OPTIMIZATION OF CONDITION FOR METHYL PHEOPHORBIDE A FORMATION AND CONVERSION TO CHLORIN E6

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ABSTRACT

Chlorin e6 is a stable chlorophyll derivative and can be used in photodynamic therapy (PDT) to treat cancer. One of the synthetic pathways of chlorin e6 is via the transformation of chlorophyll *a* to *methyl pheophorbide a*. In this study, we optimize the experimental condition for obtaining *methyl pheophorbide a* from chlorophyll extracted from Spirulina. The preliminary study showed that the suitable solvent system for extracting chlorophyll from the extract was dichloromethane and n-hexane in a ratio of 1:1. To determine the optimal parameters of 4 factors: time, temperature, chlorophyll/H_2SO_4 ratio (w/v), chlorophyll/MeOH ratio (w/v) to obtain the highest conversion efficiency of *methyl pheophorbide a*. The experimental matrix followed the Box-Behnken design to investigate the optimal region, with experiments for four factors at three levels to build the indicator surface. The optimal conditions for preparing *methyl pheophorbide a* from chlorophyll: Chlorophyll/H_2SO_4 ratio was 4/5 (w/v), chlorophyll/CH_3OH ratio was 6/50 (w/v), time was 4.4 hours and temperature was 44^{o}C. Chlorin e6 was successfully prepared from *methyl pheophorbide a.*

Keywords: Spirulina; Chlorin; photodynamic therapy.

1. Introduction

Spirulina is a multicellular, filamentous cyanobacterium. The genus *Spirulina* includes photosynthetic organisms, with phycocyanin as the primary photosynthetic pigment; it also has carotenoids and chlorophyll *a* [1]. Spirulina has evolved naturally in our environment for millions of years and is able to withstand harsh growing conditions; naturally growing Spirulina can be found in lakes with high alkalinity [2], [3]. Spirulina contains many pigments, including chlorophyll *a*, xanthophyll, beta carotene, echinenone…, c-phycocyanin and allophycocyanin [4]. Spirulina is an economical source of good quality natural chlorophyll bio-availability because most chlorophyll in Spirulina exists in chlorophyll *a*, unlike most plants with a mixture of chlorophyll *a* and *b*.

Chlorin e6 is a stable derivative of chlorophyll, one of the important optically active derivatives with many practical applications. Among them, we can mention the use of chlorin e6 in photodynamic therapy (PDT) to treat cancer [5], [6]. The synthesis of chlorin e6 from natural products, such as Spirulina, is illustrated in Figure 1 [7]. Extract and recover chlorophyll *a* from algae (a), reduce the magnesium nucleus with an acid solution and methylate, and replace the phytyl group with methanol to obtain *methyl pheophorbide a* (b). Next, methyl pheophorbide a's ring-opening reaction was performed to form chlorin e6 trimethyl ester via the Retro-Claisen condensation reaction (c). The study of chlorophyll extraction from spirulina algae has been carried out in previous studies with optimized extraction process conditions. In this study, we optimized the process of forming *methyl pheophorbide a* and converting it to chlorin e6, thereby evaluating the activity of the obtained components.

