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ORIGINAL PAPER

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Potential of a new biotreatment: *Sphingomonas cloacae* S-3^T degrades nonylphenol in industrial wastewater

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Abstract *Sphingomonas cloacae* S-3^T, a nonylphenol (NP)-degrading bacterium, was evaluated for its utility in the remediation of NP-contaminated wastewater. In flask-scale experiments, S-3^T cells immobilized on porous polypropylene carriers (beads) efficiently degraded NP to concentrations routinely measured in aquatic environments [a few parts per billion (ppb), or micrograms per liter). Therefore, we constructed and evaluated a laboratory-scale wastewater treatment system with a 3-l carrier-filled column. The system worked properly and consistently removed several hundred ppb of NP to ecologically safe concentrations of less than 10 ppb in industrial wastewater without the addition of nutrients. The effect of wastewater pH on the system performance was also evaluated; and wastewater samples with pH values of 6 or 8 were treated efficiently without pH adjustment. These results suggest that a biotreatment system using NP-degrading bacteria can efficiently remediate industrial wastewater and contribute to the preservation of aquatic environments.

Keywords Nonylphenol · Endocrine-disrupting chemicals · Pollutant-degrading bacteria · Biotreatment · Industrial wastewater

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Introduction

Concern over the potential of sewage treatment plant effluent to cause estrogenic effects in aquatic fauna has recently been mounting. Widespread sexual disruption or ″feminization″ has been observed in riverine fish in several developed nations and is thought to be due to endocrine-disrupting chemicals (EDCs) in the effluent [7, 8, 10, 14]. One of the likely suspects is nonylphenol (NP), an important intermediate in the production of many commercial and industrial materials, which is a ubiquitous pollutant in urban aquatic environments [1, 3, 6, 8, 9, 11, 13, 14, 15]. Recently, we isolated a novel NP-degrading bacterial strain from the genus *Sphingomonas* [4, 5] and referred to it as *S. cloacae* S-3^T. The S-3^T strain degraded 1,000 parts per million (ppm) of NP, i.e. a 10,000- to 1,000,000-fold higher concentration than levels found in urban environments, almost completely within 10 days. The main NP degradation metabolite was identified as nonanol (C9H19-OH), which might be derived from the nonyl group of NP. However, the utility of this strain in the remediation of industrial wastewater with lower concentrations of NP [at the level of parts per billion (ppb), i.e. micrograms per liter] has yet to be determined. Therefore, the capacity of S-3^T to degrade NP in industrial wastewater to environmentally acceptable concentrations should be evaluated to determine the practical utility of S-3^T in the bioremediation of NP-containing wastewater. Thus, we evaluated the degradation of NP by this strain in industrial wastewater via a flask-scale experiment and a laboratory-scale wastewater treatment system.

Materials and methods

Chemicals

NP was obtained from Kanto Chemical (Tokyo, Japan). Yeast nitrogen base without amino acids (YNB), used routinely as a minimal medium for *S. cloacae* and in our previous studies [4, 5],

was obtained from Difco Laboratories (Detroit, Mich.). Nutrient broth was purchased from Eiken Chemicals (Tokyo, Japan). Porous polypropylene carriers (IT10-93) for the immobilization of S-3^T were provided by Iwao Jiki Kogyo (Saga, Japan). Other materials and chemicals were available from commercial sources.

Bacterial strain and culture conditions

S. cloacae S-3^T (JCM 10874^T) was isolated from wastewater from a sewage treatment plant in Tokyo in 2000 [4,5]. Unless otherwise mentioned, the strain was cultivated aerobically in nutrient broth at 25 °C.

Wastewater

Industrial wastewater was obtained from a textile plant in Tokushima, Japan. The chemical and physical properties of the wastewater were: color red-brown, pH 7, chemical oxygen demand around 120 ppm, dissolved oxygen 5–7 ppm, and salinity 0.6%. Biochemical oxygen demand was not determined. In the flask-scale experiment described in the next section, we obtained wastewater from the plant which contained no NP. Thus, a 4% NP/ethanol solution was supplied to the wastewater to bring its final concentration to 200 ppb or 1 ppm just before the experiment. To evaluate the processing capability of the wastewater treatment system constructed in this study, we used wastewater originally containing less than 500 ppb of NP. We supplied NP only to the samples with NP concentrations below 200 ppb to bring the final concentrations to 200 ppb.

