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Profile of a Nonylphenol-Degrading Microflora and Its Potential for Bioremedial Applications[1]

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Nonylphenol (NP) is an important intermediate in the production of various commercial and industrial materials, but is also known as a ubiquitous pollutant in urban aquatic environments. We recently studied the NP-degrading activities of microflora in several aquatic environments, and found a notable degrading activity for wastewater from a sewage treatment plant in Tokyo. This result led us to isolate NP-degrading microbes and identify biodegradation products. Using conventional plate culture techniques and molecular biological methods, *Pseudomonas* and *Sphingomonas* species, which are known for their degradation activities of many aromatic compounds, have been isolated. But it has also been found that *Sphingomonas* sp. (S-strain) is necessary and sufficient for the degradation of NP. Although the role of *Pseudomonas* sp. (P-strain) remains unclear, P-strain seems to provide some co-nutrients for the growth of S-strain. The degradation products were analyzed by GC/MS and NMR. More than 95% of NP was degraded within 10 days and aromatic compounds other than NP were not found, suggesting that the phenolic part of NP was completely degraded. We also examined the potential of S-strain for bioremedial applications. S-strain cells immobilized on chitosan or alginate beads retain their NP-degrading activity in flask-scale experiments. Furthermore, the chitosan-bound cells in a lab-scale bioreactor have been found to be persistent for repeated use, suggesting that S-strain is applicable to the treatment of NP-contaminated wastewater.

Key words: bacteria, bioreactor, bioremediation, endocrine disrupter chemicals, nonylphenol.

Nonylphenol (NP, Fig. 1) is a well-known, important intermediate in the production of many commercial and industrial materials. Above all, the major use of NP is found in the production of nonylphenol polyethoxylates (NPnEO), nonionic surfactants used widely in industrial applications.

However, NP is also known as a ubiquitous pollutant in urban aquatic environments with concentrations in the ppb (μg/liter) order, and is found in both sediment and surface water (*1–7*). There is growing evidence that NP has a certain estrogenic activity (*8–11*). Recent studies demonstrated that NP is highly toxic and accumulative in some aquatic organisms (*12–18*). For example, in mussels, the LD_{50}-values have been found to be 3.0 ppm (96 h), 500 ppb (360 h), and 140 ppb (850 h) (*12*). The bioconcentration factors in fish and mussels have been reported, respectively, as 1,300 and 3,400 (*13*). Exposure of female juvenile rainbow trout to NP (10–50 ppb) results in growth inhibition, and the ovosomatic index is significantly elevated in fish treated with 30 ppb of NP (*17*).

The main reason that NP is found in many aquatic environments is the microbial breakdown of discharged NPnEO. Although NPnEO is easily degraded to NP by some bacterial species (*19–23*), NP is a relatively stable chemical (*24, 25*). Only a few studies refer to the slow biodegradation of NP in natural environments (*26, 27*). Therefore, information on the microbial degradation of NP is limited and many questions remain to be answered for the construction of a treatment system for NP-contaminated wastewater. It is therefore necessary to extend our knowledge of the microbial degradation of NP in more detail for bioremedial applications.

In a previous study, we reported the NP-degrading activity of microflora in several aquatic environments, and a notable activity was observed in wastewater from a sewage treatment plant in Tokyo (*28*). In this paper, we report the microbial profile of the wastewater sample, its degradation products, and the potential of its bioremedial application.

MATERIALS AND METHODS

Chemicals—Yeast Nitrogen Base without amino acids (YNB), YPD broth, and Bacto agar were purchased from

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Difco Laboratories (Detroit, MI). NP (Cat.No. 28315-02) was obtained from Kanto Chemical (Tokyo). This reagent contains various structural isomers of NP through its branched nonyl-group. Other materials and chemicals were available from commercial sources.

Culture Conditions for NP-Degrading Bacteria—YNB (pH 7.0 at 25°C) with 0.1% (w/v), *i.e.* 1,000 ppm, of NP was used as the minimal culture medium (NP/YNB medium). YNB consists of $(NH_4)_2SO_4$ as the nitrogen source, other salts (KH_2PO_4, $MgSO_4$, NaCl, and $CaCl_2$), trace metals, and a very small amount of vitamins. No other carbon sources, as described in the Difco Manual, are present and thus NP is considered to be nearly the sole carbon source in the NP/YNB medium.

