Molecular Screening of Nitrate Reductase Enzyme in Native Halophilic Bacteria of Iran

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Abstract

With the increased usage of nitrate fertilizers, the removal of their stable ionic and water-soluble end products is a challenge for human health. Several physicochemical methods have been examined for nitrate removal of water, but biological treatments are mostly preferred due to a higher efficiency and lower cost. To remove nitrogen from water, we investigated the potential of nitrate-reducing halophilic and halotolerant bacteria. A total of 50 strains from different saline and hypersaline environments of Iran, including the Incheboron wetland, Aran-Bidgol salt-lake, and Urmia endorheic salt-lake, were screened for nitrate reductase production. Among investigated bacteria, 60% and 19% of strains obtained from Urmia lake, and Incheboron wetland produced nitrate reductases, respectively. The nitrate reductase coding genes *narG,* and *napA* were analyzed in all strains with confirmed nitrate-reducing capacity. The *napA* gene was successfully amplified from a gram-negative halophilic strain, and the *narG* gene was detected in ten halophilic strains. Among nitrate-reducing isolates with the *narG* gene expression, the *Kocuria rosea* strain R3A34 showed the highest nitrate reductase production level. This strain was selected to optimize for its denitrifying activity. Results showed that 32°C, pH 7.0, NaCl 8% (w/v), and mannitol (as a carbon source) provide the optimal environmental conditions for the efficient production of nitrate reductase by the *Kocuria rosea* strain R3A34. As these are compatible with wastewaters conditions, this bacterium can be a proper candidate for bioremediation of wastewaters from nitrate pollutants.

Keywords: Halophiles, *Kocuria*, *napA, narG*, Nitrate Reductase

Introduction

Increased frequency of water eutrophication has made the issue of nitrate removal from the ecosystem more important. With the increased usage of nitrate fertilizers, the amount of nitrate uptake in the body has quadrupled compared to the past (Luvizotto et al., 2018, Pastorelli et al., 2013). As nitrate is a stable ion with a high water-solubility and low precipitation or adsorption capacity, its removal is remained as a challenge for human health. Several methods have been examined, such as reverse osmosis, ion exchange, distillation, electrodialysis, and biodenitrification. Among which, the latter one is widely applied in the removal of different pollutions such as nitrogen because of its high efficiency and low cost (Duan et al., 2015). Heterotrophic denitrification is the process of nitrate reduction to nitrite, nitric oxide, nitrous oxide, and

nitrogen (N_2). This process is carried out by denitrifying bacteria and eukaryotic microorganisms (Baggs, 2011). Nitrate reductase is one of the most important enzymes which reduces nitrate to nitrite. The membrane-bound Nar and the periplasmic Nap enzymes are two types of bacterial nitrate reductases (Argandona et al., 2006).

The previously known heterotrophic denitrifying bacteria are mostly isolated from freshwater or soil. Hence, they may not work efficiently in wastewaters with a high salinity (>1%), mostly originated from various industrial activities such as seafood processing, tanning, and petroleum production. As *i*) high salinity can cause cell plasmolysis, and *ii*) several nitrate-contaminated environments contains high amounts of salts, the utilization of salt-tolerant/halophilic microorganisms are of great significance. The majority of the reports about isolated halophilic microorganisms have focused on

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the degradation of organic substances (Paniagua-Michel et al., 2018), while halophilic strains which are capable of heterotrophic nitrification have been rarely reported.

According to above explanations, halophilic, or halotolerant strains can be considered as valuable candidates to remove nitrate from saline or hypersaline environments. To remove nitrogen from such wastes, we studied several nitrate-reducing halophilic, and halotolerant bacteria and found a novel strain with the nitrate contamination-reducing capacity of such saline environments. The newly-identified bacterial strain *Kocuria rosea* R3A34 IBRC-M 11008 with such a high level of denitrifying activity in a short time, has not been reported before. So, considering its capacity, it can be proposed as a valuable candidate for industrial treatment of saline wastes. Moreover, bacterial isolates were screened for the *narG,* and *napA* nitrate-reducing responsible genes. It was confirmed that the selected halophilic bacterium *Kocuria rosea* strain R3A34 has *narG,* and *napA* genes. Furthermore, different conditions of the culture medium affecting the heterotrophic denitrification capacity of the strain R3A34 were investigated to find the optimal condition.

