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Characterization and Nitrate Reduction Activity of Bacteria Isolates from Groundwater in Sleman, Yogyakarta as a Potential for *In-Situ* Bioremediation

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ABSTRACT. Groundwater pollution is common in densely populated areas due to the high discharge of domestic as well as agricultural waste. As a result, nitrate is one of the pollutants that is commonly found within the affected groundwater system. Sleman, Yogyakarta is especially vulnerable to nitrate pollution due to the co-occurrence of rapid urban expansion and agricultural activities. High nitrate concentrations in groundwater lead to many adverse effects on the environment and human health. Therefore, this research was conducted to assess the capacity of indigenous aerobic nitrate-reducing bacteria in groundwater in Sleman, Yogyakarta, as potential agents for in situ bioremediation. A total of 19 indigenous bacteria were isolated from groundwater samples and tested for nitrate-reduction activity. Two of the highly efficient isolates in reducing nitrate aerobically were then measured for their nitrate reduction activity every 24 hours within a 3-day incubation period. The phenotypes of the two bacteria isolates were characterized through the observation of cell morphology, colony morphology, and biochemical tests. Both isolates were identified as *Pseudomonas sp* and *Flavobacterium sp*. *Pseudomonas sp* was the most efficient at reducing nitrate, removing 95.38% of total nitrate and achieving a nitrate reduction activity of 40.67 mg L^{-1} h. Meanwhile, mixed-culture bacteria did not show positive effects on nitrate reduction due to antagonistic interactions specific to the isolates. Both isolates of indigenous nitrate-reducing bacteria, cultured individually, have great potential for use and development in in situ bioremediation of nitrate pollutants.

Keywords: Groundwater pollution · Indigenous bacteria · Nitrate reduction activity.

1 INTRODUCTION

The increasing demand for clean water in the last few decades is attributed to the skyrocketing growth of the world's population. In Indonesia, groundwater is the main source of clean water for most people (Kobayashi *et al.*, 2024). Especially in urban areas, such as Sleman, Yogyakarta, groundwater faces a significant reduction in quantity as well as quality (Abascal *et al.*, 2022; Wijayanti *et al.*, 2018). The population growth gives rise to anthropogenic activities that cause degradation in the aquifer (Kagabu *et al.*, 2010; Taufiq *et al.*, 2018).

Excessive groundwater abstraction lowers groundwater levels, thereby increasing the risk of contamination. The accumulation of pollution on the surface also heightened the risk of it leaching into the groundwater system (Adi *et al.*, 2023; Foster & Chilton, 2003; Wilopo *et*

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al., 2021, Xiong *et al.*, 2025). Nitrate becomes one of the major pollutants in groundwater due to the intensification of agricultural activities and urbanization (Abascal *et al.*, 2022; Breida *et al.*, 2020; Gutiérrez *et al.*, 2018). Its high solubility allows nitrate to leach into the waters, causing environmental problems (i.e., eutrophication) and posing a risk to human health (i.e., methemoglobinemia and cancer) (Gutiérrez *et al.*, 2018; Hurtado-Martinez *et al.*, 2021; Yang *et al.*, 2020).

Hence, efficient and sustainable nitrate removal methods have become a focus of research in recent years. Previous studies suggested bioremediation via microbes due to their availability in the natural environment. Nitrate-reducing bacteria occur naturally as part of the nitrogen cycle (Huno *et al.*, 2018), where they use nitrate as an electron acceptor during respiration. It was generally believed that this happens during anaerobic conditions (Zhang *et al.*, 2020). However, new findings suggest the existence of bacteria capable of aerobically reducing nitrate. These bacteria were isolated from sediments, wastewater, and wastewater treatment systems (Ji *et al.*, 2015).

The performance of aerobic nitrate reducers is not limited by the presence of oxygen, which makes them a great potential for in situ bioremediation (Kou *et al.*, 2021). During aerobic nitrate reduction, bacteria utilize both oxygen and nitrate in a process called co-respiration (Kou *et al.*, 2021), removing both total nitrogen (TN) and total organic nitrogen (TOC) at the same time (Zhang *et al.*, 2019). However, heterotrophic bacteria need a carbon source to support their growth. Therefore, a low TOC in groundwater is a limiting factor in nitrate removal (Huang *et al.*, 2015; Kou *et al.*, 2021). To counter this, indigenous bacteria have been proposed in treatments of micro-polluted water due to their tolerance of low nutrients (Huang *et al.*, 2015). The use of mixed cultures has also been widely observed in bioremediation due to their ability to improve microbial resistance under suboptimal conditions (Zhang *et al.*, 2022). Mix-culturing a microorganism resistance in sub-optimal conditions (Zhang *et al.*, 2022). Mix-culturing a consortium of bacteria may have a positive effect on nitrate removal due to the combination of synergistic effects and metabolic pathways (Li *et al.*, 2022). Therefore, co-culturing indigenous bacteria isolated from micro-polluted groundwater is expected to increase nitrate removal efficiency.

