A Study on A Local *Bacillus thuringiensis* SP7 To Control Mosquito Larvae of *Aedes aegypti*

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**Abstract**: *Bacillus thuringiensis* (Bt) is a gram-positive bacteria, spore-forming, and producing crystal proteins used as bioinsecticides. The purpose of the study was to know potential of local isolates of Bt in controlling mosquito larva of *Aedes aegypti*. Isolation of Bt was conducted from soil samples of Tongkoh Forest, Brastagi, North Sumatra, Indonesia. Bt spore and crystal protein were observed using compound light microscope and further using scanning electron microscope. Two suspected isolates were choosen based on their morphological and biochemical characteristic. Further identification was done using their 16S rRNA gene. To know Bt growth pattern in inexpensive C and N-source, Bt isolate was grown in culture media with molasses and urea as C and N sources. To assay on Bt isolate to control mosquito, larva instar 3 of mosquito were put in 50 ml of test media of concentration of 10, 20, 30, 40, and 50% of Bt culture in plastic cup. Observation was done after 24 hrs. Two isolates SP7 and SP15 showed to have morphological and biochemical characteristics similar to that of Bt. Identification based on 16S rRNA gene sequence showed that SP7 and SP15 were closed to *B. thuringiensis* strain MCCC 1A00395 with similarity of 99%. Since two Bt isolates seemed to be similar to *B. thuringiensis* strain MCCC 1A00395, SP7 was choosen for further study. SP7 reached its maximum growth at 30 hrs of incubation time with cell number of 17.01 log CFU/ml in culture media with molasses and urea as C and N sources. It showed to kill up to 97.5% mosquito larvae at concentration of 50% bacterial culture.

**Keywords**: *Aedes aegypti, Bacillus thuringiensis*, bacterial cell growth, crystal protein.

**Introduction**

Malaria, dengue, and several life-treathening diseases was transmitted by mosquitoes. Dengue is a disease transmitted to human through the bite of mosquito *Aedes*. The disease is common in tropical and subtropical regions. Data from around the world shows that Asia ranks as the first in the number of dengue sufferers, and Indonesia occupies the highest dengue fever case in Southeast Asia each year¹. Efforts to overcome and prevent the disease through mosquito control have been done.

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Chemical insecticides is widely used but the use of insecticides is very unfavorable since it can be harmful to humans and cause environmental pollution and insect resistance\(^2\). One control considered as safe is to use bioinsecticides. One bacteria *Bacillus thuringiensis*(Bt) is well-known for this purpose\(^6\).

The use of Bt as bioinsecticide has been widely applied to control mosquito larva and agricultural insect pests as well. This bacteria kills various mosquito larvae such as larvae of *Aedes*, *Culex*, and *Anopheles*, but no harm for non-target organisms. Special protein is produced by Bt especially toxic to larve of lepidopteran, coleopteran, and dipteran larvae. This protein is a protoxin unless is lysed by alkaline protease in gut of insects\(^4,5\).

Various strains of Bt as bioinsecticide have already been in market. However, study in effectiveness of new isolate to control insect larvae is still on-going, as there are still many disease vector and pest that can not be controlled using existing insecticides\(^5\). Therefore, this study was conducted to assay newly local Bt which might be used as bioinsecticide against *A. aegypti* mosquito larve.

**Materials and Methods**

**Soil Sampling and Isolation of Bt from Soil Samples**

Soil samples were taken from Tongkoh Forest, North Sumatra, Indonesia. A 100 g of 2-10 cm depth soil was collected aseptically using a spade from 3 randomly sites. Samples were put in a sterile plastic bag, and immediately brought to our laboratory.

To isolate Bt, modified method by Travers et al\(^6\) was utilised. Soil samples of the 3 sites were thoroughly mixed. A 25 g of soil sample was put into 250 ml of 0.25 M sodium acetate buffer (pH 6.8), shaken strongly for 15 min and heated at 80°C for 30 minutes in waterbath. Suspension was serial diluted in 0.25 M sodium acetate pH 6.8. Diluted suspension was spread in Luria Bertani (LB) agar and incubated at 30°C for 48 hrs. Selected colony appeared to have similar morphological and colony colour to Bt was transferred to LB agar and incubated at 30°C for 4-5 days for sporulation\(^7,8\).

**Cell and Crystal Morphology**

Morphology of bacterial colony was observed including colony shape, elevation, edge, and colour. Cell shape was observed using compound light microscope. Gram staining and biochemical test was conducted to bacterial cell. To observe crystal morphology, Coomassie Brilliant Blue (CBB) staining was deployed to late culture. Isolates defined as Bt indicated by its crystal protein was stored on LB agar for further study.

