

Original Research Article

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Screening of Di-2-Ethyl Hexyl Phthalate (DEHP) Degrading Bacteria and its Characterization by Liquid Chromatography Mass Spectrometry Analysis

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ABSTRACT

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DEHP (di-ethyl hexyl phthalate) is widely used as a plasticizer and it adversely affects humans. In this study, one of the promising DEHP-degrading strain T28 had been selected among isolated pure bacterial strain from rubbish landfill soil near the hospital area, Patna. The bacterial strain T28 was capable of consuming DEHP as a sole source of carbon and energy. By analysing morphological, biophysical, biochemical characteristics, gram staining technique followed by the analysis of 16s rRNA phylogenetic studies, the strain was identified as *Bacillus subtilis* (Genbank Accession no. CPO230367). The mechanism of biodegradation and its characterization at different environmental parameters influencing the degradation process in contaminated soil have also been examined. The results of this study showed the optimal pH value ranges (7.0-8.0) optimal temperature ranges (37-50 °C) while salinity could tolerate up to 5% which influenced the degradation rate in soil. We also investigated the effect of different carbon sources, nitrogen sources and different DEHP concentrations on its degradation. The isolated strain *Bacillus subtilis* degraded more than 99% of 250mg/l of DEHP within 24-48 hrs. The metabolites or end products were detected by liquid chromatography and mass spectrometry (LCMS) analysis. We hope that these findings can provide some information in the bioremediation of DEHP from contaminated soil.

Introduction

Phthalates are a group of compounds employed in the fabrication of plastics and diverse other industrial applications (Lin Y. *et al.*, 2018). Phthalates are released into various ecosystems: land, water and air because of their expanded application (Zhang L. *et*

al., 2015 and Zhang Y. *et al.*, 2015). If there is an increase in phthalate level then the ladies would had been related with endometriosis and gestational duration with precocious breast development (Toft G. *et al.*, 2012). DEHP could cause central nervous system inhibition and renal injury in mice due to its significant lethal or mutagenic effects (Gao *et*

al., 2017). DEHP is one amongst the foremost commonly used plasticizers and is typically applied in such plastic products as polyvinylchloride (PVC), toys, and medical devices. DEHP is taken into account as one of the most resistant phthalate esters because of its long hydrocarbon chain (Chang *et al.*, 2004).

It has been reported that the humans are affected by DEHP due to its exposure in our daily lives which may result in dysfunctioning of endocrine, reproductive and nervous systems (Lo *et al.*, 2014; Net *et al.*, 2015 and Chen *et al.*, 2016). Biological methods have been given more attention due to their environmental friendly nature, low cost, and contaminant mineralization. DEHP is liable to the microbial degradation and studies had shown this phthalate could be degraded by microorganisms because the sole carbon and energy sources (Chen *et al.*, 2007; Meng *et al.*, 2015 and Pradeep *et al.*, 2015).

This study aims that a bacterium which incorporates a remarkable degradation capability towards DEHP was isolated from the rubbish landfill soil near a hospital. Batch experiments were performed at different temperatures, pHs, salt concentration, different DEHP, different carbon sources and nitrogen sources to further optimize the DEHP biodegradation and its molecular characterization by liquid-chromatography mass-spectrometry analysis.

Materials and Methods

Chemicals

DEHP (99% purity) was acquired from Accu Standard Inc. The stock solution of DEHP (10 mg/ml) was prepared in corn oil and kept at 4°C until use. Higher purity grade chemicals were used throughout the experiment. The minimal salt medium (MSM) consisted of the

following chemicals (mg/l): (NH₄)₂SO₄, 1,000; KH₂PO₄, 800; K₂HPO₄, 200; MgSO₄·7H₂O, 500; FeSO₄, 10; CaCl₂, 50 and the pH was maintained to 7.0±0.1 with HCl or NaOH, unless otherwise specified (Fang *et al.*, 2010). DEHP was added solely as the carbon source. All the glassware and media were autoclaved at 121 °C and 101 kPa for 20 min before use.

