KINETICS STUDIES AND BIOREMEDIATION OF PLASTICIZER (DI-2-ETHYLHEXYL PHALATE) BY INDEGENOUS BACTERIA ACHROMOBACTER XYLOXIDANS (ACCESSION NO. KJ010764.1)

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Abstract : This study has been undertaken to determine the biological degradation potential of plasticizer (DEHP). DEHP and its different metabolites are found to affect humans, including its potential effects on reproduction. The microorganisms play the major roles in the phthalates degradation in the environment under various conditions. One of the promising DEHP degrading bacteria designated as strain T-1 was selected for the present study, and find out the optimum condition for its maximum activity with halotolerance, and wide range of temperature and pH identified as *Achromobacter xylooxidant,* with degradation potential of more than 90%.

IndexTerms **-Plasticizer, DEHP, Bacteria, Biodegradation**

I.INTRODUCTION:

Synthesizing of Phthalates are done in massive amounts for the production of various plastics and it have become widespread in environments, followed by its release in environment due to extensive usage and production. It is of an environmental concern because phthalates are hepatotoxic, teratogenic, and carcinogenic by nature. Among the phthalate esters, DEHP (Di-2-ethylhexyl phthalate) is most frequently used additives in the manufacture of flexible polyvinyl chloride, due to its stability, fluidity, and low volatility (Staples, 1997). According to an annually approximately 10^6 tons of DEHP is produced. DEHP is classified as a priority pollutant with relatively low acute toxicity but suspected mutagenic and carcinogenic effects (Nielsen et al, 1996). DEHP and its different metabolites are found to affect humans, including its potential effects on reproduction.

The metabolic breakdown of DEHP by microorganisms is one of the major routes of environmental degradation for hazardous pollutant. Numerous studies have demonstrated the biodegradation of several Phallic acids including DEHP under aerobic conditions in soil, natural waters, and wastewater (Ribbons et al. 1984). Sugatt et al. (1984) studied the biodegradation of 14 commercial phthalate esters that are commonly used as plasticizers by an acclimated shake flask CO_2 evolution. Nozawa and Maruyama (1988) found that the anaerobic metabolism of phthalate and other aromatic compounds by the denitrifying bacterium *Pseudonomas sp*. strain P136

Numerous studies have demonstrated that microorganisms play the major roles in the phthalates degradation in the environment under various conditions, which has been reviewed by Staples et al. (1997). This paper focused on summarizing the studies on DEHP biodegradation by isolating the potent bacteria, under various environments and chemical condition, one of the promising DEHP degrading bacteria designated as strain T-1 was selected for the present study, and find out the optimum condition for its maximum activity.

II **RESEARCH 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. All glassware was heated at 550°C prior to use. All other equipment was washed with analytical grade hexane prior to use.

2.1 Soil samples.

Waste soil sample was collected from municipal landfills area of Patna. Soil samples were sieved (2-mm mesh), dried to a water content of 7% (wt/wt), and then stored at 5°C until they were used.

2.2 Microorganisms

Several strains of DEHP-degrading microorganisms were isolated from waste amended soil by enrichment and acclimation shaking culture at 36.5°C. Serial dilutions from 10^{-1} to 10^{-6} were made from the soil sample separately. It was acclimated to 0.250 to 1 mg/L DEHP as the sole carbon source. Successive streak transfers on agar-plate medium (peptone 5g, beef extract 3g, sodium chloride 5g, D W 1 lit., pH 7.2-7.4) purified the microorganisms (Wang et al. 1995). The strain was identified as *Achromomonas sp*. because of its characteristics according to Bergey's Manual (Buchanna and Gibbons 1984).

2.3 Screening of Microorganism for Degradation ability.

Screening was done to find out the efficient bacterial strains capable of degrading DEHP, using modified Mineral Salt Media (MSM- sodium chloride 1g, $CaCl_2.2H_2O$ 0.1g, $MgSO_4.7H_2O$ 0.5 g, KH_2PO_4 1g, Na_2HPO_4 1g, yeast extract 4g, distilled water 1000ml, pH- 7.0) and DEHP at different concentration (50 to 1000µg/ml). The potential was determined by spectrophotometer at 490 nm. (Mahmood et al.,2011).

