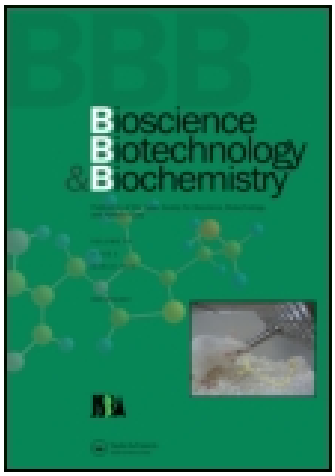


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# Production of 6-Hydroxynicotinic Acid from Nicotinic Acid by Resting Cells of *Pseudomonas fluorescens* TN5

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**Resting cells of *Pseudomonas fluorescens* TN5 catalyze the hydroxylation at the 6-position of nicotinic acid to produce 6-hydroxynicotinic acid. We optimized the reaction and culture conditions for the production of 6-hydroxynicotinic acid from nicotinic acid by using *P. fluorescens* TN5 resting cells. The addition of molybdenum and iron ions and nicotinic acid as an inducer to the culture medium significantly enhanced the formation of the hydroxylation enzyme. The supply of oxygen was important for the enhancement of hydroxylation activity. Under the optimum conditions, 98.7% of the added 1.4 M nicotinic acid was converted to 6-hydroxynicotinic acid, and the highest yield was 191 g of 6-hydroxynicotinic acid per liter of reaction mixture containing 7.43 g dry weight of cells in 45 h at 35°C.**



Microbial hydroxylation of aromatic and heterocyclic compounds catalyzes the position-specific and stereospecific reactions efficiently.[1-10] The hydroxylation reaction has been attempted to produce useful compounds in the field of medicine and for chemical industries. 6-Hydroxynicotinic acid serves as a versatile building block chiefly in the synthesis of modern insecticides.[11-15] For example, 6-hydroxynicotinic acid is an important intermediate in the synthesis of imidachloprid, a potential new insecticide.[11,13,16] Recently the accumulation of 6-hydroxynicotinic acid by *Achromobacter xylosoxidans* LK1 has also been reported.[17] Studies on the microbial production of 6-hydroxynicotinic acid seem to be in progress in a Swiss firm on an industrial scale.[18-19]

We surveyed the conversion of nicotinic acid into 6-hydroxynicotinic acid by various bacteria. *Serratia marcescens*, *Comamonas acidovorans*, and *Pseudomonas fluorescens* were found to accumulate much 6-hydroxynicotinic acid.[20] In this study, we searched for optimum reaction and culture conditions for *P. fluorescens* TN5, and described the microbial conversion of nicotinic acid into 6-hydroxynicotinic acid using *P. fluorescens* TN5 resting cells.

## Materials and Methods

*Microorganism, media, and growth conditions.* *P. fluorescens* TN5 was isolated from a soil sample and then identified in our laboratory for use in this study.[20] Subculture was done at 28°C for 24 h with reciprocal shaking in a test tube containing 3 ml of medium comprising 0.5 g of Polypepton (Daigo, Osaka, Japan), 50 mg of yeast extract (Oriental Yeast, Tokyo, Japan), 0.5 g of meat extract (Mikuni, Tokyo, Japan), and 0.2 g of NaCl per 100 ml of tap water (pH 7.0). Six ml of subculture was then inoculated into a 3-liter shaking flask containing 300 ml of the medium to be examined. Each cultivation was done at 28°C with rotary shaking (120 rpm). The following media were used: Medium I comprised 0.5 g of yeast extract, 0.3 g of nicotinic acid, 0.1 g of $K_2HPO_4$, and 1 ml of metal solution per 100 ml of tap water; Medium II comprised 1.0 g of malic acid, 0.3 g of nicotinic acid, 50 mg of yeast extract, 0.1 g of $K_2HPO_4$, and 1 ml of metal solution per 100 ml of tap water; Medium III comprised 1.0 g of nicotinic acid, 1.0 g of meat extract, 0.1 g of yeast extract, 1.0 g of malic acid, 0.1 g of $K_2HPO_4$, and 1 ml of metal solution per 100 ml of tap water. Medium IV comprised 1.0 g of sodium glutamate, 1.0 g of malic acid, 1.0 g of nicotinic acid, 0.1 g of yeast extract, 0.1 g of $K_2HPO_4$, and 1 ml of metal solution per 100 ml of distilled water. The pH of each medium was adjusted to 7.0 with 3 N NaOH. The metal solution comprised 40 mg of $CaCl_2 \cdot 2H_2O$, 50 mg of $H_3BO_3$, 4 mg of $CuSO_4 \cdot 5H_2O$, 10 mg of KI, 20 mg of $FeSO_4 \cdot 7H_2O$, 40 mg of $MnSO_4 \cdot 7H_2O$, 40 mg of $ZnSO_4 \cdot 7H_2O$, 20 mg of $H_2MoO_4 \cdot 2H_2O$, 5 g of $MgSO_4 \cdot 7H_2O$, and 2 ml of conc. HCl per 100 ml of distilled water.

