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Optimization of Microwave Ultrasound Extraction of Ulvan Biopolymer from *Ulva lactuca* using Response Surface Methodology

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Abstract

This study aims to determine the effect of combining the two extraction methods Microwave Assisted Extraction (MAE) and Ultrasound-Assisted Extraction (UAE) on the yield and content of ulvan polysaccharides from *Ulva lactuca* with the best operation condition. Algae biomass *U. lactuca* is abundant, according to its content, *U. lactuca* contains polysaccharides which can be used as biodegradable plastic materials. The extraction was carried out using Microwave Ultrasound Extraction by varying the microwave power from 100 to 800 watts, the microwave time variations from 10 to 50 minutes, the ultrasonic wave temperature range from 40-80 ^{o}C, and the ultrasonic wave time variations from 20 to 50 minutes. Optimization was performed using the response surface methodology center composite design methodology for a total of 28 runs. Several tests were carried out to determine the character of the ulvan polysaccharide, including water content, FT-IR spectroscopy, High-Performance Liquid Chromatography, and its antioxidant activity. The effect of combining Microwave Assisted Extraction (MAE) and Ultrasound-Assisted Extraction (UAE) provides an optimal extraction condition with higher yields and good quality ulvan.

Keywords: *microwave ultrasound extraction, ulvan, Ulva lactuca*

1 Introduction

In general, *Ulva lactuca* is known as sea lettuce. Morphologically the color of *U. lactuca* Varies from light green to dark green depending on where the ulva originates [1–3]. Based on data from the Ministry of Maritime Affairs and Fisheries, Indonesia the total seaweed production reached 11.6 million tons in 2016 [4]. *U. lactuca* produces translucent sheet fronds that are irregular in shape with wrinkled edges and belong to the macroalgae division Chlorophyta [5]. *U. lactuca* cells contain a lot of chlorophyll. In tropical areas, *U. lactuca* grows in shallow waters and lives at temperatures of 28 – 31°C. This algae contains polysaccharides which have many benefits. In general, *U. lactuca* contains 15% protein, 12% fiber, 1% lipid, and 45% polysaccharides in the cell wall, i.e. ulvan (9–36%), cellulose (1–15%), xyloglucan, and glucuronos [6,7]. Ulvan is a sulfated anionic polysaccharide consisting primarily of xylose and rhamnose 3-sulfate linked to uronic acid [8,9]. Ulvan can form thermoreversible films because it has hydrophilic (OH, COOH, SO_4) and hydrophobic functional groups [10].

Bioplastic comes from natural polymers such as polysaccharides, proteins, and lipids [11]. Which generally involves intra- and intermolecular interactions as well as cross-linking between polymer constituents, forming three-dimensional polymers [12]. Ulvan has a repeating disaccharide structure consisting mostly of uronic acid. In this case, ulvan has the potential to be applied in bioplastics. Population growth and consumption habits are increasing and causing several negative impacts on the environment. This requires consideration of pollution, such as the siltation of soil and the accumulation of plastic waste. About 12% of plastic waste is included in the world's total solid waste [13]. This is due to the

continued increase in production so that since 1950 until now more than 6 billion tons of plastic waste have been produced [11]. Therefore, it is necessary to have alternative technology and bioproducts in the form of biodegradable bioplastics. Bioplastics do not take a long time to degrade, are non-toxic, and reduce the waste produced or the space needed to manage waste [14].

