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RESEARCH ARTICLE

Bioremediation

Screening of potential aerobic denitrifying bacteria for nitrate removal from water

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Abstract: Nitrate pollution in groundwater is a common problem in areas where inorganic fertilizer is used to a large extent. This situation seriously affects communities that use ground water as their main source of drinking water and for many other purposes. Therefore, finding an efficient and cost effective system for the removal of nitrate from groundwater is an urgent necessity. The present study was aimed at identifying aerobic bacteria isolated from various soils and water sources and to test their potential for reducing nitrate in groundwater. The bacterial isolates (n = 128) were screened for nitrate reduction by various processes in nutrient broth and in mineral salt medium containing glucose and starch, using KNO_3 as the nitrate substrate. Liberated gases during nitrate reduction were analyzed using gas chromatography. Out of 128 morphologically different isolates, two strains, namely *Paracoccus* sp. (A2) and *Bacillus* sp. (A19), were selected for further analysis on the basis of their performance for water treatment. The nitrate reduction percentages of A2 and A19 were within the range of 59.63-100% and 86.67-100%, respectively. Gas chromatography results indicated that these two strains liberated a higher percentage of N_2 (68 - 90%) compared to N_2O (5-13%) and CO_2 (traces), while reducing the amount of nitrate. These results confirmed that A2 and A19 have the potential to be used in bioremediation of nitrate contaminated groundwater.

Keywords: Bioremediation, contamination, groundwater , isolates, nitrate, treatments

INTRODUCTION

Nitrate is a harmful pollutant that has become a common water contaminant in many parts of the world (Rajta *et al*., 2019; Zhang *et al*., 2019b; Abascal *et al*., 2021). Excessive use of nitrogen-rich fertilizers used for agricultural purposes, discharge of poorly treated domestic and industrial wastewater, livestock manure, and leachate from landfill sites are the main anthropogenic sources for nitrate pollution of groundwater (Gutierrez *et al*., 2018; Tokazhanov *et al*., 2020). The World Health Organization (WHO) reconfirmed the safe level of nitrate as below 50 mg/L for drinking water, which was set to protect against methaemoglobinaemia (WHO, 2017). Excessive consumption of nitrates can cause health effects in humans and animals alike, especially methaemoglobinaemia (blue baby syndrome) in infants and gastrointestinal cancer in adults (Ren *et al*., 2018; Cotruvo, 2017). There are reports of other health disorders, including increased infant mortality, hypertension, central nervous system birth defects, diabetes, spontaneous abortions, respiratory tract infections, and changes to the immune system due to the consumption of high levels of nitrates (Kotopoulou *et al*., 2022).

Nitrate contamination of groundwater is a burning issue in many areas in the world such as UK (Neal *et al*., 2006), Australia (Rasiah *et al*., 2013), North America (Power & Schepers, 1989), Morocco, Changshu in China (Sadeq *et al*., 2008), and Toyserkan in western Iran (Jalali, 2011). The problem is more severe in some regions of South East Asia Karunanidhi *et al*., (2021) reported that synthetic fertilizers, cow dung and sheep manure, industrial discharge, septic tank leakage, and

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municipal solid waste disposal are the major sources of nitrate pollution in India. They reported that around 117.93 million people drink water contaminated with nitrate levels between 45-100 mg/L and 108.2 million people consume water with levels more than 100 mg/L in India. It is also reported that nitrate contamination in groundwater is a major concern in the coastal region of Bangladesh (Jannat *et al*., 2022).

Similar to many parts of the seasonally dry tropical areas of the world, the Jaffna peninsula in Sri Lanka also experiences minimal periods of rain, while ground water is the only source of water for drinking (Prabagar *et al*., 2020). The water quality in the area has been drastically reduced due to intensive inorganic fertilizer use, resettlement and urbanization within the last decade (Piyathilake *et al*., 2022).

