Cell surface hydrophobicity (CSH) of *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger* and the biodegradation of Diethyl Phthalate (DEP) via Microcalorimetry.

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Abstract: This work was focused on investigating the occurrence of cell surface hydrophobic (CSH) character among diethyl phthalate (DEP)-degrading microbes (*Escherichia coli*, *Staphylococcus aureus*, and *Aspergillus niger*) by evaluating the effect of DEP on microbial cell surface hydrophobicity and to investigate any relationship between cell surface hydrophobicity and the ability of such microbes to degrade DEP using microcalorimetry and other methods. In this study, a TAM III multi-channel microcalorimeter, at 28 ^{o}C, was used to measure the minimum inhibitory concentration (MIC) of DEP and the DEP biodegradation efficiency by fitting the thermogenic curves and integrating the area limited by these curves, respectively. Using MATHS (microbial adhesion to hydrocarbons) assay, CSH of the microbial cells was determined as a measure of their adherence to the hydrophobic n-octane. From the experimental data, *S. aureus* was found to be the most efficient DEP degrader and *E. coli* the least and that *S. aureus* showed high, whilst *E. coli* and *A. niger* showed moderate hydrophobicity and autoaggregation abilities. There were positive correlations between microbial cell surface hydrophobicity and autoaggregation ability, DEP biodegradability, IC_{50} values for the tested strains. [Journal of American Science 2010;6(7):78-88]. (ISSN: 1545-1003).

Keywords: Hydrophobicity, *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, Microcalorimetry, Diethyl phthalate, Autoaggregation, IC_{50}.

1. Introduction

To satisfactorily define hydrophobicity has not been very easy, though this effect has been investigated for so many decades ago. Many researchers have tried to proffer its definition: the thermodynamists defined it from a thermodynamic view (Edsall, 1992) and the microbiologists defined it from a phenomenological view (Kauzmann, 1959; Doyle, 2000). Breslow (1991) observed that, hydrophobicity is the tendency of apolar species to aggregate in aqueous medium so as to decrease the hydrocarbon-water/oil-water interfacial area. The inability of non-polar compounds like phthalic acid esters (PAEs) to dissolve in aqueous medium has been associated with the hydrophobicity of these compounds, in fact, making the term misleading; the London dispersion interactions between molecules of water and non-polar compounds are favourable and quite substantial. Reviews by Doyle (2000) and Karplus (1997) attempted to throw light on hydrophobicity in terms of both biology and chemistry. Adherence of a microorganism to a surface may occur through hydrophobic effect provided the associating sites have sufficiently high densities of non-polar areas. Microorganisms, including bacteria and fungi have adopted ways to use the hydrophobic effect to adhere to surfaces. Hydrophobic microorganisms, in particular, are capable of adhering to the oil-water interface, and of utilizing oil components as a source of energy for growth and metabolism (Marshall, 1991). Duncan-Hewitt, (1990) observed that, there exist compelling rationales to believe that the hydrophobic effect (attributed to cell wall proteins and lipids) may be the driving force for the adhesion of most microbes.

Miyoshi (1895) was the first to describe the ability of microorganisms to consume organic compounds as sole carbon source, by reporting the microbial utilization of paraffins. A great diversity of different microbes (bacteria, fungi, yeast and moulds) capable of degrading organic compounds (such as PAEs, and other oily-liquids) in the environment

occurs. Bacteria belong to a group of the 'best bio-degraders' of organic compounds (Olivera et al., 2003; Chao et al., 2006) including oily-liquids and other hydrocarbons; in this study, diethyl phthalate, DEP, and n-octane. Unfortunately, little or no information is available on the simultaneous degradation of both pollutants and without focusing on the relationship between extent of degradation achieved and cell surface hydrophobicity (CSH), which seems to be an avoided topic especially in systems containing more than one carbon source.

CSH influences the direct contact of cell with hydrocarbon droplets and is hence, one of the major factors affecting the degradation of hydrophobic compounds (Chao et al., 2006; Prabhu and Phale, 2003; Al-Tahhan et al., 2000). This factor is of paramount importance for cell survival, as it controls the process of association between the cells and other surfaces. The rate of biodegradation of organic compounds is dependent upon several other physicochemical and biological parameters. For instance, PAEs with shorter alkyl-chains (like diethyl phthalate, DEP; dibutyl phthalate, DBP) are relatively easily biodegraded than phthalate esters with longer alkyl-chains, such as di-(2-ethylhexyl)-phthalate (DEHP) (Chang et al., 2004; Chao et al., 2006). So it's expected that DEP is more easily biodegraded than DBP. The poor solubility of PAEs in water is potentially one of the commonest physicochemical properties which hinders their rates of biodegradation in the environment, which effect makes PAEs poorly bio-available, and thus hampers their microbial degradation (Rosenberg et al., 1992; Bouchez et al., 1995; Efroymson and Alexander, 1995; Churchill et al., 1999). Bioavailability of hydrophobic organic compounds, a physicochemical parameter critical in the overall rate of compound degradation, is a function of phase solubility and solution transport processes. For chemical and physical compatibility between and/or among the organisms and the hydrophobic DEP and n-octane substrates, the microbes must possess cell surface hydrophobicity, hence, leading to enhanced mutual interactions between and/or among them (Busscher et al., 1995), which consequently, would lead to increased dispersion and greater bioavailability of DEP substrates, and enhanced utilization. Therefore, the ubiquitous occurrence of hydrophobic cell surface property among strains of DEP-degrading population of microorganisms will be a favourable feature for DEP contamination bioremediation activity in any environmental matrix. Potentially, the rate-limiting step in biodegradation of hydrophobic compounds is the ability of the compounds to be solubilized and transported into microbial cells capable of metabolizing them. Though there exist among microorganisms, direct contact and DEP-solubilization mechanisms for substrate uptake but their relative contribution in a population of DEP degraders is yet unknown. Therefore, the aim of this study was to investigate the effect of microbial CSH on the degradation of DEP by exploring any existing relationship between CSH and the ability of such microbes (two bacterial strains, *Escherichia coli* and *Staphylococcus aureus*; and a fungal strain, *Aspergillus niger*) to degrade this compound by monitoring their growth on media amended with DEP, using a combination of microcalorimetry and other methods.

