Response surface method optimization of ectoine fermentation medium with moderate halophilic bacteria *Halomonas* sp. H02

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**Abstract.** Moderate halophilic bacteria are of halophilic bacteria whose suitable growth of NaCl is 5-10%. When the moderate halophilic bacteria response to high osmotic stress, the intracellular will synthesize small organic molecule compatible solutes. Ectoine, which is the major synthetic osmotic compatible solutes for moderate halophilic bacteria, can help microbial enzymes, nucleic acids and the whole cell resist to hypertonic, high temperature, freezing and other inverse environment. In order to increase the Ectoine production of Moderate halophilic bacteria *Halomonas* sp. H02, the Ectoine fermentation medium component was optimized by Plackett-Burman (PB) and Response Surface Methodology (RSM) based on the principle of non-complete equilibrium. The results of PB experiments showed that the three main influencing factors of Moderate halophilic bacteria *Halomonas* sp. H02 synthesis Ectoine culture medium were C5H8NNaO4 concentration, NaCl concentration and initial pH. According to the center point of the steepest climbing experiment, the central combination design experiment was used to show that the model is consistent with the actual situation. The optimum combination of three influencing factors were C5H8NNaO4 41 g/L, NaCl 87.2 g/L and initial pH 5.9, and the predicted amount of Ectoine was 1835.8 mg/L, increased by 41.6%.

1. Introduction

Ectoine (1, 4, 5, 6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most important compatible solutes prevalent in most moderate halophilic bacteria [1-4]. Ectoine can not only equilibrate the osmotic pressure of cells, but also provide a reversible assistance on enzymes, DNA and the whole cell in inverse environments [5]. Therefore, it causes extensive attention from scholars, and the optimization of Ectoine synthesis conditions, especially the effect of fermentation medium on Ectoine synthesis has become a research hotspot in recent years.

The effect of different carbon sources on Ectoine synthesis is related to the synthesis pathway of Ectoine. When C5H8NNaO4 is the sole carbon source, it can provide a rich amino group for the reaction of transforming oxaloacetic acid into aspartic acid and transforming aspartic acid-β-semialdehyde into L-2,4-diaminobutyric acid during the Ectoine synthesis pathway, making the Ectoine synthesis higher [6]. The osmotic pressure of the culture medium has an important effect on the microbial growth activity, and the osmotic pressure of the appropriate cell growth is generally adjusted by adding an inorganic salt in the culture medium. In the range of NaCl concentration suitable for Ectoine synthesis, the higher the concentration of NaCl, the higher the concentration threshold, the higher the concentration of Ectoine in the intracellular [7, 8]. Therefore, higher concentrations of NaCl...
have a positive effect on the synthesis of Ectoine [9]. While each micro-organisms have their optimum pH, that pH is too low or too high not only affects the growth of micro-organisms, but also affects the product synthesis. The Deamination process of synthesis of Ectoine with C₃H₂NNaO₄ as the sole carbon source in the medium is a process of pH increase, so adjusting the appropriate initial pH has a certain effect on the synthesis of Ectoine.

In this paper, we used the response surface optimization method to establish the continuous variable surface model, at the same time optimize and evaluate the factors affecting the yielkl and their interactions. Therefore, the optimal conditions of the multi-factor system could be determined quickly and effectively [10]. In this paper, the main influencing factors of Ectoine fermentation medium were screened by PB design. Then, the optimum Ectoine synthesis area was selected according to the steepest climbing experiment. Finally, the optimal Ectoine fermentation medium was designed by center combination design, and the Ectoine synthesis was predicted under optimal conditions.

2. Materials and methods

2.1. Strains
Sample of strain: The strain Halomonas sp. H02 was isolated and identified by this laboratory.

2.2. Medium
Enrichment medium (LB medium) (g/L): peptone 10, yeast powder 5, NaCl 60, pH 7.2, 121 °C, 20 min sterilization. The medium was autoclaved at 121 °C for 20 min.

