academicJournals

Vol. 12(16), pp. 2019-2026, 17 April, 2013
DOI: 10.5897/AJB11.3462
ISSN 1684–5315 © 2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Demulsification capabilities of a Microbacterium species for breaking water-in-crude oil emulsions

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Accepted 28 March, 2013

A bacterium strain belonging to *Microbacterium* sp., isolated from oily sludge samples of Siri Island in the south of Iran, produced a strong, thermo stable microbial demulsifier ($Y_{x/s}$=0.663, $Y_{p/s}$=0.204, productivity=0.185 g L^{-1} h^{-1}) on glucose as a sole carbon source supplemented with yeast extract. The optimum values of temperature, inoculum concentration, pH and culture age for microbial demulsifier production were 25°C, 10^8 CFU mL^{-1}, 7 and 24 h, respectively. The maximum demulsification activity and the half-life value ($t_{1/2}$) of culture broth measured for a water-in-crude oil (W/CO) emulsion were 96.4% and 36 h at 80°C in flask. The demulsifier was purified to homogeneity using cold ethanol. For 4.33 mg mL^{-1} of partially purified microbial demulsifier, the half-life value for the W/CO model emulsion was 3 h.

Key words: Biopolymer, demulsification, *Microbacterium*, demulsifier, petroleum emulsion, water-in-crude oil.

INTRODUCTION

An emulsion is a thermodynamically unstable system in which liquid drops are dispersed in another immiscible liquid phase. Petroleum emulsions are generally formed in reservoirs or during refining processes and in oil transportation through pipelines. They are commonly classified, based on the continuous phase, into two groups: i) water-in-oil emulsions (W/O), and ii) oil-in-water emulsions (O/W) (Manning and Thampson, 1995; Scharmam, 2005). The formation of oilfield emulsions at various stages of exploration, production and recovery leads to many problems, such as corrosion and scaling on pipelines and equipment used in production or recovery (Mouraille et al.,1998). Recently, microbial demulsifiers have been attracting more attention due to their excellent surface properties, low toxicity, biodegradability, low cost, high specificity at extreme temperatures, and environmental compatibility (Desai and Banat, 1997). So far, microbial demulsifiers have been commonly produced by pure cultures such as *Alcaligenes* sp., *Corynebacterium petrophilum, Nocardia amarae, Rhodococcus aurantiacus, Mycobacterium* sp., *Bacillus subtilis, Torulopsis bombicola, Acinetobacter calcoaceticus, Arthrobacter* sp., *Micrococcus* sp. (Carins et al., 1982; Cooper et al., 1982; Stewart et al., 1983; Das et al., 2001; Huang et al., 2009; Wen et al., 2010; Li et al., 2012, Long et al., 2012), and mixed bacterial culture (Kosaric and Duvanjak, 1987; Nadarajah et al., 2002) for demulsifying, or at least destabilizing, O/W and W/O emulsions.

This work aimed to investigate the behavior of a demulsifier isolated from *Microbacterium* sp. on water and crude oil (W/CO) model emulsions, optimization of growth conditions and factors influencing microbial demulsifier production as well as the effect of the W/CO demulsification, assay temperature and inoculum concentration on the model emulsion.

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Table 1. Properties of crude oil used for preparing the model emulsion.

Property	Value
API[1] gravity	30.2
Kinematic viscosity at 20°C (cP)	11.93
Density (Kg m^{-3})	875
BS&W[2] (%v)	0.5
Sulfur content (%w)	1.3
Asphaltene content (%w)	2
Wax content (%w)	11
Vanadium and nickel (%w)	1.22

[1]American petroleum institute; [2]Bottom sediment and water.

MATERIALS AND METHODS

Screening of microorganism

Samples of polluted soil and oily sludge were collected from the Siri Island and Aghajari oil field located in the south of Iran, in the area near the Esfahan Oil and the Tehran Oil Refineries. 1 mL of oily sample was added to 99 mL of mineral salt (MS) solution in a 250 mL glass flask containing nutrient broth (Merck, Germany). This mixture was shaken at 160 rpm and 30°C for 12 h. Screening was carried out by transferring serial dilutions of samples onto nutrient agar plates, incubating at 30°C for 48 h and selection of individual colonies for culturing. The capability of microbial demulsifier production was determined by measuring the demulsification activity of water-in-oil emulsions.

