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EFFICIENT DEGRADATION OF DIBUTYL PHTHALATE AND UTILIZATION OF PHTHALIC ACID ESTERS (PAES) BY *ACINETOBACTER* SPECIES ISOLATED FROM MSW (MUNICIPAL SOLID WASTE) LEACHATE

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ABSTRACT

Bacteria from the genus *Acinetobacter* are opportunistic pathogens and cause harm to humans. In this study a new bacterial strain designated as *Acinetobacter* sp. 33F was isolated from municipal solid waste (MSW) leachate. This bacterium is useful as it degrades and utilizes dibutyl phthalate (DBP) as sole carbon source. *Acinetobacter* sp. 33F degraded 82.45% of the initial 2000 mg/L of DBP in minimal salt medium. It degraded 54.36 % of initial DBP in samples collected from phthalate esters contaminated site. Monod model explained the growth kinetics of *Acinetobacter* sp. 33F. The calculated values of μ_{max}, K_s and yield were 0.01616 h^{-1}, 382.92 mg/L and 0.43 respectively. Stoichiometry of DBP degradation suggested that 43 g of microbial biomass was produced for every 100 g DBP consumed. *Acinetobacter* sp. 33F also grows on Di-Ethyl Phthalate (DEP), Di-Octyl Phthalate (DOP), Benzyl Butyl Phthalate (BBP), Mono-Butyl Phthalate (MBP) and Di–isodecyl Phthalate (DIDP) as sole carbon source. Five genes responsible for phthalate esters (PAEs) degradation were amplified from the genome of *Acinetobacter* sp. 33F. *Acinetobacter* sp. 33F can be considered as an important tool for remediation of sites contaminated with phthalate esters.

Keywords: Efficient DBP degradation, growth kinetics, stoichiometry of DBP degradation, phthalate esters degrading genes.

1. Introduction

Phthalic acid esters (PAEs) are widely used as plasticizers (Bauer & Herrmann, 1997). They are responsible for cancers, developmental defects and also affects male reproductive system (Blount *et al.*, 2000; Mo, Cai, Li, & Zeng, 2008; Wu *et al.*, 2011). Dibutyl phthalate is the most widely used phthalate esters (Yangyang Wang, Miao, Hou, Wu, & Peng, 2012). It is classified as top priority toxicant by both United States Environmental Protection Agency (Niazi, Prasad, & Karegoudar, 2001) and China National Environmental Monitoring Center (Niazi *et al.*, 2001) . Landfill sites are used for deposit Municipal Solid Waste (MSW) and also plastic items containing dibutyl phthalate (DBP) and other phthalate esters (Zhang, Wang, Tan, Niu & Su, 2013). There is always possibility to find the PAEs degrading bacteria in landfill. Transformation of PAEs by microorganisms is one of the important methods to remove them from the polluted environments. Bacteria from various genera, including *Sphingomonas, Comamonas, Burkholderia, Viarovorax, Pseudomonas* etc. are known to be able degrade of these pollutants (Chang & Zylstra, 1998; Kong *et al.*, 2012; Shailaja, Ramakrishna, Mohan, &

Sarma, 2007; Vamsee-Krishna, Mohan, & Phale, 2006; Vega & Bastide, 2003; Y Wang, Y Fan, & J-D Gu, 2003; Y. P. Wang & Gu, 2006; X.-R. Xu, Li, and Gu, 2005).

The aim of the study is to isolate and characterize DBP degrading bacteria and examine the efficiency of DBP degradation by bacteria in contaminated samples. Genes responsible for PAEs degradation has been mapped in the total genome of *Acinetobacter* sp. 33F in this study and this is first time report of an efficient degradation by bacteria from the genus *Acinetobacter*.

2. Materials and Methods

2.1 Chemicals

HPLC grade monobutyl phthalate, dioctyl phthalate, protocatechuate, di-isodecyl phthalate, benzyl butyl phthalate, diethyl phthalate and dibutyl phthalate were used as substrate for growth of the bacteria. HPLC grade acetonitrile was used as solvent in the analysis of DBP in HPLC. The chemicals were purchased from Sigma Aldrich and Hichem Lifesciences.

