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Assessment of Nitrate Reduction by Microbes in Artificial Groundwater Medium

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ABSTRACT

There are significant reasons for nitrate contamination in groundwater (Delhi, India): sewage, runoff from landfill sites, nitrogenous chemical fertilisers, and pesticides from agricultural lands. The highest recorded concentration of nitrate in Delhi's groundwater is reported to be 1500 mg/l. Consumption of high nitrate through water may pose serious health problems in humans, especially children (below five years). The study's primary objective was to isolate and identify nitrate-remediating microbes from the nitrate-contaminated site Okhla Barrage, located on the Yamuna River in Delhi, India. A total of 11 different strains were isolated from this site. Among these four strains exhibited 40%–50% remediation efficiency at a nitrate concentration of 1000 mg/l. Molecular characterisation revealed that these four strains, *Enterobacter aerogenes*, *E. coli* K12, *Klebsiella oxytoca* and *Lelliottia amnigena*, belong to the Enterobacteriaceae family. This study assessed the nitrate remediation potential of isolated microbes in groundwater with 1000 and 1500 mg/l nitrate concentrations. By using a 2% inoculum, the microbes were incubated anaerobically at room temperature for ten days. Nitrate concentrations were monitored every 48 hours. *Lelliottia*, *E. coli*, and *Enterobacter* reduced nitrate (1500 mg/l) by approximately 42%, 24%, and 29%, respectively, while *K. oxytoca* showed minimal reduction. *L. amnigena* exhibited superior nitrate removal efficiency compared to other strains. According to the reported data, these strains are known to reduce nitrate concentrations of 620 mg/l. However, our findings demonstrate a remarkable nitrate remediation capacity of 1500 mg/l, showcasing a novel contribution to this study. Further detailed analysis for condition optimisation and association of microbe-microbe could be more helpful.

Keywords: Enterobacteriaceae, groundwater, microbe-microbe association, nitrate, remediation

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INTRODUCTION

Many pollutants, such as heavy metals and organic and inorganic pollutants, cause water pollution. Among them, a significant contribution is from inorganic pollutants. Nitrate is one of those inorganic pollutants with a substantial presence in groundwater and has become one of the leading problems worldwide (Zhang et al., 2018; Zhao et al., 2022). In India, its high concentration in drinking water is regulated by an Indian agency, BIS, which sets the permissible limit (ideal for use) at 45 mg/l (Tyagi et al., 2020; Raja & Neelakantan, 2021). Excess nitrate concentration in groundwater causes significant effects on human health and the environment (Addiscott & Benjamin, 2004; Karunanidhi et al., 2021). These include respiratory problems (Fewtrell, 2004), hypertension (Malberg et al., 1978), goiter (Morris et al., 2011), thyroid cancer (Tariqi & Naughton, 2021), genetic mutations, gastrointestinal cancer (Hameed et al., 2021), congenital disabilities, and Blue Baby syndrome (Majumdar, 2003). The main reason for the rising nitrate concentration is the excessive use of nitrogenous chemical fertilisers for agricultural activity (Singh & Craswell, 2021). Additionally, inadequate treatment of domestic and industrial wastewaters, leachate from landfill sites and livestock manure contribute to nitrate pollution.

Delhi, the capital of India, is heavily affected by nitrate contamination of groundwater (Tirkey et al., 2017). Some parts of Delhi, like Chattarpur, Inderlok, and Inderapuri, are affected by a high nitrate concentration of 1500mg/l according to 'groundwater yearbook 2011-12' by CGWB (Central Ground Water Board). Consequently, BIS (Bureau of Indian standard) is allowed to set the maximum limit (this concentration can be treated, but above this level, water can be shut down until corrected) for nitrate at 100 mg/l for drinking water (Reddy, 2023). Furthermore, several other states, including Bihar, Haryana, Uttar Pradesh, Punjab, Rajasthan, Karnataka, and Kerala, also contend with elevated nitrate concentrations, surpassing the safety benchmark of 45 mg/l (Sikdar, 2018).

A high nitrate concentration in drinking water creates many health issues in humans. Approximately 85% of people depend on groundwater. Nitrate in water makes it unsafe for human consumption (Ward et al., 2018). It is, therefore, necessary to treat the polluted groundwater. Several technologies have been developed to remove nitrate from drinking water. It includes ion exchange (Jaeshin & Benjamin, 2004), reverse osmosis (Schoeman & Steyn, 2003; Kim et al., 2007), electrodialysis (Elmidaoui et al., 2003; Sahli et al., 2006), chemical denitrification (Lin & Wu, 1996), ion exchange (Matos et al., 2006) and catalytic reduction (Reddy & Lin, 2000; Maia et al., 2007). These techniques are effective in nitrate removal, but the commercial application of these techniques is pretty expensive.

