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ORIGINAL ARTICLE

Effect of trace elements and optimization of their composition for the nitrification of a heterotrophic nitrifying bacterium, *Acinetobacter harbinensis* HITLi7^T, at low temperature

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Abstract The effects of trace elements on ammonium degradation performance and extracellular polymeric substances (EPS) secretion of *Acinetobacter harbinensis* HITLi7^T at low temperature were investigated. Response surface methodology (RSM) was applied to obtain the optimal composition of trace elements and analyze their correlation. In this study, the results indicated that the ammonium removal performance could be enhanced by the presence of 0.1 mg L^{-1} Fe, Mn, or B in pure cultivation. When the concentrations of Fe and Mn were 0.2 mg L^{-1}, the ammonium removal rates of the novel strain HITLi7^T were 0.49 ± 0.01 mg L^{-1}·h^{-1} and 0.58 ± 0.01 mg L^{-1}·h^{-1}, respectively, while it was the low concentration of 0.05 mg L^{-1} B that showed the maximum ammonium removal rate (0.56 ± 0.02 mg L^{-1}·h^{-1}) of strain HITLi7^T. The regression model was obtained and the optimal formulation of trace elements was: B 0.064 mg L^{-1}, Fe 0.12 mg L^{-1}, and Mn 0.1 mg L^{-1}. Based on these values, the experimental ammonium removal rate could reach 0.59 mg L^{-1}·h^{-1}, which matched well with the predicted response. The study also found that the addition of trace elements, causing high ammonium removal rates, resulted in a high polysaccharide (PS) ratio in the EPS secreted by *Acinetobacter harbinensis* HITLi7^T. Especially under the optimal conditions, the PS ratio reached the highest value of 49.9%.

Keywords Ammonium removal · Trace element · Response surface methodology · *Acinetobacter harbinensis* HITLi7^T · Polysaccharide

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Introduction

Nitrogen excess is a serious issue faced by source water in China. The traditional biological method to remove ammonium mainly relies on the biological degradation ability of the nitrification bacteria at the appropriate temperature (Zhu et al. 2012). Recently, bacteria that are capable of heterotrophic nitrification and aerobic denitrification simultaneously have attracted wide attention. So far, many species, such as *Alcaligenes faecalis* strain NR (Zhao et al. 2012), *Acinetobacter* sp. HA2 (Yao et al. 2013), *Pseudomonas stutzeri* YZN-001 (Zhang et al. 2011), and *Bacillus methylotrophicus* strain L7 (Zhang et al. 2012), with high ammonium removal efficiency have been isolated from nature. However, most bacteria preferred high ammonium concentration (> 100 mg L^{-1}) and high temperature (> 15 °C), which were not suitable for source water in northern China during winter time (low temperature and poor nutrition) (Zilouei et al. 2006). In a previous study, a heterotrophic bacterium, strain HITLi7^T with ammonium removal ability at 2 °C, was isolated from the Songhua River in Harbin, China. It was identified as a new strain of *Acinetobacter* and named as *Acinetobacter harbinensis*, with international recognition (Huang et al.

2013; Li et al. 2014; Oren and Garrity 2014). In the study of the environmental impact factors, strain HITLi7^T achieved a maximum removal rate of 0.18 mg $L^{-1} \cdot h^{-1}$ at 8 °C, pH = 6.0, C/N = 2, while shaking at 100 rpm (Qin et al. 2016a). In our previous study, the potential of strain HITLi7^T in the drinking water treatment system was investigated by constructing a biologically enhanced activated carbon (BEAC) filter with strain HITLi7^T as the dominant microflora at 2 °C (Qin et al. 2016b). The results showed that the ammonium removal efficiency in the BEAC filter was 2.8-fold higher than in a granular activated carbon (GAC) filter, indicating that BEAC filters with strain HITLi7^T were more suitable for ammonium removal at low temperature (Qin et al. 2016b).

Due to the great potential of strain HITLi7^T for ammonium degradation in the drinking water system at low temperature, strain HITLi7^T has been applied to remove ammonium in both groundwater and surface water in northern China. When dealing with surface water in Songhua River, we found that the ammonium removal ratio was only 21% in winter, and it was difficult improve further (Qin et al. 2017). Compared to surface water, groundwater is relatively more concentrated in terms of trace metals (Winter et al. 1998), which might cause a difference in ammonium removal performance. Microbiological activity was strongly influenced by metal elements, which were usually used as cofactors of enzymes. Nitrification in biological rapid sand filters in water plants is limited by the deficiency of Cu (Wagner et al. 2016). Fe was reported to influence the nitrification rate, as organic carbon does (Krishnan and Loka Bharathi 2009). In previous studies, people paid more attention to the conditions, like temperature, pH value, and toxicity. In terms of nutrition, nitrogen and phosphorus were often taken into consideration (Qin et al. 2016a), but trace elements have been overlooked consistently. Although trace elements were crucial to the growth and metabolism of microorganisms, different concentrations might cause completely opposite results (Sato et al. 1986). The excessive, insufficient, and inappropriate proportion of them would have a negative influence or even lead to a collapse in the biological water treatment systems. Therefore, it is essential to find an optimal composition of trace elements to promote ammonium removal efficiency of strain HITLi7^T.

