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Degradation of Acetonitrile by *Pseudomonas putida*

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A bacterium capable of utilizing high concentrations of acetonitrile as the sole source of carbon and nitrogen was isolated from soil and identified as *Pseudomonas putida*. This bacterium could also utilize butyronitrile, glutaronitrile, isobutyronitrile, methacrylonitrile, propionitrile, succinonitrile, valeronitrile, and some of their corresponding amides, such as acetamide, butyramide, isobutyramide, methacrylamide, propionamide, and succinamide as growth substrates. Acetonitrile-grown cells oxidized acetonitrile with a K_m of 40.61 mM. Mass balance studies with [^{14}C]acetonitrile indicated that nearly 66% of carbon of acetonitrile was released as $^{14}CO_2$ and 14% was associated with the biomass. Metabolites of acetonitrile in the culture medium were acetic acid and ammonia. The acetate formed in the early stages of growth completely disappeared in the later stages. Cell extracts of acetonitrile-grown cells contained activities corresponding to nitrile hydratase and amidase, which mediate the breakdown of actonitrile into acetic acid and ammonia. Both enzymes were intracellular and inducible and hydrolyzed a wide range of substrates. The specific activity of amidase was at least 150-fold higher than the activity of the enzyme nitrile hydratase.

Nitrile compounds and their derivatives are extensively used in many industrial operations. These compounds are cyanide-substituted carboxylic acids and are produced naturally and synthetically. Naturally occurring nitriles are found in higher plants, bone oils, and insects (21), and it has been proven that microorganisms have the capacity to synthesize them (12). Synthetic nitriles, on the other hand, have been used extensively in the manufacture of herbicides such as dichlobenil (2,6-dichlorobenzonitrile) and bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) (4) and the synthesis of polymers and plastics, and they are widely used as organic solvents (10).

Very little information is available on the ecological impact of organic nitriles and their derivatives. It is highly conceivable that the direct discharge of wastewater containing some of these nitrile compounds could cause severe health hazards, since most of them are highly toxic and some are mutagenic and carcinogenic.

Only a few microorganisms have been reported to metabolize nitrile compounds and their derivatives. Robinson and Hook (17) isolated a *Pseudomonas* strain capable of utilizing the naturally occurring nitrile ricinine as a sole source of carbon and further purified an enzyme, ricinine nitrilase, responsible for the hydrolysis of ricinine to ammonia and the corresponding carboxylic acid. DiGeronimo and Antoine (7) isolated a strain of *Nocardia rhodochrous* that metabolized a selected number of nitrile compounds and their derivatives to carboxylic acids and ammonia. Selected strains of *Arthrobacter* (28) and *Brevibacterium* (6) have also been reported to metabolize certain nitrile compounds to their corresponding carboxylic acids and ammonia. Earlier, Yamada et al. (29) isolated a bacterium, identified as *Pseudomonas* sp. strain K-9, that was capable of utilizing only glutaronitrile and failed to utilize other nitrile compounds as growth substrates. The present investigation describes the isolation and characterization of a strain of *Pseudomonas putida* capable of utilizing acetonitrile and a variety of other lower-molecular-weight nitrile compounds and amides as the sole sources of carbon and nitrogen.

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MATERIALS AND METHODS

Chemicals. All nitrile compounds and their corresponding amides were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. [^{14}C]acetonitrile (specific activity, 3.0 mCi/mmol; purity, 99.0%) was purchased from Pathfinder Laboratories, St. Louis, Mo. Aquasol-2 and acetohydroxamate were purchased from Dupont, NEN Research Products, Boston, Mass., and Sigma Chemical Co., St. Louis, Mo., respectively.

Media and culture conditions. The minimal medium used for the isolation of acetonitrile-utilizing microorganisms contained the following (in grams per liter): 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 the trace element solution containing the following (in milligrams per liter): $MnCl_2 \cdot 4H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot H_2O$, 2.6; $NaMoO_4 \cdot 2H_2O$, 6.0. The pH was adjusted to 7.0, and the medium was autoclaved.

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

Isolation of bacteria. The bacteria used in this study were isolated from contaminated soil and water samples collected from industrial sites. A 1:10 dilution of each sample was made with sterile minimal medium, and the suspension was incubated at room temperature for 1 h. One milliliter of the suspension was inoculated into sterile Pyrex test tubes containing 9 ml of the medium supplemented with different concentrations of acetonitrile (ranging from 2.5 to 1,200 mmol/liter). The tubes were then incubated at 25°C. After 7 days of incubation, the tubes were examined for turbidity. After 5 to 7 transfers, the turbid samples were streaked out on plates containing acetonitrile as the sole source of carbon and nitrogen. Colonies that grew on agar plates containing acetonitrile but not on control plates (without acetonitrile) were then selected for identification.

