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# Denitrification of high salinity, high nitrate wastewater using clinoptilite in a packed bed bioreactor

Abbas Rezaee<sup>1\*</sup>, Hatam Godini<sup>2</sup>, Reza Darvish Cheshma Soltani<sup>1</sup>,  
Hooshyar Hossini<sup>1</sup>, Afshin Nili-Ahmadabadi<sup>1</sup>

<sup>1</sup> Department of Environmental Health, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

<sup>2</sup> Department of Environmental Health, Lorestan university of medical sciences, Khoramabbad, Iran

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The aim of the present work was to study the treatment of high strength nitrate, high salinity wastewater using a packed bed bioreactor filled with clinoptilolite as a carrier. Biological denitrification was monitored at different loadings rates ( $0.8\text{--}4\text{ kg NO}_3^-/\text{m}^3\cdot\text{d}$ ) and salinities ( $10\text{--}40\text{ g L}^{-1}$ ). The experiments were carried out at an ambient temperature of  $25\text{ }^\circ\text{C}$  with synthetic wastewater. The denitrification rate, COD consumption, and nitrite accumulations were response parameters. In the experimental conditions, a maximum denitrification rate of  $3.72\text{ kg NO}_3^-/(\text{m}^3\cdot\text{d})$  was achieved with ethanol as a carbon source and salinity at  $10\text{ g L}^{-1}$ . During denitrification, no more than  $0.1\text{ mg NO}_2^- \text{ L}^{-1}$  could be accumulated. This work demonstrates that biological denitrification with the denitrifier attached to clinoptilolite in a packed bed bioreactor can be a promising method for denitrifying brine wastewater.

**Key words:** Denitrification, Wastewater, Packed bed, Clinoptilolite, Salinity

## INTRODUCTION

Nitrate in surface and groundwater has been an increasing concern in recent years. It is the primary cause of methemoglobinemia in infants, also known as "blue baby disease", and the formation of nitrosamines from reduced nitrate in the stomach has been suspected to cause cancer (Emamjomeh and Sivakumar, 2009). To avoid adverse health effects, the European Community and the US Environmental Protection Agency set nitrate limits to  $5.6\text{ mg NO}_3^- \text{ N/L}$  and  $10\text{ mg NO}_3^- \text{ N /L}$  (Aslan and Cakici, 2007).

Several methods with different removal performances and costs are available for denitrification, including ion exchange, reverse osmosis, electrodialysis, and biological denitrification (Cortez et al, 2010; Rezaee et al, 2010). In recent years, biological denitrification has been

shown to be the most economical, practical, and versatile approach for water and wastewater treatment (Moon et al, 2008). However, streams with high nitrate concentrations can also contain large amounts of other ions such as chloride (from the fish canning industry, wet lime-gypsum desulphurization process, and regeneration liquid from ion exchange columns) and sulfate (from tannery wastes). High saline concentrations in wastewater have negative effects on biological denitrification (Uygur and Karg, 2004). However, several halo-tolerant denitrifying bacteria have been isolated and identified in hypersaline waters (Kim *et al*, 2006).

The application of immobilization techniques to industry, especially in the biological wastewater treatment systems, not only offers a high cell concentration in the reactor tank for increasing efficiency, but also facilitates the separation of liquid and solid in the settling tank (Arnaz et al, 2000; González et al, 2009). Wastewater treatment by fixed-film filter technology requires the formation of a biofilm around an inert substance. In urban

