EFFECT OF TEMPERATURE ON HYDROCARBON DEGRADATION ABILITY BY BACTERIAL ISOLATES : A BIOSTIMULATION STUDY

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Received September 29, 2014 Accepted January 20, 2015

ABSTRACT

Bioremediation of highly contaminated petroleum waste is one of the critical challenges of environmental biotechnology. Nutrient deficiency, especially nitrogen has been reported to be of vital significance affecting rate of hydrocarbon degradation by microorganisms. In this study, we report a novel method for enrichment of bacteria from oily sludge sample collected from Indian oil cooperation limited refinery Guwahati, Assam, India. Under various nitrogen amendment conditions. In this method, we use multiple nitrogen sources for nitrate, nitrite and ammonium (concentration range of 1-12mM) along with sodium pyrophosphate as dislodging chemical. Further to estimate the biodegradation ability of these isolates effect of temperature (30°C, 40°C, 50°C) on their growth has been studied using aliphatic (pentadecane) and aromatic (naphthalene) compounds as carbon sources. Microcosm based studies on original sludge sample showed a correlation between the three factors are temperature, growth and biodegradability of bacteria with increasing temperature having a detrimental effect on microbial growth. Crucially our findings suggest that response of N amended bacteria to increase in temperature was less drastic as compared to unamended bacteria.

Key Words : Biostimulation, Temperature, Hydrocarbon, Aliphatic, Aromatic, Naphthalene

INTRODUCTION

Petroleum refineries unavoidably generate enormous quantity of waste oily sludge, in terms of tank bottom and Effluent Treatment Plant (ETP) oily sludge and oil contaminated soil in their day to day refining process. These waste oily sludge and oil contaminated soil (termed as oily waste) are stored in sludge pits inside the refinery premises and due to stringent regulatory norms, the disposal of these oily waste in an environment friendly manner is a serious problem. Indian Pollution Control Board (CPCB), USEPA (United States Environmental Protection Agency) and OECD (Organization for Economic Co-operation and Development) organizations have designated oily sludge wastes as hazardous.¹⁻⁵ The hazardous oily waste is composed of particulates mixture of highly variable carbon chain length hydrocarbons and metals. The TPH constitutes a complex mixture of alkane, aromatic, Nitrogen, Sulphur and Oxygen containing compounds (NSO) and asphaltene fractions.⁶⁻⁸ Oil contamination has severe impacts in the plant and animal ecosystem including human health. Crude oil exposure may cause damage to lungs, liver, kidneys, intestines and other internal organs. Polycyclic Aromatic Hydrocarbons (PAH) may lead to cancer Inhalation leads to headache, nausea, dizziness and respiratory irritation. BTEX (Benzene, Toluene, Ethyl benzene and Xylene) cause mutations, cancers, birth defects, nervous disorders, liver disease, depression, irregular heartbeats etc. Oil contaminated soil loose its fertility and have impact on seed germination.⁹⁻¹⁰ Hence disposal
of the oily waste in an improper manner may cause a serious environmental problem. Various conventional methods like land filling incineration, air sparging etc. have been applied since early times for remediation of oily waste. It is observed that none of the conventional methods is environment friendly solution. The common drawback is that they are not the permanent solution for the environmental pollution and sometimes they are not cost effective. It is established that virtually all types of hydrocarbons are susceptible to microbial degradation and hence the relevance of using the biotechnological approach using the microbial capability for bioremediation of the hazardous waste is justified. Bioremediation has been applied as a cost effective, ecologically friendly and efficient treatment technology for the contamination of hydrocarbon polluted soils. Laboratory studies and field tests have shown that bioremediation can enhance oil biodegradation on contaminated shorelines. The success of bioremediation depends on having the appropriate microorganisms in place under suitable environmental conditions and composition of the contaminant.\textsuperscript{11} It is well accepted that microorganisms require nutrients like Nitrogen (N), Phosphorus (P) and micronutrients to degrade organic soil contaminants. Several reports document beneficial effects of N addition on biodegradation rates. Studies indicate that proper N management can elevate the speed with which populations of degrading microorganisms increase and maintain long-term microbial populations at high activity levels. Many researchers have examined the fate of oil in various environments including soils, water and seawater and concluded that nutrient addition, especially nitrogen and phosphorus, can stimulate oil biodegradation. Mineral nutrients (e.g. KNO\textsubscript{3}, NH\textsubscript{4}NO\textsubscript{3}, K\textsubscript{2}HPO\textsubscript{4}, MgNH\textsubscript{4}PO\textsubscript{4}) and organic nutrients, such as urea paraaffin-supported mineral nutrients and octyl phosphate are the most common compounds used for bioremediation.\textsuperscript{12} In most of these studies only one type of nutrient compound was used. Few researchers have studied different nutrient types concurrently.\textsuperscript{13-17} While some studies showed addition of mineral nutrients stimulated biodegradation more than the organic nutrients, other studies reported effect of a proprietary organic nutrient was equivalent to nitrate in closed systems.\textsuperscript{18-20} The source of nitrogen added to oil-degrading enrichment cultures may have a powerful effect on degradative ability of the microbial community.

