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Characteristics of native entomopathogenic *Bacillus* sp. BK5.2 as an environmental friendly potential agent for disease vectors and plant diseases control

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ABSTRACTS

Biological control is one way to overcome the problem of disease vectors and plant diseases. Biological control can be carried out by utilizing living organisms, for example, group of *Bacillus* bacteria. This study aimed to determine the name of species of the native entomopathogenic *Bacillus* sp. BK5.2 isolated from natural breeding sites of *Ae. aegypti* in Baluran National Park East Java Indonesia, genetic similarity with other species of *Bacillus*, detect a toxin encoding gene, and hemolytic activity test. The species name was determined by DNA isolation of the genome *Bacillus* sp. BK5.2 and visualized by electrophoresis. Amplification of 16S rRNA gene was done by the Polymerase Chain Reaction (PCR) method. The resulting nucleotide sequences were analyzed for genetic similarity with another *Bacillus*. Detection of toxin encoding gene was done through PCR method visualized by electrophoresis. The hemolytic activity test was done to analyze its biolarvacidal activity. The results showed that *Bacillus* sp. BK5.2 has 99% genetic similarity with *Bacillus thuringiensis* strain B16. The results of the detection of toxin encoding gene was a *Cry*11 coding gene. The Hemolytic activity test on blood agar plate showed that positive results. *B. thuringiensis* (BK5.2) can be developed as an environmental friendly biological control agent against disease vectors and plant diseases.

Key words *: Bacillus thuringiensis, Entomopathogent, Genetic characteristics, Hemolytic activity.*

Introduction

The controlling insect vectors and plant diseases using natural enemies is recommended compared to insecticides with chemically active ingredients (Amin, 2016; Lubis *et al.*, 2018; Safni *et al.*, 2018; Sutriono *et al.*, 2019). Entomopathogens from microorganism's act as natural enemies that can produce toxic metabolites towards insects. The advantages of this natural entomopathogenic are relatively high specificity and selectivity towards the host, therefore it does not cause pollution and is safe in the environment (Thomas, 2017).

The use of organisms including *Bacillus* in vector and plant disease control has been sufficiently developed (Syaharuddin *et al.*, 2018; Permata *et al.*, 2015; Novasari and Sasongkowati, 2017). Use of *Bacillus thuringiensis var. israeliensis* (Bti) as a biological

agent was reported to be effective in controlling the *Ae. aegypti* larvae vector of Dengue Hemorrhagic Fever (Boyce *et al.*, 2013). *B. thuringiensis* was capable to produce inclusions of *Cry* protein when sporulation and the resulting toxin can cause death of several types of insects (Soares-da-Silva *et al.*, 2015; Aramideh *et al.*, 2016).

B. thuringiensis can kill mosquito larvae from the *Aedes*, *Culex* and *Anopheles*, but is not safe againts non-target organisms. Isolation, identification and development studies of entomopathogenic *Bacillus* are still being carried out, because there are still many disease vectors caused by mosquitoes (Purnawati *et al.*, 2015).

Salamun *et al.* (2020a) reported that the entomopathogenics *Bacillus* sp. isolated from domestics breeding sites of *Ae. aegypti* was variative of divercity against *Ae. aegypti* larvae, from 133 isolates of *Bacillus* sp., 88(66.2%) toxic against *Ae. aegypti* larvae, with toxicity status 51% low toxic, 30,6% moderate toxic, and 18.1% high toxic. Native *Bacillus* sp. also isolated from natural breeding sites of *Ae. aegypti* in Baluran National Park, East Java, Indonesia and entomopathogenic *Bacillus* sp. BK5.2 was one of the bacterial isolates ahigh toxic status against *Ae. aegypti* larvae, with larval mortality rate 70% in 48 hour exposure. Based on phenotypic characteristics, *Bacillus* sp. BK5.2 has a similarity with *B. thuringiensis* (Salamun *et al.*, 2020b). Further studies need to be done through genetic characteristics to determine species name and phylogenetic, detect toxin encoding gene, and hemolytic activity.

