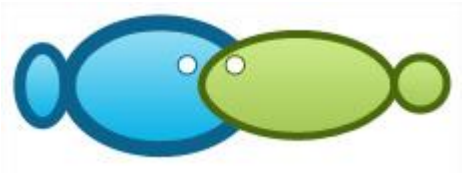

Density and types of probiotic bacterial filter media with different number of bioballs in silver pompano (*Trachinotus blochii*) culture with recirculation system

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Abstract. Cultivation intensification with a recirculation system tends to have a high stocking density and a large amount of feed, so that feed residue, metabolic waste, and oxygen consumption also increase. To speed up the decomposition of feed residue and metabolic waste, filtering is necessary, namely by means of a bioball. This study aimed to analyze the types and numbers of bacteria present in the bioball, to improve water quality and the optimal number of bioballs for bacterial living media. The study used a completely randomized design, with 1 factor, 5 levels of treatment and 3 replications. The treatment levels are: A=without bioballs (control), B=35 bioball filters per container, C=45 bioball filters per container, D=55 bioball filters per container and E=65 bioball filters per container. Silver pompano (*Trachinotus blochii)* fish specimens measuring 10-12.55 cm and a body weight of 24.3-28.9 g, were cultivated for 56 days, at a stocking density of 1 fish for a water volume of 4 L (a total of 20 fish in a volume of 80 L). During the rearing, fish were fed with Megami GR2 pellets with a composition of 46% protein, 10% fat, 2% crude fiber and 10% moisture content, 3 times a day adlibitum. The type and density of bacteria in the bioball was observed, together with the water quality parameters, such as: temperature, pH, dissolved oxygen, total ammonium nitrogen, nitrite and nitrate. The research data were analyzed statistically using ANOVA ($P<0.05$), Newman Keuls's advanced test and multiple regression models, with the SPSS 17.0 software. The best treatment was found in the use of 55 bioball filters per containers, with a volume of 9.8 L, which gave a bacterial density of 667.67±1.53x105 CFU mL^{-1}. The number of bioballs affected the bacterial density by 31.1%, the nitrogen absorption in fish by 16.5% and the filter media by 85.2%. The bacteriocidal types found in the filter media are *Acinetobacter* sp., *Shewanella* sp. and *Nitratireductor* sp. A salinity of 15‰ in the culturing media with a filter substrate of 55 bioball filters per container in the recirculation system gave the best bacterial density and water quality: temperature 28.6-29.1^{0}C, pH 7.6-7.8, dissolved oxygen (DO) 6.3-6.7 mg L^{-1}, total ammonia nitrogen (TAN) 0.06-1.57 mg L^{-1}, nitrite (NO_2) 0.120-0.861 mg L^{-1} and nitrate (NO_3) 0.93-3.07 mg L^{-1}.

Key Words: bioball, bacterial density, probiotics, water quality.

Introduction. Silver pompano (*Trachinotus blochii*) fish is a marine aquaculture species that is relatively new in Indonesia. However, the demand for this kind of fish continues to increase, especially from the international market. *T. blochii* is a highly adaptable fish species, easy to cultivate and with a considerable economic value. Its consumption price reaches USD 4.25 kg^{-1} on the local markets and USD 14.144 kg^{-1} as export commodity (Mo 2017). Since 2015, *T. blochii* has become a leading commodity in marine cultivation fisheries, with a production of 1900 tons in 2015 and the target to increase production with 31.5% $year^{-1}$ (Prahadi 2015).

The production target for the *T. blochii* commodity can be achieved through the culture intensification, but the high stocking density results in a higher potential for stressing, due to a narrower space and to an increased competition for oxygen and feed, so that the fish growth will be hampered. High density also accelerates the decline in water quality due to the accumulation of metabolic waste and feed residue, shaving a major effect on growth.

Nitrogen from fish culture waste produces ammonia (NH_3), due to feed residue and fish metabolism, which can result in a buildup of organic matter causing a decrease

in water quality (Putra & Pamukas 2011; Prayogo et al 2012). Recirculation systems are technologies used to maintain water quality in fisheries, in order to remain suitable for aquatic organisms and to support the optimization of water utilization. The recirculation system is able to reduce the level of ammonia concentration, restraining it within the range of 31-43% (Djokosetiyanto et al 2006; Putra & Pamukas 2011).

According to Lekang (2008) and Fadhil et al (2010), the use of a recirculation system has several advantages including: more efficient water use, flexibility in culture locations, more hygiene, relatively small space or land requirements, ease of controlling and maintaining cultured organisms, ease of maintaining water quality, environmentally friendly, pollution prevention and it can be functional permanently, without disturbing the aquatic environment.

Recirculating aquaculture system technology can also be adapted to the aquaculture system and to the filter substrate in order to control the dissolved solids (Fadhil et al 2010). The use of the right type of filter material will determine the success of fish rearing in the recirculation system because it will determine the growth of non-pathogenic bacteria on the material. Currently, biological filter substrates are widely used, being more environmentally friendly.

There are many biological filters that can be used, one of which is the bioball. Bioball is a sphere with a diameter of 4 cm, a specific area of ±230 m^2 m^{-3} with a width of each hole in a bioball of 0.92 mm, made of PVC material (Said 2002). Bioball functions as a physiological filter which is a growing medium for bacteria that can remove ammonia contained in water (Alfia et al 2013). Nelvia et al (2015) reported that the bioball filtered *Nitrosomonas* sp. bacteria, which function to oxidize ammonia to nitrite, and *Nitrobacter* sp., which function to oxidize nitrite to nitrate.

Several studies related to improving the water quality in aquaculture with recirculation and hydroponic systems have been carried out, such as: Mulyadi & Pamukas (2011) used a recirculation system in nursery and rearing of fish (*Mystus nemurus*); Alfia et al (2013) used a recirculation system with a bioball filter in tilapia (*Oreochromis niloticus*) culture; Nelvia et al (2015) studied the addition of a bioball to the maintenance media filter on the survival and growth of goldfish (*Carassius auratus*) fry. Based on the description above, a series of studies on the number and types of bacteria cultured with different numbers of bioballs and on the improvement of water quality in the cultiring and recirculation systems are needed. This research was aimed at analyzing the types and numbers of bacteria present in the bioball, to measure the water quality improvement (temperature, pH, DO, TAN, nitrite and nitrate) and to determine the optimal number of bioballs.

Material and Method

Location and time of research. This research was conducted between January and July 2019, in several laboratories, as follows: the fish rearing was carried out at the Batam Marine Cultivation Fishery Center (BPBL), the water quality analysis was carried out at the Laboratory of Fish and Environmental Health Testing of the Batam Marine Aquaculture Fisheries Agency, the bacteria types identification and densities calculation were performed at the Fish and Environmental Health Examiners Lab of the BPBL, the polymerase chain reaction (PCR) test was performed at PT Genetics Science Indonesia, West Jakarta.

Materials and tools. The main ingredients used in this research were 300 seeds of *Trachinotus blochii* (Lacepede) with a size of 10-11 cm, originating from the BPBL, fed with commercial pellets "GR-2" (46% protein, 10% fat, 13% ash content, 2% crude fiber and 10% moisture content). The test feed was obtained from the BPBL. A round bucket with a diameter of 60 cm, a height of 45 cm and a volume of 100 L was used as a research vessel. Bioballs have a diameter of 4 cm, a specific area ±230 m^2 m^{-3} and a witdth of each hole in a bioball of 0.92 mm, and are made of PVC, as a filter substrate (Figure 1a).

