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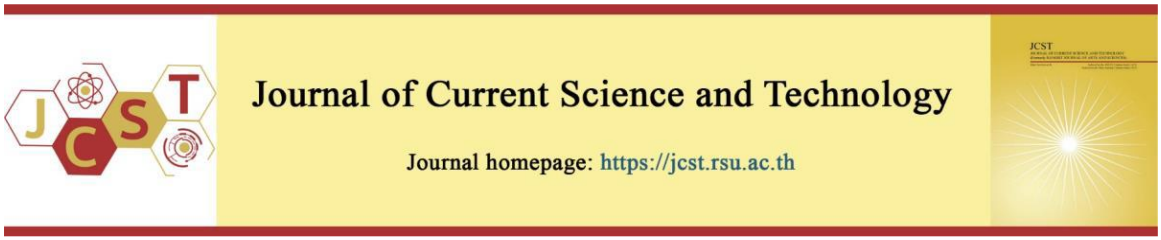

Optimization of Sulfated Polysaccharides Extraction from *Gracilaria fisheri* Obtained Through Microwave-Assisted Extraction

Ratchakrich Patthamasopasakul[1], Chaowalit Monton[2], Thanapat Songsak[2], Supachai Kunaratnpruk[3] and Apirada Sucontphunt[2*]

[1]Graduate Program, College of Pharmacy, Rangsit University, Pathum Thani 12000, Thailand
[2]College of Pharmacy, Rangsit University, Pathum Thani 12000, Thailand
[3]Medical College and Health Science, Rangsit University, Pathum Thani 12000, Thailand

*Corresponding author; E-mail: apirada@rsu.ac.th

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Abstract

Gracilaria fisheri is a red seaweed that possesses several biological activities. The major important compounds comprising this seaweed are sulfated polysaccharides. However, limited data exists on the optimal extraction conditions for this seaweed using eco-friendly techniques like microwave-assisted extraction (MAE). This work aimed to optimize the MAE conditions for extracting sulfated polysaccharides from *G. fisheri*. Three factors influencing MAE were investigated: microwave time, solvent-to-solid ratio, and microwave power. These factors were initially screened by varying one factor at a time to identify levels leading to high extraction yields. These levels were then included in a Box-Behnken design. Microwave time was varied from 20 to 40 s, solvent-to-solid ratio from 8:1 to 10:1 mL/g, and microwave power from 300 to 600 W. Two responses were monitored: extraction yield and sulfate content. The optimal condition for both high extraction yield and sulfate content was found to be 30 s of microwave time, a solvent-to-solid ratio of 9.5:1 mL/g, and 450 W of microwave power. This condition yielded 20.32-20.93% extraction yield and 48.28-49.95 µg/10 mg sulfate content. The computer program's predictions were accurate and reliable, with a very low percentage error of less than 3%. Therefore, the Box-Behnken design proved to be an effective tool for optimizing the extraction of sulfated polysaccharides from *G. fisheri* using the MAE method.

Keywords**:** *Sulfated polysaccharide; Gracilaria fisheri; Box-Behnken Design; Design of Experiments*

1. Introduction

Gracilaria fisheri, a red seaweed belonging to the Gracilariaceae family, is widely distributed. It is commonly found along the coasts of the Gulf of Thailand and the Andaman Sea in Thailand. A significant component of *G. fisheri* is agar (Praiboon et al., 2006), which is used as a raw material in various industries (Praseptiangga et al., 2023). Sulfated polysaccharides are anionic polysaccharide constituents of the cell wall of marine algae (seaweeds) including genus *Gracilaria* (Castro et al., 2018; Chattopadhyay et al., 2008). Primarily composed of cellulose and hemicellulose, these biomolecules exhibit high carbohydrate content while remaining low in calories and fat (Muthukumar et al., 2021). Sulfated polysaccharides possess several biological activities such as anticancer, anticoagulant, antidiabetic, antioxidant, antiviral, anti-allergic, and immunomodulatory activities (Olasehinde et al., 2020).

