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EFFECT OF NAPL COMPOSITION ON CELL SURFACE CHARACTERISTICS OF NAPHTHALENE DEGRADING CULTURES

Mukherji S.[1] , S. Chakraborty [2] and S. Mukherji[3]

Abstract: *In the environment, naphthalene typically exists as a component of non-aqueous phase liquids (NAPLs), such as, oil and tar. Cell surface hydrophobicity and cell surface charge are characteristics that can potentially impact adhesion to NAPLs and uptake of components from NAPL phases. This study was designed to explore the impact of NAPL composition on cell surface characteristics of two naphthalene degrading Burkholderia multivorans strains (NG1 and HN1) isolated from contaminated sites. Model NAPLs composed of n-hexadecane, naphthalene, phenanthrene and pyrene were used. Changes in cell surface characteristics was measured by determining cell surface water contact angle, adhesion to n-hexadecane in BATH (Bacterial Adhesion to Hydrocarbon) assay and zeta potential of cells for cultures grown on model NAPLs varying in composition. The water contact angle for HN1 grown on NAPLs was in the range of 65-75^o whereas it was in the range of 49-63^o for the NG1 strain. At neutral pH, the zeta potential of both the cultures was less than -35 mV and less than 30% adherence to n-hexadecane was observed in the BATH assay. A significant increase in adherence (43-75%) was observed at pH 2 when zeta potential was in the range of +5 to -10mV. Change in composition of the NAPL substrate altered the zeta potential at pH 7 and a decrease in adherence with increase in negative zeta potential was evident. The negative charge on cells at physiological pH thus, hinders attachment to NAPLs, even for conditions where contact angle is high.*

Keywords: *Cell surface hydrophobicity; direct uptake; hydrocarbon degradation; surface charge.*

INTRODUCTION

Polynuclear aromatic hydrocarbon (PAHs) commonly exist as components of oil and tar which are non aqueous phase liquids (NAPLs) immiscible in water. Naphthalene is the simplest PAH with two fused benzene rings and is also characterized with the highest aqueous solubility of 31 mg/L. Its biodegradation by pure and mixed bacterial cultures is widely reported. The high molecular weight PAHs characterized by three or more rings and low aqueous solubility are less biodegradable due to bioavailability limitations (Cerniglia, 1993). Although bacterial cultures

[1] Professor, Centre for Environmental Science and Engineering, Indian Institute of Technology Bombay, Mumbai 400076, India, Email: mitras@iitb.ac.in.
[2] MTech Student, Centre for Environmental Science and Engineering, Indian Institute of Technology Bombay, Mumbai 400076, India, Email: sichakraborty@deloitte.com.
[3] Professor, Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai 400076, India, Email: mukherji@iitb.ac.in.

typically uptake compounds from the aqueous phase after dissolution some cultures are reported to be capable of uptaking directly from the interface by attachment to solid crystals or by attachment to the NAPL-water interface. Such cultures capable of direct uptake are typically characterized by high cell surface hydrophobicity (CSH) (Mohanty and Mukherji, 2008). It is generally believed high CSH is not a prerequisite for naphthalene degradation (Mukherji and Weber, 1998). In contrast, either high CSH or biosurfactant production is typically considered a prerequisite for cultures showing significant degradation of three and four ring PAHs such as, anthracene and pyrene (Johnsen and Karlson, 2004, Wick et al., 2003).

The effectiveness of attachment of bacteria to NAPLs can be tested using the bacterial adhesion to hydrocarbon (BATH) assay, where n-hexadecane is commonly used as the test NAPL (Rosenberg et al., 1980). In this assay a bacterial suspension is contacted with a test NAPL, mixed vigorously by vortexing and subsequently the phases are allowed to separate. Adherence to NAPL is determined based on reduction in absorbance of the aqueous phase. This test has also been widely used as a measure of CSH of bacterial cultures (Achouak et al., 1994; Whyte et al., 1999). In addition to BATH assay, water contact angle measurement on bacterial cell surfaces is also used as a measure of CSH. In this test the CSH is determined by depositing a drop of water on a uniform layer of bacterial cells (Busscher, et al., 1984). However, % adherence determined based on BATH assay often provides a poor correlation with contact angle measurements (Mohanty and Mukherji, 2008) due to electrostatic interactions between bacteria and NAPL droplets. Surface charge on colloidal particles is manifested as negative zeta potential in electrophoretic mobility experiments (Wilson et al., 2001). Researchers have reported that n-hexadecane droplets in dilute aqueous solution are negatively charged (Medrzyka, 1991). Bacterial cells belonging to various genus are also reported to carry negative charge at physiological pH (Achouak et al., 1994; Ahimou et al., 2001). The electrostatic repulsion between the droplets of the test NAPL and bacterial cultures may hinder adherence due to like charges on the NAPL and bacterial cultures. It is well established that higher ionic strength facilitates coagulation of a colloidal dispersion due to double layer compression. Thus, adherence is expected to increase at higher ionic strength due to reduction in repulsion potential (Hermansson, 1999). The solution pH may also impact the speciation of charged functional groups on bacterial cell surfaces and may thus impact adherence to NAPLs.

