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Journal of Biobased Chemicals

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PREFACE

We would like to present the second volume and edition of our journal, Journal of Biobased Chemicals published by the Department of Chemical Engineering, University of Jember, Indonesia. This pioneer is expected to enhance the findings and research about natural product and their derivatives, mostly in energy, chemicals, and materials. We present articles related to the products, processes, and management of biobased chemicals.

This new journal was envisioned and founded to represent the growing needs of biobased chemicals research as an emerging and increasingly vital field, now widely recognized as an ideal substitution for fossil-based chemicals. The journal has an objective to deliver and provide notable and standardized research and finding through journal reporting. The journal is intended as a window or a library for practitioners and researchers to share their works, identify new issues, and organize further research, while industrial users could apply the invention for scale-up, problem-solving, and application.

Hopefully, this edition would contribute valuable thought for the readers and enhance future research related to biobased chemicals product. Finally, we send gratitude to all participants including authors, reviewers, and editors due for their contribution.

December 2022

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Effect of Time, pH, and Yeast Concentration on Bioethanol Levels in the *Ulva* sp. Fermentation Process

Aina Christalia Rinastiti[a], Dianita Ivana Permata[a] Bekti Palupi[ab*], Zuhriah Mumtazah[a], Meta Fitri Rizkiana[ab], Atiqa Rahmawati[c]

[a] Department of Chemical Engineering, University of Jember, Indonesia 68121
[b] Research Center for Biobased Chemical Product, University of Jember, Indonesia
[c] Department of Leather Engineering and Skin Preservation, Politeknik ATK Yogyakarta, Indonesia

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Abstract. Bioethanol is a form of renewable energy that is used to reduce dependence on the use of fossil fuels which cause various negative impacts on the environment. *Ulva* sp. contains high carbohydrates so it has the potential as a raw material for bioethanol production. This study aims to determine the optimum conditions of the fermentation process with the variables used time, pH, and yeast concentration. This study used the results of hydrolysis of *Ulva* sp. with optimum operating conditions of 0.1 N HCl concentration, 80 mesh particle size, and 450 watt microwave power. Measurement of bioethanol levels was carried out using an alcoholmeter. The results showed that the optimal conditions for fermentation were 7 days of fermentation, pH 5.5, and yeast concentration of 1.5% which resulted in a bioethanol content of 7.55%.

Keywords: *Bioethanol, fermentation, yeast, Ulva sp.*

1. Introduction

Fuel is the energy that is needed by all countries in the world at this time and will increase over time. Meanwhile, oil and natural gas depend on fossil resources whose rate of formation is inversely proportional to the level of consumption. These fossil resources are included in non-renewable resources (non-renewable) if they are taken continuously one day their availability will run out [1]. Oil production in the last six months up to June 2022 shows a figure of 616.6 thousand barrels per day according to the Special Task Force for Upstream Oil and Gas Business Activities (SKK Migas). The use of motorized vehicles continues to increase every year, this is what makes energy scarce. Based on the Indonesian Statistics Center (BPS) in 2021 the use of motorized vehicles in Indonesia will reach around 270,688,529 vehicles. Alternative fuels are needed to be a solution to these problems, namely fuels that can

*Corresponding author: bekti.palupi@unej.ac.id

be renewed so that they can be used optimally. Bioethanol is one of the potential that can be used as an alternative fuel [2]. Indonesia is a tropical country that has abundant biodiversity, agricultural products, and plantation products, so it has great opportunities for the bioethanol development process [3].

Ethanol has a high octane number, high laminar speed, and high heat of vaporization, so ethanol can be used as a transportation fuel [4]. Ethanol can be used as a substitute for transportation fuel, either directly or mixed with gasoline. Gasoline mixed with ethanol up to a maximum of 15% can be burned in a transportation combustion engine [5]. The level of carbon and hydrocarbon emissions in a mixture of gasoline and ethanol is lower than premium and pertamax because ethanol contains 35% oxygen which can increase combustion efficiency in vehicles [6]. Bioethanol with the chemical formula C_2H_5O is a single-chain alcohol with an octane number of 108, is difficult to evaporate, has a low calorie value, and is flammable [7]. Bioethanol production for the first generation generally still uses food as a raw material [8]. Sugar derived from carbohydrates (starch) is fermented with the help of microorganisms to produce bioethanol [9]. These microorganisms are *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli* because they can convert simple sugars into ethanol [10]. Bioethanol is obtained by converting carbohydrates to form glucose using several processes such as acid or enzymatic hydrolysis [11]. Bioethanol can be produced from a variety of natural products, therefore it can be referred to as bioethanol [12]. Residue biomass of aquatic plants can be used as a raw material for making bioethanol, as is the case with the use of Algae *Ulva* sp.

Ulva sp. is a macroalga belonging to the class Chlorophyta. This is because *Ulva lactuca* contains quite a lot of chlorophyll cells that give the algae a green color. Green algae generally store starch as a food reserve [13]. Ulva sp. is a type of macroalgae that is widespread in the sea and Indonesian fresh waters [14]. It generally inhabits rocks on dead coral fragments and varies in shape and size with changes in environmental factors [13]. The content contained in *Ulva* sp. per 100 grams of net weight, namely water 18.7%, protein 15.26%, fat 0.1-0.7%, carbohydrates 46-51%, fiber 2-5%, and ash content 16-23%, and contains vitamins B1, B2, B12, vitamin C, and vitamin E [15]. *Ulva* sp. They range up to 100 cm in length and are bright apple green in color, and have the shape of a folded sword with smooth but wavy edges. The middle of each strand is pale and dark if it gets to the edges [16]. Ulva sp. can survive in the temperature range of 28-31°C [15]. *Ulva* sp. is one of the species of the *Ulvaceae tribe*, systematically classified as *Ulva* sp. can be seen in table 1 as follows [17]

Table 1. Classification of *Ulva lactuca*

Kingdom	***Plantae***
Division	*Chlorophyta*
Class	*Ulvophyceae*
Order	*Ulvales*
Family	*Ulvaceae*
genus	*Ulva*
Species	*Ulva lactuca*

The manufacture of bioethanol goes through several stages, the first of which is hydrolysis which converts polysaccharides into simple sugars and then converts them into ethanol [18]. The hydrolysis process generally uses an enzymatic chemical method with an acid catalyst [19]. The hydrolysis process can use a catalyst in the form of an acid or an enzyme [20]. The acid used is like a strong acid, namely hydrochloric acid (HCl). The effectiveness of the HCl catalyst type was higher in producing glucose at the same temperature, concentration, and time compared to H_2SO_4. This is because the nature of HCl is stronger with higher reactivity compared to H_2SO_4 [21]. The process of hydrolysis of cellulose with acid using the microwave method can convert starch into simple sugars in a relatively short period. Based on this, it can produce higher bioethanol production with lower acid concentrations so that it becomes environmentally friendly, saves costs, and shortens production time [22]. Hydrolysis is a reactant process with water capable of breaking down a compound to form its constituents [23]. The hydrolysis process can use a catalyst in the form of an acid or an enzyme [20]. One of them is hydrochloric acid (HCl) which can be used in the starch hydrolysis process because HCl is very easy to obtain, but hydrochloric acid must go through proper handling because this liquid is corrosive [24]. Although this compound is acidic, it contains chloride ions which are non-toxic and non-reactive. Hydrochloric acid with an intermediate concentration of 30% to 34% is stable enough to be stored and continues to maintain its concentration [25]. Therefore, the hydrolysis process is important in the manufacture of bioethanol, because this process determines the amount of glucose obtained, then fermented into bioethanol. Termination of starch polymer chains to form dextrose or monosaccharide units, namely glucose, is the principle of starch hydrolysis [26]. The following reaction equation for the hydrolysis process is shown in equation 1[27].

$$(C_6H_{10}O_5)n_{(s)} + nH_2O \xrightarrow[HCl_{(l)}]{} nC_6H_{12}O_{6(l)} \quad (1)$$

The second stage is the fermentation process, at this stage, it functions to convert glucose into ethanol and carbon dioxide (CO_2). The fermentation process is carried out by

adding yeast to work at optimal temperatures [18]. Factors that influence the fermentation method are temperature, nutrition, pH, and fermentation time. The optimal temperature for the fermentation process ranges from 27-30ºC and the optimal pH ranges from 4 – 7 [28]. Fermentation using ingredients that have ingredients such as glucose, starch, and fiber can produce liquid bioethanol [29]. The fermentation process is one of the ways to produce alcohol by precipitating a carbohydrate-containing substance in an anaerobic state [30]. Fermentation is a process in which chemical changes occur in an organic substrate from the activity of enzymes produced by microorganisms [31]. Fermentation usually uses microorganisms such as yeast or mold, but it can be done with bacteria and a mixture of various microorganisms [32]. The microorganism commonly used in bioethanol production is *Saccharomyces cerevisiae* because it can easily produce alcohol in large quantities and has a fairly high response to alcohol content [33]. *Saccharomyces cerevisiae* yeast is used to increase the yield of the bioethanol production process because the process does not require sunlight. The alcohol produced from the fermentation process can contain up to 8-10% alcohol content [34]. In the fermentation process, yeast has the role to convert glucose into ethanol and carbon dioxide gas [35]. The fermentation reaction for the formation of alcohol is shown in equation 2 [36]

$$C_6H_{12}O_{6\,(l)} + \textit{Saccharomyces cerevisiae} \longrightarrow C_2H_5OH_{\,(l)} + 2CO_{2\,(g)} \qquad (2)$$

The next process is the distillation process, this stage functions to obtain purer ethanol with the help of a distillation apparatus. Then do a test on the determination of ethanol levels [11]. Measurement of ethanol levels can be done using an alcoholmeter.

In this study, *Ulva* sp. hydrolyzed using hydrochloric acid to form simpler molecules. Then the hydrolysis results are carried out by the ethanol fermentation process with yeast. In several previous studies with various kinds of raw materials, it has been shown that the fermentation time and the concentration of *Saccharomyces cerevisiae* affect the bioethanol fermentation process. The longer the fermentation time, the higher the bioethanol content produced. However, bioethanol levels decreased when they reached the optimal point because the productivity of *Saccharomyces cerevisiae* decreased and nutrients were running out. High levels of bioethanol are also obtained from favorable environmental conditions, one of which is the influence of pH. This study aims to determine the optimum conditions of the fermentation process on *Ulva* sp. with the variables used, namely time, pH, and yeast concentration. References for this study were obtained from several journals which are summarized in Table 2.

Table 2. Research on Bioethanol Production

Raw Materials	Methods and Results	Ref
Ulva reticulata	Fermentation of *Saccharomyces cerevisiae* at pH 4.5 and temperature 30°C for 6 days. The results of the analysis of ethanol content have a purity of 5.02%	[37]
Microalgae *Nannochloropsis sp.*	*Saccharomyces cerevisiae* fermentation at pH 4.5. The best fermentation time to produce ethanol is 72 hours, which is 8.9%	[27]
Tetraselmis chuii Microalgae	*Saccharomyces cerevisiae* fermentation at 30°C for 5 days. The resulting ethanol content of 1%	[38]
Codium geppiorum Algae	Ferment at 27-30°C. The highest ethanol content was in fermentation with 20% yeast concentration for 7 days, namely 3.03%.	[39]
Green Algae *Spirogyra sp.*	*Saccharomyces cerevisiae* fermentation at pH 4.5 and temperature 30°C. The highest yield of ethanol was 0.0613 in 5 days of fermentation with 1% fermipan content	[40]
Elephant Grass (*Pennisetum purpureum* Schumach)	*Saccharomyces cerevisiae* fermentation at pH 4. The highest bioethanol yield was 17.30% with 11% starter content for 6 days	[36]
Microalgae *Chlorella pyrenoidosa*	Fermentation *Saccharomyces cerevisiae* at pH 5 was fermented for 3 days. The highest bioethanol concentration produced was 0.280% with 25% yeast concentration	[41]
Sargassum crassifolium	*Saccharomyces cerevisiae* fermentation at 30°C. The highest yield of bioethanol was obtained at pH 7 which was fermented for 72 hours, namely 67 ml	[1]
Powder Agar *Gracilaria verrucosa*	Fermentation of *Saccharomyces cerevisiae* at pH 7. Optimum ethanol content was achieved at 120 hours of incubation with 0.1M H2SO4 concentration of 0.77%	[42]
Nira Aren	*Saccharomyces cerevisiae* fermentation. The highest ethanol content was 45.70% in 6 days of fermentation using 7.5 ml of starter	[43]

2. Research Methods

2.1 Materials

The materials used in this study included hydrolysis of *Ulva* sp., yeast (*Saccharomyces cerevisiae*), distilled water, HCl, NaOH, and urea.

2.2 Material preparation

Algae *Ulva* sp. washed thoroughly to remove sand and other impurities. Furthermore, the material is dried using an oven, after which the material is mashed with a blender and sieved using an 80 mesh sieve to obtain *Ulva* sp. powder.

2.3 Hydrolysis Process

The hydrolysis process used 0.1N HCl, *Ulva* sp. powder. weighed and added 0.1 N HCl. Furthermore, the hydrolysis process was carried out using a microwave with a power of 450 watts. After the hydrolysis process is complete, the hydrolysate results are cooled in the beaker

glass until it reaches room temperature. The hydrolysate is then filtered using filter paper to separate the filtrate from the residue. The filtrate obtained was then put into a vial for further testing of reducing sugar levels.

