

Larvicidal Effect of Mixture of *Beauveria bassiana* Crude Metabolite and Chitinase Enzyme against *Aedes aegypti* Larvae

Efek Larvasida Campuran Metabolit *Beauveria bassiana* dan Enzim Kitinase terhadap Larva *Aedes aegypti*

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Abstract

Aedes aegypti is a major vector of dengue, a deadly disease causing deaths of millions of people in developing countries. *Aedes aegypti* control with chemical insecticide is widely used, and affects on a widespread insecticide resistance. Mosquito biological control was needed to replace the use of chemical insecticide. This study aimed to evaluate larvicidal effect of mixture of *Beauveria bassiana* crude metabolite and chitinase enzyme against *Aedes aegypti* larvae. An experimental study using completely randomized design was conducted in March-April 2016 at Banjarnegara Research and Development Unit for Zoonosis Control. Biolarvacide formula was made of mixture with 2 : 1 ratio between *Beauveria bassiana* crude metabolite and chitinase isolated from chitinolytic bacteria, which was propagated by the Agency's Bacteriology Laboratory. In experimental method, 120 *Aedes aegypti* larvae (the third instar) were exposed with four concentrations of biolarvacide formula (4%, 8%, 16%, and 32%) in 3 replicates. Results showed that exposure of biolarvacide formula caused the third instar larval mortality which started on the first day of exposure. Probit analysis showed LC₅₀ value was obtained at concentration of 53.2 ppm. This shows that larvacide formula of *Beauveria bassiana* and chitinase enzyme compounds are effective to be used as larvacides against *Aedes aegypti* larvae.

Keywords: *Aedes aegypti*, *Beauveria bassiana*, larvacide

Abstrak

Aedes aegypti merupakan vektor utama demam berdarah dengue (DBD) penyakit mematikan yang menyebabkan kematian jutaan orang di negara berkembang. Pengendalian *Aedes aegypti* dengan insektisida kimia banyak digunakan dan menimbulkan dampak resistensi insektisida yang meluas. Pengendalian nyamuk secara hayati diperlukan untuk menggantikan penggunaan insektisida kimia. Penelitian ini bertujuan untuk mengetahui efek larvasida campuran metabolit sekunder *Beauveria bassiana* dan enzim kitinase terhadap larva *Aedes aegypti*. Sebuah penelitian eksperimental dengan desain rancangan acak lengkap dilaksanakan pada bulan Maret-April 2016 di Balai Litbang P2B2 Banjarnegara. Formula larvasida hayati dibuat dari campuran dengan perbandingan 2:1 antara metabolit sekunder jamur *Beauveria bassiana* dan enzim kitinase yang diisolasi dari bakteri kitinolitik biakan Instalasi Bakteriologi Balai Litbang P2B2 Banjarnegara. Dalam metode eksperimen, sebanyak 120 ekor larva *Aedes aegypti* (instar III) dipaparkan dengan empat konsentrasi formula larvasida hayati (4%, 8%, 16% dan 32%) dengan tiga buah ulangan. Hasil penelitian menunjukkan bahwa formula larvasida hayati menyebabkan kematian larva instar III *Aedes aegypti* yang dimulai pada hari pertama paparan. Hasil analisis probit menunjukkan nilai LC₅₀ campuran *Beauveria bassiana* dan enzim kitinase sebesar 53,2 ppm. Hal ini menunjukkan bahwa formula larvasida dari campuran *Beauveria bassiana* dan enzim kitinase efektif untuk digunakan sebagai larvasida terhadap jentik *Aedes aegypti*.

Kata kunci: *Aedes aegypti*, *Beauveria bassiana*, larvasida

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Introduction

Dengue is currently considered globally as the most important arboviral disease that occurred in tropical and sub-tropical climates worldwide. This infection is transmitted by the biting of female adult *Aedes aegypti* or *Ae. albopictus*.¹ Currently, the only method of preventing or controlling dengue virus transmission is to effectively wipe out the vector mosquitoes. It consists of environmental management, biological, genetic, and chemical control using synthetic insecticides. The synthetic insecticides have been widely used to control vector species of mosquitoes. These insecticides have caused adverse effects on nontarget organisms and high operational cost in addition to physiological resistance. Previous studies have reported the resistance of *Aedes aegypti* toward various synthetic insecticides in Java.²⁻⁴

