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Original Paper

Efficient separation of water-in-oil petroleum emulsions by a newly isolated biodemulsifier producing bacterium, *Delftia* sp. strain HS3

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Abstract

Problems related with refining of water-in-oil (W/O) petroleum field emulsions and also produced water evacuation limitation have created a need for more research in practical demulsification. Among the most commonly used demulsification techniques (e.g., chemical, biological, membrane, electrical, and microwave irradiation) used in the separation of water from crude oil, biological demulsification with bacterial demulsifiers or biodemulsifiers is a new biocompatible method with relatively efficient performance. The main goal of this study was to obtain demulsifying bacteria. Following a multi-step screening and enrichment method and with the aid of biochemical and physiological tests and 16S rDNA sequencing analysis a demulsifying bacterium, *Delftia* sp. strain HS3 (accession number: MF139710) was isolated from crude oil samples of an oil refinery located in southwest Iran. The presence of hydrocarbon material in growth medium was found to be unnecessary for production of the biodemulsifier. The demulsifying ability of the isolated bacterium was investigated through demulsification tests on W/O model emulsions. The demulsifying activity of the produced biodemulsifier was thermally stable and it increased with bacterial culture age. This bacterium has potential as a biodemulsifier producer for future studies and industrial demulsification applications.

Key words: biodemulsifier, demulsification, demulsifying bacteria, water/oil emulsions.
Abbreviations: BATH, bacterial adherence to hydrocarbons; MMSM, modified mineral salt medium; OD, optical density; PCR, polymerase chain reaction; W/O, water-in-oil.

Introduction

Water-in-oil emulsion formations in petroleum fields is a common problem, with water as a dispersed phase being uniformly disseminated in crude oil as continuous phase (Issaka et al. 2015; Lim et al. 2015). Colloidal structures of asphaltene and resin in crude oil act as a natural emulsifier in these emulsions and lead to their long-term stability (Sullivan, Kilpatrick 2002; Mousavi 2016). In upstream operations of oil industries, pipeline emulsion flow is a common occurrence (Rossi et al. 2017). Demulsification is used for elimination of undesirable emulsions in order to achieve better efficiency in refining operations and less damage to equipment and facilities. In this process, through eliminating the intermediate layer between the two phases, the dispersed and continuous phases of an emulsion are separated (Issaka et al. 2015). Demulsification can be achieved by chemical, physical and biological treatments. The best demulsification process must operate with desirable separation efficiency and regard environmental regulations, while imposing the least economic burden on the petroleum industry. Due to disadvantages of chemical and physical demulsification processes, e.g., high energy demand and environmental pollution, biological demulsification as a green method will be a promising and efficient method for practical application in the future and must be investigated (Zolfaghari et al. 2016; Coutinho et al. 2013; Hou et al. 2014a, Issaka et al. 2015).

Several studies have been performed on biological demulsification, where microbial demulsification ability has been reported as a phenomenon associated with whole cells (Liu et al. 2009; Huang et al. 2012; Mohebali et al. 2012; Amirabadi et al. 2013), but specific bacterial metabolites have also been studied (Long et al. 2013; Hou et al. 2014a; Hou et al. 2014b; Li et al. 2017).

Biodemulsifiers are environmental compatible agents that make them ideal substitutes for chemical demulsifiers. Furthermore, their preparation is easy and have good efficiency in demulsification, which promote their practical application in various industries such as in the petroleum industry (Mohebali et al. 2015), but cost of production, limited application and problems related to large-scale have restricted their application. Research regarding biological demulsification has been started since early the 1980s

(Zolfaghari et al. 2016) and since then several bacteria like as *Dietzia* sp. (Liu et al. 2009), *Alcaligenes* sp. (Huang et al. 2012), *Pseudomonas* sp. (Yan et al, 2012), *Paenibacillus* sp. (Amirabadi et al. 2013), *Ochrobactrum* (Mohebali et al. 2012), *Bacillus* sp. (Hou et al. 2014a; Hou et al. 2014b) *Achromobacter* sp. (Li et al. 2017) have been reported as promising demulsifying strains.

