# Microbial Degradation of Nitrate: Put Microbes to Work

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## ABSTRACT

Three nitrate degrading bacteria, namely S1, S2 and S3 strains, were isolated from soil samples collected from agricultural sites at Polonnaruwa, Oruwala and Gampaha, Sri Lanka respectively. Among the isolated strains, S1 showed a maximum nitrate removal rate of 4.20±0.08 mg/L/day whereas S2 and S3 showed nitrate removal rates of 3.45±0.57 mg/L/day and 3.72±0.19 mg/L/day, respectively. The nitrate removal abilities of all three strains were measured at different temperatures (25°C, 30°C, 32°C), different pH (7.0, 7.5, 8.0) and for different nitrate concentrations (15, 30, 45 mg/L) in vitro. The maximum nitrate degrading rate by the bacterium S1 (4.52±0.01 mg/L/day), S2 (4.25±0.47 mg/L/day) and S3 (4.04±0.09 mg/L/day) were detected at 32°C whereas the highest degradation rate for S1 (4.6±0.05 mg/L/day), S2 (4.38±0.03 mg/L/day) and S3 (4.14±0.25 mg/L/day) bacteria strains was reported when the pH was 8.0. The maximum nitrate degradation was found to be 5.88±0.17, 5.67±0.45 and 5.71±0.43 mg/L/day for S1, S2 and S3 bacteria strains when 45 mg/L of nitrate was present in the medium. A biochemical test tentatively identified the isolates S1, S2 and S3 as *Pseudomonas* sp., *Bacillus* sp. and *Proteus* sp. The bio sand filter developed in co-operating all three bacterial strains showed 6.12±0.06 mg/L/day of nitrate degradation rate within 24 hours.

## 1. INTRODUCTION

Groundwater and surface water are widely used as drinking water sources in most countries of the world (Mahagamage et al., 2015; Idroos et al., 2017). However, many studies have recorded a remarkable increase of nitrates in groundwater aquifers and surface water bodies (Wang et al., 2009; Aopreeya et al., 2013). Nitrate, being a highly mobile and readily water-soluble ionic chemical species, is naturally available in water resources. Nitrate pollution is caused by the intensive use of nitrogen fertilizers in agriculture, industrial effluents, domestic wastewater and sewage (Mohammadi et al., 2011).Hence, nitrate contamination is a global problem and stands as second most dangerous pollutant after pesticides (Wakida and Lerner, 2006). Presence of high nitrate concentration in water leads to serious ecological and social issues such as eutrophication (Hettiarachchi et al., 2014), deterioration of water quality and potential hazard to human health (Cambra et al., 2010). High concentrations of nitrate in drinking water is a threat, especially to infants, inducing the formation of methemoglobinemia in blood, also called "blue baby syndrome" (Gao et al., 2004). The carcinogenic effect of nitrate is also reported due to consumption of nitrate-contaminated water (Wakida and Lerner, 2006). The presence of over 100 µg/mL of nitrate in drinking water has resulted in stomach cancers, especially in infants (Gao et al., 2004). Hence, the Environment Protection Agency (EPA) has demarcated the maximum permissible level of nitrate in drinking water to be 45 µg/mL for nitrate and a similar guideline of 50 µg/mL as nitrate has been set by the WHO and the European Community (EC) (Gao et al., 2004) and Sri Lanka Standard Institute (SLSI) as well (SLSI, 2013). Sri Lanka, being an agricultural country, uses large amounts of fertilizer, which contains nitrogen sources, which are transformed to nitrate and other nitrogen species under environmental conditions (Mahagamage et al., 2015). These species flow down-stream in rivers/streams, and a fraction is mixed with groundwater. Excess levels of nitrogen and phosphorus in water resources cause algae to grow faster than ecosystems can safely handle (Idroos et al., 2014). Significant increases in algae deteriorates the quality of water, decreasing oxygen levels

required by fish and other aquatic life for their survival, which leads to health problems in fish and eventual death of fish (Idroos et al., 2014). An alarming fact is that some algal blooms are harmful to humans because they produce toxins and bacterial growth that could severely affect humans if they come into contact with polluted water, consume tainted fish or shellfish, or drink contaminated water.

Physical and chemical methods such as ion exchange, reverse osmosis, nanofiltration and electrodialysis, have shown poor selectivity for nitrate removal in water (Choi et al., 2009; Bonse et al., 1994; Wenske, 1997). In addition, the utility of these processes have been limited due to their expensive operational and subsequent disposal problem of the generated nitrate waste (Rezaei et al., 2009). Several conventional technologies adopted for nitrate removal are ion exchange resins, electrodialysis, reverse osmosis and distillation which substantially increase the cost of operation and are inaccessible for most part of the world due to high operation cost (Shrimali and Singh, 2001). Therefore, the cost-effective alternative lies in the biological denitrification process (Eckford and Fedorak, 2002).