Due to the above reasons protection of groundwater quality is an important concern confronting much of the world's population. Though sophisticated technologies such as chemical denitrification (Xu *et al*., 2017), ion exchange (Vandekerckhove *et al*., 2018), reverse osmosis (Epsztein *et al*., 2015), electro-dialysis and catalytic denitrification (Zhang *et al*., 2016) can be used to remove nitrate from groundwater, proper, cost effective and environmentally friendly systems need to be adopted for remediation of nitrate from ground water in the developing countries. Therefore, finding appropriate treatment technologies for nitrate removal is critical. Biological denitrification is the most promising approach currently investigated for treatment of nitrate contaminated water.

Microbial denitrification has been proven to be an advanced, high performance, and highly selective method for complete nitrate elimination (Gomez *et al*., 2000b). Biological denitrification is the most important and widely used method to treat nitrate wastes as it enables the conversion of nitrogen compounds into harmless dinitrogen (N_2) gas (Costa *et al*., 2018). A microbial consortium composed of *Cellulosimicrobium* sp., *Aeromonas veronii*, *Lysinibacillus sphaericus,* and *Rhodococcus rhodochrous* was found to be the most efficient bacterial consortium for reducing nitrate in rubber latex wastewater (Dey *et al*., 2019). Biological denitrification utilizes the anaerobic reduction of oxidized nitrogen compounds through the sequential activity of microbial reductase enzymes, finally converting them to harmless nitrogen gas. Four enzymes, namely, *nitrate reductase*, *nitrite reductase*, *nitric oxide reductase*, and *nitrous oxide reductase*, are responsible for the complete reduction of nitrate ion to dinitrogen gas (Pang & Wang, 2021). A variety of incomplete denitrification pathways exists. Some denitrifying bacteria reduce both nitrates and nitrites, while others reduce only nitrite. Some produce only dinitrogen, some produce a mixture of dinitrogen and nitrous oxide, while others produce only nitrous oxide.

Even within a single species such as *Pseudomonas fluorescens*, the biotypes differ in the end product of the pathway. Although nitrate reduction activity is exhibited by diverse microbial genera, with a range of heterotrophic and autotrophic metabolisms, the aerobic nitrate reducers belong to a variety of groups of heterotrophs (Guo *et al*., 2013). Aerobic denitrification gained attention due to its easier operation and higher nitrate reduction efficiency compared to anaerobic denitrification (Chen *et al*., 2012). The most predominant denitrifying bacteria that are reported in our environment belong to the genus *Pseudomonas.* There are reports on aerobic denitrifying species isolated from environmental samples such as ponds, canals, soils, and activated sludge (Patureau *et al*., 2000; Huang *et al*., 2020). *Pseudomonas aeruginosa* has been extensively studied genetically (Wu *et al*., 2013) and therefore is usually considered a favorable organism to be used in studies on denitrification in wastewater treatment plants. However, as it is an opportunistic pathogen, it could not be utilized for drinking water treatment processes.

The present study investigates the possibility of utilizing microorganisms isolated from the environment to recover NO_3^- contaminated ground water from Jaffna, Sri Lanka. We hypothesized that microorganisms isolated from different environments would be efficient and capable for this purpose. The expected result would be applicable for the remediation of groundwater resources in Sri Lanka and other countries in the same region of South Asia.

MATERIALS AND METHODS

Sources for the isolation of bacteria

Soil and water from submerged paddy fields and ponds, and wet soil enriched with partially decomposed manure from the Jaffna peninsula (9.6615°N, 80.0255°E) were used for the isolation of bacteria.

Isolation and screening of aerobic denitrifiers

Nitrate rich modified nutrient broth consisting of (g/L): Peptone 5.0, NaCl 5.0, KNO_3 1.0, glucose 1.0, yeast extract 5.0, and beef extract 5.0 with pH 7.2 was used for the

enrichment of nitrate reducers. The enrichment cultures were plated on modified bromothymol blue (BTB) medium containing 0.1% L-asparagine, 0.1% KNO_3, 0.1% KH_2PO_4, 0.005% $FeCl_2.6H_2O$, 0.02% $CaCl_2.2H_2O$, 0.1% $MgSO_4.7H_2O$, 1 mL of BTB (1% in ethanol), 2% agar, and 0.5% glucose at pH 7.0, to isolate and screen the denitrifier under aerobic conditions (Takaya *et al*., 2003). Culture plates were incubated at 30 ℃ for 3 ds. Well defined bacterial strains on the basis of their colony and different morphological characteristics were selected for further screening. A nutrient broth with KNO_3 was used to determine denitrification activity at the initial screening stage. Each strain was inoculated into a 15 mL screw cap tube containing the sterile nutrient broth and Durham's tube, and was incubated at 30 ℃ for 48 hours (Guo *et al*., 2013).

