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The Potential of Local Chitinolitic Bacteria Isolates as Larvacide of *Aedes aegypti* L.

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Abstract

The purpose of the research is to identify the potential of the local chitinolitic bacteria as biocontrol of *Aedes aegypti* L. This research has been conducted in the Microbiology Laboratory of Mathematics and Science Faculty, University of Syiah Kuala. The chitinolitic bacteria were isolated from water that taking in some area in Banda Aceh and Greater Aceh. The method used was an experimental method using completely randomize factorial designed (CRFD) with two factorial and consists of 6 isolates of chitinolitic bacteria and 4 concentrations of bacteria (0.0 mL, 0.5 mL, 1.0 mL dan 1.5 mL). The results show that the isolates bacteria do not have any effect on the *Aedes aegypti* L. Larvae death in the transformation from larvae to pupa until the seventh observation day. The concentration of the bacteria influences the *Aedes aegypti* L. larvae death during the transformation larvae to pupa.

Abstrak

Potensi Bakteri Kitinolitik Isolat Lokal sebagai Larvasida *Aedes aegypti* L. Penelitian ini bertujuan untuk mengetahui potensi dari bakteri kitinolitik isolat lokal sebagai biokontrol *Aedes aegypti* L. Penelitian ini telah dilakukan di laboratorium Mikrobiologi Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Syiah Kuala. Bakteri kitinolitik diisolasi dari perairan di daerah Banda Aceh dan Aceh Besar. Metode yang digunakan adalah metode eksperimental menggunakan rancangan acak lengkap dengan 2 faktor, yang terdiri dari 6 isolat bakteri kitinolitik dan 4 konsentrasi dari bakteri (0,0 mL, 0,5 mL, 1,0 mL dan 1,5 mL). Hasil penelitian menunjukkan bahwa isolat bakteri tidak memberikan pengaruh yang nyata terhadap kematian larva *Aedes aegypti* L. maupun perubahan bentuk dari larva menjadi pupa selama tujuh hari pengamatan. Konsentrasi memberikan pengaruh yang nyata terhadap kematian larva *Aedes aegypti* L. maupun perubahan bentuk dari larva menjadi pupa.

Keywords: Aedes aegypti L., *chitinolitic bacteria, larvacide, larvae, local isolate*

Introduction

Dengue fever is a disease caused by the dengue virus and transmitted by *Aedes aegypti* L. mosquitos, which belong to the insect class. This disease has become a major issue for public health in Indonesia.[1] Until now the spreading of the virus can only be controlled by killing the vector.[2] According to Gafur (2006),[3] the extermination of dengue fever's vector can be done when the mosquito is in the larval or adult stage.

The extermination of adult mosquitos is typically done by fogging and spreading ultra-low volume (ULV) sprayers.[1] Another way to reduce the vector increase is by modifying their habitats. For example, the container in which they usually breed can be managed naturally and also the extermination its nest.[4] Mosquitos also tend to breed in bathtubs, used tires, used goods,[5] and other places or objects that can collect water.[6] The extermination process in the larval stage of a mosquito's life can be accomplished by modifying habitats or controlled by using synthetic pesticide. The use of synthetic pesticide continuously showed many effects for environment.[7] The control by this method is not effective if implemented continuously because it cause the resistance to the larvae, the death of larvae predator and environment pollution.

Because of the negative effects of using larvaeide synthetic, it is very important to look for another

alternative that is better for the environment. The reported organisms that can replace synthetic pesticide are the extracts of *Eupatorium riparu'*s leaves,[8] water mushrooms Metharrhizium,[9] and *Mesocyclops aspericornis*, which has a high level of predation.[10]

In addition to plants and animals, bacteria can be used as natural larvacide for *Aedes aegypti* L. One example is clitinolitik bacteria. This bacteria can produce a clitinolitik enzyme that destroys the chitin substances.[11-12] The use of this bacteria based on the component of exoskeleton of adults mosquito that formed by chitinase enzyme. The damage to the larva's exoskeleton from this enzyme causes the death of the mosquito, which reduces the population.[13]

