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Isolation of Chitinolytic Bacteria from Shrimp Waste as a Control of *Aedes aegypti* Instar III Mosquito Larvae

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Abstract

Insect-borne diseases remain a serious health problem in Indonesia, especially Dengue Hemorrhagic Fever (DHF), which can cause mass mortality in developing countries. Biological control, such as using chitinolytic bacteria, is needed to contain the spread of this disease. The methods used include sampling and preparation of shrimp waste, manufacture of chitin agar media, isolation of chitinolytic bacteria, macroscopic and biochemical characterization, maintenance of *Aedes aegypti* larvae, observation of effects on mosquito larvae, and data analysis. The results obtained there are six isolates that have different morphological characteristics: IS1 to IS6 isolates have a round shape and milky white color, with wavy edges on IS1, IS2, and IS4, and flattened on other isolates. At elevation, IS1 and IS5 are flat, while the other four isolates are convex. Microscopic tests found 2 isolates contained Gram-negative and 4 isolates Gram-positive. The biochemical test of all six isolates was positive. Effect of administration of the concentration that caused the highest time of death of larvae on the fourth day, with a concentration of 15% and an average number of deaths of 4.66. Based on the results of the study, it can be concluded that six isolates of chitinolytic bacteria were successfully isolated, with diverse morphological characteristics, including differences in shape and color. Microscopic tests identified two isolates as Gram-negative and four isolates as Gram-positive. The biochemical test results showed that the six isolates were positive in the catalase, Simmons Citrate Agar (SCA), and Triple Sugar Iron Agar (TSIA) tests. Concentration administration affected the time of larval death, with a concentration of 15% showing the highest mortality rate on the fourth day, with an average death toll of 4.66.

Keywords: Chitinolytic Bacteria, Shrimp Waste, Mosquito Larvae

INTRODUCTION

Diseases spread by insects are still a serious health problem in Indonesia, especially Dengue Hemorrhagic Fever (DHF). This disease is caused by the dengue virus, transmitted by the *Aedes aegypti* mosquito as a vector. DHF can result in mass mortality in developing countries, both in urban and rural areas. With its rapid spread, it is necessary to control the vector of *Aedes aegypti* physically, chemically, and biologically. One of the control approaches taken is biological control (Indrayani & Wahyudi, 2018).

Biological control involves the natural enemies of *Aedes aegypti* by utilizing animals, plants, and bacteria. In the context of the use of bacteria as controllers, the main focus is on chitinolytic bacteria. These bacteria have the ability to produce chitinase enzymes that play a role in the degradation process of chitin into chitin derivatives (Yuniarti & Blondine, 2005; Prayogo *et al*., 2017). Bacteria that are able to break down chitin can be found in various environments, such as lakes, seas, soil, as well as organic waste, including shrimp waste susceptible to decay and pollutes the environment. To combat this, shrimp waste can be recycled into useful materials. The main components of shrimp waste, such as protein,

calcium carbonate, and chitin, make it a potential habitat for bacteria capable of degrading chitin (Darmawan *et al*., 2007; Dompeipen *et al*., 2016).

Research conducted using chitinolytic bacteria as biological controllers of *Aedes aegypti* insects has been widely conducted. The results of Pujiyanto et al.'s (2011) research isolating aquatic chitinolytic bacteria in several regions of Central Java obtained the B6 isolate, and it was known that the test results with a bacterial volume of 1 mL caused 97% larval death within 108 hours. Meanwhile, the results of research from Widiastuti & Marbawati (2016) obtained isolates of chitinolytic bacteria from shrimp shell baths which have potential as biological agents in dengue vector control, because they can cause the death of *Aedes aegypti* larvae by 53.4% at a volume of 8 mL, 56.6% at a volume of 16 mL, 70% at a volume of 32 mL, and 76.6% at a volume of 64 mL with an LC50 value of 2% at a volume of 20000 ppm.

This study aims to determine the characteristics of chitinolytic bacteria isolated from shrimp waste in TTU Regency and determine the concentration of chitinolytic bacteria isolates that can affect the mortality rate of *Aedes aegypti* instar III mosquito larvae.

METHOD

Tools and Materials

The tools used in this study include glassware, autoclaves, Laminar Air Flow (LAF), micropipettes, digital scales, stirring rods, magnetic stirrers, blenders, hot plates, Bunsen, measuring pipettes, pH meters, ose needles, filter paper, incubators, vortexes, L Glass, stationery, and digital cameras.

