A Robust Oxysalt-Tolerant Bacterium *Marinobacter* sp. for Simultaneous Nitrification and Denitrification of Hypersaline Wastewater

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Article

A Robust Oxysalt-Tolerant Bacterium *Marinobacter* sp. for Simultaneous Nitrification and Denitrification of Hypersaline Wastewater

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Abstract: Robust strains with high simultaneous nitrification and denitrification (SND) capabilities in hypersaline wastewater, particularly those containing different oxysalts are rarely reported. Here an isolated oxysalt-tolerant bacterium *Marinobacter* sp. showed excellent nitrogen removal capabilities of around 98% at 11% salinity of NaCl or oxysalts such as Na_2SO_4, Na_2HPO_4, $NaHCO_3$, and $NaNO_3$ through response surface methodology optimization. At > 5% salinities, *Marinobacter* sp. performed superior nitrogen removal performance in oxysalt-laden wastewater compared to chloride-based wastewater. In contrast, other SND strains including *Pseudomonas* sp. and *Halomonas* sp. experienced significant activity inhibition and even bacterium demise in oxysalt-rich wastewater, despite their high halotolerance to NaCl. The excellent SND activities of the oxysalt-tolerant strain were further validated using single and mixed nitrogen sources at 11% Na_2SO_4 salinity. Moreover, amplification of nitrogen removal functional genes and the corresponding enzyme activities elucidated the nitrogen metabolism pathway of the strain in harsh oxysalt environments.

Keywords: hypersaline wastewater; oxysalt-tolerant; simultaneous nitrification and denitrification; nitrogen removal characteristic; nitrogen metabolism pathway

1. Introduction

Microbial treatment especially simultaneous nitrification and denitrification (SND) processes for saline wastewater that is being ceaselessly created by industries, is of significant interest for mitigating water eutrophication and maintaining the natural nitrogen balance [1–5]. For instance, wastewater generated from processes such as the steaming of ammonia in the coking industry, crude oil electro-desalting in the refining sector, pharmaceutical manufacturing, and the production of pickling and tartaric acid, typically exhibits elevated salinity levels ranging from 1% to over 10% [6–10]. The elevated osmotic pressure of saline wastewater leads to the inhibition of microbial activity, induces plasmolysis, gives rise to bacterial demise. Up to now, most present halotolerant SND strains can only effectively remove nitrogen from wastewater with ≤ 5% NaCl [11–13]. Recently, a robust bacterium *Exiguobacterium mexicanum* demonstrated the ability to perform SND beyond 5% NaCl salinity but its growth was greatly inhibited [14]. In addition, our previous work identified that *Halomonas salifodinae* exhibited substantial SND capabilities at 15% salinity. This was achieved through the accumulation of intracellular compatible solutes, which facilitate water retention and enable the bacterium to withstand high osmotic pressure of the hypersaline wastewater [15,16].

Besides chloride salts, industrial wastewater usually contains various oxysalts such as sulfates (e.g., SO_4^{2-}), phosphates (e.g., HPO_4^{2-}), carbonates (e.g., HCO_3^-), and nitrates (e.g., NO_3^-) [17–20]. For example, wastewater from the hydrolysis of imidazole aldehyde in the pharmaceutical industry and

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from metal processing contain more than 10% phosphates and sulfates, respectively. In addition, swine wastewater contains ca. 1% carbonates, while wastewater from the nuclear industry contains over 5% nitrates due to the use of nitric acid in metal cleaning processes [21–23]. Thus, it is desirable to remove nitrogen of industrial wastewater containing these oxysalts via SND processes. At present, research is mainly concentrated on determining the influences of sulfate salinity on the SND activities of mixed bacterial communities [6,11,23–25]. When the concentration of Na_2SO_4 reached ca. 1.5% in the parallel bench reactors, the removal efficiency of ammonium nitrogen (NH_4^+-N) exceeded 90%. In contrast, ca. 60% of total nitrogen (TN) was removed, attributed to the inhibition of nitrite oxidizing bacteria activity and the accumulation of nitrite nitrogen (NO_2^--N) under the stress induced by Na_2SO_4 [25]. In the sequenced batch reactors at 3% salinity, the exposure of halophilic autotrophic nitrification sludge system to SO_4^{2-} instead of Cl^- resulted in an increased relative abundance of an ammonia oxidizing bacterium *Nitrosomonas*, accompanied by a rise in extracellular polymeric substances to tolerate elevated osmotic pressure [11]. In addition, half inhibitory concentrations of NaCl and Na_2SO_4 in freshwater Anammox system were determined to be 0.106 and 0.063 M, respectively, indicating that SO_4^{2-} has higher microbial activity inhibition than Cl^- [6]. To date, efficient nitrogen removal has been reported in mixed bacterial systems containing $\leqslant$ 3% SO_4^{2-} [11,25,26]. However, there is a paucity of robust single strains capable of the SND in saline wastewater with > 3% SO_4^{2-}, as well as even other oxysalts. A SND bacterium *Acinetobacter* sp. TAC-1 isolated from pig farm wastewater exhibited half inhibition concentrations of 0.205 M for NaCl, 0.238 M for KCl, and 0.110 M for Na_2SO_4 [27].

In this study, a robust bacterium *Marinobacter* sp. was separated from aerobic sludge in a saline wastewater treatment plant (WWTP). The SND activity of the separated bacterium at 11% Na_2SO_4 salinity was optimized via response surface methodology. The strain's exceptional tolerance to oxysalts was confirmed through comparative analysis with *Pseudomonas* sp. and *Halomonas* sp., which were isolated from aerobic sludges in petrochemical and pharmaceutical WWTP, respectively. A systematic investigation was conducted to assess the SND efficiencies in saline wastewater containing NaCl or oxysalts such as Na_2SO_4, Na_2HPO_4, $NaHCO_3$, and $NaNO_3$ at salinities ranging from 1% to 11%. Moreover, the SND efficiency of the separated bacterium at 11% Na_2SO_4 salinity, along with the associated functional genes and key enzymatic activities were studied to elucidate the nitrogen removal pathway and mechanism.

