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Isolation for bacteria and fungi for the hydrolysis of phthalate and terephthalate esters

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Bacterial and fungal strains were isolated from enrichment cultures using diethylphthalate, diethylterephthalate, or ethylene glycol dibenzoate as sole carbon sources. *Aureobacterium, Flavobacterium,* and *Micrococcus* species were isolated from diethylphthalate enrichments; *Rhodococcus* and *Xanthomonas* species were isolated from diethylterephthalate enrichments; *Rhodococcus* and *Fusarium* species were isolated from ethylene glycol dibenzoate enrichments.

Keywords: phthalate esters; biodegradation; *Rhodococcus; Aureobacterium; Flavobacterium; Fusarium*

The wide use of polyesters, from textiles to soft drink containers, has led to steady increases in production of these materials to a current rate of about 1.5 million tons annually in the United States alone [10]. Predictably, phthalate esters from polymer synthesis have been widely found in the environment [4,12,13]. Concern over the environmental impact of phthalate esters has led to several studies of the microbial degradation of these xenobiotic compounds. Karegoudar and Pujar [5] described a *Micrococcus* sp which mineralized diethylphthalate. Englehard *et al* [1,2] and Kurane [6] reported that species of *Nocardia, Arthrobacter,* and *Pseudomonas* degraded several phthalate esters while Lewis and Holm [7] reported the degradation of diethylphthalate by 'aufwuchs' or periphytic microorganisms in aquatic ecosystems. One report of the hydroylsis of dimethylterephthalate by a fungus has also appeared [11].

On-site composting of waste streams is an attractive method of waste reduction, eliminating by-products before they become pollutants [3]. However, cost-effective disposal often requires organisms capable of rapid transformation of unreactive compounds. In this paper we report on our search for organisms with high activity toward aromatic esters. The choice of diethylphthalate (DEP) and diethylterephthalate (DETP) as substrates was made based on the potential health threats from these compounds, while ethylene glycol dibenzoate (EGDB) was chosen because of its use as a model for the synthesis of polyester [9].

Materials and methods

Diethylterephthalate was from TCI American Organic Chemicals (Portland, OR, USA) and ethylene glycol dibenzoate was obtained from CTC Organics (Atlanta, GA, USA). All other reagents were from Aldrich (Milwaukee, WI, USA). The mineral salts solution consisted of 10 g $(NH_4)_2HPO_4$, 5 g K_2HPO_4, 0.5 g Na_2SO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 20 mg each $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 2H_2O$, and NaCl, 0.5 mg H_3BO_3, 0.04 mg $CuSO \cdot 5H_2O$, 0.2 mg $Na_2MO_4 \cdot H_2O$, 50 mg $CaCl_2$, and 0.2 mg $CoCl_2 \cdot 6H_2O$ per liter.

Soil samples were collected locally and in the state of Florida. For the enrichments, a 5-g soil sample was suspended in 30 ml mineral salts medium contained in a 50-ml conical flask. The suspensions were charged with 300 mg carbon source and the flasks were stoppered with a cotton plug and were shaken at 200 rpm at 28°C for 10–20 days. After this time the supernatant fluid from the enrichments was diluted and spread over mineral salts agar plates amended with 0.2% carbon source. These plates were incubated at 28°C until clear zones and colonies appeared (7–30 days). These colonies were then streaked on TGY agar (tryptone, 5 g L^{-1}; glucose, 1 g L^{-1}; yeast extract, 5 g L^{-1}; K_2HPO_4, 1 g L^{-1}; pH 7.0 solidified with 15 g L^{-1} agar) plates for further isolation. Fungi were isolated by placing mycelium onto YM agar (yeast extract, 3 g L^{-1}; malt extract 3 g L^{-1}; peptone, 5 g L^{-1}; glucose, 10 g L^{-1}; agar, 15 g L^{-1}; pH 6.2) plates.

Bacteria were identified from fatty acid profiles of cells grown for 24 h at 28°C on trypticase soy broth (BBL, Cockeyville, MD, USA) solidified with 1.5% agar by comparison to the AEROBE library of the Microbial Identification System. Fatty acid methyl esters were obtained from the biomass by saponification, methylation, and extraction, using the method of Miller and Berger [8]. The fatty acid methyl esters were separated and quantified using a Hewlett-Packard Model 5980A gas chromatograph/Microbial Identification System (MIDI, Newark, DE, USA) fitted with a 5% phenylmethyl silicone capillary column (0.2 mm × 25 m). Strains were identified by comparing their fatty acid profiles to those in the MIDI AEROBE library.