Isolation of plasticizer degrading bacterial strain and culture conditions

Microbes using DEHP as a sole source of carbon was isolated through an MSM-culture procedure. The initial culture was established by adding 1 g of fresh soil taken from rubbish landfill contaminated with plastics near the hospital area of Patna, Bihar (India) with 100 ml minimal salt media (MSM) in the flask (Zhao *et al.*, 2016).

The flask was kept static for 30 min after shaking at 125 rpm for 30 min. 1.0 ml of supernatant was inoculated into a new flask containing 100 ml of sterilized MSM supplemented with DEHP from the flask to enrich the culture. The flasks were shaken at 125 rpm and 35 °C for 48 h (Li *et al.*, 2012). Then 150 µl of medium with OD 600 1.0 was moved from the flask and inoculated by the spread plate method into a nutrient agar medium. After incubation at 37°C, few well-separated individual colonies of different morphological types appeared and purify it with further repeated streaking on agar plates. Among the isolates, strain T28 was able to significantly degrade DEHP.

Identification of isolated strain

The strain T28 was identified on the basis of its morphology, physicochemical characteristics, and analysis of the 16S rRNA gene sequencing. The 16S ribosomal RNA gene from the bacterial isolate was amplified

by specific universal primers: 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3') (Edger *et al.*, 2004). The sequencing of amplification products was done by Yaazh Xenomics (Coimbatore, India) and the similarity of the nucleotide sequence was determined by the BLAST search in NCBI from NT database. The multiple sequence alignments were performed by program MUSCLE 3.7.

The resulting aligned sequences were cured by employing the program G block 0.91b. This G blocks eliminates poorly aligned positions and alignment noise was removed by divergent regions (Talavera and Castresana 2007). The phylogeny analysis and Program PhyML 3.0 was performed. The program Tree Dyn 198.3 was employed for the rendering of tree (Dreeper *et al.*, 2007).

Effect of different physiological conditions

The degradation of DEHP (20 mg/l) by T28 under different pH (5.5, 7.0, 8.5 and 10.5), temperature (24, 37 and 50 °C) and salinity (5%, 10% and 15%) conditions was examined to determine the optimal conditions for biodegradation. After 48 h of incubation, taken OD at 600nm of the samples. Experiments were performed in triplicate (Ting Yang *et al.*, 2018).

Effect of different chemical conditions

The degradation of DEHP by T28 at different glucose sources (sucrose, lactose, mannose and dextrose) and different nitrogen sources (peptone, beef extract, yeast extract and casein) was done to determine optimal conditions for degradation.

Substrate utilization test

To examine the ability of T28 to utilize DEHP, T28 was cultured in MSM broth

which was supplemented with 200 mg/l DEHP as a sole source of carbon and energy. The cell growth was measured by taking optical density at 600 nm by using a UV-spectrophotometer.

Analytical method

The intermediate metabolites were analyzed by liquid chromatography-mass spectrometry (LC-MS) with the electrospray ionization (ESI) source, employing a full scan for the mass range of 50e400.

ESI mass spectral data were obtained in positive mode and the probe temperature was 300 °C. The optical density of the culture was measured at 600 nm (OD600) using a UV spectrophotometer.

Results and Discussion

Identification and characterization of the bacterial strain T28

After the screening process, the most potent bacterial isolate named T28 was selected. The strain was gram-negative, rod-shaped and flagellated with non-spore forming. After cultured for 18–24 h at 37 °C, this strain T28 formed red, opaque and irregular elevated colonies with smooth margin on nutrient agar.

The strain T28 identified as *Bacillus cereus* through its morphological characteristics, biochemical characteristics and 16s rRNA gene sequencing. According to the NCBI BLAST analysis of its 16S rRNA gene sequence, strain T28 belonged to the genus *Bacillus* of *ceres* family (GenBank accession no. CPO230367).

Fig. 1 illustrates the phylogenetic relationship of T28 with its close relatives. This isolate belongs to the same genus as the one isolated by Quan CS *et al.*, (2005).