The study of biological nitrate removal from drinking water supply first began operation in 1981, at the Chateau-Landon in France (Mohseni et al., 2013) and the study was based on the natural nitrogen cycle. Nitrogen cycling involves four key microbiological processes: nitrogen fixation, mineralization (decay), nitrification and denitrification. Microorganisms play very important roles in the nitrogen cycles and denitrifying fungi, anammox bacteria, nitrifying archaea aerobic denitrifying bacteria and heterotrophic nitrifying microorganisms are key players in the nitrogen cycle (Cabello et al., 2004). For this reason, denitrification is considered to be a process that removes bioavailable nitrogen forms from the Earths' surface. Moreover, excess nitrate or nitrite in soil or water ecosystems leads to environmental and health problems (Wick et al., 2012). This excess nitrate in water or soil can be converted to its acid forms, which consequently reduce soil and water quality (Gomez et al., 2002). In addition to ecological problems, excess nitrate stimulates denitrification, resulting in the loss of bioavailable nitrogen used by plants in the form of the gaseous nitrogen compounds such as $N_2O$ and $N_2$ (Ergas and Reuss,

2001). Despite these detrimental effects, denitrification is beneficial in sewage treatment because it converts nitrate to its gaseous forms, thus reducing the amount of available nitrogen in the sewage (Gomez et al., 2002).

Denitrifying bacteria have been isolated from diverse environments (agricultural soils, deep sea sediments, wastewater treatment plants) and belong to diverse bacterial genera (Zumft, 1997). *Pseudomonas* species are generally presumed to be the predominant microorganisms through which denitrification is achieved (Janda et al., 1998). However, other studies have shown that various species, of bacteria; *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium* and *Hyphomicrobium*, are responsible for denitrification (Lim et al., 2005). Thus, under anoxic conditions, heterotraphic soil bacteria are capable of reducing nitrogenous oxides by oxidizing carbonaceous substrates (Gomez et al., 2002). Autotrophic denitrification based on an inorganic carbon source involves sulfur or hydrogen gas as the electron donor (Wang et al., 2009). Influence of microbial composition and denitrifying microbiota in natural biofilms are also recorded as significant factors that affect this technology in treating nitrate-contaminated water (Mousavi et al., 2011). Therefore, many full scale heterotrophic and autotrophic biological nitrate removal processes are currently under operation (Mohseni et al., 2013). Hence, the present study was carried out to isolate nitrate degrading bacteria from agricultural sites and to develop a laboratory scale sand filter using efficient nitrate degrading bacteria to treat nitrate-contaminated water.

## 2. METHODOLOGY
### 2.1 Chemicals
Chemicals used for microbiological and nitrate analysis, were purchased from Hardy diagnostics while Molecular grade chemicals were purchased from Promega, USA and Thermo Fisher Scientific, USA.

### 2.2 Sampling
Soil samples were collected from Paddy fields and sewage contaminated farm sites of Polonnaruwa, Oruwala and Gampaha areas to isolate nitrate degrading bacteria. A core sampler was used to obtain soil samples and freshly collected samples were aseptically transferred into sterile clean black

polyethylene bags. Collected samples were transported to laboratory under chilled conditions and stored at 4ºC until use for bacterial isolation.

## 2.3 Data analysis

An aliquot of 5.0 g of soil from each collected sample was transferred into 100 mL Erlenmeyer flasks (200 mL) and nitrate solution (Potassium nitrate) was added at a final concentration of 15 mg/L. Flasks were maintained at 28ºC at 100 rpm in a shaking incubator for 8 days. Following 8 days of incubation, 1 mL of sample aliquots were removed followed by tenfold serial dilution and a pour plate method was employed to isolate bacteria colonies with different morphological features (Manage et al., 2009). LB agar media (Tryptone 9.3 g, NaCl 4.5 g, Yeast extract powder 4.5 g, Bacteriological agar 13.1 g/L) was used as the culture medium. Following three days of incubation, bacterial colonies with different colony color, shape, elevation and margin were picked up and re-suspended in liquid LB medium. The liquid cultures were incubated at 28ºC for 3 days at 50 rpm and they were used to prepare pure bacterial cultures by repeated streaking on LB agar slants. Subsequently, pure bacterial cultures were subcultured and stored in LB-glycerol at -80ºC.

## 2.4 Screening of nitrate degrading bacteria through degradation studies

Five bacterial strains, namely S1, S2, S3, S4, and S5, with different morphological characters were selected for nitrate degradation studies. Overnight grown bacterial cultures were subjected to repeated washing with 0.01 M PBS at least three times and kept overnight for carbon depletion. Subsequently, the turbidity of carbon depleted bacteria culture was equalized at $A_{590nm}=0.35$. This bacteria culture was inoculated into filter-sterile aged tap water with nitrate at a final concentration of 15 mg/L (Manage et al., 2009). Flasks were incubated at 28ºC with shaking at 100 rpm for 8 days and 10 mL of subsample was removed from each flask under sterile conditions at two days intervals and nitrate concentration was measured using the APHA 1996 standard method. The following equation was employed to calculate nitrate degradation rate (h):

$$h \ (mg/L/day) = -\ln\left[\frac{C/C_0}{t}\right]$$

where $C_0$ (mg/L) and C (mg/L is the nitrate concentrations at the beginning and at the end of the time interval t (days), respectively.