2.4 Effect of Different Physiological and Chemicals condition

DEHP biodegradation by isolated strains was affected by different environmental factors, such as pH, temperature, salinity, and glucose concentration. To determine the optimal conditions for DEHP degradation by strain T1, single-factor optimization experiments were performed in this study, including pH (5.5, 7.0, 8.5, and 10.5), temperature (15, 25, 37, and 50°C), salinity (1, 5, 10, 15%,), and glucose concentration (Glucose, Mannose, Lactose, Sucrose and Dextrose). We set the initial concentration of DEHP to 10 mg/L in the optimization tests. The control was used as stated above without inoculation of the seeds. All cultures were incubated in a shaker (180 r/min) at 30°C. All experiments were conducted in triplicates (Ting Yang et al, 2018).

2.5 Efficient Degradation of DEHP

In natural environments, the concentration of DEHP is very low. Thus, it is important for the strain to be able to have degradation ability at low DEHP concentrations. Conversely, the strain's bioremediation application ability against high concentration pollutants must be determined. Maximum and minimum concentration tests were performed with different initial concentrations of DEHP (max, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mg/L; min, 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 (Ting Yang et al, 2018).

III. RESULT AND DISCUSSION

3.1 **Isolation and Screening of DEHP degrading Bacteria:**

Isolation of strains on agar plates containing DEHP (50g/ml) as a sole source of carbon results in 28 isolates from 3 different soil samples. Among 8 selected isolates with degradation ability more than 250g/ml, strain T-1 was selected for further study.

Culture and Biochemical characteristics of selected isolateT-1 from a convex circular, greyish white colony on nutrient agar plate (fig1), Gram staining and microscopic view revealed a Gram-ve, rod shape bacteria (fig 2). The specified biochemical test was performed on isolates shows positive result for, catalase, oxidase, citrate and identified as Achromobacter sp. According to according to Bergey's Manual (Buchanna and Gibbons 1984). Which was further confirmed on the basis of 16s rRNA sequencing as *Achromobacter xylooxidans* (Acession no. KJ010764.1) at Yaazh Xenomics (Madurai, Tamilnadu, India). The phylogenic neighbor-joining tree was constructed for T-1 (fig 3), using complete 16S rRNA gene sequence analysis.

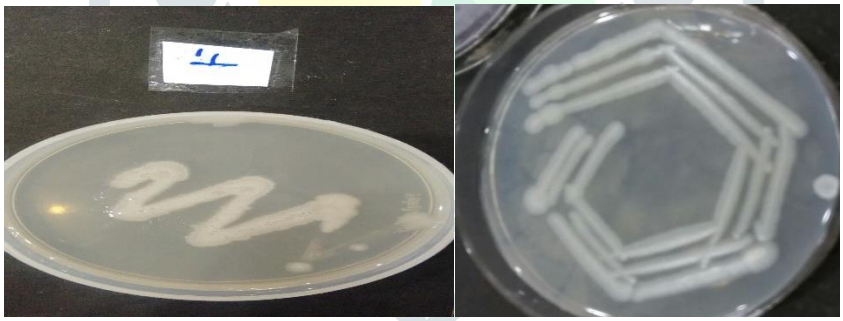

Fig 1: Colony Morphology of T-1

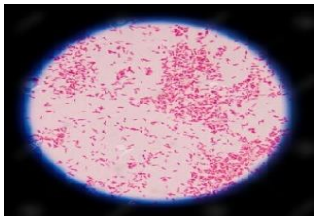

Fig 2: Microscopic view of T-1

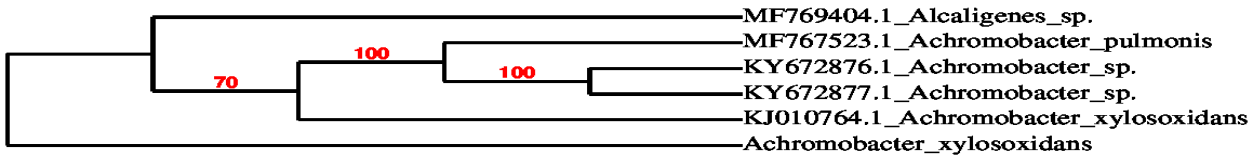

Figure 3. Phylogenic analysis of strain T-1 based on 16s r RNA analysis. The sequence bar equals 0.02changes per nucleotide position

3.2 Effect of physical and chemical parameter on DEHP degradation

a) Effect of Temperature and pH:

Different temperature and pH, play an important role in affecting cell growth, the efficiency of biodegradation, and the enzymatic activation involved in these metabolic pathways. As shown in Figure 4, strain T1 showed different degradation rates at pH values under ranging from 5.5 to 10.5, showing maximum activity at pH 8.5. Similarly, the optical density of the strain T-1 was recorded at temperature 15°, 26, 37, 50 C, on 2nd, 4th, 6th, and 8th day of incubation. The data are provided in figure 5. The highest activity was obtained at 6th day of incubation 37 C.

