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THE ACTIVITY ASSAY OF PROTEASE, CELLULASE, AMYLASE, XYLANASE AND MANNANASE FROM *BACILLUS* SP. AS A CANDIDATE OF PROBIOTICS

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ABSTRACT

The purpose of this study was determineted the potential for five strains of *Bacillus* sp. KPP212, *Bacillus* sp IP121, *Bacillus* sp UJ131, *Bacillus* sp UJ132, and *Bacillus* sp SB141 isolated from the mangrove forest ecosystem. The bacteria were selected to produce hydrolase enzymes (proteases, cellulases, amylases, xylanases, and mannanase s). Enzyme production was also carried out on production media with shrimp and fish feed as main substrate. The method consisted of assay of selection and enzyme activity. The data analyzed descriptively. The research showed there are four strains that produce protease and xylanase enzyme namely *Bacillus* sp. KPP212, *Bacillus* sp. IP121, *Bacillus* sp. UJ131, and *Bacillus* sp. UJ132. The highest protease enzyme activity is *Bacillus* sp UJ131 i.e. 0.07 U ml^{-1}. The highest xylanase enzyme is *Bacillus* sp UJ131 i.e. 0.05 U ml^{-1}. *Bacillus* sp. UJ132 is a bacteria that produced three kind of enzyme with the highest cellulase activity of 0.06 U ml^{-1}. The five bacteria did not produce amylase and mannanase. The production of enzyme from shrimp feeds medium yielded protease activity of 0.05 U ml^{-1}, xylanase activity of 0.2 U ml^{-1}, and cellulase activity of 0.07 U ml^{-1}. While, enzyme activity from fish feeds medium as follows: protease: 0.08 U ml^{-1} xylanase: 0.31 U ml^{-1}; cellulase: 0.15 U ml^{-1}. While, production of enzyme from fish feeds medium yielded protease activity i.e. 0.08 U ml^{-1}; xylanase activity i.e. 0.31 U ml^{-1}; and cellulase activity i.e. 0.15 U ml^{-1}.

KEYWORDS: *Bacillus* sp, enzyme, probiotics, and shrimp feed.

I. INTRODUCTION

The used of microbial probiotics in the shrimp farming sector have become a popular thing at the moment. Microbial probiotics were benefits of the host by increasing the absorption rate of nutrient value of feed, and improve host immune response to a disease and improve the quality of the surrounding environment.[1]

One common type of probiotic bacteria is *Bacillus* sp. Some *Bacillus* has been shown to suppress the population of *Vibrio* bacteria.[2] *Bacillus* sp. control the pathogen bacteria by producing antimicrobials in the stationary phase when the bacteria produced secondary metabolites.[3] This is also supported by the results of else the study that stating the bacteria *Bacillus* sp. produce immunity and antimicrobials such as bacteriocin.[4] Other researchers also concluded that *Shewanella algae* was as candidate probiotic of juvenile *Penaeus monodon* to antagonistic against *Vibrio harveyi*, *V. parahaemolyticus* and *V. alginolyticus*.[5]

The ability of bacteria in producing enzymes is useful to increase chemical reactions of 10^8 to 10^{11} times by decreasing the energy of activation.[6] The ability of bacteria in producing hydrolase enzyme will help the process of breaking the nutrients contained in the feed thus absorption of nutritional value of animals can occur optimally.

Based on the description earlier, efforts should be made to solve the problems found in the aquaculture sector. Therefore, it was needed to select of microbial ability in producing hydrolase enzymes; proteases, cellulases, amylases, xylanases and mannanases. This study was conducted to determine the ability of bacteria *Bacillus* sp. where will be candidates for probiotics to help shrimp digestibility and increase competition in bacterial cell growth.

