Chapter 5

Experimental
Part I

Biodegradation of waste lubricating oils

IA: Biodegradation using biostimulation strategy
IB: Biodegradation using bioaugmentation strategy
Part IA: Biodegradation using biostimulation strategy

5.1 Isolation of microorganisms

Bushnell Haas (BH) media with the composition (g/L): K$_2$HPO$_4$ (1.0g), KH$_2$PO$_4$ (1.0g), NH$_4$NO$_3$ (1.0g), MgSO$_4$.7H$_2$O (0.2g), FeCl$_3$.6H$_2$O (0.05g), CaCl$_2$.2H$_2$O (0.02g), was used as enrichment medium with WTO and WEO as the sole carbon source to isolate waste lubricating oil degrading bacteria. Oil contaminated soil samples were collected from 24 different locations including local automobile workshops and various industry effluent areas in and around Kolkata, India. Soil samples (~10 gm) were added to 50 ml BH media taken in 250 ml Erlenmeyer culture flasks with 2% (v/v) waste oil (WEO and WTO separately) and incubated at 37°C at 100 rpm in a rotary shaker incubator (ORBITEK-LJE, Scigenics Biotech Pvt.ltd., Chennai, India) for 7 days. After 7 days incubation, the cultures were isolated as single colony on to Nutrient Agar (NA) media by streak-plate method. They were maintained in slant cultures by preserving at 4°C.

5.2 Selection of microorganisms

For selection of microorganism, the isolated cultures were screened for effective waste lubricating oil degrading capability and tolerance limit towards both WTO and WEO. Fresh overnight cultures (OD$_{600} = 1.0$) suspended in BH media were used as inoculum at 2% (v/v) level for all the experiments and inoculated aseptically in culture flasks with same concentration (2% v/v) of carbon source used for isolation at same culture conditions. After completion of the incubation period (7 days), the culture broth samples were centrifuged at 5,000 rpm (REMI R24) for 20 minutes and the culture supernatant was separated from the oil phase in a separating funnel. The waste oil percent degradation was determined by gravimetric analysis as well as gas-chromatographic analysis for evaluating the degradation efficiency of the isolated microorganism. All the experiments were performed in triplicate and a control devoid of the bacterial isolates was prepared for each set of experiments.

5.3 Identification of the selected microorganisms

The genomic identification of the selected bacterial isolates K1 and C1 was carried out by preliminary biochemical tests according to Bergey’s Manual for Determinative Bacteriology.
(1984) and 16S rRNA gene sequencing method from Bhat Biotech India Pvt. Ltd. Bangalore, India.

5.3.1 **Biochemical test procedures**

5.3.1.1 **Methyl red test**

It is used to identify enteric bacteria based on their pattern of glucose metabolism. Isolated bacterial strains were inoculated in test tubes containing Glucose-Phosphate Broth and incubated at 30°C for 24 hrs. After incubation, 5 drops of methyl red indicator was added to each test tube and the color change was observed. If the color changes to red, the test is positive and if the color changes to yellow, the test is negative.

5.3.1.2 **Voges-Proskauer test**

Loop full of isolated bacterial strains were inoculated in test tubes containing MR-VP broth and incubated for 24 hrs at 30°C. After incubation 0.5 ml of α-napthol was added to each tube followed by few drops of 40% KOH and kept at room temperature for 20-30 mins and color change was observed. A cherry red color indicates a positive result, while a yellow-brown color indicates a negative result.

5.3.1.3 **Catalase test**

The H₂O₂ solution was added to the test tubes containing the bacterial culture and was kept in undisturbed condition for few minutes to check the bubble formation. The lack of catalase is evident by a lack of or weak bubble production. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes.

5.3.1.4 **Oxidase test**

A fresh culture (18 to 24 hours) of bacteria was grown in test tubes containing nutrient broth and 0.2 ml of 1% α-naphthol was added along with 0.3 ml of 1% p-aminodimethylaniline oxalate (Gaby and Hadley reagents). Microorganisms are oxidase positive when the color changes to blue within 15 to 30 seconds. Microorganisms are delayed oxidase positive when the
color changes to purple within 2 to 3 minutes. Microorganisms are oxidase negative if the color does not change.

5.3.1.5 Indole test

A culture tube containing tryptone broth were inoculated with the isolated bacterial strains and incubated at 30°C for 24 hrs. After incubation 0.5ml of Kovac’s reagent was added to the tube and checked for the formation of pink color of the reagent layer. A negative result appears yellow.

5.3.1.6 Urease test

18 to 24 hours grown culture was inoculated in test tubes containing urease test broth and incubated with loosened caps at 35°C. The color change within the broth was observed at 8, 12, 24 and 48 hours. Urease production is indicated by a bright pink (fuchsia) color throughout the broth.

5.3.1.7 Triple sugar iron (TSI) test

The TSI slant was inoculated with the bacterial isolates and was observed for color change after incubation at 37°C for 18 to 24 h. If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator both in butt and in the slant. If lactose is not fermented but the small amount of glucose is, the oxygen deficient butt will be yellow. If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red.

5.3.1.8 Simmon’s citrate agar test

Simmons Citrate Agar slant was inoculated lightly with the bacterial strains and incubated at 37°C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium. The development of blue color was observed; denoting alkalinisation.
5.3.2 16s rRNA gene sequencing procedure

DNA extraction was done using genomic DNA extraction Kit (Bhat Biotech, India) and PCR amplification of the 16s rRNA gene was performed using the universal primers. The PCR products were sequenced by automated DNA sequencer -3037xl DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems, India). Sequence data were aligned and dendrograms were generated using Sequence analysis software version 5.2 from Applied Biosystems. Sequences were compared to the non-redundant NCBI database by using BLASTN, to find the most similar sequence, sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignment with the default settings. The multiple-alignment file was then used to create phylogram using MEGA5 software (Nei and Kumar 2000).

5.4 Analytical procedures

5.4.1 Bacterial growth determination

The biomass concentration in the culture broth was determined by dry weight method. In this method, the broth was centrifuged at 5,000 rpm for 20 min. The bacterial mass was then transferred to a pre-weighted aluminum cup and dried at 50°C overnight. The exact weight of the bacterial mass was determined by subtracting the weight of dry cup from that of the cup containing dry bacterial mass (Guchhait et al., 2005).

