International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 2 (2014) pp. 179-197**
http://www.ijcmas.com

Review Article

Safe Strategy to Control Mosquito: The Potential of *Bacillus thuringiensis* Isolated Indigenously from East Java as a Natural Agents of Mosquito: *Aedes aegypti*

Zulfaidah Penata Gama[1,2,*] and Nobukazu Nakagoshi[1]

[1]Graduate School for International Development and Cooperation, Hiroshima University, 1-5-1, Kagamiyama Higashi Hiroshima, 739-8529, Japan
[2]Department of Biology, Faculty of Science, Brawijaya University, Jl. Veteran, Malang, 65145, Indonesia
Corresponding author

A B S T R A C T

K e y w o r d s

Biocontrol; Dengue Haemorrhagic Fever; Natural Enemy; Toxicity.

Bacillus thuringiensis provides effective alternatives to broad-spectrum larvicides in many situations with little or no environmental impacts. The advantages of microbial control agents' usage are numerous. These include safety for humans and other non-target organisms, reduction of pesticide residues in the aquatic environment, increased activity of most other natural enemies and increased biodiversity in aquatic ecosystem. The toxins of *Bacillus thuringiensis* affect structure of ephitel and intestine tissue of *Aedes aegypti* larvae. This phenomenon indicates that indigenous *Bacillus thuringiensis* from East Java isolates have its potential to become bio control of *Aedes aegypti* larvae.

Introduction

Mosquitoes are insect vectors responsible for the transmission of many diseases. Mosquitoes cause more human sufferings than any other organism; over one million people worldwide die from mosquito-borne diseases every year. Mosquito borne diseases include malaria, filariasis, yellow fever, chikungunya and dengue fever cause extensive morbidity and mortality and are a major economic burden within disease-endemic countries (Boutayeb, 2006; Poopathi and Abidha, 2010; Nareshkumar et al., 2012). *Aedes aegypty* (L.) is the main dengue vector worldwide because of its close association with humans in tropical and sub-tropical urbanized areas (Jansen and Beebe, 2010; Cox et al., 2007). The *Aedes aegypti* mosquito, the principal vector for DHF, which is considered the most pressing vector-borne viral disease in the world, is particularly susceptible to climate variability and climatic change. These mosquitoes are well adapted to urban environment and successfully breed in containers where water is allowed to accumulated, such as discarded can, bottles, plastic containers and tires. The

presence and abundance of *Aedes aegypti* is vital to the transmission of DHF. Changes in mean climatic conditions and climate variability also can affect human health via indirect pathways, particularly in the changing of biological and ecological processes that influence infectious disease transmission and food yields (Barrett *et al.,* 1998). As in the study conducted by Zulfaidah and Nakagoshi (2013) also mentioned that environmental conditions strongly control the geographic distribution and abundance of *Aedes aegypti.* The result of this study indicated that the climatic variability is clearly associated with the dengue incidence rate. The maximum air temperature, humidity, rainfall and light duration have played an important role in the transmission of DHF in Nganjuk district, East Java, Indonesia. Based on statistical analysis in this study, it showed that humidity and rainfall have positive correlation with DHF incidence, on the contrary a decreased value of maximum air temperature and light duration would have impact on increased IR. Another regression analysis resulted that IR of DHF was affected by the maximum air temperature, minimum air temperature and rainfall in the rainy season; however in the dry season, the IR was affected by wind velocity and rainfall.

Dengue haemorrhagic fever is one of the serious arboviral diseases in the tropical America, Asia, and Africa. DHF has been the most significant vector borne disease with increasing distribution and incidence of cases in the recent decades. An estimated 2.5 billion people in the world are at risk of dengue haemorrhagic fever, of which 1.3 billion people live in South East Asian Countries (Sankari et al., 2012). The incidences of Dengue Hemorrhagic Fever have been increasing in the last five years both in rural and urban areas of the world. The incidence of dengue has grown dramatically in recent decades. WHO currently estimates there may be 50 million dengue infections worldwide every year. An estimated 500,000 people with severe dengue require hospitalization each year; a large proportion of them are children. About 2.5% of those affected die. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-east Asia and the Western Pacific regions. Southeast Asia and the Western Pacific are the most seriously affected (WHO, 2010).

Indonesia as one of the countries in the tropics is very susceptible to diseases spread by the *Aedes aegypti* mosquito (Suci et al., 2010). DHF outbreaks have occurred in Indonesia since 1986; the first case was in Surabaya city. In 2010, there were more than 26,059 reported DHF cases in East Java (Provincial Health Office, 2011). In the past 50 years, the incidence of dengue has increased 30-fold with a widespread geographic area well into the new state or from urban to rural (WHO, 2009). According to Basic Health Research 2007, in Indonesia dengue cases that were diagnosed by health officers and the existence of symptoms stated by respondents showed the prevalence of 0.6% (range: 0.3 ‰ - 2.5%) within a period of 12 months (Malang City Health Office (2008). In addition, the prevalence in East Java is only 0.24%; but monitoring is still needed, considering that East Java is one of the densely populated provinces in Indonesia (Badan Pusat Statistik, 2012). According to the Basic Health Research 2007 of East Java Province, the prevalence of dengue and prevalence of the dengue fever symptoms in rural areas is smaller than in urban areas (0.23% vs. 0.27%).

Recently, uncontrolled use of chemical insecticides has resulted in irreparable damage to environment. Continuous use of chemical insecticides has led to the emergence and spread of resistance in vectors of human diseases and agricultural pest (Georghiou, 1990; El-Kersh et al., 2012). A major alternative to chemical control is biological control, which is a crucial part of integrated pest management (Aramideh et al., 2010; El-kersh et al., 2012). Interestingly, *Bacillus thuringiensis* is an important insect pathogen that is highly toxic to mosquito larvae and related dipterans (Poopathi, S. and Abidha, S 2010; Zulfaidah, et al. 2013). *Bacillus thuringiensis* is a Gram-positive, facultative anaerobe and spore forming saprophyte soil bacterium that was first isolated from diseased larvae of *Bombyx mori* (an economically important insect, being a primary producer of silk, called the silkworm) in Japan (Ishiwata, 1901; Baig and Samina, 2010). The toxicity is attributed to δ-endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate (Sanahuja et al., 2011; Haggag and Yousef, 2010). *Bacillus thuringiensis* during the sporulation produces one or more proteinaceous parasporal crystals (Cry), recognized as delta-endotoxin. This crystal protein under alkaline condition of midgut of insects, gets solubilized, and then activated by intrinsic protease into an active toxin that selectively binds specific receptor in the cell membrane, leading to pore formation and consequent insect death (Soberon et al., 2000; Eswarapriya et al., 2010; El-kersh et al., 2012). In Indonesia, some insecticides use active microbial *B. thuringiensis* imported from the countries such as Belgium (Bactospeine), the United States (mop) and Switzerland (Thuricide). The original *B. thuringiensis* exploration efforts in Indonesia were carried out because the *B. thuringiensis* crystal protein had an arrow host spectrum. Therefore, the ideal effort for controlling Indonesian mosquitoes would be using *B. thuringiensis* isolated from Indonesia (Zulfaidah et al., 2013).

The purpose of this review paper is to introduce the potential of *Bacillus thuringiensis* isolate indigenous from East Java as a natural agents of mosquito (*Aedes aegypti*) and to summarize the current status and developmental trends of biological control based on the published reports. Parts of the texts, tables and figures in this study were directly copied from previously published papers and reports.

