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Characterization of Novel 4-*n*-Butylphenol-Degrading *Pseudomonas veronii* Strains Isolated from Rhizosphere of Giant Duckweed, *Spirodela polyrrhiza*

HOANG HAI[1], DAISUKE INOUE[1], NAONORI MOMOTANI[1], NING YU[1], TADASHI TOYAMA[2], KAZUNARI SEI[1], and MICHIHIKO IKE[1]*

[1]Division of Sustainable Energy and Environmental Engineering, Osaka University /2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[2]Division of Applied Sciences, Muroran Institute of Technology /27-1 Mizumoto, Muroran 050-8585, Japan

Abstract

Novel bacteria capable of utilizing 4-*n*-butylphenol (4-*n*-BP) as the sole carbon source were isolated from rhizosphere of giant duckweed, *Spirodela polyrrhiza*. These bacteria were identified as *Pseudomonas veronii*. One of the isolates, *P. veronii* designated strain nBP5, could completely degrade 4-*n*-BP up to 1.0 mM. The strain was capable of growing on 4-*n*-BP at temperature of 10-30 ℃, pH of 5-8 and NaCl concentration of 1.5 % or less, and the optimal temperature, pH and NaCl concentration were 25 ℃, 6.0 and 0.1 %, respectively. Specific 4-*n*-BP-degrading activity of strain nBP5 followed the Michaelis-Menten kinetics, with the maximal degradation rate of 0.258 mmol/mg-cell/h although the activity declined at ≥0.075 mM probably due to inhibitory effect of high substrate concentration. Based on the metabolites identified by GC-MS, the pathway of 4-*n*-BP degradation by strain nBP5 was proposed: 4-*n*-BP is initially hydroxylated to 4-*n*-butylcatechol, and subsequently 4-*n*-butylcatechol is metabolized via the *meta*-cleavage pathway. 4-*n*-BP-degrading enzyme of strain nBP5 was very effective to 4-*n*-alkylphenols (4-*n*-APs) with short and medium alkyl chain (C2-C5) and effective to 4-*n*-APs with long alkyl chains (C6-C9) although the degradation ratio declined with increase of length of alkyl chain.

Key words: 4-*n*-butylphenol (BP), 4-*n*-BP-degrading bacteria, *Spirodela polyrrhiza*, *Pseudomonas veronii*, *meta*-cleavage pathway, alkylphenols

INTRODUCTION

Alkylphenols (APs), a group of chemical compounds that consist of phenol with alkyl chain (C1 to C9), are ubiquitous pollutants in the aquatic environment. APs result from the partial biodegradation of nonionic surfactants, alkylphenol polyethoxylates, which are used worldwide in large quantities for both industrial and domestic uses[1] and the use as antioxidants and plasticizers[2,3]. APs exhibit acute and chronic toxicities[4] as well as estrogenic activity[5-8] to human and wild animals. Therefore, the environmental fate of APs merits close attention.

Biodegradation is considered as a major mechanism for eliminating APs in the aquatic environment. Microbial degradation of APs has been extensively studied, especially focusing on two major APs, nonylphenols (NPs) and octylphenols (OPs), and phylogenetically different APs-degrading bacteria

*Corresponding author

have been isolated from activated sludge and soil[9-17]. Despite such extensive studies on biodegradation of NPs and OPs, butylphenols (BPs)-degrading bacteria isolated so far were limited to those from activated sludge[18,19] or soil[20], and no BPs degrading bacterium has been isolated from the natural aquatic environment. Since BPs can exhibit acute and chronic toxicity and estrogenic activity[7], it is of great importance to further understand the fate of BPs via biodegradation in the aquatic environment.

