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Biodegradation of indole at high concentration by persolvent fermentation with *Pseudomonas* sp. ST-200

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Abstract *Pseudomonas* sp. strain ST-200 grew on indole as a sole carbon source. The minimal inhibitory concentration of indole was 0.3 mg/ml for ST-200. However, ST-200 grew in a persolvent fermentation system containing a large amount of indole (a medium containing 20% by vol. diphenylmethane and 4 mg/ml indole), because most of the indole was partitioned in the organic solvent layer. When the organism was grown in the medium containing indole at 1 mg/ml in the presence of diphenylmethane, more than 98% of the indole was consumed after 48 h. Isatic acid (0.4 mg/ml) and isatin (0.03 mg/ml) were produced as the metabolites in the aqueous medium layer.

Key words Biodegradation of indole · Indole · *Pseudomonas* sp. · Persolvent fermentation · Organic solvent

Introduction

Aromatic *N*-heterocyclic compounds, including substituted indoles, are often found in aqueous waste effluents associated with oil shale and coal mining operations (Blum et al. 1986). Members of this chemical class are toxic (Wilkes, 1981). For example, 3-methylindole has been shown to induce pneumotoxicosis in ruminants (Carlson et al. 1983). Chemical disinfection of indole-containing waste-waters with dilute aqueous solutions of chlorine, chlorine dioxide, and chloramine leads to the formation of other toxic chlorinated aromatic products (Lin and Carlson 1984).

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Biodegradation of heterocyclic aromatic compounds has been extensively investigated (Madsen and Bollag 1989; Madsen et al. 1988). The concentrations of these compounds that can be supplied in the medium are low because of the toxicity for microorganisms (Eaton and Chapman 1995; Madsen and Bollag 1989; Madsen et al. 1988; Mermod et al. 1986). Aromatic compounds are generally lipophilic. When lipophilic compounds are added to a water–organic solvent two-phase system, concentrations of the compounds in the water phase are kept low because most of the compounds partition into the organic solvent layer. In such a two-phase system, the toxic effects of heterocyclic aromatic compounds might be reduced, and it might be possible to supply a large amount of substrate in a small volume of the medium at one time. This conversion system is made feasible by the use of appropriate organic-solvent-tolerant microbes capable of degrading toxic aromatic compounds.

We have isolated a cyclohexane-tolerant, cholesterol-converting bacterium, *Pseudomonas* sp. strain ST-200, from humus soil. We showed that this organism effectively oxidized cholesterol dissolved in an organic solvent overlying the medium (Aono et al. 1994). During characterization of strain ST-200, we noticed that this organism assimilated indole dissolved in organic solvent. In this report, we examined the effect of organic solvent on the consumption of indole by the organism.

Materials and methods

Organism and culture conditions

Pseudomonas sp. strain ST-200 was used in this study (Aono and Doukyu 1996; Aono et al. 1994; Doukyu et al. 1996). This organism was grown in 10 ml of a mineral basal salts (MBS) medium (Stanier et al. 1966) overlaid with 2 ml of an appropriate organic solvent containing 1.25–80 mg of indole as the sole carbon and energy source. This culture was shaken at 150 rpm with 4-cm strokes at 30°C. When the

organism was grown in MBS medium without an organic solvent, indole was added directly to the medium. Viable cells were counted on LB agar medium consisting of 1% Bacto tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% yeast extract (Difco), 1% NaCl, and 1.5% agar.

Purification of the products derived from indole

Strain ST-200 was grown in 400 ml of MBS medium overlaid with 80 ml of diphenylmethane containing 0.5% indole. The culture was shaken (120 rpm) at 30°C. An aqueous layer was recovered with a separating funnel after 3 days and centrifuged at 8000 × *g* for 10 min at 4°C. The supernatant was concentrated in a rotary evaporator to ca. 5 ml at 25°C. This solution was applied to a column (20 by 2.2 cm) of Diaion HP-10 (Mitsubishi Chemicals, Tokyo, Japan). The column was eluted with 400 ml of a linear gradient of 0%–20% (vol/vol) methanol. The absorbance of fractions (5 ml) was measured at 270 or 370 nm. The absorbance-positive fractions were pooled and concentrated in a rotary evaporator to ca. 1 ml at 25°C.