On the other side, many researchers and scientists focus on bioremediation. Bioremediation of nitrate became practically possible when using exogenous carbon sources (Li et al., 2021). A large number of organic carbon sources are required in the denitrification process, where microbial strains use organic carbon as electron donors (Qin

et al., 2017). Several strains with known nitrate remediation potential are listed in Table 1. Their remediation capacity typically extends only to concentrations up to 620 mg/l. As per the literature, it is known that these isolates (*E. coli*, *Enterobacter aerogenes*, *Lelliottia amnigena*) have nitrate remediation potential. However, our study showed that these strains now remediate high nitrate concentration.

Table 1
Nitrate remediation potential of reported microbial strains

Microbial strain/ consortium	Initial nitrate concentration (mg/l)	Percentage of nitrate remediation/reduced up to (%)	References
1. *Escherichia coli*	620	90	Bing & Hollocher, 1988
2. *Enterobacter aerogenes*	620	90	Bing & Hollocher, 1988
3. *Enterobacter amnigenus*	138	50	Fazzolari et al., 1990
4. *Pseudomonas* sp. KW1 and *Bacillus* sp. YW4	100	99.4	Rajakumar et al., 2008
5. *Pseudomonas putida* AD-2	254.6	95.9	Kim et al., 2008
6. *Bacillus subtilis JD-014*	100	98.99	Yang et al., 2021
7. *Enterobacter cloacae DK-6*	101.70	86.98	Liao et al., 2022
8. *Pseudomonas aeruginosa*	100	93	Rajta et al., 2020

All isolated strains had the potential to remediate nitrate (Thakur & Gauba, 2021). Additionally, these microbes also showed nitrate remediation in groundwater except *K. oxytoca*. An *in-silico* study was also done of two microbes where *L. amnigena* has assimilatory and respiratory nitrate reductase genes while *E. coli* K12 has only the respiratory nitrate reductase gene (Thakur & Gauba, 2023). As per the literature, *Enterobacter aerogenes* had only the respiratory nitrate reductase gene, a membrane-bound enzyme (Riet & Planta, 1975).

The assimilation of the nitrate reductase gene found in the cytoplasm is involved in the nitrate assimilation process. It converts the nitrate into amino acids, which are finally uptake by microbes to increase their number. Conversely, the respiratory nitrate gene is a membrane-bound enzyme involved in denitrification, which converts the nitrate into nitrogen. Therefore, the presence of both processes in *L. amnigena* makes this bacterium more potent in nitrate remediation in comparison with the other two (*E. coli* K12 and *Enterobacter aerogenes*) (Thakur & Gauba, 2024). Besides this, all microbes showed nitrate remediation under strict anaerobic conditions, whereas *Enterobacter aerogenes* remediated nitrate under aerobic and anaerobic conditions (Madmanang & Sriwiriyarat, 2019).

In the previous study, four microbes were isolated belonging to the Enterobacteriaceae family, which were subsequently molecularly characterised and identified as *Lelliottia amnigena*, *E. coli* K12, *K. oxytoca* and *Enterobacter aerogenes* (Thakur & Gauba, 2021).

This paper assessed the potential of these isolated microbes in a groundwater medium containing nitrate concentrations of 1000 mg/l and 1500 mg/l. The microbes exhibited 25%–42% nitrate remediation except *K. oxytoca* at the 1500 mg/l concentration. *L. amnigena* demonstrated the highest remediation potential compared to the other two strains.

MATERIALS AND METHODS

Study Area

Water samples were collected from the polluted Yamuna River site (Okhla Barrage) as per the reported data (CPCB, 2015; Srivastava et al., 2015), as presented in Figure 1.

