J. Microbiol. Biotechnol. (1998), **8**(4), 347–352

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Isolation of a *Pseudomonas aeruginosa* Strain Capable of Degrading Acrylamide

KUMAR, ARVIND AND ASHOK KUMAR*

School of Biotechnology, Banaras Hindu University, Varanasi-221 005, India

Received: March 20, 1998

Abstract A new strain of *Pseudomonas aeruginosa* growing in a rice field contaminated with herbicide and effluents of a factory manufacturing explosives was isolated. This isolate showed excellent growth in unusually high concentration of acrylamide (60 mM). It utilized acrylamide as the sole source of carbon and nitrogen for growth. Other amides such as acetamide, butyramide, isobutyramide, and methacrylamide were also utilized for the growth by this isolate. Acrylamide was degraded into acrylic acid and ammonia by the enzyme amidase. More than 65% of added acrylamide (40 mM) was converted into acrylic acid after 40 h of growth of the culture. Amidase activity was inducible, the highest activity being observed with isobutyramide (12.5 μM ammonia/mg protein/min). These results demonstrate that this bacterium can degrade a variety of amides.

Key words: Acrylamide, acrylic acid, amidase, *Pseudomonas aeruginosa*, GLC

Acrylamide, a derivative of acrylonitrile, is widely used as an agent in the manufacture of acrylic and methacrylic resins and as a solvent in petrochemical and dye-stuff industries [9]. Acrylamide and certain other amides are also used in the manufacture of herbicides and pesticides. Both acrylonitrile and acrylamide are neurotoxic, mutagenic, and carcinogenic in nature [8]. The extensive and indiscriminate use of highly dangerous compounds such as acrylamide and polyacrylamide have led to the contamination of soil, water, and plants at an alarming rate [7, 16, 25]. It has been demonstrated that unreacted acrylamide from the polymer manufacturing process may be transported into plants from contaminated soil and water [22]. Acute acrylamide poisoning from contaminated water has also been reported [11]. It has been established that acrylamide is degraded rapidly at low concentrations (10 ppm) in surface water, higher concentrations are slower to degrade [7], and may remain stable for more than 60 days in tap water [3].

Very little is known about the microbial metabolism of acrylamide [6, 14]. Shanker *et al.* [24] reported a very slow rate of degradation of acrylamide to acrylic acid and ammonia by a *Pseudomonas* sp. Nawaz *et al.* [20] made serious attempts to isolate acrylamide degrading bacteria from amide contaminated soils. But they found only two species which were potential degraders of acrylamide and other amides, namely, a *Pseudomonas* sp. and *Xanthomonas maltophilia*. They demonstrated that the rate of degradation could be enhanced following immobilization of cells on calcium alginate.

Amidase, the initial enzyme responsible for degradation of acrylamide, has been isolated and purified from *Pseudomonas chlororaphis* [5]. However, no report is available on the factors involved in acrylamide degradation, because of the failure in isolating sufficient number of amide-degrader species.

The main objective of this study was to isolate acrylamide degrading bacteria from sites having a history of amide contamination. It was also desirable to test whether the bacteria capable of degrading acrylamide possess the physiological machinery needed to degrade other related amides.

MATERIALS AND METHODS

Test Organisms and Culture Conditions

Acrylamide degrading bacteria were isolated from soil samples receiving regular treatment of herbicides or from the effluent of a factory near Gomia (Bihar, India) that manufactures explosives. Twenty soil samples from different rice fields were collected and 5 g of each soil sample was placed in 50 ml of phosphate buffered medium in screw-capped flasks. Phosphate buffered medium (PBM) contained: K_2HPO_4, 1.0; KH_2PO_4, 1.0;

*Corresponding author
Phone: 91-542-317331/318151; Fax: 91-542-316693/317074;
E-mail: kashok@banaras.ernet.in

$MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; and D-glucose, 1.0 (g/l), and 1.0 ml of a trace elements solution free of nitrogen source as per White *et al.* [26]. Acrylamide (10 mM) was added in place of glucose in the above medium so as to test its utilization as the sole source of carbon and nitrogen. All the flasks were incubated at 30°C and after 10 days of incubation, 2 ml suspension was transferred into fresh medium (50 ml). After 10 days of incubation, 2 ml suspension was again retransferred into fresh PBM. After five consecutive transfers, 10 ml suspension was removed from each flask and after centrifugation, the supernatant was tested for ammonia, which would reveal acrylamide degradation [18, 20]. Samples showing a positive test for ammonia were retained for the isolation of bacteria.

