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Potential of the Alfalfa (*Medicago Sativa*) Rhizobium Bacteria for Nitrate and Phosphate Removal from the Sugarcane Industry Wastewater

Shahrzad GolabKesh[1], Shahrzad Khoramnezhadian[1]*, Ebrahim Rajabzadeh Ghatrami[1,2] and Mohammad Rashno[1,3]

[1]Department of Environment, Damavand Branch, Islamic Azad University, Damavand, Iran

[2]Department of Marine Natural Resources, Khorramshahr University of Marine Sciences and Technology, Khorramshahr, Iran

[3]Department of Immunology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; Cellular and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences Ahvaz, Iran

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*to whom all correspondence should be addressed: e-mail: khoramnezhadian@yahoo.com

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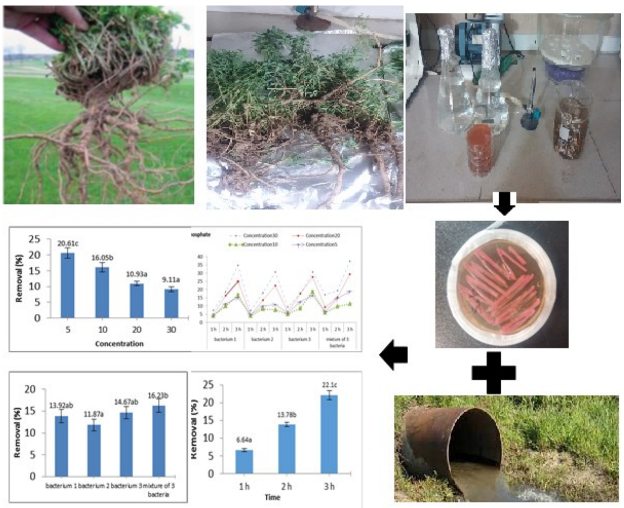
Graphical abstract



Abstract

To conduct this experiment, rhizospheric bacteria were isolated from the root nodules of alfalfa plants (*Medicago sativa*) in the Khuzestan province during the spring season. For the molecular identification of superior isolates, DNA extraction and expression of 16S rDNA were performed. To investigate nitrate (NO_3^-) and phosphate (PO_4^{3-}) removal, 120 mL vials were filled with 50 mL of laboratory wastewater containing specified concentrations of nitrate (25, 50, 100, and 200 mgL^{-1}) and phosphate (5, 10, 20, and 30 mgL^{-1}). The removal percentage was examined over 1, 2, and 3 hours. Results revealed that three rhizobial bacteria, including *Sinorhizobium meliloti* (bacterium 3) (Query ID= DQ-145546.1), *Sinorhizobium medicae* (bacterium 2) (Query ID= CP-000738.1), and *Sinorhizobium meliloti* (bacterium 1) (Query ID= CP 021215.1), were isolated and identified. The highest removal efficiency was recorded within 3 hours at 44.61%. With an increase in concentration, the removal efficiency decreased, reaching its lowest value (14.82%) at 200 mg L^{-1} compared to other concentrations ($p < 0.05$). Besides, the bacterial type or mixture did not significantly affect the nitrate removal percentage ($p > 0.05$). At a concentration of 25 mg L^{-1}, the highest removal percentage (63.08%) was measured in the presence of bacteria at 3 hours. Furthermore, the highest phosphate removal percentage was obtained at 37.4% at 5%, within 3 hours, and a mixture of 3 bacteria. Furthermore, the efficiency of the mixture of 3 bacteria was higher compared to bacterium 2 ($p < 0.05$). Besides bacteria 1 and 3 with a removal percentage of 16.23% were ranked higher ($p > 0.05$). At higher concentrations and increased time, the nitrate and phosphate removal percentages exhibited a consistent and fluctuating trend, respectively ($p > 0.05$). Ribosome bacteria performed better in nitrate removal compared to phosphate ($p < 0.05$). Considering that wastewater from agricultural and industrial centers contains a mixture of nitrogenous and phosphorous substances, it appears that the use of a consortium of these bacteria with different characteristics and compatibilities enhances the efficiency of purification and removal of these parameters.

