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OPTIMIZING ALLOPHYCOCYANIN EXTRACTION FROM *Chaetomorpha* sp. ALGAE WITH ENZYME CELLULASE

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ABSTRACT

Allophycocyanin (APC) is a pigment-carrying protein belonging to the phycobiliprotein group, with many specific biological activities. This study was carried out to obtain APC from *Chaetomorpha* sp. with the support of cellulase enzyme by experimental method. The surveyed influencing factors include enzyme treatment time (30; 60; 90; 120; 180 min), material/solvent ratio surveyed (1:10; 1:15; 1:20); 1:25; 1:30, w/v), and enzyme/substrate ratio (1%; 2%; 3%; 4%; 5%). Response surface methodology (RMS) was performed to optimize the factors affecting the extraction process. The analysis results of JMP 10 software showed that the optimal APC content was 0.27 mg/mL with an enzyme/substrate ratio of 3.21%; the material/solvent ratio is 1:20.89 w/v; extraction time 102.68 min.

Keywords: Allophycocyanin, *Chaetomorpha* sp., cellulase, optimization.

1. INTRODUCTION

Allophycocyanin (APC) is composed of two subunits α and β with a molecular mass of 17.8 kDa and 17.9 kDa, respectively, and a molecular mass of intact APC of 107 kDa [1]. The α subunit consists of 160 amino acids with a site for binding a Phycocyanobilin (PCB) at α-Cys84. The β subunit has 161 amino acids and a covalently linked PCB at the β-Cys84 position [2]. APC is a major component of the core of Phycobiliproteins (PBPs) and depending on the solvent, pH, and concentration of APC present in the protein, Allophycocyanin can exist in the core as an α and β monomer, a trimer $(\alpha\beta)^3$ or a hexamer $(\alpha\beta)^6$, identified and isolated using electron microscopy [3]. Although there are many applications in many fields such as making fluorescent probes, antioxidants, antibacterial agents... [4, 5] the collection of APC in water has not been studied yet, opening up the potential of research to obtain APC from many different raw materials.

Chaetomorpha sp. has filamentous algae belonging to the phylum Chlorophyta that grows in clusters that are interwoven like blankets on the water surface, commonly present in extensive brackish water shrimp ponds. *Chaetomorpha* sp. with high protein content ranging from 11-23% w/w dry matter, especially bioactive proteins and peptides, is a new and very promising approach to exploit this sustainable biomass source [6]. Enzyme-assisted extraction is a commonly used extraction method for plant materials [7, 8]. Various enzymes such as cellulase, pectinase, and hemicellulase are commonly used to break down the plant cell wall structure, facilitating the enhanced extraction of biologically active substances from the plant. These enzymes hydrolyze the components of the cell wall by increasing the permeability of the cell wall, facilitating the penetration of solvents into the internal structure to dissolve the compounds to be extracted. Previous studies focused on PBP extraction mainly focused on R-

Phycoerythrin or C-Phycocyanin extraction methods, while APC extraction preparations were few. Enzyme-assisted extraction of biomolecules from plants is a potential alternative to solvent extraction methods and is gaining more attention as an efficient, benign extraction technology, sustainable, and environmentally friendly. The application of enzymes to the extraction of plant compounds is based on their catalytic properties and modes of action, optimal operating conditions, and combinations of enzymes [8].

This study aimed to investigate the impact of cellulase enzyme on the content of APC derived from *Chaetomorpha* sp.. The response surface method (RMS) was used for optimization. The results of this provide the foundation for further research on other phycobiliprotein extracts from algae. Cellulase enzymes can destroy plant cell walls and facilitate the extraction of biologically active substances. The price is relatively cheap and easy to buy compared to other enzymes. In addition, the optimum temperatures of the effective enzyme (50-60 °C) are close to the stable temperature of APC (45-50 °C).

2. MATERIALS AND METHODS

2.1. Materials

Chaetomorpha sp. algae were received after 20-25 days of discharge from extensive shrimp ponds in Vinh Chau town, Soc Trang province, Vietnam. It was dark green with algae fibers from 2 to 4m. The algae were transported in styrofoam to the laboratory within the day. In the laboratory, the alga was washed through tap water to remove impurities, dried in an oven to less than 10% moisture, finely ground with a stainless steel blender, and sieved with an 80-mesh sieve. Samples were kept frozen and used throughout the study.