Ngemplak Subdistrict, Sleman Regency. Yogyakarta experienced a population increase in recent years, accompanied by a shift in land use, transforming land into urban settlements (Partoyo & Shrestha, 2017). The area is located in one of the most rapidly growing regencies in Java, which accounted for 62% of the population growth each year and has high agricultural productivity (Subkhi & Mardiansjah, 2019). Additionally, the aquifer comprises unconsolidated materials, which have a high potential for groundwater pollution (Neritarani & Sejati, 2021). In addition, agricultural activities made up a large share of that area's income (Badan Pusat Statistik Provinsi DI Yogyakarta, 2016). Agricultural activity has become one of the most prominent causes of nitrate pollution due to the use of nitrogen-rich fertilisers. Those aforementioned factors put Yogyakarta's groundwater at high risk for nitrate pollution.

Several previous studies have reported the nitrate removal capability of nitrate-reducing bacteria from various samples, including paddy soil in China, lakes in India, and wastewater in China, each in single and mixed cultures (Jiang *et al.*, 2021; Meng *et al.*, 2024; Mir and Rather, 2024; Zang *et al.*, 2026). However, no studies comparing the nitrate removal capability of nitrate-reducing bacteria isolated from groundwater in Sleman, Yogyakarta, Indonesia, in single and mixed cultures have been reported. Therefore, it is crucial to isolate indigenous bacteria from groundwater samples collected in Ngemplak, Sleman, Yogyakarta, and determine their ability to reduce nitrate aerobically.

The purpose of this study was to: 1) confirm the existence of indigenous aerobic nitrate-reducing microorganisms in the groundwater of Ngemplak, Sleman; 2) measure the activity of nitrate removal by indigenous aerobic nitrate-reducing bacteria within a 3-day incubation period; 3) understand the phenotypes of indigenous bacteria most efficient in reducing nitrate aerobically; 4) observe the difference in nitrate removal efficiency between single cultures and mixed cultures.

2 Geological Setting

The Yogyakarta-Sleman groundwater basin is situated within the Special Region of Yogyakarta, which stretches from Mount Merapi to the south towards the Indian Ocean. The groundwater basin spans across three regions: Sleman Regency, Yogyakarta City, and Bantul Regency, serving as a zone for groundwater recharge and discharge (Wilopo *et al.*, 2021). The hydrogeological characteristics of the Yogyakarta-Sleman groundwater basin are linked to volcanic processes of Mount Merapi, where the deposits form layers of an aquifer called the Merapi Aquifer System (Razi *et al.*, 2024). The material which constitutes the aquifer are mostly gravel, silt, and clay (Razi *et al.*, 2023). Most of the aquifers in the area are unconfined aquifers that are not protected by a confining layer, such as an aquitard, and are hence more vulnerable to pollution (Neritarani & Sejati, 2021; Razi *et al.*, 2023). According to Adi *et al.* (2023). The groundwater on the southern slope of Mount Merapi is susceptible to contamination due to high recharge and discharge rates, suggesting high aquifer permeability. Moreover, other factors such as groundwater depth and, more importantly, anthropogenic factors affect the susceptibility of groundwater to contamination.

Special Region of Yogyakarta, as a province, experienced a rapid growth of population and economy in the last few decades, exceeding its surrounding areas (Kintoro *et al.*, 2025; Partoyo & Shrestha, 2017; Razi *et al.*, 2023; Sutardi *et al.*, 2017; Wijayanti *et al.*, 2018; Wilopo *et al.*, 2021). Urbanization is observed in Yogyakarta as many people pursue higher education or retire (Partoyo & Shrestha, 2017). The increase in population size puts pressure on the provision of clean water for the residents of Yogyakarta (Wijayanti *et al.*, 2018). As most people still rely greatly on groundwater for their daily needs, the Yogyakarta-Sleman groundwater basin becomes one of the main sources of clean water. Dependence on groundwater is linked to the poor quality and unavailability of surface water, limiting its usage. Many people extract groundwater directly from dug or bored wells due to the limited coverage of the municipal waterworks (PDAM) (Wilopo *et al.*, 2021). The rising number of inhabitants is also responsible for contamination within the groundwater of Yogyakarta. High demand for settlements drives land-use conversion from agricultural lands to urban areas (Partoyo & Shrestha, 2017), increasing leachate from domestic waste entering the groundwater system.

