The Morphology of Contaminant Organism in Kappaphycus alvarezii Tissue Culture

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Abstract: The main problem in increasing the production of seaweed cultivation of *Kappaphycus alvarezii* is the availability of quality seeds. One of the causes is because the seeds are susceptible to infectious diseases. Tissue culture is one of the techniques to produce Specific Pathogen and Epiphyte Free /*SPE* Nevertheless, the presence or absence of contamination needs to be analyzed to determine the cause of contamination,, morphology of contaminating organisms, and changes of morphological cell of tissue culture which can be used to prevent contamination during the next tissue culture and cultivation at the sea. Based on the results, it could be revealed that the occurring contamination caused by bacteria and fungi as well as caused by the less sterill culture process. Thallus morphology affected by the disease has slower growth. There were also black spots, cotton-like substance as contaminated fungi (*Saprolegnia sp* and *phytopthora*), and the fading of green and slimy pigment as bacteria contamination. In addition, the morpology of ill seaweed cells has smaller cells and shrinked tissue compared to the healthy ones with their bigger cells and no shrinkage.

1 INTRODUCTION

The species of seaweed widely cultivated in Madura is Euchema Cottonii which is also known as *Kappaphycus alvarezii*. It is the most important seaweed and the largest on production volume in Indonesia. This type contains Karaginan which is useful as a Gelling agent, solidified agent, anda fertilizer (Suryaningrum, 1998). The main problem in increasing production of seaweed (Kappaphycus Alvarezii) cultivation is the availability of good seeds. One of the cause is that the seeds are susceptible to the disease. Epiphytic attack caused the decrease on seeds quality;, hence, the resistance of seaweeds toward the disease is an indicator of seaweed cultivation accomplishment. A widely applied plain and low-cost technologyof seaweed cultivation is not supported by the availability of seeds which are unrestrained from disease and epiphytic (specific pathogen and epiphyte free / *spef*).

Tissue culture is one of techniques to produce quality seeds stock. Research that has been carried by Parenrengi, *et. a*. in 2007; Hurtado and Biter in 2007; Hurtado, *et. al*. 2009; and Yunque, *et.al* in 2010 revealed that seaweed *K. alvarezii* can be cultivated by using tissue culture. However, those research did not specifically examine occuring contamination and identify the contaminant species. One of the problems occurring in tissue culture is the contamination. The condition of *in vitro* favored by *Eksplan* contains sucrose and nutrient in high concentration, high moisture, and suitable temperature. These situations are also preferred by microorganisms that grow more rapidly than *eksplan*. A source of contaminating organisms can come from unsterile environment, or they have existed in the cell when eksplan of *Kappaphycus alvarezii* will be cultivated through tissue culture. Therefore, it needs to be controlled. The first step is by identifying the types of contaminated microorganisms and by acknowledging those symptoms.

2 LITERATURE REVIEW

2.1 Biology *kappaphycus alvarezii*

Kappaphycus alvarezii is a seaweed of class Rhodophyceae. Based on the identification of the karaginan fraction produced by *Kappaphycus alvarezii*, kappa karaginan type, it is taxonomically changed its name from *Eucheuma alvarezii* become

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Kappaphycus alvarezii (Patadjai 2007). The name "alvarezii" given to *Kappaphycus alvarezii* comes from the name of the Vicente (Vic) Alvarez. Vic is a pioneer in *cottonii* cultivation method (Patadjai 2007). The world of seaweed trade is more familiar with the name *Eucheuma cottonii* or *cottonii* only. Meanwhile, seawater disease is defined as disruption of structures and normal functions such as changes in growth rate and appearance (color and shape) that can affect productivity levels. Culture or tissue culture *in vitro* is a method to isolate parts of the plants grown on sterile artificial media, in sterile culture bottles, and in aseptic conditions. Thus the parts can proliferate and regenerate into a complete plant. Tissue culture is a series of activities undertaken to make plant parts (roots, buds, plant growing tissues) grow into the whole (perfect) condition *in vitro* (in glass) plants (Indrianto 2002).