Experimental design. The research method used in this study was an experimental method completely randomized design, with 1 factor, 5 levels of treatment and 3 replications (Steel & Torrie 1993). As a factor in the study was the difference in the number of bioballs. The treatment level determination in this study is based on the study of Nelvia et al (2015) on the goldfish fry (*Carassius auratus*) in closed recirculation system, stating that the use of 50 bioballs gutter^{-1} can improve the water quality, providing 100% survival rates and a growth performance up to a weight of 2.605 g and to a length of 1.48 mm. Therefore, the treatment in the present study was established as follows: A=0 (control), B=35, C=45, D=55 and E=65 bioball filters per container.

Research procedure. *T. blochii* measuring 10-12.55 cm in length and having a body weight of 24.3-28.9 g were obtained from the BPBL and were adapted for 7 days before being used as test specimens. Fish rearing containers were filled with sea water with a salinity of 15‰, using a closed recirculation system. The rearing container was connected to a 50 x 14 x 14 cm PVC gutter, as a filter container placed at the top of the fish-raising containers. Then the water from the filter gutter flowed back through the PVC pipe with a diameter of 2.5 cm to the rearing tub of *T. blochii*. Water from the fish rearing containers flowed into the filter media (gutters), each gutter containing a number of bioballs corresponding to the tested treatment (Figure 1b) and according to Nelvia et al (2015), by using a water pump with a power of 18 watts. After passing through the filter media (gutter), the water returned to the fish-raising tub through the drain pipe in the filter container. In the rearing medium (KP-SUPER N, with *Nitrosomonas* sp. and *Nitrobacter* sp.), trademark starter bacteria were added at a dose of 2.5 mL 200 L^{-1} week^{-1} (Hartini et al 2013), to accelerate bacterial growth in the bioball.

(a) (b)

Figure 1. (a) Bioball used as a filter substrate, (b) The design of the *Trachinotus blochii* aquaculture recirculation system (original).

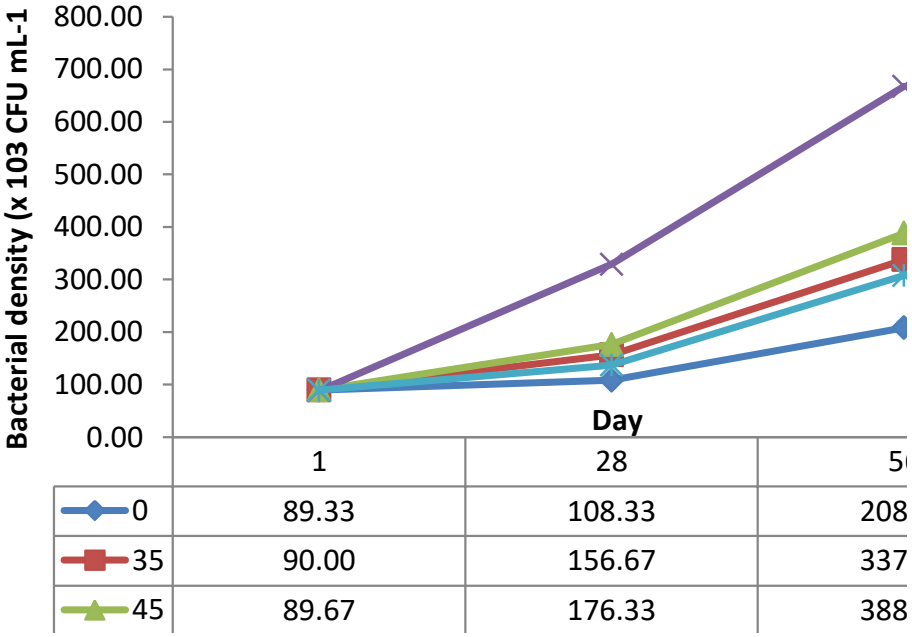
The test specimens that have been adapted to the culturing media were then randomly placed into 15 rearing containers. The fish were given commercial feed at satiation, containing: 46% protein content, 10% fat, 13% ash, 2% crude fiber, and 10% moisture, at 07:00, 12:00 and 17:00 WIB.

Measured response. The response variables measured in this study were: density and type of bacteria in the filter container were observed at the beginning, in the middle and at the end of the study (SNI.01-2332.3-2006 and SNI.01-2332.3-2006); the temperature, pH and salinity were observed every day; the dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite (NO_2) and nitrate (NO_3) were observed every week. Water quality measurement procedures refer to SNI in the Public Works Service (2013).

Data analysis. Bacterial density data were analyzed according to the RAL model (Steel & Torrie 1993). To determine the effect of salinity on each measured variable, a diversity analysis was performed using the SPSS 17.0 software. If $P<0.05$, the Newman-Keuls further test was performed to see the differences between treatments. Water quality data were analyzed descriptively using the Microsoft Excel application and displayed in tables and graphs. To see the relationship of the number of bioballs and the density of bacteria with the water quality, a regression analysis was carried out.

Results and Discussion

Density of bacteria in the filter container. The results of bacterial density observations carried out on plate count agar (PCA) media using the total plate count method can be seen in Figure 2.



	1	28	5
0	89.33	108.33	208
35	90.00	156.67	337
45	89.67	176.33	388

Figure 2. Bacterial density in all treatments during the study.

Figure 2 shows that the bacterial density from day 1 to day 28 in treatments with 0, 35, 45 and 65 bioball filters per container are relatively similar, in contrast to the treatment with 55 bioball filters per container, which caused a significant increase of bacteria, compared to the other treatments. This is due to the fact that the bacteria and *T. blochii* fish are permanently adapting to the media in which they live and to the fact that the nutrients resulting from the remaining feed and fish feces are still not sufficient to support the maximum bacterial growth. After the 28th day the number of bacteria in all treatments continued to increase, reaching a maximum peak on the 56th day. This is due to the increase in organic matter from fecal matter and fish feed along with the growth of the *T. blochii* specimens, which is a source of bacterial nutrition, thereby increasing the rate of bacterial development. In accordance with the opinion of Sunarto (2003), dissolved organic matter is needed by living bacteria, then Sidharta (2000) explains that organic material contains carbon, nitrate, phosphate, sulfur, ammonia, and several minerals which are nutrients for bacterial growth. Administering KP SUPER N probiotics (routinely added in culture containers) causes continuous bacterial growth due to the availability of organic materials that can be used optimally. The highest bacterial density was found in the treatment of 55 bioball filters per container.

The highest bacterial density was found in the treatment with 55 bioball filters per container, at the end of the study, namely 667.7 x 10^5 CFU mL^{-1}, demonstrating the optimality of a configuration consisting of: 55 bioballs for the given size of the filter (50 x 14 x 14 cm) and for the given volume of the culture media (80 L). These parameters are balanced in such a manner that bacteria receive a sufficient amount of oxygen, through the bioball cavity. According to Nelvia et al (2015), the bioball structure (not too compact) provides an opportunity for oxygen to enter the bioball cavity and to reach bacteria. If the oxygen is sufficient, then the bacteria growth determines an optimal functioning of the bioball.

The lowest bacterial density, 208.3 x 10^5 CFU mL^{-1}, was found in treatment A without using bioballs, starter bacteria (KP SUPER N) and additional nutrients, resulting in a limited media for the growth and development of bacteria. The low density of

bacteria when using the highest number of bioball filters (65 container^{-1}) is due a number of bioballs almost exceeding the container capacity. According to Nelvia et al (2015) bioball too densely disposed in the gutter and lacking of oxygen supply can cause extinction of the bacteria in the bioball cavity. Consequently, the performance of the bioball filter decreases, suspending the process of decomposing organic matter by nitrifying bacteria.

