BASIC 2019

by Bambang Fajar Suryadi

IOP Conf. Series: Materials Science and Engineering **546** (2019) 022031 doi:10.1088/1757-899X/546/2/022031

Utilization of lablab bean (*Lablab purpureus*) and palm sugar (*arengapinnata*) as natural medium to grow Mataram indigenous isolateof entomopathogenic *Bacillus thuringiensis* for controlling *Aedes aegypti* larvae

Bambang Fajar Suryadi[1*], Muhammad Masyari[1], and Galuh Tresnani[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Mataram, Jl. Majapahit No. 62, Mataram 83125Mataram - West Nusa Tenggara – Indonesia

*Corresponding author: bambangfajar@unram.ac.id

Abstract. The purpose of the study was to grow Lombok Island indigenous isolate of entomopathogenic Bacillus thuringiensis using some combinations of lablab bean (Lablab purpureus) as protein source and palm sugar (Arengapinnata) as carbohydrate source and to test its toxicity against Aedes aegipty 3rd instar larvae. This study used entomopathogenic B. thuringiensis isolate Bt-TP2B, locally isolated from soil in Mataram City. Lablab bean was mixed with palm sugar and water to form liquid medium. The based concentration for lablab bean and palm sugar was 50 g/L each. The medium was made into four weight-based concentration combinations, which were 1:1; 1:3: 1:5 and 1:7, respectively. NYSM (Nutrient broth Yeast Extract Salts Medium) supplemented with Amoxicillin was used as standard medium. Six hundred of 3rd Aedes aegypti instar were used as target in toxicity test. The test was performed in 5 culture dilutions (10-1 to 10-5 dilutions) against A. aegypti larvae. Larvae mortality was recorded in 24, 48 and 72 hours observation. Lethal concentration values were calculated using Probit Analysis. The highest cell concentration was reached by B. thuringiensis grown in 1:1 combination medium (5.95 x 107 cell/mL). Almost all B. thuringiensis grown in natural and NYSM medium showed decreasing trend within 72 hours incubation. Only 1:1 combination medium showed increasing trend from 24 to 72 hours incubation. The highest endospore concentration was reached at 4.32 x 108 endospore/mL by 1:1 combination medium in 72 hours incubation. All medium showed increasing trend within 72 hours incubation. The lowest LC50 value was reached by 1:7 combination medium (1.44 x 105 cell/mL) and the lowest LC90 value was reached by 1:5 combination medium (9.06 x 105 cell/mL). We concluded that lablab bean mixed with palm sugar can be used to grow Lombok indigenous isolate of entomopathogenic B. thuringiensis to substitute standard/industrial medium with almost similar toxicity.

1. Introduction

Dengue Haemorrhagic Fever (DHF) or Dengue Frever is still becoming health problem in Lombok Island, Indonesia. disease is caused by Dengue Virus, spread widely by *Aedes aegypti* mosquito. In 2017, 1,605 people were reported suffering from this disease in this island [1]. One approach to eliminate the disease is by eliminating *A. aegypti* mosquito spread. The recommended procedure is using integrated mosquito management. One component of the approach is applying biological control

using bacterial agent, namely *Bacillus thuringiensis*[2]. *B. thuringiensis* is soil bacterium that is widely spread almost all over the world. Some strain of this bacterium is capable of killing mosquito larvae, caused by specific endotoxin they harbour[3]. Since 1980s *B. thuringiensis* is used to control mosquito larvae in US and other countries. The most popular strain is *B. thuringiensis* serovar Israelensis, as it was discovered in Israel dessert [4].

Until recent time, there is no local bacterial-based biopesticide product available in Indonesia. Such product must be imported from other countries. This situation will hamper moaquito control program. To develop local biopesticide product, it is very important to have indigenous entomopathogenic isolate of bacterial strain/isolate for developing the product. Isolation of entomopathogenic isolate of *B. thuringiensis* from sewage sediment at Mataram, Lombok was previously done by some researchers [5,6]. Local material (preferably from natural source) is also needed for producing the cell and toxin needed for the biopesticide preparation. The isolate was able to kill *C. quinquefasciatus* and*A. aegypti* larvae in their laboratory experiment.

