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RESEARCH ARTICLE

Simultaneous removal of urea nitrogen and inorganic nitrogen from high-salinity wastewater by *Halomonas* sp. H36

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Abstract

To treat high-salt urea wastewater by microbial hydrolysis, it is necessary to overcome the dual problems of incomplete removal of nitrogen (N) from mixed strains and inhibition of microbial activity by high salt (NaCl) concentrations. In this paper, the mechanism of NaCl tolerance of *Halomonas* sp. H36 was investigated. Using molecular biology and enzymatic methods, it was proven that the strain's N-removal enzymes (urease; ammonia monooxygenase, AMO; nitrite reductase, NIR; nitrate reductase, NAR) played a key role in the removal of N, and the N-removal pathway was clarified. For the strain used to treat simulated ship domestic sewage, the urea nitrogen ($CO(NH_2)_2$-N)-removal rate was 88.52%, the ammonia nitrogen (NH_4^+-N)-removal rate was 91.16%, the total nitrogen (TN)-removal rate was 90.25%, and nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) did not accumulate. It was proven for the first time that *Halomonas* sp. H36 has the function of simultaneous urea hydrolysis-nitrification–denitrification with urea as the initial substrate and can simultaneously remove urea nitrogen and inorganic nitrogen from high-salt urea wastewater.

Keywords High-salt urea wastewater · Microbial hydrolysis · *Halomonas* · N-removal enzymes · Urea nitrogen · Inorganic nitrogen

Introduction

Urea (H_2N-CO-NH_2) is a simple nitrogen (N)-containing organic compound that is widely used in agricultural fertilizers, deicers, foaming agents, herbicide and pesticide production, automobile exhaust treatment, and other fields (Urbańczyk et al. 2016). Agricultural N fertilizer leaching, industrial production with urea as a raw material, and human life produce large amounts of urea wastewater (Li et al. 2015). Studies have shown that 80% of N pollution in domestic sewage comes from urea (Jimenez et al. 2012). The urea content in urine is as high as 25 g/L (Lee 1971). Urea-N ($CO(NH_2)_2$-N) easily leads to coastal eutrophication (Tzilkowski et al. 2018). According to the 2020 China Marine Ecological Environment Bulletin, a total of 31 red tides were found in China's waters in 2020, with a cumulative area of 1,748 km^2. Among them, there were two toxic red tides, with a cumulative area of 81 km^2 (Ministry of Ecology and Environment, PRC 2020). Untreated urea wastewater poses a serious threat to biological survival, human health, and the ecological environment (Hu et al. 2020a, b; Vaneeckhaute and Fazli 2020). Therefore, there is an urgent need for the effective treatment of urea wastewater.

At present, microbial hydrolysis is one of the most economical and effective methods to remove urea from wastewater (Ilgrande et al. 2019; Jia et al. 2020). Traditionally, the complete removal of $CO(NH_2)_2$-N into gaseous N requires three steps: urea hydrolysis, nitrification, and denitrification (Garrido et al. 2001). However, the multistage urea treatment process has problems such as high cost and large space requirements. To overcome this drawback, researchers have used mixed strains for urea hydrolysis in a reactor. Hu et al. (2020a, b) treated urea-containing wastewater with mixed strains in a reactor. The results showed that urea-degrading bacteria, phylum *Gemmatimonadetes*, hydrolyzed urea to NH_3, and then NH_3 was oxidized to NO_2^- by ammonia-oxidizing bacteria. During this process, the high concentration of NH_4^+ inhibited the activity of nitrite-oxidizing bacteria,

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resulting in accumulation of NO_2^-, at a rate of 0.985 kg/(m^3·d); Chen et al. (2022) used a mixed flora dominated by urea-degrading bacteria (*Nitrosomonas*) and anaerobic ammonia-oxidizing bacteria (*Candidatus Brocadia*) to undergo urea hydrolysis ($CO(NH_2)_2$-N → NH_4^+-N), part of the nitrification (PN) (NH_4^+-N → NO_2^--N) and anammox (NH_4^+-N + NO_2^--N → $N_2 + 2H_2O$) pathways to complete the removal of total nitrogen (TN). However, in the existing mixed-bacteria urea-removal process, the type, activity, and ratio of urea-hydrolyzing bacteria to N-removing bacteria in the biofilm are not easy to control, and the accumulation of NO_2^--N and NO_3^--N makes it difficult to convert $CO(NH_2)_2$-N to gaseous N and completely remove it (Rittstieg et al. 2001). It is necessary to develop a simple, reliable, and efficient technology for the complete removal of $CO(NH_2)_2$-N.

In addition, urea wastewater is often accompanied by high salt (NaCl > 10 g/L) problems. Urea containing azodicarbonamide foaming agent industrial sewage (NaCl = 56 g/L), toilet flushing wastewater in coastal cities (NaCl = 10–17 g/L), and domestic sewage from ships (NaCl = 10–20 g/L) have high salt contents (Jiang et al. 2019; Li 2018; Yang et al. 2015). The resulting high-salt urea wastewater also needs to be treated urgently. However, high salt concentrations can inhibit the respiration of microorganisms and decrease the urea removal rate. When the NaCl concentration was increased from 0.37 to 30.70 g/L, the respiratory inhibition rate of microorganisms increased from 4 to 84% (Pernetti and Palma 2005). When the NaCl concentration increased from 0.00 to 0.04 g/L, the removal rate of $CO(NH_2)_2$-N by mixed bacteria decreased from 94.2 to 67.0% (Wang et al. 2020a, b).

