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Biological Denitrification of Ground Water Using Various Carbon Sources by *Pseudomonas Fluorescens* and *Pseudomonas Stutzeri* in a Hetrotrophic Denitrification Reactor

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Abstract: A pilot plant study has been performed on biological removal of nitrate using heterotrophic denitrification reactor. The system behaviour has been analysed with the usage of – (i) three carbon sources (cotton, wheat straw and wood shavings), (ii) two microbial strains (*Pseudomonas fluorescens* NCIM 5059 and *Pseudomonas stutzeri* NCIM 5136) and (iii) at different temperatures. An optimal operating mode for 90% nitrate removal has been achieved by *Pseudomonas fluorescens* with inlet nitrate concentration of 200 ppm, flow rate 2.5 ml/min, carbon source cotton, temperature 30°C±0.5°C and for *Pseudomonas stutzeri* at same process conditions but with the temperature of 40°C±0.5°C. The feasibility of nitrate removal was also tested with carbon source as wood shavings and wheat straw.

Key words: Denitrification, heterotrophic denitrification reactor (HDR), trickling sand filter (TSF), solid natural carbon source.

Introduction

The contamination of ground water by agricultural practices and meteorological conditions has been rapidly increasing nitrate levels in water. The possible health consequences of nitrate contamination include Methenoglobinemia, blue baby syndrome in infants (Mary et al., 2005) and the possible formation of n-nitroso compounds in the gastric system (Janos et al., 2001). The World Health Organization (WHO) has set a safe limit of 45 mg NO_3^- mg/l to regulate nitrate concentration in drinking waters. Several treatment methods, with different performance levels have been available in the drinking water treatment to reach WHO standards (Mateju et al., 1992; Kapoor and Viraraghavan, 1997) but the most economical process has been the biological denitrification. Denitrification is the dissimilative reduction of nitrate (NO_3^-) to nitrogen gas (N_2), through the production of nitrite (NO_2^-) and gaseous nitric oxide (NO) and nitrous oxide (N_2O) intermediates (Metcalf and Eddy, 2003).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Bacterial denitrification, as a heterotrophic process requires an external source of organic carbon to develop the metabolism of bacterial species. Many different simple carbon compounds have been used to support denitrification processes. These are methanol, ethanol,

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acetic acid, glucose, acetate, aspartate, formic acid and different industrial wastes including molasses, whey, distillery still age, and sulphite waste liquor (Mateju et al., 1992).

Recently, a number of studies have been conducted to evaluate the potential use of solid natural carbon sources in biological denitrification process (Soares et al., 1988; Mateju et al., 1992; Ovez et al., 2006; Ovez, 2006; Oskar et al., 2007; Willie et al., 2007; Mary and Brennan, 2009; Xu et al., 2009; Prashant et al., 2010). The present work deals with a pilot plant study of denitrifying ground water in HDR with different cellulose based carbon sources using *Pseudomonas fluorescens* and *Pseudomonas stutzeri*. The amount of denitrification along with carbon source consumption has been investigated at various temperatures by maintaining constant values of the process parameters like inlet nitrate concentration and flow rate.

Materials and Methods

Microorganism

The lyophilized culture of *Pseudomonas fluorescens* NCIM 5059 and *Pseudomonas stutzeri* NCIM 5136 have been procured from the National Chemical Laboratory, Pune, India. The organism was maintained on nutrient agar slants and sub cultured every 2–3 weeks. The inoculum was prepared from the microbial strains.

Heterotrophic Denitrification Reactor

The heterotrophic denitrification reactor followed by a trickling sand filter, as shown in Figure 1, consisted of cylindrical PVC columns 50 cm high and 10 cm in diameter (Soares, 2000). For first run column was packed with unprocessed 150 gms cotton fibre, previously weighed and inoculated. A thin layer of PVC fine net was placed at each end of the packing (Della Rocca, 2005). Water supplemented with 200 mg/l of nitrate (as KNO_3) and 3 mg/l of phosphate (as K_2HPO_4) was fed to the column. The flow to the HDR was regulated at 2.5 ml/min (unpublished results from preliminary studies) using the peristaltic pump. The temperature of the nitrate rich feed water was regulated between 15°C to 35°C for *Pseudomonas fluorescens* NCIM 5059 and 15°C to 45°C for *Pseudomonas stutzeri* NCIM 5136, using a thermostat. The water samples collected from the HDR were analysed using a digital ion-meter having a nitrate probe (ORION Benchtop ION Meters 720A, Thermo Fisher Scientific, Inc., USA). The above process was repeated with 120 gms fresh shredded wheat straw and 120 gms wood shavings.