The research in applying natural material for *B. thuringiensis* culture and toxin production medium have been accomplished by several researchers [7-9]. Other study showed that *B. thuringiensis* could grow and produced the highest amount of toxin when grown in natural medium with high content of carbohydrate [10].

Many materials potentially used for *B. thuringiensis* growing medium are widely available in Lombok. Some of them are lablab bean (*Lablab purpureus*) as protein source and palm sugar as carbohydrate source. They are all easy to get or buy almost at many places in this area. The aims of the study were to examine the affectivity of lablab bean and palm sugar to grow *B. thuringiensis* and to induce the toxicity of this bacterium against 3rd instar larvae of *A. aegypti*.

2. Material and Method

2.1. B. thuringiensis Isolate and Culture Refreshing

B. thuringiensis isolate used in this research was Bt-TP2B provided kindly by Ms. Herfiyanti. Culture refreshing was done by subculturing*B. thuringiensis* culture stock onto Nutrient Agar solid medium supplemented with 30 μg/L Amoxicillin to avoid contamination. The culture was incubated aerobically at 35^{o}C for 24 hours.

2.2. Medium Preparation

Medium used in this study were grounded lablab bean mixed with palm sugar in 4 combinations, which were 1:1 (1 part of lablab bean and 1 part of palm sugar); 1:3; 1:5 and 1:7, respectively. All medium combinations were added with aquades to meet 1 L final volume. Based concentration was 50 g/L for every solid ingredient. Standard medium used was NYSM (Nutrient Broth-Yeast Extract-Salt-Medium) liquid medium [11], supplemented with 30 μg/L of Amoxicillin.

2.3. Fermentation

One full loop from *B. thuringiensis* single colony was transferred into fermentation medium (4 combinations of lablab bean-palm sugar medium and standard NYSM medium). The cultures were then incubated in 35^{o}C with shaking for 72 hours. Within 72 hours incubation, cell, endospore and crystal protein concentration were observed every 24hours. At the end of fermentation, all cultures were subjected to bioassay against *A. aegypti* 3rd instar larvae.

2.4. Larval Rearing of A. aegypti

A. aegypti larvae were hatched from eggs obtained from Institute of Tropical Disease, Airlangga University, Surabaya. All eggs were submerged in untreated water with 12 hours light and 12 hours dark periods. After 1 week submerging, all eggs were hatched and 3rd instar larvae were ready in 6 days. When 3rd instar larvae were ready, bioassay can be performed shortly.

2.5. Bioassay

One thousand five hundred larvae were prepared for all groups (4 lablab bean-palm sugar medium combinations and NYSM standard medium). Each experiment consisted of 5 ten-fold concentration difference in 3 replications. Three hundred larvae were used in every group. Larval death was recorded every 24 hours (within 72 hours) from all experiment. Data obtained was analysed using Probit Analysis [12] to gain Lethal Concentration (LC) value using Minitab V16 for windows [13].

3. Result and Discussion

B. thuringiensis is one member of Genus Bacillus that (some strains/isolates) has ability to kill mosquito larvae. Along with other species (*B. sphaericus*), *B. thuringiensis* becomes popular as biological control agent used to control mosquito larvae. The ability to kill mosquito is caused by toxin (δ-endotoxin) it harbour in its chromosome [14]. Cell, endospore and crystal toxin morphology of *B. thuringiensis* is presented in **Figure 1.**

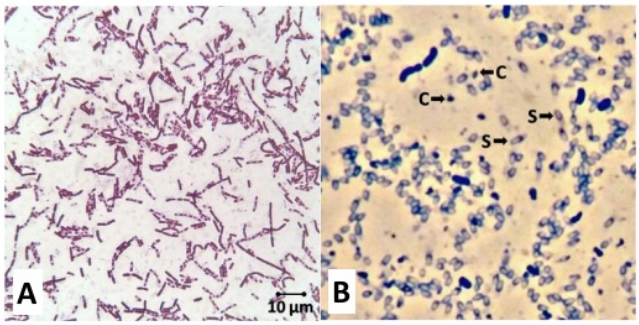

Figure 1. (A) Cell and endospore morphology of *B. Thuringiensis* (B) Endospore and toxin crystal morphology of *B. Thuringiensis*; C: Crystal; S: Spore (Endospore) (1,000x magnification)