Isolation and Identification

Isolation, purification, and identification of pure cultures from mixed cultures showing a positive test for ammonia production were made by employing standard microbiological techniques [10]. The purity of a culture was based on morphological characteristics and biochemical tests (Table 1).

Estimation of Growth

1 ml aliquots (OD = 0.8) from exponentially growing cultures were inoculated into 50 ml of the growth medium containing acrylamide or other amides and incubated at 30°C. Absorbance at 600 nm was determined at desired time intervals in a Milton Roy 1201 Spectrophotometer (Milton Roy, Rochester, U.S.A.).

Test for the Degradation of Acrylamide

After the determination of growth, the cells were centrifuged at 15,000 × g for 10 min at 4°C in a Sorvall RC-5 B refrigerated superspeed centrifuge. The supernatant was analyzed for ammonia, acrylic acid, and acrylamide. Ammonia was estimated according to Kaplan [13]. Acrylamide and acrylic acid were identified and quantified by gas liquid chromatography using a 5700 Nucon gas chromatograph (Nucon Engineers Ltd., New Delhi) equipped with a flame ionization detector and silica gel column. The injection and detector temperature were 180 and 250°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.5 ml/min. Peaks were identified by the retention times and quantified with reference to a standard prepared from known quantities of pure acrylamide and acrylic acid in PBM.

Table 1. Morphological and biochemical characters of the bacterial isolate.

Characters	Nature/Response
Colony morphology	Creamy
Cell shape	Rod
Cell size	0.8-2.0 μm
Number of flagella	1-5
Movement	Motile
Pigment production	Green (pyoverdin)
Biochemical tests:	
Catalase	+
Oxidase	+
Urease	+
Nitrate reductase	+
Arginine dihydrolase	+
Ornithine decarboxylase	-
Carbohydrate utilization:	
Glucose	Acid
Xylose	Acid

-, negative reaction; +, positive reaction.

Enzyme Assay

Amidase activity was determind by measuring the ammonia released from acrylamide [1]. Activity was based on the crude enzyme present in cell extract. Cell extract was prepared after Nawaz *et al.* [19]. Culture was grown in a 2-l flask containing 600 ml of PBM supplemented with desired amides. After 80 h of growth, cells were centrifuged (15,000 × g, 15 min, 4°C) and the resulting pellet was suspended in 10 ml phosphate buffer (50 mM, pH 7.5). After one wash the suspension was sonicated for 5 min at intervals of 15 sec in a sonicator (Branson Sonifier 450). The extract made was centrifuged (15,000 × g, 20 min, 4°C). The supernatant served as a source of crude enzyme.

Enzyme activity was measured in a reaction mixture containing 0.9 ml phosphate buffer (100 mM, pH 8.5), 20 mM acrylamide and 100 μl of crude extract (approx. 40~50 μg of protein). Samples were incubated at 30°C for 30 min. Reaction mixtures containing crude extract but no substrate served as controls. One unit of amidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of ammonia/min. Protein was measured according to Lowry *et al.* [17].

SDS-PAGE of Proteins

SDS-polyacrylamide gel electrophoresis was carried out in a vertical system (2001, Pharmacia, LKB, Uppsala, Sweden) with gels of 155 × 130 mm, 1.5 mm thick, using the method described by Laemmli [15]. Gels were stained with Coomassie Brilliant Blue R250 and dried in a gel dryer (Bio-Rad, Richmond, U.S.A.).

Chemicals

All the amides, acrylic acid, and chemicals used in SDS-PAGE and enzyme assay were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. All other chemicals used were of highest purity level and purchased from SISCO Research Laboratories, Mumbai, India.

