NOTES

Encapsulated Cells of Carbaryl and Hippuric Acid-Degrading Bacterium *Pseudomonas cepacia* in Polyacrylamide

In the course of our investigation on the biotransformation of organic compounds by *Pseudomonas cepacia,* we have reported that representative antiseptics parabens, e.g., methyl, ethyl, *n*-propyl, and *n*-butyl *p*-hydroxybenzoates, were almost completely degraded by the bacterium after 3 weeks of culturing and *p*-hydroxybenzoic acid and methyl *p*-hydroxybenzoate were identified as metabolites.[1]

Mankind faces many serious environmental pollution problems. The environmental fate of agricultural chemicals is of great concern because some of them might be the cause of environmental pollution. For instance, effluence from farms or golf courses involves us all. This study has been attempted to degrade such chemicals using encapsulated cells of bacteria. We examined the biotransformation activity of *P. cepacia* on carbaryl, 1-naphthyl methylcarbamate, one of the most commonly used agricultural chemicals.

Some reports are available on the encapsulation of microorganisms on the solid supports.[2,3] In this study, commonly used polyacrylamide was selected and examined because other solid supports such as sodium alginate, urethane prepolymers PU-3, PU-6 and ENT were found to be unsuitable because of the leak of bacterium from the gel.

On the other hand, considering acylase activity of *P. cepacia,* hippuric acid having an amide group in it was selected and examined whether it was degraded by the bacterium. In the cases of both carbaryl and hippuric acids, the reaction velocity of encapsulated cells and free cells with them were compared and investigated (Fig. 1).

EXPERIMENTAL

Material

P. cepacia used in this experiment was given from Institute for Fermentation (Osaka), strain 15124. Commercially available carbaryl, hippuric acid, and some chemicals for polymerization of acrylamide were used.

Culture and Administration of Substrates

A 10-ppm saline solution of carbaryl and hippuric acid was used as a culture medium. In each case, a number of 500-mL Erlenmeyer flasks containing 250 mL saline solution were autoclaved for 20 min at 2.0 bar and 120°C, to which carbaryl or hippuric acid was added. A few loops of bacteria, subcultured on SCD agar medium, were inoculated into flasks, which were allowed to stand for 1–5 weeks at 30°C.

Performance of High-Performance Liquid Chromatography

As the mobile phase, 0.01 *M* $NH_4H_2PO_4$ (adjusted to pH 2.5 with H_3PO_4)—CH_3CN (6 : 4 for carbaryl, 15 : 85 for hippuric acid) was used. The column used was Shiseido Capcell Pak C_{18} SG-120 (150 × 6.0 mm I.D.) commercially packed with reversed-phase octadecylsilica, through which the above mobile phase was run at a flow rate of 1.0 mL/min. The operation was carried out at 254 nm and samples of 10 μL were injected onto the column. The determination of compounds was carried out according to the internal standard method.

Isolation and Identification of the Conversion Product from Carbaryl

Ten ppm saline solution of carbaryl (2.5 L) was used as a substrate. After culturing for 14 days, culture liquid was extracted with EtOAc. Evaporation of the solvent gave the dark purple materials, which were subjected to the preparative thin-layer chromatography (TLC) on silica gel (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of $CHCl_3$—MeOH—NH_3 (aq.) (90 : 10 : 1). A well-separated band, R_f 0.86, was collected and stripped with MeOH to colorless materials (4 mg). The substance was identified as 1-naphthol by comparison of its IR and mass spectroscopy (MS) with those of the authentic sample.

Isolation and Identification of the Conversion Product from Carbaryl

Ten ppm saline solution of hippuric acid (2.5 L) was used as a substrate. After culturing for 1 week, culture liquid was extracted with EtOAc. Evaporation of the solvent gave

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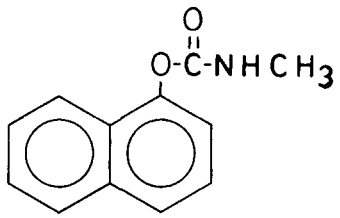
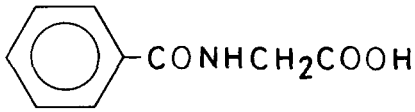


Carbaryl



Hippuric acid

Figure 1 Structures of carbaryl and hippuric acid.

colorless materials, which were subjected to the preparative TLC on silica gel (Merck, Art 5715 Kieselgel 60 F_{254}) with a solvent system of $CHCl_3$—MeOH—AcOH (90 : 10 : 1). A band, R_f 0.6, was collected and stripped with MeOH to colorless materials (1.8 mg). The 100-ppm methanol solution of the substance was subjected to high-performance liquid chromatography (HPLC) analysis. When 10 μL of the solution was injected onto the column under the conditions of HPLC mentioned above, the retention time (t_R 18.9) was obtained (benzoic acid t_R 19.1). Electron impact ms (EI-MS) of the substance showed m/z 122 (M^+, MW of benzoic acid 122) and the same fragment pattern as that of authentic benzoic acid.

