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Production and Properties of 3-Cyanopyridine Hydratase in *Rhodococcus equi* SHB-121

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ABSTRACT

A strain of *Rhodococcus equi* SHB-121 forming 3-cyanopyridine hydratase was screened from nitrile-polluted soil. The optimum conditions for the formation of 3-cyanopyridine hydratase by the strain SHB-121 have been studied. Under the optimum conditions, the specific activity of the enzyme reached 5.32 U/mg of dry cell, 95 times higher than that cultured in screening medium. In addition, the activity of coexistent amidase was very low.

3-Cyanopyridine hydratase was purified from methylacrylamide-induced cells of *Rh. equi* SHB-121 by procedures including ultrasonic oscillation, ammonium sulfate precipitation, and column chromatographies on DEAE-cellulose DE52, hydroxyapatite, and Sephadex G-25. The overall purification was 31-fold. The molecular weight of the enzyme was about 30 kDA by SDS-PAGE. The pI value was 4.1. The transition temperature and pH were 7.0°C and 6.0, respectively, resulting from the differential spectra.

The optimum pH and temperature for the enzyme reaction were 8.0 and 30°C. The enzyme activity was strongly inhibited by Ag^+, Hg^{2+}, Cu^{2+}, and NH_4^+, whereas it was enhanced by Fe^{3+} slightly. The enzyme catalyzed the hydration of 3-cyanopyridine to nicotinamide, and its *Km* value was 0.1 mol/L. Uncompetitive inhibitor sodium cyanide has a K_i value of 5 mmol/L.

Index Entries: *Rhodococcus equi* SHB-121; nicotinamide; nitrile hydratase; microbial conversion.

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INTRODUCTION

Many microorganisms can use nitriles as sources of carbon and/or nitrogen for growth, and microbial nitrile metabolism were now well understood (*1–5*). Various kinds of nitriles can be converted to corresponding amides and acids by nitrile-utilizing microorganisms via nitrile hydratase and nitrilase.

Researchers from H. Yamada's lab have carried out the industrial-scale conversion of acrylamide from acrylonitrile (*6*). They have also reported the bioconversion of nicotinamide (*7*) and nicotinic acid (*8,9*) from 3-cyanopyridine.

Many kinds of nitrile hydratase have been purified from *Brevibacterium* (*10*), *P. chlororaphis* (*11*), and *Rh. rhodochrous* J1 (*10*). Results showed that properties of nitrile hydratase were different when bacteria and inducers changed.

Nitrile hydratase from *Rh. equi* SHB-121 can catalyze the hydration of 3-cyanopyridine to a useful vitamin nicotinamide. This article describes the optimization of the enzyme formation conditions, and purification and characterization of 3-cyanopyridine hydratase from *Rh. equi* SHB-121.

MATERIALS AND METHODS

Microorganism and Cultivation

Rh. equi SHB-121, which was isolated from soil as a 3-cyanopyridine-utilizing microorganism and was identified in our laboratory, was used. The culture was carried out at 28°C for 48 h in a medium containing KH_2PO_4 0.75 g, K_2HPO_4 0.75 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and $FeSO_4 \cdot 7H_2O$ 0.01 g, supplemented with various carbon and nitrogen sources as indicated in each experiment per liter of tap water, pH 7.0.

Chemicals

Nicotinic acid, nicotinamide, and 3-cyanopyridine were purchased from Aldrich. DEAE-cellulose DE52 and Sephadex G-25 were obtained from Pharmacia. Standard proteins were from Sigma, and Ampholine from LKB. All the other chemicals were commercial products.

Biomass

The biomass was estimated turbidimetrically by a dry cell calibration curve. 1.0 U of A_{460nm} = 7.47 mg of dry cell wt/mL.

Enzyme Assay

Five milliliters of reaction mixture contained an appropriate amount of 3-cyanopyridine in 0.1*M* potassium phosphate buffer (pH 7.5) and an

Table 1
Effect of Carbon Sources on Enzyme Formation of *Rh. equi* SHB-121

Carbon sources	Biomass, mg dry cell/mL	Specific activity U/mg	
		3-Cyanopyridine hydratase	Nicotinamide amidase
Glucose	2.28	0.09	0.01
Frucose	4.63	0.02	0.06
Sucrose	4.93	0.00	0.01
Maltose	4.11	0.02	0.05
Soluble dextrin	5.15	0.04	0.05
Soluble starch	4.67	0.03	0.05
Mannitol	4.18	0.00	0.02
Sorbitol	4.07	0.00	0.01
Glycerol	5.19	0.01	0.02
Citrate, Na	4.26	1.79	0.31
Succinate, Na	4.03	1.69	0.31
None	2.46	0.74	0.25

appropriate amount of enzyme or dry cell weight. The reaction was incubated at 25°C for 20 min with moderate shaking. The reaction was stopped by adding 0.1 mL of 2.0*N* HCl. The nicotinamide formed was assayed by HPLC (*12*). One unit of hydratase activity was defined as the amount of the enzyme that catalyzed the formation of 1.0 μm nicotinamide/min. The activity of nicotinamide amidase was assayed as above, except that the substrate used was 0.4*M* nicotinamide.