TABLE 1. Growth of *P. putida* on nitriles and related amides[a]

Substrate	Growth (A_{660})	Final pH	Concn of ammonia (mmol/liter)
Acetonitrile	0.657	7.87	31.6
Butyronitrile	0.461	7.67	20.3
Glutaronitrile	0.401	7.52	18.7
Propionitrile	0.442	7.60	19.2
Methacrylonitrile	0.292	7.23	8.1
Isobutyronitrile	0.311	7.29	9.4
Succinonitrile	0.227	7.16	5.29
Valeronitrile	0.201	7.14	5.18
Acrylonitrile	0.127	7.01	
Crotononitrile	0.118	7.03	
Benzonitrile	0.120	7.00	
Phenylacetonitrile	0.123	7.00	
Acetamide	0.722	8.01	36.3
Propionamide	0.461	7.69	20.8
Butyramide	0.479	7.76	21.8
Methacrylamide	0.339	7.37	10.01
Isobutyramide	0.341	7.38	10.14
Succinamide	0.230	7.20	5.31
Acrylamide	0.123	7.00	
2,4-Dicyanobutene	0.120	7.01	
2-Cyanoacetamide	0.122	7.00	

[a] *P. putida* was grown on minimal medium (pH 7.0) containing 0.2% of each compound as a sole source of carbon and nitrogen at 30°C. The initial A_{660} of the culture medium was 0.120. Growth (A_{660}), pH, and ammonia concentration were determined after 72 h of incubation.

Identification of the organism. Identification of bacteria was based on the classification scheme described in *Bergey's Manual of Systematic Bacteriology* (16). Tests were performed as described in the manual (16) or as described by Smibert and Krieg (20). Subsequently, the isolates were biochemically characterized by using commercially available diagnostic kits from Flow Laboratories, Inc., McLean, Va.), and the API 20E test kit (Analytab Products, Plainview, N.Y.). The results were also confirmed by a computer survey available from these two suppliers.

Physiology. A 50-ml sample of the sterile medium containing 120 mM acetonitrile was inoculated with 2 ml of the bacterial suspension. (Suspensions having an A_{660} [turbidity] of 1.0 contained 653 mg of dry cells per liter of the medium. The dry cell weight was determined by the method described earlier [19].) Growth was measured every 24 h from 0 to 120 h with a Bausch and Lomb Spectronic 20. Uninoculated medium served as a reference.

The optimal temperature for the growth of the bacteria was determined by inoculating 50 ml of the minimal medium containing 120 mM of acetonitrile into 250-ml Pyrex flasks, which were then incubated at 5, 15, 25, 35, 45, and 55°C. Growth was monitored after 72 h of incubation. The optimal pH was determined by inoculating the medium adjusted to pH 3.0 to 9.0. The inoculated cultures were then incubated at 25°C. The growth was measured after 72 h of incubation.

Screening of substrates. Several nitrile compounds and some of their corresponding amides (Table 1) were tested for their ability to support growth of *Pseudomonas putida* at an initial concentration of 0.2% (vol/vol) in 50 ml of filter-sterilized medium dispensed in 250-ml Erlenmeyer flasks on a rotary shaker at 200 rpm (Forma Scientific, Marietta, Ohio). Bacterial cells (36 h old) were harvested by centrifugation at 15,000 × *g* for 10 min, washed twice in 25 mM phosphate buffer (pH 7.0), sedimented again by centrifugation, suspended, and stirred in 9 ml of the buffer to give a final A_{660} (turbidity) of 1.0. Two milliliters of this suspension was used as the inoculum. Growth, pH, and ammonia were determined after 72 h of incubation.

Measurement of substrate oxidation. Oxygen uptake was measured with a Gilson 5/6 Oxygraph (Gilson Medical Electronics, Inc., Middleton, Wis.) 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 at 15,000 × *g* for 10 min. The pellet was washed twice in 25 mM phosphate buffer (pH 7.0), sedimented again by centrifugation, and suspended in buffer to give a final concentration equivalent to an A_{660} (turbidity) of 0.25. One milliliter of the suspension was then injected into the cell chamber, followed by 1 ml of the substrate (ranging from 5 to 100 mmol/liter). The suspension was then stirred and equilibrated at 30°C for 20 min before readings were recorded. The oxygen uptake with different concentrations of acetonitrile was measured for 10 min. All data were corrected for endogenous oxygen uptake.

Accumulation of metabolites. A 1-liter flask containing 200 ml of the medium supplemented with 120 mM of acetonitrile was inoculated with the bacterial isolate and placed on a shaker (200 rpm). Samples (5 ml) were removed from the culture broth at periodic intervals starting from 0 to 120 h. After determination of growth at 660 nm, the cells in the samples were centrifuged. The pH of supernatant fluid was measured with a pH meter (Orion Research, Inc., Cambridge, Mass.), and the presence of ammonia and acetic acid was determined.

