Microbial Metabolism of Di-*n*-butyl Phthalate by Bacterium *Bacillus Natto*

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Microbial metabolism behavior of di-*n*-butyl phthalate (DBP) in aqueous solution by bacterium *Bacillus natto* was quantitatively characterized by HPLC. Under the optimal conditions, the degradation percentage of DBP was 72% after 24 h of cultivation. Concerning the metabolites of DBP degradation, dipropyl phthalate, monobutyl phthalate, and phthalic acid were identified by GC/MS.

Dialkyl phthalates are widely used as plasticizers in different resins, especially polyvinyl chloride (PVC) resins.[1] In addition, di-*n*-butyl phthalate (DBP) is an important additive of special paints and adhesives. DBP is the main plasticizers (3%) of all plasticizers used in Japan.[2] Through production, manufacturing, use, and disposal, dialkyl phthalates are potentially released, and found in the environmental samples such as natural waters, marine sediments, and biota,[3,4] where they appear as toxic compounds. Toxicity of DBP cause serious problems, due to its high bioaccumulation rate in different organisms.[5] The effects of environmental chemicals on transactivation of the estrogen receptor and breast cancer cell growth in vitro were pointed out and phthalic acid esters (PAEs) such as di-*n*-butyl phthalate (DBP) was estrogenic.[6]

Because the rate of hydrolysis and photolysis of PAEs are very low, metabolic breakdown by microorganisms is considered to be one of the major routes during environmental degradation for PAEs.[5,7] Until now, numerous studies have been reported on the biodegradation of PAEs under aerobic conditions in natural water, wastewater, and soil.[8] Jonson and Lulves[9] studied the biodegradation of DBP and di-2-ethylhexyl phthalate in freshwater hydrosoil. Walker et al.[10] investigated the degradation of DBP in estuarine and freshwater sites. Sugatt et al.[11] examined the biodegradation of 14 commercial PAEs that are commonly empolyed as plasticizers by an acclimated shake flask CO_2 evolution. Inman et al.[12] carried out incubation experiments to investigate the factors affecting the decomposition of carboxyl-labeled (^{14}C) PAEs. Wang et al.[13] published the kinetics of PAEs degradation by acclimated activated sludge.

In the present study, we examined the biodegradation of di-*n*-butyl phthalate by *Bacillus natto*. The objectives of this study are to assess the influence of various factors on the capability of degradation of DBP, and to describe the degradation pathway by this bacterium.

DBP was commercial grade at least 99% (Nacalai Tesque), ammonium dihydrogen phosphate (ADP) was of analytical grade (Nacalai Tesque). Lyophilized *B. natto* was purchased from a grocery shop. Water used in this study was purified by an ultra pure water system (Advantec MFS) resulting in a resistivity $>18\,M\Omega\,cm$. All other chemicals and solvents were of analytical grade, and were used without further purification.

B. natto 0.2–10.0 mg mL^{-1} was incubated in 0–2.0 mg mL^{-1} of ADP solution (cultivation medium) at 40 °C for 60 min. After incubation for 5 mL of DBP added at a concentration of 3.0 mg L^{-1}. The cultivation was performed at 20–50 °C. The cultivation medium with *B. natto* was periodically withdrawn. DBP was tested in triplicate.

The DBP concentrations of all samples were determined by HPLC (Model TRI ROTAR-V, JASCO Co.) equipped with a UV detector (254 nm, Model UVDEC-100-VI, JASCO Co.). The separation column used was a MIGHTYSIL RP-18 GP 150 (Kanto Chemicals). The mobile phase was ethanol/water (4/1, v/v) and the flow rate 0.7 mL min^{-1}. The degradation of DBP was defined as C/C_0, where C_0 and C are DBP concentration in the sample solution before and after degradation, respectively.

The sample solution taken after 24 h course of the process, initially containing 3.0 mg L^{-1} DBP was extracted with 30 mL of hexane, then hexane was concentrated to 50 μL under nitrogen flow. Identification of intermediates was carried out by comparison of peak retention times and mass spectra with those of standard compounds or NIST library records.

The effect of culture temperature on DBP degradation was investigated in the range from 10 to 50 °C, at an incubation time of 30 min, and bacterium concentration of 2.0 mg mL^{-1}. As shown in Figure 1, the degradation of DBP was significantly influenced by the culture temperature. The degradation of DBP through bacterium was enhanced with increasing culture temperature up to 40 °C, and then reached a plateau. Therefore, the optimal cultivation temperature of 40 °C was chosen for subsequent degradation purposes.

The effect of the bacterium concentration demonstrated quite interesting dependence. Bacterium concentrations in the range from 0.2 to 10.0 mg mL^{-1} were investigated. The degradation percentage of DBP increased with increasing the bacterium concentration, that is, the bacterium concentration above

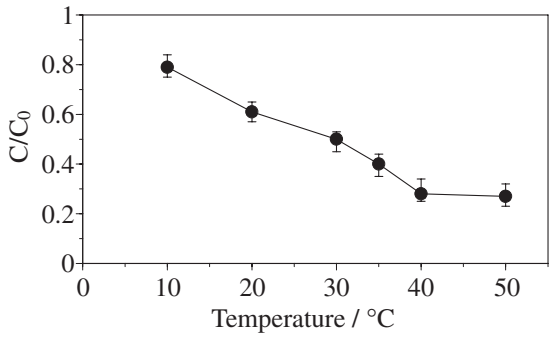

Figure 1. Effect of culture temperature on the biodegradation of DBP by *B. natto* after 24 h. DBP: 3.0 mg L^{-1}; Bacterium concentration: 2 mg mL^{-1}; Bacterium incubation time: 30 min; ADP concentration: 0.5 mg mL^{-1}. C/C_0: DBP concentration after degradation/initial concentration.

