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Orthogonal Test Design to Optimize on Ammonium Removal by *Pseudomonas stutzeri* ZH-1

Caifang Wen[1] and Qingping Hu[1]*

[1]College of Life Science, Shanxi Normal University, 1 Gongyuan Street, Linfen City, 041004, P.R. China.

Authors' contributions

This work was carried out in collaboration between both authors. Author CW managed the literature searches, analyses of the study, wrote the protocol and carried out laboratory experiments under author QH supervision. Author QH designed the study, performed the statistical analysis. Both authors read and approved the final manuscript.

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ABSTRACT

To give reference for the application of nitrobacteria in actual wastewater treatment, the impact factors of C/N ratio, shaking speed (DO) and carbon source on ammonium removal by *Pseudomonas stutzeri* ZH-1 was investigated. Through orthogonal test, the optimal conditions for NH_4^+-N removal were C/N 12, shaking speed 100 r/min and sodium citrate as carbon source. The strain ZH-1 exhibited efficient nitrifying ability and maximum NH_4^+-N removal efficiencies were 99.66%, and removal rate was 4.27 mg $L^{-1}h^{-1}$. The results demonstrated that C/N and DO were the most important factors influencing the performance of heterotrophic nitrification. Also, these findings stated that the strain ZH-1 might be an effective and suitable candidate for ammonium nitrogen removal in industrial wastewater treatment.

Keywords: Orthogonal test; ammonium nitrogen; optimization; Pseudomonas stutzeri.

**Corresponding author: E-mail: hqp72@163.com;*

1. INTRODUCTION

Emission of nitrogen into aquatic ecosystems can cause eutrophication and other environmental problems. In the past few decades, nitrogen removal, especially ammonia removal, had always been the core of wastewater treatment [1]. Biological nitrogen removal (BNR) involving nitrification and denitrification has been still adopted in wastewater treatment because it is inexpensive and causes little secondary pollution of the environment in contrast to physico-chemical treatments [2]. Biological nitrification-denitrification is one of the most effective and economical processes for nitrogen removal from wastewaters [3]. Nitrification, the first step in BNR, involves two processes: ammonium oxidation to nitrite by ammonia oxidizing bacteria (AOB); and nitrite oxidation to nitrate by nitrite oxidizing bacteria (NOB) [4]. Ammonia oxidation is thought to be the rate-limiting step in nitrification, because AOB have lower growth rates than NOB, and are more sensitive to inhibition by environmental factors [5].

In recent years, autotrophic nitrification and anoxic denitrification have become a dominant approach of nitrogen removal. However, Metcalf and Eddy found that nitrifying bacteria grow slowly and require different conditions and cost-intensive and time-consuming [6]. Compared with autotrophs, heterotrophic nitrification-aerobic denitrifiers were widely focused for its much higher growth rate and more efficient substrate removal with simultaneous nitrogen removal and organic carbon degradation. With the advancement of research, a large number of heterotrophic nitrification-aerobic denitrification strains were isolated and characterized, such as *Alcaligenes faecalis* No. 4 [7], *Bacillus* sp. strains [8], *Pseudomonas* sp. [9], *Pseudomonas stutzeri* [10], *Providencia rettgeri* and *Klebsiella pneumonia* [11] and so on. These special heterotrophic nitrification-aerobic denitrifying bacteria could use various carbon sources and different inorganic nitrogen, such as ammonium, nitrate and nitrite nitrogen into nitrogenous gas [12,13]. Significantly, the great complexity and in stability of actual wastewater usually leads to much lower nitrogen removal efficiency than synthetic wastewater [14], which limits its application in actual wastewater treatment. Several studies have focused on nutritional and physical factors that adjust the ammonia nitrogen removal characteristics of heterotrophic nitrification-aerobic denitrifying bacteria. Nevertheless, information on the comprehensive impact resistance study of these bacteria is still very limited.

As a typical heterotrophic nitrification bacterium, *Pseudomonas stutzeri* ZH-1 enables simultaneous removal organic carbon and nitrogen at high efficiencies. In this paper, orthogonal test was used to study the effect of different factors affecting the nitrification performance of the strain ZH-1, including carbon sources, C/N ratio, and shaking speed had been researched. The optimal conditions had been gained through orthogonal test, and which could help promoting the use of heterotrophic nitrification bacteria in actual wastewater treatment.

