Original Research Article

Characteristics of *Bacillus* CgM22 and The Effect of Induction on The Growth Rate in Carp (*Cyprinus carpio*)

ABSTRACT

Bacillus is a potential probiotic candidate which is currently widely applied in aquaculture. *Bacillus* can be found in various hosts, one of which is found in the intestines of fish. This study contains the genus *Bacillus subtilis* intestinal fish *Bacillus* CgM22. The steps taken were the characterization of *Bacillus* CgM22 with gram staining test, inhibition test, and proteolytic test. The method at the fish rearing stage is an experimental method with a Completely Randomized Design (CRD) model that uses four treatments and three replications. Treatment A was control with no addition of antimicrobials in the feed. Treatment B was the addition of 10^8 CFU *Bacillus* CgM22 at a dose of 15 ml/kg of feed. Treatment C was the addition of supernatant from *Bacillus* CgM22 at a dose of 5 ml/kg of feed. Treatment D was the addition of supernatant from *Bacillus* CgM22 at a dose of 10 ml/kg of feed. Furthermore, *Bacillus* CgM22 was induced in carp (*Cyprinus carpio*) to see its effect on absolute weight. The results showed that the gene characteristics of the bacteria genus *Bacillus* CgM22, including gram-positive bacteria, had the shape of a rod or *Bacillus*. It was also found that the growing *Bacillus* CgM22 could inhibit the growth of *Aeromonas hydrophilla*. There are proteolytic test results that can be seen from the formation of a clear zone with a proteolytic index of IP = 2.19. Based on statistical tests, it was shown that the addition of probiotics was not significantly different ($P > 0.05$) on the growth rate of carp *(Cyprinus carpio).*

Keywords: Absolute Weight, Bacillus subtilis, Carp, Proteolytic

1. INTRODUCTION

Probiotics are live microorganisms that provide health benefits to the host (1). Probiotics have the function of increasing the host's response to disease and can be used as biocontrol agents to reduce disease attacks. One of the probiotics that can be used is *Bacillus subtilis* because this bacterium can stimulate immunity both in vivo and in vitro (2). Induction of *B. subtilis* added to feed can express genes from the immune system and physiologically will increase the body's resistance to disease.

Currently, there have been many studies regarding the potential of *Bacillus* sp. Based on the results of research Mulyani *et al.* (3), several types of *Bacillus* bacteria were obtained which were isolated from the intestines of carp. Furthermore, *Bacillus* bacteria were also identified to select alternative sources of immunostimulant candidates and one of them was found in *Bacillus* CgM22 that identified as *Bacillus subtilis* (4).

Bacillus are also given to fish as probiotics and can have a better effect on the growth and health status of carp (5). Several probiotics have been shown to be growth promoters in several carp species. The results showed that supplementation of *B. subtilis* significantly improved the growth performance than other groups whereas feed efficiency was unaffected by dietary treatments (6). *B. subtilis* can increasing the digestive enzymes activities and improving the intestinal morphology. β-glucan and B. subtilis supplementation significantly improved the fillet quality, immune responses and antioxidant status of Pengze crucian carp (7).

Based on this description, it is necessary to conduct research on *Bacillus* CgM22 which is a bacterium derived from fish intestines to analyze its gene characteristics by gram staining test, inhibition test, proteolytic test, and its effect on growth rate.

2. METODOLOGY

2.1 Methods

The steps taken were the characterization of *Bacillus* CgM22 by gram staining test, inhibition test, and proteolytic test. Next, fish rearing is carried out. The method at the fish rearing stage is an experimental method with a Completely Randomized Design (CRD) model that uses four treatments and three replications. The treatment given was different at each dose concentration of *Bacillus* CgM22 supernatant mixed into artificial feed. Treatment A was control with no addition of antimicrobials in the feed. Treatment B was the addition of 10^8 CFU *Bacillus* CgM22 at a dose of 15 ml/kg of feed. Treatment C was the addition of supernatant from *Bacillus* CgM22 at a dose of 5 ml/kg of feed. Treatment D was the addition of supernatant from *Bacillus* CgM22 at a dose of 10 ml/kg of feed. Furthermore, the weight of the fish is observed until the end of fish rearing period.

