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Evaluation of a microbial consortium for crude oil spill bioremediation and its potential uses in enhanced oil recovery

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ABSTRACT

Microbial enhanced oil recovery (MEOR) process utilizes reservoir microorganisms (*in situ*) or specially selected bacteria to use their metabolic products (*ex-situ*) for extra oil recovery from depleted oil reservoirs. Biodegradation of crude oil and simultaneous production of bioemulsifiers by a mixed culture consisting of *Ochrobactrum pseudintermedium* C1 and *Bacillus cereus* K1 have been investigated in this study along with the potential *ex-situ* application of bioemulsifier in MEOR process. The culture parameters namely pH, temperature, aeration rate and agitation rate have been optimized in a lab scale bioreactor using Taguchi's L9 orthogonal array approach to increase the rate of biodegradation of crude oil. At optimized culture condition (pH 8.0, temperature 35 °C, aeration rate 1.5Nl/min and agitation rate 150 rpm), the bacterial consortium degrade up to 70.54% of total petroleum hydrocarbons (TPH) after 72 h incubation using crude oil (4% v/v) as the sole source of carbon and produced two different exopolysaccharides (EPS), which showed very good emulsification activity (EI) towards crude oil up to 80.5% and also reduced the interfacial tension of crude-oil water system from 46 mN/m to 14.5 mN/m. FT-IR, NMR and chemical analyses revealed that EPS formed by C1 contained 61.5% (w/w) carbohydrates, 29.2% protein and 9.3% lipid whereas, the EPS formed by K1 comprises of 43.6% (w/w) carbohydrates, 51.7% lipids and 6.3% protein. The crude bioemulsifier mixture was found to be pseudoplastic, Non-Newtonian in nature and was stable at a wide range of pH (2 − 10), temperature (40–121 °C) and salinity (0–15% w/v), hence signifies its applicability in MEOR process. The results of the sand pack column flooding tests at simulated reservoir conditions demonstrated that the additional oil recovery efficiency due to the *ex-situ* injection of the cell-free bioemulsifier solution was 40.93% and 46.85% at 40 °C and 70 °C temperatures respectively. The mixed culture presented a great potential application in bioremediation of oil polluted sites owing to its degradation ability to crude oil and also in enhanced oil recovery process using the crude bioemulsifier having combined surface and emulsification activity.

1. Introduction

Crude petroleum plays a vital job as the significant vitality asset in this day and age because of quick industrialization everywhere throughout the world. Previous field studies have demonstrated that about 66% of the total crude petroleum reserves remains unrecovered or caught after conventional oil recovery applications by primary technique utilizing common pressure drive of the reservoir and the auxiliary strategy including the infusion of water to enhance the stream of oil and gas to the well bore (Lazar et al., 2007; Suthar et al., 2008; Brown, 2010). The challenge to meet the ever growing energy consumption along with increasing petroleum prices, various tertiary oil recovery techniques are being evaluated in depleted oil reservoirs including the use of surfactants, polymers and solvents to mobilize the entrapped oil known as chemically enhanced oil recovery (CEOR) process. It is generally accepted that CEOR technology has the potential for recovery of significant portion of the entrapped oil contained in the reservoirs (Youssef et al., 2013). However, these procedures are earth perilous, costly, and leave bothersome buildups which are hard to discard off, without antagonistically influencing the environment (Gudiña et al., 2012).

Then again, crude petroleum as the primary wellspring of vitality on the planet, with its wide scale transportation and utilization builds the danger of spillage, prompting soil and water pollution (Darvishi et al., 2011). The hydrocarbon components of crude oil are of environmental concern due to their toxic hazards (Bejarano and Michel, 2010) and are

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detrimental to a wide range of plants, animals and microbial communities. As compared to physical and chemical methods, such as utilizing booms, skimmers, adsorbents, chemical surfactants, oxidants, etc., the technique of microbial remediation has been proved to be a viable treatment option to minimize the adverse effects caused by oil spillage (Kumari et al., 2012). Nevertheless, the mostly insoluble oil hydrocarbon can possibly confine the limit of microorganisms to avail and degrade the substrate. Consequently, microorganisms frequently produce biosurfactant/bioemulsifiers to build the bioavailability of hydrocarbons and to accelerate their uptake as carbon source (Banat et al., 2010). Low toxicity and high biodegradability compared to their chemical counterparts make them environmentally acceptable and potential candidates for enhanced oil recovery and bioremediation besides various other industrial applications (Sen, 2008; Banat et al., 2010).

Microbial Enhanced Oil Recovery (MEOR) is an essential tertiary oil recovery innovative technology that corresponds to a more affordable and eco-accommodating option to CEOR. In MEOR, specific microbial strains are utilized to produce compounds closely resembling those utilized in CEOR procedures to expand the recuperation of oil from drained and marginal oil reservoirs (Sen, 2008; Brown, 2010). MEOR is a less expensive process when compared with CEOR because microorganisms can synthesize EOR agents by fermenting low-cost substrates or raw materials (Gudiña et al., 2012). There are three general systems exist for the execution of MEOR (Al-Wahaibi et al., 2014). In the primary way, just supplements were infused to influence the growth of the indigenous microorganisms to debase heavy oil portions. Thus the oil consistency diminishes and it turns out to be increasingly portable, lighter and economically important. It also generates water flow diversion by generating biomass that plugs high permeability flow paths (Bauer et al., 2011; Sunde et al., 2012). In the second method, injection of exogenous microorganisms(s) and nutrients were done to produce various bacterial metabolites such as biosurfactants, bioemulsifiers, extracellular polymeric substances (EPS) etc. which will act as EOR agents. The third strategy is the injection of *ex-situ* produced microbial products to the reservoir for enhancing oil recovery. The first two strategies *(in-situ*) have the added difficulty of dealing with subsurface bacterial transport, competition for nutrients among the desired organism and other indigenous microorganisms, and maintaining nutrient levels throughout a reservoir for extended periods of time (Gray et al., 2008). In some cases, incorporating multiple procedures by utilizing consortia of microorganisms with distinct properties (capacity to debase heavy oil portions and biosurfactant generation) might be a compelling technique for enhanced oil recuperation or recovery (Jinfeng et al., 2005; Darvishi et al., 2011).

Several investigations have been carried out on the *in-situ* bacteria flooding (Bordoloi and Konwar, 2008; Youssef et al., 2013), and the *ex-situ* biosurfactant production and addition to the sand pack as agents for MEOR (Amani et al., 2013). Few researches have been reported about combining multiple microbial mechanisms by the use of a consortium of microbes to increase the biodegradation rate of hydrocarbons in the contaminated sites with simultaneous production of biosurfactants under extreme environmental conditions and further its application in enhanced oil recovery process (Darvishi et al., 2011; Gudiña et al., 2012). In the two instances of microbial enhanced oil recovery and biodegradation of hydrocarbons, it is admitted that a variety of environmental factors, for example, high temperature, salinity, pressure, hydrocarbon toxicity and absence of oxygen would negatively influence the development of microorganisms, henceforth the measures of their bioproducts. The combination of these factors can be relied upon to confine the number of appropriate microorganisms that could develop and deliver bioproducts (Plaza et al., 2006).

Since the rates of microbial population development and biosurfactant synthesis in nature are to a great extent dictated by the neighborhood ecological conditions, this investigation is aimed at exploring the capability of two isolated bioemulsifier producing strains in a consortium for crude oil degradation and bioemulsifier production at varied temperatures, pH, aeration and agitation rate under controlled bioreactor system and determining the optimum criteria for this process by Taguchi's design experimental methodology. The underlying objective of this study was to investigate whether the bioemulsifer mixture could be produced in optimized condition through biodegradation of crude oil and to prove its potential uses as an *ex-situ* MEOR agent for application in enhanced oil recovery process. The chemical characterization of the bioemulsifiers was investigated by Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance spectroscopy (NMR). The emulsification activity and inter-facial activity of oil/water biphasic system have also been measured. Finally, the crude oil degradation was measured by gas chromatography (GC) and the evaluation of possible application of this bioemulsifier in enhanced oil recovery using sand pack columns have also been investigated.

