Optimization of Enzymatic Cell Disruption for Improving Lipid Extraction from *Schizochytrium* sp. through Response Surface Methodology

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Abstract: This study is aimed to explore the optimal conditions of cell disruption in the extraction algae oil process, using alkaline protease to disrupt cell of *Schizochytrium* sp. to extract oil in this paper. The effects of enzymatic lysis temperature, enzymatic lysis time, enzyme dosage and pH value on oil yield and DHA yield were studied. Through the combination of single factor test and response surface design, the optimal cell disruption conditions were screened out. The fatty acid composition of algal oil was analyzed by gas chromatography-massspectrometry (GC-MS). The results showed that when the conditions were: enzymatic lysis temperature 55°C, enzymatic lysis time 9 h, enzyme dosage 3% of biomass and pH 8, oil yield and DHA yield reached the highest 14.52 g/L and 7.12 g/L, respectively. When the strains were cultured in 50 L fermentor, oil yield reached 26.27 g/L and DHA yield reached 12.89 g/L. They were 1.81 times higher than that in shake-flask cultivation. The optimization experiment provides the basis for the industrial production of *Schizochytrium* sp.

Key words: *Schizochytrium* sp., enzymatic cell disruption, response surface methodology, GC-MS

1 Introduction

*Schizochytrium* sp. is a kind of marine fungi which morphological characteristic is single cell globose. The strain is rich in DHA and other polyunsaturated fatty acid¹. In industry, *Crypthecodinium cohnii* and *Schizochytrium* sp. were the important oleaginous microorganisms. However, because of the high yield and easy extraction *Schizochytrium* sp. was the main oleaginous microorganisms, such as Cabio biotech co., Ltd mainly use *Schizochytrium* sp. to produce DHA. As we all know, DHA is docosahexaenoic acid. DHA is an essential polyunsaturated fatty acid for most vertebrates which, in humans, is involved in the prevention of several diseases³. It is not only important for the normal development of infants’ brain and vision, but also has many physiological functions such as treatment of cardiovascular diseases, tumor suppression and enhancement of human immunity⁴-

The oil of the *Schizochytrium* sp. exists in cells wrapped by cell wall. It causes that the effect of direct extracting oil is unsatisfactory. To get higher product recovery and quality lipids with lower operating costs from microbial cells, a suitable cell disruption method is required. Cell disruption enhances the release of intracellular lipids from microalgae by improving the access of the extracting solvent to fatty acid⁵. There are many cell disruption methods such as microwave, ultrasonication, bead mill, drying, and supercritical fluid extraction⁶-

Most microalgal lipid extraction studies are based on sole technique, and the disruption degrees and lipid recoveries obtained are not acceptably high, but the energy consumptions for the lipid extraction are still too much¹⁰. Enzymatic process, as a biochemical process, requires much lower energy than mechanical processes. Because the constitution of green microalgae cell wall and the contained cellulose are similar to most plant cells¹¹, it is expected that the algal cell wall can be weakened and loosened by degradiation of the protein, cellulose and pectin of the wall. Researchers have reported many results of enzymatic degra-
dation\textsuperscript{12, 13}, however, the enzymatic process has not achieved very high cell disruption degree in the reports. Most of reported studies have used enzymes separately, and have also had limited lipid extraction; not more than 72\% of lipid recovery has been achieved\textsuperscript{14, 15}. The cell wall of \textit{Schizochytrium} sp. is mainly composed of protein and pectin. Alkaline protease can degrade complex macromolecular proteins into small peptides or fatty acids and it was the most widely used in industry. So in this paper, cell wall of \textit{Schizochytrium} was disrupted by alkaline protease. Intracellular cell was extracted and the optimum condition of enzymatic cell disruption was screened, which can be used as a reference for industrial production.

2 Materials and methods

2.1 Materials

\textit{Schizochytrium} sp. ATCC20888 was obtained from American Type Culture Collection (ATCC) and was preserved in 20\% (v/v) glycerol at \textdegree{}80C.

