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ORIGINAL ARTICLE

Growth and nitrogen removal characteristics of *Halomonas* sp. B01 under high salinity

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Abstract

Purpose At present, the nitrogen (N) removal efficiency of the microbial treatment in the high-salinity nitrogenous wastewaters is relatively low. Study on the N removal behavior and properties of moderately halophilic bacteria *Halomonas* under high salinity is of great significance for the microbial treatment of high-salinity nitrogenous wastewater.

Methods The response mechanism of *Halomonas* sp. B01 to high osmotic pressure stress was investigated by measuring the compatible solute ectoine concentration and superoxide dismutase (SOD) activity. The salt tolerance during growth and N removal of the strain was evaluated by measuring the activities of growth-related and N removal–related enzymes and the mRNA expression abundance of ammonia monooxygenase-encoding gene (*amoA*). The process of simultaneous heterotrophic nitrification and aerobic denitrification (SND) under high salinity was described by measuring the concentration of inorganic N.

Result *Halomonas* sp. B01 synthesized ectoine under NaCl stress, and the intracellular ectoine concentration increased with increased NaCl concentration in the growth medium. When the NaCl concentration of the medium reached 120 g L^{-1}, the malondialdehyde concentration and SOD activity were significantly increased to 576.1 μg mg^{-1} and 1.7 U mg^{-1}, respectively. The growth-related and N removal–related enzymes of the strain were active or most active in medium with 30–60 g L^{-1} NaCl. The *amoA* of the strain cultured in medium with 60 g L^{-1} NaCl had the highest mRNA expression abundance. In the N removal medium containing 60 g L^{-1} NaCl and 2121 mg L^{-1} NH_4^+-N, SND by *Halomonas* sp. B01 was performed over 96 h and the N removal rate reached 98.8%.

Conclusion In addition to the protective mechanism of synthetic compatible solutes, *Halomonas* sp. B01 had the repair mechanism of SOD for lipid peroxidation. The growth-related and N removal–related enzymes of the strain were most active at a certain salt concentration; *amoA* also had the highest mRNA expression abundance under high salinity. *Halomonas* sp. B01 could efficiently perform N removal by SND under high salinity.

Keywords *Halomonas* · High salinity · Salt tolerance · Nitrogen removal

Introduction

Simultaneous heterotrophic nitrification (SND) and aerobic denitrification constitute a nitrogen (N) removal method in which nitrification and denitrification are performed simultaneously in the same reaction system with a supplied organic carbon source and oxygen. SND has advantages such as fast microbial growth, self-adjusting pH balance in the N removal system, simple processing mechanisms, and high N removal efficiency (Shoda 2017). Some industries generate high-salinity nitrogenous wastewater, such as those undertaking leather processing and marine aquaculture; using synthetic ammonia and chemical fertilizer; maintaining landfills; and producing nickel batteries. The N removal efficiency of the microbial treatment, especially that of SND processes, in these high-salinity nitrogenous wastewaters is relatively low. The main reason is that high salinity levels inhibit microbial growth and metabolic activity. Therefore, theoretical and technical research on SND in high-salinity nitrogenous wastewater is of great significance. Research on N removal by moderately halophilic bacteria from high-salinity nitrogenous wastewater has been of interest in recent years. In the family

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Halomonadaceae, *Halomonas* is a genus of moderately halophilic bacteria that currently includes 79 species (de la Haba et al. 2014). Some of these strains have been reported to be capable of nitrification, denitrification, or simultaneous nitrification and denitrification at certain salt concentrations (Berendes et al. 1996; Mormile et al. 1999; Guo et al. 2013; Wang et al. 2017). Te Wang reported that *Halomonas* sp. B01 can simultaneously tolerate high concentrations of both NaCl and NH_4^+-N and efficiently perform SND. In a N removal solution containing 60 g L^{-1} NaCl and 4000 mg L^{-1} NH_4^+-N, SND was performed over 180 h under optimal conditions and resulted in a residual total inorganic N concentration of 21.7 mg L^{-1}, and the N removal rate reached 99.2% (Wang et al. 2017). *Halomonas* spp. show unique potential in the treatment of wastewater with respect to N removal. In addition to their strong growth and metabolism and N removal while significantly reducing the COD of wastewater, the most notable feature is their resistance to high salinity growth and N removal.

It is of great theoretical and practical significance to study the resistance of *Halomonas* bacteria to high salinity growth and N removal. Most *Halomonas* bacteria can resist environmental osmotic stress by synthesizing compatible solute ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (Pastor et al. 2010). However, neither lipid peroxidation caused by high salinity nor a superoxide dismutase (SOD) repair mechanism has been reported for *Halomonas*. Numerous studies have described *Halomonas* growth (including reports of the optimal salt concentration for growth) and ectoine synthesis of different strains of *Halomonas* in different NaCl concentrations (de la Haba et al. 2014; Yin et al. 2015), but the relationship between the activities of growth-related and N removal–related enzymes in *Halomonas* and NaCl concentration in the medium has not been reported. Likewise, the effect of NaCl concentration in the medium on the mRNA expression abundance of N removal–related enzyme genes has not been reported. However, the studies cited above help reveal the mechanism of salt tolerance during the growth and N removal by *Halomonas*.

