Identification of extracellular enzyme-producing bacteria (proteolytic, cellulolytic, and amylolytic) in the sediment of extensive ponds in Tanggulrejo, Gresik

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Abstract. The process of aquaculture is an activity that potentially generates organic waste primarily derived from residual feed and the results of excretion (feces). Heterotrophic bacteria have the ability to produce extracellular enzymes (protease enzymes, amylases, and cellulases) that are necessary for the bioremediation of organic waste. The purpose of this study was to investigate the identifiable extracellular enzyme bacteria from extensive pond sediments in Tanggulrejo Village, Gresik. Twenty bacterial isolates were found from pond sediments that had the ability to produce extracellular enzymes and proteolytic, amylolytic and cellulolytic bacteria. The four isolates were identified as genera Bacillus thuringiensis, Bacillus lentus, Bacillus sphaericus and Corynbacterium pilosum. Bacillus thuringiensis bacteria have a hydrolysis zone of protein of 11 mm, a hydrolysis zone of amylum of 10 mm, and a hydrolysis zone of cellulose of 8 mm. Bacillus lentus bacteria have a hydrolysis zone of protein of 14 mm, a hydrolysis zone of amylum of 12 mm and a hydrolysis zone of cellulose of 7 mm. Bacillus sphaericus bacteria have a hydrolysis zone of protein 14 mm, a hydrolysis zone of amylum of 11 mm and a hydrolysis zone of cellulose of 6 mm. Corynebacterium pilosum bacteria have a hydrolysis zone of protein of 10 mm, a hydrolysis zone of amylum of 14 mm and a hydrolysis zone of cellulose of 16 mm.

1. Introduction

Bacteria have the ability to produce the extracellular enzymes that are needed for the bioremediation of organic waste, known as heterotrophic bacteria [1]. The decomposition process of the organic material consists of various types of bacteria such as proteolytic, cellulolytic, amylolytic, nitrification and denitrification [2]. Heterotrophic bacteria utilize ammonia as an energy source for cell regeneration and use organic compounds such as acetate, pyruvate and oxaloacetate as a carbon source in pond water [3].

Gresik is one of the regencies in East Java which is still dominated by an extensive system of aquaculture. The lack of available water sources causes most farmers in Gresik to rarely change the water, which can lead to a decrease in the water quality of the aquaculture pond. The remaining feed and metabolic waste in the cultivation system can cause the pond water’s quality parameters to decrease.

Organic matter from the remaining feed, feces and added organisms that die and settle at the bottom of the pond are used as substrates for the growth of microorganisms. The accumulation and overhauling of organic matter is carried out by the bacteria that occurs at the bottom of pond sediments [4].
2. Methodology

2.1 Location and time
Sediment sampling was carried out in extensive pond systems in Tanggulrejo Village, Manyar District, Gresik Regency, East Java. Observations and trials were conducted in the Airlangga University Microbiology Laboratory and in the Bacteriology Laboratory in the Class 1 Perak Quarantine Fish Center, Surabaya. This was done from February 2018 to May 2018.

2.2 Methods and materials
The tools used include an incubator, autoclave, oven, centrifuge, refrigerator, vortexmicropiper, and microscope. The materials used in this study were the sediments of an extensive pond system from Gresik, Bacto-agar, Bacto-peptone, yeast extract, Bacteriological agar, skimmed milk, CMC (carboxymethyl cellulose), starch/starch, paper disc blanks, sterile quads, 1% Lugol iodine, Congo red, 70% alcohol and physiological NaCl.

2.3 Procedures
This research was conducted in several stages, beginning with the sterilization of the tools and materials by autoclave at 121°C for 15 minutes. The media of bacterial isolation used was an NA medium containing 15 grams of Bacto agar, 5 grams of Bacto peptone, 1 gram of yeast extract and sterile seawater to reach a volume of 1 liter.