For liquid cultures, a single strain colony or mixed culture (consisting of microbes that have not been isolated from each other) from NP/YNB agar plates was selected with a sterile toothpick, and inoculated into fresh NP/YNB medium. The medium was subsequently incubated in a rotary shaker at a speed of 90 rpm at 25°C in the dark.

NP Degradation Assay—A reverse-phase HPLC system (TOSOH, Tokyo) with a Mightysil RP-18GP HPLC column (Kanto Chemical) was used for the NP biodegradation assay. Cultures (respectively, 0, 2, 4, 6, 8, and 10 days old, 15 ml per sample) were diluted with 60 ml of deionized water and mixed with 225 ml of acetonitrile. The mixture was subsequently filtered through a 0.2 μm Omnipore Membrane Filter (Millipore Corporation, Bedford, MA) and subjected to the HPLC system. As the mobile phase (flow-rate: 1.0 ml/min), a mixture of acetonitrile/water (75/25 vol%) was used. NP was detected by UV absorption at 277 nm.

Total Biomass of NP-Degrading Bacterium—A 10-day-old culture sample (30 ml) was centrifuged at 4,000 ×*g* for 10 min at 4°C. The precipitated bacterial cells were resuspended in 150 μl 0.85% NaCl and recovered into a weighing bottle. The suspension was then incubated at 105°C for 5 h to complete drying. The total biomass of the bacteria was measured using a AEG-120 electronic microbalance (Shimadzu, Tokyo).

Cloning and Phylogenic Analysis of Total 16S-Ribosomal DNAs (16S-rDNAs) of NP-Degrading Microflora—An outline of the 16S-rDNA cloning approach used for analyzing NP-degrading microflora is shown in Fig. 2. Mixed cultures (15 ml) were centrifuged at 4,000 ×*g* for 10 min at 4°C, and the bacterial cells were harvested. Total bacterial DNA was obtained by lysis with proteinase-K and sodium dodecyl sulfate, followed by phenol–chloroform extraction and ethanol precipitation. Subsequently, a partial region of the 16S-rDNA was amplified by PCR using *Taq* DNA polymerase (Takara Shuzo, Kyoto) and two general bacterial primers (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′; 530R, 5′-GTATTACCGCGGCTGCTGGC-3′) in an AB-1820 thermal cycler (ATTO Corporation, Tokyo). The thermal profile involved 25 cycles: 94°C for 60 s, 58°C for 60 s, and 72°C for 90 s, with a final polymerization at 72°C for 7 min. Using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA), the amplified PCR products and the pCR2.1 vector were ligated and the resulting plasmids were introduced into *Escherichia coli*. Transformed cells were chosen at random from an LB agar plate (to which 50 μg/ml ampicillin had been added), and the plasmids contained were independently recovered by the alkaline method. Each of the 16S-rDNA fragments inserted in the plasmids was analyzed using a model 373A DNA sequencer (Perkin-Elmer, Foster City, CA). The obtained nucleotide sequences were subjected to the similarity search program BLAST (Basic Local Alignment Search Tool), which can be found at http://www.ncbi.nlm.nih.gov/BLAST (*29*). The 16S-rDNA nucleotide sequences of known species were obtained from the Entrez nucleotide sequences database (http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html). Phylogenetic trees based on the 16S-rDNA sequences were thus constructed using the Clustal W program of the so-called neighbor-joining method (*30, 31*).

Gas Chromatography/Mass Spectrometry (GC/MS)—The identity of the biodegradation products was analyzed by GC/MS using a GC-17A gas chromatograph interfaced with a QP-5000 Mass Spectrometer (Shimadzu, Tokyo). The conditions were as follows: column, HiCap-CBP-20M fused silica column (20 m by 0.25 mm, Shimadzu); injection volume, 1 μl; carrier gas, helium (2 ml/min); temperature gradient, 50°C for 3 min, 50 to 150°C at 5°C/min, 150 to 220°C at 10°C/min, 220°C for 20 min; total run time was 50 min. The ionization energy and temperature for electron impact ionization were 70 eV and 260°C, respectively. The samples for analysis were extracted with hexane from 0 to 20-day-old cultures at intervals of 1 day, and the resulting supernatants (organic phase) were concentrated on a rotary evaporator. The gas phase of 20-day-old cultures in headspace bottles were also analyzed using a Carbowax/DVB-SPME unit (SUPELCO, Bellefonte, PA). Total ion chromatograms of each sample were monitored, and the degradation products were identified by analyzing their molecular weights and fragmentation patterns on mass spectra using a QP-5000 Mass Spectrometer.

