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## IMPORTANT DATES

| | |
|---|---|
| Abstract Submission Deadline | : **June 4, 2019** |
| Accepted Abstract Announcement | : **June 18, 2019** |
| Early Bird Registration Deadline | : **July 2, 2019** |
| Regular Registration | : **July 3 - August 2, 2019** |
| Conference date | : **September 4 - 5, 2019** |
| Full-text Manuscript Submission | : **August 6, 2019** |

## TOPICS

1. **Botany**: plant conservation, ethnobotany, taxonomy, development & physiology
2. **Zoology**: animal conservation, taxonomy, development, physiology & reproduction
3. **Ecology**: environmental conservation, bioremediation, biocontrol & toxicology
4. **Sustainable Bioeconomy**: economization of renewal resources, bio-based products, bioenergy & ecotourism
5. **Conservation Education & Policy**: environmental education, curriculum development, teaching technique and environmental law.

## REGISTRATION FEE

| Presenter/Attendee | Early Bird | Standard |
|---|---|---|
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| Non-student Presenter | IDR 1.000.000 | IDR 1.500.000 |
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| Non-student Presenter | USD 150 | USD 200 |
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# Utilization of Coconut Milk and Cane Sugar to Grow Indigenous Entomopathogenic *Bacillus thuringiensis* for Controlling *Aedesaegypti* Larvae

# Utilization of Coconut Milk and Cane Sugar to Grow Indigenous Entomopathogenic *Bacillus thuringiensis* for Controlling *Aedesaegypti* Larvae

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**Abstract.** *Bacillus thuringiensis* is bacteria utilised to control *Aedes aegypti* larvae for many years. However, there is no *B. thuringiensis* commercial products are commercially available in Indonesia. Therefore, development of *B. thuringiensis*-based biolarvicide using indigenous strain and local medium is very important. The aim of the study were to evaluate growth and toxicity of Mataram indigenous *Bacillus thuringiensis* isolate when grown on coconut milk and cane sugar as natural culture. Mataram indigenous *B. thuringiensis* isolate (namely Bt-TP2B) was grown in liquid medium consisted of coconut milk and cane sugar in 4 ratios (1:1; 1:3; 1:5 and 1:7) for 7x24 hours. Cell, endospore, and parasporal crystal (toxin protein) concentration were measured every 24 hours for 7x24 hours. Toxicity of the cultures was evaluated against 3rd instar of *A. aegypti* larvae. This study showed that the highest cell, endospore and toxin protein crystal concentration was shown by *B. thuringiensis* grown in ratio of 1:7, which were $2.20x10^7$ cell/mL, $2.09x10^7$ endospore/mL and $9.85x10^6$ crystal/mL, repectively. The highest toxicity against 3rd instar *A. aegypti* larvae was shown by *B. thuringiensis* grown in ratio of 1:7 which was 90-100% (in 3-day observation). From this study It was concluded that natural medium consisted of coconut milk and cane sugar can be used to grow *B. thuringiensis* and to stimulate toxicity against 3rd instar of *A. aegypti* larvae.

## 1. Introduction

*Bacillus thuringiensis* is one popular species of bacteria used to control *A. aegypti* [1]. This rod and endospore-forming bacteria can be found on soil almost everywhere throughout the world. The ability of *B. thuringiensis* in killing some larvae mosquito species was found firstly in 1977, although *B. thuringiensis* was widely used to control other insects [2]. After this discovery, hundreds of mosquito-killing strains were isolated [3]. The ability of *B. thuringiensis* in killing some species of mosquito was caused by its toxins (delta endotoxins) produces when *B. thuringiensis* enters sporulating stage [4]. Based on those discoveries, several companies developed *B. thuringiensis*-based biolarvicidal products in some formulation forms, such as VectoBac® [5], Teknar® [6] and many more [7]. However, none of them are commercially available in Indonesia. This situation will impede biological mosquito control program in Indonesia. Therefore, it is important to develop local biolarvicidal product using indigenous *B. thuringiensis* strain and applying local natural medium material. Local biolarvicide product

doubtlessly will support mosquito control program and local biolarvicidal industry capability in Indonesia.

