Biospectra : ISSN: 0973-7057, Vol. 16(1), March, 2021, pp. 143-148
An International Biannual Refereed Journal of Life Sciences

29(PS).
Plant Science

ISSN : 0973-7057

Int. Database Index: 616 www.mjl.clarivate.com

Indigenous isolate *Bacillus subtilis* strain mp1 mediated degradation of di -(2-ethyl hexyl) phthalate and analysis of the degradation intermediates by lc-ms.

Tanuja[a], Madhavi Rashmi[b]* & Shivanand Singh[c]

[a]University Department of Botany, Patliputra University, Patna, Bihar, India

[b]Department of Biotechnology, Magadh University, Bodh Gaya, Bihar, India

[c]Department of Zoology, B.R.A Bihar University, Muzaffarpur, Bihar, India

Received : 11th January, 2021 ; Revised : 12th February, 2021

Abstract- DEHP (di-ethyl hexyl phthalate) is an endocrine disrupting chemical that is commonly used as a plasticizer worldwide and is reported to adversely affect humans as well as wildlife. In the present study, strain MP1, an indigenous isolate from landfill soil has been selected which was able to grow and metabolize DEHP as sole carbon and energy sources in the MSM liquid media and showed DEHP degrading potential . It was capable of sustaining a wide range of environmental conditions with the optimum pH for bacterial growth 7.0, temperature 28°C and tolerance upto salinity 5%. The analysis of morphological and biochemical characteristics along with gram staining technique followed by 16s rRNA sequencing, identified it as *Bacillus subtilis* (Genbank Accession no. KP241787.1). LC-MS) analysis was done for the determination of metabolic intermediates. 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 757.68 at RT value 11.27. 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),n-(2-hydroxyethyl)-n,n-dimethyl-2,3-bis(octadecanoyloxy)propan-1-aminium) acetate (mass 755.66). These findings can provide some information in the bioremediation of DEHP from contaminated soil.

Key words: DEHP, Bacteria, Degradation, *Bacillus subtilis*, 16sr RNA, LC-MS

INTRODUCTION

Phthalates are a group of compounds employed in the fabrication of plastics and diverse other industrial applications.[1] Di-ethyl hexyl phthalate (DEHP) is a kind of phthalic acid esters (PAEs), widely used as a plasticizer. It is also known as environmental secretion disruptor or environmental hormone. It is used in the manufacture of plastics, rubber, medicine, cosmetics, toys and other industries as a plasticizer.[2] As DEHP physically adhere to the polymeric matrix it can easily leak from the polymers into the environment through manufacturing, storage, use and disposal.[3] The women would have been related with endometriosis and gestational duration with precocious breast development due to increase in the level of phthalate.[4] Due to its significant lethal or mutagenic effects DEHP could cause central nervous system inhibition and

*Corresponding author :
Phone : 9661047829
E-mail : madhavi.rashmi@gmail.com

renal injury in mice.[5] DEHP is considered as one of the most resistant phthalate esters because of its long hydrocarbon chain.[6] Many researchers reported that the humans are affected by DEHP due to its exposure in daily lives which may result in adversely affecting the functioning of endocrine, reproductive and nervous systems.[7,8] Biological methods of degradation have been paid 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.[9-11]

This study focused on degradation capability of *Bacillus subtilis* towards DEHP, isolated from the landfill soil that could utilize DEHP as a sole source of carbon and energy. Batch experiments were performed at different temperatures, pH and salt concentration for optimizing the conditions for better degradation of DEHP. LC-MS analysis was done to detect metabolic intermediates of degradation.

MATERIALS & METHODS

Chemicals and media- DEHP was acquired from Accu Standard Inc. USA. The stock solution of DEHP (20 mg/ml) was prepared in corn oil and kept at 4°C until use. Corn oil used as a vehicle was purchased from Sigma Pvt. Ltd and Nieshiel Chemical Pvt. Ltd. which were of analytical grade. The minimal salt medium (MSM) consisted of the following chemicals (mg/l): $(NH_4)2SO_4$, 1,000; KH_2PO_4, 800; K_2HPO_4, 200; $MgSO_4 \cdot 7H_2O$, 500; $FeSO_4$, 10; $CaCl_2$, 50 and the pH was maintained to 7.0±0.1 with HCl or NaOH.[12]

