Biodegradation of Dimethyl Phthalate, Diethyl Phthalate, Dibutyl Phthalate and Their Mixture by *Variovorax* Sp.

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Abstract—Biodegradation of dimethyl phthalate (DMP), diethyl phthalate (DEP) and dibutyl phthalate (DBP) and their mixture by a bacterial strain is reported. The strain was isolated by enrichment technique from garbage dumped soil nearby Indian Institute of Technology Bombay. The isolate had the ability to utilize mixture of DMP, DEP and DBP in the growth medium as well as the individual phthalate esters as the sole source of carbon and energy. The bacterial isolate was identified as *Variovorax* sp. based on 16S r-DNA sequence analysis. *Variovorax* sp. degraded >99% of 300 mg/l of DMP, DEP, DBP or a mixture containing 100 mg/l of each within 30 h. Metabolites or end products were not detected by HPLC analyses. Biodegradation of phthalate esters followed first-order kinetics. The rates of biodegradation of individual esters followed the order: DEP>DBP>DMP while in the mixture, rates followed the order: DEP>DMP>DBP. Crude cell free extracts prepared from bacterial isolates contained cocktail of inducible enzymes responsible for the degradation of DMP or DEP.

Index Terms—Biodegradation, enzyme, phthalate esters, *variovorax*.

I. INTRODUCTION

Phthalate esters (PAEs) are a group of compounds, which include dialkyl or alkyl aryl esters of 1, 2 -benzendicarboxylic acid (phthalic acid). Among phthalates esters, dimethyl phthalate (DMP), diethyl phthalate (DEP) and dibutyl phthalate (DBP) are mostly used for a variety of commercial purposes and have been listed as priority pollutants by agencies such as U.S. Environmental Protection Agency [1]. Humans are regularly exposed to PAEs through food, medicines, plastics, dairy products, cosmetics, etc. PAEs and their metabolites have been reported to cause reproductive and developmental disorders. PAEs have also been reported to be hepatotoxic, teratogenic and carcinogenic [2]. PAEs are not tightly bonded to the polymers (such as plastics), hence leach out into the environment and pose serious risks to ecosystem components.

DMP is used in cellulose ester based plastics, paints, adhesives, printing inks and coatings. Due to its wide applications in industries, DMP has been recognized as a significant environmental contaminant. DMP is a relatively stable compound in the natural environment with a half life of ≈ 20 years [3]. DMP promotes chromosomal injuries in human leucocytes, also known to be an endocrine disrupting

chemical (EDC) that interferes with the reproductive system and normal development of animals and humans [4]. DEP is used as a solvent vehicle for fragrance and in cellulose ester based plastics. DEP has been reported to cause abnormality in sexual differentiation and also act as an endocrine disrupting chemical [5], [6]. DBP is used in cosmetics, children's toys and child care products. DBP has been reported to reduce human sperm production and its motility, decrease rates of pregnancy and promote miscarriages [2], [7], [8].

Removal and degradation of PAEs by abiotic processes such as hydrolysis and photodecomposition has been reported to be slow and insignificant. However, breakdown by microorganisms is considered to be one of the major routes of environmental degradation for these widespread pollutants [9]. Number of investigators has demonstrated successful degradation of several PAEs by microbes under aerobic conditions in soil, water and wastewater [10]. Although there are number of reports on the biodegradation of individual PAEs, there is scanty literature available on degradation of mixture of PAEs. Industrial wastes frequently contain mixture of PAEs and therefore it is important to investigate the biodegradation of their mixtures rather than individual PAEs. The goal of present investigation was to demonstrate biodegradation of individual phthalate esters and their mixture using whole cells of a newly isolated bacterial strain as well as cell free crude enzyme preparations from the isolate.

II. MATERIALS AND METHODS

A. Chemicals

Dimethyl phthalate, diethyl phthalate and dibutyl phthalate were procured from Sigma-Aldrich, USA. HPLC grade methanol and acetonitrile were purchased from Merck India Limited, Mumbai. All chemicals were of high purity (>99%). All experiments were performed in duplicate unless otherwise specified.

