Degradation and Biosorption of Envirinmental Endocrine Discruptor Di-(2-ethylhexyl) phthalate (DEHP) by *Gordonia* sp. YK1

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Abstract

In this study, the isolation of DEHP-degrading microorganism was successfully achieved by establishing highly enriched aerobic cultures capable of degrading DEHP. DEHP in the culture with the isolated microorganism was rapidly degraded with the growth of cells and disappeared in 48 hr (more than 90% degradation). Based on 16S rDNA analysis, the isolated DEHP-degrading microorganism belongs to the *Gordonia.* Because the DEHP-degrading microorganism, *Gordonia* sp. YK1, was attached to DEHP droplets and formed aggregation in the liquid medium during the degradation of DEHP, biosorption of DEHP by *Gordonia* sp. YK1 was investigated with 50 ppm DEHP by detecting DEHP concentration in supernatant and biomass phases. With 11 mg biomass and 50 ppm DEHP, more than 60% of DEHP was removed by biosorption in 15 min, and adsorbed DEHP was degraded rapidly (73% degradation of DEHP in 30 min). Interestingly, cell attachment to DEHP oil drops was observed even with the autoclaved biomass of *Gordonia* sp. YK1. Since it is known that there are long aliphatic chains of the mycolic acids in cell wall for the *Gordonia* genera, strong hydrophobicity of the cell surface of *Gordonia* sp. YK1 due to mycolic acids is likely to be involved in DEHP biosorption and, consequently, effective DEHP biodegradation by facilitating DEHP uptake.

Keywords: Di-(2-ethylhexyl) phthalate (DEHP), biodegradation, *Gordonia* sp. YK1

Introduction

The phthalic acid esters (PAEs) are a group of chemical compounds that are mainly used as plasticizers to make a hard plastic into a flexible plastic. The common PAEs used in plastic industry are di-(2-ethylhexyl) phthalate (DEHP), di-phentyl phthalate (DPP), di-butyl phthalate (DBP), and di-ethyl phthalate (DEP). DEHP has been classified as a priority pollutant with relatively low acute toxicity. Exposure to high concentration of DEHP has been found to cause a wide range of adverse effects in experimental animals, including cancer, liver damage and reproductive system.

Microbial degradation is believed to be the main degradation process for DEHP in aquatic and terrestrial environments, such as sewage, soils, sediments, and surface waters [1]. In recent years, a few researches have reported on DEHP degradation by *Microbacterium* [2], *Rhodococcus* [3], and *Gordoina* [4]. In this study, we report the isolation of a DEHP-degrading bacterium from environmental samples. Simultaneous biosorption and degradation of DEHP by *Gordonia* sp. YK1 was also investigated

Materials and methods

Reagents and chemicals

Di-(2-ethylhexyl) phthalate (DEHP) was purchased from Sigma-Aldrich (Dioctyl phthalate, 99.9%, Aldrich). Di-butyl phthalate (DBP) and di-ethyl phthalate (DEP) were bought from Junsei chemical (99.9%, Japan). The *n*-hexane and ethyl acetate were bought from J. T. Baker. All solvents used were HPLC-grade and all other chemicals were reagent-grade.

Media and culture conditions

Sediment sample was collected from Han River in Seoul, Korea. Enrichment cultures were established in M9 mineral medium containing (per liter): 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.015 g $CaCl_2$ ($2H_20$), and 0.5 g $MgSO_4$ ($7H_20$) [5]. The final pH of the medium was adjusted to pH 7.0. After the medium was sterilized, cultures were incubated at 30℃ in a shaking incubator at 200 rpm.

Enrichment cultures for DEHP degradation

Ten grams (milliliters) of sediment was added into a 250 ml flask with 30 ml M9 mineral medium and 500 mg/L DEHP. The cultivation was prepared in several replicates and incubated at 30℃ in a shaking incubator at 200 rpm. The enrichment cultures were transferred several times, and the fourth transferred one was spread on nutrient agar plates. Different types of colonies were tested for their capacity to grow on DEHP as the only carbon and energy source.

Biodegradation of DEHP by the isolated microorganism

The isolated DEHP-degrading microorganism, *Gordonia* sp. YK1, was inoculated into a 250 ml flask with 100 ml M9 mineral medium and 0.1 % yeast extract. After 24 hr of cultivation, the bacterial suspension was washed two times with M9 mineral medium (pH 7.0) and then added into a flask containing M9 mineral medium supplemented with DEHP (50 mg/L, 500 mg/L and 1000 mg/L). The residual concentration of DEHP was measured by the GC-FID method as described in analytical methods.

