Biodegradation of diesel using microbes from a clam (Meretrixmeretrix) shell

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Present study consists the potential ability of microorganisms present on clam (Meretrixmeretrix) shells to degrade diesel. Counts of crude oil degrading bacteria in oil polluted soil fortified with Meretrixmeretrix shells were higher than that of unfortified soil. Microorganism isolated from the Meretrixmeretrix shells was found to be Bacillus subtilis, which is seen to have potent lipase activity, thus capable of degrading diesel and releasing carbon dioxide. This study show that clam, Meretrixmeretrix shells efficiently degrade diesel and can help in bioremediation of oil polluted regions.

[Keywords: Carbon dioxide evolution, clam Meretrixmeretrix, Hydrocarbon, Gravimetric Analysis, Diesel extraction]

Introduction

Oil pollution is a prevalent problem since the commencement of oil exploration and development of petroleum industry\(^\text{11}\). Different methods have been used in restoring petroleum polluted soil, some of these include the use of oil degrading microorganisms, inorganic fertilizers, chicken droppings, periwinkle shell, melon shell, liming and tilling\(^\text{10,6,7}\). Diesel is made up of harmful compounds like benzene, cycloalkanes, polycyclic aromatic hydrocarbons which are toxic in relatively high concentrations. Since these compounds are insoluble in water due to their high molecular weight, they seep into the soil and persist in the environment and limit the oxygen and water transportation for organic processes to occur, thus limiting biodegradation\(^\text{7}\). Oil spilled in the soil or water can cause extensive threat to local ecosystems, since accumulation of saturated hydrocarbons in plant and animal tissues may lead to death or mutation.\(^\text{10}\) Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. It uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site\(^\text{10}\). Thus in respect of petroleum or diesel polluted soils, the technique for bioremediation aims at preventing further spread of the contaminants and also its removal from the soil. Naturally present microbial populations that enzymatically breakdown hydrocarbons and convert it to harmless organic compounds can be used for the bioremediation of soil polluted with diesel. The present study is an attempt to identify the microorganism from Meretrixmeretrix shell powderand determine their ability to degrade diesel.

Materials and Methods

Clams or marine bivalves Meretrixmeretrix shells were obtained from local market in Mumbai.

Clam shells were opened using a knife and the muscular insides were discarded. Shells were sun-dried for about 1 week, after which they were ground to a fine powder using traditional methods including grinding on stone and then using an electronic mixer-grinder\(^\text{12}\). Diesel used was sterilized by passing...
through syringe filter of 0.25 microns under sterile conditions. For the standardization, garden soil was taken from an available source, sieved through muslin cloth for fine soil and then sterilized in the oven at 140 degrees Celsius for 24 hours.

The pH of the sample was found using pH meter. Moisture content was found out gravimetrically. Estimation of nitrogen content was carried out by micro-Kjedhal method. Estimation of phosphorus was done using TCA extraction followed by acid molybdate reagent and determined spectrophotometrically\(^2,18\).

Isolation of microorganism from *Meretrix-meretrix*, shell powder was carried out on sterile nutrient agar. The growth of the organisms was examined on selective medium Oil Agar Medium\(^7,8,9\).

The colonies obtained on oil agar were identified by, Gram staining, endospore staining, MRVP, indole, urease, nitrate reductase, oxidase, catalase, gelatin liquefaction, lipase, starch hydrolysis, citrate and carbon utilization (glucose, fructose, lactose, xylose, galactose, sucrose, inositol and mannitol)\(^2\). Microorganism isolated had a potent lipase activity.

The degradation of diesel by the sample was checked over five weeks. Five sets of 4 tubes each were labeled as sample, duplicate, positive control and negative control\(^9\). Under sterile conditions, to every tube 10 gms of standard soil was added, 1gm of shell sample was added to the sample and duplicate test tube only. 1% w/v of diesel was added to the sample, duplicate and positive control tubes and 5mL of distilled water was added to all the tubes. Durham’s tubes containing 0.1gm of barium peroxide and distilled water up to 3/4th of the Durham’s tube was gently slid into sterile test tubes. Test tubes were then covered with sterile cotton and incubated at room temperature for 5 weeks. After every week the Durham’s tubes were examined for an air bubble, indicating the degradation of the hydrocarbons in the diesel\(^9,17,10\).

Every week, one set of experimental tubes were examined, test tube containing Durham’s tubes were removed, covered with para film and stored for further analysis. Soil content of the test tube were then transferred to sterile petri plates and heated in the oven for 48hrs at 52 degrees Celsius (below the flash point of diesel, ~60 degrees Celsius) to get rid of the moisture content for gravimetric analysis\(^10\). Heated soil from the test tube was put into crucibles and weighed. As the diesel from the soil is used up by the microorganisms in the shell sample the weight of the test tube contents reduced over the 5 weeks\(^3\).