II. MATERIALS AND METHODS

The material used in this research is *Bacillus* sp. which is a collection of the Laboratory of Microbiology FMIPA University of Lampung. Five strains of probiotic *Bacillus* sp. (KPP212, IP121, UJ131, UJ132, and SB141) isolated from mangrove ecosystems, shrimp feed and artificial fish feed, Alcohol, Physiological Salt, Skim Milk,

Carboxmethyl Cellulose (CMC), NaCl, H_2O, Starch, Casein, Folin Reagent, Buffer Phosphate, Buffer Citrate, Tricholoroacetic Acid (TCA), Na_2CO_3, Standard Tyrosine, Dinitro Salicylic Acid (DNS), Xilan, Locust Bean Gum, Pepton, Beef Extract, Na_2CO_3, Sea Water Complete (SWC), NaCl, Congo red, Spiritus, Lugol, and Distilled water.

Selection of proteolytic bacteria. Bacillus sp. was grown on the Sea Water Complete (SWC)solid medium with the composition: bacto peptone 0.5 g, 0.1 g yeast extract, 0.3 ml glycerol, 75 ml of seawater, 25 ml distilled water, and 1.5 g agar + 2 grams of skim milk. Inoculated bacteria are incubated for 24 hours, proteolytic activity is characterized by the formation of clear zones around the colony.[7]

Selection of cellulotic bacteria. Bacillus sp. was grown on 100 ml of Sea Water Complete (SWC) medium + 2 grams of CMC (Carboxmethyl cellulose). The growing colonies were stained with 1% congo red when it was 24 hours old. After that it washed with 10% NaCl solution. Clear zones were made of around the colonies indicate the existence of cellulotic activity.[8]

Selection of amylolytic bacteria. Bacillus sp. was grown on 100 ml media Sea Water Complete (SWC) solid + 2 grams of starch. After the media was incubated for 24 hours, the growing colony was stained with iodine solution. Clear zones were made of around the colony indicate the existence of amilolytic activity.[9,10]

Selection of xylanolytic bacteria. Bacillus sp. was grown on 100 ml media of Sea Water Complete (SWC) + 0.5 gram of Xylan beechwood, after incubation for 24 hours, the growing colonies were stained with 1% congo red and then washed with 10% NaCl solution. Clear zones were made of around the colony signify the existence of xylanolytic activity.[8]

Selection of mannanolytic bacteria. Bacillus sp. was grown on 100 ml media of Sea Water Complete (SWC) + 2 grams Locust bean gum, after incubation for 24 hours, growing colonies stained with congo red 1% then washed with 10% NaCl solution. Clear zones were made of around colonies indicate mannanolytic activity around the colony.[8]

Calculation of enzymatic index. The result of enzyme selective test is calculated by enzymatic index with the following formula.[11]

$$IP = \frac{diameters\ of\ clear\ zone - diameters\ of\ colony}{diameters\ of\ colony}$$

Preparation of bacterial starter. Strains of *Bacillus* sp. which has been regenerated on the SWC medium taken 1 ose and inoculated into 250 ml erlenmeyer containing 50 ml of SWC media. The bacteria starter medium was incubated for 24 hours of the orbital shaker at room temperature at 120 rpm.

Preparation of crude extract of the enzyme. Bacteria *Bacillus* sp. which has been incubated for 24 hours is taken as 5 ml, then inoculated into each erlenmeyer containing SWC media 45 ml + each of the substrate for the five enzymes (skim milk CMC, starch, xylan and Locust bean gum). The bacterial-inoculated medium was incubated above the orbital shaker at a rate of 120 rpms for 24 hours. Then, production media in centrifuge with a temperature of 4^o C with a speed of 10000 rpms for 15 minutes. The supernatant formed are the crude extract of the enzyme.

Activity assay of protease enzyme. The crude extracts protease enzyme were assay by making 3 series of test tubes containing the blank, standard, and sample solutions. The assay step is 0.5 ml of 2 mM phosphate buffer + casein is added into the three series of tubes. For the blank tube was added aquades 0.1 ml, the standard tube was added 0.1 ml standard tyrosine, and in the sample tube are added 0.1 ml of crude extract of enzyme. All test tubes were incubated at 25°C for 10 minutes. After incubation, TCA is added into all 0.5 ml tube series. For the blank and standard tubes were added a crude extract from enzyme as much as 0.1 ml, then on the sample tube is added aquades as much as 0.1 ml. All series of solutions were centrifuged at 4 ° C. The supernatant was taken 0.375 ml then added by Na_2CO_3 and folin reagents each of them 1.25 ml and 0.25 ml. After that it was incubated for 20 minutes, then read the absorbance at 578 nm wavelengths.

