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Growth characteristics and antagonistic potential of *Bacillus cereus* and *Bacillus zhangzhouensis* against pathogenic bacteria *Aeromonas hydrophila* in vitro

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ABSTRACT

This study aims to examine the growth characteristics of *B. cereus* and *B. zhangzhouensis* and to see their potential in preventing the growth of *Aeromonas hydrophila* in vitro. This research was conducted in June 2020 - May 2021 at the Laboratory of Microbiology and Biotechnology, Faculty of Fisheries and Marine Sciences, Padjadjaran University. The method used is the exploratory method and the data obtained were analyzed descriptively. The growth curve was performed using the turbidimetric method. The inhibition test used the disc diffusion method with 5 treatments, chloramphenicol antibiotics (4000 ppm), distilled water, bacterial culture, 100% supernatant and 50% supernatant. The curve results showed that the growth of *B. cereus* and *B. zhangzhouensis* was slow. The stationary phase of *B. cereus* occurred at 40-72 hours, while in *B. zhangzhouensis* the stationary phase occurred at 48-72 hours. The results of the inhibition test showed that the antimicrobial activity of *B. cereus* and *B. zhangzhouensis* was weak with a value range of 0.71 - 1.6mm, besides that the results of the inhibition zone produced by these two bacteria have different inhibitory abilities in each treatment

***Keywords*:** *Aeromonas hydrophila, Bacillus cereus, Bacillus zhangzhouensis*, pathogenic bacteria, antimicrobial activity

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1. INTRODUCTION

Intensive fish farming cannot be separated from the problem of disease attacks. Diseases that attack fish can be caused by fungi, parasites, bacteria, and even viruses. Diseases caused by bacteria are one of the obstacles in goldfish cultivation because they can result in death resulting in significant economic losses. Motile Aeromonas Septicemia (MAS) disease in goldfish occurs due to the presence of *Aeromonas* sp. if the growth of bacteria cannot be controlled, it will result in mass death so that cultivation activities will suffer a great loss.

Various attempts have been made to control the growth of *A. hydrophila* bacteria in fish cultivation ponds, one of which is using antibiotics. However, the use of chemicals in the long term can result in the emergence of strains of bacteria that are resistant to the drug. In addition, antibiotics can produce toxins and are residual for consumers so that they can cause food allergies and even poisoning. The development of novel antibacterial alternatives is the most obvious approach in combating increased resistance to pathogenic bacteria. Therefore, one of the actions that can be taken to reduce the use of antibiotics in fish farming is to use natural ingredients, one of which is probiotics and the latest is to use antimicrobial peptides. This antimicrobial peptide is a natural antibiotic so it can provide a promising alternative to the new generation of antibiotics. Antimicrobial peptides are encoded by genes everywhere, one of which is the bacterium Bacillus. Bacillus itself is classified as a probiotic bacteria that can be found in fresh and marine waters and is present in the digestive system of animals. The genus Bacillus consists of many species, some of which are *B. cereus* and *B. zhangzhouensis*. Based on previous research, Bacillus can produce antimicrobial peptides so that it can inhibit the growth of *A. hydrophila* because it can form an inhibition zone of 6.5 - 12.6 mm. In addition, *B. cereus* is known to produce antimicrobial substances ericyn, subtilin, and sublancyn. The potential of Bacillus as bacteria that can produce antimicrobial substances encourages the need for further research on the optimization of the growth of *B. cereus* and *B. zhangzhouensis* and the ability of these bacteria to inhibit the growth of pathogens *A. hydrophila*.

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 (*B. cereus* and *B. zhangzhouensis*) from the intestines of common carp that have been characterized and Aeromonas hydrophila from laboratory stocks. The medium used for the bacterial growth curve was nutrient broth (NB) and nutrient agar (NA) for the inhibition zone test.

2. 2. Research Methods

This study used a turbidimetric method for growth curves with optical density OD_{600} for Bacillus bacteria and cultured at a temperature of 30 °C and a speed of 150 rpm. The inhibition zone uses the disc diffusion method with five treatments, and cultured at a temperature of 30°C.