In laboratory experiment, *B. thuringiensis* is usually grown in NYSM (Nutrient Broth Yeast Salt Medium) or LBB (Lurian Bertani Broth) medium [11]. Despite those medium can give high degree in growth and toxicity, price of the medium is very expensive. Therefore, it is necessary find alternative ingredient for its culture medium. Lablab bean and palm sugar that are highly abundant in Lombok Island can be alternative for growing *B. thuringiensis*. Although not staple food, lablab bean is consumed by Lombok people as alternative food for protein source. Palm sugar is sweetener substituting cane sugar.

When grown in medium consisted of lablab bean mixed with palm sugar, almost all medium combinations and NYSM standard medium showed similar trend (**Figure 2**). On the first 24 hours observation, almost all lablab bean-palm sugar combinations and standard medium showed increasing trend, with concentration value ranging from 1.30×10^7 to 7.00×10^7 cell/mL.The next 24 hours, all combinations showed decreasing in concentration, ranging from 5.50×10^6 to 1.35×10^7 cell/mL. Increasing trend only occurred on bean-palm sugar medium combination 1:1, which reached 5.95×10^7 cell/mL. The cell concentration decreased almost in all medium.

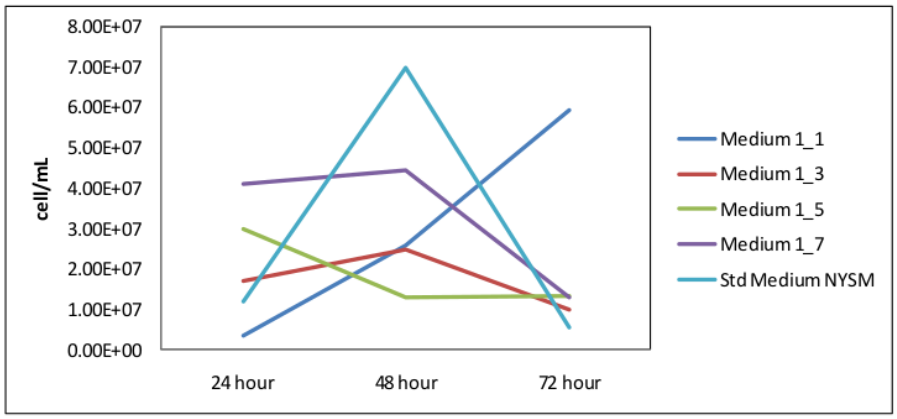

Figure 2. Cell concentration from *B. thuringiensis* grown in all medium combinations and NYSM standard medium within 72 hours observation

Endospore formation within 72 hours incubation was presented in chart in **Figure 3.** *B. thuringiensis* grown in all combinations of lablab bean-palm sugar medium did not show any endospore in the first 24 hours observation. Only *B. thuringiensis* grown in NYSM standard medium showed endospore formation (the endospore concentration was 6.50 x 10^6 endospore/mL). In 48hours incubation, *Bacillus thuringiensis* grown in all combinations of lablab bean-palm sugar medium showed endospore formation. Within 72 hours observation, the highest endospore concentration was reached by *B. thuringiensis* grown in Lablab bean – palm sugar 1:1 medium combination (4.32 x 10^8 endospore/mL), while 3 other combinations showed 2.90 x 10^7 to 7.00 x 10^7 endospore/mL.

