Biodegradation of bisphenol A and 4-alkylphenols by *Novosphingobium* sp. strain TYA-1 and its potential for treatment of polluted water

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ABSTRACT

We investigated the use of *Novosphingobium* sp. strain TYA-1 for the simultaneous removal of bisphenol A (BPA) and 4-alkylphenols (4-APs) from complex polluted waters. Strain TYA-1 degraded BPA and utilized it as a sole carbon and energy source via oxidative skeletal rearrangement involving the cytochrome p450 monooxygenase system. Strain TYA-1 also degraded 4-APs with branched side alkyl chains (4-tert-butylphenol [4-tert-BP], 4-sec-butylphenol, 4-tert-pentylphenol, 4-tert-octylphenol [4-tert-OP], and branched nonylphenol mixture) via 4-alkylcatechols but could not degrade 4-APs with linear side alkyl chains. Degradation of 4-APs, like that of BPA, involved the cytochrome p450 monooxygenase system in strain TYA-1. A sequencing batch bioreactor (100 mL of polluted water [50 mg/L BPA, 50 mg/L 4-tert-BP, and 5 mg/L 4-tert-OP]; 6 h of reaction time/cycle; 12 cycles in total) containing alginate-immobilized TYA-1 cells (15 mg dry cells) simultaneously removed BPA, 4-tert-BP, and 4-tert-OP from complex polluted waters. These immobilized TYA-1 cells could be reused for a total of 9 cycles without any loss of degradation activity. Our results support the potential of using immobilized TYA-1 cells for the simultaneous removal of BPA and 4-APs from complex polluted waters.

Key words | alkylphenol, biodegradation, bisphenol A, immobilized cells, *Novosphingobium* sp. TYA-1, polluted water treatment

INTRODUCTION

Bisphenol A (BPA; 2,2-bis[4-hydroxyphenyl]propane) is a high-production-volume chemical widely used in the generation of polycarbonate plastics, epoxy resins, flame retardants, and lacquer coatings on food cans (Charles et al. 1998). The widespread use of BPA has caused it to be released into aquatic environments via municipal and industrial wastewater treatment effluent and landfill leachate and from the natural degradation of polycarbonate plastics (Charles et al. 1998; Crain et al. 2007). Indeed, BPA is found frequently in various aquatic environments, including river waters, seawaters, sewages, wastewater treatment plant effluents, wastewater sludge samples, and landfill leachates (Charles et al. 1998; Crain et al. 2007). BPA is a known endocrine-disrupting chemical and exhibits other toxic effects on aquatic organisms and humans (Crain et al. 2007). Therefore, it is necessary to establish technologies to treat BPA-polluted waters and to remove BPA from contaminated aquatic environments.

Biodegradation plays a key role in the removal of BPA from aquatic environments. Several BPA-degrading bacteria have been isolated, including the Gram-negative bacterial strains MV1 (Lobos et al. 1992), *Sphingomonas paucimobilis* FJ4 (Ike et al. 1995), and *S. bisphenolicum* AO1 (Oshiman et al. 2007). These bacteria degrade BPA by major and minor pathways involving the oxidative skeletal rearrangement of an aliphatic methyl group in the BPA molecule (Spivack et al. 1994). Biodegradation of BPA has been well studied in regard to the degradation mechanisms and environmental fate of BPA, but the potential of applying BPA-degrading bacteria in bioreactor systems to treat BPA-polluted waters has scarcely been addressed.

Like BPA, 4-alkylphenols (4-APs) – including 4-nonylphenol (4-NP), 4-tert-octylphenol (4-tert-OP) and 4-tert-butylphenol (4-tert-BP) – are well known as serious endocrine-disrupting pollutants (Ying et al. 2002). Often,
BPA and 4-APs are detected concurrently in surface waters, sediments, and effluents from sewage and wastewater treatment plants (Heemken et al. 2001; Ko et al. 2007; Jonkers et al. 2010). Therefore, a method for simultaneously removing BPA and 4-APs from complex polluted waters is an attractive tool.