Sulfated polysaccharides can be extracted using various methods. Traditional solvent-based extraction methods, like methanol and acetone, are widely employed (Archana et al., 2013; Wu et al., 2022). However, these methods have limitations, such as low efficiency in separating low molecular weight substances and the use of large quantities of organic solvents, which can be environmentally hazardous. Additionally, certain solvents, like methanol, are restricted in the food industry. To address these issues, a more efficient method for extracting sulfated polysaccharides from seaweed involves water extraction (Bhuyar et al., 2021; Chattopadhyay et al., 2008). Nevertheless, various factors such as extraction methods, extraction time, ratio, and temperature influence the quantity of sulfated polysaccharides. Therefore, studying the techniques of extraction and the duration is a crucial step in the bioactive substance extraction process, as it significantly impacts the efficiency and quality of sulfated polysaccharide extraction (Lomartire, & Gonçalves, 2022; Wassie et al., 2021). Sulfated polysaccharides are often extracted using large amounts of water at temperatures ranging from room temperature to 100°C for several hours (Bhuyar et al., 2021; Rudtanatip et al., 2022; Yang, & Yang, 2020). Recently, the use of microwave-assisted extraction (MAE) has been extensively studied and reported as an eco-friendly, faster and more efficient method (Kanchanathawornviboon et al, 2021; Monton et al., 2022; Monton et al., 2023).

The interaction of microwave radiation with polar constituents, such as water and specific organic components within the plant matrix, is harnessed by MAE. This interaction, driven by ionic conduction and dipole rotation, induces volumetric heating throughout the sample. Notably, both heat and mass transfer processes in MAE operate in a co-linear fashion, generating a synergistic effect that significantly accelerates extraction and enhances analyte yield. Compared to traditional techniques, MAE offers several advantages, including increased extract yield, minimized thermal degradation due to rapid processing, and selective heating of target analytes within the plant material (Zhang et al., 2018). Developing a significantly faster extraction method would solve challenges like storing plant materials without degrading their properties, enabling the efficient production of large quantities for industrial use (Belokurov et al., 2019).

Traditional experimentation often employs an unstructured trial-and-error approach, which suffers from inefficacy and presents significant challenges when expertise is limited. While the one-factor-at-a-time (OFAT) method is commonly used method, however, it cannot identify crucial factor interactions and struggles to identify optimal conditions in multi-variable scenarios. Fortunately, Design of Experiments (DOE) offers a compelling solution (JMP Statistical Discovery LLC, 2022). Leveraging DOE can lead to significant time, money, and resource savings compared to traditional methods (JMP Statistical Discovery LLC, 2022). In the realm of research, the enhancement of factor interaction identification and the nuanced characterization of the response surface is notably facilitated by the utilization of this method, leading to a more holistic understanding of the experimental system (Gibson, 2016; Steele, 2018). Moreover, DOE allows for the development of predictive statistical models, enabling researchers to anticipate the combined effects of multiple changes, thereby enhancing the design and efficiency of their experiments (JMP Statistical Discovery LLC, 2022). In conclusion, while traditional experimentation approaches have limitations, DOE presents a powerful framework for researchers seeking efficient, insightful, and predictive experimentation across diverse academic disciplines.

2. Objectives

The objective of this work was to optimize the conditions for MAE of sulfated polysaccharides from *G. fisheri*. The effects of microwave time, solvent-to-solid ratio, and microwave power on the yield and sulfate content of the extracted polysaccharides were examined. The authors expect that the optimal conditions obtained from this work will be beneficial as a guide for extracting high-yield sulfated polysaccharides from *G. fisheri*.

3. Materials and methods

3.1 Materials

Potassium sulfate was purchased from CT Laboratory Co., Ltd. (Ajax Finechem, Australia). Barium chloride was purchased from CT Laboratory Co., Ltd. (Kemaus, Australia). Hydrochloric acid was purchased from CT Laboratory Co., Ltd. (Macron Chemical, China). Dried *G. fisheri* was obtained from Koh Yao Subdistrict, Mueang Songkhla District, Songkhla Province, Thailand in June 2022 and were pulverized using grinder and stored in dry place until use.

