and rhamnolipid synthesis. In present work, *Pseudomonas* sp. SWP-4 also possessed good adaptability in heavy crude oil degradation, and it was able to produce rhamnolipid when degraded the hydrocarbons. Moreover, with the addition of WCO in cultivation, *Pseudomonas* sp. SWP-4 exhibited better growth and greater rhamnolipid production. Therefore, it was concluded that the addition of WCO can accelerate the process of degradation and increase the solubilization of crude oil.

Fig. 4. Typical time course profiles of the crude oil degradation by *Pseudomonas* sp. SWP-4, cultivated in : (a) 50 mL MSM and 0.2±0.01 g crude oil; (b) 50 mL MSM and 0.2±0.01 g crude oil, with the addition of 0.2 mL WCO. Incubation was performed at 30℃, 150 rpm for 7 days. The error bars represents standard deviation values of three independent experiments (n = 3).

3.3.2 Viscosity reduction of crude oil

Fig. 5 illustrates the viscosity reduction of crude oil versus degradation time. *Pseudomonas* sp. SWP-4 showed a high crude oil viscosity reduction efficiency (＞ 90%) after cultivation of 7 days. It was able to reduce the viscosity of crude oil from 26,300 mPa·s to 1,350 mPa·s (measured at 40℃). Meanwhile, we have investigated the effect of the addition of WCO on crude oil viscosity reduction, and it indicated obvious role to decrease the crude oil viscosity to a lower value, 550 mPa·s (measured at 40℃). Moreover, cultivation with the addition of WCO shortened the crude oil degradation period, which was conducive to the heavy crude oil recovery and transportation. Fig. 5c and Fig. 5d shows the surface views of crude oil before and

after degradation. It can be seen that crude oil was emulsified obviously and its fluidity had been improved to a great extent after being degraded by 7 days.

One of the primary mechanisms of heavy crude oil viscosity reduction is that degradation of heavy oil fractions through microbial activities, and the other one is that the produced rhamnolipid could enhance the stability of oil/water emulsion system, eventually forming oil-in-water [2, 4, 24]. The high contents of the employed heavy oil fractions resulted in its high viscosity and poor fluidity. What's worse, these bad conditions usually brought negative effects on the oil's biodegradation. However, the high CSH capacity of *Pseudomonas* sp. SWP-4 could promote the uptake and metabolism of the hydrocarbons with very low aqueous solubility. Moreover, it was demonstrated that *Pseudomonas* sp. SWP-4 grew vigorously and produced more rhamnolipid in crude oil with the addition of WCO. Thus, it can be concluded that the addition of WCO promoted the biodegradation process and the better bacterial activities helped the greater viscosity reduction.

Fig. 5. Viscosity reduction of the crude oil after being degraded by *Pseudomonas* sp. SWP-4: (a) cultivation without the addition of WCO; (b) cultivation with the addition of WCO; (c) surface view of the crude oil before degradation; (d) surface view of the crude oil after degradation by 7 days. All the viscosity measurements were conducted at 40℃. The error bars represents standard deviation values of three independent experiments (n = 3).

3.3.3 Analysis of crude oil degradation by GC-MS

Fig. 6 shows a more detailed evaluation of crude oil degradation before and after flask incubation for 7 days. As Fig. 6a shown, the employed crude oil had n-alkanes ranging from C_{13} to C_{33}. Meanwhile, it can be observed from Fig. 6b and Fig. 6c that that *Pseudomonas* sp. SWP-4 could successfully degrade the crude oil samples and most of the n-alkanes were highly degraded after 7 days. The percentages of n-alkanes degradation were shown in Table 3, from which we can see that a large proportion of n-alkanes were degraded larger than 90%. However, C_{20}, C_{22}, C_{24} and C_{26} were relatively poorly degraded compared to the other n-alkanes, but the addition of WCO enhanced the degradation efficiency of these three hydrocarbons. Isoprenoid alkanes (pristane (Pr) and phytane (Ph)), as the biomarkers, was hard to degrade by bacteria. The less the solubilization, the less the bioavailability [25]. However, the addition of WCO resulted in the further degradation of Pr and Ph, which was mainly because the solubilization of isoprenoid alkanes increased through rhamnolipid emulsification. These improvements were assigned to the better bacterial growth and rhamnolipid

accumulation with the addition of WCO. On the one hand, the better bacterial growth helped the biodegradation of the heavy crude oil as well as the viscosity reduction; on the other hand, the accumulated rhamnolipid promoted the oil's solubilization and mobilization, thus bacteria could more easily take and degrade the crude oil. Therefore, it was believed that the addition of WCO could enhance the heavy crude oil biodegradation, and there was a synergistic effect between the crude oil degradation and WCO consumption.

