ELUCIDATION OF PETROLEUM HYDROCARBON DEGRADATION BY *Burkholderia cepacia* (ES1) IN MODEL SYSTEMS AND EFFECT OF NONIONIC CHEMICAL SURFACTANTS

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ABSTRACT

Two key factors have a significant impact on bioremediation of petroleum hydrocarbon contamination, i.e., the microbial cultures present and measures used for enhancement of bioavailability. This work reports petroleum hydrocarbon degrading potential of *Burkholderia cepacia* (ES1) isolated and enriched from Arabian sea sediment using diesel as the sole substrate. The ability of the organism to utilize different hydrocarbon group types as sole carbon source was studied by applying them as single substrates, binary mixtures and complex mixtures. Further, a synthetic non-aqueous phase liquid (NAPL) representing a model for petroleum fractions such as diesel, was prepared and extent of degradation of the NAPL was determined in the absence and presence of three nonionic surfactants, Triton-X 100, Igepal CA 630 and Tween 80. Culture growth and degradation rate studies were also conducted using Triton X-100. *B. cepacia* (ES1) did not utilize the polynuclear aromatic hydrocarbons and its growth was inhibited when equimolar naphthalene and n-hexadecane were present. However, its growth was not hindered by the presence of naphthalene (mole fraction ($\chi$) = 0.125) in the model NAPL. The overall loss over 60 hrs increased from 33.8% without surfactant to 82.7%, 62.2% and 37.2% in presence of Triton X-100, Igepal CA 630 and Tween 80, respectively. Surfactants favored the utilization of aliphatics present in the NAPL. The biotic loss increased from 26.5% to 74.4% in the presence of Triton X-100 over 168 hrs.

**Key Words**: *Burkholderia cepacia*, Petroleum hydrocarbons, Surfactants, NAPLs

INTRODUCTION

Petroleum hydrocarbons are high volume global pollutants which are known to support microbial growth. Thus, bioremediation is a feasible treatment option. Complete removal or uptake of petroleum hydrocarbons in a complex mixture is rarely achieved due to bioavailability limitation and substrate specificity of the available microbial cultures. Bioavailability limitation of petroleum hydrocarbons due to the aqueous-non-aqueous biphasic nature of the system can be overcome by the use of surfactants which alter the properties of solution interfaces and facilitate transfer of non aqueous phase liquids (NAPLs) to the aqueous phase. However, chemical surfactants have been found to have both...
beneficial as well as inhibitory effects on biodegradation. Most biodegradation studies have used either pure petroleum hydrocarbons or commercially available complex mixtures, such as, fuel oils, that cannot be characterized completely. Synthetic NAPLs designed using representative hydrocarbons typically present in complex petroleum mixtures can serve as representative models for complex environmental mixtures. Studies using model NAPLs can elucidate the effect of surfactants on factors affecting degradation kinetics and would be useful for planning and implementation of surfactant aided bioremediation on a large scale. *Burkholderia* sps. are known to degrade a wide range of petroleum hydrocarbons. Surfactant aided biodegradation by this species has not been unraveled extensively. Thus, the objective of this study was to elucidate the petroleum hydrocarbon degrading ability of *Burkholderia cepacia* (ES1) in model systems in the presence of nonionic chemical surfactants.