Salt-tolerant or halophilic N-removal strains have attracted much attention in the field of high-salt nitrogenous wastewater treatment (Wang et al. 2019; Man et al. 2022). Most strains of *Halomonas* can synthesize ectoine without interfering with the normal life process of cells while balancing the osmotic pressure between the cytoplasm and the environment (Zhao et al. 2018). *Halomonas meridiana* SCSIO 43,005 can survive with urea as the sole N source (Zhou et al. 2020). *Halomonas venusta* TJPU05 has an obvious treatment effect on high-salt N-containing wastewater. Removal rates of 50.96%, 47.28%, and 43.19% were observed for NH_4^+-N, NO_3^-N, and TN, respectively (Man et al. 2022). Therefore, *Halomonas* has great application potential in the treatment of high-salt urea wastewater. To our knowledge, no one has carried out research on these issues in depth or systematically. (1) The process for removal of urea by *Halomonas*. (2) The removal effect of $CO(NH_2)_2$-N and inorganic N simultaneously by *Halomonas* in high-salinity environment.

In a previous study, *Halomonas* sp. H36 had the ability to hydrolyze urea (Li et al. 2020). In this paper, the mechanism of salt tolerance of *Halomonas* sp. H36 was investigated. We further evaluated the salt tolerance of N-removal enzymes of the strain. And the N-removal pathway of the strain with urea as the initial substrate was clarified. Finally, the strain was used for the treatment of simulating domestic sewage from ships. These findings provide a theoretical and methodological foundation for the bioremediation of high-salt urea wastewater in the future.

Materials and methods

Materials

Strain: Strain H36 was isolated from a salt-drying pond in a salt factory in Dalian City, Liaoning Province, China. This specimen was determined to be *Halomonas* sp. H36 (16S rRNA sequence GenBank No. ON935442) (Li et al. 2020).

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

Growth medium (g/L): glucose 15, monosodium glutamate 15, $(NH_4)_2SO_4$ 10, urea 5, yeast powder 0.5, $K_2HPO_4·3H_2O$ 9, KH_2PO_4 3, $MgSO_4·7H_2O$ 0.4, $MnSO_4·H_2O$ 0.01. The NaCl concentration was determined by the experimental conditions (ectoine-induced synthesis experiment: 30, 60, 90, 120 g/L NaCl; effects of different NaCl concentrations in the enzymatic reaction system on the N-removal enzyme activities of *Halomonas* sp. H36: 30 g/L NaCl), pH 7.2. The medium was autoclaved at 121 °C for 20 min. The urea solution was sterilized by filtration (0.22 μm pore size, Millipore Express, USA).

Inducing medium (g/L): glucose 30, KNO_3 0.4 (N element 50 mg/L), yeast extract 0.5, $K_2HPO_4·3H_2O$ 9, KH_2PO_4 3, $MgSO_4·7H_2O$ 0.4, $MnSO_4·H_2O$ 0.01, NaCl 60, pH 7.2. The medium was autoclaved at 121 °C for 20 min.

Trace mineral solution (g/L): EDTA-2Na 63.7, $ZnSO_4$ 2.2, $CaCl_2$ 5.5, $MnCl_2·4H_2O$ 5.1, $FeSO_4·7H_2O$ 5, $Na_2MO_4·2H_2O$ 1.1, $CuSO_4·5H_2O$ 1.6, $CoCl_2·6H_2O$ 1.6 (Vyrides and Stuckey 2009), pH 7.2. The trace mineral solution was sterilized by filtration (0.22 μm pore size, Millipore Express, USA).

N-removal medium (g/L): glucose 40, urea 6 (2,800 mg/L, $CO(NH_2)_2$-N), $K_2HPO_4·3H_2O$ 9, KH_2PO_4 3, $MgSO_4·7H_2O$ 0.4, $MnSO_4·H_2O$ 0.01, trace mineral solution 2 mL, NaCl 30. The N-removal medium was autoclaved at 121 °C for 20 min. The urea solution and the trace mineral solution were sterilized by filtration, respectively (0.22 μm pore size, Millipore Express, USA).

Simulated ship domestic sewage (mg/L): starch 100, glucose 800, urea 100, $(NH_4)_2SO_4$ 550, KH_2PO_4 31, $K_2HPO_4·3H_2O$ 9, trace mineral solution 0.1 mL, NaCl 30 g/L. The simulated ship domestic sewage was autoclaved at 121 °C for 20 min. The urea solution and the trace mineral

solution were sterilized by filtration, respectively (0.22 μm pore size, Millipore Express, USA).

Induction of ectoine synthesis and estimation of ectoine content

Induction of ectoine synthesis: The strains were cultivated in 5 mL LB medium at 30 °C and 120 rpm in a rotary shaker for 24 h. Then, 1% aliquots of each these cultures were inoculated in shake flasks (300 mL) containing 30 mL growth medium, followed by the strains being grown and cultured in 30, 60, 90, and 120 g/L NaCl growth medium at 30 °C and 120 rpm for 48 h in a rotary shaker (Wang et al. 2017).

Ectoine concentration determination method. Extracellular ectoine estimation: The culture solution induced with ectoine synthesis was centrifuged at 14,000 × *g*, and the supernatant was diluted tenfold with distilled water for high-performance liquid chromatography (HPLC) measurement. The ectoine concentration obtained by HPLC analysis was defined as the concentration of extracellular ectoine. Intracellular ectoine estimation: Cells were collected by the centrifugation method described above, and the pellets were washed with 100 mM potassium phosphate (KPi) buffer (pH 7.2) containing NaCl at the same concentration as that of the medium. After centrifugation, the pellets were extracted with ethanol (80%, v/v) of the same volume as the culture medium, resuspended, and then stored at 25 °C overnight. The suspension was centrifuged again, and the supernatant was subsequently used for HPLC analysis. The ectoine concentration as estimated by HPLC analysis was defined as the concentration of intracellular ectoine. The total concentration of ectoine was the sum of the concentrations of intracellular and extracellular ectoine. The concentration of ectoine was measured by an HPLC setup with a C18 column, Hypersil ODS2 column (Elite, Dalian, China), and a UV detector (Elite, Dalian, China). A wavelength of 210 nm was used (Zhang et al. 2009).