Table 1
Characterization of crude oil.

Sl. No.	Properties
1.	Specific gravity – 0.880
2.	API gravity – 31.5
3.	Viscosity at 40 °C–9.6cp at 70 °C–7.2cp
4.	Initial boiling point (IBP) – 65 °C
5.	Sediment – 0.08
6.	Pore point – −8 °C
7.	Water content – Nil
8.	Sulphur content – 2.6 (wt%)
9.	Ash content – 3.2 (wt%)
10.	Carbon residue – 0.35 (wt%)

2. Materials and methods

2.1. Chemicals

Crude oil having the specifications represented in Table 1 was collected from IOCL Oil Refinery, Haldia, India. N-hexane of liquid chromatography grade and standard n-alkanes were purchased from E. Merck Co. (Germany) was used. Other chemicals and solvents were of analytical reagent grade and purchased from local suppliers. Bushnell-Haas (BH) media and Nutrient agar media of Hi-Media Laboratories Pvt. Ltd were used for isolation, cultivation and maintenance of culture.

2.2. Microorganisms and consortium culture preparation

The two hydrocarbon degraders as well as bioemulsifier producer organisms used in this present study were *Ochrobactrum pseudintermedium* C1 and *Bacillus cereus* K1, isolated previously from waste oil contaminated soil in our laboratory (Bhattacharya et al., 2014a, 2014c). Both the organisms were identified by 16S rRNA gene sequencing method from Bhat Biotech India Pvt. Ltd. Bangalore, India and submitted to NCBI GenBank database under the accession numbers KJ094035 and KJ922989 respectively. For isolation of the microorganisms, Bushnell Haas (BH) media was used as enrichment medium with the following composition (g/L): K_2HPO_4 (1.0 g), KH_2PO_4 (1.0 g), NH_4NO_3 (1.0 g), $MgSO_4.7H_2O$ (0.2 g), $FeCl_3.6H_2O$ (0.05 g), $CaCl_2.2H_2O$ (0.02 g). Nutrient agar media was used for maintenance of the culture. The composition of the nutrient agar used was as follows: beef extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, agar 15.0 g in a liter of distilled water. To prepare the consortium culture for biodegradation of crude oil, the two isolates C1 and K1 were mixed at 2:3 proportions and used as inoculum at 10% (v/v) level throughout the study, which was selected according to maximum degradation potential of the consortium.

2.3. Cultivation procedure

To determine the biodegradation of crude oil in accordance with

bioemulsifier production at varied environmental conditions, cultivations were performed in a 1.5 L fully mixed batch scale bioreactor (Eyela Co., Tokyo, Japan) equipped with constant temperature water circulator and monitoring devices for temperature, dissolved oxygen and pH was used for this purpose. Cultivations were performed in triplicates using BH medium (500 ml) with 4% (v/v) of crude oil as carbon source. In this section we have considered the effects of four factors (chosen from preliminary experimental trials) including pH, temperature, aeration rate and agitation rate on crude oil biodegradation by the consortium culture. Emulsification activity (EI) was used as a measure of bioemulsifier production in the culture broth for each experiment to find out a possible correlation between crude oil biodegradation and bioemulsifier production.

2.4. Optimization of fermentation conditions by Taguchi's methodology

Taguchi's experimental design techniques has been recently employed in bioprocess applications (Mohan et al., 2005; Rao et al., 2008) due to its advantage in synchronous optimization of various factors (independent variables) to accomplish the best response (dependent variable) with least number of perceptions. Taguchi investigation can give authoritative data for mostly to single response reaction frameworks (Rao et al., 2008), subsequently for the present investigation the standard orthogonal array of L9 (Roy, 2001) was utilized to look at the four factors at three levels (all tests were performed in triplicate) so as to enhance the biodegradation of crude oil by the consortium culture in terms of TPH removal efficiency (single response). The L and the subscript 9 correspond to the Latin square and the number of exploratory runs, consequently. Rather than leading 81 test runs, for general factorial design configuration including four parameters, just 9 test runs were required in the Taguchi's technique to optimize the parameter settings for the current investigation. Table 2 enlists the four independent process factors (pH, aeration rate, agitation rate and temperature) and their respective levels for the present study. The range of the parameters was specified based on preliminary laboratory experiments.

The resulting data obtained from the experiments were processed using Minitab-16 software (Minitab Inc. USA for Windows7) to evaluate the influence of individual factors, the multiple interactions of the selected factors, the determination of the optimal conditions and the process performance on crude oil biodegradation. Table 3 represents the experimental design matrix along with the mean TPH removal efficiency values and the calculated signal-to-noise (S/N) ratios. The corresponding emulsification activity (EI) of the cell free culture broth after each experimental run was also showed in Table 3. The S/N ratio values corresponding to the TPH removal efficiency values were estimated, using the 'larger-the-better' characteristics, since the point of the work was to maximize the response (crude oil biodegradation). The S/N ratio for each run was calculated according to the following equation (Roy, 2001):

$$\frac{S}{N} = -10\mathrm{Log}\left(\frac{1}{k}\sum_{i=1}^{k}\frac{1}{y_i^2}\right) \quad (1)$$

where y is the TPH removal efficiency for corresponding run, i is the number of replicate and k is the number of trial experiments performed in any particular parametric combinations as per Table 3. The predicted S/N ratio at the optimal process conditions for achieving maximum TPH removal was estimated from the following equation (Taguchi, 1986):

$$\frac{S}{N_{predicted}} = \frac{\bar{S}}{N} + \sum_{j=1}^{n}\left(\frac{S}{N_j} - \frac{\bar{S}}{N}\right) \quad (2)$$

Where $\bar{S}/N$ is the mean of all S/N ratios, S/N_j is the S/N ratio at optimal level for each parameter and n is the number of the process parameters that significantly affect the process.

Table 2
Experimental levels of the studied factors for biodegradation of crude oil.

Process parameters	pH	Aeration rate (Nl/min)	Agitation rate (rpm)	Temperature (°C)
Level 1 (L1)	6	0.5	100	30
Level 2 (L2)	7	1.0	150	35
Level 3 (L3)	8	1.5	200	40

2.5. Isolation, purification and characterization of the bioemulsifier (BE)

The crude bioemulsifiers were isolated as extracellular polymeric substances (EPS) from the culture broth following the method described by Cooper and Goldenberg (1987) and Calvo et al. (2008) with slight modifications. At the optimized fermentation condition, the 72 h grown culture broth was centrifuged at 4 °C and 10,000 rpm in a REMI C24 (Chennai, India) centrifuge for 20 min. The emulsifier agents were extracted from the supernatant with successive extraction using chloroform-methanol mixture (2:1) followed by precipitation with chilled ethanol [ethanol: medium ratio, 2:1 (v/v)] at 4 °C for 16 h. The biopolymers (EPS) precipitated from the supernatant were further centrifuged at 10,000 rpm at 4 °C. The pellets were dissolved in distilled water and dialyzed using 14 kDa cut off dialysis membrane (HiMedia, India). The dialyzed biopolymers (EPS) were then freeze-dried (EYELA FDU-1200, Japan) and finally weighed. The biopolymers (EPS) obtained were subjected to carbohydrate, protein and lipid analysis. Carbohydrate content was determined at 490 nm following phenol-sulphuric acid method according to Dubois et al. (1956) and the protein content was quantified at 595 nm according to Lowry's method (1951) using a UV–Vis spectrophotometer (CECIL, UK). Lipid content was estimated adopting the procedure of Folch et al. (1957). Functional groups of the biopolymers (EPS) were determined by Fourier transform infrared spectroscopy using JASCO FT/IR-6300 (USA) and the spectrum was recorded in the range of 4000–400 cm^{-1} with 32 scans. NMR data was obtained to further elucidate the chemical structure of the biopolymers (EPS) using Bruker Avance DPX 400 spectrometer (400 MHz FT-NMR). The ^1H NMR spectra were recorded from the sample solutions in D_2O and the chemical shifts were expressed in ppm relative to the resonance of TMS as internal standard.

