ELSEVIER

Volume 14 • 2015

ISSN 1876-6196

Procedia Chemistry

2nd Humboldt Kolleg in Conjunction with International Conference on Natural Sciences 2014, HK-ICONS 2014

Editors:

Roy Hendroko Setyobudi, Hugo Scheer, Leenawaty Limantara, Yuzo Shioi, Leszek Fiedor, Tatas H.P. Brotosudarmo and Monika N.U. Prihastyanti

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry 14 (2015) i

Procedia Chemistry

2nd Humboldt Kolleg in Conjunction with International Conference on Natural Sciences 2014, HK-ICONS 2014

Editors:
Roy Hendroko Setyobudi, Hugo Scheer,
Leenawaty Limantara, Yuzo Shioi,
Leszek Fiedor, Tatas H.P. Brotosudarmo
and Monika N.U. Prihastyanti

ELSEVIER

Amsterdam - Boston - London - New York - Oxford - Paris - Philadelphia - San Diego - St Louis

Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Procedia Chemistry 14 (2015) iii–v
Procedia Chemistry

Contents

of the Republic of Indonesia for
...al malaria with artesunate-mefloquine
...om Andrographis paniculata and its
...iculata: a brief review. Pharmacology
...aphis panuculata Nees. Journal of
...Chemical Pharmaceutical Bulletin
...m plants-experimental study, kinetics
...ane. Evidence Based Complementary
...ulata: effect of the solvent flow rate,
...aphis paniculata Nees. International
...ic microwave assisted extraction of
...oil extraction from citronella grass by
...33):173–177.
...om leaves of Ocimum sanctum Linn
...kinetics. American Journal of Applied

Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry

Procedia Chemistry 14 (2015) 193 – 201

2nd Humboldt Kolleg in conjunction with International Conference on Natural Sciences, HK-ICONS 2014

Composition of Photosynthetic Pigments in A Red Alga *Kappaphycus alvarezi* Cultivated in Different Depths

Indriatmoko[a], Heriyanto[a,b], Leenawaty Limantara[a,*], Tatas Hardo Panintingjati Brotosudarmo[a]

[a]*Ma Chung Research Center for Photosynthetic Pigment, Universitas Ma Chung, Malang 65151, East Java, Indonesia*
[b]*Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, ul. Gronostajowa 7 30-387, Poland*

Abstract

The red alga *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva has been introduced and mono cultivated in Indonesia as a seaweed commodity. This species is specifically grown in shallow and clear seawater, although there are several reports concerning the cultivation in deep seawater. It is interesting to know compositional changes of chlorophylls and carotenoids when *K. alvarezii* is grown at different depths. In this investigation, therefore *K. alvarezii* green and brown variants were cultivated at about 0.2 m (normal grown condition), 1 m, and 2 m depths and successfully obtained different ratios of chlorophyll and carotenoid composition at different depths. Quantitative analyses of chlorophylls to carotenoids ratio were carried out using data of chromatogram peak area. This investigation subsequently evaluated the photo and thermo-stability of the pigment extracts to examine the effects of pigment composition on the degradation rate of the pigments. This investigation was aimed to provide information regarding compositional change of the pigments by acclimation in terms of cultivation depths and pigment stability in vitro at condition of natural pigment composition in this alga.

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014

Keywords: Chlorophyll/carotenoid ratio; *Kappaphycus alvarezii*; photo-stability; pigment acclimation; pigment composition; thermo-stability.

* Corresponding author. Tel: +62 813 2636 0303, Fax+62 341 550 175
E-mail address: leenawaty.limantara@machung.ac.id

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014
doi:10.1016/j.proche.2015.03.028

1. Introduction

Marine organisms have been known as high potential natural resources. Seaweed as one of the most imp marine organisms plays a role as primary producer in water ecosystem. In industrial view, seaweed becom important commodity since it has been widely described as a source of agar, carrageenan, and alginate follow this bio product economic values[1–4]. Red alga, *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva originally co from Philippines and, currently, its spread all over the world[5]. This species are firstly introduced in Indone 1985 and became popular as one of the potential marine aquaculture products[6]. In 2010, Indonesia has reache second position as the highest productivity of seaweed in the world. In order to make alternative optio developing *K. alvarezii* economical value by different products, its biomass availability is critical especiall Indonesia.

It has been reported that *K. alvarezii* classified into red, brown, and green variants from their diffe pigmentation[7], although most seaweed had been classified conventionally based on thallus coloration[8]. This va coloration had been studied for decades and reported regarding differences in growth characteristics, photosynthe and carrageenan yield[7,9]. In this study, it is considered to use brown and green variant due to its availability a preferentially used as field and cultivation trials studies[10]. Regionally, *K. alvarezii* were grown in mostly coastal in Indonesia. Madura has become one of the most potential product areas in East Java. Introduction of *K. alvare* Java has begun in 1990. At that time (1990 to 1991), Madura has shown their potential ability by produ 100 ton to 500 ton at annum, while some region in South Sulawesi, currently as the main *K. alvarezii* produce Indonesia, only produced less than 100 ton at annum[11].

The world consumption of *K. alvarezii* are mainly supplied by Philippines and Indonesia[12], and i exponentially increasing due to the highly demand of kappa-carrageenan for industrial purposes. Prese *K. alvarezii* is being used as potential source of carrageenan. The pigment-destructive methods were usually appl to produce total bleached carrageenan product. It is interesting to minimize the degradation of pigments, and util the pigments into valuable products. Some reports[13,14] described that *K. alvarezii* is potential producer photosynthetic pigments such as zeaxanthin, chlorophyll *a*, and β-carotene. Those pigments are not only potenti to be developed into human-safe food colorant, but also it has an important health functional benefit, i.e. zeaxanth which can be used as anti-prostate cancer and anti-age related macular degeneration[15,16], Chlorophyll *a* can be us as the main material for photodynamic therapy against cancer[17], and β-carotene as the antioxidant and precursor vitamin A[18].

Unlike most species of Chlorophyta and Phaeophyta, as the members of Rhodophyta, *K. alvarezii* contai phycoerythrin as a protein-pigment complex, in addition to chlorophylls/carotenoids-containing complexes[19]. The complex systems allow this seaweed species to enhance its ability in harvesting green to yellow lights. There we several investigations on this species ability in triggering pigment production through different depth cultivation however complete composition of chlorophyll and carotenoid pigments had not been reported. In this repor information about pigment composition of *K. alvarezii* var. green and brown which grown in different depths and i vitro study on the stability of crude pigments extracts with different chlorophylls/carotenoids ratios against heat an irradiation were provided. Considering its pigment application prospect, *K. alvarezii* was proved that is not onl important in producing kappa-carrageenan but also potential in providing natural pigments for any useful purposes.

2. Materials and methods

2.1. Algae and cultivation

Red alga *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva var. brown and var. green were cultivated i Sumenep seashore (± 50 m from coastline), Padike village, Talango Island, Madura, East Java (S 7° 5' 18.0636", E 113° 56' 20.1804") (Fig. 1). Cultivation was carried out in a floating raft which had different depth positions of 0. m, 1 m and 2 m. *K. alvarezii* green and brown variants were measured 50 g in weight and tightened in raft wit ropes and cultivated for 40 d. After cultivation, these samples were harvested and collected into dark plastic bag and kept at low temperature under the dark in an ice and sealed dark box during transportations.

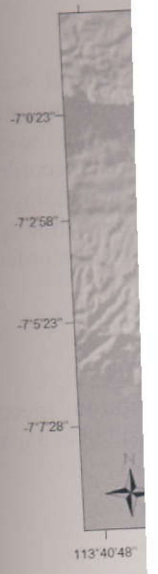

Fig. 1. *K. alvarezii*

2.2. Chemicals

GR or HPLC g were filtered using to use. Pre-injectio acetate which was

2.3. Pigment extra

Sample (4 g w calcium carbonate of *K. alvarezii* gr acetone:methanol was repeated thr partitioned using fraction) was the light at room tem atmosphere.

2.4. HPLC analy

HPLC analys array detector, S Shim-pack VP- μL. HPLC analy ammonium acet 80 : 10 : 10; 10 min[-1] at a tempe

aweed as one of the most important
dustrial view, seaweed becomes an
rrageenan, and alginate followed by
Doty ex P.C. Silva originally comes
e firstly introduced in Indonesia in
In 2010, Indonesia has reached the
der to make alternative option in
availability is critical especially in

green variants from their different
l on thallus coloration[8]. This varied
wth characteristics, photosynthesis,
n variant due to its availability and
ii were grown in mostly coastal area
Java. Introduction of *K. alvarezii* in
heir potential ability by producing
s the main *K. alvarezii* producer in

ppines and Indonesia[12], and it is
for industrial purposes. Presently,
uctive methods were usually applied
degradation of pigments, and utilize
alvarezii is potential producer of
se pigments are not only potentially
th functional benefit, i.e. zeaxanthin
ation[15,16], Chlorophyll *a* can be used
as the antioxidant and precursor of

Rhodophyta, *K. alvarezii* contains
oids-containing complexes[19]. These
green to yellow lights. There were
ough different depth cultivation[20,21],
not been reported. In this report,
ich grown in different depths and in
s/carotenoids ratios against heat and
varezii was proved that is not only
pigments for any useful purposes.

and var. green were cultivated in
lura, East Java (S 7° 5' 18.0636", E
had different depth positions of 0.2
n weight and tightened in raft with
l collected into dark plastic bag and
portations.

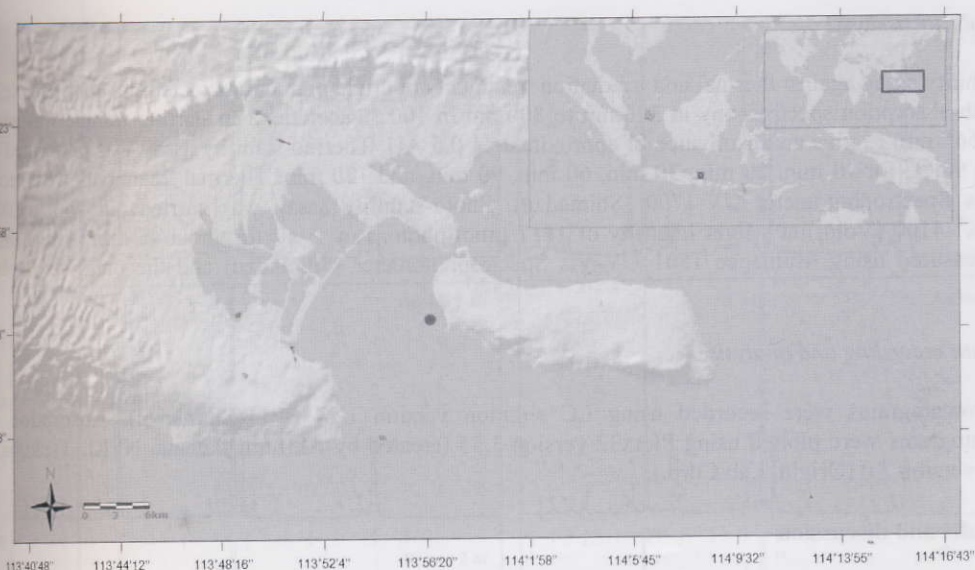

Fig. 1. *K. alvarezii* cultivation site (solid circle) was located in Padike village, Talango Island, Madura, East Java, Indonesia.