RESULTS

Of the 20 soil samples tested, four showed growth of bacteria in PBM supplemented with acrylamide. Their culture filtrates were found to be positive for ammonia. This enrichment procedure allowed us to isolate putative acrylamide degrading bacteria. Cells from all the four cultures showed identical morphological features and biochemical responses and were identified as *Pseudomonas aeruginosa* (Table 1). They produced the greenish pigment which is characteristic for *P. aeruginosa* strains. Identification was also based on its growth on *P. aeruginosa* isolation agar and King's medium [10].

Test of Growth on Acrylamide

Growth of one of the isolates was tested in PBM supplemented with varying concentrations of acrylamide. This isolate grew in as high as 60 mM of acrylamide; its complete growth inhibition was noticed at 80 mM (Fig. 1a). Growth with 20 mM acrylamide was more or less similar to that in control (PBM+glucose). Since PBM does not contain any other source of carbon and nitrogen, acrylamide addition meets the requirement of both carbon and nitrogen source for growth and development of the cells.

Once it was evident that *P. aeruginosa* utilized acrylamide, its growth was tested with a few other amides. This isolate grew in acetamide, butyramide, isobutyramide, and methacrylamide (Fig. 1b). Out of all the above amides, maximum growth was observed with acrylamide followed by butyramide and isobutyramide. Growth was very poor with acetamide.

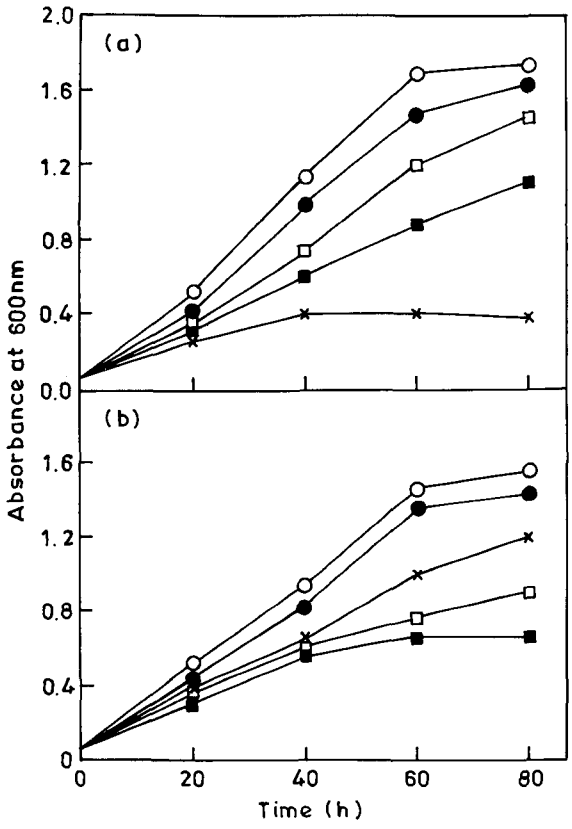

Fig. 1. Growth pattern of *P. aeruginosa* with different amides.

(a) Growth with varying concentrations of acrylamide: PBM control ○ — ○; 20 ● — ●; 40 ■ — ■; and 80 mM x — x acrylamide in PBM. (b) Growth with various other amides: acrylamide ○ — ○; butyramide ● — ●; isobutyramide x — x; methacrylamide □ — □; and acetamide ■ — ■. Each amide was added in PBM at 20 mM final concentration. Equal inoculum was added in each set and the culture was incubated at 30°C.

Products of Acrylamide Degradation

Gas chromatographic analysis of culture filtrates collected after 60 h growth of *P. aeruginosa* with acrylamide revealed that acrylamide was transformed to a compound with a retention time of 4.3 min. This was identified as acrylic acid (Fig. 2). Another peak which appeared after 7.4 min was detected as acrylamide. The culture filtrates also showed accumulation of ammonia.