Flask-scale experiment

To prepare the S-3^T-immobilizing carriers (S-3^T beads), 1 ml of S-3^T culture (24 h cultivation) was inoculated into 100 ml of nutrient broth containing 12 g of autoclaved fresh IT10-93 carriers and cultivated at 120 rpm, 25 °C. After 24 h of cultivation, the beads were recovered with a tea strainer, washed gently with 0.85% NaCl, and 4.0 g of the beads were added to 30 ml of YNB or wastewater containing NP at 200 ppb or 1 ppm, followed by incubation at 120 rpm, 25 °C. Each culture (respectively 0, 3, 6, 9 days old) was extracted using 30 ml of hexane and a SR-2 Recipro shaker (TAITEC, Tokyo, Japan) without removal of the beads. It was concentrated to 0.5 ml at 25 °C using a N-1000 rotary evaporator (EYELA, Tokyo, Japan) and then subjected directly to HPLC. From a preliminary experiment with wastewater containing a known concentration of NP, our extraction method recovered 95–99% of NP in YNB and wastewater.

A wastewater treatment system using S-3^T beads

Figure 1 shows the wastewater treatment system constructed in this study. The system consists of a 3 l column filled with 2 l of S-3^T-beads, a peristaltic pump providing industrial wastewater to the column, and a MAU-1 aeration pump (EYELA, Tokyo, Japan) to maintain aerobic conditions in the column.

To prepare the S-3^T beads, 200 g of autoclaved fresh carriers, 1.6 l of nutrient broth, and 100 ml of S-3^T culture (24 h cultivation) were added to the column and incubated with aeration at room temperature (20–25 °C). After 24 h of incubation, the broth was drained and 0.85% NaCl was added gently and drained immediately, to rinse the beads. Next, NP-containing wastewater was introduced into the column and the treatment was started. The aeration flow was 500 ml/min, controlled automatically by a MAU-1 aeration unit.

The treated wastewater (100 ml) was sampled every 3 days, extracted using 100 ml of hexane and a SR-2 Recipro shaker, concentrated to 0.5 ml at 25 °C with a N-1000 rotary evaporator, and then subjected directly to HPLC to determine the NP concentration.

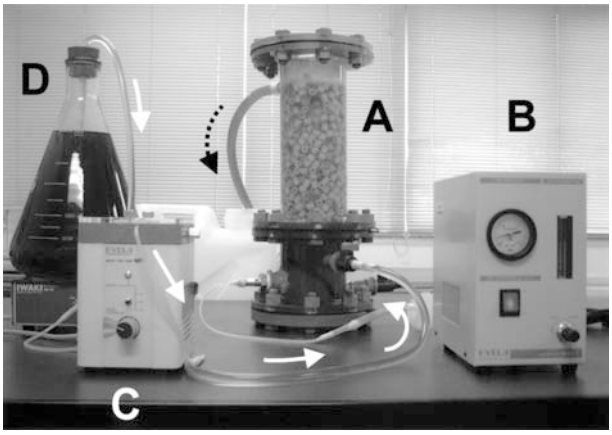

Fig. 1 The wastewater treatment system constructed in this study. *A* A 3-l laboratory-scale column filled with S-3^T beads, *B* an aeration unit, *C* a peristaltic pump, *D* nonylphenol (NP)-containing wastewater from a textile plant. *White arrows* and *dotted black arrow* indicate the flow of pre-treated and treated wastewater, respectively

Bacterial biomass on S-3^T beads

Since S-3^T cells immobilized on the porous carriers are difficult to estimate accurately by the plate-counting method, we estimated their biomass based on protein weight. Ten S-3^T beads were boiled in 5 ml of 1 N NaOH for 3 min and the resulting extract was analyzed by the Lowry method [12].

High-performance liquid chromatography

A HPLC system (Shimadzu, Kyoto, Japan) with an Inertsil ODS-3 V column (GL Sciences, Tokyo, Japan) was used to determine NP concentrations in wastewater. For the mobile phase (flow-rate 1.0 ml/min), an acetonitrile/water mixture (90/10 vol%) was used. NP was detected by UV absorption at 210 nm. The detection limit of this method was 500 ppb.