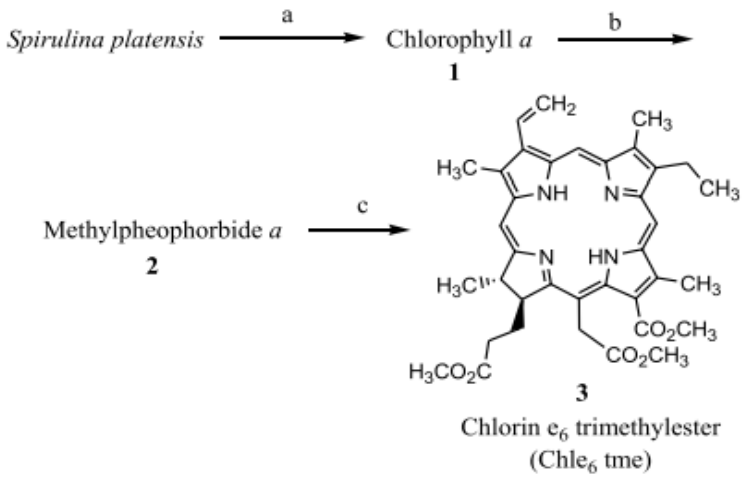


Figure 1: *Transformation pathway of chlorin e6 from Spirulina*

2. Materials and method

2.1. Materials and chemicals

Dried spirulina samples in powder form were obtained from Vinh Hao Algae Joint Stock Company in Tuy Phong, Binh Thuan province. All chemicals such as absolute alcohol (C_2H_5OH 99.7%), concentrated sulfuric acid (H_2SO_4), methanol (CH_3OH), n-hexane (C_6H_{14}), Dichloromethane (CH_2Cl_2), Tetrahydrofuran (THF), Potassium oxide (KOH), Chloroform ($CHCl_3$), Acetone (C_3H_6O), Silicagel 60 (0.063 - 0.200 mm) were of analytical grade and obtained from Xilong Scientific- China, QreC (Asia)- Malaysia, Merck- Germany.

2.2. Preparation of pheophobide a and chlorin e6

Extraction of chlorophyll in Spirulina: Chlorophyll was extracted from dried algae samples by ethanol at 50^{o}C for 3 hours and 30 minutes, then filtered through filter paper to remove the residues. Separation of chlorophyll from the extract mixture by column chromatography was carried out with the stationary phase being silica gel and the mobile phase being n-hexane (non-polar), dichloromethane (polar) and a mixture of n-hexane and dichloromethane in a 1:1 ratio (medium polarity) [8].

Preparation of methyl pheophorbide a: Chlorophyll samples, after being separated by column chromatography with the solvent system, were transferred to a glass cup that was covered with aluminium foil to avoid light and concentrated on an electric stove with a temperature control of about 60-70^{o}C to evaporate all the solvent. A certain amount of methanol was added to the concentrated solution at a controlled temperature; then, concentrated sulfuric acid was added to the solution until it was finished. Leave the reaction in the thermostatic bath and shake it every 15 minutes.

Purification of methyl pheophorbide a by column chromatography: The solution after the reaction was evaporated at 60-70^{o}C to remove the solvent. Crude *methyl pheophorbide a* was dissolved in dichloromethane, and separation was done in a silica gel column. When this fraction flows out almost wholly, a solvent system with a higher polarity was added, a mixture of dichloromethane and acetone at a ratio of 30:1. The blue-black fraction containing *methyl pheophorbide a* was a relatively clean product. Remove the solvent from the *methyl pheophorbide* solution by evaporating it in an oven at 60-70^{o}C and drying the *methyl pheophorbide* product at 70^{o}C until the mass is constant. Weigh the resulting solid to determine the mass of the substance formed, thereby determining the efficiency of the reaction on 1 g of dry algae.

The mass of dry algae extracted to collect chlorophyll in each experiment is 4 g. Based on the available mass of chlorophyll (approximately 0.12 g), the volume of H_2SO_4 and methanol in each experiment can be determined. The efficiency is determined by the ratio of the mass of *methyl pheophorbide a* (MPPB) formed to the mass of dry algae. According to the previous survey of dry algae materials, the moisture content is 8.85% [8], from which the dry matter mass can be calculated to be 3.646 g.

2.3. Optimizing the preparation process of methyl pheophorbide a

To determine the optimal parameters of 4 factors: time, temperature, chlorophyll/H_2SO_4 ratio (w/v), and chlorophyll/MeOH ratio (w/v) to obtain the highest conversion efficiency of *methyl pheophorbide a.* The experimental matrix followed the Box-Behnken design to investigate the optimal region, with experiments for four factors at three levels to build the indicator surface. Box-Behnken designs are usually rotational, with a small number of experiments in each iteration, and no experimental point falls outside the interval between the two established levels for each variable.

The mathematical model describing the influence of independent variables on dependent variables has the form of a quadratic polynomial function with the following general form: $Y = bo + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{11}X^2_1 + b_{22}X^2_2 + b_{33}X^2_3 + b_{44}X^2_4$. Where b_0: constant b_1, b_2, b_3, b_4: are first-order simple linear regression coefficients b_{11}, b_{22}, b_{33}, b_{44}: are second-order simple linear regression coefficients b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34}: are pairwise interaction regression coefficients. Each coefficient b characterizes the influence of each factor on the objective function. The coefficients are calculated according to the experimental data of the objective function, which is the reaction efficiency. The acceptance level of the model is expressed by the coefficient of determination R2 - the correlation coefficient of the regression function.

2.4. Data processing

Data was statistically processed on Megastat software version 10.4 and Microsoft Excel in Office 365. An optimal experimental strategy was developed, and results were processed on Minitab 16 software.

