Enhancement of waste engine oil biodegradation by optimization of media using factorial design study

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Biodegradation of spilled or dumped waste engine oil is a challenging task due to their toxicity and persistency in the environment. Waste engine oil biodegradation can be improved by optimizing media constituents that may stimulate the microbial degradation phenomenon and contribute to the progress of oil spill bioremediation process. In the present study, the effect of different nutrients, such as, carbon, nitrogen and inorganic ion source, added externally to Bushnell-Haas (BH) medium were investigated for waste engine oil biodegradation using a novel hydrocarbon degrading bacterium, Ochrobactrum pseudintermedium C1. The bacterial strain was isolated in our laboratory from oil contaminated soil and identified by 16S rRNA gene sequencing. The results showed that the most significant variables influencing waste engine oil biodegradation were glucose, yeast extract and FeCl$_3$. A full factorial central composite design was applied for experimental work and analysis of the data. Moreover, these three factors interacted with each other and combined to produce positive effects on waste engine oil degradation. The experimental data also allowed the development of an empirical model ($P < 0.00672$) describing the inter-relationship between independent and dependent variables. By solving the regression equation, the optimal values of the variables were determined as (g/L): glucose 22.028, yeast extract 1.949 and FeCl$_3$ 0.225. The BH medium with the above constituents resulted in significant enhancement of waste engine oil percent degradation from 51.24 to 71.52% within 7 d.

Keywords: Biodegradation, central composite design, Ochrobactrum pseudintermedium, optimization, waste engine oil

Introduction

Waste engine oil (WEO) which is also known as used motor oil is produced when fresh engine oil (or motor oil) is subjected to high temperature and high mechanical strain during running of the vehicle for a stipulated time. It is a brown-to-black liquid mixture consisting of low to high mol wt (C$_{16}$ to C$_{36}$) aliphatic and aromatic hydrocarbons, polychlorinated biphenyls, chlorodibenzoofurans, lubricative additives, and decomposition products$^{12}$ along with heavy metal contaminants, such as, zinc, lead and chromium, coming from engine parts. Thousand million gallons of WEO are generated annually from mechanical workshops, which is not recycled but spilled and dumped by automobile and generator mechanics into runoff, gutters, water drains and open vacant plots and farmlands$^{3}$. Out of this, 1 L is enough to contaminate 1 million gallons of fresh water$^{4}$. The illegal dumping of used engine oil is dangerous to the environment and constitutes a potential threat to human, animals and vegetations$^{5,7}$. Most mechanical methods like incineration and/or burial in secure landfills to reduce hydrocarbon pollution are expensive and time consuming. These are effective treatments but, after burning, the soil gets depleted of nutritional value and structure. These methods do not remove the contamination but only relocate the problem$^8$. Therefore, it is needed to find out efficient, affordable and more environment-friendly methods of WEO treatment, especially in the developing countries.

Biodegradation of oil contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as one of the efficient, economic, versatile and environmentally sound treatment. These methods can be used to treat soils contaminated with WEO or degrade these waste oils in places where waste oil recycling is not a feasible option, and to supplement existing waste recycling treatment technologies$^9$. Biodegradation of petroleum hydrocarbon pollutants and petrochemicals by bacteria have been extensively investigated$^{10-12}$. Investigations have been carried out on the bioremediation of engine oil-contaminated soils using selected bacterial and fungal agents, mixed bacterial consortium and organic wastes with some success$^{13-15}$. Since oil degradation is a natural process

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limited by different environmental and nutritional factors, optimization of the culture media conditions is often needed for better degrading efficiency of the microorganisms.

Response surface methodology (RSM) is an efficient statistical technique for the optimization of multiple variables to predict the best performance conditions with minimum number of experiments and has already been successfully applied in many fields, such as, optimization of media, fermentation conditions, and enzyme-catalyzed conditions in lab experiments. In the present investigation, *Ochrobactrum pseudintermedium* C1 was selected for the adaptation, which degraded WEO with higher tolerance limit. Therefore, it was worthwhile to study the effect of different media constituents that affect the biodegradation rate of WEO and to find out the optimum levels of the important factors considered for WEO degradation by the strain C1 in order to maximize degradation rate of WEO and to determine the mutual interactions between these factors simultaneously using RSM.