Nitrate reduction in synthetic medium

Nitrate removal activity of five selected strains based on nitrate reduction in the nitrate broth medium was evaluated in a synthetic mineral salt medium (MSM) consisting of potassium dihydrogen phosphate (0.1 g/L), dipotassium hydrogen phosphate (1 g/L), ammonium chloride (0.5 g/L), calcium chloride (0.005 g/L), magnesium sulphate (0.1 g/L), and sodium silicate (0.05 g/L), the pH being adjusted to 7.2, with either glucose or starch as the carbon source in three different percentages, *viz;* 0.25 %, 0.5 % and 1.0 % (Ayyasamy *et al*., 2007).

Analytical methods

Nitrate content of the bacteria inoculated sample was determined according to Anderson & Ingram (1993) via reaction with salicylic acid and sodium hydroxide followed by spectrophotometry at 410 nm. The amount of nitrite was measured through the reaction with sulphanilic acid and N,N-dimethyl-1-naphthylamine, followed by spectrophotometry at 520 nm, as described by Blaszczyk (1993). Ammonium ion content was determined by the method described by Guo *et al.* (2013).

Gas chromatographic analysis

Gases evolved during denitrification were analyzed for N_2, N_2O, and CO_2 by a gas chromatograph equipped with Shimadzu GC 9 AM analyzer and a thermal conductivity detector with helium as the carrier (Green *et al*., 2010). Column temperature, detector temperature and injector temperature were maintained at 50-200 °C, 200 °C and 175 °C respectively and the gas flow rate was kept at 30 mL/min. The mixture of gases evolved during nitrate reduction was collected and identified by comparing the retention time of the peaks with standards.

Identification of isolated bacteria

Standard physiological and biochemical characteristics, such as colony morphology, cell shape, gram reaction, catalase reaction, oxidase reaction, motility, nitrate reduction test, anaerobic growth, glucose acid test and starch hydrolysis were used for the identification of bacteria according to the methods described by Bergey (1994).

Evaluation of selected bacterial strains for removal of nitrate from contaminated well water with starch as the carbon source

Based on efficient nitrate removal in the carbon sources and negative growth on Maconkey agar medium, two bacterial strains (A2 and A19) were selected for further study with five nitrate contaminated water samples. Based on optimization of two different carbon sources and different percentages of the synthetic medium, 0.5% starch was selected for water treatment. Conical flasks with 100 mL of 0.5% (0.25 g) starch were sterilized by autoclaving at 120 °C for 20 min. A water sample of 50 mL was filter sterilized using 0.45 µm syringe filter and was added into each flask containing 0.5% (0.25 g) sterile starch and mixed well. Bacterial isolates were cultured on nutrient agar for 24 – 48 h and cell suspensions were prepared by suspending the cultures in 5 mL of sterile distilled water and the turbidity was adjusted to OD 0.5. Each culture suspension (0.5 mL) was added aseptically into each flask and incubated at 30 °C and kept at 120 rpm in a shaking incubator for 72 h. The same conditions were provided without the cultures as the control. Nitrate and nitrite concentrations were analyzed every 12 h in all samples.

Data analysis

All the experiments were carried out in triplicate and the results were analyzed by analysis of variance (ANOVA) using SAS statistical software version 9.1. Treatment means were compared using Duncan's multiple-range test at a significance level of 0.05.

RESULTS AND DISCUSSION

Screening of aerobic denitrifying bacteria

Out of 128 morphologically different strains isolated from different sources, 70 strains were capable of forming blue colonies on the BTB agar plates (Table 1) due to an increase of pH on the medium (Wu *et al*., 2013).

