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A Kinetic Study on the Bioremediation of Sodium Cyanide and Acetonitrile by Free and Immobilized Cells of *Pseudomonas putida*

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ABSTRACT

Pseudomonas putida capable of utilizing organic nitrile (acetonitrile) and inorganic cyanide (sodium cyanide) as the sole source of carbon and nitrogen was isolated from contaminated industrial sites and waste water. The bacterium possesses nitrile aminohydrolase (EC 3.5.5.1) and amidase (EC 3.5.1.4), which are involved in the transformation of cyanides and nitriles into ammonia and CO_2 through the formation of amide as an intermediate. Both of the enzymes have a high selectivity and affinity toward the ^-CN group. The rate of degradation of acetonitrile and sodium cyanide to ammonia and CO_2 by the calcium-alginate immobilized cells of *P. putida* was studied. The rate of reaction during the biodegradation of acetonitrile and sodium cyanide, and the substrate- and product-dependent kinetics of these toxic compounds were studied using free and immobilized cells of *P. putida* and modeled using a simple Michaelis-Menten equation.

Index Entries: *Pseudomonas putida;* nitrile; cyanide; immobilized cells; degradation; nitrile aminohydrolase; amidase.

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INTRODUCTION

Cyanide, a well-known metabolic inhibitor, is used extensively in industries involved in the production of chemical intermediates, synthetic fibers, rubbers and pharmaceuticals, and in ore leaching, coal processing, and metal plating (*1*). Nitriles, on the other hand, are found both naturally and synthetically (*2–8*). Synthetic nitrile compounds are highly toxic, mutagenic, and carcinogenic (*9–11*), and are used extensively as primary organic solvents and also in the manufacture of herbicides, polymers, and plastics (*7,8*).

In industry, the cyanide and nitrile compounds are generally detoxified prior to discharge. The standard methods used for the detoxification of cyanides and nitriles in industral waste water are chemical oxidation by alkaline chlorination, by using potent oxidants, like hydrogen peroxide or ozone, or by activated sludge systems (*12–14*). Such conventional techniques are universally applicable because of their insensitivity toward the nature of the waste materials and also by their relatively low costs. However, their main drawbacks are the need of hazardous reagents and their potentials for creating toxic residues requiring a posttreatment.

Biological treatment can be considered an important removal mechanism for consumer product chemicals released in large volumes to aquatic and terrestrial environments. It results in a decrease in the mass or load of chemicals present in the environment, and also is a key in preventing the accumulation and persistence of chemicals (*15*). It is known that certain types of microorganisms can decompose such toxic compounds as cyanides and nitriles to their respective acids and/or to further degradation products like CO_2 and H_2O (*1,16–22*). Studies have addressed the use of free and immobilized cells of bacterial sp.s to degrade toxic compounds into ammonia and carbon dioxide (*16–21*). We recently presented the data on the degradation of acetonitrile and sodium cyanide by free and alginate immobilized cells of *Pseudomonas putida* (*22–26*). Complete mineralization of these chemicals into ammonia and carbon dioxide was accomplished by immobilized cells in a batch culture reactor (*23–26*). Our studies have also reported that this biocatalyst decomposes cyanides and nitriles to ammonia and respective acid in a two-step reaction (Eqs. [1] and [2]). The acid is further degraded into CO_2 and H_2O through Krebs cycle (*16,17,19,21,22*).

$$\text{R-CN} + \text{H}_2\text{O} \xrightarrow[\text{(EC. 3.5.5.1)}]{\text{Nitrile Aminohydrolase}} \text{R-C(=O)-NH}_2 \qquad (1)$$

$$\text{R-C(=O)-NH}_2 + \text{H}_2\text{O} \xrightarrow[\text{(EC. 3.5.14)}]{\text{Amidase}} \text{R-COOH} + \text{NH}_3 \qquad (2)$$

In the present investigation, the degradation kinetics of nitrile (acetonitrile) and cyanide (sodium cyanide) and their degradative products (CO_2 and NH_3) for the free and immobilized cells of *P. putida* were examined.

MATERIALS AND METHODS

Chemicals

Acetonitrile was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. All other chemicals, including sodium cyanide of 99% purity, were purchased from Sigma Chemical Co., St. Louis, MO.

Isolation and Identification of the Bacterium

The bacterium used in these experiments was isolated from the contaminated industrial soil sites and waste water by using enrichment culture techniques (22). The soil sample was diluted (1:1) with sterile minimal mineral salt medium, and the suspension was incubated at room temperature for 1 h. One milliliter of the suspension was inoculated into 9 mL of medium supplemented with different concentrations of acetonitrile (20–640 mM/L) as the sole source of C and N. Successive streaking of the colonies onto acetonitrile plates allowed isolation of a pure culture that could utilize acetonitrile as its sole source of C and N. The bacterium was gram-negative, small rod-shaped, motile, nonspore-former, and noncapsulated. 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. Based on these characteristics, the organism was identified and confirmed as *P. putida*. The optimal temperature and pH for the growth *P. putida* were 25°C after 72 h of incubation at pH 7.0 (22).