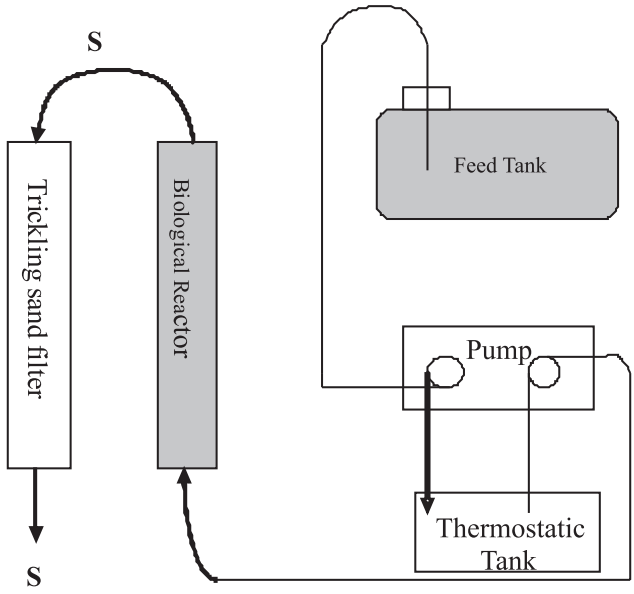

Figure 1: Schematic representation of the experimental setup (S – Sampling port).

Results and Discussion

The system was operated for a period of 90 days with a start-up phase of one week. In the start-up phase the columns packed with the different carbon sources were operated on a recycle for a week so that the micro organism could grow and spread in the entire column. The columns were replenished with inoculums everyday. After one week the columns were operated on a continuous flow basis for a period of 90 days during which the flow rate was maintained at 2.5 ml/min, the inlet nitrate concentration of water was maintained at 200 mg/l and the temperature was varied between 15°C and 45ºC.

The temperature increase from 15ºC to 30ºC showed a progressive increase in nitrate removal for the strain of *Pseudomonas fluorescens*. The maximum nitrate removal of 678.2 mg/day was achieved at 30ºC on the 40th day of operation of the column with cotton as the carbon source. Similarly, for *Pseudomonas stutzeri* it was observed that nitrate removal increased as the temperature was varied from 15ºC to 40ºC and maximum nitrate removal of 597.6 mg/day was observed at 40ºC on the 35th day of operation of the column with cotton as the carbon source.

Denitrification rates in a freshly packed column increased steadily with time and continued up to 40th day of operation. It suggests that colonization of the substrate by bacteria was the rate limiting factor in the removal of nitrate. The distribution of the denitrification

activity in the column varied with time at all the temperatures for all the carbon sources and for both the strains of *Pseudomonas* as shown in Figures 2 to 7. For a period of 70 days nitrate removal was evenly distributed with a maximum at 35 to 40 day but after 70 days time the amount of nitrate removal decreased drastically suggesting channelling in the carbon source bed; this was confirmed by visual observations. By 78th day, the carbon source bed was pushed upwards, the lower part of the column was occupied by water only, and removal of nitrate took place in a narrow section of the column. At this stage, the amount of nitrate removal did not show any variation. After observing for a few more days the operation of HDR was stopped at 90 days.

The maximum removal of the nitrate was observed for cotton as the carbon source which is in accordance

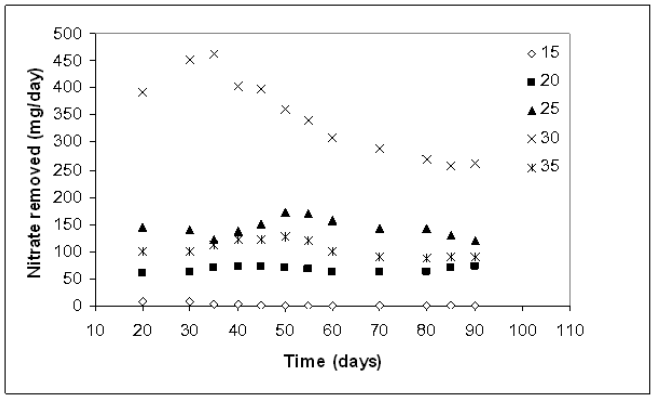

Figure 4: Amount of nitrate removed at different temperatures in °C by *Pseudomonas fluorescens* NCIM 5059 using wood shavings as the carbon source at a flow rate of 2.5 ml/min and inlet nitrate concentration of 200 mg/l.