3.2 Screening factor level using OFAT method

The OFAT method was used to screen the effect of three factors on MAE: microwave time, solvent-to-solid ratio, and microwave power. For each factor, separate experiments were conducted. For microwave time, 10 g of dried *G. fisheri* powder was weighed into a 250-mL beaker and mixed with 50 mL of distilled water (solvent-to-solid ratio of 5:1 mL/g). The mixture was subjected to microwave extraction in a microwave oven (Samsung, MS23K3513AW) at 450 W for 20, 30, 40, 50, or 60 s. Each condition was performed in triplicate. After the predetermined time, samples were filtered using Whatman® filter paper No. 1 and the filtrates were evaporated under vacuum using a rotary evaporator (Heidolph, Germany) at 50°C until dry. The extraction yield for each condition was then collected. For solvent-to-solid ratio, the same procedure as described above was followed, but with varying solvent-to-solid ratios. Ratios of 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, and 10:1 mL/g were achieved by using 40, 50, 60, 70, 80, 90, and 100 mL of distilled water per 10 g of *G. fisheri* powder, respectively. All samples were microwaved at 450 W for 30 s. According to microwave power, the same basic procedure was used, but with varying microwave powers. Ten grams of *G. fisheri* powder were mixed with 90 mL of distilled water (9:1 solvent-to-solid ratio) and exposed to 100, 300, 450, 600, and 800 W for 30 s each.

3.3 Box-Behnken experimental design

An experiment was designed to evaluate the effects of three factors of MAE: microwave time, solvent-to-solid ratio, and microwave power. The Box-Behnken design (Table 1) was used. Microwave times were varied at 20, 30, and 40 s; solvent-to-solid ratios were varied at 8:1, 9:1, and 10:1 mL/g; and microwave powers were varied at 300, 450, and 600 W. The extraction yield and sulfate content were monitored as responses. They were analyzed using Design-Expert® v. 11 (Stat-Ease, Inc., USA) and then the response surfaces were generated. Additionally, plots of perturbation, predicted vs. actual values, and externally studentized residuals vs. run number were generated for each response. In this analysis, the coefficient of determination (R^2), adjusted R^2, predicted R^2, and adequate precision were also reported.

Table 1 Factors of the Box-Behnken design for the optimization of MAE of *G. fisheri*

Standard order	Run order	Coded value			Uncoded value		
		Microwave time (s)	Solvent-to-solid ratio (mL/g)	Microwave power (W)	Microwave time (s)	Solvent-to-solid ratio (mL/g)	Microwave power (W)
7	1	-1	0	+1	20	9:1	600
13	2	0	0	0	30	9:1	450
11	3	0	-1	+1	30	8:1	600
9	4	0	-1	-1	30	8:1	300
3	5	-1	+1	0	20	10:1	450
15	6	0	0	0	30	9:1	450
16	7	0	0	0	30	9:1	450
12	8	0	+1	+1	30	10:1	600
6	9	+1	0	-1	40	9:1	300
8	10	+1	0	+1	40	9:1	600
14	11	0	0	0	30	9:1	450
4	12	+1	+1	0	40	10:1	450
5	13	-1	0	-1	20	9:1	300
10	14	0	+1	-1	30	10:1	300
1	15	-1	-1	0	20	8:1	450
17	16	0	0	0	30	9:1	450
2	17	+1	-1	0	40	8:1	450

3.4 Optimization and verification

The optimal MAE condition, determined through the application of a desirability function, was chosen for its simultaneous maximization of extraction yield and sulfate content. The optimal condition was used to verify the accuracy of the data predicted by Design-Expert®. Three batches of extract were prepared using the optimal MAE condition. Each batch was performed in triplicate. The obtained extraction yield and sulfate content were compared with predicted value and calculated as percent error (Equation 1).

$$\text{Error (\%)} = \left(\frac{\text{Experimental value-Predicted value})\times 100}{\text{Experimental value}}\right) \quad \text{Eq. 1}$$

3.5 Sulfate turbidity assay

Sulfate content of *G. fisheri* extract was determined by the turbidity assay adapted from Dodgson & Price (Dodgson, & Price, 1962). Briefly, various concentrations of sulfated polysaccharides and a series of standard potassium sulfate solutions with concentrations of 20, 40, 60, 80, 100, 200, 300, 400, and 500 µg/mL were prepared. Samples were prepared by dissolving them in 5 mL of 4 M hydrochloric acid and boiling for 2 h. Then, 15 mL of distilled water and 5 mL of sulfate turbidity conditioning reagent were added. The solution was mixed, followed by the addition of 5 mL of 6% v/v barium chloride with 10 s of mixing. Turbidity values for both the samples and standard substances were determined at a wavelength of 420 nm using a UV-Visible spectrophotometer (Genesys 10s UV-Vis, Thermo Fisher, China).

3.6 Statistical analysis

The analysis of differences among more than two groups was conducted through one-way analysis of variance (one-way ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post hoc analysis, utilizing SPSS Statistics 22.0 (IBM, New York, USA). Statistical significance was determined with a p-value below 0.05 at a 95% confidence interval.