2.4 Fermentation Process

The fermentation process uses the help of *Saccharomyces cerevisiae* with varying concentrations of 0.5%; 1%; 1.5% of the total hydrolysate volume, [40] and added 2M NaOH until the pH becomes 4; 5.5; 7 for variations in pH. After that, 1% of the total hydrolysate volume was added as a nutrient for the culture into the fermentation vessel. Fermentation is carried out anaerobically. Tightly cover the fermentation container, then the container is perforated and given a hose which is flowed into a container filled with water and fermented with variations of 3, 5, and 7 days [39] at room temperature (30°C).

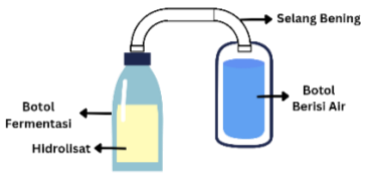

Figure 1. Series of fermentation equipment

2.5 Distillation Process

The results of the fermentation are then filtered so that the filtrate is separated from the residue. After that, the liquid is put into the distillation flask for the distillation process. Then heated at a temperature of 78˚C according to the boiling point of ethanol.

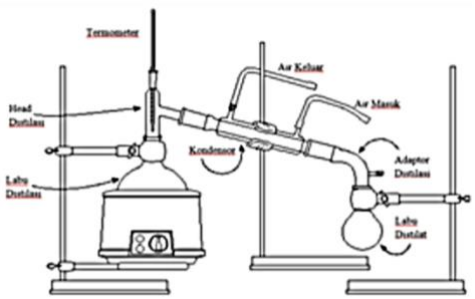

Figure 2. A series of distillation equipment [44]

2.6 Sample Analysis

This study tested the levels of ethanol produced from the process of making bioethanol. After carrying out the distillation process, cool the pure ethanol produced into a beaker glass and then test the levels by inserting an alcoholmeter into the beaker glass. Then let stand for 5-10 minutes and see the scale read on the alcoholmeter.

2.7 Data Analysis

Data analysis used Design Expert software version 13, adapted to the Response Surface Methodology (RSM) approach and the 17 running Box Behnken Design (BBD) model according to Table 3.

Table 3. Variation of *Ulva* sp. fermentation data.

No.	Fermentation time (days)	pH	Yeast Concentration (%)
1	7	4	1
2	3	5.5	1.5
3	3	4	1
4	5	7	1,5
5	7	7	1
6	5	5.5	1
7	5	5.5	1
8	3	5.5	0.5
9	7	5.5	1.5
10	5	5.5	1
11	5	4	0.5
12	3	7	1
13	5	5.5	1
14	5	5.5	1
15	5	4	1.5
16	5	7	0.5
17	7	5.5	0.5

3. Results and Discussion

3.1 Analysis of Bioethanol Content Results

Based on the bioethanol content test measured using an alcoholmeter, the highest bioethanol content was 7.55% with parameters of operating conditions namely 7 days fermentation time, pH 5.5, and yeast concentration 1.5%. While the lowest bioethanol content was 1.50% with parameters of operating conditions namely 3 days fermentation time, pH 4, and 1% yeast concentration.

Table 4. Results of Reducing Sugar Levels

No.	Fermentation time (days)	pH	Yeast Concentration (%)	Bioethanol Content (%)
1.	7	4	1	3.55
2.	3	5.5	1.5	1.55
3.	3	4	1	1.50
4.	5	7	1.5	3.10
5.	7	7	1	5.35
6.	5	5.5	1	4.60
7.	5	5.5	1	4.50
8.	3	5.5	0.5	3.85
9.	7	5.5	1.5	7.55
10.	5	5.5	1	4.10
11.	5	4	0.5	2.20
12.	3	7	1	1.75
13.	5	5.5	1	4.30
14.	5	5.5	1	3.95
15.	5	4	1.5	2.40
16.	5	7	0.5	2.55
17.	7	5.5	0.5	3.70

3.2 *Analysis of Variance* (ANOVA)

Analysis of Variance (ANOVA) is a form of statistical hypothesis testing by drawing conclusions based on inferential statistical data or groups. Significant results can be seen from the p-value (probability value) <0.05. The results of the ANOVA can be seen in table 4, and the results obtained are p-value <0.05. This shows that the variables used in this study affect the levels of bioethanol. Lack of Fit is a deviation from the model. The p-value for Lack of Fit was >0.05 and showed insignificant results, in this study the Lack of Fit value was 0.5177. This shows the suitability of the model. A significant relationship between the variables and the yield of bioethanol content can be seen from the R^2 value. Table 5 shows that the R2 value is 0.9872, which indicates that there is a significant relationship between fermentation time, pH, and yeast concentration on bioethanol levels. In addition, there is a difference between the Predicted R^2 value and the Adjusted R^2 value <0.2 which indicates that the data is reasonable.

Table 5. Results of Analysis of Variance (ANOVA)

Source	*Sum of Squares*	*df*	*Mean square*	*F-value*	*p-value*	
Model	37.87	9	4.21	60.40	< 0.0001	*significant*
A- Fermentation time	16.68	1	16.68	239.38	< 0.0001	
B-pH	1.16	1	1.16	16.69	0.0047	
C- Yeast Concentration	0.6612	1	0.6612	9.49	0.0178	
AB	0.6400	1	0.6400	9.19	0.0191	

Source	*Sum of Squares*	*df*	*Mean square*	*F-value*	*p-value*	
AC	9.46	1	9.46	135.74	< 0.0001	
BC	0.0306	1	0.0306	0.4396	0.5285	
A^2	0.1181	1	0.1181	1.70	0.2340	
B^2	8.64	1	8.64	124.03	< 0.0001	
C^2	0.3664	1	0.3664	5.26	0.0555	
Residual	0.4876	7	0.0697			
Lack of Fit	0.1956	3	0.0652	0.8933	0.5177	*not significant*
Pure Error	0.2920	4	0.0730			
Cor Total	38.35	16				

The suitability between the experimental data and the model can be seen based on the parity plot graph in Figure 3. The straight lines on the graph are the predicted data, while the actual data from each run is shown as dots on the graph. The experimental data values spread around the line indicating that there is a match between the model and the experimental data so that the model is significant.

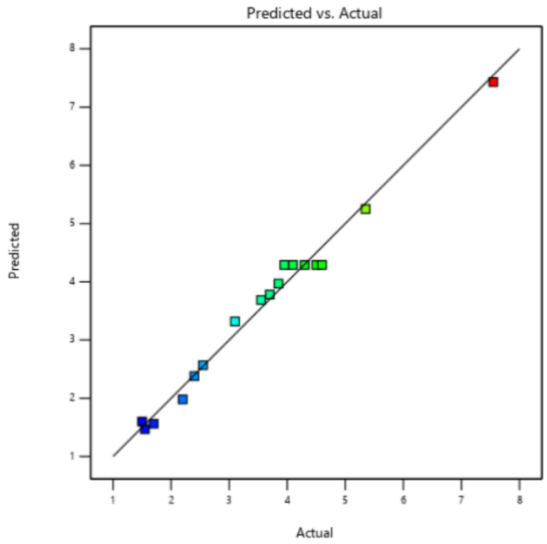

Figure 3. Graph of comparison of model data with experimental data on ANOVA

3.3 Effect of Parameters on Bioethanol Levels

3.3.1 Effect of Fermentation Time and pH on Bioethanol Levels

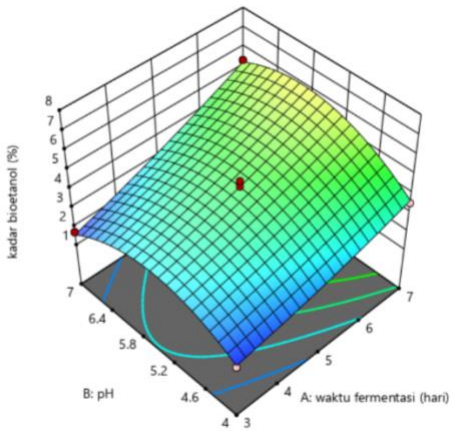

Figure 4. Effect of bioethanol content on fermentation time (days) and pH

Figure 4 is a graph of the effect of the variable fermentation time and pH on the levels of bioethanol produced. The effect of fermentation time on significant bioethanol levels is shown in table 5 that p-value = <0.0001. The effect of pH on bioethanol levels is also significant as shown in Table 5 that p-value = 0.0047. The interaction between fermentation time and pH for bioethanol is significant because p-value = 0.0191 <0.005.

The graph in Figure 4 shows that the longer the fermentation time and the higher the pH, the higher the bioethanol content. High levels of bioethanol can be influenced by favorable environmental conditions, one of which is pH. However, the optimal pH condition is pH 5.5 because *Saccharomyces cerevisiae* has an optimal pH for the growth process, namely pH 4-5 [45]. Enzyme performance in yeast is affected by pH, if the pH is too acidic or alkaline it will disrupt enzyme activity. High pH conditions can cause the value of bioethanol levels to decrease because when the fermentation media conditions lead to a neutral pH *Saccharomyces cerevisiae* enters a stationary phase or is no longer working and is experiencing growth again. During the fermentation process, the pH can decrease in the presence of organic acids produced by microorganisms [46]. This is what causes the condition of pH 4, yeast can not work optimally so the level of bioethanol produced is lower. The length of time of fermentation also affects the levels of bioethanol, because the yeast will continue to reproduce over time in a fermenting solution medium that is capable of converting glucose into bioethanol [36]. It can be seen in Figure 4 that for 3 days, yeast concentration of 1.5% and pH 5.5 produced a bioethanol content of 1.55%. Meanwhile, 7 days of fermentation, yeast concentration of 1.5% and pH 5.5 produced a bioethanol content of 7.55%.

3.3.2 Effect of Fermentation Time and Yeast Concentration on Bioethanol Levels

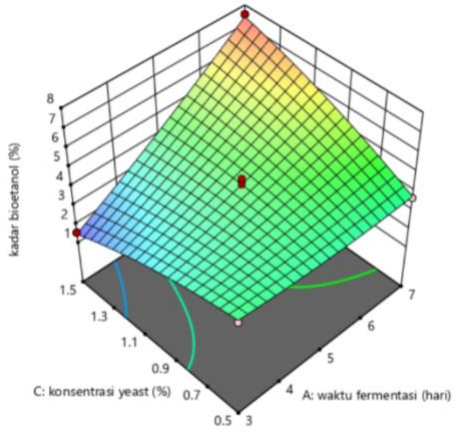

Figure 5. Effect of bioethanol content on fermentation time (days) and yeast concentration (%)

Figure 5 is a graph of the effect of the variable fermentation time and yeast concentration on the levels of bioethanol produced. The effect of fermentation time on significant bioethanol levels is shown in table 5 that p-value = <0.0001. The effect of yeast concentration on bioethanol levels is also significant as shown in table 5 that p-value = 0.0178. The interaction between fermentation time and pH towards bioethanol is significant because p-value = <0.0001.

The bioethanol content with the operating conditions of 7 days fermentation time, pH 5.5 and 1.5% yeast concentration is 7.55%, whereas, in the operating conditions of 3 days fermentation time, pH 5.5 and 0.5% yeast concentration produces bioethanol 3.85%. Figure 5 shows that the bioethanol content increases with the length of time of fermentation and the high concentration of yeast. Yeast can develop and grow in fermented solution media so that it can convert glucose into bioethanol. Thus a high concentration of yeast will increase the level of bioethanol formed in the fermentation process. However, the fermentation time has a maximum limit of 7 days, but the fermentation time that exceeds the maximum number does not affect the increase in bioethanol levels because the yeast undergoes a death phase so that the activity of the yeast in converting glucose to bioethanol decreases [47].

3.3.3 Effect of pH and Yeast Concentration on Bioethanol Levels

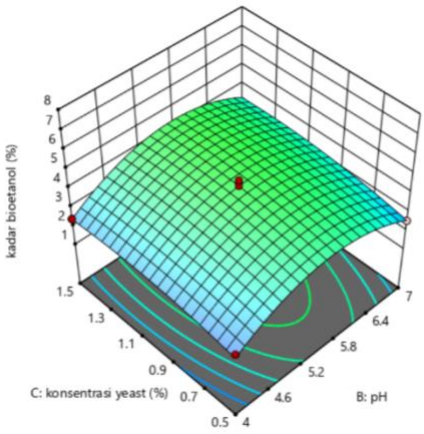

Figure 6. Effect of bioethanol content on pH and yeast concentration (%)

Figure 6 is a graph of the effect of the variable pH and concentration of yeast on the levels of bioethanol produced. The effect of pH on significant bioethanol levels is shown in Table 5 that p-value = 0.0047. The effect of yeast concentration on bioethanol levels is also significant as shown in table 5 that p-value = 0.0178. However, the interaction between fermentation time and pH for bioethanol was not significant because of the p-value = 0.5285 > 0.05.

The bioethanol content at 5 days, pH 5.5, and 1% yeast concentration was 4.6%, while at 5 days, pH 4 and 0.5% yeast concentration was 2.2%. The high concentration of yeast accelerates the occurrence of fermentation so that microorganisms can decompose glucose into ethanol maximally. This is because the higher concentration of yeast will increase the population of microorganisms that work in it so that the level of bioethanol produced is greater [41]. The pH variable in the fermentation process is very important for yeast growth because yeast can only grow at certain pH conditions. Optimal growth of *Saccharomyces cerevisiae* takes place in media with a pH of 4-5. However, the speed of the bioethanol fermentation process will decrease if the pH is below 3. Therefore, the level of bioethanol produced will be low if the pH is below 4 [45].