Concepts of biological control agents

Global use of insecticides for mosquito vector control in recent decades has caused environmental pollution of aqueous ecosystem and resulted in insecticide resistance in many mosquito species. The last decade has witnessed and increased interest in biological control agents. Biological means to control vectors, based on entomopathogenic bacteria has been studied for more than 20 years. More number of biological control agents was screened for their efficacy, mammalian safety and environmental impact. Many organisms have been investigated as potential agents for vector mosquito control, including viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fish. Only a few spore forming bacteria, copepods and fish have reached operational use and are undergoing extensive field trials. The discovery of bacteria like *B. thuringiensis* serovar *israelensis* deBarjac, which are highly toxic to dipteran larvae have opened up the possibility of its use as

potential biolarvicides in mosquito eradication programs in the over the world (Poopathi and Tyagi, 2002; Poopathi et al., 2002). The larvicidal substances of these preparations are based on endotoxin proteins accumulated as parasporal crystals produced by the bacterial cells during the sporulation growth phase. These biological preparations have some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organism including human beings. Also, it is harmless to the environment (Prabakaran and Balaraman, 2006). The *B. thuringiensis* serovar *israelensis* has been used operationally for the control of mosquitoes for over two decades and its formulations are highly effective against *Anopheles*, *Aedes* and *Culex* mosquitoes (Mahmood, 1998; Poopathi and Abidha, 2010).

The potential of *Bacillus thuringiensis* isolated indigenously from East Java as a natural agents of *Aedes aegypti*

Research using *B. thuringiensis* var. *israelensis* is often done to control mosquitoes, either the formulation of *B. thuringiensis* as commercial products or culturing soil bacteria isolates. However *B. thuringiensis* isolate indigenous from East Java rarely used as a natural enemy. The previous study (Zulfaidah et al., 2010) showed that the efficacy of *B. thuringiensis* Madura isolates higher than *B. thuringiensis* var. *israelensis* for killing mosquito larvae. The ANOVA results showed that this experiment has significantly different for stage of mosquito larvae and dilution factors in the toxicity test of *B. thuringiensis* isolated from Madura Island against *Aedes aegypti* larvae, as well as the interaction between the dilution and stadia larvae also have different influences. The results of the study showed that the higher density of the bacteria (before dilution) would cause high mortality of mosquito larvae because the amount of protein crystals formed also increased. These results were indicated that the density of bacteria depended on the dilution, which increasing the dilution of bacteria could reduce the density of bacteria.

Mosquitoes are insect vectors responsible for the transmission of many diseases. Mosquitoes cause more human sufferings than any other organism; over one million people worldwide die from mosquito-borne diseases every year. Mosquito borne diseases include malaria, filariasis, yellow fever, chikungunya and dengue fever cause extensive morbidity and mortality and are a major economic burden within disease-endemic countries (Boutayeb, 2006; Poopathi and Abidha, 2010; Nareshkumar et al., 2012). *Aedes aegypty* (L.) is the main dengue vector worldwide because of its close association with humans in tropical and sub-tropical urbanized areas (Jansen and Beebe, 2010; Cox et al., 2007). The *Aedes aegypti* mosquito, the principal vector for DHF, which is considered the most pressing vector-borne viral disease in the world, is particularly susceptible to climate variability and climatic change. These mosquitoes are well adapted to urban environment and successfully breed in containers where water is allowed to accumulated, such as discarded can, bottles, plastic containers and tires. The presence and abundance of *Aedes aegypti* is vital to the transmission of DHF. Changes in mean climatic conditions and climate variability also can affect human health via indirect pathways, particularly in the changing of biological and ecological processes that influence

infectious disease transmission and food yields (Barrett *et al.,* 1998). As in the study conducted by Zulfaidah and Nakagoshi (2013) also mentioned that environmental conditions strongly control the geographic distribution and abundance of *Aedes aegypti.* The result of this study indicated that the climatic variability is clearly associated with the dengue incidence rate. The maximum air temperature, humidity, rainfall and light duration have played an important role in the transmission of DHF in Nganjuk district, East Java, Indonesia. Based on statistical analysis in this study, it showed that humidity and rainfall have positive correlation with DHF incidence, on the contrary a decreased value of maximum air temperature and light duration would have impact on increased IR. Another regression analysis resulted that IR of DHF was affected by the maximum air temperature, minimum air temperature and rainfall in the rainy season; however in the dry season, the IR was affected by wind velocity and rainfall.

Dengue haemorrhagic fever is one of the serious arboviral diseases in the tropical America, Asia, and Africa. DHF has been the most significant vector borne disease with increasing distribution and incidence of cases in the recent decades. An estimated 2.5 billion people in the world are at risk of dengue haemorrhagic fever, of which 1.3 billion people live in South East Asian Countries (Sankari et al., 2012). The incidences of Dengue Hemorrhagic Fever have been increasing in the last five years both in rural and urban areas of the world. The incidence of dengue has grown dramatically in recent decades. WHO currently estimates there may be 50 million dengue infections worldwide every year. An estimated 500,000 people with severe dengue require hospitalization each year; a large proportion of them are children. About 2.5% of those affected die. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-east Asia and the Western Pacific regions. Southeast Asia and the Western Pacific are the most seriously affected (WHO, 2010).

Indonesia as one of the countries in the tropics is very susceptible to diseases spread by the *Aedes aegypti* mosquito (Suci et al., 2010). DHF outbreaks have occurred in Indonesia since 1986; the first case was in Surabaya city. In 2010, there were more than 26,059 reported DHF cases in East Java (Provincial Health Office, 2011). In the past 50 years, the incidence of dengue has increased 30-fold with a widespread geographic area well into the new state or from urban to rural (WHO, 2009). According to Basic Health Research 2007, in Indonesia dengue cases that were diagnosed by health officers and the existence of symptoms stated by respondents showed the prevalence of 0.6% (range: 0.3 ‰ - 2.5%) within a period of 12 months (Malang City Health Office (2008). In addition, the prevalence in East Java is only 0.24%; but monitoring is still needed, considering that East Java is one of the densely populated provinces in Indonesia (Badan Pusat Statistik, 2012). According to the Basic Health Research 2007 of East Java Province, the prevalence of dengue and prevalence of the dengue fever symptoms in rural areas is smaller than in urban areas (0.23% vs. 0.27%).