Keywords Rhizobia, Alfalfa (*Medicago sativa*), Nitrate and phosphate removal, Wastewater

1. Introduction

Eutrophication is a global problem occurring due to the uncontrolled growth of cyanobacteria and eukaryotic algae. This phenomenon arises from the entry of high volumes of nitrogen and phosphorus-rich effluents into the oligotrophic water body at a higher level than the growth limiting concentration for photosynthetic organisms.

The uncontrolled discharge of industrial heavy metals continuously contaminates both air and aquatic environments. These toxic metals are non-biodegradable and exhibit an undesirable tendency to accumulate in biological systems. There are various remediation techniques for removing toxic ions, such as precipitation, coagulation, membrane processes, and electrolytic methods. However, the ultimate implementation of the final metal and toxic ion removal system is inevitable in these methods. Besides, biological adsorption has been proposed as an efficient, cost-effective, and effective alternative for pollutant removal, owing to its capability to transform or store pollutants within biological structures (Zafar *et al.* 2019). In recent years, biological adsorption processes using microbial biomass as a bio-adsorbent for pollutant removal have been extensively studied. This technique shows superior performance compared to others due to its strong compositional affinity for pollutants derived from various functional groups on the microbial cell wall, such as phosphate, carboxyl, and hydroxyl (Rasheed *et al.* 2020).

Leguminous plants, comprising 19,300 species and 750 genera, hold significant importance globally due to their high protein production and the stabilization of nitrogen levels through symbiosis with rhizobia (Andrews and Andrews 2017). Most of these plants, including peas, beans, and clovers, possess nodules or tumor-like structures on their roots. These nodes are formed in nitrogen-deficient conditions, leading to the contamination of the root nodules with a group of bacteria known as rhizobia (Ghorbani 2010). Alfalfa, as one of the most important native forage plants, plays a crucial role in soil fertility due to its strong root system and resistance to adverse environmental conditions, aided by rhizobial symbiotic bacteria. Rhizobial bacteria are the most important microorganisms in the soil, playing a crucial role in converting atmospheric nitrogen into ammonium and organic nitrogen and nitrogen fixation compared to other soil microorganisms (Fuskhah and Darmawati 2019). Rhizobia are a group of bacteria capable of forming nodules on the roots of plants in the legume family. These microorganisms are aerobic and can utilize various carbohydrate sources such as pentoses and hexoses as carbon sources while using ammonium and nitrate as nitrogen sources. The presence of these bacteria in the plant may increase the plant's resilience in adverse environmental conditions such as salinity and high temperatures. This symbiosis is also promoted by processes such as the dissolution of phosphate and iron in the soil, and the absorption of micronutrients, particularly the regulation of pathogenic factors. In addition, this symbiotic relationship stimulates the overall growth of the plant (Sarr *et al.* 2016). The mentioned bacteria can be adapted to soil stress conditions. This ability plays a fundamental role in their survival and symbiosis with bacteria and plants. Some of these symbiotic bacteria exhibit higher resilience against biological, physical, and chemical stresses (Andrews and Andrews 2017).

Due to the diversity of climate and soil type in Iran, there are probably unique strains in terms of phylogeny and symbiotic efficiency with native Iranian alfalfa. Biological denitrification is a proposed method for removing nitrogenous compounds by bacteria under anaerobic conditions, called bacterial denitrification. In this process, nitrate acts as an electron acceptor and replaces oxygen in the electron transport chain (Du *et al.* 2014).

In addition to nitrogen, phosphorus is one of the essential elements for the growth of legumes. The need of host plants for phosphorus to have optimal growth and the symbiotic nitrogen fixation has been measured by controlling nodule development and its function, and the effect of this element on the symbiotic nitrogen fixation in plants from Leguminosae family is significant (Tsvetkova and Georgiev 2003). Increasing the dissolved phosphorus by some bacteria in the rhizosphere can increase plant growth. Phosphate solubilizing bacteria play the main role in dissolving the unavailable form of phosphorus in the soil (Andrews and Andrews 2017). The high resistance of bacterial colonies to acute conditions, their survival in high concentrations of nitrate and phosphate, and their potential to decrease the level of these compounds profoundly motivate the use of these bacteria in wastewater treatment (Rossi *et al.* 2015). Paul *et al.* (2015) reported the biological phosphorus removal (74.15%-82.5%) from the sewage using a consortium of bacteria. Jia and Yuan (2016) reported the high efficacy of algae and bacteria and their consortium in removing nitrogen from the sewage. According to Zhang *et al.* (2020), heterotrophic bacteria can use the wastewater nitrogen source without using ammonium as a nitrogen source. Among many agricultural-related industries, the sugarcane industry is usual in more than 130 countries (Podadar and Sahu 2017). In this industry, sugarcane milling and processing are two main steps producing wastewater with a high level of COD and BOD due to having a high amount of organic compounds. Thus, the entrance of this wastewater into water sources deplete dissolved oxygen in the water and cause the death of aquatic organisms through the stimulation of microbial growth (Fito *et al.* 2019). Regarding the adsorption of soil nitrogen and phosphorus by symbiotic bacteria, there is the potential to reduce water pollution by using these bacteria in contaminated surface waters to uptake nitrate and phosphate. Therefore, this study aimed to evaluate the potential of rhizobial strains for the removal of nitrate and phosphate pollutants in wastewater from the sugarcane factory in Khuzestan province, Iran.