Isolation of DEHP degrading bacterial strain and enrichment-

An aliquot of 1g of fresh soil was added into 100 ml minimal salt medium (MSM) supplemented with 100 mg/l of DEHP to enrich the culture and the culture was incubated in a shaker (30°C, 125rpm).[13,14] After 7 days of incubation, 2 ml of the culture was incubated further for another 7 days. There was repetition of process by increasing the DEHP concentration. Finally, the samples were streaked onto nutrient agar plates supplemented with 100 mg/l DEHP as sole carbon sources, and plates were incubated under 37°C in the dark. After incubation at 37°C, few well-separated individual colonies of different morphological types appeared and it pure culture was obtained by further repeated streaking on agar plates. Among the isolates, strain MP1 was able to significantly degrade DEHP.

Microbial Identification and Phylogenetic Analysis

The genomic DNA of the bacterial isolate was extracted using Bacterial Genomic DNA Extraction kit (Yaazh xenomics, Coimbatore, India) and subjected to PCR amplification of 16S rRNAgene sequence using 8F and 1541R. The PCR products were sequenced and deposited into Gen Bank, and related sequences were obtained by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). 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. The phylogeny analysis and Program PhyML 3.0 was performed. The program Tree Dyn 198.3 was employed for the rendering of tree.

Biodegradation tests:

To determine the effect of environmental factors,optimization experiments were performed, as pH (5.5, 7.0 and 10.5), temperature (28, 37 and 50°C)and salinity (5%, 10% and 15%).After 48 hrs of incubation interval, up to the period of 8 days, the response was recorded in the terms of absorbance at 600nm. Experiments were performed in triplicate.[15]

Analysis of metabolic intermediates

DEHP degradation intermediateswere analysed using 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 350ºC and capillary voltage was 4000 V.[16]

RESULT & DISCUSSION

1. Microbial Isolation, Identification and Phylogenetic Analysis-

The most potent bacterial isolate named MP1 was selected after several screening. The strain was gram-positive, rod-shaped and flagellated with non-spore forming. After cultured for 18-24 h at 37°C, this strain MP1 formed off white, opaque and irregular flat colonies with smooth margin on nutrient agar. The strain MP1 identified as *Bacillus subtilis* through its morphological

characteristics, biochemical characteristics and phylogenetic analysis. According to the NCBI BLAST analysis of its 16S rRNA gene sequence, strain MP1 belonged to the *Bacillus subtilis* (GenBank accession no. KPO241787.1). Fig. 1 illustrates the phylogenetic relationship of MP1 with its close relatives.[17]

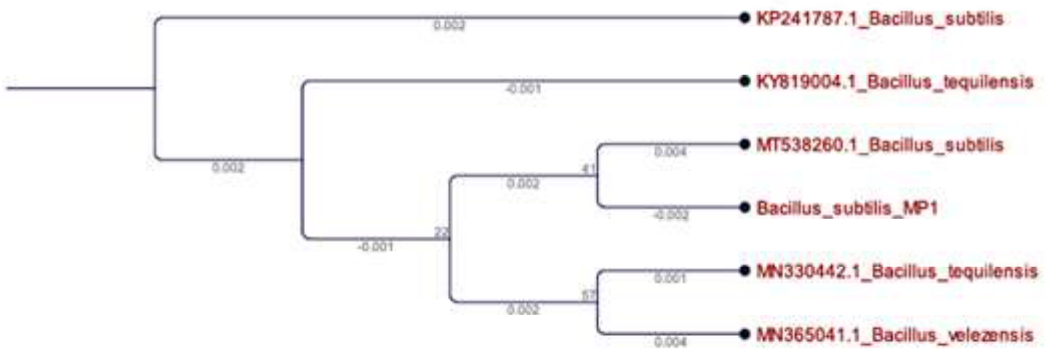

Fig. 1.Phylogenetic tree of the isolated strain MP1.

2. Biodegradation Experiments

Environmental factors such as pH, temperature, salinity and pollutant affect biodegradation of phthalates.[18] In this study, strain MP1 was able to grow and metabolize DEHP as sole carbon and energy sources in the MSM liquid media. It was capable of sustaining in a wide range of environmental conditions.

2.1 Effect of pH on DEHP degradation

The effect of pH on DEHP degradation by strain is shown in Fig. 2a. The degradation rate increased rapidly when pH was increased from 5.5 to 10.5. The highest DEHP degradation for MP1 was achieved at pH 10.5. Some other microorganisms such as *Rhodococcus* sp.HS-D2[19],*Gordonia alkanivorans* YC-RL2[19], *Acinetobacter* sp. SN13[14] and *Pseudomonas fluorescens* FS1[13] have been reported a wide pH range at 5-10, 6-11, 3-9, and 4-9 respectively.