In this study, we aimed to remove BPA and 4-APs simultaneously from complex polluted waters by using *Novosphingobium* sp. strain TYA-1. Strain TYA-1 has recently been isolated from the rhizosphere of *Phragmites australis* (common reed) and can utilize BPA for cell growth as a sole carbon and energy source via oxidative skeletal rearrangement (Toyama et al. 2009). First, we assessed the BPA-degrading enzyme in strain TYA-1. Previous studies have demonstrated that cytochrome p450 monooxygenase system in strain AO1 is involved in the initial hydroxylation of BPA biodegradation (Sasaki et al. 2005). Here we confirmed the involvement of a cytochrome p450 monooxygenase in BPA degradation by strain TYA-1. Second, we examined the ability of strain TYA-1 to degrade various 4-APs. Third, we conducted flask-scale sequencing batch bioreactor (SBR) experiments to simultaneously remove BPA, 4-tert-BP, and 4-tert-OP by using TYA-1 cells immobilized on alginate beads. Because BPA, 4-tert-BP (a typical branched 4-AP with a short alkyl side chain), and 4-tert-OP (a typical branched 4-AP with a long alkyl side chain) are detected concurrently in various aquatic environments, we combined these three compounds to use as the complex pollutant in this study.

**METHODS**

**Chemicals**

BPA was purchased from Kanto Chemical Industry (Tokyo, Japan). Metyrapone was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-n-BP, 4-sec-BP, 4-tert-BP, 4-n-pentylphenol, (4-n-PenP), 4-tert-PenP, 4-n-OP, 4-tert-OP, 4-n-NP, and technical NP (tNP; a mixture of branched NP isomers) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

**Bacterium and culture media**

*Novosphingobium* sp. strain TYA-1 and basal salts medium (BSM; pH 7.2) (Toyama et al. 2010) were used in this study. BSM containing 0.5 mM BPA (BPA–BSM) as the sole carbon source and BPA–BSM supplemented with peptone (0.5 g/L) and yeast extract (0.25 g/L) (BPA–PY–BSM) were both used to culture strain TYA-1.

**Environmental water**

The sample of environmental water used in the SBR experiments was collected from the Fuefuki River (Kofu, Yamanashi, Japan). The water quality of the sample was: pH 7.3; 9.2 mg/L dissolved O₂; 0.06 mg/L ammonium-N; below the detection limit nitrite-N; 1.22 mg/L nitrate-N; 0.11 mg/L phosphate-P; and 12.6 mg/L total dissolved organic carbon. The bacterial count was measured by using 0.1× LB plates (1.0 g/L Bacto peptone, 0.5 g/L yeast extract, 1.0 g/L NaCl; pH 7.0; 2.0% agar) and yielded 5.6×10⁴ colony-forming units/mL total heterotrophic bacteria. The water sample was stored at 4 °C until use.

**BPA degradation experiments in the presence and absence of metyrapone**

To assess the involvement of cytochrome p450 monooxygenase in the biodegradation of BPA by strain TYA-1, BPA degradation experiments were conducted in the presence and absence of metyrapone, a specific inhibitor of cytochrome p450 monooxygenase (Sasaki et al. 2005). Strain TYA-1 was grown overnight in BPA–BSM. Cells were harvested by centrifugation (9,600×g at 4 °C for 10 min), washed twice with BSM, and inoculated to a cell density (as determined by the optical density at a wavelength of 600 nm [OD₆₀₀] of 0.05 (i.e. OD₆₀₀ = 0.05) into 100 mL BPA–BSM. Cells also were inoculated at OD₆₀₀ = 0.05 into 100 mL BPA–BSM supplemented with 0.2 mM metyrapone. The BPA degradation reaction flasks were incubated at 28 °C and 120 rpm in triplicate. Cell density and BPA concentration were monitored over the 12-h experimental period.