Extracellular polymeric substances (EPS) is a kind of high molecular polymer mainly composed of extracellular proteins (PN) and extracellular polysaccharide (PS) (Tsuneda et al. 2003). It is usually used to help microorganisms absorb soluble nutrients, reduce the sensitivity to bacteriophage, and can also be used as a medium to promote the cooperation and signal transmission between bacteria (Wingender et al. 1999). It was reported that extracellular polysaccharide, usually with anionic characteristics, could not only be used as a physiological barrier from oxygen, but also absorb some positively charged ions, such as some metal ions (Mohamed 2001). As we know, ammonia nitrogen exists in the form of ammonia ion in water which is positively charged, and we want to find whether its degradation by bacteria have some connection with extracellular polysaccharide.

The aim of this study was to investigate the impacts of trace elements on ammonium removal capacity and EPS secretion of strain HITLi7^T during pure cultivation process and find a way to solve the problem of low activity caused by low temperature and deficient nutrient. Response surface methodology (RSM) can be used to estimate the maximum production of a special substance by the optimization of operational factors, and the interaction among process variables can be determined by statistical techniques. It is widely used as an effective statistical method in various fields (Asadi and Zilouei 2017). It was adopted in this study to evaluate the optimal composition to promote ammonium degradation ability of strain HITLi7^T.

Materials and methods

Strain and medium

Strain HITLi7^T was isolated from the Songhua River in Harbin (126°38′E, 45°45′N) during winter. It is a new strain of bacteria belonging to the genus *Acinetobacter*, named *Acinetobacter harbinensis* (Li et al. 2014). The cultures of strain HITLi7^T were maintained in heterotrophic nitrification medium, containing 0.382 g L^{-1} NH_4Cl, 2 g L^{-1} CH_3COONa, 0.2 g L^{-1} K_2HPO_4, 0.05 g L^{-1} $MgSO_4$, and 0.12 g L^{-1} NaCl, at pH 7.0 under 4 °C.

Shaking culture experiment

Strain HITLi7^T was pre-cultured in the heterotrophic nitrification medium at 4 °C, while shaking at 100 rpm. When the turbidity of bacteria exceeded OD_{600} = 0.5, 2 L of the liquid was centrifuged at 8000 × g for 5 min. The deposit was washed with sterile water three times, and then re-suspended in 10 mL sterile deionized water as an inoculum for experiment cultivation. One milliliter of re-suspended bacteria was then inoculated into a 300-mL conical flask which contained 200 mL basal culture medium with 0.0382 g L^{-1} NH_4Cl, 0.1 g L^{-1} glucose, 0.2 g L^{-1} K_2HPO_4, 0.05 g L^{-1} $MgSO_4$, and 0.12 g L^{-1} NaCl. The cultivation was maintained at 4 °C, pH 7.0, and 100 rpm, and samples were analyzed periodically for OD_{600} and ammonium concentration.

Effect of trace elements on ammonium removal

In order to investigate the impact of trace elements on the ammonium removal ability of strain HITLi7^T, 0.1 mg L^{-1} B

(H_3BO_3), 0.1 mg L^{-1} Zn ($ZnSO_4 \cdot 7H_2O$), 0.1 mg L^{-1} Fe ($FeSO_4 \cdot 7H_2O$), 0.1 mg L^{-1} Cu ($CuSO_4 \cdot 5H_2O$), 0.1 mg L^{-1} Mn ($MnSO_4 \cdot 4H_2O$), and 0.1 mg L^{-1} Mo ($Na_2MoO_4 \cdot 2H_2O$) were separately added into six 300-mL flasks with 200 mL basal culture medium. A flask with no trace metal added was used as a control. After being cultured for 4 h at 4 °C, pH 7.0, and 100 rpm, the remaining ammonium concentration was measured. Further experiments were conducted to obtain the advisable concentration of B, Fe, and Mn to promote the ammonium reduction rate. Three different concentrations (0.05, 0.1, and 0.2 mg L^{-1}) were prepared for each of the trace elements mentioned above. The remaining ammonium concentration was determined after shaking for 2 h at 4 °C, pH 7.0, and 100 rpm. All experiments were run in triplicate simultaneously.

Investigation of EPS secretion of strain HITLi7^T

Bacteria were cultivated in the basal culture medium with different additions: (a) 0.05 mg L^{-1} B, (b) 0.15 mg L^{-1} B, (c) 0.1 mg L^{-1} Fe, (d) 0.3 mg L^{-1} Fe, (e) 0.2 mg L^{-1} Mn, (f) 0.6 mg L^{-1} Mn, (g) 0.064 mg L^{-1} B, 0.12 mg L^{-1} Fe, and 0.1 mg L^{-1} Mn. Additions (a), (c), and (e) were the concentrations of B, Fe, and Mn, respectively, that caused the highest ammonia removal rate of strain HITLi7^T under single-factor experiments. Addition (g) was the optimal composition of trace elements, which resulted in the most effective removal capability. After shaking for 24 h at 4 °C, pH 7.0, and 100 rpm, EPS was extracted. Cultivation with no trace metal added was used as a control. All experiments were carried out three times and expressed as the average value.

Analytical methods

The ammonium concentration was measured colorimetrically by using Nessler's reagent colorimetric method at a wavelength of 420 nm. The OD_{600} value of the strain was measured by spectrophotometry at a wavelength of 600 nm. The EPS was extracted by the method using formaldehyde plus NaOH (Liu and Fang 2002). The protein concentration was measured by following the instructions of the Bicinchoninic Acid (BCA) Protein Assay Kit (Sangon Biotech, Shanghai, China). The polysaccharide content was determined according to the phenol-sulfuric acid method.

