Optimization of *Kappaphycus alvarezii* seaweed seeding system by aquaculture

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**Abstract.** *Kappaphycus alvarezii* is the main commodity of the cultivation in Madura-East Java. The preparation of qualified seeds of seaweed is one of the efforts in increasing its production. Conventional method of water nursery that has been carried out has several weaknesses, which are susceptible to pest and fluctuationg climate. The application of aquaculture method is one of the main ways to improve the quality and quantity of seaweed seeds through nutrient enrichment and the optimization of environmental factors measured in seawater as media. Therefore, in this research, the media optimization of *K. alvarezii* seaweed seeding was carried out by aquaculture. The condition in optimizing seaweed seeding was determined by setting the culture condition according to the environmental parameters where seaweed was taken. The method used in this study was TMAMQ test to measure the antioxidant capacity, the total of phenol, biomass and TPC test. This result of this research was obtained through morphological observation of seaweed growth with the provision of a recirculation system, 0.05M urea and UV-filter showed the most excellent performance observed from the number of talus growth, in addition, UV-filter could help it inhibit the growth of microbes that were proven through total colony test in the medium of seawater after entering UV, it did not show microbial growth.

1. **Introduction**

*Kappaphycus alvarezii* is a species of seaweed from the Rhodophyceae class (red algae) which has polysaccharide on its cell wall and can be processed into kappa carrageenan which is widely used as thickener, gelling agent, stabilizer and emulsifier [1,2,3,4]. A variety of products that can be produced from kappa karaginan triggers high value of market demand for *K. alvarezii* and has an impact on the improvement of socio-economic in the community so that this type of seaweed is produced in many tropical regions [4,5].

However, there are several obstacles in the process of seaweed production, one of them is caused by the association between microorganisms and seaweed that leads to the emergence of a disease called ice-ice disease [6]. In addition, one of the limiting factors in seaweed cultivation is the growing season where the growth response of seaweed is different in times and seasons of the year [7,8,9].

Ice-ice disease is very influential in reducing biomass production and carrageenan quality. The factors causing ice-ice disease in Kappaphycus/Eucheuma are triggered by unconducive environmental conditions such as extreme temperature, radiation, salinity, and pathogenic bacteria Vibrio sp. (P11) and Cytophaga sp. (P25) [10]. In overcoming this problem, superior seed is needed to support the productivity of the seed, its quality and resistance of seaweed in controlling ice-ice disease [11]. In addition, the cultivation management of aquaculture seaweed and the control of ice-ice infection become...
the important components in seaweed production [12]. However, the study on the optimization of *K. alvarezii* seaweed seeding system has not been developed much in aquaculture. Therefore, in this research, the value of *K. alvarezii* seeding system optimization by using aquaculture method would be determined. The information obtained from this research is expected to be the reference in determining the optimum condition for the cultivation and production of *K. alvarezii* seeds through aquaculture.

2. Methodology

2.1 Time and Place
This research was conducted in December 2017 to July 2018 in the Laboratory of Microbiology and Biotechnology, Laboratory of Biosciences and Plant Technology and the Zoology and Animal Engineering Laboratory at the Department of Biology, Faculty of Natural Science, Sepuluh Nopember Institute of Technology, Surabaya. Seed sampling and acclimatization were carried out in Padike Village, Talango District, Poteran Sumenep Island, Madura.

2.2 The Methods

2.2.1 Sampling
The sample was packed through semi-dry method by wrapping the sample of *K. alvarezii* seeds by using a newspaper covered with cool gel and stored in styrofoam. This was done to maintain overall low temperature and reduce the damage caused by bacteria and the process of autolysis [13].

2.2.2 The optimization of *K. alvarezii* seed samples
The obtained *K. alvarezii* seeds were acclimatized for 3 days and the optimization period was for 25 days in the aquarium. The method used in acclimatization was filter recirculation and aeration systems [10,14]. *K. alvarezii* was grown in aquaculture tank with different depths using the hanging method or "top down" [15]. *K. alvarezii* seed optimization was influenced by several controlled physical and chemical factors which were salinity, temperature, light, pH, depth, current velocity, fertilizer application (N and P sources). pH measurement was carried out once a week while the other parameters was measured every day for in 25 days. *K. alvarezii* which would be acclimatized was taken with an initial weight of 26-30 grams in the sea water as the media. Each aquaculture tub was equipped with pumping machine and filter for circulating the water consisting of: a) physical filter which were cotton and coral; b) chemical filters in the form of activated carbon; and c) biological filter in bioball form.