2. Materials and Methods

2.1 Microorganisms

The DEP-degrading microorganisms employed in this study were wild strains of *Escherichia coli* (a Gram-negative, facultative anaerobic and non-sporulating bacterium), *Staphylococcus aureus* (a Gram-positive, facultative anaerobic coccus), and *Aspergillus niger* (a filamentous ascomycete fungus) and were all provided by the National Key Laboratory of Agromicrobiology, Huazhong Agricultural University (Wuhan, PR China).

2.2 Media preparations

Peptone culture medium (commonly known as Luria Broth, LB, culture medium) was used to incubate the bacteria, *E. coli* and *S. aureus* and was prepared by adding 10.0g Peptone and 5.0g sodium chloride (NaCl) to a 1000mL measuring cylinder containing 5.0g Beef Extract and was made to the mark with distilled water. The medium, at pH 7.2, was then sterilized in high-pressure steam in an autoclave at 121^{o}C for 30 minutes.

Potato sucrose medium (PSM), used to incubate the fungus, *A. niger*, was prepared by adding 20.0mg D-Glucose to a filtrate, obtained by boiling 200.0mg peeled potato slices in 1000mL distilled water for 30 minutes. The volume of the medium was adjusted to 1000mL with distilled water, with no pH adjustment; and was also sterilized in high-pressure steam in an autoclave at 121^{o}C for 30 minutes.

2.3 Preparation of microbial cell suspensions

For inoculum preparations, a loop full of each of the microorganisms was subcultured by inoculating them into 10 mL each of their respective sterile medium and incubated on a horizontal orbital shaker (FM FUMA, PR China) at 37^{o}C with orbital shaking 200 rev min^{-1} (rpm) for 12–18 hours, prior each measurement. The culture was then used as a source of inoculum for the experiments carried out in this work.

2.4 Reagents and their preparations

n-octane (obtained from Sinopharm Chemical Reagent Company Limited, PR China) and diethyl phthalate (DEP); obtained from Tianjin Standard Science and Technology Company Limited, PR China] were used in this work. A 5mL stock solution of 5000 μg mL^{-1} of an analytical grade 99.0% DEP was prepared by thoroughly mixing 0.0223mL DEP with 4.978mL ethanol (95%±5) and stored in the refrigerator. Ethanol was used as solvent because of the low solubility of DEP in water and that it has been widely used as a depolymerizing agent catalyzing the depolymerization of compounds of the DEP family. All chemicals were used as received without further purification. In each microcalorimetric determination, five concentrations (S_o) of DEP (0, 50, 100, 200 and 300 μg mL^{-1}) were prepared as standards from the stock DEP solutions and pipetted into separate conical flasks and/or test ampoules (whereupon most of the added ethanol was removed through 15 minutes volatilization with the aid of HDL APPARATUS clean bench).

2.5 Minimum Inhibitory concentration (MIC) of DEP

The MIC of DEP (defined as the minimum concentration of DEP at which microbial growth was inhibited) for each of the three microbial cultures was separately determined from the half inhibitory concentration, IC_{50}, values (inhibitory concentrations of causing a 50% decrease of microbial growth rate) obtained microcalorimetrically (see Section 2.6) by inoculating a series of 4 mL test ampoules each containing 2 mL air, 2 mL of each respective medium and various concentrations (0 – 300 μg mL^{-1}) of DEP with individual culture prepared as above. The IC_{50} value is determined at 50% of the growth inhibitory ratio (I) obtained by

$$I = [(k_0 - k_C)/k_0] \times 100\%, \quad (1)$$

where k_0 and k_C are the rate constants of the blank/control (0 μg mL^{-1}) and the DEP-containing sample of dose C, respectively. DEP at 25 % of the MIC concentrations was used to determine its effect on cell surface hydrophobicity (CSH) and autoaggregation of the three microbial cultures, and DEP degradation.

2.6 Calorimetry and analytical methods

Enthalpy changes of microbial growth processes can be computed through microcalorimetry, quantifying continuously the exchange of heat between the growth system and the environment. In this study, a TAM III (the third generation thermal activity monitor) multi-channel microcalorimeter (Thermometric AB, Järfälla, Sweden), in the isothermal (constant temperature) mode, was employed to measure continuously the heat production rate from microbial respiration (which is measured as microbial activity) or thermal activities of the wide variety of processes taking place in the test sample (mixture of culture medium and varied concentrations of the compound) contained in the cleaned and sterilized 4mL stainless steel ampoules placed in the instrument in terms of heat, heat flow and heat capacity (Wadsö, 1997; Yao et al., 2008) at 28 ^oC. Each of the hermetically closed (by teflon sealing discs) sample ampoules contained 2mL of nutrient medium, varied doses of DEP, a loop of cell suspension and 2mL of headspace (air). The aim of the ampoule closure was to ensure the control of evaporation yet allowing oxygen and carbondioxide transfer (Critter and Airoldi, 2001). The cell concentration of inoculums for bacteria and fungus was controlled to be equal for the purpose of comparison. The culture medium (i.e., 2mL nutrient medium plus microorganisms) in the reference ampoule serves as blank/control in all the determinations. The performance and details of the instrument's construction are described in its users' manual.

The values of the total heat generated or total thermal effect (Q_T), peak-heat output power (P_{Max}), and peak-time (t_{Max}, related to the maximum position in the P-T curves, Figure 1(a)-(c),) of the growth process for each experiment (from the start of the growth-process until data collection was ended) was computed by integrating the area limited by the P-T curves (Prado and Airoldi, 2000; Critter et al., 2002); also computed from the curves were the value of the microbial growth rate constant (k) and the generation time (t_G). The quantification procedures for these parameters are well established in Hashimoto and Takahashi (1982). The thermogenic (P-T) curves generated from all the experiments illustrate typical patterns of all the phases of the exponential microbial growth-process reactions in environmental matrices (Kimura and Takahashi, 1985; Nuñez et al., 1994): *lag*, *exponential/log growth* (exponential increase of the heat evolution rate), *stationary* and *death phases* (decline of the P–T curve due to the microbial death) and hence obey the first-order thermokinetic relation (Equation 2) at the log phase of growth,

$$S_t = S_o \exp(-kt) \quad (2)$$

where t is the time period, k is the growth rate constant, S_o and S_t represent concentrations of DEP at times 0 and t, respectively. The biodegradation kinetics of organic compounds has been described by so many models and the first-order kinetics has been utilized often to describe their biodegradation process

at low dosages (Zeng et al., 2004). The degradation rate of the DEP by the strains, expressed as relative rate of degradation (δ), could be determined using S_t and S_0 [Equation (2)] via Equation (3) as the DEP dose utilized divided by the original DEP dose, multiplied by 100.

