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EVALUATION OF BIOREMEDIATION POTENTIAL OF *BACILLUS CEREUS* AND ITS OPTIMISATION BY TWO-WAY ANOVA STATISTICAL ANALYSIS

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ABSTRACT

To investigate the biodegradation of di-2-ethyl phthalate (DEHP) by an isolated bacterial strain *Bacillus cereus spp.* from soil contaminated with phthalate, collected from a garbage dumping site in Patna. The strain named T20 was isolated by serial dilution method in minimal salt media (MSM) supplemented with DEHP. We studied the effects of various environmental and chemical factors for optimising the conditions for degradation. The strain T20 was identified 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, liquid chromatography and mass spectrometry (LC-MS) analysis was done. The bacterial isolate was identified as *Bacillus cereus (*accession no.CP026375.1) by 16sr RNA gene sequencing analysis. According to Tukey post-hoc test the optimal temperature for degradation was 37 °C and pH was 7.0 and it could tolerate up to 0-5% NaCl. The most preferable carbon source was lactose and nitrogen source was yeast extract to optimize the conditions for DEHP degradation. Liquid chromatography and mass spectrometry (LC-MS) was done to estimate degradation intermediates of DEHP. Strain T20 showed potential characteristics making it a promising strain that can be implemented in the bioremediation of soil contaminated with phthalate.

Keywords: Bioremediation, DEHP, LC-MS, phthalate degradation, 16s r-RNA

INTRODUCTION

Phthalic acid esters (PAEs) are synthetic compounds commonly used as plasticizers in a wide range of industrial, domestic and medical fields. Di 2-(ethylhexyl) phthalate (DEHP) is an endocrine disrupting chemicals (EDCs) and associated with the reproductive and developmental toxicities to animals **[1]**. In many researches, it has been reported that the leaching of phthalates occur from the plastics dumped landfill area **[2, 3]**. Phthalic acid esters leaches out into the environment easily as they are physically adhered to the matrix of plastic products and therefore remains present in natural waters, wastewaters, soils, sediments and it is also present in air because of their relatively high vapour pressures **[4]**. The phthalic acid esters in microplastics belong to refractory organic compounds, which are detected in soil **[5]**. Among the PAEs, DEHP is believed to be the most resistant and commonly used phthalic acid esters because of its long hydrocarbon chain **[6]**. The European Community in 2000, listed DEHP as hazardous substances to be controlled in surface water **[7]**. It has been reported that the human exposure to DEHP cause liver damage and is linked with the reproductive and developmental abnormalities **[8-12]**. In a recent study, a number of PAEs metabolites have been detected in hair of pregnant women that were most likely associated with the use of cosmetics and plastics **[13]**. In the United States and Europe so much effort has been taken to control this compound **[14]**. Recently, it has been reported that the plastic mulch film which is applied for the cultivation of vegetables allows DEHP to be taken up by plants and hence enter the human food chain, through which human health is at a potential risk **[15]**. Many studies of the degradation reported that the DEHP microbial mechanism of action is the principal source for the degradation of DEHP in aquatic and terrestrial systems, such as soils, sediments and surface waters **[16]**. Various analytical methods have been developed to determine phthalates in different matrices **[17]**. To determine the risk of phthalate contamination in foodstuffs, though many studies correlated with phthalate contamination have been carried out but still there are several diseases which are associated with phthalate contamination, are controversial **[18]**. Rather than physicochemical methods, biological processes are considered more applicable and environmental friendly and cost effective **[19]**. *Bacillus cereus spp.* is one of the most potent strains for DEHP degradation in contaminated soil and the strain has a strong ability to degrade phthalate completely **[20]**. In the present study, we aimed at isolating the microbial strains capable of degrading phthalate from soil contaminated with DEHP. This strain was recognized to have the ability to utilize DEHP. Besides this, some biochemical tests along with various environmental and chemical factors have also been employed to further optimize the conditions for degradation of DEHP. The optimized parameters will help us in deciding the practical feasibility of the application of efficient microbes in bioremediation.

