Available online at www.worldscientificnews.com

World Scientific News

An International Scientific Journal

WSN 158 (2021) 91-104 EISSN 2392-2192

Growth Characteristics and Tracing Antagonistic Properties of *Bacillus flexus* and *Bacillus subtilis* as Antibacterials to Overcome the Attack of *Aeromonas hydrophila* Bacteria on Fish

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ABSTRACT

This research was conducted to assess the antibacterial potential of *Bacillus flexus* and *Bacillus subtilis* against *Aeromonas hydrophila*. Bacterial samples came from the stock of the FPIK Unpad Biotechnology Laboratory. This research was conducted in July 2020 - May 2021 in Laboratorium Biotechnology FPIK Padjadjaran University. This research was conducted using qualitative and quantitative descriptive analysis. Bacteria were collected using the purposive sampling method, Observation of the growth curve using the turbidimetric method, and the disc diffusion method for the inhibition zone test with bacterial supernatant treatment with a concentration of 50%, a concentration of 100%, bacterial culture, chloramphenicol antibiotics with a concentration of 4000 ppm as a positive control. The results showed that *B. flexus* bacteria experienced an exponential phase at 6 to 12 hours, after which it entered a stationary phase at 14 to 72 hours. *B. subtilis* bacteria experienced an exponential phase at 20 hours. Until 40 hours, then experiencing a stationary phase at 42 hours to 72 hours, and the growth of *A. hydrophila* bacteria experiences an exponential phase at 6 hours to 28 hours, the stationary phase at 30 to 28 hours 72. Based on the results of the inhibition zone test, it showed the formation of the inhibition zone only in the positive control treatment, while the supernatant and bacterial cultures of *B. flexus* and *B. subtilis* didn't form an inhibition zone.

***Keywords*:** *Bacillus flexus, Bacillus subtilis, Bacillus tequilensis, Bacillus carboniphilus, Bacillus haynesii, Bacillus zhangzhouensis, Aeromonas hydrophila,* Inhibition Zone Test, fish

(Received 09 May 2021; Accepted 28 May 2021; Date of Publication 29 May 2021)

1. INTRODUCTION

Cultivation is one of the fastest-growing activities in fisheries. In addition to the ever-increasing market demand, this activity can provide great benefits for the owner. This activity has several obstacles that can occur at any time. One of the obstacles in cultivation is management. Poor management can cause fish to be easily attacked by microbes such as bacteria. One example of a bacterium that attacks farmed fish is the bacterium *Aeromonas hydrophyla. Aeromonas hydrophila* is a bacteria that is harmful to freshwater fish farming. These bacteria can infect various sizes of fish which can cause mortality up to 80%. These bacteria cause Motile Aeromonas Septicemia (MAS) disease or red spot disease. These bacteria attack various freshwater fish such as goldfish, catfish, gourami, and galangal shrimp and cause disease outbreaks with a mortality rate of 80% - 100% within 2 weeks. These bacteria are found in gills, skin, liver, kidneys, and digestive tract.

By looking at the impact caused by the attack of the bacterium *Aeromonas hydrophyla*, it is necessary to do countermeasures. One of the efforts to overcome the negative impact of the use of chemicals and antibiotics is to use alternative materials that are safer, environmentally friendly, easy to apply, and easily biodegradable in waters. Alternative medicinal materials that can be used to combat *Aeromonas hydrophila* attacks are Bacillus bacteria such as *Bacillus flexus* and *Bacillus subtilis.* Bacillus has anti-microbial resistance and can produce antimicrobials so that these bacteria can survive in the digestive tract. Bacillus is resistant to erythromycin, lincomycin, cephalosporins, cycloserine, chloramphenicol, tetracyclines, streptomycin, and neomycin.

