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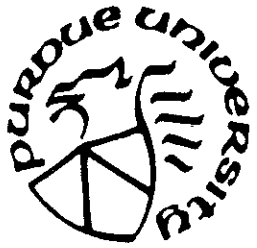

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Section Four
BIOLOGICAL SYSTEMS – A. GENERAL

31 DEGRADATION OF ACETONITRILE BY *PSEUDOMONAS AERUGINOSA*

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INTRODUCTION

Nitrile compounds and their derivatives are used in increasing amounts in a number of industrial operations as chemical solvents, extractants and recrystallizing agents. Consequently, there is also a concomitant increase in the dissemination of these chemicals into the environment via the industrial waste water streams. Increasing accumulation of such compounds in the ecosystem may cause deleterious effects, as most of them are highly toxic and tend to destabilize the ecological balance, by inhibiting beneficial microbial growth. Currently, a number of these compounds are listed as priority pollutants by the Environmental Protection Agency.[1] Biodegradation, the microbial transformation of organic compounds, has been recognized as an effective process for the removal of toxic chemicals from the environment.

Microbial ecosystems exposed to cyanide in the soil especially in sewage plants treating industrial waste water have been studied.[2] However, only a few reports have appeared, on pure cultures utilizing cyanide as sole carbon substrate. A majority of these microorganisms were strictly autotrophic actinomycetes[3] and just a few microbial strains such as *Arthobacter*[4,5] *Brevibacterium*[6-8] and *Rhodococcus* sp[9] were shown to hydrolyse nitrile compounds into amides. Little is known about microorganisms utilizing acetonitrile, a methyl cyanide of increasing industrial use and having potential of becoming a major pollutant, as a substrate. Our present study was undertaken to isolate and characterize various bacteria that were able to use acetonitrile, as the sole carbon source and to define the optimal conditions for growth of such organisms. This report describes one such isolate.

MATERIALS AND METHODS

Chemicals

All nitrile compounds were purchased from Aldrich Chemical Company, Milwaukee, WI. Acetonitrile (^{14}C) (sp.act. 3.0 mCi/mM, purity 99.0%) was purchased from Pathfinder Laboratories, Inc., St.Louis, MO. Aquasol-2 was purchased from NEN Research Products, MA.

Cultures and Media

Acetonitrile-utilizing bacteria were isolated from contaminated industrial soil and water, by use of a phosphate buffer medium (PBM), consisting of the following (g/l): K_2HPO_4, 4.3; KH_2PO_4, 3.4; $(NH_4)_2SO_4$, 2.0; $MgCl_2 \cdot H_2O$, 0.3; amended with 0.5 ml of the trace element solution containing

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(mg/L), $MnCl_2 \cdot 4H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot 2H_2O$, 2.6; and $NaMoO_4 \cdot 2H_2O$, 6.0. Unless otherwise stated, all isolates were grown in medium containing acetonitrile as the sole carbon sou

Isolation of Bacteria

A total of 60 (serially numbered) contaminated soil and water samples around industrial sites were collected, stored in polythene bags and transported to the laboratory on ice. A 1:10 dilution of each sample was made with sterile PBM and the suspension was incubated at room temperature for one hour. One ml of the suspension was then transferred into tubes, containing nine ml of sterile PBM. Different concentrations of acetonitrile, ranging from 0.1 to 50 μl/ml were then added. A loopful of the diluted sample was also streaked on PBM agar plates (1.5% Difco Agar) containing acetonitrile. The tubes and plates were then incubated at 25°C. After seven days of incubation, the tubes and plates were examined for turbidity or colony development. Loopful of turbid solution or streaks of colonies were subcultured into fresh broth or phosphate buffer agar plates containing acetonitrile. Three to four subcultures were made to ensure pure cultures, before the final identification was undertaken.

Diagnostic Tests and the Identification of the Organism

The isolate was characterized using commercially available diagnostic kits from Flow Laboratories (Flow Lab Inc, VA) and API systems (API Analytab Products, NY). The results were confirmed by a computer survey available from these two suppliers. The compiled results were compared with the descriptions in Bergey's Manual.

Determination of Optimal Growing Conditions

One hundred ml of the sterile phosphate buffer medium (pH 7.0) containing 1.0 μl/ml of acetonitrile was inoculated with the bacterial isolate. Unless otherwise stated, the initial inoculum always contained approximately 10^3 per ml of the bacterial suspension. Serial dilutions (10^1 to 10^{12}) were made with 1 ml of the inoculated culture at periodic intervals starting from 0-96 h. One ml of the various dilutions were then mixed with 9 ml of the sterile phosphate buffered medium contai 1.5% agar (Difco Lab, MI) and 1.0 μl/ml of acetonitrile. The suspension was then poured on sterile petri dishes and incubated at 25°C. The bacterial colonies were then enumerated after 96 h with the help of Gallenkamp colony counter.

