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Simultaneous Nitrification and Denitrification under Aerobic Atmosphere by Newly Isolated *Pseudomona aeruginosa* LS82

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Abstract: Discharge of wastewater contained high amount of nitrogen would cause eutrophication to water bodies. Simultaneous nitrification and denitrification (SND) has been confirmed as an effective process, the isolation of SND bacteria is crucial for its successful operation. In this study, an SND strain was isolated and identified as *Pseudomona aeruginosa* LS82, which exhibited a rapid growth rate (0.385 h^{-1}) and good nitrogen removal performance (4.96 mg $N{\cdot}L^{-1}{\cdot}h^{-1}$). Response surface methodology was applied to optimize the TN removal conditions, at which nearly complete nitrogen (99.8 ± 0.9%) removal were obtained within 18 h at the condition: pH 8.47, 100 rpm and the C/N ratio of 19.7. The saddle-shaped contours confirmed that the interaction of pH and inoculum size would influence the removal of total nitrogen significantly. Kinetic analyses indicated that the reduction of nitrite was the rate-limiting step in the SND process. Our research suggested strain LS82 can serve as a promising candidate for the treatment of ammonium rich wastewater, and expended our understanding the nitrogen removal mechanism in the SND process.

Keywords: simultaneous nitrification and denitrification (SND); new isolation strain; response surface methodology; nitrogen removal; kinetics

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1. Introduction

The conventional nitrogen removal processes, including autotrophic nitrification and heterotrophic denitrification, have both efficient and cost-effective advantages leading to their widespread use around the world. Nevertheless, a large spatial occupation and long retention time are required in a wastewater treatment plant because these two processes have different demands for dissolved oxygen concentration and carbon [1,2]. Recently, many novel, effective nitrogen removal processes have been announced, such as short-cut nitrification and denitrification, simultaneous nitrification and denitrification (SND), and partial nitrification coupled with anammox. SND integrates nitrification and denitrification in the same unit. Within only a single bioreactor, ammonia can be oxidized to nitrite/nitrate and then further reduced to N_2 under relatively high dissolved oxygen (DO) conditions. This can avoid some shortcomings of the traditional process, such as the larger spatial occupation, complicated oxygen operation, and growth limitation of autotrophic nitrifiers [3].

However, the SND process still has some limitations, with one of the most important being the effective culture of SND bacteria.

To date, many SND bacteria have been isolated from different environments, e.g., marine sediments [4], aeration tank [5], swine wastewater [6], and landfill leachate [7]. The nitrogen removal performance of the SND bacteria varied remarkedly under different culturing conditions. Many studies systematically investigated the influencing effects of pH, DO concentration, and carbon source. He et al. [5] found that 10% of inoculum and a pH of 8 were favorable for TN removal by *Pseudomonas aeruginosa* PCN-2. Zhou et al. [8] isolated SND strain *Pseudomonas stutzeri* KTB and found that the optimum initial pH and C/N ratio were 7–8 and 10, respectively. Su et al. [9] also isolated strain *Acinetobacter* H36 and found that the optimized pH and inoculum size were 7.5 and 10%. Chen and Ni [7] investigated the pathway of nitrogen removal and the optimum conditions of *Agrobacterium* sp. LAD9, a strain that is capable of heterotrophic nitrification–aerobic denitrification, reporting that the highest nitrogen removal efficiency occurred at a C/N ratio of 8.28 and pH of 8.46. Moreover, nitrogen could be eliminated through both assimilation and dissimilation by LAD9. Zhang et al. [10] applied orthogonal tests to assess the effect of C/N ratio, pH, and DO on aerobic denitrification by *Bacillus methylotrophicus* strain L7 and found that the optimal conditions were a C/N ratio of 20, pH of 7–8, and DO of 4.82 mg/L.

Generally, the optimization process of bacterial growth is conducted using a one-factor-at-a-time approach. However, bacterial growth is not controlled by a single factor but rather regulated by the comprehensive interactions of all key factors, i.e., C/N ratio, pH, and DO. It is, thus, necessary to screen the significant factors and consider their interactions.

The objectives of this study were to (1) isolate an SND strain and identify it using both the morphological and taxonomic methods, (2) optimize the growth of the strain via response surface methodology, and (3) investigate the pathway of nitrogen conversion and the kinetic characteristics. Response surface methodology can not only help to screen the significant factors but also determine the effects of the square terms. This work is expected to provide information on a new isolated SND bacterium and its dynamic model, which can help in understanding the mechanisms of nitrogen removal from the SND process during wastewater treatment.

