OPTIMIZATION OF LECTIN EXTRACTION BY ENZYME-ASSISTED METHOD FROM ALGAE *Chaetomorpha linum*

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ABSTRACT

Chaetomorpha linum is a brackish algae widely distributed in the Mekong Delta. This study used the cellulase enzyme to enhance lectin extraction from *C. linum.* Response surface methodology (RSM) was employed to investigate the impact of enzyme concentration (0, 0.5, 1, 1.5, 2, 2.5%, w/w), temperature (30, 40, 50, 60 °C), and extraction time (2, 4, 6, 8 hours) on lectin extraction from *C. linum* algae. The RSM method was employed to optimize the conditions for enzyme-assisted lectin extraction. The obtained optimal extraction conditions were enzyme concentration of 2.55% w/w, temperature 49.98 °C, and extraction time of 6.9 hours. Under the optimum conditions, the obtained protein content of 0.134 g/g dry mass had the highest lectin activity of 61946.79 HU/g. The enzyme-assisted extraction technique has proven to be an efficient approach for enhancing the extraction yield of lectins from *C. linum.*

Keywords: Chaetomorpha linum, cellulase enzyme, lectin, optimization, extraction.

1. INTRODUCTION

Lectins are proteins capable of binding specifically to carbohydrates, demonstrating high-affinity interactions with glycans found in glycoproteins, glycolipids, and polysaccharides [1]. Lectins have been isolated from a wide range of sources, including viruses, fungi, bacteria, invertebrates, unicellular organisms, and animals. Lectins are associated with various properties, including anti-fungal, anti-inflammatory, anti-viral (HIV, hepatitis B...), anti-tumor, etc. [2]. Thus, lectins are currently the focus of extensive research due to their significant applications in medicine and technology. Lectins have been isolated from a variety of algae. Lectins extracted from cyanobacterial species, such as the freshwater cyanobacteria *Oscillatoria agardhii*, *Nostoc ellipsosporum*, and *Scytonema varium*, as well as the red algae *Griffithsia* sp., have antiviral properties and may therefore be strong candidates for preventing HIV transmission [3]. Lectins isolated from *Microcystis viridis* and *Scytonema varium* also inhibit viral infection and prevent the entry of Hepatitis C into human hepatocytes [4].

Green algae are widely distributed in the provinces of the Mekong Delta, Vietnam [5]. *C. linum* is a brackish water alga belonging to the genus *Chaetomorpha*. There are various biologically active substances: fucoidan, C-phycocyanin, A-phycocyanin, R-phycoerythrin, and lectin... have been found in *C. linum* algae. Lectin has been less concerned with extracting, although there was a significant protein content in this alga (14-19% dry mass) [6]. Moreover, the overgrowth of *C. linum* consumes oxygen and blocks sunlight from underwater plants, especially in aquaculture ponds. Ultimately, when the algae die, the bacteria responsible for decomposing the dead algae consume oxygen from the water. This depletion of oxygen makes it impossible for aquatic life to survive. Thus, it is essential to exploit valuable components like lectin from *C. linum* for further practical applications to enhance the value of this material and reduce the environmentally negative effects of its decomposition [6]. *C. linum* is an abundant but underutilized resource. To expand our understanding of the phytochemical potential of *C. linum*, the present study focused on the isolation of lectin-rich extracts.

The extraction of lectins is influenced by various factors, including temperature, time, and the meal/solvent ratio, all of which can significantly impact the extraction process. When interactions among these factors affect the desired outcomes, response surface methodology (RSM) serves as a

powerful tool for process optimization. RSM enables the efficient determination of optimal conditions by providing relevant information within a short time frame and requiring a minimal number of experiments, making it a highly effective approach for optimizing lectin extraction parameters.

This study used cellulase enzyme to enhance lectin extraction from *C. linum*. Response surface methodology (RSM) was used to optimize the effects of enzyme concentration, temperature, and extraction time. This study provides a foundation for further research on the application of these compounds in functional foods and pharmaceutical purposes.

2. MATERIALS AND METHODS

2.1. Materials

C. linum algae were collected from abandoned ponds in Bac Lieu province - Vietnam in mid-October 2020 and transported to the laboratory on the same day. The morphology and structure of the collected algae samples were compared with the identification of *C.linum* algae as documented by Huang [7, 8]. *C. linum* was washed with distilled water, impurities (gravel, sand and snails) were removed, dried until moisture content <10%, ground into fine powder and sieved through an 80 mesh sieve. The fine powder was then stored at −20 °C.

