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Study on Enhancement of Denitrification Performance of *Alcaligenes faecalis*

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Abstract: Nitrogen pollution in water bodies presents a serious threat to ecosystems due to its role in eutrophication. In this study, the aerobic denitrifying bacterium *Alcaligenes faecalis* was used as a model microorganism to investigate the optimal operating conditions for nitrogen removal from nitrogen-containing wastewater by *Alcaligenes faecalis* under different aeration modes, microbial dosages and C/N ratios. The results showed that the optimal aeration mode for efficient bacterial denitrification was 10 min of aeration with a 30 min interval, and the total nitrogen removal reached 87.82%. At different bacterial doses, NO_3^-–N was completely denitrified and NO_2^-–N accumulation levels were reduced, all of which resulted in significant denitrification, and the final total nitrogen removal efficiencies reached 86.39–98.50%. With an increase in the C/N ratio, the pollutant removal performance of denitrifying bacteria increased. When the C/N ratio was 17, the final rates of NO_3^-–N, TN and COD removal were 100%, 98.50% and 96.13%, respectively. At lower C/N ratios, the growth and metabolism of microorganisms were inhibited and fewer electron acceptors were available during the denitrification process, which seriously affected denitrification performance. In this study, the denitrification performance of aerobic denitrifying bacterium *Alcaligenes faecalis* was explored in experiments using changes in aeration mode, microbial dosage and C/N ratio, and the optimal operating conditions of *Alcaligenes faecalis* for treating nitrogenous wastewater were indicated. This provides technical support for *Alcaligenes faecalis* in improving the remediation effect of nitrogenous wastewater and provides a theoretical basis for further in-depth research on the performance of *Alcaligenes faecalis* in the future.

Keywords: aerobic denitrification; heterotrophic nitrifiers; nitrate reduction; nitrogen removal; nitrogen-containing wastewater

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1. Introduction

Due to the effects of climate change and human activities, excessive concentrations of nutrients required by organisms, such as nitrogen and phosphorus, have entered water bodies such as rivers and lakes [1]. The excess loading of these nutrients has had serious impacts on aquatic ecosystems, causing the death of aquatic organisms such as fish and plankton, while also intensifying algal reproduction in water bodies, leading to frequent eutrophication events, which not only pose a serious threat to the affected ecosystem but may also have an impact on the global environment [2–4]. Therefore, determining how to effectively remove nitrogen pollution from water bodies has become an urgent research focus.

To solve the problem of excessive nitrogen loading in aquatic ecosystems and avoid the onset of eutrophication, it is necessary to control the exogenous input of pollutants and remediate the pollutants already present within water bodies (endogenous pollution).

At present, treatment methods used for nitrogen-containing wastewater mainly include physical, chemical and bioremediation methods. Bioremediation refers to the addition of functional microorganisms to eutrophic water bodies in order to purify water by absorbing and degrading pollutants. Due to the relatively low cost of bioremediation systems and their inherently low environmental risk, with no secondary pollution concerns, this approach has received widespread attention for nitrogen-containing wastewater treatment [5–7]. The biological removal of nitrogen from nitrogen-containing wastewater mainly utilizes nitrifying bacteria to convert ammonia nitrogen (NH_4^+–N) into nitrate nitrogen (NO_3^-–N) under aerobic conditions, allowing complete nitrogen removal to be achieved through denitrification. The functional microorganisms used in the denitrification process are heterotrophic bacteria, and generally, under anoxic or anaerobic conditions, a carbon source is used as the electron acceptor for the reduction of nitrate nitrogen to N_2 or N_2O [8,9]. Therefore, microorganisms play a vital role in remediation performance. When a water body is polluted, the type and number of indigenous microorganisms within the water body are often significantly reduced and studies have shown that the addition of functional microbes is an effective method to increase denitrification in nitrogen-containing water bodies. Through a process of isolation and screening, Zhang et al. [10] obtained culturable aerobic denitrifying bacteria and applied them to nitrogen removal, effectively increasing the rate of total nitrogen (TN) removal from 64.45% to 89.59%. Sun et al. [11] used a highly efficient denitrifying strain of Pseudomonas (*Pseudomonas stutzeri* sp. GF2) to prepare fungal pellets, which under low-C/N conditions could achieve a NO_3^-–N removal rate of 100% after 6 h while also playing a protective role, providing a habitat and nutrients for denitrifying bacteria, improving their carbohydrate, amino acid and nitrogen metabolism capabilities.

