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Introductory Study towards the Extraction of Chlorophyll Pigment from Sargassum

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Abstract

The research aims to draft a protocol for extracting chlorophyll from *Sargassum*, a brown seaweed purchased in Garut, West Java, Indonesia. Three solvents were examined; acetone, ethanol and dematerialized water. The first two give the same yield and extract the same pigments. The concentrations are approximated using the absorbency measured with a spectrophotometer. The spectra show that the pigment being extracted is pheophytin, the derivative of chlorophyll. With decrease in sample size the yield would increase proportionally. Acetone was chosen as optimal solvent after a first experiment. T-test was performed on the results and showed a higher yield for acetone as for ethanol. The high concentration of salt in the seaweed does not interfere with the measured concentrations, does washing the seaweed before extraction improve the yield. There are still pigments left in the sample after extraction as the seaweed still has color, even after repetitive extractions on the same sample. To make sure the samples could be stored before analysis without any change in the results the degradation of the Chl was studied. After three days the calculated yield was increased from 32,69 mg/100 g to 47,06 mg/100 g what implies the degradation can be reversible. The spectra on the other hand looked more like chlorophyllide then pheophytin what stated an increase in degradation.

Keywords: Chlorophyll; pheophytin; pigment; *Sargassum.*

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1. Introduction

The major pigment of brown seaweeds is fucoxanthin, which is one of the most abundant carotenoids in nature [1]. It is an orange colored pigment, found in brown seaweeds along with chlorophyll, to give a brown or olive green color [2]. Chlorophylls (Chl) are greenish pigments that contain a porphyrin ring around which electrons are free to migrate. The ring coordinates a magnesium atom at its center, a fused, 5-membered ring and a C20 phytyl side chain [3]. The non-polar hydrocarbon side chain enhances the solubility of chlorophyll in non-polar solvents. The ring has the potential to gain or lose electrons easily because they move freely. So after absorption of light the pigment can provide energized electrons to an acceptor molecule to initiate the photosynthetic process [4].

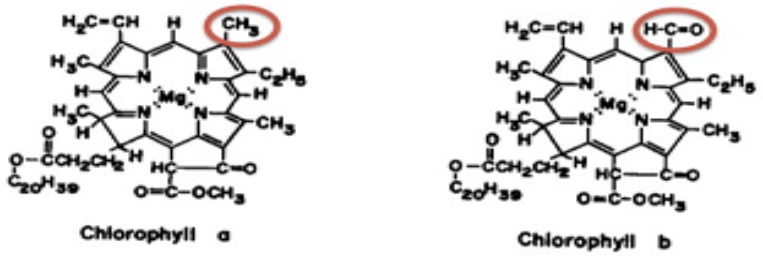

Figure 1: Structures of chlorophyll a (chl a) and chlorophyll b (chl b) [33].

The main difference between Chl a and CHL b is the substituent on position 3 (ring 3). Chl a has a methyl group, whereas Chl b has an aldehyde. It is an oxidized derivative of Chl a. Chlorophylls are classified as lipids because of their solubility characteristics. The pigments are present in the chloroplast, where they are bound to proteins in the thylakoids. To release pigments in a protein-free form they need to be grinded in solvents such as acetone and ethanol [3]. The grinding will not brake the bounds, but it will destroy the cell walls and make it easier for the pigments to be extracted. In order to use the pigments in the Sargassum extract (waste) produced during fucoidan extraction, the types of pigments present in the seaweed extract must be determined first and its characteristics and stability should be studied. Hence, this research was aimed to determine the types of pigment present in the extract of Sargassum from Garut, West Java and the stability of the pigment.

2. Materials and methods

Materials

The seaweed is purchased from the beach in Garut, a town in the West Java province of Indonesia. Local fisherman harvest the seaweed, wash it to remove adhering soil particles and dry the samples in the sun. Every single experiment is done with seaweed from the same batch, so the influences of the harvesting process can be ignored. The dried material is stored in a plastic bag in a closet in the laboratory. No measures have been taken to prevent the seaweed from absorbing moisture from the air. The experiments are done with ambient temperature (30-35◦C)

