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PROTEOLITIC POTENTIAL OF *Bacillus sp*. FROM FISH GUT AND NUTRIENT CONTENT OF SUBSTRATE

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Abstract

Proteolytic bacteria are bacteria that can hydrolyze proteins into smaller peptides or amino acid units. The existence of extracellular protease-producing bacteria is very important for life because it provides the need for nitrogenous compounds and can be used as a probiotic agent. This study was conducted to determine the proteolytic activity of Bacillus sp. Isolates. From the results of the collection and isolation of indigenous bacteria, the gut of carp. The method used is purposive sampling method, the data is analyzed quantitatively descriptive. The material used is the bacterium Bacillus sp. selected from the digestive tract of omnivore fish. Proteolytic test using clear zone method in skim milk agar (SMA) culture was measured at 12th, 24th, and 48th hours. The results showed that nine isolates of Bacillus sp. selected has proteolytic activity. The diameter of the clear zone (14.33-18.33.mm) Bacillus isolates from the 12th hour showed qualitatively the high proteolytic ability of the protease enzyme produced or also the high number of enzymes produced and released out. The optimum pattern of production time is in line with the general bacterial growth curve pattern. The results showed that at the 48th hour, bacterial isolates CP013984_s Bacillus sp. produce the highest protease activity, which is equal to 46.84 mm. The use of bacillus isolates in making probiotics can increase protein content and reduce crude fiber substrate, with a crude protein composition of 31.23%, extract ether 4.38% and crude fiber 8.64%.

Key words*: Bacillus sp., proteolytic activity, fishgut, nutrient content of substrate.*

INTRODUCTION

The digestive tract, especially the intestine, is an important part of the habitat of the bacteria that live in it. According to Fidyandini (2015) compared to gills, mucus in scales and the surrounding aquatic environment, the highest number of microorganisms is found in fish intestines. Interactions that occur between bacteria in the intestine can be negative and positive. These negative interactions can cause fish health problems but can also produce positive interactions that affect the increase in digestibility and the fish's immune system (as immunostimulants). Therefore, microbial communities of each aquatic species need to be identified to provide a more effective development opportunity for probiotics or immunostimulants. One type of bacteria in many probiotic products used today in the field of aquaculture is the *Bacillus* genus. Gupta et al. (2002) state that the genus *Bacillus* is suitable as a probiotic for aquaculture as it is commonly found as part of microbiota in fresh and marine water, and in the digestive tract of animals. *Bacillus* inhibition activity on the growth of *A. hydrophila* due to this bacterium produces enzymes including esterase lipase, leucine aryl amidase, acid phosphatase, lipase, and Naphthol-AS-BI-phospholipase. Whereas according to Susanti et al. (2002) that probiotics from the *Bacillus* group are widely applied for biotechnological purposes including the types of enzymes and amino acids produced and the production of antibiotics for fermentation and pathogen control.

Microorganisms are the most potential source of enzymes compared to plants and animals. The use of microorganisms is more beneficial because of their fast growth, can grow on a cheap substrate, the results are easier to increase through regulation of growth conditions and genetic engineering. Some genera of bacteria known to produce proteases

include *Bacillus, Lactococcus, Streptomyces*, and *Pseudomonas* (Rao et al., 1998).

The results of the research by Susanti et al. (2003) stated that four *Bacillus* strains isolated from the digestive tract of healthy white shrimp and applied through water at a concentration of 10^5 CFU/mL could improve the health of white shrimp larvae (*Litopenaeus vannamei*). Wijaya (2011) stated that probiotics *Bacillus* P4I1 applied to tilapia (*Oreochromis niloticus*) culture media at a concentration of 10^9 CFU/mL showed a survival value of 51.67% higher than control (21.67%) after being infected with Streptococcus agalactiae. The application of the superiority of *Bacillus* probiotics has emerged in the market especially in the form of drugs and food.

Based on the background explanation, a problem can be formulated regarding the extent to which bacteria originating from the genus *Bacillus* isolated from the gut of carp (*Cyprinus carpio*) can potentially be bacteria that have proteolytic activity. Based on the research background described, the objectives of this study are:Identify bacteria from the genus *Bacillus* that are found and Knowing proteolytic activity through the measurement of clear zones and change in the composition of the bioprocess substrate in making probiotic products. The usefulness of this study is to provide scientific information for farmers regarding indigenous bacteria species from the intestine that have proteolytic abilities so that they can function as a source of microbial and probiotic enzymes.