The majority of microbial demulsification assessments have been conducted using chemically stabilized emulsion models, which consist of water, hydrocarbon material and surfactant(s). These emulsions can be used for rapid screening of demulsifying performance. Although emulsion models do not imitate emulsions in field conditions, there is an association between demulsification results in emulsion models and these in field emulsions. Most of the previous studies considered the kerosene-water emulsion model for investigating the demulsification performance of the isolated strains (Liu et al. 2009; Huang et al. 2012; Mohebali et al. 2012; Hou et al. 2014a), while the heavy crude oil emulsion model was rarely considered in microbial demulsification (Amirabadi et al. 2013; Long et al. 2013). Thus, the kerosene-water emulsion system was used for evaluation of the demulsification ability of isolated biodemulsifier producing bacteria (Huang et al. 2009). It has been shown that *Bacillus mojavensis* XH1 was able to produce biodemulsifier in order to demulsify kerosene-in-water emulsions (Hou et al. 2014b). The aim of this study was to achieve native biodemulsifier producing strains that could be used for efficient demulsification of water in crude oil emulsions and introducing them as a natural source for substitution of chemical demulsifiers.

Materials and methods

Sampling and initial preparation

Crude oil samples were collected from an oil refinery located in Abadan (30°20'45.57"N 48°16'29.3"E), a city in south-west of Iran, and transferred to laboratory immediately. The initial preparations (pre-enrichment and enrichment) were made in physiological saline (9% sodium chloride solution) and Mueller-Hinton broth (Biolife, Italy), respectively.

Culture medium and cultivation conditions

Modified mineral salt medium (MMSM) was prepared as follows (amounts per L): NH_4NO_3 4.0 g, K_2HPO_4 4.0 g, KH_2PO_4 6.0 g, $MgSO_4$ $7H_2O$ 0.2 g, trace mineral solution 1 mL with 4% (v/v) liquid paraffin as the sole carbon source. Trace mineral contents per L were $CaCl_2$ $2H_2O$ 1.0 g, $FeSO_4$ $7H_2O$ 1.0 g and EDTA 1.4 g. The pH of the medium was adjusted to 7 using 1 N HCl/1 N NaOH. In addition, an agar slant culture medium was used to store the isolates. This medium was prepared by adding 15 g L^{-1} of bacteriological agar powder (Bio-lab, Hungary) to the culture medium (Huang et al. 2009). All of the chemicals were purchased from Merck Company and the crude oil and kerosene were obtained from an oil refinery located in southwest Iran.

Screening and isolation of biodemulsifier-producing bacteria

The isolation and screening were done based on a modified protocol (Huang et al. 2009). About 10 ml of crude oil was inoculated into 90 mL MMSM and cultivated for 7 days in a rotary shaker (120 rpm) at 35 °C. Then, a 100-µL aliquot was inoculated to 100 mL of MMSM and incubated for 7 days. The above process was repeated twice and then biodemulsifier producing bacteria were selected according to demulsification assay.

Demulsification assay and biosurfactant screening methods

The water-in-oil (W/O) emulsion model was prepared by mixing in a 1:1 ratio (v/v) of sterile deionized water and kerosene (or fresh crude oil) containing 1.67% (v/v) of non-ionic surfactant, span 80. The mixture was homogenized with sonication at maximum level for 5 min (ESM, Germany; Huang et al. 2009). The emulsion type was verified by an oil red O test (Lee and Lee 2000). The prepared emulsion was very stable, with an oil separation ratio less than 5% at 35 °C within 7 days.

Demulsification experiments were based on a process for breaking up a W/O emulsion model, which included: (i) contacting the emulsion model with a whole cell suspension and (ii) permitting the reaction mixture to release an aqueous layer. For this purpose, 2 mL of broth culture was added to 10 mL colorimetric tubes containing 5 mL of the W/O emulsion and manually inverted 20 times to produce a uniform culture emulsion mixture. Unless otherwise mentioned, the colorimetric tubes were kept static at 35 °C for 48 h with continuous monitoring and measurement. Controls including culture emulsion mixture and 5 mL emulsions alone were also run in parallel in each experiment. To determine the intra- and extracellular nature of biodemulsifier, demulsification assays was performed with cell-free supernatant as well as cell suspension. Over a prolonged demulsification period, we observed that the emulsion droplets started to coalesce and form larger droplets. After the demulsification was complete, the upper kerosene phase formed a clear layer without any emulsion droplets. The demulsifying ratio (%) of the emulsion model was determined by comparing the volume of the upper kerosene (crude oil) phase and the kerosene (crude oil) volume in the original emulsion using the following equation (Huang et al. 2009; Huang et al. 2012):

$$\text{Demulsification ratio (\%)} = 1 - \text{volume of remaining emulsion} / \text{volume of original emulsion} + \text{volume of added culture} \times 100.$$