Materials and Methods

Isolation of *Bacillus* sp. BK5.2 DNA genome

The DNA genome of *Bacillus* sp. BK5.2 was isolated using a DNA Purification Kit (Promega), confirmed by electrophoresis and evaluated for purity and concentration with a Thermo Scientific Multiskan GO Microdroplet Spectrophotometer. The DNA electrophoresis was visualized under the UV Transluminator. The level of genomic DNA purity was determined by calculating the ratio between the values of 260 nm and 280 nm in DNA samples.

Analysis of the 16S rRNA gene and the toxin encoding gene

Amplification of 16S rRNA gene used universal primers P0 and P6, then electrophoresed and visualized under UV Transluminator. The results of 16S rRNA gene amplification was confirmed by electrophoresis and then purified and sequenced. Purification and sequencing of the 16S rRNA gene was carried out by sending PCR results to the 1st Base Sequencing Service in Singapore. The sequencing results were then analyzed using the Bio Edit Sequence Alignment Editor software version 7.2.5.

The 16S rRNA nucleotide sequence from *Bacillus* sp. BK5.2 was aligned with 16S rRNA gene sequences from other microorganisms that have been published in GenBank. The 16S rRNA gene homology was observed with the Basic Native Alignment Search Tools (BLAST) program and accessed through the National Center for Biotechnology Information at The National Library of Medicine in Washington DC. Detection of the toxin encoding gene was done according to procedure as mentioned above by replacing the 16S rRNA gene with the toxin encoding gene and using *Cry*IV primers.

Phylogenetic tree analysis

Determining genetic similarity by making a phylogenetic tree using the Program of Mega 7. The phylogenetic tree was created by inputting FASTAs from BLAST species. The whole species were chosen based on the 16S rRNA gene nucleotide sequence, because the genetic similarity of one species with the other can be seen from the sequence.

Hemolytic activity test

Hemolytic activity test using bloodagar media. *Bacillus* sp. BK5.2 was inoculated on bloodagar media by the spotted method. Then incubated at room temperature for 2 days. After incubation, the isolate was observed to see the clear zone formed around the colony.

Results and Discussion

Species name

The result of DNA isolation of *Bacillus* sp. BK5.2 dna genome with DNA purity = 1.86(A260/A280), DNA concentration of 26.4(ng/µL) and after being confirmed with gel electrophoresis in Fig. 1.

Besides the agarose gel pit there is one of DNA band. This position shows that the size of DNA is very large, above 1500bp. Several factors can influence DNA migration during electrophoresis including agarose concentration, DNA molecular size,

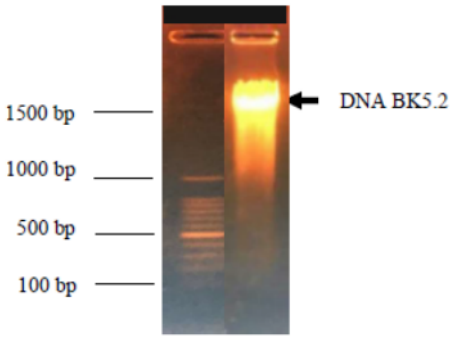

Fig. 1. The electrophoresis results of DNA genome of native entomopathogenic *Bacillus* sp. BK5.2 on 1% agarose gel. Note: M=Marker DNA 100bp, S=Sample

voltage, and temperature (Brown, 2016). The electrophoresis results from 16S rRNA gene amplification of *Bacillus* sp. BK5.2 in Fig. 2. The analysis result shows that there is a band over 1500bp in size. The 16S rRNA gene is about 1550 base pairs and about 500 bases at the end of the sequence are hypervariable regions. This area is the part that distinguishes between organisms. The primers used in sequence amplification will recognize sustainable regions and amplify hypervariable regions, thereby obtaining sequences that are specific to the organism. The 16S rRNA gene is one of the genes that has been well characterized so that it can be used to identify microorganisms (Pearson, 2014).

The 16S rRNA nucleotide sequence from *Bacillus* sp. BK5.2 has a size of 1379 base pairs. Based on BLAST analysis of 16S rRNA and BLAST of encoding *Cry* gene of *Bacillus* sp. BK5.2 sequence has similarities with *B. thuringiensis* strain B16 with a homology level of 99% (Table 1) and 100% *B. thuringiensis* strain BT62 (Table 2), respectively.

