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Biodegradation of Phthalic Acid Esters by Bakery Yeast *Saccharomyces cerevisiae*

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Phthalic acid esters (PAEs) are widely used industrial chemicals, serving as important additives impart flexibility in polyvinyl chloride (PVC) resins. They have been found widespread in environmental samples such as sediments, natural waters, soils, plants and aquatic organisms (Atlas and Giam 1980; Giam *et al.* 1984; Staple *et al.* 1997). PAEs have become ubiquitous environmental pollutants. Jobling *et al.* (1995) assessed the effects of environmental chemicals on transactivation of the estrogen receptor and breast cancer cell growth in vitro, and reported that PAEs such as di-*n*-butyl phthalate (DBP) and butyl benzyl phthalate (BBP) were estrogenic. Therefore, it is important to develop a technology for elimination or degradation of those compounds.

Because the rate of hydrolysis and photolysis of PAEs are very low, metabolic breakdown by microorganisms is considered one of the major routes of environmental degradation for PAEs (Giam *et al.* 1984; Staples *et al.* 1997). Numerous studies have reported the biodegradation of PAEs under aerobic conditions in natural water, wastewater and soil (Ribbons *et al.* 1984). Jonson and Lulves (1975) studied the biodegradation of DBP and di-2-ethylhexyl phthalate in freshwater hydrosoil. Walker *et al.* (1984) investigated the degradation of DBP in estuarine and freshwater sites. Sugatt *et al.* (1984) examined the biodegradation of 14 commercial PAEs that are commonly used as plasticizers by an acclimated shake flask CO_2 evolution method. Inman *et al.* (1984) conducted incubation experiments to investigate the factors affecting the decomposition of carboxyl-labeled (^{14}C) PAEs. Wang *et al.* (1997) studied the kinetics of PAEs degradation by acclimated activated sludge.

In recent years, increasing knowledge has been gained concerning the ability of yeast and moulds to degrade aromatic pollutants (Clauben *et al.* 1996; Middelhoven 1993). For example, the yeast *Rhodotorula mucilaginosa* could degrade di-*n*-octyl phthalate (Peciulyte 1997). Nozawa and Maruyama (1988) investigated the anaerobic metabolism of phthalate and other aromatic compounds by the denitrifying bacterium *Pseudomonas* sp. strain P 136. Microbial catabolic pathways offer great promise for the accelerated, in expansive and safe removal of chemical pollutants from the environment. However, the bakery yeast *Saccharomyces cerevisiae* has not ever been applied to the biodegradation of PAEs and other chemical pollutants. The yeast has

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advantage that include it is easily cultivation compared with other microorganisms and can inexpensively obtain.

In the present study, we examined the biodegradation of three PAEs, namely, diethyl phthalate (DEP), DBP and BBP by *S. cerevisiae*. The objectives of this study are to investigate the capability of degradation of the PAEs and to describe their biodegradation characteristics by this yeast.

MATERIALS AND METHODS

PAEs used in this study, namely, DEP, DBP and BBP, were commercial grade (Nacalai Tesque, Inc., Kyoto, Japan) and had a purity % at least 99%. Diammonium hydrogen phosphate used was of analytical grade (Nacalai Tesque, Inc., Kyoto, Japan). Lyophilized *S. cerevisiae* was purchased from S. I. Lesaffre, Marcq, France. The water employed in this study was purified by an ultra pure water system (Advantec MFS, Inc., Tokyo, Japan) resulting in a resistivity > 18 MΩ cm. All other chemicals and solvent were of analytical grade and were used without further purification.

