Pigment concentration of red algae, *Kappaphycus alvarezii* (Doty) Doty ex Silva during the cultivation in the coastal waters of Nain Island, North Sulawesi, Indonesia

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Abstract. Cultivation of brownish red algae *Kappaphycus alvarezii* in controled conditions was conducted in order to obtain informations about the growth and concentration of chlorophyll and carotenoid pigments. The cultivation was carried out for 9 weeks namely from March to April 2020 in coastal waters of Nain Island that was quite far from the mainland of Manado City. Pigment concentrations were obtained through extraction based on different polarity in the total pigment extract in petroleum ether added with methanol, KOH in methanol and diethyl ether. Red algae as much as 17 thalli with an initial weight of 100 g were cultured using floating method. The distance between thallus was 25 cm. A total of three thalli was taken every two weeks, namely at week 3, 5, 7, and 9 and grouped into 3 periods. After measuring the weight gain, the algae were then exposed to pigment extraction. *K. alvarezii* experienced weight gain, but the relative growth rate showed a decrease in the 3rd period. The pattern of algae relative growth was identical with the pattern of temperature fluctuation during cultivation. Pigments obtained from the separation using different solvents were chlorophyll a, chlorophyll d, ß-carotene and xanthophyll. It was found that the concentration of chlorophyll a and d increased as the weight gain increased. Likewise, the concentration and quantity of ß-carotene and xanthophyll pigments were in line with the growth rate pattern of *K. alvarezii*.

Key Words: macroalgae, chlorophyll, carotenoid, carotene, xanthophyll.

Introduction. *Kappaphycus alvarezii* is a red macroalgae or seaweed that has an important economic value. This macroalgae is a producer of kappa-carrageenan so that it can be a source of raw material for carrageenan extraction to be used in various industries of food, cosmetics, pharmaceuticals and other industrial applications (Pereira 2016). The carrageenan content of *K. alvarezii* originating from Arakan Village, North Sulawesi has immunostimulating activity (Kreckhoff et al 2019). Nain Island, located close to the capital city of North Sulawesi Province, is one of the macroalgae cultivation areas in North Sulawesi. One type of macroalgae cultivated in this area is *K. alvarezii*.

K. alvarezii has round thalli, cylindrical and flattened, smooth surface, branched irregularly, and has spines that vary in length from 1 to 17 mm (Pereira 2016). Thallus is gelatinous or soft cartilogenous like cartilage (Trono 1997; Prasetyowati et al 2008). In nature, *K. alvarezii* algae generally have two colors, namely brown and green (Calumpong & Meñez 1997). *K. alvarezii* cultivated in the Philippines and Indonesia was also found to be green and brown (Indriatmoko et al 2015; Pereira 2016). The habitat of *K. alvarezii* is in the sublittoral zone in the area of coral reef flats and lives on sandy to rocky substrates where water flow in these areas is slow to moderate (Qin 2018).

The chemical composition of *K. alvarezii* is carbohydrate 65.20%, protein 3.40%, fat 1.10% and ash 11.57% (Khalil et al 2018). According to Rajasulochana et al (2012), *K. alvarezii* has antioxidant potential which comes from the content of vitamin C, vitamin E and metals (selenium and magnesium). Furthermore, according to Prabha et al (2013), *K. alvarezii* has antibacterial activity because it has several compounds including alkaloids, flavonoids, terpenoids, tannins and saponins.

K. alvarezii cultivation has the potential to be developed because it contains beneficial chemical compounds especially pigments. According to Thirumaran et al (2009), *K. alvarezii* contains carotenoid and chlorophyll pigments. Its main pigment contents are carotene, xanthophyll and xanthophyll derivatives as well as chlorophyll a and chlorophyll derivatives. According to de Fretes et al (2012), the dominant pigments in red algae are chlorophyll a, chlorophyll d, zeaxantin, lycopene, cryptoxanthine, α-carotene, β-carotene, lutein, and picobillin.