2. Materials and methods

Root nodules of alfalfa (*Medicago sativa*) were collected from Pashmineh Zar croplands (before Karkheh Dam), Andimeshk, southwest Iran, in spring. Firstly, the soil around the plant (with an approximate radius of 15 cm and a depth of 20 cm) was removed, and the root along with soil was slowly separated (not to damage the sub-roots with nodules). Then, the roots of alfalfa were placed in plastic bags to retain moisture and avoid cross-contamination and transferred to the lab. The roots were gently washed in a dish containing water and soil around the nodules were cleaned under tap water. The nodules were disinfected using 95% ethanol (5-10 s) and 2.5% sodium hypochlorite (2-3 min), washed 5-6 times with distilled water near a flame, and kept in sterile water in the last three washes for 15 min. Sterile nodules were transferred to a sterile porcelain mortar containing 8.5 g/L salt solution (0.5 mL) and crushed. The suspension (0.1 mL) of salt solution and the nodule was spread onto YMA culture medium (0.5 g K_2HPO_4, 0.2 g MgSO4, 0.1 g NaCl, 10 g $C_6H_{14}O_6$, 0.5 g yeast extract, and 15 g agar) (Beck *et al.*, 1993) and plates were incubated at 25-28°C. After growing the bacterial colony along the streak line, they were re-cultured and purified after several stages. For the long-term maintenance of purified cultures, isolates were cultured in culture tubes containing YMA in a diagonal orientation and kept in a refrigerator. Four to five samples were prepared for each strain (Fuskhah and Darmawati, 2019).

2.1. Identification of bacterial isolates

Identification based on morphological features was carried out according to guidelines described in detail by Claus & Berkeley (1986). Briefly, colonies were cultured on the nutrient agar plates and incubated for 3 days. Then, the microscopic characteristics of colonies were evaluated, including morphology and gram staining reaction. Molecular identification of superior isolates, DNA extraction, and 16S rDNA amplification were carried out using a DNA extraction kit (CinnaGen Co., Iran). Briefly, after extracting the genomic DNA and amplification by 16S rDNA primer using the PCR method, the products were assessed on agarose gel electrophoresis. The obtained sequences were edited using ChromasPro software and analyzed in the NCBI BALST database

2.2. Experimental design

The experiment of nitrate and phosphate removal was carried out in vials (120 mL) containing wastewater (50 mL) (obtained from Hakim Farabi Agriculture and Industry Company of Khuzestan) with the certain concentrations of nitrate (25, 50, 100, and 200 mg/L) and phosphate (5, 10, 20, and 30 mg/L). Each bacterium was inoculated into vials to match the density of No.3 MacFarland standard (3×10^8 CFU/mL) (Goodini *et al.*, 2012). Vials were shaken at 28°C and nitrate and phosphate concentrations were measured after 1, 2, and 3 h (according to the standard method, 2005). The wastewaters were filtered (Watman filter paper No. 42) and then evaluated to measure nitrate and phosphate levels. Briefly, nitrate was measured using a spectrophotometer (Hach DR/2800). Moreover, soluble reactive phosphorus was measured by changing the color of the solution in the presence of molybdic acid, ascorbic acid, and antimony to the blue complex of molybdenum using the spectrophotometer. The phosphate and nitrate removal efficiency was calculated using the following equation (Aslan and Kapdan, 2006)(Eq. 1):

$$\text{Removal efficiency \%} = (\text{initial concentration} - \text{final concentration}/\text{initial concentration}) \times 100 \quad (1)$$

