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Nitrile Hydratase-Catalyzed Production of Nicotinamide from 3-Cyanopyridine in *Rhodococcus rhodochrous* J1

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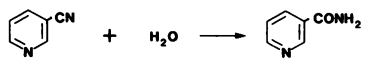
Nitrile hydratase, which occurs abundantly in cells of *Rhodococcus rhodochrous* J1 isolated from soil samples, catalyzes the hydration of 3-cyanopyridine to nicotinamide. By using resting cells, the reaction conditions for nicotinamide production were optimized. Under the optimum conditions, 100% of the added 12 M 3-cyanopyridine was converted to nicotinamide without the formation of nicotinic acid, and the highest yield achieved was 1,465 g of nicotinamide per liter of reaction mixture containing resting cells (1.48 g as dry cell weight) in 9 h. The nicotinamide produced was crystallized and then identified physicochemically. The further conversion of the nicotinamide to nicotinic acid was due to the low activity of nicotinamide as a substrate for the amidase(s) present in this organism.

Nitrile hydratase catalyzes the hydration of nitriles to amides, whereas nitrilase catalyzes the direct cleavage of nitriles to the corresponding acids and ammonia. Thus, nitrile hydratase can be clearly distinguished from nitrilase by the mode of nitrile degradation. We previously proposed a new enzymatic production process for acrylamide, applicable on an industrial scale, involving nitrile hydratase as a catalyst (3). *Pseudomonas chlororaphis* B23 and *Brevibacterium* sp. strain R312 were selected as favorable strains (3, 6). Indeed, *P. chlororaphis* B23 produces more than 400 g of acrylamide per liter of reaction mixture from acrylonitrile under suitable conditions. Thus, very recently, the use of the *P. chlororaphis* B23 nitrile hydratase as a catalyst for the industrial production of acrylamide, an important chemical commodity, was started.

Recently, we found that *Rhodococcus rhodochrous* J1 produces nitrile hydratase or nitrilase selectively, depending on the culture conditions (T. Nagasawa, M. Kobayashi, and H. Yamada, Arch. Microbiol., in press). Although the nitrile hydratases from *P. chlororaphis* B23 and *Brevibacterium* sp. strain R312 hardly act on aromatic nitrile compounds, we have found that the *Rhodococcus* nitrile hydratase exhibits high activity toward 3-cyanopyridine and produces nicotinamide (equation I).



(I)

In the present study, we optimized the reaction conditions for the production of a useful vitamin, nicotinamide.

MATERIALS AND METHODS

Screening for nicotinamide-producing strains. Nitrile-utilizing microorganisms were isolated from soil samples by an enrichment culture technique involving medium containing 0.2% (wt/vol) nitrile and 0.001% (wt/vol) $FeSO_4 \cdot 7H_2O$ or $CoCl_2$ in basal media I and II; medium I consisted of 4 g of glycerol, 2 g of K_2HPO_4, 1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 mg of biotin, and 1 mg of thiamine hydrochloride per liter of tap water. Medium II consisted of 2 g of yeast extract (Oriental Yeast, Tokyo), 0.5 g of Polypepton (Daigo, Osaka), 0.5 g of K_2HPO_4, 0.2 g of $MgSo_4 \cdot 7H_2O$, and 2 g of NaCl per liter of tap water. Microorganisms which utilize acetonitrile, propionitrile, succinonitrile, adiponitrile, glutaronitrile, isobutyronitrile, isovaleronitrile, β,β′-oxydipropionitrile, iminodiacetonitrile, 3,3′-iminopropionitrile, benzylcyanide, and phenylglycinonitrile were isolated. The isolated strains were cultured aerobically at 28°C for 3 to 4 days on the isolation medium. The cells were centrifuged, washed with physiological saline, and then suspended in 0.1 M potassium phosphate buffer, pH 7.5. The reaction mixture for the screening of nicotinamide-producing strains contained 50 μmol of potassium phosphate buffer, pH 7.5, 200 μmol of 3-cyanopyridine as a substrate, and washed cells from 2 to 10 ml of culture broth, in a total volume of 2.0 ml. The reaction was carried out at 30°C for 3 h with reciprocal shaking (80 strokes per min) and terminated by the addition of 0.2 ml of 1 N HCl. The amount of nicotinamide formed in the reaction mixture was determined.

