

Extraction optimization of polysaccharides from *Acanthophora* spp. with antioxidant activity

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Abstract

Acanthophora spp. is a wild, underutilized red seaweed in the Philippines with potential antioxidant properties. Local studies on the utilization of this red alga are scarce, so optimization of the extraction process needs streamlining. This study intended to explore the antioxidant properties of polysaccharides extracted from *Acanthophora* spp. for its broader application in food, cosmetics, or biotechnological industries. Using central composite design (CCD) of response surface methodology (RSM), varying 5-level factors (% KOH, temperature, and time) were combined for optimizing extraction conditions using yield, galactose, total sulfate, and in vitro antioxidant activity (DPPH, ABTS, and FRAP) as responses. Our findings revealed eight optimum extraction conditions (with a desirability value of 1.00) for producing a highly sulfated polysaccharide with good antioxidant properties. Based on our results, the optimum extraction conditions at 3.00–3.01% KOH at 65.00–65.06°C for 2.97–3.00 h could predict a 29.00–29.04% yield, 3.18–3.19% GAL, and 38.05–38.25% total sulfate, 31.28–31.65% DPPH and 64.02–64.38% ABTS inhibition activities, and 0.09 FRAP value. The findings in this study could provide baseline information for the extraction of sulfated polysaccharide from *Acanthophora* spp. with potential antioxidant properties.

1. Introduction

Carrageenan is a linear polysaccharide from the Rhodophyceae family built with 3-linked β -D-galactopyranose (Unit A) and 4-linked α -D-galactopyranose (Unit B), while the Unit B in agar is linked with α -L-galactopyranose (Usov, 1998). The hydroxyl groups may be replaced by sulfate, methyl, or pyruvate, or may also exist in the form of 3,6-anhydrogalactose (3,6-AG) (Usov, 1998). Understanding the variations of the substituents sulfate and 3,6-AG groups could help identify different types of carrageenan such as kappa (κ) (22%, 33%), iota (ι) (32%, 26%), and lambda (λ) (37% sulfate group, little or no 3,6-AG) (van de Velde *et al.*, 2002; Pradhan and Ki, 2023). The highly sulfated λ -carrageenans are non-gelling, but their bioactivities may be promising, especially when applied to food, cosmetics, pharmaceutical, and biomedical industries (Joint FAO/WHO Expert Committee on Food Additives, 2014; European Food Safety Authority, 2018; Liu *et al.*, 2021). While the side effects of synthetic antioxidants are concerning (Augustyniak *et al.*, 2010), natural highly sulfated λ -carrageenans from red

seaweeds can be explored as an alternative (Sokolova *et al.*, 2011a; Sokolova *et al.*, 2011b; Anand *et al.*, 2018; Saluri and Tuvikene, 2020). Currently, many studies have investigated the antioxidant, immune-stimulating, and pharmacological properties of sulfated polysaccharides from red seaweeds (Anand *et al.*, 2018; Liu *et al.*, 2021; Budiyo *et al.*, 2022; Pradhan and Ki, 2023).

Acanthophora spp. is a red seaweed rich in structurally heterogeneous sulfated polysaccharides, which are strongly influenced by environmental conditions and seasonality (Rodrigues *et al.*, 2016). Studies showed that the genus *Acanthophora* contains λ -carrageenan (Parekh *et al.*, 1989; Pettongkhao *et al.*, 2019; Muthulakshmi *et al.*, 2021; Budiyo *et al.*, 2022; Pradhan and Ki, 2023); however, there are reports documenting the presence of highly sulfated agar in *Acanthophora* species as well (Duarte *et al.*, 2004; Pereira Júnior *et al.*, 2021). Many parts of *Acanthophora* spp. may contain different types of polysaccharides, and the substituent present, especially the sulfate and 3,6-AG groups, and linkages, which are influenced by

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environmental and intrinsic factors (Usov, 1998; Duarte *et al.*, 2004; Budiyanto *et al.*, 2022). In the Philippines, *Acanthophora* species (*A. orientalis*, *A. thierryi*, and *A. spicifera*) (Lastimoso and Santiañez, 2020) naturally grow in inter- and shallow subtidal zones and estuaries of tropical and sub-tropical waters (Kilar and McLachlan, 1986). Its high adaptive trait to broad abiotic conditions allows *Acanthophora* spp. to become invasive in Hawaii, the Pacific coasts of Mexico, and other countries (<https://www.iucngisd.org/gisd/>). Harvesting *Acanthophora* spp. is a viable measure in mitigating ecological impacts while diversifying potential products, thereby developing a local bioeconomy. However, reports on the utilization of *Acanthophora* spp. in the Philippines are limited to domestic consumption as salads and viands, flavoring in soups, thickening or emulsifying agent, and as a plant-growth promoter (Trono, 1999). Recently, Budiyanto *et al.* (2022) reported that crude extracts of *A. spicifera* convey anti-bacterial, anti-coagulant, anti-cancer, anti-fungal, anti-inflammatory, antioxidant, and anti-viral properties. Sulfated polysaccharides from *A. spicifera* stimulated defense response as bio-elicitor against plant infection caused by *Phytophthora palmivora* (Pettonghkao *et al.*, 2019). Meanwhile, purified and fractionated sulfated polysaccharides from *A. spicifera* exhibited antioxidant and anticancer (Anand *et al.*, 2018), anticoagulant and antiviral properties (Duarte *et al.*, 2004). Extracts from *A. spicifera* also displayed immune-stimulating activity against a fish pathogen, *Aeromonas hydrophila*, providing a 60% better survival rate (Muthukrishnan and Raja, 2021). Therefore, *Acanthophora* spp. from the Philippines can be explored as a potential source of bioactive sulfated polysaccharides for pharmaceutical, nutraceutical, or biotechnological product development.

Various methods have also been proposed to improve yield, bioactivity, minimize chemicals, and shorten extraction time on sulfated polysaccharides from *A. spicifera*, using either water, alcohol, buffer, enzyme, alkaline, or acid (Budiyanto *et al.*, 2022). However, studies on determining the yield, physicochemical, and functional properties of polysaccharides from *A. spicifera* using alkaline treatment are still limited (Anand *et al.*, 2018; Schnoller *et al.*, 2020). *Acanthophora spicifera* after alkali treatment possessed antimicrobial activity against plant pathogens (*Xanthomonas campestris* pv. *vesicatoria* and *Alternaria solani*) and promoted the growth and pigment contents in tomatoes and sweet peppers (Ali *et al.*, 2021; Ali *et al.*, 2022). Despite potential biotechnological applications of polysaccharides from *A. spicifera*, industry application could be hampered due to constraints at scale-up production levels. Thus, optimization of the alkaline extraction condition is fundamental to enhance the yield,

chemical properties, and bioactivity of polysaccharides from *Acanthophora* spp. The application of response surface methodology (RSM) could tailor-fit the extraction of polysaccharides from *Acanthophora* spp. with antioxidant properties. However, currently, no studies have reported using RSM to optimize the extraction of polysaccharides from *Acanthophora* spp. Understanding the interactions of alkaline concentration, extraction time, and temperature is also important in the extraction optimization of polysaccharides from *Acanthophora* spp.

2. Materials and methods

2.1 Preparation and proximate composition analysis of *Acanthophora* spp.

The red seaweed, *Acanthophora* spp., collected from Batac, Ilocos Norte, Philippines, was morphologically identified based on Hurtado *et al.* (2006). The seaweed sample was then sun-dried, and 50 g of dried seaweed samples were packed, labeled in polyethylene bags, and stored at 4°C for the extraction of crude polysaccharides.

For proximate composition analysis, the moisture content was measured using Halogen Rapid Moisture Analyzer (Shimadzu MOC63u). A dried seaweed sample (2 g) was heated at 120.0°C, and the moisture content (%) was calculated gravimetrically. The moisture contents of extracted polysaccharides were determined at 13.03±1.02%. The total lipid content was measured using the chloroform: methanol (1:2, v/v) method after rehydration with distilled water overnight at room temperature (28–30°C), following the Bligh and Dyer (1959) method. For total protein content determination, seaweed powder (1 g) was digested using H₂SO₄ and a copper-based catalyst, and the samples were distilled and titrated based on the Kjeldahl method (AOAC Method 978.02; Abrams *et al.*, 2014). For total ash content determination, seaweed powder (1 g) was heated at 550±10°C in a muffle furnace for 4–6 h and the white residue was measured gravimetrically to calculate the percent ash (total inorganic matter/ mineral composition) (AOAC Method 942.05; Thiex *et al.* (2012). The total carbohydrate content was then calculated by difference. All samples were analyzed in triplicate.

2.2 Experimental design

RSM with randomized central composite design (CCD) of Design Expert 13.0 Software® (StatEase) was used to formulate the experimental design for the alkali extraction optimization experiment. The 5-level independent variables were: (A) potassium hydroxide (KOH% w/v); (B) temperature (°C); and (C) time (h) (Table 1). The dependent or response variables included yield (%), total galactose (GAL%), total 3,6-

Table 1. Hydrolysis conditions using three independent variables at five levels and their actual values using a randomized central composite design (CCD) of response surface methodology (RSM).

1	Coded and actual values				
	-α	-1	0	+1	+α
A: KOH concentration (%)	1.98	3.00	4.50	6.00	7.02
B: Hydrolysis temperature (°C)	58.18	65.00	75.00	85.00	91.82
C: Extraction time (h)	1.66	2.00	2.50	3.00	3.34

*The CCD has four (4) center points and generated a total of eighteen (18) runs.

anhydrogalactose (3,6-AG%), total sulfate (%), and *in vitro* antioxidant activity assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and ferric reducing antioxidant power (FRAP) assays, which were used to determine the interactions of the independent variables for evaluating optimum extraction of polysaccharide. The axial points (Run 1–6), factorial points (Run 7–14), and center points (Run 15–18) are shown in Table 2.

2.3 Extraction of polysaccharides from *Acanthophora* spp.

The dried seaweed was subjected to depigmentation and alkaline hydrolysis following the method in Schnoller *et al.* (2020), but with modifications, producing a semi-refined KOH-treated seaweed. During extraction, 50 g of the dried seaweed was rehydrated with 100 mL of distilled water (1:2, $\frac{w}{v}$) and kept at room temperature overnight for the removal of water-soluble pigments and other contaminants. The rehydrated seaweed was drained and de-pigmented using a 100 mL methanol: acetone mixture (1:1, $\frac{v}{v}$), and then the liquid was drained thereafter. The seaweed was then treated with 400 mL KOH at varying concentrations (1.98–7.02%), temperature (58.18–91.82°C), and time (1.66–3.34 h) combinations as shown in Table 2. The semi-refined polysaccharide was then filtered using a 3-ply cheesecloth, and the residue was washed several times with distilled water to remove excess alkali residues. The semi-refined polysaccharide extract was dried using a forced air draft oven at 60–65°C until reaching a moisture content below 15%. The dried semi-refined polysaccharide extracts were milled, and the powdered samples were stored at 4–7°C for subsequent analyses.

2.4 Physico-chemical analysis of polysaccharide from *Acanthophora* spp.

2.4.1 Yield

The yield of the crude polysaccharide extract was calculated by dividing the weight (g) of the dried polysaccharide extract (W_E) by the weight (g) of the dried seaweed used for extraction (W_S) shown in the equation. The results were then expressed as a percentage of yield. All samples were recorded in triplicate. The percentage of yield was calculated using

$$\%Yield = (W_E/W_S) \times 100$$

the equation:

2.4.2 Galactose and 3,6-anhydrogalactose content

The galactose (GAL) (%) content of the extracted polysaccharide was measured using the anthrone reagent-based method of Yaphe (1960). A stock solution of anthrone reagent was prepared by dissolving 200 mg anthrone in 100 mL of 83.6% sulfuric acid and stored in an amber bottle at 4°C. During analysis, GAL standard and polysaccharide samples were added with anthrone reagent (at 1:10, $\frac{v}{v}$) in 30 mL test tubes and then heated at 95°C for 11 min in a water bath. The test tubes were then rapidly cooled in an ice bath. The absorbances of the samples (200 μ L) were read in a 96-well microplate using a BMG Labtech UV Vis spectrophotometer at 630 nm. All analyses were conducted in triplicate. Meanwhile, the 3,6-anhydrogalactose (3,6-AG) (%) contents of crude polysaccharide extracts were measured according to the method of Yaphe (1960), using 0.1 M D-fructose as a standard. A stock solution of resorcinol reagent was prepared by dissolving 130 mg of resorcinol in 100 mL of absolute ethyl alcohol. Then, 10 mL of the stock solution was added to 100 mL of 12 M hydrochloric acid and stored in a brown bottle for further use. Crude polysaccharide extracts were added with freshly prepared resorcinol reagent (2:10, $\frac{v}{v}$), transferred to 30 mL Pyrex test tubes, and placed in an ice bath for 5 min. The tubes were then heated for 10 min at 80°C in a water bath and cooled in an ice bath. The standard and samples (200 μ L) were dispensed in a 96-well microplate, and the absorbance was read at 550 nm. All analyses were conducted in triplicate.

2.4.3 Total sulfate content

A simple and rapid turbidimetric method of Tabatabai (1974) was used to determine the total sulfate (%) contents of polysaccharide extracts. The barium chloride ($BaCl_2$)-gelatin reagent was freshly prepared by dissolving 0.6 g gelatin powder (Bacteriological Grade) in 200 mL warm distilled water and stored at 4°C for 16 h. $BaCl_2$ (2.0 g) was then dissolved in 0.30% gelatin solution, and the mixture was incubated for 2 h at 25–28°C. During analysis, an aliquot of the polysaccharide sample (5 mL) was added with 500 μ L of 0.5N

Table 2. The experimental runs generated by central composite design (CCD) of response surface methodology (RSM).

Run	Design space	Coded values			Actual values		
		A: KOH (%)	B: Temp (°C)	C: Time (h)	A: KOH (%)	B: Temp (°C)	C: Time (h)
1	Axial	0.00	-1.68	0.00	4.50	58.18	2.50
2	Axial	-1.68	0.00	0.00	1.98	75.00	2.50
3	Axial	0.00	0.00	+1.68	4.50	75.00	3.34
4	Axial	0.00	+1.68	0.00	4.50	91.82	2.50
5	Axial	+1.68	0.00	0.00	7.02	75.00	2.50
6	Axial	0.00	0.00	-1.68	4.50	75.00	1.66
7	Factorial	+1.00	-1.00	-1.00	6.00	65.00	2.00
8	Factorial	+1.00	+1.00	+1.00	6.00	85.00	3.00
9	Factorial	+1.00	-1.00	+1.00	6.00	65.00	3.00
10	Factorial	-1.00	-1.00	+1.00	3.00	65.00	3.00
11	Factorial	-1.00	-1.00	-1.00	3.00	65.00	2.00
12	Factorial	-1.00	+1.00	+1.00	3.00	85.00	3.00
13	Factorial	-1.00	+1.00	-1.00	3.00	85.00	2.00
14	Factorial	+1.00	+1.00	-1.00	6.00	85.00	2.00
15	Centre	0.00	0.00	0.00	4.50	75.00	2.50
16	Centre	0.00	0.00	0.00	4.50	75.00	2.50
17	Centre	0.00	0.00	0.00	4.50	75.00	2.50
18	Centre	0.00	0.00	0.00	4.50	75.00	2.50

A factor of +1.68 and -1.68 was used for axial points.

hydrochloric acid (HCl) and mixed with 250 μL of BaCl_2 -gelatin reagent, followed by incubation for 30 min at room temperature. Sample absorbances were then measured in cuvettes using a UV Vis spectrophotometer at 500 nm against the reagent blank. The sulfate contents of the polysaccharide extracts from *Acanthophora* spp. were determined based on the linear regression curve of the standard potassium sulfate (0.1 M K_2SO_4). All analyses were conducted in triplicate.

2.4.4 Mild acid hydrolysis of polysaccharide from *Acanthophora* spp.

The extracted polysaccharide was hydrolyzed using the method of Yu *et al.* (2002) with slight modifications. Polysaccharide extracts from *Acanthophora* spp. were dissolved in 0.1 M HCl (at 10 mg/mL) and hydrolyzed in a water bath for 3 h at 65°C. The hydrolysis was terminated by neutralization with 0.1M NaOH added dropwise until pH 7.0 was reached at 25°C. The mixture was filtered using Whatman Filter No. 1, and the supernatant containing the acid-hydrolyzed polysaccharide labelled and kept at 4°C. All analyses were conducted in triplicate.

2.4.5 Antioxidant activity of the hydrolyzed polysaccharide from *Acanthophora* spp.

2.4.5.1 DPPH inhibition activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition activity of acid-hydrolyzed polysaccharide extracts was determined according to Maleki *et al.* (2023). Hydrolyzed polysaccharide samples (0.05 mL)

were added with 2.95 mL 0.15 mM DPPH solution in 80% methanol in test tubes, and vortexed briefly. Test tubes were then incubated in the dark for 30 min at room temperature. The sample absorbances were then measured at 517 nm using a UV Vis spectrophotometer to assess the reduction in absorbance by free radical scavenging activity. The positive control used was ascorbic acid. The percentage DPPH inhibition (%) was

$$\text{DPPH Inhibition (\%)}(A_{517 \text{ nm}}) = ((A_{\text{Blank}} - A_{\text{Sample}})/A_{\text{Blank}}) \times 100$$

calculated using the equation. All analyses were conducted in triplicate.

2.4.5.2 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) inhibition activity

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) inhibition activity was conducted based on the procedure used by Re *et al.* (1999). The ABTS radical cation solution was prepared by mixing 7 mM ABTS solution and 2.45 mM potassium persulfate. The mixture was placed for 12–16 h in the dark at room temperature. The ABTS solution was equilibrated at 30°C diluted with 95% methanol until reaching 0.70 ± 0.02 absorbance at 734 nm. After 16 h, the polysaccharide sample (10 μL) was mixed with the ABTS solution (1 mL), vortexed, and incubated again for 30 min in the dark at room temperature. The aliquot (0.2 mL) was dispensed to a UV Vis spectrophotometer equipped with a 96-well microplate, and the sample

$$\text{ABTS Inhibition (\%)}(A_{734 \text{ nm}}) = ((A_{\text{Blank}} - A_{\text{Sample}})/A_{\text{Blank}}) \times 100$$

absorbances were read at 734 nm. The percentage ABTS inhibition was calculated using the equation. All analyses were conducted in triplicate.

2.4.5.3 Ferric reducing acid power assay

The Ferric reducing acid power (FRAP) assay was performed based on the procedure described by Benzie and Strain (1996). The FRAP reagent was prepared using 25 mL 300 mM pH 3.6 acetate buffer (sodium acetate and glacial acetic acid), 2.5 mL 20 mM FeCl₃, and 2.5 mL 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl. The FRAP reagent was acclimatized to 37°C before use. Aliquots of polysaccharide samples (5 µL) and distilled water (blank) were added to 70 µL of FRAP reagent and incubated at 37°C for 4 min. Each sample (200 µL) was dispensed to a 96-well microplate, and the absorbance was read at 593 nm. All analyses were conducted in triplicate.

2.5 Statistical analysis

The statistics of the response variables, such as yield (%), GAL (%), total sulfate (%), DPPH and ABTS inhibition activities (%), FRAP, and the combined effects of the model terms were analyzed using RSM Design Expert® version 13. Analysis of variance (ANOVA) was carried out to determine significant interactions of independent variables or their combined effects. Independent variables (A, B, and C) in Table 1 were standardized by scaling the original factors and were transformed into -α, -1, 0, +1, and +α. In creating a mathematical model, a higher-order regression model was simplified by eliminating terms that gave the highest *p*-value to the model, thereby improving the fitness of the model. Model term reduction was applied to improve the accuracy in describing the behavior of the responses on a surface graph.

The mathematical model should achieve an insignificant Lack of Fit (LoF) value (*p* value ≥ 0.05), and the combination of terms that gave the highest R²,

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i < j} \beta_{ij} X_i X_j$$

adjusted R², and adequate precision was used to describe an ideally fit model for each response variable. These tests were used to correct for the over- (or under) estimation of the response variable so it could navigate the design space. From each response, an empirical model was generated as shown in the equation:

Where Y is any response, β is the coefficient, β₀ is the coefficient at the center point (when all coded values were set at 0), ∑ β_iX_i and ∑ β_{ij}X_iX_j were coefficients from various model terms. A desirability test was also performed in determining the optimum condition, considering the individual and combined effects of independent and response variables when performed simultaneously. The target desirability value of 1.00 should be achieved based on the combined set of constraints, including weights per variable, and the desired outcome or predicted characteristics of polysaccharide which significantly influenced the optimum condition.

3. Results and discussion

3.1 Proximate composition of *Acanthophora* spp.

The proximate composition of dried *Acanthophora* spp. in powder form was determined and compared with other studies (Table 3). Results showed that the majority of the dry matter content of *Acanthophora* spp. is composed of total carbohydrates and ash (total minerals), followed by proteins and lipids. Similar findings were observed in Abomohra *et al.* (2018), Dixit *et al.* (2018), D'Armas *et al.* (2019), Herrera *et al.* (2019), and Premarathna *et al.* (2022).

Fresh seaweeds usually contain 80–90% moisture, and the residual 20% or lower after drying is important to delay growth of deteriorative microorganisms (D'Armas *et al.*, 2019). The high total ash content is indicative of the ecological interaction of *Acanthophora* spp., which grows in rocky and sandy substrates of the inter- and shallow subtidal zones (Kilar and McLachlan, 1986; Guillén *et al.*, 2022). Meanwhile, the total carbohydrate content of *A. spicifera* is mainly attributed to sulfated galactans, which could be either agar (Duarte *et al.*, 2004; Pereira Júnior *et al.*, 2021) or carrageenan

Table 3. Proximate composition of *Acanthophora spicifera*.

Composition (g/100 g sample, %)					Reference
Moisture	Protein	Lipid	Carbohydrate	Ash	
19.63±0.65	4.60±0.04	1.65±0.30	30.92±1.72	43.10±1.90	This study
95.02±0.54	28.89±0.35*	3.49±0.13*	48.83±0.91*	13.32±0.01*	Premarathna <i>et al.</i> (2022)
18.84±0.13	5.07±0.01	0.55±0.01	44.76±0.01	28.38±0.39	D'Armas <i>et al.</i> (2019)
10.09±0.09	8.12±0.07	0.30±0.06	33.50±1.53	31.09±1.12	Herrera <i>et al.</i> (2019)
-	12.58±2.60*	5.43±0.42*	40.22±3.45*	-	Abomohra <i>et al.</i> (2018)
8.12±0.19	6.55±0.22	1.40±0.14	48.51±1.81	47.04±1.97	Dixit <i>et al.</i> (2018)

Values are presented as mean±SD. Values in asterisks are expressed on dry basis.

(Parekh *et al.*, 1989; Pettongkhao *et al.*, 2019; Muthulakshmi *et al.*, 2021; Budiyanto *et al.*, 2022; Pradhan and Ki, 2023).

3.2 Factors affecting yield and chemical properties

The current study evaluated the combined effects of KOH (%), time (h), and temperature (°C) using the conventional extraction method on the yield and chemical properties of the polysaccharide. Results showed that the extraction yield of the polysaccharide ranged from 12.00 to 30.84%. Based on the generated

RSM model, variables KOH (%) (A), temperature (°C) (B), time (h) (C), and linear (AB, BC), quadratic (A², B²), and higher polynomial interactions (ABC, A²B, AB², A²B²) significantly affected the extraction yield of polysaccharides. The model showed a coefficient of determination (R²) value of 0.9892, indicating that the model is precise in predicting results with good correlation to the actual data, as shown in Table 4.

High polysaccharide yields (30.84%) can be obtained using lower KOH (3%), lower extraction temperature (65°C), and lower time (2 h). Conversely, low polysaccharide yields (12%) can be obtained at higher KOH (%), temperature, and time combinations as shown in Figure 1A. The reduction in yield is primarily

$$\text{Yield (\%)} = 21.04 - 1.98A - 2.88B - 0.79C + 1.75AB + 0.221AC - 0.2575BC + 0.8948A^2 + 1.75B^2 + 1.24ABC - 2.43A^2B + 1.54AB^2 - 2.63A^2B^2$$

Table 4. ANOVA for the RSM models.

Source	DF	SS	MS	F value	p value
Model 1 (Yield, %)					
Model	12	391.61	32.63	38.32	0.0004
Residual (Error)	5	4.26	0.8516		
Lack of Fit	2	1.5	0.7511	0.8177	0.5207
Total	17	395.87			
R ² = 0.9892; Adjusted R ² = 0.9634					
Model 2 (Galactose content, %)					
Model	13	1.21	0.0929	263.69	<0.0001
Residual (Error)	4	0.0014	0.0004		
Lack of Fit	1	0.0001	0.0001	0.1123	0.7596
Total	17	1.21			
R ² = 0.9988; Adjusted R ² = 0.995					
Model 3 (Sulfate content, %)					
Model	9	1424.11	158.23	17.07	0.0003
Residual (Error)	8	74.15	9.27		
Lack of Fit	5	47.65	9.53	1.08	0.5077
Total	17	1498.26			
R ² = 0.9505; Adjusted R ² = 0.8948					
Model 4 (DPPH free radical scavenging activity, %)					
Model	13	1177.47	90.57	12.37	0.0131
Residual (Error)	4	29.29	7.32		
Lack of Fit	1	7.49	7.49	1.03	0.3847
Total	17	1206.76			
R ² = 0.9757; Adjusted R ² = 0.8969					
Model 5 (Ferric reducing acid powder, Absorbance value)					
Model	11	0.0054	0.0005	19.34	0.0008
Residual (Error)	6	0.0002	0		
Lack of Fit	3	0.0001	0	3.32	0.1753
Total	17	0.0055			
R ² = 0.9726; Adjusted R ² = 0.9223					
Model 6 (ABTS free radical scavenging activity, %)					
Model	12	1058.39	88.2	8.4	0.0144
Residual (Error)	5	52.49	10.5		
Lack of Fit	2	43.16	21.58	6.94	0.0749
Total	17	1110.88			
R ² = 0.9528; Adjusted R ² = 0.8394					

MS: Mean square, DF: Degrees of freedom, SS: Sum of squares.

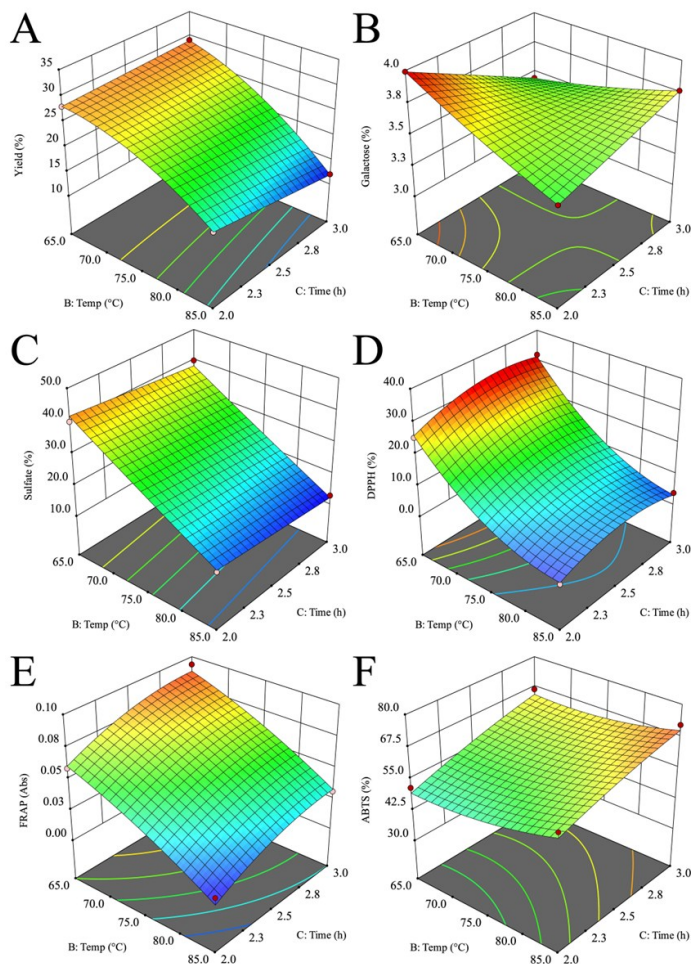


Figure 1. Surface response graphs of the combined effects of KOH (3%), temperature (65–85°C), and time (2–3 h) on various responses for extracting polysaccharides. Responses: A (Yield, %), B (GAL, %), C (Sulfate, %), D (DPPH, %), E (FRAP), F (ABTS, %)

attributed to the degradation of polysaccharides at higher alkaline concentration, higher extraction temperature, and longer hydrolysis time. KOH is commonly used in the carrageenan industry to remove extraneous matter, break down the seaweed, and perform chemical transformation of precursor polysaccharides (Usov, 1998; Freile-Pelegrín *et al.*, 2006). Meanwhile, increased extraction temperature and time may reach the melting point of the polysaccharide, which could allow degradation or diffusion of the polysaccharide from the thallus into the alkaline solution (Freile-Pelegrín and Murano, 2005). Moreover, similar findings were observed in Schnoller *et al.* (2020), where polysaccharide yield from *A. spicifera* after native extraction (24.70–39.30%) was higher than after KOH treatment (9.10–14.90%). Alkali treatment was also employed on *A. spicifera*, having reported yields of 18.82% (Parekh *et al.*, 1989). Anand *et al.* (2018) also used alkaline treatment in *A. spicifera* and extracted 48.10%. However, Duarte *et al.* (2004) reported low yields in *A. spicifera* after alcohol precipitation (3.60–6.60%). In Webber *et al.* (2012), alkaline treatment for the extraction optimization of *K. alvarezii* found that

higher extraction temperature (°C) could predict higher carrageenan yield (%). However, Nurmiah *et al.* (2017) reported that at pilot plant scale production, lower extraction temperature (85°C) and higher extraction time (4 h) were recommended in predicting high carrageenan yields.

Concurrently, there is a significant increase in the GAL and 3,6-AG contents of the extracted polysaccharide after high KOH (6%) treatment. Results showed that the GAL content (Figure 1B) was strongly influenced by KOH (%) (A) and extraction temperature (°C) (B), and the linear (BC) and quadratic (A²) interactions based on the equation. Meanwhile, the 3,6-

$$\begin{aligned} GAL (\%) = & 3.39 + 0.1417A + 0.0307B - 0.0472C - 0.0697AB - 0.1228AC + 0.1597BC \\ & + 0.1326A^2 + 0.0684B^2 + 0.0466AB - 0.0089A^2B + 0.1054A^2C \\ & + 0.0893AB^2 - 0.1356A^2B^2 \end{aligned}$$

AG contents in the polysaccharide extracts were found to be negligible and have a narrow range between 0.99 and 1.92%. Based on Table 4, the model for GAL content showed a coefficient of determination (R²) value of 0.9988, which indicates high precision of the model showing good correlation with the actual data and could predict sulfate contents with high reproducibility. Similarly, the model for 3,6-AG content showed an R² value of 0.9956.

The increase in GAL and 3,6-AG contents after using high KOH (%) is due to the alkali conversion of 4-linked- α -D/L-galactopyranose into 3,6-anhydro-D/L-galactose (Ciancia *et al.*, 1993; Usov, 1998). Moreover, the 4-linked residues of the sulfated polysaccharide may be present in part as 3,6-AG after alkali treatment (Usov, 1998). Compared to Parekh *et al.* (1989) and Schnoller *et al.* (2020), the GAL (3.00–3.99%) and 3,6-AG (0.99–1.92%) contents of the polysaccharide extract from *A. spicifera* were lower. Moreover, Schnoller *et al.* (2020) reported that between 6% KOH and native extraction, the 3,6-AG contents of the polysaccharide from *A. spicifera* did not significantly vary. Meanwhile, Anisuzzaman *et al.* (2014) reported that in extracting carrageenan from *Kappaphycus alvarezii*, GAL (%) content was strongly influenced by the interactions of

$$\begin{aligned} Sulphate (\%) = & 22.61 - 2.37A + 7.1B - 1.79C + 5.18AB + 1.73A^2 + 4.19B^2 - 14.94A^2B \\ & - 1.31AB^2 - 5.29A^2B^2 \end{aligned}$$

KOH (%) and extraction temperature (°C), while 3,6-AG (%) was affected only by the increasing KOH (%).

The total sulfate contents of the extracted polysaccharides ranged from 12.70 to 46.40%. Results showed that the total sulfate contents of polysaccharide extracts were strongly influenced by KOH (%) (A), temperature (°C) (B), and their linear (AB), quadratic (B²), and higher polynomial (A²B, A²B²) interactions as shown in the equation.

Results also showed that sulfate levels are strongly influenced by extraction temperature (B). Moreover, the R^2 value of the model for the total sulfate content of the polysaccharides is 0.9505, which could indicate high precision. Based on our findings, highly sulfated polysaccharides can be obtained at a lower KOH (3%) concentration, lower temperature (65°C), and shorter extraction time (2 h) as shown in Figure 1C. However, in Anisuzzaman *et al.* (2014), as KOH (9%) increased while extracting at 70–75°C, the degree of sulfation of semi-refined carrageenan from *K. alvarezii* increased. Moreover, high D-galactose 4-sulfate contents can be predicted using combinations of high KOH (9%) but low temperature (60°C), or low KOH (6%) but high extraction temperature (80°C). Similarly, Sormin *et al.* (2019) also reported that at low KOH (0.35%) levels, sulfate contents of carrageenan from *E. cottonii* are lower (at 35.20%) compared to higher KOH (0.75%) levels, which could obtain higher sulfate contents of 40.20%. Meanwhile, Schnoller *et al.* (2020) observed that native polysaccharide extraction (0% KOH) resulted in high sulfate content (8.8–13.8%) while 6% KOH treatment obtained low sulfate contents (4.5–8.9%). Diharmi *et al.* (2020) also reported that 4% KOH treatment produces highly sulfated carrageenan (29.64%) while 12% KOH treatment leads to lower sulfate content (21.47%). Meanwhile, Anand *et al.* (2018) reported low sulfate levels in alkali-treated *A. spicifera* (8.9%) as compared to the sulfate levels of polysaccharides after acid treatment (21.9%). Thus, the interactions of KOH (%), temperature (°C), and time (h) on sulfate content of polysaccharide extract vary per species. The industrial processing of red seaweeds usually applies KOH ranging from 0.4 to 4.0 M (or 2.24–22.44%) for efficient extraction and modification of the chemical structure of carrageenan (Ciancia *et al.*, 1993). In addition to seasonal fluctuations and species type, extraction conditions are major factors affecting carrageenan quality (Hasizah *et al.*, 2021). Alkali treatment often leads to *in vitro* chemical conversion of seaweed galactans, particularly the 'precursor' Unit B α -4-linked L/D galactose-6-sulfate into 3,6-L/D-AG. In effect, the reduction of sulfates as they are being replaced by OH groups may increase the concentration of GAL and 3,6-AG. Freile-Pelegri *et al.* (2006) reported that alkali treatment could reduce the carrageenan sulfate content by 22–40% after 3–4 h extraction time, with values reaching 16.6–24.5%.

Despite the presence of GAL and 3,6-AG and high sulfate contents, the extracted polysaccharide does not gel and form viscous solutions even after the addition of potassium (KCl) or calcium (CaCl₂) cations, unlike other carrageenans (Usov, 1998). Ciancia *et al.* (1993) reported that the behavior of chemical reactions between

carrageenan types varies due to the heterogeneity of the substituents and functional groups present from different sources. Having high sulfate levels even after KOH treatment, this study agrees with Schnoller *et al.* (2020) that the extracted polysaccharide differs from true agar, where sulfate contents should be negligible or not present at all. Moreover, this study showed that polysaccharides may be considered as D/L hybrids, deviants from true agar and true λ - or θ (theta)-carrageenan (Schnoller *et al.*, 2020). Furthermore, the findings in this study showed that polysaccharide extracts from *A. spicifera* have high sulfate (%), low GAL and negligible 3,6-AG (%) contents, which are characteristics of λ -carrageenan (Campo *et al.*, 2009).

3.2 Factors affecting *in vitro* antioxidant activities

Seaweed polysaccharides and their oligosaccharides may play crucial protective roles from oxidative damage caused by reactive oxygen species (Guo *et al.*, 2022). Acid-hydrolyzed polysaccharide extracts may give rise to oligosaccharide fractions having improved bioactive properties (Guo *et al.*, 2022). In this study, the antioxidant activities of polysaccharide extracts after

$$\begin{aligned} \text{DPPH Inhibition (\%)} &= 17.97 + 2.66A - 0.1038B + 1.04C + 5.36AB - 0.6273AC - 3.24BC \\ &- 1.77A^2 - 4.37B^2 - 3.68C^2 - 2.16ABC - 7.64A^2B - 4.15AB^2 + 5.91A^2B^2 \end{aligned}$$

mild acid hydrolysis were measured based on *in vitro* DPPH and ABTS free radical scavenging activity and FRAP capability. Acid-hydrolyzed polysaccharide showed low DPPH inhibition activity, ranging from 1.29–31.99%. Results denote significant linear (AB, BC), quadratic (B², C²), and higher polynomial (A²B, AB², A²B²) interactions based on the equation and showed a coefficient of determination (R²) value of 0.9757, indicating that the model could predict highly precise results (Table 4).

Moreover, high DPPH inhibition activity can be predicted when extracting at a lower temperature (65°C) and using lower KOH (3%). Conversely, higher extraction temperature (85°C) and higher KOH (6%)

$$\begin{aligned} \text{ABTS Inhibition (\%)} &= 54.22 - 3.7A - 2.67B - 2.83C + 0.1391AB - 3.94AC + 4.39BC + 1.16A^2 \\ &+ 2.54B^2 - 1.08C^2 + 5.15ABC + 6.36A^2B + 5.52A^2C \end{aligned}$$

lead to low DPPH inhibition activity, as shown in Figure 1D. Meanwhile, the results in the ABTS inhibition activity of acid-hydrolyzed polysaccharide extracts of *A. spicifera* ranged from 38.31 to 72.07%. Based on our findings, KOH (%) (A), linear (AC, AB), quadratic (B²), and higher polynomial (ABC, A²B, A²C) interactions strongly influenced the ABTS inhibition activity of acid-hydrolyzed polysaccharide. The coefficient of determination (R²) value of 0.9757, as shown in Table 4, indicates that the model can predict precise results based

on the equation.

Results also showed that high ABTS inhibition

$$FRAP = 0.0474 - 0.0074A - 0.0075B - 0.0003C + 0.0148ABC - 0.0055AC + 0.0029A^2 + 0.001B^2 - 0.0067C^2 - 0.0053A^2B + 0.0085A^2C + 0.0053AB^2$$

activity (72.07%) can be obtained when extracting using low KOH (3%), but favors higher temperature (85°C), and longer extraction time (3 h). Whereas low ABTS inhibition activity (38.31%) can be obtained after 6% KOH at 65°C for 3 h (Figure 1E). Lastly, the FRAP assay of acid-hydrolyzed polysaccharide ranged between 0.01 and 0.09. High FRAP values were obtained when extracting polysaccharides using 3% KOH at 65°C for 3 h. Whereas negligible FRAP values are obtained using 3% KOH at 85°C for 2 h, as shown in Figure 1F. These findings generated a model with an R² value of 0.9762, which also indicates good precision to predict results based on the equation.

