Wastewater treatment by amidohydrolase activity of agar gel immobilized resting cells of *Nocardia globerula*

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INTRODUCTION

Elimination of toxic amides from wastewaters and other industrial wastes has become a very important environmental issue. Many amides and nitriles are extensively manufactured and used in industry as organic solvents, herbicides, organic feed stocks, extractants, and precursors in the synthesis of emulsifiers and pharmaceuticals (Ramakrishna *et al.* 1999, Hoyle *et al.* 1998). They are extremely harmful when directly discharged into natural habitats. Additionally, many of these compounds are highly toxic and some are mutagenic as well as carcinogenic (Nawaz *et al.*1989).
At present, world- wide annual demand for acrylamide is over 200,000 tons (Nagasawa & Yamada 1989). On the other hand, acrylamide is a well known neurotoxicant, carcinogen and teratogen and is extensively used in numerous industrial processes and the extensive use of acrylamide, its widespread and indiscriminate discharge has led to the contamination of terrestrial and aquatic ecosystems ((Cherry *et al.*1956; Nawaz *et al.* 1994). Hence, removal of acrylamide is of paramount importance, because of its deleterious effects on health and environment.
Here, we describe the successful degradation of amides in wastewater by *Nocardia globerula*. The influence of various conditions, including pH, temperature, and immobilization of resting cells of *Nocardia globerula* in agar were investigated. Furthermore, substrate specificity was assessed. A highly compact five-stage plug flow reactor was used for the treatment of wastewater. The wastewater treated contained acrylamide, acetamide and propionamide.

MATERIALS AND METHODS

Chemicals

All amides and carboxylic acids used in the present study were purchased from Lancaster Inc. (United Kingdom). The media components and miscellaneous reagents were of highest analytical grade available and purchased from Merck (India and Germany) and Sisco Research Lab. Chemical Ltd. (India) and HiMedia, Mumbai, India.

Microorganism, culture conditions and preparation of resting cells

The bacterial isolate *Nocardia globerula* had been procured from the culture collection of the Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla-5, India. It had been isolated earlier from the soils of Himachal Pradesh as a nitrile-metabolizing bacterium (Bhalla *et al.* 2005).
Nocardia globerula was seeded to 2 ml of modified nutrient broth containing 5 g peptone, 3 g beaf extract, 1 g yeast extract, 10 g glucose per litre of distilled water pH 7.0 and incubated at 30 ^{0}C for 24 h in an incubator shaker at 175 rpm. These 24 h precultures were added to 50 ml of production medium containing 30 g tryptone, 15 g yeast extract, 5 g NaCl per litre of water, pH 7.5 (Piotraschke *et al.* 1994) and 0.2% acetonitrile (v/v) as an inducer, followed by incubation at 30 ^{0}C for 24 h in an incubator shaker at 175 rev/min. Then cultures were centrifuged at 5,000 ×g for 15 min at 0 – 4 ^{0}C. The cell pellets were suspended in 0.1 M sodium phosphate buffer pH 7.0 after two

washings with the same buffer. These cell suspensions were referred to as 'whole resting cells'. The whole resting cells were assayed for amidohydrolase activity and used for further investigations.

Amidohydrolase assay

The amidohydrolase activity was assayed with different substrates by the method described by Fawcett & Scott (1960). If not stated otherwise, around 120 µg dry cell weight (dcw) per ml of reaction mixture was applied for the degradation of acetamide that was taken as standard substrate at a concentration of 312.5 mM. 100 mM potassium phosphate buffer pH 8.5 was used to carry out the reactions and the assays were performed at 55 ^{0}C. A unit of amidohydrolase activity was defined as the amount of enzyme that released 1 µmole of product per min. The absorption was measured at 640 nm and related to the amount of product formed in reaction by comparison with a standard.

HPLC analysis

Acetamide, acrylamide and propionamide and their respective carboxylic acids present in the reaction mixture were quantitatively analyzed by high performance liquid chromatography (HPLC), as described by Goldlust & Bohak (1989) using a Perkin Elmer System (Series 200 Ic Pump, US Instruments Division, Norwalk, USA) equipped with a C-18 reverse phase column (4.6 X 1.50 mm) with mobile phase of 0.5 % acetonitrile and 0.07 % orthophosphoric acid at a flow rate of 1 ml min^{-1}. The temperature applied was between 20 to 25 ^{0}C. The sample volume injected was 5 µl and compounds were detected at a wavelength of 210 nm and quantitated by area under curve of the chromatograms. HPLC calibration standard curves were prepared for acetamide (20 to 200 mM), acetic acid (50 to 500 mM), acrylamide (1 to 10 mM), acrylic acid (1 to 10 mM), propionamide (20 to 200 mM) and propionic acid (50 to 500 mM).

Five-stage plug flow reactor

A highly compact five-stage packed-bed plug flow reactor was fabricated according to Fig.1 for the treatment of the wastewater, which contained 100 mM each of acetamide, propionamide, and 10 mM of acrylamide.

Immobilization of Nocardia globerula in agar gel discs

Whole resting cells were immobilized by the method of agar gel immobilization as described by Kierstan & Coughlan (1985). However, concentration of agar was optimized with respect to optimal amidohydrolase activity. In contrast to the standard protocol, 2.5% agar was applied.