Time Course Degradation of Acrylamide

Time course analysis of culture filtrates showed higest accumulation of acrylic acid (26 mM) at 40 h and

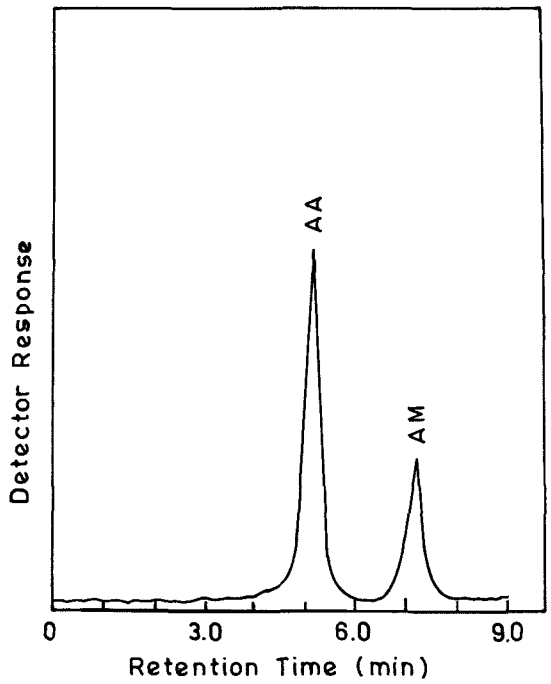

Fig. 2. Gas chromatographic response of acrylamide degradation product by *P. aeruginosa*.

Peaks shown as AA stand for acrylic acid and AM for acrylamide. Culture filtrate from 60 h old culture grown on acrylamide was used for GLC analysis. Identification of peak was based on standard acrylic acid and acrylamide run separately.

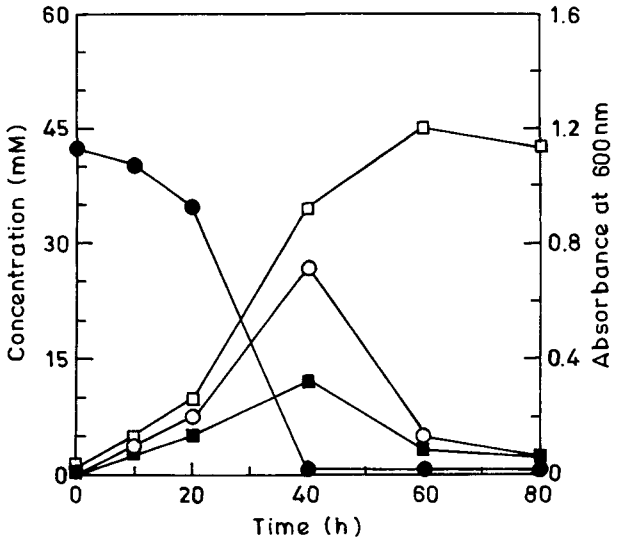

Fig. 3. Time course of cell growth with acrylamide and the formation of products.

Culture was grown at 30°C in PBM containing 40 mM acrylamide. Symbols: acrylamide ● — ●; acrylic acid ○ — ○; ammonia ■ — ■; and cell growth (A_{600}) □ — □.

thereafter there was an abrupt decline. Disappearance of acrylamide coincided with the highest accumulation of acrylic acid in the growth medium (Fig. 3). On the other hand, the decrease in the concentration of acrylic acid elicited close correlation with the increase in the bacterial growth. The concentration of acrylic acid decreased to 3 mM at 80 h whereas, the absorbance (A_{600}) of culture increased from 0.04 to 1.15. In addition to acrylic acid, ammonia accumulated in the medium. The highest concentration of ammonia was observed at 40 h and gradually declined. The trend of decrease was identical to that for acrylic acid.

Amidase Activity

Possible involvement of amidase in the conversion of acrylamide to acrylic acid and ammonia was tested in cell extract of *P. aeruginosa*. Cultures grown with acrylamide or other amides showed a significant level of amidase activity. Highest activity was noticed with isobutyramide followed by acrylamide (Table 2). The activity was optimum at 30°C and pH 7.0.