5.4.2 Determination of waste oil biodegradation

The WTO and WEO degradation was determined by gravimetric analysis as well as gas chromatography analysis.

5.4.2.1 Gravimetric analysis

At the end of each experiment, the supernatant phase was separated in a separating funnel after centrifuging the culture broth at 5,000 rpm (REMI R24) for 20 minutes. The residual waste oil was extracted by adding 20 ml of hexane and shaking thoroughly as described by Mandri and Lin (2007). The extracted phase was then collected in a pre-weighed beaker and the final weight
was noted after evaporating the solvent. The amount of residual oil was found out from the weight difference. Then the percentage of oil degraded was calculated as follows:

\[
\text{% of oil degraded} = \left( \frac{\text{weight of test oil sample} - \text{weight of residual oil sample}}{\text{weight of test oil sample}} \right) \times 100
\]

5.4.2.2 WTO analysis by gas chromatography

After completion of each experiment, the culture broth samples were centrifuged at 5,000 rpm (REMI R24) for 20 minutes and the culture supernatant was extracted with hexane 2 times to separate the residual oil phase. After passing the extract through anhydrous sodium sulfate (Na₂SO₄), it was concentrated down to 2 mL by applying air blowdown technique as suggested by USEPA (2000) and was taken in GC glass vials prior to GC analysis. PCB analysis was done using a Thermo Scientific Trace 1300 series gas chromatograph equipped with ECD detector and 30mm long TR-1 column (internal diameter 0.32 mm and film thickness 1.0 µm) according to the method described by Kaya et al. (2013). GC experimental conditions for PCB analyses were as follows: helium was the carrier gas at 1.5 mL/min; nitrogen was the make-up gas at 20 mL/min; injection mode/temperature was splitless/250 °C; detector temperature was 350 °C; temperature program was: initial temperature 100 °C; 100–160 °C at 20°C /min; 160°C for 2 min; 160–200°C at 3°C /min; 200–240°C at 8°C /min; 240°C for 5 min; 240–290°C at 30°C/min; 290°C for 3 min. 1 µL sample was injected for each analysis. The relative percent degradation of WTO was calculated by the differences in summation of peak area with respect to biphenyl and PCB analytical standard (Bhattacharya et al., 2015).

5.4.2.3 WEO analysis by gas chromatography

The residual oil sample from inoculated and un-inoculated culture flasks were extracted three times with hexane after each biodegradation experiment according to Adebusoye et al. (2007). The organic phase was concentrated by evaporation of the solvent after drying over anhydrous Na₂SO₄ and analyzed by gas chromatography according to the condition described by Ghazali et al. (2004). Hexane extracts of residual oil sample (1µl) were injected for analysis by using a Polaris Q Mass Spectrometer coupled with Thermo Scientific Trace 1300 series gas chromatograph and TR-1 column (30×10³ cm length; 0.032 cm id; and 1×10⁻³ cm film
thickness). Helium was used as carrier gas at 1.2 ml/min. The injector and detector temperatures were maintained at 280°C and 290°C respectively. The oven was programmed at an initial temperature of 75°C; this was held for 2 min, then ramped at 5°C/min to 330°C and held for 5 min. The relative percent degradation of WEO was calculated by the differences in summation of peak area of total petroleum hydrocarbons (TPH) present in the residual oil compared to that from un-inoculated control flasks (Bhattacharya and Biswas 2014a). Chromatographs were analyzed by Chromeleon 7.0 program and a library (NIST 2007) search was performed for identification of chromatogram peaks.

5.5 Biostimulation strategy for WTO biodegradation by bacterial isolate K1

5.5.1 Study of optimization of physicochemical parameters by OVAT approach

The physicochemical parameters were evaluated in a series of experiments to obtain higher degradation efficiency of WTO by the bacterial isolate K1. The independent parameters and their ranges used for this study were incubation time (0 to 168 hour), WTO concentration (2% to 10% v/v), pH (5 to 9) and incubation temperature (32°C to 42°C). Bacterial growth (OD\textsubscript{600}) and WTO percent degradation were monitored with varying parameters under study, keeping other parameters constant at a time i.e. one-variable-at-a-time (OVAT) approach. All the experiments were performed in duplicate and a control devoid of the bacterial isolates was prepared for each set of experiments.

5.5.2 Study of supplementation and limitation of nutrients in the growth media

Supplementation or limitation of growth media (BH media) component concentrations and addition of supplementary carbon or nitrogen source may enhance biodegradability of recalcitrant compounds (Xu and Lu 2010). Glucose was used as a supplementary carbon source within the range between 5 to 20g/L. Yeast extract, beef extract and peptone within the range of 0.5 to 2 g/L were used as supplementary organic nitrogen source. BH media components were also varied within the following ranges: NH\textsubscript{4}NO\textsubscript{3} (0.5 to 2 g/L), MgSO\textsubscript{4} (0.1 to 0.4 g/L), CaCl\textsubscript{2} (0.01 to 0.04 g/L) and FeCl\textsubscript{3} (0.025 to 0.1 g/L). Addition of chemical surfactant Tween 80 (25 to 100 µL) and NaCl (1 to 5 g/L) in BH media was also evaluated for enhanced biodegradation of WTO. All the experiments were performed in duplicate and with previously optimized culture
parameters. A control devoid of the bacterial isolates was prepared for each set of experiments. Bacterial culture density (OD\textsubscript{600}) and WTO percent degradation were monitored with varying parameters under study, keeping other parameters constant at a time.

5.6 Biostimulation strategy for WEO biodegradation by bacterial isolate C1

5.6.1 Study of optimization of physicochemical parameters by OVAT approach

WEO was treated by the isolate C1 with culture condition variations such as, incubation period (0 to 168 hours), pH (5, 7 and 9), incubation temperature (32\textdegree C, 37\textdegree C, and 42\textdegree C) and WEO concentration (2%, 5%, and 10% v/v) to study the effect on waste oil percent degradation ability of the culture.