In this study, we report the isolation and characterization of novel bacteria capable of utilizing 4-*n*-butylphenol (4-*n*-BP) as its sole carbon source. This is the first study describing 4-*n*-BP-degrading bacteria isolated from the aquatic environment. For the isolation of the BPs-degrading bacteria, first we constructed an enrichment culture of an aquatic plant, *Spirodela polyrrhiza*, and bacteria in its rhizosphere which is capable of 4-*t*-butylphenol (4-*t*-BP). The enrichment culture lost the 4-*t*-BP-degrading ability by eliminating *S. polyrrhiza*; however, it showed the 4-*n*-BP-degrading ability. Thus, we isolated 4-*n*-BP-degraging bacteria, and characterized the 4-*n*-BP-degrading ability of an isolate designated *Pseudomonas veronii* strain nBP5.

MATERIALS AND METHODS

Chemicals 4-ethylphenol (4-EP), 4-*n*-propylphenol (4-*n*-ProP), 4-*i*-ProP, 3-*i*-ProP, 2-*i*-ProP, 4-*s*-BP, 4-*t*-BP, 3-*t*-BP, 2-*s*-BP, 2-*t*-BP, 4-*n*-amylphenol (4-*n*-AmyP), 4-*t*-AmyP, 4-*n*-hexylphenol (4-*n*-HexP), 4-*n*-heptylphenol (4-*n*-HepP), 4-*t*-octylphenol (4-*t*-OP), technical nonylphenol (t-NP) and BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 4-*n*-NP was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-*n*-ProP and 4-*n*-OP were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of highest grade commercially available.

Aquatic plant The sterile *S. polyrrhiza* was maintained in 300 m*l* Erlenmeyer flasks containing 200 m*l* of sterile modified Hoagland nutrient solution until used for experiments[21]. They were statically grown in an incubation chamber at 28±1 ℃ under fluorescent lamp at 8000 lux (16 h of light and 8 h of dark condition).

Culture media Mineral salts medium (MSM), which was used for enrichment and isolation of 4-*t*-BP and 4-*n*-BP degrading bacteria from aquatic plant rhizosphere and biodegradation tests of APs, contained (per liter of water) 1.0 g of $(NH_4)_2SO_4$, 1.0 g of K_2HPO_4, 0.2 g of NaH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of NaCl, 0.05 g of $CaCl_2$, 8.3 mg of $FeCl_3 \cdot 6H_2O$, 1.4 mg of $MnCl_2 \cdot 4H_2O$, 1.17 mg of $NaMoO_4 \cdot 2H_2O$, and 1 mg of $ZnCl_2$. Unless otherwise noted, the pH of MSM was adjusted to 7.2. The isolates were routinely maintained on MSM containing 1.0 mM 4-*n*-BP as the sole carbon source. Solid agar medium was prepared with 20 g of agar per liter.

4-*t*-BP degradation tests in river water microcosms with and without *S. polyrrhiza* A river water sample was collected from the subsurface zone (30- to 50-cm depth) of Kita River (Fukui, Japan). The quality of the river water sample was as follows: pH, 7.3; dissolved oxygen, 7.3 mg/*l*; total nitrogen, 2.5 mg/*l*; total phosphorus, 0.18 mg/*l*; heterotrophic bacteria, 1.3×10^5 CFU/m*l*. The water sample after membrane filteration (pore size 10.0 μm; Millipore, Tokyo, Japan) was used for 4-*t*-BP degradation experiments.

Degradation tests of 4-*t*-BP were conducted in the river water microcosms with and without 20 fronds of *S. polyrrhiza*. Control microcosm made up of sterilized river water sample was also prepared. All microcosms were constructed using 100 m*l* of river water sample in 300 m*l* Erlenmeyer flasks. 4-*t*-BP was added to give a final concentration of 0.03 mM. Microcosms were statically incubated at 28℃ under fluorescent lamp at 8000 lux (16 h light and 8 h dark condition) for 3 days × 7 cycles (test microcosms A and B) or 7 days × 1 cycle (control microcosm). Aliquot (1 m*l*) of each microcosm was periodically sampled and subjected to high-performance liquid chromatography (HPLC) analysis to measure the concentration of 4-*t*-BP.

