Effects of Organic Solvents on Indigo Formation by *Pseudomonas* sp. strain ST-200 Grown with High Levels of Indole

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The indole tolerance level of *Pseudomonas* sp. strain ST-200 was 0.25 mg/ml. The level was raised to 4 mg/ml when diphenylmethane was added to the medium to 20% by volume. ST-200 grown in this two-phase culture system containing indole (1 mg/ml) and diphenylmethane (0.2 ml/ml) produced a water-soluble yellow pigment, isatic acid, and two water-insoluble and diphenylmethane-soluble pigments, blue indigo and purple indirubin. The amounts of the water-insoluble pigments corresponded to 0.5% (indigo) and 0.2% (indirubin) of the indole added to the medium. Of the conditions tried, indigo and indirubin were formed only when ST-200 was grown in the two-phase system overlaid with organic solvents with appropriate polarity.

Key words: bioconversion of toxic compound; indigo formation; organic solvents; persolvent fermentation; *Pseudomonas* sp.

Indigo is one of the world’s largest-selling textile dye and is used on cotton and wool fabrics. Several *Pseudomonas* strains produce indigo from indole or its derivatives. 1,2 Escherichia coli recombinants that carry dioxygenase genes of *Pseudomonas* can synthesize indigo. 3,4 One difficulty hampering the biosynthetic production of indigo involves the toxicity of the substrate, indole. Aromatic N-heterocyclic compounds, including indole, harm microorganisms. 1-7 Therefore, the concentration of indole in the medium must be kept low. Microorganisms can grow in the presence of toxic substrates, including indole, provided that the concentration is low. However, the substrates are readily consumed because little is supplied.

Two-phase bioconversion systems consisting of water and an organic solvent have been used for the conversion of several compounds with low solubility in water. 8-12 Although indole is high polar and soluble in water, aromatic compounds are generally more soluble in organic solvents than in water. When a large volume of an appropriate hydrophobic organic solvent is added to a medium containing indole, the indole concentration in the medium phase decreases because of partition of the indole into the organic solvent phase. As a result, the toxic effect of indole is lessened in two-phase systems with an appropriate organic solvent with a high partition power for indole. 13

The toxicity of organic solvents used in the two-phase system is, as a rule, inversely correlated with the log $P_{ow}$ of the organic solvent. 15,16 We have isolated *Pseudomonas* sp. strain ST-200. 17 This organism is tolerant of cyclohexane (log $P_{ow}$, 3.4) and grows in the presence of an organic solvent with log $P_{ow}$ $\geq$ 3.4. The log $P_{ow}$ is the common logarithm of $P_{ow}$, a partition coefficient of the organic solvent between n-octanol and water. 18 ST-200 grows on indole as the sole carbon source when indole is added to a low concentration. 19 In the presence of an appropriate organic solvent, this organism can grow at levels of indole higher than the minimum inhibitory concentration. In this study, we found that ST-200 produced yellow isatic acid, blue indigo, and purple indirubin when grown in an appropriate two-phase system containing high concentration of indole.

In this report, we describe that indigo formation by ST-200 grown on indole is revealed in the presence of organic solvent phase that weakens the toxic effect of indole.

Materials and Methods

Organism and culture conditions. *Pseudomonas* sp. strain ST-200 17 was grown at 30°C in conversion medium (medium C) consisting of 0.2% Bacto Yeast Extract (Difco Laboratories, Detroit, MI, USA), 1% (w/v) NaCl, 0.05% (NH₄)₂SO₄, and 10 mM MgSO₄. Indole was added to this medium as a major carbon and energy source. The medium was overlaid with a 20% volume of an organic solvent. ST-200 was grown also in mineral basal salts medium (MBS medium) 18 and in LB medium composed of 1% (w/v) Bacto Tryptone (Difco), 0.5% Bacto Yeast Extract and 1% NaCl, and in LBG medium (LB medium with 0.1% glucose). The cultures were shaken at 150 oscillations/min with 4-cm strokes at 30°C. The number of viable cells was counted from colonies grown on LB medium solidified with 1.5% (w/v) agar.