2.2 Research Materials

The materials used for gram-bacteria staining were distilled water, *Bacillus* CgM22 bacterial isolate, Nutrient agar (NA), iodine, gentian violet dye, safranin/water fuchsin dye. In addition, the materials used for the inhibition zone test included chloramphenicol antibiotics, cotton buds, isolates of Aeromonas hydrophila bacteria, Nutrient agar (NA), Nutrient broth (NB). Materials for proteolytic assay are agar, Nutrient Broth (NB), skim milk. Materials for feed induction include carp seeds as test 120 carp, *Bacillus* CgM22, artificial feed and The aquarium used is 15 with each aquarium containing 10 carp.

2.3 Working Procedure

Based on Agustina *et al.* (8), Gram staining is carried out through several stages, namely the bacterial sample is scratched on the object glass. 1 drop of gentian violet dye is placed on the smear area and left for 20 seconds. Then washed gently using distilled water and left for 2 seconds. Next, 1 drop of Iodine is placed on the smear area for 30 seconds – 1 minute and then washed gently with alcohol and left for 10 – 20

seconds. After that, the Object glass was washed gently using distilled water, left for 2 seconds. Safranin dye / fuchsin water as much as 1 drop is dripped over the smear area and left for 20 seconds and washed slowly using distilled water and left for 2 seconds. The bacterial smear on the object glass was allowed to dry at room temperature then covered with a cover glass, and the sample was observed under a microscope with the help of immersion oil with a 100x objective magnification.

The zone of inhibition test was carried out by the well diffusion method. The zone of inhibition test against the pathogen *Aeromonas hydrophila* was carried out by growing bacterial isolates on agar medium. The steps taken were as follows (9): Sterile petri dishes were prepared and filled with starter culture, 50% and 100% supernatants, chloramphenicol antibiotics, and sterile distilled water, respectively. Then, 10 ml of NA was poured into a petri dish. Petri dishes were divided into 4 quadrants and Aeromonas hydrophila bacteria were planted on NA media by pouring 0.1 ml of bacteria. After that, the Aeromonas hydrophila bacteria were flattened using a sterile lab cottonbud and waited for the bacteria to absorb into the media. Furthermore, the media was made wells with each treatment inserted into the well as much as 15 l, and then incubated at a temperature of 30°C for 24 hours.

The proteolytic test was carried out using skim milk agar media. The proteolytic test on the *Bacillus* CgM22 was carried out by growing bacterial isolates on agar media. The skim milk agar medium was prepared by dissolving 1 g of Nutrient Broth (1%), 2 g of agar (2%), and 2 g of skim milk (2%) into 100 ml of distilled water. Then the media was sterilized using an autoclave at 121^{o}C for 60 minutes. Aseptically, the media was poured into sterile petri dishes evenly until all surfaces of the petri dishes were filled with media. After the media hardened, each bacterial isolate was streaked with an ose needle on the skim milk media. The samples were then incubated for 24-48 hours at 30^{o}C. The presence of protease enzyme activity was then observed with the formation of a clear zone around the bacterial colonies after incubation (10).

Induction was carried out by weighing the feed and adding *Bacillus* CgM22 culture using a density of 10^8 with a dose of 10 ml/kg and the supernatant with a dose of 5 ml/kg and 10 ml/kg of feed, respectively. Before being mixed into the feed, a binder (egg white) was added as much as 2% of the weight of the feed. Then the feed is mixed and stirred until evenly distributed until dry. Feeding per day only 2 times a day using the method of feeding by calculating feed requirements based on body weight (ad libitum). Before being fed fish from each aquarium, they were weighed in order to know their weight and to be able to determine the weight of feed that should be given per day. Provision of probiotic feed was carried out for 2 months of the maintenance period (11).