Activity Assay of cellulase enzyme, xylanase and mannanase

The activity of cellulase enzyme was assayed using DNS (Dinitro-salicylic acid) method by quantification of reducing sugars released from CMC solubilised in 0.05M phosphate buffer pH 7.0. Crude enzyme was added to 1% CMC in 0.05 M phosphate buffer and incubated at 25^oC for 30 minutes. The reaction was stopped by addition of 1 ml of DNS reagent in the reaction mixture. Then the mixture was boiled in water at 100^oC for 15 minutes. Reduction sugars liberated were determined by measuring absorbance at 575 nm spectrophotometer. One international unit (IU) of cellulase enzyme activity refers to the amount of enzyme that released 1 µM of glucose per ml of the sample per minute under assay conditions. The same method was also performed on the mannanase and xylanase enzyme assays. However, xylanase assay use xylan and mannanase assay use locust bean gum as substrate of enzyme

Production of enzymes used shrimp and fish feed as substrate. The fifth starter of *Bacillus* sp. which has been incubated for 24 hours is taken as much as 5 ml, then inserted the starter into elenmeyer, each erlenmeyer containing media SWC 45 ml + shrimp feeds substrate as much as 1% of media volume. The culture of SWC

media was incubated on the orbital shaker at 120 rpms for 24 hours. After finish, the culture centrifuged with a temperature of 4^{o} C with a speed of 10000 rpms for 15 minutes.

The supernatant are the crude extract of the enzyme. The production process of enzyme use fish feeds substrate is done same with step earlier. The same enzyme assay was also done with substrate of shrimp feed.

III. RESULTS AND DISCUSSIONS

Base of the proteolytic and xylanolytic assay, there is 4 bacteria *Bacillus* sp that shows enzyme activity marked by the formation of clear zone around the colony. The four bacteria are *Bacillus* sp. KPP212, *Bacillus* sp. IP121, *Bacillus* sp. UJ131, and *Bacillus* sp. UJ132 (Figures 1 and 2). In cellulolytic assay media, there is only 1 bacterium that produces cellulolytic activity namely *Bacillus* sp. UJ132 (Figure 3). While, the amilolytic and mannanolytic assay media, all the bacteria did not produce clear zones (Figures 4 and 5). The results of this selective assay of enzymatic activity will be used to determine the isolates to be assayed for their enzyme activities. *Bacillus* sp produced hydrolase enzyme namely protease, xylanase, cellulase, amylase, and mannanase (the following figure).