The ammonium removal rate is calculated by a mathematical formula, as follows: $(C_0 - C_t)/t$, where C_0 is the initial concentration of NH_4^+-N, C_t is the final concentration at a given time, and t is the time that strain HITLi7^T was cultivated in the experiments.

The polysaccharide ratio is defined as: $Q_s/(Q_s + Q_n) \times 100\%$, where Q_s and Q_n are the concentrations of extracellular proteins (PN) and extracellular polysaccharide (PS), respectively.

Experimental design and statistical analysis by RSM

In this study, for the purpose of identifying the optimal formulation of the three effective elements, a three-factor and three-level (−1, 0, +1) central composite design (CCD) was adopted. A total of 17 experiments were done to study the effects of the three significant variables [B (X_1), Fe (X_2), and Mn (X_3)] on the response of the ammonium removal rate (Y) of strain HITLi7^T. The ammonium removal rate of all experimental tests were the average of the triplicates after 2 h of incubation. The experimental parameters were selected according to the advisable concentration and are summarized in Table 1.

The results were analyzed by Design-Expert 8.0 software. A second-order polynomial formula was fitted to simulate the relationship between variables (B, Fe, and Mn) and response (ammonium removal rate), and then to predict the optimal point. The ammonium removal rate was analyzed based on multiple linear regression combining the least-squares method to fit Eq. (1), where Y is the predicted response, X_i are the independent variables, β_0 is the intercept term, β_i are the linear coefficients, β_{ij} are the interaction coefficients, and β_{ii} are quadratic coefficients:

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

Results and discussion

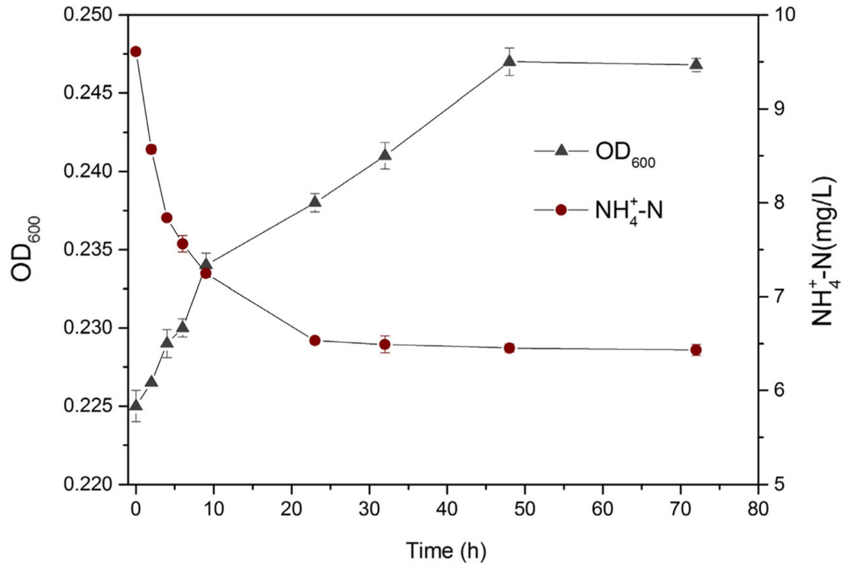
Shaking culture experiment

Low temperature is a factor that affects nitrifiers by slowing down or inhibiting their growth and function (Rodriguez-Caballero et al. 2012). The aerobic growth and ammonium removal characters were investigated in this experiment. As shown in Fig. 1, the concentration of ammonium decreased rapidly in the first 9 h, with a maximum removal rate of 0.52 ± 0.01 mg $L^{-1} \cdot h^{-1}$ achieved in the initial 2 h. Afterwards, a gradually slowing down ammonium degradation was observed and eventually remained stable after 48 h. Simultaneously, the increase

Table 1 Experimental ranges of the independent variables

Independent variables	Factors, X_i	Ranges and levels		
		−1	0	1
B (mg/L)	X_1	0	0.05	0.1
Fe (mg/L)	X_2	0.05	0.125	0.2
Mn (mg/L)	X_3	0.1	0.2	0.3

Fig. 1 Ammonium removal and microbial growth performances of the strain HITLi7^T in basal culture medium at 4 °C, pH 7.0, and 100 rpm