Likewise, Pasumarthi *et al.* [5] also reported *Pseudomonas aeruginosa* could highly degraded the n-alkanes, but the degradation period was quite longer than present *Pseudomonas* sp. SWP-4. Al-Hadhrami *et al.* [12] have proved that the addition of organic carbon sources such as cane sugar molasses could result in significant n-alkanes breakdown. However, their degradation efficiency was not as good as *Pseudomonas* sp. SWP-4 with the addition of WCO.

Fig. 6. GC-MS analysis of the crude oil degradation by *Pseudomonas* sp. SWP-4: (a) blank test; (b) cultivation without the addition of WCO; (c) cultivation with the addition of WCO. Incubation was performed at 30℃, 150 rpm for 7 days.

Table 3
Percentages of n-alkanes degradation by 7 days at 30℃, 150 rpm: (a) cultivation without the addition of WCO; (b) cultivation with the addition of WCO.

3.4 Analysis of core displacement experiment

All of those characteristics described above demonstrate the potential application of *Pseudomonas* sp. SWP-4 in MEOR, thus we employed the core displacement experiment to further prove its potential application. The physical parameters of these two man-made cores and the oil recovery efficiency were shown in Table 4. Because of the high viscosity and poor fluidity of the heavy crude oil, water flooding just recovered 5.8% of oil. Control test demonstrated that after the first stage of water flooding, flooding the cores again with formation water couldn't enhance the oil recovery efficiency any more. However, after incubation for 24 h with the addition of WCO, the microbial flooding produced by *Pseudomonas* sp. SWP-4 had a rhamnolipid concentration around 7.0 g/L, and the OD600 value of the diluted (6-fold) microbial flooding was measured to be 0.757, which indicated the biomass was very large and bacterial growth entered stationary phase [11]. Unsurprisingly, the microbial flooding effectively mobilized the oil in the core models, giving an additional oil recovery efficiency of 24.4%.

The mechanisms of oil recovery fall into two broad categories: one of them is the alteration of interfacial /surface properties among oil, water and sand; and the other one is the changes of the crude oil in flow behavior. Firstly, the injected microbial flooding increased the capillary number through decreasing the viscosity of oil and the interfacial tension among oil, water and sand. Then, it was able to decrease the oil sweep efficiency by improving the cores' physicochemical characteristics [6]. Meanwhile, the promotion of crude oil's solubilization and mobilization helped the bacterial transportation and metabolism. Moreover, some of the heavy oil fractions' biodegradation also resulted in oil's solubilization and mobilization. Therefore, it was believed that each role promoted and supplemented to each other. In present work, *Pseudomonas* sp. SWP-4 exhibited excellent physiological activities with the addition of WCO, so it can be inferred that the increase of oil recovery was attributed to the better biodegradation and solubilization and mobilization of oil. In addition, biomass plugging might also result in the enhancement of oil recovery.

Similarly, Xia *et al.* [7] reported about 23.02% oil recovery efficiency with the injection of the strain *Pseudomonas aeruginosa* WJ-1 and its biosurfactant rhamnolipid. However, our injected microbial flooding avoided the costly and complex process of biosurfactant purification, and no additional biosurfactant solution needed being injected in present work. In addition, Zou *et al.* [6] reported that the lipopeptide biosurfactant produced by *Acinetobacter baylyi* ZJ2 presented nearly 28% additional oil recovery efficiency, but their employed oil just have an average viscosity of 26.9 mPa·s, which indicated that the solubilization and mobilization of light crude oil itself was superior to our employed heavy oil. As we know, recovering the heavy crude oil is much more difficult than the light crude oil. What's more, the lack of detailed report about heavy crude oil recovery through biotechnology demonstrated the significance of *Pseudomonas* sp. SWP-4 to be applied in heavy crude oil recovery with the addition of WCO.

Table 4
Oil recovery in core displacement experiment using the formation water flooding and microbial flooding produced by *Pseudomonas* sp. SWP-4[a].