Materials and Methods

Bacterial strains, and culture medium conditions

A Total of 50 halophilic bacterial strains were received from the Iranian Biological Resource Center (IBRC) (Supplementary Table 1). The strains belong to the different saline environments of Iran, including Incheboron wetland, Aran-Bidgol lake, and Urmia lake. Halophilic bacterial strains were recovered in the moderate halophilic medium with total salt of 8% (MH 8%) (Biswas et al., 2018). The strains were then cultured and incubated at 34°C for 5 days. Pure cultures of each strain were then streaked on slants of MH 8% media, and stored at 4°C for further studies.

Measurement of nitrate reductase production, a point-assay

Nitrate broth medium with total salt concentration of 8% (w/v), which was applied to perform a point-assay enzyme production for all strains with nitrite positive results, contains the following compounds: NaCl (67.33g/L), $MgCl_2.6H_2O$ (4.6g/L), KCl (1.33g/L), $NaHCO_3$ (0.04g/L), $CaCl_2.2H_2O$ (0.17g/L), $MgSO_4.7H_2O$ (6.4g/L), potassium nitrate (1g/L), yeast extract (3g/L), peptone (5g/L), and glucose (1g/L).

1ml of 0.5 McFarland suspension of each of the strains was transferred into the nitrate-containing broth medium and incubated at 28°C for 72h. Then, a crude extract of the cells was prepared by breaking washed cells in a French pressure cell press, and unbroken cells were removed by sedimentation. Then, the supernatant was mixed with sodium formate (1M) (1:20), and 2 µg nitrate was added to the mixture (1.5 ml). 4h later the reaction was stopped using the equal volumes of sulfanilamide (1%, w/v) in HCl (1.5N), and N-(1-Naphthyl) ethylene diamine dihydrochloride (0.02%, w/v; Griess reagent) (Boon et al., 2018). The tubes that developed a red/pink color in 15min belongs to the strains with the nitrate-reductase activity. Thereafter, a toothpick full zinc powder was added to non-pink color tubes incubated for 15min. Tubes with no color changes were assumed as further denitrification, which means that no nitrate was present. No color change at this point considered as positive result. Strains with positive result, were selected for the point assay of nitrate reductase production. The test was repeated three times for all strains with nitrite positive results.

In order to measure the nitrate reductase production of different bacterial isolates quantitatively, the above mentioned steps were repeated again to prepare red/pink color tubes. Then, all the tubes were examined by the UV-vis spectrophotometry at 540 nm (Boon et al., 2018). The nitrite concentration was calculated by comparing the absorbance of each sample (at 540 nm) to the standard curve which was obtained for known nitrite concentrations. One unit of the nitrate reductase activity is considered equal to a 1 µmol of nitrite release per minute at pH 7.0 in the room temperature (Kroneck et al., 2018).

To obtain the growth curve for the most potent nitrate-reducing strain, and to determine the optimum time of reducing nitrate to nitrite, the selected strain was cultured in the nitrate broth medium with a total salt concentration of 8% (w/v), and incubated at 28°C for 90h. The monitoring period was 4h.

Effects of different factors on the growth and heterotrophic denitrification of R3A34 strain

To study the heterotrophic denitrification characteristics of the bacterial strain R3A34 under different circumstances it was cultured in replicates by changing one factor at a time method (Javaheri-Kermani and Asoodeh 2019). To do so, they were inoculated into the nitrate broth medium (150 ml), and incubated at 28°C for 72 h. Studying the effects

of salinity on the nitrite release was conducted by the adjustment of the NaCl concentration in the medium to provide different salinity percents (0-20%, w/v). The effect of the initial pH on the denitrification was conducted by adjusting pH in the medium between 5 to 10. Different ranges of incubation temperatures (20-50°C) were also examined. In addition, the consequences of the utilization of different carbon sources, including glucose, mannitol, citrate, acetate, and succinate (1 g/L) were investigated on the nitrite release. All experiments were carried out in triplicates. Analysis of variance (ANOVA) was used to study the variation of the data (with a 5% level of significance).