**Growth on and degradation of 4-APs**

Nine 4-APs (4-n-BP, 4-sec-BP, 4-tert-BP, 4-n-PenP, 4-tert-PenP, 4-n-OP, 4-tert-OP, 4-n-NP, and tNP) were used for growth and degradation experiments. In the growth experiments, cells of strain TYA-1 grown in BPA–BSM were washed and inoculated at OD₆₀₀ = 0.05 into 100 mL BSM supplemented at 0.5 mM with one of the previously mentioned 4-APs as the sole carbon and energy source. The cultures were incubated at 28 °C and 120 rpm. Cell density and concentrations of 4-APs were monitored over the 120-h experimental period. In the degradation experiments,
washed cells of strain TYA-1 were suspended at OD\textsubscript{600} = 0.5 into 10 mL BSM containing 0.5 mM of one of the previously mentioned 4-APs. The whole-cell mixtures were incubated in vials at 28 °C and 120 rpm. Triplicate vials were prepared for each sampling time, and the concentrations of 4-APs were determined at 0 and 30 min and 3, 12, and 24 h after the start of the experiment. In addition, degradation inhibition experiments using 0.2 mM metyrapone were conducted.

**Immobilization of strain TYA-1 cells in alginate beads**

For immobilization on alginate beads, cells of strain TYA-1 grown in BPA–PY–BSM were washed with deionized water, suspended into 2 mL deionized water, and mixed with 18 mL of 4% (weight/vol) room-temperature sodium alginate solution that had been autoclaved (121 °C, 10 min). The cell density of the cell–alginate mixture was adjusted to 0 to 2 mg dry cells/mL. For each experiment, 20 mL of the cell–alginate mixture was dripped through a syringe into 500 mL of 0.2 M CaCl\textsubscript{2} on a magnetic stirrer. After 30 min, the alginate beads carrying immobilized TYA-1 cells (spherical form, 3–5 mm diameter) were harvested, washed with deionized water, and used for the experiment.

**Simultaneous removal of BPA, 4-tert-BP, and 4-tert-OP from complex polluted waters by immobilized TYA-1 cells**

First, to evaluate the effect of the cell density in the alginate beads, 20 mL of alginate-immobilized TYA-1 cells (0 to 2 mg dry cells/mL) were added to 100 mL BSM containing 50 mg/L BPA, 50 mg/L 4-tert-BP, and 5 mg/L 4-tert-OP (BPA–BP–OP mixture). The experiments were conducted at 28 °C and 120 rpm in duplicate. Concentrations of BPA, 4-tert-BP, and 4-tert-OP were monitored over the 12-h experimental period.

Second, additional SBR experiments were conducted to assess the reuse potential of the alginate-immobilized TYA-1 cells. BSM containing BPA–BP–OP and environmental water containing BPA–BP–OP were both used in SBR experiments. The environmental water mixture was prepared by dissolving 50 mg/L BPA, 50 mg/L 4-tert-BP, and 5 mg/L 4-tert-OP into the previously described environmental water sample and was used as simulated complex polluted water. Alginate-immobilized TYA-1 cells (20 mL; 0.75 mg dry cells/mL; 15 mg dry cells in total) were added into 100 mL of each BPA–BP–OP-containing mixture and incubated at 28 °C and 120 rpm in duplicate. After 6 h, the alginate beads were transferred into fresh BPA–BP–OP-containing mixtures. The SBR was operated for 12 cycles (i.e. 6 h of reaction time/cycle; 12 cycles; 72 h in total).

**Analytical procedures**

Bacterial cell density was measured as OD\textsubscript{600} and dry-weight of cells. In this study, OD\textsubscript{600} = 1 of washed cells of strain TYA-1 was equivalent to 0.46 mg dry cells/mL.

Concentrations of BPA and 4-APs were determined by high-performance liquid chromatography (HPLC). In the BPA and BPA–BP–OP degradation experiments, the collected samples were acidified with 1 mol/L HCl to pH 2 to 3 and centrifuged (15,000 × g, 4 °C, 10 min) and the supernatant analyzed by HPLC as described previously (Toyama et al. 2010). In the 4-AP degradation experiments, each culture sample was acidified with 1 mol/L HCl to pH 2 to 3, shaken for 3 min with an equal volume of an ethyl acetate–n-hexane mixture (2:1, vol/vol), and centrifuged (3,200 × g, 4 °C, 10 min). The organic layer was collected, 500 μL of the organic phase was collected, and the dry extract was dissolved in 500 μL acetonitrile. The sample was analyzed by HPLC as described previously (Toyama et al. 2010). In addition, the metabolites produced during degradation of 4-APs were analyzed by gas chromatography–mass spectrometry (GC–MS). For GC–MS analysis, 5 mL of the organic extract was dried under nitrogen flow and then subjected to trimethylsilylation (TMS) at 60 °C for 1 h by using 100 μL of a N,O-bis(trimethylsilyl)trifluoroacetamide-acetonitrile mixture (1:1, vol/vol). The trimethylsilylated sample was analyzed by GC–MS as described previously (Toyama et al. 2010).

