Chapter – 2

MATERIALS & METHODS
2. MATERIAL AND METHODS

2.1 Efficiency of denitrification in denitrifying reactor of GNFC (DNR)

Samples were drawn from different parts of DNR, and 0.5ml of each sample was used as an inoculum. The nitrate containing effluent entering, DNR-I was sterilized and supplemented with 0.05% yeast extract and 0.08% fusel oil and used as a medium. Yeast extract provided the essential nutrients for the growth of microorganisms. Tubs were prepared in duplicates. One set was incubated aerobically and the second anaerobically. Sterile paraffin oil was layered on the medium to create anaerobic conditions. NO$_3^-$, NO$_2^-$, and NH$_4^+$ were monitored qualitatively after every 24h incubation time.

**Qualitative analysis**

(A) NO$_3^-$ estimation : 0.5% Naphathylamine and 0.8% Sulphanilic acid + Zinc
(B) NO$_2^-$ estimation : 0.5% Naphthylamine and 0.8% Sulphanilic acid
(C) NH$_4^+$ estimation : Nessler’s Reagent

2.2 Enumeration of denitrifiers from DNR samples

Samples were withdrawn from DNR-I and DNR-II after interval of every two days for a month. NO$_3^-$, NO$_2^-$, and NH$_4^+$ were monitored qualitatively. Enumeration studies were carried out using Most Probable Number (MPN) and Standard Plate Count (SPC) technique described by (Gamble, et al., 1977).

2.3 Composition of nitrate containing effluent of GNFC

After chemical treatment for phosphate removal, the effluent of the fertilizer industry had following composition (mg l$^{-1}$): NO$_3^-$-N (1000-1200), NO$_2^-$-N (60-120), NH$_4^+$-N (40-60), Ca$^{2+}$ (800-1000), Al$^{3+}$ (<4), COD (<40-60), SO$_4^{2-}$ (500-700) and PO$_4^{3-}$ (<2), which was used as influent into the reactor for the optimization of carbon requirement. The COD of the influent was adjusted to the desired level by fusel oil addition which was a mixture of methanol (42-45%), butanol (<1%) and propane-2-ol (<0.4%).
2.4. Analytical methods
Nitrate was determined by method of Tenkins & Medsker (1964) and ammonia release estimated by the method of Fawcett & Scott (1960). COD (5220-C), nitrite (4500-NO₂⁻-B) and biomass (2540-G) were estimated as per the standard methods (APHA, 1995). Gases evolved during denitrification were analyzed using a molecular sieve column (5A) for N₂ and Unibed C column for CO₂, each 3m by 4mm, connected to thermal conductivity detector (80mA).

2.5. Isolation of denitrifiers
2.5.1. Isolation of denitrifiers from DNR
Samples were taken from different parts of DNR-I and DNR-II. Samples were diluted to 10⁻², 10⁻³ and 10⁻⁴ which were spread on nitrate agar and Molecular Hydrogen Nitrate (MHN) plates which were prepared in duplicates. One set was incubated aerobically and the other anaerobically at 30°C for 48h (Gamble et al., 1977). Colonies of bacteria showing different morphological features were isolated on nitrate agar plates. They were further inoculated separately on to nitrate broth having Durham’s vial. The tubes were layered with sterile paraffin oil and incubated at 30°C for 48h. NO₃⁻, NO₂⁻, and NH₄⁺ were monitored qualitatively and gas formation in Durham’s vial was also checked.