Figure 1. Signs ↙ produce protease enzymes

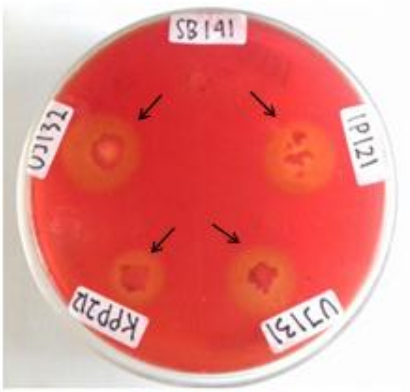

Figure 2. Signs ↙ produce xylananase enzymes

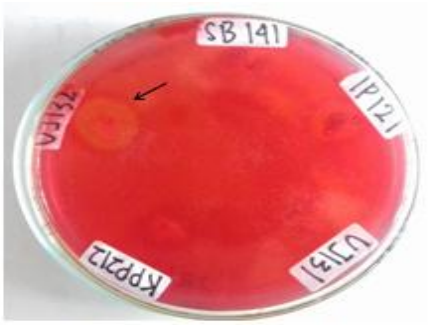

Figure 3. Signs ↙ produce cellulase enzymes

Figure 4. No isolates produced amilase enzyme

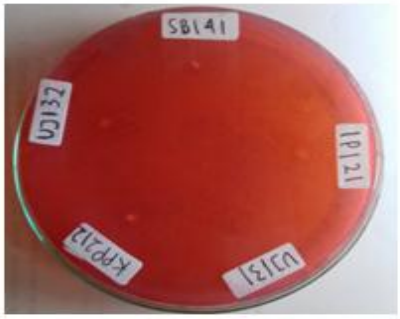

Figure 5. No isolated produced mannanase enzyme.

The enzymatic activity produced on each strain of *Bacillus* sp. is different. The largest proteolytic index was *Bacillus* sp. KPP212 with proteolytic index of 0.06. Xylanolytic activity in *Bacillus* sp. UJ131 produces an index of 0.07 which is also fairly high. While the highest cellulolytic index was produced by *Bacillus* sp. UJ132 of 0.03. From the results of qualitative assay is known that there will only be four bacteria that assayed the activity of enzymes with liquid media. The four bacteria are *Bacillus* sp. KPP212, *Bacillus* sp. IP121, *Bacillus* sp. UJ131, and *Bacillus* sp. UJ132. *Bacillus* sp. SB141 was not assayed for its enzyme activity because it did not show positive result of selective assay (Table 1).

Table 1: Proteolytic, cellulolytic and Xylanolytic Index.

Bacteria	Origin of isolates	Index value				
		Proteolytic	Xylanolytic	cellulolytic	Amylolytic	Mannanolytic
Bacillus sp. KPP212	Krab	0.06 ± 0.02	0.06 ± 0.01	0 ± 0	0 ±0	0 ±0
Bacillus sp. IP121	Fish	0.02 ± 0.01	0.03 ± 0.01	0 ± 0	0 ±0	0 ±0
Bacillus sp. UJ131	Shrimp	0.05 ± 0.01	0.07 ± 0.01	0 ± 0	0 ±0	0 ±0
Bacillus sp. UJ132	Shrimp	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0 ±0	0 ±0
Bacillus sp. SB141	Snail	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

The production of protease and xylanase enzyme used bacteria i.e. *Bacillus* sp. KPP212, *Bacillus* sp. IP121, *Bacillus* sp. UJ131 and *Bacillus* sp UJ132 showed different result (Table 2 and Table 4). While, the cellulase enzyme was produced by *Bacillus* sp. UJ132 (Table 3). Based on data activity of protease enzyme has the higgest activity from *Bacillus* sp. UJ131 of 0.06 U ml^{-1} at all production medium (Table 2).

Table 2: The production of protease enzyme from *Bacillus* with various substrates.

Bacteria	Origin of isolates	Activity of protease (U ml^{-1})		
		skim milk medium	Shrimp feed medium	Fish feed medium
Bacillus sp. KPP212	Krab	0.05 ± 0.01	0.03 ± 0.01	0.06 ± 0.01
Bacillus sp. IP121	Fish	0.04 ± 0.01	0.02 ± 0.01	0.05 ± 0.02
Bacillus sp. UJ131	Shrimp	0.07 ± 0.06	0.05 ± 0.01	0.08 ± 0.08
Bacillus sp. UJ132	Shrimp	0.05 ± 0.02	0.04 ± 0.02	0.06 ± 0.03

The assay of xylanase activity showed that the highest activity was produced from *Bacillus* sp. UJI131 namely 0.15 U ml^{-1} (Table 3).

Table 3: The production of xylanase enzyme from *Bacillus* with various substrates.