This paper describes the stress effects of high osmotic pressure on the growth of *Halomonas* sp. B01 and its response mechanism. The effects of the NaCl concentration in the medium on the activity levels of growth-related enzymes (glucokinase (GK); dehydrogenase (DHA); and succinate dehydrogenase (SDH)) and N removal–related enzymes (ammonia monooxygenase (AMO) and nitrite reductase (NIR)) in *Halomonas* sp. B01 were characterized. Real-time quantitative PCR (RT-PCR) was used to investigate the effect of NaCl concentration in the medium on the mRNA expression abundance of AMO-encoding gene (*amoA*) in this strain. The N removal process of the strain under high salinity was also investigated. Thus, the growth and N removal of *Halomonas* sp. B01 under high salinity were characterized.

Materials and methods

Materials

Strain *Halomonas* sp. B01 was isolated, screened, and identified by the author's previous study (16S rRNA sequence GenBank No. KJ778559) (Wang et al. 2017), and the strain was deposited in the China Center for Type Culture Collection (CCTCC) under the accession number CCTCC AB 2014335.

Drugs and kits Ectoine standard was purchased from Biomol GmbH (Germany). TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa Code: 9767), TaKaRa PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa Code: RR047), and SYBR *Premix Ex Taq™* II (TliRNaseH Plus) (TaKaRa Code: RR820) were purchased from Takara Biotechnology (Dalian) Co., Ltd. Malondialdehyde Determination Kit (BC0020), BCA Protein Concentration Assay Kit (PC0020), glucose-6-phosphate dehydrogenase, and reduced coenzyme II (nicotinamide adenine dinucleotide phosphate) were purchased from Beijing Solarbio Science&Technology Co., Ltd.

Medium

LB medium (g L^{-1}): peptone 10, yeast extract powder 5, NaCl 60. The medium was autoclaved at 121 °C for 20 min.

Growth medium (g L^{-1}): glucose 40, $(NH_4)_2SO_4$ 10, yeast extract powder 0.5, $K_2HPO_4{\cdot}3H_2O$ 9, KH_2PO_4 3, $MgSO_4{\cdot}7H_2O$ 0.4, $MnSO_4{\cdot}H_2O$ 0.01, NaCl (30, 60, 90, 120), pH 7.2. The medium was autoclaved at 121 °C for 20 min.

N removal medium (g L^{-1}): $(NH_4)_2SO_4$ 10, sodium succinate 35.8, $K_2HPO_4{\cdot}3H_2O$ 9, KH_2PO_4 3, $MgSO_4{\cdot}7H_2O$ 0.1, NaCl (30, 60, 90, 120 respectively), pH 8. The N removal medium was autoclaved at 121 °C for 20 min. Trace mineral solution (EDTA-2Na 63.7, $ZnSO_4$ 2.2, $CaCl_2$ 5.5, $MnCl_2{\cdot}4H_2O$ 5.1, $FeSO_4{\cdot}7H_2O$ 5, $Na_2MO_4{\cdot}2H_2O$ 1.1, $CuSO_4{\cdot}5H_2O$ 1.6, $CoCl_2{\cdot}6H_2O$ 1.6, g L^{-1}) was at 2 mL. The trace mineral solution was sterilized by filtration (0.22 μm pore size, Millipore Express, USA).

SND method

The N removal by SND in this paper was simultaneous heterotrophic nitrification and aerobic denitrification, so the N removal conditions were set to heterotrophic (N removal medium containing organic carbon compound sodium succinate) and aerobic (using rotary shaker ventilation to provide oxygen in the N removal process). The strains were cultivated in 5 mL of LB medium at 30 °C in a rotary shaker at 120 rpm for 24 h. Then, 1% of the cultures were inoculated in shake flasks (300 mL) containing 30 mL of N removal medium, and the SND was performed at 30 °C in a rotary shaker at 90 rpm.

Methods for assaying strain growth and metabolic stress in cultures with different salt concentrations and ability of strain to respond to the stress

The stress of growth and metabolism of the strain in cultures with different salt concentrations was investigated: The inhibition of cell growth by different salt concentrations was evaluated by measuring the amount of cell growth; the lipid peroxidation under high salinity was evaluated by measuring the concentration of malondialdehyde (MDA). The ability of strains to respond to stress under high salinity was investigated: The salt-tolerant growth ability of the strain was evaluated by measuring intracellular ectoine concentration; the repair ability of the strain to lipid peroxidation was evaluated by measuring the SOD activity. The samples to be determined were the cultures which incubated for 36 h in the growth medium with different NaCl concentrations.

