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Abstract. Efficient simultaneous nitrogen removal and phosphorus accumulation microorganisms are great significance for the purification treatment of high salt wastewaters containing N and P. In this paper, the processes of nitrogen removal and phosphorus accumulation of *Halomonas* sp. H12 strain under high salt conditions were investigated. The optimal conditions of nitrogen removal and phosphorus accumulation were examined separately by using response surface methodology. The results showed that the *Halomonas* sp. H12 strain had high efficiency competent of nitrogen removal and phosphorus accumulation under high salt conditions under both growth stage and non-growth stage. Within 108 h, the rates of the nitrogen removal and phosphorus accumulation reached 78.51% and 49.18% respectively. During 0-84 h, the strain was in the growth stage, and its nitrogen removal rate and phosphorus accumulating rate increased gradually. At 84-108 h, the strain then entered the non-growth stage with its growth reached the maximum. Interestingly in this equilibrium phase, high efficient nitrogen removal and phosphorus accumulation were still carried out with the prolongation of time. Results from this work are of great significance for the purification of industrial wastewaters containing N, P and high salt.

1. Introduction

The discharge of wastewater containing nitrogen and phosphorus will result in water eutrophication, cause water blooms and red tide [1]. In addition to nitrogen or phosphorus, some industrial wastewater(such as printing and dyeing wastewaters, tannery, seafood breeding and processing, pesticide, medicine wastewaters, or discharged sewage that is formed after we use seawater as life water, etc) has the high salt (NaCl ≥ 2%) characteristics [2-5]. It is one of the effective ways to solve the problem of this kind of wastewater by the microbial remediation technology with strong adaptability, low cost and no secondary pollution and wide application. However, high salt has an inhibitory effect on microbial growth and metabolism [6]. Up to now, there is no report about the simultaneous removal of nitrogen and phosphorus by high salt. It is of great significance to filter bacterial strains that are efficiently and simultaneously capable of nitrogen removal/phosphorus accumulation.

In this paper, a preliminary study was conducted to screen out *Halomonas* sp. H12, a novel strain capable of simultaneous nitrogen removal and phosphorus accumulation from a sediment of a salt pond in Dalian, Liaoning Province, China. In this paper, response surface methodology was used to optimize the conditions of nitrogen removal and phosphorus accumulation of *Halomonas* sp. H12, respectively. At the same time, under the condition of comprehensive optimization, the process of

simultaneous nitrogen removal and phosphorus accumulation of the strain was investigated. It provides theoretical and methodological basis for the treatment of nitrogen, phosphorus and high salt wastewaters.

2. Materials and methods

2.1. Strain sample

Laboratory screening and identification of preserved strains *Halomonas* sp. H12.

2.2. Culture medium

Activated medium (g/L): monosodium glutamate 30, yeast powder 5, K_2HPO_4 3, $MgSO_4·7H_2O$ 0.4, $MnSO_4·H_2O$ 0.01, NaCl 60. The medium was autoclaved at 121℃ for 20 min. (the inoculation quantity is 1%)

Polyphosphate medium (g/L): glucose 20, monosodium glutamate 20, $(NH_4)_2SO_4$ 10, yeast powder 0.5, phosphate $K_2HPO_4·3H_2O$ 9, KH_2PO_4 3) 12, $MgSO_4·7H_2O$ 0.4, $MnSO_4·H_2O$ 0.01, NaCl 60, Trace mineral solution (EDTA-2Na 63.7, $ZnSO_4$ 2.2, $CaCl_2$ 5.5, $MnCl_2·4H_2O$ 5.1, $FeSO_4·7H_2O$ 5, $Na_2MO_4·2H_2O$ 1.1, $CuSO_4·5H_2O$ 1.6, $CoCl_2·6H_2O$ 1.6) 2 mL. pH 7.2, 121℃, The medium was autoclaved at 121℃ for 20 min. Glucose is sterilized separately, 15 min sterilization.

