

19

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Submission date: 05-Nov-2022 12:48PM (UTC+0800)

Submission ID: 1945140149

File name: 19_JAFH_10_3_2021.doc (345.5K)

Word count: 2965

Character count: 16238



DNase and Gelatinase Activities Of β -Hemolysin *Aeromonas hydrophila* Isolated from Catfish (*Clarias batrachus*)

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Received : 2021-03-12
Accepted : 2021-05-09

Keywords :
 β -hemolysin, DNase, Gelatinase

Abstract

Aeromonas hydrophila infection can cause mass mortality in fish and result in economic losses for farmers. These are bacterium that secrete substances or compounds that support its virulence by producing toxins and extracellular proteins in the form of enzymes. This study aimed to determine the activity of DNase enzyme, and gelatinase enzyme of β -hemolysin toxin *A. hydrophila* isolated from catfish (*Clarias batrachus*). This research was carried out in November 2020 in Dukuh Tengah Village, Buduran District, Wedoro Village, Waru District, Wadung Asri Village, Waru District and Keboan Sikep Village, Gedangan District. The sampling technique was carried out by researchers by following activities in the field and visiting correspondents directly in the catfish cultivator ponds, then the catfish samples were carried out by biochemical tests and toxin and enzyme activity tests to obtain the information and data needed. The results showed that *A. hydrophila* bacteria in catfish produced positive β -hemolysin toxin, DNase enzyme, and gelatinase enzyme. The research concluded that 10 isolates of *A. hydrophila* bacteria isolated from catfish (*C. batrachus*) in Sidoarjo showed the activity of β hemolysin toxin, DNase enzyme, and gelatinase enzyme.

INTRODUCTION

The production of aquaculture in Indonesia has increased by an average of 27.84% each year. The percentage increase in the average aquaculture production in Indonesia is the highest compared to the top ten world aquaculture-producing countries. China, as a world fishery-producing country, has an average production increase of only 5.29%. The percentage of aquaculture production is still below the average world aquaculture production (Direktorat Jenderal Perikanan Budidaya, 2015). In 2017-2018 the production of catfish

farming in Indonesia increased from 841.75 thousand tons to 1.81 million tons with an increase of 114.82% (Kementerian Kelautan dan Perikanan, 2018). East Java is the second largest province for catfish aquaculture production. Sidoarjo is a contributor to catfish production of 75,720 tons in 2018 (Badan Pusat Statistik Jawa Timur, 2018).

Many pathogenic bacteria are of concern in fish diseases, but only a few species of pathogenic bacteria are responsible for the economic losses in fish farming worldwide (Sudheesh *et al.*,

2012). *Aeromonas hydrophila* is a pathogenic bacterium that causes mass death of fish worldwide, resulting in economic losses for farmers (Janda and Abbott, 2010). *A. hydrophila* and other strains are known to be capable of producing extracellular toxins such as hemolysin which greatly determine the pathogenicity and virulence level of bacteria. *A. hydrophila* produces a β -hemolysin toxin which can lyse red blood cells in a fast time (Mangunwardoyo *et al.*, 2009). The activity of the hemolysin toxin causes a reddish color (hemorrhage) on the surface of the fish's skin (Li *et al.*, 2013). Several pathogenic bacteria have been known to produce extracellular DNase, but the benefits of utilizing their enzyme activity are not fully understood (Sunita *et al.*, 2015).

Fox and Holtman (1968) stated that DNase helps in the spread of infectious pathogenic bacteria to the host and provides nucleotides for the rapid growth of bacteria through DNA hydrolysis. Gelatinase enzymes are produced by several species of bacteria to hydrolyze gelatin into compounds (polypeptides, peptides and amino acids), this enzyme is used by bacteria to be able to pass through the host cell membrane and the results of gelatin hydrolysis are used by bacteria for metabolic processes. (Balan *et al.*, 2012). Chandrarathna *et al.* (2018) stated that the activity of β -hemolysin toxin, DNase and gelatinase determines the pathogenicity of *A. hydrophila* bacteria in fish. Based on this background, a study was conducted to provide information about isolates that showed β -hemolysin, DNase and gelatinase activity in *A. hydrophila* so that they could help in disease management in catfish in Sidoarjo.