2.2 Chemicals

GR or HPLC grade chemicals and solvents were obtained from MERCK (Darmstadt, Germany). The solvents were filtered using polypropylene backed membrane filter (0.5 µm) (Whatman, Maidstone, UK) and degassed prior to use. Pre-injection pigment samples were filtered through a nylon membrane (0.2 µm) (Whatman). Ammonium acetate which was used in HPLC system was analytical reagent grade (Chameleon Reagent, Osaka, Japan).

2.3 Pigment extraction

Sample (4 g wet weight) was ground in a mortar after addition of a trace portion of sodium L-ascorbate and calcium carbonate to minimize oxidative reaction and reduce acidification, respectively, due to cell lysis. Pigments of *K. alvarezii* green and brown variants from different depths were extracted using 20 mL solvent mixture of acetone:methanol (3 : 7, in volume) and recovered by centrifuge at 550 rpm (60 rpm = 1 hertz) for 15 min. This step was repeated three times until the residue becomes colorless. The extracts were collected and filtered, then partitioned using diethyl ether, petroleum benzene, and saturated sodium chloride. Non-polar fraction (upper fraction) was then collected and concentrated using a rotary evaporator. All of these steps were done in dimmed light at room temperature (± 25 °C), and under nitrogen (ultra-high purity grade) (SAMATOR, Surabaya, Indonesia) atmosphere.

2.4 HPLC analysis

HPLC analysis was carried out with Liquid Chromatography (LC) 20AD which was equipped by photodiode array detector, SPD–M20A and column oven CTO–20A (Shimadzu, Kyoto, Japan). The analytical column was Shim-pack VP–ODS C18 (5 µm, 4 i.d. × 250 mm) column protected by guard column. The injection volume was 20 µL. HPLC analysis was performed using a tertiary solvent system consisted of methanol (A), acetone (B), and 1 M ammonium acetate (C) and gradient elution with a time program in the following: 0 min to 10 min, A : B : C = 80 : 10 : 10; 10 min to 25 min, 80 : 16 : 4; and 25 min to 80 min, 80 : 20 : 0, by volume. The flow rate was 1 mL · min^{-1} at a temperature of 30 °C and pigments were detected in the range of 190 nm to 800 nm.

2.5. Stability assays

Stability assay against thermal and irradiation treatments of crude pigment extracts from each sample was carried out with absorption spectroscopy at 300 nm to 800 nm in 100 % acetone. The starting sample was adjusted at Q band (665 nm) to give an absorbance of approximately 0.5 AU. Thermo stability assay was performed using water bath at 90 °C for 10 min, 20 min, 30 min, 60 min, 90 min, and 120 min. Thermal treatments were recorded using UV-Vis Spectrophotometer UV-1700 (Shimadzu). Photo-stability assay was carried out with a halogen lamp Intralux® 4100 (Volpi) at a light intensity of 1417 µmol photons $m^{-2} \cdot s^{-1}$ for about 45 min. A real time spectrum was measured using Multispec 1501 UV-Vis Spectrophotometer (Shimadzu) and the data were recorded every 5 min.

2.6. Data recording and analysis

Chromatograms were recorded using LC solution version 1.24 SP1 (Shimadzu). Absorption spectra and chromatograms were plotted using Plotx32 version 1.35 (created by Akifumi Ikehata, NFRI, Tsukuba, Japan) and Origin version 7.0 (Origin Lab Corp.).

3. Results and discussion

The advantage of isograms is to be able to easily compare the separation and concentration of some pigments in parallel without limiting selection to a given detection wavelength. As can be seen in Fig. 2, the blue color indicates chromatogram baseline (low intensity), while yellow to red color scales indicate absorption intensity. The isograms of purified standard pigments, such as zeaxanthin, chlorophyll *a*, and *β*-carotene, are shown in Fig. 2A. The separation of chlorophyll *a* is indicated by the increasing intensity of the color contours at 400 nm to 500 nm for Soret band and 550 nm to 650 nm for Qx and Qy bands. The carotenoid groups can also be identified by the color contour at 400 nm to 500 nm. The first peak-line (3.27 min) with absorbance peak range at 300 nm to 350 nm indicated solvent peak position. The second, the third and the fourth peak lines were the positions of zeaxanthin (19.65 min), chlorophyll *a* (37.85 min), and β-carotene (60.24 min), respectively. These pigments can be detected clearly by the isograms (Fig. 2. B, C, D, E) by observing the color contours.

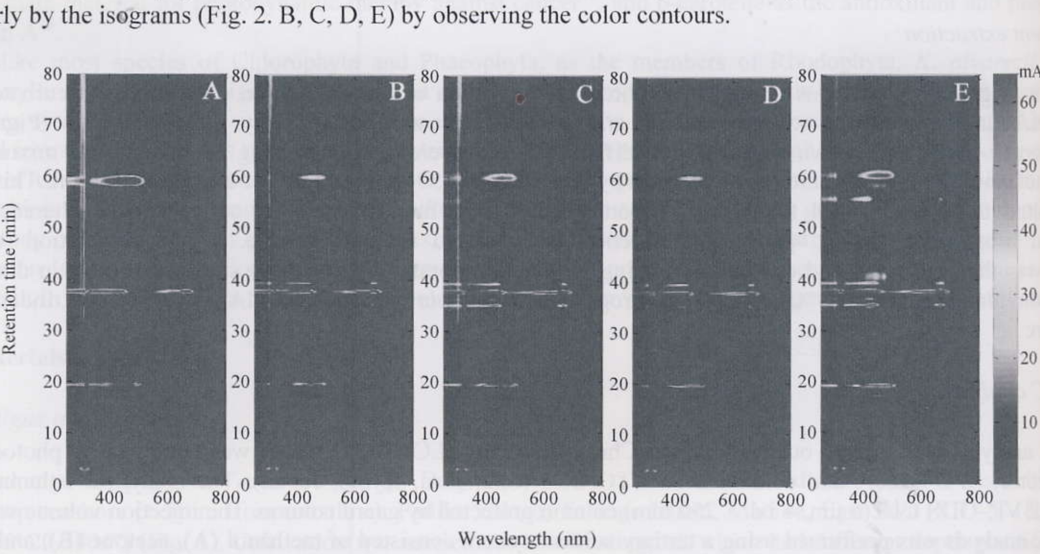

Fig. 2. HPLC isograms of the standard pigments, zeaxanthin, chlorophyll *a*, and β-carotene in order of elution (A), pigment extracts from *K. alvarezii* brown (B and C) and green (D and E) variants, which were cultivated in 1 m and 2 m depths, respectively. The red color in contour map indicates the apex of an absorption peak (in mAU), while the blue indicates baseline.

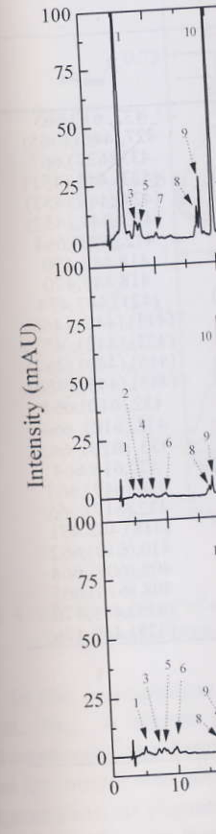

Fig. 3. HPLC Chromatogr
calculated from sel

In order to identify
been chosen. There a
major pigments were
carotene (44.72 min)
major pigments, i
carotenes/xanthophy
to be similar based c
in agreement with th
Table 1 lists the
pigments, but there
Table 1. Unidentifi
addition, derivative
antheraxanthin (16.
Fig. 3 shows th
depth of water co
culture depth posit
by solar irradiance
according to the p
variant were obtai
when it was grow
chlorophyll and ca

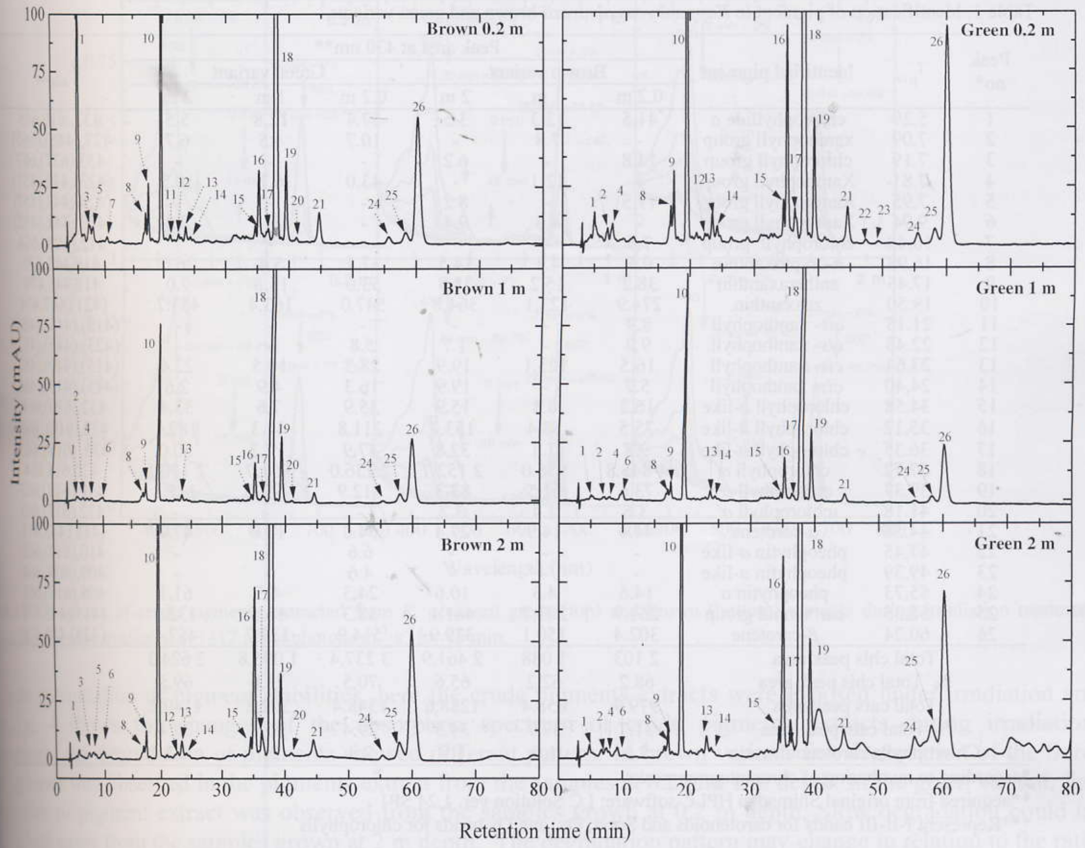

Fig. 3. HPLC Chromatograms of pigment extracts from *K. alvarezii* brown (left) and green (right) variants in different depths. Peak areas were calculated from selected wavelength at 430 nm.