Nitrogen removal medium (g/L): glucose 20, monosodium glutamate 10, sodium succinate 10, $(NH_4)_2SO_4$ 10, yeast powder 0.5, phosphate ($K_2HPO_4·3H_2O$ 9, KH_2PO_4 3) 12, $MgSO_4·7H_2O$ 0.4, $MnSO_4·H_2O$ 0.01, NaCl 60, Trace mineral solution 2 mL. pH 7.2, 121℃, the medium was autoclaved at 121℃ for 20 min. Glucose is sterilized separately, 15 min sterilization.

2.3. Determination of ammonia concentration

Total inorganic nitrogen (TN) included NH_4^+-N, NH_2OH-N, NO_2^--N, NO_3^--N. The nitrogen (N) removal rate was defined as the percentage reduction in total inorganic N of the total inorganic N in the nitrogen removal system. The nitrogen removal rate = $(TN_0 - CN - TN_t)/(TN_0 - CN) \times 100\%$. NT_0 was the total inorganic nitrogen at the initial nitrogen removal, CN was the total cell nitrogen, TNt was the total inorganic nitrogen at a time during the nitrogen removal process.

- *Determination method of NH_4^+-N.* NH_4^+-N was determined by Nessler's reagent method [7].
- *Determination method of NO_2^--N.* NO_2^--N was determined by diazotization-coupling reaction method [7].
- *Determination method of nitrate NO_3^--N.* NO_3^--N was determined by zinc-cadmium reduction method [8].

2.4. Determination of inorganic phosphorus concentration

The inorganic phosphorus concentration was determined by photocolorimetrical method with ammonium molybdate [9].

2.5. Response surface methodology design

In this study, the response surface method was used to optimize the conditions of nitrogen removal and phosphorus accumulation, and conduct modeling and factor effects assessment for significant evaluation [10]. Through the Plackett-Burman experimental design [11], the effects of 7 factors on nitrogen removal and phosphorus accumulation were analyzed. Three factors affecting the nitrogen removal rate, i.e. sodium succinate, ammonium sulfate, and pH were screened out, and phosphate, sodium chloride, and time were screened out to be the significant factors affecting phosphorus accumulation rate. Based on these results, the steepest climbing experiment was adopted to approach the maximum response area. Combined with the Central composite rotatable design (CCD) and response surface analysis, the best level range of the key factors affecting nitrogen removal rate and phosphorus accumulation rate was further studied [12]. Two regression equation models, which took the nitrogen removal rate and the phosphorus accumulation rate as the response values, were set up,

and the optimal conditions were analyzed.

3. Results and discussion

3.1. Denitrification response surface methodology optimization of halomonas sp. H12 strain

The response surface method was used to optimize *Halomonas* sp. H12 nitrogen removal conditions and establish a model of nitrogen removal. First of all, single factor experiments were carried out. Glucose(Glu), monosodium glutamate(MSG), Sodium succinate(ACSNa), Yeast(YE), $(NH_4)_2SO_4$, NaCl, pH were selected as the seven single factors, and Design expert 8.0 software was employed to set them to high, medium and low levels. According to the method of nitrogen removal test of SND for determining the nitrogen removal rate, the best concentrations of these seven factors were determined. Then Plackett-Burman test and analysis of variance were carried out and the results were shown in table 1. As can be seen from the table 1, the main factors affecting the nitrogen removal rate of *Halomonas* sp. H12 were as follows: pH (X_3), ammonium sulfate (X_2) and sodium succinate (X_1). Sodium succinate and pH showed a positive effect on the nitrogen removal rate of *Halomonas* sp. H12 strain, while ammonium sulfate exhibited a negative effect.

Table 1. Nitrogen removal Plackett-Burman test design and calculations.