**MATERIAL AND METHODS**

*Burkholderia cepacia* (ES1) was isolated and enriched on diesel from Arabian Sea sediment, and has been reported to utilize aliphatic hydrocarbons as the sole source of carbon and energy. The ability of the culture to utilize various group types of petroleum hydrocarbons was investigated by using n-hexadecane, n-octadecene, cyclohexane (1% v/v), naphthalene (500 mg/L) and phthalic acid (500 mg/L) provided as single substrate. Equimolar n-hexadecane: naphthalene, equimolar n-hexadecane : 1-methyl-naphthalene and n-hexadecane: pyrene at a molar ratio of 5:1 were applied as binary substrates and diesel and lubricating oil (1% v/v) were applied as complex petroleum hydrocarbon mixtures. The batch cultures with single substrate, binary substrate and complex mixtures were incubated for 15 days and enhancement in turbidity was used as an indication of culture growth. Subsequently, experiments were conducted with a model non-aqueous phase liquid (NAPL) composed of petroleum hydrocarbons belonging to different group types, i.e., 3 aliphatic compounds, n-hexadecane, n-octadecane and n-nonadecane; 2 polyaromatic hydrocarbons (PAHs), naphthalene and pyrene; and 1 substituted PAH, 1-methylnaphthalene. The naphthalene: n-hexadecane molar ratio was maintained as 1:3 in order to represent the aromatic: aliphatic ratio typically present in a complex mixture such as diesel. Based on the fugacity ratios of the compounds, the design mole fraction of each component was chosen and subsequently the corresponding mass fraction and mass of each component to be added was determined. The model NAPL had the following composition (mole fraction), n-hexadecane (0.375); n-octadecane (0.09); n-nonadecane (0.11); naphthalene (0.125); 1-methylnaphthalene (0.25); and pyrene (0.05). The solid components were added to the liquid components and heated to 100°C for 30 minutes to obtain a uniform homogenous liquid mixture.

Systematic studies were designed to assess the effect of three non-ionic chemical surfactants namely, Triton X-100, Igepal CA 630 and Tween 80 on the ability of *B. cepacia* (ES1) to degrade the model NAPL over a period of 60 hrs. Following this, the surfactant facilitating maximum degradation was selected for growth study over a period of 168 hrs. A degradation rate study with the selected surfactant was also conducted in multiple batch flasks and the residual NAPL was estimated at various time intervals over a period of 168 hrs. An independent set of experiments without any surfactant was also conducted.

All the batch experiments were conducted in Erlenmeyer flasks with 50 ml
mineral media, 0.1% (v/v) model NAPL, surfactants at a dose twice the critical micelle concentration (CMC) and 1% (v/v) inoculum having an optical density of unity. The CMC of Triton X-100, Igepal CA 630 and Tween 80 are 0.24mM, 0.083mM, 0.012mM respectively. For the growth studies, the biomass was estimated at different time intervals and a specific protocol was adopted to avoid interferences in estimation due to emulsification. The biomass was harvested from the culture broth (10 ml) and resuspended in an equal volume of phosphate buffer. While harvesting, the supernatant was carefully removed to ensure that no hydrocarbons were remaining in the centrifuge tubes. The absorbance of the culture suspension was measured in a spectrophotometer at a wavelength of 600 nm to estimate the biomass content. Degradation of the model NAPL was assessed by estimating the residual mass of NAPL after a specified time interval. Residual mass of NAPL was estimated by extracting the NAPL from the mineral media by liquid- liquid extraction using dichloromethane at an extraction ratio of 1:1. Internal standard 5 α-androstane (40 mg/L) was added to the extract for sample preparation for gas chromatography (GC). The samples (1μL) were analyzed in a GC (Clarus 500, Perkin Elmer) equipped with a flame ionization detector using Elite-5 capillary column and a temperature programme with total run time of 29.75 minutes. 

RESULTS AND DISCUSSION

As illustrated in Table 1, B.cepacia (ES1) only demonstrated growth on n-alkane and n-alkene but could not grow on aromatics present as sole substrate. Inspite of its ability to degrade n-hexadecane, degradation of n-hexadecane was hindered in presence of equimolar naphthalene although 1-methylnaphthalene and pyrene did not cause such a growth inhibitory effect in binary combinations. Contrasting results have been observed by Kim et al. (2003) where B.cepacia 2A-12 utilized naphthalene as sole carbon source and pyrene in the presence of yeast extract as co substrate. Thus, growth inhibition to naphthalene is dependent on the strain of B.cepacia used.

Table 1: Substrate utilization matrix for Burkholderia cepacia (ES1)