In order to identify the carotenoid and chlorophyll groups in details, a single wavelength detection at 430 nm has been chosen. There are 26 peaks that can be clearly detected from the sample of *K. alvarezii* (Fig. 3, Table 1). The major pigments were identified as antheraxanthin (17.56 min), zeaxanthin (19.65 min), chlorophyll *a* (37.85 min), α-carotene (44.72 min), and *β*-carotene (60.24 min). The minor peaks were expected to be alteration products of the major pigments, i.e., chlorophylide *a* (5.29 min), pheophytin *a* (55.73 min), *cis* derivatives of carotenes/xanthophylls, and unidentified trace pigments. The composition of pigment from both variants has shown to be similar based on the number and the retention time of the peaks. This carotenoid composition in *K. alvarezii* is in agreement with those of other red algae[22–24].

Table 1 lists the identified pigments from the 26 detected peaks. In general, both variants had similar major pigments, but there were small differences in the presence of minor pigments and derivatives as can be seen in Table 1. Unidentified xanthophylls, chlorophylls, and their derivatives were mostly recorded up to 25 min. In addition, derivatives of some major pigments were also found from all samples, i.e., chlorophylide *a* (5.29 min), *cis*-antheraxanthin (16.99 min), and pheophytin *a* (55.73 min).

Fig. 3 shows that *K. alvarezii* contained similar composition of pigments even when it is cultured in different depth of water column. However, it is expected that concentrations of pigments were varied depending on the culture depth position in both variants. It is known that the different pigment composition of red algae is influenced by solar irradiance that penetrates into the water level[25]. Here the relative concentration of pigments is presented according to the peak areas (Table 1). The highest total concentration of chlorophylls and carotenoids in the green variant were obtained from the algae cultivated at 0.2 m depth. Relative concentration of total pigments decreased when it was grown deeper at 1 m depth, but it increased when algae were grown at 2 m depth. The ratio between chlorophyll and carotenoid pigments appears to decrease along with decreasing their cultivation positions.

Table 1. Identification of pigment in *Kappaphycus alvarezii* brown and green variants

Peak no*	t_{R}**	Identified pigment	Peak area at 430 nm**						λ_{mak}***
			Brown variant			Green variant			
			0.2 m	1 m	2 m	0.2 m	1 m	2 m	
1	5.29	chlorophyllide *a*	41.5	12.3	3.5	57.4	12.8	5.5	432, 610,665
2	7.09	xanthophyll group	-	7.8	-	10.7	4.5	6.7	427,(446),(665)
3	7.19	chlorophyll group	14.8	-	6.2	-	-	-	433,(627),667
4	7.81	Xanthophyll group	-	12.1	-	43.0	6.7	10.5	(423),442,(451)
5	7.95	Xanthophyll group	17.5	-	8.2	-	-	-	(424),443,(457)
6	9.94	Xanthophyll group	-	4.3	9.4	-	13.4	2.8	(420),442,(452)
7	10.48	chlorophyll group	7.6	-	-	-	-	-	412,(608),664
8	16.98	*α*-cryptoxanthin	0.5	4.1	14.5	37.1	5.4	26.6	418,443,470
9	17.45	antheraxanthin	38.2	15.2	21.8	53.0	15.8	7.0	418,443,470
10	19.50	zeaxanthin	274.9	123.1	364.8	547.0	163.4	433.7	(421),447,474
11	21.15	*cis*- xanthophyll	8.9	-	-	-	-	-	(413),(441),(466)
12	22.48	*cis*- xanthophyll	9.0	-	1.7	5.8	-	-	(423),(445),(457)
13	23.64	*cis*- xanthophyll	16.5	123.1	19.9	28.3	10.5	22.4	(415),(440),(467)
14	24.40	*cis*- xanthophyll	5.7	-	19.9	16.3	4.9	2.6	(415),(443),(466)
15	34.58	chlorophyll *a*-like	16.2	6.8	15.9	35.9	7.6	33.4	432,(610),664
16	35.17	chlorophyll *a*-like	75.5	38.4	153.7	211.8	41.3	189.1	432,(610), 664
17	36.35	chlorophyll *a*-like	9.8	7.3	32.8	47.9	12.2	50.0	430, (616), 665
18	37.72	chlorophyll *a*	1 845.8	956.0	2 152.7	2 736.0	951.7	2 170.1	430,618,664
19	39.37	chlorophyll *a'*	73.6	61.5	83.3	112.9	65.7	115.1	430,(617),663
20	41.18	chlorophyll *a'*	3.6	1.4	3.2	-	-	-	432,(617),665
21	44.58	*α*-carotene	44.4	14.9	27.4	54.0	12.0	47.0	(418),443,471
22	47.45	pheophytin *a*-like	-	-	-	6.6	-	-	410,(610),662
23	49.39	pheophytin *a*-like	-	-	-	4.6	-	-	409,(609),664
24	55.73	pheophytin *a*	14.6	4.3	10.6	24.3	4.5	61.1	408,(610),665
25	58.35	carotenoid group	261.9	203.7	461.1	38.3	16.5	132.5	(415),445,470
26	60.24	*β*-carotene	302.4	150.1	339.9	514.9	139.7	457.4	(428),450,476
Total chls peak area			2 103	1 088	2 461.9	3 237.4	1 095.8	2 624.3	
% Total chls peak area			68.2	62.2	65.6	70.5	73.6	69.5	
Total cars peak area			979.9	658.4	1288.6	1348.4	392.8	1149.2	
% Total cars peak area			31.7	37.7	34.3	29.4	26.3	30.4	
Chlorophylls/carotenoids ratio			2.1	1.6	1.9	2.4	2.7	2.2	

*according to numbering of chromatogram peaks
**acquired from original Shimadzu HPLC software: LC Solution ver. 1.24 SP1
***Represent I-II-III bands for carotenoids and Soret, Qx, and Qy bands for chlorophylls

K. alvarezii variants showed different behaviour. In brown variant grown at 0.2 m depth, relative concentration of total chlorophylls was higher than the carotenoids (chls 68.2 % and cars 31.7 %). At 1.0 m depth, the total chlorophylls decreased, but the total carotenoid concentrations increased (chls 62.2 % and cars 37.7 %). At 2.0 m depth, the total chlorophyll increased and the total carotenoid decreased (chls 65.6 % and cars 34.3 %). A similar fluctuation was also observed for the ratio between chlorophylls and carotenoids. Table 1 shows that total chlorophyll and carotenoid can reached to highest concentration, when the green variant was grown at 0.2 m depth or when the brown variant was grown at 2 m depth. The ratio between total chlorophyll and carotenoid seems to vary as a function of different depth of growth conditions. Accessory pigment such as phycoerythrin is also produced in red algae[26,27], although this experiments focused only on chlorophylls and carotenoids distribution. Phycoerythrin is important for absorption of light in the spectrum region where chlorophylls and carotenoids are unable to absorb efficiently. It is reported that *K. alvarezii* brown variant has higher phycoerythrin concentration than green variant[28].

While small amount of light can penetrate into the depth of seawater, some photosynthetic organisms are capable of controlling their light capture by producing different photosynthetic pigments[29,30]. This mechanism is very important in order to maintain their photosynthetic activities working effectively. In deeper positions under the water, light intensity and quality have been filtered. Blue to green lights (400 nm to 450 nm) penetrate further into the seawater, while the rest of visible lights have been scattered or absorbed[31]. This might explain that the total chlorophyll content was increased significantly when *K. alvarezii* was grown at 2 m depth. In this case, the Soret band (350 nm to 450 nm) of the chlorophylls plays the role of utilizing the blue light. In addition the total carotenoids content was also increased to play the role in light harvesting at blue-green region and photoprotection.

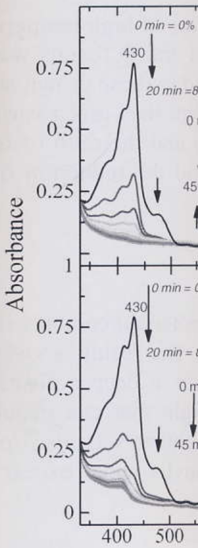

Fig. 4. UV–Vis spectra of crude pigme
Samples were irradiated at 1417

In the examination of pigmen
heat. Fig. 4 shows the chang
Interestingly, the degradation of
and Qy bands was observed in th
degradation of pigment extract v
seen in both cases from the samp
between total chlorophylls and
chlorophylls.

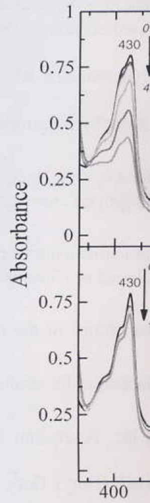

Fig. 5. UV–Vis spectra of crude pigment
were heated in 90 °C for 120 mi

2 m	λ_{mak***}
5.5	432, 610,665
6.7	427,(446),(665)
-	433,(627),667
10.5	(423),442,(451)
-	(424),443,(457)
2.8	(420),442,(452)
-	412,(608),664
26.6	418,443,470
7.0	418,443,470
433.7	(421),447,474
-	(413),(441),(466)
-	(423),(445),(457)
22.4	(415),(440),(467)
2.6	(415),(443),(466)
33.4	432,(610),664
189.1	432,(610), 664
50.0	430, (616), 665
2 170.1	430,618,664
115.1	430,(617),663
-	432,(617),665
47.0	(418),443,471
-	410,(610),662
-	409,(609),664
61.1	408,(610),665
132.5	(415),445,470
457.4	(428),450,476
2 624.3	
69.5	
1149.2	
30.4	
2.2	

m depth, relative concentration %). At 1.0 m depth, the total % and cars 37.7 %). At 2.0 m % and cars 34.3 %). A similar oids. Table 1 shows that total ariant was grown at 0.2 m depth rophyll and carotenoid seems to t such as phycoerythrin is also lls and carotenoids distribution. chlorophylls and carotenoids are gher phycoerythrin concentration

tosynthetic organisms are capable ts[29,30]. This mechanism is very y. In deeper positions under the to 450 nm) penetrate further into This might explain that the total 2 m depth. In this case, the Soret blue light. In addition the total reen region and photoprotection.