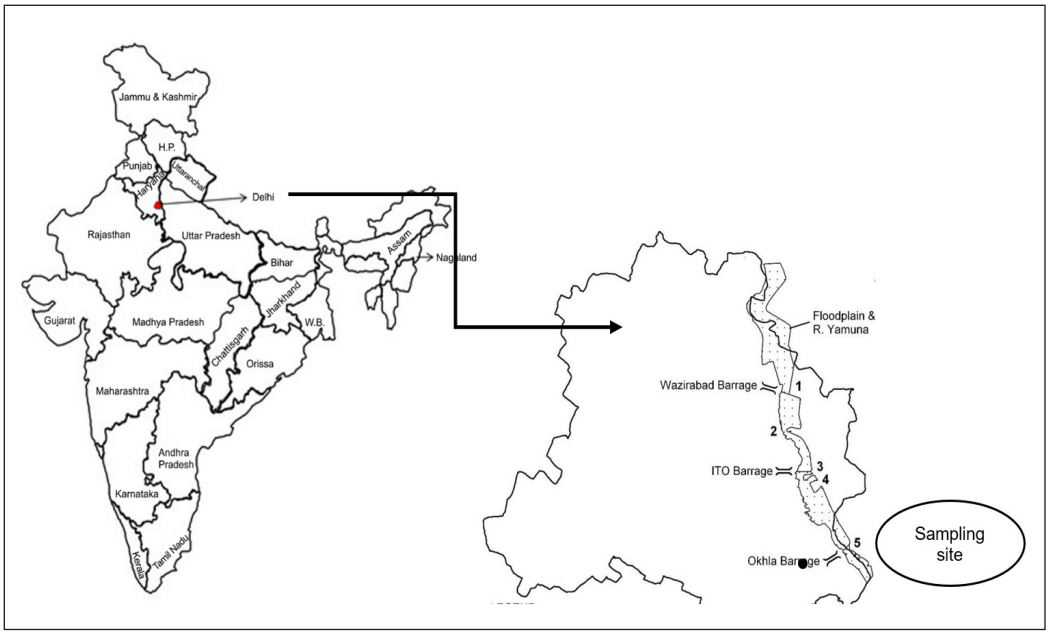

Figure 1. Sampling site of Yamuna River (Okhla Barrage) Delhi

Isolation of Microbes from Polluted Yamuna River Site

The stock solution was prepared where 1 ml water sample was taken in 10 ml demineralised water; further, the samples underwent serial dilution ranging from 10^{-1} to 10^{-9}, and 100µl aliquots were transferred from dilutions 10^{-7} to 10^{-9} onto nutrient agar petriplates containing 500 mg/l nitrate concentration. These plates were then incubated at 37°C for 48 hours. Following incubation, each colony was carefully picked up using a sterile inoculating loop and transferred to fresh petri plates containing nitrate. Quadrant streaking was performed until pure colonies were obtained. Concurrently, Gram staining was conducted repeatedly until consistent morphology was observed on two consecutive occasions. The remediation

rate of isolated microbes was calculated at 500–1000 mg/l nitrate concentration, and they were further Molecular characterised (Thakur & Gauba, 2021).

Assessment of Nitrate Remediation Rate of Isolated Microbes in Groundwater Medium

Preparation of Groundwater Medium

Groundwater media was designed with composition KH_2PO_4- 0.235 g/l, K_2HPO_4 - 0.09 g/l, NH_4Cl - 0.1 g/l, $MgCl_2$ - 0.0248 g/l, $CaCl_2$- 0.141 g/l (Pan et al., 2014) and 5 ml/l Vitamin and minerals mixture (Lovley et al., 1988), amended with acetate (10 mM concentration) and nitrate (1000 and 1500 mg/l) dissolved in demineralised water where acetate prepared from sodium acetate and nitrate from potassium nitrate.

Preparation of Nitrate Standard Solution

The process involved drying 3.6 g of KNO_3 at 105°C for 24 hours, then dissolved in 1000 ml of deionised water, resulting in a solution labelled 1000 mg/L nitrate-nitrogen. Subsequent calculations converting between nitrate and nitrate-nitrogen were conducted using the Equations 1 and 2:

$$\text{Nitrate} = \text{Nitrate-nitrogen} \times 4.43 \quad [1]$$

$$\text{Nitrate-nitrogen} = \text{Nitrate} \times 0.226 \quad [2]$$

Inoculation of Enterobacteriaceae Strains in Groundwater Medium

Eleven microbes were isolated from a polluted site (DebRoy et al., 2012). Among these, four bacterial strains exhibited promising capabilities in nitrate remediation, effectively reducing it to a concentration of 1000 mg/l. All four strains belong to the Enterobacteriaceae family. These isolates were then inoculated in a groundwater medium containing 1000 mg/l and 1500 mg/l of nitrate concentration. Each strain was inoculated (2% inoculum) in a groundwater medium and incubated at room temperature (27°C), in an anaerobic state, and kept under dark conditions for 10 days (192–240 hours). Following the incubation period, samples were extracted to assess the residual nitrate concentration in the media at 420 nm (LABMAN UV-VIS), concurrently with the measurement of bacterial growth at 600 nm. Both the optical density of the microbes and the nitrate concentration in the media were monitored at regular intervals. The optical density was primarily measured by bacteria, followed by centrifugation to separate the cells from the supernatant. The supernatant was then collected to determine the nitrate concentration. In both conditions, media is used as blank and set as zero. Then, a sample reading was taken. LABMAN UV-VISIBLE spectro was used to measure the optical density.