2.6. Analytical methods

2.6.1. Determination of total petroleum hydrocarbons (TPH)

At the end of each experiment, the residual oil samples were extracted using hexane and chloroform in succession according to Kumari et al. (2012). The organic phase was concentrated by evaporation of the solvent after drying over anhydrous Na_2SO_4 and analyzed by gas chromatography according to USEPA 8015B (1986) test methods. 1.0 µl of sample were injected for analysis by using a Thermo Scientific Trace 1300 series gas chromatograph equipped with flame ionization detector and TR-5 column (30×10^3 cm length; 0.032 cm id; and 1×10^{-3} cm film thickness). Nitrogen was used as carrier gas. The injector and detector temperatures were maintained at 300 °C and 280 °C respectively. The oven was programmed at an initial temperature of 40 °C; this was held for 2 min, then ramped at 15 °C/min to 300 °C and held for 10 min. The relative percent degradation of crude oil was calculated by the differences in summation of peak area of total petroleum hydrocarbons (TPH) present in the residual oil from test samples compared to that from un-inoculated control samples (Bhattacharya and Biswas, 2014). The component analysis of crude oil samples were done by using a standard n-alkane mixture composed of C8, C9, C10, C12,C14, C16, C18, C20, C28, C34 and C40.

Table 3
L9 orthogonal array design matrix with calculated S/N ratios for mean TPH removal efficiency values, along with corresponding emulsification activity (EI) of the culture broth.

Run No.	Parameters						Corresponding emulsification activity (EI %)
	pH	Aeration rate (Nl/min)	Agitation rate (rpm)	Temperature (°C)	TPH removal efficiency (%)	S/N ratio	
1	6	0.5	100	30	42.67	32.60	45.72
2	6	1.0	150	35	47.25	33.49	50.95
3	6	1.5	200	40	48.34	33.68	52.25
4	7	0.5	150	40	55.12	34.82	60.35
5	7	1.0	200	30	56.82	35.09	62.5
6	7	1.5	100	35	60.25	35.59	65.5
7	8	0.5	200	35	59.85	35.54	65.34
8	8	1.0	100	40	65.24	36.29	73.85
9	8	1.5	150	30	70.25	36.93	80.24

2.6.2. Bacterial growth determination

At the end of each biodegradation experiment, the culture broth was centrifuged at 10,000 rpm for 20 min. The biomass concentration in the culture broth was determined by dry weight method as well as by measuring optical density at 600 nm. After centrifugation, the bacterial mass was transferred to a pre-weighted aluminum cup and dried at 50 °C overnight to obtain the yield of biomass (Guchhait et al., 2005).

2.6.3. Emulsification index measurements

Emulsifying activity of the culture was assessed by a modified method (Cooper and Goldenberg, 1987) as follows. The cell free culture broth and crude oil (2 mL) were added into each test tube. The tubes were then vortexed at high speed for 2 min. The emulsion stability was determined after 24 h, and the emulsification index (EI) was estimated as the height of the emulsion layer divided by the total height and multiplied by 100. All the results were repeated three times to obtain the average value.

2.6.4. Surface tension and interfacial tension measurements

Surface tension (ST) of cell free and oil free culture supernatant was measured by the application of a digital tensiometer (Dataphysics DCAT 11, Germany) at 30 °C using Du Nouy ring method (Lunkenheimer and Wantke, 1981). For the calibration of the instrument, the surface tension of pure water was first measured, which was repeated three times to obtain the average value to express the surface activity of the sample. Interfacial tension (IFT) measurements were carried out against crude oil in the same way.

2.7. Stability studies

The cell free culture broth containing crude bioemulsifier mixture was used to determine the effect of temperature, pH, and salinity on emulsification activity (EI) and interfacial tension (IFT) reduction property. The bioemulsifier sample was incubated in a water bath for 30 min from 25 to 100 °C and also kept at 121 °C for 15 min during autoclaving. The pH stability of the emulsifying agent was assessed by adjusting the bioemulsifier to different pH values (2–10) with diluted HCl or NaOH. To determine the effect of salinity, different concentrations of NaCl were added (2–15%, w/v) to the sample and mixed until complete dissolution was achieved. The EI and IFT measurements of each treatment were assessed as described above, with crude oil used as the substrate.

2.8. Rheology studies

The viscosity of the crude extracellular polymeric substances (EPS mixture and the individual EPS samples) produced by the strains C1 and K1 were measured by a Brookfield Digital DV-II Pro Viscometer (M/s Brookfield Engineering Company, Middleborough, MA) with spindle S-21 in a speed range of 3–200 rpm. The rheological behavior and apparent viscosity of the EPS solutions were obtained using the modified Casson equation for non-Newtonian fluids:

$$\log \tau = \log \mu_{app} + n \log(\partial u/\partial y) \quad (3)$$

where, τ is shear stress, μ_{app} is apparent viscosity, n is flow behavior index and $\partial u/\partial y$ is shear rate.

The instrumental parameters were as follows:

$$\text{Shearstress } (\tau) = M/(2\pi R_b^2 L) \quad (4)$$

where M is the torque input by the instrument (maximum torque is 673.30 dyne-cm), $R_b = 0.58$ cm and L is the effective length of the spindle (5.00 cm).

$$\text{Shear rate } (\partial u/\partial y) = (2\omega R_c^2 R_b^2)/(R_c^2 R_b^2) \quad (5)$$

where ω is angular velocity of the spindle (s^{-1}) and R_c is the radius of the container (1.00 cm). The log–log plots of shear rate vs. shear stress were used to evaluate the visco-elastic parameters of the emulsifying agent such as apparent viscosity (μ_{app}) and flow behavior index (n) from the modified Casson equation. The flow behavior index (n) was used to identify the type of fluid flow ($n < 1$ pseudoplastic, $n = 1$ Newtonian and $n > 1$ dilatants).

2.9. Sand-pack flooding studies

The execution of sand pack column flooding tests for enhanced oil recovery has been performed adopting the strategy portrayed by Bera et al. (2014). The experimental set up (see in Fig. 1) is made out of a sand pack holder, cylinders for holding chemical slugs and crude petroleum, positive displacement pump, and measuring cylinders for collecting the samples. The displacement pump is one set of Teledyne Isco (USA) syringe pumps. The control and measuring system is composed of different pressure transducers and a computer. The physical model is a homogeneous sand packing model with vertically positive rhythm. The model geometry estimate is L = 35.3 cm and r = 2.8 cm. The sand pack holder was firmly stuffed with uniform sands (60–100 mesh) and saturated with 3% brine solution. It was flooded with the brine at a pressure of 30 psig and the absolute permeability was calculated from the flow rate through the sand pack. The sand pack was then flooded with the crude oil at a pressure of 200 psig to irreducible water saturation. The initial water saturation was determined on the basis of mass balance. Water flooding was conducted by placing the core holder horizontally at a constant injection pressure at 100 psig. After water flooding, when the water-cut reached above 95%, an approximate 1.0 pore volume (PV) of crude bioemulsifier slug was injected followed by chasing water. The experiments were repeated using two different temperatures at 40 °C and 70 °C. The additional recoveries were calculated by material balance.

The effective permeability to oil (k_o) and effective permeability to water (k_w) were measured at irreducible water saturation (S_{wi}) and residual oil saturation (S_{or}), respectively, using Darcy's equation (Eq.