2.5.2. Isolation of denitrifiers from cow dung
10g of cow dung was suspended in 250ml distilled water. The slurry was filtered and centrifuged to get a clear suspension. Dilutions 10⁻², 10⁻³ and 10⁻⁴ of cow dung were plated by spreading them on MHN and nitrate agar plates, which were prepared in duplicates. One set was incubated aerobically and the other anaerobically at 30°C for 48h (Gamble et al., 1977). Colonies of bacteria showing different morphological features were picked, isolated on fresh nitrate agar plates. They were further inoculated separately on to nitrate broth having durhams vials. The tubes were layered with sterile paraffin oil and incubated at °C for 48h. NO₃⁻, NO₂⁻, and NH₄⁺ were monitored qualitatively and gas formation in Durham’s vial was also checked.
2.5.2.1. Composition of Molecular Hydrogen Nitrate Medium (MHN) (Van Vessereld, 1992)

Solution 1 (g l⁻¹):
Na₂HPO₄, 9; KH₂PO₄, 1.5; NH₄Cl, 1.0; MgSO₄, 0.2; KNO₃, 1.0 and Trace element solution 2.0 ml

Trace element solution (mg l⁻¹):
ZnSO₄, 100; MnSO₄, 30; H₃BO₃, 300; CaCl₂, 200; NiCl₂, 20 and Na₂MoO₄, 30.

Solution II (g 100ml⁻¹): NaHCO₃, 5.0

Solution III (mg 100ml⁻¹): CaCl₂, 100; Ferrous Ammonium citrate, 50.

2.5.2.2. Composition of Nitrate agar medium

Peptone Nitrate: Peptone: 5g. Beef extract: 3g. Potassium nitrate: 3g, pH 7.0, Distilled water: 1000 ml.

2.5.3. Isolation of denitrifiers by column enrichment technique

Two types of enrichment columns having different nutrient media were set up.

2.5.3.1. Static column

100ml measuring cylinders were used for static column (Fig. 3.5). 90ml of three media (a, b & c) were used in three separate columns. 10ml DNR sample was used as the source of microorganisms. Columns were kept at ambient temperature for one week. Aliquots drawn after a week from each column were plated on nitrate agar medium (Alexander, 1978).

(a) 1% (w/v) Tartarate and 1% (w/v) sodium succinate were added in to basal medium as source of carbon.

(b) 1% (w/v) glucose was added to basal medium.

(c) 1% (v/v) alcohol and 1% (w/v) meat extract were added to basal medium.

2.5.3.1.1. Composition of Basal Medium (g l⁻¹)

MgSO₄·7H₂O, 0.2; K₂HPO₄, 1.0; FeSO₄, 0.05; CaCl₂, 0.02; MnCl₂·4H₂O, 0.002; NaMoO₄·2H₂O, 0.001; pH 7.0
2.53.2. Percolation column

A glass column of 64 cm × 18 cm dimensions was packed with small pebbles and thermocol pieces up to 48 cm from bottom (Fig. 3.4). The column was equipped with an outlet part at the bottom. One liter of sterile medium was circulated through the column with the help of peristaltic pump. Different nutrient media used were run twice through column. First run was with basal mineral medium (Van Vessereid, 1992) second with sterile DNR inlet (supplemented with 0.05% yeast extract). Both the media were supplemented with 0.08% fusel oil as carbon source. 10g rhizosphere soil suspended in one liter of respective medium was used as the source of microorganisms. In a six day run at ambient temperature, aliquots were drawn at 48 hr interval and checked for NO$_3^-$, NO$_2^-$ and NH$_4^+$ productions qualitatively. After 72 h aliquots drawn from the bottom and pellicle from top were plated on nitrate agar medium. Plates were incubated at 30°C for 48 h.

