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Biodegradation of seven phthalate esters by *Bacillus mojavensis* B1811

Jingfan Zhang[a,b], Chengnan Zhang[a,c], Yunping Zhu[a,c], Jinlong Li[a,b,*,1], Xiuting Li[a,b]

[a] Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology and Business University, Beijing 100048, PR China
[b] Beijing Higher Institution Engineering Research Center of Food Additives and Ingredients, Beijing 100048, PR China
[c] Beijing Key Laboratory of Flavor Chemistry, Beijing 100048, PR China

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ABSTRACT

Phthalate esters (PAEs) are one of the most widely used groups of plasticizers and have been considered threats to the environment and human health. This study investigated the efficient biodegradation of seven phthalate esters (PAEs) by *Bacillus mojavensis* B1811. The results showed that di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP) and dipentyl phthalate (DPP) could be almost completely degraded by strain B1811 within four days in mineral salt medium (MSM) under shaking conditions, while only 5.9% of the dimethyl phthalate (DMP) and 42.9% of the diethyl phthalate (DEP) present, which both have short alkyl chains, were degraded by strain B1811 under the same conditions. An esterase activity assay also indicated that the specific activities of esterases induced by PAEs with longer alkyl chains were much higher than those of esterases induced by PAEs with short alkyl chains. High-performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) was applied to identify the major metabolites of the seven PAEs. The PAEs were first degraded to the corresponding phthalate monoesters and then degraded to phthalic acid; phthalic acid was rapidly degraded to produce benzoic acid which was subsequently converted to protocatechuate and ultimately transformed to CO_2 and H_2O. The optimal conditions for biodegradation were also obtained and an exponential model was the best model to represent the PAEs depletion. It is therefore concluded that *B. mojavensis* B1811 offers great application potential in the bioremediation of environments polluted with PAEs.

1. Introduction

Phthalate esters (PAEs) are the most commonly used plasticizers in a broad range of industrial and consumer products because of their excellent properties of improving product flexibility, durability and longevity. It has been reported that more than 8 million tons of PAEs are produced worldwide (Xu et al., 2008). However, a major problem with the widespread application of PAEs is environmental pollution. Because PAEs are not chemically bound to the polymer molecules, they can be gradually released and migrate from the host polymers into the environment (Xu et al., 2005). Previous studies have shown that PAEs have been found in various samples, such as soil, water, air and food (Kong et al., 2012; Liu et al., 2014; Wang et al., 2012; Sakhi et al., 2014). The China National Environmental Monitoring Center, the European Union and the U.S. Environmental Protection Agency now have listed PAEs as top priority pollutants in view of the mutagenicity, teratogenicity and carcinogenicity of these compounds (European Union, 1993; Niazi et al., 2001).

Since the natural processes that degrade PAEs occur on long time scales, biodegradation is more likely to be the most effective process to remove PAEs from aquatic and terrestrial environments (Wang et al., 2008). Numerous studies have demonstrated that bacteria, fungi, algae and even yeasts can effectively degrade PAEs under aerobic and anaerobic conditions (Li and Gu, 2007; Gao and Wen, 2016). Because of their more recalcitrant character and low biodegradation rates compared with those of short-alkyl-chain phthalates, long-alkyl-chain phthalates (such as DEHP, DNOP and DPP) have been considered as key risk factors that affect the environment and human health (Cousins et al., 2003). It has been shown that 63% of DEHP could be efficiently degraded by *Achromobacter denitrificans* SP1 within 72 h (10 mM), where DEHP was degraded to mono(2-ethyl-hexyl) phthalate and 2-ethyl hexanol, but the expected intermediate phthalic acid was not detected (Pradeep et al., 2015). Xu et al. (2017) also have reported that 90% of DEHP was degraded by *Acinetobacter* sp. SN13 within five days of incubation.