NMR Analysis—NMR spectra were obtained with a JEOL GX-270 NMR spectrometer on sample solutions in perdeuterated solvent in 5-mm diameter tubes. ^{1}H-NMR spectra were recorded at 270 MHz. Spectral size was 37,000 points and acquisition time was 2 s.

Preparation of Chitosan- or Alginate-Bound S-Strain—S-strain was pre-cultured in 10 ml of Nutrient Broth (Eiken, Tokyo) at 25°C for 3 days. After cultivation, 1.0 ml of the pre-culture was added to two flasks containing 100 ml of Nutrient Broth (one for the preparation of free S-strain cells and the other for the preparation of alginate-encapsulated S-strain cells) and one flask containing 100 ml of Nutrient Broth and 12 ml of sterile chitosan beads (CHITOPEARL 5010-SH; Fuji Spinning, Tokyo) (for the preparation of chitosan-bound S-strain cells). The flasks were then cultivated for 3 days. For the preparation of free cells and alginate-encapsulated cells, cultured cells were harvested by centrifugation and suspended in 12 ml of YNB or 1.5% sodium alginate/YNB, respectively. The S-strain cell-alginate mixture was dropped and stirred for 30 min in 1.0% $CaCl_2$, and then washed with saline. For chitosan-bound cells, the chitosan beads were recovered from the culture medium using a tea strainer, and the cells were washed with saline. Three milliliters of free cell suspension or immobilized cells (chitosan or alginate) were added to flasks containing 30 ml of NP/YNB, "hypothetical" wastewater. Although we did our best to obtain "actual" NP-contaminated industrial wastewater from several companies, none would provide us with wastewater.

Construction of a Lab-Scale NP-Degrading Bioreactor—

An MBR-053-F aerobic bioreactor system (Tokyo Rikakikai, Tokyo) was used to construct an NP-degrading bioreactor. This system consists of a batch-type bioreactor unit (500 ml), an EPC-1000 process controller, a MAU-1 aeration unit, and a UC-55N cooling unit. In the system, the pH and temperature of the wastewater (NP/YNB) were adjusted to 7.0 and 25°C, respectively.

RESULTS AND DISCUSSION

Microbial Profile of NP-Degrading Microflora—We first examined whether yeast or fungi are present among the NP-degrading microflora by (i) microscopic analyses and (ii) growth studies on YPD agar, which is suitable for the growth of yeast. However, no yeast or fungi were found, suggesting that the microflora consist mainly of bacteria.

When the microflora were inoculated on Nutrient agar, many bacterial colonies emerged within 1-week. However, no isolate showed NP-degrading activity in NP/YNB medium. The NP used in our study is a mixture of various structural isomers through the highly branched nonyl-group (Fig. 1). Therefore, we assumed that several kinds of bacteria are involved cooperatively in the degradation of NP. Since the NP-degrading microflora degraded 1,000 ppm of NP almost completely in 10 days with stable degrading activity, the total 16S-rDNAs in 2, 7, and 10-day-old microflora samples were cloned (Fig. 2), and the obtained sequences were subjected to the BLAST similarity search program. Table I shows the results obtained from the analysis of 7-day-old microflora culture (Table I). The 45 randomly selected clones were divided into 2 groups (group-I and group-II in Table I). The clones in group-I were found to be highly homologous to the partial 16S-rDNA sequences of *Pseudomonas* species, and those in group-II were nearly identical to *Sphingomonas* species. Similar experiments with 2-day-old and 10-day-old microflora cultures were also performed, with similar results (data not shown). Phylogenic trees based on the 16S-rDNA sequences were constructed from known species and representative clones of each group, and the clones in group-I and group-II show a close phylogenic relationship with known *Pseudomonas* or *Sphingomonas* species, respectively (Fig. 3). Thus the NP-degrading microflora were found to comprise mainly *Pseudomonas* and *Sphingomonas* species. The genera *Pseudomonas* and *Sphingomonas* are known to include many species that utilize biodegradation-resistant compounds as carbon sources (*23, 32–48*).

