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The Use of Fish Flour and Palm Sugar Medium to Grow Mataram Indigenous Isolate of Entomopathogenic *Bacillus thuringiensis* for Controlling *Aedes aegypti* Larvae

Bambang Fajar Suryadi *, Hilal Aisyah Hidayat, Galuh Tresnani

Department of Biology, Faculty of Mathematics and Natural Sciences, University of Mataram, Mataram 83125, Indonesia

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*Corresponding author:

E-mail: bambangfajar@unram.ac.id

ABSTRACT

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To grow Mataram indigenous local isolate of entomopathogenic *Bacillus thuringiensis* using 4 combinations of fish flour and palm sugar natural liquid medium. The culture was then tested against 3rd instar *Aedes aegypti* larvae to gain lethal concentration (LC) value within 72-hour test. Four combinations of fish flour and palm sugar liquid medium (in 1:1; 1:3; 1:5 and 1:7 weight ratio) were made to grow *B. thuringiensis* isolate Bt-TP2B. Cell, endospore and protein crystal were recorded every 24 hours within 72-hour incubation. Seventy-two-hour-old *B. thuringiensis* cultures from those media were serially diluted, then tested against 3rd instar *B. thuringiensis* larvae. Larval death was recorded every 24 hours within 72-hour observation to obtain lethal concentration (LC) values. Results from all medium were compared to those of NYSM standar medium. *B. thuringiensis* isolate BT-TP2B could grow in fish flour and palm sugar natural medium. The highest toxicity (the lowest LC) was seen on *B. thuringiensis* grown on fish flour and palm sugar natural medium in 1:1 weight ratio. The LC50 values were 1.98x10³ cell/mL (in 24-hour observation) and 1.49x10³ cell/mL (in 48-hour and 72-hour observation), while LC90 values were 1.35x10³ cell/mL in 24-hour observation and 1.58x10³ cell/mL in 48 and 72-hour observations). These values were lower (more toxic) compared to those of NYSM standard medium. Fish flour and palm sugar natural medium could be used to grow entomopathogenic *B. thuringiensis*. Moreover, this natural medium could increase toxicity of *B. thuringiensis* against *B. thuringiensis* 3rd instar larvae.

Keywords: *Bacillus thuringiensis*, *Aedes aegypti*, fish flour, palm sugar, toxicity

Introduction

Dengue Haemorrhagic Fever (and some other infectious disease) is still becoming health problem in Mataram, Indonesia. Statistic shows 1,605 people are suffering from the the disease [1]. DHF is caused by Dengue Virus (*Flavivirus*) and spread among people by *Aedes aegypti* mosquito [2]. *B. thuringiensis* control can be achieved in many ways, however many studies recommend integrated mosquito managements to deal with the problem [3]. One component of this integrated approach is by controlling *B. thuringiensis* larvae either with chemical agent or biological agent. The

most popular biological used is *B. thuringiensis*, a soil bacterium widely spread throughout the world. The ability of the bacterium to kill mosquito larvae caused by some toxin protein exist in their cell (i.e.-endotoxins) [4].

Up to recent time, bio-pesticide products based on *B. thuringiensis* in Mataram/Indonesia market are still imported from other countries. This situation hampers mosquito control program that's making it inefficient. To reduce dependence against importing such products, it is important to encourage research in exploring local *B. thuringiensis* isolates and local material to grow the bac-

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teria.

Fish (including its byproduct) and palm sugar are some of many local commodities of Mataram [5]. Fish flour (fishmeal) is made from remaining/unused fish widely abundant in surrounding waters. Palm sugar is well known as cooking sugar in this place and its tree is widely spread throughout Mataram as well. These two materials have potential to be utilized for growing *B. thuringiensis*. Fish flour can be used as nitrogen source, while palm sugar can be used as carbon source to fulfill the bacteria needs during its growth. Therefore, the aims of this study were to obtain the best combination fish flour and palm sugar (four combinations were applied) which resulting the highest toxicity of *B. thuringiensis* against *B. thuringiensis* 3rd-instar larvae.