Induction medium (g/L): C₃H₂NNaO₄·H₂O 40, (NH₄)₂SO₄ 8, KH₂PO₄ 3, K₂HPO₄ 9, MgSO₄·7H₂O 0.4, MnSO₄·H₂O 0.01, FePO₄·2H₂O 0.01, CH₃COONa·3H₂O 0.2, Na₂C₆H₅O₂·2H₂O 0.05, NaCl 60, pH 7.2, trace mineral solution(EDTA-2Na 63.7, ZnSO₄ 2.2, CaCl 5.5, MnCl·4H₂O 5.1, FeSO₄·7H₂O 5, Na₂MoO₄·2H₂O 1.1, CuSO₄·5H₂O 1.6, CoCl₂·6H₂O 1.6) 2 mL, which contains 30% of the filtered seawater (the salt concentration of seawater is calculated as 2.75%). The medium was autoclaved at 121 °C for 20 min. The trace mineral solution was sterilized by filtration (0.22 μm pore size, Millipore Express, USA)

2.3. Ectoine induction and release

2.3.1. Ectoine induction. The strain Halomonas sp. H02 was inoculated into LB medium, and cultured at 30 °C and 120 rpm for 24 h. 1% inoculation quantity access to 30 mL induction medium, then at 30 °C, 120 rpm shake flask to induce culture for 72 h.

2.3.2. Release of Ectoine. Separate cells from fermentation broth. Using Induction medium, fermentation broth at the exponential phase of growth (72 h) was centrifuged at 4 °C and 12,000 rpm for 15 min.

Preparation of Ectoine sample: Cells were collected by the method described above and add 1 mL of 80% ethanol (v/v) resuspension, standing overnight. The suspension was centrifuged again (12000 rpm, 4 °C, 15 min) and the supernatant was stored for HPLC analysis.

2.4. Determination of Ectoine concentration
For the preparation of Ectoine samples, see "2.3.2".

Ectoine was determined by high performance liquid chromatography (HPLC) method. The sample for determining ectoine was prepared by ethanol extraction method [11]. We used TSK-GEL reversed phase column (TOSOH Corporation, Japan) with 50 mmol/L KPi buffer (pH 6) as the mobile phase at 35 °C. The flow rate of 1 mL/min and a UV detector wavelength of 210 nm was used [12]. The retention time of Ectoine was obtained by testing the Ectoine standard (the extracellular sample was water-soluble sample, and the intracellular sample was alcohol-soluble samples). Ectoine standards came from Biomol GmbH company (Germany).
Ectoine content calculation:

\[ X = 2 \times \frac{S}{S_0} \times 142.2 \]  

(1)

Where \( X \) represents Ectoine content, unit (mg/L), \( S \) represents Ectoine sample peak area and \( S_0 \) represents Ectoine standard peak area.

The standard concentration of Ectoine is 2 mol/L and the molecular weight of Ectoine is 142.2 g/mol.

2.5. 2PB experiment

According to the related literature and the results of the previous study of the laboratory, this experiment used N=12 PB design, selected seven factors (g/L), each factor selected high (+1) low (-1) two Level: \( X_1 \): \( \text{C}_6\text{H}_8\text{NNaO}_4 \cdot \text{H}_2\text{O} \) (20, 40), \( X_2 \): \( \text{NH}_4\text{SO}_4 \) (6, 12), \( X_3 \): \( \text{FePO}_4 \cdot 2\text{H}_2\text{O} \) (0.4, 0.8), \( X_4 \): \( \text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O} \) (0.06, 0.12), \( X_5 \): \( \text{Na}_2\text{C}_2\text{H}_3\text{O}_7 \cdot 2\text{H}_2\text{O} \) (0.3, 0.6), \( X_6 \): \( \text{NaCl} \) (75, 150), \( X_7 \): initial pH (6, 8), for other factors, see "2.2" induction medium. Experiment design was performed with Design-Expert 8.0 software. According to the results obtained, the effect of each factor on Ectoine level was analyzed and the significance was evaluated, then the factors which had a significant impact on Ectoine level were obtained.