Table 1: Number of isolates and nitrate reducers from different sources

Sources of isolation	Number of strains isolated	Number of nitrate reducers
Municipal compost (GMC)	12	11
Pond soil	8	1
Fish waste (GFW)	15	1
Municipal solid waste dumping place (MSW)	17	4
Manure (swine, poultry)	17	6
Paddy soil (KPS)	23	17
Paddy water (KPW)	18	14
Unutilized well water (PSW)	8	8
Compost (COM)	10	8
Total	128	70

Among the 70 strains, 38 showed gas bubble formation in Durham's tubes. After rescreening, the nitrate removal efficiency was examined in nutrient broth with nitrate under aerobic conditions. Out of 38 strains, 5 bacterial isolates, capable of reducing either nitrate or nitrite efficiently (more than 50%) were selected by quantitative screening. Since most of the denitrifiers were heterotrophs, they required carbon sources for energy consumption (Pang & Wang 2021). Although five strains had higher nitrate reduction capacity (more than 70 %) either with glucose or starch, three of them were grown on Maconky agar medium, hence, cannot be used for treatment of water samples. The other two, A2 and A19 were tested with five nitrate contaminated water samples for their nitrate removal efficiency.

Table 2: Physical and biochemical identification of bacterial strains

Identification test	A2 (*Paracoccus* sp.)	A19 (*Bacillus* sp.)
Shape	Round	Rod
Gram staining	Negative	Positive
Endospore formation	Negative	Positive
Motility test	Negative	Positive
Catalase test	Positive	Positive
Oxidase test	Positive	Positive
Anaerobic growth	Positive	Positive
Nitrate reduction test	Positive	Positive
Growth on Maconkey agar	Negative	Negative
Glucose acid test	Positive	Positive
Starch hydrolysis test	Positive	Positive

Identification of the selected bacterial strains

According to the physical and biochemical identification, strains A2 and A19 were identified as *Paracoccus* sp. and *Bacillus* sp. (Table 2).

Optimization of the percentage of the carbon sources for aerobic nitrate removal

The two carbon sources used, namely glucose and starch at the levels of 0.25%, 0.5%, and 1%, were tested for carbon source optimization for the strains A2 and A19. The optimum carbon percentage was decided based on efficient nitrate removal as well as lowest intermediate accumulation (nitrite) in the medium.

Figure 1 shows the effect of glucose (a) and starch (b) on nitrate removal and nitrite accumulation by the strain A2. The nitrate nitrogen concentration was lowered with both carbon sources at all three levels tested. Although nitrate reduction was observed with time, after 24 hours, nitrite began to accumulate with glucose at concentrations of 0.25%, 0.5%, and 1% up to 60 hours. However, when starch was used as the carbon source, nitrite accumulation was not observed at all three levels.

Strain A2, grown with 0.5% and 1% starch reduced nitrate to 11 mg/L after 36 hours of incubation (Figure 1). Although nitrate reduction was observed with glucose as the carbon source, nitrite accumulation also was recorded at all three levels. When the two carbon sources were compared at three different levels, 0.5% starch exhibited higher nitrate reduction and nitrite was not detected. Ammonium ion was not detected at any levels of the two carbon sources. Therefore, it could be concluded that the strain A19 has a higher potential to remove nitrate with 0.5% of starch, without accumulation of the nitrite intermediate. Further, the nitrate reduction efficiency and intermediate accumulation of strain A2 could be controlled by the organic carbon sources, nitrate concentration, and C/N ratio. A study conducted by Blaszczyk (1993) clearly stated that the denitrification performance of *Paracoccus denitrificans* strongly depended on the quality of the medium.