Isolation of NP-Degrading Bacteria—An aliquot of a 7-day-old microflora culture was inoculated on a NP/YNB agar plate in order to isolate and separate not only the *Pseudomonas* sp. strains (P-strains) but also the *Sphingomonas* sp. strains (S-strains). Only P-strains could be isolated after 1 week of incubation. S-strains finally emerged as tiny colonies after 1 month of incubation. That is why NP-degrading colonies could not be isolated after 1 week of cultivation on Nutrient agar.

Random single colonies of P-strains and/or S-strains were inoculated into NP/YNB medium and cultivated for 7 days to examine whether they are necessary and sufficient for the degradation of NP. NP (1,000 ppm) was degraded by S-strain as well as by the mixed culture, while P-strain cells did not degrade NP (Fig. 4). This important result

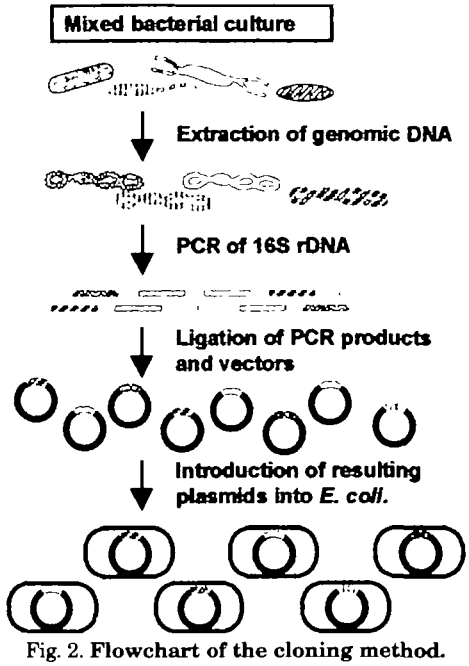

Fig. 2. **Flowchart of the cloning method.**

TABLE I. **Results of 16S-rDNA cloning and BLAST similarity search.**

Group	No. of clones	Results of BLAST similarity search
I	1–12, 15–19, 22–27, 29–31, 33, 35–37, 40–45 (36 clones)	Highly homologous (≧ 93%) to many *Pseudomonas* species.
II	13, 14, 20, 21, 28, 32, 34, 38, 39 (9 clones)	Highly homologous (≧ 95%) to many *Sphingomonas* species.

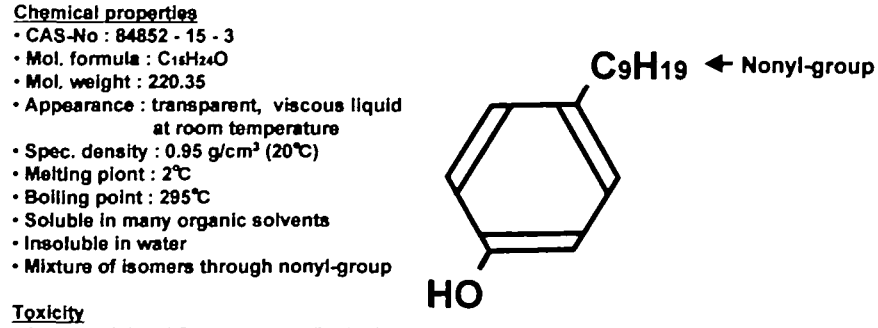

Fig. 1. **The chemical structure of NP.**

indicates that S-strains are necessary and also sufficient for the degradation of NP.

The NP-degrading activity of S-strain was examined in detail by inoculating a single colony into NP/YNB medium (Fig. 5). In independent assays, with an initial concentration of 1,000 ppm NP, NP began to be degraded within 2 days and degradation was almost complete within 10 days. The total biomass of S-strain in liquid culture was found to increase significantly after 10 days of cultivation (Fig. 6), indicating that NP is utilized as a sole carbon source. Since NP/YNB is a so-called minimal growth medium, the microbial growth is not remarkable. Thus it might be advantageous to apply S-strain in an NP-degrading bioreactor, since the disposal of surplus bacterial sludge would not be required so frequently during operation. We have found that S-strain is a novel species, and its phylogenetic data have been submitted to the *International Journal of Systematic and Evolutionary Microbiology*, an official journal of the International Committee of Systematic Bacteriology.

