Enhanced Microbial Adaptation to p-Nitrophenol Using Activated Sludge Retained in Porous Carrier Particles and Simultaneous Removal of Nitrite Released from Degradation of p-Nitrophenol

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In order to examine the microbial degradation of p-nitrophenol (PNP) by a mixed culture system and simultaneous removal of nitrite released via the degradation, an activated sludge retained in porous carrier particles and a suspension culture as a control were acclimated to artificial sewage containing PNP as the sole carbon source. The adaptation of microbes retained in porous carrier particles to PNP was faster than that of suspended microbes by more than 20 d. After microbial adaptation to PNP, it was degraded completely without significant accumulation of intermediate metabolites. The PNP degradation activity of the retained microbes was more than 2 times higher than that of the suspended microbes. By increasing the retained microbial concentration, nitrite released from the degraded PNP was removed by denitrification. This research demonstrates that using microbes retained in porous carrier particles is not only effective for reduction of acclimation time but also enables simultaneous removal of the nitrogen compounds resulting from the degradation of nitroaromatics.

[Key words: microbial adaptation, p-nitrophenol degradation, mixed culture, porous carrier particles, retained microbes, suspended microbes, nitrification, denitrification]

Synthetic chemicals have been utilized widely in industry and agriculture. Environmental pollution caused by the use of such chemicals has attracted much attention in recent years. Nitroaromatic compounds, such as nitrophenol, nitrobenzene, nitrotoluene and nitrobenzoates, have been of considerable industrial importance as the main raw materials for the production of dyes, pesticides and explosives. These compounds are widely distributed in the environment and have been detected in wastewater, rivers and soils (1–3). In order to treat the nitroaromatic compounds effectively, a mixed culture is considered to be a promising method for both industrial wastewater and polluted sites that need remediation. Until recently, many studies have investigated degradation pathways with pure cultures of microbes such as Pseudomonas sp., Alcaligenes eutrophus and Nocardia sp. (1, 4, 5). However, the majority of the nitroaromatic compounds are highly toxic to microorganisms (4), and thus are difficult to be degraded microbiologically unless the microbes are acclimated. A rapid acclimation is thus needed for the biological treatment process.

On the other hand, from the point of view of pollution control, the removal of nitrogen compounds released during the degradation of the nitroaromatic compounds are also desired due to their contribution to eutrophication. For example, it is reported that the concentration of nitrobenzene in industrial wastewater is more than 400 mg/l (6), which contains nitrite nitrogen of more than 40 mg/l. Biological nitrogen removal traditionally consists of two main steps, aerobic nitrification and anaerobic denitrification. In our previous study, it has been demonstrated that by using porous carrier particles to retain an activated sludge, organic oxidizing bacteria, aerobic nitrifiers and anaerobic denitrifiers were combined into one bioreactor, resulting in simultaneous removal of carbonaceous and nitrogenous substances in wastewater (7, 8). Using the porous carriers in a fluidized bed bioreactor retains the microbes in the carriers, and an apparent "aerobic denitrification" is obtained by the coexistence of both aerobic and anaerobic regions in the porous carrier. The anaerobic zone appears because of the depletion of dissolved oxygen (DO) by the aerobic microbes inhabiting the surface of the carrier. From these observations, it was considered possible to treat the nitroaromatic compounds and nitrites released from their degradation by the retained microbes. In addition, in the retained microbial community there exists a concentration gradient of toxic chemicals, and the microbes are highly concentrated inside the particle, which can be expected to affect the interaction between the microbes for the effective removal of chemicals (9, 10).

In the present paper, in order to devise a mixed culture system for effective degradation of p-nitrophenol (PNP), widely used in production of dyes and pesticides, and for simultaneous removal of the released nitrates, an activated sludge retained in porous carrier particles was acclimated to artificial sewage containing PNP as the sole carbon source. Microbial adaptation to PNP and the reaction characteristics were examined in comparison with a suspension culture system as a control.

MATERIALS AND METHODS

Experimental apparatus and procedures Two acrylic resin reactors, Reactor-A and Reactor-B, of rectangular cross section with a tapering tetrahedral base were used (Fig. 1). Each reactor was equipped with an air sparger at the bottom and had an effective volume of 4 l. Porous 20-mm polyurethane carrier cubes (average pore size: 1.47 mm; density: 30 g/l) were placed in Reactor-A
at a volume ratio of 20%, and a suspension culture in Reactor-B without carrier particles was used as the control.

