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OPTIMIZATION OF CHLOROPHYLL EXTRACTION FROM ULVA SP. WITH ULTRASOUND-ASSISTED LIQUID BIPHASIC SYSTEMS METHOD

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ABSTRACT

Ulva sp. is a type of green algae that is easily found in the shallow seas of Indonesia and contains various bioactive compounds. Chlorophyll is a bioactive compound that functions as a natural dye, free radical scavenger, and antioxidant in the body. Chlorophyll extraction with conventional methods requires a relatively long time and large amount of solvent. In this research, chlorophyll extraction from *Ulva* sp. with the Ultrasound-Assisted Liquid Biphasic System (UALBS) method. *Ulva* sp. as much as 2.5 grams mixed in acetone solvent, added K_2HPO_4 solution, and carried out the sonification process in a dark room. The extraction process performs at a parameter range of 5-15 minutes, ratio 0.05-0.1 g/mL, and 60-100 mesh particle size. The extracted filtrate was added with petroleum ether and distilled water to form a biphasic condition. The extracted chlorophyll was analyzed with chromatography and spectrophotometry method. Antioxidant activity was analyzed using the DPPH method. The optimum result of *Ulva* sp. chlorophyll extraction at an extraction time of 10 minutes, the ratio of solids to solvent was 0.1, the particle size of 60 mesh produced a yield of 1.88% with chlorophyll a 20.12%, chlorophyll b 21.59% and total chlorophyll 41.71%, and the percentage inhibition 45.32%.

Keywords: *Ulva* sp., Chlorophyll, Ultrasound-Assisted Liquid Biphasic Systems (UALBS)

INTRODUCTION

The quantity of green algae in Indonesia is very abundant. One of its types is *Ulva* sp., also known as sea lettuce (Gazali *et al*., 2019). *Ulva* sp. is a type of algae from the class of green algae (*Chlorophyceae*) (Nome *et al*., 2019) which has had speedy and seasonal growth for almost a year (Chemodanov *et al*., 2017). Habitat of *Ulva* sp. lots are found on the eastern coast of Indonesia, such as Bali, the beaches of Sanur, Serangan, Sawangan, Nusa Dua, and Nusa Penida. Indonesia is an archipelagic country with a broader sea area than land, with a ratio of 3:1 (Adha Ardinata & Manguntungi, 2020). One potential wealth of Indonesia's oceans, namely algae, provides advantages in terms of productivity, abundant raw materials, no seasonal variations, and ease of extraction *(Windyaswari et al*., 2019).

Ulva sp. contains bioactive compounds such as vitamins B1, B2, B12, C, phenols, tocopherols, and chlorophyll (Yunita *et al*., 2018). Specifically, *Ulva* sp. contains chlorophyll a, chlorophyll b, and β-carotene with successive levels of 2.18%, 4.64%, and 0.72% (Magdugo et al., 2020). In addition, *Ulva* sp. can produce antioxidants with levels of 11.82% (Pangestuti *et al*., 2021).

Chlorophyll is a green dye pigment in the chloroplasts of living things that can carry out photosynthesis (Aryanti *et al*., 2016). Green algae themselves have the characteristics of containing dominant chlorophyll a and b pigments, are cosmopolitan, and can be found in semi-aquatic environments (Harmoko & Sepriyaningsih, 2020). Chlorophyll a has a less polar nature with a blue-green color, while chlorophyll b is polar with a yellow-green color (Novitasari & Adawiyah, 2018). Chlorophyll is a natural coloring agent in food, a free radical scavenger, and an antioxidant. Recently, algae processing has begun to be developed into various types of medicinal ingredients, organic fertilizers, fish feed, animal feed, heavy metal absorbents, food supplements, cosmetics, biodiesel, alternative sources of bio-pigments, antioxidants, and also has the potential to replace plastic base materials (Dwimayasanti & Kurnianto, 2018; Ghazali & Nurhayati, 2018; Suparmi & Sahri, 2009).