The nitrate removal efficiency percentage (E) of bacterial strains was calculated using the following equation.

$$E = [(C_0-C)/C_0] \times 100$$

where $C_0$ (mg/L) and C (mg/L) is the nitrate concentrations at the beginning and at the end of the time interval respectively.

## 2.5 Optimization of nitrate degradation by selected bacteria

Five bacterial strains were selected as potential candidates for nitrate degradation study by the preliminary screening test. Among the five bacterial strains, three strains were isolated which showed a pronounced degradation of nitrates; S1 from the Polonnaruwa sampling site, S2 from Oruwala, and S3 from the Gampaha sampling site. Thus, S1, S2 and, S3 were selected for further studies. The nitrate removal abilities of all three strains were optimized for different incubating temperatures (25ºC, 30ºC, 32ºC), pH (7.0, 7.5, 8.0) and for different concentrations of nitrate (15, 30, 45 mg/L). The optimization studies were carried out according to the method described in 2.4.

## 2.6 Identification and characterization of nitrate degrading bacteria

As an initial identification, the gram staining was carried out according to the method described by Della Valle et al. (1999). Following the gram staining, biochemical tests (Citrate test, starch hydrolysis, catalase test, Urease test, Methyl red test, Vogus Proskauer test, and Gelatin test) were carried out as described in the Cowans and Steels' manual for tentative identification (Cowan and Steel, 2004).

## 2.7 Development of a nitrate removing sand filter

The experimental setup of the sand filter consisted of a sand filter column and an output column each with a diameter of 50 mm and a height of 210 cm (Figure 1). Sterilized coral particles were employed as the substrate for the attachment of bacteria.

The overnight grown bacterial culture of all three bacterial strains (S1, S2 and, S3) were starved

in 0.01 M of PBS and each suspension was equalized at $A_{590nm}$=0.35. Ten mL of bacterial inoculum from each suspension was introduced to the substrate in order to form a biofilm.
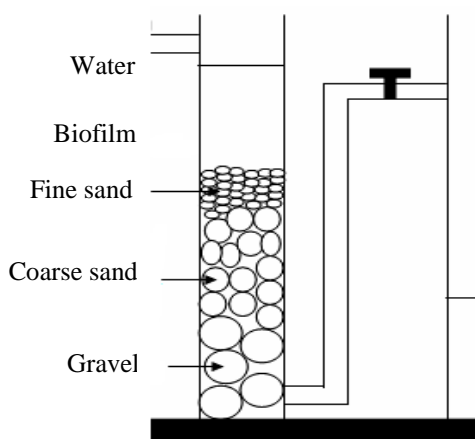


**Figure 1.** Structure of the sand filter

The filter bed of the sand filter column was supported by gravel layers. Sand and gravel used for the experiment were washed in distilled water and autoclaved at 121°C for 15 minutes at 1.5 atm. Then they were dried in an oven at 100°C for 40 minutes prior to introduction to the sand filter column. The column was made of PVC tubes and the minimum supernatant level was controlled by an outflow weir. The flow rate was maintained at 0.4 L/min using valves set up to the sand filter column. A minimum supernatant level of 30 cm was maintained above the sand bed. Filter design, material and operating conditions are listed in Table 1. The retention time in the biological layer was 30 minutes. The control filter setup was also developed according to the above conditions excluding bacterial inoculums in the coral substrate. Freshly prepared Potassium nitrate was spiked at a final concentration of 45 μg/mL in the water reservoirs of both control and experimental setups. Five mL aliquots were collected from the outlet tank at 0, 3, 6, 12, 24, 48 and 72 hours. These sample aliquots were used to analyze remaining nitrate concentration.

**Table 1.** Filter design, material and operating conditions of the sand filter column

| Components | Size (mm) | Depth (cm) |
|---|---|---|
| Fine sand | <2.0 | 50.0 |
| Coarse sand | 2.0-4.0 | 5.0 |
| Gravel | 4.0-8.0 | 5.0 |

## 2.8 Statically analysis

Two-way ANOVA was employed to see the significant difference of nitrate removal by all three bacterial strains at different temperature, pH and Nitrate concentrations.

## 3. RESULTS

Among bacterial strains (S1, S2, S3, S4, S5) which were used to screen nitrate reduction, three bacteria strains (S1, S2 and, S3) showed a pronounced reduction of nitrates. Figure 2 represents the nitrate reduction by S1, S2 and, S3 within 8 days of incubation.
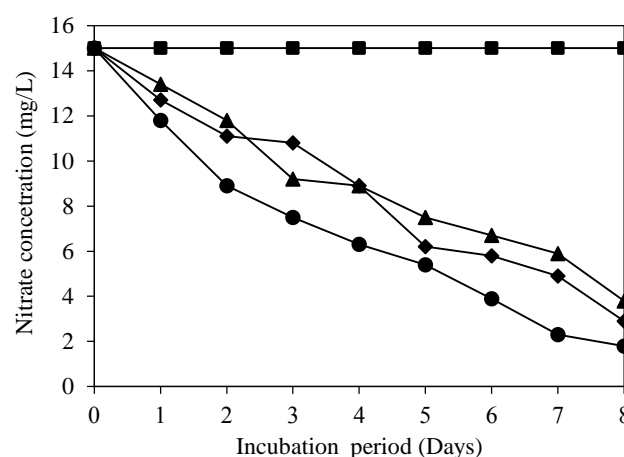


**Figure 2.** Nitrate reduction by S1, S2 and, S3 strains (closed squares- control, closed circle- S1, closed triangle- S2, closed diamond- S3). When error bars are not shown, the standard errors are less than the width of the symbol.