Purification of pigments derived from indole. The organism was grown in a two-phase system consisting of diphenylmethane (80 ml) and medium C (400 ml) containing indole (400 mg). After 3 days, the water and organic solvent phases were separated from each other with a separating funnel. The solvent phase was recovered and dehydrated with anhydrous Na₂SO₄ overnight. A portion (15 ml) of the solution was put on a column (2.2 by 20 cm) of silica gel (90-230 mesh; E.
Merck AG) equilibrated with n-hexane. The column was washed with 200 ml of n-hexane and then eluted with 200 ml of diethyl ether. The eluate was concentrated to 2 ml in a rotary evaporator at 25°C. Compounds in the residual solution were developed zonally on a 2-mm thick silica gel plate (no. 60F254; Merck) in diethyl ether. The pigments were recovered with chloroform from a zone colored blue or purple.

**Estimation of molecular weight of the conversion product.** Mass spectra of samples were measured by low-resolution electron-impact mass spectrometry with a mass spectrometer (model QP-5000; Shimadzu). The samples were ionized at 230°C and at 70 eV.

**Measurement of absorption spectra of indole conversion products.** Samples were dissolved in 95% (v/v) ethanol to 20 μg/ml. Absorption spectra of the solutions were recorded at room temperature.

**Measurement of indole and its conversion products.** A sample obtained from the organic solvent phase was analyzed directly by reverse-phase chromatography on a column (4.6 by 200 mm) of octadecysilica gel (ODS-1201-H; Senshu Science Co. Ltd., Tokyo, Japan) attached to an HPLC apparatus. To determine indole, the column was eluted with n-hexane-isopropanol (10:0.1, v/v) at a flow rate of 1.0 ml/min and the elution was monitored by measurement of A$_{254}$. To determine indigo and indirubin, the column was eluted with chloroform and the elution was monitored by measurement of A$_{260}$ and A$_{550}$, respectively.

A sample (0.1 ml) from the water phase was centrifuged (5,000 × g, 5 min, 4°C). The supernatant was analyzed by normal-phase chromatography on a column (4.6 by 250 mm) of silica gel (Silica-1251-N; Senshu Science). The column was eluted with acetonitrile-isopropanol (10:0.1, v/v) at a flow rate of 1.0 ml/min. The elution was monitored by measurement of A$_{270}$ to determine indole and isatine acid.

**Materials.** Indole, indigo, and isatin were purchased from Nacalai Tesque, Kyoto, Japan. Isatine acid was purified from a culture in which ST-200 was grown on indole as the sole carbon source. The organic solvents used were of the highest quality commercially available. The log P$_{ow}$ values of compounds were calculated by the addition rule with the log P$_{ow}$ calculation software, Clog P version 1.0.3 (BioByte Corp., Claremont, CA, USA).

**Results**

**Identification of the pigments derived from indole**

Absorption spectra of a blue pigment A and purple pigment B purified from the diphenylmethane phase are shown in Fig. 1. Comparison of the λ$_{max}$ of indole (282 nm) and pigments A (600 nm), and B (534 nm) showed that conjugated double bonds in the products increased the π-resonance system. M, of the pigments, 262, were the same, and roughly twice that of indole (M, 117), indicating that the derivatives were produced by the condensation of two molecules of indole.

The m/z values (intensity, proposed composition of ions) of major ions found in the mass spectrum of pigment A were 263 (18%, [M + H]$^+$), 262 (100%, [M]$^+$), 234 (15%, [M−CO]$^+$), 205 (19%, [M−CO−CHO]$^+$), 179 (3%, [M−CO−CHO−C=S]$^+$), 131 (18%, [M/2]$^+$), 103 (26%, [M/2−CO]$^+$), and 76 (26%, [M/2−CO−HCN]$^+$). This mass spectrum was identical with that of authentic indigo, and similar to the spectrum reported for indigo. Major ions of m/z in the mass spectrum of pigment B were 263 (18%, [M + H]$^+$), 262 (100%, [M]$^+$), 234 (56%, [M−CO]$^+$), 205 (31%, [M−CO−CHO]$^+$), 179 (4%, [M−CO−CHO−C=S]$^+$), 131 (15%, [M/2]$^+$), 103 (24%, [M/2−CO]$^+$), and 76 (26%, [M/2−CO−HCN]$^+$). This mass spectrum was similar to that of pigment A, except for intensities of the fragment ions with m/z of 234 and 205. The spectrum was similar to the mass spectrum reported for indirubin. These results showed that the blue pigment was indole and the purple one was indirubin.