PAEs were dissolved in 0.5 mg mL^{-1} of diammonium hydrogen phosphate solution (cultivation medium) to give a concentration of 0.5 mg L^{-1} each compound. *S. cerevisiae* (10 mg) was grown in 5 mL of the cultivation medium containing each PAE at 30°C for 0 – 72 h. The cultivation medium with *S. cerevisiae* was periodically withdrawn. Each compound was tested in triplicate. After the cultivation, the PAE solution with the yeast was filtered through a membrane filter (0.5 μm) and then the yeast trapped on the filter was washed with methanol because loss of PAE might be caused by microbial adsorption. In the blank control, the recovery of PAEs by this method was about 98 %. The filtrate and methanol were combined and used for PAEs analysis.

The PAEs concentrations of all samples were determined by high performance liquid chromatography (JASCO Co., Tokyo, Japan) with a UV detector (254 nm). The separation column used was a MIGHTYSIL RP-18 GP 150 (Kanto Chemicals, Tokyo, Japan). The mobile phase was ethanol/water (8/2) and the flow rate was 0.7 mL min^{-1}.

RESULTS AND DISCUSSION

Effect of culture medium on the biodegradation of DBP was investigated at a temperature of 30°C. The culture mediums tested were diammonium hydrogen phosphate (0.5 mg mL^{-1}) and water alone. These results are shown in Fig. 1. The biodegradation process of DBP proceeded with increasing cultivation time in the presence of diammonium hydrogen phosphate. Under this condition, DBP was almost degraded within 72 h. On the other hand, in the absence of diammonium hydrogen phosphate (water alone), an almost constant degradation rate was obtained over the cultivation time range 36 - 72 h. Therefore, the suitable cultivation medium for

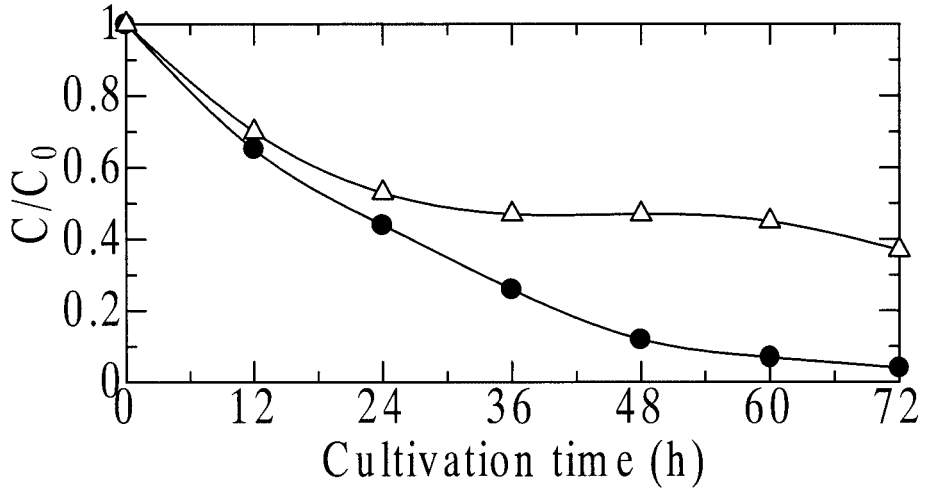

Figure 1. Effect of cultivation medium on the biodegradation of DBP by *S. cerevisiae* at 30°C.

Cultivation medium: (●); diammonium hydrogen phosphate solution, (△); water.

Initial DBP concentration: 0.5 mg L^{-1}.

biodegradation of PAEs was diammonium hydrogen phosphate. In our previous study, it has been also found that diammonium hydrogen phosphate was the best culture bed for the preconcentration of copper by *S. cerevisiae* (Ohta *et al.* 2001). Therefore, it is considered that the optimal culture medium for *S. cerevisiae* is diammonium hydrogen phosphate solution.

The biodegradation of the three PAEs, i.e., DEP, DBP and BBP by *S. cerevisiae* were tested in the presence of ADP. The results of the degradation of the phthalates are shown in Fig. 2. It can be clearly seen that two of the examined phthalates, DBP and BBP were degraded very quickly, with more than 96 % and 90 % within 3 days (72 h), respectively. Whereas the degradation of DEP was relatively slower, only less than 62 % of DEP was degraded throughout the test period (72 h).
The biodegradation of the three phthalates appeared to be related to the length of the alkyl side chains, as is evident from Fig. 2, the rate of degradation of the three phthalates was different. The long-chain PAEs, such as DBP and BBP, were degraded quicker than the shorter-chain phthalate DEP.

