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Study of Probiotic Candidate Bacteria CBL20 for Inhibiting of *Aeromonas hydrophila* with Different Concentration in Tilapia (*Oreochromis niloticus*)

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Abstract. Aeromoniasis is still a problem in tilapias culture industry since its caused mass mortality. Manipulation microbial population in the culture media using probiotic to prevent pathogenic bacterial bloom is becoming the most environmentally friendly approach. Probiotic candidate CBL20 was molecularly identified through 16s RNA method as *Bacillus methylotropicus*. The purposes of this study were to determine the ability of CBL20 to inhibit the growth of pathogenic bacteria (*Aeromonas hydrophila*) in vitro and to evaluate the performance of CBL20 to prevent Aeromoniasis outbreaks in vivo. Survival rate of experimental animals was observed. The 120 fish (*Oreochromis niloticus*) at an average weight of 11.85 ± 1.02 g were used as experimental animals. Completely randomized design with 4 treatments and 3 replications was applied. The treatments were various mixture concentration of probiotic CBL20 at 10^6 CFU ml⁻¹ with bacterial *A. hydrophila* (a) 0 CFU ml⁻¹, (b) 10^2 CFU ml⁻¹, (c) 10^3 CFU ml⁻¹, and (d) 10^4 CFU ml⁻¹. In vitro showed that CBL20 at a concentration of 10^6 CFU ml⁻¹ were inhibited *A. hydrophila* with growth inhibition area of 9.3 ± 0.9 mm. Addition of CBL20 as a candidate probiotic bacteria with concentration 10^6 CFU ml⁻¹ has not been able to inhibit *A. hydrophilla* in the treatment given. The concentration of treatment did not have a significant effect ($P > 0.05$) on the survival of tilapia. CBL20 probiotic candidates with a concentration of 10^6 CFU ml⁻¹ were able to maintain SR 33.33% for 14 days after infected by *A. hydrophilla*.

1. Introduction

Tilapia (*O. niloticus*) is one of freshwater cultivation fish which have a good prospect. tilapia has some beneficial nature, such as breed easily, and growth quickly [1]. Tilapia has been widely cultivated to meet increasing consumer demand. Increasing demand for domestic markets encourages the fish farmers to increase their production [2]. Tilapia production from aquaculture has a problem that there are *A. hydrophila*. These bacteria include pathogen bacteria which cause "Motile Aeromonas Septicemia (MAS)" disease, especially for freshwater fish species in tropical waters. Fish infected with *A. hydrophila* will show clinical indication. In several cases, the bacteria's attack will effect sudden death without any clinical indication on fish, otherwise, the fish will clinically indicate such like swelling, redness, generate skin damage, gill, and internal organ [3]. *Aeromonas hydrophila* infection can inhibit growth and sudden death. *Aeromonas hydrophila* has an effect on freshwater cultivation and often causes high mortality (80-100%) in a short time so that it causes considerable losses. however, until now there is no known density of *A. hydrophila* which causes attacks on tilapia [4]. Bacterial diseases control by using antibiotics or chemicals must be in accordance with the rules set by the government. This type of antibiotic and the choice of concentration to control the disease is still reviewed. The effects of the wrong use of antibiotics can cause resistance to pathogenic bacteria, water pollution environment and there

will be chemical residues from antibiotics consumed in aquaculture products [5]. One of the probiotic candidate bacteria that have been obtained are *B. methylotrophicus* (CBL20) which is the result of screening bacteria from mud aquaculture on central aquaculture production in Central Java [6]. The preliminary studies to determine the density of bacteria CBL20 and *A. hydrophila*. These results estimate that probiotic candidate bacteria CBL20 is able to break communication or inhibit *A. hydrophila* attacks at a concentration of 10^5 CFU ml^{-1} . However, the cultivation of bacterial growth in water cannot be ascertained the type and amount without laboratory observation. Inhibition of the growth process of *A. hydrophila* in cultivation media with space and nutrient competition. This study was conducted to find out more clearly the function of probiotic candidate bacteria CBL20 with a concentration of 10^6 CFU ml^{-1} to different concentrations of *A. hydrophila* in aquaculture.