2. Materials and Methods

2.1. Mediums

Three culture media were prepared: Czapek (CM), denitrification (DM), and LB. The Czapek medium was prepared as follows (per L): $NaNO_3$ (3 g), KH_2PO_4 (1.5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4$ (0.01 g), and sucrose (30 g). This medium was used to enrich bacteria utilizing nitrate as the only nitrogen source. The denitrification medium (per L) consisted of Na_2HPO_4 (4.2 g), KH_2PO_4 (1.5 g), $MgSO_4{\cdot}7H_2O$ (0.1 g), NH_4Cl (0.3 g), sodium succinate (1.7 g), KNO_3 (1g), and the trace elements (2 mL). It was applied for culture enrichment and isolate purification. The trace elements (per L) contained 50 g of EDTA, 2.2 g of $ZnSO_4$, 5.5 g of $CaCl_2$, 5.06 g of $MnCl_2{\cdot}4H_2O$, 5 g of $FeSO_4{\cdot}7H_2O$, 1.57 g of $CuSO_4{\cdot}5H_2O$, 1.1 g of $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, and 1.61 g of $CoCl_2{\cdot}6H_2O$. The LB medium was purchased from Huankai Microbial (Guangzhou, China), and the solid medium was prepared by adding 1.5–2% of agar. Prior to use, all media were autoclaved for 20 min at 121 °C.

2.2. Isolation and Identification of the Strain LS82

The isolates were screened from landfill leachate in Guangzhou, China. Firstly, an Erlenmeyer flask (250 mL) containing the sludge from landfill leachate (100 mL) and CM (100 mL) were incubated at 150 rpm and 30 °C. When the total nitrogen removal from the flask was over 90%, 15 mL of the bacterial suspension was transferred to another 135 mL fresh of CM, and the incubation continued. This procedure was repeated for more than 2 months. Then, 1 mL of bacterial suspension was transferred to a 250 mL flask with 100 mL of fresh DM for selective cultivation of bacterial cultures. This procedure was also

repeated several times. Next, the enriched bacterial culture was gradient-diluted, and the suspensions were spread on a DM plate using the dilution separation method. The DM plate was incubated at 30 °C for 2 days, and then single colonies formed. The colonies with different morphological features were collected and purified by the lineation method, and their nitrogen removal capacity was tested.

The DNA extraction and purification of the isolates were conducted using a Bacterial DNA Kit (Omega) referring to the manufacturer's instructions. The 16S rRNA sequence of each colony was analyzed by polymerase chain reaction (PCR) amplification with the universal primers 27f (AGAGTTTTGATCCTGGCTCAG) and 1492r (TACGGTTACCTTGTTACGACTT). The detailed PCR amplification process was described in our previous report [11]. The sequences of the isolates were detected by Biotech Co., Ltd. (Guangzhou, China) and matched with known 16S rRNA sequences using the NCBI BLAST program; then, the phylogenetic tree was drawn using the MEGA 5.0 program.

2.3. Performance in Terms of Growth and Nitrogen Removal

The isolated strain was precultured for 24 h in LB medium to the exponential growth phase and then harvested by centrifuging, before being resuspended in 2 mL of sterile water. The cell resuspension solution was inoculated in a 250 mL flask (inoculation size (v/v) of 10%) with 150 mL of DM (with ammonium as the sole nitrogen source). The solution was shaken at 150 rpm and 30 °C. The samples were withdrawn at specific time intervals to detect the concentrations of ammonium, nitrate, and nitrite. The total nitrogen (TN: the summation of ammonia, nitrite, and nitrate) removal efficiency was calculated using Equation (1).

$$Rv = (C_0/TN - C_1/TN)/C_0/TN \times 100\%, \quad (1)$$

where Rv, C_0/TN, and C_1/TN represent the TN removal efficiency and the initial and final concentrations of TN, respectively.

In addition, a completely randomized design (one-factor) was conducted to determine the influence of various parameters. The three common factors were pH (3, 4, 5, 10, and 11), C/N ratio (3, 4, 6, 20, and 25), and shaking speed (25, 50, 150, and 200 rpm), and their effects on nitrogen removal were investigated. The total nitrogen removal efficiency after 24 h of incubation was regarded as the criterion for evaluation.

2.4. Analytical Methods

The concentrations of ammonium, nitrite, nitrate, and TN, as well as the optical density (OD_{600}) of bacterial cells, were measured by a spectrophotometer (DR5000, HACH, Ralph land, CO, USA). The samples were taken at a specific time and measured after centrifugation at 8000 rpm for 5 min. Ammonium, nitrite, nitrate, and TN were measured on the basis of standard methods (APHA, 2012). Bio-N was estimated by establishing the correlativity of Bio-N and OD_{600} according to the general formula of bacteria ($C_5H_7NO_7$) and the standard curve of the dry cell weight (DCW) and OD_{600} (DCW = 40.84 OD_{600}). The gas-N level was calculated by the difference in the sum of TN and Bio-N at initial and "t" moments. The value of pH was measured using a pH-Meter (HACH, Ralph land, CO, USA).

2.5. Response Surface Methodology

As an efficient tool to identify the important factors among numerous variables, a Box–Behnken design (BBD) was utilized to select the main factors significantly impacting the TN removal efficiency of the isolated strain LS82, as well as optimize the performance of nitrogen removal of strain LS82.

