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OPTIMIZATION OF PHENOL LEVELS FROM PHYCOERYTHRIN OF GRACILARIA VERRUCOSA WITH FREEZE THAW USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Gracilaria verrucosa is a one of the seaweeds with a red pigment in the form of phycoerythrin. Phycoerythrin pigment contains phenol which is an antioxidant. The pigment can be obtained by extraction. One of the extraction methods that can be done is freeze thaw. This study uses the Response Surface Methodology to obtain the optimum sample. The purpose of this study was to determine the optimization model, the optimum point and the phenol content of the phycoerythrin Gracilaria verrucosa using the freeze thaw using Response Surface Methodology. Extraction of phycoerythrin pigment was carried out using the freeze thaw with a factor of freeze time, thawing time and solvent ratio, then the phenol content test was carried out. The results obtained are that the mathematical model used for optimization is the quadratic method with the equation y = 148.71 – 3.47(A) + 20.94(B) + 32.36(C) + 10.94(AB) + 9 .90(AC) + 6.25 (BC) + 6.52(A2) – 15.57(B2) + 30.46(C2). The optimum response obtained is the freeze duration 121,964 minutes; thawing duration 144,281 minutes; and the solvent ratio is 62.556%, and the phenol content obtained is 163.742 g GAE/g.

Keywords: *gracilaria verrucosa, phycoerythrin, freeze thaw, Response Surface Methodology, phenol content*

INTRODUCTION

Indonesia is a country with three quarters of its territory is the sea, and has a coastline of 95,161 km. (Lasabuda, 2013). Seaweed is divided into three types based on the color pigments contained, namely green algae (*Chlorophyceae*), brown algae (*Phaeophyceae*) and red algae (*Rhodophyceae*). Indonesia is one of the countries that produces quite high red seaweed, which is as much as 9 million tons in 2010 (Salim & Ernawati, 2015). The red color found in seaweed comes from the phycoerythrin pigment which is part of the fikobiliprotein. These pigments contain natural antioxidants that can be utilized by humans as a substitute for synthetic antioxidants.

One of the seaweeds with red pigment is *Gracilaria verrucosa*. *Gracilaria verrucosa* has a red color due to the presence of dominant phycobiliprotein pigments, especially phycoerythrin. Abfa *et al*. (2013) stated that *Gracilaria* is a type of microalgae that has a color pigment (anthocyanin), namely phycoerythrin. Phycoerythrin is a globular protein that has high stability compared to other pigments. Phycoerythrin is a protein that works as a complementary pigment in red algae and blue-green algae, which has the function of helping chlorophyll-*a* in the absorption of light in the photosynthesis process. After light is absorbed by phycoerythrin, it

will efficiently transfer to phycocyanin, then to allophycocyanin which will be forwarded to allophycocyanin B until it reaches chlorophyll (Chakdar, 2012).

Phenol is one of the compounds contained in *Gracilaria verrucosa*. Phenol belongs to the flavonoid group which is a secondary metabolite group. Flavonoids have the ability to scavenge free radicals and inhibit oxidation, so they play a major role as antioxidants (Zuraida *et al*., 2017). Widowati *et al*. (2014) stated that *Gracilaria verrucosa* has a phenol content of 7.86 mg GAE/g sample of methanol extract. This makes *Gracilaria verrucosa* have the opportunity to be a source of natural antioxidants based on the phenol content produced.

Phycoerythrin pigment can be obtained by extraction, which is separating the material from the mixture using an appropriate solvent (Mukhriani, 2014). One of the extraction methods that can be done is *freeze thaw.* The advantage of this method is that it can maintain the protein structure of the phycoerythrin pigment, because the *freezing* is carried out at low temperatures so that it can maintain the color and appearance from damage caused by high temperatures (Rahmawati *et al*., 2017). The concept of the *freeze thaw* is that the sample will be frozen in the *freezer* then the *thawing*, where the *freeze* and *thawing* are carried out several times with the same or different durations of time. In the research of Mayasari *et al*. (2018), *freeze thaw* with the addition of a phosphate buffer solution to damage algal cells by forming ice crystals in algal cells.