The hydrolysis of one of the two alkyl chains of an individual

* Corresponding author. 11, Fucheng Road, Haidian District, Beijing 100048, PR China.
E-mail address: lijinlong@btbu.edu.cn (J. Li).
[1] Post addresses: Room 309, Building 8, School of Food and Chemical Engineering, Beijing Technology and Business University, 11, Fucheng Road, Haidian District, Beijing 100048, PR China.

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phthalate ester to produce the corresponding monoester is the common initial step in the biodegradation of PAEs, which is mostly catalyzed by esterase (Wang and Gu, 2006). Li et al. (2005a, 2005b) have reported that two types of bacteria were necessary to transform dimethyl terephthalate (DMT) into terephthalic acid via monomethyl terephthalate, which suggested that the esterases performing the hydrolysis of the each individual alkyl chain are highly specific. However, comparative investigations of the degradation of various PAEs by a single bacterium, as well as the PAEs degradation pathways, are relatively scarce. The transformation efficiency and intermediate fate of DMP, DMT and dimethyl isophthalate (DMI) degraded by *Rhodococcus rubber* Sa were previously investigated by Li et al. (2005a, 2005b), and their results showed that the transformation of DMI was achieved in 1 day while the transformation of DMP was achieved in 9 days. Luo et al. (2012a, 2012b) found that an esterase obtained from *Fusarium* sp. DMT-5-3 showed a high specificity for DMT hydrolysis but had no influence on DMP or monomethyl phthalate ester (MMP) hydrolysis. Nevertheless, the broad spectrum and specificity characteristics of PAEs degradation and the specificity of esterases and the corresponding pathways involved in the degradation of various PAEs by a single strain of bacterium under identical conditions have been relatively neglected.

Bacillus mojavensis B1811 is a novel strain of bacteria isolated from soil in our previous research. Our preliminary results indicated that strain B1811 possessed PAE degradation activity and could serve as a potential bacterium for PAE biodegradation. Among the various PAEs, DEHP, DBP, BBP, DNOP, DMP, DPP and DEP are the relatively widely used phthalate esters, and their application and consumption worldwide have grown (Liu et al., 2014; Staples et al., 1997; Huang et al., 2008; Fang et al., 2010; Gledhill et al., 1980). The objectives of this study were to compare the degradation characteristics and the metabolic pathways of the seven PAEs with alkyl chains of different lengths (DEHP, DBP, BBP, DNOP, DMP, DPP and DEP) when degraded by *Bacillus mojavensis* B1811 and to investigate the specific esterase activities of this strain induced by the seven PAEs. In addition, the optimal conditions for the biodegradation of these seven PAEs were obtained, and the biodegradation kinetics of the seven PAEs were also studied.

2. Materials and methods

2.1. Chemicals

DMP (99.0%), DEP (99.5%), DBP (99.0%), BBP (98.0%), DNOP (99.0%), DPP (98.0%), DEHP (99.0%) and possible degradation products such as mono-methyl phthalate (MMP, 97.0%), mono-ethyl phthalate (MEP, 99.0%), mono-benzyl phthalate (MBeP, 98.0%), mono (2-ethylhexyl) phthalate (MEHP, 98.0%), phthalic acid (PA, 99.0%), benzoic acid (BA, 99.0%), and protocatechuate (3,4-dihydroxybenzoate) (PCA, 99.0%) were acquired from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). A phthalate ester standard solution containing the seven tested PAEs (1000 μg mL^{-1} in n-hexane) was purchased from J&K Scientific Ltd. (Beijing, China). Methanol, ethyl acetate, dichloromethane and acetonitrile were of HPLC grade (Mreda Technology Inc., USA). Other chemicals were all of analytical reagent grade.

