Enhancement of NAPL bioavailability by induction of cell-surface hydrophobicity in *Exiguobacterium aurantiacum* and *Burkholderia cepacia*

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Induction of cell surface hydrophobicity in bacterial cultures can facilitate the direct interfacial uptake of non-aqueous phase liquids (NAPLs). This study explores bioavailability of NAPLs for *Exiguobacterium aurantiacum* and *Burkholderia cepacia* isolated from oil-contaminated soil and sediments. Surface tension measurements and emulsification activity tests did not provide evidence for release of extracellular biosurfactants/bioemulsifiers. Contact angle measurement on cell layers and bacterial adhesion to hydrocarbon (BATH) assay was conducted for determining the cell surface hydrophobicity. While the surfaces of cultures grown on soluble substrate, dextrose, were not hydrophobic, higher water contact angle and greater adherence to n-hexadecane/diesel revealed the highly hydrophobic nature of the cell surfaces for cultures grown on NAPLs (n-hexadecane and diesel), thus providing evidence for induction in cell surface hydrophobicity. Positive results in the carboxyanine assay indicating release of lipopolysaccharides/extracellular polysaccharides was observed over the log growth phase only for the NAPL-grown cells. Transmission electron microscopy (TEM) revealed an abundance of intracellular electron transparent globules within the NAPL-grown cells. Light microscopy and TEM images together revealed differences in cell surface characteristics of *E. aurantiacum* and *B. cepacia*, which was also affected by the growth substrate.

**Keywords:** Aliphatic hydrocarbons, biodegradation, LPS, oil, TEM, uptake mechanism

**Introduction**

Petroleum hydrocarbons are characterized by low aqueous solubility and typically exist as components of complex non-aqueous phase liquids (NAPLs), such as oil and tar. Seas and oceans are often polluted with petroleum hydrocarbons due to massive oil spills during transportation of oil, oil drilling activities, seepage and run-off of wastewater. Subsequent to volatilization, dispersed oil contamination in seas and beaches is primarily reduced by intrinsic or engineered bioremediation processes, where microorganisms utilize oil as their sole source of carbon and energy. In such scenarios, bioavailability is one of the most critical factors governing the fate and persistence of NAPLs. Microorganisms that use hydrocarbons as the sole source of carbon and energy, overcome the bioavailability limitations, either through solubilization or emulsification of oil or by adhering and uptaking oil directly from the oil-water interface. Biodegradation of NAPLs has been studied extensively for cultures producing extracellular biosurfactants and bioemulsifiers. In contrast, NAPL biodegradation by bacterial cultures capable of direct interfacial uptake have not been studied extensively. The presence of extracellular biosurfactants is manifested as lowering in surface tension of the culture medium in the range of 27-36 mN/m. Some bacteria produce extracellular bioemulsifiers, which forms minute hydrocarbon droplets and significantly increases the interfacial area for mass transfer.

Direct interfacial uptake of aliphatic hydrocarbons facilitated by hydrophobic cell surfaces have been reported for several bacteria belonging to the genus *Acinetobacter*, *Rhodococcus* and *Pseudomonas*. Microorganisms capable of changing the structure of their outer membrane and enhancing cell surface hydrophobicity to facilitate direct uptake of oil/hydrocarbons may employ various mechanisms: changes in extracellular polymeric substances (EPS), accumulation of lipopolysaccharides (LPS)/nonionic biosurfactants on the cell wall and release/structural modification in LPS present in the outer membrane. A *Rhodococcus* strain depicted enhanced cell surface hydrophobicity when the growth substrate was changed from glucose-acetate to diesel. The EPS produced by this strain was a complex mixture of glycoconjugates and their composition was specific to the growth substrate and growth temperature. In *P. aeruginosa* loss of LPS

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and loss of the O-antigen [containing 2-keto 3-deoxyoctononic acid (KDO)] from LPS was found to increase the cell surface hydrophobicity\textsuperscript{15,16}. The release of LPS and O-antigen was induced by addition of low concentration of rhamnolipids and by growth on n-alkanes. In \textit{Acinetobacter} strains alkane uptake and cell surface hydrophobicity is linked with extracellular release of membrane bound vesicles, while reduction of cell surface hydrophobicity in the stationary phase is linked with release of bioemulsifiers, such as emulsan, comprised of capsular heteropolysaccharides\textsuperscript{17}.

Fuel oil/n-alkane degradation and uptake mechanism by \textit{Exiguobacterium} strains has not been reported in the literature, although they are commonly reported to withstand extreme environmental stresses\textsuperscript{18}. Based on phylogenetic analysis, Dojka \textit{et al}\textsuperscript{19} reported the presence of \textit{E. aurantiacum} in the 94 membered soil bacterial community in an aquifer undergoing intrinsic bioremediation. This aquifer in Michigan (USA) was contaminated with jet-fuel and chlorinated hydrocarbons. Yoshida \textit{et al}\textsuperscript{20} reported \textit{Burkholderia cepacia} as one of the most predominant culturable species in crude oil. \textit{Burkholderia} strains have been reported for their ability to degrade both aliphatic and aromatic hydrocarbons\textsuperscript{21,22}, however, the uptake mechanisms employed/bioavailability aspects have not been studied specifically.

