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Efficiency of Rice Bran for Removal of Di-n-Butyl Phthalate and Its Effect on the Growth Inhibition of *Selenastrum capricornutum* by Di-n-Butyl Phthalate

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Di-n-butyl phthalate (DBP) is used as a plasticizer for cellulose plastics and as a solvent for dyes. Since the phthalates are not covalently bound to the plastics, they can leach into the environment. DBP with long ester chains are less susceptible to degradation (Alartriste et al. 2003). DBP are the potential to interfere with reproduction and development by endocrine-mediated processes (Gray et al. 2000). Wellejus et al. (2002) reported that oxidative DNA damage was measured in terms of the premutagenic modified nucleoside 7,8-dihydro-8-oxo-2'-deoxyguanosine in nuclear DNA from liver, kidneys, and testes from rats exposed to DBP in the perinatal or preadult period. Saillenfait et al (1998) showed that the testicular effects of DBP could be reproduced using mono-n-butyl phthalate (MBP), the major metabolite of DBP. Environmentally friendly technologies for the removal of DBP from groundwater and surface water are needed. However, few detailed reports have been conducted on the removal of this compound.

The present study describes efficiency of rice bran for removal of DBP and its effect on the growth inhibition of *Selenastrum Capricornutum* by DBP. A standard procedure for the use of an algal bioassay to determine toxicity has been adopted by the American Society for Testing and Materials (USEPA 1989).

MATERIALS AND METHODS

To study the removal reaction, batch tests were carried out. Sample 100 ml DBP solutions (dissolved in distilled water) were placed into 100-ml glass-stoppered Erlenmeyer flasks, to which rice bran or spherosome was then added. The samples were mixed with a magnetic stirrer for 1.5 h. During mixing, the flasks were stopperd to prevent evaporayive loss. The reaction mixture was filtered through filter paper (quantitative ashless no. 5A, Toyo Roshi, Ltd., Japan) to remove the rice bran or spherosome. The initial 10 ml of filtrate was discarded because of the adsorption of DBP by the filter paper. In control samples lacking rice bran or spherosome, the subsequent filtrate after the discarded portion contained the same amount of chemical compounds as those in the original solution. Fifty mL of this filtrate was placed in a separatory funnel and 5 mL of

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Table 1. Composition of rice bran.

Constituent	Content (g/100 g)
Water	13.5
Protein	13.2
Lipid	18.3
Carbohydrate	
Glucide	38.3
Fiber	7.8
Ash	8.9

m-xylene was added to the solution. The mixture was shaken for 1 min. The separated m-xylene layer was subjected to GC to analyze DBP. A blank containing only DBP solution was used to monitor the stability of DBP with respect to time. The determination of DBP was carried out by a Shimadzu Model GC-14A gas chromatograph equipped with a flame ionization detector and a capillary column (ULBON HR-52, 30 m x 0.53 mm). Both the column and injection port were maintained at 230 ℃ with the detector maintained at 280 ℃.
Rice bran was purchased at the local market (Daiei, Kobe, Japan). The composition of rice bran is shown in Table 1. DBP standard was purchased from Wako Pure Chemical Industries (Amagasaki, Japan).
Spherosomes were isolated by using an improved method based on that of Moreau et al. (1980). Rice bran (1g) was ground with a mortar and pestle in a 40 mL grinding medium of 20 mM sodium succinate, pH 5.6, containing 10 mM $CaCl_2$. The paste was filtered through eight layers of cheesecloth and the filtrate centrifuged at 30,000 g for 20 min. The spherosome pad was removed from the surface with a spatula.

The alga used was the green alga *Selenastrum capricormutum* purchased from the Global Environmental Forum (Tsukuba, Japan). The cells were grown for 5 days in medium containing 12.1 mg/L magnesium chloride, 4.4 mg/L calcium chloride, 0.19 mg/L boric acid and 0.42 mg/L manganese chloride proposed by Suzuki et al. (1996). The cultures were incubated at room temperature (22 ± 2 ℃) with illumination of 4,000 - 5,000 lux for 24 hrs. Cell density was determined using a hemacytometer under a light microscope in order to maintain density in the range 1 - 2 x 10^6 cells / mL.