2. Materials and Methods

2.1. Isolation and Identification

Saline aerobic sludge supplied by Hubei Huisheng pharmaceutical WWTP (China) was used to isolate the SND bacteria. The control SND bacterium was separated from saline aerobic sludge sample in petrochemical refinery WWTP (China). The SND capabilities of the separated bacteria were assessed in heterotrophic nitrification (HN) media and aerobic denitrification (AD) media, the components of which are detailed in the Supplementary Material. Strain Y2 with high SND efficiencies and distinctive oxysalt-tolerant properties was selected for subsequent assays. The 16S rRNA of strain Y2 was amplified by polymerase chain reaction (PCR) and sequenced by TSINGKE Biotechnology Co., Ltd (Wuhan, China). The results were submitted to GenBank database in comparison with other microorganisms by BLAST. MEGA 7.0 software was applied to create a phylogenetic tree through NJ algorithm.

2.2. Response Surface Methodology Optimization

The influences of C/N ratio (8, 12 and 16), initial pH value (6, 8 and 10), temperature (20, 30 and 40 °C), and shaking speed (50, 150 and 250 rpm) on the SND activities of the isolated strain Y2 at 11% Na_2SO_4 salinity were investigated through response surface methodology with the Box-Behnken design. In these 29 experiments, bacterial suspensions were transferred into the HN media containing

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11% Na_2SO_4, which were cultured for 72 h. The nitrogen removal efficiencies were measured by the equation:

$$R_N = (C_0 - C_t)/C_0 \times 100\% \quad (1)$$

where R_N is the nitrogen removal efficiencies, C_0 and C_t are the NH_4^+-N concentrations before and after the SND processes, respectively. Response surface plots along with the associated contour plots were generated according to the experimental models.

2.3. Assessment of Salt Tolerance

To evaluate the tolerance of microorganisms to different inorganic salts, including NaCl, Na_2SO_4, Na_2HPO_4, $NaHCO_3$, and $NaNO_3$, the isolated strain *Marinobacter* sp. as well as *Pseudomonas* sp. and *Halomonas* sp. were cultivated at different salinities to determine bacterial growth and nitrogen removal efficiencies. In addition, *Marinobacter* sp., *Pseudomonas* sp., and *Halomonas* sp. were cultivated at 11%, 3%, and 11% NaCl for 96 h, 72 h, and 60 h, respectively. Subsequently, all the strains were collected and were individually inoculated into HN media containing 11%, 3%, and 11% $NaNO_3$ for further cultivation. The bacterial growth was measured at intervals and the bacterial morphology was observed by SEM (Regulus 8100, Hitachi). The salinity activity inhibition ratios were calculated according to the equation:

$$IR_S = (v_{opt} - v_s)/v_{opt} \quad (2)$$

where IR_S is the salinity activity inhibition ratios, v_{opt} and v_s are the nitrogen removal rates at the optimal and other salinities, respectively.

2.4. Effects of Different Oxysalts on Bacterial Growth and Nitrogen Removal

Batch experiments were conducted to estimate the effects of Cl^- and various oxysalts including SO_4^{2-}, HPO_4^{2-}, HCO_3^-, and NO_3^- on bacterial growth and nitrogen removal efficiencies of strain Y2. Suspensions of strain Y2 were transferred into either HN or AD media including NaCl, Na_2SO_4, Na_2HPO_4, $NaHCO_3$ or $NaNO_3$ with the initial pH of 7.2. The concentrations of these adscititious salts were 1%, 3%, 5%, 7%, 9%, and 11%. During cultivation under 150 rpm at 30 °C, the bacterial growth and NH_4^+-N or nitrate nitrogen (NO_3^--N) concentrations were measured at intervals.

2.5. SND Capability at 11% Na_2SO_4

To evaluate SND performance of the oxysalt-tolerant strain Y2, bacterial suspensions were added into the sterilized media containing different nitrogen sources and 11% Na_2SO_4. Sodium succinate was used as carbon source to achieve a C/N ratio of 15, the initial concentrations of nitrogen sources and pH values were 200 mg/L and 7.2. Strain Y2 was incubated at 150 rpm and 30 °C. Samples were collected at intervals to measure the bacterial growth and various nitrogen source concentrations.

2.6. Nitrogen Removal Enzymes and Functional Genes for the SND

Nitrogen removal enzymes for the SND mainly include ammonia monooxygenase (AMO), hydroxylamine oxidase (HAO), periplasm nitrate reductase (NAP), nitrite reductase (NIR). Ultrasonication was utilized to prepare acellular crude enzyme extracts [28,29]. The activities of AMO and HAO were determined in Tris-HCl buffer solutions containing the crude enzyme extracts, cytochrome *c*, and specific substrates. The activities of NIR and NAP were determined in phosphate buffer solutions containing the crude enzyme extracts, NADH, and target substrates. Control experiments were conducted in the absence of either the crude enzyme extracts or cytochrome *c*/NADH. Functional genes associated with nitrogen removal in the SND were detected. Genomic DNA of strain Y2 was extracted through the manufacturer's protocol and was used for functional genes amplification. The primers and conditions for PCR amplification were based on previously established methodologies [15].

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2.7. Analytical Methods

The bacterial growth (OD_{600}) was estimated through determining the optical density of bacterial suspension at 600 nm. The concentrations of NH_4^+-N, NO_2^--N, NO_3^--N, and TN were quantified through the standard methods [30]. The concentrations of NH_2OH were determined referring to the reported spectrophotometric method [31]. The concentrations of IN were determined by deducting the TN of centrifuged bacterial supernatants from the TN of uncentrifuged bacterial suspensions.

3. Results and Discussion

3.1. Identifying the Isolated Strain Y2

After a repeated-batch acclimation, eight robust salt-tolerant bacterial strains were isolated from saline aerobic sludge from a pharmaceutical WWTP. Among these isolated bacteria, strain Y2 with unique oxysalt-tolerant capabilities and high SND activities was used for further investigation. On the agar plate, strain Y2 forms beige colonies with a diameter of ca. 1 mm, and smooth, slightly raised surfaces (Figure S1A). SEM image shows that strain Y2 appears short rods with the size of 0.4~0.6 × 2.0~4.0 μm (Figure S1B).