The development of macroalgae in aquaculture activities is not only influenced by the cultivation technique and water quality, but also by photosynthetic pigment, namely chlorophyll-a. Chlorophyll-a is important for the survival of algae or for competing with other organisms in a certain habitat (Ming-Li et al 2010). Meanwhile, carotenoid pigments contained in *K. alvarezii*, have a role as accessory pigments in the photosynthesis process.

According to Lila (2004), each type of pigment has various health benefits. Mantiri & Kepel (1999) stated that carotenoid pigments functioned as natural dyes, could be used as immune materials and as raw materials for pharmaceutical, cosmetic, and foodstuff industries. Β-carotene pigments have a physiological function as a precursor of vitamin A. According to Britton et al (2008), β-carotene pigments are useful for the human body and physiological activities especially eye, protecting skin surface tissues from stinging sunlight, as antioxidants and anticancer.

In North Sulawesi, the red algae *K. alvarezii* is abundant because it has been widely cultivated however this alga also was found growing naturally on the Minahasa peninsula which is only a few miles from Nain Island (Kepel et al 2019, 2020). However, there is no research conducted yet concerning pigments based on growth. Therefore, this study aimed to analyze the concentration of carotenoid and chlorophyll pigments in *K. alvarezii* algae cultivated on Nain Island.

Material and Method

Culture of K. alvarezii. In general, this type of algae is widely cultivated in the coastal waters of Nain Island, which was used as the research location (Figure 1). Nain Island has a huge coral reef zone that provides a supply of nutrients needed by these algae to grow. According to Trono (1997), coral reef areas were good places for algal growth. This algae cultivation was carried out in March - April 2020 in hot and rainy condition interspersed. The culture was conducted under controled conditions aiming to measure the the weight gain of the thallus.

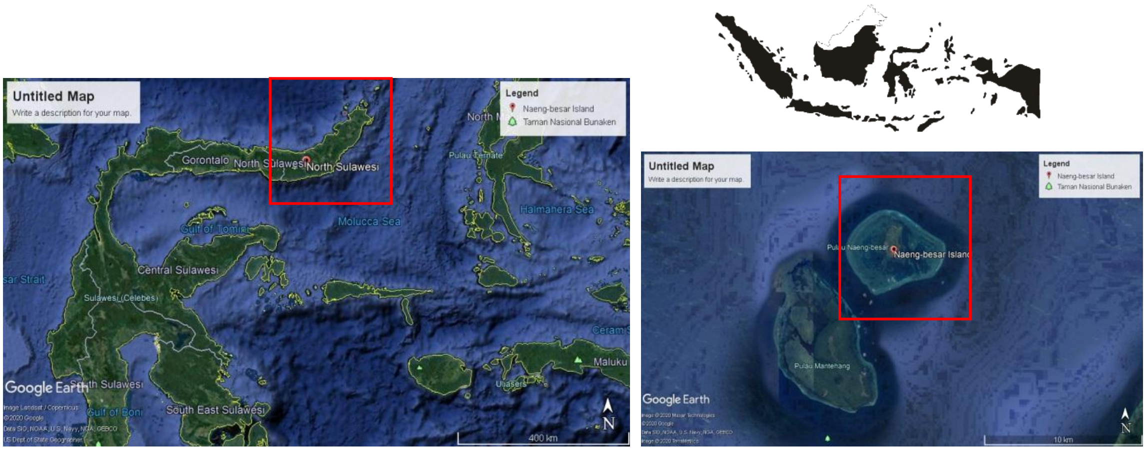

Figure 1. Nain Island of North Sulawesi, Indonesia.

K. alvarezii was cultured using the floating method as described by Qin (2018). The culture site had a water depth between 1.5 and 2.0 m during the lowest tide. The culture was adjusted in accordance to the tidal conditions in the coastal waters of Nain Island. A total of 17 thalli were tied to a rope with an initial weight of 100 g and the distance between the thallus was 25 cm. At the end of each rope a ballast was placed (Figure 2).

The culture was carried out for 9 weeks. Measurement of weight gain and collection of algal thalli for pigment analysis were carried out every two weeks.

