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Degradation of Organic Cyanides by *Pseudomonas aeruginosa*

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ABSTRACT

A bacterium capable of utilizing acetonitrile (methyl cyanide) as the sole source of carbon and nitrogen was isolated from soil and identified as *Pseudomonas aeruginosa*. This bacterium could also utilize and oxidize numerous lower-mol-wt nitrile compounds and their corresponding amides as growth substrates. A metabolite of acetonitrile in the culture medium was determined to be ammonia. The accumulation of ammonia in the culture medium was proportional to the concentration of the substrate and the inoculum. Cell extracts of the bacterium contained activities corresponding to nitrile aminohydrolase (E C 3.5.5.1) and amidase (E C 3.5.1.4), which regulate the degradation of acetonitrile. Both enzymes were inducible and hydrolyzed a wide range of substrates, and it was determined that the specific activity of amidase was far greater than the activity of nitrile aminohydrolase.

Index Entries: Acetonitrile; biodegradation; nitrile aminohydrolase; amidase.

INTRODUCTION

Nitrile compounds are cyanide-substituted carboxylic acids. These compounds are produced naturally and synthetically. Naturally occurring nitriles are found in higher plants and in bone oils; in addition, insects and a variety of microorganisms have the capacity to produce them (*1,2*).

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Recently, it has been reported that certain types of shale oil also contain high concentrations of nitriles (*3*). Synthetic nitriles, on the other hand, have been extensively used in the manufacture of herbicides, polymers, and plastics, and as premier organic solvents (*4*). The direct discharge of wastewater containing some of these nitrile compounds could possibly cause severe health hazards, since most of them are highly toxic and some are mutagenic and carcinogenic. Earlier, a report (*5*) had indicated the removal of nitriles by activated sludge systems. However, the cost of applying this technology is prohibitive. Bioremediation, an inexpensive technology, can eliminate such compounds by degrading them to harmless intermediates or, ultimately, to carbon dioxide and water.

Despite the widespread use of nitrile compounds in numerous industrial processes, only a few microorganisms have been reported to metabolize nitrile compounds and their derivatives. Selected strains of *Pseudomonas* (*6*), *Arthrobacter* (*7*), *Nocardia rhodochrous* (*8*), and *Brevibacterium* (*9*) have been reported to metabolize a certain number of nitrile compounds and their derivatives, predominantly to ammonia and traces of carboxylic acids. Earlier, Yamada et al. (*10*) reported the isolation of a *Pseudomonas* sp. strain, K-9, that was capable of utilizing only glutaronitrile. This strain failed to utilize other nitrile compounds as growth substrates. In this report we describe a strain of *Pseudomonas aeruginosa* capable of utilizing acetonitrile and a variety of other low-mol-wt nitrile compounds and amides as the sole source of carbon and nitrogen. In addition, we have determined the optimal conditions of the nitrile-degrading enzymes, viz. nitrile hydratase and amidase, because of their commercial applications (*11*).

MATERIALS AND METHODS

Chemicals

Nitrile compounds and their derivatives were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. All other reagents were of the highest purity and were purchased from Sigma Chemical Company, St. Louis, MO.

Media and Culture Conditions

The minimal medium used for the isolation of acetonitrile-utilizing microorganisms contained the following (g/L): K_2HPO_4, 4.3; KH_2PO_4, 3.4; $MgCl_2 \cdot H_2O$, 0.3; the medium was amended with 0.5 mL of trace element solution containing the following (g/L): $MnCl_2 \cdot 4H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot H_2O$, 2.6; $NaMoO_4 \cdot 2H_2O$, 6.0.

The minimal-medium plates were prepared by adding 15 g of agar (Difco Laboratories, Detroit, MI) to 1 L of the medium. Unless otherwise stated, the microorganisms were grown in medium containing acetonitrile as the sole source of carbon and nitrogen.

Table 1
Growth of *Pseudomonas aeruginosa* on Nitriles and Related Compounds

Substrate	Protein (mg/L)	Ammonia (mM/L)
Acetonitrile	8.05	24.8
Butyronitrile	7.15	16.7
Glutaronitrile	6.77	16.0
Propionitrile	7.09	16.6
Methacrylonitrile	5.71	15.9
Isobutyronitrile	5.96	16.0
Succinonitrile	5.29	14.8
Valeronitrile	5.07	9.21
Acrylonitrile	0.11	-
Benzonitrile	0.11	-
Phenylacetonitrile	0.11	-
Acetamide	9.11	26.9
Propionamide	7.36	17.3
Butyramide	7.47	17.5
Methacrylamide	6.21	16.8
Isobutyramide	6.13	17.2
Succinamide	5.47	14.8
Acrylamide	0.11	-
2,4-Dicyanobutene	0.11	-
2-Cyanobutene	0.11	-

Pseudomonas aeruginosa was grown on minimal medium containing 0.2% (V/V) of each compound as sole source of carbon and nitrogen. Biomass (protein content) and ammonia was determined after 48 h of incubation.

