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Influence of selected physical parameters on the biodegradation of acrylamide by immobilized cells of *Rhodococcus* sp.

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Abstract

The influences of concentration of acrylamide, pH, temperature, duration of storage of encapsulated cells and presence of different metals and chelators on the ability of immobilized cells of a *Rhodococcus* sp. to degrade acrylamide were evaluated. Immobilized cells (3 g) rapidly degraded 64 and 128 mM acrylamide in 3 and 5 h, respectively, whereas free cells took more than 24 h to degrade 64 mM acrylamide. An acrylamide concentration of 128 mM inhibited the growth of the free cells. Immobilized bacteria were slow to degrade acrylamide at 10 °C. Less than 60% of acrylamide was degraded in 4 h. However, 100% of the compound was degraded in less than 3 h at 28 °C and 45 °C. The optimum pH for the degradation of acrylamide by encapsulated cells was pH 7.0. Less than 10% of acrylamide was degraded at pH 6.0, while ca. 60% of acrylamide was degraded at pH 8.0 and 8.5. Copper and nickel inhibited the degradation, suggesting the presence of sulfhydryl (-SH) groups in the active sites of the acrylamide degrading amidase. Iron enhanced the rates of degradation and chelators (EDTA and 1,10 phenanthroline) reduced the rates of degradation suggesting the involvement of iron in its active site(s) of the acrylamide-degrading-amidase. Immobilized cells could be stored up to 10 days without any detectable loss of acrylamide-degrading activity.

Introduction

Acrylamide (Figure 1), an aliphatic amide, is a compound of intense industrial use. The annual global production of acrylamide is estimated to be over 200,000 tons (Nagasawa & Yamada 1989). Extensive usage and indiscriminate discharge of the compound has led to the contamination of terrestrial, aquatic and plant ecosystems (Croll et al. 1974; Nishikawa et al. 1978). Biodegradation of acrylamide is of paramount importance because of its carcinogenicity, teratogenecity and its neurotoxic properties (Dearfield et al. 1987).

Several microorganisms (Friedrick & Mitrenga 1981; Grant & Wilson 1973; Hynes & Pateman 1970; Kagayama & Ohe 1990) are known to degrade an array of aliphatic and aromatic amides. However, acrylamide, because of its inhibitory effect on sulfhydryl proteins (Cavins & Friedman 1968) inhibits the growth of these microorganisms. Several strains of *Rhodococcus* sp. are known to degrade acrylamide. A strain of *Rhodococcus rhodocrhous* J1 isolated from soil is capable of degrading acrylonitrile and acrylamide (Nagasawa et al. 1993). A strain of *R. erythropolis* that degrades 2-arylpropionamides could also degrade acrylamide to acrylic acid and ammonia (Hirrlinger et al. 1996). Nawaz et al. (1994) described an isolate of *Rhodococcus* sp. that was highly specific in degrading acrylamide. Earlier a strain of *Pseudomonas chlororaphis* B23 capable of degrading acrylamide to acrylic acid and ammonia was reported (Asano et al. 1986).

Immobilized bacterial technology (IBT) has been successfully used to degrade pollutants (Cassidy et al. 1996). This technology offers several advantages over the application of free cells in the destruction of xenobiotics. Immobilized cells retain high catabolic activity, can be reused, efficiently mineralize substrates and offers physical protection from predators

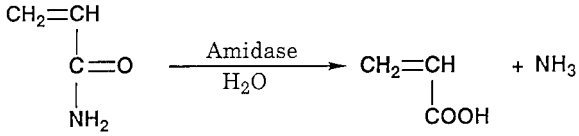

Figure 1. Structure of acrylamide and its metabolism.

and parasites. This technology has been successfully used in the commercial production of acrylamide from acrylonitrile with immobilized *Rhodococcus* sp. (Nagasawa et al. 1993) and *P. chlororaphis* B23 (Asano et al. 1986).