The biofilter system removes ammonia where it accumulates and reaches toxic levels if it is not transferred by a nitrification process where the process, first oxidizing ammonia to toxic nitrite, then to nitrate, which is relatively non-toxic. The above process involves *Nitrosomonas* and *Nitrobacter* bacteria. Bacteria grow like a film on the surface of the material (biofilter container) or adhere to organic particles. Biofilter consists of a medium with a large surface for the growth of nitrite bacteria. In the recirculation system, bacteria play a role in converting toxic substances (ammonia) to non-toxic substances (nitrate). The performance of bacteria in the biofilter system is marked by an increase in the average biochemical oxygen demand (BOD) value in the culture medium (Nurhidayat et al 2012). The more bioballs, the more opportunities for bacteria to stick, when a viable oxygen level is still preserved (Nelvia et al 2015).

ANOVA results showed that the number of bioballs had an effect on the density of bacteria on day 28 and 56. The Newman Keuls range study test identifies the bacterial density differences between treatments. The results of the regression analysis between the number of bioballs and bacterial density provide the following regression equation:

$$Y = 227.9 + 3.851X$$

Where:
Y-bacterial density;
X-number of bioballs;
The calculated r was 0.557 and the R^2 was 0.311.

The results of the regression analysis showed that the number of bioballs had a positive correlation with the bacterial density (r=0.557). The increase in the number of bacteria in the filter container was influenced by the number of bioballs by 31.1 and 68.9%, which is thought to be influenced by the content of organic matter (Stepwise regression analysis). Nelvia et al (2015) stated that the bioball is a breeding ground for various bacteria needed in the breakdown of organic matter. According to Sunarto (2003), dissolved organic matter is needed by bacteria to live. Furthermore, according to Sidharta (2000) organic matter contains carbon, nitrate, phosphate, sulfur, ammonia and several minerals which are nutrients for bacterial growth.

Identification of bacteria in filter containers. Based on the observations, there were 7 pure bacterial isolates. The identification of bacteria against the isolates found in the filter media was carried out by physical, biochemical and sensitivity tests to see which bacteria had the greatest inhibition zone against pathogenic bacteria.

Physics test. The results of observations of color, shape, edge, elevation and gram of the seven isolates found are presented in Table 1.

Table 1

Observation results of bacterial characteristics

No	*Isolate*	*Colony*				*Cell*		*Treatment*
		Color	*Shape*	*Edge*	*Elevation*	*Gram type*	*Shape*	
1	A_1	Yellow	Circular	Entire	Umbonate	Negative	Stem	P_2U_1
2	A_2	Beige	Circular	Entire	Raised	Negative	Stem	P_0U_3
3	A_3	White	Circular	Entire	Raised	Negative	Stem	P_3U_2
4	A_4	White	Circular	Entire	Raised	Negative	Stem	P_4U_1
5	A_5	Beige	Filamentous	Entire	Filiform	Negative	Stem	P_1U_1
6	A_6	White	Circular	Entire	Raised	Negative	Stem	P_4U_2
7	A_7	White	Circular	Entire	Raised	Negative	Stem	P_3U_3

Table 1 shows that all bacterial isolates have gram-negative, entire edges and are rod-shaped. The dominant bacterial colony was white (4 isolates), 2 isolates were beige and 1 isolate was yellow. Colonies were circular or round (6 isolates) and filamentous (1 isolate). The elevation of the dominant bacterial colony was raised (5 isolates), 1 umbonate isolate and 1 filiform isolate.

Biochemical test. The results of observations of the Catalase, Oxidase, Motility, oxidative/fermentation (O/F) glucose, Indole and triple sugar iron agar (TSIA) tests are presented in Table 2.

Table 2

Bacterial biochemical test results

No.	*Isolate*	*Biochemical characteristics*						*Treatment*
		Catalase	*Oxidase*	*Motility*	*O/F*	*Indole*	*TSIA*	
1	A_1	+	+	+	-	-	A/K	P_2U_1
2	A_2	+	+	-	F	-	K/K	P_0U_3
3	A_3	+	-	+	O	-	A/K	P_3U_2
4	A_4	+	+	+	F	-	A/A	P_4U_1
5	A_5	+	+	+	F	+	A/A	P_1U_1
6	A_6	+	+	+	F	-	A/A	P_4U_2
7	A_7	+	+	-	F	-	A/A	P_3U_3

O-oxidative; F-fermentative; A/K-alkaline acid; K/K-alkaline; A/A-acid.

Table 2 shows a positive catalase enzyme test in all bacterial isolates, indicated by the gas bubbles formation during their reaction with the H_2O_2 drops. According to Stoica (2016), catalase is an enzyme contained in the majority of bacteria and it is involved in the decomposition of hydrogen peroxide into water and oxygen. Hydrogen peroxide and oxygen are used for electron transport during fermentation in both aerobic and facultatively anaerobic bacteria.

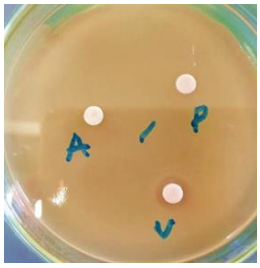
Oxidase tests A_1, A_2, A_3, A_4, A_5, A_6 and A_7 are positive, as shown by the color change on the oxidase paper, which indicates activity, while A_3 is negative. Five isolates (A_1, A_3, A_4, A_5, A_6) showed positive results in the motility test, due to the spread of bacterial growth on the Sulfide Indole Motility (SIM) medium, and did not grow on the part of the stick site bacteria. The O/F test results showed no color change in the paraffin-covered media and a color change on the exposed media. Five bacterial isolates (A_2, A_4, A_5, A_6, A_7) were observed to be fermentative because the tube without paraffin and the tube filled with paraffin changed their color to yellow. According to Fahri (2008), in the O/F test, oxidative organisms occur at a color change in the open media, while fermentative organisms can be indicated by no color change in the closed media.

The indole test shows 6 isolates (A_1, A_2, A_3, A_4, A_6 and A_7) giving negative results, marked with a yellow color on the surface of the media, which means that the bacteria are unable to break down the amino acid tryptophan, and no red ring after dropping the Kovacs reagent in the sulfur, indole, motility (SIM) media. According to Acharya (2012), the indole test was carried out to determine the ability of bacteria to break down amino acid tryptophan and to form indole compounds. Tryptophan is hydrolyzed by tryptophanase to produce three possible end products, one of which being the indole. Indole production is detected by the Kovacs or Ehrlich's reagent, composed of 4-(dimethylamino)benzaldehyde, which reacts with the indole to produce a red compound. A positive reaction is indicated if there is a red color on the surface of the medium, which indicates that the bacteria are able to break down the amino acid tryptophan. In the TSIA test, 4 isolates (A_4, A_5, A_6 and A_7) showed acidic properties (A/A), 2 isolates (A_1 and A_3) were acid alkaline and 1 isolate (A_2) was alkaline.

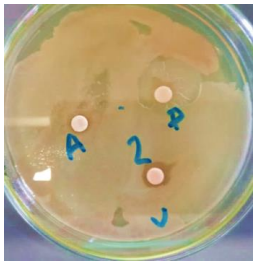
Sensitivity test. The sensitivity test to pathogenic bacteria was carried out after observing the physical and biochemical tests of the seven isolates. This test aimed to find probiotic bacteria candidates for polymerase chain reaction (PCR) testing. The results of the sensitivity test on the 7 isolates are presented in Figure 3. Inhibition zones produced

by the seven bacterial isolates were found in different filter containers. Bacteria from isolate A_1 were able to inhibit the growth of *Vibrio* bacteria during the 24-hour incubation period at a temperature of 37^0C, as indicated by the formation of a clear area around the blank disk, but they were unable to inhibit the growth of *Aeromonas* and *Pseudomonas* bacteria. After an incubation period of 48 hours, the inhibition zone remains clear, which indicates that these bacteria can kill (bacteriocidal) *Vibrio* bacteria. Similarly, for the bacteria from isolate A_2, a clear zone was formed around the blank disk, indicating an inhibition of *Pseudomonas* and *Vibrio* bacteria during the 48-hour incubation period, meaning that A_2 bacteria are bacteriocidal against *Pseudomonas* and *Vibrio*, but they cannot inhibit the growth of *Aeromonas* bacteria.