Measurement of molecular weight of the conversion product

Molecular weights of samples were measured by low-resolution electron impact mass spectrometry using a mass spectrometer (Shimadzu Tokyo, Japan; model QP-5000). The samples were ionized at 230°C and 70 eV.

Measurement of NMR spectrum of the conversion product

The sample was dissolved in deuterated acetone or deuterated water to a concentration of 2–20 mg/ml. ^{1}H-nuclear magnetic resonance (NMR) spectra were recorded at 20°C with a 500-MHz NMR spectrometer (Varian; model VXR-500S, Tokyo, Japan). ^{13}C-NMR spectra were measured with a 50-MHz NMR spectrometer (Varian; model Gemini-200) with complete decoupling. Tetramethylsilane was used as an internal standard for the spectra.

Determination of indole and its conversion products

A sample was obtained periodically from the organic solvent or water layer of the culture. The sample obtained from the organic solvent layer was directly analyzed by reverse-phase chromatography on a column of ODS-1201-H (4.6 by 200 mm; Senshu Science, Tokyo, Japan) attached to a high-pressure liquid chromatograph. The column was eluted with *n*-hexane-isopropanol (10:0.1, vol/vol) at a flow rate of 1.0 ml/min. A sample (0.1 ml) from the water phase was centrifuged (5000 × *g*, 5 min, 4°C). The supernatant solution was analyzed by normal-phase chromatography on a column of Silica-1251-N (4.6 by 250 mm; Senshu Science). The column was eluted with acetonitrile-isopropanol (10:0.1, vol/vol) at a flow rate of 1.0 ml/min. The elutions were monitored by measuring A_{270}. To measure each substrate, the relevant authentic compound was used as a standard.

Table 1. Amount of indole partitioned into water phase in the persolvent fermentation system

Solvent	Amount of indole added to the system (mg)[a]						
	1.2	2.5	5	10	20	40	80
	Indole concentration in water phase (mg/ml)[b]						
None[c]	0.12	0.25	0.50	1.0	2.0	4.0	8.0
n-Octane	0.08	0.17	0.37	0.71	1.4	2.8	5.2
Cyclooctane	0.08	0.15	0.28	0.57	0.98	1.9	3.7
Diphenylmethane	0.01	0.03	0.06	0.11	0.18	0.28	0.48
Propylbenzene	0.01	0.03	0.06	0.12	0.18	0.25	0.42

[a] Indole (the amount shown) was added to 10 ml of fresh MBS medium overlaid with 2 ml of each organic solvent.
[b] Concentration of indole in water phase was measured at 24 h after shaking at 30°C.
[c] Solubility of indole in the medium was 8.6 mg/ml at 30°C.

Materials

Indole, isatin, and *o*-aminobenzoate were purchased from Nacalai Tesque, Kyoto, Japan. Organic solvents used were the highest qualities that were commercially available. The logarithm of partition coefficient (log P_{ow}) values of compounds were calculated by the addition rule (Leo et al. 1971).

Results and discussion

Effects of organic solvents and indole concentration on growth of strain ST-200

To represent the toxicity of a particular organic solvent, we use the logarithm of the partition coefficient (P_{ow}) of the solvent between *n*-octanol and water (Inoue and Horikoshi 1989). We represent the tolerance level of a particular microorganism by using two terms, the index-solvent and index-value (Aono et al. 1991; Inoue and Horikoshi 1989). The index-solvent is the most toxic solvent among the organic solvents to which the organism is tolerant. The index-value is the log P_{ow} value of the index-solvent. The index-solvent for strain ST-200 is cyclohexane, and the index-value for the tolerance level of the organism is 3.4 (Aono and Doukyu 1996). In this study, we used *n*-octane (log P_{ow} 5.0), cyclooctane (log P_{ow} 4.5), diphenylmethane (log P_{ow} 4.2), and propylbenzene (log P_{ow} 3.8), none of which were assimilated by the organism.

