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CONCENTRATION OF LIQUID PES MEDIA ON THE GROWTH AND PHOTOSYNTHETIC PIGMENTS OF SEAWEEDS COTONII PROPAGULE (KAPPAPHYCUS ALVAREZII DOTY) THROUGH TISSUE CULTURE

Lumbessy Salnida Yuniarti[1,3]*, **Andayani Sri**[2], **Nursyam Happy**[2], **Firdaus Muhammad**[2]
[1]Graduate School, University of Brawijaya, Indonesia
[2]Faculty of Fisheries and Marine Studies, University of Brawijaya, Indonesia
[3]Marine Cultivation Study Program, University of Mataram, Indonesia
*E-mail: alyachali@gmail.com

ABSTRACT
In vitro tissue culture of *Kappaphycus alvarezii*, the required nutrients are derived from the culture medium. The use of liquid PES medium as a culture medium has been widely applied to increase the growth rate of seaweeds. Seaweeds growth is also associated with photosynthetic pigments. If the absorption of light by chlorophyll a is sufficient, the process of photosynthesis will take place optimally, so that the growth of seaweed can increase. This study aims to examine the effect of liquid PES medium on the growth and photosynthetic pigments of *Kappaphycus alvarezii* propagule. The treatments used included 10 ml and 20 ml liquid PES media. Parameters measured include weight gain, daily growth rate, photosynthetic pigment contents (chlorophyll a and phycoerythrin) and absorption of nitrate (N) and phosphorus (P). The results showed that the use of 10 ml liquid PES medium gave better results than the use of 20 ml liquid PES medium on all parameters measured.

KEY WORDS
Growth, photosynthetic pigments, *K.alvarezii*, tissue culture.

Every living creature, including small creature seaweed, requires both macro element and microelement nutrients to live and grow. In the real habitat, seaweed obtains nutrients from sea water containing various chemical components. In addition, seaweed also gains the nutrients from organic materials flown by the ocean. In order to survive in vitro cultures, cultivation media that serves as a provider of nutrients of the seaweed should contain the nutrients needed by the seaweed. In various types of algae, liquid PES media have been widely reported to be excellent for callus morphogenesis (Reddy et al., 2003; Munoz et al., 2006; Kumar et al., 2007; Baweja et al., 2009 & Yong et al. 2011).

Studies on seaweed in vitro tissue culture, particularly on *Eucheuma cottonii* or *K. alvarezii* species in recent decades have provided new discoveries in seaweed cultivation, such as the discovery of improved varieties as seeds and better carrageenan production (Hayashi et al. 2008; Reddy et al.2008, Hurtado et al 2014, Yong et al 2014, & Yong et al 2015). Meanwhile, some studies on metabolite and biological activities of macro algae also show that in addition to having polysaccharide content such as carrageenan and bioactive compounds, seaweed also contains other compounds namely pigments (Liu et al., 2005; Andersson et al., 2006; Schubert et al ., 2006; & Indriatmoko et al., 2015).

Several studies on photosynthetic pigments in red seaweed have been conducted (Yocum & Blinks, 1957; Saenger, 1969; Giuseppe & Felicini, 1973; Mary & Dawes 1981; Luning & Schmitz, 1988; David & Rowan, 1989; Ojala; 1993; Dagmar & Mathew; 1998; Reeta & Kulandaivelu; 2000; Aguilera et al., 2002; Gudrun and Wincke; 2005; Naguit & Tisera; 2009; Sarojini & Narayanan, 2009; Schmidt et al., 2010; Vanitha & Chandra, 2012). However, most of the results of these studies only provide information about photosynthetic pigments with the limitation only to the cultivated red seaweed (*in vivo*), and no information about photosynthetic pigment in seaweed tissue culture (*in vitro*). Therefore, this research is intended to examine the extent to which liquid PES media as a tissue culture medium can give effect to the growth and content of photosynthetic pigments consisting of chlorophyll a and *phycoerythrin* on *K. alvarezii* propagule resulted from the tissue culture.

MATERIALS AND METHODS OF RESEARCH

The materials used in this research are *K. alvarezii* callus in the form of micropropagules obtained from SEAMEO-BIOTROP in Bogor. Fertilizer used as a treatment is PES fertilizer obtained from the Tissue Culture Laboratory of Marine Aquaculture Hall Lombok.