Analytical Methods

Protein was assayed by the Coomassie Brilliant Blue G-250 dye-binding method of Bradford (*13*), with bovin serum albumin as standard protein. Polyacrylamide disk gel electrophoresis (PAGE) was carried out according to Davis (*14*). SDS-gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide slab gel by the method of King et al. (*15*). Isoelectric focusing of protein was performed according to Vesterberg (*16*).

RESULTS

Effect of Carbon Sources on Enzyme Formation

The strain *Rh. equi* SHB-121 was cultivated in the basal medium containing 0.5% (w/v) of various carbon sources and 0.25% (w/v) of butyronitrile as nitrogen source at 28°C for 72 h. The results (Table 1) showed that sodium citrate was the best carbon source for growth and enzyme formation. The optimum concentration of sodium citrate was 1% (w/v).

Table 2
Effect of Inducers on Enzyme Formation of *Rh. equi* SHB-121

Inducers	Biomass, mg dry cell/mL	Specific activity U/mg	
		3-Cyanopyridine hydratase	Nicotinamide amidase
Propionitrile	3.32	0.20	0.02
Methacrylonitrile	4.11	1.53	0.32
Iso-Butyronitrile	4.55	0.05	0.04
n-Butyronitrile	4.09	2.94	0.06
Acetamide	1.10	0.00	0.05
Oxamide	2.31	0.00	0.01
Methacrylamide	3.14	3.00	0.39
Nicotinamide	1.57	0.01	0.00
Acrylic acid	0.25	0.00	0.00
Nicotinic acid	0.37	0.00	0.01
3-Cyanopyridine	1.81	0.00	0.01
None	0.40	0.00	0.00

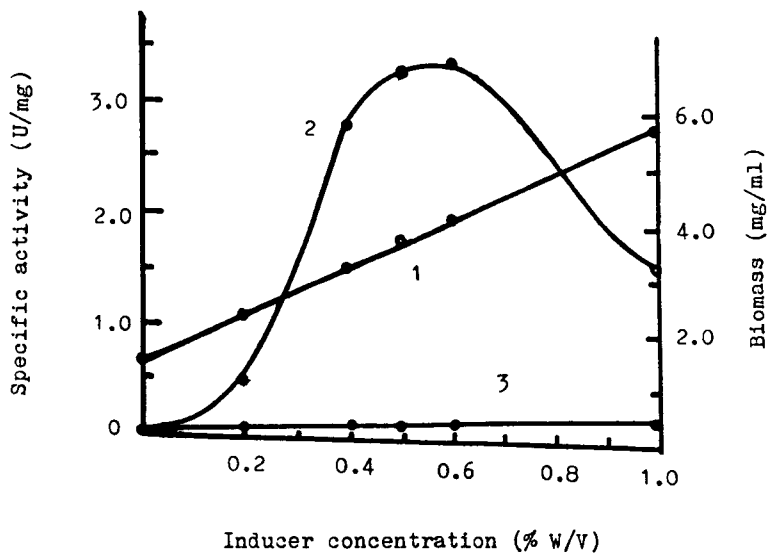

Fig. 1. Effect of inducer concentration on enzyme formation of *Rh. equi* SHB-121. 1. Biomass; 2. 3-cyanopyridine hydratase; 3. nicotinamide amidase.

Effect of Inducers on Enzyme Formation

Various nitriles, amides, and acids were tested to find the best inducer for the formation of 3-cyanopyridine hydratase. The results were shown in Table 2. Both methacrylamide and *n*-butyronitrile were the best inducers. In view of the volatility of *n*-butyronitrile, methacrylamide was used as an inducer, and its optimum concentration was 0.6% (w/v) (Fig. 1).