Among the three strains tested S1 showed the highest rate of 4.2±0.08 mg/L/day for nitrate removal within eight days of incubation whereas nitrate removal rates of strain S2 and S3 were 3.45±0.57 and 3.72±0.19 mg/L/day, respectively. Nitrate reduction by S1, S2 and, S3 were optimized for temperature (25°C, 30°C, 32°C) and, pH (7, 7.5, 8). Nitrate degradation by S1 at 25°C, 30°C, and 32°C was 4.2±0.14, 4.25±0.06 and 4.52±0.01 mg/L/day, respectively (Figure 3(a)). S2 strain showed an optimum nitrate degradation of 4.25±0.47 mg/L/day at 32°C, while the nitrate degradation rate at 25°C and 30°C was 3.45±0.1 mg/L/day and 4.04±0.09 mg/L/day, respectively (Figure 3(b)). Nitrate degradation by S3 at 25°C, 30°C, and 32°C was 3.78±0.04, 3.87±0.29 and 4.04±0.09 mg/L/day, respectively (Figure 3(c)). Hence, the maximum nitrate reduction at 32°C was

shown by S1 strain in comparison to S2 and S3 strains. Table 2 shows the nitrate removal

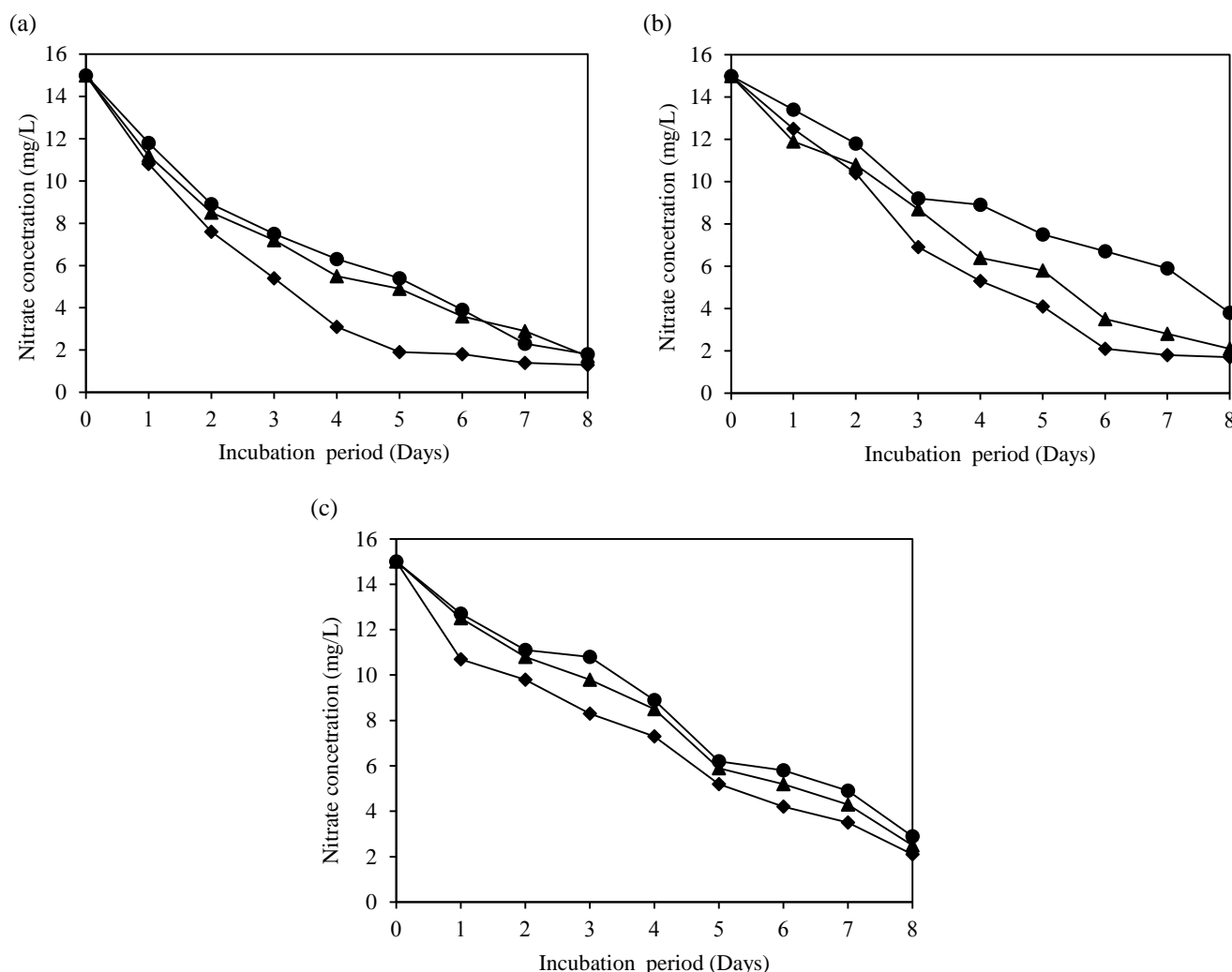percentages of all three bacterial strains at 25ºC, 30ºC, and 32ºC.