According to the preliminary experiment, C/N ratio, initial pH, initial inoculation of the strain, and shaking speed had obvious influences on the nitrogen removal of LS82. Thus, with C/N ratio (A), shaking speed (B), inoculation level (C), and pH (D) as the factors, and total nitrogen removal after inoculation for 24 h as the response, a four-factor three-level BBD was performed. Each factor was set at three levels: −1, 0, and 1, indicating low, medium, and high levels, respectively. The specific values of each factor were determined

according to the preliminary experiment. According to this design, 27 experimental runs were performed, as described in Table 1. All experiments were conducted at 30 °C and 150 rpm with an identical DM medium containing 80 mg/L ammonium nitrogen.

Table 1. Kinetic parameters of growth and nitrogen removal by *P. aeruginosa* LS82.

Factor		Growth		Reaction Kinetics of Nitrate				Reaction Kinetics of Gas-N		
		K	R^2	A_1	k_1/h^{-1}	k_2/h^{-1}	R^2	A_2	$k_3 \times 10^{-4}/h^{-1}$	R^2
pH	5	0.1257	0.9721	15.71	0.07686	0.00279	0.9943	190.38	5.4	0.9804
	6	0.1718	0.9890	23.98	0.09363	0.06473	0.8929	568.5	6.2	0.9802
	7	0.2250	0.9838	99.17	0.12946	0.12948	0.7397	276.93	4.94	
	8	0.4099	0.9954	35.91	0.14901	0.14903	0.9280	61.41	654.4	0.9694
	9	0.3517	0.9961	29.43	0.15539	0.15536	0.8261	74.32	331.1	
Shaking speed (rpm)	40	0.2989	0.9903	31.08	0.14344	0.14348	0.8364	112.70	32.58	0.9529
	75	0.3317	0.9960	64.69	0.13509	0.13511	0.7837	1916.4	3.08	0.9752
	110	0.3509	0.9746	86.98	0.13068	0.13070	0.7429	282.71	6.15	0.9812
	150	0.3177	0.9984	45.47	0.14187	0.14190	0.8230	2810.3	4.53	0.9662
C/N	4	0.3584	0.9778	34.14	0.01569	0.01568	0.9800	-	-	-
	8	0.3513	0.9888	17.82	0.11643	0.02832	0.9959	3987.1	0.54	0.8039
	12	0.3053	0.9932	19.29	0.13472	0.13470	0.6561	1671.4	2.69	0.8965
	16	0.3755	0.9870	31.73	0.14559	0.14546	0.8808	2972.2	2.03	0.8763
	20	0.3554	0.9902	37.35	0.14831	0.14833	0.9018	59854	1.82	0.9091

The mean values of dependent parameters obtained from triplicate runs were fitted to a second-order polynomial model as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j>1}^{k} \beta_{ij} X_i X_j, \quad (2)$$

where Y is the response variable (the TN removal efficiency), X_i and X_j are independent variables, and k is the number of tested variables (k = 4). The regression coefficients β_0, β_i, β_{ii}, and β_{ij} were defined as the intercept for linear, quadratic, and cross-product terms, respectively.

2.6. Kinetic Characteristics

The growth kinetics of *P. aeruginosa* LS82 were fitted with the logistic equation (Equation (3)) to describe the relationship between microbial growth rate and time. Both microbial growth rate and time were integrated to obtain Equation (4). In this study, the biomass of bacteria was represented by OD_{600}.

$$\frac{dX}{dt} = \mu X = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X, \quad (3)$$

$$X = \frac{X_{max}}{1 + e^{\ln(X_{max}/X_0) - Kt}}, \quad (4)$$

where μ and μmax refer to the specific growth rate and the maximum value, respectively, X is the bacterial concentration, X_0 and X_{max} are the initial and maximum bacterial concentrations, respectively, K is the growth kinetic constant, and t is the cultivation time.

The apparent first-order kinetic equation (Equation (5)) was applied to describe the change of intermates (nitrite, nitrate, and gaseous nitrogen) over time.

$$C_N = \frac{Ak_1}{k_1 - k_2}\left(e^{-k_2 t} - k_1 t\right), \quad (5)$$

where CN refers to the concentration of nitrate, nitrite, or gas-N, A is the pre-exponential factor, k_1 and k_2 are the rate constants of the formation reaction and decomposition reaction of nitrite, nitrate, or gas-N, and t is the cultivation time.

The kinetic model of substrate utilization was obtained by combining the growth kinetics and nitrogen removal kinetics. Due to there being no generation of nitrite throughout the process, the model was simplified as Equation (6).

$$\Delta c_{NH_4^+-N} = K_{Bio-N}\frac{X_{max}}{1+e^{\ln(X_{max}/X_0)-Kt}} + \frac{A_1k_1}{k_1-k_2}\left(e^{-k_2t}-e^{-k_1t}\right) + A_2\left(1-e^{-k_3t}\right), \quad (6)$$

where X is the bacterial concentration, X_0 and X_{max} are the initial and maximum bacterial concentrations, respectively, A_1 and A_2 are the pre-exponential factors of nitrate and gas-N, k_1 and k_2 are the rate constants of nitrate formation and decomposition reaction, k_3 is the rate constant of gas-N formation, K_{Bio-N} is the conversion coefficient of Bio-N and OD_{600}, K is the growth kinetic constant, and t is the cultivation time.