Coconut and cane sugar are widely available in Indonesia [8]. Those materials have potentials to be utilized as bacterial culture medium, replacing industrial-standard culture medium, since coconut milk is source of fat, carbohydrate, vitamins and minerals [9]. Cane sugar is main source of carbohydrate (sucrose) [10]. In this study, coconut milk (derivative product of coconut) and cane sugar were used to grow local strain of entomopathogenic *B. thuringiensis* as mosquito biological control agent. Growth of *B. thuringiensis* was then evaluated and its toxicity against 3rd-instar *A. aegypti* larvae was measured.

## 2. Material and Methods

### *2.1 Material*

Indigenous strain *B. thuringiensis* (isolate Bt-TP2B) that previously isolated in Mataram, Indonesia [11] was used in this study. This bacterium was cultured as stock in Nutrient Agar added with enrichment with antibiotic Amoxicillin 100 µg/mL. The bacterium was incubated in aerobic incubator at 33ºC. Coconut milk was from from old coconut bought from local market and cane sugar was bought from local market as well.

### *2.2 Fermentation*

Coconut milk and cane sugar were prepared in 100 g/L standard concentration. Four ratios of fermentation medium consisting of coconut milk and cane sugar were prepared. They were 1:1 (1 part coconut milk + 1 part cane sugar); 1:3; 1:5 and 1:7, respectively.

Stock of *B. thuringiensis* isolate Bt-TP2B was initially grown on Nutrient Agar (Oxoid, UK) enriched with 30 µg/mL Amoxicillin at 33ºC for 1x24 hour as described in other study [12]. One full loop of pure colony of *B. thuringiensis* was transferred to each ratio of coconut milk and cane sugar natural medium. Fermentation was done at 33ºC for 7x24 hours aerobically [13]. During fermentation, all medium were sampled for measuring cell, endospore and toxin protein crystal concentration. The measurements were performed every 24 hours for 7x24 hours (7 days), then recorded and transformed into graph using Microsoft Excel for Windows.

### *2.3 Toxicity test*

Toxicity test was conducted based on standard procedure [13][14]. *A. aegypti* eggs were hatched in well water and reared for 6-8 days to reach 3rd instar in laboratory. Seventy-five containers were prepared for toxicity testing. *B. thuringiensis* from each ratio of natural medium prepared in 5 dilutions ($10^{-1}$ to $10^{-5}$ dilution) in 3 replications. Twenty individual of 3rd instar of *A. aegypti* larvae were put in every testing container. In total, there were 1,500 *A. aegypti* larvae used in this test. Larval death was recorded every 24 hours for 3x24 hours. Larval death observed from natural medium was compared to that of NYSM standard medium. Mortality data was calculated using Microsoft Excel for Windows.

### *2.4. Data Analysis*

Mortality data from 4 ratios of coconut milk-cane sugar natural medium were analyzed using Single Factor Analysis of Variance using $\alpha = 0.05$. Analysis of Variance was performed using Statistical ToolPak in Microft Excell [15].

## 3. Result and Discussion

Cell, endospore and toxin protein crystal morphology of local isolate of *B. thuringiensis* is presented on Figure 1 as follows.

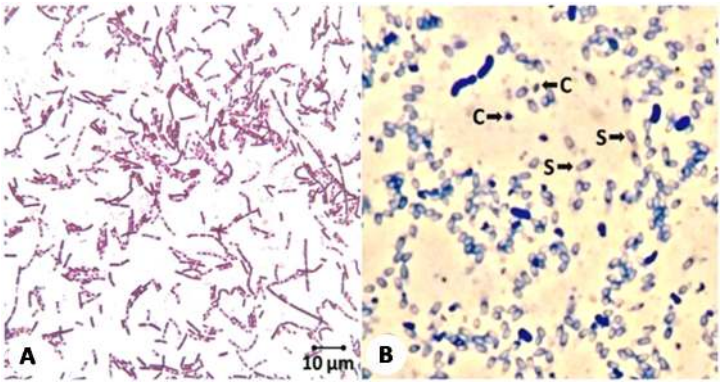


**Figure 1**(A) Cell and endospore morphology of *B. thuringiensis*Bt-TP2B (B) Endospore and toxin protein crystal (1,000x magnification)

Culture characteristic of the indigenous isolate of *B. thuringiensis* used in study is presented in Table 1.