The optimal temperature range for the bacterial isolate was determined by inoculating 100 ml of the PBM containing 1.0 μl/ml of acetonitrile in 150 ml Nalgene dilution bottles (Nalge Company, NY). The inoculated bottles were then incubated at the following temperatures viz: 5, 15, 25, 30, 35, 45 and 55°C. Viable cell counts were performed after 96 h of incubation. The optimal pH for growth was determined by inoculating the medium adjusted to pH 3.0 to 9.0. The inoculated culture was then incubated at 25°C. Viable cell counts were performed after 96 h of incubation.

Measurement of Oxygen Uptake

Bacterial cells were harvested by centrifugation at 12,000 g for 15 minutes at 4°C after 96 h of incubation. The pellet was then washed twice in 25 mM of phosphate buffer, (pH 7.0), and sedimented again by centrifugation. Respirometeric studies were carried out using Gilson's Differential Respirometer. A 0.5 ml of the bacterial suspension, in 25 mM of phosphate buffer, (pH 7.0) reading 57-59% transmission (having approximately 10^5 cells per mL) was added to the flasks containing 2.2 ml of 25 mM of phosphate buffer. Oxygen uptake was measured after absorbing CO_2 with 0.2 ml of 40% KOH (w/v), in the center well of the flask. Various concentrations of acetonitrile: 0.1, 1.0, 10.0, 25.0, 30.0 μl/ml were each added separately to the side arm flask. After equilibration was attained at 25°C, the respiration process was initiated by tipping 0.3 ml of the substrate from the side arm into the main compartment. Autoclaved bacterial cells served as control. Observations were recorded at 15 minutes intervals.

Aerobic Degradation of Acetonitrile

It was determined at different incubation periods (0-96 h) in triplicate samples by trapping $^{14}CO_2$ that was formed during bacterial growth on ^{14}C acetonitrile. Air was drawn through 20 n the culture containing 20 μl/ml of acetonitrile, supplemented with ^{14}C acetonitrile (200,000 cpm/ml) and subsequently through 5 ml of 1 N NaOH (to entrap CO_2). The amount of radioactivity present in NaOH was determined by adding 0.25 ml of NaOH to 9.75 ml of Aquasol-2 scintillation fluid. The radioactivity in the culture media was determined by filtering the culture media (0.5 mL) through 0.45

Table I. Morphological and Physiological Characteristic of the Bacterial Isolate

Characters[a]	Isolate 16
Morphology	Rod shaped
Gram Reaction	Gram Negative
Motility	Motile
Growth on MacConkey agar	+
Growth on Benzoate agar	+
Fluorescent Pigments	+
Catalase Production	+
Arginine Metabolism	+
Lysine Metabolism	-
Citrate Utilization	+
H_2S production	-
Tryptophane Metabolism	-
Indole production	-
Gelatin Liquefaction	+
Glucose reaction	-
Oxidase, Kovacs	+
Growth at 25 μl/mL of acetonitrile	Abundant

[a] *Identification*: The above characteristics of isolates 16 are consistent with the characteristics of the genus *Pseudomonas aeruginosa*.

μm GN 6 Metricel membrane filter paper (Gelman Science Inc, MI) in a sampling manifold filter system (Millipore Corporation, MA) and washing the filter paper with 5 ml of the sterile phosphate buffer media (25 mM, pH 7.0). The filter paper was dissolved in 1 ml of ethylene glycol monoethyl ether for 5 min and 9 ml of the scintillation fluid was added. Similarly, 0.5 ml of the filtrate was added to 9.5 ml of the scintillation fluid and the radioactivity was determined with a liquid scintillation counter (model LS 6800; Beckman Instruments, Inc., Irvine, Calif). Uninoculated cultures served as the control.

RESULTS AND DISCUSSION

Isolation and Characterization of Bacteria

Among the 60 soil and water samples drawn from different ecosystems, sample No. 16 yielded bacteria capable of growth on acetonitrile as sole source of carbon and energy. Repeated transfers from single colonies into fresh plates and tubes containing phosphate buffer agar with acetonitrile were made to check the purity of isolate. The isolate was rod shaped, gram negative bacteria (Table I) and identified as *Pseudomonas aeruginosa*.