Cell growth of *P. fluorescens* TN5 was estimated turbidimetrically using a dry cell calibration curve with absorbance at 610 nm: 0.69 mg dry cell weight/ml was equivalent to 1.0 unit of optical density at 610 nm.

*Enzyme assay.* Hydroxylation of nicotinic acid to 6-hydroxynicotinic acid was done in a 50-ml beaker (3.5 × 6.0 cm) containing 2 ml of reaction mixture. The reaction mixture comprised of 400 μmol of nicotinic acid, 170 μmol of potassium phosphate buffer (pH 6.5), and an appropriate amount of cell suspension. The reaction was done at 35°C for 30 min with reciprocal shaking (160 strokes per min at 3.5 cm amplitude) and stopped by adding 1 ml of acetonitrile to the reaction mixture.

One unit of hydroxylation activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of 6-hydroxynicotinic acid per min under the standard assay conditions. Specific activity was expressed as units per mg dry cells.

However, for the optimization of the reaction conditions, pH, frequency of shaking, substrate concentration, and cell concentration in each experiment were varied, depending upon the reaction conditions to be examined. The pH of the reaction mixture was adjusted to 5.4 with 0.1 M sodium acetate buffer, to 6.0, 6.5, and 7.0 with 0.1 M potassium phosphate buffer, to 8.3 and 9 with 0.1 M Tris/HCl buffer, and to 10 with 0.1 M sodium borate buffer.

Nicotinic acid and 6-hydroxynicotinic acid were measured by analytical HPLC done with a Jasco (Tokyo, Japan) 880-PU system with an M & S pack $C_{18}$ column (reverse-phase, 4.6 × 150 mm; M & S Instruments, Tokyo, Japan) at the rate of 1.0 ml/min by use of the following solvent system: 10 mM $KH_2PO_4$–$H_3PO_4$ (pH 2.5)/acetonitrile (98/2, by volume). The eluate was monitored at 210 nm with a Jasco Uvidec 100-V detector.

## Results and Discussion

### *Optimization of resting cells reaction for the production of 6-hydroxynicotinic acid*

Various reaction conditions for the efficient production of 6-hydroxynicotinic acid were studied using resting cells prepared from the culture broth of medium I with 1.0% (w/v) malic acid (specific activity: 1.10 units/mg dry cell weight).

The hydroxylation reaction was done under various pHs as described in Materials and Methods. The optimum pH was between 6.5 and 7.0 with 0.1 M potassium phosphate

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buffer. The effects of various kinds of buffer on the hydroxylation reaction were examined using 0.1 M Hepes/NaOH buffer (pH 6.5), 0.1 M Mes/NaOH buffer (pH 6.5), and 0.1 M citric acid/$Na_2HPO_4$ buffer (pH 6.5). No prominent influence was observed using the different kinds of buffers.

To discover the effects of the supply of oxygen on the hydroxylation reaction, both the amount of cells in the reaction mixture and the shaking frequency were varied. The formation of 6-hydroxynicotinic acid increased in proportion to the amount of resting cells in the reaction mixture, only when frequency of skaking was 160 strokes per min (Fig. 1). On the other hand, even when a great quantity of resting cells was added to the reaction mixture, the formation of 6-hydroxynicotinic acid was not enhanced as long as the aeration was not enough. It is strongly suggested that oxygen in the air is very important for the hydroxylation reaction of nicotinic acid and a full supply of oxygen is required for the full hydroxylation activity.

The effects of nicotinic acid concentration on hydroxylation activity were examined (Fig. 2). The initial synthesis rate of 6-hydroxynicotinic acid was gradually inhibited at concentrations higher than 200 mM. Thus, 200 mM nicotinic acid was selected as the most suitable concentration for the reaction.