3. Results and Discussion

3.1. Isolation and Identification of Strain LS82

Five isolates with different colonial morphologies were isolated on a DM agar plate and tested for their nitrogen removal capability in the fresh DM. Strain LS82 had the highest removal efficiency of TN (89.3% ± 0.8%) with the lowest residual ammonium (no detection) and nitrate (5.40 ± 0.53 mg·L^{-1}), as well as a minimal accumulation of nitrite (Table S1). Accordingly, strain LS82 was selected for further experiments.

The time courses of ammonium, nitrite, nitrate, TN, and OD_{600} of strain LS82 are displayed in Figure 1a. The results show that 95.9% of ammonium could be eliminated within 24 h. The maximum removal rate of nitrogen reached 4.96 mg N·L^{-1}·h^{-1}, which is higher than most strains reported in the literature, such as *Acinetobacter* sp. Y16 (1.13 mg N·L^{-1}·h^{-1}), *Pseudomonas tolaasii* Y-11 (2.04 mg N·L^{-1}·h^{-1}), *Bacillus methylotrophicus* strain L7 (2.15 mg N·L^{-1}·h^{-1}), *Bacillus subtilis* A1 (3.52 mg N·L^{-1}·h^{-1}), *Enterobacter cloacae* HW-15 (3.52 mg N·L^{-1}·h^{-1}), and *Ochrobactrum anthropic* LJ81 (3.8 mg N·L^{-1}·h^{-1}) [10–15]. Concomitantly, the biomass of LS82 maintained rapid growth without the stagnation period.

Slogicals1 was utilized to fit the growth curves, and the maximum growth rate was calculated as 0.385 h^{-1}, which was not only much higher than that of autotrophic nitrifiers (0.03–0.05 h^{-1}) [16], but also larger than that of many heterotrophic nitrifiers (0.20–0.31 h^{-1}) [17,18]. Meanwhile, an increase in nitrate was observed with the consumption of ammonium within 6.5 h, indicating that heterotrophic nitrification occurred. Subsequently, the simultaneous decline in ammonium and nitrate confirmed that heterotrophic nitrification–aerobic denitrification occurred. Notably, no detection of nitrite was observed throughout the process, overcoming the shortcoming of nitrite accumulation during aerobic denitrification mediated by other aerobic denitrification bacteria [17,19,20]. High accumulation of nitrite would inhibit the respiration and proliferation of nitrifiers and denitrifying bacteria. These results verified that strain LS82 had excellent capability of SND with no nitrite accumulation, while it followed the complete nitrification and denitrification process.

The morphology and heredity characteristics of strain LS82 were analyzed and identified. On the agar plate, the semitransparent colony of LS82 was round and oyster-white with an irregular edge. The SEM image (Figure 1b) revealed strain LS82 to be a short rod with a length of 200–400 nm. The 16S rRNA sequence result was used for homology comparison via BLAST, and the phylogenetic tree (Figure 1c) indicated that strain LS82 was closely related to *Pseudomonas aeruginosa* KR349544.1 with a similarity above 95%, suggesting that it belonged to this species. According to Bergry's manual of systematic bacteriology, the morphology of LS82 matched well with the description of *Pseudomona aeruginosa*. The accession number MF784614 of this isolate was obtained upon submitting the nucleotide sequence to the GenBank database.

3.2. Response Surface Methodology

3.2.1. Model Parameters

A completely randomized design (BBD, one-factor) was used to conduct the preliminary experiment in order to determine the parameter levels of the follow-up factorial

design experiment. According to the results in Figure 2, the specific values of each factor at three levels in the BBD were set as follows: pH (5, 7.5, 10), inoculate size (2%, 6%, 10%), shaking speed (50, 100, 150 rpm), and C/N (5, 10, 25).