\*Corresponding Author E-mail: [rezaee@modares.ac.ir](mailto:rezaee@modares.ac.ir);  
Tel/Fax: 98 21 82883575

wastewater it is possible to form a biofilm from the influent to be treated, owing to the presence of a high microbial loading. However, with industrial wastewater containing extreme concentrations of pollutants, it is necessary to prepare the biofilm previously, and on occasion to allow a period of time for acclimation of the microbial mass. Many different materials have been studied as filter support media including biological materials, baked clay, RPF sheets, plastic, PVC rings, and ceramic rings (Kocadagistan et al, 2005; Rezaee et al, 2008<sub>b</sub>). The surface state of filter bed material, pore size, and media geometries are important factors in the treatment efficiencies of packed bed reactors (Elmitwalli et al, 2002). In this context, the use of clinoptilolite as carrier is of great interest. Clinoptilolite is a natural zeolite comprising a microporous arrangement of silica and alumina tetrahedral with the complex formula,  $(\text{Na}, \text{K}, \text{Ca})_{2-3} \text{Al}_3(\text{Al}, \text{Si})_2\text{Si}_{13}\text{O}_{36} \cdot 12(\text{H}_2\text{O})$ . It forms as white to reddish crystals with a Moh's hardness of 3.5 to 4 and a specific gravity of 2.1 to 2.2 and commonly occurs as a devitrification product of volcanic glass shards in tuff and as vesicle fillings in basalts, andesites and rhyolites (Englert and Rubio, 2005). It has interesting and potentially useful properties, such as a large surface area (200-1000 m<sup>2</sup>/g), hydrophobic or hydrophilic properties that create electrostatic interactions, different ion-exchanged forms, and exert mechanical and chemical resistances (Nikolaeva et al, 2002; Ratiu et al, 2009). Despite these properties, reports on wastewater treatment for high nitrate and salinity levels using clinoptilolite in a packed bed bioreactor system are rare. The main objective of this study is to investigate the performance of a packed bed biofilm reactor packed by clinoptilolite with regards to two main operational parameters: the high nitrate loading rate and salinity concentrations.

## MATERIALS AND METHODS

### Isolation And Identification Of Denitrifying Bacterium

The grab samples were collected from an up flow anaerobic sludge blanket (UASB) of dairy processing wastewater treatment plant, Tehran, Iran. Wastewater was analyzed according to the standard methods of the American Public Health Association (APHA, 2005). Samples were subjected to basal salt medium (BSM) contained per liter of distilled water:  $\text{KB}_{2\text{B}}\text{HPOB}_{4\text{B}}$ , 0.9 g;  $\text{KHB}_{2\text{B}}\text{POB}_{4\text{B}}$ , 0.45 g;  $\text{NHB}_{4\text{B}}\text{Cl}$ , 0.45 g;  $\text{MgSOB}_{4\text{B}}$ , 0.2 g;  $\text{CaClB}_{2\text{B}} \cdot 2\text{HB}_{2\text{B}}\text{O}$ , 0.02 g;  $\text{FeClB}_{3\text{B}}$ , 0.005 g; and trace elements solution, 1 ml containing (mg L<sup>-1</sup>):  $\text{HB}_{3\text{B}}\text{BOB}_{3\text{B}}$ , 400;  $\text{ZnSOB}_{4\text{B}} \cdot 7\text{HB}_{2\text{B}}\text{O}$ , 400;  $\text{CoClB}_{2\text{B}}$ , 50;  $\text{NiClB}_{2\text{B}} \cdot 6\text{HB}_{2\text{B}}\text{O}$ , 200;  $\text{NaB}_{2\text{B}}\text{MoOB}_{4\text{B}} \cdot 2\text{HB}_{2\text{B}}\text{O}$ , 300;  $\text{CuSOB}_{4\text{B}} \cdot 5\text{HB}_{2\text{B}}\text{O}$ , 10;  $\text{MnSOB}_{4\text{B}} \cdot \text{HB}_{2\text{B}}\text{O}$ , 500. Ethanol was added as the carbon source, and the ratio of nitrate

to ethanol was 1:4 to keep the nitrogen as the limiting substrate.

The inoculated media were incubated with constant shaking (120 rpm) at room temperature. Serial dilutions were then made and plated on the synthetic agar medium, and the solid media were incubated at 30 °C. To obtain pure cultures, single colonies were picked and streaked on slant solid media.