Alteration in growth substrate is reported to alter the CSH of cultures. Hydrocarbon grown cultures are typically more hydrophobic compared to cultures grown on rich media (Whyte et al., 1999, Mohanty and Mukherji, 2008). Change in growth substrate is also reported to affect the production of extracelular polymeric substances by bacterial cultures. However, researchers have not explored the impact of changes in composition of the oil used as growth substrate on CSH and zeta potential. Thus, zeta potential and CSH may vary during growth on NAPLs, as composition of the NAPL phase changes, i.e., with depletion of substrate or with enrichment of toxic components in the NAPL phase. The objective of this study was to determine the impact of NAPL composition on cell surface contact angle, adherence to n-hexadecane in BATH assay and zeta potential for naphthalene degrading *Burkholderia* cultures.

MATERIALS & METHODS

Two strains of *Burkholderia multivorans,* HN1 and NG1 were isolated from HPCL refinery sludge and from a motor garage soil, respectively, using naphthalene as the sole substrate. Strain NG1 was identified based on microseq analysis (Applied Biosystems, Gurgaon, India) while strain HN1 was identified based on 16S r-RNA analysis (Macrogen Inc., Korea) followed by maximum similarity match obtained based on nucleotite BLAST at NCBI site.

Model NAPLs of varying composition was prepared using the hydrocarbons, n-hexadecane, naphthalene, phenanthrene and pyrene. The solid PAHs were dissolved in n-hexadecane, such that, n-hexadecane: naphthalene ratio was 4:1 for all the NAPLs. Mole fractions of the solid phase pure components were always kept at less than their fugacity ratio at room temperature so as to ensure stable NAPL formulations (Peters et al., 1997). In NAPL-1 mole fraction of naphthalene, phenanthrene and pyrene, were 0.18, 0.05 and 0.05, respectively. NAPL-II consisted of only naphthalene (0.19) and pyrene (0.05) while NAPL-III consisted of only naphthalene (0.19) and phenanthrene (0.05) apart from n-hexadecane which was the dominant component in all the NAPLs (mole fraction range 0.72-0.76). The cultures were grown on these NAPLs (0.5% v/v) provided as sole source of carbon and energy in a 100 ml mineral medium (Mukherji et al., 2004) contained in 500 mL flasks. The flasks were incubated in a rotary shaker set at 120 rpm and 30^{o}C. Culture growth was measured as absorbance at 600 nm and growth profiles were obtained by sampling an aliquot over a period of time until the cultures reached end of log growth phase. Cultures grown up to the end of log growth phase (24 hrs) were harvested (15500 rpm for 45 minutes) and washed twice with phosphate buffer for determining the impact of the growth substrate on cell surface characteristics.