**Materials and Methods**

**Chemicals**

WEO were collected from local automobile workshops nearby Kolkata, India. Bushnell-Haas (BH) medium and Nutrient agar medium of Hi-Media Laboratories Pvt. Ltd. were used for isolation, cultivation and maintenance of culture. Other chemicals and solvents were of LR grade and purchased from local suppliers.

**Organism and Cultivation Conditions**

The bacterium used in this present study, *O. pseudintermedium* C1 was isolated in our laboratory from oil contaminated soil of ore handling plant of IISCO Steel Plant, Burnpur, India and identified by 16S rRNA gene sequencing method from Bhat Biotech India Pvt. Ltd., Bangalore, India. Yeast extract was found as the most suitable nitrogen source. Yeast extract was as the most suitable nitrogen source and was further employed at varying concentration range (between 0.1 to 0.3 g/L) effective inorganic salt FeCl₃ was further employed at varying concentrations from 0.5 to 2 g/L for determining its optimal level. To evaluate the effect of various inorganic salts, BH medium was formulated with different concentrations of the salts, such as, MgSO, CaCl, FeCl, NH₄NO₃, MnSO₄, and NaCl.

**Media Optimization Studies**

The medium optimization was conducted with an optimum WEO concentration of 4% (v/v) (34.4 g/L) in a series of experiments changing one variable at a time and keeping the other factors constant. Three factors, viz., carbon source (C), nitrogen source (N) and various inorganic salts, were chosen to obtain higher percent biodegradation of waste engine oil. For evaluation of carbon source, glucose was added as a co-substrate for degradation of WEO in varying concentrations, in the range of 5 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 found as the most suitable nitrogen source and was further employed at varying concentrations from 0.5 to 2 g/L for determining its optimal level. To evaluate the effect of various inorganic salts, BH medium was formulated with different concentrations of the salts, such as, MgSO₄, CaCl₂, FeCl₃, NH₄NO₃, MnSO₄, and NaCl.

**Experimental Design and Statistical Analysis**

A full factorial central composite design (CCD) was applied with the three experimental factors chosen from previous optimization studies, namely, glucose, yeast extract, and FeCl₃. A total of 16 runs, performed in duplicate, were required for this procedure. Table 1 lists the coded (Xᵢ) and actual (xᵢ) levels of each variable. For statistical calculation, the

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coded variables</th>
<th>Variable levels</th>
<th>Step change value Δxᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose conc. (g/L)</td>
<td>X₁</td>
<td>10</td>
<td>-1</td>
</tr>
<tr>
<td>Yeast extract conc. (g/L)</td>
<td>X₂</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>FeCl₃ conc. (g/L)</td>
<td>X₃</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>
The test factors were coded according to the following equation:

\[ X_i = (x_i - x_0)/\Delta x_i \quad i = 1, 2 \ldots \ldots \ldots \ldots k \]

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 real value of the independent variable at the centre point; \( \Delta x_i \) is the step change value. Response surface methodology (RSM) allows modeling of the results using a quadratic equation. The experimental data obtained was fitted to the following quadratic polynomial equation:

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

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. The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface plots.

### WEO Degradation Analysis

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

#### 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 min. The residual waste oil was extracted by adding 20 mL of hexane and shaking thoroughly as described by Mandri and Lin. 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:

\[ \text{% of oil degraded} = \frac{[\text{wt of test oil sample} - \text{wt of residual oil sample}]}{\text{wt of test oil sample}} \times 100 \]

#### Gas Chromatography Analysis

The residual waste oil sample after each biodegradation experiment was analyzed by gas chromatography according to the condition described by Ghazali et al.\(^2\). Hexane extracts of residual oil sample (1 µL) were injected for analysis by using a Polaris Q Mass Spectrometer coupled with Thermo Scientific Trace 1300 series gas chromatograph and TR-5 column (30 x 10\(^3\) cm length; 0.032 cm id; and 1 x 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 column 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 WEO was calculated by the differences in summation of peak area of total petroleum hydrocarbons (TPH) present in the waste oil samples. Chromatographs were analyzed by Chromeleon 7.0 program and a library (NIST 2007) search was performed for identification of chromatogram peaks.