Sequencing of the PCR-amplified 16S rRNA (1413 bp) of strain Y2 were conducted, and 16S rRNA were submitted to GenBank database (accession number: PQ241653). BLAST homology analysis indicates that strain Y2 shares over 99% similarity with *Marinobacter* sp. A phylogenetic tree created using the NJ algorithm further classifies that strain Y2 is affiliated to *Marinobacter shengliensis* (Figure S1C). While *Marinobacter* sp. are commonly found in wastewater treatment systems [32–34], but there are few reports on their role in the SND of saline wastewater.

3.2. Optimization of the SND at High Salinity

To assess the impacts of different culture conditions on NH_4^+-N removal efficiency of strain Y2 at 11% Na_2SO_4 salinity, the response surface methodology was carried out in combination with a four-factor, three-level Box-Behnken design. This approach was employed to explore the interactions among the independent variables and to identify the optimal condition for nitrogen removal. The correlation among the four independent variables and NH_4^+-N removal efficiency was represented by the following equation:

$$Y = 92.15 + 14.25X_1 + 1.64X_2 + 1.04X_3 + 8.62X_4 + 0.44X_1X_2 + 0.34X_1X_3 + 6.77X_1X_4 + 1.23X_2X_3 + 2.17X_2X_4 + 4.67X_3X_4 - 15.41X_1^2 - 4.22X_2^2 - 6.88X_3^2 - 13.68X_4^2 \quad (3)$$

where Y is the NH_4^+-N removal efficiency (%), X_1, X_2, X_3, and X_4 indicate the coded values of C/N ratio, initial pH value, temperature, and shaking speed, respectively.

Variance analysis and significance testing were used to assess the rationality and validity of the response surface quadratic model (Table S1). The model's significance is corroborated by *F-value* of 43.21 and low *p-value* of < 0.0001. For the Lack of Fit term, the calculated *F-value* and *p-value* are 5.64 and 0.055, evidencing that the model exhibits a satisfactory degree of fit. The coefficient of determination (R^2 = 0.9774) illustrates that the fitted model accounts for 97.74% of the variability, thereby demonstrating the model's high accuracy. In addition, according to the *F-value* and *p-value* of four independent variables, the impact of C/N ratio and shaking speed on the NH_4^+-N removal performance is more substantial compared to that of initial pH value and temperature.

To further elucidate the relationship between NH_4^+-N removal efficiencies and four independent variables, the response surface plots and corresponding contour plots were constructed to represent the influence of the SND conditions on nitrogen removal efficiency. Figure 1A and 1D show that C/N ratio (8-16) has significantly more positive influence on NH_4^+-N removal efficiency than temperature (20-40 °C), which is in accordance with the previously reported studies [35,36]. Under relatively high shaking speed (ca. 150-200 rpm), strain Y2 can well grow at 11% Na_2SO_4 salinity and efficiently

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remove NH_4^+-N (> 90%) at relatively wide pH value range of 7-10 (Figure 1B and 1E). Figure 1C and 1F show that the interaction between C/N ratio and shaking speed is significant.

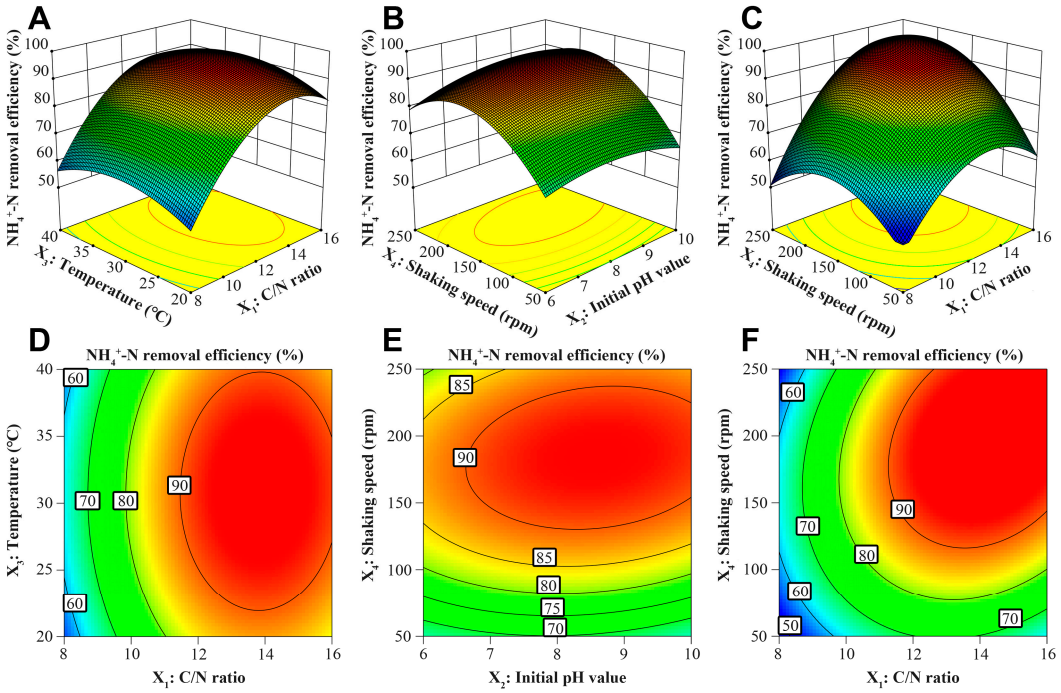

Figure 1. Response surface plots along with the contour plots for NH_4^+-N removal efficiency of strain Y2 at 11% Na_2SO_4 salinity. The interaction between (**A**, **D**) C/N ratio and temperature, (**B**, **E**) initial pH value and shaking speed, (**C**, **F**) C/N ratio and shaking speed for NH_4^+-N removal efficiency.

The optimal conditions for nitrogen removal by strain Y2 at 11% Na_2SO_4 salinity is C/N ratio of 13.97, initial pH value of 8.82, temperature of 30.96 °C, shaking speed of 171.89 rpm, corresponding to the predicted NH_4^+-N removal efficiency of 97.8%. To verify the precision of the prediction, nitrogen removal experiments were performed in triplicate under the optimal condition. The measured NH_4^+-N removal efficiency was 97.4±1.1%, demonstrating great concordance with the prediction.