The contact angle measurement and BATH assay were performed as suggested by Mohanty & Mukherji (2008). For the contact angle measurement, the harvested cells were resuspended in phosphate buffer to unit absorbance (at 600 nm) and a fixed volume of this suspension was filtered through a 0.45 μm cellulose acetate membrane (Millipore). The membrane filter with the bacterial cell mat was first stored in a petri plate on the surface of an agar (1% wt/v) –glycerol (10% v/v) layer for about 2 hours for standardizing the moisture content,. The membrane filter containing the bacterial cell layer was cut into strips and a strip was fixed on to a glass slide with double sided adhesive tape and was allowed to air dry. The drying time was standardized over 30-90 minutes using the naphthalene grown NG1 culture. The measurements were performed using Digidrop (GBX instruments, France) within 1-2 second after depositing a drop of water on the bacterial mat. The contact angle between the film surface and the tangent to the drop at the solid-liquid-air meeting point was measured at 2-3 different locations on the cell layer, and for each culture-substrate combination measurements were made by growing two sets of cultures. For conducting the BATH assay the harvested and washed cell pellet was resuspended in phosphate buffer/ 1 mM KCl to obtain an uniform cell suspension with absorbance value adjusted to unity. The phosphate buffer consisted of Na_2HPO_4 $7H_2O$ (668 mg/L), K_2HPO_4 (435 mg/L) and KH_2PO_4 (170 mg/L). For one set of studies the pH was adjusted to pH 7 while in another set the pH was adjusted to pH 2. Equal volumes of the cell suspension (5 mL) were distributed into multiple tubes containing varying volumes (0-0.5 mL) of n-hexadecane. The phases were contacted in a vortex mixer set at 2000 rpm for 2 minutes. After allowing the phases to separate over a 15 minutes 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 with no n-hexadecane. For later studies only n-hexadecane to aqueous phase ratio of 1:10 was used. Surface charge of the microbial cultures was determined by measuring the zeta potential after resuspending the cells in phosphate buffer/ 1 mM KCl and adjusting the pH to 7 and 2, respectively. The culture suspension was sonicated for 3 min in an ultrasonic bath and subsequently Zeta Potential was analyzed using the Zeta Potential Analyzer (Brookhaven Instrument Corp, NY, USA).

RESULTS AND DISCUSSION

Growth of both the cultures on naphthalene and on the model NAPLs is depicted in Figure 1a and b.

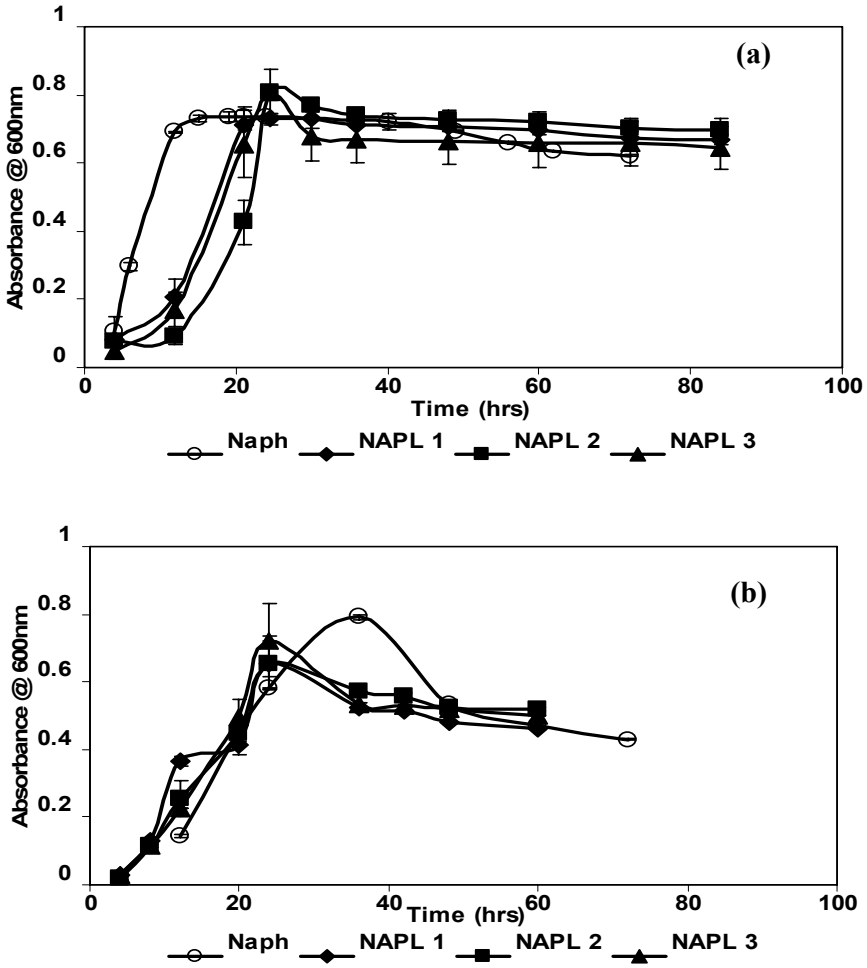

Figure 1. Growth of *Burkholderia multivorans* (a) strain HN1 and (b) strain NG1on naphthalene and model NAPLs

When grown on model NAPLs, both the cultures were found to reach end of log growth phase within 24 hrs. The HN1 culture exhibited a greater lag time but faster growth rate compared to the NG1 culture. Growth on the three NAPLs was almost comparable for both the cultures.