2. MATERIALS AND METHODS

2.1 Microorganism and Medium

P. stutzeri ZH-1 was originally isolated and identified from Fenhe River (in Shanxi Province, China). After being grown on the solidified of Beef extract-peptone medium containing (per liter): 5.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, 18.0 g bacto-Agar and 1000 ml tap water, the bacteria strain was stored at -80℃ in 30% (w/v) glycerol. In the heterotrophic nitrification medium (HNM) [15] study consisted of the following components (per liter): 0.5 g of $(NH_4)_2SO_4$, 4.78 g of Sodium citrate, and 1 mL mineral solution. The mineral solution contained $(g\cdot L^{-1})$: $FeSO_4\cdot 7H_2O$ 3; H_3BO_3, 0.01; $Na_2MoO_4\cdot 2H_2O$, 0.01; $MnSO_4\cdot H_2O$, 0.02; $CuSO_4\cdot 5H_2O$, 0.01; the concentrations of carbon and culture conditions were adjusted following the subsequent experiments. The initial pH of all the mediums which mentioned above was adjusted to 7.2-7.5.

2.2 Analysis Methods

Growth of the bacteria was monitored by measuring the optical density at 600 nm (OD_{600}) of the culture broth using a spectrophotometer. Culture samples were centrifuged at 8,000 rpm for 10 min. The concentration of ammonium was analyzed by Nasser's reagent photometry at 420 nm (GB 7497-87). The nitrification ratio was calculated according to the method of Joo et al. [7]. The ammonium removal efficiencies were calculated by the equation: $R_V = (T_1 - T_2)/T_1 \times 100\ \%$ to assess the nitrification ability of strain ZH-1. Note that Rv, T1 and T2 represent ammonium removal efficiency, the initial concentration of ammonium in medium and the final concentration of ammonium, respectively. All tests were conduct-triplicate. Statistical analyses were performed by the SPSS Statistics and differences were considered significant at $p < 0.05$. And drawing software was used Origin 9.0. The results were presented as means ± SD (standard deviation).

2.3 Single-factor Experiments to Study the Factors Influencing the Nitrification Performance of Strain ZH-1

Single-factor experiments were conducted for studying the heterotrophic nitrification characteristics of the strain ZH-1 under different culturing conditions, including carbon source, C/N ratio and shaking speeds (DO).

In carbon source experiments, glucose, sucrose, glycerol, sodium acetate, sodium citrate, sodium succinate, sodium potassium tartrate, lactose and lactose were employed as sole carbon source, respectively. In C/N ratio experiments, the content of carbon source was changed in order to adjust C/N ratio to 2, 4, 8, 12, 16, and 20, respectively. The other culturing conditions were the same as the carbon source experiments. The effects shaking speed, was also investigated in the optimum medium from carbon source test and C/N test. The shaking speed was adjusted to 0, 50, 100, 150, 200 and 250 r/min, respectively. All of the above experiments were conducted in triplicate with inoculation size of 2% (v/v) and the non-seeded samples were also conducted as controls. Unless otherwise stated, all the heterotrophic nitrification experiments were conducted at initial nitrogen concentration 100 mg/L, C/N 8, initial pH 7.5, culturing temperature 30℃ and shaking speed 120 rpm. Then strain growth (OD_{600}), and the contents of NH_4^+-N was determined. All the experiments were carried out in triplicate. And all the NH_4^+-N efficiencies and removal rates were calculated at 24 h.

2.4 Orthogonal Test to Study the Factors Influencing the Denitrification Performance of Strain ZH-1

Orthogonal tests were designed and analyzed with SPSS statistical software (SPSS 16.0 version for windows, SPSS, Inc., Chicago, IL, USA) to illustrate the effect of different factors on the nitrification performance of strain ZH-1. The factors and their levels were detailed in Table 1. Differences were considered to be significant when $P < 0.05$. Cells of the strain ZH-1 were grown in Beef extract-peptone medium over night and harvested by centrifugation at 8000 r/min for 5 min, washed twice with sterile saline solution, and then re-suspended in sterile saline solution. Two ml of cell suspension was inoculated into 100 ml HNM in 250 ml flask. All the experiments were done in triplicates.