**Scanning Electron Microscope (SEM)**

SEM observation were conducted at Zoological Laboratory, Research Center of Biology, Indonesian Institute of Science, Cibinong, West Java, Indonesia. Bt isolate was cultured on LB medium incubated in incubator shaker at 30°C for 3 days. Bt culture was spun at 10,000xg for 10 minutes. Supernatant was discarded and added with 2% coccodylate buffer, then centrifuged again. The fixation solution was discarded. Add coccodylate centrifuged plus 1% tetratoxide, soaked for one hour with this solution. Centrifuged back, tetroxide solution was discarded and 70%, 80%, 90%, and ethanol absolute ethanol were added. The re-centrifuged solution was discarded and added butanol and then suspension was made in butanol. The slip cover piece was frozen and made a suspension review on the cover slip and then dried with freeze-dryer, then coated with gold on vacuum dryer. Observed with JSM-5310LV scanning electron microscope (Japan)\(^9\).

**Identification Bt Isolate Based on 16S rRNA Gene Sequence**

Bt isolate was cultured in LB agar for 24 hrs. One loop of Bt culture was picked and suspended with sterilised water in microtube. Cell suspension was frozen at -10°C and heated at 90°C for 10 minutes. This was done for 5 times. Broken cell was spun at 10,000xg for 10 minutes. Supernatant contained bacterial DNA was stored at 4°C for further study. DNA in supernatant was amplified using a PCR machine (Sensoquest Labcyler). Amplification of the 16S rRNA gene was conducted using primer 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′), which is a universal primer for various strains bacteria\(^10\). PCR reaction was run in mixture of 2 μl DNA template; 12.5 Master Mix 2x.
GoTaqGreen; 1 μl (10 pmol) of each primary; 8.5 μl Nuclease Free Water. PCR machine was run at 94°C for 2 minutes, denaturation at 92°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute, and post PCR at 72°C for 5 minutes. The reaction was conducted for 40 cycles.

The PCR result were visualized on 1% agarose gel (1 g agarose in 100 ml TAE 1X) using minigel electrophoresis. Electrophoresis was performed at 80 volts of 400 mA for 60 minutes. The amplified DNA was visualized with UV-transluminator.

The amplified DNA was purified and commercially sequenced to determine the sequence of its DNA bases. The sequence data was compared to similar data in GenBank from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov), using the Local Local Alignment Search Tool (BLAST) program.

Bt Growth on Molasses Urea Medium

Since SP7 and SP15 were actually similar only one isolate of Bt SP7 was chosen for further study. A 2.5 ml of Bt culture (OD600=0.5) was inoculated in molasses urea salt (MUS) broth composed of 1.0 g/l CaCO3; 0.03 g/l MgSO4.7H2O; 0.02 g/l MnSO4.7H2O; 0.02 g/l ZnSO4.7H2O; and 0.02 g/l FeSO4.7H2O. For appropriate growth, 20% molasses (w/v) and 3% urea (w/v) was used. Bt cell number was counted using plate count agar method for every 6 hrs.

Assay of Bt to Control A. aegypti Larvae

Larvae of instar 3 of A. aegypti was obtain from Balai Teknik Kesehatan Lingkungan dan Pengendalian Penyakit Kelas I, Medan, North Sumatra, Indonesia. Bt culture of MUS previous cultured was poured into plastic cups at concentration of 10, 20, 30, 40, and 50% of 50 ml of well-water test medium with 10 larvae of instar 3 of A. aegypti in it. Larvae death was counted in percentage.

Results And Discussions

Isolation of bacteria from soil samples obtained 2 potential isolates of SP7 and SP15 (Table 1). These 2 isolates showed to have similar morphological and biochemical characteristics to Bt. These two isolates showed to have catalase, motility, hydrogen sulfide production, gelatin hydrolysis, starch hydrolysis but no citrate metabolism which was also observed by Jung et al\(^1\)\(^2\), and distincted to other Bacillus in which they produced crystal protein.

Table 1: Morphological and biochemical characterization of SP7 and SP15 isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Colony shape</th>
<th>Colony edge</th>
<th>Colony elevation</th>
<th>Colony color</th>
<th>Gram</th>
<th>Catalase</th>
<th>Motility</th>
<th>Hydrogen sulfide</th>
<th>Gelatin Hydrolysis</th>
<th>Citrate metabolism</th>
<th>Starch Hydrolysis</th>
<th>Crystal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP 7</td>
<td>Circular</td>
<td>Entire</td>
<td>Flat</td>
<td>Cream</td>
<td>G(^+)</td>
<td>+</td>
<td>+</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SP 15</td>
<td>Circular</td>
<td>Entire</td>
<td>Flat</td>
<td>Cream</td>
<td>G(^+)</td>
<td>+</td>
<td>+</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Notes: + = positive reaction; - = negative reaction; G\(^+\) = Gram-positive

Observation of Crystal Protein

SP7 and SP15 cells of late culture were stained with CBB dyes. Microscopic observations showed that SP7 and SP15 have spores with solid-shaped crystal protein next to it (Figure 1) which was colored in dark black\(^1\)\(^4\).
Figure 1. Compound light microscope photograph of (A) SP7 isolate and (B) SP15 isolate with its spore (s) and crystal protein (c)

Further examination was carried out using SEM techniqueto see more clearly crystal protein shape(Figure 2). The observations using SEM with magnification of 10,000 times showed clearly the shape of spores and crystal proteins. SP7 isolate has spherical crystal protein form, SP15 from observation shows spherical crystal protein form. Other observation showed crystal proteins of spherical shape15,16,17.