Extraction is a technique of separating a substance into solid or liquid materials using the help of a solvent. The factors that affect extraction include temperature, time, particle size, number of process steps, solvent flow rate, solvent viscosity, solvent type, and solvent concentration, as well as the ratio between solvent and material (Yunita *et al*., 2018). There are two methods that are often used in extraction, namely conventional and modern methods. Conventional methods consist of maceration and Soxhlet while modern methods consist of Ultrasound Assisted Extraction (UAE) and Microwave Assisted Extraction (MAE). Using conventional extraction methods requires longer time and requires large amounts of organic solvent (Ramadhan *et al*., 2022). Apart from that, extraction processes using high temperatures such as microwave assisted extraction (MAE) can cause problems for several types of thermolabile algae *(Essa et al*., 2018). This can occur because the extraction conditions are too extreme in temperature, pH and time which causes significant depolymerization in the Ulva sp extraction process (Mo'o *et al*., 2020). Based on these problems, an extraction technique using the Ultrasound Assisted Extraction (UAE) method was used. which is able to bind chlorophyll in Ulva sp. by maintaining good quality in a short time and producing high yields while still maintaining the concept of energy efficiency by using as little organic solvent as possible.

UAE is an extraction technique using the help of ultrasonic waves for some time which results in the formation of bubbles and cavitation (Chemat *et al*., 2016). The cavitation process periodically deforms the cell wall so that the release of chlorophyll compounds occurs more easily and quickly to the solvent (Essa *et al*., 2018). Ultrasound Assisted Extraction (UAE) is also widely used to extract the bioactive compounds in *Ulva* sp. presented in table 1 as follows:

Table 1. Extraction of UAE on *Ulva* sp.

Raw materials	Solvent	Extraction Conditions	Target Compound	Reference
Ulva lactuta	Ethanol	T = 25˚ C, raw material and solvent ratio = 1 : 5 g/mL, t = 60 minutes.	Quercetin	(Rashad et al., 2021)
Ulva sp.	NaOH 1M	T = 25˚ C, raw material and solvent ratio = 1 : 10 g/mL, t =120 minutes.	Proteins and phenolics	(Kazir et al., 2019)
Ulva lactuta	NaOH 0.05 M	T = 70˚ C, raw material and solvent ratio = 1 : 20 g/mL, t = 90 minutes, frequency = 40 kHz.	*Ulva*n	(Ramadhan et al., 2022)
Ulva rigida	methanol	T = 37˚ C, raw material and solvent ratio = 1 : 10 g/mL, t = 120 minutes, frequency = 40 kHz.	Phenolics and Flavonoids	(Kumar et al., 2020)
Ulva lactuta	Hexane and methyl tertbutyl ether	T = 55˚ C, raw material and solvent ratio = 1 : 6 g/mL, t = 140 minutes, size 0.15 mm, moisture content 5%.	Fatty acids and fatty acid methyl esters	(Kalavathy & Baskar, 2019)

Liquid Biphasic System (LBS) is a type of method for separating two phases of a solution using the addition of salt to speed up the process. The chlorophyll content that comes out of the cell wall from the bottom liquid phase will be selectively adsorbed on the surface of the gas bubbles and carried up to the surface (Sankaran *et al*., 2018). The common salt solvent used is K_2HPO_4, a type of organic solvent that can reduce the solubility of chlorophyll (Elise *et al*., 2021). The biphasic method can quickly and efficiently separate chlorophyll from the solvent compared to conventional extraction methodsm (Khoo *et al*., 2020) .

Ultrasound Assisted Extraction (UAE) method in extracting bioactive compounds, which has been widely studied, needs to be studied regarding the development of a combination of extraction methods to increase chlorophyll activity in *Ulva* sp.. The combination of these extraction methods is Ultrasound-Assisted Liquid Biphasic Systems. This method is the latest modified extraction technique combining ultrasonic extraction with the biphasic separation method, which can provide an extraction and recovery process for bioactive compounds that is more efficient, fast, environmentally friendly, and produces high yields as a simple process. (Khoo *et al*., 2020; Sankaran *et al*., 2018). Extraction studies that have been carried out using the Ultrasound-Assisted Liquid Biphasic Systems (UALBS) method are shown in table 2 as follows:

Table 2. *Ultrasound-Assisted Liquid Biphasic Systems* Extraction of various feedstocks