The resulting antimicrobial is bacteriocin. The phosphorus produced by Bacillus has high resistance to chemical a physical factors such as extreme temperatures, alcohol, and so on. Bacillus can control pathogenic bacteria and suppress the growth of other bacteria through the antibiotics it produces / competition in terms of nutrition and space. Any species of the genus Bacillus capable of producing antimicrobial substances such as bacteriocins, and potentially result in the form of lipopeptide antibacterial compound called bacitracin that can kill pathogenic bacteria. Research results show that *Bacillus* bacteria can inhibit the pathogenic bacteria *Aeromonas hydrophila*, including *Bacillus carboniphilus, Bacillus haynesii, Bacillus zhangzhouensis, Bacillus* sp., *Bacillus tequilensis,* and *Bacillus subtilis*.

The purpose of this study was to study the growth characteristics and tracing the antagonistic properties of *Bacillus flexus* and *Bacillus subtilis* against *Aeromonas hydrophyla* in fish through growth curves and inhibition zone tests.

2. MATERIALS AND METHODS

This research was conducted in July 2020 - May 2021 at the Laboratory of Microbiology and Biotechnology of Fisheries and Marine, Building 3, Faculty of Fisheries and Marine Sciences, Padjadjaran University.

2. 1. Research Materials

The materials used in this study were the bacteria *Bacillus flexus, Bacillus subtilis, and Aeromonas hydrophila* from laboratory stocks. Isolate Bacillus bacteria from the intestines of common carp that have been characterized. The density of Aeromonas used was 10^8 CFU / ml.

The medium used for the bacterial growth curve was broth media and agar medium for the inhibition zone test.

2. 2. Research Methods

This study used a turbidimetric method for growth curves with optical density OD_{540} for Aeromonas bacteria and OD_{600} for Bacillus bacteria and cultured at a temperature of 37 ℃ and a speed of 180 rpm. The inhibition zone uses the disc diffusion method with four treatments, each treatment is repeated 3 times.

2. 3. Growth Curves

The growth curve is made by inoculation. The bacteria regenerated culture was taken as much as 1 ml and inoculated into 200 ml of NB media and incubated in a shaker incubator at 30 ℃ at a speed of 150 rpm for 72 hours. During the incubation period, the absorbance of bacteria was measured every two hours with a spectrophotometer.

2. 4. Zone of Obstacle Test

Stock intestinal bacterial isolates stored in glycerol were reactivated and grown in NA medium. The test against the pathogen *Aeromonas hydrophila was* carried out by growing bacterial isolates in a liquid medium. NA was poured into 10 ml of Petri dishes, then the pathogenic bacteria *Aeromonas hydrophila were* added with a concentration of 10^8 CFU / ml. *Aeromonas hydrophila* bacteria were leveled using a *sterile* Cotton Bu lab and wait for 10 minutes.

Sterile disc paper was immersed in bacterial culture and bacterial isolate supernatant at each concentration of 50% and 100%. Disc paper was also immersed in 4000 ppm chloramphenicol antibiotic as a positive control and distilled water as a negative control. The soaking process is carried out for 15 minutes. Then the disc paper is placed on the NA medium using sterile tweezers. This process is repeated 3 times. The agar medium was incubated at 30 °C for 72 hours and the inhibition zone formed around the *paper disc was* measured using a caliper and observed every 24 hours.

2. 5. Data Analysis

Data from the zone inhibition in this study were analyzed using Microsoft Excel. The collected data will be interpreted and analyzed in descriptive.

3. RESULT

Based on the research activities, the following results were obtained:

3. 1. Bacterial Growth Curves

Observation Results The growth curves of *Bacillus flexus, Bacillus subtilis,* and *Aeromonas hydropila* can be seen in Figures 1, 2, and 3.