Authentic indigo and pigment A were developed on a silica gel thin-layer plate at room temperature in the following solvents: diethyl ether, chloroform, or chloroform-methanol (15:1, v/v). Rf values of the authentic indigo and pigment A were identical in each of the solvent systems: Rf values were 0.43 (diethyl ether), 0.50 (chloroform), and 0.66 (chloroform-methanol). Retention times for the authentic indigo and pigment A were identical; 2.6 min when the compounds were eluted by reverse-phase chromatography with chloroform as the eluent. By silica-gel thin-layer chromatography, Rf values of pigment B were 0.02, 0.29, and 0.35 in these solvent systems respectively, although authentic indirubin was not available. The results described here indicated that ST-200 produced indigo and indirubin as known in strains of *Pseudomonas.*
### Table 1. Effects of Medium Compositions on Indole Consumption and Production of Indole Derivatives

<table>
<thead>
<tr>
<th>Mediuma</th>
<th>Residual indole (mg)</th>
<th>Isistic acid (mg)</th>
<th>Indigo (µg)</th>
<th>Indirubin (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>&lt;0.1</td>
<td>4.6</td>
<td>54</td>
<td>23</td>
</tr>
<tr>
<td>MBS</td>
<td>&lt;0.1</td>
<td>4.0</td>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>LB</td>
<td>7.5</td>
<td>&lt;0.2</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>LBG</td>
<td>7.6</td>
<td>&lt;0.1</td>
<td>2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*a Pseudomonas* sp. strain ST-200 was grown in 10 ml of a medium containing 1 mg/ml indole and 20% (v/v) diphenylmethane at 30°C.  
*b After 72 h, indole and its derivatives were measured as described in Materials and Methods.

Effects of culture conditions on production of indigo and indirubin

(i). Medium composition. The consumption of indole and production of indole derivatives were influenced by the medium composition (Table 1). When ST-200 was grown in a nutritionally poor medium (C or MBS) in the presence of 20% (v/v) diphenylmethane, more than 99% of the indole initially added was consumed by 72 h of culture. About half was assimilated and the other half was converted, mainly to isatic acid. The organism grown in a medium with an abundant carbon source (LB or LBG) consumed less of the indole: About 75% of the indole remained even after 72 h. Production of indole derivatives was low. Moderate amounts of indigo and indirubin were formed only when ST-200 was grown in medium C. We previously noticed the toxicity-lessening effect when certain organic solvents overlay MBS medium containing indole. However, ST-200 hardly produced any pigments in MBS medium. Preliminary results suggested that manganese ions inhibited indigo formation in MBS medium.

(ii). Polarity of organic solvent overlaying the medium. When ST-200 was grown in medium C containing 1 mg/ml indole, indigo and indirubin were produced in the presence of cyclooctane and diphenylmethane (1:1, v/v), diphenylmethane, diphenylmethane and propylbenzene (1:1, v/v) or propylbenzene (Fig. 2). The organism did not grow in the medium overlaid with p-xylene (log $P_{ow}$, 3.1) probably because of toxicity of p-xylene. When the medium was overlaid with no solvent, or a solvent of low toxicity, n-octane (log $P_{ow}$, 4.9) or cyclooctane (log $P_{ow}$, 4.5), the concentration of indole in the medium phase was 1 mg/ml without solvent, 0.7 mg/ml with n-octane, and 0.6 mg/ml with cyclooctane. These indole concentrations exceeded the minimum inhibitory concentration. Therefore, the organism did not grow because of the toxicity of indole partitioned in the medium.

The log $P_{ow}$ of diphenylmethane is 4.2. The log $P_{ow}$ values were estimated to be 4.0 and 4.3 for the solvent mixtures, cyclooctane-diphenylmethane and diphenylmethane-propylbenzene, respectively, on the basis of the calculation rule. ST-200 grew well in the two-phase system containing 1 mg/ml indole when the medium was overlaid with organic solvents with log $P_{ow}$ of 4.0 to 4.3. ST-200 grown under these conditions produced indigo and indirubin from indole. During the cultivation, the organic solvent phases became blue with the indigo converted from indole (Fig. 2, “1 day”). Then, the organic solvent phases turned purple because of the accumulation of indirubin (Fig. 2, “3 days”). The phases of medium became dark yellow because of the formation of isatic acid.

