



Protease Production from *Bacillus* sp. Isolated from Gastrointestinal Tract of Catfish (*Clarias* sp.) with Different Medium

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Abstract

The need for protease in the industrial field has been increasing. Candidates for producing these enzymes can be isolated from the digestive tract of catfish (*Clarias* sp.). The purpose of this study was to obtain bacterial isolates that produce proteolytic from the gastrointestinal tract of catfish and determine the effect of different production media on the activity of proteolytic. The first step of this study was isolation, screening, and identification of bacteria. The second step was to test the effect of the media Luria Bertani, trypticase soy broth, and skim milk broth on proteolytic activity. Nineteen isolates were obtained from the screening process of proteolytic bacteria. Isolate no 1, was known as the best isolate in producing enzymes and was known as *Bacillus* sp. Tests with different growth media gave results that semi-quantitative, nutrient growth media produced the highest activity with a proteolytic index value of 2.09 ± 0.41 . In addition, based on quantitative tests, the media Luria Bertani Broth produced the highest specific activity with a value of 36.479 U/mg. The conclusion of this study, *Bacillus* sp. from the gastrointestinal tract of catfish that cultured on the Luria Bertani Broth medium produced the best activity.

INTRODUCTION

Protease is a proteolytic enzyme that catalyzes the breakdown of peptide bonds in proteins. Application of protease enzymes, in the detergent making industry, the leather tanning industry, food additives industry, and therapeutic substances in the pharmaceutical field (Gupta *et al.*, 2002). Protease can be produced by plants, animals, and microorganisms. The use of plants as a source of protease is limited by the availability of arable land, suitable growth conditions, and requires a long time to

produce enzymes. Protease production from animals is also limited by the availability of enzyme-producing animals (Poernomo *et al.*, 2017).

Microorganisms are the most potent source of enzymes compared to plants and animals. The use of microorganisms is more beneficial because of their rapid growth, can grow on inexpensive substrates, more efficiently, the results are improved through the regulation of growth conditions and genetic engineering (Efendi *et al.*, 2017).

Proteolytic bacteria can be found in the digestive tract of fish. In this study, catfish is used because it is a carnivore fish so that it is assumed the gastrointestinal tract is rich in proteolytic bacteria. Bacteria found in the digestive tract of fish include *Citrobacter* sp., and *Bacillus* sp. (Artika, 2010). Bacteria that produce extracellular enzymes can be obtained from the digestive tract (stomach, hepatopancreas, and intestine) and aquaculture pond sediments (Kurniawati, 2015). In the digestive tract, there are proteases and other digestive enzymes that catalyze the breakdown of complex nutrients (protein, carbohydrates, and fats) into simple nutrients. This study aimed to obtain candidates of bacterial proteolytic isolates from the gastrointestinal tract of catfish and determine the effect of different production media on the activity of proteolytic enzymes.

METHODOLOGY

Place and Time

This research was conducted from October to December 2019 in the Faculty of Fisheries and Marine Science, Brawijaya University, Malang, East Java, Indonesia.

Research Materials

The materials used in this research were Catfish (*Clarias* sp.) obtained from local fish farmers in Dinoyo, Malang, East Java, Luria Bertani Broth (LBB) and Luria Bertani Agar (LBA), Skimmed Milk Agar (SMA), Nutrient Broth (NB), Trypticase Soy Broth (TSB).

The main equipment used were shaker incubator (Memmert, USA), UV-VIS spectrophotometer (Thermo Scientific, USA), Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA).

Research Design

The experimental design was explorative using descriptive. Only for the experimental on the effect of media on the enzyme production and activity, the

Complete Randomized Design (CRD) was applied. The treatment used were different mediums namely LBB, NA, TSB, and SMB for the production of protease.

Work Procedure

Isolation of Proteolytic Bacteria

The method for bacterial isolation followed Sivasubramanian *et al.* (2012) with modification. The stomach and intestine were obtained from catfish. One-gram sample added to 9 ml NaCl 0.9% sterile and homogenized using vortex. Samples with proper dilution were plated on LB Agar. This culture was then incubated at 37 °C for 24 hours until the bacterial colony grew. The pure colony was isolated based on differences in color, shape, and size.

Isolate Identification

The identification of the isolates was using Microbact™ system. The identification of the isolate based on biochemical characteristics namely Methyl Red (MR) test, Indole test, Voges Proskauer test (VP), Catalase test, and Citrate test for genus identification.