<table>
<thead>
<tr>
<th>Substrate Type</th>
<th>Representative group type</th>
<th>Substrate</th>
<th>Growth in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single substrate</td>
<td>n-alkanes</td>
<td>n-Hexadecane</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>n-alkene</td>
<td>Octadecene</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Cycloalkane</td>
<td>Cyclohexane</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Polyaromatic hydrocarbon</td>
<td>Naphthalene</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Polar Aromatic</td>
<td>Phthalic Acid</td>
<td>-</td>
</tr>
<tr>
<td>Binary substrates</td>
<td>Aliphatic + Aromatic</td>
<td>n-Hexadecane + Naphthalene (molar ratio = 1:1)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Aliphatic + Substituted polyaromatic hydrocarbon</td>
<td>n-Hexadecane + 1-methylnaphthalene (molar ratio = 1:1)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Aliphatic + High molecular weight</td>
<td>n-Hexadecane + Pyrene (molar ratio = 5:1)</td>
<td>+</td>
</tr>
<tr>
<td>Complex mixture</td>
<td>Polyaromatic hydrocarbon</td>
<td>Diesel Oil</td>
<td>+</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------</td>
<td>------------</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Aliphatic + Aromatic +</td>
<td>Lubricating Oil</td>
<td>+</td>
</tr>
<tr>
<td>Polar substitutes</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ Indicates ability to grow on the substrate under consideration
– Indicates inability to grow on the substrate under consideration (over 15 days)

Of the nonionic surfactants chosen in this study, Triton X-100 and Igepal CA 630 are both structurally similar polyethoxylates with Triton X-100 being slightly more hydrophilic than Igepal CA 630 (ethylene oxide (EO) units, 9.5 and 9 respectively). In contrast Tween 80 is structurally different (sorbitan oleate) and is highly hydrophilic (EO unit = 20). Studies on degradation of model NAPL in the presence of these nonionic surfactants suggested that all the surfactants enhanced the degradation of model NAPL. The enhancement in degradation was highest in the presence of Triton X-100. The overall loss of model NAPL over 60 hrs showed the following decreasing trend, i.e., 82.7%<62.2%<37.2%<33.8% for Triton X-100<Igepal CA 630<Tween 80<no surfactant, respectively (Figure 1) while losses in the controls (extraction and abiotic losses) were 20.1%, 16%, 17.4% and 15% in the presence of Triton X-100, Igepal CA 630, Tween 80 and no surfactant, respectively. While Triton X-100 is observed to cause enhancement in NAPL degradation for *B.cepacia* (ES1), it is also known to inhibit degradation of phenanthrene by *Bacillus* sps. B- UM" and degradation of fluoranthene by *Mycobacterium* sps. and *Sphingomonas* sps. In contrast, Tween 80 while demonstrated only a marginal increase in degradation of the model NAPL by *B.cepacia* (ES1) has been reported to significantly enhance degradation of fluoranthene by *Sphingomonas* sp. Thus, the effect of a particular surfactant on degradation can vary depending on the microorganism used. It has also been reported that the bioavailability of a hydrocarbon trapped in surfactant micelles varies depending on the surfactant used. Moreover, different surfactants can act by different mechanism. The most common mechanisms are pseudosolubilization in surfactant micelles and emulsification of NAPLs by surfactants. Thus, an important criterion for successful surfactant aided bioremediation of hydrocarbon contaminated site is selection of appropriate surfactant-microorganism combination.

Component-wise degradation of the model NAPL, indicated that *B.cepacia* (ES1) was capable of utilizing naphthalene and 1-methylnaphthalene from the NAPL phase (Fig. 1) while it was unable to utilize naphthalene as sole source of carbon and energy from the aqueous phase when applied either as a single substrate or as binary mixtures. Typically, most cultures utilize naphthalene subsequent to its dissolution into the aqueous phase; hence, bioavailability limitations cannot explain the inability of the cultures to utilize naphthalene when present in the form of crystals. However, Mohanty and Mukherji (2008), indicated that *B.cepacia* (ES1) takes up NAPLs by direct uptake. Thus, direct uptake could have facilitated the utilization of naphthalene from NAPLs although its utilization subsequent to dissolution cannot be ruled out. It is also
possible that naphthalene at its aqueous solubility limit is toxic to B. cepacia (ES1), whereas when present in the model NAPL ($\chi = 0.125 < 0.287$) the aqueous solubility of naphthalene is reduced, such that the toxic effect is lower. In the absence of any surfactant, the growth of the organism within 60 hrs was due to the utilization of naphthalene and 1-methylnaphthalene as is evidenced by low overall loss of n-hexadecane (8.2%), n-octadecane (10.1%) and n-nonadecane (14.1%) and pyrene (39.3%) comparable to the controls. The presence of surfactants facilitated the utilization of aliphatic components present in the model NAPL and there was a maximum increase in the overall loss (%) of n-hexadecane (79.5%), n-octadecane (84%) and n-nonadecane (82%) in the presence of Triton X-100. Thus, the enhancement in overall degradation of the model NAPL by surfactants is primarily due to the enhancement in utilization of the aliphatic components (Figure 1). In the absence of surfactant, B. cepacia (ES1) could utilize n-alkanes present as sole carbon source at an initial concentration of 1%, however, due to the significantly lower interfacial area in the studies with 0.1% model NAPL, significant degradation of n-alkanes was not observed. Thus, the substrate utilization pattern of an organism is largely dependent on the initial concentration at which the substrate is applied. Prediction of degradation of complex mixtures cannot be done based on single substrate utilization pattern as the scenario may change completely when the substrate exists as a component in a complex non-aqueous phase liquid. Based on these results, Triton X-100 was selected for further studies.