B. Isolation of PAE Degrading Bacterial Strain and Culture Conditions

Microorganisms capable of utilizing the three phthalate esters as a mixture (DMP, DEP and DBP) were isolated via enrichment culture technique. Samples were taken from soil contaminated with electronic and plastic wastes situated near Powai Lake, Indian Institute of Technology Bombay (IITB). The initial enrichment culture was started by inoculating 100 ml of sterile mineral salts medium (MSM) with 1 g of fresh soil sample and 100 mg/l each of DMP, DEP and DBP in a 250 ml Erlenmeyer flask. The MSM consisted of: 3.5 g

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K₂HPO₄, 1.5 g KH₂PO₄, 0.27 g MgSO₄, 1 g NH₄Cl, 0.03 g Fe₂(SO₄)₃ · 7H₂O and 0.03 g of CaCl₂ dissolved in 1 l of water [11]. The pH of MSM was adjusted to ~ 6.8 and sterilized by autoclaving. Iron sulphate and magnesium sulphate were filter-sterilized and added from respective stock solutions to the cooled sterile medium. No other source of carbon other than phthalate esters was added. Inoculated flasks were incubated on an orbital rotary incubator shaker maintained at 180 RPM and 30°C. The cell growth was monitored by visual inspection. PAE degrading microbial population was obtained following five successive enrichments which involved repeated transfer of 1 ml of culture into fresh MSM containing mixture of PAEs. This was to ensure the elimination of growth supportive constituents contributed from soil and to gradually enrich biodegrader population capable of utilizing PAEs as their sole source of carbon and energy. Finally a single bacterial colony which had the ability to grow rapidly on agar plates containing mixture of PAEs (100 mg/l each of DMP, DEP and DBP) was selected, grown in MSM for 24 h culture, pelleted at 4°C and analysed for 16S r-DNA sequence. The pure culture of bacterial strain was also subjected to wet mounting, Gram staining and observed under oil immersion lens using the Zeiss phase contrast microscope.

C. 16S r-DNA Sequencing for the Identification of Isolated Bacterial Strain

16S r-DNA analysis was performed by Bangalore Genei, India. Genomic DNA was isolated from the pure culture pellet. Primers were designed based on conserved regions of bacterial 16S r-DNA sequence. Using consensus primers, ~1.5 kb r-DNA fragment was amplified using high-fidelity Xt5 polymerase (a combination of *Taq* and *Pfu* polymerase). The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. 16S r-DNA sequence data was aligned and analyzed for identifying the closest homology for the isolated bacterial strain. Phylogenetic tree was constructed in MEGA 3.1 software using neighbour joining method to analyze the relationship between the isolated bacterial strain BS1 with the other species from neighboring taxa.

D. Growth Profiles of the Isolated Bacterial Culture

Overnight grown starter culture (O.D. of 0.4) of the isolated bacterial strain was inoculated into 150 ml of fresh MSM containing 300 mg/l of DMP or DEP or DBP or a mixture containing all the three PAEs (100 mg/l of each) and incubated at 30°C on a rotary shaker (180 RPM). Five ml aliquots of the culture medium were withdrawn at regular intervals of time to record the cell optical density values at 600 nm. Plots of $\ln(X_t/X_0)$ versus time were used to calculate specific growth rates (μ) on individual PAEs and their mixture. X_t and X_0 represent the absorbance values at 600 nm at time t and zero, respectively.

E. Biodegradation of DMP, DEP, DBP and Their Mixture

The conditions for the cultivation of bacterial strain were similar to those described under section D. Following addition of 1.5 ml of overnight grown starter culture (O. D. of 0.4), aliquots of the spent growth medium containing DMP or DEP were aliquoted at chosen time points, centrifuged at

11,000 RPM for 15 min at 25°C to separate cells and the clear cell free supernatant was subjected to HPLC analyses. An equal volume of methanol was added into the spent growth medium which contained DBP or mixture of PAEs and mixed vigorously for 1 min to aid complete solubilization of DBP in the aqueous phase. Thereafter the cells were pelleted and discarded while the clarified supernatant was subjected to HPLC analyses. A control flask with un-inoculated growth medium was also maintained under same conditions on the test flasks and analyzed for the abiotic loss of PAEs.

F. Preparation of Cell-Free Extracts

The method of Prathibha and Suresh was used with some modifications [12]. Bacterial cells grown on phthalate esters were harvested at mid exponential phase (~17 h) by centrifugation at 4°C. The cell pellet was washed twice using 20 mM sodium phosphate buffer (pH 7.0) and subsequently resuspended in three volumes of the same buffer containing 20 mM 2-mercaptoethanol and 10% glycerol. The cell suspension was maintained at 0 to 4°C and subjected to pulse mode ultra-sonic cell disruption at 50 duty cycle for 7 minutes using a Branson sonifier. Sonicated cell suspension was then centrifuged at 11,000 RPM for 45 minutes at 4°C. The clear cell free supernatant was designated as the crude cell free extract.

