Assistance of Ectoine on Acinetobacter sp. A06 of simultaneous heterotrophic nitrification and aerobic denitrification denitrifying at stress condition

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Abstract. The simultaneous heterotrophic nitrification and aerobic denitrification (SND) performed by Acinetobacter sp. A06 could be inhibited by high salt, high ammonia nitrogen concentrations and extreme pH. To improve the SND nitrogen removal efficiency of Acinetobacter sp. A06 in adverse environment, we investigated the effects on nitrogen removal by Acinetobacter sp. A06 of five compatible solutes. The results show that Ectoine was the best effect in five osmotic compensation solutes when the salt concentration was 15 g/L, 30 g/L, 45 g/L, and 60 g/L, adding Ectoine, nitrogen removal were increased by 21.10%, 26.94%, 14.67% and 11.21%, respectively. When the NH4+-N concentration was 1.5 g/L, 2 g/L and 2.5 g/L, adding Ectoine, the nitrogen removal rate increased by 15.93%, 10.07% and 7.11%, respectively. When the extreme pH of pH was 5 or 9, adding Ectoine, the nitrogen removal rate was increased by 20.70% and 10.44%, respectively.

1. Introduction

With the large discharge of ammonia nitrogen wastewater, ammonia pollution also became serious in water. At present, the technical methods of ammonia nitrogen waste water purification are mainly physical, chemical and biological methods [1]. Simultaneous heterotrophic nitrification and aerobic denitrification (SND) was one of the biological nitrogen removal methods [2, 3]. SND has the advantages of rapid nitrification process, high nitrogen removal efficiency and pH stability of nitrogen removal system [4, 5], so it has broad application prospects. However, when we use SND to deal with high salt, high ammonia nitrogen wastewater, the nitrogen removal rate of the strain often is affected. Therefore, how to improve the efficiency of nitrogen removal in high salt, high ammonia nitrogen concentration wastewater had important significance.

In 1985, Ectoaine (1, 4, 5, 6-tetrahydro-2-methyl-4-pyrimidin ecarboxylic acid, C6H10N2O2) was first identified by Galinski et al. in Halorhodospira halochloris (the original chlorophilus vulgaris Ectothiorhodospira halochloris) [6]. Ectoine can maintain intracellular osmotic pressure within the bacteria, and can resist the adverse environment. So in high temperature, high pressure, extreme pH, freezing and drying conditions, enzymes, nucleic acids, cells have a protective effect [7-9]. SND bacteria combined with Ectoine suitable for the purification of sewage wastewater with high salt and high concentration of ammonia nitrogen, which has important theoretical and practical significance.

In this paper, we investigated the effects of different osmotic pressure solutes and Ectoine assistance to Acinetobacter sp. A06 nitrogen removal. The effects of different osmotic pressure solutes
and different concentrations of Ectoine on the nitrogen removal of Acinetobacter sp. A06 are investigated, and the effects of different NaCl concentration, different NH$_4^+$-N concentration, different pH Ectoine on Acinetobacter sp. A06 nitrogen removal assistance are investigated.

2. Materials and methods

2.1. Experimental materials

2.1.1. Bacterium. Acinetobacter sp. A06 derived from the biological laboratory.

2.1.2. Medium. LB medium (g/L): peptone 10, yeast powder 5, NaCl 5. The medium was autoclaved at 121°C for 20 min.

Nitrogen removal medium (g/L): (NH$_4$)$_2$SO$_4$ 2.83, Na$_3$C$_6$H$_5$O$_7$•2H$_2$O 18.38, MgSO$_4$•7H$_2$O 0.05, K$_2$HPO$_4$ 2, KH$_2$PO$_4$ 6, MnSO$_4$•4H$_2$O 0.01, FeSO$_4$ 0.01, pH 8. The medium was autoclaved at 121°C for 20 min.

Ectoine standard: Ectoine standard was purchased from Biomol GmbH, Germany.

2.2. Experimental methods

2.2.1. Nitrogen removal experiment. The strains were cultivated in 5 mL LB medium at 30°C and 120 rpm in a rotary shaker for 24 h. Then 1% of the cultures were inoculated in shake flasks (30/300 mL) containing nitrogen removal medium or solution at 30°C and 120 rpm in a rotary shaker for 48 h.

2.2.2. Determination of NH$_4^+$-N, NO$_3^-$-N, NO$_2^-$-N concentration and calculation of nitrogen removal rate in inorganic nitrogen. NH$_4^+$-N was determined by Nessler’s reagent method. A reddish brown complex was formed by the reaction of NH$_4^+$-N present in the form of free ammonia or ammonium and Nessler’s reagent. According to the standard curve of NH$_4^+$-N standard sample and the absorbance of the measured sample, calculate the NH$_4^+$-N concentration of the tested sample [10].