METHODOLOGY

Place and Time

This research was conducted in November 2020 in Dukuh Tengah Village, Buduran District, Wedoro Village, Waru District, Wadung Asri Village, Waru

District and Keboan Sikep Village, Gedangan District, Sidoarjo Regency.

Research Materials

The tools used in this study were section set, syringe, laminar airflow, tray, analytical scale (Scout Pro), loop needle (Usebeck 5431), petri dish (Iwaki), incubator (Memmert in 55), test tube rack, test tube (Iwaki), bunsen (OMM), dropper (Pyrex), spatula, Erlenmeyer (Pyrex), measuring cup (Pyrex), object glass, autoclave (Hirayama), microscope, optilab, label, matches, refrigerator (Ikeda), hot plate (Cimarec), magnetic stirrer, measuring pipette (Iwaki), bulb (D&N), syringe (OneMed), nets, and vacutainer (Vacculab).

The materials used in this study were 70% alcohol, *Aeromonas* selective agar medium, blood agar medium, sheep blood 5%, DNase agar medium, methyl green 0.05%, nutrient gelatin medium, spirits, phosphate-buffered saline, gram staining reagent, plastic wrap, packing plastic, and immersion oil.

Research Design

This study used a survey method by taking samples at the location directly to obtain *A. hydrophila* bacterial isolates in catfish (*Clarias batrachus*). Sampling was carried out on catfish cultivation pond plots. The samples taken were catfish showing clinical symptoms of *A. hydrophila* infection, then isolation, identification by biochemical tests and activity tests of hemolysin, DNase and gelatinase were taken.

Work Procedure

Sampling

The catfish taken for the sample is catfish measuring 25-35 cm, aged 2-3 months and weighing 100-150 grams. The fish used are catfish that show clinical symptoms such as red spots (hemorrhagic) on the skin surface, flatulence (dropsy), protruding eyes (exophthalmia), scaly fins and slow-moving fish ulcers and hemorrhage on the surface of the fish's

body and fish swim slowly on the surface of the water (Lili *et al.*, 2019).

Equipment Sterilization

The initial stage of this research is sterilization. The equipment used is a petri dish, test tube, Erlenmeyer, section set, wrapped in heat-resistant plastic (HDPE) and put in an autoclave at a temperature of 121 °C with a pressure of 15 psi for 15 minutes. All surfaces and walls of laminar airflow are cleaned with 70% alcohol spray. The fan is turned on to circulate the air to keep it sterile. The media that has been made is put into a sterile Erlenmeyer which is tightly closed with cotton, then put in an autoclave at a temperature of 121 °C with a pressure of 15 psi for 15 minutes (Anggraini *et al.*, 2016).

Isolation of Bacteria on Catfish (*Clarias batrachus*)

Bacteria were isolated using a loop needle then planted on ASA (*Aeromonas* Selective Agar) media and incubated at 37°C for 24 hours. Testing of isolates was carried out using a 1000x magnification microscope after Gram staining of the bacterial isolates. Observations were carried out with a microscope that had been added with immersion oil and then observed under a 1000x magnification microscope to see the morphology of bacterial cells.

Identification of *Aeromonas hydrophila* on Catfish (*Clarias batrachus*)

Identification was carried out by macroscopic test with colony morphology observation, microscopic test in the form of gram stain and biochemical test. The gram stain test was carried out with reagent gram stain using a microscope that had been installed with oil immersion to observe the color and shape of the bacteria, and continued with biochemical tests such as catalase test, oxidase test, indole test, motility test, TSIA test, and others.

Aeromonas hydrophila Hemolysin Activity Test

A hemolysin test is carried out using blood agar media. Isolates were planted in blood agar base with the addition of sheep blood that had been fibrinated as much as 5% of blood agar base, then incubated at 37 °C for 18-24 hours. A positive result if a clear zone is formed around the colony on the media, indicates that the microbe is pathogenic (Sukmadewi *et al.*, 2017).