Identification of Bt Isolates Based on 16S rRNA Gene Sequences

Bt is one of insect pathogenic bacteria. These bacteria belong to the Bacilli class, the Bacillales order, in the Bacillaceae family. These bacteria are aerobes, rodshape, Gram-positive, and spore-forming. They growth in various media. Special characteristics of Bt is that its ability to form crystal proteins along with spore formation18,19. Two isolates SP7 and SP15 were identified by using 16S rRNA gene. SP7 and SP15 isolates were closely related to B. thuringiensis strain of MCCC 1A00395 strain with 99% similarity (Table 2).
Table 2: Similarity of SP7 and SP15 to *Bacillus thuringiensis* strain MCCC 1A00395 based on 16S rRNA gene sequence

<table>
<thead>
<tr>
<th>Code</th>
<th>Isolates</th>
<th>Results of Blast</th>
<th>Similarities</th>
<th>Number of AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP7</td>
<td><em>Bacillus thuringiensis</em> strain MCCC 1A00395</td>
<td>99%</td>
<td>KJ812420.1</td>
<td></td>
</tr>
<tr>
<td>SP15</td>
<td><em>Bacillus thuringiensis</em> strain MCCC 1A00395</td>
<td>99%</td>
<td>KJ812420.1</td>
<td></td>
</tr>
</tbody>
</table>

**Bt Growth in Mollasse Urea Salt Medium**

SP7 cell was grown exponentially until 30 hrs of incubation time which reached cell number of 17.01 log CFU/ml, following by its stationary phase (Figure 3). It started to decline after 36 hrs of incubation time. *B. thuringiensis* 47 and Lot2 showed to have exponential phase at 4 hrs and reached its stationary phase at 36 hrs of incubation time in nutrient broth.

![Cell growth profile of SP7 in mollasse urea salt medium](image)

Figure 3. Cell growth profile of SP7 in mollasse urea salt medium

**Assay of Bt in Controlling *A. aegypti* Larvae**

Assay of Bt in controlling mosquito larvae showed that SP7 was able to kill the larvae up to 97.5% in 24 hrs of SP7 culture concentration of 50% (Figure 4). It was shown that the lower bacterial culture concentration the lower larvae death indicating its toxicity was concentration dependent.
It was said that Bt potential isolate showed to have ability to kill more larvae at concentration below 50%\textsuperscript{21}. Another study showed that the percentage of mortality in Bt exposure was 80\% over 24 hrs with cell concentration of 4.5 ppm\textsuperscript{22}. Gama et al\textsuperscript{23} showed that \textit{B. thuringiensis} of Madura killed mosquito larvae instar 1 up to 88.89\%, and high toxicity was found in bacterial density of $1.51 \times 10^8$ cells/ml. Lantang et al\textsuperscript{24} reported that 19 Bt isolates killed 50\% and 3 isolates of which killed more than 80\% of mosquito larvae.

Mosquito larvae exposed with Bt showed to a damage in its gastrointestinal tract shown by black color (Figure 5). Other indications can be seen from decreasing feeding activity of the larvae\textsuperscript{25}. Bt exposure to insect larvae cause insect epithelial organelles to swell and mid intestinal tissue disturbed so that the ability of insect to eat decrease and eventually stopped within a few days\textsuperscript{26}. The crystal proteins ingested by the insects are dissolved in the base environment of the target insect intestine andto be activated by an alkaline protease in insect gut. Activated proteins attach to receptor proteins located on the surface of intestinal epithelial cells resulting in the formation of pores causing the cells undergo lysis, and eventually the insects die\textsuperscript{27}.
Conclusion

Two isolates SP7 and SP15 were shown to have similarities to *B. thuringiensis*. The results of crystal protein observation using CBB and SEM technique knew that the crystal protein shape was spherical. SP7 and SP15 were closely related to *Bacillus thuringiensis* MCCC 1A00395 with 99% similarity. Molasses urea salt medium could be used as growth media of Bt. SP7 showed its maximum growth at 30 hrs of incubation time with cell number of 17.01 log CFU/ml. It showed to kill up to 97.5% *A. aegypti* larvae at concentration of 50% of Bt culture in 24 hour.

Acknowledgment

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References:


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