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Microflora Contribution to Cellulase and Digestion Enzyme in Mud Crab Digestive Tract

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Abstract: Microflora in the digestive tract of aquatic animals is thought to be not only the microbes that contribute to the production of cellulase enzymes but also contribute to other digestive enzymes, such as protease, amylase and lipase. This study aims to examine the microflora contribution to the mud crab's digestive tract. Scylla sp. were maintained in the crab box individually and placed in a pond. Two groups of crabs were fed without antibiotics and containing antibiotics at a dose of 100 IU mL^{-1} penicillin G and 100 IU mL^{-1} streptomycin, respectively. Each treatment had two replications. Crabs were kept for eight days and fed 5% of body weight per day in the afternoon. The results showed that the cellulitis, amylolytic, proteolytic and lipolytic microbial population in the digestive tract of mud crabs that received feed added with antibiotics decreased significantly compared to those feed without antibiotics. Furthermore, the α-amylase, protease and lipase enzyme activity also decreased. The decrease in cellulase activity, α-amylase, protease and lipase enzymes were 89.55, 41.90, 26.50 and 37.26%, respectively. The decreasing percentage of cellulase, α-amylase, protease and lipase enzymes indicated a significant microflora contribution in the mud crab digestive tract.

Keywords: Mud Crab, Microflora, Cellulitis, Proteolytic, Amylolytic, Lipolytic

Introduction

The indigenous microflora of fish in aquaculture has previously been studied for many purposes, including descriptions of microbial spoilage, the correlation between environment and fish microflora (Horsley, 1973), the monitoring of changes in fish farms (Allen *et al*., 1983), the nutritional role of the intestinal flora (Hansen *et al*., 1992) and the antibiotic resistance profile of the indigenous flora (Spanggaard *et al*., 2000).

It is generally recognized that the intestinal flora of endothermic animals serves both as a digestive function and as a protective barrier against disease (Sissons, 1989). The intestinal flora of fish has, as a consequence, received much attention from several authors (Ringø *et al*., 1995; Sugita *et al*., 1997). The composition of the intestinal microflora in fish is influenced by, or directly derived from, the microflora of the food (Grisez *et al*., 1997). Fish with abundant and diverse microflora have considerable opportunities to adapt to changing nutritional substrates, assimilate food better and enhance their adaptive possibilities (Al-Harbi and Uddin, 2005).

The main component in animal feed is protein, carbohydrate and fat. To increase absorption and circulation throughout the body through the circulatory system required a simplification process into smaller molecules. According to (Bakke *et al*., 2010), a chemical feed simplification process is also called hydrolysis, involving digestive enzymes as biological catalysts. The main feed components in protein, fat and carbohydrates are broken down into simple compounds, which are the constituent components. Steinberg (2017) explained that the hydrolysis of macronutrients into micronutrients in the digestive system was occurred due to the presence of digestive enzymes, namely protease, amylase, carbohydrase, lipase and stomach acid. Those compounds are produced by the stomach, intestines, liver and pancreas. In addition, several researchers reported that there were cellulase enzyme activities in several species of aquatic animals. The presence of the cellulase enzyme is closely related to the microflora in the digestive tract (Bui and Lee, 2015; Xue *et al*., 1999). The discovery of the enzyme cellulase in the digestive tract of aquatic animals allows these animals to digest feed containing fiber.

Previous studies showed that in addition to endogenous digestive enzymes, exogenous digestive enzymes are also found from microflora that lives in mutualism symbiosis with aquatic animals in their digestive tract (Das *et al*., 2014; Ganguly and Prasad, 2012; Pond *et al*., 2006; Xue *et al*., 1999). Microflora in the digestive tract of aquatic animals is thought to be the microbes that contribute to the production of cellulase enzymes and contribute to other digestive enzymes, such as protease, amylase and lipase. In fish, the intestinal microflora has been regarded as fulfilling several roles. A nutritional function has been suggested, in which bacteria break down ingested foods into individual components such as vitamins or amino acids (Pond *et al*., 2006). However, there is a lack of study conducted related to microflora activities in the mud crab digestive tract. This experiment was carried out with reference to the experiments that had been conducted by previous researchers. The aim of study was to examine the microflora contribution in the mud crab's digestive tract., namely in contributing of cellulase enzymes and digestive enzymes including α-amylase, protease and exogenous lipase.