2.2 Effect of temperature on DEHP Degradation

Strain MP1 was able to grow and degrade DEHP in temperatures of 28-50^{o}C with the optimum being 28^{o}C as shown in Fig. 2b. At 37^{o}C, there was little or minimal growth and degradation. As the temperature was increased from 37^{o}C to 50^{o}C, the rate of degradation increased drastically. The maximum rate of degradation was at 50^{o}C. The capability of MP1 to degrade DEHP at higher temperatures in accordance with the ability of *B. subtilis* 3C3.[20] There are few other strains reported which are capable of degrading DEHP at a wide range of temperatures.[21]

2.3 Effect of NaCl concentration on DEHP degradation

Strain MP1 was able to grow and degrade DEHP in salt concentrations ranging from 0 to 5%. There was a relatively slow growth in higher salt concentrations of 5-15%. At 5% salt concentration the microbial growth was highest as in Fig.2c. Salinity is an important factor ofwaste water treatment as many industries such as the leather industry and landfill leachate releases waste water containing high salt concentrations.[22] A study has identified halo tolerant isolate *Sphingobium* sp. which degraded DBP at the salinity ranging from 0 to 4%.[23] In biological waste treatment, salinity content of wastes is a major factor affecting bacterial growth and hence enzymatic activity.[24]