Effect of different physiological conditions

Effects of pH and temperature on biomass and DEHP biodegradation

The effect of pH and temperature on DEHP degradation by *Bacillus subtilis* is shown in Figure-2 & Figure-3 respectively. Hydrogen ion concentration in the culture medium greatly influence bacterial growth as pH limited enzyme activities, degradation of PAEs was particularly sensitive to low pH (Fan *et al.*, 2004). The accumulation of intermediates of degradation such as phthalic acid makes the culture medium acidic and inhibits further bacterial degradation of the intermediates (Xu *et al.*, 2005a). In the present investigation, the OD 600 increased rapidly when the culture pH was increased from 5.5 to 7.0. When the pH increased from 7.0 to 8.5 DEHP degradation rate decreased. The DEHP degradation rate and the OD 600 increased rapidly with the increase of temperature from 37°C to 50°C. However, at 28°C temperature, the microbial biomass did not very much, the DEHP degradation rate decreased notably. Based on the above results, the optimal pH and the temperature selected for DEHP degradation *Bacillus sp.* T28 for subsequent experiments was selected as 7.0 and 50°C respectively.

Effect of salinity or NaCl concentration on DEHP degradation

Salinity is an important factor affecting microbial growth and pollutant degradation, but there are just a few halo tolerant strains in degrading PAEs that have been published in previous reports. Burkholderiacepacia DA2 was isolated by Wang *et al.*, (2003) from marine sediments for degradation of DMP, which could tolerate a salt concentration from 0% to 1%. In the present investigation, the bacterial strain T28 could degrade DEHP with the salinity ranging from 0% to 5% as

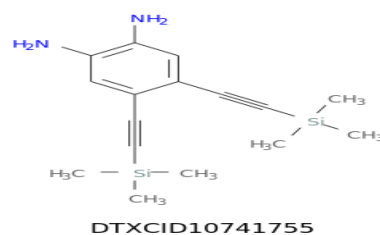
illustrated in Figure 4 (Wang *et al.*, 2003). Its high degradation efficiency made T28, a potent degrader.

Effect of chemical factors on DEHP degradation

The highest rate of degradation was seen on dextrose among the selected glucose sources (sucrose, mannose, lactose, and dextrose) and on yeast extract, it showed the highest degradation among chosen nitrogen sources (peptone, beef extract, yeast extract and casein) as shown in Figure 5. and Figure 6.

Identification of metabolite by LCMS analysis

The mass spectral analysis of the compound showed the parent ion peak at m/z 321.25 as shown in Figure 7. The fragment peaks patterns showed at m/z ion peaks at m/z 301.15 base peaks Figure 7. presents the metabolic intermediated identified in DEHP degradation by *Bacillus subtilis*. The metabolites of DEHP degradation by *Bacillus cereus* were extracted at different time intervals and identified by LC-MS (liquid chromatography- mass spectrometry) method. The metabolites were identified by comparing the mass spectrum at a particular retention time (RT) with published mass spectra from the database. The molecular mass of the degraded compound is 301.15 at retention time 9.01 min. as depicted in Figure 8. and the degraded end product was 4,5B is [(trimethylsilylethynyl)] benzene-1,2-diamine. Molecular Formula C₁₆H₂₄N₂Si₂



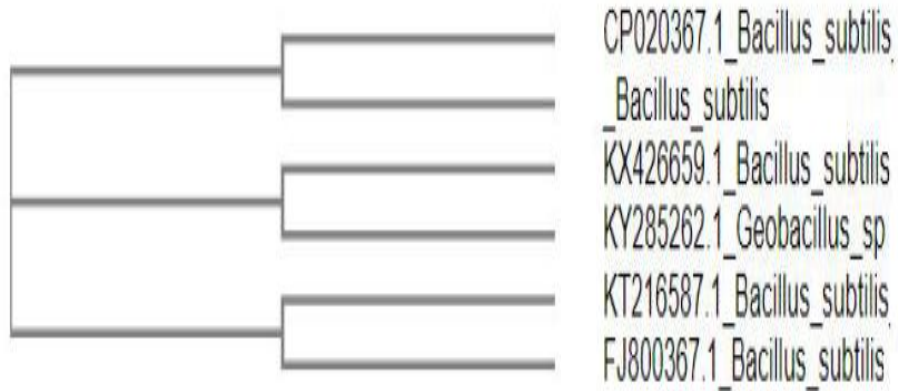


Figure.1 Phylogenetic tree of *Bacillus_subtilis*; The sequence bar equals 0.02 changes per nucleotide position