5.6.2 Optimization studies using response surface methodology (RSM) approach

Preliminary trials indicated that pH, temperature and waste oil concentration in the culture medium were the significant variables for the percentage biodegradation of waste oils. Hence pH, temperature and waste oil concentration were chosen as the three variables for optimization studies. The three levels of each variable are given in Table 3. Percent degradation of WEO was taken as single response.

A $3^3$ Box-Behnken experimental design was carried out with three replicates at the center points leading to 15 runs. The variables were coded according to the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X} \quad i = 1, 2,3 \ldots \text{k}$$

where, $x_i$ is the dimensionless value of an independent variable, $X_i$ is the real value of an independent variable, $X_0$ is the value of $x_i$ at the centre point and $\Delta X$ is the step change value.

The second-order polynomial model was fitted to response giving an equation term:

$$\hat{Y}_i = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i,j=1}^{3} \beta_{ij} X_i X_j$$ (2)
where \( \hat{Y}_i \) is the predicted response, \( x_i \) and \( x_j \) are the input variables, \( \beta_0 \) is the intercept term, \( \beta_i \) is the linear effects, \( \beta_{ii} \) is the squared effects and \( \beta_{ij} \) is the interaction term.

‘Statistica v.10’ software was used for regression and graphical analysis of the data. The optimum values of culture condition variables (pH, temperature and oil concentration) were obtained by solving the regression equation and analyzing the response surface contour plots.

Table 3 Variables and their levels used in the Box-Behnken experimental design for optimization of physicochemical parameters

<table>
<thead>
<tr>
<th>Variables</th>
<th>Low level (-1)</th>
<th>Center point (0)</th>
<th>High level (+1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( X_1 ): Temperature</td>
<td>34°C</td>
<td>37°C</td>
<td>40°C</td>
</tr>
<tr>
<td>( X_2 ): pH</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>( X_3 ): Oil concentration (v/v)</td>
<td>3%</td>
<td>5%</td>
<td>7%</td>
</tr>
</tbody>
</table>

5.6.3 Media optimization studies by OVAT approach

The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed, with a constant WEO concentration of 4% (v/v) i.e. 34.4g/L (which was selected from previous optimization studies), incubated at 37°C, pH 7.0 and 80 rpm agitation for 7 days. Three factors were chosen aiming to obtain higher percent biodegradation of waste engine oil: carbon source (C), nitrogen source (N) and various inorganic salts. For evaluation of carbon source, glucose was used as added co-substrate for degradation of WEO in varying concentrations, in the range of 5 g/L to 20 g/L. For evaluation of nitrogen sources, NaNO₃, urea and yeast extract were employed at a concentration of 1 g/L with the optimum carbon source. Yeast extract was chosen as the most suitable nitrogen source and was further employed at varying concentrations from 0.5 g/L to 2 g/L for determining its optimal level. To evaluate the effects of various inorganic salts, BH media was formulated with different concentrations of the salts such as MgSO₄, CaCl₂, FeCl₃, NH₄NO₃, MnSO₄ and NaCl. The most effective inorganic salt FeCl₃ was further employed at varying concentration range between 0.1 g/L to 0.3 g/L for determining its optimal level. Carbon and nitrogen sources were added separately at their optimal levels. All the experiments were performed in triplicate and a control devoid of the bacterial isolates were prepared for each set of experiments.
5.6.4 Experimental design and statistical analysis

A full factorial central composite design (CCD) was applied with three experimental factors chosen from previous optimization studies, namely the glucose ($X_1$), yeast extract ($X_2$) and FeCl₃ ($X_3$). A total of 16 runs, performed in duplicate, were required for this procedure. Table 4 listed the coded ($X_i$) and actual ($x_i$) levels of each variable. For statistical calculation, the test factors were coded according to the same equation (1) given in section 5.6.2. CCD allows modeling of the results using a quadratic equation similar to the equation (2), but it is advantageous due to its orthogonality function ‘α’, which allows the model to expand beyond the set levels of the factors and predict accurate optimization levels. The experimental data obtained was fitted to the following quadratic polynomial equation:

\[
Y = A_0 + A_1 X_1 + A_2 X_2 + A_3 X_3 + A_4 X_1^2 + A_5 X_2^2 + A_6 X_3^2 + A_7 X_1 X_2 + A_8 X_2 X_3 + A_9 X_3 X_1
\]  

(3)

Where, $Y$ is the predicted response value; $A_0$ is the intercept term; $A_1$, $A_2$, $A_3$ are the linear coefficients; $A_4$, $A_5$, $A_6$ are the quadratic coefficients; $A_7$, $A_8$, $A_9$ are the interactive coefficients. Statistica software (Version 10.0) was used for regression and graphical analysis of the experimental data obtained similar to the previous section 5.6.2.

**Table 4** Coded and uncoded values of experimental variables in Central-composite design for optimization of media

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coded variables</th>
<th>Variable levels</th>
<th>Step change value $\Delta x_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose conc. (g/L)</td>
<td>$X_1$</td>
<td>10 20 30</td>
<td>10</td>
</tr>
<tr>
<td>Yeast Extract conc. (g/L)</td>
<td>$X_2$</td>
<td>1 2 3</td>
<td>1</td>
</tr>
<tr>
<td>FeCl₃ conc. (g/L)</td>
<td>$X_3$</td>
<td>0.1 0.2 0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Part IB: Biodegradation using bioaugmentation strategy

5.7 Microorganisms

The two hydrocarbon degrader organisms used in this study were Ochrobactrum pseudintermedium sp. C1 and Bacillus cereus sp. K1, isolated previously from waste oil contaminated soil in our laboratory (Bhattacharya et al., 2014b; 2015). 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.

5.8 Determination of degradation ability on different hydrocarbons

Several hydrocarbons of napthene-paraffin-aromatic (N-P-A) series like nonane, dodecane, tetradecane, hexadecane, octadecane, eicosane, octacosane, decalin, tetralin, xylene, naphthalene, phenanthrene and anthracene were tested with both the bacterial isolates to study its degradation ability of the type and range of hydrocarbons. Various petroleum fractions like lubricating oil base stock (LOBS), vacuum gas oil (VGO), diesel, kerosene and crude oil were also tested with the bacterial isolate to establish its degradation ability of different petroleum fractions from low boiling fraction kerosene to high boiling fraction lubricating oils. All the hydrocarbons tested were at 2% (v/v) concentration level at same culture parameters mentioned in section 5.1.