3.3. Oxysalt-Tolerant Capabilities

The proposed bacterium *Marinobacter* sp. and other SND strains *Pseudomonas* sp. and *Halomonas* sp. were utilized to examine the oxysalt-tolerant capabilities. At 3% salinity, *Pseudomonas* sp. can endure high osmotic pressure induced by NaCl, exhibiting vigorous growth with a short-term adaptation period (Figure 2A). After 84 h, NH_4^+-N is almost completely removed (Figure 2B). When Na_2SO_4, Na_2HPO_4, $NaHCO_3$, and $NaNO_3$ replace NaCl, the duration of the adaptive phases is extended and the growth vitalities diminishes in sequence. Correspondingly, the rates and efficiencies of NH_4^+-N removal decline owing to the toxicities of these oxysalts, with the respective inhibition ratios of 0.72, 0.82, 0.94, and 0.99. As another robust SND bacterium, *Halomonas* sp. can more rapidly grow and efficiently remove NH_4^+-N at 11% NaCl or Na_2SO_4 salinities (Figure 2C and 2D), achieving high removal efficiencies of > 99% after 72 h. However, when Na_2HPO_4, $NaHCO_3$, and $NaNO_3$ replace NaCl and Na_2SO_4, both bacterial growth and NH_4^+-N removal rates and efficiencies are significantly decreased. After 96 h, only 41% NH_4^+-N can be removed by *Halomonas* sp. at 11% Na_2HPO_4 salinity. In addition, *Halomonas* sp. cannot grow at 11% $NaHCO_3$ or $NaNO_3$ salinities.

To further study the influence of oxysalts on these mature bacteria, *Pseudomonas* sp., *Halomonas* sp., and *Marinobacter* sp. were cultivated in the HN media containing 3%, 11%, and 11% NaCl, respectively. Subsequently, the grown bacteria were collected and were transferred to the HN media containing $NaNO_3$ at the same concentrations. As shown in Figure 2E, the bacterial concentrations of

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Pseudomonas sp. and *Halomonas* sp. decreases immediately within 12 h. SEM images of the microbial samples acquired at the half decay period reveal that *Halomonas* sp. is being wizened owing to water loss and *Pseudomonas* sp. suffers from cell apoptosis and aggregation. In contrast, *Marinobacter* sp. continues to grow, until nearly doubling the bacterial number while maintaining its initial shape, showing high oxysalt-tolerant capability. Notably, most of the reported halotolerant SND bacteria cannot efficiently remove nitrogen at > 3% Na_2SO_4 or Na_2HPO_4 salinities, and the SND bacteria that can work in saline wastewater containing $NaHCO_3$ and $NaNO_3$ are not reported (Figure 2F) [6,23–25,27,37].

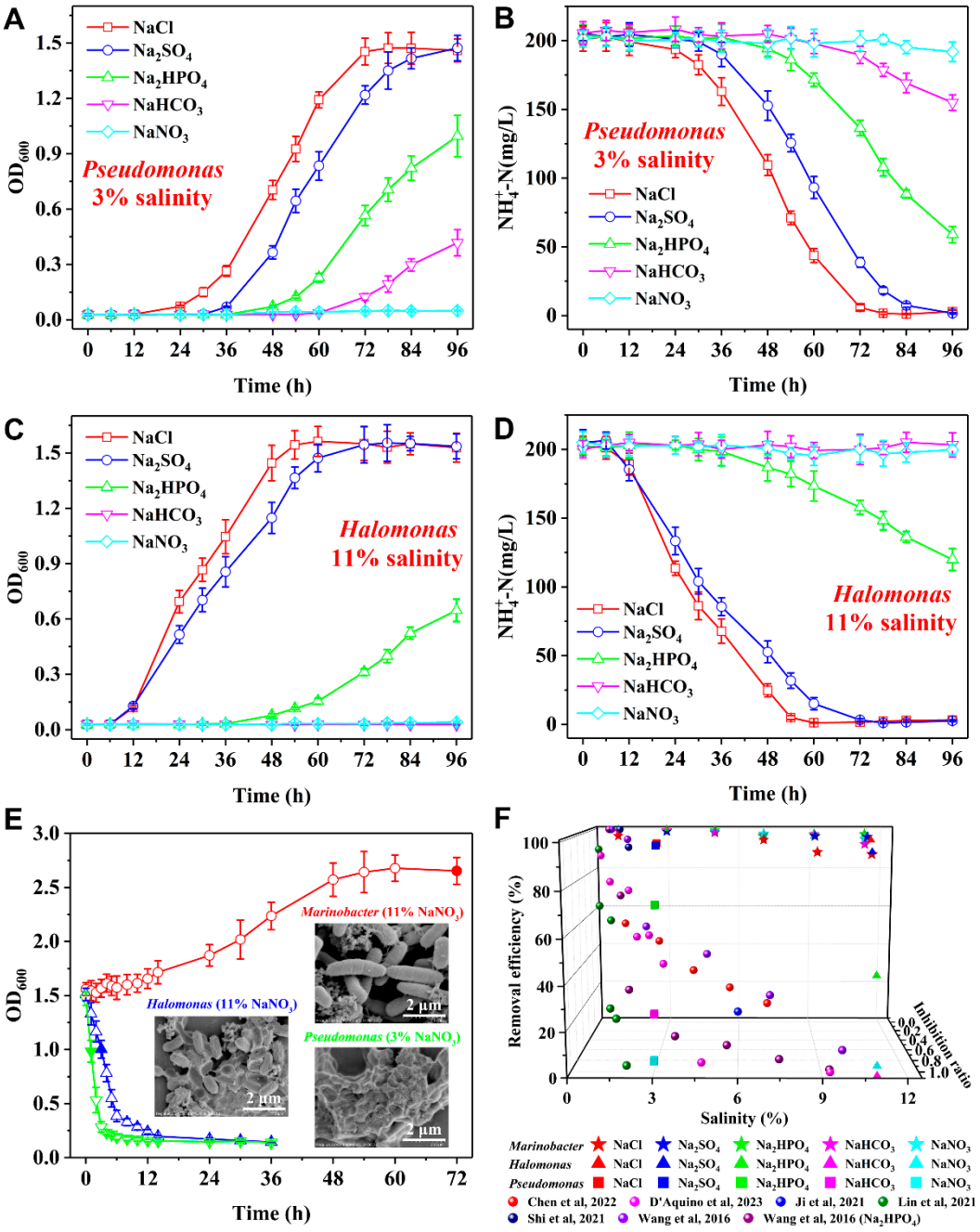

Figure 2. (**A**, **C**) Bacterial growth and (**B**, **D**) NH_4^+-N removal of *Pseudomonas* sp. (**A**, **B**) and *Halomonas* sp. (**C**, **D**) at 3% (**A**, **B**) and 11% (**C**, **D**) salinities. The salts include NaCl, Na_2SO_4, Na_2HPO_4, $NaHCO_3$, and $NaNO_3$. (**E**) Bacterial growth of *Marinobacter* sp., *Pseudomonas* sp., and *Halomonas* sp. after being transferred to the HN media containing $NaNO_3$. Insets are SEM images of *Marinobacter* sp., *Pseudomonas* sp., and *Halomonas* sp. after cultivation in the HN media containing $NaNO_3$ for 72 h, 1 h, and 3 h, respectively. (**F**) Nitrogen removal performance of the reported nitrogen removal bacteria at various salinities of oxysalts.