Cell Growth

The bacterium was grown in minimal mineral salt medium (pH 7.0) containing 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 a trace element solution containing (mg/L): $MnCl_2 \cdot 4H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$; $CaCl_2 \cdot H_2O$, 2.6; $NaMoO_4 \cdot 2H_2O$, 6.0, and acetonitrile or sodium cyanide as carbon and nitrogen sources. Bacterial cells (48-h-old) were harvested by centrifugation at 10,000*g* for 10 min, washed twice in 0.85% normal saline, sedimented again by centrifugation, suspended, stirred in normal saline to give a final A_{660} (turbidity) of 1.0, and used for subsequent studies.

Immobilization of *P. putida* in Alginate

A 1:1 mixture of cell paste (200–400 mg) suspended in 0.85% normal saline and sterile sodium alginate was added to bring the mixture to the final concentration of 4% sodium alginate. The alginate-cell mixture was added dropwise to cold 0.2*M* $CaCl_2$ solution, and each drop hardened into a bead containing entrapped *P. putida* cells. The beads were allowed to harden further in $CaCl_2$ solution at 5°C for 24 h. The typical yield following immobilization of cells with alginate was about 0.5–0.6 g of beads/mL of cell-alginate suspension. Individual beads had a diameter of 1–2 mm and a wet and dry wt of 12–15 and 0.5–0.8 mg, respectively. At the time of immobilization, each bead contained approx 1–2 × 10^8 viable cells, as determined by pour-plate method of disrupted beads (*20*).

Kinetic Study by Free and Immobilized Cells of *P. putida*

Measurement of Substrate Oxidation by Free and Immobilized Cells of P. putida

The oxidation of acetonitrile or sodium cyanide by the cells of *P. putida* was studied by measuring the oxygen uptake with an Oxygraph (Gilson Medical Electronics, Inc., Middleton, WI). One milliliter of the cell suspension (A_{660} 1.0) was injected into the cell chamber followed by the substrate (1 mL). The suspension was then stirred and equilibrated at 30°C for 20 min before readings were recorded. The oxygen uptake with different concentrations of the substrate was recorded for 15 min. The oxidation of substrates by immobilized cells of *P. putida* was also studied using the Oxygraph. All data were corrected for endogenous respiration.

Measurement of Substrate Transformation by Immobilized Cells of P. putida

Kinetic experiments were performed in an 800-mL air-uplift-type fluidized bed reactor. Five hundred milliliters of immobilized cells of *P. putida* and 250 mL of normal saline supplemented with substrate (acetonitrile or sodium cyanide) were taken in the bioreactor. The immobilized beads were aerated with 200 mL/min of CO_2 and NH_3 free air at 25°C. Transformation rates of the substrate degradation products, such as CO_2 and NH_3, were investigated. Degradation rates were calculated by using the linear regression of time vs concentration. The kinetic constants were determined by double-reciprocal plots drawn manually according to the method of Lineweaver and Burk (*27*), using the Henri-Michaelis-Menten equation (*28,29*).

Analytical Methods

Dissolved NH_3 was determined calorimetrically by the Berthelot's procedure as described by Kaplan (*30*). The amount of gaseous NH_3 in 20

mL of boric acid was determined by dissolving 10 mL boric acid in 10 mL of 0.5*M* KOH, and back-titrated for free KOH against 0.5*M* HCl. The gaseous CO_2 in 20 mL of KOH was determined by titrating 10 mL KOH against 0.5*M* HCl (*31*).

All experiments were repeated three times, and the mean of the results obtained are reported in this article. The values were found to be significant at $P < 0.05$.

RESULTS

Kinetics of Substrate (Acetonitrile and Sodium Cyanide) Oxidation

The respirometric data on the degradation of acetonitrile and of sodium cyanide by the free and immobilized cells of *P. putida* are presented in Figs. 1 and 2. Figures 1A and 2A illustrate that the bacterium is able to degrade both acetonitrile and sodium cyanide, and these substrates are biologically oxidizable. The cell suspensions of *P. putida* readily oxidized different concentrations of acetonitrile (5–100 m*M*) and sodium cyanide (5–50 m*M*) (Figs. 1A and 2A). The K_m value of acetonitrile was found to be 41 m*M* and the V_{max} value was 84 nmol of oxygen consumed/mg protein/min, respectively, where as the K_m and V_{max} values of sodium cyanide were 23 m*M* and 56 nmol of oxygen consumed/mg protein/min, respectively. Figures 1A and 2A also show the oxidation of acetonitrile or sodium cyanide by immobilized cells of *P. putida*. The immobilized cells oxidized acetonitrile and sodium cyanide over the range of 10 to 100 m*M* (Figs. 1A and 2A). The K_m and V_{max} values of acetonitrile/sodium cyanide were 33/17 m*M* and 71/37 nmol of oxygen consumed/min, respectively. Cells subcultured on glucose as a carbon source failed to oxidize acetonitrile or sodium cyanide immediately (data not shown).