The role of P-strain in the NP-degrading pathway remains unclear. The NP-degrading activity of S-strain was not affected by P-strain as shown in Fig. 3. However, P-strain was isolated by way of enriching cultures in which NP was a sole carbon source. One possibility is that S-strain and P-strain form a symbiotic relationship. As described above, many *Pseudomonas* sp. can assimilate some aromatic compounds that are potential intermediary metabolites. On the other hand, some species of the genus *Sphingomonas* are known to require trace co-nutrients such as vitamins for growth (Eguchi, M., personal communication). Therefore, it seems that P-strain may provide some co-nutrients for the growth of S-strain and S-strain excretes intermediary metabolites in the NP-degrading pathway in exchange for the gift from P-strain. Since, as noted in "MATERIALS AND METHODS," trace vitamins were added to the YNB, it was not possible to observe the contribution of P-strain to the degradation of NP.

Degradation Products of NP—When the aromatic part of NP was monitored by UV absorption following HPLC as described, neither benzene, phenol, nor alkylphenols with short alkyl chains were detected in the cultures. Therefore, GC/MS was used to identify biodegradation products in both the culture medium and gas phase. It has been found that NP was degraded and that alcohols, mainly nonanol, were detected in 1-day to 20-day-old cultures. In the degradation of NP, alcohol peaks are found to increase day by day. Figure 7 and Table II show the total ion chromatogram of a 7-day-old culture and the identities of unique peaks, respectively. Alcohols were also found in the gas phase of the cultures (data not shown). Nonanols with different retention times were detected in both the culture medium and the gas phase, indicating that various structural isomers of nonanol through the branched hydrocarbon chain exist (Table II). Since the NP used in our study also contained various isomers through the branched nonyl-group, the results suggest that the alcohols are derived from the nonyl-group of NP. This conclusion may be controversial, because it conflicts with the biodegradation mechanism of nonylbenzene (*49*). However, that proposed mechanism was based on studies using nonylbenzene with a linear nonyl chain. Moreover, the observation that alkylphenols with shorter alkyl chains were not degraded by S-strain strongly suggests differences in the degradation mechanisms of NP and nonylbenzene.

Phenolic compounds are often highly toxic to many organisms. Furthermore, some have recently been found to be endocrine disrupter chemicals. Thus, it is very important

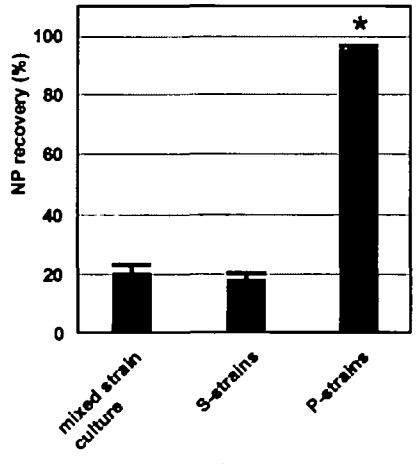

Fig. 4. **Degradation of NP by S-strain, P-strain, and mixed strain (S and P) cultures.** NP recovery is the percentage of NP remaining in solution. NP recovery in a 7-day-old pure culture of S-strain is shown relative to that of P-strain or mixed cultures, as means ± SEM (4 samples examined). $^{*}p < 0.01$ by Student's *t*-test.