The method used to isolate the bacteria was the pour plate method. Inoculant material obtained from the sediments was obtained from the extensive pond systems. The sediment pond samples, as much as 1 gram, were placed in 9 ml of physiological NaCl (0.85%) in a test tube and centrifuged for 20 minutes at a speed of 3500 rpm. 1ml of supernatant was taken and put in physiological NaCl, before being vortexed for about 1 minute. Multilevel dilution was carried out to obtain a 10^-5 dilution, 10^-6 dilution and 10^-7 dilution. At each dilution, 1 ml of the bacterial suspension was taken using a sterile pipette before being put into each sterile petri dish.

The purification of the bacterial isolates was carried out using the streak method. The pure bacterial isolates were tested for proteolytic, amylolytic and cellulolytic enzymatic activity. The bacterial isolates were dissolved in physiological NaCl up to 10^8 CFU / ml and the researcher then dripped 10 μl onto blank paper disks.

Isolates in the liquid NA media were used to test proteolytic activity, inoculated onto paper disc blanks which had been placed on NA enriched with skimmed milk (1%) and incubated at 32 °C for 15 hours. Proteolytic activity was indicated by the formation of clear zones around the paper disk with a white background.

The activity test for extracellular amylase enzyme production was carried out with the medium enriched with starch. Insulation liquid culture was inoculated onto paper disc blanks which had been placed on NA media enriched with starch (1%). This was incubated at 32 °C for 48 hours. Lugol's 1% iodine solution was poured onto the culture to identify amylase activity, shown by the formation of clear zones around the paper disc with a dark blue background.

The cellulase enzyme production activity test was carried out with the medium enriched with CMC. Liquid isolates inoculated onto paper disc blanks that placed on 1% NA enriched CMC enriched media. Congo red solution was poured onto the culture to identify cellulolytic activity. The presence of cellulase enzyme activity was indicated by the formation of clear zones around the paper disc with a pink background.

2.4 Data analysis
Data from the isolation, bacterial identification and clear zone observation through to the tests on the extracellular enzyme-producing bacteria (proteolytic, cellulolytic and amylolytic) were analyzed descriptively and presented in the form of figures and tables.
3. Results and discussion
3.1. Isolat bacteria
Bacteria obtained from the pond sediment amounted to 20 isolates (Table 1). Bacteria isolated from the sediments of the extensive pond systems were then tested for extracellular enzymes to determine the ability of the bacteria to hydrolyze proteins, starch and cellulose. Qualitative testing of the extracellular enzyme-producing bacteria was done by observing the clear zones around the bacterial colonies and then dividing the diameter of the clear zone with the diameter of the bacterial colony (Figure 1). The results for the diameter were expressed as relative enzymatic activities.

The results of the observations of the enzymatic activity of the 20 isolates (Figure 2) revealed that 3 isolates had one type of enzymatic activity, 9 isolates had two enzymatic activities and 8 isolates had three enzymatic activities (protease, amylase, and cellulase). The isolates which had the three largest enzymatic activity zones and the largest diameter of the clear zone (hydrolysis zone) were submitted to undertake biochemical tests in order to be identified. The diameter of the hydrolysis zone showed the concentration and enzymatic activity produced [5]. Isolates which had the highest diameter of hydrolysis zones were IS-11, IS-12, IS-13, and the IS-19. IS-11 isolates had a 35 mm protein hydrolysis zone, a 34 mm starch hydrolysis zone and a 41 mm cellulose hydrolysis zone respectively. IS-12 isolates had a 42 mm protein hydrolysis zone, a 34 mm starch hydrolysis zone and a 31 mm cellulose hydrolysis zone. The IS-13 isolates had a 34 mm protein hydrolysis zone, a 43 mm starch hydrolysis zone and a 44 mm cellulose hydrolysis zone. The IS-19 isolates have a 32 mm protein hydrolysis zone, a 36 mm starch hydrolysis zone and a 28 mm cellulose hydrolysis zone.

Figure 1. The clear zone of activity a) protease b) amylase c) cellulase.