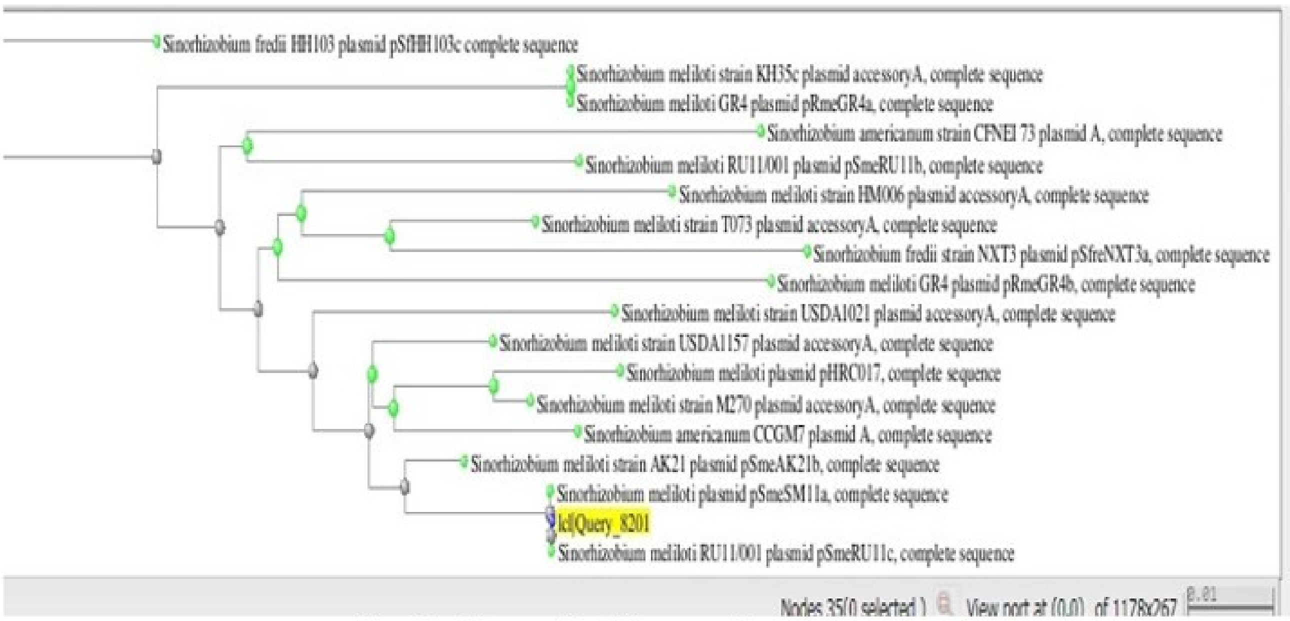
The pH of the effluent remained constant within the range of 8-7 throughout the experiment.

3. Results and discussion

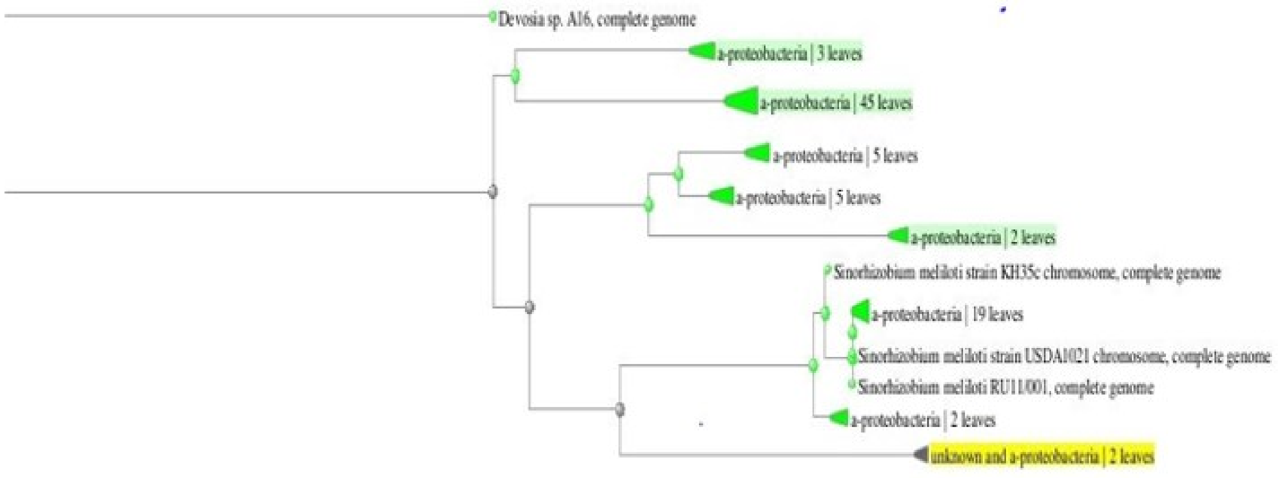
Colonies obtained from culturing rhizobial strains isolated from alfalfa had beige (one colony) and red-pink color (two colonies; this color was obtained from the medium) (Figure 1).