Method of PCR

The primer sequences of N-removal enzymes (urease; ammonia monooxygenase, AMO; nitrite reductase, NIR; nitrate reductase, NAR) were designed by a similar comparison method (Zhang et al. 2012). The sequence design information of the N-removal enzymes of *Halomonas* sp. H36 are shown in Table 1. Genomic DNA using TaKaRa DNAiso Reagent (Code: D305A). The PCR product was refined and recovered using the TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (Code: DV805A) and was then sequenced by Takara Biotechnology (DALIAN) Co., Ltd.

Determination of N-removal enzyme activities

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 (300 mL) containing 30 mL growth medium at 30 °C and 120 rpm in a rotary shaker for 36 h. The cells were collected by centrifugation (at 4 °C and 14,000 × *g* for 15 min) and transferred to the inducing medium at 30 °C and 120 rpm for 36 h.

Urease is a cytoplasmic enzyme (Mulrooney et al. 1989), AMO and NAR are cell membrane-bound enzymes (Hyman and Arp 1992; Blasco et al. 1992), and NIR is a periplasmic space enzyme (Blackmore et al. 1986).

Preparation of crude extracts of N-removal enzyme activities for enzyme activity determination: A crude extract of each enzyme prepared for this study was used for enzyme activity assays. The cytoplasmic enzyme (including the periplasmic enzyme) was prepared in 20 mL of the cell culture medium, which was also used to determine enzyme activity, centrifuged at 14,000 × *g* for 15 min at 4 °C, and the supernatant was discarded. Then, 100 mM phosphate buffer (pH 7.2) was added to the centrifuged pellet to resuspend it. A freeze–thaw cycle (at − 20 °C ≥ 2 h and at 30 °C for 30 min) was repeated 4 times. Ultrasound was used to disrupt cells in an ice bath (sonicated at 400 W for 3 s and stopped for 3 s) continuously through 30 cycles to obtain a solution of disrupted cells. The disrupted-cell solution was centrifuged at 14,000 × *g* for 15 min at 4 °C, the supernatant was collected, and the sample was used for cytoplasmic and periplasmic enzyme assays. The cell membrane debris was in the centrifuged pellet. The membrane-bound enzyme was prepared as follows: Dodecyl-β-D-maltoside is an alkylglycoside nonionic surfactant that promotes the disintegration of

Table 1 Reference strains and primers designed to amplify the gene sequence of the N-removal enzymes of *Halomonas* sp. H36

Reference strains (GenBank)	Target gene	Primers
Halomonas taeanensis strain USBA-857 (NZ_QLSX00000000.1)	*ureA*	F-GTGATCACCAATGCGCTGAT R-CCCTTCGGTGTGATAGGTGT
Halomonas campaniensis strain LS21 (NZ_CP007757.1)	*amoA*	F-ATGAAAGGCATCCGTCTCAGCG R-TTAAGAAGATTGGCGACGCAAT
Halomonas venusta strain MA-ZP17-13 (NZ_CP034367.1)	*nirS*	F-TTACCCCGCCACCCAGACCT R-ATGACCGCGACCGCACTCAA
	narH	F-TGACAAGGTGCAGGCAGATA R-CAATGGCCAAGTAGCGGTAC

lipid membranes to release membrane proteins and provides a hydrophobic environment for membrane proteins in the state of membrane removal in solution. It maintains and protects the hydrophobic transmembrane structure of membrane proteins, thereby maintaining the structure and function of membrane proteins (Wang et al. 2020a). Therefore, decyl-β-D-maltoside is often used to extract proteins from cell membranes. In this paper, dodecyl-β-D-maltoside was used to dissolve the cytoplasmic membrane and release the membrane-bound proteins. The disrupted-cell centrifuge pellet, as described above, was resuspended in 100 mM phosphate buffer (pH 7.2) containing 1.0% dodecyl-β-D-maltoside, incubated for 1 h at 4 °C in the dark, and centrifuged at 14,000 × *g* for 15 min at 4 °C. Then, the supernatant was collected, and the sample was used for the cell membrane-bound enzyme assay. The total protein concentration of the enzyme solution to be determined was assayed as follows: Protein was determined using the BCA Protein Concentration Assay Kit (PC0020, Solarbio, Beijing, China). The amount of the enzyme solution to be determined was based on the total protein (mg) (Wang et al. 2020a).

Except for the enzyme reaction system (NaCl = 30 60 90 120 g/L in **3.3**), NaCl = 0 g/L in the enzyme reaction system in other cases.

The urease activity was assayed as follows: Urease hydrolyzes $CO(NH_2)_2$-N to NH_4^+-N (Wang 2009). One unit of urease activity (U) is the amount of urease that hydrolyzes 1 mmol $CO(NH_2)_2$ per min at the reaction substrate of $CO(NH_2)_2$ (40 g/L), pH 7.2, and 37 °C under the specified reaction conditions.

The AMO activity was assayed as follows: AMO oxidizes NH_4^+-N to NH_2OH (Ensign et al. 1993). One unit of AMO (U) is the amount of AMO that oxidizes 1 μmol NH_4^+ per min at the reaction substrate of NH_4^+-N (1,000 mg/L), pH 7.2, and 30 °C under the specified reaction conditions.

The NIR activity was assayed as follows: NIR reduces NO_2^- to NO (Rosa et al. 2001). One unit of NIR (U) is the amount of NIR that reduces that 1 μmol NO_2^- per min at the reaction substrate of NO_2^--N (1,000 mg/L), pH 7.2, and 30 °C under the specified reaction conditions.