Growth studies revealed that presence of the surfactant Triton X-100 (2 CMC), enhanced the growth rate of B. cepacia (ES1). The end of log growth phase was reached within a period of 67 hrs. Conversely, in the absence of any surfactant, growth was slow and an extended stationary phase was observed (Fig. 2).

Degradation rate studies revealed a sequential pattern of utilization of aromatics followed by aliphatics upon addition of surfactant Triton X-100. Surfactants are known to increase the rate of dissolution of PAHs. Hence, the rate of degradation of naphthalene was increased and it was completely depleted within 12 hrs followed by 1-methylnaphthalene between 12-36 hrs. Subsequently, Triton X-100 induced the uptake of the aliphatic components completely within 36–168 hrs. Mohanty and Mukherji (2007) attributed the enhanced uptake of n-alkanes from diesel oil in presence of Triton X-100 to emulsification of diesel. A similar mechanism could have enhanced degradation of aliphatics from the model NAPL. Pyrene remained unutilized in both the cases. The loss of pyrene is attributed to extraction losses and abiotic losses. Mass balance at 168 hr depicted that the biotic loss increased from 26.51% to 74.44% after addition of Triton X-100 (Table 2). The extent of degradation of model NAPL was low within 60 hrs and it was almost completely depleted within 168 hrs suggesting that complex mixtures with high aliphatic content would require sufficient time for complete depletion.
Fig. 1: Component wise overall loss of model NAPL by \textit{B. cepacia} (ES1) in the presence and absence of surfactants in 60 hrs: (a) in control set up; (b) in experimental set up.
Table 2: Mass balance of model NAPL after degradation by *B. cepacia* (ES1) over 168 hrs in the absence and presence of Triton X-100 at 2CMC

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Triton X-100 (0 CMC)</th>
<th>Triton X-100 (2 CMC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R (%)</td>
<td>E (%)</td>
</tr>
<tr>
<td>0</td>
<td>82.7</td>
<td>12.8</td>
</tr>
<tr>
<td>36</td>
<td>58.3</td>
<td>12.8</td>
</tr>
<tr>
<td>60</td>
<td>61.5</td>
<td>12.8</td>
</tr>
<tr>
<td>168</td>
<td>56.2</td>
<td>12.8</td>
</tr>
</tbody>
</table>

CONCLUSION

In the present study, nonionic chemical surfactants were found to be useful for enhancement of biodegradation of a model NAPL by *B. cepacia* (ES1). The model NAPL represented environmental mixtures such as diesel which have a relatively high aliphatic fraction. A compositionally defined model NAPL was used for elucidation of surfactant aided biodegradation to obtain appropriate quantitative estimates. Such estimates cannot be obtained using environmental complex mixtures such as diesel with undefined composition. Enhancement in degradation was due to induction of utilization of aliphatic components in the presence of surfactants and this was highest for Triton X-100. The substrate utilization pattern determined using pure/ binary substrate was found to be
different for the model NAPL. Hence, prediction of consequences of surfactant aided biodegradation in the environment needs to be made based on studies conducted with multicomponent mixtures. Thus, from the studies reported here it can be concluded that *Burkholderia cepacia* (ES1) can be used for surfactant aided biodegradation of complex NAPLs with high aliphatic content using nonionic chemical surfactants.

**ACKNOWLEDGEMENT**

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**REFERENCES**


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