To investigate the effects of pH on DEHP degradation, pH was adjusted to 3.0 to 9.0 with 5N HCl or 5N NaOH. The effect of temperature on DEHP degradation was investigated by incubating cultures at 20, 25, 30, 35, and 40℃ and the residual concentration of DEHP was measured periodically.

Biosorption and Biodegradation of DEHP by *Gordonia* sp. YK1

The cultivated *Gordonia* sp. YK1 washed two times with M9 mineral medium (pH 7.0). Ten milliliters of DEHP-degrading cultures (OD_{600}=1.0) were incubated with 50 mg/L of DEHP in a shaking incubator (200 rpm) at 30 ℃. The cultures were centrifuged periodically to analyze the concentration of DEHP in supernatant and biomass phase.

Scanning electron microscopy (SEM)

The isolated strain was placed on PTFE membrane filter (Whatman, Maidstone), washed three times with 0.2 M phosphate buffer (pH 7.3) and fixed with 100 mM HEPES buffer and 2% (v/v) glutaraldehyde for 2h at room temperature. After gradual dehydration with ethyl alcohol (30%, 50%, 70%, 80%, 90%, 95%, and 100%), dehydration was done again with iso-amyl acetate (70%, 80%, 90, 95%, and 100%). The sample was dried for overnight at the room temperature [6]. The dried sample was coated on gold (Hitachi E1030 auto fime coater). For observation, a FEI XL-30 FEG Scanning electron microscopy (FEI) was used.

16S rDNA analysis

The isolated strain was identified by the amplification and sequencing of bacterial 16S rDNA. After extracting whole genomic DNA, bacterial 16S rDNA was amplified using the universal primer 27F (*E.coli* numbering 8-27: 5'-AGA TGA TGA TCC TGG CTC AG-3') and 1492R *(E. coli* numbering 1492-1513: 5'-GGT TAC CTT GTT ACG ACT T-3') [7]. The 16S rDNA gene was sequenced and aligned to the Ribosomal Database Project (RDP Database) [8] and NCBI blast search (National Center for Biotechnology Information) to identify the isolated microorganism.

Analytical methods

Twenty milliliters volume of DEHP-degrading cultures were acidified with 6N HCl to attain pH 2.0~4.0. The samples were then extracted with ethyl acetate using two times volume of cultures (i.e, 40 ml). After shaking it for 10 mi, the ethyl acetate layer was separated from the water phase and filtered with anhydrous sodium sulfate (Na_2SO_4, Sigma). N_2 gas was used to concentrate ethyl acetate layer until the volume reached to 20 ml. The ethyl acetate layer was collected in 2 ml GC vials and DEHP concentration was analyzed by GC-FID (ACME 6000 gas chromatograph with a flame ionization detector, Yonglin instruments, Korea). The column used was the HP-1 capillary column (length, 30 m; inner diameter, 0. 32 mm; film thickness, 0.25 ㎛). GC/TOF-MS conditions were as follows: the initial temperature was set at 40 ℃ for 3 min, increased by 10 ℃/min to 300 ℃, and it was maintained 2 min at 300 ℃. Injector and detector temperatures were 250 ℃ and 300 ℃, respectively. Helium was used as carrier gas at 3 ml/min. The inlet was set to split mode (1:10) and 2 μl of extracted sample was injected.

To analyze intermediates, the rest of ethyl acetate layer was dried with N_2 gas completely and the residue was dissolved 1 mL ethyl acetate to inject to GC/TOF-MS (HP-6890N, Agilent Technology; Leco Pegasus Ⅲ TOF-MS (Time-of-Flight-mass spectrometer), Leco corporation) with DB-5 capillary column (length, 17 m; inner diameter, 0. 25mm; film thickness, 0.25 ㎛) and the same temperature conditions as described above. The DEHP intermediates were identified with the score matching to the standard library and retention time.

Results and Discussion

Isolation and identification of a DEHP-degrading microorganism

Isolation of DEHP-degrading microorganism was successfully achieved by establishing highly enriched aerobic cultures capable of degrading DEHP. DEHP in the culture with the isolated microorganism was rapidly degraded with the growth of cells and disappeared in 48 hr (more than 90% degradation). The cell was an aerobic, gram-positive bacterium with a shape of rods (1.0-2.0 um) (Fig. 1).