4. Conclusions

Pseudomonas sp. SWP-4 was able to grow on heavy crude oil and produce rhamnolipid. Cultivation with the addition of WCO could stimulate the bacterial growth and rhamnolipid accumulation. Meanwhile, the degradation period was shortened and the degradation efficiency was improved to a great extent by adding

WCO. Core displacement experiment showed the oil recovery efficiency of water flooding is relatively low, but the microbial flooding produced by *Pseudomonas* sp. SWP-4 was quite effective. On the one hand, the vigorous bacteria growth resulted in the better degradation of crude oil and viscosity reduction. On the other hand, the produced rhamnolipid altered the oil's solubilization and mobilization. Meanwhile, the addition of WCO helped to produce abundant biomass, so the biomass plugging might also contribute to the increase of oil recovery. Therefore, all of these positive effects manifest that *Pseudomonas* sp. SWP-4 has great potential to be applied in heavy crude oil biodegradation and MEOR process with the addition of WCO.

Acknowledgements

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Table captions:

Table 1

Abilities of *Pseudomonas* sp. SWP-4 to grow on specific hydrocarbon substrates, cultivated at 30℃, 150 rpm for 3 days[a].

Table 2

Physicochemical properties of the employed heavy crude oil[a].

Table 3

Table 4

Oil recovery in core displacement experiment using the formation water flooding and microbial flooding produced by *Pseudomonas* sp. SWP-4[a].

Table 1

Abilities of *Pseudomonas* sp. SWP-4 to grow on specific hydrocarbon substrates, cultivated at 30℃, 150 rpm for 3 days[a].

Substrate	Growth	
	Surface view	DCW (g/L)
n-hexane	++	0.65 ± 0.02
n-dodecane	+++	0.83 ± 0.01
n-hexadecane	+++	0.98 ± 0.02
paraffin wax	+	0.38 ± 0.01

methybenzene	+	0.25 ± 0.03
dimethylbenzene	+	0.15 ± 0.02
naphthalene	+	0.13 ± 0.01

[a] +++: luxuriant growth; ++: good growth; +: poor growth; —: no growth. Results represent the average of three independent experiments ± standard deviation.

Table 2

Physicochemical properties of the employed heavy crude oil[a].

Reservoir	Water content %	Wax %	Resins %	Asphaltene %
Ji-2-Ping-8	3.66±0.07	11±0.05	4.32±0.12	23.86±0.24
	Solidifying point ℃	Viscosity mPa·s (30℃)	Density g/cm^3 (30℃)	Temperature ℃
	11±0	220,000±1,200	0.9488±0.004	25.4–30.7

[a] Results represent the average of three independent experiments ± standard deviation.

Table 3

Percentages of n-alkanes degradation by 7 days at 30℃, 150 rpm: (a) cultivation without the addition of WCO; (b) cultivation with the addition of WCO.

n-alkanes	% Of degradation		n-alkanes	% Of degradation	
	a	b		a	b
C13	99.0	99.4	C24	67.3	87.9
C14	99.3	99.8	C25	95.8	97.1

C15	99.0	99.2	C26	37.1	83.4
C16	97.8	97.9	C27	98.4	99.1
C17	98.8	98.7	C28	96.6	97.9
C18	99.2	99.2	C29	86.3	91.2
C19	99.0	99.1	C30	87.2	86.7
C20	79.6	89.3	C31	99.0	99.4
C21	99.0	99.6	C32	96.9	98.3
C22	90.9	91.9	C33	97.2	97.4
C23	97.6	98.7			

Table 4

Oil recovery in core displacement experiment using the formation water flooding and microbial flooding produced by *Pseudomonas* sp. SWP-4[a].

Core	Flooding type	V_P (mL)	Φ_t (%)	K ($10^{-3}\mu m^2$)	Oil recovery (%)
1#	Water flooding	44.1	30.0	0.64	5.8 ± 3.7
	Microbial flooding				24.4 ± 0.9
2#	Water flooding	44.5	30.2	0.73	6.4 ± 1.9
	Water flooding				0.1 ± 0.1

[a] Results represent the average of three independent experiments ± standard deviation.

Figure captions:

Fig. 1. Schematic view of the core displacement experiment.

Fig. 2. CSH capacity of *Pseudomonas* SWP-4 against specific hydrocarbons. The error bars represents standard deviation values of three independent experiments (n = 3).

Fig. 3. Viscosity−temperature curve of the employed heavy crude oil. The error bars represents standard deviation values of three independent experiments (n = 3).

Fig. 4. Typical time course profiles of the crude oil degradation by *Pseudomonas* SWP-4, cultivated in : (a) 50 mL MSM and 0.2±0.01 g crude oil; (b) 50 mL MSM and 0.2±0.01 g crude oil, with the addition of 0.2 mL WCO. Incubation was performed at 35℃, 150 rpm for 7 days. The error bars represents standard deviation values of three independent experiments (n = 3).