This study were to determine the effect of probiotic candidate bacteria (CBL20) on the survival rate and concentration which was optimally prevent from *A. hydrophila* bacteria with different concentrations based on preliminary studies. This study conducted from January to April 2018 at the Laboratory of Aquaculture, Faculty of Fisheries and Marine Sciences, Diponegoro University.

2. Research Methods

This study used tilapia with an average weight of $13,85 \pm 1,02$ g. Tilapia was obtained from UPR Mina Muncul, Ambarawa. With used 12 aquariums with a size of $40 \times 30 \times 40$ cm^3 and put 10 fish in the entire aquarium. This research used commercial feed with *ad satiation* method on feeding. Pathogen bacteria which used in this research is pure isolate of *A. hydrophila* bacteria from Fisheries and Marine Science Faculty, Diponegoro University, Semarang, Central Java. The probiotic candidate bacteria found by screening which code is CBL20 from the best productivity fish cultivation in Boyolali, Central Java [6,7]. TSA (Merck) was used as an agar medium in the in vitro test process, then tilted TSA as a pure culture medium of isolate, GSP (Glutamate Starch Phenol) media as a culture medium and collective growth test or pasase. Furthermore, TSB (*Tryptic Soy Broth* - Merck) media used as an agar culture medium for *A. hydrophila* and probiotic candidate bacteria CBL20. The procedure in the study consists of the preparation phase and the implementation phase. The preparation stage consists of sterilizing microbiological [3]. The Fishes acclimatization were cultivated for almost 2 months. After that, making TSA (*Trypticase Soya Agar*) media, GSP media (*Glutamate Starch Phenile*), and TSB media (*Trypticase Soy Broth*). The culture proces of *A. hydrophila* bacteria is taking pure purified bacterial colonies (TSA, Merck) using ose, then cultured in TSB liquid media using a 10 ml volume and incubated in a shaker for 1x24 hours [3,6,7].

The determination of *A. hydrophila* pathogenicity was carried out to improve the virulence of bacteria against tilapia as the object of this study [7]. It's carried out three times. The isolation results were cultured on GSP media and it become yellow (if *Aeromonas* sp.) then will be purified on TSA media. Pure isolates will then be cultured in TSB media for use in the passage process until the in vivo test. The implementation phase consists of three phases. The first step is a preliminary test, the second step is antimicrobial test and mixture test [6,11]. The third step is the in vivo test using probiotic candidate bacteria with pathogenic bacteria in the cultivation medium. The first step is a preliminary test to determine the concentration of probiotic bacteria. Preliminary tests were carried out with the application of probiotic candidates in culture media that contained *A. hydrphila* pathogenic bacteria and observed tilapia response for 14 days of maintenance. The parameters observed were survival,

clinical indications and bacterial density in the culture medium. The second stage is the test phase of the probiotic candidate bacteria in vivo which is a test from the growth inhibition area to see the growth inhibition area around the disc paper and the diameter of the measured growth inhibition area. This test is to determine the potential of probiotic candidate bacteria (CBL20) which can inhibit the growth of *A. hydrophila*. Test the growth inhibition area using disc paper that contains specific probiotic candidate bacteria. The results will be compared between mixed probiotic bacteria and pathogenic bacteria with controls that only contain pathogenic bacteria. The best results are fewer bacteria in the mixed media than the control media. This will be a reference in determining the application of probiotic candidate bacteria in vivo. The third step is the in vivo test.

