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Nitrate removal from the effluent of a fertilizer industry using a bioreactor packed with immobilized cells of *Pseudomonas stutzeri* and *Comamonas testosteroni*

Swati L. Zala[1], Jayalexmy Ayyer[2] and Anjana J. Desai[1,*]
[1]*Department of Microbiology and Biotechnology Centre, Faculty of Science, M.S. University of Baroda, 390 002 Gujarat, India*
[2]*Gujarat Narmada Valley Fertilizer Company Ltd., Narmadanagar, Bharuch, Gujarat, India*
**Author for correspondence: Tel.: +91-265-2794396, Fax: +91-265-2792508, E-mail: desai_aj@yahoo.com*

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Summary

A bioreactor for the removal of nitrate nitrogen (NO_3-N) from industrial effluent is described which is comprised of a glass column (60 cm × 6 cm) packed with alginate beads containing denitrifying organisms *Pseudomonas stutzeri* and *Comamonas testosteroni*. The effluent containing high concentrations of nitrate (600–950 mg l^{-1}) from the fertilizer industry and fusel oil (methanol as a major component) as organic carbon were used in the process. The reactor is operated in the continuous mode by injecting the pretreated nitrate-containing effluent at the top of the column. The Hydraulic retention time (HRT) was adjusted by changing the flow rates. When nitrate-containing wastewater was treated with immobilized cells, the nitrate removal rate reached a maximum 1.66 ± 0.07 Kg NO_3-N m^{-3} d^{-1} at an influent NO_3-N concentration of 850 mg NO_3-N l^{-1} within 12 h. The denitrification activity of the immobilized cells was compared with that of the free cells.

Introduction

Elevated levels of nitrate in drinking water are a hazard to humans and animals. The permissible limit of nitrate in potable water is not more than 10 mg l^{-1} NO_3-N. Microbiological contribution offers a radical solution, since bacteria can effectively reduce nitrate to molecular nitrogen (N_2) (Tiedje 1994). Up to 95–100% nitrate removal was achieved by using a consortium of *Pseudomonas stutzeri* and *Comamonas testosteroni* by the suspended growth technique (Zala *et al.* 1999). Immobilized bacterial bioreactors have a higher productivity per unit biomass and are ideal for small manufacturers and commercial laboratories, which lack space for a conventional free cell treatment plant (Chibata *et al.* 1983). A number of studies (Santos *et al.* 1993; Uemoto & Saiki 1996, 1999; Kesseri *et al.* 2002) have shown an effective denitrification with attached and immobilized cells on a different supporting matrix. Uemoto & Saiki (2000) had achieved nitrogen removal from wastewater by use of a tubular gel containing *Nitrosomonas europaea* and *Paracoccus denitrificans*. Gas-permeable silicone tubing was used as the basis of a bioreactor for autotrophic denitrification (Ho *et al.* 2001). A hollow fibre membrane bioreactor (HFMB) was operated for hydrogenotrophic denitrification (Sarina & Andreas 2001). The matrix used in an immobilized bioreactor should be able to take sufficient organic load with effective mass transfer, which increases the efficiency of the process.

Here we report nitrate removal from an industrial effluent using a consortium of bacteria immobilized on to alginate beads. Immobilized cells are convenient to handle, appear to be less susceptible to microbial contamination and permit easy separation of products from the biocatalyst. The denitrification efficiency of an immobilized bacterial consortium is compared here with the free cell culture.

Materials and methods

Cell immobilization procedure

The bacterial isolates used for this experiment, *P. stutzeri* and *C. testosteroni,* were isolated from the denitrifying reactor of a fertilizer company (Zala *et al.* 1999). Cell cultures were separately grown aerobically in peptone nitrate broth; peptone, 5 g l^{-1}; beef extract, 3 g l^{-1}; potassium nitrate, 1 g l^{-1}, incubated on a shaker for 24 h at 37 °C ± 2. Following growth, cells were collected by centrifugation, washed twice with saline and a 10% (v/v) cell suspension (0.55 g l^{-1} dry weight of *P. stutzeri* and 0.34 g l^{-1} dry weight of *C. testosteroni*) added into a solution of 3% (w/v) sodium alginate. The mixture was extruded drop wisevia syringe into 0.1 M

$CaCl_2$ solutions. When the entire mixture was entrapped, the beads along with the $CaCl_2$ mixture were placed in the refrigerator for 1 h for hardening and the beads were then transferred to nitrate-containing effluent and incubated overnight (Chibata *et al.* 1983). Finally the beads were washed with effluent and used for nitrate removal in batch and continuous bioreactor studies.