The carbon source can provide the energy for the aerobic denitrifier and electron donors for their growth and metabolism (Li *et al*., 2020b). Nitrogen transformation of strain A19 with the carbon sources glucose (a) and starch (b) is shown in Figure 2. The results indicate that, after 60 hours of incubation, complete reduction of nitrate was observed with 0.5% and 1% of starch, without the accumulation of nitrite. However, with glucose,

nitrate was reduced to below the permissible level with high amount of nitrite accumulation. Intermediate accumulation mainly depends on the carbon sources. In a study, N_2O and NO accumulation in the presence of nitrite during denitrification was observed with acetate-fed denitrifying cultures, but not in methanol- or ethanol-fed denitrifying reactors with excessive carbon source supply (Lu *et al*., 2014). At the end of 60 hours of incubation, almost all the nitrate was found to be in the form of nitrite with glulose as the carbon source. Therefore, it could be concluded that nitrite concentration also varied with the types and levels of carbon sources for each strain. Among the two carbon sources, starch possessed significantly higher nitrate reduction efficiency for both strains. It may be due to the presence of amylolytic enzymes in the two strains (A2 and A19) and the ability to utilize starch as the carbon source. Ayyasamy *et al*. (2007) also stated that the amylolytic enzymes present in organisms can utilize the starch well. In another study conducted by Rajakumar *et al*. (2008), it is reported that the denitrification rate was higher for starch than glucose, acetic acid, cellulose, and sucrose.

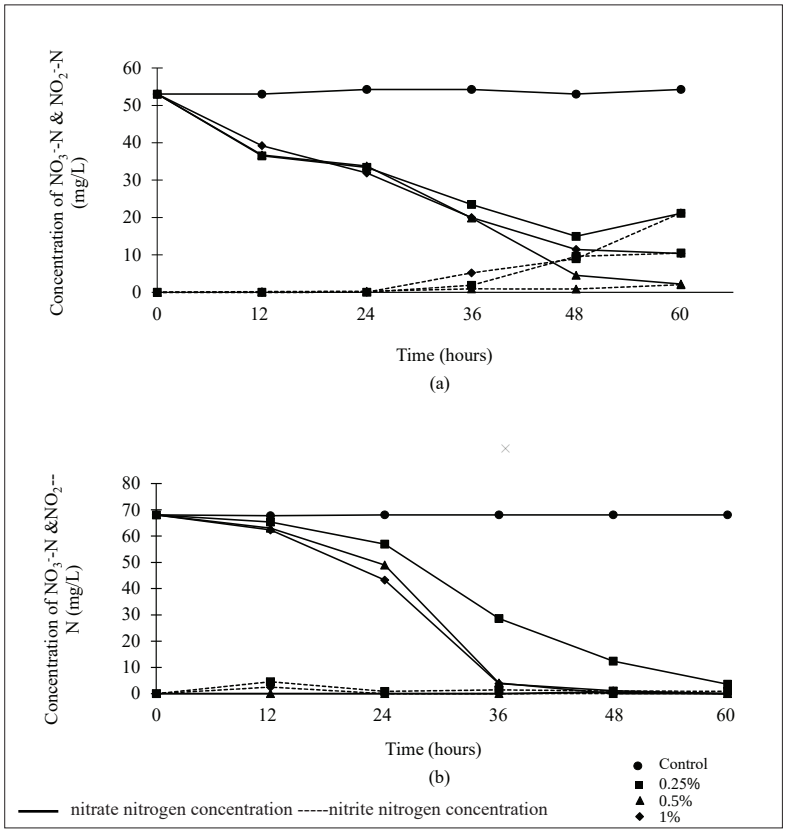

Figure 1: Effect of glucose (a) and starch (b) on nitrate and nitrite concentration in MSM inoculated with strain A2 up to 60 hours of incubation

Therefore, the results indicate that the process of complete denitrification is not a stable process and depends on several factors such as bacterial strains and types and amounts of carbon sources. This unstable nature of the process is due to the influence of these factors, which have been reported in many previous studies (Li *et al*., 2020; Yin & Yan, 2020). Furthermore, nitrate removal is a highly dynamic process that can be affected by temperature, pH, C/N ratio, dissolved oxygen concentration, and bacterial population (Chen *et al*., 2006; Olaya-Abri *et al*., 2021; Zhou *et al*., 2021). In another bacterial denitrification study conducted by Gomez *et al*. (2000), the nitrite accumulation was higher when sucrose was used as the carbon source, but it was not found when methanol and ethanol were used.