Identification of the microorganism. Identification of the microorganism was carried out according to *Bergey's Manual of Systematic Bacteriology* (9). The chemical compositions of cells were analyzed by the methods of Becker et al. (4), Lechevalier and Lechevalier (10), Suzuki and Komagata (13), Alshamaony et al. (1), and Uchida and Aida (15).

Culture conditions and preparation of resting cells. The cultivation conditions for *R. rhodochrous* J1 for the production of nitrile hydratase were optimized previously (T. Nagasawa, K. Takeuchi, and H. Yamada, manuscript in preparation). The subculture was carried out at 28°C for 24 h with reciprocal shaking in a test tube containing 4 ml of medium (pH 7.2) consisting of 10 g of glycerol, 5 g of Polypepton, 3 g of malt extract, and 3 g of yeast extract per liter of tap water. Then, two test tubes were added to each 2-liter flask containing 400 ml of the culture medium (pH 7.2) containing 3 g of yeast extract, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CoCl_2$, and 2 g of crotonamide per liter of distilled water, followed by incubation with shaking at 28°C. After 26 and 56 h, 0.2% (wt/vol) crotonamide was added. The cultures (2.4 liters) were pooled after 76 h, followed by centrifugation at 10,000 × *g* for 20 min at 0°C. The cells obtained were washed with 0.85% (wt/vol) NaCl, centrifuged at 10,000 × *g*, and then

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suspended in 240 ml of the same solution (29.6 mg [dry weight] of cells per ml). This cell suspension was used for the resting-cell reaction.

Assay for the production of nicotinamide. The standard reaction mixture (2 ml) for nicotinamide production contained 400 mM 3-cyanopyridine, 10 mM K_2HPO_4-KH_2PO_4 buffer (pH 8.0), and 0.02 ml of the cell suspension (0.592 mg, dry cell weight) obtained from 0.2 ml of culture broth. The reaction was carried out at 25°C with shaking. However, for optimization of the reaction conditions, pH, temperature, time, and concentrations of added substrate and cells were varied in each experiment, depending on the reaction conditions being examined. The reaction was stopped by adding 0.2 ml of 3 N HCl.

Analytical methods. The amounts of nicotinamide and 3-cyanopyridine in the reaction mixture were assayed by analytical high-pressure liquid chromatography (HPLC). HPLC was performed with a Toyosoda CCPM system equipped with a pack C18 column (reverse-phase column, 4.6 by 150 mm; M&S Instruments Inc., Osaka, Japan) at a flow rate of 1.0 ml/min at 30°C, with the following solvent system: acetonitrile–10 mM KH_2PO_4-H_3PO_4 buffer (pH 2.8), 1:4 (vol/vol). In some cases, a Hibar LiChrosorb-NH_2 column (4 by 250 mm; Cica-Merck, West Germany) was used to detect the formation of nicotinic acid at a flow rate of 1.0 ml/min at 30°C, with the following solvent system: acetonitrile–10 mM KH_2PO_4-H_3PO_4 buffer (pH 2.8), 3:1 (vol/vol). The absorbance was measured at 230 nm.

The ^1H and ^{13}C nuclear magnetic resonance (NMR) and infrared and mass spectra were recorded with a Nihondenshi JNN-GX270, Perkin Elmer 1710-FTIR, and Hitachi M-80, respectively. Elemental analysis was carried out with a Perkin Elmer 240-B.

Assay for amidase activity. The *R. rhodococcus* J1 cells were suspended in 0.1 M K_2HPO_4-KH_2PO_4 buffer (pH 7.0) containing 1 mM dithiothreitol and then disrupted with an ultrasonic oscillator (19 kHz, below 10°C for 45 min). The sonicate was centrifuged at 12,000 × *g* for 30 min, and the supernatant solution was used as the cell-free extract.