Commercial cellulase enzyme preparation Cellulast (Novozymes, Denmark), anhydrous and active 700 EUG/g; BSA Bovine Serum Albumin, Folin–Ciocalteu reagent by Ciocalteu (Merck, Germany). Other reagents of the highest purity available were obtained from local suppliers.

UV-VIS spectrophotometer V-730 (Kern - Germany), cold centrifuge Hermle, Z 216 MK (Germany), thermostatic tank Memmert WNB22, 22 Liters (Germany).

2.2. Method

2.2.1. Effects of cellulase enzyme on the lectin extraction process

The mixture consisted of 1 g of biomass sample (based on dry mass) and phosphate buffer (0.025 M, pH 7.3) in a ratio of 1/20 (w/v). Then, cellulase enzyme was added to the mixture at the studied concentrations (0.5; 1; 1.5; 2; 2.5% based on dry mass) and placed in a thermostatic bath for 6 h at the studied temperatures (30, 40, 50, 60 °C), after incubation (2, 4, 6 and 8 h) in the thermostatic bath [9]. The mixture was centrifuged at 5000 rpm for 30 minutes to collect the supernatant to determine the total protein content and lectin-specific activity.

2.2.2. Optimization of enzyme-assisted extraction by response surface method (RSM)

RSM is used to determine the combined effect of three independent variables [10], including the enzyme concentration (X_1, %), temperature (X_2, °C), and extraction time (X_3, hours). The experiment used RSM combining layout according to the CCD model and experimental matrix built with JMP10 software to optimize the synthesis process. The marginal values of the extracted factors have been determined, as shown in Table 1.

Table 1. The scope of independent variables and their associated levels

Independent variables	Coding	Coded factor level				
		-α	-1	0	+1	+α
Enzyme concentration (%)	X_1	0.68	1	2	3	3.68
Temperature (°C)	X_2	33.20	40	50	60	76.81
Time (hours)	X_3	2.64	4	6	8	9.36

The design comprised 16 experimental points and was conducted in a random order. Two center-point replicates were incorporated into the experimental design to aid in estimating the pure error sum of squares. The quadratic model used to predict the optimal point was formulated as represented by the following equation:

$$Y = b_0 + \Sigma b_i X_i + \Sigma b_{ii} X_i^2 + \Sigma b_{ij} X_i X_j \qquad \text{(Eq. 1)}$$

Y represents the response function, where b0 denotes the system's center point. The coefficients bi, bii, and bij correspond to the linear, quadratic, and interaction terms, respectively, while Xi and Xj are the variables involved.

2.2.3. Analysis methods

Protein concentration was determined according to the method described by Lowry et al. (1951), with bovine serum albumin (BSA) as the standard [11].

The hemagglutination (HA) test was performed by measuring the maximum visible agglutination on a 2% suspension of rabbit red blood cells and the hemagglutination activity (HU/mg) was expressed as the number of hemagglutinins per mg protein [12, 13]. HA was performed by preparing serial twofold dilutions of the lectin solution in microplates, with each well containing 50 µL of diluent. An equal volume (50 µL) of a 2% suspension of rabbit erythrocytes in phosphate buffer (pH 7.2) was then added to each well. The assay was conducted at 20 °C, with results recorded after roughly one hour, once the erythrocytes in the control well had fully settled. The hemagglutination titer was determined as the reciprocal of the highest dilution that exhibited hemagglutination, representing a single hemagglutination unit. Specific activity was measured by calculating the number of hemagglutination units per milligram of protein [12, 13].

$$\mathrm{HA} = \frac{\mathrm{V} \times 2^{\mathrm{n}}}{\mathrm{protein}_{\mathrm{total}}} \quad (Eq.\,2)$$

In this context, V represents the total volume in milliliters (mL), n denotes the number of dilutions, and proteintotal refers to the total protein content.

2.2.4. Data analysis

The experiments were conducted in triplicate, with the results expressed as mean ± SD. Statistical analysis, including arrangement and treatment comparisons, was performed using JMP 10 software and analysis of variance (ANOVA) at a significance level of $p < 0.05$ to assess differences between treatments. The chart was created using Microsoft Excel 2019.

3. RESULTS AND DISCUSSION

3.1. Effects of enzyme concentration, temperature, and treatment time on lectin extraction

The influence of three independent variables—enzyme concentration, extraction temperature, and extraction time—on protein content and hemagglutinating activity has been analyzed. The impact of these factors is illustrated in Fig. 1.