Standard Assay of Biotransformation Activities of Encapsulated Cells

The encapsulation of *P. cepacia* was performed according to Chibata and Tosa's method.[4] The saline solution of carbaryl or hippuric acid (10 ppm) was incubated with the encapsulated cells (7.5 g wet wt) and allowed to stand for 1–4 weeks at 30°C. As a comparative assay, the substrate solution with polyacrylamide gel (blank test) was also incubated and subjected to HPLC assay.

RESULTS AND DISCUSSION

Degradation of Carbaryl by Free Cells and Identification of Metabolite

After culturing for 25 days, almost half quantity of carbaryl was degraded as shown in Figure 2. As shown in the ex-

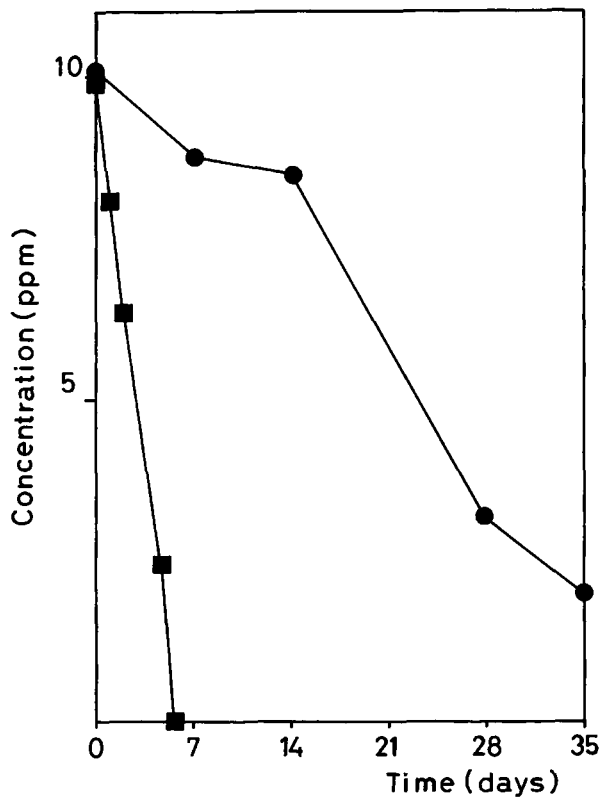


Figure 2 Degree of degradation of carbaryl and hippuric acid with time, using free cells: (●) carbaryl; (■) hippuric acid.

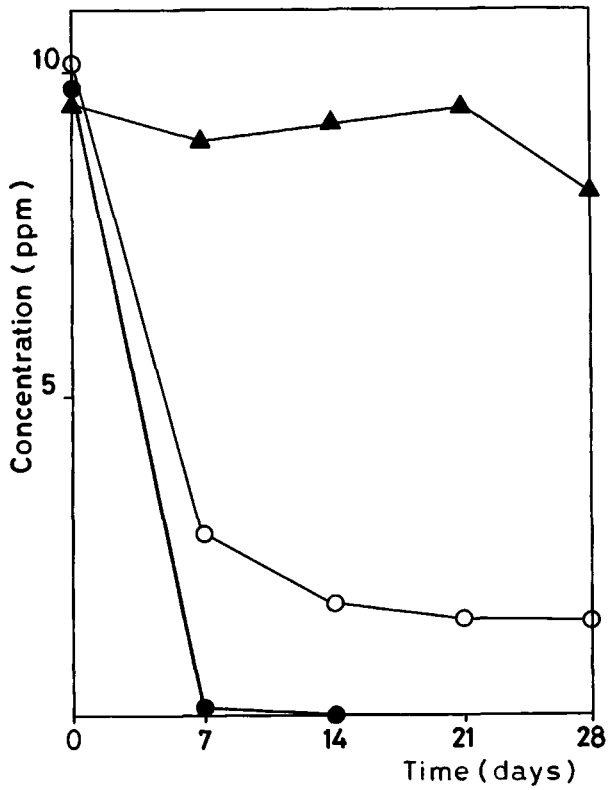


Figure 3 Comparative degree of degradation of carbaryl with time, using encapsulated cells, second-use encapsulated cells, and polyacrylamide gel: (●) encapsulated cells; (○) second-used encapsulated cells; (▲) polyacrylamide gel.

perimental section, 1-naphthol was identified as a metabolite, showing the bacteria's ability to cleave the C—O linkage in ester bond of carbaryl.