Research shows that chitinolitic bacteria can be isolated from many sources, such as the soil, breeding locations [14] and other areas that accumulate.[13] The areas with a high potential for gathering water in Banda Aceh and Greater Aceh are very large, so there is a lot of opportunity to isolate chitinolitic bacteria that can be used as a natural larvaeide for the *Aedes aegypti L.* mosquito. Most importantly, it is environmentally friendly. Therefore, research should be conducted to isolate chitinolitic bacteria in areas with bodies of water in Banda Aceh and Greater Aceh such as fishponds, rivers, and seawater.

Methods

Materials. Water samples (from the sea, fishponds, and rivers), the second instars' larva of *Aedes aegypti* L., chitin agar medium (0.5 % chitin colloid, 0.1% MgSO4.7H2O, 0.02% K2HPO4, 0.1% yeast extract, 1.5% agar), liquid chitin medium (0.3 % chitin colloid, 1% pepton, 0.5% yeast extract, 0.1% NaCl, 0.1% K2HPO4, 0.05% MgSO4.7H2O, 0.001% FeSO4.7H2O, 0.001% ZnSO4.7H2O, 0.0001% CuSO4.5H2O, 0.0001% MnSO4.nH2O, and 0.0001% CaCl2.2H2O, pH 7).

Breeding of *Aedes aegypti L.*'s larva. Larvae from the field was collected with ovitrap. Larva was bred until pupa stage and fed with yeast. Once the larvae reached the pupa stage they were kept in a container and put in a cage for breeding in Imago's stage. Male mosquitos in the imago stage were fed with a 10% sugar solution, but females in the imago stage were fed mouse blood by placing shaven mice into the cage for ±30 minutes. After 7 days, filter-moistened papers were placed in the cage for laying eggs. The eggs then breed until they reach the second instars stage for treatments.[9]

Chitin forming. Deproteination process. One hundred g of powdered shrimp shells were placed inside the Erlenmeyer, and 500 mL of NaOH 3.5% was added. This solution was then mixed on a magnetic stirrer for 2 hours at under 60 °C. The deposition was taken and washed with aquadest until it reached a neutral pH level and was then dried in the oven for 4 hours at under 60 °C.[15-16]

Demineralization process. The dried shrimp shell as product of deproteination (64 g) is dissolved into HCl 2 N for 640 mL. The compound is put into the room temperature during 2 days. The residue will washed by aquadest up to neutral pH and dried into oven during 4 hours in temperature 60 ºC to obtain chitin.[15-16]

Preparing of colloidal chitin. Twenty g of powdered chitin were dissolved in 400 mL of concentrated HCL and kept in the refrigerator overnight. The deposition that formed was filtered, and 200 mL of cold distilled water was added. The solution was then centrifuged at 4000 rpm for 15 minutes. The deposition was taken, and 200 mL of cold distilled water was added. The solution was neutralized with NaOH 10N until it reached a neutral pH level.

Next, the solution was centrifuged again at 4000 rpm for 15 minutes. The supernatant that formed were discarded, and 200 mL of distilled water was added to the deposition again. The solution was stirred to dissolve the residual salt and then centrifuged again at 4000 rpm for 15 minutes. The deposition that formed at this stage was a colloidal chitin.

Preparation of Chitin agar medium. The chitin agar media consist of 0.5% chitin colloid, 0.1% MgSO4. 7H2O, 0.02% K2HO4, 0.1% yeast extract and 1.5% agar. The material of chitin agar media and chitin colloid are sterilized by using autoclave during 15 minutes in temperature of 121 C but in the different media. And after be cool, the chitin colloid mixed to the other chitin agar media in sterile condition. The sterile chitin agar media pour into Petri saucer. The media stored into incubator in the reverse position before using.[13]

Isolation and selection of chitinolitic bacteria. Chitinolitic bacteria were isolated from the water samples collected from the seawater, rivers, and fishponds around Banda Aceh and Greater Aceh, and 20 samples were taken from each source.

