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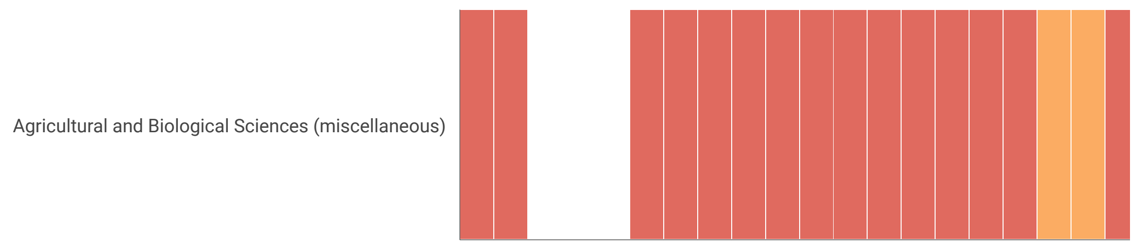

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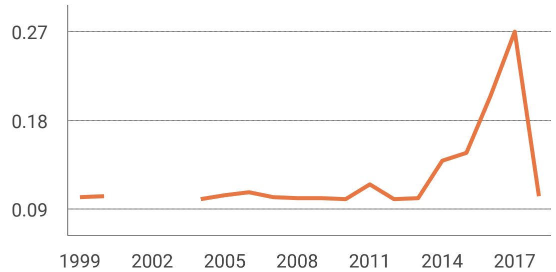

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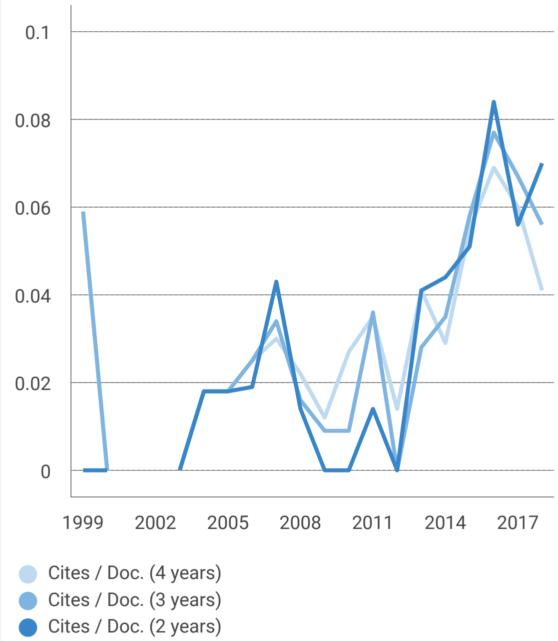

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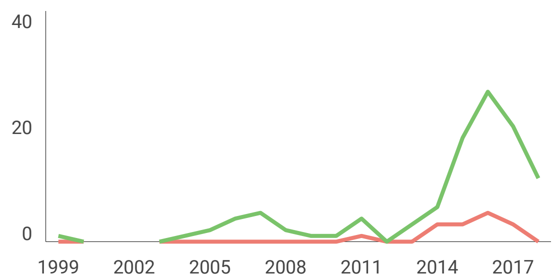

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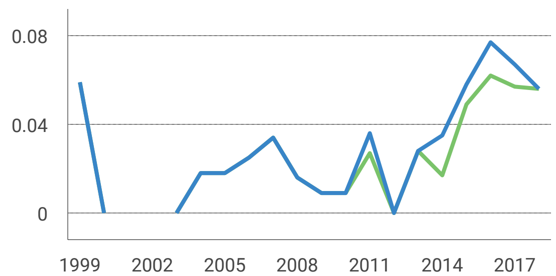

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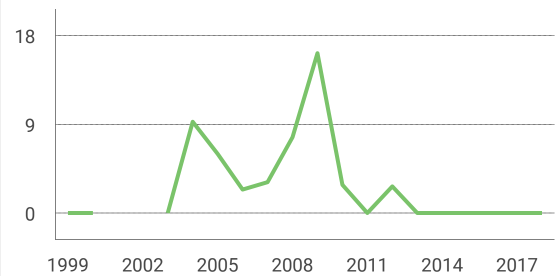