The materials used in this study were shrimp waste, HCL, NaCl, yeast extract, MgSO4, KH2PO4, bacterial agar, MGMK media, chitin, aquadest, 95% alcohol, spiritus, parafilm, tissue, well water, and *Aedes aegypti* instar III mosquito larvae.

Research Design

The method used in this research is an experimental method using a Completely Randomized Design (CRD). The treatment consisted of 3 concentrations (5%, 10%, and 15%) with 3 repetitions. Observations were carried out for 4 days after treatment.

Table 1. Experiment Design

Deuteronomy	**Isolate Concentration Treatment**		
	IS_1	**IS_2**	**IS_3**
U_1	U_1IS_1	U_1IS_2	U_1IS_3
U_2	U_2IS_1	U_2IS_2	U_2IS_3
U_3	U_3IS_1	U_3IS_2	U_3IS_3

Table 2. Isolate Concentration

Isolate Concentration Treatment (%)					
5%		10%		15%	
Isolate (mL)	Well Water (mL)	Isolate (mL)	WellWater (mL)	Isolate (mL)	Well Water (mL)
5	95	10	90	15	85

Work Procedure

Sterilization tools. Before isolating chitinolytic bacteria, all tools used must be sterilized using an oven at 1800C for 2 hours.

Shrimp waste sampling and sample preparation. Samples of shrimp waste taken from Pasar Baru, Kefamenanu, in the form of shrimp. The process of preparing shrimp waste samples begins with cleaning them using water, then drying them in the sun for one day, and finally grinding them using a blender and the process of laying to get shrimp waste powder. The powder will be used for the isolation of bacteria capable of degrading chitin.

Chitin agar media creation. Chitin media is made by weighing 2 grams of chitin powder, 0.12 grams of yeast extract, 4 grams of bacterial agar, 0.1 grams of MgSO4, and 0.1 grams of K2HPO4. All materials then dissolve using a hot plate to produce a medium. The formed medium is then sterilized using an autoclave for 15 minutes at a temperature of 121°C. After that, the medium is poured into a petri dish and allowed to stand for 15 minutes before use (Herdyastuti *et al*., 2009).

Chitinolytic bacteria isolation. Chitinolytic bacteria were isolated from 1 gram of shrimp waste samples. The sample is put into a test tube containing 9 mL of physiologically sterile NaCl solution, then diluted to the desired suspension (10-3) and homogenized using a vortex. Then, 0.1 mL of diluted solution is taken using a micropipette, and the sample is inserted into a petri dish containing chitin agar medium. After that, the surface of the media was flattened using L. Glass, and the petri dish was incubated in an inverted position for 48 hours (Chasanah *et al*., 2009). After the bacteria grow, selection is carried out by observing the clear zone formed. The isolates with the largest clear zones are purified to obtain pure isolates. The pure isolates obtained were then cultured in chitin media to reach absorbance (OD) 0.5 at a wavelength of 600 nm (Widiastuti & Marbawati, 2016).

Macroscopic and biochemical characterization of chitinolytic bacteria. Colony isolates are macroscopically characterized to determine their color, size, elevation, and shape. Microscopic characterization includes Gram staining. Biochemical tests performed include the catalase test, Simmons Citrate Agar (SCA) test, and Triple Sugar Iron Agar (TSIA) test.

Rearing of *Aedes aegypti* larvae. To maintain the larvae of *Aedes aegypti*, one black bucket is prepared by adding enough clean water. Then, filter paper is laid out on the water, and the bucket is closed. The bucket that has been covered with filter paper is then perforated and placed in several moist locations with minimal light. Within two to three days, the filter paper containing mosquito eggs is taken and transferred to a container filled with clean water. After two days, the eggs will hatch into larvae. The larva will develop from instar I to instar III. When the larvae reach instar III, and then transferred to the test container for further experiments (Buni, 2013).

Observation of the effects of *Aedes aegypti* mosquito larvae. Bacterial isolates that had been grown in chitin media with an OD600 value of 0.5 were inoculated in several proportions into a test container 5 mL of isolate culture was added to 95 mL of well water, 10 mL of isolate culture was added to 90 mL of well water, and 15 mL of isolate culture was added to 85 mL of well water. Then, 15 instar III larvae were put into each test container. Observations were made every 24 hours for 4 days. The parameters observed were the time of death of instar III larvae caused by the time concentration and the number of bacterial colonies that caused death (*Lethal Time Lethal Concentration*).