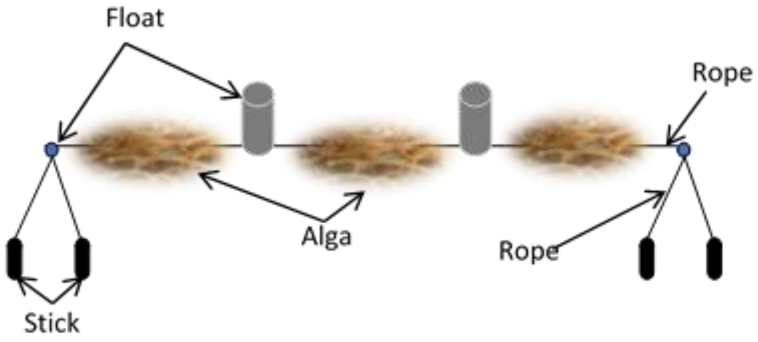

Figure 2. Culture technique of *Kappaphycus alvarezii*.

Pigment extraction. Algal thalli were taken every two weeks, namely week 3, 5, 7 and 9, each with three replications. Furthermore, the carotenoid and chlorophyll pigments were extracted to determine the pigment concentration during the culture period. The initial extraction was Total Pigment Extract (EPT) from each thallus according to the culture time. Each thallus weighing as much as 50 g was crushed with acetone PA and added with Petroleum ether PA, forming EPT. The total pigment extract in petroleum ether was continued to an extraction step based on different polarity with the addition of a PA methanol solution, so that two layers were formed, the upper layer (PE) and the lower layer (Met):

A. The top part of the pigment was added with 15 mL of KOH in methanol, so that two layers were formed namely: (1) The yellow top layer was the carotene pigment in Petroleum ether and (2) The green bottom layer was the chlorophyll a in methanol pigment.

B. The lower part of the pigment was added with diethyl ether and KOH in methanol, then it was divided into two layers, namely (3) the yellow upper layer was the xanthophyll pigment in diethyl ether and (4) the green bottom layer was the chlorophyll pigment in methanol.

The results of each extract were then analysed using a UV-VIS GBC CINTRA 10 spectrophotometer with a wave length range of 380-550 nm for carotenoids (Britton et al 1995) and absorption spectrophotometer with a wave length of 350-700 nm for chlorophyll (Harborne 1987). The maximum absorption peak of the spectrophotometer formed was used to obtain the concentration of carotenoid pigments (Britton et al 1995) and chlorophyll pigments based on the mathematical formula of Jeffrey & Humprey (1975) and Harborne (1987).

Analysis of growth data. The formula to determine the growth rate was:

$$RGR = \frac{\text{In Wt} - \text{In Wo}}{t}$$

Where:
RGR = relative growth rate
Wo = initial weight of the thallus
Wt = final weight of thallus
t = time

Results and Discussion

Macroalgae growth. The cultivated *K. alvarezii* had a brownish colour as shown in Figure 3. According to Ahda et al (2005), the colour diversity of *K. alvarezii* is caused by environmental factors and is a chromatic adaptation process, namely the adjustment of the proportion of pigments with various lighting qualities.

Figure 3. Macroalgae *Kappaphycus alvarezii* with greenish brown color.

The growth of *K. alvarezii* in the coastal waters of Nain Island within two weeks showed an increase in weight gain. For the 17 thalli, the initial weight of each thallus was 100 g at week 1. At week 3, it was found that the mean weight of thallus was 135 g. Furthermore, at week 5, the average weight was 186 g. At the 7th week, the average weight was 260 g. At the 9th week, the average weight was 355 g. The growth of macroalgae is shown in Figure 4A.