Aeromonas hydrophila DNase Activity Test

DNase test is performed using DNase agar. Bacteria to be tested are inoculated on DNase agar plate which has been added with 0.005% methyl green, then incubated at 37 °C for 24 hours. After incubation, the DNase agar plate was immersed with 1 N HCl for 5 minutes (Chu *et al.*, 2013). A positive result if a clear zone is formed around the colony indicates that there is DNase activity which hydrolyzes deoxyribonuclease (Kateete *et al.*, 2010).

Aeromonas hydrophila Gelatinase Activity Test

The gelatinase test was carried out using bacteria that had been pure culture using a loop needle, then inserted into the nutrient gelatin media in the middle of the media. Bacteria that have been inoculated into gelatin media are stored in an incubator at 37 °C for ± 3 days. After waiting for ± 3 days, observe utilizing a test tube containing bacterial isolates stored in the refrigerator at 4 °C (Prihanto *et al.*, 2018). Wait 25 minutes for the jar in the refrigerator. Furthermore, observations were made including whether or not the gelatin was melted compared to the control. If there is melting of gelatin, it indicates that the bacteria are capable of producing gelatinase exoenzymes (Putri *et al.*, 2017).

Data Analysis

The data obtained were analyzed descriptively, namely about the activity of Hemolysin, DNase and Gelatinase from

Aeromonas hydrophila isolates that had been isolated in catfish (*C. batrachus*) in Sidoarjo.

RESULTS AND DISCUSSION

Aeromonas hydrophila Identification

Ten isolates were identified by the bacterium *Aeromonas hydrophila* by

macroscopic and microscopic observations. Macroscopic observations include the shape and color of the *Aeromonas hydrophila* colony and microscopic observations including the shape of the gram stain, the form of short stem bacteria measuring 1.90-3.42 μm , red-colored using a 1000x magnification microscope can be seen in Figure 1.

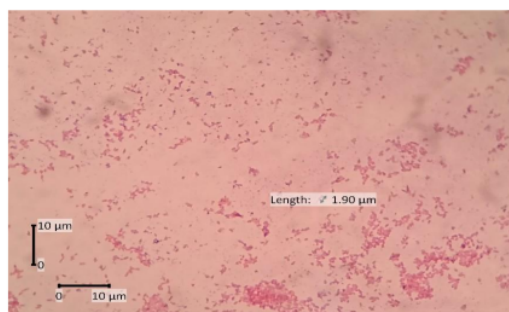


Figure 1. Cell morphology of *A. hydrophila*.

Hemolysin Activity

The results of observations on 10 bacterial isolates identified as *A. hydrophila* showed hemolysin activity. Zones were formed on the agar blood medium from the inoculation of *A.*

hydrophila bacteria which had been incubated for 24 hours at 37 °C. According to Mangunwardoyo *et al.* (2010), perfect hemolysin (β -hemolysin) can be seen from the formation of a clear zone around the *A. hydrophila* colony which can be seen in Figure 2.

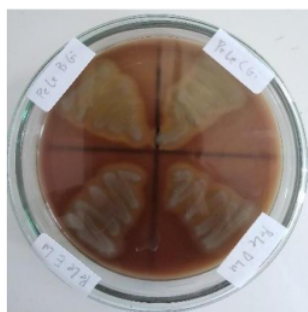


Figure 2. β -hemolysin activity of *A. hydrophila*.

DNase Activity

The results of observations on 10 bacterial isolates were identified as *A. hydrophila* positively showed DNase

activity. Zones were formed on DNase agar from the inoculation of *A. hydrophila* which had been incubated for 24 hours at 37 °C (Majumdar *et al.*, 2007). The clear zone formed can be seen in Figure 3.