2. 3. Cultures of *B. cereus* and *B. zhangzhouensis*

Nutrient Agar (NA) which has been weighted was dissolved with 100 ml distilled water in an erlenmeyer, covered with a cotton plug, then heated using a hot plate equipped with a magnetic stirrer to homogenize the solution. Then the solution is sterilized using an autoclave for 15 minutes at a temperature of 121 °C. The sterile NA medium was poured aseptically into a petri dish. *B. cereus* and *B. zhangzhouensis* isolates were inoculated on NA media aseptically, then incubated at 30 °C for 24 hours. Then stored at –20 °C and the bacterial culture is ready for use.

2. 4. Making Inoculum / Starter Culture

Nutrient Broth (NB) which has been weighted was dissolved with 200 ml of distilled water in an erlenmeyer, covered with a cotton plug, then heated using a hot plate equipped with a magnetic stirrer to homogenize the solution. Then the solution is sterilized using an autoclave for 15 minutes at a temperature of 121 °C. The *B. cereus* and *B. zhangzhouensis* bacteria from NA culture were harvested using a loop needle and put in an erlenmeyer containing NB. Then incubated in a shaker incubator at a temperature of 30 °C with a shaker speed of 150 rpm for 24 hours.

2. 5. Growth Curve

Growth of *B. cereus* and *B. zhangzhouensis* was carried out in a nutrient broth (NB) medium. The cultures were incubated at 30 °C with a shaker speed of 150 rpm. Bacterial growth was measured by the turbidity method using a spectrophotometer (λ = 600 nm). Sampling was carried out every 2 hours for 24 hours. Then continue sampling every 8 hours until the growth curve shows a stationary phase.

2. 6. Making of *A. hydrophila* bacteria 10^8 CFU/ml

A. hydrophila culture was taken using a loop needle, then put into an erlenmeyer containing 200 ml NB solution. Erlenmeyer was covered with a cotton plug, then the bacteria were incubated at 30 °C with a shaker speed of 150 rpm. Bacterial culture was inserted into a 2 ml cuvette and calculated using a spectrophotometer (λ = 540 nm) and an absorbance value of 0.235 to obtain a density of 10^8 CFU/ml.

2. 7. Inhibition Zone Test (In Vitro)

The inhibition zone test against the pathogen *A. hydrophila* was carried out by growing bacterial isolates on a nutrient agar (NA) medium. Sterile petri disc were prepared and filled with each treatment control + (antibiotic chloramphenicol 1000 ppm), control – (sterile distilled water), supernatant 100%, 50%, and culture (not centrifuged). Sterile 6 mm disc paper was immersed in each treatment. NA is poured into 10 ml of petri dishes. Petri dishes are divided into 4 quadrants.

Planting *A. hydrophila* bacteria on NA medium was carried out by pouring 0.1 ml of bacteria. *A. hydrophila* bacteria were flattened using sterile lab cotton buds. Soaked sterile disc paper, then placed and gently pressed on top of the NA medium. Then incubated at 30 °C. Inhibition zone measurements are carried out every 24 hours.

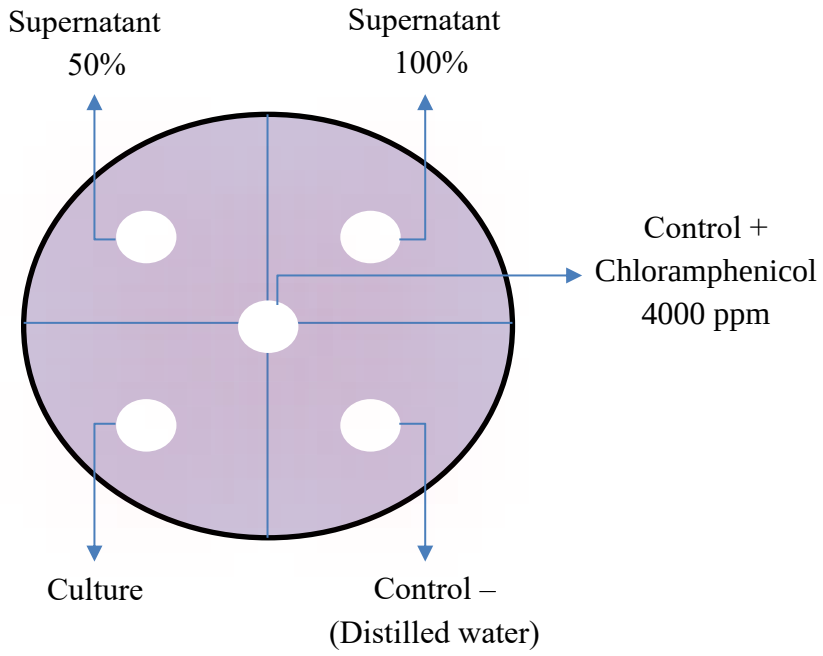

Fig. 1. Design of inhibition zone

2. 8. Data analysis

The results of the measurement of the growth curve were calculated using MS. Excel and the results of the inhibition zone measurements were interpreted using the category of antibacterial activity.