NO$_3^-$-N was determined by diazotization-coupling reaction method. A reddish purple azo dye was formed by coupling diazotized sulfanilamide with N-(1-naphthyl)-ethylenediamine dihydrochloride under acidic conditions. According to the standard curve of NO$_3^-$-N standard sample and the absorbance of the measured sample, calculate the NO$_3^-$-N concentration of the tested sample [10].

Phenol-disulfonic acid spectrophotometric determination of NO$_2^-$-N concentration, nitrate in the absence of water reaction with phenol disulfonic acid to produce nitro - disulfonic acid phenol, in alkaline solution to generate yellow compounds. According to the standard curve of NO$_2^-$-N standard sample and the absorbance of the measured sample, calculate the NO$_2^-$-N concentration of the tested sample [11].

Calculation of nitrogen removal rate in inorganic nitrogen: In this paper, the total inorganic nitrogen is defined as the sum of the N concentrations of NH$_4^+$-N, NO$_3^-$-N and NO$_2^-$-N. The nitrogen removal rate is defined as the percentage of the initial total inorganic nitrogen concentration in the reaction system and the initial total inorganic nitrogen concentration.

3. Results and analysis

3.1. Effect of different osmotic compensation solutes on SND by Acinetobacter sp. A06

The effect of different osmotic compensation solutes on SND by Acinetobacter sp. A06 was investigated. The nitrogen removal medium added Ectoine, proline, trehalose, glycerol, mannitol with a concentration of 200 mg/L and 30 g/L NaCl. After 48 h, we determined NH$_4^+$-N, NO$_3^-$-N, NO$_2^-$-N concentration. NO$_3^-$-N, NO$_2^-$-N were not detected Results of SND under these conditions are shown in figure 1. Nitrogen removal for 48 h, the growth and nitrogen removal rates of Acinetobacter sp. A06 were significantly increased after adding different osmotic compensation solutes in the nitrogen
removal medium. Among them, Added Ectoine in the nitrogen removal medium, that had best nitrogen removal efficiency, and the nitrogen removal rate increased from 3.73% to 46.35%.

![Figure 1](image1.png)

**Figure 1.** Effect of different osmotic pressure compensating solutes on growth and nitrogen removal.

3.2. *Effects of different concentrations of Ectoine on SND by Acinetobacter sp. A06*

The effect of different concentrations of Ectoine on SND by *Acinetobacter* sp. A06 was investigated. The nitrogen removal medium added Ectoine and 30 g/L NaCl, and Ectoine concentration was set to 0, 150 200 250, 300 mg/L. After 48 h, we determined NH$_4^+$ -N, NO$_2^-$ -N, NO$_3^-$ -N concentration. NO$_3^-$ -N, NO$_2^-$ -N were not detected. Results of SND under these conditions are shown in figure 2. Nitrogen removal for 48 h, the growth and nitrogen removal rate of *Acinetobacter* sp. A06 increased with the increase of Ectoine concentration and have significantly improved. When the concentration of Ectoine was more than 250 mg/L, the increase of strain growth and nitrogen removal was stable.

![Figure 2](image2.png)

**Figure 2.** Effects of different concentrations of Ectoine on growth and nitrogen removal.

3.3. *Effect of Ectoine on SND by Acinetobacter sp. A06 at different salt concentrations*

The effect of Ectoine on SND by *Acinetobacter* sp. A06 under different salt concentrations was investigated. The nitrogen removal medium added Ectoine 250 mg/L and NaCl, and the NaCl concentration was set to 0, 15, 30, 45 and 60 g/L. After 48 h, we determined NH$_4^+$ -N, NO$_3^-$ -N, NO$_2^-$ -N...
concentration. NO$_3^-$-N, NO$_2^-$-N were not detected. Results of SND under these conditions are shown in figure 3. Nitrogen removal for 48 h, with the increase of salt concentration Acinetobacter sp. A06 increment and nitrogen removal rate is lower and lower so that medium had not Ectoine, and add Ectoine medium Acinetobacter sp. A06 nitrogen removal rate and growth had increased significantly. When the concentration of NaCl was 30 g/L, the nitrogen removal rate increased the most, and the nitrogen removal rate increased from 19.41% to 46.35%.

