Identification of Proteolytic Bacterial Isolates in Sediment Ecosystem of Gunung Anyar Mangrove Forest, Surabaya

P B Utomo, Sudarno and B S Rahardja

Abstract. Mangrove ecosystem is a system consisting of biotic and abiotic environments that interact with each other in a mangrove habitat. There are also biological aspects in mangroves, which are influenced by litter production, decomposition, mineral extraction by plants, and other biological activities of all biota in mangrove forests. Litter is an organic material that undergoes several stages of the decomposition process, producing substances that are important for life and productivity of waters, especially in food chain events. Litter is leaves, twigs, fruit, and other parts of mangrove trees that fall. The process of decomposing organic material in ponds involves multiple species of bacteria. These bacteria are composed of various types of bacteria, such as proteolytic, cellulolytic, amylolytic, nitrifying and denitrification bacteria. Organic waste with high protein content will decompose into simpler compounds with the help of proteolytic bacteria. Naturally, proteolytic bacteria are already present in pond waters even with limited quantity and quality. Therefore, it is necessary to have a mediating bacterial inoculum which contains proteolytic bacteria with high ability to tackle organic waste in ponds. This research aims to obtain information on proteolytic bacteria species in the mangrove ecosystem of Gunung Anyar, Surabaya. This is a descriptive research with sampling and data collection. The research activity consists of station research continued with isolation, enzymatic testing, bacterial identification and data analysis. The results show that out of four mangrove sediment samples from Gunung Anyar, Surabaya, two species of bacteria was obtained, namely Yersinia enterocolotica and Enterobacter agglomerans.

Keywords. Mangrove Forest, Proteolytic Bacteria, and Protease Enzyme.

1. Introduction

Indonesia is an archipelago country with a total of around 17,508 islands and coast length of approximately 81,000 km and abundant coastal resources, both biological and non-biological. Coastal is a border area between land and sea, therefore affected by processes occurring on land and in the sea. This kind of region is called ecotone, a transition area that is very different between two or more communities (Odum, 1993).

Mangrove forest as a typical natural resource of tropical coastal areas has a strategic function for coastal ecosystems, namely as a connector and balancer of land and sea ecosystems. The high level of organic material in the waters of the mangrove forest allows this forest to be used as a nursery for biota living in mangrove ecosystems. It also functions as feeding ground since mangroves are primary producers capable of producing a large amount of detritus from leaves and branches of mangrove trees where a lot of food is available for biota foraging for the ecosystem. The third function is as spawning ground for certain fish to be protected from predatory fish while looking for an optimal environment to separate and raise their fries. Additionally, it also functions as a supplier of shrimp, fish and crab larvae (Claridge and Burnett, 1993).

The existence of bacteria in the mangrove ecosystem has a highly important role in decomposing mangrove leaves litter into organic materials as a source of nutrition for organisms that inhabit
mangrove forests (Yahya et al., 2014 in Nizar et al., 2015). The active role of bacteria is absolutely necessary in the process of decomposition in mangrove waters. The bacteria will decompose the litter enzymatically through the active role of proteolytic, cellulolytic and chitinoclastic enzymes. Proteolytic group bacteria that play a role in the process of protein decomposition is Pseudomonas, while the group of bacteria playing a role in the cellulose decomposition process is the Cytophaga bacterium, Sporocytophaga bacterium, a group of bacteria that decompose chitin, which includes Bacillus, Pseudomonas and Vibrio (Lyla and Ajmal, 2006).

The main sources of organic material in the waters of mangrove forests are the litter produced by mangrove plants such as leaves, twigs, fruit and flowers; thus, one way of knowing how much organic matter contributes to an estuary is to calculate the total litter production (Knight, 1984 in Brown, 1996). Proteolytic bacteria are bacteria that can hydrolyze proteins into smaller peptides or amino acid units (Yuniati et al., 2015). Proteases are enzymes that catalyze the breakdown of peptide bonds in proteins (Gupta et al., 2002).

The benefit of this research is that it will provide scientific information about species of proteolytic bacteria that can be found in Gunung Anyar mangrove forest sediments and developed for industrial and aquaculture purposes.

2. Methodology
2.1. Research methods
This research used descriptive, factual and accurate methods regarding the facts, characteristics and relationships between the phenomena being investigated (Nazir, 2003). Determination of sampling stations in each of these stations was done using a purposive sampling method based on temperature and salinity differences (Hadi, 2007).