Denitrifying bacteria play an important role in the degradation and transformation of organic matter, with the traditional biological denitrification process generally involving anoxic conditions. Therefore, different aerobic and anoxic environments must be constructed throughout the denitrification process to achieve complete deoxygenation, affecting the denitrification performance of systems. In contrast to traditional denitrification theory, aerobic denitrifying bacteria can perform denitrification under aerobic conditions. Aerobic denitrifying bacteria are conducive to the purification of eutrophic water bodies, solving the problem that eutrophic water bodies are prone to insufficient provision of oxygen [12]. Research by Zhang et al. [10] showed that aerobic denitrifying bacteria can exert a better treatment effect on nitrogen-containing pollutants and can overcome the shortcomings of traditional denitrification processes, which require different reactors. Wen [13] studied the aerobic denitrification performance of *Acinetobacter johnsonii* strain WGX-9 in actual reservoir water, showing good denitrification efficiency when the C/N ratio was 5, at pH levels 5–11 and temperatures as low as 8 °C. Sun et al. [14] investigated the heterotrophic nitrification and aerobic denitrification capabilities of a newly isolated heterotrophic nitrifying metal-resistant bacteria, *Cupriavidus* sp. S1, achieving ammonium, nitrate and nitrite removal rates of 99.68%, 98.03% and 99.81%, respectively. Ren et al. [15] isolated a novel salt-tolerant aerobic denitrifying bacterium, *Halomonas Alkaliphile* HRL-9, from a marine recirculating aquaculture system and explored the optimal operating conditions for the bacterium. The results showed that the highest NO_3^-–N and TN removal rates of 98.0% and 77.3% were achieved at a C/N of ratio 10, DO = 7.48 $\pm$ 0.39 mg/L and a temperature of 30 °C. Wei et al. [16] isolated an aerobic denitrifying bacterial strain, *Acinetobacter* strain WZL728, with a population burst function. The optimal operating conditions were pH = 7.1, temperature = 32.0 °C, C/N = ratio 7.4 and shaker speed = 108.0 rpm, and the NO_3^-–N removal rate reached 98.35%. It is worth noting that Acinetobacter strain WZL728 has high population burst functional activity, which can effectively control biofilm contamination when treating wastewater.

In summary, aerobic denitrifying bacteria have been shown to have the capacity for good denitrification performance in the treatment of nitrogen-containing wastewater. This shows that the use of aerobic denitrifying bacteria to purify nitrogenous wastewater has research prospects, and at the same time, we need to focus on the study of the optimal operating conditions of different aerobic denitrifying bacteria in order to improve the purification effect of nitrogenous wastewater in future work. Therefore, this study aimed to investigate the denitrification performance of highly efficient denitrifying bacteria under optimal conditions through simulation experiments of *Alcaligenes faecalis* in nitrogenous wastewater. The results of this experiment can help to promote the innovation and development of wastewater treatment and provide new references and ideas to improve the performance of nitrogenous wastewater treatment.

2. Materials and Methods

2.1. Experimental Materials and Instruments

2.1.1. Preparation of Bacterial Suspensions

For the preparation of bacterial suspensions, freeze-dried *Alcaligenes faecalis* bacteria were maintained at 4 °C in the dark. A pipette was used to transfer 5 mL of sterilized LB culture solution into a glass tube, which was then shaken, sealed with sterile sealing film and cultivated in a biochemical incubator at 28 °C with agitation at 150 r/min. After the cultivation process was repeated twice, the bacteria were inoculated into enriched culture medium at an inoculation dose of 1% and then cultured for 36 h, after which the enriched bacterial suspension was obtained and centrifuged [8]. The bacterial suspension was then diluted to an OD_{600} of approximately 1 and stored in a 4 °C refrigerator until further use.

2.1.2. Culture Medium

The main culture media used in this study included (1) LB culture medium, consisting of peptone 5.0 g/L, beef extract 3.0 g/L and NaCl 5.0 g/L (at pH 7.0) and (2) enriched culture medium, consisting of KNO_3 2g/L, sodium citrate 5 g/L, peptone 5.0 g, beef extract 3.0 g/L and NaCl 5.0 g/L (at pH 7.0–7.2). All reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.1.3. Simulated Nitrogen-Containing Wastewater

An artificially formulated nitrogenous water body with NH_4^+–N, NO_3^-–N and COD as the main pollutants was created for simulation experiments; the main components were NH_4Cl, KNO_3, sodium citrate, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$, and 2 mL/L of trace elements was included. The water quality parameters of the nitrogen-containing wastewater are shown in Table 1.