The cultures were examined microscopically for purity. Initial identification schemes were performed with biochemical tests as suggested by the Bergeys Manual of Systematic Bacteriology. For final and specific identification, 16S rRNA sequencing was performed (Rezaee et al, 2008). The 16S rRNA was amplified by polymerase chain reaction (PCR) using primers (5'-GCGAGGAAATGAAGCTG-3'; 3'-AAGGTGATCGACGAGGTC-5'). PCR amplification was carried out for 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. A PCR product purification kit (PCR-M Clean-Up System; Viogene, Sunnyvale, CA, USA) was used for purification of the amplified DNA. The 16S rRNA sequences were determined with a SQ5500E DNA sequencer (Hitachi Chemical, Tokyo), and the sequences were compared with the 16S rRNA sequences in the GenBank database using BLAST, which is available at NCBI.

### Experimental Procedure

The experimental set-up consisted of one upflow column. The column consisted of a 70 cm Plexiglas tube with an 80 mm inner diameter (empty bed volume of 2.5 L). The upper end of the column was closed with a Plexiglas flange to maintain anoxic conditions and prevent media from washing out. Sampling ports were installed 0.1 m apart along the column to obtain liquid and solid samples. The end of the column was equipped with a screen layer of 2 mm diameter ports to obtain uniform fluid flow in the bed.

The reactor was loaded with uniformly clinoptilolite particles as a carrier (fraction passing USA Sieve Number 18 and retained on USA Sieve Number 25). The clinoptilolite had a geometric mean diameter of 0.54 mm, a specific gravity of 1.85, and a specific surface area of 3900 m<sup>2</sup>/m<sup>3</sup>. Synthetic wastewater was pumped through a perforated flange to the bottom of the reactor from a 20 L sealed plastic tank using a peristaltic pump. The effluent was collected from the top of the column in a 20 L plastic tank. The reactor operated with nitrate concentrations of 100-500 mg NO<sub>3</sub><sup>-</sup> L<sup>-1</sup> and NaCl concentrations of 10-40 g/L. Hydraulic retention times maintained at 1-3 h. The pH of the reactors was maintained at 7.2. The experiments were operated at room temperature (25 °C). To inoculate the biofilter media with bacteria, the bioreactor was filled with synthetic media and inoculated with 1.5 × 10<sup>8</sup> CFU/mL of

*Pseudomonas stutzeri* for 48 h. After the static period, wastewater was circulated through the reactors in a closed loop, returning to the storage tank.

This recirculation was continued until there was a substantial decline in the nitrate concentration of wastewater in the storage tank. During this acclimation period, wastewater in the storage tank was amended with the addition of nitrate and ethanol to improve bacterial growth. After recirculating the wastewater for 3 d, synthetic wastewater was fed into the system at an influent nitrate concentration of 100-500 mg/ L. After the start up period, the entire study was conducted by nitrate loading rates (0.8, 1.6, 2.4, 3.2 and 4 kg NO<sub>3</sub><sup>-</sup>/(m<sup>3</sup>.d) and NaCl (10, 20, 30 and 40 g L<sup>-1</sup>).

The nitrate eliminated by the bioreactor was calculated as follows:

$$\text{Denitrification rate} = \frac{[\text{NO}_3^-]_{\text{input}} - [\text{NO}_3^-]_{\text{output}} \times R}{V}$$

Where R is the wastewater flow rate, [NO<sub>3</sub><sup>-</sup>]<sub>input</sub> is the influent and [NO<sub>3</sub><sup>-</sup>]<sub>output</sub> the effluent NO<sub>3</sub><sup>-</sup> concentrations (g NO<sub>3</sub><sup>-</sup> L<sup>-1</sup>), and V is the reactor volume.