The water contact angle indicates the cell surface hydrophobicity (CSH) of bacterial cultures. This method is relatively unaffected by solution phase conditions but is impacted by process variables such as time for moisture standardization and drying time of the bacterial mats. Initial standardization studies were performed with *B. multivorans*-NG1 grown on naphthalene In the absence of moisture standardization, contact angle of naphthalene grown *B. multivorans*-NG1 was found to be 57.2° after an hour of air drying. It was lowered to 48.3° when the moisture content of the cellular mat was standardized as suggested by Busscher et al., (1984). Variation in drying time caused variation in the contact angle as shown in Fig 2(a). A drying time of 60 minutes was found to be optimal. Fig 2(b) reveals the impact of growth substrate on contact angle. *B. multivorans* HN1 had higher contact angle than the NG1 strain irrespective of growth substrate indicating greater hydrophobicity associated with its cell surface. Both the cultures depicted the lowest hydrophobicity when grown on naphthalene. Hydrophobicity of the HN1 strain was highest when grown on NAPL 2 (75^o) while that of strain NG1 was highest when grown on NAPL 3 (63^o). The classification of microorganisms based on water contact angle has been proposed as follows: 30°- 40° is hydrophilic, 50°- 60° is moderately hydrophobic and 80°-90° is hydrophobic (Mozes and Rouxhet (1987)).

The results for BATH assay performed with 0.5 ml n-hexadecane and corresponding zeta potential values are depicted in Fig 3a for cultures grown on various substrates and resuspended in phosphate buffer at pH 7. Similar studies conducted for cultures resuspended in 1 mM KCl at pH 7 is depicted in Fig 3b. Negative zeta potential is indicative of negative charge on the bacterial cell surfaces. It is evident from Fig 3a & b that the growth substrate and the solution phase affects both BATH assay results and the zeta potential values. For these cultures, zeta potential is in the range of -7 to -35 mV and adherence is less than 30%. For cultures resuspended in phosphate buffer at pH 7 (Fig 3a), the NAPL-1 grown cultures depict the highest negative zeta potential and correspondingly depicts the least adherence to n-hexadecane. For HN1 culture grown on NAPL-2 and NAPL-3 the zeta potential is low and comparable, however adherence in higher for the NAPL 2 grown cultures. This may be attributed to the higher contact angle of these cultures. The reverse trend between % adherence and zeta potential is evident from Fig 3a & b. Thus, BATH assay is significantly affected by solution phase electrostatic interactions. Electrostatic repulsion hinders attachment of negatively charged bacteria to n-hexadecane droplets that also carry negative charges (Busscher et al., 1984; Medrzyka, 1991). Researchers have also reported that specific ions in the aqueous phase and ionic strength of the aqueous solution can also impact adherence to NAPLs by affecting the charge on bacterial surfaces (Busscher et al., 1984; Hermansson, 1999). Thus, differences in %adherences observed

for the cells resuspended in phosphate buffer and 1 mM KCl may be explained by the differences in zeta potential of the bacterial cells.

The effect of charge on adherence was further illustrated by measurements carried out at pH 2. The zeta potential of these cultures grown on the various NAPLs was in the range of +1 to +5 mV after resuspension in phosphate buffer at pH 2 and in the range of 0 to -10 mV after

