The axenic treatments for Kappaphycus alvarezii (Rhodophyta) seedling in laboratory culture

R I Adharini¹*, A R Setyawan¹, A D Jayanti¹, Suadi¹ and E A Suyono²

¹ Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora 1A, Bulaksumur, Sleman, Yogyakarta, Indonesia
² Department of Biotechnology, Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Sleman, Yogyakarta, Indonesia

*Email: ratih.adharini@ugm.ac.id

Abstract. Obtaining an algae axenic culture in the culture medium are challenging. This study aims to determine the effectiveness of the application of several methods to reduce bacterial contamination in culture medium. The study was conducted using a complete randomized design in 3 stages, stage 1 using iodine immersion method with 4 treatments, stage 2 using immersion of antibiotics mixture with 3 different concentrations, stage 3 using mixture of antibiotic and medium with 3 concentration. The results showed that in stage 1, the lowest bacteria density was in treatment 1 (15 % betadine with 60') (675.27 idv·mm⁻²), stage 2 showed that treatment 1 (50 mg·L⁻¹) had the lowest bacteria density (265.62 idv·mm⁻²). Stage 3 showed that treatment 3 (10 mg·L⁻¹) had the lowest density of bacteria (24.78 idv·mm⁻²). Based on the ANOVA test, stage 1 has no significant difference (> 0.05), in stage 2 there was a significant difference with treatment 1 was the best treatment, in stage 3 there was a significant difference and treatment 4 was the best result. Iodine 15 % with 60' immersion time; immersion with 50 mg·L⁻¹ antibiotic, and mixture of medium with 10 mg·L⁻¹ antibiotic gave the best results in reducing bacterial contamination.

1. Introduction
Seaweed is one of the leading commodities of aquaculture fisheries that have high economic value both in local and export markets. Seaweed is one of the three main commodities in the revitalization of fisheries other than fish and salt. Based on Ministry of Fisheries and Marine Affairs production data of 2015, the production volume of seaweed cultivation is 11.68 million tons or reach 66.87 % of total aquaculture production [1]. This shows that seaweed is the most dominant commodity in the production of aquaculture fishery.

Kappaphycus alvarezii is a seaweed that has a very high economic value. Kappaphycus alvarezii seaweed contains carrageenan that has been used in several industries. The use of carrageen in the industry is almost equal to the agar, among others, as a regulator of balance, thickening, gelling, emulsifying and so on. Utilization of carrageenan by industry includes food industry, pharmaceuticals, cosmetics, textiles and organic fertilizer [2]. Kappaphycus species are also highly sought after by coastal communities due to relatively easy cultivation technology, short maintenance time and relatively cheap production costs [3].

Provision of good seed is one of the activities that determine the success of seaweed cultivation. Currently the seeds are used and developed by the community is still obtained from the results of vegetative development by way of setting aside thallus cultivation of his own, so the availability of good quality seeds are sometimes limited. The low technological mastery ranging from
seeding, cultivation, harvesting and drying of seaweed is also a crucial problem in increasing the productivity of seaweed farming [4]. Efforts to provide superior seaweed seedlings can be done with the application of biotechnology. Technology which has been developed in seaweed in general is a technique of tissue culture, protoplast culture, embryogenesis techniques and culture of the spore [2].

Seed propagation through in vitro as tissue culture and culture is one seedling that can be done at any time and does not depend on the season. However, one of the obstacles in the application of biotechnology in the development of seaweed seedling is the difficulty to make an axenic culture. Axenic culture is a culture that obtained from an algal species, without any contamination from other living organisms [5]. The contamination in culture is common, generally by diatoms and bacteria. Development of the axenic culture of seaweed is very important in the preparation of seed stock and mass culture from seeds so that it will determine the success and quality of the seed produced. This study aims to determine the effectiveness of the application of several methods of antibiotic treatments to reduce bacterial contamination in the culture medium.