### Biofilm Estimation

For quantification of the biofilm biomass, total protein concentrations in packed bed biofilm reactors were determined by the Bradford method using the Coomassie blue protein assay reagent (Li et al, 2006). Bovine serum albumin was used as a standard. To release cell proteins, 1.2 mL of 1M NaOH was added to 10 mL of normal saline containing packing materials with biofilm attached. After incubation at 30°C for 30 min, test tubes containing samples were heated in boiling water for 15 min. After cooling, 0.86 mL of 32% HCl was added to each tube to neutralize the pH. Samples were then centrifuged at 10000 g for 5 min and 0.05 mL of each supernatant was mixed with 1 mL of protein assay reagent. After 10 min the absorbance at 595 nm was determined. The total protein contents of biofilms were determined by the same method.

### ANALYTICAL METHODS

The chemicals used were of analytical grade quality. All data reported in our study refer to steady state conditions. Influent and effluent samples were collected and filtered through membranes of 0.45µm pore size and filtrates were analyzed for NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, COD, and pH. Analyses were carried out according to the recommendations of the standard method (APHA 2005).

### Statistical Evaluations

Analysis of variance (ANOVA) of the various parameters was used to compare differences among treatment groups. If significance was determined ( $P < 0.05$ ), Fisher's protected least-significant difference was used to determine differences among treatment means.

## RESULTS AND DISCUSSION

### Characterization Of The Isolated Denitrifying Bacterium

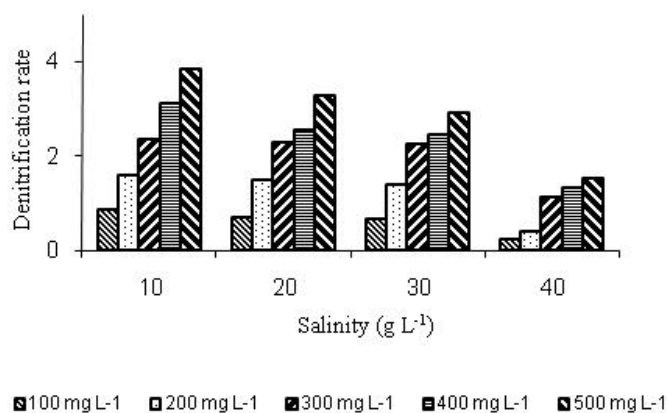
Initially, some bacterial were grown on the BSM medium after incubation. The colonies were chosen for additional analysis. Because all of the characteristics studied were found to be identical for the isolates, only one representative isolate (*P. stutzeri*) is discussed below. The bacterium is able to remove nitrate under anoxic conditions. The isolate is short to very short gram-negative rods. Growth on solid media is fast, resulting in yellow colonies after 18 h.

The bacterium showed these characteristics: gram-negative, small cells, motile, catalase and oxidase positive, yellow colonies with regular outline on agar plate. In 16S rRNA sequence analysis, more than 98% similarity was observed between the sequences of the isolate bacterium and the sequences of *P. stutzeri* in database.

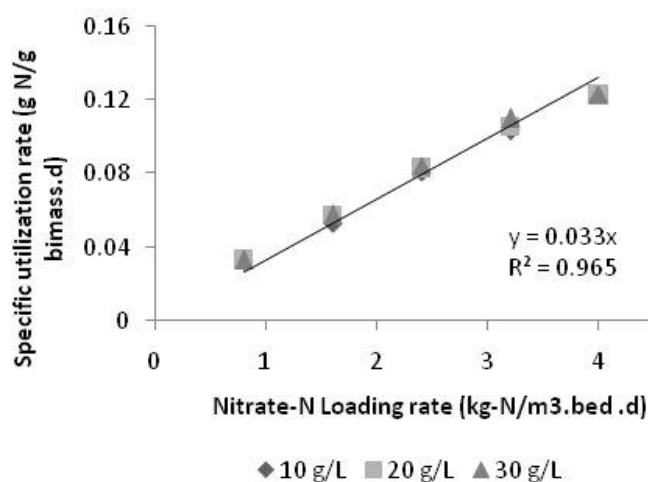
The results of the biochemical tests along with the molecular identification of the bacterium using 16S rRNA sequence analysis proved that the genus of the bacterium was *P. stutzeri*.