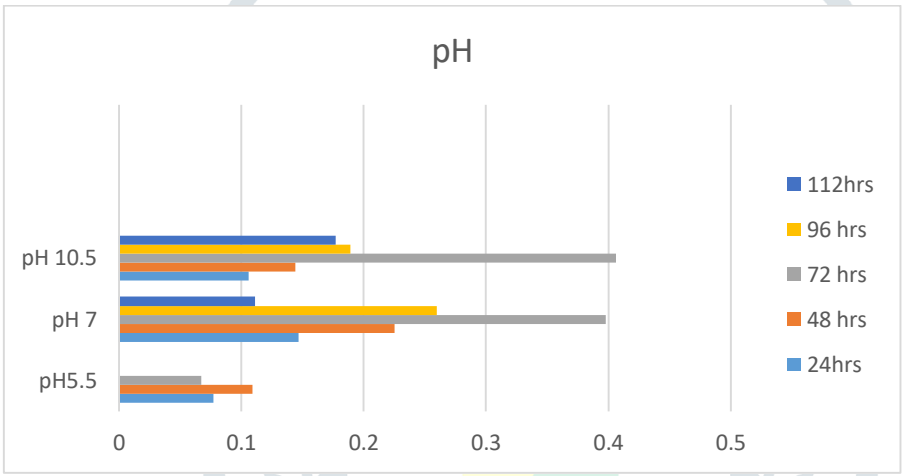

Figure 4: Showing effect of pH

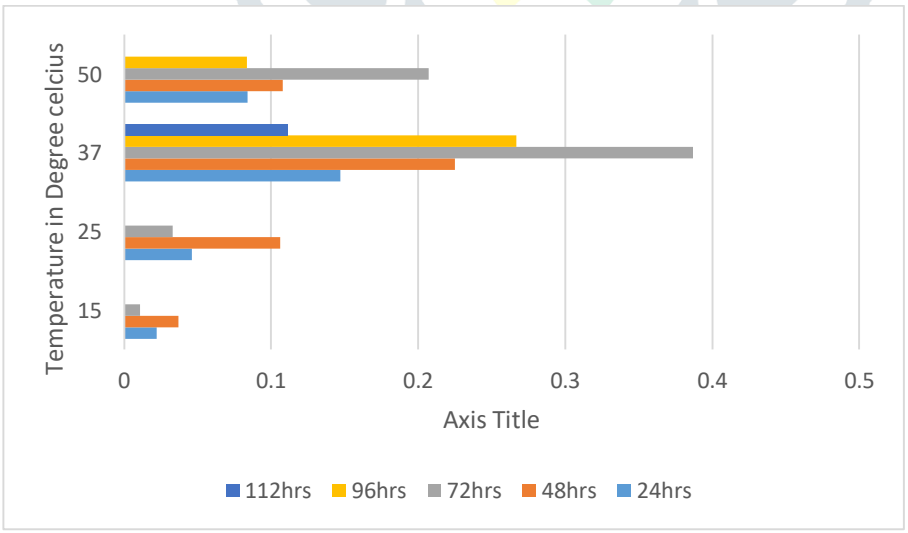

Figure 5: Effect of Temperature

b) Effect of Carbon, Nitrogen source and NaCl concentration 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 (figure 6,7 and 8).

Most DEHP-degraders, such as Arthrobacter sp. C21 [Wan et al, 2014], Sphingomonas sp., Acinetobacter sp, Gordonia sp., and Rhodococcus WJ4 [Wang J, 2015], had a high degradation rate at pH 7.0, but could not tolerate high or low pH. Some other studies showed a wide pH range for Pseudomonas fluorescens FS1 at 6.5–8.0, ordonia alkanivorans YC-RL2 at 6.0–11.0 (Nahurira R, 2017),

