0273-2289/93/3940—0655$03.40

Degradative Capability of *Pseudomonas putida* on Acetonitrile

KIRIT D. CHAPATWALA,* G. R. V. BABU, CHARLES DUDLEY, RITA WILLIAMS, AND KAZEEM AREMU

Division of Natural Sciences, Selma University, Selma, AL 36701

ABSTRACT

Pseudomonas putida, capable of utilizing acetonitrile as a sole source of carbon and nitrogen, was isolated from contaminated soil and water samples collected from industrial sites. The *P. putida* cells were immobilized in calcium alginate beads. The degradation of acetonitrile by the immobilized cells of *P. putida* was investigated. The immobilized cells degraded different concentrations of acetonitrile into ammonia and carbon dioxide. The effect of aeration on the degradation rate was also studied. Oxygen limitation was suggested in the alginate-immobilized system. The rate of degradation of acetonitrile increased with increase in the rate of aeration.

Index Entries: Acetonitrile; air-uplift-type bioreactor; biodegradation; *Pseudomonas putida*; immobilization; aeration.

INTRODUCTION

Nitrile compounds are cyanide-substituted carboxylic acids (R-CN) that occur naturally and synthetically. These compounds are widely used in a number of industrial operations as chemical solvents, extractants, and recrystallizing agents (*1*). Consequently, there is a concomitant increase in the dissemination of these chemicals into the environment via industrial waste waters. Increased accumulation of such compounds in the ecosystem could cause deleterious effects, since most of them are highly toxic, mutagenic, and carcinogenic (*2,3*).

Microorganisms can degrade and, thereby, detoxify a wide range of these toxic chemcials (*4–7*). A promising method to harness these abilities

*Author to whom all correspondence and reprint requests should be addressed.

to remove the toxic chemicals from the polluted waters lies in the application of immobilized cell technology (*8*). Immobilization of bacteria, plant, or animal cells and enzymes (*9–13*) in different matrices has been shown to provide advantages over the use of free cells or enzymes in various biotechnological applications. For example, the immobilization may increase the cell loading capacity (*14–16*) and/or increase rates of production of microbial products (*17–19*) in bioreactors. One potential disadvantage of the immobilization is the limitation of oxygen to the aerobic cells. Owing to the low solubility of oxygen in water and the high cell density, oxygen transfer often becomes the rate-limiting factor in the performance of aerobic immobilized cell systems (*20*). Thus, when aerobic cells are used, aeration techniques become a very important consideration in bioreactor design (*8*). Hence, in the present investigation, the degradation of acetonitrile by immobilized cells of *Pseudomonas putida* and the effects of aeration rate on its degradation in an air-uplift-type fluidized bed batch reactor were studied.

MATERIALS AND METHODS

Chemicals

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

Media and Culture Conditions

The minimal medium used for the isolation of acetonitrile-utilizing microorganisms contained the following (in 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 the trace element solution containing the following (in mg/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 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, MI) to 1 L of the medium. Unless otherwise stated, the microorganisms were grown in the medium containing acetonitrile as the sole source of carbon and nitrogen.

Isolation of Acetonitrile-Degrading Microorganisms

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 (20–640 m*M*) of acetonitrile. The tubes were then incubated at 25°C. After 7 d of incubation, the tubes were examined for turbidity. After five to seven transfers, the turbid samples were streaked onto 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.

Identification of the Organism

Identification of bacteria was based on the classification scheme described in *Bergey's Manual of Systematic Bacteriology* (*21*). Tests were performed as described in the manual (*21*) or as described by Smibert and Krieg (*22*). Subsequently, the isolates were biochemically characterized by using the API 20E test kit (Analytab Products, Plainview, NY). The results were also confirmed by a computer survey available from the supplier.