We have isolated a *Pseudomonas* sp., *Xanthomonas maltophilia* (Nawaz et al. 1994; Nawaz et al. 1993), *Rhodococcus* sp. (Nawaz et al. 1994), and *Klebsiella pneumoniae* (Nawaz et al. 1996) that are capable of utilizing acrylamide as the sole sources of carbon and nitrogen. An aliphatic amidase (amidohydrolase, EC 3.5.1.4) is known to participate in the deamination of acrylamide to acrylic acid and ammonia (Figure 1) in these microbes (Nawaz et al. 1994, Nawaz et al. 1996). We reported the degradation of acrylamide by immobilized *Pseudomonas* sp. and *X. maltophilia* (Nawaz et al. 1993) under optimal physical parameters (pH 7.5, 28 °C). However, successful bioremediation of acrylamide depends on the ability of the microbe to adapt to a wide range of temperature, pH, the presence of metals, toxicants and the availability of active and stable chemical degrading bacteria. In this report we examine the effects of temperature, pH, substrate concentration and the presence of metal ions on the rate of degradation of acrylamide by immobilized cells of *Rhodococcus* sp.

Materials and methods

Chemicals

HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer and alginic acid were purchased from Sigma Chemical Co., St. Louis, MO. Acrylamide and acrylic acid were purchased from Aldrich Chemical Co., Milwaukee, WI. All chemicals were of analytical grade, available commercially, and used without further purification.

Immobilization of bacteria

Five grams (wet weight) of *Rhodococcus* sp. cells, harvested after 36 h of incubation, were immobilized in 100 mL of sterile 3% sodium alginate solution (O'Reilly & Crawford 1989). The mixture was added dropwise to cold (4 °C) sterile 100 mM $CaCl_2$. The immobilized cells were stored at 4 °C in HEPES buffer (50 mM, pH 7.5) amended with 1.5 mM acrylamide. The diameter of each immobilized cell was ca. 2–3 mm. The number of viable cells per bead was estimated to be 1.75×10^5.

Degradation of acrylamide by immobilized bacteria

Unless otherwise stated, flasks (125-mL) containing 45 mL of filter-sterilized HEPES buffer (50 mM, pH 7.5) amended with 64 mM acrylamide as the sole growth substrate, were inoculated with 3 g of biobeads. Acrylamide and acrylic acid were identified and quantified by gas chromatography-Fourier transform infrared (GC-FTIR). Sterile growth medium supplemented with acrylamide served as the control.

Degradation of acrylamide by free cells

Flasks (125-mL) containing 45 mL of the growth medium, supplemented with 64.0 mM acrylamide were inoculated with 1 mL of an overnight bacterial suspension (A_{600} = 1.0). Samples (2 mL) were removed periodically. After determination of bacterial growth and ammonia production, the cells were centrifuged at 10,000 × g for 15 min. Acrylamide, and acrylic acid in the supernatant were identified and quantified by gas chromatography.

Analytical methods

Ammonia liberated was measured colorimetrically (Kaplan 1969). Viable cell counts of immobilized beads were determined by dissolving two biobeads in 1 mL sterile potassium phosphate buffer (100 mM, pH 7.2) and plating the dilutions on trypticase soy agar (Kearney et al 1990). Bacterial growth was determined by measuring the A_{600} with a DU-7 spectrophotometer (Beckman Instruments, Fullerton, CA). Acrylamide and acrylic acid were identified and quantified by GC-FTIR after extracting 2 mL of the filter-sterilized culture medium with equal quantities of ethyl acetate in the presence of sodium sulfate. The ethyl acetate extract was concentrated to 0.5 mL before analysis by GC-FTIR. Ethyl acetate extract (2 μL) was injected into a Hewlett Packard HP 5890 gas chromatograph equipped with a 2 mm i.d. splitless capillary injector operated at 250 °C. A 30 × 0.32 mm i.d. fused-silica J&W DE-1701 capillary column was programmed from 105 °C (1 min hold) to 220 °C at 70 °C per

mm. The GC column was connected to a BioRad Digilab FTS-40 FTIR spectrometer. The IR chromatographic data were processed on a 166 MHz IBM compatible computer. FTIR spectra were taken using 8 cm^{-1} resolution and together with Gram-Schmidt chromatograms were plotted by an HP 7550A.

All experiments were repeated at least three times and the averages are reported.