The isolate was identified in the National Laboratory of Industrial Microbiology (NLIM) at Alzahra University according to Bergey's Manual of Systematic Bacteriology and other literature (Holt and Williams, 1989; Evtushenko and Takeuchi, 2006).

Medium and culture conditions

The medium for microbial demulsifier production included MS solution (g L^{-1}): NH_4NO_3, 4; K_2HPO_4, 4; KH_2PO_4, 6; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.0001; $FeSO_4$, 0.0001, supplemented with yeast extract (1.0 g L^{-1}) and glucose (30 g L^{-1}) as the carbon source. Production of the microbial demulsifier was optimized using the Taguchi experimental design (Qualitek 4, Demo version) software. The effect of various carbon sources on microbial demulsifier production was examined by culturing with addition of various separate sugars (glucose, sucrose, molasses) at 3% (w/v) and hydrocarbon substrates (hexadecane, kerosene, crude oil) at final concentration of 0.4% (v/v) to the MS solution. Hydrocarbons were sterilized by filtration using Millipore membranes (0.22 µm). All chemicals used were of analytical grade obtained from Merck, Germany.

Preparation of W/CO emulsion

W/CO model emulsion was prepared according to the method described by Nadarajah et al. (2002) with some modifications. The stock solution of Tween 80 in water was prepared by dissolving 40 mL of Tween 80 (HLB=15) in 1 L of de-ionized water on a stirring plate for 1 min. To prepare the W/CO emulsion, 8 mL of crude oil was added dropwise to 2 mL Tween 80 in water solution in a test tube and vortexed at maximum speed for 3 min before each use. Crude oil was obtained from the Esfahan Oil Refinery, Esfahan. Characteristics of the crude oil sample are presented in Table 1.

Measurement of demulsification activity

1 mL of the culture broth was added to 9 mL W/CO emulsion in a test tube and vortexed for 30 s to form a homogeneous culture emulsion mixture, then transferred to a 10 mL graduated cylinder, covered with aluminum foil and incubated at a defined temperature. For all experiments, the controls used 1 mL uninoculated production medium and 9 mL W/CO emulsion. The demulsification activity of the microbial demulsifier was determined as follows: demulsification activity (%) = ([Initial emulsion volume – final emulsion volume at interface]/Initial emulsion volume) ×100 (Nadarajah et al., 2002). All experiments were conducted in triplicate and the mean of the results was reported. W/CO demulsification assay temperature was 80°C for 48 h.

Optimization of microbial demulsifier production using the Taguchi method

Taguchi experimental design software (Qualitek4, Demo version) was used to optimize the demulsification activity of the culture. A standard array (L16) was applied to evaluate the effect of four factors and four levels (Table 2). Analysis of data was done using the analysis of variance (ANOVA) method in order to determine which factors were statistically significant.

Extraction and biochemical analysis of microbial demulsifier

Extraction of the crude microbial demulsifier was carried out according to the method of Peat et al. (1961) with some modifications. The culture broth was centrifuged at 5000 rpm for 10 min. The precipitate was washed with a phosphate buffer (5%, w/v) and then suspended in a solution containing 20% (v/v) potassium citrate (0.1 M) and potassium meta bisulfate (0.02 M). The pH of the solution was adjusted to 6, and autoclaved at 121°C for 20 min, then centrifuged at 4000 rpm for 10 min. To obtain the precipitate of the microbial demulsifier, three volumes of 95% cold ethanol containing 1% (v/v) acetic acid were added. The solution was kept at 4°C for 12 h, after which the microbial demulsifier was centrifuged and freeze dried.

The phenol/sulphuric acid method was used to determine the

Table 2. Comparison of microbial demulsifier production using glucose (3%) and molasses (3%) in the presence of yeast extract (1.0 g L^{-1}) in 5 L fermenter.