This paper explores the NAPL uptake mechanism employed by two naturally occurring bacterial strains identified as \textit{E. aurantiacum} and \textit{B. cepacia} isolated from oil contaminated soil and sediments. The capability of these cultures to grow on and degrade n-hexadecane and diesel oil as sole source of carbon and energy was demonstrated. Subsequent studies were aimed at elucidating the hydrocarbon uptake mechanism. Changes in cell surface hydrophobicity with change in growth substrate was measured using the BATH assay and contact angle measurements to compare conformity between the two assays and to specifically understand the changes induced by growth on hydrocarbon.

**Materials and Methods**

**Culture Isolation, Growth and Identification**

Diesel obtained from a re-fuelling station (Mumbai, India) was stored in air-tight containers and was used as received. The cultures were enriched and isolated using diesel as the sole substrate. The AS1 culture was isolated from soil contaminated with diesel oil obtained from a tanker re-fuelling station (International Airport, Mumbai, India). The ES1 culture was isolated from Arabian Sea sediment obtained from the vicinity of an oil field in the Bombay High region (Mumbai, India). Method for culture enrichment using diesel oil was reported by Mukherji \textit{et al}\textsuperscript{23}. After multiple transfers in diesel oil, these cultures consisted of uniform colony types when observed in mineral media plates with diesel as substrate. For isolation, one colony was picked up and grown in diesel through multiple transfers and was subsequently identified. Culture purity was assessed microscopically and by streaking on plates. After obtaining an axenic culture, it was identified by sequencing of the 16S rDNA gene (Bangalore Genei, Bangalore, India) The resultant sequence was matched with the Genebank database using the BLAST based homology analysis protocol. The isolated cultures were maintained using diesel as the sole substrate through successive transfers over several years.

Growth studies using NAPL substrates were conducted in 500 mL conical flasks containing 100 mL of mineral medium, 1 mL NAPL (n-hexadecane/diesel oil) and 1 mL inoculum from late log-growth phase. The primary constituents in the mineral medium were as specified by Mukherji \textit{et al}\textsuperscript{23} (conc. in mg/L): KH\textsubscript{2}PO\textsubscript{4} (170), K\textsubscript{2}HPO\textsubscript{4} (435), Na\textsubscript{2}HPO\textsubscript{4}.7H\textsubscript{2}O (668), NH\textsubscript{4}Cl (850), MgSO\textsubscript{4}.7H\textsubscript{2}O (22.5), CaCl\textsubscript{2}.2H\textsubscript{2}O (27.5), and FeCl\textsubscript{3} (0.25). The medium was also supplemented with trace nutrients (conc. in µg/L): CoCl\textsubscript{2} (38.2); H\textsubscript{2}BO\textsubscript{3} (61.8); Na\textsubscript{2}MoO\textsubscript{4} (25.4); CuCl\textsubscript{2}.2H\textsubscript{2}O (39.2); ZnCl\textsubscript{2} (136.3); NiCl\textsubscript{2} (13); FeCl\textsubscript{3}.4H\textsubscript{2}O (701.6); MnCl\textsubscript{2}.4H\textsubscript{2}O (280.7); and Na\textsubscript{2}SO\textsubscript{4} (14.2). Growth of the microorganisms was observed as increase in absorbance (at 600 nm) of the culture broth over time, which was converted to viable counts using predetermined culture specific calibration curves\textsuperscript{24}. The viable count of diesel grown cultures was determined using the five tube MPN technique as reported by Biswas \textit{et al}\textsuperscript{25}. The dextrose grown cultures were grown on the same mineral media by replacing 1% NAPL (v/v) with 1% dextrose (w/v). The differential impact of various concentrations of EDTA on growth of the two bacterial cultures was observed using 1% of diesel (v/v)/dextrose(w/v) as growth substrate. EDTA concentration was varied over the range of 2 to 8 mM by supplementing the growth medium before autoclaving. In experiments with dextrose grown cultures, additional CaCl\textsubscript{2} and MgSO\textsubscript{4} were added after 109 h. EDTA is an effective complexing agent.
that reacts with divalent metal ions. The toxic effect of EDTA may, thus, be due to unavailability of chelated metal ions for bacterial growth or due to a direct toxic effect of EDTA on the cultures. To distinguish between these two mechanisms of growth inhibition, the medium was supplemented with additional Ca\textsuperscript{2+} and Mg\textsuperscript{2+} ions.

**Microscopic Observations**

All microscopic observations were made with harvested and washed cells re-suspended in buffer. Gram staining was performed with standard reagents and negative staining for capsule was performed against a nigrosin background. Stained cells were observed using a Zeiss Axiostar Plus microscope equipped with optical image analysis software at an overall magnification of 1000× (Zeiss, Germany, objective 100× and eyepiece 10×). TEM observations of both the cultures grown on various substrates were made directly on cells harvested at the end of log growth phase. The harvested cells were washed twice and re-suspended in phosphate buffer. The phosphate buffer consisted of (conc. in mg/L): KH\textsubscript{2}PO\textsubscript{4} (170), K\textsubscript{2}HPO\textsubscript{4} (435) and Na\textsubscript{2}HPO\textsubscript{4}.7H\textsubscript{2}O (668). An aliquot (5-10 µL) of the cell suspension was taken on a carbon coated copper grid of 200 mesh size, air-dried for a few min and the sample containing grid was subsequently observed in the TEM (FEI TECNAI G2 12, Netherlands) with an acceleration voltage of 120 kV. Samples were observed at room temperature.