Material and Methods

B. thuringiensis isolate and *B. thuringiensis* larvae preparation

In this study, we used *B. thuringiensis* isolate BT-TP2B generously provided by Ms. Herfianti from her study [6]. *B. thuringiensis* stock culture was refreshed on Nutrient Agar solid medium supplemented with 30 µg/mL Penicillin and incubated on 35°C for 24 hours [7]. When growth is observed, Gram, endospore and crystal characteristic observation was made to ensure there was not any contamination in culture. *B. thuringiensis* larva was reared in Medical Research Unit - West Nusa Tenggara General Hospital. The larva used in this study was 3rd-instar larvae which obtained from 6-7 days rearing procedure.

Natural medium and *B. thuringiensis* production

Natural fermentation medium used were local-produced fish flour (as nitrogen source) and palm sugar (as carbon source). Both materials were purchased at local market on Mataram City.

The medium in fermentation process were made in four combination based on component ratios. The combinations were 1 : 1 (i.e. 1-part fish flour: 1-part palm sugar); 1 : 3; 1 : 5 and 1 : 7. The based concentration was 50 g/L medium.

The medium was made by mixing fish flour, palm sugar and aquadest (in accordance to the component ratio combination). The mix was heated to boil for 3 minutes and let it sit to the room temperature. Standard medium used was

NYSM liquid medium as described by El-Bendary [8].

One full loop of *B. thuringiensis* isolate TP2B from NA solid culture was added to four natural tested medium combination and NYSM control medium. Fermentation was conducted on 35°C with rigorous shaking for 72 hours. Measurement on cell, endospore and crystal concentration were done every 24 hours. All measurements were made using Naubauer counting chamber in three replications.

Bioassay

Bioassay was performed to obtain Lethal Concentration (LC) value. The procedure was done as described by McLaughlin et al. [9]. Four natural media tested in 10-fold concentration differences and control medium were prepared. One thousand five hundred 3rd-instar *B. thuringiensis* larvae were distributed evenly to 75 containers (20 larvae per container). Mortality of larvae was recorded every 24 hours for 72 hours. LC values were calculated using Probit Analysis [10] applying MINITAB V16 Statistical Software for Windows [11].

Results and Discussion

Cell concentration

Comparison of cell concentration among *B. thuringiensis* cultured in 4 combinations of fish flour - palm sugar medium within 72-hour incubation was presented in Figure 1.

The highest cell concentration reached by *B. thuringiensis* grown in fish flour – palm sugar natural medium was from 1 : 5 ratio medium (6.50×10^7 cell/mL), followed by 1 : 3 ratio, 1 : 7 ratio and 1:1 ratio. *B. thuringiensis* grown in NYSM standard medium showed the highest cell concentration (7.00×10^7 cell/mL) from all medium, whereas the lowest concentration was reached by *B. thuringiensis* grown in fish flour – palm sugar natural medium in 1 : 1 ratio (1.20×10^5 cell/mL). In this observation, NYSM standard medium could give the highest *B. thuringiensis* cell concentration in the first 24 hours then decreased rapidly after 48-hour incubation.

All *B. thuringiensis* cultured in 4 combinations (1 : 1 to 1 : 7 ratios) of fish flour – palm sugar natural medium and NYSM standard medium showed significantly different cell concentration ($F = 4495.01$, $p < 0.05$). *B. thuringiensis* cultures

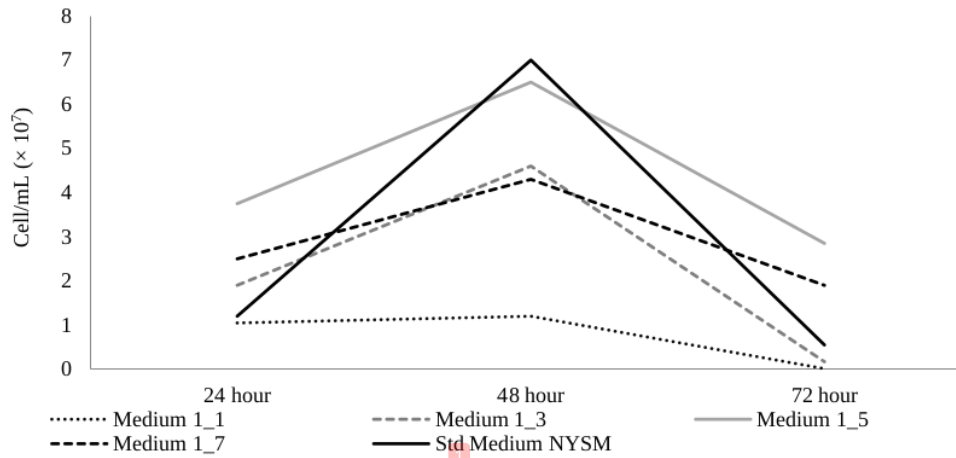


Figure 1. Comparison of cell concentration among *B. thuringiensis* grown in 4 combinations of fish flour –palm sugar medium and NYSM standard medium within 72-hour incubation

