RESEARCH ARTICLE

Microbial Treatment of di (2-ethyl hexyl) Phthalate by *Lysinibacillus xylanilyticus* Isolated from Landfill Soil

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Abstract

The bacterial strains were isolated from landfill soil contaminated with phthalate, collected from dump yard near Byepass, Patna. The strain named T23 was chosen among the isolated strains due to its high efficacy towards the degradation. We observed the effects of various environmental and chemical factors for optimising the conditions for degradation. The strain T23 was identified as *Lysinibacillus xylanilyticus* based on its phenotypic as well as phylogenetic characteristics by performing 16S rRNA gene sequencing and for the determination of the metabolic end products after degradation. Gas chromatography and mass spectrometry (GC-MS) analysis was done and the degradable intermediates obtained were 7, 10, 13-Hexadecatrienoic acid, Cyclotrisiloxane, n-Hexadecanoic acid, Oleic Acid and Erucic acid. The strain T23 showed maximum degradation at pH 8.5 and temperature was 10.5 and it could tolerate up to 0-15% NaCl. Maximum degradation was exhibited at the carbon source, dextrose, and nitrogen source, casein. The 23 strain was having maximum potential for degradation which can be used for various remediation purposes.

Keywords: DEHP degradation, 16s r-RNA, GC-MS, *Lysinibacillus xylanilyticus.*

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Introduction

Phthalates are manmade compounds ordinarily used as plasticizers in a very wide selection of commercial, domestic and medical fields. Di 2-(ethylhexyl) phthalate (DEHP) is reportable to be associated with endocrine-disrupting chemicals (EDCs) that cause reproductive and developmental disorders in animals (Liu *et al.,* 2014). It has been found in many researches that the leaching of phthalates occurs from the plastics dumped landfill areas (Gao and Wen, 2016). Phthalates leaches out into the atmosphere as they are physically adhered to the matrix of plastic things and so it is abundant in natural waters, wastewaters, soils, sediments and it is additionally present in air because of comparatively high vapor pressures (Latorre *et al.,* 2012). The micro plastics present in soil containing acid esters belong to refractory organic compounds (Wei *et al.,* 2020). Among the PAEs, DEHP is taken into account because the most resistant and ordinarily used acid esters as a result of its long organic compound chain (Chang *et al.,* 2004). As per the previous reports the exposure of human to DEHP cause liver harm and is related to the reproductive and developmental anomalies (Dargnat *et al.,* 2009). In a study which was done recently, few PAEs metabolites have been found in hair of pregnant women that were most likely linked with the use of cosmetics and plastic products (Lomenick *et al.,* 2010). In the United States and Europe so many control measures have been taken to control this hazardous compound (Katsikantami *et al.,* 2020. In a very recent report, it's been reported that the plastic mulch film that is employed for the cultivation of vegetables permit DEHP to be absorb by plants and hence enter the human organic phenomenon, have an effect on human health (Magdouli *et al.,* 2013). Several studies done on degradation reportable that the DEHP microorganism mechanism of action is the principal supply for the degradation of DEHP in aquatic and terrestrial systems, like soils, sediments and surface waters (Fu and du, 2011). Numerous analytical strategies are developed to detect phthalates in various matrices (Notardonato *et al.,* 2019). To detect the chance of phthalate contamination in foodstuffs, although several studies correlate with phthalate contamination are disbursed however still there are many diseases that are related to phthalate contamination, are debatable (Mu *et al.,* 2015). Rather than physiochemical methodology, biological processes are thought of additional applicable and environmental friendly and effective in cost (Zolfaghari *et al.,* 2014). During this analysis, our aim was to isolate the microorganism strains capable of degrading phthalate from soil contaminated with DEHP. The isolated strain was having the flexibility to utilize DEHP as a sole carbon supply. Besides this some biochemical tests and numerous environmental and chemical factors have additionally been utilized to optimize the conditions for higher degradation.

