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Isolation and characterization of acetonitrile utilizing bacteria

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SUMMARY

Bacteria utilizing high concentrations of acetonitrile as the sole carbon source were isolated and identified as *Chromobacterium* sp. and *Pseudomonas aeruginosa*. Maximum growth was attained after 96 h of incubation and *P. aeruginosa* grew slightly faster than *Chromobacterium* sp. The strains were able to grow and oxidize acetonitrile at concentrations as high as 600 mM. However, higher concentrations inhibited growth and oxygen uptake. Degradation studies with (^{14}C)acetonitrile indicated 57% of acetonitrile was degraded by *Pseudomonas aeruginosa* as compared to 43% by *Chromobacterium*. The isolates utilized different nitrile compounds as carbon substrates.

INTRODUCTION

Nitrile compounds and their derivatives are used in a number of industrial operations as chemical solvents, extractants and recrystallizing agents [8]. Consequently, there is a concomitant increase in the dissemination of these chemicals into the environment via industrial wastewater. Increasing accumulation of such compounds in the ecosystem may cause deleterious effects, as most of them are highly toxic, mutagenic and carcinogenic.

Arthrobacter [1,2,4,14,15], *Brevibacterium* [5,10, 11] and *Nocardia rhodochrous* [6,7,9,12,13] hydrolyze some of the nitrile compounds into carboxylic acid and ammonia. Yamada et al. [15] isolated a bacterium identified as *Pseudomonas* K-9 that was capable of utilizing glutaronitrile. Our present study describes the isolation of two bacterial strains capable of utilizing higher concentrations of acetonitrile than reported previously and related compounds as the sole source of carbon and energy.

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MATERIALS AND METHODS

Chemicals

All nitrile compounds were from Aldrich Chemical Company, Milwaukee, WI. Acetonitrile (^{14}C)

(sp. act. 3.0 mCi/mM, purity 99.0%) was from Pathfinder laboratories, Inc., St. Louis, MO. Aquasol-2 was from NEN Research Products, Boston, 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 a trace element solution containing (mg/l), $MnCl_2 \cdot 4H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.6; $CaCl_2O$, 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 source.

Isolation of bacteria

Soil and water samples around industrial sites were collected, stored in polyethylene 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 1 h. One ml of the suspension was then transferred into tubes, containing 9 ml of sterile PBM. Different concentrations of acetonitrile, ranging from 2.5 to 1200 mM 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 7 days incubation, the tubes and plates were examined for turbidity or colony development. Loopfuls of turbid solution or streaks of colonies were subcultured into fresh broth or phosphate buffered agar plates containing acetonitrile. Three to four subcultures were made to ensure purity of cultures before the final identification was undertaken.

Diagnostic tests and the identification of the organism

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

Measurement of oxygen uptake

After 96 h of incubation, bacterial cells were harvested by centrifugation at 12 000 × *g* for 15 min at 4°C. The pellet was then washed twice in 25 mM phosphate buffer (pH 7.0), and sedimented again by centrifugation. Respirometric studies were carried out using Gilson's Differential Respirometer (Gilson Medical Electronics, Milwaukee, WI). A 0.5-ml aliquot of the bacterial suspension in 25 mM phosphate buffer (pH 7.0), reading 57–59% transmission at 540 nm (approximately 10^5 cells per ml) was added to the flasks containing 2.2 ml of 25 mM 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. Acetonitrile at 2.5, 25, 250, 600 or 730 mM was added separately to the side arm flask. After equilibration 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 cells served as control. Observations were recorded at 15-min intervals.

Aerobic degradation of acetonitrile

Degradation rates were determined at different incubation periods (0–96 h) in triplicate samples by trapping the $^{14}CO_2$ that was formed during bacterial growth on ^{14}C acetonitrile. Air was drawn through 20 ml of the culture containing 120 mM of acetonitrile, supplemented with ^{14}C acetonitrile (200 000 cpm/ml) and subsequently through 5 ml of 1 N NaOH to trap CO_2. The amount of radioactivity present in the NaOH was determined by adding 0.25 ml of NaOH to 9.75 ml of Aquasol-2 scintillation fluid. The radioactivity in the culture medium was determined by filtering 0.5 ml through 0.45-μm GN 6 metricel membrane filter paper (Gelman Science Inc, Ann Arbor, MI) in a sampling manifold filter system (Millipore Corporation, Boston, MA) and washing the filter paper with 5 ml of sterile phosphate buffer medium (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, CA). Uninoculated cultures served as the control.

