Eco. Env. & Cons. 26 (April Suppl. Issue) : 2020; pp. (S21-S26)
Copyright@ EM International
ISSN 0971–765X

Diversity of Indigenous entomopathogenic bacilli from domestics breeding sites of dengue Hemorrhagic fever vector based on the toxicity against *Aedes aegypti* Larvae

Salamun*, Ni'matuzahroh, Fatimah, M. I. F. Maswantari, M.U. Rizka, T. Nurhariyati and A. Supriyanto

Department of Biology, Faculty of Science and Technology, Airlangga University, Jl. Dr. Ir. Soekarno, Surabaya, 60115, Indonesia

(Received 27 September, 2019; Accepted 10 January, 2020)

ABSTRACTS

Dengue Hemorrhagic Fever (DHF) is an endemic disease caused by Dengue Virus and transmitted by *Ae. aegypti* mosquitoes. The cities of Surabaya and Sidoarjo and Gresik have been the highest density of *Ae. aegypti*. The research aimed to show the diversity of indigenous entomopathogenic bacilli from domestics breeding sites of vector of DHF based on toxicity against *Ae. aegypti* larvae. The 30 soil samples from breeding sites of *Ae. aegypti* were taken from 10 sites in Surabaya and Sidoarjo and Gresik, respectively. Isolation of bacteria was performed using *Bacillus* sp. growth media, identified with Gram and spore staining, and toxicity test was performed on the third instar larval of *Ae. aegypti*. The result of toxicity test shown that the indigenous entomopathogenics *Bacillus* sp. was variative of divercity against *Ae. aegypti* larvae. The 133 isolates of *Bacillus* sp. was isolated, 45(33.8%) non toxic and 88(66.2%) toxic against *Ae. aegypti* larvae. From 88 isolates, 45(51%) isolates low toxic, 27(30.6%) isolates moderate toxic, and 16(18.1%) isolates high toxic against *Ae. aegypti* larvae. The 16 isolates of *Bacillus* sp. was high toxic to the third instar larvae of *Ae. aegypti* more than 50% mortality. The three highest potency of these *Bacillus* sp. were isolate code EG6.4 from Gresik and isolate codes ES7.3 and ES4.3 from Surabaya. Base on macroscopic, microscopic, and physiological characterizations the isolate code EG6.4 and ES7.3 were *B. thuringiensis* with similarity indexs 80.6%, and 78.12% respectively, and isolate code ES4.3 was *Bacillus sphaericus* with similarity index 71.4%.

Key words *: Diversity, Indigenous bacillus sp., Toxicity test, Aedes aegypti*

Introduction

Bacteria *Bacillus* sp. is abundant soil bacteria which ranging from 10^6 to 10^7 cells per gram of soil (Paul, 2007). *B. thuringiensis* which has insecticidal activity, was first discovered by Ishiwata in 1901, then Berliner in 1915, and had a major role in plant pest control programs in agriculture (Balaraman and Pillai, 1990). The following period Kellen and colleagues in 1965 reported that *B. sphaericus* which had the first insecticidal activity was isolated from fourth instar larvae of *Culiceta*, in California USA (Balaraman and Pillai, 1990). Since the discovery of *B. thuringiensis* var. *israelensis* serotype H-14 by Golberg and Margalit (1977) in Israel and has been shown to have bioinsecticidal activity against mosquito larvae. Lately there have been many attempts to search for indigenous *B. thuringiensis* bacteria in

Asia, including Indonesia.

Dengue Hemorrhagic Fever (DHF) is an infectious disease caused by the Dengue Virus transmitted through the *Ae. aegypti* vector. The cities of Surabaya, Sidoarjo, and Gresik are 3 cities in East Java Indonesia that have become DHF endemic areas. Based on data from the Ministry of Health of the Republic of Indonesia (2016), in 2015 in Indonesia, there were 129,650 sufferers, 1,071 died and in 2016 there were 202,314 sufferers, 1,593 died. Population control of *Ae. aegypti* is a very important to prevent DHF outbreaks. Community actions to prevent DHF outbreaks are carried out through fogging and abatization. But until now the DHF outbreak has not been resolved, so it needs to control *Ae. aegypti* with environmentally friendly methods, namely biological control using mosquito natural enemies of disease vectors (Thomas, 2018).

