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A new enzymatic method of nitrile synthesis by *Rhodococcus* sp. strain YH3-3 [1]

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Abstract

The substrate specificity of a novel aldoxime dehydratase from *E*-pyridine-3-aldoxime assimilating bacterium, *Rhodococcus* sp. strain YH3-3, was examined. The enzyme catalyzed a dehydration reaction of various aryl- and alkyl-aldoximes to form the corresponding nitriles, but did not act on arylalkyl- and substituted alkyl-aldoximes. Of various aldoximes tested, *E*-pyridine-3-aldoxime was the most suitable substrate for the enzyme. *E*-Pyridine-3-aldoxime analogs such as *O*-acetyl-*E*-pyridine-3-aldoxime, *Z*-pyridine-3-aldoxime, and *E*/*Z*-pyridine-3-aldehyde-hydrazone also acted as substrates and were converted to 3-cyanopyridine. Heat-treatment of the cells increased the accumulation of 3-cyanopyridine from *E*-pyridine-3-aldoxime because the nitrile degrading enzyme, nitrile hydratase was inactivated. Under the optimized reaction conditions (pH 7.0, 30°C), various nitriles were synthesized from the corresponding aldoximes in preparative scales with heat-treated cells of the strain. This is the first report on the microbial synthesis of nitriles from aldoximes. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Enzymatic synthesis; Aldoxime; Nitrile; Oxime dehydratase; *Rhodococcus* sp.; Screening

1. Introduction

In recent years, the microbial transformation has been extensively evaluated from the viewpoint of synthetic organic chemistry [1–5]. The industrial production of acrylamide from acrylonitrile using nitrile hydratase [6], which had been discovered and named by Asano et al. [7], is one of the typical examples. Although nitrile hydratase has become one of the most important industrial enzymes, there has been no report concerning the biosynthesis of nitriles and their physiological function. We have been interested in the microbial metabolism of aldoximes in nature and its relationship with nitrile degrading enzymes. In the course of our investigations, we isolated *E*-pyridine-3-aldoxime utilizing bacterium, strain YH3-3, from soil and identified it as *Rhodococcus* sp. [8]. The strain contained a novel dehydratase catalyzing the stoichiometric dehydration of *E*-pyridine-3-aldoxime to form 3-cyanopyridine.

The synthetic importance of dehydration of aldoximes, easily prepared from aldehydes and hydroxylamine, to their corresponding nitriles has been documented well in literatures [9–14]. Most of these reported methods, however, re-

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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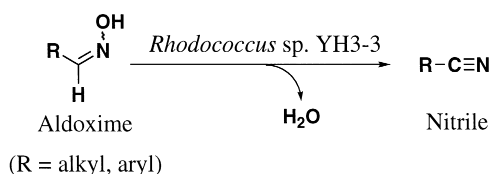

Fig. 1. Microbial dehydration of aldoximes into nitriles with *Rhodococcus* sp. strain YH3-3.

quire harsh reaction conditions. Clearly the microbial dehydration reaction of aldoximes to nitriles under mild conditions would provide an alternative attractive synthetic tool.

In this report, we clarified the substrate specificity of the dehydratase and optimized the reaction conditions for the dehydration reaction. We applied the enzyme to the synthesis of several nitriles from the corresponding aldoximes in preparative scales (Fig. 1).

2. Materials and methods

2.1. Materials

^{1}H- and ^{13}C-NMR spectra were recorded using a JEOL JNM-LA400 spectrometer (Tokyo, Japan) with tetramethylsilane as an internal standard. HPLC columns ODS-80Ts and Hibar LiChrosorb-NH_2 were purchased from Tosoh (Tokyo, Japan) and Kanto Chemicals (Tokyo, Japan), respectively. *E*-Pyridine-3-aldoxime-*N*-oxide, 3-cyanopyridine-*N*-oxide, *O*-methyl-pyridine-3-aldoxime, and *O*-benzyl-pyridine-3-aldoxime were prepared as described [8]. All other chemicals were from commercial sources and used without further purification.

2.2. Bacterial strain and cultivation

Rhodococcus sp. strain YH3-3, utilizing *E*-pyridine-3-aldoxime as a sole source of nitrogen, was isolated from soil and identified in our laboratory [8].