Optimal Conditions

The optimal temperature for the growth of the bacteria was determined in 250-mL Pyrex™ flasks containing 50 mL of the minimal medium supplemented with 120 m*M* acetonitrile and bacterial suspension. The samples were incubated at 5, 15, 25, 35, 45, or 55°C. After 72 h of incubation, the growth of bacteria was monitored at A_{546} using a 10-mm cuvet (Gilford Spectrophotometer, Ciba-Corning, USA). The optimal pH was determined by inoculating the medium adjusted to pH 3.0–9.0 with 2 mL bacterial suspension. The inoculated cultures were then incubated at 25°C, and the growth was measured after 72 h of incubation.

Immobilization of *P. putida* in Alginate

Three hundred to 400 mg (wet wt of the cell paste) of cells were suspended in 100 mL of 0.85% normal saline and mixed with 100 mL of sterile sodium alginate (Type VII: Sigma Chemical Co. [St. Louis, MO]) to a 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 (1–2 mm in diameter) were allowed to harden further in $CaCl_2$ solution at 5°C for 24 h in a refrigerator.

Batch Bioreactor Experiment

The batch bioreactor experiment was performed using an 800-mL capacity air-uplift-type reactor (Fig. 1). Seven hundred and fifty milliliters of the reactor volume were filled with 375 mL bead volume and 375 mL of 0.85% normal saline supplemented with 20–160 m*M* of acetonitrile. The immobilized beads were aerated with different amounts (100, 150, and 200 mL/min) of CO_2- and NH_3-free air. The experiment was performed at 25°C, and the samples (5 mL) for the analysis of both pH and dissolved

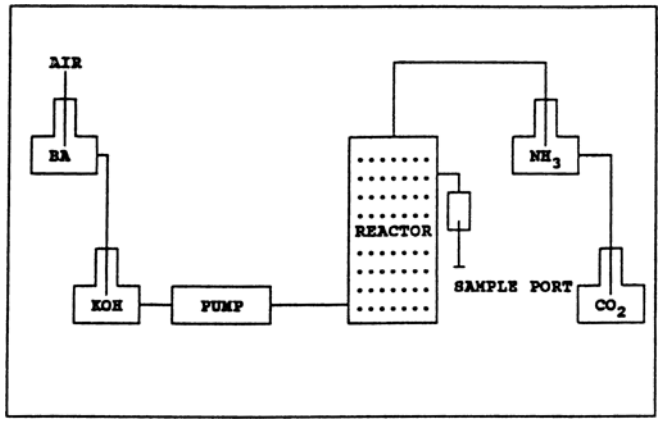

Fig. 1. Schematic diagram of the air-uplift-type fluidized-bed batch bioreactor.

NH_3 were collected from the sample port at regular intervals starting from 0 to 168 h. Both gaseous NH_3 and CO_2 were trapped in 20 mL each of 0.5*M* boric acid and 0.5*M* potassium hydroxide, respectively. At each sampling time, 10 mL of each sample were taken twice for the determination of CO_2 and NH_3. The batch bioreactor experiments were repeated three times using the same bioreactor and beads, and the mean values are reported in the present investigation.

Analytical Methods

The pH of the sample was measured with a pH meter (Orion Research Inc., Cambridge, MA). Dissolved NH_3 was determined colorimetrically by the Berthelot's procedure as described by Kaplan (*23*). The gaseous NH_3 in 20 mL of boric acid was determined by dissolving 10 mL boric acid in known concentration (0.5*M*) and volume (10 mL) of KOH, and back-titrated for free KOH using known concentration of HCl (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.

RESULTS AND DISCUSSION

Isolation of Acetonitrile-Degrading Microorganisms

Acetonitrile-utilizing bacteria were isolated from contaminated soil and water samples collected from industrial ecosystems. Contaminated soil and water samples were transferred and incubated aerobically (with head space) at 25°C. Bacterial growth (development of turbidity) was observed in the soil samples after 5–7 d. However, one sample was found

to produce turbidity within 36–48 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. The colonies obtained on minimal medium plates were small (1–3 mm in diameter), circular, convex with entire margin, beige and creamy in color, and fluorescent. 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

The cellular morphology of the bacteria was gram-negative, small rod-shaped, motile, nonspore former, and noncapsulated. 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 and confirmed as *Pseudomonas putida*.