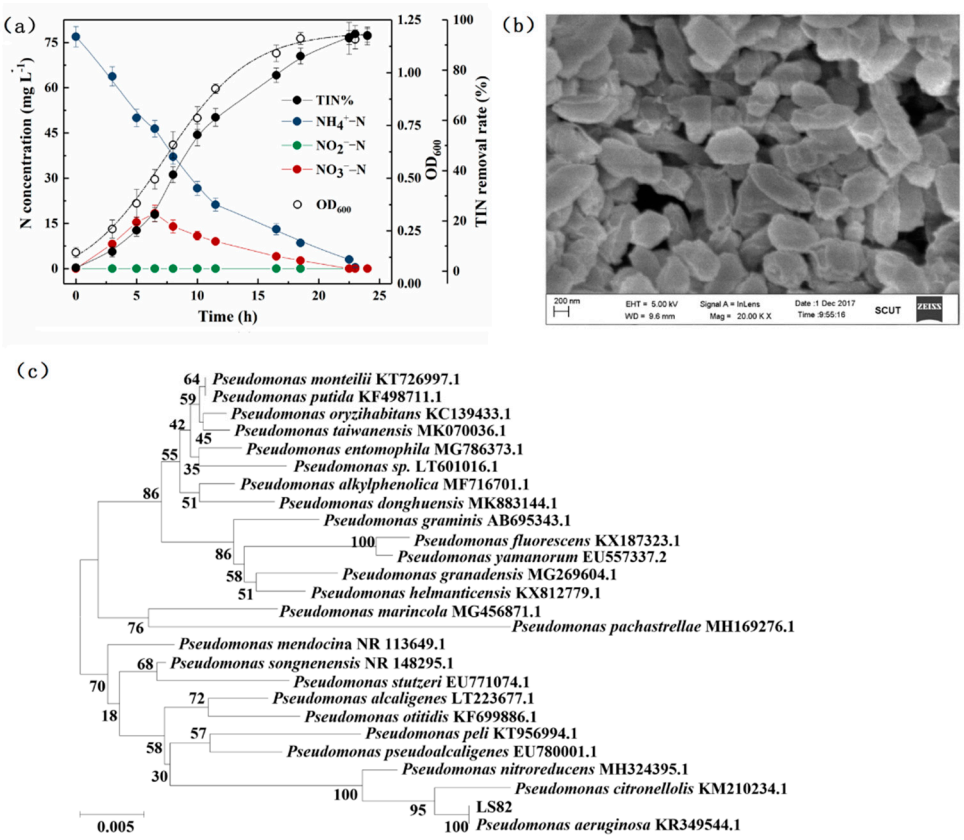

Figure 1. (**a**) Performance in terms of nitrogen removal; (**b**) SEM image; (**c**) phylogenetic tree based on 16S rRNA sequence of strain LS82.

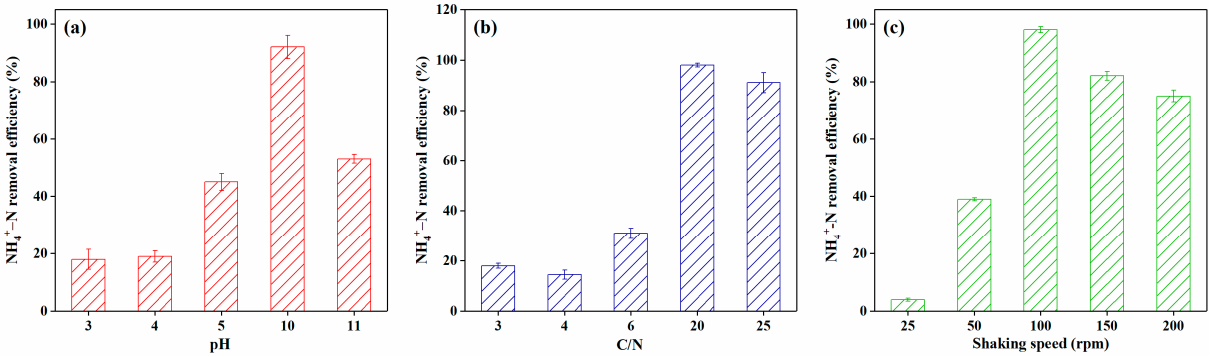

Figure 2. Ammonium removal rate as a function of key factors: (**a**) pH, (**b**) C/N ratio, and (**c**) shaking speed.

Using C/N ratio (A), shaking speed (B), inoculation level (C), and pH (D) to fit the pareto diagram model, the results illustrated that all terms (A, C, D, AA, BB, and DD) in the second modified model could display strong effects on the response. The significance degrees followed the order DD > D > A > AA > BB > C (Figure 3). Residual analysis was also employed to evaluate the fitness of the final model (Table S2 and Figure S1). According to the model, pH was the most crucial factor for LS82. These results are similar to a previous report [7](Chen & Ni 2012), where pH and C/N had significantly positive effects on ammonium removal, while DO was an insignificant factor. A possible reason is that the key enzyme in SND bacteria, periplasmic nitrate reductase, is insensitive to DO

concentration. Our results are similar to previous research indicating that the C/N ratio enhanced the reduced/oxidized nicotinamide adenine dinucleotide ($NADH/NAD^+$) ratio and then enhanced the heterotrophic nitrification–aerobic denitrification process [21].

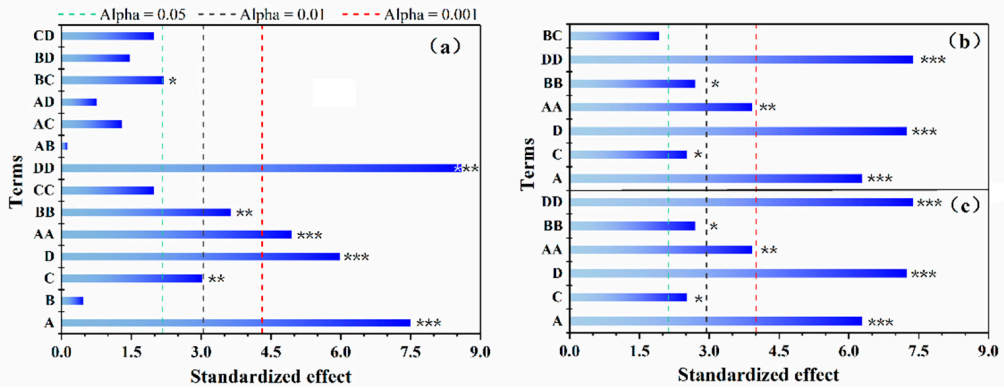

Figure 3. The pareto diagram of standardized effects on the TN removal of the (**a**) initial model, (**b**) first modified model, and (**c**) second modified model. (A) C/N, (B) shaking speed, (C) inoculation level, and (D) pH. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The fitted second-order polynomial equation of the TN removal efficiency of strain LS82 in terms of actual factors was as follows:

$$Rv = -394.5 + 87.5\,A + 1.813B + 6.82\,D - 5.233\,AA - 0.00493\,CC - 0.1718\,DD. \quad (7)$$