### Results and Discussion

#### Experimental Optimization Using One Factor at a Time

\( O. \) pseudintermedium Cl was inoculated into BH medium with addition of different nutrient supplements, such as, glucose (10 g/L), NaNO\(_3\) (1 g/L), Urea (1 g/L), yeast extract (1 g/L), NH\(_4\)NO\(_3\) (1 g/L), MgSO\(_4\) (1 g/L), CaCl\(_2\) (0.1 g/L), FeCl\(_3\) (0.2 g/L), NaCl (1 g/L) and MnSO\(_4\) (0.1 g/L). The most favourable carbon source, nitrogen source and inorganic salt were found to be glucose, yeast extract and FeCl\(_3\) according to the effect on enhancement of percent degradation of WEO as shown in Fig 1a. Supplementation of glucose as an added carbon source showed the maximum positive effect probably due to promoting growth of the bacterial species, thus enhancing ability to uptake hydrocarbons more easily.\(^24,25\) The nature and concentrations of nitrogen sources are the factors stimulating the biodegradation of hydrocarbon compounds\(^31\). Of the various nitrogen sources tested (Fig. 1c), organic nitrogen source (yeast extract) showed positive effect compared to the inorganic nitrogen sources (NaNO\(_3\), NH\(_4\)NO\(_3\) & Urea). Addition of FeCl\(_3\) in the growth medium showed inducing effect on biodegradation of WEO compared to other inorganic ion sources. Fe as an ion source might induce the oxidative enzymes of the bacterial species for metabolizing the hydrocarbons.\(^29,30\) The optimum concentrations of the chosen variables, glucose, yeast extract and FeCl\(_3\), were determined from Figs 1(b-d), corresponding to maximum percentage of WEO degradation.
Optimization Using Central Composite Design (CCD) Regression Modeling

In order to approach the optimum response region of maximal degradation rate of WEO, a total of 16 experiments were conducted following the CCD method using three factors with three levels of glucose (10, 20, 30 g/L), yeast extract (1, 2, 3 g/L) and FeCl$_3$ (0.1, 0.2, 0.3 g/L). A regression model containing three linear ($X_1$, $X_2$, $X_3$), three quadratic ($X_1^2$, $X_2^2$, $X_3^2$) and three interaction ($X_1X_2$, $X_2X_3$, $X_3X_1$) terms plus one block term was employed by using STATISTICA version (10.0). The experimental results were analyzed through RSM to obtain an empirical model for the best response. The results of experimentally obtained and theoretically predicted response are shown in Table 2. The results show that the predicted data of the response from the empirical model is in good agreement with the experimentally obtained data. The quadratic model was used to explain the mathematical relationship between the independent variable and dependent responses. The mathematical expression of relationship to the WEO degradation with variables like glucose, yeast extract and FeCl$_3$ are shown below as in terms of coded factors. All terms regardless of their significance are included in the following equation:

$$Y = 70.52085 + 3.95581X_1 - 9.66808X_2 - 0.82952X_3 - 7.62765X_1^2 + 2.78294X_2^2 - 5.35804X_3^2 - 0.25925X_1X_2 - 0.18425X_2X_3 + 0.41975X_3X_1$$

Where, $Y$ (%) is the percentage of WEO degradation, $X_1$, $X_2$ and $X_3$ are the coded values of glucose, yeast extract and FeCl$_3$, respectively.

Analysis of Variance

The results of ANOVA are summarized in Table 3. The value of the determination coefficient ($R^2$), being a measure of the goodness of fit of the polynomial model, was 0.987, which indicates that only 0.02% of the variability in the responses cannot be explained by

![Image of a figure showing effects of medium constituents on percent degradation of WEO by O. pseudintermedium C1]
However, the lack of fit was observed to be insignificant ($p_{\text{lack of fit}} = 0.07$), implying that the obtained model is adequate to represent the experimental data. A higher $R^2$ value of 0.987 also shows that the equation is highly reliable and depicts the model to be adequate for prediction within the range of variable chosen. The significance of each coefficient of the model was determined by F-test and P-value. The smaller P-value and higher F value represent the corresponding coefficient to be more significant. A P value less than 0.05 indicates that the model is statistically significant. In the present case, it was found that the three linear coefficients and all quadratic coefficients were highly significant.