The reaction mixture consisted of 20 μmol of K_2HPO_4-KH_2PO_4 buffer (pH 7.0), 20 nmol of dithiothreitol, 20 μmol of amide, and an appropriate amount of enzyme solution, in a final volume of 1 ml. The mixture was incubated at 30°C for 20 min, and the reaction was terminated by adding 0.2 ml of 1 N HCl. Ammonia formed was estimated by the indophenol method (8).

Protein was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford (5) with dye reagent supplied by Bio-Rad Laboratories.

RESULTS

Isolation and identification of nicotinamide-producing strains. The ability to produce nicotinamide from 3-cyanopyridine was examined in 131 strains which had been isolated as utilizers of various nitrile compounds. This activity was shown by acetonitrile-, succinonitrile-, glutaronitrile-, β,β′-oxydipropionitrile-, and benzylcyanide-utilizing strains. One of the acetonitrile-utilizing strains, strain J1, exhibited the highest activity and thus was chosen as the best producer of nicotinamide.

Strain J1, the best nicotinamide producer, was identified taxonomically. The microbiological characteristics of strain J1 were as follows. Morphology: gram-positive, aerobic, non-endospore forming, not acid-fast, and non-motile. Cocci germinated and gave rise to branched filaments which underwent fragmentation into rods (0.5 to 1.1 μm by 1.5 to 5.0 μm). Elongated cells and snapping division were observed frequently. Cultural characteristics: nutrient agar colonies were round, regular, entire, smooth, opaque, pale orange-pink, low convex, and about 1 mm in diameter after 48 h. Nutrient agar slant gave moderate growth, orange surface smooth, convex with a drying tendency, and pale pink. Nutrient broth gave vigorous growth, slight turbidity, and the formation of a precipitate with growth. Nutrient gelatin stab gave good growth on the surface on nutrient gelatin stab and no liquefaction of gelatin. Litmus milk was not changed. Physiological characteristics: reduction of nitrate was positive. Denitrification, methyl red test, indole production, hydrogen sulfide production, and Voges-Proskauer test were negative. Oxidase was negative, and catalase and phosphatase were positive. Growth range was pH 5 to 10; growth temperature was 10 to 41°C. Growth in 7% NaCl was positive. Oxygen relation was aerobic. Hugh-Leifson test was weakly oxidative. Acid production from glucose was positive. Gas production from glucose was negative. Decomposition of adenine, tyrosine, and urea was positive. Growth on sole carbon sources was positive with 1% (wt/vol) maltose, mannitol, sorbitol, trehalose, 0.1% *m*- and *p*-hydroxybenzoic acid, sodium adipate, sodium benzoate, sodium citrate, sodium lactate, testosterone, acetamide, and sebacic acid and negative with 1% inositol and rhamnose. Hydrolysis of starch was negative. Tween 80 hydrolysis was positive. *o*-Nitrophenyl-β-D-galactopyranoside hydrolysis was negative. Chemical composition of cells: *meso*-diaminopimelic acid, arabinose, and galactose were present. Major fatty acid components were *n*-hexadecanoic acid, 21.0%; *n*-hexadecenoic acid, 19.8%; *n*-octadecenoic acid, 6.7%; and methyloctadecanoic acid (tuberculostearic acid), 25.8%. C_{32} to C_{46} mycolic acids of the mycolic acid type were present. Glycolate test was positive. The G+C content of the DNA was 67 to 70% mol(*Tm*).

Thus, strain J1 is pleomorphic and has some typical characteristics of a coryneform bacterium. Recently, chemical testing of the cell wall provided data useful for distinguishing coryneform bacteria at the generic level. From its morphology and physiology and the presence of *meso*-diaminopimelic acid, arabinose, and galactose (10), fatty acids of the straight-chain saturated and monounsaturated types, and 10-methyl branched-chain acids (14), strain J1 was thought to belong to the genus *Rhodococcus*, *Corynebacterium*, or *Nocardia*. To clearly differentiate between *Corynebacterium* and related taxa, the types of mycolic acids and the acyl type in the cell wall were determined (7, 16). Strain J1 possessed mycolic acids with 32 to 46 carbon atoms and glycolyl residues of the glycan moiety. From these results, it was assumed that strain J1 might belong to the genus *Rhodococcus*. Then, according to references 13 and 9, strain J1 was classified as *Rhodococcus rhodochrous*.