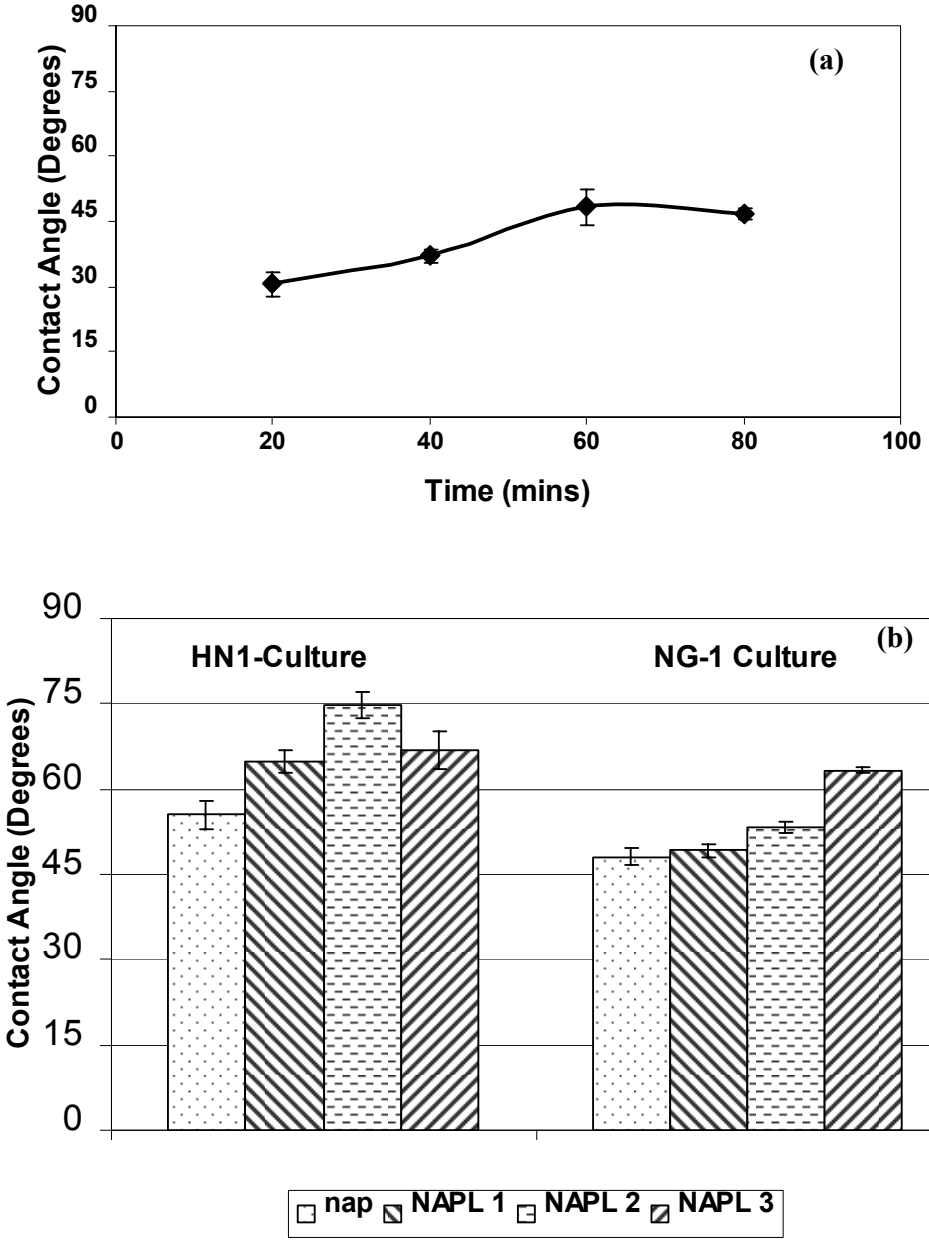

Figure 2. Variation in water contact angle with (a) drying time for *B. multivorans* NG1 and (b) growth substrate for *B. multivorans* strains HN1 and NG1