2.4 Analysis Method

Inhibition zone test analysis was carried out by measuring the transparent zone after 24 hours of incubation from bacterial cultivation and disc placement. The results obtained are divided by two to get the average as in the following formula (12).

$$D = \frac{(D1 - A) + (D2 - A)}{2}$$

Description:
D : average inhibition zone diameter (mm)
D1 : horizontal diameter of clear zone (mm)
D2 : vertical diameter of clear zone (mm)
A : well diameter (mm)

The analysis of the proteolytic test is to calculate the proteolic index, namely the comparison between the diameter of the clear zone and the diameter of the colony to obtain potential isolates (13). The proteolytic index is obtained by the formula based on (14) as follow:

$$IP = \frac{Clear\ zone\ diameter}{Colony\ diameter}$$

Description :

IP : Proteolytic Index

3. RESULTS AND DISCUSSION

The results of *Bacillus* CgM22 staining can be seen in Figure 1.

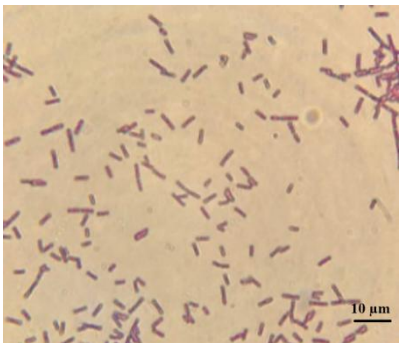

Fig 1. Gram *Bacillus* CgM22 staining results with 100x magnification

Based on the results obtained, the results showed that the bacterial isolates had the shape of a rod or *Bacillus* and included gram-positive bacteria. Gram-positive bacteria cells look purple because they can form complex bonds with the main dye (crystal violet), namely purple. The cell wall of Gram-negative bacteria consists of 5-20% peptidoglycan, the rest is polysaccharide. Giving 95% alcohol solution to the cell can increase the porosity of the cell wall by dissolving the lipids in the outer membrane, so that the purple color will be released and the cell will become colorless. Furthermore, the cells will be red because they are colored by the comparison color, namely safranin (15).

Tests for the inhibition of *Bacillus* CgM22 against *Aeromonas hydrophilla* were carried out with distilled water as a negative control and the antibiotic chloramphenicol as a positive control. The method used is the well diffusion method using 5 treatments, namely chloramphenicol, distilled water, culture, and bacterial supernatants 50% and 100%. This test was carried out for 3 days and the results were as shown in Figure 2.

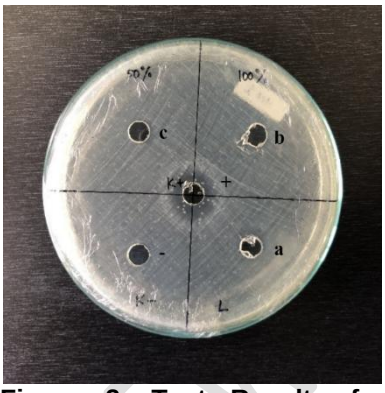

Figure 2. Test Results for *Bacillus* CgM22 . Inhibitory Zone Description: (a) Culture, (b) 50% supernatant, (c) 100% supernatant, (+) Chloramphenicol 1000ppm,(-) Aquades

Based on the results obtained, there is an inhibitory activity on *Bacillus* CgM22. According to Morales *et al.* (16), the inhibitory zone activity was grouped into four categories, namely: weak (<5mm), moderate (5–10mm), strong (>10–20mm), very strong (>20–30mm). Bacterial inhibitory activity was expressed based on the clear zone produced around the well. The diameter of the zone of inhibition of bacterial growth was measured in mm. The larger the clear zone formed, the greater the inhibitory activity of lactic acid bacteria isolates against pathogenic bacteria (17). This inhibition zone activity is indicated by the presence of a clear zone around the well. Based on the results obtained, the activity of this bacterium was proven to inhibit *Aeromonas hydrophilla* bacteria.