In addition to the contamination from urban settlements, high agricultural activity is also observed in Yogyakarta. As much as 76% (2411.13 km^2) of the area is comprised of agricultural areas: rice fields, plantations, fields, and ponds (Badan Pusat Statistik Provinsi DI Yogyakarta, 2016). It is reported that agricultural activities contributed to 10% of the Gross Regional Domestic Product (GRDP) in 2024 (Badan Pusat Statistik Provinsi DI Yogyakarta, 2025). This shows that even though urbanization converts many arable lands into urban settlements, it remains prominent in Yogyakarta.

These two factors became major drivers of nitrate pollution, especially in the Ngemplak Subdistrict. The population growth of 62.48% from 2000 to 2010, as well as 7.6% land conversion rate, puts Ngemplak Subdistrict as one of the fastest growing Subdistricts in Sleman Regency (Subkhi & Mardiansjah, 2019). Additionally, Ngemplak Subdistrict has notable productivity in agricultural yields and rice production, with the largest harvest area (38.65 km^2) and one of the largest irrigated land areas (23.13 km^2). These risks are significant, given that potential groundwater pollution in Ngemplak Subdistrict is classified as extreme based on the aquifer type, overlying lithology, and depth to the water table (Neritarani & Sejati, 2021). According to Neritarani & Sejati (2021), the groundwater in Ngemplak Subdistrict is at a high risk of pollution due to it being an unconfined aquifer composed of loose unconsolidated material such as sand and gravel.

Previous studies have measured nitrate concentrations in groundwater at Ngemplak. Nitrate concentration is recorded as the highest around agricultural areas at 10–18 mg/L around the headwaters of the Kuning River and the Tepus River, according to one study by (Sutardi *et al.*, 2017). However, another study by Adi *et al.* (2023) follows the trend of an increasing nitrate concentration gradient towards the southern part of Ngemplak Subdistrict, near the

more densely populated area, reaching up to 30 mg/L during rainy seasons.

3 METHODOLOGY

3.1 Sample collection

Sample collection was conducted in April 2025 during the dry season, in Ngemplak Subdistrict, Sleman Regency. Groundwater samples were collected from 5 springs with high nitrate concentrations, as determined in a preliminary survey. Physicochemical parameters such as temperature and dissolved oxygen (DO) were measured with a DO meter (DO-5510, Lutron, USA). Elevation was recorded using GPS (*Avenza Maps*). Samples were stored in amber bottles at 4 °C and carried to the laboratory for further analysis of nitrate concentrations.

3.2 Nitrate concentration measurements

Nitrate concentrations were measured using a rapid colourimetric method (Cataldo *et al.*, 1975) with a spectrophotometer (Genesys 150, Thermo Fisher, USA). This method uses two reagents: a salicylic acid-H_2SO_4 reagent and a NaOH reagent. Standard solutions were prepared by dissolving 0.0361 g of KNO_3 in 100 mL of distilled water, yielding a stock solution containing 50 mg/L NO_3–N. This stock solution was diluted to create a series of standards. Standards containing 10, 20, 30, 40, 50 mg/L NO_3–N were prepared in a 0.25 mL aliquot. Standards, blanks, and samples were then treated with the reagents and measured at the peak absorbance (λ_{max}) within the range of 400–800 nm, which in this research was 411 nm. The concentration results of nitrate nitrogen (NO_3–N) were then multiplied by 4.43 to produce nitrate (NO_3^-) concentrations.

3.3 Isolation of nitrate-reducing bacteria

Samples were serially diluted in distilled water, and 0.1 mL of each aliquot was both poured and spread-plated onto sterile nutrient agar (NA). Colonies with distinct morphological features were selected and streaked onto agar plates to obtain pure isolates. The pure isolates were then used for further experimentation. Isolates were tested for their ability to reduce nitrate by growing them in nitrate broth with a Durham tube. After incubation for 24 hours at 37 °C, the formed nitrites were tested using nitrate reagent A (sulfanilic acid) and nitrate reagent B (α-naphthylamine). The formation of a cherry-red complex accompanied by gas is indicative of a positive result. Zinc powder was added to the colourless results to test for false negatives. Nitrate reducers were then subcultured in nutrient broth (NB).