Raw Material	Solvent	Extraction Conditions	Target Compound	Reference
Olive leaf	$(NH_4)_2SO_4$ and etanol	T = 45˚ C, raw material and solvent ratio = 1:20 g/mL, t = 15 minutes, pH = 6.7	Polyphenols	(W. Wang *et al.*, 2021)
Spirulina platensis	PEG 4000, K_2HPO_4, and KH_2PO_4	Frequency=20 kHz, power=750 W, amplitude=20-30%, t sonification=3-12 minutes, ratio of solvent to raw material=1:0.5 – 1:1.5, air rate=75-175 cc /minute, t flotation =3-12 minutes, biomass loading =0.1-0.6 g.	Pigment-Proteins	(Chia *et al.*, 2020)
Spirulina platensis	PEG and K_2HPO_4	PEG concentration=200-400 g/L, salt concentration 200-350 g/L, ratio of solvent to substance=1:0.7 – 1:1.5, pH=6-9, t flotation = 1.5-9 min, frequency=35 kHz, amplitude=40%.	C-phycocyanin	(Chew *et al.*, 2019)

Chlorophyll can be obtained through the extraction process. The effectiveness of the extraction process depends on the type of solvent used (Agustina *et al*., 2018). Chlorophyll is insoluble in water but has good solubility in ether, acetone, ethanol, methanol, benzene, and chloroform (Sumiati, 2021). Using conventional extraction methods such as maceration requires a longer time and a large amount of organic solvent (Ramadhan *et al*., 2022). In addition, the extraction process uses high temperatures, such as Microwave assisted extraction (MAE) can cause problems for some types of thermolabile algae (Essa *et al*., 2018). This can occur because the extraction conditions are too extreme at temperature, pH, and time which causes significant degradation in the *Ulva* sp. extraction process (Mo'o *et al*., 2020). Based on these problems, an extraction technique is needed that can bind chlorophyll in *Ulva* sp. by maintaining good quality in a short time and producing high yields while still maintaining the concept of energy efficiency by using as few organic solvents as possible.

Based on a literature search, increased chlorophyll extraction from *Ulva* sp. using the Ultrasound-Assisted Liquid Biphasic Systems method has yet to be widely studied. This study aims to determine the time, the ratio of raw materials *Ulva* sp. to solvents, and optimal particle size to produce statistical models and yields of *Ulva* sp. chlorophyll extract.

METHOD

Materials

The tools used were a sonicator (CSBJZQFS-150N0001V2), analytical balance (MSA225S-100-DU), UV-Vis spectrophotometer (752AP), drying oven (WGL-125B), blender (PHILIPS HR-2115), sieve (CBN) 60, 80 and 100 mesh. The material used is *Ulva* sp. obtained from the northern coastal waters of Situbondo, East Java which harvest during the dry season in August 2022, acetone (technical Smart-Lab) as a solvent, K_2HPO_4 as a biphasic agent, petroleum ether (technical Smart-Lab) and aquadest.

Procedure

Material Preparation

Material preparation is done by washing, draining, and baking *Ulva* sp. at 60°C for 5 hours to dry (Puspita *et al*., 2021). After the baking, size reduction is carried out using a blender and sieved at 60, 80, and 100 mesh (Widyaningsih *et al*., 2016).

Ultrasound-Assisted Liquid Biphasic Systems (UALBS) Extraction

Ultrasound-Assisted Liquid Biphasic Systems extraction was carried out by adding 2.5 grams of *Ulva* sp. powder and 0.3 g/mL of K_2HPO_4 solution into acetone (25, 33.3, or 50 mL) and connected to a sonicator. The extraction process is carried out in a dark room. The extraction results were filtered to separate the filtrate and residue. The filtrate was added 8 mL of petroleum ether solution and 15 mL of distilled water to form 2 liquid phases. The upper phase, rich in chlorophyll content, is separated and put in the oven to remove the solvent (L. Wang *et al.*, 2021). The chlorophyll extract was baked at 45℃, referring to research (Puspita *et al.*, 2021) with modifications to keep the chlorophyll content from being degraded.

Parameters

The parameters observed in this study were extraction time (5 - 15 minutes) *(Nazarudin et al.*, 2020), the ratio of *Ulva* sp. to acetone (0.05 – 0.1 g/mL) (Cadar et al., 2022; Nazarudin *et al.*, 2020), and the particle size of *Ulva* sp. (60, 80, and 100 mesh) (Yan *et al.*, 2021). The effect of these parameters was observed on the yield response of chlorophyll extract.