Larval control would seem to be an ideal approach to mosquito control as it combats mosquitoes before they reach the stage at which they can transmit the disease. Larviciding using chemical compound such as Temephos remains to be the major method of larval control. This method was regarded to be able to rapidly reduce the vector population. However, these repeated applications may potentially lead to larvae resistance to insecticides, kill the larvae predators and environmental pollution.⁵ Resistance of *Aedes aegypti* larvae to Temephos has been reported in many areas.²⁻⁴ Therefore, it requires an eco-friendly alternative method of *Aedes aegypti* control such as biological control using mosquitoes' natural enemies.⁶

Chitinolytic bacteria have some important advantages over conventional insecticides in mosquito control operations.⁷ The larvicidal effect derived from its important role in the degradation of chitin, a major component of insect exoskeletons. Suryanto *et al.*,⁸ stated that chitinolytic bacteria can be used as larvacide because they are capable of decomposing chitin. Chitin plays an important role in cell wall morphogenesis and exoskeleton development. Exoskeletons stabilize cell, tissue, and body morphology in many living organisms including fungi, plants, and arthropods.

Exoskeleton damage can inhibit growth and cause mortality of mosquito larvae. Widiastuti,⁹ has isolated entomopathogen chitinolytic bacteria from shrimp waste (isolate BKUd01).⁸ When the fourth instar of *Aedes aegypti* larvae was exposed to this isolate, LC₅₀ value was reached within eight days. However, this study still needs an improvement due to its limitation i.e. the high value of LC₅₀ = 20,000 ppm. Addition of 20,000 ppm chitinolytic bacteria results the larval growth medium turbid and stinky. This condition will reduce the acceptability of isolate BKUd01 usage as larvacide in society. Study by Pratiwi,¹⁰ showed that larvacide application was acceptable to be applied among society if the larvacide was eco-

friendly and did not pollute the water bodies. Therefore, the use of isolate BKUd01 as natural larvacide still needs more improvement in order to obtain the higher effectivity.

Beauveria bassiana is entomopathogenic fungi which can be used to control pest in agriculture and health. Ikawati,¹¹ reported that application of *Beauveria bassiana* to control *An. maculatus* larvae showed a slow larvicidal effect. LC₅₀ was reached at 1x10⁷ concentration with 11 days time of exposure. Study conducted by Putri *et al.*,¹² reported that the average density of *Beauveria bassiana* spores that can kill 50% of the third instar *Aedes aegypti* larvae during 24 hours of exposure was 49.0x10⁹ spora/mL. Whilst for 48-hour exposure, the average density of *Beauveria bassiana* spores that can kill 50% of third instar *Aedes aegypti* larvae was 19.0x10⁸ spora/mL.¹¹

The potential use of metabolites of *Beauveria bassiana* as a biological control agent for *Aedes aegypti* requires an initial study. Singh and Prakash,¹³ reported that the use of *Beauveria bassiana* metabolites can be an alternative method that is inexpensive and eco-friendly in the control of larvae of Anopheles. Widiastuti and Kalimah,¹⁴ also reported the use of *Beauveria bassiana* metabolites as larvacide against *Aedes aegypti*, but the highest mortality rate was only 63.3% obtained within 8 days. That study still needs more improvement to obtain the higher effectivity. In this study, a larvicidal test was conducted using a formula made from mixture of *Beauveria bassiana* metabolites and chitinase enzyme produced by isolates BKUd01 against *Aedes aegypti* larvae. The study question was if the formula could be more effective to be used as larvacide against *Aedes aegypti* larvae. The aim of this study was to obtain the more effective formula of biological larvacide as a biopesticide for controlling *Aedes aegypti* larvae.

Method

This study was conducted in The Microbiology, Molecular Biology and Immunology Laboratory and Entomology Laboratory in Research and Development Unit for Zoonosis Control, Banjarnegara, Indonesia in March-April 2016. Chitin liquid medium was prepared and sterilized in autoclave for 14 minutes at 121°C. This isolate was cultured in liquid chitin medium and harvested when it reached log phase (OD=0.5 at 600 nm).¹⁶ Chitinase enzyme was obtained by centrifuging the isolate at 6000 rpm (4°C) for 20 minutes, then supernatant was collected.

Beauveria bassiana spores suspension was diluted in 1 ml aquadest to achieve dilution rates 1x10⁸. Collected spores were aseptically inoculated into 200 mL of *Potato Dextrose Broth* (PDB) and incubated at room temperature for eight days.¹⁴ Culture filtrate was obtained by fil-

tering the culture medium *Beauveria bassiana* in PDB medium using filter paper (Whatmann no 42).