3.4 Measurement of nitrate removal rate

All isolated nitrate-reducing bacteria were assessed for their nitrate-reducing capacity, and two of the best-performing isolates were selected for characterization. As much as 3 mL of isolates confirmed to be nitrate reducers were adjusted to the same optical density (0.3) at 600 nm (OD_{600}). Suspensions were centrifuged at 5000 rpm for 15 minutes. Pellets were collected and resuspended in 40 mL of denitrifying medium (DM). The denitrifying medium (DM) consisted of 10 g CH_3COONa; 5 g KNO_3; 3 g Yeast Extract; 0,5 g $(NH_4)_2SO_4$; 0,9 g K_2HPO_4; 0,5 g $MgSO_4 \cdot 7H_2O$; 0,2 g KH_2PO_4; and 0,1 g $CaCl_2 \cdot 2H_2O$ (per liter) (Syahputra *et al.*, 2011). Inoculums were incubated aerobically at 25 °C for 72 hours. After incubation, the nitrate concentration in the denitrifying medium was analyzed for its nitrate concentration using a rapid colourimetric method. The stock solution was adjusted to contain 700 mg/L NO3–N. The series of standard solutions is as follows: 700 mg/L, 560 mg/L, 420 mg/L, 280 mg/L, 140 mg/L. For this step, a blank of denitrifying medium without nitrate was used as the extractant. The two best-performing nitrate reducers were named bacteria isolate 1 and bacteria isolate 2.

Bacteria isolates 1 and 2 were sub-cultured in nutrient broth (NB) and inoculated in denitrifying medium (DM). Isolates were inoculated in combinations of single isolates (bacteria isolate 1, bacteria isolate 2) and two isolates (bacteria isolate 1 and bacteria isolate 2), or as co-cultures. All bacterial isolates were adjusted to the same optical density of 0.3. In cultures containing single isolates, 3 mL of each isolate was adjusted. In cultures containing two isolates, 1.5 mL of each isolate was adjusted. These suspensions were centrifuged at 5000 rpm for 15 minutes, and the pellets were resuspended in 40 mL denitrifying medium (DM) according to the combinations previously mentioned. Inocu-

lums were incubated at 25 °C for 72 hours. Nitrate was removed, and optical density (OD_{600}) was measured every 24 hours using a rapid colourimetric method. Daily nitrate removal rate was determined using the following formula:

$$x_n = \frac{C_{n-1} - C_n}{h} \quad (1)$$

Note:
n = day of measurement
x_n = nitrate removal rate on the day of measurement ($mgL^{-1}h$)
C_{n-1} = nitrate concentration on the previous day (mg/L)
C_n = nitrate concentration on the day of measurement (mg/L)
h = time consumed (hour)

Daily nitrate removal rates were then averaged over the number of measurements to get an estimation of the nitrate removal rate of each bacteria isolate.

$$\underline{x} = \frac{\sum x}{p} \quad (2)$$

Note:
$\underline{x}$ = average of nitrate removal rate ($mgL^{-1}h$)
$\sum x$ = total of nitrate removal rate on day 1, 2, and 3 ($mgL^{-1}h$)
p = number of measurements (in this study, p = 3)

3.5 Morphological and biochemical characterization of bacteria isolates

Bacteria isolates 1 and 2 were characterized based on their cell morphology, colony morphology, and their biochemical characteristics. Cell morphology of isolates, such as cell shape and gram staining, was observed under the microscope (400–1000x magnification). Colony morphology of isolates, such as colony shape, elevation, margin, surface, texture, and pigmentation, was observed through streak plates of pure isolates. Biochemical testing was performed through methyl red (MR) tests, catalase tests, coagulase tests, gelatine hydrolysis tests, casein hydrolysis tests, citrate utilization tests, and oxidase tests.

4 Results and Discussion

4.1 Isolation of indigenous bacteria

Samples were collected from 5 springs within three villages in Ngemplak Subdistrict, Sleman Regency with a high nitrate content which were assigned the name MA1, MA3, MA7, MA13, and MA15. Measurement of nitrate concentration shows that nitrate (NO_3^-) is within the range of 7.07–34.85 mg/L (Table 1). Based on regional water quality standards in the Special Region of Yogyakarta, this number was well within the established range that is acceptable to use (<44.2 mg/L for drinking water) (Governor's Regulation) of the Special Region of Yogyakarta Province Number 20 of 2008 Concerning Water Quality Standards in the Special Region of Yogyakarta Province, 2008). This number was also acceptable according to the WHO standards of <50 mg/L (World Health Organization, 2022). However, many springs were located near or adjacent to urban settlements or agricultural lands, increasing their vulnerability to nitrate contamination. In this study, MA7 and MA15, which were in proximity to urban settlements, had the highest nitrate concentrations (Table 1). This can be explained by the time of sample collection, which was conducted in April during the transition towards the dry season. According to Wang *et al.* (2025), there is a difference in the sources of nitrate pollution in groundwater between dry and wet seasons. In dry seasons, nitrate pollution is dominated by manure and domestic sewage. In contrast, fertilizer and soil nitrogen leachate became the dominant source of nitrate pollution in wet seasons.