The cell growth was assayed as follows: Cell dry weight (CDW) with different OD_{600} was determined. The relationship between CDW and OD_{600} was calculated. CDW was calculated by determining the OD_{600} in experiment. Cell growth was defined as CDW per liter culture broth (g L^{-1}).

The ectoine concentration was assayed as follows: The sample for determining ectoine was prepared by ethanol extraction method (Zhang et al. 2009). Ectoine was determined by high-performance liquid chromatography (HPLC) method (Zhang et al. 2009). An HPLC was equipped with a TSK-GEL reversed-phase column (TOSOH Corporation, Japan). A UV detector wavelength of 210 nm was used.

The MDA concentration was assayed as follows: MDA was determined using Malondialdehyde Determination Kit (BC0020, Solarbio, Beijing, China).

The SOD activity was assayed as follows: Pyrogallol rapidly autoxidizes under alkaline conditions. SOD can inhibit the autoxidation of pyrogallol (Wu et al. 2011). One unit of SOD activity (U) is the amount of SOD that catalyze the inhibition of pyrogallol autoxidation at a rate of 50% per min at the reaction substrate of pyrogallol (21 mmol L^{-1}), pH 8.2 and 30 °C under the specified reaction conditions. The sample is to be determined using the preparation liquid of cytoplasmic enzyme (including the periplasmic enzyme). Methods for preparing enzyme liquid to be determined and assayed protein concentration see "Methods for assaying growth-related and N removal–related enzyme activity."

Methods for assaying growth-related and N removal–related enzyme activity

The salt tolerance during the growth and N removal of the strain was evaluated by measuring the activities of growth-related and N removal–related enzymes in cultures with different salt concentrations. Growth-related enzymes selected GK, DHA, and DHA as markers for growth-related enzyme activity (Szeto et al. 2007; Zhang et al. 2008; Wolińska and Stępniewska 2012). The N removal–related enzymes selected AMO and NIR, which are key enzymes in the heterotrophic nitrification process and aerobic denitrification process, respectively (Ensign et al. 1993; Tavares et al. 2006).

The enzyme solution to be assayed was prepared as follows: SOD and growth-related enzymes (GK, DHA, and DHA) were from cultures which incubated for 36 h in growth medium with different NaCl concentrations. N removal–related enzymes (AMO and NIR) were from cultures which incubated for 36 h in N removal medium with different NaCl concentrations. A crude extract of each enzyme prepared for this study was used for enzyme activity assays. The following enzymes were studied: SOD and GK are cytoplasmic enzymes (Dols et al. 1997; Valdivia et al. 2009), NIR is a cell periplasmic enzyme (Blackmore et al. 1986), and SDH and AMO are cell membrane–bound enzymes (Hyman and Arp 1992; Miyadera et al. 2003). 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, which was centrifuged at 14000×*g* for 15 min at 4 °C, and the supernatant was discarded. Then, 100 mM pH 7.2 phosphate buffer 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 14000×*g* for 15 min at 4 °C, the supernatant was collected, and the sample was used for cytoplasmic and periplasmic enzyme assays. The membrane-bound enzyme was prepared as follows: 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 14000×*g* for 15 min at 4 °C. Then, the supernatant was collected, and the sample was used for the cell membrane–bound enzyme assay (Zhang et al. 2015). 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).

The GK activity was assayed as follows: GK converts glucose into glucose-6-phosphate, and then, glucose-6-phosphate dehydrogenase (G-6-PDH) converts glucose-6-phosphate into glucose acid 6-phosphate and reductive coenzyme II (Algar and Scopes 1985; Miyata and Yonehara 1999). One unit of GK activity (U) is the amount of GK that produces 1 μmol of reductive coenzyme II per min at the reaction substrate of glucose (1 mol L^{-1}), pH 7.8 and 30 °C, under the specified reaction conditions. The sample is to be determined using the preparation liquid of cytoplasmic enzyme (including the periplasmic enzyme).

The DHA activity was assayed as follows: The activity of DHA was measured using triphenyl tetrazolium chloride (TTC) salt as a hydrogen acceptor. TTC is a colorless salt, but in the presence of DHA, it is reduced to triphenylformazan (TPF), a red compound insoluble in water. TPF is extracted with $CHCl_3$ and measured at 485 nm (Jafari et al. 2015). One unit of DHA activity (U) is the amount of DHA that produces 1 μmol of TPF per min at the reaction substrate of TTC-glucose solution (0.7 mmol L^{-1}), pH 8.4 and 30 °C, under the specified reaction conditions. The samples to be determined were the cell obtained by centrifugation of the cultures which incubated for 36 h in the growth medium with different NaCl concentrations. The amount of the cell to be determined was based on the CDW (g).