5.9 Preparation of consortium culture

To prepare the consortium culture for biodegradation of waste oil, we have used crude oil as the sole carbon source (4% v/v level) because all the petroleum fractions including lubricating oils are derived from the base crude oil. The initial inoculum concentrations were varied from 2% to 10 % level upon mixing both the bacterial isolates in equal proportions (1:1) to achieve maximum possible degradation of oil within minimum incubation period. The bacterial consortium was prepared according to maximum degradation efficiency achieved with varying the ratio of individual isolates at all possible combinations having the initial inoculum...
concentration fixed at 10% (v/v) level with the same culture parameters in shake flask experiments mentioned in section 5.1.

5.10 Experimental setup for batch experiments

A 1.5 L fully mixed bench scale bioreactor (Eyela Co., Tokyo, Japan) equipped with constant temperature water circulating system and monitoring devices for temperature, dissolved oxygen and pH was used for batch biodegradation experiments. The reactor was manufactured from glass having a water jacket for controlling the bioreactor inside temperature which was kept constant at 37°C for all experiments. The air flow rate to the reactor and stirring speed were optimized to 1.5 Nl/min and 150 rpm respectively, from control trial runs to ensure that the volumetric rate of oxygen mass transfer to the liquid phase was not rate limiting (Schuler and Kargi 2000). Dissolved oxygen (DO) concentration was measured continuously throughout the experiments, so that it does not fall below 5 mg/L ensuring adequate DO for hydrocarbon oxidation and also preventing emulsification of hydrocarbons during agitation. The reactor was operated under batch operation and samples were taken from the reactor at predetermined time intervals using a peristaltic pump. All the cultivations were performed consecutively in triplicates with total media volume of 500 ml and a control without bacteria was prepared for each set of experiments.

5.11 Batch experiments

5.11.1 Waste lubricating oil degradation in pure and consortium culture

The WEO and WTO biodegradation capacity by bacterial isolates was evaluated individually and in consortium by cultivation in BH medium with 4% (v/v) of carbon source at 37°C incubation temperature and 150 rpm agitation speed for 72 hours. The concentration of waste oil was chosen from previous experimentation on optimization of physicochemical parameters (Sections 5.5.1 and 5.6.1). The two isolates C1 and K1 were mixed at 2:3 proportions (v/v) and used as inoculum at 10% (v/v) level throughout the study, which was selected according to the maximum degradation potential of the consortium.
5.11.2 Kinetic studies with model hydrocarbons

To determine the growth kinetics of the microorganisms with respect to different model hydrocarbon compounds such as hexadecane, eicosane, octacosane, phenanthrene, dibenzothiophene and their mixture (in equal proportions), batch experiments were conducted in the bioreactor for a period of 72 h. For each compound, the initial concentration was 1000 mg/L. Cell mass and the substrate concentration were determined at an interval of 4 h throughout the study.

5.11.3 Kinetic studies with waste lubricating oils

For both the waste lubricant samples WEO and WTO, a set of experiments was performed for a period of 72 h varying the initial concentration in the range of 1-10 % (v/v). The initial concentrations of waste oil were chosen from our previous experimental trials in shake flask cultivation (Sections 5.5.1 and 5.6.1). For each initial substrate concentration, cell mass and the substrate concentration were determined at an interval of 4 h throughout the study.

5.12 Analytical procedures

5.12.1 Bacterial growth determination

The biomass concentration in the culture broth was determined as described in section 5.4.1.

5.12.2 Hydrocarbon analysis using gas chromatography

Hexane extracts of residual oil sample (1µl) were injected for analysis by using a Polaris Q Mass Spectrometer coupled with Thermo Scientific Trace 1300 series gas chromatograph and TR-1 column (30× 10\(^3\) cm length; 0.032 cm id; and 1×10\(^{-3}\) cm film thickness) as described for WTO and WEO analysis in previous sections 5.4.2.2 and 5.4.2.3. The relative percent degradation of WEO and WTO 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 following USEPA method 8015B (1986). The major metabolites from the residual hydrocarbon compounds were also analyzed in this section by GC-
MS. For this purpose, the injector and detector temperatures were maintained at 240°C and 250°C respectively. Helium was the carrier gas with flow rate of 1.5 ml/min. The oven was programmed at an initial temperature of 100°C; this was held for 1 min, then ramped at 10°C/min to 125°C and held for 1 min, again ramped at 10°C/min to 250°C and held for 10 min and final ramp was set at 10°C/min to 300°C and held for 8 min. Degradation efficiency of pure hydrocarbons were also calculated by the differences in summation of peak area compared to their respective standards. Chromatographs were analyzed by Chromelone 7.0 program and a library (NIST 2007) search was performed for identification of chromatogram peaks.

5.13 Theoretical analysis

The key assumptions were: 1) substrate utilization takes place in the dissolved phase and all other mechanisms are negligible; 2) aeration provided oxygen levels at sufficient concentration and does not limit growth rate. Hence, the influence of oxygen was not considered and it was assumed that the growth and substrate degradation rate of the consortium culture was only inhibited by substrate concentration at given initial pH, temperature, and aeration rate (Wang et al., 2010; Schuler and Kargi, 2000). In a batch reactor, for modelling of substrate (S) and microorganism concentration (X) change with time (T), a simple biokinetic Monod model with two components was chosen. The mathematical expressions for Monod kinetics is as follows:

\[
\mu = \frac{\mu_{\text{max}} S}{K_s + S} \quad (4)
\]

Where,
\( \mu \) is specific growth rate (h\(^{-1}\)),
\( \mu_{\text{max}} \) is maximum specific growth rate (h\(^{-1}\)),
S is the substrate concentration (mg/L) and
K\(_s\) is the half saturation constant (mg/L)

Microorganism concentration change is given by:

\[
\frac{dX}{dT} = \mu X \quad (5)
\]

\[
\mu = \frac{1}{X} \frac{dX}{dT} \quad (6)
\]
Where,

\( X \) is the biomass concentration (mg/L) and

\( T \) is the time (h)

Substrate concentration change is given by:

\[
\frac{dS}{dT} = - \frac{1}{Y_{X/S}} \frac{dX}{dT}
\]  

(7)

Where, \( Y_{X/S} \) is the ratio of cell mass growth and substrate concentration used for cell growth.