Microbes prepared by acclimating activated sludge obtained from a municipal sewage treatment plant with artificial sewage (G sewage) containing glucose, urea and trace elements (Table 1) for more than 3 months, were inoculated in each reactor and precultured for 2 weeks with the same sewage (8). By this operation, almost all the microbes were retained in the carrier particles in Reactor-A, and in both reactors, the activated sludge concentration reached about 3000 mg-MLSS (mixed liquor suspended solids)/l. Thereafter, P sewage (Table 1), consisting of PNP as the sole carbon source, was used instead of G sewage to examine the microbial acclimation to PNP. The initial PNP concentration was 10 mg/l, and was raised step-wise by 10 mg/l after the PNP in the reactors was confirmed to be degraded completely. The final PNP concentration was 100 mg/l.

In order to study simultaneous nitrogen removal by the retained microbes, another experiment, where P+G sewage (Table 1) containing 100 mg/l PNP (51.8 mg-C/l; C = carbon) and 50 mg-C/l glucose was used to acclimate above activated sludge inoculum in Reactor-A with fresh porous carriers and in Reactor-B without carriers was carried out using the same procedure as described above.

In all the experiments, the cultivation was carried out using a fill-and-draw method, by which all the water was drawn from the reactor after PNP was completely degraded followed by addition of the fresh artificial sewage. The water was drawn after 30 min of sedimentation. The aeration rate was 1.2 l/min, and the temperature was maintained at 296 K.

The microbial adaptation process was evaluated by measuring PNP concentration, total organic carbon (TOC), biomass concentration. The adaptation was considered to be complete when neither lag phase for PNP degradation nor accumulation of the intermediate metabolites were observed. After the microbial concentration was at a constant level and PNP degradation was confirmed to be complete, the time course of one cycle was measured in terms of PNP and nitrogen concentrations.

**Analysis** The suspended and retained microbial concentrations were measured by the method described previously (8).

PNP concentration of the sample filtered with a 0.2 μm-pore-size nitrocellulose filter was measured spectrophotometrically according to Spain and Gibson's method (11) with a modification. The light absorbance of the filtered sample was detected at 400 nm using a spectrophotometer (Hitachi 557, Hitachi, Tokyo) after adjusting the pH to higher than 9.5 with 1N NaOH. TOC was measured using a TOC analyzer (TOC-500, Shimadsu, Kyoto) (8). Ammonia-N (NH4-N), nitrite-N (NO2-N) and nitrate-N (NO3-N) were detected using an ionic chromatograph (SCL-10A, Shimadzu) with a conductivity detector (DDC-6A, Shimadsu).

**RESULTS AND DISCUSSION**

**Acclimation of the activated sludge** Figure 2 shows the daily variation in PNP concentration in Reactor-A and Reactor-B operated by the fill-and-draw method during the acclimation process. After feeding sewage containing PNP at the initial concentration of 10 mg-l PNP/l, the PNP was gradually degraded in one cycle. PNP degradability increased with the progression of the culture and step-wise increase in PNP concentration. The PNP concentration in the sewage reached 100 mg/l after 64-d of acclimation. In all the experiments, the PNP degradation by retained microbes in Reactor-A was significantly faster than that by suspended microbes in Reactor-B.

Figure 3 shows the daily variation in the retained and suspended microbial concentrations in the two types of bioreactors. In Reactor-A, the concentration of suspended microbes was approximately 0 because almost all the microbes were retained in the porous carriers. During the acclimation, the microbial concentrations in both reactors showed a gradual decrease, and reached stable

