Study on the Properties of *Halomonas* Urease and Optimization of its Production Conditions

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**Abstract.** It is of great significance to treat the wastewater of high salt urea by using the halophilic microorganism containing urease. In this paper, *Halomonas* containing urease was selected to study its urease properties and optimize its urease production conditions. *Halomonas* sp. H36 was selected, and its growth reached its peak at 36 h. The maximum value was 7.42, and the urease activity was 63.38 U. In the study on urease properties of this strain, when pH = 8, temperature was 50°C, salt concentration was 60 g/L, and urea concentration was 50 g/L, urease activity reached 137.52 U, increasing by 116.98%. It was found that the urease activity remained at 73.53% when the salt concentration reached 150 g/L. It was found by response surface method that glucose, ammonium sulfate and sodium chloride were the key factors affecting urease activity of the strain. After response surface optimization, the urease activity reached 93.628 U, increasing by 47.72%. This is of great significance to the wastewater treatment of high-salt urea and has a broad application prospect in the sewage treatment of ships.

1. Introduction

The unreasonable discharge of urea wastewater will promote eutrophication of coastal waters. High salt inhibits the growth and metabolism of microorganisms and activity of related enzymes, resulting in slow microbial growth and low pollutant removal efficiency[1]. Therefore, the application of traditional biological methods in the purification of wastewater containing high-salt urea is limited. In this paper, *Halomonas* strains capable of producing urease and tolerant to high salinity were screened and their urease properties and urease production conditions were studied, which is of great significance for the purification and treatment of wastewater containing high salinity urea.

In this paper, from the 13 strains of *Halomonas* stored in the laboratory, the strains with high urease activity *Halomonas* sp. H36 were screened out, and the effects of pH, temperature, salt concentration and urea concentration on the activity of *Halomonas* sp. H36 urease were investigated, and the urease production conditions were optimized by response surface method. It provides a theoretical and technical method for microbial treatment of wastewater from high salt urea plant.

2. Materials and methods

2.1. Medium

Growth medium (g/L): glucose 20, urea 15, yeast powder 0.5, KH₂PO₄·3H₂O 9, KH₂PO₄ 3, MgSO₄·7H₂O 0.4, MnSO₄·H₂O 0.01, NaCl 30, pH 7.2. It was sterilized at 121 °C for 20 min. Glucose was sterilized separately at 115 °C for 15 min. Urea is sterilized by separate filtration.

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Urease producing medium (g/L): glucose 15, monosodium glutamate 15, ammonium sulfate 5, urea 5, K2HPO4·3H2O 9, KH2PO4 3, MgSO4·7H2O 0.4, MnSO4·H2O 0.01, NaCl 30, microelement 2 mL/L, pH 7.2. It was sterilized at 121 °C for 20 min. Glucose was sterilized separately at 115 °C for 15 min. Urea is sterilized by separate filtration.

2.2. Urease activity determination method
The urease activity was determined by p-dimethylaminobenzaldehyde (PDAB) method[2-3]. The urea substrate of 40 g/L was mixed with the bacterial liquid, and the enzymatic hydrolysis reaction was carried out at 37 °C. The diluted urease hydrolysate was mixed with PDAB chromogenic agent. After 5 min of chromogenic reaction, the colorimetry was carried out with a spectrophotometer at the wavelength of 430 nm.

The activity of urease was defined as the millimole of urea hydrolysis per milliliter of bacterial liquid and per minute. Urease activity formula: \( U = (C_1 - C_2) \times N/(V \times T) \). \( U \): urease activity (mM/mL·min), \( C_1 \): urea concentration before enzymatic hydrolysis (g/L), \( C_2 \): urea concentration after enzymatic hydrolysis (g/L), \( T \): reaction time of enzymatic hydrolysis (min), \( V \): enzyme liquid product (mL), \( N \): 1000/60.

2.3. Experimental design of response surface method
According to the response surface method[4], In this study, the urease production conditions were optimized. The single factor test was used to determine factors and their levels. Through the two-level design Plackett-Burman experiment, the key factors affecting urease activity were found. The direction and step length of the key factors were determined by the steepest climb experiment, closing the optimal response area, combining with the experiments of central composite rotatable design (CCD) and response surface analysis research on the interaction of the key factors for further discussion, according to the urease activity in response to a quadratic regression equation model of value, it is concluded that the optimal conditions.

3. Results and discussions

3.1. Screening of Halomonas strain containing urease
The 13 \( H. \) strains stored in the laboratory were inoculated into the growth medium with urea as the sole nitrogen source, and their growth was measured. \( H. \) sp. H36 with the highest growth was selected, up to 7.42. And its urease activity reached its maximum value of 63.38 U at 36 h. The enzyme production curve of \( H. \) sp. H36 was obtained, as shown in Figure 1. The strain was identified as Halomonas taeanensis NY-3 by 16S rDNA.