In order to emphasize production of the biodemulsifier compounds by the isolated strains, biosurfactant production screening methods [blood-plate hemolysis test, oil-spreading technique, drop collapse method and bacterial adherence to hydrocarbons (BATH) assay] were used. For the blood-plate hemolysis test, a broth culture of

the strain was inoculated on blood agar plates (24 h, 35 °C) and the clear hollow zone around colonies was regarded as biosurfactant activity (Hausmann 201; Hassanshahian 2014).

Similarly, in the oil spreading assay, 20 mL of distilled water was placed in a Petri dish (diameter ~ 80 mm), and 100 µL crude oil was added to the water. A drop (10 µL) of enrichment culture was then transferred onto the oil. The diameter of oil spreading observed indicated the strength of the biosurfactant (Hausmann 2010; Hassanshahian 2014).

The drop collapse assay was performed following the procedure described by Jain et al. (1991) and modified by Bodour and Miller (1998). A sample of of crude oil (100 µL) was applied to the well regions delimited on a 96-well micro-plate and allowed to equilibrate for 1 h. Ten microliters of the cell free broth culture were transferred to the oil-coated regions and the drop size was observed 1 min later with the aid of a magnifying glass. A result was considered positive for biosurfactant production when the drop was flatter than that produced by deionized water (negative control), which is convex (Hausmann 2010; Hassanshahian 2014).

Cell surface hydrophobicity was measured by bacterial adherence to hydrocarbons (BATH) according to a method described by Rosenberg et al. (1980) The cell pellets collected from broth culture medium were rinsed with phosphate-buffered saline (PBS) pH 7.0 twice and then diluted to obtain initial OD_{490} of around 0.8 to 1.0 (original OD_{490}). Then 3 mL of this cell suspension was mixed with 3 ml of kerosene in a test tube on a vortex mixer at 2500 rpm for 5 min. The mixture was left undisturbed for 5 min and then the final OD_{490} of the aqueous phase was measured using an ELISA reader (Bio-Rad, USA). BATH was calculated as follows:

$$\text{BATH (\%)} = 1 - OD_{490}\ \text{final} / OD_{490}\ \text{original} \times 100$$

High BATH values indicate high affinity of the cells for oils (Hausmann 2010; Hassanshahian 2014).

Identification and characterization of isolates

The identification of isolates was done based on phenotypic methods according to Bergey's Manual of Systematic Bacteriology (Bergey et al. 2005). For genotypic identification, sequencing of 16S rRNA using fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGCTCAG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') was also used. Amplification was done at initial denaturation (94 °C, 3 min), followed by 35 cycles of denaturation (94 °C, 30 s), annealing (56 °C, 30 s) and extension (72 °C, 80 s), and a final extension (72 °C, 10 min) (Weisburg et al. 1991). The PCR product was documented through electrophoresis on 1% agarose gel and then sequenced by the Macrogene biotechnology company (Seoul, South Korea). The obtained sequence was edited by Bioedit (version 7.2.5) and compared with the available sequences in the GenBank database using the the BLAST Program.

Effect of culture age on demulsification efficiency

A modified protocol (Coutinho et al. 2013) was used to determine the effect of culture age on demulsification ratio. To investigate this effect, a cell suspension (OD = 0.05, 655 nm) of a selected strain was inoculated to 100 mL Mueller- Hinton broth medium containing 1% liquid paraffin and incubated for 120 h at 35 °C with continuous shaking at 120 rpm. Every 24 h, the bacterial growth as well as the demulsification activity of bacterial suspension was evaluated and recorded and the relation of bacterial growth with demulsification was determined (Coutinho et al. 2013).