The low concentration of nitrate as nitrogen (NO3–N) across all samples (<10 mg/L) places the groundwater sources in Ngemplak, Sleman, in the classification of micro-polluted water (Kou *et al.*, 2021), which could be growth-limiting for heterotrophic microorganisms such as aerobic nitrate reducers. In this study, it is found that there is an equal number of isolates between the two sample collection points: the one with the highest (MA7) and the one with the lowest (MA3) nitrate concentration. Therefore, it can be assumed that the abundance or diversity of microorganisms is not directly linked to denitrification performance,

TABLE 1. Bacteria isolates collected in each sample collection point.

Sample collection point	Physicochemical characteristics				Bacteria isolate
	Elevation (m)	DO (mg/L)	Temperature (°C)	NO_3^- concentration (mg/L)	
MA1	346.86	3.9	26.6	9.09	1, 2, 3, 4, 5, 6, 13
MA3	392.52	4.5	28.3	7.07	2, 4, 7, 8, 9, 14, 15, 16
MA7	354.2	3.4	27.2	34.85	3, 4, 5, 10, 11, 12, 13
MA13	331.5	3.5	27.5	7.18	3, 9, 10, 17
MA15	279.9	5.3	27.7	24.32	4, 9, 10, 18, 19
Total of isolates					19

as mentioned by Dandie *et al.* (2011). There seems to be no direct correlation between nitrate concentration and microbial diversity. The measured physicochemical characteristics are then observed to explain the relationship between nitrate concentration and isolates found in groundwater samples.

Dissolved Oxygen (DO) was measured within the range of 3.4–5.3 mg/L (Table 1). DO indicates water quality and correlates with nitrate concentration (Espinosa-Díaz *et al.*, 2021). According to Table 1, although MA3 and MA7 have the same number of isolates, their DO levels differ. DO levels affect the occurrence of microorganisms in the isolation process. More bacteria are isolated from MA7 using the pour plate method, in which DO levels are lower. In contrast, more bacteria are isolated via the spread plate method from MA3, where DO levels are high (MA3). This suggests that more microorganisms can grow aerobically in samples with high DO levels, and vice versa.

DO levels also affect microbial community structure and functional genes relating to nitrate reduction. In general, respiratory nitrate reductase (Nar) is a gene that codes for nitrate reductase used in anaerobic nitrate reduction, meanwhile periplasmic nitrate reductases (Nap) codes for nitrate reductase used in aerobic nitrate reduction (Ji *et al.*, 2015). However, it was found that both Nap and Nar coexist and interact with each other during aerobic conditions, which supports nitrate reduction, thus proving the positive correlation between DO levels and nitrate reduction (Liu *et al.*, 2021). In a study by Liu *et al.* (2021)It is shown that aerobic conditions (DO $\geq$2 mg/L) support interactions within microbial communities that drive nitrate transformation. In contrast, low-oxygen conditions disrupt patterns of co-occurrence and reorganize microbial community structures, explaining the dominance of nitrate transformation at high DO levels. This suggests an inverse relationship between DO levels and nitrate concentration, where, in areas with lower DO levels, nitrate concentrations are higher due to the slower rate of transformation. It is observed in MA7, where DO is the lowest (3.4 mg/L), nitrate concentration is the highest (34.85 mg/L) (Table 1). In contrast, DO is high where nitrate concentration is the lowest (7.07 mg/L).

It should also be noted that MA7 has the lowest temperature (27.2 °C) among the rest. Though the variance between samples is small, temperature is one of the main parameters affecting the nitrate reduction process, with nitrate reduction optimally carried out within the range of 25–37 °C (Rajta *et al.*, 2020). Low temperature affects nitrate removal by inhibiting enzyme activity, resulting in the low efficiency of nitrate reduction (Kou *et al.*, 2021). The correlation between DO levels, temperature and nitrate concentration could also be associated with the result for the sample collection point MA3 (Table 1). Sample collected in MA3 shows one of the lowest concentrations of nitrate (7.07 mg/L), accompanied by higher DO levels (4.5 mg/L) than most, and one of the highest recorded temperatures (28.3 °C) to match.

However, the highest DO level is recorded at sample collection point MA15, at 5.3 mg/L, where the nitrate concentration is also among the highest, despite the previously mentioned negative correlation between DO and nitrate concentrations. This could be associated with the low elevation of MA15 (279.90 m). DO level is dynamic and can be influenced by many factors, including elevation. Water located at a

TABLE 2. Nitrate removal of bacteria isolates.