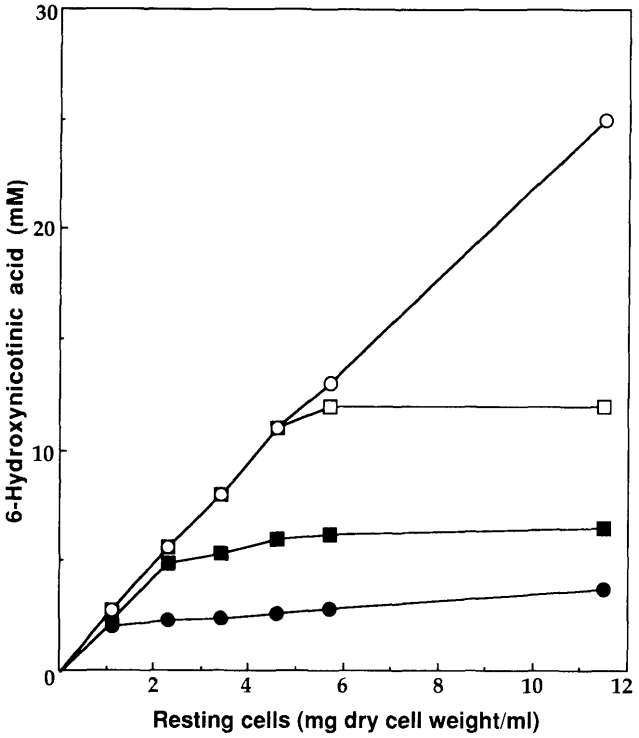


**Fig. 1.** Effects of Aeration on Nicotinic Acid Hydroxylation by Resting Cells of *P. fluorescens* TN5.

Reactions were done under the standard assay conditions except that frequency of shaking was varied. (○), 160; (□), 100; (■), 50; (●), 0 (strokes per min at 3.5 cm amplitude).

*Optimization of culture medium*

We examined the effects of various carbon sources on the formation of the hydroxylation enzyme. The following carbon sources were tested: malic acid, fumaric acid, acetic acid, citric acid, succinic acid, tartaric acid, glucose, sucrose, maltose, fructose, sorbose, soluble starch, dextrin, glycerol, ethanol, and methanol. Each carbon source was added to medium I at a final concentration of 1.0% (w/v) and cultivation was done at 28°C for 28 h. The addition

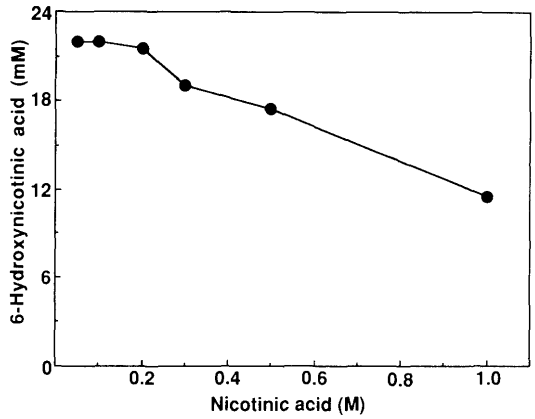


**Fig. 2.** Effects of Concentration of Nicotinic Acid on 6-Hydroxynicotinic Acid Production.

The concentration of nicotinic acid in the standard reaction mixture (2 ml) containing 1.47 unit, was varied as indicated. After the incubation for 30 min at 35 C, 6-hydroxynicotinic acid formed was compared.