4. Result and Discussion

4.1 Effect of MAE conditions on extraction yield based on OFAT method

The preliminary test for selecting factor levels employed the OFAT method, focusing on factors influencing the quantity of sulfated polysaccharides: microwave time, solvent-to-solid ratio, and microwave power. The extraction yield obtained using the OFAT method with varying levels of these factors is shown in Figure 1. When the solvent-to-solid ratio was fixed at 5:1 mL/g and the microwave power at 450 W, increasing microwave time from 20 s to 30 s significantly increased the extraction yield. Conversely, increasing the microwave time further from 30 s to 60 s significantly decreased the extraction yield. Therefore, a microwave time of 30 s provided the highest extraction yield (Figure 1a). Subsequently, to investigate the effect of the solvent-to-solid ratio, the microwave time at 30 s and the microwave power at 450 W. Increasing the solvent-to-solid ratio from 4:1 to 9:1 significantly increased the extraction yield. No significant difference was observed when the ratio was further increased from 9:1 to 10:1 mL/g (Figure 1b). To examine the effect of microwave power, the microwave time was set at 30 s and the solvent-to-solid ratio at 9:1 mL/g. Increasing the microwave power from 100 W to 300 W had no significant impact on the extraction yield. However, enhancing the power from 300 W to 450 W significantly increased the extraction yield, while a further increase from 450 W to 800 W resulted in a significant decrease. Notably, there was no significant difference between the extraction yields obtained at 600 W and 800 W (Figure 1c).

Increasing extraction time increases extraction yield. Increased microwave power and time likely elevate plant cell pressure, leading to cell rupture and enhanced extraction yield (Taqi et al., 2020). However, prolonged exposure to high-power microwaves, potentially leading to the decomposition of target compounds, resulted in a decrease in extraction yield (Figures 1a and 1c). The yield of crude extract from *G. fisheri* increased with increasing microwave power due to the resulting rise in temperature. Higher temperatures enhance solubility and diffusion in water, leading to greater initial extract production. However, this trend eventually reverses as further temperature increases disrupt interactions between polymer chain segments and substituent groups like sulfates and methoxyl groups, resulting in a decline in extract yield (Imjongjairak et al., 2016).

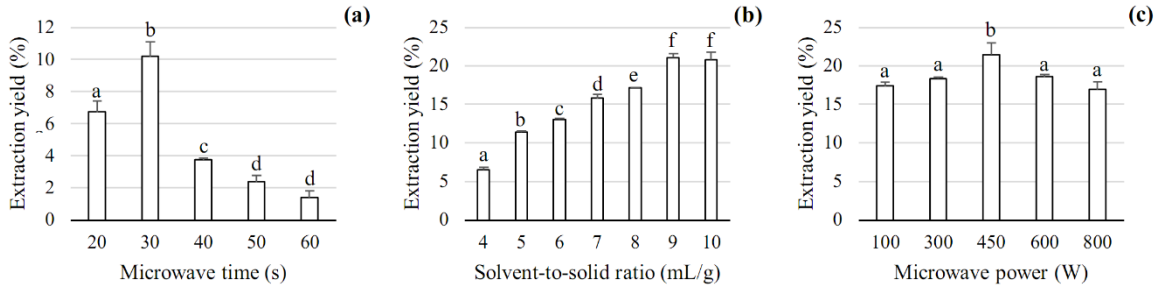

Figure 1 Extraction yield obtained using the OFAT method varied with changes in (a) microwave time, (b) solvent-to-solid ratio, and (c) microwave power. Different symbols (a-f) denote statistically significant differences when analysis by one-way ANOVA followed by Tukey's HSD post hoc analysis ($p < 0.05$)

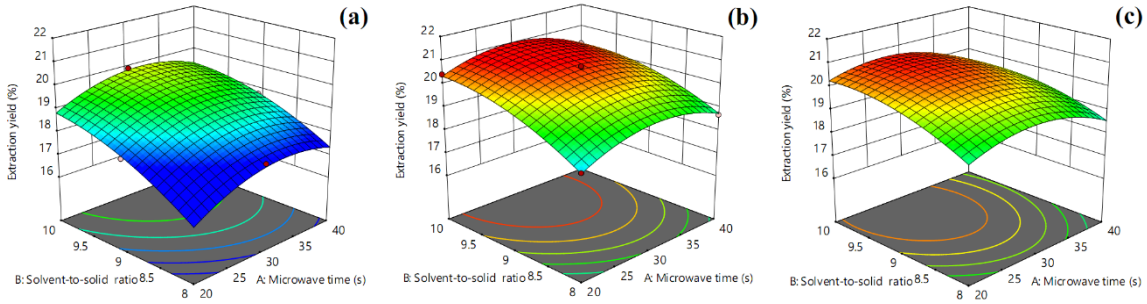

Figure 2 Response surfaces of the extraction yield when different microwave powers were applied: (a) 300 W, (b) 450 W, and (c) 600 W