Materials and Methods

The experiment was conducted at Education Pond, Universitas Hasanuddin, Barru Regency, on May-June 2020. We only conducted the experiment for two months due to limited time and other sources of research, including funds and labor. Crab feed making, analysis for microbial population and enzyme activity were conducted at Biotechnology Fisheries and Marine Laboratory, Center for Research Activities, Universitas Hasanuddin.

The number of mud crab (Scylla spp.) samples were 60 crabs. There were two treatments and three replicates for each treatment. There were 30 crabs for each treatment and replicate. The initial crab weight was 95.56±2.87 g and carapace width was 8.69±2.95 mm. Crab samples were obtained from crab fishers and local crab suppliers. Before the experiment started, crab samples were acclimated to the pond for one week. After the acclimatization process, crabs were fasted for 24 h. The fasting process for crab samples was removing the remaining feed in the crab body. Before treatment, the crab was weighted for recorded an initial body weight using electric balance. Carapace width was measured using a caliper. Crab samples were then tagged in carapace dorsal using a marker for easy-to-do observation. After that, the crab was put into the crab box.

The crab was growing out individually into the crab box (the dimension of the box: 21×15×8 cm). Crabs box was put into polyethylene pipa and set on the surface pond with the depth of pond was ±100 cm. The water replacement was done daily following daily high and low tide (minimal 10% of water replacement). Water quality parameters (temperature, salinity, dissolved oxygen and pH) were measured daily.

The experiment method was referred to (Xue *et al*., 1999; Aslamyah, 2006). Crab samples were divided into two groups, namely crab fed with feed with antibiotic penicillin G with the dose of 100 and 100 IU mL^{-1} streptomycin kg^{-1} feed and crab samples fed with feed without antibiotic. The crab was fed twice a day. The crab was maintained in the crab box for eight days and fed as an amount of 5% of total body weight. We used pellet form for crab feed, with the nutritional contents of the feed, namely protein 41.93%, Nitrogen Free Extract/NFE 29,33%, fiber 7.82%, fat 7.43% and Digestible Energy/DE 2767.63 kcal/kg.

Parameters that were measured in this study were microbial population, cellulose enzyme and digestive enzyme activity that were conducted at the end of the experiment.

Measurement Microbial Population

The method for measuring the microbial population of cellulitis, amylolytic, proteolytic and lipolytic referred to (Aslamyah, 2006) as the detailed method: Measurement of the microbial population begins with preparing the source of the inoculum from the digestive tract of the crabs. Digestive tract samples were crushed and every 10 g of the sample was diluted with 90 mL of sterile physiological solution (0.85% NaCl). The inoculum source of 0.5 mL was inoculated into 10 mL of standard liquid media, namely Trypticase Soy Broth (TSB, Merck), which added 1% NaCl and cellulose for cellulitis, casein as an energy source for proteolytic, TSB plus 1% NaCl and starch for amylolytic and TSB plus 1% NaCl and fish oil for lipolytic. The culture was then incubated at 29°C for 24 h. Serial dilution is carried out from 10^{-2} to 10^{-10} by taking 0.05 mL from the microbial culture in liquid media and putting it in 4.95 mL of the first diluent medium, then 0.05 mL of the first diluent medium is taken and put into in 4.95 mL of second diluent medium and so on until the last diluting medium. Each dilution series is transferred as much as 0.1 mL into solid media, which consists of a mixture of TSB, 1% NaCl, agar and its energy source. This preparation was incubated again at 29°C for 24 to 48 h. The microbial colonies that grew were counted in colony count (CFU/mL).

Measurement Enzyme Activity

Analysis for cellulase enzyme and digestive enzyme (α-amylase, protease and lipase) activity begins with preparing a crude enzyme extract which refers to (Aslamyah, 2006). Activities carried out at a temperature of 0 to 4°C with the aim of the enzyme in an inactive condition. Digestive tract samples with distilled water and dried with suction paper. Samples were taken as

much as 1 g and crushed with mortar until smooth and homogenized with 10 mL of cold distilled water, then centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was taken as crude enzyme extract and used as samples for enzyme activity testing.

Cellulase enzyme activity was determined using the DNS method (3,5-dinitrosalicylic acid). A total of 80 µL of crude enzyme extract plus 720 µL of 1% CMC solution substrate (in citrate phosphate buffer pH 7) was put into the Eppendorf tube and then incubated a water bath at 37°C for 60 min. The incubation results were added with 1200 µL of DNS reagent then put in a boiling water bath for 15 min. After that, put in an ice bath for 20 min. In this test, control was used in the form of 80 µL crude extract of the enzyme, which had been heated for 15 min, then added 720 µL of the substrate and 1200 µL of DNS reagent. The solution mixture is treated the same as the sample conditions above. After that, the reducing sugar formed was measured by a UV-Vis spectrophotometer at λ = 540 nm (Miller, 1959).