Figure 1. Bacteria isolated from the nodule were cultured on the YMA medium.

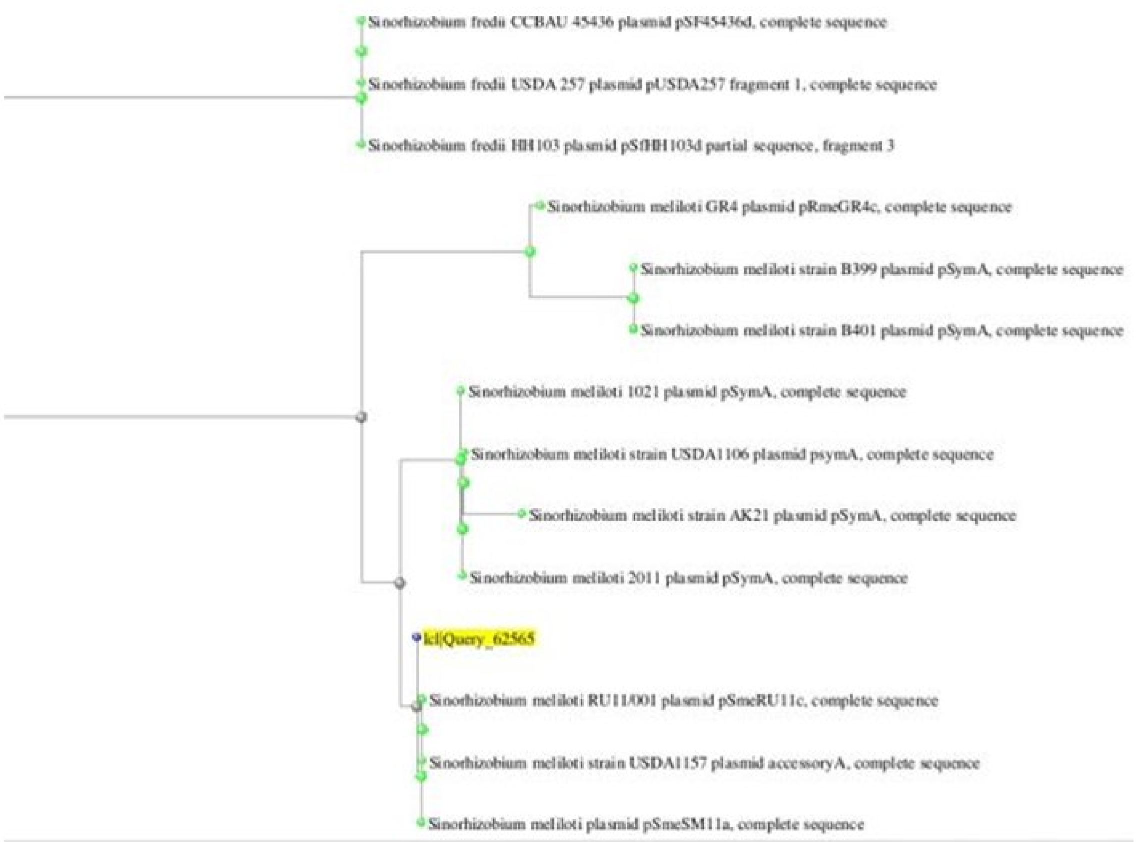
Figure 2 shows the phylogenetic tree of isolates. Based on the results, three rhizobial strains were isolated and identified, including *Sinorhizobium meliloti* (Query ID=DQ-145546.1) (bacterium 3), *Sinorhizobium medicae* (Query ID= CP-000738.1) (bacterium 2), and *Sinorhizobium meliloti* (Query ID= CP- 021215.1) (bacterium 1).