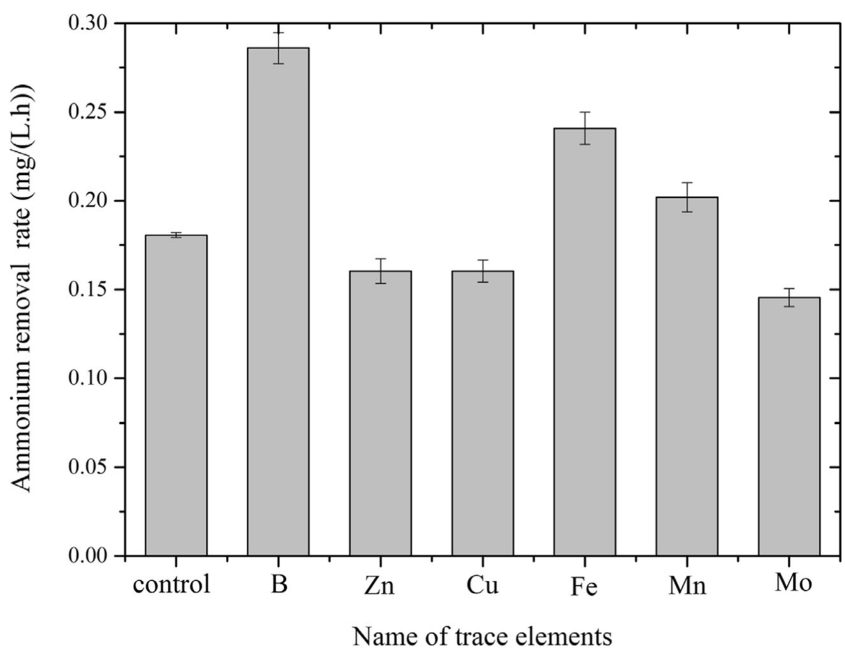


of OD_{600} shared the same trend over time. The cell OD_{600} increased dramatically in the first 9 h, then the increase gradually slowed down and eventually remained stable after 48 h. This implied that the growth of strain HITLi7^T synchronized with ammonium removal at low temperature. A similar relationship between cell growth and ammonium removal of stain AN-1 (Qu et al. 2015) was observed at 10 °C. Meanwhile, there was a lack of a lag period in ammonium removal of strain HITLi7^T at 4 °C, indicating that the shaking experiment could be well described by a zero-order reaction. The same phenomenon that ammonium degradation was rapid at the beginning was also reported for strain HA2 at 10 °C (Yao et al. 2013).

Effects of trace elements on the ammonium removal capability of strain HITLi7^T

Since trace elements are essential for the growth and activity of microorganisms, it is indispensable to study the influence of trace elements on the ammonium removal ability of strain HITLi7^T. *Acinetobacter harbinensis* HITLi7^T was cultivated in basal culture medium with the addition of different trace elements (0.1 mg L^{-1}) at 4 °C, pH 6.0, with 100 rpm shaking (DO ≈ 3.5 mg L^{-1}). The ammonium concentration was tested after 4 h. As shown in Fig. 2, the ammonium concentration had an obvious decrease in 4 h under the application of strain HITLi7^T. The addition of

Fig. 2 Effects of different trace elements on the ammonium removal rate of strain HITLi7^T. Cultivations were maintained for 4 h in basal culture medium with different additives at 4 °C, pH 7.0, and 100 rpm



0.1 mg L^{-1} Cu, Zn, or Mo showed no remarkable impacts on the ammonium removal efficiency and the other three elements had an obvious promoting effect. The ammonium removal rate could reach up to 0.29 mg $L^{-1}·h^{-1}$ (approximately 1.6 times that of the control) with the addition of boron (0.1 mg L^{-1}). The presence of Fe and Mn yielded a maximum ammonium removal rate of 0.24 mg $L^{-1}·h^{-1}$ and 0.20 mg $L^{-1}·h^{-1}$, respectively. Both of them were higher than the control. In the studies of *Providencia rettgeri* strain YL (Zhao et al. 2010) and *Alcaligenes faecalis* strain NR (Zhao et al. 2017), the ammonium degradation ability was highly promoted by the addition of 10 mg L^{-1} Mn, Zn, and Mg. The different results might be related to the bacteria types and all these studies demonstrated that trace elements did have a significant influence on ammonium removal.

Fe was one of the essential trace elements for microorganisms and is considered as an active site of AMO. Many proteins, especially some enzymes, such as catalase, peroxidase, superoxide dismutase, and ribonucleotide reductase, were Fe-containing proteins. Fe and related compounds were significant enzyme cofactors (Guerinot 1994). Whittaker et al. (2000) found that more than 2% of the genes in *Nitrosomonas europaea* were coded for heme synthesis and heme-containing proteins and for proteins with Fe-S centers, indicating the value of Fe to the energy metabolism of ammonia-oxidizing bacteria. However, it was also reported that Fe (II) or Fe (III) showed no stimulating effect on AMO activity nor bacteria growth during a pure *Nitrosomonas europaea* cultivation (Ensign et al. 1993). Converse results were obtained in this paper; with the presence of 0.1 mg L^{-1} Fe, the ammonium removal rate of strain HITLi7^T was increased by 33% in a pure culture.

Tekerlekopoulou and Vayenas (2007) found that manganese was the rate-limiting pollutant in optimal ammonia removal efficiency in trickling filters. Some researchers also found that ammonium removal could be promoted by utilizing an Fe–Mn co-oxide filter film to remove ammonium and Mn simultaneously (Guo et al. 2017). According to these results, Mn might play a role that potentially inhibits or stimulate the ammonium removal activity of nitrifiers. Then, in this study, a stimulating effect was demonstrated by the result that the ammonium removal rate of strain HITLi7^T was increased by adding 0.1 mg L^{-1} Mn.

In spite of the fact that B was not involved in the structure and composition of enzymes, many studies showed that B could affect the activity and have physiological and biochemical effects of many enzymes (Kobayashi et al. 1996). In addition, B had a certain role in maintaining the stability of cell membrane (Novati and Zannini 1957). These observations might explain why B had a great stimulating effect on the ammonium removal ability of strain HITLi7^T at low temperature.