Degradation of ^{14}C acetonitrile. Twenty-five milliliters of the filter-sterilized medium (pH 7.0) containing 120 mM of acetonitrile and supplemented with [^{14}C]acetonitrile (30,000 cpm/ml) was dispensed into a 125-ml Warburg flask (Gilson Medical Electronics, Inc., Milwaukee, Wis.) on a rotary shaker. The mouth of the flask was fitted with an airtight stopper. The $^{14}CO_2$ that was formed during the bacterial growth on [^{14}C]acetonitrile was trapped in 2.5 ml of 1 N NaOH contained in the center well of the flask. The amount of radioactivity present in the NaOH solution was determined by addition of 0.25 ml of NaOH solution to 9.75 ml of Aquasol-2 scintillation fluid. The radioactivity in the culture medium was determined by filtering the culture medium (1.0 ml) through a 0.45-μm-pore-size GN 6 Metricel membrane filter paper (Gelman Sciences, Inc., Ann Arbor, Mich.) in a sampling manifold filter system (Millipore Corp., Bedford, Mass.) and washing the filter paper with 5.0 ml of the sterile phosphate buffer medium (25 mM, pH 7.0). The filter paper was dissolved in 1.0 ml of ethylene glycol monoethyl ether (Sigma) for 5 min, and 9.0 ml of the scintillation fluid was added. Similarly, 1.0 ml of the filtrate was added to 9.0 ml of the scintillation fluid and the radioactivity was determined with a liquid scintillation counter (model LS 6800; Beckman Instruments, Inc., Irvine, Calif.). Uninoculated medium served as a control.

Preparation of cell extract. Batch cultures of the bacterial isolate were cultivated in 4-liter Fernbach flasks containing 120 mM acetonitrile. Cells were harvested by centrifugation (15,000 × *g*) for 10 min at 24-h intervals starting from 24 to 120 h. The resulting pellet was washed twice with 25 mM phosphate buffer (pH 7.0), sedimented at 15,000 × *g* for 10 min, and homogenized with 25 mM phosphate buffer (pH 7.0). Unbroken cells and debris were removed by centrifugation at 15,000 × *g* for 10 min, and the cell-free supernatant fluid thus obtained served as a crude enzyme source for all subsequent studies.

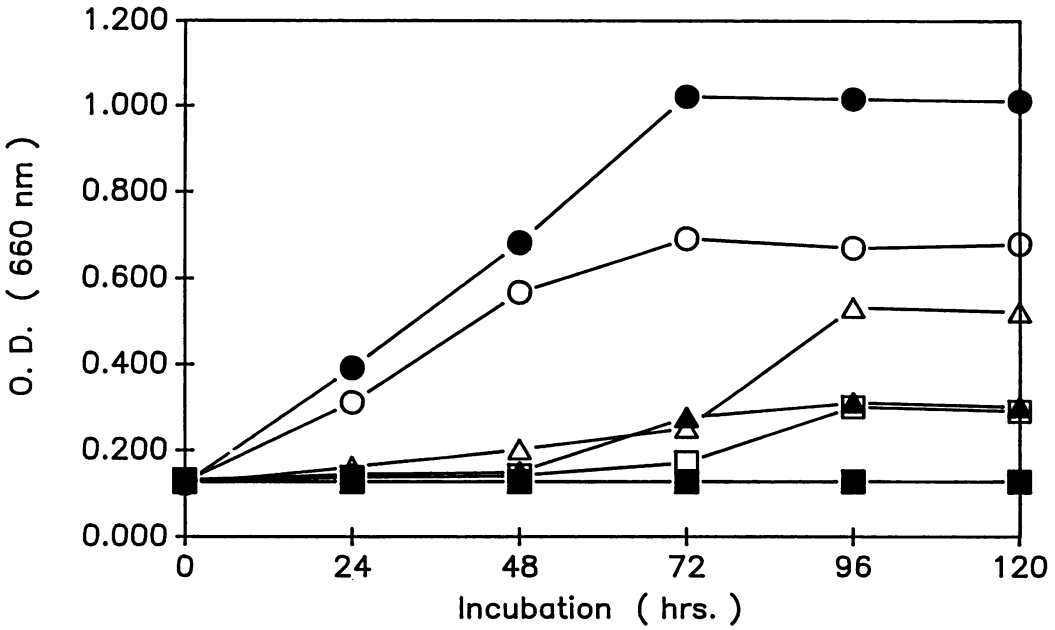

FIG. 1. Effect of acetonitrile concentrations on the growth of *P. putida*. Cultivation was carried out at 30°C in 1-liter flasks containing 200 ml of the minimal medium supplemented with 60 (○), 120 (●), 240 (△), 480 (▲), 600 (□), or 720 (■) mM acetonitrile. O.D., Optical density.

Enzyme assays. (i) Nitrile hydratase activity was routinely assayed by measuring the production of ammonia from the nitriles used as substrates (1). The standard reaction mixture contained 120 mM acetonitrile in phosphate buffer (pH 7.0) and enzyme solution in a total volume of 1.0 ml contained in a 15-ml test tube (Pyrex). The reaction was started by the addition of acetonitrile and carried out at 37°C for 30 min. After incubation, the reaction was terminated by boiling at 65°C for 1 min and the amount of ammonia released was determined. (ii) Amidase activity was determined by the production of ammonia in the reaction mixture containing 85 mM of acetamide in phosphate buffer (pH 7.0) and enzyme solution in a total volume of 1.0 ml (2). The reaction was started by the addition of acetamide and incubated at 37°C for 30 min. After incubation, the reaction was 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 hydratase 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 a standard (13). Acetate formed was determined enzymatically by the method described by Rose (18), with acetohydroxamate as a standard. Ammonia was determined colorimetrically by the Berthelot procedure as described by Kaplan (11).