Isolation and identification of the bacteria were performed by methods described elsewhere (*6*).

Screening of Substrates

Several nitrile compounds and some of their corresponding amides (Table 1) were tested for their ability to support growth of *Pseudomonas aeruginosa* at an initial concentration of 0.1% (v/v) in 50 mL of filter-sterilized medium dispensed in 250-mL Erlenmeyer flasks on a rotary shaker at 200 rpm. Bacterial cells (24 h old) were harvested by centrifugation, washed twice in 25 m*M* phosphate buffer (pH 7.0), sedimented again by centrifugation, suspended, and stirred in 9 mL of the buffer to give a final A_{600} (turbidity) of 1.0. One milliliter of this suspension was used as the inoculum. Growth (protein), pH, and ammonia were determined after 48 h of incubation.

Measurement of Oxygen Uptake

Oxygen uptake was measured with a Gilson 5/6 Oxygraph (Gilson medical Electronics, Inc., Middleton, WI) containing a semipermeable membrane-coated Clark electrode, which was attached to a water-jacketed cell and linked to a chart recorder. Bacterial cells were harvested by centrifugation, washed twice in 25 m*M* phosphate buffer (pH 7.0), sedimented again by centrifugation, and suspended in buffer to give a final concen-

tration equivalent to an A_{600} (turbidity) of 1.0. Of the cell suspension, μL was then injected into the cell chamber, following which the substrate (1.5 mL, 0.2% v/v) was injected into the cell chamber. The suspension was then stirred and equilibrated at 30°C for 20 min before readings were recorded. The oxygen uptake with different substrates was recorded for 10 min. All data were corrected for endogenous respiration.

Preparation of Cell Extract

Batch cultures of the bacterial isolate were cultivated in 4-L Fernbach flasks containing 120 m*M* acetonitrile. Bacterial cells (48 h old) were harvested by centrifugation (18,000*g*) for 15 min, washed twice in 23 m*M* phosphate buffer (pH 7.0), and sedimented again by centrifugation. The resulting pellet was homogenized with 25 m*M* phosphate buffer (pH 7.0). Unbroken cells and debris were removed by centrifugation at 18,000*g* for 15 min, and the cell-free supernatant fluid thus obtained served as a crude enzyme source for all subsequent studies.

Enzyme Assays

Nitrile aminohydrolase and amidase activities were assayed by measuring the production of ammonia by the method described elsewhere (*12,13*). Nitrile aminohydrolase assay was started by the addition of substrate (acetonitrile) and carried out at 37°C for 30 min. Amidase activity was determined by using acetamide as substrate and incubating at 37°C for 30 min (*12,13*). Both enzyme assays were terminated by boiling at 65°C for 1 min, and the amount of ammonia released was determined. Boiled enzyme solution served as a blank for both assays. Both reactions (nitrile aminohydrolase and amidase) were found to be linear with time and enzyme concentrations.

Analytical Methods

Protein content was determined by the Lowry method, with bovine serum albumin as standard (*14*). Ammonia was determined colorimetrically by the Berthelot procedure, as described by Kaplan (*15*).

RESULTS AND DISCUSSION

Isolation, Characterization, and Identification of Bacteria

Acetonitrile-utilizing bacteria were isolated from a variety of soil and water samples collected from different industrial ecosystems. Bacterial growth (development of turbidity) and simultaneous increase in the pH of the medium (caused by ammonia accumulation as a result of CN cleavage) was observed after 2–7 d of incubation. A pure culture of this organism

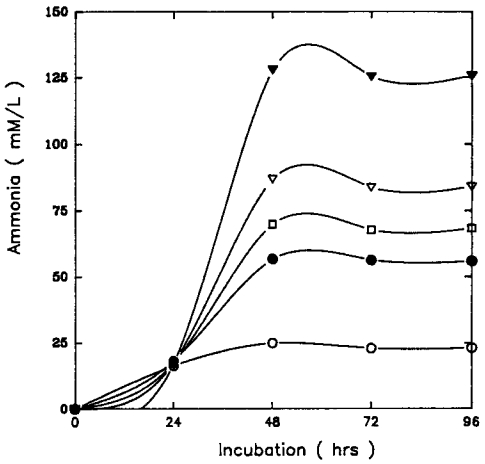

Fig. 1. Effect of acetonitrile concentration on ammonia accumulation. Cells of *Pseudomonas aeruginosa* were grown and suspended as described in the Materials and Methods section. Different concentrations of acetonitrile was added to each flask, and the quantity of ammonia accumulated in the culture medium was determined at 24-h intervals. Symbols: 37 m*M*, ○——○; 75 m*M*, ●——●; 112 m*M*, □——□; 150 m*M*, ▼——▼; 187 m*M*, ▽——▽.