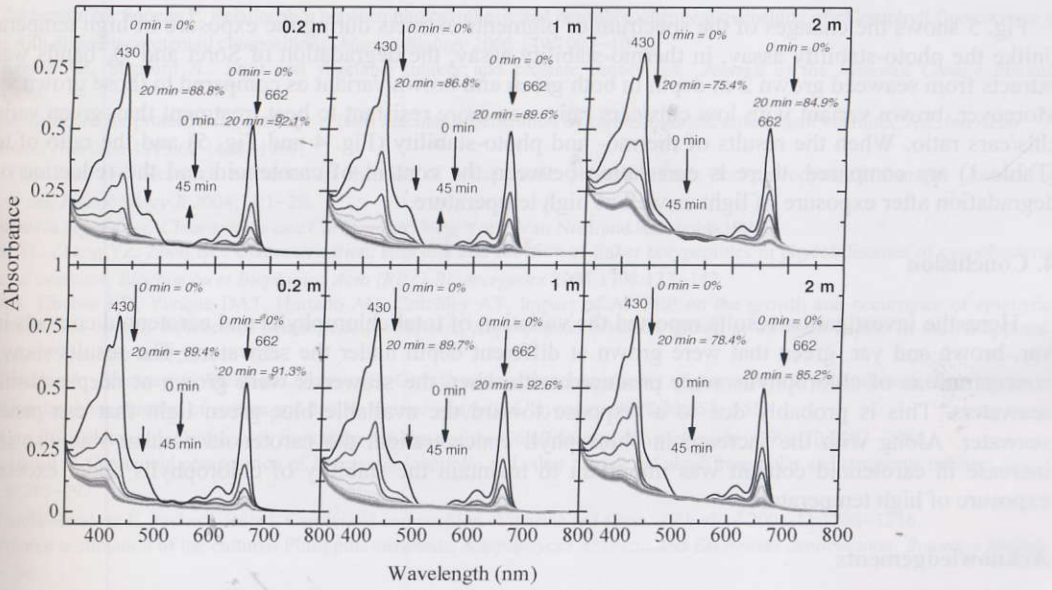

Fig. 4. UV–Vis spectra of crude pigments extracted from *K. alvarezii* green (top) and brown (bottom) variants during irradiation treatment. Samples were irradiated at 1417 µmol photons $m^{-2} \cdot s^{-1}$ for 45 min

In the examination of pigment stabilities, here the crude pigments extracts were exposed under irradiation and heat. Fig. 4 shows the changes of the absorption spectrum of crude pigments extracts during irradiation. Interestingly, the degradation of pigments showed different pattern. In brown variant, fast degradation of the Soret and Qy bands was observed in the pigments extract from the samples at 0.2 and 1 m depth. In the green variant, fast degradation of pigment extract was observed from the samples grown at 0.2 m depth. Slow degradation could be seen in both cases from the samples grown at 2 m depth. The degradation pattern may change in relation to the ratio between total chlorophylls and carotenoids. As shown in Table 1, the seaweed produced more carotenoids than chlorophylls.

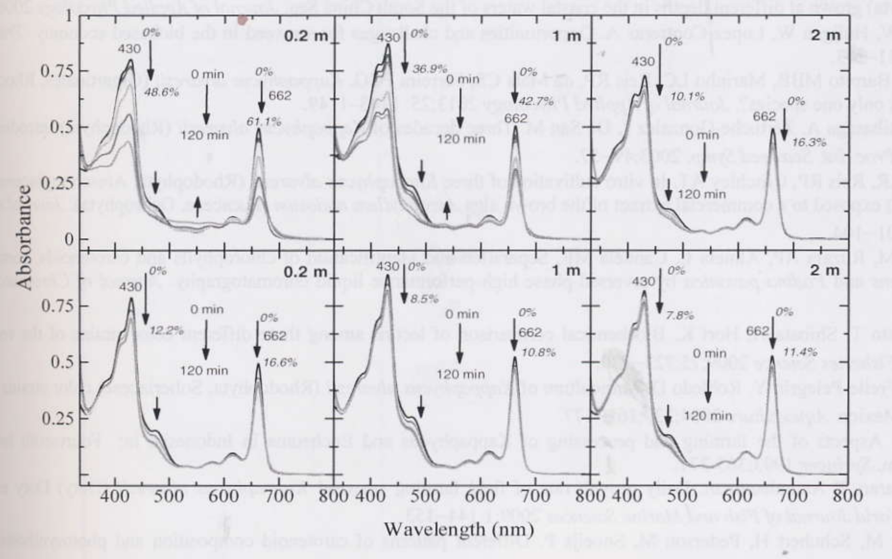

Fig. 5. UV–Vis spectra of crude pigments extracted from *K. alvarezii* brown (bottom) and green (top) variants during thermal treatment. Samples were heated in 90 °C for 120 min.

Fig. 5 shows the changes of the spectrum of pigments extracts during the exposure of high temperature (90 °C). Unlike the photo-stability assay, in thermo-stability assay, the degradation of Soret and Q_y bands was slow in the extracts from seaweed grown 2 m depth in both green and brown variant as compared to those grown at 0.2 m depth. Moreover, brown variant with low chls/cars ratio was more resistant to heat treatment than green variant with high chls/cars ratio. When the results of thermo- and photo-stability (Fig. 4 and Fig. 5) and the ratio of total pigments (Table 1) are compared, there is correlation between the content of carotenoid and the reduction of chlorophyll degradation after exposure of light as well as high temperature.

4. Conclusion

Here, the investigation results reported the variation of total chlorophylls and carotenoid contents in *K. alvarezii* var. brown and var. green that were grown at different depth under the seawaters. The results show that the higher concentrations of chlorophylls were produced with when the seaweeds were grown at deeper position under the seawaters. This is probably due to a response toward the available blue-green light that can penetrate into the seawater. Along with the increase in chlorophyll concentration, the carotenoid content was also increased. The increase in carotenoid content was important to maintain the stability of chlorophylls under excess of light and exposure of high temperature.

Acknowledgements

This project was supported by National Innovation System Research Grant (RT-2013-0172, No: 187/M/Kp/XI/2012 and RT-2014-0432, No: 288/M/Kp/XII/2013), National Research Centre of Excellence (Pusat Unggulan Iptek) Program (SK No. 284/M/Kp/XI/2013) provided by Indonesian Ministry of Research and Technology, and Ma Chung Research Grant IV, Grant number 05/MACHUNG/LPPM–MRGIV/II/2012. Heriyanto acknowledges the scholarship from the Foundation for Polish Science (TEAM/2010-5/3).

References

1. Phang S. Seaweed resources in Malaysia: current status and future prospects. *Aquatic Ecosystem Health & Management* 2006;9:185–202.
2. Msuya FE, Buriyo A, Omar I, et al. Cultivation and utilisation of red seaweeds in the Western Indian Ocean (WIO) region. *Journal of Applied Phycology* 20014;26:699–705.
3. Xu J, Gao K. Growth, pigments, UV–absorbing compounds and agar yield of the economic red seaweed Gracilaria lemaneiformis (Rhodophyta) grown at different depths in the coastal waters of the South China Sea. *Journal of Applied Phycology* 2008;20:681–686.
4. van Hal JW, Huijgen W, Lopez-Contreras A. Opportunities and challenges for seaweed in the biobased economy. *Trends in Biotechnology* 2014;32:231–233.
5. de Barros–Barreto MBB, Marinho LC, Reis RP, da Mata CS, Ferreira PCG. *Kappaphycus alvarezii* (Gigartinales, Rhodophyta) cultivated in Brazil: is it only one species?. *Journal of Applied Phycology* 2013;25: 1143–1149.
6. Ask E, Batibasaga A, Zertuche-Gonzalez J, De San M. Three decades of *Kappaphycus alvarezii* (Rhodophyta) introduction to non-endemic locations. *Proc. Int. Seaweed Symp.* 2003;49–57.
7. Loureiro RR, Reis RP, Critchley AT. In vitro cultivation of three *Kappaphycus alvarezii* (Rhodophyta, Areschougiaceae) variants (green, red and brown) exposed to a commercial extract of the brown alga *Ascophyllum nodosum* (Fucaceae, Ochrophyta). *Journal of Applied Phycology* 2010;22:101–104.
8. Hegazi MM, Ruzafa AP, Almela L, Candela ME. Separation and identification of chlorophylls and carotenoids from *Caulerpa prolifera*, *Jania rubens* and *Padina pavonica* by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* 1998;829: 153–159.
9. Le HD, Sato T, Shibata H, Hori K. Biochemical comparison of lectins among three different color strains of the red alga *Kappaphycus alvarezii*. *Fisheries Science* 2009;75:723–730.
10. Munoz J, Freile-Pelegrin Y, Robledo D. Mariculture of *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae) color strains in tropical waters of Yucatan, Mexico. *Aquaculture* 2004;239:161–177.
11. Luxton D. Aspects of the farming and processing of Kappaphycus and Eucheuma in Indonesia, in: Fourteenth International Seaweed Symposium. *Springer* 1993;365-371.
12. G Thirumaran, P Anantharaman. Daily growth rate of field farming seaweed *Kappaphycus alvarezii* (Doty) Doty ex P. Silva in Vellar Estuary. *World Journal of Fish and Marine Sciences* 2009;1:144–153.
13. Andersson M, Schubert H, Pederson M, Snoeijs P. Different patterns of carotenoid composition and photosynthesis acclimation in two tropical red algae. *Marine Biology* 2006;149:653–665.