2.6. Confirmation of denitrifiers

PNB tubes with durham vials were inoculated from the same isolates obtained from various sources. Counts of nitrite accumulators were based on those tubes in which nitrite were detected. Counts of anaerobes were based on tubes in which visible growth of production of nitrite occurred. Counts of denitrifiers were based on tubes in which no NO$_3^-$ or NO$_2^-$ was detected. Quantitative NO$_3^-$ and NO$_2^-$ were checked with PNB and nitrate containing effluent under aerobic and anaerobic conditions. After 72h of incubation in the nitrate containing tubes, colonies that had produced gas were presumed to be denitrifiers. A scheme used for confirmation of denitrifiers by using different medium and growth conditions illustrated in Fig. 2.1. To confirm this, we streaked gas producers on nitrate agar to obtain isolated colonies. We re-inoculated purified isolates into nitrate tubes containing modified Durham tubes (large lengths of 6 to 7 cm glass tubing 5 to 7 mm ID), with silicon rubber stopper inserted in one end. The stopper allowed us to sample the gases collected in the tubes (Fig. 3.7). Any isolates producing N$_2$ or N$_2$O after additional incubation was classified as denitrifiers. The final confirmation of denitrifiers should be done by identifying the gas as N$_2$ by GC analysis.
2.7. Effect of temperature on consortium of *Pseudomonas stutzeri* and *Comamonas testosteroni*.

50 ml of sterile nitrate containing effluent supplemented with 3% (v/v) of fusel oil and 0.02% (w/v) yeast extract was used as a medium. pH was adjusted to 7.0 in each of the flasks. 3% (v/v) consortium of *Pseudomonas stutzeri* and *Comamonas testosteroni* was inoculated to the medium. The flasks were incubated at 20°C, 25°C, 30°C and 37°C under static condition.

Initially before inoculation the NO$_3^-$-N, NO$_2^-$-N and NH$_4^+$-N concentration was checked quantitatively from the effluent to find out their initial concentrations.

Samples were withdrawn from each of the flasks at the end of 24h, 48h, 60h and 72 h and monitored quantitatively for NO$_3^-$-N, NO$_2^-$-N and NH$_4^+$-N and graphs were plotted to find out the optimum temperature supporting denitrification.

The mean of three reading was taken for all the flask level and bioreactor studies.
Isolates obtained from various sources were inoculated in PNB and nitrate containing effluent tubes; incubated for 72h

(Non-Gas producers)
Broth of non-gas producers were qualitatively tested for $\text{NO}_3^-$, $\text{NO}_2^-$ and $\text{NH}_4^+$

(Gas producers)
$\text{NO}_3^-$, $\text{NO}_2^-$ and $\text{NH}_4^+$ were checked qualitatively. Gas producers streaked for purity on nitrate agar plate

Stocks made of purified gas producers

Colonies picked and transferred to durham nitrate containing effluent tubes; incubated for 48h under aerobic and anaerobic conditions

(Non-Gas producers)
Broth of non-gas producers were qualitatively tested for $\text{NO}_3^-$, $\text{NO}_2^-$ and $\text{NH}_4^+$

(Gas producers)
$\text{NO}_3^-$, $\text{NO}_2^-$ and $\text{NH}_4^+$ were checked qualitatively. Gas producers streaked for purity on nitrate agar plate and

Stocks made of purified gas producers

Isolates which produced gas within 48h were picked and transferred to nitrate agar plate and further confirmed by Gas Chromatography analysis.

Fig. 2.1. Scheme used for confirmation of denitrifiers.
2.8. **Effect of different carbon sources on denitrification efficiency**

The objective of this experiment was to determine the denitrification efficiency with acetate, butanol, butyrate, glucose, methanol, propionate and sucrose. Nitrate containing effluent of GNFC was used to check effect of various carbon compounds. The treatments consisted of 200mg C kg\(^{-1}\) as acetate, butanol, glucose, methanol, propionate and sucrose, as well as controls unamended with C. Acetate, propionate, and butyrate were prepared by neutralizing the respective acids to pH 7 with KOH. Samples were withdrawn from each of the flasks at the end of 24h, 48h, 60h and 72 h and monitored quantitatively for NO\(_3^\)\(-\)N, NO\(_2^\)\(-\)N and NH\(_4^+\)-N.

2.9. **Effect of hydrogen ion concentration (pH) on denitrification efficiency of consortium.**

50 ml of sterile nitrate containing effluent supplemented with 3% (v/v) of fusel oil and 0.02% (w/v) of yeast extract was used as a medium. The pH of the respective flasks was adjusted to 6.5, 7 and 7.5. Initially before inoculation the NO\(_3^\)\(-\)N, NO\(_2^\)\(-\)N and NH\(_4^+\)-N concentration of the effluent was checked quantitatively to find out their initial concentrations. 3% (v/v) of actively grown consortium was inoculated on to each of the flasks. The flasks were incubated at 30°C under static condition.