G. Assay of Enzyme Activities Using Cell Free Crude Extracts

1) Esterase assay using *p*-nitrophenyl acetate as the substrate

Esterase activity was determined using *p*-nitrophenyl acetate (pNPA) as the substrate. The method of Xu *et al.*, was used with some modifications [13]. The test reaction mixture (1 ml) consisted of freshly prepared 0.064 mM of pNPA (0.2 ml added from a stock solution prepared in methanol), 0.2 ml of 50 mM potassium phosphate buffer (pH 7.0), 0.1 ml of cell free extract (protein concentration of ~2 mg/ml), and 0.5 ml of distilled water. was incubated in a water bath maintained at 30°C for 3 min. Absorbance was recorded at 400 nm which is the λ_{max} of the product, *p*-nitrophenol. The control reaction mixture consisted of all the components present in the test reaction mixture except that the cell free extract was omitted and corresponding volume of plain phosphate buffer was added. An additional control reaction mixture consisted of all the components of test reaction mixture and denatured (prior incubation at 100°C for 5 min) cell free extract. One unit of enzyme activity was defined as the amount of protein required to form 1 nmole of the end product, *p*-nitrophenol per min and specific enzyme activity was defined as nmoles of *p*-nitrophenol formed per min per mg of protein. Calibration plots for the desired range of concentrations of *p*-nitrophenol were obtained.

2) Enzyme Activity Using DMP or DEP as the Substrates

The test reaction mixture (10 ml) consisted of 1 mM of DMP or DEP and 3 ml of crude cell free extract (protein concentration of ~2 mg/ml) in 20 mM sodium phosphate buffer (pH 6.8). Protein concentrations in cell free extracts were measured using Bradford's method [14]. The control reaction mixture consisted of all the components present in the test reaction mixture except that the cell free extract was omitted and corresponding volume of plain phosphate buffer

was added. An additional control reaction mixture consisted of all the components of test reaction mixture and denatured (prior incubation at 100°C for 5 min) cell free extract. The tubes containing reactions mixtures were incubated in a water bath shaker for one hour at 30°C. Aliquots of the reaction mixtures were withdrawn at regular intervals of time, acidified using acid (2 N HCl) to terminate the reaction and centrifuged to remove the precipitated protein. The clarified supernatant was further filtered through 0.2 µm nylon membrane and subjected to HPLC analyses. One unit (U) of enzyme was defined as the amount of protein that was able to catalyze the removal of 1 nmol of target PAE per minute. Specific activity of cell free crude extract was calculated as the number of enzyme units per mg of protein.

H. Determination of Thermostability of Cell Free Extracts as a Function of Storage Time and Temperature

Enzyme assays were performed to examine the stability of esterase in cell free extracts using DMP or *p*-nitrophenyl acetate as the substrates. The protocols used for enzymatic assays were similar to those described in the previous sections (G-1 and G-2). A portion of the freshly prepared cell free extracts was assayed immediately for the enzyme activity (day 0). The remaining cell free extract was dispensed into 18 sample tubes closed tightly and divided into three groups. One group was stored at room temperature (approximately 25°C) while the other two groups were stored at 4°C and -20°C. Aliquoted extracts stored at three different temperatures were assayed for residual enzymatic activities as a function of storage time (for example at 1, 2, 4, 6, 8 and 10 d). Aliquots of the stored crude extract were allowed to pre-equilibrate for a short time to attain room temperature and mixed well prior to assays at the designated time points.

I. Effect of Glycerol Concentrations on Esterase Activity

Cell free extracts were prepared from the isolated bacterial strain using extraction buffer containing 1, 5 or 10% of glycerol. Esterase activity in the cell free extracts solution was measured using DMP as the substrate. The method used for this enzyme assay was according to the protocol described under section G-2.

J. Analytical Methods

Phthalate esters were analyzed using Shimadzu HPLC, equipped with a high pressure pump (LC-20AD), degasser (DGU-20A₅), photo diode array detector (PDA) (SPD-M20A) and an auto sampler (SIL-20AHT). A kromasil C18 column (5 µm particle size, 250 mm x 4.6 mm i.d.) was used with a mobile phase consisting of methanol/water (80:20, v/v) at a flow rate of 1 ml/min. Sample injection volume was 10 µl. Calibration plots for the desired range of concentrations of individual phthalate esters were obtained.

