Determining of water bioremediation dosage in recirculating water system for Cantik Grouper (*Epinephelus fuscoguttatus* × *Epinephelus microdon*) nurseries

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**Abstract.** The use of recirculating aquaculture system (RAS) in nurseries of cantik grouper (*Epinephelus fuscoguttatus* × *Epinephelus microdon*) has the potential to make the culture environment more stable and maximize production performance, but the work of RAS decreases with the accumulation of waste in the water used. Remediation of water with microorganisms (bioremediation) can improve the performance of RAS. This research aims to determine the best dose of commercial probiotics in water bioremediation to be used in nurseries of cantik grouper seeds. This research was conducted for 7 days using a completely randomized design with four treatments and three replications. Treatment doses of probiotics tried: 3 ml/m3, 6 ml/m3, 9 ml/m3 and 12 ml/m3. The container used was a concrete tank measuring 1 m × 2 m × 0.8 m filled with 1 m3 of seawater and 3.2±0.3 cm of cantik grouper seeds with a density of 500 fish/m3. The addition of probiotics up to 12 mL/m3 had no significant effect (P>0.05) on the survival rate of cantik grouper fish for 7 days of rearing, ranging from 99.1% to 99.3%. The best results of the bioremediation process were obtained with the addition of 6 mL/m3 probiotics with the lowest range of ammonia and nitrite about 0.0896–0.9303 mg/L and 0.0977–8.4880 mg/L. The addition of a probiotic dose of 6 mL/m3 can inhibit the growth of pathogenic bacteria such as *Vibrio* spp. in water at the beginning, middle, and end of maintenance reached 2, 48, and 140 CFU/mL.

1. **Introduction**

Grouper *Epinephelus* sp. known as marine commodities that have high economic value. One of the grouper fish that is in demand for aquaculture is the cantik grouper, namely the hybridization between tiger grouper (*Epinephelus fuscoguttatus*) and batik grouper (*Epinephelus microdon*) [1]. The grouper production system is integrated into several business segments including egg production, hatchery, nursery, and aquaculture businesses. Grouper hatcheries usually only maintain larvae in one production cycle until they are ready for sale with a size of ± 3.0 cm with a rearing age of 40-60 days [2]. Nursery is the maintenance of seeds from a size of ± 3.0 cm to larger sizes that are ready to be stocked, according to demand for aquaculture [3]. The benefit of nursery is the ease of aquaculture efforts to get grouper seeds in the right size, quantity and time as desired.

Nurseries are carried out with high stocking of small grouper seeds that are susceptible to stress and disease, so there needs to be an appropriate nursery system to make it more profitable [4]. Nursery is usually done with regular water changes every day and requires a lot of replacement water. The system is susceptible to changes in water conditions that give rise to bacterial diseases [5]. Symptoms of the disease that attack such as appearing red in the mouth and fins. If not treated immediately, it will cause injuries to the body and many deaths that occur in fish [6]. Disease control in fish that are
Generally used are antibiotics and disinfectants. However, long-term use of chemicals can cause bacterial resistance [7].

Recirculating aquaculture system (RAS) is a way to reduce water requirements, energy consumption, and create a more stable fish rearing environment. The use of water in the RAS is reused by rotating the water continuously so that it can save water needs [8]. The filter on the RAS causes a decrease in ammonia in the water in the recirculation system due to the presence of pores where bacteria live for the nitrification process [9]. Solid particles dissolved in water can also be filtered and stick to the filter surface [10]. RAS is one solution in intensive grouper aquaculture. However, along with the growth of fish and accumulation of waste, the work of RAS decreases. Increased metabolic waste, increased concentrations of various water elements, and decreased water quality in RAS can affect fish growth and health [11]. Water remediation in the RAS system is needed to improve the performance of the RAS. This research aims to determine the best dose of commercial probiotics in water bioremediation to be used in nurseries of cantik grouper seeds.

2. Methodology

2.1. Research time and place

This research was conducted for 7 days at CV Jaya Utama Abadi, Kaliasem Village, Banjar District, Buleleng Regency, Bali. Ammonia and nitrite measurements were carried out at the water quality laboratory and total bacteria and *Vibrio* spp. at the pathology laboratory, Mariculture Research and Fisheries Extension, Gondol, Bali.

