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Microbial Production of Optically Active β-Phenylalanine through Stereoselective Degradation of Racemic β-Phenylalanine

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The ability to produce (*R*)- or (*S*)-β-phenylalanine from racemic β-phenylalanine through stereoselective degradation was screened for. *Variovorax* sp. JH2 and *Arthrobacter* sp. the faculty of Agriculture, Kyoto University (AKU) 638 were found to be potential catalysts for (*R*)- and (*S*)-β-phenylalanine production respectively. On 192 h cultivation of *Variovorax* sp. in medium containing 1.0% (w/v) racemic β-phenylalanine, 0.46% (w/v) (*R*)-β-phenylalanine with an enantiomeric purity of 99% *e.e.* was obtained. The initial step of the (*S*)-isomer degradation was stereoselective transamination. On 312 h cultivation of *Arthrobacter* sp. in medium containing 1.0% (w/v) racemic β-phenylalanine, 0.51% (w/v) (*R*)-β-phenylalanine with an enantiomeric purity of 90% *e.e.* was obtained. The initial step of the (*R*)-isomer degradation was supposed to be oxidative deamination. Resting cell reaction with vigorous shaking, with cells of *Arthrobacter* sp. as the catalyst, resulted in production of 0.49% (w/v) of (*S*)-β-phenylalanine with an enantiomeric purity of 99% *e.e.* from 1.0% (w/v) racemic β-phenylalanine in 45 h.

Key words: β-amino acid; β-phenylalanine; chiral resolution; *Variovorax*; *Arthrobacter*

β-Phenylalanine (3-amino-3-phenylpropionic acid, BPA) is a β-amino acid present in several bioactive molecules, such as antibiotics and enzyme inhibitors.[1,2] Because of its bioactivity, optically active BPA and its derivatives are becoming important chiral building blocks for the synthesis of pharmaceutical agents. For example, (*S*)-BPA is a chiral building block of fibrinogen receptor antagonists.[3,4]

We have reported screening for microorganisms that hydrolyze BPA ethyl ester stereoselectively and have developed a process for the production of optically pure BPA ethyl ester with cells of the selected strains as biocatalysts.[5] By this method, the maximal yield of optically pure BPA is theoretically 50%, which limits the efficiency of the process. We expected that there must be a more efficient microbial process for optically pure BPA-production that gives a higher production yield, and hence investigated microbial reactions involving BPA. We collected BPA-assimilating microorganisms and analyzed their BPA metabolism. Through this investigation, we discovered microorganisms that assimilate BPA with high enantioselectivity. Their BPA-degradation did not permit optically active BPA production with a yield of more than 50%, but, they were useful for convenient production of optically active BPA through stereoselective degradation of racemic BPA without the tedious chemical esterification step required for the enzymatic chemical resolution of BPA esters.[6–8] Furthermore, their BPA-degradation was expected to involve unique enzymes that would give a higher production yield.

In this report, we describe the results of screening of microorganisms degrading BPA stereoselectively, application of the potential strains for the microbial production of optically active BPA, and biochemical analysis of the reactions involved in their BPA-degradation.

Materials and Methods

Chemicals. DL-3-Amino-3-phenylpropionic acid (BPA) was purchased from Ardrich Chemical (Milwaukee, WI). 3-Oxo-3-phenylpropionic acid (benzoyl acetic acid, BAA) was a kind gift from Kaneka (Osaka, Japan). All other chemicals used were of analytical grade and were commercially available.

Isolation of BPA-assimilating microorganisms. Small amounts of soil samples were added to test tubes (16 × 165 mm) containing 5 ml of isolation liquid medium comprising 0.1% (w/v) KH_2PO_4, 0.1% (w/v) K_2HPO_4,

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Abbreviations: AKU, the faculty of Agriculture, Kyoto University; BAA, benzoyl acetic acid; BPA, β-phenylalanine; *e.e.*, enantiomer excess; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate; HPLC, high-performance liquid chromatography; 2-OG, 2-oxoglutaric acid; PLP, pyridoxal-5′-phosphate; UV, ultraviolet