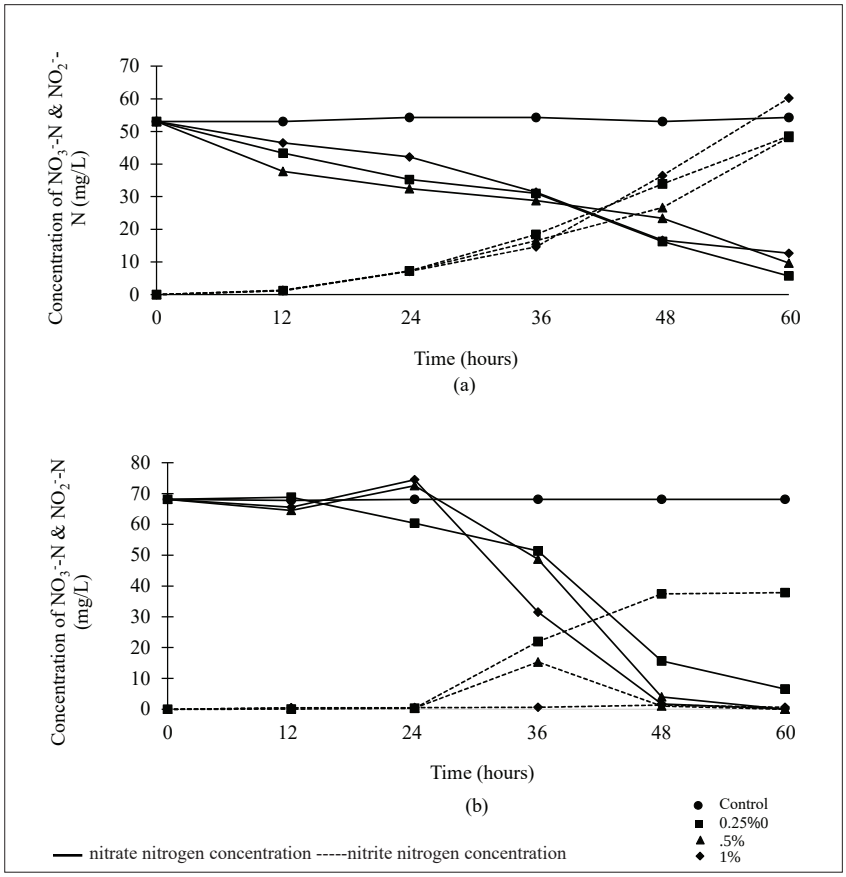

Figure 2: Effect of glucose (a) and starch (b) on nitrate and nitrite concentrations in MSM inoculated with strain A19

Aerobic nitrate removal in well water contaminated with nitrate

Five water samples having initial nitrate nitrogen concentrations of 57.37 mg/L (S1), 32.72 mg/L (S2), 26.70 mg/L (S3), 20.81 mg/L (S4) and 15.91 mg/L (S5) were tested with either A2 or A19. Changes in the nitrate nitrogen concentration of different water samples treated with A2 and A19 with 0.5% of starch are shown in Figures 3.

Figure (3a) shows the nitrate reduction profile of water sample S1, having an initial nitrate nitrogen concentration of 57 mg/L, treated either with A2 or A19. With the sample treated with A19, nitrate was found to be at a safe level after 60 hours and not detected at 72 hours. During nitrate reduction by A19, nitrite formation increased at a high rate up to 48 hours and declined thereafter. Although, nitrate was not detected at 72 hours, nitrite was not at a safe level (3.89 mg/L). Accumulation of nitrate at early stage by aerobic denitrifiers such as *Paracoccus denitrificans*, which had 14.07 mg/L initially and reduced to zero at 40 hours (Zhang *et al*., 2020) has been reported. The accumulation of NO_2^--N in S1 treated with strain A19 is possibly due to high initial nitrate content of the water and the consecutive lag of nitrite reduction (Chen *et al*., 2020), which could be possibly removed with more time. Significant difference was observed in final nitrate concentration in the sample S1 treated with either A2 or A19. Further, A2 reduced nitrate nitrogen to the level of 9.1 mg/L at 72 hours without accumulation of nitrite. *Paracoccus* sp. (A2) expressed greater aerobic nitrate removal capacity similar to numerous aerobic bacteria. For instance, *Paracoccus denitrifcans* strain removed 90.00% of NO_3^--N in a 250 mg/L initial NO_3^{--N} medium (Medhi *et al*., 2018). Similar aerobic nitrate removal (87.63%) was achieved by *Paracoccus denitrifcans* strain Z195 (Zhang *et al*., 2020). *Paracoccus thiophilus* strain LSL 251 had an aerobic denitrification rate of 5.90 mg/L/h in nitrate rich medium (Chen *et al.,* 2020).