Enzyme cellulase purchased at Vietnam Biogreen Pharmaceutical and Biotechnology Joint Stock Company (Cellulase works between pH 6.5 and 8.5 in the temperature range from 45 to 65 °C); Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) and disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$); Other analytical chemicals that meet the laboratory's technical requirements were purchased at Doan Le chemical company.

2.2. Methods

2.2.1. Effects of enzyme/substrate concentration ratio on APC extraction

A quantity of 2 g of sample (calculated by the mass of dry matter) was weighed and supplemented with 0.05 M phosphate buffer pH=7 with the ratio of material/solvent 1:20 (w/v). The extraction was conducted at 50 ℃ in a thermostatic bath (Memmert WNB22, Germany) for 90 min. The investigated enzyme/substrate concentration ratios were changed (1%; 2%; 3%; 4%; 5%) to record APC content. After the extraction process was completed, the sample was centrifugated at 5,500 rpm for 20 minutes to isolate the supernatant. Then the supernatant was subjected to the UV-Vis measurement at 620 nm, and 650 nm [9].

2.2.2. Effects of raw material/solvent ratio on APC extraction

The enzyme/substrate ratio was selected from the results of the above experiment (2.2.1), while the temperature and time were maintained at 50 ℃ and 90 min, respectively. The influence of the material/solvent ratio (1:10; 1:15; 1:20; 1:25; 1:30, w/v) in 0.05 M phosphate buffer pH 7 on the APC content was studied. At the end of the extraction, the mixtures were centrifuged at 5,500 rpm for 20 min to collect the supernatant for the UV-Vis measurement at 620 nm, and 650 nm [9].

2.2.3. Effects of enzyme treatment time on APC extraction

The enzyme/substrate concentration ratio (results in 2.2.1) and material/solvent ratio

(results in 2.2.2) in 0.05 M phosphate buffer pH 7 were selected. The influence of incubation times (30, 60, 90, 120, and 150 min) was investigated at a fixed 50 °C. At the end of the extraction process, the mixtures were centrifuged at 5,500 rpm for 20 min to collect the supernatant for the UV-Vis measurement at 620 nm, and 650 nm [9].

2.2.4. Optimization of APC extraction process by RSM

To optimize the APC extraction process by the experimental method, the second-order orthogonal model with the center of rotation with 19 experiments was applied. 3 influencing factors included enzyme/substrate ratio (%, X_1), raw material/solvent ratio (w/v, X_2), and enzyme treatment time (min, X_3). The response was APC content (Y). The coding and experimental values were shown in Table 1.

Table 1. Coding and experimental values of empirical factors

Variables	Factors	Unit	Levels				
			$-\alpha$	-1	0	1	α
Enzyme/substrate ratio	X_1	%	1.32	2	3	4	4.68
Raw material/solvent ratio	X_2	g/mL	11.59	15	20	25	28.41
Enzyme treatment time	X_3	min	39.55	60	90	120	140.45

In this study, the optimal experimental factors were enzyme/substrate ratio (1.32% - 4.68%), material/solvent ratio (1 g/11.59 mL - 1 g/28.41 mL), enzyme treatment time (39.55 min - 140.45 min), Optimal experiment by quadratic experimental planning method, centered structure with three influencing factors: enzyme/substrate ratio (X_1), raw material/solvent ratio (X_2), enzyme treatment time (X_3) with an objective function: APC content (Y, mg/mL), with the regression equation shown:

$$Y= \alpha_0 + \alpha_1X_1 + \alpha_2X_2 + \alpha_3X_3 + \alpha_4X_1X_1 + \alpha_5X_2X_2 + \alpha_6X_3X_3 + \alpha_7X_1X_2 + \alpha_8X_1X_3 + \alpha_9X_2X_3$$

Where: α_0, α_1... α_9 were parameters of the equation.

The extraction process of APC with the support of cellulase enzyme and optimization of all 3 survey factors, with that goal, there are 2 common data processing layouts for experimental optimization, namely Central composite (CCD) and Box Behnken (BBD). CCD contains combinations of extreme factors and BBD does not. According to the DoE method, mathematical models should be used to test the theory of experimental space to determine the optimal position. However, the BBD models do not show a reliable estimation of the new data points and therefore cannot be used for our study.