0.03% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) yeast extract, 0.2% (w/v) NH_4Cl, and 0.15% (w/v) racemic BPA (9.1 mM) (pH 7.0), followed by incubation at 28 °C with shaking (300 strokes/min) for 1–2 d. Fifty µl of medium, in which the growth of microorganisms was observed, was transferred to another test tube containing the same medium, followed by further cultivation at 28 °C with shaking (300 strokes/min) for 1–2 d. The culture media were streaked onto agar plates with the same composition as the isolation medium and cultivated at 28 °C for 2–3 d. After cultivation, the colonies that appeared were isolated as BPA-assimilating microorganisms. Microorganisms obtained from the culture collection of the Faculty of Agriculture of Kyoto University (AKU) were also inoculated onto the isolation media, and strains with the ability to assimilate BPA were selected by the same methods. These BPA-assimilating strains from soil samples and the culture collection were used for the subsequent experiments.

Screening method. The medium used for screening was the same as the isolation liquid medium except that the concentration of racemic BPA was 1.0% (w/v) (61 mM). BPA-assimilating microorganisms were inoculated into 5 ml of the medium and then cultivated at 28 °C with shaking (300 strokes/min) for 1–10 d. During cultivation, aliquots of the media were withdrawn and the supernatants obtained on cetrifugation (15,000 rpm, 3 min, MX-150; Tomy Seiko, Tokyo) were analyzed as described below.

Preparation of cell-free extracts. Cells of the selected strains, *Variovorax* sp. JH2 and *Arthrobacter* sp. AKU 638 (about 1 g of wet cells obtained by cultivation in 1-liter of the isolation medium), were suspended in 3.3 ml of buffer and then disrupted for 10 min with an ultrasonic oscillator (Kubota, Osaka, Japan). Samples were ultracentrifuged at 40,000 rpm for 1 h (L-80 Ultracentrifuge; Beckman Coulter, Fullerton, CA). The supernatants were used as soluble cell-free extracts. The pellets obtained on ultracentrifugation were suspended in 3.3 ml of 50 mM potassium phosphate buffer (pH 7.0) and used as the insoluble fraction.

Effects of pyridoxal-5′-phosphate (PLP) and 2-oxoglutaric acid (2-OG) on (S)-BPA degradation. A reaction mixture comprising, in 100 µl, 50 µl of the soluble cell-free extract of *Variovorax* sp. JH2, 10 mM BPA, and 5 mM each chemical mentioned below was incubated at 28 °C with shaking (300 strokes/min). The chemicals examined were as follows: NAD^+, $NADP^+$, 2-OG, pyruvate, and PLP. After 10 h reaction, the decomposition of BPA was assayed as described below.

Effect of agitation on (R)-BPA degradation. A reaction mixture comprising, in 100 µl, 50 µl of the insoluble fraction of *Arthrobacter* sp. AKU 638 and 20 mM BPA was incubated at 28 °C with or without shaking (300 strokes/min). After 10 h reaction, the decomposition of BPA was assayed as described below.

Analytical methods. Qualitative analysis of BPA, BAA, and L-glutamic acid was performed by thin-layer chromatography (TLC) with Kieselgel 60 F_{254} (Merck, Rahway, NJ). The developing system consisted of *n*-butanol, acetic acid, and H_2O in a ratio of 4:1:1 (v/v/v). Detection was carried out with 0.2% (v/v) ninhydrin in acetone and ultra-violet (254 nm). Quantitative analysis of BPA was performed by reverse-phase high-performance liquid chromatography (HPLC) with a Shimadzu LC-VP system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil $5C_8$-MS column (0.46 × 250 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase was acetonitrile–water (9:1 by vol., pH 2.0, adjusted with phosphoric acid) at a flow rate of 1.0 ml/min, and the effluent was monitored by means of 210 nm ultraviolet (UV) detection. For detection of BAA, the same HPLC method was used. Liquid chromatography–mass spectrometry (LC–MS) analysis was carried out under the condition described above with LC–MS 2010A (Shimadzu).

Determination of the enantiomeric purity of BPA was carried out by reverse-phase HPLC with a Shimadzu LC-VP system equipped with a Cosmosil $5C_{18}$-AR-II column (0.46 × 250 mm; Nacalai Tesque) after derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC).[9–11] The mobile phase was methanol–water (55:45 by vol., pH 2.5, adjusted with phosphoric acid) at a flow rate of 1.0 ml/min, and the effluent was monitored by means of 250 nm UV detection. For detection of ammonia, the same HPLC method was used after derivatization with GITC. For detection of L-glutamic acid, the same HPLC method was used after derivatization with GITC except that methanol–water (40:60 by vol., pH 2.5, adjusted with phosphoric acid) was used as the mobile phase.

