Journal of Functional Materials and Applied Engineering

Research Paper

Screening of Heterotrophic Nitrification-Aerobic Denitrification Strains and Their Application in Nitrogen Removal from Soybean Product Wastewater

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Abstract. Soybean product wastewater (SPW) is organic wastewater generated during the processing of soybean products. Untreated discharge of this wastewater would inflict severe damage on the receiving environment and pose significant risks to human health. However, to date, no studies have been reported on the application of Heterotrophic Nitrification-Aerobic Denitrification (HN-AD) bacterial strains for the treatment of SPW. To obtain HN-AD strains that can directly treat undiluted SPW, enrichment culture, gradient dilution-plate streaking method were used to screen, isolate, and purify epiphytic aggregates of cyanobacteria in Taihu Lake. Morphological analysis and 16S rDNA sequencing results showed that the isolated HN-AD strain was Gram-negative and belonged to the genus Enterobacter (Enterobacter CS-1). Gradient concentration acclimation results showed that the strain can grow normally in undiluted SPW. Single-factor optimization results showed that the optimal conditions for strain CS-1 to treat simulated wastewater were: using sodium succinate as carbon source, C/N=15, temperature 30°C, pH 6.50, shaking speed 180 rpm. Under these conditions, the removal rate of NH_4^+-N in simulated wastewater reached 96.2% within 48 h, with a maximum removal rate of 2.95 mg L^{-1} h^{-1}. Whole-genome scanning results showed that strain CS-1 mainly achieves nitrogen removal through two metabolic pathways: NH_4^+-N is assimilated into glutamate via the GDH or GS-GOGAT process, while NO_3^--N is first dissimilated and reduced stepwise to generate NO_2^--N and NH_4^+-N, which is then assimilated and utilized. Response surface methodology (RSM) was employed to analyze the influence of pH, temperature, and agitation rate on NH_4^+-N removal from undiluted SPW by strain CS-1. The model identified optimal operational parameters as pH 6.85, a temperature of 32.1°C, and a shaking speed of 189 revolutions per minute, without the supplementation of an external carbon source. Under these optimized conditions, Enterobacter sp. CS-1 accomplished the concurrent removal of 90.1% NH_4^+-N, 42.0% COD, 53.4% DTN, and 67.6% DTP from raw SPW within a 48-hour period. These results demonstrate the considerable practical potential of this strain for the treatment of SPW.

Keywords: *Heterotrophic Nitrification-Aerobic Denitrification; Soybean Product Wastewater; Acinetobacter*

Received on 03 Sep 2025, Accepted on 05 Nov 2025, Published on 15 Dec 2025

1 Introduction

Nitrogen is a key limiting factor for water eutrophication. Nitrogen pollution in water bodies mainly originates from point and non-point sources. Point source nitrogen pollution refers to concentrated discharges of nitrogen-containing pollutants through fixed outlets. If discharged without advanced treatment, it will significantly increase the nitrogen load of receiving water bodies. Industrial wastewater, originating from equipment cleaning and material synthesis in manufacturing sectors, is a significant source of organic pollution. Effluents from industries such as tanning, petrochemicals, and pharmaceuticals are key contributors to the increasing nitrogen levels observed in aquatic environments [1]. Conventional activated sludge (CAS) systems, however, face challenges in treating such industrial wastewater due to issues like high energy demand, substantial carbon emissions, and the production of surplus sludge [2]. Concurrently, recent trends in the livestock industry toward intensive, large-scale operations—driven by diverse consumer needs and quality-focused development—have become prevalent. This expansion in production scale inevitably leads to the generation of substantial volumes of wastewater [3]. According to relevant research, 24 billion tons of nutrient-rich livestock and poultry

wastewater annually need to be recycled and valorized through microalgae bioabsorption [4]. Driven by rapid urbanisation and population rise, annual global municipal wastewater now totals 380 billion t with ~40 mg L^{-1} NH_4^+-N ; nitrification–denitrification bioprocesses remain the dominant N-removal technology worldwide. Although effective in nitrogen removal, these systems require high-intensity aeration and sufficient organic donors to ensure treatment performance [6], which greatly increases operational costs. Hence, growing emphasis on energy savings and sustainability fuels demand for efficient, low-cost nitrogen-removal options tailored to high-strength organic-N wastewaters.

Traditional biological nitrogen removal methods have some disadvantages, including long treatment time, large footprint, high energy loss, and high investment and operating costs. In contrast, novel biological nitrogen removal technologies such as iron-amended ammonia oxidation (Feammox), partial nitrification coupled with anaerobic ammonium oxidation (PN/Anammox), and partial denitrification coupled with anaerobic ammonium oxidation (PD/Anammox) improve nitrogen removal efficiency while shortening the treatment time of traditional processes, reducing operation and maintenance costs, and belong to environmentally friendly, low-energy consumption advanced nitrogen removal technologies [7]. Feammox represents an emerging autotrophic biological nitrogen removal process. It utilizes cost-effective and widely available iron as an electron donor for microorganisms, achieving nitrogen elimination through the synergistic coupling of Fe(III) reduction and anaerobic ammonium oxidation (Anammox) [36]. Recent studies have found that anaerobic ammonium-oxidizing bacteria (AnAOB) have low cell yield and high sensitivity, which limit the large-scale application of Anammox processes. Dai et al. [8] investigated the performance and underlying mechanisms of Feammox processes across multiple scales, from macro-level system behavior to micro-level interactions. Their work provides a systematic review of how three distinct forms of iron—ionic iron, zero-valent iron, and iron-containing minerals—influence the efficiency of Anammox processes. Comparative findings showed that the enhancing effect of Fe(II) on AnAOB seems more prominent. Chen Xiaofeng et al. [38] collected sediments from different areas of Taihu Lake for anaerobic incubation simulation experiments. The study found that adding a certain amount of Fe(III) to the system can promote the oxidation of NH_4^+-N to NO_3^--N, while adding a certain amount of NH_4^+-N can promote the reduction of Fe(III). They developed a process that utilizes NO_3^--N byproducts to in situ oxidize zero-valent iron (ZVI). In this process, the main AnAOB promoted the formation of iron-phosphate minerals, which is beneficial for the phosphorus removal process of the system, improved particle settleability, and achieved simultaneous nitrogen and phosphorus removal from wastewater.