2.2.5. APC content determination

The APC content was determined by measuring the UV-Vis absorbance values (A) at 620 nm (A_{620}) and 650 nm (A_{650}). APC concentration (C) was calculated by the following formula:

$$C = \frac{A_{650} - 0.2*A_{620}}{5.73} (mg/mL)$$ [10]

2.2.6. Data analysis

In this study, all of the experiments and investigations were conducted in triplicates. The results were presented as mean ± error values. Data processing and evaluation of significant differences between treatments were performed by statistical method ANOVA with MiniTab 19 software with α = 5%. The RSM was carried out by JMP 10 software.

3. RESULTS AND DISCUSSION

3.1. Effects of enzyme/substrate ratio on APC extraction

In this study, the enzyme/substrate ratios of 0%, 1%; 2%; 3%; 4%; 5% (w/w) with 0.05 M phosphate buffer/material mass ratio of 2/20 w/v, pH 7 were incubated for 90 min at 50 ℃ in the thermostatic bath. The results of the survey were shown in Table 2.

Table 2. Effects of enzyme/substrate ratio on APC content

Experiment	Enzyme/substrate ratio (%)	APC content (mg/mL)
1	0	0.053 ± 0.005^{d}
2	1	0.132 ± 0.010^{c}
3	2	0.245 ± 0.004^{b}
4	3	0.278 ± 0.003^{a}
5	4	0.281 ± 0.014^{a}
6	5	0.284 ± 0.004^{a}

Different letters in the same column represent a statistically significant difference according to ANOVA analysis (α = 0.05)

Cellulase enzyme breaks down the cell wall of *Chaetomorpha* sp. algae, increasing the release of active substances inside. Enzyme activity is affected by its concentration and substrate concentration [11]. Low enzyme concentration reduces the reaction rate, resulting in a slow and long extraction process. If high enzyme concentration and low substrate concentration, the reaction rate is prompt and thorough, but the product formed does not reach the maximum level. The obtained APC content increased in terms of expanding the enzyme/material ratio. The APC content no longer increased when enzyme concentration reached 3% (Table 2). It may be due to the intense enzyme activity causing the raw cell wall to be completely broken, extracting phycocoloids compounds, which hinders the determination of APC content [11]. Simultaneously with a high degree of cell disruption of the raw material, a decrease in APC purity (due to the release of undesirable biomolecules located in the cytoplasm and other cells) and denaturation of APC may occur [12, 13]. There was no significant difference in the APC content obtained at 3% and 4% enzyme/substrate ratios. Therefore, the enzyme/substrate ratio of 3% was selected for further experiments.

3.2. Effect of material/solvent ratio on the content of APC

In this study, the mass ratio of the materials was 1; 1.5; 2; 2.5; 3 (g) in 20 mL of solvent with enzyme/substrate ratio (3%) selected from the above survey results and incubated for 90 min at 50 ℃ in a thermostatic bath. The survey results were shown in Table 3.

Table 3. Effects of material/solvent mass ratio on APC content

Experiment	Material/solvent ratio (w/v)	APC content (mg/mL)
1	1:20	0.084 ± 0.006^{e}
2	1.5:20	0.156 ± 0.004^{d}
3	2:20	0.288 ± 0.003^{a}
4	2.5:20	0.235 ± 0.007^{b}
5	3:20	0.185 ± 0.015^{c}

Different letters in the same column represent a statistically significant difference according to ANOVA analysis (α = 0.05).

Table 3 showed that the APC content increased from the material/solvent 1:20 to 2:20 (w/v) but the APC content decreased from further the material/solvent ratio (2.5:20 and 3:20 w/v). According to Tavanandi H.A. *et al.* (2018), a high solvent ratio was advantageous in APC extraction, i.e.,a high solvent ratio could promote an increasing concentration gradient, resulting in increased diffusion rates and improved extraction efficiency [12, 14]. Nonetheless, an increased volume of extraction solvent can also lead to the simultaneous extraction of other proteins from the cytoplasm, which have similar absorption wavelengths to APC, thereby diminishing the purity of APC. Copper extraction under a discounting condition (solvent system, time, temperature, energy) will be influenced by the structure of the substances to be extracted. Thereby changing the purity of the substance of interest. In this study, the highest APC content was 0.288 ± 0.003 mg/mL at 2/20 w/v, which was in line with the results from the study providing an efficient extraction method for extraction of high-purity PC from *Arthrospira maxima* was 0.28 ± 0.003 (mg/mL) with the same material-to-solvent ratio of 0.5/5 (w/v) phosphate-buffered solvent pH 7 with the support of cellulase enzyme. Thus, the material/solvent ratio of 2:20 w/v was chosen for the next experiment for reasonable and economical prospects.