Table 6. Optimization of Maximum Bioethanol Content Expert Design

Fermentation time (days)	**pH**	**Yeast Concentration (%)**	**Bioethanol Content (%)**	***Desirability***
7	5,5	1,5	7,283	1,000

Table 6 shows the optimal results in response to bioethanol content of 7.283% when the operating conditions were 7 days of fermentation, pH 5.5 and yeast concentration of 1.5%, and the desirability value reached 1.000. The suitability of the model for the optimization value is obtained when the desirability value is close to one.

Table 7. Comparison of bioethanol levels with previous studies

No.	Raw material	Operating Conditions	Bioethanol Content Results	Ref
1.	*Ulva reticulata*	Fermentation time 6 days, pH 4.5	5.02%	[37]
2.	Mikroalga *Chlorella pyrenoidosa*	Fermentation time 3 days, pH 5	0.280%	[27]
3.	Alga Hijau *Spyrogyra* sp	Fermentation time 5 days, pH 4.5	0.281%	[40]
4.	Alga Merah *Gracilaria verrucosa*	Fermentation time 5 days, pH 7	0.77%	[39]
5.	*Ulva* sp.	Fermentation time 7 days, pH 5.5	7.55%	In this research

Table 7 shows the results from previous studies using different operating conditions. From the table above, the lowest bioethanol content resulted from operating conditions, namely 3 days of fermentation with a pH of 0.280%. Meanwhile, the highest bioethanol content resulted from the operating conditions of 6 days of fermentation with a pH of 4.5 of 5.02%. However, judging from the operating conditions in Table 7, it is known that previous studies produced lower levels of bioethanol compared to this study. This can be due to differences in operating conditions used in the fermentation process.

4. Conclusion

This research uses variables that include fermentation time (days), pH, and yeast concentration (%). This variable has a significant effect on the bioethanol content because it has a p-value <0.05. The results of the bioethanol content test are supported by the Analysis of Variance (ANOVA). The results show that the value of R2 is 0.9872. In this study, the bioethanol content was obtained at 7.55% under operating conditions of 7 days of fermentation, pH of 5.5, and yeast concentration of 1.5%.

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RESEARCH ARTICLE

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Extraction Method of Ultrasound-Assisted Extraction (UAE) of Robusta Coffee Skin Waste using 96% Ethanol Solution in Tanah Wulan Village, Maesan District, Bondowoso Regency

Miftakhul Ulum[a], Fitriah Novita Sari[b], Helda Wika Amini[a*], Hanggara Sudrajat[c]

[a] Department of Chemical Engineering, University of Jember, Indonesia
[b] SMA Ra'iyaul Husna Bondowoso, Indonesia
[c] National Research and Innovation Agency (BRIN), Indonesia

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Abstract. Argopuro Mountains, Tanah Wulan Village, Maesan District, Bondowoso Regency, East Java, Indonesia is one of the Robusta coffee-producing areas. Robusta coffee beans that are processed can produce quite a lot of by-products in the form of underutilized waste. It is known that coffee skin waste still has a lot of remaining content and it is possible to take these bioactive compounds using extraction methods. Bioactive compounds in natural materials can be done by extraction. Extraction is the process of separating substances in a sample based on different solubilities. The extraction method used is the ultrasonication method (nonconventional). Extraction of the ultrasonication method only requires a relatively shorter time with the help of an ultrasonicator. There is a lot of content in the coffee skin waste powder that needs further testing. The results of this study are expected to provide economic value to robusta coffee husk waste.

Keywords: *Coffee husk waste powder, ultrasonication*

1. Introduction

Indonesia is one of the largest coffee-producing countries in the world. Indonesia is also in fourth place after Brazil, Vietnam, and Colombia. Coffee production in Bondowoso Regency, East Java Province reached 2,900 tons in 2019 (BPS 2019). In the coffee processing process, coffee husk waste reaches 30-35% and is the largest waste from the coffee fruit processing process. Utilization of coffee husk waste is only used simply, some of which are as animal feed and fertilizer. The use of coffee skin waste needs attention because there is still minimal information about the use and content of coffee skin waste and a lack of attention to the economic value of coffee skin waste (Pujianto, 2007; Juwita, et al., 2017).

Coffee plants produced in Indonesia generally consist of two types of coffee, namely Robusta coffee and arabica coffee. Indonesia has an increase in coffee production from year to

*Corresponding author: heldawikaamini@unej.ac.id

year. All regions of Indonesia are capable of producing coffee, except for the DKI Jakarta area. South Sumatra Province has the largest coffee area, which is 277,542 Ha, and produces coffee production of 140,812 tons per year. Meanwhile, the highest coffee production was produced by Lampung Province, which was 142,599 tons with a land area of 166,058 Ha (Adline et al., 2013). This study uses coffee husk waste from Robusta coffee plantations in the Argopuro Mountains area, Tanah Wulan Village, Maesan District, Bondowoso Regency because of its closer location making it easier for researchers to obtain samples of coffee husk waste.

Coffee husk waste is a by-product that is underutilized to the maximum (Adeline et al., 2013). There is a lot of content left in the coffee husk waste so it is possible to reuse it by extracting the coffee skin waste. Extraction is a process of separating substances in materials based on different solubilities. Two factors can affect the content of bioactive compounds in the resulting extract, namely solvents concentration, and temperature. Based on this, the higher the concentration of the solvent used, the higher its ability to remove bioactive compounds in the material to be extracted. This can facilitate the contact that occurs between the solvent and the extracted material.

Extraction is the process of separating and withdrawing the chemical content contained in a material that is easily soluble so that it is clear if there is an insoluble material using a liquid solvent. The extraction process can be carried out using several methods, one of which is the ultrasonication method (Ministry of Health, 2000). The Ultrasonic-Assisted Extraction (UAE) method is an ultrasonication-assisted extraction method. Ultrasonic-assisted extraction can increase yield, and antioxidant effectiveness and reduce the extraction time of coffee husk waste.

Ultrasonic-assisted extraction (UAE) is one of the extraction methods assisted by ultrasonication. Ultrasonic waves are sound waves that have a frequency above human hearing ($\geq$ 20 kHz). The ultrasonication method is non-destructive and non-invasive so it can be easily adapted to various applications (Rochmani, 2009). With the help of ultrasonication, the process of extracting organic compounds in plants using organic solvents can take place more quickly. The cell wall of the material is broken down by ultrasonication vibrations so that the contents in it can come out properly (Rochman, 2005).

UAE studies are used to produce higher yield values and many extraction activities have been carried out. (Balachdran et al., 2006) performed ultrasonication-assisted extraction which can increase the yield by 30% and reduce the extraction time. The quality of the extract in the extraction process is influenced by the extraction technique, extraction time, temperature, type

of solvent, solvent concentration, and material-solvent ratio. The time or length of the extraction process determines the content of compounds that come out of the material. as well as the ratio of the material-solvent, and the amount of extractant involved in the diffusion process which will affect the content of the compound. Because the extraction process is influenced by various factors, it is necessary to research the extraction of coffee husk waste using the maceration method and the ultrasonication method.

This research was conducted by extracting coffee husk waste using the ultrasonication method. So this research was conducted to determine the value of the percent yield of the ultrasonic extraction method with variations in time and variations in particle size.

2. Materials And Methods

2.1 Materials

The raw material used in this research is robusta coffee husk waste obtained directly from coffee farmers in Tanah Wulan Village, Maesan District, Bondowoso Regency, East Java, Indonesia. The other ingredient is 96% ethanol.

2.2 Simplified Preparation of Coffee Peel Waste

Simplicia dried coffee husk waste was obtained directly from robusta coffee farmers, precisely in the Tanah Wulan area, Bondowoso Regency, East Java Province, Indonesia. Then the simplicia is dried in the sun for approximately 1 day to avoid wet coffee husk waste, after making sure it is completely dry then mashed using a grinder (selep), after the coffee husk waste becomes powder then it is sifted with various sieves, namely by sizes of 60, 80, and 100 mesh because the principle of the maceration and ultrasonication method is to apply the concentration equilibrium principle, namely between the concentration of the solvent and the concentration of the sample cells so that if it reaches a fixed equilibrium (constant) then the extraction process will stop or even decrease. The optimum limit for the particle size of coffee husk waste is below 60 mesh, so variations in particle size of 60, 80, and 100 mesh are used.

2.3 Water Content Analysis

The water content of coffee skin waste was determined using the oven method (Noorhamdani, et al., 2012). A total of 5 grams of the sample was put in a weighing dish and then dried in an oven at 65°C for 3 hours then weighed, put in the oven again for 10 minutes, then weighed again, and repeated twice. Drying was carried out until a constant weight was obtained. The determination of water content is calculated by equation (1)

$$\text{Water Content (\%)} = \frac{\text{initial mass(g)} - \text{final mass (g)}}{\text{initial mass of coffee skin (g)}} \text{x100\%} \quad (1)$$

2.4 Extraction of Coffee Peel Waste

Extraction was carried out by dissolving 50 grams of coffee husk waste in 100 ml of 96% ethanol in a beaker. There are several variations used, namely: (a) Variations in time starting from 0, 5, 15, 30, and 45 minutes. This time variation was chosen to determine the percent yield value without any ultrasonication treatment (0 minutes) and to determine the effect of ultrasonication over the length of time used. (b) Variations in particle size of 60, 80, and 100 mesh as a comparison in the ultrasonication method. The amplitude used is 30 which is set through the generator panel with the converter, probe, and temperature sensor dipped in the solution. The extraction results were filtered and concentrated using an oven at a temperature of 65^{o}C to remove the solvent.

3. Results

3.1 Simplified Preparation of Coffee Peel Waste

Simplicia coffee husk waste was obtained directly from robusta coffee farmers in Tanah Wulan Village, Bondowoso Regency, East Java Province, Indonesia. Simplicia is a natural ingredient that is used and has not undergone any process changes (Ministry of Health, 2019). The simplicia preparation process was carried out at the Basic and Process Laboratory, Faculty of Engineering, University of Jember. Simplicia was obtained in a semi-dry condition. Then the drying treatment was carried out for 8 hours under the hot sun to dry the coffee skin waste. Figure 1 shows the waste of coffee husks when dried.

Figure 1. Coffee Peel Waste When Drying

After the simplicia, the coffee husk waste is dried in the sun and then mashed using a grinder (selep). The coffee husk waste that has been smoothed and turned into powder as shown in Figure 2 is sieved with various sieves, namely 60, 80, and 100 mesh.

Figure 2. Coffee Peel Waste Powder

3.2 Analysis of Water Content Simplicia Coffee Peel Waste

Moisture content is one of the simplicia standardization. The presence of water in the simplicia extract of coffee skin waste will allow the growth of microbes. In addition, the water content also affects the shelf life. Therefore, water content is one important factor that must be considered. Determination of water content was carried out to determine the water content contained in simplicia and coffee skin waste extract, which could affect the growth of microorganisms and adversely affect the content of active compounds during the storage process. To measure the water content, a sample of coffee husk waste was used with variations in the size of a 60 mesh sieve of 5 grams and put in an oven with a temperature of 65ºC for 3 hours. Then the sample was weighed using an analytical balance, in the oven again for 10 minutes as shown in Figure 4.3. This treatment was carried out until the sample mass was constant. If the sample has a constant mass, it indicates that all the water in the sample has evaporated. The final mass of coffee skin waste after being in the oven was 4.53 grams. So the water content can be calculated as follows :

$$\text{Water Content (\%)} = \frac{5{,}00 - 4{,}53\ (\text{g})}{5{,}00\ (\text{g})} \text{x } 100\% = \frac{0{,}47\ (\text{g})}{5{,}00\ (\text{g})} \text{x } 100\% = 9{,}4\%$$

Coffee husk waste samples have a moisture content of 9.4%. The water content value is following the simplicia standard, which is below 10% (Prastuwo et al., 2010).

3.3 Extraction of Coffee Peel Waste

Extraction is a process of withdrawing secondary metabolites with the help of a solvent. Extraction will be faster at high temperatures, but this can result in some components being damaged (Harborne, 2007). The extraction method used in this study is the ultrasonication

method (non-conventional). The ultrasonication method is one of the modern methods that can speed up the extraction process.

3.3.1 Extraction Method Ultrasonic Assisted Extraction (UAE)

The ultrasonication method is a method that uses ultrasonic waves, namely waves with a frequency greater than 16-20 kHz (Suslick, 2008). According to Kuldiloke (2002) one of the benefits of the ultrasonication, extraction method is to speed up the extraction process. This is evidenced by the research of Cameron and Wang (2006) on extraction using ultrasonication can take place more quickly. The cell wall of the material is broken down by ultrasonication vibrations so that the contents in it can come out easily (Mason, 2000). Some of the advantages of using ultrasonication technology in its application (Lida, 2002) are: the ultrasonication process does not require the addition of chemicals and other additives, the process is fast and easy, which means the process does not require high costs, the process does not result in significant changes to the chemical structure, particles, and the compounds used.