![Figure 3](image3.png)

**Figure 3.** Effect of Ectoine on the strain growth and nitrogen removal rate under different salt concentrations.

3.4. Effect of Ectoine on SND by Acinetobacter sp. A06 at different NH$_4^+$-N concentrations

![Figure 4](image4.png)

**Figure 4.** Effect of Ectoine on the strain nitrogen removal strains under different nitrogen ammonia concentrations.

The effect of Ectoine on SND by Acinetobacter sp. A06 under different NH$_4^+$-N concentrations was investigated. The nitrogen removal medium added Ectoine 250 mg/L and NH$_4^+$-N, and the NH$_4^+$-N concentration was set to 0.6, 1, 1.5, 2 and 2.5 g/L. After 48 h, we determined NH$_4^+$-N, NO$_2^-$-N, NO$_3^-$-N
concentration. NO$_3$ -N, NO$_2$ -N were not detected. Results of SND under these conditions are shown in figure 4. Nitrogen removal for 48 h, with the increase of NH$_4$ -N concentrations Acinetobacter sp. A06 increment and nitrogen removal rate is more and more low that medium had not Ectoine medium, and add Ectoine medium Acinetobacter sp. A06 nitrogen removal rate and growth had increased significantly. When the concentration of NH$_4$ -N was 1.5 g/L, the nitrogen removal rate increased the most, and the nitrogen removal rate increased from 27.38% to 46.35%.

3.5. Effect of Ectoine on SND by Acinetobacter sp. A06 at different pH

The effect of Ectoine on SND by Acinetobacter sp. A06 under different pH was investigated. The nitrogen removal medium added Ectoine 250 mg/L, and the pH was set to 5, 6, 7, 8 and 9. After 48 h, we determined NH$_4$ -N, NO$_x$ -N, NO$_2$ -N concentration. NO$_3$ -N, NO$_2$ -N were not detected. Results of SND under these conditions are shown in figure 5. Nitrogen removal for 48 h, the growth and nitrogen removal rate of the strain increased gradually, with the increase of pH. And the growth and nitrogen removal began to decrease when pH was more than 8, when Ectoine was added, Acinetobacter sp. A06 nitrogen removal rate and growth have improved significantly. When the concentration of pH was 6, the nitrogen removal rate increased the most, and the nitrogen removal rate increased from 22.57% to 49.78%.

![Figure 5](image)

**Figure 5.** Effect of Ectoine on the strain growth and nitrogen removal rate under different pH.

4. Conclusion

- Different osmotic compensation solutes have a synergistic effect on the removal of Acinetobacter sp. A06, among of them, Ectoine contributes best to nitrogen removal.
- Increasing the concentration of Ectoine could increase the nitrogen removal rate of Acinetobacter sp. A06, but the rate of nitrogen removal could not be increased when Ectoine concentration was higher than or equal to 250 mg/L.
- The addition of 250 mg/L Ectoine could increase the nitrogen removal rate of Acinetobacter sp. A06 at high NaCl concentration, high ammonia concentration and extreme pH. When the concentration of NaCl 30 g/L, concentration of NH$_4$ -N 1500 mg/L, pH 6, nitrogen removal rate increased by 26.94%, 18.97%, 27.21%, respectively. And the protective mechanism of Ectoine on Acinetobacter sp. A06 in the reverse environment needs further study.

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References
[1] Tian S S and Liu Q L 2013 Treatment of raw water ammonia nitrogen pollution in water supply plant Resour. Conserv. Environ. Prot. 7 172
[2] Pochana K and Keller J 1999 Study of factors affecting simultaneous nitrification and denitrification (SND) Water Sci. Technol. 39 61-8
[3] Chen H H, Liu S T, et al 2009 The development of simultaneous partial nitrification, anammox and denitrification (SNAD) process in a single reactor for denitrification Bioresour. Technol. 100 1548-54
[4] Huang T L, Bai S Y, et al 2015 Experimental identification and denitrification of a non-nutrient heterotrophic nitrification-aerobic denitrifying bacteria Chin. J. Environ. Eng. 12 5666
[5] Zhao B, He Y L, et al 2010 Heterotrophic nitrogen removal by a newly isolated Acinetobacter calcoaceticus HNR Bioresour. Technol. 101 5194-200
[6] Zhao B Q, Yang L F, et al 2007 Application of moderate halophilic bacteria in biotechnology Acta Microbiologica Sinica 34 359-62
[7] Jan B, Anne U K, et al 2008 Synthesis and up take of the compatible solutes Ectoine and 5-Hydroxyectoine by Streptomyces coelicolor A3(2) in response to salt and heat stresses, Appl. Environ. Microbiol. 74 7286-96
[8] Zhang L H, Wang Y, et al 2006 Supplementation effect of ectoine on thermostability of phytase Biosci. Bioeng. 102 560-3
[9] Lentzen G and Schwarz T 2006 Extremolytes: Natural compounds from extremophiles for versatile applications Appl. Microbiol. Biotechnol. 72 623-34
[10] 1999 Standard Methods for the Examination of Water and Wastewater 20th ed (Washington, D.C.: APHA)
[11] Sun H F, Wang H W and Yuan C Y 2013 Optimization of zinc-cadmium reduction method for determination of nitrate in seawater Adv. Mater. Res. 864-867 1004-7