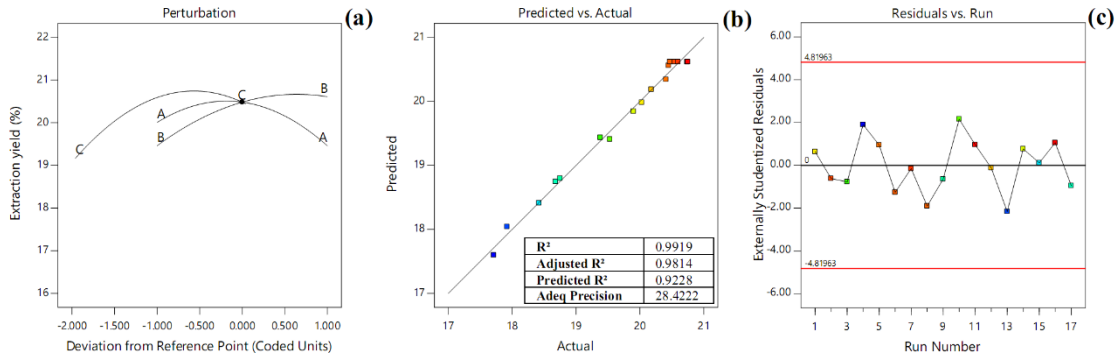

Figure 3 The plots of (a) perturbation (b) predicted vs actual values, and (c) externally studentized residuals vs. run number of the extraction yield

Elevation of the solvent-to-solid ratio demonstrably enhances extraction yield from the raw material. This phenomenon can be ascribed to the amplification of the concentration gradient established by the increased solvent volume, consequently facilitating mass diffusion (Hibbert et al., 2019). Therefore, Figure 1b demonstrates that the extraction yield increases with a rising solvent-to-solid ratio, reaching a plateau at a ratio of at least 9:1 mL/g. This finding differs from previous work, where a 35:1 or 50:1 solvent-to-solid ratio yielded the highest extraction using the decoction technique (Rormwong et al., 2023).

4.2 Effect of MAE conditions on extraction yield based on the Box-Behnken design

The OFAT method was initially employed to screen factors influencing MAE yield. Subsequent optimization was conducted using a Box-Behnken design, incorporating three factors: microwave time

(20, 30, and 40 s), solvent-to-solid ratio (8:1, 9:1, and 10:1 mL/g), and microwave power (300, 450, and 600 W), coded as low, medium, and high, respectively. Figure 2 displays the response surfaces illustrating the extraction yield as generated through the experimental design. The analysis revealed optimal values for all three factors, with both microwave time and power exhibiting maxima at the medium levels. The optimal solvent-to-solid ratio fell between the medium and high settings (Figures 2 and 3a). Additionally, high R^2 values indicated good agreement between predicted and actual values. The predicted R^2 closely matched the adjusted R^2, with a difference less than 0.2, demonstrating model validity. The adequate precision, a measure of signal-to-noise ratio, exceeded 4, indicating a reliable model (Figure 3b). Visual inspection of the externally studentized residuals plot against run number confirmed random data distribution within the red confidence interval (Figure 3c), suggesting that all data points fall within the 95% confidence range (Duangjit et al., 2014; Duangjit et al., 2012).

The ANOVA analysis of extraction yield data (Table 2) indicates a significant model, suggesting that the chosen factors collectively influence extraction yield significantly. Furthermore, the insignificant lack of fit confirms that the predicted values adequately fit the actual data. Examining the individual terms, it is evident that most factors significantly affect extraction yield. These include linear, interaction, and quadratic terms of factors other than microwave time and the interplay of microwave time and solvent-to-solid ratio, which were not statistically significant.