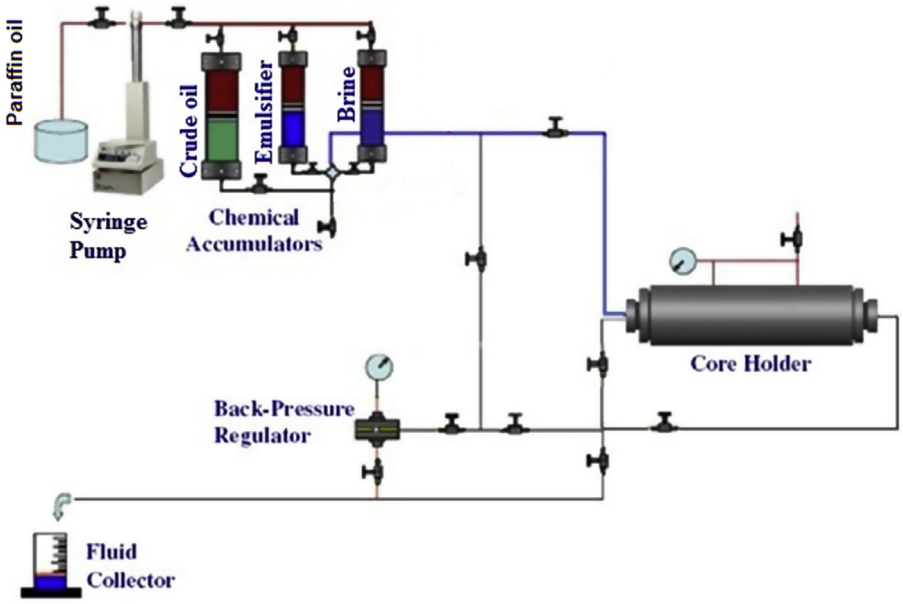

Fig. 1. Schematic representation of sand pack flooding experiments for EOR.

Table 4
ANOVA results for parameters affecting TPH removal efficiency.

Factor	DF (Degrees of freedom)	Sum of squares	Mean squares	*F*-value	*P*-value
pH	1	549.477	549.477	92.63	0.001
Aeration rate	1	75.161	75.161	11.67	0.024
Agitation rate	1	9.747	9.747	1.657	0.062
Temperature	1	0.957	0.957	0.112	0.236
Residual error	4	22.649	22.649		
Total	8	657.991			

Table 5
Response table for signal-to-noise (S/N) ratio.

Level	pH	Aeration rate	Agitation rate	Temperature
1	33.26	34.32	34.83	34.88
2	35.17	34.96	35.08	34.93
3	36.25	35.41	34.77	34.89
Delta	3.00	1.08	0.31	0.06
Rank	1	2	3	4

(6)), used for fluid flow in porous media. For a horizontal laminar system, flow rate is related with permeability as per Darcy:

$$q = -\frac{kA}{\mu}\frac{dp}{dx} \tag{6}$$

where q is volumetric flow rate (cm^3/sec), A is total cross-sectional area of the sand pack (cm^2), μ is the fluid viscosity (centipoise), dp/dx is the pressure gradient (atm/cm), and k is permeability in Darcy.

The recovery factor is obtained by summing up the amounts of oil recovered in each step (secondary and tertiary oil displacement process) and is expressed in percentage (%)

$$RF_{Total} = RF_{SM} + RF_{TM}$$

where, RF_{Total} = total recovery factor (%), RF_{SM} = recovery factor obtained by secondary method (%), RF_{TM} = recovery factor obtained by tertiary method (%).

3. Results and discussion

3.1. Taguchi design analysis and prediction of optimal condition for TPH removal

The ANOVA (Analysis of Variance) results for the L9ortho-gonal

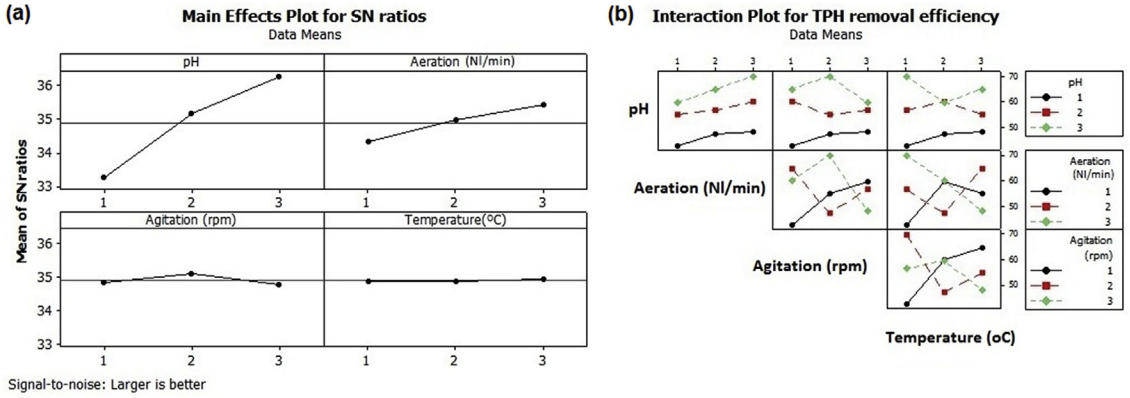

Fig. 2. Optimization of crude oil biodegradation process parameters using Taguchi's design methodology (a) S/N ratio plots for four process control factors studied; (b) Interaction plot for maximum TPH removal efficiency.