Optimal Conditions

Maximum growth was obtained after 72 h of incubation at 25°C. No growth was seen at 10 or 55°C. The isolate was found to grow well between pH 5.0 and 8.0. However, maximum cell growth was obtained at pH 7.0. No growth was observed below pH 5.0 or above pH 8.5.

Immobilization

The typical yield following immobilization of cells with alginate was about 0.6 g of beads/mL of cell-alginate suspension. Individual beads had a diameter of 1–2 mm and an average wet and dry wt of 15 and 0.8 mg, respectively. At the time of immobilization, each bead contained approx 1×10^8 to 2×10^8 viable cells, as determined by pour plate counts of disrupted beads.

Batch Reactor Experiment

The data on the degradation of acetonitrile by the immobilized cells of *P. putida* and the effect of aeration on its degradation are presented in Figs. 2–5. The immobilized *P. putida* cells were able to degrade different concentrations of acetonitrile into CO_2 and NH_3. In the presence of acetonitrile, the initial pH of the medium (pH 6.7) increased rapidly with an increase in incubation period and then remained constant after 96–120 h. (Fig. 2). Prolonging the incubation period failed to elicit any change in the pH of the medium (Fig. 2). The highest pH values were 8.26, 8.60, 9.19, and 9.38 at 20, 40, 80, and 160 m*M* acetonitrile at 100 mL/min of aeration, respectively (Fig. 2). The data in Fig. 3 indicate that the CO_2 levels were increased with increase in acetonitrile concentration. A similar increase in