The samples obtained were plated on a chitin agar medium for 1 mL and incubated for 48-72 hours at under 30 ºC. The colonies that grew and formed a clear zone were chitinolitic bacteria isolates. The isolates obtained were stored at 4 ºC for maintainance.[13] The selection of isolates had been done by regrowing all of the isolates on a chitin agar medium. After being incubated for 48 hours at under 30 ºC, the 6 best isolates were selected, which measured from the size of bacterial colonies that grow after spotted on chitin agar medium.

Multiplication cells of chitinolitic bacteria. Starter 1 scratch from each isolates, and inoculated in 100 mL of

culture medium and were incubated using a rotary shaker at a speed of 120.[13]

Biological test. The larvae of mosquito *Aedes aegypti* L. and *Culex* spp. put into the cultivation media for 10 individuals into 150 mL of water. Bacterial cultures that had been obtained were added to each maintenance container in the amounts of 0.5 mL, 1.0 mL, and 1.5 mL. The number of larvae's deaths was observed every day until the seventh day following the treatment. The *Aedes aegypti* L.'s larvae served as a control group and were not given the Chitinolitic bacteria's culture (0.0 mL).

Results and Discussion

Isolating chitinolitic bacteria. The chitinolitic bacteria isolates obtained for this research were collected from water sources in Banda Aceh and Greater Aceh. Sixty samples were taken, which included 20 seawater samples, 20 river water samples, and 20 fishpond water samples. The development of the water samples on the chitin agar medium showed slow growth, which is confirmed by the small size of bacterial colonies which grew until the third day of observation.

The number of isolates that were able to grow on the chitin agar medium was 24 isolates. After spotted on chitin agar medium in the same time to knew the chitinolitical index of each bacteria, nine isolates were obtained that were able to form a clear zone. According to Dewi (2008),[17] the clear zone formed on the chitin agar medium is caused by chitinase enzymes produced by the bacteria, which is an uncolored metabolite.

The number of local isolates of citinolitic bacteria selected to be applied as a treatment was 6 isolates. The selection of the 6 local isolates as larvacide has been done based on the size of the largest colonies of bacteria. The size of bacterial colonies ranged from 0.1–0.2 cm. The six local isolates use for the treatment represented each water sample. There were two local isolates from the seawater samples, two local isolates from the fishpond samples, and two isolates from the river samples.

The average size of the clear zone formed by bacteria was <0.1 mm. According to Dewi (2008),[17] the small, clear zone formed on the chitin agar medium could be caused by the condition of environmental temperatures required by each bacteria was not optimum. The six isolates selected for the larvae treatment were incubated for 18 hours on a rotary shaker before being cultured on the chitin agar medium. The objectives were to combine the bacteria into one cell (not colonized) and to increase the active bacterial cells to sufficient quantities for treatment.

The mortality of *Aedes aegypti L.*'s larvae after treatment. The variant analysis results obtained after seven days of treatment showed that the dosage of the different local bacterial isolates created a marked difference in causing the death of the larvae ($p > 0.05$). These results also reveal that the six local isolates used as larvaeide did not create a significant effect in causing the death of larvae ($p > 0.05$). The combination between six local isolates with different doses of larvaeide also did not affect the larvae mortality rate ($p < 0.05$).

Table 1 shows that the larvae that were treated with a dosage of 1.5 mL had a significantly higher mortality rate than the larvae treated with a different dosage. The treatment with a dosage of 0.5 mL and 1 mL shows that there was no significant difference in the mortality of the larvae. Table 1 illustrates that the best dosage of larvaeide is the highest dose concentration (1.5 mL). The research of Pujiyanto *et al.* (2008),[13] revealed similar results and concluded that a higher concentration of bacteria used as a larvaeide causes a higher mortality rate in larvae.