The third step was the in vivo test study [7] to determine the effect of probiotic candidate bacteria on inhibition or prevent attacks of *A. hydrophila* in tilapia. In this phase the water culture media prepared for each aquarium is 10 L, the water must be sterilized. After that, administration of *A. hydrophila* and probiotic bacterial candidate CBL20 on water media. For *A. hydrophila* concentration of 10^2 , 10^3 , 10^4 CFU ml⁻¹ and for 10^6 CFU ml⁻¹ probiotic candidate bacteria.

Experimental design in this research used **6** complete random design with used 4 treatments and 3 times repetition. The treatments used:

- A : Probiotic candidate bacteria 10^6 CFU ml⁻¹ density on water media
- B : *A. hydrophila* bacteria 10^2 CFU ml⁻¹ density and probiotic candidate bacteria with 10^6 CFU ml⁻¹ density on water media
- C : *A. hydrophila* bacteria 10^3 CFU ml⁻¹ density and probiotic candidate bacteria with 10^6 CFU ml⁻¹ density on water media
- D : *A. hydrophila* bacteria 10^4 CFU ml⁻¹ density and probiotic candidate bacteria with 10^6 CFU ml⁻¹ density on water media

2.1. Clinical Symptoms

Clinical symptoms observed visually after the tilapia placed on maintenance media. The observed parameters are behavior change and morphology on the tilapia. The observation held everyday as long 14 days.

2.2. Survival Rate

Survival rate the tilapia can be counted by using this formula [8]:

$$SR = \frac{N_t}{N_o} \times 100\% \quad (1)$$

SR = Survival Rate (%); N_t = Survive fish; N_o = an initial fish

2.3. Data Analysis

Data on survival rates were analyzed using diversity analysis (ANOVA) method. The results data will be tested by normality test, homogeneity test and before additive analysis (ANOVA) additivity test to the observed variables. While clinical indications were analyzed descriptively.

3. Results and Discussion

The in vitro test result shown that bacteria density with the highest growth inhibition area was 10^6 CFU ml⁻¹. This test result become consideration to determinate probiotic candidate bacteria which will be used in in vivo. The best result is when the bacteria quantity in mixed

media less than control media with probiotic candidate bacteria CBL20 density is 10^6 CFU ml^{-1} with *A. hydrophila* density is 10^2 CFU ml^{-1} . Inhibition area result shown on Table 1.

Table 1. Inhibition zone CBL20 against *A. hydrophilla*

No	Density (CFU ml^{-1})	Inhibition zone (mm)			Average \pm SD (mm)
		1	2	3	
1.	10^4	9	10	8	9.0 \pm 1.0
2.	10^5	9	8	8,6	8.5 \pm 0.5
3.	10^6	10.3	8.5	9	9.3 \pm 0.9

The highest inhibition area is on 10^6 CFU ml^{-1} density (9.3 \pm 0.9 mm), followed by 10^4 CFU ml^{-1} density (9.0 \pm 1.0mm) and the lowest is on 10^5 CFU ml^{-1} density with inhibition area (8.5 \pm 0.5 mm). In vitro result shown that bacteria density with the highest inhibition area is 10^6 CFU ml^{-1} . This dose is the inhibition area on 5-10 mm classified as medium categorized [9]. The highest results will be used on the next step of research. The mixture test bacteria result on Table 2.

Table 2. Mixture test bacteria

<i>A. hydrophila</i>	CBL20	Result (GSP Media)		
		1	2	Average
10^2	10^6	+	+	+
10^3	10^6	++	++	++
10^4	10^6	+++	+++	+++
10^2 (control)	-	+++	+++	+++

+++ =CBL20 can not preventing *A. hydrophila* growth

++ =CBL20 start able to dominate *A. hydrophila*

+ =CBL20 dominate and start to preventing *A. hydrophila*

-- =CBL20 is not growing

The best results were treatment B with bacterial quality in the mixed media less than the control media with probiotic bacteria candidate CBL20 10^6 CFU ml^{-1} density and density of *A. hydrophila* was 10^2 CFU ml^{-1} . Probiotic candidate bacteria CBL20 dominate and suspect it can prevent the growth of *A. hydrophila*. Behavioral changes in the behavior of tilapia (*O. niloticus*) given by *A. hydrophila* and probiotic candidate bacteria CBL20 in water medium for 14 days of maintenance are shown in Figure 1.