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Figures 3 and S2 show the bacterial growth of *Marinobacter* sp. in the HN media containing various salts, including NaCl, Na_2SO_4, Na_2HPO_4, $NaHCO_3$, and $NaNO_3$ at different salinities, as well as the corresponding NH_4^+-N removal. It can be found that strain Y2 can vigorously grow and efficiently remove NH_4^+-N at 1%-11% salinities of different salts. At 1%-5% salinities, the rates of bacterial growth and NH_4^+-N removal are almost consistent. The rates of NH_4^+-N removal are slightly higher in the presence of HPO_4^{2-}, HCO_3^-, and NO_3^- compared to Cl^- and SO_4^{2-}. After 36 h, all the NH_4^+-N removal efficiencies exceed 90%, expect for SO_4^{2-}, which requires approximately 48 h. Beyond 5% salinity, both bacterial growth and NH_4^+-N removal are inhibited by oxysalts, particularly HPO_4^{2-}, HCO_3^-, and NO_3^-, exhibiting significantly lower inhibition ratios. For example, after 48 h cultivation at 11% salinity, the NH_4^+-N removal efficiencies of *Marinobacter* sp. increase markedly when substituting from NaCl and Na_2SO_4 to Na_2HPO_4, $NaHCO_3$, and $NaNO_3$ (Figure 3F). In contrast, the respective NH_4^+-N removal efficiencies of *Pseudomonas* sp. and *Halomonas* sp. at 3% and 11% salinities are dramatically decreased owing to the toxicity of oxysalts.

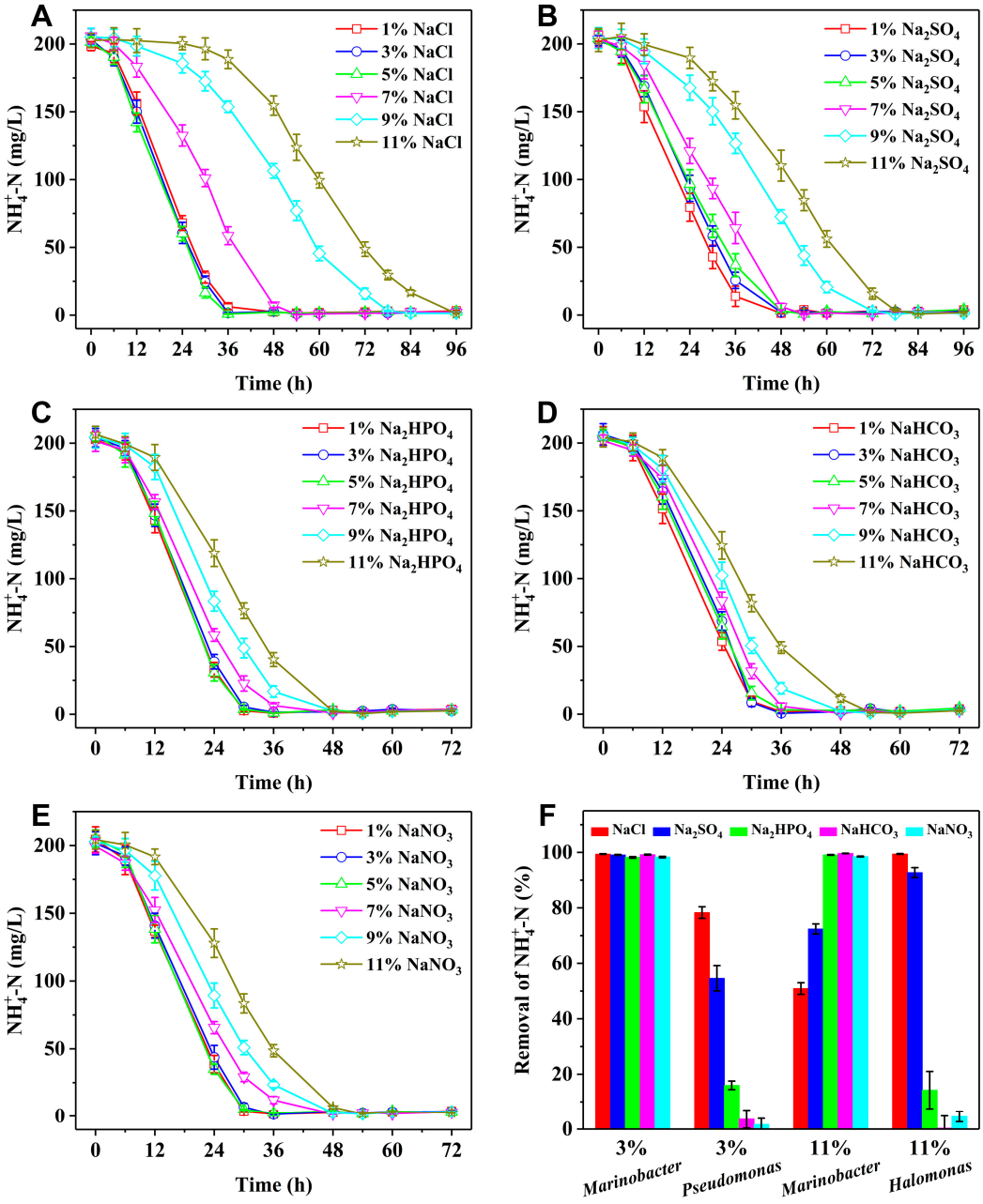

Figure 3. (**A**-**E**) NH_4^+-N removal of *Marinobacter* sp. at various salinities. Salts include NaCl (**A**), Na_2SO_4 (**B**), Na_2HPO_4 (**C**), $NaHCO_3$ (**D**), and $NaNO_3$ (**E**). (**F**) NH_4^+-N removal efficiencies of *Marinobacter* sp., *Pseudomonas*

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sp., and *Halomonas* sp. after 48 h cultivation at various salinities. Salts include NaCl, Na_2SO_4, Na_2HPO_4, $NaHCO_3$, and $NaNO_3$.