During the optimization period, seaweed seeds in the aquarium were used as recirculation system, which was done by 50% water change within two weeks [16]. The seawater flow in the filter recirculation system are as follows [16]: a) The sea water enters the filter space through the outlet channel; b) The sea water pass by the physical, chemical and biological filters in the filter room; c) The sea water enters the filter pump engine room; and the sea water is pumped through an inlet channel in the form of pvc pipes at a speed of 1,200 ml/minute.

2.3 Antioxidant Capacity Test.

2.3.1 Drying and extraction of *K. alvarezii* seed
The seaweed that has been taken, is washed first by using synthetic seawater which is added by 1% of betadine solution into the synthetic seawater to remove microbes and epiphytes that attached to the surface [17]. The seaweed sample was soaked in 1% of betadine for 3 minutes. The betadine solution was made by dissolving 2 mL of betadine in 200 mL of sterile seawater. Then continued by re-rinsing using sterile seawater until the betadine solution was rinsed. The *K. alvarezii* seaweed extraction method is done with several modifications [18]. This *K. alvarezii* seaweed extract was obtained by weighing 2 grams of seaweed then mashed using a mashed tool and mixed with 7 ml of 96% ethanol then macerated for 6 hours. Then homogenized by centrifuge with a speed of 3,000 rpm for 20 minutes.
2.3.2 Tetramethoxy azobismethylene quinone (TMAMQ) preparation
This method was done with modifications of the researches [19, 20]. The TMAMQ stock reagent production is done by incubating 3.19 mM of syringaldeazine compound with 20 ml of acetone and 40 ml of citrate buffer with pH 4.5. Then added by 20 ml of laccase and is incubated at 30°C for 12 hours/night while shaken at 140 rpm in thermomixer (Eppendorf AG, Germany). The TMAMQ manufacturing process was monitored using Hitachi U-2001 UV-vis spectrophotometer with a wavelength of 530 nm in a single-use cuvette. In addition, the compound mixture was filtered using Buchner flask and Buchner funnel on Whatmann filter paper number 1 until all of the compounds were on filter paper. The TMAMQ compounds that had been filtered were then rinsed with 2 liters of distilled water and dried in an incubator at 37°C for 5 hours. The TMAMQ which had been dried then stretched out and placed in a micro-tube and stored at 4°C.

2.3.3 The determination of antioxidant capacity using TMAMQ reagent
The seaweed extract was taken according to the serial dilution then added by dimethyl sulfoxide (DMSO) solution, then added by 0.7 ml of TMAMQ reagent that was made. The number of compounds used was serialized dilution starting at 0.02; 0.04; 0.06; 0.02 and 0.1 ml of dilution using 96% of ethanol. The mixture was then measured with a spectrophotometer with a wavelength of 530 nm. The measurement of the decrease in absorbance is done with the existence of the level color fading of TMAMQ with this K. alvarezi seaweed extract which indicating the presence of ionic withdrawals when adding antioxidant sample (K. alvarezi seaweed extract) [19, 20]. The results of absorbance measurements are done using the following formula [19]:

\[ \Delta \text{Absorbance} = \text{Initial Absorbance} - \text{Final Absorbance} \]  

The initial absorbance was the absorbance value of TMAMQ reagent (by 0.7-0.8), and the final absorbance was the absorbance of the extract solution mixture with TMAMQ reagent in the 5th minute. After obtaining the value of absorbance reduction, next was made a graph between the concentration of seaweed extract K. alvarezi (x) and \( \Delta \text{Absorbance} \) (y), so that the linear regression equation \( y = ax + b \) was obtained. These equation obtained by using Microsoft Excel program. The abscissa value of the results is then included in the linear regression equation on the TMAMQ standard curve in order to obtain antioxidant capacity [20].

2.4 Total Phenol Analysis
The analysis of total phenol in this research was done by extracting a sample of K. alvarezi with Follin-Ciocalteu reagent. This analysis was done by taking 1 gram of talus K. alvarezi, then adding 10 ml of 96% ethanol and 5 ml of HCL. The seaweed sample was centrifuged for 20 minutes at speed of 2,500 rpm, then taken 1 ml of seaweed extract that had been centrifuged and then added 100 ml of follin reagent and 1 ml of sodium carbonate 15% (w/v). After incubated for 1 hour, the sample absorbance was measured using a spectrophotometer at a wavelength of 760 nm. The total phenol was calculated based on the tannic acid standard curve.