The effects of enzyme concentration on the protein content and hemagglutinating activity (HA) performed at 50 ℃ for 6 hours were presented in Fig. 1A. At 2% enzyme concentration, the protein content increased significantly, reaching 0.131 ± 0.005 g/g dry mass (DM) (2.7 times higher than the control sample) and the HA also was 55848.592 ± 2104.022 HU/g (2.97 times higher than the control sample). 2.5% enzyme resulted in a close maximum result; however, it had no significant difference compared to 2% enzyme according to ANOVA analysis ($p < 0.05$). Cellulase enzyme breaks the cell wall structure of *C. linum*, increasing protein extraction efficiency, but higher enzyme concentration also could not result in more extraction effectiveness due to the limit of target compounds. Thus, this study chose a 2% enzyme concentration for further experiments. Ramakrishnan *et al.* (2013) also studied the effects of enzyme concentration on protein extraction from mackerel fish processing waste which resulted in the optimal enzyme concentration of 2%, which was close to the current result.

The effects of temperature on the protein content and hemagglutinating activity (HA) performed for 6 hours were presented in Fig. 1B. Proteins exhibit temperature sensitivity, with their structure and functionality being significantly influenced by thermal conditions. The temperature was below 40 ℃, and the protein content increased insignificantly. In contrast, with increasing temperature up to 50 ℃, the protein content increased promptly, reaching 0.132 ± 0.006 g/g DM and HA reaching 44287.983 ± 2541.445 HU/g. This phenomenon occurs because elevated temperatures enhance the solubility of proteins. However, as the temperature is over 50 ℃, the protein content decreases due to the lower solubility of protein stemming from protein denaturation [14]. Jebor *et al.* (2013) also studied the effects

of temperature on protein extraction from *Phaseolus vulgaris* L. and resulted in the optimal temperature of 50 °C, which was close to the current result.

As shown in Fig. 1C, protein contents and HA are influenced by extraction. Prolonged soaking durations were observed to enhance protein solubility, resulting in increased protein content and HA levels. Protein content and HA reached 0.133 ± 0.011 g/g DM and 60122.992 ± 4783.595 HU/g after 6 hours of extraction. Duc *et al.* (2022) also studied the effects of time on protein and lectin extraction from *Ceratophyllum demersum* which resulted in the optimal time of 6 hours, which was close to the current result. The longer the extraction time, the more protein and lectin content was released, thereby increasing HA activity.

