Optimization of *Halomonas* Denitrification in Seawater Substrate

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**Abstract.** This paper studied the effects of carbon source types, carbon-nitrogen ratio, pH and salt concentration on the denitrification of *Halomonas* bacteria under high-salt conditions to optimize the denitrification performance of mixed *Halomonas* bacteria. When the initial carbon source is glucose, the carbon to nitrogen ratio is 5:1, the pH is 7.2, and the salt concentration is 30 g/L, the maximum denitrification rate of mixed bacteria is only 19.92%. By optimizing the carbon source, the nitrogen removal rate can reach 69.25% at 72 h, which is about 49% higher than that before optimization. Under the optimal conditions with trisodium citrate as the carbon source, the carbon-to-nitrogen ratio is 5:1, the pH is 8 and the salt concentration is 60 g/L, the denitrification rate of the mixed bacteria in the seawater matrix is higher than before optimization 282%. Carbon source, carbon-nitrogen ratio, pH and salt concentration will all affect the denitrification of *Halomonas* bacteria. By optimizing them, it can greatly improve the denitrification of *Halomonas* bacteria under seawater substrate conditions.

1. Introduction

Calculated by NaCl content, wastewater with a mass fraction of total salt greater than or equal to 1% is called high-salt wastewater\(^1\). In the food processing industry, leather industry, chemical and aquaculture industries, a large amount of high-salt nitrogen-containing wastewater will be generated\(^2\). Compared with the physical and chemical methods, biological nitrogen removal process has been widely used in wastewater denitrification due to its low cost and no secondary pollution. Since high salt will inhibit the growth and metabolism of microorganisms, the traditional process is not only complicated and costly, but also cannot effectively remove nitrogen in high-salt wastewater. At the same time, the ammonia (NH\(_4^+\)), nitrate (NO\(_3^-\)) and nitrite (NO\(_2^-\)) in wastewater will threaten human health and the ecological stability of lakes and rivers, so an effective method for removing nitrogen from high-salinity wastewater is essential\(^3\). Moderately halophilic bacteria can grow in the NaCl concentration range of 0.1%-32.5%\(^4\). Therefore, its application in the denitrification treatment of high-salt wastewater has attracted attention in recent years. Rongpeng Li reported that the isolated and screened *arionobacter hydrocarbonoclasticus* bacteria can grow in the salt concentration range of 20-120 g/L NaCl and have good denitrification ability. The nitrate removal rate can reach 94.2% within 48 h\(^5\). As a moderately halophilic bacterium, *Halomonas* bacterium, its denitrification ability under high-salt conditions has long been discovered\(^6\text{-}\text{7}\). In addition, Te Wang isolated strains that can denitrify under high-salt conditions by means of SND. *Halomonas* sp. B01 can reach 99.2% nitrogen removal rate in 180 h under optimized conditions\(^8\). In this paper, by mixing a variety of
Halomonas bacteria as denitrification bacteria in a seawater matrix, with the help of the characteristics of Halomonas that can grow under high salt conditions, its denitrification ability is optimized in the seawater matrix to improve its denitrification ability under high salt conditions.

2. Materials and methods

2.1. Strains

Strains: Halomonas sp. H41, Halomonas sp. H50, Halomonas sp. H46, Halomonas sp. H55, Halomonas sp. H56, Halomonas sp. H43, Halomonas sp. H44, Halomonas sp. H47, Halomonas sp. H48 used in the experiment, Halomonas sp. H34, Halomonas sp. H33, Halomonas sp. H36, Halomonas sp. B01. From the preserved strains of our laboratory. In the experiment, Halomonas sp. H41 was used as pure bacteria, and all the strains were mixed as mixed bacteria.

2.2. Medium

LB medium (g/L): peptone 10, yeast powder 5, NaCl 30, pH 7.2, sterilized at 121 °C for 20 min.

Growth medium (g/L): glucose 15, monosodium glutamate 15, \((\text{NH}_4)_2\text{SO}_4\) 10, yeast powder 0.5, \(\text{KH}_2\text{PO}_4\) 3, \(\text{K}_2\text{HPO}_4\cdot3\text{H}_2\text{O}\) 9, \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) 0.4, \(\text{MnSO}_4\cdot\text{H}_2\text{O}\) 0.01, NaCl 30. Sterilize at a pH of 7.2 and 121 °C for 20 minutes. Sterilize with glucose at 115 °C for 15 min.