**TABLE 1. Artificial sewage composition for activated sludge**

<table>
<thead>
<tr>
<th>Preculture sewage</th>
<th>Acclimation sewage</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G)</td>
<td>(P)</td>
</tr>
<tr>
<td>Glucose 100 mg-C/l</td>
<td>PNP 10-100 mg/l</td>
</tr>
<tr>
<td>Urea 30 mg-N/l</td>
<td>Urea 10 mg-N/l</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>KH2PO4</td>
<td>15.0 mg/l</td>
</tr>
<tr>
<td>MgSO4</td>
<td>11.3 mg/l</td>
</tr>
<tr>
<td>CaCl2·2H2O</td>
<td>1.4 mg/l</td>
</tr>
<tr>
<td>FeCl3·6H2O</td>
<td>0.1 mg/l</td>
</tr>
</tbody>
</table>

a Carbon.  
^b p-Nitrophenol.  
^c Nitrogen.
levels after around 76 and 98 d in Reactor-A and Reactor-B, respectively. The decrease in the microbial concentration was indicative of microbial selection for individuals capable of PNP degradation, wherein PNP non-degraders would have died out. After the microbial concentration reached the constant level, the retained microbial concentration per carrier volume was about 5500 mg-MLSS/ carriers.

Figure 4 shows the time courses of PNP degradation by retained and suspended microbes in the 77-d (Fig. 4-I) and 98-d (Fig. 4-II) cultures. The experimental data shown in Fig. 4-I were obtained during the 3rd cycle after the initial PNP concentration was raised to 100 mg/l. As observed from the time course of PNP concentration, PNP was degraded rapidly after the start of the culture in Reactor-A and the degradation was completed after around 25 h, while in Reactor-B, a lag period of about 50 h for PNP degradation was observed. The pH decrease was due to the formation of acids such as maleylacetic acid during the degradation of PNP (11). Figure 4-II shows that as the acclimation progressed, even though the PNP degradation in both reactors was accelerated as compared with that after 77-d acclimation (Fig. 4-I), the PNP degradation by retained microbes was still faster than that by suspended microbes. Incidentally, the rapid decrease of PNP in Reactor-A in the initial step after the start of the experiment was also related to PNP absorption to the porous carriers which is discussed later.

Figure 5 shows variation in TOC and PNP removal in both reactors calculated from the data shown in Fig. 4. The removal ratio of PNP was higher than that of TOC prior to the 24-h and 64-h cultivation in Reactor-A and Reactor-B, respectively, but no difference between TOC and PNP removal in Reactor-A was smaller than in Reactor-B (Fig. 5-I). This indicated the presence of intermediate metabolites of PNP by 77-d acclimation, which was specially remarkable in Reactor-B. However, as shown in Fig. 5-II, after the 98-d acclimation, almost no significant difference between TOC and PNP removal was observed in both reactors, which suggested that PNP was degraded to the end product, CO2, by the sufficiently acclimated microbes.

As shown in Fig. 4, the bulk PNP concentration in Reactor-A was significantly decreased in the initial step after the start of the experiment. This was presumably due to the absorption of PNP to the polyurethane fibers which was observed in our pre-experiment and was also reported by Enkiri et al. (14). To confirm whether the absorption phenomenon occurred, nitrogen concentrations were measured and an analysis based on the nitrogen balance was carried out. Figure 6 shows the time courses of various nitrogen concentrations during PNP degradation in Reactor-A after 77-d acclimation. NH4-N was formed from urea-N, and NO2-N was formed from PNP degradation because the first step in aerobic PNP degradation releases NO2-, and/or from nitrification of NH4-N. NO3-N was formed from nitrification of NO2-N. The total-N remained almost constant during one cycle, in-
indicating that no denitrification occurred, and nitrogen uptake for microbial growth was negligibly small. Thus, the nitrogen oxide, i.e., NO$_x$-N (NO$_2$-N+NO$_3$-N) released solely from PNP degradation, could be estimated as follows by excluding the effect of nitrification from the ammonium which remained in the reactor after exchanging the wastewater and which was released from urea degradation.

The nitrogen oxide concentration ($C_{NOx-N}$) resulting from PNP degradation is given by Eq. 1.

$$C_{NOx-N} = C_{NOx-N,0} - C_{NOx-N,t} - (C_{N,0} - C_{N,t})$$

where,

- $C_{N,0}$ = NH$_4$-N concentration, mg/l,
- $C_{N,t}$ = urea-N concentration, mg/l,
- $C_{NOx-N,0}$ = N concentration of nitrogen oxide, mg/l,
- $C_{NOx-N,t}$ = N concentration of nitrogen oxide resulting from PNP degradation, mg/l,
- subscripts of t and 0 =time t and 0.