Data Analysis

The optimization and statistical analysis process are carried out with the help of Design Expert 13 software. The method used is the response surface method with Box Behnken Design for three process parameters: extraction time, the ratio of solids to solvent, and the particle size of *Ulva* sp.. A total of 14 experiments will be carried out for process optimization. The effect of extraction parameters on extract yield will be observed using analysis of variance (ANOVA) with Design Expert's application. The extracted yield is a function of the process parameters.

Water Content Analysis

Testing the percentage of water content in the powder sample *Ulva* sp. can be done using the thermogravimetric method, namely the determination of water content using heating in an oven with a temperature range of 105-110℃. This test was observed from the weight of the sample powder produced after heating to show a constant weight. After heating, the powder is weighed when the sample has been cooled in a closed container for ± 30 minutes. Initial weight (A) used *Ulva* sp. powder as much as 1 gram as a sample. While the final weight (B) of *Ulva* sp. when reaches constant. The following is an equation for calculating the percentage of water content (Syahidah et al., 2022):

$$\text{Water Content}(\%) = \frac{(\text{A} - \text{B})}{\text{B}} \times 100 \quad (1)$$

Chlorophyll Analysis

Measurement of chlorophyll in *Ulva* sp. performed using a UV-Vis spectrophotometer. The wavelength of the chlorophyll extract was measured using the Arnon method at 645 nm and 663 nm (Sumiati, 2021). The following equation does calculation of chlorophyll content:

$$\text{Chlorophyll a (mg/L)} = (12.7 \times \text{OD } 663) - (2.69 \times \text{OD } 645) \quad (2)$$

$$\text{Chlorophyll b (mg/L)} = (22.9 \times \text{OD } 645) - (4.68 \times \text{OD } 663) \quad (3)$$

$$\text{Total chlorophyll (mg/L)} = 20.2 \text{ (OD } 645) + 8.02 \text{ (OD } 663) \quad (4)$$

Chlorophyll analysis using the Thin Layer Chromatography (TLC) method was carried out using a 2×8 cm TLC plate. *Ulva* sp. extract results were taken a few milliliters and then spotted on the TLC plate using a capillary tube. Furthermore, the color and density of each spot are recorded and analyzed qualitatively (Aisoi, 2019).

Antioxidant Activity Test

Antioxidant analysis refers to (J. R. Hidayati *et al.*, 2020), 3 mL of chlorophyll extract was added to 1 mL of 0.1 mM DPPH and incubated at room temperature for 30 minutes in the dark. After incubation, a UV-Vis spectrophotometer measured the absorbance at a wavelength of 515 nm. An antioxidant analysis is formulated as follows:

$$\%\text{Inhibition} = \frac{\text{A}_{\text{control}} - \text{A}_{\text{sample}}}{\text{A}_{\text{sample}}} \quad (5)$$

$A_{control}$ is the absorbance of the control (DPPH without sample), and A_{sample} is the absorbance of the test sample (extract sample and DPPH solution). Antioxidant activity can be seen from the percentage of inhibition. The higher the percentage of inhibition indicates the higher the content of antioxidant activity in the sample (Latief *et al*., 2013).

RESULTS AND DISCUSSION

Ultrasound-Assisted Liquid Biphasic System (UALBS) Extraction Method

The process of extracting chlorophyll from *Ulva* sp. has two stages: a solid-liquid extraction stage of extracting the chlorophyll core content from the raw material and a liquid-liquid extraction stage to separate the chlorophyll from xanthophyll in the solvent. Chlorophyll extraction on *Ulva* sp. was performed using the Ultrasound Assisted Extraction (UAE) method with acetone solvent. Research (Sumiati, 2021) showed that chlorophyll extraction using acetone solvents gave higher levels of total chlorophyll than ethanol solvents. This extraction method is assisted by ultrasonic waves, which can cause the formation of bubbles in the solvent resulting in accelerated rupture of the cell wall to release intracellular components in the solvent (L. Wang *et al*., 2021). Adding K_2HPO_4 solution is carried out in the extraction process as a chlorophyll solubility-reducing agent that can create a biphasic condition. Furthermore, the extraction process is carried out in an ultrasonic box under dark room conditions, so the chlorophyll content is not degraded.