Results

Screening for microorganisms that stereoselectively degrade BPA

Three hundred-thirty BPA-assimilating microorganisms were obtained from soil samples and the AKU culture collection. Three hundred of them were bacteria and the others were molds. BPA was degraded in various portions of enantiomers by them (data not shown), and some strains exhibited stereoselectivity as to BPA degradation. (*R*)-BPA production through (*S*)-BPA selective degradation was observed for six strains. Among them, the highest optical purity and yield of (*R*)-BPA were observed with strain JH2 (Table 1A). (*S*)-BPA production through (*R*)-BPA selective degradation was observed for three strains. Among them, the highest optical purity and yield of (*S*)-BPA were observed for

Table 1. Microorganisms Producing (*R*)-BPA through (*S*)-BPA Selective Degradation (A), and Microorganisms Producing (*S*)-BPA through (*R*)-BPA Selective Degradation (B)

A	Strain	Type of strain	Cultivation time (h)	Remaining BPA	
				% (w/v)	*e.e.* (%) for (*R*)-BPA
	JH1	Bacterium	192	0.41	97
	JH2	Bacterium	192	0.46	98
	JR1	Bacterium	192	0.43	97
	JT1	Bacterium	192	0.40	96
	JT2	Bacterium	192	0.43	96
	JT3	Bacterium	192	0.38	96

B	Strain	Type of strain	Cultivation time (h)	Remaining BPA	
				% (w/v)	*e.e.* (%) for (*S*)-BPA
	JW1	Mold	120	0.52	33
	JW2	Mold	120	0.40	63
	Arthrobacter sp. AKU 638	Bacterium	240	0.55	76

e.e., enantiomer excess

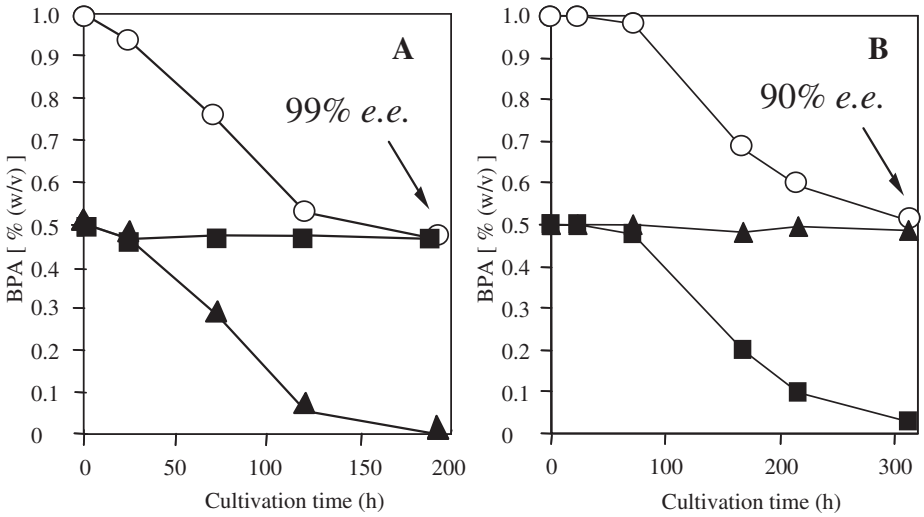

Fig. 1. Preparative Production of (*R*)-BPA during Cultivation of *Variovorax* sp. JH2 (A), and of (*S*)-BPA during Cultivation of *Arthrobacter* sp. AKU 638 (B).

The cultivations were carried out under the same conditions as those for screening, as described in "Materials and Methods." The % *e.e.* values for obtained BPA are presented with arrows. The averages of three separate experiments, the results of which were reproducible within ±10%, are presented in the figure. ■, (*R*)-BPA; ▲, (*S*)-BPA; ○, total BPA.

