Screening and Denitrification Characteristics of Simultaneous Heterotrophic Nitrification and Aerobic Denitrification Bacteria

Ang Qu¹, Xiaoya Liang¹, Xiaoshuang Deng¹, Yaoting Wu¹, Linghua Zhang¹*, Weifeng Liu¹ and Yimin Zhu¹

¹Environmental Science and Engineering College, Dalian Maritime University, Dalian, Liaoning, 116026, China
*Corresponding author’s e-mail: dlzlh2008@163.com

Abstract. Two highly efficient simultaneous heterotrophic nitrification and aerobic denitrification (SND) bacteria, isolated from activated sludge samples which domesticated in laboratory, were identified by 16S rDNA as Pseudomonas sp. QU02 and Paracoccus sp. QU07. The factors influencing the denitrification of Pseudomonas sp. QU02 and Paracoccus sp. QU07. SND were investigated. For Pseudomonas sp. QU02, the optimum carbon source was sodium succinate, the optimum C/N was 10, the optimum initial pH was 8, and the optimum NaCl concentration was 5 g/L. The optimum carbon source for denitrification of Paracoccus sp. QU07 was sodium succinate, the optimum C/N was 10, and the optimum initial pH was 7, the optimum NaCl concentration was found to be 5 g/L, and salt tolerance response in two strains. The SND process was characterized under optimized conditions. The N removal rate of two strains were higher than 90% after 72 h SND. The enhanced denitrification of industrial wastewater by mixed SND strains was investigated. After 144 h SND, N removal rate is obviously better than unstrengthened. The mixed SND strains have potential practical application value in enhancing SND of industrial wastewater.

1. Introduction
Ammonia nitrogen is one of the main pollutants in water body. Its pollution will cause great harm to aquatic organisms, water ecosystem and human health. Therefore, the treatment of high concentration of ammonia nitrogen in wastewater is particularly urgent[1]. The treatment methods of ammonia nitrogen pollution can be divided into physical method, chemical method and biological method. Compared with physical and chemical method, biological method has the advantages of no secondary pollution and low cost[2]. Among them, nitrification and denitrification need to be separated in the treatment of ammonia nitrogen wastewater by traditional biological method, which is complex and covers a large area. SND is a simultaneous denitrification method by heterotrophic nitrification and aerobic denitrification under the same operating conditions in the same reactor. It has the advantages of high denitrification efficiency, stable system pH, suitable for the treatment of ammonia-nitrogen wastewater containing organic matter, simple process operation, etc[3]. Although SND method has obvious advantages in denitrification, the high concentration of salt and high ammonia wastewater produced in some industries can inhibit the growth of microorganisms, resulting in a reduction in N removal rate of SND[4,5]. In order to improve N removal rate in industrial wastewater, this paper intends to isolate and screen SND strains from activated sludge samples, and study the denitrification
characteristics of the strains with high N removal rate from four aspects: carbon source type, C/N, initial pH and NaCl concentration. The strains are cultured and used to enhance SND.

2. Materials and methods

2.1. Experimental materials

2.1.1. Samples for strain isolation and industrial wastewater. Samples for strains isolated: activated sludge samples which domesticated for 3 months by SND denitrification in our laboratory. Industrial wastewater: The concentration of NaCl is 5 g/L and the initial concentration of NH$_4^+$-N is 600 mg/L in wastewater with activated sludge from an enterprise.

2.1.2. Medium and Nitrogen removal solution. LB medium (g/L): peptone 10, yeast powder 5, NaCl 5, pH 7. The medium was autoclaved at 121 °C for 20 min. Growth medium (g/L): glucose 40, (NH$_4$)$_2$SO$_4$ 10, 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, NaCl 5, pH 7.0. The medium was autoclaved at 121 °C for 20 min. Trace mineral solution 2 mL (EDTA-2Na 63.7, ZnSO$_4$ 2.2, CaCl$_2$ 5.5, MnCl$_2$•H$_2$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). The trace mineral solution was sterilized by filtration (0.22 µm pore size, Millipore Express, USA). Glucose was autoclaved at 115 °C and 15 min separately.

Nitrogen removal solution (g/L): (NH$_4$)$_2$SO$_4$ 2.83 (concentration of N is 600 mg/L), sodium succinate 33.75 (concentration of C is 6000 mg/L), K$_2$HPO$_4$•3H$_2$O 0.3, KH$_2$PO$_4$ 0.1, MgSO$_4$•7H$_2$O 0.4, MnSO$_4$•H$_2$O 0.01, NaCl 5, pH 7.0. The medium was autoclaved at 121 °C for 20 min.