% DEP degradation (δ) = [1-(S_t/S_0)] x 100% = ($\Delta S/S_0$) x 100%, (3)

2.7 DEP treatment and preparations of blanks and samples.

In order to investigate the extent to which 50 µg mL^{-1} (i.e., about 25 % of the MIC concentrations) of DEP could be used as a source of carbon and energy for the microbes under aerobic growth conditions, shake flask experiments were performed. Each culture was grown individually in 150 mL of respective medium in 250 mL Erlenmeyer flasks on the same shaker used for inoculum preparation, with and without 50 µg mL^{-1} DEP for the evaluation of the effect of these compounds on growth, dissolved oxygen (DO), and autoaggregation. At the end of the 24 hours' incubation of the flasks in dark at 28^{o}C, flasks were taken in turn at predetermined times and assayed for cell autoaggregation ability, and DO concentration as described Section 2.8 and the remaining culture medium was centrifuged, using TGL–16M (PR China), at 5000 rpm for 20 minutes to obtain cell pellet, which was used to evaluate the effect of DEP on microbial cell surface hydrophobicity (CSH), as described in Section 2.9.

2.8 Cells autoaggregation assay.

The microbial strains were inoculated in 250 mL Erlenmeyer flasks containing 150 mL each of either LB (for the bacteria) or PSM (for the fungus) culture and initial concentration of DEP made at 50 µg mL^{-1}. Absorbance of the cultured media of the microbial cells, grown with (as test samples) or without (as controls or blanks) 50 µg mL^{-1} DEP, was monitored as described below to observe the effect of DEP on autoaggregation ability. After 24-hour incubation at 28^{o}C in the dark, the cultured medium was vortexed for 15s and allowed to stand at room temperature for 120 minutes, and changes in absorbance were monitored, by taking 3.5 mL inoculated culture medium into quartz cuvette cell at 660 nm using the Spectrumlab 725s Spectrophotometer (Lingguang Ltd., Shanghai, PR China). Autoaggregation ability was determined as percent autoaggregation ($\%A_{Ag}$) using the formula (Equation 4)

$\%A_{Ag}$ = [1-(A/A_o) x 100, (4)

where A_o and A are the absorbances of the cultured media at 0 and 120 minute intervals, respectively. Strains with $\%A_{Ag}$ ≥70%, between 50 and 70% and <50% can arbitrarily been designated as highly, moderately and low autoaggregating, respectively.

2.9 Microbial cell surface hydrophobicity (CSH) assay

Hydrophobicity of the microbial cell suspensions prepared as above was determined, using MATHS (microbial adhesion to hydrocarbons) assay, as a measure of their adherence to the hydrophobic hydrocarbon (n-octane) following the procedure described previously by Rosenberg et al., (1983). After 24-h incubation, microbial cells were concentrated and harvested during the exponential growth phase by centrifugation (5000 x rpm for 20 minutes, TGL–16M, PR China); washed twice with phosphate buffered saline (PBS: 7.6g NaCl, 1.9g $Na_2HPO_4.7H_2O$, 0.7g $NaH_2PO_4.2H_2O$ per liter, pH 7.2, which is a hydrophilic solution), resuspended in the same buffer and the absorbance measured at 660 nm (A_1). Five milliliters (5 mL) of microbial suspension and 1 mL n-octane (i.e., the assay mixture) were mixed for 120s by vortexing to ensure thorough mixing and then incubated for 1h without shaking to insure that the two solutions had separated into biphasic state. The absorbance of the lower hydrophilic (aqueous) layer was measured again (A_2) by recording the changes in absorbance of microbial suspensions due to microbial adhesion to n-octane at 660 nm using the Spectrumlab 725s Spectrophotometer. Microbial cell surface hydrophobicity was expressed as percentage adherence ($\%Adh$) and calculated using the formula (Equation 5)

$\%Adh$ = [1-(A_2/A_1)] x 100, (5)

Strains with $\%Adh$ ≥70%, between 50 and 70% and <50% can arbitrarily been classified as highly, moderately and low hydrophobic, respectively.

2.10 Statistical analyses

Experimental values were obtained through three parallel experiments. Means and standard deviations of generated triplicate data were determined using Originlab Scientific Graphing and Analysis software (OriginPro 7.5). This tool was also used to evaluate the differences between cultures, and the effect of DEP by statistically determining the significance at the P<0.0001 level of difference between treatments and the correlation coefficient (R>0.99).

3. Results and Discussions

In this study, the minimum inhibitory concentration (MIC) for the three microbial cultures, ranging between 127.84 and 197.37 µg mL^{-1} (Table 1), is considered as the least concentration of DEP at which microbial growth was inhibited. Twenty-five percent (25%) of the highest MIC value (i.e., sub-inhibitory dose) was approximated to 50 µg mL^{-1}.