2.6. The steepest climbing experiment

The response surface fitting equation was similar to the real situation in the immediate neighborhood of the study, therefore, it was necessary to approximate the maximum Ectoine production region to establish an effective response surface fitting equation [13]. The direction of the steepest climbing experiment and the step of change were determined by the three main influencing factors of \( \text{C}_6\text{H}_8\text{NNaO}_4 \) concentration, \( \text{NaCl} \) concentration and initial pH according to the results of PB experiment, and the area of Ectoine maximum production could be approached rapidly.

2.7. Center combination experiment

According to the factors determined by the PB experiment and the center point of the steepest climbing experiment, three factors \((X_1, \text{sodium chloride (C}_6\text{H}_8\text{NNaO}_4), X_2, \text{sodium acetate (NaCl), } X_7, \text{initial pH})\) were designed by using the Central composite design (CCD) in Design-Expert 8.0 software. The experiment took the level of Ectoine as a response value, while taking five levels, encoded as (-1.68, -1, 0, 1, 1.68). The experiment design was shown in table 1.

| Variable | Coded level |
|----------|-------------|
|          | -1.68 | -1 | 0 | 1 | 1.68 |
| \( X_1 \) (g/L) | 23.18 | 30.00 | 40.00 | 50.00 | 56.82 |
| \( X_2 \) (g/L) | 64.77 | 75.00 | 90.00 | 105.00 | 115.23 |
| \( X_3 \) | 5.16 | 5.50 | 6.00 | 6.50 | 6.84 |

3. Result and analysis

3.1. Ectoine synthesis of key influencing factors

According to the method "2.5" design PB experiment, PB experiment results were shown in table 2. The variance analysis of table 2 experiment data was carried out by Design-Expert 8.0 software. The results were shown in table 3.
Increased with the concentration of the central combination experiment. The design and results of the steepest climbing experiment, the change range of the concentration of C₈H₁₅NNaO₄, NaCl and initial pH were selected as 10 g/L, 15 g/L and 0.5, and the combination of the maximum concentration of Ectoine was selected. The design and results of the steepest climbing experiment were shown in table 4.

The results showed that the Ectoine level reached 1679.2 mg/L under No. 3 experiment, and the level of Ectoine decreased with the concentration. Therefore, No. 3 experiment was selected as the central point of the central combination experiment.

### Table 2. Plackett-Burman experiment design and results.

| Serial number | X₁ | X₂ | X₃ | X₄ | X₅ | X₆ | X₇ | X₈ | X₉ | X₁₀ | X₁₁ | Ectoine (mg/L) |
|---------------|----|----|----|----|----|----|----|----|----|-----|-----|----------------|
| 1             | 1  | 1  | 1  | -1 | -1 | -1 | -1 | 1  | 1  | -1  | -1  | 976.5          |
| 2             | -1 | 1  | 1  | -1 | 1  | 1  | -1 | -1 | -1 | 1   | 426.6         |
| 3             | -1 | -1 | -1 | 1  | 1  | -1 | -1 | 1  | 1  | 1   | 417.0         |
| 4             | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1   | 830.8         |
| 5             | 1  | -1 | 1  | 1  | 1  | -1 | -1 | -1 | 1  | 1   | 1268.7        |
| 6             | 1  | -1 | -1 | -1 | 1  | 1  | -1 | -1 | 1  | 1   | 1028.4        |
| 7             | -1 | -1 | 1  | -1 | 1  | 1  | -1 | -1 | 1  | 1   | 593.1         |
| 8             | 1  | 1  | -1 | 1  | 1  | -1 | -1 | -1 | 1  | 1   | 785.3         |
| 9             | 1  | -1 | 1  | 1  | -1 | 1  | 1  | 1  | 1   | 641.5         |
| 10            | 1  | 1  | -1 | -1 | 1  | -1 | 1  | 1  | 1   | 1   | 984.2         |
| 11            | -1 | 1  | -1 | 1  | 1  | 1  | 1  | 1  | 1   | 1   | 649.8         |
| 12            | -1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1   | 1   | 752.4         |