Table 1. Water quality parameters of nitrogen-containing wastewater.

Item	COD/(mg/L)	ρ/(mg/L)		pH
		NO_3^-–N	TN	
Content	400~1100	65	65	7.0~7.2

2.1.4. Experimental Instruments

The instruments used in this study included a UV spectrophotometer (UV-2600i, Shimadzu, Japan), a microwave digestion system (FUP FG40, Qingdao, China), a digital ultrasonic cleaner (KQ-50DB, Shanghai, China), a digital thermostatic shaking water bath (SHZ-A, Foshan, China), a biochemical incubator (LRH-250CL, Shanghai, China) and a portable pressure steam sterilizer (YX-18LD, Jinan, China).

2.2. Experimental Design

A single-factor analysis approach was adopted in this study, assessing each parameter in isolation, with the specific parameters of each system described in Table 2. (1) Aeration mode: 150 mL of sterilized simulated nitrogen-containing wastewater was added to a 300 mL conical flask with 5 mL of bacterial suspension. Four different flasks were prepared and different aeration modes were applied (A1, A2, A3 and A4). The conical flasks were placed in a digital thermostatic shaking water bath for 36 h to allow the reaction to proceed, with samples collected every 6 h. The supernatant solution was collected at 10,000 r/min and the concentrations of NO_3^-–N, NO_2^-–N, TN and COD were determined. (2) Dosage of bacterial suspension: 150 mL of sterilized simulated nitrogen-containing wastewater was added to a 300 mL conical flask along with different volumes of bacterial suspension. The experiment was carried out under optimal aeration conditions as determined previously. All other experimental conditions and the sample collection procedure were the same as those described for aeration mode above. (3) C/N ratio: 150 mL of sterilized simulated nitrogen-containing wastewater was added to a 300 mL conical flask along with the previously determined optimal bacterial dosage and aeration mode. While the NO_3^-–N concentration remained unchanged, the COD concentration was adjusted to achieve different C/N ratios. All other experimental conditions and the sample collection procedure were the same as those described for aeration mode above.

Table 2. Operating conditions during whole experiment.

No.	Factors	Experimental Condition	NO_3^-–N (mg/L)	COD (mg/L)
1	Aeration method	A1 (Continuous aeration)	65 ± 1	1100 ± 50
		A2 (Aeration: 10 min/intermittent 30 min)	65 ± 1	1100 ± 50
		A3 (Aeration: 10 min/intermittent 60 min)	65 ± 1	1100 ± 50
		A4 (Aeration: 10 min/intermittent 90 min)	65 ± 1	1100 ± 50
2	Dosage of bacterial suspension/mL	1	65 ± 1	1100 ± 50
		5	65 ± 1	1100 ± 50
		15	65 ± 1	1100 ± 50
3	C/N	6	65 ± 1	400 ± 20
		11	65 ± 1	700 ± 20
		14	65 ± 1	900 ± 20
		17	65 ± 1	1100 ± 50

2.3. Analytical Methods

The optical density (OD_{600}) of samples was determined using spectrophotometry. The methods used to measure NO_3^-–N, NO_2^-–N, TN and COD concentrations referred to the fourth edition of "*Water and Wastewater Monitoring and Analysis Methods*" compiled by the former State Environmental Protection Administration.

3. Results and Discussion

3.1. Effects of Different Aeration Modes on Denitrification Performance

At an initial C/N ratio of 17, the performance of *Alcaligenes faecalis* in the removal of NO_3^-–N, NO_2^-–N and TN was assessed under conditions of different aeration modes. It can be seen from Figure 1a that after 6 h of treatment, the denitrification rate accelerated and NO_3^-–N removal rates reached 78.26–87.82% under all four tested aeration modes. Since *Alcaligenes faecalis* is an aerobic denitrifying bacterium, the NO_3^-–N concentration reduced significantly under A1 and A2 aeration modes, achieving final removal rates that stabilized at 85.71% and 87.82%, respectively, which is similar to other reported results. For example, Wang et al. [17] significantly improved the nitrogen removal efficiency by increasing DO concentrations during treatment.