Number	Glu (g/L)	Msg (g/L)	ACSNa (g/L)	YE (g/L)	$(NH_4)_2SO_4$ (g/L)	NaCl (g/L)	pH	Nitrogen removal rate (%)
1	15	20	15	0.1	20	60	9	58.60±0.91
2	15	10	15	0.1	20	60	7	47.80±0.78
3	25	20	15	0.1	10	30	9	36.21±0.79
4	25	10	15	1	20	30	7	44.25±1.85
5	25	20	5	1	20	60	7	23.14±0.10
6	25	10	5	0.1	20	30	9	37.17±0.25
7	15	20	5	1	20	30	9	48.29±1.00
8	15	10	5	0.1	10	30	7	45.02±0.16
9	15	10	5	1	10	60	9	57.69±0.15
10	15	20	15	1	10	30	7	47.73±0.42
11	25	10	15	1	10	60	9	27.48±0.41
12	25	20	5	0.1	10	60	7	57.24±1.70
Effect	2.76	0.66	9.42	-1.04	-11.52	3.70	12.52	
F	0.86	0.049	10.05	0.12	15.05	1.55	17.76	
Prob>F	0.406	0.836	0.034	0.743	0.018	0.281	0.014	

Based on the above results, a three-factor five levels steepest ascent experiment was design and the results were shown in table 2. At the sodium succinate and ammonium sulfate concentrations of 15.0 g/L and 13.0 g/L respectively and pH 8.4, the nitrogen removal rate reached the highest level 61.41%. Therefore, these conditions were selected as the CCD's center point. The experimental design and variance analyses were displayed in table 3-1 and table 3-2.

Table 2. Nitrogen removal rate steepest ascent test design and calculations.

Number	1	2	3	4	5
ACSNa (g/L)	5.00	10.00	15.00	20.00	25.00
$(NH_4)_2SO_4$ (g/L)	21.00	17.00	13.00	9.00	5.00
pH	7.2	7.8	8.4	9.0	9.6
Nitrogen removal rate (%)	25.93±0.57	47.31±1.87	61.41±1.96	60.15±1.05	12.40±0.41

Table 3-1. Design and result of nitrogen removal rate of *Halomonas* sp. H12 strain by central combination test.

Number	ACSNa X_1 (g/L)	$(NH_4)_2SO_4$ X_2 (g/L)	pH X_3	Nitrogen removal rate (%)
1	20	17	9	28.65±1.09
2	15	13	8.4	51.21±2.01
3	15	13	8.4	60.14±0.04
4	10	17	9	32.65±0.15
5	10	9	7.8	30.55±0.98
6	10	17	7.8	24.51±0.99
7	10	9	9	34.64±1.00
8	15	6.3	8.4	33.78±0.88
9	15	19.7	8.4	25.08±0.60
10	6.6	13	8.4	25.25±0.93
11	15	13	8.4	51.31±1.12
12	23.4	13	8.4	28.62±1.09
13	15	13	8.4	51.01±0.32
14	15	13	7.4	16.79±0.70
15	15	13	8.4	41. 98±1.08
(Cont)				
16	15	13	9.4	26.88±1.16
17	15	13	8.4	51.06±0.06
18	20	9	9	31.10±0.88
19	20	17	7.8	21.92±0.82
20	20	9	7.8	26.06±0.87

Table 3-2. Variance analysis of nitrogen removal rate of *Halomonas* sp. H12 strain by center combination test results.

Source	Degree of freedom	Variance	F	Prob>F
Model	9	2.698E+005	56.89	< 0.0001
X_1	1	5748.95	0.81	0.3905
X_2	1	81279.71	4.92	0.0508
X_3	1	54599.79	19.20	0.0014
X_1X_2	1	1841.03	0.25	0.6262
X_1X_3	1	76081.20	0.028	0.8709
X_2X_3	1	83836.94	1.93	0.1954
X_{12}	1	8.885E+005	230.62	< 0.0001
X_{22}	1	1.225E+006	123.80	< 0.0001
X_{32}	1	3.944E+005	223.31	< 0.0001
Residual	10	6560.35		
Lack of fit	10	5.48		
Pure error	5	10.93	559.33	< 0.0001
Cor total	5	0.020		
R^2=0.9808				