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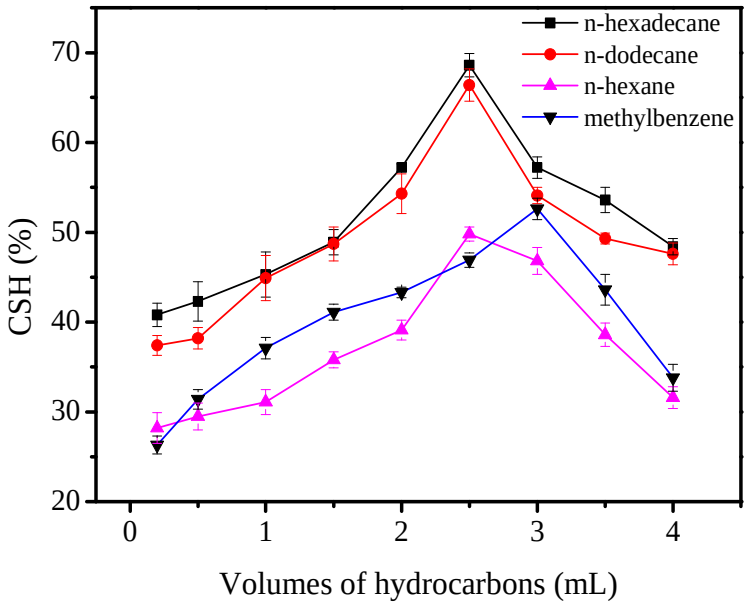

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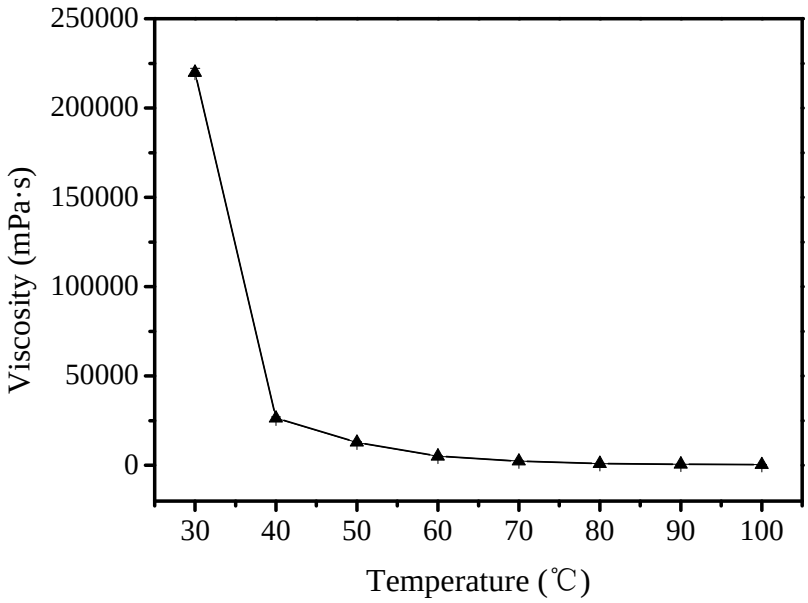