MATERIAL AND METHODS

Chemicals and media:

Di -2 ethyl hexyl phthalate was procured from Accu standard Inc.USA. Corn oil used as a vehicle was purchased from Sigma Pvt. Ltd and Nieshiel Chemical Pvt. Ltd. which were of analytical grade. The initial enrichment culture is initiated on minimal salt media (MSM media) supplemented with DEHP. The MSM consisted of the following chemicals (mg l^{-1}): (NH4)2SO4, 1,000 mg l^{-1}; KH2PO4, 800 mg l^{-1}; K2HPO4, 200 mg l^{-1}; MgSO4·7H2O, 500 mg l^{-1} ; FeSO4, 10 mg l^{-1}; CaCl2, 5 mg l^{-1} and the pH were maintained 7.0±0.1 with the help of HCl or NaOH **[21]**.

Isolation of DEHP degrading bacterial strain:

Microbes using DEHP as a sole source of carbon were isolated through an MSM-culture procedure. The initial culture was prepared by the addition of 10 g of fresh soil with 100 ml sterile water in the flask **[21]**. The flask was left static for 30 min after shaking at 125 rpm for 30 min, 1.0 ml of supernatant was moved to a new flask containing 100 ml of sterilized MSM supplemented with DEHP (10 mg l^{-1}) to enrich the culture. The flasks were shaken at 125 rpm and 35 °C for 48 hrs **[22]**. Then 100 µl of medium was moved from the flask and inoculated by the spread plate method into a solid nutrient agar medium. After 48hrs incubation, various well-separated independent colonies of diverse morphological characteristics showed up and pure culture colonies of the bacterial isolates were conducted in MSM, with this purifying process conducted in excess of five times. The dominant DEHP-degrading bacterium was confirmed and named T20. All experimental works have been performed in triplicates **[23]**.

Identification of isolated strain T20:

The strain T20 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') and 1541R (5'AAGGAGGTGATCCAGCCGCA3') **[24]**. 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 **[25]**. The phylogeny analysis and Program PhyML 3.0 was performed. The program Tree Dyn 198.3 was employed for the rendering of tree **[26]**.

Optimization of conditions for DEHP degradation:

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

Analytical method employed for degradation:

The intermediate metabolites were analyzed by liquid chromatography-mass spectrometry (LC-MS). The mass spectrometry was operated with ESI ionization source, positive mode, mass scanner range of 100~300 amu, fragment at 100 V; drying gas flow was 13.0 $L^{-\,min}$; nebulizer pressure was 60 Psig; drying gas temperature was 350 ºC and capillary voltage was 4000 V **[27]**.

RESULT AND DISCUSSION

Identification and characterization of isolated strain T20-

After the screening process, the most potent bacterial isolate named T20 was selected. Through various investigations we found that the isolated bacterial strain was gram-negative, rod-shaped and non-spore forming flagellated. After culturing for 18–24 h at 37°C, this strain T20 formed was off white in colour, opaque, oval and flat colonies with irregular margin on nutrient agar. The strain T20 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 T20 identified as *Bacillus cereus* (GenBank accession no. CP026375.1). **Figure 1** illustrates the phylogenetic relationship of T20 with its close relatives **[28]**.

Optimization of the conditions at different environmental and chemical conditions

Effect of pH

The initial descriptive analysis of effect of pH on DEHP degradation by *Bacillus cereus* is shown in **Figure 2 (a)**. Highest DEHP degradation can be observed at pH 7.0 level on 4th day which is 97%. At pH 5.5 level the DEHP degradation for *Bacillus cereus* was the lowest. A two-way ANOVA test showed a statistical significant difference in DEHP degradation by both pH (f(pH) = 12.58, p< 0.005) and by no of days (f(day) = 36.19, p <0.005), the interaction between these terms was also significant (f (pH: day) = 2.02, p < 0.005). A Tukey post-hoc test revealed significant pair wise difference between pH 5.5 level and pH 7.0 levels. Likewise, there is significant pair wise difference among all days except between day 2 and day 8, day 6 and day 8.Therefore, the study shows that pH range 7.0 was optimal for DEHP degradation by *Bacillus cereus* **[20]**. These findings are consistent which was 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 **[28]**. The optimum pH in degradation of various pollutants by other genus has been reported is range from 7.0 to 8.0 **[29, 30, 31]**. Some other microorganisms such as *Rhodococcus* sp. HS-D2 **[32]** *Gordonia alkanivorans* YC-RL2 **[33]**, *Acinetobacter* sp. SN13 **[34]** and *Pseudomonas fluorescens* FS1 **[22]** have been reported a wide pH range at 5–10, 6–11, 3–9, and 4–9 respectively.