### Table 3. Analysis of variance of PB results.

| Source | Value | F  | Prob>F |
|--------|-------|----|--------|
| X₁     | 167.92| 40.07| 0.0032 |
| X₂     | -17.06| 0.41 | 0.5551 |
| X₃     | -3.06 | 0.013| 0.9138 |
| X₄     | -27.08| 1.04 | 0.3651 |
| X₅     | 12.48 | 0.22 | 0.6626 |
| X₆     | -138.24| 27.15| 0.0065 |
| X₇     | -89.57| 11.40| 0.0279 |

It can be seen from table 3 that according to the variance analysis results of PB design, the main influencing factors of Ectoine synthesis were determined as C₈H₁₅NNaO₄ concentration, NaCl concentration and initial pH of the culture medium. Which C₈H₁₅NNaO₄, NaCl on the confidence of Ectoine synthesis in more than 95% had a significant impact. The initial pH of the culture medium had a significant effect on the confidence of Ectoine synthesis in more than 90%. Meanwhile, according to table 3, in addition to C₈H₁₅NNaO₄ and NaCl, other factors showed a negative effect on Ectoine synthesis. The next step in the optimization should increase the concentration of C₈H₁₅NNaO₄, reduced the concentration of NaCl and initial pH, in addition to Na₂C₆H₅O₇, other factors selected the low level of concentration.

### 3.2. The steepest climbing experiment

In the steepest climbing experiment, the change range of the concentration of C₈H₁₅NNaO₄, NaCl and initial pH were selected as 10 g/L, 15 g/L and 0.5, and the combination of the maximum concentration of Ectoine was selected. The design and results of the steepest climbing experiment were shown in table 4.

The results showed that the Ectoine level reached 1679.2 mg/L under No. 3 experiment, and the level of Ectoine decreased with the concentration. Therefore, No. 3 experiment was selected as the central point of the central combination experiment.

### Table 4. The design and results of the steepest climbing test.

| Serial number | X₁ (g/L) | X₆ (g/L) | X₇ | Ectoine (mg/L) |
|---------------|----------|----------|----|----------------|
| 1             | 20       | 60       | 5.5| 867.8          |
| 2             | 30       | 75       | 6  | 1114.7         |
| 3             | 40       | 90       | 6.5| 1679.2         |
| 4             | 50       | 105      | 7  | 1322.8         |
| 5             | 60       | 120      | 7.5| 931.8          |
3.3. Response surface analysis of Ectoine fermentation medium components

3.3.1. Quadratic regression model fitting and verification.

The C\textsubscript{5}H\textsubscript{8}NNaO\textsubscript{4} Concentration, NaCl Concentration and initial pH of the culture medium were determined in 3.2, and the amount of Ectoine synthesis was used as the response value. The experiment results were shown in tables 5 and 6.

Table 5. Design and results of the center combination experiment.

| Serial number | X\textsubscript{1} | X\textsubscript{2} | X\textsubscript{3} | Ectoine (mg/L) |
|---------------|----------------|----------------|----------------|---------------|
| 1             | 30.00          | 75.00          | 5.50           | 1300.3        |
| 2             | 40.00          | 90.00          | 6.00           | 1887.8        |
| 3             | 30.00          | 105.00         | 5.50           | 912.8         |
| 4             | 56.82          | 90.00          | 6.00           | 1341.6        |
| 5             | 30.00          | 105.00         | 6.50           | 1240.9        |
| 6             | 50.00          | 105.00         | 6.50           | 947.6         |
| 7             | 40.00          | 90.00          | 6.00           | 1900.0        |
| 8             | 50.00          | 105.00         | 5.50           | 1132.8        |
| 9             | 40.00          | 64.77          | 6.00           | 1849.0        |
| 10            | 40.00          | 90.00          | 6.00           | 1240.9        |
| 11            | 40.00          | 115.23         | 6.00           | 995.4         |
| 12            | 40.00          | 90.00          | 6.00           | 1870.0        |
| 13            | 50.00          | 75.00          | 5.50           | 1457.7        |
| 14            | 40.00          | 90.00          | 5.16           | 1583.3        |
| 15            | 40.00          | 90.00          | 6.84           | 1387.0        |
| 16            | 30.00          | 75.00          | 6.50           | 1095.6        |
| 17            | 40.00          | 90.00          | 6.00           | 1876.6        |
| 18            | 23.18          | 90.00          | 6.00           | 1159.9        |
| 19            | 40.00          | 90.00          | 6.00           | 1912.9        |
| 20            | 50.00          | 75.00          | 6.50           | 986.2         |