The NAR activity was assayed as follows: NAR reduced NO_3^- to NO_2^- in the presence of a reduced coenzyme (Kushner 1985). One unit of NAR activity (U) is the amount of NAR that reduces 1 μmol NO_3^- per min at the reaction substrate KNO_3 (50.55 mg/L), pH 7.2, and 30 °C under the specified reaction conditions.

Determination method of N

The TN is the sum of cell total N (CN), $CO(NH_2)_2$-N, NH_4^+-N, NO_2^--N, NO_3^--N.

$CO(NH_2)_2$-N-removal rate (%) = $(TUN_0 - CN - TUN_t) \times 100\% / (TUN_0 - CN)$, where TUN_0 is the initial $CO(NH_2)_2$-N, CN is the cell total N, and TUN_t is the $CO(NH_2)_2$-N at a certain point in the N-removal process.

NH_4^+-N-removal rate (%) = $(TAN_0 - CN - TAN_t) \times 100\% / (TAN_0 - CN)$, where TAN_0 is the initial NH_4^+-N, CN is the total cellular N, and TAN_t is the NH_4^+-N at a certain point in the N-removal process.

TN-removal rate (%) = $(TN_0 - CN - TN_t) \times 100\% / (TN_0 - CN)$, where TN_0 is the initial TN, CN is the total cellular N, and TN_t is the TN at a certain point in the N-removal process.

CN was determined by the Kjeldahl method (Wang et al. 2020a). Cell growth was defined as the cell dry weight per liter of culture medium (CDW, g/L). The CDW of different qualities was taken to determine CN, and the relationship between CDW and CN was fitted. The urea concentration was determined by the p-dime-thylam inobenzaldehyde colorimetric method (Jiang and Bao 2005). NH_4^+-N was determined by Nessler's reagent method (Wang et al. 2020a). NO_2^--N was determined by the diazo-azo reaction method (Eaton et al. 1966). NO_3^--N was determined by the zinc-cadmium reduction method (Sun et al. 2013).

Results and discussion

Effect of the medium NaCl concentration on the urease activity of Halomonas sp. H36

A previous study obtained a strain of *Halomonas* sp. H36 with high urease activity (Li et al. 2020). This experiment investigated the effect of NaCl concentration in the medium on the urease activity of *Halomonas* sp. H36. The results are shown in Fig. 1.

Under different NaCl concentrations, the cell growth and urease production of *Halomonas* sp. H36 had the same trend. When NaCl = 60 g/L, the strain growth amount was 10.26 g/L, and the urease activity was 75.28 U, both of which reached maximum values. When NaCl > 60 g/L, the urease activity of the strain decreased with increasing NaCl concentration. This shows that the excessive NaCl concentration in the medium affects the urease-producing ability of the strain. When NaCl = 120 g/L, the strain growth amount still reached 7.05 g/L, which was 68.71% of the maximum growth amount, the urease activity was 37.89 U, and 50.33% of the enzyme activity was still retained. Thus, *Halomonas* sp. H36 could still produce urease under high salinity.

The intracellular ectoine concentration increased with increasing NaCl concentration in the growth medium, which was similar to the ectoine synthesis in *Halomonas* sp. B01 (Wang et al. 2020a). In this experiment, the lowest intracellular ectoine concentration was 283.04 mg/L (30 g/L NaCl), and the highest intracellular ectoine concentration was 1179.89 mg/L (120 g/L NaCl). The extracellular ectoine

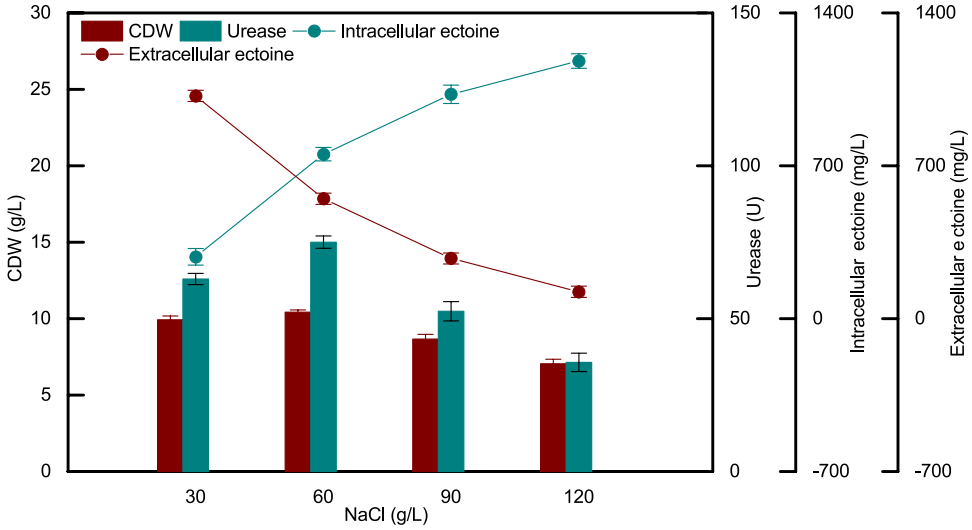


Fig. 1 Growth, urease production, and ectoine synthesis by *Halomonas* sp. H36 under different NaCl concentrations

concentration decreased with increasing NaCl concentration. The highest extracellular ectoine concentration was 1019.98 mg/L (30 g/L NaCl), and the lowest extracellular ectoine concentration was 123.13 mg/L (120 g/L NaCl). The highest secretion rate of ectoine was 78.28%, which was 1.28 times that of *Halomonas organivorans* D227 (Thuoc et al. 2019). The results indicated that *Halomonas* sp. H36 could initiate the protective mechanism of synthesizing ectoine in response to high-salt stress when NaCl = 30 g/L. *Halomonas* sp. H36 ectoine, which is used to regulate osmotic pressure, further protects the strain activity and allows the strain to produce urease.