Kinetics of Substrate Transformation

Immobilized *P. putida* cells were able to degrade either acetonitrile or sodium cyanide into CO_2 and NH_3. The product-dependent kinetics of sodium cyanide and acetonitrile show that the K_m and V_{max} values of products (NH_3 and CO_2) of sodium cyanide and acetonitile were 7 m*M*/L and 9 m*M*/L/min for carbon and 7 m*M*/L and 14 m*M*/L/min for nitrogen of acetonitrile (Fig. 1B), and 7 m*M*/L and 8 m*M*/L/min for carbon and 7 m*M*/L/min and 10 m*M*/L/min for nitrogen of sodium cyanide (Fig. 2B).

DISCUSSION

The results obtained from the present study indicate that both free and immobilized cells of *P. putida* are able to oxidize acetonitrile and

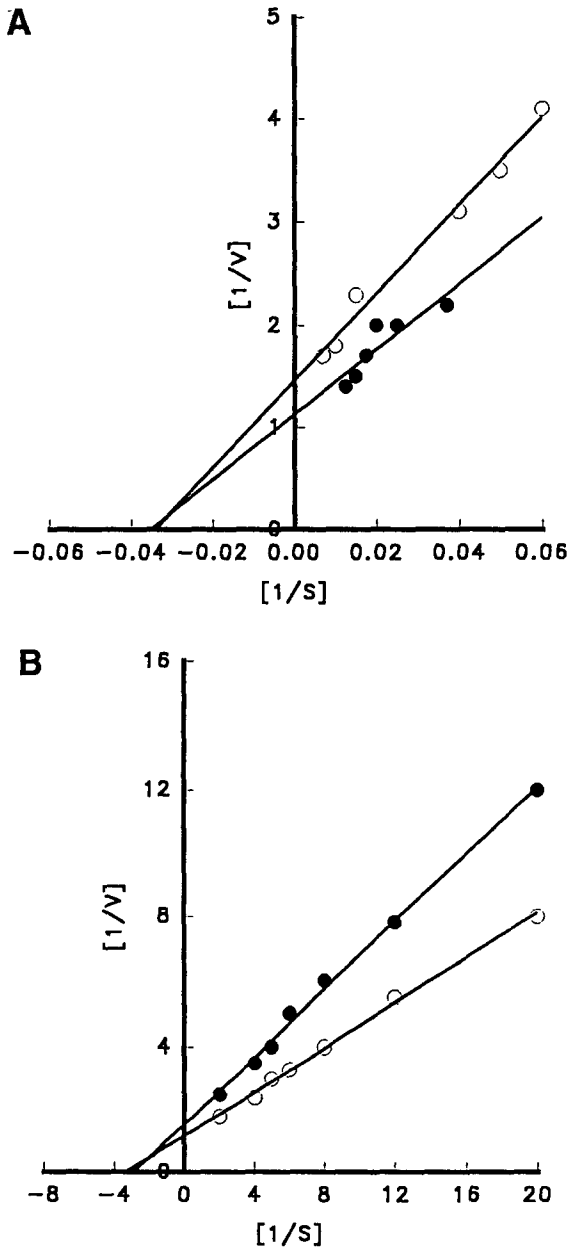

Fig. 1. (**A**) Kinetics of acetonitrile oxidation by free [○—○] and immobilized [●—●] cells of *P. putida*. Oxidation rates—V_{max} and K_m were expressed as nmol of oxygen consumed/min/mg protein and m*M*, respectively. (**B**) Kinetics of NH_3 (as N) [○—○] and CO_2 (as C) [●—●] production during the degradation of acetonitrile by immobilized cells of *P. putida*. V_{max} and K_m were expressed as m*M*/L/min and m*M*, respectively.

