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Experimental breaking of water/oil emulsions aimed at development of a water separation bacterial process in oil industries

G. Mohebali*, A. Kaytash and N. Etemadi

Microbiology and Biotechnology Research Group, Research Institute of Petroleum Industry, Tehran, Iran
E-mail: mohebaligh@ripi.ir

Abstract

Microbial demulsifiers represent a potentially important product for the petroleum industry due to their specific modes of action, relative ease of preparation, biodegradability and low toxicity. An extracellular demulsifier, capable of the rapid break up of model multiple water-crude oil emulsions was obtained from the bacterial isolate *Ochrobactrum anthropi* RIPI5-1. Demulsifying activity of the cell-free culture filtrate was not age dependent. The time course of demulsification by the whole culture and cell-free culture filtrate showed a similar pattern; the rate of demulsification was 5.21 h^{-1} and the emulsion half-life time ($t_{1/2}$) was 5 h. The potential activity of the filtrate was also examined using a complex oilfield emulsion; demulsifying activity (DeI_{16}) was calculated as 42%. Demulsifying activity of whole culture, cell-free culture filtrate and whole cells of strain RIPI5-1 was found to be associated with both the cell surface and extracellularly. Fractionation of the filtrate showed that demulsifying activity was a function of several compounds. The presence of various active demulsifying compounds may explain the ability of the filtrate to break up various crude oil emulsions and therefore represents a promising new technology.

Keywords: Demulsification; biosurfactants; water/oil (w/o) emulsions; water separation; bacteria

1. Introduction

Water-oil emulsions occur throughout oil production, transportation and processing. Problems associated with water in oil include corrosion, scale formation, sludge accumulation in storage tanks, altered viscosity and flow properties and reduced distillation efficiency (Kirkwood et al., 2004). Regardless of the source of water, the emulsions have to be broken to separate out the water at some point before refinery.

The breaking up of water-in-oil (w/o) emulsions improves the quality of oil and is therefore economically driven. This is commonly accomplished through a combination of physical and chemical treatment methods. These physicochemical demulsification processes are capital intensive. In addition, a major disadvantage with chemical demulsification is the requirement for the subsequent disposal of the chemical demulsifier(s) in the aqueous phase, which may represent an ecological issue. Microbial demulsification is a potential alternative; the potential advantages of microbial demulsifiers include (i) biodegradability, (ii) selectivity and specificity towards hydrocarbon substrates, (iii) compatibility with chemical products leading to novel formulations, (v) relative ease of preparation and (VI) widespread applicability (Bach and Gutnick, 2004, Singh et al., 2007).

Several researchers have studied microbial demulsification and reported some microorganisms as demulsifying agents. Beckman (1930) issued a patent suggesting a method of treating emulsions with microorganisms. Since then, bacterial whole cells have received the most attention (Cairns et al., 1982; Coutinho et al., 2013; Das, 2001; Duvnjak and Kosaric, 1987; Huang et al., 2009; Mohebali et al., 2012; Nadarajah et al., 2002a, b; Wen et al., 2010; Wilkinson and Cooper, 1985). However, the potential application of bacterial metabolites has also been studied (Coutinho et al., 2013; Huang et al., 2010; Janiyani et al., 1994; Li et al., 2012; Neufeld and Zajic, 1984). The aim of this work was to study emulsifier(s) extracellularly produced by a recently reported demulsifying bacterium, *Ochrobactrum anthropi* strain RIPI5-1 (Mohebali et al., 2012). We succeeded in obtaining several fractions of cell-free culture filtrate capable of breaking up two experimental (model) emulsions, a multiple water-crude oil emulsion and a water/kerosene emulsion. The use of cell-free culture filtrate to carry out demulsification offers greater stability and applicability when compared with whole cultures.

*Corresponding author
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2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Tween 80 and Spam 80 were obtained from SigmaAldrich, USA.

2.2. Microorganism

Following a multi-step enrichment programme *Ochrobactrum anthropi* strain RIPI5-1 was isolated from the oil-polluted sandy bank of Siri Island, Iran (Mohebali et al., 2012) and selected for further study with respect to its efficient demulsifying activity. The strain has been deposited in the Petroleum Biotechnology Culture Collection (PBCC), Research Institute of Petroleum Industry, Iran.