Cells from isolated bacterial colonies were grown in TGY broth for 18 h. Two hundred microliters of this inoculum were added to 1.80 ml of mineral salts medium containing approximately 5 mg DEP, DETP, or EGDB. After 48 h the reaction mixtures were diluted with 4.0 ml aceto-

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nitrile. High pressure liquid chromatography (HPLC) analyses were performed using a Spectra-Physics SP8700 pump and SP8440 UV/Vis detector (San Jose, CA, USA) operating at 238 nm. Components from the hydrolyses were separated on a Waters Bondapak C18 column (Milford, MA, USA) with a mobile phase of 70/30 methanol and 0.2% aqueous acetic acid at pH 2.7 with a flow of 1 ml min^{-1}. Aromatic rings were considered to be utilized when their loss was determined from this analysis.

Reaction products were identified by gas chromatography-electron ionization mass spectroscopy: monoethylterephthalate; *m/e* M^+ 194, 166, 149, 121, 65. Monomethylterephthalate [11] and monoethylphthalate [5] matched published spectra. Derivatization of ethylene glycol monobenzoate for mass spectral analyses was accomplished using bis(trimethylsilyl)trifluoroacetamide; ethylene glycol monobenzoate trimethylsilane; *m/e* 237, 207, 105, 77, 73.

Results and discussion

Thirteen soil samples were used in the enrichment cultures. Though the soil samples were collected without consideration of potential activity, all of the samples yielded organisms capable of utilizing either the ethyl group or the aromatic ring of the substrate molecules. Table 1 shows representative active strains isolated from 13 of these cultures (seven soil samples).

DEP enrichments gave three species of bacteria capable of utilizing DEP. Two *Aureobacterium saperdae* strains were isolated from soil samples 2 and 4. Sample 3 gave two strains of *Flavobacterium aquatile* and one of *Micrococcus kristinae*. Sample 5 also gave a strain of *Micrococcus kristinae*. Hydrolysis of DEP, leading to utilization, was limited to bacteria isolated from DEP enrichment cultures. Figure 1 shows the time course for the hydrolysis and consumption of a 0.5% solution of DEP by *A. saperdae* NRRL B-14840. Hydrolysis of the substrate was complete in 30 h. Along with phthalic acid, a transient level of monoethylphthalate was also detected. This strain was able to grow on phthalic acid and ethanol as well as DEP (data not shown). The four strains which were able to hydrolyze DEP had limited activity toward DETP and virtually no activity toward EGDB (Table 2).

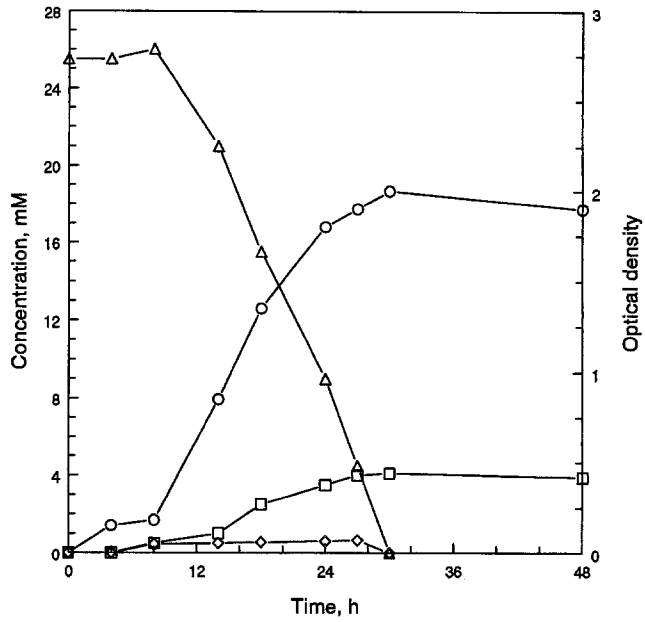

Figure 1 Time course of hydrolysis of 5% (w/v) diethylphthalate (DEP) by *Aureobacterium saperdae* NRRL B-14840. Legend: △ = diethyl phthalate concentration, ○ = optical density at 600 nm, □ = phthalate concentration, ◇ = monoester concentration.

The DETP enrichments gave 13 strains of *Rhodococcus rhodochrous* and one *Xanthomonas maltophila* capable of utilizing this ester. Hydrolysis of DETP and dimethylterephthalate led to the corresponding monoesters as final products. This was the case for all bacteria and fungi whether the microorganisms were isolated from DETP enrichments or from enrichments using DEP or EGDB.

The EGDB enrichments were unique in selecting for *Fusarium* species. *R. rhodochrous, Pseudomonas diminuta*, and *Flavobacterium indologenes* strains were also isolated from these enrichments though only the *R. rhodochrous* strains possess high levels of hydrolytic activity.