As shown in Figure 1b, under the aeration modes of A3 and A4, no accumulation of NO_2^-–N occurred. However, due to the long interval time and insufficient DO supply, the microorganisms were aerobic bacteria, which was not conducive to the aerobic denitrification of microorganisms. Therefore, the denitrification effect was poor but better than that with continuous aeration.

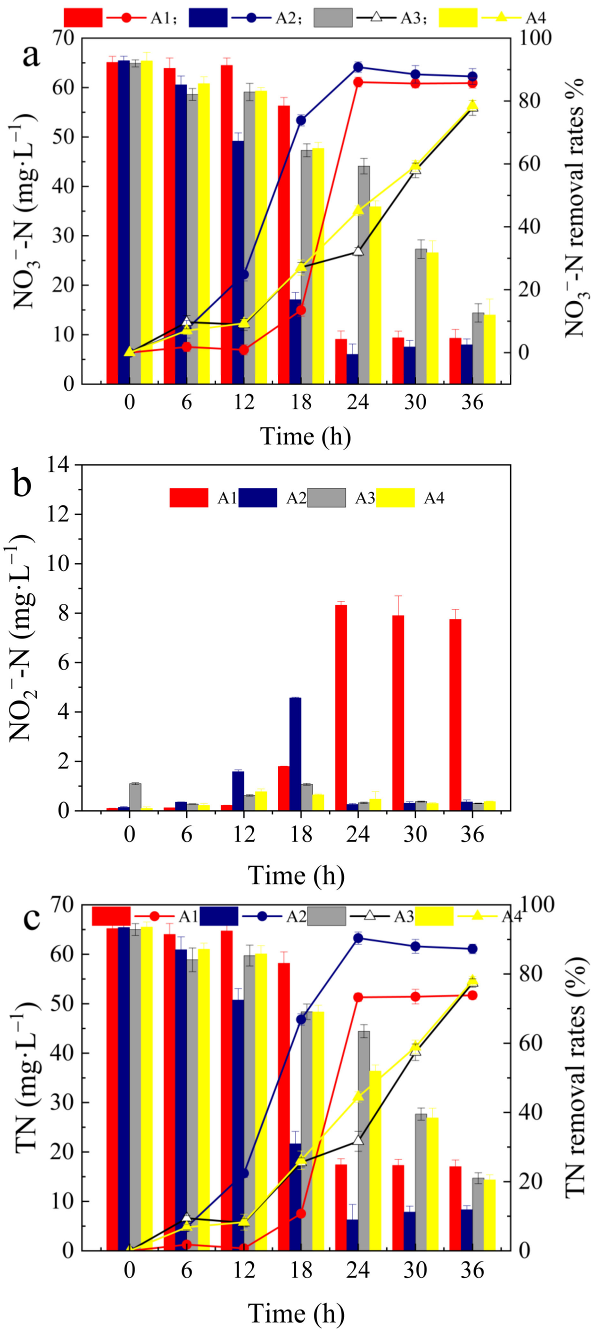

Figure 1. Removal of nitrogen-containing pollutants by *Alcaligenes faecalis* under different aeration conditions: (**a**) NO_3^--N, (**b**) NO_2^--N, (**c**) TN. Values are means ± SD (error bars) of three replicates.

The TN removal effect under the intermittent aeration conditions was consistent with that of NO_3^-–N, as shown in Figure 1c. However, the accumulation of NO_2^-–N caused by continuous aeration resulted in a decrease in the TN removal rate. Studies have shown that intermittent aeration stimulates microorganisms to release tryptophan and simple aromatic proteins, while this situation does not occur under continuous aeration conditions [7,18]. Bian et al. [19] showed that when the initial DO concentration increased from 0.5 mg/L to 1.5 mg/L, the TN removal rate increased by 20% accordingly. However, further increases in DO did not promote the removal of TN, with excessive DO concentrations having no significant effect on denitrification efficiency, although they did promote electron utilization and respiratory proliferation. The A2 aeration mode achieved the best denitrification effect after 36 h, with a final TN removal rate of 87.27%. Therefore, the A2 aeration mode not only provided the aerobic conditions required by microorganisms but also allowed them to alternate between aerobic and anoxic phases during treatment, which is more conducive to a high microbial denitrification performance [20,21].