2.3. Media

A medium containing (grams per liter of distilled water) NH_4Cl (1.4), KH_2PO_4 (4.0), Na_2HPO_4 (6.0), $FeCl_3$ (0.001), $MnCl_2{\cdot}4H_2O$ (0.004), $MgSO_4{\cdot}7H_2O$ (0.4), $CaCl_2{\cdot}2H_2O$ (0.002) and yeast extract (1.0) was used as the base of the growth medium; the pH was adjusted prior to autoclaving to 8.0; filter-sterilized ethanol (4.0 g l^{-1}) was added to the medium as the sole carbon source (medium C). Medium C without yeast extract and ethanol was used as the whole cell suspension (WCS) buffer solution.

Enriched nutrient agar (ENA) medium containing nutrient broth (13.0 g), yeast extract (2.0 g), tryptone (4.0 g), agar (15.0 g), and distilled water (960 ml), was used when necessary.

2.4. Preparation of cell-free culture filtrate and cell suspension

Whole culture was obtained at the late-exponential growth phase. The whole culture supernatant was prepared by centrifugation (5,000 rpm, 30 min). In order to prepare the cell-free culture filtrate the resultant supernatant was filtered through a 0.22 μm pore-size filter (Schleicher and Schuell, Germany). This filtrate was stored at 1 ºC for up to 48 h. The cell suspension was prepared by re-suspending the pellet in WCS buffer solution and then adjusting the cell concentration (OD_{660}) to a suitable value.

2.5. Effect of strain RIPI5-1 on surface tension of its environment

In order to investigate the effect of strain RIPI5-1 and its product on surface tension, various samples containing whole culture, whole culture supernatant and cell-free culture filtrate were prepared as described above. The uninoculated medium C and de-ionized water were used as controls. The surface tension of the samples was measured using a Krüss tensiometer K9.

2.6. Organic phase

In this work the demulsification activity of samples was studied using model oil emulsions containing crude oil or kerosene as the oil phase. The characteristics of crude oil have recently been described (Mohebali et al., 2012). Kerosene was obtained from Tehran refinery, Iran. Strain RIPI5-1 was found to be resistant to the crude oil and kerosene (data not shown)

2.7. Model oil emulsions preparation

In this work two kinds of model (surfactant-stabilized) emulsions were used: (I) multiple water-crude oil and (II) water/kerosene emulsions.

Multiple emulsions are often stabilized using a combination of hydrophilic and hydrophobic surfactants; a hydrophobic surfactant is added to the oil phase and a hydrophilic surfactant is added to the aqueous phase (Jiao and Burgess, 2003). The concentration ratio of these surfactants is important to obtain stable, high yields of emulsions. In this study, multiple emulsions containing Span 80 as the hydrophobic surfactant and Tween 80 as the hydrophilic surfactant were prepared as described recently (Mohebali et al., 2012). Long-term stability of the resultant emulsion was confirmed following 72 h of incubation at 50 ºC. Fresh stock solutions were prepared before each experiment.

A water/kerosene emulsion was prepared as described recently (Mohebali et al., 2012). The long-term stability of the emulsion was confirmed following 24 h of incubation at 50 ºC.

2.8. Demulsification assessment

Assessment of demulsification was based on a process for breaking up a w-o emulsion comprising (i) contacting the w-o emulsion with the sample being tested e.g. whole culture and (ii) permitting the reaction mixture to form a released aqueous layer. The sample being studied (3.0 ml) was transferred to sterile, graduated and disposable conical tubes (15 ml, NUNC, USA) and then mixed with 6.0 ml of the w-o emulsion vigorously by vortex (VORTEX-T GENIE2, Scientific Industries Inc., USA) at full speed for 1 min to produce a uniform emulsion. The tube was incubated for up to 24 h at 50 ºC under static conditions; the tube was checked at defined time intervals for an aqueous

phase released from the emulsions. Controls tubes containing medium C/WCS buffer solution (3 ml) together with model emulsion (6 ml), or model emulsion alone (9 ml) were run under the same conditions. Each experiment was performed in triplicate. The phase volume ratio and reaction mixture volume used was as previously optimized (Mohebali et al., 2012).