Recently, uncontrolled use of chemical insecticides has resulted in irreparable damage to environment. Continuous use of chemical insecticides has led to the emergence and spread of resistance in vectors of human diseases and agricultural pest (Georghiou, 1990; El-Kersh et al.,

2012). A major alternative to chemical control is biological control, which is a crucial part of integrated pest management (Aramideh et al., 2010; El-kersh et al., 2012). Interestingly, *Bacillus thuringiensis* is an important insect pathogen that is highly toxic to mosquito larvae and related dipterans (Poopathi, S. and Abidha, S 2010; Zulfaidah, et al. 2013). *Bacillus thuringiensis* is a Gram-positive, facultative anaerobe and spore forming saprophyte soil bacterium that was first isolated from diseased larvae of *Bombyx mori* (an economically important insect, being a primary producer of silk, called the silkworm) in Japan (Ishiwata, 1901; Baig and Samina, 2010). The toxicity is attributed to δ-endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate (Sanahuja et al., 2011; Haggag and Yousef, 2010). *Bacillus thuringiensis* during the sporulation produces one or more proteinaceous parasporal crystals (Cry), recognized as delta-endotoxin. This crystal protein under alkaline condition of midgut of insects, gets solubilized, and then activated by intrinsic protease into an active toxin that selectively binds specific receptor in the cell membrane, leading to pore formation and consequent insect death (Soberon et al., 2000; Eswarapriya et al., 2010; El-kersh et al., 2012). In Indonesia, some insecticides use active microbial *B. thuringiensis* imported from the countries such as Belgium (Bactospeine), the United States (mop) and Switzerland (Thuricide). The original *B. thuringiensis* exploration efforts in Indonesia were carried out because the *B. thuringiensis* crystal protein had an arrow host spectrum. Therefore, the ideal effort for controlling Indonesian mosquitoes would be using *B. thuringiensis* isolated from Indonesia (Zulfaidah et al., 2013).

The purpose of this review paper is to introduce the potential of *Bacillus thuringiensis* isolate indigenous from East Java as a natural agents of mosquito (*Aedes aegypti*) and to summarize the current status and developmental trends of biological control based on the published reports. Parts of the texts, tables and figures in this study were directly copied from previously published papers and reports.

Concepts of biological control agents

Global use of insecticides for mosquito vector control in recent decades has caused environmental pollution of aqueous ecosystem and resulted in insecticide resistance in many mosquito species. The last decade has witnessed and increased interest in biological control agents. Biological means to control vectors, based on entomopathogenic bacteria has been studied for more than 20 years. More number of biological control agents was screened for their efficacy, mammalian safety and environmental impact. Many organisms have been investigated as potential agents for vector mosquito control, including viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fish. Only a few spore forming bacteria, copepods and fish have reached operational use and are undergoing extensive field trials. The discovery of bacteria like *B. thuringiensis* serovar *israelensis* deBarjac, which are highly toxic to dipteran larvae have opened up the possibility of its use as potential biolarvicides in mosquito eradication programs in the over the world (Poopathi and Tyagi, 2002; Poopathi et al., 2002). The larvicidal substances of these preparations are based on endotoxin proteins accumulated as parasporal

crystals produced by the bacterial cells during the sporulation growth phase. These biological preparations have some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organism including human beings. Also, it is harmless to the environment (Prabakaran and Balaraman, 2006). The *B. thuringiensis* serovar *israelensis* has been used operationally for the control of mosquitoes for over two decades and its formulations are highly effective against *Anopheles, Aedes* and *Culex* mosquitoes (Mahmood, 1998; Poopathi and Abidha, 2010).

The potential of *Bacillus thuringiensis* isolated indigenously from East Java as a natural agents of *Aedes aegypti*

Research using *B. thuringiensis* var. *israelensis* is often done to control mosquitoes, either the formulation of *B. thuringiensis* as commercial products or culturing soil bacteria isolates. However *B. thuringiensis* isolate indigenous from East Java rarely used as a natural enemy. The previous study (Zulfaidah et al., 2010) showed that the efficacy of *B. thuringiensis* Madura isolates higher than *B. thuringiensis* var. *israelensis* for killing mosquito larvae. The ANOVA results showed that this experiment has significantly different for stage of mosquito larvae and dilution factors in the toxicity test of *B. thuringiensis* isolated from Madura Island against *Aedes aegypti* larvae, as well as the interaction between the dilution and stadia larvae also have different influences. The results of the study showed that the higher density of the bacteria (before dilution) would cause high mortality of mosquito larvae because the amount of protein crystals formed also increased. These results were indicated that the density of bacteria depended on the dilution, which increasing the dilution of bacteria could reduce the density of bacteria.

The honesty testing results showed that the *Bacillus thuringiensis* isolated from Madura was able to kill 50% of each instar mosquito larvae which were tested within 24 hours. $LC_{50\text{-}24\ hours}$ values for each instar were relatively similar, although for the first instars was a smallest compared to the older age. This was because the first instar was more sensitive to the crystal toxin produced by *Bacillus thuringiensis* than the second instar *Aedes aegypti* larvae, third and fourth instar larvae.

The same thing also happened in the study conducted by Zulfaidah et al., (2013), using indigenous *Bacillus thuringiensis* isolates from Malang city and Madiun city, East Java on the third instar *Aedes aegypti* larvae (Lidwina et al., 2013). Based on phenotypic characteristics and literature searches, it was found that indigenous *Bacillus thuringiensis* isolated from Malang had similar characteristics to the *Bacillus thuringiensis* subspecies aizawai. Based on the percentage of spore prevalence in *Bacillus thuringiensis* isolates at 48 hours, it was known that if bacterial spore prevalence was increasing, the amount of toxin produced was also growing. As the number of bacterial toxins increases, one may expect the bacteria to be more effective at killing mosquito larvae. There are differences in spore prevalence, which is associated with individual characteristics of the spore-forming isolates. *Bacillus thuringiensis* has two developmental phases: germination and sporulation (Manonmani et al., 2011). During sporulation, parasporal crystals are released by

Table.1 ANOVA of toxicity test of *Bacillus thuringiensis* isolated from Madura on *Aedes aegypti* larvae

Source	DF	SS	MS	F	P
Instar larvae (L)	3	1807.40	602.46	13.56	0.00
Dilution (D)	6	222271.90	3711.90	83.50	0.00
L * D	18	9651.80	536.21	12.07	0.00
Error	57	2488.80	44.44		
Total	84	41511.10			

Table.2 The cell density of *Bacillus thuringiensis* isolated from Madura (cell/ml) in each dilution series

Dilution series	The cell density of bacteria in each dilution (cell/ml)					
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Average	1.51×10^{8}	9×10^{6}	1.27×10^{6}	6.17×10^{5}	4×10^{5}	5×10^{4}

Table.3 Honesty test results on interaction between dilution series of *Bacillus thuringiensis* isolated from Madura and the mortality percentage of *Aedes aegypti* larvae (for each instar larvae)

Dilution series	Percentage of larvae mortality on			
	First instar	Second instar	Third instar	Fourth instar
10^{0}	88.89 d	64.44 c	26.67 b	11.11 ab
10^{-1}	2.22 a	0.00 a	2.22 a	4.44 ab
10^{-2}	2.22 a	2.22 a	4.44 a	0.00 a
10^{-3}	0.00 a	0.00 a	2.22 a	0.00 a
10^{-4}	6.67 ab	0.00 a	0.00 a	0.00 a
10^{-5}	2.22 a	0.00 a	2.22 a	0.00 a

The same letters after the number denote no significant difference (significant at the 0.05 level).

Table.4 Honesty testing results for each instar *Aedes aegypti* larvae based on LC50-24 hours

Instar	N	Subset for $\alpha = 0.05$
1	3	80765494^{a}
2	3	$1.24 \times 10^{8\,a}$
3	3	$3.94 \times 10^{8\,a}$
4	2	$2.66 \times 10^{8\,a}$

The same letters after the number denote no significant difference of LC50-24 hours (significant at the 0.05 level).

autolysis. These crystals are toxic and will damage the mosquito larval digestive tract, thus causing larval mortality.