III. RESULTS AND DISCUSSION

A. Isolation and Characterization of PAEs Degrading Strain

Microscopic examination revealed that the isolated bacterial strain was motile, rod shaped and Gram-negative. Colonies produced on MSM agar plates containing PAEs (as the sole source of carbon and energy) were pale white and smooth. The partial 16S r-DNA sequence alignment from the isolated strain, BS1 revealed that it was most closely related to *Variovorax* sp. (GenBank Accession Number AB513921) with complete homology (100% similarity based on ≈ 15kb). The next closest homolog was *Variovorax paradoxus* (GenBank Accession Number DQ335121) with 99.8 % similarity. Figure 1 illustrates the phylogenetic relationship of strain BS1 based on 16S r-DNA sequence alignments. *Variovorax* species are known to be widespread in nature and have been reported to degrade dimethyl terephthalate [15], phenol, trichloroethylene [16], 2, 2' dithiodibenzoic acid [17], 2, 4 dichlorophenoxy acetate [18] and phenylurea herbicide diuron [19].

Fig. 1. Phylogenetic tree based on the 16S r-DNA sequence alignments showing the relationship of PAEs degrading bacterial isolate, *Variovorax* sp. strain BS1 (indicated in a bold letter) to other *Variovorax* species and sequences of bacteria from neighboring taxa. Sequence was aligned using Kimura 2 parameter method. The tree was constructed using neighbour joining method

B. Bacterial Growth on PAEs and Biodegradation of DMP, DEP, DBP and Their Mixture

The isolated bacterial strain, identified as *Variovorax* sp. was able to utilize DMP, DEP, DBP or their mixture as the sole sources of carbon and energy. Figure 2 demonstrates the time dependent reduction in the concentrations of DMP (a), DEP (b), DBP (c) and their mixtures (d) with the concomitant increase in the cell density of *Variovorax* species. The control flasks (without bacteria) did not show significant changes in the concentrations of PAEs which indicated negligible abiotic loss of the phthalate esters. The specific growth rates (μ) of *Variovorax* sp. in 300 mg/l of DMP, DEP, DBP and a mixture containing 100 mg/l of each phthalate ester were computed to be 0.085 per h, 0.107 per h, 0.087 per h and 0.077 per h, respectively.

Fig. 2. Time course profiles for the removal of 300 mg L⁻¹ of (a) DMP; (b) DEP; (c) DBP; (d) mixture containing 100 mg L⁻¹ of each by *Variovorax* sp. and corresponding growth profiles

TABLE I: RATE CONSTANTS AND HALF LIVES OF PHTHALATE ESTERS BASED ON THE FIRST ORDER BIODEGRADATION KINETIC PLOTS OBTAINED USING WHOLE CELLS OF *VARIOVORAX* sp.

PAEs used in the growth medium	PAEs monitored	First order rate constant (per h)	Half life (h)	R ²
DMP alone (300 mg/l)	DMP	0.3	2.4	0.85
DEP alone (300 mg/l)	DEP	0.44	1.6	0.87
DBP alone (300 mg/l)	DBP	0.36	1.9	0.97
Mixture of DMP + DEP + DBP (100 mg/l each)	DMP	0.27	2.6	0.94
Mixture of DMP + DEP + DBP (100 mg/l each)	DEP	0.34	2.1	0.96
Mixture of DMP + DEP + DBP (100 mg/l each)	DBP	0.24	2.8	0.93

Biodegradation of DMP, DEP, DBP and their mixture containing all the three PAEs followed first-order kinetics corresponding to the equation: $dC/dt = -kC$ where C and k represent residual concentration of the target PAE at any time t and degradation rate constant, respectively. Results presented in Table I suggest that the rates of degradation of individual PAEs followed the order: DEP>DBP>DMP while in the mixture the order was: DEP>DMP>DBP. However in the current investigations, extra-cellular accumulation of metabolites or end product could not be detected at any time point by HPLC analyses.

C. Biodegradation of DMP and DEP Using Cell Free Crude Extracts

Fig. 3 demonstrates time dependent biodegradation of 1 mM of DMP and DEP by cell free extracts derived from

Variovorax sp. The extent of biodegradation of DMP and DEP were ~ 82 % and ~ 90%, respectively after 60 min of incubation. HPLC profiles did not reveal the presence of intermediates or products. These results suggested that the cell free crude extracts derived from *Variovorax* sp. contained the necessary cocktail of enzymes for the biodegradation of DMP or DEP. Specific activities of DMP esterase and DEP esterase were ~ 89 nmole/min/mg and 170 nmole/min/mg, respectively, using 1 mM DMP or DEP. Removal of DMP or DEP was insignificant in control experiments wherein denatured cell free extracts were used. These results indicated that the abiotic loss of PAEs was negligible under the experimental conditions used in this investigation.