The SDH activity was assayed as follows: SDH can catalyze the dehydrogenation of succinic acid to create fumaric acid while reducing methylene blue to methylene white (colorless) (Moller-Zinkhan and Thauer 1988). One unit of SDH activity (U) is the amount of SDH that reduces the A_{665} value of the reaction by 0.01 per min at the reaction substrate of sodium succinate (0.1 mol L^{-1}), pH 7.3 and 30 °C, under the specified reaction conditions. The sample is to be determined using the preparation liquid of cell membrane–bound enzyme.

The AMO activity was assayed as follows: AMO oxidization of NH_4^+ to generate hydroxylamine (Ensign et al. 1993). One unit of AMO activity (U) is the amount of AMO that oxidizes 1 μmol NH_4^+ per min at the reaction substrate of $(NH_4)_2SO_4$ (0.2 mmol L^{-1}), pH 7.2 and 30 °C, under the specified reaction conditions. The sample is to be determined using the preparation liquid of cell membrane–bound enzyme.

The NIR activity was assayed as follows: One unit of NIR activity (U) is the amount of NIR that reduces 1 μmol NO_2^- per min at the reaction substrate of KNO_2 (0.5 mmol L^{-1}), pH 7.2 and 30 °C, under the specified reaction conditions (Martínez-Espinosa 2001). The sample is to be determined using the preparation liquid of cytoplasmic enzyme (including the periplasmic enzyme).

Method of RT-PCR

RT-PCR was performed by SYBR Green I fluorescent dye method (Marino et al. 2003). The 16S rDNA sequence primers of *Halomonas* sp. B01 were 16S rDNA universal primers for bacterial, which were F_1- ACATCCTG CGAACTTGTGAGAG and R_1- CCGCTGGCAAATAA GGACA. The *amoA* primers of *Halomonas* sp. B01 were F_2- GCTGGTATCGGCAAAAGGAA and R_2- CCCAGCACCAATCCCATAAC. The nucleotide sequence of *Halomonas* sp. B01 *amoA* had been registered on GenBank (KT809509). The total RNA of samples were extracted using TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa Code: 9767). RT-PCR was performed using TaKaRa PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa Code: RR047) and SYBR *Premix Ex Taq*™ II (TliRNaseHPlus)(TaKaRa Code: RR820) in apparatus of TaKaRa PCR Thermal Cycler Dice™ (TaKaRa Code: TP600) and TaKaRa PCR Thermal Cycler Dice™ Real-Time System (TaKaRa Code: TP800). RT-PCR results were processed using Delta-delta Ct method. The mRNA expression abundance of *amoA* was expressed by $2^{-\triangle\triangle Ct}$.

Methods for determining inorganic N concentration

The inorganic N herein include NH_4^+-N, NO_2^-N, and NO_3^--N, and the N removal rate of the strain was evaluated by measuring the concentration of inorganic N. NH_4^+-N was determined by Nessler's reagent method (APHA 1999). NO_2^--N was determined by diazotization-coupling reaction method (APHA 1999). NO_3^--N was determined by zinc-cadmium reduction method (Sun et al. 2013).

Three parallel samples were set up for all the above experiments. The average of the three measurements with standard deviation values is presented in the results section.

Results and discussion

Response of *Halomonas* sp. B01 to stress from high concentrations of NaCl

The concentration of compatible solute ectoine synthesized by *Halomonas* sp. B01 was investigated under NaCl stress. This study assessed cell growth and the intracellular ectoine concentration in *Halomonas* sp. B01 after being cultured for 36 h in growth medium containing 30, 60, 90, and 120 g L^{-1} NaCl, respectively; the results are shown in Fig. 1b. At a NaCl concentration of 120 g L^{-1}, the growth still reached 7.0 g L^{-1}, and the intracellular ectoine concentration was 921.2 mg L^{-1}. The intracellular ectoine concentration in *Halomonas* sp. B01 increased with increased NaCl concentration in the growth medium. *Halomonas* sp. B01 responded to high salt stress by synthesizing a compatible solute protection mechanism.