Enrichment, isolation and identification of 4-*n*-butylphenol degrading bacteria First degradation tests revealed that 4-*t*-BP was

degraded only in the presence of *S. polyrrhiza* (test microcosm A). Thus, enrichment of 4-*t*-BP and 4-*n*-BP degrading bacteria was carried out using the test microcosm A. The *S. polyrrhiza* roots and 10 % of the river water from the 7th cycle of test microcosm A were inoculated in 300 m*l* Erlenmeyer flasks containing 100 m*l* of MSM supplemented with 0.2 mM of 4-*t*-BP or 4-*n*-BP. The enrichment culture was incubated at 28 ℃ on a rotary shaker at 120 rpm. After degradation of BP was confirmed, 10 m*l* of the culture was transferred to fresh MSM. In the second, third and forth subculturings, BP was added at 0.2, 0.3 and 0.5 mM, respectively. Because no obvious degradation was observed for 4-*t*-BP in the first enrichment culture, these subculturings were carried out only for 4-*n*-BP.

The final subculture was serially diluted and spread onto MSM plates amended with 1.0 mM of 4-*n*-BP. The plates were incubated at 28 ℃, and morphologically different colonies were screened for their ability to degrade 4-*n*-BP in an axenic culture. Consequently, three bacterial strains, designated strains nBP2, nBP3 and nBP5, were isolated as 4-*n*-BP-degrading bacteria. The isolated bacterial strains were characterized and identified by physiologic and phylogenetic analyses as described previously[22].

Biodegradation assays using isolated 4-*n*-BP-degrading bacteria Biodegradation assays were conducted using the growing cells and whole cells. Unless otherwise indicated, cultivation was carried out on a rotary shaker (120 rpm) at 28 ℃. Cells of isolated 4-*n*-BP-degrading bacteria were grown to the late logarithmic phase in MSM containing 1.0 mM of 4-*n*-BP as the sole carbon source. The cells were harvested by centrifugation (9,600×*g*, 4 ℃, 10 min) and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The cells were added to the turbidity, that is, the optical density at 600 nm (OD_{600}), of 0.01 (for the growing cell assays) or 0.5 (for whole cell assays) to MSM containing 1.0 mM of 4-*n*-BP or one of the following compounds as the sole carbon source: 4-EP, 4-*n*-ProP, 4-*i*-ProP, 3-*i*-ProP, 2-*n*-ProP, 2-*i*-ProP, 4-*s*-BP, 4-*t*-BP, 3-*t*-BP, 2-*s*-BP, 2-*t*-BP, 4-*n*-AmyP, 4-*t*-AmyP, 4-*n*-HexP, 4-*n*-HepP, 4-*n*-OP, 4-*t*-OP, 4-*n*-NP or t-NP. To examine the effects of temperature, pH and salinity on the growth of isolated 4-*n*-BP-degrading bacteria, conditions in the degradation assays were changed as follows: temperature was set at 10, 15, 20, 25, 30, 35 and 40 ℃; pH of MSM was adjusted to 4-8; NaCl concentration was adjusted to 0.1, 0.5, 1, 1.5, 2 and 3 % (w/v). Effect of 4-*n*-BP concentratin on the 4-*n*-BP degradation activity was examined using the whole cells at 4-*n*-BP concentrations of 0.001 to 1.0 mM. At appropriate intervals during the degradation assays, the cell density and the concentrations of substrate were measured. All tests were performed in triplicate.