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Conflict of interest: None

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Materials And Methods

Enrichment Culture Media and Chemicals

Di -2 ethyl hexyl phthalate was obtained from Accu standard Inc. USA. Sigma Pvt. Ltd provided the analytical grade oil made of

corn was used as a medium. The initial enrichment culture was initiated on minimal salt media (MSM media) supplemented with DEHP, having following chemicals (mg l^{-1}): ammonium sulfate 1,000 mg l^{-1}; potassium dihydrogen phosphate, 800 mg l^{-1}; dipotassium hydrogen phosphate, 200 mg l^{-1}; hydrated magnesium sulfate, 500 mg l^{-1}; ferrous sulfate, 10 mg l^{-1}; Calcium chloride, 5 mg l^{-1} and the pH were maintained 7.0 with the aid of hydrogen chloride or sodium hydroxide (Fang *et al.,* 2010).

Isolation of Bacterial Strains for DEHP Degradation

Microorganisms consuming DEHP as a sole supply of carbon were isolated through the MSM-culture procedure. The initial culture was ready by adding 10 g of recent soil with 100 ml of distilled water within the flask (Fang *et al.,* 2010). The flask was kept stationary for 30 min when shaking at 125 revolutions per minute for 30 min, and 1.0 mL of supernatant was placed to an another flask containing 100 mL of sterilized MSM supplemented with DEHP (10 mg l -1) to complement the culture. The flasks were centrifuged at 120 revolutions per minute at 37°C for 48 hours (Zhao *et al.,* 2016). Then 100 µL of the medium was transferred from the flask and inoculated by the spread plate technique into a solid nutrient agar culture medium. When 48hrs incubation, varied well-separated colonies of various morphological characteristics showed up and pure culture colonies of the microorganism isolates were conducted in MSM, with this purifying process conducted more than 5 times. DEHP-degrading microorganism was approved and entitled T23. All experimental works have been performed in triplicates (Çevik *et al.,* 2019).

Identification of Isolated Strain T23

The strain T23 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 primers 8F (5'AGAGTTTGATCCTGGCTCAG3') and1541R ((5'AAGGAGGTGATCCAGCCGCA3') (Edgar, 2004). The nucleotide sequence similarity was determined by the BLAST search in NCBI from database. The multiple sequence alignments were performed and the aligned sequences were cured with G block 0.91b program (Talavera and Castresana, 2007).The program PhyML 3.0 was performed for phylogenetic analysis (Dereeper *et al.,* 2008).

Optimization of Conditions for DEHP Degradation

The degradation of DEHP under different experimental parameters as pH 5.5, pH 7.0, pH 8.5 and pH 10.5, the temperature at 24°C, 37°C and 50°C, 5% salt concentration, 10% salt concentration and 15% salt concentration, Various carbon sources as sucrose, lactose, dextrose and mannose and various nitrogen sources as peptone, beef extract, casein and yeast extract were investigated to determine the optimal conditions for better biodegradation. The response was recorded in terms of absorbance (OD) at 600nm after 48 hrs of incubation interval, up to the period of 8 days.

Analytical Method Employed for Degradation

DEHP degradation metabolites were known employing a Gas chromatography- mass spectrum analysis (GC-MS) attached with an Agilent mass selective detector (Agilent, USA). The column was silicone-coated, fused-silica capillary column, which was used. The temperature maintained at 50°C for 5 min hold, a rise to 280°C at 10°C min^{-1}, and a 15 min hold at 280°C. Comparison with accessible authentic compounds in library searches, and mass fragmentation pattern were applied to establish the probable metabolic products.

Results And Discussion

Identification and Characterization of Isolated Strain T23

The foremost potent microorganism isolate named T23 was picked after the screening method. Through varied investigations we tend to found that the isolated microorganism strain was gram-negative, rod-shaped and non -sporing. Once culturing for 24 hours at 37°C, this strain T23 was pale yellow in color, semitransparent, oval and rough colonies with irregular margin on culture medium. The strain T23 known as *Lysinibacillus xylanilyticus* through its morphological characteristics, biochemical characteristics and 16s rRNA sequencing. In keeping with the NCBI BLAST search of its 16S rRNA sequence, strain T23 belonged to the Lysinibacillus genus of xylanilyticus family (Gen Bank accession no. KF804075.1). Fig. 1 illustrates the phylogenetic relationship of T26 with its close relatives (Quan *et al.*, 2005).