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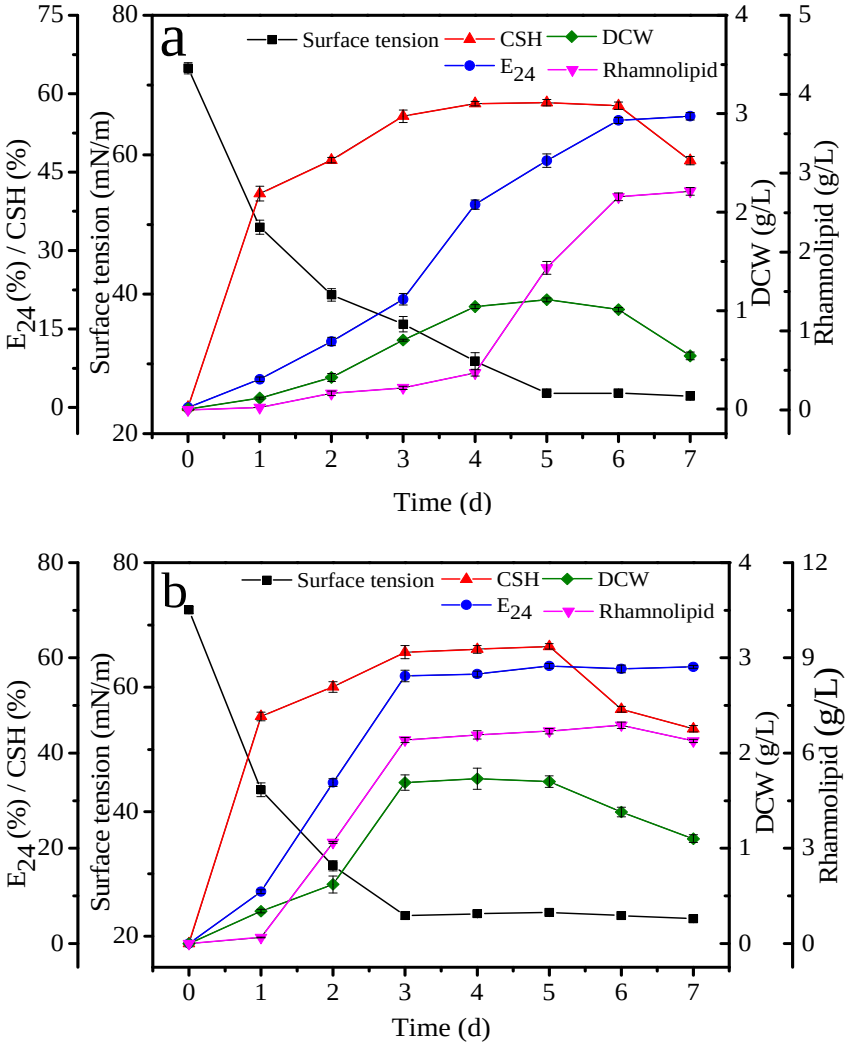

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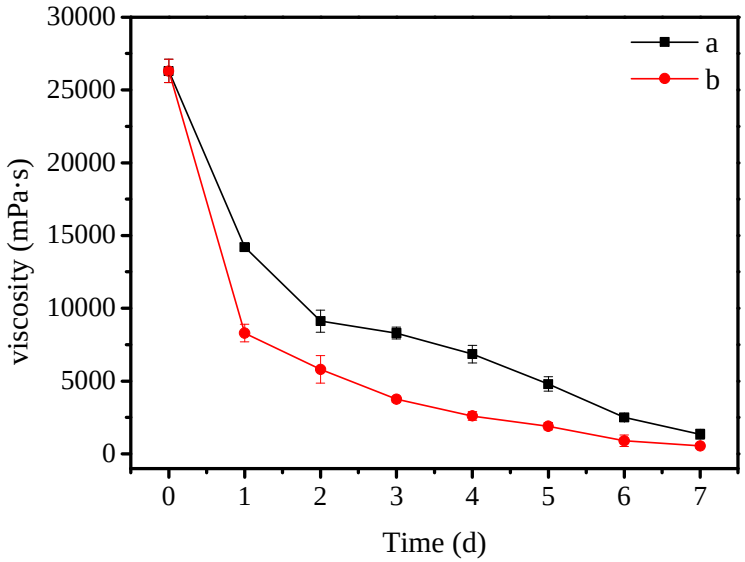

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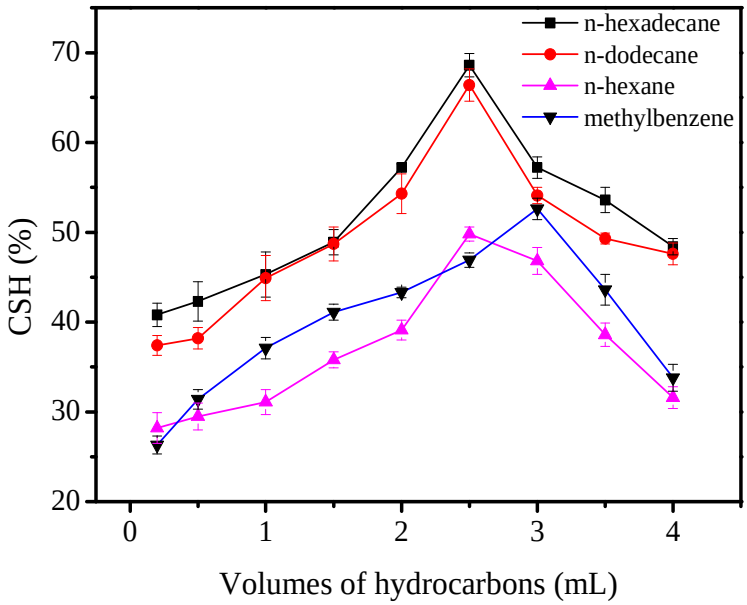