The subculture was carried out at 30°C for 24 h with reciprocal shaking in a medium consisted of 1% of meat extract (Kyokuto, Tokyo, Japan), 1% of Polypepton (Nippon Seiyaku, Tokyo, Japan), and 0.5% of NaCl (pH 7.0). Then 4 ml of the subculture was inoculated into a 2-l Sakaguchi flask containing 400 ml of a medium (pH 8.0) containing 0.2% of K_2HPO_4, 0.1% of NaCl, 0.02% of $MgSO_4 \cdot 7H_2O$, 0.05% of yeast extract (Nippon Seiyaku, Tokyo, Japan), 1.0% of sodium acetate, 0.2% of NZ amine (Type-A, Wako, Osaka, Japan), 0.15% of *E*-pyridine-3-aldoxime, and 1.0% each of vitamin mixture and trace element solution [8], and cultivated at 30°C for 48 h with reciprocal shaking.

2.3. Enzyme assay

E-Pyridine-3-aldoxime dehydratase, nitrile hydratase, and amidase activities were measured as described previously [8]. One unit of the enzyme activities were defined as the amount of the enzyme that catalyzed the conversion of 1 μmol of substrates into the products per minute.

2.4. Preparation of heat-treated cells of Rhodococcus sp. strain YH3-3

Washed cells of the strain from 50 ml culture were suspended in 5 ml of 0.1 M potassium phosphate buffer (KPB, pH 7.0) containing 5 mM of 2-mercaptoethanol and 1 mM of DTT, and incubated 40°C for 1 h. Cells harvested by centrifugation were used for the enzyme reaction.

2.5. Preparation of acetone-dried cells of the strain

The washed cells of the strain from 50 ml culture were suspended in 50 ml of saline, and to this was added 100 ml of acetone, which had been cooled to −20°C, with stirring. The cells were filtrated, washed with the same volume of cold acetone, evacuated for 2 h, and then stored at −20°C until use.

2.6. Chemical synthesis of aldoximes and nitriles [9]

2.6.1. Preparation of aldoximes [8]

Aryl- and alkyl-aldoximes were obtained by the reaction of aryl- and alkyl-aldehydes with hydroxylamine, respectively. Arylacetaldoximes were synthesized by condensing hydroxylamine with arylacetaldehydes, which had been prepared by diisobutylaluminium hydride (DIBAL–H) reduction of arylacetonitriles.

2.6.2. Preparation of E/Z-pyridine-3-aldehyde hydrazone

E/Z-Pyridine-3-carboxaldehyde hydrazone was prepared from hydrazine and pyridine-3-aldehyde (46.3 mmol), and the compound was obtained as pale yellow crystals (0.98 g, 17.4%, $E/Z = 1.63/1$); ^{1}H-NMR($CDCl_3$) δ_{ppm} 11.893 (br, 2H), 8.991 (d, 0.62H, $J = 1.5$ Hz, *E*), 8.778 (d, 0.38H, $J = 3.5$ Hz, *Z*), 8.686–8.716 (m, 1.24H, *E*), 8.516 (dd, 0.38H, $J = 1.5$, 4.9 Hz, *Z*), 8.230 (dt, 0.62H, $J = 2.0$, 7.8 Hz, *E*), 7.915 (dt, 0.38H, $J = 1.9$, 7.8 Hz, *Z*), 7.731 (s, 0.38H, *Z*), 7.414 (dd, 0.62H, $J = 4.9$, 7.8 Hz, *E*), 7.286 (m, 0.38H, *Z*); ^{13}C-NMR($CDCl_3$) δ_{ppm} 159.84, 152.10, 150.49, 149.41, 148.02, 138.95, 134.87, 132.50, 131.09, 129.68, 123.83, 123.57.