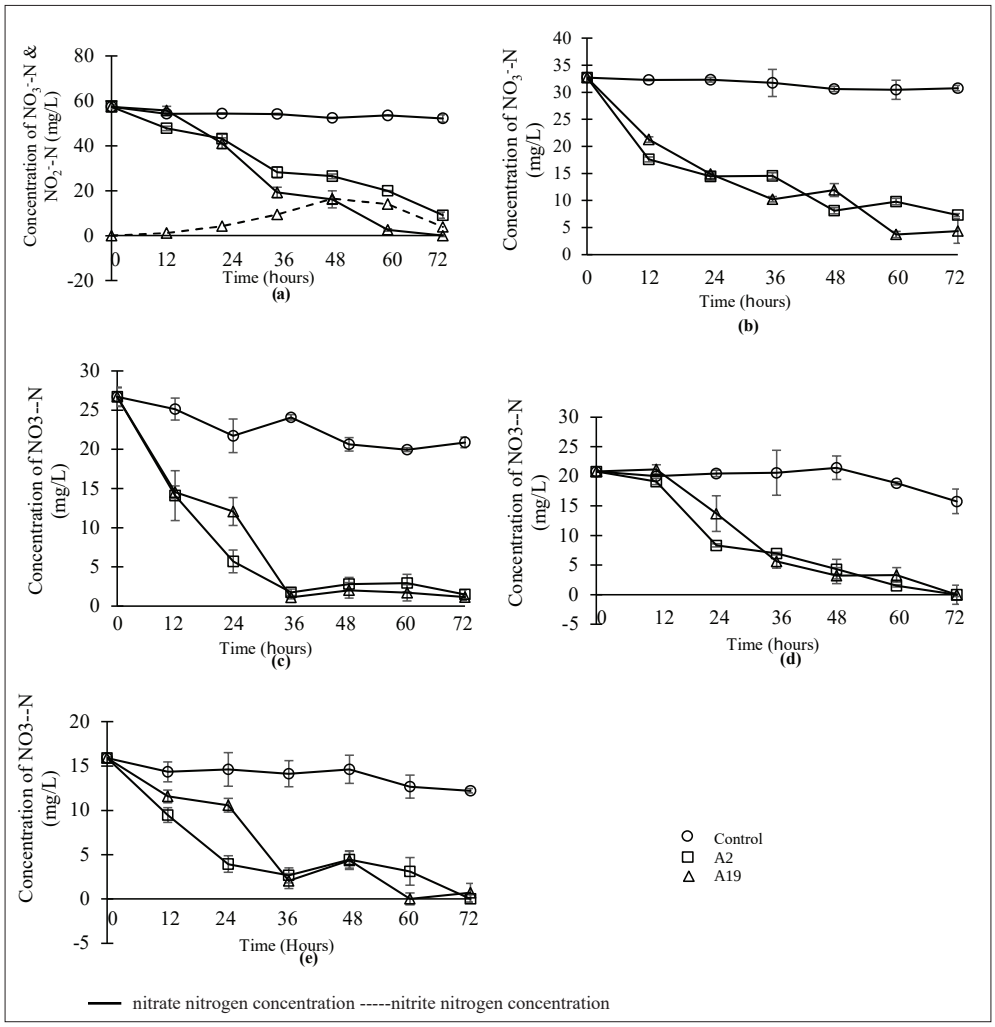

Figure 3: Bacterial reduction of nitrate nitrogen in well water in the presence of 0.5 % starch a) S1, b) S2, c) S3, d) S4 and e) S5