Degradation of Carbaryl by Encapsulated Cells

As shown in Figure 3, carbaryl was completely degraded within 2 weeks. Interestingly, the degradation activity of bacteria was found to be remarkably increased after the encapsulation, showing that polyacrylamide is an efficient solid supporter in this case.

Degradation of Hippuric Acid by Free and Encapsulated Cells and Identification of Metabolite

Hippuric acid was completely degraded for 6 days' culturing as shown in Figure 2. As shown in the experimental section, benzoic acid was identified as a metabolite of hippuric acid, showing the bacterial ability to cleave the CONH linkage. Meanwhile, as shown in Figure 4, 9.89 ppm of the initial concentration of hippuric acid was reduced to 3.41 ppm after culturing for 12 days. This fact shows that the activity was decreased a little after encapsulation.

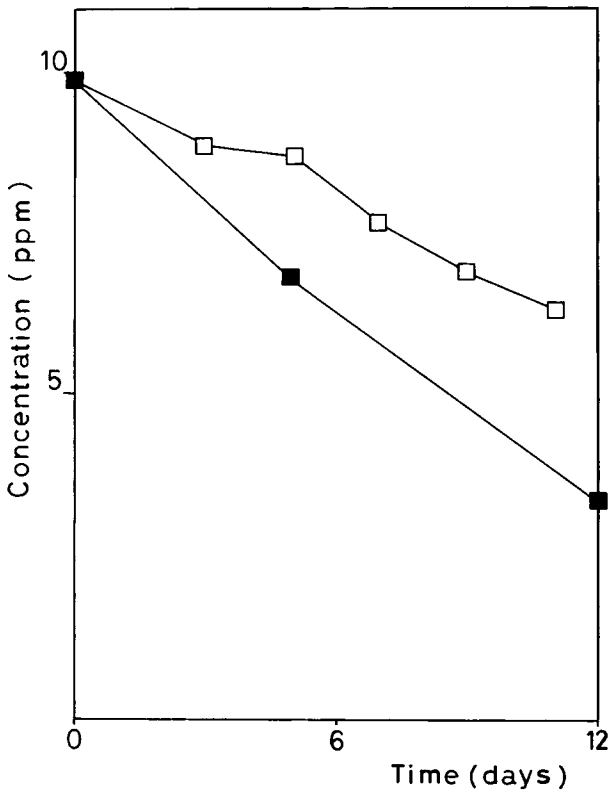


Figure 4 Comparative degree of degradation of hippuric acid with time, using encapsulated cells and second-used encapsulated cells: (■) hippuric acid; (□) hippuric acid (second-used encapsulated cells were used).

Repeated Use and Stability of Encapsulated Cells

The relative activity of the encapsulated cells after repeated use was examined. The encapsulated cells were washed thoroughly with the saline solution each time before the addition of a new substrate solution. It was found that the activity remained unchanged when the repeated cells were used, as shown in Figures 3 and 4. Meanwhile, the encapsulated cells were stored in saline solution at 5 and 30°C for 3 months. In both cases, it was found that the activity remained unchanged, showing the considerable stability as a biocatalyst.

Consideration on Effective Activity of Encapsulated Cells Concerning the Degradation of Carbaryl

As shown in Figure 3, the activity was remarkably increased after encapsulation concerning the degradation of carbaryl. To explore the reason, the reaction of 1-naphthol, a metabolite of carbaryl, with the encapsulated cells was examined. The results showed that 9.79 ppm of the initial concentration of 1-naphthol was found to be reduced to 0.09 ppm after culturing for 1 week. Interestingly, 9.34 ppm of the initial concentration of the substrate was reduced to 4.01 ppm after incubation for 1 week when polyacrylamide gel was used instead of encapsulated cells (blank test). This phenomenon can be ascribable to the absorption of 1-naphthol on the polyacrylamide gel. Therefore, the reaction velocity of the substrate with encapsulated cells was accelerated by the consumption of 1-naphthol.

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