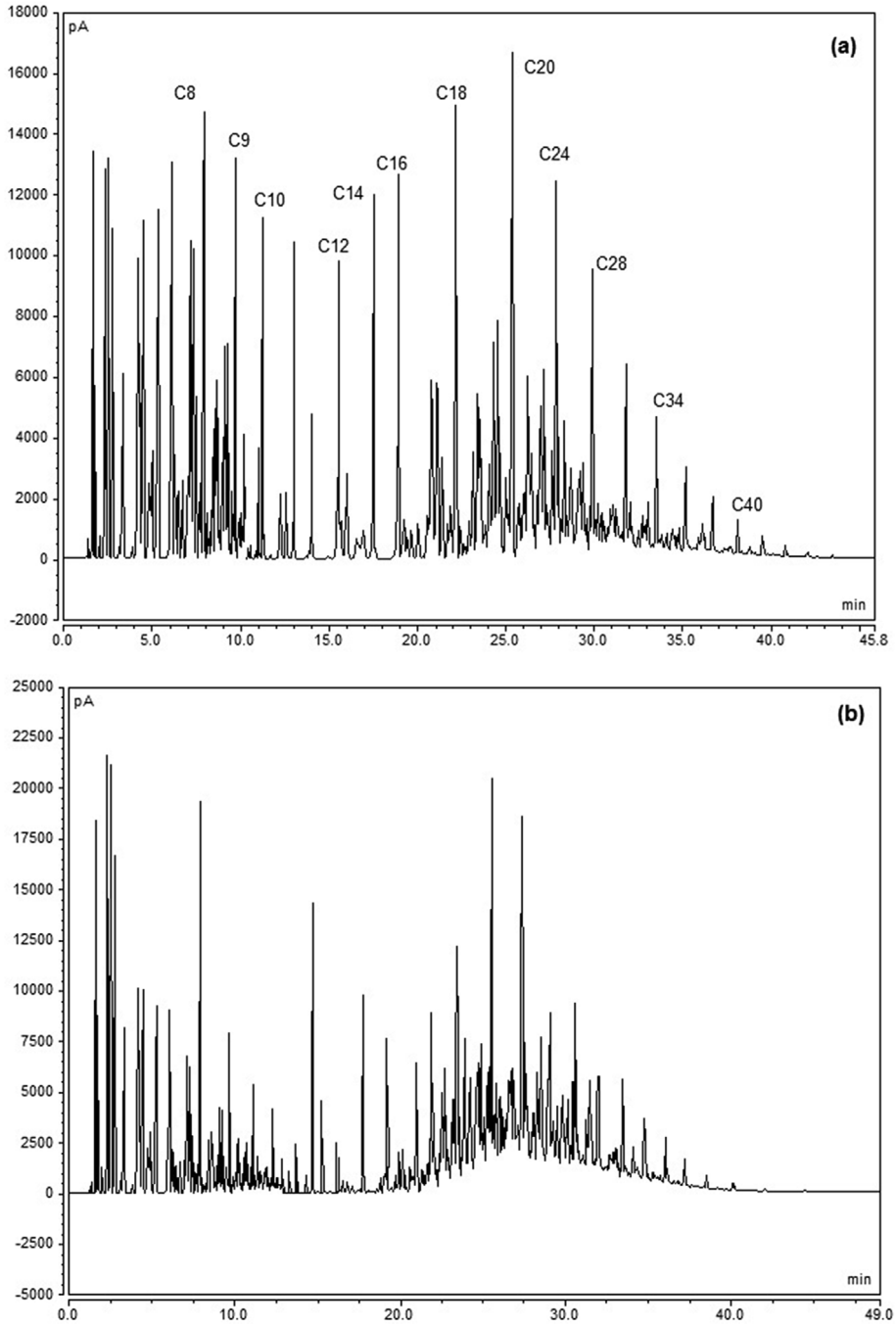

Fig. 3. Crude oil biodegradation analysis by gas chromatography (a) Crude oil sample before biodegradation; (b) Recovered crude oil sample after biodegradation.

array are depicted in Table 4. The outcomes demonstrated that out of the four process control factors examined, pH and aeration rate have critical impact on the biodegradation response with their *p* value estimates (likelihood or probability) under 0.05. The highest *F* value (Fischer's F-test) for the pH indicates its highest influence on the extent of TPH removal followed by aeration rate, agitation rate and temperature. The S/N ratio was determined utilizing "the larger the better" paradigm, as the key point was to amplify the TPH removal efficiency of the culture. Table 5 shows the positioning of the parameters dependent on the delta value estimates (the difference of the S/N ratio values between the most elevated and the lowest level of process factors). The higher the delta estimation of each factor, the higher is its impact on the removal of TPH by biodegradation of crude oil. Thus, the results (Table 5) once again confirmed, that the factor with the most significant effect on biodegradation of crude oil is pH followed by aeration rate, agitation rate and temperature. Fig. 2(a) demonstrated the plots for the S/N ratios and each of the control parameter. The most significant S/N ratio relating to the highest TPH removal recommended the best level for each of the significant parameters viz. pH (L3) and aeration rate (L3). Although agitation rate and incubation temperature have less impact on the response according to ANOVA analysis, agitation rate at L2 and temperature at L2 have been optimized (Fig. 2(a)) to maximize the TPH removal efficiency. The predicted optimal S/N ratio for the optimized conditions was computed from Eq. (2) (Taguchi, 1986) and the TPH removal efficiency was predicted to be 70.85%. In order to validate the optimal conditions confirmatory runs (triplicate) were conducted that gave 70.54% TPH removal efficiency.

Aeration rate has a basic impact in raising the biodegradation

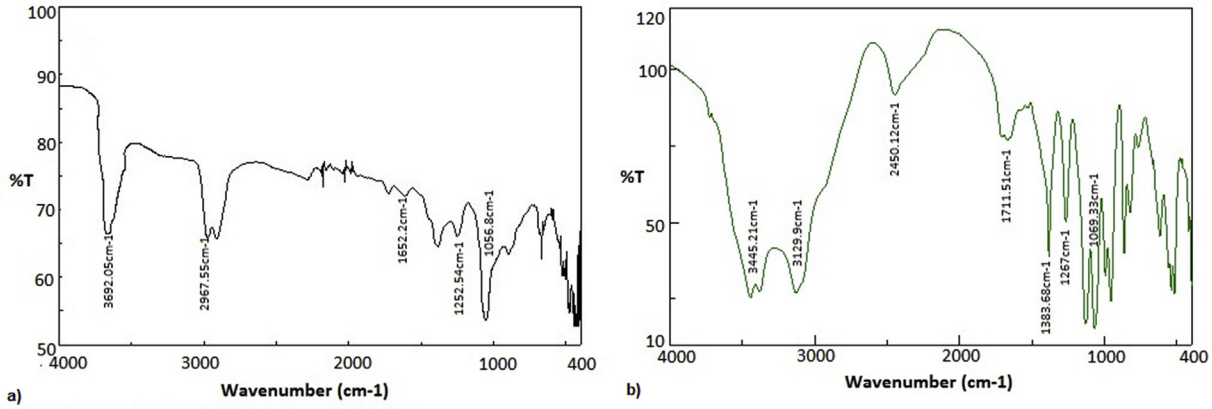

Fig. 4. FT-IR spectra of the biopolymers (a) EPS produced by *Ochrobactrum pseudintermedium* C1; (b) EPS produced by *Bacillus cereus* K1.

kinetics of hydrocarbons and thereby bringing remarkable TPH reduction from crude oil. Biodegradation increased at higher aeration rates, being maximized at 1.5 Nl/min (optimal). This might be due to the fact that high aeration rate increased diffusion process of substrates and favoring biodegradation of hydrocarbons present in crude oil (Gargouri et al., 2011). The dependence on pH in any biodegradation process varies between hydrocarbon degrading microorganisms (Megharaj et al., 2011). The mixed culture used in this study favors alkaline pH (pH 8.0) for optimal growth and biodegradation of crude oil, which further signifies its suitability for oil spill bioremediation in marine environments. pH and aeration rate have been found to affect the biodegradation reaction most significantly. The increase in TPH removal with increasing agitation rate (100–200 rpm) is probably due to reducing the retention time or contact area between biomass and substrates thereby increasing the rate of biodegradation (Wang et al., 2008). However, higher agitation rates (200 rpm) might have caused damage to the bacterial cells due to the shearing effects of the impellers, which could not significantly enhance the TPH removal any further, so the intermediate level of agitation rate was found to be optimal (150 rpm). Though there was negligible difference in biodegradation with changing temperatures from 30° to 40 °C (Fig. 2(a)), temperature (35 °C) at intermediate level was optimized by Taguchi analysis corresponding to its highest S/N ratio obtained for TPH removal efficiency (Table 5). It has also been reported that the optimal working temperature for biodegradation process is between 30°C-40 °C for (Das and Chandran, 2011), which is mostly suitable for various environmental bioremediation.

3.2. Interaction among process variables

Fig. 2(b) exhibits the interaction between different parameters contemplated for biodegradation of crude oil by the mixed culture. The interactions between two factors were assessed keeping the other two factors at their optimal levels (Mohan et al., 2005). The interaction between pH and aeration rate showed that at all levels of pH, an increase in aeration rate could enhance the removal of TPH during biodegradation. At the lowest level of pH there was a monotonic increment in TPH removal with consequent increase in agitation rate, and maximum TPH removal could be accomplished at the highest level of pH and medium level of agitation rate. Similarly, pH and temperature interactions confirmed that maximum TPH removal could be achieved at the highest level of pH and intermediate level of temperature keeping the aeration rate and agitation rate at their optimal levels. It is noteworthy to mention here that maximum TPH removal could only be achieved at the highest level of pH for all other combinations of parametric values studied. Keeping pH and temperature constant, maximum TPH removal was obtained at the highest level of aeration rate and medium level of agitation rate. Whereas for interaction between aeration rate and temperature, the optimal level of aeration could bring about maximum TPH removal in minimum temperature (30 °C). Finally keeping pH and the aeration rate fixed at the optimum level, the TPH removal was found to gradually increase with increase in incubation temperature from 30° to 40 °C for the minimum agitation rate. However, maximum TPH removal could be obtained at 35 °C with the intermediate level of agitation rate.