2.9. Determination of demulsification index (DeI_n)

The DeI_n was used to explain demulsifying activity of the sample being tested, e.g. whole culture where n represents the assay incubation time (h). The DeI_n was determined using a modified method described elsewhere (Bodour et al., 2004; Mohebali et al., 2007). The sample was mixed with a model emulsion as described above. The tube was incubated for a defined time, e.g. 12 h (n= 12) at 50 ºC; following incubation the tube was checked for the presence of an aqueous phase. The volume of the aqueous phase released from the emulsion was divided by the primary water content of the reaction mixture and multiplied by 100; the resulting figure (%) was used as DeI_n. Every index was calculated from triplicate samples.

2.10. Effect of assay temperature on demulsification

The effect of assay temperature on the rate of demulsification by both whole culture and cell-free culture filtrate was investigated as follows: a whole culture sample was taken at the late-exponential growth phase (OD_{660}= 1.08) from which cell-free culture filtrate was obtained; demulsification assessment was carried out using five aliquots (3 ml) of each sample together with the multiple water-crude oil emulsions (6 ml). The reaction mixtures were incubated at 30, 40, 50, 60 and 70 ºC for 24 h. During incubation at a defined time interval all tubes were examined and the results were used to calculate the initial rate (DeI_1) and extent (DeI_{24}) of demulsification, and also $t_{1/2}$ for every assay temperature. Each of the experiments was performed in triplicate.

2.11. Demulsification activity during various growth stages

In order to examine the change in demulsifying activity during various growth stages, demulsifying activity and growth of strain RIPI5-1 were monitored simultaneously. The strain was inoculated in medium C and incubated on a rotary shaker (120 rpm) at 30 ºC for 36 h; at a defined time interval a whole culture sample was taken and its concentration was measured (dry cell weight, DCW, g l^{-1}). Cell-free culture filtrate was prepared using the whole culture sample as described above. Demulsifying activity (DeI_{12}) of the whole culture and cell-free culture filtrate was monitored. All tests were carried out using triplicate samples. Every sample was checked for purity using ENA medium. From growth curves, the specific growth rate was calculated as follows: $\mu = (\text{Ln } X_2 - \text{Ln } X_1)(t_2 - t_1)$, where X_2 and X_1 are cell densities at time t_2 and t_1, respectively.

2.12. Breaking up water/kerosene emulsion

In order to examine the capability of strain RIPI5-1 for breaking up water/kerosene emulsions the strain grown in medium C (OD_{660}= 1.02) was used to prepared the cell-free culture filtrate. The initial rate (DeI_1) of demulsification by cell-free culture filtrate (3 ml) was determined using a water/kerosene emulsion (6 ml) as described above. Each of the experiments was performed in triplicate.

2.13. Breaking up an oilfield emulsion

In order to study the potential demulsifying activity of strain RIPI5-1 an oilfield emulsion consisting of Siri crude oil and its produced water was used. The characteristics of the crude oil and its produced water have been recently described (Mohebali et al., 2012). The crude oil and the produced water were mixed vigorously (100:20; v/v) until a stable emulsion was created. Stability of the emulsion was confirmed by incubation at 50ºC for 24 h. Three aliquots (3 ml) of whole culture or cell-free culture filtrate were mixed vigorously by vortex with the field emulsion (6 ml) for 1 min and then incubated at 50 ºC for 48 h. Control tubes containing medium C (3 ml) together with the field emulsion (6 ml), or the field emulsion alone (9 ml) were run under the same conditions. DeI_n (n= 16) was calculated and used to estimate the potential activity of the strain to break up the oilfield emulsions. Each of the experiments was performed in triplicate.