2.6.3. Preparation of E-O-acetyl-pyridine-3-aldoxime

E-Pyridine-3-aldoxime was acetylated with Ac_2O and gave *E-O*-acetyl pyridine-3-aldoxime as pale yellow crystals (1.3 g, 79.3%); ^{1}H-NMR($CDCl_3$) δ_{ppm} 8.845 (d, 1H, $J = 2.0$ Hz), 8.707 (dd, 1H, $J = 1.6$, 4.7 Hz), 8.420 (s, 1H), 8.178 (dt, 1H, $J = 0.85$, 8.3 Hz), 7.381 (dd, 1H, $J = 4.9$, 8.3 Hz), 2.252 (s, 3H); ^{13}C-NMR($CDCl_3$) δ_{ppm} 168.30, 153.18, 152.54, 150.02, 134.49, 126.39, 123.87, 19.51.

2.6.4. Preparation of Z-pyridine-3-aldoxime

Z-Pyridine-3-aldoxime was prepared from picoline-*N*-oxide by the methods as described by Tagawa et al. [15] and the compound was obtained as pale yellow crystals (0.97 g, 45.6%, 3 steps); ^{1}H-NMR(DMSO-d_6) δ_{ppm} 11.950 (s, 1H), 9.046 (s, 1H), 8.566 (t, 1H, $J = 4.5$ Hz), 8.398 (d, 1H, $J = 7.8$ Hz), 7.504 (s, 1H), 7.459 (dd, 1H, $J = 4.5$, 8.0 Hz); ^{13}C-NMR(DMSO-d_6) δ_{ppm} 150.87, 149.92, 142.37, 137.34, 126.94, 123.63.

2.7. Microbial synthesis of nitriles

2.7.1. Synthesis of 3-cyanopyridine from E-pyridine-3-aldoxime

The reaction mixture contained 25 mmol of KPB (pH 7.0), the heat-treated cells from 2.5 l culture, and 12.5 mmol (50 mM) of *E*-pyridine-3-aldoxime in a total volume of 250 ml. When the reaction mixture was incubated with shaking at 30°C for 105 min, the product formed in the mixture was extracted with AcOEt. The organic layer was combined and dried over anhydrous Na_2SO_4. Recrystallization with hexane/AcOEt afforded 3-cyanopyridine as colorless crystals (1.27 g, 97.6%); ^{1}H- and ^{13}C-NMR spectra and melting points of the compound were identical with those of 3-cyanopyridine which were described previously [8].

2.7.2. Synthesis of 2-cyanopyridine from E-pyridine-2-aldoxime

The reaction mixture contained 30 mmol of KPB (pH 7.0), the heat-treated cells from 3-l culture, and 30 mmol (100 mM) of *E*-pyridine-2-aldoxime in a total volume of 300 ml. After incubation with shaking at 30°C for 3 h, the product formed in the mixture was extracted with AcOEt. The organic layer was combined and dried over anhydrous Na_2SO_4. Purification with column chromatographies on silica gel (hexane/AcOEt = 4/1) and recrystallization with hexane/AcOEt gave 2-cyanopyridine as colorless crystals (945 mg, 30.3%); ^{1}H-NMR ($CDCl_3$) δ_{ppm} 8.744 (d, 1H, $J = 4.6$ Hz), 7.902 (dt, 1H, $J = 0.5$, 7.0 Hz), 7.864 (dd, 1H, $J = 1.0$, 7.8 Hz), 7.565 (dd, 1H, $J = 4.8$, 7.5 Hz); ^{13}C-NMR ($CDCl_3$) δ_{ppm} 151.06, 137.00, 133.89, 128.48, 126.90, 117.11; mp. 27–29°C.

2.7.3. Synthesis of anisonitrile from E-anisaldoxime

The reaction mixture (300 ml) contained 30 mmol of KPB (pH 7.0), the heat-treated cells from 3-l culture, and 3 mmol (10 mM) of *E*-anisaldoxime, and the reaction was carried out with shaking at 30°C for 3 h. After the same workup procedure as described above, anisonitrile was obtained in a yield of 23.9 mg (5.95%) as colorless crystals; ^{1}H-NMR ($CDCl_3$) δ_{ppm} 7.589 (d, 2H, $J = 8.8$ Hz), 6.954 (d, 2H, $J = 8.8$ Hz), 3.864 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ_{ppm} 162.87, 134.00, 119.27, 114.78, 103.95, 55.57; mp. 57–60°C.