Callus Acclimatization. Micropropagules obtained from SEAMEO-BIOTROP were firstly placed on the rotary shaker to be shaken for one week to adapt the culture. Rotary shaker was placed in the culture room with the room temperature between 22-25 ^{o}C, given the irradiation of TL lamp with the light intensity was ± 1500 lux, the duration of irradiation was set 12 hours on and 12 hours off.

In Vitro Culture. After the acclimatization of micropropagules placed on the rotary shaker, it was sub-cultured to a 1L bottle containing liquid PES media with two treatments of 10 ml and 20 ml of PES in 500 mL of sterile seawater. The culture was aerated by aerator. After one week, the media was replaced with new media. The culture bottles were stored in the culture room with room temperature between 22-25 ^{o}C, light intensity of ± 1500 lux with the irradiation time of 12 hours on and 12 hours off. After six weeks, the volume of the PES media was added to 20 ml of PES in 1 liter and 40 ml of PES in 1 liter of sterile sea water. The cultivation of the micropropagules was performed for eight weeks (about 2 months).

Measurement of Propagule Growth. The measurements of propagule growth includes weight gain and daily growth rate which were measured every two weeks for two months of cultivation, using the formula proposed by Dawes et al. (1994). The weight gain was calculated based on the formula:

$$\Delta W = Wt - Wo$$

Where: Wt = total weight of propagule at time of t (gram); Wo = total initial weight of propagule (gram).

The daily growth rate was calculated based on the following formula:

$$\alpha = \frac{\text{Ln Wt} - \text{Ln Wo}}{\text{t}} \text{ x } 100\%$$

Where: W_t = final weight (gram); W_o = initial weight (gram); t = observation duration (days).

Measurement of Photosynthetic Pigments (Chlorophyll a and Phycoerythrin). The measurement of photosynthetic pigment includes the content of chlorophyll a and *phycoerythrin*. The measurements were taken every four weeks for eight weeks of cultivation. The samples of the seaweed were smoothed using a blender then weighed 2 gram and crushed using mortar. Samples were added with 10 ml 100% acetone (for chlorophyll) and 10 ml 0.1M phosphate buffer (for *phycoerythrin*). The samples were inserted in a test tube to be centrifuged and filtered. The centrifuged samples were measured on their absorbance by using a spectrophotometer at wavelengths of 664 nm and 647 nm for chlorophyll a and 592, 564 and 455 nm for *phycoerythrin*. Chlorophyll concentration was calculated based on the Sterman's equation (1988) while the concentration of *phycoerythrin* was calculated based on the Beer and Eshel equation (1985) as follows:

a) Chlorophyll a = 11.93 (A_{664}) – 1,93 (A_{647})
b) *Phycoerythrin* (mg/L) = [(A_{564} – A_{592}) – (A_{455} – A_{592}) 0.20] * 0.12

Furthermore, the concentration of pigment per gram of seaweed was calculated based on Naguit and Tisera equation (2009) as follow:

$$\frac{\text{concentration (mg.·L)} \times \text{solvent volume (ml)}}{\text{talus weight (gr)}} \text{ X } \frac{1000\ \mu\text{g}}{\text{mg}}$$

Absorption of N and P on the Seaweed. Absorption of nitrogen and phosphorus by seaweed can be seen from the results of measurement of water quality and proximate analysis. Here are the formula to calculate the nitrogen and phosphorus absorbed by measuring the water quality proposed by Zhou et. al (2006):

$$\text{N uptake (mg/g)} = \frac{|\,[\text{N}]t - [\text{N}]0\,| \text{ x } 1\text{ kg}}{W\ (g)}$$

$$\text{P uptake (mg/g)} = \frac{|\,[\text{P}]t - [\text{P}]0\,| \text{ x } 1\text{ kg}}{W\ (g)}$$

The absorption of nitrogen and phosphorus by the seaweed talus based on the results of proximate analysis with Kjeldahl method was calculated based on the equation of Zhou et. al (2006):

$$\text{N uptake (µmol/g/day)} = \frac{\text{daily growth rate (\%/day) x N } tissue\ \text{(g/100g)}}{100}$$

$$\text{P uptake (µmol/g/day)} = \frac{\text{daily growth rate (\%/day) x P } tissue\ \text{(g/100g)}}{100}$$

Chemical and Physical Measurement of Water Quality. The measurements of the water quality were conducted every four weeks for eight weeks of the cultivation. The measurements were carried out physically which included some aspects, e.g. salinity, pH, DO and temperature.