Bacillus sp. reported has the potential to kill certain types of insects and is known as entomopathogenic bacteria. Research by Gama *et al.,* (2010), showed that the mortality of mosquito larvae was 88.89% using indigenous isolates of *B. thuringiensis* from Madura with a bacterial density of $1.51x10^8$ cells/mL. El-Kersh *et al.,* (2016), isolated from 300 soil samples and characterized the native strains of *B. thuringiensis* from Saudi Arabia, a number of 68 isolates have a larvisidal toxicity to mosquito vector of Malaria *Anopheles gambiens*, and recommended that the isolates of *B. thuringiensis* strains 63 has highest larvisidal toxicity and potential to developed as a biological control for mosquito vector disease in the future. Ammouneh *et al.* (2011) also isolated and characterized the native strains of *B. thuringiensis* derived from soil in Syria that could be developed as a bioinsecticide against agricultural pest insects. Researchers are interested in isolation and characterization of *Bacillus* sp. as well as examining the diversity of indigenous entomopathogenic *Bacillus* sp. from mosquito breeding sites based on their toxicity against *Ae. aegypti* larvae vector of DHF disease.

Materials and Methods

Sampling

Sediment soil samples were taken from mosquito breeding sites of *Ae. aegypti*. Sediment soil was taken in water reservoirs that have larvae in them. Based on, *Bacillus* could be isolated from soil, aquatic environments, herbivorous droppings, soil in the forest, dead insects and mosquito breeding sites (Schünemann's *et al.,* 2014; Chatterjee *et al.,* 2007). Sediment soil was taken using a rubber pipette and put into a sterile glass bottle.

Isolation of *Bacillus* sp.

Isolation of *Bacillus* sp. conducted at the Microbiology Laboratory of the Department of Biology, Faculty of Science and Technology, Airlangga University based on the method of Ohba and Aizawa (1986). The media used was *Bacillus* sp. growth media Nutrien Yeast Salt Media (NYSM) (Suryadi *et al.,* 2016). The media was poured into a Petri dish and then homogenized, allowed to condense and incubated at 37 ºC for 48 hours. Growing bacterial colonies were observed morphological characters of the colonies. Isolates which have characteristics similar to *Bacillus* sp. were purified by the quadrant streak plate method, and incubated for 72 hours at 30º C. The pure isolates were stored on slant agar at 4º C.

Preparation of *Ae. aegypti* larvae

The trial larvae were third instar larvae of *Ae. aegypti*. The egg of *Ae. aegypti* was obtained from Tropical Diseases Center (TDC) Airlangga University Surabaya, hatched in a plastic pan. The eggs hatched into third instar larvae for 6-8 days. Third instar larvae is more resistant to mechanical factors and has a longer time than other instar on the life cycle of *Ae. aegypti*.

Bacillus sp. toxicity test

Pure isolate of *Bacillus* sp. as much as one ose was taken inoculated in a sterile glass bottle containing 10 mL NYSM and incubated at room temperature for 24 hours on a rotary shaker. *Bacillus* sp. suspension absorbance values were measured with a wavelength spectrophotometer 600 nm (A_{600}). The preliminary toxicity test was carried out by suspension of *Bacillus* sp. as much as 5 mL inoculated in a glass bottle filled with 45 mL of tap water, 10 tailed test larvae. The control treatment was 45 mL of tap water, 5 mL of NYSM and 10 larvae. Larval mortality (%) was calculated at 24 and 48 hours exposure. Toxicity criteria determined with non-toxic, low toxic, moderate toxic and high toxic by mortalitas larva 0%, 10-20%, 30-50%, and >50% respectively. Diversity index calculated with Formula as used by Chatterjee *et al.* (2007). An advanced toxicity test with a high toxic would be carried out with 3 repli-

cation, further toxicity test with an equal absorbance value of A_{600} = 0.8.