Results and discussion

Degrading activity of S-3^T beads in a flask-scale experiment

First, the NP-degrading activity of the S-3^T beads was evaluated in the flask-scale experiment. The beads degraded NP efficiently in the NP-containing YNB (Fig. 2A). NP at 200 ppb or 1 ppm was degraded almost completely within several days. Interestingly, the immobilized S-3^T degraded trace concentrations of NP; in fact, the NP concentration in YNB was 3–4 orders of magnitude lower than the concentration of carbon sources generally added to microbiological media. Next, the degrading activity in actual industrial wastewater was assessed. Surprisingly, the beads also degraded both 200 ppb and 1 ppm of NP in the wastewater without the addition of nutrients or adjustment of pH (Fig. 2B). We also performed a control experiment using carriers not colonized by S-3^T. As a result, NP degradation was not observed, indicating that the disappearance of NP was

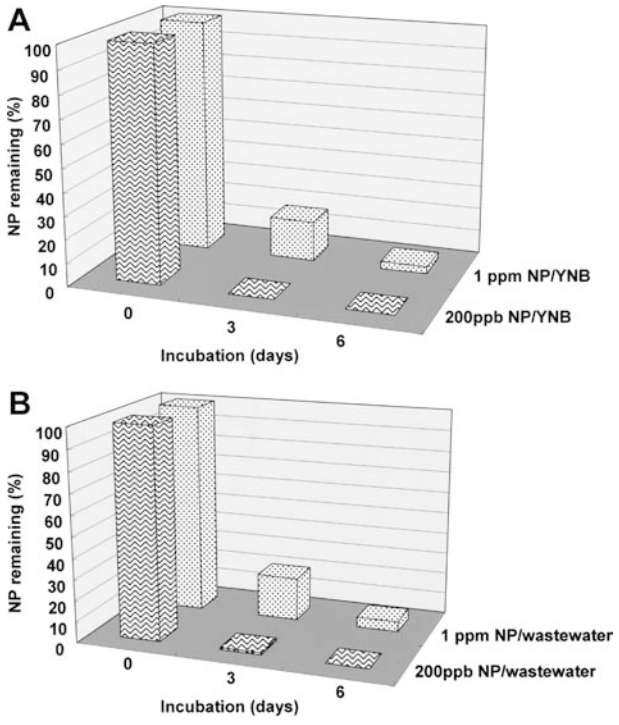

Fig. 2A, B Results of the flask-scale experiments. S-3^T beads were incubated with a NP-containing yeast nitrogen base medium (*NP/YNB* in **A**) or NP-containing wastewater (*NP/wastewater* in **B**) to examine their NP-degrading activity. Each result is representative of three independent experiments

not due to adsorption of NP onto the carriers (data not shown). We then examined whether addition of 1 g KH_2PO_4/l, 0.5 g $MgSO_4$/l or 5 g $(NH_4)_2SO_4$/l (the main components of YNB [2]) to wastewater stimulated degradation. However, none of these nutrients significantly increased the degradation rate (data not shown), suggesting that nutrients do not need to be supplied to wastewater for NP degradation.

Construction and test-run of a wastewater treatment system using S-3^T beads

We constructed a wastewater treatment system with S-3^T beads that did not require supplemental nutrition. Figure 1 shows the system constructed in this study. First, 2 l of the S-3^T beads were poured into the 3-l laboratory-scale column. The system was operated continuously for 1 month and the flow rate of the inflowing wastewater was increased step-by-step (from 135 ml/h to 270 ml/h, or from 135 ml/h to 540 ml/h) during this period. The NP concentration in the treated wastewater was monitored by HPLC, as described in the Materials and methods. During the 1-month running period, approximately 90% of the 200 ppb of NP was removed, without addition of nutrients, pH adjustment, or temperature control of the pre-treated wastewater (Fig. 3). The system was operated for 1 month, but this does not mean that the ability of S-3^T beads to remove NP reached a limit. Although we have not yet operated for more than 1 month, the system might work properly for longer than that running period, because there was no decline in the NP-degrading activity through the test-run (Fig. 3).

Although 20 ppb of NP remained in the treated wastewater, concentrations of less than 10 ppb do not appear to cause feminization in riverine fish [9]. We next increased the bed volume of S-3^T beads to 3 l. As a result of this improvement, NP was degraded more efficiently and the NP concentration in treated wastewater was reduced to less than 10 ppb at a stable flow rate of 360 ml/h (Fig. 4). The system was run at flow rates between 500 ml/h and 750 ml/h and functioned properly at 500 ml/h (Fig. 4).