Figure 4 presents the response surfaces for sulfate content generated by the experimental design. Similar to the extraction yield results, the analysis identified optimal values for all three factors. Both microwave time and power exhibited maxima at the medium levels, while the optimal solvent-to-solid ratio fell between the medium and high settings (Figures 4 and 5a). High R^2 values indicate good agreement between predicted and actual values, further supported by the minimal difference (<0.2) between predicted and adjusted R^2, demonstrating model validity. The adequate precision, exceeding 4, signifies a reliable model with a strong signal-to-noise ratio (Figure 5b). Upon visual inspection of the externally studentized residuals plot against run number, it is confirmed that the data distribution appears random within the red confidence interval (Figure 5c). This observation suggests that all data points fall within the 95% confidence range (Duangjit et al., 2014; Duangjit et al., 2012).

Table 2 ANOVA for the quadratic model of extraction yield

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	16.4300	9	1.8300	94.98	< 0.0001*
A-Microwave time	0.0162	1	0.0162	0.84	0.3892
B-Solvent-to-solid ratio	5.7000	1	5.7000	296.25	< 0.0001*
C-Power	3.2600	1	3.2600	169.78	< 0.0001*
AB	0.0600	1	0.0600	3.12	0.1206
AC	0.4422	1	0.4422	23.00	0.0020*
BC	0.3136	1	0.3136	16.31	0.0049*
A^2	2.3700	1	2.3700	123.19	< 0.0001*
B^2	0.8432	1	0.8432	43.86	0.0003*
C^2	2.7800	1	2.7800	144.58	< 0.0001*
Residual	0.1346	7	0.0192		
Lack of Fit	0.0740	3	0.0247	1.63	0.3172
Pure Error	0.0606	4	0.0152		
Cor Total	16.5700	16			

An asterisk (*) denote significant values

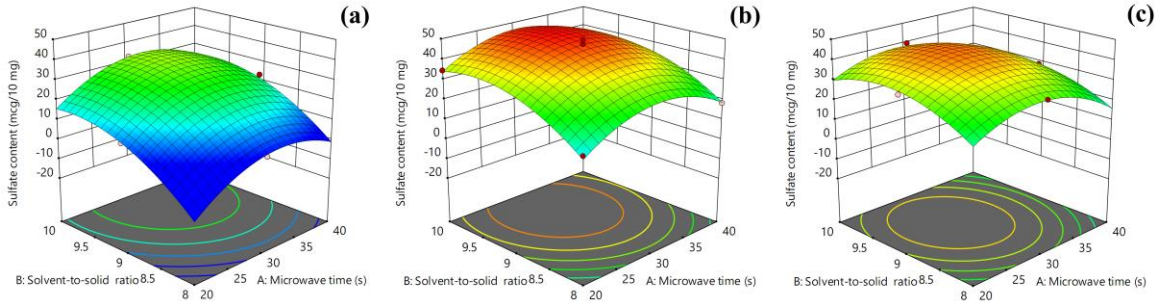

Figure 4 Response surfaces of the sulfate content when different microwave powers were applied: (a) 300 W, (b) 450 W, and (c) 600 W

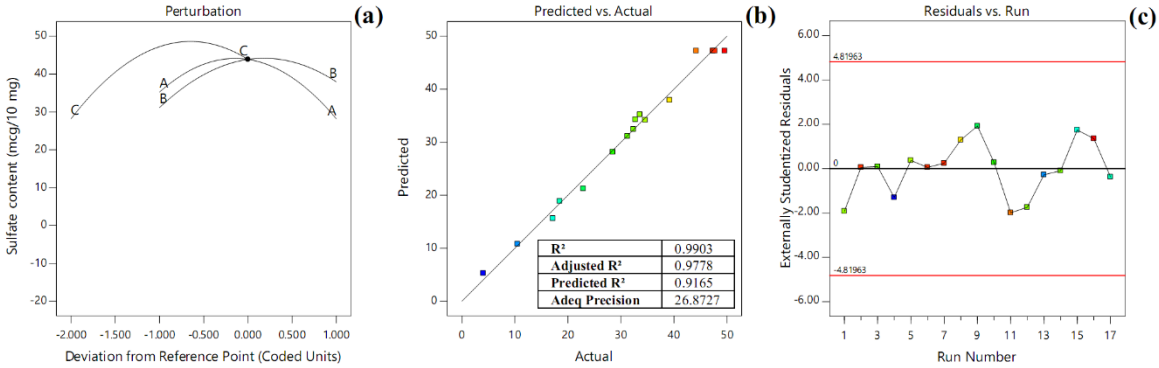

Figure 5 The plots of (a) perturbation (b) predicted vs actual values, and (c) externally studentized residuals vs. run number of the sulfate content