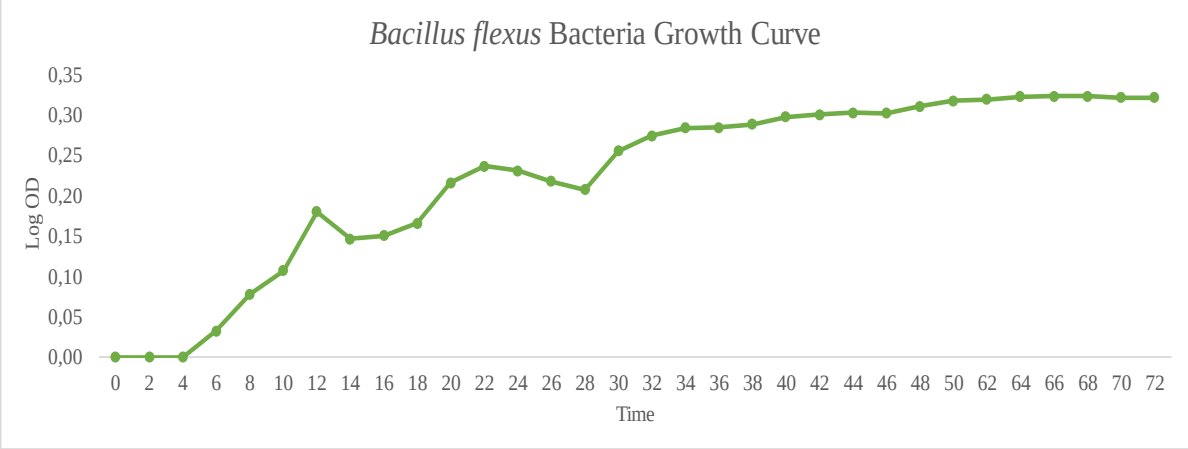

Figure 1. Graph of *Bacillus flexus* Bacteria Growth Curve

Based on Figure 1, it can be seen that the growth curve of *B. flexus* bacteria experiences a lag phase or an adaptation phase at 0 to 4 hours, followed by an exponential phase at 6 to 12 hours, after which the bacteria begin to enter the phase stationary which is characterized by the growth of bacterial cells that are almost constant or experience very little growth and tend to experience a decrease in the number of cells, namely at 14 to 72 hours and there is no death phase because the bacterial growth process is still ongoing. The results of this study are different from those of Xiong *et al.* (2020), the results of Xiong *et al.* research showed that the bacteria *Bacillus flexus* experienced a lag phase at 0 to 6 hours then an exponential phase at 12 to 60 hours, continued with a stationary phase at 60 -72 and the death phase at 96 hours to 120 hours. These observations were carried out every 24 hours using OD_{600} and cultured at a temperature of 37 ℃ and a speed of 180 rpm.

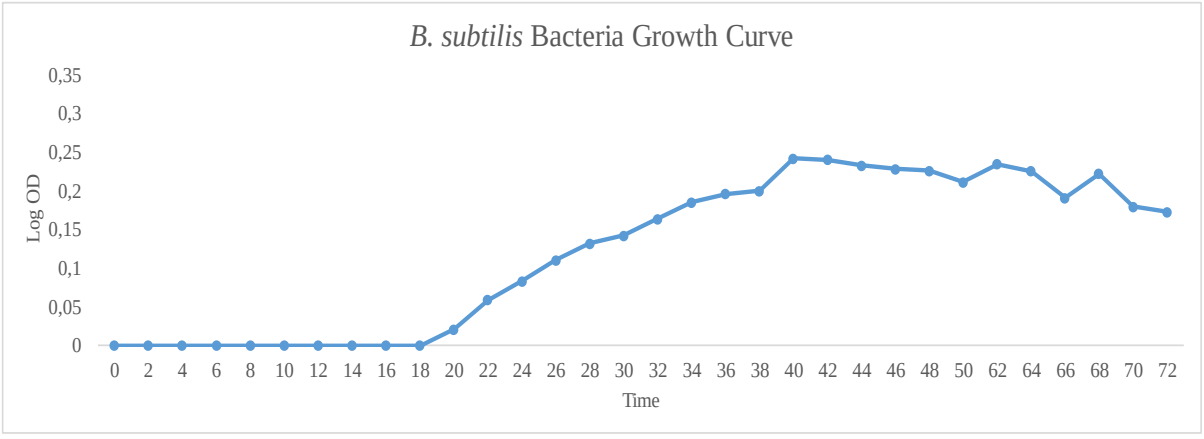

Figure 2. Graph of the Growth Curve for *Bacillus subtilis*

Based on Figure 2, it can be seen that *B. subtilis* bacteria experience a lag phase at 0 to 18 hours, an exponential phase at 20 to 40 hours, then experiences a stationary phase at 42 hours

to 72 hours and there be no death phase because the bacterial growth process is still ongoing. Meanwhile, the results of research conducted showed that *B. subtilis* experienced an exponential phase or log phase from the first day or the 24th hour to the 72nd hour, then the stationary phase on the fourth day and experienced a death phase as indicated by the presence of decreases in absorbance values which decreased on the fifth and sixth day.