2.7.4. Synthesis of 2-furonitrile from E-furfurylaldoxime

The reaction mixture contained 17.5 mmol of KPB (pH 7.0), the heat-treated cells from 1.75-l culture, and 17.5 mmol (100 mM) of *E*-furfurylaldoxime in a total volume of 175 ml. When the reaction mixture was incubated with shaking at 30°C for 75 min, the product formed was extracted with AcOEt. The organic layer was combined and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified with Kugelrohr distillation (bath temperature, 160°C/20 mmHg) to give 2-furonitrile as a colorless oil (1.0 g, 62.0%); ^{1}*H*-NMR ($CDCl_3$) δ_{ppm} 7.599 (m, 1H), 7.119 (dt, 1H, $J = 1.0$, 3.7 Hz), 6.549 (m, 1H); ^{13}*C*-NMR ($CDCl_3$) δ_{ppm} 147.37, 126.35, 122.02, 111.48.

2.7.5. Synthesis of cyanopyrazine from E-pyrazinealdoxime

The reaction was carried out in a similar manner as described in the synthesis of 2-cyanopyridine using *E*-pyrazinealdoxime (30 mmol) as a substrate. Purification by preparative TLC (AcOEt/acetone = 3/2) afforded cyanopyrazine as colorless crystals (696 mg, 22.1%); ^{1}H-NMR ($CDCl_3$) δ_{ppm} 9.228 (m, 1H), 8.970 (m, 1H), 8.865 (m, 1H); ^{13}C-NMR (400 MHz, $CDCl_3$) δ_{ppm} 148.77, 148.33, 145.87, 129.84, 116.00.

2.7.6. Synthesis of n-butyronitrile from E/Z-n-butyraldoxime

The reaction was carried out similarly as described in the synthesis of 2-cyanopyridine using *E*/*Z*-*n*-butyraldoxime (30 mmol) as a substrate. The reaction product was purified with Kugelrohr distillation (bath temperature, 130°C/20 mmHg) to afford *n*-butyronitrile as a colorless oil (939 mg, 45.4%); ^{1}H-NMR ($CDCl_3$) δ_{ppm} 2.329 (t, 2H, $J = 6.1$ Hz), 1.695 (m, 2H), 1.084 (t, 3H, $J = 7.2$ Hz); ^{13}C-NMR (400 MHz, $CDCl_3$) δ_{ppm} 119.72, 19.15, 19.05, 13.31.

3. Results and discussion

3.1. Substrate specificity of E-pyridine-3-aldoxime dehydratase shown by the cells of Rhodococcus sp. strain YH3-3

Various aldoximes were incubated for 15–90 min with intact cells of *Rhodococcus* sp. strain YH3-3 to investigate the substrate specificity of the dehydratase. As shown in Table 1, analogs of pyridine-3-aldoxime, such as *E*-pyridine-2-aldoxime, *E*-pyrazine-aldoxime, *O*-acetyl-*E*-pyridine-3-aldoxime, and *E*-pyridine-3-aldoxime-*N*-oxide acted as substrates. Substituted benzaldoximes were also accepted as substrates but the relative activities were low. *Z*-Pyridine-3-aldoxime was also dehydrated to form 3-cyanopyridine at a rate of 18% to that for the *E*-isomer. DeMaster et al. [16] and Boucher et al. [17] reported a dehydration of aldoximes, such as *E*/*Z*-*n*-butyraldoxime, *Z*-benzaldoxime, *E*/*Z*-phenylacetaldoxime, and *E*/*Z*-heptanaldoxime, to their corresponding nitriles by cytochrome P-450s under anaerobic conditions. In these reports, only *Z* isomers of aldoximes were dehydrated, but *Rhodococcus* enzyme appeared to catalyze the reaction with both geometrical isomers of the aldoxime under aerobic condition. Of analogs of *E*-pyridine-3-aldoxime, *O*-alkyl and *N*-methyl derivatives of the aldoxime and *E*-pyridine-4-aldoxime were not dehydrated

Table 1
Substrate specificity of *E*-Pyridine-3-aldoxime dehydratase activity in *Rhodococcus* sp. YH3-3