**Extracellular Biosurfactant/Bioemulsifier Activity and Cell Surface Hydrophobicity**

To avoid interferences in determination of biosurfactant and emulsification activity, the residual oil was first extracted using n-hexane and subsequently the culture filtrate was obtained by membrane filtration. External biosurfactant activity was determined by measuring the surface tension of the culture filtrate and its dilutions using a DuNoy ring tensiometer equipped with a 4 cm platinum ring. The emulsification activity of the culture filtrate was measured following the method outlined by Das et al\textsuperscript{[26]}. Sterile diesel (2 mL) and equal volume of culture filtrate were vortexed for 2 min. Subsequently, the height of emulsion in the tube was noted after 1 min and 48 h.

The BATH assay (adapted from Rosenberg et al\textsuperscript{[11]}) and contact angle measurement were performed with cells in the early stationary phase for cultures grown on various substrates. The generation time and duration of log growth phase of the cultures grown on dextrose was significantly shorter than for those grown on NAPL substrates. For the hydrocarbon grown cultures and dextrose grown cultures, the measurements were performed with cells harvested on the 2\textsuperscript{nd} and 10\textsuperscript{th} d of incubation, respectively, so as to represent the early stationary phase. Contact angle measurement was also performed with late stationary phase NAPL grown cultures harvested at 15\textsuperscript{th} d. The harvested and washed cell pellet (15500 rpm for 45 min) was resuspended to obtain an uniform cell suspension with absorbance value adjusted to unity. For the BATH assay, equal volumes of the cell suspension (5 mL) were distributed into multiple tubes containing varying volumes (0-0.5 mL) of a NAPL (n-hexadecane/diesel). The phases were contacted in a vortex mixer set at 2000 rpm for 2 min. After allowing the phases to separate over a 15 min time interval, the absorbance of the lower aqueous phase of each tube was determined at 600 nm. Adherence was calculated as change in absorbance relative to that of the control devoid of NAPL. For the contact angle measurements, fixed volume of the cell suspension adjusted to unit absorbance, was filtered through a 0.45 µm cellulose acetate membrane (Millipore). To standardize the moisture content, the membrane filter with the bacterial cell mat was stored in a Petri plate on the surface of an agar (1% wt/v)–glycerol (10% v/v) layer for up to 3 h\textsuperscript{[27]}. A strip was cut from the filter paper containing the bacterial cell layer, fixed onto a glass slide with double sided adhesive tape and were allowed to air dry for 30-90 min as suggested by Busscher et al\textsuperscript{[28]}. The measurements were performed using Digidrop (GBX instruments, France) within 1-2 sec after depositing a drop of water (polar liquid)/α-bromonaphthalene (apolar liquid). The contact angle between the film surface and the tangent to the drop at the solid-liquid-air meeting point was measured at different locations on the cell layer (5-12 replicates were obtained).

**Carbocyanine Colorimetric Assay**

The carbocyanine colorimetric assay\textsuperscript{[29]} was conducted to obtain a quantitative estimate of lipopolysaccharide (LPS) content in the culture broth over the active growth phase. LPS obtained from P. aeruginosa (Sigma-Aldrich) was used to prepare a standard curve over the range 1-50 µg/mL. The carbocyanine dye reagent was prepared using 10 mg of the dye in a 20 mL mixture comprised of equal parts of 1,4-dioxane and 0.03 M sodium acetate buffer.
(pH 4) and the final volume was made up to 100 mL with the buffer. For the estimation of LPS, 0.5 mL of sample containing LPS was added to 0.3 mL of dye reagent, 0.2 mL buffer (0.03 M) and 0.2 mL ascorbic acid (0.1 M), and the mixture was kept at 4°C for 5 to 10 min in the dark. A spectral shift was observed due to LPS and the absorbance was measured in a UV/VIS spectrophotometer at 472 nm (Jasco-V-530, Japan).

2-Keto 3-deoxy octonic Acid (KDO) Assay
KDO release into the supernatant was measured on the basis of absorbance at 548 nm using a spectrophotometer (Spectronic 20, Genesys, USA) as per the thiobarbituric acid assay. A calibration curve was prepared using KDO standards derived from P. aeruginosa, serotype 10 (Sigma-Aldrich) over the conc. range 1-30 µg/mL. At less than 1 µg/mL KDO, no detectable change in absorbance with respect to the blank was observed.

Results
Culture Identification and Morphology
Gram staining and microscopic observations revealed the ES1 culture as Gram-negative and rod shaped, while the AS1 culture was also rod shaped but Gram-positive. Negative staining with nigrosin revealed the presence of a colourless capsule surrounding the diesel grown AS1 culture only, while no capsule was observed for AS1 cultures grown on n-hexadecane or dextrose. No capsule was observed for the ES1 culture irrespective of growth substrate. Searches in the GenBank database using the sequence data determined for the 16S rDNA gene of the AS1 isolate resulted in 99.3% match with Burkholderia cepacia LMG 12614T. The sequence data determined for the 16S rDNA gene of the ES1 isolate resulted in 99.6% match with Exiguobacterium aurantiacum NCDO 2321 (T). The similarity of the 16S rDNA gene sequences of both isolates suggested that they belong to the same species. Thus, the isolates were identified as B. cepacia and E. aurantiacum, respectively. For identification of the culture putatively identified as E. aurantiacum on the basis of sequence data, 16S rDNA sequencing was carried out. The sequence data determined for the 16S rDNA gene of the ES1 isolate resulted in 99.6% match with Exiguobacterium aurantiacum NCDO 2321 (T). The similarity of the 16S rDNA gene sequences of both isolates suggested that they belong to the same species. Thus, the isolates were identified as B. cepacia and E. aurantiacum, respectively.