Change in PNP-containing N concentration ($C_{PNP-N}$) and NO$_x$-N concentration originating from PNP degradation between time t and 0 was expressed by Eqs. 2 and 3.

$$\Delta C_{PNP-N} = C_{PNP-N,0} - C_{PNP-N,t}$$

$$\Delta C_{NOx-N} = C_{NOx-N,0} - C_{NOx-N,t}$$

where,

- $C_{PNP-N}$ = PNP-containing N concentration, mg/l,
- $\Delta C_{PNP-N}$ = difference in PNP-containing N concentration between time t and 0, mg/l,
- $C_{PNP-N,0}$ = PNP-containing N concentration at time 0, mg/l,
- $C_{PNP-N,t}$ = PNP-containing N concentration at time t, mg/l,
- $C_{NOx-N}$ = nitrogen oxide concentration.

If the ratio of Eq. 2 to Eq. 3 equals 1, PNP degradation is considered to occur, otherwise, the PNP adsorption is present. The change in $\Delta C_{PNP-N}/\Delta C_{NOx-N}$ with cultivation time, calculated from the data shown in Fig. 6, is shown in Fig. 7. Even though PNP decreased significantly in the initial step (0-8 h), the ratio was 0, which indicated the presence of PNP adsorption. After the 8-h cultivation, $\Delta C_{PNP-N}/\Delta C_{NOx-N}$ began increasing gradually with the decrease in PNP, and eventually reached 1 after 24 h. This result demonstrates that PNP adsorption occurred quickly, and all of the added PNP was degraded eventually.

The above results indicate that the microbial adaptation to PNP for retained microbes was significantly enhanced and the adaptation period was largely reduced compared with those for suspended microbes. To examine the reason for the difference in PNP degradation between the two types of bioreactors, PNP degradation activities were measured. To measure the overall degradation activity of PNP by retained microbes, the microbes in the porous carriers of a predetermined number were squeezed out completely by a pair of tweezers. The measurements were carried out using a 500-ml bottle contain-
ing sewage stirred at 100 rpm with an aeration rate of 0.4/l/min. The PNP concentration in the sewage was maintained as low as 10 mg/l to avoid substrate inhibition because PNP is toxic to the microbes at high concentration. Suspended microbes were withdrawn from the Reactor-B for comparison. The initial microbial concentrations were 80.0 and 68.4 mg-MLSS/l for retained and suspended microbes, respectively. As a result, the PNP degradation activities of retained and suspended microbes were 23.8 and 10.8 mg-PNP/g-MLSS/h, respectively, the former being about two times higher than the latter.

The actual reasons for the enhanced microbial adaptation to PNP degradation by retained microbes remain unclear at the present, but compared with the suspension culture (Reactor-B) the following events in Reactor-A were considered to be significant: (i) microbial interaction due to the retained microbes localized inside the porous carriers, such as gene transfer and/or cascade metabolization of PNP by different microbes distributed in the carriers; (ii) existence of a PNP concentration gradient across the porous carriers which would reduce the chemical toxicity to the microbes located in the inner region of carriers; and (iii) combination of (i) and (ii). The above-mentioned PNP adsorption to the carrier particles would also have affected the microbial adaptation. The adaptation mechanism of retained microbes to PNP is presently under investigation.

Simultaneous nitrogen conversion Under appropriate conditions, porous carriers enable aerobic and anaerobic regions to coexist in the carriers, and can thus achieve simultaneous removal of nitrogenous and carbonaceous substances even under apparent aerobic conditions (8). To examine if denitrification occurred after the microbial adaptation reached a stable state, the time course of the various nitrogen concentrations during the PNP degradation in the retained microbial reactor after a 98-d cultivation was measured. The DO varied between 7.0-8.0 mg/l during the cultivation. As mentioned above, the retained microbial concentration per carrier volume was about 5500 mg-MLSS/l-carriers. With the PNP degradation, nitrite-N was formed gradually and reached a peak at about 12 h. Similar to the results shown in Fig. 6, the total nitrogen concentration in one cycle of cultivation after the 98-d acclimation still remained almost constant, which indicated the absence of denitrification by the retained microbes. Even when pH was controlled at 7.0±0.1, no denitrification was detected. In our previous research where glucose or acetate was used as the carbon source, cubic porous carrier particles of edge length larger than 7 mm retained both aerobic and anaerobic microbes under the same DO level (7, 8). In these studies, microbial concentration (more than 7500 mg-MLSS/l-carriers) and TOC removal rate were much higher than those obtained in the present study. From these results, it was suggested that the lower retained microbial concentration and PNP degradation rate in Reactor-A resulted in the absence of anaerobic regions inside the carriers, and thus no denitrification occurred.