Cells grown on glucose did not show amidase activity, suggesting an inducible nature of this enzyme. Addition of acrylamide to cultures did not lead to instant appearance of amidase activity; rather, the activity appeared after 4 h. This activity was blocked if cultures received treatment of chloramphenicol (25 μg/ml) plus acrylamide. However, cells grown on any of the above amide sources showed full activity in cell extracts irrespective of the specific substrate used in the assay mixture.

Table 2. Amidase activity in *P. aeruginosa* with various substrates.

Substrate	Amidase activity (μM ammonia/mg protein/min)
Acrylamide	12.0
Methacrylamide	8.5
Butyramide	10.5
Isobutyramide	12.5
Acetamide	11.2
Glucose	Nil

Cultures were grown with 40 mM of each amide separately. Amidase activity was estimated after 96 h of growth. Activities are based on averages of three replicates each.

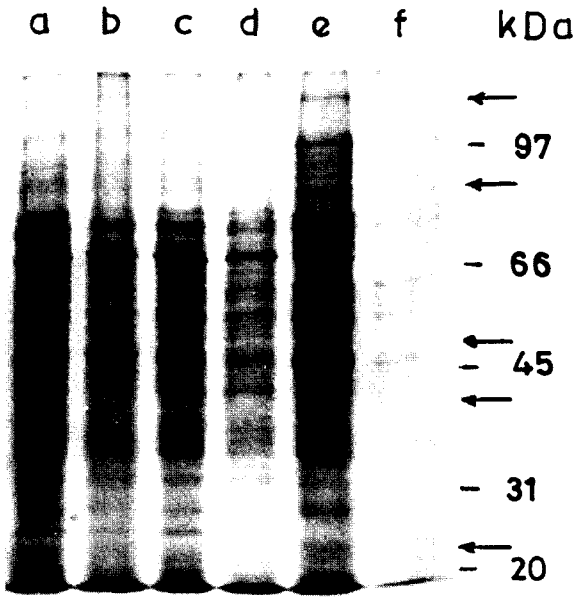

Fig. 4. SDS-polyacrylamide gel electrophoretic pattern of total protein of *P. aeruginosa* after growth on varying concentrations of acrylamide.

Lane a, 20 mM; b, 40 mM; c, 60 mM; d, 80 mM acrylamide in PBM; e, only PBM; f, molecular weight marker proteins. Numbers on the right denote MW in kDa. Arrows indicate the appearance/loss of protein bands.

Protein Profile

SDS-PAGE showed minor alterations in intensity as well as number of protein bands (Fig. 4). High molecular weight protein bands started disappearing following growth of cultures at 40 mM of acrylamide. Certain new bands appeared specifically after growth on acrylamide (55 and 22 kDa).

DISCUSSION

Except for the reports of Nawaz *et al.* [20, 21] and Shanker *et al.* [24], there is no information about the isolation of acrylamide-degrading bacteria. We have isolated a strain of *P. aeruginosa* which quite effectively

degrades acrylamide. Our results indicate that acrylamide-degraders could be readily found in soils contaminated with amides or their derivatives such as herbicides. Nawaz *et al.* [20] also reported the isolation of acrylamide-degraders from soils contaminated with propanil (acrylamilide). It has been demonstrated that herbicides are transformed to amides by soil microflora [23]. Obviously, amides released in the soil after breakdown may support growth of acrylamide degrading microorganisms.