2.2 Isolation and characterization of DBP degrading bacteria

To isolate the DBP degrading bacteria, leachate samples were obtained from municipal solid waste landfill site at Ghazipur, New Delhi, India. The location co-ordinates of Ghazipur landfill site are 28◦ 37’ 22.4’’N and 77◦ 19’ 25.7’’E. The physical parameters of the site are: pH 8.4, TDS 29700, COD 31600, Fe 9.81 and Cl 1174.2. After sampling the samples were stored at 4 °C. Enrichment culture with increasing DBP concentration was used for isolating bacteria. For isolation and screening of DBP degrading bacteria a minimal salt medium (MSM), magnesium sulphate and iron sulphate solution and DBP as sole carbon source was used. MSM was prepared by dissolving 3.5 g K_2HPO_4, 1.5 g KH_2PO_4, 0.27 g $MgSO_4$, 1 g H_4Cl, 0.03 g $Fe_2(SO_4)_3.7H_2O$ and 0.03 g $CaCl_2$ in 1 liter distilled water. The pH of MSM was adjusted to 6.8 and sterilized by autoclaving at 121 °C and 15 psi for 20 minutes. A separate solution of iron sulphate and magnesium sulphate was prepared, filter sterilized with 0.22 µm membrane filter and added to MSM to avoid precipitate formation (Vega and Bastide, 2003). To isolate bacteria, 100 ml MSM with one milliliter magnesium sulphate and iron sulphate solution and 10 mg/L. DBP was inoculated with one milliliter leachate in an Erlenmeyer flask. The flask was incubated in an incubator shaker at 30 °C and 180 rpm for 72 hours. After 72 hours 10 % of the culture was transferred to fresh medium with increasing DBP concentration. DBP concentration was increased in further enrichments till the concentration reached up to 2000 mg/L. Culture from 2000 mg/L flask was streaked on MSM-agar plate containing DBP. A single colony from the plate was cultured in fresh medium and again streaked on fresh MSM-agar plate. The process was repeated six times to get pure cultures. A colony numbered 33, which was able to grow in presence of DBP was designated as strain 33C.

Scanning electron microscopy (SEM) and Transmission Electron Microscopy (TEM) of strain 33C were performed at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi. For electron microscopy, bacterial cells were fixed in 3 % glutaraldehyde at room temperature for 3 hours. After fixation bacterial cells were washed with 0.1 M phosphate buffer thrice for 10 min. Post fixation was performed in 1-2% osmium tetroxide solution followed by dehydration in gradation of ethanol with water (K C A Smith, 1955) Characterization of *Acinetobacter sp. 33F* was performed using biochemical tests (Vos *et al.*, 2011). To identify whether the isolated species is a single bacterial species or a consortium of bacteria, Denaturing Gradient Gel Electrophoresis (DGGE) was performed. For DGGE, a denaturing gradient ranging from 22 % to 55 % in 6 % acrylamide was formed. Acrylamide was formed by using 7 M urea and 40 % deionized formamide. Electrophoresis of the 16S-rRNA gene fragment was performed at 60 °C and 130 V for 4 h(Ercolini, 2004). To assess the capability of *Acinetobacter* sp. 33F to cleave the benzene ring, Rothera`s test was performed. For the test 5 ml of bacterial culture was saturated with solid ammonium sulfate and

mixed with few drops of 2% sodium nitroprusside solution and liquor ammonia. The mixture was left for 15 min. A bluish-purple ring indicates the presence of the ketone bodies in it (Rothera, 1908).

2.3 16S-rRNA gene PCR, sequencing and phylogenetic studies

The identification of the isolated bacterial strain was performed by amplifying the 16S-rRNA gene from the genome of strain 33C. The genomic DNA of the strain 33C was isolated using Fast DNA® SPIN Kit for soil from MP Bio. The 16S-rRNA gene was amplified using bacterial universal primers: 27F and 1492R by PCR. For PCR a 50 µl reaction was used, which contained 25 µl PCR master mix (Thermo Scientific), 2 µl forward primer, 2 µl reverse primer, 23 µl sterile water and 1 µl genomic DNA. Time programming used for the thermo cycler (Applied Biosystems Gene Amp PCR system 9700) was: 10 min at 95 °C, 35 cycles, 60 sec at 95 °C, 90 sec at 54 °C, and 60 sec at 72 °C and 5 min at 72 °C. After amplification the PCR products were purified using HiYield™ PCR DNA Mini Kit from Real Genomics™ (Ref catalogue no. YDF100). The gel purified amplification products of the isolated bacterial strains were sequenced at DNA sequencing facility of UDSC, University of Delhi, New Delhi, India. The sequencing was performed by dideoxy termination method using Applied Biosystems with bacterial universal primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). Forward and reverse sequences were combined using BioEdit program. The sequences obtained were submitted to NCBI with accession number KM107909.1. The sequencing results were analyzed for phylogenetic studies using Basic Local Alignment Search Tool (BLAST) from NCBI and compared with the sequences present in the database.

2.4 Substrate utilization test

To examine the ability of *Acinetobacter* sp. 33F to grow and utilize other PAEs substrate utilization test was performed. The substrate used were: di-ethyl phthalate (DEP), monobutyl phthalate (MBP), di-isodecyl phthalate (DIDP), di-octyl phthalate (DOP), benzyl butyl phthalate (BBP) and protocatechuic acid (PC). The substrates were used at a concentration of 2000 mg/L and no other source of carbon was provided. A negative control without any substrate was also used. Biomass was measured in the form of optical density at 600 nm using Perkin Elmer Lambda 25 uv/vis spectrophotometer.