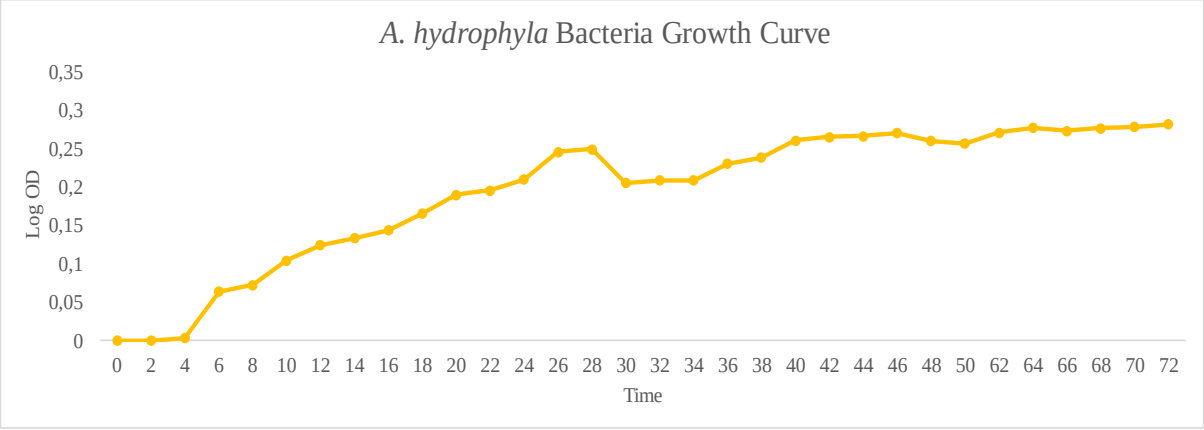

Figure 3. Graph of *Aeromonas hydrophila* Bacteria Growth Curve

The growth curve of *A. hydrophila* bacteria (Figure 3) experiences a lag phase at 0 to 4 hours, then experiences an exponential phase at 6 to 28 hours, followed by a stationary phase at 30 to 72nd there is no death phase because the process of bacterial growth is still ongoing. While the research results showed that the bacteria *A. hydrophila* through a phase lag on the clock to-0 up to 2 nd hour later exponential phase at the 4th hour up to an hour to-22 continued with phase Stationer and death on the 24th hour.

Each phase in bacterial growth is determined by several factors. The length of the lag phase is influenced by factors such as bacterial species, changes in environmental conditions, and conditions of culture bacteria in the previous medium, besides that the available nutrient content in the medium also affects the time in the lag phase. If culture transferred from a medium rich in nutrients to a medium that has little nutritional value then the culture will undergo a phase lag because bacterial cells are a very necessary complement of enzymes that are fully equipped to adapt to the new environment and phase lag doesn't occur when the bacteria were transferred into a new medium with environmental conditions or the same nutrient content as the previous medium.

The length or shortness of the adaptation phase or the lag phase is determined by the number of cells inoculated, the appropriate physiological and morphological conditions, and the media required. Factors that influence the log phase or exponential phase are the media where it grows such as nutrient content and pH, as well as environmental conditions including temperature and air humidity. There is a stationary phase of secondary metabolites, many are produced because bacteria defend themselves to survive by removing their secondary metabolites and some are poisoned by changing environmental conditions because of the metabolites produced. Factors affecting not seem to stationary phase on a growth curve of bacteria that the carbon content in the medium. Bacteria more easily utilize the simple carbon source instead of complex carbon source, the stationary phase does not appear on a growth

curve in the medium CMC and looked on NB medium as a carbon source in the medium NB simpler than cellulose. The rate of growth or length of time in each phase of growth can be caused by different bacteria species environmental conditions, if the environmental conditions have little nutritional value then growth bacteria will be slower. Bacterial growth factors are influenced by nutrition, environmental factors, and genetic factors.