2. Materials and methods

2.1. Experimental design

The experiments were performed using a complete randomized design divided into 3 stages with one control (without treatment). Stage 1 was the application of iodine immersion with treatments: control (C); T1 (10% betadine for 30'); T2 (10% betadine for 60'); T3 (15% betadine for 30') and T4 (15% betadine for 60') with three replications. Stage 2 was the application of antibiotic (Penicillin and streptomycin) immersion with 3 different concentrations: control (C); T1 (50 mg·L⁻¹); T2 (100 mg·L⁻¹); T3 (150 mg·L⁻¹) for 24 hours with three replication. Stage 3 was the application of medium and antibiotic mixture (the mixture of medium f/2 with penicillin and streptomycin antibiotics) with 4 different concentrations: control (C); T1 (6 mg·L⁻¹); T2 (8 mg·L⁻¹); T3 (10 mg·L⁻¹). The samples were cultured in the room temperature and light intensity of 4.617 µmol·m⁻²·s⁻¹.

2.2. Sample preparation, betadine and antibiotics

The sample was *Kappaphycus alvarezii*. The samples were collected from seaweed farming site in Popayato, Pohuwato district, Province of Gorontalo, Sulawesi, Indonesia 0° 30' 53" North and 121° 28' 24" East. Before the experiment, the samples were cleaned by washing using sterile sea water and the sample was cleaned from the dirt and epiphytes on the thallus surface using a paintbrush and a razor blade. Once clean, then thallus cut into small pieces of 3–5 mm in size. Making betadine by mixing 100 % betadine with sterile seawater with the ratio according to treatment that is equal to 10 % and 15 %. Preparation of antibiotics by mixing Penicillin and streptomycin with sterile sea water by comparison according to treatment i.e. 50 mg·L⁻¹, 100 mg·L⁻¹ and 150 mg·L⁻¹. A mixture of medium and antibiotics was prepared by mixing the f/2 medium and the antibiotics with concentrations of 6 mg·L⁻¹, 8 mg·L⁻¹ and 10 mg·L⁻¹.

2.3. Data collection

In the first stage, iodine immersion was applied, the concentration used was 10 % and 15 % with 30' and 60' immersion period respectively. The samples used in each well were 18 pieces of thallus. Observations were made after 24 hours of immersion. The data taken is the bacterial density of each well from the microplate.

In stage 2 with the application of antibiotic placement, the concentrations used were 50 mg·L⁻¹, 100 mg·L⁻¹, and 150 mg·L⁻¹. The numbers of thallus used in each well were 3 thallus with 5 mm size per well. Observation of bacterial density was done after 24 hours of immersion using microscopic observation with 100x magnification.

In stage 3, the application of a mixture of medium f/2 with antibiotics, the concentration used was without the addition of antibiotics, 6 mg·L⁻¹, 8 mg·L⁻¹ and 10 mg·L⁻¹. The number of thallus used in
each well is 3 thallus. Observation of bacterial density was done at 24 hours, 48 hours and 72 hours of immersion.

2.4. Data analysis
The obtained bacterial density data were then descriptively graphed and one way ANOVA test was performed using SPSS program to find out the best treatment.

3. Results and Discussion

3.1. Stage 1 (Iodine immersion)

Based on the observation of bacterial density in stage 1 by immersion on betadine, can be seen the influence between the amount of concentration of betadine and the duration of immersion to bacterial density. The results showed that in 10% betadine (1% provine-iodine) immersion (T2 and T3) had the highest bacterial density value and there was no difference between immersion time. The magnitude of bacterial density at T2 and T3 is higher than control, this may be possible with concentrations used still under betadine concentrations to kill bacteria. Figure 1 shows that the best treatment is in T4 which was immersion with 15% betadine (1.5% provine-iodine) with 60’ immersion time. However, based on ANOVA test, there was no significant difference in each treatment, but T4 treatment showed the best result. Application of higher concentrations and longer periods of time is possible to kill the thallus and spores of seaweed. Betadine contains provine-iodine which is commonly used as a disinfectant. Application of iodine as a disinfectant in the culture of seaweed has been done to some researchers, such as [6] using provine-iodine 2% to obtain pure cultures, [7] provine-iodine using a mixture of 3 g·L⁻¹ and 15% ethanol, [8] using provine-iodine of 0.5%. Iodine works by reducing the oxygen needs of aerobic microorganisms. Iodine would disrupt the rate of the respiratory chain of microorganisms by blocking the transport of electrons through electrophilic reactions with respiratory chain enzymes [9].
3.2. Stage 2 (Antibiotic immersion)