The results of nitrogen removal experiments of *Halomonas* sp. H12 strain were analyzed by design expert 8.0 software, and a quadratic equation model was obtained as:

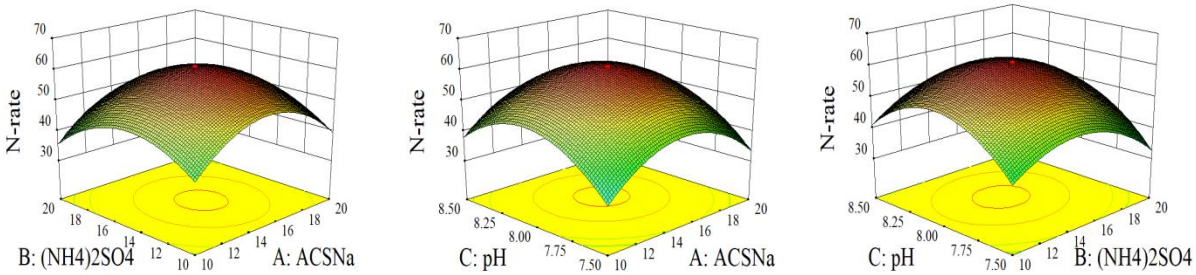
$$Y(g/L) = 51.06 + 0.57X_1 - 1.41X_2 + 2.77X_3 + 0.42X_1X_2 - 0.14X_1X_3 + 0.15X_2X_3 - 9.36X_1^2 - 6.86X_2^2 - 9.21X_3^2 \quad (1)$$

The variance of the model P < 0.0001, suggesting that the impact of the items in the model was significant. As shown in tables 3-1 and 3-2, the model had a confidence of 99.99%, indicating significant regression equation and a higher degree of fit. The regression equation predicted value was

close to the actual value, which can make relatively accurate prediction. Through the analysis and calculation, the coefficient of the nitrogen removal model of *Halomonas* sp. H12 strain was R^2 = 0.9808, which suggested that the model was in good agreement with the actual situation. Based on the above results the constructed model can be used to accurately predict the nitrogen removal rate of *Halomonas* sp. H12 strain.

3.2. Response surface optimization of denitrification conditions and the best point to find variables

The denitrification response surface response surface contour map is shown in figure 1, with each response surface separately representing the interaction between two independent variables.



(a) (b) (c)

Figure 1. Denitrification response surface contour map. (a) Effect of ACSNa and $(NH_4)_2SO_4$ interaction on N-rate; (b) Effect of pH and ACSNa interaction on N-rate; and (c) Effect of pH and $(NH_4)_2SO_4$ on N-rate.

From the response surface contour map analysis, the largest nitrogen removal rate can be obtained by optimizing the values of the three significant factors. In order to determine the maximum nitrogen removal rate, the first-order partial derivative of the regression equation was solved to make it equal to zero, and the results were as follows:

$$0.57 + 0.42X_2 - 0.14X_3 - 18.72X_1 = 0 \quad (2)$$

$$-1.41 - 0.42X_1 + 1.15X_3 - 13.72X_2 = 0 \quad (3)$$

$$2.77 - 0.14X_1 + 1.15X_2 - 18.42X_3 = 0 \quad (4)$$

The nitrogen removal rate equations were solved to obtain model extreme points: X_1 = -0.02735, X_2 = 0.089808, X_3 = -0.14464. Substituting into the regression equation, the predicted nitrogen removal rate was 61.08%. At this point, the concentrations of sodium succinate and $(NH_4)_2SO_4$ were 15.14 g/L and 14.47 g/L respectively, and the pH was 7.79.

3.3. Optimization of polyprophore response conditions of halomonas sp. H12 strain

The conditions of phosphorus accumulation ewere optimized according to method 2.5. The process and means of response surface analyses are the same as the nitrogen removal optimization analysis of section 3.1.

