



Identification of Entomopathogenic Nematode-Associated Bacteria Originating from Mojokerto

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ABSTRACT

Entomopathogenic nematodes (EPN) are one of the soil worms that have been widely used as a natural pest control. EPN has its pathogenic capability because of the mutualistic interactions between nematodes and symbiotic bacteria inside the digestive tract of nematodes. Symbiotic bacteria capable of producing exoenzymes that are toxic to insects. The isolation of symbiotic bacteria accomplished by infection of obtained EPN (Belik II isolate) into *Tenebrio molitor* larvae. Symbiotic bacteria were isolated from the hemolymph of dead larvae on NBTA media. Isolation of symbiotic bacteria was successfully obtained two morphologically distinct bacteria: B 3.1 isolate and B 4 isolate. Both bacteria were further identified using PCR analysis of the 16S rRNA gene. Based on the sequencing results, the B 3.1 isolate was in accordance with *Acinetobacter pittii* strain ATCC 19004, while B 4 isolate was in accordance with bacteria *Enterobacter aerogenes* strain KCTC 2190. The characterization of B 3.1 isolate was shown to have similarities with *Acinetobacter* sp., i.e.: gram-negative, non - motile, rod-shaped, and some other characteristics of biochemical tests. While the characterization of B 4 isolate was shown to have similarities with *E. aerogenes* i.e.: gram-negative, rod-shaped, motile, and some other characteristics of biochemical tests. These findings will be the potential to be applied as biological agents in pest control.

Keywords: Entomopathogenic Nematodes, Symbiotic Bacteria, 16S rRNA gene, *Acinetobacter pittii*, *Enterobacter aerogenes*

INTRODUCTION

Indonesia is the second country in the world after Brazil which is known to have a very large source of biological wealth [1]. This great potential should be utilized to obtain the desired plant products. However, one of the limiting factors in the effort to increase the production of

quality food crops is the presence of pests. Losses obtained from pest attacks reach 13% of total crop production in the world [2].

Currently, various ways have been applied to obtain healthy and quality food crop products. One way is to use biological agents as natural pest control in addition to the use of synthetic insecticides. One example of a biological agent

that has been widely used today is entomopathogenic nematodes. As the name implies, entomopathogenic nematodes (EPN) can cause disease (pathogenic) to insects. Nematodes of the genus *Steinernema* and *Heterorhabditis* are pathogenic nematodes that have very strong virulence against various insects [3]. The pathogenic ability of EPN is the result of mutualistic interactions between nematodes and bacteria in the digestive tract of these nematodes [4]. *Xenorhabdus* spp. is a bacterium that can have a specific symbiosis with the nematode *Steinernema* spp. while the bacteria that are symbiotic with the nematodes *Heterorhabditis* spp. are *Photorhabdus* spp. [5,6].

However, apart from bacteria make a specific symbiosis with NPS, several other types of bacteria are also associated with NPS. According to Razia et al. [7], even though there are no symbiont bacteria present in the nematodes, the presence of these associated bacteria helps EPN to reproduce and obtain nutrition.

Based on preliminary research conducted by Askitosari [8], it showed that from 27 EPN isolates from soil samples in 13 villages in Trawas, a district in Mojokerto, one of EPN isolates has the potential as a bioinsecticide, namely Belik II. Based on the observations, the EPN Belik II isolate showed the best viability after years of storage in the cold room. Therefore, in this study, the NPS of Belik II isolates were selected to be further identified and characterized. Thus, this study aims to identify and characterize the associated bacteria present in the EPN Belik II isolate. These findings will be fruitful to investigate further the pathogenicity level of the associated bacteria against host insects through the pathogenicity test.

METHODS

Infection of EPN into *Tenebrio molitor*

EPN Belik II isolate was rejuvenated using insect larvae of *T. molitor* to obtain infective juvenile of EPN Belik II isolate. Subsequently, the infective juveniles were reinfected into *T. molitor* (30 larvae) by dropping 1 ml of EPN Belik II infective juvenile suspension (700 JI / ml) in a small petri dish coated with wet filter paper. The same treatment was also carried out on the control experiment using 1 ml of sterile distilled water. Petri dishes were incubated at room temperature until all 30 *T. molitor* larvae died [9, 10].