Data Analysis. The data related to the growth and photosynthetic pigment were analyzed by ANOVA variance analysis, while the N and P absorption data and water quality were integrated as the supporting data analyzed by using the mean and graph. Data processing through the graph was done with Microsoft Excel program

RESULTS OF STUDY

Micropropagule Growth. The observed weight gain of propagules during the 8 week cultivation increased along with longer duration for all treatments (Figure 1).

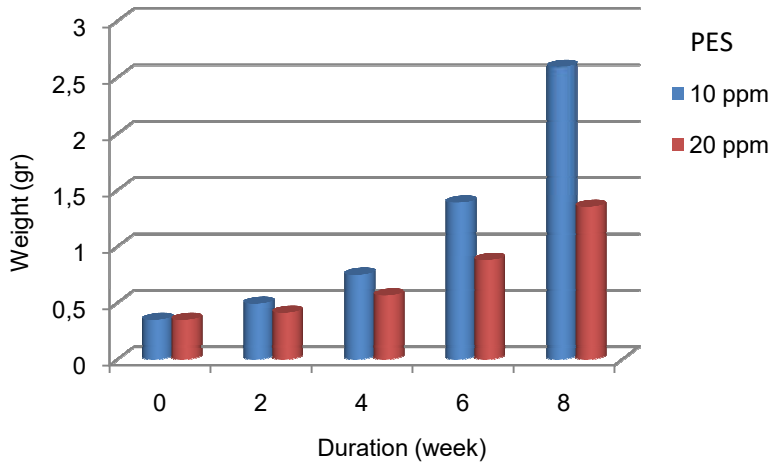

Figure 1 – The weight gain of *K. alvarezii* propagule in 8 weeks of cultivation

The treatment of 10 ml liquid PES medium in 500 ml of sterile seawater gave a better average on weight gain compared to the 20 ml liquid PES medium treatment in the same 500 mL of sterile seawater. The highest average weight gain was obtained at 10 ml liquid

PES medium treatment, which was 2.24 gram, while the lowest average weight gain was in the 20 ml liquid PES medium treatment, amounted to 1.00 gram (see Table 1).

Table 1 – The average weight gain of *K. alvarezii* propagule in various concentrations of liquid PES media

Liquid PES (ml)	Initial weight (gram)	Final weight (gram)	Absolute weight (gram)
10	0.35 ± 0.02	2.59 ± 0.072	2.24 ± 0.073
20	0.35 ± 0.01	1.35 ± 0.042	1.00 ± 0.035

This is also in line with the daily growth rate of propagules, in which the range of daily growth rate in the 10 ml liquid PES medium treatment results in a higher yield of 2.42 - 4.46% per day compared with the 20 ml liquid PES medium treatment at the range of daily growth rate is 1.18 - 3.072% per day (see Figure 2).

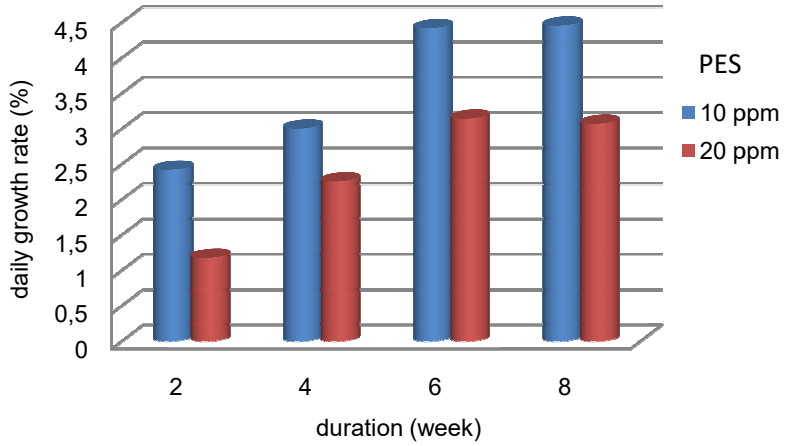

Figure 2 – Daily growth rate of *K. alvarezii* propagule in eight weeks of cultivation

The results of the variance analysis showed that the different concentration of the liquid PES media contribute very real effect (F statistic > F table) to the daily growth rate of seaweed *K. alvarezii* propagule.