Table 1. Inhibitory Zone Test Results on *Bacillus* CgM22

Test	Hours of-	Inhibition Zone Diameter (mm) Treatment				
		Control +	Control –	Culture	Supernatant 100%	Supernatant 50%
1	24	10,3	0	1,26	1,49	0,85
	48	10,73	0	3,06	2,59	2,18

	72	11,31	0	4,33	2,94	2,19
	24	10,11	0	1,5	0,97	0,92
2	48	10,46	0	5,11	1,02	1,68
	72	11,58	0	3,95	3,3	2,57
	24	9,87	0	1,24	1	1
3	48	10,89	0	2,55	1,01	1,16
	72	10,99	0	4,37	3,43	3,63
Average		10,69	0	3,04	1,97	1,80
Interpretation		Strong	–	Weak	Weak	Weak

Based on the results of the completely randomized design test (Table 1), it was found that the results obtained from the complete design test were significantly different from each treatment $P < 0.05$). The notation results obtained are treatment C 2.0^{a}, treatment B 2.68^{ab}, treatment A 3.24^{b}, treatment D 10.69^{c}, seen from the highest average obtained, and the best inhibition zone test for *Bacillus* CgM22 in culture treatment with an average 3.04mm. Utilization of *B. subtilis* bacteria in cultivation systems has been used as a solution to prevent the development of pathogens, increase nutrient assimilation and improve environmental parameters (18).

The average yield in the clear zone formed around the isolates was caused by the presence of extracellular antimicrobial compounds released. The formation of the diameter of the inhibition zone at each concentration can be caused by differences in the size of the concentration or the amount of antibacterial active substances contained therein and the rate of diffusion of these antibacterial compounds. Other factors affect the size of the inhibition zone, namely the length of time the sample is stored in the refrigerator and whether the container used to store bacterial samples is tight or not (4).

Furthermore, *Bacillus* CgM22 was tested for its proteolytic activity using skim milk agar media and incubated for 24 hours. Based on Figure 3, it is known that there is a clear zone around the bacterial colonies. The presence of bacterial extracellular protease activity causes casein in CCA media to hydrolyze into peptides and amino acids. The clear zone is an indicator that bacterial isolates can utilize casein in the media as a source of nutrition (19).

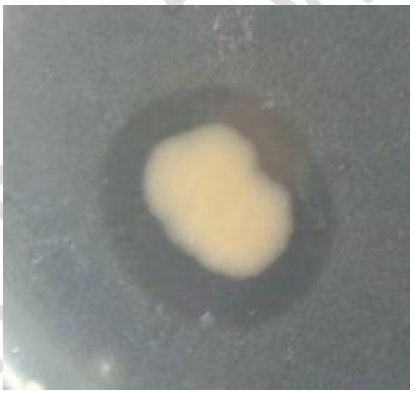

Figure 3. Proteolytic Test Results

Based on the results of the study, it was found that the identified *Bacillus* CgM22 could produce extracellular proteolytic enzymes. Proteolytic bacteria are bacteria that can degrade proteins because they produce extracellular protease enzymes. Protease is an enzyme that is widely used in the animal feed industry and has almost reached 65% of the total sales of enzymes in the world (20). One of the functions of proteases is to play a role in the degradation of protein into amino acids which makes fish feed more easily absorbed by the digestive system (21).

Most of the genus *Bacillus* are major producers of extracellular proteases. The advantages of the *Bacillus* genus are that it does not produce toxins, is easy to grow, does not require expensive substrates, and can withstand high temperatures (22). Among the various protease producers, *Bacillus* sp. is commercially recognized for exploiting microbes for

protease production (23). Several alkaline proteases derived from *Bacillus* with apparent activity, stability, wide pH, temperature, short fermentation time, and modest but high efficiency are getting more consideration for isolation and facilitating the study of the enzymes they produce (24).