Carbon source	Culture age (h)	$Y_{x/s}$ (g g^{-1})	$Y_{P/S}$ (g g^{-1})	Productivitiy (g $L^{-1}h^{-1}$)	Demulsification activity[1] (%)	Half-life[2] $t_{1/2}$ (h)
Glucose	24	0.663	0.204	0.185	98.6	3
Molasses	64	0.92	0.124	0.0469	64.7	18

[1]Data obtained using culture broth; [2]data obtained by adding 1 mL of the partially purified microbial demulsifier (4.33 g/L) to 9 mL 20:80 W/CO emulsion. W/CO demulsification assay time was 48 h, and W/CO demulsification assay temperature was 80°C.

Table 3. Relationship between W/CO mixing ratio and the added Tween 80 volume on stability of model emulsion.

Tween 80 volume (µl)	W/CO ratio 40:60 (v/v)	W/CO ratio 30:70 (v/v)	W/CO ratio 20:80 (v/v)
800	- - - -	- - - -	- - - -
600	- - - -	- - - -	- -
400	- - - -	- - - -	- -
200	- - - -	- - -	-
80	- - -	- - -	+
60	- - -	-	-
40	- - -	-	-
20	- - -	- - -	- -

W/CO phase separation percent: stable emulsion 0% (+); 20% (-); 30 (--); 40% (---); more than 50% phase separation (----).

total sugar content of the microbial demulsifier (Dubois et al., 1956). The protein content of the microbial demulsifier was measured by the Lowry-Folin (1951). The Lipid content of the microbial demulsifier was determined according to the method of Floch (1975).

RESULTS

Screening of microorganism

Out of the 11 strains that were isolated from polluted soil and oily sludge samples, only one strain (isolate S2) exhibited considerable demulsification activity and grew in a liquid medium containing glucose as the sole carbon source. Emulsification activity of culture broth was growth dependent achieving its highest value at the beginning of stationary phase. Isolate S2 was aerobic, rod-shaped, gram-positive, motile, catalase-positive and oxidase-negative. Based on the morphological and biochemical properties in NLIM's report, the isolate belongs to *Microbacterium* sp.

Demulsification characteristics of emulsion

Although W/CO emulsion was not the best model to test due to non-uniformity in crude oil, it was used in all experiments as the most closely approximated actual field emulsions. The stability of different volumetric ratios of the W/CO emulsions (20:80, 30:70 and 40:60) was studied. The stability of the W/CO emulsion was found to decrease with increasing dispersed phase (water) content from a relative percentage of 20 to 40%. The W/CO ratio of 20:80 exhibited the highest emulsion stability (Table 3). The effect of the Tween 80 concentration in the range of 0.02 to 0.8 mL on the stability of the W/CO emulsions was also examined. Results show that the most stable emulsions were achieved by adding 80 µl Tween 80 to 2 mL of water and mixing it with 8 mL of crude oil. Stability increased as pH increased from 5 towards the higher pHs and the best results were obtained at pH 6.3. Concomitantly, the pH of the W/CO emulsion was adjusted to 6.3 in all experiments. The uninoculated control emulsion was stable for 30 days at 80°C and 240 days at 25°C.

Effect of demulsification assay temperature on demulsification of the W/CO model emulsion

The influence of the demulsification assay temperature in breaking the W/CO emulsions using the culture broth of *Microbacterium* sp. was evaluated at five different temperatures. According to Figure 1, the demulsification rate enhanced linearly with increasing W/CO demulsification temperature. Demulsification activity was negligible at 25°C. The highest percentages of demulsification were observed at 80 and 100°C. Since the difference in the demulsification activity at 80 and100°C was small, the W/CO demulsification tempera-