Optimization of the Conditions at Different Environmental and Chemical Conditions

Effect of pH

The pH effect on consumption of DEHP by *Lysinibacillus xylanilyticus* is depicted in Fig. 2 (a). The DEHP consumption enhanced gradually when pH rose from 7.0 to 10.5. The DEHP consumption was maximum at pH 10.5 for *Lysinibacillus xylanilyticus*. Therefore, our study found that pH range 7.0-10.5 was optimal for DEHP degradation by *Lysinibacillus xylanilyticus* (Zhao *et al*., 2016). Similar findings has been reported at the suitable range of pH 6-10 while degrading DEHP by Acinetobacter sp. SN13 due to the adaptability of strain to neutral and alkaline condition (Toledo *et al*., 2017). Various pollutants degraders have been reported ranges from pH 7.0 to 8.0 (Lu *et al*., 2009). Some other microorganisms like Rhodococcus sp.HS-D2 (Khleifat, 2006). Gordonia alkanivorans YC-RL2 (Zhang, *et al*., 2016) (Acinetobacter sp. SN13 (Li *et al*., 2012) and Pseudomonas fluorescens FS1 (Zhao *et al*., 2016), Bacillus cereus T20 Rashmi and Tanuja, (2022) have been reported a wide pH range at 5–11.

Effect of Temperature

Temperature affects PAE consumption which is one of the important factors as reported in many studies. As shown in Fig.2 (b), the DEHP degradation rate enhanced gradually as the temperature rose from 28°C to 37°C. The highest biomass and maximum DEHP degradation for strain T23 were attained at 50°C. The present finding is consistent with Rhodococcus ruber YC-YT1 which was isolated from contaminated soil (Ren *et al*., 2018).

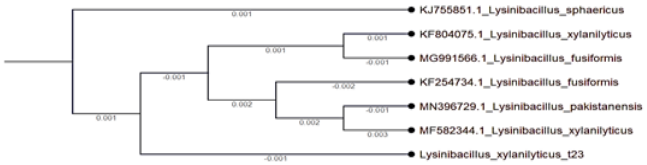

Fig. 1: Phylogenetic tree of isolated bacterium (T23) designated as *Lysinibacillus xylanilyticus* based on 16sr RNA sequence alignment

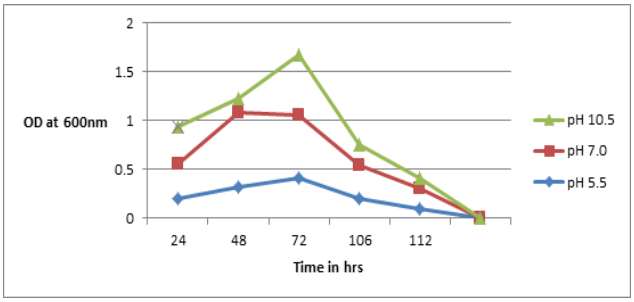

Fig. 2 (a): Effect of pH on degradation of DEHP

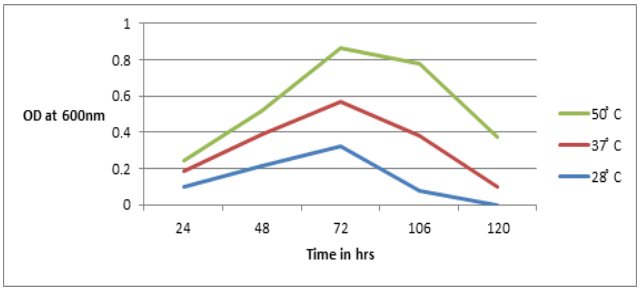

Fig. 2 (b): Effect of temperature on degradation of DEHP

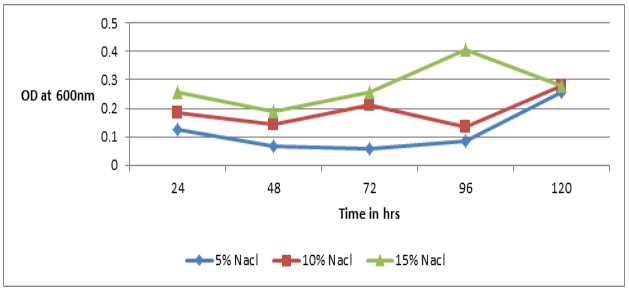

Fig. 2(c): Effect of salt concentration on DEHP degradation.