Regression analysis of the phosphorus accumulation test results of *Halomonas* sp. H12 by Design expert 8.0 software fitted quadratic equation (5) as follows:

$$Y(g/L) = 47.85 + 1.79X_1 + 1.13X_2 + 1.96X_3 - 0.76X_1X_2 - 0.49X_1X_3 - 3.03X_2X_3 - 5.19X_1^2 - 6.45X_2^2 - 5.26X_3^2 \quad (5)$$

Analysis results of the variance of the model in the confidence interval of 99% confidence level was 99.99%, indicating that the regression equation was significant, and has a high degree of fit. The regression equation predicted values were close to the actual values, indicative of a relatively accurate prediction. The coefficient of determination (R^2) of *Halomonas* sp. H12 strain was 0.9752, indicating

that the two models are in good agreement with the actual situation. The above results demonstrated that the model can be used to predict P-rate in *Halomonas* sp. H12 strain.

3.3.1. Response surface optimization of poly-phosphorus analysis and find the best point of variable. he polyphosphate rate response surface contour lines are depicted in figure 2, with each response surface representing the interaction between two independent variables.

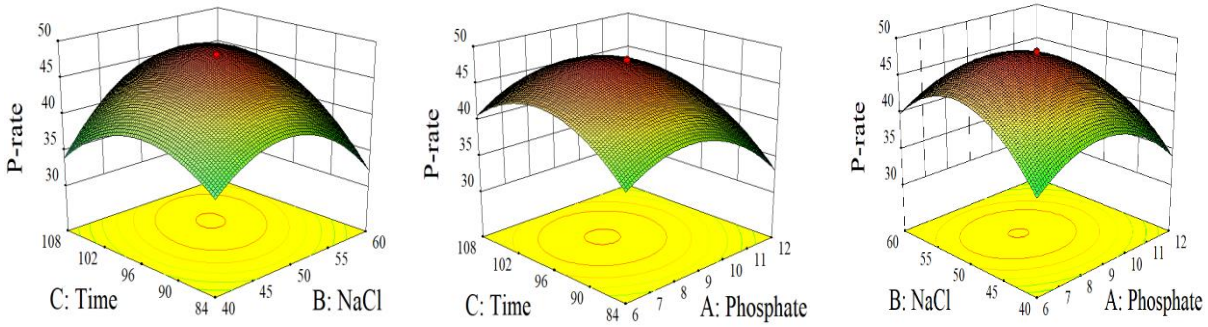


Figure 2. Polyphosphate rate response surface contour map. (a) Effect of NaCl and t-interaction on P-rate; (b) Effect of phosphate and t-interaction on P-rate; and (c) Effect of Phosphate and NaCl interaction on P-rate.

As seen from figure 2, there was a true maximum of the fitting surface, that is, all three factors should have an optimal value. In order to further determine the stability point of the maximum phosphorus accumulation rate, the first-order partial derivative of equation (5) was solved to make it equal to zero, and the results were as follows:

$$-1.79 - 0.76X_2 + 0.49X_3 - 10.38X_1 = 0 \quad (6)$$

$$1.13 - 0.76X_1 + 3.03X_3 - 12.9X_2 = 0 \quad (7)$$

$$1.96 + 0.49X_1 + 3.03X_2 - 10.52X_3 = 0 \quad (8)$$

The extremum points of the model equation were obtained as: X_1 = -0.173, X_2 = 0.150, X_3 = 0.221. Substituting equation (5), the predicted maximum phosphorous recovery rate was 46.48%. At this point, the concentrations of phosphate and sodium chloride were 8.48 g/L and 51.50 g/L respectively, and the phosphorus accumulation time was 98.65 h.