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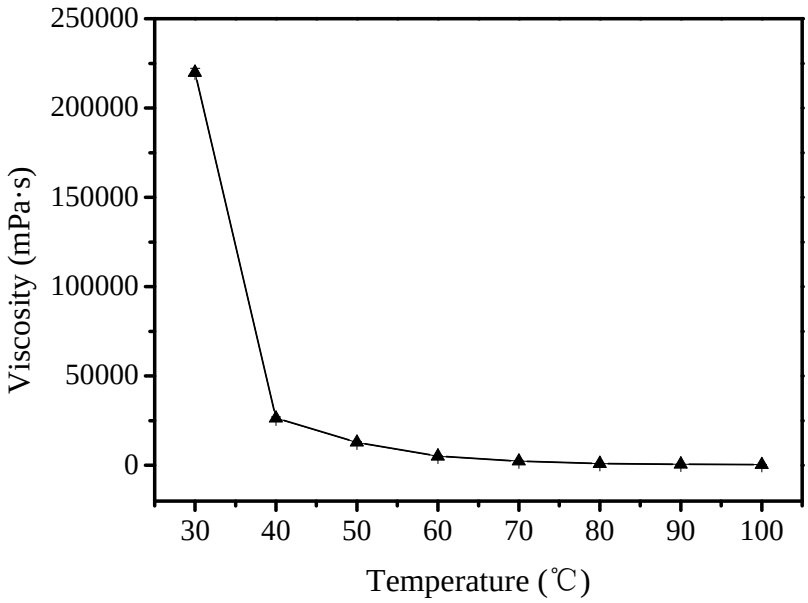

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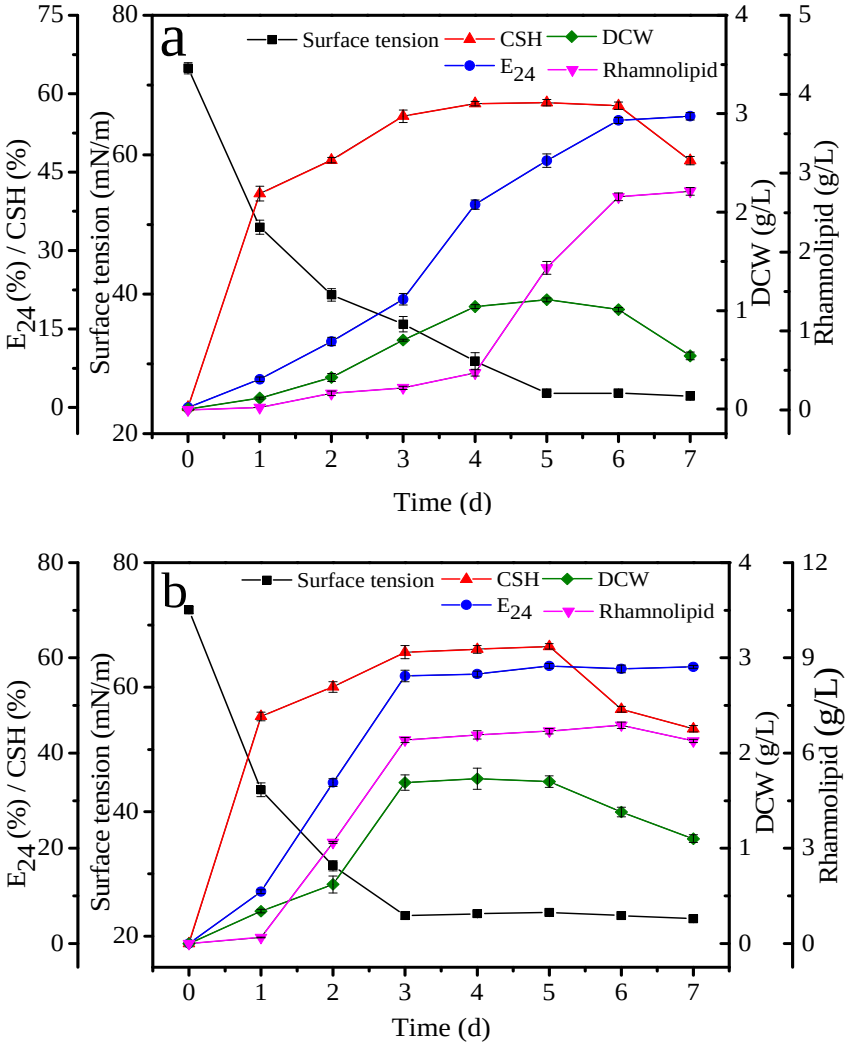