3. RESULTS AND DISCUSSION

3.1 Nitrification Characteristics of Strain ZH-1 under Various Conditions

3.1.1 Effect of carbon source

Carbon source is an important factor affecting heterotrophic nitrification ability and cell growth. The organic and small molecular weight carbon sources are comfortable to serve as electron donors and promote the growth of microorganisms [16]. In addition, carbon source were indispensable for the growth of microorganisms and energy-producing, and optimum certain carbon source at a concentration range is beneficial to the bacterial growth and the higher nitrification rate [17]. The results in Fig. 1 illustrated that the ammonium removal efficiencies were significantly different among the tested carbon sources ($p < 0.05$). Sodium citrate was the most suitable carbon source for the type of nitrogen removal, presenting a removal efficiency of 57.85% in 24 h. The possible reason was that sodium citrate could involve in the tricarboxylic acid cycle, which was an important way to microbial oxidation. When acetate, glycerol and succinate were used as sole carbon source, strain ZH-1 also exhibited efficient ammonium removal ability. The suitable carbon source for nitrogen removal might be a strain or species characteristic [18]. Citrate has been reported to directly insert into the metabolic process without modification, and make the medium more alkaline during nitrification, which was better for nitrification process [19]. The result was consistent with previous finding [20], that higher NH_4^+-N removal efficiency was observed in *P. stutzeri* YG-24 with sodium citrate as carbon source. Therefore, for strain ZH-1, sodium citrate was selected as the optimal exogenous carbon source in subsequent experiments.

3.1.2 Effect of DO

The essential difference between the emerging heterotrophic nitrification and the traditional one was whether the denitrifying microbes were vulnerable to oxygen [21]. To investigate the effect of dissolved oxygen content on nitrogen removal, aeration was controlled by adjusting the shaking speed. As shown in Fig. 2 the maximum NH^{+}_{4}-N removal efficiency was 92.09% at a shaking speed of 100 r/min and lesser NH_4^+-N removal rates when soluble oxygen augmented. But *Microbacterium* sp. SFA13 [22] and *Marinobacter* sp. F6 [23] exhibited higher NH_4^+-N removal as dissolved oxygen increased. The data showed that approximate 77.64% of NH_4^+-N could be removed at the shaking speeds of 250 rpm. Besides, these results demonstrated that, to remove various types of nitrogenous compounds effectively, the aeration condition must be adjusted according to different sources of wastewater in practical application when using strain ZH-1.

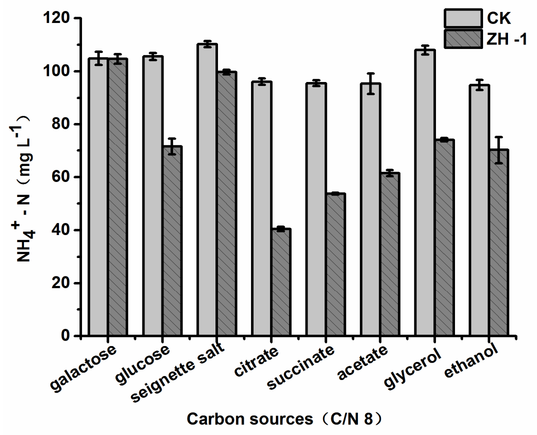

Fig. 1. The ability of ammonium removal under different carbon source. CK means the initial concentration of ammonium in medium

3.1.3 Effect of C/N ratio

To determine the influence of different C/N ratio on ammonium removal in the basic media, a set of experiments were devised. The ammonium removal percentage was not significantly different among C/N 12-20 as shown in Fig. 3(a) ($p < 0.05$). Maximal ammonium consumption occurred at C/N ratio of 12 with the ammonium removal percentage of 98.56%, but further increase in C/N ratio yielded a slight decrease in nitrogen removal efficiency. Even if the C/N ratio was as high as 20, which was too high for the growth of autotrophic nitrifying bacteria, strain ZH-1 still exhibited satisfying nitrification ability with the ammonium removal percentage of 97.16%. As showed in Fig. 3(b), the remnant of ammonium at low C/N ratios was mainly due to the insufficient carbon supply, which would impair both microbial growth and electron donors for denitrification [24]. Our results indicated that strain ZH-1 could use or convert ammonium during the growing phase and had a high capability for ammonium removal in the presence of high organic load. The ratio of C/N 12 seems to be advantageous for achieving cost-effective heterotrophic nitrification. The appropriate C/N also was an important factor affecting heterotrophic nitrification ability [16].

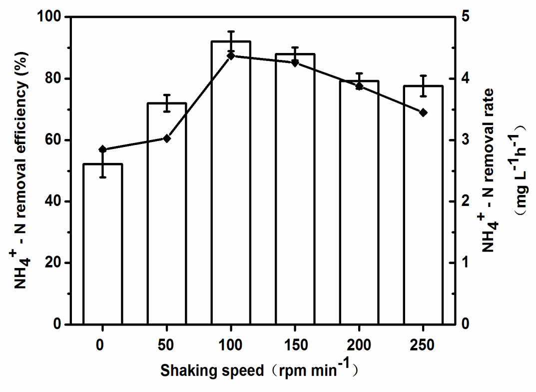

Fig. 2. Ammonium removal rate and efficiency of ZH-1 at different temperature. Columns with blank, NH_4^+-N efficiency; ◆, removal rate of NH_4^+-N