was obtained by repeated subculturing and simultaneous streaking of isolated colonies on minimal-medium plates containing acetonitrile as the sole source of carbon and nitrogen.

Individual colonies on minimal-medium plates were small (about 1 mm in diameter), circular, convex with entire margin, beige, and creamy in texture. Cells of the bacteria were rod-shaped, gram-negative, and motile. Spores were not observed. Colonies were fluorescent. Oxidase, catalase, and arginine dihydrolase reactions were positive. Succulent growth was obtained on sodium benzoate, MacConkey, and glucose plates, but not on xylose and maltose. The isolate failed to hydrolyze gelatin. On the basis of these reactions, the organism was identified as *Pseudomonas aeruginosa*.

Maximal growth was obtained after 72 h of incubation at 30°C. No growth occurred at either 10 or 55°C. The isolate was found to grow well between pH 5.0 and pH 8.0; however, maximal cell yields were obtained at pH 7.0. No growth occurred below pH 5.0 or above pH 8.5.

Effect of Acetonitrile Concentration on Ammonia Accumulation

Figure 1 indicates the effects of different concentrations of acetonitrile on ammonia accumulation (an indication of acetonitrile degradation) in the culture medium when inoculated with 1-mL cells at an A_{600} (turbidity) of 1.0. Ammonia accumulation was proportional to the concentration of

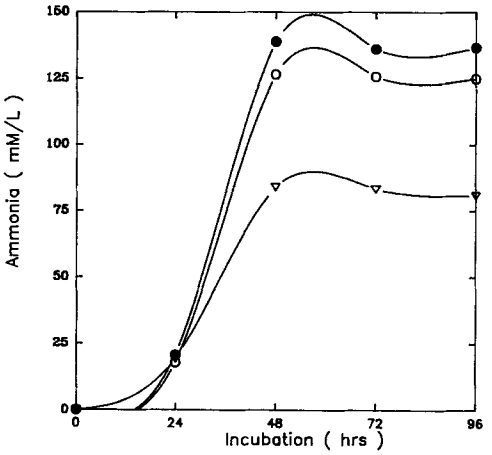

Fig. 2. Dependence of ammonia accumulation on cell density. Cells of *Pseudomonas aeruginosa* grown as described in the Materials and Methods section were incubated with acetonitrile (150 m*M*) at the following cell densities (A_{600}): 1.0, ∇——∇; 2.0, ●——●; 3.0, ○——○.

acetonitrile in the medium, up to 150 m*M*, whereas at concentrations higher than 150 m*M*, a lag in ammonia production was observed. Consequently, culture media in succeeding experiments were always supplied with 150 m*M* of acetonitrile as a growth substrate.

Effect of *Pseudomonas aeruginosa* Cell Density on Ammonia Accumulation

Figure 2 shows the effect of different cell densities on the accumulation of ammonia (sign of acetonitrile degradation). Although ammonia concentrations were similar up to 24 h of incubation, after that point, a significant increase was seen in the cells at a turbidity of 2.0 (at 600 nm) compared with those at a turbidity of 1.0. Hence, in succeeding experiments, inoculum densities were always maintained at a turbidity of 2.0. This corresponded to a protein concentration of 110 μg/mL.

Growth and Utilization of Acetonitrile and Other Nitrile Compounds by *Pseudomonas aeruginosa*

The growth and utilization of different nitrile compounds by *Pseudomonas aeruginosa* was determined by measuring bacterial protein concentrations and ammonia accumulation in the culture medium after 48 h of incubation at 30°C (Table 1). Among the nitriles, acetonitrile was the best, and butyronitrile, propionitrile, and glutaronitrile were better than the other nitriles tested. All the tested nitriles supported growth and utilization (as indicated by higher protein concentration and ammonia accumulation). Among the amides, acetamide induced higher growth rates and

Table 2
Measurement of Oxygen Uptake by *Pseudomonas aeruginosa*

Substrate	Oxygen Uptake (nmol/min/mg of protein)
Acetonitrile	65.09
Propionitrile	61.11
Crotononitrile	-
Butyronitrile	63.01
Methacrylonitrile	52.91
Isobutyronitrile	56.61
Succinonitrile	51.29
Acrylonitrile	-
Benzonitrile	-
Acetamide	74.77
Butyramide	71.01
Propionamide	69.83
Methacrylamide	59.92
Isobutyramide	63.09
Succinamide	58.29
Acrylamide	-
2,4-Dicyanobutene	-
2-Cyanobutene	-

ammonia accumulation than other amides tested. Acrylonitrile, benzonitrile, crotonitrile, phenylacetonitrile, acrylamide, 2-cyanobutene, and 2,4-dicyanobutene inhibited the growth of the bacteria.

