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RESEARCH ARTICLE

Investigation of the relationship between cell surface hydrophobicity and demulsifying capability of biodemulsifier-producing bacteria

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BACKGROUND: Cell surface hydrophobicity (CSH) is one of the key physicochemical features of biodemulsifier-producing bacteria that influence their demulsification capability maintenance in petroleum contaminated environments.
METHODS: In present study, biodemulsifier-producing bacteria were isolated from petroleum contaminated environments using different isolation media and the correlation between their CSH and demulsifying ability was investigated. The demulsifying ability of isolates was measured through demulsification tests on water in kerosene emulsions. The microbial adhesion to the hydrocarbon (MATH) assay was used to denote their CSH.
RESULTS: The evaluation of CSH showed that majority of biodemulsifier producing bacteria have high CSH which indicating a positive correlation between CSH and demulsifying capability.
CONCLUSIONS: According to these results it can be concluded that CSH can be used as an indicator for assessment of biodemulsifier-producing bacteria and screening of new isolates for their biodemulsifier production.

Keywords bacterial adhesion, biodemulsifier, biodemulsifier-producing bacteria, demulsification, hydrophobicity, MATH assay

Introduction

"Biosurfactants are a variety of amphipathic or surface-active compounds" (Khan and Butt, 2016) that some microorganisms such as bacteria (e.g. *Bacillus subtilis* and *Pseudomonas aeruginosa*), yeasts (e.g. *Wickerhamiella domercqiae* and *Candida batistae*) and some filamentous fungi (e.g. *Aspergillus*, *Ustilago maydis* and *Pseudozyma flocculosa*) can produce them during their metabolism on water immiscible substrates (Luna et al., 2014). They can be found as cell membrane associated or as secreted extracellular metabolite (Sekhon et al., 2012). Main prominent properties of biosurfactants in comparison with synthetic surfactants which promote their application in different industries are structural diversity, biocompatibility, excellent surface properties and low toxicity (Fakruddin, 2012; Oliveira et al., 2015). To explore heavy oil in petroleum industry applying of biosurfactants have been suggested advantages over the synthetic surfactants in the entire petroleum processing chain. Main application fields of biosurfactants in petroleum industry are facilitation of heavy crude oil transport through pipelines, microbial-enhanced oil recovery (MEOR) and clearance of contaminated vessels (Luna et al., 2014; Silva et al., 2014). However, the demulsification potential of biosurfactants for separation of crude oil emulsions has been less paid attention than the other applications of them e.g., bioremediation. The double function of some biosurfactants, i.e., emulsification and demulsification ability has been reported in previous studies (Huang et al., 2009). The bioproducts that were able to break emulsion were entitled as biological demulsifiers or microbial demulsifiers (Liu et al., 2009).

Biodemulsifier is a type of biosurfactant that due to its unique functional groups has high efficiency in breaking industrial emulsions including petroleum emulsions (Huang et al., 2009). The main privilege of biodemulsifiers than chemical demulsifiers is their environmental compatibility.

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So, this unique feature made biodemulsifiers as potent candidates to be used in many commercial applications such as petroleum industry. Demulsification of produced emulsions during oil drilling process is one prominent field of application of biodemulsifiers in oil industry (Liu et al., 2009). It has been shown in different researches that most of the bacteria originated from oil contaminated habitats have the ability of demulsification of water in oil and/or oil in water emulsions. The native strains can survive and become predominate in such environments due to their ability in production of either surface-active substances such as biodemulsifiers or by altering CSH increase their affinity to oil which facilitate hydrocarbons utilization by them through appropriate their positioning between hydrophobic (oil) and hydrophilic (water) phases and efficient demulsification (Huang et al., 2010; Amirabadi et al., 2013; Salehizadeh et al., 2013; Hou et al., 2014a, 2014b). So, CSH and the ability to producing biodemulsifiers are main factors that affect the ability of bacteria to demulsify petroleum emulsions (Liu et al., 2011b). Therefore, knowing the CSH of bacterial cells facilitates the biological demulsification process of petroleum emulsions through biodemulsifiers.