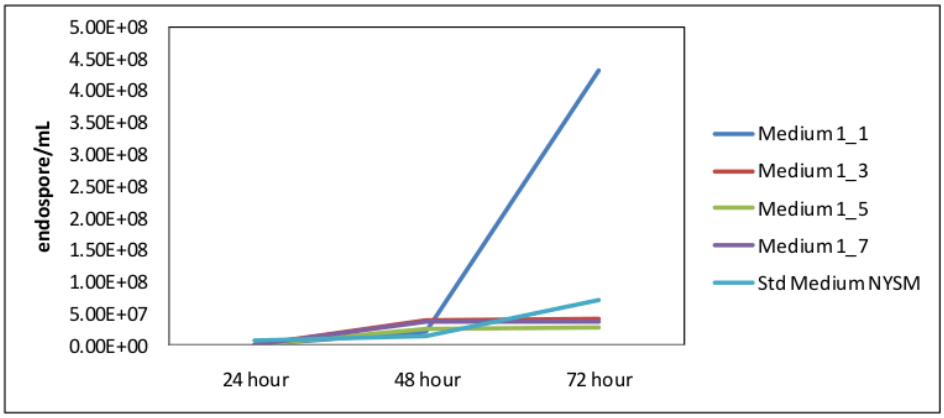

Figure 3. Endospore concentration from *B. thuringiensis* grown in all medium combinationsand NYSM standard medium within 72 hours observation

Toxin crystal concentration within 72 hours observation is presented in **Figure 4.** The highest crystal concentration in 72 hours was reached by *B. thuringiensis* grown in lablab bean-palm sugar medium 1:7 combinations (which was 2.55 x 10^7 crystal/mL). Other combination and NYSM standard medium only showed 1.50 x 10^6 to 2.55 x 10^7 crystal/mL. This observation was negatively correlated with endospore concentration observation in 72 hours.

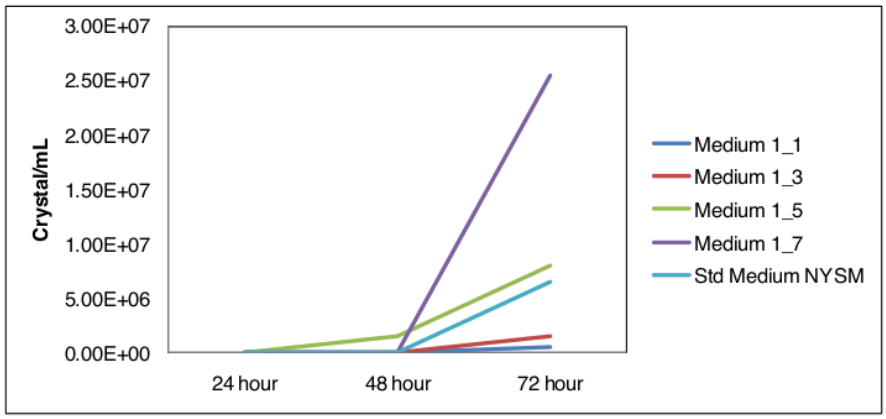

Figure 4. Crystal toxin concentration from *B. thuringiensis* grown in all medium combinations and NYSM standard medium within 72 hours observation

Lethal Concentration (LC) values (LC_{50} and LC_{90}) from all lablab bean-palm sugar medium combinations are presented in **Figure 5.** The lowest LC_{50} value of *B. thuringiensis* grown in lablab bean-palm sugar medium was reached by 1:7 medium combination (which was 1.97 x 10^5 cell/mL in 24 hours, 1.71 x 10^5 cell/mL in 48 hours and 1.44 x 10^5 cell/mL in 72 hours, respectively). These valued were followed by 1:5 combination (which was 4.66 x 10^6 cell/mL in 24 hours, 3.84 x 10^5 cell/mL in 48 hours and 1.52x10^5 cell/mL in 72 hours, respectively). The lowest LC_{90} value of *B. thuringiensis* culture was only reached by *B. thuringiensis* grown in NYSM standard medium (which was 7.47 x 10^5 in 24 hours and 2.67 x 10^5 in 48 and 72 hours, respectively).