Every two weeks the thallus *K. alvarezii* experienced an increase in weight gain, but the relative weight gain rate showed a decrease in the 3rd period (Figure 4B). The relative weight gain rate showed an increase of 2.26% day^{-1} during the culture. The relative weight gain rate increased from the 1st period (between the 1st and 3rd week) with 2.14% day^{-1}, from the 2nd period (between the 3rd and 3rd week 5) as much as 2.28% day^{-1} and continued to increase to 2.39% day^{-1} in the 3rd period (between the 5th and 7th week). Furthermore, it decreased in the 4th period (between the 7th and 9th week) to be 2.20% day^{-1}. In the fourth period, although the relatively weight gain rate decreased, the absolute weight gain of the thallus continued.

According to Salisbury & Ross (1995), growth is the increase in the size of an individual in terms of volume, weight and number of cells. Growth occurs due to cell division of the organism's body and the results of division will increase in volume or size, so that the stem cell will experience aging. Kasim et al (2019) reported a weight gain of *K. alvarezii* with 50 g of initial weight (Wo) to 262 g during 50 days of cultivation. In that study, the water quality for chemical concentrations including ammonia, nitrate + nitrite and phosphate were in normal conditions.

The rate of relative weight gain of *K. alvarezii* in coastal waters of Nain Island supposed to be influenced by fluctuations in light intensity during cultivation. This was indicated by an increase in temperature in the first three weeks and decreased in week 4. The pattern of relative growth rate of algae was similar to the pattern of temperature fluctuation during cultivation, where a decrease in temperature occurred at week 4 was in line with a decrease in the relative weight gain of the algae (Figure 4C). Jamal et al (2011) stated that the complex interaction between light radiation, temperature, nutrients and current strength at the cultivation site played an important role for the growth of *Kappaphycus*. According to Hurd et al (2014), changes in sea water temperature were directly related to the amount of light reaching the sea. Qin (2018) stated that optimal growth of macroalgae required sunlight in shallow waters, ranging from 30-50 cm. In this research activity, algae culture was carried out at the optimal range of sunlight. Furthermore, Irfan et al (2020) reported that the growth of *K. alvarezii* depended on ecological factors including temperature and brightness suitable for its cultivation activities. Ecological impacts on the sustainability of environmental resources and ecosystems provide benefits for sustainable macroalgae culture activities.

The temperature at the coastal waters of Nain Island during the research fluctuated from 26 to 30°C. Temperature has an important role in the metabolic process of *K. alvarezii*. Organisms are able to perform optimal metabolism at a proper water temperature. According to Lüning (1990), the temperature of water is very specific in influencing the growth of macroalgae. High water temperature, which is more than 36ºC, causes mortality to macroalgae. This can affect the photosynthesis process, enzyme damage, and membrane instability. At the low water temperature, which is below 20ºC, the protein and fat membranes will be damaged and form crystals. This is very influential for macroalgae life. According to Barsanti & Gualtieri (2006), the most important parameters that regulate macroalgae growth are the quantity and quality of nutrients, light, pH, turbulence, salinity, and temperature as well as the photosynthesis process. The algae will be able to synthesize all the biochemical compounds essential for growth.