2.5 Total Plate Count (TPC Test)
TPC test was done at the recirculation stage and after using the UV filter. The sea water sample was taken before and after entering the UV filter by using sterile centrifuge tube. The water sample was taken as much as 10 ml and serial dilution was made from 10-1 until 10-6 media of sea water sterile. The sample was taken as much as 0.1 ml and was inoculated into a Petri dish containing NA + sterile sea water media and flattened by using drigalski spatula. Then, it was incubated for 24 up to 48 hours at room temperature. The number of growing colonies were about 30-300 counted by using colony counter [21]. Based on the total bacteria that grew were calculated by using the calculation formula
which had been determined by BPOM Regency Regulation number HK.03.1.23.08.11.07331 in 2011, as follows:

\[
N = \frac{\Sigma c}{(V(n1 + 0.1n2)xd)}
\]  

(2)

2.6 Data Analysis

The data obtained from the measurement of antioxidant level, total phenol, and ALT/TPC test on the seaweed seed \( K.\ alvarezii \) and was analysed descriptively qualitative. The capacity data of extract \( K.\ alvarezii \) was known from the decrease of the absorbance value, then was calculated the antioxidant capacity by using Microsoft Excel program and compared with the TMAMQ standard curve (the measurement using reagent TMAMQ).

3. Results and Discussions

3.1 The Acclimatization of \( K.\ alvarezii \) in the Aquaculture Container

The acclimatization of seaweed was done in 3 days in the plastic container completed with recirculation. In the Figure 1 could be seen the appearance of the seaweed after acclimatization.

![Figure 1. The appearance of \( K.\ alvarezii \) after the acclimatization](image)

The whole samples of \( K.\ alvarezii \) came from Talango District, Sumenep Regency, Madura with the morphology characteristic that was the talus was tapered, long, and close altogether. The color of the seaweed used was all dark green with the smooth talus surface and formed a thick clump. The characteristic of the seaweed used was adjusted with SNI standard for Kotoni Seaweed seed [23]. The recirculation system in acclimatization containers and maintenance of \( K.\ alvarezii \) seaweed seed as shown in the Figure 2.

The multilevel height in the Figure 2 used to produce the equal incoming water debit with the outcoming water debit, then the water was filtered in the aquarium containing cotton and coral as the bio-filter media to produce the qualified sea water called Recirculating Aquaculture System (RAS) compared to use Batch system in which the sea water circulation occurred only in each plastic container without any circulation to the whole parts of aquaculture system. The comparison between Batch system and the circulation system could be seen in [24,25]. Furthermore, the sea water quality parameter in this research was done by measuring the sea water media covering the temperature, pH, lightness, salinity every 2 hours for 10×24 hours before sea weed planting was done. The \( K.\ alvarezii \) seaweed seed was tied to ancak bamboo with 20 cm spacing and in a plastic container consisted of 9 seeds.
3.2 The Influence of Recirculation, UV and Fertilizer Giving on the Seaweed Growth

The seaweed growth on the sea water media with the different treatment variations was shown in the Figure 3. Based on the Figure 3A showed that the *K. alvarezii* seaweed seed was in a healthy condition and protected from the virus attack, so that it was suitable to be cultivated. This was in line with the statement says that the good performance of seed has several criteria that are has many branches, lush and pointed tip, there are no spots or cuts, and looks fresh [26]. Then, in the Figure 3B could be seen that the seaweed growth with no recirculation treatment, UV and fertilizer giving showed the bad growth. This was indicated by the changing color of the talus into fade (bleaching) and the texture talus soften. The change of seed in the day 7. In the Figure 3C could be seen that the seaweed seed experienced the discoloration on the tip and the base of the talus contained white spots. The quality declining of the seed was also seen in the Figure 3D that was the talus development was not perfect indicated by several broken parts, but in some parts, there was still a growth of talus. Whereas, in the Figure 3E through the
use of urea fertilizer showed the good quality performance of the seaweed, indicated by the abundance of the talus and the seaweed seed’s color still looked fresh.

Figure 3. The influence of aquaculture system differences on the *K. alvarezii* seaweed growth. A. initial seed; B. no recirculation, UV, and fertilizer group; C. the group with recirculation, without UV and fertilizer; D. the group with recirculation and UV, without fertilizer; E. the group with recirculation, UV and urea fertilizer 0.05M; without arrow showed the quality changing growth of *K. alvarezii* which was observed morphologically.