Method to Calculate Nitrate Remediation Rate (Sodium Salicylate Method)

Cultures were centrifuged for 10 mins at 8609RCF (Relative Centrifugal Force). The supernatant (40 µl) and 5% salicylic acid in sulphuric acid (200 µl) were added. The mixture was vortexed and incubated in the dark for 10 minutes (DebRoy et al., 2012). The reaction was stopped after adding 2 ml NaOH (4N NaOH). The absorbance was taken at 420 nm after 20 mins of incubation. The nitrate remediation rate was calculated using Equation 3.

$$Degree\ of\ remediation = \frac{(X_0 - X_1) \times 100}{X_0} \quad [3]$$

Where X_0 is the initial amount of nitrate, and X_1 is the amount after remediation. The final results are calculated using control samples (media without microorganisms) (Chouhan et al., 2012).

A control sample (media with nitrate concentration but devoid of microbes) was included for each experimental set, and its initial optical density was recorded at 420 nm. Following incubation, the optical density of samples containing both nitrate concentration and microbes was measured. The percentage change in nitrate concentration was calculated using Equation 4.

$$\frac{(Initial\ concentration\ of\ nitrate - Final\ concentration\ of\ nitrate)}{Initial\ concentration\ of\ nitrate} \times 100 \quad [4]$$

Initial Concentration of Nitrate

This method uses 5% salicylic acid in sulphuric acid and sodium hydroxide chemical. This method is based on an electrophilic aromatic substitution reaction in which Sulfosalicylic acid (5% salicylic acid in sulphuric acid) reacts with nitrate and forms nitrobenzoic compounds. After adding sodium hydroxide (alkaline condition), this nitrobenzoic compound converts into a quinoid compound, which gives it a yellow colour. The darkness of the yellow colour shows the presence of high nitrate in media and vice versa. It is a colourimetric method. This yellow complex gives maximum absorption at 420 nm wavelength, and the absorbance is directly proportional to the nitrate content.

Molecular Characterisation of Microbes by 16S rRNA Gene

Genomic DNA Extraction from Microbes

Each isolated colony was transferred into 5 ml of Nitrate Broth and incubated at 37°C with agitation at 200 rpm in a shaker incubator. After growth, 1.5 ml of bacterial culture was centrifuged at 4°C for 10 minutes at 4000 rpm. Following centrifugation,

the supernatant was carefully removed, and the pellet was resuspended in 180 µl of lysozyme solution (pH 8.0) before incubating at 37°C for 30 minutes until lysis occurred. The lysed cells were then briefly kept at -80°C for 10 minutes. Subsequently, 30µl of lysis solution (pH 8.0) was added, and the mixture was incubated on ice for 10 minutes. A volume of Phenol-Chloroform (1:1) was added to the tube to facilitate DNA separation from proteins (1). After centrifugation at 8000 rpm for 5 minutes at 4°C, the upper layer containing DNA was carefully transferred to a new tube. Chloroform (500 µl) was added to this upper layer, followed by another round of centrifugation. After centrifugation, the upper layer was collected, and 1/10th part of 1M NaCl and 2–2.5 volumes of absolute ethanol were added. The tube was then placed at -80°C for 30 minutes and subsequently centrifuged at 10,000 rpm for 30 minutes at 4°C. After centrifugation, the supernatant was completely decanted, and the DNA pellet was washed with 70% chilled ethanol. The washed pellet was centrifuged at 8000 rpm for 5 minutes at 4°C, dried, and dissolved in 20 µl of TE buffer.

Amplification of 16S rRNA Gene

Microbes were identified using the polymerase chain reaction (PCR) method, with bacterial genomic DNA isolated following the Sambrook protocol. Universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492r (5-CGGTTACCTTGTTACGACTT-3) were amplified. The reaction mix was prepared in a total volume of 20 µl, comprising 10µl of PCR master mix (Genei), 0.4 µl of Primer F, 0.4 µl of Primer R, 2 µl of DNA template (50 ng/µl), and 7.2µl of nuclease-free water (Hyclone). The amplification was carried out with a thermal profile consisting of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 2 minutes. A final extension step at 72°C for 10 minutes was performed. The PCR products were then analysed using 1% agarose gel electrophoresis. After electrophoresis, the gel was stained with ethidium bromide and visualised under UV light to detect the amplified DNA bands.