PN/Anammox is a nitrogen removal process combining partial nitrification and Anammox. In the initial stage, both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) facilitate the oxidation of NH_4^+-N to NO_2^--N under aerobic conditions. In the second step, the remaining NH_4^+-N and the produced NO_2^--N serve as electron acceptors and are converted to N_2 via Anammox under anaerobic conditions. Hendrickx et al. [10] screened an AnAOB applicable for autotrophic nitrogen removal in municipal wastewater treatment plants. Through the autotrophic (Anammox) process, it not only removes NH_4^+-N from municipal wastewater but also reduces aeration energy for BOD removal and nitrification under similar TN removal rates, further reducing the energy demand for wastewater treatment [11]. Li et al. [12] improved the nitrogen removal efficiency and operational stability of the PN/Anammox process by regulating reasonable influent organic matter concentration to reduce the enrichment risk of AnAOB, and discussed the impact of organic matter on AnAOB metabolism and the competitive relationship between AnAOB and heterotrophic bacteria. Regarding the energy-saving and emission-reduction advantages of the PN/Anammox process, Feng et al. [13] had similar research conclusions. They found that the process does not require a carbon source, can reduce aeration demand by about 60%, simultaneously reduce sludge production by about 70%, and lower alkalinity demand to 45%. Furthermore, this nitrogen removal process produces minimal carbon dioxide (CO_2) during Anammox and does not produce nitrous oxide (N_2O), thus it can reduce greenhouse gas (GHG) emissions.

The PD/Anammox process is a novel nitrogen removal technology that utilizes denitrification-generated NO_2^--N for Anammox reaction, thereby simultaneously removing NO_3^--N and NH_4^+-N. Du et al. [14] proposed from an engineering practice perspective that the partial denitrification process (terminating NO_3^--N reduction at the NO_2^--N stage) has advantages such as stable NO_2^--N production, alleviated organic matter inhibition, and greenhouse gas emission reduction. This finding provides a feasible and effective pathway for the Anammox process. In the Anammox process, NH_4^+-N and the produced NO_2^--N are converted to N_2 under anaerobic conditions. The PD/Anammox process offers a great opportunity for sustainable wastewater treatment because

it can simultaneously remove NO_3^--N and NH_4^+-N, reduce organic carbon demand, improve effluent quality, and since N_2O is not produced during Anammox, the PD/Anammox process can reduce emissions of nitrogen oxides (NO and N_2O). Therefore, PD/Anammox has broad prospects and receives special attention for treating mainstream municipal wastewater, ammonia-rich watersheds, and industrial NO_3^--N wastewater. However, the PD/Anammox process still faces challenges. Du et al. [15] observed severe sludge flotation in an UASB under high NO_2^--N build-up during PD/anammox synergy, suppressing activity; optimising hydrodynamics by tuning the recycle ratio, however, restored N-removal efficiency.

The combination of novel processes provides more possibilities for achieving microbial advanced nitrogen removal. Qiu et al. [16] established a two-stage combined process of PN/Anammox and PD/Anammox to treat landfill leachate, achieving advanced nitrogen removal and providing innovative insights for the practical application and modification of leachate treatment systems in waste treatment plants. Currently, nitrogen removal processes are developing towards high efficiency, energy saving, and environmental protection. Although traditional processes are still widely used, novel processes are gradually being promoted. Future needs include further process optimization and development of new materials to meet the demands of complex wastewater treatment.

Classical nitrogen removal hinges on aerobic nitrification coupled with anaerobic denitrification. Within this system, nitrification is carried out by autotrophic microorganisms under aerobic conditions, while denitrification is performed by heterotrophic microorganisms under anaerobic conditions. Because nitrifiers and denitrifiers differ in oxygen tolerance, the two steps are normally housed in separate tanks to secure efficient nitrogen removal. However, the nitrogen removal efficiency of conventional nitrification-denitrification systems is constrained by the slow proliferation rate of autotrophic nitrifying bacteria and their high sensitivity to environmental variables and operational parameters. In contrast, under the condition of organic carbon presence, heterotrophic nitrification-aerobic denitrifying bacteria (HNADMs) can convert NH_4^+-N to hydroxylamine (NH_2OH), NO_2^--N, or NO_3^--N under aerobic conditions, and then immediately reduce these products to N_2O or N_2 [17]. Therefore, HNADMs have great potential in wastewater nitrogen removal. Single-factor tests assessed carbon source, C/N, temperature, pH, shaking speed and nitrogen source on the isolate's nitrogen-removal performance, while molecular tools elucidated the underlying pathway. Adaptive concentration gradient acclimation cultivation was implemented on the target strain, ultimately enabling the strain to grow in undiluted SPW. Through experiments designed with response surface methodology, the impact of pH, temperature, and agitation rate on the NH_4^+-N removal efficiency of the strain was elucidated, and the optimal process conditions were identified.

2 Experimental Process

2.1 Single-Factor Controlled Variable Condition Optimization。

Previous research found that high concentrations of SPW might inhibit the growth of strain CW-1. Therefore, the solution obtained by mixing centrifuged and filtered SPW with ultrapure water at a ratio of 3:7 was used as the experimental wastewater. The isolated strain CW-1 was used to treat SPW. Using the single-factor controlled variable method, the effects of five factors—carbon source type, carbon-to-nitrogen ratio (C/N), temperature, pH, and shaking speed—on strain growth and nitrogen removal efficiency were investigated. Specific experimental factor levels are shown in Table 2. Compared to SPW1, SPW2 has higher COD and TN levels but lower pH (Table 1). Using SPW2 as the experimental substrate, nitrogen removal tests were carried out under optimized parameters to evaluate the strain's efficacy in treating SPW across various phases. A diluted and sterilized SPW sample (3.3-fold dilution) was inoculated with the bacterial suspension and incubated under optimal conditions for 48 hours. Samples were collected at 12-hour intervals to measure optical density at 600 nm (OD_{600}) and the concentrations of various inorganic nitrogen species. Furthermore, the concentrations of total nitrogen (TN), dissolved total nitrogen (DTN), dissolved total phosphorus (DTP), and chemical oxygen demand (COD) were analyzed at both the start and conclusion of the experiment. All assays were conducted in triplicate, with results reported as mean (Avg.) ± standard deviation (SD).