Substrate[a]	Relative activity (%)
E-Pyridine-3-aldoxime	100
Z-Pyridine-3-aldoxime	18
E-Pyridine-2-aldoxime	2.4
E-Pyrazinealdoxime	5.5
E-*p*-Tolualdoxime	1.1
E-*p*-Chlorobenzaldoxime	1.0
E-*p*-Methoxybenzaldoxime	0.22
E-*p*-Nitrobenzaldoxime	0.08
E-2-Furfurylaldoxime	1.8
E-Indole-3-aldoxime	0.27
E/*Z*-Pyridine-3-aldehyde hydrazone	0.35
O-Acetyl-*E*-pyridine-3-aldoxime	5.5
E-Pyridine-3-aldoxime-*N*-oxide	5.1
E/*Z*-Acetaldoxime	5.7
E/*Z*-*n*-Butyraldoxime	33

[a] No transformation of the following substrates was detected in 24 h even when cells from 250 ml culture were added to the reaction mixture: *E*-benzaldoxime, *E*-pyridine-4-aldoxime, *E*-quinoline-2-aldoxime, *E*-thiophene-2-aldoxime, *E*/*Z*-phenylacetaldoxime, *E*/*Z*-2-phenylpropionaldoxime, *Z*-3-phenylpropionaldoxime, *Z*-cinnamaldoxime, *E*-1-naphtoaldoxime, *E*-*o*, *m*, *p*-phthalodialdoximes, *Z*-*p*-toluacetaldoxime, *Z*-*p*-chlorophenylacetaldoxime, *Z*-*p*-anisylacetaldoxime, *Z*-*p*-hydroxyphenylacetaldoxime, *E*-thiophene-2-acetaldoxime, *E*/*Z*-indole-3-acetaldoxime, *E*/*Z*-diphenylacetaldoxime, *E*-*O*-methyl-pyridine-3-aldoxime, *E*-*O*-benzyl-pyridine-3-aldoxime, *E*-*N*-methyl-pyridine-3-aldoxime iodide, *E*/*Z*-3-acetylpyridine oxime, *E*/*Z*-cyclohexylaldoxime, *E*/*Z*-methacrylaldoxime, *Z*-crotonaldoxime.
Cells from 25 ml culture harvested by centrifugation (3,500 × *g*, 25 min) were suspended to 2.5 ml of 0.1 M KPB (pH 7.0) and to this was added 2.5 μmol of aldoxime, as a substrate. The reaction was carried out at 30°C for 15 to 90 min with shaking. The reaction mixture was sampled, centrifugated (18,000 × *g*, 15 min), and the supernatant obtained were analyzed with high-performance liquid chromatography (HPLC; Waters, Bedford, USA) or gas-liquid chromatography (GLC; Shimazu, Kyoto, Japan). Aromatic compounds were measured with HPLC at a flow rate of 1 ml/min equipped with a ODS-80Ts column (4.6 × 150 mm) using an elution solvent containing H_3PO_4 or KPB at various pHs in aqueous CH_3CN, and the A_{254} was measured. Aliphatic compounds were analyzed with GLC equipped with a flame ionization detector with a glass column (2 mm by 2 m) packed with polyethyleneglycol (PEG20M, 60–80 mesh; GL-Science, Tokyo, Japan). The enzyme activity for *E*-pyridine-3-aldoxime was expressed as 100%.

by the enzyme. *E*/*Z*-Pyridine-3-aldehyde hydrazone, whose conversion to 3-cyanopyridine by chemical synthesis requires a very harsh condition [9], was transformed to the nitrile by the enzyme. Indole and furan ring containing heterocyclic aldoximes were active as substrates, but those with quinoline and thiophene ring were not dehydrated. The enzyme did not act on non-substituted benzaldoxime and various arylalkylaldoximes. Although straight chain alkylaldoximes such as *E*/*Z*-acetaldoxime and *E*/*Z*-butyraldoxime were active as substrates, unsaturated or substituted alkyl aldoximes were not. Ketoxime such as *E*/*Z*-3-acetylpyridine oxime was not transformed by the enzyme.