**Table 1.** Characteristics of *B. thuringiensis* isolate Bt-TP2B

| Characteristics | Results |
|---|---|
| Colony morphology characteristics | |
| • Form | Circular |
| • Elevation | Convex |
| • Margin | Irregular |
| • Translucency | Non-transparent |
| • Colour | Creme |
| Cell morphology characteristics | |
| • Structure | Rod |
| • Size | 2,0-4,0 µm (L) x 0,8-1,2 µm (W) |
| • Gram | Positive |
| • Endospore | Present |
| • Endospore position | Subterminal (mainly) and terminal |
| Biochemical characteristics | |
| • Glucose | Positive |
| • Sucrose | Positive |
| • Lactose | Negative |
| • Maltose | Positive |
| • Manitoll | Negative |
| • Voges Proskauer | Positive |
| • Starch hydrolysis | Positive |
| • Catalase | Positive |
| • Oxsidase | Positive |
| Physiology characteristics | |
| Growth on | |

| | |
|---|---|
| • 30°C | Positive |
| • 35°C | Positive |
| • 40°C | Positive |
| Growth on pH | |
| • 5 | Positive |
| • 7 | Positive |
| • 9 | Positive |
| Antibiotic Resistance characteristics | |
| • Ampicillin | Resistant |
| • Amoxycillin | Resistant |
| • Sulphamethoxazole | Sensitive |
| • Tetracycline | Resistant |
| • Amikacin | Sensitive |
| • Gentamicin | Sensitive |
| • Cyprofloxacin | Sensitive |
| • Cefotaxime | Resistant |
| • Chloramphenicol | Sensitive |
| • Clindamycin | Sensitive |

The cell concentration measurement of *B. thuringiensis* isolate Bt-TP2B grown in 4 ratio combination of coconut milk-cane sugar medium is presented on Figure 2.

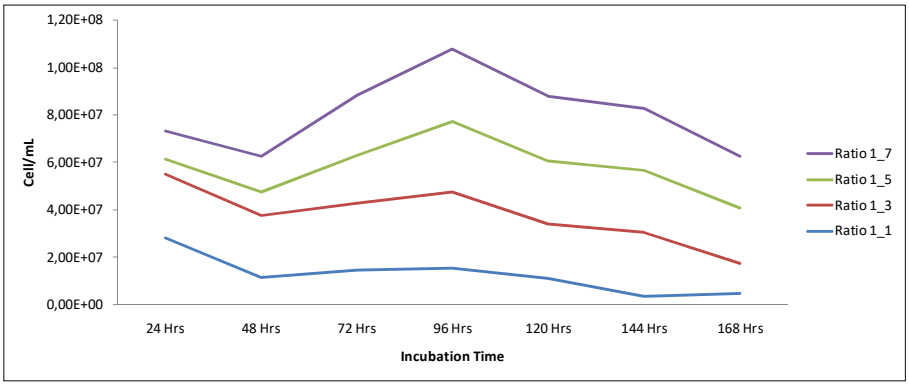


**Figure 2.** Cell concentration of *B. thuringiensis* isolate Bt-TP2B grown in all medium ratio combination in 7x24 hour-incubation

All *B. thuringiensis* grown on all ratios of coconut milk-cane sugar natural medium showed decreased cell concentration on the first 24 hours of incubation. From 48 to 96 hours, all ratios showed increasing cell concentration. After 96 hours of incubation, cell concentration from all ratios decreased dramatically until the end of the experiment.