Figure 4. The appearance of total colony in the petri disc: A. the sea water sample before entering the UV filter dilution, B. the sea water sample after entering the UV filter dilution.
The quality of seed greatly determined the productivity and virus-resistant, so that by the influence of recirculation, UV and fertilizer giving influenced the nutrient availability. Nutrition was able to influence the metabolism process by providing the nutrition sources which consisted of the important element such as nutrient (N and P) and triggered the diffusion process that was the entry of nutrients in the tissue on the whole parts of the seaweed surface so that if the diffusion continued, then it accelerated the metabolism process and increased the growth rate, on the contrary, when the water was lack of nutrients, it affected the seaweed growth became slow and unhealthy [27], while the influence of UV filter was able to suppress the microbial growth in the seawater media as shown in the Figure 4.

Based on the Figure 4 could be seen that there was a difference in the media NA + sea water before and after UV filter installation On the second measurement, there was no microbial colony obtained that grew, while on the first measurement, the number of microbial colonies reached 1.6×10^9 CFU/gr with 10^-6 dilution. The average temperature and light intensity in the sea water during the recirculation period was shown in the Figure 5.

![Figure 5](image.png)

**Figure 5.** The average light intensity and daily temperature during sea water recirculation period

![Figure 6](image.png)

**Figure 6.** The light intensity average and daily temperature during the seawater recirculation period

Based on Figure 5, it could be explained that the light intensity average got during the recirculation period was about 540-1920 Lux and the daily temperature average during the recirculation period was about 27-31°C. Moreover, the salinity and daily pH (Figure 6) was in the optimum range of 26-31 ppt.
and 6-8. The average range of temperature, pH, salinity and light intensity that was measured during the recirculation period was a good range to the growth of seaweed *K. alvarezii*, this was based on the BSNI and SNI KKP value number SNI 01-6492-2010 which said that the optimum temperature for the growth of seaweed was about 27-30°C, optimum pH was about 7-8 and the salinity as much a 27-30 ppt.

Based on the fluctuations result on seawater quality, there needed an effort in conditioning the physicochemical factor in this research by diluting the seawater regularly to reduce the salinity level, it aimed to protect the optimization of seawater media and to support the seaweed *K. alvarezii*’s growth on aquaculture container [28]. The salinity fluctuation could influence the osmolarity of the cell and caused stress to the organism [29].

### 3.3 The Effect of Sea Water Condition on *K alvarezii* Growth Profile

The antioxidant level calculation was done twice to know the antioxidant gripped condition. The total phenol was measured four times to know the change happened on the seaweed sample with differentiation on the depth and urea fertilization, while the seaweed biomass calculation was done after 6 days of cultivation, it was shown at Figure 7.

![Figure 7](image)

**Figure 7.** The effect of seawater condition on *K. alvarezii* growth during the optimization:  
A. the total phenol average, B. the antioxidant level average, C. the daily growth rate average

In Figure 7A, it could be seen that the phenol content on each measurement had decreased and it was marked by the high concentration of the total phenol in the first measurement that reached 7.78 on the
control seaweed sample (+) while in the last measurement it could be seen that the value of the obtained total phenol was inversely proportional with the antioxidant acreage which tends to increase.

Figure 7B. in the seaweed sample with fertilizer dosage, where the first measurement reached 3.15 mM while in the last measurement reached 3.974 mM. The size of phenolic compounds content was influenced by the environment, this was because the secondary metabolite compounds were formed to protect the plant from environment gripped, provided enzyme, energy, substrate and cellular machinery that played a role to maintain long-term survival [30].

The seaweed K. alvarezii growth rate (after 10 days of cultivation) on Figure 7C increased on some planted seedling involved the talus length, the branch number, and mass. However, after 10 days, the seaweed quality in terms of biomass was decreased until bleaching occurred. This happened because there was a nutrition limitation and unstable environment parameter got during the optimization period until trigger the tissue damage of the seaweed. When the seaweed experienced stress, it would produce halocarbon toxic that was easy to evaporate and caused necrosis on the plant tissue [31].

4. Conclusions
Based on the research that had been conducted, it can be concluded that the performance of seaweed was best observed morphologically after having recirculation treatment, UV-filter, and urea 0.05 M fertilization. The highest antioxidant capacity was in the first measurement that was on 35 cm seedlings depth with reduction average of TMAMQ as much as 3.197 mM. In addition, the highest total phenol concentration was gotten on the first day of the measurement as much as 3.13 ppm on the seedlings with 35 cm depth.

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