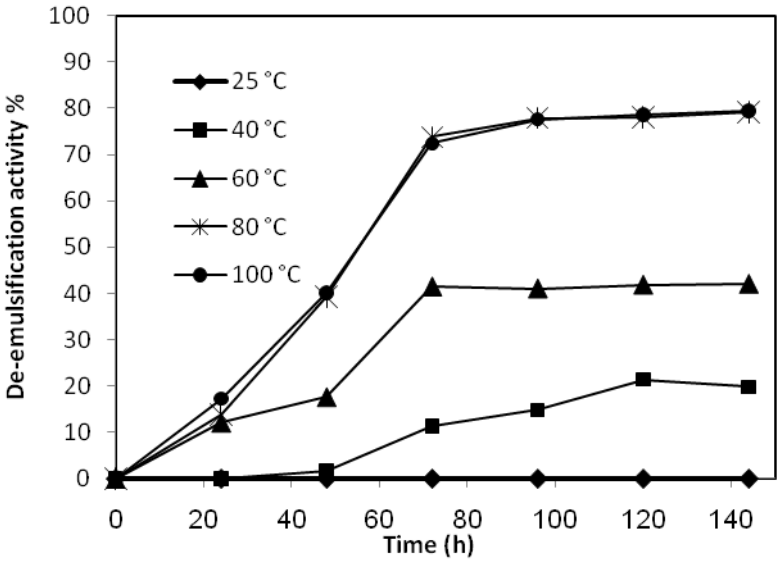

Figure 1. Effect of W/CO demulsification assay temperature on demulsifying the model emulsion by *Microbacterium* culture under unoptimized production. Inoculum concentration was 10% v/v (bacterial counts: 5×10^8 CFU mL^{-1}). Cultivation temperature was 30°C. W/CO demulsification assay time was 48 h. Control experiment carried out by adding uninoculated production medium was negligible.

ture was selected as 80°C throughout this research. For control test, medium containing yeast extract was added to the emulsion and its effect was negligible and the emulsion was stable.

Effect of inoculum concentration on demulsification of the W/CO model emulsion

Figure 2 shows that demulsification activity intensified by increasing the culture broth concentrations in the assay, but correlation was not linear. Both 10 and 15% (bacterial counts: 5×10^8 CFU mL^{-1}) of the culture broth samples indicated similar demulsification activity. After 48 h of W/CO demulsification time, the maximum demulsification activity of the model W/CO emulsion was 82 with 10% inoculum under unoptimized conditions, while only 11% of the phase separation was achieved using 1% culture broth.

Optimization of demulsifier production

Production of the microbial demulsifier by the *Microbacterium* sp. in flasks was optimized using the Taguchi experimental design statistical method by evaluating demulsification activity when varying four factors (inoculum concentration, culture age, pH, and temperature). The effect of each factor on demulsification was determined by ANOVA. The effect of temperature was the most important factor followed by inoculum concentration, pH, and culture age and was determined as 70.4, 12.7, 7.1 and 5.8%, respectively. The temperature and inoculum concentration were the most significant interactive factors in the production of the demulsifier. The optimum values of temperature, inoculum concentration, pH, and culture age were 25°C, 10^8 CFU mL^{-1}, pH 7 and 24 h, respectively. The value error was 4% and the selected values in the experimental design were significant at more than a 95% confidence limit. To evaluate the proposed optimum conditions, repeated experiments on microbial demulsifier production were conducted under optimum conditions. The demulsification activity obtained was always close to the expected value predicted by the Taguchi software. Cell free supernatant of *Microbacterium* did not exhibit demulsification activity while culture broth and washed cells displayed considerable demulsification activity.

The best demulsification activity by the culture broth of *Microbacterium* sp. growing on glucose (3% w/v) and yeast extract (1.0 g L^{-1}) was 96.4 % ($t_{1/2}$=36 h) (in flask) at W/CO demulsification assay temperature of 80°C and pH 6.3. With addition of 4.33 mg mL^{-1} of purified microbial demulsifier, half-life value ($t_{1/2}$) of the W/CO model emulsion was 3 h at 80°C. Demulsification of water-in kerosene system (30:70 v/v) using 24 old culture broth was also examined and the half-life value ($t_{1/2}$) was reduced to 1 h at 25°C.