The highest cell concentration at the end of the experiment was showed by natural medium ratio of 1:7 ($2{,}20 \times 10^7$ cell/mL), followed by ratio of 1:5 ($2{,}32 \times 10^7$ cell/mL); 1:3 ($1{,}28 \times 10^7$ cell/mL) and 1:1 ($4{,}60 \times 10^6$ cell/mL), respectively. All *B. thuringiensis* cultured in 4 combinations of coconut milk-cane sugar natural medium showed significantly different cell concentration ($F = 4{,}448$; $p < 0.05$).

In this figure, it was clear that the addition of cane sugar was able to increase bacterial cell concentration within all ratios of coconut milk-cane sugar natural medium. After 96 hours of incubation,

cell concentration of all ratios were decreased, as all bacterial cell seemed to enter sporulating stage. This stage was clearly seen in **Figure 3**, where 96 hours was the peak of sporulation.

The endospore concentration measurement of *B. thuringiensis* isolate Bt-TP2B grown in 4 ratio combination of coconut milk-cane sugar medium is presented on **Figure 3**.

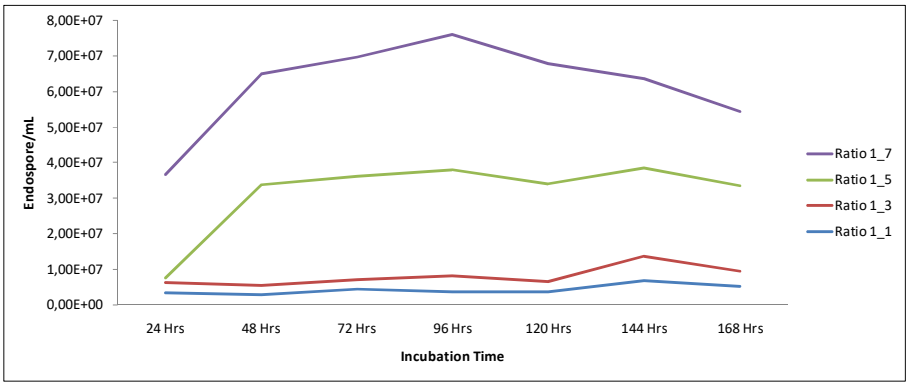


**Figure 3**. Endospore concentration of *B. thuringiensis* Isolate Bt-TP2B grown in all medium ratio combination in 7x24 hour-incubation

All *B. thuringiensis* cultures grown on all ratios of coconut milk-cane sugar natural medium showed increase endospore concentration on the first 24 hours of incubation to 48 hours of incubation. From 48 to 96 hours, all ratios showed increasing endospore concentration. After 96 hours of incubation, endospore concentration from all ratios decreased slightly until the end of the experiment.

*B. thuringiensis* grown in coconut milk-cane sugar natural medium on ratio of 1:7 showed the highest endospore concentration ($2{,}09 \times 10^7$ endospore/mL), followed by ratio of 1:5 ($2{,}41 \times 10^7$ endospore/mL), 1:3 ($4{,}15 \times 10^6$ endospore/mL) and 1:1 ($5{,}20 \times 10^6$ endospore/mL), respectively. All *B. thuringiensis* cultured in 4 combinations of coconut milk-cane sugar natural medium showed significantly different endospore concentration ($F = 2826{,}678$; $p < 0.05$).

Once again, the addition of sugar clearly caused the increase of endospore concentration during incubation. On ratio of 1:5 and 1:7, the increase of endospore concentration was very drastic. We suggest that, the higher concentration of sugar in the medium caused faster growth of the bacterial cell. This situation was causing sporulation occured earlier than *B. thuringiensis* grown in medium with lower cane sugar concentration.

The toxin protein crystal concentration measurement of *B. thuringiensis* Bt-TP2B grown in some ratio combination of coconut milk-cane sugar medium is presented on **Figure 4**.