Thermal stability of the produced biodemulsifier

To investigate the effect of temperature on the stability of the produced biodemulsifier, the selected strain was cultured in Mueller-Hinton broth containing 1% liquid paraffin for 72 at 35 °C. Then four different treatments (5, 25, 45 and 65 °C) were used for different aliquots of the obtained bacterial suspension and following 1 h treatment, the demulsification activity of the treated suspensions was evaluated. Thermal stability analysis of the biodemulsifier was estimated by the demulsification ratio of fermented broth maintained at a constant temperature in the range of 5 to 65 °C for 60 min at 20 °C intervals (Amirabadi et al. 2013).

Results

In screening and identification of a highly effective demulsifying bacterial strains from crude oil samples a total of four strains were isolated. Strain HS3 had the highest demulsifying capability (95.56%) and was selected for characterization and further analysis. The percentage of demulsification by other isolates was: 47.85, 26.43 and 60.71%. All isolates had an alpha hemolytic zone and showed different results in oil spreading and drop collapse assays (Table 1). HS3 showed high homology (over 99 %) in the 16S rRNA sequence to *Delftia* sp. and based on this and also phenotypic results, it was identified as *Delftia* sp. strain HS3 (accession number: MF139710).

HS3 exhibited better demulsification performance in the water-in-kerosene emulsion model than the water-in-crude oil emulsion model (Fig. 1A, B). In the case of the water-in-kerosene emulsion model, following 24 h incubation, more than 90% demulsification and after 72 h almost 100% demulsification was demonstrated, while demulsification was reduced to half in the water-in-crude oil emulsion model at the above times (Fig. 1C). The comparison of the results of demulsification potential of cell suspension and cell-free supernatant of *Delftia* sp. strain HS3 (75.71 and 35.71%, respectively) revealed that the biodemulsifier of

Table 1. Demulsification and screening assay results of the isolated strains. +, positive; – negative; ++, moderate oil spreading and drop collapse performance; +++, high oil spreading and drop collapse performance. BATH data are reported as an average of two series of test ± standard deviation

Strain code	BATH	Hemolytic activity	Oil spreading performance	Drop collapse assay	The average percentage of demulsification ratio (%)
Control	–	–	–	–	7.14
HS1	70.2 ± 0.0	Alpha	+	+	47.85
HS2	78.7 ± 1.0	Alpha	++	+	26.43
HS3	83.0 ± 2.0	Alpha	+++	+++	95.56
HS4	81.4 ± 3.0	Alpha	++	++	60.71

this bacterium is a cell-wall associated compound and the best demulsification performance will be obtained when the whole cells of the strain used for demulsification. An increase in the demulsification rate occurred in parallel to increase in culture age. Thermal stability analysis suggested that the produced biodemulsifier by *Delftia* sp. strain HS3 can efficiently break water-in-oil emulsions under extreme thermal conditions due to its stability over 5 to 65 °C (Fig. 2).

Discussion

In the present study, the demulsifying activity of isolated strains was measured using a stabilized W/O emulsion containing kerosene and sterile deionized water as the oil and aqueous phases, respectively and non-ionic surfactant, Span80, which acts as an emulsifier between these two phases. The demulsification efficiency of HS3 reached 98.14% in separating water in the kerosene emulsion model under optimal conditions (35 °C, pH 7), which reflected the highly efficient demulsification activity of HS3. This demulsifying performance is comparable to the performance of demulsifying bacteria that have previously been reported. For example, in previous studies, a 96.5% demulsification ratio was shown for the demulsifying bacterium, *Alcaligenes* sp. S-XJ-1 in the W/O emulsion model (Huang et al. 2009), while other biodemulsifiers from *Achromobacter* sp. LH-1 and *Bacillus cereus* LH-6 cells reached 95.40 and 95.6% efficiency in the same emulsion model, respectively (Hou et al. 2014a; Li et al. 2017).

Despite good demulsification performance of the strain HS3 in water-in-kerosene emulsion models, this strain had poor performance in demulsifying water-in-crude oil emulsion models (58.45%; Fig. 1C) due to the presence of colloidal structures of asphaltene-resin in crude oil, which act as a natural emulsifier and stabilizer of emulsion. Presence of these structures led to more stability of emulsions and made their demulsifying more difficult (Issaka et al. 2015). This low efficiency of demulsification was in accordance with the demulsifying performance of biodemulsifier producing bacteria in breaking petroleum field emulsions (Amirabadi et al. 2013).