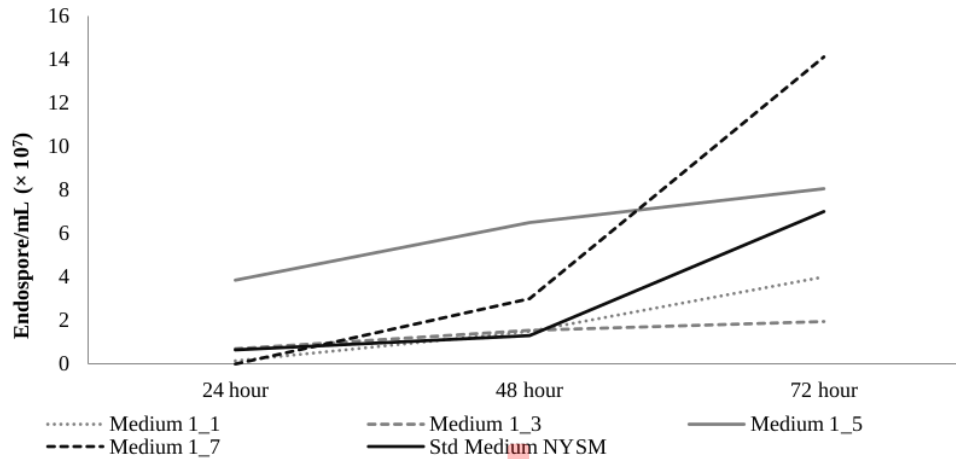


Figure 2. Comparison of endospore concentration among *B. thuringiensis* grown in 4 combinations of fish flour – palm sugar medium and NYSM standard medium within 72-hour incubation

from all medium applied showed similar growth pattern. Increase in cell concentration was seen from 24-hour incubation until 48-hour incubation. From 48 hours, *B. thuringiensis* cell concentration grown in fish flour – palm sugar natural medium from all ratios decreased rapidly. However, there was no correlation among protein-carbohydrate ratios with final bacterium cell concentration.

Cell concentration decrease after 48-hour incubation presumably was due to consumed carbohydrate in all medium used. This condition becomes a limiting factor to *B. thuringiensis* growth. In addition to the consumed carbohydrate, some

acid might be produced from carbohydrate metabolism that decreases pH of the medium, as low pH will suppress cell growth and trigger sporulation [12].

Endospore concentration

Comparison of endospore concentration among *B. thuringiensis* cultured in 4 combinations of fish flour - palm natural sugar medium within 72-hour incubation and NYSM standard medium was presented in Figure 2. All *B. thuringiensis* grown in fish flour medium (in all ratios) and NYSM standard medium showed significantly

different endospore concentration ($F = 384.52$, $P < 0.05$). The highest endospore concentration in 72 hours was seen on *B. thuringiensis* cultured in fish flour – palm sugar natural medium on 1 : 7 ratio (1.41×10^8 endospore/mL) followed by 1 : 5 ratio medium (8.05×10^7 endospore/mL) and 1 : 1 ratio medium (4.00×10^7 endospore/mL). The lowest was *B. thuringiensis* cultured in NYSM standard medium (7.00×10^7 endospore/mL), it was lower than *B. thuringiensis* cultured on 1 : 1 ratio.

From this observation, *B. thuringiensis* cultured in all medium showed increasing endospore concentration trend from the beginning of the incubation. After 48-hour incubation, almost all *B. thuringiensis* endospore concentrations were increased drastically (except those from 1:3 and 1:5 ratio). Once again, there was no correlation among protein-carbohydrate ratios with final bacterium endospore concentration.

The raising on endospore concentration trend is due to lower carbohydrate concentration that consumed by *B. thuringiensis* after 48-hour incubation, as it triggered sporulation process of the bacterium cell. Besides nutrient limitation, some acid might be produced from carbohydrate catabolism [12].