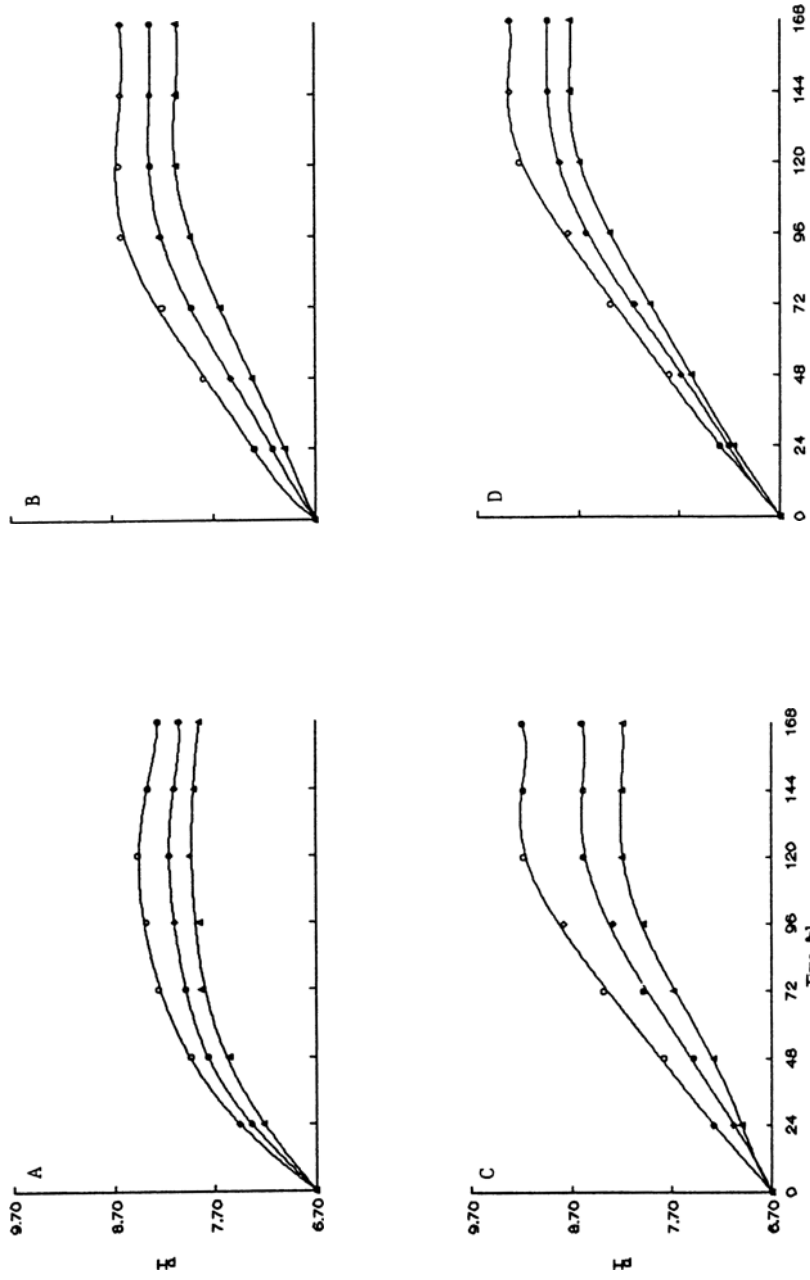

Fig. 2. Changes in pH at different concentrations of acetonitrile (A:20 m*M*, B:40 m*M*, C:80 m*M*, D:160 m*M*) and different rates of aeration (mL/min) 100 ○—○ 150 ●—● 200 △—△.