In order to examine the operating conditions for denitrification by the retained microbes, activated sludge was acclimated in Reactor-A using the P + G sewage containing 100 mg/l PNP and 50 mg-TOC/l glucose (Table 1) by fill-and-draw operation of one cycle per day to increase the microbial concentration. Acclimation of suspended microbes in Reactor-B was also carried out as a control. By using this glucose-containing sewage, the microbial concentration in Reactors-A and -B finally reached about 3000 mg-MLSS/l reactor after a 30-d acclimation, and the retained microbial concentration per carrier volume was approximately 15,000 mg-MLSS/l-carrier. Figure 8 shows time course of PNP degradation and nitrogen conversion in one cycle of cultivation. To prevent the effect of pH decrease, all the reactors were controlled at pH 6.9-7.1 by an on-line pH controller. After the cultivation, DO decreased to 5.0 and 2.0 mg/l in Reactor-A and Reactor-B, respectively, due to the organic oxidation, and gradually rose to 7.3 and 7.2 mg/l. During 24-h cultivation, the total-N concentration decreased from 16.0 to 5.0, and from 16.0 to 13.0 mg/l in Reactor-A and Reactor-B, respectively, from the wastewater decreased by 1 mg/l in the retained microbial reactor. In the initial 1 h of cultivation, the NO3-N which remained after aerobic regions to coexist in the carriers, and can thus achieve simultaneous removal of nitrogenous and carbonaceous substances even under apparent aerobic conditions (8). To examine if denitrification occurred after the microbial adaptation reached a stable state, the time course of the various nitrogen concentrations during the PNP degradation in the retained microbial reactor after a 98-d cultivation was measured. The DO varied between 7.0-8.0 mg/l during the cultivation. As mentioned above, the retained microbial concentration per carrier volume was about 5500 mg-MLSS/l-carriers. With the PNP degradation, nitrite-N was formed gradually and reached a peak at about 12 h. Similar to the results shown in Fig. 6, the total nitrogen concentration in one cycle of cultivation after the 98-d acclimation still remained almost constant, which indicated the absence of denitrification by the retained microbes. Even when pH was controlled at 7.0±0.1, no denitrification was detected. In our previous research where glucose or acetate was used as the carbon source, cubic porous carrier particles of edge length larger than 7 mm retained both aerobic and anaerobic microbes under the same DO level (7, 8). In these studies, microbial concentration (more than 7500 mg-MLSS/l-carriers) and TOC removal rate were much higher than those obtained in the present study. From these results, it was suggested that the lower retained microbial concentration and PNP degradation rate in Reactor-A resulted in the absence of anaerobic regions inside the carriers, and thus no denitrification occurred.

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in the initial 1 h of cultivation in Reactor-A was considered to be related to the glucose added to the sewage which was used as the proton donor for denitrification. Glucose was found to be exhausted after 1 h of cultivation according to the measured TOC and PNP concentrations. These results indicated that by supplying glucose the retained microbial concentration was maintained at a high level, and denitrification due to the formation of anaerobic regions in the porous carriers occurred with the degradation of PNP. The optimal conditions for PNP degradation and the simultaneous removal of the released nitrite need to be examined further.

In summary, the adaptation of microbes retained in porous carrier particles to PNP was faster than that of suspended microbes by more than 20 d. The PNP was degraded completely by the adapted microbes without accumulation of intermediate metabolites. Furthermore, by increasing the retained microbial concentration, nitrite released from the degraded PNP was removed by denitrification. This research demonstrates that using microbes retained in porous carrier particles is not only effective in reduction of acclimation time but also enables simultaneous removal of the nitrogen compounds resulting from the degradation of nitroaromatics.

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