DNA extraction and amplification of *napA*, and *narG* genes

The genomic DNA of the selected halophilic strain with the best reductase activity was extracted as described by Marmur (Marmur, 1961). Degenerated Primer pairs S.F1173d (5'-TGGT/AG/CG/CATGGGT/G/CT/ATT/G/CAACC-3'), and S.R2294 (5'-GT/AG/ATGCCAG/ATGA/T/C/GTC -3') (Klatte et al., 2011) and degenerated primer pairs NarG1960F (5' TAT/CGTG/CGGG/CCAG/AGAG/AAA -3'), and NarG2650R (5'-TTT/CTCG/ATACCAT/G/AGTT/G/AGC -3') (Ma et al., 2019) were used respectively for the *napA*, and *narG* amplification. The reaction mix (25 μl) contained 50ng of genomic DNA, 12.5 μl of Taq Master Mix (2X), 0.5 mM of each primer, and 5% (v/v) DMSO. The reaction was started with an initial denaturation at 95°C for 5min followed by 30 cycles of denaturation at 94°C for 60s, annealing at 53°C for 60s, and 72°C for 60s with a final extension at 72°C for 420s. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea). To identify phylogenetic neighbors and to calculate pairwise *16S rDNA* sequence similarities, the BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. To align sequences, the Clustal X software (version 2.0, Conway Institute, USA) was used. The Phylogenetic tree was constructed by the neighbor-joining method using the MEGA software (version 6.0, Biodesign Institute, USA). The bootstrap value was calculated from 1000 replicates.

Results

Selection of nitrate reductase-producing strains

In the current study, 50 halophilic isolates were screened for their nitrate reductase production capacity. Twelve isolates produced a red-pink color in the medium from the nitrite test, and 5 strains were colorless in the zinc powder test. They were selected for comparing their nitrate reductase production (supplementary table 1).

Among bacteria from different regions, 60% and 19% of the strains which were isolated from the Urmia lake, and the Incheboron wetland produced nitrate reductase, respectively. None of the strains from the Aran-Bidgol lake produce this enzyme. Among all nitrate-reducing strains, 69% were gram-positive bacilli form, 19% were gram-negative bacilli form, and 12% were coccus.

The *presence of napA* and *narG* genes in the nitrate-reducing strains

The *napA*, and *narG* genes were analyzed in all nitrate-reducing strains. In this study, the *napA* gene (1121bp) was amplified successfully from *Marinobacter adhaerens* as a gram-negative strain. The *narG* gene (690bp) was detected in 7 gram-positive and 3 gram-negative strains belonging to *Oceanobacillus caeni*, *Staphylococcus saprophyticus, Bacillus daliensis, Oceanobacillus pictuare, Kocuria rosea, Virgibacillus necropolis, Saliteribacillus persicus*, and *Halomonas fontilapidosi* species. The amplified genes from two different strains are shown in the figure 1.
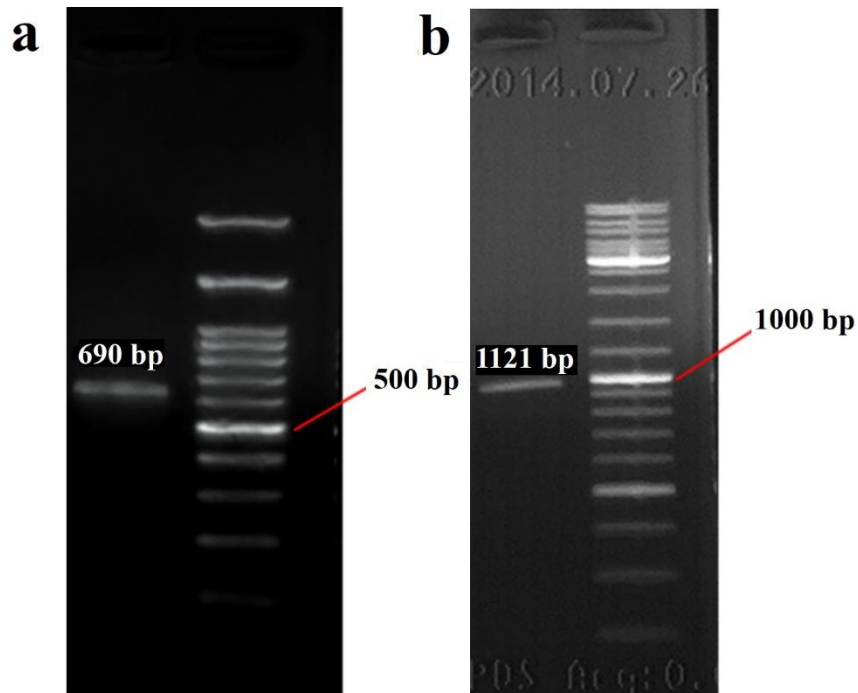
**Figure 1.** The amplified *narG* gene of *K. rosea* (a), and *napA* gene of *M. adhaerens* (b).