The early stationary phase is marked by vegetative cell death, followed by toxin accumulation, because the cells metabolise the available nutrients, resulting in nutrient shortage and competition. The bacteria will then synthesise secondary metabolites that are used to maintain life. In *Bacillus thuringiensis*, this stationary phase is associated with spore and toxin formation. Toxins from *Bacillus thuringiensis* cells are formed after the cells have formed endospores (Muniady et al., 2011).

The study by Zulfaidah et al., (2013) showed that this experiment also conducted toxicity tests using various dilutions of the bacterial suspension (1:0, 1:1,1:3, 1:5, 1:7, 1:10, 1:15, and 1:20) and exposure times (24, 48, and 72h). The toxin effectiveness of the *Bacillus thuringiensis* isolates was determined. The bacteria form spores and parasporal crystals during the stationary phase, which is a nutritionally deficient state; at that time, the parasporal crystals will be toxic and can kill the *Aedes aegypti* larvae (Renganatan et al., 2011). The mosquito third instar larvae are selected because at this stage, the larvae have a complete anatomical structure and the body is divided into three parts (head, thoracic, abdomen); therefore, damage to the larvae can be easily observed within each section. A previous study demonstrated the numbers of intestinal epithelial cells and peritrophic cells increase in accordance within creasing larval toxin resistance (Poopathi, 2010).

Based on the study conducted by Zulfaidah et al., (2013) it was indicated that the statistical analysis of the LC_{50} test (lethal concentration) have a significant effect ($p<0.05$) among the tested isolates. The three isolates that are indigenous to Malang City (PWR4-32, SWJ 4-4b, SWJ 5-1) killed *Aedes aegypti* larvae. Among those, the PWR4-32 isolates were the most effective, as 22.79 x 10^7cells/ml were required to kill fifty percent of the *Aedes aegypti* larvae within 72 hs. The 72-hs exposure time was more effective than the 24-hs and 48-hs exposure times. Once the bacterial toxin enters the mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with exposure. The toxin binds to receptors located on the epithelial midgut cell wall's apical microvillus membrane. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing toxin insertion into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralysed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after two hours and die within six hours of toxin injection (Poopathi et al., 2002; Poopathi, 2010). Mosquito larvae mortality is also influenced by factors such as bacterial concentration, larval age and the bacterial strain used (Ramirez-Suero et al., 2011; Poopathi and Tyagi, 2006).

A negative relationship can be observed between the exposure time and the LC_{50} for the *Bacillus thuringiensis* indigenously from Malang City and the reference *Bacillus thuringiensis*. This means that with a longer exposure time, the LC_{50} value will decrease and the larval mortality level will increase (Arivoli and Tennyson, 2011; Valadez-Lira et al.,

2011). All of the tested and reference isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. Increased mortality was also observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate. Some researchers also stated that among mosquitoes, different preparations of *Bacillus thuringiensis israelensis* have shown different levels of toxicity to host species. Others factors influencing the susceptibility of mosquito larva to *Bacillus thuringiensis israelensis*. For example; the effect of given dosage of toxin could produce different results depending on weather the lethal dose is administered all at once or in same doses over a long period (Aly et al., 1988; Ramirez-Lepe and Montserrat, 2012).

The current status and developmental trends of biological control using *Bacillus thuringiensis*

Over the past decades, the trend has been towards abatement in the use of chemical insecticides, progressively replaced by emerging environment-friendly pesticides such as bacterio-insecticides, strongly recommended by the World Health Organization (WHO, 1976). *Bacillus thuringiensis* is one of the most famed spore forming bacterium, which was first isolated in 1901 in Japan by Shigetane Ishiwata as the cause of the sudden ("sotto") death disease of silkworms, larvae of the silkworm moth, *Bombyx mori* (Federici et al., 2010). After Ishiwata's discovery, Ernst Berliner, the German bacteriologist, unaware of Ishiwata's publication, described a similar bacterium as cause of disease in larvae of the flour moth, *Ephestia kuhniella*. The species name "*thuringiensis*" is derived from Thuringia, the German state where the diseased flour moth larvae were found. It was later isolated from Mediterranean flour moths and named *Bacillus thuringiensis* in 1911. It was not until 1958 than *Bacillus thuringiensis* was used commercially in the United States (Jenkins, 1992; Poopathi and Abidha, 2010). Federici et al., (2010) argues that *Bacillus thuringiensis* is a species of bacteria that has insecticidal properties affecting a selective range of insect orders. There are at least 34 subspecies of *Bacillus thuringiensis* (also called serotype or varieties) and probably over 800 strain isolates By 1989, *Bacillus thuringiensis* products had captured 90-95 per cent of the biopesticide market. *Bacillus thuringiensis* products available in the United States are comprised of one of five varieties of *B. thuringiensis; Bacillus thuringiensis* var. kurstaki and var. morrisoni which cause disease in moth and butterfly caterpillars; *Bacillus thuringiensis* var.israelensis which causes disease in mosquito and blackfly larvae; *Bacillus thuringiensis* var. aizawai which causes disease in wax moth catterpillars; and *Bacillus thuringiensis* var. *tenebrionis*, also called var. *san diego*, which causes disesase in beetle larvae. Other strains of *Bacillus thuringiensis* have been discovered that exhibit pesticidal activity against nematodes, mites, flatworms and protozoa (Feitelson, et al., 1992).

Two general groups of insecticidal crystal proteins made this wide variety of subspecies have been identified by Cyt (cytolysins) and Cry (crystal delta-endotoxins). Höfte and Whiteley (1989), reviewed systematic nomenclature and classified the crystal proteins in five major groups according to their insecticidal and

molecular relationship (Cry I, Cry II, Cry III, Cry IV and Cry V, Cyt). As new strains are discovered, a need for a new nomenclature arose. According to new nomenclature which is used today, roman numerals have been exchanged with the Arabic numerals and the strains are named on the basis of their evolutionary divergence. Additionally, beneath the capital letters which were present at the first nomenclature as well, small letters have been brought indicating the minor amino acid differences like the capital letters denoted for the major differences. It is also noted that most *Bt* strains produce more than one type of crystal protein that act in combination. Hastowo et al., (1992) have been set up several screening programs that aim to isolate new strains producing novel mosquitocidal crystal proteins that could replace or be used in combination with *Bacillus thuringiensis* subsp. *israelensis. Bacillus thuringiensis* subsp. *entomocidus* INA288 has been isolated from Indonesia soil, and it produces large cuboidal crystals. Although known mosquitocidal *cry* genes, such as *cry2A, cry4A, cry4B* and *cry cry11A*, were not detected by PCR, this strain showed a toxicity comparable to that of *Bacillus thuringiensis* subsp. *israelensis* against second-instar *Aedes aegypti*. This result indicated the presence of a novel mosquitocidal *cry* gene(s). When the structural and sequential similarities are considered, conserved amino acid sequences drew attention among most of the Cry toxins. According to these similarities and insecticidal activities, the properties of the Cry proteins differ and the members of the same group share a number of common features. The types of crystal proteins and the insect orders to which they are active were showed in table 6.