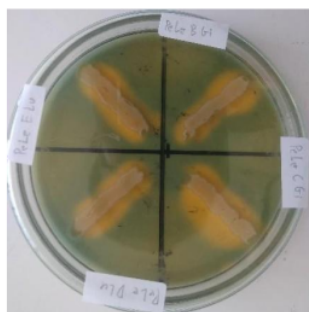


Figure 3. DNase activity of *A. hydrophila*.

Gelatinase Activity

The results of observations on 10 bacterial isolates identified as *A. hydrophila* showed positive gelatinase activity. Gelatin liquefaction occurs in nutrient gelatin media from the

inoculation of *A. hydrophila* bacteria which has been incubated for 72 hours at 37 °C, then the test tube containing bacterial isolates is stored in a refrigerator at 4 °C (Prihanto *et al.*, 2018). Gelatin melting can be seen in Figure 4.



Figure 4. Gelatinase activity of *A. hydrophila*, A (Control: there is no melting of gelatin); B (Treatment: there is melting of gelatin).

A. hydrophila is a pathogen that attacks various types of freshwater fish and causes Motile Aeromonas Septicemia (MAS) worldwide. MAS causes pathological changes in fish such as tail/fin rot, hemorrhagic septicemia all over the body surface, local bleeding especially in the gills and anus, ulcers, abscesses, exophthalmia and flatulence. Internally, there may be ascitic fluid accumulation, anemia and damage to organs, especially the kidneys and liver (Austin and Austin, 2016). The virulence factors produced by *A. hydrophila* include hemolysin, DNase, and Gelatinase, these factors affect the virulence level of *A. hydrophila* (Hossain *et al.*, 2020).

Catfish samples suspected of being infected with *A. hydrophila* were isolated from four areas in Sidoarjo, East Java, namely Dukuh Tengah Buduran, Wedoran Waru, Wadung Asri Waru and Keboan Sikep Gedangan. Ten isolates from 25 isolates, as much as 40% were identified as *A. hydrophila* by biochemical tests according to Austin and Austin (2007). Ten *A. hydrophila* isolates showed bacterial colonies of round shape, flat edges, slightly convex elevation, yellow color, gram-negative with rod-shaped cells ranging in size from 1.90 to 3.37 μm . Austin and Austin (2016) stated that *A. hydrophila* is a gram-negative bacterium with rod-shaped cells measuring 1.0–3.5

μm and Himedia (2016) that *A. hydrophila* colonies are large round and yellow.

Hemolysin test for *A. hydrophila* bacteria (10 isolates) showed hemolysin activity with the formation of a bright zone (β -hemolysin) around the colony. This is supported by research by Pandey *et al.* (2010) which states that *A. hydrophila* bacteria produce β hemolysin toxin which is one of the important virulence factors of pathogenic bacteria. In the study by Majumdar *et al.* (2007), *A. hydrophila* isolates isolated from catfish (*C. batrachus*) showed β hemolysin activity. Abd-El-Malek (2017) also stated that 100% *A. hydrophila* isolates produced β hemolysin toxin. Rahim *et al.* (2004) added that *A. hydrophila* bacteria produces two types of hemolysin toxins, namely α hemolysin and β hemolysin. Angka (2001) states that hemolysin is an enzyme capable of lysing red blood cells and freeing hemoglobin (a protein in blood that gives red blood cells their red color).

A. hydrophila also produce hemolysin toxin which plays a role in the infection and spread of bacteria in the host body which causes cell damage and cell mortality (Qosimah *et al.*, 2020). Bhakdi *et al.* (1996) added that in addition to being able to lyse red blood cells, this toxin also destroys tissue cells and the first immune system of the host cell (fish), thus hemolysin toxin directly increases the infection of these bacteria. Mangunwardoyo *et al.* (2009) added that *A. hydrophila* produces β hemolysin toxin which can lyse blood quickly and completely, and plays a role in infecting fish bodies (hosts) through the mechanism of action of β -hemolysin toxin which is released when *A. hydrophila* bacteria attaches to the surface. fish body. This β hemolysin toxin causes lesions and fatal hemorrhage in catfish (Majumdar *et al.*, 2007).