3.3.1.1 Particle Size Variations

The results of the variation in particle size of the ultrasonication method are as follows:

Table 1. Results of Particle Size Variations in the Ultrasonic Method

Mass (g)	Particle Variation (mesh)	Volume of Ethanol 96%	Ultrasound time (minutes)	Extracted Mass (g)	% Yield
50	60	100	15	0.75	1.50
50	80	100	15	2.18	4.36
50	100	100	15	2.24	4.48
			Average	1.72	3.44

Table 1 shows the % yield of ultrasonication extraction with variations in particle size in the ultrasonication method with a simplicia mass of 50 g, ethanol volume of 100 mL, and an ultrasonication time of 15 minutes. The average yield of the extraction results was 3.44%. The highest yield was at the particle variation of 100 mesh, which was 4.48%, while the lowest yield was at the particle variation of 60 mesh, which was 1.5%. This shows that the smaller the particle size, the greater the % yield. This happens because in the ultrasonication method, the smaller the particle size can cause the breakdown of cell walls and membranes in the coffee husk waste powder, resulting in many damaged cell walls which can then make it easier for compounds in the material to rise to the surface. So that the finer the coffee skin waste powder used, the greater the percentage of yield produced (Alfian and Susanti., 2012).

3.3.1.2 Time variation

The results of the time variation of the ultrasonication method are as follows:

Table 2. Results of Time Variations of Ultrasonic Method

Mass (g)	Particle Variation (mesh)	Volume of Ethanol 96%	Ultrasound time (minutes)	Extracted Mass (g)	% Yield
50	60	100	0	0.27	0.54
50	60	100	5	0.48	0.96
50	60	100	15	0.75	1.50
50	60	100	30	1.07	2.14
50	60	100	1545	1.40	2.80
			Average	0.79	1.58

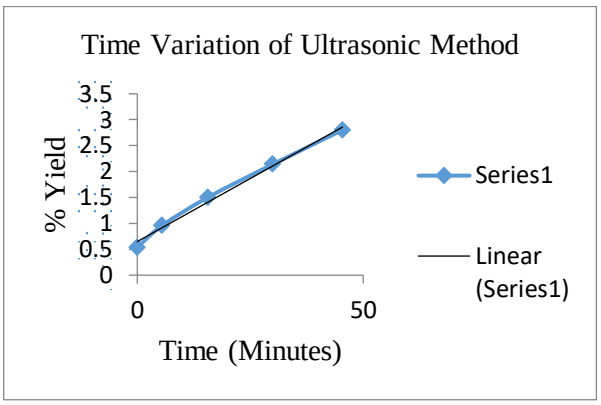

Figure 3. Time Variation of Ultrasonic Method

Table 2 shows the % yield of ultrasonication extraction with variations in the size of the ultrasonication time with a mass of 50 g simplicia, 100 mL ethanol volume, and a particle size of 60 mesh. The average yield of the extraction results was 1.58%. The percentage yield without ultrasonication treatment (0 minutes) showed the lowest yield, which was 0.54%. Figure 3 shows that the longer ultrasonication time (0-45 minutes) results in an increase in the yield of coffee husk waste extraction obtained. The highest yield occurred in the 45 minute ultrasonication time variation, which was 2.80%. This happens because the longer the ultrasonication time causes the longer the contact between the solid and the solvent (solvent) so that it increases the number of broken cells. So the longer the extraction time used, the greater the percentage of yield obtained (Agoes, 2007).

4. Conclusion

The percentage of the average yield of the extraction method of ultrasonication of particle size variation is 3.44%. The percentage yield of the ultrasonication extraction with a variation of the average time of the extraction yield is 1.58%. The finer the coffee husk waste

powder used, the greater the percentage of yield produced, and the longer the extraction time used, the greater the percentage of yield obtained.

5. Suggestions

Suggestions for this research are to do further research on the Test antioxidant robusta coffee husk waste.

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Effect of Ethanol Solvents in the Extraction Process of Bioactive Compounds from Brown Seaweed (*Sargassum* sp.) with the Ultrasound Assisted Extraction Method

Slamet Pujianto[1*], Boy Arief Fachri[1], Erna Subroto[2], and Istiqomah Rahmawati[1]

[1]Department of Chemical Engineering, University of Jember, Indonesia 68121
[2]Crop Protection Pte, Ltd, Singapore

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Abstract. *Sargassum* sp., part of brown algae, is traditionally used as animal feed. Nonetheless, *Sargassum* contains phenolic compounds that promise to be the raw material of natural antioxidants. The work is to extract phenolic compounds from *Sargassum* sp. in ethanol and to investigate the effect of the process on phenol yields. Several dried *Sargassum* sp. are milled in a certain size and extraction using ultrasound assisted extraction with ethanol solvent. Process variables are the extraction time (30-50 minutes) and the Ratio of *Sargassum* sp. to ethanol which is 5:100-15:100 (b/v), and power of 170-190 watts. Phenol compounds are quantified using the error acid method. The DPPH method is performed to check the antioxidant activity. To investigate the influence of the process, surface response methods based on central composite designs are applied in this work. 153.334 mgGAE/g in 30-minute extraction conditions, 170 watts of power, and a ratio between masses and solvents of 0.05. The antioxidant activity (IC_{50}) of *Sargassum* sp. extract is 87.57 ppm.

Keywords: *Sargassum sp., phenolic compound, ethanol, ultrasound assisted extraction*

1. Introduction

Indonesia is a country rich in natural wealth that has the potential to be developed. The natural wealth in Indonesia is seaweed. Seaweed production in Indonesia in 2018 reached 10.18 million tons. Production in 2020 the Ministry of Marine Affairs and Fisheries (KKP) targets seaweed production can reach 10.99 million tons and is projected by 2024 seaweed production to reach 12.33 million tons. The utilization of brown algae has not been developed optimally by the community.

Brown seaweed contains many chemical compounds such as carbohydrates 54.3-73.8%, vitamins (vitamins B1, B2, B6, B16, C, and niacin), protein 0.3-5.9%, minerals especially calcium, sodium, magnesium, potassium, iodine, iron, and contain bioactive compounds namely phenolic compounds, natural pigments, fibers, polysaccharides sulfate, and other

*Corresponding author: slametpujianto373@gmail.com

bioactive compounds [1]. Extracts from brown seaweed contain phenols including eckol, phlorotannin, gallic acid, catechins, epicatechin, and phlorotannin [2]. Brown seaweed has bioactive compounds such as polyphenolics, terpenoids, polysaccharides, carotenoids, phenolic acids, chlorophyll, steroids, and glycolipids [3]. Seaweed extraction results show phytochemical components in the form of alkaloid compounds, flavonoids, phenols, and tannins [4].

The biological activity of seaweed bioactive compounds is antioxidant, anticancer, antitumor, anti-inflammatory, antihypertensive, anti-obesity, antidiabetic, antibacterial, antifungal, antiviral, antiallergic (ovalbumin and shrimp), hypocholesterolemia, neuroprotective, skin lightening and intracellular ROS protection [3]. In addition to containing many bioactive substances, seaweed also has antioxidants. One application of antioxidant activity in seaweed is as a sunscreen [5]. Antioxidants are compounds that can neutralize free radicals to inhibit oxidation [6]. Free radicals will unwittingly form continuously in the human body in the form of normal cell metabolic processes, malnutrition, and such as the result of the response to the influence that exists outside the body [7].

2. Materials and Methods

2.1 Materials

The ingredients used are brown seaweed (*Sargassum* sp.), aquades, ethanol p.a (Smartlab), Folin-Ciocalteu reagent, standard antioxidant (gallic acid), and DPPH solution (1,1-diphenyl-2-picrylhydrazyl). Branson sonifer 250, Hitachi CR 21 GIII High-Speed Refrigerated Centrifuge, and Rotary Evaporator Steroglas Strike 300.

2.2 Methods

The research stages consisted of preparation, extraction, and total phenolic and antioxidant tests. The variables used are the ratio, time, and size of the particles.

2.2.1 Preparation

The material used in the study is brown seaweed (*Sargassum* sp.) from Gedong Tataan, Pesawaran Lampung Regency. Brown seaweed is dried aerated and not exposed to sunlight for 3 days, then mashed using a blender, and then scrambled according to the specified size.

2.2.2 Extraction

Extraction of bioactive compounds on brown seaweed (*Sargassum* sp.) is carried out using ultrasonic extraction methods with ethanol solvents. Experimental design is used in research conducted to facilitate the research process. Based on the experimental design that has

been designed there are 20 treatments for extraction using ultrasonic methods. The variables used are the ratio between samples: solvents are 5:100 to 15:100 (b/v). Then the power used is 170-190 at room temperature. The time used is for 30 to 50 minutes.

2.2.3 Identification of Total Phenols

The standard error acid manufacturing process used in the total test of brown seaweed extraction phenol (*Sargassum* sp.) is a solution of gallic acid stock containing 1000 µg/mL in ethanol. Then the stock solution is diluted to get a working solution with a rate of 10 µg / mL. Then a series of standard solutions are made with levels: 10, 20, 40, 60, 80, 100, and 120 µg/mL. The total phenol test process from the extraction of brown seaweed (Sargassum Sp.) is to dissolve 500 µL residue in 5 mL of 50% ethanol and put it into a test tube. Then added 0.5 ml of Folin-Ciocalteau solution that has been diluted 10 times and left to mix for 2 minutes and then added 2 mL sodium carbonate solution 7.5%. Next, aquades are added up to a volume of 10 mL and incubated at 45ºC for 30 minutes at room temperature. It then measured the uptake at a wavelength of 765 nm.

3. Result and Discussion

To find out the effect of process treatment on *Sargassum* sp. extract, 20 experiments were conducted using the experimental design. The variables used are the ratio (0.05-0.15), the particle size of 80 mesh, the power used are 170-190 watts and the time of 30-50 minutes. The results are presented in Table 1. In experiments, the *Sargassum* extract that had the highest total phenol was about 153.334 mgGAE/g under 30-minute extraction conditions, 170 watts of power, and a ratio between mass and solvent of 0.05.

Table 1. Overview of experiments for extraction of *Sargassum* sp.

Run	Ratio (g/mL)	Power (Watt)	Time (Menit)	Total Phenol (mg GAE/g)
1	0.10	180	23.18	69.026
2	0.15	170	30	78.633
3	0.10	180	40	70.214
4	0.10	180	40	61.493
5	0.15	190	50	122.086
6	0.10	180	40	79.643
7	0.10	180	40	70.962
8	0.01	180	40	142.147
9	0.10	180	40	80.322
10	0.05	190	30	71.453
11	0.05	170	30	153.334
12	0.10	180	40	75.334
13	0.18	180	40	76.028
14	0.10	180	56.81	74.274
15	0.15	190	30	49.247
16	0.05	190	50	95.234

Run	Ratio (g/mL)	Power (Watt)	Time (Menit)	Total Phenol (mg GAE/g)
17	0.10	163.18	40	66.026
18	0.10	196.81	40	83.275
19	0.15	170	50	45.725
20	0.05	170	50	110. 892

The value of R^2 (coefficient of determination) of 0.9344 shows data supporting the model of 93.44%. Model variance analysis is given in Table 2.

Table 2. Results of Variety Analysis (ANOVA) for Total Phenolic Response

Source	F-value	P-value	Description
Model	15.82	< 0.0001	*significant*
A-Ratio	45.10	< 0.0001	
B-Power	0.3413	0.5720	
C-Time	0.6793	0.4290	
AB	24.46	0.0004	
AC	4.34	0.0639	
BC	37.48	0.0001	
A^2	27.88	0.0004	
B^2	0.3882	0.5472	
C^2	0.0472	0.8324	
Residual			
Lack of Fit	2.98	0.1281	*not significant*

Total phenolics are indicators of the efficiency of the brown seaweed extraction process (*Sargassum* sp.). The effect of ratio (g/mL) and power (watt) to total phenolics is expressed in the graph model presented in figure 1.

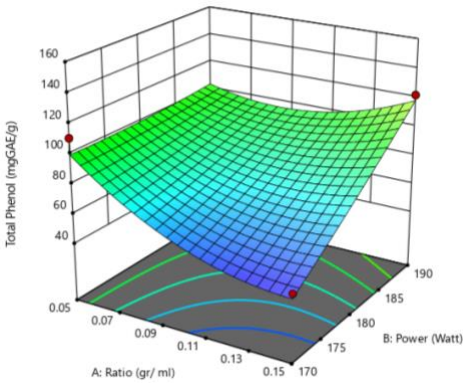

Figure 1. Surface model graphic to total phenolic ratio (gr/ml) to power (watt) at the constant time (50 minutes)

From figure 1 it is seen that the graph model's acquisition of the total phenolic expressed in mg gallic acid equivalent (GAE) per gram of brown seaweed (*Sargassum* sp.) is seen to be affected by the ratio between weight per solvent volume and the power used at a constant time. The greater the ratio and power used at a constant time of 50 minutes can be seen the total acquisition of phenolics will also be greater. However, there is an optimum point that slows the

total value of phenolics to decrease. So that if it has reached the optimum value or power above 170 watts will cause the total value of phenol to decrease.

The effect ratio (g/ml) and time (minutes) to the total phenolic are expressed in the graph model presented in figure 2.