RESULTS AND DISCUSSION

Isolation and identification of bacteria

Two bacterial isolates capable of utilizing acetonitrile as sole source of carbon and energy were isolated from soil. Both isolates were rod shaped, Gram-negative and identified as *Chromobacterium* sp. and *Pseudomonas aeruginosa*, respectively. Growth patterns of the two acetonitrile-utilizing isolates were similar, although *P. aeruginosa* grew faster than the *Chromobacterium* isolate in basal medium supplemented with acetonitrile. Maximal growth was attained in 96 h. The optimal temperature range for growth was 25–30°C for both isolates tested with no growth at 10°C or 55°C. The isolates grew well between pH 5.0–8.0; however, maximal cell yields were obtained between pH 6.8–7.0, with no growth below 5.0 or above 8.0 (data not shown).

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 (600 mM); however, higher concentrations (730 mM) strongly inhibited growth.

Growth on other nitrile compounds

Each isolate utilized acetonitrile, acetamide, butyronitrile, isobutyronitrile, methacrylonitrile, methacrylamide, propionitrile, propionamide, succionitrile and valeronitrile; however acrylonitrile, benzonitrile, malononitrile and acrylamide inhibited growth even at low concentrations. Phenylacetonitrile suppressed the growth of *Chromobacterium* whereas the growth of *P. aeruginosa* was uninhibited (data not shown). These nitrile substrates as sole carbon and energy source supported growth comparable to phosphate buffer agar plates containing acetonitrile.

Oxidation of acetonitrile

With acetonitrile as substrate oxygen uptake was proportional to the concentration of the substrate (Fig. 1a, 1b). The isolates readily oxidized acetonitrile up to 600 mM; however, respiration was strongly inhibited at 730 mM.

Aerobic degradation of acetonitrile

The isolates were cultured on 20 ml PBM with 480 mM of acetonitrile and supplemented with (^{14}C) acetonitrile (200 000 cpm/ml) as the sole carbon substrate. Degradation increased rapidly after

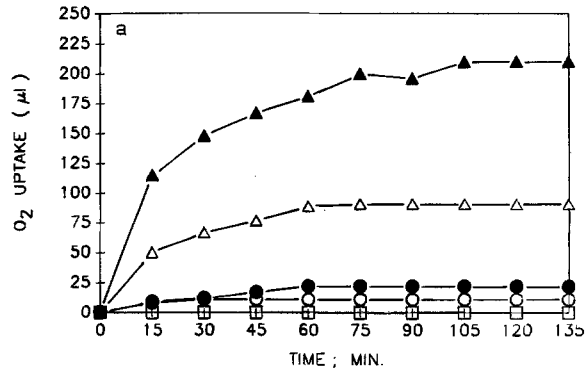

Fig. 1a. Effect of acetonitrile; 2.5 mM (○-○), 25 mM (●-●), 250 mM (△-△), 600 mM (▲-▲) and 730 mM (□-□) on oxygen uptake by *Chromobacterium* sp. Each respirometer flask contained 0.5 ml of cell suspension (approximately 10^5 cells per ml), 2.2 ml of 25 mM phosphate buffer (pH 7.0), 0.3 ml of the substrate and 0.2 ml of 40% KOH in center well.