Analytical procedures Bacterial growth was monitored as the increase in OD_{600} of culture with a plate reader (Viento XS, Dainippon Sumitomo Pharma, Osaka, Japan). Concentration of substrates was determined by HPLC, and metabolites of 4-*n*-BP were analyzed by gas chromatography-mass spectrometry (GC-MS). For HPLC, the collected culture was acidified with phosphoric acid and centrifuged (9,600×*g*, 4 ℃, 10 min); the supernatant was then used. HPLC analysis was conducted using a Shimadzu LC-10A*vp* HPLC system consisting of an SIL-10AF automatic sampler, LC10AD*vp* solvent delivery units, a CTO-10A*vp* column oven, and an SPD-10A*vp* UV/VIS detector (Shimadzu, Kyoto, Japan). The instrument was equipped with a Shim-pack VP-ODS column (4.6×250 mm; particle size, 5 μm; Shimadzu). Acetonitrile and water at a ratio of 8:2 was used as the mobile phase. The detection was carried out at 277 nm. For GC-MS analysis of metabolites produced by 4-*n*-BP biodegradation, the collected culture was acidified with 1 N HCl, shaken for 3 min with an equal volume of 1:1 (vol/vol) ethyl acetate/*n*-hexane and centrifuged (9,600×*g*, 4 ℃, 10 min); the organic layer was then extracted. The extract was dried under nitrogen flow and trimethylsilylated (TMS) by a BSTFA-acetonitrile solution at 60 ℃ for 1 h. The GC-MS analysis was conducted using a Shimadzu GC-MS system (GCMS-QP2010; Shimadzu) and a Rxi-5ms capillary column (30 m, 0.25 mm ID, 1.00 μm df;

Nonpolar 5 % diphenyl 95 % dimetylpolysiloxane phase; Restek, Bellefonte, PA, USA). Samples were injected in splitless mode for 1 min. Helium gas (99.9999 %) was used as the carrier gas at a flow rate of 1.2 m*l*/min. The oven temperature programs were as follows: 60 ℃ isothermal for 2 min, 60 to 300 ℃ at 20 ℃/min and 300 ℃ hold for 6 min. The MS analysis was performed at 70 eV.

Nucleotide sequence accession numbers The partial 16S rRNA gene sequences of strains nBP2, nBP3 and nBP5 were registered in GenBank/EMBL/DDBJ as accession numbers AB494443, AB494444 and AB494445, respectively.

RESULTS

Accelerated degradation of 4-*t*-BP in the presence of *S. polyrrhiza* Seven cycle-batch degradation experiments of 4-*t*-BP in river water microcosms with and without *S. polyrrhiza* revealed that approximately 20% of the amended 4-*t*-BP was degraded in the presence of *S. polyrrhiza*, while no significant decrease of 4-*t*-BP was observed in microcosms without *S. polyrrhiza* (Fig. 1). This suggests that 4-*t*-BP can be acceleratedly degraded in the presence of *S. polyrrhiza* possibly due to the selective accumulation of organic pollutants degrading bacteria in the rhizosphere of *S. polyrrhiza*, as observed in our previous studies[21,23]. Such selective accumulation of specific bacteria in the *S. polyrrhiza* rhizosphere would be attributable to the rhizosphere effects such as transportation of oxygen and secretion of physiologically active root exudates.

Enrichment, isolation and identification of BP-degrading bacteria To enrich BP-degrading bacteria from the microcosm with *S. polyrrhiza* where significant 4-*t*-BP degradation was observed, roots of *S. polyrrhiza* and 10 m*l* of liquid were transferred to MSM supplemented with 0.2 mM of 4-*t*-BP or 4-*n*-BP at the end of the 7th cycle of degradation experiment. In the enrichment culture, no obvious reduction of 4-*t*-BP was detected, although 4-*n*-BP decreased rapidly and disappeared within 48 h (Fig. 2). The results suggested that the 4-*t*-BP degrading ability of our test system

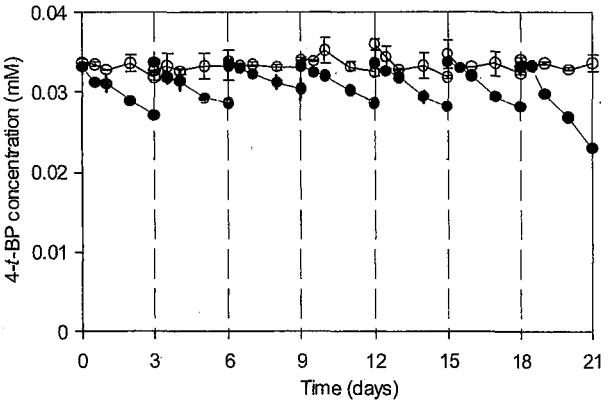

Fig. 1 4-*t*-BP degradation profile in river water microcosms with (closed circle) and without (open circle) *S. polyrrhiza*. Experiments were carried out for 3 days × 7 cycles. Error bars represent 95 % confidence intervals.