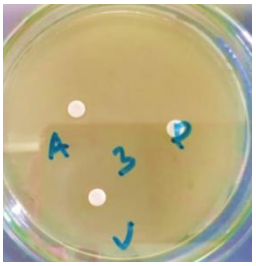
Bacteria in A_3 and A_4 isolates were unable to inhibit the growth of Aeromonas, *Pseudomonas* and *Vibrio* bacteria, as shown by a no inhibition zone formed on the blank disk. In A_5 and A_7 isolates, an inhibition zone occurred against *Aeromonas*, *Pseudomonas* and *Vibrio* bacteria during the 24-hour incubation period, but during the 48-hour incubation period, the clear area became cloudy, indicating that these bacteria were only able to inhibit (bacteriostatic) the three pathogenic bacteria . In A_6 isolate, a clear zone was also formed during the 48-hour incubation period against *Aeromonas* and *Vibrio* bacteria, but no inhibition zone was formed against *Pseudomonas* bacteria. This indicates that the A_6 bacteria are able to kill (bacteriocidal) *Aeromonas* and *Vibrio* bacteria.This is in line with Azaldin et al (2020), who states that sensitivity of a material to bacteria is characterized by the presence of an inhibition zone around the blank disk. Furthermore, according to Dwyana & Murniati (2020), this inhibition area is formed due to bioactive compounds contained in microbes that produce exoenzymes which break down organic matter and secrete hydrolytic enzymes, proteases, lipases, amylases, and cellulose so that they are able to hydrolyze polysaccharides as a carbon source and electron donors.



A_1 - Inhibition
A = 0, P = 0 and
V = 3.74 mm
(bacteriocidal)



A_2 - Inhibition
A = 0, P = 8,88 mm and
V = 3.49 mm
(bacteriocidal)

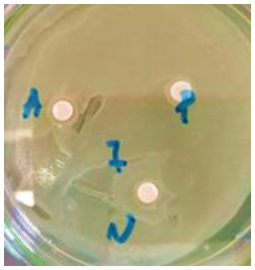


A_3 - Inhibition
A = 0, P = 0 and
V = 0

A_4 - Inhibition
A = 0, P = 0
and V = 0

A_5 - Inhibition
A = 4.32 mm,
P = 3.87 mm and
V = 5.24 mm

A_6 - Inhibition
A = 1.62 mm, P = 0
and V = 3.8 mm
(bacteriocidal)



A_7 - Inhibition
A = 3.15 mm,
P = 2.58 mm and
V = 2.20 mm

Figure 3. Sensitivity test of bacterial isolates against pathogenic bacteria (A: *Aeromonas*, P: *Pseudomonas*, V: *Vibrio*).

Madigan et al (2012) grouped bacteria based on their selective toxicity. According to them, antimicrobial compounds had 3 kinds of effects on the microbial growth: 1). Bacteriostatic inhibit growth but they do not kill. Bacteriostatic compounds inhibit protein synthesis or bind to ribosomes; 2). Bacteriocides kill cells but not cause cell lysis or cell

breakdown. 3). Bacteriolytic cause cells lysis or cell breakdown. Furthermore, Dwyana & Murniati (2020) stated that the sensitivity test on bacteria indicated that these were bacteriostatic after 24 hours of incubation period (clear zone formed on disc paper), while after 48 hours they were classified as bacteriocidal.

According to Jannata (2014), the responses related to the bacterial growth inhibition can be classified based on the inhibition zone diameter, as follows: weak (≤5 mm), medium (5-10 mm), strong (10-20 mm) and very strong (≥20 mm). Based on this scale, isolates A_5, A_6 and A_7 were classified as having a weak response to *Aeromonas*, isolate A_2 had a moderate response and isolates A_5 and A_7 had a weak response to *Pseudomonas* bacteria. Furthermore, A_5 isolate had a moderate response to *Vibrio* bacteria, while isolates A_1, A_2, A_6 and A_7 were classified as having a weak response. According to Sugita et al (1996), these bacteria are able to produce antimicrobial compounds, such as: siderophores, bacteriocins, lysozymes, proteases, hydrogen peroxidase, and organic acids, as a mechanism of competition for nutrients and energy, inhibiting the growth of pathogens in the filter container. According to Rengpipat et al (2000), the siderophore is a compound with a low molecular weight (<1500) and it is also a specific agent for ferric ions binding, which can dissolve iron precipitates and change them into the form required for microbial growth. An organism's ability to form siderophores causes inhibition in other organisms, especially in aquatic animal pathogens, due to their incapacity to obtain the required ferric ions.

Based on the results of observations on the inhibition zone diameter and inhibition power, the bacterial isolates selected as candidates for probiotic bacteria were: A_1, A_2 and A_6, classified as bacteriocidal, then coded MUL_1, MUL_2 and MUL_3. Furthermore, to determine the type of the three bacteria, a DNA analysis was carried out using PCR.

Polymerase chain reaction (PCR) test

The results of amplification and visualization of DNA fragments of the 16S rRNA gene. The genomic DNA had been isolated and extracted from isolates MUL_1, MUL_2 and MUL_3, and then the 16S rRNA markers were amplified using PCR technique. PCR analysis of the 3 bacteria isolates used a 27F forward primer: AGAGTTTGATCMTGGCTCAG and reverse primer 1492R: TACGGYTACCTTGTTACGACTT. The analysis results showed positive with the formation of bands with a total molecular weight (in base pairs - bp) of 1,000–1,500 bp. The results of amplified DNA visualization, using 27F and 1,492R primers electrophoresed with UV light, are presented in Figure 4. The amplified fragment has a molecular weight of 1,227-1,372 bp. Sequence analysis performed with Geneious reveals that isolates produce single clearly visible bands with a molecular weight of 1,331 bp (MUL_1), 1,227 bp (MUL_2) and 1,372 bp (MUL_3), according to the primary amplification size with 27F and 1,492R primers, by comparison using 1 Kb Ladder DNA. The size is in accordance with the expected size of the bacterial 16S rRNA genes, which is between 1,300–1,500 bp (Seprianto et al 2017).