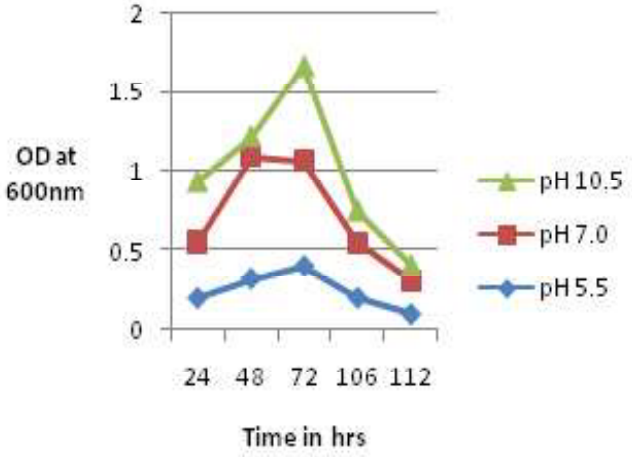

Fig.2a: Effect of pH on DEHP degradation

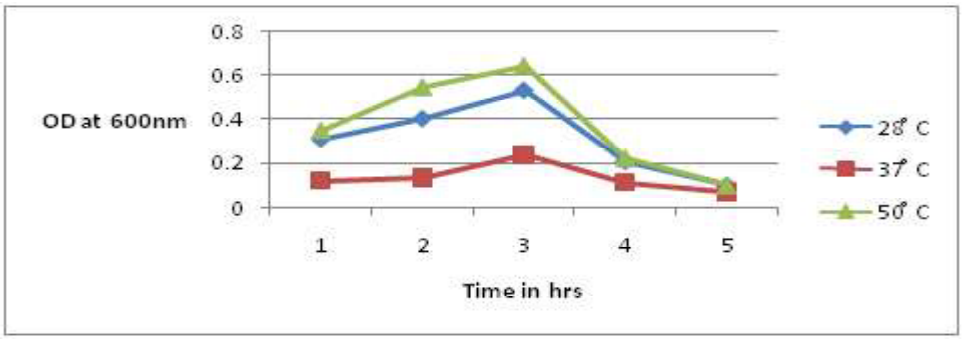

Fig. 2 b: Effect of temperature on DEHP degradation

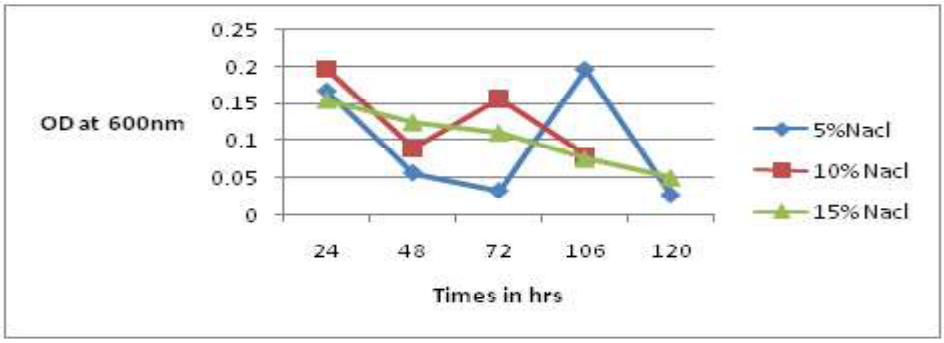

Fig 2 c: Effect of salinity on DEHP degradation

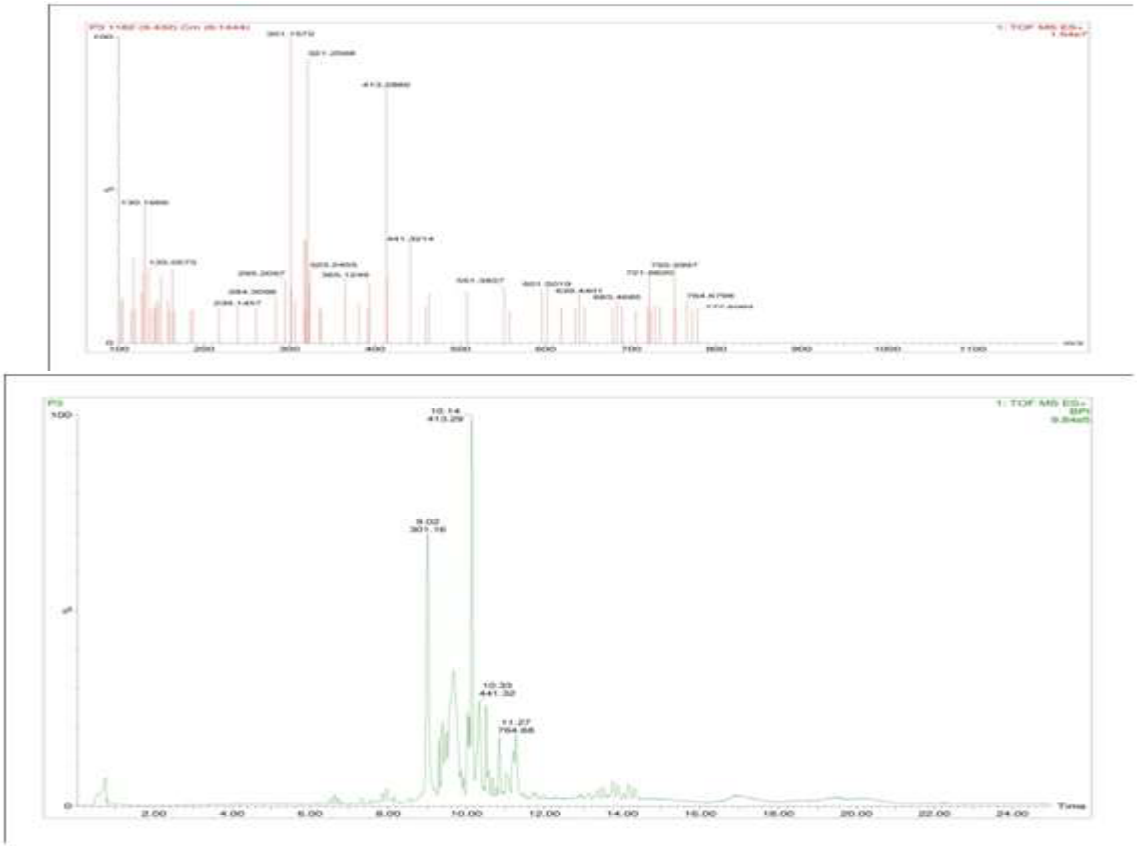

Fig.3 - The mass spectrum of DEHP degraded intermediate compounds, X-Axis contains mass in relative to charge ratio (m/z) while y-axis contains a relative abundance

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	VogesProskuer	-
10	Lactose	-
11	Dextrose	+
12	Sucrose	+
13	H_2S production	-

Identification of DEHP degradation intermediates

The mass spectral analysis of the compound showed the parent ion peak at m/z 321.25 as shown in Fig.3. The fragment peaks patterns showed at m/z ion peaks at m/z 301.15 base peaks Fig.3 presents the metabolic intermediated identified in DEHP degradation by *Bacillus subtilis*.