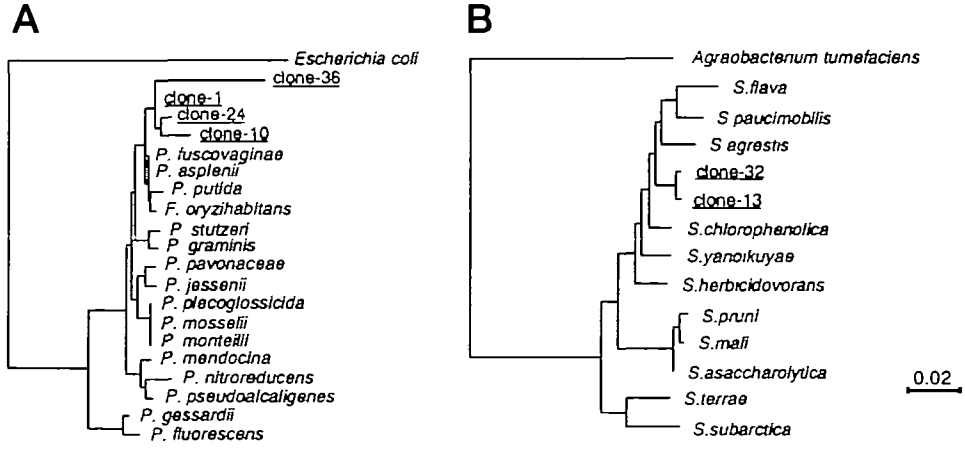

Fig. 3. **Phylogenic trees of NP-degrading microflora.** The phylogenic rooted trees for group I (A) and II (B) were constructed based on 16S-rDNA sequences from representative clones and known species. Scale bar = 2 nucleotide substitutions per 100 nucleotides.

to know whether the aromatic part of NP remains intact upon biodegradation. We could not detect any aromatic compounds other than NP by HPLC and GC/MS. We also analyzed the 20-day-old cultures using ^{1}H-NMR, but the signal for the aromatic ring was found to have disappeared almost completely (Fig. 8). Therefore, it is highly likely that the aromatic part of NP was degraded.

Construction of a Lab-Scale NP-Degrading Bioreactor System—Next, we examined the potential of S-strain to treat NP-contaminated wastewater. First we prepared chitosan- or alginate-bound S-strain and analyzed their NP-degrading activities in a flask-scale experiment, and the results are shown in Fig. 9. The chitosan-bound cells were found to degrade NP as efficiently as free cells. After 6 days of incubation, approximately 90% of the NP was degraded. After 9 days of incubation, NP was found to be degraded completely. Interestingly, the alginate-encapsulated cells were also found to degrade NP, although the time course of NP-degradation was not so steep as with free or chitosan-bound cells. The detailed mechanism of this phenomenon remains unknown, but is probably due to cell leakage from the surface of the Ca^{2+}-alginate beads due to abrasion.

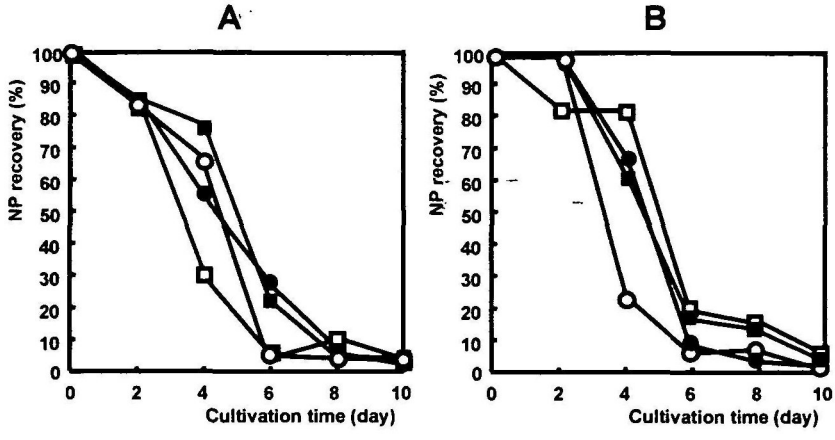

Fig. 5. **Typical time courses for the degradation of NP by mixed (A) or S-strain (B) cultures.** NP recovery is the percentage NP remaining in solution. Results of 4 independent experiments (○, ●, □, and ■) are shown.

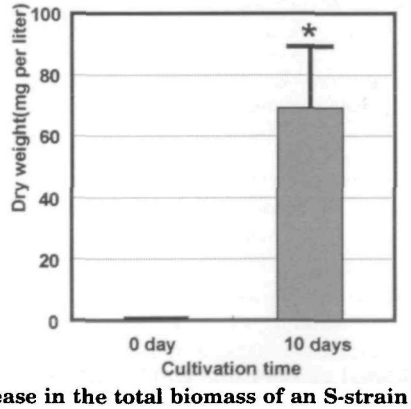

Fig. 6. **Increase in the total biomass of an S-strain after a 10-day cultivation.** The dry weights of the bacterial biomass of 10-day-old cultures are shown relative to those of a 0-day-old culture, as means ± SEM (4 samples examined). $^*p < 0.01$ by Student's *t*-test.