The 16S rDNA sequence of the isolated DEHP-degrading microorganism, designed as YK1, was determined and compared with the sequences of previously reported 16s rDNA genes. The isolated microorganism (YK1) revealed a 99.6% (1483 bp out of 1499 bp) similarity with 16S rDNA sequences of *Gordonia sihwensis* (DSM44576) [9]. As shown in the phylogenetic tree in Fig.2**,** the isolated bacterium and all of the representatives of the genus *Gordonia* were clearly grouped in a single cluster. Based on the 16s rDNA gene sequence, the isolated bacteirum belong to the genus *Gordonia*.

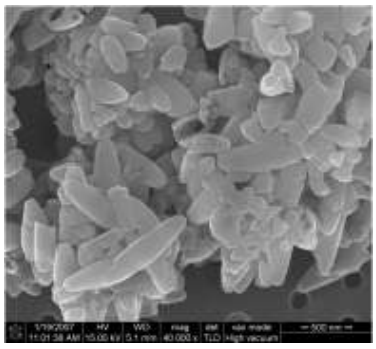

Fig. 1. SEM photograph of *Gordonia* sp. YK1

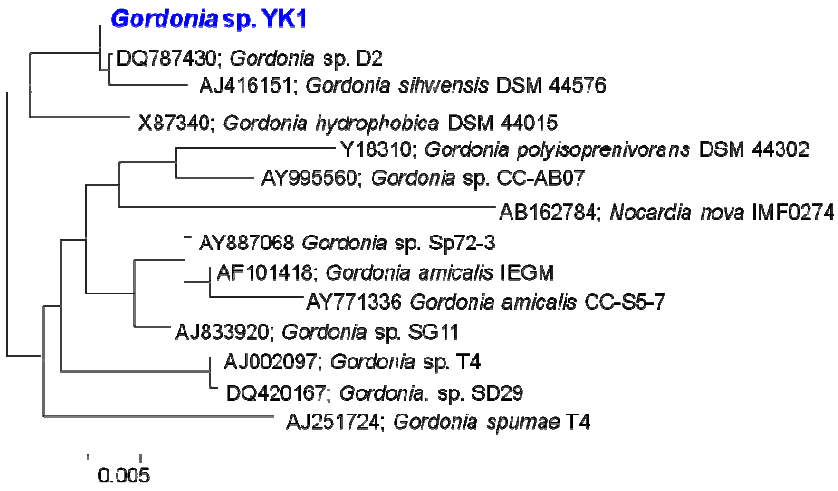

Fig. 2. Phylogenetic tree based on Neighbor-joining method with 16S rDNA sequences of *Gordonia* sp.YK1

Biodegradation of DEHP by the *Gordonia* sp. YK1

As shown in Fig. 3, the isolated microorganism, *Gordonia* sp. YK1 was capable of degrading DEHP as a sole carbon source in M9 medium with the growth of cells under aerobic condition. *Gordonia* sp. YK1 degraded more than 90% of the 50 mg/L, 500 mg/L and 1000 mg/L DEHP after 36 and 48 hours, respectively. The half life of DEHP by *Gordonia* sp. YK1 was 1 hr with 50 mg/L, 15 hr with 500 mg/L and 24 hr with 1000 mg/L. Other phthalate diesters, such as DBP and DEP, were tested for degradability as a sole carbon source. In M9 mineral medium supplemented with each of 500 mg/L DBP and DEP, *Gordonia* sp.YK1 exhibited rapid degradation activity and it was able to degrade more than 90% of the added DBP and DEP in three days under the same conditions that were used for DEHP (data not shown).