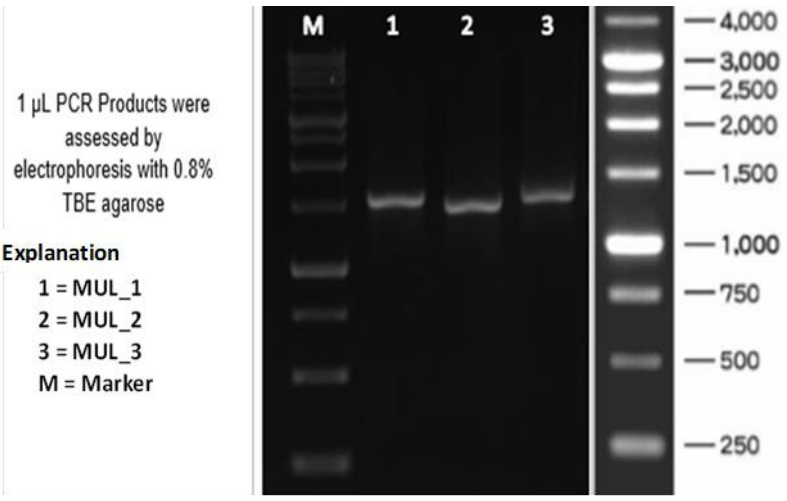
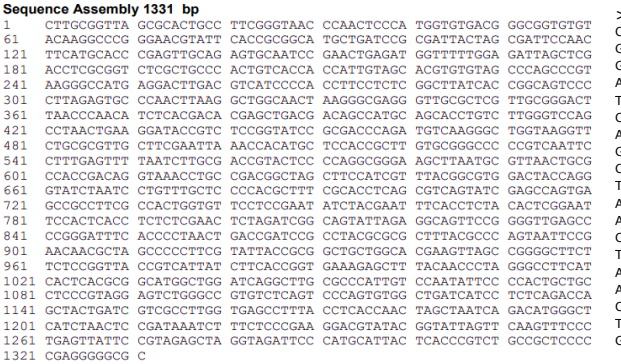


Figure 4. Results of universal DNA-PCR amplification on agarose gel.

The base pairs obtained were combined and trimmed using the Bioedit 7.0 software. DNA sequencing analysis gives the following results for the nucleotide and protein FASTA sequences (Figure 5).



Sequence Assembly 1331 bp

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121  TTCATGCACC CGAGTTGCAG AGTGCAATCC GAACTGAGAT GGTTTTTGGA GATTAGCTCG
181  ACCTCGCGGT CTCGCTGCCC ACTGTCACCA CCATTGTAGC ACGTGTGTAG CCCAGCCCGT
241  AAGGGCCATG AGGACTTGAC GTCATCCCCA CCTTCCTCTC GGCTTATCAC CGGCAGTCCC
301  CTTAGAGTGC CCAACTTAAG GCTGGCAACT AAGGGCGAGG GTTGCGCTCG TTGCGGGACT
361  TAACCCAACA TCTCACGACA CGAGCTGACG ACAGCCATGC AGCACCTGTC TTGGGTCCAG
421  CCTAACTGAA GGATACCGTC TCCGGTATCC GCGACCCAGA TGTCAAGGGC TGGTAAGGTT
481  CTGCGCGTTG CTTCGAATTA AACCACATGC TCCACCGCTT GTGCGGGCCC CCGTCAATTC
541  CTTTGAGTTT TAATCTTGCG ACCGTACTCC CCAGGCGGGA AGCTTAATGC GTTAACTGCG
601  CCACCGACAG GTAAACCTGC CGACGGCTAG CTTCCATCGT TTACGGCGTG GACTACCAGG
661  GTATCTAATC CTGTTTGCTC CCCACGCTTT CGCACCTCAG CGTCAGTATC GAGCCAGTGA
721  GCCGCCTTCG CCACTGGTGT TCCTCCGAAT ATCTACGAAT TTCACCTCTA CACTCGGAAT
781  TCCACTCACC TCTCTCGAAC TCTAGATCGG CAGTATTAGA GGCAGTTCCG GGGTTGAGCC
841  CCGGGATTTC ACCCCTAACT GACCGATCCG CCTACGCGCG CTTTACGCCC AGTAATTCCG
901  AACAACGCTA GCCCCCTTCG TATTACCGCG GCTGCTGGCA CGAAGTTAGC CGGGGCTTCT
961  TCTCCGGTTA CCGTCATTAT CTTCACCGGT GAAAGAGCTT TACAACCCTA GGGCCTTCAT
1021 CACTCACGCG GCATGGCTGG ATCAGGCTTG CGCCCATTGT CCAATATTCC CCACTGCTGC
1081 CTCCCGTAGG AGTCTGGGCC GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTCAGACCA
1141 GCTACTGATC GTCGCCTTGG TGAGCCTTTA CCTCACCAAC TAGCTAATCA GACATGGGCT
1201 CATCTAACTC CGATAAATCT TTCTCCCGAA GGACGTATAC GGTATTAGTT CAAGTTTCCC
1261 TGAGTTATTC CGTAGAGCTA GGTAGATTCC CATGCATTAC TCACCCGTCT GCCGCTCCCC
1321 CGAGGGGGCG C
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> MUL_1
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TTATCACCGGCAGTCCCCTTAGAGTGCCCAACTTAAGGCTGGCAACTAAGGGCGAGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTCTTGGGTCCAGCCTA
ACTGAAGGATACCGTCTCCGGTATCCGCGACCCAGATGTCAAGGGCTGGTAAGGTTCTGCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTA
CTCCCCAGGCGGGAAGCTTAATGCGTTAACTGCGCCACCGACAGGTAAACCTGCCGACGGCTAGCTTCCA
TCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGT
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ATTCCACTCACCTCTCTCGAACTCTAGATCGGCAGTATTAGAGGCAGTTCCGGGGTTGAGCCCCGGGATTT
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TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTACCGTCATTATCTTCACCGGTG
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CAGACCAGCTACTGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCAGACATGGGCTCATC
TAACTCCGATAAATCTTTCTCCCGAAGGACGTATACGGTATTAGTTCAAGTTTCCCTGAGTTATTCCGTAGA
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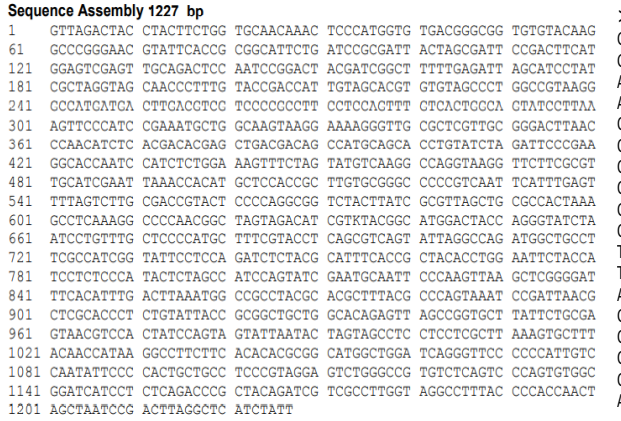
The nucleotide sequence and fasta MUL_1