Nitrate reductase production of *K. rosea* strain R3A34 during the growth phases

Among five selected nitrate reductase-producing strains, a halophilic strain, called *K. rosea* strain R3A34 displayed the highest nitrate reductase production (0.006 U/ml, 7.8mg nitrite/L) in three days as it was measured by the spectrophotometric assay. Nitrate reductase activity of the other four selected strains including *Oceanobacillus* IBRC-M 11331, *S. saprophyticus* IBRC-M 10635, *Marinobacter sp.* IBRC-M 10904 and

Gracilibacillus sp. IBRC-M 4252 were 0.005, 0.004, 0.004, 0.003 U/ml, respectively.

Figure 2 illustrates the growth curve, and the nitrite concentration curve of the R3A34 strain during 90h in the nitrate broth medium under aerobic conditions.
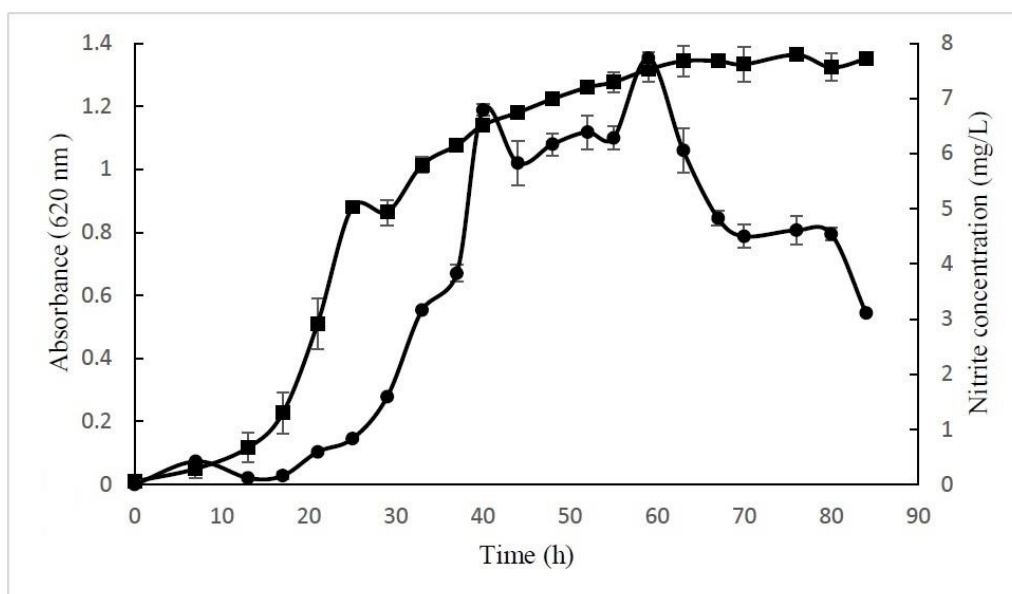
Figure 2. The growth curve (square), and nitrite production (circle) of *K. rosea* R3A34 in nitrate broth medium at 28°C for 90h.

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As it is shown in the figure, along with its growth curve, the concentration of the nitrate reductase enzyme increases during the time, and reaches to its maximum concentration in the middle of the stationary phase (40h). Furthermore, as it was indicated in the figure, the growth was approximately remained constant after 60h, while the reduction ability dropped at 65h (Figure 2).

Different factors affect the growth and denitrification

Investigating the growth rate, and enzyme production of strain R3A34 in the MH medium with an extensive range of 0 to 20% (w/v) of salt concentrations demonstrated that this strain grows well in the presence of 3.5 to 8% (w/v) of NaCl, while the maximum denitrification (5.95 mg/L) occurs in 8% (w/v) of NaCl (Figure 3a). Also, the effect of initial pH was checked on the growth and enzyme production. It was revealed that the maximum growth, and denitrification occurs in pH 7.0 (Figure 3b).