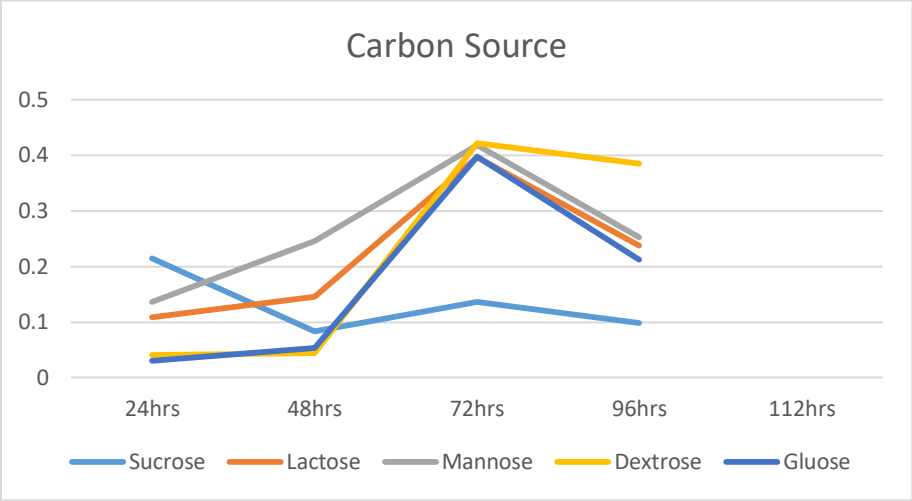

Figure 6: Showing effect of Carbon source on growth of Strain T-1

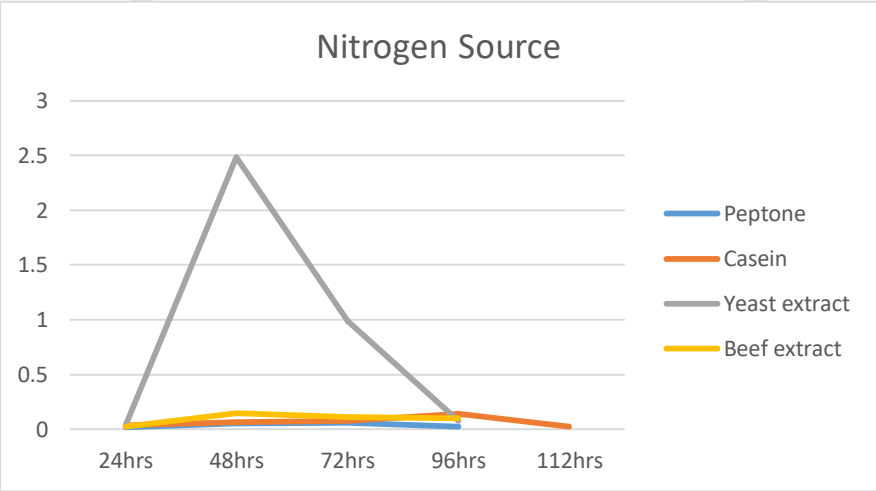

Figure 7: Showing effect of Nitrogen source on growth of Strain T-1

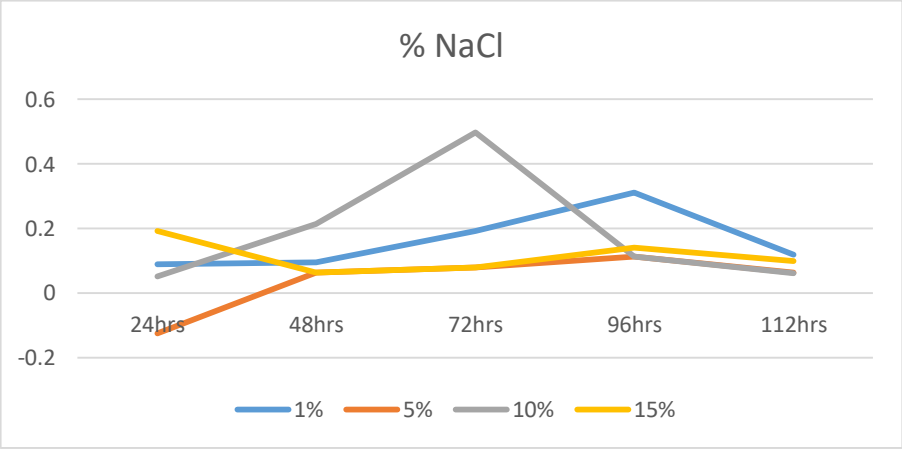

Figure 8: Showing effect of NaCl % on growth of Strain T-1

The osmotic potential of strains with superior salinity tolerance increases, which might affect their metabolic activities [Cortés-Lorenzo et al, 2014]. Reports have shown that pH and temperature always impact microbial DEHP degradation [Ren et al, 2018]. Efficient microbial degradation is generally mediated by enzymes, which prefer to occur under neutral or mildly acidic/alkaline conditions (Nahurira R, 2017).