Sequence Assembly 1227 bp

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121  GGAGTCGAGT TGCAGACTCC AATCCGGACT ACGATCGGCT TTTTGAGATT AGCATCCTAT
181  CGCTAGGTAG CAACCCTTTG TACCGACCAT TGTAGCACGT GTGTAGCCCT GGCCGTAAGG
241  CCCATCATCA CTTCACGTCC TCCCCCCCTT CCTCCAGTTT GTCACTCCCA CTATCCTTAA
301  AGTTCCCATC CGAAATGCTG GCAAGTAAGG AAAAGGGTTG CGCTCGTTGC GGGACTTAAC
361  CCAACATCTC ACGACACGAG CTGACGACAG CCATGCAGCA CCTGTATCTA GATTCCCGAA
421  GGCACCAATC CATCTCTGGA AAGTTTCTAG TATGTCAAGG CCAGGTAAGG TTCTTCGCGT
481  TGCATCGAAT TAAACCACAT GCTCCACCGC TTGTGCGGGC CCCCGTCAAT TCATTTGAGT
541  TTTAGTCTTG CGACCGTACT CCCCAGGCGG TCTACTTATC GCGTTAGCTG CGCCACTAAA
601  GCCTCAAAGG CCCCAACGGC TAGTAGACAT CGTKTACGGC ATGGACTACC AGGGTATCTA
661  ATCCTGTTTG CTCCCCATGC TTTCGTACCT CAGCGTCAGT ATTAGGCCAG ATGGCTGCCT
721  TCGCCATCGG TATTCCTCCA GATCTCTACG CATTTCACCG CTACACCTGG AATTCTACCA
781  TCCTCTCCCA TACTCTAGCC ATCCAGTATC GAATGCAATT CCCAAGTTAA GCTCGGGGAT
841  TTCACATTTG ACTTAAATGG CCGCCTACGC ACGCTTTACG CCCAGTAAAT CCGATTAACG
901  CTCGCACCCT CTGTATTACC GCGGCTGCTG GCACAGAGTT AGCCGGTGCT TATTCTGCGA
961  GTAACGTCCA CTATCCAGTA GTATTAATAC TAGTAGCCTC CTCCTCGCTT AAAGTGCTTT
1021 ACAACCATAA GGCCTTCTTC ACACACGCGG CATGGCTGGA TCAGGGTTCC CCCCATTGTC
1081 CAATATTCCC CACTGCTGCC TCCCGTAGGA GTCTGGGCCG TGTCTCAGTC CCAGTGTGGC
1141 GGATCATCCT CTCAGACCCG CTACAGATCG TCGCCTTGGT AGGCCTTTAC CCCACCAACT
1201 AGCTAATCCG ACTTAGGCTC ATCTATT
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> MUL_2
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AGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCGTCCCCGCCTTCCTCCAGTTTGTCA
CTGGCAGTATCCTTAAAGTTCCCATCCGAAATGCTGGCAAGTAAGGAAAAGGGTTGCGCTCGTTGCGGGA
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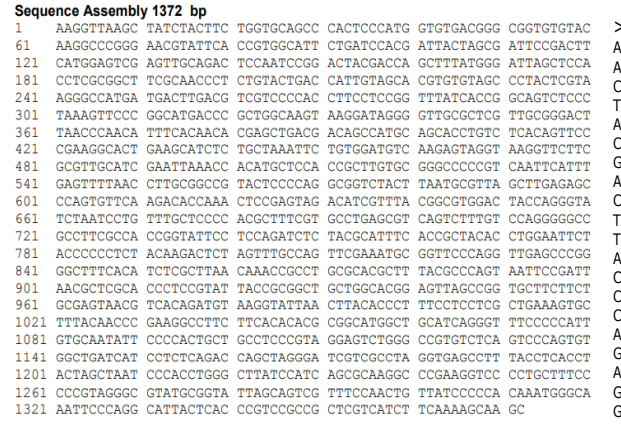
The nucleotide sequence and fasta MUL_2



Sequence Assembly 1372 bp

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121  CATGGAGTCG AGTTGCAGAC TCCAATCCGG ACTACGACCA GCTTTATGGG ATTAGCTCCA
181  CCTCGCGGCT TCGCAACCCT CTGTACTGAC CATTGTAGCA CGTGTGTAGC CCTACTCGTA
241  AGGGCCATGA TGACTTGACG TCGTCCCCAC CTTCCTCCGG TTTATCACCG GCAGTCTCCC
301  TAAAGTTCCC GGCATGACCC GCTGGCAAGT AAGGATAGGG GTTGCGCTCG TTGCGGGACT
361  TAACCCAACA TTTCACAACA CGAGCTGACG ACAGCCATGC AGCACCTGTC TCACAGTTCC
421  CGAAGGCACT GAAGCATCTC TGCTAAATTC TGTGGATGTC AAGAGTAGGT AAGGTTCTTC
481  GCGTTGCATC GAATTAAACC ACATGCTCCA CCGCTTGTGC GGGCCCCCGT CAATTCATTT
541  GAGTTTTAAC CTTGCGGCCG TACTCCCCAG GCGGTCTACT TAATGCGTTA GCTTGAGAGC
601  CCAGTGTTCA AGACACCAAA CTCCGAGTAG ACATCGTTTA CGGCGTGGAC TACCAGGGTA
661  TCTAATCCTG TTTGCTCCCC ACGCTTTCGT GCCTGAGCGT CAGTCTTTGT CCAGGGGGCC
721  GCCTTCGCCA CCGGTATTCC TCCAGATCTC TACGCATTTC ACCGCTACAC CTGGAATTCT
781  ACCCCCCTCT ACAAGACTCT AGTTTGCCAG TTCGAAATGC GGTTCCCAGG TTGAGCCCGG
841  GGCTTTCACA TCTCGCTTAA CAAACCGCCT GCGCACGCTT TACGCCCAGT AATTCCGATT
901  AACGCTCGCA CCCTCCGTAT TACCGCGGCT GCTGGCACGG AGTTAGCCGG TGCTTCTTCT
961  GCGAGTAACG TCACAGATGT AAGGTATTAA CTTACACCCT TTCCTCCTCG CTGAAAGTGC
1021 TTTACAACCC GAAGGCCTTC TTCACACACG CGGCATGGCT GCATCAGGGT TTCCCCCATT
1081 GTGCAATATT CCCCACTGCT GCCTCCCGTA GGAGTCTGGG CCGTGTCTCA GTCCCAGTGT
1141 GGCTGATCAT CCTCTCAGAC CAGCTAGGGA TCGTCGCCTA GGTGAGCCTT TACCTCACCT
1201 ACTAGCTAAT CCCACCTGGG CTTATCCATC AGCGCAAGGC CCGAAGGTCC CCTGCTTTCC
1261 CCCGTAGGGC GTATGCGGTA TTAGCAGTCG TTTCCAACTG TTATCCCCCA CAAATGGGCA
1321 AATTCCCAGG CATTACTCAC CCGTCCGCCG CTCGTCATCT TCAAAAGCAA GC
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>MUL_3
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CCAATCCGGACTACGACCAGCTTTATGGGATTAGCTCCACCTCGCGGCTTCGCAACCCTCTGTACTGACCA
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GTGTGGCTGATCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCTTTACCTCACCTACTAGCT
AATCCCACCTGGGCTTATCCATCAGCGCAAGGCCCGAAGGTCCCCTGCTTTCCCCCGTAGGGCGTATGCG
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GCTCGTCATCTTCAAAAGCAAGC
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The nucleotide sequence and fasta MUL_3

Figure 5. The nucleotide sequence and fasta MUL_1, MUL_2 and MUL_3.

16S rRNA gene sequence alignment with basic local alignment search tool (BLAST). BLAST is accessible online via: http://blast.ncbi.nlm.nih.gov/Blast.cgi and provides the results of the DNA sequencing. A search was carried out for species identification, based on the percentage of the sequenced DNA homology with existing databases on GenBank. The identification results of probiotic bacterial isolates using BLAST correspond to the highest homology (closest relationship) with the bacteria in GenBank. Based on the nucleotides registered with the National Center for Biotechnology

Information (NCBI), the species, strains and accession numbers of bacterial isolates identified in this study are presented in Table 3.

Table 3

Results of tracing the 16S rRNA sequencing of bacterial isolates with the BLAST system

Isolate	*Species*	*Strain*	*Accession number*	*Query coverage*	*Homology*
MUL_1	*Acinetobacter* sp.	MUL37	MT229070	100%	100%
MUL_2	*Shewanella* sp.	MUL31	MT229068	100%	100%
MUL_3	*Nitratireductor* sp.	MUL35	MT229069	100%	100%

The alignment results of 16S rRNA gene sequences with BLAST (Table 3) showed that the bacterial isolate MUL_1 was identified as *Acinetobacter* sp. strain MUL37 with 100% homology and 100% query coverage, MUL_2 isolate was identified as *Shewanella* sp. strain MUL31 with 100% homology and 100% query coverage and bacterial isolate MUL_3 was identified as *Nitratireductor* sp. strain MUL35 with 100% homology and 100% query coverage. The types of bacteria found are thought to have come from the waters of the Batam Sea which are used as a medium for culturing *T. blochii*, because the starter bacteria added to the media do not contain these types of bacteria.