The low number of deaths of the larvae might be due to the type of chitinolitic bacteria that were isolated from bodies of water in Banda Aceh and Greater Aceh had a low number of Chitinolitic, it showed form the small size of clear zone formed after spotted on chitin agar medium. According to Dewi (2008),[17] that difference in chitinolitic can be related to the type of bacteria, the speed of growth from each bacteria on medium, and also the chitinase enzyme produced by bacteria. Nasran *et al.* (2003),[11] explain that the ability of bacteria to produce the enzyme is based on the type of bacteria and the environmental conditions, so bacteria may produce enzymes of various types and chitin structures.

Variant analysis results show that six local isolates that were used for treatment did not have any significant effect on the death of larvae. The most effective isolate was from the sample water of Lamnyong River (local isolate coding L4). The other isolates did not have a significant effect. The number of deaths of the larvae with each local isolate is shown on the Figure 1.

The figure above shows that the highest number of deaths of larvae occurred on the fourth and fifth day following the treatment. The highest number of the deaths occurred with the L4 isolate, and the lowest number of deaths occurred with B5 and AN1 isolates.

Table 1. The Influence of Concentration on the Average *Aedes aegypti* L.'s Larvae Mortality

Larvaeide doses	The average mortality of larvae
0.0 mL	0.00^{c}
0.5 mL	0.22^{b}
1.0 mL	0.22^{b}
1.5 mL	0.72^{a}

Caption: Superskrip the different letters (a,b,c) show a real difference ($p < 0.05$)

The death of the larvae with L4 is shown to begin on the first day and increase on the fourth and fiveth day following treatment.

A decrease in the mortality of larvae occurred on the sixth day presumably because the bacteria's ability to survive decreased due to the reduction of nutrients in the breeding water of the larva. This conclusion is also mentioned by Pelczar and Chan (2008),[18] they state that the cell of bacteria can be decreased or dead within a few days or weeks depending on the species of bacteria and the nutrient availability in environment.

The death of the larvae in this study revealed damage to the exoskeleton's structure. Figure 2(a) shows the structure of the exoskeleton of a larva in which the thorax was destroyed, while Figure 2(b) shows a normal larva exoskeleton. According to Borror *et al*. (1996),[19] the main compound of an insect's exoskeleton is chitin polysaccharide, it is cover with the protein matrix. Pujiyanto *et al*. (2008),[13] claim the damage that occurred on the larva's exoskeleton was caused by the degradation of chitin compounds, which is the main component of a larva exoskeleton. Polymer chitin degradation caused by chitinase activity produced by chitinolitic bacteria.

Chitinase enzymes produced by chitinolitic bacteria that were applied as a larvacide known to break the larva's eksoskleton that is formed by a chitin compound. According to Suryanto and Munir (2006),[11] chitinase

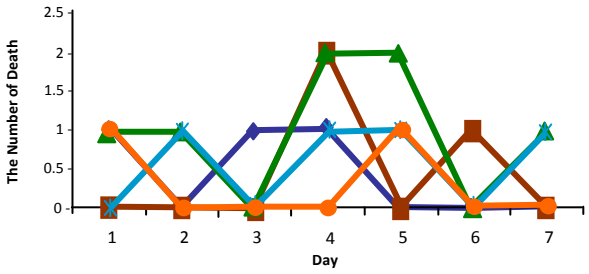
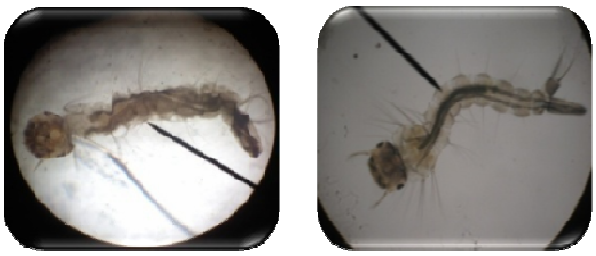


Figure 1. The Number of Deaths of the Larvae with Each Local Isolate: B5 (♦), DR4 (■), L4 (▲), SK3 (X), KR3 (X), AN1 (●)



(a) (b)

Figure 2. The Death of a Larva with the Treatment (a) and the Living Larva (b) (Magnification 50x)

enzymes change chitin polymers into monomers of N-Asetil-glukosamin by breaking the glycoside bonds on chitin. Furthermore, the monomers of N-Asetilglukosamin will be dismantled slowly by bacteria until carbon and nitrogen are obtained for the survival needs of bacteria.