Effect of temperature

Temperature is also an important factor influencing PAE degradation as reported in many studies. A two-way ANOVA test showed a statistically significant difference in DEHP degradation by both Temperature (f (Temp) = 43.22, p < 0.005) and by no of days (f (day) = 71.64, p < 0.005), the interaction between these terms was also significant (f (Temp: day) = 3.98, p < 0.005). A Tukey post-hoc test revealed significant pair wise difference between temperature 28°C and 37°C and temperature 37°C and 50°C. Likewise, there is significant pair wise difference among all days except between day 0 and day 8, day 2 and day 4, day 2 and day 6, day 4 and day 6. Therefore, the study shows that the rate of degradation by strain T20 increased significantly when the temperature was increased to 37 °C from 28

°C. The degradation was highest on 4th day of incubation which is 94% followed by 5th day which is 90%. As depicted in **Figure 2(b)**. Hence, the study found that 37 °C and 50 °C are the optimum temperatures for degradation. The present finding is consistent with *Rhodococcus ruber* YC-YT1 which was isolated from contaminated soil **[35]**.

Effect of NaCl concentration

Salinity is a significant parameter; the effect of salt concentration on DEHP biodegradation was also examined. As shown in **Figure 2 (c)**, strain T20 was sensitive to the salinity level. A two-way ANOVA test showed a statistical significant difference in DEHP degradation by NaCl concentration (f (NaCl conc) = 85.72, $p < 0.005$) and by no of days (f (day) = 14.24, $p < 0.005$), the interaction between these terms was also significant (f (NaCl:day) = 4.76, $p < 0.005$).A Tukey post-hoc test revealed significant pair wise difference between NaCl 10 % and 5% and NaCl 15% and 5%. Likewise, there is significant pair wise difference between day 0 and day 4 & day 4 and day 8. Therefore, the analysis shows that the rate of DEHP degradation is highest at 5% NaCl on 4th day which is 98% as in **Figure 2(c)**. Virtually very slow degradation or bacterial growth was observed at 10% and 15% concentration of NaCl. There is very little research which has been focused on phthalate degradation in the presence of NaCl **[36]**. Although strain T20 exhibited a similar trend in that higher salinity levels reduced the bacterial growth rates, resulting in longer degradation times, it could tolerate a NaCl concentration upto 5%. This characteristic suggests that strain T20 would be a promising candidate for bioremediation of DEHP-contaminated soil containing salts. *Rhodococcus ruber* YC-YT1 isolated from water which containing NaCl could tolerate up to 120 g/l of NaCl **[37]**. A study has identified halotolerant isolate *Sphingobium* sp. which degraded DBP at the salinity ranging from 0 to 4% **[38, 39]**.

Effect of various carbon and nitrogen sources-

DEHP degradation was affected by the nature of the carbon source differently. As the results indicated, the best activity of DEHP was obtained with culture containing lactose as a sole source of carbon as illustrated in **Figure 2(d)**. A two-way ANOVA test showed a statistical significant difference in DEHP degradation by carbon source (f (C-source) = 324.21, $p < 0.005$) and by no of days (f (day) = 407.51, $p < 0.005$), the interaction between these terms was also significant (f (C-source: day) = 203.99, $p < 0.005$). A Tukey post-hoc test revealed significant pair wise difference between C-source dextrose and lactose, lactose and mannose, lactose and sucrose. Likewise, there is significant pair wise difference between day 0 and day 4, day 2 and day 4 , day 4 and day 6&day 4 and day 8. Therefore, from the analysis it can be interpreted that the rate of degradation is significantly highest when lactose is used as carbon source compared to other carbon sources used and highest degradation is observed at 4^{th} day which is 95% as in **Figure 2 (d)**. In case of *Rhodococcusruber*YC-YT1 strain which was capable of degrading DEHP due to utilization as a sole source of carbon **[38]**.