3. Preparations of sample

Choice of Solvent

Protocol is based on [9]. The seaweed is taken from the same batch. Extractions are performed on different days. The experiments were done with dried Sargasum. Fresh Sargasum is more difficult to purchase, to keep it fresh and to process it. The dried seaweed was cut (average size = 0,25-0,50cm) to enlarge the surface and to brake the rigid cellulose cell-walls to ease the diffusion. The cutting will optimize the extraction and prevent the magnetic stirrer gets stuck. After cutting, 10g of dried seaweed was weighed and added in a beaker of 500ml. The solvent was added with ratios of 1:6, 1:8 and 1:10 for ethanol and acetone. The usage of demineralized water needed bigger ratios; 1:10, 1:15, 1:20. This due to characteristic of seaweed to absorb water, with the use of the lower ratios there was no solvent left and the stirrer got stuck in the sample. The beaker is covered with aluminum foil to prevent the pigment from degrading [3]. The sample is stirred with a magnetic stirrer for 2, 3 or 4 hours (in the fume hood depending on the kind of solvent). Before storage in the refrigerator the extract is filtered with fast filtration paper. The residual seaweed is dried and recycled.

Characterisation of chlorophyll

Uv-vis Spectrophotometric Analysis

UV-VIS (Ultraviolet-visible Spectrophotometry) is used to determine the concentration of the Chl. The wavelengths are selected based on published articles [7, 5, 3]. Use a 3 ml quarts cuvet with a 1cm path length containing the solvent to zero the single beam spectrophotometer (HewlettPackard 8453). The absorbency value never exceeded 1 to assure the accuracy and the absorbency at 750 nm is used as indicator for the turbidity. The measurements at the other wavelengths are decreased with this value in order to take the influence of the turbidity into account [8]. After analyzing the blank, the cuvet is rinsed and filled with sample for measurement. Specific wavelengths for concentration calculations and a complete spectrum (400-800nm) will be analyzed. This procedure will be repeated 3 times per sample.

Wash Sample with Tap Water

Protocol The seaweed is washed with tap water before cutting. The sample was not soaked in water just rinsed, to prevent other substances will be extracted from the seaweed. After washing it was dried over night in the laboratory. The day after the sample was cut and shred before extraction. 5g of sample was extracted with 30ml of acetone for 1 hour in the incubator (rpm=200, T=30-35◦C). The extraction was perfor med by subdued light and filtered with fast filtration paper. The sample is diluted (1:25) and analyzed with the spectrophotometer.

Influence of dissolved salts on UV-VIS analysis

As mentioned before there is a possibility the dissolved salts of the unwashed sample will influence the absorbency from the spectrophotometer analysis. The result of the T-test on previous data was insignificant, more research was necessary. Protocol 5g of cut sample is extracted for 1 hour in 30 ml of acetone. The samples

are filtered with fast filtration paper and divided in two tubes, in one of them a little of NaCl will be dissolved. The same sample is extracted multiple times.

Analysis of the wash water

Analysis of the wash water was necessary to make sure there was no pigment extracted during rinsing of the sample. Protocol 5g of sample is washed with tap water before shredding. The water is collected and evaporated with a rota-evaporator. The solid is dissolved in acetone and analyzed with the spectrophotometer. The sample is dried over night in the laboratory, shredded and extracted with 30 ml of acetone for 1 hour in the incubator (T=30-35◦, rpm=200).

4. Degradation pigment

Literature study states degradation of pigment must be taken into account [3]. Some of the suggested remedies are to protect the samples from light, to store them in the refrigerator and to add additives to preserve the Chl. The preservatives were not available for this study, but it was possible to pack the beakers used for extraction with alumina foil. Next experiment is executed to determine the influence of storage on the measured yield Chl a+b in the extract. Protocol 5g of grind sample was extracted in the incubator for 1 hour with a 1:6 ratio of acetone. After the extraction the samples are stored in 4 different bottles in the refrigerator and measured with intervals of 1 day.

Influence solvent

To check the influence of the solvent two extractions were done, one with acetone and one with ethanol. The solvents are evaporated by use of a rota-evaporator and the remaining solid is solved in the other solvent. The spectra and concentrations are measured and compared. Protocol 5g of shredded seaweed is extracted for 1 hour in 30 ml of acetone/ethanol in an incubator (rpm=200, T=30-35◦C). The extract is filtrated and diluted (1:25) before it is analyzed with the spectrophotometer. Wavelengths are measured for concentration calculations and the spectra are copied. The rest of the sample is evaporated in a rota-evaporator. The solids are resolved in ethanol/acetone and filtered with fast filtration paper. The sample is diluted again (1:25) and analyzed with the spectrophotometer.