2.5 Degradation kinetics, growth kinetics and stoichiometry of DBP degradation by Acinetobacter sp. 33F

Biodegradation experiments were conducted by inoculating *Acinetobacter* sp. 33F in MSM medium supplemented with DBP at a concentration of 2000 mg/L. After inoculation, the flasks were incubated at 30 °C and 180 rpm in incubator shaker for 192 hours. Sterile controls of MSM without inoculation of *Acinetobacter* sp. 33F were also used. For analysis of the residual amount of DBP, samples were collected every 24 hours. After collection, the samples were mixed with ethyl acetate in a ratio of 1:1 and centrifugation at 12,000 rpm for 3 min was performed to separate the organic and aqueous phases. The aqueous samples were extracted thrice, ethyl acetate was evaporate to dryness and the residue was dissolved in 10 ml of methanol. 1 ml of the mixture was filtered through a 0.22 µm membrane filter and filtrate was transferred to auto sampler vials for Gas Chromatography analysis (Jin *et al.*, 2011).

To determine the efficiency of DBP degradation by *Acinetobacter* sp. 33F, phthalates contaminated samples in the form of leachate were collected from the landfill site at Ghazipur, New Delhi. This landfill site is contaminated with domestic wastes for decades. Before starting the degradation experiments, the amount of DBP was quantified in the leachate. In the experiments, leachate was used as medium for inoculation of *Acinetobacter* sp. 33F and no other nutrients were added. The bacteria were inoculated in leachate in Erlenmeyer flasks and flasks were incubated at 30 °C and 180 rpm. Samples were collected every 24 hours for further analysis. A standard curve for calculation of residual amount of DBP was prepared using HPLC. Samples at 0 hour, 24 hours, 36 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours, 168 hours and 172 hours were analyzed to determine DBP degradation. The residual DBP was determined using Shimadzu HPLC system. 20 µl of filtrate was injected to the HPLC system. Analysis of the samples was performed using C18

column Ascentis® C 18, 5µm, 25cm x 4.6cm from Sigma Aldrich. In analytical method, a gradient programing was used. Gradient program consisted of two mobile phases: mobile phase A consisting of water/acetonitrile (15:85) v/v and mobile phase B consisting of 100 % acetonitrile. The gradient programing was as follow: 0 - 3 min a 100 % of A, 6.5 – 19.5 min 100 % of B. A total flow rate of 0.6 ml/min was maintained. Run time of the samples was 45 min. DBP was detected using a UV detector at 225 nm (Thuren, 1986). Identification of the intermediates was performed using Shimadzu Gas Chromatography – Mass Spectrometry (GC –MS) system from the Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, India, with column temperature of 100 °C, injection temperature 250 °C, total flow 16.3 ml/min. Kinetics studies were performed by growing *Acinetobacter* sp. 33F in MSM supplemented with DBP at initial concentrations of 100 mg/L, 200 mg/L, 500 mg/L, 1000 mg/L, 1200 mg/L and 1500 mg/L. Samples for the growth measurement and degradation experiments were collected every 24 hours. Growth of the bacteria was measured by measuring the optical density of the bacterial culture and samples were prepared for HPLC analysis as described in the analytical methods. To calculate the biomass in terms of dry weight, *Acinetobacter* sp. 33F was grown in MSM supplemented with DBP. Dry weight of bacterial biomass was measured by inoculating *Acinetobacter* sp. 33F in 1 liter MSM supplemented with 2000 mg/L of DBP. Then bacterial cells were separated from supernatant by centrifugation. Bacterial cells were filtered in a membrane filter of 0.45 µm membrane filter and dried in an oven at 100 °C. Dried biomass was measured on weighing balance (Bratbak and Dundas, 1984).

2.6 Identification of phthalate esters degrading genes

Identification of the phthalates degrading genes was performed by selecting primers from the literature and these primers were used to amplify the genes from the genomic DNA of strain 33C *using* PCR (Table1).

Table 1. List of primers used for amplification of phthalate esters degrading genes from the genome of strain 33C.

Primer name	Gene name	References
Oph A1	3,4-dioxygenase oxygenase component large subunit	(Han 2008)
Oph A2	3,4-dioxygenase oxygenase component small	(Han 2008)
Oph B	Phthalate dihydrodiol dehydrogenase	(Han 2008)
Oph C	3,4-Dihydroxyphthalate decarboxylase	(Han 2008)
Oph D	d-galactonate transporter	(Han 2008)
Oph H	Hemerythrin like metal binding protein	(Han 2008)
Oph R	transcriptional regulator, MarR family	(Han 2008)
FEH	Phthalic ester hydrolase	(Stanislauskienė *et al.*, 2011)
HFDH	3,4-dihydroxy-3,4-dihidrophthalate dehidrogenase	(Stanislauskienė *et al.*, 2011)
FOXG	phthalate dioxygenase large and small subunits	(Stanislauskienė *et al.*, 2011)
FOXGS	ferredoxin and reductase subunits	(Stanislauskienė *et al.*, 2011)
FDK	3,4-dihidroxyphthalate-2-decarboxylase	(Stanislauskienė *et al.*, 2011)
Ptr A	Transpoeter ATPase	(Choi *et al.*, 2005)
Tph A2	Terephthalate 1,2-dioxygenase oxygenase component large subunit	(Han 2008)
Tph A3	Terephthalate 1,2-dioxygenase oxygenase component small subunit	(Han 2008)
Tph B	Terephthalate dihydrodiol dehydrogenase	(Han 2008)