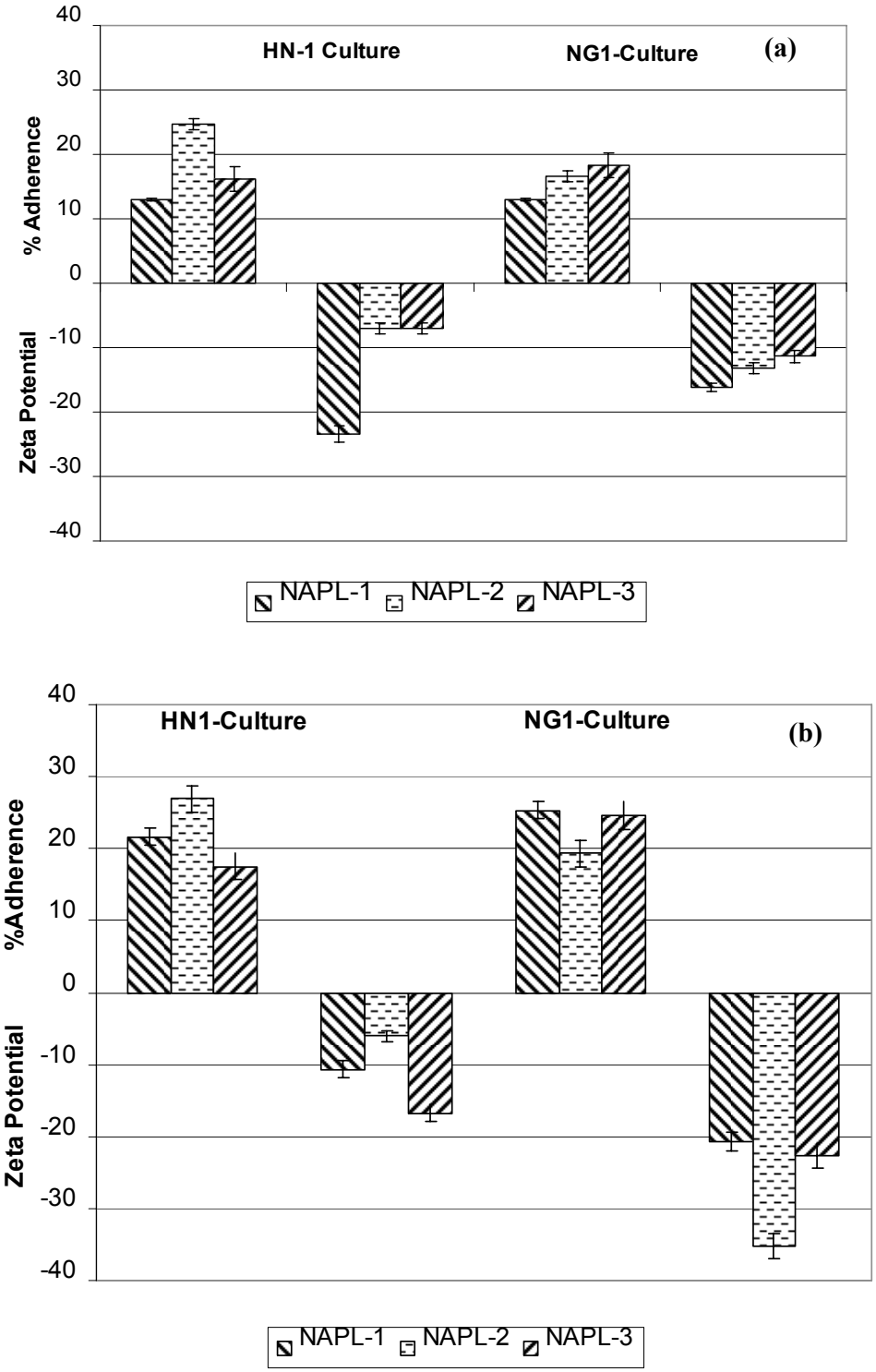

Figure 3. Variation in %adherence and zeta potential for *B. multivorans* strains HN1 and NG1with variation in growth substrate and resuspension solution (a) Phosphate buffer at pH 7 and (b) 1 mM KCl at pH 7

resuspension in 1 mM KCl at pH 2. The corresponding adherence values in phosphate buffer and 1 mM KCl was in the range of 48- 61% and 43-75%, respectively. Lowering in pH facilitated adherence by reducing the electrostatic repulsion.

Although the water contact angle values clearly depicts greater hydrophobicity of the HN1 cultures compared to NG1 culture irrespective of growth substrate, it is not evident from the % adherence values. Low adherence (less than 30%) was observed even when water contact angle was 75^{o}. Thus % adherence to hexadecane does not adequately depict the hydrophobicity of bacterial cell surfaces. Although BATH assay and contact angle are both used as measures of hydrophobicity, BATH assay is not a good measure of CSH.

The results of this study have important implications on bacterial uptake of hydrocarbons from NAPL phases. Both the cultures are found to vary their cell surface characteristics in response to change in composition of the growth substrate. Such variations in cell surface characteristics have also been reported for a strain of *Burkholderia cepacia* capable of utilizing n-hexadecane as sole source of carbon and energy (Chakraborty et al., 2010). Cultures that take up naphthalene from the aqueous phase after dissolution may alter their cell surface hydrophobicity in response to growth on a NAPL phase containing naphthalene so as to utilize naphthalene by direct interfacial uptake. Such alteration in cell surface hydrophobicity and surface charge may also be utilized for detaching from used oil droplets.