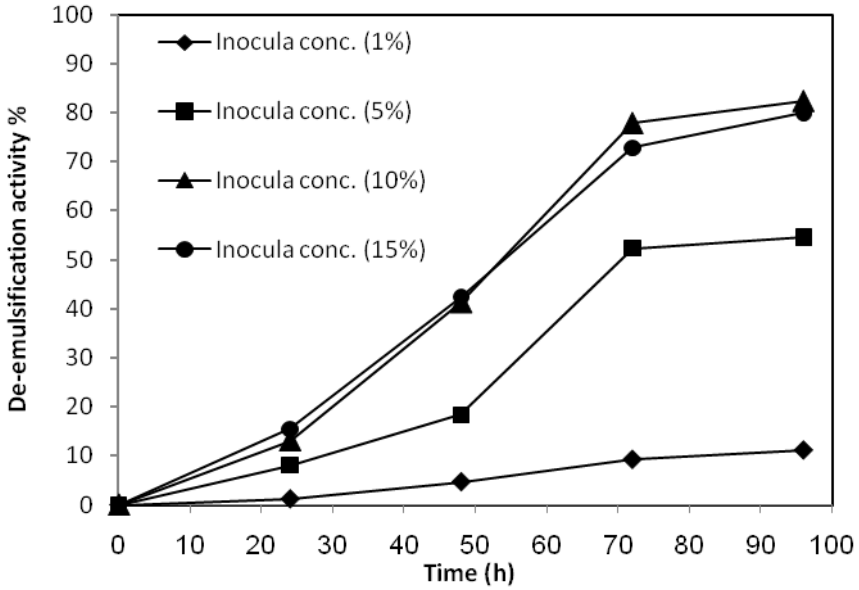

Figure 2. Effect of inoculum concentration on the demulsification of the model emulsion by *Microbacterium* culture under unoptimized production. Bacterial counts was 5×10^8 CFU mL^{-1}. W/CO demulsification assay temperature was 80 °C for 48 h. Control experiment carried out by adding uninoculated medium was negligible.

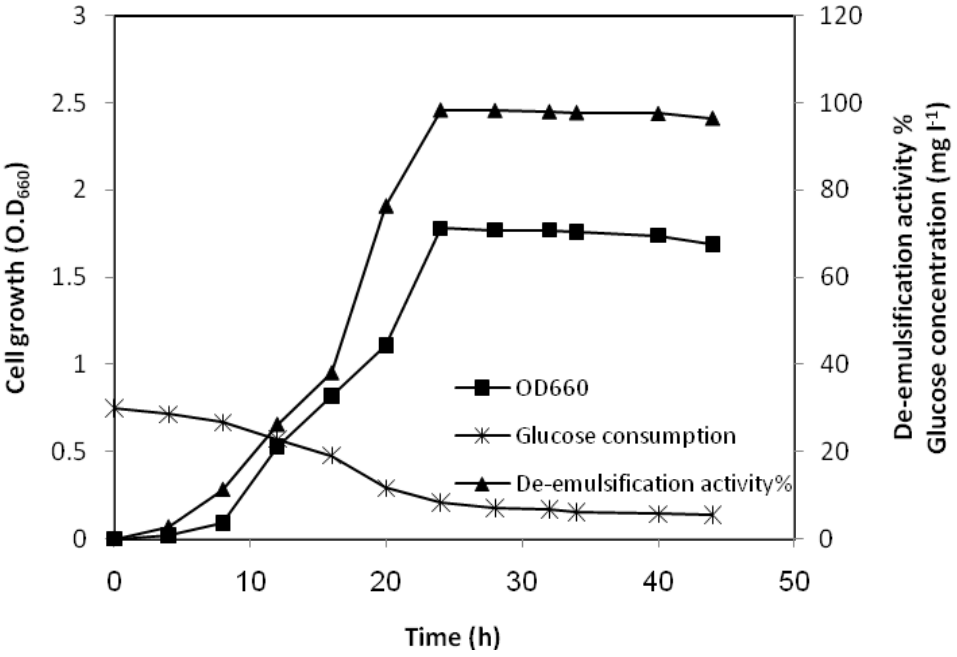

Figure 3. The cell growth, glucose consumption, demulsification activity of *Microbacterium* in a 5 L fermenter at 25°C, 200 rpm, and 0.5 vvm. Glucose: 30 g L^{-1}, yeast extract: 1.0 g L^{-1}.