The extraction of ulvan biopolymer from green algae can be done in various ways. The ulvan polysaccharide extraction method can be carried out using conventional methods with a yield of 8% within 24 hours at room temperature, and 6% for 8 hours at 80ºC [15]. More yield was produced by the hot water extraction method, which was 22.90% [5]However, this extraction requires a long time in the autoclave with continuous stirring at 120ºC. In addition to these methods, green extraction or extraction with the help of enzymes produces a higher yield of 17.14% [16], but the use of this method requires special attention to the culturing of the enzymes and the maintaining of the extraction condition is hard to apply. Special treatment of enzymes is one of the weaknesses in the application of enzyme extraction, because of the high risk of failure during the extraction [12,17]. In a study by Ramadhan, et al. using ultrasound extraction which showed a yield of 16.6% within 90 minutes [18]. Whereas in a study conducted by Kidgell, et al. ulvan extraction using the microwave-assisted extraction method obtained a yield of 14.4% in a shorter time [10]. The Microwave Assisted Extraction (MAE) and Ultrasound Assisted Extraction (UAE) methods produce more yield at a shorter extraction time, whereas there are no studies that discuss the combination of these two non-conventional methods[19,20]. Therefore, a study is needed to discuss the combination of Microwave Assisted Extraction (MAE) and Ultrasound Assisted Extraction (UAE) in extracting ulvan polysaccharides from *U. lactuca*. The way this combination method works is carrying out extraction with microwave followed by a second extraction with ultrasound, this method is called Microwave Ultrasound Extraction (MUE).

The yield produced in the extraction will depend on the treatment carried out during the extraction. From the study by Le, et al., the extraction yield will increase as the extraction time increases, it is also mentioned that microwave power will control the temperature during extraction, which will affect the physiochemical properties of water and will increase the solubility of polar compounds present in water [21]. Another study conducted by Rahimi, et al. showed that the ratio of raw materials and solvents will affect the yield obtained, a greater amount of solvent will produce a higher yield [22]. In addition, the difference in power used in extraction will provide a difference in extraction temperature [21], higher power will increase the temperature, and the temperature increase is proportional to the increase in yield up to a certain point. As explained by Michalak, et al. temperature affects the extraction efficiency, and the optimal extraction temperature will depend on what substance is to be extracted [23]. Thus, by varying the variables in the form of extraction time, power used, and temperature during extraction, it will be possible to determine the effect of these factors on the yield produced in the extraction of ulvan polysaccharides, so that the most optimal conditions for extraction can be obtained. Optimization is carried out as an effort to find optimal conditions for extraction. Optimization can be used to determine the effect of each variable and the relation between variables on the yield extraction.

The purpose of this study is to determine the effect of combining two extraction methods and find the optimal operation condition (microwave power, microwave extraction time, ultrasound temperature, and ultrasound extraction time) for ulvan extraction towards the yield and content of ulvan polysaccharide, which will then be used as a raw material in the manufacture of biodegradable plastic. The characterization used is the extraction yield, calculation of molecular weight, FT-IR spectroscopy, and High-Performance Liquid Chromatography (HPLC).

2 Method

2.1 Materials and Tools

U. lactuca algae biomass was taken from Paras village, Duwet, Panarukan, Situbondo Regency, East Java on July 30 2022. *U. lactuca* was taken in dry condition and stored at room temperature. The materials used in the pre-treatment are dichloromethane (pro-analysis), 99% ethanol (pro-analysis) and acetone (technical). While the solvent in the extraction is aquadest. In the characterization carried out, 1M H_2SO_4 (pro-analysis), $CaCO_3$, KBr, 1-2-diphenylpicrylhydrazyl and 70% ethanol (technical) were used. The extraction was carried out using two main tools, namely a microwave (SAMSUNG MS23K3515AS) with a maximum

power of 800 watts and an ultrasound bath type BAKU BK-1200 Ultrasonic Cleaner with a maximum power of 60 W and a maximum capacity of 1.47 L.

2.2 Pre-treatment

The dried *U. lactuca* seaweed is washed thoroughly until there are no impurities present on the ulva such as shells, sand, and others. Ulva was dried in an oven at 60ºC for ± 2 hours. Dried *U.* lactuca was blended and sieved using a 60-mesh sieve. Ulva which had been sifted was soaked with dichloromethane for 16 hours, then stirred for 8 hours constantly at 250 rpm. Then the ulva was filtered and soaked using 99% ethanol.

