Simultaneous Heterotrophic Nitrification and Aerobic Denitrification at High Concentrations of NaCl by \textit{Halomonas} Bacteria

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Abstract. \textit{Halomonas} bacteria can remove nitrogen (N) by simultaneous heterotrophic nitrification and aerobic denitrification (SND) in high salt nitrogenous matrix was reported in this paper. The \textit{Halomonas} strains were isolated, screened and identified from the sediment of a saltern pool in the city of Dalian, China. Ectoine synthesis of \textit{Halomonas} strains under high salt were investigated. The cloning and analysis of N-removal-related enzyme-encoding genes were performed. The N removal rates were determined and the typical strain was selected to describe its SND process. 14 \textit{Halomonas} strains were able to synthesize ectoine at 60 g/L NaCl concentration. The encoding genes \textit{amo A}, \textit{nar H} and \textit{nir S} fragments of ammonia monoxygenase, nitrate reductase and nitrite reductase were cloned in the genomic DNA of some \textit{Halomonas} strains. The N removal rates of 14 \textit{Halomonas} strains by SND were in the range of 21.7% to 91.8%. The N removal rate of \textit{Halomonas venusta} DSM 4743 was maximum, and it reached 91.8%. The N removal of \textit{H. venusta} DSM 4743 by SND was performed under the conditions of 60 g/L NaCl and 4000 mg/L initial NH$_4^+$-N, after 192 h the residual total inorganic N concentration was 218.7 mg/L. This study provides theoretical and technical support for microbial denitrification technology for high salt nitrogenous wastewater.

1. Introduction

The \textit{Halomonas} bacteria are taxonomically belonging to the \textit{Gammaproteobacteria}, \textit{Oceanospirillales}, \textit{Halomonadaceae} and \textit{Halomonas}[1]. \textit{Halomonas} bacteria can salt-tolerant grow and generally grow at 5-10% NaCl concentration. Some strains such as \textit{Halomonas organivorans}, \textit{Halomonas ramblicola}, \textit{Halomonas sinaiensis} can grow at 30% NaCl concentration[1]. The reason why the \textit{Halomonas} bacteria can tolerate high salt is the ability to synthesize compatible solute (mainly ectoine, 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) under NaCl stress[2]. Ectoine provides stress protection to the cell itself or to biological macromolecular substances in the cell’s internal and external environment, thus resisting the adverse effects of environmental such as osmotic pressure (and extreme temperature, extreme pH, desiccation, radiation, etc.), and then allows for cell growth and metabolism to be maintained in a high osmotic pressure environment[2].

In recent years, some researches have reported that certain strains of the genus \textit{Halomonas} can be subjected to nitrification, denitrification or simultaneous nitrification and denitrification. Such as the report by Te Wang, \textit{Halomonas} sp. strain B01 could simultaneously tolerate high concentrations of...
NaCl and NH$_4^+$ –N and efficiently perform nitrogen (N) removal by simultaneous heterotrophic nitrification and aerobic denitrification (SND). The SND by *Halomonas* sp. strain B01 under optimized conditions was performed in N removal medium containing 60 g/L NaCl and 4000 mg/L NH$_4^+$ –N; after 180 h the residual total inorganic N concentration was 21.7 mg/L and the N removal rate was 99.2%[3]. These strains have characteristics of tolerating high NaCl and NH$_4^+$ –N concentrations for using N removal treatment of high-salt nitrogenous wastewater (such as wastewaters from leather processing, marine aquaculture, synthetic ammonia, landfill and nickel battery production). It has important theoretical and practical significance.

In this paper, some *Halomonas* standard strains and *Halomonas* strains isolated, screened and identified by our laboratory were selected. The salt-tolerant growth and ectoine synthesis of *Halomonas* strains under high salt were investigated; The cloning and analysis of N-removal-related enzyme-encoding genes were performed; The N removal rates of 14 *Halomonas* strains by SND were determined and *Halomonas venusta* DSM 4743 was selected to describe its SND process. To provide theoretical and technical support for the N removal treatment of high-salt nitrogenous wastewater by using *Halomonas* strain.

