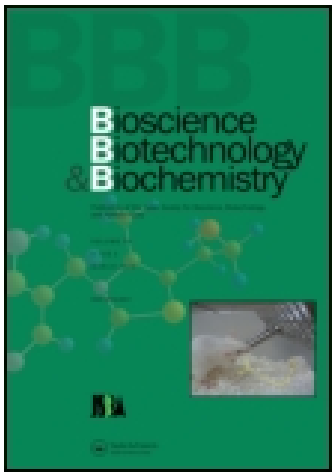


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# Formation of 5,10-Dihydrophenazine from Phenazine by Pseudomonas cepacia IFO 15124 at Low Oxygen Tensions

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*Note*

# Formation of 5,10-Dihydrophenazine from Phenazine by *Pseudomonas cepacia* IFO 15124 at Low Oxygen Tensions

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**5,10-Dihydrophenazine ($H_2$Phen) was formed from phenazine (Phen) by *Pseudomonas cepacia* IFO 15124 in growing cultures at low oxygen tensions. Effects of culture conditions on microbial reduction of Phen with this strain were investigated. Under optimized conditions, the transformation of Phen to $H_2$Phen by this strain gave the molar conversion yield of 30%. However, $H_2$Phen was not detected in the culture medium when the strain was incubated with Phen with sufficient aeration.**

**Key words:** phenazine; 5,10-dihydrophenazine; *Pseudomonas cepacia*; low oxygen tensions

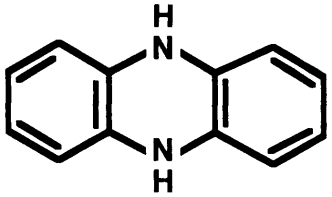


Phenazine (Phen) and its derivatives have antibiotic activity[1] or antiviral and antitumor activities,[2] and are quite resistant to microbial attack. There have been a few reports on the microbial oxidation of Phen and its derivatives[3] but little has been known about the microbial reduction of Phen except for its derivatives.[4] Therefore, we attempted to transform heterocyclic aromatic hydrocarbons and nondegrable molecules, such as Phen and carbazole. Through studies on microbial transformation of Phen, we found that 5,10-dihydrophenazine ($H_2$Phen) was formed from Phen as a product of *Pseudomonas cepacia*. Although a number of workers[5–8] have reported the photochemical and chemical reactions of Phen and its derivatives, no study on the microbial formation of $H_2$Phen from Phen has been done. $H_2$Phen has been used as an interesting starting material for synthesis of polymers such as a charge transferring dye of organic solar cells and an electric rectifier.[9–11] $H_2$Phen derivatives are also interesting compounds for their potential photochemical use. In this paper, we report the identification of the product as $H_2$Phen and some factors affecting the formation of $H_2$Phen from Phen by this strain.

*Pseudomonas cepacia* IFO 15124 was obtained from the Institute for Fermentation, Osaka (Japan). Cultures were maintained on nutrient agar slants and transferred monthly. The compositions of the various media are as follows (per liter of distilled water). TYG: tryptone (Difco), 5 g/liter; yeast extract, 5 g/liter; glucose, 1 g/liter; and $K_2HPO_4$, 2 g/liter (pH 7.2). GM: glucose, 10 g/liter; $NH_4Cl$, 1 g/liter; $Na_2HPO_4$, 4 g/liter; $KH_2PO_4$, 15 g/liter; and $MgSO_4 \cdot 7H_2O$, 0.2 g/liter (pH 7.2). NB: meat extract, 5 g/liter, peptone, 5 g/liter; NaCl, 15 g/liter; and $K_2HPO_4$, 5 g/liter (pH 7.2). Microbial transformation of Phen was done in TYG, NB, and GM media with Phen. *P. cepacia* was grown in 10 ml of TYG, NB, and GM media in a 50 ml Erlenmeyer flask with a sealed cap or cotton plug at 30°C in the dark on a rotary shaker operating at 150 rpm. Seed cultures were prepared by inoculating cells grown on an agar slant into a 50-ml Erlenmeyer flask containing 10 ml of a seed culture medium (TYG, NB, or GM medium) followed by incubation at 30°C for 2 days on a rotary shaker operating at 150 rpm. One ml of seed culture was transferred to each of 50-ml Erlenmeyer flasks containing 10 ml of TYG, NB, or GM medium with 0.4% (w/v) Phen and incubated on a rotary shaker in the dark at 30°C for 5 days. The effects of oxygen concentration on the formation of $H_2$Phen were examined by changing the volume of the culture medium.