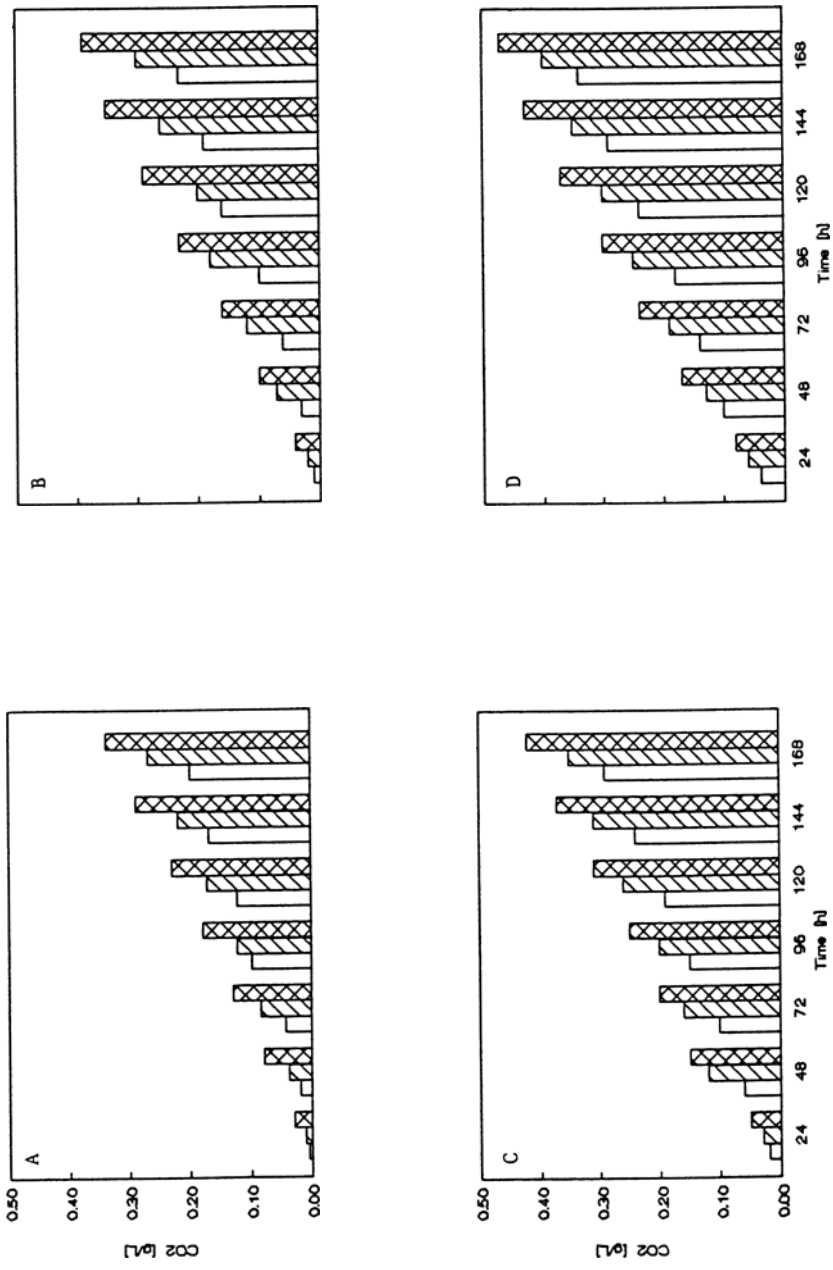

Fig. 3. Effect of acetonitrile concentrations (A:20 m*M*, B:40 m*M*, C:80 m*M*, D:160 m*M*) and aeration (mL/min) 100 □ 150 ▨ 200 ▩ on the level of CO_2.