In general, rates of biological reactions tend to be hyperbolic saturation functions of substrate concentration, and can be expressed by

$$r = \frac{r_m C}{k + C} \qquad (1)$$

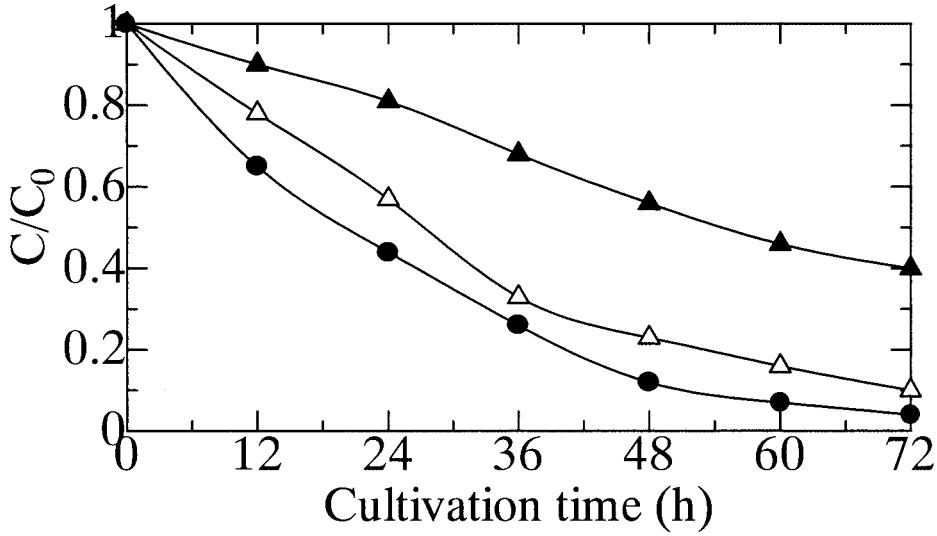

Figure 2. Biodegradation of PAEs by *S. cerevisiae* at 30°C.

Cultivation medium: diammonium hydrogen phosphate solution.

(▲); DEP, (●); DBP, (△); BBP.

Initial PAEs concentration: 0.5 mg L^{-1}.

where r is reaction rate, i.e., the degradation rate of the pollutant, r_m the maximum rate, C is the concentration of the pollutant and k is the half-saturation coefficient. When $C << k$, Eq. (1) reduces to form

$$r = \frac{r_m C}{k}, \qquad (2)$$

and the degradation process conforms to a first-order reaction with

$$k_1 = \frac{r_m C}{k} \qquad (3)$$

When $C >> k$, Eq. (1) becomes $r = r_m$, and degradation approaches a zero-order reaction with $k_0 = r_m$ as the zero-order rate constant.

First-order kinetics have been used frequently to describe biodegradation at low substrate concentrations. The half-lives for PAEs in the environment is often based on assumption of first-order kinetics (Staples *et al.* 1997). The half-lives of first-order reaction can be calculated according to the equation