2. Materials and methods

2.1. Strains and kits
Strain: *Halomonas alimentaria* DSM 15356, *Halomonas elongata* DSM 2581$^T$, *Halomonas halodentificans* DSM 735, *Halomonas marina* DSM 4741, *Halomonas pacifica* DSM 4742, *Halomonas salina* DSM 5928, *Halomonas ventosae* DSM 15911, *Halomonas venusta* DSM 4743, were preserved in our laboratory.

Kit: TaKaRa DNAiso Reagent (Code No. 9770Q), TaKaRa 16S rDNA Bacterial Identification PCR Kit (Code No. RR176), TaKaRa Taq$^{TM}$ (Code No. R001B), TaKaRa DL2,000 DNA Marker (Code No. 3427A), TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (Code No. 9762), purchased from Takara Biotechnology (Dalian) Co., Ltd.

2.2. Medium
LB medium (g/L): peptone 10, yeast powder 5, the NaCl concentrations determined by the experimental conditions, pH 7. The medium was autoclaved at 121 °C for 20 min.

Ectoine induced synthesized medium (g/L): monosodium glutamate 30, K$_2$HPO$_4$·3H$_2$O 9, KH$_2$PO$_4$ 3, MgSO$_4$·7H$_2$O 0.4, MnSO$_4$·H$_2$O 0.01, yeast powder 0.5, NaCl 60. pH 7. The medium was autoclaved at 121 °C for 20 min.

N removal medium (g/L): (NH$_4$)$_2$SO$_4$ 18.9 (N element concentration was 4000 mg/L), glucose 40, K$_2$HPO$_4$·3H$_2$O 9, KH$_2$PO$_4$ 3, MgSO$_4$·7H$_2$O 0.1, the NaCl concentrations determined by the experimental conditions. pH 7. The medium was autoclaved at 121 °C for 20 min. Trace mineral solution (EDTA-2Na 63.7, ZnSO$_4$ 2.2, CaCl$_2$ 5.5, MnCl$_2$·4H$_2$O 5.1, FeSO$_4$·7H$_2$O 5, Na$_2$MoO$_4$·2H$_2$O 1.1, CuSO$_4$·5H$_2$O 1.6, CoCl$_2$·6H$_2$O 1.6) 2 mL. The trace mineral solution was sterilized by filtration (0.22 µm pore size, Millipore Express, USA).

2.3. Isolation and screening of strains
*Halomonas* strains were isolated from the sediment of a salt pan pool in the city of Dalian, Liaoning Province, China. The sample to be separated was made into a bacterial suspension with 30 g/L sterile NaCl solution, and then pretreated using gradient dilution method and coated on LB plates containing 30, 60, 90, 120, 150 and 180 g/L NaCl, and inverted cultured at 30 °C for 48 h. The selected single colony was performed repeatedly by streak plate technique using LB medium (the concentrations of NaCl corresponded to LB media of strain screening). Gram staining, microscopic observing, and colony morphology observing were performed until pure bacterial colonies were obtained.
2.4. Ectoine induced synthesized method
The strains were cultivated in 5 mL LB medium containing 60 g/L NaCl at 30 °C and 120 rpm in a rotary shaker for 24 h. Then 1% of the cultures were inoculated in 300 mL shake flasks containing 30 mL ectoine induced synthesized medium at 30 °C and 120 rpm in a rotary shaker for 48 h.

2.5. Determination method of ectoine
The sample for determining ectoine was prepared by ethanol extraction method[4]. Ectoine was determined by high performance liquid chromatography (HPLC) method[4].

2.6. SND method
The strains were cultivated in 5 mL LB medium containing 60 g/L NaCl at 30 °C and 120 rpm in a rotary shaker for 24 h. Then 1% of the cultures were inoculated in 300 mL shake flasks containing 30 mL N removal medium at 30 °C and 120 rpm in a rotary shaker. The N removal medium with NH$_4^+$-N as substrate contained an organic C source (40 g/L glucose) and oxygen was provided in the N removal process by shaking (120 rpm in a rotary shaker). Therefore, N removal by the strains was attributed to SND.