All reagents were as follows: Glucose, Na\textsubscript{2}SO\textsubscript{4}, NaCl, KH\textsubscript{2}PO\textsubscript{4}, MgSO\textsubscript{4}, NaHCO\textsubscript{3}, CaCl\textsubscript{2}, yeast extract, agar, monosodium glutamate, alkaline protease, absolute ethanol and n-hexane from Sinopharm Chemical Reagent Co., Ltd. These chemicals were of analytical grade and the N-hexane we use for GC-MS was HPGC grade.

2.2 Culture conditions

The composition of solid culture medium was (g/L or mL/L): glucose, 50; monosodium glutamate, 30; yeast extract, 6; NaCl, 8; KH\textsubscript{2}PO\textsubscript{4}, 2; MgSO\textsubscript{4}, 6; NaHCO\textsubscript{3}, 0.2; CaCl\textsubscript{2}, 0.5; Agar, 20.

The composition of seed culture medium was (g/L or mL/L): glucose, 60; monosodium glutamate, 40; yeast extract, 6; NaCl, 20; KH\textsubscript{2}PO\textsubscript{4}, 6; MgSO\textsubscript{4}, 22; NaSO\textsubscript{4}, 0.3; NaHCO\textsubscript{3}, 0.23; CaCl\textsubscript{2}, 0.3; trace element solution, 2; vitamin solution, 1.4. The trace element solution contained (g/L): CoCl\textsubscript{2}, 0.0065; MnCl\textsubscript{2}, 0.1623; ZnCl\textsubscript{2}, 0.0122; H\textsubscript{2}BO\textsubscript{3}, 0.1020; FeCl\textsubscript{3}, 0.0556; EDTA, 0.15; CuSO\textsubscript{4}, 0.0492. The vitamin solution was filter-sterilized and contained (g/L): thiamine, 0.9973; biotin, 0.110; cobalamin, 0.103.

The composition of fermentation culture medium was (g/L or mL/L): glucose, 75; monosodium glutamate, 15; yeast extract, 6; NaCl, 2.4; KH\textsubscript{2}PO\textsubscript{4}, 4.8; MgSO\textsubscript{4}, 6; KCl, 0.4; NaHCO\textsubscript{3}, 0.6; CaCl\textsubscript{2}, 0.6; trace element solution, 2.8; vitamin solution, 2. The compositions of trace element and vitamin solutions were the same as described above.

\textit{Schizochytrium} sp. ATCC20888 were inoculated into a 250-mL flask containing 100 mL of seed culture medium and cultivated for 48 h. After cultivation the activated seeds in mid-exponential phase were inoculated to a 100 mL of medium in 500-mL flask with 20\% (v/v) inoculums size and incubated at 26\degree{}C for 7 d with a speed of 180 rpm.

2.3 Determination of biomass

After fermentation, 100 mL of the fermentation broth was centrifuged for 10 min with a speed of 5000 rpm. The cell pellet was washed with distilled water for three times and dried at 60\degree{}C to a constant weight. Biomass (g/L) = dry cell mass (g)/ broth volume (L).

2.4 Extraction and yield determination of oil

Cells were harvested by centrifugation for twice. The cell pellet was resuspended with distilled water as 1:1 ratio (w/w). The suspension was stirred by magnetic stirrer (DF-101S magnetic stirring from GongYi YuHua instrument Co., Ltd, China). After enzymatic lysis, the cell suspension was put into freeze dryer (FD-8 freeze dryer from Beijing Boyikang experimental instrument Co., Ltd, China). After that it was ground to algae powder. Then oil was extracted from the algae powder by soxhlet extraction method. The formula for calculating oil yield is as follows: oil yield (g/L) = oil (g)/ broth volume (L).

2.5 Analytical methods

Fatty acid methyl esters (FAMES) were prepared by the modified method\textsuperscript{16, 17} as follows: 0.2 g oil samples were taken in 20 mL test tube with 3 mL 0.5 mol/L NaOH-methanol solution and heated in water bath at 60\degree{}C for about 30 min. After it cooled, 2 mL 25\% BF\textsubscript{3} solution was added into it and heated in water bath at 60\degree{}C for about 20 min. After it cooled, 3 mL n-hexane and 2 mL saturated NaCl solution were added into it and shooked, then it was set for about 30 min. The upper organic phase was extracted into test tube. The relative content of DHA was analyzed by gas chromatography mass spectrometer. The formula for calculating DHA yield is as follows: DHA yield (g/L) = oil yield (g) \times DHA content (%).