Morphological characterization

Morphological characterization was carried out to determine the characteristics of the colony and microscopic characteristics. Three isolates of *Bacillus* sp. with the high toxic was grown on a Petri dish containing 15 mL NYSM media by the streak method, then observed its macroscopic characteristics directly after 48 hours of incubation. Macroscopic characteristics observed included the size of the bacterial colony, the color of the bacterial colony, the shape of the colony, the edge of the colony, and the elevation of the colony. Gram staining and spore staining used to identify microscopic characterization.

Physiological Characterization

Physiological characterizations ware done by tests of indol, motility, oxidase, starch hydrolysis, salinity, and additional tests using Microbact 12A12B. The additional test was carried out by means of a 225 µL bacterial suspension taken using a micropipette and inserted into each well on Microbact 12A12B. One drop of emersion oil was added to the well. The seal of the well was closed again if all suspensions had been entered. The results were observed after incubated in a 37 °C for 24 hours.

Data analysis

The data of the isolation and the toxicity test of *Bacillus* sp. against *Ae. aegypti* larva were analyzed with descriptive statistics. Data on morphological and physiological characteristics of selected isolates of indigenous entomopathogenic *Bacillus* sp. then identified with the Bergey's Manual of Systematic Bacteriology. The percentage of the proportional coefficient was determined based on the positive and negative similarities of each character to determine the bacterial species of the genus *Bacillus* (Stainer *et al.*, 1986).

Results and Discussion

Results

The results of the isolation obtained 45 isolates from Surabaya city, 42 isolates from Gresik city, and 46 isolates from Sidoarjo city. The results of Gram staining and spores staining of 133 isolates determined that all as Gram-positive bacteria, rod bacteria, and spores bacteria.

Bacillus sp. toxicity test

Total of 133 isolates of *Bacillus* sp., 88 of them were *Bacillus* sp. Entomopathogenic bacteria, while 45 other isolates were *Bacillus* sp. non-entomopathogenic bacteria. The preliminary toxicity test in Table 1.

In the preliminary toxicity test obtained 16 isolates high toxic status. Isolates ES6.1, ES9.1, ES9.2, ES8.2, and ESDA1.5 were the 5 high toxic with 100% larval mortality, then isolates EG6.4 with 90% larval mortality. Isolates ES1.2, ES7.1 and EG6.1 with 80% larval mortality, isolates ES3.4, ES4.5, ES4.2 and ESDA2.4 with 70% larval mortality, while ES7.3, ES10.3 and ES4.3 isolates with 60% larval mortality. Sixteen high toxic isolates were followed by advanced toxicity tests in Figure 1 and Figure 2.

Table 1. The results of the preliminary toxicity test for indigenous entomopathogenic *Bacillus* sp. against third instar larvae of *Ae. aegypti*.

	Surabaya city	Sidoarjo city	Gresik city
Non toxic	10	20	15
Low toxic	13	13	19
Moderate toxic	10	11	6
High toxic	12	2	2
Total isolates	45	46	42
Diversity index	1.38*	1.20*	1.15*

(* Moderate diversity)

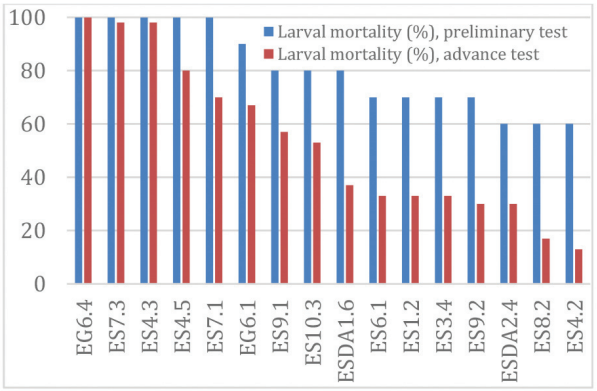

Fig. 1. Comparison of the results betwen preliminary toxicity tests with advanced toxicity tests for 16 indigenous entomopathogenic *Bacillus* sp. against *Ae. aegypti* larvae (Optical Density, A_{600}=0.8).