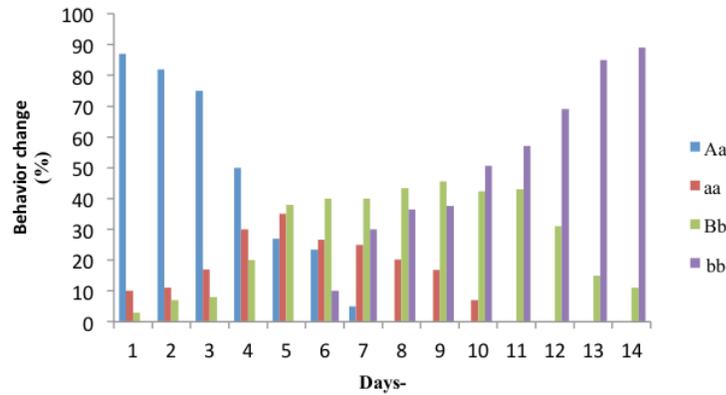


Figure 1. Tilapia's Behavior

- (Aa) : Fast Response to feed
- (aa) : Slow response to feed, swim normally
- (Bb) : Very slow response to feed, swimming movement tend to be weak
- (bb) : No response to feed, swim weakly, keep away from the aeration, the dorsal fins being stretch

After being given *A. hydrophila* bacteria and probiotic candidate bacteria CBL20 on water media Tilapia responds to eat quickly and swim normally from day 1 to day 7, the percentage decreases by 87% to 5%. Then the fish responds to eating slowly from day 1 to day 10, the percentage of decline is 35% to 7%. The fish began to experience a very slow feed response and a slightly weak swimming movement until the 14th day there was an increase in the percentage of 3-86.25%. On the 6th day until the 14th day, the fish did not respond to feed, swam weakly, and tended to stay away from aeration, which increased by 10-89%. All repetitions showed morphological changes on the 4th and 5th days, fish experienced morphological changes such as flushed mouth (16.66%), wound (12.49%), thin caudal fin (29.16). %, pale skin (58%) and excessive mucus on the surface of the body (12.49%). Starting from the 6th to the 14th day the fish experienced an increase in the percentage of morphological changes such as mouth flushing (16.66%), wounds (15.26%), thin caudal fins (66.66%), pale skin (76.86%), swelling in the eyeball (33.33%) and excessive mucus on the body surface (61.76%). Morphological changes in Tilapia are shown in Figure 2.

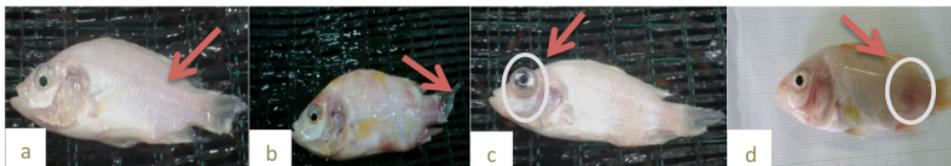


Figure 2. Tilapia's Clinical Symptoms

- A.) Pale skin
- B.) Tail-fin rot
- C.) Exophthalmia
- D.) Wound and bleeding on the scale

Based on survival results, probiotic bacterial candidate CBL20 with *A. hydrophila* bacteria at different concentrations did not significantly affect the survival of tilapia (*O. niloticus*). At a density of 10^6 CFU ml⁻¹ from probiotic candidate bacteria with *A. hydrophila* 10^4 CFU ml⁻¹ bacteria had the lowest survival rate of tilapia. The lowest survival rate of tilapia treatment at the end of the study (16.67%), while in treatment A, B and C the survival of tilapia at the end of the study (23.33%, 33.33% and 23.33%). The following diagram shows the survival rate of tilapia that has been given by *A. hydrophila* bacteria and CBL20 probiotic candidate bacteria on water medium for 14 days of maintenance can be seen in Figure 3.