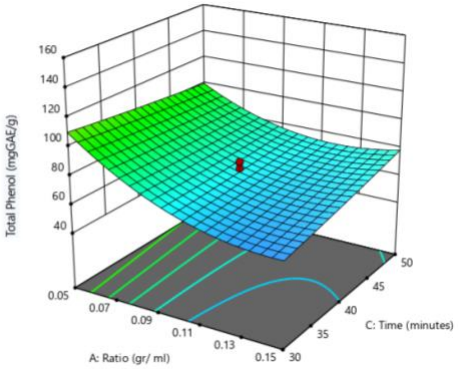

Figure 2. The surface model graph to the total phenolic ratio (gr/ml) with time at constant power (180 watts)

From figure 2 it is seen that the graph model's acquisition of the total phenolic expressed in mg gallic acid equivalent (GAE) per gram of brown seaweed (*Sargassum* sp.) is seen to be affected by the ratio between weight per solvent volume and time used at constant power. This is supported by his research [8] that the longer the time, the more bioactive compounds are obtained but, there is an optimum point that increases the total value of phenolics to decrease. So that if it has reached an optimum value above 30 minutes will result in the total value of phenols decreased.

The effect of power (watt) and time (minutes) on the total phenolic are expressed in the graph model presented in figure 3

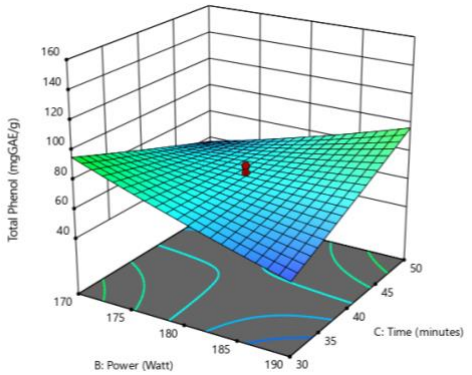

Figure 3. The surface model graph to total phenolic between power (watt) and time (minutes) at a ratio (0.1)

From figure 3 it is seen that the greater the ratio and power used can be seen the total acquisition of phenolics will also be greater as well. However, there is an optimum point that slows the total value of phenolics to decrease. So that if it has reached the optimum value or power above 180 watts will result in the total value of the phenol decreased. In addition, the longer the extraction time, the more levels of extracted compounds.

To check the antioxidant activity of the extracted *Sargassum* sp., a DPPH test was carried out. The results imply that fucoxanthin extracted from *Sargassum* sp. has radical cleaning activity. This is indicated by the IC_{50} value. IC_{50} value (50% inhibitory concentration) indicates the concentration of the sample needed to find 50% of DPPH free radicals. IC_{50} value is inversely proportional to antioxidant activity. The IC_{50} value calculated in this work was 87.57 ppm.

4. Conclusions

The effect of variables on the total phenolic is the greater the ratio of weight: volume, power, and time used then the total phenolic obtained will be greater but, there is an optimum point on the power that causes the total value of phenolics to decrease. The total phenolic test results found that the highest value was located at A11 which was 153.334 mgGAE /g in the operating conditions of 30 minutes, power of 170 watts, and the ratio between mass and solvent of 0.05. The results of antioxidant activity testing obtained an IC_{50} value of 87.57 ppm.

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Microwave Assisted Hydrolysis Ulva sp. Using HCl for the Production of Bioethanol Raw Materials

Bekti Palupi[ab*], Nadia Ayumna Fa'iqoh[a], Alifia Rahma Putri Neysella[a], Boy Arief Fachri[ab], Ditta Kharisma Yolanda Putri[a] , Lukman Nulhakim[c], Maulida Septiyana[d]

[a] Department of Chemical Engineering, University of Jember, Indonesia 68121
[b] Research Center for Biobased Chemical Product, University of Jember, Indonesia
[c] Department of Chemical Engineering, Industrial Technology Faculty, Universitas Jayabaya, Indonesia
[d] School of chemistry, the University of Edinburgh, United Kingdom

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Abstract. The challenges faced by the Indonesian state are increasing greenhouse gases, climate change, and depleting fossil fuel reserves. This requires the exploration of alternative energy that is environmentally friendly and sustainable. Algae biomass, especially *Ulva* sp. is one of the resources that have the potential for bioethanol production as an alternative energy producer. The purpose of this study is to determine the potential of *Ulva* sp. as raw material for bioethanol and to determine the effect of particle size, solvent concentration, and power on the hydrolysis process. This study used *Ulva* sp. as raw material. hydrolyzed with HCl solvent using the microwave assisted hydrolysis method. Hydrolysis with HCl concentrations of 0.1 N, 1 N, 2 N, variations of microwave power 150 watts, 300 watts, 450 watts, and particle sizes of 60 mesh, 80 mesh, and 100 mesh. Measurement of reducing sugar levels was carried out using the dinitro salicylic acid (DNS) method. The results showed that the best conditions for hydrolysis were when the HCl concentration was 0.1 N, the microwave power was 450 watts, and the particle size was 80 mesh which resulted in a reducing sugar content of 20.751 mg/mL.

Keywords: *Bioethanol, reducing sugar, hydrolysis, microwave assisted hydrolysis, Ulva sp.*

1. Introduction

Energy needs increase every year along with the increasing population and economy of a country, including Indonesia. This also has an impact on increasing the use of transportation and industrial activities which results in the use of fuel oil (BBM) increasing. The world's energy needs still rely on fossil energy [1]. Meanwhile, non-renewable energy supplies, such as coal and oil, are running low. Therefore the need for alternative energy that can replace the availability of non-renewable energy that is environmentally friendly and renewable. The National Energy Policy issued by the government in Presidential Decree No. 5 of 2006 states

*Corresponding author: bekti.palupi@unej.ac.id

that the target for the use of biofuels in 2010 is 2% and in 2025 it is 5% [2]. Bioethanol is an alternative that can replace fuel oil derived from natural resources. Bioethanol is obtained from cellulose, starch, and simple sugars [3]. Bioethanol is more widely used as an alternative energy due to its environmentally friendly use [4], [5].

Bioethanol with the molecular formula C_2H_5OH can be produced using biomass containing starch, cellulose, and sugar [6]. Generally, the main processes in the conversion of biomass to ethanol consist of two processes, namely hydrolysis, and fermentation. Hydrolysis aims to break down polysaccharides into monosaccharides [7]. Polysaccharides become ethanol through chemical and biological processes. One of the potential biomass that can be utilized as bioethanol is seaweed. The development of marine resources owned by Indonesia has great and promising potential. The development of Indonesia's marine cultivation land is 80,929 ha with a beach area of 46,734,300 tons/year [8]. However, only about a quarter of seaweed cultivation can be used as industrial raw materials and processed products. Seaweed cultivation is influenced by chemical factors, namely the quality of the water at the location of the aquaculture waters [9]. Seaweed contains essential amino acids, crude fiber, unsaturated fats (omega 3 and omega 6), polysaccharides (non-starch), minerals, and vitamins [10], [11]. Seaweed cultivation as an alternative energy is the latest innovation and must be supported. Data from the Inha University of Korea states that 58700 L of biodiesel can be produced in one hectare of seaweed with an estimated oil content of 30% [12]. The content of macroalgae consists of 60% carbohydrates, 10-47% protein, 1-3% lipids, and 7-38% other minerals [13]–[16]. This causes macroalgae to be considered as a future method to become an alternative to sustainable biomass production. The high carbohydrate fraction includes a wide variety of soluble polysaccharides, such as laminarian and alginate in the brown type, starch and sulfate gelectan in the red type, and *Ulva* in the green type [17].

Ulva sp. is a type of green macroalgae that has the potential as a raw material for biomass [18]. This is due to its fast growth rate and adaptability to a variety of habitats with different abiotic conditions. Carbohydrates in *Ulva* sp. consists of C5 and C6 monosaccharides, iduronic acid, and glucuronic acid [19]. Ulva sp. carbohydrates. mostly in the form of complex hydrocolloid *Ulva* and with cellulose which is a structural component of the cell wall and starch as a place of intracellular energy storage [20], [21]. Acid hydrolysis is often used to hydrolyze cellulose [22], [23]. Acid hydrolysis is divided into two types, namely hydrolysis of dilute acids and concentrated acids. The temperature used for the hydrolysis of concentrated acids is lower than that of dilute acids. This can minimize the degradation of sugar. Acid functions as a catalyst

which can accelerate the rate of the hydrolysis process [24], [25]. The resulting sugar conversion will be high when using concentrated acids up to 90% [26]. The use of acid in the hydrolysis process can produce a greater yield compared to hydrolysis with enzymes [27]. Acid hydrolysis has advantages, including the yield of more ethanol compared to using enzymatic hydrolysis. The hydrolysis process is also faster and more on the random breaking of glycosidic bonds [28]. The effectiveness of acid hydrolysis can be increased by using microwave irradiation [29], [30].

Alkaline and acid treatment at 100 –120ºC can only digest hemicellulose, so cellulose must be hydrolyzed further with the addition of acid so that it can be converted into fermentable sugar. However, it cannot effectively hydrolyze some types of algae due to different cell wall compositions [31]. A study conducted by Rabelo, et al [32] showed that the maximum release of glucose reached 68.2% after being hydrolyzed using Chlorococcum humicola with 20 mg of cellulose at 40ºC for 72 hours. Enzymatic hydrolysis shows a suitable potential in microalgae biomass. However, the disadvantages of enzymatic hydrolysis such as long processing time and high costs indicate the need for more processing and treatment to produce optimal bioethanol [33]. Lignin binding compounds that protect cellulose make cellulose difficult to hydrolyze [34]. Pre-treatment aims to break down and reduce the amount of lignin and hemicellulose, increase the porosity of the material, and destroy the crystal structure contained in cellulose. Several methods in the pre-treatment process, including chemically by adding acids or bases and enzymatically [35]. Enzymatic pretreatment has higher complexity and is more expensive than acid pretreatment [36]. Hydrolysis which is commonly used on the type of seaweed *Ulva* sp. for bioethanol is enzymatic polysaccharide hydrolysis and fermentation with the help of microorganisms (*Saccharomyces cerevisiae*) [37].

Table 1. Hydrolysis of Ulva that has been carried out

Material	Standard Method and Results	Ref
Ulva reticulata	Acid hydrolysis without delignification using H_2SO_4 2% (v/v) at 75-150°C for 30 minutes (microwave), obtained a reducing sugar content of 23.7 g/L	[27]
Ulva reticulata	Acid hydrolysis by delignification using H_2SO_4 2% (v/v) at 75-150°C for 30 minutes (microwave), obtained a reducing sugar content of 27.3 g/L	[27]
Ulva lactuca	Hydrothermal hydrolysis at 121°C for 30 minutes using an autoclave, obtained a reducing sugar content of 2.24%	[38]
Ulva reticulata	Acid hydrolysis using 2% H_2SO_4 for 30-50 minutes (microwave irradiation), obtained reducing sugar levels of 16.41-27.97 g/L	[39]
Ulva reticulata	Acid hydrolysis using 2% H_2SO_4 with a temperature variation of 75-150°C (microwave irradiation), obtained reducing sugar content of 5.80-27.30 g/L	[39]

Material	Standard Method and Results	Ref
Ulva lactuca	Hydrothermal hydrolysis at 180°C for 60 minutes, obtained a reducing sugar content of 7.83 mg/g	[40]
Ulva prolifera	Thermal acid hydrolysis with 0.9M H_2SO_4 using a temperature of 121°C for 50 minutes, obtained a reducing sugar content of 11.07%	[41]
Ulva lactuca	Acid hydrolysis with 1N H_2SO_4 using a temperature of 121℃ for 30 minutes, obtained a reducing sugar content of 158 mg/g	[42]
Ulva lactuca	Acid hydrolysis with 1% HCL using a temperature of 121℃ for 60 minutes (autoclave), obtained a reducing sugar content of 13.17%	[43]
Ulva sp.	Hydrolysis using polyoxometalate (POM) with microwave irradiation for 4-10 minutes at 140℃, obtained reducing sugar levels of 349-435 mg/g	[44]

This research was conducted to determine the potency of *Ulva* sp. as raw material for bioethanol and to determine the effect of particle size, solvent concentration, and power on the hydrolysis process. The use of hydrochloric acid in the process of making bioethanol because hydrochloric acid produces glucose higher than sulfuric acid. The variables used in this study were particle size, solvent concentration, and microwave power.

2. Materials and Methods

2.1 Materials

The materials in this study were *Ulva* sp., hydrochloric acid (HCl), aquadest, glucose, 2M NaOH, potassium sodium tartrate, and dinitro salicylic acid (DNS).

2.2 Material preparation

Ulva sp. washed to remove sand and other impurities. Then the material is dried using an oven with a temperature of 70℃ for 3 hours. Physical pretreatment was carried out by pulverizing the material using a blender, which was then sieved using 60 mesh, 80 mesh, and 100 mesh sieves to produce *Ulva* sp. powder. Then an analysis of the water content was carried out. The purpose of the water content analysis is to determine the water content in the sample. This is because water is very influential on metabolic activity both enzymatic, microbial, and chemical activity. The principle of water content analysis is heating and weighing using an oven at a temperature of 105℃ until a constant weight is obtained. The temperature used is above the boiling point of water 100℃ for maximum evaporation of water [45]. This research resulted in a water content of 8.1%.

2.3 Hydrolysis Process

Ulva sp powder. Weigh 3 grams using an analytical balance. Then it was put into an erlenmeyer plus 100 mL HCl with various concentrations, namely 0.1 N, 1 N, and 2 N. The hydrolysis process took place with several microwave powers of 150 watts, 300 watts, and

450 watts. The optimum time for the hydrolysis process is 15 minutes [46]. Furthermore, the cooling process in the hydrolyzed solution until it reaches room temperature. The resulting hydrolysis solution is filtered using filter paper to separate the filtrate from the residue. Then the next process is the analysis of reducing sugars.