3. Results and discussion

3.1. Selection of experimental condition

Selection of solvent: Based on the images of the chromatographic columns when using different solvents, all three solvents gave clear separation between chlorophyll and β-carotene. However, with the extraction by the mixture of dichloromethane and dichloromethane: n-hexane (with the ratio of 1:1), the colour of chlorophyll was relatively clear, while with the extraction by n-hexane, the chlorophyll colour was pale and unclear. Because n-hexane is an almost non-polar solvent, the ability to dissolve chlorophyll was poor, and less chlorophyll flowed through the column than with the other two solvents. When extracting chlorophyll, we need a solvent system that harmoniously balances removing the impurity component, β-carotene (the main impurity mixed in chlorophyll) and the maximum amount of chlorophyll obtained. When dichloromethane was used as the solvent for the extraction, the chlorophyll volume was the largest. However, the β-carotene volume was relatively modest, resulting in a large amount of β-carotene remaining in the obtained chlorophyll solution. On the contrary, when using n-hexane, the β-carotene volume was significant, but the chlorophyll obtained was very little. Thus, using the solvent system of dichloromethane and n-hexane (with a 1:1 ratio) gave the best efficiency in extracting chlorophyll.

Table 1: *The volume of chlorophyll and β - carotene obtained from dried algae extract when using mobile phase dichloromethane and n-hexane in a ratio of 1:1*

Solvent	Chlorophyll (mL)	β - carotene (mL)
dichloromethane and n-hexane	36.3 32.5 43	25 26 15
dichloromethane	40 45 47.5	10 7 12
n - hexane	16.8 24 22	40 30 32.5

3.2. Building a regression equation

The optimized efficiency of *methyl pheophorbide a* formation with the input factors of chlorophyll/H_2SO_4 ratio (1/5-10/5, w/v), chlorophyll/CH_3OH (5/50-10/50, w/v), time 2-6 hours and temperature 30-70^oC are presented in Table 2 based on a previous study [5]. The maximum yield of *methyl pheophorbide* formation was 3.264% (based on dry algae) at 0.11 mL H_2SO_4, 1.2 mL CH_3OH in 4 hours and at a temperature of 50^oC. The

minimum conversion efficiency was 0.234% at 0.11 mL H_2SO_4 and 1.2 mL CH_3OH in 2 hours at 70°C. The effects of factors such as the volume of H_2SO_4 and CH_3OH, time and temperature, and the interaction effects of factors were tested through the significance of coefficients in the regression model.

Table 2: *Experimental design and performance results according to the Box-Behnken model*

STT	Coding				True values				MPPB weight (g)	Efficiency (%)
	X1	X2	X3	X4	Volume H_2SO_4 (mL)	Volume CH_3OH (mL)	Time (h)	Temp (°C)		
1	-1	-1	0	0	0.6	6	4	50	0.0210	0.576
2	1	-1	0	0	0.06	6	4	50	0.0530	1.454
3	-1	1	0	0	0.6	0.6	4	50	0.0759	2.082
4	1	1	0	0	0.06	0.6	4	50	0.0166	0.455
5	0	0	-1	-1	0.11	1.2	2	30	0.0503	1.38
6	0	0	1	-1	0.11	1.2	6	30	0.0732	2.008
7	0	0	-1	1	0.11	1.2	2	70	0.0085	0.234
8	0	0	1	1	0.11	1.2	6	70	0.0465	1.278
9	-1	0	0	-1	0.6	1.2	4	30	0.0867	2.38
10	1	0	0	-1	0.06	1.2	4	30	0.0759	2.082
11	-1	0	0	1	0.6	1.2	4	70	0.0466	1.278
12	1	0	0	1	0.06	1.2	4	70	0.0174	0.477
13	0	-1	-1	0	0.11	6	2	50	0.0210	0.576
14	0	1	-1	0	0.11	0.6	2	50	0.0604	1.656
15	0	-1	1	0	0.11	6	6	50	0.0732	2.008
16	0	1	1	0	0.11	0.6	6	50	0.0653	1.791
17	-1	0	-1	0	0.6	1.2	2	50	0.0749	2.054
18	1	0	-1	0	0.06	1.2	2	50	0.0315	0.863
19	-1	0	1	0	0.6	1.2	6	50	0.0816	2.237
20	1	0	1	0	0.06	1.2	6	50	0.0575	1.579
21	0	-1	0	-1	0.11	6	4	30	0.0491	1.348
22	0	1	0	-1	0.11	0.6	4	30	0.0766	2.102
23	0	-1	0	1	0.11	6	4	70	0.0223	0.612
24	0	1	0	1	0.11	0.6	4	70	0.0580	1.592
25	0	0	0	0	0.11	1.2	4	50	0.0971	2.664
26	0	0	0	0	0.11	1.2	4	50	0.1142	3.132
27	0	0	0	0	0.11	1.2	4	50	0.1190	3.264