3.2.2. Effects of the Variables on the Nitrogen Removal by Strain LS82

The 3D response surfaces and 2D contour plots were drawn to intuitively display the results of the statistical analyses (Figure 4). The TN removal efficiency increased linearly with the increase in inoculum size, whereas the TN removal efficiency initially increased to a peak and then decreased as the pH was varied from 5 to 10 (Figure 4a). A similar phenomenon was discovered by Li et al. [4], who showed that both acidic (pH 5–6) and alkaline (pH 9–10) conditions suppressed the growth of *Vibrio* sp. Y1–5 and caused a sharp decrease in TN removal efficiency. Strain LS82 exhibited excellent nitrogen removal ability (more than 90%) at the initial pH of 7.5–9, indicating that LS82 could tolerant slightly alkaline conditions, whereas highly alkaline conditions would be conducive to SND due to the more release of more free ammonia.

Figure 4b shows the clearly saddle-shaped contours of pH and inoculum size, indicating that their interaction had a strong effect on the TN removal efficiency. Another notable result of Figure 4b is that the optimum value of inoculum size was outside the experimental boundary. Although ridge analysis could allow a further optimization of inoculum size, we still achieved great nitrogen removal performance using a 10% inoculum size; thus, this size was applied in further tests.

As shown in Figure 4c, the nitrogen removal efficiency declined when using an extremely poor or rich carbon source. Cell growth needs carbon as a basic element. Under a low C/N ratio, the electron flow cannot provide abundant energy for cell proliferation [22]. Moreover, TN removal efficiency fluctuated slightly under different shaking speed conditions (50–150 rpm), indicating a great adaptation of strain LS82 to DO level. Zhao et al. [18] obtained a different result, whereby an extremely high DO concentration (under 150 rpm) markedly restrained the nitrogen removal performance of strain XL-2. Compared to their results, our *Pseudomonas* LS82 showed excellent tolerance and adaptability to a high DO level, suggesting that it performed better under an SND process. Furthermore, Figure 4d shows a nearly circular contour, indicating that the two-way interaction of shaking speed and C/N ratio had no significant effect on the response. Moreover, an obvious peak appeared in the 3D response surface curve, further confirming that the optimum values of C/N ratio and shaking speed were inside the experimental boundary.

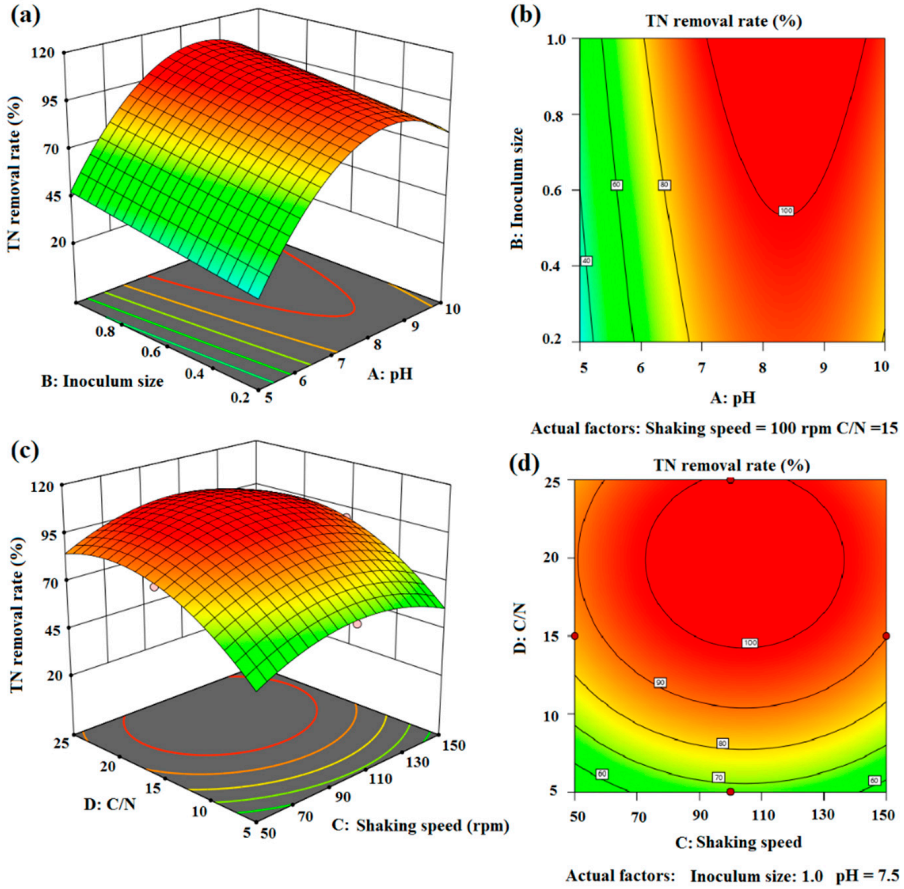

Figure 4. Response surface methodology results: (**a**) response surface and (**b**) contour plot of pH and inoculum size; (**c**) response surface and (**d**) contour plot of C/N ratio and shaking speed.