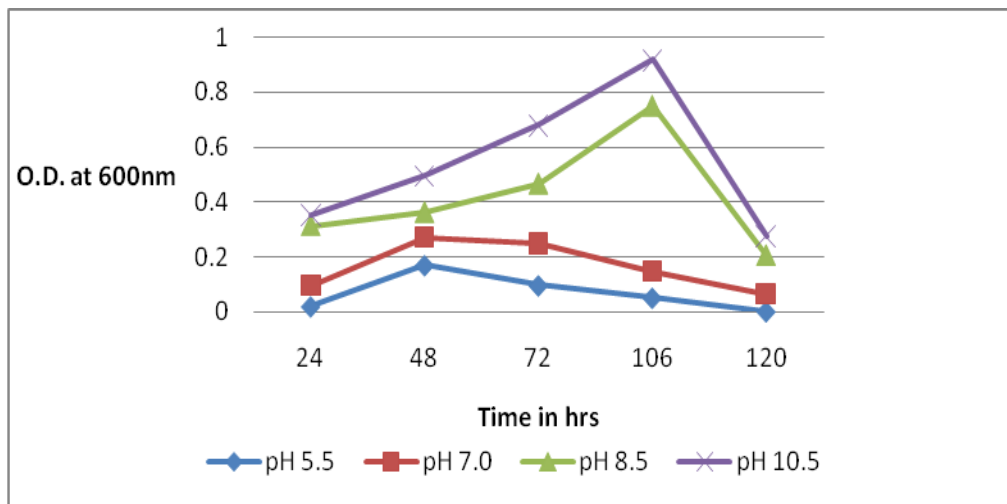


Figure.2 Degradation of DEHP at different pH

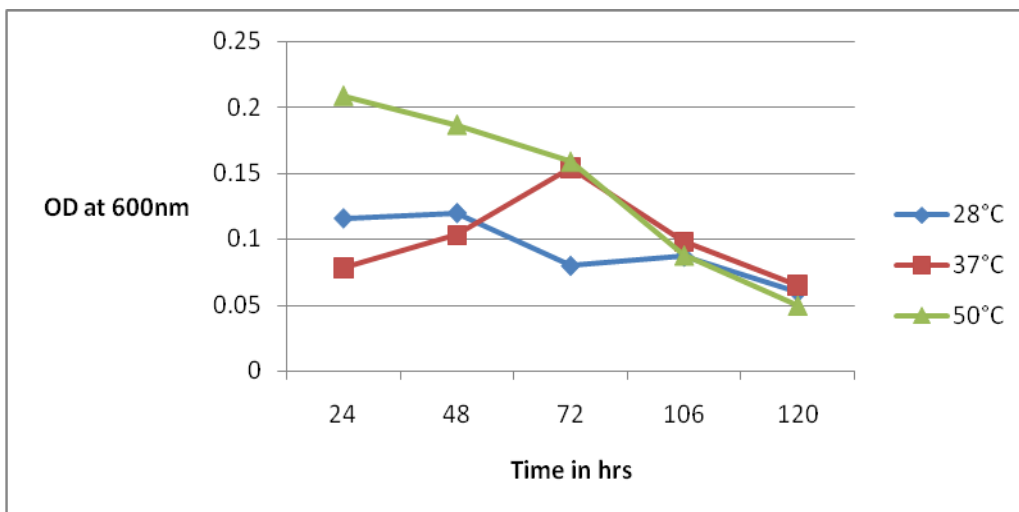


Figure.3 Degradation of DEHP at different temperature

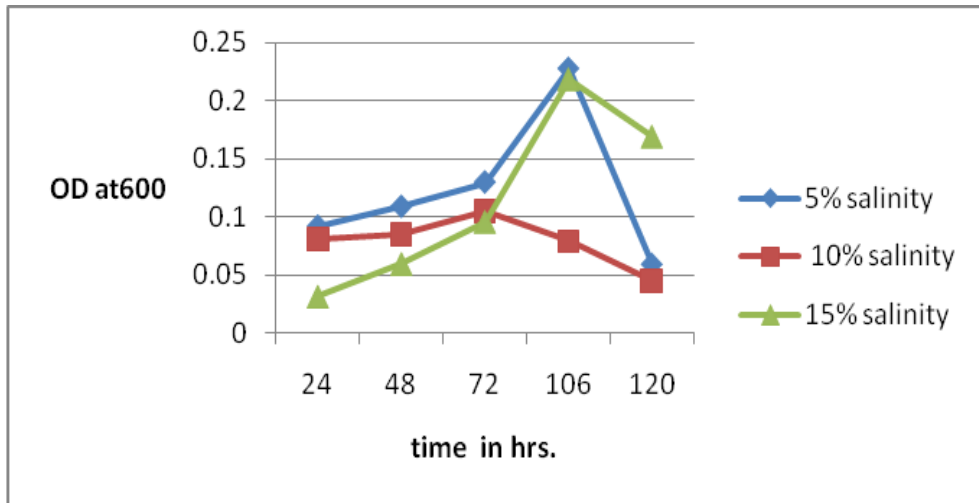


Figure.4 Degradation of DEHP at different salt concentrations

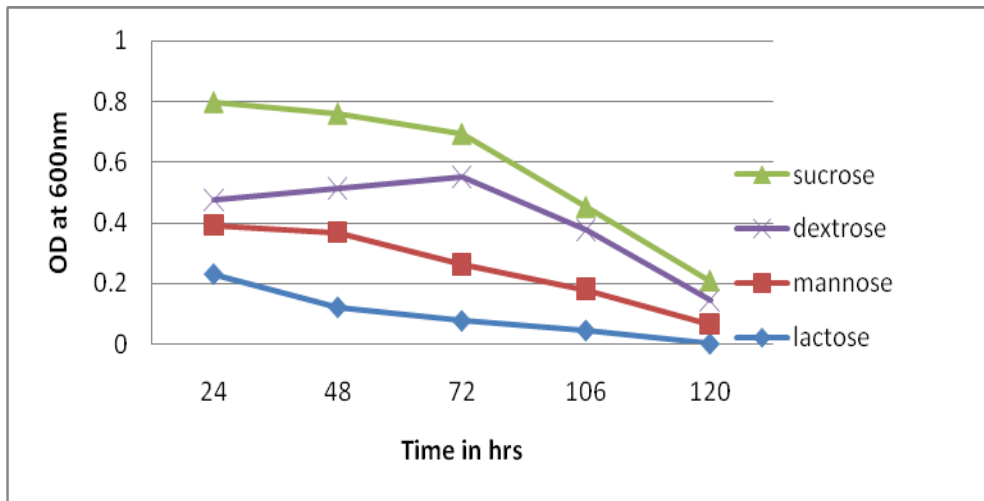


Figure.5 Degradation of DEHP at different glucose sources