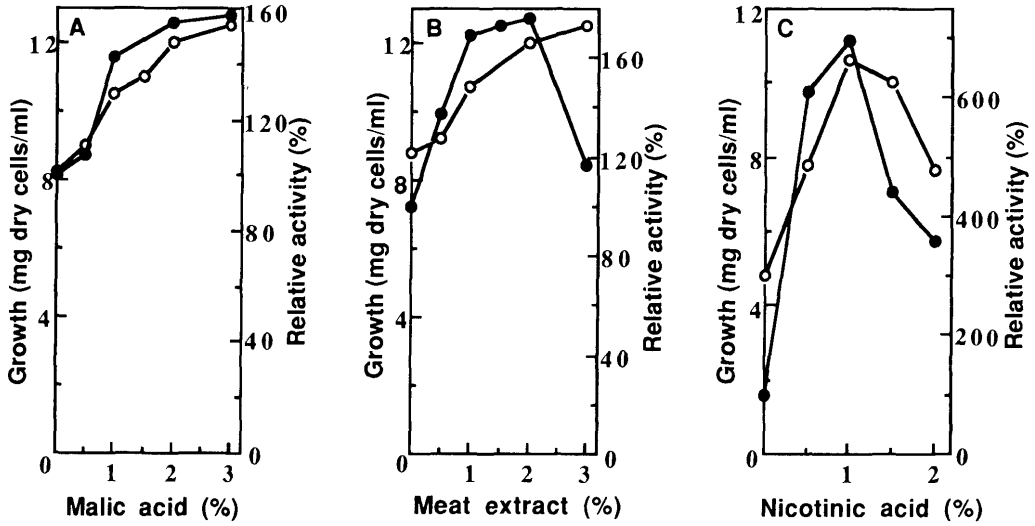


**Fig. 3.** Effects of Various Concentrations of (A) Malic Acid, (B) Meat Extract, and (C) Nicotinic Acid on the Formation of Hydroxylation Enzyme by *P. fluorescens* TN5.

The concentrations of malic acid, meat extract, and nicotinic acid in medium III were varied as indicated in (A), (B), and (C), respectively. Growth, (○); relative activity, (●).

of malic acid, fumaric acid, or succinic acid promoted both growth and total activity. Among them, the addition of malic acid resulted in the highest total and specific activity. These results may be related to fact that malic acid is an intermediate in the degradation pathway of nicotinic acid.[1] Although glucose and methanol promoted the growth, the formation of the hydroxylation enzyme was significantly repressed. The optimum concentration of malic acid in the medium was examined. As shown in Fig. 3A, hydroxylation activity increased with an increase in malic acid concentration, reaching a maximum at 3% (w/v). However, the concentration of malic acid was decided to be 1.0% (w/v), since there was no significant difference in hydrolyation activity between 1.0% (w/v) and 3.0% (w/v).

The effects of various nitrogen sources on the formation of the hydroxylation enzyme were examined using medium II. Cultivation was done at 28°C for 28 h. The addition of meat extract, yeast extract, or sodium L-glutamate promoted enzyme formation. Among them, the addition of meat extract gave the highest total activity. Inorganic nitrogen sources such as $(NH_4)_2SO_4$, $NH_4Cl$, and $NH_4NO_3$ and corn steep liquor were not suitable for the enzyme formation, although *P. fluorescens* TN5 grew well on these media. The optimum concentration of meat extract was examined (Fig. 3B). Hydroxylation activity increased with an increase in meat extract concentration, reaching a maximum at 2.0% (w/v). However, the concentration of meat extract was decided to be 1.0% (w/v), since there was no significant difference in hydroxylation activity between 1.0% (w/v) and 2.0% (w/v).

We examined the effects of various pyridine-related compounds as an inducer on the formation of hydroxylation enzyme. As shown in Table I, 6-hydroxynicotinic acid was the best inducer with respect to the specific activity. However, the highest enzyme activity per culture broth (units/ml) was observed with the addition of nicotinic acid. Higher specific activity with 6-hydroxynicotinic acid compared to nicotinic acid, is ascribed to its low growth rather than its high enzyme activity. It is interesting that nicotinamide and 6-hydroxynicotinic acid are good inducers despite the fact they do not act as substrates for hydroxylation enzyme of *P. fluorescens* TN5, including the fact that nicotinamide was not readily hydrolyzed to nicotinic acid by this strain. The optimum concentration of nicotinic acid was examined (Fig. 3C). The hydroxylation activity increased with an increase in nicotinic acid concentration, reaching a maximum at 1.0% (w/v).

To examine the effects of various metal ions on hydroxylation activity, *P. fluorescens* TN5 was cultivated in medium IV from which each indicated metal ion was omitted, and hydroxylation activity was assayed. As shown in Table II, the omission of magnesium or iron ion inhibited growth and the omission of magnesium, molybdenum, or iron ion in the medium significantly decreased hydroxylation activity. Particularly, the osmission of iron or molybdenum ion provoked the low specific activity. The omission of molybdenum ion seems to influence the hydroxylation activity rather than the growth. These results reflect the fact that molybdenum and iron ions are essential to the hydroxylation reaction. A similar effect of molybdenum ion in the culture medium on the hydroxylation activity was reported with *Bacillus niacini*.[21]

As for the preparation of the resting cells with high hydroxylation activity, optimum medium (medium III) comprising 1.0 g of nicotinic acid, 1.0 g of meat extract 0.1 g of yeast extract, 1.0 g of malic acid, 0.1 g of $K_2HPO_4$, and 1 ml of metal solution per 100 ml of tap water (pH 7.0) has been established.