2.2. Place and time of research
This research was conducted from July to August 2017 at the Microbiology Laboratory of Faculty of Fisheries and Marine, Airlangga University, Surabaya, East Java.

2.3. Research equipment and materials
Tools used included laminar air flow, incubator, trigalsky, autoclave, Erlenmeyer flask, test tube, heater, aluminum foil, Bunsen burner, petri dish, inoculating loop and needle, Ohaus balance with 0.1 gr accuracy, measuring cup, test tube, cotton, micro pipette (100-1000 µl), binocular microscope, object glass, vortex, styrofoam, bucket, crob cylinder, DL-96 II Auto Microbial ID/AST system, and heat-resistant plastic. Materials used in this research were the sediment sample of Gunung Anyar mangrove forest Surabaya, physiological NaCl, methylated, non-selective agar media Tryptone Soya Agar (TSA), Skim Milk Agar (SMA) media, and DL-96E Test card.

2.4. Sampling
Sources of sediments were taken from the mangrove sediments of Gunung Anyar, Surabaya. The sediment samples were taken directly and randomly using cylinder crob up to a depth of 10 cm below the surface of the sediment and 10 grams were taken from each station. The samples were stored in plastic that had been sterilized using 70% alcohol and the samples’ number label was based on the sampling location group (A1, A2, A3, and A4). They were then put in a cooler and after they arrived at the laboratory, the snippet was stored in a cooling box at a temperature of 5º-10º C until usage (Triyanto et al., 2009).

2.5. Isolation of bacteria
Each sediment sample was weighed by 1 gram and then homogenized in 9 ml of physiological solution (0.9% NaCl) with the help of vortex. Bacterial isolation was performed on TSA culture media by dripping 1 ml suspension of sediment isolates with micro pipette and trigalsky to flatten it on the media and purify it.
2.6. Test of the proteolytic enzyme production activity
This test was conducted by isolate obtained from the isolation results inoculated by streak on the Skim Milk Agar (SMA) media and incubated at 32°C for 15 hours. The production of proteolytic enzymes was indicated by the formation of clear zones around the paper disk (Triyanto, 2009).

2.7. Identification of bacteria
Bacterial identification was performed conventionally by morphological observation and biochemical tests. Morphology was the science used to recognize a type of bacteria from the shape and structure outside the organism's body. The series of test could be seen in the attachment, then was identified using the DL-96 II Auto Microbial ID/AST system and results of identification of proteolytic bacteria was then concluded.

2.8. Parameter
2.8.1. Main Parameter
The main parameters observed were the diversity of proteolytic bacteria in the mangrove forest in Gunung Anyar, Surabaya. The method used was direct sampling of sediment at five different points, namely ST1, ST2, ST3 and ST4.

2.8.2. Supporting parameters
The supporting parameters of this research were temperature, soil pH, and water salinity at the time of sampling.

2.9. Data analysis
Analysis of the data was conducted using descriptive form, i.e. the delivery of data by presenting it on the distribution of proteolytic bacteria at each point in the area of mangrove sediment sampling. The diversity of bacterial species obtained from the identification results was correlated with their presence in the sampling point group.

3. Results and Discussion
3.1. Results of identification of bacteria
From the four sediment samples taken and isolated in the media, 22 isolates of bacterial colonies was obtained.