PCR protocol used was as follow: 5 min at 95 °C, 30 cycles of 30 sec at 95 °C, 30 sec at Tm of corresponding primers, 90 sec at 72 °C and final extension for 7 min at 72 °C (Han, 2008). The amplification products were gel purified and sequenced at DNA sequencing facility of UDSC, University of Delhi, New Delhi, India. Sequencing of the amplicons was performed by using specific primers for each gene and amplicons were cloned in M13 vector in the sequencing facility.

2.7 Statistical analysis

Statistical analysis of DBP degradation was performed by F-test and one way ANOVA with three replicates data by using data analysis tool pack in Microsoft excel. For analysis the hypothesis made at 5 % level of significance and P values and F values were calculated.

3. Results and Discussions

3.1 Isolation and characterization of DBP-degrading bacteria

The strain 33C was aerobic, gram negative, transparent and forms round colonies on MSM agar plates. Biochemical characterization of strain 33C is presented in table 2. From the table 2 it was observed that the presented characteristics are specific for the bacteria from the genus *Acinetobacter (*Constantiniu, Romaniuc, Iancu, Filimon, & Taraşi*,* 2004*).*

Table 2. Biochemical tests used for characterization of strain 33C.

S. N.	Test	Principle	*Acinetobacter* sp. 33F
1.	Gram staining	Ability to retain stain	-
2.	Citrate utilization	Detects capability of organism to utilize citrate as a sole carbon source	-
3.	Indole	Detects deamination of tryptophan	-
4.	Malonate utilization	Detects capability of organism to utilize sodium malonate as a role carbon source	+
5.	Glucose	Glucose utilization	-
6.	Oxidase	Detects oxidation capability	-

+ positive reaction, - negative reaction.

Strain 33C was resistant for growth on antibiotics such as penicillin, streptomycin, kanamycin and chloramphenicol (Table 3).

The strain showed only a single band of 16S-rRNA gene on DGGE which confirm it to be a single strain and not the consortium of bacteria. Therefore this culture was considered as pure culture.

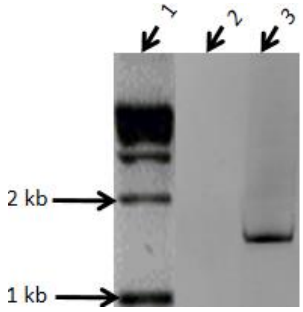


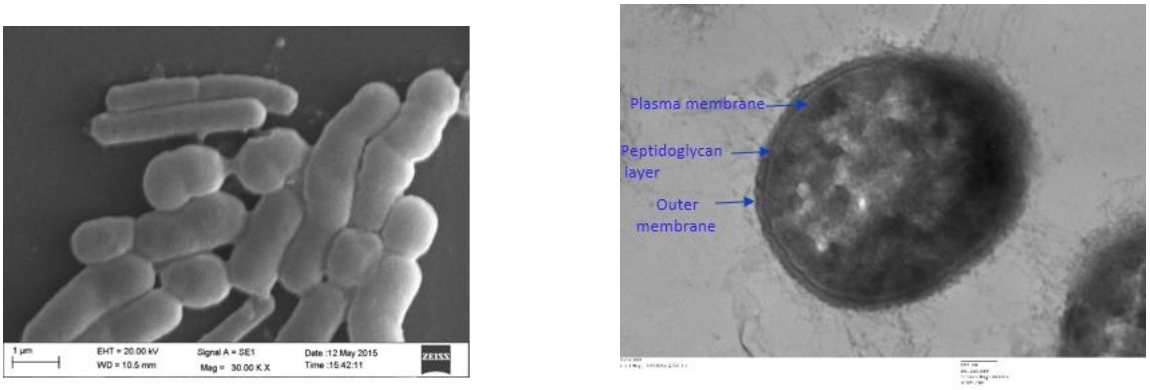
Figure 1. 16S-rRNA-DGGE profile of *Acinetobacter* sp. 33F. Negative control was represented by C. No band was observed in the negative control. Lane 1 – DNA ladder, lane 2- negative control and lane 3 – 16S-rRNA of *Acinetobacter* sp. 33F

Table 3. Antibiotics sensitivity tests of strain 33C.