Table 2. Proteolytic Index of *Bacillus* CgM22

Test	Clear zone diameter (mm)	Colony diameter (mm)	IP
1	13,14	6,33	2,35
2	10,96	5,21	2,1
3	10,37	4,9	2,12
Average	12,05	5,48	2,19

Based on Table 2, it was found that the results of calculating the proteolytic index (IP) based on the clear zone obtained value IP ≥ 2. This index indicates that *Bacillus* CgM22 has protease activity (25). The ability of microorganisms to secrete proteases is due to the enzyme degrading proteins. The medium included with skim milk containing casein is a milk protein that can be broken down by proteolytic microorganisms into dissolved nitrogen compounds so that the colonies are surrounded by a clear zone, that these microorganisms have proteolytic activity (26).

Fish feed contains high enough protein as the main component of feed and a source of energy for fish. Feed that enters the digestive tract of fish will be degraded by digestive enzymes. Based on the results *Bacillus* CgM22 has proteolytic properties, which can secrete protease enzymes to hydrolyze peptide bonds in proteins into oligopeptides and amino acids.

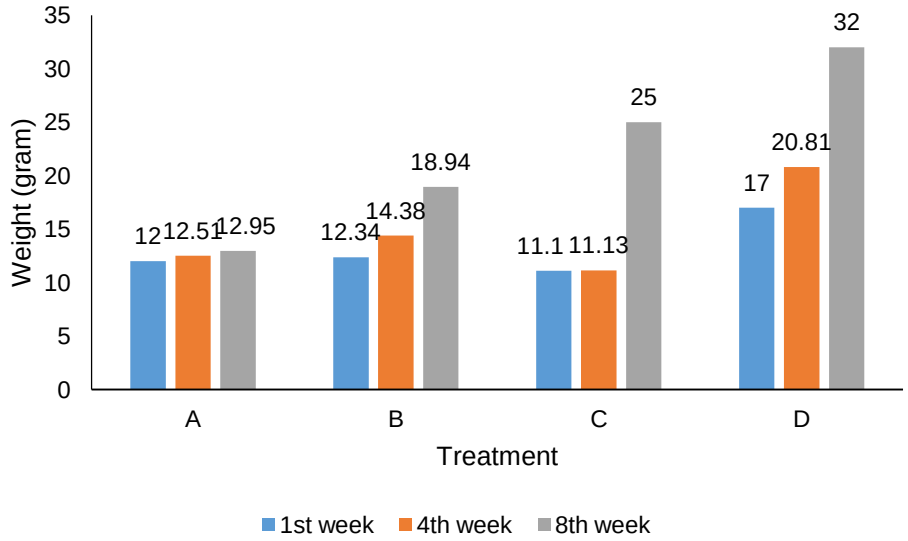

Figure 4. Growth Rate After Induction of *Bacillus* CgM22
(a) Control, (b) Addition of culture *Bacillus* CgM22 10^8 CFU as much as 10 ml/kg feed, (c) addition of Supernatant from *Bacillus* CgM22 as much as 5 ml/kg feed (d) addition of Supernatant from *Bacillus* CgM22 as much as 10 ml/kg feed

Based on the results of statistical tests, there was no significant difference between the adition of probiotics ($P > 0.05$). As the results of research giving *Bacillus* showed weight gain, increased growth performance, and feed conversion ratio (27). The value of fish weight gain in each treatment was higher than the control treatment, presumably because the probiotic *Bacillus* given entered the digestive tract and was able to increase fish appetite. Furthermore, these bacteria in the digestive tract of fish will secrete digestive enzymes such as proteases

and amylase (28). (29) stated that the presence of enzymes produced by *Bacillus* probiotics such as amylase, lipase and protease that can trigger fish growth

4. CONCLUSION

The characteristics of the gene for bacteria of the genus *Bacillus* CgM22, including gram-positive bacteria, have the shape of a rod or *Bacillus*. It was also found that the growing *Bacillus* CgM22 could inhibit the growth of *Aeromonas hydrophilla*. There are proteolytic test results with a proteolytic index of IP = 2.19. Based on the results of statistical tests showed that the addition of probiotics was not significantly different (P > 0.05) to the growth rate of carp (*Cyprinus carpio*).

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