\( Y_{X/S} \) can be expressed as:

\[
Y_{X/S} = - \frac{dX}{dS}
\]  

(8)

\( Y_{X/S} \) was calculated from experimental data using the Eq. (9).

\[
Y_{X/S} = \frac{X - X_0}{S_0 - S}
\]  

(9)

Where, \( X_0 \) and \( S_0 \) represent initial biomass and substrate concentration respectively.

The Haldane's kinetic model [Eq. (10)] has been frequently used to describe growth rates of microorganisms on inhibitory substrates (Guchhait et al., 2005; Nakhla et al., 2006; Wang et al., 2010).

\[
\mu = \frac{\mu_{max}S}{KS + S + \frac{S^2}{Ki}}
\]  

(10)

Where, \( K_i \) is the inhibition constant (mg/L)

For each hydrocarbon compound and WEO, WTO the value of maximum substrate concentration, \( S_{max} \) corresponding to the maximum substrate consumption rate has been determined using the following theoretical concept:

At \( S = S_{max} \), \( d\mu/dS = 0 \),

By putting \( d\mu/dS = 0 \) in Eq. (10), \( S_{max} \) can be derived as:

\[
S_{max} = \frac{K_s * K_i}{K_s + S_{max}}
\]  

(11)

The kinetic parameters, \( \mu_{max} \), \( K_s \), \( K_i \) and \( S_{max} \) have been determined both graphically and by non-linear regression analysis using STATISTICA v.10 (Statsoft, Oklahoma, USA) and MATLAB v 7.10 (R2010a).
Part II

Production of microbial surface active agents

IIA: Production, isolation and characterization of the surface active agents

IIB: Sustainable development on bioemulsifier production
Part IIA: Production, isolation and characterization of the surface active agents

5.14 Analysis of surface active agent production

5.14.1 Surface tension measurement

Surface tension 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).

5.14.2 Emulsification index measurement

Emulsification index (EI) was measured following the method described by Cooper and Goldenberg (1987). 2 ml of engine oil was added to 2 ml of cell free extract and vortexed at high speed for 2 min. Diesel, kerosene, crude oil and transformer oil were also used as substrate for emulsification. Measurements were taken after 24 hours as follows:

\[ EI = \left( \frac{\text{height of the emulsion (H)}}{\text{total height (T)}} \right) \times 100 \]

5.15 Isolation of the biopolymers

The crude biopolymers were isolated as extracellular polymeric substances (EPS) from the culture broth. The 5 days grown culture of C1 strain and 3 days grown culture of K1 strain were centrifuged at 4°C and 10,000 rpm in a REMI C24 (Chennai, India) for 20 minutes. The emulsifier agents produced by the bacterial isolates C1 and K1 were precipitated from the culture supernatant with ethanol [ethanol: medium ratio 2:1 (v/v)] and by using chloroform-methanol mixture (2:1) respectively at 4°C for 16 h, following the method described by Calvo et al. (2008) and Cooper and Goldenberg (1987). The biopolymer precipitated from the supernatant was further centrifuged at 10,000 rpm at 4°C. The pellet was dissolved in distilled water and dialyzed using 14kDa cut off dialysis membrane (HiMedia, India). The dialyzed biopolymer was then freeze-dried (EYELA FDU-1200, Japan) and finally weighed.
5.16 Characterization of the biopolymers

5.16.1 Chemical analysis

The biopolymer obtained was 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).

5.16.2 FT-IR analysis

Functional groups of the biopolymer 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.

5.16.3 NMR Spectroscopy

NMR data was obtained to further elucidate the chemical structure of the bioemulsifier using Bruker Avance DPX 400 spectrometer (400 MHz FT-NMR). The \(^1\)H NMR spectra was recorded from the sample solution in D\(_2\)O and the chemical shifts were expressed in ppm relative to the resonance of TMS (Tri-methyl silane) as internal standard.

5.16.4 Scanning electron microscopy

Surface morphology of the biopolymer was studied under scanning electron microscope (SEM). Sample was washed three times with aqueous 1% osmium tetroxide solution for 2 hours at 4\(^\circ\)C and dehydrated in ethanol series (50, 80, 90 and saturated) followed by critical point drying. Gold sputtering on the sample was done using a sputter coater under vacuum and the sample was examined under JEOL EVO18 SEM (Japan) at 2 kV.
5.17 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 surface tension (ST) reduction property. The bioemulsifier sample was incubated in a water bath for 30 mins from 25 to 100°C and also kept at 121°C for 15 mins 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 ST measurements of each treatment were assessed as described above, with crude oil used as the substrate.

5.18 Rheology studies

The viscosity of the crude extracellular polymeric substances 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:

\[
\log \tau = \log \mu_{\text{app}} + n \log (\partial u/\partial y)
\]

where,

\( \tau \) is shear stress,

\( \mu_{\text{app}} \) is apparent viscosity,

\( n \) is flow behavior index and

\( \partial u/\partial y \) is shear rate.
The instrumental parameters were as follows:

Shear stress \( (\tau) = \frac{M}{2\pi R_b^2 L} \)  \hspace{1cm} (13)

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).

Shear rate \( (\frac{\partial u}{\partial y}) = \frac{2\omega R_c R_b^2}{(R_c^2 - R_b^2)} \)  \hspace{1cm} (14)

Where,

\( \omega \) 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 \( (\mu_{app}) \) 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).
Part IIB: Sustainable development on bioemulsifier production

5.19 The microorganism, its maintenance and culture conditions

The microorganism used in this study was *Ochrobactrum pseudintermedium* sp. C1 which has showed higher tolerance to waste engine oil. The strain was conserved in slants containing nutrient agar media with the composition (g/L): beef extract 5.0, peptone 10.0, NaCl 5.0 and agar 15.0. Growth and BE production studies by the strain C1 were evaluated using BH medium [in g/L: 1.0 KH$_2$PO$_4$, 1.0 K$_2$HPO$_4$, 1.0 NH$_4$NO$_3$, 0.2 MgSO$_4$7H$_2$O, 0.02 CaCl$_2$.2H$_2$O, 0.05 FeCl$_3$.6H$_2$O; pH 7.0±0.2] with 4% (v/v) WEO as sole carbon source, grown in an orbital shaker at 100 rpm and 37\(^\circ\)C for 7 days. The basal production of the bioemulsifier detected in this medium was used as control or reference.