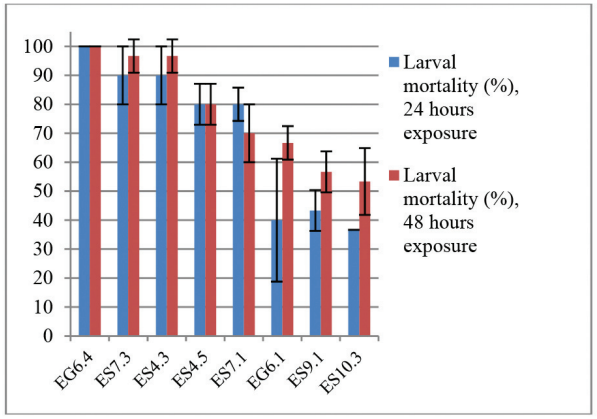

Fig. 2. The results of advanced toxicity test for 8 indigenous entomopathogenic *Bacillus* sp. against *Ae. aegypti* larvae (Optical Density, A_{600}=0.8 and 3 replication).

Characteristics of *Bacillus* sp isolate

Three high toxic of the indigenous entomopathogenic *Bacillus* sp. from the advanced toxicity test was EG6.4, ES4.3, and ES7.3 isolate. Based on the characterizations of colonies, the three isolate were irregular colonies and milky white color. EG6.4 isolate are small size, lobulated edges, and raised elevation, while ES4.3 and ES7.3 are moderate size, serrated edges and flat elevation. Based on Gram and spore staining, the three isolates included Gram-positive length 3-4 µm, oval spore shape, and subterminal spores for isolates EG6.4 and ES7.3, but terminal spore for ES4.3 isolate. Visualization of morphological characteristics of the three isolates in Figure 3.

The physiological characterization of EG6.4, ES4.3, and ES7.3 isolates showed that there were similarities and differences between the three isolates. The three isolates showed positive reactions in the lysine, ONPG, VP, gelatine, arabinose, arginine, motility, catalase, and salinity 5% test, but there were showed negative reactions in the ornithine, H_2S, glucose, indole, urease, citrate, TDA, malonate, inositol, rhamnose, lactose, adonitol, salicin, oxidase, and salinity 10% test. The three isolates showed diversity in the mannitol, xylose, sorbitol, sucrose, raffinose, and arginine tests. EG6.4 isolate was showed positive reactions in the sorbitol, sucrase, and arginine test, but was showed negative reactions in the xylose and raffinose test. ES4.3 isolate was showed positive reactions in the xylose and raffinose test, but was showed negative reactions in the sorbitol, sucrase, and arginine test. ES7.3 isolate was showed positive reactions in the mannitol and sucrose test, but was showed negative reactions in the xylose, sorbitol, raffinose, and arginine test. Based on similarities and differences in morphological and physiological characteristics, the results of the identification of the three isolates in Table 2.

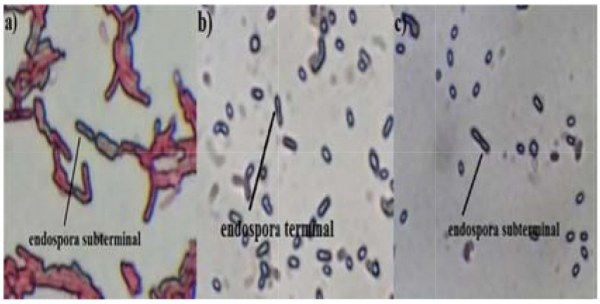

Fig. 3. Visualization of the shape and location of endospores in each bacterial isolate by spore staining a) EG6.4 isolate b) ES4.3 isolate c) ES7.3 isolate. (Magnification 1000x)

Table 2. The results of the identification of the three indigenous entomopathogenic *Bacillus sp.* from the breeding sites of *Ae. aegypti* mosquitoes.