The basic theories of tissue culture are: a. Cells of a multicellular organism wherever it is located is actually the same as a zygote cell because it originates from one cell (omne *cellula ex cellula)*. b. The Cell Totipotency Theory by Schwann and Schleiden (1898) states that the cell has Totipotency nature that every living plant cell is equipped with genetic information and complete physiological devices to grow and develop into whole plants if the conditions are appropriate. This theory believes that every part of the plant can breed because all parts of the plant are made up of living tissues. According on Thorpe, (1981) , there are three main principles in tissue culture:

a. Isolation of plant parts of whole plants (organs, roots, leaves, stems);

b. Maintainance of the plant's parts in the appropriate environment and conditions of the culture;

c. Maintenance under aseptic conditions.

3 METHOD

The implementation of tissue culture was carried out in the Tissue Culture Laboratory Agroecotechnology and research on contaminant organisms was conducted at Marine Science Laboratory of Trunojoyo University Madura. Tools used for tissue culture process included Bottle Culture, Filter Paper, Funnel, Glass Beaker 250 ml and 500 ml, Autoclave, Laminar Flow, Microscope, Glass preparation + cover glass and other tools.

Seaweed materials used were *Kappaphycus alvarezii* cultivated by farmer groups in Aengdake Village, Bluto District, Sumenep Regency. C*onway* was utilized for tissue culture media and the seawater was also sterilized.

The selected explant source had the following criteria: (a) had many branches, dense and spiky leaves, (b) no spots found and peeling (c) had specific bright color (d) was live less 35 days, (e) weighed between 50-100 grams per rumpon and was not exposed to *ice-ice* disease. Tool sterilization used 70% alcohol and autoclave; while the material for eksplan sterilization utilized 0,5% betadine and 25 ppt sterile sea water. The stages of tissue culture implementation to explant observation was conducted by methods from Hurtado and Biter (2007) as mentioned below:

a. Preparation of tools and materials

1. Preparing the tool
   - Washing the appliance with running water.
   - Sterilizing equipment to be used with alcohol, wet sterilization using *autoclave* with temperature of 1210C at 1.5 atm pressure for 20 minutes

2. Preparing materials
   - Filtering sea water into culture bottle ± 100ml
   - Saving seawater that had been in autoclave in the sterile room
   - Filtering the media *conway* and sterilizing it into an *autoclave* with a temperature of 121^0C at a pressure of 1.5 atm for 15 minutes.

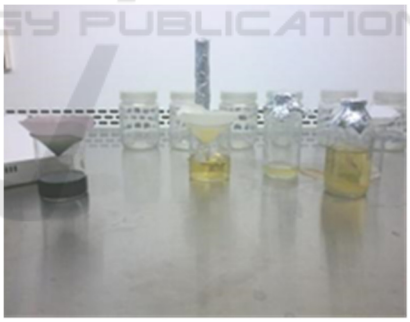


Figure 3.1 Media Conway filter.

b. Planting stage culture

Bottles containing media, as well as other planting tools, were sprayed first with 70% alcohol. Cut where *Thallus* was taken and put it into a disinfectant solution I (1% betadine solution where concentration 1 ml per 100ml seawater). Ten minutes later, rinsed with sterile distilled water for subsequent inclusion in a solution of disinfectant II (0.5% betadine solution where 0.5 ml per 100ml seawater) for 10 minutes. Then rinsed with sterile aquades for 3 times, aiming for no remnants of disinfectant material stick on eksplan then drained in petridish.

Implant planting was done in *laminar air flow*. Eksplan should be planted in culture bottle containing media; there was an eksplan planted on each bottle by using tweezers clamp. This activity was carried out in *a laminar flow,* placed in a room temperature of 25 oC. The media was of 25 ppt salinity, pH 7.5 and the explants were cultured in a culture bottle amounted to 120 units. Then the bottle was closed, removed, and arranged on a culture rack in accordance with the placement plan, given 10 watts fluorescent lighting.