Cry genes *Bacillus* sp. BK5.2 native bacterial isolates detection uses PCR techniques with Cry specific primers (Aramideh, 2016), in Fig. 3. Based on the visualization of *Cry* gene detection from *Bacillus* sp. BK5.2, there is a single band in sizes ranging from 300bp. These results are not in accordance with the report of Ben-Dov *et al.*, (1997) which is the primary reference in the conducted research. Based on the Ben-Dov*et al.*, (1997) report the *Cry* gene size amplified using a *Cry*IV primer was 439bp. In this study had a size ranging from 300bp, where the gene size was the *Cry*11 gene (305bp).

Several other *Bacillus* species are reported to pro-

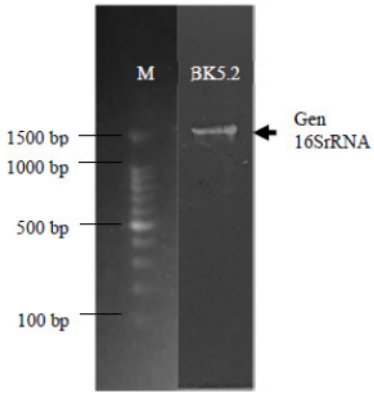

Fig. 2. The electrophoresis results of 16S rRNA gene amplification of native entomopathogenic *Bacillus* sp. BK5.2. Description: M=100bp DNA marker; BK5.2=16S rRNA gene sample.

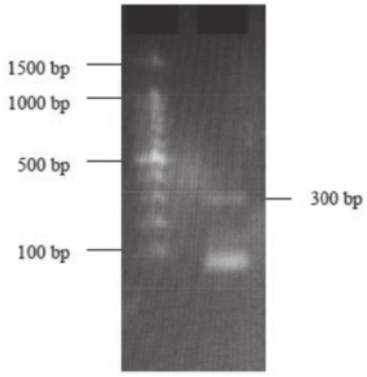

Fig. 3. The electrophoresis results of nativeentomo pathogenic *Bacillus* sp. BK5.2*Cry* gene on agarose gel 0.8%. M=Marker DNA 100bp; S=Sample

Table 1. Species name of native entomopathogenic *Bacillus* sp. BK5.2 based on analysis the 16S rRNA gene with Basic Native Alignment Search Tools (BLAST) program

No	Species Name	Accession	E value	%ID	Query Cover
1	*Bacillus thuringiensis* strain B16	KX977387.1	0.0	100%	99%
2	*Bacillus thuringiensis* strain IARI-UPS 6	KT441072.1	0.0	100%	99%
3	*Bacillus thuringiensis* strain 2110	JF947357.1	0.0	100%	99%

Table 2. Species name of native entomopathogenic *Bacillus* sp. BK5.2 based on encoding geneanalysis with Basic Native Alignment Search Tools (BLAST) program

No	Species Name	Accession	E value	%ID	Query Cover
1	*Bacillus thuringiensis* strain BT62	CP044978.1	0.0	99.12%	100%
2	*Bacillus thuringiensis* strain c25	CP022345.1	0.0	99.12%	100%
3	*Bacillus thuringiensis* strain GA-A07	CP042270.1	0.0	98.68%	100%

duce toxins that have an entomopathogent ability for larvae and insects. El-Kersh *et al*. (2016) reported *Cry*11 toxin with weight 68-72 kDa to 99 kDa are produced by *B. thuringiensis*. Mani *et al*. (2017) reported the 51.7 kDa protein toxin is produced by *B. cereus* VCRC B540 acts as a mosquitocidal toxin. Camacho-Millán *et al*. (2017) reported *Cry*1Ac toxin weighing 65 kDa is produced by *B. thuringiensis*.

Phylogenetic tree analysis

The phylogenetic analysis results of *Bacillus* sp. BK5.2 in Fig. 4. *Bacillus* sp. BK5.2 is the closest relative to *Bacillus thuringiensis* strain B16, seen from the branching location of the two bacteria, *Bacillus thuringiensis* strain IARI-UPS 6 and *Bacillus thuringiensis* strain 2110.