Table 3 shows the results of the regression coefficients of the model before and after removing the insignificant factors. The independent variables strongly affecting the model are X_1, X_2, X_3, X_4. The *p*-values of these variables are much smaller than the significance level $\alpha = 0.05$. The second-order factors all strongly impact the model (X_{11}, X_{22}, X_{33}, X_{44}) because the p-values of these variables are less than or equal to 0.001. Among the two-level interaction factors, only the interaction pair between variables X_1 and X_2 has a significant impact ($p = 0.005$), while the other interaction pairs have very weak impacts on the model. We eliminate these interaction pairs, including chlorophyll/H_2SO_4 ratio and time (b_{13}); the interaction pair chlorophyll/H_2SO_4 ratio and temperature (b_{14}); The interaction pair of chlorophyll/CH_3OH ratio and time (b_{23}); the interaction pair of chlorophyll/CH_3OH ratio and temperature (b_{24}); the interaction pair of time ratio and temperature (b_{34}) out of the model and rerunning the regression model, we have the table of regression coefficient values.

Table 3: *Regression coefficient values of the model before and after removing insignificant interaction factors*

Regression coefficient	***Before***		***After***	
	Regression coefficient value	***p*-value**	**Regression coefficient value**	**p-value**
bo	3.020	0.000	3.02	0.000
b1	-0.308	0.013	-0.308	0.009
b2	0.259	0.03	0.259	0.023
b3	0.345	0.007	0.345	0.004
b4	-0.486	0.001	-0.486	0.000
b11	-0.741	0.001	-0.741	0.000
b22	-0.899	0.000	-0.899	0.000
b33	-0.723	0.001	-0.723	0.000
b44	-0.834	0.000	-0.834	0.000
b12	-0.626	0.005	3.02	0.000
b13	0.133	0.478	-0.308	0.009
b14	-0.126	0.503	0.259	0.023
b23	-0.324	0.100		
b24	0.056	0.762		
b34	0.104	0.578		
R2	0.906		0.8701	
R^2 adjusted	0.797		0.801	
Lack of fit		0.487		0.508

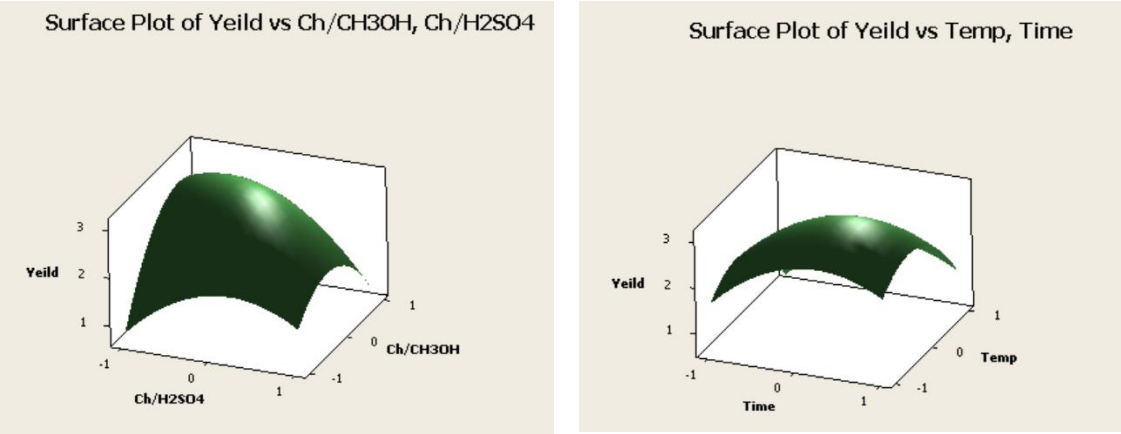
After removing the insignificant interaction factors from the regression model, we see that the *p*-value of "Lack of fit" increases and the adjusted R^2 and R^2 coefficients are closer to each other than the original. This shows that after removing the insignificant interaction factors, the model is more suitable than the original one. The fitting coefficient