Among the various methods for assessment of the CSH of microbial cells the best method is microbial adhesion to hydrocarbons or MATH assay which is simple, rapid and easy to operate. This assay was first developed by Rosenberg and applies a simple procedure based on the percentage of bacterial cells adhering to the hydrocarbons to measure the bacterial relative hydrophobicity (Rosenberg et al., 1980; Rosenberg, 2006; Chao et al., 2014). In this assay the bacterial suspension is vortexed in the presence of a test liquid hydrocarbon. In this process the microorganisms adhere to the droplets formed as a result of hydrocarbon breaking into small droplets and then rise with the hydrocarbon during phase separation. Then via comparing the initial and final absorbance of the microbial suspension, the fraction of adhering microorganisms will be evaluated (Zoueki et al., 2010). Till now, several studies have paid attention to the relationship between cell surface properties and demulsifying effectiveness (Liu et al., 2011a).

The aims of this study were to (i) isolate bacterial strains with demulsification activity for breaking water in kerosene model emulsions; (ii) evaluation of their CSH; and (iii) find the relationship between CSH of demulsifying strains and their demulsification activity.

Materials and methods

Environmental samples from crude oil polluted regions were collected from an oil refinery located in the south-west of Iran and immediately transferred to laboratory. The enrichment and screening were carried out in Mueller-Hinton broth (Biolife, Italy) and modified mineral salt medium (MMSM), respectively (Huang et al., 2009). The broth culture of purified isolates was screened for biodemulsifier production through measuring the demulsification activity on water in kerosene-stabilized emulsions. To prepare water in kerosene emulsion model, 50 ml of sterile deionized water and 50 ml of kerosene containing 1.67% (w/v) of span 80 were mixed and homogenized through 10 min sonication at maximum power (ESM, Germany) (Huang et al., 2010). The prepared emulsion was stable and showed less than 5% of demulsification ratio at room temperature within 48 h. The kerosene was obtained from the mentioned refinery with the specification presented in Table 1.

Two ml of the bacterial culture grown in Mueller-Hinton broth was added in graduated test tubes containing 5 mL of the water in kerosene emulsion and manually inverted for 3 min to produce a homogeneous emulsion and incubated at 35°C in a water bath. For all experiments, two ml of non-inoculated broth culture and five ml of water in kerosene emulsion was used as control. All demulsification tests were carried out in duplicate and the mean of results was reported. At the end of demulsification, the volume of residual emulsion (middle layer) was recorded and the demulsification performance of isolates was evaluated by calculating demulsification ratio, using following equation (Liu et al., 2011a):

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

Table 1 Properties of kerosene used for preparing the emulsion model

Property	Unit	NIORDC [a] standard
Freezing point (max)	°C	–47
Density at 15°C (max.)	kg/m^3	775-830
Distillation recovered at 185°C (max.)	Vol%	50
Distillation FBP (max.)	°C	275
Total aromatics content (max)	Vol%	25
Smoke point (min)	mm	25
Flash point (min)	°C	43

[a] National Iranian Oil Refining & Distribution Company

Measurement of CSH through MATH assay

The MATH assay was used to measure the CSH of the bacterial strains. This indirect method quantifies the surface hydrophobicity of bacteria by quantifying the relative percentage of retained bacteria in a hydrophobic phase after mixing with an aqueous phase containing the initial bacterial culture. The obtained cell pellet was twice rinsed with phosphate-buffered saline (PBS) pH 7.0 and then diluted to obtain initial OD_{490} around 0.8-1.0. Then, three ml of this cell suspension was vortex-mixed with three ml of kerosene in a test tube at 2500 r/min for 5 min. The mixture was left undisturbed at room temperature for 5 min and then final OD_{490} of the aqueous phase was measured. MATH was calculated using following equation:

$$\mathrm{MATH}(\%) = \frac{1 - \mathrm{OD}_{490}(\mathrm{final})}{\mathrm{OD}_{490}(\mathrm{initial})} \times 100$$

This value varies from zero to 100 percentage. A higher value indicates higher cell hydrophobicity. For all experiments, three ml of PBS and three ml of kerosene was used as control (Liu et al., 2011a; de Wouters et al., 2015).