2.2. Bacterium and medium

Bacillus mojavensis strain B1811 was isolated from soil and had been deposited in the China General Microbiological Culture Collection Center (CGMCC, No.:12805). The isolation and enrichment of strain B1811 were performed using a sequential subculture technique in culture medium containing rhodamine B for plate screening. Strain B1811 was isolated from oil-polluted soil collected at a vegetable oil factory collected in Handan City, China. Approximately 0.2 g of the soil sample was suspended in 10 mL water. After the soil was precipitated, 100 μL of the sample was added to culture medium for plate screening containing (L^{-1}): 0.5 g $(NH_4)_2SO_4$, 3 g NaCl, 0.5 g K_2HPO_4, 0.4 g $MgSO_4$, 18 g agar, 120 mL olive oil and 1 mL rhodamine B. The *Bacillus mojavensis* strain B1811 was identified and characterized using 16S rRNA gene sequencing. The primers F27 and R1541 were used to amplify the 16S rRNA, DNA sequencing was carried out by the Beijing Haocheng Mingtai Technology Co., Ltd. The obtained 16S rRNA was aligned in GenBank (NCBI) using the BLAST program on the NCBI website. The sequence data of the closest relatives in GenBank and aligned with CLUSTALW with all parameters set at their default values. A phylogenetic tree was then constructed using the neighbor-joining method with MEGA 4.1 software. The tree was validated using bootstrap analysis performed with 1000 replicates.

The Luria-Bertani (LB) medium contained 20.0 g L^{-1} glucose, 10.0 g L^{-1} peptone, 5.0 g L^{-1} yeast extract and 10.0 g L^{-1} NaCl. The mineral salt medium (MSM) contained 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} K_2HPO_4, 0.01 g L^{-1} $CaCl_2$, 0.001 g L^{-1} $Fe_2(SO_4)_3{\cdot}3H_2O$, 1.0 g L^{-1} $(NH_4)_2SO_4$, and 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$. The MSM was adjusted to pH 7.0 using HCl or NaOH. After the MSM was autoclaved at 121 °C for 20 min, the various phthalate esters were directly added to the MSM using a sterile 0.22 μm membrane (Mreda, USA).

2.3. Biodegradation of phthalates esters by B. mojavensis B1811

Bacillus mojavensis B1811 growing in LB medium for 16 h was harvested and resuspended in mineral salt medium after washing with phosphate-buffered saline and was then used as the inoculum for all experiments. All the degradation tests were conducted in 250 mL Erlenmeyer flasks containing 49 mL mineral salt medium with the PAEs, and 1 mL of the bacteria. The biodegradation tests were conducted at 40 °C with shaking at 210 rpm. Sterile controls and inoculated controls were set up as above but without inoculation of the bacteria or addition of the PAEs, respectively. All tests were conducted in triplicate. For quantification of the residual phthalate esters after incubation for 4 days, 20 mL of dichloromethane was used to extract the aqueous phase by adding it directly into each flask. The dichloromethane phase was then dried by nitrogen gas, and dissolved in 5 mL of methanol for further high-performance liquid chromatography (HPLC) analysis.

2.4. Determination of the influence of different factors on PAEs biodegradation

Factors in the biodegradation of PAEs such as the pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0), temperature (10, 20, 30, 40, 50 °C), added yeast extract (0.25%, 0.5%, 0.75%, 1.0%) and initial PAEs concentration (100, 500, 1000, 2000 mg/L) were investigated to find the optimal conditions. The samples were collected for HPLC analysis after 96 h of incubation.

The PAEs degradation kinetics of strain B1811 were investigated, and the initial PAEs concentrations were 100 and 500 mg/L. Samples were collected for HPLC analysis at 0 h, 20 h, 40 h, 80 h, and 96 h of incubation.

2.5. HPLC analytical methods

The concentrations of residual phthalate esters in the flasks were investigated using an HPLC system (Agilent 1260 Infinity, Agilent Technology, USA) equipped with a ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5.0 μm). Gradient elution was performed to obtain a well-separated compounds profile. Three mobile phases were used: A, methanol; B, ultrapure water; and C, acetonitrile. The gradient program was as follows: 0–4 min B:C (30:70, v/v), 9–14 min 100% A, and 14–18 min A:B (65:35, v/v). The column temperature was maintained at 25 °C throughout the run. Qualitative data were obtained by comparing the sample peaks to peaks of standard compounds, and the concentrations of PAEs in the samples were calculated using external standards. The detection limits and the recovery rates of the seven PAEs