Antibiotic	Concentration (µg/ml)	*Acinetobacter* sp. 33F
Ampicillin	10	Do not grow
	25	Do not grow
	50	Do not grow
	100	Do not grow
Penicillin	10	Grows
	25	Grows
	50	Grows
	100	Do not grow
Streptomycin	10	Grows
	25	Grows
	50	Grows
	100	Grows
Kanamycin	10	Grows
	25	Grows
	50	Grows
	100	Do not grow
Tetracycline	10	Do not grow
	25	Do not grow
	50	Do not grow
	100	Do not grow
Chloramphenicol	10	Grows
	25	Grows
	50	Grows
	100	Grows

SEM at 30 KX revealed that *Acinetobacter sp. 33F* was bacillus in shape, appears smooth, without flagella with a length of ~ 2.5 µm in length and 1 µm in breadth. TEM at 20 KX revealed the presence of outer membrane, peptidoglycan layer and plasma membrane.

16S-rRNA gene region of strain 33C found maximum similarity with *Acinetobacter baumanii*. It is interesting to note that the *Acinetobacter baumanii* is responsible for many opportunistic infections in humans (Visca, Seifert, & Towner, 2011). But in this study, *Acinetobacter sp. 33F* from genus Acinetobacter proved to be useful as it has the potential to utilize and degrade DBP. This bacterial strain was designated as *Acinetobacter* sp. 33F. 16S-rRNA gene sequence was submitted to nucleotide database of NCBI with the accession number: KM107909.1. The phylogenetic relationship of *Acinetobacter sp. 33F* is represented in figure 3.



(a) (b)

Figure 2 (a) Scanning electron microscope image of strain 33C. The image was captured at a magnification of 30 KX. The bacteria in the image were rode shaped. Some of the bacteria are in dividing stage and they are clumped together, (b)Transmission electron microscope image of strain 33C. The image shown in the transverse section of scanning electron microscope image of *Acinetobacter* sp. 33F at a magnification of 20 KX.

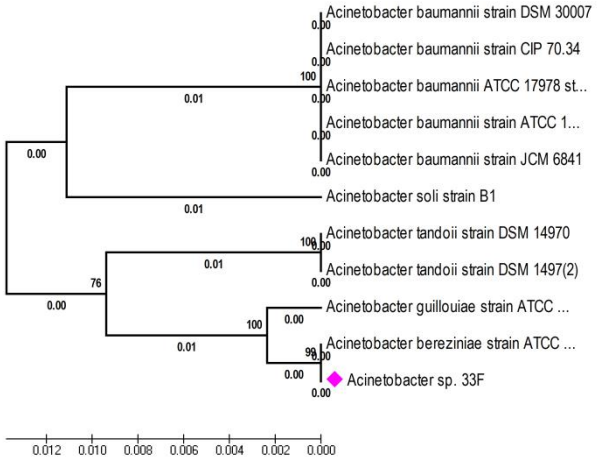


Figure 3. Phylogenetic tree of *Acinetobacter* sp. 33F

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.05032880 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown above the branches. The tree is drawn to scale, with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1281 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. The values like 0.002, 0.004 denotes evolutionary distance between different species and values such as 99 and 100 denotes the similarities between different species.

3.2 Substrate utilization test

Acinetobacter sp. 33F was grown in diethyl phthalate (DEP) , dioctyl phthalate (DOP), benzyl butyl phthalate (BBP) , mono - butyl phthalate (MBP), di – isodecyl phthalate (DIDP) and protocatechuic acid (PC) at a concentration of 2000 mg/L. *Acinetobacter sp. 33F* was able to grow on all the six compound in addition to DBP (Table 4).

Table 4. Growth of *Acinetobacter sp.* 33F on substrates other than DBP.

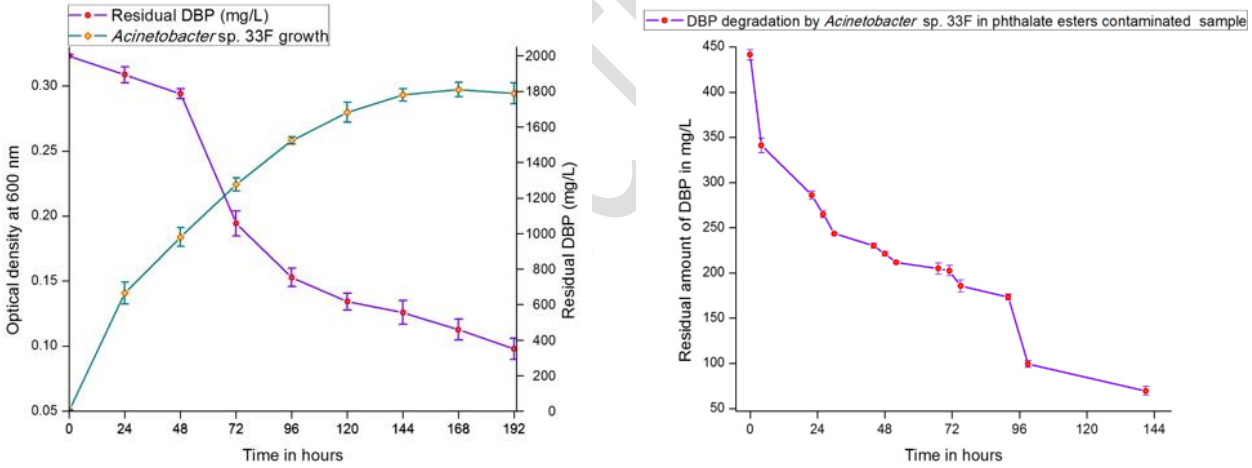
Substrates	Growth of *Acinetobacter* sp. 33F
Protocatechuate (PC)	+++
Mono-butyl phthalate (MBP)	+++
Di-ethyl phthalate (DEP)	++
Benzyl butyl phthalate (BBP)	+
Di-isodecyl phthalate (DIDP)	+
Di-octyl phthalate (DOP)	+