On the other hand, various nitrogen sources peptone, beef extract, yeast extract and casein were added by equivalent amounts at a concentration of 1% (w/v) to MSM medium, one at a time. **Figure 2 (e)** illustrated that DEHP worked differently with different nitrogen sources. A two-way ANOVA test showed a statistical significant difference in DEHP degradation by nitrogen source (f (N-source) = 1312.80, $p < 0.005$) and by no of days (f (day) = 984.02, $p < 0.005$), the interaction between these terms was also significant (f (N-source: day) = 224.62, $p < 0.005$).A Tukey post-hoc test revealed significant pair wise difference between N-source beef extract and peptone, beef extract and yeast extract, casein and peptone and casein and yeast extract. Likewise, there is significant pair wise difference between day 0 and day 2, day 0 and day 4, day 0 and day 6, day 2 and day 4 & day 4 and day 8. Peptone and Yeast extract were the most preferable nitrogen source with increase in DEHP degradation rate 79% and 92% respectively compared to the control as in **Figure 2 (e)**. On the other hand, the lowest microbial activity was obtained in the cultures containing beef extract and casein as a nitrogen source with decrease in DEHP compared to the control. Therefore, from the analysis it can be interpreted that the rate of degradation is significantly highest when yeast extract is used as nitrogen source compared to other nitrogen sources used and highest degradation is observed at 4^{th} day. Based on the previous results, the best microbial activity was obtained with the culture broth containing yeast extract as a nitrogen source **[40]**.

Analytical method for the identification of metabolic intermediates-

LC-MS method was employed for the identification of the metabolic intermediates.

The mass spectral analysis of the compound showed the parent ion peak at m/z 301.15 as shown in **Figure 3 (b)**. The fragment peaks patterns showed at m/z ion peaks at m/z

321.25 base peaks presents the metabolic intermediates identified in DEHP degradation by *Bacillus cereus* T20. In the present study, peak was obtained at 10.14 min. in the chromatogram which exhibited mass spectrum depicted in **Figure 4 (a)**. Increase in the other peaks within chromatogram revealed the degradation products of DEHP by *Bacillus cereus* T20. The metabolites were identified by comparing the mass spectrum at a particular retention time (RT) with published mass spectra from the database.

There are four main peaks identified having different RT values and mass. These are 301.15 at RT value 9.02, 413.29 at RT value 10.14, 441.32 at RT value 10.33 and 454.39 at RT value 10.5. These peaks are degradation intermediates identified as Sodium isooctyl phthalate (mass 300.13), 2-(Dodec-1-yn-1-yl)-1,1,1,3,3,3-hexamethyl-2-(trimethylsilyl)trisilane (mass 412.28), bis[4-(oxolan-2-yl) butan-2-yl] nonanedioate (mass440.31), 1-[Bis(2-hydroxyethyl) amino] -3- (octadecyloxy) propan-2-ol (mass 431.39).

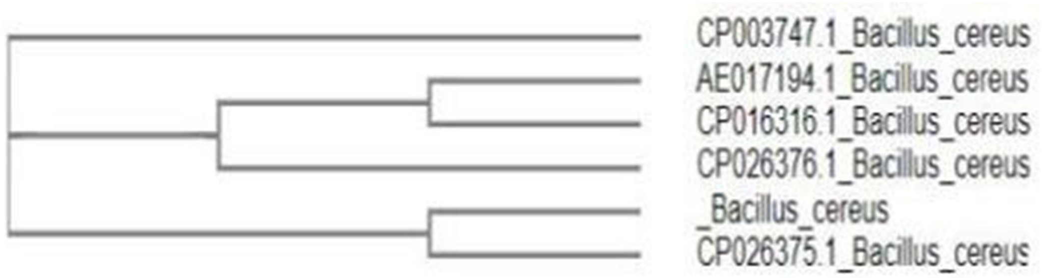

Figure 1: Phylogenetic tree of isolated bacterium (T20) designated as *Bacillus cereus spp.* based on 16sr RNA sequence alignment