Sinorhizobium meliloti (Sequence ID= CP 021215.1)



Sinorhizobium medicae (Sequence ID= CP-000738.1)



Sinorhizobium meliloti (Sequence ID= DQ-145546.1)

Figure 2. The phylogenetic tree of rhizobia identified in alfalfa (*Medicago sativa*)

The results showed that the three variables including time, concentration, and strain were effective factors on the percentage removal of phosphate and nitrate (P <0.05). Moreover, based on F-test, concentration (phosphate and nitrate) and exposure time had a higher effect on the percentage removal of phosphate and nitrate from the wastewater compared to bacterial strain (Tables 1 and 2).

Figure 3 shows the effect of the three factors, including rhizobial strain, concentration, and exposure time separately and simultaneously on the percentage removal of nitrate. Based on the central diagram showing the three factors simultaneously, the increased rate of removal was observable in 4 groups (P<0.05) and the highest removal rate (44.61%) was obtained after 3 h. Regarding concentration, the removal rate decreased with increasing concentration and reached its lowest level (14.82%) at a concentration of 200 mg/L (P<0.05). Although the presence of bacteria was an effective factor in the removal of nitrate from the wastewater, bacterial strain and/or the mixture of bacteria did not have a significant effect on the percentage of removal (P> 0.05). The data showed that bacterium 2 was more effective in the removal of nitrate (26.46%) compared to other bacteria (especially bacterium 3). The bacterial mixture (3 bacteria) with a removal rate of 24.72% was in third place in terms of performance (P <0.05). Moreover, the maximum rate of nitrate removal (63.08%) was obtained in bacterium 1 at the concentration of 25 mg/L after 3 h compared to other groups.

Table 1. Analysis of variance (ANOVA) test to evaluate the rate of phosphate removal by rhizobial strains isolated from alfalfa (*Medicago sativa*)

Factor	Mean squared error	Degrees of freedom	F-value	P-value
Model	240.97	47	7052.8	0.0
Bacteria	2.98	3	87.3	0.0
Phosphate concentration	3730.49	3	109185.29	0.0
Exposure time	47.38	2	1386.74	0.0
Bacteria*concentration	0.67	9	19.65	0.0
Bacteria*exposure time	0.64	6	18.78	0.0
Exposure time*concentration	2.40	6	70.45	0.0
Bacteria*exposure time*concentration	0.33	18	9.89	0.0

Table 2. Analysis of variance (ANOVA) test to evaluate the rate of nitrate removal by rhizobial strains isolated from alfalfa (*Medicago sativa*)

Factor	Mean squared error	Degrees of freedom	F-value	P-value
Model	11241.60	47	7052.80	0.0
Bacteria	95.84	3	87.30	0.0
Nitrate concentration	167262.82	3	109185.29	0.0
Exposure time	9892.89	2	1386.74	0.0
Bacteria*concentration	72.54	9	19.65	0.0
Bacteria*exposure time	5.65	6	18.78	0.0
Exposure time*concentration	865.77	6	70.45	0.0
Bacteria*exposure time*concentration	34.02	18	9.89	0.0

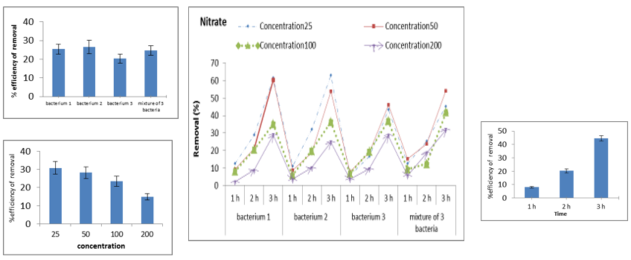


Figure 3. Effect of main factors related to rhizobial strains including *Sinorhizobium meliloti* (Query ID=DQ-145546.1) (bacterium 3), *Sinorhizobium medicae* (Query ID= CP-000738.1) (bacterium 2), and *Sinorhizobium meliloti* (Query ID= CP-021215.1) (bacterium 1) isolated from alfalfa (*Medicago sativa*) on the efficiency of nitrate removal.