Figures S3 and S4 show the bacterial growth of *Marinobacter* sp. in the AD media containing NaCl, Na_2SO_4, Na_2HPO_4, and $NaHCO_3$ at different salinities, and the corresponding NO_3^--N removal efficiencies. Being consistent with the NH_4^+-N removal results, the inhibition ratios of NO_3^--N removal induced by oxysalts especially HPO_4^{2-} and HCO_3^- are obviously lower than that induced by NaCl. After 60 h cultivation at 11% Na_2HPO_4 or $NaHCO_3$ salinities, strain Y2 achieves the NO_3^--N removal efficiencies exceeding 90%. In contrast, achieving a NO_3^--N removal efficiency of > 90% requires 96 h at 11% NaCl salinity. These results demonstrate that the robust SND bacterium *Marinobacter* sp. possesses excellent oxysalt-tolerant capabilities.

3.4. SND Performance Using Single Nitrogen Sources

The SND performance of the oxysalt-tolerant bacterium *Marinobacter* sp. at 11% Na_2SO_4 salinity was evaluated using NH_4^+-N, NH_2OH, NO_2^--N, NO_3^--N as single nitrogen sources with initial concentration of ca. 200 mg/L. Figure 4 shows that strain Y2 can grow well in single nitrogen species and all the inorganic nitrogen species are barely detected in the SND systems after 96 h, with the TN removal rates exceeding 95%. In addition, NH_4^+-N, NH_2OH, NO_3^--N are removed at slightly higher rates than NO_2^--N. For example, after 72 h, the respective removal efficiencies for NH_4^+-N, NH_2OH, NO_3^--N are 93.3%, 96.4%, and 94.3%, while the NO_2^--N removal efficiency is 86.7%.

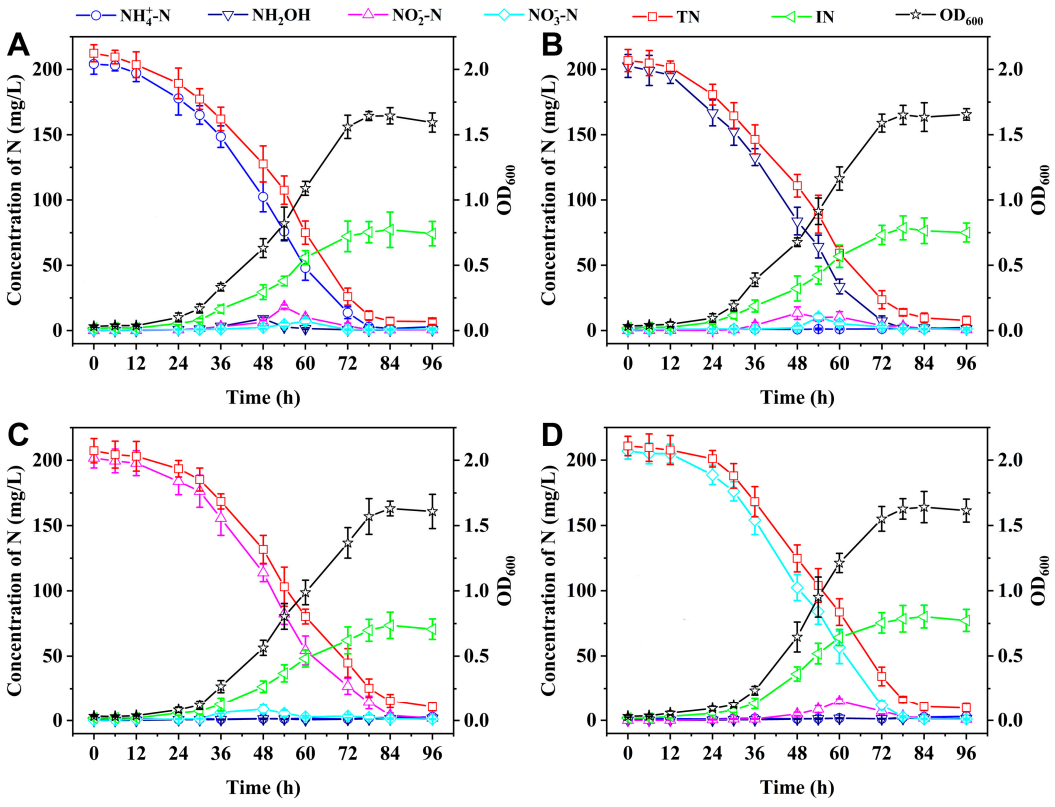

Figure 4. SND performance of strain Y2 at 11% Na_2SO_4 with (**A**) NH_4^+-N, (**B**) NH_2OH, (**C**) NO_2^--N, and (**D**) NO_3^--N as single nitrogen sources.

During the SND processes involving NH_4^+-N, NH_2OH, NO_3^--N as single nitrogen species, a minor accumulation of NO_2^--N (< 20 mg/L) can be observed after 48-60 h followed by its rapid removal, which could be ascribed to slower removal rate of NO_2^--N than those of NH_4^+-N, NH_2OH, and NO_3^--N. Similarly, more NO_2^--N was accumulated during the denitrification-anammox process at high salinity, which was ascribed to the enriched denitrifying community [38,39]. When *Halomonas*

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sp. was utilized to remove NH_4^+-N, NH_2OH, or NO_3^--N at 15% NaCl salinity, considerable NO_2^--N (ca. 50 mg/L) was progressively amassed until the complete elimination of these single nitrogen sources [15]. In addition, a minor accretion of NO_3^--N is detected after 48 h during the NO_2^--N denitrification, indicating the conversion of NO_2^--N to NO_3^--N under aerobic condition. Nitrogen balance analysis reveals that 35%-38% of initial nitrogen sources are assimilated as the IN, while 55%-60% are removed through the SND processes.