(iii). Effects of amount of indole added to the two-phase system. Pigment formation by ST-200 grown in medium C with different concentrations and organic solvents is summarized in Table II. When the medium was overlaid with cyclooctane, diphenylmethane, or propylbenzene, indigo and indirubin were produced with the amounts depending on the organic solvent used. When ST-200 was grown with no solvent or n-octane, no pigment was formed whatever the amount of indole. Growth yields of ST-200 grown under the different conditions are shown in Table III. It is interesting that ST-200 grown in the presence of propylbenzene and 1.2 mg of indole produced some indigo although the growth yield was low. It was likely that the production of indigo and indirubin depended on the polarity of the organic solvent and the amount of indole rather than the growth yield. Strain ST-200 grown in the two-phase system containing indole (1 mg/ml) and diphenylmethane (0.2 ml/ml) produced more indigo and indirubin than the other...
Table II. Production of Indigo and Indirubin by Strain ST-200

<table>
<thead>
<tr>
<th>Solventa</th>
<th>( \log P_{cw} )</th>
<th>Indole added to 10 ml of medium (mg) ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>(&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01)</td>
</tr>
<tr>
<td>(n)-Octane</td>
<td>4.9</td>
<td>(&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01)</td>
</tr>
<tr>
<td>Cyclooctane</td>
<td>4.5</td>
<td>0.5  0.6  0.01  0.01  0.01  0.01  0.01</td>
</tr>
<tr>
<td>Diphenylmethane</td>
<td>4.2</td>
<td>5.5  2.0  0.01  0.01  0.01  0.01  0.01</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>3.7</td>
<td>(&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01) (&lt; 0.01)</td>
</tr>
</tbody>
</table>

\(^a\) Indole (amounts shown) was added to 10 ml of medium C overlaid with 2 ml of an organic solvent. \(Pseudomonas\) sp. strain ST-200 was grown as described in Table I.

\(^b\) Indigo and indirubin were assayed after 72 h of incubation. The amounts of indigo and indirubin are shown in brackets and parentheses, respectively.

Table III. Growth of Strain ST-200 in the Presence of Indole and Organic Solvents

<table>
<thead>
<tr>
<th>Solventa</th>
<th>( \log P_{cw} )</th>
<th>Indole added to 10 ml medium (mg) ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1.50  1.42  0.62  —  —  —  —</td>
</tr>
<tr>
<td>(n)-Octane</td>
<td>4.9</td>
<td>1.42  1.42  0.56  —  —  —  —</td>
</tr>
<tr>
<td>Cyclooctane</td>
<td>4.5</td>
<td>1.40  1.40  1.42  0.55  —  —  —</td>
</tr>
<tr>
<td>Diphenylmethane</td>
<td>4.2</td>
<td>1.40  1.42  1.40  1.42  1.35  0.60  0.16</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>3.7</td>
<td>1.00  0.75  0.40  0.26  0.19  0.08  —  —</td>
</tr>
</tbody>
</table>

\(^a\) \(Pseudomonas\) sp. strain ST-200 was grown in 10 ml of medium C containing indole and with an organic solvent as described in footnote of Table II.

\(^b\) The growth was measured in terms of \(OD_{660}\) after 72 h incubation at 30°C. — shows no growth (\(< OD_{660}\) of 0.01).

conditions used.

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

The viable cell density increased to \(4 \times 10^8/\text{ml}\) in medium C containing 1 mg/ml indole and 20% (v/v) diphenylmethane (Fig. 3). The density increased to \(9 \times 10^8/\text{ml}\) without indole in the presence or absence of diphenylmethane. The number of viable cells decreased with prolonged incubation. The decrease was greatest when ST-200 was grown with indole: at 96 h, there were only \(9 \times 10^8\) viable cells per milliliter. The decrease in the number of viable cells was less when indole was absent.

ST-200 grown with indole and diphenylmethane accumulated isatic acid in the aqueous phase (Fig. 4). This product reached 4.6 mg/ml by 72 h. The amount accounted for 33% of the indole initially added. A minor product, isatin, also was found in the aqueous phase, at a concentration of less than 0.03 mg/ml.