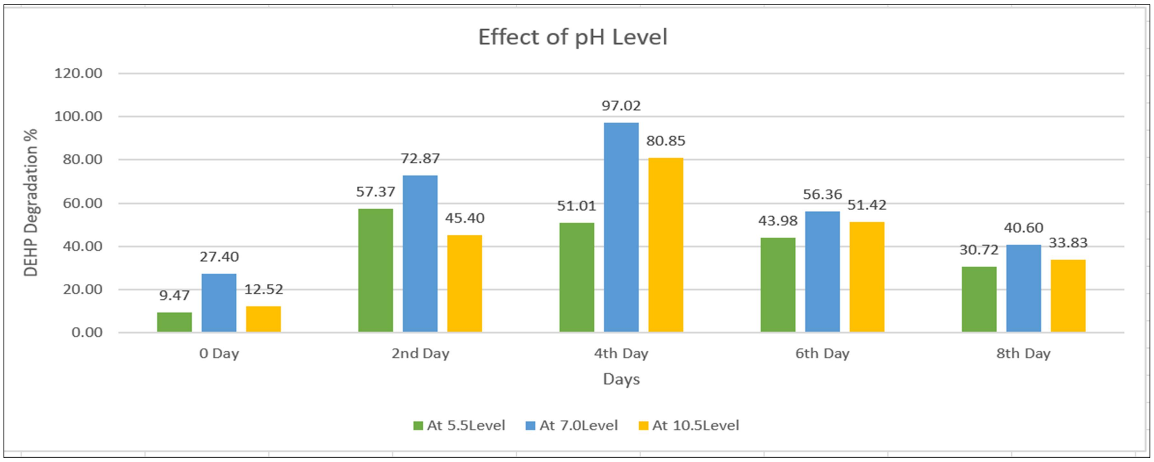

Figure 2 (a): Effect of pH on degradation of DEHP

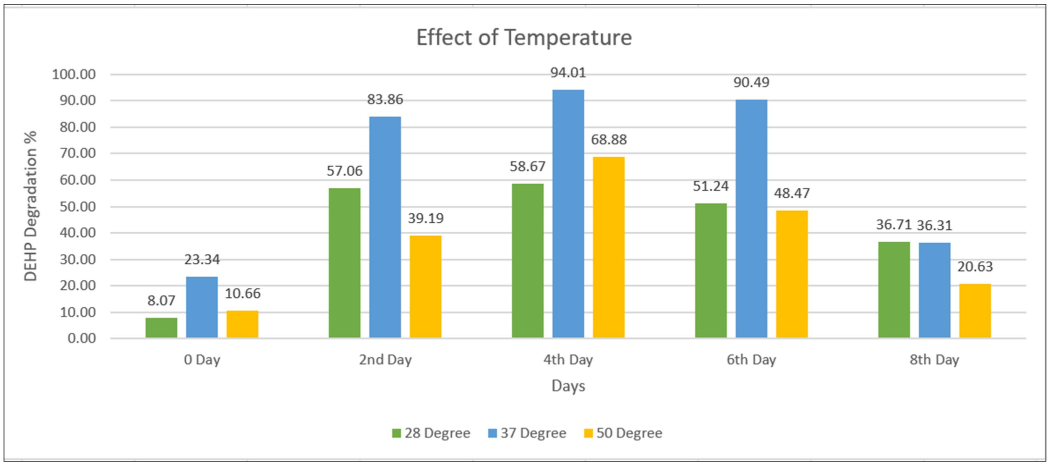

Figure 2 (b): Effect of temperature on degradation of DEHP

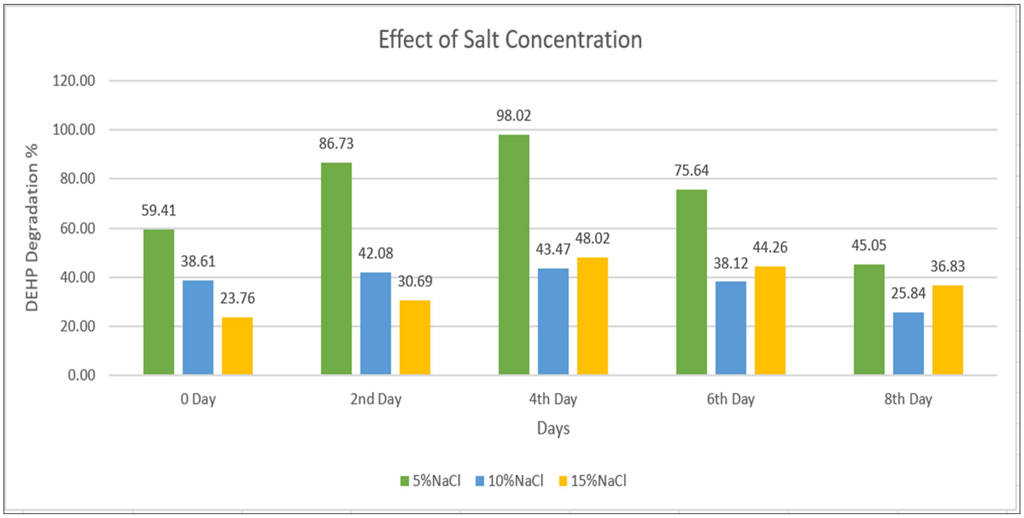

Figure 2(c): Effect of salt concentration on DEHP degradation

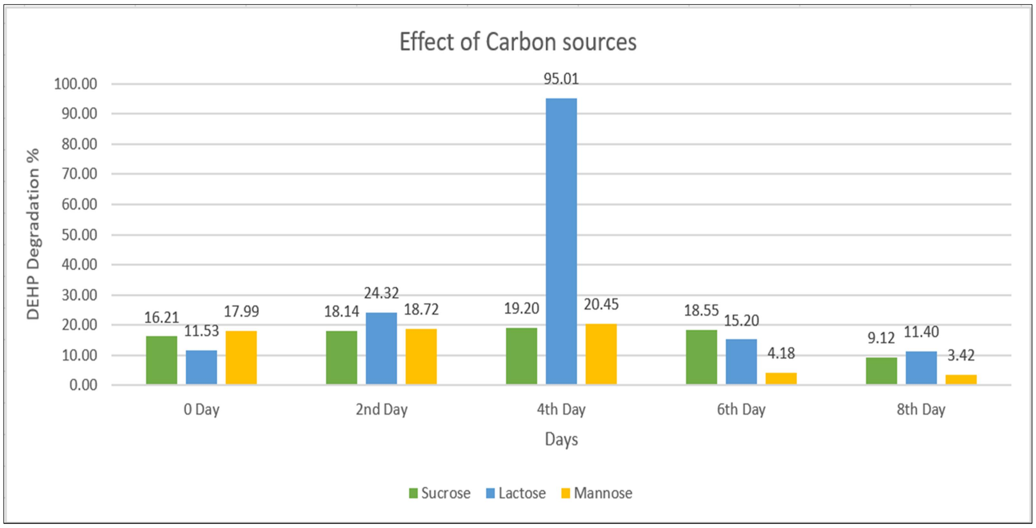

Figure 2 (d): Effect of different carbon sources on DEHP degradation