Data analysis. Data analysis in this study used quantitative descriptive, qualitative descriptive analysis, and SPSS.

RESULTS AND DISCUSSION

Isolation and Characteristics of Chitinolytic Bacteria

The results of the isolation of chitinolytic bacteria from shrimp waste samples resulted in six isolates. All bacterial isolates were characterized by colony morphology. The results of the isolation and characterization can be seen in Table 1.

Table 1. Morphological Characteristics of Chitinolytic Bacterial Isolate Colonies from Shrimp Waste

Bacterial Isolate	Colony Morphology			
	Form	Color	Edge	Elevation
IS_1	Round	Milk White	Wavy	Flat
IS_2	Round	Milk White	Wavy	Convex
IS_3	Round	Milk White	Flat	Convex
IS_4	Round	Milk White	Wavy	Convex
IS_5	Round	Milk White	Flat	Flat
IS_6	Round	Milk White	Flat	Convex

Based on Table 1, the morphological characterization of the colonies of the six isolates from shrimp waste has the same round shape and milky white color. The difference is seen in the edges and elevation of IS_1, IS_2, and IS_4 isolates have wavy edges, while IS_3, IS_5, and IS_6 have flat edges. Elevation is also different, with IS_1 and IS_5 having flat elevation, while IS_2, IS_3, IS_4, and IS_6 have convex elevation.

Pardosi (2018) found that of the 20 isolated bacterial colonies, 13 had irregular shapes and 7 were circular. The edges of the colony on 9 isolates are wave-shaped, 9 isolates are whole-shaped, and 2 isolates are lobate-shaped. A total of 16 isolates had flat elevation, while 4 colonies had elevated elevation, and 19 isolates were cream-colored, while 1 isolate was white. Fitri & Yasmin (2011) noted that all isolates of chitinolytic bacteria are round, with flat colony edges, and colony sizes are generally between 1.0 mm to 3.5 mm. Ruma *et al*. (2020) isolated chitinolytic bacteria from Vaname shrimp waste (Litopenaeus vannamei) and found that the five isolates had a spherical colony shape.

Microscopic Test and Biochemical Test

The results of microscopic tests and biochemical tests of the six isolates can be seen in Table 2.

Table 2. Gram Staining Test Results and Biochemical Test

Isolate Code	Gram stain	Biochemical Test		
		Catalase	*Simmons Citrate Agar* (SCA)	*Triple Sugar Iron Agar* (TSIA)
IS_1	Positive	Positive	Positive	Positive
IS_2	Positive	Positive	Positive	Positive
IS_3	Negative	Positive	Positive	Positive
IS_4	Positive	Positive	Positive	Positive
IS_5	Positive	Positive	Positive	Positive
IS_6	Negative	Positive	Positive	Positive

Based on Table 2, of the six isolates, four of them (IS_1, IS_2, IS_4, IS_5) are classified as gram-positive bacteria, while the other two isolates (IS_3, IS_6) are gram-negative bacteria. All six isolates showed positive results on biochemical assays, including the catalase test, Simmons Citrate Agar (SCA), and Triple Sugar Iron Agar (TSIA). A positive result in the catalase test indicates the presence of an enzyme that decomposes hydrogen peroxide (H2O2) into H2O and O2, which is characterized by the appearance of air bubbles. Biochemical tests on SCA show that all isolates can use citrate as a carbon and energy source. Meanwhile, a positive result on the TSIA test shows the isolate's ability to ferment lactose and sucrose.

Lay (1994) explains that the difference in color of gram-positive and negative bacteria is due to differences in the complex reaction of dyes to acetone alcohol bleaching solutions. Gram-positive bacteria remain purple while Gram-negative bacteria are pink. Rostinawati (2008) added that this difference shows a different cell wall structure between the two types of bacteria, where gram-positive bacteria have a cell wall with thick peptidoglycan, while gram-negative bacteria have a high lipid content.