As can be seen from Figure 1b, under intermittent aeration conditions, a small amount of NO_2^-–N accumulation occurred, with the concentration remaining below 0.5 mg/L throughout treatment. However, under continuous aeration conditions, the NO_2^-–N concentration significantly increased from 18–36 h, reaching an average concentration of 6.44 mg/L. Sun [11] explored the factors affecting nitrogen metabolism and nitrite accumulation of aerobic denitrifying bacteria, showing that high concentrations of DO had little effect on nitrate reduction but had a significant effect on NO_2^-–N reduction. Therefore, the accumulation of NO_2^-–N was observed under continuous aeration conditions.

3.2. Effects of Different Microbial Dosages on Denitrification Performance

Different microbial dosages could affect the survival status and quantity of strains in the system, thereby affecting the denitrification effect. In this research, when the C/N ratio was fixed at 17, the effects of microbial dosage on denitrification performance were examined under optimal aeration mode conditions, as determined previously (A2). The results indicated that all three dosages can have a certain removal effect on NO_3^-–N and TN.

The removal performance of *Alcaligenes faecalis* at different inoculation dosages is shown in Figure 2. As can be seen from Figure 2a, after 6 h of treatment, NO_3^-–N and TN concentrations began to show a downward trend and then reached a stable state after 24 h. As the *Alcaligenes faecalis* dosage increased, the pollutant removal efficiency of the system gradually increased. All three dosages showed good removal of NO_3^-–N with removal rates of 88.33%, 87.82% and 100%, respectively. When the *Alcaligenes faecalis* dosage was 15 mL, the NO_3^-–N removal rate was close to 100% as sufficient bacteria directly participated in denitrification. The TN removal effect of the system under intermittent aeration conditions was similar to that of NO_3^-–N, reaching a final TN removal rate of 98.50%. Similar to our research results, Elkarrach et al. [7] showed that as the concentration of the strain increased, the NO_3^-–N removal rate also gradually increased, and denitrification occurred simultaneously with bacterial growth. The results indicate that the denitrification characteristics of heterotrophic nitrification–aerobic denitrification bacteria are directly proportional to the dosage of microorganisms. Zhang et al. [8] explored the denitrification characteristics of heterotrophic nitrification–aerobic denitrification bacteria, showing that the dosage of functional microbes was directly proportional to the system's denitrification efficiency.

Figure 2b shows that under intermittent aeration conditions, the level of NO_2^-–N accumulation is low, irrespective of the microbial inoculation dose applied. The accumulation of NO_2^-–N at the three dosages was very small, and the final concentrations were 1.28 mg/L, 0.36 mg/L and 0.96 mg/L. Even when the dosage was 5 mL, the accumulation of NO_2^-–N was the highest after 18 h, but it quickly decreased to 0.36 mg/L as the reaction progressed. This confirms that NO_3^-–N is fully available under the conditions of sufficient oxygen and carbon source availability, allowing complete denitrification to be achieved.

Figure 2c shows the changes in TN under different dosages of microorganisms. From the figure, it can be seen that the TN removal effect under the condition of intermittent aeration seems to be consistent with the NO_3^-–N removal effect. This is because there is no significant difference in the accumulation of NO_2^-–N under different microbial dosages. The TN removal rate increased with the increase in microbial dosage, and when the microbial dosage was 15 mL, the TN removal rate reached 98.5%, and the reaction reached a steady state in 24 h.