2.14. Time course of demulsification

In order to determine the time course for demulsification the whole culture sample was taken at the late-exponential growth phase (OD_{660}= 1.05) from which triplicate samples of the cell-free culture filtrate were obtained. Demulsifying activity (DeI_n, n= 0.5-8.5 h, at interval of 0.5) of the whole culture and cell-free culture filtrate was studied using multiple water-crude oil emulsions. The resultant figures (expressed as a %) were used to prepare the time course of emulsion breaking. The time course curve is a plot of demulsifying activity

(DeI_n) versus time (h).

2.15. Demulsifying activity of solvent extracts of strain RIPI5-1

In order to explain the presence of extractable demulsifying agent(s) in whole culture, whole cells, and cell-free culture filtrate of strain RIPI5-1 50 ml of whole culture was taken at the late-exponential growth phase (OD_{660}= 1.02) from which whole cells were harvested by centrifugation (5000 rpm, 30 min). Cell-free culture filtrate was prepared as described above. The pH of these samples (whole culture, whole cell suspension, and cell-free culture filtrate) was adjusted to 2 using HCl (10 M) and the acidified samples (50 ml) were extracted using chloroform (v/v; 50:50) three times. The total volume of the extract was evaporated at room temperature and the dried sample was dissolved in WCS buffer solution (25 ml) using a magnetic stirrer. The resultant solutions were assessed to determine their demulsifying activity as DeI_n (n= 0-6 h at time interval of 0.5). Each of the experiments was repeated five times.

2.16. Effect of demulsifying product concentrations on demulsification

In order to explain the effect of demulsifier concentrations on demulsification RIPI5-1 whole culture (200 ml) was taken at the late-exponential growth phase (OD_{660}= 1.02) from which cell-free culture filtrate was obtained. Following adjustment of pH to 2 (10 M HCl) the culture filtrate was extracted using 50 ml of chloroform (50:50; v/v) three times. The total volume of the extract was evaporated at room temperature. The resultant dried sample was then dissolved in 100 ml of WCS buffer solution using a magnetic stirrer; this demulsifier-containing solution was serially diluted using WCS buffer solution (0.0-100% of demulsifier at intervals of 6.66%; v/v). The initial rate (DeI_1), extent (DeI_{24}) and $t_{1/2}$ were determined using the multiple water-crude oil emulsion and triplicate samples as described above.

2.17. Demulsifying activity of culture filtrate fractions

In order to fractionate cell-free culture filtrate RIPI5-1 whole culture samples were taken at the late-exponential growth phase (OD_{660}= 1.09) from which the cell-free culture filtrate was obtained. The filtrate was fractionated using a glass packed column; this column (1.6×80 cm, working volume 160.76 cm^3) was packed with Sephacryl S400-HR (Sigma-Aldrich, USA). The column was supplemented with a fraction collector (Fraction Collector Model II, Waters, USA) and a peristaltic pump (Model 505S, Watson Marlow, England). The column was eluted with WCS buffer solution at 2 ml min^{-1}. Ten ml of the filtrate was loaded onto the packed column; forty fractions (5 ml) were collected and their demulsifying activity (DeI_{24}) was examined using multiple water-crude oil emulsion. The fractions with demulsifying activity were extracted by chloroform (50:50, v/v) three times as described above. The total volume of each solvent extract was evaporated at room temperature. The resultant dried samples were dissolved in WCS buffer solution. Demulsifying activity (DeI_{24}) of the solutions was then studied using multiple water-crude oil emulsions. All experiments were performed in triplicate.

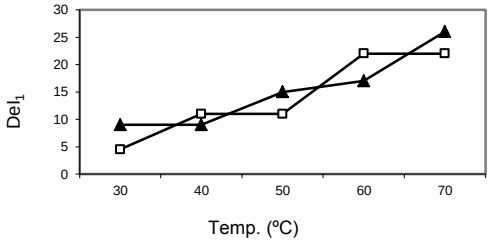
3. Results

3.1. Effect of strain RIPI5-1 on surface tension of its environment

The effect of strain RIPI5-1 on the surface tension of its environment was studied. The surface tension of whole culture, whole culture supernatant, cell-free culture filtrate and medium C was detected as 40.4, 43.2, 46.1 and 45.9 mN m^{-1}, respectively, while de-ionized water had surface tension of 74 mN m^{-1}.