2.7. 16S rDNA analysis and identification and making phylogenetic tree method
Genomic DNA was extracted using TaKaRa DNAiso Reagent kit from Takara Biotechnology (Dalian) Co., Ltd., the strain was identified by 16S rDNA PCR using TaKaRa 16S rDNA Bacterial Identification PCR Kit, the DNA fragment was recovered using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0, and the PCR product was sequenced by the above company. Comparison of 16S rDNA sequence of the strain with the sequences in GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The 16S rDNA gene phylogenetic tree was prepared using MEGA6

2.8. N-removal-related enzyme-encoding gene cloning method
PCR Primers of N-removal-related enzyme-encoding genes were designed by the similarity comparison method. Genomic DNA was extracted using TaKaRa DNAiso Reagent kit. PCR was performed using TaKaRa Taq™ kit. PCR product was refined and recovered using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0. The DNA fragment was sequenced by Takara Biotechnology (Dalian) Co., Ltd.

2.9. Determination method of inorganic N
The total concentration of inorganic N (TN) was the sum of concentrations of ammonia N (NH$_4^+$-N), hydroxylamine N (NH$_2$OH-N), nitrite N (NO$_2^-$-N), and nitrate N (NO$_3^-$-N). The N removal rate was defined as the percentage reduction in TN of the TN in the N removal system. N removal rate = (TN$_0$ − CN − TN$_t$) / (TN$_0$ − CN) × 100%. Where NT$_0$ was total inorganic N at the beginning of N removal, CN was total cell N and NT$_t$ was TN at the end of N removal. The N balance equation in the N removal system was TN$_0$ = CN + TN$_t$ + GN, where TN$_0$ is initial total inorganic N, CN is total cell N, TN$_t$ is final total inorganic N and GN is the inorganic N emission from the system as gaseous N[5]. NH$_4^+$-N was determined by Nessler’s reagent method[6]. NH$_2$OH-N was determined by 8-hydroxyquinoline reagent method[7]. NO$_2^-$-N was determined by diazotization-coupling reaction method[6]. NO$_3^-$-N was determined by zinc-cadmium reduction method[8]. Determination method of cell total N (CN). CN was determined by Kjeldahl method[9]. CN with different cell dry weight (CDW) was determined. The relationship between CDW and CN was calculated. CN was calculated by determining the CDW in experiment.
3. Results and discussions

3.1. Isolating, screening and identification of Halomonas strains

The Halomonas strains were isolated, screened and identified from the sediment of a saltern pool in the city of Dalian, Liaoning Province, China. Single colonies of Gram-negative strains were selected from LB separation plates (30-180 g/L NaCl) according to method 2.3. These single colony strains were subjected to ectoine induced synthesized experiments according to method 2.4, and the concentrations of ectoine synthesized by strains were determined according to method 2.5. N removal by SND was then tested in strains positive for ectoine synthesis according to method 2.6. The 16S rDNA gene sequences of the strains positive for N removal by SND were analyzed according to method 2.7. 7 Halomonas strains with SND ability under 30-120 g/L NaCl were obtained and named as Halomonas sp. B01, Halomonas sp. B11, Halomonas sp. B17, Halomonas sp. B31, Halomonas sp. B35, Halomonas sp. B36, Halomonas sp. B61, with the sequence registered in GenBank under the accession no. KJ778559, MG561375-MG561380. The study of Halomonas sp. B01 was reported in another article[3]. 8 Halomonas strains with SND ability were selected from the standard strains preserved in our laboratory. The above 6 Halomonas strains and 8 Halomonas standard strains were used to prepare 16S rDNA phylogenetic tree according to method 2.7. The results were shown in Figure 1. Figure 1 showed the phylogenetic relationship of the above 14 Halomonas strains with SND ability.

