Pedosphere **25**(2): 202–211, 2015
ISSN 1002-0160/CN 32-1315/P

Published by Elsevier B.V. and Science Press

PEDOSPHERE

www.elsevier.com/locate/pedosphere

Isolation and Identification of a Di-(2-Ethylhexyl) Phthalate-Degrading Bacterium and Its Role in the Bioremediation of a Contaminated Soil

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(Received July 8, 2014; revised January 12, 2015)

ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) is a high-molecular-weight phthalate ester (PAE) that has been widely used in the manufacture of polyvinylchloride and contributes to environmental pollution. The objectives of the present study were to isolate a DEHP degrader that can utilize DEHP as a carbon source and to investigate its capacity to biodegrade DEHP in both liquid culture and soil. A bacterial strain WJ4 was isolated from an intensively managed vegetable soil, which was contaminated with PAEs. The strain WJ4 was affiliated to the genus *Rhodococcus* and was able to remove DEHP from soil effectively. A period of only 7 d was required to degrade about 96.4% of DEHP (200 mg L^{-1}) in the liquid culture, and more than 55% of DEHP (1.0 g kg^{-1}) in the artificially contaminated soil was removed within 21 d. Furthermore, *Rhodococcus* sp. strain WJ4 had a strong ability to degrade DEHP without additional nutrients in liquid minimal medium culture and DEHP-contaminated soil and to degrade the homologue of DEHP in both liquid culture and soil. Strain WJ4 represents a novel tool for removing PAEs from contaminated soils and it may have great potential for application in the remediation of environmental pollution by PAEs.

Key Words: biodegradation, environmental pollution, microbial community, *Rhodococcus*, soil enzyme activities, 16S rDNA phylogeny

Citation: Wang, J., Zhang, M. Y., Chen, T., Zhu, Y., Teng, Y., Luo, Y. M. and Christie, P. 2015. Isolation and identification of a di-(2-ethylhexyl) phthalate-degrading bacterium and its role in the bioremediation of a contaminated soil. *Pedosphere*. **25**(2): 202–211.

INTRODUCTION

Phthalate esters (PAEs) are a class of refractory organic compounds largely used as additives and plasticizers to increase the flexibility and workability of diverse products such as personal care products, food packaging, medical products and plastic film (Hauser and Calafat, 2005; Abdel daiem *et al.*, 2012; Wang *et al.*, 2013). Di-(2-ethylhexyl) phthalate (DEHP) is one of the most commonly used PAEs in polyvinyl chloride (PVC) products (Fromme *et al.*, 2002; Gómez-Hens and Aguilar-Caballos, 2003; Chou and Wright, 2006; Latorre *et al.*, 2012; Pradeep *et al.*, 2013), but is not bound chemically to the polymer matrix and thus readily migrates to the environment to become the most abundant phthalate in water, air and soils during the manufacture, use and disposal of PVC products (Xie *et al.*, 2007; Wang *et al.*, 2012; Wang *et al.*, 2013). Moreover, DEHP has a low aqueous solubility (0.285 mg L^{-1} at 24 °C), long side chain, and high octanol-water partitioning coefficient ($\log K_{ow} = 7.5$) (Fromme *et al.*, 2002; Gómez-Hens and Aguilar-Caballos, 2003; Hauser and Calafat, 2005). These properties make DEHP more difficult to biodegrade than PAEs with shorter ester chains (Chang *et al.*, 2004, 2007; He *et al.*, 2013; Wu *et al.*, 2013). DEHP has been listed as a priority pollutant by the United States Environmental Protection Agency and by China National Environmental Monitoring because it is an endocrine disrupting compound and may lead to reproductive abnormalities in males as characterized by malformations of some organs in both rodents and humans (Foster, 2006; Swan, 2008). Repeated exposure to DEHP may result in DNA damage, altered mitotic rate, apoptosis and cell proliferation (Caldwell, 2012) and adverse interference with hormones responsible for the maintenance of homeostasis, behavior, reproduction, and the development of regulatory processes (USEPA, 1997; López de Alda and

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Barceló, 2000).