![Figure 2. Density of bacterial phase II.](image)

Based on the observation in stage 2 that by immersion using three antibiotic concentration there was a significant difference between without immersion (control) and with antibiotic immersion (T1 (50 mg·L⁻¹), T2 (100 mg·L⁻¹), T3 (150 mg·L⁻¹)). The growth of bacteria in control in density was 991 idv·mm⁻², compared with the treatment of T3 immersion which is the higher density between antibiotic immersion treatments having density 544 idv·mm⁻². It is showed that the antibiotic immersion treatment inhibits the bacterial growth effectively. It is also indicated by ANOVA test which has a significance value of 0.04 or below 0.05 which shows that there are different in between treatment and there was an effect of immersion antibiotic with bacterial growth. The best treatment in stage 2 was T1 (50 mg·L⁻¹) with a density of 266 ind·mm⁻² (figure 2). The best treatment is the smallest concentration because in the larger concentration the bacteria become more resistant to antibiotics therefore ineffective in reducing bacterial growth. Antibiotics have been widely applied to remove or inhibit bacterial growth. Penicillin G, streptomycin sulfate, chloramphenicol and gentamycin are commonly used antibiotics [10]. Antibiotics immersion has been widely applied, as in [7] used a mixture of penicillin and streptomycin of 200 mg·L⁻¹ to obtain the axenic culture. While, [11] using a rifampicin (300 mg·L⁻¹), streptomycin/penicillin (25 mg·L⁻¹) and nystatin (10 mg·L⁻¹) to obtain axenic culture.
3.3. Stage 3 (Medium and antibiotic mixture)

![Figure 3. Density of bacterial phase III.](image)

Based on the observation at stage 3 that is, using the mixture of medium f/2 and antibiotic, showed the influence of different concentration with bacterial growth. Figure 3 shows that treatment of C (control) which is a sample without antibiotics had the highest bacterial growth value with the density of 58.11 idv·mm$^{-2}$. This is different from the treatment of 1 (6 mg·L$^{-1}$), 2 (8 mg·L$^{-1}$) and 3 (10 mg·L$^{-1}$) which is a mixture of medium and antibiotics. The best treatment was found in the treatment of T3 which was the highest concentration of medium and antibiotic (10 mg·L$^{-1}$) with the lowest bacterial growth of 25 idv·mm$^{-2}$. Based on the ANOVA test, it shows a significance value of 0.000 or below 0.05 so there is a real difference between treatments, with the best treatment being at T3. Penicillin G. and streptomycin sulfate are included in B-lactam antibiotics, which have a broad spectrum and have an effect on cell wall synthesis in both gram-positive and negative bacteria [11]. This makes both types of antibiotics are effectively used in inhibiting the growth of bacteria in seaweed culture. The application of medium and antibiotic mixtures was shown to be effective in inhibiting bacterial growth, [12] used a mixture of antibiotics (Penicillin G., Streptomycin sulfate, Polymyxin B sulfate and Chloramphenicol) on the medium for ten days to obtain axenic culture and [10] stated an addition of 2–5 drops of antibiotic mixture on 10 mL of medium for three days can inhibit bacterial growth and produce axenic culture.

4. Conclusion
Application of several methods to reduce bacterial growth can be done by using immersion of 15% betadine for 60 minutes, followed by a mixture of penicillin G. and streptomycin sulfate immersion at concentrations of 50 mg·L$^{-1}$ for 24 hours and using a medium and antibiotic mixture of 10 mg·L$^{-1}$.

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