Results

As a result of primary isolation 20-four demulsifying bacterial strains were isolated from environmental samples. Their demulsification performance was measured in two series of demulsification test conducted for destabilizing water in kerosene emulsion after 48 h at 35°C. The blank with two ml of non-inoculated culture broth showed 7±1 percentage demulsification after 48 h. The cell surface hydrophobicity of 24 isolates was measured according to MATH evaluation in duplicates. The blank with three ml of PBS and three ml of kerosene showed no demulsification activity. The recorded results were presented in Table 2.

Discussion

The ability of bacterial cells to adhere to different surfaces is greatly dependent to bacterial CSH (Chakraborty et al., 2010). This property has been investigated in various processes including wastewater treatment (Chao et al., 2014), bioremediation for hydrocarbon biodegradation (Abbasnezhad et al., 2011) and adhesion ability and demulsifying activity of biodemulsifier-producing bacteria (Liu et al., 2011b; Fernandes et al., 2014). Therefore, measuring the CSH of biodemulsifier-producing bacteria to find their adhesion property to the oil-water interfaces is necessary.

The results of various studies have also pointed out that the MATH assay provides a clue for cell adhesion to hydrophobic

Table 2 Demulsification ratio and MATH percentage of the isolated strains from environmental samples of the refinery [a]

Code of isolated	Source	Demulsification ratio (%)	MATH (%)
1	Crude oil	47.85±3	70.19±0
2	Crude oil	26.43±2	78.71±1
3	Crude oil	95.71±1	83.02±2
4	Crude oil	64.29±2	81.36±3
5	Drainage	49.66±0.4	78.52±0
6	Drainage	49±0	75.80±0
7	Drainage	97.14±2	85.96±2
8	Drainage	42.86±2	77.24±1
9	Crude oil contaminated soil	25±3	64.43±0
10	Crude oil contaminated soil	28.57±5	73.76±1
11	Crude oil contaminated soil	27.14±1	62.22±0
12	Crude oil contaminated soil	51.57±1	67.82±4
13	Crude oil sludge	30.71±2	69.71±1
14	Crude oil sludge	30.71±2	74.05±2
15	Crude oil sludge	50.43±2	69.27±1
16	Crude oil sludge	33.56±2	75.23±1
17	Crude oil sludge	28.57±0	66.38±0
18	Crude oil precipitate	32.14±6	76.11±2
19	Crude oil precipitate	92.85±3	76.11±1
20	Crude oil precipitate	71.43±1	72.99±0
21	Crude oil precipitate	38.57±2	65.38±2
22	Crude oil precipitate	85.71±3	72.24±1
23	Crude oil precipitate	50.14±1	69.58±1
24	Crude oil precipitate	50.28±1	69.89±2