Screening of Potential Bacterial Isolates

Based on the results of bacterial observations on selective media containing chitin, namely the minimum salt media chitin (MGMK), after 24 hours incubation of 6 isolates, there was one isolate, namely IS_2, which had a clear zone that showed chitinase enzyme activity

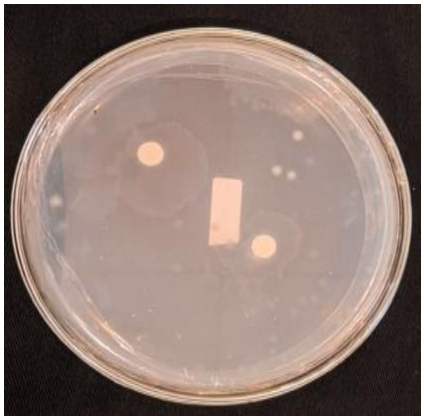

Figure 1. Growth of IS_2 Bacterial Isolate on MGMK Media shows a clear zone

Figure 1 shows the clear zone around IS_2 bacterial isolates, signaling chitinolytic activity in those bacteria. According to Suryanto (2005), the ability of bacterial isolates to form clear zones on MGMK media indicates their ability to produce chitinase enzymes. This suggests that IS_2 isolates have the ability to produce extracellular chitinase, which is responsible for breaking down chitin molecules into smaller N-acetyl-D-glucosamine monomers.

The effect of IS_2 isolate concentration on larval mortality of *Aedes aegypti* instar III mosquitoes can be seen in Table 3.

Table 3. Larval Mortality of *Aedes aegypti* Instar III Mosquitoes

Treatment	Average Mortality			
	Day 1	Day 2	Day 3	Day 4
IS_2 (5%)	$0,00^a$	$1,00^a$	$2,66^a$	$4,00^a$
IS_2 (10%)	$1,33^b$	$1,66^a$	$3,00^b$	$4,33^a$
IS_2 (15%)	$1,66^b$	$2,66^b$	$4,00^c$	$4,66^a$

Based on Table 3, the results of IS_2 isolate tests on *Aedes aegypti* instar III mosquito larvae with different concentration treatments, it was found that on the first day, there had

been deaths in larvae. The highest mortality occurred at a concentration of 15%, with an average mortality of 1.66%, while at a concentration of 10% the mortality rate was 1.33%, and larvae at the 5% treatment had not died. On the second day, the larval mortality rate further increased, where the 5% concentration had a mortality rate of 1.00%, the 10% concentration increased to 1.66%, and the 15% concentration increased to 2.66%. On the third day, the larval mortality rate continues to increase, reaching an average mortality of 4.00 at a concentration of 15%. On the fourth day, the entire treatment leads to the death of all remaining larvae. From the results of this test, a concentration of 15% has the highest average larval mortality compared to concentrations of 5% and 10%.

Before being given treatment, the behavior of *Aedes aegypti* larvae is generally no different, actively swimming on the surface of the water and circling. However, after treatment, differences begin to show. *Aedes aegypti* larvae were seen to be less active on the first day of observation, with movements being slow, according to the findings of Yasmin & Fitri (2010), which stated that *Aedes aegypti* larvae became slowed down after exposure to microorganisms as larvicide. This shows the potential of IS_2 bacterial isolates as degrading agents for mosquito larvae. The use of higher concentrations of chitinolytic bacteria results in a higher mortality rate of *Aedes aegypti* larvae.

Research by Pujiyanto *et al*. (2008) showed that IS_2 isolates caused 82.1% mortality on the fifth day, while other chitinolytic bacterial isolates caused 86.7% mortality of *Aedes aegypti* larvae during seven days of observation. Damage to the larval exoskeleton is caused by degradation of chitin, the main polymer of the exoskeleton, by chitinase activity produced by chitinolytic bacteria (Pujiyanto *et al*., 2011). This is in line with Jumar's (2000) findings that exoskeletons are body walls that function as the outer skeleton of insects.

CONCLUSION

Based on this study, it was concluded that six isolates of chitinolytic bacteria were successfully isolated, each exhibiting different morphological characteristics. IS_1 to IS_6 isolates have a round shape and milky white color, with wavy edges in IS_1, IS_2, and IS_4, and flattened in other isolates. At elevation, IS_1 and IS_5 are flat, while the other four isolates are convex. Through microscopic tests, it was found that two isolates were Gram-negative and four were Gram-positive. Biochemical tests showed that all six isolates tested positive with catalase, Simmons Citrate Agar (SCA), and Triple Sugar Iron Agar (TSIA). Concentration administration affects the time of death of larvae, with the highest time of death occurring on

the fourth day. A concentration of 15% indicates the highest number of deaths, with the average number of deaths reaching 4.66.

SUGGESTION

More in-depth molecular research and analysis are needed to study microorganisms as biological control agents in reducing the population of *Aedes aegypti* instar III mosquito larvae. More research is needed to understand the morphological damage that occurs in *Aedes aegypti* instar III larvae. Observation of the symptoms leading to the death of *Aedes aegypti* instar III larvae is also necessary.

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