Reaction conditions for the production of nicotinamide by resting cells. Various reaction conditions for the efficient production of nicotinamide were studied with resting cells of *R. rhodochrous* J1.

(i) Effect of pH. The effect of different pHs on the production of nicotinamide by resting cells was studied. The initial pH of the reaction mixture was adjusted to 5 and 6 with 0.1 M CH_3COOH-CH_3COONa buffer, 7 and 8 with 0.1 M K_2HPO_4-KH_2PO_4 buffer, 9 with 0.1 M Tris hydrochloride buffer, and 10 with 0.1 M CAPS (cyclohexylaminopropanesulfonic acid)-KOH buffer. The reaction was carried out at 25°C for 20 min as described under Materials and Methods. The optimum pH was found to be between 7 and 9, maxi-

mum activity being observed at pH 8.0. The nitrile hydratase showed a sharp decrease in activity at low pHs. During the reaction, a change in pH of the reaction mixture was hardly observed.

The enzyme activity did not change on changing the buffer at pH 8.0 from K_2PO_4-KH_2PO_4 to Tris hydrochloride, H_3BO_3-NaOH, or HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH buffer.

After the resting cells had been incubated with 0.02 M glycine hydrochloride at pH 2, 3, and 4, 0.02 M CH_3COONa-CH_3COOH at pH 5 and 6, 0.02 M K_2HPO_4-KH_2PO_4 at pH 7, HEPES-KOH at pH 8 and 9, and CAPS-KOH at pH 10 and 11 for 15 h at 30°C, activity was measured under the standard conditions except that 75 mM K_2HPO_4-KH_2PO_4 buffer (pH 8.0) was used. A sharp decrease in stability was seen only after incubation at pH 2 and 3. About 80 and 70% of the initial activity remained after incubation at pH 10 and 11, respectively. Thus, the nitrile hydratase in the *R. rhodochrous* cells seemed to be stable in a wide pH range.

(ii) Effect of temperature. The effect of temperature on the synthesis of nicotinamide was investigated. Each reaction was carried out in the standard reaction mixture at various temperatures. The enzyme activity increased linearly from 0.63 μmol/min at 5°C to 4 μmol/min at 35°C. The increase in activity was less between 35 and 40°C, and the activity decreased at 50°C.

Thermal stability of the *Rhodococcus* nitrile hydratase was examined. After the cell suspension was incubated in 10 mM K_2HPO_4-KH_2PO_4 at different temperatures for 3 h, nicotinamide synthesis was determined under the standard reaction conditions. The enzyme was stable up to 30°C, with a sharp decrease in stability from 40 to 60°C.

(iii) Production of nicotinamide. To determine the maximum production of nicotinamide from 3-cyanopyridine, the concentration of 3-cyanopyridine was varied. The reaction mixtures (4 ml) consisted of various amounts of 3-cyanopyridine, 10 mM K_2HPO_4-KH_2PO_4 buffer (pH 8.0), and the resting cells at two different concentrations. The incubation was carried out at 25°C with shaking. Nicotinamide, nicotinic acid, and 3-cyanopyridine were measured, and the conversion ratio of 3-cyanopyridine added to nicotinamide formed was determined. With the addition of the resting cells (2.96 mg of cells [dry weight]) from 4 ml of culture broth, 6 to 8 M 3-cyanopyridine was converted to nicotinamide completely in 9 h and 9 M 3-cyanopyridine was completely converted to nicotinamide after 22 h of incubation (Fig. 1). In the case of the enzymatic production of acrylamide, acrylonitrile was added in portions successively so as not to exceed a concentration of 0.4 M due to its high inhibitory effect on the *Pseudomonas* enzyme (3). On the other hand, in this case, a high amount of 3-cyanopyridine can be added at one time. Probably 3-cyanopyridine is not so inhibitory toward the *Rhodococcus* nitrile hydratase contained in the cells as it is to acrylonitrile. The use of resting cells (4.92 mg [dry weight]) from 8 ml of culture broth resulted in the production of 12 M nicotinamide, which is equivalent to 1,465 g of nicotinamide per liter of reaction mixture, after 9 h of incubation. No formation of nicotinic acid was detected. As shown in Fig. 2, the 3-cyanopyridine added did not dissolve completely in the reaction mixture. In the course of the reaction, however, the 3-cyanopyridine precipitate dissolved completely, and crystals of nicotinamide appeared gradually after 6 h of incubation and the reaction mixture solidified after 18 h of incubation. Thus, this may be an example of pseudocrystal fermentation (i.e., crystalline substrate → solution of substrate → solution of product → crystalline