Effects of the concentration of trace elements on the ammonium removal capability of strain HITLi7^T

The aforementioned data showed that B, Fe, and Mn had a positive influence on the ammonium removal capability of strain HITLi7^T. The influence of the concentration of three effective elements on the ammonium degradation ability of strain HITLi7^T was investigated in the shaking cultures of duration 2 h, as shown in Fig. 3. When the concentration of B was 0.05 mg L^{-1}, the removal rate reached 0.56 mg $L^{-1}·h^{-1}$. However, as the concentration increased to 0.2 mg L^{-1}, the

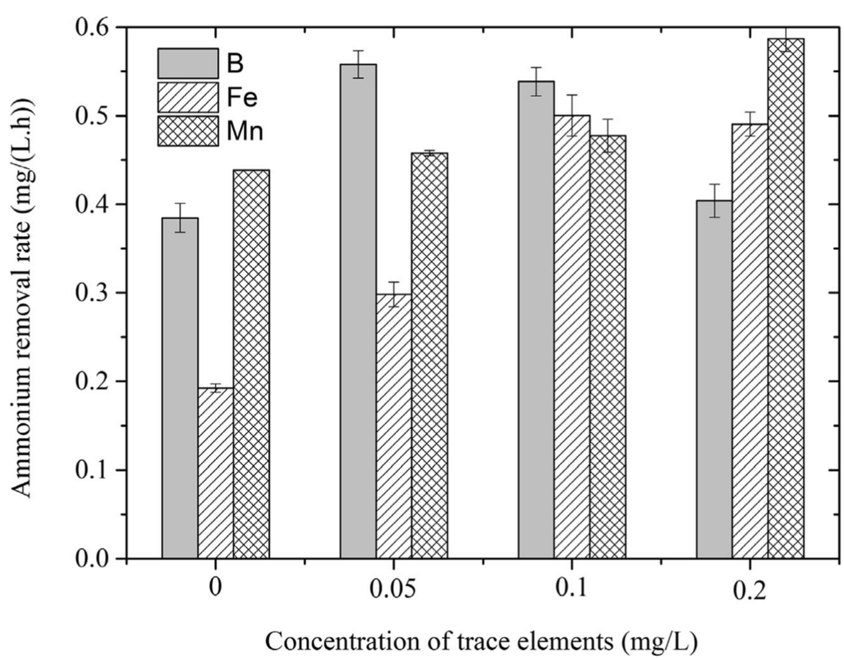


Fig. 3 Effects of the concentration of trace elements on the ammonium removal rate of strain HITLi7^T. Cultivations were maintained for 2 h in basal culture medium with different additives at 4 °C, pH 7.0, and 100 rpm

removal rate decreased to 0.40 mg $L^{-1}\cdot h^{-1}$, which was only marginally higher than the control group (0.38 mg $L^{-1}\cdot h^{-1}$). A low concentration of B could motivate ammonium degradation at low temperature. On the other hand, when the concentration of Fe increased from 0.05 mg L^{-1} to 0.1 mg L^{-1}, the ammonium removal rate increased rapidly from 0.30 mg $L^{-1}\cdot h^{-1}$ to 0.50 mg $L^{-1}\cdot h^{-1}$. Despite a slight reduction observed with a further increase to 0.2 mg L^{-1}, it was still higher than the control rate of 0.19 mg $L^{-1}\cdot h^{-1}$. As the concentration of Mn was gradually increased from 0 mg L^{-1} to 0.2 mg L^{-1}, the degradation rate increased consequently and reached a maximum value of 0.59 mg $L^{-1}\cdot h^{-1}$. It was the higher concentrations of Fe and Mn that had an obvious promotion of the ammonium removal ability of strain HITLi7^T. According to the results, the most suitable concentrations of B, Fe, and Mn for strain HITLi7^T were near 0.05, 0.1, and 0.2 mg L^{-1}, respectively.

The most appropriate trace elements concentrations differed among microorganisms. At present, only a few studies have paid attention to the potential effect of B on the ammonium removal of nitrifiers at low temperature. In terms of Fe, it was reported that the maximum cell mass could be reached with the addition of 0.56 mg L^{-1} Fe (Wei et al. 2006). Concentrations ranging from 0.56 mg L^{-1} to 14 mg L^{-1} Fe can lead to a normal growth of *Nitrosomonas europaea*, while 67.2 mg L^{-1} Fe severely inhibited its growth (Keyer et al. 1995). In this study, the best ranges of Fe, B, and Mn concentrations for strain HITLi7^T to remove ammonium were 0.05–0.2, 0–0.1, and 0.1–0.2 mg L^{-1}, respectively. According to these ranges, 0.125 mg L^{-1} Fe, 0.05 mg L^{-1} B, and 0.2 mg L^{-1} Mn were used as the center points of response surface experiments.

Optimization of the optimal composition of trace elements

On the basis of single-factor experiments, B, Fe, and Mn were selected as the significant variables, and the ammonium removal rate was the response, which means that B, Fe, and Mn had significant influence on the ammonium removal rate of strain HITLi7^T. According to the experiment design of Design-Expert, 17 experiments were conducted to detect the optimal formulation of the three effective elements. The high and low levels of each variable are shown in Table 2. Under the experimental conditions, the ammonium removal rate ranged from 0.25 mg $L^{-1}\cdot h^{-1}$ to 0.60 mg $L^{-1}\cdot h^{-1}$.