The effect of NaCl stress on lipid peroxidation in *Halomonas* sp. B01 cells was also investigated. The intracellular MDA concentration and SOD activity in *Halomonas* sp. B01 after being cultured for 36 h in growth medium containing 30, 60, 90, and 120 g L^{-1} NaCl are shown in Fig. 1c. With the increase in NaCl concentration, synchronous changes in the activity of SOD and in the cytoplasmic concentration of MDA were observed. When the concentration of NaCl reached 120 g L^{-1}, the intracellular MDA concentration increased significantly (576.1 μg mg^{-1}), indicating that significant lipid peroxidation transpired in *Halomonas* sp. B01 under

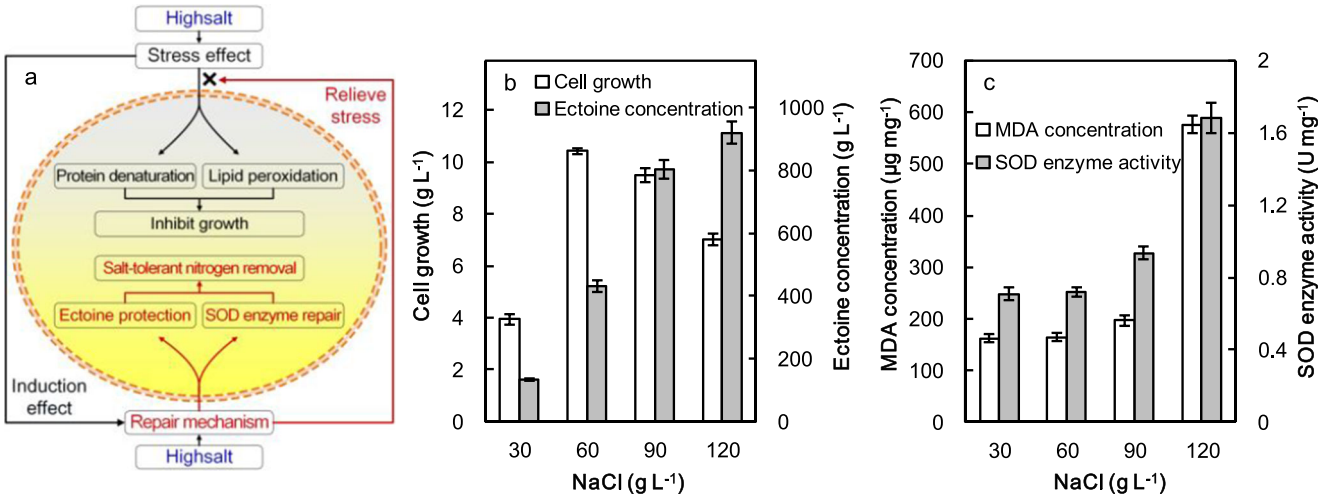

Fig. 1 **a** Response of *Halomonas* sp. B01 to stress from high concentrations of NaCl. **b** Cell growth and induced ectoine concentration in cultures at different NaCl concentrations. **c** MDA concentration and SOD activity in cells cultured with different NaCl concentrations. Cells were incubated in growth medium at 30 °C while shaking at 120 rpm for 36 h

this high osmotic pressure. In addition, the SOD activity was also significantly increased (1.7 U mg^{-1}), indicating that while *Halomonas* sp. B01 was undergoing lipid peroxidation, the SOD repair mechanism was initiated, allowing the cells to tolerate the salt and be able to grow and metabolize.

The above results indicated that *Halomonas* sp. B01 responds to high osmotic pressure stress in two ways. One was the protective mechanism of synthetic compatible solutes; the other was the repair mechanism of SOD for lipid peroxidation. The amount of ectoine synthesis and the increase of NaCl concentration increased substantially simultaneously. The concentration of MDA and SOD activity increased significantly when the NaCl concentration reached 120 g L^{-1}, indicating that lipid peroxidation was severe at this time and the corresponding SOD repair effect was also significantly increased.

Based on existing research (Komanapalli and Lau 1996; Uygur and Kargı 2004; Valdivia et al. 2009; Vyrides and Stuckey 2009; Pastor et al. 2010; Wang et al. 2017) and studies in this paper, the response mechanism of *Halomonas* sp. B01 to high osmotic pressure stress could be described as follows (Fig. 1a): High salt-induced osmotic pressure had at least two stress effects on *Halomonas* sp. B01, the first being the denaturation and reduction biological macromolecule activities (such as proteins) and the second being lipid peroxidation. These two types of stress could lead to a decrease of cell growth and growth rate and a decrease of metabolic activity. The response of *Halomonas* sp. B01 to these two osmotic pressure stress effects was the synthetic compatible solute protection mechanism and the SOD repair mechanism. The synthesis of compatible solute ectoine increased in line with cell growth, while the large amount of MDA and the significant increase of SOD activity both occurred at 120 g L^{-1} NaCl in the medium, indicating that the synthesis of compatible solute was the first protective mechanism initiated by *Halomonas* sp. B01, and the SOD repair mechanism was the protective mechanism activated by this strain in response to increasingly extreme conditions. The two response mechanisms to high osmotic pressure stress in *Halomonas* sp. B01 enabled it to show significant salt-tolerant growth characteristics for a wide range of NaCl concentrations (30–120 g L^{-1}), such that, at 120 g L^{-1}, the cell growth of the strain was 7.0 g L^{-1}, and it reached 67.3% of the greatest level of growth (10.4 g L^{-1}) in 60 g L^{-1} NaCl in the medium.