### Denitrification Activity

In this study, the highest observed denitrification rate was 3.72 kg NO<sub>3</sub><sup>-</sup>/(m<sup>3</sup>. d) at a nitrate load of 4 kg NO<sub>3</sub><sup>-</sup>/(m<sup>3</sup>.d) and 10 g L<sup>-1</sup> NaCl. The denitrification rates of the packed bed bioreactor is within or higher than the range reported by other researchers (Kim et al. 2002; Suzuki et al. 2003). Hirata et al. (2001) reported a maximum nitrogen volumetric rate of 0.24 kg NO<sub>3</sub><sup>-</sup>/(m<sup>3</sup>.d) using an anaerobic aerobic circulating bioreactor system to remove ammonia and nitrate from two- to five-fold diluted industrial wastewater discharged of metal recovery processes. Typical volumetric nitrate loadings for up flow denitrification filters are in the range from 3.0 to 4.0 kg NO<sub>3</sub><sup>-</sup> to achieve effluent concentrations below 5.0 g NO<sub>3</sub><sup>-</sup>/m<sup>3</sup> (Pujol et al. 1994). The bioreactor showed lower rates of denitrification than the other salinity concentrations at 40 g/L ( $P < 0.05$ ). Significant nitrate reduction was observed at salinities lower than 30 g L<sup>-1</sup> ( $P > 0.05$ ). Figure.1 shows the denitrification rates obtained during the processes.



**Figure 1.** Comparison of denitrification rates in the different concentration of nitrate (100-500 mg L<sup>-1</sup>) and salinity (10-40 g L<sup>-1</sup>).



**Figure 2.** Effect of nitrate loading rate on the nitrogen specific utilization rate

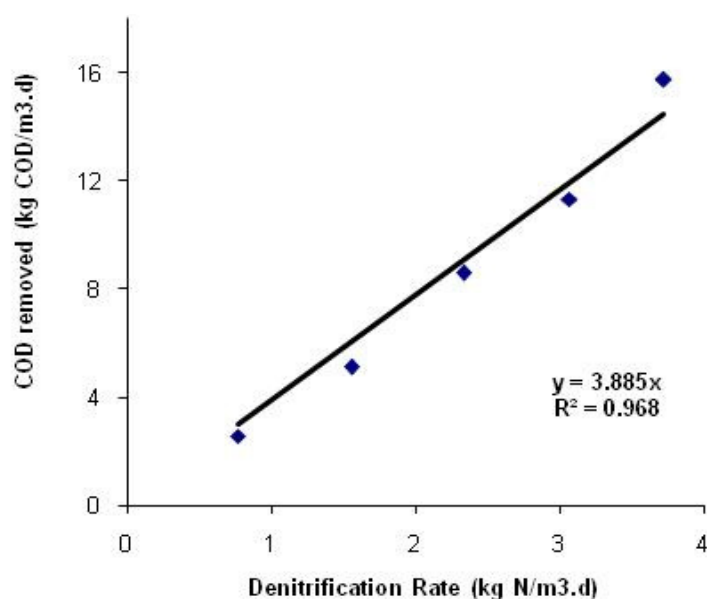
These results were in agreement with a previous study that performed nitrate removal with bacterial cells attached from salty wastewater (Foglar et al. 2007). High salt concentrations have negative effects on organic matter, as well as nitrogen and phosphorous removal. However, several halo-tolerant nitrifying and denitrifying bacteria have been isolated and identified in hypersaline waters. In this study, the acclimated bacterium for denitrification was isolated from a high salinity and high nitrate wastewater.