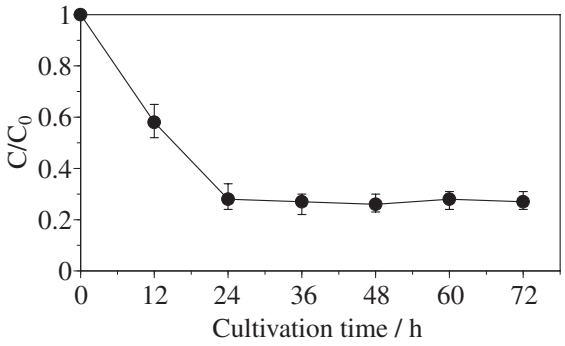

Figure 2. Biodegradation of DBP by *B. natto*. Culture temperature: 40 °C; Bacterium concentration: 2 mg mL^{-1}; Bacterium incubation time: 60 min; ADP concentration: 0.5 mg mL^{-1}. C/C_0: DBP concentration after degradation/initial concentration.

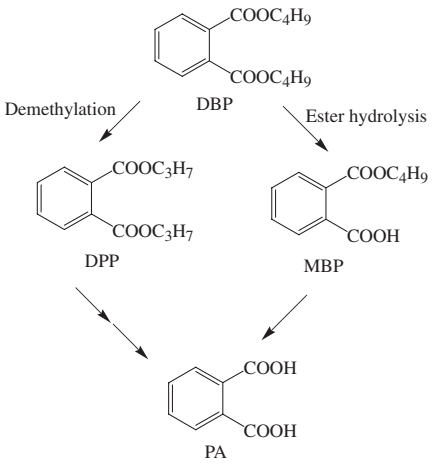

Figure 3. Degradation pathway of DBP by *B. natto*.

0.2 mg mL^{-1} degraded DBP up to 2.0 mg mL^{-1}, and then the percentages reached plateau. Therefore, 2.0 mg mL^{-1} of bacterium concentration was recommended for DBP degradation purposes.

In our previous publication, it has been found that ADP was the best culture medium for the biodegradation of DBP.[14] In order to determine the effect of cultivation medium concentration on the growth of microbial cells and the aerobic degradation of the DBP, *B. natto* was cultivated with different initial ADP solutions ranging from 0 to 2.0 mg mL^{-1}. The degradation percentage of DBP was enhanced with increasing concentration of ADP up to 0.5 mg mL^{-1}, and then reached a plateau. The optimum ADP concentration was 0.5 mg mL^{-1}. Therefore, 0.5 mg mL^{-1} was selected for the degradation of DBP.

The biodegradation of DBP by *B. natto* was tested under the optimum conditions. DBP (3.0 mg L^{-1}) could be degraded by *B. natto* with 72% after 24 h, and then reached an almost constant degradation rate was observed with increasing cultivation time up to 72 h as depicted in Figure 2.

In order to identify intermediate products, the DBP sample solution degraded by the bacterium over 24 h was analyzed by GC/MS. The DBP sample solution contained dipropyl phthalate (DPP), monobutyl phthalate (MBP), and phthalic acid (PA) eluting at $t_{ret} = 27.08$, 25.56, and 9.55 min, respectively. During GC/MS analysis, the phthalates were predicted to be cleaved at the carbon–carbon bonds between the aromatic ring and the carbonyl groups and the carbon–oxygen bonds linking the carboxyl groups to the alkyl chains. The cleavage of these bonds in DBP generated fragments of similar molecular mass with peaks at m/z 149 and 167 present in the mass spectra of DPP, MBP, and m/z 104 and 148 detected in the mass spectrum of PA. Based on these observations, the comparison of the retention times with authentic standards, the peaks were identified as DPP, MBP, and PA. The comparison of mass spectra of experimental samples with those of the standards and NIST library records showed a strong similarity with the parent ions (250, 222, and 166) for each of the traces matching the molecular masses of DPP (250), MBP (222), and PA (166). These results suggest that DPP, MBP, and PA are the degradation products formed during the degradation of DBP by *B. natto*.

The pathway for the degradation of DBP may be proposed by cleavage of a side chains sequentially, forming di-phthalate ester by demethylation or mono-phthalate ester by ester hydrolysis, yielding subsequently PA (Figure 3). It might be considered that the biodegradation pathway of DBP also follows the above reaction in the present study. The degradation pathway of DBP involves sequential cleavage of the ester bond to yield the phthalate monoester, and then PA, which is further metabolized to produce carbon dioxide and water.[15]

The degradation method of DBP by *B. natto* has many advantages as an economic, simple, and environmentally preferable, and was faster than that by *Saccharomyces cerevisiae* (0.5 mg L^{-1}; 55% degradation after 24 h).[14] Furthermore, *B. natto* could be useful for other PAEs.

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