Figure 3 shows bacterial growth, glucose consumption, and demulsification activity of *Microbacterium* sp. under optimum batch conditions when grown in a 5 L fermenter (Minifors, Infors, Switzerland, aeration at 0.5 vvm with turbine impellers stirring at 200 rpm) using 3% glucose in the presence of yeast extract (1.0 g L^{-1}) as an organic nitrogen source. Table 2 summaries the result of the production of the microbial demulsifier using glucose and

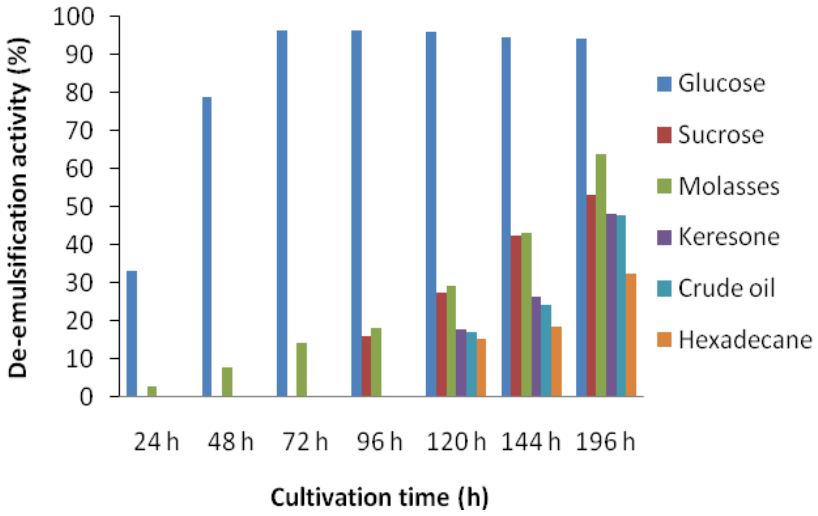

Figure 4. Effect of carbon source on demulsification activity of *Microbacterium* under optimum conditions. Initial nitrogen content in medium included NH_4NO_3 (4 g L^{-1}) and yeast extract (1.0 g L^{-1}).

molasses supplemented with yeast extract in the 5 L fermenter. Using molasses as the carbon source, demulsification activity of approximately 65% was achieved after 64 h. As with glucose, activity was growth associated and was maximum at the beginning of the stationary phase.

Effect of carbon and nitrogen sources on demulsifier production

Additional tests were run in shake flasks to evaluate demulsification activity of *Microbacterium* sp. using a variety of carbon sources such as glucose, molasses, sucrose, kerosene, crude oil, and hexadecane with and without supplementary yeast extract (Figure 4). *Microbacterium* sp. grown on sugar substrates displayed higher demulsification activities as compared with hydrocarbon substrates with best results obtained with glucose. Therefore, glucose was selected as the carbon source for further experiments. In fact, no significant demulsification activity was observed by *Microbacterium* sp. in the absence of yeast extract in the growth media.

Characterization of microbial demulsifier

Following extraction of the crude demulsifier, it was found to have a high sugar component of 73% and protein content of 15.6%. The infra-red (IR) spectrum of the microbial demulsifier (Figure 5) showed absorptions at 1539 and 1641.5 cm^{-1}, representing peptide groups. The peaks at 1641 and 1402 cm^{-1} indicate carboxylate groups. The peaks at 2914 and 2956 cm^{-1} confirm the presence of aliphatic carbon-hydrogen bonds. The peaks at 3278 and 3396 cm^{-1} represent broad amine and hydroxyl groups. The strong adsorption at 1090 exhibits the C–O stretching and the presence of a methoxyl group. The demulsifier produced by *Microbacterium* sp. is a thermo stable, cell-surface associated component and 38% of its activity remained after heating at 100°C for 30 min.