[a] Data were reported as an average of two series of test±standard deviation

hydrocarbon surfaces. In spite that MATH assay is widely used as a measure of hydrophobicity, some studies have shown that it correlates poorly with other hydrophobicity assays. For instance, Zoueki et al. (2010) have shown that it measures a combination of forces, such as electrostatic and van der Waals interactions, as well as various short-range forces. It has been found that if the surfaces in question are uncharged or if the system be at isoelectric point, the MATH assay is only a measurement of hydrophobicity (Zoueki et al., 2010). Hence this, Rosenberg has suggested doing experiments at high ionic strengths to minimize electrostatic effects. Therefore, the MATH assay can be regarded as hydrophobicity assay if it has been performed at the isoelectric point, with a high ionic strength, or when the surfaces are not charged. Otherwise, the MATH assay is simply valid as an adhesion test (Rosenberg et al., 1980; Rosenberg, 2006). Also Van der Mei et al. (1995) found that besides surface hydrophobicity, the adhesion of microorganisms to hydrocarbon droplets is pH dependent. This indicates that, electrostatic forces are also involved in the adhesion process (Van der Mei et al., 1995). Using phosphate urea magnesium sulfate (PUM) buffer in MATH assay, due to its high ionic strength (more than 150 mM) can diminish electrostatic effects and thus only accentuated the hydrophobicity interactions. Therefore, in MATH assay for evaluating microbial surface hydrophobicity, PUM or other buffers with high ionic strength are strongly recommended (Chao et al., 2014). As it has been seen in the present study, adhesion percentage to hydrocarbons (e.g. kerosene) obtained in the MATH assays carried out using PBS as an aqueous phase were higher for the most of biodemulsifier-producing bacterial strains. Lower cell surface hydrophobicity detected in assays carried out with PUM buffer in previous studies could be attributable to different ionic strength of this buffer (Kadam et al., 2009). A report suggested that in comparison with PBS, PUM buffer due to its magnesium sulfate and urea, could interfere with the adhesion of bacterial cells to hydrocarbons, thus yielding lower percentages of cell surface hydrophobicity (Balebona et al., 2001). To eliminate this problem, the present study recommends using PBS buffer in the measurement of CSH. The evaluation of CSH showed that the majority of biodemulsifier-producing bacteria from petroleum-polluted origins have medium to high CSH (Fig. 1 and Fig. 2).

Linear regression of CSH and the demulsification efficiency shown in Fig. 3 demonstrated a positive relationship

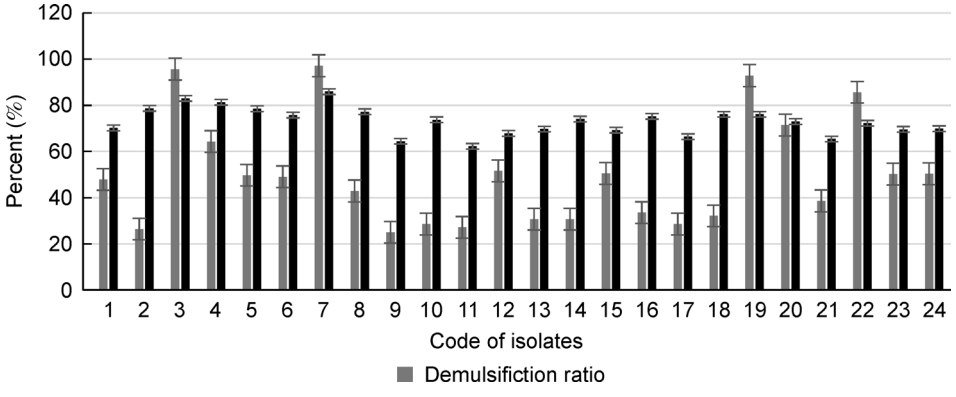

Figure 1 Demulsification capability and cell surface hydrophobicity of the demulsifying bacteria with taking into account the standard deviation of the experiments. The cell surface hydrophobicity of the isolated bacterial strains was determined by evaluation of their adhesion to the hydrocarbons according to MATH assay.

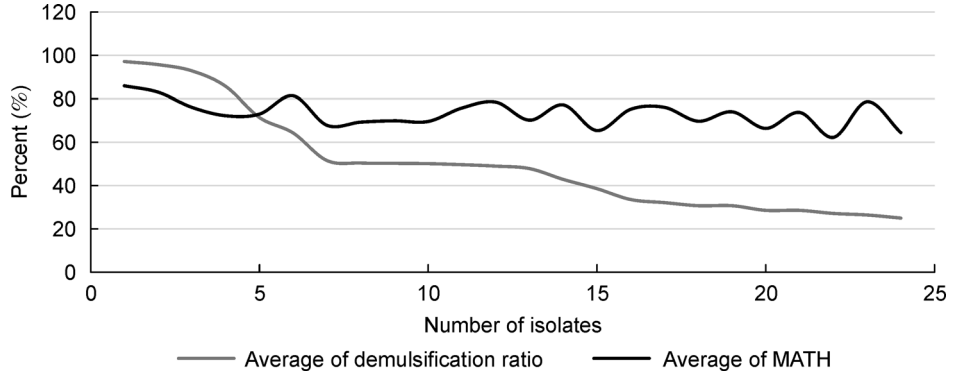

Figure 2 Linear relationship between the demulsification capability and cell surface hydrophobicity of the demulsifying bacteria. The positive relationship between these two variables is quite evident.