Pre-treatment using 99% ethanol serves to remove pigments and low molecular weight compounds [21]. Soaking using ethanol was carried out for 24 hours. After being soaked in ethanol, the sample was filtered and then rinsed using acetone. Ulvan is dried at room temperature to a moisture content of ±0.3% in the ulva before further extraction.

2.3 Microwave Ultrasound Extraction

The extraction process is carried out in a SAMSUNG MS23K3515AS microwave which is equipped with a power and time regulator. Extraction was carried out with a time variation of 10 – 50 minutes at a power variation of 100 – 800 watts. Each time and temperature variation was carried out with aquadest solvent.

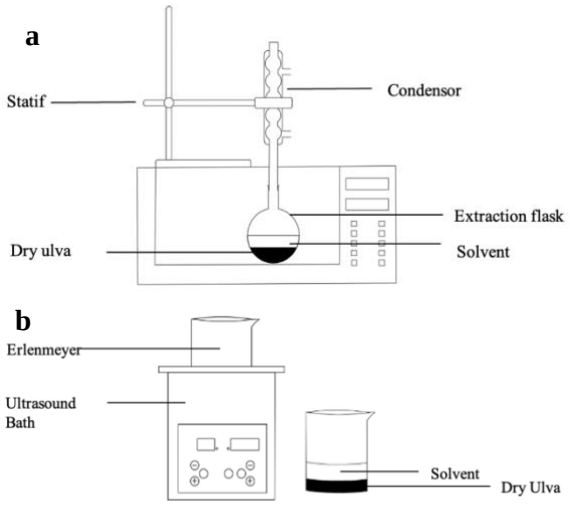


Figure 1. Microwave Ultrasound Extraction Scheme, a) Microwave Extraction; b) Ultrasound Extraction

Samples that had been extracted using a microwave were then extracted again using a BAKU BK-1200 Ultrasonic Cleaner with variations in extraction time of 20 – 50 minutes at various temperatures of 40–80ºC. The extracted substrate is then filtered with a 100-mesh filter cloth. The extract was then centrifuged at 4000 rpm for 20 minutes. Extract in the oven at 80ºC for 20 minutes. The dry extract was then stored at room temperature to wait for the next treatment.

2.4 Experimental Design

After determining the lower limit and upper limit on the single factor extraction variable to be used, the Response Surface Methodology (RSM) of the Central Composite Design (CCD) type is used to determine the optimal combination of extraction variables [21]. Ultrasound temperature (X_1 40 – 80ºC), ultrasound time (X_2 20 – 50 minutes), microwave power (X_3 100 – 800 watts) and microwave time (X_4 10 – 50 minutes). Central Composite Design Matrix is shown in **Table 1.**

Table 1. Central Composite Design Matrix with Yield as Response

Run	X_1	X_2	X_3	X_4
1	40	50	100	50
2	60	20	450	30
3	80	50	800	10
4	40	20	100	50
5	80	20	800	10
6	80	20	100	10
7	60	35	450	30
8	40	20	800	10
9	80	50	800	50
10	80	20	100	50
11	80	50	100	10
12	40	50	800	50
13	60	35	450	30
14	80	35	450	30
15	40	50	800	10
16	60	50	450	30
17	40	35	450	30
18	60	35	450	30
19	40	20	100	10
20	80	20	800	50
21	40	20	800	50
22	60	35	450	10
23	60	35	800	30
24	80	50	100	50
25	60	35	450	30
26	60	35	100	30
27	40	50	100	10
28	60	35	450	50