Table 6. Analysis of variance of the results of the central combination experiment.

| Source            | df | Mean Square | F      | Prob>F |
|-------------------|----|-------------|--------|--------|
| Model             | 9  | 2.698E+005  | 41.13  | <0.0001|
| X\textsubscript{1} | 1  | 5748.95     | 0.88   | 0.3713 |
| X\textsubscript{6} | 1  | 81279.71    | 12.39  | 0.0055 |
| X\textsubscript{7} | 1  | 54599.79    | 8.32   | 0.0162 |
| X\textsubscript{1}\times X\textsubscript{6} | 1  | 1841.03     | 0.28   | 0.6078 |
| X\textsubscript{1}\times X\textsubscript{7} | 1  | 76081.20    | 11.60  | 0.0067 |
| X\textsubscript{6}\times X\textsubscript{7} | 1  | 3.944E+005  | 60.11  | <0.0001|
| Residual          | 10 | 6560.35     |        |        |
| Lack of fit       | 5  | 12604.75    | 24.43  | 0.0016 |
| Pure error        | 5  | 515.96      |        |        |
| Cor total         | 19 | 2.494E+006  |        |        |

The experiment results regression analysis was performed by Design-Expert.8.0 software to fit out a quadratic equation: \(Y(mg/L)=1886.52 + 20.52 X_1 - 77.15 X_2 - 63.23 X_3 - 15.17 X_1X_2 - 97.52 X_1X_3 + 102.37 X_2X_3 - 248.31 X_1^2 - 291.52 X_2^2 - 165.42 X_3^2\).

The variance analysis of the model was shown as table 6. The confidence interval of the model was
within 99%, the confidence is 99.99%, and the regression equation is significant, indicating that the equation has a good fit degree; the equation is consistent with the actual situation. In this model, the confidence levels of \( X_1, X_1^2, X_2, X_2^2, X_3, X_3^2 \) were all above 95%, indicating that they were all significant items of this model. The coefficient of determination of the model was \( R^2 = 0.9737 \), indicating that the model was in good agreement with the actual situation.

3.3.2. Response surface analysis and determination of the best medium components.

Combined with the regression equation, the response surface analysis diagram was plotted by the Design-Expert.8.0 software. Each response surface plot represented the effect of two independent variables, holding the other variables at zero [14].

From the three-dimensional response surface (figures 1-3) showed that there was a real maximum for the fitting surface, that all the three factors had a more appropriate concentration. In order to further determine the stability of the Ectoine maximum production, the first order partial derivative of the regression equation is made equal to zero. The results show as:

\[
20.52 - 15.17X_2 - 95.72X_3 - 496.62X_1 = 0 \\
-71.15 - 15.17X_1 + 102.37X_2 - 583.04X_2 = 0 \\
-63.23 - 95.72X_1 + 102.37X_2 - 330.84X_3 = 0
\]

(Figures 1-3)

The extreme point of the model was obtained by solving the equation group: \( X_1 = -0.102, X_2 = 0.184, X_3 = 0.278 \); Substituting the regression equation, the predicted Ectoine production was 1835.8 mg/L. The concentrations of \( \text{C}_5\text{H}_8\text{NNaO}_4 \) and NaCl were 41 g/L, 87.2 g/L, respectively, and the initial pH
was 5.9.

In order to confirm the predicted results of the experiment, the fermentation experiments were carried out with the optimum medium conditions obtained by response surface experiment. Repeat the experiment twice, the concentrations of Ectoine were 1765.5 mg/L and 1862.4 mg/L, respectively, and the average value was 1813.9 mg/L. The difference between the experimental value and the predicted value is very small, indicating that the model can predict the actual fermentation situation appropriately.