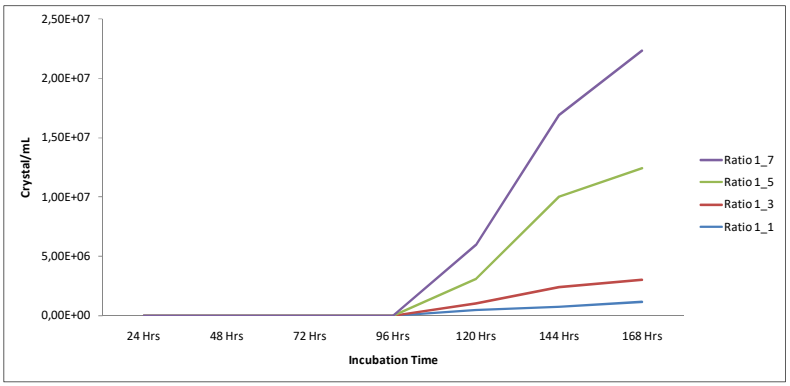

**Figure 4**. Toxin protein crystal concentration of *B. thuringiensis* Isolate Bt-TP2B grown in all medium ratio combination in 7x24 hour-incubation

All ratios of coconut milk-cane sugar natural medium showed detected toxin protein crystal on 96 hours of incubation and afterward. There was not any toxin protein crystal detected before 96 hours of incubation. The highest toxin protein was showed by ratio of 1:7 (9,85x$10^6$ crystal/mL), followed by ratio of 1:5 (9,45x$10^6$ cystal/mL), 1:3 (1,85x$10^6$ crystal/mL) and 1:1 (1,15x$10^6$ crystal/mL), respectively. All ratios of the natural medium showed increasing toxin protein synthesis until the end of the experiment. All *B. thuringiensis* cultured in 4 combinations of coconut milk-cane sugar natural medium showed significantly different toxin protein crystal concentration ($F = 26699$; $p < 0.05$).

When comparing endospore concentration graph (Figure 3) and toxin protein crystal concentration graph (Figure 4), the toxin protein crystal from all ratios of coconut milk-sugar cane natural medium was produced when endospore concentration decreased (started at 96 hours). The decrease of endospore is caused by lyses of endospore into medium. When the cell lyses, toxin protein crystals were released into the medium as well. The toxin protein crystal synthesis in this natural medium was very slow if compared to protein-rich material with similar concentration [16] where toxin protein crystal synthesis is faster (started at 48 hours of incubation). This condition was caused by the low content of total protein in coconut milk (about 3-7% per 100 gram) [17]. The low content of total protein will be focused mainly for growth of bacterial cell, instead of synthesizing toxin protein crystal [18] [19].

The toxicity measurement of *B. thuringiensis* Bt-TP2B grown in some ratio combination of coconut milk-cane sugar medium is presented on Figure 5. The highest toxicity was showed by medium ratio of 1:7. The toxicities were reaching 90-100% in 10% bacterial culture, 60-90% in 1% bacterial culture and 10-30% in 0.1% bacterial culture, respectively. The second highest is from ratio of 1:5 (60-100% in 10% bacterial culture, 36-83% in 1% bacterial culture and 3-23% in 1% bacterial culture, respectively). The larval death in both ratios of natural medium was better than that of showed by standard medium NYSM. Standard medium NYSM resulted in very high larval death only on 10% bacterial culture in 3 day test.