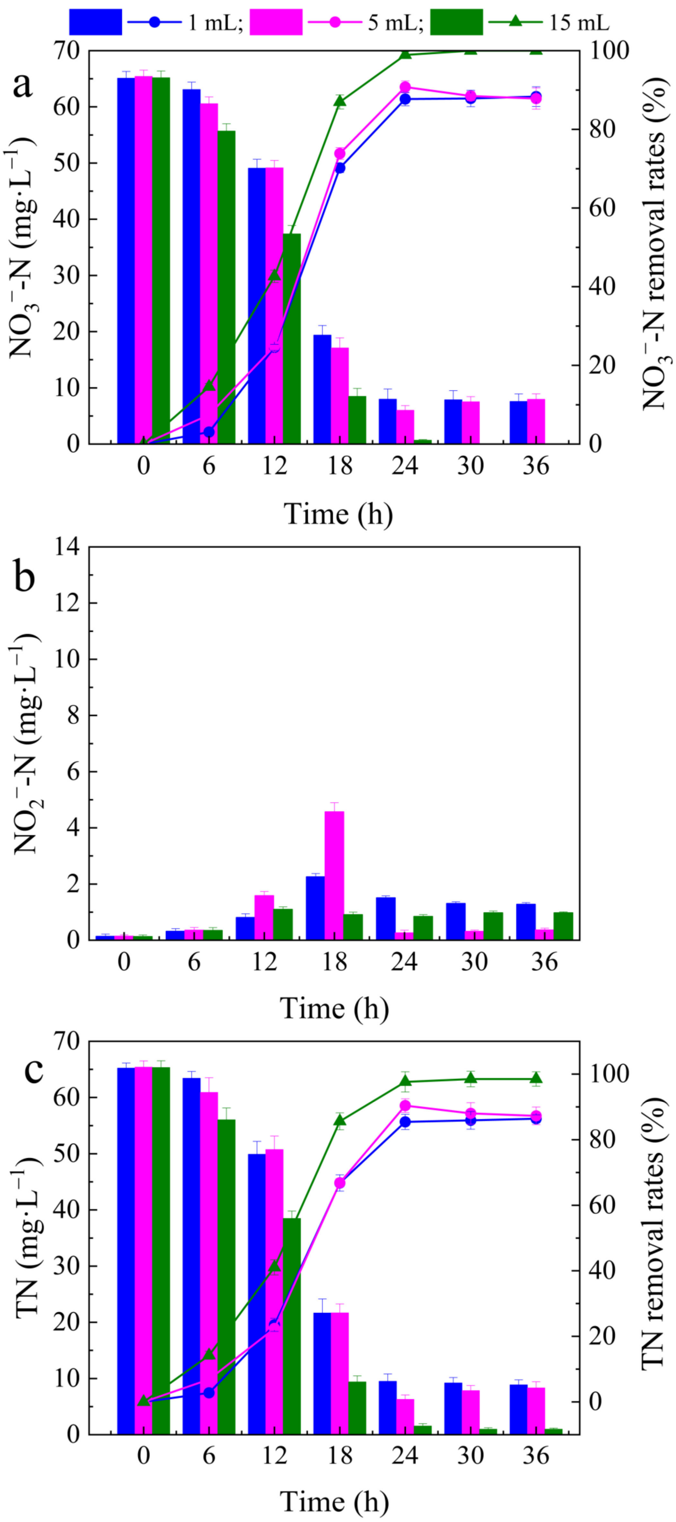

Figure 2. The effect of different *Alcaligenes faecalis* dosages on the removal of nitrogen-containing pollutants: (**a**) NO_3^--N, (**b**) NO_2^--N, (**c**) TN. Values are means ± SD (error bars) of three replicates.

3.3. Effect of Different C/N Ratios on Denitrification Performance

The C/N ratio has a significant impact on the performance of denitrifying microorganisms, as carbon and nitrogen are essential nutrients for microbial growth and metabolism. The performance of *Alcaligenes faecalis* in the removal of NO_3^-–N, NO_2^-–N, TN and COD at a C/N ratio of 4 is shown in Figure 3. As the C/N ratio increased, the NO_3^-–N and

TN removal efficiency also gradually increased (Figure 3a,c). The removal efficiency of NO_3^-–N and TN for the four different C/N ratios was poor within 12 h before the reaction. And then within 12–24 h, the concentrations of NO_3^-–N and TN rapidly decreased and then remained stable. After 24 h, there was a slight upward trend, possibly due to the mutual transformation of NO_3^-–N and NO_2^-–N under sufficient DO.