Results

Calcium alginate beads containing cells of *Rhodococcus* sp. were globular with an external diameter of 2–4 mm. The biobeads could degrade various concentrations of acrylamide. Acrylamide concentrations of 8–32 mM were degraded within an hour of incubation. However higher concentrations of acrylamide (64 and 128 mM) were completely degraded within 3 and 5 h of incubation, respectively.

GC-FTIR analysis of concentrated extracts of culture filtrates containing 64 mM of acrylamide after 3 h of incubation indicated the complete transformation of the compound by immobilized cells to stoichiometric amounts of acrylic acid and ammonia (Figure 2A). The pH of the culture medium increased from 7.5 to 7.7. The complete transformation of acrylamide was concomitant with the increase in the number of immobilized cells (from 1.75×10^5 to 2.0×10^5 per bead). Very little change in acrylic acid, ammonia or viable cell numbers in the beads was observed after 3 h of incubation. Culture media inoculated with free cells were slow to degrade acrylamide. Complete degradation of acrylamide was observed in culture media after 36 h of incubation (Figure 2B). Maximum accumulation of acrylic acid and ammonia were determined to be 18 mM after 24 h of incubation. Free cells of *Rhodococcus* sp. increased from an initial A_{600} of 0.07 (26.7×10^3 cells/mL) to a maximum of 1.9 (13.6×10^9 cells/mL) after 36 h of incubation.

Since *in situ* biodegradation of a xenobiotic is influenced by temperature and pH, the effects of these two physical parameters on the biodegradation of acrylamide were determined. Immobilized cells were slow in degrading the xenobiotic at 10 °C, reaching a maximum of 65% after 4 h of incubation (Figure 3A). Complete transformation of acrylamide was obtained after 8 h of incubation at 10 °C (data not shown). Faster removal of acrylamide was achieved at an incubation temperature of 28 °C. The aliphatic amide was totally transformed to its metabolites within 3 h (Figure 3A). Interestingly, a higher temperature of 45 °C had no inhibitory influence on the biodegradation of acrylamide. Acrylamide was totally degraded to its metabolites after 3 h of incubation at 45 °C. An incubation temperature of 55 °C completely inhibited the degradation of acrylamide (data not shown).

Neutral pH (pH 7.0) favoured maximum degradation of acrylamide (Figure 3B). The aliphatic amide was totally degraded to its metabolites within 3 h of incubation at pH 7.0. Although biodegradation of acrylamide was slower at pH 8.0 and 8.5 than at pH 7.0, approximately 70% of the xenobiotic was degraded to its corresponding metabolites within 4 h of incubation. Total transformation of acrylamide was observed after 6 h of incubation at pH 8.0 and 8.5 (data not shown). Very little degradation of acrylamide was observed at pH 6.0. A maximum of 11% of acrylamide was degraded after 4 h of incubation at pH 6.0.

The effect of storage of immobilized cells at 4 °C was evaluated. Encapsulated cells retained their catabolic activity up to a 360 h (Figure 4). Storage of immobilized cells for more than 360 h resulted in slight loss of acrylamide-degrading capability. More than 80% of the acrylamide-degrading capability was lost in cells stored for 480 h.

The effects of two different concentrations (5 and 10 mM) of metals and chelators (EDTA and 1,10 phenanthroline) on the rates of degradation of acrylamide was investigated (Table 1). Lower concentrations (5 mM) of nickel and cobalt slowed the rates of degradation, whereas higher concentrations (10 mM) inhibited the biodegradation of acrylamide. Lower and higher concentrations of copper inhibited acrylamide degradation. Regardless of the concentration, barium did not affect the degradation of acrylamide. The highest rate of degradation of acrylamide was obtained in the presence of iron. However, iron concentrations higher than 10 mM inhibited acrylamide degradation (data not shown) Although lower concentrations of EDTA had no significant effect on the rates of degradation, higher concentrations of iron inhibited the biodegradation of acrylamide. Regardless of the concentration, 1,10-phenanthroline significantly reduced the rates of degradation.