MATERIALS AND METHODS

Collecting Intestine Samples

- Incubator for incubating bacteria.
- Ruler to measure the diameter of the clear zone
- Disc paper to be dipped in the supernatant isolate

Isolation and Purification of Bacteria

- Fish gut
- Physiological NaCl and H_2O
- Cotton and gauze
- Plastic and plastic wrap
- foil alumina
- Medium skim milk

Proteolytic Characterization of Intestinal Bacteria with a Clear Zone

- Isolates pure bacteria of the genus *Bacillus*
- Medium SIM
- H_2O_2 solution 3%
- Erich reagent
- Skim medium for agar milk (SMA)
- Laminar as a place to conduct test activities
- Petri dishes as a medium place and testing
- Bunsen to avoid contamination when testing
- Incubator for incubating bacteria.
- Ruler to measure the diameter of the clear zone
- Paper discs to be dipped in supernatant isolates

Research methods

This research was conducted using qualitative and quantitative descriptive analysis. Sampling of fish and bacteria was carried out using purposive sampling method, is a technique of determining samples with certain considerations. The research was conducted at the Aquaculture Laboratory, Faculty of Fisheries and Marine Sciences, Padjadjaran University.

Carp Intestine Sample Preparation

The intestine of goldfish as the research sample is derived from carp cultivated in the floating net cage of the Cirata Reservoir in Gandasoli Village, West Bandung. The fish is brought to life as an aseptic intestine in the laboratory for laminar. Goldfish intestine is taken through several stages, namely: (a) Turn off the fish by piercing the brain using a sonde needle; (b) Washing the outside of the fish with sterile distilled water, then spraying alcohol on the entire surface of the fish's body and on a cloth using sterile cotton; (c) Place the fish in the tray and make an incision in the belly of the fish until the internal organs are visible; (d) Separating the digestive organs of the fish and cutting the intestines of the fish from the stomach to the anus; (e) Measuring the length of the intestine using a ruler, then divided into three parts (front, middle, and back of the intestine), each in a separate analysis; (f) Taking the contents of the intestine as much as 0.5-1 grams by splitting the fish intestine using scissors then scraping the intestinal contents; (g) Store the contents of the intestine in a sterile vial bottle that has added 10 ml of physio-

logical NaCl; (h) Take 1.0 ml of the solution using a micropipette and put it in the second test tube. And so on until 10-7 dilutions and obtained a single isolate.

As much as 1 ml of each dilution 10-2-10-10 by taking 1 ml of each dilution, put on the Skim Milk Agar media using the pour plate method.

The isolates of protease producing bacteria from the genus *Bacillus*, are characterized and biochemical identified by reference to the Manual of Determinative Bacteriology, which includes microscopic and macroscopic observations.

The results of bacterial isolates that have been isolated from goldfish intestine, then identified on agar culture, then carried out qualitatively proteolytic tests. Using a needle in the SMA media center on the petri dish. The media is first incubated at 50^{o}C for 48 hours. Qualitative protease activity is indicated by the formation of clear zones around the colonies. Diameter of colonies and clear zones using a ruler. The greater the clear zone formed, the greater the protease activity produced.

Proteolytic measurement through a clear zone

After obtaining a single isolate a proteolytic activity test was conducted. This screening test is carried out on the agar media with the addition of skim milk from the agar volume. In this test, the clear zone is produced. This test is carried out through several stages, namely: a) Taking bacteria that are present in a single isolate using an ose needle; b) Move the bacteria using an osseous needle into another petri dish containing medium so that it is skimmed; c) Perform bacterial cultivation for 2×24 hours; d) Observe and measure the distance of the diameter of the clear zone produced.

Proteolytic activity was determined by the size of the clear zone formed. Clear zone is a response from bacteria to TSB and skim milk and soy flour added to the medium. Clear zone formation activity was calculated from the difference in the diameter of the clear zone with the diameter of the bacterial colonies (Isnansetyo and Kamei, 2009).

A total of 20 µl of *Bacillus* probiotic inoculum was inserted into a well on NA media which was inoculated with Aeromonas bacteria which was planted on a pour plate with 0.5 McFarland concentration. Then incubated for 24 hours at 37^{0}C. The inhibitory zone (clear) formed on the test media was measured using a caliper. The variable measured is the diameter of the inhibitory zone (clear) formed in each well hole in mm.