3. 2. Bacterial Inhibition Zone Test

The results of the inhibition zone test for *Bacillus flexus* and *Bacillus subtilis* against *Aeromonas hydrophila are* shown in Figures 4 and 5.

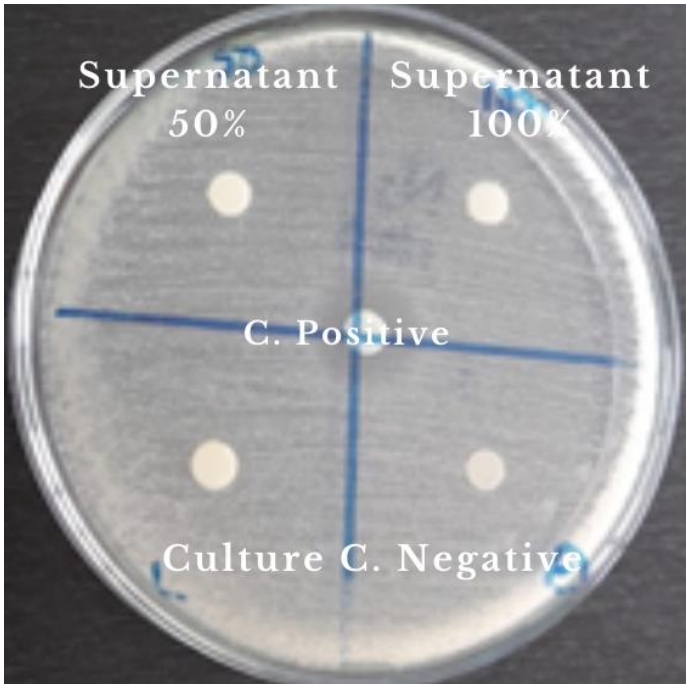

Figure 4. Inhibition Zone Test for *Bacillus flexus* bacteria

Figure 4 shows the formation of an inhibition zone only in the positive control treatment, namely on a *paper disc* dripping with chloramphenicol antibiotics with a concentration of 4000 ppm, while in the treatment with a drop of *Bacillus flexus* bacteria culture, bacterial isolate supernatant with a concentration of 50%, 100%, and negative control not the inhibition zone is formed. The results showed that the negative control of distilled water did not provide an inhibition zone in the antibacterial test, compared to the positive control which had a wide inhibition zone effect, so that distilled water as a solvent couldn't inhibit bacterial growth. The antibiotic chloramphenicol was used as a positive control because it is a broad-spectrum antibiotic that is effective against several types of bacteria and anaerobes. Chloramphenicol is a broad-spectrum antibiotic that is effective against a prokaryotic either kills or inhibits the growth of gram-negative and gram-positive.

This antibiotic was used as a positive control for tests carried out as comparative data. The negative control used is sterile distilled water, negative controls are r function to compare

the presence or absence of a solvent effect on the growth of the battery so that it can be seen that the activity has antibacterial is the test substance is not solvent. The results of this study are by the results of research conducted by Mulyani (2018), these results indicate that no inhibition zone is formed in the antagonistic test of *Bacillus flexus* bacteria against *Aeromonas hydrophila* bacteria.

The results of the measurement of the inhibitory power obtained are the results of measurements three times and the diameter of the formed zone is measured using a caliper at 24 hours, 48 hours, and 72 hours. The antimicrobial inhibitory activity is expressed based on the clear zone produced around disc paper. The diameter of the growth inhibition zone bacteria measured in mm. Observation committed against clear inhibition zone or zones are formed is done by measuring the diameter of a clear zone is reduced by diameter filter paper used. The filter paper size used is 6 mm. The formation of the inhibition zone of *Bacillus flexus* bacteria against *Aeromonas hydrophila* can be seen in Table 1.