3. RESULT

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

3. 1. Growth Curves of *B. cereus* and *B. zhangzhouensis*

The growth curve is a piece of information about the life phase of a bacterium. This growth phase reflects the state of the bacteria in the culture at any given time. The development of the growth curve also functions as an optimization of the production time of secondary metabolites that are antimicrobial by sampling the starter culture. The existence of this time optimization can help to determine the length of incubation time needed in order to know the right time to harvest bacteria. The bacterial suspension will become cloudier (Figure 2) with increasing incubation time, this shows that the more the number of bacteria grows.

This study used nutrient broth (NB) as a medium in making starter cultures. This is because the media has the carbon and minerals needed for bacterial growth. The starter culture was incubated in a shaker incubator with a temperature of 30 °C and a shaker speed of 150 rpm. This is because *B. cereus* and *B. zhangzhouensis* are thermophilic bacteria that can live in a

temperature range of 20 - 40 ° C, while the function of the media shaker during incubation is to influence the mixing of nutrients in the media so that the metabolite yield can increase. The wavelength used for measuring OD with a spectrophotometer is a wavelength of 600 nm. The use of this 600 nm wavelength is because Bacillus is able to absorb at that wavelength.

Fig. 2. Bacterial suspension in starter cultures (incubation time **a.** 0 hours; **b.** 24 hours; **c.** 48 hours; **d.** 72 hours)

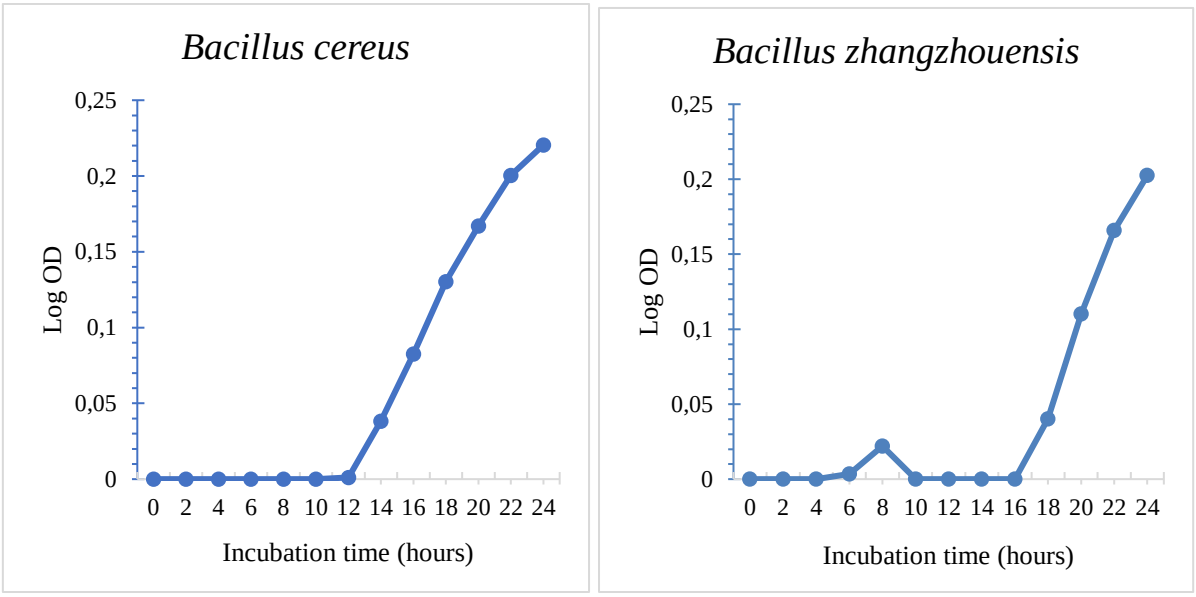

Fig. 3. Growth curve of Bacillus bacteria

Growth curves were made based on the results of OD measurements every 2 hours for 24 hours, which were then converted into LOG OD. The results of the growth curve above, the growth of the two Bacillus bacteria for 24 hours still increased (Figure 3). This is consistent with the growth of other Bacillus, one of which is *Bacillus pumilus* which also has slow growth so that the growth curve is continued with culture OD measurements every 24 hours.