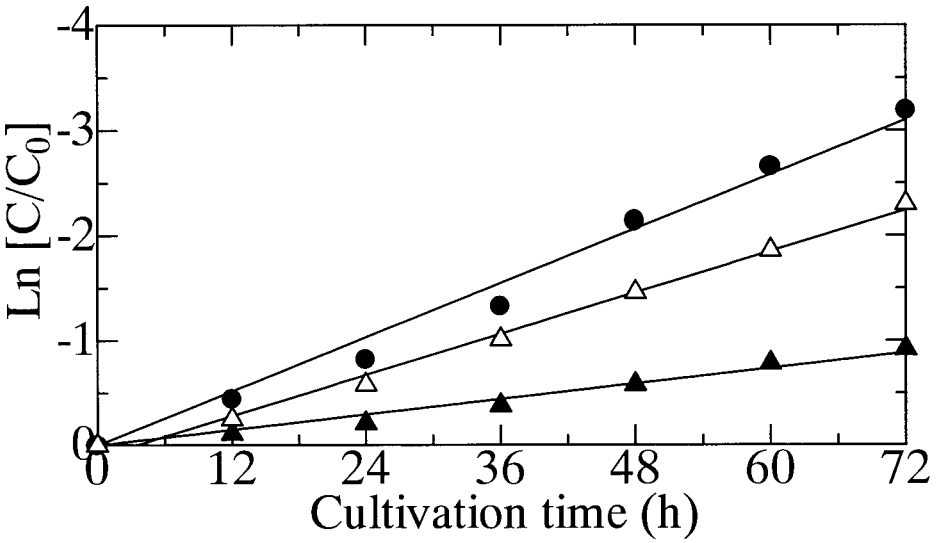

Figure 3. Plot of $Ln(C/C_0)$ versus cultivation time in the biodegradation of PAEs by *S. cerevisiae* at 30°C.

Cultivation medium: diammonium hydrogen phosphate solution.

(▲); DEP, (●); DBP, (△); BBP.

$$t_{1/2} = \frac{0.693}{k_1} \qquad (4)$$

Linear regression was applied to the experimental data shown in Fig. 2 according to the equation

$$\ln (C/C_0) = k_1 t \qquad (5)$$

$$(C/C_0) = k_0 t \qquad (6)$$

where C and C_0 are PAEs concentration and initial one, respectively, t express cultivation time, and k_1 and k_0 are the first- and zero-order rate constant, respectively. The calculated results indicated that the first-order model gives a better fit (Fig. 3) than the zero-order model (not shown).

The kinetic equations of DEP, DBP and BBP degradation are summarized in Table 1. These results were consistent with those of Wang *et al.* (2000) in the viewpoint of first-order degradation reaction of PAEs. However, for the rate of degradation, their findings were opposite to our results. Wang *et al.* (2000) reported that the short-chain

Table 1. Kinetic equations for PAE degradation.

Phthalates	Kinetic equation	Half-life (*h*)	r^2
DEP	$\ln (C/C_0) = 0.0123t$	56	0.987
DBP	$\ln (C/C_0) = 0.0431t$	16	0.992
BBP	$\ln (C/C_0) = 0.0306t$	22	0.993

PAEs were degraded at a higher rate than the longer-chain PAE. This may result from different species of microorganisms. They performed biodegradation of PAEs by anaerobic sludge. Shelton *et al.* (1984) also indicated that although degradation results were consistent among organisms from four wastewater treatment plants, rates were variable.

The biodegradation pathway of PAEs was proposed by Engelhardt *et al.* (1975) and Keyser *et al.* (1976) using pure cultures studies. Some organisms selectively hydrolyze only one ester bond to give the monoalkyl phthalate plus an alcohol; the alcohol such as butanol and benzyl alcohol is then used for growth. Other organisms are capable of the complete mineralization of either the monoalkyl or dialkyl phthalate. The pathway of degradation is as follows:

dialkyl phthalate ⟶ monoalkyl phthalate
⟶ phthalic acid ⟶ protocatechuic acid
⟶ ring cleavage and mineralization.

It might be considered that the biodegradation pathway of PAEs also follows the above reaction in this study. This will become clear by analyzing the intermediate products.

In the present study, we clarified the biodegradation characteristics of PAEs by *S. cerevisiae*. The degradation reaction of PAEs followed a first-order kinetic low and the degradation rate was DBP > BBP > DEP. The present method would be able to be applied to other kind of PAEs as well as many toxic chemicals.

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