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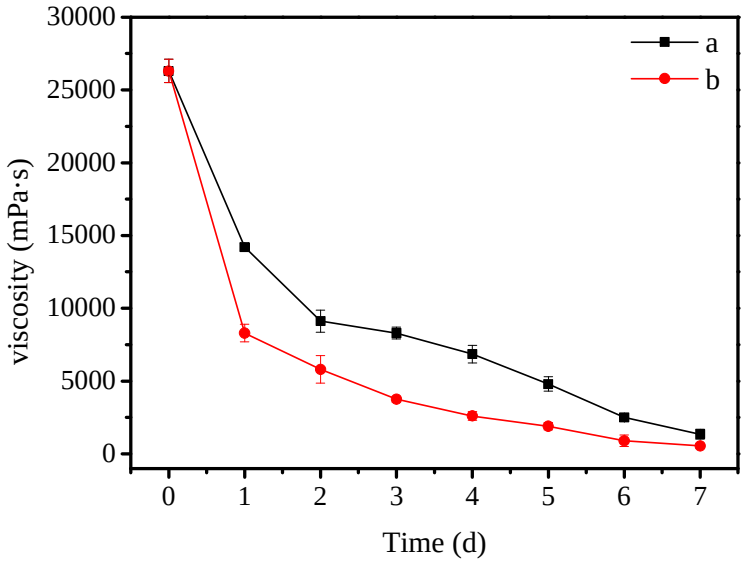

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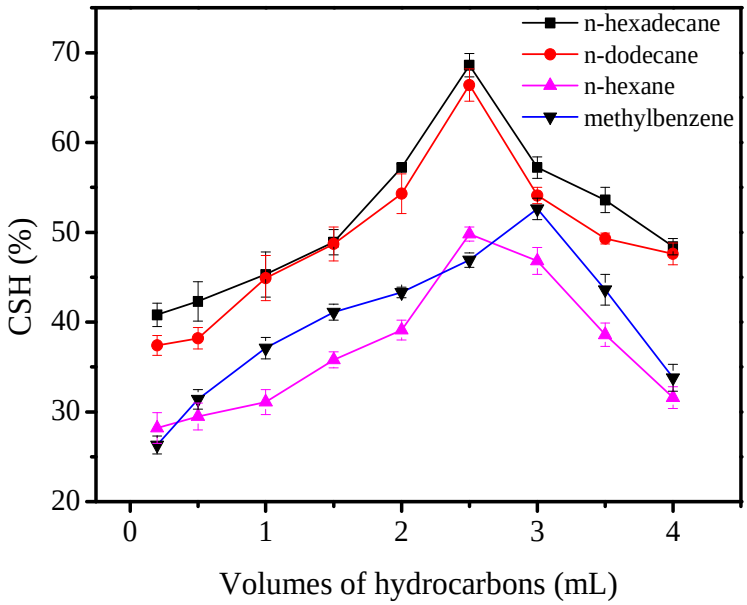

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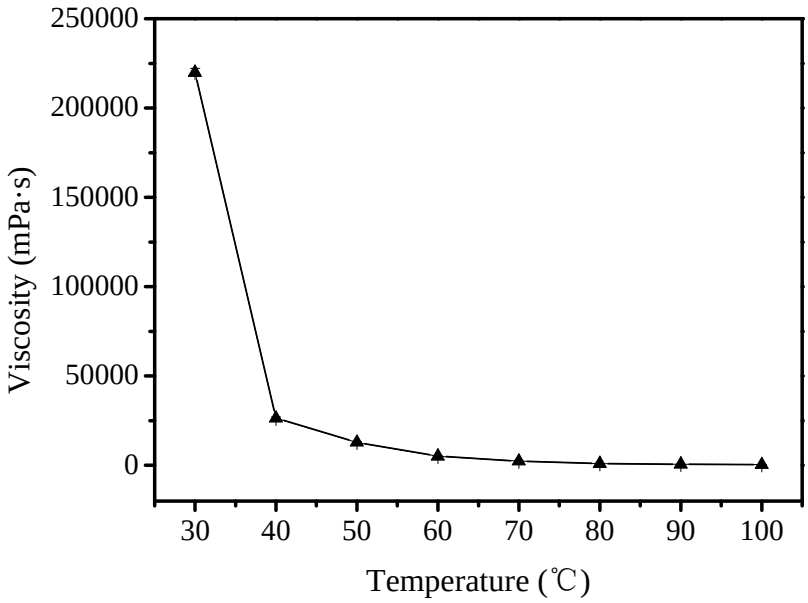