14. Schubert H, Andersson M,
two red algae with different
15. Cohen JH, Kristal AR, St
2000;92:61–68.
16. Snodderly DM. Evidence fo
Journal of Clinical Nutrition
17. Nyman ES, Hynninen PH
Photochemistry and Photobi
18. Gross J. *Pigments in vegetal*
19. Liu LN, Chen XL, Zhang Y
and red algae: an overview.
20. Borlongan IAG, Tibubos K
Neosiphonia infestation on
Journal of Applied Phycolog
21. Hurtado A, Critchley A, Tre
different stocking densities,
22. Palermo JA, Gros EG, Seldes
23. Marquardt Jr, Hanelt D. Caro
Phycol 2004;39:285–292.
24. Schubert N, Garcia-Mendoza
25. Dawes C. Irradiance acclima
1992;35:189–196.
26. Aguirre-von-Wobeser E, Fig
(Rhodophyta) from the Philip
27. Zhao S, He P. Effects of light
28. Naguit MRA, Tisera WL. P
cultured at different depths. T
29. Scheuring S, Goncalves R
Organization. *Journal of Mol*
30. Sineshchekov OA, Jung KH,
Proceedings of the National A
31. Wozniak B, Dera J. *Light abs*

14. Schubert H, Andersson M, Snoeijs P. Relationship between photosynthesis and non-photochemical quenching of chlorophyll fluorescence in two red algae with different carotenoid compositions. *Marine Biology* 2006;149:1003–1013.
15. Cohen JH, Kristal AR, Stanford JL. Fruit and vegetable intakes and prostate cancer risk. *Journal of the National Cancer Institute* 2000;92:61–68.
16. Snodderly DM. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *The American Journal of Clinical Nutrition* 1995;62:1448–1461.
17. Nyman ES, Hynninen PH. Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy. *Journal of Photochemistry and Photobiology B* 2004;73:1–28.
18. Gross J. *Pigments in vegetables: Chlorophylls and Carotenoids*. New York: Van Nostrand Reinhold; 1991.
19. Liu LN, Chen XL, Zhang YZ, Zhou BC. Characterization, structure and function of linker polypeptides in phycobilisomes of cyanobacteria and red algae: an overview. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 2005;1708:133–142.
20. Borlongan IAG, Tibubos KR, Yunque DAT, Hurtado AQ, Critchley AT. Impact of AMPEP on the growth and occurrence of epiphytic Neosiphonia infestation on two varieties of commercially cultivated *Kappaphycus alvarezii* grown at different depths in the Philippines. *Journal of Applied Phycology* 2011;23:615–621.
21. Hurtado A, Critchley A, Trespoey A, Bleicher-Lhonneur G. Growth and carrageenan quality of *Kappaphycus striatum* var. sacol grown at different stocking densities, duration of culture and depth. *Journal of Applied Phycology* 2008;20:551–555.
22. Palermo JA, Gros EG, Seldes AM. Carotenoids from three red algae of the Corallinaceae. *Phytochemistry* 1991;30:2983–2986.
23. Marquardt Jr, Hanelt D. Carotenoid composition of *Delesseria lancifolia* and other marine red algae from polar and temperate habitats. *Eur J Phycol* 2004;39:285–292.
24. Schubert N, Garcia-Mendoza E, Pacheco-Ruiz I. Carotenoid composition of marine red algae. *J Physicol* 2006;42:1208–1216.
25. Dawes C. Irradiance acclimation of the cultured Philippine seaweeds, *Kappaphycus alvarezii* and *Eucheuma denticulatum*. *Botanica Marina* 1992;35:189–196.
26. Aguirre-von-Wobeser E, Figueroa F, Cabello-Pasini A. Photosynthesis and growth of red and green morphotypes of *Kappaphycus alvarezii* (Rhodophyta) from the Philippines. *Marine Biology* 2001;138:679–686.
27. Zhao S, He P. Effects of light intensity and salinity on growth of *Kappaphycus alvarezii*. *Journal of Tropical Oceanography* 2009;28:24–29.
28. Naguit MRA, Tisera WL. Pigment analysis on *Eucheuma denticulatum* (collins & hervey) and *Kappaphycus alvarezii* (doty) cultivars cultured at different depths. *Threshold* 2009;4:29–37.
29. Scheuring S, Goncalves RP, Prima V, Sturgis JN. The Photosynthetic Apparatus of *Rhodopseudomonas palustris*: Structures and Organization. *Journal of Molecular Biology* 2006;358:83–96.
30. Sineshchekov OA, Jung KH, Spudich JL. Two rhodopsins mediate phototaxis to low-and high-intensity light in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences USA* 2002;99:8689–8694.
31. Wozniak B, Dera J. *Light absorption in sea water*. New York: Springer; 2007.

Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry 14 (2015) 193 – 201

Procedia Chemistry

2nd Humboldt Kolleg in conjunction with International Conference on Natural Sciences, HK-ICONS 2014

Composition of Photosynthetic Pigments in A Red Alga *Kappaphycus alvarezi* Cultivated in Different Depths

Indriatmoko[a], Heriyanto[a,b], Leenawaty Limantara[a,]*, Tatas Hardo Panintingjati Brotosudarmo[a]

[a]*Ma Chung Research Center for Photosynthetic Pigment, Universitas Ma Chung, Malang 65151, East Java, Indonesia*
[b]*Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, ul. Gronostajowa 7 30-387, Poland*

Abstract

The red alga *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva has been introduced and mono cultivated in Indonesia as a seaweed commodity. This species is specifically grown in shallow and clear seawater, although there are several reports concerning the cultivation in deep seawater. It is interesting to know compositional changes of chlorophylls and carotenoids when *K. alvarezii* is grown at different depths. In this investigation, therefore *K. alvarezii* green and brown variants were cultivated at about 0.2 m (normal grown condition), 1 m, and 2 m depths and successfully obtained different ratios of chlorophyll and carotenoid composition at different depths. Quantitative analyses of chlorophylls to carotenoids ratio were carried out using data of chromatogram peak area. This investigation subsequently evaluated the photo and thermo-stability of the pigment extracts to examine the effects of pigment composition on the degradation rate of the pigments. This investigation was aimed to provide information regarding compositional change of the pigments by acclimation in terms of cultivation depths and pigment stability in vitro at condition of natural pigment composition in this alga.

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014

Keyword: Chlorophyll/carotenoid ratio; *Kappaphycus alvarezii*; photo-stability; pigment acclimation; pigment composition; thermo-stability.

*Corresponding author. Tel: +62 813 2636 0303, Fax+62 341 550 175
E-mail address: leenawaty.limantara@machung.ac.id

Peer-review under responsibility of the Scientific Committee of HK-ICONS 2014
doi:10.1016/j.proche.2015.03.028

1. Introduction

Marine organisms have been known as high potential natural resources. Seaweed as one of the most important marine organisms plays a role as primary producer in water ecosystem. In industrial view, seaweed becomes an important commodity since it has been widely described as a source of agar, carrageenan, and alginate followed by this bio product economic values[1–4]. Red alga, *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva originally comes from Philippines and, currently, its spread all over the world[5]. This species are firstly introduced in Indonesia in 1985 and became popular as one of the potential marine aquaculture products[6]. In 2010, Indonesia has reached the second position as the highest productivity of seaweed in the world. In order to make alternative option in developing *K. alvarezii* economical value by different products, its biomass availability is critical especially in Indonesia.

It has been reported that *K. alvarezii* classified into red, brown, and green variants from their different pigmentation[7], although most seaweed had been classified conventionally based on thallus coloration[8]. This varied coloration had been studied for decades and reported regarding differences in growth characteristics, photosynthesis, and carrageenan yield[7,9]. In this study, it is considered to use brown and green variant due to its availability and preferentially used as field and cultivation trials studies[10]. Regionally, *K. alvarezii* were grown in mostly coastal area in Indonesia. Madura has become one of the most potential product areas in East Java. Introduction of *K. alvarezii* in Java has begun in 1990. At that time (1990 to 1991), Madura has shown their potential ability by producing 100 ton to 500 ton at annum, while some region in South Sulawesi, currently as the main *K. alvarezii* producer in Indonesia, only produced less than 100 ton at annum[11].

The world consumption of *K. alvarezii* are mainly supplied by Philippines and Indonesia[12], and it is exponentially increasing due to the highly demand of kappa–carrageenan for industrial purposes. Presently, *K. alvarezii* is being used as potential source of carrageenan. The pigment-destructive methods were usually applied to produce total bleached carrageenan product. It is interesting to minimize the degradation of pigments, and utilize the pigments into valuable products. Some reports[13,14] described that *K. alvarezii* is potential producer of photosynthetic pigments such as zeaxanthin, chlorophyll *a*, and β-carotene. Those pigments are not only potentially to be developed into human-safe food colorant, but also it has an important health functional benefit, i.e. zeaxanthin which can be used as anti-prostate cancer and anti-age related macular degeneration[15,16], Chlorophyll *a* can be used as the main material for photodynamic therapy against cancer[17], and β-carotene as the antioxidant and precursor of vitamin A[18].

Unlike most species of Chlorophyta and Phaeophyta, as the members of Rhodophyta, *K. alvarezii* contains phycoerythrin as a protein-pigment complex, in addition to chlorophylls/carotenoids-containing complexes[19]. These complex systems allow this seaweed species to enhance its ability in harvesting green to yellow lights. There were several investigations on this species ability in triggering pigment production through different depth cultivation[20,21], however complete composition of chlorophyll and carotenoid pigments had not been reported. In this report, information about pigment composition of *K. alvarezii* var. green and brown which grown in different depths and in vitro study on the stability of crude pigments extracts with different chlorophylls/carotenoids ratios against heat and irradiation were provided. Considering its pigment application prospect, *K. alvarezii* was proved that is not only important in producing kappa-carrageenan but also potential in providing natural pigments for any useful purposes.

2. Materials and methods

2.1. Algae and cultivation

Red alga *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva var. brown and var. green were cultivated in Sumenep seashore (± 50 m from coastline), Padike village, Talango Island, Madura, East Java (S 7° 5' 18.0636", E 113° 56' 20.1804") (Fig. 1). Cultivation was carried out in a floating raft which had different depth positions of 0.2 m, 1 m and 2 m. *K. alvarezii* green and brown variants were measured 50 g in weight and tightened in raft with ropes and cultivated for 40 d. After cultivation, these samples were harvested and collected into dark plastic bag and kept at low temperature under the dark in an ice and sealed dark box during transportations.