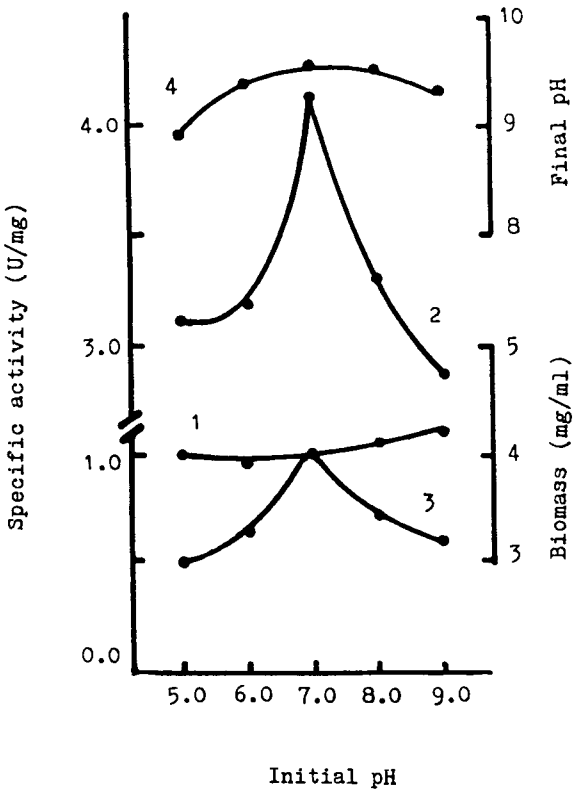

Fig. 2. Effect of initial pH on enzyme formation of *Rh. equi* SHB-121. 1. Biomass; 2. 3-cyanopyridine hydratase; 3. nicotinamide amidase; 4. final pH.

Effect of Metal Ions on Enzyme Formation

The effect of various metal ions on the formation of 3-cyanopyridine hydratase was examined with the basal medium containing 1.0% sodium citrate and 0.6% methacrylamide. The results showed that Ba^{2+}, Ni^{2+}, Mg^{2+}, and Fe^{2+} improved the hydratase activity, whereas the coexistent nicotinamide amidase activity changed little.

Effect of Initial pH and Growth Temperature on Enzyme Formation

The optimum pH for hydratase formation was pH 7.0 (Fig. 2). The optimum temperature was 28°C. The hydratase activity decreased above 28°C.

Time-Course of Enzyme Formation

The activity of 3-cyanopyridine hydratase came to its climax at 34 h, whereas the activity of amidase was very low. The above-mentioned results showed that the optimum culture medium for the formation of 3-cyanopyridine hydratase was as follows (in 1 L tap water): KH_2PO_4 0.75 g, K_2HPO_4 0.75 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, sodium citrate 10 g, polypepton 0.5

g, methacrylamide 6 g (pH 7.0). When the strain was cultivated at 28°C for 34 h in this medium, the specific activity of the hydratase reached 5.32 U/mg dry cell.

Purification of 3-Cyanopyridine Hydratase

All of the purification operations were performed at about 4°C.

Step 1. Crude extract: The washed cells were suspended in 0.06*M* potassium phosphate buffer, pH 7.0, and disrupted for 4 min on ice with ultrasonic oscillator (Labsonic 2000, B. Braun) at 19 kHZ, 200 W. The cell debris was removed by centrifugation at 10,000 *g* for 30 min.

Step 2. Ammonium sulfate fractionation: Solid ammonium sulfate was added to the crude extract to 50% saturation. After the precipitate was removed by centrifugation, ammonium sulfate was added up to 75% saturation. The precipitate was collected and dissolved in the proper amount of 0.06*M* potassium phosphate buffer, pH 7.0, and then dialyzed against the same buffer.

Step 3. DEAE-cellulose DE52 column chromatography: The desalted sample from step 2 was applied to a column of DEAE-cellulose DE52 (2.6 × 25 cm), which had been equilibrated with 0.06*M* potassium phosphate buffer, pH 8.0, and eluted with the same buffer containing 0.2*M* NaCl at 30 mL/h. After 1 h, it was eluted further with the same buffer containing 0.35*M* NaCl at the same flow rate, and 4-mL fractions were collected.

Step 4. Hydroxyapatite column chromatography: The desalted sample from step 3 was applied to a hydroxyapatite column (2.6 × 10 cm) equilibrated with 0.02*M* phosphate buffer, pH 7.0. The hydratase was eluted with a linear gradient (0.02–0.1*M*) of phosphate buffer, pH 7.0, at a flow rate of 10 mL/h, and 5-mL fractions were collected. Then the sample was dialyzed against 0.06*M* potassium phosphate buffer, pH 7.0.

Then the enzyme was desalted on Sephadex G-25 column (1.7 × 100 cm) with 0.06*M* phosphate buffer. The activity fractions were pooled. The sample was found to contain one protein band with hydratase activity on PAGE (Fig. 3).