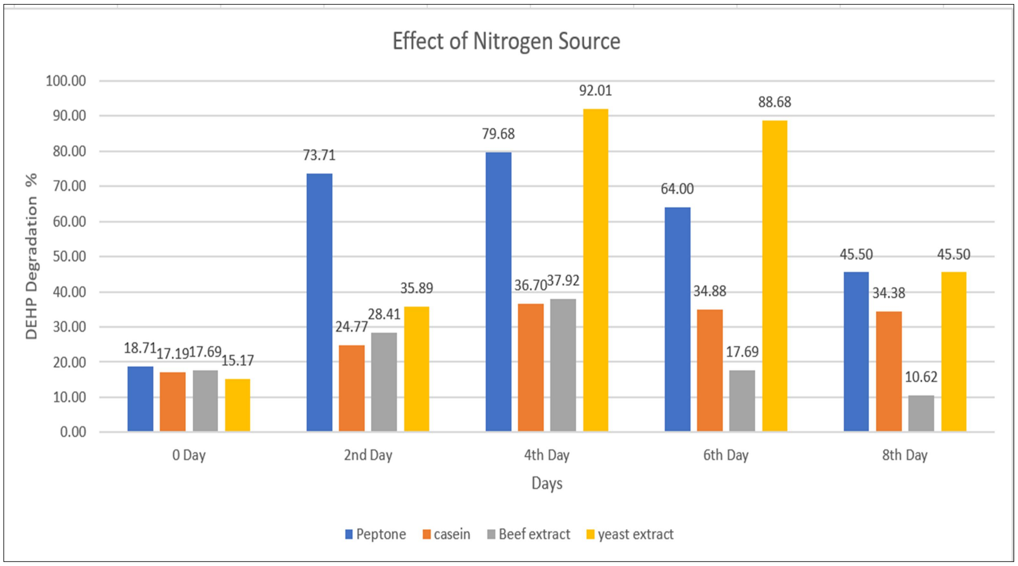

Figure 2 (e): Effect of different nitrogen sources on DEHP degradation

Table 1: Biochemical characterization result-

	Biochemical tests	Bacterial strain (T20)
1	Amylase	-
2	Casein Hydrolysis	+
3	Catalase	+
4	Gelatin Hydrolysis	+
5	Nitrate Reduction	+
6	Citrate Utilization	-
7	Indole Production	+
8	Methyl Red	+
9	Voges Proskuer	-
10	Lactose	-
11	Dextrose	-
12	Sucrose	-
13	H_2S production	+