Barium peroxide from the Durham’s tubes was transferred to a conical flask and titrated against hydrochloric acid (0.1 N)\(^7,10\). The amount of carbon dioxide was determined by titrating the barium carbonate formed with 0.1 N HCl.

5ml toluene was added to soil content of all the experimental tubes fresh for extraction of diesel using toluene. Test tubes were then incubated at 48 hrs at room temperature. After 48 hrs the toluene was filtered out from the mixture using Whatmanfilter.

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Table 1–Isolation of microorganism

<table>
<thead>
<tr>
<th>Media</th>
<th>No. of Colony forming units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient agar</td>
<td>(5.2 \times 10^5)</td>
</tr>
<tr>
<td>Zajic and Supplisson media(oil agar)</td>
<td>(3.6 \times 10^5)</td>
</tr>
</tbody>
</table>

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Fig. 1–Determination of Phosphorus content in *Meretrixmeretrix*, shell powder.
Results and Discussion

The pH of the sample was found to be 8.1. Moisture content 3%. Nitrogen 4.2%, phosphorus 0.56%. Microorganism isolated was identified as *Bacillus subtilis* by biochemical and staining techniques. It was also found to have potent lipase activity.

Results of biodegradation by *Bacillus subtilis* in terms of carbon dioxide evolved is proportional to the amount of diesel degraded. Amount of carbon dioxide evolved was found to increase from the first week to the fifth week (Fig 2). Microorganisms present in the shell powder degrade diesel to release carbon dioxide, which gets converted to barium carbonate on reaction with the barium peroxide present in the fusion tubes. Degradation of diesel in the fifth week was seen to be maximum as compared to the first week indicating biodegradation of diesel was taking place.

The positive and negative control weighed approximately the same over the 5 week period (Fig3). This may be attributed to absence of shell powder. Since the clam powder was absent in the control tubes the diesel was not degraded, thus maintaining the weight to be fairly constant over the experimental time.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gram staining</td>
<td>Gram positive bacilli</td>
</tr>
<tr>
<td>2. Endospore staining</td>
<td>Green spores in pink rods</td>
</tr>
<tr>
<td>3. Indole</td>
<td>-</td>
</tr>
<tr>
<td>4. Urease</td>
<td>+</td>
</tr>
<tr>
<td>5. Nitrate Reductase</td>
<td>+</td>
</tr>
<tr>
<td>6. Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>7. Catalase</td>
<td>+</td>
</tr>
<tr>
<td>8. Methyl red</td>
<td>-</td>
</tr>
<tr>
<td>9. VP</td>
<td>+</td>
</tr>
<tr>
<td>10. Gelatin liquefaction</td>
<td>+</td>
</tr>
<tr>
<td>11. Lipase</td>
<td>+</td>
</tr>
<tr>
<td>12. Starch hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>13. Citrate</td>
<td>+</td>
</tr>
<tr>
<td>14. Carbon sources:</td>
<td></td>
</tr>
<tr>
<td>- Glucose</td>
<td></td>
</tr>
<tr>
<td>+ Fructose</td>
<td></td>
</tr>
<tr>
<td>- Lactose</td>
<td></td>
</tr>
<tr>
<td>+ Xylose</td>
<td></td>
</tr>
<tr>
<td>- Galactose</td>
<td></td>
</tr>
<tr>
<td>+ Sucrose</td>
<td></td>
</tr>
<tr>
<td>+ Inositol</td>
<td></td>
</tr>
<tr>
<td>- Mannitol</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 2–The percentage carbon dioxide evolved](image)

![Fig. 3–Graph to determine moisture content](image)
period of five weeks. Weight of the sample and the
duplicate tubes decreased from the first to the fifth
week, as compared to the control tubes. Loss of weight
indicates the utilization of the diesel by the
microorganisms present in the sample (clam shell
powder) (Fig 3).

**Fig. 4–Graph from determination of diesel content.**

The percentage concentration of diesel extracted
decreases from the first week to the fifth week
(Fig 3). Presence of the microorganisms in the sample
may have degraded the diesel and thus the amount
of diesel also decrease with time. Percentage of diesel
degraded was found to be 26% in 5 weeks. (Fig 4).

**Conclusion**

The results from the study indicate that the
microorganism *Bacillus subtilis* found in the shell of
*Meretrix meretrix* can degrade diesel up to 20-30%
over the time period of 5 weeks. It was seen from the
experiments conducted that the microorganism
*Bacillus subtilis* found is capable of breaking down
diesel which can be due its potent lipase activity.
Hence, it is a novel methodology for biodegradation
of diesel. Bioremediation of diesel is feasible using
*Meretrix meretrix* shell powder and the microorganism
isolated from the powder can prove to be useful in
clearing up of oil spills and can degrade oil or
petroleum products like petrol and diesel to a great
extent.

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