Table 1 Levels of the factors in the condition optimization experiment

	Factors			
Carbon source	Glucose	Sucrose	Sodium Acetate	Sodium Succinate
C/N	0	1	5	10
Temp/ºC	15	20	25	30
pH	5.26	6.50	7.50	8.50
Shaking /rpm	60	120	180	240

2.2 Whole-Genome Scanning

To study the HN-AD pathway of strain Enterobacter CS-1, whole-genome analysis was performed. By comparing with the COG and KEGG databases, a detailed nitrogen cycle pathway map was constructed.

2.3 Nitrogen Removal Performance Detection

NH_4^+-N, NO_3^--N, and NO_2^--N were determined by salicylic acid-spectrophotometry, UV spectrophotometry, and diazotization-spectrophotometry, respectively; TN and TP were determined by alkaline persulfate–spectrophotometry and persulfate–molybdate spectrophotometry, respectively; COD was determined by potassium dichromate digestion-ferrous sulfate titration; pH was measured by electrode method; bacterial count was represented by absorbance at 600 nm (OD_{600}).

3 Results and Discussion

3.1 Environmental Factor Effects。

HNADMs are unique microbes capable of concurrent nitrification and denitrification under aerobic conditions. However, their growth and metabolic efficiency are affected by various factors. It is reported that different HNADMs utilize different carbon sources to varying degrees. Most strains can use easily degradable organic carbon (low molecular weight organic matter) and inorganic carbon as electron donors. Alongside carbon-source identity, its dosage must also be regulated. Insufficient carbon source will limit the denitrification process of the strain, while excessive concentration may lead to overgrowth of the strain and inhibit nitrogen removal efficiency. Therefore, appropriate C/N is key to strain growth. The shaking speed is closely related to the DO concentration in the strain culture medium. High DO concentration may inhibit the activity of denitrifying enzymes of the strain. Although some studies have found that some HNADMs exhibit excellent nitrogen removal performance under low oxygen conditions, aeration is still needed in most nitrogen removal processes to regulate DO to ensure normal growth and nitrogen removal of the strain. Research shows that most HNADMs are mesophilic (25-35°C) because too low temperature will reduce the enzyme activity in the strain, and too high temperature may cause protein denaturation. Therefore, selecting an appropriate growth temperature for the strain is also crucial. HNADMs may increase pH during denitrification, so a buffer system in the culture medium is needed to maintain stability. Meanwhile, extreme pH environments are unfavorable for strain growth as they affect cell membrane function. Usually, the initial pH is adjusted to neutral or weakly alkaline (pH about 7.0-8.5) to start the experiment. In addition to the above-mentioned influencing factors, medium salinity and osmotic pressure, trace element content, heavy metals, and organic matter toxicity also have certain effects on the growth and nitrogen removal efficiency of HNADMs.

Glucose, sucrose, sodium acetate, and sodium succinate were used as the sole carbon sources for strain growth to prepare heterotrophic nitrification liquid medium, with NH_4^+-N content of 100 mg L^{-1}, C/N=5, and other substances in the medium unchanged. With the exception of the medium containing glucose as a carbon source, which was sterilized at 115°C for 15 minutes, all other media underwent sterilization at 121°C for 30 minutes. Each medium with different carbon sources was set up with 3 parallel groups, all cultured in a constant temperature shaker at 25°C, 180 rpm for 48 h, sampled every 12 h, and the OD_{600} and concentrations of different forms of nitrogen were measured.

As electron donors for heterotrophic bacteria, carbon sources can provide energy for strain growth. As shown in Figure 1a, the growth of strain Enterobacter CS-1 and its efficiency in removing NH_4^+-N varied depending on the carbon source type. Compared to organic matters like glucose and sucrose, the strain utilized sodium acetate and sodium succinate better. When sodium acetate and sodium succinate were used as carbon sources, the maximum OD_{600} values were 0.837 and 1.002, respectively, and the NH_4^+-N removal rates reached 56% and 72%. When glucose and sucrose were used as carbon sources for 48 h, the NH_4^+-N removal rates were both less than 40%, indicating that strain Enterobacter CS-1 more easily utilizes inorganic carbon as carbon source for nitrogen removal. Sodium succinate was therefore chosen as the sole carbon source for *Enterobacter* CS-1 cultivation.

Medium pH is a key determinant of the strain's denitrification performance. The imbalance between nitrification (producing H^+) and denitrification (consuming H^+) leads to changes in pH in the system. Therefore, pH conditions significantly affect bacterial activity. Growth and NH_4^+-N removal were examined at pH 6.5, 7.5, 8.5 and 9.5; results are plotted in Figure 1c. When the initial pH was 6.50 and 8.50, the strain showed similar growth and nitrogen removal efficiency, with a maximum OD_{600} of 0.797, and the highest NH_4^+-N removal rate reached 67%. Since the system with initial pH of 6.50 had the highest NH_4^+-N removal concentration in simulated wastewater within 48 h, 6.50 was selected as the initial pH for subsequent strain culture experiments.