Indole was partitioned preferentially into polar organic solvents (Table 1) due to its high polarity (log P_{ow} 2.1). By the addition of diphenylmethane or propylbenzene, about 90% of the added indole was partitioned into these organic solvents. The minimal inhibitory concentration (MIC) of indole was 0.3 mg/ml for strain ST-200 (results not shown). The organism grew in MBS medium containing less than

Table 2. Growth of strain ST-200 in the presence of indole

Solvent	Amount of indole added to the system (mg)[a]							
	0	1.2	2.5	5	10	20	40	80
	Growth (OD_{660})[b]							
None	<0.01	0.12	0.19	<0.01	<0.01	<0.01	<0.01	<0.01
n-Octane	<0.01	0.14	0.19	<0.01	<0.01	<0.01	<0.01	<0.01
Cyclooctane	<0.01	0.14	0.20	0.14	<0.01	<0.01	<0.01	<0.01
Diphenylmethane	<0.01	0.15	0.22	0.40	0.47	0.18	0.10	<0.01
Propylbenzene	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Indole (amount shown) was added to 10 ml of fresh MBS medium overlaid with 2 ml of each organic solvent.
[b] ST-200 was grown in the media with shaking at 30°C for 72 h. The growth was measured by optical density at 660 nm (OD_{660}).

Table 3. Consumption of indole by strain ST-200

Solvent	Amount of indole added to the system (mg)[a]						
	1.2	2.5	5	10	20	40	80
	Indole consumption (%)[b]						
None	97	95	0	0	0	0	0
n-Octane	94	93	0	0	0	0	0
Cyclooctane	96	95	58	0	0	0	0
Diphenylmethane	98	99	99	98	25	4	0
Propylbenzene	0	0	0	0	0	0	0

[a] Indole (amount shown) was added to 10 ml of fresh MBS medium overlaid with 2 ml of each organic solvent.
[b] The organism was grown as shown in Table 2. The concentration of residual indole was measured after 72 h of the incubation. Indole consumption is represented as a percentage of the amount of indole added to the medium.

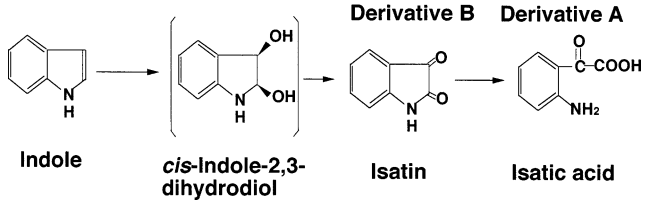


Fig. 1. A putative scheme for indole conversion by *Pseudomonas* sp. strain ST-200

0.3 mg indole/ml without regard to presence of organic solvent, except for propylbenzene, which did not support growth (Tables 1 and 2). The final cell density was suppressed when the initial concentration of aqueous indole was near its MIC. The organism grew even in the presence of a large amount of indole when an appropriate organic solvent was added. In particular, in the presence of diphenylmethane, which has a high partitioning power for indole, the organism grew even in medium containing apparently 4 mg indole per ml. This concentration was 16-fold the concentration tolerated in the absence of organic solvent (0.25 mg/ml). The indole tolerance level was apparently improved because most of the indole was partitioned into diphenylmethane. The organism consumed apparently 1 mg indole per ml after 72 h, when the medium was overlaid with diphenylmethane (Table 3). This concentration was four times that without organic solvent (0.25 mg/ml).