Effect of Media on Enzyme Production

Based on isolates identification, each bacterial isolate that had proteolytic activity was grown on LBB, NB, TSB, and SMB media. The isolates were incubated in a 120-rpm incubator shaker for 24 hours at temperature 37 °C. Cell-free extracts were harvested using centrifugation at 10,000 rpm for 10 min.

Protease Assay with Semi-quantitative Method

Qualitative proteolytic activity was characterized by the presence of clear zones around isolates grown on skim milk agar media. The casein hydrolysis test was carried out by preparing skim milk agar media containing 2% casein in a petri dish. The culture was incubated at 37 °C for 24 hours. Positive isolates produced proteases characterized by the formation

of clear zones around the colony (Mazzucotelli *et al.*, 2013). Clear zone indicated the length of clear zone diameter which was measured by caliper. Meanwhile, clear zone index was the result of the clear zone diameter minus bacterial colony diameter. Proteolytic index (PI) indicated the result of diameter of clear zone minus diameter of well.

Protease Assay with Quantitative Method

Protease assay has followed the Method of Prihanto *et al.* (2016) with modification. A total of 0.5 ml of H₂O was mixed with 0.5 ml of casein 1.5% and put in a 30 °C temperature water bath for 5 minutes. Then 0.5 ml of sample was added to the mixture; then, the mixture was put in the water bath again at 30 °C for 10 minutes. After that, 1.5 ml of TCA was added to the mixture and centrifuged at 2,000 rpm for 10 minutes. Supernatant from the mixture was taken as much as 1 ml and then added a solution of 2.5 ml of 0.55M Na₂CO₃ solution and 0.5 ml of Folin reagent. The supernatant was put into a water bath at 30 °C for 30 min.

Quantitative protease activity can be measured based on the liberated tyrosine on the reaction. The tyrosine on the reaction was read with an absorbance of 660 nm.

Protein Calculation

The amount of protein was calculated by the spectrophotometric method using Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). The absorbance was measured at 280 nm.

Data Analysis

Analysis of variance (ANOVA) was used for analyzing the significance of the data result. Further, Duncan's test was used for analyzing the differences among treatments.

RESULTS AND DISCUSSION

Proteolytic Bacteria

In this study, nineteen isolates of proteolytic bacteria from the digestive tract of catfish (*Clarias* sp.) with different clear zone diameters were shown in Table 1.

Table 1. Positive proteolytic bacteria.

Isolates	Clear Zone (mm)	Clear Zone Index
1	19.29	2.25
2	9.75	1.15
3	2.75	0.32
4	11.75	1.38
5	4.00	0.47
6	5.25	0.62
7	0.75	0.09
8	16.25	1.91
9	7.00	0.82
10	2.01	0.25
11	0.25	0.03
12	4.75	0.56
13	8.01	0.96
14	14.5	1.71
15	2.25	0.26
16	2.25	0.3
17	7.25	0.85
18	1.75	0.21
19	0.25	0.03

It was found that isolate code 1 was the isolate with the highest ability to

produce the proteolytic enzyme. The clear zone was the primary selection for

determining the bacteria which able to produce the proteolytic enzyme. Only the highest clear zone index was checked for its capability for protease production. Hence, the rest of the isolates was not further explored. The clear zone indicated the bacteria had the ability to hydrolyze protein into simple protein such as amino acids.

Positive isolates that produced proteases form clear zones around the colonies in the media. It designated that bacteria used skim milk in the medium as their nutrient. According to Kurniasih *et al.*

(2013), the width of the hydrolysis zone can be used as a primary reference in the selection of proteolytic bacteria, this indicated the ability of a bacterium to utilize proteins by hydrolyzing proteins into amino acids.

Isolate Species

Based on the Microbact™ result, it was indicated that the isolates were Gram-positive and rod bacteria (Table 2) and it has similarities with groups of *Bacillus sp.*

Table 2. Morphology and biochemical characteristic of isolate no 1.