3.5. SND Performance Using Mixed Nitrogen Sources

The SND capabilities of strain Y2 at 11% Na_2SO_4 salinity was further investigated using NH_4^+-N, NO_2^--N, NO_3^--N as mixed nitrogen sources with initial TN concentration of ca. 200 mg/L. When NH_4^+-N and NO_2^--N are used as mixed nitrogen source, they are simultaneously removed and NH_4^+-N is removed at a higher rate than NO_2^--N (Figure 5A). A minor quantity of NO_3^--N is accumulated after 60 h, which can be eliminated completely after 72 h. During simultaneous removal of NH_4^+-N and NO_3^--N, they are nearly completely removed after 72 and 84 h, respectively, and NO_2^--N reaches the maximum accumulation of 21.0 mg/L after 48 h (Figure 5B). When NO_2^--N and NO_3^--N are used as mixed nitrogen source, the removal rates of NO_2^--N and NO_3^--N are almost same, accomplishing complete removal after 84 h (Figure 5C). When NH_4^+-N, NO_2^--N, NO_3^--N are used as mixed nitrogen source, NH_4^+-N and NO_3^--N are preferentially removed and NH_4^+-N is removed at a higher rate than NO_3^--N. Until NH_4^+-N is nearly entirely removed, NO_2^--N begins to undergo rapid denitrification under aerobic condition (Figure 5D). The maximum assimilated IN falls within the range of 70-80 mg/L, indicating that the TN of 34%-36% contributes to the bacterial growth.

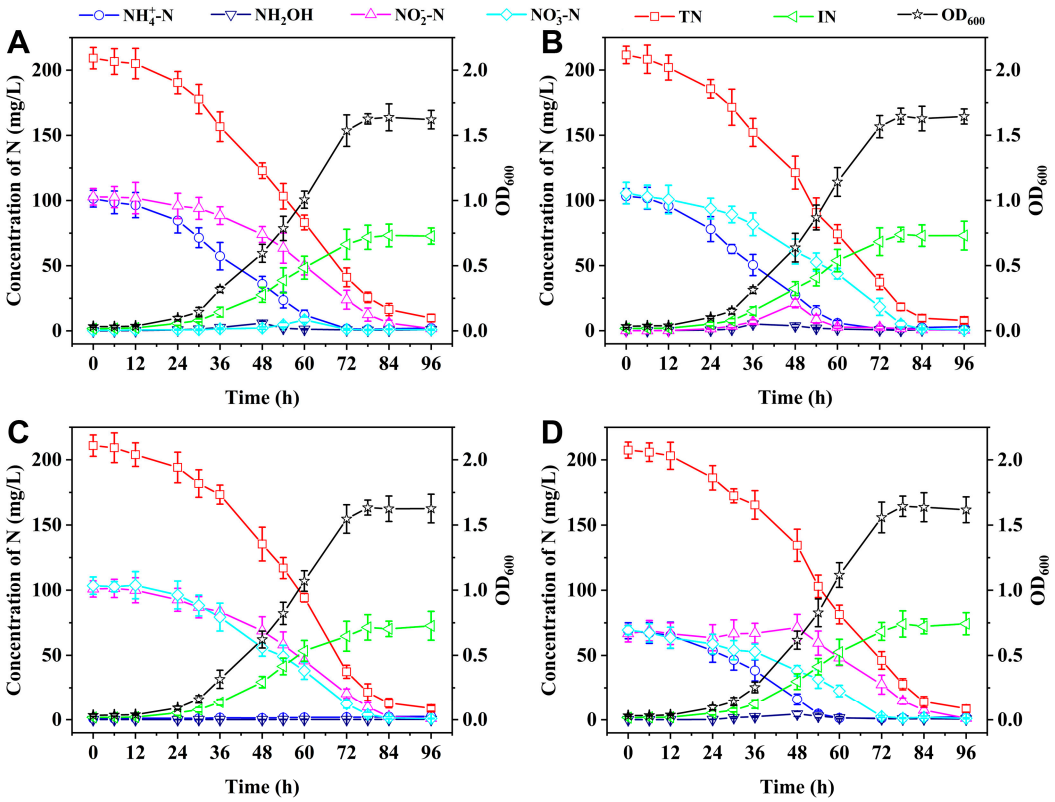

Figure 5. SND performance of strain Y2 at 11% Na_2SO_4 with (**A**) NH_4^+-N and NO_2^--N, (**B**) NH_4+-N and NO_3^--N, (**C**) NO_2^--N and NO_3^--N, (**D**) NH_4^+-N, NO_2^--N and NO_3^--N as mixed nitrogen sources.

The results demonstrate that the isolated robust bacterium *Marinobacter* sp. is capable of efficiently conducting the SND processes in hypersaline wastewater containing oxysalts such as Na_2SO_4. During the SND processes, NH_4^+-N is oxidized to NH_2OH, NO_2^--N, and NO_3^--N. Subsequently, the generated NO_2^--N and NO_3^--N can be transformed to N_2, in which a small amount

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of NO_2^--N is accumulated. The SND-related enzymes determine nitrogen removal efficiencies of the oxysalt-tolerant bacterium in such harsh oxysalt environments.

3.6. Nitrogen Removal Enzymes and Functional Genes for the SND

Enzymes associated with the SND including AMO, HAO, NAP, and NIR were extracted from strain Y2 cultivated at 11% Na_2SO_4 salinity and the enzyme activities and specific enzyme activities were measured. In the enzyme activity assays, cytochrome *c* functioned as the electron acceptor, while NADH acted as the electron donor for target substrate removal, respectively. When only cytochrome *c*/NADH and crude enzyme extracts coexist, the target substrates are removed significantly. During the nitrification process, AMO and HAO are the key enzymes responsible for NH_4^+-N and NH_2OH oxidation. During the subsequent denitrification process, NO_3^--N and NO_2^--N are removed by the key enzymes of NAP and NIR, respectively [28,40–42].