DISCUSSION

In this study, an aerobic and gram positive strain belonging to *Microbacterium* sp. was isolated from an oily sludge sample from Siri Island in the south of Iran. Growth conditions play a key role on the production of microbial demulsifier produced by *Microbacterium* sp. *Microbacterium* was able to grow on glucose as a sole carbon source and produced microbial demulsifier only in the presence of yeast extract. Kosaric (1996) obtained similar results and showed that *Rhodococcus auranticus*, *Rhodococcus rubropertinctus* and *Nocardia amarae* had better demulsifying ability when grown on carbohydrates such as glucose than by growing on hydrocarbons such as hexadecane in the presence of yeast extract.

Demulsification activity of the culture broth obtained from the 5 L fermenter increased in parallel with the cell growth (10 to 20 h) and reached a maximum value of98.6% at the end of the exponential growth phase (approximately 24 h) and did not change during the stationary phase which was stopped after 48 h (Figure 3). Similar results have been reported by other researchers (Das et al., 2001; Nadarajah et al., 2002). Demulsifier produced by *Alcaligenes* sp. which was a hydrophilic cell-wall associated bioproduct, achieved 96.5%

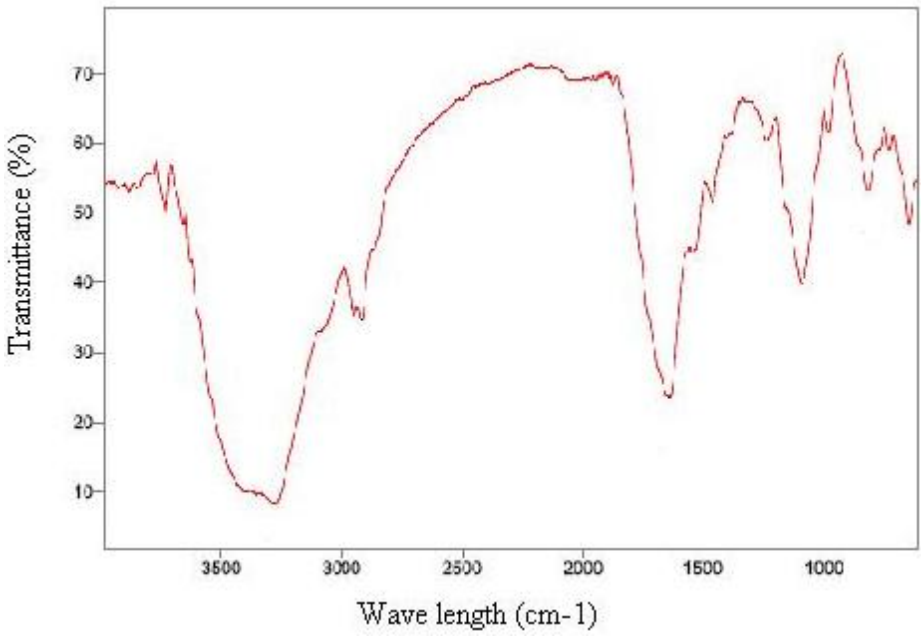

Figure 5. The IR spectrum of the partially purified microbial demulsifier produced by *Microbacterium* sp.

demulsification in W/O emulsion after 24 h ($t_{1/2}$= 2 h) (Das et al., 2001). Nadarhajah reported the culture containing *Acientobacter calcoaceticus* exhibited high demulsification activity (96%) in W/O emulsion after a 24 h incubation time which corresponded to the beginning of the stationary phase. *Acientobacter radioresistans* were capable of providing more than 90% demulsification, while *P. aeruginosa*, *P. carboxydohydrogena* and *Alcaligenes latus* demonstrated up to 80% demulsification (Nadarajah et al., 2002). *Alcaligenes* sp. S-XJ-1 showed demulsification activity of 81.3% for W/O emulsion within 24 h of incubation with cell concentration of 500 mg L^{-1} (Wen et al., 2010).

In summary, *Microbacterium* sp. was found to produce a highly effective, thermostable microbial demulsifier using glucose at the end of the exponential growth phase. *Microbacterium* sp. demulsifier promises potential biotechnological application for use in the oil industry and for environmental remediation.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Petroleum Engineering and Development Company (PEDEC) and University of Isfahan for their financial supports.

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