RESULTS

Four different microbes were isolated and identified from water samples of the polluted site of Yamuna River (Okhla Barrage). After molecular characterisation, the sequence of the 16S rRNA gene of each strain was submitted to GenBank. They belong to the Enterobacteriaceae family and are identified as *Enterobacter aerogenes* (MN252552), *E. coli* K12 (MN754025), *K. oxytoca* (MT457847) and *Lelliotia amnigena* (MN647560). Further, the remediation rate of microbes was checked in a Groundwater medium containing various nitrates.

Remediation Rate of Strains in Groundwater Medium

Following an initial incubation period of 2 days, a sample was extracted after 48 hours to assess the remaining nitrate concentration. This process was repeated at 48-hour intervals. Figures 2 to 7 illustrate the remediation rates of microbes at different nitrate concentrations of 1000 mg/l and 1500 mg/l. Analysis of the readings suggests that *Lelliottia amnigena*, *E. coli* K12, and *Enterobacter aerogenes* exhibit significant potential for remediating nitrate in groundwater medium. The comparative remediation rates of the isolated microbes are presented in Table 2.

Figure 2 represents the optical density and remediation rate with time. Per our experimental observation, at least 48 hours are required for microbial growth at those provided conditions (room temperature, anaerobic and dark conditions) Table 3. In Figure 2 (*L. amnigena*), the optical density increases steadily over time, yet after 200 hours (8 days), a slight downturn is observed, although the remediation rate continues to rise consistently.

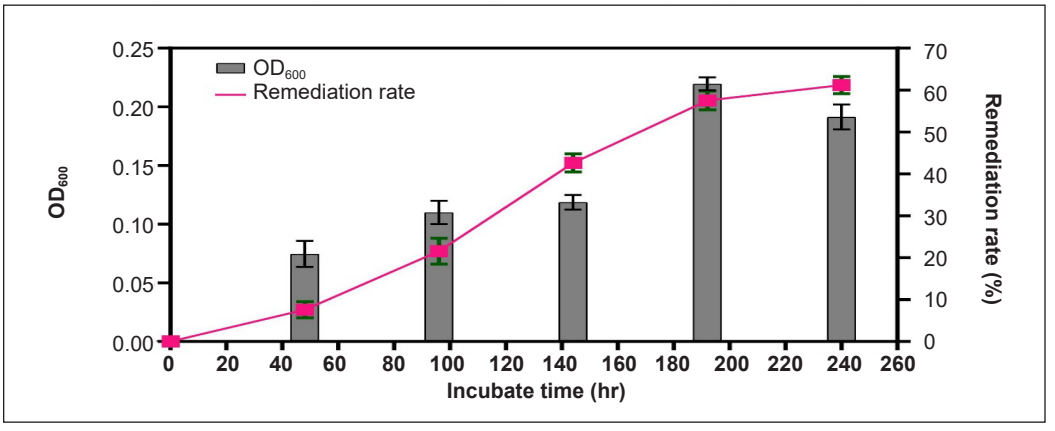

Figure 2. Absorbance and nitrate reduction by *Lelliottia amnigena* at 1000 mg/l nitrate concentration in groundwater medium

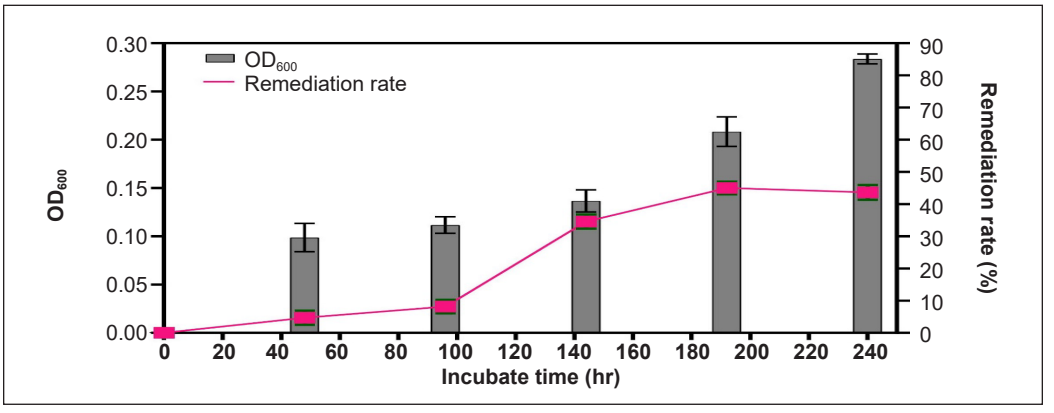

Figure 3. Absorbance and nitrate reduction by *Escherichia coli* K12 at 1000 mg/l nitrate concentration in groundwater medium