2.4 Sample Analysis

Reducing sugar levels can be analyzed using the dinitro salicylic acid or 3,5-dinitro salicylic acid (DNS) method using a spectrophotometer [47]. DNS reagent was prepared by dissolving 1 gram of DNS powder, 20 mL of 2M NaOH, and 30 grams of Ka-Na tartrate until the volume reached 100 mL. Ka-Na tartrate was dissolved separately using sufficient distilled water, then 1 gram of DNS powder was dissolved using 20 mL of 2 M NaOH. After the powder dissolves, the two solutions are homogenized using a stirrer. The solution is stored in the refrigerator solution.

Preparation of the glucose standard curve begins with the preparation of a 1000 ppm glucose mother liquor made by dissolving 100 mg of anhydrous glucose in 100 mL of distilled water. The standard glucose solutions were made with concentrations of 50 ppm, 200 ppm, 400 ppm, 600 ppm, 800 ppm, and 1000 ppm. Each standard glucose solution was taken 1 mL and filled in each test tube. Then 1 mL of Reagent DNS and 1 mL of distilled water were added. The solution was then homogenized and heated with a water bath for 5 minutes. After that, the solution was cooled to room temperature and added distilled water to a final volume of 5 mL and homogenized again. The adsorption value was measured using a spectrophotometer with a wavelength of 540 nm. The standard curve obtained will be used to determine the concentration of the sample.

Reducing sugar was tested by taking 1 mL of the sample and putting it into a test tube, then adding 1 mL of DNS reagent and 2 mL of distilled water. Additions were made to each test tube using a pipette. The filled test tube was heated using a water bath for 5 minutes. The purpose of heating is for a reaction to occur between glucose and DNS. After that, the solution was cooled to room temperature, then added distilled water to a final volume of 5 mL and homogenized again. Next, the absorbance of each solution was measured at a wavelength of 540 nm. With a wavelength of 540 nm, the dinitro salicylic acid compound is able to absorb these waves strongly [48]. The measured values are then plotted on a standard curve. The reduced sugar content is obtained from the standard curve equation where y is the absorbance value and x is the reduced sugar content [49].

2.5 Data Analysis

Data analysis was used using Design Expert version 13 software with the Box-Behnken Design (BBD) Surface Response Method (BBD) 17 times as shown in table 2. The choice of method was based on the variables used, namely three variables, which were considered more effective compared to other methods [50], [51].

Table 2. *Hydrolysis Run Order*

No.	Particle Size (mesh)	Concentration of HCl (N)	Microwave Power (*watt*)
1.	80	0.1	450
2.	100	2	300
3.	60	2	300
4.	60	1	450
5.	100	0.1	300
6.	100	1	450
7.	80	2	150
8.	60	1	150
9.	80	1	300
10.	80	2	450
11.	60	0.1	300
12.	80	1	300
13.	80	0.1	150
14.	100	1	150
15.	80	1	300
16	80	1	300
17.	80	1	300

3. Results and Discussion

3.1 Standard Glucose Curve

The glucose standard curve gives results as shown in Figure 1.

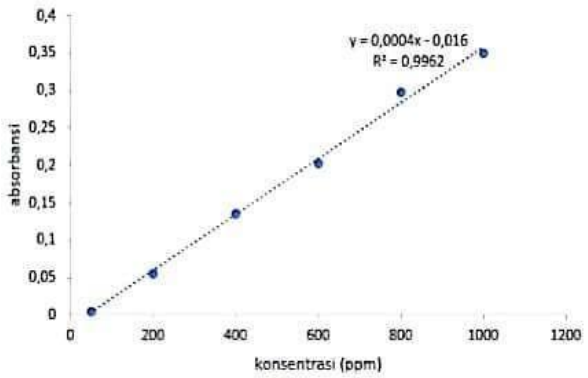


Figure 1. Glucose Standard Curve

Glucose standard curve results with $R^2 = 0.9962$ and linear equations $y = 0.0004x – 0.016$ (1) for determining the concentration of glucose as the determination of reducing sugars.

3.2 Quantitative Analysis of Reducing Sugar Content

Based on the reducing sugar test on the *Ulva* sp. measured using a UV-Vis spectrophotometer, the highest reducing sugar content was 20.751 mg/mL with operating conditions parameters, namely particle size of 80 mesh, HCl concentration of 0.1 N, and microwave power of 450 watts. The results of a study conducted by Dave, et al [41] yielded a reducing sugar content of 1.61 mg/mL when hydrolyzed using H_2SO_4 at 121°C where these results were lower than this study. Differences in results may occur due to differences in operating conditions.

Table 3. Results of Reducing Sugar Levels

No.	Particle Size (mesh)	Concentration of HCl (N)	Microwave Power (watt)	Reducing Sugar Levels (mg/mL)
1.	80	0.1	450	20.751
2.	100	2	300	11.083
3.	60	2	300	5.672
4.	60	1	450	7.584
5.	100	0.1	300	16.172
6.	100	1	450	11.253
7.	80	2	150	7.252
8.	60	1	150	3.021
9.	80	1	300	5.674
10.	80	2	450	8.333
11.	60	0.1	300	14.751
12.	80	1	300	6.012
13.	80	0.1	150	9.924
14.	100	1	150	4.331
15.	80	1	300	6.252
16.	80	1	300	6.083
17.	80	1	300	5.834

3.1 *Analysis of Variance* (ANOVA)

Analysis of Variance (ANOVA) is a form of statistical hypothesis testing in which conclusions are based on inferential statistical data or groups. Significant results can be seen from the p-value (probability value) <0.05. The results of the ANOVA can be seen in table 4. In the table, the p-value obtained is <0.0001. This shows that the variables used in this study affect the levels of reducing sugars. Lack of Fit is a deviation from the model. The p-value and lack of fit which were > 0.05 showed insignificant results and in this study the lack of fit value was 0.1230 where the results indicated suitability for the model. A significant relationship between the variable and the yield of reducing sugar can be seen from the R^2

value obtained of 0.9979. In table 4 you can see the difference between the predicted R^2 value and the adjusted R^2 value <0.2 which shows reasonable data.

Table 4. Results of Analysis of Variance (ANOVA)

Source	*Sum of Squares*	*df*	*Mean square*	*F-value*	*p-value*	
Model	352.11	9	39.12	366.94	< 0.0001	*significant*
A- Particle Size	17.49	1	17.49	164.07	< 0.0001	
B- Concentration of HCl	107.02	1	107.02	1003.73	< 0.0001	
C- Microwave Power	68.50	1	68.50	642.50	< 0.0001	
AB	3.98	1	3.98	37.33	0.0005	
AC	1.37	1	1.37	12.84	0.0089	
BC	23.77	1	23.77	222.90	< 0.0001	
A^2	0.9085	1	0.9085	8.52	0.0224	
B^2	126.77	1	126.77	1188.95	< 0.0001	
C^2	0.0505	1	0.0505	0.4735	0.5135	
Residual	0.7463	7	0.1066			
Lack of Fit	0.5454	3	0.1818	3.65	0.1230	*not significant*
Pure Error	0.2009	4	0.0502			
Cor Total	352.86	16				

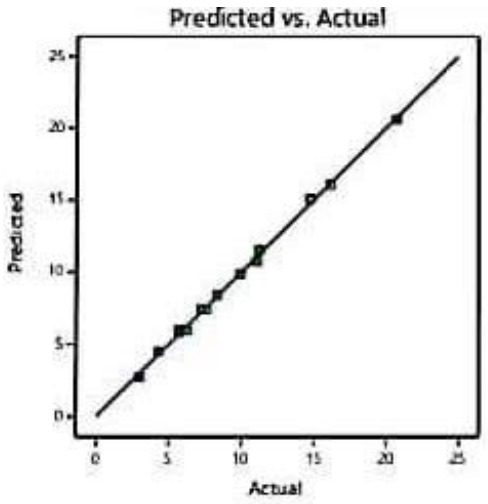


Figure 2. Graph of comparison of model data with experimental data

The fit between the experimental data and the model can be seen based on the porosity plot graph in Figure 2. The response of reducing sugar levels will increase in direct

proportion to particle size, microwave power, the interaction between particle size and HCl concentration, and the interaction between particle size and microwave power. This is indicated by a positive constant value. The response of reducing sugar levels will decrease along with decreasing HCl concentration, the interaction between HCl concentration and microwave power. This is indicated by a negative constant value

$$Y = 5.97 + 1.48A - 3.66B + 2.93C + 0.9975AB + 0.5850AC - 2.44BC + 0.46456A^2 + 5.49B^2 + 0.1095C^2 \quad (1)$$

where,

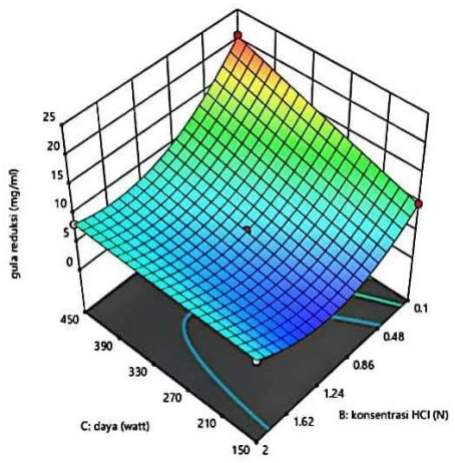
Y = Reducing sugar content (mg/mL)

A = Particle Size (mesh)

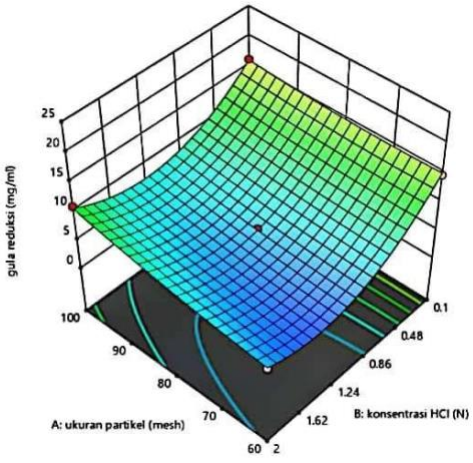
B = Concentration of HCl (N)

C = Microwave Power (W)

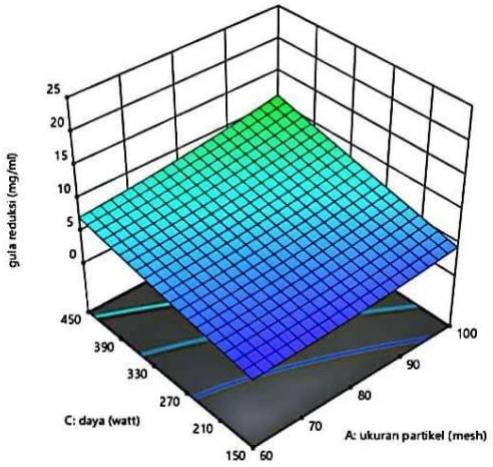
3.2 Effect of Parameters on Reducing Sugar Levels



(a)



(b)



(c)

Figure 3. Variable relationship to reducing sugar content between (a) microwave power (watts) and HCl concentration (N); (b) particle size (mesh) and concentration of HCl (N); (c) particle size (mesh) and microwave power (watts)

Figure 3 is a graph of each variable (microwave power, concentration, and particle size) on the resulting reducing sugar content. The reducing sugar content is affected by the HCl concentration as shown in Figure 3(a) and Figure 3(b) which shows that at a concentration of 0.1 N, 450 watt microwave power, and 80 mesh particle size, a reducing sugar content of 20.75 mg/mL. Meanwhile, at a concentration of 2 N, microwave power of 450 watts, and a particle size of 80 mesh, a reducing sugar content of 8.33 mg/mL was obtained. This is because the use of acids with low concentrations can reduce the decomposition of glucose by acids, while the use of acids with high concentrations can accelerate hydrolysis, but the resulting reducing sugars will be less [28].

Figure 3(a) shows that a concentration of 0.1 N can produce the maximum reducing sugar from Ulva sp. N, and 2 N which showed an increase in reducing sugar levels as the concentration of HCl decreased. This decrease is due to an increase in acid concentration in the hydrolysis process so that glucose is degraded into other compounds [28].

Figures 3(a) and 3(c) show that the higher the microwave power used, the higher the reduced sugar content obtained. The effect of power on the process of hydrolyzing carbohydrates is that the higher the conversion obtained will be higher, but if the power is too high the conversion obtained will decrease [52]. The microwave power used affects the reducing sugar content where there is a significant release of organic matter in the range of 360 watts to 630 watts as research has been conducted by Kumar, et al [46].

Figures 3(b) and 3(c) show that at a particle size of 100 mesh, a concentration of 0.1, and a microwave power of 300 watts, a reducing sugar content of 16.17 mg/mL was obtained.

Meanwhile, at a particle size of 60 mesh, a concentration of 0.1, and a microwave power of 300 watts, a reducing sugar content of 14.75 mg/mL was obtained. The finer the particle size of the raw material, the greater the ethanol content produced, which is also directly proportional to the reduced sugar content produced [53]. This is also because the smaller the particle size, the greater the surface area in contact with the HCl solution so the greater the reducing sugar produced [54].

Table 5 shows the optimal results for a reducing sugar content response of 20.584 mg/mL when the particle size is 80 mesh, the HCl concentration is 0.1 N, the microwave power is 450 watts and the desirability value is 1,000. The suitability of the model for the optimization value is obtained when the desirability value is close to one [55].