No.	High Toxic Isolates	Species Name, (Similarity Indexs)
1	EG6.4	*B. thuringiensis* (80.6%)
2	ES4.3	*B. sphaericus* (76.12%)
3	ES7.3	*B. thuringiensis* (71.4%)

Discussion

The difference in larval mortality in the preliminary toxicity test with advanced toxicity test can be caused by differences in the activation of the toxin in the intestinal larvae. The reduced affinity of Bt-toxin in epithelial receptor cells will reduce the potential of *Bacillus* in killing larvae (Oppert *et al.*, 1997). Ibrahim *et al.* (2010) reported that larval mortalities caused δ-endotoxins released by *B. thuringiensis* during the sporulation phase. The ä-endotoxin in the form of protoxin is produced during the sporulation and is insecticidal when it enters the digestive tract of the larvae. The activity of protein crystals produced by *B. thuringiensis* can damage the larval midgut because the digestive tracts of mosquito larvae are alkaline and produce minerals and protease enzymes that can break down protoxin into toxins. A few minutes after entering

the larval digestive tract, the toxin will be bound to special receptors found in the microvilli of intestinal epithelial cells. After binding, the toxin caused small pores that disrupt the osmotic balance, so that ions and water easily enter the cell and cause damage to the structure and function of the intestine causing paralysis and larval death. Death of the larvae depend on the concentration of bacteria and varian of bacteria.

The larvacidal of *B. thuringiensis* is influenced by the presence of toxins in the larval breeding sites, eating behavior and usually takes food of *Ae. aegypti* larvae at the bottom and on the walls of water reservoirs (Dylo *et al.,* 2014). The results of the preliminary toxicity test, isolates from Surabaya, Gresik and Sidoarjo cities showed moderate diversity. Most of the isolates were nontoxic until moderate toxic and only 16 isolates with high toxic were obtained. A total of 16 isolates, 12 isolates from Surabaya city, 2 isolates from Gresik city and 2 isolates from Sidoarjo city. In the advanced toxicity test, 8 isolates of high toxic were obtained, namely EG6.4, ES4.3, ES7.3, ES4.5, ES7.1, EG6.1, ES9.1 and ES10.3 with successive test larval mortality 100%; 96.67%; 96.67%; 80%; 70%; 66.67%; 56.67% and 53.33% in the 48 hour exposure. Further advanced toxicity test on 16 potential isolates had different effects on the same bacterial density (A_{600}=0.8). The difference in toxicity of each isolate due to *Bacillus* sp. isolated from sediment soil has a diversity of pathogenic properties. Besides, the potential for toxicity from protein crystal of certain *B. thuringiensis* is influenced by solubility, an affinity for receptors and the breakdown of protoxin into toxins (Swadener, 1994). The *B. thuringiensis* effectiveness is also influenced by mosquito larvae instar, food, exposure period, water quality, bacterial strains, water temperature, presence of toxins in the substrate and larval feeding behavior (Dambach *et al.,* 2014).

The Surabaya city has the most number of *Bacillus sp.* isolates with high toxic compared to Sidoarjo and Gresik cities. The discovery of entomopathogenic bacteria at a certain time is influenced by many factors including rain and erosion, epizootic and enzootic, so it is possible at one time to find entomopathogenic bacteria in a certain place but at other times cannot be found again and vice versa (Salaki *et al.,* 2010).