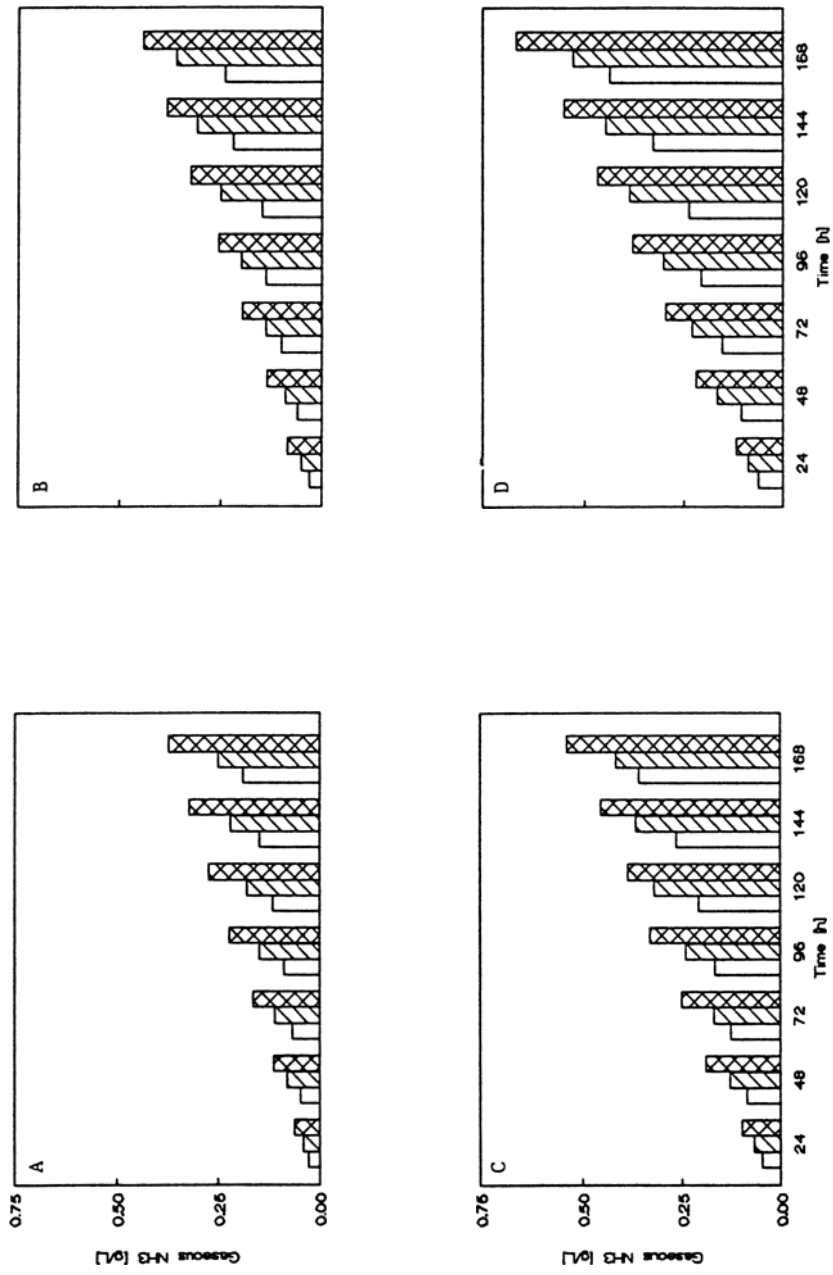

Fig. 4. Effect of acetonitrile concentrations (A:20 m*M*, B:40 m*M*, C:80 m*M*, D:160 m*M*) and aeration (mL/min) 100 □ 150 ☒ 200 ☒ on the level of gaseous NH_3.