Isolate	Percentage of nitrate removal (%)
1	18.46
2	6.99
3	5.99
4	1.57
5	2.61
6	5.20
7	4.41
8	5.41
9	4.90
10	2.92
11	2.77
12	1.01
13	5.74
14	4.84
15	2.53
16	4.21
17	5.00
18	4.89
19	2.26

high elevation will generally have a lower DO level, and vice versa (Chapra *et al.*, 2021). Therefore, the low elevation could contribute to the high DO level at MA15. Most importantly, elevation also affects nitrate pollution. It is reported by Fantong *et al.* (2013) The springs located at lower elevations will be more vulnerable to nitrate pollution.

A total of 19 morphologically distinct bacteria isolates were found and tested for their capability of reducing nitrate via the Griess assay test. It was found that all of the isolates could reduce nitrate. Isolates were then screened for their efficiency in reducing nitrate aerobically within 24 hours. The control is a denitrification medium without treatment. All isolates show nitrate reduction ranging from 2.26% to 18.46%, except for one, which shows no capability for aerobic nitrate reduction. Two bacteria isolates, isolates 1 and 2, were selected based on their efficiency in reducing nitrate (Table 2).

4.2 Nitrate reduction rate of indigenous aerobic nitrate-reducing bacteria

These two bacteria isolates were then inoculated in single cultures and mixed cultures. The correlation between nitrate reduction and cell growth is shown in Figure 1. In nitrate-reducing microorganisms, cell growth should be inversely proportional to nitrate concentration (Syahputra *et al.*, 2011). This correlation

TABLE 3. Nitrate removal of bacteria isolates.

Bacteria isolate	NO_3^- reduction rate ($mgL^{-1}h$)			
	Day 1	Day 2	Day 3	Average
1	27.24	47.93	46.83	40.67
2	17.6	4.11	-4.78	5.64

is evident in the decline in nitrate concentration over the course of cell growth in Figure 1. Within the first 24 hours, all the cultures experienced periods of significant growth (log phase). After 24 hours, cell growth in all cultures had stagnated, with a slight decline afterwards, signifying a shift towards the stationary phase and eventual death phase. The prolific cell growth during the first 24 hours also coincided with the notable decline in nitrate concentration, especially in bacteria isolate 1. Similar results were reported by Zhang *et al.* (2019), where the log phase of mix-cultured aerobic denitrifying bacteria occurred before the first 36–48 hours. In another study by Syahputra *et al.* (2011), most nitrate reduction occurs within 12 hours of incubation period, which is also in line with the results in this research.

According to Table 3, it is also evident that a single culture of bacteria isolates 1 shows the best nitrate reduction rate in comparison to bacteria isolate 2. This could be further explained by the species of isolated bacteria which requires further phenotypical characterization.

4.3 Characterization of aerobic nitrate reducing bacteria

Characterization bacteria isolate is conducted by observation of cell morphology, colony morphology, and biochemical tests. As shown in Table 4, it can be summarized that all isolated bacteria resulted in negative gram staining with rod or round cell shape. Colony shape, margin, surface, texture varied; and colour ranged from white to yellow. Biochemical tests conducted also showed varied results (Table 5). The characteristics of the isolates were matched with Bergey's Manual of Systematic Bacteriology 2nd Edition (Brenner *et al.*, 2005; Krieg *et al.*, 2010). Bacteria isolate 1 proved to have similar characteristics to *Pseudomonas sp.*, and isolate 2 has similar characteristics to *Flavobacterium sp.*

Pseudomonas sp is said to have a vigorous denitrification ability and is one of the most

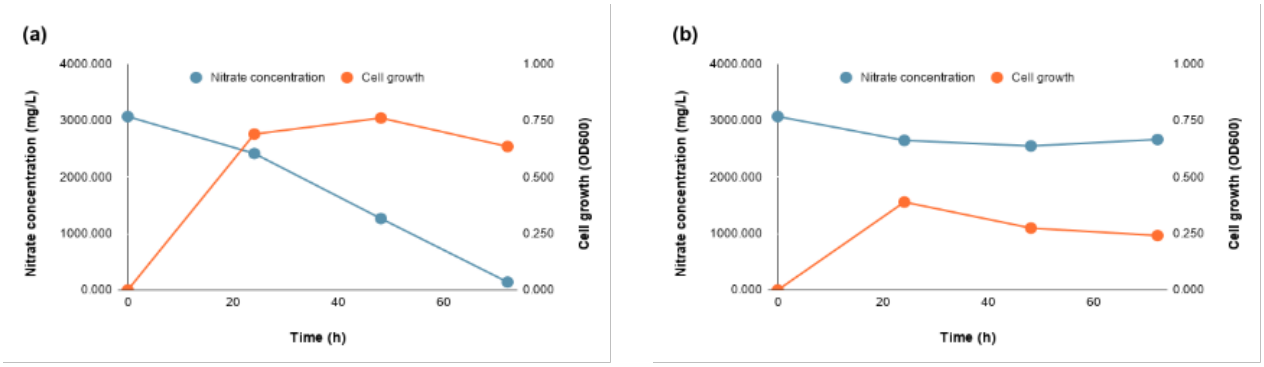

FIGURE 1. Nitrate reduction and cell growth of bacteria isolates in 72 hours: a) Bacteria isolate 1; b) Bacteria isolate 2.