Table 1. *Inhibition* zone diameter of *B. flexus* bacteria against *A. hydrophila* bacteria.

Treatment	Deuteronomy	24th hour	48th hour	72nd hour
A (4000 ppm)	1	8.61 mm	8, 13 mm	7, 56 mm
	2	-	-	-
	3	-	-	-
	Average	8.61 mm	8, 13 mm	7, 56 mm
B (Aquades)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-
C (Supernatant 100%)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-
D (Supernatant 50%)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-

Treatment	Deuteronomy	24th hour	48th hour	72nd hour
E (Culture)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-

Based on Table 1, it can be seen that the average diameter of the inhibition zone formed by chloramphenicol antibiotics as a positive control was 8.61 mm at 24 hours after incubation and decreased the diameter of the inhibition zone meter to 8.13 mm after 48 hours of incubation and There was a decrease in the diameter of the inhibition zone after 72 hours of incubation to 7.56 mm. The average diameter of the inhibitory zone in the treatment of bacterial isolates with a concentration of 50%, 100%, and the bacterial culture treatment was not present or equal to 0, meaning that in the treatment there was no inhibition zone formed on the *paper disc.*

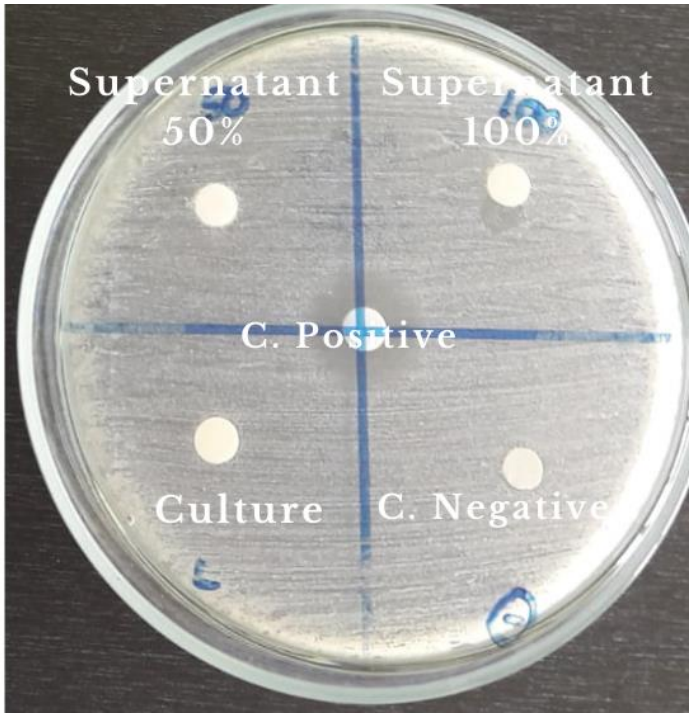

Figure 5. Inhibition Zone Test for *Bacillus subtilis* bacteria

The decrease in the average number in the positive control treatment was due to the reduced ability of antibiotics to fight *Aeromonas hydrophila* bacteria. This can happen because of inhibition zone formed influenced by the density of the culture medium, the speed of

diffusion that occurs in antibiotic, the concentration of antibiotics on paper discs, the sensitivity of bacteria to antibiotics, and the interaction of antibiotics with the media, so that the resistance zone formed on test no inhibition zone in suppressing or killing the bacteria *Aeromonas hydrophila* or occur decrease the ability to kill the bacteria *Aeromonas hydrophila*. The activity of the antimicrobial inhibition zone is grouped into three categories, namely: an inhibition zone ≤ 10 mm means weak, 11-14 mm means strong, and ≥ 15 mm means very strong. So the test results inhibition zone *Bacillus flexus* against bacteria *Aeromonas hydrophila* into the category of weak.