(a)



(b)



(c)



**Figure 3.** Nitrate reduction by (a) S1, (b) S2 and (c) S3 strains at 25ºC, 30ºC, and 32ºC (closed circle- 25ºC, closed triangle- 30ºC, closed diamond- 32 ºC). When error bars are not shown, the standard errors are less than the width of the symbol

**Table 2.** Nitrate removal percentages of all three bacterial strains at 25, 30, 32ºC.

| Bacterial strain | Nitrate removal% at 25ºC | Nitrate removal% at 30ºC | Nitrate removal% at 32ºC |
|---|---|---|---|
| S1 | 88.1±2.12 | 88.3±2.45 | 91.2±2.41 |
| S2 | 75.2±3.78 | 86.4 ±1.26 | 88.5 ±1.32 |
| S3 | 81.6±2.03 | 83.5 ±1.19 | 86.4±2.09 |

The pH optimization studies recorded that Nitrate degradation by S1 at pH 7, 7.5 and 8 was 4.12±0.09, 4.25±0.31 and 4.6±0.05 mg/L/day, respectively (Figure 4(a)). S2 strain showed an optimum nitrate degradation of 4.38±0.03 mg/L/day at pH 8, while the nitrate degradation rate at pH 7 and pH 7.5 was 4.15±0.105 and 4.24±0.79

mg/L/day, respectively (Figure 4(b)). Nitrate degradation by S3 at pH 7, 7.5 and 8 was 4.04±0.14, 4.09±0.06 and 4.14±0.25 mg/L/day, respectively (Figure 4(c)). Hence, all three bacterial strains showed highest nitrate degradation at pH 8. Table 3 shows the nitrate removal percentages of all three bacterial strains at pH 7, 7.5 and 8.
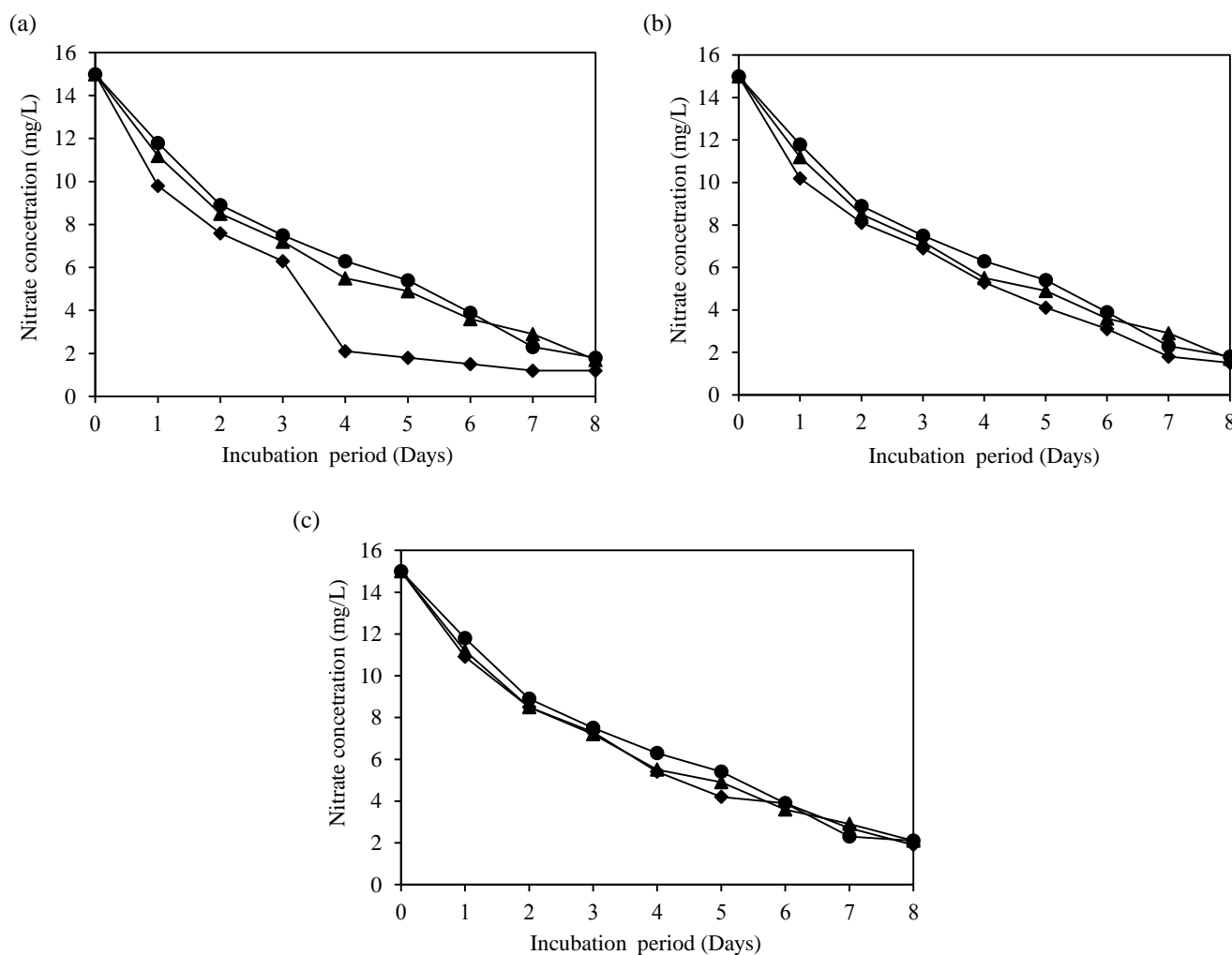
(a)