The cell growth was measured as OD at 600 nm with a colorimeter after the culture broth was diluted 10-fold with distilled water. Shake flask cultures were diluted 5-fold with dimethyl sulfoxide (DMSO) and centrifuged. Distilled water was added to supernatants to give dark purple crystals. The crystals were collected by centrifugation, washed several times with distilled water, and dried in a vacuum oven at 40°C. The structure of the crystals was analyzed by $^1$H-NMR and $^{13}$C-NMR with a Bruker AC-400 spectrometer in DMSO-$d_6$. Since purification of each Phen to measure its molar absorbance coefficients was impossible, we confirmed only the formation of $H_2$Phen by NMR or UV spectral analyses. Therefore, the amounts of Phen and $H_2$Phen in the culture broth were analyzed by $^1$H-NMR with a Bruker AC-400 spectrometer in DMSO-$d_6$. Organic acids in the culture were analyzed by a Hitachi HPLC with a Shodex Ionpak KC-811 column at flow rate 1.0 ml/min with 10 mM phosphoric acid, and the $A_{210}$ was measured.[12]

Sixty-five strains of microorganisms maintained in our laboratory were screened for transformation of Phen. Among all the microbes examined, only one strain, *P. cepacia* IFO 15124 was found to transform Phen to $H_2$Phen by UV spectral analyses. We found the formation of dark purple crystals of *P. cepacia* IFO 15124 with Phen in growing cultures. The product was identified by $^1$H- and $^{13}$C-NMR spectra. NMR data were as follows. $^1$H-NMR (400 MHz, DMSO-$d_6$): $\delta = 7.27$ (2H, s, NH), 6.24 (4H, dd, $J = 5.5$, 3.4 Hz, 1,4,6,9-ArH), 6.00 (4H, dd, $J = 5.5$, 3.4 Hz, 2,3,7,8-ArH). $^{13}$C-NMR (101 MHz, DMSO-$d_6$): $\delta = 133.9$ (C4a, C5a, C9a, C10a), 120.4 (C1, C4, C6, C9), 111.5 (C2, C3, C7, C8). Furthermore, upon treatment of the crystals with acid, such as HCl or acetic acid, green solution of protonated semiquinone radical, 5,10-dihydrophenazinium radical cation, characterized by its UV spectra was produced.[13] From the above results, the dark




$H_2$Phen

**Fig.**

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*Abbreviations*: Phen, phenazine; $H_2$Phen, 5,10-dihydrophenazine.

**Table I.** Effects of Oxygen Concentration on $H_2$Phen Formation from Phen

| Vol. (ml) | Final pH | $OD_{600}$ | $H_2$Phen (mg/ml)[a] | Mol. yield (%) |
|---|---|---|---|---|
| 5 | 7.1 | 4.31 | 0.5 | 12.3 |
| 10 | 6.9 | 2.12 | 1.1 | 27.2 |
| 20 | 6.9 | 1.53 | 0.9 | 22.1 |
| 30 | 6.5 | 1.04 | 0.8 | 19.7 |
| 50 | 6.1 | 0.51 | 0.6 | 14.8 |

[a] The cultivation was done by varying the volumes of the culture medium containing 4 mg/ml Phen from 5 to 50 ml in every 50-ml Erlenmeyer flask with shaking in the dark. The flask was sealed with a screw cap. The culture was incubated at 30 °C for 5 days.