The process of separating chlorophyll from the solvent was carried out using liquid-liquid extraction with petroleum ether and distilled water as solvents. The extraction results will separate the acetone solution, which is rich in chlorophyll, into the upper phase and distilled water as the lower phase, as shown in Figure 1 below:

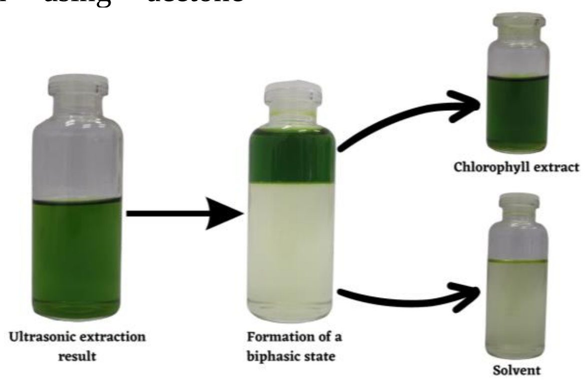


Figure 1. Biphasic condition on extraction

The petroleum ether will rise to bind chlorophyll in the upper phase, and acetone will dissolve in the lower phase along with distilled water. Separation of the two phases occurred with the upper phase containing chlorophyll and the lower phase containing xanthophyll. This separation occurs because chlorophyll has more hydrophobic properties, so it tends to dissolve in non-polar solvents such as petroleum ether (Santos *et al*., 2018). In contrast, less hydrophobic xanthophyll will dissolve in more polar solvents such as acetone and aquadest. The chlorophyll extract that has been separated is then heated in the oven at 45°C to evaporate the solvent to obtain a thick chlorophyll extract.

The results of the extraction process are referred to as yields analyzed and show that the highest yield content was achieved at 1.88%, which was carried out under 10 minutes extraction time, the raw material to solvent ratio of 0.1, and particle size of *Ulva* sp. 60 mesh. Research on chlorophyll extraction on *Ulva* sp. that has been carried out can be compared according to table 3 below:

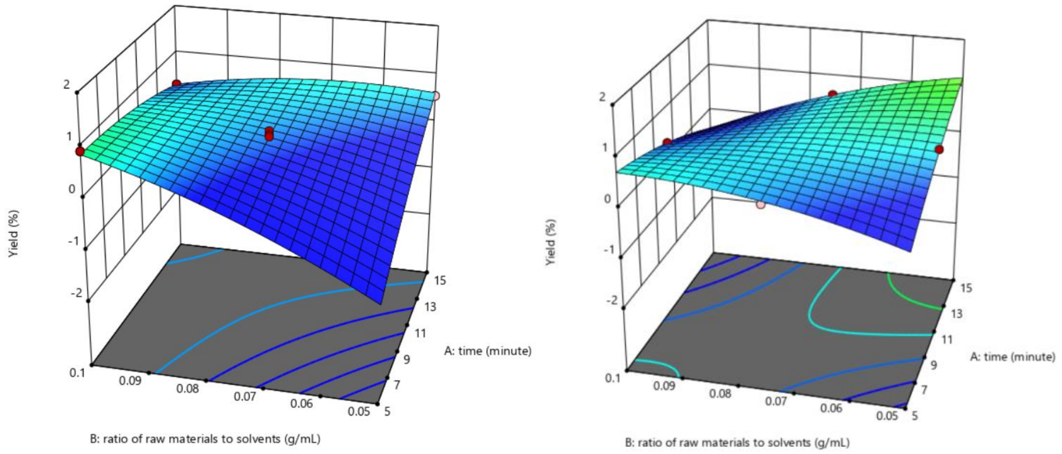
Table 3. Results of chlorophyll extraction from *Ulva* sp. on various sources