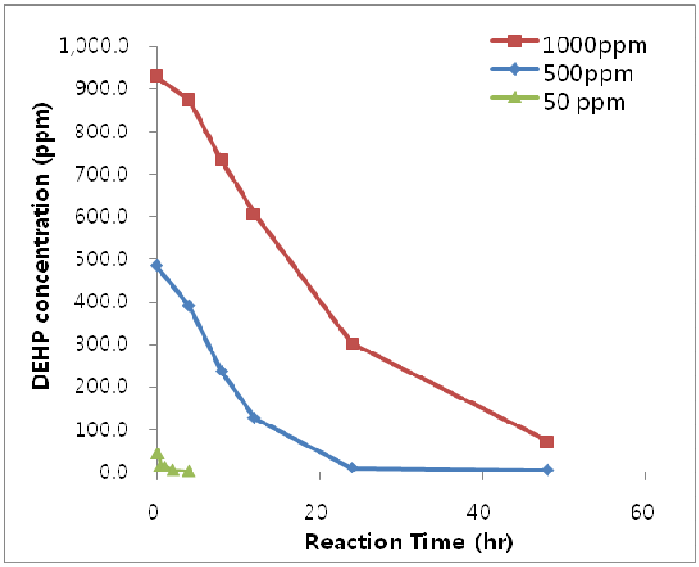

Fig. 3. Biodegradation of DEHP by *Gordonia* sp. YK1

Effect of pH and temperature on DEHP degradation by *Gordonia* sp. YK1

Gordonia sp. YK1 degraded more than 95% of the 500 mg/L at pH ranging from 5.0 to 9.0 after 48 hours (Fig. 4). As shown in Fig. 5, the *Grodoina* sp. YK1 degraded well at

temperatures ranging from 20 to 40℃. Based on the results, *Gordonia* sp. YK1 can degrade DEHP effectively in a wide range of pH and temperature.