5.20 Effects of different nutritional supplements

An univariate analysis using dose–response experiments was performed for the effects of adding supplementary carbon source (glucose, 5-30 g/L), nitrogen source (as NaNO$_3$, urea and yeast extract, 0.5-3 g/L) and inorganic ion source (as MgSO$_4$, CaCl$_2$, FeCl$_3$, NH$_4$NO$_3$, MnSO$_4$ and NaCl, 0.05-0.3 g/L) over BH medium for bioemulsifier production. Based on these experiments the independent variables (glucose, yeast extract, FeCl$_3$) and their initial ranges were chosen to perform the optimization studies.

5.21 Optimization of BE production

In order to identify the main effects and interactions of the selected variables on bioemulsifier production, a three-level, three-factor factorial central composite design (CCD) leading to 16 runs (with 2 center points, 6 star points having orthogonality \(\alpha\) of 1.7638 and 8 factorial points. The series of experiments designed are shown in Table 5 in coded and uncoded terms. The ‘‘+, 0 and −’’ notation is used to represent the highest, centre and lowest levels for each factor. ‘\(\alpha\)’ was denoted as star points for each factors with lowest (-) and highest levels (+). Statistical analysis was performed using STATISTICA v.10.0 software (Statsoft, OK, USA). All
the data were represented as the mean (±standard deviation) of triplicate results. Statistical significance values for the means were evaluated using one-way analysis of variance. Subsequent comparisons were performed using Student’s t-test. Differences were accepted as significant when p<0.05. Associations between variables were assessed by using Pearson’s correlation coefficient. The optimization of yield of the bioemulsifier was conducted by generation of response surfaces followed by their characterization by regression modeling.

Table 5 Experimental design using CCD for optimization of bioemulsifier production

<table>
<thead>
<tr>
<th>No. of runs</th>
<th>Coded variables</th>
<th>Glucose (g/L) (X₁)</th>
<th>Yeast Extract (g/L) (X₂)</th>
<th>FeCl₃ (g/L) (X₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10(-)</td>
<td>1(-)</td>
<td>0.1(-)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10(-)</td>
<td>1(-)</td>
<td>0.3(+1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10(-)</td>
<td>3(+)</td>
<td>0.1(-)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10(-)</td>
<td>3(+)</td>
<td>0.3(+1)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>30(+1)</td>
<td>1(-)</td>
<td>0.1(-)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>30(+1)</td>
<td>1(-)</td>
<td>0.3(+1)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>30(+1)</td>
<td>3(+)</td>
<td>0.1(-)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>30(+1)</td>
<td>3(+)</td>
<td>0.3(+1)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>20(0)</td>
<td>2(0)</td>
<td>0.2(0)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.362(-α)</td>
<td>2(0)</td>
<td>0.2(0)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>37.638(+α)</td>
<td>2(0)</td>
<td>0.2(0)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20(0)</td>
<td>0.236(-α)</td>
<td>0.2(0)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>20(0)</td>
<td>3.764(+α)</td>
<td>0.2(0)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>20(0)</td>
<td>2(0)</td>
<td>0.024(-α)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>20(0)</td>
<td>2(0)</td>
<td>0.376(+α)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>20(0)</td>
<td>2(0)</td>
<td>0.2(0)</td>
<td></td>
</tr>
</tbody>
</table>

5.22 Growth and BE production kinetics

To define efficient strategies for bioprocesses, it is important to elucidate the degree of interdependence between biomass growth and product formation. For this purpose, the experimental data were analyzed according to the Luedeking–Piret’s model (Luedeking and Piret 1959) and Kono–Asai classification (Kono and Asai 1969). Microbial growth kinetics can be described by an empirical model (Eq. (4)) originally proposed by Monod (1949), which is related to the substrate limited biomass growth and product formation during biodegradation process.
Growth yield coefficient ($Y_{X/S}$) and product yield coefficient ($Y_{P/S}$) can also be represented by this model. However, it does not consider the fact that cells may need substrate or may consume their own synthesized products even when they do not grow (Wang et al., 2006). Luedeking and Piret (1959) proposed another model to correlate product formation and growth. According to this model, the product formation rate depends on both the instantaneous biomass concentration and the growth rate in a linear manner. The kinetics of product formation was analyzed based on the Luedeking–Piret’s equation (Eq. (15)).

\begin{equation}
\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X 
\end{equation} 

(15)

where, $\alpha$ and $\beta$ are the product formation constants which may differ under different fermentation conditions. It is easier to verify this assumed relationship and to evaluate the constants if the equation (Eq. (15)) can be modified dividing by $X$ to give

\begin{equation}
\frac{1}{X} \frac{dP}{dt} = \frac{\alpha}{X} \frac{dX}{dt} + \beta 
\end{equation} 

(16)

Since, by definition, $k = \frac{1}{X} \frac{dX}{dt}$, the equation (Eq. (16)) finally simplifies to

\begin{equation}
\frac{1}{X} \frac{dP}{dt} = \alpha k + \beta 
\end{equation} 

(17)

The constants $\alpha$ and $\beta$ were determined from the plots, where $\alpha$ is equal to the slope of the straight line and $\beta$ is equal to the $(1/X)\frac{dP}{dt}$ intercept. The relationship between $dP/dt$ (rate of product accumulation) and $dX/dt$ (rate of biomass formation) as a function of biomass concentration ($X$) was determined following the clarification proposed by Kono and Asai (1969).