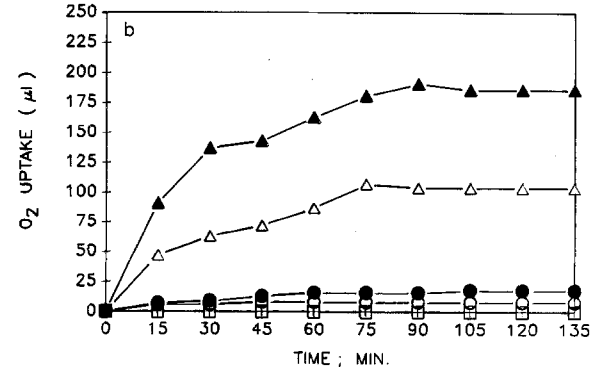

Fig. 1b. Effect of acetonitrile; 2.5 mM (○-○), 25 mM (●-●), 250 mM (△-△), 600 mM (▲-▲), and 730 mM (□-□) on oxygen uptake by *Pseudomonas aeruginosa*. Each respirometer flask contained 0.5 ml of the cell suspension (approximately 10^5 cells per ml), 2.2 ml of 25 mM phosphate buffer (pH 7.0), 0.3 ml of substrate and 0.2 ml of 40% KOH in center well.

24 h incubation, (Fig. 2a, 2b) and peaked at 96 h of incubation. A higher percentage of degradation was noted in the case of *P. aeruginosa* (57%) compared to *Chromobacterium* sp. (43%). Production of $^{14}CO_2$ was not increased by extending the incubation period. At least 20% of the radioactive material added initially was found in the cytoplasmic fraction.

Degradation of nitrile compounds by *Nocardia rhodochrous* [6,7], *Arthrobacter* [1,2,4], and *Brevibacterium* sp. R312 [5,10,11] have been well documented. Asano et al [3] reported a *Pseudomonas chlororaphis* which utilized acrylonitrile as sole source of carbon and nitrogen. The same workers earlier reported another organism identified as *Pseudomonas* K-9 that could utilize only glutaronitrile [15]. Further, this bacterium failed to utilize acetonitrile, butyronitrile, propionitrile or succinonitrile even at lower concentrations. Our results demonstrate that both isolates are equipped with the enzymatic mechanism for the degradation of nitrile compounds and some related amides. *Chromobacterium* sp. and *Pseudomonas aeruginosa* might be exploited to grow on nitrile pollutants, particularly where the pollutant concentrations are high.

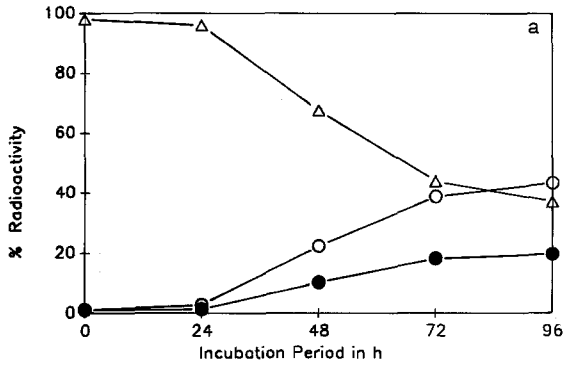

Fig. 2a. Degradation of ^{14}C acetonitrile by *Chromobacterium* sp. Cultivation was carried out with 20 ml of phosphate buffer medium (pH 7.0) containing 120 mM of acetonitrile and supplemented with 200 000 cpm per ml of ^{14}C acetonitrile. Symbols (●-●), cell bound ^{14}C; (○-○), $^{14}CO_2$; (△-△), unutilized ^{14}C remaining in the culture filtrate.

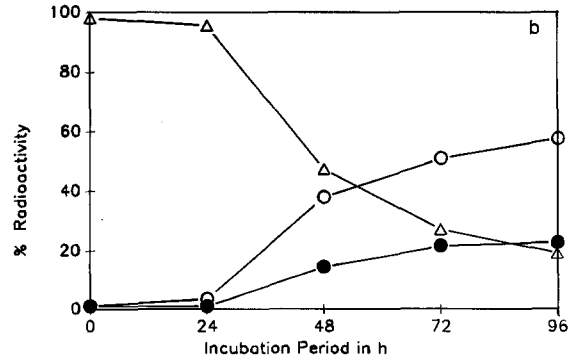

Fig. 2b. Degradation of ^{14}C acetonitrile by *Pseudomonas aeruginosa*. Cultivation was carried out with 20 ml of phosphate buffer medium (pH 7.0) containing 120 mM of acetonitrile and supplemented with 200 000 cpm per ml of ^{14}C acetonitrile. Symbols (●-●), cell bound ^{14}C; (○-○), $^{14}CO_2$; (△-△) unutilized ^{14}C remaining in the culture filtrate.

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