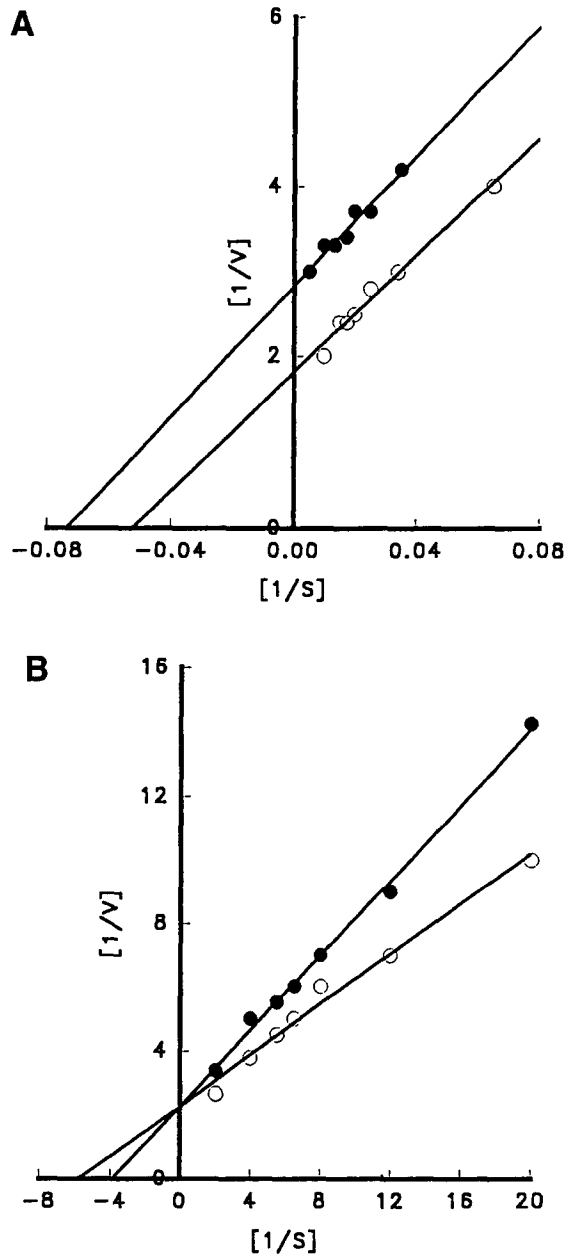

Fig. 2. (**A**) Kinetics of sodium cyanide oxidation by free [○—○] and immobilized [●—●] cells of *P. putida*. Oxidation rates—V_{max} and K_m were expressed as nmol of oxygen consumed/min/mg protein and m*M*, respectively. (**B**) Kinetics of NH_3 (as N) [○—○] and CO_2 (as C) [●—●] production during the degradation of sodium cyanide by immobilized cells of *P. putida*. V_{max} and K_m were expressed as m*M*/L/min and m*M*, respectively.

sodium cyanide. The rates of oxygen uptake are used as an indirect and approximate measure of enzyme specificity in *P. putida* (*32*). Cells grown in the presence of acetonitrile or sodium cyanide showed significant oxygen uptake. However, cells that were repeatedly subcultured on glucose as a sole source of carbon showed negligible levels of oxygen uptake when incubated in the presence of acetonitrile and sodium cyanide. It is possible that the catabolic enzymes responsible for the breakdown of acetonitrile and sodium cyanide are inducible because no significant oxygen uptake could be measured when whole cells grown on glucose were incubated in the presence of nitrile/cyanide compounds.

The products detected during the degradation of acetonitrile by immobilized cells of *P. putida* show that the *P. putida* can degrade this toxic chemical into ammonia and CO_2 through its hydrolysis to acetamide by nitrile aminohydrolase (E .C. 3.5.5.1), and the amide is later degraded by amidase (E.C. 3.5.1.4) to acetic acid and ammonia (*33,34*). On the other hand, degradation of sodium cyanide to CO_2 and NH_3 could be one of the following metabolic pathways: (1) the hydrolysis of cyanide to formamide (*35*) or formate and ammonia (*36–38*), or (2) the direct formation of bicarbonate and ammonia via cyanide oxidase (*39*).

Although reports have been published on the use of immobilized bacterial cells to degrade toxic environmental pollutants (*18–21,24,25*), this is the first instance in which immobilized cell degradation kinetics both of a parent compound (acetonitrile and sodium cyanide) and two of its degradative products (NH_3 and CO_2) have been investigated simultaneously. Understanding the kinetics of transformation of toxic compounds is important in optimizing the bioreactor design. Also, the degradative kinetics of products could be important in the environmental application of immobilized cell technology, since the products formed during the degradation of some xenobiotic molecules can be more toxic than the original contaminant (*40*).

The present study thus concludes that the free and immobilized cells of *P. putida* are able to oxidize different concentrations of acetonitrile or sodium cyanide, and these compounds are found to be oxidizable. The V_{max} and K_m values of acetonitrile and sodium cyanide for free cells are higher than those of immobilized cells of *P. putida.* Also, both free and immobilized cells of *P. putida* degraded acetonitrile or sodium cyanide effectively into CO_2 and NH_3.

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