The location of the lamp was 30 cm on the top of the shelf away from the culture bottle. The lighting was programmed for 24 hours continuously.

c. Subculture stage

Every 5 days, eksplants grew in subculture to a new culture bottle containing tissue culture media with the same concentration and parameters with previous culture media.

d. Maintenance stage

Maintenance is conducted on the culture room by maintaining cleanliness and room temperature. Culture bottles containing media and explants were sprayed with 70% alcohol every day and contaminated plants and media were immediately removed from the room and observed.

e. Stage of data collection

Observation was carried out every day to see the contamination of bacteria or fungi. Contaminated media and explants were removed from the room and their cells as well as their contaminants (Hayashi *et al.* 2008) were observed.

The data taken include:

a. Percentage of contamination

The percentage of contamination is calculated using the formula previously performed by Amiluddin (2007) as listed below:

$$C\ (\%) = \frac{A}{T} \times 100\%$$

description:
C: percentage of infected seaweed (%)
A: number of explants or infected seaweed
T: number of explants or bonded seaweed points observed

b. Morphology cell

Cell of morphology was observed using the microscope 100L Olympus with 100 times magnification. It was photographed on size 1600 x 1200 with ISO 100 (Yulianto 1993).

c. Contaminant agent

The presence of epiphytic diseases and epiphytes that infect the explants (seaweed) was observed based on signs of morphological abnormalities. Observed morphological abnormalities were identified by looking at and comparing with the images contained in the literature. The morphology of contaminant organisms was observed using the Olympus 100L microscope with magnification 100 times. It was photographed at the size of 1600 x 1200 with ISO 100 (Yulianto 1993).

d. Survival rate

The rate of seedling survival explants of K. *alvarezii.* Based on Amiluddin (2007) was calculated using the following formula:

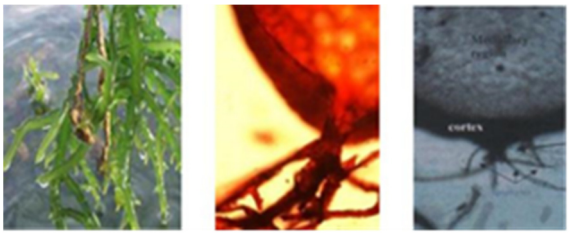
$$SR\ (\%) = \frac{S}{\overline{S + M}} \times 100$$

description: SR = Survival Rate (%)
S = Number of living seaweed
M = Number of dead seaweed

4 RESULTS

Epifit conditions on control treatment

Seaweed affected epiphytes in the sea is characterized by the presence of algae filament attached to the epidermis of the *thallus* of *appaphycus alvarezii* as seen in Figure 4.1. Based on the identification in the type and characteristics of filament algae in accordance with Largo (2002), the species identified are *polysiphonia* sp which is an epiphytic competitor algae.



(a) (b) (c)

Figure 4.1 *Polysiphonia* sp. on *Kappaphycus alvarezii. a)* The state of *Thallus* the contaminated. b) morphology of *Polysiphonic* attached to the epidermis *thallus* in a magnification microscope 100x. c) *Polysiphonia* according on Largo (2002).

The existence of competitor algae can result in disruption of the *thallus* in obtaining nutrients and light. If the components needed in metabolism are reduced; then over time, the seaweed *Kappaphycus alvarezii* can become thin, flabby, pale and can cause death.

The observation showed the presence of abnormalities in tissue are characterized by: a dangling lump in the epidermis, morphological observations through a microscope found that *Polysiponia* had afilament dangling and the base through the part of the cell wall of *Kappaphycus alvarezii.* According to Darmayati *et. al.* (2001), *Polyshiponia* sp. is type of *Rhodophyta-rhinoceros* and is a competitor algae in seaweed that can cause disturbance in seaweed photosynthesis resulting from the covering of *thallus* surface by *Polyshiponia.*

Epiphytic organisms in tissue culture

Organisms that contaminate seaweeds resulting from tissue culture which consists of fungi and bacteria.

Contamination of fungus

Contaminated fungi consists of *Saprolegnia* sp. and *Phythopthora* sp.,mushrooms of Oomycota class that can only grow in environment with high moisture or aqueous.

a. *Saprolegnia* sp.

The contaminated seaweed *Saprolegnia* sp. is characterized by the whitening of *thallus,* mucus covered by dirt like white flour, the peeled outer skin or epidermis to reveal deep cell or medullaryin of the *tissue thallus*, and the presence of white hifa on the surface of the media.