Bacteria	Origin of isolates	Activity of Xilanase (U ml^{-1})		
		Xilan medium	Shrimp feed medium	Fish feed medium
Bacillus sp. KPP212	Krab	0.07 ± 0.02	0.08 ± 0.03	0.18 ± 0.03
Bacillus sp. IP121	Fish	0.02 ± 0.02	0.05 ± 0.05	0.09 ± 0.01
Bacillus sp. UJ131	Shrimp	0.15 ± 0.03	0.20 ± 0.04	0.31 ± 0.04
Bacillus sp. UJ132	Shrimp	0.10 ± 0.04	0.20 ± 0.04	0.23 ± 0.04

The assay of cellulase activity showed that only *Bacillus* sp. UJI132 produced cellulose. Fish feeds medium was best substrate to cellulase production i.e. 0.15 U ml^{-1} (Table 4).

Table 4: The production of cellulase enzyme from *Bacillus* with various substrates.

Bacteria	Origin of isolates	Activity of cellulase (U ml^{-1})		
		CMC medium	Shrimp feed medium	Fish feed medium
Bacillus sp. UJ132	Shrimp	0.06 ± 0.03	0.07 ± 0.07	0.15 ± 0.10

The results showed that the enzyme of protease and xylanase were produced by bacteria i.e : *Bacillus* sp. KPP212, Bacillus sp. IP121, Bacillus sp. UJ131, and Bacillus sp.

UJ132. Whereas, cellulolytic only produced by *Bacillus* sp. UJ132.

It is signed by the appear of clear zones around the colonies of *Bacillus* . So, *Bacillus* sp. capable of hydrolyzing the substrate contained in the growing medium.

The xylan backbone was composed a xylose which it was bound by the β-1,4-glycosidic bond. The main

monomers in most xylan are D-xylose, D-mannose, D-galactose, and L-arabinose.[12]

When, the xylanolytic, cellulolytic, and mannanolytic assay are done, the red color is formed from the xylan substrate having the β-1.4 glycosidic bond attached to the congo red. Degradation of β-1,4 glycosidic bonds to form new molecules in the form of xylose, glucose, and mannose cause congo red no joined β-1,4 glycosidic. So, the media around of the colony of bacteria made clear zones.[13] On the other hand, a clear zone made on the SWC + skim milk medium occurs when the skim substrate contains a cloudy white casein protein. Casein protein is degraded by protease to peptides and amino acids. So, the media around the colony will develop clear zones.[14]

Based on the selective assay, there is one isolate from *Bacillus* sp. SB141 which showed no activity in all hydrolase enzymes. It is known that the bacteria do not produce amylase and mannanase enzymes. It does not show clear zones around the Bacillus sp. In the amilolytic assay, α-1,4 glucosidic bonds of starch do not degrade into glucose, maltose and dextrin molecules. So, it was the blue colors when iodine is bound to amilum on the media and no clear zones are formed [15]. It based on data onto assay of selective and hydrolase enzyme activity, *Bacillus* sp. KPP212 can be used as probiotics to improve nutrient absorption of feed in aquaculture. This is similar with else research that the use of probiotics containing proteolytic bacteria producing protease enzymes can improve the normal microflora in the intestine. The bacteria can improve fish growth.[16]

The bacteria can improve fish growth. *Bacillus* sp. KPP212 can use to degrade pollution in pond bottom and mangrove. *Bacillus* sp. UJ132 producing cellulase helps to degrade fiber of feed of the nutrients. The cellulase capable of hydrolyzing cellulose into glucose that it can be well absorbed by host cell also.[17] The use of *Bacillus* sp. KPP212 and *Bacillus* sp. UJ132 can increase productivity of the host animal by optimizing of nutrition absorption and increasing their metabolism.

IV. CONCLUSION

The research is known that from 5 isolates of *Bacillus* sp. there are 4 *Bacillus* that produced protease and xylanase enzyme i.e. *Bacillus* sp. KPP212, *Bacillus* sp. IP121, *Bacillus* sp. UJ131, and *Bacillus* sp. UJ132. *Bacillus* sp. UJ131 has the highest protease and xylanase activity. *Bacillus* sp. UJ132 produces three enzymes: protease, cellulases and xylanases enzymes. The fish feed substrate is best substrate to produce many hydrolase enzyme.

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