Discussion

Immobilized cells have been used for the degradation of phenol (Dwyer et al. 1986), cresol (O'Reilly & Crawford 1989) and pentachlorophenol (O'Reilly

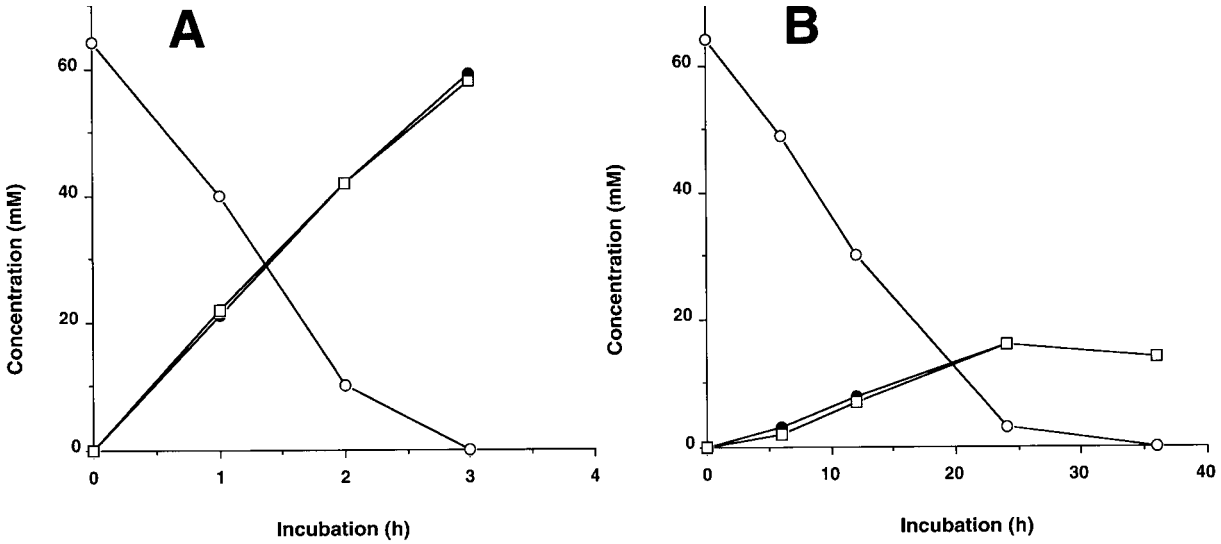

Figure 2. Biodegradation of acrylamide by immobilized and free cells of *Rhodococcus* sp. Growth media were amended with 64 mM of acrylamide and the culture media was inoculated with immobilized (A) and free cells (B). Immobilized cells degraded acrylamide within 3 h of incubation. Free cells degraded acrylamide in 36 h of incubation. The concentrations of acrylamide (○) , acrylic acid (●) and ammonia (□) were estimated at various incubation periods.

