ISSN (Print): 0972-4397
ISSN (Online): 0974-0805
Journal of Ecophysiology and Occupational Health, *Vol 20(3&4),DOI: 10.18311/jeoh/2020/25041, 140–144, July–December 2020*

Biodegradation of Di-(2-Ethylhexyl Phalate) by *Bacillus antracis* (Accession no. KJ085972.1)

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Abstract

DEHP is one of the most broadly used PAEs (Phthalic Acid Esters) as a plasticizer in Polyvinyl Chloride (PVC) manufacturing. The DEHP and its other monoester metabolites are considered as very harmful for animal and human and affect the endocrine system. The strain isolated in this study is very efficient in degrading the DEHP. The degrading bacterial strain *Bacillus anthracis* is used in this study to determine the biological degradation potential of DEHP. Bacteria play very important roles in DEHP degradation in the environment under various conditions. The selected strain in for DEHP degrading bacteria designated as strain T-10 is optimized at the different conditions for its maximum activity at different temperatures, pH, chemicals like carbon and nitrogen source identified with degradation potential of more than 85%.

Keywords: Bacteria, Biodegradation, DEHP, Plasticizer

1. Introduction

Phthalic Acid Esters (PAEs) are very widely used as plasticizers for manufacturing of Polyvinyl Chloride (PVC) and other plasticizers which belong to family of refractory organic compounds. These PAEs are xenoestrogenic and can disrupt the endocrine so it is classified as endocrine-disrupting chemicals[1]. Among all the plasticizers Di-(2-ethylhexyl)-phthalate (DEHP) is the most widely-used. Previously it is reported that DEHP and its others metabolites like mono (2-ethylehxyl) phthalate (MEHP) and Phthalic Acid (PA), which are among the major intermediate produced during the degradation of DEHP and these metabolites are adverse effect on neurological, respiratory and immune system of the living organism. As reported 10[6] tons of DEHP is produced annually and it is having acute toxicity with mutagenic and carcinogenic effects[2]. So, finding out an alternative method for DEHP degradation which is having more environmental persistence and other remediation methods for removing DEHP and its metabolites from the environment is currently needed.

The breakdown of the DEHP and its metabolites by the different bacterial strains is the most efficient method of the degradation of such hazardous pollutants. Various research studies have reported the biodegradation of Phallic acids like the DEHP in the aerobic condition in different environments including soil and water[3]. In a study of 14 commercially used phthalate biodegradation by an acclimated shake flask CO_2 evolution, it was found that some bacterial strains like denitrifying bacterium *Pseudomonas sp*. strain P136 have the properties of anaerobically degrading the phthalate and other aromatic compounds[4,5].

Several studies have shown that bacteria have very important role in the degradation of the phthalates in the variable environment conditions[6]. The primary goal of this work is to focused on the DEHP biodegradation by isolating the potent bacteria, under various environments and chemical condition, one of the promising DEHP degrading bacteria, the selected strain in this study is designated as strain T-10 and it is further optimize at different environmental conditions for the maximum degradation potential.

2. Materials and Methodology

Analytical grade DEHP was obtained from Merck; other chemicals used for the preparation of the media were obtained from Sigma Chemical, Merck, Hi media and Qualigens. Prior of using all the glassware were kept in hot air oven at the temperature of 550°C and other equipments were cleaned by washing with analytical grade.

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2.1 Samples Collection

Waste soil sample was collected from municipal landfills area of Patna. The collected waste samples were sieved by using 2-mm mesh and were further dried in sterilized condition up to the water content of 7% (wt/wt) and it was further kept in aseptic condition at 5°C to be used in further study.

2.2 Microorganisms

By using the enrichment and shaking culture method bacterial strains were isolated with the potential of DEHP-degrading from the waste amended soil sample. Serial dilutions from 10^{-1} to 10^{-6} were made from the soil sample using 0.85% double sterilized saline. It was acclimated to 0.250 to 1 mg/L DEHP as the only source of carbon. The isolated culture were successively streaked transfers on agar-plate medium (peptone 5 g, beef extract 3 g, sodium chloride 5 g, D W 1 lit., pH 7.2-7.4 at temperature 36.5°C) for the purification of the bacteria culture (Wang *et al.,* 1995). The purified bacterial strain was further identified after the series of biochemical tests as *Bacillus* sp. as per the Bergey's Manual[7].