2.5 Characterization

a) Yield of Ulvan

To calculate the yield obtained, according to Ramadhan et al [18], it can be calculated by dividing the resulting mass by the initial mass multiplied by 100%, or written as follows by equation 1:

$$\text{yield (\%)} = \frac{\text{Dry ulvan mass}}{\text{Dry ulva mass}} \text{ x } 100\% \quad (1)$$

b) Water Content

A total of 2 g of 60 mesh ulva was placed in a cup, then in the oven at 105°C for 2 hours. Repeat the oven until you find a constant mass. To calculate the water content obtained from the equation 2:

$$\text{water content (\%)} = \frac{\text{initial mass} - \text{constant mass}}{\text{constant mass}} \text{ x } 100\% \quad (2)$$

c) Molecular Weight

The molecular weight is calculated with Oswald viscometer to find out the viscosity, then the results will be calculated by the equation 3:

$$\eta \text{ sample} = \frac{(\text{t sample} - \rho \text{ sample})}{(\text{t water} - \rho \text{ water})} . \eta \text{ water} \quad (3)$$

η sample = sample viscosity,
η water = water viscosity,
ρ sample = sample density,
ρ water = water density.

Meanwhile, the relation between viscosity and molecular weight is based on Mark Houwink's equation [18]:

$$[\eta] = K_{MH} M^a \quad (4)$$

$$M = \sqrt[a]{\left(\frac{[\eta]}{k_{MH}}\right)} \quad (5)$$

[η] = intrinsic viscosity (dL/g);
kMH = 1.69 . 10-5; a = 2.03;
M = molecular weight (kDa)

d) Chemical Composition

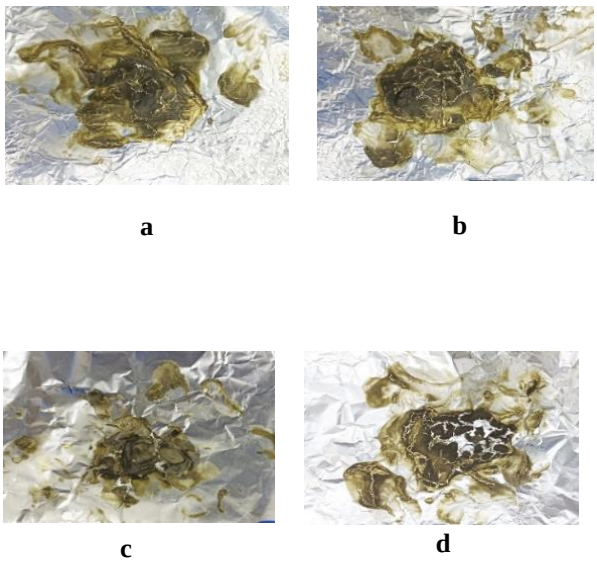
Sugar content was analyzed by High Performance Liquid Chromatography (HPLC). As much as 0.2 gram of dry ulvan was mixed with 10 mL of 1 M sulfuric acid, the mixture was then hydrolyzed with autoclave at 120°C for 1 hour. The hydrolate is neutralized by adding $CaCO_3$ and then filtered through Whatman filter paper No. 47. The resulting solution will be analyzed using High Performance Liquid Chromatography (HPLC) [24].

Functional groups in ulvan were analyzed by FT-IR spectroscopy with the help of KBr. Polysaccharide samples were added with KBr and then pulverized until homogeneous. The powder was thinned with the help of 7000 Pa pressure, the results obtained were analyzed with FT-IR (Perkin Elmer's spectrum one) at a wave of 500-4000 cm^{-1}, the results will be compared with the literature spectrum of sulfated polysaccharide standard compounds [18].