The analysis of variance (ANOVA) for the regression parameters are shown in Table 3. *p*-Values less than 0.0500 indicate that the model terms are significant. In this case, the linear coefficients (X_1, X_2, and X_3), interaction term coefficients (X_1X_3 and X_2X_3), and the coefficients of the quadratic term (X_1^2, X_2^2, and X_3^2) were significant. All of them exerted single and multiple effects on the response. According to these data, a second-order polynomial equation model was applied to fit the central composition models. Equation (2) shows the equation of coded factors for the response, where Y is the predicted ammonium removal rate, and X_1, X_2, and X_3 are the coded values of B, Fe, and Mn, respectively. As shown in

Table 2 Three-factor central composite design (CCD) matrix and the value of response

No.	Real variables, X_i			Response	
	B (mg/L)	Fe (mg/L)	Mn (mg/L)	Ammonium removal rate ($mg\cdot L^{-1}\cdot h^{-1}$)	
	X_1	X_2	X_3	Observed	Predicted
1	0.05	0.05	0.1	0.48	0.48
2	0.05	0.125	0.2	0.50	0.52
3	0.05	0.125	0.2	0.50	0.52
4	0.05	0.2	0.3	0.42	0.42
5	0.1	0.125	0.3	0.58	0.58
6	0	0.125	0.1	0.56	0.56
7	0.05	0.125	0.2	0.53	0.52
8	0.1	0.2	0.2	0.41	0.41
9	0.1	0.05	0.2	0.37	0.39
10	0.05	0.2	0.1	0.43	0.44
11	0	0.125	0.3	0.35	0.37
12	0.1	0.125	0.1	0.60	0.59
13	0.05	0.125	0.2	0.55	0.52
14	0.05	0.05	0.3	0.31	0.30
15	0.05	0.125	0.2	0.49	0.52
16	0	0.2	0.2	0.32	0.31
17	0	0.05	0.2	0.25	0.25

Table 3 Analysis of variance (ANOVA) for the ammonium removal rate according to the response surface quadratic model

Source	Statistical analysis				
	Sum of squares	df	Mean square	*F*-Value	*p*-Value
Model	0.165739	9	0.018415	37.07101	< 0.0001
X_1	0.028668	1	0.028668	57.71011	0.0001
X_2	0.003677	1	0.003677	7.401001	0.0297
X_3	0.02018	1	0.02018	40.62394	0.0004
X_1X_2	0.000212	1	0.000212	0.426165	0.5347
X_1X_3	0.009216	1	0.009216	18.55217	0.0035
X_2X_3	0.005929	1	0.005929	11.93531	0.0106
X_1^2	0.004061	1	0.004061	8.175636	0.0244
X_2^2	0.087744	1	0.087744	176.6311	< 0.0001
X_3^2	0.006642	1	0.006642	13.37065	0.0081
Residual	0.003477	7	0.000497		
Lack of fit	0.001043	3	0.000348	0.571036	0.6634
Pure error	0.002435	4	0.000609		
Cor. total	0.169216	16			

the equation, the main effect belonged to X_1, whose coefficient was the highest, at +0.06. The most significant interaction effect was X_1X_3, with a coefficient of +0.048, and the highest square effect of the factors pertained to X_2^2, with a coefficient of −0.14:

$$Y = 0.52 + 0.06X_1 + 0.021X_2 - 0.05X_3 - 7.275 \times 10^{-3}X_1X_2 + 0.048X_1X_3 + 0.039X_2X_3 - 0.031X_1^2 - 0.14X_2^2 + 0.04X_3^2 \quad (2)$$

The *F*-value of the model was 37.07, which implied that the model was significant, and the lack of fit *F*-value was 0.57, which implied that the lack of fit was not significantly relative to the pure error. Non-significant lack of fit is expected (Im et al. 2012). The value of the regression coefficient R^2 was 0.9795, which indicated that 98.0% variations for ammonium removal was caused by the independent variables. The "adjusted R^2" and the "predicted R^2" were 0.9530 and 0.8789, respectively, indicating that the model was credible.

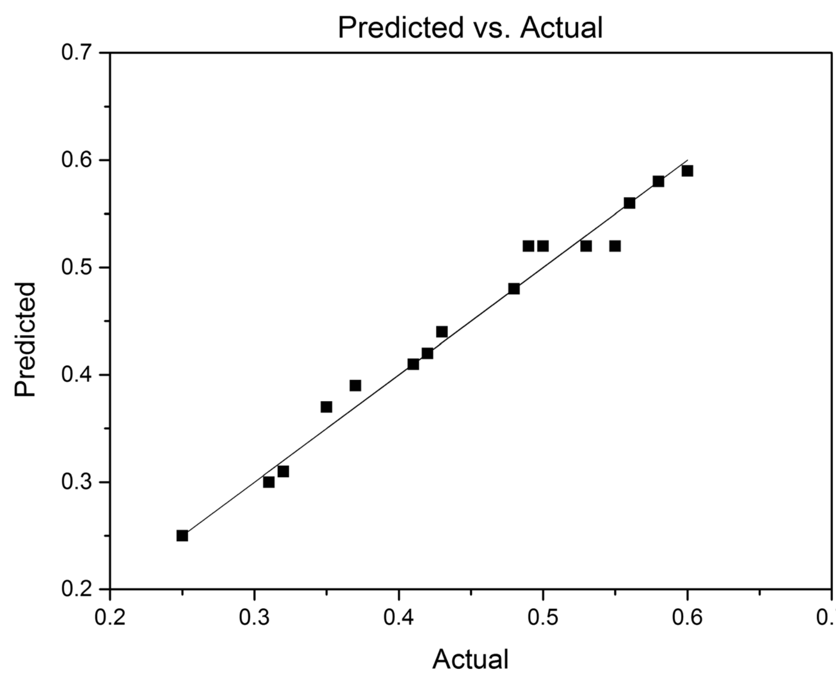


Fig. 4 Comparison between experimental results and predicted values by the proposed model