All experiments were repeated three times with four replicates. The averages of the results obtained are reported in this communication.

RESULTS

Isolation of bacteria. Acetonitrile-utilizing bacteria were isolated from 60 different soil and water samples collected from several industrial ecosystems. Contaminated soil and water samples were transferred and incubated either aerobically (with head space) or anaerobically (without head space and flushed with nitrogen) at 30°C. Bacterial growth (development of turbidity) was observed after 2 to 7 days of incubation. Although none of the cultures incubated under anaerobic conditions produced turbidity of the medium, three of the soil samples incubated under aerobic conditions developed turbidity after 5 to 7 days of incubation. However, one sample was found to produce turbidity within 36 to 48 h of incubation. A pure culture of this organism was obtained by repeated subculturing on minimal medium plates and transferring isolated colonies to liquid broth. Colonies obtained were small (1 to 3 mm in diameter), circular, convex with entire margin, beige, and creamy in texture. Each isolated colony was able to grow aerobically in minimal medium containing acetonitrile as a sole source of carbon and nitrogen.

Characterization and identification of isolate. 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. Growth was observed on sodium benzoate, MacConkey, and glucose plates but not on xylose and maltose. The isolate failed to hydrolyze gelatin. On the basis of these characteristics, the organism was identified as *P. putida*. All experiments described in this investigation were performed with this isolate.

Maximal growth was attained after 72 h of incubation at 25°C. No growth occurred at 10 or at 55°C. The isolate was found to grow well between pH 5.0 and 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 the growth of *P. putida*. Figure 1 shows the effect of different concentrations of acetonitrile on the bacterial growth. Growth was proportional to the concentration of acetonitrile up to 120 mM acetonitrile in the medium. However, concentrations above 120 mM induced a lag phase in bacterial growth. This effect was more pronounced at acetonitrile concentrations above 480 mM. Concentrations higher than 650 mM totally suppressed the growth.

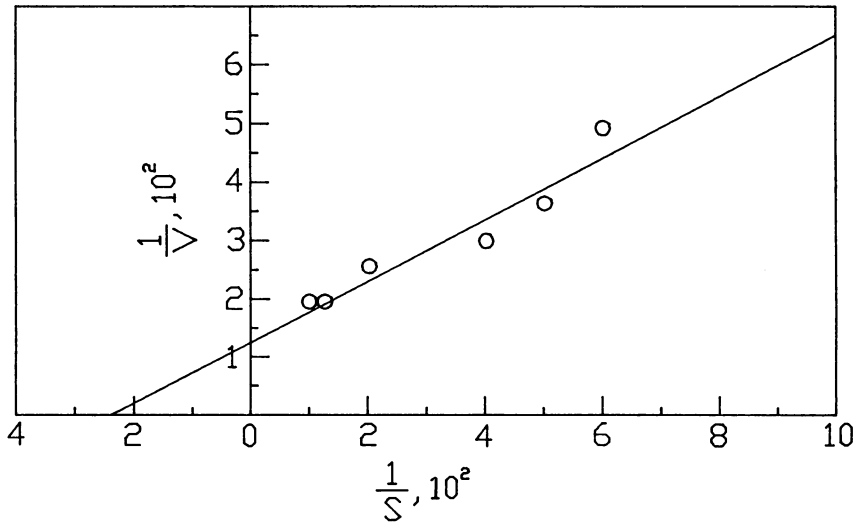

FIG. 2. Kinetics of acetonitrile oxidation. The reaction mixture contained 1.0 ml of the bacterial suspension (equivalent to an A_{660} of 0.25) and 1.0 ml of acetonitrile (S) (5 to 100 mM). Oxidation rates (V) were expressed as nanomoles of oxygen consumed per minute per milliliter. All data were corrected for endogenous oxygen uptake.

Growth of *P. putida* on acetonitrile and related compounds. Table 1 shows the growth of *P. putida* in media containing different nitrile compounds and some of their corresponding amides. The bacterium utilized acetonitrile, butyronitrile, glutaronitrile, isobutyronitrile, methacrylonitrile, propionitrile, succinonitrile, and valeronitrile as sole sources of carbon and nitrogen. Among the nitriles, acetonitrile, followed by butyronitrile, propionitrile, and glutaronitrile, supported more growth (in terms of absorbance [turbidity]) than the other nitriles. Similarly, acetamide was far superior as a growth substrate when compared with other amides tested. Additionally, it induced a higher accumulation of ammonia and increased the pH as a result of ammonia accumulation more than any other substrate tested. Acrylonitrile, benzonitrile, crotononitrile, phenylacetonitrile, acrylamide, 2-cyanobutene, and 2,4-dicyanobutene did not support growth, even after 10 days of incubation.

Kinetics of acetonitrile oxidation. The present study illustrates that the bacterium has the capability to degrade acetonitrile. Results of the respirometric studies indicate that acetonitrile is a biologically oxidizable substrate. Furthermore, oxidation of acetonitrile proceeded without a lag. Suspensions of the bacterial isolate readily oxidized acetonitrile over the range 5 to 100 mM (Fig. 2). The K_m value was found to be 40.61 mM, and the V_{max} was 83.73 nmol of oxygen consumed per min per ml of the cell suspension. Cells subcultured on acetate or glucose as a carbon source failed to oxidize acetonitrile immediately (data not shown).