Microorganisms are involved in synthesis and utilization of cyanide. Cyanide production by bacteria has been restricted to some members of *Pseudomonads* and to *Chromobacterium violaceum*, under certain growth conditions.[10] *Pseudomonas aeruginosa* had been reported to produce cyanide under certain growth conditions and that the formation of cyanide was influenced by the availability of molecular oxygen.[11] There is little information indicating that *P.aeruginosa* utilize cyanide compound as the sole carbon source for growth.

Maximal growth of *Pseudomonas aeruginosa* in basal medium supplemented with acetonitrile was attained in 96 h (Figure 1). The optimal temperature range for growth was found to be 25–30°C at (Figure 2) with no growth occurring at temperature as low as 10°C or as high as 55°C. These results are in accord with the earlier report that indicated the temperature optimal of 25–30°C for cultivation of *Arthobacter* and *Rhodococcus* sp that converted nitrile compounds to acrylamide[4,9,12] and to two facultatively autotrophic actinomycetes that utilized cyanide as the sole carbon and nitrogen source.[3] Also, this isolate was found to have an appreciable growth in the range of 5.0–8.0 pH with maximum cell yield at 6.8–7.0 pH. No significant growth was observed at or below 5.0 pH and above a pH of 8.0. (Figure 3).

Growth rates were directly proportional to the concentration of acetonitrile in the medium (data not shown). Both bacterial isolates grew profusely at high acetonitrile concentration (25 μl/ml); however, higher concentration (30 μl/ml) strongly inhibited the growth of the two strains. Although a few reports had indicated[5,9,12] the cultivation of bacteria for the conversion of nitrile compounds to acrylamide, the carbon substrate supplied was always glucose supplemented with low concentration of

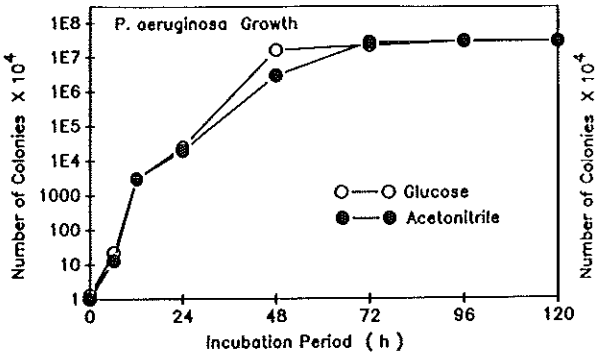

Figure 1. Time course of *Pseudomonas aeruginosa* cell growth.

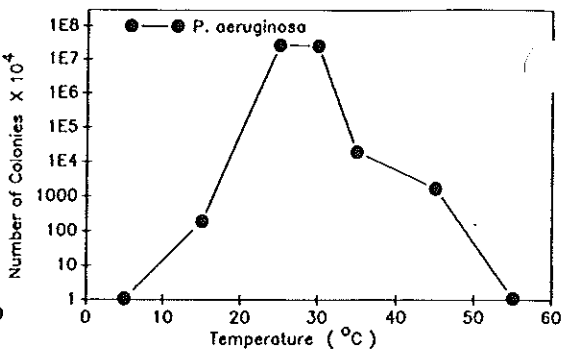

Figure 2. Effect of temperature on cell growth.

nitrile compounds. Howe[13] reported the growth of the blue green algae (Cyanophyceae) and green algae (Chlorophyceae) in a medium containing sodium cyanide increased daily from an initial cyanide concentration of 0.01 μg/ml to a final concentration of 0.2 μg/ml of cyanide. The report also indicated, that a few other organisms, viz: *Bacillus megatherium, Flavobacterium devoraus, Bacillus subtilis* and *Escherichia coli,* were found uninhibited by cyanide. Another report[14] indicated, a strain of *Bacillus pumilus* was recovered from clay soil which had been planted over many seasons with a cyanogenic crop, flax; the organism was tolerant to high concentration of cyanide (2.5 M).

Growth on Various Nitrile Compounds

The bacterial isolate was able to utilize various nitrile compounds (Table II), however acrylonitrile, benzonitrile, malononitrile and acrylamide strongly inhibited growth even at lower concentration. These nitrile substrates (sole carbon and energy source) supported uniform distinct growth very similar to the growth on the phosphate buffer agar plates containing acetonitrile. Control agar pla without an added nitrile substrate were also inoculated with the organism but failed to produce a colonies even after 7 days of incubation. Observations indicated, concentration of nitrile substrate higher than the amount indicated in this study strongly inhibited the growth of the bacteria. Watanabe et al.[12] isolated a strain belonging to the genus *Rhodococcus* was able to utilize most of the nitrile substrates reported in our study, however the concentration of the substrate added was low when compared to our findings. Earlier reports had indicated that *Aspergillus niger* in a nitrogen free sugar medium would utilize 0.1 μg/ml HCN, particularly when the organism was "sufficiently starved" for nitrogen.[15,16] Betts et al.[17] described an organism as *Arthobacter* capable of using ammonium thiocyanate (0.01 μg/ml) as a source of nitrogen. Another report[18] indicated that ten out of thirty cultures of yeast like fungus were capable of utilizing potassium thiocyanate (0.05 μg/ml) as sole source of carbon and energy. Our isolate, was capable of growing at a much higher concentration of cyanide substrate, than the cyanide utilizing microorganism as mentioned above.

Oxidation of Acetonitrile by *Pseudomonas aeruginosa*

Microorganisms require energy to maintain themselves; that is, they must carry out a basal metabolism to obtain sufficient energy to carry out their essential functions.[19] Also should the organism produce enough enzymes to make use of the compound, the responsible population is able to metabolize the chemical and in the process replicate itself faster resulting in the degradation of the particular

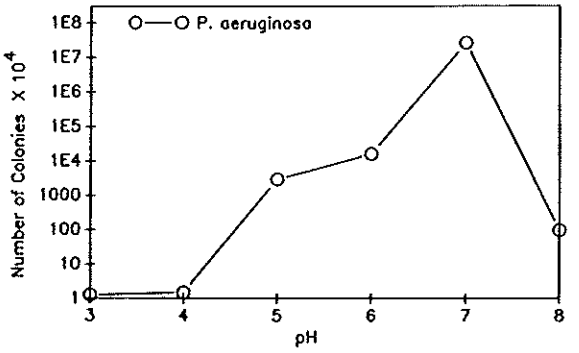

Figure 3. Effect of pH on cell growth.

Table II. Growth of Bacteria in Media Containing Different Substrates

Substrate	Concentration (μL/mL)	*P. aeruginosa*
Acetonitrile	25.0	+ +
Acetamide	30.0	+ +
Acrylonitrile	0.1	-
Acrylamide	0.1	-
Benzonitrile	0.1	-
Butyronitrile	5.0	+ +
Isobutyronitrile	5.0	+ +
Malononitrile	0.1	-
Methacrylonitrile	5.0	+ +
Methacrylamide	10.0	+ +
Propionitrile	10.0	+ +
Propionamide	10.0	+ +
Succiononitrile	10.0	+ +
Valeronitrile	5.0	+ +
Phenylnitrile	0.1	+ +

\+ positive growth on PBA plates containing the particular substrate tested within 4 days
\+ + abundant growth on PBA plates within 4 days
\- no growth even after 7 days of incubation.

compound. The rates of oxygen uptake as determined by the use of manometry are a convenient measure of the various physiological reactions taking place inside the cell. The results of oxygen uptake presented in (Figure 4) indicate that the oxygen uptake was proportional to the concentration of the substrate (acetonitrile). The bacterial isolates readily oxidized the acetonitrile up to 25 μl/ml, however, respiration was strongly inhibited at 30 μl/ml of the substrate.

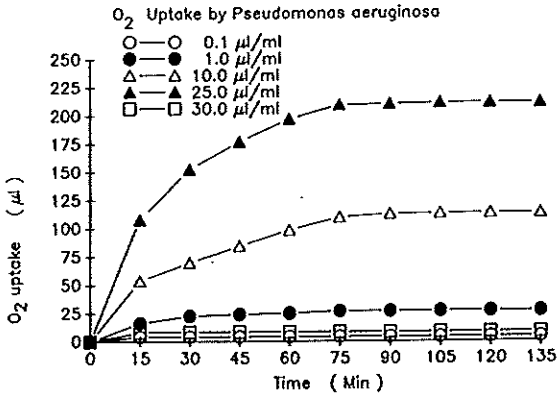

Figure 4. Oxygen uptake by *Pseudomonas aeruginosa*.

Table III. Degradation of Acetonitrile by *Pseudomonas aeruginosa*

Analysis for Radioactivity[a]	Incubation Period (h)				
	0	24	48	72	96
Radioactivity entrapped in lN NaOH	< 1.0%	3.5%	38.0%	51.1%	57.8%
Radioactivity in washed cells	< 1.0%	1.0%	14.6%	21.7%	22.9%
Total radioactivity remaining in the filtrate	98.0	95.5	47.4	27.2	19.3

[a] Increased radioactivity was noticed in the NaOH along with reduced radioactivity in the culture filtrate of the uninoculated cultures. However, these values were subtracted from the results quoted in this report.