3.2. Effect of assay temperature on demulsification

In this work the effect of assay temperature on the rate and extent of demulsification was studied using whole cultures and cell-free culture filtrates of strain RIPI5-1. The results showed that the initial rate (DeI_1) of demulsification by whole cultures increased concomitant with assay temperature up to 60ºC. The initial rate (DeI_1) of demulsification by cell-free culture filtrate also increased concomitant with assay temperature (Fig 1). The extent of demulsification (DeI_{24}) of whole culture and cell-free culture filtrate increased with assay temperature up to 50 ºC; beyond this temperature the activity remained almost constant (Fig 1).



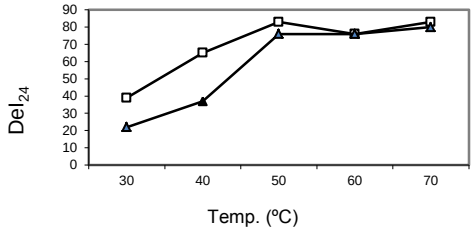


Fig 1. Effect of assay temperature on the initial rate (DeI_1) and extent (DeI_{24}) of demulsification by whole culture and cell-free culture filtrate of strain RIPI5-1. (□), whole culture; (▲), cell-free culture filtrate

3.3. Demulsifying activity during various growth stages

In order to study the production of extracellular demulsifier(s) and their demulsifying activity (DeI_{12}) during various growth stages whole culture of strain RIPI5-1 was taken during growth stages from which cell-free culture filtrate was obtained. The demulsifying activity (DeI_{12}) of the whole culture and culture filtrate was detected using multiple w-o emulsion through the exponential and stationary phases of growth (Fig. 2). The results suggested that the extracellular compound(s) were produced during the exponential growth phase and remained constant during the stationary phase. The activity (DeI_{12}) of the whole culture was at its highest level during the early-exponential growth phase; the activity decreased at the late-exponential growth phase and at the end of the exponential growth phase the activity increased and remained constant during the stationary phase. pH decreased during the growth phase from 7.56 to 7.15 and remained constant during the stationary phase.

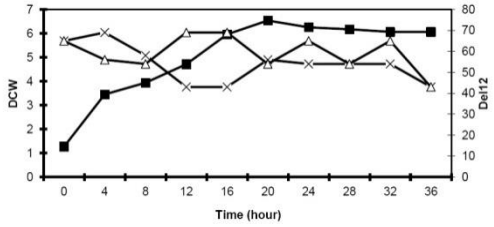


Fig 2. Demulsifying activity (DeI_{12}) of whole culture and cell-free culture filtrate of strain RIPI5-1 during various growth stages. (■), growth (gDCW l^{-1}); (×), demulsifying activity of whole culture; (Δ), de-emulsifying activity of culture filtrate

3.4. The time course of demulsification

The time course of demulsifying activity of whole culture and cell-free culture filtrate of strain RIPI5-1 was prepared using multiple water-crude oil emulsions. A linear increase in the activity of both whole culture and cell-free culture filtrate during assay incubation time from 0 to 3.5 h was observed (Fig. 3); the demulsification rate was calculated as 5.21 (h^{-1}).

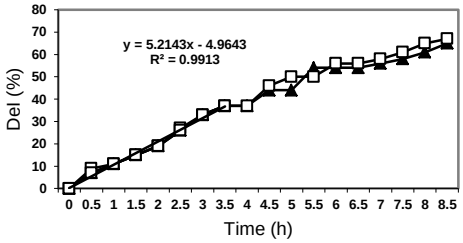


Fig 3. Time course of demulsification by whole culture and cell-free culture filtrate of strain RIPI5-1. During 0.0-3.5 hours of assay incubation time changes of DeI_n were linear. (▲), whole culture; (□), cell-free culture filtrate

3.5. Breaking up water/kerosene and oilfield emulsions

In order to study the potential demulsifying activity of strain RIPI5-1 a water/kerosene emulsion was prepared. Following the assessment DeI_1 was calculated as 80% (+/- 3) for triplicate samples of cell-free culture filtrate (Fig. 4). The treated tubes showed a clear separation of phases, namely the top kerosene phase, mid interface and bottom aqueous phase.