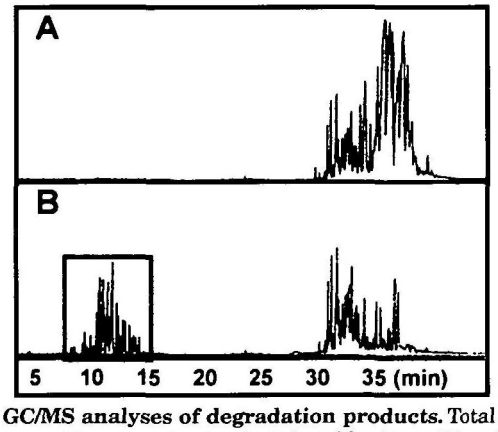

Fig. 7. ***GC/MS* analyses of degradation products.** Total ion chromatograms of hexane extracts of a 0-day-old culture (A) and a 7-day-old culture (B) are shown. Unique peaks in the 7-day-old culture are enclosed in a square and their identities are listed in Table II.

TABLE II. **Unique gas chromatograph-peaks in a 7-day-old culture and their identities.**

Time (min)	Identity	Time (min)	Identity
8.192	2,3,3-trimethyl-2-heptanol (octanol)	10.758	2-methyl-5-octanol (nonanol)
8.912	2-methyl-2-heptanol (octanol)	10.950	2,4-dimethyl-4-heptanol (nonanol)
9.083	2-methyl-2-octanol (nonanol)	11.042	2-methyl-2-octanol (nonanol)
9.308	2,4-dimethyl-3-heptanol (nonanol)	11.200	3-methyl-3-octanol (nonanol)
9.375	3-ethyl-2-methyl-2-pentanol (octanol)	11.300	3-methyl-3-octanol (nonanol)
9.658	2,4-dimethyl-4-heptanol (nonanol)	11.392	2,3-dimethyl-2-pentanol (heptanol)
9.900	2-methyl-2-octanol (nonanol)	11.908	3-methyl-3-octanol (nonanol)
9.950	2-methyl-2-octanol (nonanol)	12.067	2-methyl-2-octanol (nonanol)
10.150	2-methyl-2-octanol (nonanol)	13.708	2-methyl-2-octanol (nonanol)
10.342	3,5-dimethyl-3-heptanol (nonanol)	14.525	3-methyl-3-octanol (nonanol)
10.642	3,6-dimethyl-3-heptanol (nonanol)	14.783	3,6-dimethyl-3-heptanol (nonanol)

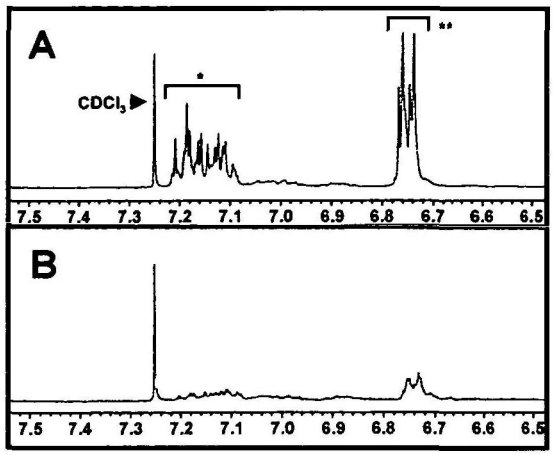

Fig. 8. **^{1}H-NMR spectra of a 0-day-old culture (A) and a 20-day-old culture (B) are shown.** Signals corresponding to the aromatic proton are indicated by "*" and "**". Unit = ppm.

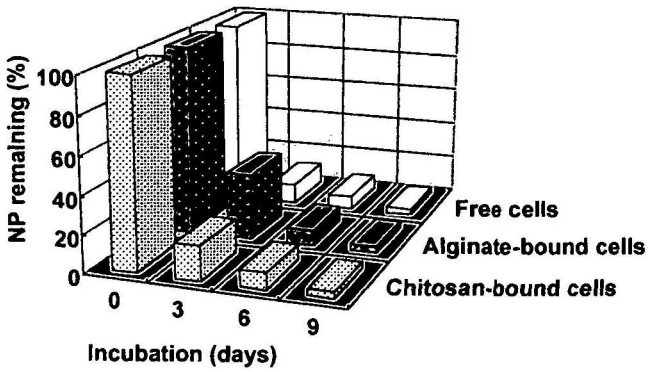

Fig. 9. **Degradation of NP by immobilized S-strain cells in flask-scale experiments.** The results are representative of three independent experiments.