For the determination of fatty acid compositions of FAMES samples, a GC–MS system (Agilent GC7890A-MS5975C, Agilent Technologies, Santa Clara, CA, USA) equipped with a HP-FFAP capillary column (30m \times 0.25mm, 0.25 \textmu m film thickness) was employed. The injector was maintained at 250\degree{}C with an inject volume of 1 \textmu L, the inject mode was split injection with a split ratio of 30:1. The column temperature was held at 140\degree{}C for 1 min, and increased to 190\degree{}C with the rate of 5\degree{}C/min, then raised to 220\degree{}C at the rate of 2\degree{}C/min, holding for 10 min. Nitrogen was used as the carrier gas and the flow rate was constantly at 1 mL/min. MS parameters were set as follows: ionization source, electron impact ion source; ionization energy, 70 eV; ion source temperature, 230\degree{}C; transfer line temperature, 250\degree{}C; quadrupole temperature, 150\degree{}C; scanning mode: full scan mode; mass scan range: 70–500 m/z. AMDIS software was used to process the raw data files for fatty acids information. The proportion for each component in lipid sample was determined by area normalization.
method.

Through the chemical workstation G1701BA data processing system, the Nist11 standard mass spectrometry library was used to retrieve and identify the oil components. The relative oil content was measured by area normalization method.

2.6 Experiment design and data analysis

The effects of dosage of enzyme (1%, 2%, 3%, 4%, 5%), enzymatic lysis temperature (45 °C, 50 °C, 55 °C, 60 °C and 65 °C), enzymatic lysis pH (7, 8, 9, 10, 11) and enzymatic lysis time (6h, 7h, 8h, 9h, 10h) on oil and DHA yield were investigated. Each experiment was repeated 3 times, taking the average value.

On the basis of the results of single factor experiments, the test was designed by using Box-Behnken method of Design Expert 8.0.6 software. The dosage of enzyme, enzymatic lysis temperature, enzymatic lysis pH and enzymatic lysis time were investigated variables. Oil yield and DHA yield were response values. The optimum formulation of factors was obtained by experimental analysis.

3 Results and discussion

3.1 Single factor test of enzymatic lysis conditions

3.1.1 Effect of enzyme dosage

The dosage of enzyme had influence on cell wall breaking. Excessive dosage of enzymes may reduce enzyme activity and increase the cost of oil extraction. When the dosage of enzyme used is too little, the cells wall cannot be broken completely, which lead to the decreasing of oil yield. The dosage of enzyme was set to 1, 2, 3, 4 and 5 of biomass. Enzymatic lysis temperature, enzymatic lysis pH and enzymatic lysis time were investigated variables. Oil yield and DHA yield were response values. The optimum formulation of factors was obtained by experimental analysis.

As results shown in Fig. 1, when the enzyme dosage was between 1% and 3%, oil yield and DHA yield increased with the increasing of enzyme dosage and reached the maximum at 3%. At this time, the yield of oil was (14.15 ± 0.12) g/L, and the yield of DHA was (7.01 ± 0.12) g/L. As the dosage of enzyme continued to increase, oil yield and DHA yield began to decrease. It illustrated that the optimum enzyme dosage was 3%.

3.1.2 Effect of enzymatic lysis temperature

Studies have shown that under extreme high temperature or low temperature conditions, the dosage of fatty acids synthesized from microalgae would decrease, moreover, the synthesis process was limited at extreme temperatures, possibly because of irreversible damage to the enzyme. The effect of temperature on enzyme activity was necessary. The enzyme had the strongest enzyme activity at the optimum temperature. Enzymatic lysis temperature was set to 45 °C, 50 °C, 55 °C, 60 °C, 65 °C. Enzymatic lysis pH, dosage of enzyme and enzymatic lysis time were adjusted to 10, 3% and 9 h, respectively. The effects of enzymatic lysis temperature on oil yield and DHA yield were studied.