Raw Material	Target Compound	Solvent	Method	Operating Conditions	Yield , mg/L	Reference
Ulva	Chlorophyll	96% alcohol	Maceration	The ratio of material to solvent=1:5, t=5 days.	6,79	(Samad *et al*., 2021)
Ulva flexuosa	Chlorophyll a Chlorophyll b	Ethanol 96%	UAE	T=27˚C, t=1 hour,	17.6±0.9 20.1±1.0	(Fabrowska *et al*., 2017)
Ulvalactuca	Chlorophyll a	n-hexane, ethyl acetate, and methanol	Maceration	T=27˚C, t=3x24 hours	9.216±0.103	(J. R. Hidayati *et al*., 2020)
Ulva sp.	Chlorophyll a Chlorophyll b Total Chlorophyll	Acetone	UALBS	The ratio of material to solvent = 1:10, t=10 minutes, particle size 60 mesh.	20.13664 21.58672 41.71012	(This research)

Based on Table 3 shows that the extraction of chlorophyll on *Ulva* sp. the ultrasound-Assisted Liquid Biphasic System (UALBS) method, gives a higher yield and the fastest extraction time compared to other methods. This happens because the effectiveness of ultrasonic waves in extraction is combined with a liquid-liquid separation called biphasic which makes the chlorophyll purification process in solvents faster (Martins *et al*., 2021).

Effect of extraction process parameters on extraction yield

The results of data analysis of the effect of extraction process parameters (time, ratio, and particle size) on the extraction yield are presented in the graph below:



(a) (b)

Figure 2. Relationship between raw material to solvent ratio (g/mL) and extraction time (minutes) to yield (%) at (a) 80 mesh ; (b) 100 mesh

Effect of time parameter on yield

Operation parameters of chlorophyll extraction from *Ulva* sp. were analyzed using the Design Expert 13 application, including time (minutes), the ratio of substance to solvent (g/mL), and particle size of *Ulva* sp.. The graph shows the correlation between time, substance to solvent ratio, and extraction results yield. The extraction conditions were kept constant at the mass of *Ulva* sp. 2.5 grams, 100% amplitude, 0.3 g/mL K_2HPO_4 solution, and 45℃ oven temperature to evaporate the acetone solvent in the extract to obtain a thick chlorophyll extract. The longer the extraction time, the effect of increasing the extraction yield. This is also according to research from (Ananingsih *et al*., 2020; Budiastra et al., 2018) which states that the longer the extraction time, the longer the contact between the powder and the solvent which results in more rupture of the *Ulva* sp. cell wall. Bubbles formed by ultrasonic waves cause this rupture. This is what causes the resulting extraction yield to increase with increasing time.

The effect of the ratio of raw materials to solvents on yield

Based on the graph shows that the greater the ratio of raw materials to solvents used, the effect of increasing the resulting extraction yield. This is consistent with the theory that the intensity of ultrasonic waves imposed on the intracellular tissue of higher materials can cause fragmentation so that the resulting yield increases. This effect is also in line with research conducted by (Buanasari *et al*., 2019; Cadar *et al*., 2022; Fachri *et al*., 2022).

Effect of particle size parameters on yield

Ulva sp. particle size as an extract material has a significant effect on the extraction yield. The higher the particle size, the smaller the size of the *Ulva* sp. powder. Based on the graph, the higher the particle size has a decreasing effect on the yield produced. This decrease in extraction yield is in line with research (Sayoga *et al*., 2020) which states that the extracted chlorophyll yield reaches a maximum at 60 mesh size and decreases at 80 mesh. This decrease is caused by the excessive amount of solids obstructing the flow of the interaction process between solvent and solid (Sayoga *et al*., 2020).

Water Content Analysis

Water content is a parameter used to determine the dryness of *Ulva* sp. powder. to be extracted. Testing the water content using the thermogravimetric method with a temperature range of 105-110˚C until a constant sample mass is obtained (Syahidah *et al*., 2022). Figure 3 shows a graph of the mass of *Ulva* sp powder samples which has reached a constant so that the water content obtained at particle sizes of 60, 80, and 100 mesh, respectively is 14.548%, 15.207%, and 16%. The results of *Ulva* sp.'s water content from testing follow the general average water content used in the extraction process of 15% (Borsali *et al*., 2020).