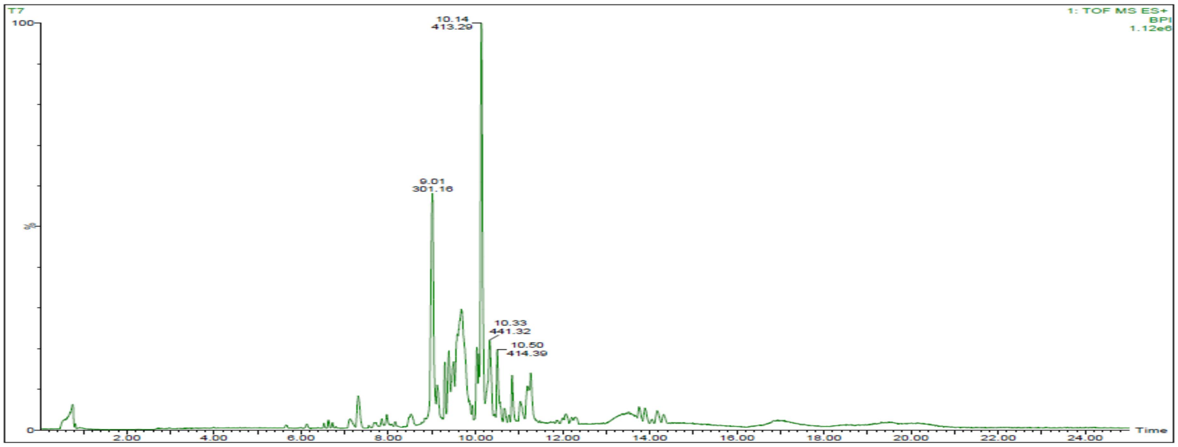

Figure 3 (a): Total ion chromatogram, x-axis showing intensity or abundance while y-axis showing retention time

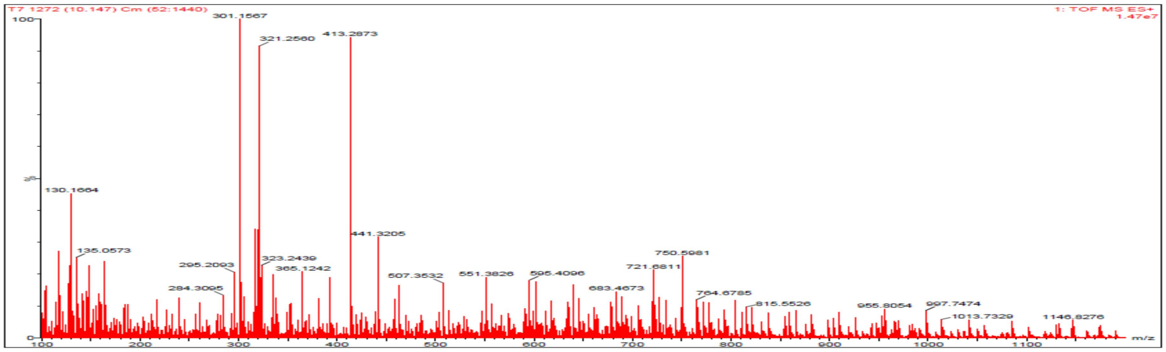

Figure 3 (b): The mass spectrum of DEHP degraded intermediate compounds, X-Axis contains mass in relative to charge ratio (m/z) while y-axis contains a relative abundance

CONCLUSION

In summary, the bacterial isolate T20 capable of utilizing DEHP was isolated from soil samples contaminated with phthalate which was identified as *Bacillus cereus* accession no. (CP026375.1) by 16srRNA gene sequencing. To our knowledge, this is the first report on DEHP degradation by *Bacillus cereus* accession no. (CP026375.1) in which the different chemical conditions for degradation were used. The newly developed technique of LC-MS using the MRM mode has proved to be a selective and reliable approach for the quantitative determination of degradable product. These results may have direct relevance for the bioremediation of DEHP pollutants in contaminated soil **[42]**.

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