5. Results and discussion

Choice of Solvent

The positive is representative for the highest value and negative for the lowest value. In case of three variables we need 9 extractions to get the possible combinations and 3 times the zero points to verify the variation. It was not known if acetone or ethanol would give the highest concentration of chlorophyll. So for the first optimization the variables are all changed and analyzed with a T-test to decide what solvent will give the optimal yield.

Table 1: Results experiment choice of solvent.

Variables			Solvent	acetone		Solvent	ethanol	
Name sample	Solvent (ml)	Time (h)	C*a+b* (μg/ml)	Yield Chl a+b (mg/100g)	Days storage	C*a+b* (μg/ml)	Yield Chl a+b (mg/100g)	Days storage
XAA 1	60	2	4.34	2.60	6	2.71	1.63	7
XAA 2	60	2	5.64	3.38	0	7.01	4.21	0
XAB 1	60	3	10.25	6.15	6	2.50	1.50	7
XAB 2	60	3	13.07	7.84	0	13.19	7.91	0
XAC 1	60	4	14.86	8.92	0	2.85	1.71	6
XAC 2	60	4	10.73	6.44	0	6.33	3.80	0
XBA 1	80	2	5.16	4.13	6	2.44	1.95	6
XBA 2	80	2	3.90	3.12	0	5.97	4.78	0
XBB 1	80	3	6.61	5.29	7	2.89	2.31	7
XBB 2	80	3	9.96	7.97	0	7.43	5.94	0
XBC 1	80	4	8.01	6.41	5	4.34	3.47	6
XBC 2	80	4	6.42	5.14	0	3.87	3.10	0
XCA 1	100	2	3.34	3.34	6	1.18	1.18	7
XCA 2	100	2	4.07	4.07	0	4.76	4.76	0
XCB 1	100	3	4.42	4.42	8	3.91	3.91	8
XCB 2	100	3	6.53	6.53	1	6.49	6.49	1
XCC 1	100	4	5.81	5.81	5	2.48	2.48	5
XCC 2	100	4	4.67	4.67	0	4.19	4.19	0

Extraction of the seaweed with dematerialized water was difficult because it absorbed half of the solvent and it resulted in a brown extract. Because of the difficulties during the extraction the decision was made to stop the experiments with this solvent. No analysis method was available for dematerialized water with UV-VIS so there are no results of the few samples that were made.

Figure 2: From left to right: demineralized water solvent, ethanol solvent, acetone solvent (pictureis taken after a few days of storage in the refrigerator in subdued light).

The first extractions resulted in 36 concentrations of Chl a+b all obtained in different conditions. It seemed like the overall yield of the acetone samples is higher than that in the ethanol samples. To proof this assumption a T-test needed to be done.

Table 2: Average yield Chl a+b (mg/100g) for all of the samples.

	Aceton	Ethanol
Mean	5.35	3.60

The data is paired as every sample in acetone has a sample with ethanol made with the same conditions. The T-test is performed using Excel software. The assumption was there was no difference in concentration using acetone or ethanol (Null hypothesis; H0) and the alternative was the difference was not zero (Alternative hypothesis; HA).

Wash Sample with Tap Water

Results The calculated concentrations and yield are shown in Table 3. The peaks of the spectra are shown in Table 5. The experiments are executed on two different days. For the first experiment the yield after washing is not significant different to the one without extra rinsing (respectively 40,24±0,64 and 39,85±1,71), the second experiment shows a lower yield of Chl after washing (without washing: 37,90±1,83; with: 29,00±0,64).

Table 3: Results absorbency of diluted samples and calculated Chl content for washing experiment

Date analysis	Name sample	C*a+b* (μg/ml)	Chl a+b (μg)	Chl a+b (mg/100g)
27-May	20	66.41	1992	39.85
27-May	W	67.07	2012	40.24
28-May	20	63.17	1895	37.90
28-May	W	48.34	1450	29.00

As we compare the two experiments among each other the difference in the wash sample is way higher than the 4,5% error calculated in 2.5.

Table 4: Mean and variance of Chl a+b (mg/100g)

	20	W
Mean	38.88	34.62

We assume that the yield will be will be confirmed with a T-test similar as done in 2.3.1. The P-value is to high and states that the possibility that this results are due to chance is to high. So we accept he null hypothesis and assume the washing has no effect on the extraction or analysis.