Degradation of [^{14}C]acetonitrile. Studies with [^{14}C]acetonitrile indicate that degradation (evolution of $^{14}CO_2$) increased rapidly after 24 h and peaked at 72 h of incubation (66%). Prolonging the incubation period failed to elicit appreciable changes in the $^{14}CO_2$ yield (Fig. 3). Less than 10% of the radioactive material initially added was present in the filtrate of the cultures, and a significant amount (14%) of radioactive material was taken up by the bacterial cells. About 10% of the initially added [^{14}C]acetonitrile volatilized, as indicated by the increased radioactivity in NaOH along with reduced radioactivity in the filtrate of uninoculated medium (data not shown).

Identification of the products from acetonitrile degradation. The pH of the culture medium increased with the increase in the cell population. An initial pH of 7.0 increased rapidly to a final pH of 8.9 within 72 h of incubation and then remained constant. Prolonging the incubation period failed to elicit any change in the pH (Fig. 4). At this stage, an intense browning of the medium was observed and the culture broth tested positive for ammonia, which suggested the probable release of acetic acid as well. Interestingly, the start of the logarithmic growth phase of *P. putida* was concomitant with the accumulation of ammonia and acetic acid in the growth medium. Although the accumulation of ammonia peaked after 72 h of incubation, the acetic acid that was initially produced disappeared after 36 h of incubation. Uninoculated culture medium tested negative for both ammonia and acetic acid production. The results confirm that the hydrolysis of acetonitrile resulted in the formation of ammonia and acetic acid.

Characterization of cell extracts. The crude cell extracts contained both the hydrolytic enzymes (nitrile hydratase and amidase) which are known to participate in the hydrolysis of acetonitrile in a sequential manner. Enzyme activities increased with increasing incubation periods, with the maximum activity occurring after 72 h of incubation, irrespective of the enzyme tested (Fig. 5). Prolonging the incubation periods resulted in lower enzyme activities. Nitrile hydratase, the primary enzyme involved in the degradation, had an optimal pH of 7.0. Enzyme activities were greatly reduced at lower and higher pH (data not shown). In contrast, the crude enzyme preparation of amidase had a broad pH profile, with the maximum activity centering around pH 7.0. Both enzyme activities increased with increasing incubation temperatures; the maximum activity occurred at 37°C (data not shown). Both enzymes were inactivated within 5 min after incubation at 60°C.

The substrate specificities of nitrile hydratase and amidase were investigated with a cell extract of the bacterium. As shown in Table 2, acetonitrile, butyronitrile, propionitrile, glutaronitrile, and methacrylonitrile were hydrolyzed as more suitable substrates than isobutyronitrile and succinonitrile. However, the enzyme failed to hydrolyze acrylonitrile, benzonitrile, and phenylacetonitrile even at lower concentrations. Similarly, the amidase activity also exhibited a broad range of substrate specificities (Table 3). This

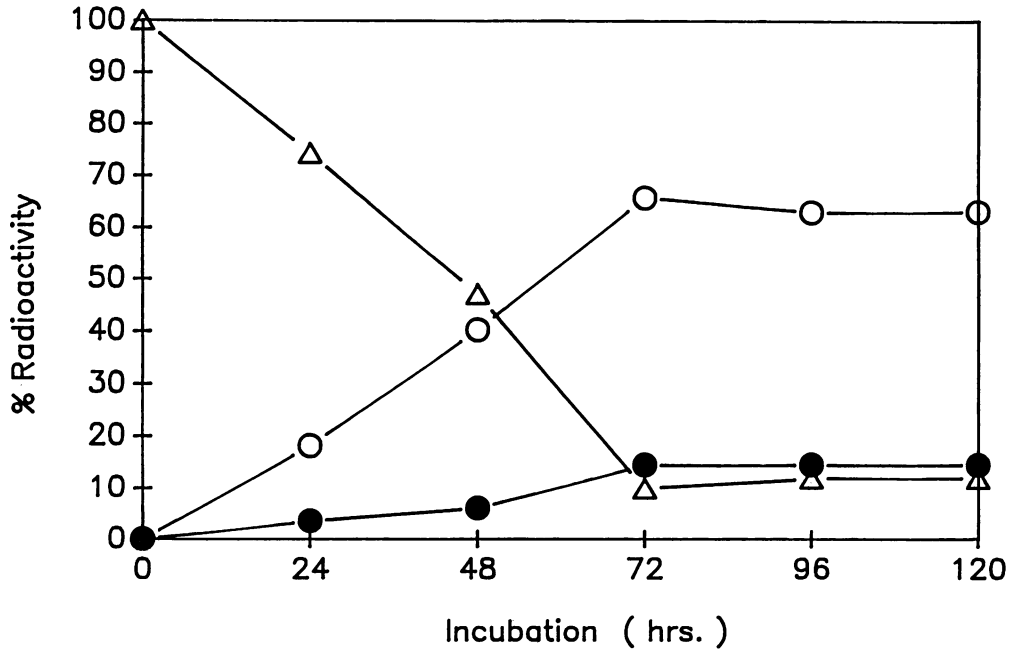

FIG. 3. Degradation of [^{14}C]acetonitrile by *P. putida*. Cultivation was carried out at 30°C in 125-ml Warburg flasks containing 25 ml of the minimal medium with 120 mM acetonitrile and supplemented with 30,000 cpm of [^{14}C]acetonitrile per ml. Symbols: ●, cell-bound ^{14}C; ○, $^{14}CO_2$; △, unutilized [^{14}C]acetonitrile remaining in the culture filtrate.