Figure 2. Graph of the test results of the protein hydrolysis ability, starch and cellulose.
Table 1. Characteristics of the bacterial isolates.

| Isolates Code | Cell Shape | Gram Test | Motility | Colony Color |
|---------------|------------|-----------|----------|--------------|
| IS-1          | Basil      | +         | Motile   | White        |
| IS-2          | Basil      | +         | Motile   | White        |
| IS-3          | Basil      | -         | Motile   | White        |
| IS-4          | Basil      | +         | Nonmotile| White        |
| IS-5          | Basil      | +         | Motile   | White        |
| IS-6          | Basil      | -         | Motile   | White        |
| IS-7          | Basil      | +         | Motile   | White        |
| IS-8          | Basil      | +         | Motile   | White        |
| IS-9          | Basil      | +         | Nonmotile| White        |
| IS-10         | Basil      | -         | Nonmotile| White        |
| IS-11         | Basil      | +         | Motile   | Cream        |
| IS-12         | Basil      | +         | Motile   | Cream        |
| IS-13         | Basil      | +         | Nonmotile| Cream        |
| IS-14         | Basil      | +         | Nonmotile| White        |
| IS-15         | Basil      | -         | Nonmotile| Red          |
| IS-16         | Cocus      | -         | Nonmotile| White        |
| IS-17         | Basil      | +         | Motile   | White        |
| IS-18         | Basil      | +         | Nonmotile| White        |
| IS-19         | Basil      | +         | Motile   | Cream        |
| IS-20         | Basil      | +         | Motile   | White        |

Table 2. The results of the identification of the extracellular enzyme-producing bacteria from extensive pond sediments.

| Isolates Code | Species of Bacterial |
|---------------|----------------------|
| IS-11         | *Bacillus thuringiensis* |
| IS-12         | *Bacillus lentus*     |
| IS-13         | *Corynebacterium pilosum* |
| IS-19         | *Bacillus sphaericus* |

3.2. Identification of bacteria

The bacterial isolates with protease, amylase and cellulase enzyme activity are then identified in order to determine the bacterial species. Bacteria from the pond sediments had 4 isolates namely IS-11, IS-12, IS-13, and IS-19. The isolates were then identified through colony observation, Gram staining, biochemical tests (TSIA, Gas, H 2S, catalase, oxidase and O / F), carbohydrate fermentation /sugar tests (glucose, lactose, sucrose, mannitol, salicin, maltose, dulcitol, trehalose, sorbitol, arabinose, xylose and inositol) and other biochemical tests (motility, indole, simonscitrate, urea, MR-VP, malonate, lysine decarboxylase and ornithine decarboxylase). The results of the bacterial identification can be seen in Table 2.

3.3. Discussion

The cultivation process is an activity that has the potential to produce organic waste, mainly derived from food waste and excretion (feces) [1]. Proteins, carbohydrates, and fats are the main components of the feed and have a large percentage of feed formulation [6]. Remover microorganisms of organic material are a biological activator that grows naturally or is intentionally given to accelerate the transformation of organic matter [7]. Heterotrophic bacteria participate in energy transformation [8] which can be utilized by cultivated organisms. Heterotrophic bacteria can be obtained by a series of nutrient hydrolysis tests using media mixed with protein, starch, and cellulose.

Twenty bacterial isolates found from the pond sediments had the ability to produce proteolytic, amylolytic and cellulolytic extracellular enzymes, and the researcher was able to obtain 4 bacterial
isolates. The four isolates were identified as *Bacillus thuringiensis*, *Bacillus lentus*, *Bacillus sphaericus* and *Corynbacterium pilosum* (Table 2).

Based on the results of the hydrolysis test, NA media - added to skimmed milk - supports the growth of proteolytic bacteria because it contains casein, which functions as a substrate for protease enzymes [9]. The clear zone produced by the proteolytic bacteria occurs due to protease activity which breaks down the peptide bonds of the casein in skimmed milk [7]. Proteolytic enzymes derived from microorganisms in the form of proteases contain proteinase and peptidase. Proteinase catalyzes the hydrolysis of the protein molecules into large fragments and peptidases the hydrolyze polypeptide fragments into amino acids. The main role of the extracellular protease in nature, as with other extracellular enzymes, is to hydrolyze the large polymer substrates (polypeptides) into smaller molecules so then they can be absorbed by the cells [10]. The bacteria that has the largest protein hydrolysis zone is *Bacillus lentus* (42 mm).