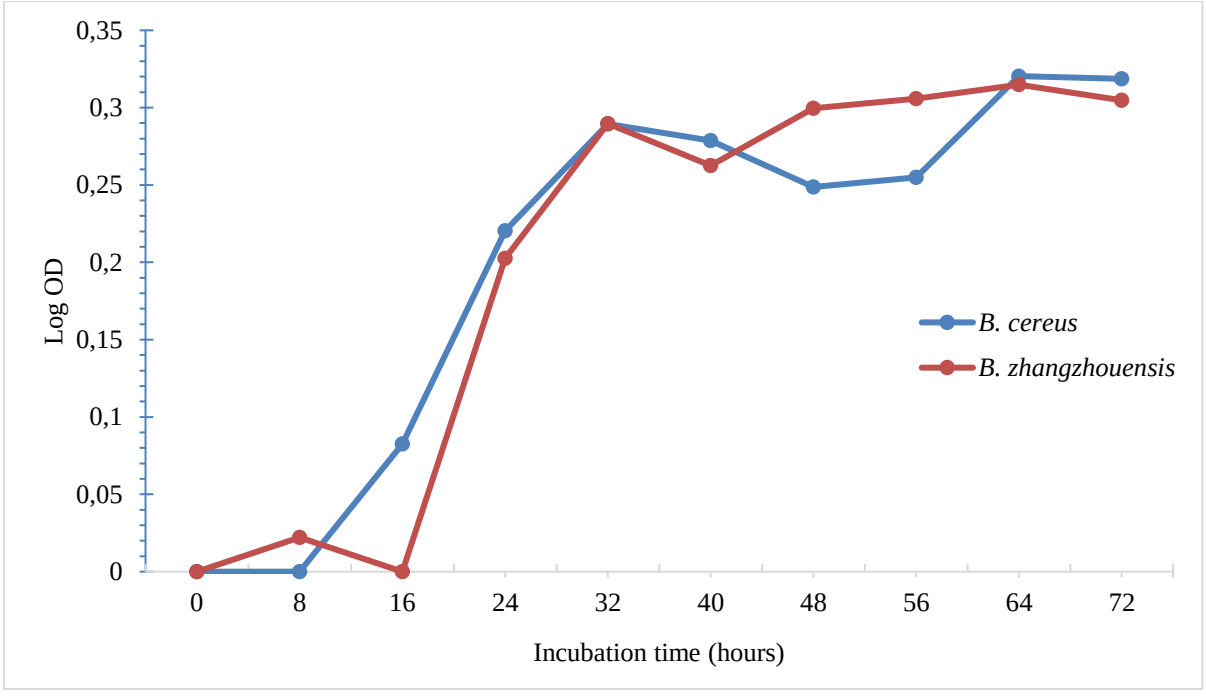

Fig. 4. Growth curve for 72 hours

Based on the curve (Figure 4) shows that the growth phases of *B. cereus* and *B. zhangzhouensis* consist of a lag phase, a log phase (exponential), and a stationary phase. The growth of *B. cereus* and *B. zhangzhouensis* begins with a lag phase (adaptation phase) which takes place at 0 - 8 hours and 0 - 16 hours respectively. The lag phase in *B. cereus* and *B. zhangzhouensis* was slow. The shortness or delay of the lag phase is largely determined by the number of cells inoculated, the appropriate physiological and morphological conditions, and the required cultivation medium.

The log phase of *B. cereus* occurs at 8 - 32 hours whereas, in *B. zhangzhouensis* it occurs at 16 - 32 hours. The log phase is the phase where cells divide at a constant rate (have been able to adapt to their environment), doubling. with the same rate, constant metabolic activity, and a state of balanced growth. The growth rate in this phase is influenced by the growth medium, pH, nutrient content, temperature, and air humidity.