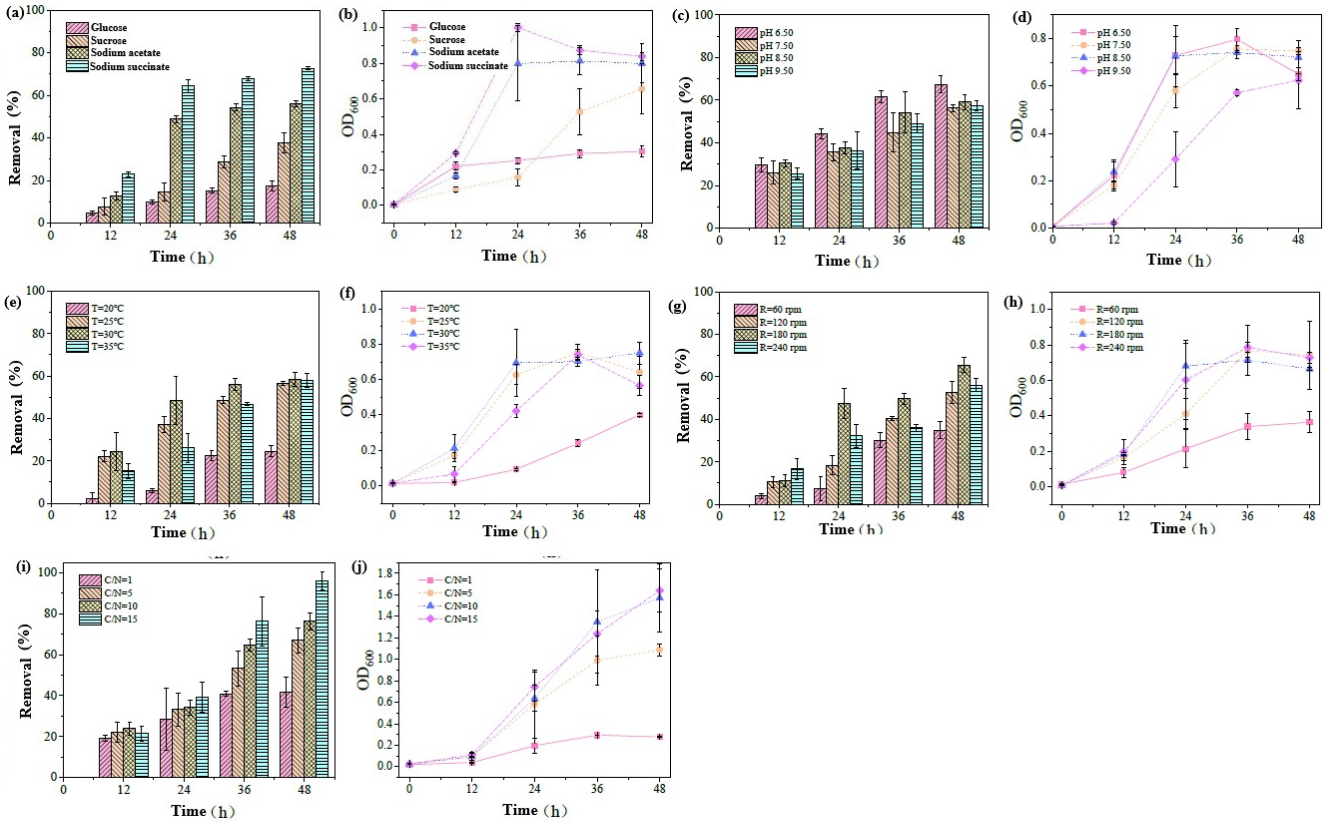

Figure 1 Figure 1 Effects of different carbon sources on strain (a, b); Effects of different pH levels on strain (c, d); Effects of different temperatures on strain (e, f); Effects of different shaking speeds on strain (g, h); Effects of different carbon nitrogen ratios on strain (I, j)

Figure 1e illustrates the influence of temperature on the growth of Enterobacter sp. CS-1 and its corresponding NH_4^+-N removal performance. At 20°C, both microbial growth and nitrogen removal efficiency were constrained. After 48 h of culture, OD_{600} was 0.401, and the NH_4^+-N removal rate was only 24%. Under conditions of 25-35°C, the final NH_4^+-N removal rates of the strain were relatively close, with the maximum removal rate about 58%. The strain attained its peak nitrogen removal efficiency when incubated at 30°C, with an OD_{600} value reaching 0.750. This optical density exceeded the maximum levels recorded at both 25°C and 35°C. Consequently, 30°C was designated as the baseline incubation temperature for all subsequent experimental procedures.

Varying shaker speed alters dissolved-oxygen (DO) levels, thereby modulating both growth and NH_4^+-N removal. This experiment examined the growth behavior of the strain and its NH_4^+-N removal efficiency across agitation speeds of 60, 120, 180, and 240 rpm (Figure 1g). Raising shaker speed from 60 to 180 rpm lifted NH_4^+-N removal from 35 % to 66 % while OD_{600} peaked at 0.665. At 240 rpm biomass rose marginally, yet NH_4^+-N removal dropped to 56 %—below the optimum recorded at 180 rpm. Hence 180 rpm delivers peak nitrogen-removal performance and was set as the standard shaker speed hereafter.

C/N significantly affects the nitrogen removal performance of HNADMs. Low C/N cannot provide sufficient energy and electron donors for cell growth and denitrification, but high C/N may lead to secondary pollution due to residual organic matter. This experiment studied the growth status and nitrogen removal performance of strain Enterobacter CS-1 under four carbon-to-nitrogen ratios (1:1, 5:1, 10:1, 15:1). The results are presented in Figure 1i. Both the optical density (OD_{600}) of the strain and its NH_4^+-N removal efficiency increased with a rising carbon-to‑nitrogen (C/N) ratio. Maximum growth and 96.2 % NH_4^+-N removal coincided at C/N 15:1. This indicates that the strain's aerobic denitrification performance is most effective at C/N=15:1. Consequently, a C/N ratio of 15 was selected as the standard condition for all subsequent experiments.