2.2. Experimental design

The research was conducted using a completely randomized design with four treatments and two replications. The treatment was carried out by maintaining grouper seeds with a recirculation system and the addition of different doses of probiotics: 3 mL/m$^3$, 6 mL/m$^3$, 9 mL/m$^3$ and 12 mL/m$^3$. The fish used were grouper seeds measuring 3.2±0.3 cm with a density of 500 fish/m$^3$.

2.3. Preparation of cultivation containers and recirculation systems

The container used is a concrete tank measuring 1 m × 2 m × 0.7 m containing 1 m$^3$ of seawater. Each tub is equipped with aeration using a blower to supply oxygen. Before being used in the research, the container was disinfected with 100 mg/L chlorine then the bottom and walls of the tub were brushed, rinsed with fresh water and dried.

The filter for the recirculation system used is made using 3 containers measuring 25 L. Water is flowed from the maintenance container into the filter using a power-heads water pump with a flow rate of 1,600 L/hour. The first filter consists of synthetic foam (dacron), sand, zeolite and activated charcoal. Then the water enters the 25 L filter containing the bioball. The water passes through a 25 L settling tank before re-entering the rearing tank.

2.4. Nursery Process

2.4.1. Fish stocking

The seeds came from a household-scale hatchery (HRST) located in Kalianget Village, Banyuglugur District, Situbondo Regency, East Java. Seeds are transported by car in a closed system. Acclimatization is done so that the fish are not stressed by changes in the media during transportation to the rearing medium. The method of acclimatization is to insert a plastic bag containing fish into the rearing medium to be used. The plastic bag were allowed to float for 15 minutes, then the ties were opened and the fish were allowed to come out of the plastic by submerging the plastic bags halfway so that the fish came out by itself.

2.4.2. Feeding

The feed given is in the form of commercial pellets with a protein content of 48-55% with a size adjusted for the mouth opening of the cantik grouper. Feed is given at satiation by spreading the feed gradually continuously until the fish do not approach the feed. Feeding time is at 06.00, 12.00 and 17.00 WITA.
2.4.3. Water quality management

The water used is sea water that has passed through a water filter consisting of stone, coral, and sand. The water is then collected into 2 reservoirs measuring 3 m × 2 m × 3 m and distributed to the maintenance tank. The water continues to flow and is filtered by the filter. Liquid commercial probiotics as much as 3 mL/m$^3$, 6 mL/m$^3$, 9 mL/m$^3$ and 12 mL/m$^3$ were added to the rearing water at the beginning of maintenance. The commercial probiotics used contain Lactobacillus casei and Saccharomyces cerevisiae bacteria.

2.4.4. Sampling

Sampling of cantik grouper in this research was carried out by counting the number of fish to get survival rate parameters. Survival rate (SR) is the ratio of the total number of fish that live until the end of the rearing with the number of fish at the beginning of stocking [12]. Water quality data collection including dissolved oxygen, salinity, temperature, and pH was carried out every day, while ammonia (NH$_3$-) and nitrite (NO$_2$-) were carried out on day 0, 4, and day 7 of maintenance. Total bacteria and total bacteria Vibrio spp. isolated from the water for each treatment. Sampling was carried out on day 0, 4, and day 7 of maintenance. Sample water placed in a 100 mL sterile bottle was taken 0.1 mL and put into thiosulphate citrate bile salt sucrose agar (TCBSA) media for examination of Vibrio spp. and 0.1 mL was put into trypsin soy agar (TSA) media for total bacterial examination, then incubated for 48 hours. The calculation is based on the colony forming unit (CFU) the number of bacteria per mL can be determined by multiplying the result of the calculation by the dilution factor. Counts were made on the total bacteria and Vibrio spp [13].

2.5. Data analysis

Parameter data for the survival rate of cantik grouper seeds, total bacteria and Vibrio spp. in water was analyzed for variance (ANOVA) at 95% confidence interval. Analysis of variance was performed to determine whether or not the treatment had a significant effect on the test parameters. If it has a significant effect, then it is continued with Duncan's test to determine the difference between treatments at a 95% confidence interval. Water quality parameters were analyzed descriptively to explain changes in water conditions during maintenance by presenting in tabular form. Data analysis was carried out using Microsoft Excel 2013 and SPSS version 23 software.

3. Result and discussion

3.1. Grouper seed survival rate

The survival rate of cantik grouper with the addition of different doses of probiotics for 7 days of rearing is presented in Figure 1. The results contained in each parameter value in the table are the average value and standard deviation. Based on Figure 1, it is known that the addition of probiotics with different doses had no significant effect (P>0.05) on the survival rate of grouper fish for 7 days of rearing. The mean survival rate for all treatments ranged from 99.1% to 99.3%.