In order to study the potential demulsifying activity of strain RIPI5-1 an oilfield emulsion consisting of Siri crude oil and its produced water was used. DeI_{16} were calculated as 42% for both whole culture and cell-free culture filtrate samples.

Fig 4. Breaking of water/Kerosene emulsion by whole culture of *Ochrobacterum anthropi* strain RIPI5-1 following assay incubation time (h). A, model water/Kerosene emulsion; B, the mixture consisting of model water/Kerosene emulsion and medium C; C, reaction mixture consisting of model water/Kerosene emulsion and cell-free culture filtrate of strain RIPI5-1

3.6. Demulsifying activity of solvent extract of strain RIPI5-1

In order to explain the presence of extractable demulsifying agent(s) of strain RIPI5-1 whole culture, whole cells and cell-free culture filtrate were prepared, extracted using chloroform and then evaporated as described earlier; dried samples were then dissolved in a buffer solution. The resultant solutions were assessed to determine their demulsifying activity using multiple water-crude oil emulsions (Fig. 5). The highest demulsifying activity (DeI_2) of solvent extract of whole cells and cell-free culture filtrate was about 60%; the activity further increased over time (DeI_6= 80%). The pattern of the changes for whole culture and cell-free culture filtrate was similar. Demulsifying activity of solvent extract of whole cells increased over time (DeI_6= 35%).

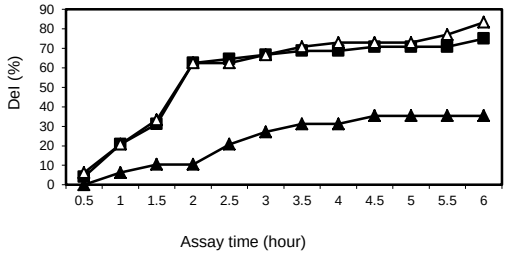


Fig. 5. Demulsifying activity (DeI_n) of solvent extract of whole culture (■), whole cells (▲), and cell-free culture filtrate (Δ)

3.7. Effect of demulsifying product concentration on demulsification

In order to explain the effect of demulsifier concentrations on demulsification solvent extract of RIPI5-1 culture filtrate was obtained by which demulsifier-containing solution was prepared; this solution was serially diluted as described above. The initial rate (DeI_1) and extent (DeI_{24}) of demulsification of the diluted samples and also their $t_{1/2}$ were determined (Fig. 6). The $t_{1/2}$ remained constant from 0.0 to 26.6% concentrations of demulsifier and beyond this point the value decreased. The change in the initial rate (DeI_1) of demulsification showed a similar pattern; at higher concentrations the rate of demulsification increased. However, the extent of demulsification (DeI_{24}) remained almost constant, irrespective of the demulsifier concentrations increase.

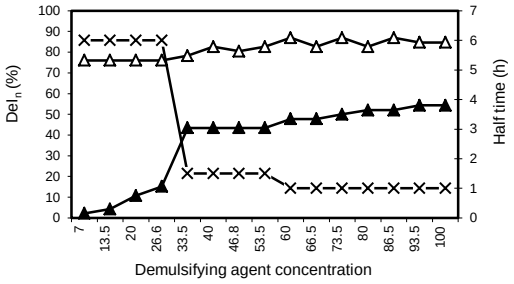


Fig 6. Relationship between demulsifying product concentrations (in term of serially diluted cell-free culture filtrate) and demulsifying activity. (▲), DeI_1; (Δ), DeI_{24}; (×), $t_{1/2}$

3.8. Demulsification activity of culture filtrate fractions

In order to determine potential variability of demulsifying compounds present in culture filtrate the cell-free culture filtrate was fractionated in 40 samples and demulsifying activity (DeI_{24}) of the fractions was examined. The results showed that the six groups of fractions (fractions No. 5, 8-11, 13, 15-18, 23-24, and 27) expressed demulsifying activity (DeI_{24}= 60-75%). The solvent extracts of the fractions expressed demulsifying activity (DeI_{24}) as 45%, 33%, 45%, 33%, 20%, and 20%, respectively. The results showed that the demulsifying activity was a function of several compounds.