(b)



(c)



**Figure 4.** Nitrate reduction by (a) S1, (b) S2 and (c) S3 strains at pH 7 (closed circle) pH 7.5 (closed triangle) and pH 8 (closed diamond). When error bars are not shown as the standard errors are less than the width of the symbol.

**Table 3.** Nitrate removal percentages of all three bacterial strains at pH 7, 7.5 and 8.

| Bacterial strain | Nitrate removal% at pH 7 | Nitrate removal% at pH 7.5 | Nitrate removal% at pH 8 |
|---|---|---|---|
| S1 | 88.3±1.08 | 88.6±2.87 | 92.3±5.41 |
| S2 | 87.2±2.31 | 88.4 ±3.08 | 90.6 ±3.32 |
| S3 | 86±3.59 | 86.2 ±2.76 | 87.4±2.09 |

When different nitrate concentrations were used to study the bacterial reduction, S1 bacterial strain showed a maximum reduction rate of 10.08±0.17 mg/L/day when 45 mg/L of nitrate was used while strain S2 showed a reduced rate of 5.67±0.45 mg/L/day and the strain S3 was 5.71±0.43 mg/L/day (Figures 5(a)-(c)). Table 4 records nitrate removal percentages of S1, S2 and, S3 bacterial strains at different nitrate concentrations.

**Table 4.** Nitrate removal percentages of all three bacterial strains at 15, 30 and 45 mg/L of nitrate.

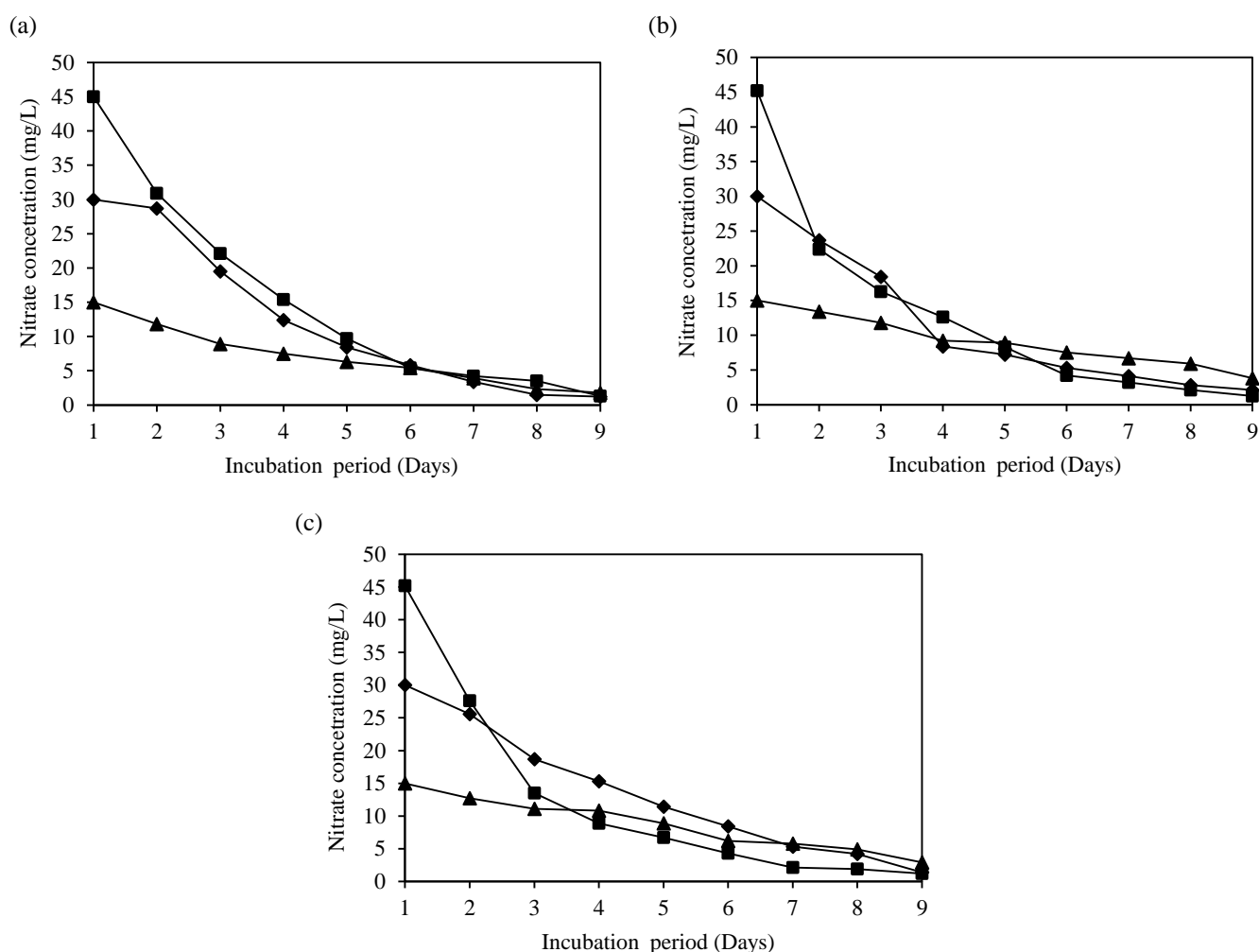| Bacterial strain | Nitrate removal% at 15 mg/L | Nitrate removal% at 30 mg/L | Nitrate removal% at 45 mg/L |
|---|---|---|---|
| S1 | 88.1 ±2.87 | 96±1.78 | 97±2.98 |
| S2 | 74±1.08 | 93±3.09 | 97 ± 1.56 |
| S3 | 81.4±2.95 | 95.4 ±6.54 | 97.2± 5.98 |