Morphology Saprolegnia sp. as contained in Figure 4.3.

Saprolegnia sp. has a characteristic feature that can grow at a temperature range of 0-35° C, with an optimum growth interval of 15-30°C. It is a fungus group of *Oomycota* also called a water fungus that can live in aqueous / high humidity environment. *Saprolegnia* sp. commonly attacks the injured part, and may subsequently spread to other healthy tissues. According to Wilfred *et al.* (1965) in Ningsih (2011), the fungus of the family *Saprolegniaceae* can live in freshwater and saltwater. Zoospora groups of these fungi search for fertile substrate, then settle down and start producing *hypha. Mycelia* grow over the wounded tissue or the site of infection, and then spread to normal tissue around the site of infection. The fungal enzyme secreted by the fungus destroys the surrounding tissue, kills cells, and progresses *mycelia*. It is very dense and sticking out into the water, making it look likes cotton.

(a)

(b)

Figure 4.3. Morphology of saprolegnia sp. a) Thallus affected by fungi b) Endogenous of Saprolegnia sp. in thallus tissue.

***b. Phytophthora* sp**

Contaminated *Thallus* is pale green and has specks of black spots. The morphological observations of the tissues show the presence regions of dark and rounded as shown in Figure 4.4 below:

Figure 4.4 Contamination *Phytophthora* sp in *thallus a)* Contaminated Morphology *thallus*, b) Morphology of contaminated tissue *Phytophthora* sp at 100x magnification microscope.

Diagnosis done in laboratories by taking mycelia wasplaced on the surface of the glass slide and given a little water for further observation under the microscope. Mycelia cause saprolegniasis to have branching with aseptate hypha structure.

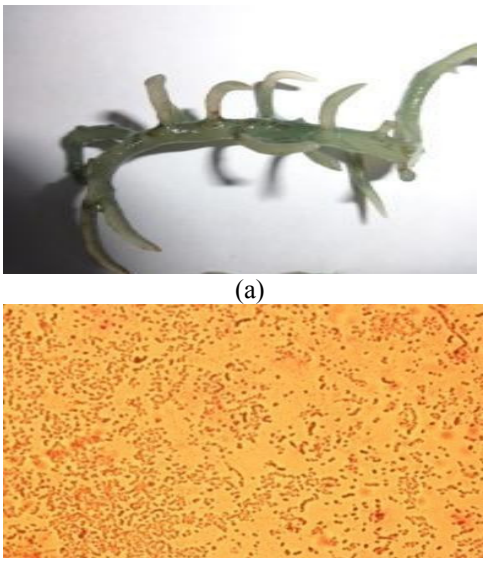
The morphology of *Phytophthora* sp., sporangium, is oval to slightly rounded or pear-shaped, whieh its spore has a whip feather (flagella) that can move in water.

The pathogens may form in round chlamydospores (Directorate of Holticulture Protection 2011).

Contamination of bacteria

Contamination due to bacteria characterized by exsplants showed symptoms of wilting and stingy media smells. The observation of the *thallus* tissue shows the presence of a ruptured cell due to a bacterial infection. Observation of bacterial morphology is done by first breeding bacteria attached to wall of *thallus* in media agar and given gram staining, then identified through electron microscope. After doing bacterial culture in media agar and identification under microscope, the result showed that morphology of bacteria is single coccus, paired, chained, and in the form of gram negative. Thus, it indicated that the bacterium was *Streptococcuss* sp.

The morphology is as shown in Figure 4.5 below:



(a)

(b)

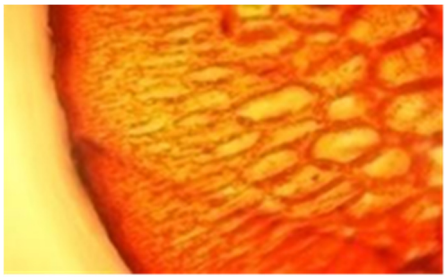
Figure 4.5 Contamination on thallus by bacteria a) morphology *Thallus* b) bacterial on a 100x magnification through microscope.