3.2.3. Response Optimization and Verification

According to the results above, the response optimization of pH, shaking speed, and C/N ratio with a fixed inoculum size (10%) was conducted via statistical analysis using MINITAB 17. Figure 5 displays the results of the response optimization and validation experiment. As shown in Figure 5a, the optimum pH, shaking speed, and C/N ratio were obtained at 8.47, 100 rpm (4.87 mg·L^{-1}), and 19.7, respectively. Under these conditions, the predicted TN removal efficiency was nearly complete.

The verified experiment was performed under optimized conditions in triplicate, and the concentrations of ammonium, nitrite, and nitrate, as well as the OD_{600} and pH, are shown in Figure 5b. With the sharp decrease in ammonium and the appearance of nitrate, *P. aeruginosa* LS82 multiplied rapidly with a larger growth rate (0.427 h^{-1}) and reached the stationary phase within 12 h. Subsequently, the denitrification process resulted in an increase in pH and TN removal efficiency within 18 h, reaching 99.8% ± 0.9%. The experimental values matched the predicted values within the margin of error, indicating that the built model could satisfactorily describe the relationship between the factors and response.

3.3. Kinetic Analysis

The kinetics of strain growth and nitrogen removal under all experimental conditions are shown in Table 1. The kinetic model was evaluated by *t*-test and Pearson's correlation. The predicted results and actual values under various conditions are shown in Figure 6a–c. Pearson's correlation coefficients under all conditions were in the range of 0.8–1.0, indicating that the predicted values of the model were strongly correlated with the actual values.

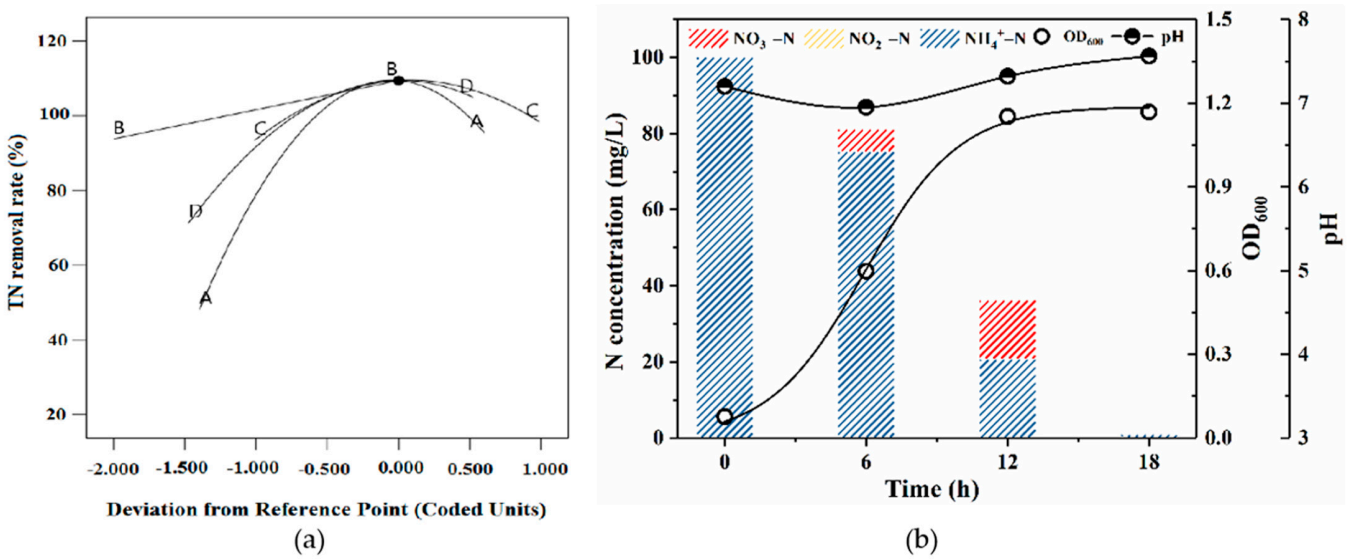

Figure 5. The response of the (**a**) optimization and (**b**) validation experiments. (A) C/N, (B) shaking speed, (C) inoculation level, and (D) pH.