Bacillus thuringiensis crystals are composed of four major polypeptides with molecular weights of 125, 135, 68 and 28 kDa, now referred to as Cry IVA, Cry IVB, Cry IVD and CytA, respectively. De Maagd et al. (2003) stated that insecticidal crystal toxins (Cry) are pore-forming toxins (PFT) produced by this bacterium as crystalline inclusions during the sporulation phase of growth. Crystal development during sporulation of *Bacillus thuringiensis* strains has been studied extensively. These crystals are predominantly comprised of one or more crystal (Cry) and Cytolytic (Cyt) toxins. Cyt proteins are parasporal inclusion protein from *Bacillus thuringiensis* exhibits hemolytic activity. For example *Bacillus thuringiensis* subsp. *israelensis* produces four protein/polypeptides ranging from 135-27kDa referred to as CryIVA, CryIVB, CryIVD and CytA (Lakxmy et al., 2011). These genes, encoding this Cry toxin, are located on 72 kDa resident plasmid and they have been cloned and expressed in various hosts. Chromosomal Cry genes have also been reported in some *Bacillus thuringiensis* strains and the role, structure and molecular organization of genes coding for the parasporal delta endotoxin of *Bacillus thuringiensis* biochemical mechanisms of insects' resistance to *Bacillus thuringiensis* indicates that altered proteolysis processing of *Bacillus thuringiensis* crystal proteins involved in one case of resistance in mosquitoes. The presence of IS240 elements responsible for mosquitocidal action was investigated in sixty-nine *Bacillus thuringiensis* strains. A PCR-based approach for detection of Cry genes in *Bacillus thuringiensis* has been reported. Since the toxins of this bacterium are highly potent for mosquito control, evaluation of the activity of *Bacillus thuringiensis* preparations is currently

Table.5 Analysis of spore prevalence in *Bacillus thuringiensis* isolates at 48 hours

Isolate	Prevalence of spores (%)
PWR4-31	9.28 ± 11.49 (a)
PWR4-32	52.44 ± 40.09 (a)
SWJ4-2b	0.82 ± 0.55 (a)
SWJ4-4b	23.59 ± 9.91 (a)
SWJ4-4k	4.34 ± 21.38 (a)
SWJ5-1	34.46 ± 12.28 (a)
Reference *Bacillus thuringiensis*	10.02 ± 36.96 (a)

The same letters after the numbers denote no significant difference (α = 0.05).

Table.6 The Cry protein groups and the orders they are pathogenic for

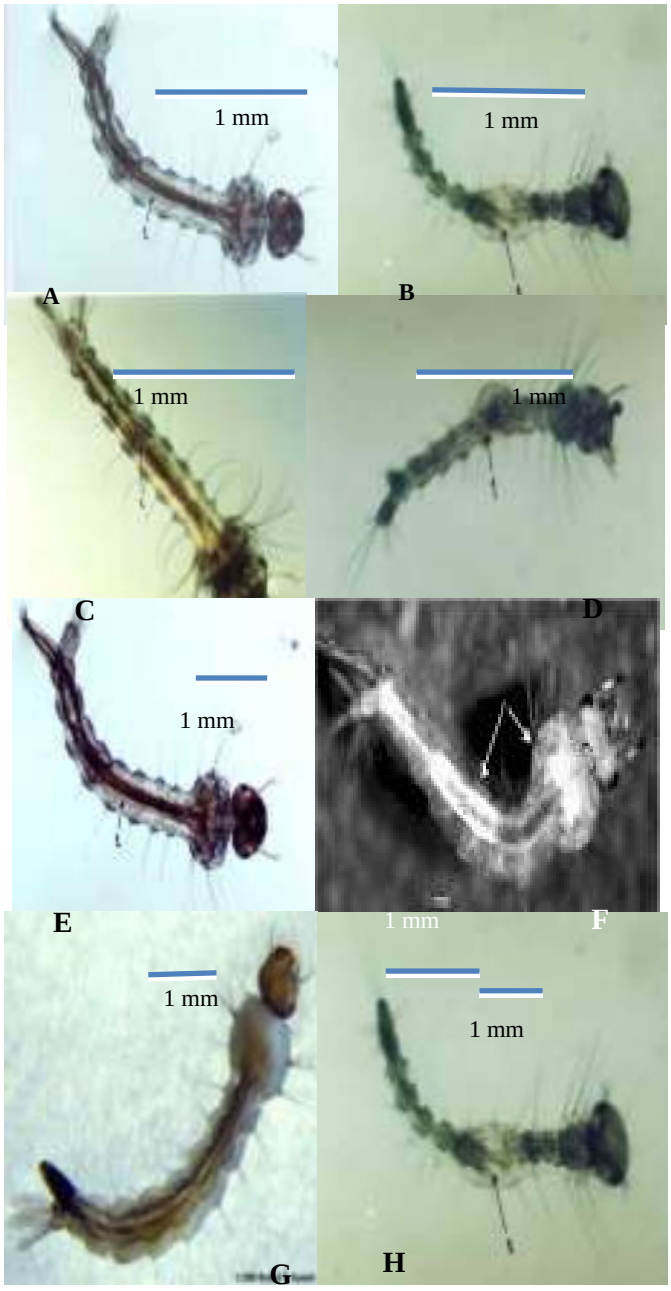
No.	The Cry protein groups	The orders for
1.	Cry1, Cry9, Cry15	Lepidopteran larvicidal
2.	Cry3, Cry7, Cry8, Cry14, Cry34, Cry35, Cry36, Cry38	Coleopteran larvicidal
3.	Cry4, Cry10, Cry11, Cry16 (Cry17), Cry19, Cry20, Cry24, Cry25, Cry27, Cry29, Cry30, Cry39, Cry40	Dipteran larvicidal
4.	Cry2	Lepidopteran and Dipteran larvicidal
5.	Cry5, Cry6, Cry12, Cry13, Cry21	Nematicidal
6.	Cry22	Active on Hymenopteran

carried out by bioassay with a target insect and compared to a defined standard (Poopathi and Abidha, 2010). Federici et al., (2010) argued that *Bacillus thuringiensis* produces a variety of insecticidal proteins produced during vegetative growth and sporulation that determines its activity for insect species belonging to different orders, with the most important of these being the Cry protein active against Lepidoptera and Coleopteran pest and a combination of Cry and Cyt proteins for mosquitoes and blackflies. After intoxication by these proteins, spores typically germinate and invade larvae, contributing to insect mortality. Whereas strains wild type isolates have been commercialized and are now used worldwide, the use of recombinant DNA technique, genetic engineering, has been used over the past decade to recombine the proteins of different *Bacillus thuringiensis* strains with those of *Bacillus sphaericus* to generate recombinant larvicides as much as ten-fold more toxic than the parental strains.

Mode of action

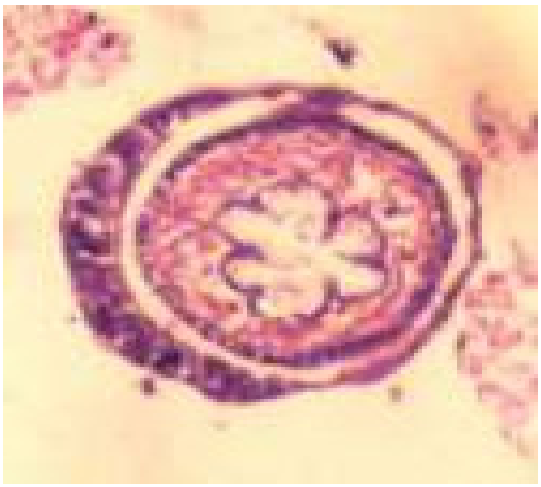
Bacillus thuringiensis israelensis products contain the spores and parasporal crystals of *Bacillus thuringiensis* var. *israelensis* H-14 serotype that must be ingested by the larval stage of the mosquito to cause mortality. This multi-step toxicity process

Figure.1 *Aedes aegypti* larvae



Note: A. The first instar larvae before toxicity test; B. The first instar larvae after toxicity test; C. The second instar larvae before toxicity test; D. The second instar larvae after toxicity test; E. The third instar larvae before toxicity test; F. The third instar larvae after toxicity test; G. The fourth instar larvae before toxicity test; H. The fourth instar larvae after toxicity test; 1. Digestive tract.