![Figure 1. H. sp. H36 urease production curve.](image)

3.2. Properties of \( H. \) sp. H36 urease
The effects of pH, temperature (T), salt concentration (NaCl) and urea concentration (CO(NH2)2) on the activity of \( H. \) sp. H36 urease were investigated. The results are shown in Figure 2. Firstly, the effect of pH on urease activity was investigated (\( T = 37°C, \) NaCl = 0 g/L, CO(NH2)2 = 40 g/L). \( H. \) sp. H36 has high urease activity in pH 5-10. The urease properties of \( H. \) sp. H36 were studied by using...
buffer solution (citrate-trisodium citrate buffer solution with pH = 5, 6; Potassium dihydrogen phosphate-dipotassium phosphate buffer solution with pH = 7; Tris-HCl buffer solution with pH = 8 and 9). At pH = 8, urease activity reached 98.90 U. When pH = 10, the urease activity remained at 87.61%, indicating that the urease had certain alkali resistance.

The effect of temperature (T) on urease activity was investigated (pH = 8, NaCl = 0 g/L, CO(NH2)2 = 40 g/L). It can be seen that the highest urease activity was 111.96 U when T = 50℃. At 20-50℃, the urease activity increased significantly, while at 50-70℃, the urease activity decreased steadily as the temperature increased.

The effect of salt concentration (NaCl) on urease activity (pH = 8, T = 50℃, CO(NH2)2 = 40 g/L) was investigated. When the NaCl = 60 g/L, the urease activity reached a maximum of 118.00 U. It was also found that when NaCl > 90 g/L, the urease activity tended to be stable. When the salt concentration reached 150 g/L, 73.53% urease activity was retained. This indicates that the urease has a certain salt tolerance.

The effect of urea concentration on urease activity was investigated (pH = 8, T = 50℃, NaCl = 60 g/L). When CO(NH2)2 = 50 g/L, the urease activity was the highest, reaching 137.52 U.

![Figure 2. The property of H. sp. H36 urease.](image)

Note: the x-coordinate of (a) (b) (c) (d) is the influencing factor, and the y-coordinate is the urease activity (U). (a) Effect of pH on urease activity; (b) Effect of temperature on urease activity; (c) Effect of NaCl on urease activity; (d) Effect of CO(NH2)2 on urease activity.

When pH = 8, temperature was 50℃, salt concentration was 60 g/L, and urea concentration was 50 g/L, the urease of H. sp. H36 at 137.52 U, increasing by 116.98%.

### 3.3. Optimization of H. sp. H36 urease production conditions

#### 3.3.1. Plackett-Burman Experimental design and results

First, a single factor experiment was carried out. Seven single factors including A-glucose, B-glutamic acid monosodium, C-ammonium sulfate, D-urea, E-yeast powder, F-pH, and G-sodium chloride were selected. The Design Expert. V8.0.6 software was used to set them to three levels respectively, and then Plackett-Burman experiment was carried out. The experimental design and results are shown in Table 1.

The results showed that the model was reasonable $P = 0.0018 < 0.05$. After F test, the P values of glucose (A), ammonium sulfate (C) and sodium chloride (G) were 0.0001, 0.0119 and 0.0149, respectively. Which were all less than 0.05. The above three factors were all key factors. The results showed that the urease activity of A and C showed positive effects, and G showed negative effect. Therefore, the concentration of A and C should be appropriately increased, while the concentrations of G should be reduced.

| Number | A (g/L) | B (g/L) | C (g/L) | D (g/L) | E (g/L) | F (g/L) | G (g/L) | Urease (U) |
|--------|---------|---------|---------|---------|---------|---------|---------|------------|
| 1      | 15      | 25      | 3       | 7       | 1.5     | 7       | 60      | 45.58      |
| 2      | 15      | 25      | 7       | 3       | 1.5     | 9       | 60      | 50.64      |
| 3      | 25      | 25      | 7       | 3       | 0.5     | 7       | 60      | 88.3       |
| 4      | 15      | 15      | 3       | 3       | 0.5     | 7       | 30      | 46.84      |
| 5      | 25      | 25      | 3       | 7       | 1.5     | 9       | 30      | 85.14      |

Table 1. Plackett-Burman test results.
3.3.2. Experiment of the steepest climb

To approximate the optimal response region, determine the direction and step size. According to Plackett-Burman test results, the steepest climb experiment was carried out. The concentration of A and C was gradually increased, and the concentration of G was gradually decreased, and 5 groups of experiments were designed with 5 g/L as the step and the experimental.

The results showed that the urease activity reached 90.65 U, when the A, C and G concentrations were 40 g/L, 15 g/L and 35 g/L, respectively. The concentration was used as the central point of the Central composite rotatable design.