Measurement of protease activity was carried out based on the method of Bergmeyer and Grassi (1983). A total of 1 mL of 2% casein solution mixed with 1 mL of borate buffer (0.01 M) pH 8.0, 0.20 mL 0.05 M hydrochloric acid and 0.20 mL crude enzyme extract, which activity will be determined. Then incubated in a water bath at 37°C for 10 min, then added 2 mL of 0.1 M Trichloroacetic Acid (TCA). Incubated for 10 min, then centrifuged. The 1.5 mL portion of the filtrate was mixed with 5 mL of 0.4 M disodium carbonate and 1 mL of Folin Ciocalteu's reagent and let stand for 20 min. Then read the absorbance at a wavelength of 578 nm.

Determination of the amylase enzyme activity value was carried out by mixing 1 mL of the enzyme with 1% starch in 1 mL of 0.05 M citrate buffer pH 5.7. Then the incubation was carried out at 37°C for 30 min. The reaction was stopped by adding 2 mL of DNS (3.5 dinitro salicylic acid), then heated in boiling water for 5 min and, after chilling, centrifuged at 3,000 rpm for 5 min. Furthermore, reducing sugar can be measured using a spectrophotometer at a wavelength of 540 nm. One unit of enzyme activity is defined as the number of enzymes that produce 1 µmol of glucose/per minute

Analysis method for lipase enzyme activity (Tietz and Friedreck 1966; Borlongan, 1990), a stable lipase substrate (olive oil) 1.5 mL plus 1 mL Tris-HCl 0.1 M as a buffer with a pH of 8.0. Then add 1.0 mL of crude enzyme extract. The mixture was homogenized and incubated for 6 h at 37°C. The reaction is stopped by adding 3 mL of 95% ethyl alcohol. Titrate the sample with 0.01 N NaOH, using 0.9% (w/v) thymolphthalein in ethanol as an indicator. The same procedure is carried out on the blank. One lipase activity unit was defined as the volume of 0.05 N NaOH needed to neutralize the fatty acids released during 6 h of incubation with the substrate, after correction with blanks.

Data were analyzed descriptively using mean and standard deviation.

Results

The measurement results for microbial population (cellulitis, amylolytic, proteolytic and lipolytic CFU mL^{-1}) and enzyme activity (cellulase, α-amylase, protease, dan lipase IU g^{-1} $minute^{-1}$) was shown in Table 1 and 2. The Paired-Samples T results showed significant differences in the microbial population and enzyme activity between the two groups of tested crabs. Table 1 showed that there was decreasing significantly of the microbial population (cellulitis, amylolytic, proteolytic and lipolytic) in mud crab digestive tract who give a feed with antibiotic than those of feed without antibiotic. This trend also occurs for the enzymatic activity of cellulose, α-amylase, protease and lipase, as shown in Table 2. The decreasing of cellulose, α-amylase, protease and lipase accounted for 89.55, 41.90, 26.50 and 37.26%, respectively.

The regression analysis showed that the correlation between the microbial population and enzyme activity was linear with the regression equation: y = 1E-10× +10.424; R = 0.5252. Figure 1 showed that the increasing microbial population followed by increasing the enzyme activity.

Table 1: The microbial population of cellulitis, amylolytic, proteolytic and lipolytic (CFU mL^{-1}) in mud crab at the end of the experiment

		Microbial population (CFU mL^{-1})			
Treatment	Replicate	Cellulitis	Amylolytic	Proteolytic	Lipolytic
Feed without antibiotic	1	1.3×10^{11}	8.3×10^{10}	9.2×10^{10}	7.8×10^{10}
	2	9.6×10^{10}	8.7×10^{10}	9.8×10^{10}	5.5×10^{10}
	Mean	1.13×10^{11}	8.5×10^{10}	9.5×10^{10}	6.65×10^{10}
Feed with antibiotic	1	4.3×10^{3}	9.8×10^{4}	1.2×10^{5}	3.4×10^{4}
	2	3.9×10^{3}	6.8×10^{5}	4.2×10^{6}	8.2×10^{5}
	Mean	4.1×10^{3}	3.9×10^{5}	2.2×10^{6}	4.3×10^{5}

Table 2: Digestive enzyme activity of cellulase, α-amylase, protease and lipase (IU g^{-1} $minute^{-1}$) of mud crab at the end of the experiment