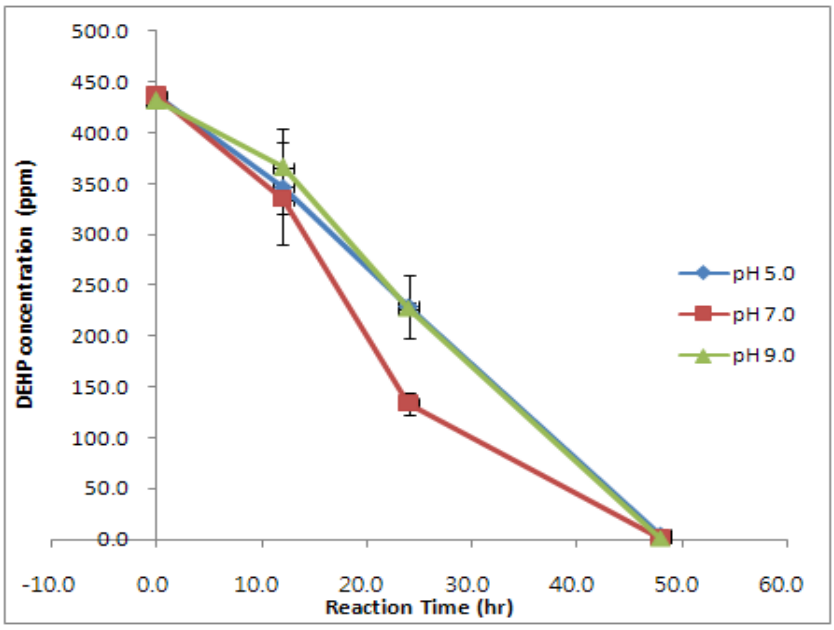

Fig. 4. Effect of pH on DEHP biodegradation by *Gordonia* sp. YK1

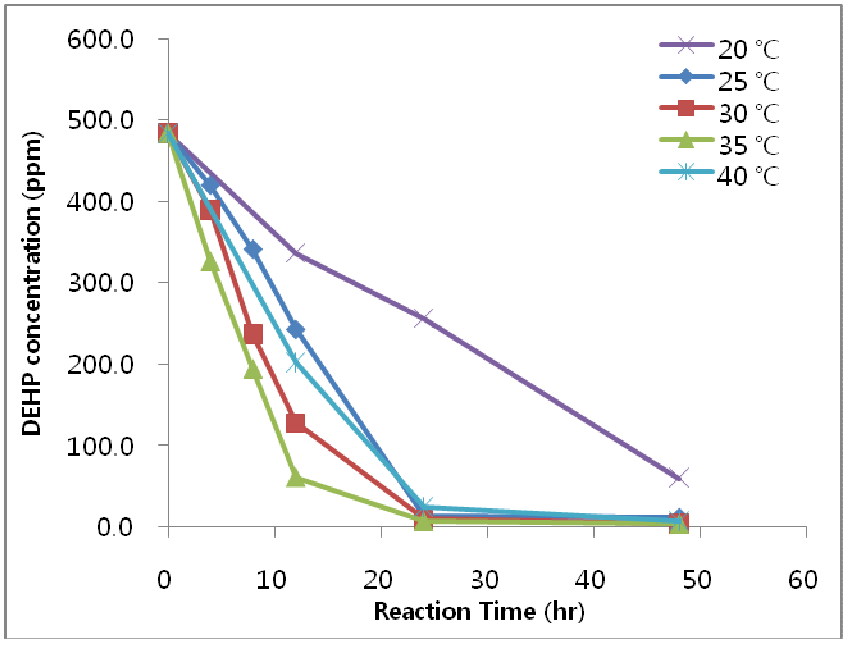

Fig. 5. Effect of temperature on DEHP biodegradation by *Gordonia* sp. YK1

Biosorption and Biodegradation of DEHP by *Gordonia* sp. YK1

Simultaneous biosorption and degradation of DEHP by *Gordonia* sp. YK1 was investigated with 50 ppm DEHP by detecting DEHP concentration in supernatant and biomass phases (Fig. 6). With 11 mg biomass and 50 ppm DEHP, more than 60% of DEHP

was removed by biosorption in 15 min, and adsorbed DEHP was degraded rapidly (73% degradation of DEHP in 30 min).

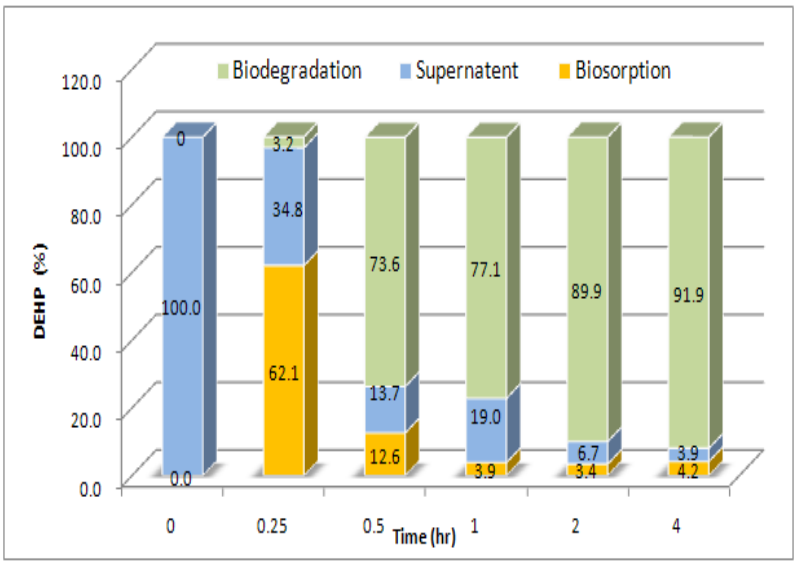

Fig. 6. Biodegradation and Biosorption of DEHP by *Gordonia* sp. YK1

Interestingly, cell attachment to DEHP oil drops was observed even with the autoclaved biomass of *Gordonia* sp. YK1. As shown in Fig. 7, with 11 mg biomass and 50 ppm DEHP, more than 50% of DEHP was removed by the autoclaved cells in 30 min. Since it is known that there are long aliphatic chains of the mycolic acids in cell wall for the *Gordonia* genera, strong hydrophobicity of the cell surface of *Gordonia* sp. YK1 due to mycolic acids is likely to be involved in DEHP biosorption and, consequently, effective DEHP biodegradation by facilitating DEHP uptake.

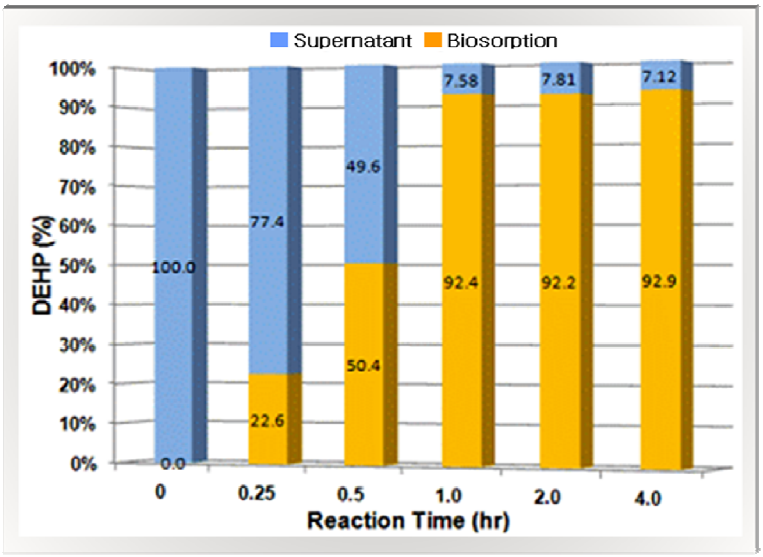

Fig. 7. Biosorption of DEHP by autoclaved *Gordonia* sp. YK1

Characterization of aerobic DEHP degradation intermediates

Intermediates were extracted from cultures and characterized by GC/TOF-MS analysis. The intermediates were identified to be MEHP and phthalic acid. Fig.7 shows the

GC/TOF-MS spectra with the extracted intermediates from 1day cultures.