3 Result and Discussion

3.1 Extraction yield

The main research conducted was to determine the effect of each extraction variable including microwave power, microwave time, ultrasound temperature and ultrasound time. As shown in **Table 1,** it can be said that the four variables have an effect on the ulvan yield. To determine the effect of the four variables used, the microwave power was varied from 100-800 watts, microwave time 10-50 minutes, ultrasound temperature 40-80°C and ultrasound time 20-50 minutes. Ulvan extraction yields range from 1.112 to 5.188%. The difference in yield is caused by the variables used in the different extraction processes. Dried ulvan is shown in **Figure 2**.



a **b**

c **d**

Figure 2. Dried Ulvan, a) Run 4; b) Run 6; c) Run 12; d) Run 27

The highest yield was from Microwave Ultrasound Extraction (MUE), which was 5.188% with the operating conditions of microwave extraction at 450 watts for 30 minutes and ultrasound at 60°C and 20 minutes. The microwave power used will affect the temperature during extraction, which affects the physiochemical properties of water thereby increasing the solubility of low polar compounds in water, the diffusion coefficient will increase as the temperature is increased by using microwave control [21]. At variations in ultrasound temperature, an increase in temperature is directly proportional to an increase in the solubility of polysaccharides which will increase the molecular diffusion coefficient [22], but this increase will be at the saturation point, where the yield will decrease. The choice of temperature to be used in extraction depends on the type of compound to be taken and its destructive effect [23]. From the results it can be seen that ultrasound temperature above 60°C will reduce the extraction yield, but the effect of ultrasound temperature in this study also depends on the three previous variables. The length of extraction time is directly proportional to the yield obtained to its maximum limit, if the extraction is carried out too long it will cause a reverse impact on the yield due to damage to the polysaccharides taken. This is evidenced by the lowest yield results when the variables of temperature, power, and time are the highest, namely 800 watts at 80°C and 50 minutes of time. Where the lowest result obtained is 1.112%.

Other than the four variables used, another thing that affects extraction is the sample particle size which will also affect the diffusion ability of the molecule [25]. The treatment of the material also affects the results. In the study, pre-treatment was carried out to remove unwanted compounds. In the process of removing these compounds, some of the polysaccharides to be extracted are wasted [5].

3.2 Statistical analytic and model fitting

There are 28 total runs for the optimization of the four variables which were carried out with CCD. Data were analyzed by multiple regression analysis using Design-Expert 11 and a polynomial equation was derived to represent the yield of polysaccharides as a function of the variables used, where Y is the yield of polysaccharides and X_1, X_2, X_3, and X_4 are codes for the extraction value (microwave power, time microwave, ultrasound temperature and ultrasound time). **Table 1** shows the process variables and experimental data. The yield percentage ranges from 1.112 to 5.118% where the optimum yield is obtained at the operating conditions of 450°C microwave power, 30 minutes of microwave time, 80°C of ultrasound temperature and 20 minutes of ultrasound time. Analysis for Quadratic Polynomial Model used in Extraction of Polysaccharide Ulvan from *U. lactuca* is shown in **Table 2.**

The application of RSM will show the empirical relation between the response variable and the test variable. The application of multiple regression analysis to the data associated with a second-order polynomial equation (eq. 6)

$$Y = 1.230 + 0.972X_1 + 4.820x_2 - 0.842X_3 - 0.001X_4 + 0.080X_1X_2 - 0.253X_1X_3 - 0.248X_1X_4 - 2.93X_2X_3 - 1.21X_2X_4 + 3.61X_3X_4 - 0.039X_1^2 + 0.003X_2^2 + 0.005_3^2 - 0.0.625X_4^2 \quad (6)$$

Table 2. Analysis for Quadratic Polynomial Model used in Extraction of Polysaccharide Ulvan from *U. lactuca*