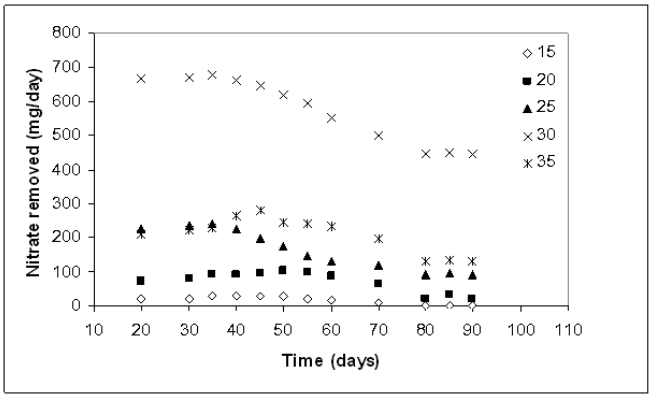

Figure 2: Amount of nitrate removed at different temperatures in °C by *Pseudomonas fluorescens* NCIM 5059 using cotton as the carbon source at a flow rate of 2.5 ml/min and inlet nitrate concentration of 200 mg/l.

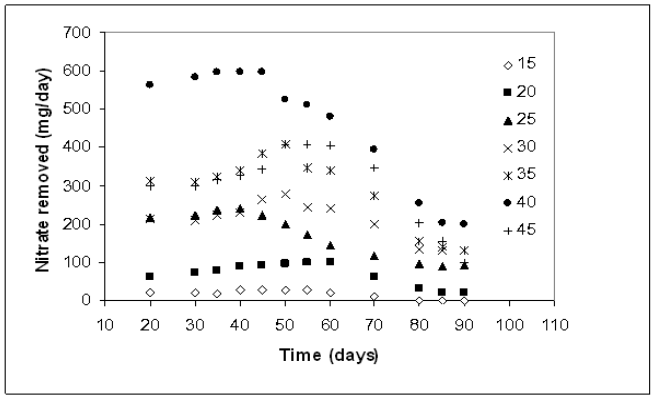

Figure 5: Amount of nitrate removed at different temperatures in °C by *Pseudomonas stutzeri* NCIM 5136 using cotton as the carbon source at a flow rate of 2.5 ml/min and inlet nitrate concentration of 200 mg/l.

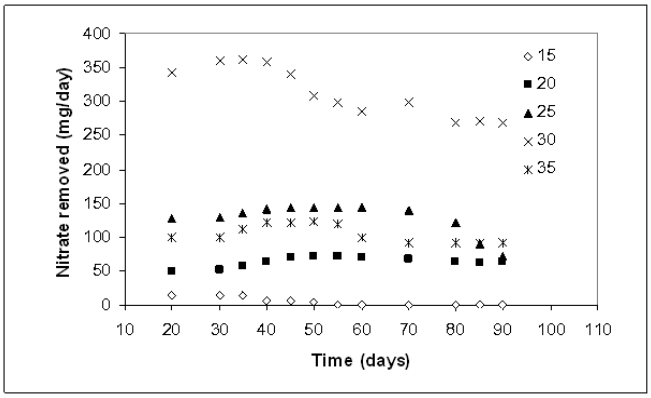

Figure 3: Amount of nitrate removed at different temperatures in °C by *Pseudomonas fluorescens* NCIM 5059 using wheat straw as the carbon source at a flow rate of 2.5 ml/min and inlet nitrate concentration of 200 mg/l.

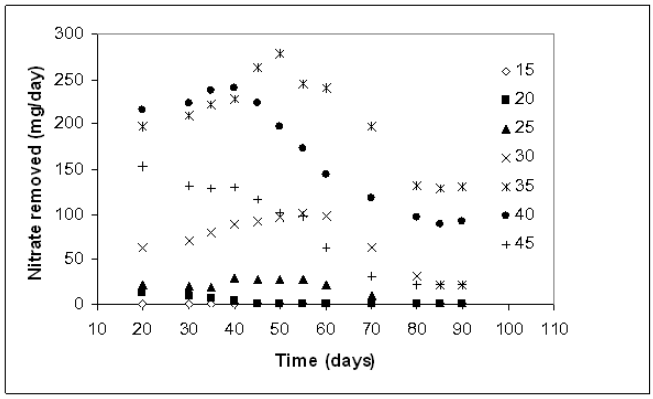

Figure 6: Amount of nitrate removed at different temperatures in °C by *Pseudomonas stutzeri* NCIM 5136 using wheat straw as the carbon source at a flow rate of 2.5 ml/min and inlet nitrate concentration of 200 mg/l.