TEM Images Illustrating Effect of Carbon Source
There was good match between observations in cell shape, relative size and cellular aggregation pattern observed in the images obtained in the light microscope and in TEM images. TEM images revealed distinct changes, such as, variation in cell size, cell surface characteristics and intracellular appearance induced by the various growth substrates, viz. dextrose, n-hexadecane and diesel oil in both the cultures. Table 1 summarizes the dimensions of E. aurantiacum and B. cepacia cultures grown on various substrates. They represent average cell size based on cell dimension observed in multiple frames. It was possible that the stress imposed during the process of drying samples on the grids and viewing them under vacuum may had caused some variation in cell size. However, all cells irrespective of growth substrate were likely to be equally affected by this stress. The growth substrate induced size variations were also apparent based on observations in the light microscope.

The sizes of the individual cells were comparatively smaller, when grown on diesel, in comparison to dextrose. However, n-hexadecane grown cells of E. aurantiacum were found to be much larger than the corresponding dextrose grown cells. For B. cepacia culture the n-hexadecane and dextrose grown cells were almost comparable in size. The dextrose grown E. aurantiacum cell was 1.64 µm in length and 0.56 µm in width. In diesel grown E. aurantiacum cells, the reduction in length and width was found to be 31 and 19%, respectively in comparison to the dextrose grown. The n-hexadecane grown E. aurantiacum cell was 4.91 µm in length and 1.41 µm in width, i.e., 3 times larger and 2.5 times wider compared to the corresponding dextrose grown cells. The dextrose grown B. cepacia cell was 2.51 µm in length and 0.571 µm in width. For diesel grown B. cepacia culture, two different types of cells were observed, long-thin and short-thick cells. Since care was taken to maintain axenic cultures, these possibly represent two distinct phenotypes. For the long and thin phenotype, the reduction in length was found to be 32%, and reduction in width was found to be 59%, in comparison to the dextrose grown B. cepacia cells. For

<table>
<thead>
<tr>
<th>Culture</th>
<th>Growth substrate</th>
<th>Mean (SE) Length</th>
<th>Mean (SE) Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. aurantiacum</td>
<td>Dextrose</td>
<td>1.64 (0.07)</td>
<td>0.56 (0.01)</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>n-Hexadecane</td>
<td>4.99 (0.31)</td>
<td>1.44 (0.13)</td>
</tr>
<tr>
<td></td>
<td>Diesel</td>
<td>1.14 (0.14)</td>
<td>0.46 (0.05)</td>
</tr>
<tr>
<td></td>
<td>Dextrose</td>
<td>2.56 (0.15)</td>
<td>0.99 (0.14)</td>
</tr>
<tr>
<td></td>
<td>n-Hexadecane</td>
<td>2.30 (0.07)</td>
<td>0.46 (0.09)</td>
</tr>
<tr>
<td></td>
<td>Diesel: long and thin</td>
<td>1.73 (0.04)</td>
<td>0.40 (0.04)</td>
</tr>
<tr>
<td></td>
<td>Diesel: short and thick</td>
<td>1.22 (0.02)</td>
<td>0.57 (0.02)</td>
</tr>
</tbody>
</table>

SE = Standard Error
the short and thick phenotype, reduction in length was found to be 52% and reduction in width was found to be 43%, in comparison to the dextrose grown *B. cepacia* cells. The n-hexadecane-grown *B. cepacia* cells were of similar dimension as the dextrose grown cells.

Figs 1 and 2 show TEM image of *E. aurantiacum* and *B. cepacia* cultures, respectively, grown on various growth substrates. The zone of lower electron density (about 70 nm thick) surrounding the diesel grown *E. aurantiacum* cell (Fig. 1f) possibly represents the EPS capsule, which was observed through negative staining with nigrosin. Such a zone was not observed for the dextrose grown *E. aurantiacum* cells (Fig. 1a), which were lined by a layer of uniform thickness (22 nm). In n-hexadecane grown *E. aurantiacum* cells (Fig. 1b), loosely attached slimes were observed in parts of the cells. The arrangement of cells was also found to vary with the growth substrate. For example, a chain like arrangement, i.e., end to end joining by a slimy material, was observed for dextrose grown *E. aurantiacum* cells (Fig. not shown). Such a chain formation was also observed under light microscope for cells harvested at early stationary phase (48 h). Subsequently, segregation of cells was observed for cells harvested in the late stationary phase. In contrast, n-hexadecane and diesel grown *E. aurantiacum* cells were observed to form clumps (Figs not shown). The *B. cepacia* cells were found to form clumps and were surrounded by a loosely bound slime layer of EPS irrespective of growth substrate (Figs 2a-c). It may be noted that *B. cepacia* cells irrespective of growth substrate did not reveal presence of capsule in negative staining with nigrosin. Similar clumping of cells through EPS was demonstrated in TEM images of oil degrading bacterial cultures forming association with clay materials.30

A large number of electron transparent irregular-shaped globules possibly composed of lipids and unmodified hydrocarbon substrates were observed within the diesel grown cells of *E. aurantiacum*.

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**Fig. 1**—TEM images of rod shaped *E. aurantiacum* cells grown on various substrates: (a) single cell grown on dextrose, (b) single cell grown on n-hexadecane, (c) intracellular globular agglomerates within a cell grown on n-hexadecane, (d) a large solitary intracellular globular agglomerate within a cell grown on n-hexadecane, (e) a magnified view of d, (f) single cell grown on diesel surrounded by EPS capsule, and (g) single cell grown on diesel demonstrating numerous intracellular electron transparent globules; scale bar in a, c, e, f, g is 200 nm and scale bar in b and d is 1000 nm and 500 nm, respectively.