In summary, the optimal conditions for NH_4^+-N removal by strain Enterobacter CS-1 are: using sodium succinate as carbon source, C/N=15, temperature 30°C, pH 6.50, oscillation speed 180 rpm. In subsequent experiments, these conditions were used for culturing strain Enterobacter CS-1.

The NH_4^+-N removal pathway of strain CS-1 was elucidated by whole-genome sequencing.. The assembled genome size was 4.24 Mb, containing 4516 coding sequences (CDS), with a GC content of 56.1%. KAAS annotation (Fig. 2a) identified genes encoding nitrite reductases (EC 1.7.1.5/1.7.1.4), nitrate reductases (EC 1.7.5.1/1.7.99.-) and glutamate dehydrogenases (EC 1.4.1.3/1.4.1.4) involved in nitrogen assimilation, dissimilatory reduction, denitrification and ammonium assimilation or organic-N mineralisation. After 16S rDNA sequencing identification, the HN-AD functional strain CS-1 isolated from cyanobacterial epiphytic aggregates was identified as belonging to the genus Enterobacter. Its removal efficiencies for NH_4^+-N and NO_3^--N reached 99.6% and 86.2%, respectively. Currently, researchers have isolated and purified multiple HNADMs from the genus Enterobacter. For example, Wang et al. [18] isolated an Enterobacter strain FL from activated sludge with good self-aggregation ability and aerobic denitrification characteristics. Under batch culture conditions, strain FL achieved removal rates of 94.6% for NO_3^--N, 63.9% for TN, and 72.5% for TOC within 24 h. After multiple operation cycles in a sequencing batch reactor, the NO_3^--N removal rate by strain FL was 90.2-99.7%. Wan et al. [19] screened a strain Enterobacter cloquillum sp. HW-15 from phosphorus-rich wastewater. The strain's consumption order for nitrogen sources was NH_4^+-N > NO_3^--N > NO_2^--N. Results of treating actual wastewater with strain HW-15 showed removal rates of 99% for NH_4^+-N, 88% for NO_3^--N, 59% for NO_2^--N, and 73% for PO_4^{3-}-P. Relative to reported *Enterobacter* isolates, CS-1 delivers similar NH_4^+-N and NO_3^--N removal efficiencies.

During the Heterotrophic Nitrification-Aerobic Denitrification (HN-AD) process, inorganic nitrogen is ultimately transformed into organic nitrogen and gaseous nitrogenous compounds through the assimilatory and dissimilatory metabolic activities of the involved microorganisms. This process involves the transformation of multiple nitrogen forms, including NH_4^+-N, NH_2OH, NO_2^--N, NO_3^--N, NO, N_2O, and N_2. Although the HN-AD process metabolic network is complex, most HNADMs follow the typical nitrogen removal pathway [20], i.e., NH_4^+-N is oxidized stepwise to NO_2^--N and NO_3^--N via the NH_2OH intermediate, then NO_3^--N is reduced stepwise through dissimilatory reduction reactions via intermediates such as NO_2^--N, NO, and N_2O, ultimately converted to N_2 (Figure 2b). Different from this pathway, strain CS-1 exhibits unique nitrogen metabolism characteristics. Its nitrogen removal mainly relies on the assimilation pathway, where NH_4^+-N is directly assimilated into glutamate via the GDH or GS-GOGAT pathway, while NO_3^--N is first dissimilated and reduced stepwise to generate NO_2^--N and NH_4^+-N, which is then assimilated and utilized (Figure 2c). In the traditional aerobic denitrification pathway, NO_3^--N is reduced to NO_2^--N under aerobic conditions by the periplasmic-located Nap enzyme (Figure 3-11 a).

The oxygen-insensitivity of this enzyme makes it a key marker enzyme for HN-AD function [21]. In this study, when strain CS-1 treated simulated wastewater with 200 mg L^{-1} NO_3^--N, the NO_2^--N accumulation reached 14.2 mg L^{-1} after 48 h, confirming its aerobic NO_3^--N reduction capability. However, genomic analysis showed that strain CS-1 only carries nitrate reductase-related genes NarGHI and NasBDE, and no Nap gene was detected.

Similar phenomena have been reported in studies of multiple HNADMs, such as Klebsiella sp. KSND, Halomonas venusta SND-01, and Acinetobacter oleivorans AHP123. These HNADMs all lack Nap genes but can achieve NO_3^--N reduction under aerobic conditions. Malm et al. [22] found that the narGHJI enzyme in Mycobacterium tuberculosis can mediate NO_3^--N assimilation under aerobic conditions. This mechanism may explain the NO_2^--N accumulation phenomenon when strain CS-1 uses NO_3^--N as the sole nitrogen source. Nor and Nos convert NO → N_2O and N_2O → N_2, respectively, allowing nitrogen to escape as gas (Fig. 1a). Strain CS-1 theoretically can only remove nitrogen through assimilation due to the lack of related coding genes. In the heterotrophic nitrification process, ammonia monooxygenase gene (amoA) and hydroxylamine oxidase gene (hao) usually mediate the key transformation of NH_4^+-N → NH_2OH → NO_2^--N. KEGG annotation results showed that strain CS-1 lacks these two types of genes, and its nitrogen removal pathway mainly assimilates NH_4^+-N into glutamate via the GDH or GS-GOGAT process. However, in simulated wastewater with NH_4^+-N as the sole nitrogen source, significant accumulation of NO_3^--N and NO_2^--N content was observed, indicating the existence of atypical nitrification during the culture process. According to relevant reports, the heterotrophic nitrification strain Pseudomonas aeruginosa SNDPR-01 also exhibited similar phenomena. Although it lacks the nitrite oxidoreductase gene (Nxr), NO_3^--N generation was detected when NO_2^--N was used as the sole nitrogen source for strain growth [23]. The above results suggest that strain CS-1 may have nitrification-related functional genes not annotated in the KEGG database, or the strain may indirectly drive the nitrification process through non-enzymatic reactions or alternative metabolic pathways. Subsequent experiments combining proteomics, enzyme activity determination, etc., are needed to further analyze its nitrogen removal mechanism.