Photosynthetic Pigment Content. The results showed that the difference of concentration of liquid PES media give obvious effect (F statistic > F table) to the photosynthetic pigment content of *K. alvarezii* propagule, both for chlorophyll a and *phycoerythrin*. The content of chlorophyll a on *K. alvarezii* propagule increases along with the increasing cultivation duration at all treatments (Figure 3). On the contrary, the *phycoerythrin* content decreases along with the increasing cultivation time at all treatments (Figure 4).

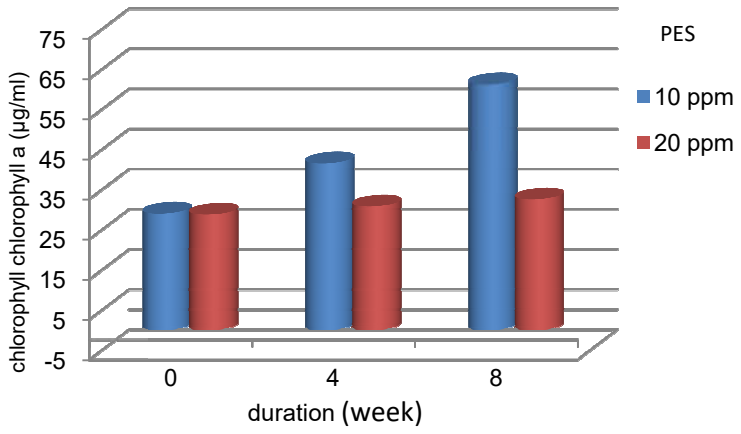

Figure 3 – Content of Chlorophyll a of *K. alvarezii* propagule in eight weeks of cultivation

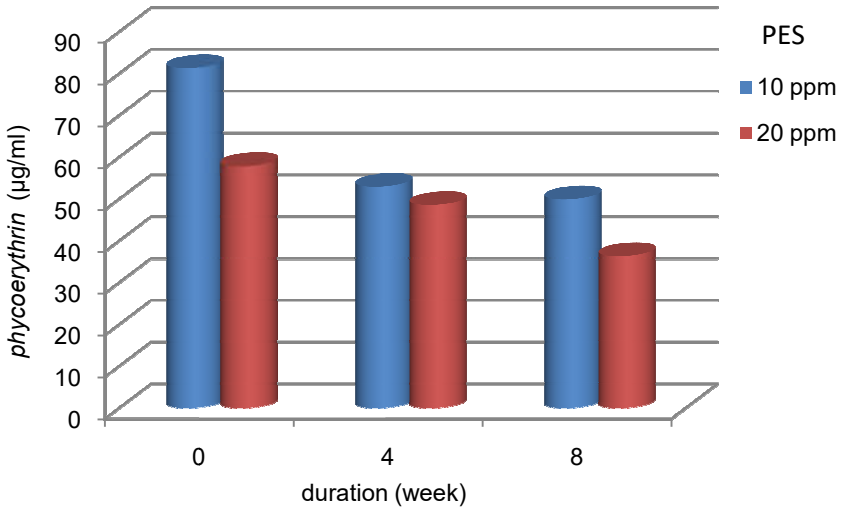

Figure 4 – Content of *phycoerythrin* a of *K. alvarezii* propagule in eight weeks of cultivation

At the end of the cultivation period (8 weeks), it was found that the 10 ml liquid PES treatment in 500 ml of sterile seawater gave chlorophyll a and *phycoerythrin* pigments of 61.308 µg/ml and 49.916 µg / ml, respectively. On the other hand, the liquid PES medium treatment 20 ml in 500 ml of sterile seawater gives chlorophyll a and *phycoerythrin* content of 32.608 µg/ml and 36.314 µg/ml (Table 2), respectively.

Table 2 – The average content of chlorophyll and *phycoerythrin* of *K. alvarezii* propagule at several doses of liquid PES

Liquid PES (ml)	Duration of cultivation (week)	Photosynthetic pigments (µg/ml)	
		Chlorophyll a	*phycoerythrin*
10	0	29.102 ± 0.575	81.301 ± 3.185
	4	41.598 ± 1.074	52.961 ± 2.501
	8	61.308 ± 0.330	49.916 ± 4.107
20	0	28.849 ± 0.983	57.925 ± 1.203
	4	30.866 ± 0.093	48.573 ± 7.266
	8	32.608 ± 0.648	36.314 ± 0.989

N and P Absorption. Nitrogen in the waters is generally in the form of nitrate (NO2), nitrite (NO3) and ammonia (NH3), whereas the phosphorus in the waters is often abundant in various forms of phosphate compounds, including total phosphate (PO4). The results of calculations on the N and P absorption based on the water quality (Table 3) show that *K. alvarezii* propagules absorbs more nitrate than nitrites and ammonia.