| Isolates | Colony Morphology | Form | Colour | Edge | Elevation |
|----------|-------------------|------|--------|------|-----------|
| ST1      | 10^4.1 Circular   | Circular | Cream | Entire | Effuse   |
|          | 10^4.2 Curled     | Curled  | Cream | Lobate| Umbonate |
|          | 10^4.3 Irregular  | Irregular| Transparent | Undulate| Raised |
|          | 10^4.1 Circular   | Circular | Pink  | Entire | Effuse   |
| ST2      | 10^4.1 Irregular  | Irregular| Cream | Undulate| Convex  |
|          | 10^4.2 Circular   | Circular | Cream | Entire | Effuse   |
|          | 10^4.3 Circular   | Circular | Brown | Entire | Effuse   |
|          | 10^5.2 Circular   | Circular | White | Entire | Effuse   |
| ST3      | 10^4.1 Filamentous| Filamentous| Cream | Lobate| Convex rugose |
|          | 10^4.2 Circular   | Circular | White | Entire | Effuse   |
|          | 10^4.3 Curled     | Curled  | Cream | Undulate| Convex  |
|          | 10^4.4 Circular   | Circular | Cream | Entire | Effuse   |
|          | 10^5.1 Circular   | Circular | Pink  | Entire | Effuse   |
| ST4      | 10^4.1 Filamentous| Filamentous| Cream | Lobate| Convex rugose |
The isolates thought to be proteolytic bacteria were tested for qualitative proteolytic activity using methods (Naiola and Widhyastuti, 2002). Qualitatively the tests were performed by growing a "loopful" bacteria selected on the same surface of the medium. After growing for 2-3 days at room temperature, protease activity was indicated by the presence of a clear circle zone around the colony. Results for the diameter of the clear circle divided with the diameter of the colony are expressed as relative protease activity. The results of the protease enzyme production activity test show that there are 2 bacterial isolates that produce clear zones, namely ST1.10\textsuperscript{4}.1 and ST1.10\textsuperscript{4}.2.

Table 2. Test results for proteolytic enzyme production activities.

| Isolates | Activities of Protease |
|----------|------------------------|
| 10\textsuperscript{4}.1 | + |
| 10\textsuperscript{4}.2 | + |
| 10\textsuperscript{4}.3 | - |
| 10\textsuperscript{4}.4 | - |
| 10\textsuperscript{5}.1 | - |
| 10\textsuperscript{5}.2 | - |
| 10\textsuperscript{5}.3 | - |
| 10\textsuperscript{5}.4 | - |

Notes: +: there is a clear zone  
- : there is no clear zone

The biochemical and identification tests were performed using the DL-96 II Auto Microbial ID/AST system. The results show that there is *Yersinia enterocolotica* bacterial species in ST1.10\textsuperscript{4}.1 samples and *Enterobacter agglomerans* bacterial species in ST1.10\textsuperscript{4}.2 samples. The results of bacterial identification can be seen in Appendix 3. The following table shows the results of identification of proteolytic bacteria in the samples of Gunung Anyar’s mangrove forest mud.

Table 3. The results of identification of proteolytic bacteria in the samples of Gunung Anyar’s mangrove forest mud.
3.2. Results of supporting parameter measurement

From the five mud samples taken at five different sampling points in Gunung Anyar mangrove forest, Surabaya, namely point T1 (sampling point at the coordinate point 7°19’50,1456” LS and 112°49’20,20” BT), T2 (sampling point at the coordinate point 7°19’50,2248” LS and 112°49’25,2688” BT), T3 (sampling point at the coordinate point 7°19’54,9192” LS and 112°49’9,9372” BT), and T4 (sampling point at the coordinate point 7°19’51,3012” LS and 112°48’52,3152” BT), the obtained supporting parameters are as follows:

Table 4. Supporting parameters for sampling mangrove forest mud in Gunung Anyar

| Parameter          | Mud Sampling Point |
|--------------------|--------------------|
|                    | T1 | T2 | T3 | T4 |
| pH of Mud          | 7  | 7  | 8  | 7  |
| Water salinity (ppt)| 9  | 8  | 9  | 9  |
| temperature (C)    | 33 | 27 | 29 | 28 |

3.3. Discussion

Sampling was performed in the afternoon where the sea water began to tide. It was performed using crob cylinder which was plugged in up to 10 cm depth into 10 grams of mangrove sediment. According to Hanafiah (2007), soil has a very important part for plant growth and soil organisms. Soil with a thickness below 30 cm has the availability of litter, soil organic materials and high level of minerals needed by plants and microorganisms. Rao (1994) claimed that bacteria are the most dominant group of microorganisms in the soil, covering half of microbial biomass in the soil. Bacteria are present in various types of soil but the population decreases along with the more depth of the soil. The water salinity data at ST1 is 9 ppt, 8 ppt at ST2, 9 ppt at ST3 and 9 ppt at ST4. According to Hutabarat and Evans (1985) in Suryanti (2008), river estuaries experience salinity fluctuations caused by tides. At high tide, salinity in the estuary area rises since the water in the estuary mixes with sea water, while at low tide, it is low since the water in the estuary is dominated by freshwater.