The low LC_{50} value of *B. thuringiensis* grown in lablab bean-palm sugar medium 1:7 combination seemed to have positive correlation with very high crystal toxin showed by *B. thuringiensis* grown in this medium. However, *B. thuringiensis* grown in lablab bean-palm sugar medium 1:7 combination did not show very high endospore concentration within 72 hours observation. Endospore production rate presumably did not positively-correlated with crystal toxin production.

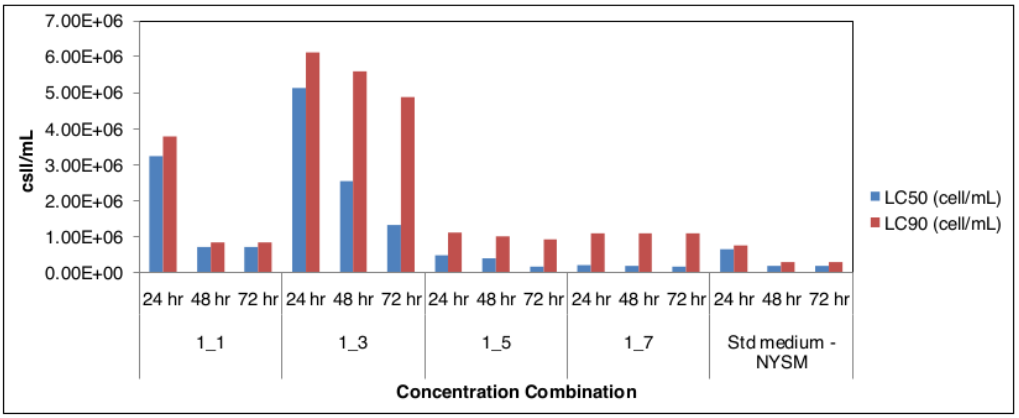

Figure 5. Toxicity of *B. thuringiensis* grown in all medium combinations and NYSM standard medium against *A. aegypti* 3rd instar larvae within 72 hours observation

Lablab bean has 23% protein content and 21% carbohydrate contents, respectively [15]. We

suggest that low protein content was not sufficient for *B. thuringiensis* to produce potent toxin protein against tested larvae in 72 hours incubation. These were showed by 1:1, 1:3 and 1:5 ratios. Therefore, more carbohydrate was needed by the bacterium to be able produce enough toxin to kill the larvae. The results were in agreement with study by Ferrera et al.[10] demonstrated that 1:7 ratio on Carbon and Nitrogen was needed by *B. thuringiensis* to produce large amount of toxin to effectively kill mosquito larvae when grown in natural plant-based medium.

Besides certain protein and carbohydrate ratio, more incubation time is needed to produce large amount toxin protein. In this study, incubation time was limited to 72 hours. In 72 hours incubation, sporulation and protein production was not taken place completely. Therefore, longer incubation time is needed to gain maximum amount of *B. thuringiensis* toxin. We suggest that for higher toxin production, incubation should be done in 5-7 days.

In killing mosquito larvae, toxin produced by *B. thuringiensis* works as follows. After being ingested by larvae, the protein crystal is solubilised and activated by high pH and protease in mosquito larval midgut. The activated toxins then bind to apical microvilli of midgut cells. Then, a part of the toxin inserts into the membrane lipid bilayer forming ionic-selective channel or pore. This will cause entry of water into the cell, exit of ions and other larger components. This condition leads to swelling and lysis of the cell [2].

Price comparison between laboratory medium (NYSM) and lablab bean-palm sugar medium is presented in **Table 1.**

Table 1. Price comparison between NYSM standard medium and lablab bean-palm sugar combinations medium for 1 L medium based on recent market price

Medium	Price/L
NYSM	Rp 92,750 (7.13 US$)
Lablab bean-palm sugar	Rp 45,200 (3.47 US$)

The price of lab-lab bean-palm sugar medium was closed to 1/2 that of NYSM standard medium. Besides cheaper, culture medium based on lablab bean and palm sugar easier to get/buy at almost any local market in Lombok Island and some places in Indonesia at any season. This will ease *B. thuringiensis*-based biopesticide producing effort using local material in Lombok Island and Indonesia.

