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# Biodegradation Study of Diethyl Phthalate by Bacteria Isolated from NIT Plastic Garbage Dump Site

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***Abstract*— Waste generated from municipal area is a major reason of pollution, and it leads to environmental degradation, destruction of the ecosystem and pretences great risks to public health. Organic pollutants are potential hazardous chemicals for human health and toxic to living things in the receiving natural bodies. Phthalate esters (PEs) are among the most common organic pollutant since they have become widespread in the environment and found in sediments, natural waters, soils, plants, landfill leachates, biota including human tissue and aquatic organisms. In the present work, degradation of Diethyl Phthalate (DEP) by isolated bacterial strain is studied. The bacterial strain was isolated from garbage dumpsite at NIT, Rourkela, Odisha, India. It was identified by biochemical and morphological tests. Furthermore, the process parameters affecting the bacterial growth (pH, inoculum size and temperature) and the degradation of the compound were optimized using one factor at a time. The biodegradation of DEP was studied at various concentrations (250-1000 mg/l). Mass transfer study was performed to determine the Oxygen Transfer Rate (OTR) and mass transfer coefficient.**

***Keywords* — Pollution, Phthalate esters, Diethyl phthalate, Biodegradation, Oxygen Transfer Rate, Mass transfer coefficient.**

## I. INTRODUCTION

Phthalate are esters of phthalic acid and are mostly used as plasticizers, which are materials additional to plastics to increase their elasticity, transparency, resilience, and durability. The plasticizers are existing in market in several varieties with specifications for composition, dosages etc. Phthalates are generally used as additives in various plastics and other substances that are present in many consumer products. Plastics such as PVC can be made soft and flexible by the use of them. They can be released from consumer products into the environment because they are not chemically bound to plastics. There is public anxiety regarding phthalates because of their extensive use and existence in the environment as well as their probable effects on human health. The extensive use of phthalates in consumer products leads to universal and continuous exposure of humans to these harmful chemicals. Phthalates were assumed to produce endocrine-disrupting effects in rodents, where regular exposure to these compounds was observed to bring developmental and reproductive toxicity [1]. Diethyl phthalate (DEP) is the diethyl ester of phthalic acid, which is at room temperature in the form of liquid and looks like clear substance. Diethyl phthalate is a colourless liquid with a pungent odor. It is commonly used for increasing the flexibility of plastics. While Diethyl phthalate is not a part of the chain of chemicals (polymers) which makes up the plastics, it can be released easily from these products into the environment. These plastics are commonly found in products such as toothbrushes, automobile parts, tools, toys, and food packaging [2]. It enters the environment through industrial effluent, by evaporation into the air from disposal sites, through consumer products, from the burning of plastic products, and by leaking from landfills into soil or water including groundwater. As DEP is consisting of many elements, in air it breaks down to its constituents. Diethyl phthalate due to its adhesive nature may enter the environment by sticking to dust particles. In stagnant or sluggish water bodies the DEP can be broken down by industrial bacteria into non-toxic products. In soils containing organic matter (matter with high levels of carbon), diethyl phthalate may adhere to particles where it may eventually break down and helps it travel. Minor amounts of toxic DEP can build up in aquatic animals [3]. There are numerous ways by DEP can enter our body such as eating, drinking, through the skin. It is easily exposable and occurs near hazardous waste sites, manufacturing facilities, or by consumer products containing the substance. Our body has very less absorptive capacity towards DEP. As it gets into the body it breaks down to other products and can be fatal. Small amounts of the compound remain in the tissues [4]. Several studies indicate that DEP can cause damage to the nervous system as well as to the reproductive organs in males and females [5]. The government has issued certain guidelines for the acceptable limits of DEP that can be released into the atmosphere for the safe exposure of chemical to the public. EPA has classified DEP as a hazardous chemical based on the massive amounts of DEP present in the superfund sites. The Occupational Safety and Health Administration (OSHA) supervises the allowable levels of diethyl phthalate in the workplace in workroom air during an 8-hour workday, 40-hour workweek, as 5 milligrams per cubic meter (mg/m3)

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[6]. The objectives of the present work are isolation and characterization of DEP degrading efficient bacterial strain. Tolerance study of isolated bacterial strain. Optimization of process parameters. Degradation study at various initial concentrations. Mass transfer study in stirred flask

## II. MATERIALS AND METHODS

### *2.1 Chemicals*

Diethyl Phthalate (DEP) was procured from Sigma Aldrich, Nutrient Broth and Nutrient Agar were procured from High Media, Methanol and all other chemicals were procured from Fisher Scientific.

### *2.2 Soil sample collection*

Soil sample was collected from the garbage dump site at NIT Rourkela. Top layer of soil was removed up to 1-2cm and sterile scoop was used for collection of soil sample. Four different samples were taken and mixed in a plastic bag.

### *2.3 Enrichment of DEP degrading strain*

For enrichment of dibutyl degrading strain, standard enrichment technique was used. 1 g of soil was transferred in 50 ppm concentration of DEP containing nutrient broth solution. The flasks were placed in orbital shaker incubator for 12 days until growth was observed at 30°C and 120 rpm. After incubation, 5 ml of enriched culture was taken and transferred to fresh 50 ppm of DEP containing nutrient broth. Same procedure was repeated for two more times for further enrichment of desired bacteria. After completion of enrichment culture, it was transferred onto nutrient agar petri plates and spread on the dish after dilution two times in NaCl solution. The dish was sealed and placed in incubator for 24 hours at 30°C. After 24 hours, different bacterial colonies were grown on the petri plates. Prominent grown colony was selected and transferred into different petri plates to found pure culture. This process was repeated for two more times. The strain was designated as strain CS1.

### *2.4 Study of tolerance capability of isolate for various concentration of DEP*

After getting pure culture, it was transferred into nutrient broth containing various initial concentrations. The flasks were placed in orbital shaker incubator till growth was observed at 30°C and 120 rpm. Same process was repeated for different concentrations of 100-600 ppm of DEP.

### *2.5 Identification of isolated DEP degrading strain*

The isolated strain CS1 was identified on the basis of their morphological, biochemical and molecular characteristics. For SEM analysis of strain CS1, it is required to fix them on glass slide. The overnight grown cultures of strain CS1 was individually centrifuged at 8000 rpm for 10 minutes. The pellet was washed twice with phosphate buffer (pH 7.4) and subsequently fixation done in 2% glutaraldehyde for period of one hour [7]. Fixed specimens were washed with the phosphate buffer (pH 7.4) and dehydrated in ethanol series i.e. 30, 50, 75, 90 and 100% ethanol. The Scanning Electron Microscopy was performed using JEOL (JSM, Japan) Scanning Electron Microscopy attached to an EDX unit, with magnification from 10X up to 400,000X and resolution 3.5 nm [8].

### *2.6 Analytical methods*

After 24 hours 1 ml sample was taken from each flask in small tubes. These tubes were centrifuged at 10000 rpm for 10 minutes. After centrifugation process, biomass and supernatant solutions were separated in different tubes. Tube with biomass part was taken for absorbance study in a spectrophotometer at 600 nm. In the centrifuge tubes of supernatant solution 500µL hexane was added and these tubes were further centrifuged at 5000 rpm for 5 minutes. After centrifugation organic and aqueous phase were separated, organic phase was kept for drying in an oven and aqueous part was discarded. After overnight drying of organic phase 1 ml of methanol was added and it's absorbance was studied in a spectrophotometer at 275 nm.

### *2.7 Optimization of process parameters*

Optimization of medium components and physiological conditions is of primary importance in biodegradation processes. Parameters like initial DEP concentration, pH, temperature, inoculum size and media components have effect on DEP degradation.

#### *2.7.1 pH*

Optimization study was started with optimizing pH and for that three 50 ml minimal salt media (MSM) flasks were prepared. In these three flasks three different pH i.e 6, 7 and 8 were set. 4 ml inoculum and DEP (100 ppm) was added to each flask. All three flasks were kept in incubator shaker for 24 hours.

#### *2.7.2 Inoculum size*

In these three different flasks inoculums concentration i.e 4%, 8% and 12% were taken. pH was maintained between 7. 100ppm DEP was added to each flask. All three flasks were kept in incubator shaker for 24 hours and samples were analysed for DEP degradation.

#### *2.7.3 Temperature*

Three different flasks were kept in incubator shaker for 24 hours at different temperatures 20, 30 and 35°C containing 100 ppm of DEP.

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***2.8 Biodegradation study of DEP at various concentrations***

DEP degradation study by isolated strain CS1 was done at its obtained optimum level of parameters for various initial DEP concentrations (250-1000 ppm). The experiments were performed in 250 ml Erlenmeyer flask containing 100 ml mineral salt medium with various initial DEP concentrations in batch mode and at 120 rpm. Freshly prepared inoculum was used. Each experiment was performed until the remaining concentration of DEP in flask was found to saturate with time. Each experiment was done in triplicate under the same operating conditions and average values of residual DEP concentrations of three independent experiments were reported. The reaction mixture containing all media components except bacterial inoculums were used as control.

***2.9 Mass transfer study in stirred flask***

Minimal salt media containing 100 and 200 ppm concentration of DEP and 4 ml inoculum was taken. It was kept upon a magnetic stirrer and a magnetic bead was kept inside flask. Dissolve oxygen measuring probe was inserted in the mouth of flask to measure dissolved oxygen. This flask was stirred continuously at 1000 rpm and reading of dissolved oxygen was taken at an interval of 3 hours for next 24 hours. The experimental set up for mass transfer study is as shown in Fig. 1.

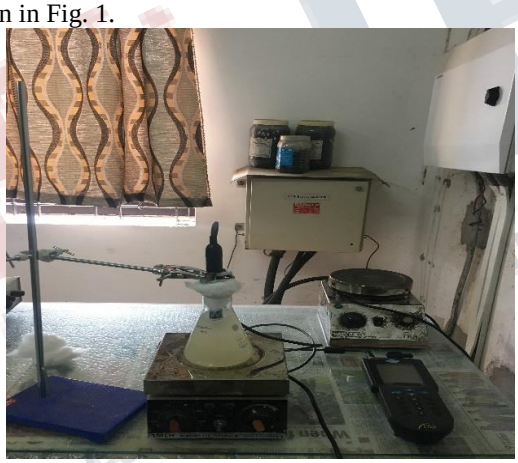

***Fig. 1. Experimental set up for mass transfer study***

OTR was calculated as per following equation [9]

OTR = KL*a*$LO_2$*($PO_2$, gas – $PO_2$, liquid)

Where, KL is mass transfer coefficient, A is specific mass transfer area, $LO_2$ is oxygen solubility and (PO2, gas – PO2, liquid) is driving pressure difference across the gas-liquid inter-face. $PO_2$ value can be calculated by using Henry's law i.e C = KH* $PO_2$, where KH was taken as1.2*10^7 atm/mg/l. KL*a can be calculated by using the correlation given as follows:

$$KLa = n*(V/V_L)^{0.845}$$

where, n is stirring frequency in rpm, V is flask volume (ml), $V_L$ is filling volume (ml).

## III. RESULTS AND DISCUSSION

***3.1 Isolation & Identification of DEP degrading strains***

The obtained pure culture of bacteria was designated as CS1. Biochemical tests was done for the isolated strain. Fig.2 shows the colony morphology of the isolated strain CS1. Morphological and biochemical characteristics of isolates are enlisted in Table 1. The isolated strain CS1 was found to be rod shaped bacillus as shown in Fig. 3.

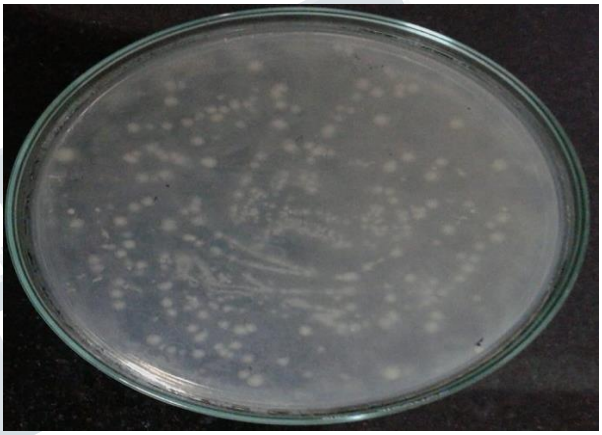

***Fig 2. Colonies observed for strain CS1***

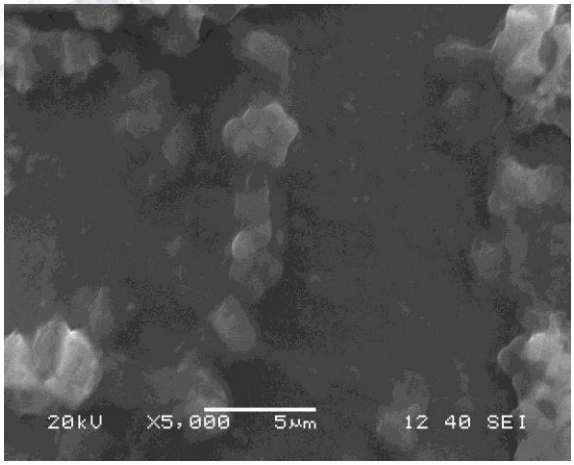


***Fig 3. SEM image of strain CS1***

**3.2 Optimization of process parameters**

Maximum growth of bacteria as well as maximum DEP degradation was observed on pH 7 (Fig. 4). It showed isolate was neutrophilic in nature.

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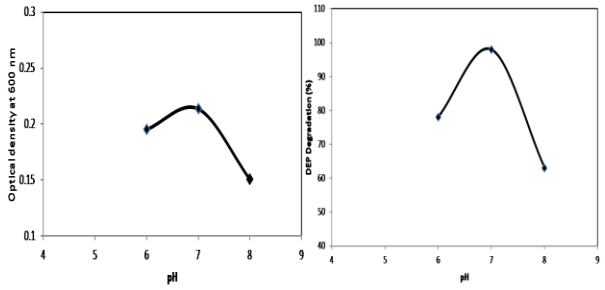
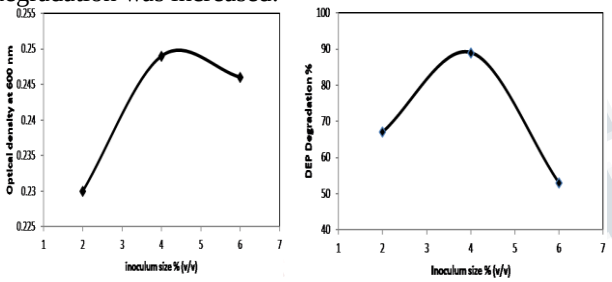



*(A)* *(B)*

***Fig. 4. Optimization of pH (A) Bacterial Growth, (B) DEP degradation***

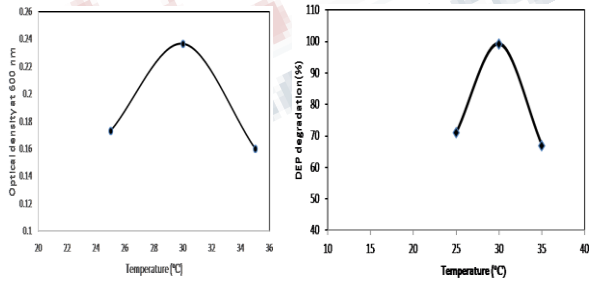
Maximum growth of bacteria as well as maximum DEP degradation was observed at 8% inoculum concentration (Fig. 5). It showed as bacterial inoculum was increased DEP degradation was increased.




*(A)* *(B)*

***Fig. 5. Optimization of inoculum size (A) Bacterial Growth, (B) DEP degradation***

Maximum growth of bacteria as well as maximum DEP degradation was observed at 30ºC (Fig. 6). It indicates that the isolate was mesophilic in nature.