In the current study, we have reported an isolation technique for extracting culturable bacterial community from the oil contaminated refinery sludge sample using sodium pyrophosphate as dislodging agent. Bacteria thus isolated were further tested for their ability to withstand higher temperatures.\textsuperscript{21,22} These were further characterized based on their ability to grow in a representative hydrocarbon viz., alkane (pentadecane) and aromatic (naphthalene) compounds. Microcosm based study was further undertaken to analyze their effect of temperature on degradation and growth in the sludge sample.\textsuperscript{23-28}

**AIMS AND OBJECTIVES**

Isolation of bacterial community and selection of ten isolates, five from unamended samples and five from nitrogen amended samples. Estimating the ability of these isolates to grow and survive in higher temperatures in presence of glucose as well as representative hydrocarbon compounds as sole carbon source. Estimation of effect of higher temperatures on degradability and survival of one N amended one amended and a control isolate using microcosm based study.

**MATERIAL AND METHODS**

**Sample collection and isolation of bacteria**

Petroleum refinery waste sludge was collected from sludge waste lagoon of an oil refinery of Indian Oil Corporation Limited (IOCL) Guwahati, Assam, India (26.14°N, 91.73°E) and transported to laboratory at 4°C. Conventional bacterial isolation techniques demonstrated low bacterial count as well as morphological diversity. Thus, a new protocol was used for isolation of bacterial community from the sludge sample. Briefly 5g of sample was mixed with 22.5ml of 1% sodium pyrophosphate solution along with 4g of glass beads vortexed for 5 mins and incubated at 30°C for 1 hr. This step was followed by freezing the sample in ice for 2 mins and the whole process repeated twice. The treated sample was then centrifuged at
1000g for 10mins at 8°C supernatant stored and
the whole process repeated with the pellet. The
supernatants from the two steps was pooled and
centrifuged at 10000g for 30mins at 8°C and the
pellet resuspended in 2ml of normal sterilized
saline solution. Dilutions of this was plated in
R2A agar plates (Table 1) and incubated at 30°C
for 48-72 hours. Morphologically distinct
colonies were isolated and maintained
(Table 2). This protocol was used to isolate
bacteria from unamended samples as well as this
bacterial consortia was further enriched with
10mM of various nitrogen sources. Five
morphologically different bacteria were then isolated from these enrichments
(Table 2).

Table 1 : Composition of media used for aerobic culturable bacterial estimation and trace
element solution

<table>
<thead>
<tr>
<th>Reasoners 2A(R2A) (g/L)</th>
<th>Minimal Salts Media (MSM) (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>K$_2$HPO$_4$</td>
</tr>
<tr>
<td>Peptone</td>
<td>KH$_2$PO$_4$</td>
</tr>
<tr>
<td>Casein acid hydrolysate</td>
<td>NaCl</td>
</tr>
<tr>
<td>Glucose</td>
<td>NH$_4$Cl</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>KCl</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>Na$_2$SO$_4$</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>MgCl$_2$·2H$_2$O</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>CaCl$_2$·2H$_2$O</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 2 : Designation for bacterial isolates used in this study

<table>
<thead>
<tr>
<th>Nitrogen amended</th>
<th>Nitrogen unamended</th>
</tr>
</thead>
<tbody>
<tr>
<td>NyNCl2-1</td>
<td>J/GR3-4</td>
</tr>
<tr>
<td>NyNC11-1</td>
<td>J/GR3-7</td>
</tr>
<tr>
<td>NyNN5-1</td>
<td>J/GR3-10</td>
</tr>
<tr>
<td>NyNa5-1</td>
<td>J/GR3-8</td>
</tr>
<tr>
<td>NyNa10-1</td>
<td>J/GR3-11</td>
</tr>
</tbody>
</table>

Estimation of effect of temperature on
growth of isolates in hydrocarbons

To estimate the effect of higher temperature on
the growth of bacterial isolates each of them
were grown in a minimal media (Table 1) with
hydrocarbon compounds, pentadecane (Alkane)
and naphthalene (Aromatic) as sole carbon
sources and their growth (as OD at 600nm)
monitored over a time scale of 7 days at 24
hours interval each. Three sets of experiment
were set up at three different temperatures.