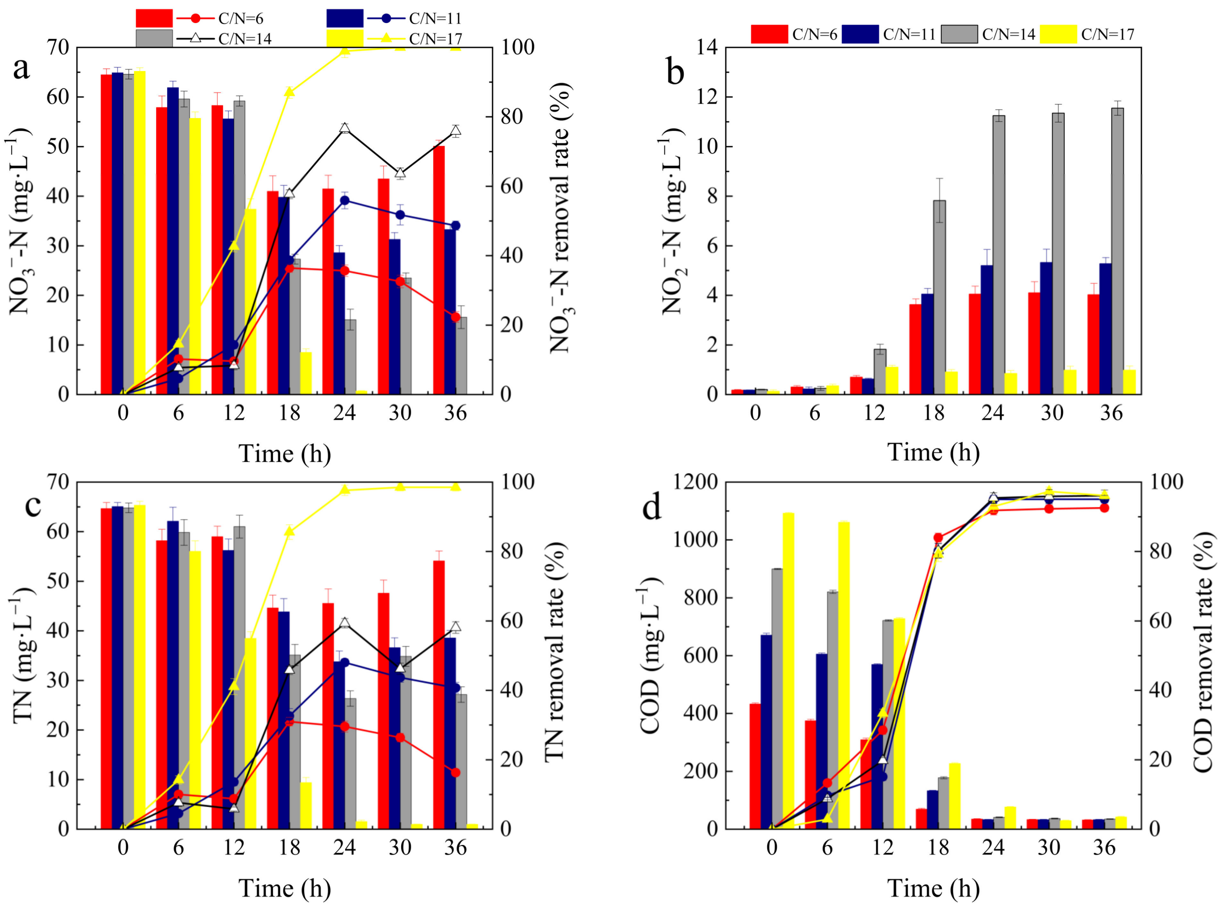

Figure 3. The removal of nitrogen-containing pollutants and COD by *Alcaligenes faecalis* under conditions of different C/N ratios: (**a**) NO_3^--N, (**b**) NO_2^--N, (**c**) TN, (**d**) COD. Values are means ± SD (error bars) of three replicates.

When the C/N ratio was 17, sufficient energy was available for microbial growth and the final NO_3^-–N and TN removal rates reached 100% and 98.50%, respectively. However, when the C/N ratio was low, insufficient carbon source availability caused denitrification to slow down, making it difficult to efficiently remove pollutants from the water. This is supported by a screening study by Chen et al. [8], showing that *Alcaligenes faecalis WT14* not only exhibited high resistance to NH_4^+–N but also had a strong nitrate/nitrite removal capability. However, when the C/N ratio was low and carbon source availability was insufficient, the denitrification rate slowed down, making it difficult to efficiently remove pollutants from wastewater. Allen et al. [22] investigated the removal effect of different C/N ratios on average total dissolved nitrogen (TDN) in the laboratory, and the results showed that at the highest C/N ratio, TDN was almost completely removed. A higher C/N ratio enhanced denitrification and improved TN removal efficiency. Therefore, sufficient carbon source availability is necessary for aerobic denitrification. Excessively low C/N ratios are not only detrimental to the survival of aerobic denitrifying bacteria but also affect the aerobic denitrification performance of the treatment system [23].