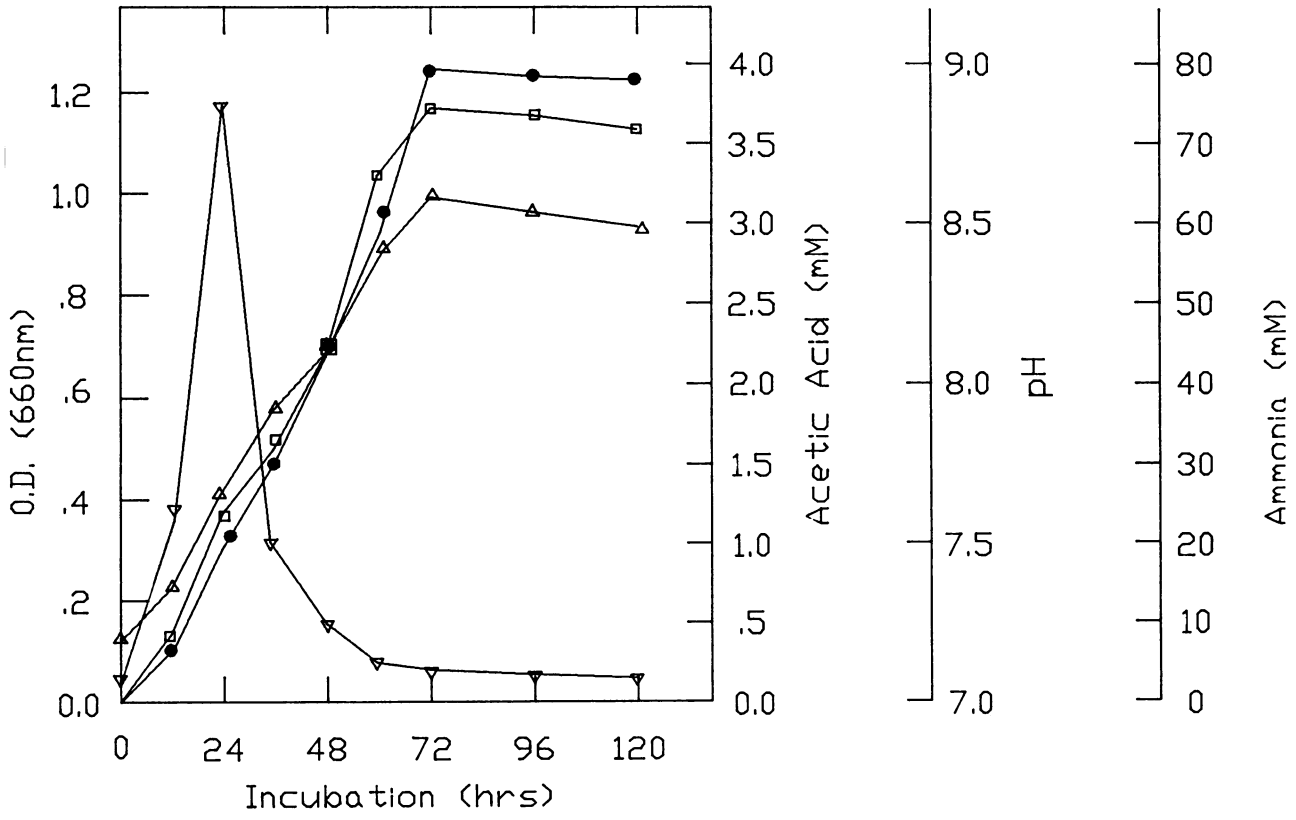

FIG. 4. Time course of cultivation of *P. putida* and product formation. Cultivation was carried out at 30°C in 1-liter Pyrex flasks containing 200 ml of the medium supplemented with 120 mM acetonitrile on a reciprocal shaker. Growth at A_{660} (△), ammonia concentration (□), acetic acid concentration (▽), and pH (●) were determined at different incubation periods. O.D., Optical density.