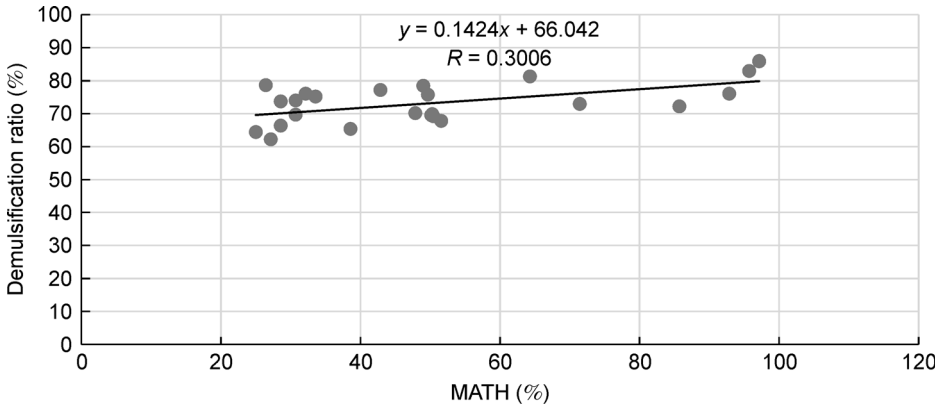

Figure 3 Correlation between the demulsification ratio and MATH of the demulsifying bacteria. The demulsification ratio was plotted as a function of the MATH. This figure showed that the demulsification ability of the demulsifying strains was largely dependent on their cell surface hydrophobicity.

between these two variables (R^2 = 0.30). This result demonstrated that the demulsification ability of the demulsifying strains was largely determined by their physicochemical properties such as CSH. Rare experiments have been focused on the relationship of physicochemical properties like as CSH and demulsification potential of bacteria. Cairns et al. (1982) which made the first report in this field speculated that the demulsifying ability of *Nocardia amarae* was induced by the properties of the cell surface (Cairns et al., 1982).

Huang et al. (2012) reported that physicochemical properties of Alcaligenes sp. S-XJ-1 is largely affect its demulsification ability and find a strong linear relationship between the MATH and demulsification efficiency of this strain which indicates a correlation between the CSH and demulsification ability (Huang et al., 2012). This finding is completely correlated with the research of Liu et al. (2011) which indicated that initial culture pH can influence the surface properties of demulsifier producing bacteria (Liu et al., 2011b). The results of the present study are in accordance with these previous reports. Therefore, CSH can positively affect demulsification progress.

As it has been shown in Fig. 4 the adsorption capability of demulsifying cells in the oil phase and the aggregation of dispersed droplets would be strengthened using demulsifying cells with higher CSH which in return improving demulsification activity. In the demulsification process, a higher CSH of demulsifying cells could promote cell transfer to the water-oil interface due to an improved affinity with kerosene. The alteration of interfacial properties would generate the occurrence of emulsion breaking. Better demulsification results will be expected as the CSH will increase.

As a result, highest demulsification ratios have been seen in strains that have high CSH (Fig. 5) but high levels of CSH are not necessarily mean higher demulsification (Fig. 6). Previous studies showed that a good demulsifier have three traits: good miscibility in dispersed phase, good diffusivity and high surface activity. Due to amphipathic property of biodemulsifier it can adsorbed on oil/water interface and act to alter the