4. Discussion

Crude oil emulsification may originate from various agents including (i) emulsifiers that are used to form an emulsion to improve the recovery rate and to increase fluidity and movement, and (ii) amphiphilic molecules from the oil, especially the resin fraction. Many crude oil emulsions are stabilized by fine solids, including clays, scale, or wax crystals. After association with the interface, asphaltenes agglomerate to form a film, which prevents coalescence of droplets (Kirkwood et al., 2004; Long et al., 2013). Bacteria can also stabilize oil emulsions (Dorobantu et al., 2004; Mohebali et al., 2007), which present a barrier to droplet coalescence.

Salt contaminants in fossil fuels can create problems in refinery processes which can be costly to rectify. For example, these contaminants can plug downstream equipment, form products that are corrosive to refinery equipment and interfere with chemical processing (Monticello, 1996). Since the presence of water and sediments in oil causes corrosion and scaling in tanks and pipelines, basic sediment and water (BS & W) content of 0.5 to 2.0% has been specified as the maximum allowable

in crude oil for transportation through the pipelines (Singh et al., 2007). In oilfield production units, during the desalting step the water content of crude oil can be increased.

Breaking water-in-oil emulsion is accomplished through physicochemical treatment methods involving centrifugation, filtration, heating, settling, electrical dehydration and the addition of chemicals containing soap, fatty acids and long-chain alcohols (Kirkwood et al., 2004; Nadarajah et al., 2002a,b). Microbial demulsifiers are potential alternatives to synthesized chemicals.

Several researchers have studied microbial demulsification; microbial demulsification has been reported as a phenomenon associated with whole cells, but bacterial metabolites have also been studied (Mohebali et al., 2012). Our microbiological analysis revealed that *Ochrobactrum anthropi* strain RIPI5-1 exhibits a demulsifying trait exhibited by both whole cells and culture supernatant (Mohebali et al., 2012).

The majority of microbial demulsification experiments have examined model emulsions, chemically-stabilized emulsions consisting of water, hydrocarbon and commercial surfactant(s). Model emulsions can be used to quickly screen demulsifying cultures. Although model emulsions do not mimic field emulsions, a correlation to predict the demulsifying activity can be established (Nadarajah et al., 2002a,b). Since using crude oil causes various difficulties, in demulsification experiments kerosene as the hydrophobic phase has been widely used to prepare model emulsions (Cairns et al., 1982; Das, 2001; Huang et al., 2009, 2010, 2013; Nadarajah et al., 2002a,b; Neufeld and Zajic, 1984; Wen et al., 2010;). However, crude oil has been used (Janiyani et al., 1994). Complex water-in-crude oil (field) emulsions have also been used (Duvnjak and Kosaric, 1987; Wilkinson and Cooper, 1985). In this work, demulsifying activity of samples being tested were measured using a multiple water-oil emulsion containing crude oil and its produced water as its oil and aqueous phases, respectively. This multiple emulsion mimics field emulsions with a more stabilized status. To the authors' knowledge the use of this model emulsion has not previously been reported in the literature by other researchers.

Elevating the assay temperature generally accelerates demulsification by reducing the viscosity of the oil phase, increasing density differences between the phases, weakening the stabilizing interfacial film and causing an increased rate of droplet collision leading to coalescence (Singh et al., 2007). The effect of temperature on the rate of microbial demulsification has been reported (Nadarajah et al., 2002a,b). In this work it was found that the whole culture and cell-free culture filtrate of strain RIPI5-1 was not inhibited by assay temperature up to 70 ºC; maximum demulsification extent was achieved at 50 ºC and their demulsification rate was dependent on assay temperature.