As results shown in Fig. 2, when the enzymatic lysis temperature was between 45 °C and 55 °C, oil yield and DHA yield increased with the increasing of enzymatic lysis temperature and reached the maximum at 55 °C. At this time, the yield of oil was (13.88 ± 0.11) g/L, and the yield of DHA was (6.69 ± 0.11) g/L. As the enzymatic lysis temperature continued to increase, oil yield and DHA yield began to decrease. It illustrated that the optimum enzymatic lysis temperature was 55 °C and at this condition the enzyme activity was highest.

3.1.3 Effect of enzymatic lysis pH

pH would affect the activity of alkaline protease, enzymatic lysis pH was 7, 8, 9, 10, 11. Enzymatic lysis tempera-
ture, dosage of enzyme and enzymatic lysis time was adjusted to 55°C, 3% and 9 h, respectively. The effects of enzymatic pH on oil yield and DHA yield were studied.

As results shown in Fig. 3, when the enzymatic lysis pH was between 7 and 10, oil yield and DHA yield increased with the increasing of enzymatic lysis pH and reached the maximum at 10. At this time, the yield of oil was \((12.05 \pm 0.06)\) g/L, and the yield of DHA was \((6.51 \pm 0.06)\) g/L. As the enzymatic lysis pH continued to increase, oil yield and DHA yield began to decrease.

3.1.4 Effect of enzymatic lysis time

The long time of enzyme hydrolysis lead to the increasing of impurity content in oil and the increasing cost. When the hydrolysis time is not enough, the effect of cell wall breaking will be affected. Enzymatic lysis time was set to 6 h, 7 h, 8 h, 9 h, 10 h. Enzymatic lysis temperature, dosage of enzyme and enzymatic lysis pH was adjusted to 55°C, 3% and 10, respectively. The effects of enzymatic time on oil yield and DHA yield were studied.

As results shown in Fig. 4, oil yield and DHA yield increased with the increasing of enzymatic lysis time and reached the maximum at 9 h. At this time, the yield of oil was \((13.94 \pm 0.09)\) g/L, and the yield of DHA was \((6.84 \pm 0.09)\) g/L. As the enzymatic lysis time continued to increase, oil yield and DHA yield began to decrease. It illustrated that the optimum enzymatic lysis time was 9 h.

3.2 Response surface test results and analysis

3.2.1 Response surface analysis of factor level selection

According to single factor test, the optimum enzyme lysis conditions were as follows: dosage of enzyme 3%, enzymatic lysis temperature 55°C, enzymatic lysis pH 10 and enzymatic lysis time 9 h. The Box-Behnken experiment design was used to optimize the 4 parameters of enzyme dosage, enzymatic lysis temperature, enzymatic lysis pH and enzymatic lysis time. Enzyme dosage \((X_1)\), enzymatic lysis temperature \((X_2)\), enzymatic lysis pH \((X_3)\) and enzymatic hydrolysis time \((X_4)\) were used as variables. Each variable was encoded at \(-1, 0\) and \(+1\) under three levels: low, medium and high. Test factors and levels were shown in Table 1.

3.2.2 Response surface analysis results

According to factor levels in Table 1, \(X_1, X_2, X_3\) and \(X_4\) were taken as independent variables. Oil yield \((Y_1)\) and DHA yield \((Y_2)\) were the response values. The test plan and results were shown in Table 2.

Oil yield was the response value. The quadratic regression analysis of data in Table 2 was carried out by using Design-Expert V 8.0.6 software and got the quadratic regression equation:

\[
Y_1 = 12.47 + 0.74X_1 - 0.52X_2 + 0.053X_3 + 0.34X_4 + 0.2X_2X_3 - 0.083X_2X_4 + 0.095X_4X_1 - 0.072X_2X_1 + 0.42X_3X_4 - 0.15X_3X_1 + 0.28X_1 - 0.82X_2^2 + 0.48X_3^2 + 0.42X_4^2
\]
DHA yield was the response value. The quadratic regression analysis of data in Table 2 was carried out by using Design-Expert V8.0.6 software and got the quadratic regression equation:

\[ Y_2 = 6.19 + 0.43X_1 - 0.31X_2 + 0.047X_3 + 0.21X_4 + 0.12X_1X_2 + 0.032X_1X_3 + 0.065X_1X_4 + 0.058X_2X_3 + 0.23X_2X_4 - 0.097X_3X_4 - 0.076X_1^2 - 0.73X_2^2 + 0.12X_3^2 + 0.16X_4^2 \]