Figure 3b shows the NO_2^-–N removal performance of *Alcaligenes faecalis* with a C/N ratio of 4. As the reaction proceeded within the initial 0–24 h, NO_2^-–N gradually accumulated and then increased and decreased to varying degrees before gradually stabilizing. When the C/N ratio was increased to 17, the lowest level of NO_2^-–N accumulation was observed and was similar to the previously discussed experimental results. When a sufficient carbon source and sufficient DO concentrations were provided, NO_2^-–N accumulated to a certain extent in the early stage of treatment. However, as the reaction proceeded, the extent of accumulation gradually decreased, with the concentration remaining at about 1 mg/L. During the entire experimental period, the NO_2^-–N concentration remained high when the C/N ratio was 14, due to the mutual conversion of NO_3^-–N and NO_2^-–N during the denitrification process, resulting in significant NO_2^-–N accumulation. When the C/N ratio was low, carbon source availability became the limiting factor in the denitrification process, resulting in less electron flow and incomplete denitrification, which in turn affected the removal of TN (Figure 3c). Sun et al. [24] used a composite membrane aerated biofilm reactor to treat wastewater with different C/N ratios. The results showed that when the C/N ratio was 6, the TN removal rate in the system was 73.21%. As the C/N ratio decreased, the TN removal rate also significantly decreased.

Figure 3d shows the COD removal performance of *Alcaligenes faecalis* with four different C/N ratios. All four C/N ratios showed good treatment effects on COD, which was reduced to 32.20 mg/L, 33.19 mg/L, 35.20 mg/L and 32.02 mg/L. The removal rate reached more than 92%, which was in line with the emission standard Class I A, achieving a good treatment effect and a similar overall trend. As the reaction proceeded, a large amount of COD was consumed in the early stage of the denitrification process, while after 24 h, the COD content gradually stabilized as the denitrification process slowed down. Therefore, the final COD removal rate reached 92%, with the treatment performance synchronized with the removal of nitrogen-containing pollutants.

3.4. Mechanism of Nitrogen Removal by Aerobic Denitrifying Bacteria

According to the results of this study, among the studied parameters, the C/N ratio has the greatest impact on the denitrification performance of aerobic denitrifying bacteria. Due to *Alcaligenes faecalis* having a high carbon source demand, the competitiveness of NO_3^-–N as the electron acceptor was enhanced as the proportion of electrons transferred to NO_3^-–N increased. Although O_2 has a higher electron affinity than NO_3^-–N, NO_3^-–N was still able to participate in microbial metabolism together with O_2, e.g., aerobic denitrification occurred. When an intermittent aeration mode was applied, the proportion of electrons allocated to O_2 and NO_3^-–N remained relatively stable, which is consistent with the "co-breathing" theory of oxygen and nitrate in the aerobic denitrification principle, as proposed by Robertson et al. [25]. Aerobic denitrifying bacteria can overcome the electron transmission barrier between cytochrome C and cytochrome aa3, achieving better denitrification effects by transferring electrons to both O_2 and NO_3^-–N. However, excessive DO concentrations (continuous aeration) affect the synthesis and activities of microbial denitrification enzymes, reducing the system's overall denitrification efficiency.

4. Conclusions

(1) The optimal aeration mode for denitrification by *Alcaligenes faecalis* is intermittent aeration for 10 min, at 30 min intervals, achieving NO_3^-–N and TN removal rates of 87.82% and 87.18%, respectively;

(2) When the bacterial suspension dosage was 15 mL, NO_3^-–N could be completely removed by denitrification, resulting in low levels of NO_2^-–N accumulation and a good denitrification performance by *Alcaligenes faecalis*, achieving NO_3^-–N and TN removal rates of 100% and 98.50%, respectively;

(3) Under the determined optimal aeration conditions and bacterial suspension dosage, the denitrification performance of *Alcaligenes faecalis* gradually increased in accordance with the C/N ratio, achieving high rates of NO_3^-–N, TN and COD removal of 100%, 98.50% and 96.13%, respectively, at an optimal C/N ratio of 17;

(4) In the future, we will continue to explore the operating conditions of different aerobic denitrifying microorganisms and compare the performance of different aerobic denitrifying microorganisms to find aerobic denitrifying microorganisms suitable for the treatment of nitrogenous wastewater with different physicochemical parameters. Meanwhile, we will continue to explore the combination of aerobic denitrifying microorganisms with other wastewater treatment facilities, such as constructed wetlands, in order to find a highly efficient and green method to purify nitrogenous wastewater.

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