Table 3 – The absorption of nitrate, nitrite, ammonia, and total phosphate by *K. alvarezii* propagule in different concentrations of liquid PES media

Absorption (mg/g)	Liquid PES	
	10 ml	20 ml
NO_2	1.316602	0.332046
NO_3	*	*
NH_3	0.06564	0.05405
PO_4	0.5444	0.42471

*Note: * not detected.*

The amount of nitrate absorbed in the 10 ml liquid PES medium treatment showed the highest value of 1.316602 mg/g, whereas in the treatment of 20% liquid PES medium showed the value of 0.332046 mg/g. The absorbed nitrite cannot be detected because the nitrite content in waters < 0.01 mg/L. Meanwhile, the ammonia absorbed in the 10 ml liquid PES medium treatment also showed the highest value of 0.06564 mg/g, whereas in the

treatment of 20% liquid PES medium only absorbed 0.05405 mg/g. In the result of calculations of the total phosphate showed that *K. alvarezii* propagule on treatment of 10 ml liquid PES media absorbed the highest total phosphate absorption that is equal to 0.5444 mg/g, whereas in the treatment of 20 mL liquid PES medium was only able to absorb the total phosphate equal to 0.42471 mg/g.

The results of calculations on the N and P uptake based on the proximate analysis show that the *K. alvarezii* propagule in the 10 ppm liquid PES medium treatment was capable of absorbing the highest nitrogen and phosphorus, i.e. 0.16 µmol/g per day and 0.03 µmol/g per day, while for the *K. alvarezii* propagule with 20 ppm liquid PES medium treatment was only able to absorb nitrogen and phosphorus of 0.11 µmol/g per day and 0.017 µmol/g per day respectively.

Parameters of Water Quality. The results of measurement of the water quality during the eight weeks of cultivation can be seen in Table 4.

Table 4 – Parameters of Water Quality

No.	Parameters	Range of observation		References
		Liquid 10 mL PES	20 mL Liquid PES	
1	Temperature (^{o}C)	22.18 – 26.81	23.5 – 26.12	22- 33 (Lideman *et al.,* 2013).
2	Acidity (pH)	7.87 – 8.67	8.13 – 8.17	6 – 8 (Semedi *et al.,* 2016)
3	DO (mg/L)	5.03 – 5.3	5.15 – 5.46	5 – 8 (Semedi *et al.,* 2016)
4	Salinity (ppm)	33 – 34.2	36.2 – 38.8	30 - 35 (Dawes, 1981)

DISCUSSION OF RESULTS

PES fertilizer is a complete fertilizer as it contains macronutrient and micronutrient elements which are very complete and needed by seaweeds. Reddy et al., reported that PES is typical fertilizer which is widely used for algae growth because of its complete nutrients contents and suitable for algae species, especially seaweed at the enlargement of the talus up to 3-5 cm in size. Mansilla et al., (2007) stated that during the cultivation of seaweed seeds in the laboratory, fertilizers containing macronutrients (nitrogen, phosphorus, potassium) and micronutrients (Mo, Ni, Mn, B, Cu, Zn, Co, Cl, and Na, S), give higher growth rates on the seaweed compared to fertilizers containing only macronutrients.

The results of this study indicate that in general the 10 ml liquid PES medium gives the highest average weight of *K. alvarezii* propagule (Table 1) compared to the 20 ml liquid PES medium. This indicates that the nutrient content of 10 ml liquid PES medium is more suitable for the growth of *K. alvarezii* propagule cells. The absorption of nutrients contained in a 10 ml liquid PES medium can be utilized well by propagule through optimal water movement by aeration. Lobban & Harrison (1994) state that water movement is a factor affecting the growth of *K. alvarezii* because the movement or current plays an important role in improving nutrient exchange conditions and avoiding deposition to support growth which is also a means of nutrient transport. Water movement serves to supply nutrients and clean the dirt on the surface of the talus.