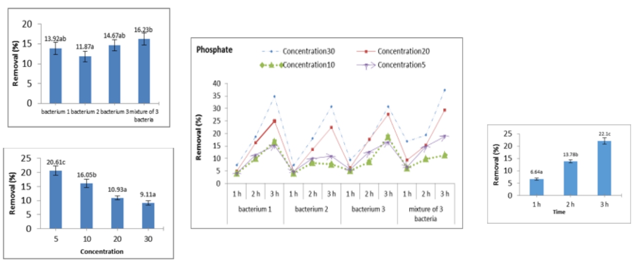


Figure 4. Effect of main factors related to rhizobial strains including *Sinorhizobium meliloti* (Query ID=DQ-145546.1) (bacterium 3), *Sinorhizobium medicae* (Query ID= CP-000738.1) (bacterium 2), and *Sinorhizobium meliloti* (Query ID= CP-021215.1) (bacterium 1) isolated from alfalfa (*Medicago sativa*) on the efficiency of phosphate removal.

The effect of factors on the percentage removal of phosphate and nitrate was evaluated simultaneously and separately (Figure 4). The highest percentage removal of phosphate (37.4%) was obtained in the mixture of 3 bacteria at the concentration of 5 mg/mL after 3 h, and this efficiency was more than that in bacterium 2 as well as in bacteria 1 and 3 with the removal rate of 16.23%. Based on the exposure time and the concentration, the removal rate of phosphate and nitrate was the same and increasing concentration and exposure time decreased (9.11%) and increased (22.1%) the removal rate (P<0.05), respectively.

The rates of nitrate and phosphate removal from wastewater by 3 bacteria and their mixture were compared in Figure 5. Rhizobial bacteria had a higher ability to remove nitrate than phosphate (P <0.05). Another significant difference in the performance was observed in bacteria 2 and 3, in which bacterium 2 had the highest and lowest efficiency to remove nitrate and phosphate, respectively. On the contrary, bacterium 3 similar to the bacterial mixture showed the highest and lowest efficiency to remove phosphate and nitrate, respectively (P<0.05).

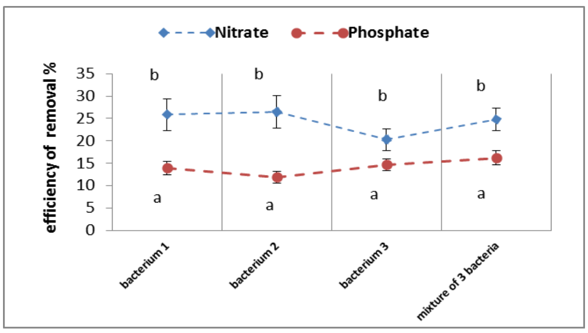


Figure 5. Comparing the efficiency of rhizobial bacteria in the removal of nitrate and phosphate from wastewater.

Nitrate and phosphate are the most important environmental pollutants, which even in small amounts, disrupt the growth of a plant in the aquatic environment, destroy the ecosystem balance, and even can threat humans (DebRoy *et al.*, 2012). This study aimed to use a component of the environment at the lowest possible level (bacteria) to remove these pollutants. In the present study, rhizobia isolated from the root of the alfalfa (Sinorhizobium sp.) included two species (*Sinorhizobium meliloti* and *Sinorhizobium medicae*) and two strains (CP-021215.1 and DQ-145546.1). Rahmani (2001) introduced *Rhizobium meliloti* as a dominant rhizobium of alfalfa. Zhang *et al.* (1991) studied bacteria isolated from the nodules of leguminous plants and introduced these two bacteria as fast-growing rhizobia. This characteristic can be an advantage for the use of these bacteria in the treatment of wastewater.

Rhizobia living in a symbiotic relationship with legumes are the most important microorganisms in the soil that play a role in the nitrogen fixation (due to soil nitrogen deficiency) rather than phosphate fixation (Fuskhah and Darmawati, 2019). In the present study, these bacteria had the potential to remove 4.2-37.4% of phosphate and 5.88-63.08% of nitrate in wastewater after 1 to 3 h, indicating a higher ability of rhizobia in nitrate removal compared to phosphate removal ($P<0.05$). In other words, the present study showed that nitrate and phosphate are differently removed by the rhizobium ($P<0.05$). DebRoy *et al.* (2012) reported that bacteria with the potential of nitrate removal were not necessarily effective in phosphate removal, which was confirmed in the present study (especially for bacteria 2 and 3). This event may result from the special mechanism and active uptake by which inorganic orthophosphate is absorbed (Delgadillo-Mirquez *et al.*, 2016) that reduces the ability of some bacteria for the absorption. A possible mechanism of phosphate uptake into the cell is the formation of polyphosphate granules as an energy source (Sidat *et al.*, 1999), which requires repeated cycles of anaerobic and aerobic processes (Plackett and Buman, 1946; Mulkerrins *et al.*, 2004). Thus, the decreased removal rate of phosphate in the present study can be related to the absence of anaerobic phase in the experiment.