TABLE 4. Nitrate removal of bacteria isolates.

Characteristics	Isolate 1	Isolate 2
Gram staining	–	–
Cell shape	Rod	Rod
Shape	Irregular	Circular
Margin	Lobate	Entire
Surface	Wrinkled - Rough	Smooth
Elevation	Flat	Flat
Color	Creamy white	Yellow
Texture	Butyrous	Slimy

TABLE 5. Nitrate removal of bacteria isolates.

Characteristics	Isolate 1	Isolate 2
MR	–	–
Catalase	+	+
Coagulase	–	–
Gelatinase	–	+
Caseinase	–	+
Citrate utilization	+	–
Oxidase	+	–

+ = positive; – = negative

active denitrifiers (Lalucat *et al.*, 2006). *Pseudomonas sp* is a facultative anaerobe, allowing it to perform denitrification under anaerobic conditions. However, previous studies suggest that it exhibits denitrifying activities when cultured aerobically (Miyahara *et al.*, 2010; Wang *et al.*, 2022; Zhang *et al.*, 2019; Zhao et al., 2018). This is attributed to the napA gene, which codes for Nap enzyme, which can reduce nitrate aerobically and has a high affinity towards nitrate (Zhao *et al.*, 2018).

Based on the research conducted, bacterial isolate 1, which shows high similarity to *Pseudomonas sp.*, has a nitrate removal percentage of 95.38% and a nitrate reduction rate of 40.67 mg L^{-1} h. According to previous studies conducted on *Pseudomonas sp.*, it is found that they are capable of aerobic nitrate removal of 97.9% (Zhao *et al.*, 2018) and 93.0–94.5% (Ji *et al.*, 2014) in 24 hours. The difference between the nitrate reduction rate in this research may be due to the high concentration of nitrate, a difference in the C/N ratio, which has been proven to affect nitrate removal efficiency (Zhao *et al.*, 2018), and the simpler composition of trace elements in the denitrifying medium.

Throughout the course of nitrate reduction by bacteria isolate 1, plenty of gas bubbles were observed daily within the medium, attached to the interior of the conical tube. These gas bubbles indicate the transformation of nitrogen into its gaseous form, N_2O and N_2, as a form of denitrification. In a previous study using nitrogen balance analysis, it was reported that *Pseudomonas sp.* converted more nitrate to N_2 than to N_2O (Zhao *et al.*, 2018), which is attributed to the expression of the nosZ gene encoding nitrous oxide reductase. This enzyme catalyzes the conversion of N_2O, a greenhouse gas, into N_2, thereby completing the denitrification process and limiting the release of dangerous gases into the atmosphere (Hurtado-Martinez *et al.*, 2021).

Flavobacterium sp. is reported to be a denitrifier, demonstrating the ability to reduce nitrate to N_2 as its final product fully (Krieg *et al.*, 2010). Nitrate reduction efficiency of *Flavobacterium sp* was measured by Horn *et al.*, (2005), where nitrate was reduced by >70% in 72 hours, the same time that it took in this experiment for bacteria isolate 2 to reduce 13.23% of nitrate at the rate of 5.64 $mgL^{-1}h$. This difference in efficiency can be attributed to the de-

sign of the experiment by Horn *et al.* (2005) conducted in anoxic conditions. Despite the ability to use oxygen as an electron acceptor, the nitrate reductase enzyme in *Flavobacterium sp.* may be sensitive to oxygen. There hasn't been any further research on nitrate reductase gene expression in *Flavobacterium sp.*, thereby limiting our knowledge of nitrate metabolic pathways. *Flavobacterium sp.* grows optimally at 25 °C (Horn *et al.*, 2005) Thus, it can also be assumed that repeated inoculation at 37°C might reduce the viability of the cells and lower the nitrate reduction efficiency.

4.4 Application of mixed culture in aerobic nitrate reduction

In mixed-culture inoculation, effects arise from interactions between microorganisms. Theoretically, these interactions affect nitrate reduction positively, especially in less favourable conditions (Kou *et al.*, 2021; Li *et al.*, 2022; Zhang *et al.*, 2019). However, in this research, it is observed that the application of mixed culture has no significant effect on nitrate reduction.