Table 3 ANOVA for the quadratic model of sulfate content

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	2952.78	9	328.09	79.14	< 0.0001*
A-Microwave time	5.68	1	5.68	1.37	0.2799
B-Solvent-to-solid ratio	577.32	1	577.32	139.27	< 0.0001*
C-Microwave power	491.40	1	491.40	118.54	< 0.0001*
AB	2.48	1	2.48	0.60	0.4648
AC	76.69	1	76.69	18.50	0.0036*
BC	103.77	1	103.77	25.03	0.0016*
A^2	624.23	1	624.23	150.58	< 0.0001*
B^2	366.78	1	366.78	88.48	< 0.0001*
C^2	528.60	1	528.60	127.51	< 0.0001*
Residual	29.02	7	4.15		
Lack of Fit	14.10	3	4.70	1.26	0.4003
Pure Error	14.92	4	3.73		
Cor Total	2981.80	16			

An asterisk (*) denote significant values

Table 4 Predicted value, experimental value, and percent error of prediction for response of extraction yield and sulfate content

Response	Predicted value	Batch	Experimental value (n = 3)	Error (%)
Extraction yield (%)	20.93	1	20.93 ± 0.14	0.00
		2	20.32 ± 0.28	-3.00
		3	20.68 ± 0.28	-1.21
Sulfate content (μg/10 mg)	49.16	1	49.95 ± 2.55	1.58
		2	48.28 ± 1.67	-1.82
		3	48.28 ± 2.55	-1.82

The ANOVA analysis of sulfate content data (Table 3) indicates a significant model, suggesting that the chosen factors collectively influence sulfate content significantly. Furthermore, the insignificant lack of fit confirms that the predicted values adequately fit the actual data. Examining the individual terms, it is evident that most factors significantly affect sulfate content. These include linear, interaction, and quadratic terms of factors other than microwave time and the interplay of microwave time and solvent-to-solid ratio, which were not statistically significant similar to extraction yield analysis.

The optimal condition was selected employing a desirability function, gave the simultaneous highest extraction yield and sulfate content was microwave time of 30.32 s, solvent-to-solid ratio of 9.42:1 mL/g, and microwave power of 471.72 W. This condition had desirability value of 1.000. However, to easy operation, the optimal condition was adjusted to microwave time of 30 s, solvent-to-solid ratio of 9.5:1 mL/g. Due to the limitation of the microwave oven setting, therefore, microwave power of 450 W was selected. Three batch of extract were prepared using this optimal MAE condition. Results showed that the experimental value was closed to the predicted value with a very low percent error of less than 3% for all batches (Table 4). These results proved that the prediction by the Design-Expert® was accurate and reliable. The authors emphasized that their optimized MAE technique achieves not only high extraction yield and sulfate content, but also does so using an eco-friendly method and significantly shorter extraction time compared to previously reported decoction techniques (Imjongjairak et al., 2016; Rormwong et al., 2023; Sakaew et al., 2022; Wongprasert et al., 2014). This method has the potential to reduce extraction time from hours to seconds. However, their biological activities will be investigated in future work to evaluate their potential for pharmaceutical and cosmetic applications.

6. Conclusion

This study successfully optimized the MAE of sulfated polysaccharides from *G. fisheri*, a red seaweed with promising biological activities. Using a Box-Behnken design, the effect of three crucial factors: microwave time, solvent-to-solid ratio, and microwave power was investigated. The optimal conditions identified for maximizing both extraction yield (20.32-20.93%) and sulfate content (48.28-49.95 μg/10 mg) were 30 s of microwave time, a solvent-to-solid ratio of 9.5:1 mL/g, and 450 W of microwave power. These findings demonstrate the effectiveness of the Box-Behnken design for optimizing eco-friendly MAE processes and highlight its potential for sustainable production of valuable sulfated polysaccharides from *G. fisheri*. Moreover, the accuracy of the computer program predictions with a very low percentage error strengthens the reliability of this approach for further optimization and process control. This optimized MAE method facilitates exploring the full potential of *G. fisheri*'s bioactive compounds for various applications in various fields.

7. Acknowledgements

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