R^2 = 0.8701 indicates that 87.01% of the change in the value of the objective function Y is explained by the coefficients of the variables in the model. The adjusted R^2 and R^2 coefficients are close to each other, which shows that the change in the value of Y is mainly due to the coefficients X without the need for additional factors. Test the model's suitability through the p-value of "Lack of fit"; the *p*-value = 0.508 > α. Therefore, the model is entirely suitable to describe the data. The quadratic regression equation with four input factors (4 coded variables are X_1, X_2, X_3, X_4) of the objective function (*methyl pheophorbide a* conversion efficiency) according to the selected optimal model has the following form:

$$Y = 3.020 - 0.308\ X_1 + 0.259\ X_2 + 0.345\ X_3 - 0.486\ X_4 - 0.741\ X^2_1 - 0.899\ X^2_2 - 0.723\ X^2_3 - 0.834\ X^2_4 + 3.02\ X_1X_2 - 0.308\ X_1X_3 + 0.259\ X_1X_4$$

3.3. Interactive effects of factors on the conversion efficiency of methyl pheophorbide a

Some factors interacted with the conversion efficiency of *methyl pheophorbide a*. Figure 2 shows the interactions between the factors investigated on the conversion efficiency. The interaction between the chlorophyll/H_2SO_4 ratio (w/v) and the chlorophyll/CH_3OH ratio (w/v) had a strong influence on the conversion efficiency of *methyl pheophorbide a* ($p<0.05$). The response surface plots representing the 3-dimensional space visually represent the relationship between the conversion efficiency of *methyl pheophorbide a* and the factors of chlorophyll/H_2SO_4 ratio (w/v) and chlorophyll/CH_3OH ratio (w/v) are shown in Figure 2. The conversion efficiency values did not change much, fluctuating between 1.8-3.1%. The graph had a slightly curved surface.



(a) *(b)*

Figure 2: *Response surface model of interaction between two factors on conversion efficiency: (a) chlorophyll/H_2SO_4 ratio and chlorophyll/CH_3OH ratio, (b) time and temperature*

3.4. Optimization of factors to obtain the highest methyl pheophorbide conversion efficiency

The conversion efficiency of *methyl pheophorbide a* from chlorophyll *a* was affected by four factors: chlorophyll/H_2SO_4 ratio (w/v), chlorophyll/CH_3OH ratio, temperature, and time. Providing optimal conditions for this intermediate compound would

limit unwanted compounds, save time and be effective in the following conversion steps. The results of statistical processing by determining the extreme value for the objective function in Mintab 16 are expressed in Figure 3.

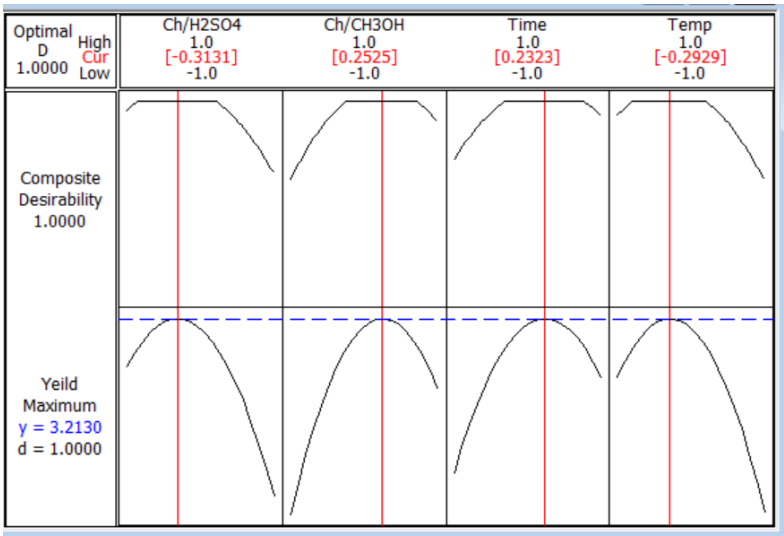


Figure 3: *Optimization of factors affecting methyl pheophorbide a conversion efficiency*

The highest *methyl pheophorbide* conversion efficiency was 3.21% when the chlorophyll/H_2SO_4 ratio was - 0.3, equivalent to 4/5 (w/v), the chlorophyll/CH_3OH ratio was 0.2, equivalent to 6/50 (w/v), the time was 0.2 equivalent to 4.4 hours, and the temperature was - 0.3 equivalent to 44^{o}C. The conversion efficiency was low if the chlorophyll/H_2SO_4 ratio was too large or smaller than the optimal value. Indeed, if the chlorophyll/H_2SO_4 ratio was significant while the mass of chlorophyll remained constant, the volume of H_2SO_4 was small. The small volume of H_2SO_4 was not enough for chlorophyll to ultimately convert to pheophytin, leading to a decrease in the conversion efficiency to methyl pheophorbide. If the chlorophyll/H_2SO_4 ratio was significant while the mass of chlorophyll remained constant, the volume of H_2SO_4 was large. This is disadvantageous for the chlorophyllase enzyme - an enzyme available in the cytosol which converts chlorophyll in organic reactions.