+++ vigorous (OD_{600} 1.22), ++ good (OD_{600} 1.18) , + moderate (OD_{600} 1.10).

3.3 Biodegradation of DBP by Acinetobacter sp. 33F

Biodegradation of DBP was assessed in samples collected from culture flasks every 24 hours.

From the growth curve of *Acinetobacter* sp. 33F it was observed that *Acinetobacter* sp. 33F grows at faster rate during initial 144 hours and it grows exponentially in this period (Figure 4(a)).



(a) (b)

Figure 4. (a) DBP degradation by *Acinetobacter* sp. 33F in MSM supplemented with 2000 mg/L, (b) DBP degradation by *Acinetobacter* sp. 33F in samples contaminated with phthalate esters

After 144 hours there is a decrease in the growth rate of *Acinetobacter* sp. 33F and it is clearly observed from the figure that there is no distinct demarcation between stationary and decline phases of *Acinetobacter* sp. 33F. Therefore, it can be concluded that at the time point 168 hours is the point where the exponential phase ends and decline phase starts. It is also observed from the figure that during 24 hours there is very rapid growth of *Acinetobacter* sp. 33F. Such that maximum specific growth of 0.08966 was observed during during first 24 hours. The residual amount of DBP after 192 hours was 351.52 mg/L from an initial amount of 2000 mg/L. Therefore, *Acinetobacter* sp. 33F degraded 82.45 % of DBP in 192 hours. It was observed that the

maximum amount of DBP i.e. 729.46 mg/L of DBP was degraded between 48-72 hours. It should be expected from the figure 5 that because maximum specific growth rate was observed during first 24 h therefore maximum amount of DBP should be degraded during first 24 h. *Acinetobacter* sp. 33F first adapt itself to produce sufficient population that is enough to degrade the DBP. After 72 h it was observed from the figure that as the rate of growth of *Acinetobacter* sp. 33F increases the rate of degradation of DBP also increases. No growth was observed in the sterile controls where no *Acinetobacter* sp. 33F was inoculated. Similarly no change in the amount of DBP was observed in controls. P value, F value and F critical calculated were 0.96, .0375 and 3.40 respectively. Statistical analysis shows that study was statistically significant. When *Acinetobacter* sp. 33F was grown in phthalate esters contaminated samples, it was observed that the residual DBP at the end of 144 h was only 15.64 % of the initial amount (Figure 4(b)). The amount of DBP measured in the phthalate ester contaminated sample was 441 mg/L and after degradation by *Acinetobacter* sp. 33F it remained only 69.63 mg/L. Therefore, *Acinetobacter* sp. 33F degraded 84.35 % of DBP. P value, F value and F critical calculated for DBP degradation in contaminated samples were 0.99, .008 and 3.40 respectively. Statistical analysis shows study is significant.

3.4 Biodegradation kinetics and stoichiometry of DBP degradation

In kinetics studies the specific growth rate (μ) of *Acinetobacter* sp. 33F was calculated for different initial concentrations of DBP viz. 100 mg/L, 200 mg/L, 500 mg/L, 1000 mg/L, 1200 mg/L and 1500 mg/L. A plot of μ and S_{av} was drawn. From the plot it was observed that there was no effect on the specific growth of the bacteria after substrate concentration of 1000 mg/L of DBP. Growth pattern was explained by a saturation model and Monod model fits the growth pattern of *Acinetobacter* sp. 33F (Figure 5).