The pH data of mud in ST1 sample is 7, 7.5 on ST2, 8 on ST3, and 7 on ST4, thus in general the pH value (acidity) of mud samples at all sampling points is in low or neutral pH conditions. This is influenced by the occurrence of rain at the time before sampling. According to Johnson (2001), one of the main causes of acidic soil is rainwater. High rainfall most effectively causes the soil to become acidic if a lot of water is absorbed quickly.

The obtained samples were then stored in the cool box with ice cubes already added to maintain its temperature and they were taken to the laboratory to be isolated. After bacterial colonies were obtained in the isolation media, the process continued with testing the protease enzyme production activity qualitatively by the method used by Dakwah et al. (2005), in which isolates obtained from isolation were inoculated on Skim Milk Agar (SMA) media and then incubated at 32°C for 1-3 days. Proteolytic activity was indicated by the formation of a clear zone around the paper disc with a white background. Clear zone was the initial indication to determine the ability of bacteria to decompose proteins present in the media; the wider the clear zone formed, the more it was qualitatively regarded as greater potential for proteolytic bacteria. The results of the protease enzyme activity test performed on 22 isolates show that there are 2 isolates with clear zones, which were found in samples ST1.10^4 and ST1.10^2.

Two bacterial isolates that showed positive results on the protease enzyme production activity test were identified using the DL-96 II Auto Microbial ID/AST system. The results of the identification show that there are two species of bacteria, namely *Yersinia enterocolitica* in ST1.10^4 and...
Enterobacter agglomerans in ST1.10^{-4}.2. Both species of proteolytic bacteria were found at the location of ST1 which had environmental conditions at 7 pH, 9 ppt salinity, and 33°C temperature.

Yersinia enterocolitica belongs to the Yersinia genus which originally included the Pasteurella genus, the name of French bacterial researcher A. J. E. Yersin, and belonged to the Bacillus genus (Solomon, 1995). The Yersinia genus was classified into the Enterobacteriaceae family, negative Gram group, negative oxidase, and facultative anaerobic. Yersinia enterocolitica is a positive catalase bacterium, cannot form stem spores, has negative lactose, positive urease, and can grow at temperatures in the range of 0-40°C (Bercovier and Mollaret, 1984). Yersinia enterocolitica is one of three bacterial species of the food-borne genus of Yersinia, they are Y. enterocolitica, Y. pseudotuberculosis, and Y. pestis which are immune to the nonspecific immune system (Cornelis et al., 1998).

Kingdom : Bacteria  
Phylum : Proteobacteria  
Class : Gamma proteobacteria  
Ordo : Enterobacteriales  
Family : Enterobacteriaceae  
Genus : Yersinia  
Species : Yersinia enterocolitica  
(Schleifstein & Coleman, 1939)

Enterobacter agglomerans is a negative gram bacteria, anaerobic, rod-shaped, and able to move (motile), its means of movement are peritric flagella, a flagella that is evenly spread throughout the cell surface. Enterobacter agglomerans can show glucose activity, form acids and gases, and can reduce nitrate to nitrite. These bacteria can form capsules, citrate, and acetate which can be used as the only carbon source (Pelezar and Chan, 1986).

Mohapatra et al. (2003) stated that Enterobacter agglomerans is a bacterium that produces protease, amylase and cellulose enzymes. The original habitat of Enterobacter agglomerans is unknown until now, but it is widely spread in the environment, food, water, soil and vegetables. Enterobacter agglomerans develops well in warm-blooded animals and is usually absent from the intestines of fish and cold-blooded animals (Grimont and Grimont, 2006).

Kingdom : Bacteria  
Phylum : Proteobacteria  
Class : Gamma proteobacteria  
Ordo : Enterobacteriales  
Family : Enterobacteriaceae  
Genus : Enterobacter  
Species : Enterobacter agglomerans  
(Garrity et al., 2004)

4. Conclusion  
Based on the results of the research, it can be concluded that out of four Gunung Anyar Surabaya mangrove sediments samples, there are two species of bacteria obtained, namely Yersinia enterocolitica and Enterobacter agglomerans.

5. Suggestion  
Based on the results of the research, it is expected that further research is conducted to determine the potential of Yersinia enterocolitica and Enterobacter agglomerans that can be utilized by human.
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