Indigo and indirubin accumulated in the diphenylmethane phase. The amount of indigo stopped increasing when the indole was almost consumed. The amount of indigo reached 62 µg by 48 h and decreased thereafter. This reduction of indigo might be due to further conversion of indigo to form isatin.\(^4\) ST-200 grown with indole decomposed authentic indigo to isatic acid slowly (results not shown). At 48 h, the amount of indigo was equivalent to 0.6% of the indole initially added; indirubin was found at 48 h. At the time, only 0.2 mg/ml of indole remained. Formation of indirubin continued although the concentration of indole was low. By 72 h, the amount reached 23 µg and corresponded to 0.2% of the indole initially added.

Discussion

We propose the pathway from indole to the pigments to be as shown in Fig. 5, based on structural analysis. Indole is oxygenated first at the 2- and 3-positions to form cis-indole-2,3-dihydrodiol. This hydroxylated compound is oxidized mainly to form isatin, and subsequently cleaved between the 2- and 3-positions to form isatic acid. Microbial conversions of indole progress through formation of cis-indole-2,3-dihydrodiol.\(^4\) Spontaneous dehydration of cis-indole-2,3-dihydrodiol results in the formation of 3-hydroxyindole (indoxyl) and 2-hydroxyindole (oxindole). Condensation of two molecules of indoxyl followed by oxidation in the air yields indigo, and condensation of indoxyl with oxindole yields indirubin.\(^4\) We attempted but failed to detect the putative intermediates cis-indole-2,3-dihydrodiol, oxindole, or indoxyl. These compounds might be unstable or immediately oxidized to indigo and indirubin.

Several microorganisms produce indigo from indole or its derivatives. These toxic substrates must be sup-
Fig. 3. Growth of \textit{Pseudomonas} sp. Strain ST-200 in the Presence of Diphenylmethane and Indole.

Strain ST-200 was grown in 10 ml of medium C containing 10 mg of indole and 2 ml of diphenylmethane. This culture was shaken at 150 oscillations/min with 4-cm strokes at 30°C. At times, a 0.1-ml sample was taken from the water phase of the culture. Viable cells in the sample were counted in terms of the number of colonies that grew on LBG agar medium. Symbols: ○, without a solvent nor indole; ●, with diphenylmethane but without indole; ▲, with both diphenylmethane and indole.

Fig. 4. Production of Indigo and Indirubin in the Presence of Indole and Diphenylmethane.

Strain ST-200 was grown at 30°C in 10 ml of medium C containing 10 mg of indole and 2 ml of diphenylmethane. At times, sample was taken from the organic solvent (0.01 ml) and water (0.1 ml) phases of the culture. These samples were assayed for pigments. Symbols: ●, indole; ○, isatic acid; ▲, indigo; △, indirubin.

Fig. 5. Putative Pathway in \textit{Pseudomonas} sp. Strain ST-200 from Indole to Pigments.

PLIED at low concentrations to avoid toxic effect. In two-phase systems, indole dissolved in the water phase can be partitioned between the water and organic solvent phases. Among hydrophobic organic solvents, high polar one reduced the concentration of indole in the water phase by such partitioning.\(^\text{10}\) However, such organic solvents can be toxic to microorganisms when added to the medium in large amounts.\(^\text{15,16}\) Diphenylmethane is an appropriate solvent when ST-200 is grown in a two-phase system containing a high concentration of indole, because the solvent partitions indole well and is not strongly toxic to ST-200.\(^\text{14}\)

In this study, diphenylmethane was used as a reservoir for a high polar substrate, indole (log \(P_{ow}\), 2.1), to reduce its concentration in the medium. A large amount of indole was supplied in a small volume of medium at one time. Without any organic solvent, ST-200 cannot grow on indole at such a high concentration. ST-200 grown at low levels of indole without an added organic solvent produced isatic acid (results not shown) but not indigo or indirubin. The organism produced indigo and indirubin when a much indole was added to the two-phase system consisting of medium C and diphenylmethane. The further modifications are needed if indigo is to be produced effectively.

The results described here give two important suggestions. First, two-phase fermentation with a suitable organic solvent can bring out some dormant property of microorganisms. Various toxic substrates have been converted microbiologically. Most of these toxic substrates is high polar and soluble in water. The next suggestion is that the two-phase fermentation system with an appropriate organic solvent could be applied for biodesorption of polar toxic substrates because the system could weaken the toxic effects by lowering concentrations of the substrates in an aqueous medium.

References