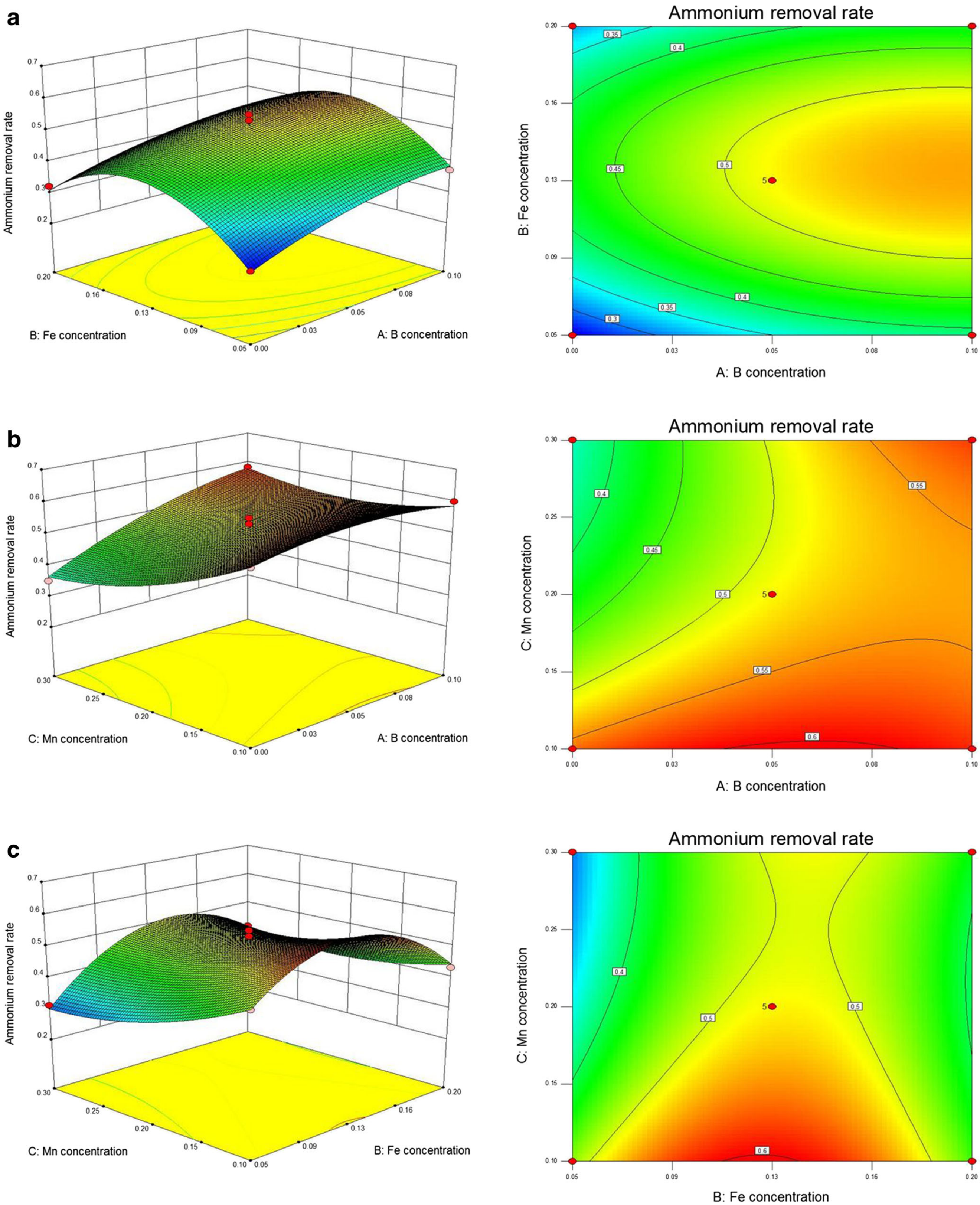


Fig. 5 Response surface (3D) and contour (2D) map for the ammonium removal rate between independent variables. **a** The interactional effects of B concentration and Fe concentration on the ammonium removal rate. **b** The interactional effects of B concentration and Mn concentration on the ammonium removal rate. **c** The interactional effects of Fe concentration and Mn concentration on the ammonium removal rate

Table 4 Effects of trace elements on the extracellular polymeric substances (EPS) secretion of strain HITLi7^T

Addition mode	a	b	c	d	e	f	g	Control
Proteins ratio (%)	51.0	53.7	51.8	55.3	50.7	58.5	50.1	56.4
Polysaccharides ratio (%)	49.0	46.3	48.2	44.7	49.3	41.5	49.9	43.6

a: 0.05 mg L^{-1} B
b: 0.15 mg L^{-1} B
c: 0.1 mg L^{-1} Fe
d: 0.3 mg L^{-1} Fe
e: 0.2 mg L^{-1} Mn
f: 0.6 mg L^{-1} Mn
g: 0.064 mg L^{-1} B, 0.12 mg L^{-1} Fe, and 0.1 mg L^{-1} Mn

The closer the value is to 1.0, the better the predicted value. “Adequate precision” measures the signal to noise ratio. Normally, a ratio greater than 4 is considered desirable. In this study, the “adequate precision” value was 19.582, which suggested that the model could be used to navigate the design space.

Figure 4 shows a comparison between the predicted and actual values of the response. The predicted values were calculated by the model mentioned above. As presented in Fig. 4, these two kinds of values clustered around the diagonal line and there was no large differences between the predicted and actual values, which indicated that the model fitted well and could be used to predict outcomes.

RSM was further applied to analyze the interactions of the three elements and their optimal levels for the ammonium removal rate. The three-dimensional response surface curves and contour plots constructed by the relationship of the response (ammonium removal rate) and any two independent variables, while maintaining the other variable at their zero level, are shown in Fig. 5a–c.