The specific nitrate utilization rate ( $r_{sp}$ ) represents the mass of nitrate removed per unit mass of biomass in the bioreactor and it better represents the rate of nitrate removal than the volumetric removal rate expressed as the mass of nitrate removed per unit volume of the

reactor. The specific denitrification rates were 0.025-0.13 g NO<sub>3</sub><sup>-</sup>/ (g biomass.d). The  $r_{sp}$  increased linearly as the applied nitrate loading rate increased in the range of 0.8 – 4 kg NO<sub>3</sub><sup>-</sup>/(m<sup>3</sup> bed) (Figure.2).

### Biofilm Estimation

Microbial fixation and biofilm formation on the support surface are two of the most important factors because they affect the levels of elimination of every pollutant (Ramos et al. 2007). To determine the effectiveness of biocatalysts in the reaction system, a biomass estimation method based on the determination of cell protein content in the clinoptilolite was used (Li et al. 2006). In two weeks



**Figure 3.** Relation between COD removal and denitrification rate

of operation, the average biomass concentration in reactor reached  $29.36 \text{ g L}^{-1}$ .

The growth of the biofilm on the clinoptilolite particles in the reactor was visually observed after three days of operation. Estimating the amount of biomass present in the reactor is essential for operational control of the immobilized cell process.

The measurements of MLSS and MLVSS are commonly used as a substitute for expressing the amount of biomass in the biological wastewater treatment of a suspended growth system. However, these methods are unavailable for the immobilized cell system because most biomass is trapped on the clinoptilolite surface.

### Nitrite Formation

When ethanol was used as carbon source, nitrate production was lower or near the proposed maximum targeted contaminant level of  $0.1 \text{ mg NO}_2^- \text{ L}^{-1}$ . Nitrite generation is one of the main problems of direct biological denitrification. Under aerobic conditions, it is energetically more favorable for bacteria to utilize molecular oxygen in the presence of organic electron donors.

Under anoxic conditions, nitrate becomes the most favorable terminal electron acceptor, releasing one nitrite ion for each nitrate ion and resulting in an undesirable release of nitrite (Gee and Kim 2004). Different mechanisms are responsible for nitrite accumulation, including the repression of the nitrite synthesis in the

presence of oxygen or the pH inhibition of enzymatic activity. In the presence of excess organic electron donors, both nitrate and nitrite can be utilized. This results in the production of nitrogen gas that enters the atmosphere and thereby exits the system.

### Utilization Of The Carbon Source

During the operation, ethanol was used as carbon source. Ethanol concentration decreased in the samples taken from the reactor. Figure. 3 shows the simultaneous denitrification rate and COD removal. The slope of the liner regression indicates the stoichiometric coefficient. Mateju et al. (1992) defined the theoretical stoichiometric equations for denitrification with ethanol as carbon source.

This equation estimates that a C/N ratio of 0.71 is necessary for complete nitrate reduction to molecular nitrogen. Our studies suggested a C/N ratio of 1.01 for denitrification with ethanol.

These higher consumptions of the carbon source showed that heterotrophic denitrification processes were not the exclusive processes occurring in the biofilm. Due to the biofilm increase found in all our tests, we believe that assimilatory reduction processes occurred, causing biomass growth in the biological reactor. Constantin and Fick (1997) indicated that bacterial growth could be most favored when ethanol was used as carbon source because ethanol catabolism allows the formation of  $\text{NADH}_2$ , an energy source for the microorganisms.

This could explain the increase in denitrifying bacterial growth observed when ethanol was used as carbon source.

## CONCLUSIONS

The following results were reached in the experiment: 1) high removal efficiencies were achieved for nitrate loads up to 500 g NO<sub>3</sub><sup>-</sup> in the high saline wastewater; 2) nitrate reduction was followed by the accumulation of low nitrite; 3) ethanol is the most suitable carbon source for *Pseudomonas stutzeri* in the packed bed bioreactor with clinoptilolite as a carrier. These results could be expanded in future work on other factors affecting denitrification in a packed bed bioreactor.

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