Phosphorus-limited denitrification medium (g/L): The type and concentration of carbon source are determined by experiments, \((\text{NH}_4)_2\text{SO}_4\) 5, inorganic phosphorus 0.064 (\(\text{K}_2\text{HPO}_4\cdot3\text{H}_2\text{O}\) 0.3, \(\text{KH}_2\text{PO}_4\) 0.1), \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) 0.1, NaCl is determined according to the experimental situation, trace elements 2 ml/L[8], the pH is determined according to the experimental conditions. Sterilize at 121 °C for 20 min, and filter and sterilize trace elements with 0.22 µm pore filter membrane (Millipore Express, USA).

2.3. SND method

Inoculate the strains in 5 mL of LB medium, activate them in a shaker at 120 r/min and 30 °C for 20 h, measure the OD600 after growth, mix the strains in equal amounts, and then mix the activated bacteria solution with 1% of the inoculation amount. Inoculate into the denitrification culture, denitrify in a shaker at 120 r/min and 30 °C. Since oxygen is supplied by shaking of the shaker in this process, it is considered that SND denitrification under this condition.

2.4. Determination of ammonia nitrogen and cell nitrogen

Using Kjeldahl method to determine total cell nitrogen, first measure cell nitrogen with different cell dry weights, and then establish a calculation relationship between cell dry weight and cell nitrogen, and then calculate cell nitrogen from cell dry weight[8]. The determination of ammonia nitrogen uses Nessler's reagent spectrophotometry. The principle of determination is that ammonia nitrogen in the form of free ammonia or ammonium ions reacts with Nessler's reagent to form a reddish-brown complex. The absorbance of the complex is proportional to the concentration of ammonia nitrogen. Calculate the ammonia nitrogen concentration based on this measurement. The denitrification rate is defined as the percentage of ammonia nitrogen reduced from the system to the ammonia nitrogen in the phosphorus-limited denitrification medium. The calculation is shown in formula (1):

\[
N_{\text{removal}} = \frac{(\text{TN}_0 - \text{CN} - \text{TN}_t)}{(\text{TN}_0 - \text{CN})} \times 100\%	ag{1}
\]

\(N_{\text{removal}}\) - Nitrogen removal rate, \(\text{TN}_0\) - Initial ammonia nitrogen concentration, \(\text{CN}\) - Total cell nitrogen, \(\text{TN}_t\) - Ammonia nitrogen concentration at a certain time in the denitrification process.
3. Results and discussions

3.1. Comparison of denitrification of pure bacteria and mixed bacteria in seawater medium

Compare the denitrification rate of pure bacteria and mixed strains under seawater substrate. As shown in Figure 1, pure bacteria and mixed bacteria reached the maximum denitrification rate at 24 h and 48 h, respectively, and the denitrification rate of mixed bacteria was 158% higher than that of pure bacteria. Through determination, there is no significant difference between the growth of pure bacteria and mixed bacteria at the same time. Therefore, compared to a single *Halomonas* strain, a mixture of multiple *Halomonas* strains is more suitable as a denitrification strain.

![Denitrification rate of pure and mixed bacteria.](image)

3.2. Optimization of denitrification conditions for mixed bacteria

3.2.1. Effect of carbon sources on nitrogen removal

Five carbon sources, glucose, sucrose, soluble starch, trisodium citrate, and sodium acetate were selected to explore the effect of carbon sources on the denitrification of *Halomonas*. The results are shown in Figure 2. The initial carbon to nitrogen ratio of the phosphorus-limited denitrification medium is 5, the pH is 7.2, and the salt concentration is 30 g/L. When the carbon source is sugar (glucose, sucrose and soluble starch), the nitrogen removal rate is significantly lower than that of non-sugar carbon sources (trisodium citrate, sodium acetate). The denitrification rate of mixed bacteria in seawater matrix using starch as the carbon source is almost 0%, and when sucrose is used as the carbon source, the denitrification rate is only 14.5%. Among all the carbon sources, when trisodium citrate is used as the carbon source, the denitrification rate of mixed bacteria in seawater matrix is the highest. At 72 h, it can be clearly seen that the denitrification effect of using trisodium citrate as the carbon source is better than other carbon sources, and the denitrification rate can reach 69.25% at this time.
3.2.2. Effect of C/N on nitrogen removal