CONCLUSIONS

The study reveals significant differences in cell surface characteristics of two *Burkholderia multivorans* strains capable of degrading naphthalene as the sole source of carbon and energy. Strain HN1 has higher cell surface hydrophobicity compared to strain NG1. The cultures depict good growth on naphthalene present in model NAPLs varying in composition. The composition of the oil phase has a significant impact on cell surface characteristics as revealed through contact angle and zeta potential measurements. Even hydrophobic to moderately hydrophobic cultures depict less than 30% adherence to n-hexadecane due to high negative charge on the bacterial cell surfaces at pH 7. The solution phase interactions significantly affects adherence and zeta potential while water contact angle is unaffected by such interactions. The results have important implications on attachment and detachment of cultures to NAPLs and on uptake of hydrocarbons from oil.

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REFERENCES

Achouak, W., Thomas, F. and Heulin. T. 1994. Physico-chemical surface properties of rhizobacteria and their adhesion to rice roots. *Colloids and Surfaces B. Biointerfaces*, 3, 131-137.

Ahimou, F., Paquot, M., Jacques, P., Thonart P. and Rouxhet, P.G. 2001. Influence of electrical properties on the evaluation of the surface hydrophobicity of *Bacillus subtilis*. *Journal of Microbiological Methods*, 45: 119-126.

Busscher, H.J., Weerkamp, A.H., van der Mei, H.C., van Pilt, A.W.J., de Jong, H.P. and Arends, J. 1984. Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. *Applied and Environmental Microbiology*, 48, 980-993.

Cerniglia, C. E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Current Opinion in Biotechnology*, 4, 331-338.

Chakraborty, S., Mukherji, S. and Mukherji S. 2010. Surface Hydrophobicity of Petroleum Hydrocarbon Degrading *Burkholderia* strains and their Interactions with NAPLs and Surfaces. Colloids & Surfaces B: Biointerfaces , 78, 101-108.

Hermansson, M. 1999. The DLVO theory in microbial adhesion, *Colloids and Surfaces., B. Biointerfaces*, 14, 105-119.

Johnsen A. R. and Karlson U. 2004. Evaluation of bacterial strategies to promote the bioavailibility of polycyclic aromatic hydrocarbons. *Applied Microbial Technology*, 63, 452-459.

Medrzyka, K.B. 1991. The effect of particle concentration on zeta potential in extremely dilute solutions. *Colloid and Polymer Science*, 269, 85-90.

Mohanty, G. and Mukherji, S. 2008. Enhancement of NAPL bioavailability by induction of cell-surface hydrophobicity in *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. *Indian J of Biotechnology*, 7, 295-306.

Mukherji S. and Weber W. J., Jr. 1998. Mass transfer effects on microbial uptake of naphthalene from complex NAPLs. *Biotechnology & Bioengineering*, 60, 750–760.

Mukherji, S., Jagadevan, S., Mohapatro, G. and Vijay, A. 2004. Biodegradation of diesel oil by an Arabian sea sediment culture isolated from the vicinity of an oil field" *Bioresource Technology*, 95, 281-286.

Peters, C.A., Mukherji, S., Knightes, C.D., and Weber, W.J.Jr. 1997. Phase stability of multicomponent NAPLs containing PAHs. Environmental Science and Technology, 31, 2540-2546.

Rosenberg, M., Gutnic, D. and Rosenberg, E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity, *FEMS Microbiology Letters,* 9: 29-33.

Whyte, L. G., Slagman, S. J., Pietrantonio, F., Bourbonniere, L., Koval , S. F., Lawrence, J. R., Inniss, W. E. and Greer, C. W. 1999. Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus sp*. Strain Q15. *Applied and Environmental Microbioogyl*, 65: 2961-2968.

Wick L.Y., Pasche N., Bernasconi S.M., Pelz O. and Harms H. 2003. Characterization of

multiple-substrate utilization by anthracene-degrading *Mycobacterium frederiksbergense* LB501T. *Applied and Environmental Microbiology*, 59 (10), 6133-6142.

Wilson, W.W., Wade, M.M., Holman S.C. and Champlin F.R. 2001. Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements, *Journal of Microbiological Methods*, 43, 153-164.