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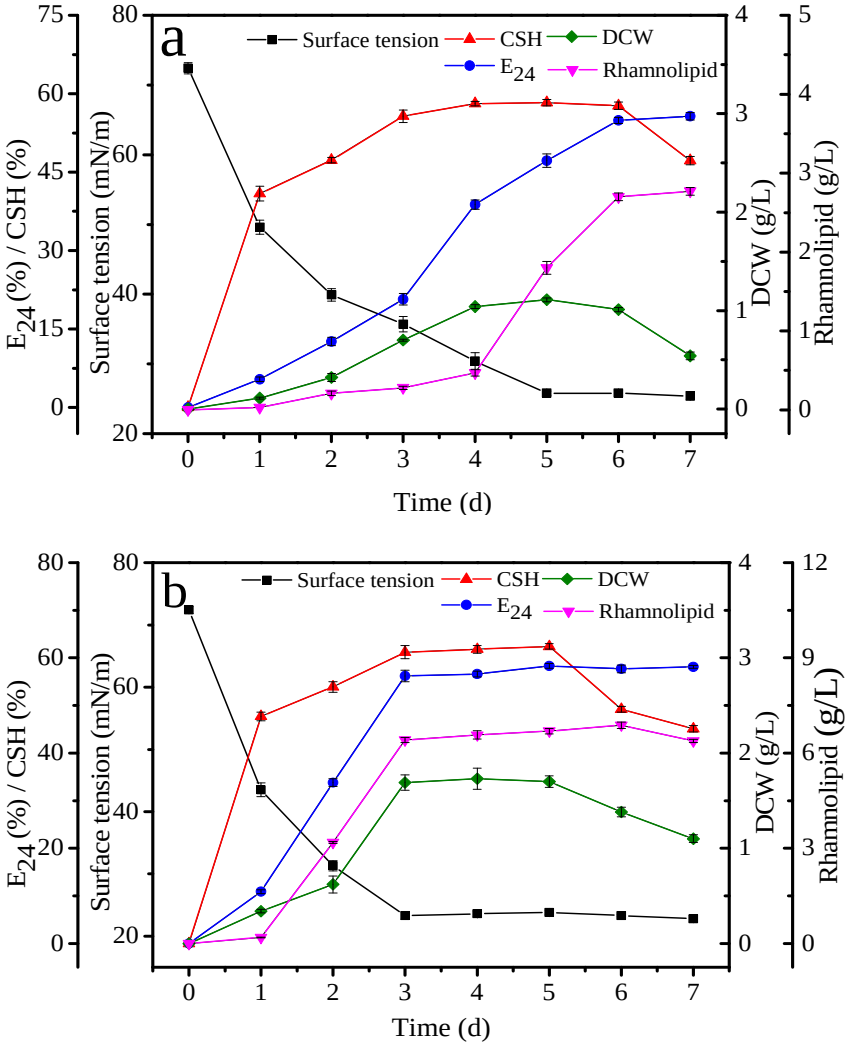

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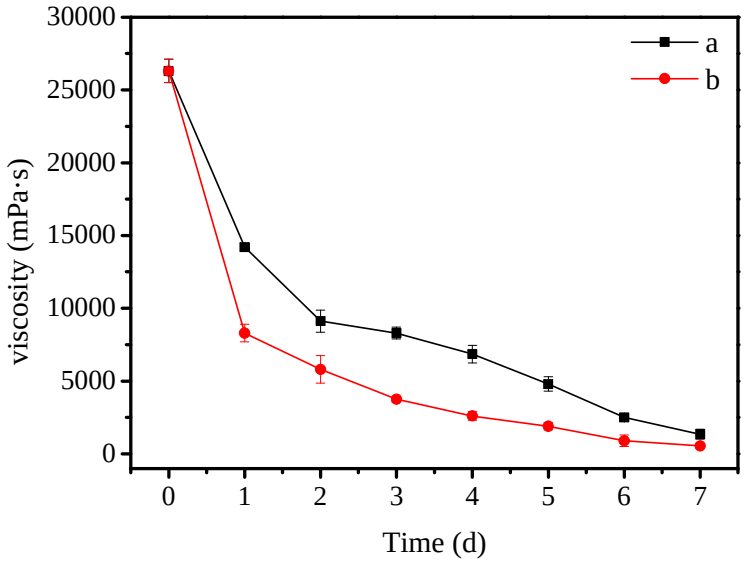

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