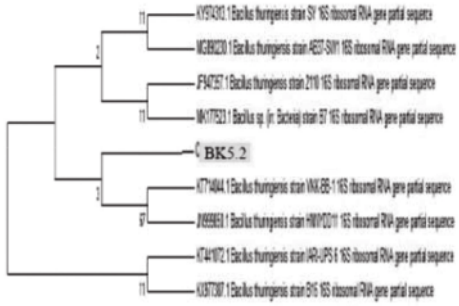

Fig. 4. Native entomopathogen *Bacillus* sp. BK5.2 based on phylogenetic tree analysis of genetic similarity using the Program of Mega7

Hemolytic Activity

Carrillo *et al*. (1996) found an association between hemolytic activity and biosurfactant production, and recommended a blood hemolytic test as a primary method for detecting biosurfactant activity. Test results for blood agar hemolytic activity in Fig.5.

Several studies have shown that biosurfactant-producing bacteria is suitable for controlling plant pathogens and controlling insects (Bais *et al*., 2004; Zhao *et al*., 2014). But there are not many studies have studied the toxin or its potential as a biological controlagents. *B. subtilis* has been reported to have the ability to produce mosquitosidal toxins (Manonmani *et al*., 2011)). Biosurfactants have been introduced as an alternative to synthetic chemical for controlling insects. Mosquitosidal biosurfactant

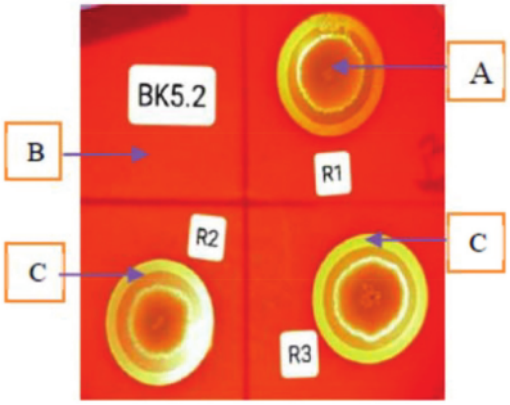

Fig. 5. Hemolytic activity of native entomopathogenic of *Bacillus* sp. BK5.2 on blood agar plate media. Note: A=Isolate of*Bacillus* sp. BK5.2; B=Blood agar plate; C=Clear zone around *Bacillus* sp. BK5.2; R=Replicates

activity in adult mosquitoes which is produced by the *Bacillus* strain has been reported to kill adult mosquitoes (Geetha *et al*., 2012).

The action mechanism of biosurfactants as insecticides also has been reported. Biosurfactants which are produced by *B. subtilis* strains were reportedly composed by a mixture of molecules such as fatty acids, peptides, polysaccharides and can be in the form of lipopeptides, lipoproteins, glycolipids, phospholipids and lipopolysacharides. Some of these compounds are toxic to arthropod pests and vectors (Geetha *et al*., 2010; Manonmani *et al*., 2011). Larvae, pupae, and adult mosquitoes need oxygen from the atmosphere for respiration, due to a decrease in surface tension due to lipopeptides, larvae and pupae are unable to get oxygen, and lead to death. The action of biosurfactants as mosquitocidal

toxins against larvae and pupae is possible because biosurfactants trigger a decrease in surface tension of the water, thereby causing a lack of oxygen under water. Low oxigene concentration causes insect spiracles to keep opening and can cause the insect to die. Biosurfactants are able to influence insect cuticles, due to their amphiphilic character with the presence of hydrophobic and hydrophilic molecules. Research has shown that biosurfactant-producing bacteria can be used to control pathogens in plants and insects (Zhao *et al*., 2014).

Conclusion

Native entomopathogenic *Bacillus* sp. BK5.2 isolated from the natural breeding sites of *Ae. aegypti* larvae in Baluran National Park East Java Indonesia was 99% close to the *Bacillus thuringiensis* strain B16. The encoding gene of protein toxin detected sizes range 300bp, assummed as *Cry*11 gene. Hemolytic activity showed that positive results on blood agar plate. This nativeentomopathogenic *B. thuringiensis* (BK5.2) has biological control potential to be developed as an environmental friendly agent against disease vectors and plant diseases.

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