The efficiency of the demulsification process depends on microbial growth medium (Kosaric et al., 1987). It has been reported that some organisms grown on non-petroleum hydrocarbon substrates demulsified petroleum emulsions (Duvnjak and Kosaric, 1987; Janiyani et al., 1994). However, there are some reports about the presence of hydrocarbons in the basal medium as the carbon source for growth (Liu et al., 2010; Ward and Singh, 2001). Liu et al. (2010) suggested that hydrophilic carbon sources were not efficient in the production of biodemulsificants. The presence of an oil phase in growth medium was found unnecessary for demulsifying activity of strain RIPI5-1.

Demulsifiers, which are surface active, promote growth of drops and emulsion disruption (Park et al., 2000). Liu et al. (2010) suggested that biodemulsifiers produced by *Alcaligenes* sp. S-XJ-1 had high surface activity. Our results showed that cell-free culture filtrate of strain RIPI5-1 had no effect on the surface tension of its environment.

The efficiency of the demulsification process depends on a number of factors including quantity and nature of the microbial demulsifier agent and culture age (Kosaric et al., 1987). In this work a clear correlation was observed between demulsification rate (initial rate and emulsion half life, $t_{1/2}$) and demulsifier concentrations, while the extent of demulsification was not dependent on demulsifier concentrations. A critical concentration point was observed in which the initial rate of demulsification was obviously shifted. The demulsifying activity was studied during various growth stages.

It has been found that the cell-surface demulsifying activity of strain RIPI5-1 was a function of the age-dependent changes in cell surface structure (Mohebali et al., 2012). The results of this work showed that demulsifying activity of cell-free culture filtrate was not a function of the age-dependent changes in extracellular secretion.

Emulsifying agents found in water-crude oil emulsions include the asphalthene and resin components of the oil, oil-soluble organic acids or other chemicals originally present in the oil (Long et al., 2013; Ward and Singh, 2001). The influence of asphaltenes and resins on the stability of emulsions has been reported (Xia et al., 2004). The efficiency of the demulsification process depends on the chemical composition of the emulsion and the presence of clay or other inorganic particles in

the emulsion (Kosaric et al., 1987). The capability of bacterial strains to break up complex water-crude oil emulsion and their resistance to the high concentrations of NaCl is of practical importance for oilfield applications; complex water-in-crude oil (field) emulsions have been used (Duvnjak and Kosaric, 1987; Wilkinson and Cooper, 1985). In this work the oilfield emulsion was successfully broken by the whole culture of strain RIPI5-1 and cell-free culture filtrate thereof. NaCl concentration of the oilfield emulsion was measured as 10.3% (Mohebali et al., 2012).

Crude oil emulsions are complex and vary from location to location. Demulsification in the oil industry is challenging due to the variety and chemical treatments are currently tailored to each site and adapted over time. The ability of bacterial cells to demulsify both model and oilfield emulsions has been demonstrated, but the potential for treating the true spectrum of real crude oil emulsions has not been rigorously tested (Kirkwood et al., 2004). Due to variability in the properties of crude oil emulsions, inconsistencies are experienced in performance of the different demulsification processes including microbial processes (Singh et al., 2007). In this work several cell-free culture filtrate fractions were shown to be capable of breaking the model emulsion. Therefore, demulsifying activity was attributed to the presence of various compounds. This offers promise as the various components of the demulsifying activity may be able to be effective over a range of crude oil-water emulsions. Further research on the microbial demulsification processes using various fractions/pure cultures need to be performed to prepare various combinations of the fractions/pure cultures leading to the development of universally effective systems.

5. Conclusion

Microbial surfactants including demulsifiers are potential alternatives to chemically-synthesized compounds that are currently used in the petroleum industries. The worldwide attention being paid to this concept can be attributed to several factors including the biodegradability of the compounds. In this study a recently reported demulsifying bacterium, *Ochrobacterum anthropi* RIPI5-1 was used to investigate its capability to produce extracellular demulsifiers; the whole culture, whole cells and cell-free culture filtrate all showed efficient breaking activity against the model and complex field emulsions. The extracellular demulsifier produced by the strain is expected to be promising for future studies aims to oilfield application. It is evident from the results reported here that the microbial demulsifier may be prepared industrially. However, the development of bioprocess for break up the large volumes of w/o emulsions in oilfield is one of the greatest challenges addressed by biotechnology.

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