From a practical point of view, however, the chitosan-bound cells are convenient for use because their preparation is easy, as described above. Moreover, chitosan, a marine waste product, is available at a low price.

Next, we tried to construct a lab-scale bioreactor using the chitosan-bound cells. Five days of operation of the bioreactor brought about the nearly complete (more than 95%) dissipation of NP (Fig. 10, A and B). Furthermore, the chitosan-bound cells were found to be persistent for at least five repeated uses (Fig. 10C). In our recent study, we found that S-strain can assimilate NP, while none of the other 50 carbon sources tested in the study was assimilated (Fujii *et al.*, submitted). This implies that S-strain can degrade NP even in heterogenous wastewater. Indeed we added glucose, sucrose, and maltose (final concentration of each; 0.1% w/v) as coexisting carbon sources to NP/YNB, but the degrading activity of the immobilized cells was not affected (Fig. 10D). We also examined the degradation products of NP. GC/MS and NMR analyses revealed that nonanol is the main degradation product and that no aromatic compounds other than NP were detected (Fujii *et al.*, submitted). Thus, wastewater treated by our bioreactor system seems to be safe for direct release into a sewer.

The results shown in this report suggest that an NP-

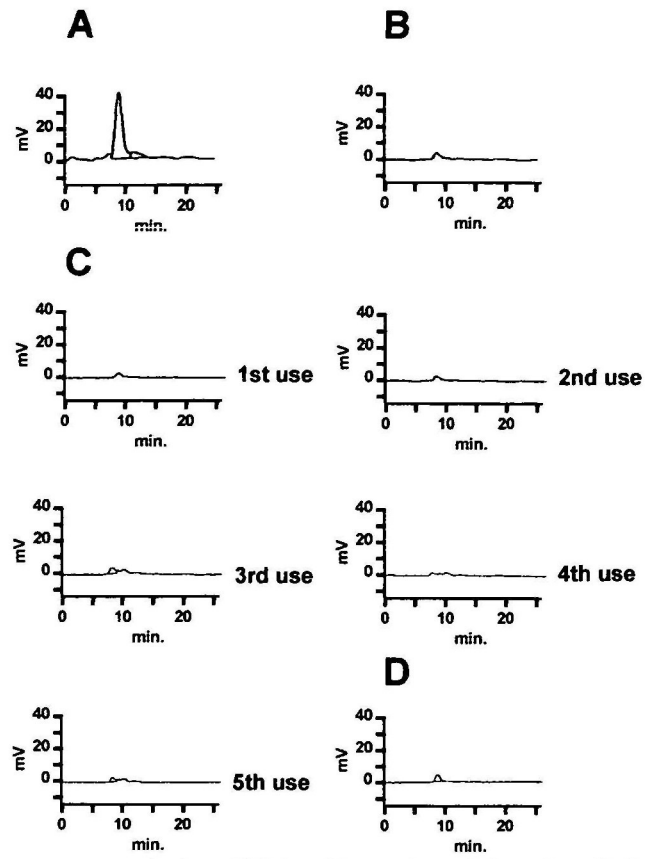

Fig. 10. **Degradation of NP by chitosan-bound S-strain cells in a bioreactor.** The HPLC profiles of NP in untreated and treated NP/YNB are shown in (A) and (B), respectively. The chitosan-bound cells could be used repeatedly at least five times for the treatment of NP/YNB (C). The degrading activity of chitosan-bound cells was not affected by the addition of sugars (glucose, sucrose, and maltose) (D).

degrading bioreactor system with chitosan-bound S-strain cells can be a useful tool for the treatment of NP-contaminated industrial wastewater. However, some problems remain for the construction of practical bioreactor systems. One is the difficulty in obtaining "actual" NP-contaminated industrial wastewater from industrial companies. However, the Law of Pollutant Release and Transfer Register (PRTR) was promulgated in 1999, and will be enforced within some years. Therefore, it will become easy to obtain and use actual wastewater for our studies in the near future.

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