The stationary phase of *B. cereus* occurred at 40 - 72 hours whereas, in *B. zhangzhouensis* it occurred at 48 - 72 hours of incubation. The stationary phase occurs when the number of cell growth equals the number of cell deaths. In the stationary phase, secondary metabolite compounds are produced because the bacteria defend themselves to survive. The production of secondary metabolites is strongly influenced by environmental conditions, namely a source of nutrients that function functionally in cells.

The results of the growth curve showed that the growth of *B. cereus* and *B. zhangzhouensis* was slow. Based on previous studies, the growth phase of *B. cereus* was slow and the death phase began to show its activity at the 80th hour of incubation. While *B. zhangzhouensis* has the same growth phase as *B. pumilus*, where the death phase begins to show its activity at 192 incubation hours.

The increase in the amount of bacterial biomass is influenced by several factors, one of the factors being measured is temperature. *B. cereus* is a bacterium that can live with a temperature range of 5 – 50 °C and is able to adapt to various environmental conditions, while *B.zhangzhouensis* can live with a temperature range of 8 – 45 °C, with temperatures for optimal growth in the range of 30 – 37 °C. The temperature used during the measurement of the growth curve was 30 °C. The temperature range used in the study was included in the temperature required for *B. cereus* and *B. zhangzhouensis* to grow.

3. 2. Inhibition Zone Test (In Vitro)

In vitro test was conducted to determine the ability of *B. cereus* and *B. zhangzhouensis* as a source of antibacterial in inhibiting the growth of *A. hydrophila*. The ability of antibacterial compounds to inhibit the growth of *A. hydrophila* is known by the presence of a clear zone or also known as the inhibition zone around the disc paper as a bacterial inhibition zone, which is measured using a calipers in millimeters (mm). The larger the diameter of the inhibition zone, it means the greater the potential possessed by the antibacterial compound (in this case derived from the bacteria *B. cereus* and *B. zhangzhouensis*) to kill or inhibit the growth of *A. hydrophila*. The zone of inhibition is caused by bacteria producing metabolites which are antibacterial compounds, where these compounds are able to inhibit the development of pathogenic bacteria. Antibacterial compounds work by disrupting cell wall components, causing plasmolysis which results in inhibition of growth or death of pathogenic bacteria. Inhibition zone activity is grouped into four categories, namely <5 mm of weak activity, 5 – 10 mm of moderate activity, >10 – 20 mm of vigorous activity, and >20 – 30 mm mm of very strong activity.