5.23 Analyses of biomass and residual waste oil

At the end of each biodegradation experiment, the culture broth was centrifuged at 10,000 rpm for 20 minutes. The biomass concentration in the culture broth was determined by dry weight method as well as by measuring optical density at 600 nm as described previously in section 5.4.1. The residual oil samples from test and control culture flasks were extracted three times with hexane and analyzed by gas chromatography to determine the relative percent degradation of waste oil as described earlier in section 5.4.2.3.
Part III

Application of the microbial product in suitable fields

IIIA: Microbial consortia as bioremediation agent for crude oil spill bioremediation

IIIB: Bioemulsifier mediated enhanced oil recovery
Part IIIA: Microbial consortia as bioremediation agent for crude oil spill bioremediation

5.24 Consortium culture and its cultivation procedure

The consortium culture for biodegradation of crude oil was prepared as described in earlier section 5.9, the two isolates C1 and K1 were mixed at 2:3 proportions (v/v) and used as inoculum at 10% (v/v) level throughout the study, which was selected according to maximum degradation potential of the consortium. To determine the biodegradation of crude oil at varied process 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.

5.25 Optimization of fermentation conditions by Taguchi’s methodology

5.25.1 Taguchi’s experimental design

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 simultaneous optimization of multiple variables (independent factors) to achieve the best response (dependent factor) with minimum number of observations. Taguchi analysis can provide definitive information mainly for single response systems (Rao et al., 2008), thus for the present study the standard orthogonal array of L9 (Roy 2001) was employed to examine the four factors at three levels (all experiments were performed in triplicate) in order to maximize the biodegradation of crude oil by the consortium culture in terms of TPH removal efficiency (single response). The L and the subscript 9 represent the Latin square and the number of experimental runs, respectively. Instead of conducting 81 experiments, for general factorial experimental design involving four
parameters, only 9 experimental runs were required in the Taguchi’s design method to optimize the parameter settings for the present study. Table 6 enlists the four independent process factors (pH, aeration rate, agitation rate and temperature) and their corresponding levels for the present study. The range of the parameters was specified based on preliminary laboratory experiments.

<table>
<thead>
<tr>
<th>Process parameters</th>
<th>pH</th>
<th>Aeration rate (NL/min)</th>
<th>Agitation rate (rpm)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1 (L1)</td>
<td>6</td>
<td>0.5</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>Level 2 (L2)</td>
<td>7</td>
<td>1.0</td>
<td>150</td>
<td>35</td>
</tr>
<tr>
<td>Level 3 (L3)</td>
<td>8</td>
<td>1.5</td>
<td>200</td>
<td>40</td>
</tr>
</tbody>
</table>

**5.25.2 Data analysis and prediction of performance**

The 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. The S/N (signal to noise) ratio values corresponding to the TPH removal efficiency values were calculated, using the ‘larger-the-better’ characteristics, since the aim 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):

\[
\frac{S}{N} = -10 \log \left( \frac{1}{k} \sum_{i=1}^{k} \frac{1}{y_i} \right)
\]

(18)

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 7.

The predicted S/N ratio at the optimal process conditions for achieving maximum TPH removal was estimated from the following equation (Taguchi, 1986):
\[
\frac{S}{N_{predicted}} = \frac{S}{N} + n \sum_{j=1}^{n} \frac{S}{N_j} - \frac{S}{N}
\]  
(19)

Where \( S/N \) 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>
<thead>
<tr>
<th>Run No.</th>
<th>Parameters</th>
<th>pH</th>
<th>Aeration rate (Nl/min)</th>
<th>Agitation rate (rpm)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>6</td>
<td>0.5</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6</td>
<td>1.0</td>
<td>150</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>6</td>
<td>1.5</td>
<td>200</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>7</td>
<td>0.5</td>
<td>150</td>
<td>40</td>
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<tr>
<td>5</td>
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<td>7</td>
<td>1.0</td>
<td>200</td>
<td>30</td>
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<tr>
<td>6</td>
<td></td>
<td>7</td>
<td>1.5</td>
<td>100</td>
<td>35</td>
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<td></td>
<td>8</td>
<td>0.5</td>
<td>200</td>
<td>35</td>
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<td>8</td>
<td></td>
<td>8</td>
<td>1.0</td>
<td>100</td>
<td>40</td>
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<tr>
<td>9</td>
<td></td>
<td>8</td>
<td>1.5</td>
<td>150</td>
<td>30</td>
</tr>
</tbody>
</table>

### 5.26 Batch Experiments

A set of experiments were performed for a period of 72 h varying the initial concentration of crude oil in the range of 1-10 % (v/v) at optimized cultivation condition. The main components of the bioreactor system were described in earlier section 5.10. The air flow rate to the reactor and stirring speed were optimized to 1.5 Nl/min and 150 rpm respectively, from control trial runs to ensure that the volumetric rate of oxygen mass transfer to the liquid phase was not rate limiting (Schuler and Kargi, 2000). Dissolved oxygen concentration was measured continuously throughout the experiments, so that it does not fall below 5 mg/L as mentioned in section 5.10. The reactor was operated under batch operation and samples were taken from the reactor at predetermined time intervals using a peristaltic pump. For each initial substrate concentration, cell mass and the substrate concentration were determined as an average of triplicate assays at an interval of 4 h throughout the study. All the cultivations were performed with total media volume of 500 ml and a control devoid of the bacterial isolates was prepared for each set of experiments.
5.27 Growth and biodegradation kinetics

5.27.1 Basic assumptions

The key assumptions taken into consideration for this study were: 1) substrate utilization takes place in the dissolved phase and all other mechanisms are negligible; 2) aeration provided oxygen levels at sufficient concentration and does not limit growth rate. Hence, the influence of oxygen was not considered and it was assumed that the growth and substrate degradation rate of the consortium culture was only inhibited by substrate concentration at given initial pH, temperature, and aeration rate (Guchhait et al., 2005; Schuler and Kargi 2000).

5.27.2 Applied models

Although biodegradability of crude oil is usually explained by first order kinetics (Zahed et al., 2011), to conduct an extensive investigation, several kinetic models for crude oil degradation have been evaluated. In a batch reactor, for modeling of crude oil and microorganism concentration change with time, a simple bio-kinetic Monod model (Monod 1949) with two components was chosen (Schuler and Kargi 2000) as previously described in section 5.13 (Eq. (4)). However, the application of the rate equation at high crude oil concentration was unsatisfactory, which was due to high concentrations of inhibitory compounds. In this case, Haldane’s model or Andrews’s model (Haldane 1968; Edwards 1970) was a well-fitted model for determination of the kinetic parameters, even at inhibitory levels of the substrate as stated earlier in section 5.13 (Eq. (10)).