2.3 Screening of Microorganism for Degradation Ability

The screening was done to find out the efficient bacterial strains capable of degrading DEHP, using modified Mineral Salt Media (MSM-sodium chloride 1 g, $CaCl_2.2H_2O$ 0.1 g, $MgSO_4.7H_2O$ 0.5 g, KH_2PO_4 1 g, Na_2HPO_4 1 g, yeast extract 4 g, distilled water 1000 ml, pH-7.0) and DEHP at different concentration (50 to 1000 µg/ml). The potential was determined by spectrophotometer at 490 nm[8].

2.4 Effect of Different Physiological and Chemicals Condition

The degradation of the DEHP biodegradation by isolated strains is highly influenced by the different environmental factors (pH, temperature, salinity and carbon percentage). To know the best physio-chemical condition for the degradation of the DEHP by isolated strain T-10 by using the single-factor optimization experiments for different factors like pH (5.5, 7.0, 8.5 and 10.5), temperature (15, 25, 37 and 50°C), salinity (1, 5, 10, 15%,) and different carbon source (Glucose, Mannose, Lactose, Sucrose and Dextrose). In the optimal condition, the initial concentration of DEHP used is 10 mg/L, similarly, the control was also maintained for reference which was without inoculation of the isolate. All experiments were conducted in triplets and the tubes were further incubated in a shaker (120 r/min) at 37°C[9].

2.5 Efficient Degradation of DEHP

In normal natural conditions, the concentration of DEHP is very low. So, it is essential to isolate the strain with the capability to degrade the DEHP at very low concentrations. Conversely, the strain's with the bioremediation capability at a wide range is to be determined. Maximum and minimum concentration tests were performed at different range of DEHP concentrations with min at 0.5 mg/l and maximum at 10 mg/l (Range: 0.5, 1, 2, 5 and 10 mg/L). After incubating at optimum condition, the DEHP concentration was analyzed using HPLC, and the degradation rate was calculated[9].

3. Result

3.1 Isolation, Screening and Characterization of DEHP Degrading Bacteria

The strain with degradation potential was islotated on the agar plates with DEHP (50 g/ml) as a sole source of carbon results in 28 isolates from 3 different soil samples. Among 5 selected isolates with degradation ability more than 250 g/ml, strain T-10 was selected for further study.

Coloney and Biochemical characteristics of the isolated bacteria T-10 is convex circular, greyish white colony on nutrient agar plate (Figure 1). As per the result of Gram staining and shape under 100X microscopic view is G-ve, rod shape bacteria (Figure 2). The series of biochemical tests were carried out on the isolates and shows positive result for, catalase, oxidase, citrate and according to Bergey's Manual (Buchanna and Gibbons 1984) it is identified as *Bacillus sp.* which was further confirmed as *Bacillus antracis* (Acession no. KY 085972.1) on the basis of 16s rRNA sequencing carried at Yaazh Xenomics (Madurai, Tamilnadu, India). The phylogenic neighbor-joining tree was constructed for T-10 (Figure 3), using complete 16S rRNA gene sequence analysis.

3.2 Effect of Physical and Chemical Parameter on DEHP Degradation

3.2.1 Effect of Temperature and pH

Different temperature and pH along with other physiological conditions have a very vital role on the bacterial growth and its biodegradation capability as it effects the enzymatic activation and other metabolic pathways. As depicted in the (Figure 4), strain T10 have variable degradation potential at different pH values ranging from 5.5 to 10.5, showing maximum activity at pH 10.5. Similarly, the O.D (Optical Density) of the strain T-10 was also taken at different temperature i.e. 15, 26, 37, 50°C, after every 24 hours till 8th day of incubation. The data are mentioned in (Figure 5). The highest activity was obtained at 8th day of incubation 37°C.

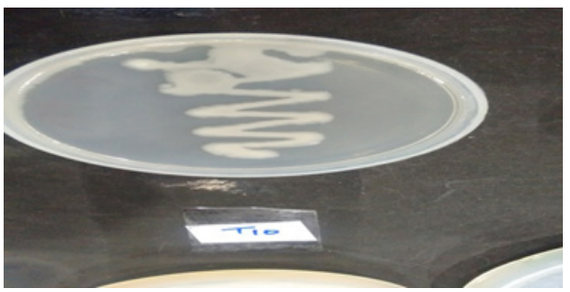

Figure 1. Colony morphology of T-10.

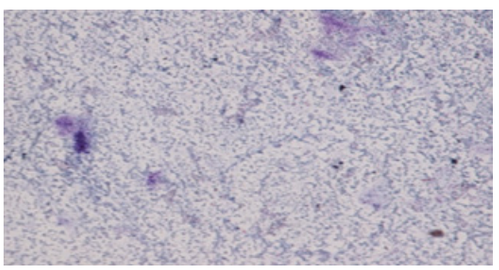

Figure 2. Microscopic view of T-10.

Figure 3. Phylogenic analysis of strain T-10 based on 16s r RNA analysis (sequence bar equals 0.02 changes per nucleotide position).

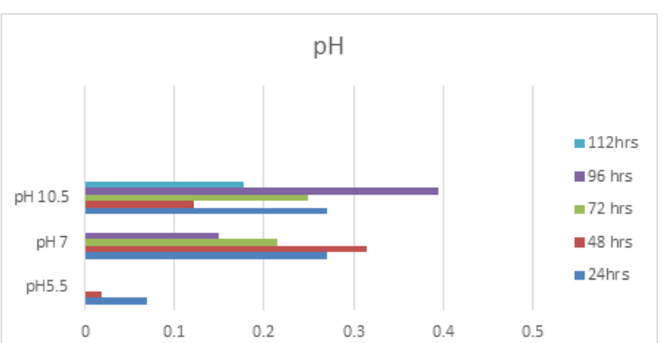

Figure 4. Showing effect of pH.

3.2.2 Effect of Carbon, Nitrogen Source and Salinity Percentage on Growth of Isolates

To characterize the growth condition the selected isolate, was growth on specific pH and temperature with varying carbon (Lactose, Glucose, Mannose and Dextrose); Nitrogen (peptone, yeast extract, beef extract and casein); and NaCl (1%, 5%, 10% and 15%) condition and monitored on regular interval till 10th day (Figures 6, 7 and 8). For sucrose highest growth was found on 2th day, and for nitrozen casein show the highest on third day.

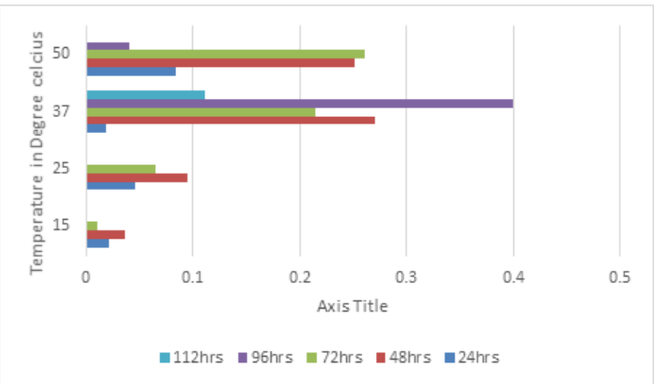

Figure 5. Effect of temperature.

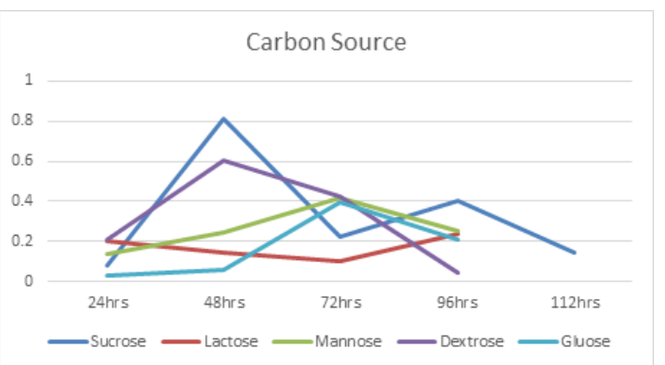

Figure 6. Showing effect of carbon source on growth of strain T-10.

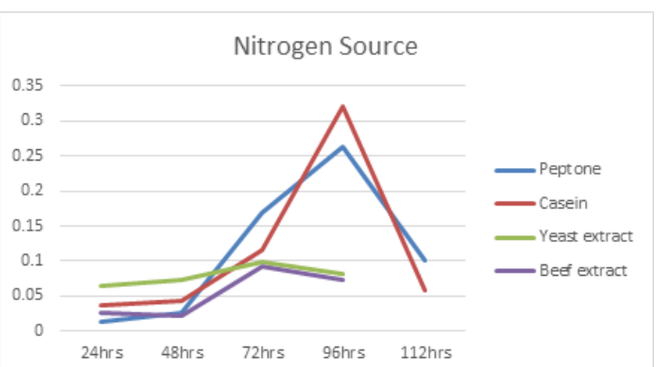

Figure 7. Showing effect of nitrogen source on growth of strain T-10.

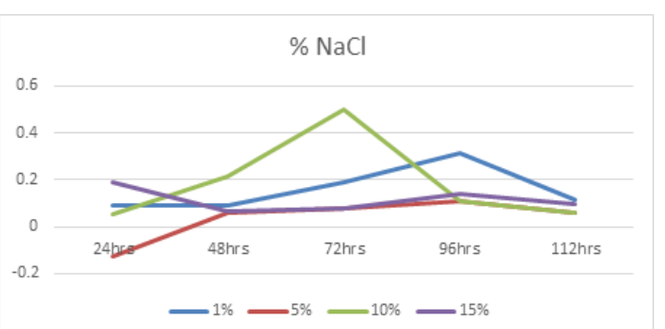

Figure 8. Showing effect of NaCl % on growth of strain T-10.

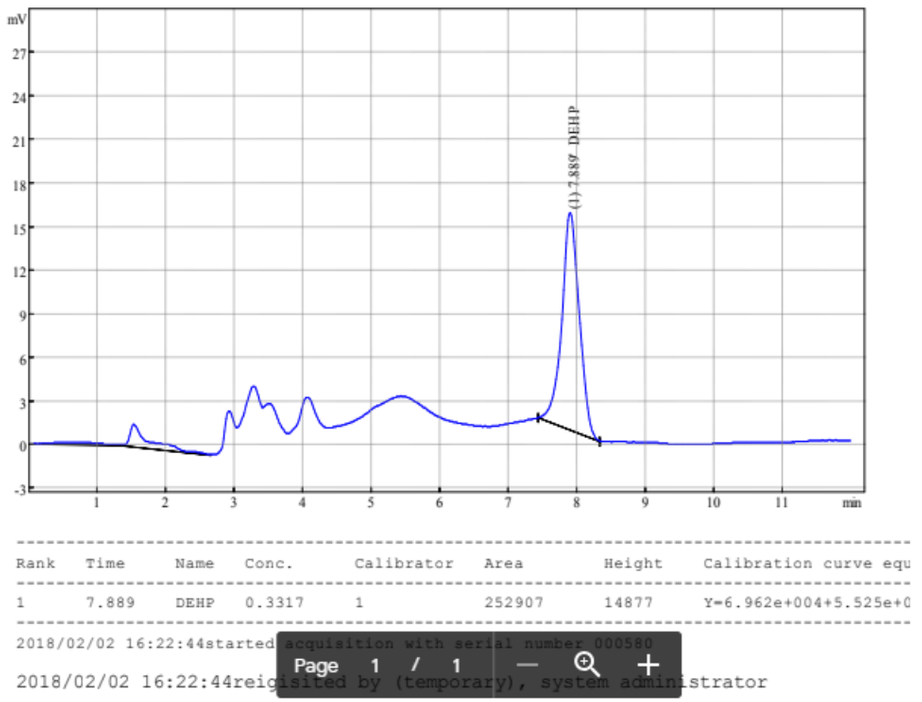

Figure 9. Graphical representation of DEHP degradation (1000 µg/ml) by HPLC of isolates T10.

Table 1. Degradation of DEHP of isolate T10

Code	Spike Sample (µg/ml)	Dilution Factor (DF)	HPLC area of dilution	Concentration of dilution found (µg/ml)	Concentration of Sample (C*DF)	% Degraded
T1	1000	100	23900	0.417381509	30.73815086	85 (approx.)

3.3 Biodegradation of DEHP by HPLC at Optimum Physical and Chemical Condition

The degradation efficiencies under optimum condition of pH, temperature, carbon nitrogen and alkalinity condition of degradation are shown. The degradation efficiency was above 85% as depicted in (Figure 9) and (Table 1).

The degradation study by the strain T-10 was found around 85% with the initial concentration of 1000 µg/ml after the HPLC analysis.

4. Discussion

Several bacterial with the potential of degradation of DEHP have been reported. Bioremediation by using bacteria and its metabolic activity is one of the most successful and economic way to degrade such pollutant.[10] The awareness about the DEHP and its metabolites is need of the present. The microorganisms with the high potential to degrade the DEHP have been isolated from the pollutant dumping area and similar environment. As far we know, strain T10 is one of the most high potent bacteria capable of DEHP-degradation form the isolated from waste soil, containing plastic debris, which can grow in 1–10% NaCl concentration in MSM medium and with the DEHP degradation rate above 85%. Some of the reported bacteria with the potential of DEHP-degradation, are *Arthrobacter sp*. C21, *Sphingomonas sp*., *Acinetobacter sp, Gordonia sp.* and *Rhodococcus* WJ4, can degrade the DEHP at the pH 7.0 and cannot tolerate very high or low pH[11,12]. Other report shows the organism like *Pseudomonas fluorescens* FS1 can tolerate the pH range 6.5–8.0 and *Ordonia alkanivorans* YC-RL2 can tolerate the pH 6.0–11.0[13]. The osmotic potential of strains with decent salinity tolerance increases, which might affect their metabolic activities[14]. Reports have shown that pH and temperature always impact microbial DEHP degradation[15]. The degradation by the help of bacteria is mainly mediated by enzymes system, which is more active at neutral or mildly acidic or alkaline conditions.

However, in this research work, we isolated and identified a halotolerant strain with high potential to degrade the DEHP-degrading strain, *Bacillus antracis* which can grow at wide range of pH, temperature and utilizes several carbon sources for its growth and showing the degradation more than 85% as depicted from the HPLC study (Table 1). However, till now reported strain for DEHP-degradation are limited.

5. Conclusions

The selected isolate T-10 named *Bacillus antracis*, is very efficient in degrading DEHP, it is a halotolerance and can also tolerate a wide range of temperature and pH. The isolate have degradation potential of more than 85% (at concentration of 1000 µg/ml). The pollution cause because of deposition and use of plastic debris is a serious threat to the environment. As reported high-salinity wastewater containing around 5000–6000 mg/L NaCl is produced by the domestic and industrial effluent[13, 16,17]. The bioremediation of such pollutant and it's as compared to any other methods is more efficient as the degradation by this mean can irreversibly depolymerize these compounds. The environment contaminated by the DEHP and similar pollutant are yet to be broadly explored; its application potential has been demonstrated by the bioprocess with various environmental samples. The strain T10 also effectively metabolized 1000 µg/ml DEHP in contaminated agricultural soils, landfills and others.

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