The survival rate is the main parameter that shows the success of the cantik grouper nursery business. The final product in the nursery business is based on the individual fish produced. The high survival rate obtained in the research was due to the condition of the water with recirculation and bioremediation systems used optimally for grouper life. Water conditions that are not suitable can be an obstacle to grouper nursery efforts during high tides, big waves, cloudy, and muddy. This clogs the sand filter so that it is no longer effective and the water to be used becomes dirty so that the seeds in the nursery tanks appear diseases from bacteria such as red coloration on the fins and mouth, injuries to the body, and many deaths [14].

3.2. Water quality

The results of water quality parameters of cantik grouper reared for 7 days with a recirculation system at each additional dose of different bioremediators are presented in Table 1. Water quality parameters include temperature, salinity, pH, dissolved oxygen (DO), ammonia (NH$_3$-), and nitrite (NH$_2$-). The quality value of each tested parameter shows the minimum and maximum values in the treatment.
Figure 1. The survival rate of cantik grouper in each additional dose of probiotics for 7 days of rearing.

The temperature during maintenance in all treatments was in the optimal range of 28.7–31.2°C. Temperature is one of the physical factors that affect the growth of fish. This happens because fish are cold-blooded animals where changes in temperature can affect changes in the metabolic rate of fish and the survival rate of fish [15]. Low seawater temperatures (<27°C) and high daily temperature fluctuations (>2°C) affect grouper survival [14]. The salinity value obtained is close to the optimal value range between 30–34 ppt. Salinity is a chemical parameter of water that affects the osmoregulation system of fish so that changes in salinity will interfere with fish growth and metabolism [16]. The pH values obtained in all treatments were close to the optimal value range between 7.3–7.8. DO values in all treatments were in the range of optimal values between 5.82–6.55 mg/L. pH and dissolved oxygen (DO) are chemical parameters of water. pH is an indicator of the balance between acid and base in water. Changes in water pH greater than 0.5 affect fish appetite. A pH value greater than 8 will increase the ammonia content in the water which can affect the metabolism and growth of fish [17]. DO is an influential parameter in helping the oxidation of waste materials and food combustion to produce energy for the life and growth of groupers. DO in the maintenance media with a recirculation system plays a role in nitrifying bacteria that live in systems that require oxygen [18].

The highest range of ammonia values during maintenance was obtained in the 3 mL/m³ treatment between 0.1316–2.4078 mg/L. The lowest ammonia range value during maintenance was obtained at 6 mL/m³ treatment of 0.0896–0.9303 mg/L. The highest nitrite range value during maintenance was obtained at 3 mL/m³ treatment between 0.1080–9.4860 mg/L. The lowest nitrite range value during maintenance was obtained at 6 mL/m³ treatment of 0.0977–8.4880 mg/L.

Table 1. The value of the water quality of the cantik grouper rearing media for each treatment at different doses for 7 days of rearing.

| Parameters       | 3 mL/m³ | 6 mL/m³ | 9 mL/m³ | 12 mL/m³ | Optimal value |
|------------------|---------|---------|---------|----------|---------------|
| Temperature (°C) | 28.7–31.1 | 28.8–31.1 | 28.7–31.2 | 28.8–31.2 | 28–32         |
| Salinity (ppt)   | 30–34   | 31–34   | 30–34   | 31–34   | 28–33         |
| pH               | 7.4–7.8 | 7.5–7.8 | 7.4–7.8 | 7.3–7.8 | 7.5–8.5       |
| DO (mg/L)        | 5.83–6.34 | 5.89–6.55 | 5.94–6.47 | 5.82–6.25 | >4            |
| NH₃ (mg/L)       | 0.1316–2.4078 | 0.0896–0.9303 | 0.1473–1.9230 | 0.0985–1.7982 | <0.01         |
| NO₂⁻ (mg/L)      | 0.1080–9.4860 | 0.0977–8.4880 | 0.1346–9.0500 | 0.0092–8.5520 | <1            |

Dangerous increase in ammonia because it is considered to exceed the optimal range. Sewage waste in maintenance water can reduce filter performance in the recirculation system, so water remediation is needed [20]. In this research, a dose of 6 mL/m³ was able to inhibit the increase in ammonia in the best care treatment. Bioremediation in the recirculation system contains Lactobacillus casei bacteria. Consists of lactic acid which can ferment organic matter so that the overhaul of organic matter in the water. Saccharomyces cerevisiae can activate protease enzymes that play a role in
solving protein problems including ammonia, nitrite, and nitrate [21]. Ammonia that accumulates in the water maintenance medium will be oxidized to nitrite. Nitrite is less toxic than ammonia [18].