The results of the identification showed that 2 isolates, namely EG6.4 and ES7.3 isolates have similarities with *B. thuringiensis* at 80.6% and 78.1%, respectively, while ES4.3 isolate have similarities with *B. sphaericus* at 71.4 %. Various studies have been carried out to explore the *B. sphaericus* bacteria which have the killing power of larvae of *Culex*, *Anopheles*, and *Aedes* mosquitoes which become vectors of several types due to protozoan and viral parasites, such as DHF disease through *Aedes* vectors (Thanabalu *et al.,* 1991). According to Suryadi *et al.,* (2016) who explores *B. sphaericus* from a coastal environment on the Lombok Island, *B. sphaericus* have larvicidal activity and are safe for non-target organisms. *B. sphaericus* isolate from the Lombok Island caused 72.5% to 100% larval mortality against *Anopheles aconitus*. *B. thuringiensis* is effective and toxic to *Ae. aegypti* mosquitoes and also safe for human and non-target organisms (Faraline, 2013). Gama *et al.,* (2010), reported that indigenous *B. thuringiensis* from Madura East Java caused mortality of mosquito larvae at 88.89%, whereas in Faraline research (2013) indigenous *B. thuringiensis* from Madiun East Java caused 100% larval mortality of *Ae. aegypti* larvae.

Conclusion

Based on the toxicity test against *Ae. aegypti* larvae, indigenous entomopathogenic *Bacillus sp.* have moderate diversity on breeding sites of *Ae. aegypti* DHF vector in Surabaya, Gresik, and Sidoarjo cities. Most of the isolates were non-toxic until moderate toxic status, only 16 isolates were high toxic in the preliminary toxicity test. In the advanced toxicity test, obtained 8 isolates with high toxic. The highest toxicity level of the indigenous entomopathogenic *Bacillus sp.* was EG6.4 isolate with 100% larval mortality, followed by ES7.3 and ES4.3 isolates with 96.7% larval mortality. Based on the morphological and physiological characteristics, EG6.4 and ES7.3 isolates had similarities with *B. thuringiensis* 80.6% and 78.12%, respectively, while ES4.3 isolate had a similarity of 71.4% with *Bacillus sphaericus*. Researcher recomended that three isolates have been developing the biolarvicidal agents for biological control of vectors, especially DHF disease vector.

Acknowledgment

The researcher would like to thank the Dean of the Faculty of Science and Technology and Chancellor of Airlangga University. This research was carried

out based on the Annual Activity Plan on Penelitian Unggulan Fakultas (Faculty of Excellence Research) of the Faculty of Science and Technology of Airlangga University in 2018. The researcher also thanked all parties who participated in this research.

References

Ammouneh, H., Harba, M., Idris, E. and Makee, H. 2011. Isolation and characterization of native *B. thuringiensis* isolates from Syrian soil and testing of their insecticidal activities against some insect pests. *Turk. J. Agric. For.* 35 : 421-431.

Balaraman, R.E. and Pillai, J.S. 1990. *Review of Biological Control Research at Vector Control Research Centre, Pondicherry*. Indian Council of Medical Research, New Delhi.

Chatterjee, S.N., Bhattacharya, T., Dangar, T.K. and Chandra, G. 2007. Ecology and Diversity of *B. thuringiensis* in soil Environment. *African J. Biotechnol*. 6 (13): 1587-1591.

Dambach, P., Louis V.R., Kaiser, A., Ouedraogo, S., Sie, A., Sauerborn, A. and Becker, N. 2014. Efficacy of *B. thuringiensis* var. *israelensis* against Malaria Mosquitoes in Northwestern Burkina Faso. *Parasites and Vectors*. 7 : 371.

Dylo, P., Martin, C. and Mhango, M. 2014. *Efficacy of B. thuringiensis var israelensis (Bti) on Culex and Anopheline mosquito larvae in Zomba. Malawi Journal of Science and Technology.* 10 : 141–152.