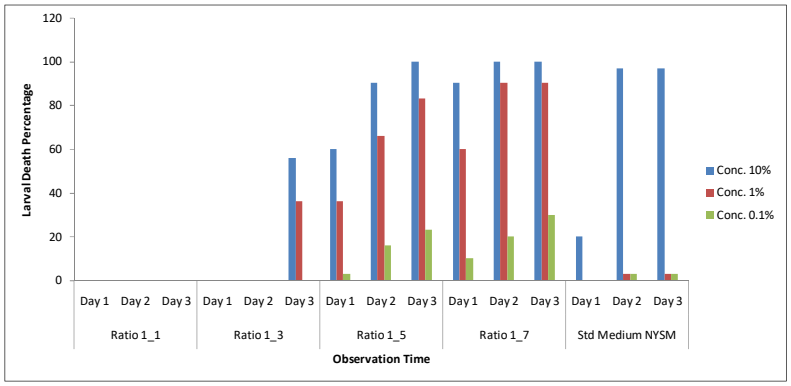


**Figure 5.** Toxicity measurement of*B. thuringiensis* grown in all medium combinations in 3x24 hour-observation

In this study we demonstrated that coconut milk-cane sugar medium could stimulate production of toxin protein in local isolate of entomopathogenic*B. thuringiensis*. However, if compared to other studies using protein-rich materials [16,20], the use of coconut milk-cane sugar will need prolong incubating time, since toxin protein crystalis produced after 96 hours of incubation. The use of protein-rich material will ensure faster production of toxin protein of *B. thuringiensis*.

Toxicity in *B. thuringiensis* is mostly caused by its toxin protein crystalsynthesised during sporulating stage. *B. thuringiensis* synthesises single or multiple types of parasporal crystalline protein

(δ-endotoxins/delta endotoxins). δ-endotoxins are consisted of crystal (Cry) and/or cytolytic (Cyt) proteins. Both areparasporalcrystalline protein produced when *B. thuringiensis*undergoes sporulation. These toxins have highly specific targeted insects. However, they are harmless to human and other vertebrates. These toxins are completely biodegradable[21]. These crystalline proteins are mainly encoded by extra-chromosomal genes located on the plasmids. The parasporal crystalline proteins produced during the stationary phase of *B. thuringiensis* growth cycle account for 20-30% of the dry weight of the cells of this phase[22]. Expression of most Cry genes (such as*cry1Aa*, *cry 2A*, *cry 4A*, etc.) is well regulated in the sporulation phase of growth [23].

*B. thuringiensis*'s capability of mosquito larvae killing works as follows. When ingested by mosquito larvae, toxin protein crystalis solubilised and activated by protease and high pH inside the mosquito larvae midgut. The activated toxin will bind to apical microvilli of midgut cells. Then ion-selective channel or pore will be formed as result of toxin part inserted into membrane lipid bilayer. Water will enter the cell, while ions and other components will exit from the cell. This condition will cause swelling and lysis of the cell [1]

The price comparison of ratio combination of coconut milk-cane sugar medium and standard medium is as followed.

**Table 2.** Price comparison between NYSM standard medium and coconut milk-cane sugar medium combination medium for 1 L medium based on recent market price

| Materials | Price in IDR | Price in US$ |
|---|---|---|
| Coconut (grated) (for 1 L vol) | 7,000 | 0.54 |
| Cane Sugar (for 1 L vol) | 12,500 | 0.96 |
| Std Medium NYSM | 30,000 | 2.31 |

Coconut milk-cane sugar natural medium was almost 2/3 cheaper than standard NYSM medium. Besides cheaper, coconut milk-cane sugar medium was available at any season locally (there is no need to import the medium) compared to standard medium. This condition will lead easy production of *B. thuringiensis*-based biolarvicidal agent in small and low tech facilities.

## 4 Conclusion

In this study we demonstrated that natural medium made of coconut milk-cane sugar could be used to grow local isolate *B. thuringiensis* and to stimulate toxicity against 3rd instar *A. aegypti* larvae in 1:7 ratio. Natural medium coconut milk-cane sugar medium cost about 2/3 cheaper compared to standard medium.

**Acknowledgement**

We would like express our gratitude to Faculty of Mathematics and Natural Sciences, Mataram University for funding support for this study and West Nusa Tenggara General Hospital, Mataram for permission to use all facilities in Biomedik Research Unit.

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**Utilization of Coconut Milk and Cane Sugar to Grow Indigenous Entomopathogenic *Bacillus thuringiensis* for Controlling *Aedes aegypti* Larvae**

*Malang, September 4 - 5, 2019*

**Prof. Dr. Ir. Nuhfil Hanani A.R., M.S**
Rector of Universitas Brawijaya

**Irfan Mustafa, S.Si, M.Si, Ph.D**
Conference Chairperson