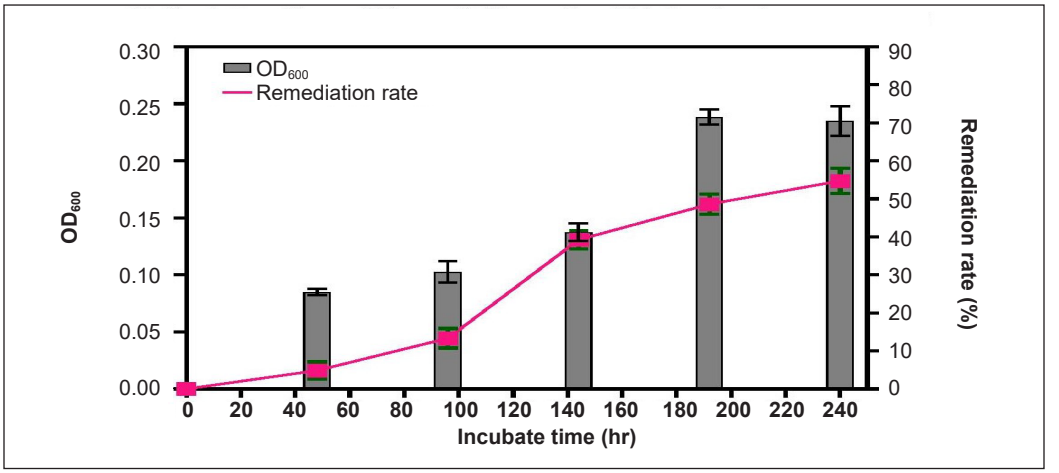

Figure 4. Absorbance and nitrate reduction by *Enterobacter aerogenes* at 1000 mg/l nitrate concentration in groundwater medium

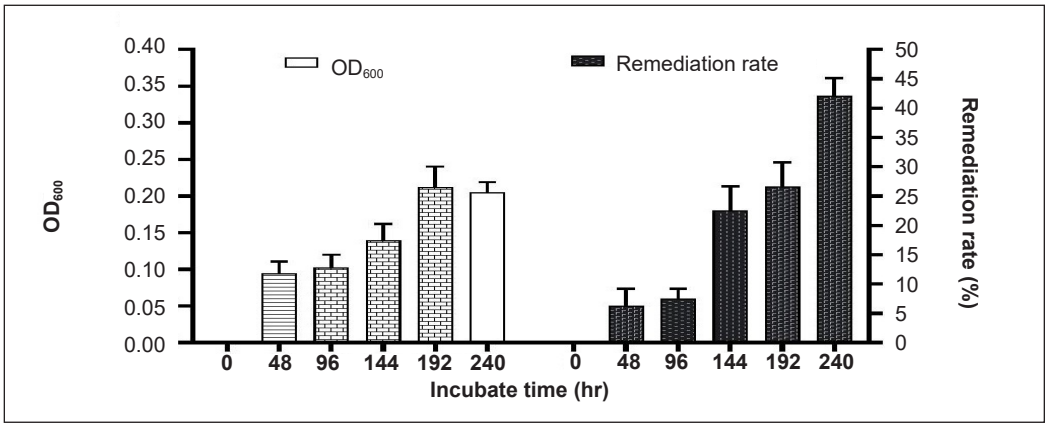

Figure 5. Absorbance and nitrate reduction by *Lelliottia amnigena* at 1500 mg/l nitrate concentration in groundwater medium

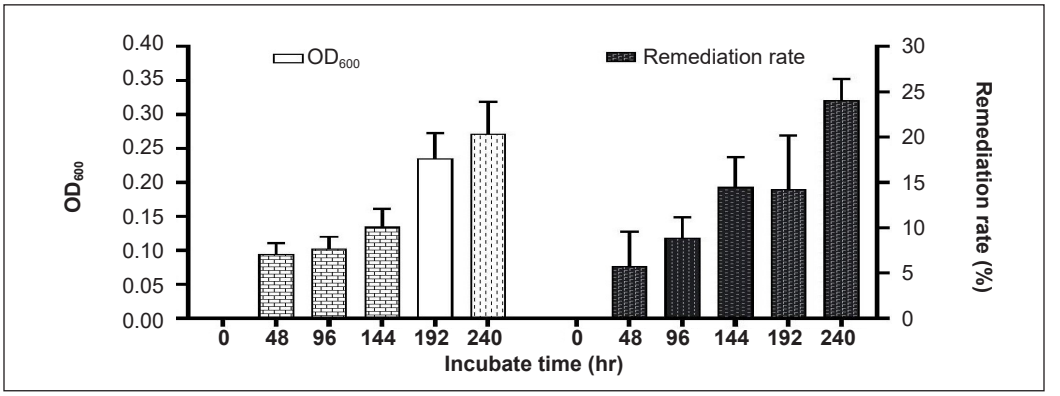

Figure 6. Absorbance and nitrate reduction by *Escherichia coli* K12 at 1500 mg/l nitrate concentration in groundwater medium