El-kersh, T.A., Ashraf, M.A., Al-sheikh, Y.A., Tripet F.M., Ibrahim, M.A. and Metwalli, A.A.M. 2016. Isolation and Characterization of Native *B. thuringiensis* strains from Saudi Arabia with Enhanced Larvicidal Toxicity against the Mosquito Vector *Anopheles gambiae*. *Parasites & Vectors.* 9 : 1-14.

Faraline, L. 2013. Studi Toksisitas *B. thuringiensis* Isolat Indigenous Jawa Timur Berdasarkan Ketinggian Tempat Terhadap Larva *Ae. aegypti*, *Jurnal Biotropika Edisi* 1 No.3

Gama, Z.P., Yanuwiadi, B. and Kurniati, T.H. 2010. Strategi Pemberantasan Nyamuk Aman Lingkungan: Potensi *B. thuringiensis* Isolat Madura Sebagai Musuh Alami Nyamuk *Ae. aegypti*. *Jurnal Pembangunan and Alam Lestari*. 1 : 2087-3522.

Goldberg, L.J. and Margalit, J. 1977. A Bacterial spore Demonstrating Rapid Larvicidal Activity against *Anophels sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti,* and *Culex pipiens. Mosq. News.* 37 (3): 355-358.

Ibrahim, M.A., Griko, N., Junker, M. and Bulla, L.A. 2010. *Bacillus thuringiensis*, A Genomics and Proteomics Perspective, Bioengineered *Bug.* 1 (1) : 31-50.

Kemenkes, R.I. 2016. Situasi DBD di Indonesia. *Pusat Data dan Informasi Kesehatan RI*, Ditjen Pencegahan dan Penularan Penyakit, Kementerian Kesehatan RI, Jakarta, Indonesia.

Ohba, M. and Aizawa, K. 1986. Insect toxicity of *B. thuringiensis* isolated from soils of Japan. *Journal of Invertebrate Pathology*. 47 : 12-20.

Oppert, B., Kramer, K.J., Beeman, R.W. and McGaughey, W.H. 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *J. Biol. Chem*. 272 : 23473-23476.

Paul, A. 2007. *Soil Microbiology, Ecology, and Biochemistry*. Academic Press, Elsevier Inc. Burlington, USA

Salaki, C. 2010. *Keanekaragaman Genetik Isolat B. thuringiensis Berliner Endogen Indonesia sebagai Agen Pengendali Hayati Hama Crocidolomia binotalis Zell. pada Tanaman Kubis*. Disertasi. Fakultas Biologi Universitas Gadjah Mada. Yogyakarta.

Schünemann, R., Knaak, N. and Fiuza, L.M. 2014. Mode of Action and Specificity of *B. thuringiensis* Toxins in the Control of Caterpillars and Stink Bugs in Soybean Culture. *Microbiology*. 1-12.

Stanier, R.Y., Adelberg, E.A. and Ingraham, J.L. 1986. *The Microbial World 5th Ed*. New Jersey: Prentice-Hall

Suryadi, B.F., Yanuwiadi, B., Ardyati, T. and Suharjono, 2016. Evaluation of Entomopathogenic *Bacillus sphaericus* Isolated from Lombok Beach Area against Mosquito Larvae. *Asian Pac. J. Trop. Biomed*. 6(2): 148–154.

Swadener, C. 1994. *B. thuringiensis. Journal of Pesticides Reform.* 14(3): 13-20. Northwest Coalition for Alternative to Pesticides. Ottawa.

Thanabalu, T., Hindley, J., Jackson-Yao, J. and Berry, C. 1991. Cloning, Sequencing, and Expression of a Gene Encoding a 100-kilodalton Mosquitocidal Toxin From *Bacillus sphaericus* SSII. *Bacteriology*. 173 : 2776-2785.

Thomas, M.B. 2018. Biological Control of Human Disease Vectors: A Perspective on challenges and Opportunities. *BioControl.* 63 : 61-69.