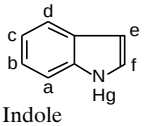
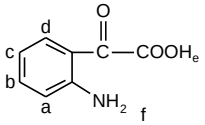
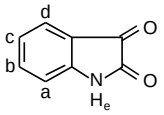
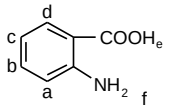
The organism did not grow in MBS medium overlaid with propylbenzene (Table 2), although the organism grew in LB medium or MBS medium supplemented with 0.1% glucose overlaid with propylbenzene containing or not containing indole (results not shown). This deviation suggests that indole and propylbenzene synergistically inhibited growth of ST-200 in a nutritionally poor medium.

Identification of the indole derivatives

Strain ST-200 grown on indole with diphenylmethane produced water-soluble products but not water-insoluble ones. The products were purified from the aqueous medium layer. Final recoveries of these products were 79 mg (derivative A) and 2 mg (derivative B) from 400 mg of indole as the substrate. The R_f values on silica gel thin-layer chromatography of derivatives A and B were 0.41 and 0.71, respectively, in chloroform-methanol-25% (wt/vol) ammonia water (65:25:4, vol/vol/vol). The molecular weights and absorbance peak (λ_{max}) measured in 95% (vol/vol) ethanol were as follows: indole, MW 117 and λ_{max} 282 nm; derivative A, MW 165 and λ_{max} 372 nm; and derivative B, MW 147 and λ_{max} 420 nm. The molecular weights of the derivatives were 30 and 48 larger than that of the starting material, indole, indicating that the derivatives were probably oxide compounds but not degraded products. Comparison of the λ_{max} values between indole and the derivatives indicated that π-resonance systems were increased by the introduction of conjugated double bounds.

The ^{1}H-NMR spectrum of derivative B was identical to that of authentic isatin (Table 4) (Madsen and Bollag 1989). The chemical shift values of signals derived from Ha to Hd in the ^{1}H-NMR spectrum of derivative A were similar to those of *o*-aminobenzoate (Sasaki 1985). Derivative A gave a positive ninhydrin reaction, indicating that derivative A had a free amino group. The chemical shift values of signals in the ^{13}C-NMR spectra of *o*-aminobenzoate and derivative A were as follows: *o*-aminobenzoate, 112.4, 119.4, 119.7, 135.1, 138.0, 154.0, 176.0 ppm; and derivative A, 116.0, 119.6, 120.4, 136.4, 139.0, 154.6, 176.5, 201.3 ppm. The chemical shift values

Table 4. ^{1}H nuclear magnetic resonance (^{1}H-NMR) characteristics of indole derivatives

Compound	Chemical shift (ppm)	Splitting[a] (integral)	Proton assignments	Coupling constant (Hz)
	6.56	– (1H)	He	–
	7.00–7.23	– (3H)	Hb, Hc, Hf	–
	7.34	d (1H)	Ha	8.2
	7.64	d (1H)	Hd	8.4
Indole	8.00	– (1H)	Hg	–
	6.78	t (1H)	Hc	7.2
	6.88	d (1H)	Ha	8.2
	7.43	t (1H)	Hb	7.2
	7.53	d (1H)	Hd	8.2
Isatic acid	unobserved		He	
(Derivative A)	unobserved		Hf	
	7.02	d (1H)	Hc	8.0
	7.12	t (1H)	Hb	7.6
	7.62	t (1H)	Ha	7.7
	7.54	d (1H)	Hd	7.6
Isatin	unobserved		He	
(Derivative B)				
	6.67	t (1H)	Hc	7.0
	6.71	d (1H)	Ha	8.2
	7.32	t (1H)	Hb	7.0
	7.74	d (1H)	Hd	8.2
	unobserved		He	
o-Aminobenzoate	unobserved		Hf	

[a] d, doublet; t, triplet.

of derivative A were the same as those of *o*-aminobenzoate except for 201.3 ppm. These results showed that derivative A had the same structure in the aromatic carbon atoms as *o*-aminobenzoate. Signals with chemical shift values of 176.5 and 201.3 indicated that this compound had a carboxyl group and a ketone group. These results showed that this compound was isatic acid (2-aminobenzoylformic acid).