In addition, at high substrate concentration, the cell growth was also inhibited. The cell growth rate was evaluated by the logistic equation which was a reasonable kinetic model (Schmidt et al., 1985) for the prediction of the growth curve. The specific growth rate was predicted by the logistic model as given by Eq. (20):

$$ \mu = \mu_m \left(1 - \frac{x}{x_m}\right) $$

(20)

where, $x_m$ is the maximum cell dry weight concentration (mg/L).
By substitution of Eq. (20) into Eq. (10) and performing integration, the following equation for the cell concentration is obtained (Schuler and Kargi 2000):

$$x = \frac{x_0 e^{\mu_m t}}{1 - \left(\frac{x_0}{x_m}\right) (1 - e^{\mu_m t})}$$  \hspace{1cm} (21)

The kinetic parameters have been determined both graphically and by non-linear regression analysis using STATISTICA v.10 (Statsoft, OK, USA) and MATLAB v 7.10 (R2010a).

5.28 Analytical methods

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$_2$SO$_4$ 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; 0.032cm 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 2014a). 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. The biomass concentration in the culture broth was determined by dry weight method as mentioned earlier in section 5.4.1.
Part IIIB: Bioemulsifier mediated enhanced oil recovery

5.29 Evaluation of bioemulsifier production by the consortium culture

Emulsification activity (EI) was used as a measure of bioemulsifier production in the culture broth for each experimental runs from previously discussed Taguchi’s design experiment to find out a possible correlation between crude oil biodegradation and bioemulsifier production by the consortium culture. Surface tension and interfacial tension between crude oil water system was also measured for evaluating the surface active properties of the culture broth.

5.29.1 Emulsification index measurements

Emulsifying activity of the cell free culture broth was assessed using crude oil as substrate by the method described earlier in section 5.14.2. All the results were repeated three times to obtain the average value.

5.29.2 Surface tension and Interfacial tension measurements

Surface tension (ST) of cell free and oil free culture supernatant was measured as described earlier in section 5.14.1. 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.

5.30 Isolation of the bioemulsifier (BE)

At the optimized fermentation condition, the 72 hours grown culture broth was centrifuged at 4°C and 10,000 rpm in a REMI C24 (Chennai, India) centrifuge for 20 minutes. The emulsifying 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 precipitated as extracellular
polysaccharides (EPS) from the supernatant were further centrifuged at 10,000 rpm at 4°C. The pellets were dissolved in distilled water and then freeze-dried (EYELA FDU-1200, Japan) and finally weighed.

5.31 Stability studies

The cell free culture broth containing crude bioemulsifier mixture was used to determine the effect of temperature (25 to 100°C, also kept at 121°C for 15 mins during autoclaving), pH (2-10), and salinity (2–15%) on emulsification activity (EI) and interfacial tension (IFT) reduction property. The EI and IFT measurements of each treatment were assessed as described above, with crude oil used as the substrate.

5.32 Rheology studies

The viscosity of the crude extracellular polysaccharides (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 as described in earlier section 5.18.

5.33 Core flooding studies

The execution of core flooding experiments for enhanced oil recovery has been done following the method described by Bera et al. (2014). The experimental apparatus (represented in Fig. 2) is composed of a sand pack holder, cylinders for holding chemical slugs and crude oil, positive displacement pump, and measuring cylinders for collecting the samples. The positive 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 size is \( L = 35.3 \) cm and \( r = 2.8 \) cm. The core holder was tightly packed 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. Pore volume (PV), defined as the empty volume of the model, was calculated by measuring the volume of water required to saturate the column. The porosity (\%) of the column was calculated as the PV divided by the total volume of the column (217.36 cc). The sand pack was then flooded with the crude oil at a pressure of 200 psig to irreducible water saturation. Original oil in place (OOIP) was calculated as the oil left in core after oil flooding. 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 necessary calculations made were as follows:

\[
\text{Porosity of core } \bar{\Omega} = \frac{\text{PV}}{\text{Bulk Volume}} \tag{22}
\]

\[
\text{Initial oil Saturation } S_{oi} = \frac{\text{OOIP}}{\text{PV}} \times 100 \tag{23}
\]

\[
\text{Irreducible water saturation } S_{wi} = \frac{(\text{PV} - \text{OOIP})}{\text{PV}} \tag{24}
\]

\[
\text{Residual oil saturation } S_{or} = \frac{[(1 - Swi) \times PV - V_1]}{PV} \tag{25}
\]

Where, \( V_1 \) is the oil produce after brine flooding

\[
\text{Residual oil saturation after bioemulsifier flooding } S_{or(A)} = \frac{[(S_{or} \times PV) - V_2]}{PV} \tag{26}
\]

Where, \( V_2 \) is the oil produce after BE flooding

\[
\text{Residual oil saturation after chase water flooding } S_{or(w)} = \frac{(S_{or(A)} \times PV - V_3)}{PV} \tag{27}
\]

Where, \( V_3 \) is the oil produce after brine flooding in end
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. 28), used for fluid flow in porous media. For a horizontal laminar system, flow rate is related with permeability as per Darcy:

$$q = \frac{kA dp}{\mu dx}$$  \hfill (28)

Where,

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

The oil recoveries were calculated as follows:

Oil Recovery by brine flooding = \( \frac{V_1}{OOIP} \)*100 \hfill (29)

Extra oil recovery by bioemulsifier flooding = \( \frac{V_2}{OOIP} \)*100 \hfill (30)

Extra oil recovery by chase water flooding = \( \frac{V_3}{OOIP} \)*100 \hfill (31)

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

$$RF_{Total} = RF_{SM} + RF_{TM}$$ \hfill (32)

where,

$RF_{Total}$ = total recovery factor (%),
$RF_{SM}$ = recovery factor obtained by secondary oil displacement method (%),
$RF_{TM}$ = recovery factor obtained by tertiary oil displacement method (%).
Fig. 2 Schematic representation of sand-pack flooding experiments for EOR

References:


