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OPTIMIZATION AND CHARACTERIZATION OF SULPHATE BIOPOLYSACCHARIDE PRODUCTION FROM EDIBLE GREEN ALGAE *Ulva Lactuca* USING SURFACE RESPONSE METHOD

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ABSTRACT

This research focuses on optimizing the production of sulfate biopolysaccharides from the edible green algae *Ulva lactuca* using surface response analysis. The objective was to identify the ideal conditions for extracting Ulvan, a sulfated polysaccharide from Ulvophyceae, by analyzing critical process factors-namely, temperature, HCl concentration, and heating duration. The study found the best extraction conditions at a temperature of 73°C, HCl concentration of 0.3 M, and heating duration of 4 hours, resulting in a 25.07% yield of Ulvan. The extracted Ulvan had a total sugar content of 0.1767 mg/mL and a sulfate content of 0.3143 mg/mL. Structural analysis conducted through FTIR and NMR spectroscopy confirmed the presence of typical sulfated polysaccharide signatures. Physical properties assessed using SEM, XRD, and TGA indicated that Ulvan is an amorphous material with substantial thermal stability. In biological evaluations, Ulvan demonstrated notable antioxidant properties, with an inhibition rate of 30.52% and a toxicity concentration (LC_{50}) of 288.978 ppm. These findings highlight the potential of Ulvan in biopolymer production and suggest its applicability in developing algae-based products. The results contribute valuable insights into the biopolysaccharide extraction process, paving the way for future applications in the pharmaceutical and food industries.

Keywords: *Ulva Lactuca*, Sulfate Biopolysaccharides, Response Surface Methodology, Ulvan Extraction, Thermal Stability, Antioxidant Properties, Algae-Based Biopolymers.

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INTRODUCTION

Indonesia is abundant in natural resources, particularly in its diverse seaweed biodiversity. Seaweed, which can generally be classified into three main categories-green, red, and brown-is a well-known source of macromolecules with unique physicochemical properties. These properties, many of which remain underexplored, hold great potential for further scientific and industrial investigation. Among these macromolecules, polysaccharides are one of the most abundant and have attracted considerable research interest due to their extensive applications in the food industry, healthcare sector, and materials science field.[2,9,10,14] Despite this, the potential of ulvan, a sulfurated complex polysaccharide derived from marine green algae, particularly in Indonesia, remains largely untapped, presenting a substantial opportunity for future exploration.[11]Polysaccharides isolated from red and brown seaweed, among them agar, carrageenan, and fucoidan, have been extensively studied. However, those derived from green seaweed, especially ulvan, remain significantly underexplored and underutilized. Ulvan, a sulfated polysaccharide derived from *Ulva lactuca* (commonly known as sea lettuce), exhibits considerable potential for industrial applications due to

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its high abundance and rapid growth rate, which can reach up to 30% per day.[8,12,13] *Ulva lactuca* is widely found in coastal areas, particularly in Eastern Indonesia, and has been cultivated through inland farming technology by Indonesia's Institute of Sciences (LIPI). This cultivation method ensures a sustainable supply of ulvan, independent of natural harvests.[15] The chemical composition of ulvan comprises 18.4% rhamnose, 4.4% glucose, 1.9% xylose, 0.9% mannose, 0.9% galactose, 15.2% uronic acid, 15.8% sulfate, and 23.7% ash content, all expressed as a percentage dry.[5] Ulvan has been shown to demonstrate diverse biological properties, including anticoagulant, antioxidant, tumor-inhibiting, immunomodulatory, and antidyslipidemic properties.[1] Additionally, it serves as a base material for the production of nanofibers, hydrogels, and anti-aging products, as well as in the realm of plant cultivation.[6,7] These diverse properties make ulvan a promising candidate for wide-ranging applications in sectors such as healthcare, beauty, and other industries. The biological efficacy of ulvan is strongly influenced by the methodologies used in its extraction. Studies indicate that sulfated polysaccharides extracted using acidic solvents demonstrate superior antioxidant activity compared to those extracted using aqueous or alkaline solvents.[4] Moreover, using hydrochloric acid in the extraction process can enhance the purity, yield, and biological activity of the polysaccharides, although higher concentrations of acid may accelerate polysaccharide degradation.[3] Thus, understanding and optimizing the extraction process is crucial for maximizing the quality and efficacy of ulvan for various applications. Given ulvan's promising potential, there is a pressing need for research to delve deeper into its extraction process. This study aims to determine the optimal conditions for producing ulvan with superior physical and chemical characteristics. Additionally, it will evaluate the biological activities of ulvan, particularly its antioxidant and toxicity properties. In the future, ulvan is expected to become a valuable raw material for various industries, including healthcare, beauty, and other sectors.

EXPERIMENTAL

Material and Methods

Green algae or *Ulva lactuca*, ethanol 96% (C_2H_5OH) (Merck), methanol p.a (Merck), potassium bromide (KBr) (Merck), dimethyl sulfoxide (DMSO) (Merck), aquabides (Jayamas Medica Industri) Barium chloride ($BaCl_2.2H_2O$) (Merck), phenol (C_6H_5OH) (Merck), aquades, DPPH powder(2,2-diphenyl-1-picrylhydrazil) (Sigma), ascorbic acid (Merck), shrimp larvae *Artemia salina Leach*, filter paper, sulfuric acid (H_2SO_4) (Merck), hydrochloric acid (HCl) (Merck), sodium sulfate (Na_2SO_4) (Merck), Tricloroacetic Acid (TCA) 4% (Merck), glucose ($C_6H_{12}O_6$) (Merck), sodium hydroxide p.a (NaOH) (Merck), gelatin and sterile seawater.

Sample Preparation

The *Ulva lactuca* sample, which played a crucial role in this research, was collected from the waters near Barrang Lompo Island, Makassar, South Sulawesi. The preparation started by washing and drying 2 kg of fresh samples in an oven at 60°C. Once dried, the sample was finely ground and sieved using an 80-mesh screen. The resulting powder was soaked in ethanol for around 24 hours to enhance its purity, followed by an additional drying step before proceeding to the next stages of the process.

Proximate Analysis by AOAC Method

The ash content (%) was determined by heating *Ulva lactuca* in a muffle furnace at 550°C for approximately four hours. Following this, the sample was removed from the furnace, the sample was promptly reweighed after cooling to room temperature in a desiccator. The carbohydrate content (%) was carried out by formula 100 – (moisture + ash + protein + fat) %. The fat content (%) determination involved placing two grams of *U. lactuca* in a thimble attached to a round-bottom flask filled with 120 mL of petroleum ether, heating and refluxing for 5 hours, and then weighing the samples.

The Moisture Content (%) determination included drying two grams of *U. lactuca* for three hours at 105°C and then cooling in a desiccator, a crucial piece of equipment, before re-weighing to ensure accuracy.

Optimization ulvan Extraction using Response Surface Methode (RSM)

The optimization process was performed using the Box-Behnken Design (BBD) with three distinct independent variables: HCl concentration level, temperature, and the duration of extraction heating, all aimed at responding to a fixed variable, which is the ulvan mass percentage (w/w).

Biopolysaccharide Production of the Ulvan

Extracting 25 g of dry green algae powder using 0.3 M hydrochloric acid solvent (500 mL, 1:20 w/v) at 73°C for 4 hours. Centrifuging the mixture at 10,000 rpm for 30 minutes, separating it into supernatant and precipitate. Precipitating the supernatant with 96% ethanol (1:3 sample: ethanol), then incubating for 24 hours. Centrifuging at 6000 rpm for 20 minutes, resulting in a precipitate, then drying it in an oven at 50°C for 48 hours. Finally, crushing and weighing the precipitate to determine the yield of ulvan produced yield (%) was estimated by using the following equation formula:

$$\text{Yield (\%)} = \frac{\text{weight of extracted yield (g)}}{\text{weight of dry biomass (g)}} \times 100 \quad (1)$$

Characterization of ulvan Biopolysaccharides

Structural Characterization by Fourier Transform Infrared (FTIR) and Nuclear Magnetic (1H-NMR) Spectroscopy

0.001 g of dried ulvan and 0,1 g of KBr were combined and ground until homogenous. FTIR device (Prestidge-21 Shimadzu) was used for the analysis, with wavenumbers ranging from 4.000 – 400 cm^{-1}. NMR analyses were performed using a Varian Agilent 500 MHz NMR spectrometer DDR2 console.

Physical Characterization by Scanning Electron Microscopy (SEM) X-ray diffraction (XRD), and Thermal Gravity Activity (TGA) Spectroscopy

0,1 g dried ulvan was performed for SEM analyses (JEOL JSM 6510 LA, EDS), XRD analyses (X-Ray Diffractometer (XRD) Bruker D8 Advance), and 0,5 g sample powder was used for TGA analyses (TG/DTA Hitachi STA7300) at 30-600°C.

Determination of ulvan Biopolysaccharides

Total Sugar Content

Following the dissolution of a 0.01 g sample in 10 mL of water, the mixture was supplemented with 0.5 mL of 5% phenol and 2.5 mL of 98% sulfuric acid, then left undisturbed at room temperature for 20 minutes until a clear color change became visible. Absorbance was then quantified at 481 nm using a UV-Vis spectrophotometer (T60 UV-VIS), and the results were derived from a standard glucose calibration curve.

The Sulfate Content

The 0.01 g of ulvan powder is dissolved in 10 mL of aquabides. Subsequently, 1 mL of the solution is combined with 1.4 mL of 4% TCA and 1 mL of the barium chloride-gelatin reagent. After incubation for 10-20 minutes at room temperature, measure the absorbance at 397 nm using a UV-Vis spectrophotometer (T60 UV-VIS). Calculate the result based on the sodium sulfate curve for accurate analysis.

Antioxidant and Toxicity Assay

The antioxidant capacity of Ulvan was assessed using the DPPH assay (1,1-diphenyl-2-pycryhydrazil), while its cytotoxicity was determined through the Brine Shrimp Lethality assay (BSLT) involving *Artemia salina* larvae.

RESULTS AND DISCUSSION

Ulvan Extraction

The chemical components of *U. lactuca* were investigated using proximate assay, the results were obtained as follows (Table-1):

Table-1: The Results of *Ulva Lactuca* Proximate Analysis

Analysis	Percent Analysis (%)	References of Percent Analysis (%)
Ash	23.02	28.9 [16]
Carbohydrate	62.30	58.1 [17] and 62 [16]
Fat	0.45	0.19 [17] and 0.45 [16]
Moisture	16.65	16.9 [17]

The proximate results closely match the reference, except for the lower ash content, which can be attributed to various factors such as seasons, exposure to waves, harvesting locations, residency periods in the ocean, physiological traits, processing techniques, and mineralization methods.[16] The optimization used the surface response technique for determining the ideal conditions to produce the highest yield of ulvan.

Table-2: Analysis of Variance (ANOVA) Response Optimization of Ulvan Production

Source	DF	Adj Sum of Square	P-Value
HCl concentration (*M*)	1	282.047	0.000
Temperature (°C) * Temperature (°C)	1	12.063	0.113
Heating hour (*h*) * Heating hour (*h*)	3	7.784	0.547

Further analysis using ANOVA obtained a correlation coefficient (R^2) of 94,82% and a value of P = 0,0 (P<0,05) which suggests that the model formed is statistically significant for use in predicting output values (Table-2). A larger R^2 value signifies a more pronounced effect of all X process parameters on the Y response variable.

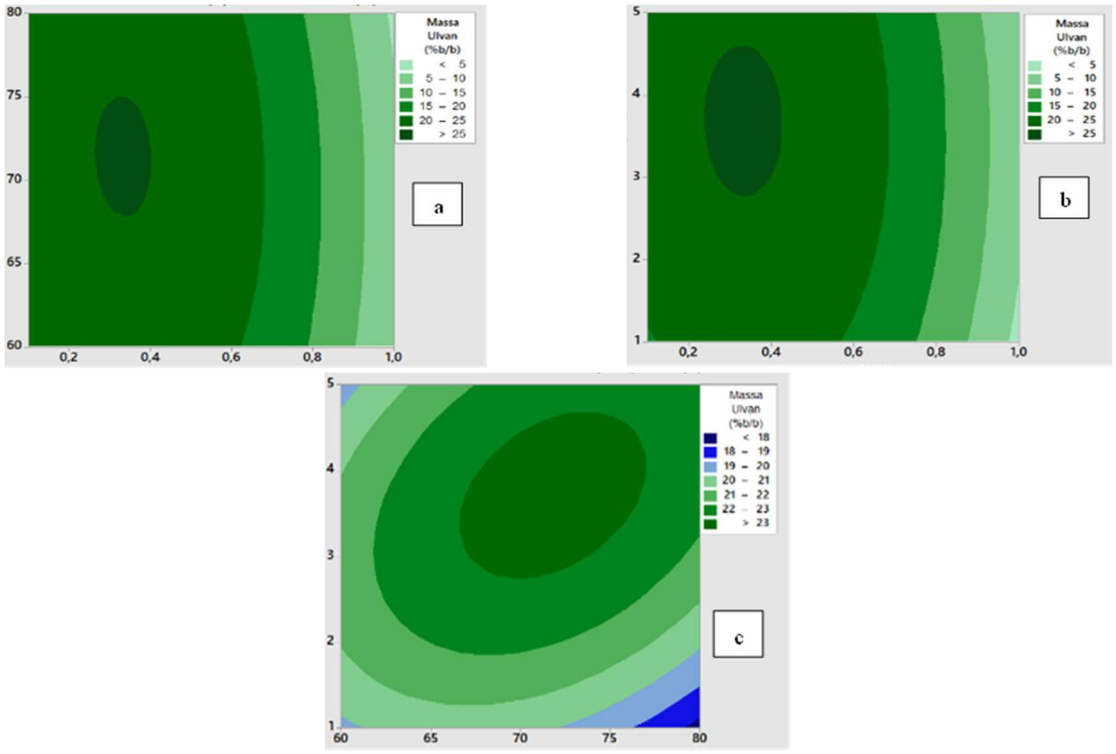


Fig.-1: Contour Plot the Influence of HCl concentration level, temperature, and heating duration on ulvan production. Interaction contour plot of (a) Temperature*HCl concentration level, (b) Heating duration*HCl concentration level, and (c) Heating duration*Temperature

The plot (Fig.-1) shows that the ideal HCl concentration level for ulvan yield production range is 0.2 M–0.4 M. Higher concentrations result in lower yields due to accelerated polysaccharide degradation. Additionally, a temperature range of 70–75°C with a heating time of 3–4 hours yield the highest ulvan production. The determination of the optimum point was carried out by determining the zero-gradient value in the three contour plots. The obtained prediction value of 25.52% w/w at the optimum concentration condition of 0.3M, temperature at 73°C, and heating time at 4 hours is shown in Fig.-2.
Based on the production results, the ulvan mass is 25.07% w/w. These results indicate that ulvan production is close to the model prediction results so this model is good to use in determining the effect of these three variables on ulvan production. Ulvan extraction uses hydrochloric acid (HCl) solvent. Acid extraction enhances antioxidant activity compared to water or base. Acid treatment is a widely recognized method for breaking down diverse polysaccharide structures into monosaccharide units.[20] Meanwhile, the ethanol was used to precipitate from the extraction results.

Ulvan Characterization

The FTIR spectra of the isolated ulvans reveal distinct peaks that signify the presence of various functional groups typical of polysaccharides. A broad peak observed at 3404.36 cm^{-1} is attributed to the O-H bond

vibrational stretching pattern in hydroxyl units. Peaks at 1651.07 cm^{-1} and 1460.11 cm^{-1} are indicative of carboxyl groups within the uronic acid component. Furthermore, a prominent absorption peak at 1112.93 cm^{-1} is characteristic of sulfate polysaccharides, representing the S=O sulfate group, complemented by an absorption peak at 850.61 cm^{-1}, which denotes the C-O-S linkage.

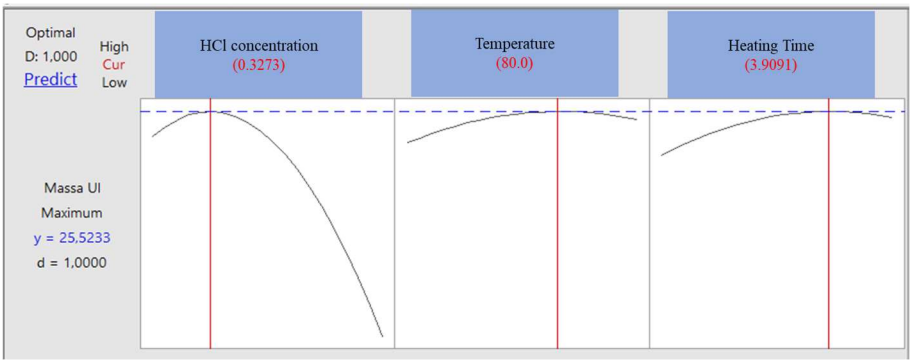


Fig.-2: Determination of the Optimum Point of Ulvan Production

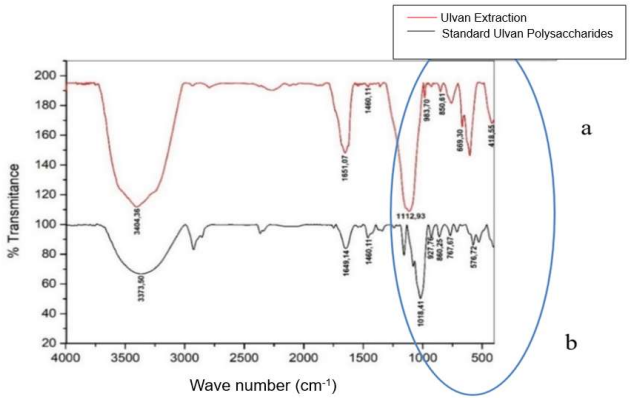


Fig.-3: FTIR Spectrum (a) Ulvan extracted and (b) Standard Polysaccharide Ulvan

Based on prior research, the C-O-S bond stretch in ulvan appears at 848 cm^{-1}, and the S=O stretching vibration in the sulfate group appears at 1.215 cm^{-1}. The extracted ulvans displayed similar absorbance profiles to the standard spectra of sulfate polysaccharides and standard ulvans.

Table-3: Wavenumber and Functional Group of Ulvan by FTIR Analysis

No.	References of Ulvan Wavenumber	Functional Group
1	3500-3200 cm^{-1} [18]	-OH (hydroxyl group)
2	1020-1050 cm^{-1} [18]	C-O-C strech (ether group)
3	1620-1600 cm^{-1} [18]and 1420 cm^{-1} [18]	Carboxyl group strech symmetric and asymmetric
4	1220 cm^{-1} [18]	S=O strech
5	840 cm^{-1} [18]	C-O-S strech

In the ^{1}H-NMR spectrum (Fig.-4), the ulvan shows signal characteristics with chemical shift values between 1.1-5.2 ppm, including anomeric protons at 5.2 and 4.4 ppm, protons around the rings of glucuronic acid and rhamnose at 4.0-3.1 ppm, and protons in the methyl group at 1.1 ppm. These values are similar to previous references.

The ^{1}H-NMR spectrum, with chemical shift values ranging from 1.3 to 5.4 ppm, is a distinctive indicator for sulfate polysaccharides from seaweed. Proton rings are typically found between 3.2 and 5.4 ppm, while methyl groups can be found between 1.2 and 2.3 ppm. The anomeric signal from 3.35 ppm to 4.26 ppm

corresponds to protons from C2 and C5 in the sugar residue, uronic acid at 3.7 ppm, and a sugar residue containing sulfate at 4.5 to 4.8 ppm.

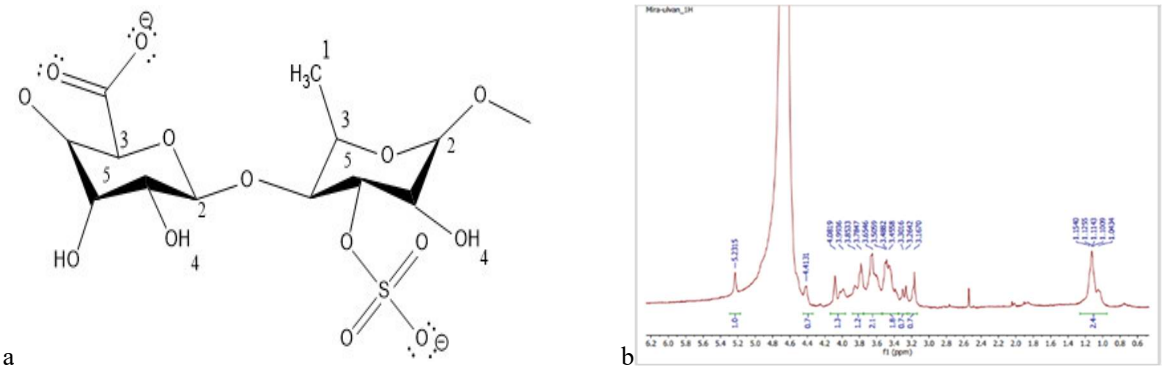


Fig.-4: (a) Ulvan Structure; (b) 1H-NMR Ulvan Spectrum

Table-4: Results of Identification of Chemical Shift Value of 1H-NMR Ulvan

	Chemical Shift		
Symbol	Ulvan Extraction Results	References of *U. Lactuca* Wavenumber	Signal Characteristics
1	1.12-1.11	1.17 [19]	Methyl group
2	4.41	4.3 [19]	Anomeric protons
2*	5.23	5.2 [19]	Anomeric protons
3	3.16	3.2 [19]	Proton ring
4	4.08	4.2 [19]	Proton ring
5	3.99	3.8 [19]	Proton ring

The results of XRD ulvan can be seen in this diffractogram graphs that appear at 2θ, 20.27°, and 29.14°, having sharp and thin peaks, identifying that ulvans have large, stacked particles (Fig.-5). The XRD diffraction pattern of ulvans shows two sharp peaks, indicating large particles, whereas standard sulfate polysaccharides have multiple widened peaks, indicating smaller particles than ulvans. This identification's structure or form points to an uneven or amorphous crystal lattice.

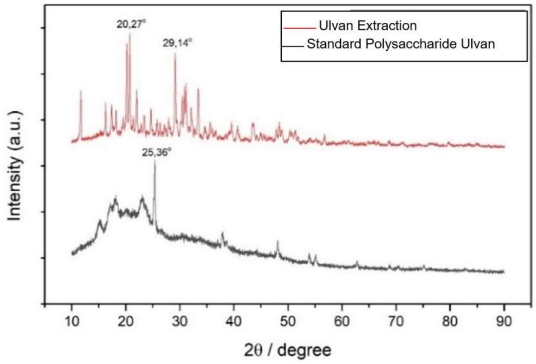
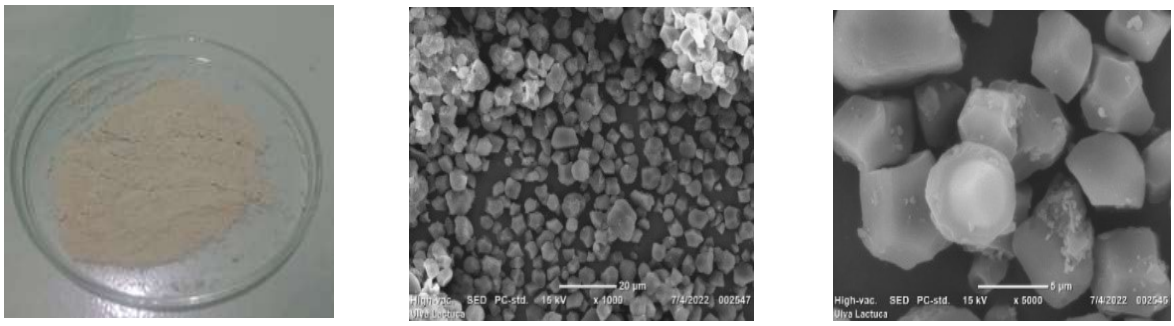


Fig.-5: Ulvan XRD Results and Ulvan Polysaccharide Standards

Ulvan XRD diffraction usually refers to four characterization peaks at 14.6°; 16.5°; 22.9° and 34.7°.[13] Based on the physicochemical characterization of biodegradable polysaccharides derived from green algae, ulvans—identified as semicrystalline polymers—display five primary crystalline peaks at 13.1°, 23.2°, 29.4°, 32.6°, and 39.4°. For comparison, the standard polysaccharide sulfate exhibits a characteristic peak at 2θ = 25.36°. Ulvan's physical properties, including strength, solubility, and flexibility, are influenced by its degree of regularity. Its water-solubility and hygroscopic characteristics are attributed to the presence of water bonds with hydrophilic groups. Ulvan's irregular pattern is influenced by environmental conditions, extraction methods, and solvents. The ulvan products with physical and morphological characteristics can

be seen in Fig.-6. The results of ulvan extraction show that the physical shape of ulvan is in the form of white powder, it looks like the morphology of the ulvan surface is arranged more neatly, and looks like granules or grains with various shapes such as circles, pentagons, squares, and triangles. The pattern of particles is flat and does not stick to each other.



(a) (b) (c)

Fig.-6: (a) Ulvan Extraction Results; (b) Morphological Structure Analysis using Scanning Electron Microscopy Ulvan Magnification 1000x and 5000x

The thermal analysis using TGA on extracted ulvans is shown in Fig.-7, demonstrating the percentage of mass decrease and stability effects.

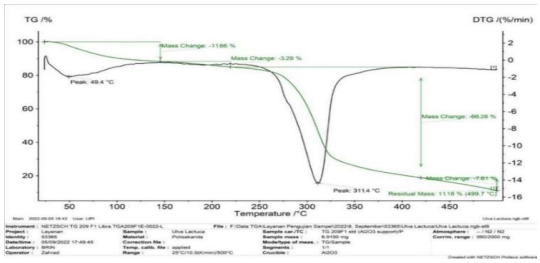


Fig.-7: The Curve of TGA Ulvan Analysis Results Extracted from Ulva Lactuca

The ulvan thermogram data revealed three distinct phases of mass reduction. The first decrease of 11.66% at 23.7-145.7°C is due to evaporation of water and ethanol. The second decrease of 3.29% at 145-219.2°C is from the decomposition of organic compounds. The third, significant decrease of 66.26% above 219.2°C is due to the decomposition of sulfate polysaccharides. Studies show that ulvan remains stable up to 180°C, but starts to break down at 200°C, with a marked decrease at 205°C.[20] These findings indicate a phase of mass reduction in ulvan production.

Ulvan Total Sugar and Sulfate Content

The total sugar (TS) method, also known as the phenol-sulfuric acid assay is employed to quantify sugar content through a colorimetric approach. This method can swiftly detect nearly any class of carbohydrates using phenol-sulfuric acid. Polysaccharides, oligosaccharides, and disaccharides are transformed into monosaccharides by sulfuric acid. Dehydrated pentose compounds (5-carbon) become furfural, while hexose sugars (containing 6 carbon atoms) become hydroxymethyl-furan compounds. The interaction of the compound with the phenolic compound leads to a shift in color to golden yellow. Complex compounds responsible for color absorb visible light, and the absorbance values are linearly related to the sugar concentration. Hexose and pentose clusters are observed at maximum wavelengths.[21]

The barium chloride-gelatin method is used to analyze sulfate content in seaweed polysaccharide samples. Ulvan has higher sulfate but lower sugar content compared to the standard polysaccharide. Factors such as extraction method and location influence sugar and sulfate concentrations. Sulfate polysaccharides, like ulvan, have the potential for biological activity and offer various positive effects on health, including free radical scavenging, inflammation reduction, and microbial inhibition properties. Ulvan's high sulfate

content and polysaccharide composition make it suitable for biomedical formulations and tissue engineering, including cartilage production in combination with poly D-lactic acid (PDLLA).[22,23]