3.3. Effects of enzyme treatment time on APC extraction

An enzyme reaction is formed when its active site combines with a substrate.The reaction rate of the decomposition process may vary, occurring either rapidly or slowly, depending on the concentrations of the enzyme and substrate. Under the same reaction rate conditions, it takes time for the enzyme and the substrate to contact and complete the decomposition. The catalyst time is not enough, so the substrate is not completely resolved. Since the catalysis time is too long, the substrate has been resolved to the extreme, the resulting product is almost unchanged. The effects of APC extraction time on APC content were shown in Table 4 with enzyme/substrate ratio (3%) and material/solvent ratio 2/20 (w/v) selected from the surveyed results.

Table 4. Effects of enzyme treatment time on APC Content

Experiment	Enzyme treatment time (min)	APC content (mg/mL)
1	30	0.148 ± 0.006^{c}
2	60	0.177 ± 0.005^{b}
3	90	0.273 ± 0.007^{a}
4	120	0.277 ± 0.004^{a}
5	150	0.280 ± 0.003^{a}

Different letters in the same column represent a statistically significant difference according to ANOVA analysis (α=0.05)

According to Table 4, the higher the enzyme treatment time, the higher the APC content would be until 90 min extraction time, and the cellulase enzyme had almost completely decomposed the plant's cell walls. The APC contents at longer extraction times (120 and 150 min) resulted in no statistically significant difference. These results in the study were consistent with the findings of Tavanandi H.A. *et al*. (2018) related to APC extraction from green algae biomass *Arthrospira platensis* with lysozyme enzyme [15]. The extraction time depended much on the structure of PBPs, PBPs, and the biliproteins were arranged in the order: R-phycoerythrin, C-Phycocyanin, and APC so extraction of APC requires a longer time than C-Phycocyanin (CPC). So, the factors of enzyme/substrate ratio, raw material/solvent ratio, and enzyme treatment time all significantly affected ANOVA on the APC content. Then, the response surface method would be applied to optimize the APC extraction conditions [14].

3.4. Optimization of APC extraction conditions

In this study, 3 factors of enzyme/substrate ratio, solvent/material ratio, and enzyme treatment time were optimized by the CCD design model with the response of APC content. After the above single-factor experiments, the centers included the enzyme e/substrate ratio of 3%, the ratio of material/substance 1:20 w/v, and the enzyme treatment time of 90 min Table 5.

Table 5. Experimental matrix and experimental results

No.	Encoding variables			Real variables			APC content (Y)	No.	Encoding variables			Real variables			APC content (Y)
	X_1	X_2	X_3	Z_1	Z_2	Z_3			X_1	X_2	X_3	Z_1	Z_2	Z_3	
1	-1	-1	-1	2	15	60	0.159	11	0	-1.68	0	3	11.59	90	0.145
2	-1	-1	1	2	15	120	0.168	12	0	1.68	0	3	28.41	90	0.198
3	-1	1	-1	2	25	60	0.174	13	0	0	-1.68	3	20	39.55	0.159
4	-1	1	1	2	25	120	0.185	14	0	0	1.68	3	20	140.45	0.256
5	1	-1	-1	4	15	60	0.179	15	0	0	0	3	20	90	0.252
6	1	-1	1	4	15	120	0.193	16	0	0	0	3	20	90	0.254
7	1	1	-1	4	25	60	0.188	17	0	0	0	3	20	90	0.269
8	1	1	1	4	25	120	0.206	18	0	0	0	3	20	90	0.263
9	-1.68	0	0	1.32	20	90	0.132	19	0	0	0	3	20	90	0.272
10	1.68	0	0	4.68	20	90	0.193								

From the experimental results, regression analysis was calculated to find meaningful regression equations and set up response surfaces for the objective functions. The coefficients of the regression equation were shown in Table 6.