Effects of salt concentration in the medium on growth-related enzyme activity in *Halomonas* sp. B01

The three core pathways of cell growth and metabolism include Embden-Meyerhof-Parnas pathway (EMP), hexose monophosphate pathway (HMP), and tricarboxylic acid cycle (TCA). GK (EC 2.7.1.2) is a limiting enzyme for the use of glucose in the EMP pathway, and its catalytic reaction is D-glucose + ATP → D-glucose 6-phosphate + ADP (Zhang et al. 2008). DHA is involved in redox reactions in cells and passes electrons through the respiratory chain to hydrogen receptors under certain conditions. DHA activity, especially which in the three core metabolic pathways, is considered to be a good indicator of microbial oxidation activity (Wolińska and Stępniewska 2012). SDH (EC 1.3.5.1) in the TCA cycle has been used as an indicator to evaluate the degree of tricarboxylic acid cycling, and its catalytic reaction is succinate + a quinone → fumarate + a quinol (Szeto et al. 2007). In this study, the activities of *Halomonas* sp. B01 GK, DHA, and SDH were used as markers of growth and metabolic related enzyme activity. The activities of the three enzymes in *Halomonas* sp. B01 were investigated in medium with

concentrations in the range of 30–120 g L^{-1} NaCl. The results are shown in Fig. 2. The GK and SDH activity levels were the highest in 60 g L^{-1} NaCl medium, at 6.0 U mg^{-1} and 4.3 U mg^{-1}, respectively. The DHA activity level was higher in 30 and 60 g L^{-1} NaCl medium (8.1 U g^{-1} and 7.7 U g^{-1}, respectively) than in the other salt concentrations assayed. Thus, the three enzymes, which are related to the growth and metabolism of *Halomonas* sp. B01, showed significant high salt adaptability.

Effects of salt concentration in the medium on N removal–related enzyme activity levels in *Halomonas* sp. B01

The process of microbial N removal is generally $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, in which $NH_4^+ \rightarrow NO_2^-$ is catalyzed by AMO and $NO_2^- \rightarrow NO$ is catalyzed by NIR. These two enzymes belong to the nitrification and denitrification enzymes of the N removal process, respectively. AMO is the first key enzyme in the process of heterotrophic nitrification, and NIR is also one of the key enzymes in the process of aerobic denitrification (Ensign et al. 1993; Tavares et al. 2006). The salt tolerance of these two enzymes has an effect on the N removal efficiency of SND under high salinity. The effects of salt concentration in the culture medium on the N removal–related enzyme activity of *Halomonas* sp. B01 were investigated. *Halomonas* sp. B01 was cultured for 36 h in a growth medium containing 30, 60, 90, and 120 g L^{-1} NaCl, respectively, and the enzyme solution was prepared according to the method described in "Methods for assaying growth-related and N removal–related enzyme activity" to determine the activity levels of AMO and NIR. The results are shown in Fig. 3. Figure 3 showed that the optimum salt concentration in the culture to activate AMO and NIR was 60 g L^{-1} NaCl, and the two enzyme activity levels were 14.9 U mg^{-1} and 20.3 U mg^{-1}, respectively. AMO had higher activity (14.6 U mg^{-1} and 14.9 U mg^{-1}, respectively) in culture media with 30 and 60 g L^{-1} NaCl, while NIR had significantly higher activity in the culture medium with 60 g L^{-1} NaCl. At the extreme salt concentration condition (120 g L^{-1} NaCl), cells retained a certain level of enzyme activity.

The results of this study demonstrated for the first time that the salt tolerance of *Halomonas* strains on growth and N removal is not only due to the protective effect of compatible solute on intracellular macromolecules (Pastor et al. 2010), but also directly related on the salt tolerance (in vitro enzyme activity) of its growth-related and N removal–related enzymes.

Effect of salt concentration in the medium on *amoA* mRNA expression abundance in *Halomonas* sp. B01

RT-PCR was used to investigate the effect of salt concentration in the medium on mRNA expression abundance of the AMO-encoding gene *amoA* in *Halomonas* sp. B01. Experimental samples were prepared by extracting total mRNA at values of $A_{260/280} \geq 2.0$. The amplification curves for the samples were S-type curves, and the melting point for a specific amplified primer was visualized as a single peak. The standard deviation of expression for parallel samples was less than or equal to 0.3%.

The mRNA expression abundance of *amoA* in growth medium at different NaCl concentrations for 36 h is shown in Fig. 4. The mRNA abundance was highest (3.5) at 60 g L^{-1} NaCl (within the concentration range of 30–120 g L^{-1} NaCl). The salt tolerance of the *Halomonas* strain on grow and N removal is also reflected in the gene expression level.