**RESULTS AND DISCUSSION**

**BPA degradation by strain TYA-1 in the presence and absence of metyrapone**

Strain TYA-1 grown on BPA completely degraded 1.0 mM BPA within 12 h in the absence of metyrapone, and the cell density increased in parallel with BPA degradation (Figure 1). In contrast, strain TYA-1 degraded BPA only slightly in the presence of metyrapone; about 10% of the 1.0 mM BPA was removed within 12 h, and cell density did not increase at all in the presence of metyrapone (Figure 1). Metyrapone clearly inhibited BPA degradation by strain TYA-1. The results suggest that, like strain AO1
(Sasaki et al. 2005), strain TYA-1 degrades BPA via an oxidative skeletal rearrangement mechanism involving the cytochrome p450 monooxygenase system.

Degradability of various 4-APs

The ability of strain TYA-1 to degrade various 4-APs is summarized in Table 1. Strain TYA-1 was unable to use any of the 4-APs tested in this study as a sole carbon and energy source for growth. In contrast, whole cells of strain TYA-1 grown on BPA degraded 4-APs with branched alkyl side chains, including 4-sec-BP, 4-tert-BP, 4-tert-PenP, 4-tert-OP, and tNP. However, strain TYA-1 failed to degrade 4-n-AP with linear alkyl side chains. An important factor determining whether strain TYA-1 can degrade 4-AP seems to be the presence of branched side chains in 4-APs. 4-APs with branched alkyl side chains are much more estrogenic than are those with linear alkyl side chains (Routledge & Sumpner 1997). The current results suggest that the ability of strain TYA-1 to degrade 4-APs is potentially useful for the treatment of water polluted with branched 4-APs.

The metabolites of 4-sec-BP, 4-tert-BP, 4-tert-PenP, 4-tert-OP, and tNP in degradation experiments underwent TMS derivatization and were analyzed by GC–MS. As an example, Figure 2 shows the GC–MS total ion chromatogram (Figure 2(a)) and electroionization MS spectral data (Figure 2(b)) obtained from the 4-tert-BP degradation experiment. A single metabolite peak with the same retention time and MS spectrum as authentic 4-tert-butylcatechol (4-tert-BC) with TMS derivatization was detected at a retention time of 14.5 min, thus identifying the metabolite as 4-tert-BC. The peaks of 4-tert-BP and 4-tert-BC decreased to below the limit of detection within 12 h. In the degradation experiments with 4-sec-BP, 4-tert-PenP, 4-tert-OP, and tNP, hydroxylated metabolites of the original 4-APs were detected. The electroionization MS spectral data showed the addition of a single hydroxyl group to each parent 4-AP (data not shown). The metabolites were identified provisionally as 4-alkylcatechols, as was the case for 4-tert-BC. In addition, the inhibition experiments with metyrapone showed no substantial degradation of the branched 4-APs. These results suggest that the branched 4-APs initially are hydroxylated to 4-alkylcatechols by the cytochrome p450 monooxygenase system in strain TYA-1.
were not the dead-end products of the degradation of 4-APs, because the catechols did not accumulate in the cultures. However, identification of the metabolites and degradation pathways after 4-alkylcatechols was beyond the scope of this study.

Two 4-NP-utilizing bacteria, *Sphingomonas* sp. strain TTNP3 (Kolvenbach et al. 2007) and *Sphingobium xenopha-gum* Bayram (Gabriel et al. 2007), degrade BPA and some 4-APs by an *ipso*-substitution mechanism. In contrast to these previous studies, our current study is the first to report that the BPA-utilizing *Novosphingobium* sp. strain TYA-1 can degrade branched 4-APs via 4-alkylcatechols by the cytochrome p450 monoxygenase system. The BPA degradation pathway of strain TYA-1 began with the hydroxylation of the alkyl group, which binds the two phenolic rings of BPA, and produced 4-hydroxyacetophenol, 4-hydroxyacetophenone, and 4-hydroxybenzoic acid as intermediate metabolites (Toyama et al. 2013), as do strain AO1 (Sasaki et al. 2005) and other BPA-utilizing strains. In contrast, interestingly, the 4-AP degradation pathway of strain TYA-1 began with hydroxylation at a phenolic ring rather than an alkyl side chain.