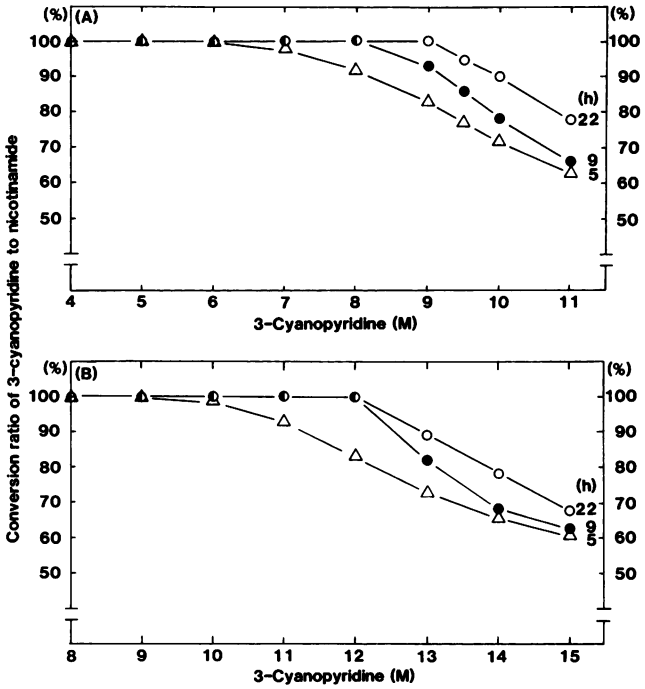

FIG. 1. Enzymatic conversion of various amounts of 3-cyanopyridine to nicotinamide. The reaction mixture (4 ml) contained 10 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0), various amounts of 3-cyanopyridine, and resting cells (2.96 mg [dry weight]) from 4 ml of culture broth (A) or resting cells (4.92 mg [dry weight]) from 8 ml of culture broth (B). The incubation was carried out at 25°C with shaking for 5 h (△), 9 h (●), or 22 h (○).

product). Figure 3 shows the enzymatic conversion of 9 and 12 M 3-cyanopyridine to nicotinamide plotted against time. Once formed, nicotinamide did not decrease with incubation for a long time, and no formation of nicotinic acid was detected.

Isolation and identification of nicotinamide. To isolate the nicotinamide produced, the reaction mixture was filtered and the filtrate was evaporated in vacuo. The solid obtained was dissolved in hot methanol and then kept in an ice bath overnight. Recrystallization was carried out in a similar manner. The crystals formed were separated by filtration and then dried in a vacuum desiccator over P_2O_5.

Identification of the product as nicotinamide was performed through comparison with the infrared, ^1H, and ^{13}C

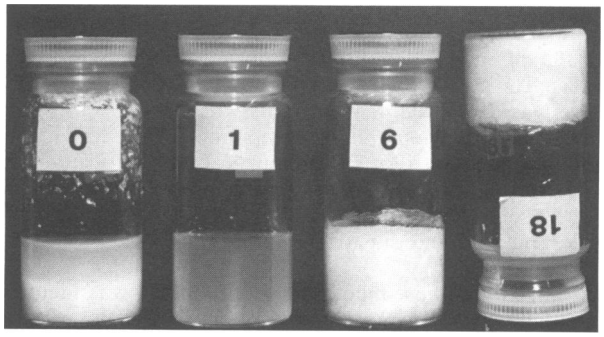

FIG. 2. Appearance of nicotinamide crystals during enzymatic synthesis. Photographs (from the left) were taken after 0, 1, 6, and 18 h of incubation.