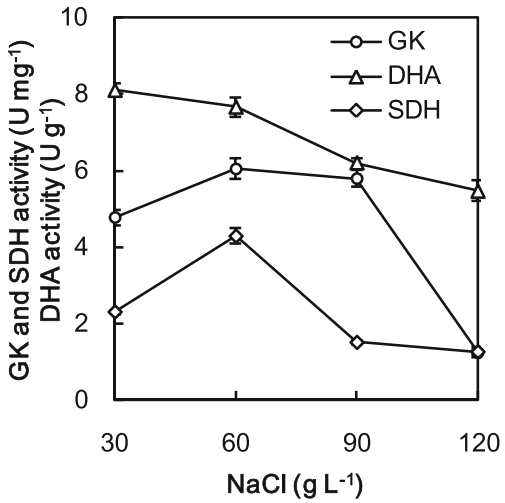

Fig. 2 Effects of salt concentration in the medium on growth-related enzyme activities in *Halomonas* sp. B01. The temperature of enzyme reaction system was 30 °C, and reaction time was 1 h

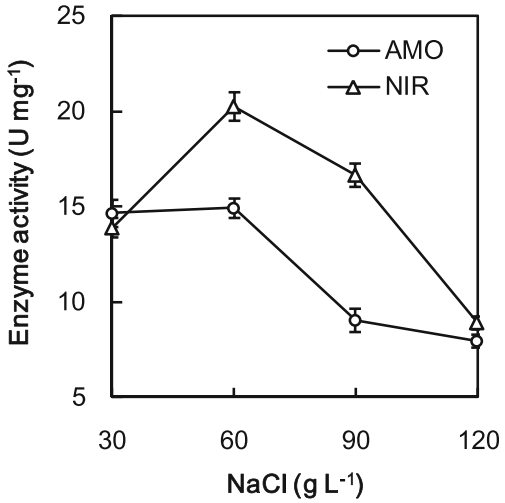

Fig. 3 Effects of salt concentration in the medium on the N removal–related enzyme activity in *Halomonas* sp. B01. The temperature of enzyme reaction system was 30 °C, and reaction time was 1 h

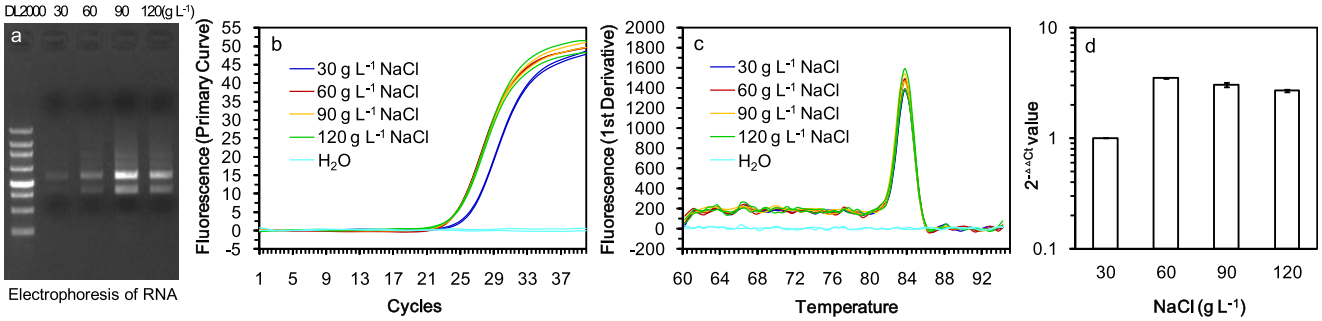

Fig. 4 Effect of salt concentration in the medium on *amoA* mRNA expression abundance in *Halomonas* sp. B01. **a** Agarose gel electrophoresis pattern of total RNA extracted from the strain samples cultured at different NaCl concentrations. **b** RT-PCR amplification curves of *amoA* from strain samples cultured at different NaCl concentrations. **c** Dissolution curves of *amoA* amplification products from strain samples cultured at different NaCl concentrations. **d** mRNA expression abundance of *amoA* from strain samples cultured at different NaCl concentrations

SND process under different NaCl concentrations

The N removal process of *Halomonas* sp. B01 at different NaCl concentrations was investigated. The results are shown in Fig. 5. In the concentration range of 30–120 g L^{-1} NaCl, the strain was able to remove N through SND, and the N removal rate increased with time. At a 60 g L^{-1} NaCl concentration, the N removal process had the highest N removal rate, which reached 98.8% in 96 h. Under the extremely high salt level of 120 g L^{-1} NaCl, the strain showed high N removal efficiency, and the N removal rate reached 89.8% at 96 h. The N removal with SND by *Halomonas* sp. B01 had significant salt tolerance (the initial NH_4^+-N concentration was 2121 mg L^{-1}).