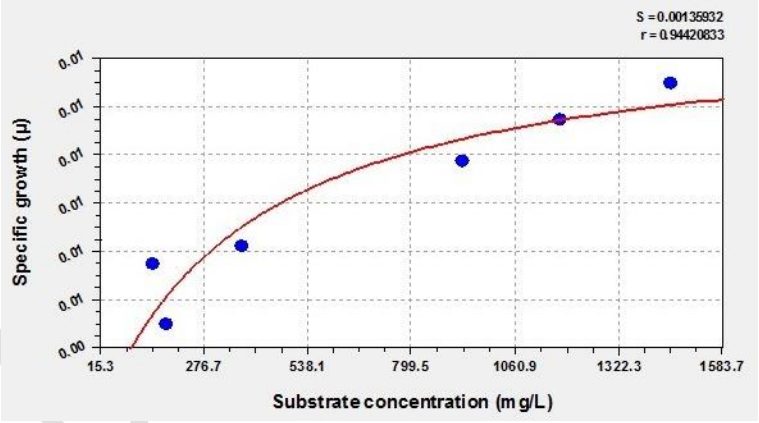


Figure 5. Kinetics of DBP degradation by *Acinetobacter* sp. 33F

Equation for the Monod model is as follows:

$$\mu = \frac{\mu_{max} * S}{(K_s + S)}$$

Values of the coefficient are as follows:

μ_{max} = 0.01616 h^{-1}

K_s = 382.92 mg/L

Yield = 0.43

Various researcher explained degradation kinetics of organic pollutants by first order equations (Lu *et al.*, 2009; X.-R. Xu *et al.*, 2005; Zeng, Cui, Li, Fu, and Sheng, 2004). A second order equation was also reported for

degradation phthalate esters by algae, *Chlorella pyrenoidosa (Yan, Ye, & Yin, 1995).*In this study neither first order nor second order equations were able to explain the degradation kinetics of DBP by *Acinetobacter* sp. 33F, but Monod model explained the degradation of DBP successfully.

Stoichiometry is required to explain biomass production from the substrate. Equation (1) represents the general equation for degradation of DBP (Shuler and Kargi, 2002). Equation (2) represents the balanced stoichiometry for DBP.

$$aC_{16}H_{22}O_4 + bO_2 + cNH_3 \rightarrow dC_5H_7O_2N + eH_2O + fCO_2 \quad (1)$$

DBP Biomass

Balanced equation for stoichiometry of DBP:

$$100C_{16}H_{22}O_4 + 1740O_2 + 43NH_3 \rightarrow 43C_5H_7O_2N + 1014H_2O + 1390CO_2 \quad (2)$$

$$\text{Yield} = \frac{\text{Biomass}}{\text{Substrate}} = \frac{\Delta X}{\Delta S} = 0.43 \text{ g/g}$$

Therefore for every 100 g of DBP consumed by *Acinetobacter* sp. 33F, 43 g of new microbial cells are produced.

3.5 Identification of the metabolic intermediates

Biodegradation of phthalate esters follow a series of consecutive steps where phthalate ester is first mineralized to its monoester by esterases. The sequential cleavage of ester bond lead to formation of phthalate monoester and to phthalic acid (PA). PA is converted to protocatechuate (PC) and PC get metabolized to carbon dioxide and water (Benckiser and Ottow, 1982; Eaton and Ribbons, 1982; Engelhardt & Wallnöfer, 1978; J. Wang, Liu, & Qian, 1995; Yingying Wang, Yanzhen Fan, & Ji-Dong Gu, 2003; X.-R. Xu *et al.*, 2005; X.-R. Xu, Li, Gu, & Li, 2007; X. R. Xu, Li, & Gu, 2006). The two ester linkages in phthalate esters are cleaved by two different bacteria (Cartwright, Owen, Thompson, and Burns, 2000; Jiaxi Li, Gu, and Yao, 2005; J. Li & Gu, 2007). In this study two degradation intermediates of DBP were identified using GC-MS. These intermediates were isobutyl propyl phthalate ester and di-ethyl phthalate ester (Figure 6).

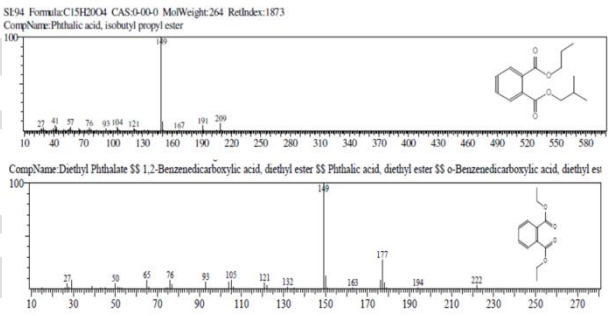


Figure 6. Metabolic intermediates of DBP degradation identified through GC-MS

Samples for 48 hours and 72 hours were analyzed on GC-MS were identified as isobutyl propyl phthalate ester and di-ethyl phthalate ester. A few very small peaks were also detected in the samples but their further characterization was not possible because they could not be isolated. In other studies it was reported that DBP was first converted to MBP and then to phthalic acid (PA) (X.-R. Xu *et al.*, 2005). Presence of MBP and PA were also reported in other studies of DBP degradation (Benckiser and Ottow, 1982; Gu, Li, and Wang, 2004; Yingying Wang, Fan, and Gu, 2004).