3.2. Microbial dehydration of E-pyridine-3-aldoxime by the heat-treated cells of Rhodococcus sp. strain YH3-3

We have observed a successive decomposition of 3-cyanopyridine to nicotinamide, nicotinic acid, and ammonia by the intact cells of *Rhodococcus* sp. strain YH3-3 which contains both nitrile hydratase and amidase [8]. To inhibit the nitrile degrading enzymes, heat-treated cells or acetone-dried cells of the strain were prepared and the dehydration reaction was examined. The yield of the nitrile was increased because the degradation of 3-cyanopyridine was suppressed by the heat-treatment. The highest enzyme activity was seen in the cells which had been incubated at 40°C for 1 h in 0.1 M KPB (pH 7.0) containing 1 mM of DTT and 5 mM of 2-mercaptoethanol, and the yield of the nitrile was reached up to 100% (Fig. 2). On the other hand, when the incubation time was longer than 2 h or the treatment temperature was higher than 40°C, the yield was no more than 60%. Omission of DTT and 2-mercaptoethanol from the buffer also lowered the yield to 70%. On the other hand, the yield of the nitrile was very low when acetone-dried cells were used as catalysts (Fig. 2). The aldoxime dehydratase and the nitrile degrading enzyme activities in intact, heat-treated, and acetone-dried cells were measured. In the heat-treated cells, nitrile hydratase activity was decreased to 55%, while no change in the aldoxime dehydratase and amidase activities were seen (data not shown). These would be the reason for the suppression of the nitrile

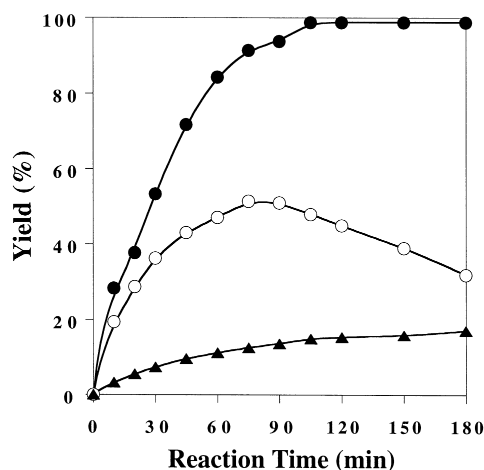

Fig. 2. Microbial synthesis of 3-cyanopyridine with the cells of *Rhodococcus* sp. strain YH3-3. The reaction mixture (2.5 ml) containing 250 μmol of KPB (pH 7.0), 125 μmol of *E*-pyridine-3-aldoxime, and intact (○), heat-treated (●), and acetone-dried (▲) cells of the strain from 25 ml culture, were incubated at 30°C with shaking. Concentrations of *E*-pyridine-3-aldoxime and 3-cyanopyridine in the supernatant obtained by centrifugation were analyzed with HPLC at a flow rate of 1 ml/min equipped with a ODS-80Ts column (4.6 × 150 mm) using an elution solvent consisted of 10 mM H_3PO_4 in 10% CH_3CN at 30°C by measuring an absorbance at 254 nm.

degradation by the heat-treatment. The low productivity of the nitrile by acetone-dried cells might be due to an inactivation of the dehydratase which was decreased to 47% (data not shown).

3.3. Reaction conditions for the dehydration of E-pyridine-3-aldoxime by heat-treated cells of the strain

Various reaction conditions for the dehydration of *E*-pyridine-3-aldoxime were studied with heat-treated cells of *Rhodococcus* sp. strain YH3-3.

3.3.1. Effects of pH

The dehydration reaction was carried out in 0.1 M buffers of various pHs. As shown in Fig. 3a), optimum pH of the reaction was found to be between 6.5 to 7.5. The enzyme was inactivated at below pH 5.0 and above 10.0.