Lowest demulsification capability in the W/O emulsion model occurred when the hydrocarbon (liquid paraffin) was used alone as the carbon and energy source in culture medium and highest demulsification capability in the W/O emulsion model was when liquid paraffin was used along with Mueller-Hinton broth. Therefore, we can conclude that the presence of a hydrocarbon material in growth medium may be unnecessary for production of the biodemulsifier, but it strengthens the demulsifying efficiency of the strain. This can be explained as follows: surface substances such as biodemulsifiers with different compositions and contents of hydrophobic side chains

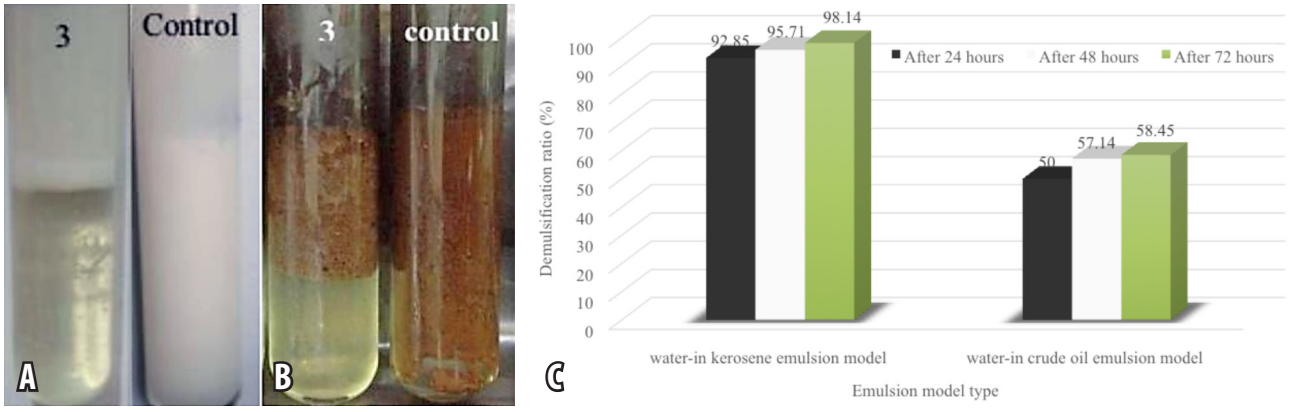

Fig. 1. The demulsification performance of *Delftia* sp. strain HS3 in demulsifying water-in-kerosene emulsion model (A) and water-in-crude oil emulsion model (B) after 24, 48 and 72 h (C). The controls have only contained emulsion model and non-inoculated cultures.

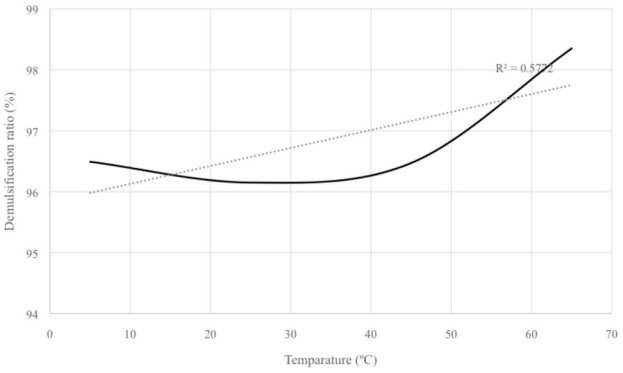

Fig. 2. Demulsifying efficiency of HS3 over a wide range of temperatures.