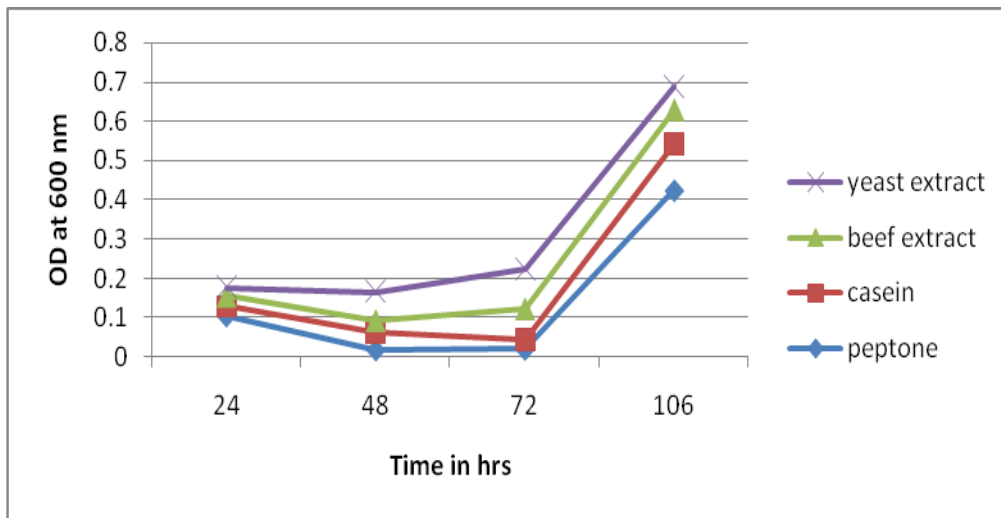


Figure.6 Degradation of DEHP at different nitrogen sources

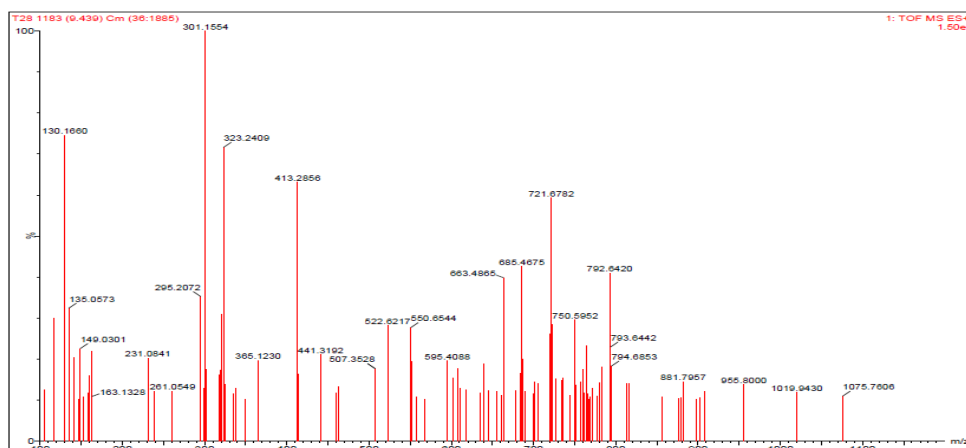


Figure.7 The mass spectrum of DEHP degraded intermediate compounds, X-Axis contains mass in relative to charge ratio (m/z) while y-axis contains a relative abundance

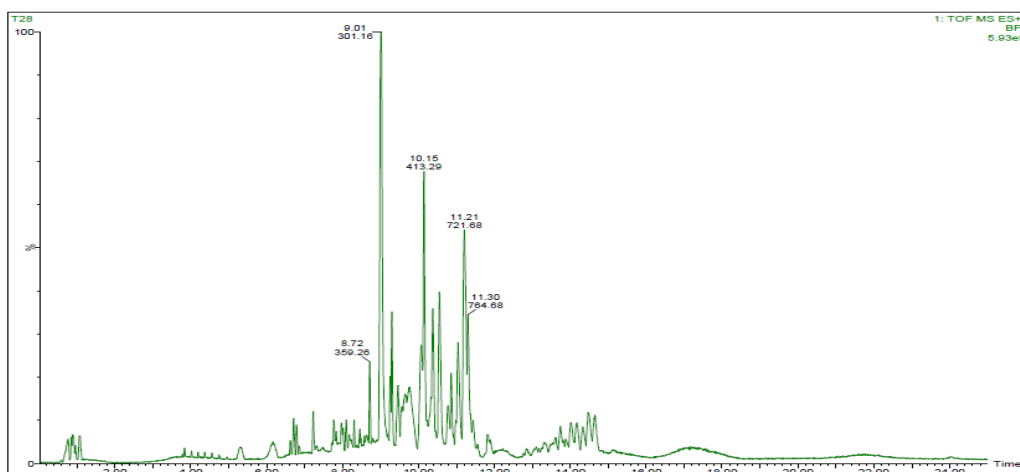


Figure.8 DEHP degradation metabolic intermediates identified by LC-MS

The results obtained in the present study showed that DEHP could be rapidly degraded by *Bacillus subtilis* isolated from rubbish landfill soil. The optimum pH for the degradation was 7.0. and optimum temperature was 37° C and the end product obtained after the LC-MS analysis was 4,5-Bis [(trimethylsilylethynyl)] benzene-1,2-diamine.

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