According to Adithiya et al (2017), the sequence equation between 93-97% can represent the identity of bacteria at the genus level but different at the species level. Furthermore, Hagstrom et al (2000) and Seprianto et al (2017) state that bacteria that have a 16S rRNA sequence equation greater than 97% are the same species, while the equation between 93-97% can represent identity at the genus level but differ at the species level. According to Dancourt et al (2000), if the similarity of the sequences is less than 97%, it is likely either a new species, since there is no data in the database, or the the sequencing results are too short to be relevant, when compared to the database.

Phylogenetic tree analysis. The analysis of phylogenetic tree formation was carried out by looking at the similarities that exist in the genetic isolates of MUL_1, MUL_2 and MUL_3 bacteria with a database of the results of alignment of nucleotide sequences in GenBank using the Mega X 10.0.5 WIN 64 application. Phylogenetic trees analysis used the UPGMA (Unweighted Pair Group Method with Arithmetic Average), a clustering method based on the pairwise distance arithmetic means. Phylogenetic analysis used 17 sequences with 3 sample sequences and 14 comparison sequences, the alignment process used 1000 bootstraps and the Kimura 2 model parameters. Branches that connect the dots (nodes) are taxonomic units, such as species or genes and tree roots are the ancestors for all organisms. The dendogram is presented in Figure 5.

Figure 5 shows that the bacteria MUL_1, MUL_2 and MUL_3 are in 1 cluster and that they are separated from the type of control bacteria, which means thatMUL_1 and MUL_2 have close relationships, tending even to be of the same species or to belong to the same subspecies, while MUL_3 bacteria are from the same genus. Based on the the branching point's location, it could be considered that the three isolates were closely related to *Nitratireductor* and *Shewanella*.

According to the calculation results of the genetic distance (using Mega X 10.0.5 WIN 64), it was found that the distances between species varied, ranging from 0.00 to 1.6659. The smallest genetic distance, of 0.2005, was found between bacteria MUL_1 and MUL_2, while between MUL_1 and MUL_3 it was of 0.2081. The genetic distances found were the following: 1.2531 between bacteria MUL_1 and *Acinetobacter venetianus* (GenBank: NR.042049.1), 1.2955 between MUL_2 and *Shewanella algae* (GenBank: NR117771.1) and 1.2468 between MUL_3 and *Nitratireductor aquimarinus* (GenBank: 117929.1). According to Tallei et al (2016), the smaller the genetic distance between two organisms, the closer their kinship.

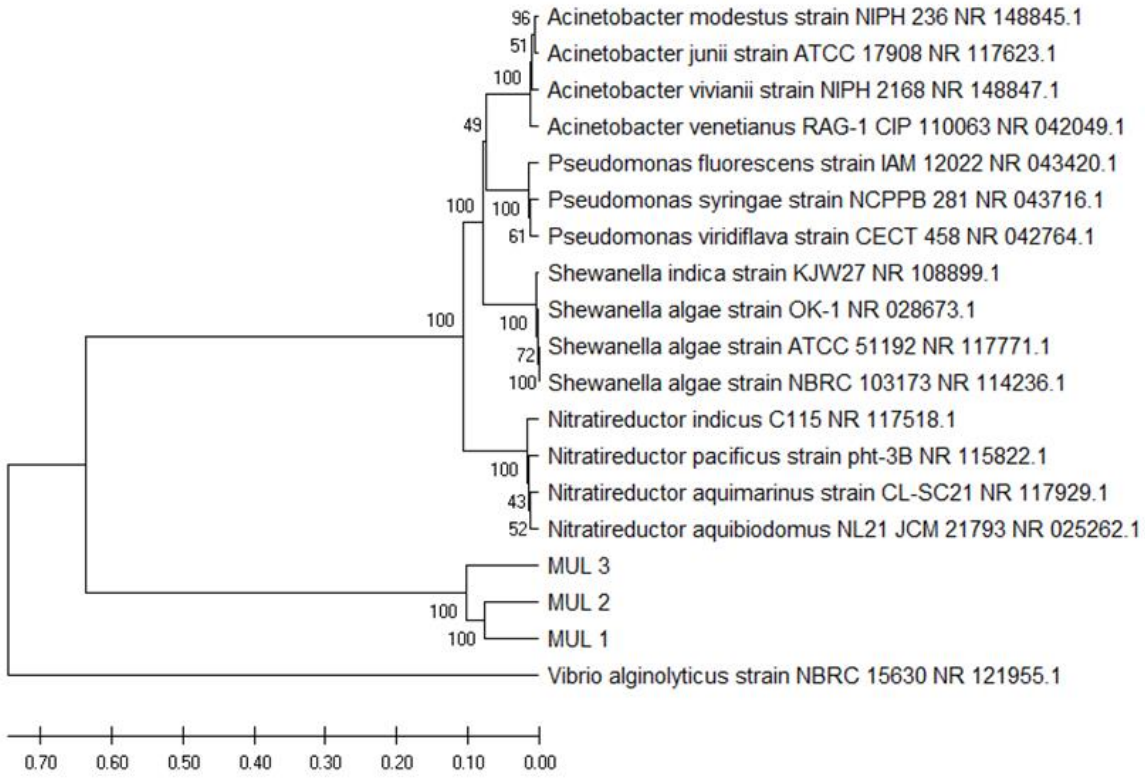

Figure 5. Phylogenetic tree dendrogram of isolates MUL_1, MUL_2 and MUL_3 with 16 comparative species.

Bauvet & Grimont (1986) stated that *Acinetobacter* (Isolate MUL_1) belongs to the kingdom *Acinetobacter*, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Moraxellaceae, genus *Acinetobacter*. In general, these bacteria are rod-shaped, gram-negative, non-motile, catalase positive and oxidase negative, live aerobically, and are opportunistic. Beleneva & Maslennikova (2004) and Soslau et al (2011) reported that the *Acinetobacter* bacteria were found to be associated with several invertebrates such as mollusks, sea cucumbers, sea urchins, starfish and crustaceans.

Shewanella bacteria (Isolate MUL_2) belong to the Bacteria kingdom, the Proteobacteria phylum, the Gamma Proteobacteria class, the Alteromonadales order, the family Shewanellaceae and the genus *Shewanella*. The special characteristics of Shewanellae are their ability to capture electrons in oxygen deficient conditions and their ability to survive in various habitats (Gralnick & Newman 2007). The genus *Shewanella* is a gram-negative rod-shaped proteobacteria, 2-3 μm long and 0.4-0.7 μm in diameter. These bacteria are facultative anaerobic bacteria that are commonly found in marine sediments and can swim with the help of a single polar flagellum (Venkateswaran et al 1999). *Shewanella* is a genus of metal reducing bacteria found in marine environments, freshwater, lakes, land or terrestrial, rivers, Arctic and Antarctic oceans, corroded oil pipes and aquifer environments contaminated with uranium. These bacteria are widely used for bioremediation or environmental cleaning of pollutants such as chlorinated compounds, radionuclides and other environmental pollutants (Venkateswaran et al 1999).