Effect of NaCl Concentration

Salinity is a significant parameter; the impact of salt concentration on DEHP biodegradation was tested too. As shown in Fig. 2 (c), strain T23 was affected with the salinity level. The degradation rate increased when salt concentrations were increased to 5 to 15%, nearly no DEHP degradation or microorganism growth was determined before 48 hours. The lag section for DEHP degradation becomes longer as NaCl concentration raised. As shown in Fig. 2 (c), when induction by NaCl, the flexibility of strain T23 to degrade DEHP within the presence of NaCl

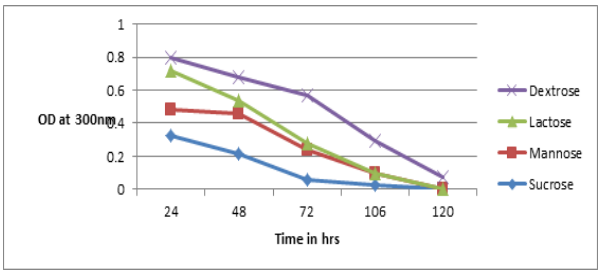

Fig 2. (d): Effect of different carbon sources on DEHP degradation.

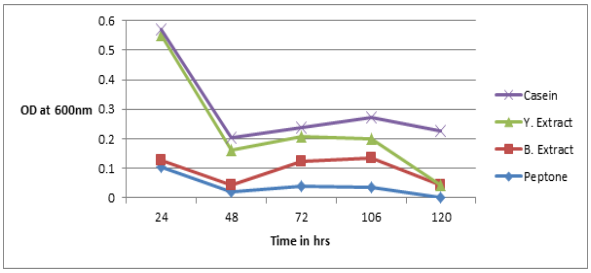

Fig 2. (e): Effect of different nitrogen sources on DEHP degradation

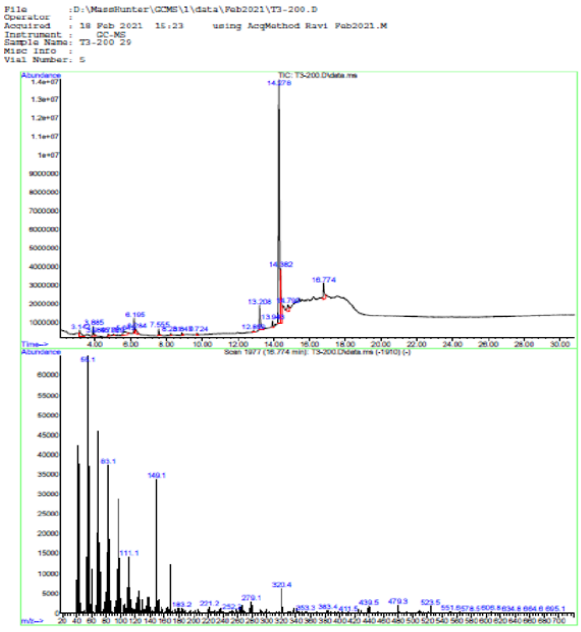

Fig. 3(a, b): GC–MS chromatogram and GC-MS spectra of DEHP and its biodegradation intermediates of strain T23.

improved significantly; all of the DEHP was degraded among 96 hours. Though strain T23 exhibited an analogous trend in this higher salinity levels reduced the microorganism growth rates, leading to longer degradation times, it may tolerate a NaCl concentration. There are very few researches which have been focused on phthalate degradation in the presence of NaCl up to 15% (Yang *et al.*, 2018). Rhodococcus ruber YC-YT1 isolated from water which containing NaCl could tolerate up to 120 g/l of NaCl (Wang *et al.*, 2008). A study has known halotolerant isolate Sphingobium sp. that degraded DBP at the salinity starting from 0 to 4% (Jin *et al.*, 2013).