**Response Surface Analysis**

**Effect of glucose**

Fig. 2a represents maximum percentage of WEO degradation against glucose and yeast extract. The maximum percentage of WEO degradation was 70% at a particular range of glucose (20-25 g/L) and yeast extract (1.5-2.0 g/L), which is also clearly illustrated in Fig. 1b. The optimum level of WEO degradation of 70.98% occurred at glucose concentration of 22.028 g/L and yeast extract concentration of 1.949 g/L, calculated by derivatization of the regression equation and by solving the inverse matrix. The effect of glucose as shown in Fig. 1(b) indicates that increase in the concentration of glucose above (20 g/L) showed...

**Table 3—ANOVA for regression model**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Co-efficient value</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean squares</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean/Interaction</td>
<td>70.52085</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.000672*</td>
</tr>
<tr>
<td>(1) Glucose conc. (L)</td>
<td>3.95581</td>
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<td>55.6389</td>
<td>5012.29</td>
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<td>243.9181</td>
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<td>243.9181</td>
<td>21973.62</td>
<td>0.004295*</td>
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<td>(2) Yeast extract conc. (L)</td>
<td>-0.82952</td>
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<td>1</td>
<td>2.4466</td>
<td>220.41</td>
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<td>Yeast extract conc. (Q)</td>
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<td>151.8257</td>
<td>13677.38</td>
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<tr>
<td>(3) FeCl$_3$ conc. (L)</td>
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<td>27.5370</td>
<td>2480.70</td>
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<td>6748.91</td>
<td>0.007749*</td>
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<tr>
<td>1L by 2L</td>
<td>-0.25925</td>
<td>0.1344</td>
<td>1</td>
<td>0.1344</td>
<td>12.11</td>
<td>0.178144</td>
</tr>
<tr>
<td>1L by 3L</td>
<td>-0.18425</td>
<td>0.0679</td>
<td>1</td>
<td>0.0679</td>
<td>6.12</td>
<td>0.244617</td>
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<tr>
<td>2L by 3L</td>
<td>0.41975</td>
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<td>1</td>
<td>0.3524</td>
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<td>0.111827</td>
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<tr>
<td>Lack of Fit</td>
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<td>1</td>
<td>1.0485</td>
<td>94.45</td>
<td>0.077953</td>
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<tr>
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<td>1</td>
<td>0.0111</td>
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<tr>
<td>Total SS</td>
<td>368.7333</td>
<td>15</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

$R^2=0.98575$; Adj. $R^2=0.96438$

*Significant for $p$ value <0.05

**Fig. 2**—Response surface plots for degradation of WEO: (a) Effects of glucose and yeast extract; (b) Effects of yeast extract and FeCl$_3$; & (c) Effects of FeCl$_3$ and glucose.
repressive effect, whereas (20 g/L) glucose resulted in only 68.14% degradation. Glucose, as a simple alternative carbon source, can enhance biodegradation of recalcitrant compounds due to its tendency to improve cell growth as reported by Wang et al.²⁴ and Saimmai et al.³⁰. Our results also show that an appropriate concentration of glucose could enhance the degradation rate of waste engine oil. The synthesis of surface active compounds, exopolysachharides etc. to enhance the hydrocarbon utilization by the cells could be taking place in presence of glucose as reported earlier by Leonie et al.²⁵.

Effect of Yeast Extract

The maximum percentage of WEO degradation was found to occur with yeast extract (1.5-2.0 g/L) and FeCl₃ (0.2–0.3 g/L) (Fig. 2b). Optimum level of yeast extract (1.949 g/L) and FeCl₃ (0.225 g/L) showed the maximum percentage of WEO degradation (70.98%). The concentration of yeast extract in BH medium (Fig. 1c) varied from 0.5 to 3.0 g/L and there was no considerable increase in the degradation beyond 2.0 g/L. However, increase in the concentration of yeast extract from 0.5 to 2.0 g/L increased the degradation from 53.47 to 62.97%. Presence of yeast extract in BH medium not only increased the bacterial yield, but also reduced the time required for completion of the degradation. A number of reports are available regarding the enhancement of growth of microorganisms on hydrocarbons using organic nitrogen source like yeast extract¹¹,²⁶.