(a)



(b)



(c)



**Figure 5.** Nitrate reduction by (a) S1, (b) S2 and (c) S3 at different concentrations (closed triangle- 15 mg/L/day, closed diamond 30 mg/L/day, closed square 45 mg/L/day). When error bars are not shown, the standard errors are less than the width of the symbol.

Isolated bacteria were identified into genus level using their morphological characters and biochemical tests (Table 5). Accordingly, bacteria isolates S1, S2, and S3 were identified as *Pseudomonas* sp., *Bacillus* sp. and *Proteus* sp. Among them *Pseudomonas* sp. was found to be the most efficient bacteria.

**Table 5.** Biochemical tests results used for the identification of bacteria

| Bacterial strain | Citrate | Starch hydrolysis | Catalase | Urease | Methyl red | Vogus proskauer | Gelatin |
|---|---|---|---|---|---|---|---|
| S1 | + | - | + | + | + | + | + |
| S2 | - | - | + | - | - | - | + |
| S3 | + | + | + | + | + | + | + |

A laboratory scale sand filter was developed in co-operating S1, S2 and S3 bacterial strains to study nitrate removal efficiency. Figure 6 represents the nitrate removal by the mixed bacterial culture. The experimental set up has been able to remove initial nitrate at a rate of 6.12±0.06 mg/L/day within 24 hours whereas the control filter without bacterial inoculation did not show a significant removal of nitrate.
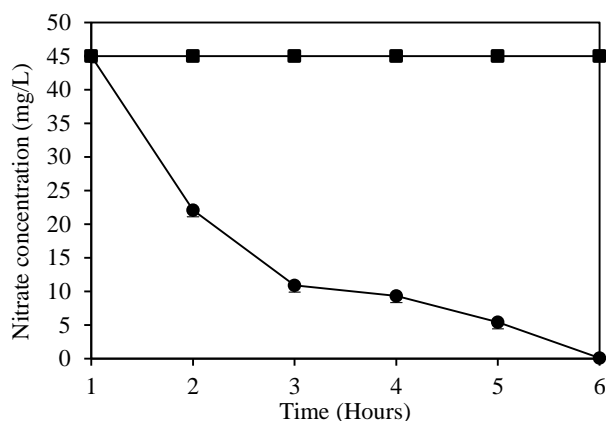
**Figure 6.** Nitrate removal by the experimental and control sand filters (closed squares- control, closed circle-experiment). When error bars are not shown, the standard errors are less than the width of the symbol.

**Table 6.** Results of two way ANOVA analysis

| Category | P-value for temperature (ºC) | P-value pH | P-value nitrate concentration (mg/L) |
|---|---|---|---|
| Bacteria strain | 0.166 | 0.018 | 0.280 |
| Treatment | 0.131 | 0.021 | 0.012 |

Table 6 presents the two way ANOVA analysis results. According to the results, there is no significant difference of nitrate degradation by all three bacterial strains at different temperature and nitrate concentrations (Temperature P=0.166, Nitrate concentration P=0.280). However, the P values obtained for pH (P=0.018) showed that there is a significant difference in nitrate degradation by all three strains. When considering the nitrate degradation rates of individual bacteria strains at different treatment methods it is evident that temperature has no significant effect (P=0.08) whereas pH (P=0.021) and nitrate concentrations (P=0.012) have a significant effect.