In the present study, peak was obtained at 10.14 min. in the chromatogram which exhibited mass spectrum depicted in figure 3. Increase in the other peaks within chromatogram revealed the degradation products of DEHP by *Bacillus subtilis* MP1. 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 757.68 at RT value 11.27. These peaks are degradation intermediates identified asSodium isooctyl phthalate (mass 300.13), 2-(Dodec-1-yn-1-yl)-1,1,1,3,3,3-hexamethyl-2-(trime thylsilyl) trisilane (mass 412.28), bis[4-(oxolan-2-yl) butan-2-yl] nonanedioate (mass440.31), n-(2-hydro xyethyl) -n, n-dimethyl-2,3-bis (octadecanoyloxy) propan-1-aminium) acetate (mass 755.66).

Strain MP1 showed an outstanding ability to degrade DEHP in a wide range of temperatures (20-50^{o}C), wide range of pH (5.5-10.5) and superior salinity tolerance (0-5% NaCl) and therefore it could be used for remediating various phthalate contaminated environment.

ACKNOWLEDGEMENT

The authors are thankful to Principal and Department of Botany, T.P.S. College, Patliputra University Patna, for providing infrastructural facilities.

REFERENCES

1. **Lin Y., Wang L., Li R., Hu S., Wang Y., Xue Y., Y. H., Jiao Y., Wang Y., and Zhang Y. 2018.** How do root exudates of bokchoy promote dibutyl phthalate adsorption on mollisol? *Ecotoxicol. Environ. Saf.* **161:**129–136.
2. **Nahurira R., Ren L., Song J.L., Jia Y., Wang J.H., Fan S.H., Wang H.S., Yan Y.C. 2017.** Degradation of Di(2-Ethylhexyl) Phthalate by a Novel *Gordonia alkanivorans* Strain YC-RL2, *Curr Microbiol*. **74:**309-319.
3. **Zhang L., Liu J., Liu H., Wang and Zhang S. 2015.** The occurrence and ecological risk assessment of phthalate esters (PAEs) in urban aquatic environments of China. *Ecotoxicol*. **24(5):**967–984.
4. **Toft G., BO A., Jonsson G., and J., P., B. 2012.** Association between pregnancy loss and urinary phthalate levels around the time of conception, *Environ. Health Perspec*. **120(3):** 458–463.
5. **Gao H. T., Xu R., Cao W. X., QianL. L., Wang M., Lu L., Xu Q., and Yu S. Q. 2017.** Effects of six priority controlled phthalate esters with long-term low dose integrated exposure on male reproductive toxicity in rats, *Food Chem. Toxicol.* **101:** 94–104.
6. **Chang B. V., Yang C. M., Cheng C. H., and Yuan S. Y. 2004.** Biodegradation of phthalate esters by two bacteria strains. *Chemosph*. **55:** 533e538.
7. **Net S., Semp R., Delmon T. A., Paluselli A., and Ouddane B. 2015.** Occurrence, fate, behaviour and ecotoxicological state of phthalates in different environmental matrices. *Environ. Sci. Technol*. **49:** 4019e4035
8. **Chen H., Zhang W., Rui B. B., Yang S. M, Xu W. P., and Wei W. 2016.** Di (2-ethylhexyl) phthalate exacerbates non-alcoholic fatty liver in rats and its potential mechanisms. *Environ. Toxicol. Pharmacol*. **42:** 38-44

9. **Chen J. A., Li X., Li J., Cao J., QiuZ., Qing Z., and Shu W. 2007.** Degradation of environmental endocrine disruptor di-2-ethylhexyl phthalate by a newly discovered bacterium, *Microbacterium sp. strain CQ0110Y. Appl. Microbiol.* **74(3):** 676-82.

10. **Meng X., Niu G., Yang W., and Cao X. 2015.** Di (2-ethylhexyl) phthalate biodegradation and denitrification by *Pseudo xanthomonas sp*. Strain. *Bioresour. Technol*. **180:** 356e359.

11. **Pradeep S., Josh M. S., Binod P., Devi R. S., Balachandran S., Anderson R. C., Benjamin S. 2015.** *Achromobacter denitrificans strain* SP1 efficiently remediates di (2-ethylhexyl) phthalate. *Ecotoxicol. Environ. Saf*. **112:** 114e121.