DNase test for *A. hydrophila* that had been inoculated on DNase agar media, all (10 isolates) showed DNase activity with the formation of a bright zone around the colony. This is following the research of

Majumdar *et al.* (2007) which stated that *A. hydrophila* bacteria isolated from catfish (*C. batrachus*) showed that *A. hydrophila* was positive for producing the DNase enzyme. In the study of Yadav *et al.* (2014), *A. hydrophila* isolates were 100% positive to produce the DNase enzyme. The DNase activity of *A. hydrophila* bacteria isolates can cause disease in fish and humans. Pemberton *et al.* (1997) added the role of DNase *Aeromonas* sp. It is considered important for bacterial nutrition because the DNase enzyme regenerates nucleic acids into phosphorus and nitrogen which will be used as a source of nutrition for *Aeromonas* sp., the DNase enzyme is a virulence factor of *A. hydrophila* bacteria (Chu *et al.*, 2013).

The gelatinase test for *A. hydrophila* bacteria as many as 10 isolates on nutrient gelatin medium showed the presence of gelatinase activity which was indicated by the occurrence of liquefaction in the gelatin medium (Fitriyah, 2017). This is in accordance with the research of Majumdar *et al.* (2007) which stated that *A. hydrophila* bacteria isolated from catfish (*C. batrachus*) showed positive results in producing gelatinase enzymes. In a study by Yadav *et al.* (2014), *A. hydrophila* isolates were 100% positive to produce gelatinase enzymes. The gelatinase enzyme in *A. hydrophila* can break down gelatin in fish into amino acids which are then used by bacteria to be used in metabolic processes (Fitriyah, 2017).

Murwani *et al.* (2017) state that *A. hydrophila* bacteria has a fimbria that acts as an attachment to the surface of the host cell and acts as an adhesin (a medium for bacteria to infect the host), then *A. hydrophila* bacteria produce several extracellular proteins and toxins that play a role in infection in the host. Degradation of tissues and proteins in the host due to extracellular protein and hemolysin toxin produced by *A. hydrophila* bacteria is used as an energy source for multiplication. *A. hydrophila* by attaching the fimbriae directly to the host cell and mucosal receptors. According to Murwani *et al.* (2017), these bacteria then spread

through blood circulation, multiplication, biofilm formation and release toxins, causing damage to the host's tissues.

According to Arwin *et al.* (2016), extracellular proteins are produced by *A. hydrophila* such as DNase and gelatinase enzymes. Fox and Holtman (1968) stated that the DNase enzyme helps pathogenic bacteria in infecting the host and provides nucleic acid for the rapid growth of bacteria through DNA hydrolysis. Buchanan *et al.* (2006) added that pathogenic bacteria produce the DNase enzyme to escape from extracellular traps. Citterio and Biavasco (2015) added that extracellular traps such as neutrophils, eosinophils and macrophages function to capture and kill pathogenic bacteria, thereby reducing their spread to infected hosts (fish).

Meanwhile, the gelatinase enzyme is produced by pathogenic bacteria to hydrolyze gelatin into polypeptides, peptides and amino acids (Petersen and McLaughlin, 2016). Balan *et al.* (2012) added that the gelatinase enzyme is used by bacteria to be able to pass through the host cell membrane and the results of gelatin hydrolysis are used by bacteria for metabolic processes. So, it can be said that β hemolysin toxin, DNase enzyme, and gelatinase enzyme produced by *A. hydrophila* are important virulence factors in the infection of *A. hydrophila* bacteria to fish (hosts).

CONCLUSION

Based on the results of the research that has been done, catfish (*C. batrachus*) cultivated in Buduran Middle Hamlet, Wedoro Waru, Wadung Asri Waru and Keboan Sikep Gedangan Sidoarjo showed β hemolysin toxin, DNase enzyme and gelatinase enzyme by 100% in all isolates.

ACKNOWLEDGMENT

The researchers would like to thank colleagues who have helped with the completion of this research.

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