In addition, it has been reported that nonsalt-tolerant strains in a hyperosmotic environment can maintain their own stability by ingesting external ectoine (Ono et al. 1999). In the future, the ectoine-secreting strain *Halomonas* sp. H36 may be used as a bioenhanced strain to hydrolyze urea and confer ectoine to other nonsalt-tolerant strains with additional function. This is of great significance for the comprehensive treatment of high-salt N-containing wastewater.

Halomonas sp. H36 N-removal enzyme gene cloning

At the molecular level, it has been proven that *Halomonas* sp. H36 N-removal enzymes exist, including urease, AMO, NIR, and NAR. The DNA of *Halomonas* sp. H36 was amplified by PCR to detect N-removal enzyme genes (*ureA*, *amoA*, *nirS*, *narH*) or fragments of *Halomonas* sp. H36. The agarose gel electrophoresis of the obtained amplification product is shown in Fig. 2. BLAST comparison was performed between the sequencing and translation results, and the results are shown in Table 2. Additional data are given in Online Resource 1.

In this study, the gene fragments (*ureA*, *amoA*, *nirS*, and *narH*) of urease, AMO, NIR, and NAR were successfully amplified from a strain of *Halomonas* for the first time. This provides a molecular theoretical basis for the simultaneous removal of $CO(NH_2)_2$-N and inorganic N from high-salt N-containing wastewater by *Halomonas*.

Effects of different NaCl concentrations in the enzymatic reaction system on the N-removal enzyme activities of Halomonas sp. H36

At the enzymology level, under different NaCl concentrations (0, 30, 60, 90, and 120 g/L) in the enzyme reaction system, without adding exogenous ectoine, the activities of the N-removal enzymes of *Halomonas* sp. H36 were investigated and the results are shown in Fig. 3.

The N-removal enzymes of *Halomonas* sp. H36 still had good activity under high NaCl concentrations in the enzymatic reaction system. With the increase in NaCl concentration in the enzyme reaction system, the activities of the N-removal enzymes decreased to varying degrees. When NaCl = 120 g/L, the enzyme activities of urease, AMO, NIR, and NAR still retained 74.25%, 22.26%, 32.01%, and 26.82%, respectively. Compared with *Halomonas* sp. B01 NAR, there was no enzymatic activity under 120 g/L NaCl (Wang et al. 2020a, b). In this study, the N-removal enzymes (urease, AMO, NIR, and NAR) of *Halomonas* sp. H36 still had enzymatic activities under high-NaCl concentrations.

The effects of salinity on the activities of N-removal enzymes (urease, AMO, NIR, and NAR) in the enzymatic reaction system were investigated. N-removal enzyme activity is the key factor for the high-salt N-removal action of the strain (Baddam et al. 2016). Cortes-Lorenzo et al. (2012) found that high salinity affects the activity of denitrification-related enzymes, which is the same phenomenon observed in

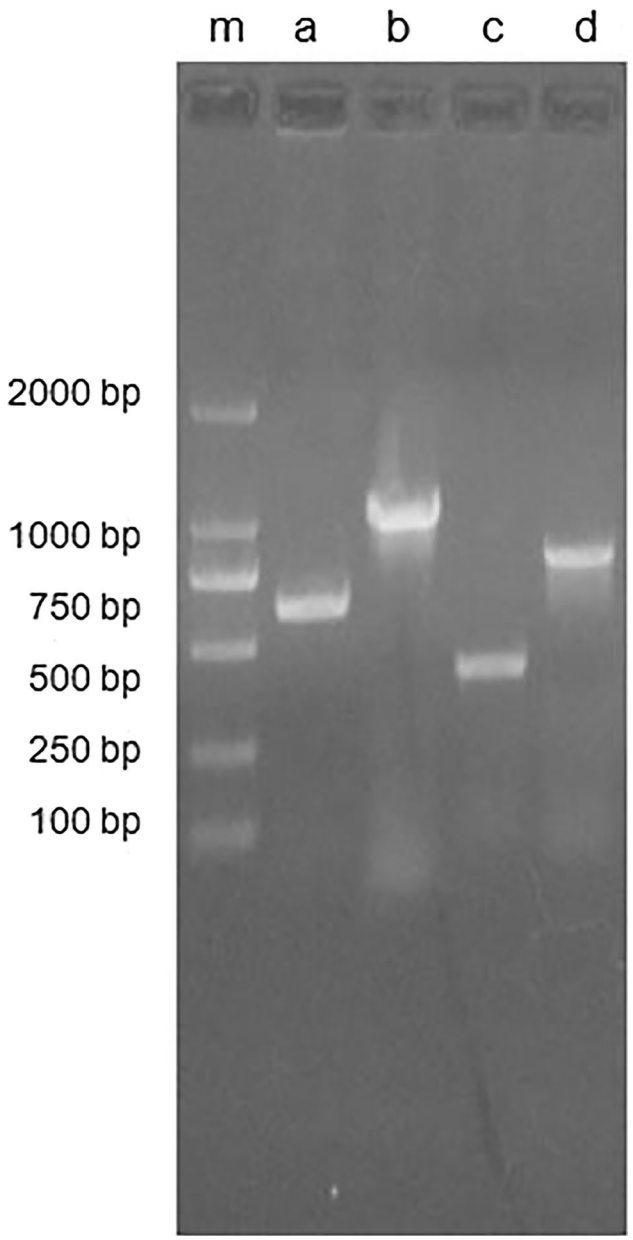


Fig. 2 Agarose gel electrophoresis of PCR products of N-removal enzymes. Note m: DL2000 Marker, a: *ureA*, b: *amoA*, c: *nirS*, and d: *narH*

Table 2 N-removal enzyme-encoding genes of *Halomonas* sp. H36

Gene		*Halomonas* sp. H36			
		ureA	*amoA*	*nirS*	*narH*
Similarity	Nucleotide	96%	99%	95%	99%
	Amino acid	99%	99%	95%	98%

this study. *Halomonas* sp. H36 urease still had high activity under high-salt conditions, which was similar to *Halomonas venusta* TJPU05 AMO (Man et al. 2022). AMO, NIR, and NAR of *Halomonas* sp. H36 were still active under high-salt conditions. The results showed that the N-removal enzymes of *Halomonas* sp. H36 could still function in a high-salinity environment. These findings lay a theoretical foundation for *Halomonas* sp. H36 to treat high-salt noxious wastewater.