12. **Fang C. R., Yao J., Zheng Y. G., Jiang C., J., Hu L. F., Wu Y. Y., and Shen D. S. 2010.** Dibutylphthalate degradation by *Enterobacter sp.* T5 isolated from municipal solid waste in landfill bioreactor. *Int. Biodeter. Biodeg*. 64: 442e446.

13. **Zhao H., Du H., Lin J., Chen X., Li Y., Li H., Cai Q, Mo C, Qin H and Wong M. 2016.** Complete degradation of the endocrine disruptor di-(2-ethylhexyl) phthalate by a novel *Agromyces sp.* MT-O strain and its application to bioremediation of contaminated soil. *Sci. Tot. Environ*. **562:**170e178

14. **Li C., Chian X. L., Chen Z., X., Deng Z. Y., and Xu H., 2012.** Biodegradation of an endocrine-disrupting chemical di n-butyl phthalate by *Serratia marcescens* C9 isolated from activated sludge. *Afr. J. Microbiol. Res*. **6(11):** 2686–2693, 702e710.

15. **Ting Y., Lei R., Yang J., Shuanghu F., Junhuan W., Jiayi W. R., Nahurira , H., Wang and Yanchun Y. 2018.** Biodegradation of Di-(2-ethylhexyl) Phthalate by *Rhodococcus ruber YC-YT1* in Contaminated Water and Soil. *Int. Journal of Environ. Res. Public Health*. **1(5):** 15-3

16. **Wu N. and Thompson R. 2006.** Fast and efficient separations using reversed phase liquid chromatography. *J. Liq. Chrom. Related Tech*. **29(7-8):** 949-988. doi:10.1080/10826070600574903

17. **Quan C. S., Liu Q., Tian W. J., Kikuchi J., and Fan S. D. 2005.** Biodegradation of an endocrine-disrupting chemical ethylhexyl phthalate, by *Bacillus subtilis* No. 66. *Appl. Microbiol. Biotechnol*. **66(6):**702-10

18. **Thomas Johnson B., Heitkamp M. A., Jones J. R. 1984**. Environmental and chemical factors influencing the biodegradation of phthalic acid esters in freshwater sediments. *Environ Pollut Ser B. Chem Phys.* **8:**101–118. doi:10.1016/0143-148X(84)90021-1

19. **Zhang Y., Chen H., Liu J., Geng G., Liu D., Geng H. and Xiong L. 2016.** Genome sequencing and biodegradation characteristics of the *n-* butyl benzyl phthalate degrading bacterium-*Rhodococcus* sp. HS-D2. *Inter. Biodetr. & Biodegr.* Doi:10.1016/j.ibiod.2016.08.024

20. **Navacharoen A., Vangnai A.S. 2011.** Biodegradation of diethyl phthalate by an organic-solvent-tolerant *Bacillus subtilis* strain 3C3 and effect of phthalate ester coexistence. *Int. Biodeterior. Biodegrad.* **65:**818–826. doi:10.1016/j.ibiod.2011.05.005

21. **Ren L., Jia Y., Ruth N.., Qiao C., Wang J., Zhao B., Yan Y. 2016.** Biodegradation of phthalic acid esters by a newly isolated Mycobacterium sp YC-RL4 and the bioprocess with environmental samples. *Environ. Sci. Pollut. Res.* **23:**1–11. doi:10.1007/s11356- 016-6829-4

22. **Kartal B., Koleva M., Arsov R., vander Star W., Jetten MSM, Strous M. 2006.** Adaptation of a freshwater anammox population to high salinity wastewater. *J Biotechnol.* **126:**546–553. doi:10. 1016/j.jbiotec.2006.05.012

23. **Jin D., Kong X., Cui Z., Bai H. and Zhang. 2013.** Biodegradation of Di-*n*-Butyl Phthalate by a Newly Isolated Halotolerant *Sphingobium* sp. *Inter. J. Mol. Sci. **14*:** 24046-24054.

24. **Cortes-Lorenzo C., Corte´s-Lorenzo C., Rodrý´guez-Dý´az M., Lo´pez- Lopez C., Sa´nchez-Peinado M., Rodelas B., Gonza´lez-Lo´pez J.2012.** Effect of salinity on enzymatic activities in a submerged fixed bed biofilm reactor for municipal sewage treatment. *Bioresour. Technol*. **121:**312–319. doi:10.1016/j.biortech.2012.06

***