4. Conclusion

In this study, growth medium consisted of lablab bean and palm sugar medium in 1:7 combination can be used to grow entomopathogenic indigenous *B. thuringiensis* isolates from Lombok and retained its toxicity against 3rd-instar *A. aegypti* larvae.

Acknowledgement

The authors would like to thank West Nusa Tenggara General Hospital for providing research facilities during this study. The authors also thank Biomedika Hospital Mataram for providing funding to support this study.

References

[1] Dinas Kesehatan Provinsi NTB 2018 *Profil Kesehatan Provinsi Nusa Tenggara Barat Tahun 2017* (Mataram, Indonesia: Dinas Kesehatan Provinsi NTB)
[2] Poopathi S and Tyagi Brij K 2004 Mosquitocidal toxins of spore forming bacteria: recent advancement *African Journal of Biotechnology* **3** 643–50
[3] Ibrahim M A, Griko N, Junker M and Bulla L A 2010 Bacillus thuringiensis A genomics and proteomics perspective *Bioeng Bugs* **1** 31–50
[4] Brownbridge M and Margalit J 1986 New Bacillus thuringiensis Strains Isolated in Israel Are Highly Toxic to Mosquito Larvae *Journal of Invertabrate Pathology* **48** 216–22

[5] Herfiyanti N F 2018 *Isolasi Bacillus thuringiensis Entomopatogenik Terhadap Culex quinquefasciatus dari Beberapa Lokasi Potensial Perindukan Nyamuk di Kota Mataram* Skripsi S1 (Mataram, Indonesia: Universitas Mataram)
[6] Adriani D F 2018 *Eksplorasi Bacillus thuringiensis Entomopatogenik Terhadap Aedes aegypti dari Beberapa Lokasi Potensial Perindukan Nyamuk di Kota Madya Mataram, Nusa Tenggara Barat* Skripsi S1 (Mataram, Indonesia: Universitas Mataram)
[7] Dulmage H T 1970 Production of the spore- endotoxin complex by variants of Bacillus thuringiensis in two fermentation media *Journal of Invertebrate Pathology* **16** 385–9
[8] Obeta J A N and Okafor N 1984 Medium for the Production of Primary Powder of Bacillus thuringiensis subsp. israelensis *Applied and Environmental Microbiology,* **47** 863–7
[9] Angelo E A, Vilas-Bôas G T, Santos C A dos, Lopes J and Arantes O M N 2012 Development of a low-cost medium for the culture of Bacillus thuringiensis subsp. israelensis Ciências Biológicas e da Saúde, Londrina vol 33 (Saúde, Londrina) pp 161–70
[10] Farrera R R, Perez-Guevara F and Torre M de la 1998 Carbon:nitrogen ratio interacts with initial concentration of total solids on insecticidal crystal rotein and spore production in Bacillus thuringiensis HD-73 *Appl Microbiol Biotechnol* **49** 758–65
[11] El-Bendary M A 2006 Bacillus thuringiensis and Bacillus sphaericus biopesticides production *J. Basic Microbiol.* **46** 158–170
[12] Finney D J 1971 *Probit Analysis* (London: Cambridge University Press)
[13] Minitab Inc. 2014 *Minitab Statistical Software V16* (Pennsylvania)
[14] Poopathi S and Abidha S 2010 Mosquitocidal bacterial toxins (Bacillus sphaericus and Bacillus thuringiensis serovar israelensis): Mode of action, cytopathological effects and mechanism of resistance *Journal of Physiology and Pathophysiology* **1** 22–38
[15] Murphi A and Colucci P 1999 A tropical forage solution to poor quality ruminant diets: A review of Lablab purpureus *Livestock Research for Rural Development* **11** 10

BASIC 2019

ORIGINALITY REPORT

15%	13%	10%	9%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

5%

★ pureapps2.hw.ac.uk

Internet Source

Exclude quotes	On	Exclude matches	< 1%
Exclude bibliography	On		

BASIC 2019

GRADEMARK REPORT

FINAL GRADE

/0

GENERAL COMMENTS

Instructor

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8