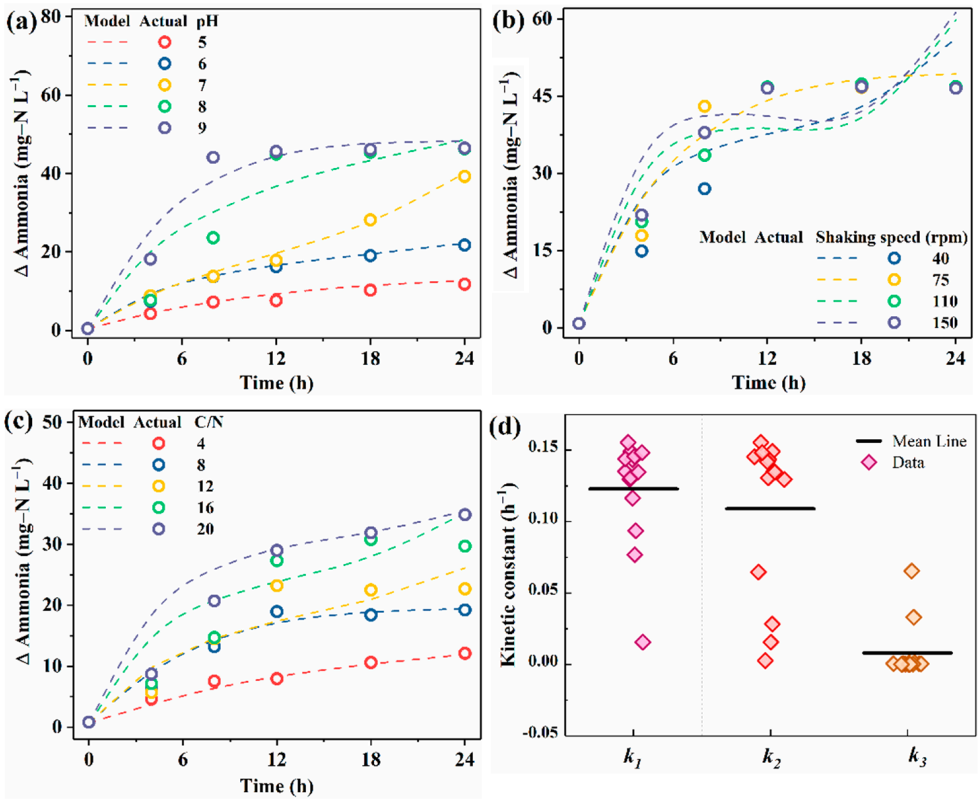

Figure 6. Kinetics of substrate utilization as a function of (**a**) pH, (**b**) speaking speed, and (**c**) C/N ratio; (**d**) kinetic constants of nitrogen removal of *P. aeruginosa* strain LS82 under various conditions.

As the pH increased from 5 to 8, the K value increased from 0.1257 to 0.4099, before declining to 0.3517 with the further increase in pH, indicating that slightly alkaline conditions were favorable for the growth of *P. aeruginosa* LS82. At the same time, both k_1 and k_2 increased with the increase in pH, confirming that the initial alkaline environment was also conducive to the occurrence of the SND process. It is notable that k_3 decreased at pH 9, suggesting that highly alkaline conditions inhibited the reduction of nitrite. These results are similar to the report by Li et al. [4].

In addition, consistent with the above results, K, k_1, and k_2 values under different shaking speed conditions were comparable, suggesting that DO level was not a limiting factor for the growth of bacteria during the nitrification and denitrification process. *P. aeruginosa* LS82 may exhibit great tolerance at high DO. Moreover, k_3 decreased sharply at a higher shaking speed, suggesting that the denitrification of nitrite to gas-N was inhibited. In another study [23], nitrite reductase was shown to be sensitive to O_2, as a sharp decrease in bacterial activity was observed in the presence of high DO.

Interestingly, in contrast to the lack of an obvious influence of C/N ratio on the growth of strain LS82 (similar K values), both k_2 and k_3 remarkably increased with the increase in C/N ratio from 4 to 12, indicating that SND reaction activity could improve with an abundant carbon source. This could be explained by the fact that sufficient carbon releasing more electrons, thereby providing sufficient electron flow to nitrate and accelerating the SND process.

In addition, the means of both k_1 and k_2 were a few orders of magnitude higher than the formation constant of gas-N (k_3), indicating that the reduction of nitrite was the rate-limiting step in the SND process (Figure 6d).

4. Conclusions

The study demonstrated that the simultaneous nitrification and denitrification could be achieved aerobically by the isolated SND bacteria *P. aeruginosa* LS82. The response surface methodology model could well describe the relation of factors and the response. Moreover, the logistic equation and the apparent first-order kinetic equation were employed to describe the bacteria growth and SND process. The kinetics analysis revealed that nitrogen conversion pathway, and the reduction of nitrite to gas-N involved in the denitrification was the rate-limiting step in the SND process.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w14091452/s1: Figure S1. Residual analysis of the final model: (a) normal probability plot; (b) versus fits; (c) versus order; (d) comparation of predicted results and the actual values; Table S1. Comparison of the nitrogen removal efficiencies of different isolates; Table S2. Surface quadratic model fitness of TN removal efficiency (second modification).

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