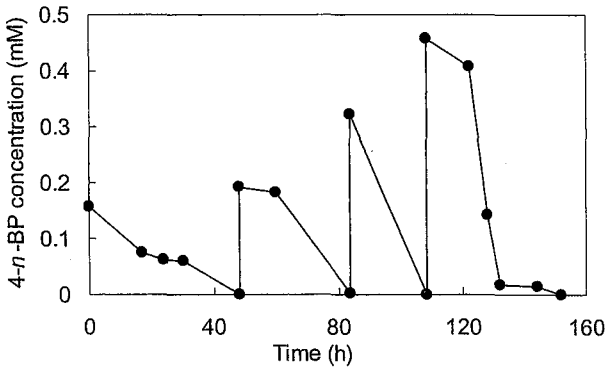

Fig. 2 4-*n*-BP degradation profile in enrichment culture. 4-*n*-BP concentration in the 1st, 2nd, 3rd and 4th subculturings were 0.2, 0.2, 0.3 and 0.5 mM, respectively.

was largely dependent on the presence of *S. polyrrhiza* and thus the enrichment lost the 4-*t*-BP-degrading ability by eliminating *S. polyrrhiza*. Therefore, we tried to obtain pure cultures of 4-*n*-BP-degrading bacteria. Enrichment by successive transfers to fresh MSM containing 4-*n*-BP at 0.2, 0.3 and 0.5 mM showed that 4-*n*-BP was completely degraded without lag period (Fig. 2). Finally, three morphologically different colonies that were capable of utilizing 4-*n*-BP as the sole carbon source were isolated and designated strains nBP2, nBP3 and nBP5.

Three isolated strains nBP2, nBP3 and nBP5 were gram-negative, rod-shaped, motile, oxidase- and catalase-positive bacteria. Phylogenetic analysis based on partial 16S rRNA gene sequence showed that the