3.3. Biodegradation of crude oil

The ability of the microorganisms to degrade the crude oil enables them to grow with crude oil as sole carbon source and produce various bio-products which may be useful in the MEOR process (Youssef et al., 2009; Zou et al., 2014). The total petroleum hydrocarbons (TPH) present in the original crude oil (before biodegradation) and in the recovered crude oil (after biodegradation) was analyzed at the end of each biodegradation experiment to determine TPH removal efficiency as a measure of the extent of biodegradation. Fig. 3(a) and (b) depicted the changes in hydrocarbon distribution that occurred at the end of biodegradation process during validation experiments with optimized condition. The recovered crude oil showed significant modifications in the hydrocarbon distribution compared to the original crude oil, probably due to bioconversion of hydrocarbons by the microorganisms during biodegradation process (Darvishi et al., 2011). In the medium fractions, a decrease in C14, C16 and C18 were detected along with an increase in C8 and C12 which is indicative of changes or bioconversion of hydrocarbons. In the heavy fractions also, an increase in C20 and C24 was found as well as degradation of C28, C34 and C40 compounds were observed. Fig. 9 depicts the content percentage change calculated from difference in peak area, before and after biodegradation of crude oil with respect to the standard n-alkanes. However, the biochemical conversion of hydrocarbons depends on substrate specificity of microorganisms and the relative distribution of hydrocarbons (Castorena-Cortés et al., 2012; Zou et al., 2014). These results indicate that microorganisms in the mixed culture used were capable of degrading different hydrocarbons fractions present in the crude oil. It has been reported that the degradation of n-alkanes causes the breakdown of hydrocarbon chains, making them lighter and therefore increasing oil mobility (Sen, 2008). Moreover, the crude oil degrading culture exhibit very high emulsification activity towards crude oil and a significant correlation was found between the extent of biodegradation and emulsification activity (EI) of the culture supernatant (R^2 = 0.994) from the Taguchi experimental runs (Table 2). This implicates further the production of biosurfactants/bioemulsifiers which could reduce the interfacial tension of water/oil biphasic system as well as control the mobility of the crude oil to improve the oil recovery process.

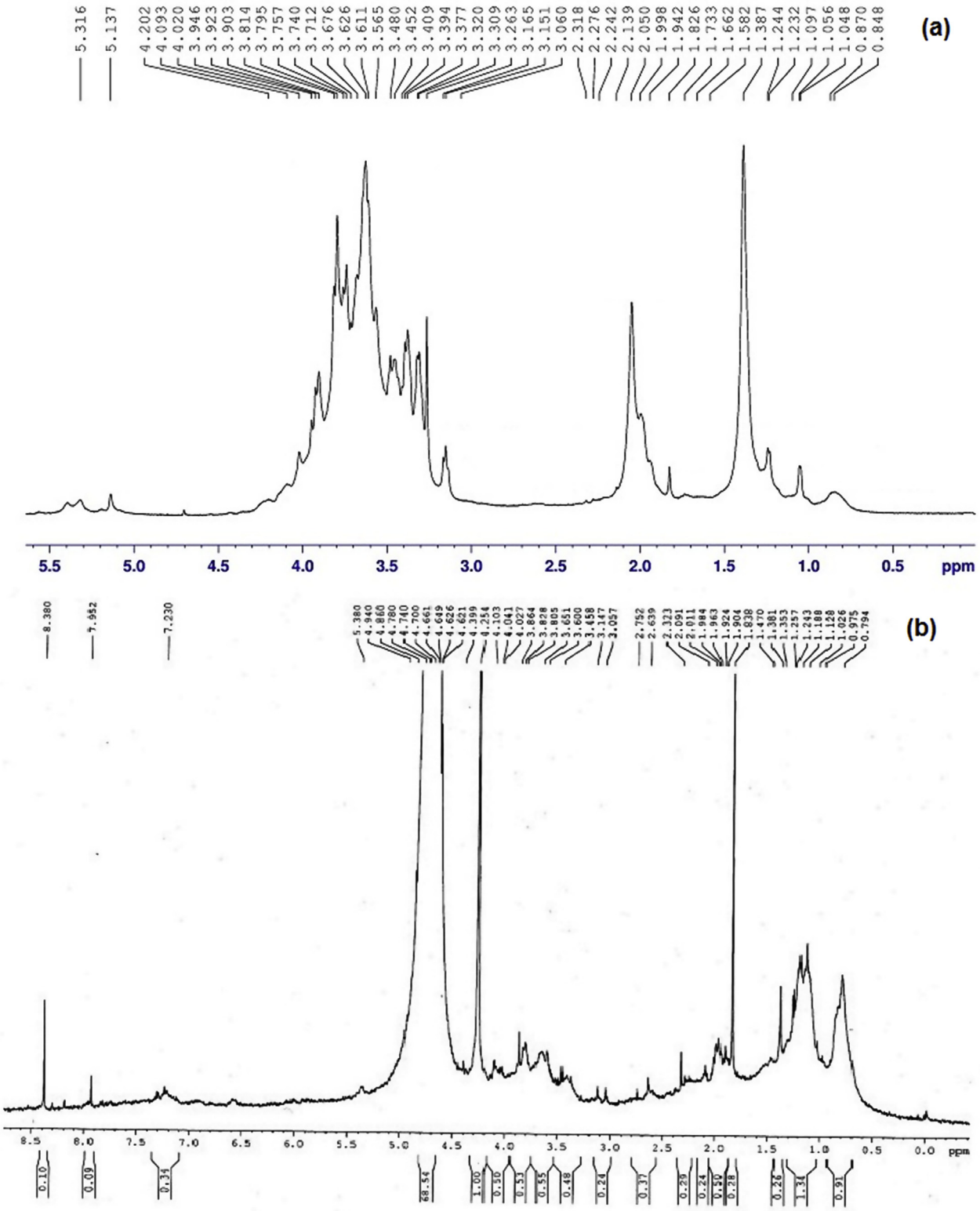

Fig. 5. NMR spectra of the biopolymers (a) EPS produced by *Ochrobactrum pseudintermedium* C1; (b) EPS produced by *Bacillus cereus* K1.

3.4. Physicochemical properties of the isolated bioemulsifier

The infrared spectroscopy, ^{1}H NMR and chemical analysis of the isolated microbial product showed that *O. pseudintermedium* C1 and *B. cereus* K1 strains produced two different exopolysaccharides (EPS) as bioemulsifiers, one is glycoprotein type and the other is a glycolipid in nature respectively. From chemical analysis, it is revealed that EPS formed by C1 (C1 EPS) contained 61.5% (w/w) carbohydrates, 29.2% protein and 9.3% lipid whereas, the EPS formed by K1 (K1 EPS) comprises of 43.6% (w/w) carbohydrates, 51.7% lipids and 6.3% protein. Some typical polysaccharide absorption peaks were observed in both the FT-IR spectrum of the biopolymer (Fig. 4(a) and (b)). C1 EPS

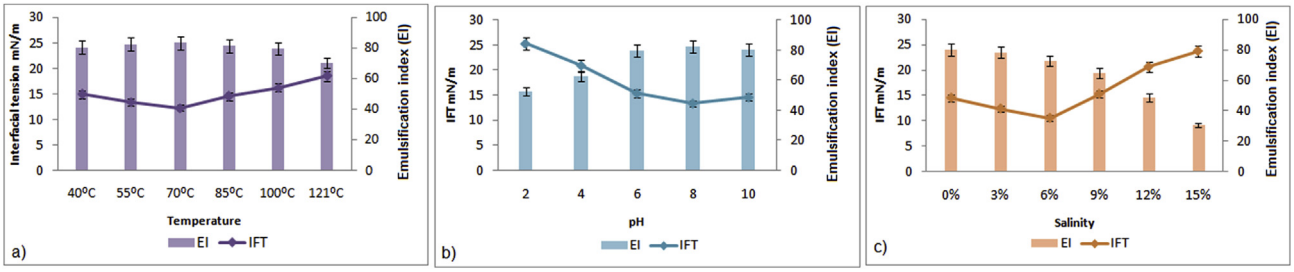

Fig. 6. Emulsification activity (EI) and interfacial tension (IFT) reduction property of the crude bioemulsifier mixture solution (a) Effect of temperature; (b) Effect of pH; (c) Effect of salinity.