Determination of Oxygen Uptake

Results of the respirometric studies (Table 2) indicate that the bacterial isolate has the ability to degrade a majority of nitrile compounds and amides. Acetonitrile-grown cells readily oxidized acetonitrile, butyronitrile, and propionitrile; succinonitrile, isobutyronitrile, and methacrylonitrile initially induced a lag in the rates of oxygen uptake. Though bacterium failed to oxidize acrylonitrile, benzonitrile, and crotonitrile, it exhibited higher rates of oxygen consumption in the presence of amides. The results demonstrate that the bacterial isolate is capable of degrading various nitriles and amides.

Characterization of Cell Extract

Cell extracts of the bacterial suspension contained both of the hydrolytic enzymes (nitrile aminohydrolase and amidase) that are known to participate in the metabolism of acetonitrile. Both enzymes peaked after 48 h of incubation (Fig. 3). Prolonging the incubation periods resulted in lower enzyme activities. Both enzyme activities increased with increasing incubation temperatures, the maximum activity centered around 35°C (Fig. 4). Altering pH vs activity demonstrated maximal enzyme activity at pH, 7.0 (Fig. 5).

The substrate specificity of nitrile aminohydrolase indicated that acetonitrile, butyronitrile, propionitrile, and glutaronitrile were more

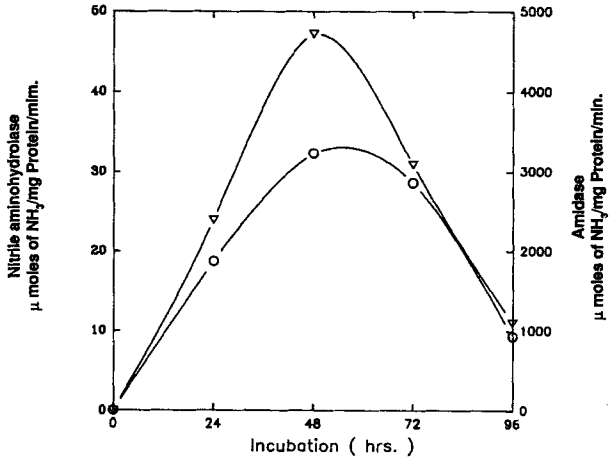

Fig. 3. Formation of nitrile aminohydrolase and amidase during cultivation of *Pseudomonas aeruginosa*. Cultivation was carried out at 30°C with 150 m*M* of acetonitrile. Enzyme activities were assayed by measuring the ammonia accumulated in the culture medium. Symbols: nitrile aminohydrolase, O——O; amidase, ∇——∇.

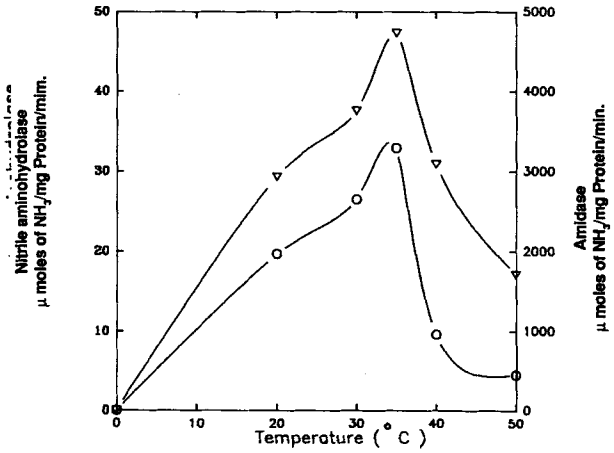

Fig. 4. Determination of optimal temperature for nitrile aminohydrolase and amidase. Cultivation was carried out at 30°C in 4-L Fernbach flasks with 1 L of the minimal medium containing 150 m*M* of acetonitrile. Symbols: nitrile aminohydrolase, O——O; amidase, ∇——∇.

suitable substrates than methacrylonitrile and succinonitrile, whereas acrylonitrile, benzonitrile, and phenylacetonitrile inhibited the nitrile aminohydrolase activity even at very low concentration (Table 3). Amidase activity also exhibited a broad range of substrate specificities (Table 4). Acetamide, butyramide, and propionamide were better substrates than isobutyramide and methacrylamide, but better than and succinamide. Acrylamide and 2,4-dicyanobutene inhibited the enzyme activity. It was determined that both enzymes were inducible.