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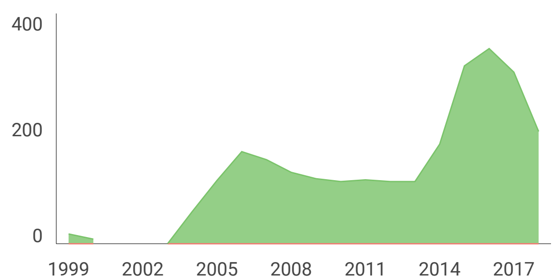

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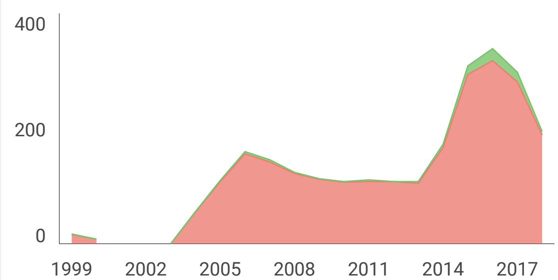

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Annals of Biology **36** (2) : 232-237, 2020

Prospect of Native Entomopathogenic *Bacilli* from Baluran National Park as Biological Control of Dengue Fever Vector

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ABSTRACT

The research was aimed at the exploration of native entomopathogenic *Bacilli* from natural breeding sites of Dengue Fever Vector in Baluran National Park, East Java, Indonesia. Soil samples were collected from 30 sites at three different locations. Isolation of bacteria used selected growth media for *Bacillus* sp. and characterized based on spore staining method. The isolates tested the mortality rates against *Aedes aegypti* larvae and observed 24 and 48 h after exposure. The potential isolates status was based on the mortality rates (%) of *Ae. aegypti* larvae. Furthermore, the characterization of macroscopic and microscopic and physiologic was identified as species name of *Bacillus* sp. by similarity index. The results of the initial potential test showed that 68 of 107 isolates of *Bacillus* sp. were obtained as native entomopathogenic *Bacillus* sp. with variations of the potential status. In the advanced potential test, there were three isolates with highest potential status, coded as BK7.1, BK7.2 and BK5.2, with the mortality rates 93, 87 and 70%, respectively. Based on macroscopic, microscopic and physiological characterizations *Bacillus* sp. BK7.1 had similarity with *Bacillus sphaericus* and *Bacillus* sp. BK7.2 had similarity with *Bacillus subtilis* and *Bacillus* sp. BK5.2 isolate had similarity with *Bacillus thuringiensis*.

Key words : Entomophatogenic, *Bacillus* sp., *Aedes aegypti,* breeding places, Baluran National Park

INTRODUCTION

Dengue Fever (DF), a disease caused by dengue virus and transmitted by vector is a serious problem in public health in South East Asia, including Indonesia (WHO, 2016; Kemenkes, 2016). The effort to solve DF problem has been done, but the results are still unsatisfactory. Main effort to control the disease was by controlling the population of the disease's vector, *Ae. aegypti* (Blasberg *et al.*, 2016). The use of chemical insecticides was the first choice, but it was proven as it has negative impact to the environmental quality and dispatch non-target organism. Biological control expert recommendation is to develop bioinsecticide and search any biological control agent as the alternative of the biological disease vector's control (Thomas, 2018). *Bacillus* spp. is one of biological control agents that have been developed as entomopatogenic bacteria.