Based on Figure 4.5, it was found that the morphology and color of bacteria originating from the culture room on the control are relatively the same as the result of identified contaminant in the contaminated explants. So, the contaminant organism is indicated to have been present in the source of the explants.

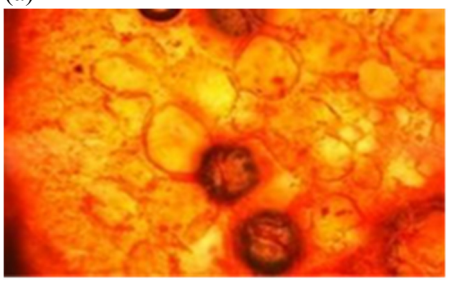
Changes in cell morphology

Cells observed in healthy *thallus* tissue appear more clearly with scattered prevalence of staining (safranin) throughout the surface of seaweed tissue. The vividly dispersed safranin indicates that the liquid cell is absorbed evenly by every space within the cell because the state of the cell wall is intact and no cytoplasm is broken while the contaminated *thallus* tissue has an incomplete cell shape and looks uneven due to the damage on cell components or rupture of the cytoplasm.

According to Juwono and Juniarto (2002) and Lakitan 2011 in Arisandi (2011), the cell wall of the plant has a main function as a protector of the cell framework. When the cell wall is damaged by the disease, it can lead to changes on shape and size of the cell. Damage to the cell wall can interfere with the absorption of nutrients into cells; it will also disrupt metabolism and inhibit cell division. The worsening damage causes the cell wall to burst, leaving the fluid out and causing the cell to become irregular and shrink (plasmolysis) subsequently as a beginning to death (Musa and Wei 2008 *in* Arisandi 2011). The difference between healthy and contaminated cells can be seen in Figure 4.6



(a)



(b)

Figure 4.6. The difference between healthy and contaminated cells in a 100x magnification. a) Healthy cells, b) cells are contaminated.

Contamination percentage

The percentage of contamination is the ratio of *thallus* of infected seaweed to cultured seaweed, calculated in percent. Based on the calculation of the contamination, percentage on culture tissue activity is 11.7% and the percentage of healthy seaweed is 88.3% with the amount of contamination per day is as shown in Figure 4.7 below

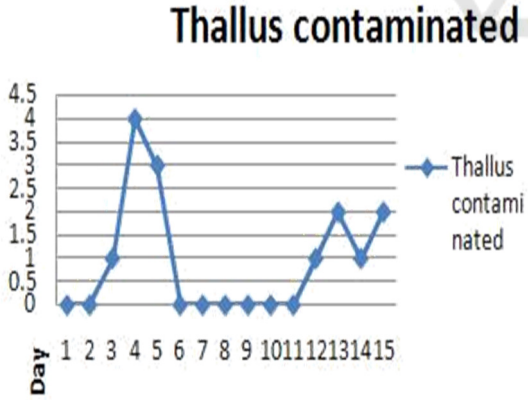


Figure 4.7 Number of Contaminated Culture Bottles.

In the first week, contaminated grass sea culture were in among 8 bottles; where the 6 bottles were characterized by bleaching *thallus,* mucus covered by dirt such as flour white, and the outer skin or epidermis flaked. While in two other bottles, there were black spots and brown line on the wall of the *thallus*. Contamination in the first week was occurred on day 3, day 4, and day 5; with the highest number occurred on day 4. In the next week, the characteristics of contamination were among in 6 bottles. Three bottles illustrated bleaching *thallus* and three other bottles obtained black spots and brown line on the wall of the *thallus.* Semangun (2001) said that between the infection and the seemingly occasional symptom, there is a long period of time yet usually the symptoms of the disease will appear after infection.