The N-removal process of *Halomonas sp. H36* using urea as a substrate

We explored the N contents of various nitrogen species ($CO(NH_2)_2$-N; NH_4^+-N; NO_2^--N; NO_3^--N) and their relationship with each other in the N-removal process of *Halomonas* sp. H36 with urea as the sole N source. The results are shown in Fig. 4.

Halomonas sp. H36 cells were in the logarithmic growth stage from 0 to 48 h, and 219 mg/L N was used for cell growth. At this stage, the $CO(NH_2)_2$-N-removal rate was 73.11%, and the content of NH_4^+-N produced by the hydrolysis of $CO(NH_2)_2$-N gradually increased. This stage is dominated by the hydrolysis of urea by *Halomonas* sp. H36 ($CO(NH_2)_2$-N → NH_4^+-N).

Halomonas sp. H36 was in the quiescent growth stage from 48 to 192 h. At this stage, the $CO(NH_2)_2$-N-removal rate was 6.21%, the NH_4^+-N-removal rate was 73.44%, and the TN-removal rate was 53.62%. Very low levels of NO_3^--N (≤ 40 mg/L) and NO_2^--N (≤ 10 mg/L) were detected, and at 156 h, NO_3^--N and NO_2^--N were basically removed indicating that the removal of N by *Halomonas* sp. H36 was dominant at this stage. Cells in the resting state converted NH_4^+-N into gaseous N through simultaneous nitrification and denitrification (SND), avoiding the negative impact of the accumulation of NO_2^--N and NO_3^--N on the cells.

The results show that in the presence of only $CO(NH_2)_2$-N, the cells in the growing state first converted $CO(NH_2)_2$-N into NH_4^+-N under the catalysis of urease, and the removal of N was not obvious at this stage. In the resting state, under the catalysis of AMO, NIR, and NAR, cells converted NH_4^+-N into gaseous N, and the removal of N was mainly completed at this stage. During the whole process (0–192 h), the removal rate of $CO(NH_2)_2$-N was 74.77%, and the N-removal rate was 57.67%.

In this experiment, with 2,800 mg/L of initial $CO(NH_2)_2$-N, the $CO(NH_2)_2$-N degradation rate of *Halomonas* sp. H36 was 74.77%, and the TN-removal rate was 57.67%. This value was 1.62 times that of *Pseudomonas mendocina* sp. DM01, and with an initial $CO(NH_2)_2$-N concentration of 400 mg/L, the TN-removal rate was 35.50% (Chen 2018). Compared with mixed strains, it had stronger salt tolerance (Wang et al. 2020a, b).

The activity of N-removal enzymes was determined, and the N-removal enzymes of *Halomonas* sp. H36 were localized, as shown in Table 3. Most ureases are cytoplasmic enzymes (Mulrooney et al. 1989), and NIR is a periplasmic

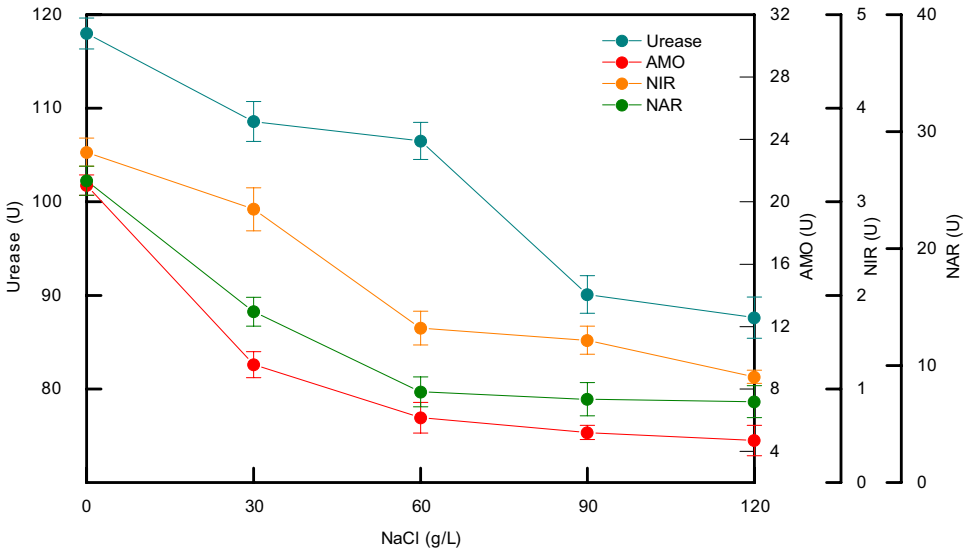


Fig. 3 Characterization of the N-removal enzyme activities of *Halomonas* sp. H36 under different NaCl concentrations