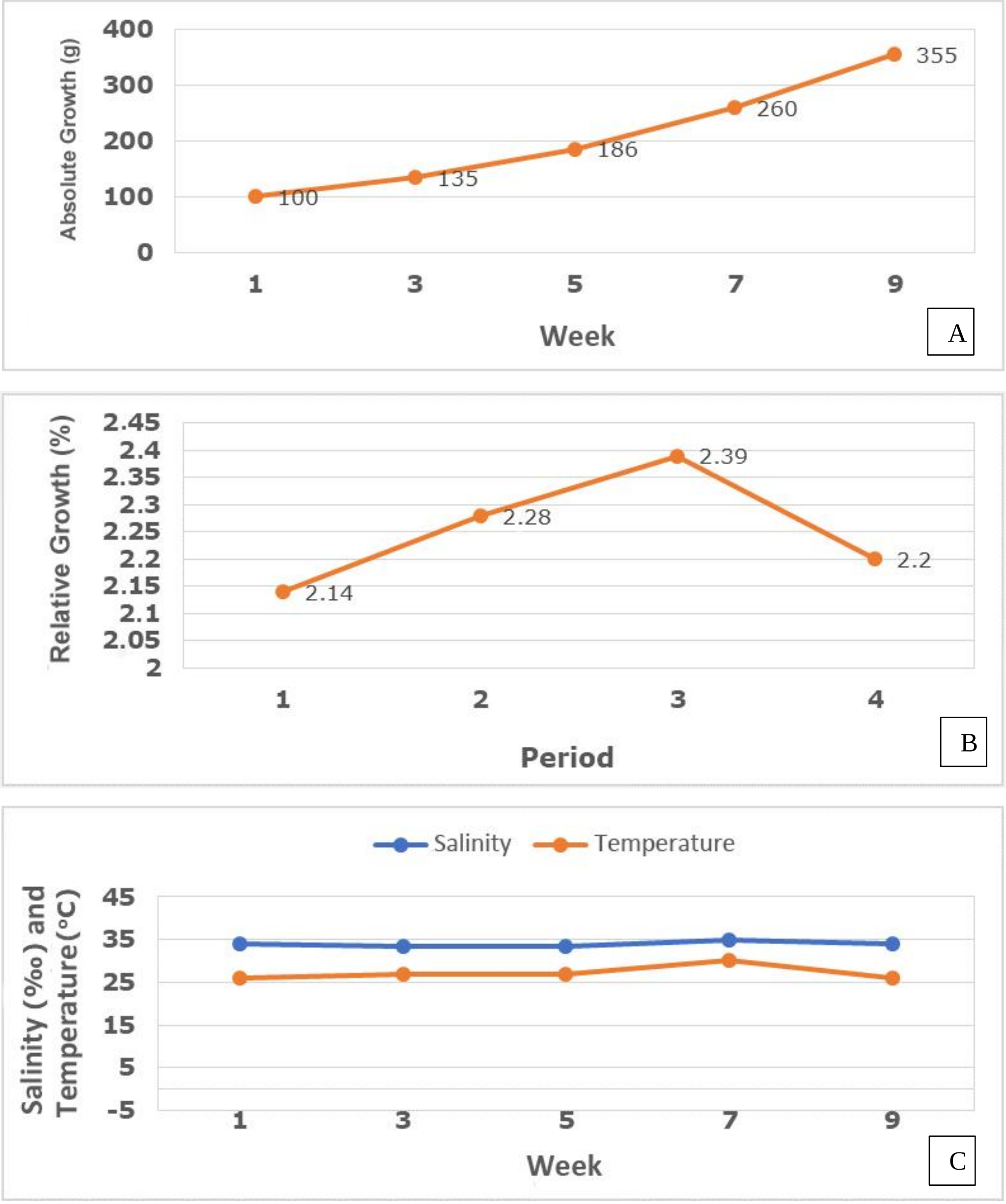

Figure 4. (A) Absolute growth of *Kappaphycus alvarezii*, (B) Relative growth of *K. alvarezii* and (C) Temperature (ºC) and salinity (‰) ranges during the research.

Temperature is an important physical factor. Its changes tend to influence various chemical processes that occur simultaneously in plant and animal tissues and can affect the biota as a whole. Ain et al (2014) reported that water temperature can affect several physiological functions of macroalgae such as photosynthesis, respiration, metabolism, growth and reproduction. According to Poncomulyo et al (2006), sea water temperature is affected by sunlight, depth, currents and tides. According to Parenrengi et al (2006) and Setiyanto et al (2008), the water temperature range for *K. alvarezii* growth is 27-30°C.

The salinity in the coastal waters of Nain Island was 33.5-35‰ and the optimum salinity in the present study occurred at the time when the maximum growth was achieved in the 7th week, namely 35‰ (Figure 4C). This indicated that the salinity conditions of the coastal waters of Nain Island could support the growth of *K. alvarezii* during the culture. According to Choi et al (2010), macroalgae will experience slow growth, if the salinity is too low (less than 15 ppt) or too high (more than 35 ppt) from the salinity range that corresponds to its life requirements for a certain period of time. Qin (2018) stated that the main factor determining the success or failure is selecting the appropriate site. Occasionally, those far from fresh water sources such as small river, etc., have been proven to be good sites. According to Kasim et al (2019), the most prominent physical factor affecting the growth of *K. alvarezii* is salinity because salinity greatly affects the macroalgae metabolism.