or hydrophobic ends is affected by the growth medium composition, especially by the carbon source. Presence of hydrocarbons or hydrophobic materials in growth medium could lead to more hydrophobic ends in the biodemulsifier structure, which affects the cell surface hydrophobicity of biodemulsifier producing bacteria and improving their demulsifying performance. As shown in Table 1, bacterial adherence to hydrocarbons (BATH) and diameter of clearing zones on oil film of tested isolates showed some correlation with their demulsification efficiency. This correlation indicates a positive effect of cell surface properties, especially surface hydrophobicity of cells, in demulsification. The adherence capability of demulsifying cells to the oil phase and the aggregation of dispersed droplets would be strengthened using demulsifying cells with higher cell surface hydrophobicity. Regarding the demulsification rate, it is well known that bacteria with a hydrophobic cell surface prefer hydrophobic materials. In W/O emulsions, demulsifying strains, due to their biodemulsifier production, have a tendency to be located in the hydrophobic phase, i.e., in the oil phase, and hence they are easily dispersed in W/O emulsions. A quicker dispersion rate and demulsification srate will be achieved with greater cell surface hydrophobicity of the demulsifying strain. This phenomenon is completely consistent with previous studies regarding the influence of surface properties of demulsifying bacteria on demulsification efficiency (Liu et al. 2011). The fact that the demulsifying bacterium HS3 exhibited its best demulsification capability in the presence of hydrocarbon material in culture medium might simply reflect the long-term adaptation of this bacterium to hydrocarbon pollution in petroleum fields. This result was similar to the findings of the previous studies (Hou et al. 2014a; Hou et al. 2014b).

In this study, an increase in demulsification was observed with an increase in culture age. Maximum values, almost 100% activity, were obtained for cells of a 72 h old culture. This period corresponded to a higher cell concentration of the strain (OD_{655} = 1.2). The bacterial growth was monitored during the culture period, and an increase in OD was observed for up to 72 h. After this period, OD values decreased, remaining approximately close to 1 after 120 h. This decrease can be explained by cell lysis or because the cells became more hydrophobic and were present on the surface of the medium with the liquid paraffin. The demulsification ratio in the W/O emulsion model increased in proportion to the amount of bacterial cells, which represents a clear positive correlation between the demulsification ratio and cell concentrations. For pure bacterial cultures, the relationship between initial rate of demulsification and cell concentration has been found to be linear. These results are consistent with previous studies. For example, Mohebali et al. (2012) also observed an increase in demulsification rate with an increase in culture age (Mohebali et al. 2012). Correlation has been shown between culture age and demulsification of the biodemulsifier producing strain, *Pseudomonas aeruginosa* MSJ and the highest activities were observed for cells and supernatants from 96 h cultures (Coutinho et al. 2013).

After incubation of HS3 fermented broth under optimal conditions for 72 h, thermal stability of the biodemulsifier and its efficiency was investigated over a wide range of temperatures. The experiments showed that increasing the temperature of the fermented broth up to 65 °C and decreasing it to 5 °C did not show any significant effect on the demulsification activity of the biodemulsifier and its thermal stability. The obtained results suggests that the produced biodemulsifier can efficiently break water-in-oil emulsions under extreme thermal conditions due to its stability over a wide range of temperatures. As a consequence, the produced biodemulsifier is able to be employed under extreme industrial conditions with no significant changes in its properties. A less positive slope of the curve (R^2 = 0.5772) showed the impact of high temperatures on demulsificaion performance. A slight increase was observed in the demulsification ratio at 65 °C treatment, which might have resulted from damage to the bacterial cell wall and release of the cell wall associated biodemulsifier, which could improve the demulsification ratio (Fig. 2). This result was similar to the findings of the previous studies (Amirabadi et al. 2013; Coutinho et al. 2013).

Conclusions

In upstream applications of drilling industry, the effective separation of water from W/O emulsions is very challenging for achieving high quality crude oil with low content of water. The toxic chemicals applied in traditional demulsification have harmful effects on the environment, thereby safe and effective substitutes are demanded. Bacterial demulsifiers are potential alternatives for chemical demulsifiers used in the petroleum industries. The worldwide increase in attention paid to biological demulsification can be attributed to several factors, including non-toxic and renewable nature

of the biodemulsifiers. In this study a new demulsifying bacterium, *Delftia* sp. strain HS31 was isolated; the results of our study indicated that fermented culture of this bacterium showed efficient demulsifying activity in the emulsion models. The produced biodemulsifier was shown to be a thermally stable demulsifying agent for demulsifying W/O emulsions in extreme thermal conditions of the drilling process. This strain therefore shows potential as a demulsifier producer for future studies and industrial application.

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