The results in the optimization of process parameters to produce polysaccharide extracts with high antioxidant properties are shown in Table 5. Considering ideal conditions of all response variables, lower KOH (3–4%), mid-range extraction temperature (65–70°C), and shorter extraction time (2–3 h) were selected as constraints for determining the optimum conditions for extracting polysaccharides. Based on the model, the extraction conditions recommend using KOH at 3.00–3.01%, extraction temperature at 65.00–65.06°C, and extraction time of 2.97–3.00 h. Meanwhile, constraints for the response values include yield (29–30.838%), GAL (3.007–3.9903%), total sulfate content (38–46.3978%), DPPH (29–31.992%) and ABTS (64–72.0685%) inhibition activities and FRAP values (0.08–0.0935).

The specific mechanism for the antioxidative roles of seaweed poly- and its acid-hydrolyzed oligosaccharides has not been elucidated yet. Unlike other polymers, such as proteins or DNA, which have distinct structures, poly- and oligosaccharides have no precise structures which may be composed of homo- or copolymers (Amicucci *et al.*, 2020). The antioxidant capacity of seaweed poly- and oligosaccharides depends on various factors, including the degree of polymerization or molecular weight, the presence, type, and position of functional groups, type of monosaccharide present, linkages and chain branching (Gómez-Ordóñez *et al.*, 2014). The presence of hydroxyl and sulfate groups in poly- or oligosaccharide which exhibit better ion chelating ability may scavenge free radicals through H-abstraction or H-donating ability (Sun *et al.*, 2015). The presence of reducing sugars and reducing ends of low molecular weight poly- and oligosaccharides may also enhance antioxidant roles (Sun *et al.*, 2015). Rocha de Souza *et al.* (2007) reported that λ-carrageenan (*Gigartina acicularis*, *G. pisillata*) possess high antioxidant scavenging activities compared to κ- and ι-carrageenan. However, in Abad *et al.* (2013), the DPPH inhibition activities were higher in κ- and ι- than in λ-carrageenan even with increasing irradiation doses of 5–50 kGy. λ-carrageenan also showed lower antioxidant activity compared to κ- and ι-carrageenan based on nitric oxide, superoxide anion, and hydroxyl radical scavenging activities, which suggested that the antioxidant activities may be due to the reducing ends present (Sokolova *et al.*, 2011a; Sokolova *et al.*, 2011b). Meanwhile, in Anand *et al.* (2018), the DPPH activities of sulfated polysaccharides from *A. spicifera* may reach >50% at 150 µg/mL concentration. In Maleki *et al.* (2023), polysaccharide extracts from *G. acerosa* exhibited high

Table 5. Constraints used to optimize the extraction of polysaccharide and the optimum conditions and predicted results from the model with their desirability values.

Variables	Limit	Optimum conditions							
		1	2	3	4	5	6	7	8
Independent									
A: KOH (%)	3–4	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.01
B: Temperature (°C)	65–70	65.00	65.02	65.06	65.00	65.04	65.02	65.01	65.02
C: Time (h)	2–3	3.00	2.98	2.99	2.97	2.98	2.99	2.97	2.99
Dependent		Predicted results							
1. Yield (%)	29.00–30.84	29.04	29.00	29.01	29.01	29.00	29.03	29.01	29.00
2. Galactose (%)	3.01–3.99	3.19	3.18	3.19	3.18	3.19	3.19	3.18	3.19
3. Sulfate (%)	38.00–46.40	38.14	38.13	38.06	38.25	38.11	38.11	38.18	38.05
4. DPPH (%)	29.00–31.99	31.37	31.46	31.28	31.65	31.39	31.36	31.55	31.34
5. ABTS (%)	64.00–72.07	64.38	64.11	64.25	64.02	64.16	64.30	64.07	64.19
6. FRAP (abs)	0.08–0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Desirability Value	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Where goals are in range of the set limits. The lower and upper weights were set to 1, and the level of importance was set to 3. The constraints generated eight (8) solutions with a desirability value of 1.0.

ABTS (80%), DPPH (81.48%), and FRAP (1.72) antioxidant activities. In its purified and fractioned form, sulfated polysaccharides of *A. spicifera* were reported to exhibit high reducing capability (71.2–97.3%), which can be obtained at 125 µg/mL concentration (Anand *et al.*, 2018). Saluri and Tuvikene (2020) reported that aside from sulfate groups, the presence of 3,6-AG in the polysaccharide synergistically enhances antioxidant activity of λ- and θ-carrageenan. In addition, Samrit *et al.* (2024) also noted that *A. spicifera* possesses high antioxidant properties due to the presence of phenolic substances in the extract, which could quench free radicals. The high antioxidant activities of polysaccharides could be due to the level of sulfate groups present, which play crucial roles in the biological activity of the polysaccharides that scavenge free radicals (Nguyen *et al.*, 2024). Rocha de Souza *et al.* (2007) reported that sulfate content is positively correlated to antioxidant activity of polysaccharides from red and brown seaweeds.

4. Conclusion

In conclusion, the findings in the study showed the potential of CCD of RSM as a tool to optimize the extraction of highly sulfated polysaccharides from *Acanthophora* spp. with antioxidant potential. Model-based evaluation of the constraints from the independent and dependent variables was tailored to fit as desired process parameters and ideal characteristics of extracted polysaccharides. The study revealed eight optimum conditions with a desirability of 1.00 that could predict a polysaccharide yield of 29.00–29.04%, having 3.18–3.19% GAL, and 38.05–38.25% total sulfate, 31.28–31.65% DPPH, 64.02–64.38% ABTS inhibition activities, and 0.09 FRAP value. The results in this study could serve as a basis for extracting bioactive polysaccharide fractions from *Acanthophora* spp. while elucidating its chemical structure.

Conflict of interest

The authors declare no conflict of interest.

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