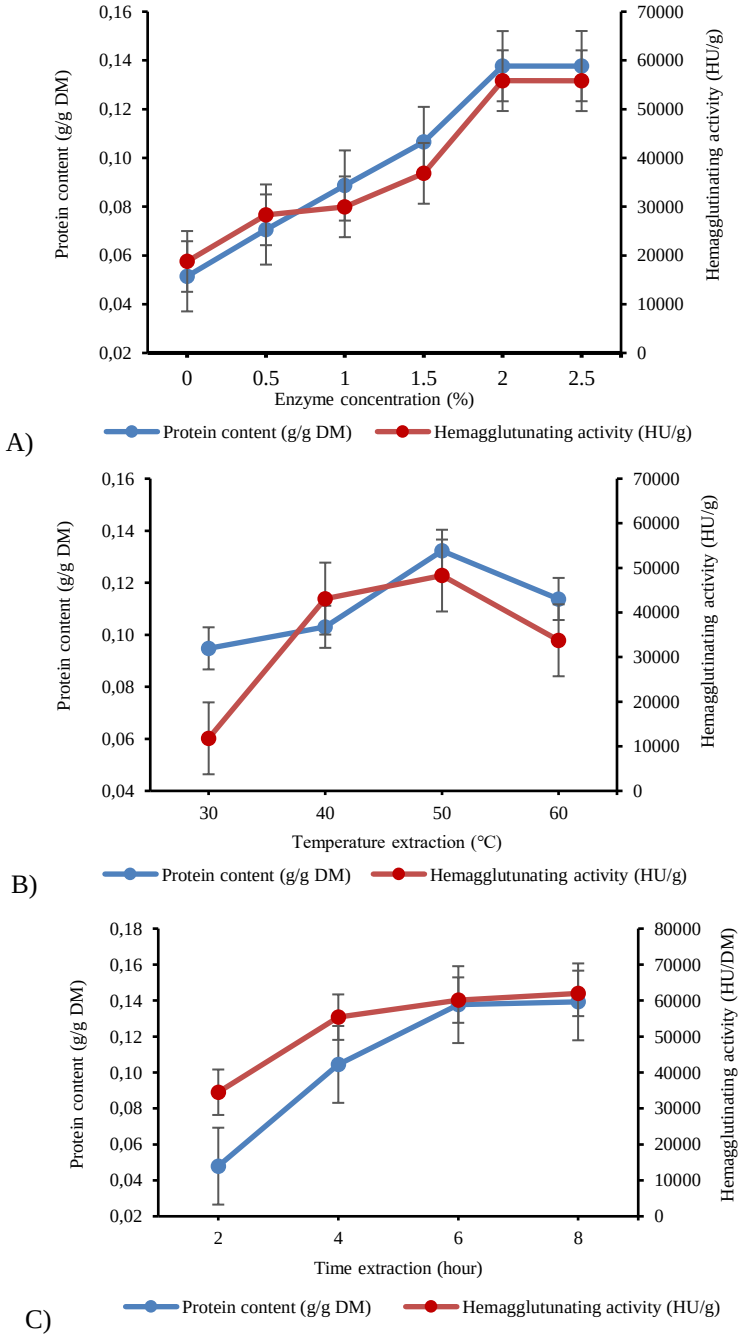


Figure 1. Influence of temperature, enzyme concentration, and extraction duration on the protein and HA content in lectin

Based on these observations, the three variables significantly influenced the protein content and HA. Based on the results of the single-factor test, the appropriate range of variables was determined for the optimal experimental design.

3.2. Optimization of enzyme-assisted extraction conditions

Effects of extraction factors: enzyme concentration (X_1), temperature (X_2), and time (X_3) on total protein content and hemagglutinating activity (HA) of lectins are shown in Table 2.

Table 2. Full experimental design results according to the CCD model

No.	Coded factor level			Actual values			Total protein content (g/g dry matter) Y_1	HA (HU/g) Y_2
	X_1	X_2	X_3	Enzyme concentration (% w/v)	Temperature (°C)	Time (hours)		
1	-1.682	0	0	0.682	50	6	0.093	48945.243
2	-1	-1	-1	1	40	4	0.092	28826.202
3	-1	-1	1	1	40	8	0.097	39582.329
4	-1	1	-1	1	60	4	0.090	25383.475
5	-1	1	1	1	60	8	0.093	41109.489
6	0	-1.682	0	2	33.2	6	0.073	26195.349
7	0	0	-1.682	2	50	2.64	0.088	43490.347
8	0	0	0	2	50	6	0.130	59291.641
9	0	0	0	2	50	6	0.130	59143.393
10	0	0	1.682	2	50	9.36	0.127	60691.212
11	0	1.682	0	2	76.8	6	0.077	28780.241
12	1	-1	-1	3	40	4	0.103	37315.665
13	1	-1	1	3	40	8	0.115	43255.167
14	1	1	-1	3	60	4	0.093	41130.934
15	1	1	1	3	60	8	0.099	48924.469
16	1.682	0	0	3.682	50	6	0.130	58128.609

3.2.1. Total protein content

The results of the analysis of variance via JMP 10 software, and the values of the coefficients of the model are shown in Table 3.

Table 3. Analysis of variance (ANOVA) for response surface quadratic model

Coefficient	Values	Standard deviation	Ratio t	Prob>|t|
The coefficient of freedom	0.130	0.006	21.020	<0.0001*
X_1	0.007	0.002	3.100	0.021*
X_2	-0.002	0.002	-0.780	0.465
X_3	0.007	0.002	2.830	0.030*
$X_1 \times X_2$	-0.003	0.003	-0.810	0.450
$X_1 \times X_3$	0.001	0.003	0.400	0.701
$X_2 \times X_3$	-0.001	0.003	-0.320	0.758
$X_1 \times X_1$	-0.006	0.003	-2.140	0.077
$X_2 \times X_2$	-0.019	0.003	-6.620	0.001*
$X_3 \times X_3$	-0.008	0.003	-2.630	0.039*

According to Joglekar and May, R^2 indicated model fit and should be at least 0.8 [15]. Additionally, the model's goodness of fit was assessed using the F-value for lack of fit. R^2 was 90.22%, indicating a high level of fit between the model and the experimental data. Numerous studies have reported R^2 values within the range of 0.71 to 0.95 for pigeon peas [16] and tomato seeds [17]. Generally, the closer the R^2 value is to one, the better the empirical model aligns with the observed data.