RESULTS AND DISCUSSIONS

Isolation of *Bacillus sp*. on the digestive tract

The initial stage of the bacterial isolation process is to do the grinding of the mid gut, dilution, planting bacteria on agar media and purification of bacteria to obtain a single colony (Figure 1). Average of weight common carp fish was 76.95 g with a length of 18.5 cm. The intestinal weight of the fish is 1.3 g with a length of 20 cm and the degree of acidity (pH) of the intestine 6.5. The contents of the intestine were taken as much as 0.5-1 grams.

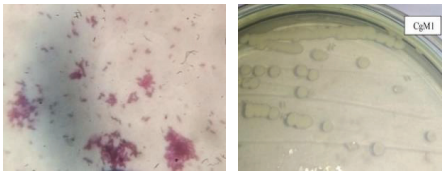

Figure 1.Colony morphology of isolated bacteria

In Figure 1 it appears that the main key character of the *Bacillus* genus is basil-shaped cells, gram-positive and forming endospores. These purified bacteria were then used as a single isolate and were used to test proteolytic activity. The morphological identification results obtained nine types of *Bacillus* bacterial isolates in the digestive tract (gut) (Table 1).

Table 1. Purified *Bacillus* isolates in nutrient agar and MRS cultures

No.	Code	Genus Bacillus
1.	I_1 (CgN2)	*Bacillus flexus*
2.	I_2 (CgN3)	*Bacillus flexus*
3.	I_3 (CgN4)	*Bacillus cereus*
4.	I_4 (CgN6)	*Bacillus carboniphilus*
5.	I_5 (CgM1)	*Bacillus haynesii*
6.	I_6 (CgM8)	CP013984_s *Bacillus* sp.
7.	I_7 (CgM18)	*Bacillus zhangzhouensis*
8.	I_8 (CgM22)	CP013984_s *Bacillus* sp.
9.	I_9 (CgM38)	CP013984_s *Bacillus* sp.

Genus *Bacillus* is a bacterium that has a large stem shape, gram-positive cell type, can grow in aerobic conditions that form a chain. Most

members of this genus are saprophytic organisms commonly found in soil, water, air, and plants. Fuller (1992) states that the genus *Bacillus* can grow at temperatures over 50^0C, is able to grow at high salt concentrations (>10%) and can produce spores. *Bacillus* bacteria can grow at high salt concentrations above 10 ppt, and still work well at pH fluctuations between 7.3-10.5. Some species are even able to live at very high pH conditions up to >11. Seeing the properties possessed by *Bacillus*, microbial cultures can be used both inside and outside the digestive tract by growing the right number of microbial populations so that it can be an alternative because used to suppress the growth of pathogenic bacteria.

Selected single isolates were as many as nine genera of *Bacillus* bacteria, then screening was performed on skim milk medium (SMA) with additional TSB, and soy flour from agar volume. In this test, the clear zone is produced. The bacteria that were successfully isolated were grown on media suitable for microbial growth because they were rich in nutrients. The results of isolation have been carried out, obtained nine isolates who have morphological characteristics of colonies that are different from each other, and able to grow in high school media (Figure 2).

Figure 2 shows that the *Bacillus* genus has a qualitative proteolytic ability isolates were obtained which had proteolytic activity. Skim milk contains casein which is included in the bacterial growth medium which functions as an enzyme substrate. Casein hydrolyses was used to show the hydrolytic activity of proteases. Proteases catalyze the degradation of casein, i.e. by breaking the CO-NH peptide bond with the entry of water into the molecule (Susanti, 2002).

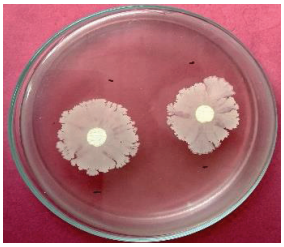

Figure 2. Qualitative Proteolytic Bacteria (a. clear zone b. colony)

Proteolytic Activity Test

The magnitude of proteolytic enzyme activity is shown by the increasing width of the clear zone, but the magnitude of proteolytic enzyme activity that plays a role in the solid medium cannot be quantified and measured quantitatively. The results of polymer protein reformation are only indicated by the presence of a clear zone which indicates that the protein has been overhauled into peptide compounds and amino acids which are dissolved in the medium. Qualitative hydrolysis activity is an illustration of the ability of proteolytic bacterial isolates to overhaul proteins by comparing the size of the clear zone around the colony with the size of the colony diameter.

The results of the proteolytic activity test showed that of the nine bacterial isolates of the bacillus genus that were obtained all produced clear zones which showed proteolytic activity at 12^{th} hour observation, 24^{th} hour, and 48^{th} hour (Table 2).