2.1.3. Kit. TaKaRa 16S rDNA Bacterial Identification PCR Kit (D310), purchased from Takara Biotechnology (Dalian) Co., Ltd.

2.2. Experimental method

2.2.1. Isolation of strains. Strains were isolated by dilution method and coated on solid LB medium. The bacteria were cultured upside down at 30 °C. Single colonies were separated repeatedly until the gram-coloring and microscopic examination were pure bacterial colonies.

2.2.2. Screening of SND bacteria and identification by 16S rDNA. Single colonies were selected to screen strains with SND capability. The 16S rDNA was amplified by the kit "2.1.3" method, the PCR products were refined and recovered, and the sequencing work was entrusted to Bao Biology (Dalian) Co., Ltd. The 16S rDNA nucleotide sequence measured was compared with the GenBank database of NCBI website.

2.2.3. Simultaneous heterotrophic nitrification and aerobic denitrification. The strain was activated and cultured for 24 h, and centrifuged at 7200 ×g and 4 °C for 20 min. The strain was transferred to nitrogen removal solution at 30 °C and 120 rpm for 48 h.

2.2.4. Determination method of inorganic N. The total concentration of inorganic N (TN) was the sum of concentrations of ammonia N (NH$_4^+$-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. NH$_4^+$-N was determined by Nessler’s reagent method[6]. NO$_2^-$-N was determined by diazotization-coupling reaction method[6]. NO$_3^-$-N was determined by zinc-cadmium reduction method[7]. Determination method of cell total N (CN). CN was determined by Kjeldahl method[8]. 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 denitrifying bacteria

3.1.1. Isolating and screening and of denitrifying bacteria. In order to obtain SND denitrification strains, the strains were separated from the samples according to the method "2.2.1", and N removal rate of SND were determined and calculated according to the method "2.2.3". The results showed that N removal rate of QU02 and QU07 were significantly higher than other strains, reaching 92.2% and 99.2% respectively, and there was no significant accumulation of NO$_3^-$-N and NO$_2^-$-N during denitrification. Select strains QU02 and QU07 for further study.

3.1.2. Identification of denitrifying bacteria. The strains were identified according to the method "2.1.2" and named Pseudomonas sp. QU02 and Paracoccus sp. QU07 respectively according to the results of comparison.

3.2. Study on SND characteristics by strains.

The effects of carbon source type, C/N, initial pH and NaCl concentration on the SND characteristics of Pseudomonas sp. QU02 and Paracoccus sp. QU07 with high N removal rate were studied.

3.2.1. Effects of carbon sources on SND characteristics. The effects of different carbon sources on SND characteristics were investigated. The carbon sources in nitrogen removal solution were glucose, sucrose, trisodium citrate, sodium succinate and sodium acetate (concentration of C is set to 6 g/L). According to the method of "2.2.3" SND, the results are shown in Fig 1. The N removal rate of Pseudomonas sp. QU02 was 84.9% with sodium succinate as the carbon source. The N removal rate of Paracoccus sp. QU07 with sodium succinate as the carbon source was the highest, reaching 99.3%.

![Fig 1. Effects of carbon sources on SND](image1)

Note: C/N 10, pH 7, NaCl 5 g/L, in Nitrogen removal solution at 30 °C and 120 rpm in a rotary shaker for 48 h.

3.2.2. Effect of C/N on SND. The effects of different C/N on SND characteristics were investigated. The denitrification matrix N concentration is 600 mg/L, sodium succinate was added to C/N 2.5, 5, 7.5, 10, 12.5 and 15, respectively, according to the method of "2.2.3" SND, the results are shown in Fig 2. 10 is suitable C/N for Pseudomonas sp. QU02, and the best C/N for Paracoccus sp. QU07 is 10.
3.2.3. **Effect of initial pH on SND.** The effects of different initial pH on SND characteristics were investigated. The initial pH was set to 5, 6, 7, 8 and 9 in nitrogen removal solution. According to the method of "2.2.3" SND, the results are shown in Fig 3. The optimum pH for *Pseudomonas* sp. QU02 is 8, 7 is optimum pH for *Paracoccus* sp. QU07.

3.2.4. **Effect of NaCl concentration on SND.** The effects of different NaCl concentrations on SND characteristics were investigated. The concentration of NaCl was set to 5, 15, 30, 45 and 60 g/L in nitrogen removal solution. According to the method of "2.2.3" SND, the results are shown in Fig 4. *Pseudomonas* sp. QU02 reached 95.4% at NaCl concentration of 5 g/L, the highest can reach 93.1% for *Paracoccus* sp. QU07. N removal rate are 61.9% and 69.7% at NaCl concentration of 60 g/L, respectively.