Yuliana et al (2015) reported that salinity affected the distribution of macroalgae in the sea. *K. alvarezii* is stenohaline, which is susceptible to wide salinity fluctuations. Most macroalgae have a low tolerance to changes in salinity. Sutresno & Prihastanti (2003) explained that optimum salinity could promote macroalgae to grow optimally, because the functional balance of the cell membrane could be maintained, especially in osmotic regulation in macroalgae and environmental fluids. This balance would facilitate the absorption of nutrients to support photosynthesis, so that macroalgae growth could be optimal.

Pigment concentration

Chlorophyll. The results showed that the pigments obtained were chlorophyll a and chlorophyll d. This is consistent with the statement of Harborne (1987) and Hurd et al (2014), that red algae contains two types of pigments, namely chlorophyll a and chlorophyll d. Figure 5 showed that during the research at weeks 3, 5, 7 and 9, the concentration of chlorophyll a and chlorophyll d recorded an increase. The concentration of chlorophyll d was higher than chlorophyll a. The lowest concentration of chlorophyll a was observed at week 3 (6.14 μg g^{-1}). At week 5, the concentration of chlorophyll a increased to 7.38 μg g^{-1} and at week 7 increased to 10.02 μg g^{-1}. At the end of research at week 9, the concentration of chlorophyll a continued to rise until 18.32 μg g^{-1}. The recorded concentration of chlorophyll d at week 3 was 16.36 μg g^{-1}, and slightly increased at week 5 and 7 to be 16.41 μg g^{-1} and 16.87 μg g^{-1}, respectively. At week 9, the concentration increased up to 21.42 μg g^{-1} (Figure 5).

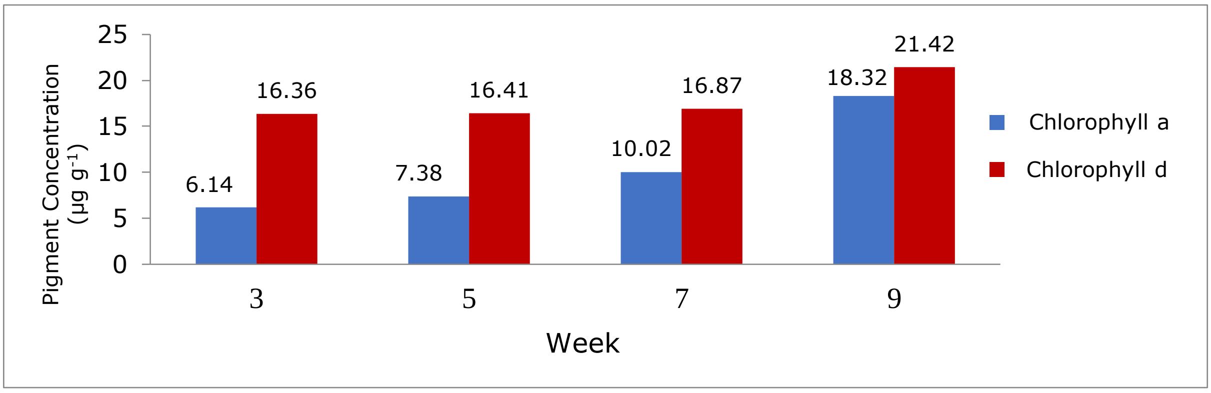

Figure 5. Concentration of of chlorophyll a and chlorophyll d of *Kappaphycus alvarezii* during the two weeks of culture.

The increasing pattern of chlorophyll a and chlorophyll d concentrations were consistent with the weight gain pattern of the *K. alvarezii* thalli. This was due to the weight of thalli which continued to increase during the cultivation. The increase in weight gain of thalli resulted in the continue growing of cells followed by the development of chloroplast cells through photosynthetic reactions. Thus, the chlorophyll a and d concentrations as pigments contained in *K. alvarezii* would also develop.