The high growth of *K. alvarezii* propagule in the 10 ml liquid PES medium treatment was also supported by the high average of daily growth rate of 3.58% per day compared with the treatment of 20 ml liquid PES medium with the average daily growth rate is 2.41% per day (Figure 2). The value of daily growth rate in the treatment of 10 ml PES medium is quite good considering that growing rate of *cottonii* seaweed at the time being cultivated in the nature ranges from 3-5% depending on the season (Thirumaran & Anantharaman, 2009). Young et al (2011) stated that in in vitro micro-propagation type *Eucheuma sp*., daily growth rate on PES medium is higher than von Stosch, F/2 and sea water media. Further, the results of Sulistiani, et al (2011) showed that the daily growth rate of propagule at 20 ml/L liquid PES was not significantly different with the concentration of 10 ml/L liquid PES, but the

second growth rate treatment was higher and significantly different with growth rate at 5 ml/L treatment. For the purpose of efficient use of chemicals, then in the enlargement of propagules should not always use the concentration of 20 ml/L, but also the use of the concentration of 10 ml/L PES.

Treatment with 10 ml liquid PES medium was not only to give the best weight gain and daily growth rate, but also provide better photosynthetic pigment (chlorophyll a) (see Table 2). Lobban & Harrison (1994) stated that sunlight directly affects the absorption of nutrients, active transport and increases the growth rate of the seaweeds. The sunlight through the photosynthesis process is able to release some energy, and this energy is used by ions and elements to pass through the cell membrane of the plants. At the time the ions enters, the ions function according to their respective functions, such as enzyme cofactors, enzyme activators, and general work of other ions. In addition, the elements that enter the cell will carry some energy.

The process of photosynthesis in the seaweeds not only utilizes chlorophyll pigment, but there are other accessories or complementary pigments, namely *phicobillyprotein* (R-*phicocyanin*, *allophicosianin* and *phycoerythrin*), (Aguirre et al., 2001; Naguit & Tisera, 2009; Zhao & He, 2009; Chakdar et al., 2012; Pugalendren et al., 2012). The complementary pigments analyzed in this study were *phycoerythrin* pigment. Based on the average value indicates that the content of *phycoerythrin* pigment inversely proportional to chlorophyll a. The higher content of *phycoerythrin* pigment at the beginning of cultivation is presumed as the amount of chlorophyll a is still low, so that it is insufficient in the absorption of light for the process of photosynthesis; this triggers the formation of more *phycoerythrin* (Kawsar et al, 2011; Pumas et al., 2012). *Phycoerythrin* is a protein that acts as a complementary pigment in red algae and blue-green algae that serves to help chlorophyll-a in absorbing light in the process of photosynthesis. The light absorbed by *phycoerythrin* is efficiently transferred to *phycocyanine*, then to *allophycocyanine* and to *allophycocyanine* B, and finally goes to the chlorophyll (Bryant, 1982; Chakdar et al. 2012; Pugalendren et al., 2012).

The increased weight and higher chlorophyll content during the 10 ml liquid PES medium treatment was believed to be strongly influenced by the nutrient content of the culture medium. In general, seaweed growth is closely related to photosynthetic pigment content, i.e. chlorophyll a. If the absorption of the light by chlorophyll a is sufficient, the process of photosynthesis may occur optimally which in turn affects the faster growth rate of the seaweeds. Therefore any growth process and pigment formation will require nutrients. The most important and indispensable nutrients needed by seaweed are nitrogen (N) and phosphorus (P). Harrison and Hurd (2001) state that the growth and development of the seaweed requires sufficient light and quality nutrients such as nitrate and phosphate. Nitrates and phosphates are needed as the basic ingredients of protein constituents and the formation of chlorophyll in the process of photosynthesis. In relation with the tissue culture activities, nitrate and phosphate can be derived from the culture medium. The results of study carried out by Dong et al. (1990) showed that the provision of N in the seaweed *Laminaria japonica* can increase the amount of chlorophyll a. Kim et al., (2007) states that the synthesis of chlorophyll a and *phycoerythrin* requires N as well.

The nutrients (N and P) enter into the body of seaweed by diffusion through the entire surface of the body of the plant. The absorption of nutrients through the diffusion process is supported by the movement of water in the aeration-assisted cultivation medium. Lobban & Harrison (1994) asserted that the elements in the waters will enter into the algae through the process of absorption, diffusion, and osmosis; there should be the balance of ions and elements between the outside and inside of the cells. The more diffusion takes place, it will accelerate the metabolism process, thus increasing the growth rate of the seaweeds.