Table 5. Optimization of Maximum Reducing Sugar Content Expert Design

Particle Size (mesh)	Concentration of HCl (N)	Microwave Power (watt)	Reducing Sugar Yield (mg/mL)	*Desirability*
80.00	0.100	450.00	20.584	1.000

3.3 Comparison of Reducing Sugar Levels with Previous Research

Table 6. Comparison of Reducing Sugar Levels with Previous Research

No.	Research Title	Method	Reducing Sugar Yield	Ref
1.	*Ulva reticula*	Microwave irradiation	23.7 mg/mL	[27]
2.	*Ulva* sp.	Autoclave, H_2SO_4	12 mg/mL	[57]
3.	*Ulva prolifera*	Enzymatic	420 mg/mL	[58]
4.	*Ulva* sp.	Microwave, HCl	20.75 mg/mL	

Table 6 shows the results from previous *Ulva* studies using different methods. From the table above the lowest reducing sugar content resulted from the autoclave method of 12 mg/mL. Meanwhile, the highest reducing sugar content resulted from the enzymatic method of 420 mg/mL. However, the enzymatic method has several drawbacks including being more expensive, the process taking longer, and being complicated.

4. Conclusion

Research variables which include particle size (mesh), HCl concentration (N), and microwave power (watts) have a significant effect on reducing sugar levels. Testing for reducing sugar content was supported by the Analysis of Variance (ANOVA) test which obtained an R^2 value of 0.9979. This research produced the highest reducing sugar content, namely 20.751 mg/mL with the conditional parameters of 80 mesh particle size, 0.1 N HCl concentration, and 450 watt microwave power.

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Effect of Ethanol Solution Concentration in the Extraction Process of *Centella asiatica* L. Bioactive Components Using Microwave-Assisted Extraction (MAE) Method

Fira Ulvatur Rohmah[a], Rina Auliyah Safitri[b], Ayu Ulum Rahmawati[c], Atiqa Rahmawati[d*]

[a] MAN 3 Jembrana, Indonesia
[b] PT. Vemci Kosmetik Banyuwangi, Indonesia
[c] PT Mitra Alam Segar Pasuruan, Indonesia
[d] Department of Leather Engineering and Skin Preservation, Politeknik ATK Yogyakarta, Indonesia

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Abstract. The Pegagan plant has a scientific name in the form of *Centella asiatica* L. which is included in the Centella genus, Apiaceae family, and kingdom Plantae. Pegagan (*Centella asiatica* L.) has distinctive bioactive components, namely triterpene ester glycoside compounds in the form of asiaticoside and madecassoside, and triterpene group compounds in the form of Asiatic acid and madecassic acid. In this study, the extraction of bioactive components from pegagan was carried out using the Microwave-Assisted Extraction (MAE) method and will study the effect of ethanol solvent concentration in the extraction of bioactive compounds. Analysis of the extracted bioactive content was carried out by analyzing the total phenol content using the Folin-Ciocalteau reagent and ANOVA analysis. The results obtained from the study were in the form of total phenol content as an indication of the presence of bioactive compounds, namely at operating conditions of 450 watts of power, 50% ethanol concentration with a radiation time of 15 minutes which resulted in a total phenol content of 21.9244 mg AGE/g sample. In the ANOVA analysis with ethanol solvent, variables that gave a significant response to the total phenol content were microwave power, radiation time, and ethanol concentration with an R-square value of 95.31%. The effect of ethanol concentration on the total phenol content produced, namely the concentration of pure ethanol solvent will produce extracts with the smallest total phenol content, the effect of extraction time on total phenol content, namely the longer extraction time will increase the total phenol content. Maximum total phenol content using ethanol solvent that is, at operating conditions of 450 watts of power, 10% ethanol concentration with a radiation time of 15 minutes which resulted in a total phenol content of 520 mg AGE/g sample.

Keywords: *Microwave-assisted extraction (MAE), pegagan, ethanol, ANOVA, Folin-Ciocalteu.*

*Corresponding author: tiqa054@gmail.com

1. Introduction

Pegagan (*Centella asiatica* L.) is a plant that has been used as a traditional medicine in the form of fresh, dry, and powdered ingredients. Pegagan is a wild plant originating from tropical Asia and spreading throughout Southeast Asia, including Indonesia, India, the Republic of China, Japan, and Australia. Pegagan (*Centella asiatica* L.) contains bioactive substances such as asiaticoside and madecassoside, triterpene ester glycosides, and triterpene group compounds such as Asiatic acid and made basic acid (Sutardi, 2016). Extracts for the extraction of bioactive compounds can be carried out using conventional extraction methods such as maceration, and non-conventional extractions which include Soxhlet extraction, enzyme-assisted extraction, subcritical water, ultrasound, supercritical fluid extraction (SFE), and Microwave-assisted Extraction (MAE). The microwave-assisted extraction method is an extraction method using microwave radiation to heat the solvent used. Microwave-assisted extraction is a method of extracting materials and liquids that uses microwave radiation in the heating process. Microwave-assisted extraction (MAE) is an extraction technique to extract bioactive compounds from plants with solvents that are safe for humans (Rinawati *et al*., 2020).

The pegagan plant has been used for hundreds of years, especially in the dermatology and cosmetology industries. The use of pagan plant extracts can be used for wound healing, skincare that is getting dull and reduces signs of premature aging accelerates the growth of collagen in the skin so that it can repair and regenerate skin when it is damaged by acne(Budi & Rahmawati, 2019). Ethnopharmacology is a branch of medical science that studies the use of plants as drugs or components in traditional medicine by people from generation to generation. The components of the pegagan plant (*Centella asiatica* L.) which are useful for traditional medicine according to research (Dharmono, 2007) include all parts of the plant from roots, stems, leaves, flowers, and fruit.

According to ethnoeconomic studies, pegagan has no significant economic value because it is not traded. This is because this plant is quite common and is only classified as a wild plant. However, the pegagan plant still has important economic value for the Indonesian people because of the availability of the pegagan plant as a medicine for diseases such as coughs and wounds. If people suffer from this disease, they do not have to spend a lot of money to get treatment. Gotu kola is used by the community as a source of animal feed other than as herbal plants. In terms of ecology, pegagan is a component that has a vital function in the ecosystem where this plant grows as one of the producers of oxygen needed by living things (Dharmono, 2007).

This research focuses on the extraction of bioactive components from pegagan (*Centella asiatica* L.) using the Microwave-Assisted Extraction (MAE) method to determine the effect of the concentration of ethanol solvent in the extraction process. This study aims to determine the effect of ethanol concentration on the extraction process using the Microwave-assisted Extraction (MAE) technique. The research findings are intended to provide insight into the relevance of the solvents used in the extraction process and allow for the development of extraction methods that produce the highest-yielding bioactive compounds while maintaining optimal operating conditions.

2. Materials and Methods

2.1 Materials

Pegagan leaf powder (*Centella asiatica* L.) with a size of 40 mesh, Na_2CO_3 (Sodium Carbonate), 96% Technical Ethanol, Folin-Ciocalteu Reagent, Gallic Acid, Aquades.

2.2 Methods

The research procedure was carried out in 3 stages, including (1) sample preparation, (2) extraction using microwaves assisted extraction (MAE), and (3) analysis of research results. The drying process is carried out under the sun for 20 hours by using a dry indicator in the form of easily breaking dry pegagan leaves. Then weigh the raw materials are mashed with a particle size of 40 mesh with a mass of 1 gram. The extraction process was carried out with several variables including a solvent concentration of 10%, 50%, and 90%, 150 watts of microwave power, 300 watts, and 450 watts, and extraction time of 5 minutes, 10 minutes, 15 minutes. The results of the extraction process were stored in a vial measuring 8 ml at a temperature of 40ºC. Then, the extraction results were analyzed using total phenol analysis.

2.2.1 Preparation of Gallic Acid Solution

Weigh 0.01 grams of gallic acid, then add 1 ml of ethanol and add distilled water until the volume becomes 100 ml.

2.2.2 Determination of Maximum Length

Take 1 ml of gallic acid mother liquor with a concentration of 100 ppm, put it in a test tube, and add 1 ml of Folin reagent, shake the two liquid mixtures until they are homogeneous, and let stand at room temperature for 4-8 minutes. Add 4 mL of 10% Na_2CO_3 solution into a test tube, shake until homogeneous, and let stand for 15 minutes. After that, a Vis-spectrophotometer with a wavelength range of 700-800 nm was used to examine the solution.

2.2.3 Create a Gallic Acid Calibration Curve

100 ppm gallic acid mother liquor, taken 1, 3, 5, and 7 ml. Then the solution was diluted using distilled water to a final volume of 10 ml, resulting in a solution with a concentration of 10, 30, 50, and 70 ppm. Then 0.2 ml of each solution was taken and put into a test tube and added 1 ml of Folin-Ciocalteu reagent was then shaken until the mixture of the two solutions is homogeneous, then allowed to stand at room temperature for 8 minutes. Then 3 ml of 10% Na_2CO_3 was added, shaken until homogeneous, and allowed to stand for 30 minutes at room temperature. The maximum wavelength absorption has been determined. Then it is used to create a calibration curve using the regression equation y = ax+b.

2.2.4 Determination of Total Phenol Content

Take 0.1 ml of extract, add 9.9 ml of distilled water (dilution 100 times), and add 1 ml of Folin-Ciocalteu reagent, then shake until the solution is homogeneous and let stand for 8 minutes. Then, 3 mL of 10% Na_2CO_3 was added to the mixture, shaken until the solution was homogeneous, then allowed to stand for 1 hour at room temperature. Measure the absorption using a vis-spectrophotometer using the maximum wavelength. The content analysis repeats 3 times so that the phenol content obtained was as mg gallic acid equivalent/gram of fresh sample.

3. Result and Discussions

3.1 Result

The research was carried out from November 2020 until December 2020 at the Basic Chemistry Laboratory and Bioprocess Laboratory, Engineering/Chemical Engineering Study Program, Department of Mechanical Engineering, Faculty of Technic, University of Jember. This study used the extracted pegagan plant to extract bioactive compounds or components using the microwave-assisted extraction method. The results of the measurement of the total phenol content of the pegagan extract can be seen in Table 1.

Table 1. Total Phenol Content of Pegagan

No.	Power (Watt)	Solvent Contentration (%)	Time (minute)	Average Absorbance	Total Phenol (mg AGE/g sample)
1.	150	10	5	0.4475	298.8461538
2.	150	10	10	0.5450	423.8461538
3.	150	10	15	0.5235	396.2820513
4.	150	50	5	0.5710	457.1794872
5.	150	50	10	0.5980	491.7948718
6.	150	50	15	0.5900	481.5384615
7.	150	90	5	0.4570	311.025641

No.	Power (Watt)	Solvent Contentration (%)	Time (minute)	Average Absorbance	Total Phenol (mg AGE/g sample)
8.	150	90	10	0.5075	375.7692308
9.	150	90	15	0.5210	393.0769231
10.	300	10	5	0.5545	436.025641
11.	300	10	10	0.5625	446.2820513
12.	300	10	15	0.5845	474.4871795
13.	300	50	5	0.6055	501.4102564
14.	300	50	10	0.5975	491.1538462
15.	300	50	15	0.6140	512.3076923
16.	300	90	5	0.5015	368.0769231
17.	300	90	10	0.5180	389.2307692
18.	300	90	15	0.5675	452.6923077
19.	450	10	5	0.5530	434.1025641
20.	450	10	10	0.5800	468.7179487
21.	450	10	15	0.6200	520
22.	450	50	5	0.6055	501.4102564
23.	450	50	10	0.5920	484.1025641
24.	450	50	15	0.5540	435.3846154
25.	450	90	5	0.5220	394.3589744
26.	450	90	10	0.5775	465.5128205
27.	450	90	15	0.5935	486.025641

3.2 Discussions

3.2.1 Maximum Wavelength Measurement Result

The maximum wavelength measurement was carried out using a vis-spectrophotometer. The purpose of determining the maximum wavelength is to determine the area of absorption in the mother liquor that has the highest absorbance. The steps taken in determining the wavelength are inserting the distilled water into a vis spectrophotometer using a cuvette to ensure the cleanliness of the equipment to be used. The next step is to set the wavelength range used around 400-800 nm. This is by following the research conducted by (Sukmawati *et al.*, 2018), with a maximum wavelength in the range of 400-800 nm for analysis using a Vis Spectrophotometer and states that the 400-800 nm range obtains the ideal wavelength results in determining the presence of bioactive compounds in herbal medicinal plants. The next step is to determine the sample content by inserting a cuvette containing a sample solution into the Vis Spectrophotometer, and the wavelength with the highest absorbance is 765 nm.

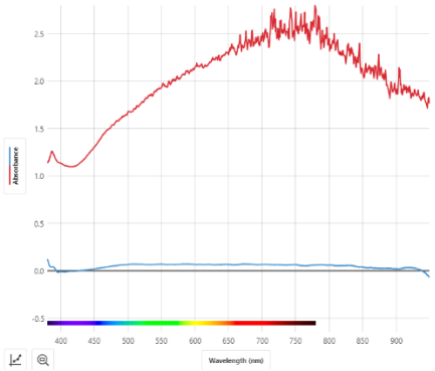

Figure 1. Maximum Wavelength Measurement

3.2.2 Gallic Acid Standard Curve

Preparation of a standard curve for gallic acid with several concentrations of gallic acid, namely 10, 30, 50, and 70 ppm. The result of measuring the maximum wavelength is 765 nm. Measurement of the absorbance of standard solutions of gallic acid from several concentrations was measured based on the maximum wavelength obtained. Based on the absorbance measurements made, a calibration curve was obtained which stated the relationship between gallic acid concentration and absorbance expressed by a linear line.