That growth was solely due to degradation of acrylamide to acrylic acid and ammonia was evident from the data of gas chromatographic analysis of culture filtrates. Growth data demonstrate that acrylamide was used as the sole source of carbon and nitrogen for the synthesis of required cell constituents. On this point, our results are in agreement with earlier reports [20, 21]. In addition to acrylamide, our isolate showed appreciable growth with a variety of amides. It appears that this strain possesses the required physiological machinery to grow sustainably on a variety of amides. Conceivably, this isolate degrades other amides to their respective carboxylic acids and ammonia which in turn support growth. Differences in growth rate and yield with various amides could be due to differences in preferential uptake of individual amides. It is pertinent to mention that although numerous bacteria can degrade a variety of aliphatic amides [4, 6, 18], only a few species can utilize acrylamide as the sole source of carbon and nitrogen. It has been demonstrated that the vinyl moiety of acrylamide has deleterious effect on the sulfhydryl proteins and this might be one of the possible reasons for rendering most bacterial strains ineffective in degrading acrylamide. In contrast, certain strains of *Arthrobacter* and *Pseudomonas* do utilize acrylamide but fail to metabolize butyramide, propionamide, isobutyramide, and methacrylamide [19, 21].

That the present isolate is indeed an efficient acrylamide degrader is evident from the rate of acrylamide disappearance from the medium. The culture filtrate showed almost negligible level (<1 mM) of acrylamide after 40 h of growth. Acrylic acid and ammonia, the products of acrylamide degradation, were also exhausted from the medium when cultures attained maximal growth. These findings indicate that the degradation of acrylamide by *P. aeruginosa* is real and occurs at a high rate.

The role of amidase in conversion of aliphatic amides to their carboxylic acids and ammonia has been previously studied in a few bacteria [1, 2, 5, 12, 20]. Asano *et al.* [1] demonstrated that nitrile hydratase converted acrylonitrile to acrylamide, and amidase transformed acrylamide to acrylic acid and ammonia. We have observed high activity of amidase in cultures grown on acrylamide. This shows that amidase hydrolyzes acrylamide to acrylic acid and ammonia. Amidase was active in cells grown on other amides (acetamide, butyramide, isobutyramide, and methacrylamide). However, the highest activity was noticed with isobutyramide. This suggests that the specific activity of the enzyme was not necessarily correlated with the growth rate. We have also demonstrated that amidase was inducible in nature since treatment of cultures with chloramphenicol abolished its activity. The presence of an inducible type of amidase has been recently demonstrated in an acetonitrile-utilizing bacterium, *Rhodococcus erythropolis* A10 [2].

The general protein profile of cells grown with acrylamide suggests that this bacterium indeed alters its biochemical properties. This is seen from the electrophoretic pattern of protein bands where a few protein bands were found to be lost and at least two new bands appeared. Most probably, the protein band of 55 kDa might be that of amidase. This is a tentative speculation since we have not characterized any protein; it is, however, known that amidase from *P. chlororaphis* B23 has a native molecular mass of about 105 kDa and is a homodimer whose subunits have a molecular mass of 54 kDa.

In summary, it may be concluded that *P. aeruginosa* is a potent degrader of a variety of amides and may be useful in bioremediation of amide contaminated soils.

Acknowledgments

This research was supported by a research grant from Department of Biotechnology (BT/R&D/2/27/94), New Delhi, Government of India. Arvind Kumar is thankful to University Grants Commission, New Delhi, for the award of a Senior Research Fellowship.

REFERENCES

1. Asano, Y. M., Y. Tachibana, Y. Tani, and H. Yamada. 1982. Purification and characterization of amidase which participates in nitrile degradation. *Agric. Biol. Chem.* **46:** 1175–1181.
2. Acharya, A. and A. J. Desai. 1997. Studies on utilization of acetonitrile by *Rhodococcus erythropolis* A10. *World. J. Microbiol. Biotech.* **13:** 175–178.
3. Brown, L., M. M. Rhead, K. C. Bancroft, and N. Allen. 1980. Model studies of degradation of acrylamide monomer. *Water Res.* **14:** 775–778.
4. Chapatwala, K. D., M. S. Nawaz, J. D. Richardson, and J. H. Wolfarm. 1990. Isolation and characterization of acetonitrile utilizing bacteria. *J. Ind. Microbiol.* **5:** 65–69.
5. Ciskanik, L. M., J. M. Wilczek, and R. D. Fallon. 1995. Purification and characterization of an enantio selective