3.1 Microcalorimetry; the growth thermogenic (power-time, P-T) curves at varied doses of DEP at 28 °C.

The third generation multi-channeled thermal activity monitor (TAM) III (Thermometric AB, Järfälla, Sweden) in the isothermal mode, was employed to generate the IC_{50} values from the metabolism of DEP by the three strains by continuously measuring the heat production rate, from the microbial respiration or thermal activities of the wide variety of processes taking place in the test sample, in terms of heat, heat flow and heat capacity at 28 °C. The P-T curves for the growth of these strains in the presence of different doses of DEP (used as the sole carbon and energy source) were shown in Figure 1; all of them depicting, more or less, lag, exponential, stationary and death phases. From these curves, important parameters, including k (the microbial growth rate constant), were computed (Table 1). From Figure 1, it can be seen that DEP has an obvious inhibitory action on the growth of these strains. The IC_{50} value (regarded as the inhibiting dose capable of causing a 50% decrease of the growth rate) was determined at 50% of the inhibitory ratio (I) obtained via Equation 1 and by extrapolating C versus I plot (linear relationship). The sub-inhibitory DEP dose (50 µg mL^{-1}) used to investigate the hydrophobicity and autoaggregation ability of the strains was determined by considering the maximum of 25% of the three sought IC_{50} values. The k, I and IC_{50} values were presented in Table 1, with correlation coefficients (R) greater than 0.99. The k values, decreasing with increase in the DEP doses, show that DEP has potential antimicrobial activity even though it was almost completely biodegraded in these experiments (see δ values in Table 1). The IC_{50} values (*E. coli*, 197.37 µg mL^{-1}; *S. aureus*, 154.43 µg mL^{-1}; and *A. niger*, 127.84 µg mL^{-1}) indicated that *A. niger* and *E. coli* are, respectively, the most sensitive and most tolerant microbes to DEP. The DEP degradation efficiencies (δ) recorded for *E. coli*, *S. aureus*, and *A. niger* ranged between 51.9 - 93.5, 91.9 – 99.8 and 59.8 – 99.9%, respectively; making *S. aureus* (mean DEP Degradation, δ, = 96.18%) the most efficient DEP degrader and *E. coli* (mean DEP Degradation, δ, = 83.83%) the least.

With increasing DEP doses the time for the appearance of the peaks on each curve (t_{max} values, Table 1) increased and their heights (P_{max} and Q_T values, Table 1) decreased correspondingly; with little or diminishing peaks on curves for DEP doses of 200 and 300 µg mL^{-1}. These trends suggest that DEP has inhibitory effect (with increasing dose) on the strains as a consequence of the increased maintenance requirements due to irreparable harm to the cellular envelope (Tiehm, 1994; Chen et al., 2000). The growth curves of these strains have two characteristic peaks; which may suggest that these strains might have adopted two ways of metabolism. The presence or absence of oxygen (O_2) is very significant for microbial growth, hence influencing the ways of microbial metabolism. In this experiment, the volume of O_2 (the headspace) in the test ampoule is approximately 2.0 mL and this is only a limited amount of O_2 to be consumed by the strain in the system. The strains adopted one way of metabolism (aerobic) at the start of the experiment and when the O_2 is used up, the microbes may have adjusted themselves and adopted another metabolic way (anaerobic); which may explain the presence of two peaks. It's worthy to note that the heights of the second peaks increased greater than the first at lower and medium DEP doses and little or shrinking peaks on curves for higher DEP doses (200 and 300 µg mL^{-1}). Therefore, we may conclude that the first way of metabolism has been greatly influenced than the second way and that in the second way of metabolism, these facultative strains must have consumed DEP or/and subsequently its related degradation intermediates (monoethyl phthalate, MEP; phthalic acid, PA; and Protocatechuic acid) that may have accumulated during the primary degradation of DEP, as sources of carbon and energy needed for their continued growth for a definite time. Biodegradation of DEP (Kurane et al., 1978) follows the proposed degradative pathway [Equation (6)].

DEP → MEP → PA → Protocatechuic acid →→ CO_2 + H_2O (through TCA Cycle) **(6)**

which entails systematic cleavage of the ester bond to produce the phthalate monoester and then phthalic acid which is further metabolized and goes through the tricarboxylic acid (TCA) cycle to produce carbon dioxide (CO_2) and water (H_2O) (Wang et al., 1995).

3.2 Microbial cell surface hydrophobicity and autoaggregation of the investigated strains.

For computing microbial CSH, a quantitative method base on adherence to liquid hydrocarbon was recently illustrated. The ease of this technique lends itself to the rapid screening of numerous microbial samples. Three microbial strains were investigated for the effect of DEP on their

Table 1. Enumerative microcalorimetric experimental results of the effects of increasing doses of DEP on the growth of the three microbial strains at 28° C.

Strains	Dose (μg mL^{-1}) (C)	moles DEP (S_o) (10^{-4} mol) L^{-1}	P_{max} (μW)	t_{max} (min)	Q_T (J)	$k(10^{-3}$ $min^{-1})$	R	t_G (min)	Moles DEP (ΔS) (10^{-4} mol) L^{-1}	DEP Degradation (δ) (%)	I (%)	IC_{50} (μg mL^{-1})
E. coli	0	0.00	193.23	1944	10.50	1.62	0.9965	428	0.00	0.0	0.00	197.37
	50	2.25	186.73	1808	10.07	1.51	0.9928	459	2.10	93.5	6.79	
	100	4.50	159.37	2090	10.29	1.26	0.9983	550	4.18	92.8	22.22	
	200	9.00	161.29	2574	14.41	1.38	0.9983	502	8.74	97.1	14.81	
	300	13.50	12.00	600	0.20	1.22	0.9960	568	7.01	51.9	24.69	
S. aureus	0	0.00	145.28	1439	9.76	2.61	0.9990	266	0.00	0.0	0.00	154.43
	50	2.25	203.81	1198	12.97	2.07	0.9913	335	2.06	91.6	20.69	
	100	4.50	191.48	1751	12.77	1.68	0.9991	413	4.26	94.7	35.63	
	200	9.00	134.10	3686	27.51	1.15	0.9905	603	8.87	98.6	55.94	
	300	13.50	39.27	4106	7.52	1.51	0.9987	459	13.47	99.8	42.15	
A. niger	0	0.00	224.25	224	13.03	4.24	0.9959	164	0.00	0.0	0.00	127.84
	50	2.25	277.01	2247	12.05	3.35	0.9986	207	2.249	99.9	20.99	
	100	4.50	239.08	1914	11.90	1.33	0.9982	521	4.15	92.2	68.63	
	200	9.00	32.06	1627	2.33	0.56	0.9978	1238	5.38	59.8	86.79	
	300	13.50	-	-	-	-	-	-	-	-	100.00	