3.3. Total bacteria and Vibrio spp.

The value of total bacteria and Vibrio spp. in water at the beginning, middle, and end of 7 days of maintenance with the addition of different doses of probiotics are presented in Table 2. Based on Table 2, the addition of probiotics with different doses had no significant effect (P>0.05) on the total bacteria in the rearing medium at the beginning and middle during 7 days of maintenance. The addition of probiotics with different doses had a significant effect (P<0.05) on the total bacteria in the rearing medium at the end of 7 days of maintenance. Total bacteria at the beginning, middle and end of maintenance for all treatments ranged from 4,800 to 58,500 CFU/mL.

The results in Table 2. show that the addition of probiotics with different doses had no significant effect (P>0.05) on the total Vibrio spp. in maintenance media at the beginning for 7 days of rearing. The addition of different probiotics had a significant effect (P<0.05) on the total Vibrio spp. in the media in the middle and at the end for 7 days of rearing. The total mean of Vibrio spp. obtained in the range of 2 to 320 CFU/mL. Total increase in Vibrio spp. at a dose of 3 mL/m³ was significantly different from all treatments. The highest total Vibrio spp. concentrations in water at the beginning, middle, and end of maintenance were shown at the probiotic dose of 3 mL/m³, namely 10, 320, and 1,040 CFU/mL. The lowest total Vibrio spp. in water at the beginning, middle, and end of maintenance indicated at 6 mL/m³ probiotic doses, namely 2, 48, and 140 CFU/mL.

| Probiotic dosage (mL/m³) | Total bacteria (CFU/mL) | Total Vibrio spp. (CFU/mL) |
|-------------------------|-------------------------|---------------------------|
|                         | Beginning | Middle | End  | Beginning | Middle | End  |
| 3                       | 37.050a  | 47.150a | 19.050a | 10a       | 320a   | 1,040a |
| 6                       | 36.100a  | 39.550a | 4.800b  | 2b        | 48b    | 140b  |
| 9                       | 58.500a  | 31.000a | 10.500ab | 9a       | 60b    | 215b  |
| 12                      | 44.050a  | 28.150a | 13.750ab | 8a       | 48b    | 175b  |

*Different letters on the same parameter indicate significantly different results between treatments at the 5% test level (Duncan’s interval test).

Measurement of total bacteria and Vibrio spp. in water the maintenance medium was carried out to determine whether there was inhibition of Vibrio spp. by probiotic bacteria [22]. The probiotic dose of 6 mL/m³ showed the best results in bioremediation inhibiting Vibrio spp. by probiotic bacteria. The prevalence of infectious diseases such as VNN (Viral Nervous Necrosis) was followed by an increase in the total bacterial population and total Vibrio spp.. Total bacteria were divided into four population groups, namely low (<10³ CFU/mL), moderate (10³-10⁶ CFU/mL), high (10⁷-10⁸ CFU/mL), and very high (10⁹-10¹¹ CFU/mL). Total Vibrio spp. also divided into four population groups, namely low (<10³ CFU/mL), moderate (10²-10¹ CFU/mL), high (10²-10⁶ CFU/mL), and very high (10¹²-10⁹ CFU/mL) [23]. The total range of water bacteria obtained during the research was in the low group. The total range of Vibrio spp. water obtained during the research included the low to moderate group.

4. Conclusion

The addition of probiotics up to 12 mL/m³ had no significant effect (P>0.05) on the survival rate of cantik grouper fish for 7 days of rearing, ranging from 99.1% to 99.3%. The best results of the bioremediation process were obtained with the addition of 6 mL/m³ probiotics with the lowest range of ammonia and nitrite about 0.0896–0.9303 mg/L and 0.977–8.4880 mg/L. The addition of a probiotic dose of 6 mL/m³ can inhibit the growth of pathogenic bacteria such as Vibrio spp. in water at the beginning, middle, and end of maintenance reached 2, 48, and 140 CFU/mL.
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