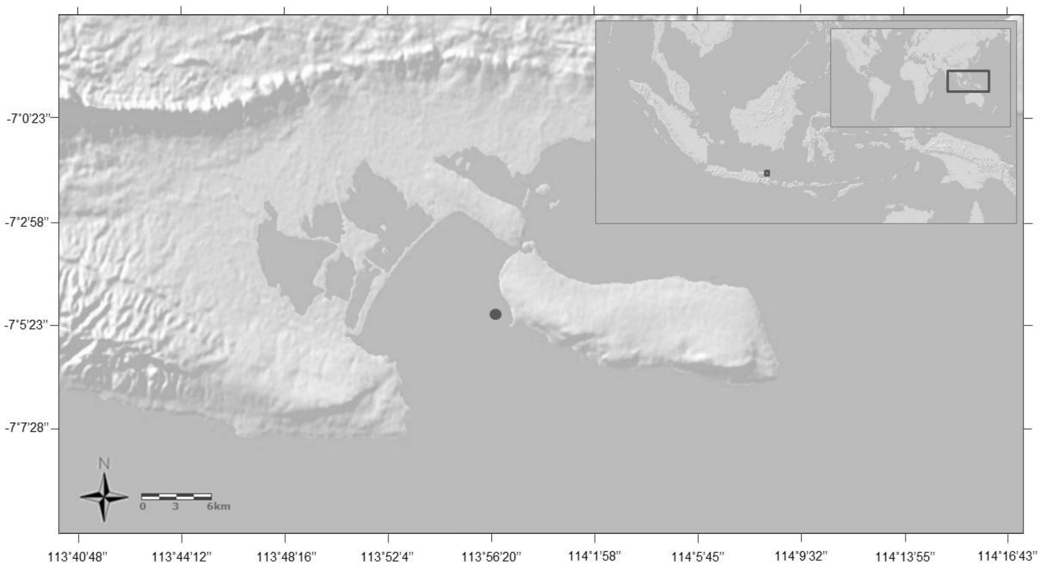

Fig. 1. *K. alvarezii* cultivation site (solid circle) was located in Padike village, Talango Island, Madura, East Java, Indonesia.

2.2. Chemicals

GR or HPLC grade chemicals and solvents were obtained from MERCK (Darmstadt, Germany). The solvents were filtered using polypropylene backed membrane filter (0.5 µm) (Whatman, Maidstone, UK) and degassed prior to use. Pre-injection pigment samples were filtered through a nylon membrane (0.2 µm) (Whatman). Ammonium acetate which was used in HPLC system was analytical reagent grade (Chameleon Reagent, Osaka, Japan).

2.3. Pigment extraction

Sample (4 g wet weight) was ground in a mortar after addition of a trace portion of sodium L-ascorbate and calcium carbonate to minimize oxidative reaction and reduce acidification, respectively, due to cell lysis. Pigments of *K. alvarezii* green and brown variants from different depths were extracted using 20 mL solvent mixture of acetone:methanol (3 : 7, in volume) and recovered by centrifuge at 550 rpm (60 rpm = 1 hertz) for 15 min. This step was repeated three times until the residue becomes colorless. The extracts were collected and filtered, then partitioned using diethyl ether, petroleum benzene, and saturated sodium chloride. Non-polar fraction (upper fraction) was then collected and concentrated using a rotary evaporator. All of these steps were done in dimmed light at room temperature (± 25 °C), and under nitrogen (ultra-high purity grade) (SAMATOR, Surabaya, Indonesia) atmosphere.

2.4. HPLC analysis

HPLC analysis was carried out with Liquid Chromatography (LC) 20AD which was equipped by photodiode array detector, SPD–M20A and column oven CTO–20A (Shimadzu, Kyoto, Japan). The analytical column was Shim–pack VP–ODS C18 (5 µm, 4 i.d. × 250 mm) column protected by guard column. The injection volume was 20 µL. HPLC analysis was performed using a tertiary solvent system consisted of methanol (A), acetone (B), and 1 M ammonium acetate (C) and gradient elution with a time program in the following: 0 min to 10 min, A : B : C = 80 : 10 : 10; 10 min to 25 min, 80 : 16 : 4; and 25 min to 80 min, 80 : 20 : 0, by volume. The flow rate was 1 mL · min^{-1} at a temperature of 30 °C and pigments were detected in the range of 190 nm to 800 nm.

2.5. Stability assays

Stability assay against thermal and irradiation treatments of crude pigment extracts from each sample was carried out with absorption spectroscopy at 300 nm to 800 nm in 100 % acetone. The starting sample was adjusted at Q_y band (665 nm) to give an absorbance of approximately 0.5 AU. Thermo stability assay was performed using water bath at 90 °C for 10 min, 20 min, 30 min, 60 min, 90 min, and 120 min. Thermal treatments were recorded using UV-Vis Spectrophotometer UV-1700 (Shimadzu). Photo-stability assay was carried out with a halogen lamp Intralux® 4100 (Volpi) at a light intensity of 1417 µmol photons $m^{-2} \cdot s^{-1}$ for about 45 min. A real time spectrum was measured using Multispec 1501 UV-Vis Spectrophotometer (Shimadzu) and the data were recorded every 5 min.

2.6. Data recording and analysis

Chromatograms were recorded using LC solution version 1.24 SP1 (Shimadzu). Absorption spectra and chromatograms were plotted using Plotx32 version 1.35 (created by Akifumi Ikehata, NFRI, Tsukuba, Japan) and Origin version 7.0 (Origin Lab Corp.).

3. Results and discussion

The advantage of isograms is to be able to easily compare the separation and concentration of some pigments in parallel without limiting selection to a given detection wavelength. As can be seen in Fig. 2, the blue color indicates chromatogram baseline (low intensity), while yellow to red color scales indicate absorption intensity. The isogram of purified standard pigments, such as zeaxanthin, chlorophyll *a*, and *β*-carotene, are shown in Fig. 2A. The separation of chlorophyll *a* is indicated by the increasing intensity of the color contours at 400 nm to 500 nm for Soret band and 550 nm to 650 nm for Qx and Qy bands. The carotenoid groups can also be identified by the color contour at 400 nm to 500 nm. The first peak-line (3.27 min) with absorbance peak range at 300 nm to 350 nm indicated solvent peak position. The second, the third and the fourth peak lines were the positions of zeaxanthin (19.65 min), chlorophyll *a* (37.85 min), and β-carotene (60.24 min), respectively. These pigments can be detected clearly by the isograms (Fig. 2. B, C, D, E) by observing the color contours.

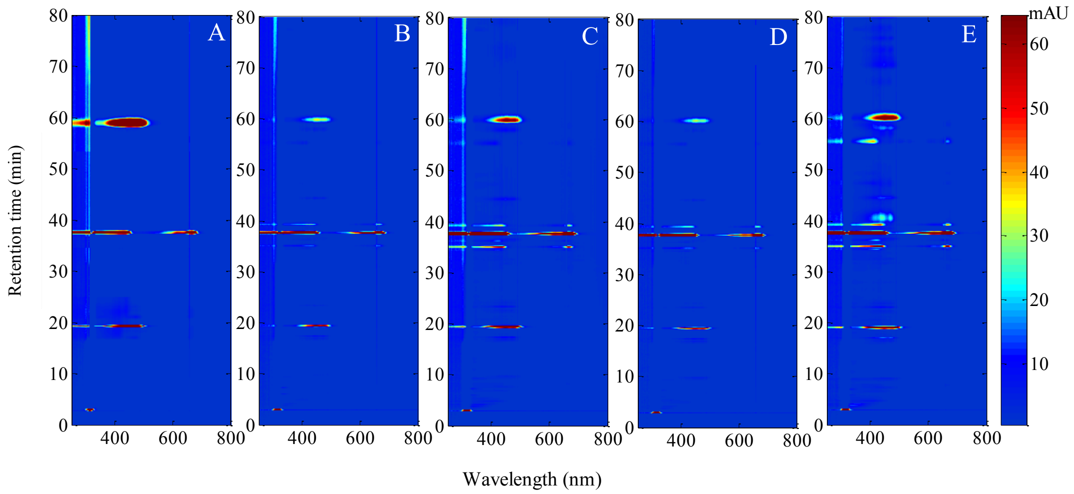

Fig. 2. HPLC isograms of the standard pigments, zeaxanthin, chlorophyll *a*, and β-carotene in order of elution (A), pigment extracts from *K. alvarezii* brown (B and C) and green (D and E) variants, which were cultivated in 1 m and 2 m depths, respectively. The red color on contour map indicates the apex of an absorption peak (in mAU), while the blue indicates baseline.

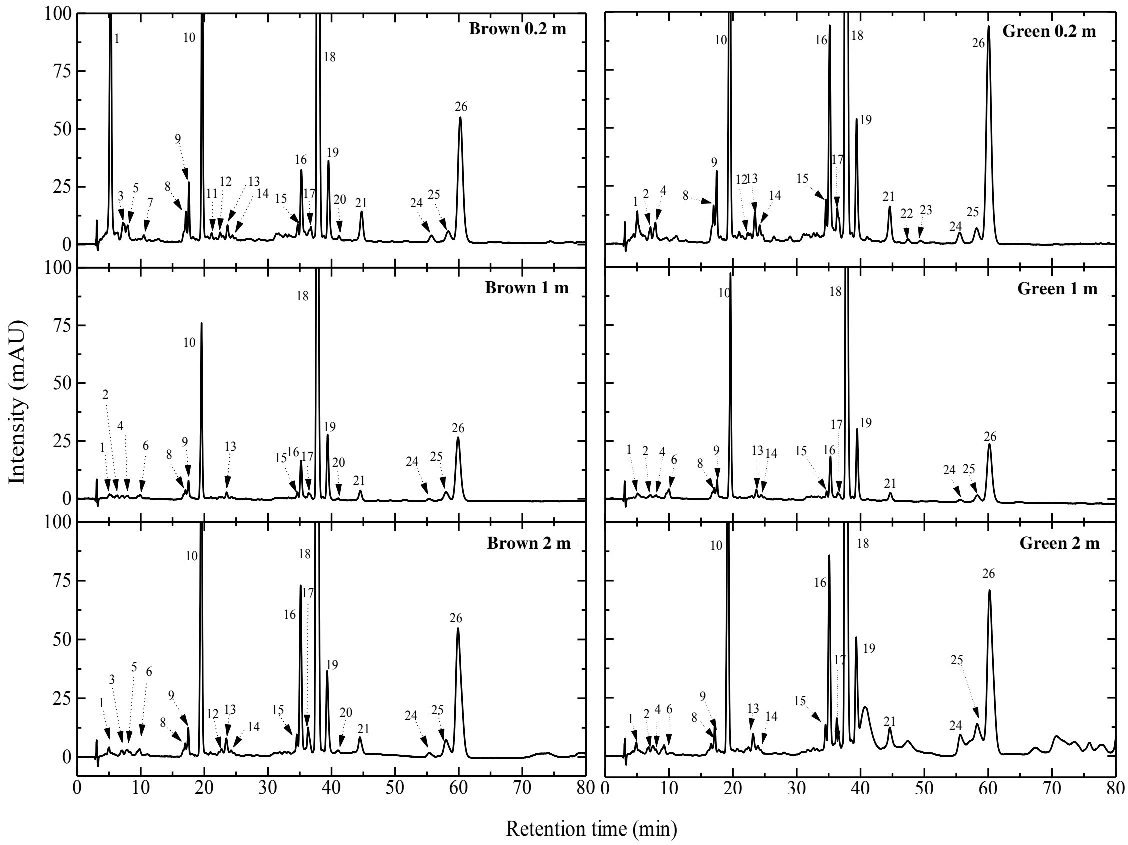

Fig. 3. HPLC Chromatograms of pigment extracts from *K. alvarezii* brown (left) and green (right) variants in different depths. Peak areas were calculated from selected wavelength at 430 nm.