Figure.2 Crossed section of the fourth instar *Aedes aegypti* larvae
(E. Ephitel; P. Peritrophic membrane)



includes ingestion of the Cry protein by a susceptible insect, solubilization, and procesing from a protoxin to an activated toxin core in the insect digestive fluid. The toxin core travels across the peritrophic matrix and binds to specific receptors called cadherins on the brush border membrane of the gut cells. Toxin binding to cadherin proteins results in activation of an oncotic cell death pathway and/or formation of toxin oligomers that bind to GPI-anchored proteins and concentrate on regions of the cell membrane called lipid rafts. Accumulation of toxin oligomers results in toxin insertion in the membrane, pore formation, osmotic cell shock, and ultimately insect death (Poopathi and Abidha, 2010). Cyt genes are active against dipteran and coleopteran pests, and additionally have shown an action against hemipterans (trus bugs) and dictyopterans (roaches and termites). Cyt, unlike Cry toxins, do not recognize specific binding sites. *Bacillus thuringiensis* directly causes mortality in insects and isolates of the toxin from different strains follow similar mode of action. After delta-endotoxin crystals are ingested, they are dissolved in the insect midgut, liberating protoxins of which they are made. These are proteolytically processed into fragments, one of which binds to cells of the midgut epithelium. The activated protein disrupts the osmotic balance of these cells by forming pores in the cell membrane causing the cell to lyse. The gut becomes paralyzed and the insect stop feeding; and as a result, most insect will die within few hours of ingestion. In order to identify the biological pathways that were activity after toxin ingestion, Cancino-Rezno et al. (2012) mapped the differential expressed protein to canonical signaling pathways found in the Kyoto Encyclopedia of Genes and Genomes (KEGG). The KEGG analysis showed the immune system NOD-like receptor pathway since the heat shock protein HSP90 participates in this pathway. In addition some protein of the pathways of glycolysis, citrate cycle and fatty acid metabolism pathways were also activated by Cry11Aa treatment, suggesting activation of carbohydrate and lipid metabolism after toxin intoxication. The binding affinity of these toxin fragments is often directly related to the toxicity, though binding does not assure

toxicity (Whalon and McGaughey, 1998). *Bacillus thuringiensis* var. *israelensis* treated mosquito larvae generally cease feeding within 1 hour, and then it shows reduced activity by two hours, extreme sluggishness by four hours and general paralysis by six hours after ingestion (Glare and Maureen, 1998).

Effect of *Bacillus thuringiensis* on no target organisms

Bacillus thuringiensis has no direct effect on aquatic organisms other than mosquitoes, blackflies, lacewing, aphid, crustacea and chironomids. Other aquatic organisms, such as shrimps, mites and oysters are generally unaffected (Eder and Iris, 2010). This large safety margin of preparations of *Bacillus thuringiensis* for non-target organisms indicated that their suitability for mosquito control programs in areas where protection of the natural ecosystem is important. Field applications have often been monitored for effects on non-target organisms but no significant non-target effects have been reported (Ramirez-Lepe and Montserrat, 2012).

Environmental fate

Bacillus thuringiensis is a biological pest control agent. A living bacteria that occurs naturally in many soils. Martin and Travers (1989) argued that *Bacillus thuringiensis* was isolated from 70 % of soil samples taken from around the world, and was most abundant in samples taken in Asia. More than half of these isolates were undescribed varieties of *Bacillus thuringiensis.* Essentially no unexpected toxicities from *Bacillus thuringiensis* sprays have been recorded (Icoz et al. 2008) probably because *Bacillus thuringiensis* does not survive or grow well in soil (Petra and Casida, 1985) and its spores are rapidly inactivated by UV radiation (Griego and Spence, 1978). Consequently, there is probably little production of toxins in soil (Vettori, et al., 2003) and the persistence of introduced toxins is a function primarily of the: (1) amount added; (2) rate of consumption and inactivation by insect larvae; (3) rate of degradation by microorganisms; and (4) rate of abiotic inactivation *Bacillus thuringiensis* has also been isolated from insect bodies, tree leaves and aquatic environments. It has even been recovered from paper (Vaisanen, *et al.*, 1991).

Bacillus thuringiensis generally persist only a short time in soil. The half-life of the insecticidal activity (the time in which half of the insecticidal activity is lost) of the crystal is about 9 days (West and Burges, 1985). However, small amount can be quite persistent. In one experiment, *Bacillus thuringiensis* spore numbers declined by one order of magnitude after 2 weeks, but then remained constant for 8 months following application (Petra and Casida, 1985). *Bacillus thuringiensis* does not appear to move readily in soil. In one study, two varieties of *Bacillus thuringiensis* were applied in adjacent plots, but did not become cross contaminated, indicating that *Bacillus thuringiensis* does not move laterally in soil (Drobniewski, 1994). Other studies found that *Bacillus thuringiensis* was not recovered past a depth of 6 centimeters after irrigation and that movement beyond the application plot was less than 10 yards (Akiba, 1991).

The indigenous *Bacillus thuringiensis* East Java isolates is very promising in killing *Aedes aegypti* larvae especially for the first to third instar within time duration from 24 to 72 hours. A negative relationship can be observed between the

exposure time and the LC_{50} for the indigenous *Bacillus thuringiensis* East Java isolates and *Bacillus thuringiensis* var. *israelensis*. It means that with a longer exposure time, the LC_{50} value will decrease and the larval mortality level will increase. All *Bacillus thuringiensis* isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. *Bacillus thuringiensis is an* effective alternative to broad spectrum larvicides in many situations with little or no environmental impact.

Acknowledgement

We are grateful for the financial support of the Directorate General of Higher Education (DGHE) scholarship batch 5th 2011 from Indonesia government and grant from the Global Environment Leaders (GELs) program, Graduate School for International Development and Cooperation (IDEC), Hiroshima University, Japan.

References

Akiba, Y. 1991. Assessment of rainwater-mediated dispersion of field-sprayed *Bacillus thuringiensis* in the soil. *Applied Entomological Zoology* 26(4), 477-483.

Aly, C., Mulla, M.S., Xu, B.Z. and Schnetter, W. 1988. Rate of ingestion by mosquito larvae (Diptera: Culicidae) as a factor in the effectiveness of bacterial stomach toxin. *Journal of Meedical Entomology* 25:191-196.

Aramideh, S., Saferalizadeh, M.H., Pourmirza, A.A., Bari, M.R., Keshavarzi, M., Mohseniazar, M. 2010. Characterization and pathogenic evaluation of *Bacillus thuringiensisi* isolates from West Azerbaijan province-Iran. *African Journal Microbiology Research* 4(12),1224-1229.