Bacillus spp. is reported having big role as bioinsecticide for insect. These are *B. thuringiensis, B. sphaericus, B. subtilis* and *B. cereus.* These bacteria have been isolated and characterized. *B. sphaericus* is isolated from Lombok Island as bioinsecticide of *Anopheles aconitus* as Malaria's disease vector (Suryadi *et al.*, 2016) and also has been successfully isolated and characterized from local strain of *B. thuringiensis* in Syria which has potential as bioinsecticide for pest insects (Ammouneh *et al.*, 2011) and local *B. thurigiensis* from Saudi Arabia as biolarvaside of Malaria's vector (El-Kersh *et al.*, 2016). Geetha *et al.* (2012) reported that secondary metabolite activity of *B. subtilis* sub-sp. *subtilis* local from India also had a good entomopathogenic activity against larvae, pupa and adult mosquitos. *B. subtilis* produced surfactin that was mosquitocidal toxin. Jayasree *et al.* (2018) succeeded to isolate 75 isolates from oil contaminated's soil. The most potential isolate was identified as *B. subtilis* B50 and produced biosurfactan that showed larvicidal activity. Research results showed that concentration of biosurfactan and exposure period took effect of larval mortality and recommended as environmental-friendly product to control disease's vector (Jayasree *et al.*, 2018). Thomas (2018) reviewed from many scientific writings about the role of biological agents and gave recommendation for control vector community to give attention to the perspective and opportunity of the biological agents on integrated control of the diseases vector.

This research has begun with isolation of native *Bacillus* sp. from the natural soils in Baluran National Park East Java Indonesia. The initial and advanced potential test was conducted to determine the potential status of native *Bacillus* sp. against *Ae. aegypti* larvae. The phenotypic characterization of the potential native *Bacillus* sp. was observed by macroscopic, microscopic and physiological characters. The results of phenotypic characterization were used to determine the species name by similarity index. The result of this research was to develop native bioinsecticide for biological control of DF vector.

MATERIALS AND METHODS

The procedure in this research was carried out as per Suryadi *et al.* (2016), to isolate and characterize native *B. sphaericus* from the coastal area of Lombok Island, entomopathogenic bacteria to *Anopheles* sp. larvae as Malaria vector. However, on this research targets, isolations, characterizations and potential status of native *Bacillus* sp. against larvae target *Ae. aegypti* as DF vector. Eggs of *Ae. aegypti* derived from mosquitoes and maintained intensively in the Institute of Tropical Diseases, Laboratory of Entomology Airlangga University Surabaya. Eggs of *Ae. aegypti* were soaked in water wells for hatching and colonization of the larvae. The larvae were kept for six days to reach the third instar larvae, ready to be used as test larvae.

Soil samples were taken from mosquito-breeding sites from three locations in the area of Baluran National Park–Bama Beach area 10 soil samples, Pesanggerahan Bekol area 10 soil samples and Batangan area 10 soil samples. Soil samples were collected then homogenized with a sterile physiological solution. The suspension was heated at 80°C for 30 min and cultured on NYSM (Nutrient Yeast Extract Salt Medium). The colony was grown and passed on to the initial potential test and advanced potential test against *Ae. aegypti* larvae.

Initial and advanced potential tests were conducted to observe toxicity of native *Bacillus* sp. isolates. The test procedure began by growing isolates in the NYSM liquid medium at 30°C for 72 h at 170 rpm. Third instar larvae of *Ae. aegypti* were inserted into 10% v/v isolates of *Bacillus* sp. from isolation results that have been grown in the NYSM liquid media. The mortality rate of larvae on each test and each replication were observed after 24 and 48 h exposure.

The characterization of high potential isolates bacteria from the advanced potential test was performed to determine phenotypic characteristics. Phenotypic features were determined by morphological and physiological characterizations. Morphological features were observed by morphology of the colony, cell structure, and endospores form and its position in the spore staining to know the microscopic structure by light microscope. Physiological characteristics were carried out with a catalase test, hydrolysis of amylum, nitrate reduction, use of sugar, indol, H_2S, ureases, oxidase, casein hydrolysis and aerobility and other additional tests. The physiological tests used Microbact 12A12B as a complement to determine the index of similarity with other species of *Bacillus* sp. (Table 1). The results obtained from morphological and physiological characterizations of native *Bacillus* sp. potential were used to determine the percentage of similarity index that included the positive and negative similarities of each bacterial species character of the *Bacillus* sp.

RESULTS AND DISCUSSION

Sampling results of the various breeding places of *Ae. aegypti* mosquitoes from Baluran National Park are detailed in Table 2.