Surival rate

In the tissue culture activities conducted, the survival rate was approximately 62.5%, which the number of dead seaweed were as many as 45 bottles and alive ones were 75 bottles as shown in Figure 4.8. This indicates that the success rate of culture is proven through the number of alive seaweed was higher than the dead ones. Based on Perkasa (2011), this survival rate is one of the determinants of success in tissue culture activities. If the number of living seaweed on the harvest is high and the number of death is low, then the value of survival will be high. Otherwise, if the number of the death is higher than the other, the survival rate might be lower or below as in the diagram:

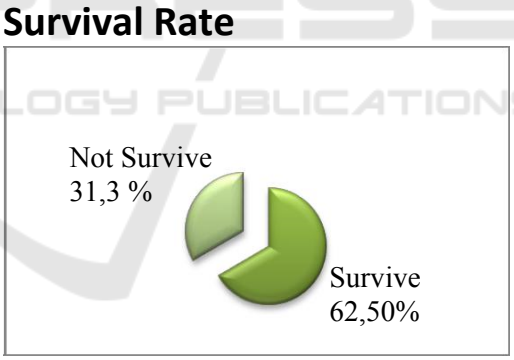


Figure 4.8 Survival rate level of tissue culture.

5 CONCLUSIONS

Based on the results obtained, it could be concluded as follows:

1. There were two species of contaminants in seawater tissue culture of *Kappaphycus alvarezii*: fungus *Saprolegnia* sp., *phythophthora* sp. and bacterium *Streptococcus* sp.
2. Fungal contamination was indicated that it is already exist in prospective explant of seaweed to be cultured, because the type of fungus is a water fungus

or Oomycota that can only grow in environments with high moisture or watery levels.

3. Contaminated cells were smaller in size and looked more shrunken than cells in tissue. A healthy *thallus* looks bigger and has more sturdy cell walls in support of its cell shape.

REFERENCES

Amiluddin, NM, 2007. Study Growth and Composition Carrageenan Seaweed Disease Affected Kappaphycus alvarezii Ice-ice in the Sea of Pari Island Thousand Islands. Thesis. IPB. Bogor. 78 p.

Anggadiredja J, S Irawati, and Kusmiyati. 1996. Potential and Benefits of Indonesian Seaweed in the Pharmaceutical Sector. National Seminar on Seaweed Industry. Jakarta. p. 49-62

Atmadja WS, A Kadi, Sulistijo and Rachmaniar.1996. Introduction of Indonesian Seaweed Types. Puslitbang Oseanologi - LIPI, Jakarta.

Atmadja WS and Sulistijo. 1977. Efforts to Utilize Euchema spinosum (L) J. AGRADH Sea Algae Cuttings Seeds in Thousand Islands to be Cultivated. In: Jakarta Bay, Resource Oceanological Properties and Problems. Editor: M. Hutomo, K Romimohtarto and Burhanudin. LON LIPI, Jakarta: pp. 433-449.

BBPP Lembang. 2008. About Network Culture, (Online), http://www.bbpp-lembang.info/ Accessed July 14, 2013. Gadjah Mada. Yogyakarta.

Darmayati, Y., A. Hatmanti, N. Farida and Surahman. 2001. Study of pests and diseases. Final report on Research on Development of Seaweed Superior Seeds, Management of Water Quality and Pests and Diseases. Deep Sea Resources Research, Development and Utilization Project. Oceanography Research Center-LIPI Jakarta. 7 things.

Ministry of Maritime Affairs and Fisheries. 2003. Profile of Indonesian Seaweed. Draft. Directorate General of Aquaculture. Jakarta. 111 p.

Directorate General of Fisheries. 2004. Seaweed Pests and Diseases.

Doty M. 1985. Euchema alvarezii sp.nov (Gigartinales, Rhodophyta) from Malaysia. Inside: Abbot IA, Norris JN (editors). Taxonomy of Economic Seaweeds. California Sea Grant College Program. P 37-45.

Haryono Semangun. 2011. Plant disease. Gadjah Mada University press: Yogyakarta.

Hayashi, L., Yokoya, N.S., Kikuchi, D.M., and Oliveira, E.C., 2008. Callus Induction and Micropropagation Improved by Colchicine and Phytoregulators in Kappaphycus alvarezii (Rhodophyta, Solieriaceae). J. Appl. Phycol., 20: 653-659.