Arthrobacter sp. AKU 638 (Table 1B). Strain JH2 and *Arthrobacter* sp. AKU 638 were thus selected as potent strains with the ability to produce (*R*)- and (*S*)-BPA respectively.

Identification of strain JH2

Strain JH2 was identified by phylogenetic analysis and from its biochemical characteristics. The partial 16S rDNA sequence of this strain showed high similarity to that of a strain of *Variovorax paradoxus* (100%), and formed a sister group with that of *V. paradoxus* in the phylogenetic tree (data not shown). Thus the isolated strain might belong to the genus *Variovorax*, probably to a group closely related to *Variovorax paradoxus*. The biological traits of this strain were determined to be as follows: methophilic (grew at 30 °C); colony shape, circular, smooth, and yellow in color; cell shape, rod-shaped (0.6–0.7 × 1.0–1.2 μm); motile; Gram-negative; and nonspore-forming. These biological traits supported our assumption that strain JH2 belonged to the genus *Variovorax*, so the strain was named *Variovorax* sp. JH2.

Preparative production of (R)- and (S)-BPA

Preparative production of (*R*)-BPA through (*S*)-isomer selective degradation during the cultivation of *Variovorax* sp. JH2 was carried out (Fig. 1A). After 192 h cultivation in medium containing 1.0% (w/v)

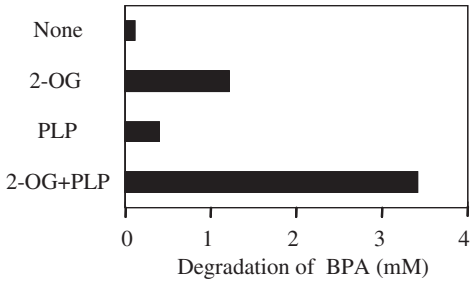

Fig. 2. Effects of PLP and 2-OG on BPA-Degradation by the Soluble Cell-Free Extract of *Variovorax* sp. JH2.

The amount of BPA (mM) degraded on 10 h reaction with 5 mM 2-OG, 5 mM PLP, or both is shown.

racemic BPA (61 mM) as the sole carbon source, 0.46% (w/v) BPA (28 mM) was obtained with an optical purity of 99% *e.e.* for the (*R*)-isomer.

Preparative production of (*S*)-BPA through (*R*)-isomer selective degradation during the cultivation of *Arthrobacter* sp. AKU 638 was also carried out (Fig. 1B). After 312 h cultivation in medium containing 1.0% (w/v) racemic BPA (61 mM) as the sole carbon source, 0.51% (w/v) BPA (31 mM) was obtained with an optical purity of 90% *e.e.* for the (*S*)-isomer.

*Reaction analysis of the (*S*)-BPA degradation by* Variovorax *sp. JH2*

The (*S*)-BPA degrading activity of *Variovorax* sp. JH2 was found in the soluble cell-free extract but not in the insoluble fraction (data not shown). The effects of various cofactors on (*S*)-BPA degradation by soluble cell-free extracts of *Variovorax* sp. JH2 were investigated. Among the five chemicals examined, PLP and 2-OG accelerated the degradation (Fig. 2). The combination of PLP and 2-OG further enhanced the degradation. The degradation products were identified by TLC, HPLC, and LC–MS. One ultra-violet positive product was observed on TLC analysis, and it showed the same R_f value as that of authentic BAA (data not shown). BAA was further identified on HPLC analysis (Fig. 3), but BAA was not detected on LC–MS, because it is not stable under ionization conditions. One ninhydrin positive product was also detected on TLC analysis, and it showed the same R_f value as that of L-glutamic acid. (data not shown). L-Glutamic acid was further identified on HPLC analysis (Fig. 4). These results suggest that the degradation products were BAA and L-glutamic acid, but the stoichiometry of the reaction was not confirmed because these products were further degraded by the cell-free extracts of *Variovorax* sp. JH2. Based on these results, we tried to produce optically active (*R*)-BPA using resting cells of *Variovorax* sp. JH2 as the catalyst in the presence of cofactors. On 36 h reaction with 1.0% (w/v) racemic BPA (61 mM), 0.44% (w/v) 2-OG (30 mM), 0.12% (w/v) PLP (5 mM), and 10% (wet cells, w/v) washed cells of *Variovorax* sp. JH2 obtained on 24 h-cultivation in the isolation liquid medium, it was