Parameter	Isolate 1	<i>Bacillus sp.</i>
Form	Rod	Rod
Gram	+	+
Methyl Red	+	+
Indol	-	-
VP	+	+
Catalase	+	+
Citric acid	+	+
Endospore	+	+

Bacillus sp., characterized by its ability to form endospores, gram-positive, movable due to the presence of peritrichous flagellum, it can be aerobic or facultative anaerobic and positive catalase or cylindrical, which is formed in vegetative cells. The endospores distinguished *Bacillus sp.* from the types of bacteria that made up the exospore. Endospores produced by *Bacillus sp.* had a high resistance to chemical and physical factors, such as extreme temperatures (Hatmanti, 2000).

Semi-Quantitative Protease Assay

The magnitude of proteolytic enzyme activity is indicated by the widening of the clear zone. Based on quantitative testing different proteolytic indices were obtained (Figure 1). The results showed that the media had different Proteolytic Index (PI) values. The highest yield was shown in the Nutrient Broth medium by showing the largest diameter of the proteolytic index (2.08 ± 0.41 mm). The lowest PI was shown in the Luria Bertani media with a yield of 1.56 ± 0.13 mm.

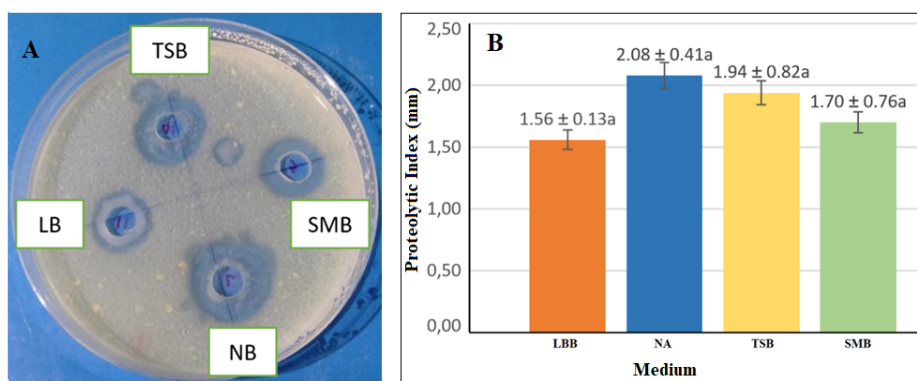


Figure 1. Proteolytic index of Isolate 1. in different mediums. A. well diffusion method. B. graphic of proteolytic index. LBB is in orange; NA is in blue; TSB is in yellow; SMB is in green color.

Quantitative Protease Assay

The results of protease activity showed that total proteolytic activity, protein content, and specific activities were different in growth media (Table 3). The best treatment of proteolytic activity

and protein content was shown in Tryptic Soy Broth media with values of 28.119 (U/ml) and 1.172 (mg/ml), respectively. As for the specific activity of the best treatment shown on Luria Bertani Broth media with a value of 36.479 (U/mg).

Table 3. Proteolytic activity of *Bacillus* sp. on different growth medium.

Media	Total activity (U/ml)	Protein (mg/ml)	Specific activity(U/mg)
LB	14.357±0,67a	0.394±0,03a	36.479±0,96a
NB	17.385±0,71b	0.928±0,07b	18.783±0,85b
TSB	28.119±0,67b	1.172±0,07c	24.035±0,89c
SMB	18.286±0,83c	1.142±0,10c	16.061±0,72d

In protease production was influenced by where the isolates came from, the incubation time and temperature of the inoculum, the pH of the buffer and the media used, the composition and concentration of the substrate which appropriate for the growth of *Bacillus* sp. It is consistent with Rosnawita *et al.* (2015) that the production of proteases has increased in line with the increase of incubation temperature, the reaching of optimum temperature for protease production, and the decreasing of enzyme catalytic rate.

The value of enzyme activity will increase in line with the addition of the substrate, but it will not increase the activity of the enzyme, because the enzyme reached its saturation point (Sutrisno, 2017).

The difference in this result revealed that the media influenced the activity and enzyme production. *Bacillus* sp. was able

to live and produce protease enzymes in each growth medium. However, the difference in output can be influenced by the composition of the growth media used. *Bacillus* sp. will produce more enzymes if the growth media have nutritional compositions that correspond with their metabolic needs. It seemed that the medium affected the production of the protease as a specific bacterial species basis.

CONCLUSION

Bacillus sp., which was isolated from the digestive tract of catfish (*Clarias* sp), was the best isolate in producing the enzyme protease among 19 isolates. The production of enzymes using Tryptic Soy Broth produced the highest proteolytic activity.

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