## 4. DISCUSSION

Among several studies carried out regarding the isolation of Nitrate-reducing bacteria, *Pseudomonas stutzeri* was first described by Burri and Stutzer in 1895. This bacterium has been capable of nitrification and denitrification processes as well as in the degradation of environmental pollutants. Strains of *Pseudomonas stutzeri* have been used successfully in many developed countries to treat wastewater (Su et al., 2009). *P. stutzeri* strains have been recorded as strong potential bacteria to remove nitrogen in wastewater in piggery wastewater (Yang et al., 2017). Su et al. (2009) reported that *Pseudomonas stutzeri* NS-2 strain, isolated from piggery wastewater systems, had excellent denitrifying capability under aerobic and anaerobic conditions. *Pseudomonus stutzeri* changes nitrate to dinitrogen ($N_2$) anaerobically (Carlson and Ingraham, 1983). The trend of nitrate removal of the *Pseudomonus stutzeri* was obtained using various carbon sources under stationary culture conditions.

Among the three strains isolated, S1 showed the highest rate of nitrate removal (4.2±0.08 mg/L/day) within eight days of incubation, whereas S2 and S3 showed 3.45±0.57 and 3.72±0.19 mg/L/day, respectively. These bacterial strains S1, S2 and S3 were identified as *Pseudomonas* sp.,

*Bacillus* sp. and *Proteus* sp. using biochemical tests. Rajkumar et al. (2006) reported 99.4% nitrate removal efficiency when a bacterial consortium of *Pseudomonas* sp., *Bacillus* sp. was used. However, in the present study, the nitrate removal efficiency of *Pseudomonas* sp. strain S1 at 15 mg/L nitrate was 88.1±2.87%. This nitrate removal efficiency is less than the value reported by Rezaei et al. (2009) which was 99% at 200 mg/L nitrate from an industrial effluent. However, when the incubation temperature was increased up to 32ºC, *Pseudomonas* sp. strain S1 showed a 91.2±2.41% removal at 15 mg/L nitrate. When the pH was increased to 8, a maximum removal percentage of 92.3±5.41% was observed at 15 mg/L. When different concentrations of nitrate were used, *Pseudomonas* sp. strain S1 showed a maximum removal of 97±2.98% at 45 mg/L of nitrate. Thus, high nitrate removal efficiency by strain *Pseudomonas* sp. strain S1 could be obtained by elevating the incubating temperature to 32ºC and maintaining the pH at 8. Although *Pseudomonas* sp. strain S1 showed a lower nitrate removal in comparison to the strain isolated by Rezaei et al. (2009), this strain is more efficient in removing nitrate than isolate WBUNB009 which was isolated by DebRoy et al. (2013) with an efficiency of 88.3%.

*Bacillus* sp. strain S2, which was isolated in the present study, showed an efficiency of 74±1.08% at 15 mg/L of nitrate. However, when incubation temperature was increased to 32ºC, *Bacillus* sp. strain S2 showed 88.5±1.32% removal at 15 mg/L nitrate. When the pH was increased to 8, a maximum removal percentage of 90.6±3.32% was observed at 15 mg/L. When different concentrations of nitrate were used, *Bacillus* sp. strain S2 showed a maximum removal of 97±1.56% at 45 mg/L of nitrate. Thus, high nitrate removal efficiency by *Bacillus* sp. strain S2 could be obtained by elevating the incubating temperature for 32ºC and maintaining the pH at 8.

When *Proteus* sp. strain S3 was used, it showed a maximum nitrate removal rate of 81.4±2.95% at 15 mg/L. The nitrate removal rate increased to 86.4±2.09% at 32ºC. When the pH was increased to 8, the nitrate removal efficiency of *Proteus* sp. strain S3 was recorded as 87.4±2.09%. However, highest nitrate removal by *Proteus* sp. strain S3 was recorded as 97.2±5.98% at 45 mg/L of nitrate. However, the two-way ANOVA suggested that there is no significant difference in nitrate degradation rates of all three bacteria strains at different nitrate concentrations, but there is an overall increase in nitrate degradation rates in all three strains. Thus, it is evident that the presence of high nitrate concentrations in the media may induce the nitrate degradation ability of all three bacterial strains as a result of high expressions of nitrate degrading genes. Mishra et al. (2015) recorded on nitrate removal by *Proteus* sp. strain S3 as 76.6% Therefore, *Proteus* sp. isolated in the present study is more efficient in nitrate removal than the strain isolated by Mishra et al. (2015).

Biofilm technology for denitrification has been proved to be one of the most advanced, selective and low cost ways to remove nitrate and nitrite ion by dissimilatory reduction (Percheron et al., 1999). Therefore, the present study focused on developing a laboratory scale sand filter using *Pseudomonas* sp. strain S1, *Bacillus* sp. strain S2 and *Proteus* sp. strain S3. The results of the study revealed that the sand filter showed 99.2±4.87% removal of nitrate. Sulfur-based autotrophic denitrification in static beds have proven successful for treating nitrate in groundwater, landfill leachate, and wastewaters (Koenig and Liu, 1996), and this approach presents a unique option for treatment of aquaculture effluents (Alves et al., 2002). Nitrogen

removal rates from previous laboratory studies are generally on the order of 0.1-0.4 g/L/day (Lampe and Zhang, 1996; Sahinkaya and Kilic, 2014). However, the biofilter developed during the present study has revealed that the nitrate removal capacity is 0.046 g/L/day. Therefore, there is a need to modify the present setup in order to increase the nitrate removal efficiency of the bacterial consortia.

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