Microcosm study

Microcosms were set up to investigate the
effect of temperature on biodegradation of
hydrocarbons and survivability of isolates in
the sludge. Aerobic microcosms were set up in
triPLICATE at each temperature 30°C, 40°C and
55°C, for three isolates one N amended
(NyNN-5), one N unamended (J/GR3-10)
and a known hydrocarbon degrading strain
(Burkholderia sp.). The intrinsic microbial
community was killed by autoclaving the
sludge at 121°C for 45mins. To account for
abiotic losses, controls were kept. Microcosms
were set up 10ml glass serum vials with 5gm
sludge each and MSM was added to maintain
water balance and allow for bacteria to grow.
The degradability and viability of the isolate
was checked at 7 days interval till 21 days as
Total Petroleum Hydrocarbon (TPH)
gravimetrically and CFU/ml. Viability of
indigenous bacterial cells within the sludge
(incubated under varied conditions) was
monitored by quantifying colony forming units
(CFU g$^{-1}$). One gram of sludge sample from
each microcosm was mixed with 9ml of pre-
sterilized 0.85 % NaCl solution, vortexed for 1
minute and incubated in an incubator shaker
for 30mins at 170rpm at 30°C. Appropriate
serial dilution of this was plated in R2a plates
and incubated for 48-72 hours at 30°C.
TPH was determined gravimetrically following a modified standard procedure. Briefly 1 gram of sludge sample was extracted twice with 10ml n-hexane, vortexed thoroughly for 15 mins and centrifuged at 3,000 rpm for 10mins to separate the particulates. The clear hexane layer was poured over pre-weighed aluminium foil made dishes and dried under nitrogen gas flow in a chemical fume hood till a constant weight is obtained. The difference in weight indicates the TPH g$^{-1}$ in the sample.

**RESULTS AND DISCUSSION**

**Sample collection and isolation of bacteria**
Comparison of conventional isolation protocols with the one reported showed increase in bacterial counts from the orders of $10^5$ to $10^8$ CFU/g sample. Ten morphologically different bacteria were isolated five directly from the sample and five after enrichment with N sources.

**Estimation of effect of temperature on growth of isolates in hydrocarbons**
For all isolates growth was found to be maximum at 30°C and isolates were found to be sensitive to higher temperatures. As evident from **Fig. 1** and **Fig. 2** increase in temperature showed pronounced decrease in growth for all bacterial isolates.

Most isolates were also found to be growing better in presence of pentadecane as compared to growth in presence of naphthalene,

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**Fig. 1 :** Effect of temperature (30°C, 40°C and 55°C) on growth of isolates after 120 hours of incubation with pentadecane (500 ppm) as sole carbon source.

**Fig. 2 :** Effect of temperature (30°C, 40°C and 55°C) on growth of isolates after 120 hours of incubation with naphthalene (500 ppm) as sole carbon source.

thus all were found to prefer aliphatic over aromatic hydrocarbons shown in **Fig. 3**. Not much difference was observed in growth pattern of isolates in naphthalene, whereas in pentadeca-
ne isolates J/GR3-8, NyNa10-1 and NyNa5-1 showed considerably higher growth at 30°C. J/GR3-8, NyNa10-1 and NyNa5-1 grew 3, 2 and 2 times better in aliphatic compared to aromatic hydrocarbon respectively at 30°C but the effect was not visible at higher temperatures. These three strains thus garners more attention for further investigation.

![Comparison of growth of isolates in aliphatic and aromatic hydrocarbons at 30°C](image)

**Microcosm study**

Fig. 4 represents the effect of isolates on degradation measured gravimetrically as TPH g⁻¹ at 30°C. The higher temperature microcosms showed no viability of added cells after 7 days and thus were not considered further. A direct correlation is found between CFU and TPH reduction higher CFU g⁻¹ leading to lower TPH in samples. The effect was more pronounced in case of nitrogen enriched isolate as compared to normal isolate. Inhibitory effects on growth on both isolates were observed after 14 days however the control bacterium could grow uninhibitedly till the end of the experiment. TPH degradation was observed to be slightly higher in N amended samples compared to the control thus drawing our attention to the isolate and its degradation ability.

![Survival and hydrocarbon degradation potential of isolates over 21 days](image)

**CONCLUSION**

Growth study of isolates on hydrocarbons at different temperature confirmed them to be sensitive to higher temperatures. Isolate was found to grow maximally in presence of hydrocarbons. Isolates were found to be better
aliphatic hydrocarbon (here pentadecane) degraders than aromatic hydrocarbon (here naphthalene) degraders.

From the microcosm study, it is seen there is a direct co-relation between the CFU/ml for the isolates with the TPH degraded. It is also seen that nitrogen amended isolates are better TPH reducers than nitrogen unamended isolates. Microcosm studies at higher temperatures i.e., 40°C and 55°C were unsuccessful owing to inability of the isolate to survive. This study provides useful insights into the effect of temperature in the degradation of hydrocarbon compounds. The positive effect of nitrogen enrichment has also been ascertained as the enriched isolates were found to be more resistive to temperature change and better degraders. Thus this study also reaffirms the effects of nitrogen as a bio stimulant and leads way for further bio augmentation based studies based on these isolates.

ACKNOWLEDGEMENT
Authors would like to thankfully acknowledge to Department of Biotechnology, Government of India under NER Twinning Project (BT/226/NE/TBP/2011) for financial aid and Indian Oil Corporation Limited, Noonmati refinery, Guwahati, Assam, India for providing the waste for generous help during sampling.

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