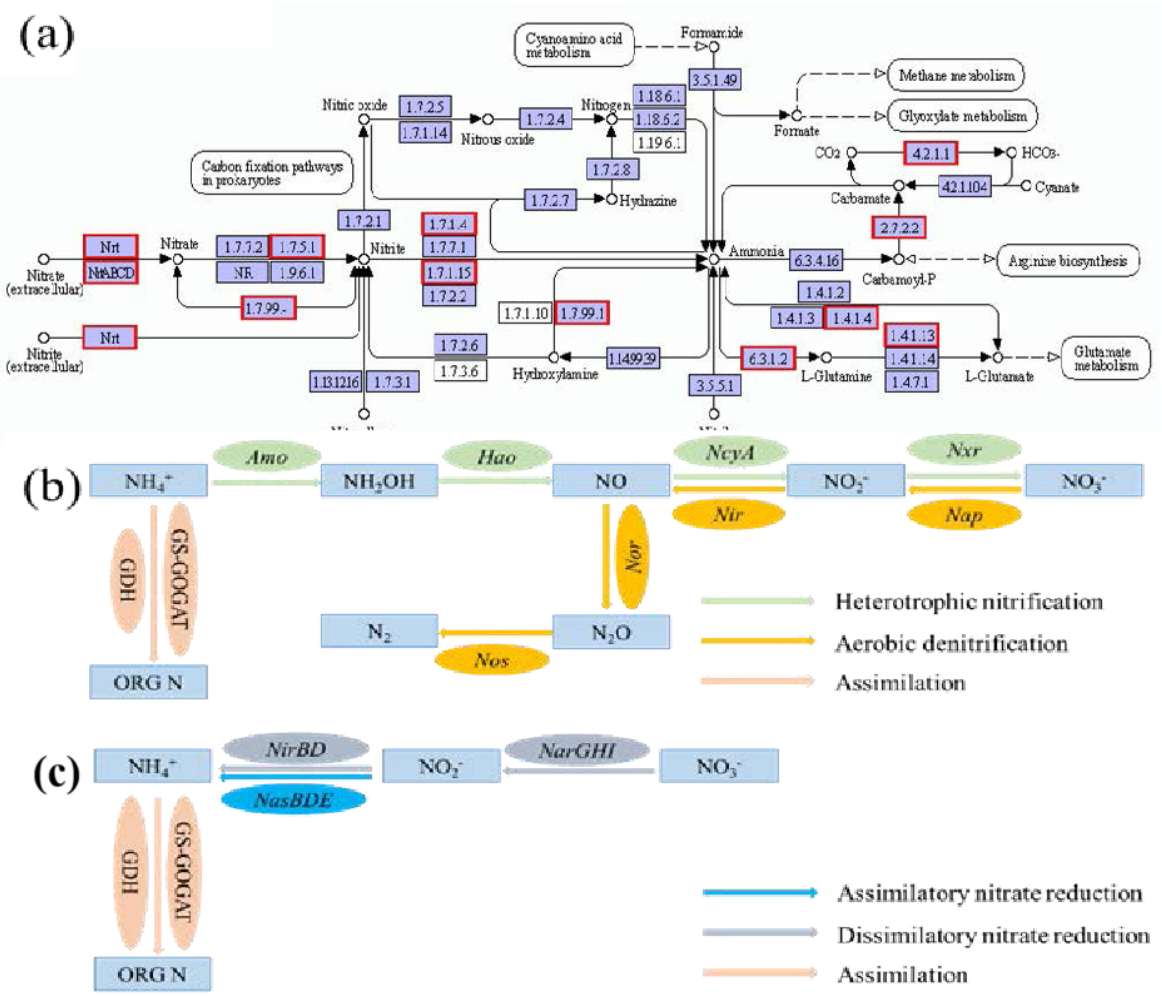

Figure 2 KEGG prediction of nitrogen metabolism pathways in strain CS-1 (genes present in the genome are highlighted with red boxes); Traditional HN-AD pathway (b) and nitrogen removal pathway of strain CS-1 (c)

3.2 Optimization Results

Based on single-factor condition optimization, a Box-Behnken design was used to conduct experiments with three factors and three levels to investigate the effects of temperature (A), pH (B), and shaking speed (C) on the NH_4^+-N removal efficiency (Y) in SPW.