Larvacide formula was prepared by mixing crude metabolites of *Beauveria bassiana* and enzyme chitinase with ratio 2:1. The solution was thoroughly mixed to get a homogeneous mixture, and serial dilutions of 2%, 4%, 8% and 16% were prepared with aquadest as control. Each experiment and control were replicated three times. Each replicate and each control group received 10 live the third instar of *Aedes aegypti*. Nutritional supplements were added during the assays. Larval mortality was registered until 192 hours (8 days) of exposure without any larvae replacement per day. The larvae were considered dead if they did not present movement. While, the larvae that had become mosquitoes were removed from the containers and counted as live samples.¹⁶

The data from mosquito larvicidal bioassay were subjected to *Saphiro Wilk* test to determine if the data were normally distributed, then followed by Levene test to assess the homogeneity of variances. If the data were normally distributed and the variance were homogen, one way anova was run and followed by post hoc test to confirm in which the differences occurred between concentration. The data from mosquito larvicidal bioassay were also subjected to manually log probit analysis for calculating the LC₅₀ and LC₉₀.

Results

The natural larvacide formula was found to have larvicidal effect against the third instar of *Aedes aegypti*. Larvicidal activity of natural larvacide formula at 8-day

exposure against *Aedes aegypti* showed the mortality rate 80%, 90%, 93%, and 100% against 2%, 4%, 8%, and 16% concentration respectively (Table 1).

The results showed that the overall mortality of *Aedes aegypti* larvae on each concentration had already begun on the first day. In general, the average of *Aedes aegypti* larvae mortality were higher along with the increasing concentration of the test. On the eight day of exposure, the highest mortality was found at concentration of 16%. Exposure to biological larvicides formula allegedly also affected the development of the larval stage test. The percentage of larvae developed into pupae and its survival in a variety concentration of biological larvicides treatments shown in Table 2.

Table 2 showed that the treatment group generally presented that pupa formation began on day three of exposure. In formula with a concentration of 8% pupa emergence occurred on the first day, but could not survive. Whilst the control group pupa emergence occurred on the fourth day. The number of pupa displayed in the table was a combination between the cumulative number of larvae that had become pupae and the pupa that had become a mosquito.

Exposure to biological larvicides formula at concentrations 2%, 4%, 8% and 16% to the third instar *Aedes aegypti* larvae cause larval death. The larvae that died on the first day due to exposure to each concentration showed a different colony density of *Beauveria bassiana* according to the high content of *Beauveria bassiana* colonies. The higher the concentration of a given larvacide formula, colonies of *Beauveria bassiana* seemed in-

Table 1. Effect of Different Concentrations of Natural Larvacide Formula on the Mortality Rate of *Aedes aegypti* Larvae

Formula	Concentration	Mean of the Number of Dead <i>Aedes aegypti</i> Larvae (%)							
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Control	0	0%	0%	0%	0%	0%	0%	0%	0%
Natural	2%	16.67	20	30	36.67	40	43.33	56.67	80
Larvacide	4%	16.67	30	43.33	50	60	63.33	73.33	90
	8%	43.33	56.67	63.33	63.33	66.67	70	90	93.3
	16%	96.67	96.67	96.67	96.67	96.67	100	100	100

Table 2. The Percentage of *Aedes aegypti* Larvae Development Become Pupae after Exposure to Various Concentrations of Natural Larvacide Formula

Group	Concentration	of Larvae	Average Number of Pupa (%) and Its Survival toward Natural Larvacide Formula Exposure															
			Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7		Day 8	
			L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
Control	0%	10	0	0	0	0	0	0	5	0	25	0	25	0	40	0	55	0
Treatment	2%	10	0	0	0	3.3	0	16.7	3.3	40	3.3	43.3	6.7	20	13.3	6.7	16.7	0
	4%	10	0	0	0	0	0	6.7	0	20	0	26.7	3.3	13.3	3.3	3.3	6.7	6.7
	8%	10	6.7	6.7	0	6.7	6.7	10	13.3	10	20	13.3	10	13.3	3.3	16.7	0	20
	16%	10	0	0	0	0	3.3	3.3	0	3.3	0	0	3.3	0	3.3	0	3.3	0

Notes:

L= Live, D= Dead

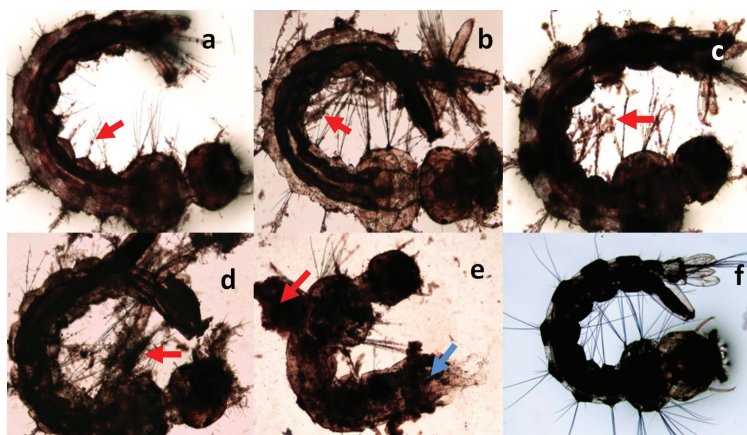


Figure 1. Dead *Aedes aegypti* Larvae on the First Day Exposure of Concentration

Notes:

(a) concentration 2%, (b) concentration 4%, (c) concentration 8%, (d) concentration 16%, dead *Aedes aegypti* larvae on the second day of exposure at concentration of 16% (e) dan control (f). Red arrows indicate a colony of *Beauveria bassiana* on the larval body. Blue arrows indicate exoskeleton damage on the larval body.

creasingly crowded even on the first day of exposure (Figure 1). The effects of exposure to chitinase enzyme on dead larvae at the second day appeared as exoskeleton degradation on larvae body.

Normality test results indicated that the data had normal distribution with $p = 1$ ($p \text{ value} > 0.05$), homogeneity test also showed a homogeneous distribution with $p = 0.057$ ($p \text{ value} > 0.05$). While the results of ANOVA test showed $p \text{ value} = 0.001$ ($p > 0.05$), which means that there were significant differences in the average of larvae mortality at different concentrations, followed by post hoc test (Table 3).

Table 3 presents significant difference in most concentrations except for a concentration of 4% and 8% which showed no difference. The results of probit analysis for exposure to biological larvicides formula indicated that a value was 1.47 and the value of b was 4.96, thus probit regression equation was $y = 4.96 + 1.47 \log c$.

Figure 2 illustrates that the exposure to biological larvicides formula on probit at value 50% mortality log concentration showed a value of 0.027, so that the value would be 1.06 mL concentration. From these calculations, the LC_{50} value after 8 days of treatment was 0.00532% equal to 53.2 ppm. Meanwhile, the exposure to biological larvicides formula on probit at value 90% mortality log concentration showed a value of 0.899, so that the value would be 7.925 ml concentration. From these calculations, the LC_{90} value after eight days of treatment was 0.039% equal to 390 ppm.

Discussion

Larviciding is one of the most common methods in dengue vector control. Larvicides target larvae in the

Table 3. Results of Post Hoc Test to Determine Differences between Various Concentrations with Death of Larvae

Concentration		p Value
2%	4%	0.015*
	8%	0.01*
	16%	0.00*
	control	0.00*
4%	8%	0.51
	16%	0.00*
	control	0.00*
8%	16%	0.01*
	control	0.00*
16 %	control	0.00*

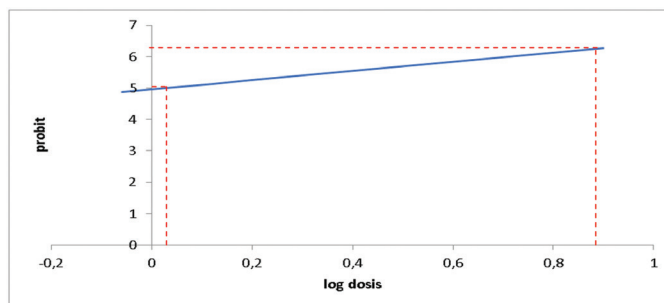


Figure 2. Graph of Probit Equation Linear Regression

breeding habitat before they can mature into adult mosquitoes and disperse. Larvicide treatment of breeding habitats helps to reduce the adult mosquito population in nearby areas. Larviciding can be more favourable because it is easier to kill the non-flying forms of the mosquitoes than going after the adults that can fly a kilometer or more.