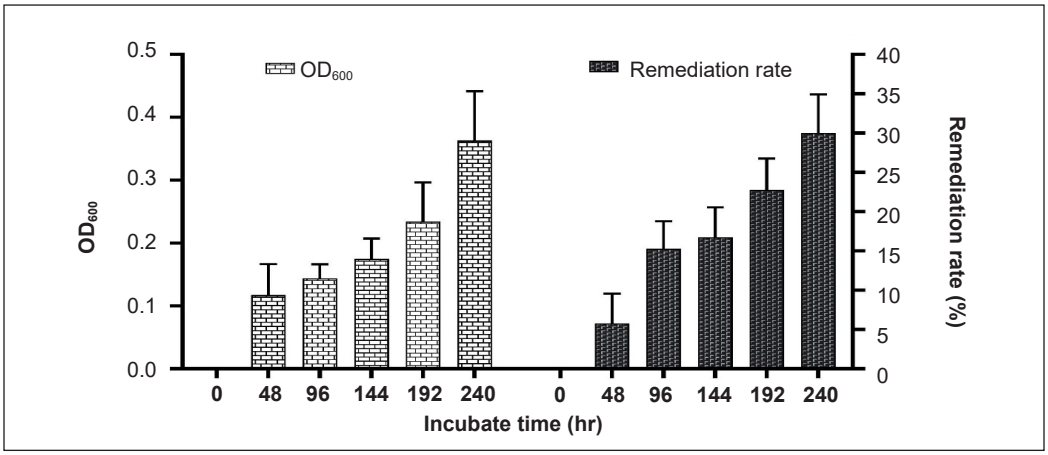

Figure 7. Absorbance and nitrate reduction by *Enterobacter aerogenes* at 1500 mg/l nitrate concentration in groundwater medium

Table 2
Comparison of nitrate remediation rate among microbes in groundwater medium

Enterobacteriaceae strains	Remediation rate at 1000 mgl^{-1} nitrate (Total 10 days incubation)	Remediation rate at 1500 mgl^{-1} nitrate (Total 10 days incubation)
Lelliottia amnigena	~61%	~42%
Escherichia coli K12	~43%	~24%
Enterobacter aerogenes	~54%	~29%

Table 3
Observed optical density of nitrate remediation of isolated strains at 420 nm

Optical Density	*Lelliottia amnigena*	*E. coli* K12	*Enterobacter aerogenes*	Optical density of control
Mean optical density at 1000 mg/l nitrate concentration	0.343	0.534	0.428	0.938
Mean optical density at 1500 mg/l nitrate concentration	0.576	0.752	0.706	0.996

Figure 3 (*E. coli* K12) exhibits growth for up to 10 days, with remediation activity persisting until day 9 before experiencing a minor decline. Conversely, in Figure 4 (*Enterobacter aerogenes*), optical density and remediation rates demonstrate continuous increases for up to 10 days. Figure 5 (*L. amnigena*) depicts a continuous rise in optical density until 192 hours, followed by a slight decline, while the remediation rate maintains an upward trend throughout. Figure 6 (*E. coli* K12) illustrates a continuous increase in optical density and remediation rate, with a notable overlap in nitrate remediation rates observed at 144 and 192 hours. Finally, in Figure 7 (*Enterobacter aerogenes*), optical density and remediation rate exhibit uninterrupted growth over time.

Genotypic Characterization of Microbes

These microbes were molecularly characterised to identify particular microbes' genus and species. Further, the similarity percentage with strains was checked using BLAST. Genomic DNA and PCR bands of isolates (initially designated as PP3, PP5, PP7 and PP10) are shown in Figures 8 and 9. The partial 16S rRNA gene sequences of strain PP10 (1399 base pairs) exhibited 99% similarity with *Enterobacter aerogenes* (KP764198). For strain PP3, the 1476 base pair sequence displayed 99% similarity with *Lelliotia amnigena*, while the 1409 base pair sequence of PP5 showed similarity with *E. coli* K12. For strain PP7, the 1436bp displayed 99% similarity with *Klebsiella oxytoca*. The nucleotide sequence of PP3, PP5, PP7 and PP10 isolates were deposited in the GenBank nucleotide sequence database under accession numbers MN647560, MN754025, MT457845 and MN252552, respectively.