The requirements for the accepted analytical method for the correlation coefficient (r) obtained from the range 0.996 ± 1 are used to determine the total phenolic content of the pegagan extract (Tahir *et al*., 2017). Based on this, the linear regression equation y = 0.0078x + 0.2144 with a correlation coefficient of R^2 is 0.9949. The correlation coefficient results obtained are by following the acceptance requirements, namely 0.996 so that it can be used to determine the total phenol content.

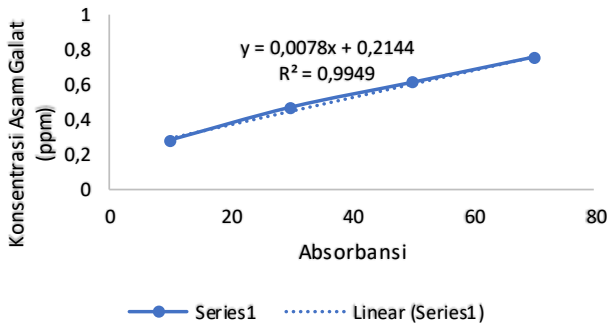

Figure 2. Gallic Acid Standart Curve

3.2.3 Effect of Variables (Power, Solvent Concentration, and Extraction Time) on Total Phenol Content

Based on the results of the study, the effect of the variables on the total phenol content can be seen in figures 3, 4, and 5. Based on Figure 3, it can be seen that at 5 minutes of extraction time with 10% and 50% ethanol concentrations the total phenol content increased, while at 90% ethanol concentration the total phenol content decreased significantly. At 150 watts the highest concentration of total phenol content was at a concentration of 50% for 10 minutes resulting in a total phenol content of 481.5384615 mg AGE/g sample, while the lowest total phenol content was at a concentration of 10% with an extraction time of 5 minutes of 298.8461538 mg AGE/g sample. In general, the concentration of ethanol solvent and extraction time affect the total phenol content, with the result that at 150 watts the total phenol content increased and decreased from the solvent concentration variable at the time of extraction.

In Figure 4 it can be seen that the graph of the total phenol content increased and decreased from the solvent concentration variable at several extraction times with a power of 300 watts. In the extraction time variable of 5 minutes, the ethanol concentration of 10% and 50% of the total phenol content increased, while the ethanol concentration of 90% of the total phenol content decreased significantly. At 300 watts the highest concentration of total phenol content was at a solvent concentration of 50% with an extraction time of 15 minutes which resulted in a total phenol content of 512.3076923 mg AGE/g sample, while the lowest total phenol content was at a concentration of 90% with an extraction time of 5 minutes of 368.0769231 mg AGE/g sample.

Based on Figure 5, it is known that the graph has a trend of increasing and decreasing the total phenol content of the variable concentration of ethanol solvent at several extraction times with a power of 450 watts. At the 5-minute extraction time variable, the ethanol concentration of 10% and 50% of the total phenol content increased, while at the 90% ethanol concentration, the total phenol content decreased significantly. At 450 watts the highest concentration of total phenol content was at 10% ethanol concentration with an extraction time of 15 minutes which resulted in a total phenol content of 520 mg AGE/g sample, while the lowest total phenol content was at a concentration of 90% with an extraction time of 5 minutes of 394.3589744 mg AGE/g sample.

The extraction process runs optimally when the polarity of the solvent is compatible with the substance to be dissolved. The yield of the extract produced during the extraction process will be higher if the polarity of the solvent is compatible with the substance. The effect

of ethanol concentration on the total phenol content produced, namely the concentration of pure ethanol solvent will produce extracts with the smallest total phenol content, this is because polyphenol extraction is very dependent on the polarity of the solvent, and using pure solvents is not effective for the separation of polyphenols whose ingredients come from plants. This is following the research conducted by (Kristanti *et al*., 2019)), which states that the increase in the concentration of ethanol solvent is inversely proportional to the total phenol content obtained. The ethanol solvent in the extraction process acts as a solute with the plant matrix. This shows that the combination of water and ethanol solvents works well for extracting bioactive chemicals from plant sources (Gunathilake *et al*., 2019). The total phenol content in this study increased when a solvent with a concentration of 10% and 50% was used, but the total phenol content decreased when a solvent with a concentration of 90% ethanol was used. The thing that needs to be known during the extraction process is that the higher the concentration of ethanol used, the higher the yield of the extract. Increasing the solvent concentration will affect the polarity of the solvent so that it can increase the solvent's ability to extract fewer polar compounds. Problems that arise when extracting a less polar solvent can cause cell walls that have the same properties to be degraded automatically so that the content of bioactive compounds from herbal plants is easier to extract. However, the use of fewer polar solvents causes a reduction in the polar compounds that can be extracted. This is following the research, so based on the results obtained, the highest yield was obtained at the time of extraction using ethanol with a concentration of 10%.

At 150 W and 300 W microwave power for 5 minutes to 10 minutes, the effect of extraction time on total phenol content resulted in findings with a tendency to increase total phenol content. According to (Kristanti *et al*., 2019), the yield of bioactive components extracted within 5 to 10 minutes experienced a significant increase while the yield obtained for more than 10 minutes was not significantly different and tended to remain constant. Based on this statement, it can be seen that the extraction time has a significant impact on the yield, and the extraction time that is too long or too short can change the physical and chemical characteristics of the extracted material. When the extraction period is too short, the solubility of phenolic compounds is less than optimal, and the material is not extracted completely, but when the extraction time is too long the phenolic compounds will be destroyed automatically.

At 450 W microwave power for 15 minutes, the effect of extraction time on the total phenol content increased. The addition of time in the extraction process can increase the penetration of the solvent into the substance, making it easier for the solvent to extract

compounds from the material. The increase in total phenol content was caused by the sample interacting directly with the solvent for a longer period so that the amount of material extracted was greater until the optimal time limit was reached. However, after the optimal duration was reached, the addition of extraction time did not affect the amount of phenolic bioactive compounds extracted. According to (Kristanti *et al*., 2019) in his research on Supercritical Fluid Extraction and Maceration Technology on Zingiber: Antioxidant Activity and Phytochemical Content, he knows the fact that the longer the extraction time, the higher the yield of the extract because solvents can penetrate the material and break down plant cell walls during the process. extraction process and causes the amount of extracted compounds to increase. This is in line with the findings of this study, which determined that 15 minutes was the best time to produce the highest total phenol content yield.

Microwave power ranges from 150 watts to 450 watts, which has enhanced the effect of total phenol concentration. With increasing microwave power, the content of bioactive compounds also increases. According to a study conducted by (Yingngam et al., 2020)when the microwave power was increased from 300 to 600 W, the content of bioactive compounds increased. This is because the increase in microwave power increases the solubility and diffusion of bioactive components out of the plant matrix, and increases the pressure in the plant matrix. However, if the microwave power is greater than 700 watts, the concentration of bioactive compounds will decrease because the power used is too high, causing the material to degrade automatically.

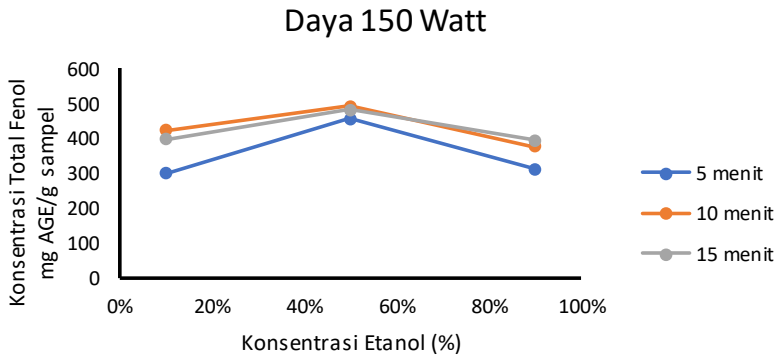

Figure 3. Effect of Variables on Total Phenol Concentration at Power 150 Watt

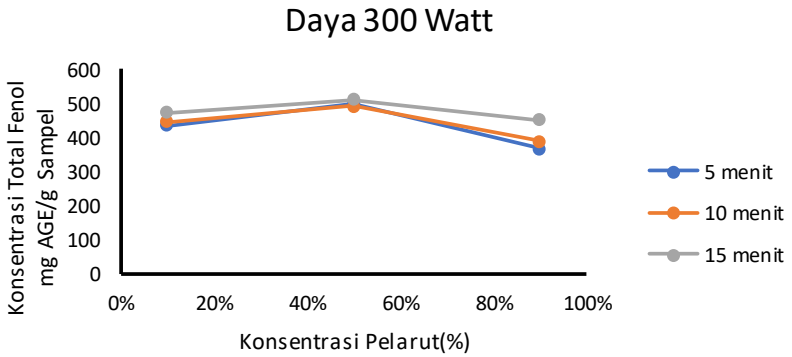

Figure 4. Effect of Variables on Total Phenol Concentration at Power 300 Watt

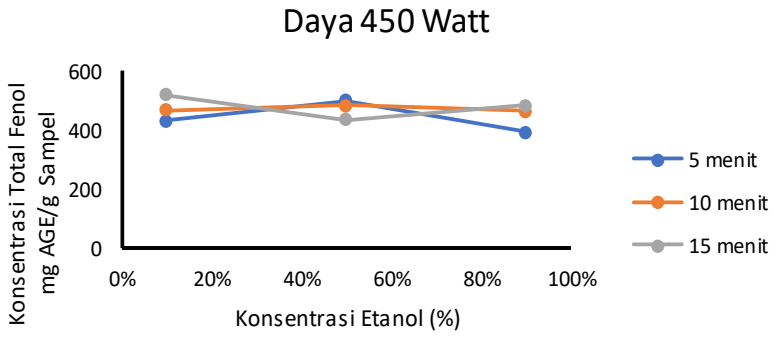

Figure 5. Effect of Variables on Total Phenol Concentration at Power 450 Watt

3.2.4 Analyze with ANOVA

The ANOVA technique was used for statistical analysis in this study. The purpose of the ANOVA analysis was to determine whether the factors used in the pegagan extraction affected the final product, and the variables were considered significant if the p-value of the

ANOVA analysis technique was less than 0.05 (5 percent). The data obtained were used to test the normal distribution as the first stage in the analysis procedure. Before running the ANOVA test, one of the assumptions is that the results of the probability plot on the ANOVA must be normally distributed. The results of the probability test carried out using the Kolmogorov-Smirnov technique can be seen in Figure 4.12 with a p-value of 0.05 and a plot that follows a diagonal line, which shows that the analysis using the Two-way ANOVA general linear model is acceptable.

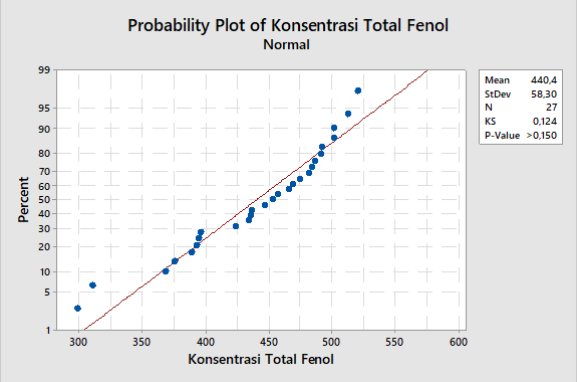

Figure 6. Graph of Normality Test for Total Phenol Content

After the data were normally distributed, ANOVA analysis was performed. The results of the ANOVA analysis are as follows:

Table 2. ANOVA Analysis Results

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Power	2	19386	9693.2	18.72	0.001
Concentration	2	29544	14772.1	28.52	0.000
Time	2	12103	6051.4	11.68	0.004
Time*Concentration	4	10845	2711.3	5.24	0.023
Power*Time	4	3923	980.8	1.89	0.025
Concentration*Time	4	8434	2108.5	4.07	0.043
Error	8	4143	517.9		
Total	26	88379			

The P-value is said to be significant or significant if the P-value <0.05. Based on Table 2, the results of the ANOVA analysis showed that the microwave power, extraction time, and ethanol concentration had a significant effect on the total phenol content with a p-value <0.05. The conclusion obtained in the research model of pegagan plant extract (*Centella asiatica* L.) is that the variables used are variables that have a significant or significant effect on the extract's total phenol content.

Based on the ANOVA analysis carried out, the R square value of 95.31% showed that the model was by following the research results. The value of R square is stated according to the model if the percentage generated is more than 75% (Yingngam *et al.,* 2020), so it can be concluded that the equations used to predict the influential variables are appropriate and can be used to predict the actual results of the study.

4. Conclusion

The effect of ethanol solvent concentration in the process of extracting the bioactive components of pegagan is the higher the concentration of ethanol solvent used does not increase the yield of the resulting extract, based on research that has been carried out the optimum concentration in research is 10%, the maximum total phenol content using ethanol solvent is at conditions operating power of 450 watts, 10% ethanol concentration with a radiation time of 15 minutes which resulted in a total phenol content of 520 mg AGE/g sample. In the ANOVA analysis with ethanol solvent, power, extraction time, and solvent concentration variables gave a significant response to the total phenol content, that is with an R square value of 95.31%.

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