Bacteria isolated from DETP enrichments and bacteria and fungi isolated from EGDB enrichments were all able to hydrolyze EGDB equally well. *R. rhodochrous* NRRL B-16909 was examined for growth on the substrates and hydrolysis products (Table 3). This strain grew as well on

Table 1 Strains isolated from soil enrichments

Soil No.	Soil location	Carbon source		
		DEP[a]	DETP[b]	EGDB[c]
1	Forest floor	–[d]	*Xanthomonas maltophilia*	*Fusarium* species
2	Florida	*Aureobacterium saperdae*	–	*Fusarium* species
3	Drainage ditch	*Flavobacterium aquatile*	–	–
4	Drainage ditch	*Aureobacterium saperdae*	–	–
5	Burn barrel	*Micrococcus kristinae*	*Rhodococcus rhodochrous*	*Rhodococcus rhodochrous*
6	Barn floor	–	*Rhodococcus rhodochrous*	*Rhodococcus rhodochrous*
7	Pasture	–	*Rhodococcus rhodochrous*	*Rhodococcus rhodochrous*

[a] DEP = diethylphthalate.
[b] DETP = diethylterephthalate.
[c] EGDB = ethylene glycol dibenzoate.
[d] No isolates taken from these samples.

Table 2 Hydrolytic activity induced by different substrates

Substrate	Strain	% Hydrolyzed[a]		
		DEP[b]	DETP[c]	EGDB[d]
DEP	*Aureobacterium saperdae* NRRL B-14389	100	–[e]	–
	Aureobacterium saperdae NRRL B-14840	100	25	–
	Flavobacterium aquatile NRRL B-14842	100	23	–
	Micrococcus kristinae NRRL B-14845	100	25	–
DETP	*Rhodococcus rhodochrous* NRRL B-16898	8	97	25
	Xanthomonas maltophila NRRL B-14846	–	25	–
	Rhodococcus rhodochrous NRRL B-16899	–	98	35
	Rhodococcus rhodochrous NRRL B-16900	–	50	68
	Rhodococcus rhodochrous NRRL B-16902	–	97	80
	Rhodococcus rhodochrous NRRL B-16903	–	71	68
	Rhodococcus rhodochrous NRRL B-16904	12	85	67
	Rhodococcus rhodochrous NRRL B-16905	–	61	25
	Rhodococcus rhodochrous NRRL B-16907	–	64	69
	Rhodococcus rhodochrous NRRL B-16911	–	10	–
EGDB	*Rhodococcus rhodochrous* NRRL B-16908	–	23	32
	Rhodococcus rhodochrous NRRL B-16909	–	99	79
	Rhodococcus rhodochrous NRRL B-16910	12	93	72
	Pseudomonas diminuta NRRL B-14847	–	13	10
	Fusarium species NRRL 22492	22	42	53

[a] Values represent percent of a 25-mM substrate sample hydrolyzed after 48 h.
[d] DEP = diethylphthalate.
[c] DETP = diethylterephthalate.
[d] EGDB = ethylene glycol dibenzoate.
[e] <5% Hydrolysis.

Table 3 Growth of *Rhodococcus rhodochrous* NRRL B-16909 on selected carbon sources

Substrate[a]	ΔOD, 24 h	ΔOD, 48 h
Glucose	2.20	4.16
Ethanol	6.31	6.33
EGDB[b]	2.74	3.72
Benzoic acid	5.11	4.90
DETP[c]	0.15	0.22
TPA[d]	0.75	3.65
Ethylene glycol	–	0.08

[a] All substrates were present at 165 mM.
[b] EGDB = ethylene glycol dibenzoate.
[c] DETP = diethylterephthalate.
[d] TPA = terephthalic acid.

EGDB, benzoic acid, and ethanol as on glucose. Growth was somewhat more limited with terephthalic acid and there was little or no growth on ethylene glycol and DETP. These results show that hydrolysis is the rate limiting step in the mineralization of terephthalate esters.

The fungi and the *R. rhodochrous* strains utilized the benzoic acid produced from the hydrolysis of EGDB. Two strains with low levels of activity, *Pseudomonas diminuta* NRRL B-14847 and *R. rhodochrous* NRRL B-16911, accumulated a transient level of ethylene glycol monobenzoate, suggesting that some organisms may use separate esterases for hydrolysis of EGDB and the monobenzoate or that some esterases hydrolyze the mono- and diester at different rates. We examined hydrolytic activity of five strains of *R. rhodochrous* taken from the ARS Culture Collection and all five exhibited activity toward DETP and EGDB comparable to that found in the soil isolates. We also screened nine *Fusarium* species for activity toward DETP and found all nine to be hydrolytic. The hydrolytic activity of *R. rhodochrous* and the *Fusarium* species is apparently general and constitutive.

American Type Culture Collection strains of *Flavobacterium aquatile* ATCC 11947, *Aureobacter saperdae* ATCC 19272, and *Micrococcus kristinae* ATCC 27570 were also examined for the ability to consume DEP. None of these strains had activity toward the ester.

In conclusion, we studied DEP, DETP, and EGDB with the aim of finding a means of eliminating them or similar compounds from industrial waste streams. Only DEP was completely mineralized. The rate of transformation was high: *A. saperdae* B-14840 hydrolyzed a 0.5% solution of DEP in 30 h. The limitation in the mineralization of DETP was the hydrolysis of monoethylterephthalate.

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