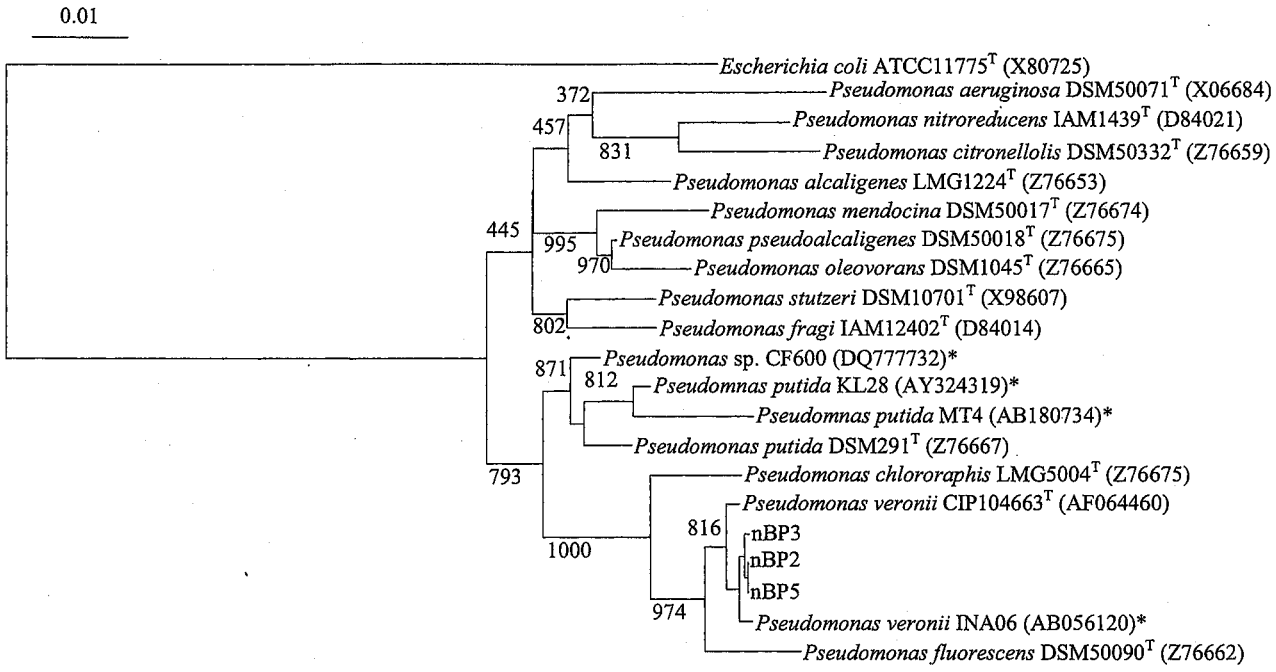

Fig. 3 A phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene sequences of strains nBP2, nBP3 and nBP5, type strains of *Pseudomonas* and *Escherichia coli* and 4-APs-degrading bacterial strains. 4-APs-degrading strains are marked with asterisks. Numbers on the branches indicate bootstrap confidence estimates obtained with 1000 replicates. The scale bar represents evolutionary distance of 0.01.

three bacteria had the greatest 16S rRNA gene similarity with *Pseudomonas veronii* CIP104663^T (99.8%; Fig. 3). Based on the result, we identified the three strains as *P. veronii*.

4-*n*-BP degradation by strain nBP5 Strain nBP5 was selected for further studies because of its highest 4-*n*-BP degrading ability among the three isolates. A typical time course of cell growth and 4-*n*-BP degradation of strain nBP5 at an initial 4-*n*-BP concentration of 0.5 mM is shown in Fig. 4. After preincubation on 4-*n*-BP, strain nBP5 completely degraded 0.5 mM of 4-*n*-BP within 5 h without any lag period. The turbidity of the culture (OD_{600}) increased from 0.03 to 0.15 concomitantly with the degradation of 4-*n*-BP.

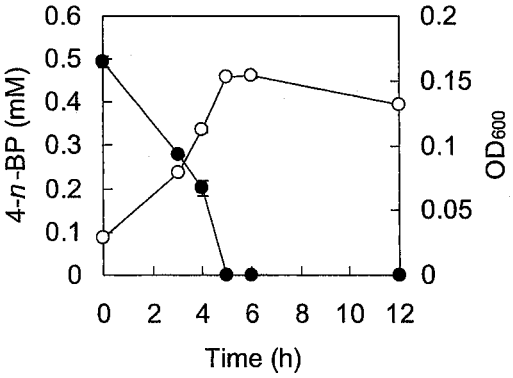

Fig. 4 Typical time course of 4-*n*-BP degradation (closed circle) and cell growth (open circle) of strain nBP5 preincubated on 4-*n*-BP. Error bars indicate the standard deviation of triplicate experiments

Effects of temperature, pH and salinity (NaCl concentration) on the growth of strain nBP5 in MSM containing 4-*n*-BP (1.0 mM) as the sole carbon source were investigated. The specific growth rate was calculated by deviding the increment of OD_{600} by time on logarithmic growth phase, and the specific growth rate under a condition was calculated as the mean in triplicate experiments. Specific growth rate of strain nBP5 under different conditions are presented in Fig. 5. Strain nBP5 was capable of growing on 4-*n*-BP at temperature of 10-30 ℃, pH of 5-8 and NaCl concentration of 1.5 % or less. The optimal temperature, pH and NaCl concentration for growth of strain nBP5 on 4-*n*-BP were 25 ℃, 6.0 and 0.1 %, respectively.