Immobilized and free cell denitrifying activity

Nitrate removal by immobilized cells in batch cultures was carried out with different concentrations of nitrate (600–950 mg l^{-1}), and COD:NO_3-N ratios. Free cell suspension and immobilized beads were inoculated in 250 ml Erlenmeyer flasks containing 100 ml nitrate-containing effluent, flasks were incubated at 37 ± 2 °C under static conditions. A negative control containing only effluent was also kept to check auto-oxidation. Fusel oil was added as an external carbon source to maintain sufficient COD concentration required for nitrate removal. Nitrate, nitrite, ammonia and COD were estimated from each flask at the interval of every 12 h.

Continuous removal of NO_3

The bioreactor used for testing the reduction of nitrate consisted of a glass cylinder [60 cm × 6 cm (ID)], which was packed with immobilized beads (up to 45 cm) and closed at the top with rubber stopper and the other end with glass wool. Two spacers were adjusted at equal intervals to minimize the gas pressure on the beads. The column was initially filled with 0.5 l effluent, and after complete removal of nitrate, the continuous process was started by running the effluent through the reactor at different flow rates. Nitrate removal rate in the bioreactor under the continuous process was checked with different COD:NO_3-N ratios (2.45 and 1.45) and retention times.

Analytical method

Nitrate was determined by the method of Tenkins & Medsker (1964) and ammonia release was estimated by the method of Fawcett & Scott (1960). COD (5220-C), nitrite (4500-NO_2^--B) were estimated as per the standard methods (APHA *et al.* 1995). Gas chromatographic analysis was used to detect N_2 and CO_2 using a molecular sieve column for N_2 and Unibed C column for CO_2, using a thermal conductivity detector.

Results and discussion

Comparison of denitrification by immobilized cells and free cells

The alginate beads immobilized with both the denitrifying strains (0.55 g l^{-1} dry weight of *P. stutzeri* and 0.34 g l^{-1} dry weight of *C. testosteroni*) were cultivated in nitrate-containing effluent. The evolution of N_2 from the consortium of these immobilized cells was checked by gas chromatographic analysis, which showed maximum evolution of N_2 (94%). After every 12 h interval, nitrate reduction was checked by measuring release of nitrite and ammonia. Comparable denitrification efficiency could be achieved by both immobilized and free cell systems within a 36 h retention time (Table 1), although inoculum size used to reach this denitrification efficiency with the immobilized system was 10% (v/v) as compared to that of 5% (v/v) used for suspended growth system.

Bead stability

From the commercial point of view, selection of supporting material and stability of immobilized beads both are equally important. After complete removal of nitrate, the beads were washed with fresh effluent and the same beads were then used with a new batch of nitrate-containing effluent. In the first run, nitrate was removed with 96% efficiency but after five successive transfers the efficiency gradually increased to 99% with a decrease in retention time to 24 h. This could be due to the inducible nature of the enzymes required for nitrate reduction (Delwiche & Bryan 1976). Plating the treated effluent on peptone nitrate agar with direct and 10^{-3} dilution checked leaching of the cells during each cycle. Absence of growth on peptone nitrate agar plate indicated that the beads were quite stable (data not shown).

Optimization of carbon in batch process with immobilized cells

In any biological system, there are minimum requirements for nutrients, the most important in practice being

Table 1. Comparison of denitrification efficiency between immobilized and suspended cells.

Growth condition	NOx-N[a] mg l^{-1}			Denitrification efficiency (%) (At 36 h HRT)
	12 h HRT[b]	24 h HRT	36 h HRT	
Suspended growth [5% (v/v) inoculum size]	534	278	18	97.82
Immobilized cells [10% (v/v) inoculum size]	502	237	24	97.09
Negative control[c] (without cells)	801	799	782	4

Initial nitrate concentration was 827 mg l^{-1} in all growth conditions.
[a] NO*x*-N = NO_3-N + NO_2-N; [b] Hydraulic retention time (HRT); [c] To check auto oxidation/reduction of nitrate.

carbon, nitrogen and phosphorus. For all denitrification systems, a source of organic carbon is necessary to provide the energy required for reduction of the nitrate ion. Methanol was found to be most economical and convenient because lower alcohols tend to be oxidized rather than synthesized into biomass. Fusel oil added as a source of organic carbon in the present study has methanol as a main component. The total methanol requirement including those needed for growth, are expressed by McCarty (1969) as follows: $Cm = 2.47No + 1.53Ni + 0.87DO$; where Cm is the methanol required, No and Ni are initial nitrate and nitrite concentration respectively and DO is the dissolved oxygen concentration. According to this equation, 80% denitrification efficiency could be achieved with 2.85 COD:NO_3-N ratio by suspended growth process within 48 h retention time (Zala *et al.* 1999). Nitrate removal efficiency of immobilized *P. stutzeri* and *C. testosteroni* was checked with different COD:NO_3-N ratios, such as 3.45, 2.85 and 2.45. Up to 96% nitrate removal efficiency could be obtained within 24 h retention time (data not shown). Nitrite was accumulated during the initial period but was reduced later to N_2O or N_2 as evidenced by the gas production and treated effluent COD reached in the range of the permissible limit. Ammonia remained constant in the range of 7–15 mg l^{-1}, indicating the absence of DNRA activity (Tiedje 1994).