Table 2. Duncan's Multiple Distance Test Clear Zone *Bacillus* Isolate at 12^{th}, 24^{th}, 48^{th}hours.

No. Isolate (Code)	Clear Zona at Hour-		
	12	24	48
	(mm)		
I_1 (CgN2)	17.67^{A}	25.03^{A}	40.72^{BCD}
I_2 (CgN3)	16.67^{AB}	25.88^{A}	43.02^{ABC}
I_3 (CgN4)	17.00^{AB}	22.64^{B}	44.73^{AB}
I_4 (CgN6)	14.33^{C}	21.01^{B}	42.57^{ABC}
I_5 (CgM1)	16.63^{B}	19.25^{B}	36.52^{CD}
I_6 (CgM8)	17.33^{AB}	23.01^{AB}	46.84^{A}
I_7 (CgM18)	18.33^{A}	20.57^{B}	42.33^{ABC}
I_8 (CgM22)	$17,67^{AB}$	$20,87^{B}$	34.27^{D}
I_9 (CgM38)	16.00^{BC}	20.62^{B}	37.03^{CD}

Description: the same letter towards the column, shows no significant difference ($P<0.05$)

Based on the results of the study, all bacteria from the genus *Bacillus* identified from the digestion of carp can produce extracellular proteolytic enzymes using casein which functions as a substrate for the protease enzyme. Casein is the main protein of milk, a micro-molecule composed of subunits of amino acids that are connected by peptide bonds.

Table 2 shows that after incubation in a high school medium for one day (24 hours), bacterial isolates I2 (CgN3) had the highest proteolytic activity (by 25.88 mm) but did not show a significant difference with isolates CgN2(25.03 mm) and CgM8 (23.01 mm). After 48 hours, isolate I6 (CgM8), showed the highest clear zone diameter, almost covered the surface of the petri dish (46.84 mm), and did not show significant differences with Isolates I2, I3, and I7. The Isolates I5 (CgM1) and I8 and isolates I8 (CgM22), both at 24th and 48th hours, yielded the clearest clear zone ($P<0.05$) at the lowest.

The diameter of the clear zone formed can quantitatively indicate the proteolytic ability of the protease enzyme produced or also the high number of enzymes produced and released out. Proteolytic bacteria are bacteria that can degrade proteins, because they produce extracellular protease enzymes.

Proteases are proteolytic enzymes that catalyze the breakdown of peptide bonds in proteins. The ability of microorganisms to secrete proteases shows a change or protein degradation in skim milk containing medium.

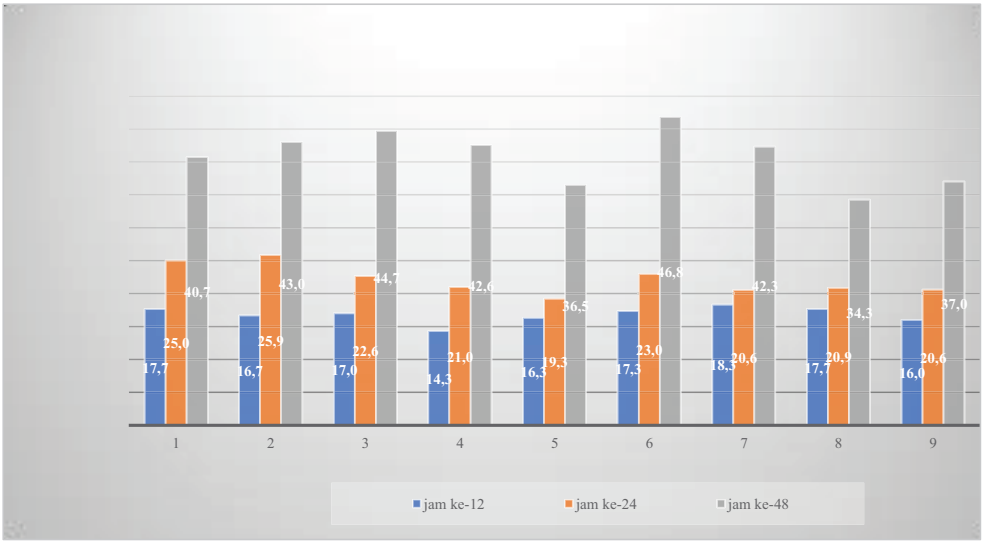

Figure 3. Proteolytic activity (Clear Zone) of ninetypes digestive isolates of fish gut

Proteolytic Index

The magnitude of the proteolytic index is related to the increase in the diameter of the inhibitory zone which is proportionally related to the increase in the diameter of the bacterial colonies, for example CgN6 and CgM8 isolates have a large bacterial diameter which shows a clear zone and high proteolytic index. The results of testing of proteolytic activity of bacterial isolates of carp samples are presented in Table 3

In Table 2 it appears that quantitatively-qualitative CgN3, CgM8 and CgM18 bacterial isolates were the largest isolates of bacteria with a proteolytic index value because almost all surfaces of the petri dishes had formed clear zones, whereas bacterial isolates I8 (CgM22) (8.70 mm) were bacterial isolates which have the smallest proteolytic index value.