**Degradation of BPA, 4-tert-BP, and 4-tert-OP by immobilized TYA-1 cells**

TYA-1 cells immobilized on alginate beads simultaneously degraded BPA, 4-tert-BP, and 4-tert-OP (Figure 3). In contrast, alginate beads without immobilized TYA-1 cells did not remove the three compounds from the reaction mixture (Figure 3(a)). These results indicate that BPA, 4-tert-BP, and 4-tert-OP were biodegraded by the immobilized TYA-1 cells rather than being removed through adsorption by the alginate beads. Degradation rates for the three compounds increased as the cell density increased. Alginate beads carrying immobilized TYA-1 cells at a cell density of greater than 0.75 mg dry cells/mL showed high degradation activity for all three compounds.

**Removal of BPA, 4-tert-BP, and 4-tert-OP from complex water samples by SBR containing alginate-immobilized TYA-1 cells**

The SBR experiments (6 h of reaction time/cycle; 12 cycle; 72 h in total) containing a 20 mL-volume of immobilized TYA-1 cells at cell densities of (a) 0, (b) 0.1, (c) 0.5, (d) 0.75, (e) 1.0, and (f) 2.0 mg dry cells/mL. Closed squares, closed circles, and open triangles indicate BPA, 4-tert-BP, and 4-tert-OP concentrations, respectively. Values are the means from duplicate experiments, and the error bars indicate 95% confidence intervals.
Near-complete removal of BPA, 4-tert-BP, and 4-tert-OP from both BPA–BP–OP mixtures occurred repeatedly during the first nine cycles; 100% of BPA, more than 90% of 4-tert-BP, and more than 90% of 4-tert-OP were removed in each cycle. Beginning with the 10th cycle, removal rates of BPA, 4-tert-BP, and 4-tert-OP from both BPA–BP–OP mixtures decreased markedly. At the 12th cycle, 79% of BPA, 57% of 4-tert-BP, and 52% of 4-tert-OP were removed from the BSM-based mixture; 77% of BPA, 45% of 4-tert-BP, and 42% of 4-tert-OP were removed from the mixture made with environmental water. These results show that alginate-immobilized TYA-1 cells could be reused for as many as nine cycles for simultaneous removal of BPA, 4-tert-BP, and 4-tert-OP without any loss of efficiency. Therefore, the current study suggests that bioreactor systems using immobilized TYA-1 cells can be a useful strategy for the simultaneous removal of BPA and 4-APs from complex polluted waters.

However, some problems remain regarding the construction of practical bioreactor systems. One key drawback is instability after prolonged incubation, as shown in Figure 4. In our study, immobilized and free strain TYA-1 cells (data not shown) lost the ability to degrade BPA, 4-tert-BP, and 4-tert-OP after long-term incubation. A similar event has been reported to occur in strain AO1 due to deletion of the DNA region containing the BPA-degrading genes (Sasaki et al. 2008). The eventual instability in the SBR with strain TYA-1 might result from the same mechanism as in strain AO1. The construction of practical bioreactor systems for treating complex polluted waters containing BPA and 4-APs likely will require improvement of the cytochrome p450 monooxygenase system in strain TYA-1 or screening for other candidate bacteria. It also will be necessary to evaluate the safety and overall toxicity of the treated water of complex polluted waters.

CONCLUSION

This study showed that the BPA-utilizing Novosphingobium sp. strain TYA-1 degraded 4-APs with branched side alkyl chains via 4-alkylcatechols by the cytochrome p450 monooxygenase system. SBR experiments containing alginate-immobilized TYA-1 cells simultaneously removed BPA, 4-tert-BP, and 4-tert-OP from complex mixtures, and the alginate-immobilized TYA-1 cells could be used for nine cycles without any loss of degradation activity. This study showed the potential of using immobilized TYA-1 cells for the simultaneous removal of BPA and 4-APs from complex polluted waters.
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