*(A)* *(B)*

***Fig.6. Optimization of temperature (A) Bacterial Growth, (B) DEP degradation***

### *3.3 Degradation study of DEP at various concentrations*

Degradation study was performed at four different concentrations namely 250, 500, 750 and 1000 mg/l. Fig. 7 shows growth profile at various concentrations. Strain showed lag phase from 250 mg/l of DEP concentration and then it was increased as concentration of DEP was increased. At 1000 mg/l, the prominent lag phase was observed.

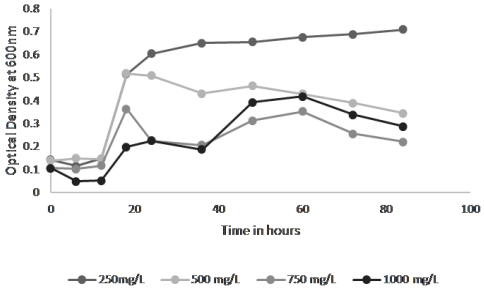



***Fig. 7. Growth profile for strain CS1 at various initial DEP concentrations***

Fig. 8 shows degradation profile at various DEP concentrations. It showed at 77, 60, 54 and 41 % degradation at 250, 500, 750 and 1000 mg/l of DEP concentration. It was observed that as concentration was increased degradation rate was decreased; this might be due to toxicity of DEP at high concentrations.

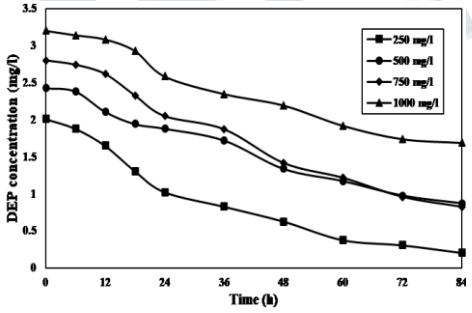
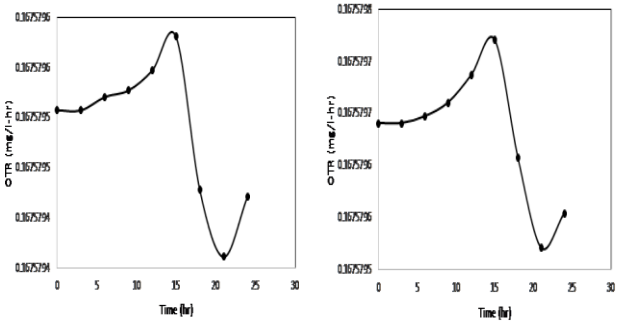



***Fig. 8. Degradation profile for strain CS1 at various initial DEP concentrations***

### *3.4 Mass transfer study*

Mass transfer study was performed in a 250 ml flask in which stirring was done by a magnetic stirrer and dissolved O2 was measured by using a DO meter.




*(A)* *(B)*

***Fig. 9. OTR at different DEP concentration (A) 200 mg/l, (B) 400 mg/l***

From these data, mass transfer coefficient was calculated and Oxygen transfer rate was calculated for two different DEP concentration namely 200 and 400 mg/l. Fig. 9 shows OTR at two different DEP concentrations. As the bacterial growth was increased, OTR was increased and after some duration, it was start to decreases. This result indicates that the isolate requires oxygen for its growth during DEP degradation.

## IV. CONCLUSION

Soil samples were collected from plastic waste dumpsite at NIT, Rourkela and further, subjected to enrichment in order to obtain potential DEP degrading strain. After enrichment, prominent grown DEP degrading bacterial strain was selected from morphologically distinct colonies and it was designated as strain CS1. It was observed that isolated strain CS1 tolerate upto 1000 ppm of DEP concentrations. It showed maximum DEP degradation at pH 7, inoculum size 8% (v/v) and 30ºC temperature. It shown 77, 60, 54 and 41 % degradation at 250, 500, 750 and 1000 mg/l of DEP concentration within 84 h. Mass transfer study in stirred flask indicated that oxygen concentration in liquid phase is limiting factor for microbial growth. The observed biodegradation study of isolate at these conditions indicates that plastic dumpsite is a possible source of potential microbe and it can efficiently degrade high concentrations of DEP present in the environment.

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