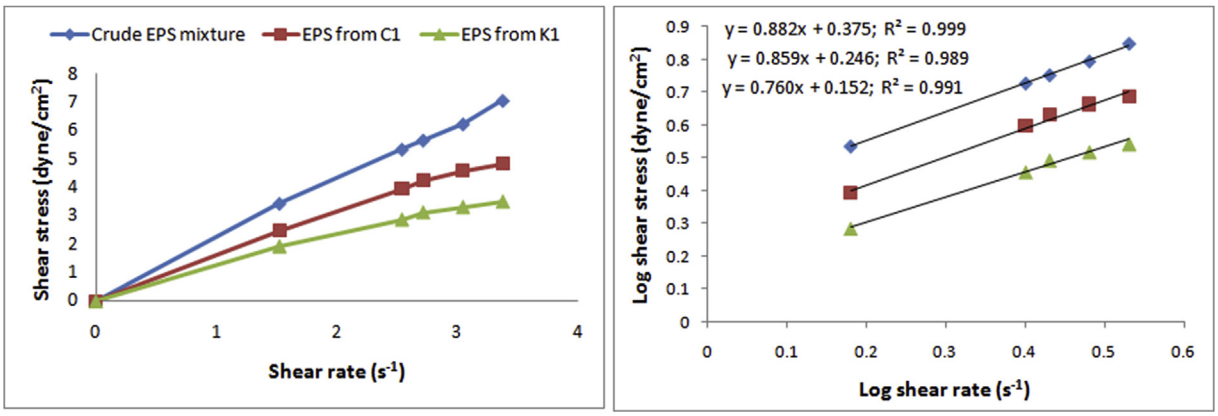

Fig. 7. Rheological analyses of the exopolysaccharide (EPS) samples by Brookfield viscometer with S-21 spindle at 25 °C.

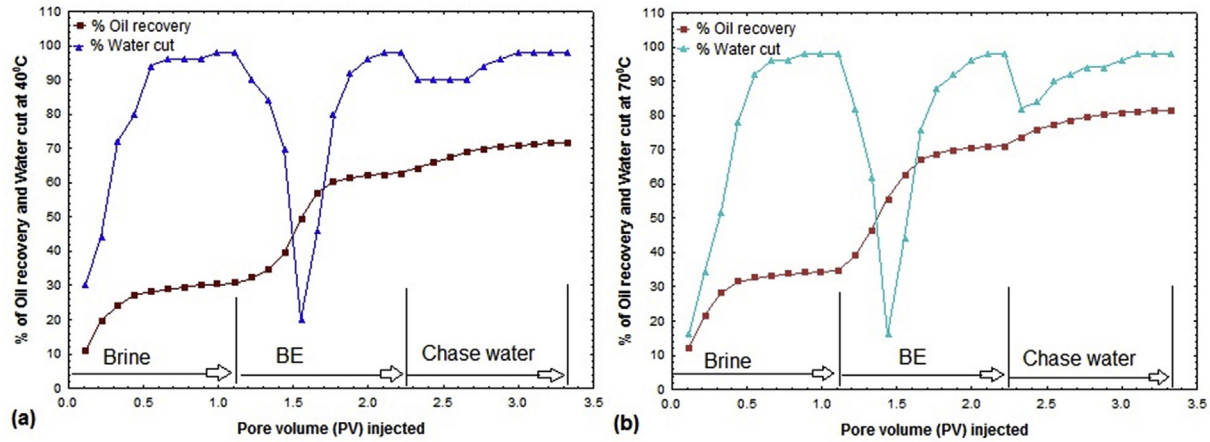

Fig. 8. Oil recovery performance of crude bioemulsifier (BE) flooding (a) at 40 °C; (b) at 70 °C.

showed four distinctive bands appeared at 3692, 2967, 1652 and 1056 cm^{-1} correspond to the stretching vibrations of −OH, C−H, NH−CO and C−O−C respectively. K1 EPS also showed five distinctive bands appeared at 3445, 3129, 2450, 1711 and 1069 cm^{-1} correspond to the stretching vibrations of N−H, −OH, N−H, C═O and C−O−C respectively. Absorption peaks in higher frequencies are the most common characteristics of polysaccharides (Stuart, 2004). The common absorption peak observed in both the EPS at 1252 and 1267 cm^{-1} is indicative of C−N functional group of amides, which in turn indicates the presence of amino sugars within the polysaccharides. The absorbance frequency in the range of 1652 cm^{-1} in C1 EPS and 1711 cm^{-1} in K1 EPS also depicts the carbonyl stretching band of amides, a typical characteristic of acetamido groups in N-acetylated sugars. In fact, most of the amino sugars found in microbial polysaccharides are usually N-acetylated (Christensen, 1989). Absorption peak at 1056 cm^{-1} and 1069 cm^{-1} in both the EPS is indicative index of ether linkage between individual glycosyl residues existed in polysaccharides (Kodali et al., 2009). Overall both the FT-IR spectrum suggested that the sample is predominantly a polysaccharide although some lipids and proteins are also present. This was confirmed by ^{1}H NMR spectrum of both the EPS samples (Fig. 5(a) and (b)) run in D_2O which was dominated by signals attributed to polysaccharide at 3.2–4.4 ppm for C1 EPS and at 4.2–4.7 ppm for K1 EPS respectively. The protons of H− C− (COOR) and H− C – (COOH) were confirmed by the peaks at 1.92 ppm for K1 EPS and at 2.20 ppm for C1 EPS respectively. The signals at 1.0–2.5 ppm for the rest of the spectrum in both samples indicated the presence of long chain −CH2 groups associated with proteins and lipids (Zou et al., 2014). It is interesting to find that either lipid or protein is able to act as the hydrophobic portion attached to the polysaccharide backbone and provide the amphiphilic structure common to surface-active agents (Satpute et al., 2010; Martínez-Checa et al., 2007). These results revealed that the composition of bioemulsifiers was in

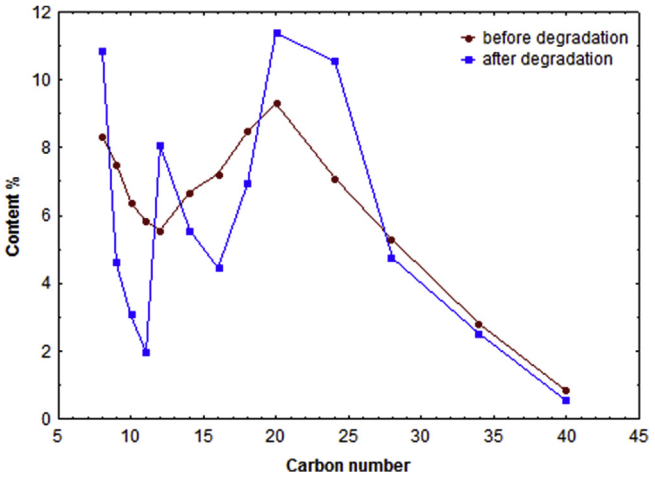

Fig. 9. The contents of different carbon number of crude oil before and after biodegradation. '●' profile of contents versus carbon number before degradation; '■' profile of contents versus carbon number after degradation.

accordance with the result from chemical analysis and the bioemulsifiers should fall into the preferred group of glycoprotein and glycolipid type microbial extracellular polysaccharides, similar to those reported previously (Markande et al., 2013; Kodali et al., 2009).