Figure 5 shows the absence of an inhibitory zone formed *on the paper disc*, both in the treatment with a drop of *Bacillus subtilis* supernatant with a concentration of 50%, 100%, bacterial culture, and negative control treatment. While the *paper discs* are in antibiotic chloramphenicol at a concentration of 4000 ppm or positive control forming inhibitory zone. The results of this study are not by the results of research conducted by Mulyani (2018), these results indicate that an inhibition zone is formed in the antagonistic test of *Bacillus subtilis* against *Aeromonas hydrophila* bacteria. The formed inhibition zone has a diameter ranging from 8 - 10 mm.

The results of the measurement of the inhibitory power obtained were the results of three replications and the diameter of the formed inhibition zone was measured using a caliper at 24 hours, 48 hours, and 72 hours. The formation of the inhibition zone of *Bacillus subtilis* against *Aeromonas hydrophila* can be seen in Table 2.

Table 2. Inhibition zone diameter of *B. subtilis* against *A. hydrophila* bacteria.

Treatment	Deuteronomy	24th hour	48th hour	72nd hour
A (4000 ppm)	1	9.26 mm	8.67 mm	8.46 mm
	2	-	-	-
	3	-	-	-
	Average	9.26 mm	8.67 mm	8.46 mm
B (Aquades)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-
C (Supernatant 100%)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-

Treatment	Deuteronomy	24th hour	48th hour	72nd hour
D (Supernatant 50%)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-
E (Culture)	1	-	-	-
	2	-	-	-
	3	-	-	-
	Average	-	-	-

Based on Table 2, it can be seen that the average diameter of the inhibition zone formed by chloramphenicol antibiotics as a positive control was 9.26 mm at 24 hours after incubation and decreased the diameter of the inhibition zone to 8.67 mm after 48 hours of incubation decreased the size of the inhibition zone diameter after 72 hours of incubation to 8.46 mm.

Antimicrobial activity is categorized as having a very strong sensitivity level if the inhibition zone diameter reaches > 20 mm, the strong sensitivity level category is given if it has an inhibition zone diameter of about 10-20 mm, the moderate sensitivity level category has an inhibition zone diameter of about 5-10 mm, and the sensitivity level category is weak when the diameter ranges from 6 mm. Based on the statement is known inhibition zone test results *Bacillus subtilis t*o the bacteria *Aeromonas hydrophila* belonging to the weaker categories.

No inhibition zone formation on *paper discs* was etched with the bacteria *Bacillus flexus* and *Bacillus subtilis* can be caused by several factors. One of these factors is the condition of the Aeromonas bacteria which is already resistant to antibiotics. One of the factors that affect the inhibition zone diameter that is the turbidity suspension of bacteria. If the bacterial suspension is less cloudy, the diameter of the inhibition zone will be bigger and the opposite is true. If the suspense is more cloudy, the diameter will be smaller.

In addition, the thickness of the agar medium can also be a factor affecting the diameter of the inhibition zone for bacterial growth. The thickness of the effective agar is about 4 mm. If it is less than 4 mm the extract diffusion will be faster. Factors that influence antibacterial activity are concentration, the content of antibacterial compounds, diffusion power, and the type of bacteria that is inhibited.

In addition, the factors that affect the work of antimicrobial substances or substances include microbial age, temperature, and antimicrobial ingredients. The formation of the inhibition zone is highly dependent on the amount of antibacterial material that is dripped into the disc, the solubility of the antibacterial agent on the media, the diffusion coefficient, and the antibacterial effectiveness.

4. CONCLUSIONS

Based on the results, it is known that *B. flexus* bacteria experience an exponential phase at the 6th to the 12th hour, after that it enters the stationary phase from the 14th hour to the 72nd hour. *B. subtili* bacteria experience an exponential phase at the 20th hour. Until the 40th hour, then experiencing a stationary phase at the 42nd hour to the 72nd hour, and the growth of *A. hydrophila* bacteria experiences an exponential phase at the 6th to the 28th hour, the stationary phase at 30 to the hour 72nd. Meanwhile, the inhibition zone test results showed the formation of the inhibition zone only in the positive control treatment in both *Bacillus flexus* and *Bacillus subtilis*, the bacterial isolates supernatant, and the bacterial cultures of *Bacillus flexus* and *Bacillus subtilis were* not formed.

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