![Figure 1. Phylogenetic tree based on 16 rRNA sequences of Halomonas SND strains.](image)

3.2. Synthesis of ectoine in Halomonas strains under NaCl stress
The synthesis of ectoine in 14 *Halomonas* strains under NaCl stress was investigated. Ectoine was induced and synthesized according to method 2.4, and the concentration of ectoine synthesized by the strain was determined according to method 2.5, and the amount of cell growth was measured by the weighing method. The results were shown in Figure 2. 14 *Halomonas* strains were able to synthesize ectoine under the induction of 60 g/L NaCl, and the highest amount was *H. elongata* DSM 2581T strain (1200.2 mg/L). The cell growth (CDW, g/L) of these strains was above 3.1 g/L at 48 h, and the maximum growth of *H. venusta* DSM 4743 was 9.2 g/L.

### 3.3. N-removal-related enzyme-encoding genes of *Halomonas* strains

The N removal process in microorganisms generally is NH$_4^+$ →NO$_2^-$ →NO$_3^-$ →NO →N$_2$O → N$_2$, and the series of enzymes catalyzing the above reactions include ammonia monooxygenase (AMO), nitrate reductase (NAR) and nitrate reductase (NIR)[10]. The genes encoding these enzymes are *amo A*, *nar H* and *nir S*. Primers were designed by the similarity comparison method according to method 2.8, and the above genes were cloned. The results were shown in Table 1. The above nucleotide sequences have been deposited in the NCBI database under accession numbers MG561375-MG561386.

| Strain               | N-removal-related enzyme-encoding gene sequences | N-removal-related enzyme-encoding gene sequences Blast comparison maximum similarity |
|----------------------|-----------------------------------------------|-----------------------------------------------------------------------------------|
| *H. elongata* DSM 2581T | *amo A*: FN869568* 100% 99.4% | *amo A*: CBV41506 100% |
| *H. halodenitrificans* DSM 735 | *amo A*: FN869568* 100% 99.4% | *amo A*: CBV41506 100% |
| *H. venosae* DSM 15911 | *amo A*: - | *amo A*: - |

* GenBank([http://www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)) number of the strain, and the others are the same.

- No results after cloning.

### 3.4. SND under highsalt conditions by *Halomonas* strains
Figure 3. N removal rates by SND of 14 *Halomonas* strains.

Note: N removal medium containing 4000 mg/L initial NH$_4^+$-N, 40 g/L glucose, 60 g/L NaCl, at 30 °C and 120 rpm in a rotary shaker for 192 h.

The SND ability of the above 14 *Halomonas* strains was investigated. N removal by SND was assessed according to method 2.6. The concentration of NaCl in the N removal medium was set to 60 g/L. The concentration of inorganic N was determined according to method 2.9, the N removal rates were calculated by N balance, and the results were shown in Figure 3. 14 *Halomonas* strains were able to remove N by SND under 60 g/L NaCl concentration, the N removal rate was in the range of 21.7% to 91.8%. The N removal rate of *H. venusta* DSM 4743 was maximum, and it reached 91.8%. The results of Figure 3 indicate that the ability of remove N by SND is a common property of *Halomonas* bacteria.

3.5. N removal processes by *H. venusta* DSM 4743

There are two types of ectoine synthetic strains, one is the ectoine-nonexcreting type, the synthetic ectoin is only accumulated in the cells, such as *H. elongata* DSM 2581T. The second is ectoine-excreting type, that is, the wild-type ectoine synthetic strain, the synthesized ectoine can be secreted extracellularly under the condition that the environmental osmotic pressure is substantially constant, and ectoine is accumulated in the medium[4]. The ectoine-secreting *Halomonas* strain not only has its own adverse environmental property such as high osmotic pressure, but also confers resistance to other microorganisms in a specific system due to secretion of ectoine. The ectoine-secreting strain *H. venusta* DSM 4743 with the highest N removal rate was selected from the above strains, and the SND process at 60 g/L NaCl concentration was investigated. The results were shown in Figure 4. *Halomonas venusta* DSM 4743 removed N by SND under the conditions of 4000 mg/L initial NH$_4^+$-N, after 192 h the residual total inorganic N concentration was 218.7 mg/L and N removal rate was 91.8%. The highest concentrations of NO$_3^-$-N and NO$_2^-$-N were 4.1 mg/L and 2.7 mg/L, respectively, during the N removal process. NH$_2$OH-N was not detected. There was almost no accumulation of intermediate products during the N removal process.
Figure 4. N removal processes by *H. venusta* DSM 4743.