amidase from *Pseudomonas chlororaphis* B23. *Appl. Environ. Microbiol.* **61:** 998–1003.
6. Clarke, P. H. 1980. The utilization of amides by microorganisms. pp. 537–562. *In* J. W. Pyne (ed.), *Microorganisms and Nitrogen Sources*. John Wiley & Sons, London, U.K.
7. Croll, B. T., G. M. Arkell, and R. P. J. Hodge. 1974. Residues of acrylamide in water. *Water Res.* **8:** 989–993.
8. DeMeester, C. F., M. Poncelet, M. Roberfriod, and M. Mervier. 1978. Mutagenesis of acrylonitrile. *Toxicology* **11:** 19–27.
9. Enviro Control Inc. 1978. Economic impact assessment for acrylonitrile. Contract Report J-9-F-602 99. Department of Labor, Occupational Safety and Health Administration, Washington, DC, U.S.A.
10. Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips. 1981. *Manual of Methods for General Bacteriology*. American Society for Microbiology, Washington, DC, U.S.A.
11. Igisu, H., I. Goto, Y. Kawamura, M. Kato, K. Izmui, and Y. Kuroiwa. 1975. Acrylamide encephaloneuropathy due to well water pollution. *J. Neurol. Neurosurg. Psychiatr.* **38:** 581–584.
12. Kagayama, T. and T. Ohe. 1990. Purification and properties of an aromatic amidase from *Pseudomonas* sp. GD 1211. *Agric. Biol. Chem.* **54:** 2565–2571.
13. Kaplan, A. 1969. The determination of urea, ammonia and urease. *Methods Biochem. Anal.* **17:** 311–324.
14. Keith, L. H. and W. A. Teliard. 1979. Priority pollutants. I. A perspective view. *Environ. Sci. Technol.* **13:** 416–423.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277:** 680–685.
16. Lande, S. S., S. J. Bosch, and P. H. Howard. 1979. Degradation and leaching of acrylamide in soil. *J. Environ. Qual.* **8:** 133–137.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-Phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Nawaz, M. S., K. D. Chapatwala, and J. H. Wolfram. 1989. Degradation of acetonitrile by *Pseudomonas putida*. *Appl. Environ. Microbiol.* **55:** 2267–2274.
19. Nawaz, M. S., W. Franklin, W. L. Campbell, T. M. Heinze, and C. E. Cerniglia. 1991. Metabolism of acrylonitrile by *Klebsiella pneumoniae*. *Arch. Microbiol.* **156:** 231–238.
20. Nawaz, M. S., W. Franklin, and C. E. Cerniglia. 1993. Degradation of acrylamide by immobilized cells of a *Pseudomonas* sp. and *Xanthomonas maltophilia*. *Can. J. Microbiol.* **39:** 207–212.
21. Nawaz, M. S., W. Franklin, and C. E. Cerniglia. 1994. Degradation of aliphatic amide mixture by immobilized and nonimmobilized cells of *Pseudomonas* sp. *Env. Sci. Technol.* **28:** 1106–1109.
22. Nishikawa, H., Y. Muto, Y. Sonada, and Y. Miyaki. 1978. Effects of high molecular coagulants on agricultural crops. *Gifu-ken kogyo Gijustu Senta kenjyu Hokoku* **10:** 5–8.
23. Pothuluri, J. V., J. A. Hinson, and C. E. Cerniglia. 1991. Propanil: Toxicological characteristics, metabolism and biodegradation potential in soil. *J. Environ. Quality* **20:** 330–347.
24. Shanker, R., C. Ramakrishna, and P. K. Sethi. 1990. Microbial degradation of acrylamide monomer. *Arch. Microbiol.* **154:** 192–198.
25. Smith, E. A., S. L. Prues, and F. W. Oehme. 1997. Environmental degradation of polyamides. II. Effects of environmental (outdoor) exposure. *Ecotoxicol. Environ. Safety* **37:** 76–91.
26. White, J. M., D. D. Jones, D. Huang, and J. J. Gauthier. 1988. Conversion of cyanide to formate and ammonia by a Pseudomonad obtained from industrial wastewater. *J. Ind. Microbiol.* **3:** 263–272.