*Course of hydroxylation activity by P. fluorescens TN5*

*P. fluorescens* TN5 was cultivated at 28°C in medium III (Fig. 4). The cell mass continued to increase for 30 h, and hydroxylation activity was highest at 28 h. The highest accumulation of 6-hydroxynicotinic acid (70 mM) was

**Table I.** Effects of Pyridine-related Compounds on the Formation of Hydroxylation Enzyme by *P. fluorescens* TN5

Nicotinic acid in medium III was replaced with various pyridine-related compounds. The indicated each compound was added at the final concentration of 0.3% (w/v). The pH of each medium was adjusted to with 3 N NaOH. *P. fluorescens* TN5 was cultivated in a 500-ml shaking flask containing 60 ml of medium, and incubation was done at 28 C for 24 h with reciprocal shaking.

| Compounds | Growth (mg dry cell weight/ml culture broth) | Enzyme activity (units/ml culture broth) | Specific activity (units/mg dry cell weight) |
|---|---|---|---|
| None | 2.5 | 0.24 | 0.096 |
| Nicotinic acid | 6.0 | 1.65 | 0.275 |
| Nicotinamide | 4.7 | 1.25 | 0.266 |
| 6-Hydroxynicotinic acid | 2.6 | 1.33 | 0.511 |
| 2-Hydroxynicotinic acid | 2.9 | 0.28 | 0.096 |
| Isonicotinic acid | 3.1 | 0.10 | 0.032 |
| Isonicotinamide | 2.8 | 0.13 | 0.046 |
| Picolinic acid | 2.4 | 0.07 | 0.029 |
| 3-Cyanopyridine | 2.8 | 0.07 | 0.025 |
| Pyrazine carboxylic acid | 3.8 | 0.11 | 0.029 |
| Pyrazinamide | 3.2 | 0.11 | 0.034 |
| Picolinamide | 2.5 | 0.15 | 0.060 |
| 3-Pyridine sulfonic acid | 2.4 | 0.22 | 0.092 |
| Quinolinic acid | 2.5 | 0.17 | 0.068 |
| 2-Hydroxypyridine | 2.5 | 0.13 | 0.052 |
| 3-Hydroxypyridine | 2.3 | 0.08 | 0.035 |

**Table II.** Effects of Metal Ions on the Formation of Nicotinic Acid Hydroxylation Enzyme by *P. fluorescens* TN5

*P. fluorescens* TN5 was cultivated in a 500-ml shaking flask containing 60 ml of medium IV from which each indicated metal ion was omitted, and incubation was done at 28°C for 28 h with reciprocal shaking.

| Omission of metal ion(s) from metal mixture | Growth (mg dry cell weight/ml culture broth) | Specific activity (units/mg dry cell weight) | Relative activity (%) |
|---|---|---|---|
| None | 13.1 | 0.244 | 100* |
| $Zn^{2+}$ | 11.5 | 0.278 | 100 |
| $Cu^{2+}$ | 12.4 | 0.258 | 100 |
| $Fe^{2+}$ | 4.32 | 0.116 | 15.6 |
| $Mg^{2+}$ | 4.61 | 0.173 | 25.0 |
| $Ca^{2+}$ | 12.7 | 0.251 | 100 |
| $Mo^{6+}$ | 13.2 | 0.144 | 25.4 |
| $Mn^{2+}$ | 13.3 | 0.180 | 75.0 |
| $Zn^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Mo^{6+}$, $Mn^{2+}$ | 2.30 | 0.173 | 12.5 |

* Corresponds to 3.2 units/ml of culture broth.