Quadratic model was analyzed by analysis of variance (ANOVA). The results were shown in Tables 3 and 4. Table 3 shows the results from the analysis of variance (ANOVA), which evaluates the significance of the predictive model. The F-value of 29.66 and the p-value < 0.0001 imply that the model is significant. The p-values of \( X_1, X_2, X_3, X_4, X_5, X_6, X_7 ^2, X_8 ^2 \) and \( X_9 ^2 \) were less than 0.01, indicating these were significant model terms. p-Value greater than 0.05 indicated that the model terms used in the model were insignificant. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. A lower CV means a higher reliability of the experiment. The lack of fit F-value of 0.2216 implied that lack of fit was not significant relative to the pure error.

According to the ANOVA in Table 4, the fit of the model was checked by the coefficient of determination \( R^2 \), which was calculated to be 0.9656, indicating that 96.56% of the
variability in the response could be explained by the model. The p-values of $X_1$, $X_2$, $X_4$, $X_2X_4$, $X_4^2$ and $X_1^2$ were less than 0.05, indicating these were significant model terms. $p$-Value greater than 0.05 indicated that the model terms used in the model were insignificant. The coefficient of variation (C.V.) indicates the degree of precision with which the treatments are compared. The lower value of C.V. (2.4759%) demonstrated the performed experiments were highly reliable.

Therefore, the regression equation could be used to analyze and predict the experimental results. According to regression equation, Design-Expert 8.0.6 software was used to make the three-dimensional surface of the response surface. The effects of enzyme dosage, enzymatic lysis temperature, enzymatic lysis pH, enzymatic lysis time and their interaction on the response surface were shown in Fig. 5.

The enzymatic lysis conditions were optimized by response surface methodology and the optimum conditions were as follows: enzyme dosage 3%, enzymatic lysis temperature 55°C, enzymatic lysis pH 8, enzymatic lysis time 9 h. The predicted oil yield and DHA yield were 14.60 g/L and 7.16 g/L, respectively. At this condition, three groups of experiments were carried out, average value of oil yield and DHA yield reached 14.52 g/L and 7.12 g/L. The actual value was similar to the predicted value, and the deviation was smaller, which showed that the model was well fitted.

4 Fatty acid analysis

According to 2.5 sections, the algal oil was pre-treated and analyzed. The results were obtained by the chemical workstation. The total ion chromatography was shown in Fig. 6, and the mass spectrum was shown in Fig. 7. The composition of fatty acids and the percentage of total oil were shown in Table 5.

GC-MS was used for analysis and determination the algae oil at the optimum condition. 10 kinds of fatty acids were identified. Among them, docosahexaenoic acid accounted for (0.16 ± 0.04) %, tetradecanoic acid accounted for (6.98 ± 0.11) %, pentadecanoic acid accounted for (0.81 ± 0.09) %, hexadecanoic acid accounted for (21.97 ± 0.23) %, stearic acid accounted for (0.45 ± 0.03) %, EPA accounted for (1.01 ± 0.07) %, n-6 DPA accounted for (18.22 ± 0.18) %, DHA accounted for (49.07 ± 0.15) % in total lipid. The mass