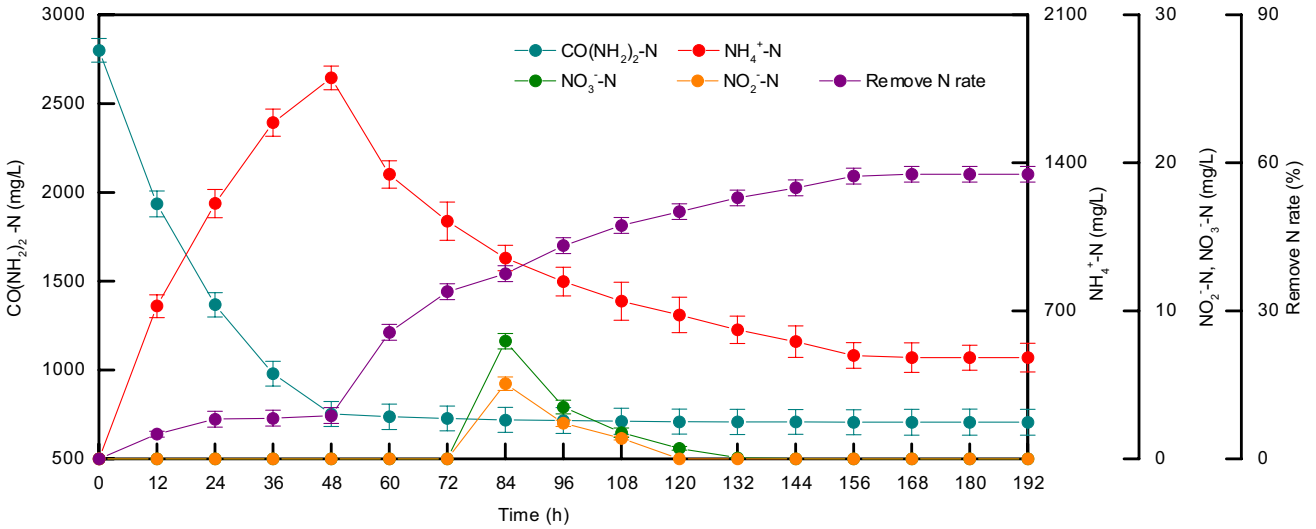


Fig. 4 Removal of N by *Halomonas* sp. H36 using urea as a substrate

Table 3 N-removal enzyme activities of *Halomonas* sp. H36

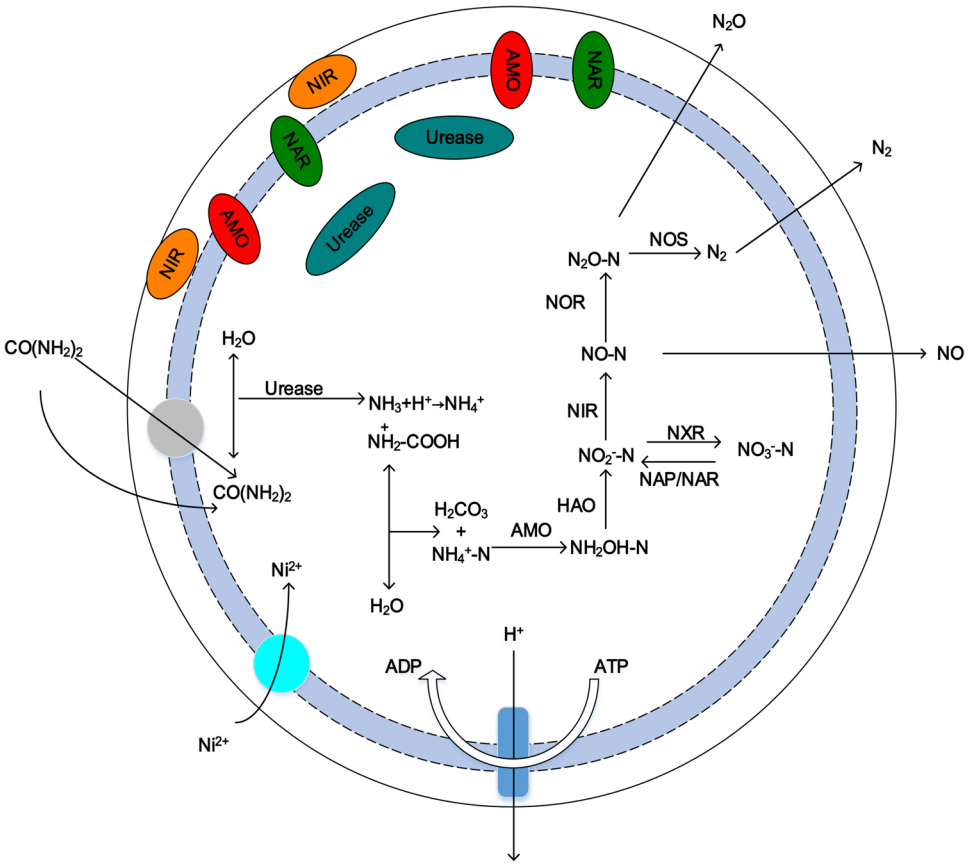
Localization		Urease	AMO	NIR	NAR
Activity (U)	Cytoplasm or periplasmic space	118.00	-	3.53	-
	Cell membrane	-	21.7	-	25.8

space enzyme (Blackmore et al. 1986). Therefore, it can be inferred that *Halomonas* sp. H36 urease is a cytoplasmic enzyme, AMO and NAR are cell membrane-bound enzymes, and NIR is a periplasmic space enzyme. Combined with the above experimental results, the N-removal pathway of *Halomonas* sp. H36 with urea as the substrate was obtained, as shown in Fig. 5.

With urea as the only N source, cells in the logarithmic growth phase mainly converted $CO(NH_2)_2$-N into NH_4^+-N under the catalysis of urease. Under the catalysis of AMO, NIR, and NAR, the cells in the resting state removed NH_4^+-N in the form of gaseous N and completed the SND. *Halomonas* sp. H36 completed simultaneous urea hydrolysis-nitrification–denitrification with urea as the initial substrate.