Continuous removal of nitrate using bioreactor

Immobilized bacterial bioreactors are ideal for small manufacturers and commercial laboratories that generally have neither the space nor the existing conventional free cell treatment plants (Chibata *et al.* 1983). A 60 cm high, 6 cm diameter radial flow column was constructed from glass material and filled with the immobilized beads containing entrapped *P. stutzeri* and *C. testosteroni*, operated as continuous reactor. The effect of different COD:NO_3-N ratio and nitrate loading rates on denitrification efficiency were checked. Denitrification rate of the continuous bioreactor with immobilized cells was determined by the following equation:

$$\text{Denitrification rate (g N-NO}_3\ l^{-1}d^{-1}) = \frac{\{[\text{N-NO}_3]_{in} - [\text{N-NO}_3]_{out}\} \times R}{V}$$

where R is the wastewater flow rate ($l\,d^{-1}$), $[NO_3\text{-N}]_{in}$ and $[NO_3\text{-N}]_{out}$ is influent and effluent NO_3 concentration (g NO_3-N l^{-1}) respectively, and V the reactor

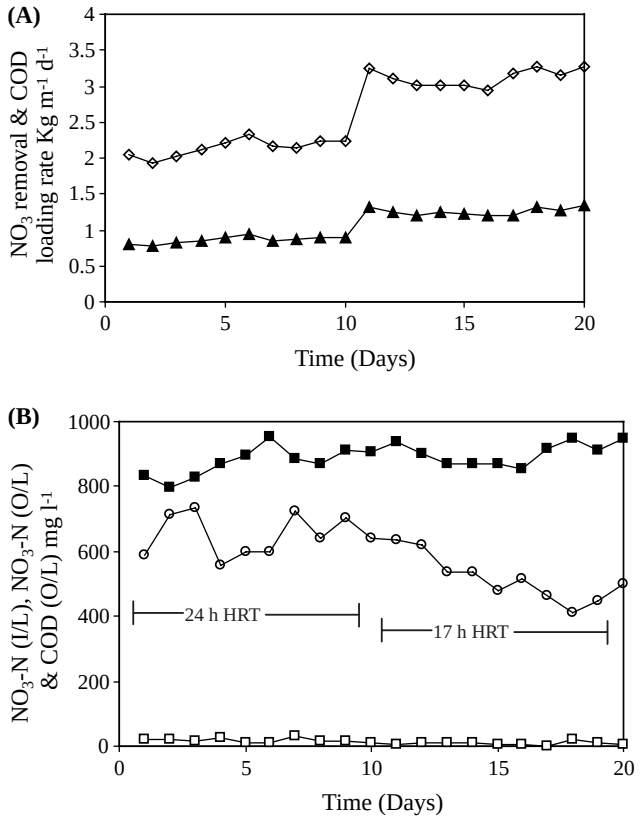

Figure 1. Performance of the continuous bioreactor with 2.45 COD:NO_3-N ratio at different HRTs (hydraulic retention time). (A) NO_3 removal and (▲) COD loading (◇) rates. (B) Inlet NO_3-N (■), outlet NO_3-N (□) and outlet COD (○) concentrations.