The results showed that the biological larvicides formula which made from a mixture of crude metabolite of

Beauveria bassiana and chitinase enzyme had larvicidal effect against the third instar *Aedes aegypti* larvae. This is in line with study by Singh and Prakash,¹³ in 2010 which reported that the crude metabolites of *Beauveria bassiana* had larvicidal effect against larvae of *Culex* and *Anopheles*.

Larvicide assay results showed that exposure to all concentrations of biological larvicides formula caused larvae death since the first day of observation. ANOVA results showed there were significant differences in larvae death average at different concentrations, whereas post hoc results showed that there were differences in mortality at different concentrations except for concentration of 4% and 8%. Concentration of 16% showed the fastest larval death effect because on the sixth day all the larvae have died (100%). This indicated a shorter time compared to the results of study by Ikawati,¹¹ on a slightly lower dose (1×10^7) which can kill 50% of *Anopheles sp* larvae at 11.69 days.

Beauveria bassiana, an entomopathogenic filamentous fungus with a high potential for insect control, because its spores are relatively easy and inexpensive to mass produce for field applications. Moreover, it is known to have nontoxic effects on nontarget organisms, including animals and humans.¹⁷ *Beauveria bassiana* kills arthropods as a result of the insect coming into contact with the conidia (fungal spores). The contact can be made in several ways. The most common and effective is the spray droplets landing on the pest or by walking on a treated surface. Once the fungal spores attach to the insect's cuticle, the fungus spores germinate sending out threaded hyphae which penetrate the insect's body and proliferate. It takes 3 to 5 days for an infected insects to die. The dead insect may serve as a source of spores for crude spread of the fungus. An infected adult male will also transmit the fungus during mating.¹⁵ The observation on Figure 1 indicates that *Beauveria bassiana* colonies were found in larvae dead body, especially in the lateral hair. In general, *Beauveria* species attack their host insects percutaneously.

Ikawati,¹¹ explained that in the initial contact, mold spores will stick to the lateral hair in larval body. The infection pathway consists of the following steps that are attachment of the spore to the insect cuticle; spore germination on cuticle; fungi that attach to the body of the insect host can germinate and grow to form a tube sprouts; penetration through the cuticle, the penetration is done mechanically or chemically by enzymes or toxins; overcoming the host immune response; proliferation within the host; saprophytic outgrowth from the dead host and production of new conidia. In the end, the spores will cover all the body and disrupt the movement of larvae that will cause death. The results showed that the body of dead larvae were covered by colonies of fun-

gus *Beauveria bassiana* with a density that is consistent with the concentration of a given larvicides formula. The higher the concentration of larvicide formula, the fungal colonies were found more solid.

Beauveria bassiana fungus has a wide distribution. This fungus grows naturally in soils throughout the world. *Beauveria bassiana* has a white or light-colored mycelium. Conidium was formed on sympodial shape from parental cells which will be stopped on its peak. As soon as a fungal spore is exposed to its favourable conditions, it changes from a dormant state to an actively metabolizing cell, and then a germ tube emerges from the spore. When the germ tube reaches a certain length, the spore is considered to be germinated. In the next steps, germ tube elongation and branching take place until the mycelium forms a colon.¹²

This fungus will further remove toxins beauverin makes insect tissue damage. Within days, the insects will die. After that, the fungal mycelia will grow throughout the body of the insect. Insects with the fungus *Beauveria bassiana* will die with a hardened body and covered by threads of white hyphae.¹¹

Biological larvicides formula used in this study was composed by chitinase enzymes and crude metabolites *Beauveria bassiana* fungus. In the preparation of crude metabolites fungus *Beauveria bassiana*, colonies grown in liquid medium filtered using filter paper with 2.5 μ m pore size. However, when applied to the test container, fungal colonies can grow (forming conidia) and infect the larvae body. This indicates that the size of the spores of *Beauveria bassiana* strain used is smaller than 2.5 μ m. Jeffs et al.,¹⁸ reported that there are differences in the size of the morphology of the various strains of *Beauveria bassiana*. One of the strains coming from New Zealand has a spore size of less than 2.5 μ m.