Table-5: Results of Analysis of Total Carbohydrate Content and Sulfate Quantification

No.	Study Sample	Total Sugars (mg/mL)	Sulfate Levels (mg/mL)
1.	Ulvan extraction yield	0,1767	0,4131
2.	Standard sulfate polysaccharide	0,2057	0,1984

Antioxidant and Toxicity Assay BSLT Method

Bioactive substances like ulvan, a sulfated polysaccharide, act as antioxidants to prevent free radical damage. The sulfate content is directly related to its capacity to neutralize reactive oxygen species. The DPPH (1,1-diphenyl-2-pycryhydrazil) method antioxidant test shows how antioxidants neutralize free radicals. The results of the ulvan antioxidant activity test can be found in Table-6.

Table-6: Results of Antioxidant Activity Measurement

Sample	IC_{50} (ppm)	Antioxidant Activity
Ulvan	313.851	Weak
Standard sulfate polysaccharide	242.060	Less
Vitamin C	2.658	Strong

Based on Table-6, polysaccharide standards have moderate antioxidant power (IC_{50} value: 242.060 ppm) compared to ulvan's weak antioxidant power (IC_{50} value: 313.851 ppm). The test compound's abundance of conjugated double bonds and hydroxyl groups contributes to its strong antioxidant activity. The sulfate ester group, a specific class of sulfate polysaccharides, along with the hydroxyl group, has the ability to prevent cell damage induced by free radicals. Ulvans with a larger molecular weight produce weak antioxidant results due to their crude extract nature. Previous studies have indicated that sulfate polysaccharides have low activity power.[13]

Table-7: BSLT Ulvan Test Results from Extraction and Polysaccharide Standards

Sample	Concentration (ppm)	Concentration log		Number of death		Death %	Probit	LC_{50} (ppm)
Ulvan	1	0	0	0	0	0	0	
	10	1	1	0	0	3	3.12	289.978
	100	2	0	2	1	10	3.72	
Standard sulfate polysaccharide	1	0	0	1	0	3	3.12	
	10	1	2	1	1	13	3.87	273.222
	100	2	5	2	3	37	4.67	

The Shrimp Lethality Test (BSLT) is a quick and cost-effective method for assessing the toxicity of compounds using shrimp larvae (Artemia salina). It provides precise measurements and is closely linked to the cytotoxic potential of anticancer drugs. The test determines toxic effects based on the observed mortality of *Artemia salina*. with the LC_{50} value classifying compounds as very toxic (<30 ppm), toxic (30-1000 ppm), or non-toxic (>1000 ppm). Table-7 shows the LC_{50} values for ulvan and sulfate polysaccharide standards 298.978 ppm and 273.222 ppm, respectively. These results suggest that the compounds fall within the toxic category with nearly identical LC_{50} values. *Artemia salina* larvae used in toxicity testing are 48 hours old, as they have fully formed mouths and gastrointestinal tracts, and increased body resistance. The mechanism of death of the larvae is related to the function of sulfate polysaccharide compounds, acting as stomach poison, leading to death by starvation.

CONCLUSION

Based on the findings of this study, it can be inferred that the ideal condition for ulvan extraction to produce the highest yield using the Response Surface Method approach is the concentration of HCl solvent of 0.3 M at a temperature of 73°C with a heating time of 4 hours. With a production yield of 25.07%, the total sugar content obtained was 0.1767 mg/mL and the sulfate content was 0.4131 mg/mL. The ulvan produced

is characterized by several instruments to determine the characteristics of the ulvan produced. Then the results of the ulvan antioxidant activity test with the DPPH method obtained an inhibition percent of 30.52% counteracting free radicals, while testing the toxicity of ulvan showed that ulvan is toxic with an LC_{50} value of 288.978 ppm.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest in preparing this paper.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profiles of the authors can be verified from their ORCID IDs, given below:

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