Isolation of Microorganisms Growing on Phthalate Esters and Degradation of Phthalate Esters by *Pseudomonas acidovorans* 256-1[†]

Ryuichiro KURANE, Tomoo SUZUKI and Yoshimasa TAKAHARA

Fermentation Research Institute,
Inage-higashi 5-8-1, Chiba City, Chiba 281, Japan

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Many microorganisms (17 strains of gram-positive bacteria, 8 strains of gram-negative bacteria, 2 strains of fungi) capable of assimilating di-2-ethyl hexyl phthalate (DEHP) were isolated from soil and other natural sources. When *Pseudomonas acidovorans* 256-1 among these microorganisms was aerobically cultured in media containing 0.5% of DEHP, DEHP disappeared completely in 72 hr when assayed gaschromatographically. Most of phthalate esters could be assimilated, regardless of their side-chain types. In addition, branched-alkyl phthalate was assimilated better than *n*-alkyl phthalate. Based on degradation rate of *n*-alkyl phthalate in relation to its side chain and carbon number, two peaks were observed in *n*-alkyl phthalate with four and seven carbon number on its side-chain.

In the past few years, considerable attention has been paid to the biodegradation of persistent organochlorine compounds such as DDT and PCB. On the contrary, little interest has been shown in non-halogenated synthetic chemical compounds such as phthalate esters, which are most commonly used as plasticizers. These chemicals are produced in large quantities. For example, total production in Japan was over 3.59 million tons until 1976. Di-2-ethyl hexyl phthalate (DEHP) so-called DOP comprises 65% of the total production in Japan. This chemical has been detected in 100-fold large quantities than organochlorine chemicals in water, soil and air according to an environmental white paper by Environment Agency, Japan.¹⁾ However, little work has been reported on the microorganisms which assimilate the phthalate esters as environmental pollutants, except for activated sludge,^{2,3)} *Bacillus*,⁴⁾ *Pseudomonads*⁵⁾ and other bacteria.⁶⁾

This paper describes the screening of the phthalate esters-assimilating microorganisms and the relationship between structures and ability of biodegradation of phthalate esters

in *Pseudomonas acidovorans* 256-1.⁷⁾

MATERIALS AND METHODS

Materials. Di-2-ethyl hexyl phthalate (DEHP) was obtained from Tokyo Kasei Co., Ltd. (Tokyo). Other phthalate esters were obtained from Gaschro Kogyo Co., Ltd. (Tokyo). These materials were gaschromatographically pure. Other chemicals used in this study were of reagent grade.

1) Screening of DEHP-assimilating microorganisms

Composition of the screening medium (DEHP medium) was as follows; 1.0 g DEHP, 1.0 g (NH₄)₂SO₄, 0.2 g KH₂PO₄, 1.6 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 0.02 g CaCl₂·2H₂O, 0.01 g FeSO₄·7H₂O, 0.5 mg Na₂MoO₄·2H₂O, 0.5 mg Na₂WO₄·2H₂O, 0.5 mg MnSO₄, 0.4 mg Ca-pantothenate, 2.0 mg inositol, 0.4 mg niacin, 0.2 mg *p*-aminobenzoate, 0.4 mg pyridoxine-HCl, 0.4 mg thiamine, 0.4 mg riboflavin, 2.0 mg biotin, 0.5 mg vitamin B₁₂, in 1 liter of distilled water, and pH was adjusted to 7.5. DEHP was diffused with a homogenizer.

Microorganisms were isolated from soil and waste water by enrichment shaking culture at 30°C, using DEHP as a sole carbon source. Microorganisms were purified by successive streak transfers on four kind of medium, i.e., DEHP agar, nutrient agar, Rich-agar and Poor-agar⁸⁾ medium.

2) Examination on the relationship between structures and biodegradation by *Pseudomonas acidovorans*

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Microorganisms. The bacterium used for this study was *Pseudomonas acidovorans* 256-1. This isolate was one of the highest DEHP-assimilating bacteria and its taxonomy was described in another paper.⁷⁾

Cultivation. After precultivation on DEHP slant agar for 2 days, three loops of cells were inoculated into 500 ml flasks containing 150 ml of the medium containing DEHP as a sole carbon source, and cultivated with rotary shaker at 30°C.

Cell growth. Emulsion of DEHP did interfere with OD reading for cell growth. Therefore, the determination of cell growth was carried out with the following solvent system; 1 ml of culture broth and 2 ml of solvent (ethanol: *n*-butanol: chloroform = 10:10:1). The solvent system did not lyse cells, and DEHP was solubilized completely and the turbid solution was clarified uniformly. Therefore, cell growth was determined by measuring optical density at 660 nm.

Estimation of phthalate esters. Phthalate esters were usually extracted with *n*-hexane (except for dimethyl phthalate (DMP) and diethyl phthalate (DEP), which were extracted with chloroform), and estimated with a Shimadzu gas chromatograph (model GC-5A). The columns used were a glass column of 3.0 mm in inside diameter, 2 m in length, packed with Silicone OV-17 on 60/80 mesh Uniport B or Silicone SE-52 on 60/80 mesh Uniport B. For example, DEHP was estimated with anthron as internal standard at column temperature of 270°C.