hydrophobicity (CSH) by measuring microbial adhesion to n-octane [Figure 2 (b)]; and on their autoaggregation abilities [Figure 2 (a)]. There were significant differences in the means of CSH (*E. coli*, 64.90%; *S. aureus*, 78.25%; and *A. niger*, 51.30%) and of the autoaggregation abilities (*E. coli*, 63.00%; *S. aureus*, 78.25%; and *A. niger*, 56.25%) amongst the strains without DEP treatment. Without DEP treatment, *S. aureus* showed high (Ljungh et al., 1985), whilst *E. coli* (Ljungh and Wadström, 1983) and *A. niger* (Wessels, 1997) showed moderate hydrophobicity and autoaggregation abilities. The fungal stain (*A. niger*) showed a lower CSH and autoaggregation values than those of the bacterial strains (*E. coli* and *S. aureus*), illustrating that remarkable differences might exist due to bacterial and fungal surface composition and structure (strain-specific characteristics). The effects of adding DEP to the respective medium on autoaggregating ability and CSH include decreased autoaggregation ability (*E. coli*, 35.00%; *S. aureus*, 58.00%; and *A. niger*, 23.00%) and a corresponding decrease in CSH of the strains (*E. coli*, 43.85%; *S. aureus*, 59.40%; and *A. niger*, 31.70%) [Figure 2 (a) and (b)]. When compared with the blank (0 μg mL^{-1}), the two moderately hydrophobic strains recorded significant reductions in autoaggregation ability (*E. coli*, 44.44%; *S. aureus*, 25.88%; and *A. niger*, 59.11%) than the strongly hydrophobic strain and similar reduction in hydrophobicity of all strains (*E. coli*, 32.90%; *S. aureus*, 24.09%; and *A. niger*, 19.64%). Such reductions made the strains less hydrophobic and less autoaggregating. These observed decreases are hard to explain as little is known about the effect of metabolic inhibitor compound (like DEP) on microbial cell surface hydrophobicity; however, Teo et al. (1998) proposed that disappearance of cell clusters can cause inhibition of proton translocation across the microbial cell surface, resulting in reduced

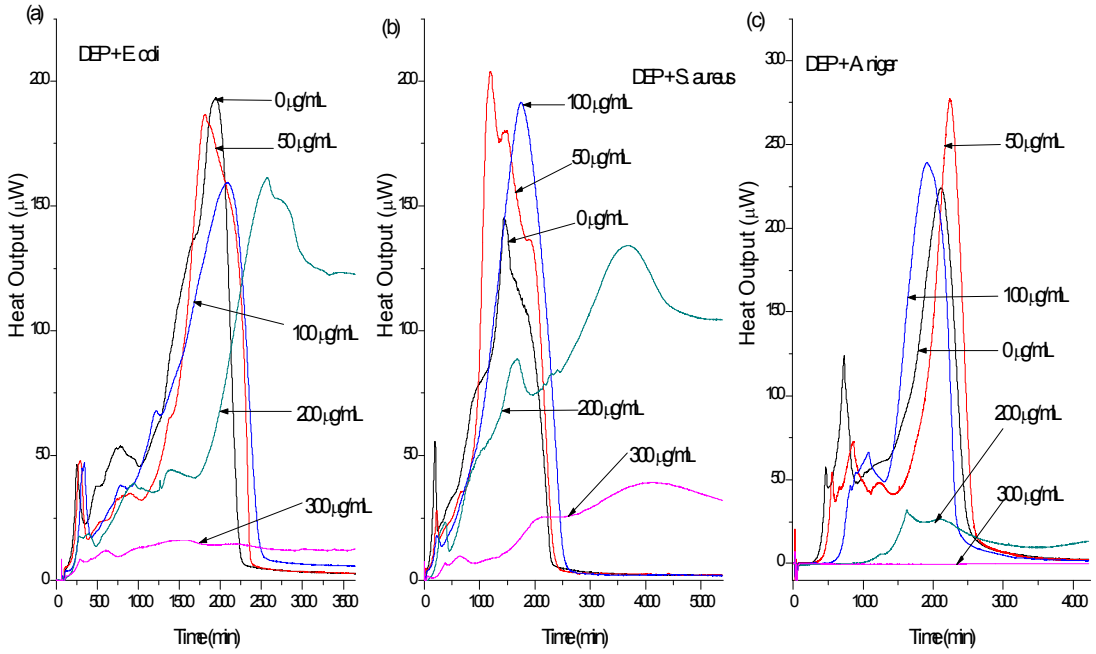

Figure 1. The growth thermogenic (power-time, P-T) curves of (a) *E. coli* in Luria Broth (LB) medium, (b) *S. aureus* in LB medium and (c) *A. niger* in potato sucrose medium (PSM) affected by varied doses of DEP at 28 ^{o}C.

dehydration of cell surface and subsequently leading to decreased hydrophobicity. This may be the likely explanation for the observed decreased in the hydrophobicity and/or adherence, thus decreasing the autoaggregation ability of the three strains. Furthermore, a possible reason for such differences could be attributed to the different compositions and origins of strains studied and the different compositions of nutrient medium; and that the surface physico-chemical characteristics of the strains are species-specific and probably decided by differences in chemical composition in the cell membrane (Christophe et al., 1997). A thorough understanding of all the significant factors, which influence the whole process, is of immense desire. Different methods to determine hydrophobicity may lead to differences to some extent. Figure 3 (b) shows the kinetics of autoaggregation followed by changes in the absorbance of the cultured medium with/without DEP. No significant decrease in absorbance was observed in all the strains. The distributions of CSH and autoaggregation values (Figures 2 and 3) shared much similarity. Regression analysis showed a significant positive linear correlation between hydrophobicity of microbial cultures and autoaggregation abilities of the strains [Figure 3 (a)], with correlation coefficient, R = 0.96826. This idea has been supported by other researchers including Jain et al. (2007). Generally, non parametric statistical analysis showed significant qualitative correlation between classes of hydrophobicity (i.e., high, moderate and low) and antibiotic resistance (IC_{50} values) at P = 0.74916, and from the regression analysis [Figure 4 (b)] we also found some kind of relationship, though a non-significant positive linear one (R = 0.3839), between CSH and the half inhibition dose (IC_{50}), which contradicted the finding of Tahmourespour et al. (2008) but supportive of those of Pan et al. (2006) and Kuntiya et al. (2005). The correlation between CSH and the DEP microbial degradation was also observed by Al-Tahhan et al. (2000) and Prabhu and Phale (2003), who observed that, as the cell surface becomes more hydrophobic, the association of the cell with the hydrophobic substrate becomes stronger, resulting in an increase in the degradation rate; but was contrary to Chao et al. (2006), who failed to observe any relationship between CSH and phthalate degradation potentials. This dichotomy, therefore, provides some information for further studies.