Note: the carbon source is sodium succinate, C/N 10, NaCl 5 g/L, at 30 °C and 120 rpm in a rotary shaker for 48 h.
3.2.5. N removal processes. The N removal process of *Pseudomonas* sp. QU02 and *Paracoccus* sp. QU07 under the optimum conditions were investigated. According to the method of "2.2.3" SND, the results are shown in Fig 5. The maximum denitrification rate of *Pseudomonas* sp. QU02 and *Paracoccus* sp. QU07 was 93.7% and 97.2% respectively at 72 hours.

![Fig 5. N removal process](image)

Note: the carbon source is sodium succinate, C/N 10, pH 8(*Pseudomonas* sp. QU02) or 7(*Paracoccus* sp. QU07), NaCl 5 g/L, at 30 °C and 120 rpm in a rotary shaker for 48 h.

3.3. SND bacteria enhanced SND removal from industrial wastewater

*Pseudomonas* sp. QU02 and *Paracoccus* sp. QU07 were cultured and centrifuged for 20 minutes at 7200 ×g and 4 °C to transfer the bacteria to industrial wastewater to enhance the N removal capacity of SND. The control group did not add bacteria. The result is shown in Fig 6. At 144 h, the enhanced N removal rate was 55.0%, which was 28.3% higher than that of the non-enhanced. At the end of denitrification, the concentration of NO$_2$ -N was 0.209 mg/L, and the concentration of NO$_3$ -N exceeded the detection limit.

![Fig 6. Enhanced N removal process of industrial wastewater by mixed SND strains](image)

4. Conclusions

(1) Two efficient SND strains were isolated from activated sludge samples which domesticated in laboratory and identified by 16S rDNA as *Pseudomonas* sp. and *Paracoccus* sp. The strains were named *Pseudomonas* sp. QU02 and *Paracoccus* sp. QU07, respectively.

(2) SND characteristics of *Pseudomonas* sp. QU02 and *Paracoccus* sp. QU07 strains with high denitrification rate were investigated from four aspects: carbon source, C/N, initial pH and NaCl concentration. Among them, the optimum carbon source for *Pseudomonas* sp. QU02 was sodium succinate, the optimum C/N was 10, the optimum initial pH was 8, and the optimum NaCl concentration was 5 g/L; the optimum carbon source for denitrification of *Paracoccus* sp.QU07 was sodium succinate, the optimum C/N was 10, the optimum initial pH was 7, and the optimum NaCl concentration was 5 g/L, and the two strains had certain salt tolerance.
(3) Under the optimum conditions, the N removal processes of strains *Pseudomonas* sp. QU02 and *Paracoccus* sp. QU07 were investigated. After 72 hours of SND, N removal rate of strain *Pseudomonas* sp. QU02 reached the maximum of 93.7% at 72 hours, and that of strain *Paracoccus* sp. QU07 reached the maximum of 97.24% at 72 hours.

(4) SND of industrial wastewater was enhanced by *Pseudomonas* sp. QU02 and *Paracoccus* sp. QU07. After 144 h, the N removal rate of mixed SND strains was 55.0%, 28.3% higher than that of non-enhanced ones.

**Acknowledgments**

This research was funded by the Fundamental Research Funds for the Central Universities and Collaborative Innovation Center for Vessel Pollution Monitoring and Control Seed Fund Project, Dalian Maritime University (20110216001).

**References**

[1] Zhang Y, Chu Y, Wang H, (2018) Operating Condition of Ammonia Nitrogen Removal in BMED Process Optimized by Response Surface Method. Environmental Science & Technology.

[2] Ramos C, Suárez-Ojeda M E, Carrera J. (2016) Denitrification in an anoxic granular reactor using phenol as sole organic carbon source. Chemical Engineering Journal, 288:289-297.

[3] Yang T, Yang Y, Liu Y X. (2017) Research progress and challenges of heterotrophic nitrification-aerobic denitrification. Microbiology China.

[4] Vyrides I. and Stuckey D. C.: (2009). Adaptation of anaerobic biomass to saline conditions: Role of compatible solutes and extracellular polysaccharides, Enzyme Microb. Technol., 44, 46–51

[5] Uygur A. and Karg F.: (2004) Salt inhibition on biological nutrient removal from saline wastewater in a sequencing batch reactor, Enzyme Microb. Technol., 34, 313–318.

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

[7] 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.

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