Estimation of water-soluble total organic carbon (TOC) concentration. Water-soluble TOC of culture broth was estimated with a Beckman model 915 TOC analyzer after extraction with *n*-hexane and centrifugation at 28,000 *g* for 30 min.

RESULTS

I. Screening of DEHP-assimilating microorganisms

The method mentioned above was used to isolate many bacteria and fungi from 90 kinds of soil and waste-water samples. 17 strains of gram-positive bacteria, 8 strains of gram-negative bacteria and 2 strains of fungi were isolated. A considerable number of strains, such as 15 strains of gram-positive bacteria, 2 strains of gram-negative bacteria and 1 strain of fungi, showed the strong activity for

DEHP assimilation. Among these bacteria, four strains: Strain S-1, 256-2, 260-2 and 256-1, showed the highest assimilating activity. Among those strains; S-1, 256-2 and 260-2, were identified to be belonging to *Nocardia erythropolis* and the strain 256-1 was *Pseudomonas acidovorans*, which was described in the another paper.⁷⁾

II. The relationship between structures and biodegradation of phthalate esters by *Pseudomonas acidovorans* 256-1

1) Time course of DEHP degradation by *Pseudomonas acidovorans* 256-1. *P. acidovorans* 256-1 was cultured in DEHP medium containing 0.5% DEHP. Time course for utilization is presented in Fig. 1. DEHP

FIG. 1. Time Course of DEHP Degradation by *Pseudomonas acidovorans* 256-1.

○—○, DEHP remained %; ●—●, growth (OD₆₆₀); △---△, TOC in water phase after *n*-hexane extraction (ppm).

Cultivation was carried out at 30°C using rotary shaking flasks in a medium containing 0.5% (5000 ppm) DEHP.

completely disappeared gaschromatographically in 72 hr with cell growth. With progress of degradation of DEHP, water soluble TOC was increased. The decrease in TOC was temporarily observed during the later period of exponential phase, while it increased again in the stationary phase. It seemed that

the former peak was caused by the accumulation of intermediates of DEHP degradation and the latter by cell lysis. Therefore, the former peak of water soluble TOC was examined by thin-layer chromatography (TLC). This water soluble TOC fraction cultured for 30 hr was extracted with acidic ethyl ether. The ether layer was evaporated and subjected to TLC. As shown in Table I,

TABLE I. *R_f* VALUES OF THIN-LAYER CHROMATOGRAPHY OF THE PRODUCTS IN THE ETHER LAYER

	TLC <i>R_f</i> Value	
	BCG	UV
Spot I		
Solvent A	0.73	0.73
Solvent B	0.37	0.37
Spot II		
Solvent A	0.84	0.84
Authentic		
Phthalic acid		
Solvent A	0.72	0.72
Solvent B	0.38	0.38
Protocatechuic acid		
Solvent A	0.85	0.85

Solvent system:

A; *n*-BuOH: Formate: H₂O=4: 1: 2

B; 96%EtOH: H₂O: 25%NH₄OH=100: 12: 16

R_f values of TLC were identical with those of authentic phthalate and protocatechuate, respectively. The pH dropped from 7.5 to around 5.2 in the early stage of exponential phase. It was caused by the accumulation of intermediate organic acid, such as phthalate and protocatechuate which were well assimilated by this strain.

2) *Effect of DEHP concentration on DEHP degradation.* Effect of DEHP concentration on DEHP degradation was shown in Fig. 2. In the case of 3000 ppm and 5000 ppm, DEHP completely disappeared gaschromatographically in 52 hr and 72 hr, respectively. The same tendency of water-soluble TOC formation as that in Fig. 1, was observed on every concentration.

3) *Relationship between structures and biodegradation of phthalate esters.* *P. acidovorans* 256-1

FIG. 2. Effect of DEHP Concentration on Degradation by *Pseudomonas acidovorans* 256-1.

▲—▲, 0.1%; △—△, 0.3%; ●—●, 0.5%; ○—○, 1.0%; ■—■, 3.0%; ⊙—⊙, 5.0%; □—□, 10%.

The strain was cultured with rotary shaking at 30°C in media supplemented with the indicated DEHP concentrations.

varans 256-1 was cultured in medium containing 6000 ppm phthalate esters, and the time (day) required for 3000 ppm microbial degradation were measured. These values might be correspond to the die-away half life.