**Fig. 2**—TEM images of rod shaped *B. cepacia* cells grown on various substrates: (a) two cells grown on dextrose embedded in a slimy layer, (b) a single cell grown on n-hexadecane surrounded by a slime layer, (c) cells grown on diesel demonstrating two different phenotype, and (d) short and thick phenotype of diesel-grown cells demonstrating numerous intracellular electron transparent globules; scale bar in a and c is 1000 nm, and that in b and d is 500 nm and 200 nm, respectively.
(Figs 1f & g) and *B. cepacia* (Fig. 2d). In dextrose grown *E. aurantiacum* and *B. cepacia* cells, these electron transparent globules were not observed. Among the two different types of cells, observed in diesel grown *B. cepacia* culture, the long and thin cells had less electron transparent globules in comparison to the shorter and thicker *B. cepacia* cells. Presence of intracellular globules was also observed for n-hexadecane grown *E. aurantiacum* (Figs 1b-e) and *B. cepacia* culture (Fig. not shown). However, the appearances of the intracellular globules were distinctly different for n-hexadecane and diesel grown *E. aurantiacum* culture. The intracellular globules in diesel grown *E. aurantiacum* cells were more numerous, irregular in shape and consists of a single globule as depicted in Figs 1f and g. In contrast, the n-hexadecane grown cells contained a fewer number of globular agglomerates of definite shape. Each of these agglomerates appears to consist of an agglomeration of smaller globules bounded by a membrane as depicted in Figs 1b-e. In some cells, only one large globular agglomerate was observed compared to multiple smaller agglomerates. Intracellular inclusions in the n-hexadecane grown *B. cepacia* culture consisted of single globules as for the diesel grown cells.

**Culture Growth and LPS/EPS Release Characteristics**

Release of LPS and other acidic extracellular polysaccharides can cause spectral shift in the carbocyanine assay with a change in $\lambda_{\text{max}}$ from 520 nm to 472 nm. The spectral shift observed with culture growth is quantified using LPS standards from *P. aeruginosa*. Figs 3a and b depict growth on n-hexadecane and diesel oil and LPS/EPS release characteristics for Gram-negative *B. cepacia* culture and Gram-positive *E. aurantiacum* culture, respectively. Growth of the microorganisms observed as increase in absorbance at 600 nm was converted to viable count (MPN/mL) based on predetermined calibration curves. Both the cultures could effectively use n-hexadecane and diesel as sole source of carbon and energy. Significant variability was observed between replicate growth curves. In general, a lag phase of 1-2 d was observed. Duration of the lag phase was affected by the age and number of microorganisms in the inoculating culture. For growth on diesel, the log phase lasted for nearly 3-4 d and, beyond the lag phase, the generation time observed was in the range of 0.25-0.3 d for the *B. cepacia* culture and 0.2-0.5 d for the *E. aurantiacum* culture. On n-hexadecane, the *B. cepacia* culture depicted a generation time of 0.2-0.3 d, while the *E. aurantiacum* culture depicted a higher generation time in the range of 0.4-0.7 d. Thus, the *B. cepacia* culture depicts marginally better growth characteristics compared to the *E. aurantiacum* culture. Independent studies confirmed that both cultures primarily degraded the aliphatic fraction of diesel. On an average, the increase in MPN count normalized to initial at the end of log growth phase was $3 \times 10^3$ - $2 \times 10^4$ MPN/mL.

For Gram-negative *B. cepacia* culture, LPS released from the outer membrane into the culture media was estimated for cultures grown on various growth substrates. For dextrose grown *B. cepacia* culture, no LPS could be detected in the culture broth. Release of LPS was observed for NAPL grown *B. cepacia* cultures (Fig. 3a) over a period of 2-3 to 8 d, i.e., essentially over the active growth phase. LPS release by n-hexadecane grown *B. cepacia* culture was significantly higher than for the diesel grown *B. cepacia* culture. The KDO assay was also conducted to determine KDO (an LPS specific sugar) release into

**Fig. 3—Growth on NAPL substrates and LPS/EPS release** quantified using LPS standards from *P. aeruginosa* for (a) *B. cepacia* (ES1) and (b) *E. aurantiacum* (AS1) cultures.
the culture broth for *B. cepacia* culture grown on diesel and n-hexadecane; however, the KDO in the supernatant was lower than the detection limit of 1 µg/mL. This may be expected since LPS released into the supernatant is in the range 6.5 to 18.5 µg/mL and KDO content in LPS is typically low, i.e., about 0.9% (for *P. aeruginosa*). However, Al-Tahhan et al.\textsuperscript{16} reported measurable KDO levels in *P. aeruginosa* cultures for lower LPS release compared to that observed in this study, i.e., 7.4 µg/mL of LPS in ATCC 27853 and 8.1 µg/mL of LPS in ATCC 9027. Analysis of cell bound KDO was also attempted; however, very high background interference caused by the lysing buffer prevented quantification.

As expected, spectral shift due to LPS was not detected in the supernatant of Gram-positive *E. aurantiacum* culture grown on dextrose and n-hexadecane. However, spectral shift to 470 nm, as observed with the LPS standards, was also observed in the supernatant of diesel grown *E. aurantiacum* culture (Fig. 3b). This observation is probably due to reaction of the cationic carbocyanine dye with some polyanion, such as, proteins, nucleic acids and acidic polysaccharides. In the LPS molecule, the polysaccharide part contains the polyanion that reacts with the cationic dye, while the lipid part of LPS does not react directly. Thus, for diesel grown *E. aurantiacum* culture, the spectral shift may have been caused by release of extracellular polysaccharides other than lipopolysaccharides. Both the phenomena of EPS release and capsule formation was specific for the diesel grown cells and were not observed for the n-hexadecane grown cells.