Different efficiencies of bacterial strains and species in phosphate removal were also observed in the study carried out by Krishnaswamy *et al.* (2011) and Paul and Sinha (2015), respectively. The cell wall of bacteria has proteins, some of which can hydrolyze phosphate, and others store phosphate as a polyphosphate, affecting the percentage removal of phosphate. This fact was reflected in bacteria 2 and 3 by different potentials in the phosphate and nitrate removal.

There was no significant difference between the bacterial mixture group and the single-strain group in the fixation and reduction of nitrate, and even the bacterial mixture group was ranked third in terms of fixation ($P>0.05$). However, the bacterial mixture group had the best performance in the phosphate removal (although this difference was not significant compared to the percentage removal of phosphate by the bacterial groups 1 and 3). Regarding various physical and chemical characteristics of wastewaters with a mixture of nitrogen- and phosphorus-containing pollutants, it seems that the use of bacterial consortium with various characteristics and adaptations increases the efficiency of the treatment, which may be due to synergistic activity among different strains as well as the activity of different enzymes (Paul and Sinha, 2015).

Data showed that increasing nitrate and phosphate concentrations decreased the potential of rhizobial strains in the removal of phosphate and nitrate. Regarding nitrate concentration, the tolerance range of bacteria to nitrate was higher than that to phosphate ($P <0.05$). It should be noted that although the percentage removal of pollutant decreased with increasing nitrate and phosphate concentration in wastewater, the difference between the percentages of nitrate removal (2.67% at 25-50 mg/L; 4.67% at 50-100 mg/L; 8.67% at 100-200 mg/L) and the percentage of phosphate removal (4.56% at 5-10 mg/L; 5.12% at 10-20 mg/L; 1.82% at 20-30 mg/L) was negligible. Thus, despite a decreased removal rate, this decrease was negligible compared to the increased nitrate and phosphate concentration. Pinar *et al.* (1997) evaluated the removal of nitrate by soil bacteria and reported that increasing the concentration of nitrate to 160 mmol also reduced the removal and conversion of nitrate. It seems that a high concentration of nitrate in the environment (Krishnamachari and Clarkson, 1993) and the presence of toxic nitrogen-derived substances such as HNO2 lead to disturbance in converting nitrate to nitrite (Hirose *et al.*, 1982). Moreover, nitrate acts as a chaotropic agent at high concentrations, leading to the removal of protein from the bacterial cell wall and killing the bacterium (Manzano *et al.*, 1976). More uptake of phosphate can also be prevented by the replenishment of bacterial phosphate storage capacity (Krishnamachari *et al.*, 2011), which is observable in the present study at concentrations of 20 and 30 mg/L.

Exposure time has also been a positive and influential factor in the removal of nitrate and phosphate, which is explainable based on the requirement of bacteria for time to adapt to new environmental conditions and increase biomass production (Castro-Barros *et al.*, 2017). Delgadillo-Mirquez *et al.* (2016) also reported the relationship between increasing percentage removal of phosphate and nitrate with time and attributed it to the increased biomass production during the exposure time. Zhang *et al.* (2020) also reported that increasing time exposure is important to adapt bacteria to wastewater conditions and remove nitrate.

3.1. Conclusion

Phosphate and nitrate-removing bacteria (*Sinorhizobium meliloti* and *Sinorhizobium medicae*) from alfalfa roots were identified in this study. These bacteria had a higher potential to remove nitrate than phosphate. The results also showed that exposure time, bacterial strains, and bacterial concentration were effective factors on this potential, and increasing exposure time and concentration were positive and negative factors, respectively. The bacterial mixture group for the removal of phosphate and this group along with bacteria 1 and 2 had the highest efficacy to remove nitrate in the wastewater from sugarcane cultivation and industry.

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