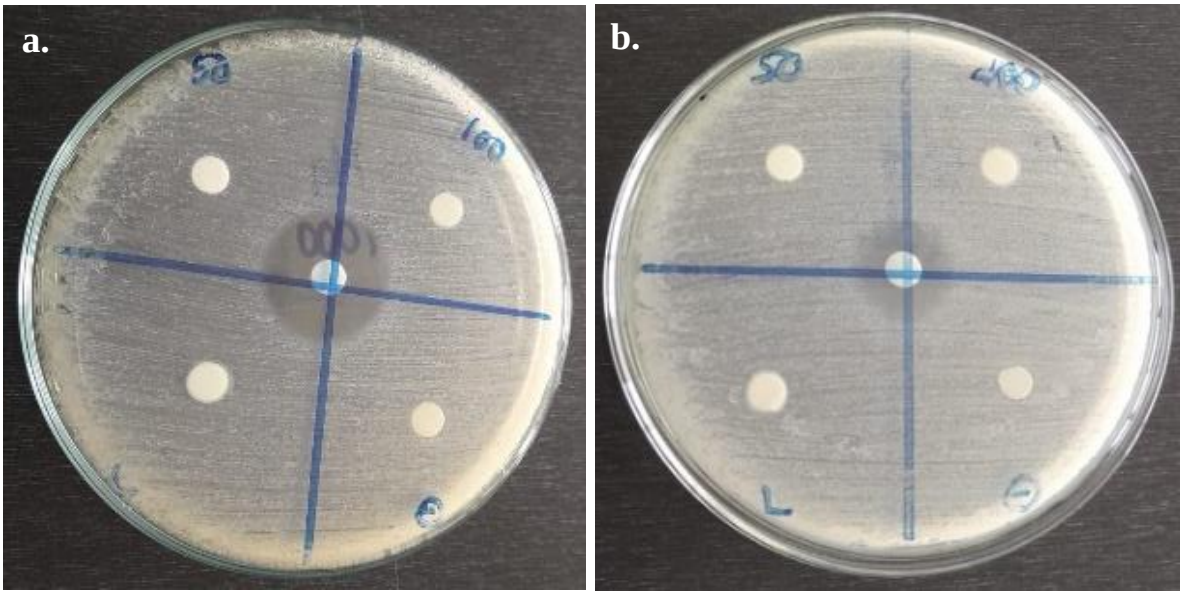

Fig. 5. Visualization inhibition zones of *B. cereus* **(a)** and *B. zhangzhouensis* **(b)**.

The results showed that there was an inhibition zone in the positive control treatment using 4000 ppm chloramphenicol antibiotics, the treatment of the *B. cereus* and *B. zhangzhouensis* test samples, while in the negative control treatment using distilled water, no inhibition zone was formed (Figure 5). Based on the image above, the negative control using distilled water does not form an inhibition zone, this shows that distilled water only functions as a solvent that does not have the ability to inhibit the growth of *A. hydrophila*.

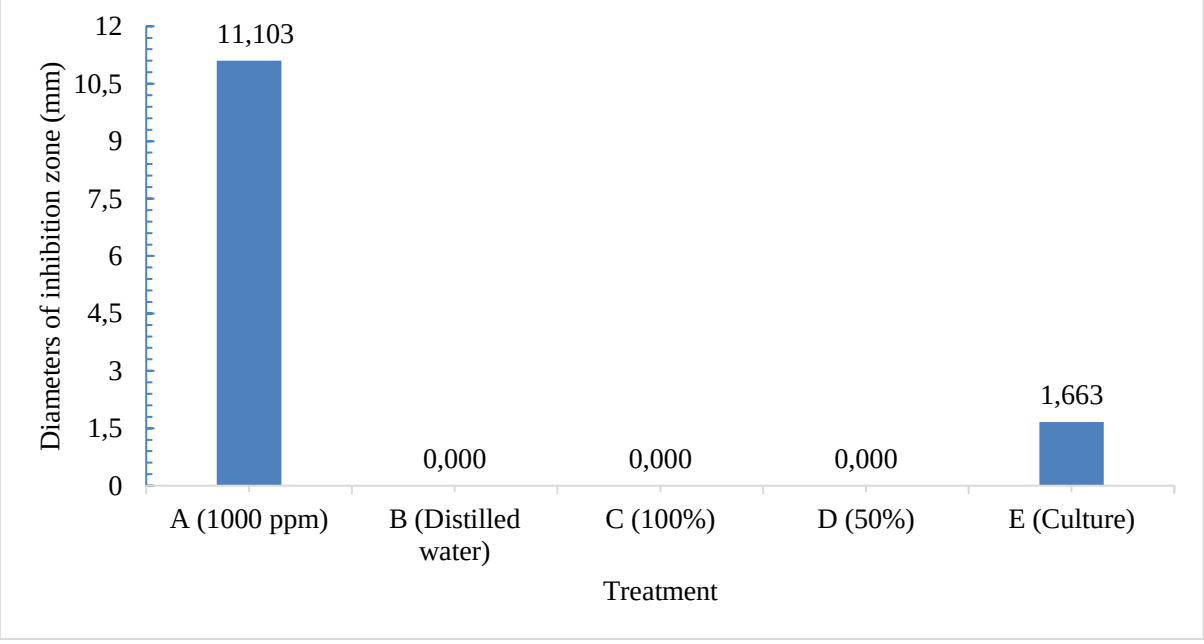

Fig. 6. Inhibition zone of *B. cereus*

The antibiotic activity of chloramphenicol from the phenicol class in inhibiting the growth of *A. hydrophila* was strong, namely 11.103 mm (Figure 6) and 10.18 mm (Figure 7). Chloramphenicol antibiotic is an antibiotic that is effective against several types of bacteria and anaerobic germs because it has a broad spectrum. These antibiotics have bacteriostatic activity and at high doses are bactericidal. Meanwhile, the antimicrobial activity of *B. cereus* in inhibiting the growth of *A. hydrophila* produced a small inhibition zone with an inhibition zone of 1.663 mm (culture), whereas in *B. zhangzhouensis* it produced an inhibition zone of 1.090 mm (culture). Based on the results, the antimicrobial activity of *B. cereus* and *B. zhangzhouensis* was in the weak category because the inhibition zone value was ≤ 5 mm, which was in the range of 1.090 - 1.663 mm.