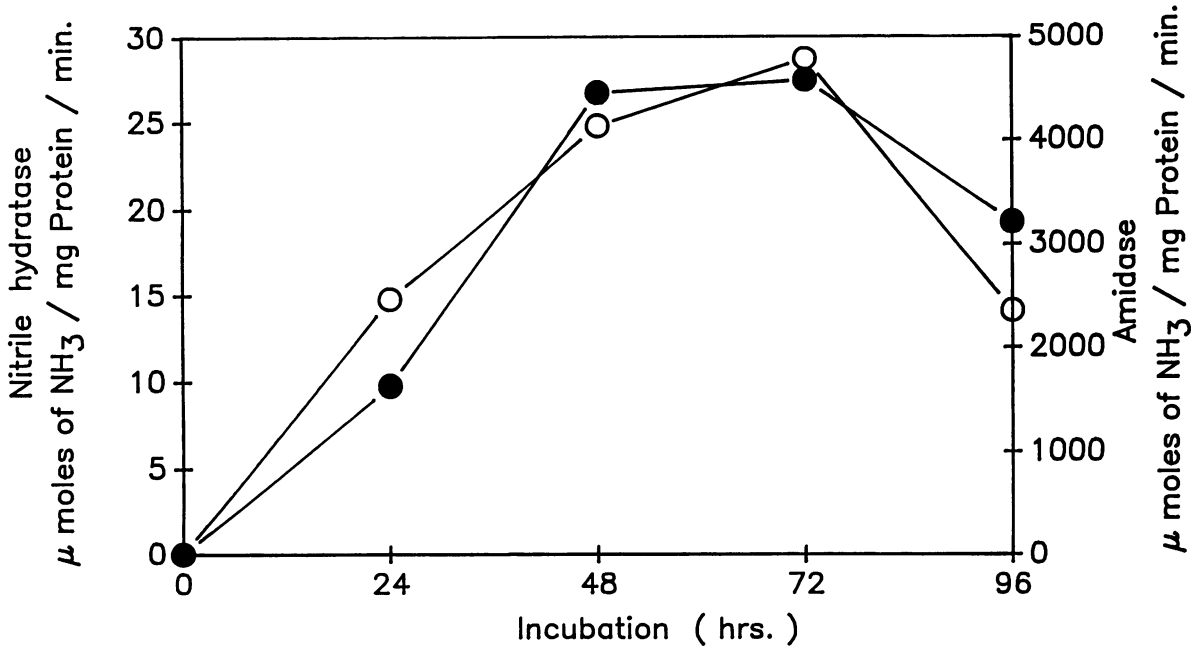

FIG. 5. Formation of nitrile hydratase and amidase during cultivation of *P. putida*. Cultivation was carried out at 30°C in 4-liter Fernbach flasks with 1 liter of the minimal medium containing 120 mM acetonitrile. The activities of the enzymes were assayed by measuring the formation of ammonia. Symbols: ○, Nitrile hydratase; ●, amidase.

enzyme exhibited relatively high affinity for acetamide, followed by propionamide, butyramide, methacrylamide, isobutyramide, and succinamide. Acrylamide and 2,4-dicyanobutene totally suppressed the enzyme activity, even at lower concentrations.

The inducible or constitutive nature of the enzyme was determined by repeatedly subculturing the bacteria on acetonitrile-free plates containing glucose as the sole source of carbon and supplemented with ammonia sulfate (2 g/liter) as a nitrogen source. The bacterium was subcultured after every 5 days of incubation. After seven such subcultures on glucose, the isolate was transferred to 50 ml of minimal medium containing 120 mM acetonitrile. The control consisted of the cells initially grown on acetonitrile as a single source of carbon and nitrogen. Cells transferred after seven subcultures on glucose failed to utilize acetonitrile (as indicated by the production of ammonia and the subsequent increase in the pH of the medium), unlike cultures which were always exposed to acetonitrile. However, cells subcultured on plates supplemented with amides as carbon and nitrogen sources induced the corresponding nitrile hydratase. Additionally, it was determined that the enzyme responsible for hydrating acetonitrile was found predominantly in the cells and not in the culture filtrate, when the cell-free culture broth was tested for the enzymes.

TABLE 2. Substrate specificity of the enzyme nitrile hydratase[a]

Substrate	Sp act (μmol of NH_3/min per mg of protein)
Acetonitrile	28.17
Butyronitrile	25.0
Glutaronitrile	22.61
Isobutyronitrile	18.25
Propionitrile	23.29
Methacrylonitrile	22.0
Succinonitrile	15.23
Acrylonitrile	
Benzonitrile	
Phenylacetonitrile	

[a] The reaction mixture contained 0.2% (vol/vol) of each nitrile in 1.0 ml of phosphate buffer and enzyme solution. The reaction was carried out as described in Materials and Methods.

DISCUSSION

The aerobic metabolism of nitrile compounds by *N. rhodochrous* (7), *Arthrobacter* species (1, 2, 5, 28), and *Brevibacterium* species (6, 22) have been well documented. Recently, Asano et al. reported a strain of *Pseudomonas chlororaphis* that utilized acrylonitrile as the sole source of carbon and nitrogen (3). The same investigators reported another organism, tentatively identified as belonging to the genus *Pseudomonas*, that could utilize only glutaronitrile as a carbon and nitrogen source (29). Further, the bacterium failed to utilize acetonitrile, butyronitrile, propionitrile, and

TABLE 3. Substrate specificity of the enzyme amidase[a]

Substrate	Sp act (μmol of NH_3/min per mg of protein)
Acetamide	4,583.2
Butyramide	2,291.7
Isobutyramide	1,562.5
Methacrylamide	1,877.9
Propionamide	2,698.9
Succinamide	145.8
Acrylamide	
2,4-Dicyanobutene	