Arivoli, S., Tennyson, S. 2011. Larvicidal and adult emergence inhibition activity of Abutilon indicum (Linn.) (Malvaceae) leaf extracts against vector mosquitoes (Diptera: Culicidae). *Journal Biopesticides* 4(1),27-35.

Badan Pusat Statistik. 2012. Kepadatan Penduduk Propinsi Jawa Timur. http://sp 2010. Bps.go.id/index.php/site?id=35&wilayah= Jawa-Timur. [Retrieved on 2 April 2012].

Baig, D.N and Samina, M. 2010. Determination and distribution of *cry*-type genes in halophilc *Bacillus thuringiensis* isolates of Arabian Sea sedimentary rocks. *Microbial Research Journal* 165, 376-383.

Barrett, R., Christopher W. K., Thomas M, and George J. A, 1998. Emerging and Re-emerging Infectious Diseases: The third epidemiologic transition. *Annual Review Anthropology* 27, 247–271.

Boutayeb, A. 2006. The double burden of communicable and non communicable diseases in developing countries. Trans. *Research of Social Tropical Medicine Hygienist* 100(3), 191-199.

Cancino-Rodezno, A., Luis L., Cris O., Julieta I.C., Humberto L.M., Sergio E., Amy E.E., Sarjeet S.G., Mario S., Juan L.J.F. 2012. Comparative Proteomic Analysis of *Aedes aegypti* Larval Midgut after Intoxication with Cry11Aa Toxin from *Bacillus thuringiensis. PloS ONE Journal* 7(5):1-9.

Cox J., Maria E. G., Olga M.R., Manuel A., Roberto B. 2007. Habitat segregation of Dengue Vectors along an urban environmental gradient. *American Journal of Tropical Medicine Hygiene* 76 (5), 820-826.

De Maagd, R.A., Bravo A., Berry C., Crickmore N.,Schnepf H.E. 2003. Structure, diversity and evolution of protein toxins from spore –forming entomopathogenic bacteria. *Annual Review of Genetic* 37:409-433.

Drobniewski, F.A. 1994. A Review: The safety of *Bacillus* species as insect vector control agents. *Bacteriol* 76, 101-109.

Eder, E. and Iris S. 2010. Toxicity of *Bacillus thuringiensis israelensis* on the Nontarget

Organisms *Triops cancriformis, Branchipus schaefferi, Leptestheria dahalacensis* (Crustacea: Branchiopoda: Notostraca, Anostraca, Spinicaudata). *The Open Environmental Pollution and Toxicology Journal* 2, 16-20.

El-kersh T.A., Al-sheikh, Y.A., Al-akeel, R.A., Alsayed, A.A. 2012. Isolation and characterization of native Bacillus thuringiensis isolates from Saudi Arabia. *African Journal of Biotechnology* 11(8), 1924-1938.

Eswarapriya, B., Gopalsamy, B., Kameswari, B., Meera, R. and Devi,P. 2010. Insecticidal activity of *Bacillus thuringiensis* IBT-15 Strain against *Plutella xylostella*. *International Journal Pharm. Technology Research* 2, 2048-2053.

Federici, B.A., Hyun-Woo, P. and Dennis K.B. 2010. Overview of the Basic Biology of *Bacillus thuringiensis* with Emphasis on Genetic Engineering of Bacterial Larvicides for Mosquito Control. *The Open Toxicology Journal* 3, 83-100.

Feitelson, J.S., Payne J. and Kim L. 1992. *Bacillus thuringiensis* Insect and beyond. *Bio/Technology Journal* 10, 271-275.

Georghiou, G. 1990. Managing resistance to agrochemicals from fundamental research to practical strategies. In Green M.B. Le Baron HM and Mobreg WK (eds). ACS Symposium series. 421. American Chemical Society, 18-41.

Glare T.R. and Maureen O. 1998. Environmental and health impacts of *Bacillus thuringiensis israelensis. Report for the Ministry of Health. Biocontrol and Biodiversity, Grassland Division, Agriculture Research*. PO Box 60, Lincoln.

Griego, V.M. and Spence K.D. 1978. Invactivation of *Bacillus thuringiensis* spores by ultraviolet and visible light. *Applied and Environmental Microbiology Journal* 35: 906-910.

Haggag, K.H.E. and Yousef, H.M.A. 2010. Differentiation among Egyptian *Bacillus thuringiensis* strains at sporulation by whole cellular protein profiles. *Journal of Agriculture Science* 6(2), 224-233.

Hastowo, S. B.W. Lay and M. Ohba. 1992. Naturally occurring *Bacillus thuringiensis* in Indonesia. *Journal Applied of Bacteriology* 73:108-113.

Hofte, H. and Whiteley, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiology Review Journal* 53, 242-255.

Icoz, I., Saxena.D, Androw,D.A., Zwahlen,C., and Stotzky, G. 2008. Microbial populations and enzyme activities in soil *in situ* under transgenic corn expressing Cry proteins from *Bacillus thuringiensis. Journal of Environmental Quality* 37:647-662.

Ishiwata,.S. 1901. On a kind of severe flacherie (sotto disease) Dainihon Sanshi Kaiho, 114: 1-5.

Ito, T., Tomonori, I., Ken, S., Hisanori, B. and Shin-ichiro,A. 2006. Cloning and Expression of Two Crystal Protein Genes, Cry 30 Ba1 and Cry 44Aa1, Obtained from a Highly Mosquitocidal Strain, *Bacillus thuringiensis* subsp. entomocidus INA 288. Applied and Environmental Microbiology 72(8): 5673-5676.

Jansen C.C. and Beebe N.W. 2010. The dengue vector *Aedes aegypti* : what comes next. *Journal Microbes Infection Diseases* 12, 272-279.

Jenkins, J. 1992. Environmental toxicology and Chemistry Memo. Subject: *Bacillus thuringiensis.* Corvallis , OR: Oregon State University Extension Service.

Lidwina F.T., Suharjono, Zulfaidah P.G. and N. Nakagoshi. 2013. Toxicity study of *Bacillus thuringiensis* isolated from East Java (based on differences of altitude) against *Aedes aegypti* larvae. (in bahasa). *Jurnal Biotropika* 1 (3), 90-94.

Mahmood, F. 1998. Laboratory bioassay to compare susceptibilities of *Aedes aegypti* and *Anopheles albimanus* to *Bacillus thuringiensis* var. *israelensis* as affected by their feeding rates. *Journal American Mosquito Control Association* 14, 69-71.

Malang City Health Office. 2008. Profile of Dengue Patients in the city of Malang in 2008. (online) http://dinkes.jatimprov.go.id/userfile/dokumen/1311839621_Profil_Kesehatan_Provi

nsi_Jawa_Timur_2008.pdf. [Accessed in 2012]

Manonmani, A.M., Geetha, I., and Bhuvaneswari, S. 2011. Enhanced production of mosquitocidal cyclic lipopeptide from Bacillus subtilis subsp. Subtilis. *Indian Journal Medical Research* 134(4), 476-82.

Martin, P.A.W. and R.S. Travers. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Applied Environmental Microbiology* 55(10), 2437-2442.

Muniady, S., Rathinam, X. and Subramaniam, S. 2011. Quick isolation and characterization for the confirmation of a novel Bacillus thuringiensis strains from chicken manure samples. *African Journal Microbiology Research* 5(20), 3131-3137.