MBP and PA were not detected in this study. The reason for nonappearance of MBP and PA in the GC-MS chromatogram may be their short half-lives or very low concentration.

3.6 Identification of phthalates degrading genes

Transformation of DBP by bacteria is mediated by crucial enzymes which are operated at each step in a degradation pathway. In the molecular analysis of PAEs degradation five genes from the genome of *Acinetobacter* sp. 33F were successfully amplified using PCR (Figure 7).

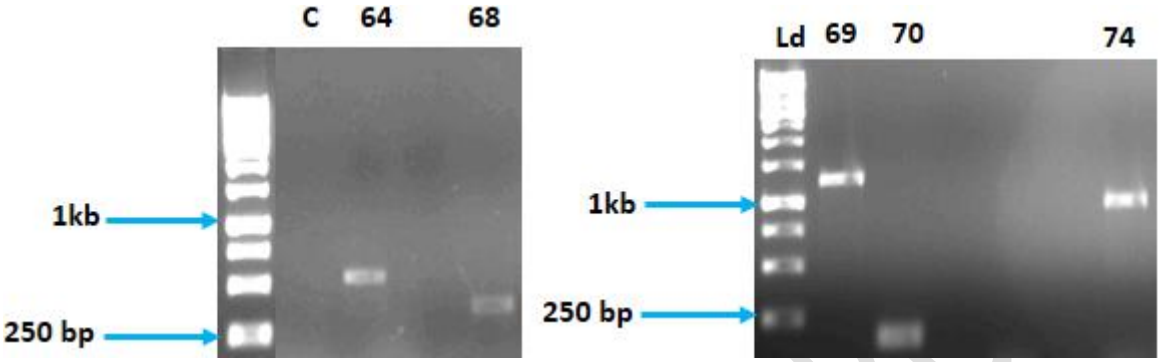


Figure 7. Genes amplified from the genome of Acinetobacter sp. 33F. C-negative control, 64- 4,5-dihydroxyphthalate decarboxylase, 68-Terephthalate dehydroxygenase, 69-Terephthalate dioxygenase, 70-Hemerythrin like metal binding protein and 74-Phthalate dehydrogenase

Table 5. Phthalate esters degrading genes amplified from the genome of *Acinetobacter sp.* 33F

Amplicon number	Prime name	Enzyme name	Amplicon size	Similarity	Identity
64	Oph-C	4,5-dihydroxyphthalate decarboxylase	1 kb	phtD gene for 4,5-dihydroxyphthalate decarboxylase from Comamonas testosteroni (Accession number: D16537.1)	99 %
68	Tph-B-F2,R3	Terephthalate dehydroxygenase	600 bp	Terephthalate 1,2 dioxygenase gene from Comamonas sp. E6 (Accession number: AB238679.1)	100 %
69	Tph-A2-N1,C1	Terephthalate dioxygenase		Reductase component of terephthalate 1,2-dioxygenase from Comamonas sp. E6 (Accession number: AB238679.1)	99 %
70	Oph-H	Hemerythrin like metal binding protein	200 bp	Sequence not available	-
74	Oph-B	Phthalate dehydrogenase	200 bp	Sequence not available	-

After amplification, the gene fragments were gel purified and sequenced. The obtained sequences were compared with nucleotide sequences using BLAST in NCBI database.

The first gene amplified was 4,5-dihydroxyphthalate decarboxylase (oph-C). This gene sequence shared 99 % identity with 4,5-dihydroxyphthalate decarboxylase gene from *Comamonas testosteroni*. The enzyme coded

by this gene converts the phthalate esters to 4,5-dihydro-4,5-dihydroxyphthalate (Lee, Omori, and Kodama, 1994). Second enzyme amplified was terephthalate dehydroxygenase (Tph-B-F2,R3). This gene sequence shared 99 % similarity with terephthalate 1,2 dioxygenase gene from Comamonas sp. Both oxygenase and reductase components of terephthalate 1,2-dioxygenase are involved in conversion of terephthalic acid to protocatechuate (PC) (Sasoh *et al.*, 2006) . The presence of a terephthalate gene in the genome of Acinetobacter sp. 33F provide evidence for possibility of Acinetobacter sp. 33F to degrade phthalates esters isomer terephthalate. Third enzyme amplified was terephthalate dioxygenase (Tph-A2-N1,C1). This gene sequence shared 99 % similarity with Reductase component of terephthalate 1,2-dioxygenase from Comamonas sp. E6. Two other genes: hemerythrin like metal binding protein and phthalate dehydrogenase were also amplified but sequencing of these genes was not possible due to poor amplification.

This study is unique as isolated strain was efficient in degradation of DBP. Also in this study the applied aspect of DBP was examined in the samples contaminated with phthalate esters. This can be a complete study where not only the degradation kinetics of *Acinetobacter* sp. 33F was performed but also the genes were identified which are responsible for degradation of phthalate esters.

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