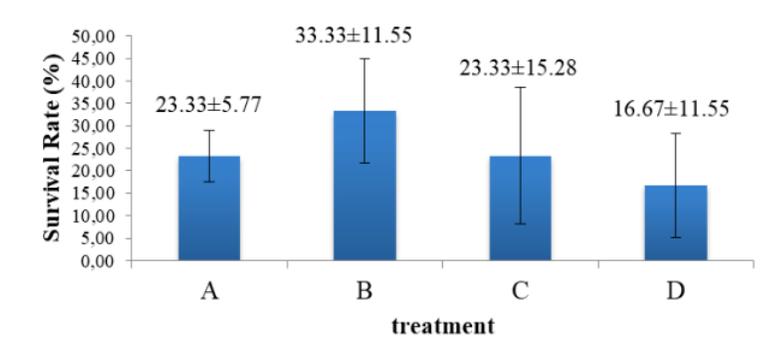


Figure 3. Survival Rate Chart of Tilapia (*O. niloticus*)

Information:

A (Probiotic candidate with density of 10^6 CFU ml⁻¹)

B (Density of *A. hydrophila* 10^2 CFU ml⁻¹)

C (Density of *A. hydrophila* 10^3 CFU ml⁻¹)

D (Density of *A. hydrophila* 10^4 CFU ml⁻¹)

Based on the in vitro test, it can be seen that the probiotic candidate bacteria *B. methylotrophicus* can inhibit the growth of *A. hydrophila* bacteria, this is evidenced by the inhibition area produced on paper disc density with the largest inhibition area of 10^6 CFU ml⁻¹ with a mean inhibition area of 9.3 ± 0.9 mm.

The ability to inhibit probiotic *B. methylotrophicus* candidate bacteria against *A. hydrophila* is classified as moderate. The criteria for the antibacterial power strength ie diameter of inhibition are ≤ 5 mm categorized as weak, diameter 5-10 mm categorized as moderate, and diameter 10-20 mm categorized as strong and inhibition area ≥ 20 mm categorized as very strong [9]. The best results from the joint culture test were the density of probiotic candidate bacteria *B. methylotrophicus* 10^6 CFU ml⁻¹ with *A. hydrophila* 10^2 CFU ml⁻¹ which had fewer bacteria in the mixed media than the control media. At higher probiotic candidate concentrations and *A. hydrophila*, the results are less than optimal because of the high competition for nutrients and oxygen, causing disturbed bacterial balance in the media. The effectiveness of probiotics is influenced by bacterial composition, the concentration used, age and quality of probiotics [10]. Behavioral changes after being given *A. hydrophila* and probiotic candidate *B. methylotrophicus* most of the treatment of tilapia experienced behavioral changes starting on the 4th day with a percentage of fish that responded to feed quickly and normal swimming by 50%, then the fish responded to feed slowly by 30% and fish begin to respond to feed very slowly and weak swimming movements by 20% (Figure 2). Fish swim slowly allegedly due to stress and the probiotic CBL20 candidate dose has not