Isolation of EPN-Associated Bacteria from the infected *Tenebrio molitor*

The EPN-associated bacteria was isolated by dissecting the dead *T. molitor* larvae by the EPN invasion symptom test. Symptom test for EPN invasion was observed through the physical characteristics of the dead *T. molitor* cuticle color change (turn into dark color). The *T. molitor* larvae cadavers were sterilized by immersing in 0.6% NaOCl solution for a few seconds, followed by 2 minutes. Afterward, the *T. molitor* larva cadavers were cut in the middle to destroy the intestines. The haemolymph fluid that comes out was dropped on the Nutrient Bromothymol Blue Tetrazolium Chloride Agar (NBTA) by using streak plate method and incubated in dark place, at 27°C temperature for 4 days. The grown colonies were then sub-cultured to obtain secondary cultures on nutrient agar (NA) medium and incubated for 4 days at 27°C temperature in the dark place. A single grown colony was then inoculated into nutrient broth (NB) media and placed in a rotary shaker at a speed of 150 rpm at 27°C temperature [8].

Bacterial Morphological and Biochemical Test

The isolated bacteria were characterized by growing bacteria on the NBTA agar to observe their shape, edges, elevation, and surface color [11]. The bacterial characterization followed by biochemical test according to the method described in the Bergey's Manual of Systematic Bacteriology. The biochemical tests carried out were catalase test, Sulfide Indol Motility (SIM) test, carbohydrate fermentation test (dextrose, lactose, sucrose), citrate utilization test, Methyl Red-Voges Proskauer test (MR-VP), nitrate reduction test, urease test, amylase test, and gelatinase test.

Genomic DNA Isolation from EPN-Associated Bacteria

Secondary cultures of isolated EPN-associated bacteria were grown on Luria Bertani (LB) medium and shook overnight at 37°C. A total of 1.5 ml of culture was centrifuged at 13,000 rpm for 1 minute. The supernatant is removed and repeats the steps until the cell pellets weigh 50-100 mg. Pellets were then rinsed with ddH₂O and subsequently used for Genomic DNA isolation by applying the protocol suggested by ZR Bacterial DNA Miniprep (Zymo Research) manufacture.

Molecular Detection of EPN-Associated Bacteria by using 16S rRNA PCR method

EPN-associated bacterial DNA that has been extracted were amplified by applying PCR method using universal primers 16S rRNA, i.e. forward primer: 5 'CACGGATCCAGACTTTG ATYMTGGCTCAG 3' and reverse primer: 5 'GTGAAGCTTACGGYTAGCTTGTTACGAC TT 3' (1st Base). The amplicon size of the 16S rRNA is approximately 1480 bp. Visualization of PCR products was carried out by electrophoresis on 0.8% agarose gel. Furthermore, the purification of PCR products was carried out using the High Pure PCR

Product Purification Kit (Roche) and ligated to the pGEM-T Easy (Promega) plasmid DNA (Promega, 2011). Subsequently, the molecular method was followed by the DNA transformation in the competent cells, blue/white colonies screening, recombinant plasmid analysis using PCR, and plasmid DNA isolation using the High Pure Plasmid Isolation Kit (Roche).

DNA Sequencing Analysis

DNA sequences obtained from the sequencing results (Macrogen-South Korea) were compared with the DNA database on NCBI (www.ncbi.nih.nlm.gov) using the BLAST nucleotide. The highest similarity between DNA sequences and the DNA database determined the bacterial species associated with EPN.

RESULTS

Isolation of EPN-Associated Bacteria

As shown in Figure 1, the colonies grown in NBTA medium showed round morphology, convex elevation, uneven colony edges, and there is a clear zone surrounding red colony. However, there is a color difference between the two colonies. The colony of B3.1 isolate was red, while the colony of isolate B4 was red accompanied by a faded red color in the middle of the colony.

Morphological and Biochemical Test of EPN-Associated Bacteria

Based on the result showed in Figure 2, it is known that the two isolated EPN-associated bacteria are both Gram negative rod-shaped bacteria. Furthermore, the recapitulation of the Biochemical test to show the physiological characterization of EPN-associated bacteria is shown in Table 1.

Molecular Detection of EPN-Associated Bacteria by using 16S rRNA PCR method

The amplification of 16S rRNA from bacterial genomic DNA was carried out by PCR using 16S rRNA universal primers. The amplicon obtained were ~ 1,500 bp in size (Figure 3). This size is in the range of expected amplicon size, which is 1480 bp. Moreover, Figure 4 showed the gel Electrophoresis of extracted-DNA Plasmid *pGEM-T* containing 16S rRNA

amplicon. Result showed the obtained bands vary in size. The plasmid conformation might affect the speed of movement of the plasmids. Furthermore, based on BLAST sequence analysis, B3.1 isolate showed highest similarity (99 %) with *Acinetobacter pittii* strain ATCC 19004, while B4 showed highest similarity (99%) with *Enterobacter aerogenes* strain KCTC 2190.