		Enzyme activity (IU g^{-1} $minute^{-1}$)			
Treatment	Replicate	Cellulose	α-Amylase	Protease	Lipase
Feed without antibiotic	1	24.360	18.550	32.580	15.360
	2	26.190	21.470	28.410	17.680
	Mean	25.275	20.010	30.495	16.520
Feed with antibiotic	1	4.700	13.650	25.610	9.600
	2	0.580	9.600	19.220	11.130
	Mean	2.640	11.625	22.415	10.365

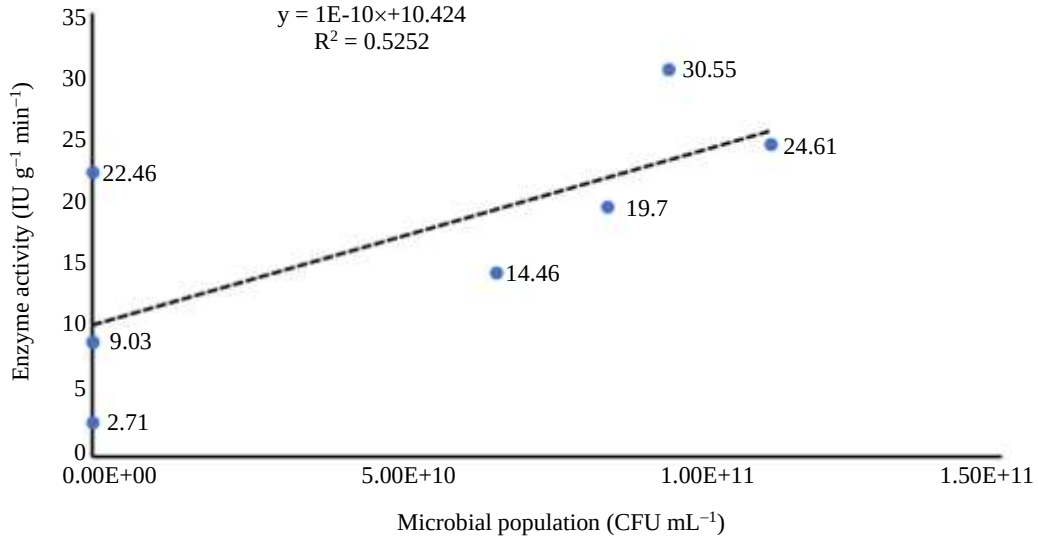

Fig. 1: The regression graph of microbial populations and enzyme activity in the digestive tract of mud crabs

Discussion

The investigation of the contribution of microflora in the digestive tract of mud crabs showed that cellulase enzymes in the digestive tract of mud crabs were thought to contribute to the microflora of the digestive tract. Our result showed that a high impact of the contribution of cellulase enzymes derived from cellulitis microbes accounting for 22.64 IU g^{-1} $minute^{-1}$ or 89.55%. The use of antibiotics to prove the role of microflora in the digestive tract of animals as reported by (Das and Tripathi, 1991; Xue *et al*., 1999). Tetracycline and penicillin are types of antibiotics that can kill gram-positive bacteria, while streptomycin is a type of antibiotic that can kill gram-negative bacteria. Das and Tripathi (1991) reported decreased cellulase activity when grass carp were fed with tetracyclines. *Cherac quadricarinatus*, which was fed with 100 IU mL^{-1} penicillin G. and 100 IU mL^{-1} streptomycin per kg of feed for eight days, showed a decrease in cellulase enzyme activity in the digestive tract by 40%, as well as a reduction of the bacterial population by 94% compared to controls (Xue *et al*., 1999). The presence of this cellulase enzyme enables the mud crab to digest feed fiber. According to (Allan and Fielder, 2003) digestibility of crabs on fiber and all vegetable feed raw materials is very high, ranging from 94.4-96.1%. Our result found a contribution of digestive enzymes α-amylase, protease and microbial lipase derived from microflora in the digestive tract of mud crabs. The contribution of amylolytic, proteolytic and lipolytic microbes to the enzymes of cellulase, α-amylase, protease and lipase in the digestive tract of mud crabs were 8.34, 8.08 and 6.16 IU g□1 minute□1. This finding indicated that in addition to the digestive enzymes α-amylase, protease and endogenous lipase secreted by the digestive tract and glands, there were also digestive enzymes α-amylase, protease and exogenous lipase originating from the microflora in the digestive tract. The contribution of fish digestive tract microflora was also reported by (Aslamyah, 2006). Microbial in the digestive tract of carp in the omnivore phase was higher than in the carnivore phase. The contribution of the digestive enzymes protease, α-amylase and microbial lipase in carnivorous gourami were 25.21, 25.22 and 18.27%, respectively. However, it was lower than the contribution