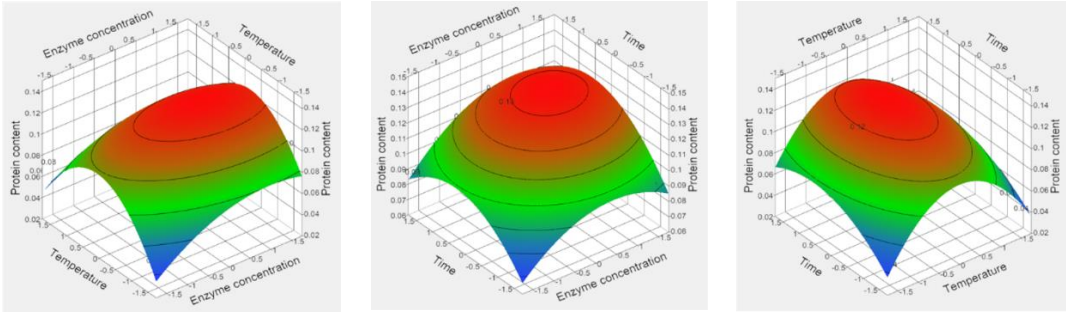
Additionally, the p-value was employed to evaluate the significance of each regression coefficient. Factors with a p-value less than 0.05 were deemed to have a significant influence on the objective function. From Table 3, the obtained multiple regression equation (in terms of coded factors) for the responses was as follows:

$$Y_1 = 0.130 + 0.007X_1 + 0.007X_3 - 0.019X_2^2 - 0.008X_3^2 \text{ (Eq. 3)}$$

Table 3 shows that enzyme concentration, temperature, and time extraction significantly affected total protein content and HA ($p < 0.05$). However, different factors had different influences. If the influence coefficient of a variable has a positive value, it means that when the value of that variable increases, the value of the objective function will increase, and vice versa. The influence coefficient of a variable is negative. The increase in the variable value will decrease the value of the responses.

The results from the model showed that the individual factors of enzyme concentration (X_1), time (X_3), and square of temperature (X_2^2), a square of time (X_3^2), had a statistically significant effect on total protein content. The effect of enzyme concentration (X_1), and time extraction (X_3) was a positive influence on increasing protein content. In contrast, the square of temperature (X_2) and time squared (X_3^2) had a negative effect on the value of Y_1. The remaining factors have no significant regression coefficients with confidence $p > 0.05$, which proved that these factors have no significant influence on the response. The regression coefficients of enzyme concentration and time were close to the same, indicating that the significant effect of enzyme concentration and time on total protein content was similar. Thus, the enzyme contributed to increasing the effectiveness of protein extraction.

Figures 2(a), 2(b), and 2(c) show the 3D response surface models for the interaction effect between X_1: enzyme concentration, X_2: temperature, and X_3: extraction time.



a) b) c)

Figure 2. Response surface models (a, b, c) indicated the influence of factors (enzyme concentration, X_1; temperature, X_2; extraction time, X_3) on total protein content.

3.2.2. Hemagglutinating activity

The results of the analysis of variance and the values of the coefficients of the model are shown in Table 4.

Table 4. Analysis of variance (ANOVA) for response surface quadratic model

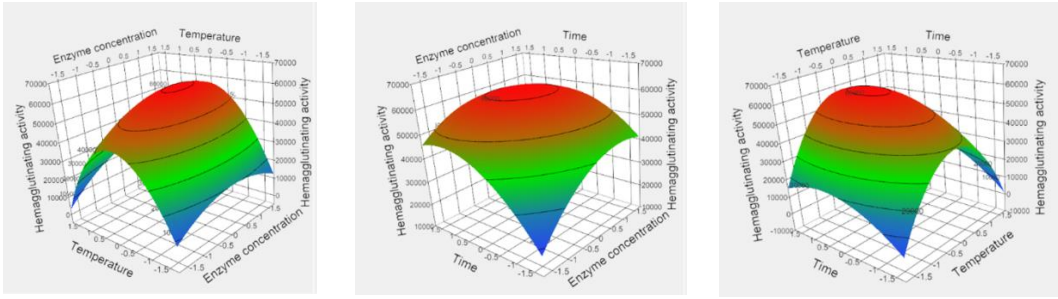
Coefficient	Values	Standard deviation	Ratio t	Prob>|t|
The coefficient of freedom	59727.01	2878.77	20.75	<0.0001*
X_1	3746.78	1104.89	3.39	0.0147*
X_2	872.55	1104.89	0.79	0.4598
X_3	5062.91	1104.89	4.58	0.0038*
$X_1 \times X_2$	1425.02	1443.61	0.99	0.3617
$X_1 \times X_3$	-1593.64	1443.61	-1.10	0.3119
$X_2 \times X_3$	852.99	1443.61	0.59	0.5762
$X_1 \times X_1$	-3238.41	1341.51	-2.41	0.0523
$X_2 \times X_2$	-12448.17	1341.51	-9.28	<0.0001*
$X_3 \times X_3$	-3749.70	1341.51	-2.80	0.0314*