Crystal concentration

Comparison of crystal concentration among *B. thuringiensis* cultured in 4 combinations of fish flour - palm sugar natural medium within 72-hour incubation was presented in Figure 3.

Observation on toxin crystal synthesis in 72 hour showed significantly different results ($F = 809.95$, $P < 0.05$). The highest concentration was of *B. thuringiensis* cultured in fish flour – palm sugar natural medium on 1 : 1 ratio (3.00×10^7 crystal/mL), followed by 1 : 3 ratio (1.90×10^7 crystal/mL), 1 : 5 ratio (1.75×10^7 crystal/mL) and 1 : 7 ratio (1.45×10^7 crystal/mL). Standard medium NYSM was the lowest of all (6.50×10^6 crystal/mL).

As we could see here, there was correlation among protein-carbohydrate ratios with final bacterium crystal concentration. The lower the carbohydrate content on medium, the higher crystal synthesis could get. There was interesting thing to see here, high concentration of cell and endospore was not necessarily followed or correlated by high toxin crystal production. *B. thuringiensis* grown on fish flour – palm sugar natural medium on 1 : 1

ratio did not show the highest cell and endospore concentration, yet it showed the highest toxin crystal concentration.

There are some factors affected the production of the bacterial crystal protein:

- (1) Carbon source. Glucose is the best carbon source for high *B. thuringiensis* growth and sporulation [13]. The decrease of glucose can trigger sporulation [14].
- (2) Nitrogen source. The best source of amino acids allows high growth rate and high sporulation for *B. thuringiensis*. Its absence delays sporulation and low yield in Cry protein production [15].
- (3) Carbon : Nitrogen ratio. Higher C : N rate glucose causes carbon does not deplete at the end of fermentation, therefore biomass yield decreases.
- (4) Oxygen. High aeration rates are important for high spore and toxin formation.
- (5) pH. Optimum pH for Bt growth is 6.8 – 7.2. If pH rises to 9.0 Cry protein can be dissolved
- (6) Temperature. Optimum temperature for *B. thuringiensis* growth is 28 – 32°C. Higher temperatures favours plasmid losses [16].

Toxicity

Lethal concentration (LC) values of *B. thuringiensis* grown in 4 combinations of fish flour – palm sugar natural medium and NYSM standard medium is presented on Figure 4.

From Figure 4, it could be seen that *B. thuringiensis* grown in fish flour – palm sugar natural medium on 1 : 1 ratio gave the lowest LC values (LC_{50} and LC_{90}). The LC_{50} 24-hour value reached 1.98×10^3 cell/mL, while LC_{50} 48 and 72 hours reached 1.49×10^3 sel/mL. LC_{90} value in 24 hours was 1.35×10^3 cell/mL, followed by LC_{90} 48 hours (1.58×10^3 cell/mL) and LC_{90} 48 hours (1.58×10^3 cell/mL). These values were lower than those from NYSM standard medium. The next higher LC value (less toxic) was from *B. thuringiensis* grown in natural medium combination on 1 : 3, 1 : 5, and 1 : 7 ratios.

Toxicity in *B. thuringiensis* is mostly causing by its toxin protein synthesized during sporulating stage. Bt synthesize single or multiple types of parasporal crystalline protein (δ -endotoxins). δ -endotoxins were consisted of crystal (Cry) and/or cytolytic (Cyt). Both are parasporal crystalline protein produced when *B. thuringiensis* enters