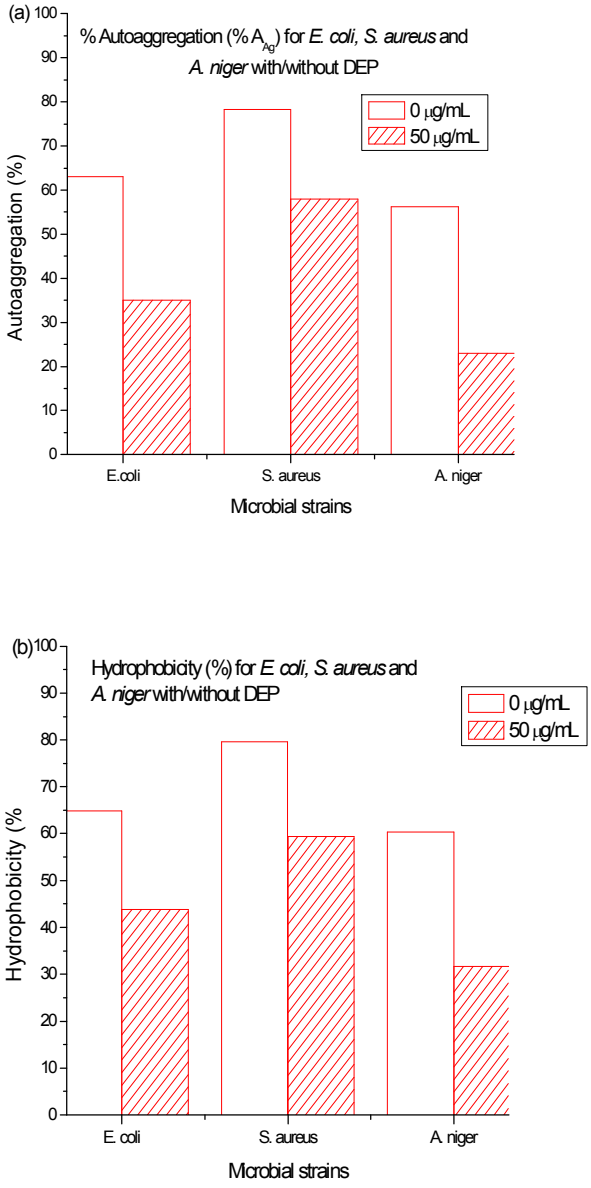

Figure 2. (a) Autoaggregation ability and (b) Surface Hydrophobicity of the three microbial strains grown in their respective medium only or a medium containing DEP.

The possible reasons for the observed trends in hydrophobicity and autoaggregation ability, as against DEP biodegradation, may include: (a) the occurrence of inhibitors enhances exopolysaccharide

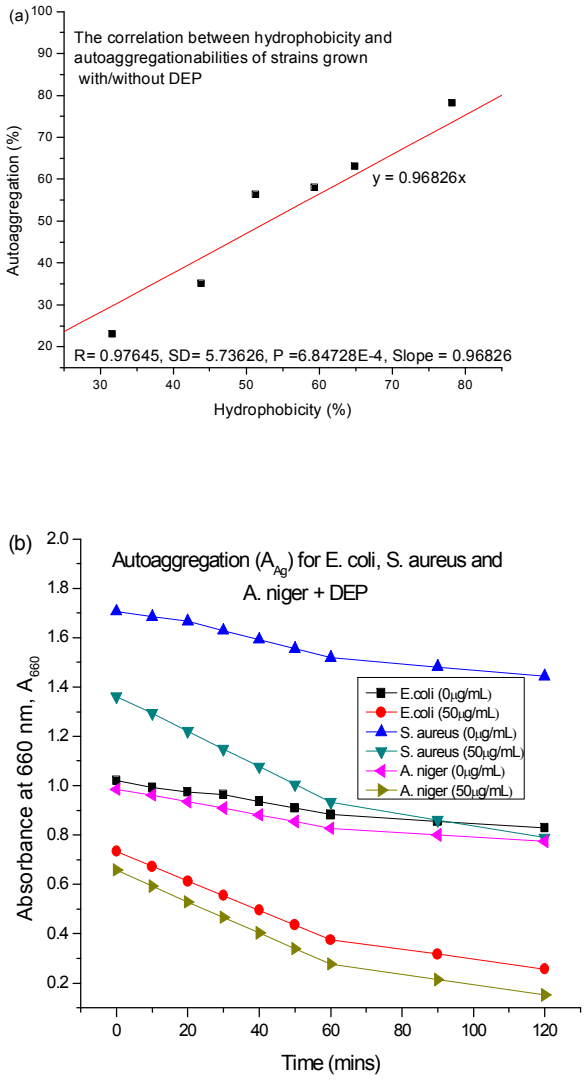

Figure 3. (a) The correlation between utoaggregation ability and the hydrophobicity and (b) autoaggregating kinetics of the three microbial strains to n-octane grown in their respective medium only or a medium containing DEP.

(EPS) production (Raihan et al., 1992; Singh and Fett, 1995) even though the mechanism hasn't been fully understood, which may, however, account for a decreased hydrophobicity (Caccavo et al., 1997), if the EPS are predominantly neutral or hydrophilic; (b) increase in the age of the cell leads to higher EPS production (Fattom and Shilo, 1984); and (c) nutrient starvation in the culture may also cause EPS production (Wrangstadh et al., 1986), thereby reducing the hydrophobicity. Additionally, Medrzycka (1991) and Busscher et al. (1995) reported that during surface hydrophobicity assay, the

hydrocarbons used are negatively charged, and this may cause repulsive (for Gram-negative cells) or attractive (for Gram-positive cells) electrostatic interactions and interfere with adherence of cells to hydrocarbons.