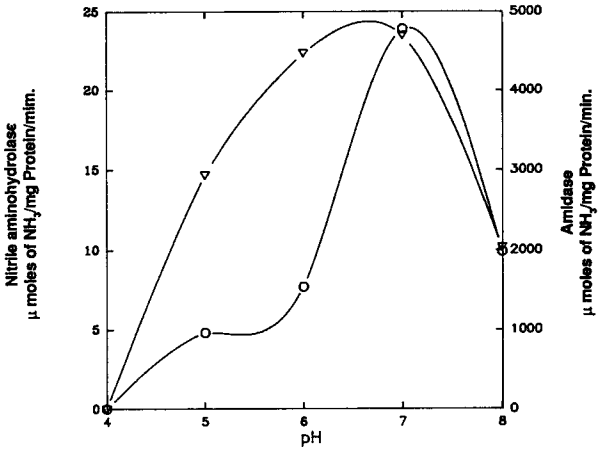

Fig. 5. Determination of optimal pH for nitrile aminohydrolase and amidase. Enzyme activities were assayed by measuring the ammonia accumulated in the reaction mixture. Symbols: nitrile aminohydrolase, ○——○; amidase, ∇——∇.

Table 3
Substrate Specificity of the Enzyme Nitrile Aminohydrolase

Substrate	Specific Activity (μmol of NH_3/min/mg protein)
Acetonitrile	32.8
Butyronitrile	27.6
Propionitrile	27.1
Glutaronitrile	27.3
Methacrylonitrile	20.9
Succinonitrile	19.9
Acrylonitrile	-
Benzonitrile	-
Phenylacetonitrile	-

The reaction mixture contained 0.2% of each nitrile compound. The reaction was carried out as described in Materials and Methods.

Table 4
Substrate Specificity of the Enzyme Amidase

Substrate	Specific Activity (μmol of NH_3/min/mg protein)
Acetamide	4730.6
Butyramide	4690.2
Propionamide	4681.3
Isobutyramide	3961.5
Methacrylamide	3300.0
Succinamide	3486.2
Acrylamide	-
2,4-Dicyanobutene	-

The reaction mixture contained 0.2% of each amide. The reaction was carried out as described in Materials and Methods.

The metabolism of nitrile compounds by several microorganisms have been documented (*6–10*). However, an earlier report (*10*) had indicated the isolation of a strain of *Pseudomonas* that utilized glutaronitrile. This strain failed to utilize acetonitrile, butyronitrile, propionitrile, crotonitrile, isobutyronitrile, and other corresponding amides as growth substrates. Our results demonstrate that the isolated bacteria, *Pseudomonas aeruginosa,* is equipped with the enzymatic mechanism for the metabolism of acetonitrile (Fig. 1 and 2) and various other nitriles and their amides as growth substrates (Table 1).

Microorganisms have been reported to break down nitriles, involving a two-step enzymatic mechanism (*12,13*). Nitrile aminohydrolase, the primary enzyme, transforms the nitrile compounds to their respective amide. The amide was later degraded by amidase to its carboxylic acid and ammonia. However, an earlier report (*16*) had indicated microbial transformation of nitrile compounds directly to their corresponding carboxylic acids and ammonia, without formation of the amide as an intermediate. Based on our results, we are led to suggest that *Pseudomonas aeruginosa* inducibly synthesizes nitrile aminohydrolase and amidase. Both enzymes have a wide range of substrates for hydration. From the rates of NH_3 production, it is evident that the activity of amidase was superior to that of nitrile aminohydrolase.

The application of microorganisms as biocatalysts for the production of amides and organic acids has attracted commercial interest (*11*). Biocatalysts are highly specific and efficient in the transformation of nitrile compounds to their corresponding amides or organic acids. Our results indicate that the nitrile aminohydrolase activity of *Pseudomonas aeruginosa* was lower than the specific activity of other microorganisms known to participate in the transformation of nitrile compounds to amides (*11,12*). However, the amidase activity of this strain was superior to any of the microorganisms known to participate in the degradation of nitrile compounds to their corresponding carboxylic acids. Hence, *Pseudomonas aeruginosa* may be utilized commercially for the production of respective organic acids.

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