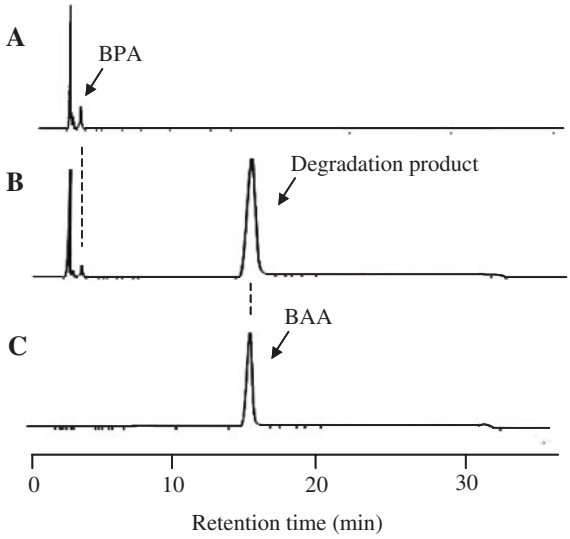

Fig. 3. HPLC Chromatograms of the Product of (*S*)-BPA Degradation by the Soluble Cell-Free Extract of *Variovorax* sp. JH2 and Authentic BAA.

HPLC analysis was performed under the conditions given in "Materials and Methods." A, HPLC chromatogram of the reaction mixture before incubation; B, HPLC chromatogram of the reaction mixture after incubation for 30 min; C, HPLC chromatogram of authentic BAA (2.5 mM).

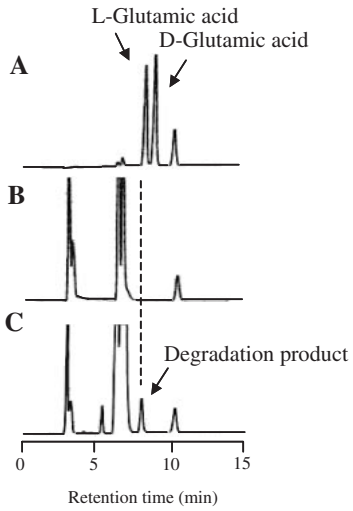

Fig. 4. HPLC Chromatograms of the GITC-Derivatized Product of (*S*)-BPA Degradation by the Soluble Cell-Free Extract of *Variovorax* sp. JH2 and a Standard Mixture of GITC-Derivatized Racemic Glutamic Acid.

HPLC analysis was performed under the conditions given in "Materials and Methods." A, HPLC chromatogram of a standard mixture containing racemic glutamic acid (10 mM); B, HPLC chromatogram of the reaction mixture before incubation; C, HPLC chromatogram of the reaction mixture after incubation for 30 min.

almost the case that only (*S*)-BPA was degraded, 0.48% (w/v) of (*R*)-BPA being obtained in a high yield and with a high enantiomeric purity (molar yield as to racemic BPA, 48%; optical purity, 99% *e.e.* for (*R*)-BPA).

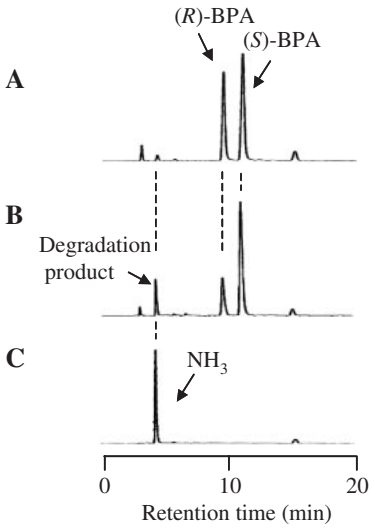

Fig. 5. HPLC Chromatograms of GITC-Derivatized Samples Containing the Product of (*R*)-BPA Degradation by the Insoluble Fraction of *Arthrobacter* sp. AKU 638 and a Standard Mixture of GITC-Derivatized Ammonia.

HPLC analysis was performed under the conditions given in "Materials and Methods." A, HPLC chromatogram of the reaction mixture before incubation; B, HPLC chromatogram of the reaction mixture after incubation for 10 h; C, HPLC chromatogram of the standard mixture containing NH_3 (10 mM).