Both strains reduced nitrate with time without nitrite accumulation in sample S2 (Figure 3b). Reduction of the nitrate nitrogen concentration from 32.71 mg/L to 7.30 mg/L was observed in 72 hours with A2, while 4.36 mg/L was observed with A19 at the same time. In another water sample having an initial nitrate nitrogen concentration of 26.7 mg/L, the nitrate level was reduced below the safe level (11 mg/L) at 24 hours by the strain A2, while it reached 1.09 mg/L with A19 at 36 hours, without nitrite being detected (Figure 3c). Moreover, when the initial nitrate concentration was about 20.81 mg/L both strains A2 and A19 lowered the nitrate concentration below the safe level after 24 and 36 hours, respectively (Figure 3d). Changes in NO_3^- nitrogen concentrations of water sample S4 and S5 treated with the strains are shown in Figures 3d and 3e respectively. Initial concentrations in water samples S4 and S5 were 20.81 mg/L and 15.91 mg/L, respectively.

During incubation, fluctuation was observed in the nitrate nitrogen concentration with the treatment of either A2 or A19, however, no nitrite was detected in either sample with strain A2. This might be due to the oxidization of the negligible amount of nitrite, resulting from the brief exposure to air during sampling (Zhang *et al*., 2011) or the simultaneous nitrification and denitrification capability of the strains (Kim *et al*., 2008; Khardenavis *et al*., 2007). The denitrification process can also be influenced by metal ions such as Fe^{3+} and Mo^{6+} (Pintathong *et al*., 2009). As can be observed from the

figures, both strains of A2 and A19 with 0.5% of starch lowered the nitrate nitrogen concentration from various initial levels of nitrogen in water. Nevertheless, the time needed to attain the permissible level or below varied with strains and the well water samples. Although the difference is not significant, it might be due to the effect of initial nitrate concentration, pH, and availability of other nutrients in the water sample (Körner & Zumft, 1989). Denitrification of synthetic waste water having a high nitrate level was inhibited at the pH values of 6.5 and 7.0. Although, higher nitrate reduction was achieved with the increased pH values of 7.5, 8.0 and 9.0, accumulation of nitrite increased significantly (Glass & Silverstein, 1998). A *Bacillus pumilus* strain removed 99.7% of NO_3 – nitrogen in a 70 mg/L initial NO_3^- containing medium (Elkarrach *et al*., 2021). In another study, 89.4% of nitrate removal was reported by *Bacillus* sp. after a 48-h cultivation in a sole N-source medium with initial nitrogen approximately 20 mg/L (Huang *et al*., 2017). *Bacillus sp*. PB8 showed excellent aerobic denitrifying ability (0.25 mg/L/h) both in artificial media and real wastewater treatment (Barman *et al*., 2018).

Gas chromatographic analysis

During denitrification by A2, a higher percentage (90.6%) of N2 , lower percentage of N2O (5.7%) and traces of CO2 were released, while A19 released 68.5 % of N2 and 12.5% of N2 O. Considering the composition of gases released, A2 would be a better strain than A19.

CONCLUSION

Among the 70 nitrate reducing bacteria strains isolated, *Paracoccus* sp. A2 and *Bacillus* sp. A19 exhibited a high nitrate reduction potential either with glucose or starch as the carbon source. However, with glucose, in all three levels (0.25%, 0.5% and 1%), nitrite accumulation was observed. Among the three percentages of starch (0.25%, 0.5% and 1%), 0.5% was the optimum level for efficient nitrate removal. Further, *Paracoccus* sp. A2 and *Bacillus* sp. A19 were capable of removing the nitrate nitrogen content in the range of 15 mg/L to 57.37 mg/L in groundwater to safe levels within 72 hours with 0.5% of starch. To the best of our knowledge, this is the first report on nitrate reduction by bacterial strains in Sri Lanka and this finding may provide useful information for the potential use of these two bacterial species. Further studies are required to validate nitrate removal efficiency of the strains under different physico-chemical conditions.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.