Isolation of native *Bacillus* sp. obtained 107 isolates, from a sample location from Bama, Bekol and Batangan. Each isolate carried out the initial potential test against larva of *Ae. aegypti*. The results of initial potential test with variety of Optical Density (OD_{600nm}) against larvae of *Ae. aegypti* were used to group into not potential, low potential, medium potential and high potential isolates (Fig. 1). Soil samples from Bama, Bekol and Batangan regions gained 1, 4 and 5 of high potential isolates, respectively. The results of the advanced potential test of isolates by OD_{600nm} = 0.8 (1.2 x 10^8 CFU/ml) as much as 10 isolates was native *Bacillus* sp. (Fig. 2). The high potential of native *Bacillus* sp. was developed as a biolarviside against *Ae. aegypti* larvae from the natural soils of Baluran National Park. *Bacillus* sp.

Table 1. Physiological characteristics of the three isolates of native Bacillus sp. from Baluran National Park [positive (+) value indicated positive test and negative (-) values indicated negative test]

S. No.	Physiological test	Native *Bacillus* sp. isolates (Code)		
		BK5.2	BK7.1	BK7.2
1.	Lysine	+	+	-
2.	Ornithin	+	-	-
3.	H_2S	-	-	-
4.	Glucose	-	-	-
5.	Mannitol	-	-	-
6.	Xylose	-	-	+
7.	ONPG	+	+	+
8.	Indole	-	-	-
9.	Urease	+	+	-
10.	VP	+	+	+
11.	Citrate	-	-	-
12.	TDA	-	-	-
13.	Gelatine	+	+	+
14.	Malonate	-	-	-
15.	Inositole	-	-	-
16.	Sorbitole	-	-	-
17.	Rhamnose	-	-	-
18.	Sucrose	-	-	-
19.	Lactose	-	-	-
20.	Arabinose	+	+	+
21.	Adonitole	-	-	-
22.	Raffinose	-	-	-
23.	Salicine	-	-	-
24.	Arginine	-	-	-
25.	Oksidase	-	-	-
26.	Motility	+	+	+
27.	HydrolisisAmylum	+	-	+
28.	Catalase	+	+	+
29.	Nitrate reduction	+	+	+
30.	Growth in NaCl 5%	+	+	+
31.	Growth in NaCl 10%	-	-	-

bacteria was one of the abundant bacteria on soil and also widely isolated from the water and the infected host. Isolation of *Bacillus* sp. bacteria from soil was carried out with special techniques; by heating the soil sample between 65-80°C for 30 min to trigger the formation of endospores and to kill bacterial cells which were not heat resistant; and could not form endospores. The medium for the

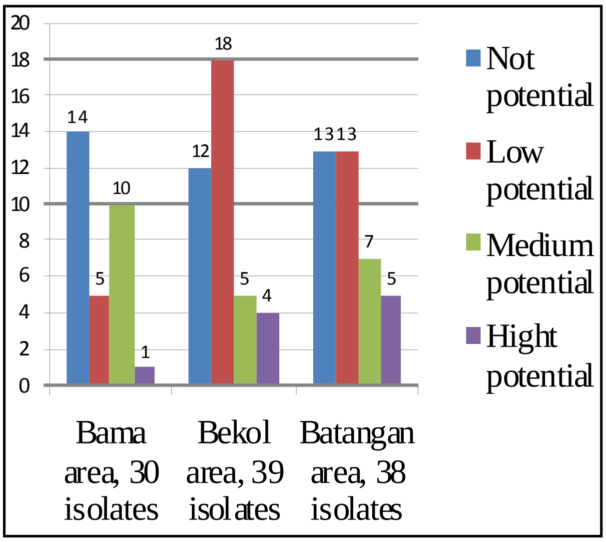

Fig. 1. The results of isolation of native *Bacillus* sp. from the region of Bama, Bekol and Batangan area, as well as initial potential test against *Ae. aegypti* larvae.