In this study, optimization was carried out to obtain the best phenol yield from the phycoerythrin pigment *Gracilaria verrucosa*. The study used the *Response Surface Methodology* (RSM) method to determine *freeze thaw* was better to obtain the best phenol content from *Gracilaria verrucosa* the most optimal *Response Surface Methodology* is used in this study because can provide better profits, and require a smaller number of treatments so as to save time and materials used (Hasan, 2012).

The purpose of this research is to find out how the optimization model, the optimum point and the phenol content of the phycoerythrin *Gracilaria verrucosa* method *freeze thaw* using *Response Surface Methodology*.

METHODS

Materials and Tools

Materials used included *Gracilaria verrucosa* obtained from Cilacap, NaH2PO4 (Merck), Na2HPO4 (Merck), 85% ammonium phosphate (Merck), distilled water, Na_2CO_3 (Merck), and folin-ciochalteu's phenol (Merck). The tools used include a *freezer* (Midea HS131CNK), *blender* (Phillips HR2115), jars, trays, spoons, digital scales (Camry), Erlenmeyer (Iwaki), beaker (Pyrex), measuring pipettes (Iwaki), dropper pipettes, test tubes. (Hach), volumetric flask (Herma), aluminum foil, UV-Vis spectrophotometry (Shimadzu), analytical balance (Sartorius), and centrifuge (Hettich).

Method

Extraction was carried out by the *freeze thaw. Gracilaria verrucosa* washed with running water, then reduced in size using *a blender* seaweed samples were weighed *Gracilaria verrucosa* and then 50 mL of distilled water was added. Then *freeze thawing* for 1 hour, 2 hours, and 3 hours for 5 cycles. Furthermore, the precipitation of the sample pigment using 85% ammonium sulfate was carried out by taking 10 mL of water extract of *Gracilaria verrucosa* and centrifuged for 30 minutes at a speed of 3500 rpm, then 5 mL of supernatant was taken and 5 mL of 85% ammonium sulfate was added, then centrifuged again for 10 minutes at 2000 rpm. Samples were precipitated for 4 hours at 4^oC in the dark. After that, it was centrifuged for 20 minutes at a speed of 3500 rpm to collect the protein precipitate of *Gracilaria verrucosa*. The protein precipitate was added with 12 mL of phosphate buffer pH 7 to obtain the phycoerythrin

extract of *Gracilaria verrucosa*. The absorbance value of the extract was observed using a UV-Vis spectrophotometer at wavelengths of 540 nm, 620 nm and 650 nm.

Optimization was carried out using *Response Surface Methodology* with factors of *freeze* (120, 180 and 240 minutes), *thawing* (120, 180 and 240 minutes), and solvent ratio (25%, 50% and 75%). the upper and lower limits of each factor to the *Design Expert V 10 software* using the *Response Surface Methodology* (RSM) method with the *Central Composite Design* (CCD) design so that 20 formula recommendations are obtained. Furthermore, verification and data validation is carried out based on the best recommendations from the *Design Expert V 10 software.* The phenol concentration test refers to Kate (2014). Total phenol analysis is carried out by determining the *Operating Time* (OT), maximum absorption wavelength, and gallic acid standard curve referring to Kate (2014). Determination of total phenol content is carried out by taking 0,5 extract sample *Gracilaria verrucosa* and put into a 10 mL volumetric flask, then added with added Folin Ciocelteau reagent. h of distilled water with a ratio of 1:10 (v/v) and 4 mL of 1 M sodium carbonate solution. Then, it was allowed to stand for 20 minutes. Then read the absorbance at the maximum wavelength of 760.5 nm.

RESULTS AND DISCUSSION

Response to Phenol Levels of The Phycoerythrin Pigment

Based on the results of research that has been carried out, the total response of phenol is obtained as shown in Table 1.