Effect of Ferric Chloride

Fig. 2c depicts the maximum percentage of WEO degradation with FeCl₃ (0.15–0.25 g/L) and glucose (20-25 g/L). Optimum level of degradation (70.9%) was observed at 0.225 g/L FeCl₃ and 22.038 g/L glucose. It was observed that the growth of the bacterial strain increased with increasing concentrations of FeCl₃. And that was probably due to its isolation from oil contaminated soil of iron ore handling plant. Earlier reports also described the heavy metal tolerance of the Ochrobactrum sp.²⁷. It was also observed that they could grow in a medium with higher Fe concentration, varying from less than 0.05 to about 0.3 g/L, but their degradation activities were impaired with increasing FeCl₃ concentration as observed from Fig. 1d.

Optimal Media Composite and Validation of Experiments

To determine the optimum media composite, the optimal values of the variables X₁, X₂ and X₃ were found out by solving the regression equation. This was achieved by putting the second order regression equation into matrix form as described by Myers and Montgomery²⁸. The optimum values of the test variables were found to be as follows: glucose 22.028 g/L, yeast extract 1.949 g/L and FeCl₃ 0.225 g/L, with the predicted biodegradation of WEO at 70.98%. In order to verify the results, waste engine oil degradation was carried out using both the optimal medium and the original BH medium (not supplemented with yeast extract, glucose and FeCl₃). Fig. 3 shows that 71.5% of WEO was degraded using the optimal medium within 7 d in comparison to 51.24% using the original BH medium. Saimmai et al.³⁰ also observed that the addition of nutrients stimulated the used lubricating oil degradation capabilities of indigenous microorganisms. These results show that the predicted data of the response from the empirical model is in good agreement with the experimentally obtained data and suggested that the model is satisfactory and practicable.

Biodegradation Analysis of WEO

WEO composition is highly variable due to variation in combustion conditions, so biodegradation analysis was performed based on TPH concentration measurement using gas chromatography³². The GC-MS spectra of TPH occurring in both the treated (at optimized condition) and untreated waste oil samples were expressed in Figs 4a and b. Benzene and naphthalene derivatives were the predominant hydrocarbon structures in the composition of the WEO sample. The WEO sample after biodegradation revealed significant reduction of major hydrocarbon peaks (from 6-40 min) compared to those in WEO from control samples. The most abundant peaks were located between 11-35 min of retention time and were identified as derivatives of benzene, naphthalene,
azulene, indole, benzopyrene, dibenzophenazine etc. Naphthalene, acenaphthylene, diphenylanthracenes, benzopyrene and benzanthracene were also detected in used motor oil samples by several other researchers. Benzonic acid and its derivatives were detected in the WEO sample recovered after biodegradation, which were presumably formed by the biodegradation of waste oil. This data suggest that the potential toxic hydrocarbon pollutants were metabolized by the *Ochrobactrum* sp. as alcohols, aldehydes and organic acids and these are common products of the so-called ‘beta-oxidation’ of long-chain aliphatic compounds.

**Conclusion**

The CCD selected as a RSM proved to be suitable for performing degradation studies. The true functional relationship between the dependent variable (carbon, nitrogen, & inorganic metal sources) and maximum percentage of WEO biodegradation have been studied. The optimum conditions for growth and degradation by *O. pseudintermedium* Cl were also found out from the empirical model, which provided good quality of predictions for the above variables in terms of effective WEO degradation and good correlation coefficient ($R^2$ 0.987) was obtained. The treatment of WEO in industrial and domestic effluents is very important due to its persistent and toxic effect. The optimum culture medium obtained in these experiments gives a basis for further study with batch or fed-batch cultivation in a bioreactor for degradation of WEO in industrial effluents.

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