Metabolic breakdown of PAEs by microorganisms is considered to be one of the major ways by which these widespread pollutants can be degraded in the environment (Liang *et al.*, 2008). A number of studies have reported the biodegradation of phthalates in different environments including aqueous solutions, sludges, wastewaters, sediments, and soils (Stales *et al.*, 1997; López de Alda and Barceló, 2000; Pham *et al.*, 2011; Erythropel *et al.*, 2013). Many bacteria or fungi have the ability to degrade phthalates under aerobic, anoxic and anaerobic conditions and have been isolated from rivers, sediments, sludges and soils and even in marine zones (Liang *et al.*, 2008; Abdel daiem *et al.*, 2012). Chang *et al.* (2004) isolated two bacterial strains that could rapidly degrade PAEs with shorter alkyl-chains but PAEs with longer alkyl-chains were poorly degraded under aerobic conditions. Wu *et al.* (2013) isolated a *Bacillus* sp. strain from shallow aquifer sediments that can degrade di-*n*-butyl phthalate (D*n*BP) under shallow aquifer conditions. Some other studies have reported that PAEs can be removed in the anaerobic digestion of sludges (Di Gennaro *et al.*, 2005; Stasinakis, 2012). Lee *et al.* (2007) and Pradeep *et al.* (2013) reported that PAEs can be removed by several types of fungi. Although DEHP has long alkyl-chains and cannot be readily degraded by bacteria, it may still be completely degraded by some strains in sediments and sludges with low concentrations of DEHP (Zeng *et al.*, 2002; Chang *et al.*, 2004, 2007). Recently, Latorre *et al.* (2012) and Pradeep *et al.* (2013) found that DEHP in PVC wastes was also degraded in a bio-augmented reactor with a high concentration of phthalate. However, only a few microorganisms isolated from soils can be used to remediate PAE-contaminated soils and there have been few studies on the biodegradation of DEHP in soils (Quan *et al.*, 2005; de Moura Carrara *et al.*, 2011).

Indigenous microorganisms offer a cost-effective approach for removing contaminants from soils because they have adapted to the contaminated soils (Liang *et al.*, 2008). Approximately 50% to 60% of the carbon in DEHP was mineralized directly to CO_2 by indigenous microbial flora and the remaining 40% to 50% was converted into cell mass in a bench-scale slurry reactor study (Irvine *et al.*, 1993). The biodegradation of the pollutants followed first-order kinetics in an aerobic slurry-phase reactor and the removal efficiencies were above 61% (Ferreira and Morita, 2012). In addition, toxic organic compounds may affect soil microbial activities (Sebiomo *et al.*, 2011; Sofo *et al.*, 2012) which may be restored when the contaminants are degraded by bacteria (Teng *et al.*, 2010). A previous study indicated that PAEs in soil can impact soil microcosm activity (Xie *et al.*, 2010). However, the bioremediation of DEHP-contaminated soils and the effects of bioremediation on soil microbial activities have not been studied extensively and it would be useful to isolate new bacterial resources to remediate DEHP-contaminated soils.

The objectives of the present study were, therefore, to isolate a DEHP-degrading bacterial strain by enrichment culture from an intensively managed vegetable soil and identify its characteristics, to investigate the ability of the bacterium to degrade DEHP and its homologue, and to assess the bioremediation potential and ecological effects of the isolate on DEHP-contaminated soil.

MATERIALS AND METHODS

Reagents and growth medium

D*n*BP, DEHP and di-*n*-octyl phthalate (D*n*OP) were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). PAE stock solutions were 1.0 mg mL^{-1} in *n*-hexane. High performance liquid chromatography grade acetone and *n*-hexane were purchased from Tedia Company Inc., Fairfield, USA. All other solvents and chemicals used were of laboratory reagent grade or higher. Minimal medium (MM) contained NH_4NO_3 1.0 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.01 g L^{-1}, $(NH_4)_2SO_4$ 0.5 g L^{-1}, KH_2PO_4 0.5 g L^{-1}, NaCl 0.5 g L^{-1}, K_2HPO_4 1.5 g L^{-1}, $MnSO_4{\cdot}H_2O$ 0.02 g L^{-1}, and trace element solution 1.0 mL ($FeSO_4{\cdot}7H_2O$ 2.0 g L^{-1}, $MnSO_4{\cdot}H_2O$ 2.0 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, $Na_2MoSO_4{\cdot}2H_2O$ 0.5 g L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.4 g L^{-1}, $NiCl_2{\cdot}6H_2O$ 0.2 g L^{-1} and H_3BO_4 0.20 g L^{-1}). Luria-Bertani (LB) medium contained 10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl (pH 7.0).

Isolation of DEHP-degrading bacteria

Five grams of fresh intensive vegetable soil was added to a 250-mL flask containing 100 mL of MM with 100 mg L^{-1} DEHP and the culture was incubated at 28 °C for 7 d in the dark on a rotary shaker at 150 r min^{-1}. Then 5 mL soil suspension was transferred to 100 mL MM containing DEHP (100 mg L^{-1}) followed by incubation with shaking at 150 r min^{-1} at 28 °C for a further 7 d in the dark. The transfer operation was repeated twice. The final enrichment was diluted with sterile water, spread on LB plates containing DEHP (100 mg L^{-1}) and incubated at 28 °C for 48 h. Several well-separated and individual colonies of different morphological types appeared and were further streaked onto fresh LB plates containing DEHP