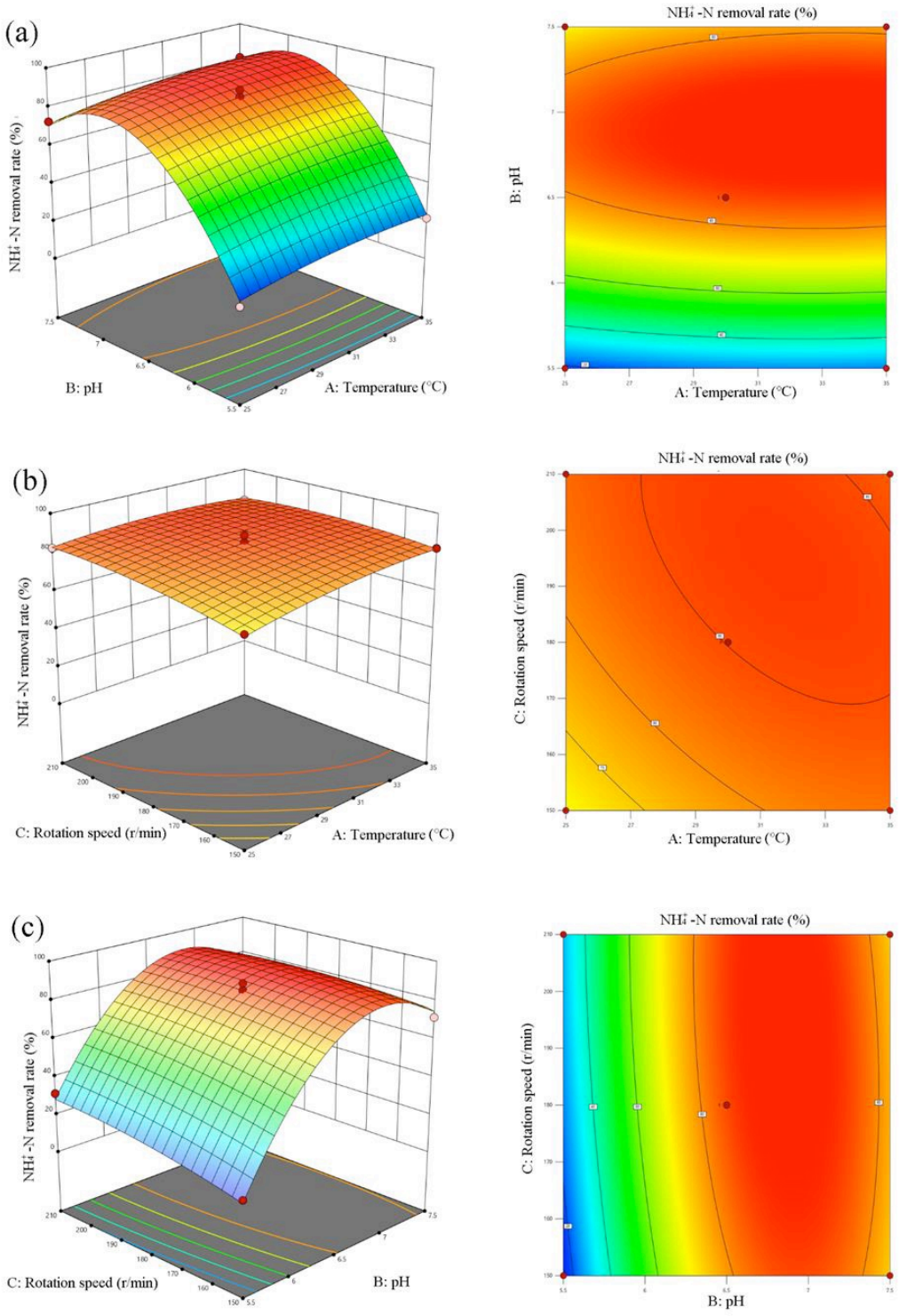

Figure 3 Response surface analysis

Multiple regression analysis yielded the following quadratic regression model equation: Y = -1997 + 9.85A + 493.23B + 2.49C + 0.112AB - 0.018AC - 0.117BC - 0.11A^2 - 34.52B^2 - 0.00295C^2. Significance test results showed that the regression model $p < 0.0001$, coefficient of determination $R^2 = 0.9935$, indicating good fit between the model and experimental data. The influence of the three factors on the response value was in the order: initial pH > shaking speed > temperature. Among them, pH had a very significant effect on the response value ($p < 0.01$), while shaking speed and temperature had significant effects ($p < 0.05$). However, among the quadratic terms or interaction terms of different factors, only pH^2 had a significant effect on nitrogen removal efficiency ($p < 0.01$). To intuitively display the comprehensive effects of each factor on NH_4^+-N removal, response surface plots of the interactions between the three factors were drawn (Figure 3).

3.3 Discussion on Strain Nitrogen Removal Efficiency

Based on the model prediction, the optimal nitrogen removal conditions for strain CS-1 were determined to be 32.1°C, pH 6.85, and an agitation speed of 189.0 rpm, achieving a predicted NH_4^+-N removal efficiency of 90.31%. To validate the model, verification experiments were performed in triplicate under these optimized conditions. The results indicated that the NH_4^+-N concentration decreased from 91.7 mg L^{-1} to 60.9 mg L^{-1} within the first 24 hours, after which the removal rate accelerated, leaving only 9.1 mg L^{-1} at the end of the cultivation period. The actual removal efficiency was 90.1% (Figure 4a), confirming the reliability of the model. Additionally, about 25.5 mg L^{-1} of NO_3^--N and 172.2 mg L^{-1} of DTN were removed, and no NO_2^--N accumulation occurred. Besides N elements, COD and DTP are also important pollutants in SPW. Based on the COD and DTP content at the beginning and end of the experiment, the removal rates of COD and DTP reached 42.0% and 67.6%, respectively (Figure 4b), indicating that strain CS-1 has strong application potential in actual SPW treatment.

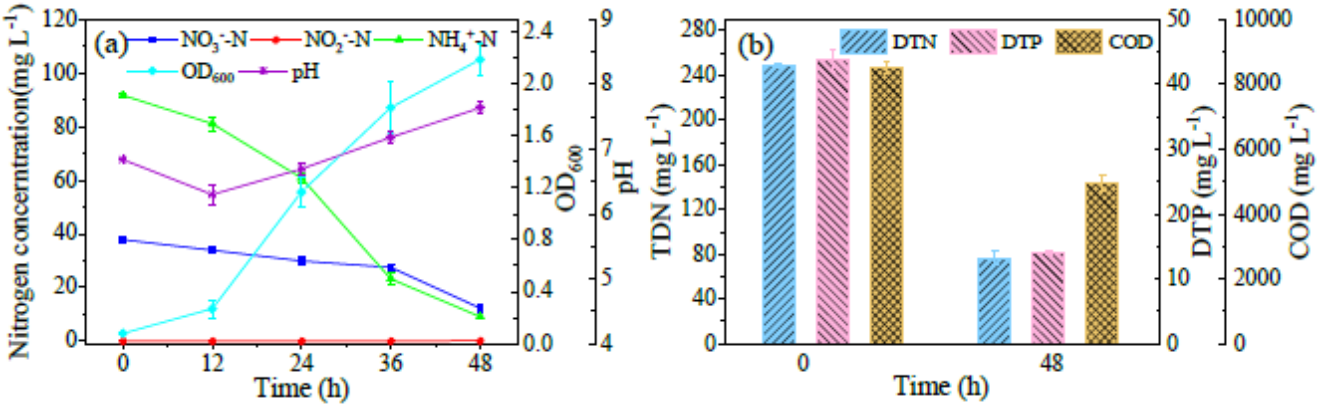

Figure 4 Capacity of strain CS-1 to remove nitrogen from soybean product wastewater

To gauge the full HN-AD capacity of CS-1, a nitrogen balance was compiled under model-optimised settings (Table 1). By comparing the initial and final values of TN and intracellular nitrogen, it was found that about 47.5% of TN was converted into intracellular nitrogen, and 21.7% of TN was removed through the HN-AD pathway. Notably, besides inorganic nitrogen, about 53.4% (64.1 mg L^{-1}) of DTN was removed, which may be why the proportion of nitrogen removed in gaseous form is relatively low.