3.3.3. Central composite rotatable design

The design and results of the central composite rotatable design are shown in Table 2., and the analysis of variance is shown in Table 3.

### Table 2. Design and results of center combination test.

| Number | A(g/L) | C(g/L) | G(g/L) | Urease(U) |
|--------|--------|--------|--------|-----------|
| 1      | 40.00  | 15.00  | 35.00  | 89.51     |
| 2      | 35.00  | 10.00  | 30.00  | 61.71     |
| 3      | 40.00  | 6.59   | 35.00  | 75.27     |
| 4      | 40.00  | 15.00  | 35.00  | 92.90     |
| 5      | 45.00  | 20.00  | 40.00  | 69.84     |
| 6      | 31.59  | 15.00  | 35.00  | 71.20     |
| 7      | 48.41  | 15.00  | 35.00  | 72.22     |
| 8      | 40.00  | 15.00  | 35.00  | 94.25     |
| 9      | 40.00  | 15.00  | 35.00  | 92.22     |
| 10     | 40.00  | 15.00  | 26.59  | 77.64     |
| 11     | 40.00  | 15.00  | 35.00  | 89.51     |
| 12     | 40.00  | 15.00  | 43.41  | 72.89     |
| 13     | 35.00  | 20.00  | 30.00  | 65.10     |
| 14     | 35.00  | 10.00  | 40.00  | 62.04     |
| 15     | 35.00  | 20.00  | 40.00  | 61.71     |
| 16     | 40.00  | 23.41  | 35.00  | 71.88     |
| 17     | 40.00  | 15.00  | 35.00  | 93.57     |
| 18     | 45.00  | 20.00  | 30.00  | 63.74     |
| 19     | 45.00  | 10.00  | 30.00  | 64.08     |
| 20     | 45.00  | 10.00  | 40.00  | 64.08     |

### Table 3. Analysis of variance of center combination test.

| Source | Sum of Squares | df | Mean Square | F Value | p-value |
|--------|----------------|----|-------------|---------|---------|
| Model  | 2521.77        | 9  | 280.2       | 9.84    | 0.0007  |
| A      | 12.18          | 1  | 12.18       | 0.43    | 0.5279  |
| B      | 0.57           | 1  | 0.57        | 0.02    | 0.8907  |
| C      | 1.79           | 1  | 1.79        | 0.063   | 0.8069  |
| AB     | 0.7            | 1  | 0.7         | 0.024   | 0.8789  |
| AC     | 10.49          | 1  | 10.49       | 0.37    | 0.5574  |
The study showed that the model $P = 0.0007 < 0.01$, showing a significant correlation, in which A, C and G were the key factors affecting urease activity, and the order of influence of each factor was A > C > G. The regression analysis of urease activity experimental results of H. sp. H36 was performed by Design Expert. V8.0.6, and the quadratic multinomial regression equation: $Y (g/L) = 92.26 + 0.94 A + 0.20 B - 0.36 C + 0.30 AB + 1.15 AC + 0.30 BC - 8.94 A^2 - 8.28 B^2 - 7.68 C^2$. The curve contour of urease activity under the interaction of each parameter is shown in Figure 3.

Software optimized urease producing medium (g/L): glucose 40.26, ammonium sulfate 15.06, sodium chloride 34.90, urea 5, monosodium glutamate 20, yeast powder 1, KH$_2$PO$_4$ 3, K$_2$HPO$_4$ 9, MgSO$_4$·7H$_2$O 0.4, MnSO$_4$·H$_2$O 0.01, and microelement 2 mL/L.

The quadratic model predicted that the maximum urease activity was 92.2925 U, and the average urease activity obtained through 5 parallel tests was 93.628 U. There is no significant difference between the model and the theoretical predictive value.

4. Conclusions

*Halomonas* sp. H36 was selected. Its growth and urease activity peaked at 36 h, the growth is 7.42, urease activity is 63.38 U. The urease properties of the strain were studied, and the results showed that pH = 8, $T = 50 ^\circ C$, NaCl = 60 g/L, CO(NH$_2$)$_2$ = 50 g/L, urease activity reached 63.38 U, with an increase of 116.98%. It was also found that NaCl = 150 g/L, the urease activity remained at 73.53%, indicating that the urease of the strain had certain salt tolerance. After response surface optimization, the urease producing medium (g/L): glucose 40.26, ammonium sulfate 15.06, sodium chloride 34.90, urea 5, monosodium glutamate 20, yeast powder 1, KH$_2$PO$_4$ 3, K$_2$HPO$_4$ 9, MgSO$_4$·7H$_2$O 0.4, MnSO$_4$·H$_2$O 0.01, and microelement 2 mL/L. *Halomonas* sp. H36 urease activity reached 93.628 U, an increase of 47.72%.

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