Morphology of dead *Aedes aegypti* larvae on the first day was different with the larvae died on the second day of exposure to concentrations of 16%. Dead larval body on the first day did not show any exoskeleton damage, while on the second day the damage began to appear, especially in the tail. This is consistent with study by Yasmin and Fitri,¹⁹ which showed that the longer the larvae exposed to the chitinolytic bacteria, the exoskeleton structure in the larval body will be damaged more. In this study, chitinase enzyme from chitinolytic bacterial was applied to the larval medium, so that the enzymes can work in all parts of the larval body without being influenced by the nature of bacteria tropism. Exoskeleton is the body wall that serves as an outer skeleton of insects. Damage to the exoskeletons of the larvae was presumably occurred because the chitinase enzyme has degraded chitin exoskeleton which are building blocks of larvae body.

Chitin in larvae serves as body protector and the pri-

mary mechanism to limit water loss through the body walls.²⁰ According Pujiyanto *et al.*,²¹ larvae eksoskeleton damage caused by degradation of chitin which is a major polymer exoskeletons by chitinase activity produced by bacteria chitinolytic. This is supported by Gooday,²² that chitin degradation is mainly carried out by microorganisms, which chitin can be a source of carbon and nitrogen for growth. In this study, exposure to biological larvicides formula led to the formation time of pupae in the treatment group lasted shorter than the control group.

The smaller the LC₅₀ value indicates that an insecticide has higher toxicity value. The results showed that the LC₅₀ value was obtained at concentration of 53.2 ppm. This value is higher of it compared to several other studies. Bramachary and Paily,²³ reported metabolite products similar to enzyme chitinase as produced by *Pseudomonas fluorescens* has LC₅₀ values of 2.29 ug/mL or 2.29 ppm against larvae of *Culex quinquefasciatus*. However, the LC₅₀ value of natural larvacide formula used in this study was lower if compared to Bucker *et al.*,²⁴ study which reported that endophytic fungi extract and basidiomiset extract indicate LC₅₀ values at 101.8 ppm and 156.8 ppm against *Aedes aegypti* larvae. In addition, the mixture of LC₅₀ *Beauveria bassiana* crude metabolites and enzyme chitinase is also lower than in study by Widastuti,⁹ stating that LC₅₀ value of isolate BKUd01 against *Aedes aegypti* was achieved at concentration 2000 ppm. These differences indicate that application of *Beauveria bassiana* crude metabolites and enzyme chitinase can produce higher larvicidal effects than live isolated applications.

Observation showed that the application of natural larvacide formula changes the water condition in larvae container, especially at a concentration of 16%. The water in larvae container become cloudy but not smell. This showed a better result compared to study by Widastuti,⁹ which stated that the application of life chitinolytic bacteria as biolarvacide causes the water in containers become muddy and stinky. Changes in water containers in the application of larvicides should be considered as the application of larvicides in community, especially for containers of water consumption should be sought not leading to a change in the water. Larvicides which change the water condition in the container will tend to be unacceptable among the public.

Study by Pratiwi mentioned that the application of larvicides in the bath tub can be tolerated by the respondent if the larvacide does not cause discoloration and odor changes in water. One of the factors that inhibit community interest in using larvicides is because the process related to the use of clean water for daily use. Thereby the community were preferred to drain their bath tubs instead of using larvicides.⁹

Some weaknesses were found in the application of bi-

ological larvicides exposure formula from a mixture of *Beauveria bassiana* crude metabolites and chitinase enzyme on *Aedes aegypti* larvae, which showed that the formula as biological larvicides still require further processing. Nevertheless, the results of this study indicated that biological larvacide formula of a mixture of crude metabolites and enzyme chitinase *Beauveria bassiana* had larvicidal effect against larvae *Aedes aegypti* and had the potential to be developed as biolarvacide. This study is an advanced stage of development of bacterial biological larvicides chitinolytic as an alternative in vector control activities.¹⁰ Some of the successful and large-scale vector control programmes using microbial insecticides have been reported worldwide. The main advantages of biological agents in vector control are their specificity to target pests, safety to the non-target organism, that they do not cause pathogenic effects on environment and human health and can be used to control vectors which develop resistance to the conventional insecticides, they fit as ideal components in integrated vector control management. *Beauveria bassiana* is not toxic or infective to mammals, and exposure to the people and the environment will be minimal to non-existent. Therefore, no adverse effects are expected on children, adults, pets, or the environment when this fungus is used as larvacide.

Conclusion

Formula of biological larvicides derived from a mixture of *Beauveria bassiana* crude metabolites and chitinase enzyme has a potential as the biocontrol agent in controlling the dengue vector. It can cause *Aedes aegypti* larval death with LC₅₀ values of 0.00532% or 53.2 ppm and LC₉₀ values of 0.039% or 390 ppm.

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