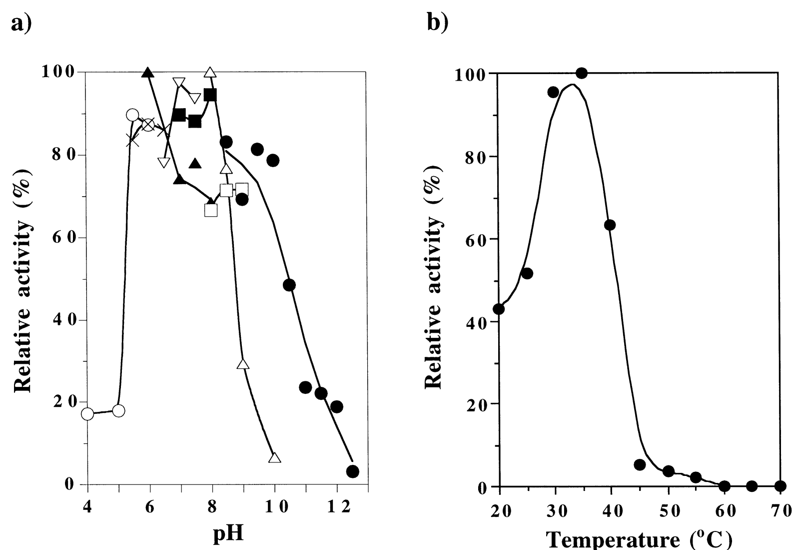

Fig. 3. Effects of a) pH and b) temperature on 3-cyanopyridine formation. A reaction mixture (0.5 ml) containing 50 μmol of a buffer, 25 μmol of *E*-pyridine-3-aldoxime, and heat-treated cells of the strain from 5 ml culture was incubated with shaking. The relative activity is expressed as the percentage of the maximum activity attained under the conditions. a) The reactions were done at 30°C for 1 hr in the following buffers: pH 3.0-6.0, AcOH-AcONa (○); pH 6.0-8.0, KPB (▲); pH 7.5-9.0, Tris-HCl (□); pH 9.0-12.0, Gly-NaCl-NaOH (●); pH 8.0-10.5, NH_4Cl-NH_4OH (△); pH 5.5-6.5, MES (×); pH 7.0-8.0, HEPES (■); and pH 7.0-8.0, MOPS (▽). b) The reactions were carried out in KPB (pH 7.0) at various temperatures for 1 hr. Concentrations of 3-cyanopyridine in the supernatant were analyzed as described in the legend to Fig. 2.

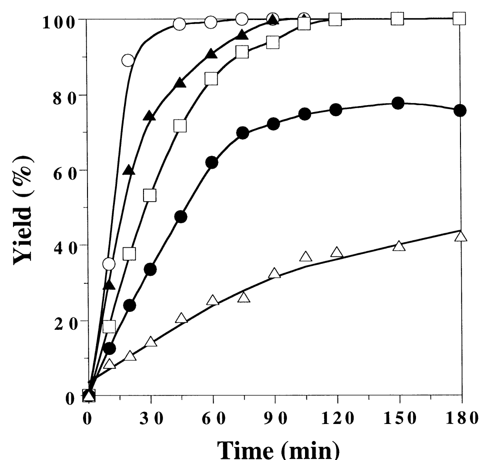

Fig. 4. Effects of substrate concentrations on 3-cyanopyridine formation. The reaction was carried out as described in the legend to Fig. 2 except 2.5 (○), 25 (▲), 125 (□), 250 (●), and 625 (△) μmol of the substrate were added, and 3-cyanopyridine concentrations were determined.

3.3.2. Effects of temperature

The reaction temperature was varied (20–70°C) and the formation of the nitrile was measured. As shown in Fig. 3b), the optimum temperature was around 30°C while the enzyme activity was decreased rapidly over 45°C.

3.3.3. Effects of metal ions and inhibitors

To a reaction mixture (0.5 ml) containing heat-treated cells from 5 ml culture, 50 μmol of KPB (pH 7.0), and 10 μmol of the aldoxime, were added 10 mM of various additives, and the enzyme reaction was done at 30°C for 1 h with shaking. The additives used were metal ions, such as Na^{+}, Mg^{2+}, Al^{3+}, K^{+}, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ag^{+}, Cd^{2+}, Sn^{2+}, Ba^{2+}, Hg^{+}, Hg^{2+}, and Pb^{2+}, metal cheating reagents, such as EDTA, EGTA, Tiron, 8-hydroxyquinoline, 2,2′-bipyridyl, and *o*-phenanthroline, sulfhydryl compounds, such as iodoacetic acid, *p*-chloromercuribenzoic acid, dithiobis-(2-nitrobenzoic acid), and *N*-ethylmaleimide, carbonyl reagents, such as KCN, NaN_3, hydroxylamine, hydrazine, and phenylhydrazine, and other compounds, such as phenylmethanesulfonyl fluoride, D-penicillamine, D-cycloserine, 2-mercaptoethanol, and DTT. None of them was effective for the enhancement of the enzyme activity, while the compounds such as heavy metal ions, NH_2OH, KCN, D-cycloserine, phenylhydrazine strongly inhibited the reaction. Purification and characterization of the enzyme should provide details for a mechanism of the inhibition.