**Table II.** Effects of Phen Concentration on $H_2$Phen Formation

| Phen (mg/ml) | Final pH | $OD_{600}$ | $H_2$Phen (mg/ml)[a] | Mol. yield (%) |
|---|---|---|---|---|
| 0 | 6.9 | 1.34 | 0 | 0 |
| 5 | 6.8 | 2.21 | 1.7 | 33.6 |
| 10 | 6.9 | 2.10 | 3.1 | 30.7 |
| 15 | 6.9 | 2.10 | 0.7 | 4.6 |
| 20 | 6.9 | 2.04 | 0.3 | 1.5 |

[a] The cultivation was done by varying Phen concentration from 5 to 20 mg/ml. The culture was incubated at 30 °C for 5 days with shaking in the dark. The flask containing 10 ml of the culture was sealed with a screw cap.

purple crystals were confirmed to be $H_2$Phen formed from Phen by *P. cepacia* IFO 15124. For this reduction of Phen by this strain the mechanism is being investigated. The formation of complexes between Phen and $H_2$Phen has been observed to occur in the solid state by Toromanoff,[14] who photolyzed concentrated solutions in alcoholic solvents, and Bailey *et al.*,[13] who examined the photochemical reaction of Phen in weakly and strongly acidic media.

Effects of various media on $H_2$Phen formation were examined. Since TYG medium gave the highest yield of $H_2$Phen formation, it was used as the basal medium in this transformation (data not shown). Effects of pH on $H_2$Phen formation were examined by using buffers (50 mM) of different pHs in TYG medium, and the maintenance of the pH of TYG medium at 6.0–7.2 was found to be essential for $H_2$Phen formation. Table I shows that low oxygen tensions were very effective in increasing the formation of $H_2$Phen. In contrast, $H_2$Phen was not formed when the cultures were incubated with Phen with sufficient aeration (data not shown). Through the analysis of culture broth, lactic acid was detected (total: 1 mM) only in the culture broth incubated in 50 ml of TYG medium in a 50-ml Erlenmeyer flask (Table I). This broth containing lactic acid without cells did not react with Phen to form $H_2$Phen under identical physical conditions. To examine for lysis of *P. cepacia* in liquid media with Phen, visible colonies of this strain on TYG plates were counted. Autolyzation of the cells to growing in the presence of Phen at low oxygen tensions was not observed. Therefore, it seemed likely that $H_2$Phen was formed from Phen by this strain at low oxygen tensions. The entrance of oxygen was restricted to initiate the microbial transformation of Phen. To start the microbial transformation, the flask was sealed to restrict the entrance of oxygen. The formation of $H_2$Phen was mostly dependent on whether the entrance of oxygen is depressed or not. Table II shows that a maximum $H_2$Phen formation of 3.1 mg/ml with the molar conversion ratio of 30% was reached at the initial concentration of 10 mg/ml of Phen after 5 days of cultivation and $H_2$Phen formation decreased with increasing the initial concentration of Phen. Further increase of cultivation time did not substantially affect the maximum amount of $H_2$Phen formed. We could not find any products derived from Phen except for $H_2$Phen. The stimulation of growth of *P. cepacia* was induced by the addition of Phen to the culture medium. However, the degradation of Phen and $H_2$Phen in the culture by this strain was negligible after 5 days of cultivation. This remains to be investigated.

Microorganisms use many types of naturally occurring organic compounds and synthetic chemicals for their growth. To our knowledge this is the first report on the microbial reduction of Phen. Further study is needed to discover correlations between Phen/$H_2$Phen and its related enzymes in this strain at low oxygen tensions. The metabolism of *Pseudomonas* is typically respiratory with oxygen as the terminal electron acceptor, but many species can also use nitrate as an alternate electron acceptor. There is some possibility that a peculiar adaptation to low oxygen stress on *P. cepacia* leads to the formation of $H_2$Phen from Phen in order to maintain its growth.

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