Hurtado A.Q., and Biter A.B., 2007. Plantlet Regeneration of Kappaphycus alvarezii var. younger siblings by tissue culture. Journal of Applied Phycology, 19: 783-786

Hurtado, A.Q., Yunque, D.A., Tibubos, K., and Critchley, A.T., 2009. Use of Acadian Marine Plant Extract Powder from *Ascophyllum nodosum* in Tissue Culture of *Kappaphycus alvarezii.*Journal of Applied Phycology, 21:633–639

Hutabarat J. 1995. Workshop on Marine Cultivation: Bio Hydrography Conditio Evaluation in Determining the Location of Aquaculture, Jepara.

Indriani H and E Sumiarsih. 1999. Aquaculture, Management and Marketing of Seaweed. PT. Penebar Swadaya, Depok. IPB (1997).

Indrianto. 2002. In Vitro Selection of Andalas Plant (Morus macroura Miq.) Tolerant Drought Stress Using Polyethylene Glycol (PEG). Proceedings of the 21st Western Conference BKS-PTN Seminar and Meeting.

Kresno Yulianto. 2004. Phenomenon of controlling factors causing losses in caraginophyte cultivation in Indonesia. OseanaISSN Journal 0216-1877 LIPI, 29:

Largo, B.D., K. Fukami and T. Nishijima. 1995. Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae Kappaphycus alvarezii and Eucheuma denticulatum (Solieriaceae, Gigartinales, Rhodophyta). Journal of Applied Phycology 7: 545 – 554

Lobban CS, PJ Harison. 1994. Seaweed Ecology and Physiology. Cambridge Univ. Press New York.

Lobban, C. S and Harrison, P. J. 1995. Seaweed Ecology and Physiology. Cambridges University Press. 366 pp.

Nasution MH 2005. Pathogenicity of Several Bacterial Isolates Against Seaweed Kappaphycus alvarezii from Pari Island, Thousand Islands. Faculty of Biology, National University of Jakarta. Jakarta.

Neish, I.C. 1989. Alkali Treatment of Carragenan Bearing Seaweeds Past, Present and Future. FMC Corporation, Marine Colloid Div. 11 pp.

Nontji 1993. Laut Nusantara. Penerbit Djambatan: Jakarta

Patadjai Hospital. 1993. Effect of TSP Fertilizer on the Growth and Quality of Seaweed Gracilaria gigas Harv. Thesis Study Program on Aquatic Sciences. IPB Postgraduate Program. Bogor.

Fish quarantine center of the marine and fisheries department. 2007. Standard method of bacterial HPIK examination: Directorate General of aquaculture.

Fish quarantine center of the marine and fisheries department. 2007. HPIK class of HPIK examination standard method: Directorate General of aquaculture.

Fish quarantine center of the marine and fisheries department. 2007. Standard method of HPIK parasitic examination: Directorate General of aquaculture.

Schwann dan Schleiden.1898. Plant Propagation by Tissue Culture 3rd Edition. Springer Publisher: Netherlands

Soegiarto A., WS Atmadja, Sulistijo and H. Mubarak. 1978. Seaweed (Algae): Benefits, Potential and Cultivation Efforts. LON-LIPI, Jakarta.

Sulistiyo. 1988. Root Induction from Leaf Leaf Explants of Three Patchouli Varieties (Pogos temon cablin Benth) in MS Media Containing Paclobutrazol In Vitro Zuriat. 19 (1): 16-31

Suryaningrum TD. 1988. Study of Quality Properties of Seaweed Commodities Cultivation of Eucheuma cottonii and Eucheuma spinosum. IPB Thesis. Bogor.

Thorpe.1981, Review: In Vitro Selection and 45Somaclonal Variation for Biotic and Abiotic stress Tolerance.Biodiversitas. 7 (3): 297-301

Widianti. 2003. Introduction to Tissue Culture Techniques and Vegetative-Modern Plant Propagation Guidelines. Yogyakarta: Kanisius.

Wetherell, D. F. 1982. Introduction to Plant Propagation in Vitro. Koensoemardiyah S. SU, translator; Semarang: IKIP Semarang Press. Translation of: Introduction to In Vitro Propagation

Yusuf M.I., 2004. Production, Growth and Content of Seaweed Carrageenan Kappaphycus alvarezii (Doty) Doty (1988) which was cultivated with Different Water Media Systems and Thallus Seeds. Dissertation: Ppps. Universitas Hasanudin. Makassar. Pp.13-15.