The purification of nitrile hydratase from *Rh. equi* SHB-121 is summarized in Table 3. About 31-fold purification and recovery of 34.1% were achieved. The specific activity of the hydratase reached 74.4 U/mg protein.

General Properties

The molecular mass of the enzyme was about 30 kDa (Fig. 4). The enzyme gave only one protein band on SDS-PAGE with 10% gel. The isoelectric point was 4.1, shown in a polyacrylamide gel isoelectric focusing. The transition temperature and pH were 7.0°C and 6.0, respectively, as shown in differential spectra of the enzyme.

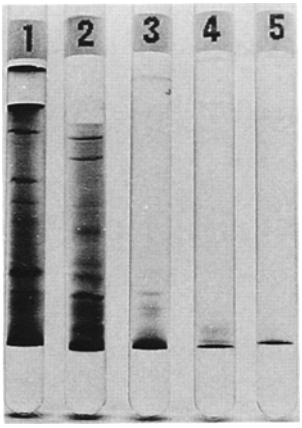

Fig. 3. Disk-PAGE of the enzyme from five purification steps. 1. Crude extract; 2. $(NH_4)_2SO_4$ precipitation; 3. DEAE-cellulose DE-52; 4. hydroxyapatite; 5. Sephadex-G25.

Table 3
Purification of 3-Cyanopyridine Hydratase from *Rh. equi* SHB-121[a]

Step	Volume, mL	Total protein, mg	Specific activity, U/mg	Purification, fold	Recovery, %
Crude extract	315	551.3	2.4	1.0	100.0
$(NH_4)_2SO_4$ Precipitation	63	76.2	11.7	4.9	13.8
DEAE-cellulose DE52	22	51.3	25.5	10.6	9.3
Hydroxyapatite	28	21.0	46.6	19.4	3.8
Sephadex-G25	25	6.1	74.4	31.0	1.1

[a] From 9 L of culture broth.

The optimum pH and temperature for hydration of 3-cyanopyridine to nicotinamide were 8.0 and 30°C. The enzyme activity was strongly inhibited by Ag^+ (0.1 m*M*), Hg^{2+} (0.1 m*M*), Cu^{2+} (1.0 m*M*), and NH_4^+ (10 m*M*), and slightly inhibited by phenyl mercuric acetate (0.1 m*M*) and iodoacetic acid (1.0 m*M*), whereas EDTA showed no effect on the enzyme activity. The enzyme activity was stable from 20 to 30°C.

The hydratase catalyzed the hydration of 3-cyanopyridine to nicotinamide. Its *Km* value was 0.1 mol/L. The enzyme activity was inhibited by sodium cyanide, as shown in Fig. 5. The inhibition was uncompetitive. The K_I value was 5 mmol/L with 3-cyanopyridine as the substrate.

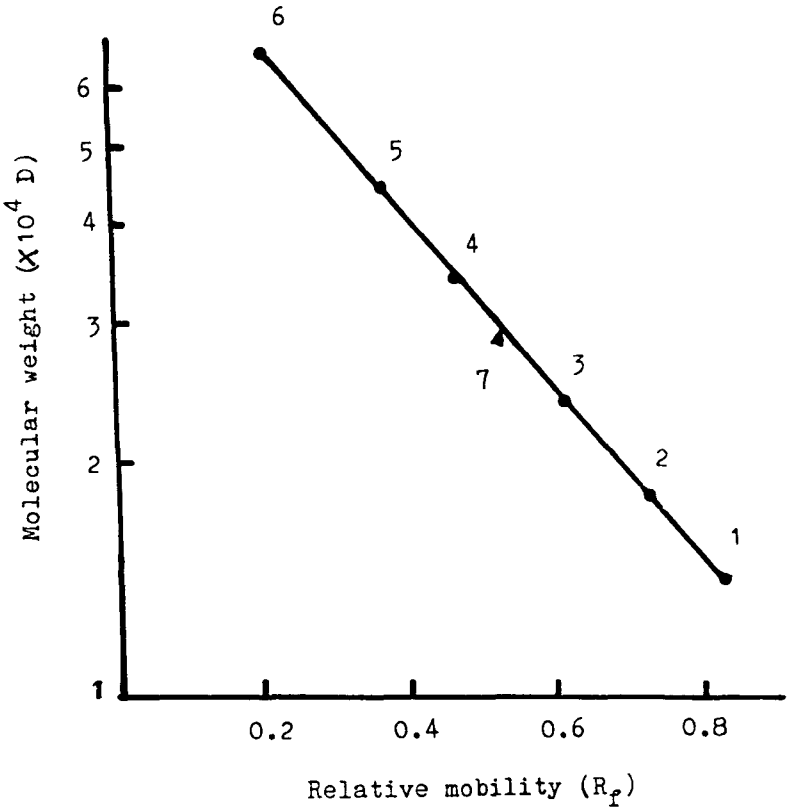

Fig. 4. Determination of molecular weight of 3-cyanopyridine hydratase. 1. Lysozyme (14,300); 2. β-lactoglobulin (18,400); 3. trypsinogen (24,000); 4. pepsin (34,700); 5. albumin (Egg, 45,000); 6. albumin (bovin, 66,000); 7. 3-cyanopyridine hydratase.