Aerobic Degradation of Acetonitrile

The bacterial isolates was cultured on 20 ml of PBM with 20 μl/ml of acetonitrile and supplemented with (^{14}C) acetonitrile (200,000 cpm/ml) as the sole carbon substrate. Degradation was found to increase rapidly after 24 h of incubation and reached a maximum at 96 h (57%); (Table III) however it was found the yield of $^{14}CO_2$ did not increase by extending the incubation period. Interestingly, 20% or more of the initially added radioactive material was found ingested into the bacterial cells. It may be possible that the bacterial cells while growing may convert the organic substrate into inorganic products and in the process the responsible population may convert some of the carbon in the substrate to cell constituents.

The results presented here suggest that the bacterial isolate has the ability to utilize high concentrations of acetonitrile, as sole source of carbon and energy. We propose that this isolate might be exploited to grow on nitrile pollutants, particularly where the pollutant concentrations are high.

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REFERENCES

1. Keith, L. H. and W. A. Telliard, "Priority Pollutant Perspective View," *Environ. Sci. Technol.*, 13:416-423 (1979).
2. Strobel, G. A., "Cyanide Utilization in Soil," *Soil Sci.*, 103:299-302 (1967).
3. Ware, G. C. and H. A. Painter, "Bacterial Utilization of Cyanide," *Nature*, 173:900-902 (1955).
4. Asano, Y., K. Fujishiro, Y. Tani and H. Yamada, "Aliphatic Nitrile Hydratase from *Arthobacter* sp. J-1. Purification and Characterization," *Agric. Biol. Chem.*, 46:1165-1174 (1982).
5. Asano, Y., M. Tachibana, Y. Tani and H. Yamada, "Purification and Properties of an Amidase Which Participates in Nitrile Degradation," *Agric. Biol. Chem.*, 46:1175-1181 (1982).
6. Arnaud, A., P. Galzy and J. C. Jallageas, "Remarques sur l'activite nitrilasique de quelques bacteries," *C. R. Acad. Sci. Paris*, 283:571-573 (1976).
7. Arnaud, A., P. Galzy and J. C. Jallageas, "Etude de l'activite nitrilasique de quelques bacteries," *Rev. Ferment. Ind. Aliment.*, 31:39-44 (1976).
8. Arnaud, A., P. Galzy and J. C. Jallageas, "Amidase Activity of Some Bacteria," *Folia Microbiol.*, 21:178-184 (1976).
9. Watanabe,I., Y. Satoh, K. Enomoto, S. Seki and K. Sakashita, "Optimal Conditions for the Cultivation of *Rhodococcus* sp. N-774 and for Conversion of Acrylonitrile to Acrylamide by Resting Cells," *Agric. Biol. Chem.*, 51:3201-3206 (1987).
10. Michaels, R. and W. A. Corpe, "Cyanide Formation by *Chromobacterium violaceum*," *J. Bacteriol.*, 89:106-112 (1965).
11. Wissing, F., "Cyanide Formation from Oxidation of Glycine by a *Pseudomonas* Species," *J. Bacteriol.*, 117:1289-1294 (1974).
12. Watanabe, I., Y. Satoh and K. Enomoto, "Screening and Taxonomical Properties of Microorganisms Having Acrylonitrile-hydrating Activity," *Agric. Biol. Chem.*, 51:3193-3199 (1987).
13. Howe, R. H. L., "Biodestruction of Cyanide Wastes Advantages and Disadvantages," *J. Air Wat.-Poll.*, 9:463-478 (1965).
14. Skowronski, B. and G. A. Strobel, "Cyanide Resistance and Cyanide Utilization by a Strain of *Bacillus pumilus*," *Can. J. Microbiol.*, 15:93-98 (1969).
15. Rangaswami, G. and A. Balasubramaniam, "Studies on the Rhizosphere Microflora of Sorghum in Relation to Hydrocyanic Acid Content of Roots," *Can. J. Microbiol.* 9:719-725 (1962).
16. Rangaswami, G. and A. Balasubramaniam, "Release of Hydrocyanic Acid by Sorghum Roots and Its Influence on the Rhizosphere Microflora and Plant Pathogenic Fungi," *Indian J. Expt. Biol.*, 1:211-217 (1963).
17. Betts, P. M., D. F. Rinder and J. R. Fleeker, "Thiocyanate Utilization by an *Arthobacter, Can. J. Microbiol.*, 25:1277-1282 (1979).
18. Knowles, C. J., "Microorganisms and Cyanide," *Bacteriol. Rev.*, 40:652-680 (1976).
19. Alexander, M., "Biodegradation of Chemicals of Environmental Concern," *Science*, 211:132-138 (1980).