According to Labbe et al (2004), *Nitratireductor* (Isolate MUL_3) belongs to the kingdom Bacteria, phylum Proteobacteria, class Alpha Proteobacteria, order Rhizobiales, family Phyllobacteriaceae and genus *Nitratireductor*. *Nitratireductor* bacteria isolated from sea water in the Indian Ocean and marine sediments in the Pacific Ocean have the ability to reduce ammonia, are rod-shaped and form white bacterial colonies. The optimum temperature for their growth is 25-35ºC and pH 5-12.

Water quality. The results of observations on temperature, pH, dissolved oxygen (DO), TAN, nitrite, (NO_2) and nitrate (NO_3) in all treatments during the study are presented in Table 4.

Table 4

Average temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite (NO_2) and nitrate (NO_3) in all treatments during the study

Parameters	*Number of bioball of each filter container*				
	0	*35*	*45*	*55*	*65*
Temp. (^{0}C)	28.6-29.1	28.4-29.1	28.7-29.1	28.6-29.1	28.6-29.1
pH	7.5-7.7	7.6-7.7	7.6-7.7	7.6-7.8	7.6-7.7
DO (mg L^{-1})	6.2-6.5	6.2-6.6	6.2-6.6	6.3-6.7	6.2-6.6
Salinity (g L^{-1})	15	15	15	15	15
TAN (mg L^{-1})	0.61.-1.58	0.28-1.59	0.18-1.56	0.06-1.57	0.12-1.56
Nitrite (mg L^{-1})	0.624-0.953	0.209-0.863	0.170-0.875	0.120-0.861	0.129-0.874
Nitrate (mg L^{-1})	0.820-1.12	0.98-1.40	0.96-2.15	0.93-3.07	0.98-1.42

Table 4 shows that all water parameters, namely: temperature, pH, dissolved oxygen (DO), ammonia (NH_3) and nitrite (NO_2) are relatively similar in all treatments, except for the nitrate content. The highest nitrate content was found in the treatment with 55 bioball filters per container. In general, temperature, pH, DO, TAN, nitrite and nitrate are in a good range for the growth of *T. blochii*.

SNI 7901.4 (2013) and Ashari et al (2014) state that the optimal temperature for the growth of *T. blochii* ranges from 28 to 32°C, and the pH ranges from 6.8 to 8.4. According to Ezraneti et al (2019), *T. blochii* fish can live optimally at a salinity of 14-19 ppt. SNI 7901.4 (2013) requires a dissolved oxygen level of at least 5 mg L^{-1} for the rearing of *T. blochii* seeds. Royan et al (2019) reported that the lethal concentration (LC50) of TAN ranges from 1.10 to 22.8 mg L^{-1} for invertebrates and from 0.56 to 2.37 mg L^{-1} for fish, within 24-96 hours after exposure. The nitrite level for the maintenance of *T. blochii* seeds is of maximum 1 mg L^{-1} (SNI 7901.4. 2013).

The highest nitrate concentration was found in the treatment with 55 bioball filters containers^{-1} for a volume of 9.8 L, optimal for nitrification bacterial culture media. The higher the number of bacteria, the faster the nitrification process, resulting in an increase in the concentration of nitrate in the media. According to Lampert & Sommer (2007), bacteria have proteolytic enzymes that can break down organic nitrogen into nitrates, which are nutrients needed by phytoplankton and other aquatic microflora. Furthermore, Nelvia et al (2015) obtained the almost the same optimal number of bioballs for the growth of goldfish, namely 50 bioball filters per container of 9.8 L. Nitrate levels obtained during the study were still in accordance with the quality standards referred to in the Government Regulation No. 82 of 2001, namely <20 mg L^{-1}. Effendi (2014) considers a nitrate content of 0-1 mg L^{-1} in the oligotrophic (low) category and a content of 1-5 mg L^{-1} in the mesotrophic (moderate) category. According to Ulqodry et al (2010), normal nitrate content in marine waters generally ranges from 0.01-50 mg L^{-1}. Furthermore, Hartoko (2010) reports that algae, especially phytoplankton, can grow optimally at a nitrate content of 0.009-3.5 mg L^{-1}.

Relationship between number of bioballs and water quality. The results of the regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO_2 and NO_3 are presented in Table 5.

Table 5 shows that the number of bioballs has the greatest effect on the TAN reduction (R^2=0.931 or 93.1%), with a very strong negative linear correlation (r=0.965), meaning that the ammonia concentration decreases by adding bioballs in the filter container. This is because in the filter media there are several types of nitrifying bacteria derived from the introduced starter bacteria, namely *Nitrosomonas* sp. and *Nitrobacter* sp., in addition to the bacteria found in the bioball, namely *Acinetobacter* sp., *Shewanella algae* and *Nitratireductor aquimarinus.* The presence of these bacteria can accelerate the

oxidation process of TAN, which is toxic to fish, into non-toxic nitrate through the nitrification process.

Table 5

Linear regression analysis between the number of bioballs with temperature, pH, DO, TAN, NO_2 and NO_3

Parameter	*Linear regression equation*	*Correlation coefficient (r)*	*Coefficient of determination (R^2)*
Temperature	Y=289.72–0.038 X	-0.767	0.589
pH	Y=759.51+0.067 X	0.444	0.197
Dissolved oxygen (DO)	Y=634.56+0.196 X	0.650	0.423
TAN	Y=58.29–0.83 X	-0.965	0.931
Nitrite (NO_2)	Y=587.55–8.345 X	-0.949	0.900
Nitrate (NO_3)	Y= 94.73+1.75 X	0.664	0.441

The value of r=0 indicates that there is no correlation between the two variables (number of bioballs and water quality), r<0.25 indicates a very weak correlation, r>0.25 and <0.5 shows a sufficient correlation, r> 0.5 and <0.75 suggest a strong correlation, r>0.75 and <0.99 demonstrate a very strong correlation and r=1 signifies a perfect correlation; R^2≥0.5 (50%) measures the ability to explain the dependent variable (water tanquality) by the independent variable (number of bioballs) (Sarwono 2012).

Relationship between bacterial density and water quality. The results of regression analysis between bacterial density and temperature, pH, DO, TAN, NO_2 and NO_3 are presented in Table 6.

Table 6

Linear regression analysis between bacterial density with temperature, pH, DO, TAN, NO_2 and NO_3

Parameter	*Linear regression equation*	*Correlation coefficient (r)*	*Coefficient of determination (R^2)*
Temperature	Y=289.50–0.003 X	-0.226	0.475
pH	Y=756.37+0.015 X	0.695	0.484
Dissolved oxygen (DO)	Y=289.50+0.036 X	0.816	0.667
TAN	Y=59.578–0.090 X	-0.725	0.526
Nitrite (NO_2)	Y=557.73–0.796 X	-0.625	0.391
Nitrate (NO_3)	Y=30.304+0.352 X	0.923	0.852

Table 6 shows that bacterial density has the greatest influence on the increase in nitrate concentration in the *T. Blochii* rearing medium (R^2=0.852 or 85.2%). This is due to the presence of *Nitrosomonas* bacteria, *Nitrobacter* which are included as a starter in the filter container, and to the heterotrophic bacteria in the bioball, which accelerate the ammonia to turn into nitrate, so that the nitrate concentration increases with the bacterial density.

Conclusions. The number of bioballs had an effect (P<0.05) on the bacterial density. The best treatment was found in the use of 55 bioball filters per container, in a volume of 9.8 L, which gave a bacterial density of 667.67±1.53 x 105 CFU mL^{-1}. The number of bioballs had an influence on the bacterial density by (R^2=31.1%). The bacteriocidal types found in the filter media are *Acinetobacter*, *Shewanella* and *Nitratireductor*. The number of bioballs strongly affected the decrease in TAN (R^2=93.1%), while the bacterial density strongly influenced the increase in nitrates (R^2=85.2%).

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