The specific activities of AMO, HAO, NAP, NIR were determined according to the removal of target substrates. As shown in Figure 6A, AMO, HAO, NAP have the specific activities of 0.046 ± 0.007, 0.048 ± 0.007, and 0.043 ± 0.007 U/mg protein, which are higher than NIR's (0.036 ± 0.007 U/mg protein). The enzyme activities of AMO, HAO, NAP for the isolated bacterium *Marinobacter* sp. at 11% Na_2SO_4 salinity are lower than those for the reported halotolerant bacterium *Halomonas* sp. at 15% NaCl salinity [15], which are consistent with the elimination rates of various nitrogen sources using different halotolerant bacteria (Figure 6B). However, the enzyme activity of NIR for *Marinobacter* sp. is higher than that for *Halomonas* sp., resulting in significantly reduced NO_2^--N accumulation for *Marinobacter* sp. compared to *Halomonas* sp.

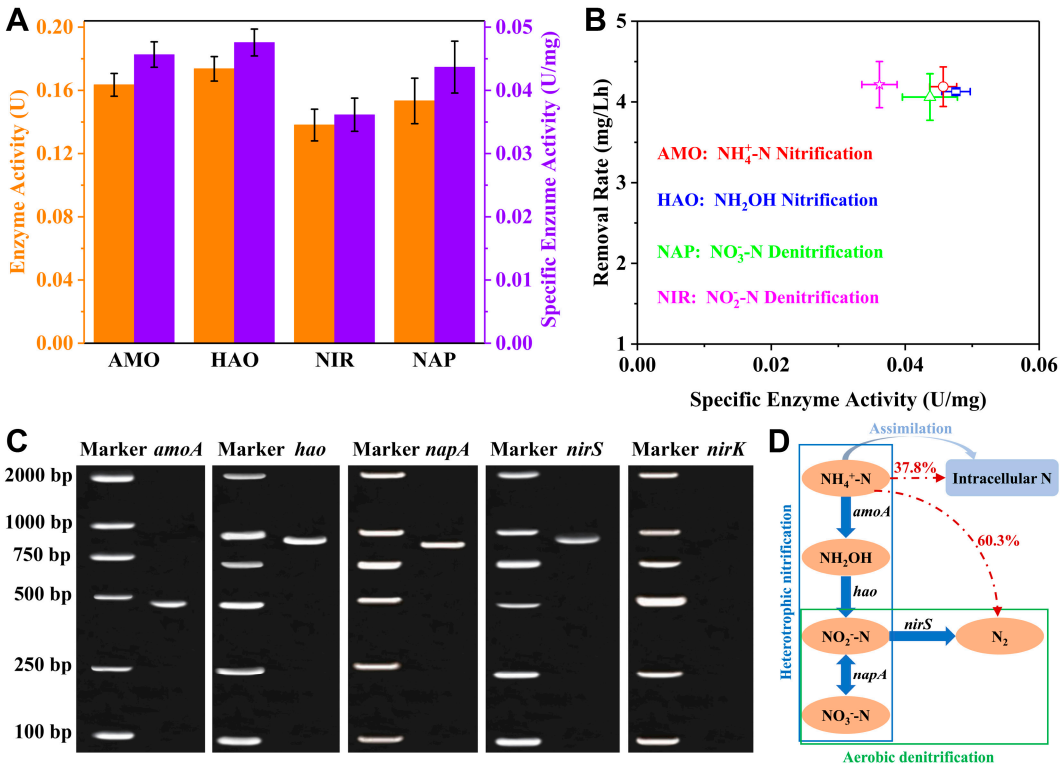

Figure 6. (**A**) AMO, HAO, NIR, NAP enzymatic activity and specific activity. (**B**) The correlation of specific enzymatic activity with the maximum rate of nitrogen removal. (**C**) PCR amplification of SND-associated functional genes. (**D**) The proposed nitrogen removal pathway of strain Y2.

The SND-associated enzymes of strain Y2 at 11% Na_2SO_4 salinity are encoded by specific functional genes, e.g., *amoA* and *hao* responsible for encoding the AMO and HAO in the nitrification process, *napA* and *nirS*/*nirK* encoding the NAP and NIR in the denitrification process [43-45. As shown in Figure 6C, the functional genes for SND, like *amoA*, *hao*, *napA*, *nirS*, are amplified

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successfully. The functional genes *amoA* and *hao* are widely detected for the SND study of heterotrophic nitrifying bacteria. The functional gene *napA* involves in the transformation of NO_3^--N to NO_2^--N under aerobic condition [36,46,47]. The successful amplification of these nitrogen removal functional genes further demonstrates the SND capabilities of strain Y2 in such harsh oxysalt environments. According to the SND-associated enzymes and functional genes, the proposed nitrogen metabolism pathway is NH_4^+-N → NH_2OH → NO_2^--N ↔ NO_3^--N → N_2 (Figure 6D).

4. Conclusions

An oxysalt-tolerant SND bacterium *Marinobacter* sp. has been isolated from saline aerobic sludge, which can achieve 98% of nitrogen removal at 11% Na_2SO_4 salinity after optimizing SND conditions via response surface methodology. At > 5% salinities, the robust bacterium exhibits significantly reduced oxysalt-induced inhibition on bacterial growth and nitrogen removal rates compared to other SND strains *Pseudomonas* sp. and *Halomonas* sp. The proposed strain can achieve nitrogen removal efficiencies exceeding 90% with both single and mixed nitrogen sources at 11% Na_2SO_4. The excellent SND performance is attributed to the SND-associated functional genes and their encoded enzymes. The isolated oxysalt-tolerant SND bacterium provides a viable approach for nitrogen removal from hypersaline wastewater containing oxysalts.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Figure S1: (A) Colony morphology and (B) SEM image of strain Y2. (C) NJ algorithm phylogenetic tree of strain Y2 based on 16S rRNA gene sequences; Figure S2: Bacterial growth of *Marinobacter* sp. in the HN media at different salinities. Salts include NaCl (A), Na_2SO_4 (B), Na_2HPO_4 (C), $NaHCO_3$ (D), and $NaNO_3$ (E); Figure S3: Bacterial growth of *Marinobacter* sp. in the AD media at different salinities. Salts include NaCl (A), Na_2SO_4 (B), Na_2HPO_4 (C), and $NaHCO_3$ (D); Figure S4: NO_3^--N removal of *Marinobacter* sp. at different salinities. Salts include NaCl (A), Na_2SO_4 (B), Na_2HPO_4 (C), and $NaHCO_3$ (D); Table S1: Analysis of variance for response surface quadratic model (Y) [a].

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