Bacteria that have extracellular amylase enzyme activity can hydrolyze starch (amylose and amylpectin) contained in the media, so then the degraded starch cannot bind to I$_2$ (iodine) and does not produce a clear zone around the bacterial colonies [7]. Starch is a component of plants, and many prokaryotic bacteria produce amylase for use as energy and carbon sources [10]. The bacteria that had the largest starch hydrolysis zone was *Corynbacterium pilosum* (43 mm).

Bacteria that have extracellular cellulase enzyme activity can hydrolyze the Carboxymethylcellulose (CMC) contained in the media. The formation of clear zones shows that the polysaccharides have been degraded into saccharides with shorter chains, so then they cannot absorb the congo red dye [12]. CMC is the best substrate for cellulase production because it can induce the bacteria to produce cellulase enzymes [12]. The bacteria that had the largest cellulase hydrolysis zone was *Corynbacterium pilosum* (44 mm).

The sediments from the extensive pond systems came from the remaining pile of feed and the results of fish metabolites,which can stimulate the growth of heterotrophic bacteria and pathogenic bacteria [13]. The results of the enzyme activity tests were that the bacteria that can produce extracellular protease, amylase, and cellulase enzymes identified as *Bacillus thuringiensis*, *Bacillus lentus*, *Bacillus sphaericus* and *Corynbacterium pilosum*.

*Bacillus thuringiensis* is one of the insect pathogenic bacteria that have been developed into a pathogenic bioinsecticide for mosquito larvae and black fly larvae. It is not toxic to the environment and non-target organisms. According to [15], economically, *Bacillus thuringiensis* is widely used for bioinsecticide production and has been widely used to control insect pest larvae.

*Bacillus lentus* is a bacterium that can produce various types of enzymes that can break down food substances such as carbohydrates, fats and proteins into simpler compounds that are easier to digest [16]. *Bacillus lentus* belongs to the category of Generally Recognized as Safe (GRAS) whose function can stabilize microbial environmental conditions in the digestive tract of cultivated organisms [17].

*Bacillus sphaericus* is a pathogenic bacterium in insects, as well as *Bacillus thuringiensis*, which is often used as a biopesticide. *Bacillus sphaericus* belongs to the category of Gram-positive bacillus bacteria with prominent endosporic forms that shows high toxicity to *Culex* and *Anopheles*. [18] stated that *Bacillus sphaericus* had potential against *Culex, Anopheles* and *Aedes* larvae.

*Corynbacterium pilosum* has the characteristics of being basil, Gram-positive and non-motile, and cannot form spores. These bacteria are classified as pathogenic in animals, especially in livestock. [19] explained that *Corynbacterium pilosum* can cause genitourinary infections (Cystitis and pyelonephritis) in animals, especially livestock. [20] also stated that *Corynbacterium pilosum* includes parasites and/or pathogens in animals.

4.Conclusion

Pursuant to the results of the research, the bacteria found in the pond sediments were drawn from 20 isolates, and out of the bacteria that could produce extracellular enzymes (proteolytic, amylolytic and cellulolytic), there were 4 isolates namely *Bacillus thuringiensis*, *Bacillus lentus*, *Bacillus sphaericus* and *Corynbacterium pilosum*. *Bacillus thuringiensis* a bacterium has a hydrolysis zone of protein 35 mm, a hydrolysis zone of amylog of 34 mm, and a hydrolysis zone of cellulose of 41 mm. *Bacillus lentus* a bacterium has a hydrolysis zone of protein 42 mm, a hydrolysis zone of amylog of 34 mm and a hydrolysis zone of cellulose of 31 mm. *Bacillus sphaericus* bacteria have a hydrolysis zone of protein of 32 mm, a hydrolysis zone of amylog of 36 mm and a hydrolysis zone of cellulose of 28
Corynebacterium pilosum bacteria have a hydrolysis zone of protein of 34 mm, a hydrolysis zone of amylum of 43 mm and a hydrolysis zone of cellulose of 44 mm.

5. References
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