Based upon the structural analyses of the products, we proposed a pathway for the indole assimilation by the organism (Fig. 1). Indole was first oxygenated at the 2- and 3-positions to form *cis*-indole-2,3-dihydrodiol, then oxidized to form isatin, and subsequently cleaved between 1- and 2-positions to form isatic acid. Microbial indole conversions involve the formation of *cis*-indole-2,3-dihydrodiol by dioxygenation (Eaton and Chapman 1995; Mermod et al. 1986). The putative intermediate, *cis*-indole-2,3-dihydrodiol, was not detected in the present study. This compound seems to be immediately oxidized to isatin by ST-200. Isatin has been reported as an intermediate metabolite during anaerobic or aerobic metabolism of indole (Eaton and Chapman 1995; Madsen and Bollag 1989). Isatic acid was not assimilated as a sole carbon source by ST-200 (results not shown). This organism might assimilate indole as a growth substrate in another degradation pathway. Isatin was unstable in MBS medium and was spontaneously converted to isatic acid. When isatin (0.5 mg/ml) was added to the medium without the organism and incubated at 30°C , more than 85% of the isatin was converted to isatic

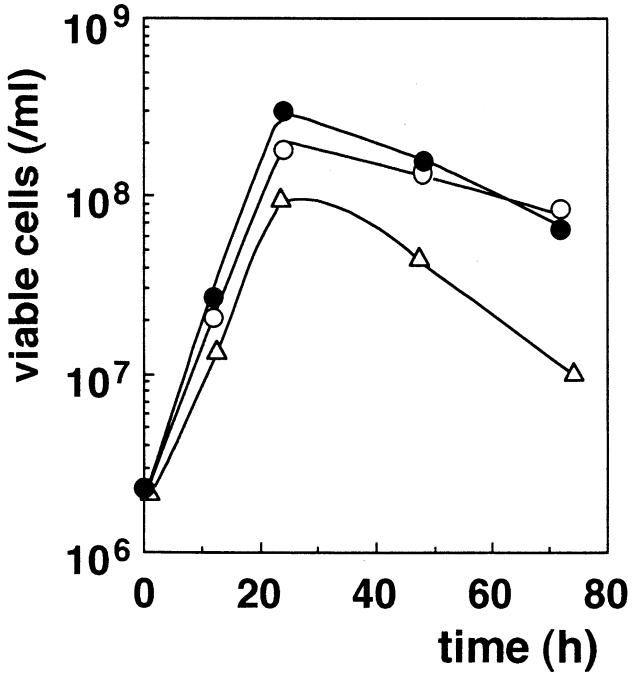


Fig. 2. Growth of *Pseudomonas* sp. ST-200 on indole in the presence of diphenylmethane. To 10 ml of MBS medium, 2 ml of diphenylmethane containing 5 (*closed circles*), 10 (*open circles*), or 20 (*triangles*) mg of indole was added. Strain ST-200 was grown in the medium at 30°C with shaking. Periodically, samples were taken from water phases (0.1 ml) of the cultures. Viable cells in the samples from the water phase were counted from the number of colonies grown on LBG agar medium