As shown in Table II, most of the phthalate esters were degraded, regardless of the type of its side-chain, that is, *n*-alkyl, branched alkyl (isoalkyl and 2-ethyl-alkyl), glycolate, oxy, benzyl and double-bond type. Among these phthalate esters, *n*-alkyl, branched-alkyl, benzyl and double-bond type of side-chain of phthalate esters were rapidly degraded. The effect of the position of side-chain on degradation was examined with ethyl ester of phthalate. As the result, *ortho*-position of phthalate ester was degraded, while *para*-ethyl ester could not. But, in the case of methyl ester, none of these was degraded. This strain well assimilated phthalate and protocatechuate which were identified to be intermediates of phthalate ester. The detail metabolism of phthalate ester will be described in future.

As shown in Fig. 3, with compounds of

TABLE II. DEGRADATION RATE OF PHTHALATE ESTERS BY *Pseudomonas acidovorans* 256-1

Degradation rate of phthalate esters was measured by the described in MATERIALS AND METHODS after *Pseudomonas acidovorans* 256-1 was cultured with rotary shaking at 30°C in media containing 6000 ppm of the phthalate esters. — indicates no growth, after 40 days of incubation at 30°C.

	Time (day) for 3000 ppm degradation
<i>n</i> -Alkyl type	
Dimethyl phthalate (DMP)	—
Diethyl phthalate (DEP)	10.5
Di- <i>n</i> -propyl phthalate (DnPP)	6.5
Di- <i>n</i> -butyl phthalate (DnBP)	3
Di- <i>n</i> -amyl phthalate (DnAMP)	9
Diheptyl phthalate (DHP)	5
Di- <i>n</i> -octyl phthalate (DnOP)	8
Dinonyl phthalate (DNP)	10.5
Didodecyl phthalate (DDP)	10
Ditridecyl phthalate (DTDP)	10.5
Branched-alkyl type	
Isoalkyl type	
Diisopropyl phthalate (DIPP)	1.5
Diisobutyl phthalate (DIBP)	3
Diisodecyl phthalate (DIDP)	4
2-Ethyl type	
Di-2-ethyl hexyl phthalate (DEHP)	1.3
Di-(2-ethyl hexyl)hexahydro phthalate (DEHHP)	4
Glycolate type	
Methyl phthalyl ethyl glycolate (MPEG)	—
Ethyl phthalyl ethyl glycolate (EPEG)	13
Butyl phthalyl butyl glycolate (BPG)	10
Oxy type	
Di(2-methoxy ethyl) phthalate (DMEP)	—
Di(2-butoxy ethyl) phthalate (DBEP)	10
Phenyl type	
Di- <i>n</i> -butyl benzyl phthalate (BBP)	3
Diphenyl phthalate (DPeP)	2
Dicyclohexyl phthalate (DCHP)	4
Double type	
Diallyl phthalate (DALP)	2
<i>ortho</i> , <i>meta</i> and <i>para</i> type	
Dimethyl phthalate (DMP)	—
Dimethyl isophthalate (DMIP)	—
Dimethyl terephthalate (DMTP)	—
Diethyl phthalate (DEP)	10.5
Diethyl terephthalate (DETP)	—
Intermediates and other chemicals	
Phthalate	1.8
Isophthalate	30
Tere-phthalate	30
Hexahydro phthalate	4
Protocatechuate	1.5

FIG. 3. Degradation Rate of Phthalate Esters, in Relation to Their Types of Side Chain and Carbon Number, by *Pseudomonas acidovorans* 256-1.

○—○, *n*-alkyl phthalate; ●—●, isoalkyl phthalate; △, di-2-ethyl hexyl phthalate.

Estimation of degradation rate of phthalate esters was mentioned in MATERIALS AND METHODS.

similar side chain numbers, branched alkyl phthalates were degraded better than *n*-alkyl phthalates. It is noteworthy that two peaks were observed in *n*-alkyl phthalate with four and seven carbon number in side-chains. This results suggest that di-*n*-butyl phthalate (DnBP) and di-heptyl phthalate (DHP) were more rapidly degraded than other *n*-alkyl phthalates. This may be due to an ester-degrading enzyme (esterase or lipase).

DISCUSSION

A considerable number of strains, which could assimilate the phthalate esters as environmental pollutants, were isolated from the soil and the other natural sources. It suggests that, such microorganisms may be abundantly present in the environment.

The degradation of some kinds of phthalate esters by *Pseudomonas acidovorans* 256-1 has been examined in detail from the point of view of the relationship between structures and degradability. Most of phthalate esters can be degraded by this strain, regardless of the type of side-chain.

And two peaks have been observed, in relation to the degradation rate of *n*-alkyl phthalate and its side-chain carbon number.

These results might suggest that the first step in degradation of phthalate esters is carried out lipase(s) or esterase(s). It is also reported that higher activity of lipases or esterases is shown with substrates having four and seven or eight carbon number of the side chain.⁹⁾ There are some reports concerning the degradation mechanisms of phthalic acid^{10,11)} which was identified as the intermediates of phthalate ester after the first degradation.

Based on our demonstrating the appearance and subsequent disappearance of TOC and well assimilation of phthalate and protocatechuate as the intermediates, it can be proposed that phthalate esters may be completely mineralized by *Pseudomonas acidovorans* 256-1.

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