**Extracellular Biosurfactant and Bioemulsifier Activities**

Lowering in surface tension of the culture broth is indicative of external biosurfactant activity. As concentration of a surfactant in aqueous media increases the surface tension falls until the minimum value of surface tension is reached at the critical micelle concentration (CMC), i.e., the concentration at which micelles just begin to form. Hydrocarbons are pseudosolubilized within the hydrophobic core of the surfactant micelles. The surface tension of culture filtrate of the *E. aurantiacum* and *B. cepacia* cultures were found to be 51.5 and 49 dynes/cm, respectively. However, even with a 2-fold dilution, the surface tension values increased, indicating negligible concentration of surfactants in the culture filtrate with respect to CMC concentration. Surface tension in controls was observed as 69-71 dynes/cm, similar to that of water. The emulsification activity in the culture filtrate was negligible both for *E. aurantiacum* and *B. cepacia* cultures. Thus, these cultures do not provide evidence for notable extracellular biosurfactant/bioemulsifier activities.

**Cell Surface Hydrophobicity**

BATH assays conducted with both the oil biodegrading bacterial cultures, grown on n-hexadecane, diesel oil and soluble substrate dextrose with two different NAPL types, are interpreted to demonstrate cell surface hydrophobicity of the microbial cultures. Figs 4a and b show the impact of growth substrate on adherence to a NAPL for two types of NAPL, e.g., n-hexadecane and diesel. In addition to BATH assay with n-hexadecane, as proposed by Rosenberg et al.\textsuperscript{11}, BATH assay was also conducted with diesel to see if the same adherence trends were observed for both the NAPLs for cultures grown on various substrates. The adherence values were only reported corresponding to 0.5 mL NAPL volume used in the assay. The data points represent average of duplicates and error bars represent standard error.

Fig. 4a demonstrates that for n-hexadecane grown cultures, adherence to n-hexadecane was higher for the *B. cepacia* cultures, i.e., 89%, than for the *E. aurantiacum* cultures, i.e., 64%. In contrast, Fig. 4b demonstrates that, for diesel grown cultures, adherence to diesel was higher for the *E. aurantiacum*
culture, i.e., 70%, in comparison to 45% for the *B. cepacia* culture. For dextran grown *E. aurantiacum* and *B. cepacia* cultures, adherence to both diesel and n-hexadecane was comparatively negligible in the range of 28-17% and 39-25%, respectively. Thus, adherence to a NAPL, which is often assumed to be a measure of cell-surface hydrophobicity is induced by growth on NAPL substrates.

In general, for the cultures grown on NAPL substrate, the adherence ranged from 31 to 90%. However, for n-hexadecane grown *B. cepacia* culture the adherence was only 17% when BATH assay was performed with diesel. The data reveals that adherence is both a function of the growth substrate and the NAPL type to which adherence is tested in the BATH assay. Figs 4a and b clearly depict that the adherence pattern changes as the NAPL type changes for cultures grown on the same substrate. Hexadecane grown cultures exhibited maximum adherence to n-hexadecane, whereas adherence to n-hexadecane was lower with cultures grown on the other two growth substrates. Similarly, adherence to diesel was the maximum for the diesel grown cultures as compared to cultures grown on the other two growth substrates. Thus, there is good correlation between culture growth on a NAPL and adherence to that specific NAPL, indicating that cell surface hydrophobicity is responsible for enhancing bioavailability for these cultures. Dextran grown cultures demonstrated low to intermediate adherence both with diesel and n-hexadecane, thus, indicating no specific evidence for enhanced cell surface hydrophobic activity.

Contact angle measurement on cell layers also provides a measure of cell surface hydrophobicity. The hydrophobicity ranges defined on the basis of water contact angle is as follows: 30-40° is hydrophilic, 50-60° is moderately hydrophobic and 80-90° is highly hydrophobic. Based on Table 2, the relative trends in hydrophobicity across the cultures grown on various substrates can be summarized for the early stationary phase cultures. The dextran grown cultures depict similar and low values of water contact angle (in the range of 29-31°) and are hydrophilic. The n-hexadecane grown cultures with intermediate water contact angle values (in the range of 50-60°; *B. cepacia* > *E. aurantiacum*) are moderately hydrophobic, and the diesel grown cultures with water contact angle greater than ~75° (*E. aurantiacum* > *B. cepacia*) are most hydrophobic. The contact angle measurement with α-bromonaphthalene on the dextran grown cultures were higher (61-72°), as expected, in comparison to the corresponding NAPL grown cultures (46-65°) based on the difference in polarity between α-bromonaphthalene (apolar and hydrophobic) and water (polar and hydrophilic). This reverse trend in contact angle measured with the apolar liquid α-bromonaphthalene confirms the hydrophobicity classification suggested by the water contact angle ranges. Both the BATH assay and contact angle measurements confirm that the growth substrate affects affinity for adherence to a NAPL and that cell surface hydrophobicity is induced by growth on NAPL substrates. The contact angle measurements based on each growth substrate was very similar for both the cultures although specific and preferential adherence affinity was observed in the BATH assay.