[a] The reaction mixture contained 0.2% (wt/vol) of each amide in 1.0 ml of phosphate buffer and enzyme solution. The reaction was carried out as described in Materials and Methods.

succinonitrile even at a low concentration (0.2%, wt/vol). Our results demonstrate that the isolated bacteria, *P. putida*, is equipped with the enzymatic mechanism for metabolizing acetonitrile (Fig. 1) and various other nitriles that serve as a source of carbon and nitrogen (Table 1). In addition, these bacteria were found to grow profusely on amides (as indicated by the higher growth rate and pH as a result of increased accumulation of ammonia). These findings appear to differ from those of an earlier study (7) that indicated marginal growth on the amides by *N. rhodochrous* when compared with the growth on the corresponding nitriles.

Microorganisms require energy to perform their essential functions. To sustain a basal metabolism, the organism produces enzymes that metabolize the substrate, resulting in the degradation of the compound. The rates of oxygen uptake by whole cells have been used as an indirect and approximate measure of enzyme specificity in *P. putida* (27). Cells grown in the presence of acetonitrile or their corresponding amides showed significant oxygen uptake (Fig. 2). However, cells that were repeatedly subcultured on glucose or acetate as a sole source of carbon or nitrogen showed negligible levels of oxygen uptake when incubated in the presence of any of the nitrile compounds or their corresponding amides (data not shown). It is possible that the catabolic enzymes responsible for the breakdown of nitriles or their amides are inducible because no significant oxygen uptake could be noticed when whole cells grown on glucose were incubated in the presence of any of the nitrile compounds.

The hydrolysis of nitrile compounds to their respective carboxylic acids and ammonia has been reported in plants (23), bacteria (5, 8, 28), and fungi (9). Similarly, reports have also indicated the sequential formation of the corresponding amide, carboxylic acid, and ammonia due to microbial transformation of nitrile compounds (1, 7, 14, 22). In our present investigation, *P. putida* transformed acetonitrile to acetic acid and ammonia (Fig. 4). Accumulation of acetic acid peaked at 24 h of incubation and later disappeared after 36 h of incubation. It is possible that the acetate formed may have been utilized as an additional source of carbon for the synthesis of cell material.

Controversy still exists about the nature and type of enzymes that are involved in the transformation of nitrile compounds. Nitrilases have been found to hydrolyze nitriles to the corresponding carboxylic acids and ammonia without forming amide as an intermediate and do not use amide as a substrate (9, 17). However, a significant amount of amide was detected in the reaction mixture of a partially purified ricinine nitrilase (17) and its accumulation was attributed to a side reaction. DiGeronimo and Antoine reported the production of acetamide as a result of the hydrolysis of acetonitrile by *N. rhodochrous* (7). These investigators concluded that acetamide was the true intermediate of acetonitrile degradation because of its total conversion to acetic acid and ammonia in reactions starting with either acetonitrile or acetamide. However, these investigators failed to detect propionamide in the culture medium supplemented with propionitrile. The failure to detect propionamide was attributed to the direct conversion of propionitrile to its carboxylic acid and ammonia involving a nitrilase or to the slow turnover of propionitrile. Asano et al. purified a nitrilase hydratase (1) and an amidase (2) from *Arthrobacter* sp. strain J-1 that participated in the degradation of acetonitrile. These investigators reported that the hydrolysis of acetonitrile was a two-step enzymatic mechanism involving a nitrile hydratase that initially transformed the nitrile compound to the amide. The amide formed was later degraded by amidase to the carboxylic acid and ammonia. Recently, Bandyopadhyay et al. (5) reported the purification of benzonitrilase from the same bacterial isolate that directly transformed benzonitrile to the corresponding carboxylic acid and ammonia, without the formation of the respective amide as an intermediate. Bui et al. (6) and Tourneix et al. (24) have reported the hydrolysis of acrylonitrile by nitrile hydratase and amidase purified from *Brevibacterium* sp. strain R312. We suggest that these enzymes are synthesized inducibly in *P. putida*. Both enzymes have a wide range of substrates for hydration. Acetonitrile was a highly preferred substrate for hydration by nitrile hydratase (Table 2), as was acetamide by amidase (Table 3). However, it was observed that amidase activity was at least 150-fold higher than nitrile hydratase activity. Although acetamide was not detected in our study, it is possible that it was produced, as indicated by the presence of the enzyme amidase.

Microbial metabolism of nitrile compounds has become a subject of considerable interest from the viewpoint of amide production. Microbial catalysts are highly specific and efficient in the transformation of nitrile compounds to the corresponding amides. Recently, numerous reports have indicated the enzymatic hydrolysis of various aliphatic nitriles to their corresponding amides (15, 25, 26). All these microorganisms synthesized nitrile hydratase that exhibited higher specific activity than the one produced by *P. putida*. This aspect makes the strain less attractive for the industrial production of amide. However, the amidase activity of *P. putida* was far superior to that of any of the microorganisms known to participate in the degradation of nitrile compounds to their corresponding organic acids. This feature makes the strain highly attractive for the production of organic acids.

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