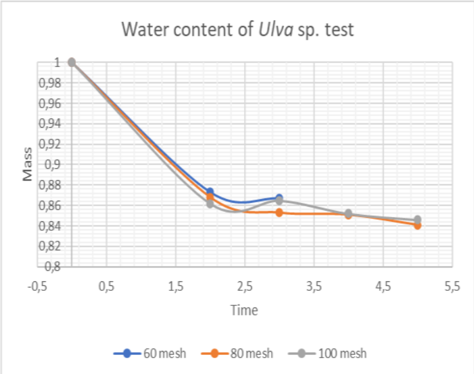


Figure 3. Graph of the mass of the water content test sample

Statistical Analysis

Analysis of Variance (ANOVA) then analyzed total chlorophyll content data to prove that the parameters used in the extraction process can affect the total chlorophyll yield. The results of ANOVA are presented in Table 4 below:

Table 4. Results of ANOVA analysis

Source	p-values	
Model	0.0067	significant
A- Time	0.0047	
B- Ingredient and Solvent Ratio	0.0049	
C- Particle Size Ratio	0.0469	
Lack of Fit	0.3594	Not significant
Pure Error		
Total Cast		

Parameters can be said to be significant if the probability value (p-value) from the results of the analysis is ≤ 0.05 or 5%, and the value of lack of fit is if the p-value is ≥ 0.05 (Sari *et al*., 2020). The resulting p-value is 0.0067, so it can be said that the parameters of extraction time, particle size, and the ratio of *Ulva* sp. with a significant solvent or effect on the response, namely chlorophyll yield (Rohmah *et al*., 2022). In comparison, the p-value for lack of fit is 0.3594 or 35.94% which indicates that it is not significant. Lack of fit is a deviation or discrepancy between experimental data and predictive model data. The p value>0.05 indicates that the model adequately describes the experimental data and there are no deviations from the model (Pertiwi, 2018; Rahmawaty & Sutanto, 2014).

R^2 is the coefficient of determination which shows the influence of variables X_1 and X_2 on variable Y (Khumaedi, 2016). R^2 value from the research results can be stated according to the model if the value exceeds 0.75 or is close to 1 (Fachri *et al*., 2015; Haryani *et al*., 2019; Marjoni *et al*., 2015; Yingngam *et al*., 2020). The ANOVA results yield an R^2 value of 0.9761, indicating that the model follows the research results. The resulting R^2 adjusted value of 0.9222 indicates a strong relationship between the parameters of extraction time, *Ulva* sp. particle size, and *Ulva* sp. ratio with dissolving to yield chlorophyll (Rohmah *et al*., 2022). The regression equation in this study can be written as follows:

$$\text{Yield} = 0{,}43 + 0{,}36A - 0{,}48B - 0{,}18C - 0{,}63AB - 0{,}29AC + 0{,}65BC - 0{,}4C^2 \quad (6)$$

Based on the existing equations, all parameters statistically affect the total yield chlorophyll content. The relationship between experimental data and model data is presented in Figure 4 below:

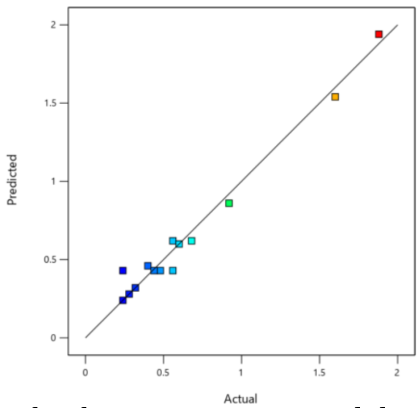


Figure 4. The relationship between experimental data and model data

The picture above shows that the model for the prediction graph of experimental data with model data is very accurate. There is a strong correlation between experimental data and model data. The distance between the data and the trendline shows the accuracy of the data. The closer the data is to the line, the more accurate the data will be (Yılmaz & Tavman, 2017). Based on this research, the actual data is obtained close to the data model, so the data plot will touch the line, as evidenced by the R^2 value of 0.9992.

Extraction yield optimization

Optimization is a search technique for operating conditions based on predetermined parameters and provides profitable results. Optimization was carried out using the Respond Surface Methodology, which obtained the optimum value at 14.23 minutes, the ratio of material to solvent was 0.08, and the particle size of *Ulva* sp., 75.25 mesh produces the most optimal and profitable yield of 0.78% and desirability of 1.000. Desirability is a determinant of the optimum value point, the closer to the value 1, the closer it is to the desired optimum condition point value (B. Hidayati et al., 2020).