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	17.23	14	1.23	2.64	0.0447
X_1	0.9721	1	0.9721	2.08	0.1728
X_2	4.82	1	4.82	10.32	0.0068
X_3	0.8420	1	0.8420	1.80	0.2024
X_4	0.0012	1	0.0012	0.0025	0.9609
X_1X_2	0.0809	1	0.0809	0.1733	0.6840
X_1X_3	0.2530	1	0.2530	0.5416	0.4748
X_1X_4	0.2485	1	0.2485	0.5320	0.4787
X_2X_3	2.93	1	2.93	6.27	0.0264
X_2X_4	1.21	1	1.21	2.59	0.1317
X_3X_4	3.61	1	3.61	7.73	0.0156
X_1^2	0.0393	1	0.0393	0.0840	0.7765
X_2^2	0.0031	1	0.0031	0.0066	0.9364
X_3^2	0.0058	1	0.0058	0.0124	0.9131
X_4^2	0.6253	1	0.6253	1.34	0.2681
Residual	6.07	13	0.4671		
Lack of Fit	6.07	10	0.6072		
Pure Error	0.0000	3	0.0000		
Total	**23.31**	**27**			

Analysis of variance (ANOVA) was performed to evaluate predictive models and

variables. In Table 2 it can be seen that the F value (F-values) is 2.64 and the P value (P-values) is less than 0.0500 indicating that the response of the quadratic model is significant and can be used to optimize variable extraction. Linear coefficient values (X_1, X_2, X_3, and X_4), squared coefficients (X_1^2, X_2^2, X_3^2), and cross product coefficients $X_1X_2, X_1X_3, X_1X_4, X_2X_3$) has a significant effect if the p-values are low. Lack of fit indicates a model failure that represents experimental data where points are not included in the regression or random errors occur [21].

The coefficient of determination (R^2) in this study is 0.7394 indicating a fairly good correlation between the original and predicted values. The results of the adjusted R^2 analysis show a value of 0.4589 which means that there are many variations in the yield extraction that can be predicted from the model. The coefficient of variation (CV%) of 19.28% indicates better precision than the experimental value.

3.3 Optimization of Ulvan Polysaccharide Extraction Conditions

The graph that represents the multiple regression equation can be seen in **Figure 3,** which is illustrated using 3D response surfaces. This graph illustrates the correlation between the response and experimental level of each variable, but it also describes the type of interaction between the two variables tested. The graph that is displayed has a variety of shapes, where each contour shape describes the interaction of different variables. The circular contour indicates a neglected interaction between the appropriate variables, while the oval contour indicates a significant relation.

Figure 3-a shows the relation between ultrasound time and ultrasound temperature, it can be seen that the yield will decrease if the ultrasound time increases, and as well as increasing the ultrasound temperature, the yield will also decrease. Extraction with a medium temperature of 60°C and a short time of 20 minutes produced a higher yield, however, at temperature 80°C and a medium time of 35 minutes produced a yield that was not much different. The increase in the ultrasound temperature will also increase the solubility of the material, therefore the longer contact of solvent and ulva will also increase the yield of ulvan until the saturation point [22]. Meanwhile, the relation between ultrasound temperature and microwave power can be seen in **Figure 3-b.** Medium microwave power of 450 watts and high ultrasound temperature of 80°C produces a yield that is not much different from using a high microwave power of 800 watts and medium ultrasound temperature of 60°C, the yield will decrease if the two variables are used in the highest range. The increase of microwave power will also increase the temperature of the extraction. Because these two variables are both related to the solubility of ulvan, the increase of these variables will also increase the yield of ulvan [12]. **Figure 3-c** shows the relation between ultrasound temperature and microwave time, the yield will increase at 10-30 min microwave time and will decrease further, the yield will increase if the ultrasound temperature used rises to 60°C. The microwave extraction time will affect the contact between the solvent and the ulva, the longer the contact will also increase the diffusion rate of ulvan and increase the yield until the saturation point, on the other hand, if the contact is too long it will cause a reverse effect because of the destruction of ulvan. [26]. **Figure 3-d shows** the relation between ultrasound time used and microwave power, as previously written, the yield will decrease as the ultrasound time increases, while the yield will increase as the power increases up to 600 watts. High power and long ultrasound times result in low yields. In **Figure 3-e** microwave time and microwave power, where high yield occurs at 30 min microwave time and 450 watt microwave power. The last graph **Figure 3-f** shows the relation between ultrasound time and microwave time showing an increase in yield occurs if these two variables are in the middle range, namely 30 min for microwave and 20 min for ultrasound.