3.5. Stability and surface activity of the produced bioemulsifier mixture

The aftereffects of the stability studies of the produced bioemulsifier mixture by the consortium culture with respect to temperature, pH and salinity are shown in Fig. 6(a)–(c) respectively. The utilization of partially purified bioemulsifiers for ex situ MEOR requires the bioemulsifier to be steady inside a range of high temperatures (around 50°–80 °C), wide scope of pH and high salt concentrations to guarantee wide appropriateness to actuate oil recovery (Darvishi et al., 2011). Incubation of cell-free broth at a given time interim of 30 min for different temperatures did not demonstrate any momentous impact on the emulsification activity of the bioemulsifier solution, IFT value reduced to minimum (12.27 mN/m) at 70 °C, therefore it was found that the bioemulsifier mixture is quite thermo-stable over a wide scope of temperatures from 40 to 100 °C, and furthermore was steady at 121 °C (EI = 72.5%). Khopade et al. (2012) also reported the stability of biosurfactants under extreme conditions of temperature. Darvishi et al. (2011) reported that heat treatment on some biosurfactants caused no appreciable change in their properties, even after autoclaving at 120 °C for 15 min. Then again, action of the bioemulsier solution was influenced by pH and saltiness, as pH was changed from 2 to 10 and saltiness differed in the range of 0–15% (w/v). By expanding pH from an acidic to a basic region (pH 2.0–8.0), IFT decreased from 25.34 to 14.54 mN/m. Emulsification activity (EI) was found to be optimum at pH 8.0, which is also the pH requirement for optimum growth conditions of the mixed culture on crude oil. Increasing salinity up to 6% IFT first decreased and then it increased from 10.54 to 23.67 mN/m within the salinity range from 6% to 15%. Under highly acidic pH (< 4.0) bioemulsifiers showed less emulsification activity, may be due to the fact that the exopolysaccharides are not soluble under highly acidic conditions and tends to precipitate (Zou et al., 2014). Several reports confirmed the stability of biosurfactant at different pH values, mostly in the alkaline medium (Khopade et al., 2012; Satpute et al., 2010). The tests were repeated for the aliquots of the individual purified EPS, and the same trend was observed. However, the emulsification activity of both the purified bioemulsifiers was lower (approximately 24% for C1 EPS and 27% for K1 EPS) than the corresponding cell-free bioemulsifier mixture solution.

3.6. Rheological behavior

Rheology of the biopolymer plays an important role in controlling the mobility ratio and hence the sweep efficiencies for *ex-situ* MEOR process (Xu et al., 2014). The basic rheological behavior of crude EPS mixture and aqueous solutions of extracted EPS (0.2% w/v) of the strains C1 and K1 has been investigated by varying shear rates. Fig. 7 represents the flow behavior (plot of shear rate vs. shear stress) of the biopolymer samples and the log–log plots of the same. The flow behavior indices n are equal to 0.882, 0.859 and 0.76 for crude EPS mixture, C1 EPS and K1 EPS respectively, indicating that the microbial exopolysaccharide sample have a shear-thinning, pseudoplastic nature. The apparent viscosities of the samples were found to be 2.86 cp, 2.64 cp and 2.43 cp respectively, which were quite significant at moderate shear rate. As the shear rate approaches toward zero the solutions show maximum viscosity. The viscosities of all samples decreased with the increasing shear rate, due to uncoiling and aligning of polymer chains when exposed to shear flow (Xu et al., 2014), suggesting that the aqueous solutions of crude mixture and partially purified microbial exopolysaccharides exhibit non-Newtonian behavior.

3.7. Emulsifier flooding and oil recovery

The sand pack was flooded with crude bioemulsifier slug after water flooding and the oil recovered with pore volume injected into sand pack at 40 °C and 70 °C was depicted in Fig. 8(a) and (b) respectively. Both the figures shows an early breakthrough and channel flow which caused much lower oil recovery by waterflood. During injection of bioemulsifier slug, water-cut decreases slowly, and afterward again comes to above 95% toward the finish of flooding. The bioemulsifier produced by the mixed culture can reduce the oil wettability of the rocks by lowering the interfacial tension and consequently facilitating the mobilization of oil by keeping the oil/water emulsion stable which prevents the mobilized oil from being re-trapped behind the oil bank.

The effectiveness of the crude bioemulsifier solution at different temperatures was tested with two sets of flooding experiments performed in the sand-pack systems. The details of the recovery performance and sand pack properties have been depicted in Table 6. In the present work, the water flood recovers 30–40% of the original oil in place (OOIP) because of higher porosity ($\varphi = 0.20$) and permeability of the sand pack system. During water flooding, as the water cut reaches above 95%, it was subsequently flooded with bioemulsifier slugs, followed by chase water. The recovery of oil and water-cut with pore volume (PV) injected for two different temperature systems is presented

Table 6
Porous media property and oil recovery by bioemulsifier (BE) flooding.

Expt. No	Porosity (%)	Permeability K (Darcy)		Recovery of oil (% OOIP)			Additional recovery (% OOIP)	% Saturation		
		k_w (At S_{wi} = 1)	k_o	Brine flooding	Bio emulsifier flooding	Chase water flooding		S_{wi}	S_{oi}	S_{or}
1. At 40 °C	20.47	1.874	0.588	30.62	32.19	8.75	40.94	28.89	71.11	49.33
2. At 70 °C	20.47	1.091	0.626	34.57	36.57	10.29	46.86	21.35	78.65	51.46

in Fig. 8(a) and (b). It has been found that water begins to break through when the injected volume of water reaches 1.45–1.5 PV, and then the water-cut sharply increases above 95% for each case. In case of water flooding, oil cut decreases as efficiency of water flooding decreases with decreasing initial oil saturation. When bioemulsifier was applied then again oil cut increases for enhanced efficiency of emulsion to recover the trapped oil in porous media. It is clear from the both the figures that the cumulative oil recovery by emulsifier flooding is higher in the case of higher (70 °C) temperature might be due to reduction in viscosity of crude oil which results in increased fluidity of the trapped oil thus improves the sweep efficiency. The additional recovery of oil over water flooding is 40.93% and 46.85% at 40 °C and 70 °C temperatures respectively. Shavandi et al. (2011) also reported that *Rhodococcus* sp. strain TA6, isolated from an Iranian oil contaminated soil, produced a mix of extracellular lipids and glycolipids capable of enhancing the residual oil recovery of oil saturated sand packs by up to 70%. Suthar et al. (2008) reported application of a bioemulsifier produced by *Bacillus licheniformis* K125 which gave 43% additional oil recovery in a sand pack column system designed to simulate an oil reservoir. However, field test conducted by Youssef et al. (2013) on a limestone petroleum reservoir illustrated that with lipopeptide biosurfactants produced in-situ by two bacilli strains, oil production increased by 10%. Hence, the proper design of the bioemulsifer slug should be performed by feasibility analysis, considering the operating costs and market value of the crude.

4. Conclusions

In this work, we have documented the dual role of a crude oil degrading microbial consortium for application in bioremediation of crude oil contaminated water as well as in microbial enhanced oil recovery (MEOR) process. The mixed culture of *O. pseudintermedium* C1 and *B. cereus* K1, which can significantly degrade crude oil in mineral medium at optimized environmental parameters in a bioreactor, also produce a mixture of extracellular polysaccharides capable of significant emulsification activity and interfacial tension reduction property towards crude oil-water system. Moreover, the stability of the produced bioemulsifier mixture at extreme environmental conditions and combination of surface and emulsification activity suggested its potential application as *ex-situ* MEOR agent for enhanced oil recovery process. Encouraging results of additional oil recovery (above 40% of OOIP) was obtained from sand pack flooding tests which suggest that *ex-situ* application of the produced bioemulsifiers by the consortium, can efficiently mobilize the trapped oil by lowering the interfacial tension of crude oil-water system as well as increasing the fluidity of the oil resulting in enhanced sweep efficiency. *Ex-situ* application of microbial products, such as bioemulsifiers, instead of *in-situ* stimulation of bacterial growth, could be cost effective and favorable for reservoirs with growth limiting conditions. However, further field trials may be needed to prove the applicability of the bioemulsifier mix in enhanced oil recovery process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101034.

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