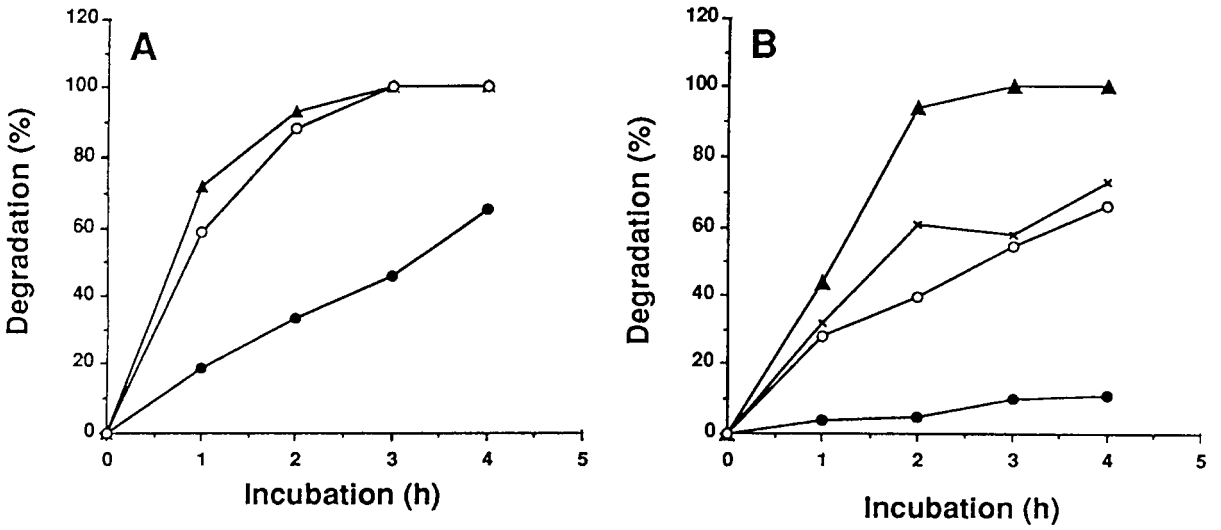

Figure 3. The effect of temperature (A) and pH (B) on the degradation of acrylamide by immobilized *Rhodococcus* sp. Flasks (125 mL) containing 40 ml of HEPES buffer amended with 64 mM acrylamide were inoculated with 3 g (wet weight) of immobilized cells and incubated at different temperatures. Efficient degradation of acrylamide was observed between 28–45 °C. Concentrations of acrylamide, acrylic acid and ammonia were determined at various incubation periods. (●) 10 °C, (▲) 28 °C and (○) 45 °C. B. The influence of pH on the degradation of acrylamide by immobilized *Rhodococcus* sp. Buffer at pH 6.0 (●), 7.0 (▲), 8.0 (×) and 8.5 (○) containing 64 mM acrylamide were inoculated with immobilized *Rhodococcus* sp. and incubated at 28 °C. The concentrations of acrylamide, acrylic acid and ammonia were determined at periodic intervals.

Table 1. Effects of selected metals on the degradation of acrylamide by immobilized cells of *Rhodococcus* sp.

Metals/Chelator	Concentrations (mM)	Rate of acrylamide degradation (mM/h)
None (Control)	0	21.3 ± 1.8
Ni^{2+}	5	8.0 ± 1.7
	10	0.0
Cu^{2+}	5	0.0
	10	0.0
Co^{2+}	5	10.6 ± 1.3*
	10	1.0 ± 0.4
Fe^{2+}	5	25.6 ± 3.9
	10	26.6 ± 3.4
Ba^{2+}	5	21.3 ± 1.8
	10	21.3 ± 1.8
EDTA	5	20.1 ± 1.9
(disodium salt)	10	14.2 ± 0.8
1,10 & Phenanthroline	5	12.8 ± 0.8
	10	8.0 ± 1.2

*± denotes one s.d.; $n = 5$.
Flasks containing 45-ml of filter sterilized HEPES buffer (50 mM, pH 7.5) containing acrylamide (64 mM) was inoculated with 3 g of immobilized cells. Degradation of acrylamide was monitored every hour. The average of five replication is reported in this table.

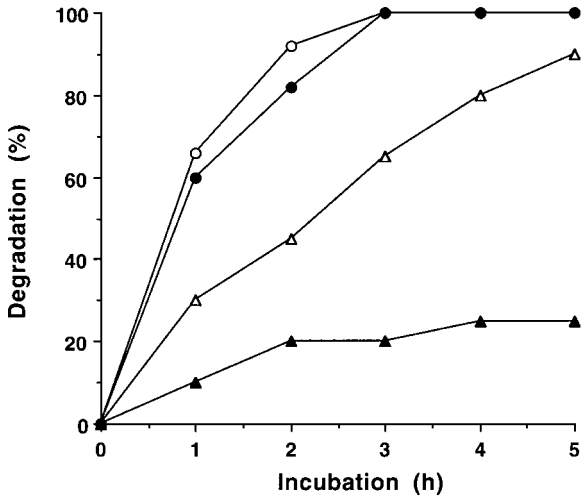

Figure 4. The effect of different periods of storage of immobilized cells on the degradation of acrylamide. Flasks (125 mL) containing the growth media amended with 64 mM acrylamide were incubated with 3 g of immobilized cells stored at different periods of incubation at 28 °C. Immobilized cells retained its catabolic activity upto 360 h. The concentrations of acrylamide, acrylic acid and ammonia were determined at various incubation periods. (○) 24 h, (●) 240 h, (△) 360 h and (▲) 480 h.

& Crawford 1989). Although immobilized cells offer several potential advantages over the application of free cells in the field of bioremediation, the implementation of this technology may be adversely affected by variation in the pH, temperature and concentration of the xenobiotic. Thus there is a need to understand the roles of these factors to aid the implementation of this technology.