Table 6. The results of regression model analysis with the objective function of APC (Y)

Coefficient	Coefficient value	Value T	Value P	Coefficient	Coefficient value	Value T	Value P
α_0	0.262	34.71	0.0000	α_{22}	0.002	0.25	0.8071
α_1	0.014	2.96	0.0160	α_{33}	0.001	0.1	0.9188
α_2	0.010	2.28	0.0482	α_{12}	-0.034	-7.4	0.0000
α_3	0.016	3.43	0.0075	α_{13}	-0.031	-6.7	0.0000
α_{11}	-0.001	-0.22	0.8291	α_{23}	-0.018	-3.96	0.0033
$R^2 = 0.93$				$R^2_{adj} = 0.86$			

The regression analysis results showed that all coefficients in the regression equation are significant ($P < 0.05$). The coefficient R^2= 92.89 shows that the regression model explained 92.89 percent of the data. The regression analysis results showed that the regression model was significant ($P < 0.05$). Thus, the APC content is represented by the second-order model as follows:

$$Y = 0.262 + 0.014X_1 + 0.01X_2 + 0.016X_3 – 0.034\ X_1X_1 – 0.031X_2X_2 – 0.018X_3X_3$$

From the regression equation, the factors of enzyme/substrate ratio, the raw material/solvent ratio, and time treatment were positive effects on APC content while the interaction of them had a negative direction. The reason for this change may bet the higher the enzyme ratio, the solvent ratio, and the treatment time would cause the cell wall of the material to be completely cut, the more solvent medium, the extraction efficiency is lower than that of

single extraction and the longer the extraction time causing the components in the cell to move to the solvent more with the amount of APC extracted, other unwanted substances are also extracted, resulting lower purity of the target compounds. Response surfaces of the influence of enzyme/substrate ratio, material/solvent ratio, and extraction time were shown in Fig. 1.

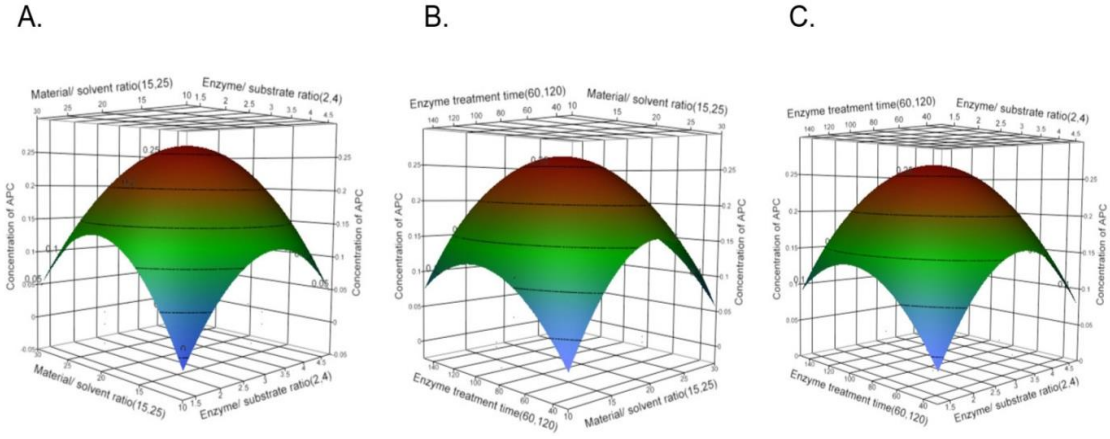

Fig. 1. Effects of raw material/solvent ratio and enzyme/substrate ratio (A); Raw material/solvent ratio and enzyme processing time (B); Raw material/solvent ratio and enzyme treatment time (C) on APC content.