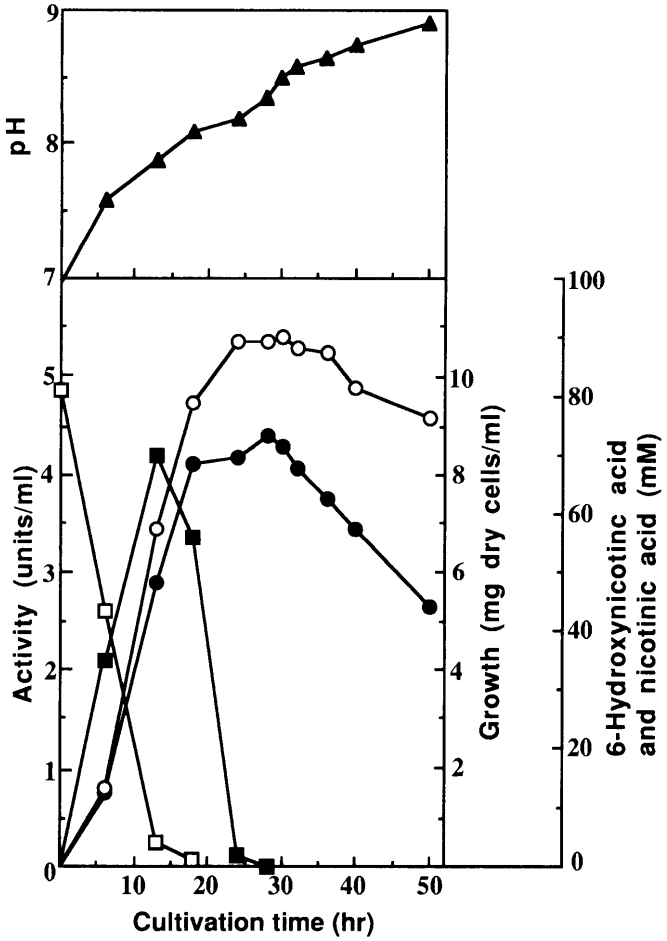


**Fig. 4.** Growth of *P. fluorescens* TN5.

*P. fluorescens* TN5 was cultivated under the optimized conditions. (○), hydroxylation activity; (▲), pH; (○), growth; (□), nicotinic acid; (■), 6-hydroxynicotinic acid were followed with time.

found at 13 h, but gradually decreased. In spite of the consumption of nicotinic acid, a rapid decrease in hydroxylation activity was not provoked. This fact reflects that 6-hydroxynicotinic acid also acts as an inducer for the formation of the hydroxylation enzyme as shown in Table I. Based on these results, the resting cells prepared from 28-h cultivation culture broth were used for the accumulation of 6-hydroxynicotinic acid.

*Accumulation of 6-hydroxynicotinic acid*

A typical production of 6-hydroxynicotinic acid from nicotinic acid was done (Fig. 5). During the course of the reaction, the concentration of nicotinic acid in the reaction mixture was monitored, and 200 mM nicotinic acid was periodically fed when the substrate was almost consumed. With 6 feedings of nicotinic acid during the 45-h reaction, 1.37 M 6-hydroxynicotinic acid accumulated with a 98.7% molar conversion yield from nicotinic acid. The amount of 6-hydroxynicotinic acid accumulated corresponded to 191 g per liter of reaction mixture.

The isolation and identification of 6-hydroxynicotinic acid formed was done as described elsewhere.[20]

Thus, *P. fluorescens* TN5 cells may produce a high molar conversion yield with large amounts of 6-hydroxynicotinic acid and no formation of by-products. In addition,

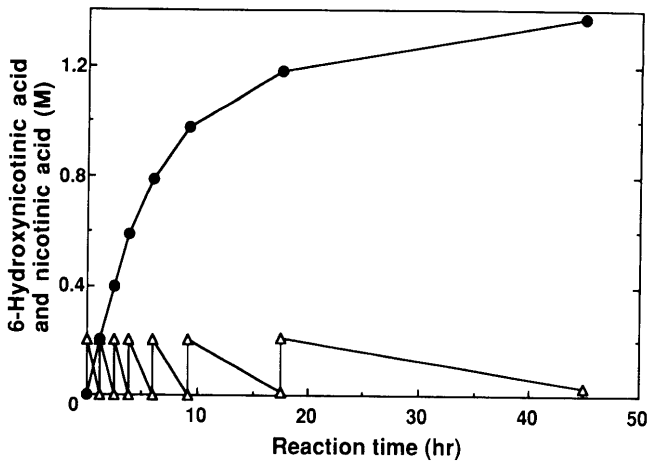


**Fig. 5.** Accumulation of 6-Hydroxynicotinic Acid by *P. fluorescens* TN5.

The reaction mixture (40 ml in a 300-ml Erlenmeyer flask) contained 60 mM potassium phosphate buffer (pH 6.5), 200 mM nicotinic acid, and resting cells (297 mg dry weight of cells, 123 units). The reaction was done at 35°C with stirring (600 rpm) by magnetic stirring bar (8 × 50 mm). (●), 6-hydroxynicotinic acid; (△), nicotinic acid.

*P. fluorescens* TN5 cells were easily prepared and kept for at least 2 years at −30°C without any loss of activity. Therefore, the application of the nicotinic acid-hydroxylation enzyme might be of value for the production of a useful intermediate in the synthesis of mordern insecticides on an industrial scale.

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