We also tested for NP removal using IT10–93 carriers not colonized with strain S-3^T. During 1 month of operation, 130–160 ppb of NP leaked constantly into the treated wastewater, indicating that 20–35% of NP in pre-treated wastewater appeared to adsorb onto the carriers (data not shown). When S-3^T-colonized beads were used, however, 200 ppb of NP were reduced to 10–20 ppb or < 10 ppb, as shown in Figs. 3, 4. These results indicate that the major contributor in successful NP removal is microbial degradation by S-3^T, although adsorption of NP onto polypropylene carriers occurs and seems to help NP removal (biodegradation) by immobilized S-3^T cells.

We also evaluated the effect of wastewater pH on system performance. Wastewater pH was adjusted to 6,

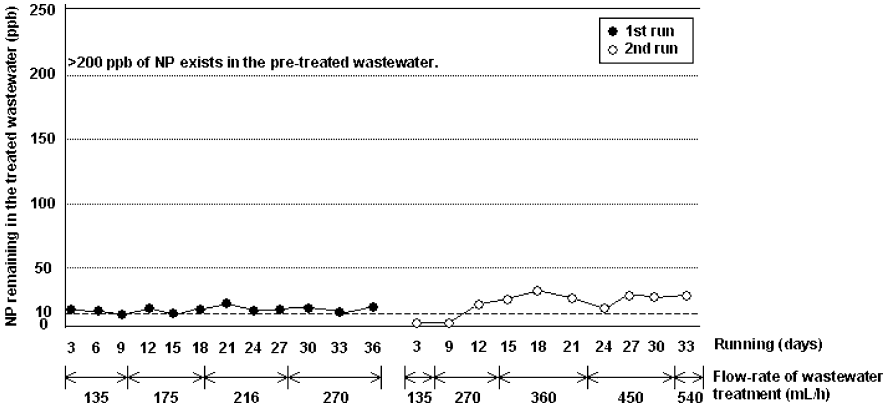

Fig. 3 Evaluation of the biotreatment system (Fig. 1) filled with 2 l of S-3^T beads. Wastewater containing more than 200 ppb was treated for 1 month. Results from two test runs are shown. The flow-rate of the treatment was increased step-by-step throughout the running. More than 200 ppb of NP were constantly degraded to 10–20 ppb by this treatment. The *broken line* indicates 10 ppb, an ecologically safe concentration of NP. *mL*·h Milliliters per hour

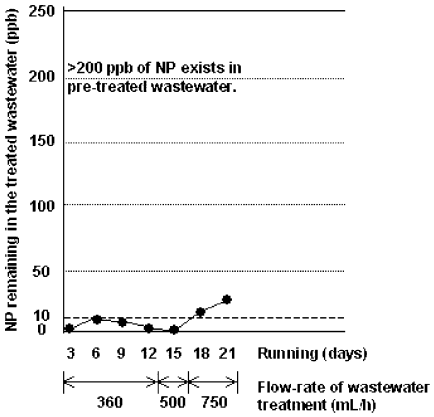

Fig. 4 Evaluation of the biotreatment system filled with 3 l of S-3^T beads. The performance of the system was improved by increasing the bed-volume of the beads

8, or 9 with HCl or NaOH before treatment. The system worked properly and NP in the treated wastewater was reduced to less than 10 ppb (Fig. 5) when the wastewater pH was 6 or 8. However, when the pH was 9, the system no longer degraded NP and NP leaked through the system (Fig. 5). Likewise, S-3^T did not form colonies on nutrient agar plates adjusted to pH 9 (data not shown), indicating that the treatment failed because S-3^T cannot tolerate such an extremely alkaline pH. In addition, NP begins to ionize to the phenolate anion at pH 9 and this polar species does not adsorb onto hydrophobic carriers. Thus, the treatment system constructed in this study might degrade NP in extremely alkaline industrial wastewater only after neutralization of the wastewater pH with acidic reagents.