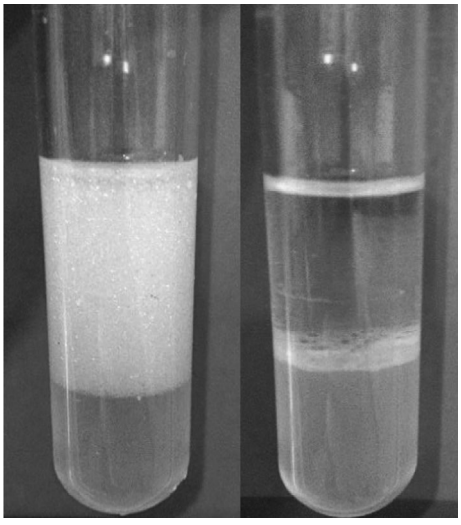

Figure 4 The adsorption capability of demulsifying cells in the oil phase (left) and the control (right). The control contained 3 ml of PBS and 3 ml of kerosene. The production of biodemulsifiers by demulsifying bacteria facilitated the dispersion of them into the oil phase.

properties of the interface (Huang et al., 2010). In the present study, it was found that the cultures of five highly efficient strains (3, 7, 19, 22, and 20) quickly disperse into the emulsion, showing good miscibility. Early studies showed that the CSH of demulsifying bacterium is an important factor determining demulsification efficiency (Ma et al., 2006). According to Liu et al. (2004) the cells with higher CSH can potentiate adsorption of demulsifying cells in the oil phase and the aggregation of dispersed droplets (Liu et al., 2004). Huang et al. (2012) suggest that, in the demulsification process, a higher CSH could accelerate the transference of the cells to the water-oil interface which is the result of improved affinity to kerosene, and the alteration of interfacial properties would contribute to emulsion breaking (Huang et al., 2012).

Microorganisms are able to modify emulsion properties

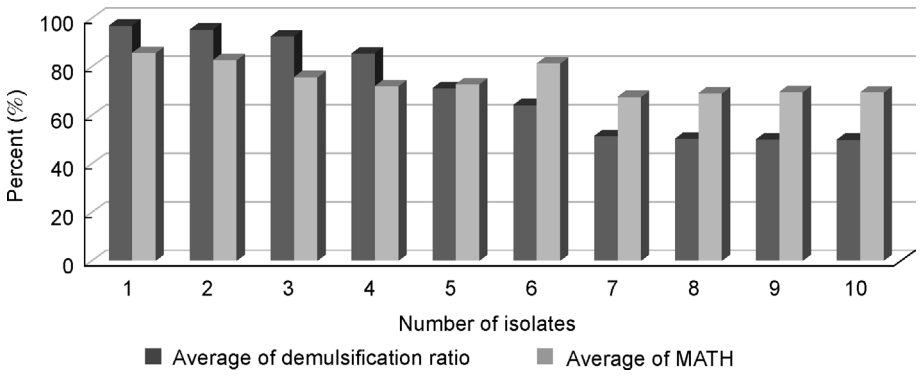

Figure 5 Relationship between the demulsification capability and cell surface hydrophobicity of the demulsifying bacteria with ≥ 50 percentage of demulsification ratio. Best demulsification performance have been seen in strains which have high cell surface hydrophobicity.

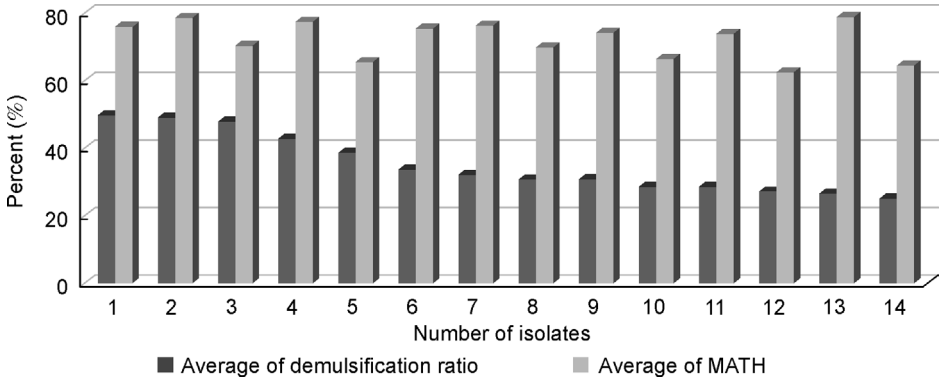

Figure 6 Relationship between the demulsification capability and cell surface hydrophobicity of the demulsifying bacteria with 50 percentage of demulsification ratio. High levels of cell surface hydrophobicity do not always mean high demulsification.