According to the results of carbon source optimization, trisodium citrate was selected as the carbon source, and the concentration of trisodium citrate was changed so that the carbon to nitrogen ratio was 0, 2.5, 5, 7.5, and 10, and the effect of carbon to nitrogen ratio on the denitrification of mixed bacteria was studied. The experimental results are shown in Figure 3. When the carbon to nitrogen ratio is 5, the denitrification effect is better than other conditions of carbon to nitrogen ratio. Because *Halomonas* is a heterotrophic nitrifying, aerobic denitrifying bacteria, when the carbon to nitrogen ratio is 0, the highest denitrification rate of the bacteria is only 11%. At this time, it may be that *Halomonas* consumes organic matter in the cells for denitrification.

3.2.3. Effect of pH on nitrogen removal

The effects of pH 7, 8, 9 and 10 on nitrogen removal were investigated by selecting the best carbon source and carbon nitrogen ratio. The experimental results are shown in Figure 4. When the pH is 8, it can be seen that the denitrification effect is better than that under other pH conditions. By measuring the pH after denitrification, it can be known that when mixed bacteria use seawater as a substrate and trisodium citrate as a carbon source for denitrification, the substrate pH will rise to a certain extent during the denitrification process, and the pH can rise to about 8.5. Therefore, the denitrification effect did not change significantly during the pH optimization process.

3.2.4. Effect of salt concentration on nitrogen removal

Seawater was used in the experiment, and 0 g/L, 33 g/L, 63 g/L and 93 g/L NaCl were added to the seawater to investigate the influence of salt concentration on the denitrification of mixed bacteria. (When the NaCl concentration is 0 g/L, 33 g/L, 63 g/L, and 93 g/L, the corresponding salt concentrations are 27 g/L, 60 g/L, 90 g/L, and 120 g/L, respectively). The results are shown in Figure 5.
5. When the salt concentration in the medium changed from 27 g/L to 60 g/L, the nitrogen removal rate increased as the salt concentration increased. When the salt concentration is greater than 60 g/L, it will have a negative impact on the denitrification rate of *Halomonas* bacteria, but even when the salt concentration is 120 g/L, the denitrification rate is only 14% lower than that under the optimal salt concentration. It shows that *Halomonas* has good application value for the denitrification of high-salt wastewater.

3.3. Denitrification process under optimal conditions

It can be seen from the above that when the carbon source is trisodium citrate, the carbon to nitrogen ratio is 5:1, the pH is 8 and the salt concentration is 60 g/L, the mixed bacteria has the best denitrification effect. Under this optimal condition, the denitrification rate of pure bacteria and mixed bacteria was compared again, and the result is shown in Figure 6. It can be seen that the denitrification effect of mixed bacteria is better than that of pure bacteria. At 120 h, the denitrification rate of mixed bacteria reaches 88.49%, which is 15.46% higher than that of pure bacteria at the same time.

4. Conclusions

Comparing the denitrification of pure bacteria and mixed bacteria, it can be seen that the denitrification effect of multiple *Halomonas* strains mixed under the same conditions is better. By optimizing the denitrification conditions of mixed *Halomonas* bacteria, the following conclusions can be drawn: (1) When glucose, sucrose, starch, trisodium citrate, and sodium acetate are used as carbon sources for denitrification of mixed bacteria, the best carbon source is trisodium citrate; (2) *Halomonas* is a kind of heterotrophic nitrifying and aerobic denitrifying bacteria, the carbon source concentration in the substrate, that is, the carbon to nitrogen ratio, will have a significant impact on nitrogen removal; (3) Since the pH of the substrate will rise to a certain extent during the denitrification process, the denitrification ability of the strains has not increased significantly when the pH is optimized; (4) *Halomonas*, as a moderately halophilic bacteria, can maintain a relatively high denitrification capacity even when the salt concentration is as high as 120 g/L. In summary, this article optimizes the type of carbon source, carbon-nitrogen ratio, pH and salt concentration of *Halomonas* bacteria in seawater substrate to improve the denitrification ability of *Halomonas* bacteria when seawater is used as substrate.

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