Effect of substrate (4-*n*-BP) concentration

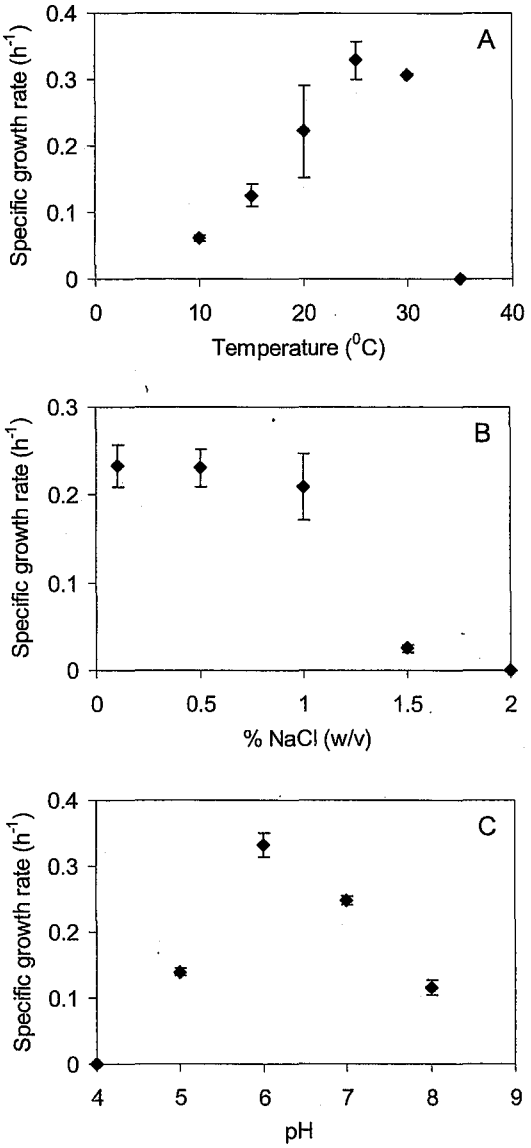

Fig. 5 Effect of temperature (A), salinity (B) and pH (C) on specific growth rate of strain nBP5 grown on 4-*n*-BP as the sole carbon source. Error bars indicate the standard deviation of triplicate experiments.

on the 4-*n*-BP degradation by strain nBP5 was examined by whole-cell assays (Fig. 6). Experiments were carried out at initial 4-*n*-BP concentrations of 0.001-1.0 mM. Strain nBP5 completely degraded 1.0 mM or less of 4-*n*-BP. The 4-*n*-BP degradation rate increased with increasing substrate concentration between 0.001 and 0.05 mM, and reached the highest value of 0.246 mmol/mg-cell/h at 0.05 mM. However, the degradation rate declined at 4-*n*-BP concentration of 0.075 mM or higher. The degradation rate at 1.0 mM was only 0.007 mmol/mg-cell/h

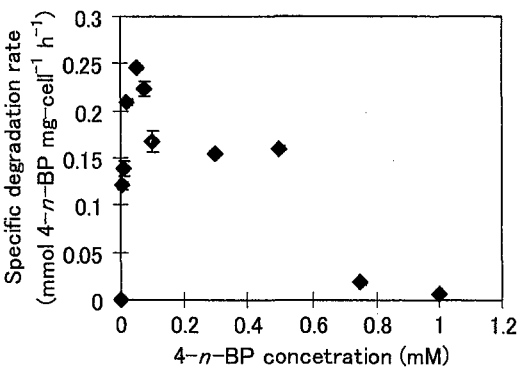

Fig. 6 Effect of initial 4-*n*-BP concentration on specific degradation rate of 4-*n*-BP by strain nBP5. Error bars indicate the standard deviation of triplicate experiments.

(2.8 % of the highest rate). These results suggested that 4-*n*-BP concentrations more than 0.075 mM were inhibitory to the 4-*n*-BP degradation by strain nBP5.