By simulating saltwater intrusion, Yang studied the effects of saltwater intrusion on microorganisms in the rhizosphere soil of riparian zone of Chongming Island, Shanghai, China. The denitrification process of these microorganisms in rhizosphere soil was also studied. The results showed that the nitrite reductase activity of the rhizosphere soil microorganism decreased significantly (down 56.5%) under the simulated saline water that contained 23.5 g L^{-1} NaCl (Yang et al. 2012). This result indicated that, in general, nitrifying bacteria in the soil do not have salt tolerance (or low salt tolerance). Guo reported that under salt conditions, the nitrification rate of *Halomonas* was significantly higher than that of *Bacillus*. The former had a nitrification efficiency of 8.3 mg NH_4^+-N L^{-1} h^{-1}, which was approximately three times greater than that of the latter (2.1 mg NH_4^+-N L^{-1} h^{-1}) (Zhang et al. 2012; Guo et al. 2013). In the study cited, *Halomonas* showed high salt tolerance during N removal. Guo reported that the nitrification and denitrification efficiency of *Halomonas campisalis* ha3 under 4% NaCl condition were 8.3 mg NH_4^+-N L^{-1} h^{-1} and 87.5 mg NO_3^--N L^{-1} h^{-1}, respectively (Guo et al. 2013). Sun reported that the optimum growth condition for a strain of *Halomonas* isolated and screened was 3% salinity, and the N removal rate at seawater salt concentration was 98.3% (Sun et al. 2012). Qu reported that the total N removal rate of a *Halomonas* strain isolated and screened at 30 g L^{-1} salt concentration was 81.5% (Qu et al. 2011). *Halomonas* sp. B01 reported in this study had an optimum NaCl concentration of 60 g L^{-1} for growth and SND N removal. Under this condition, the N removal rate reached 98.8%. This strain was the highest level reported in the genus *Halomonas* in terms of salt tolerance and N removal efficiency.

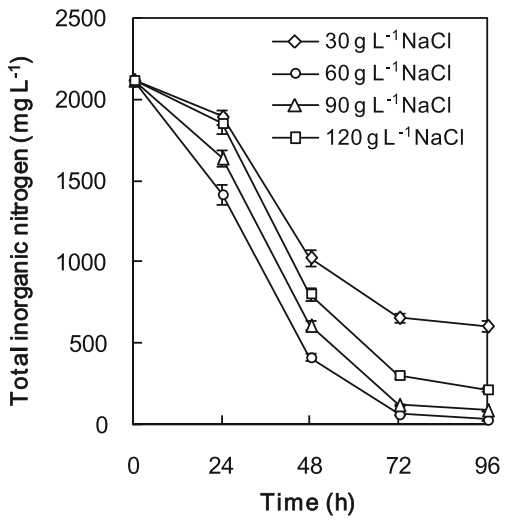

Fig. 5 The N removal process of *Halomonas* sp. B01 at different NaCl concentrations. Measurements were taken from cultures in N removal medium at 30 °C while shaking at 90 rpm in a rotary shaker

Conclusion

This study revealed that *Halomonas* sp. B01 possessed two sets of mechanisms for responding to high osmotic stress during the sequential increase of NaCl concentration in the culture environment. One is the protective mechanism of synthetic compatible solutes; the other is the repair mechanism of SOD for lipid peroxidation. The activity levels of growth- and metabolism-related and N removal–related enzymes in *Halomonas* sp. B01 were affected by NaCl concentration in the medium. The activity levels of GK and SDH showed

maximum values (6.0 U mg^{-1} and 4.3 U mg^{-1}, respectively) under conditions of 60 g L^{-1} NaCl in culture. The activity of DHA was higher (8.1 U g^{-1} and 7.7 U g^{-1}) in the culture with 30 and 60 g L^{-1} NaCl, respectively. The optimum NaCl concentration in culture for AMO and NIR activity was 60 g L^{-1} NaCl, and the enzyme activity was 14.9 U mg^{-1} and 20.3 U mg^{-1}, respectively. The mRNA expression abundance of *amoA* in *Halomonas* sp. B01 was highest at 60 g L^{-1} NaCl in the range of 30–120 g L^{-1} NaCl in the medium. At 2121 mg L^{-1} initial NH_4^+-N and 60 g L^{-1} NaCl in the medium, the highest N removal rate was obtained during the whole N removal process, and the N removal rate reached 98.8% in 96 h. Until now, the effects of different salt concentrations on the growth-related and N removal–related enzymes in *Halomonas* and the effect of salt concentration in the medium on mRNA expression abundance of N removal–related enzymes encoding genes have not been reported. In this study, the growth and N removal of *Halomonas* sp. B01 under high salinity were characterized, which is of great significance for the microbial treatment of high-salinity nitrogenous wastewater.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent N/A. This article does not involve human participants.

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