3.2 Orthogonal Test for the Heterotrophic Nitrification Performance of Strain ZH-1

The orthogonal experiment results and variance analysis were tabulated in Tables 1 and 2, respectively. The results indicated that the three factors had impact on the ammonium removal in the order of B > A > C. This result was consistent with research of Huang and Tseng, who found that C/N and DO were the most important factors influencing the performance of nitrification [25]. The optimal conditions for maximum removal according to the result were shaking speed 100 r/min, C/N 12 and acetate as carbon source, ($A_2B_2C_2$). But this result was not found in the nine experimental groups and not consistent with the group $A_2B_2C_3$. Through experiment again and found that the bacteria had the higher removal rate of ammonium nitrogen with 99.66% at the experimental group ($A_2B_2C_3$) than theory group with 99.2% ($A_2B_2C_2$). Based on the above discussion, it could be concluded that the optimal conditions for maximum removal were shaking speed 100r/min, C/N 12 and citrate as carbon source ($A_2B_2C_3$). According to the predecessors'

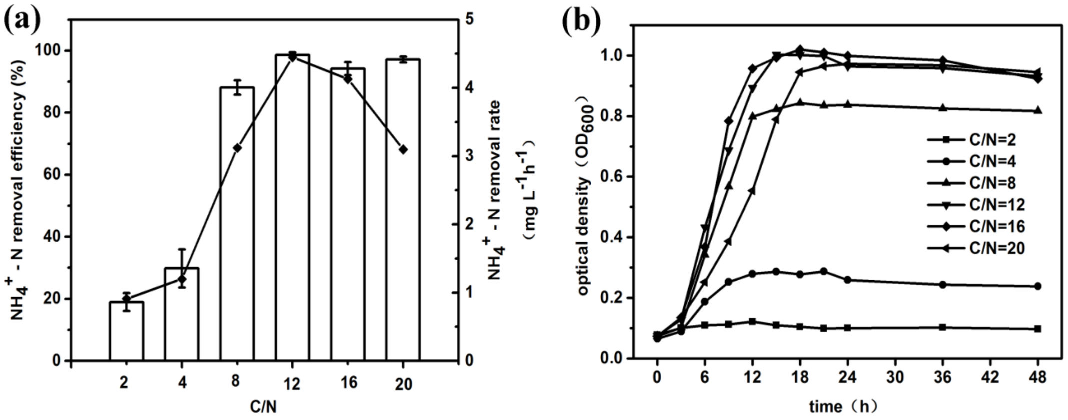

Fig. 3. (a) Ammonia removal rate and efficiency of the strain ZH-1 at different C/N; (b) the cells growth at different C/N. Columns with blank, NH_4^+-N efficiency; ◆, removal rate of NH_4^+-N

Table 1. Results and analysis of orthogonal experiment for the optimization of reaction conditions of culture of strain ZH-1

Test numbers	Factor A (shaking speed rpm)	Factor B (C/N ratio)	Factor C (carbon source)	NH_4^+-N removal rate (%)
1	8(A_1)	50(B_1)	Succinate (C_1)	91.4
2	A_1	100(B_2)	Acetate (C_2)	97.98
3	A_1	150(B_3)	Citrate (C_3)	89.07
4	12(A_2)	B_1	C_2	92.55
5	A_2	B_2	C_3	98.43
6	A_2	B_3	C_1	95.69
7	16(A_3)	B_1	C_3	92.97
8	A_3	B_2	C_1	96.73
9	A_3	B_3	C_3	95.27
K1	92.817	92.307	94.607	
K2	95.557	97.713	95.267	
K3	94.990	94.343	93.49	
R	2.740	5.406	1.777	
The optimal levels		$A_2B_2C_2$		
Major and minor factors		B ＞A ＞ C		

Table 2. Analysis results of orthogonal tests with software SPSS 16.0

Factors	DevSp	Df	F	Sig.
A	12.552	2	0.564	0.000
B	49.404	2	2.219	0.000
C	4.839	2	0.217	0.000
Error	66.80	6		

research, carbon source were indispensable for the growth of microorganisms and energy-producing. These results were also consistent with the researches by Patureau [17].

4. CONCLUSIONS

The orthogonal experiment results that the optimal conditions for NH_4^+-N removal were: citrate as carbon source, C/N ratio 12 and shaking speed 100 r/min. Under the conditions, the strain ZH-1 exhibited efficient heterotrophic nitrifying ability with maximum NH_4^+-N removal rate of 4.27 mg $L^{-1}h^{-1}$. Therefore, strain ZH-1 was a promising candidate in the extensive application of various pollution control system including industrial wastewater, municipal wastewater, etc.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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