Table 2 The results of the nitrogen balance analysis

	TN/mg	NO^{3-}-N/mg	NH^{4+}-N/mg	NO^{2-}-N/mg	*N/mg	Loss of N /mg
Before	49.77 ± 0.38	7.56 ± 0.28	18.35 ± 0.15	0.00 ± 0.00	0	-
After	38.98 ± 1.21	2.45 ± 0.32	1.82 ± 0.09	0.02 ± 0.00	23.66 ± 0.13	10.79

Under the above-obtained optimized conditions, when strain CS-1 treated undiluted SPW, after 48 h of reaction, the TN removal reached 53.9 mg L^{-1}. This phenomenon may be related to ammonia volatilization induced by

oscillation conditions. However, under the same experimental conditions, the TN reduction in the control group without inoculation of strain CS-1 was only 18.8 mg L^{-1}. Therefore, strain CS-1 may have uncharacterized nitrogen metabolic pathways. Similar contradictory phenomena occurred in Gordonia amicalis UFV4. This strain does not carry any key enzyme coding genes involved in anaerobic denitrification metabolic pathways, but the proportion of gaseous nitrogen in TN removal in nitrogen balance calculations was as high as 52.37%. Additionally, Richardson and Watmough found that during the dissimilatory reduction of NO_2^--N to NH_3, NO and N_2O may escape as intermediates under oscillation conditions, forming an invisible gaseous nitrogen removal pathway, which may be the main reason for TN reduction when strain CS-1 treats SPW.

HN-AD isolates handle diverse nitrogen sources: they assimilate inorganic-N and catabolise organic-N. Using tryptone, He et al. showed strain Y-11 channelled organic-N into biomass and inorganic-N, yet produced negligible gaseous products. Under optimized conditions, about 64.1 mg L^{-1} of dissolved organic nitrogen in SPW was removed by strain CS-1 (Table 1), confirming the organic nitrogen metabolism advantage of HN-AD strains. Draft genome analysis showed that CS-1 carries the carbamate kinase gene (arcC), whose encoded enzyme catalyzes the reaction "carbamate + ATP → carbamoyl phosphate" to provide precursors for arginine and carbamoyl aspartate synthesis, while achieving recycling of nitrogen sources in metabolites through reversible reactions. Notably, SPW is rich in arginine and aspartic acid, and the arcC-encoded enzyme can specifically degrade such amino acids, which explains the biological basis for the efficient removal of organic nitrogen in SPW by strain CS-1 at the molecular mechanism level.

Although SPW is rich in nutrients such as N and P and has no biotoxicity, its high concentration of soluble proteins and sugars may cause bacterial osmotic pressure changes, inhibiting its growth. Additionally, excessively high COD-to-microorganism ratio can cause energy uncoupling, leading to microbial metabolic imbalance. Previous research confirmed that the screened Acinetobacter HN-AD strain CW-1 could only survive in SPW diluted 3.3 times, while microalgae like Chlorella require 10-fold diluted wastewater to grow normally. In traditional aerobic processes treating SPW, the high COD of the raw wastewater significantly inhibits the activity of autotrophic nitrifying bacteria, thus requiring substantial dilution of the influent. This step greatly reduces the overall treatment efficiency. However, through the gradient acclimation method (gradually increasing from 0% to 100% to the raw water load), strain CS-1 successfully achieved a specific growth rate of 0.034 h^{-1} in undiluted wastewater, greatly improving wastewater treatment efficiency and providing an effective approach for treating high COD, high N content organic wastewater.

4 Conclusion

Under optimized conditions (C/N=15, 30°C, pH 6.50, 180 rpm), Enterobacter CS-1 achieved a NH_4^+-N removal rate of 96.2% in simulated wastewater, with a maximum removal rate of 2.95 mg L^{-1} h^{-1}. Genomic analysis revealed that the strain can assimilate NH_4^+-N via the GDH/GS-GOGAT pathway and utilize NO_3^--N by dissimilatory reduction to NH_4^+-N. Under the optimal process parameters determined by response surface optimization experiments (pH 6.85, 32.1°C, 189 rpm, without additional carbon source), Enterobacter CS-1 achieved simultaneous removal of 90.1% NH_4^+-N, 42.0% COD, 53.4% DTN, and 67.6% DTP from undiluted SPW within 48 h.

Due to limitations such as time shortage and experimental environment, some research work has not been deeply and systematically carried out. Many issues need further research: 1. Whole-genome sequencing cannot fully explain the abnormal nitrogen removal phenomena of strains CW-1 and CS-1 under different nitrogen sources. Techniques such as enzyme activity analysis, quantitative PCR, and transcriptome analysis are needed for deeper research; 2. The current experiments applying strains Acinetobacter CW-1 and Enterobacter CS-1 for nitrogen removal in SPW were conducted in the laboratory. If they are to be applied in engineering applications for actual SPW, pilot-scale experiments are necessary later; 3. Experimental results show that the two strains have their own characteristics. Strain CS-1 has higher TP removal rate in SPW, while strain CW-1 has advantages in nitrogen removal and COD degradation. This study only investigated the treatment effects of single strains on SPW. Whether co-culture of the two strains can achieve synergistic effects needs to be verified through subsequent co-culture experiments.

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