During the cultivation of *K. alvarezii*, chlorophyll a and accessories pigments plays a role in absorbing sunlight for the photosynthesis process. Chlorophyll a is the main pigment in red algae (Jupin & Lamant 1999). Hurd et al (2014) stated that the chlorophyll d concentration was found to be higher than the chlorophyll a in cyanobacterium (*Acaryochloris marina*) in waters. According to Lüning (1990), the dominant pigments found in red algae are chlorophyll a, chlorophyll d, zeaxantin, cryptoxanthine lycopene, α-carotene, β-carotene, lutein, and picobilin. Chlorophyll d as an accessory pigment can support the role of chlorophyll a as the main pigment in red algae. According to Barsanti & Gualtieri (2006), cells that were accustomed to high radiation levels generally had relatively high concentrations of carotenoid pigments compared to chlorophyll a. Carotenoid pigments such as β-carotene and zeaxanthin pigments do not transfer excitation energy to the reaction center and consequently act to filter cells from excessive light. According to Indriatmoko et al (2015), the changes of chlorophyll and carotenoid concentrations of *K. alvarezii* specifically the one cultivated at the sea in shallow and clear water was very interesting to be studied.

Carotenoids. The content of carotenoid pigments in *K. alvarezii* consists of β-carotene and xanthophyll pigments, namely zeaxantin, violaxantin, cryptoxanthine, and lutein (Fretes et al 2011; Andersson et al 2006). Pigments in diethyl ether were extracted from different polarity of the thallus. The pigment content of *K. alvarezii* in the present research was identified as β-carotene.

The results of this study showed that the concentration of β-carotene pigment taken during the research with two weeks interval tended to increase until it reached its maximum point at week 7. Subsequently there was a decrease in concentration until week 9 (Figure 6).

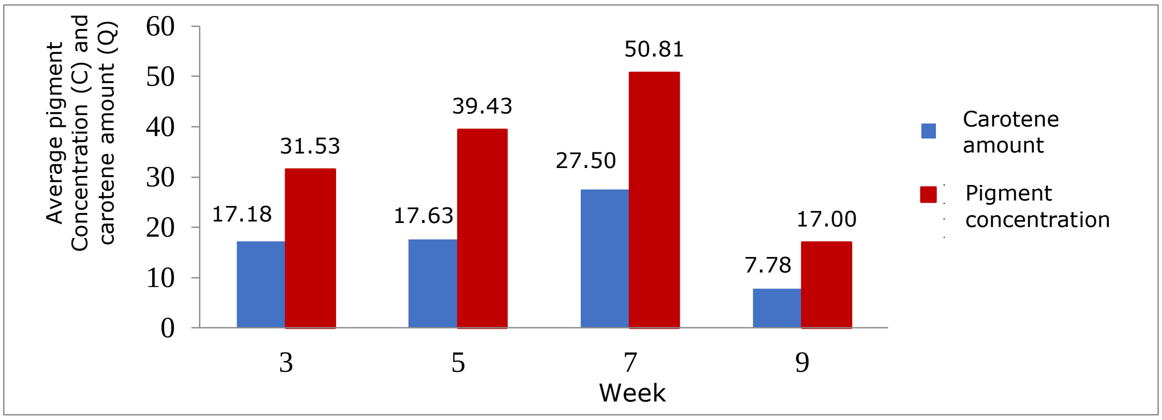

Figure 6. Pigment concentration and carotene amount of *Kappaphycus alvarezii* during the two weeks of culture.

The average concentration of β-carotene pigment extracted based on the separation of different solvents at week 3 was 17.18 μg g^{-1} of the dry residual weight. At week 3, the carotene pigment concentration was the highest compared to the pigment concentration of xanthophyll, chlorophyll a and chlorophyll d. At week 5, the carotene pigment concentration increased to 17.63 μg g^{-1}. It was supposed that at weeks 3 and 5, the carotene pigment played a major role in the photosynthesis process. At week 7, the carotene pigment concentration rose to 27.50 μg g^{-1}. At the 9th week, the carotene pigment concentration decreased to 7.78 μg g^{-1}. The concentration pattern of carotene pigments in *K. alvarezii* followed the relative growth rate pattern of the algae.