3.4. Comprehensive optimization of halomonas sp. *H12 nitrogen removal and phosphorus process*
Nitrogen removal and phosphorus accumulation response surface optimization results, combined with the optimization of the two factors, under the comprehensive conditions of the nitrogen removal and polyphosphate medium set, growth medium (g/L), as shown in table 4, optimization process shown in figure 3, it can be seen that the strain under the comprehensive conditions, at 108 hours, the rates of nitrogen removal and phosphorus accumulation of the strains were as high as 78.51% and 49.18%, respectively. The strain in 0 - 84 hours in the growth stage, the nitrogen removal rate and the rate of polyphosphorus increase accordingly. The strain entered the non-growth phase at 84 - 108 hours, its growth reached its maximum value. During this phase of equilibrium, the strain was still highly efficient in nitrogen removal and phosphorus accumulation enrichment over time. The *Halomonas* sp. H12 strain can nitrogen removal and phosphorus accumulation in both the growth phase and the non-growth phase, this provides an important theoretical basis for the future research on industrial high salt wastewater treatment.

Table 4. Responses of denitrifying and polyphosphorus to *Halomonas* sp. H12 strain optimization design.

Serial number	Glu (g/L)	MSG (g/L)	ACSNa (g/L)	$(NH_4)_2SO_4$ (g/L)	Phosp-hate (g/L)	pH	NaCl (g/L)	YE (g/L)	T (h)
Denitrification	20	20	15.14	14.47	12	7.97	60	0.5	72
Poly Phosphorus	20	10	-	10	8.48	7	51.50	0.5	98.65
Synthesis	20	15	15	12.50	8.50	8	50	0.5	96

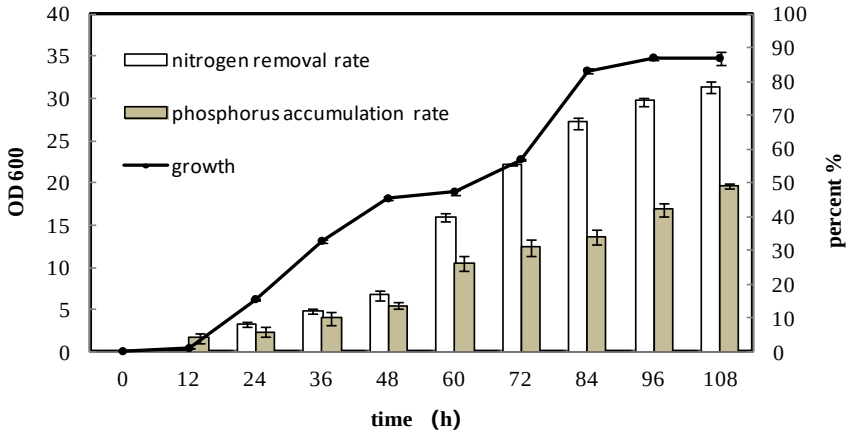


Figure 3. Processes of denitrification and polyphosphorus of *Halomonas* sp. H12 strain under comprehensive conditions.

4. Conclusions

The optimum conditions for nitrogen removal and phosphorus accumulation of the *Halomonas* sp. H12 strain were obtained by response surface method. Based on the Plackett-Burman experimental design, the nitrogen removal rate was affected by three factors, i.e. sodium succinate concentration, ammonium sulfate concentration, and pH. While the phosphate concentration, sodium chloride concentration, and time were the significant factors affecting the phosphorus accumulation. On the basis of that, the steepest climbing experiment was used to approach the maximum response area. Combined with the central composite experiment and response surface analysis, two regression equation models were established with the nitrogen removal rate and phosphorus accumulation rate as the response values respectively. The optimal conditions for nitrogen removal and phosphorus accumulation were obtained as follows： sodium succinate 15.14 g/L, ammonium sulfate 14.47 g/L, pH 7.97, phosphate 8.48 g/L, sodium chloride 51.50 g/L, phosphorus accumulation time 98.65 h. Under the above optimal conditions, the process of nitrogen removal-phosphorus accumulation was comprehensively studied, and experimental results showed that the *Halomonas* sp. H12 strain exhibited efficient nitrogen removal rate 78.51% and phosphorus accumulating rate 49.18% in high salt conditions. Results of this work are of great significance for the purification of high salt and high nitrogen and phosphorus wastewaters, and have broad application prospects in industrial high salt wastewater treatment.

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