Table 5: Results intensity peaks from spectra diluted samples for washing experiment.

Date sample	Name sample	Peak 1 (nm)	Abs	Peak 2 (nm)	Abs	Peak 3 (nm)	Abs	A(Soret)/A(Qy)
27-May	20	408.3	0.72129	666.0	0.30521	504.7	0.10349	2.36
Stdev		0.6	0.00126	0.0	0.00173	0.6	0.05837	0.02
27-May	W	406.7	0.71468	665.3	0.29457	503.0	0.06889	2.40
Stdev		0.6	0.00117	0.6	0.00861	0.0	0.00110	0.07
28-May	20	407.3	0.66793	665.0	0.27932	504.0	0.06930	2.37
Stdev		0.6	0.00364	0.0	0.00565	1.0	0.00048	0.01
28-May	W	407.0	0.52778	665.7	0.21466	503.7	0.05379	2.46
Stdev		0.0	0.00237	0.6	0.00909	0.6	0.00070	0.10

The spectra in Figure 4 shows a graph similar to the one from pheopytin (Figure 1.). The graphs look very similar and have the same peaks. The only difference between the washed and the unwashed sample is that the absorbency is lower for the washed sample as you can see in Table 5. This can indicate that some of the pigment will be lost by rinsing with the tap water. Another possible explanation is that part of the salt dissolve in the extract and influence the absorbency results of the spectrophotometer, as we know that the salts will dissolve during the extraction and will not be filtered by use of fast filtration paper [6]

Analysis of the wash water

Results As shown in Table 6 the yield of the wash water is very low, washing will not remove a significant amount of Chl.

Table 6: Yield Chl a+b for the extract of the washed sample (W) and the collected wash water (WW).

Name sample	Chl a+b (mg/100g)
W	56.85
WW	0.04

Degradation pigment

Results The results in Table 7 show that the amount of Chl would increase when the samples are stored compared with the fresh analyzed sample. This implies that the degradation is reversible or more plausible is that there is an interference between pheophytin and Chl what influences the results of the spectrophotometer, as stated in the literature study 1.6.

Table 7: Results wavelengths and concentration degradation experiment

Name sample	Chl a+b (mg/100g)
Day 0	32.69
Day 1	35.80
Day 2	37.83
Day 3	47.06

The spectra of the samples show more intense peaks around 400 and 600 as the storage time increases. The little peaks are disappearing by time and the spectra Day3 (Figure 3d) show more similarity to chlorophyllide (see Figure 3c) then pheophytin (see Figure 3b) .

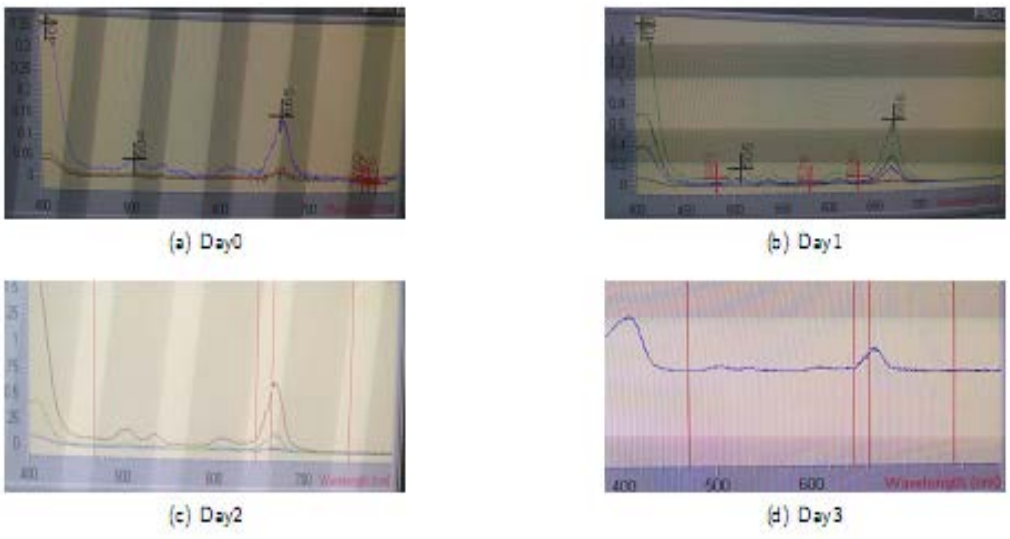

Figure 3: The little peaks are disappearing by time and the spectra Day3

Table 8: Results degradation experiment, peaks diluted samples

Name sample	Peak 1 Abs (nm)	Peak 2 Abs (nm)	Peak 3 Abs (nm)	A(Soret)/A(Qy)
Day0	407.7	665.0	504.0	2.31
Day1	0.35435	0.15346	0.04488	2.33
Day2	407.3	665.7	504.3	2.39
Day3	0.38783	0.16650	0.03377	2.42
	408.0	667.0	504.0	
	0.42430	0.17785	0.04173	
	408.0	665.0	503.0	
	0.51672	0.21390	0.05319	