| Source | Sun of Squares | df | Mean Square | F Value | P-Value | Prob>F |
|--------|---------------|----|-------------|---------|---------|--------|
| Model  | 21.01         | 14 | 1.50        | 29.66   | <0.0001 | significant |
| $X_1$  | 6.63          | 1  | 6.63        | 131.04  | <0.0001 | **     |
| $X_2$  | 3.31          | 1  | 3.31        | 65.37   | <0.0001 | **     |
| $X_3$  | 0.03          | 1  | 0.03        | 0.65    | 0.4323  |
| $X_4$  | 1.35          | 1  | 1.35        | 26.75   | 0.0001  | **     |
| $X_1X_2$ | 0.16        | 1  | 0.16        | 3.08    | 0.1009  |
| $X_1X_3$ | 2.72 × 10⁻²  | 1  | 2.72 × 10⁻² | 0.54    | 0.4753  |
| $X_1X_4$ | 0.04         | 1  | 0.04        | 0.71    | 0.4125  |
| $X_2X_3$ | 0.02         | 1  | 0.02        | 0.42    | 0.5296  |
| $X_2X_4$ | 0.69         | 1  | 0.69        | 13.62   | 0.0024  | **     |
| $X_3X_4$ | 0.09         | 1  | 0.09        | 1.84    | 0.1966  |
| $X_1^2$ | 5.15 × 10⁻³  | 1  | 5.15 × 10⁻³ | 0.10    | 0.7545  |
| $X_2^2$ | 4.38          | 1  | 4.38        | 86.59   | <0.0001 | **     |
| $X_3^2$ | 1.52          | 1  | 1.52        | 30.08   | <0.0001 | **     |
| $X_4^2$ | 1.13          | 1  | 1.13        | 22.28   | 0.0003  | **     |
| Residual| 0.71          | 14 | 0.05        |         |         |        |
| Lack of Fit | 0.60       | 10 | 0.06        | 2.28    | 0.2216  | not significant |
| Pure Error | 0.11        | 4  | 0.03        |         |         |        |

*Standard deviation = 0.2249; Mean = 12.5166; C.V. = 1.7971%; Predicated residual sum of squares (PRESS) = 3.6366; R-Squared = 0.9674; Adjusted (R²) = 0.9348; Predicted (R²) = 0.8326; Adequate Precision = 23.9761.

*Significant difference ($p < 0.05$); ** Significant differences ($p < 0.01$).
spectra of the identified chemical constituents were retrieved by gas chromatography-mass spectrometry computer spectrum library.

5 Fermentor test

At the enzymatic lysis condition optimized by response surface methodology, experiments in 50 L fermentor under the optimized conditions were conducted. The strains were cultured at the condition of 10% inoculation concentration, 28°C and 190 r/min for 96 h. The Oil yield reached 26.27 g/L, DHA yield reached 12.89 g/L and 1.81 times as high as shake-flask cultivation. It showed that the optimized scheme was also suitable for the expanded production of 50L fermentor. It had a certain reference value for industrial production.

6 Conclusion

The response surface test of 4 factors was optimized by Design-Expert 8.0.6 software, optimized enzymatic cell disruption conditions were as follows: enzyme dosage 3%, enzymatic lysis temperature 55°C, enzymatic lysis PH 8, enzymatic lysis time 9 h. The predicted oil yield were 14.60 g/L, DHA yield were 7.16 g/L. At this condition, three groups of experiments were carried out, average value of oil yield and DHA yield were 14.52 g/L and 7.12 g/L. The actual value was similar to the predicted value, and the deviation was smaller, which showed that the model was well fitted. An additional batch experiment was carried in 50L fermentor under the optimized conditions, oil yield reached 26.27 g/L, DHA yield reached 12.89 g/L and 1.81 times as high as shake-flask cultivation. Byreddy et al. reported that through solvent extraction, the lipid extraction yield of Schizochytrium sp. was 22%. Yu X. et al. reported that through acid digestion the lipid extraction yield of microalgae was 43%. Through the optimization of enzymatic cell disruption method, the lipid extraction yield of Schizochytrium sp. was 63%. Compared with the previous studies, the lipid extraction yield increased substantially.

This paper explores the enzymatic lysis conditions of algae oil extraction process and analyzed algal oil by GC-MS. It provided reference for better cell disruption technology in the future. The test results had certain refer-