*Reaction analysis of the (*R*)-BPA degradation by* Arthrobacter *sp. AKU638*

The (*R*)-BPA degrading activity of *Arthrobacter* sp. AKU 638 was found in the insoluble fraction but not in the soluble cell-free extract (data not shown). By shaking the reaction mixture, the (*R*)-BPA degradation by the insoluble fraction of *Arthrobacter* sp. AKU 638 was greatly enhanced. Under the static condition, only 0.8 mM BPA in the reaction mixture was degraded in 10 h while 6.7 mM of BPA was degraded under the shaking condition. One of the products of the reaction under the shaking condition was identified as ammonia on HPLC chromatography (Fig. 5), and further by LC–MS analysis showing the same molecular weight ($m/z = 407$; ESI positive) of that of GITC-derivatized ammonia. However, a possible deamination product, BAA, was not found in the reaction mixture. The stoichiometry of the reaction was not confirmed because these products were further degraded by the insoluble cell-free fractions of *Arthrobacter* sp. AKU 638. These results suggest that (*R*)-BPA is degraded through an oxidative deamination process, but the reaction mechanism could not be elucidated because general cofactors for enzymatic oxidation or dehydrogenation, such as NAD^+, $NADP^+$, NADH, NADPH, FAD, FMN, phenazine methosulfate, 2,6-dichlorophenolindophenol, methylene blue, potassium ferricyanide, 1,4-benzoquinone, 1,4-naphthoquinone, pyrroloquinoline quinone, cytochrome *c*, thioredoxin, and rubredxin, did not accelerate the reaction (data not shown). Based on these results, we tried to produce optically active (*S*)-BPA using resting cells of *Arthrobacter* sp. AKU 638 as the catalyst with vigorous shaking. On 45 h reaction with 1.0% (w/v) racemic BPA (61 mM) and 10% (wet cell, w/v) of washed cells of *Arthrobacter* sp. AKU 638 obtained on 24 h of cultivation in the isolation liquid medium, it was almost the case that only (*R*)-BPA was degraded, 0.49% (w/v) of (*S*)-BPA being obtained in a high yield and with a high enantiomeric purity (molar yield as to racemic BPA, 49%; optical purity, 99% *e.e.* for (*S*)-BPA).

Discussion

Variovorax sp. JH2 and *Arthrobacter* sp. AKU 638 were found to be (*S*)- and (*R*)-BPA degrading microorganisms, and selected as potential catalysts for the production of optically pure (*R*)- and (*S*)-BPA respectively.

The (*S*)-BPA degrading activity of *Variovorax* sp. JH2 was found in the soluble cell-free extract. The enzyme catalyzing the initial step of the (*S*)-BPA metabolism appeared to be a soluble protein. On analysis of the biochemical properties of the reaction, PLP and 2-OG were found to accelerate it. In addition, the products of the reaction were identified as BAA and L-glutamic acid. These facts suggest that the reaction comprises stereoselective transamination and that the enzyme degrading (*S*)-BPA can be classified as (*S*)-3-amino-3-phenylpropionic acid: 2-oxoglutaric acid aminotransferase. Concerning the microbial metabolism of β-amino acids, such as β-alanine, β-aminoisobutyrate, β-aminobutyrate and taurine, metabolism through transamination catalyzed by ω-amino acid transaminases has been reported.[12–14] Until now, there has been no report on BPA metabolism. In this study, it was found that one type of BPA metabolism involves transamination that is the same as some other β-amino acid metabolisms.

(*R*)-BPA degrading activity was found in the insoluble fraction of *Arthrobacter* sp. AKU 638, suggesting that the enzyme catalyzing the initial step of (*R*)-BPA metabolism is a membrane protein. The reaction with the insoluble fraction as the catalyst proceeded slowly under the static condition but faster with shaking, suggesting that the concentration of soluble oxygen in the reaction mixture affects the enzyme activity involved in the (*R*)-BPA degradation. Ammonia was identified as the reaction product under the shaking condition and hence it was speculated that (*R*)-BPA is degraded through an oxidative deamination process.

In summary, we report here two novel types of microbial BPA metabolism with stereoselectivity, *viz.*, transamination and oxidative deamination. This microbial degradation should allow efficient production of both isomers of BPA.

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