Growth and enzyme production assays in a range of temperatures demonstrated that the strain grows in 25 to 40°C and the maximum denitrification (5.95 mg/L) occurs at 32°C (Figure 3c). Moreover, the effects of different carbon sources were checked on the growth and enzyme production. The maximum growth and denitrification occurs in the medium containing mannitol as the carbon source (Figure 3d).

Phylogenetic analysis of nitrate reductase genes in *K. rosea*

The genomic DNA of the selected strain was isolated and its *napG* gene was PCR amplified with specific forward and reverse primers. Based on the *napG* gene sequence, it has a high level of sequence similarity (76%) with the *narG* gene of *Kocuria flava.* The phylogenetic tree based on the *napG* sequences was constructed with the neighbor-joining (NJ) method (Figure 4).

Discussion

Iran is among the countries where saline ecosystems, especially saline lakes, are found to be abundant in. Hence, many arid and semi-arid regions of the country have salty soil and water. These lakes are the origins of halophilic or halotolerant microorganisms with a high biodiversity. These microorganisms, with a biodenitrification capacity,

have the ability to produce nitrate reductase enzymes that are resistant to extreme environmental conditions, such as temperature, pH, and high salt concentrations. These microorganisms are of a great value in the fields of biotechnology, and more particularly in industrial decontamination of wastewaters since they contain a large amount of salt and nitrate (Beeler and Singh, 2016). For this reason, halophilic denitrifying bacteria investigated in our study could play a major role in the treatment of such nitrate-contaminated wastewaters. Previous studies have shown that several genes are responsible for encoding denitrifying activated enzymes in microorganisms, and detecting responsible genes (*narG* and *napA*) in halophilic bacteria can be useful as biotechnological tools for denitrification (Duan et al., 2015). Bacteria can harbor either or both of these nitrate reductases (Asamoto et al, 2021). In the present study, a membrane-bound nitrate reductase gene (*narG*) was found in ten halophilic strains, and a periplasmic nitrate reductase gene (*napA*) was found in one halophilic strain, all of which have never been reported before. We have also examined denitrification ability of 50 strains and discovered the best strain with the nitrate reductase production capacity, which is belonged to the *K. rosea* strain R3A34.

The results revealed a positive correlation among bacterial growth and nitrate reductase activity (Figure 2). The same result was reported by Eltarahony et al. (2020). It was mentioned in the results section (Figure 3) that the nitrite release by the strain R3A34 is stabilized at a relatively high level under 3.5–8% of salinity. As the highest rate of the denitrification activity was detected in 8% of NaCl concentration, it is evident that this enzyme has a better function in high-saline media. The same result was reported by Li et al. (2013) for *M. hydrocarbonoclasticus.* However, strain R3A34 does not release nitrite in the absence of NaCl. It can be explained that due to the strain's origin (i.e. the saline ecosystem) the presence of NaCl is essential for its enzymatic activity. Upon increasing the salinity to 10%, no nitrite is released by the strain; because too high salinity may cause microorganisms /cells plasmolysis and loss of activity as Duan et al., (2015) reported. Since the highest nitrite release was occurred in 3.5–8% of salinity, strain R3A34 could be identified as a halophilic bacterium (Kushner and Kamekura, 1988). This characteristic determined its application scope for the treatment of wastewater with high salinity, such as aquaculture wastewater

and seafood processing wastewater. Strain R3A34 grows in temperatures of 20-40°C and the optimum temperature of growth was recorded as 32°C, where the best nitrate-reductase production can take place.
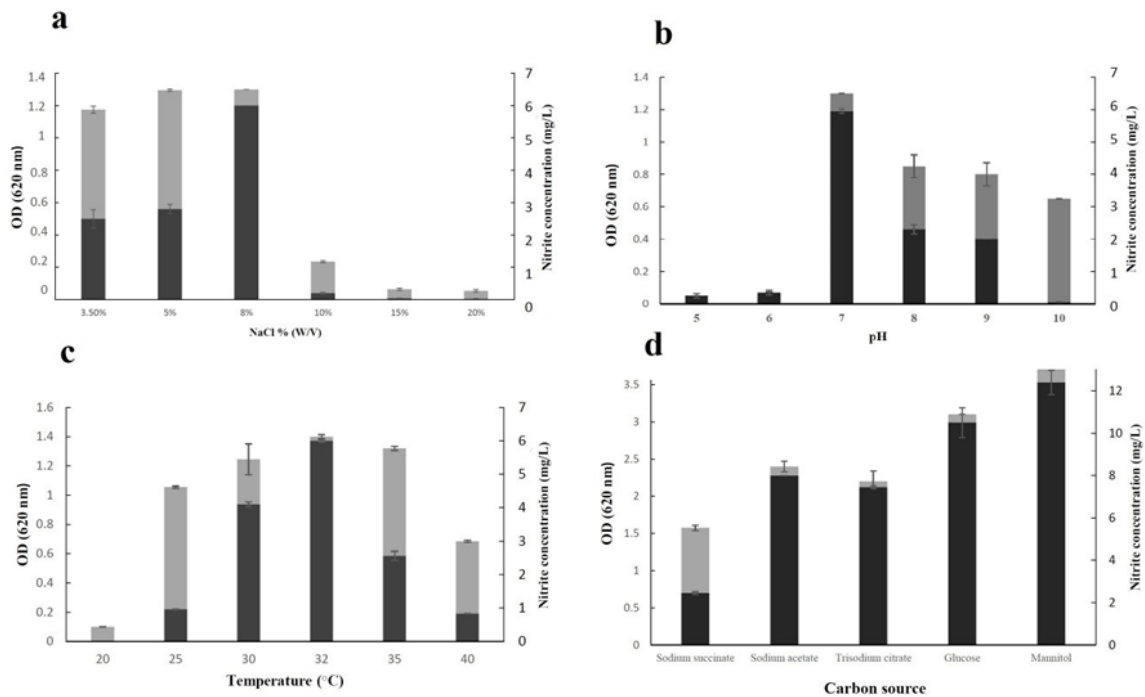
Figure 3. Effects of various culture conditions, including salinity (a), pH (b), temperature (c), and carbon source (d) on the nitrate reductase production/nitrate concentration (black color) and growth (gray color) of *K. rosea* R3A34, incubated at 28°C for 72 h. The difference between data was significant P<0.05.
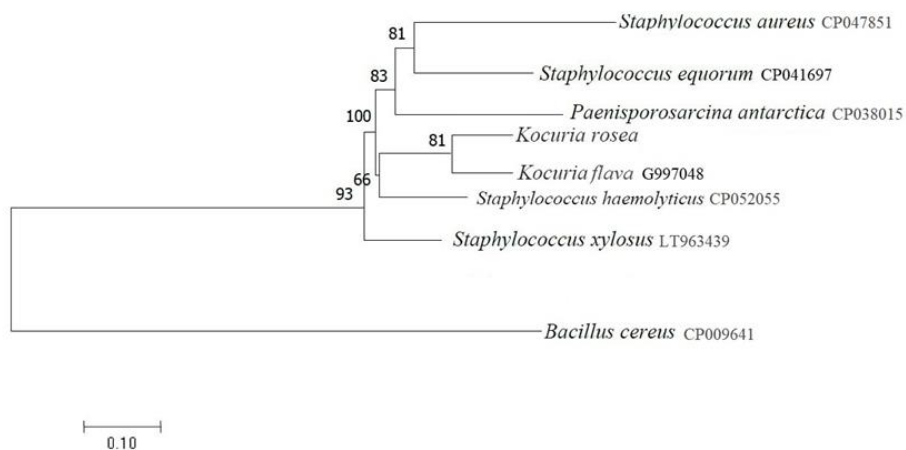
Figure 4. The phylogenetic relationship of *narG* genes. Phylogenetic distances were determined by the neighbor-joining (NJ) analysis. The *Bacillus cereus narG* gene was used as an outgroup. The bootstrap value was calculated from 1000 replicates.