Chlorophyll analysis by Thin Layer Chromatography (TLC) method

Pigment analysis using the Thin Layer Chromatography (TLC) method serves as a qualitative test to determine the presence of chlorophyll content in the extract of *Ulva* sp. From the results shown in Figure 5, the dominant color is green. The green color indicates chlorophyll content in the *Ulva* sp. extract (Aisoi, 2019; Pratiwi *et al*., 2015; Rosang & Wagey, 2016). Based on research (Aisoi, 2019), chlorophyll a has a more intense green color, while a bright green color indicates the presence of chlorophyll b with the order chlorophyll an at the top.

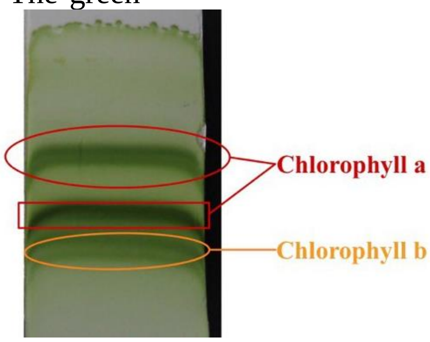


Figure 5. Results of chlorophyll analysis using the Thin Layer Chromatography (TLC) method

Arnon method chlorophyll analysis

This test aims to determine the chlorophyll content in the *Ulva* sp. extract quantitatively. The extraction process was performed at optimum conditions, namely at 10 minutes, the raw material to solvent ratio of 0.1, and a particle size of 60 mesh. The extraction solvent used is acetone, so the appropriate chlorophyll calculation method is the Arnon method. Based on the results of the analysis of chlorophyll content in the *Ulva* sp. using a spectrophotometer, the Optical Density (OD) value is obtained as follows:

Table 5. Absorbance value of chlorophyll extract *Ulva* sp. in acetone solvent

Solvent Type	Optical Density (OD) value	
	645nm	663 nm
Acetone	1.324	1.866

Analysis of the content of chlorophyll a, chlorophyll b, and total chlorophyll was carried out using the Arnon method as shown in the following table:

Table 6. Levels of chlorophyll a, chlorophyll b and total chlorophyll in the extract of *Ulva* sp.

Solvent Type	Chlorophyll Content (mg/L Fresh Weight)		
	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Acetone	20.14	21.50	41.71

Based on research on *Ulva* Lactuca L. chlorophyll extraction, which had been carried out previously using the maceration method by (Negreanu-Pirjol *et al.*, 2020), it produced 16.35 mg/L chlorophyll a and 8.59 mg/L chlorophyll b, (Samad *et al.*, 2021) produced a total chlorophyll of 6.79 mg/L. The results of extraction using UALBS method showed a higher chlorophyll content than the maceration method, so this method proved to be more optimal in the extraction of chlorophyll in *Ulva* sp..

Analysis of Antioxidant Activity

Analysis of antioxidant activity used the DPPH method, which refers to (Tristantini *et al.*, 2016). The percentage of inhibition is a parameter of high or low antioxidant activity in samples with a DPPH control solution. The higher the percentage of inhibition, the higher the antioxidant activity (Latief *et al*., 2013).

Based on the analysis, the inhibition percentage of *Ulva* sp. of 45.32%. The percentage of antioxidant activity of *Ulva* sp. obtained did not differ much from the inhibition percentage in the study (B. Hidayati *et al*., 2020) by 46% and in research (Samad *et al*., 2021) by 51%. The resulting inhibition percentage was even higher than the study (Cadar *et al*., 2022), which had a percentage of 38% with the same species.

CONCLUSION

Based on the research results, it can be concluded that the optimum conditions for chlorophyll extraction from *Ulva* sp. were achieved at an extraction time of 10 minutes, a material to solvent ratio of 0.1 g/mL, and a particle size of 60 mesh, which gave the highest yield value of 1.88%. The higher the yield produced, the more chlorophyll that can be extracted and benefited from it. Modifying the ultrasonic extraction and biphasic separation methods shows the effectiveness of being faster, more efficient, and produce higher yields than conventional methods like macerations. However, high accuracy is required in separating the upper and lower phases in biphasic conditions so that chlorophyll can be extracted optimally. Researchers suggest that further extraction and analysis be carried out regarding the effect of yield on the mass of *Ulva* sp. and more solvent volume.

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