Optimization of reaction parameters for gel entrapped resting cells

The conversion of amides to corresponding carboxylic acids was carried out using agar gel entrapped resting cells of *Nocardia globerula* in selected 100 mM potassium phosphate buffer at different pH value from 5.0 to 11, at temperature between 30 to 55 ^{0}C and varied concentrations of acetamide. Substrate affinity of amidohydrolase activity of immobilized cells was also tested using a number of substrates.

Treatment of wastewater using five-stage plug flow reactor

Degradation of acetamide, acrylamide and propionamide was performed in a five-stage plug flow reactor containing agar gel entrapped resting cells of *Nocardia globerula* (Fig. 2) using a 100 ml of wastewater at 45 ^{0}C**.** The wastewater was injected into first reactor and when the wastewater

reached the top of a reactor, samples were taken for quantitation of the amides and their degradation products, the corresponding acids. The residual amounts of amides and acids formed were determined for each cycle by high performance liquid chromatography (HPLC).

RESULTS AND DISCUSSION

Optimization of reaction parameters for gel entrapped resting cells

The maximum turnover of amide to corresponding carboxylic acids was obtained in 100 mM phosphate buffer at pH 8.5 and a temperature of 55 ^{0}C. Gel discs with 0.8 mg resting cells (dcw) showed a maximal amidohydrolase activity when acetamide was used as substrate. The maximum amide degradation was observed at 55 ^{0}C.

Fig. 1 Setting of reactor for wastewater treatment using resting cells of *Nocardia globerula* entrapped in agar gel discs.

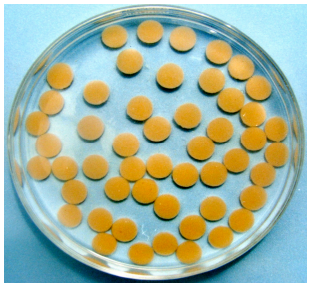

Fig.2 Whole resting cells of *Nocardia globerula* entrapped in agar gel discs.

[**Specifications of each reactor**: Total volume = 65 ml; Void volume = 22.6 ml; Agar disc volume = 38.4 ml; Weight of packed agar discs = 35 g; Dry weight of cells loaded = 53 mg; Diameter of each disc = ~1.3 cm; Thickness of each disc = ~2 mm; Wastewater flow rate = 2 ml min^{-1}; Simulated wastewater used per cycle = 0.1 l; Operational temperature = 45 ^{0}C; Operating pH = 8.5].

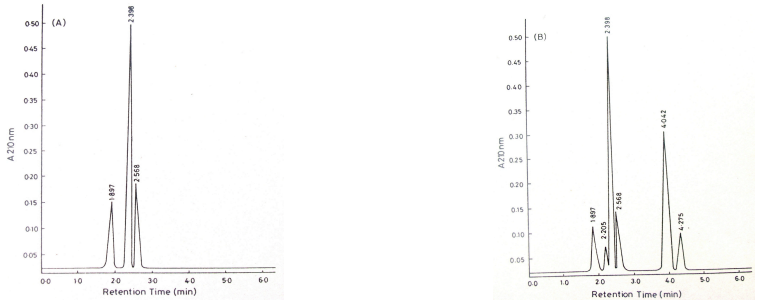

Fig. 3 HPLC chromatograms of a sample of wastewater before treatment (A), fifth reactor of second run (B).

Gel beads entrapped cells exhibited broad substrate affinity with greater turnover of aliphatic amides as compared to aromatic amides to their respective carboxylic acids.

Degradation patterns of amides

The degradation patterns of amides within wastewater by the five-stage plug-flow reactor are shown by HPLC chromatograms (Fig. 3 & 4). There was about 100 % degradation of propionamide in reactor stage No.5 during the third run (Fig. 4 C). The degradation pattern of propionamide in different runs and reactor stages was similar as recorded for acetamide and acrylamide. A gradual increase in amide degradation in consecutive runs was observed, which might be due to a decrease in gel matrix diffusion barrier and after a certain level of substrate and product is maintained within the immobilized cell system, which favors the reaction system.
Maximum degradation of acetamide (97 %) was observed in the fifth reactor stage in the third run.

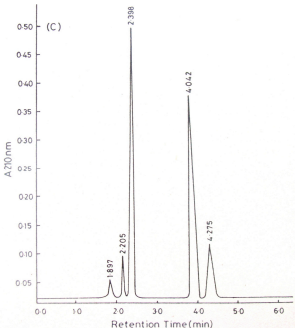

Fig. 4 HPLC chromatograms of a sample of wastewater from fifth reactor of third run

CONCLUSIONS

Due to the efficient degradation of amides of at least 90%, we believe that this microbe has a high potential for bioremediation of wastewater containing toxic amides. Moreover, no one has reported the application of nitrile-degrading organism for the treatment of wastewater containing toxic amides. Therefore, we conclude that *Nocardia globerula* or the appropriate enzymes extracted from this microbe could be successfully used for the treatment of industrial wastewater containing toxic amides.

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