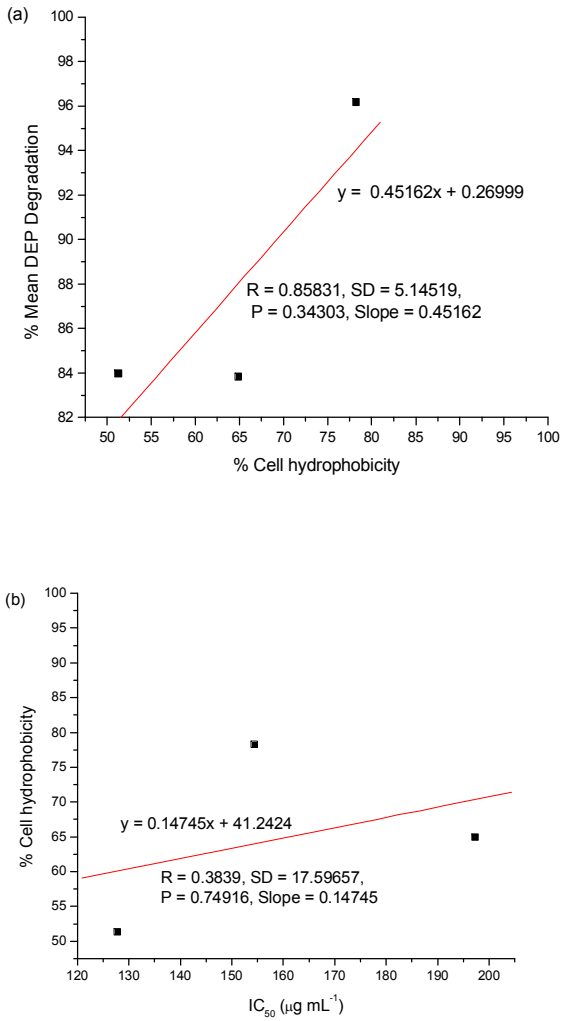

Figure 4. The correlation between the microbial cell surface hydrophobicity and (a) mean DEP degradation efficiency and, (b) inhibition (IC_{50}).

4. Conclusion:

Microcalorimetry can be a valuable supplement in the study of microbial inhibition (toxicity) and degradation of chemical compounds by determining valuable physico- and bio-chemical parameters including IC_{50}, used in this research to determine the sub-MIC value for the hydrophobicity and autoaggregation assays. There were significant positive correlations between microbial cell surface hydrophobicity and autoaggregation ability, biodegradability, IC_{50} values. This suggests the importance of hydrophobic interactions in the attachment of these cultures to chemical compounds. It was very clear that hydrophobicity had some kind of relation to autoaggregation ability [Figure 3 (a)], inhibitory effect [Figure 4 (b)] and thus DEP biodegradability [Figure 4 (a)] for the tested strains. So the substantiation of correlation between surface hydrophobicity and autoaggregation, vis-à-vis DEP biodegradation and inhibitory effect, could provide a means for characterizing these strains without complicated and protracted methods. This study also provided information to understand how these strains (*E. coli*, *S. aureus* and *A. niger*) work very well on the biodegradation of DEP, thereby, identifying these three strains as potential degraders of DEP, hence, their possible use in the DEP clean-up in the environment.

Acknowledgements:

This work was supported in parts by grants from National Outstanding Youth Research Foundation of China (40925010), International Joint Key Project from National Natural Science Foundation of China (40920134003), National Natural Science Foundation of China (40873060, 40673065), and International Joint Key Project from Chinese Ministry of Science and Technology, and the 111 Project (B08030).

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References

1. Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A., Maier, R.M., 2000. Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on cell surface properties and interaction with hydrophobic substrates. Appl. Environ. Microbiol. 66:3262-3268.
2. Bouchez, M., Blanchet, D., Vandecasteele, J.P., 1995. Substrate availability in phenanthrene biodegradation: transfer mechanisms and influence on metabolism. Appl. Microbiol. Biotechnol 43:952–960.
3. Breslow, R., 1991. in Comprehensive Organic Synthesis, Volume 7 (Eds.: B. M.