Table 1. Treatment factors and response variables to phenol levels

Treatment	Factor 1 A: Freezing Time (Minutes)	Factor 2 B: Thawing Time (Minutes)	Factor 3 C: Solvent Comparison (%)	Phenol Content (µg GAE /g)
1	120	240	25	154.167
2	79.0924	180	50	179.167
3	120	240	75	197.917
4	180	180	50	131.25
5	180	79.0924	50	70.8333
6	280.908	180	50	170.833
7	240	240	75	225
8	180	180	50	183.333
9	180	180	50	154.167
10	240	240	25	154.167
11	180	180	7.95518	164.583
12	240	120	25	102.083
13	180	180	92.0448	320.833
14	120	120	75	164.583
15	180	180	50	179.167
16	180	280.908	50	154.167
17	120	120	25	158.333
18	180	180	50	114.583
19	240	120	75	160.417
20	180	180	50	127.083

Table 2. *Analysis of Variants* quadratic model of response to phenol levels

Source	Total Squared	df	Squared Total	F value	p-value	
Model	41308.32	9	4589.81	6.70	0.0032	Significant
A- *Freeze*	164.16	1	164.16	0.24	0.6351	
B-Thawing Time *5988.65*	5988.65	1	0.0144	8.74	14301.75	
C-Comparative	Solvent	1	14 301.75	20.86	0.0010	
AB	957.03	1	957.03	1.40	0.2647	
AC	783.42	1	783.42	1.14	0.3102	
BC	312.50	1	312.50	0.46	0.5149	
A^2	613.32	1	613 ,32	0.89	0.3665	
B^2	3495.20	1	3495.20	5.10	0.0475	
C^2	13372.86	1	13372.86	19.51	0.0013	
Residual	6854.87	10	685.49			
Lack of Fit	2762.71	5	552.54	0.68	0.6615	Insignificant
Pure Error	4092.16	5	818.43			
Total	Cornol	19				

Based on Table 2, it is known that the model obtained is significant. This can be seen from the p-*value* which is less than 0.05. Then the *lack of fit* which is a deviation from a model. According to Dewi & Gapsari (2013), the *lack of fit* be seen in the analysis of variance table above with the following hypothesis:

H_0 = there *lack of fit* in the model

H_a = there is no *lack of fit* in the model

If the *lack of fit is* smaller than 0.05, then H0 is accepted and HA is rejected, whereas if it is greater than 0.05, then H0 is rejected and HA is accepted. Based on the table of analysis of phenol variance, obtained p-*value lack of fit* of 0.0819. Because the value is greater than 0.05, then H0 is rejected and HA is accepted. Therefore, the phenol response quadratic model does not have a *lack of fit* or deviation.

The mathematical model equations obtained in the optimization of *freeze time*, time *thawing*, and solvent ratio are as follows:

Phenol content (y) = 148.71 – 3.47(A) + 20.94(B) + 32.36(C) + 10.94(AB) + 9.90(AC) + 6.25(BC) + 6.52(A^2) – 15.57(B^2) + 30.46(C^2)…… ……(1)

Description:

A: *Freeze*

B: *Thawing*

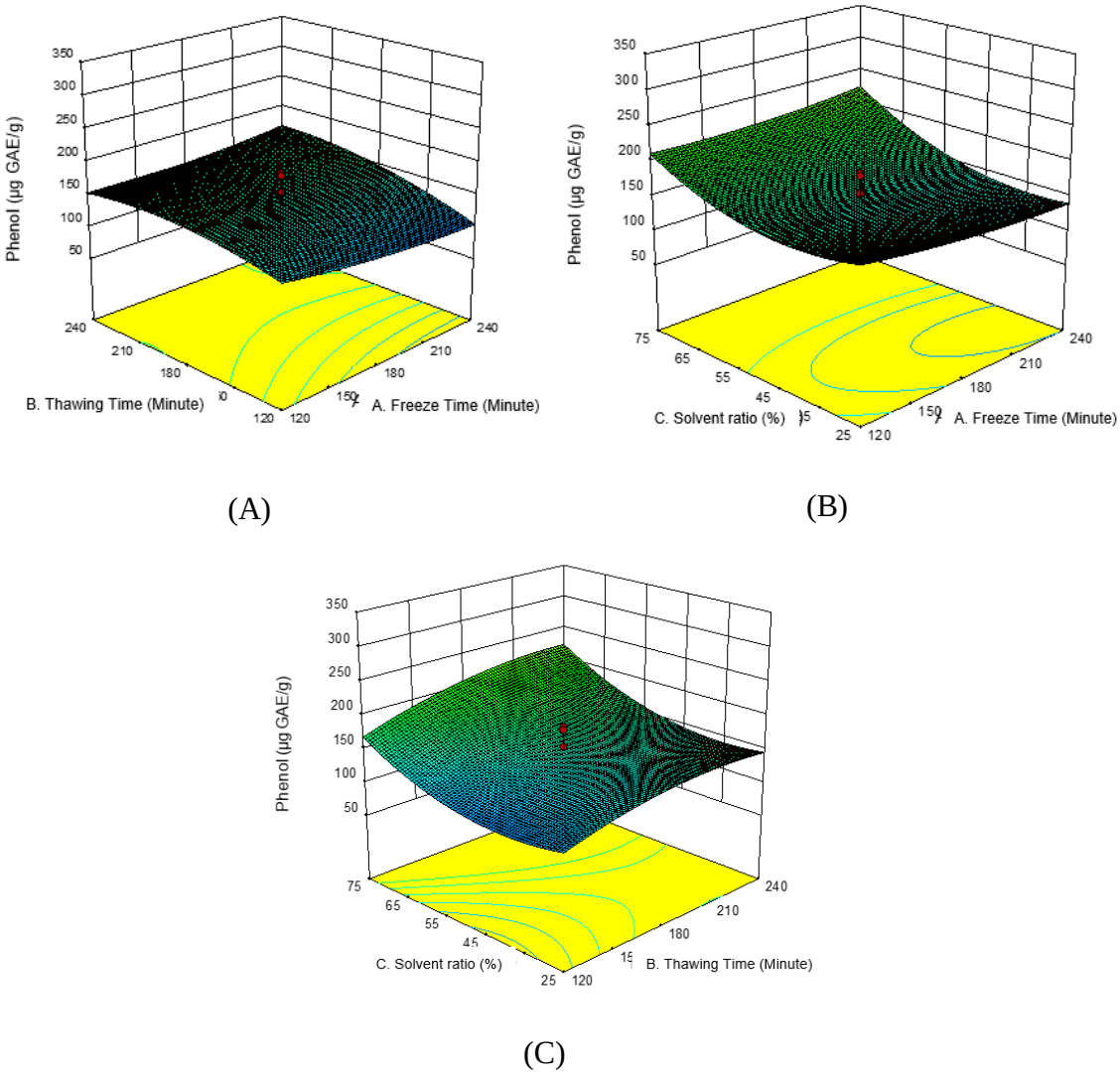
C: Comparison of ingredients and solvents

Based on the above equation, it can be seen that the *freeze* is inversely proportional to the response variable of phenol content. This is indicated by the negative constant value obtained at A. However, the *thawing* and the ratio of materials and solvents show a directly proportional relationship with the phenol content which is indicated by the positive constant value at B and C.

Furthermore, to determine the effect of the three factors on the phenol content can be seen in the following three-dimensional graph.

Based on the Figure 1, it can be seen that the phycoerythrin content decreased when the freeze time was longer. This is because when the *freezing* is carried out longer, more and more ice crystals are formed. This is in accordance with Hamidi (2010) which states that freezing can cause the development of water so that cells will be damaged. Freezing slowly or done for a long time can result in the formation of extracellular ice outside the cell, thus triggering the transport of water from inside to outside the cell, then the cell becomes dehydrated and

shriveled. in addition, extracellular which is formed in large size can also put pressure on the cell to tear the cell membrane. If too many ice crystals are formed, not only the cell wall is damaged, but other cells are also damaged, including the phycoerythrin pigment. This is because ice crystals have a sharp surface, so they can damage the surrounding cells. The longer the *freezing* is carried out, the less phycoerythrin pigment is obtained so that the less phenol content is produced.



(A) (B)

(C)

Figure 1. Three-dimensional graph of the quadratic model of the effect of the three factors on phenol content: (A) Three-dimensional graph of freeze time and thawing time. (B) three-dimensional graph between freeze time and thawing time. (C) three-dimensional graph between freeze time and solvent comparison.