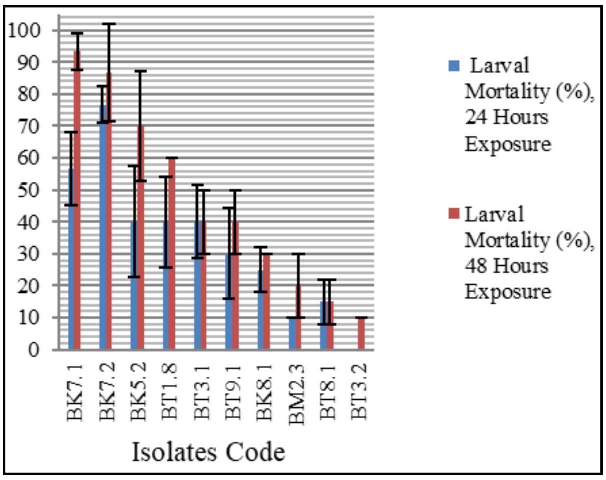

Fig. 2. The isolate code (BK, BT and BM) of native entomopathogenic *Bacillus* sp. which was high potential from the advance potential test results against the *Ae. aegypti* larva at 24 and 48 h exposure.

Table 2. The range of coordinate points and environmental conditions at soil sampling locations in Baluran National Park

Location	Coordinate point range	Soil	
		pH	Humidity (%)
Bama	S07°50.677'E114°27.604 - S07°51.033'E114°27.415	6.8-7	70-80
Bekol	S07°50.248'E114°26.402 - S07°50.358'E114°26.357	6.6-7	70
Batangan	S07°55.208'E114°23.252 - S07°55.274'E114°23.242	5-7	70-85

growth used for isolation was NYSN media (Suryadi *et al.*, 2016).

The results of the advanced potential test obtained as much as three isolates which is the native *Bacillus* sp. with a high potency level (Fig. 2). While the other isolates were native *Bacillus* sp. with low and moderate potency level (Fig. 1). The difference of larval mortality rate between isolates due to the isolation of *Bacillus* sp. had a diversity of pathogenic properties against *Ae. aegypti* larvae. Potential toxicity of protein crystals (δ-endotoxin) *B. thuringiensis* was also influenced by solubility, affinity to receptors and break down of protoxin into toxin (El-Kersh *et al.*, 2016). Effectiveness of *B. thuringiensis*is was also influenced by the presence of mosquito larvae, food, exposure period, water quality, bacterial strains, water temperature, toxin presence in substrate and the feeding behaviour of larvae (Dambach *et al.*, 2014). The discovery of entomopathogenic bacteria was influenced by many factors, such as rain and erosion, epizootic and enzootic, so there was a possibility to find entomopathogenic bacteria in a particular place.

Native *B. thuringiensis* and *B. sphaericus* in Indonesia had been isolated and also potentially developed as a biological control of the disease vector. Pratiwi *et al.* (2013) isolated and tested the toxicity of *B. thuringiensis* from Nganjuk city Indonesia with potentially as biolarvicide against *Ae. aegypti*. Blondine (2013) also carried out native strain efficacy test of *B. thuringiensis* H-10 against mosquito vector *Ae. aegypti* and *An. aconitus*. Suryadi *et al.* (2016) isolated and performed characterization of *B. shpaericus* as potential bioinsecticide for biological control of the mosquito vector of Malaria from Lombok Island. El-Kersh *et al.* (2016) isolated and characterized the native strains of *B. thuringiensis* in Saudi Arabia from 300 soil samples; 68 isolates had larvisidal toxicity to mosquito vector of Malaria *A. gambiens*, and 63 of *B. thuringiensis* had highest larvisidal toxicity and potential to be 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, this bacteria could be developed as a bioinsecticide against agricultural pest insects.

Three isolates of native *Bacillus* sp. with very high potential were isolates with code BK7.1, BK7.2 and BK5.2. The visualization of spores of the three isolates is shown in Fig. 3.