This is apparent when comparing the optical density (OD_{600}), where the most prolific cell growth is observed in a single culture of bacteria isolate 1 (Figure 2). It can be seen that the optical density of bacterial isolate 1 increased the most within 24 hours and reached a peak of 0.76 over the next 24 hours. Other cultures show similar results.

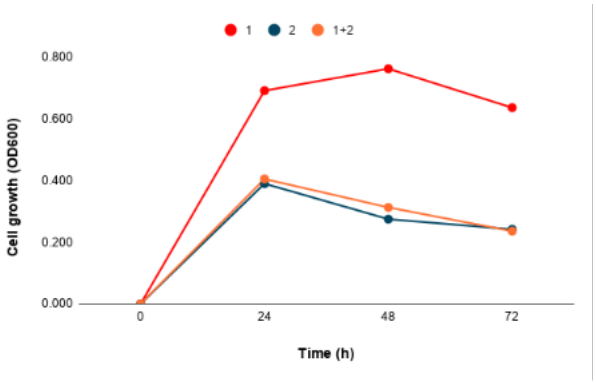

FIGURE 2. Cell growth of bacteria isolates in 72 hours: a) Bacteria isolate 1; b) Bacteria isolate 2; c) Bacteria isolate 1 and 2.

Similarly, bacteria isolate 1 also outperformed all other cultures of isolates in nitrate reduction. Looking at the final nitrate concentrations measured after 72 hours of incubation (Figure 3), a total of 95.38% nitrate was removed by bacteria isolate 1, lowering the initial NO_3^- concentration from 3070 mg/L (NO_3–N = 693 mg/L) to only 141.97 mg/L (NO_3–N = 32.04 mg/L). Other cultures showed similar results to one another, with nitrate removal of 13.23–13.57%.

TABLE 6. Nitrate removal of bacteria isolates.

Isolates	NO_3^- reduction rate ($mgL^{-1}h$)			
	Day 1	Day 2	Day 3	Average
1+2	21.56	-4.01	-0.19	5.79

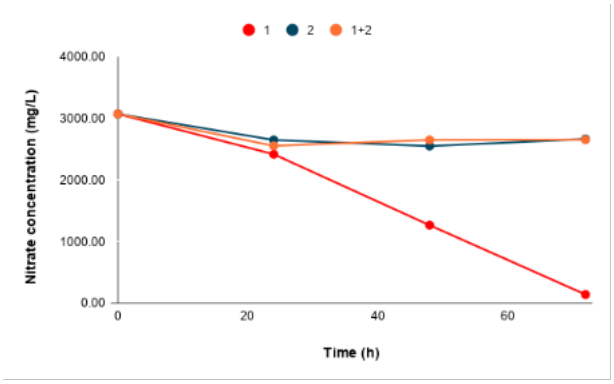

FIGURE 3. Nitrate reduction of bacteria isolates in 72 hours: a) Bacteria isolate 1; b) Bacteria isolate 2; c) Bacteria isolate 1 and 2.

According to Table 6 mix-culturing has no significant effect on the nitrate reduction rate. When compared, the single-culture isolate 1 outperformed all other cultures in nitrate reduction efficiency (Table 3). Culture containing a mix of two isolates did not provide a better result than those with a single isolate.

Compared to previous studies, mix-cultured isolates supposedly provide better results than those of single-cultured bacteria isolates (Li *et al.*, 2022; Oljira *et al.*, 2018; Zhang *et al.*, 2019). The explanation might lie in the specific dynamics among the microorganisms isolated in this experiment. The low rate of nitrate reduction and optical density in Table 6 and Figure 2 by mixed culture, especially compared to bacteria isolate 1, suggests competitive traits between bacteria species.

Mixed culture containing bacteria isolates 1 and 2 does not improve nitrate reduction due to it scoring a similar result with a single culture

containing only bacteria isolate 2, and significantly lower than bacteria isolate 2. This might be due to the competition for nutrients between bacteria isolate 1 and bacteria isolate 2. *Pseudomonas sp*. and *Flavobacterium sp*. are both reported to be competitive in a nutrient-deficient environment with minimal flow. *Flavobacterium sp*. has a faster growth rate, thus allowing an advantage in accessing more nutrients and dominating the competition (Zhang *et al.*, 2013).

5 Conclusion

Two nitrate-reducing bacteria have been isolated from groundwater in Sleman, Yogyakarta. Both isolates were identified as *Pseudomonas sp* and *Flavobacterium sp*, with nitrate reduction efficiencies of 95.38 % and 13.6 %, respectively, in single cultures. However, mixed cultures did not have a positive effect on nitrate reduction due to specific antagonistic interactions between the isolates. Due to their pollutant-reducing abilities, both isolates have great potential for in situ nitrate pollutant bioremediation.

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