One-hundred mL of medium containing 1-2 x 10^4 cells/ mL was placed in a 250-mL glass Erlenmeyer flask, to which di-n-butyl phthalate solution (dissolved in medium for cell culture) and rice bran were then added. The cultures were

incubated for 96 h at room temperature (22 ± 2℃) with illumination of 4,000 - 5,000 lux and shaking at 100 rpm. The concentration of DBP was determined by gas chromatography (GC) method and cell density was determined every 24 h.

Values are shown as means ± SD of four separate samples. Data were analyzed using one-way ANOVA and, when appropriate, by the Student-Newman-Keul test . Results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Figure 1 shows the removal efficiencies of DBP in distilled water as a function of time for rice bran at 0.1 or 0.5 g/L levels. DBP was removed from water samples with an average removal efficiency of 93 % after 60 min when rice bran (0.5 g/L) was added to distilled water containing 10 mg/L DBP. After 60 min of reaction time, the removal appeared to plateau. Without rice bran, the disappearance of DBP was very slow and prolonged. After 24 h, over 90% of the DBP still remained in the medium without rice bran. In contrast, after 24 h DBP was almost completely removed in the medium with rice bran (rice bran: 0.1 g/L or 0.5 g/L).

Next, we investigated the mechanism of removal. We have previously reported that rice bran and defatted seed were effective in removal of organochlorine compounds such as chloroform, dichloromethane and benzene. Furthermore, it was confirmed that the spherosomes isolated from these adsorbents were effective in removing these organic compounds (Adachi et al 2000). Analytical and laser microscopic data have confirmed that the removal of organochlorine compounds and benzene is dependent on the uptake of these compounds into intracellular particles called spherosomes (Adachi et al 2001). Spherosomes are widely distributed among plants and fungi (Buttrose et al. 1963). Neither the function of spherosomes nor its analysis is well understood. Table 2 shows the efficiency of removal of DBP by spherosomes isolated from 0.5 g of rice bran. The removal by spherosomes was similar to that of rice bran. Based on these results, we concluded that removal of DBP by rice bran is dependent on the uptake into spherosomes.

The growth inhibition of *Selenastrum capricornutum* exposed to different concentrations of DBP during various periods is shown in Figure 2. Significant inhibition of growth with DBP occurred at concentrations of more than 1 mg/L. This inhibition was observed in the first 24 h of exposure and was maintained until the end of the 96 h exposure period. The toxicity of DBP (10 mg/L)to the growth of the green alga *Seleastrum capricornutum* with or without rice bran (rice bran: 0.1 g/L or 0.5 g/L) is shown in Figure 3. The algal growth in the medium without rice bran showed significant ($p<0.01$) depression. In contrast, the algal growth in the medium with rice bran (both of 0.1 g/L and 0.5 g/L) was reduced a little for the first 48 h, but improved to control levels within 72 h. This result shows that the damage by DBP at the initial stage recovered with the progression of time.

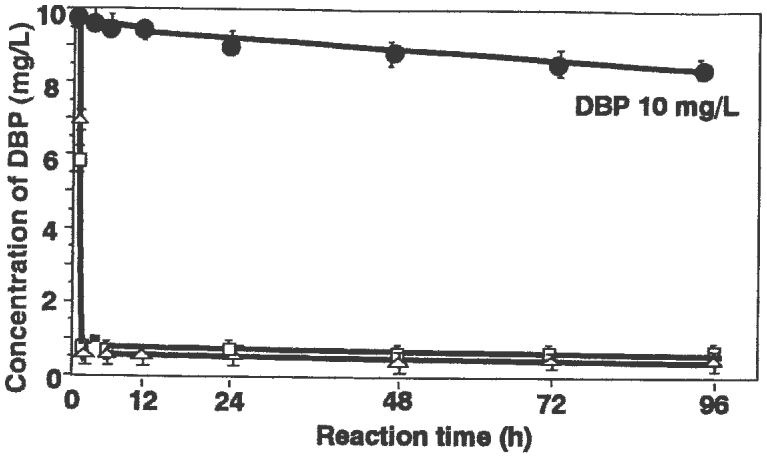

Figure 1. Time courses of the removal of di-n-butyl phthalate by rice bran. ●(control): rice bran 0 g/L, □: rice bran 0.1 g/L, △: rice bran 0.5 g/L