Lakxmy A.P., R. Xavier, C.M.R. Josephine, Y.W.Lee, K. Marimuthu, S. Kathiresan, S. Sreeramanan. 2011. Mosquitocidal activity of a native *Bacillus thuringiensis* isolate Bt ReX02 from Gunung Jerai Forest, Malaysia against *Culex quinquefasciatus* and *Aedes albopictus. European Review forMedical and Pharmacological Sciences* 15: 149-155.

Nareshkumar, A., K. Murugan, I. Baruah, P.Madhiyazhagan and T. Nataraj. 2012. Larvicidal potentiality, longevity and fecundity inhibitory activities of *Bacillus sphaericus* (Bs G3-IV) on vector mosquitoes, *Aedes aegypti* and *Culex quinquefasciatus*. *Journal of Entomological and Acarological Research* 44(15), 79-84.

Petras, S.F. and L.E. Casida, Jr. 1985. Survival of *Bacillus thuringiensis* spores in soil. *Applied Environmental Microbiology* 50, 1496-1501.

Poopathi, S. 2010. Novel fermentation media for production of mosquito pathogenic bacilli in mosquito control. *Applied Microbiology and Microbial Biotechnology* A.Mendez-Vilas (Ed.) India, 349- 358. Formatec.

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(3), 22-38.

Poopathi, S., Mani, T.R., Raghunatha, R.D., Baskaran, G. and Kabilan, L. 2002. Evaluation of synergistic interaction between *Bacillus sphaericus* and a neem based biopestiside against *Culex quinquefasciatus* larvae susceptible to *Bacillus sphaericus*. 1593M. *Insect Science Applied Journal* (Kenya) 22, 303-306.

Poopathi, S. and Tyagi, B.K. 2002. Studies on *Bacillus sphaericus* toxicity-related resistance development and biology in the filariasis vector, *Culex quinquefasciatus* (Diptera: Culicidae) from South India. *Applied Entomological Zoology* 37(3), 365-371.

Poopathi, S. and Tyagi, B.K. 2006. The challenge of mosquito control strategies: from primordial to molecular approaches. *Journal Biotechnology and Molecular Biology Review* 1(2), 51-65.

Prabakaran, G. and K. Balaraman 2006. Development of a cost-effective medium for the large scale production of *Bacillus thuringiensis* var. *israelensis*. *Biological control* 36, 288-292.

Provincial Health Office. Profile of Dengue patients in East Java in 2005-2010. Indonesia : Provincial Health Office; 2011. [Online] Available from : http : //dinkes.jatimprov.go.id/userfile/dokumen/ 1312948638_Profil_Kesehatan_Provinsi_J awa_Timur_2010.pdf. [Accessed in 2012]

Ramires Suero-Suero M., Valerio-Alfaro G., Bernal J.S. and Ramirez-Lepe, M. 2011. Synergistic effect of chitinases and *Bacillus thuringiensis israelensis* spore-toxin complex against *Aedes aegypti* larvae. *Journal of Canadian Entomologist* 148, 157-164.

Ramirez-Lepe, M and Montserrat R.S. 2012. Biological control of mosquito larvae by *Bacillus thuringiensis* subsp. *israelensis*. *Insecticides-Pest Engeneering*. First published February 2012. 239-264.

Renganathan, K., Rathinam, X., Danial, M. and Subramaniam. S. 2011. Quick isolation and characterization of novel

Bacillus thuringiensis strain from mosquito breeding sites in Malaysia. *Journal Food Agriculture* 23(01), 17-26.

Sanahuja, G., Banakar R., Twyman, R.M., Capell, T. and Christou, P. 2011. *Bacillus thuringiensis*: a century of research, development and commercial application. *Journal of Plant Biotechnology* 9, 283-300.

Sankari, T., Hoti, S.L., Singh, T.B. and Shanmugavel, J. 2012. Outbreak of dengue virus serotype-2 (DENV-2) of Cambodian origin in Manipur, India-Association with meteorological factors. *Indian Journal Medical Research* 136, 649-655.

Soberon, M. Perez, R.V., Nueznez-Valdez, M.E., Lorence, A., Gomez, I., Sanchez, J. and Bravo, A. 2000. *Evidence for intermolecular interaction as a necessary step for pore-formation activity and toxicity of Bacillus thuringiensis Cry1Ab toxin*. FEMS Microbiology Lett. 191, 221-225.

Suci, A., Bayu, R., Agustin, I., Rahma, F. and Murtini. 2010. Detection of spatial – temporal autocorrelation using multivariate moran and lisa method on Dengue Hemorrhagic Fever (DHF) incidence in East Java. *Proceedings of the Third International Conference on Mathematics and Natural Sciences* (ICMNS 2010)

Vaisanen, O.M., J. Mentu and Salkinoja-Salonen, M.S. 1991. Bacteria in food packaging paper and board. *Journal Applied Bacteriology* 71, 130-133.

Valadez-Lira, J.A., Alcocer-Gonzalez, J.M., Damas, G., Nunuz-Mejia, G., Oppert, B., Rodriques-Padilla, C. and Tamez-Guerra, P. 2011. Comparative evaluation of phenoloxidase activity in different larval stages of four lepidopteran pests after exposure to *Bacillus thuringiensis* . *Journal Insect Science* 12, 1-11.

Vettori C., Paffetti D., Stotzkyaxena D., Stotzky G., Giannini R. 2003. Persistence of toxins and cells of *Bacillus thuringiensis* subsp. *kurstaki* introducedin spray to Sardinia soils. Soil Biology Biochemistry 35 : 1635-1642.

West, A.W. and H.D. Burges. 1985. Persistence of *Bacillus thuringiensis* and *Bacillus cereus* in soil supplemented with grass or manure. *Plant and Soil Journal* 83, 389-398.

Whalon, M.E. and McGaughey, W.H. 1998. *Bacillus thuringiensis*: *Use and resistance management* . In: Insecticides with novel modes of action: mechanism and application, Ishaaya,I. and Degheele,D.,Eds.Springer, Berlin,106-137.

WHO. 1976. 22nd report of the WHO Expert Committee on Insecticides. Geneva: World Health Organization.

WHO. 2009. Dengue Guidelines for Diagnosis, Treatment, Prevention, and Control. Geneva.

WHO. 2010. Regional Office for Southeast Asia. World Health Organization. Regional Office for Southeast Asia Dengue case fatality rate. Available from: http://www.searo.who.int/LinkFiles/Dengue_85-06.pdf. Accessed on October 2, 2010.

Zulfaidah, P. G., Bagyo, Y. and Tri, H.K. 2010. Safe-strategy to control mosquito: the potential of *Bacillus thuringiensis* isolate indigenous from Madura as natural enemies of mosquito (*Aedes aegypti*). *Jurnal Pembangunan dan Alam Lestari*, 1-10. ISSN.2087-3522. (in bahasa).

Zulfaidah, P.G.and Nakagoshi, N. 2013. Climatic Variability and Dengue Haemorrhagic Fever Incidence in Nganjuk District, East Java, Indonesia. *Acta Biologica Malaysiana* 2(1): 31-39.

Zulfaidah, P.G., Nakagoshi, N., Suharjono and Faridah, S. 2013. Toxicity studies for indigenous *Bacillus thuringiensis* isolates from Malang city, East Java on *Aedes aegypti*. *Asian Pasific Journal of Tropical Biomedicine* 3(2), 111-117.