The average quantity of β-carotene pigment followed the pigment concentration pattern and the relative growth rate pattern of the algae. The average carotene amount at week 3 was 31.53 μg from the wet weight of *K. alvarezii*. At week 5, it increased to 39.43 μg and at week 7, it increased to 50.81 μg. At the 9th week there was a decrease to 17.00 μg. Thus, the average carotene amount would increase along with the increase of growth rate of these algae to carry out the photosynthesis process.

Similar pigment concentration and carotene amount patterns were found by by Paransa et al (2002), where the red algae *Kappaphycus striatum* was cultured on the surface of coastal waters of Nain Island for two months. Pigment concentration and carotene amount during the culture increased from week 3 to week 7. At the 7th week there was a decrease until the 9th week. There was a similarity in the pigment concentration and carotene amount even though the culture was carried out in different months. It was suspected that this similarity occured because algae came from the same genus, namely Kappaphycus, the same rearing location but with different months and years.

The average xanthophyll pigment concentration at week 3 was 13.74 μg g^{-1}, and increased to 16.64 μg g^{-1} at week 5. At week 7 increased to 35.55 μg g^{-1}, but at week 9 decreased to 14.78 μg g^{-1}. The average xanthophyll amount at week 3 was 25.07 μg and increased at week 5 and 7 to 36.43 μg and 65.95 μg, respectively. However, at week 9 it decreased to 35.18 μg (Figure 7).

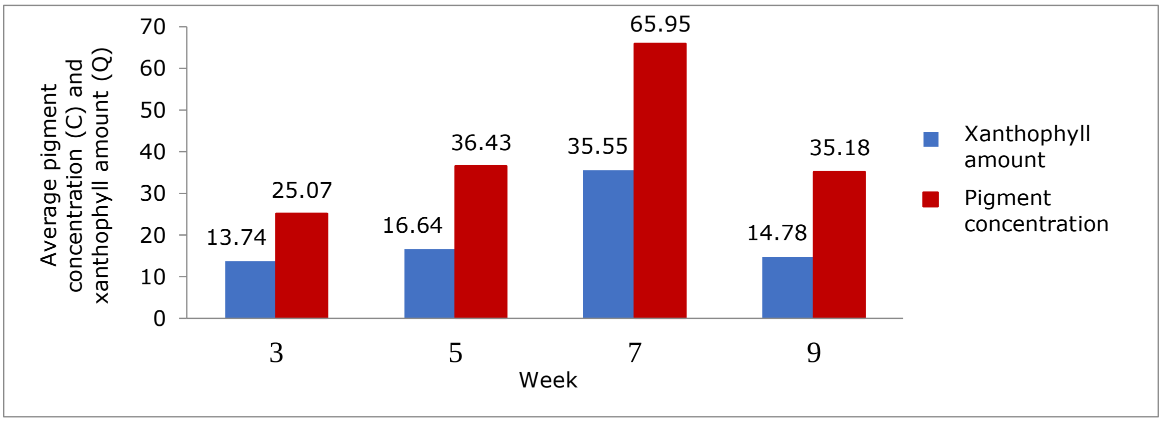

Figure 7. Concentration and amount of xanthophyll pigment of *Kappaphycus alvarezii* extract during the two weeks of culture.

The average concentration and amount of xanthophyll pigments obtained were the same, where there was an increase both in pigment concentration and xanthophyll amount at week 5 and 7, The highest average quantity was obtained at week 7, while at week 9 the average quantity and quality of xanthophyll pigments decreased due to the age of algae.

Conclusions. The concentration of chlorophyll a and d pigments increased as the weight gain of *K. alvarezii* increased. The increase in pigment concentration and carotene and xanthophyll (carotenoid) amount were in line with the growth rate pattern of *K. alvarezii*.

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