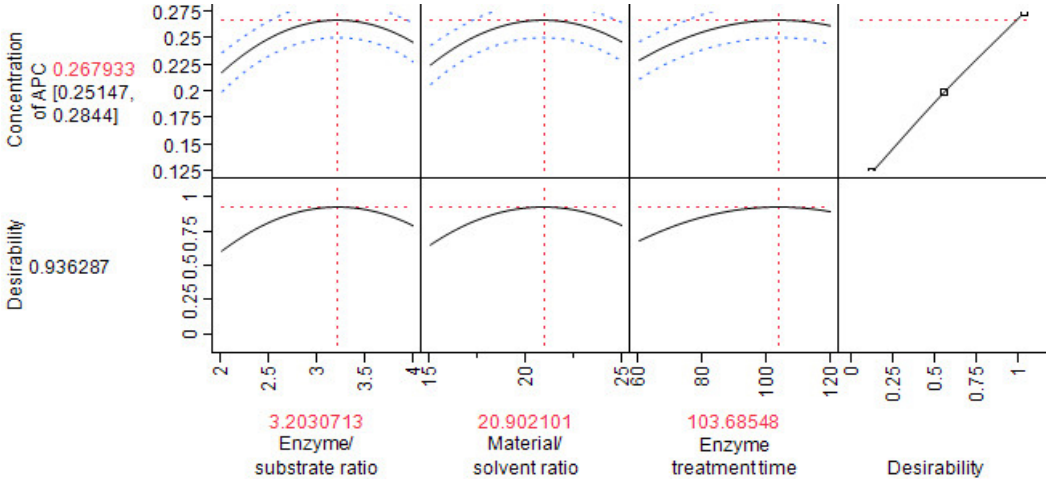

Fig. 2. Optimal parameters of the three factors

To demonstrate the parameters obtained from the analysis results of the JMP 10 software (Fig. 2), tests under optimized conditions were performed (repealed three times). The optimal APC content obtained was 0.27 mg/mL with an enzyme/substrate ratio of 3.20%; the material/solvent ratio is 20.83 (g/mL) and the enzyme processing time is 103.32 min. The findings indicate that the obtained APC content aligns with the values the quadratic regression model projected with a <5% difference. Therefore, using the quadratic equation to predict the APC content under optimal extraction and extraction conditions has practical value.

4. CONCLUSION

The study found the optimal extraction conditions for APC from *Chaetomorpha* sp. The optimal conditions for APC extraction were the enzyme/substrate concentration ratio of 3.21%; material/solvent ratio of 1:20.89 w/v and enzyme treatment time of 102.68 min,

resulting in APC content of 0.27 mg/mL, 5 times higher than the control sample without enzyme support cellulase 0.055 mg/mL. APC had economic benefits and high applicability in medical and food sectors due to its biological activities such as antioxidant, antibacterial mold, and support against cancer cells. Further studies related to purifying or characterizing it should be done to offer the basic information for practical applications.

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TÓM TẮT

TỐI ƯU HÓA QUÁ TRÌNH TRÍCH LY THU NHẬN ALLOPHYCOCYANIN TỪ RONG *Chaetomorpha* sp. CÓ SỰ HỖ TRỢ CỦA ENZYM CELLULASE

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Allophycocyanin (APC) là một loại protein mang sắc tố thuộc nhóm phycobiliprotein, được cấu tạo từ 2 tiểu đơn vị α và β, mang nhiều hoạt tính sinh học đặc trưng như tính huỳnh quang, hoạt tính kháng oxy hóa, kháng khuẩn, mốc... Nghiên cứu này được thực hiện để thu nhận APC từ rong *Chaetomorpha* sp. có sự hỗ trợ của enzyme cellulase bằng phương pháp thực nghiệm. Các yếu tố ảnh hưởng được khảo sát gồm: thời gian xử lý enzyme (30; 60; 90; 120; 180 phút), tỷ lệ nguyên liệu/dung môi khảo sát (1:10; 1:15; 1:20; 1:25; 1:30, w/v) và tỉ lệ enzyme/cơ chất khảo sát (1%; 2%; 3%; 4%; 5%). Phương pháp bề mặt đáp ứng (RMS) được sử dụng để tối ưu hóa các yếu tố ảnh hưởng đến quá trình trích ly. Kết quả thu được cho thấy khi tỉ lệ enzyme/cơ chất 3,21%; tỉ lệ nguyên liệu/dung môi là 1:20,89 w/v; thời gian trích ly 102,68 phút thu được hàm lượng APC ở điều kiện tối ưu đạt 0,27 mg/mL.

Từ khoá: Allophycocyanin, *Chaetomorpha* sp., cellulase, tối ưu.