Table 2 shows that *Bacillus sp*. and *Bacillus zhangzhouensis* has the best proteolytic index, as indigenous bacteria in the digestive tract of carp. The diameter of the clear zone formed can

qualitatively indicate the proteolytic ability of the protease enzyme produced or also the high number of enzymes produced and released out to carry out degradation activities. Indigenous bacteria are microbial bacteria obtained from the habitat of the digestive tract of fish. As for the research results of Affandi (2017), there were found the types of Bacillus subtilis proteolytic microbes which can produce extracellular proteolytic enzymes.

The proteolytic properties of microbes can be applied as probiotics in various interests. *Bacillus* in inhibiting microbial growth is a probiotic *Bacillus* with its metabolite binding to a negative carboxylic group on the surface of bacterial cells (Rabea et al., 2003). Some beneficial bacteria have proteolytic properties causing weakening or damage to membranes and other microbial cell components.

Bacillus probiotics also contain the lysozyme enzyme and amino-polysaccharide group which can inhibit microbial growth. Several mechanisms of inhibition of microbes by probiotics *Bacillus* have been proposed by several researchers, but the exact mechanism is not yet known. The most accepted mechanism is the interaction between probiotics *Bacillus* and the surface of bacteria, which causes changes in cell surface permeability.

Number of Microbial Colonies Results of Application in Feed Substrate

Table 3. Nutrient Content of Substrate and Products *Bacillus* sp.

		CFUCP	EE	CF	Ca	P
$\times 10^9$CFU				(%)		
Initial Substrate	4.01-4.42	22.19	5.91	12.82	3.41	1.44
Prebiotic *Bacillus*	15.22	31.23	4.38	8.64	4.22	2.05

CFU: colony forming units, CP: Crude Protein, EE: Ether Extracts; CF: Crude Fiber

In Table 3, it appears that there is a change in the composition of the bioprocess substrate in making probiotic products. This is in line with the opinion of Shurtleff and Aoyagi (1979), which states that in bioprocess there will be changes in complex molecules or organic compounds such as proteins, carbohydrates and fats into simpler molecules. Addition of *Bacillus* sp. and *Staphylococcus* sp. 10^3 CFU/ml each can increase the body's endurance in public waters which usually has *Aeromonas hydrophila* of 10^3 CFU/ml (Fidyandini, 2015). Feliatra et al. (2004) have also examined that in the digestive tract of carnivorous fish there are at least nine bacteria that function to help increase feed digestibility. The types of bacteria are *Lactococcus sp., Carnobacterium* sp*., Staphylococcus* sp., *Bacillus* sp., *Eubacterium* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Micrococcus* sp., and *Bifidobacterium* sp. These bacteria are often used as probiotic candidates. Based on Aslamyah's research (2006), it was found that bacteria in the digestive tract of milkfish were: (i) amylolytic (*Citrobacter* sp*., Aeromonas hydrophila, Staphylococcus* sp*., Flavobacterium* sp*., Carnobacterium* sp*., Moraxella* sp., and *Vibrio* sp.); (ii) proteolytic (*Vibrio alginoliticus, Streptococcus* sp*., Micrococcus* sp*., Proteus sp., Pseudomonas sp*., and *Bacillus* sp.); and (iii) lipolytics (*Planococcus* sp., *Kurthia* sp., *Serratia* sp., and *Plesiomonas* sp.). Amylolytic bacteria isolated from milkfish can increase the availability of carbohydrate feed, thereby reducing the use of energy sources from protein.

CONCLUSIONS

The results showed that nine isolates of *Bacillus* sp. selected has proteolytic activity and bacterial isolates CP013984_s *Bacillus sp.* produce the highest protease activity, which is diameter of clear zone equal to 46.84 mm. The use of bacillus isolates in making probiotics can increase protein content and reduce crude fiber substrate, with a crude protein composition of 31.23%, extract ether 4.38% and crude fiber 8.64%.

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