Trost, I. Fleming), Pergamon Press, Oxford, p. 39.
4. Busscher, H.J., van de Belt-Gritter, B., van der Mei, H.C., 1995. Implications of microbial adhesion to hydrocarbons for evaluating cell surface hydrophobicity 1. Zeta potentials of hydrocarbon droplets. Colloid Surf B-Biointerf 5:111-6
5. Caccavo, F.Jr, Schamberger, P.C., Keiding, K., Neilson, P.H., 1997. Role of hydrophobicity in adhesion of the dissimilatory Fe (III) oxide. Appl. Environ. Microbiol. 63(10):3837-3843.
6. Chang, B.V., Yang, C.M., Cheng, C.H., Yuan, S.Y., 2004. Biodegradation of phthalate esters by two bacteria strains. Chemosphere 55:533-538.
7. Chao, W.L., Lin, C.M., Shiung, I.I., Kuo, Y.L., 2006. Degradation of di-butyl-phthalate by soil bacteria. Chemosphere 63:1377-1383
8. Chen, P., Pickard, M.A., Gray, M.R., 2000. Surfactant inhibition of bacterial growth on solid anthracene. Biodegradation 11:341-347.
9. Christophe, P., Christine, B., Chantal, C., et al., 1997. Cell surface characteristics of *Lactobacillus casci* subsp. casci. *Lactobacillus paracasci* subsp. Paracasei, and *Lactobacillus rhamnosus* strains. Appl. Environ. Microbiol. 63(5):1725-31
10. Churchill, S.A., Harper, J.P., Churchill, P.F., 1999. Isolation and characterization of a mycobacterium species capable of degrading three-and four-ring aromatic and aliphatic hydrocarbons. Appl. Environ. Microbiol. 65:549–552.
11. Critter, S.A.M., Freitas, S.S., Airoldi, C., 2001. Calorimetry versus respirometry for the monitoring of microbial activity in a tropical soil. Appl. Soil Ecol. 18:217.
12. Critter, S.A.M., Freitas, S.S., Airoldi, C., 2002. Microbial biomass and microcalorimetric methods in tropical soils. Thermochim. Acta 394:145
13. Doyle, R.J., 2000. Contribution of the hydrophobic effect to microbial infection, Microbes and infection/Institut Pasteur 2(4):391-400.
14. Duncan-Hewitt, W.C., 1990. Predicting the relative rate of wear of pharmaceutical compacts using the mechanical properties of their constituent crystals. Powder Technol. 60:265-272.
15. Edsall, J.T., 1992. Memories of early days in protein science, 1926-1940. Protein Sci.1(11):1526-1530
16. Efroymson, R.A., Alexander, M., 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. Environ. Sci. Technol 29:515–521.
17. Hashimoto, M., Takahashi, K., 1982. Calorimetric studies of microbial growth: quantitative relation between growth thermograms and inoculum size. Agricultural and Biological Chemistry 46:1559-1564.
18. Jain, A., Nishad, K.K., Bhosle, N.B., 2007. Effects of DNP on cell surface properties of marine bacteria and its implication for adhesion to surfaces. Biofouling. 23(3-4), 171-177.
19. Karplus, P.A., 1997. Hydrophobicity regained. Protein Sci. 6(6):1302-1307
20. Kauzmann, W., 1959. Some factors in the interpretation of protein denaturation. Adv. Protein Chem. 14:1-63.
21. Kimura, T., and Takahashi, K., 1985. Calorimetric studies of soil microbes: quantitative relation between heat evolution during microbial degradation of glucose and changes in microbial activity in soil. J. Gen. Microbiol. 131:3083-3089.
22. Kuntiya, A., Nicolella, C., Pyle, L., Poosaran, N., 2005. Effect of sodium chloride on cell surface hydrophobicity and formation of biofilm in membrane bioreactor. Songklanakarin J. Sci. Technol. 27(5):1073-1082.
23. Kurane, R., Suzuki, T., Takahara, Y., 1978. Microbial degradation of phthalate esters, *Agricultural and Biological Chemistry* 42:1469-1473.
24. Ljungh, Å., Hjertén, S., Wadström, T., 1985. High surface hydrophobicity of autoaggregating *Staphylococcus aureus* isolated from human infections studied with the salt aggregation test. American Society for Microbiology 47(2):522-526.
25. Ljungh, Å., Wadström, T., 1983. Fimbriation of Escherichia coli in urinary tract infections. Comparisons between bacteria in the urine and subcultured bacterial isolates. Current Microbiol. 8:263-8.
26. Marshall, K.C., 1991. The importance of studying microbial cell surfactants. In: Mozes N (ed) Microbial cell surface

analysis, 1st edn. Wiley-VCH Publishers, New York.
27. Medrzycka, K.B., 1991. The effect of particle concentration in extremely dilute solutions. Prog Colloid Polym Sci. 269:85 – 90
28. Miyoshi, M., 1895. Die durchbohrung von membranen durch pilzfäden. Jahrb Wiss Bot 28:269–289.
29. Nuñez, L., Barros, N., Barja, I., 1994. Thermochim. Acta 237:73-81.
30. Olivera, N.L., Commendatore, M.G., Delgado, O., Esteves, JL., 2003. Microbial characterization and hydrocarbon biodegradation potential of natural bilge waste microflora. J Ind Microbiol Biotechnol 30:542–548
31. Pan, W.-H., Li, P.-L., Liu, Z., 2006. The correlation between surface hydrophoicity and adherence of *Bifidobacterium* strains from centenarians' faeces. Food microbiology. Anaerobes 12:148-152.
32. Prabhu, Y., Phale, P.S., 2003. Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: Novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. Appl. Microbiol. Biotechnol. 61:342-351.
33. Prado, A.G.S., Airoldi, C., 2000. Effects of the pesticide 2,4-D on microbial activity of the soil monitored by microcalorimetry. Thermochim. Acta 349:17-22.
34. Raihan, S., Ahmed, N., Ali, R., Khan, N., Eshaq, A., 1992. Production of exopolysaccharide by an indigenous soil isolates. JIAS. 5(4). http://www.medicaljournal-ias-org/5-4/Raihan.html
35. Rosenberg, E., Kaplan, N., Pines, O., Rosenberg, M., Gutnick, D., 1983. Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hydrocarbon. FEMS Microbiol. Lett. 17(1-3):157-160.
36. Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., Ron, E.Z., 1992. Petroleum bioremediation – a multiphase problem. Biodegradation 3:337–350.
37. Singh, S., Fett, W.F. 1995. Stimulation of exopolysaccharide production by fluorescent pseudomonads in sucrose media due to dehydration and increased osmolarity. FEMS Microbiol. Lett. 130(2-3):301-306.
38. Tahmourespour, A., Kermanshahi, R.K., Salehi, R., Nabinejad., A., 2008. The relationship between cell surface hydrophobicity and antibiotic resistance of Streptococcal strains isolated from dental plaque and caries. Iranian Journal of basic medical sciences 10(4):251-255.
39. Teo, K.C., Xu H.L., Tay, J.H., 1998. Molecular mechanism of granulation. II: Proton translocation activity. J. Environ Eng 126:411 - 418
40. Tiehm, A., 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. 60:258-263.
41. Wadsö, I., 1997. Isothermal microcalorimetry near ambient temperature: An overview and discussion. Thermochim. Acta 294:1-11.
42. Wang, J, Liu, P., Qian, Y., 1995. Microbial degradation of di-n-butly phthalate. Chemosphere. 31(9):4051-4056.
43. Wessels, J.G.H., 1997. Hydrophobins: proteins that change the nature of a fungal surface. Adv Microb Physiol. 38:1-45.
44. Wrangstadh, M., Conway, P.L., Kjellberg, S., 1986. The production and release of an extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion. Arch. Microbiol. 145(3):220-227.
45. Yao, J., Tian, L., Wang, Y., Djah, A., Wang, F., Chen, H.-L., Su, C.-L., Zhuang, R., Zhou, Y., Choi, M.M.F., Bramanti, E., 2008. Microcalorimetric study of the toxic effect of hexavalent chromium on microbial activity of Wuhan brown sandy soil: An in vitro approach. Ecotoxicology and Environmental safety 69:289-295.
46. Zeng, F., Cui, K., Li, X., Fu, J., Sheng, G., 2004. Biodegradation kinetics of phthalate esters by *Pseudomonas fluorescens* FS1. Process Biochemistry 39, 1125–1129

03/12/2010