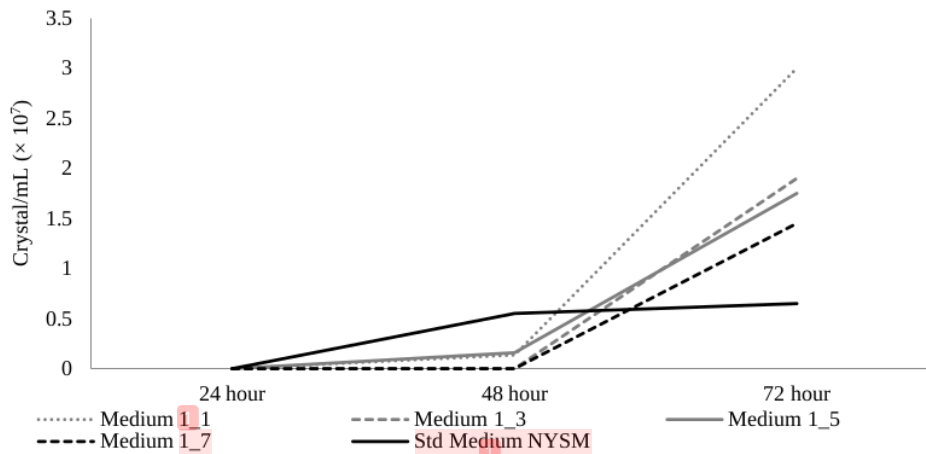


Figure 3. Comparison of crystal concentration among *B. thuringiensis* grown in 4 combinations of fish flour – palm sugar medium and NYSM standard medium within 72-hour incubation

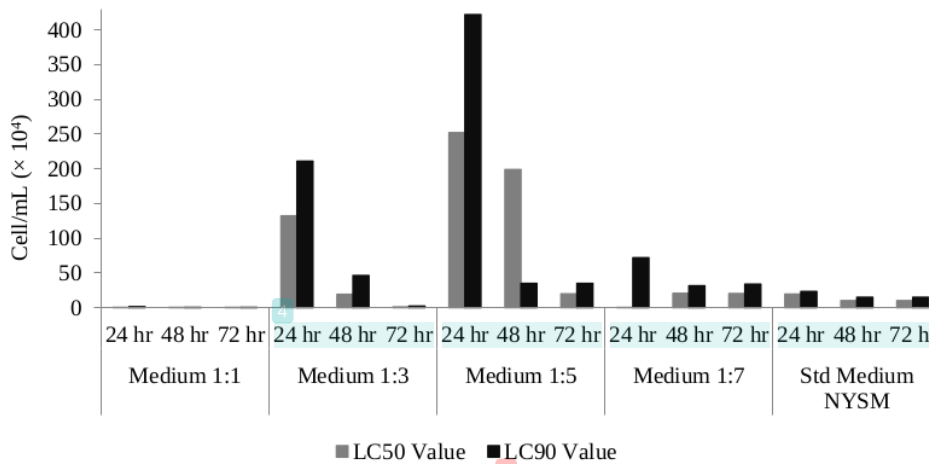


Figure 4. Comparison of LC50 and LC90 values among *B. thuringiensis* grown in 4 combinations of fish flour – palm sugar medium and NYSM standard medium within 72-hour incubation

sporulation. These toxins are highly specific targeted insects, but harmless to human, vertebrates and plants. These toxins are completely biodegradable [17]. 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 [18]. Expression of most Cry genes (e.g., cry1Aa, cry 2A, cry 4A, etc.) are well regulated in the sporulation phase of growth [19].

Larval death occurs several hours after crystal ingested by targeted larvae. Inactive protoxin will solubilize in alkaline condition inside larval midgut. Protoxin will be converted into toxic fragment proteolytically. The fragment will bind to receptor found on apical microvillus membrane of epithelial cell along midgut of targeted larvae. After binding with receptor, the toxins will cause lysis on epithelial cell by the formation of cation-selective channels. This allows free flux of ions and liquids, causing disruption of membrane transport and cell lysis leading to larval death [20, 21].

Price comparison

One problem faced in *B. thuringiensis*-based bio-pesticide production is choosing the appropriate medium to use for fermentation process. It should give maximum cell biomass and cell toxicity, yet very cheap in price [22]. If compared to NYSM standard medium, natural medium based on fish flour and palm sugar offer good potential to be used in a scale-up production scheme.

Fish flour – palm sugar natural medium cost only Rp 37,500 (2.9 US\$) /L. It is 39% cheaper than NYSM medium (Rp 94,100 or 7.2 US\$ /L). Besides cheaper in price, this natural medium is available in all seasons. In contrast, NYSM medium components should be imported from other countries, makes this medium more expensive and harder to buy or find in Mataram/Indonesia. Therefore, hopefully by using natural medium bacterium-based bio-pesticide production in higher scale will not face any constraint in the future.

Conclusion

In this study, fish flour-palm sugar medium natural medium can be used to grow *B. thuringiensis* and 1 : 1 ratio medium gave the best toxicity against *A. aegypti* 3rd-instar larvae.

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