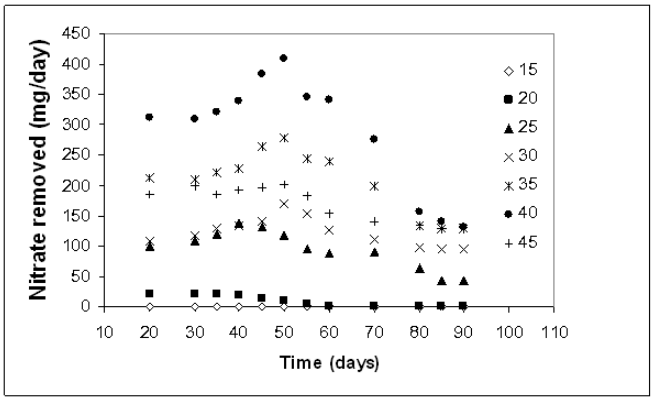

Figure 7: Amount of nitrate removed at different temperatures in °C by *Pseudomonas stutzeri* NCIM 5136 using wood shavings as the carbon source at a flow rate of 2.5 ml/min and inlet nitrate concentration of 200 mg/l.

Table 1: Composition of all the natural cellulosic substrates

Content	*Cotton (Gossypium hirsutum)*	*Wheat straw*	*Wood shavings (Tectona grandis - Teak)*
Cellulose (%)	90	41.6	49
Hemicellulose (%)	6	31.3	20
Lignin (%)	-	20.5	30
Ash (%)	1.8	3.7	-
Silica and silicates (%)	-	2	-
Others (%)	2.2	2.9	1

with the cellulose content of cotton as compared to the other carbon sources (Table 1). The denitrification is dependent on cellulose content which is the highest in cotton fibres as compared to wheat straw and wood shavings.

Table 2 shows the maximum amount of nitrate removed per day and the amount of carbon source consumed for a period of operation of 90 days. For the entire operation C:N ratio varied from 1.6 to 3.0. By the end of the experiment, marked changes were visible in the composition of the carbon source bed. The bulk of the remaining material was found to be in advance state of degradation.

Conclusion

The microbial process described in this study for the removal of nitrate from contaminated ground water uses cotton, wheat straw and wood shavings as the substrate for denitrifying microorganisms. Cotton is the most effective substrate for the removal of nitrate from water as compared to wheat straw and wood shavings on account of its high cellulose content. The post treatment in the form of sand filter and UV radiation would however be still required to reach drinking water standards for removing both the strains of microorganisms.

The results demonstrate that all three chosen carbon sources, cotton, wheat straw and wood shavings, can effectively support denitrification of nitrate contaminated water. Although cotton is the most effective supporter of denitrification, however, wheat straw and wood shavings are more economical options for the commercial applications. The treated water is characterized by no detectable flavour, odour and colour. The washout of bacteria was relatively high, requiring further disinfection of the treated water.

Temperature has a marked effect on the cellulose degradation denitrification process. The diminished capacity at lower temperatures should be taken into account when planning commercial application of the process. To compensate for lower rates of denitrification at lower temperatures, longer residence time would be required in the reactor which can be accomplished by increasing the length of the reactor or by decreasing the flowrate.

Table 2: Summary of experimental results for different carbon sources and microbial strains of *Pseudomonas* used for the process of denitrification in a HDR

Carbon source/ Microbial strain	*Initial mass of carbon source (gms)*		*Final mass of carbon source (gms)*		*Maximum nitrate removed in 90 days (mg/day)*		*C/N ratio (mg/mg)*	
	Pseudomonas fluorescens NCIM 5059	*Pseudomonas stutzeri NCIM 5136*	*Pseudomonas fluorescens NCIM 5059*	*Pseudomonas stutzeri NCIM 5136*	*Pseudomonas fluorescens NCIM 5059*	*Pseudomonas stutzeri NCIM 5136*	*Pseudomonas fluorescens NCIM 5059*	*Pseudomonas stutzeri NCIM 5136*
Cotton	150	150	110	114	678	597.6	2.9	3
Wood Shavings	120	120	100	102.6	462	410	2.1	2.1
Wheat Straw	120	120	108	109.2	363	240.3	1.6	2.2

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