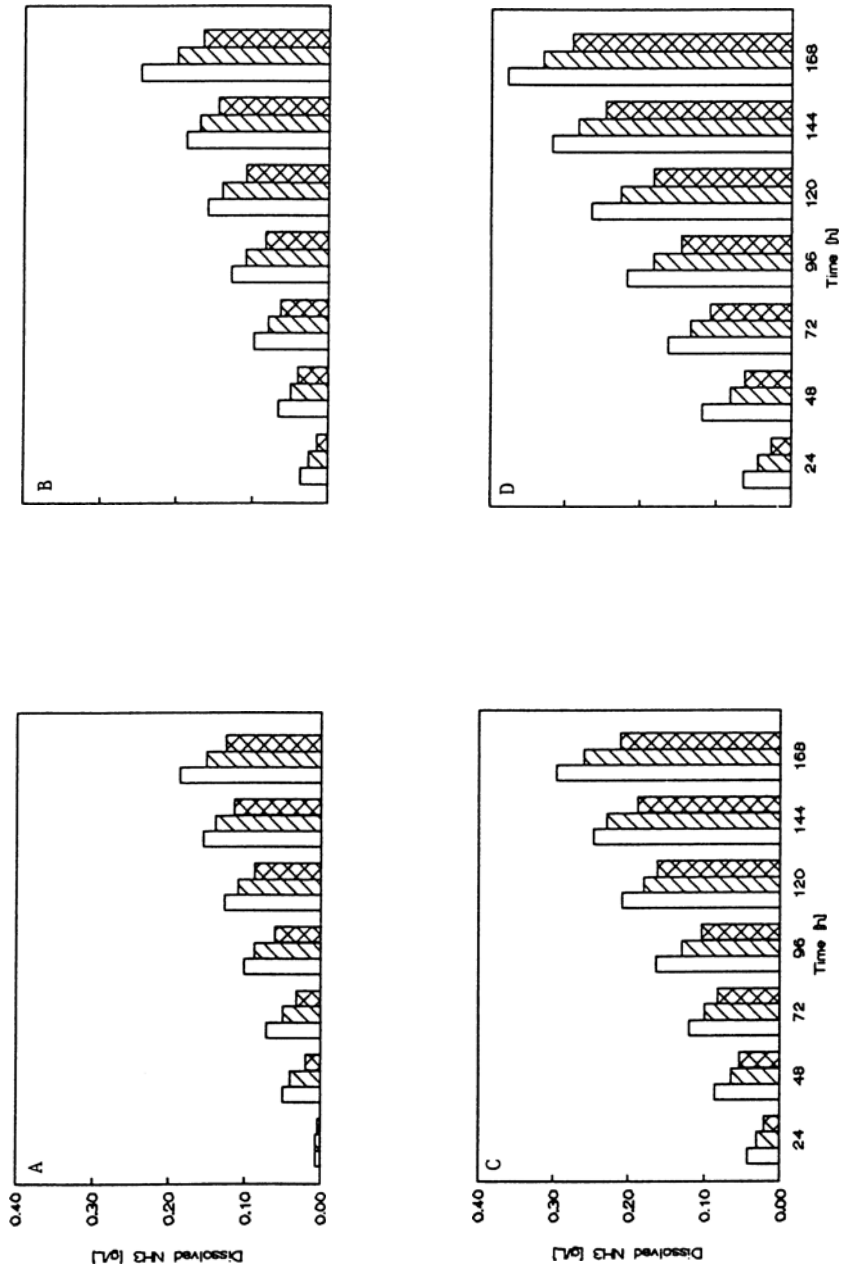

Fig. 5. Effect of acetonitrile concentrations (A:20 m*M*, B:40 m*M*, C:80 m*M*, D:160 m*M*) and aeration (mL/min) 100 □ 150 ☒ 200 ⊠ on the level of dissolved NH_3.

the levels of gaseous and dissolved NH_3 was also observed (Figs. 4 and 5). The increase in the levels of total NH_3 and total CO_2 was more pronounced at 160 m*M* acetonitrile as compared to 20, 40, and 80 m*M*.

Effect of Aeration on Acetonitrile Degradation

From the data, it is also evident that the rate of acetonitrile degradation and the concentrations of acetonitrile degradative products were influenced by the rate of aeration. At 100 mL/min aeration, the increase in pH and dissolved NH_3, but not gaseous NH_3 and CO_2, was found to be more, whereas at 200 mL/min aeration, the increase in gaseous NH_3 and CO_2 as compared to pH and dissolved NH_3 was more pronounced. The rate of degradation of acetonitrile in general was found to be higher at 200 mL/min as compared to 100 and 150 mL/min aeration.

The production of CO_2 and NH_3 observed in the present study indicates that the degradation of acetonitrile by nitrile aminohydrolase and amidase (*24–26*) by *P. putida* cells even in the state of immobilization is not altered. Similar degradation of acetonitrile to CO_2 and NH_3 by the free cells of *P. putida* was reported recently (*4*). The time-dependent increase in CO_2 and NH_3 might be attributed to the utilization of acetonitrile as a carbon and nitrogen source. An increase in pH of the medium was owing to the accumulation of NH_3 as a result of acetonitrile degradation, but the pH remained constant after 96–120 h of incubation, which could be the result of assimilation of NH_3 by bacteria or depletion of the substrate in the medium, or increased production of carboxylic acid (acetate) and/or CO_2, which might neutralize the ammonia concentration in the medium.

The data suggest that the rate of degradation is also dependent on the rate of aeration. The decreased production of CO_2 and NH_3 at 100 mL/min as compared to 150 and 200 mL/min aeration indicates the oxygen limitation of the entrapped cells in the alginate immobilization system. The rate of degradation of acetonitrile increased with increase in supply of oxygen to the medium. Since the cells are immobilized in alginate in the form of solid and spherical beads, the oxygen must diffuse through the outer cell matrix layers to reach cells deeper within the beads. Hence, the density of the cells within the matrix is also a limiting factor in the diffusion of oxygen. Further, the acetonitrile transformation enzymes might compete for oxygen at lower amounts of aeration. The reduced supply of O_2 apparently decreases the activities of O_2-dependent enzymes involved in the metabolism of acetonitrile.

The present study thus concludes that the immobilized cells of *P. putida* can degrade different concentrations of acetonitrile as effectively as free cells (*4*). Transformation of acetonitrile to CO_2 and NH_3 is limited by oxygen at a lower rate of aeration, whereas a higher rate of degradation of acetonitrile can be achieved at a higher rate of aeration. The study also

suggests that the complete remediation of contaminated soils can be possible in the presence of higher aeration. The present study supports the application of immobilized-cell technology in the field of bioremediation of toxic nitrile compounds.