through displacing or altering the emulsifiers that are present at the oil-water interfaces (Coutinho et al., 2013). During demulsification at first flocculation of droplets and then their coalescence will happen in order to form a continuous second phase. Demulsifiers can improve one or both of these steps (Al-Sabagh et al., 2011). Based on the hydrophobicity of cell surface, aggregation of cells at the water-oil interface may take place and promote flocculation and coalescence of oil droplets. In general, it seems that for treatment of water/oil emulsions more hydrophilic cells are required, while in case of oil/water emulsions relatively more hydrophobic cells are needed (Kirkwood et al., 2004). Results of Mohebali et al. (2012) showed similar pattern of demulsifying activity and cell-surface hydrophobicity of strain RIPI5-1 during various growth phases which represents that the demulsifying activity of whole cell might be cell surface-mediated (Mohebali et al., 2012).

In the present study, the strains were isolated from environmental samples of an oil refinery located in the south-west of Iran. These strains represented from a variety of sources (as shown in Table 2). As it is shown in Table 2, the cell surface hydrophobicity of the tested isolates showed some correlation with their sources. In the present study, all 20-four strains that isolated from petroleum-related sources, such as crude oil, drainage of storage tanks, oilfield soils and crude oil sludge and precipitate showed high MATH value which indicating a direct relation between cell surface hydrophobicity and presence of hydrophobic substances in the environments. It seems the presence of hydrophobic substances alter the cell surface hydrophobicity and increasing it. As a consequence of natural selection in petroleum-contaminated environments, microbial diversity will decrease only the hydrocarbon degrading strains will survive and predominate. These strains through production of amphipathic agents or increase their affinity to oil by altering cell surface hydrophobicity facilitate their metabolism in such environments (Huang et al., 2010). The importance of adhesion in biological demulsification processes may be underestimated in many studies. Most experiments without considering their counter-effect on detaching cells from the interface have focused on the role of surface-active compounds to increase mass transfer (Singh et al., 2007). Laboratory experiments are usually conducted at a small scale where sufficient mixing is available while in large-scale biological demulsification applications, this is rare. Usually the effect of various factors such as nutrients, temperature and biodemulsifiers have been regarded in determining the effectiveness of full-scale biological demulsification projects

(Gallego et al., 2007). The role of adhesion typically is neglected in field biological demulsification studies, mostly because there is not a well-established connection between adhesion and biological demulsification in laboratory studies. These conditions emphasize the importance of adhesion to the oil–water interface for the demulsification of petroleum emulsions. Consequently, microbial adhesion may offer significant advantages for large-scale demulsification, in order to overcome bioavailability limitations and play a more important role in increasing the mass transfer of hydrophobic substances to the microorganisms. Development of biological demulsification approaches that use adhesion as a property to benefit process performance may be an interesting strategy. Several studies have established some connection between adhesion, which is affected by cell surface hydrophobicity, and its possible role in biological demulsification of different kinds of petroleum emulsions (Rosenberg et al., 1980; Obuekwe et al., 2009; Chakraborty et al., 2010). Despite these studies, to the best of our knowledge the direct use of cell surface hydrophobicity parameter to improve demulsification of water in oil emulsions has not been reported.

Conclusions

In the present study, the CSH of biodemulsifier-producing bacteria isolated from environmental samples obtained from a refinery in south-west of Iran were characterized based on classical MATH assay. The results showed that most biodemulsifier-producing bacteria in such environments had high CSH which indicating a positive correlation between cell surface hydrophobicity and demulsifying activity. The present report is the first definitive example demonstrating the positive correlation between cell surface hydrophobicity and demulsifying activity. Due to the importance of the oil-water interface in demulsification of water in oil emulsions, this study suggests new strategy for biological demulsification and for optimizing existing processes. It may also help further our fundamental understanding of mechanisms and methods involved in such processes.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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