As shown in Fig. 5a, the ammonium removal rate of strain HITLi7^T had a positive correlation with B. The ammonium removal rate reached a peak level when the concentrations of B and Fe were 0.064 mg L^{-1} and 0.12 mg L^{-1}, respectively. The three-dimensional response surface curves and contour plots in Fig. 5b indicated that a low concentration of Mn led to a higher ammonium removal rate. At the central condition of Fe, the maximum ammonium removal was obtained under the conditions of 0.064 mg L^{-1} B and 0.1 mg L^{-1} Mn. Figure 5c illustrates the variation of ammonium removal with the change of Fe and Mn concentrations. Mn provided a negative effect, and the maximum rate would be reached when the concentrations of Fe and Mn were 0.12 mg L^{-1} and 0.1 mg L^{-1}, respectively.

The experimental data were fitted to the aforementioned equation, and the optimal conditions for the maximum ammonium removal rate of strain HITLi7^T were predicted to be 0.064 mg L^{-1} B, 0.12 mg L^{-1} Fe, and 0.1 mg L^{-1} Mn. The maximum rate of 0.61 mg $L^{-1}\cdot h^{-1}$ was proposed by the model.

In order to demonstrate the model’s authenticity, strain HITLi7^T was cultured in basal medium with optimized trace elements solution. The basal culture without trace elements solution served as the control. The ammonium removal rate of strain HITLi7^T after 2 h of cultivation was 0.59 ± 0.04 mg L^{-1}, marginally lower than the predicted value of 0.61 mg L^{-1}. Nonetheless, it was 2.03 times higher than the control value of 0.29 ± 0.03 mg L^{-1}.

Effects of trace elements on the EPS secretion of strain HITLi7^T

Trace elements at appropriate concentrations were key factors for the growth response and EPS production of bacteria and were always considered as significant coenzymes for enzyme catalytic action, especially enzymes that utilized ATP and synthesized DNA and RNA (Reeslev and Jensen 1995; Tang et al. 2008). The EPS secretion of strain HITLi7^T was detected under different trace elements additions. As shown in Table 4, the addition modes causing a high ammonium removal rate always resulted in a high polysaccharides ratio. When the addition mode was the optimal composition, the ammonium removal rate reached the highest of 0.59 mg $L^{-1}\cdot h^{-1}$ after 2 h of cultivation, while the polysaccharides ratio reached the highest value of 49.9%. The same results were observed in the single-factor experiments. With the addition of 0.05 mg L^{-1} B, the ammonium removal rate and polysaccharides secretion ratio reached the highest values of 0.56 mg $L^{-1}\cdot h^{-1}$ and 49.0%, respectively. The addition mode of 0.10 mg L^{-1} Fe caused the maximum removal rate (0.50 mg $L^{-1}\cdot h^{-1}$) and polysaccharides secretion ratio (48.2%) simultaneously. In terms of Mn, a concentration of 0.2 mg L^{-1} that resulted in the maximum removal rate caused the greatest polysaccharides secretion of 49.3%.

So far, only a limited number of articles studied the microbial adsorption of ammonium. Nielsen (1996) first found that ammonium adsorption did take place in activated sludge, which consists mainly of bacterial cells and EPS with a net negative surface charge. Ammonium adsorption also occurred in the biofilm, whose composition was similar to EPS, mainly protein and polysaccharide (Wik 1999). It was reported that 9% and 20% of the influent ammonium load were removed by microbial adsorption when the influent ammonium concentrations were 52 mg L^{-1} and 37 mg L^{-1}, respectively (Temmink et al. 2001). Polysaccharides were a significant component of EPS and always classified with homo- and heteropolysaccharides. Although the homopolysaccharides were neutral, most of the heteropolysaccharides, composed of a large quantity of glucuronic acid, galacturonic acid, and mannuronic acid, were polyanionic (Sutherland 1990). It was reported that the polysaccharide-containing carboxyl or uronic acid had the function of absorbing or supplying nutrients and inorganic salts for the cells (Pal and Paul 2008). Since most polysaccharides were negatively charged, the more polysaccharides strain HITLi7^T secreted, the more positively charged ammonia ions were adsorbed, causing a high ammonia removal rate. This might be the reason why more polysaccharides secretion could cause higher ammonia degradation ability of strain HITLi7^T.

Conclusion

This study was conducted to optimize the composition of trace elements in basal medium to promote the ammonium removal rate of strain HITLi7^T under pure cultivation at low temperature. Six elements were tested at a concentration of 0.1 mg L^{-1} and three elements (B, Fe, and Mn) appeared to have stimulating effects. Experiments were further carried out to precisely acquire the optimal concentration ranges of the three effective elements. In order to optimize the formulation of the trace elements (B, Fe, and Mn), response surface methodology (RSM) experiments were conducted. The optimal levels of each variable were as follows: 0.064 mg L^{-1} B, 0.12 mg L^{-1} Fe, and 0.1 mg L^{-1} Mn. An increase (2.06-fold) of the ammonium removal rate was achieved by the addition of the optimal composition. The results also showed that the polysaccharides ratio changed with different trace elements addition modes. Generally speaking, the optimal composition could increase the polysaccharides secretion ratio and ammonium removal rate of strain HITLi7^T at the same time. The optimized trace elements found in this study might propose a new approach to increase the ammonium degradation ability of strain HITLi7^T at low temperature in both pure cultivation and practical biological water treatment systems dealing with surface water.

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