ACKNOWLEDGMENT

This work was supported by NIH/DRR/MBRS Grant No. RR08169.

REFERENCES

1. Henahan, J. F. and Idol, J. F. (1971), *Chem. Eng. News* **49,** 16.
2. Milvy, P. and Wolff, M. (1977), *Mutation Res.* **48,** 271.
3. Ludzack, F. J., Schaffer, R. B., and Bloomhoff, R. N. (1961), *J. Water Poll. Cont. Fed.* **33,** 492.
4. Nawaz, M. S., Chapatwala, K. D., and Wolfram, J. H. (1989), *Appl. Environ. Microbiol.* **55,** 2267.
5. DiGeronimo, M. J. and Antoine, A. D. (1976), *Appl. Environ. Microbiol.* **130,** 900.
6. Harper, D. B. (1977), *Biochem. J.* **165,** 309.
7. Yamada, H., Asano, Y., and Tani, Y. (1979), *J. Ferment. Technol.* **5,** 8.
8. O'Reilly, K. T. and Crawford, R. L. (1989), *Appl. Environ. Microbiol.* **55,** 866.
9. Barbotin, J. N., Berry, F., Briasco, C., Huang, J., Nasri, M., Sayadi, S., and Thomas, D. (1989), *Chimica Oggi* **7,** 49.
10. Dominguez, E., Nilsson, M., and Hann Hagerdal, B. (1988), *Enzyme Microb. Technol.* **10,** 606.
11. Fukushima, Y., Okamura, K., Imai, K., and Motai, H. (1988), *Biotechnol. Bioeng.* **32,** 584.
12. Hulst, A. C., Tamper, J., Van't Riet, K., and Westerbeek, J. M. M. (1985), *Biotechnol. Bioeng.* **27,** 870.
13. Pras, N., Hesselink, P. G. M., Ten Tussiher, J., and Malingre, T. M. (1989), *Biotechnol. Bioeng.* **34,** 214.
14. McGhee, J. E., Carr, M. E., and St. Julian, G. (1984), *Cereal Chem.* **61,** 446.
15. Nilsson, H., Mosbach, R., and Mosbach, K. (1972), *Biochim. Biophys. Acta* **268,** 253.
16. Vetvicka, V. and Fornusek, L. (1987), *Biomaterials* **8,** 341.
17. Berry, F., Sayadi, S., Nasri, M., Barbolin, J. N., and Thomas, D. (1988), *Biotechnol. Lett.* **10,** 619.
18. Lawton, C. W., Herbert, E. K., Sundstrom, D. W., and Voronko, P. J. (1987), *Biotechnol. Bioeng. Symp.* **17,** 507.
19. Pundle, A., Prabhune, A., and Siva Raman, H. (1988), *Appl. Microbiol. Biotechnol.* **29,** 426.
20. Adlercreutz, P. and Mattiasson, B. (1982), *Eur. J. Appl. Microbiol. Biotechnol.* **16,** 165.

21. Palleroni, N. J. (1984), *Bergey's Manual of Systematic Bacteriology,* vol. 1., The Williams and Wilkins Co., Baltimore.
22. Smibert, R. M. and Krieg, N. R. (1981), *Manual of Methods for General Bacteriology,* American Society for Microbiology, Washington D.C.
23. Kaplan, A. (1969), *Methods Biochem. Anal.* **17,** 311.
24. Yamada, H., Asano, Y., and Tani, Y. (1980), *J. Ferment. Technol.* **58,** 495.
25. Asano, Y., Fujishiro, K., Tani, Y., and Yamada, H. (1982), *Agric. Biol. Chem.* **46,** 1165.
26. Asano, Y., Tachibana, M., Tani, Y., and Yamada, H. (1982), *Agric. Biol. Chem.* **46,** 1175.