From Table 4, the obtained multiple regression equation (in terms of coded factors) for the responses was as follows:

$$Y = 59727.01 + 3746.78X_1 + 5062.91X_3 - 12448.17X_2^2 - 3749.70X_3^2 \quad \text{(Eq. 4)}$$

The results indicated that individual factors such as enzyme concentration (X_1), time (X_3), and square of temperature (X_2^2) all had a statistically significant influence on HA. In which enzyme concentration (X_1) and time extraction (X_3) had a positive influence, and the square of temperature (X_2^2) had a negative influence on the value of Y_2. The remaining factors had a confidence $p > 0.05$ which proved that these factors had no significant influence on the HA. The regression coefficient X_3 had the most significant positive value, showing that X_3 (time) positively affected increasing HA.

Figures 3(a), 3(b), and 3(c) show the 3D response surface models for the interaction effect between X_1: enzyme concentration, X_2: temperature, and X_3: extraction time.



a) b) c)

Figure 31. Response surface models (a, b, c) show the influence of factors (enzyme concentration, X_1; temperature, X_2; extraction time, X_3) on lectin hemagglutinating activity.

The obtained optimal results were enzyme concentration of 2.55% (v/w), extraction temperature of 49.98 ºC; and extraction time of 6.9 hours. The total protein content reached 0.134 g/g dry mass (DM), and lectin hemagglutinating activity reached 61946.79 HU/g. The optimal prediction model is shown in Fig. 4. When increasing the enzyme concentration, the ability to break down the cell wall on the surface of *C. linum* will increase and help protein and lectin to escape more easily. Moreover, extended extraction times allow the target compounds to fully dissolve into the solvent. However, when the enzyme concentration is too high, it will affect the available protein content in the raw materials, cause erroneous results, and affect the HA of lectin. Besides, excessively prolonged extraction times result in significant energy consumption during the extraction process, thereby reducing economic efficiency [18].

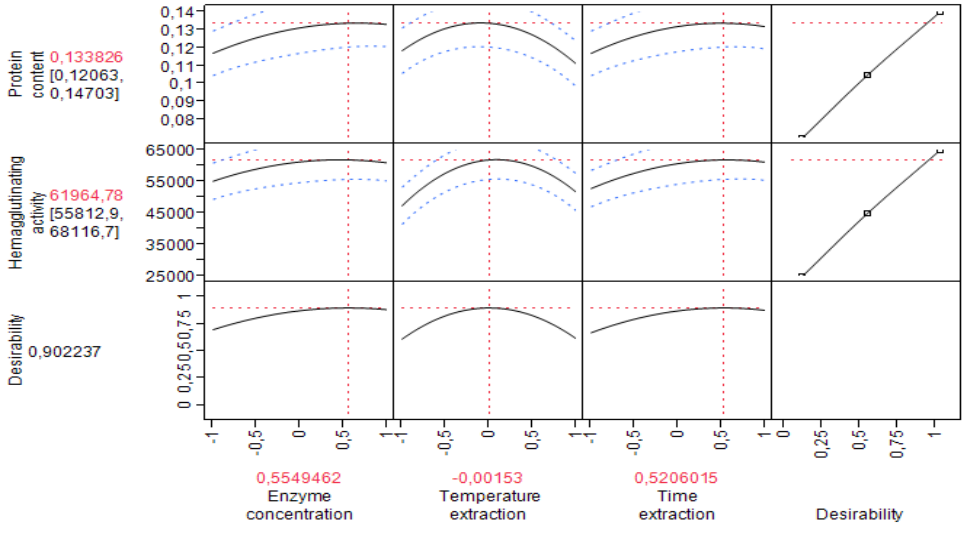


Figure 4. Response surface model of the influence of factors on total protein content and HA of extracts obtained at the optimal condition