The larvae that survive to enter the pupa stage. The Variant analysis result of the number of larvae that were survive to enter the pupa stage showed that the dose treatment were significantly effects to formed pupa ($p < 0.05$). The local isolate treatments had been known did not effect on formed of pupa ($p > 0.05$). The combination of isolates and dosages did not affect the number of larvae that reached the pupa stage, either ($p > 0.05$). The average number of larva that reached the pupa stage is illustrated in Table 2.

Table 2 shows that increasing the bacterial doses decreases the number of larvae who become a pupa. The highest number of larvae that became a pupa corresponds with the lowest concentration (0.0 mL), and the least number corresponds with the highest concentration (1.5 mL). Doses of 0.5 mL and 1.0 mL show no difference in the number of larvae becoming a pupa.

The different number of larva that formed allegedly caused by the influence of bacteria concentration which applied to larvae. The lowest number of larvae able to become a pupa was caused by the highest dose of bacteria applied, so that the chance of larvae to be exposed by bacteria were higher. The treatment with the 0.0 mL dose did not contain bacteria, so the larvae given this treatment were not exposed to bacteria. Therefore, the number of larvae that were able to become a pupa is much greater. The results obtained are consistent with those reported by Pujiyanto *et al*. (2008).[13] Chitinolitic bacteria not only affected the mortality of larvae but also influenced the morphological process of larvae when the pupa formed.

The chitinolitic bacteria treatment administered to the larvae influenced the process of metamorphosis from larvae to the pupa stage. This change occurred after chitinolitic bacteria were administered slowly. According to Gandahusada (1998) and Hamzah (2004),[20-21] the

Table 2. The Influences of Concentration to the Average Number of Pupa Formed

Larvaside dose	The average number of the larvae that survived to enter the pupa stage
0.0 mL	8.66^{a}
0.5 mL	5.38^{b}
1.0 mL	4.83^{bc}
1.5 mL	3.5^{c}

Caption: Superskrip the different letters (a,b,c) show a real difference ($p < 0.05$)

Figure 3. The Normal Pupa of *Aedes aegypti* L.

(a) (b)

Figure 4. (a) and (b) *Aedes aegypti* L. Failure to Become a Pupa

pupa stage generally begins six days after the eggs hatch. In this study, the pupa stage began nine days after the eggs hatched.

Chitinolitic bacteria thought effected the forming process of pupa's body, it is seen form pupa formation were not completely (Fig. 3). The failure to reach the pupa stage formation occurs because bacteria can hydrolyze the chitin, which results in the disruption of metabolic processes in a larva's body. Disruption of metabolism in the larval stage can affect the process of larval metamorphosis (change of larval stage) enter to the pupa stage.

Figure 4 is an illustration of the condition in which larvae cannot metamorphose into pupa. Figure 4(a) shows a larva of *Aedes aegypty* L. that died before reaching the early formation of the pupa stage Do you mean that the head and the abdomen began to form beyond the larva stage, but the insect died before the formation was complete Figure 4(b) shows the insect in between stages because it has a thorax but still has the head of a larva. Both of two morphological larvae which is failure to become a pupa is very different form a normal pupa's morphology in Fig. 3.

Conclusions

Based on this research, it can be concluded that from 60 isolated samples, 24 isolates of local chitinolitic bacteria were obtained that were able to grow on a chitin agar medium. Local isolates which is taken for treatment were 6 from 9 isolate that are able to form clear zone. Administering doses of local bacterial isolates showed significant differences in the mortality rate of larvae and in reaching the pupa stage ($p < 0.05$). Six of the local isolates used for larvacide treatments of *Aedes aegypti* L. did not show a significant effect on larvae mortality or on reaching the pupa stage ($p > 0.05$).

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