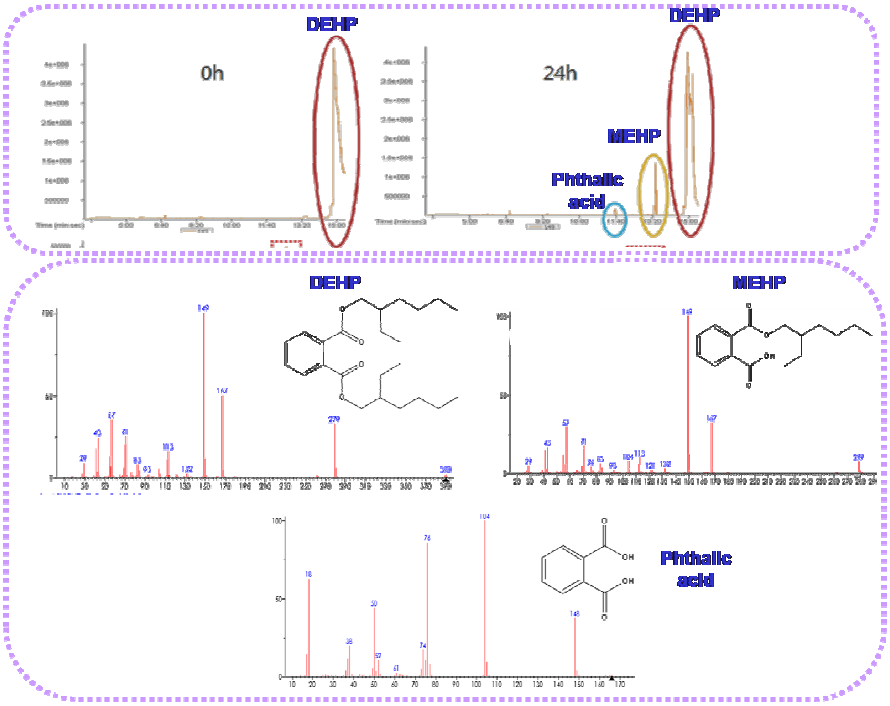

Fig. 7. GC/TOF-MS spectra of metabolic intermediates

On the basis of GC/TOF-MS results, *Gordonia* sp. YK1 is likely to degrade DEHP to produce MEHP and then PA as shown in Fig. 8.

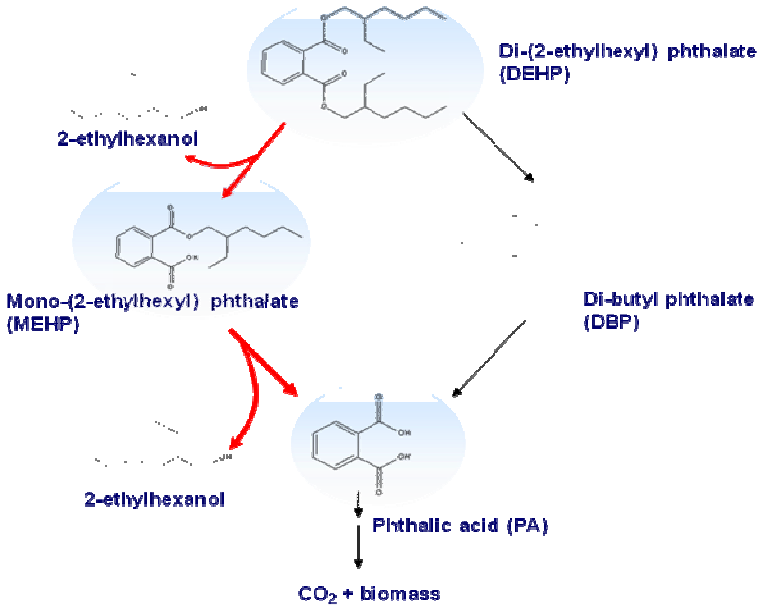

Fig. 8. Possible DEHP degradation pathway by *Gordonia* sp. YK1

Acknowledgements

This work was supported by the research program of The Korea Research Council of Fundamental Science & Technology.

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