This indicates that the growth of this strain is directly affected by its denitrification activity. Similar effects of the temperature on the nitrate reduction was examined by Cyplik et al., (2007), and the best temperature was reported as 37°C. The optimized pH for nitrate reduction of strain R3A34 was 7. Li et al, reported the optimized Ph 8 for nitrate reduction of *M. hydrocarbonoclasticus* (Li et al., 2013). The source of carbon in the culture media is one of the most critical factors affecting the denitrification capacity during which bacterial cells use a carbon source as an electron donor and finally reduce nitrate to nitrite. Since the molecular structure of carbon sources plays a significant role in the efficiency of denitrification, evidently, sources with simpler and smaller molecular structures are more favorable (Li et al., 2013). Hence, glucose, mannitol, citrate, acetate, and succinate were also investigated as carbon sources to evaluate the denitrification efficiency of the strain R3A34. Finally, it was concluded that among various sources mannitol was the best source. The best reduction was occurred following the 40h of bacterial culture, which is in the middle of the stationary phase of the growth curve, meaning that the highest reduction of nitrate to nitrite occurs when the cell mass is in its maximum level.

One of the objectives of this study was to determine bacterial strains which can grow in saline environments and reduce nitrate to nitrite efficiently. Here we report the *K. rosea* strain R3A34 as the best strain which is active in the saline media. The strain can carry out the denitrification in a proper time. Hence, it could be a suitable candidate for the biological treatment of wastewaters.

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Supplementary Table 1. Bacterial strains, accession number from the Iranian Biological Resource Center (IBRC) and nitrite absorption (A)

Bacterial species	Accession number	Nitrite absorption (A)
Alkalibacterium sp.	IBRC-M 11371	-*
Halomonas ventosae	IBRC-M 10566	0.91
Halobacillus sp.	IBRC-M 10952	0.13
Marinobacter sp.	IBRC-M 10904	2.1
Bacillus cohnii	IBRC-M 4079	-
Halomonas sp.	IBRC-M 10886	0.32
Planococcus sp	IBRC-M 11377	-
Thalassobacillus hwangdonensis	EU817571	-
Pontibacillus marinus	IBRC-M 10973	0.78
Piscibacillus salipiscarius	IBRC-M 10562	0.11
Bacillus safensis	NZ_UWJF00000000.1	-
Alkalibacterium putridalgicola	IBRC-M 11371	-
Gracilibacillus sp.	IBRC-M 4252	1.62
Bacillus sonorensis	IBRC-M 4099	-
Staphylococcus saprophyticus	IBRC-M 10635	2.1
Planococcus rifietoensis	IBRC-M 4022	-
Marinobacter hydrocarbonoclasticus	IBRC-M 10592	-
Kocuria rosea	IBRC-M 11008	3
Marinobacter sp.	IBRC-M 11306	-
Aeromocrobium halocynthiae	IBRC-M 4042	-
Virgibacillus salaries	IBRC-M 4091	-
Desmospora active	NZ_PZZP01000001.1	-
Marinobacter szutsaonensis	IBRC-M 11378	-
Bacillus mojavensis	AY212986.1	-
Rhodococcus corynebacterioides	MZ477522.1	-
Micrococcus yunnanensis	NZ_SMVL00000000.1	-
Micrococcus luteus	IBRC-M 10691	-
Martelella mediterranea	IBRC-M -4040	-
Bacillus vietnamensis	IBRC-M 10877	-
Halobacillus profundi	NR_041246.1	0.19
Salicola sp.	IBRC-M 4044	0.11
Marinobacter persicus	IBRC-M 10445	-
Alteribacillus iranensis	IBRC-M 10446	-
Halomonas andesensis	IBRC-M 4081	1.5
Kocuria Polaris	IBRC-M 10207	-
Halobacillus yeomjeoni	IBRC-M 10952	-
Halomonas sp	IBRC-M 4013	0.79
Oceanobacillus	IBRC-M 11331	2.4
Chromohalobacter israelensis	IBRC-M 10835	-
Halobacillus litoralis	IBRC-M 10222	-
Bacillus halosaccharovorans	IBRC-M 10095	-
Bacillus daliensis	MN713774.1	0.19
Bacillus circulans	IBRC-M 4069	-
Saliteribacillus sp.	IBRC-M 4457	0.24
Bacillus horikoshii	MW332512.1	-
Bacillus circulans	IBRC-M 10697	-
Bacillus pocheonensis	AB245377.1	-
Virgibacillus necropolis	IBRC-M 10959	0.25

*Negative Nitrite test