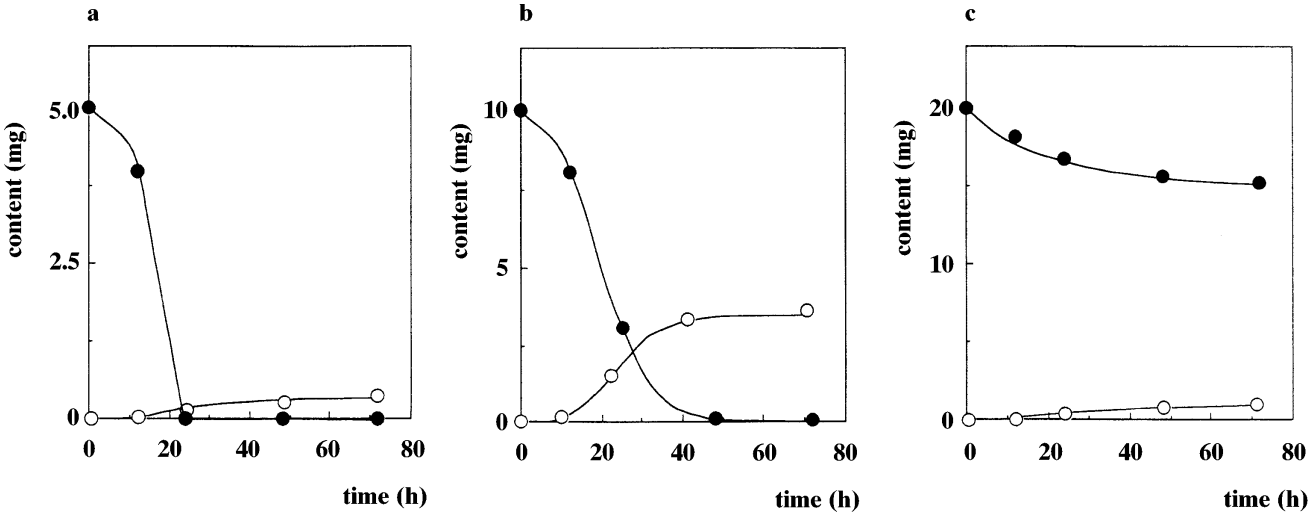


Fig. 3a–c. Consumption of indole in the presence of diphenylmethane. Strain ST-200 was grown at 30°C in 10 ml of MBS medium overlaid with 2 ml of diphenylmethane containing (**a**) 5 mg, (**b**) 10 mg, or (**c**) 20 mg of indole. Periodically, samples were taken from the organic solvent (0.01 ml) and water phases (0.1 ml) of the cultures, respectively. *Closed circles*, indole (sum of two phases); *Open circles*, isatic acid (water phase)

acid after 3 h. These results are coincident with the abundance of isatic acid among the persolvent fermentation derivatives.

Growth of strain ST-200 with indole in MBS medium overlaid with diphenylmethane

Strain ST-200 grew to 2–4 $\times$ 10^8 cells/ml in MBS medium overlaid with diphenylmethane when the initial indole concentration was equivalent to 0.5 or 1 mg/ml (Fig. 2). The number of viable cells decreased with prolonged incubation. The decrease was significant in the culture with indole initially present at 2 mg/ml, with only 1 $\times$ 10^7 cells/ml remaining after 72 h (Fig. 2). Growth was inhibited with this concentration of indole, although the indole concentration was below the MIC in the water phase.

During the indole consumption, isatic acid accumulated in the aqueous medium (Fig. 3). This product was detected after 24 h and reached maximum amounts at 48 h. The amount of isatic acid was equivalent to 9% (initial indole concentration of 0.5 mg/ml), 30% (1 mg/ml), or 4% (2 mg/ml) of the initial indole concentration. The concentration of isatin was below 0.03 mg/ml. No other indole derivative was detected in the organic solvent layer or aqueous layer.

Indole has been degraded at low concentrations to avoid toxicity effects (Eaton and Chapman 1995; Madsen and Bollag 1989; Madsen et al. 1988; Mermod et al. 1986). The indole degradation by persolvent fermentation with *Pseudomonas* sp. strain ST-200 is convenient and effective. This method uses an organic solvent such as diphenylmethane, with a high partitioning power for toxic indole, as a reservoir for indole. As a result, a large amount of indole can be supplied in a small volume of the medium. This may be applicable for microbial degradation of other toxic compounds.

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