In order to identify the carotenoid and chlorophyll groups in details, a single wavelength detection at 430 nm has been chosen. There are 26 peaks that can be clearly detected from the sample of *K. alvarezii* (Fig. 3, Table 1). The major pigments were identified as antheraxanthin (17.56 min), zeaxanthin (19.65 min), chlorophyll *a* (37.85 min), α-carotene (44.72 min), and *β*-carotene (60.24 min). The minor peaks were expected to be alteration products of the major pigments, i.e., chlorophylide *a* (5.29 min), pheophytin *a* (55.73 min), *cis* derivatives of carotenes/xanthophylls, and unidentified trace pigments. The composition of pigment from both variants has shown to be similar based on the number and the retention time of the peaks. This carotenoid composition in *K. alvarezii* is in agreement with those of other red algae[22–24].

Table 1 lists the identified pigments from the 26 detected peaks. In general, both variants had similar major pigments, but there were small differences in the presence of minor pigments and derivatives as can be seen in Table 1. Unidentified xanthophylls, chlorophylls, and their derivatives were mostly recorded up to 25 min. In addition, derivatives of some major pigments were also found from all samples, i.e., chlorophylide *a* (5.29 min), *cis*-antheraxanthin (16.99 min), and pheophytin *a* (55.73 min).

Fig. 3 shows that *K. alvarezii* contained similar composition of pigments even when it is cultured in different depth of water column. However, it is expected that concentrations of pigments were varied depending on the culture depth position in both variants. It is known that the different pigment composition of red algae is influenced by solar irradiance that penetrates into the water level[25]. Here the relative concentration of pigments is presented according to the peak areas (Table 1). The highest total concentration of chlorophylls and carotenoids in the green variant were obtained from the algae cultivated at 0.2 m depth. Relative concentration of total pigments decreased when it was grown deeper at 1 m depth, but it increased when algae were grown at 2 m depth. The ratio between chlorophyll and carotenoid pigments appears to decrease along with decreasing their cultivation positions.

Table 1. Identification of pigment in *Kappaphycus alvarezii* brown and green variants

Peak no*	t_{R**}	Identified pigment	Peak area at 430 nm**						λ_{mak***}
			Brown variant			Green variant			
			0.2 m	1 m	2 m	0.2 m	1 m	2 m	
1	5.29	chlorophyllide *a*	41.5	12.3	3.5	57.4	12.8	5.5	432, 610,665
2	7.09	xanthophyll group	-	7.8	-	10.7	4.5	6.7	427,(446),(665)
3	7.19	chlorophyll group	14.8	-	6.2	-	-	-	433,(627),667
4	7.81	Xanthophyll group	-	12.1	-	43.0	6.7	10.5	(423),442,(451)
5	7.95	Xanthophyll group	17.5	-	8.2	-	-	-	(424),443,(457)
6	9.94	Xanthophyll group	-	4.3	9.4	-	13.4	2.8	(420),442,(452)
7	10.48	chlorophyll group	7.6	-	-	-	-	-	412,(608),664
8	16.98	*α*-cryptoxanthin	0.5	4.1	14.5	37.1	5.4	26.6	418,443,470
9	17.45	antheraxanthin	38.2	15.2	21.8	53.0	15.8	7.0	418,443,470
10	19.50	zeaxanthin	274.9	123.1	364.8	547.0	163.4	433.7	(421),447,474
11	21.15	*cis*- xanthophyll	8.9	-	-	-	-	-	(413),(441),(466)
12	22.48	*cis*- xanthophyll	9.0	-	1.7	5.8	-	-	(423),(445),(457)
13	23.64	*cis*- xanthophyll	16.5	123.1	19.9	28.3	10.5	22.4	(415),(440),(467)
14	24.40	*cis*- xanthophyll	5.7	-	19.9	16.3	4.9	2.6	(415),(443),(466)
15	34.58	chlorophyll *a*-like	16.2	6.8	15.9	35.9	7.6	33.4	432,(610),664
16	35.17	chlorophyll *a*-like	75.5	38.4	153.7	211.8	41.3	189.1	432,(610), 664
17	36.35	chlorophyll *a*-like	9.8	7.3	32.8	47.9	12.2	50.0	430, (616), 665
18	37.72	chlorophyll *a*	1 845.8	956.0	2 152.7	2 736.0	951.7	2 170.1	430,618,664
19	39.37	chlorophyll *a'*	73.6	61.5	83.3	112.9	65.7	115.1	430,(617),663
20	41.18	chlorophyll *a'*	3.6	1.4	3.2	-	-	-	432,(617),665
21	44.58	*α*-carotene	44.4	14.9	27.4	54.0	12.0	47.0	(418),443,471
22	47.45	pheophytin *a*-like	-	-	-	6.6	-	-	410,(610),662
23	49.39	pheophytin *a*-like	-	-	-	4.6	-	-	409,(609),664
24	55.73	pheophytin *a*	14.6	4.3	10.6	24.3	4.5	61.1	408,(610),665
25	58.35	carotenoid group	261.9	203.7	461.1	38.3	16.5	132.5	(415),445,470
26	60.24	*β*-carotene	302.4	150.1	339.9	514.9	139.7	457.4	(428),450,476
		Total chls peak area	2 103	1 088	2 461.9	3 237.4	1 095.8	2 624.3	
		% Total chls peak area	68.2	62.2	65.6	70.5	73.6	69.5	
		Total cars peak area	979.9	658.4	1288.6	1348.4	392.8	1149.2	
		% Total cars peak area	31.7	37.7	34.3	29.4	26.3	30.4	
		Chlorophylls/carotenoids ratio	2.1	1.6	1.9	2.4	2.7	2.2	

*according to numbering of chromatogram peaks
**acquired from original Shimadzu HPLC software: LC Solution ver. 1.24 SP1
***Represent I-II-III bands for carotenoids and Soret, Qx, and Qy bands for chlorophylls

K. alvarezii variants showed different behaviour. In brown variant grown at 0.2 m depth, relative concentration of total chlorophylls was higher than the carotenoids (chls 68.2 % and cars 31.7 %). At 1.0 m depth, the total chlorophylls decreased, but the total carotenoid concentrations increased (chls 62.2 % and cars 37.7 %). At 2.0 m depth, the total chlorophyll increased and the total carotenoid decreased (chls 65.6 % and cars 34.3 %). A similar fluctuation was also observed for the ratio between chlorophylls and carotenoids. Table 1 shows that total chlorophyll and carotenoid can reached to highest concentration, when the green variant was grown at 0.2 m depth or when the brown variant was grown at 2 m depth. The ratio between total chlorophyll and carotenoid seems to vary as a function of different depth of growth conditions. Accessory pigment such as phycoerythrin is also produced in red algae[26,27], although this experiments focused only on chlorophylls and carotenoids distribution. Phycoerythrin is important for absorption of light in the spectrum region where chlorophylls and carotenoids are unable to absorb efficiently. It is reported that *K. alvarezii* brown variant has higher phycoerythrin concentration than green variant[28].

While small amount of light can penetrate into the depth of seawater, some photosynthetic organisms are capable of controlling their light capture by producing different photosynthetic pigments[29,30]. This mechanism is very important in order to maintain their photosynthetic activities working effectively. In deeper positions under the water, light intensity and quality have been filtered. Blue to green lights (400 nm to 450 nm) penetrate further into the seawater, while the rest of visible lights have been scattered or absorbed[31]. This might explain that the total chlorophyll content was increased significantly when *K. alvarezii* was grown at 2 m depth. In this case, the Soret band (350 nm to 450 nm) of the chlorophylls plays the role of utilizing the blue light. In addition the total carotenoids content was also increased to play the role in light harvesting at blue-green region and photoprotection.

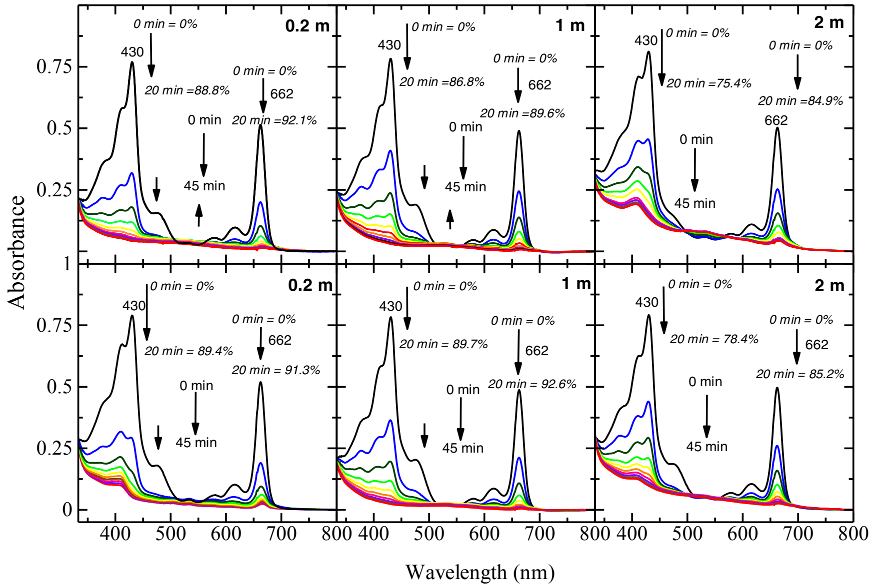

Fig. 4. UV–Vis spectra of crude pigments extracted from *K. alvarezii* green (top) and brown (bottom) variants during irradiation treatment. Samples were irradiated at 1417 μmol photons $m^{-2}\cdot s^{-1}$ for 45 min

In the examination of pigment stabilities, here the crude pigments extracts were exposed under irradiation and heat. Fig. 4 shows the changes of the absorption spectrum of crude pigments extracts during irradiation. Interestingly, the degradation of pigments showed different pattern. In brown variant, fast degradation of the Soret and Qy bands was observed in the pigments extract from the samples at 0.2 and 1 m depth. In the green variant, fast degradation of pigment extract was observed from the samples grown at 0.2 m depth. Slow degradation could be seen in both cases from the samples grown at 2 m depth. The degradation pattern may change in relation to the ratio between total chlorophylls and carotenoids. As shown in Table 1, the seaweed produced more carotenoids than chlorophylls.