Experiments were conducted at obtained optimal conditions 3 times, protein content reached 0.129 g/g DM, and HA reached 58327.637 HU/g, a difference of 3.73% < 5% compared to the optimal data predicted from regression. This indicated that the experimental protein content and lectin activity were not significantly different from those predicted by the quadratic model. Thus, the quadratic equation was consistent with reality and had practical value. It showed that the RSM was suitable for optimizing protein extraction [19, 20]. When compared to other algae, the lectin findings from *C. linum* exhibited greater HA. The red seaweed *G. comea*'s lectin extraction in the investigation had a specific activity of 49 HU/mg [21]. In a different study, lectins from *C. linum* had a higher HA than those from green alga *Halimeda renschii*, which was 32 (HU/mg) [22]. Additionally, HA was given 2.5 HU/mg by analyzing the lectin from the green seaweed *Microcystis viridis* [23]. *C. linum* seaweed-derived HA purified in the meanwhile yielded a HA of 61946.79 (HU/g). This demonstrates that the lectin activity of lectins from various sources varies.

After extracting lectins from *C. linum*, the activity is quite high compared to other seaweeds. Therefore, the lectin from *C. linum* has potential applications because of its high results. However, certain purification conditions and in-depth biomedical studies are required to determine its applicability.

The team now solely evaluated anticancer activity: The anticancer activity of the lectin from *Leguminosae* reached IC_{50} at concentrations of 20.8 g/mL when compared to the study of Barbosa *et al*. (2021), demonstrating that the anticancer level of the lectin from *Leguminosae* was higher than that of the lectin from *C. linum* and achieving good anti-cancer results [24]. The average anti-cancer level was still attained by *C. linum* lectins, with an IC_{50} of 34,556 g/mL [25].

4. CONCLUSION

The cellulase enzyme-assisted extraction method could enhance the protein content and lectin hemagglutinating activity from *C. linum*. The study indicated that the factors of enzyme concentration, temperature, and extraction time significantly affected the protein content with high HA. The optimal treatment conditions were at an enzyme concentration of 2.55% at 49.98 ^{o}C for 6.9 hours. At this condition, the total protein content was 0.134 g/g dry matter, with the lectin hemagglutinating activity of 61946.79 HU/g. Thus, cellulase enzyme was appropriate to support lectin extraction from *C. linum* algae. Further studies should be done to increase the purity of the obtained lectin for future practical applications. Furthermore, additional research on the purification and characterization of lectin from *C. linum* is essential to establish a comprehensive understanding of its properties. Such studies would support the potential application of purified lectin in the food and pharmaceutical industries.

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TÓM TẮT

TỐI ƯU HÓA QUÁ TRÌNH TRÍCH LY THU NHẬN LECTIN BẰNG PHƯƠNG PHÁP ENZYME TỪ RONG *Chaetomorpha linum*

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Rong mền *Chaetomorpha linum* là loài rong nước lợ có mật độ phân bố rộng ở các tỉnh Đồng bằng sông Cửu Long. Nghiên cứu này sử dụng enzyme Cellulase để tăng cường khả năng trích ly lectin từ loài rong *C. linum*. Phương pháp bề mặt đáp ứng (RSM) đã được sử dụng để nghiên cứu ảnh hưởng của nồng độ enzyme (0-2,5% w/w), nhiệt độ (30-60 °C) và thời gian trích ly (2-8 giờ) đối với quá trình chiết xuất lectin từ *C. linum*. Từ mô hình do RSM, các điều kiện tối ưu của quá trình chiết xuất lectin có sự hỗ trợ của enzyme được xác định bao gồm: nồng độ enzyme Cellulase 2,55% w/w, nhiệt độ 49,98 °C và thời gian ủ 6,9 giờ. Ở điều kiện tối ưu hàm lượng protein thu được 0,134 g/g CK có hoạt tính lectin cao nhất 61946,79 HU/g. Phương pháp trích ly có sự hỗ trợ của enzyme là một phương pháp hiệu quả để nâng cao hiệu quả chiết xuất lectin từ rong mền *C. linum*.

Từ khóa: Chaetomorpha linum, enzyme cellulase, lectin, tối ưu, trích ly.