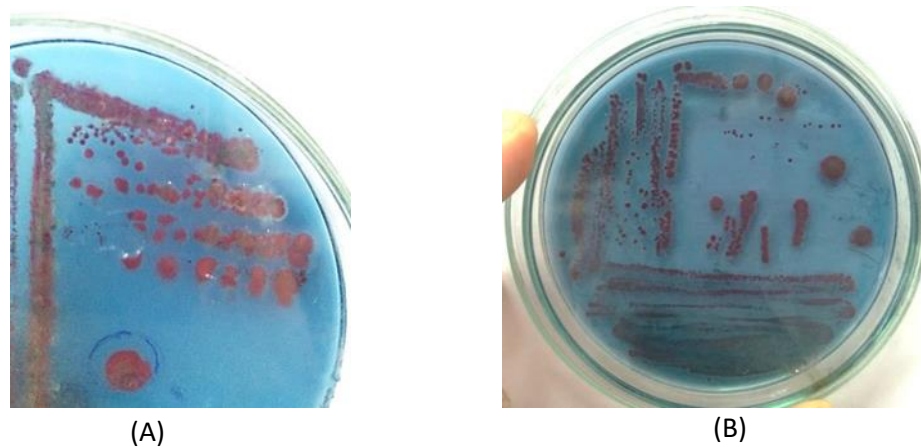


Figure 1. EPN-associated bacteria isolated from Belik (Mojokerto) soil sample grown in NBTA medium (A: B3.1 isolate, B: B4 isolate)

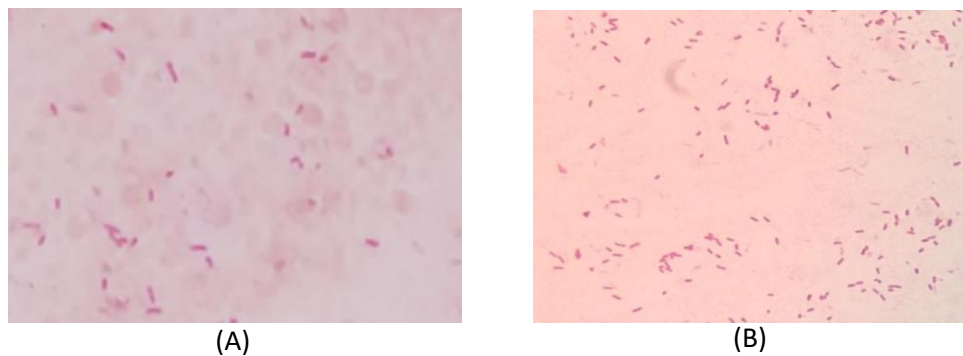


Figure 2. Gram staining of EPN-associated bacteria isolated from Belik (Mojokerto) soil sample grown in NA medium showed the rod shape observed by microscope (10x100 magnification) (A: B3.1 isolate, B: B4 isolate)

Tabel 1. Biochemical Test of EPN-Associated Bacteria Originating from Belik (Mojokerto)

Test	B3.1	B4
Amylase	+	+
Catalase	+	+
Nitrate reduction	-	+
Gelatinase	-	-
Citrate utilization	+	+
Indol production	-	-
MR	-	-
VP	-	-
Motility	-	+
Urease	-	-
Fermentation:		
Dekstrose	-	+
Sukrose	-	+
Laktose	-	+
H ₂ S production	-	-

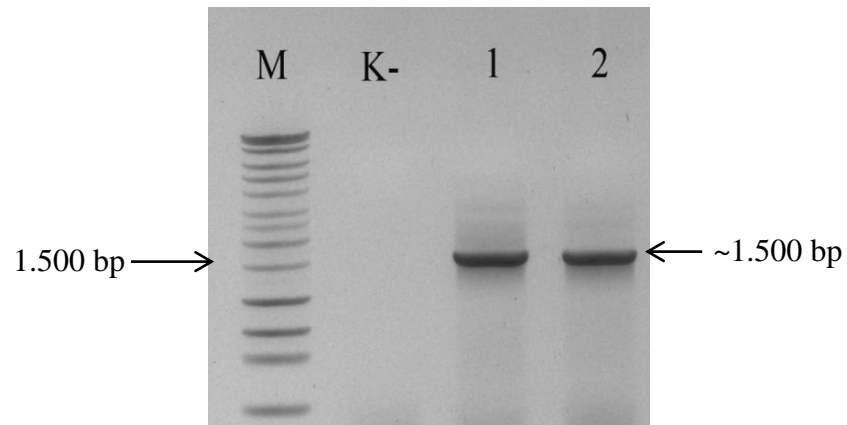


Figure 3. Gel Electrophoresis of PCR 16S rRNA amplification using 16S universal primer (Lane M: 1 Kb DNA ladder (Hyperladder Bioline); Lane 1: B3.1 isolate, Lane 2: B4 isolate; Lane K- negative control)