Comparison of the degradation of acrylamide by immobilized and free cells of *Rhodococcus* sp. indicate that the concentration of acrylamide influences its biodegradation. Data from this study indicate that immobilized cells of *Rhodococcus* sp. are faster than free cells in degrading acrylamide to acrylic acid and ammonia. The rapid transformation of acrylamide to acrylic acid and ammonia is due to the fact that immobilized cells contain a high concentration of cells at their peak catabolic activity whereas free cells take time to multiply. Immobilized cells degraded 128 mM of acrylamide within 6 h of incubation whereas the growth of the free cells was completely inhibited by higher concentrations. It is possible that the alginate matrix of the immobilized cells protects the cells from the substrate toxicity. Our results also indicate that encapsulated cells can withstand the toxicity of high concentrations of accumulated metabolites. Although numerous reports indicate higher chemical degradation rates with immobilized cells (Heitkamp et al. 1993; Heitkamp et al. 1990; Nawaz et al. 1994; Nawaz et al. 1993; O'Reilly & Crawford 1989) these cells are

prone to substrate and metabolite inhibition (Dwyer et al. 1986).

Distinct difference between immobilized and free cells was observed in the utilization pattern of the metabolites. Immobilized cells were slow in their uptake of metabolites, thus resulting in their accumulation in the mineral media. Slow cell division within the immobilized matrix may retard the uptake of the metabolites. Contrarily, free cells rapidly utilized the metabolites for the synthesis of additional cell materials.

Immobilized cells could degrade approximately 66% of the xenobiotic after just 4 h of incubation at 10 °C. Free cells grow very poorly at 10 °C. Immobilized cells could easily degrade 100% of the acrylamide within 3 h at 45 °C whereas free cells grew poorly at 45 °C. These results indicate that the alginate matrix may offer a protective shield against adverse physical conditions. Contrarily, the alginate matrix offers very little protection for the cells against the adverse pH of the growth medium. Although the optimum pH of the purified amidase (Nawaz et al. 1994) is around 8.5, less than 70% of the carcinogen was degraded by the immobilized bacteria at pH 8.5. Acidic pH (6.0) was inhibitory to both the purified amidase (Nawaz et al. 1994) and the rates of degradation of immobilized cells of *Rhodococcus* sp. The optimal pH for the degradation of acrylamide by immobilized cells was determined to be pH 7.0.

The amidase responsible for the degradation of aliphatic amides is a sulfhydryl (SH)-containing protein (Nawaz et al. 1994, Nawaz et al. 1996). The inhibition of the degradation of acrylamide by immobilized cells of *Rhodococcus* sp. by nickel and copper in this study suggests that some SH groups may be involved at the active site(s) of the enzyme or these groups may play an active role in the tertiary enzyme structure. Although barium did enhance the enzyme activity of the purified *Rhodococcus* sp. amidase (Nawaz et al. 1994) it did not enhance the degradation of acrylamide by immobilized cells. Since iron enhanced the degradation of acrylamide by purified amidase (Nawaz et al. 1994) and immobilized cells of *Rhodococcus* sp., we suggest that iron may have a crucial role in the active site(s) of the *Rhodococcus* amidase.

Immobilized bacterial technology (IBT) is a low-cost, efficient methodology for the rapid removal of chemical wastes. This technology uses highly selective xenobiotic-specific bacteria under optimal conditions (optimum concentration of the compound, temperature and pH) for the rapid removal of the xenobiotic by encapsulated bacteria. However, data on the influence of different concentration of the compound, temperature, pH and the presence of heavy metals on the rates of degradation of acrylamide by immobilized *Rhodococcus* sp. needs to be evaluated for the ready application of this technology. Results of our study indicate that immobilized cells were far superior to free cells in the rapid degradation of high concentrations of acrylamide. Unlike free cells that were inhibited by higher temperatures (>30 °C), immobilized cells are thermotolerant in degrading acrylamide at higher temperatures. Such positives traits of immobilized *Rhodococcus* sp. cells make it highly attractive for its application in the treatment of acrylamide-contaminated wastes.

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