3.3.4. Effects of substrate concentration

As shown in Fig. 4, the concentration of the aldoxime was varied and the amount of the

Table 2
Microbial synthesis of nitriles from aldoximes by heat-treated cells of *Rhodococcus* sp. strain YH3-3[a]

Substrate	Substrate conc. (mM)	Reaction time (min)	Yield (%)
E-Pyridine-3-aldoxime	50	105	98
E-2-Furfurylaldoxime	100	75	62
E/*Z*-*n*-Butyraldoxime	100	30	45
E-Pyridine-2-aldoxime	100	180	30
E-Pyrazinealdoxime	100	240	22
E-Anisaldoxime	10	180	6.0
E-*p*-Tolualdoxime	10	120	24[b]
E-*p*-Chlorobenzaldoxime	10	150	7.2[b]
E-*p*-Nitrobenzaldoxime	100	90	0.06[b]
E-Indole-3-aldoxime	100	10	0.07[b]
E-Pyridine-3-aldoxime-*N*-oxide	10	105	0.20[b]
O-Acetyl-*E*-pyridine-3-aldoxime	100	180	73[b]
Z-Pyridine-3-aldoxime	50	180	20[b]
E/*Z*-Pyridine-3-aldehyde hydrazone	10	100	2.4[b]

[a]The reaction was carried out as descried in Section 2.
[b]The yield was determined by HPLC.

formed nitrile was measured. Initial reaction rate was gradually decreased by increasing the substrate concentrations probably due to the substrate or product inhibitions. 3-Cyanopyridine was obtained in 98.7, 100, 100, 77.6, and 42.3% yields from 1, 10, 50, 100, and 250 mM of the aldoxime, respectively.

3.4. Microbial synthesis of nitriles from aldoximes by heat-treated cells of Rhodococcus sp. strain YH3-3

Various nitriles were synthesized in preparative scales from 10–100 mM of aldoximes under the optimized condition (pH 7.0, 30°C) with heat-treated cells of the strain. Table 2 shows that arylnitriles, such as 3-cyanopyridine, 2-cyanopyridine, cyanopyrazine, 3-cyanopyridine-*N*-oxide, 2-furonitrile, *p*-anisonitrile, *p*-tolunitrile, *p*-chlorobenzonitrile, *p*-nitrobenzonitrile, and 3-cyanoindole, and alkylnitrile, such as *n*-butyronitrile were synthesized from the corresponding aldoximes. The higher were the substrate concentrations than those shown in Table 2, the more the enzyme was inactivated by their substrates. 3-Cyanopyridine was also synthesized in 72.5%, 19.7%, and 2.36% yields from *E*-pyridine-3-aldoxime analogs, such as *O*-acetyl-*E*-pyridine-3-aldoxime, *Z*-pyridine-3-aldoxime, and *E/Z*-pyridine-3-aldehyde hydrazone, respectively. By the chemical dehydration reaction of aldoximes, only *Z*-isomer of aldoxime such as *Z*-*p*-bromobenzaldoxime was dehydrated into the corresponding nitrile [9,10] and the *E*-aldoxime was dehydrated via isomerization to the *Z*-isomer. It is interesting that *Rhodococcus* enzyme prefers *E*-pyridine-3-aldoxime to its *Z*-isomer as a substrate, although no isomerization of the *E*-aldoxime to the *Z*-isomer had been observed in the reaction mixture. Continuing studies are in progress, to clarify the difference between the enzyme reaction and the chemical one and to have a further insight into the mechanism of the enzyme reaction.

This report has dealt with the first microbial synthesis of nitriles from aldoximes in preparative scales. The enzymatic synthesis of nitriles was proved to be superior to the chemical reactions because of its mild reaction conditions. The results encourage us to continue investigations on screening for other oxime dehydratases with different substrate specificities, which are applicable to synthesis of various nitriles.

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