Samples were withdrawn from each of the flasks after every 24 h, 48h, 60h and 72h and monitored quantitatively for NO\(_3^\)\(-\)N, NO\(_2^\)\(-\)N and NH\(_4^+\)-N.

2.10. **Influence of dissolved oxygen on denitrification**

50 ml of sterile nitrate containing effluent supplemented with 3% (v/v) of fusel oil and 0.02% (w/v) yeast extract was used as a medium. pH of the medium was adjusted to 7.0. 3% (v/v) of consortium was inoculated on to a flask and it was incubated on a rotary shaker (80 rpm, 30°C). At the end of 24 h, 48 h, 60 h and 72 h samples were withdrawn and monitored for NO\(_3^\)\(-\)N, NO\(_2^\)\(-\)N and NH\(_4^+\)-N quantitatively and a graph was plotted to see the effect of dissolved oxygen on the denitrifying ability. Controls included an inoculated flask kept under static condition 30°C and an un-inoculated flask kept under static condition at 30°C.
2.11. Yeast extract requirement for removal of nitrate from wastewater

Different yeast extract concentrations (0.02%, 0.03% and 0.05%w/v) were used to check complete removal of nitrate in GNFC effluent. Samples were withdrawn from each of the flasks after every 24 h, 48h, 60h and 72h and monitored quantitatively for NO$_3^-$-N, NO$_2^-$-N and NH$_4^+$-N.

2.12. Inoculum optimization in batch experiment

Cultures of both *Pseudomonas stutzeri* and *Comamonas testosteroni* were grown at 37°C in PNB. A mixture (1:1) of both cells of 1.0 O.D. (600 nm) was used to checked denitrification efficiency. Nitrate removal efficiency was checked with various concentrations (2%, 3% and 4%) of consortium.

2.13. 4 and 50 l Bioreactor studies.

The 4-L [20cm x 30cm (ID)] and 50-L (25cm x 12cm x 25cm) bioreactors were designed as shown in the Fig. 4.1 & 4.6 respectively. The influent was fed at the different flow rates mentioned in the text. One peristaltic pump with two connectors was used to control the inlet and outlet flow rates to maintain the constant volume of influent in the bioreactor. The influent into the reactor was agitated with an overhead agitator at 45 rpm. Fusel oil was continuously added at a flow rate to maintain the COD within 100mg l$^{-1}$. The temperature was controlled at 28°C ± 2 and pH at 7.0 ± 0.2. The effluent coming out from the reactor was used to monitor levels of NH$_4^+$, NO$_3^-$, NO$_2^-$, COD and biomass.
2.13.1. Calculation for specific denitrifying activity and denitrification efficiency (%)

Specific denitrifying activity and denitrification efficiency were calculated as described by Bernet et al., (1992), \([\text{in}]\) represents influent substrate concentration (g/l), \([\text{out}]\) is the outlet substrate concentration (g/l), \(R\) the influent wastewater flow rate (g/l), \(V\) the reactor volume (l) and \(\text{NOx-N} = \text{N-NO}_2^- + \text{N-NO}_3^-\) (g/l), \([\text{VSS}]\) is the volatile suspended solids (g/l).

\[
\text{Volumetric denitrification rate} = \frac{\{[\text{N-NO}_3^-\text{in}] - [\text{N-NOx}]_{\text{out}}\} \times R}{V}
\]

\[
\text{Specific denitrifying activity} = \frac{\text{Volumetric rate}}{\left(\text{g N-NO}_3^- \text{ gVSS}^{-1} \text{d}^{-1}\right)}
\]

\[
\text{Denitrification efficiency (\%)} = \frac{[\text{N-NO}_3^-\text{in}] - [\text{N-NOx}]_{\text{out}}}{[\text{N-NO}_3^-\text{in}]} \times 100
\]