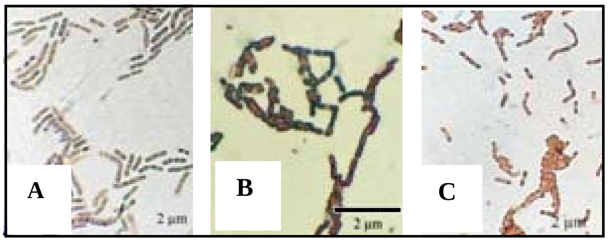

Fig. 3. Visualization of spores microscopic through spores staining (magnification : 1000x) isolates code BK5.2 (A), BK7.1 (B) and BK7.2 (C).

Conventional identification of these above three isolates (Table 3) were performed using similarity index based on the physiological characters and the morphological characters to produce similarity to three species of *Bacillus*, namely, *B. sphaericus, B. subtilis* and *B. thuringiensis*. More research was needed to ensure the species name with the identification by 16S rRNA for the three isolates. Bacterial identification using 16S rRNA had a high specificity and sensitivity level, in which a molecular-coding gene marker of the rRNA was used for taxonomy determination, phylogenism and inter-diversity distances species of bacteria.

Table 3. Characterization of native entomopathogenic *Bacillus* sp. with potentially high prospect as biolarvisides for biological control of vector isolated from Baluran National Park

S. No.	Isolate Code	Species name (Similarity indexes)
1.	BK7.1	*Bacillus sphaericus*
2	BK7.2	*Bacillus subtilis*
3.	BK5.2	*Bacillus thuringiensis*

B. subtilis subsp. *inaquosorum* was isolated from soil with low water content (desert soils), having a single rod-shaped cell and size of 0.5 x 2-3 µm, motile and cylindrical spore-ellipse forms located in the central or parasentral, facultative anaerobes, grew at 14-55°C, containing genes producing antibiotic subtylosin, basilisin, basilomycin F, lipopeptides, surfactin and fengycin (Dunlap *et al.*, 2019). *B. subtilis* produced mosquitosidal toxin; role of toxin or its potential as a biocontrol needs to be studied. Mosquitosidal toxin activity and molecular characteristics had been reported from *B. cereus* (Mani *et al.*, 2017). Several studies have shown that biosurfactant-producing bacteria were suitable for controlling plant pathogens (Zhao *et al.*, 2014). Biosurfactants have been introduced as alternatives to synthetic chemicals to control insects. Biosurfactants produced by several strains of *Bacillus* sp. can enter the cuticle or open spirits and dispatch adult mosquitoes (Geetha *et al.*, 2012). The use of *B. thuringiensis* as a bioinsecticide has been widely applied to inhibit the development of mosquitoes as vectors of various diseases (Boyce *et al.*, 2013). Ben-Dov (2014) has studied of the toxin *B. thuringiensis* subsp. *israelensis* (*Bti*) and reported that *Bti* was first discovered and used as a biological control agent of mosquito larvae and black flies. Thus, suggested that *Bti* will remain an effective biological control agent for future years. Thomas (2018) has studied various scientific writings on the role of biological control of disease vectors and hoped that the vector control community gave attention to the prospects and opportunities of biological agents role in controlling integrated disease transmitted by vectors.

CONCLUSION

Native *Bacillus* sp. as biolarvisides against *Ae. aegypti* larvae were isolated from the natural breeding sites of *Ae. aegypti* mosquitoes in Baluran National Park, East Java, Indonesia. The number of isolates of native *Bacillus* sp. with high potential of the initial potential test of *Ae. aegypti* larvae was 10 isolates. The number of isolates of native *Bacillus* sp. with high potential of the advanced potential test was three isolates, namely, *Bacillus* sp. BK7.1, *Bacillus* sp. BK7.2 and *Bacillus* sp. BK5.2, respectively. Based on phenotypic characterizations, the *Bacillus* sp. BK7.1 had a similarity with *B. sphaericus* and *Bacillus* sp. BK7.2 had a similarity with *B. subtilis* and *Bacillus* sp. BK5.2 had a similarity with *B. thuringiensis.* Native *Bacillus* sp. BK7.1, *Bacillus* sp. BK7.2 and *Bacillus* sp. BK5.2 were very potential and prospect to be developed as biolarvasides for biological control of vector, so that further research is needed in accordance with the next phase of research, which has been established, in an effort development of bioinsecticide products.

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