3.3 Biodegradation of DEHP, at optimum physical and chemical condition:

The degradation efficiencies under optimum condition of pH, temperature, carbon nitrogen and alkalinity condition the degradation. The degradation efficiency was above 90% as depicted in figure 9 and table 1.

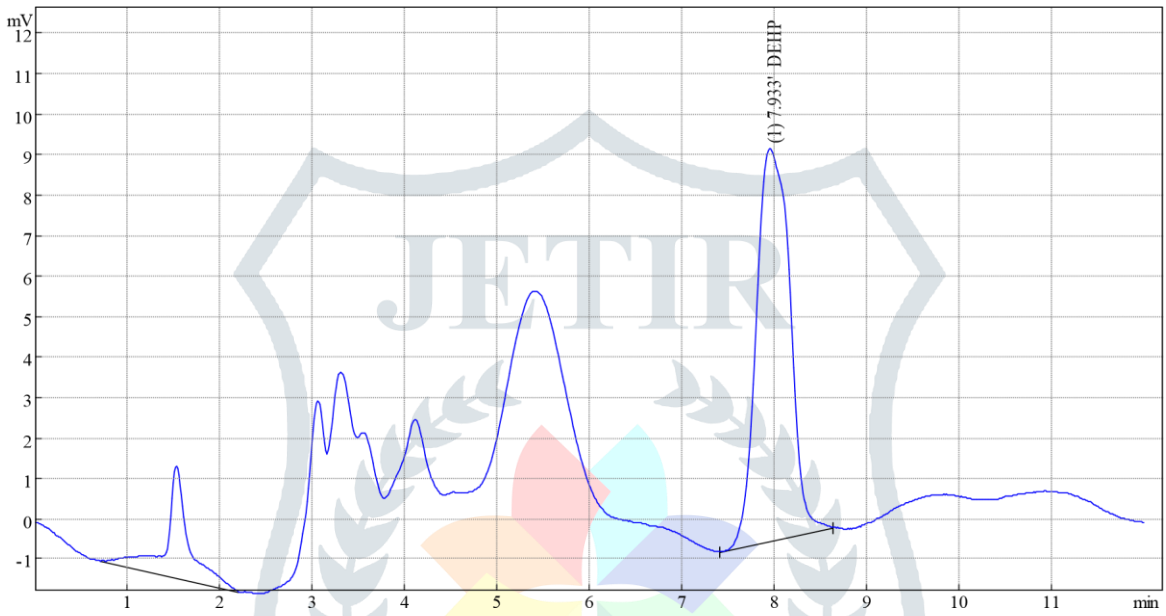

Figure 9: Graphical representation of DEHP degradation (1000 **µg/ml)** by HPLC of isolates T1.

- Slope: 552375.2795
- LOD: 0.156230696
- LOQ:0.473426351

Table 1 : Degradation of DEHP of isolate T1

Code	Spike Sample (µg/ml)	Dilution Factor (DF)	HPLC area of dillution	Concentration of dilution found (µg/ml)	Concentration of Sample (C*DF)	% Degraded
T1	1000	100	239857	0.307381509	30.73815086	95 (approx.)

Several DEHP-degrading isolates have been reported. Bioremediation has the potential to effectively restore the polluted ecosystem based on the biodegradative activities of microorganisms (Jin et al, 2012). Given the awareness of DEHP and its metabolites' toxicity, many microorganisms have been isolated from different environments. As far as we know, strain T1 is the first DEHP-degrader isolated from waste soil, containing plastic debris, which could tolerate 1–10% NaCl in MSM medium and maintained a DEHP degradation rate above 90%.

In this research, we isolated an efficient and halotolerant DEHP-degrading strain, Achromobacter xylooxidant which can grow at wide range of pH, temperature and utilizes several carbon source for its growth and showing the degradation more than 90%. However, till now reported the available DEHP-degrading strains are limited.

V. Conclusions

A highly-efficient DEHP-degrading bacterium with halotolerance, and wide range of temperature and pH named Achromobacter xylooxidant was isolated, with degradation potential of more than 90% (1000 µg/ml). However, the pollution due to plastic debris is a serious threat to the oceans [Landon-Lane,2018], and high-salinity wastewater of about 5000–6000 mg/L NaCl has been generated in domestic or industrial effluent (Nahurira R, 2017). Compared with other methods, bioremediation can irreversibly depolymerize the composition the pollutants (He L, 2018). DEHP-contaminated environments has not yet been broadly explored, the bioprocess with various environmental samples has demonstrated its application potential. The strain T1 also effectively metabolized 1000 µg/ml DEHP in contaminated agricultural soils, landfills and others.

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