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### Table 4 Analysis of Variance of quadratic model for DHA yield

| Source        | Sum of Squares | df | Mean Square | F Value | P-Value Prob>F |
|---------------|----------------|----|-------------|---------|---------------|
| Model         | 8.58           | 14 | 0.61        | 28.03   | <0.0001       |
| X₁            | 2.18           | 1  | 2.18        | 99.58   | <0.0001       ** |
| X₂            | 1.13           | 1  | 1.13        | 51.93   | <0.0001       ** |
| X₃            | 0.03           | 1  | 0.03        | 1.24    | 0.2844        |
| X₄            | 0.53           | 1  | 0.53        | 24.41   | 0.0002        ** |
| X₁X₂          | 0.05           | 1  | 0.05        | 2.42    | 0.1420        |
| X₁X₃          | 4.22×10⁻³      | 1  | 4.22×10⁻³   | 0.19    | 0.6669        |
| X₁X₄          | 0.02           | 1  | 0.02        | 0.77    | 0.3940        |
| X₂X₃          | 0.01           | 1  | 0.01        | 0.61    | 0.4495        |
| X₂X₄          | 0.22           | 1  | 0.22        | 10.11   | 0.0067        ** |
| X₃X₄          | 0.04           | 1  | 0.04        | 1.74    | 0.2083        |
| X₁²           | 0.04           | 1  | 0.04        | 1.71    | 0.2124        |
| X₂²           | 3.46           | 1  | 3.46        | 158.55  | <0.0001       ** |
| X₃²           | 0.09           | 1  | 0.09        | 4.04    | 0.0641        |
| X₄²           | 0.16           | 1  | 0.16        | 7.52    | 0.0159        * |
| Residual      | 0.31           | 14 | 0.02        |         |               |
| Lack of Fit   | 0.27           | 10 | 0.03        | 2.61    | 0.1837        not significant |
| Pure Error    | 0.11           | 4  | 0.03        |         |               |
| Cor Total     | 21.72          | 28 |            |         |               |

b Standard deviation = 0.1478; Mean = 5.9703; C.V. = 2.4759%; Predicated residual sum of squares (PRESS) = 1.5917; R-Squared = 0.9656; Adjusted (R²) = 0.9311; Predicted (R²) = 0.8208; Adequate Precision = 22.9512.

*Significant difference (p<0.05); ** Significant differences (p<0.01).
Fig. 5 The surface response plots of the effects of enzyme dosage, enzymatic lysis temperature, enzymatic lysis pH, enzymatic lysis time on the oil yield and DHA yield: (a) oil yield of the interaction between enzyme dosage and enzymatic lysis temperature. (b) DHA yield of the interaction between enzyme dosage and enzymatic lysis temperature. (c) oil yield of the interaction between enzymatic lysis pH and enzymatic lysis temperature. (d) DHA yield of the interaction between enzymatic lysis pH and enzymatic lysis temperature. (e) oil yield of the interaction between enzymatic lysis time and enzymatic lysis temperature. (f) DHA yield of the interaction between enzymatic lysis time and enzymatic lysis temperature.

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![TIC profile of fatty acids methyl ester in algae oil.](image)

**Fig. 6** TIC profile of fatty acids methyl ester in algae oil.

![Mass spectra of DHA (C22:6) methyl ester.](image)

**Fig. 7** Mass spectra of DHA (C22:6) methyl ester.

| Run | Retention time/min | Compounds                                      | content/% |
|-----|--------------------|------------------------------------------------|-----------|
| 1   | 4.420              | Dodecanoic acid, C12:0                           | 0.16 ± 0.04 |
| 2   | 6.986              | Tetradecanoic acid, C14:0                        | 6.98 ± 0.11 |
| 3   | 8.508              | Pentadecanoic acid, C15:0                        | 0.81 ± 0.09 |
| 4   | 10.153             | Hexadecanoic acid, C16:0                         | 21.97 ± 0.23 |
| 5   | 10.475             | 9-Hexadecenoic acid                              | 1.11 ± 0.06 |
| 6   | 13.708             | Stearic acid, C18:0                              | 0.45 ± 0.03 |
| 7   | 16.807             | 9-Oleic acid                                     | 0.22 ± 0.01 |
| 8   | 20.740             | 5,8,11,14-Eicosatetraenoic acid, C20:4           | 1.01 ± 0.07 |
| 9   | 27.373             | 5,8,11,14,17-Eicosapentaenoic acid, C20:5        | 18.22 ± 0.18 |
| 10  | 29.728             | 4,7,10,13,16,19-Docosahexaenoic acid, C22:6      | 49.07 ± 0.15 |

**Table 5** Fatty acid composition in algae oil.
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