The opposite occurs in the *thawing* which can be seen in Figure 1, where the longer the *thawing* is carried out, the more phycoerythrin pigment is produced, and the more phenol content is produced. This is because the longer the *thawing*, the ice crystals previously formed during the *freezing* can melt completely. When *thawing* is done in a short time, the ice crystals have not melted completely, so the phycoerythrin pigment produced is not optimal because it is blocked by ice crystals. Therefore, the longer *thawing* will produce more phycoerythrin pigment because the previously formed ice crystals have melted completely. This is in accordance with the research of Tan *et al.* (2020) regarding the extraction of phycobiliprotein *Arthrospira* sp. by the *freeze thaw*. In this study, the most phycobiliprotein extracts were obtained at *freeze* and

thawing. Tan *et al*. (2020) stated that the incubation time at room temperature was longer to produce more phycobiliprotein pigments.

In the solvent ratio factor, it can be seen in Figure 1 that the higher the solvent content used, the higher the phycoerythrin pigment produced, and the more phenol content produced. This is because the phycoerythrin pigment which has melted during the *thawing* will dissolve in a polar solvent, namely distilled water. This is in accordance with the research of Tan *et al*. (2020) which states that phycoerythrin is a water-soluble pigment, and can produce the highest pigment using a solution at pH 7, namely distilled water. The more distilled water is used, the more soluble the phycoerythrin pigment will be, so the higher the phycoerythrin pigment produced because it is more soluble with distilled water.

Optimum Formula Optimization

Table 3. Optimum formula prediction from Design Expert software V.10

No	Time *Freeze* (minutes)	Time *Thawing* (minutes)	Solvent Comparison (%)	Phenol Content (µg GAE/g)	*Desirability*	
1	121.964	144.281	62.556	163.742	1.000	selected

Table 3 shows *freeze time*, *thawing* and solvent ratio recommended by *Design Expert software V.10* to produce the most optimum phenol content of the phycoerythrin extract. The table also shows the predicted value of the phenol content produced. The formula was chosen because it has a *desirability* 1, so it is predicted to produce phycoerythrin pigment according to the optimization target. The optimum formula was repeated and analyzed again to verify and validate the sample. The results of verification and validation can be seen in Table 4.

Verification and validation of the optimum formula

Table 4. The results of the optimum phenol content were compared with the obtained correction interval

value Actual value of phenol content (µg GAE/g)	Predicted Value	95% Correction Interval	
		Low	High
186.042	163.742	149.213	196.681

Based on the data presented in Table 4, it can be seen that the phenol content of the optimum sample was 163.742 g GAE/g. The value of the phenol content produced was in accordance with the predictions of Design *Expert V.10 software* and was within the correction interval determined by the *Design Expert V.10 software*, which was between 149.213 g GAE/g to 196.681 g GAE/g. Based on these data, it can be concluded that the optimum formula has been verified and valid, because it is in accordance with the predictions of Design *Expert V.10 software*.

The phenol content of the phycoerythrin pigment *Gracilaria verrucosa*

The phenol content produced in the optimum product was 163.742 g GAE/g. This value is smaller than in the study of Lestario *et al*. (2008) conducted a total phenolic test of *Gracilaria verrucosa* using various solvents. The results showed that the highest phenol content of seaweed *Gracilaria verrucosa* was 45.29 mg GAE/g derived from acetone solvent. The high phenolic value is due to the semi-polar nature of the phenolic compounds in red seaweed, so they dissolve well in the semi-polar acetone solvent (Febrianto *et al*. 2019). In this study, distilled water was used as a solvent which has polar properties. This causes the phenolic compounds contained in *Gracilaria verrucosa* are not dissolved optimally. However, the phycoerythrin pigment has potential as a source of phenol.

CONCLUSION

The mathematical model used in the research to optimize the phenol content of the phycoerythrin pigment *Gracilaria verrucosa* is a quadratic model with the equation y = 148.71 – 3.47(A) + 20.94(B) + 32.36(C) + 10.94(AB) + 9.90(AC) + 6.25(BC) + 6.52(A^2) – 15.57(B^2) + 30.46(C^2). The optimum response is selected and recommended to get the highest phycoerythrin levels according to the recommendations from the *Design Expert software V.10* time *freeze* 121,964 minutes;time *thawing* 144.281 minutes; and the solvent ratio is 62.556%. The phenol content produced from the optimum sample was 163.742 g GAE/g. This makes the phycoerythrin pigment a potential source of phenol.

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