Table 2—Contact angle measurement on cell layers of cultures grown on various substrates EDTA toxicity

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Growth substrate</th>
<th>Early stationary phase (10/2 d)</th>
<th>Late stationary phase (15 d)</th>
<th>Early stationary phase (10/2 d)</th>
<th>Late stationary phase (15 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. aurantiacum</strong></td>
<td>Dextran</td>
<td>29.6 (0.67)</td>
<td>-</td>
<td>61.18 (0.79)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>n-Hexadecane</td>
<td>52.03 (0.98)</td>
<td>50.38 (0.34)</td>
<td>50.49 (0.81)</td>
<td>45.07 (1.26)</td>
</tr>
<tr>
<td></td>
<td>Diesel</td>
<td>82.4 (1.66)</td>
<td>60.55 (1.19)</td>
<td>51.23 (0.52)</td>
<td>50.78 (0.33)</td>
</tr>
<tr>
<td><strong>B. cepacia</strong></td>
<td>Dextran</td>
<td>31.23 (0.69)</td>
<td>-</td>
<td>72.26 (1.35)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>n-Hexadecane</td>
<td>59.17 (0.41)</td>
<td>57.7 (0.56)</td>
<td>65.27 (0.52)</td>
<td>41.79 (0.61)</td>
</tr>
<tr>
<td></td>
<td>Diesel</td>
<td>75.5 (1.40)</td>
<td>64.79 (0.64)</td>
<td>46.72 (0.37)</td>
<td>42.93 (0.62)</td>
</tr>
</tbody>
</table>

SE = Standard Error
lag phase of about 7 h and reached the end of log growth phase at 38 h. Negligible growth was recorded for the cultures containing various concentrations of EDTA in the range 2-8 mM in comparison to culture without EDTA. Similar toxic effect of EDTA was also observed for the dextrose grown

**Fig. 5—Effect of EDTA on growth of** E. aurantiacum (AS1)  
culture grown on various substrates: a) dextrose, and b) diesel

biosurfactant activities. Variation of surface tension values in the range of 27-36 dynes/cm have been reported based on chemical nature of the biosurfactant, i.e., glycolipid, rhamnolipid, sophorose lipid, trehalose lipid, lipopeptide, phospholipids, fatty acid, and the type of microorganism. Even if the observed lowering is due to presence of extracellular biosurfactants, their concentration is lower than the CMC value. Thus, these cultures do not enhance NAPL bioavailability by solubilization or emulsification aided with presence of extracellular biosurfactants or bioemulsifiers. However, low level release of biosurfactant may have facilitated the development of cell surface hydrophobicity as proposed by Al Tahhan et al.

In general, there is good correlation between culture growth, hydrophobicity (contact angle measurement) and adherence affinity (BATH assay) to a NAPL, indicating specific cell surface changes are responsible for enhancing bioavailability for these cultures. Both cultures show good growth on diesel and n-hexadecane although the generation time is higher than for growth on dextrose. Both the cell surface hydrophobicity indicators, BATH assay and contact angle, indicate that the dextrose grown cultures are hydrophilic. Based on water contact angle values, which provide a rigorous measure of hydrophobicity, the surfaces of cultures grown on diesel are highly hydrophobic, whereas the surfaces of cultures grown on n-hexadecane are of intermediate hydrophobicity. BATH assay results demonstrate that diesel and n-hexadecane grown cultures show better adherence to the NAPL used as growth substrate. Several researchers have used BATH assay results with a pure hydrocarbon, n-hexadecane, as a measure of relative hydrophobicity. However, it appears that BATH assay is a measure of specific affinity rather than a measure of hydrophobicity alone. The n-hexadecane grown B. cepacia culture of intermediate hydrophobicity demonstrates much higher adherence to n-hexadecane compared to the adherence to diesel demonstrated by the diesel grown B. cepacia culture of high hydrophobicity. For the E. aurantiacum culture the adherence trend in BATH assay correlates well with the hydrophobicity indicated by the contact angle values, i.e., diesel grown cultures show higher adherence compared to the n-hexadecane grown cultures. Although the BATH assay results and contact angle values does not always correspond, higher cell surface hydrophobicity is clearly indicated for cultures grown on insoluble substrate in comparison to cultures grown on soluble substrate. Cell-surface modifications,
when grown on a NAPL as the sole carbon source, thus, enhance the ability of these cultures to adhere and grow on NAPL substrates.

Several researchers have demonstrated that cultures capable of direct uptake can overcome the bioavailability limitation posed by low dissolution rates of alkanes from oil by induction of cell surface hydrophobicity. High adherence to NAPLs measured in the BATH assay has been correlated with cell-surface hydrophobicity, which is manifested by cultures exhibiting direct uptake of alkanes from oil, although cultures are also reported to adhere to non-growth substrates. Correlation between induced adherence to NAPLs and alkane uptake has been demonstrated for *Pseudomonas, Rhodococcus* and *Acinetobacter* strains. For aliphatic hydrocarbons with negligible aqueous solubility, microbial uptake is necessarily associated with release of biosurfactants/bioemulsifiers or by induction of cell surface hydrophobicity. For aromatic hydrocarbons and polynuclear aromatic hydrocarbons (PAHs) with intermediate aqueous solubility, although adherence to the NAPL-water interface is often reported, it may or may not facilitate the uptake of NAPL constituents. Adherence to NAPLs and biofilm formation at the NAPL-water interface was reported to decrease the biodegradation rate of naphthalene from a synthetic NAPL representative of coal tar.