D. Stability of Esterase Enzyme as a Function of Storage Temperature and Time

Table II shows esterase activities as a function of storage temperature and time using DMP and *p*-nitrophenyl acetate as substrates. DMP esterase showed highest activity in freshly prepared cell free extracts. However the enzyme was rapidly inactivated by incubation at 25°C. At -20°C, the enzyme showed ~ 25% and ~ 50% reduction in the activity after 2 d and 10 d of incubation, respectively. A similar trend was also observed using *p*NPA as the substrate (last column of Table 2). These results revealed that the storage time and temperature had significant effect on the enzyme activity. DMP and *p*NPA esterase activities were not stable and hence the crude cell free extracts could not be stored for a long time period. However, the protective effects of substrate and ammonium sulfate fractionation need to be investigated further.

E. Induction of Esterase Enzyme

One of the most crucial aspects of environmental adaptation is the role of substrates in the induction and maintenance of biodegradative enzyme activities in bacterial cells. In order to correlate the involvement of phthalate ester such as DMP in the induction of corresponding esterase, cell free extracts were prepared from *Variovorax* cells grown on three different types of carbon sources, namely, DMP (600 mg/l) or phthalic acid (641 mg/l) or glucose (927 mg/l). The concentrations of the three substrates were adjusted to achieve elemental carbon equivalence. Fig. 4 suggests that the specific activity of the esterase using DMP as the substrate was ~33 and ~16 times higher as compared to those observed using glucose and phthalic acid, respectively. Similarly, esterase activity measured using *p*NPA as the substrate was ~10 and ~6 times higher as compared to those observed using glucose and phthalic acid, respectively. These results demonstrated that the DMP degrading enzyme was significantly induced by the presence of DMP, its target substrate which was being used as the sole source of carbon and energy by the isolated bacterial strain. Activation and expression of specific biodegradative genes which in turn leads to the synthesis of inducible enzymes in the presence of environmental pollutants is a well known phenomenon [20].

Fig. 3. Time course profiles for the degradation of 1 mM DMP or DEP using cell free extracts derived from *Variovorax* sp.

F. Effect of Glycerol Concentration on Esterase Activity

DMP esterase showed highest activity (89 nmole/min/mg) in cell free extracts containing 10% glycerol. Inclusion of lower glycerol concentrations in the crude enzyme extracts led to substantial reduction in the enzyme activity. As an example, the specific activity of DMP esterase was 52 nmole/min/mg in 5% glycerol and 23 nmole/min/mg in 1% glycerol. These results indicated that the presence of glycerol facilitated in stabilizing the native 3-dimensional structure of protein and hence the functional integrity of catalytic (active) site of the enzymes involved.

Fig. 4. Comparison of esterase activities in cell free extracts obtained from *Variovorax* sp. grown on phthalic acid or glucose or DMP.

TABLE II: ESTERASE ACTIVITY OF CRUDE CELL FREE EXTRACTS AS A FUNCTION OF STORAGE TIME (PRESERVED AT 25°C, 4°C AND -20°C) USING DMP OR *P*-NITROPHENYL ACETATE AS THE SUBSTRATES

Time of storage (d)	Esterase activity using DMP (nmole/min/mg)			Esterase activity using <i>p</i> -nitrophenyl acetate (nmole/min/mg)		
	Storage temperature					
	25°C	4°C	-20°C	25°C	4°C	-20°C
0	88.8	88.8	88.8	132.7	132.7	132.7
1	36.6	46.3	75.5	47.4	66.2	113.1
2	33.1	41.8	67.1	41.4	60.5	98
4	33.1	36.6	54.8	42.7	47.9	92.3
6	32.1	34.4	49.3	35	44.2	87.3
8	28.8	31.8	45.4	37.5	46.9	78.9
10	26.9	27.5	47	33.2	47.6	69.2

IV. CONCLUSION

The newly isolated bacterial strain, *Variovorax* sp. had the ability to utilize DMP, DEP, and DBP individually or as a mixture using them as the sole source of carbon and energy. The bacterial strain was able to degrade >99% of 300 mg L⁻¹ PAEs in 30 h. The rates of biodegradation of PAEs by *Variovorax* were higher as compared to those reported in the literature using other species of bacteria. Accumulation of metabolites or end products from the degradation of PAEs was not observed in the present investigations. This observation is in contrast to those reported in the literature which suggested the production of toxic metabolites (such as monomethyl phthalate or monoethyl phthalate) during biodegradation of PAEs. Results related to degradation of DMP or DEP using cell free extracts from *Variovorax* species suggested that the enzymes were inducible and efficient in the biodegradation of phthalate esters. *Variovorax* sp. was capable of degrading mixture of PAEs and therefore the isolate may be applied for field scale bioremediation.

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