3.4 Water Content

Analysis of water content in ulvan was carried out using the thermogravimetric method, this analysis was used to determine the moisture content of the ulva before the extraction. Moisture content is the ratio between the mass of the sample after and before heating. The working principle of this thermogravimetric method is to remove water molecules by heating in an oven with a temperature of 105°C for 5 hours until the weight of the sample being tested is constant. Based on the above results, the percentage of water content in sample 1 is 0.037%, and in sample 2 is 0.034%. So, the average water content of the sample is 0.036%. The results were obtained after 3 repetitions. Where the lower the water content contained in the material, the easier the components of the active compound are analyzed.

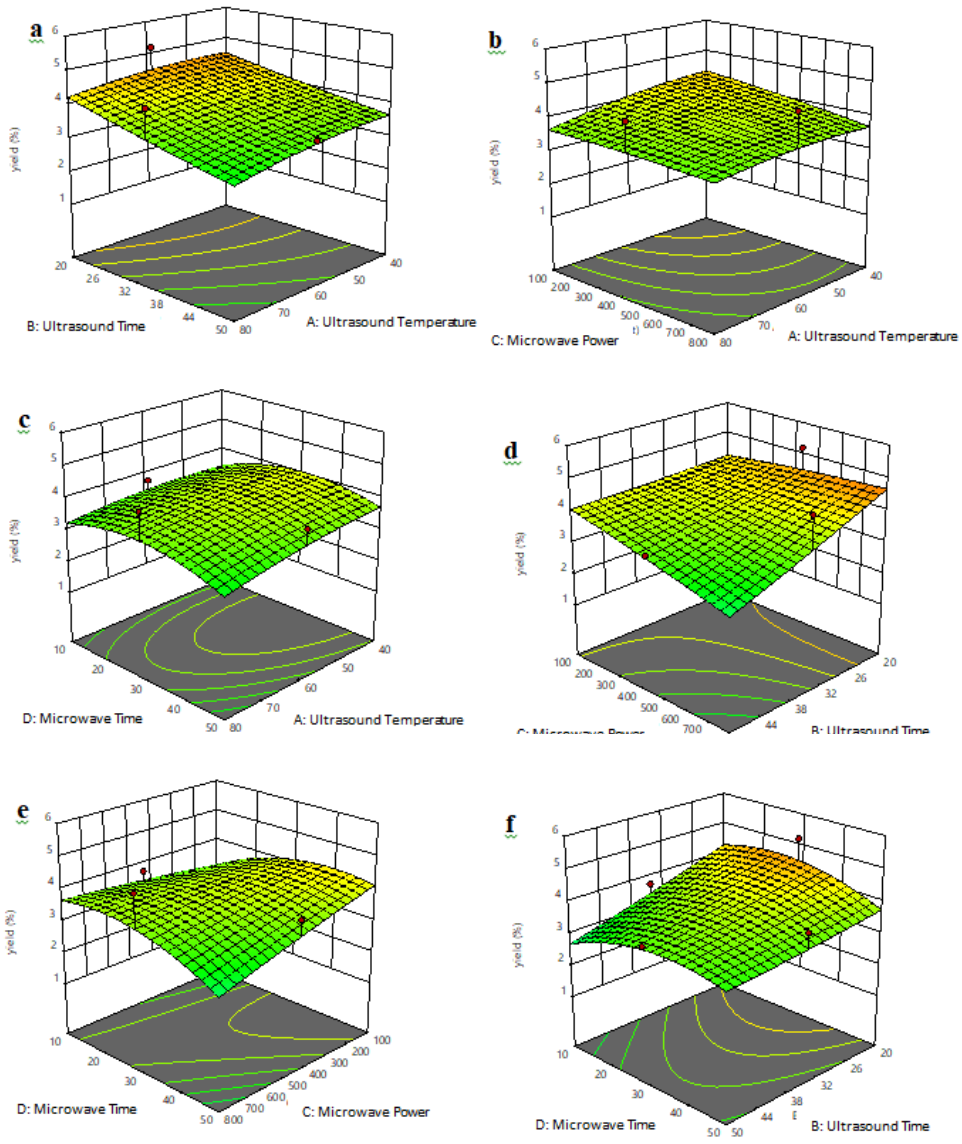


Figure 3. Response Surface Plots (a–f)