been optimal in inhibiting *A. hydrophila* for 14 days of maintenance. *Aeromonas hydrophila* that attack fish can disturb the balance of swimming so that the fish become abnormalities in swimming like fish swimming slowly or vertically [3,6]. Decreasing response to eating fish decreases, this is due to the body's metabolic processes are disrupted. Liver is one of the target organs of *A. hydrophila* where the liver is the center of the body's metabolism, so that when the liver is disrupted by toxic pathogens it can affect the body's metabolic processes [12]. Treatment A began to show clinical symptoms on day-7 passive maintenance of tilapia, whereas treatment B began to show clinical symptoms of passive behavior on day 5 maintenance. Treatment of C and D began to show clinical symptoms of passive behavior from day 1 to day 14 of maintenance. Provision of probiotic candidate bacteria CBL20 is thought to be able to inhibit the appearance of clinical symptoms in tilapia. This is because probiotic candidate bacteria CBL20 can inhibit the growth of *A. hydrophila* 10^2 CFU ml⁻¹ concentration for 5 days of maintenance so that the appearance of clinical symptoms is longer than treatment with the addition of *A. hydrophila* 10^3 and 10^4 CFU ml⁻¹. Morphological clinical symptoms seen on the 6th to 14th day of the fish have morphological changes in the form of mouth flushing (16.66%), thin caudal fins (66.66%), pale skin color (76.86%), swelling on eyeball (*exophthalmia*) (33.33%) and excessive mucus on the body surface (61.76%). The external symptoms caused by MAS vary from changes in the body color of the fish that becomes darker, the redness that extends to the abdominal part, often accompanied by necrosis of the fin and tail, ulcer, pale skin color. Other symptoms, loss of scales, injured mouth, *exophthalmia* [4,7,13].

Based on the analysis of the variety of probiotic candidate bacteria giving no effect on the survival of tilapia ($P < 0.05$). The treatment of D has the lowest survival value presumably because of probiotic candidate bacteria with a density of 10^6 CFU ml⁻¹ were not able to inhibit the growth of *A. hydrophila* with a density of 10^4 CFU ml⁻¹ in water for 14 days of maintenance. The survival rate has a low value in all treatments allegedly because the administration of probiotic candidate bacteria CBL20 can only inhibit for 7 days of maintenance and the dose of probiotic candidate bacteria that is not optimal enough to inhibit the growth of *A. hydrophila* in water media. In treatment, B is able to maintain SR of 33.33% and can increase 10% of treatment A (control). The survival rate has a low value presumably because the administration of probiotic candidate bacteria CBL20 (*B. methylotrophicus*) is not optimal so that surfactin has not been able to optimally lyse *A. hydrophila*. The effectiveness of surfactin as an antimicrobial compound depends on the amount of its concentration and the resistance of the surrounding microorganism membrane which can be inhibited [14]. There were five antimicrobial work mechanisms, namely inhibition of bacterial cell wall synthesis, inhibition of the integrity of bacterial cell wall permeability, inhibition of bacterial cell protein synthesis, inhibition of bacterial cell nucleic acid synthesis, and inhibition that inhibits microbial cell metabolism. The working system of the surfactin is the inhibition of the integrity of the cell wall permeability. In all living cells is limited by the cytoplasmic membrane which acts as a barrier with selective permeability, carrying out the active transport function so that it can control the cell structure. However, if the integrity of the function of the cytoplasmic membrane is disturbed for example by substances that are surfactin so that the permeability of the cell wall changes or becomes damaged, then important components such as proteins, nucleic acids, nucleotides, etc. come out of the cell and the cell gradually dies. Therefore, *A. hydrophila* bacteria will lysis if the CBL20 probiotic candidate bacteria is optimally administered and cannot produce extracellular enzymes that are toxic (*aerolysin* and *hemolysin*). *Bacillus methylotrophicus* bacteria produce a wide range

of cyclic lipopeptides that are active against other microorganisms. Surfactin belongs to the group of amino acids that make up the composition of lipopeptide [15]. The mechanism of action of the fractin is the inhibition of the integrity of the cell wall permeability. Surfactin lipopeptide have great potential as antibacterial [16].

4. Conclusion

Addition of CBL20 as a candidate probiotic bacteria with concentration 10^6 CFU ml⁻¹ has not been able to inhibit *A. hydrophilla* in the treatment given. The concentration of treatment did not have a significant effect ($P > 0.05$) on the survival of tilapia. CBL20 probiotic candidates with a concentration of 10^6 CFU ml⁻¹ were able to maintain SR 33.33% for 14 days after infected by *A. hydrophilla*

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