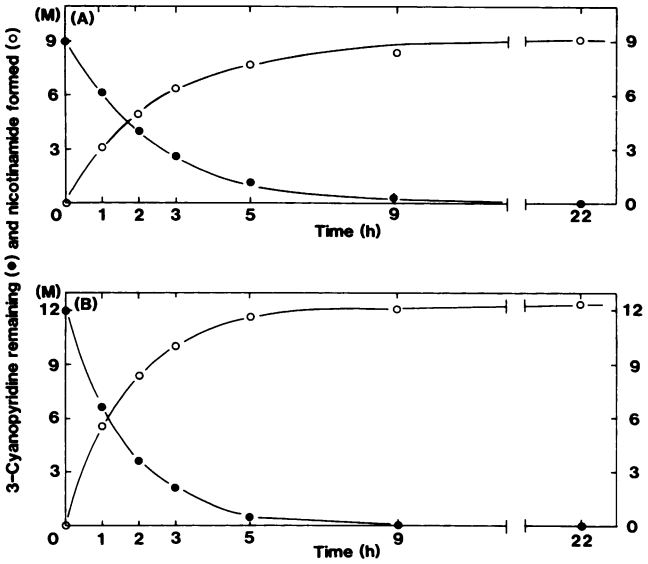

FIG. 3. Time course of the enzymatic hydration of 3-cyanopyridine to nicotinamide with resting cells of *R. rhodochrous* J1. 3-Cyanopyridine (9 and 12 M in A and B, respectively) was incubated at 25°C in the reaction mixture containing resting cells (2.46 and 4.92 mg [dry weight] in A and B, respectively).

NMR, and mass spectra of authentic nicotinamide. Analytical data were as follows: m.p., 129°C; ^{1}H NMR (dimethyl sulfoxide [DMSO]), 7.50, 7.65, 8.20, 8.70, 9.10 ppm; ^{13}C NMR (DMSO), 124, 130, 135, 149, 152, 167 ppm; mass spectrum *m/z* 51, 78, 122; elemental analysis, calculated for $C_6H_6ON_2$: C, 59.10; H, 4.95; N, 22.94. Found: C, 59.02; H, 4.95; N, 22.85.

Amidase activity of *R. rhodochrous* J1 cells. To investigate the mechanism underlying the accumulation of nicotinamide, the amidase activity of crotonamide-induced *R. rhodochrous* J1 cells was measured. When propionamide was used as the substrate, the amidase activity of the cell-free extract was 0.068 μmol/min per mg of protein at 30°C. Compared to propionamide (100%), the activities for crotonamide, acrylamide, acetamide, and nicotinamide were 2.9, 28.9, 17.7, and 0.1%, respectively. The amidase hardly acted on nicotinamide under the conditions used for the production of nicotinamide.

DISCUSSION

Nicotinamide is produced from 3-cyanopyridine obtained from picoline (2) through treatment with H_2O_2 and NaOH at 42°C (W. Kroh, German patent 828247, 1953). The yield on chemical synthesis is low due to the formation of nicotinic acid as a by-product. Thus, the enzymatic conversion of 3-cyanopyridine to nicotinamide via nitrile hydratase is attractive due to the nonformation of nicotinic acid as a by-product. However, the nitrile hydratases from *P. chlororaphis* B23 (11) and *Brevibacterium* sp. strain R312 (12), which are used for the production of acrylamide, hardly act on aromatic nitrile compounds. However, the nitrile hydratase from *R. rhodochrous* J1 exhibits high activity toward both aromatic and aliphatic nitrile compounds. In this study, with resting cells of *R. rhodochrous* J1, 1,465 g of nicotinamide per liter was accumulated in 9 h at 25°C, with a conversion rate of 100% and without the formation of nicotinic acid. Due to the high yield, this process seems to be useful for the production of nicotinamide. Other advantages of this process are that it is easy to cultivate *R. rhodochrous* J1 cells and that resting cells are stable at −20°C for more than 6 months. Nitrile hydratase activity of the cells is stable up to 30°C. Thus, the application of this enzymatic hydration process seems to be promising for the production of nicotinamide on an industrial scale.

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