We also immobilized S-3^T cells on KURAGEL polyvinyl alcohol carriers (Kurarei, Osaka, Japan) and compared their degrading activity in the system with that of the S-3^T beads. The polyvinyl alcohol beads were not as efficient as the S-3^T beads. S-3^T immobilized on KURAGEL suddenly lost their degrading activity on

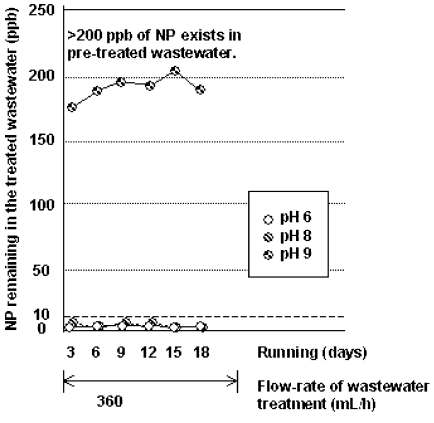

Fig. 5 Effect of wastewater pH on system performance. The wastewater pH was adjusted with 5 N HCl (for pH 6) or 5 N NaOH (for pH 8, pH 9) before the treatment. The flow-rate of the treatment was fixed at 360 ml/h

day 12 and more than 30 ppb of NP remained in the treated wastewater (Fig. 6). While the IT10-93 carrier is made of a hydrophobic, polypropylene polymer, the KURAGEL carrier is hydrophilic. Most EDCs, including NP, are hydrophobic. Thus, temporal adsorption of NP to the hydrophobic IT10-93 beads may explain the efficiency of the trapping and degrading capacity of the immobilized S-3^T cells. During a 9-day run with KURAGEL beads, NP adsorbed and accumulated on the carrier, causing a sudden, serious leakage of NP and functional loss of the treatment system (Fig. 6). Therefore, carriers made from hydrophobic polymers like IT10-93 are recommended.

Wastewater treatment using series-connected columns

When the system was run at a flow rate of 1.0 l/h, 200 ppb of NP were reduced to around 40 ppb (data not shown). Therefore, we examined the system′s performance in the treatment of 40 ppb NP in wastewater with a flow rate of 1.0 l/h. As a result, 40 ppb of NP were reduced to 14.5 ppb by day 3 and 8 ppb by day 6, suggesting that system performance can be improved easily by connecting the columns.

Bacterial biomass on the S-3^T beads

The bacterial biomass on the S-3^T beads (IT10-93) was monitored while the system ran. About 150 mg of protein per bead were measured just before the treatment began. The amount of protein decreased gradually to 30–40 mg/bead and remained stable. Since the surface area of one IT10-93 porous carrier was 600 mm^2 and the protein content in a living S-3^T cell was estimated by the Lowry method to be 10% (w/w), 50–67 mg of cells were immobilized in 1 cm^2. Table 1 shows a representative measurement from individual beads.

In summary, immobilized *S. cloacae* S-3^T cells degraded NP even at the low concentrations found in the environment. Furthermore, the cells degraded NP in

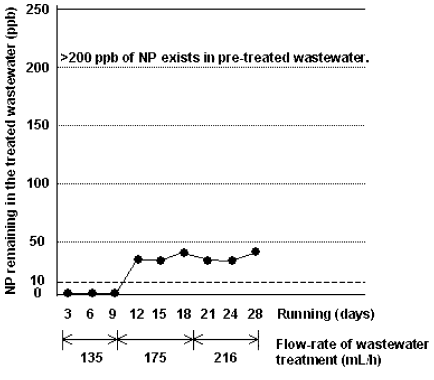

Fig. 6 Result of wastewater treatment using a column filled with 3 l of S-3^T immobilized on KURAGEL

Table 1 Bacterial biomass of *Sphingomonas cloacae* S-3^T on IT10-93 carrier beads, estimated by protein weight

Sampling stage	Protein bound (per IT10-93 carrier)
Day 0 (just after treatment system started running)	166 mg
Day 3	50 mg
Day 6	45 mg
Day 9	47 mg

actual industrial wastewater samples efficiently without the addition of nutrients. The wastewater treatment system removed >95% of NP at a concentration of 200 ppb, so that an ecologically safe concentration of less than 10 ppb of NP remained. The system performed well without the addition of nutrients or controlling the temperature of the wastewater over a range of pHs (6–8). Furthermore, the simple serial connection of columns improved the NP-degrading activity of the system during high-speed (1 l/h) treatment. The results of the present study clearly demonstrate the potential for pollutant-degraders like EDC-degrading bacteria to preserve aquatic environments via the treatment of industrial wastewater.

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