Time is a factor to consider: a short or long reaction time resulted in low conversion efficiency. A short time means the reaction was continued. However, prolonging the reaction time reduced efficiency because *methyl pheophorbide a* is unstable in environmental conditions; it can be oxidized by light and air oxygen. In addition, the temperature was important in the conversion process of *methyl pheophorbide* formation because, at high temperatures, the chlorophyllase enzyme was inactivated. Furthermore, the reaction of converting chlorophyll into *methyl pheophorbide* decreased under high temperatures. This was because *methyl pheophorbide a* continued to react and transform into *pyropheophorbide* [10]. However, the chlorophyllase enzyme was less active at low temperatures, leading to a decrease in conversion efficiency. The optimal operating temperature of the chlorophyllase enzyme was about 50^{o}C, which was also quite close to the optimal temperature of the conversion (44^{o}C). From the discussion results and the expected function value d = 1, it can be seen that the optimal value of the objective

function, as predicted in the model, could be achieved. The factors affecting the highest conversion efficiency of *methyl pheophorbide a* were the ratio of chlorophyll/H_2SO_4 4/5 (w/v), the ratio of chlorophyll/CH_3OH 6/50 (w/v) at 4.4 hours and 44^oC.

4. Conclusion

In this study, we conducted a preliminary survey of the solvent systems that can recover chlorophyll from the mixture of dried algae extract. Specifically, the solvent system of dichloromethane and n-hexane with a ratio of 1:1 was selected for the optimization design. The optimal conditions for preparing *methyl pheophorbide a* from chlorophyll *a* of dried algae extract achieved a high yield of 3.21%. The preparation conditions for the extraction were: the ratio of chlorophyll/H_2SO_4 was 4/5 (w/v), the ratio of chlorophyll/CH_3OH was 6/50 (w/v), the time was 4.4 hours, and the temperature was 44^oC.

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TÓM TẮT

TỐI ƯU HÓA QUÁ TRÌNH TẠO THÀNH *METHYL PHEOPHORBIDE A* VÀ CHUYỂN HÓA THÀNH CHLORIN E6

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Chlorin e6 là một dẫn xuất ổn định của diệp lục và có thể được sử dụng trong liệu pháp quang động (PDT) để điều trị ung thư. Một trong những con đường tổng hợp chlorin e6 là thông qua quá trình chuyển đổi diệp lục *a* thành *methyl pheophorbide a*. Trong nghiên cứu này, chúng tôi đã tối ưu hóa điều kiện thực nghiệm để thu được methyl pheophorbide a từ diệp lục chiết xuất từ tảo xoắn. Nghiên cứu sơ bộ cho thấy hệ dung môi thích hợp để chiết xuất diệp lục từ dịch chiết là dichloromethane và n-hexane theo tỷ lệ 1:1. Để xác định các thông số tối ưu của bốn yếu tố: thời gian, nhiệt độ, tỷ lệ diệp lục/H_2SO_4 (w/v), và tỷ lệ diệp lục/MeOH (w/v) nhằm đạt hiệu suất chuyển đổi *methyl pheophorbide a* cao nhất, chúng tôi đã sử dụng ma trận thực nghiệm theo thiết kế Box-Behnken. Thiết kế này bao gồm các thí nghiệm với bốn yếu tố ở ba mức để xây dựng bề mặt chỉ thị. Điều kiện tối ưu để điều chế *methyl pheophorbide a* từ diệp lục là: tỷ lệ diệp lục/H_2SO_4 là 4/5 (w/v), tỷ lệ diệp lục/CH_3OH là 6/50 (w/v), thời gian phản ứng là 4,4 giờ, và nhiệt độ là 44°C. Chlorin e6 đã được điều chế thành công từ *methyl pheophorbide a* với hiệu suất đạt 3,12% tính theo khối lượng chất khô.

Từ khóa: Tảo xoắn; Chlorin; liệu pháp quang động.