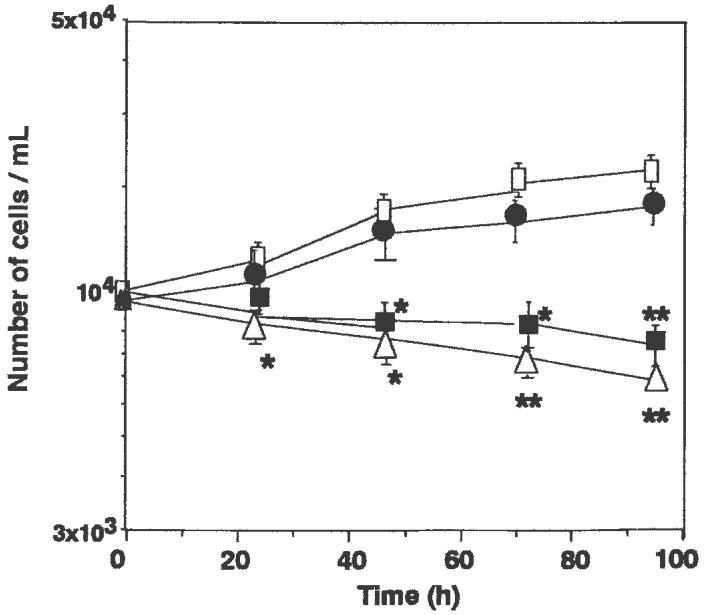

Figure 2. Growth inhibition of *Selenastrum Capricornutum* by di-n-butyl phthalate. □(control): DBP 0 mg/L, ●: DBP 0.1 mg/L, ■:DBP 1 mg/L, △: DBP 10 mg/L.Significantly different from control group : *$p<0.01$, **$p<0.001$.

Table 2. Removal of di-n-butyl phthalate by spherosome isolated from rice bran.

Substance	Concentration (mg/L)		Removal efficiency (%)
	Before treatment	After treatment	
DBP	10	1.2 ~ 2.2	83.9 ± 4.5★

★Data represent the mean ±SD of four separate determinations.
Reaction time: 90 min

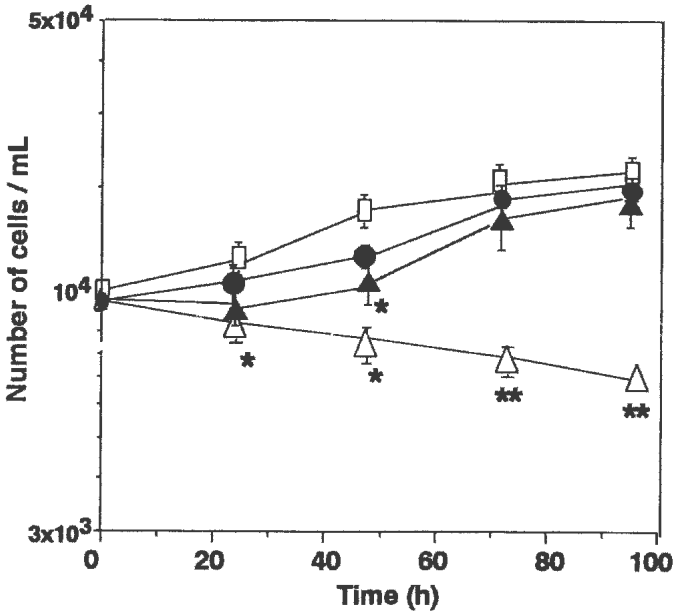

Figure 3. Effect of rice bran on growth inhibition of *Selenastrum Capricornutum* by di-n-butyl phthalate. □(control): DBP 0 mg/L , rice bran 0 g/L , △: DBP 10 mg/L, rice bran 0 g/L, ▲:DBP 10 mg/L, rice bran 0.1g/L, ●: DBP 10 mg/L, rice bran 0.5 g/L. Significantly different from control group : *$p<0.01$, **$p<0.001$.

Rice bran contains adequate nitrogen and phosphate. In our laboratory tests, it was confirmed that nitrate and phosphate ions did not dissolve from rice bran. Therefore, the use of rice bran is an efficient and cost effective method for removing DBP from environmental water. Rice bran is a by-product of making polished rice from brown rice, and is therefore a waste product. This process also

offers a significant use of lees material in terms of recycling.

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