Factors that can affect the value of the water content are temperature and sample size. Where the higher the temperature, the more water vapor will be accommodated by the air. So when the temperature of the sample being tested has a high-temperature difference, the faster the heat transfer occurs between the heating medium and the sample [27]. In addition, the particle size will also affect the rapid evaporation of water in the sample. The smaller the particle size, the faster the process of reducing the water content in the sample so that it can reduce the value of the water content quickly too [28].

3.5 Molecular Weight

Molecular weight calculations were carried out with the help of an Oswald viscometer which was then calculated by the relationship between viscosity and molecular weight. From the calculation of the molecular weight, the molecular weight of ulvan polysaccharide from *Ulva* Sp. of 278.9725 kDa.

3.6 Chemical Composition

Monosaccharides in ulvan extract were identified using HPLC characterization. The ulvan extract obtained was acid hydrolyzed first. The selected ulvan extract came from Run 2 which produced the highest yield and Run 9 which produced the lowest yield. Both extracts showed a dominant chromatogram peak at a retention time of about 7 minutes (**Figure 5).** This peak is characteristic of an oligosaccharide which is probably 4-O-β-D-glucuronosyl-L-rhamnose

[29,30]. In the Run 2 extract, the dominant monosaccharide produced was glucose with a retention time of about 9 minutes, while the dominant monosaccharide produced by the Run 9 extract was glucuronic acid with a retention time of about 8 minutes. HPLC chromatogram of ulvan extract from acid hydrolysis is shown in **Figure 4.**

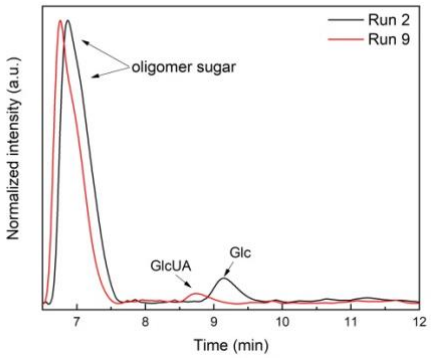


Figure 4. HPLC chromatogram of Ulvan Extract from Acid Hydrolysis

3.7 Biodegradable Plastic Prototype

The ulvan was added with solvent to make a biodegradable plastic prototype. The solvent used is 5% acetic acid and glycerol as the plasticizer. Five grams of sample was added with 50 mL of 5% acetic acid and 1.5 grams of glycerol. The mixture was then stirred at 60^{o}C until the water fully evaporated, then oven for 24 hours at 100^{o}C. The biodegradable plastic prototype from ulvan is shown in **Figure 5.**

Figure 5. Biodegradable Plastic Prototype from Ulvan

4 Conclusion

Microwave-ultrasound extraction is commonly used in the extraction of ulvan polysaccharides from *Ulva* sp. The maximum yield obtained was 5.188% under operating conditions of 450 watt microwave power for 30 minutes and at 60^{o}C ultrasound temperature for 20 minutes. The four variables used have an impact on the yield produced and are interrelated with one another, these variables will affect the physiochemical properties of the ulva, namely the rate of diffusion and product destruction or damage that occurs during the extraction process.

There are several characterizations carried out, the water content of dry ulva which has been analyzed by thermogravimetry is 0.036%, from the molecular weight test carried out it shows that the molecular weight is 278.9728 kDa.

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