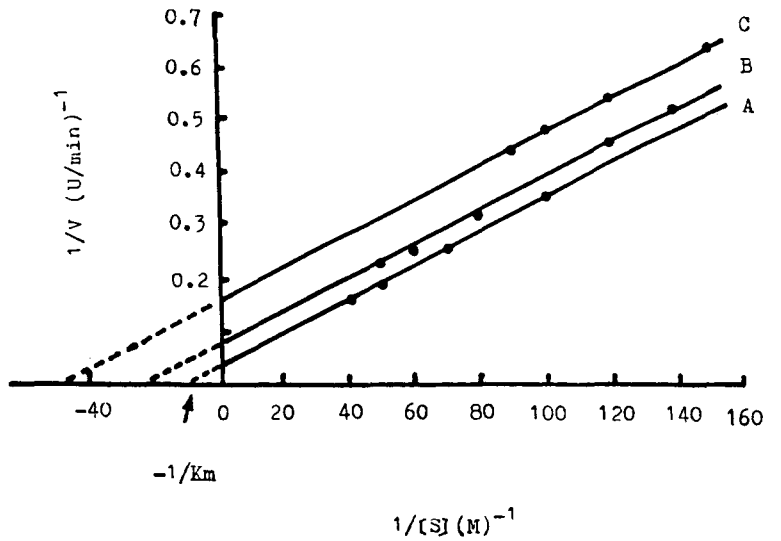

Fig. 5. Inhibition of 3-cyanopyridine hydratase by sodium cyanide. A. [NaCN] = 0; B. [NaCN] = 5 m*M*; C. [NaCN] = 10 m*M*.

DISCUSSION

In researching the enzyme specificity, we found that the hydratase from *Rh. equi* SHB-121 can act on aromatic nitriles (such as 3-cyanopyridine) and aliphatic nitriles (such as acrylonitrile). Thus, this enzyme may be applied in nitrile hydrolysis of various kinds of nitrile compounds.

Nicotinamide (vitamin pp) is an important chemical, used as medicine and in feed production. The traditional method to produce nicotinamide (*17*) needs high temperature and high alkalinity. The yield is very low because of the formation of nicotinic acid as a byproduct. The 3-cyanopyridine hydratase from *Rh. equi* SHB-121 shows high activity and a high conversion rate, with very low content of nicotinic acid.

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REFERENCES

1. Harper, D. B. (1977), *Biochem. J.* **165,** 309.
2. Harper, D. B. (1977), *Biochem. J.* **167,** 185.
3. Yamada, H., et al. (1980), *J. Ferment. Technol.* **58,** 495.
4. Bandyopadhyay, A. K., et al. (1986), *Appl. Environ. Microbiol.* **51,** 302.
5. Arnaud, A., et al. (1973), French Patent No. **73,** 33,613.
6. Nagasawa, T., et al. (1989), *Trends in Biotechnol.* **7,** 153.
7. Nagasawa, T., et al. (1988), *Appl. Environ. Microbiol.*, 1766.
8. Mathew, C. D., et al. (1988), *Appl. Environ. Microbiol.*, 1030.
9. Nagasawa, T., et al. (1988), *Arch. Microbiol.* **150,** 89.
10. Nagasawa, T., et al. (1986), *Biochem. Biophys. Res. Commun.* **139,** 1305.
11. Nagasawa, T., et al. (1987), *Eur. J. Biochem.* **162,** 691.
12. Li. Wenzhong (1993), *Chinese J. of Chromatogr.* **11(2),** 76.
13. Bradford, M. (1976), *Anal. Biochem.* **72,** 248.
14. Davis, B. J. (1964), *Ann. NY Acad. Sci.* **121,** 404.
15. King, J., et al. (1971), *J. Mol. Biol.* **62,** 465.
16. Vesterberg, O. (1971), *Methods in Enzymology,* vol. 22, Jacoby W. B., ed., Academic, NY, p. 389.
17. Kroh, W. (1953), German Patent No. 828247.