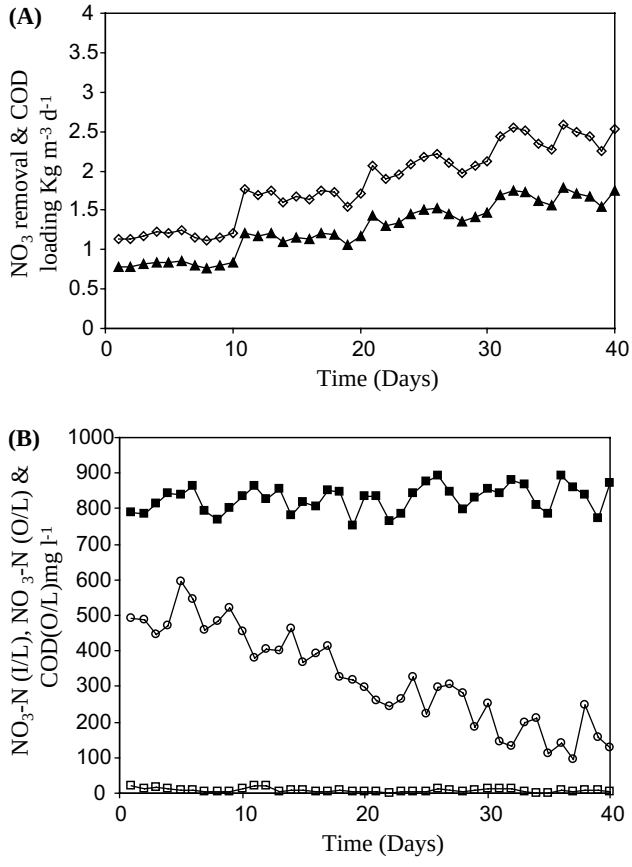

Figure 2. Performance of the continuous bioreactor with 1.45 COD:NO_3-N ratio at different retention times. (A) NO_3 removal and (▲) COD loading (◇) rates. (B) Inlet NO_3-N (■), outlet NO_3-N (□) and outlet COD (○) concentrations.

Table 2. Comparison of denitrification rate using attached/immobilized growth system.

Reactor system	Bacteria	Carrier for immobilization	Denitrification rate (kg NO_3-N m^{-3} d^{-1})
Membrane feeding substrate bioreactor (Ho *et al.* 2001)	*Alcaligenes eutrophus*	Silicone tube	0.016–0.054
Hollow fibre membrane bioreactor (Sarina & Andreas 2001)	NA[a]	Polypropylene	0.770
Packed gel envelopes (Uemoto & Saiki 1999)	*N. europaea* & *P. denitrificans*	Photocross-linkable resin (PVA-SbQ)	1.60 ($NH_4^+ \rightarrow N_2$)
(Kesseri *et al.* 2002)	*P. butanovora*	NA	1.53 & 1.63
Glass column (This study)	*P. stutzeri* & *C. testosteroni*	Na-alginate beads	1.41 ± 0.06 & 1.66 ± 0.07

[a] Not available.

volume (l). Figure 1 depicts the performance of the bioreactor at 24 and 17 h HRT with 2.45 COD:NO_3-N ratio. The load of 0.87 and 1.27 Kg NO_3-N m^{-3} d^{-1} was applied during 24 and 17 h HRT respectively. Nitrate removal rate reached almost 0.85 ± 0.05 and 1.25 ± 0.04 Kg NO_3-N m^{-3} d^{-1} (95–98%) irrespective of the retention time, with concentration of ammonia remaining constant (<5 mg l^{-1}) and nitrite not exceeding 10 mg l^{-1}. However, the effluent COD concentration remained between 600 and 450 mg l^{-1}, which was higher than the acceptable limit. When nitrate is removed by immobilized cells, no carbon is used for cell mass biosynthesis.

Since efficient COD removal could not be achieved with 2.45 COD:NO_3-N ratio, a separate run with a COD:NO_3-N ratio of 1.45 was done. Nitrate removal rate at 24, 17, 14 and 12 h HRT with 1.45 COD:NO_3-N ratio is depicted in Figure 2. At this carbon ratio, complete nitrate removal was achieved with all four HRTs, the final NO_2-N and NO_3-N concentrations achieved was <5 mg l^{-1}. The nitrate removal rate observed was 0.80 ± 0.03, 1.14 ± 0.04 and 1.41 ± 0.06 Kg NO_3-N m^{-3} d^{-1} at 24, 17 and 14 h retention times respectively (Figure 2). The average final NO_3-N concentration during 12 h HRT was <10 mg l^{-1} with the nitrate removal rate reaching a maximum 1.66 ± 0.07 Kg NO_3-N m^{-3} d^{-1} at an influent NO_3-N concentration of 850 mg NO_3-N l^{-1}.

The rates of denitrification by different reported attached growth systems are compared with our results (Table 2). Among the reactor systems listed, the immobilized *Pseudomonas butanovora* cells exhibited the highest denitrification rate with ethanol as organic carbon. Our reactor exhibited a comparable nitrate removal rate with immobilized *P. stutzeri* and *C. testosteroni* cells in a continuous bioreactor. The advantage of our system is the use of cheaper organic carbon, fusel oil as compared to the acetic acid, ethanol and succinic acid used for *P. butanovora*, making this process economically more feasible (Kesseri *et al.* 2002). The carrier (matrix) material used for immobilization in other systems is either membranes or resins, which are less cost effective compared to Na-alginate (Uemoto & Saiki 1999; Sarina & Andreas 2001). By considering the results of bioreactor studies, immobilized cells with alginate gave comparatively higher denitrification efficiency, with a bioreactors which requires less space, little capital investment and offers operational simplicity, thus appearing to be relatively cost-effective for small systems. In addition, the use of fusel oil as carbon source, which is relatively cheap, makes the process more promising.

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