of the digestive enzymes protease, α-amylase and microbial lipase derived from microflora in the digestive tract of milkfish. In milkfish, the contribution of the digestive enzymes protease, α-amylase and microbial lipase were 36.12, 41.33 and 22.51% (Aslamyah, 2006). Lazado *et al.* (2012) reported GP21 (*Pseudomonas* sp.) And GP12 (*Psychrobacter* sp.), which was two bacteria isolated from the gastrointestinal tract of Atlantic cod, GP21 capable of producing amylase, chitinase, cellulase and protease, whereas GP12 can only produce chitinase and protease. Furthermore, GP21 and GP12, potential probiotic organisms that could support the digestion of Atlantic cod.

The microflora in the digestive tract was originated from detritus consumed by mud crabs. In general, the types of microbes found in the digestive tract of mud crabs were from cultivation media and or form pond sediments. Al-Harbi and Uddin (2005) stated a positive correlation between the composition of bacteria in the gills and digestive tract of fish with the composition of bacteria in water and sediment pond.

Detritus contains many microorganisms that play a role in contributing exogenous digestive enzymes to degrade feed nutrients consumed by mud crabs. These microorganisms are also a source of additional nutrients for mud crabs. This finding was in line with a previous study by (Xue *et al*., 1999), who found that the growth of several species of freshwater *Cherac quadricarinatus* reared in soil ponds was better than those reared in tank ponds. This difference was interpreted as *Cherac quadricarinatus* had an ability to obtain additional nutrients from the detritus material at the bottom of the pond, which is not found in the tank pond and/or utilized the presented microorganisms in detritus to support digestif activities.

The microflora was in the digestive tract lives in mutualism symbiosis with the host and is in balance, namely between beneficial microbes and pathogenic microbes. These microfloras also interact with each other between various microbial species in the digestive tract, both antagonistically and synergistically. The interactions that occurred are very important in maintaining the balance of the digestive tract microflora. It will have a positive effect on the host. Douillet and Langdon (1994) reported that on oyster cultivation, there was a high growth rate which was associated with the contribution of bacteria supplying (1) essential nutrients which were not found in some individuals in the algae population and (2) enzymes that could improve the digestive process of larvae. The microflora in the intestines of bivalve larvae was obtained in optimal proportions to produce extracellular enzymes such as proteases and lipases

The estimation of a relationship between the habit of water animals eating detritus and the presence of microflora in the digestive tract was also reported by (Xue *et al*., 1999). Liu *et al*. (2016) reported that the trophic level of the host affected the structure and composition of the intestinal microbiota, metabolic capacity and enzyme activity of the intestinal content. *Clostridium*, *Citrobacter* and *Leptotrichia* cellulose-degrading bacteria were dominant in herbivores, while *Cetobacterium* and *Halomonas* protease-producing bacteria were dominant in carnivores. In addition, cellulase and amylase activity in herbivorous fish was significantly higher than in carnivorous fish, whereas trypsin activity in carnivorous fish was much higher than in herbivorous fish.

Conclusion

Cellulase, amylolytic, proteolytic and lipolytic microbes in the digestive tract of mud crabs play an important role in the physiological function of the digestive tract, namely contributing to the enzymes cellulase, α-amylase, protease, dan lipase (IU g^{-1} $minute^{-1}$) was shown 55, 41.90, 26.50 and 37.26%, respectively. Furthermore, the role of the gastrointestinal microflora in contributing to cellulase enzymes and exogenous digestive enzymes amylase, protease and lipase can be demonstrated. The microflora of the digestive tract is thought to have originated from the detritus eaten by mud crabs.

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Author's Contributions

Siti Aslamyah: Designed an experiment method, conducted experiment, data analysis and interpretation, writing a draft manuscript, submitting a manuscript.

Yushinta Fujaya: Designed an experiment method, data interpretation, final editing and proofreading of manuscript before submitting.

Nita Rukminasari: Conducted data analysis and interpretation, writing a draft manuscript, submitting a manuscript.

Conflict of Interest

Author Siti Aslamyah, Yushinta Fujaya dan Nita Rukminasari have received a research grant from Universitas Hasanuddin. The authors declare that they have no conflict of interest.

Ethics

"All applicant bale international, national and/or institutional guidelines for the care and use of animals were followed by the authors".

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