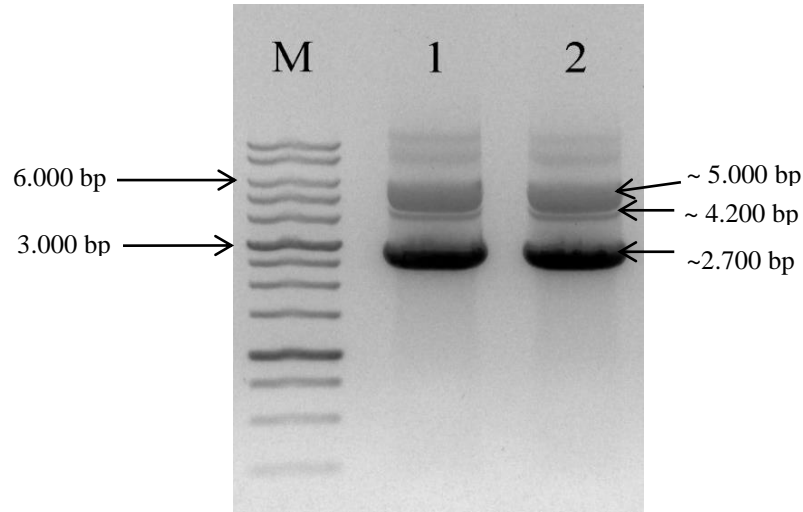


Figure 4. Gel Electrophoresis of extracted-DNA Plasmid *pGEM-T* containing 16S rRNA amplicon (Lane M: 1 Kb DNA ladder (Hyperladder Bioline); Lane 1: B3.1, Lane 2: B4)

DISCUSSION

Based on a preliminary test conducted by Askitosari (2014) [8], it has been reported that EPN Belik II is one of potential candidate for pest control. In this study, it showed EPN Belik II which was reinfected into *T. molitor* larvae, was able to kill all the 30 larvae population within 60 hours. The isolation of bacteria obtained from *T. molitor* larvae cadaver was cultivated in NBTA media showed distinctive color between two strains of bacteria. The colony of B3.1 isolate was red (Figure 1A), while the colony of isolate B4 was red with a faded color in the middle of the colony (Figure 1B). The red color is actually produced from the TTC (Tetrazolium Chloride) compound in reduced NBTA media. This compound can be reduced by bacteria with the help of the dehydrogenase enzyme to become a red compound called formazan (1,3,5-triphenylformazan) [12].

Based on the gram staining results, it is known that the bacteria obtained are both Gram negative rod-shaped bacteria. In general, most of the bacteria found to be associated with EPN are Gram-negative rod-shape bacteria [13]. Furthermore, based on the molecular detection

method (Figure 3&4), the B3.1 isolate is similar with the *Acinetobacter pittii* ATCC 19004, while the B4 isolate is similar with *Enterobacter aerogenes* KCTC 2190 strain. This result was also confirmed by colony morphological observations and biochemical tests carried out (Table 1).

The results of this study indicate the presence of bacteria other than symbiont bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) which are generally associated with EPN. According to Lysenko & Weiser (1974) and Gouge & Snyder (2006), there are several other types of associated bacteria that have been isolated from *Steinernema carpocapsae*. These bacteria include *Alcaligenes* sp., *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *Enterobacter agglomerans*, *Serratia liquefaciens*, *Acinetobacter* sp, and *Ochrobactrum* sp. In addition, there is a hypothesis that bacteria associated with EPN might be originating from the stomach and cuticle of the EPN [14, 15], the stomach of the host insect [16], or it can also be originating from secondary infection.

Although the *Acinetobacter pittii* and *Enterobacter aerogenes* strain obtained have been isolated in this study, the pathogenicity

mechanism of both bacteria is still unclear. However, *Enterobacter* species are known to produce endotoxins which could be virulence factors of these bacteria [17]. Apart from endotoxins, there is still limited information regarding the potential pathogenicity of *Enterobacter* species. In addition, there are also opinions regarding the deaths of insect larvae are not actually caused by *E. aerogenes*. This bacterium is only an opportunistic bacterium that invades larvae after the larvae died. Therefore, *E. aerogenes* only plays a role in secondary infection [18].

CONCLUSION

Based on the results of molecular identification, two types of obtained bacteria have high similarity to *Acinetobacter pittii* strain ATCC 19004 and *Enterobacter aerogenes* strain KCTC 2190. These results were also supported by the results of biochemical tests which showed match characteristics with the two bacteria. The bacteria found in this study are known as unusual associated bacteria in EPN. Therefore, it is necessary to investigate further the pathogenicity of these bacteria into insects pest, that can be useful to be applied as biological pest control agent.

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