Experimental data were further analyzed to elucidate the degradation and substrate inhibition kinetics of 4-*n*-BP degradation by strain nBP5. For less than 0.075 mM of 4-*n*-BP, substrate inhibition was negligible, and the 4-*n*-BP degradation patterns could be represented by the Michaelis-Menten equation:

$$V = \frac{V_{max}S}{K_m + S}$$

where V and V_{max} are the actual and maximum degradation rate of 4-*n*-BP degradation (mmol/mg-cell/h), S is the initial 4-*n*-BP concentration (mM) and K_m is the saturation constant (mM). From the experimental results, V_{max} and K_m were estimated to be 0.258 mmol/mg-cell/h and 0.0062 mM, respectively.

Identification of metabolites and 4-*n*-BP degradation pathway During the 4-*n*-BP degradation by strain nBP5, no peaks responsible for metabolites were detected in HPLC-UV/VIS analysis. However, in GC-MS analysis of the ethyl acetate/*n*-hexane extracts from the cultures during the 4-*n*-BP degradation by strain nBP5, several peaks responsible for the metabolites were detected in addition to the parental compound. By interpreting the mass spectral pattern, five compounds including 4-*n*-butylcatechol (Fig. 7A) and its *meta*-cleavage products (Figs. 7B

similar to that of these strains.

Strain nBP5 isolated in this study completely degraded 4-*n*-APs with short and medium alkyl chains (C2-C5) within 72 h under the inductive condition. This strain also degraded 4-*n*-APs with long alkyl chains (C6-C9) although the degradation ratio declined with increase of length of alkyl chain. In addition, 4-*n*-BP-degrading enzyme of strain nBP5 was effective (>60 % of degradation ratio within 72 h) to 3- and 4-*i*-ProPs, but not to other branched APs and *ortho*- and *meta*-substituted APs. Previous studies showed that *Pseudomonas* sp. strain KL28, *P. veronii* strain INA06 and *P. putida* strain MT4 could degrade 4-*n*-APs with alkyl chain of C1-C5[20], C3-C6[18] and C1-C4[19], respectively. Thus, the range of degradable 4-*n*-APs by our strain nBP5 appeared to be wider than that for other 4-*n*-BP-degrading bacterial strains.

Although there have been only a few precedents on the isolation of BP-degrading bacteria, we could isolate novel 4-*n*-BP-degrading bacterial strains with degradative ability to a relatively wide range of 4-*n*-APs from the aquatic environment using *S. polyrrhiza* relatively easily. Rhizosphere of *S. polyrrhiza* would be attractive as a promising source of useful bacteria for the treatment of wastewater containing recalcitrant compounds and the remediation of natural aquatic environment contaminated with recalcitrant compounds. Considering the versatile APs-degrading ability, 4-*n*-BP-degrading bacteria isolated here can be applied for the treatment of industrial wastewaters containing complex mixture of APs. Furthermore, from the results obtained in this study and our previous studies[21,23], aquatic plant (*S. polyrrhiza*)-bacterial associations seem effective for the accelerated degradation of various recalcitrant compounds, particularly aromatic compounds. Thus, although aquatic plants treatment system has been mainly used for the removal of nutrients to prevent eutrophication in the aquatic environment so far, it may be also applied for the finishing process to remove recalcitrant compounds that cannot be removed in the secondary treatment in wastewater treatment plants. Further study on the treatment system utilizing *S. polyrrhiza* is desirable for the development of a novel contamination control system especially in developing countries in the tropical region as an alternative to high-cost physical/chemical treatment technologies.

CONCLUSION

Novel bacteria capable of utilizing 4-*n*-BP as the sole carbon source were isolated from the rhizosphere of *S. polyrrhiza* and characterized. This is the first study describing 4-*n*-BP-degrading bacteria isolated from the aquatic environment. Similar to other 4-*n*-BP degrading bacteria reported previously, strain nBP5 metabolizes 4-*n*-BP via the *meta*-cleavage pathway. Strain nBP5 completely degraded 4-*n*-BP up to 1.0 mM although some inhibitory effect occurred at 0.075mM or higher of 4-*n*-BP. This strain was capable of degrading a wider range of 4-*n*-APs than other 4-*n*-BP-degrading bacteria. Therefore, our strain nBP5 can be used in bioremediation of aquatic environment contaminated with various 4-*n*-APs.

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