Effect of Various Carbon and Nitrogen Sources

Carbon sources viz. sucrose, dextrose, mannose and lactose were added by equal amounts at a concentration of 1% (w/v) MSM medium, one by one. Fig 2 (d) depicted that DEHP degradation was affected by the characteristics of the carbon source individually. Dextrose was the most superior carbon sources when compared to the control for the degradation of DEHP respectively. While the cultures containing sucrose had lowest DEHP activity was a sole supply of carbon compared to control. Similar findings were obtained in case of Rhodococcus ruber YC-YT1 strain which was capable of degrading DEHP due to utilization as a sole source of carbon (Chang *et al.*, 2005).

On the other hand, various nitrogen sources peptone, beef extract, yeast extract and casein were added in equal proportion at a concentration of 1% (w/v) to MSM medium, individually. Fig. 2 (e) shown that DEHP worked differently with different nitrogen sources. Casein extract was the most preferable nitrogen source compared to the control with rise in DEHP activity. While the lowest microbial activity was obtained in the cultures containing peptone as a nitrogen source with decrease in DEHP compared to the control. There was no any microbial activity was detected by the identified strain cultivated on MSM medium containing peptone as a nitrogen source. The findings we obtained are somewhat similar with the findings reported by a previous study (Chang *et al.*, 2005).

Analytical Method for the Identification of Metabolites

GC-MS method was employed for the identification of the metabolic end product. The metabolic products were detected by comparing the mass spectrum (RT) with the published mass spectra at a particular retention time from the database. In this study, five main peaks were obtained at different retention time as 3.885, 6.195, 13.208, 14.278, 16.774 as depicted in Fig. 3(a) in the chromatogram in which the highest peak obtained was 14.278. The degraded intermediate compounds obtained were 7, 10, 13-Hexadecatrienoic acid, Cyclotrisiloxane, n-Hexadecanoic acid, Oleic Acid and Erucic acid respectively. However, the DEHP metabolites were not detected in this research, due to the low concentration of DEHP to its detection limit. DEHP was generally degraded in two steps, the hydrolysis of DEHP to Methyl hexyl phthalate (MEHP) and then to Phthalic acid (PA) by de-esterification (Benjamin *et al.*, 2015). But in the present research it was not detected, mainly due to the efficient degradation ability of isolated potential strain to PA, which resulted in the concentration of PA below the detection limits. Similar results have been found during DEHP degradation where PA was not being detected (Pradeep *et al.*, 2015; Shailaja *et al.*, 2008). However, five new substances were found in our study as discussed above, thus the DEHP degradation mechanisms was been further explored. The mass spectrum, Fig. 3(b), X-Axis denotes mass in relative to charge ratio (m/z) while the y-axis contains a relative abundance. The mass spectral analysis of the compound showed the parent ion peak at m/z 320.4 as shown in Fig.3 (b) The fragment peaks patterns showed peaks at m/z 55.1 which represents base peak, m/z 83.1, m/z 111.1 and m/z 149.1 presents the metabolic intermediates identified in DEHP degradation by Lysinibacillus xylanilyticus. In the previous studies it has demonstrated that di-n-octyl phthalate could be transferred to phthalic acid by the Gordonia sp. strain JDC-2 via similar biodegradation pathway and that the Arthrobacter sp. strain JDC-32 degrades phthalic acid to CO_2 and H2O (Wu *et al.*, 2010). So it is very clear that in this study the isolated strain shown a complete metabolic pathway for the degradation and the metabolism of DEHP which makes it an ideal strain for the bioremediation of pollutants present in the environments contaminated with phthalate esters.

Conclusion

In summary, the bacterial isolate T23 is capable of utilizing DEHP was isolated from soil samples contaminated with phthalate which was identified as *Lysinibacillus xylanilyticus* sp by 16srRNA gene sequencing. The present study found the optimal pH 8.5, temperature 10.5°C, and salinity 15% for degradation in MSM. To our knowledge, this is the first report on DEHP degradation by *Lysinibacillus xylanilyticus* accession no. (KF804075.1). The newly developed technique of GC-MS is the one of the best method for quantitative detection of degradable products. These results are very much helpful in the remediation of DEHP pollutants in contaminated landfill soil (Lin *et al.*, 2011).

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