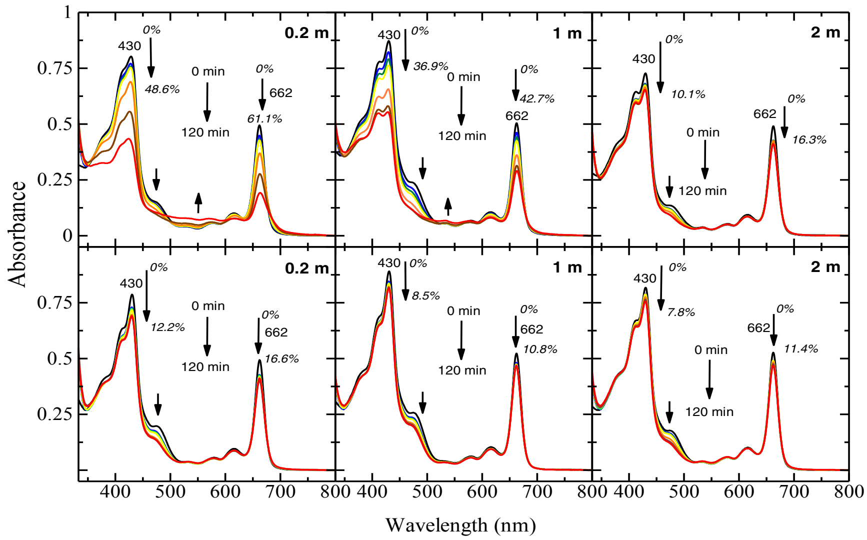

Fig. 5. UV–Vis spectra of crude pigments extracted from *K. alvarezii* brown (bottom) and green (top) variants during thermal treatment. Samples were heated in 90 °C for 120 min.

Fig. 5 shows the changes of the spectrum of pigments extracts during the exposure of high temperature (90 °C). Unlike the photo-stability assay, in thermo-stability assay, the degradation of Soret and Q_y bands was slow in the extracts from seaweed grown 2 m depth in both green and brown variant as compared to those grown at 0.2 m depth. Moreover, brown variant with low chls/cars ratio was more resistant to heat treatment than green variant with high chls/cars ratio. When the results of thermo- and photo-stability (Fig. 4 and Fig. 5) and the ratio of total pigments (Table 1) are compared, there is correlation between the content of carotenoid and the reduction of chlorophyll degradation after exposure of light as well as high temperature.

4. Conclusion

Here, the investigation results reported the variation of total chlorophylls and carotenoid contents in *K. alvarezii* var. brown and var. green that were grown at different depth under the seawaters. The results show that the high concentrations of chlorophylls were produced with when the seaweeds were grown at deeper position under the seawaters. This is probably due to a response toward the available blue-green light that can penetrate into the seawater. Along with the increase in chlorophyll concentration, the carotenoid content was also increased. The increase in carotenoid content was important to maintain the stability of chlorophylls under excess of light and exposure of high temperature.

Acknowledgements

This project was supported by National Innovation System Research Grant (RT-2013-0172, No: 187/M/Kp/XI/2012 and RT-2014-0432, No: 288/M/Kp/XII/2013), National Research Centre of Excellence (Pusat Unggulan Iptek) Program (SK No. 284/M/Kp/XI/2013) provided by Indonesian Ministry of Research and Technology, and Ma Chung Research Grant IV, Grant number 05/MACHUNG/LPPM–MRGIV/II/2012. Heriyanto acknowledges the scholarship from the Foundation for Polish Science (TEAM/2010-5/3).

References

1. Phang S. Seaweed resources in Malaysia: current status and future prospects. *Aquatic Ecosystem Health & Management* 2006;9:185–202.
2. Msuya FE, Buriyo A, Omar I, et al. Cultivation and utilisation of red seaweeds in the Western Indian Ocean (WIO) region. *Journal of Applied Phycology* 20014;26:699–705.
3. Xu J, Gao K. Growth, pigments, UV–absorbing compounds and agar yield of the economic red seaweed Gracilaria lemaneiformis (Rhodophyta) grown at different depths in the coastal waters of the South China Sea. *Journal of Applied Phycology* 2008;20:681–686.
4. van Hal JW, Huijgen W, Lopez-Contreras A. Opportunities and challenges for seaweed in the biobased economy. *Trends in Biotechnology* 2014;32:231–233.
5. de Barros–Barreto MBB, Marinho LC, Reis RP, da Mata CS, Ferreira PCG. *Kappaphycus alvarezii* (Gigartinales, Rhodophyta) cultivated in Brazil: is it only one species?. *Journal of Applied Phycology* 2013;25: 1143–1149.
6. Ask E, Batibasaga A, Zertuche-Gonzalez J, De San M. Three decades of *Kappaphycus alvarezii* (Rhodophyta) introduction to non-endemic locations. *Proc. Int. Seaweed Symp.* 2003;49–57.
7. Loureiro RR, Reis RP, Critchley AT. In vitro cultivation of three *Kappaphycus alvarezii* (Rhodophyta, Areschougiaceae) variants (green, red and brown) exposed to a commercial extract of the brown alga *Ascophyllum nodosum* (Fucaceae, Ochrophyta). *Journal of Applied Phycology* 2010;22:101–104.
8. Hegazi MM, Ruzafa AP, Almela L, Candela ME. Separation and identification of chlorophylls and carotenoids from *Caulerpa prolifera*, *Jania rubens* and *Padina pavonica* by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* 1998;829: 153–159.
9. Le HD, Sato T, Shibata H, Hori K. Biochemical comparison of lectins among three different color strains of the red alga *Kappaphycus alvarezii*. *Fisheries Science* 2009;75:723–730.
10. Munoz J, Freile-Pelegrin Y, Robledo D. Mariculture of *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae) color strains in tropical waters of Yucatan, Mexico. *Aquaculture* 2004;239:161–177.
11. Luxton D. Aspects of the farming and processing of Kappaphycus and Eucheuma in Indonesia, in: Fourteenth International Seaweed Symposium. *Springer* 1993;365-371.
12. G Thirumaran, P Anantharaman. Daily growth rate of field farming seaweed *Kappaphycus alvarezii* (Doty) Doty ex P. Silva in Vellar Estuary. *World Journal of Fish and Marine Sciences* 2009;1:144–153.
13. Andersson M, Schubert H, Pederson M, Snoeijs P. Different patterns of carotenoid composition and photosynthesis acclimation in two tropical red algae. *Marine Biology* 2006;149:653–665.

14. Schubert H, Andersson M, Snoeijs P. Relationship between photosynthesis and non-photochemical quenching of chlorophyll fluorescence in two red algae with different carotenoid compositions. *Marine Biology* 2006;149:1003–1013.
15. Cohen JH, Kristal AR, Stanford JL. Fruit and vegetable intakes and prostate cancer risk. *Journal of the National Cancer Institute* 2000;92:61–68.
16. Snodderly DM. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *The American Journal of Clinical Nutrition* 1995;62:1448–1461.
17. Nyman ES, Hynninen PH. Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy. *Journal of Photochemistry and Photobiology B* 2004;73:1–28.
18. Gross J. *Pigments in vegetables: Chlorophylls and Carotenoids*. New York: Van Nostrand Reinhold; 1991.
19. Liu LN, Chen XL, Zhang YZ, Zhou BC. Characterization, structure and function of linker polypeptides in phycobilisomes of cyanobacteria and red algae: an overview. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 2005;1708:133–142.
20. Borlongan IAG, Tibubos KR, Yunque DAT, Hurtado AQ, Critchley AT. Impact of AMPEP on the growth and occurrence of epiphytic Neosiphonia infestation on two varieties of commercially cultivated *Kappaphycus alvarezii* grown at different depths in the Philippines. *Journal of Applied Phycology* 2011;23:615–621.
21. Hurtado A, Critchley A, Trespoey A, Bleicher-Lhonneur G. Growth and carrageenan quality of *Kappaphycus striatum* var. sacol grown at different stocking densities, duration of culture and depth. *Journal of Applied Phycology* 2008;20:551–555.
22. Palermo JA, Gros EG, Seldes AM. Carotenoids from three red algae of the Corallinaceae. *Phytochemistry* 1991;30:2983–2986.
23. Marquardt Jr, Hanelt D. Carotenoid composition of *Delesseria lancifolia* and other marine red algae from polar and temperate habitats. *Eur J Phycol* 2004;39:285–292.
24. Schubert N, Garcia-Mendoza E, Pacheco-Ruiz I. Carotenoid composition of marine red algae. *J Physicol* 2006;42:1208–1216.
25. Dawes C. Irradiance acclimation of the cultured Philippine seaweeds, *Kappaphycus alvarezii* and *Eucheuma denticulatum*. *Botanica Marina* 1992;35:189–196.
26. Aguirre-von-Wobeser E, Figueroa F, Cabello-Pasini A. Photosynthesis and growth of red and green morphotypes of *Kappaphycus alvarezii* (Rhodophyta) from the Philippines. *Marine Biology* 2001;138:679–686.
27. Zhao S, He P. Effects of light intensity and salinity on growth of *Kappaphycus alvarezii*. *Journal of Tropical Oceanography* 2009;28:24–29.
28. Naguit MRA, Tisera WL. Pigment analysis on *Eucheuma denticulatum* (collins & hervey) and *Kappaphycus alvarezii* (doty) cultivars cultured at different depths. *Threshold* 2009;4:29–37.
29. Scheuring S, Goncalves RP, Prima V, Sturgis JN. The Photosynthetic Apparatus of *Rhodopseudomonas palustris*: Structures and Organization. *Journal of Molecular Biology* 2006;358:83–96.
30. Sineshchekov OA, Jung KH, Spudich JL. Two rhodopsins mediate phototaxis to low-and high-intensity light in *Chlamydomonas reinhardtii. Proceedings of the National Academy of Sciences USA* 2002;99:8689–8694.
31. Wozniak B, Dera J. *Light absorption in sea water.* New York: Springer; 2007.