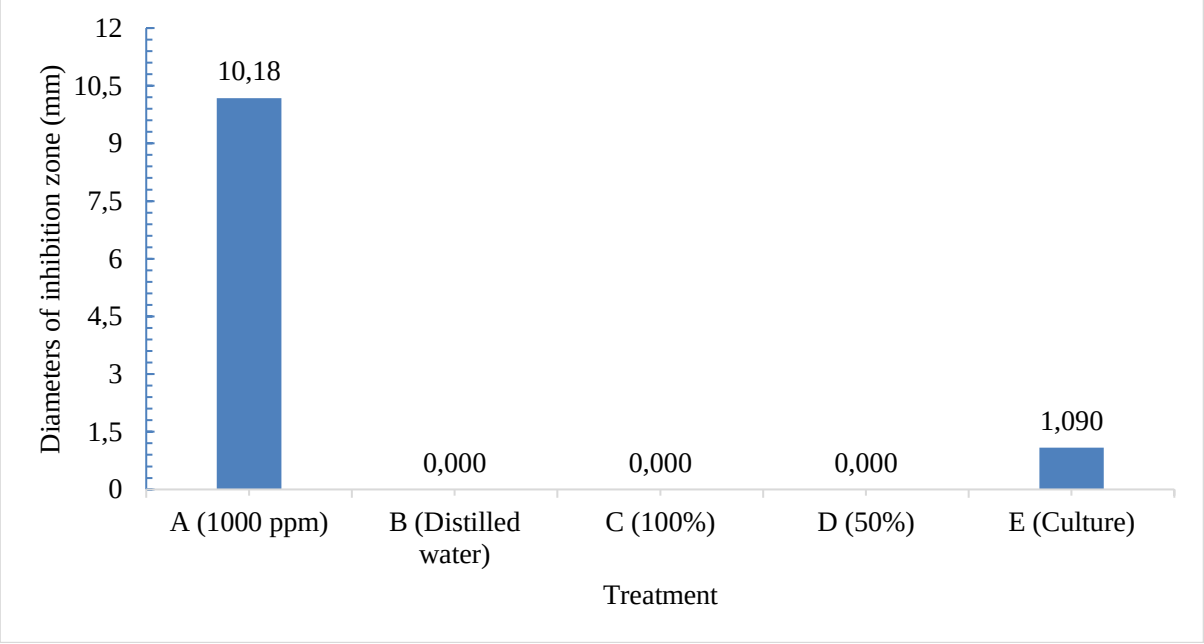

Fig. 7. Inhibition zone of *B. zhangzhouensis*

The results also showed that there was no inhibition zone in the 100% and 50% supernatant treatments, both for *B. cereus* and *B. zhangzhouensis*. This proves that the antimicrobial activity of *B. cereus* and *B. zhangzhouensis* against *A. hydrophila* shows that the inhibition zone diameter produced by the treatment on the supernatant is smaller than the culture treatment. The small zone of inhibition in the supernatant is due to the fact that the supernatant contains only secondary metabolites, this causes inhibition of pathogenic bacteria which is only supported by the results of secondary metabolites, but the bacterial cells that produce metabolites in this case have been separated. In addition, culture isolate fluid contains complete antimicrobial compounds consisting of primary metabolite compounds and secondary metabolite compounds. This is what causes the culture isolate fluid to produce a larger diameter of the inhibition zone on average.

However, based on previous research, it was found that *B. cereus* does not have an inhibition zone, which means that *B. cereus* does not have the ability to inhibit the growth of *A. hydrophila* whereas, *B. zhangzhouensis* has moderate antibacterial activity ranging from ±

8.4mm. In addition, the resulting inhibition zone values have different diameters depending on the treatment and incubation time. The resulting inhibition zone diameter at 72 hours after incubation was greater than the 24 hours and 48 hours incubation period. The length of the incubation period can determine the size of the diameter of the inhibition zone. The results of bacterial growth after 48 hours of incubation are more effective because the antibacterial activity is bacteriostatic so that it can inhibit the growth of microorganisms.