Complete mix no sludge recycle rectangular type of pilot scale denitrifying reactor was used to check denitrification efficiency at 1.5m³ level. Dimensions of bioreactor at base : 80 × 33 × 52inches and at top : 99 × 33 × 52inches. To start with the experiment the reactor (Figs. 5a.2a, 5a.2b) was filled with the nitrate containing effluent along with seed culture (5% v/v consortium of \textit{Pseudomonas stutzeri} & \textit{Comamonas testosteroni} 1:1) and agitated. A number of operational problems arise during the study, but some of the parameters were already optimized during bench scale processes. A major problem was to maintain dissolved oxygen concentration in the range of 0.2-0.6 mg l⁻¹ though out the process. Therefore the speed and size of agitator was set in such a manner that did not allow increasing DO concentration beyond certain level in the reactor. Dissolved oxygen
concentration is very important because most of the nitrate removal occurs under anoxic environments. The system was first operated for a week or 15 days to establish steady state conditions with specific COD:NO₃⁻-N ratio and flow rates. The actual size of the reactor was 1.7m³ a partition of 0.2m³ was made for sludge stabilization; therefore the working volume of the reactor was 1.5m³.

2.15. Cell immobilization procedure

The bacterial isolate used for this experiment was *Pseudomonas stutzeri* which was isolated from denitrifying reactor of Gujarat Narmada Valley Fertilizer Company. Vegetative batch culture of *Pseudomonas stutzeri* was grown aerobically in 250ml Erlenmeyer flasks containing 100ml of peptone nitrate broth; peptone, 5g l⁻¹; beef extract, 3g l⁻¹; potassium nitrate, 1g l⁻¹, incubated on shaker for 24h at 37°C ± 2. Cells following growth were collected by centrifugation, washed twice with saline and 5%(v/v) cell suspension (0.55g l⁻¹ dry weight of *Psedomonas stutzeri* and 0.34g l⁻¹ dry weight of *Comamonas testosteroni*) added in to a solution of 3%(w/v) sodium alginate. The mixture was extruded dropwise via syringe (16mm) in to 0.1M CaCl₂ solution. When the entire mixture has been entrapped, beads and CaCl₂ mixture was placed in the refrigerator for 1h for hardening and beads were transferred to nitrate containing effluent, incubated for overnight (Chibata & Wingard, 1983). Finally beads were washed with effluent and used for nitrate removal in batch and continuous bioreactor studies.

2.16. Immobilized and free cells denitrifying activity

Nitrate removal by immobilized cells in batch cultures was carried out with different concentration of nitrate (600 to 950 mg l⁻¹), and COD:NO₃-N ratios. Free cells suspension and immobilized beads were inoculated in 250ml Erlenmeyer flasks containing 100ml nitrate containing effluent, flasks were incubated at 37°C ± 2 under static condition. 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 12h.
2.17. Beads stability

Once the entire nitrate got reduced from the effluent containing alginate beads, the same beads were then washed with nitrate containing effluent and saline than reinoculated in fresh 100ml effluent. The positive control containing free cells and negative control were also kept to confirm nitrate removal efficiency of immobilized cells. After each transfer of the beads, the leaching of the cells from beads checked by plating on peptone nitrate agar plate.

2.18. Continuous operation of Bioreactor

The bioreactor used for testing the reduction of nitrate consisted [60cm × 6cm (ID)] of a glass material which was packed with immobilized beads (up to 45cm) 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 beads. The range of nitrate load from 0.5-1.5gNO₃-N l⁻¹d⁻¹ was applied during the experiment depending upon retention time. The column was initially filled with 0.350 l effluent and after complete removal of nitrate; continuous process was started with different flow rates. Nitrate removal rate in the bioreactor under continuous process was checked with different COD:NO₃-N ratios (2.45 and 1.45) and retention times.