Influence solvent

Results Concentrations of samples originated with extraction with ethanol are lower than the ones from acetone. After the evaporation the solid is difficult to dissolve in the solvent and agglomerates.

The extract had to be filtrated to avoid turbidity, this with a loss of pigment as result.

Table 9: Yield Chl a+b in different solvents.

Name sample	Chl a+b (mg/100g)
A	37.90
E(A)	14.78
E	14.63
A(E)	3.79

The Spectra of the extractions of acetone and ethanol look similar and after change of solvent there is still no change. Kind of pigment extracted will not depend on the kind of solvent used.

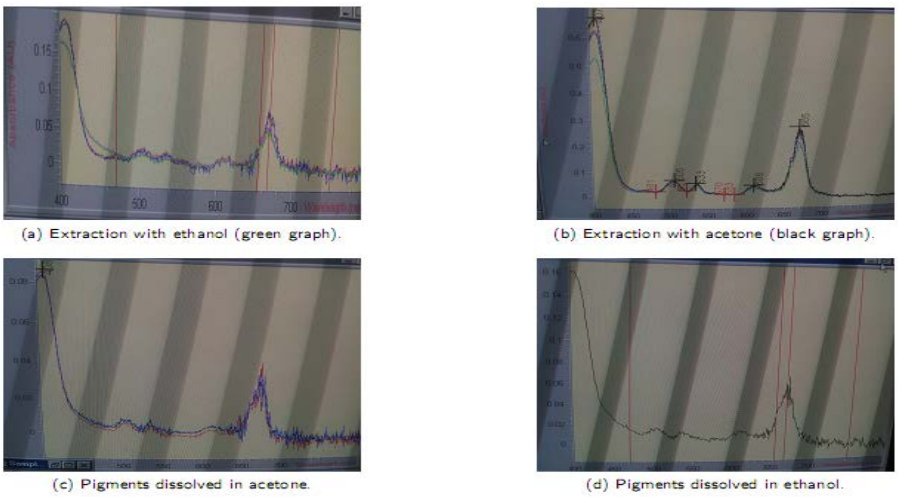

Figure 4: Spectra (a) extraction with ethanol; (b) extraction with acetone; (c) pigments dissolved in acetone after evaporation of ethanol; (d) pigments dissolved in ethanol after evaporation of acetone.

6. Conclusion

Experiments with dematerialized water were stopped before analysis because it would absorb half of the sample causing difficulties during the extraction. Acetone was chosen as optimal solvent after a first experiment. T-test was performed on the results and showed a higher yield for acetone as for ethanol. As further research made clear the storage of samples would influence the results of the spectrophotometric analysis some of these initial data had to be removed. The choice of solvent had no influence on the kind of pigment extracted. The spectra from the spectrophotometer show similarities to the one from Pheo. As the articles said Pheo can be formed while adding weak acid to a Chl solution the pH of the solvents had to be obtained. The pH of acetone and ethanol is 5 and the one from tap water is 6. To make sure the solvents would not cause the degradation bicarbonate can be added. First extractions had a very low yield, because of the rigid cell walls and diffusion smaller sample size was needed. More experiments showed that the decrease of sample size was proportional to the increase of the yield of Chl a+b from 3,60mg/100g to 39,85 mg/100g with a decrease from diameter 0,5-1 cm to 0,841 mm (factor 10). To make sure the samples could be stored before analysis without any change in the results the degradation of the Chl was studied. After three days the calculated yield was increased from 32,69 mg/100 g to 47,06 mg/100 g what implies the degradation can be reversible. The spectra on the other hand looked more like chlorophyllide then pheophytin what stated an increase in degradation. As degraded forms will influence the measurements, there can be concluded that after storage the calculations will give other results that can not be compared to fresh analyzed samples.

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