This study suggests that inducible cell surface hydrophobicity manifested by *E. aurantiacum* and *B. cepacia* cultures facilitate direct interfacial uptake of aliphatic hydrocarbons from diesel oil. Adherence is both a function of the growth substrate and the NAPL type to which adherence is tested in the BATH assay. The adherence pattern changes as the NAPL type changes for cultures grown on the same substrate. Difference in surface charges of diesel and n-hexadecane droplets due to electrostatic interactions may explain the difference in adherence pattern observed in BATH assay with the two NAPLs. n-Hexadecane grown cultures exhibit maximum adherence to n-hexadecane and poor adherence to diesel. Similarly, adherence to diesel is more for the diesel grown cultures. Different adherence trends for the two NAPLs suggest that BATH assay is a measure of adherence affinity rather than a measure of hydrophobicity.

Although, various manifestations indicate that induction of hydrophobicity in NAPL grown cells facilitated bioavailability enhancement in both the cultures, the underlying mechanisms are different for *E. aurantiacum* and *B. cepacia* culture. For *E. aurantiacum* culture, the induction of hydrophobicity is possibly facilitated by release of extracellular polysaccharides. In diesel grown *E. aurantiacum*, presence of capsule and EPS release into the culture broth was observed. This may be partially responsible for the observed increase in hydrophobicity and specific affinity. Whythe *et al.* demonstrated that the nature of EPS varies with the growth substrate glucose–acetate/diesel for *Rhodococcus* strain Q15, exhibiting high cell surface hydrophobicity when grown on diesel. A slime layer of EPS surrounding *B. cepacia* cells was observed irrespective of growth substrates; thus, in this Gram-negative organism the role of EPS in NAPL uptake is not evident. The TEM images of *E. aurantiacum* demonstrate specific changes in surface characteristics of *E. aurantiacum* cells grown on the various substrates, which also affect their aggregation characteristic. EPS with surfactant activity has been reported to facilitate growth of mucoidial *Rhodococcus* strains on crude oil.

For *B. cepacia*, release of LPS was responsible for the enhanced hydrophobicity. Norman *et al.* and Al Tahhan *et al.* also demonstrated a positive correlation between LPS release and cell surface hydrophobicity in Gram-negative bacteria. External addition of rhamnolipid biosurfactant in two *P. aeruginosa* strains grown on glucose and n-hexadecane was found to cause release of LPS from the outer membrane in a dose dependant manner at rhamnolipid concentrations below the CMC. In most of their studies, the cell surface hydrophobicity induced by addition of rhamnolipids dropped rapidly in the stationary phase; however, the LPS released persisted in the culture media. This is in sharp contrast to the results presented in our study. In the carbocyanine assay, the drop in LPS/polysaccharide at the 9th d indicates that no further release occurred beyond this time in the early stationary phase and that the LPS/polysaccharide released over the log growth phase was either disintegrated or was taken up by the cells. In the late stationary phase of diesel grown cultures, some drop in hydrophobicity (contact angle) was observed compared to the early stationary phase and this may have occurred due to uptake of LPS from the culture broth. However, a notable drop in contact angle was not observed for *B. cepacia* culture grown on n-hexadecane, although LPS disappearance from the culture broth was observed beyond 9th d.

In the present study, a significant toxic effect of EDTA was observed on the dextrose grown cultures.
EDTA has been shown to be capable of penetrating biological membranes with the help of divalent cations, like calcium and magnesium, present in the pores/channels in the cell membrane. Upon penetration, the toxic effect of EDTA is possibly due to its ability to bind to intracellular metal ligands. By virtue of enhanced cell surface hydrophobicity, the cultures grown on diesel exhibited significantly lower toxic effect due to reduced penetration of EDTA into the cells. In *B. cepacia*, enhanced hydrophobicity due to release of LPS may have disrupted the Ca$^{++}$ and Mg$^{++}$ channels that are known to facilitate EDTA transfer into the cells.

TEM images of both the cultures clearly demonstrate that diesel grown cells underwent size reduction in comparison to the dextrose grown cells. However, n-hexadecane grown cells of *E. aurantiacum* were found to be much larger than the corresponding dextrose grown cells. For *B. cepacia* culture, the n-hexadecane and dextrose grown cells were almost comparable in size. Reduction in cell length when grown in Bonny Light crude oil in comparison to glucose grown cells, were also reported by Norman et al. for two *P. aeruginosa* isolates. Change of phenotype in response to change in growth media and formation of heterogeneous phenotype population is frequently observed in bacteria. Similar observations were reported for *P. aeruginosa 57RP* grown on n-hexadecane. Intracellular electron transparent inclusions as observed in this study for cultures grown on diesel and n-hexadecane were reported by Radwan, et al. in *Nocardioform* isolates grown on crude oil and by Alvarez et al. for cultures grown on hydrocarbon substrates. These electron transparent inclusions were reported to represent lipids and unmodified hydrocarbon substrates.

The cultures used in the present study may prove beneficial in bioremediation applications. For *E. aurantiacum* and *B. cepacia*, the bioavailability of oil/hydrocarbons is increased by induction of cell-surface hydrophobicity. Cell surface hydrophobicity and adherence affinity to NAPLs exhibited by *B. cepacia* is linked with release of LPS into the culture broth, whereas for *E. aurantiacum*, these manifestations are linked with capsule formation and secretion of EPS. Growth on NAPLs also induced changes in cell size based on the type of NAPL substrate. The underlying mechanism affecting induction of cell surface hydrophobicity may differ depending on culture-type and NAPL-type.

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