The difference in the inhibition zone can also be due to the difference in inhibiting ability in each treatment which increases the diffusion power along with the increase in the concentration of the treatment. The formation of the inhibition zone diameter at each concentration can be caused by differences in the size of the concentration or the size of the content of the antibacterial active substances contained therein and the diffusion rate of the antibacterial compound. Factors that influence the size of the inhibition zone generated in the diffusion method are the diffusion rate, the nature of the agar media used, the number of organisms inoculated, the growth rate of bacteria, the concentration of chemicals, and the conditions during the incubation period. There are other factors that affect the size of the inhibition zone, namely the length of time the samples are stored in the refrigerator and whether the containers used to store bacterial samples are tight or not.

Based on the results of the study, some of the antimicrobial activities of *B. cereus* and *B. zhangzhouensis* against *A. hydrophila* showed that the diameter of the inhibition zone produced by the supernatant was smaller than the culture. The small value of the inhibition zone in the supernatant is due to the fact that the supernatant contains only secondary metabolites, this causes inhibition of pathogenic bacteria which is only supported by the results of secondary metabolites, but the bacterial cells that produce metabolites in this case have been separated. Culture isolate fluids contain complete antimicrobial compounds consisting of primary metabolite compounds and secondary metabolite compounds. This is what causes the culture isolate fluid to produce a larger diameter of the inhibition zone on average. The greater the antimicrobial concentration, the faster the diffusion occurs so that the antibacterial power will be greater and the diameter of diffusion will occur rapidly so that the antimicrobial power will be greater and the resulting inhibition zone diameter will be wider.

The small value of the inhibition zone in this study could be due to not extracting the antimicrobial compounds present in *B. cereus* and *B. zhangzhouensis*. The factor for the formation of the inhibition zone is highly dependent on the amount of antibacterial material that is dripped onto the disc, the solubility of the antibacterial agent on the media, the diffusion coefficient, and the antibacterial effectiveness. The inhibition zone is getting bigger due to the large number of extracts that have antibacterial properties that accumulate on the growing media so that they can further disrupt the process of bacterial growth. Extraction is the process of separating material from its mixture using a suitable solvent. The choice of extraction method depends on the properties of the material and compound to be isolated. The existence of this extraction can increase the results of the inhibitory power test.

The presence of an inhibition zone in *B. cereus* and *B. zhangzhouensis* proves that both bacteria have antibacterial compounds. The genus Bacillus is known to be able to produce antibacterial compounds such as surfactin, fengycin, iturin, bacitracin, subtilosin, subtilisin, bacillomycin. *B. cereus* itself is known to be able to produce the antibacterial compound basilomycin D. *B. zhangzhouensis* has a close genetic relationship with *B. pumilus* by 80%. *B. pumilus* has the ability to produce pumilacidin antibacterial which belongs to the surfactin family and bacitracin which is active against *Micrococcus luteus* and *S. aureus*.

Inhibition of pathogenic bacteria occurs by destroying the cell wall which causes lysis or inhibiting cell wall growth in growing bacteria, changing the permeability of the cytoplasmic membrane, causing leakage and nutrients to leave the cell. In addition, bacteria that produce antibacterial substances can also inhibit protein and nucleic acid synthesis by denaturing proteins and destroying nucleic acids so that their function as genetic material is lost. In addition, it can also inhibit the activity of intracellular enzymes that interfere with cell metabolism. Antibacterial compounds can also increase lysozyme activity. Lysozyme enzymes are usually used as immunogenic parameters, which work by lysing bacterial cell walls such as hydrolyzing N-acetylglucosamine and N-acetylmuramic acids in peptidoglycan, with the loss of these cell walls the bacteria will die.

4. CONCLUSIONS

The results of the growth curve showed that the stationary phase of *B. cereus* and *B. zhangzhouensis* occurred at 36 - 72 hours of incubation, so it can be concluded that the growth of the two bacteria was slow. *B. cereus* and *B. zhangzhouensis* have the ability to inhibit the growth of *A. hydrophila* in the weak category. The zone of inhibition of *B. cereus* and *B. zhangzhouensis* against *A. hydrophila* ranged from 0.7 to 1.6 mm.

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