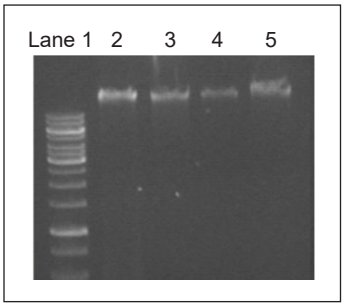

Figure 8. Extraction of Genomic DNA from all isolates (Lane 1 = DNA ladder; Lane 2 = PP3; Lane 3 = PP5; Lane 4 = PP7; and Lane 5 = PP10 strain

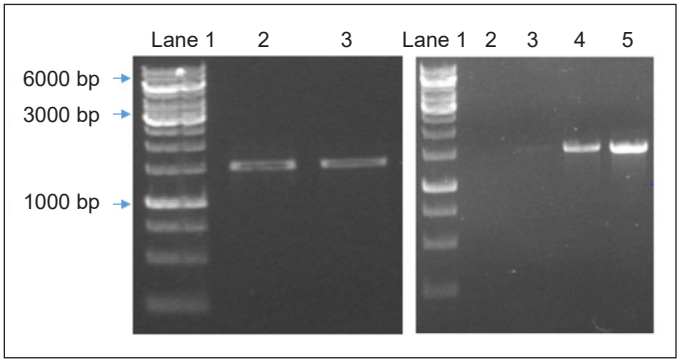

Figure 9. PCR bands of isolates PP3 and PP5 in lane 2, lane 3, PCR bands of strain PP7 and PP10 in lane 4 and lane 5, 1 kb plus DNA ladder in lane 1

Statistical Analysis

One-way ANOVA is used to compare different groups. Each experiment was repeated three times in replicates. Microsoft Excel calculated the average and standard deviation in Tables 4 and 5, and the

Table 4
Results of statistical analysis

Groups	Count	Sum	Average	Variance
Data 1	3	8	2.666667	0.33333
Data 2	3	12	4	1
Data 3	3	12	4	1

Table 5
Represent the degree of freedom, F value and P-value

Source of variation	SS	df	MS	F	p-value	F crit
Between Groups	3.55556	2	1.777778	2.285714	0.182832	5.143253
Within Groups	4.666667	6	0.777778			
Total	8.222222	8				

associated probability (P)<0.05 was statistically insignificant. The nitrate removal by isolates was within 24%–42%, the average being removal 31.86%.

DISCUSSION

According to the literature, *Lelliottia amnigena* is reported to remediate 50% of 138 mg/l nitrate-nitrogen (equivalent to 611.34 mg/l nitrate) (Fazzolari et al., 1990), while *E. coli* and *Enterobacter aerogenes* are known to remediate 90% of 10 mM nitrate (equivalent to 620 mg/l nitrate) (Bing et al., 1988). Our experimental study corroborates these findings, demonstrating the nitrate remediation potential of these isolated microbes. Our study assessed their efficacy at the maximum reported nitrate concentration in a groundwater medium supplemented with nitrate and acetate (as a carbon source). *Lelliottia*, *E. coli* K12, and *Enterobacter* exhibited remediation rates of approximately 42%, 24%, and 29%, respectively, at a nitrate concentration of 1500 mg/l. Notably, *Lelliottia* displayed the highest remediation rate among the three microbes, followed by *Enterobacter* and *E. coli*.

The *in-silico* study further validates the superior remediation potential of *L. amnigena* over *E. coli* K12, attributing it to the presence of both assimilatory and denitrification pathways (Thakur & Gauba, 2023; Thakur & Gauba, 2024). Moreover, the low nitrate reduction rate of these microbes may be influenced by various factors such as temperature, pH variations, and nutrient availability, as each microbe requires specific conditions for optimal growth, thereby enhancing nitrate reduction. Potential factors contributing to the nitrate remediation by these microbes include their slow growth, the impact of nitrate by-products, and the presence of specific metabolic pathways. Additionally, certain chemicals in the groundwater medium did not impede the nitrate reduction, as confirmed by detecting nitrite and ammonium using Griess reagent, indicating successful nitrate reduction.

CONCLUSION

This study successfully demonstrates the nitrate remediation capacity of isolates in groundwater, achieving approximately 25%–40% reduction at a concentration of 1500 mg/l. While numerous studies on nitrate bioremediation exist, they often remain confined to laboratory scales. Exploring large-scale remediation methods and determining optimal microbial conditions are critical next steps. Future research aims to refine conditions and investigate microbe-microbe interactions, which holds promise for improving the efficiency and affordability of bioremediation techniques and effectively addressing this urgent environmental concern.

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