Note: N removal medium containing 4000 mg/L initial NH$^+$-N, 40 g/L glucose, 60 g/L NaCl, at 30 °C and 120 rpm in a rotary shaker for 192 h.

4. Conclusions

In this paper, 14 *Halomonas* strains capable of remove N by SND were obtained, among them, 8 strains were the standard strains preserved in our laboratory, the others were isolated, screened and identified from the sediment of a saltern pool in the city of Dalian, Liaoning Province, China. 14 *Halomonas* strains were able to synthesize ectoine in the medium containing 60 g/L NaCl, and the highest synthesis amount of ectoine was *H. elongata* DSM 2581$^T$ strain (1200.2 mg/L). N-removal-related enzyme-encoding gene fragments of partial *Halomonas* strains were cloned: amo A (*H. elongate* DSM 2581$^T$, *H. halodenitrificans* DSM 735), nar H (*H. elongate* DSM 2581$^T$, *H. halodenitrificans* DSM 735), nir S (*H. halodenitrificans* DSM 735, *H. ventosae* DSM 15911).

The N removal rates of 14 *Halomonas* strains by SND were in the range of 21.7% to 91.8%. The N removal rate of *H. venusta* DSM 4743 was maximum, and it reached 91.8%. The N removal of *H. venusta* DSM 4743 by SND was performed under the conditions of 60 g/L NaCl and 4000 mg/L initial NH$_4^+$-N, after 192 h the residual total inorganic N concentration was 218.7 mg/L. There was almost no accumulation of intermediate products during the N removal process. The study had shown that the ability of remove N by SND is a common property of *Halomonas* bacteria.

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References

[1] Haba, R.R., Arahal, D.R., Sanchez-Porro. C., Ventosa. A. (2014) The *prokaryotes: Gammaproteobacteria*: 17 The Family *Halomonadaceae*. Springer, Heidelberg.

[2] Pastor, J.M., Salvador, M., Argandoña, M., Bernal, V., Reina-Bueno, M., Csonka, L.N., Iborra, J.L., Vargas, C., Nieto, J.J., Cánovas, M. (2010) Ectoines in cell stress protection: Uses and biotechnological production. Biotechnol. Adv., 28: 782–801.

[3] Wang, T., Li, J., Zhang, L.H., Yu, Y., Zhu, Y.M. (2017) Simultaneous heterotrophic nitrification and aerobic denitrification at high concentrations of NaCl and ammonia nitrogen by *Halomonas* bacteria. Water Sci. Technol., 76: 386–395.

[4] Zhang, L.H., Lang, Y.J., Nagata, S. (2009) Efficient production of ectoine using ectoine-excreting
strain. Extremophiles, 13: 717–724.

[5] Jin, R.F., Liu, T.Q., Liu, G.F., Zhou, J.T., Huang, J.Y., Wang, A.J. (2015) Simultaneous heterotrophic nitrification and aerobic denitrification by the marine origin bacterium Pseudomonas sp. ADN-42. Appl. Biochem. Biotechnol., 175: 2000–2011.

[6] APHA (1999) Standard methods for the examination of water and wastewater 20th edn. American Public Health Association, Washington, DC, USA.

[7] Samuni, Y., Samuni, U., Goldstein, S. (2012) The mechanism underlying nitroxyl and nitric oxide formation from hydroxamic acids. Biochim. Biophys. Acta., 1820: 1560–1566.

[8] Sun, H.F., Wang, H.W., Yuan, C.Y. (2013) Optimization of zinc-cadmium reduction method for determination of nitrate in seawater. Advanced Materials Research, 864–867: 1004–1007.

[9] AOAC (1990) Official methods of analysis 15th edn. Association of Official Analytical Chemist, Arlington, VA, USA.

[10] Jetten, M.S.M., Logemann, S., Muyzer, G., Robertson, L.A., Vries, S.D., Loosdrecht, M.C.M.V., Kuenen, J.G. (1997) Novel principles in the microbial conversion of nitrogen compounds. Antonie Van Leeuwenhoek. 71: 75–93.