4. Conclusion
This paper uses the Plackett-Burman design in the first place, with the amount of Ectoine synthesis as the response value, three main factors influencing the amount of Ectoine synthesis were screened out from seven factors, and they were C2H6NNaqO4 concentration, NaCl concentration and initial pH of the culture medium. Through the steepest climbing test, No. 3 experiment was chosen as the central point of the center combination test. The optimum combination of three influencing factors was determined as C2H6NNaqO4 41 g/L, NaCl 87.2 g/L and initial pH 5.9 through the central combination experiment.

The optimal fermentation medium for Ectoine produced by *Halomonas* sp. H02 were (g/L): C2H6NNaqO4·H2O 41, (NH4)2SO4 8, KH2PO4 3, K2HPO4 9, MgSO4·7H2O 0.4, MnSO4·H2O 0.01, FePO4·2H2O 0.4, CH3COONa·3H2O 0.06, Na2C3H6O2·2H2O 0.6, NaCl 87.2, initial pH 5.9, trace elements 2 mL. Which contains 30% of the filtered seawater (sea salt concentration by 2.75%). This medium was named as ZMG medium.

The verification experiment to this model was carried out on the optimized medium, and the amount of Ectoine synthesis reached 1835.8 mg/L, which was 41.6% higher than before optimization, indicating that it is scientifically reasonable and accurate to combine Plackett-Burman experiment and response surface in optimizing the Ectoine fermentation medium of *Halomonas* sp. H02.

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References
[1] Ventosa A, Nieto J J and Oren A 1998 Biology of moderately halophilic aerobic bacteria *Microbiol. Mol. Biol. Rev.* 62(2) 504-44
[2] Oren A 2002 Diversity of halophilic microorganisms: Environments, phylogeny, physiology, and applications *J. Ind. Microbiol. Biotechnol.* 28 56-63
[3] Ren P G and Zhou P J 2003 Advances in studies on moderate halophilic bacteria *Acta Microbiol. Sin.* 43(3) 427-31
[4] Wang Y Q and Tao T 1994 Compatible solute in microbial osmotic pressure regulation *Microbiol. Chin.* 21(5) 293-6
[5] Zheng X, Ma H and Yan X W 2010 Ectoine synthesis and release under osmotic shock in halomonas venusta DSM4743 *Microbiol. Chin.* 37(7) 1090-6
[6] Zhang L H and Lang Y J 2008 Method for producing Ectoine by microbial fermentation (China: CN101314785)
[7] Grammann K, Volke A and Kunte H J 2002 New type of osmoregulated solute transporter identified in halophilic members of the Bacteria domain: TRAP-transporter TeaABC mediates uptake of ectoine and hydroxyectoine in Halomonas elongata DSM 2581T *J. Bacteriol.* 184 3078-85
[8] Kuhlmann A U and Bremer E 2002 Osmotically regulated synthesis of compatible solute ectoine in Bacillus pasteurii and related Bacillus spp *Appl. Environ. Microb.* 68(2) 772-83
[9] Tao P, Li H, Yu Y, Gu J and Liu Y 2016 Ectoine and 5-hydroxyectoine accumulation in the halophile Virgibacillus halodenitrificans PDB-F2 in response to salt stress *Appl. Environ.
[10] Van-Thuoc D, Guzmán H, Mai T H and Hatti-Kaul R 2010 Ectoine production by Halomonas boliviensis: Optimization using response surface methodology Mar. Biotechnol. 12(5) 586-93

[11] Zhang L H, Lang Y J and Nagata S 2009 Efficient production of ectoine using ectoine-excreting strain Extremophiles 13 717-24

[12] Lang Y J, Bai L, Ren Y N, Zhang L H and Nagata S 2011 Production of ectoine through a combined process that uses both growing and resting cells of Halomonas salina DSM 5928T Extremophiles 15(2) 303-10

[13] Khuri A I and Mukhopadhyay S 2011 Response Surface Methodology (Berlin Heidelberg: Springer)

[14] Zhou J, Yu X, Ding C, Wang Z, Zhou Q, Pao H and Cai W 2011 Optimization of phenol degradation by Candida tropicalis Z-04 using Plackett-Burman design and response surface methodology J. Environ. Sci-China 23(1) 31-6