Application of *Halomonas sp. H36* in simulating domestic sewage from ships

Halomonas sp. H36 was transferred to simulated ship domestic sewage for treatment, and the results are shown in Fig. 6. In the whole process, the removal rate of $CO(NH_2)_2$-N



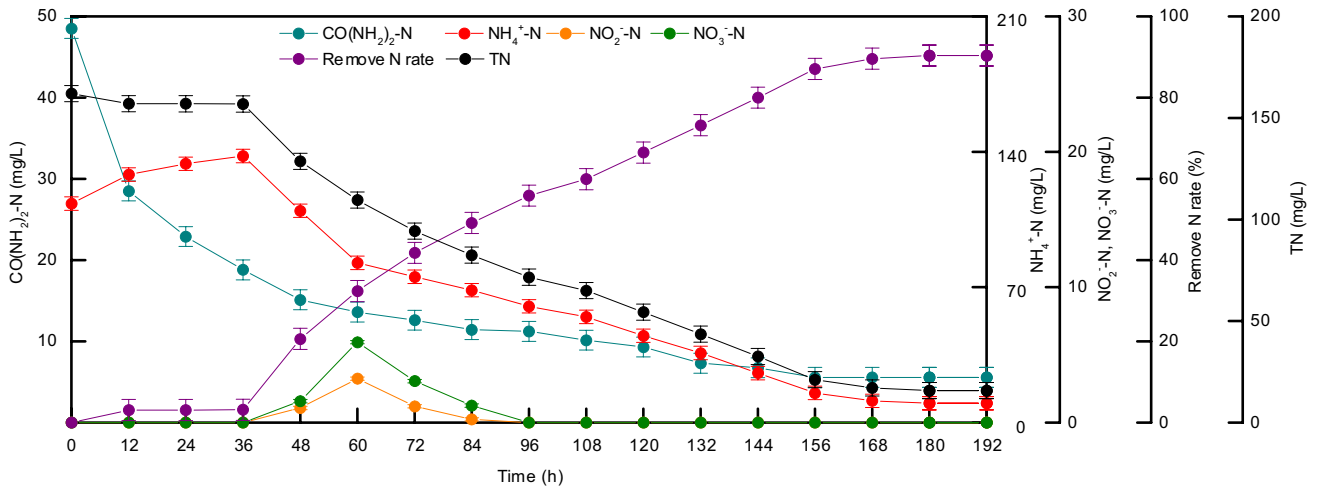


Fig. 5 N-removal pathway of *Halomonas* sp. H36 using urea as a substrate

Fig. 6 The application of *Halomonas* sp. H36 for N-removal from domestic sewage from ships

was 88.52%, the removal rate of NH_4^+-N was 91.16%, and the TN-removal rate was 90.25%.

It was found that in 0–36 h, $CO(NH_2)_2$-N decreased from 48.52 to 18.80 mg/L, and 29.72 mg/L $CO(NH_2)_2$-N was converted into NH_4^+-N. The NH_4^+-N in the matrix only increased by 24.61 mg/L, and 5.11 mg/L NH_4^+-N was removed. In this process, the degradation rate of $CO(NH_2)_2$-N was 0.83 mg/(L·h), and the degradation rate of NH_4^+-N was 0.14 mg/(L·h). This process was dominated by the degradation of $CO(NH_2)_2$-N. It was shown that urease, AMO, NIR, and NAR in cells can play roles in the presence of $CO(NH_2)_2$-N and NH_4^+-N. However, urea degrades more rapidly. At 36–192 h, the $CO(NH_2)_2$-N decreased from 18.8 to 5.57 mg/L, the NH_4^+-N decreased

from 137.84 to 10.01 mg/L, the $CO(NH_2)_2$-N-removal rate was 0.09 mg/(L·h), and the NH_4^+-N degradation rate was 0.82 mg/(L·h). The process was dominated by nitrification–denitrification. This may have been due to the low level of $CO(NH_2)_2$-N at this time, and NH_4^+-N accounted for 88.00% of the TN. This caused the enzymatic hydrolysis of urea to gradually weaken, and nitrification–denitrification became dominant.

NO_2^--N (≤ 5 mg/L) and NO_3^--N (≤ 10 mg/L) were detected. At 96 h, NO_3^--N and NO_2^--N were completely removed. It was proven that the strain avoided the negative impact of the accumulation of NO_2^--N and NO_3^--N on the cells and ensured effluent quality.

In this section, the results proved that *Halomonas* sp. H36 has the ability to simultaneously remove $CO(NH_2)_2$-N and inorganic N from ship domestic sewage. The $CO(NH_2)_2$-N-removal rate of *Halomonas* sp. H36 was 88.52%, the NH_4^+-N-removal rate was 91.16%, and the TN-removal rate was 90.25%. These results have obvious advantages compared with *Pseudoalteromonas* sp. SCSE709-6 (TN = 70 mg/L, with an TN-removal rate of approximately 50%) (Jiang et al. 2019).

Conclusions

In this paper, it was found for the first time that *Halomonas* sp. H36 has the function of simultaneous urea hydrolysis-nitrification–denitrification with urea as the initial substrate. It could simultaneously move $CO(NH_2)_2$-N and inorganic N in high-salt urea wastewater. Ectoine synthesized by *Halomonas* sp. H36, which is used to regulate osmotic pressure, protects the strain activity and allows urease production in a high-salt environment. The key roles of urease, AMO, NIR, and NAR in removing N were proven by molecular biology and enzymatic methods, and the simultaneous urea hydrolysis-nitrification–denitrification pathway was clarified by using urea as the initial substrate. The TN-removal rate of the strain in simulated ship sewage was 90.25%. In the future, *Halomonas* sp. H36 can be combined with an actual high-salt urea wastewater treatment process.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by YL, LZ, WL, and ZZ. The first draft of the manuscript was written by YL. And all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability All data are mentioned in the body of manuscript, tables, and figure.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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