Nitrogen and Phosphorus is a mineral nutrient that limits plant growth due to its large availability in media (Harrison & Hurd, 2001; Harpole et al 2011). The results of this study indicate that 10 ml liquid PES medium also provides the most nutrient absorption of nitrate and total phosphate (Table 3). After the proximate analysis was conducted, it was shown that *K. alvarezii* propagule in the 10 ml PES liquid medium treatment was the most nitrogen and phosphorus-containing seaweeds in the body that could be used for the faster growth. The

more seaweed absorbs nitrogen and phosphorus, the greater the amount of nitrogen and phosphorus contained in the seaweed, and this indicates that the better quality.

The absorption rate of nitrate and phosphate has a positive correlation with the increasing growth rate as well as synthesis of chlorophyll a and *phycoerythrin* (Dong et al., 1990; Gordillo et al. 2002; Kim et al., (2007). Lea and Azevedo (2006) stated that growth may occur as the result of the function of nitrate as an ingredient of protein. It is presumably the protein has potential to activate the enzymes in the plant body; it will change the substrate into new products as the result of duplication or multiplication of cells. The bigger amount of Nitrogen in plants may cause the bigger formation of glutamic acid. Initially nitrogen is absorbed is in the form of ammonia, and then ammonia change to glutamic acid, catalyzed by enzyme glutamine synthase. Glutamic acids serve as the base material in biosynthesis of amino acid and nucleic acid. Glutamic acid will form acid *aminolevulinate* (ALA) which acts as a *porphyrinin* precursor ring in the formation of chlorophyll. Therefore, the amount of nitrogen content can affect photosynthesis results through photosynthetic enzyme and chlorophyll content (Robinson 1995, Lea & Miflin, 2003; Suzuki & Knaff, 2005; Yaronskaya et al., 2006)

In addition to nitrate, phosphate is also a major nutrient factor to meet the needs of algae. Phosphate is a component that plays a role in the formation of DNA, lipid and energy metabolism, such as ATP and NADPH. The production of ATP and NADPH through a light reaction to the photosynthesis process will produce the energy to be used in the Calvin cycle to phosphorylate and convert 3-phosphoglycerate (PGA) to Glyceraldehyde 3-phosphate (G3P) and regenerate Ribulose 1.5-bisphosphate (RuBP) (Farguhar et al., 1980; Reich et al., 2009). The content of phosphorus in algae cells affects phosphate uptake, which is reduced in line with increased phosphate content in cells. Several types of algae are able to absorb the phosphate beyond their needs (luxury consumption) and are able to absorb phosphates at very low concentrations and have alkaline phosphatase enzymes that can convert phosphates into orthophosphate ready to use (Sahoo & Ohno, 2001).

Although nitrate and phosphate are essential for the growth and process of plant photosynthesis, plants need only this element in sufficient quantities. Therefore, based on the results of this study, it is believed that the 10ml liquid PES medium provides sufficient N and P elements for the growth and photosynthesis process of *K. alvarezii* propagules. As the concentration of liquid PES media was raised to 20 ml, the growth and the photosynthesis rate decreased. This suggests that increased concentrations of nutrients above optimal requirements will not increase the growth of the plant. This is in line with Knecht and Göransson's (2004) opinion that plants require a certain minimum concentration of nutrients to grow, and when nutrient concentration rises to optimum levels, it increases the relative growth rate. The increased concentrations of nutrients above the optimum will not increase growth, and at very high concentrations the nutrients even will become toxic, the growth and the rate of photosynthesis will decrease, and the plant may die.

Besides supported by the nutrients, the growth rate may increase if the media and the environment around the cultivation are in accordance with needs or tolerable ranges. Aks and Azansa (2002) stated that environmental factors that have an important role in the cultivation of *K. alvarezii* such as temperature, salinity, nutrition, light and several other ecologic factors. The water quality that has been measured during the cultivation indicates a value that is still in the good and tolerable range (Table 4.)

CONCLUSION

The results of this study indicate that the use of 10 ml liquid PES medium gives higher weight gain, better daily growth rate and better photosynthetic pigment content (chlorophyll a and *phycoerythrin*) than the use of 20 ml liquid PES medium for the seaweed *K. alvarezii* propagule through tissue culture. It is believed that the 10 ml liquid PES medium provides the optimum N and P elements for the growth and photosynthesis process of *K. alvarezii* propagules.

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