Exploration of Symbiotic Bacteria of Oryctes rhinoceros (Coleoptera: Scarabaeidae) Larvae from Oil Palm Empty Fruit Bunches

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INTRODUCTION

Currently, microorganisms have been widely used as biological fertilizers or for biological control in sustainable agriculture. This microorganism can also be obtained from the digestive tract of insects. Oryctes rhinoceros utilizes oil palm empty fruit bunches as a breeding ground and these insects have a bacterial symbiont inside hind gut. Identification is an activity carried out to determine the identity of a particular organism by observing, testing, recording and identifying based on test results. Sijabat, Marheni, & Bakti (2018) stated that the use of starter bacteria from the genus Bacillus isolated from the hind gut larvae of O. rhinoceros can accelerate the maturity of litter composting from 30 to 18 days. So it is necessary explore and identify other useful bacteria.

O. rhinoceros is one of the important pests in oil palm plantations (Manjeri, Muhamad, & Tan, 2014). Both female and male imago of O. rhinoceros can cause damage on oil palm and other palm types. The unutilizable stacks of oil palm stems, both standing and chopped, provides an opportunity for the insect to build the nest. The availability of organic material provides the site for the beetles to lay their eggs. The organic matters in the form rotted, piles of oil palm trunks, chopped oil palm trunks, sawdust, rubber stumps and empty bunches of oil palm also provide food for the larvae that hatched from the eggs. The O. rhinoceros can attack and give significant yield losses, kill the young plants in the nursery, replanting and productive plants. The insect sheared the leaves in mild attacks, resulted in the delay of generative phase and decreased yield.

ABSTRACT

Keywords: Exploration
Larvae Oryctes rhinoceros
Symbiotic bacteria

Oil palm empty fruit bunches (OPEFB) were utilized as the source of organic fertilizer, but become a medium of Oryctes rhinoceros proliferation in oil palm plantations. O. rhinoceros uses cellulose, hemicellulose and lignin in the OPEFB as energy sources for larvae growth and development. In the process of breaking and combining complex compounds into simple molecules, they are establishing symbiotic relationship with bacteria residing in the digestive tract of O. rhinoceros larvae. Isolation of the symbiotic bacteria of the O. rhinoceros larvae was carried out with the aim of identifying bacteria in the digestive tract of the third instar found in the OPEFB. This research was done by using a descriptive method. Exploration of symbiotic bacteria of O. rhinoceros larvae was conducted through two stages. The first step was attained by isolating, growing and multiplying the bacteria and conducting biochemical testing. The second phase was accomplished by molecular testing for bacterial identification. The results showed that bacterial isolates were found and after molecularly sequenced based on 6S rDNA, the species were identified as Bacillus stratosphericus, B. siamensis, B. cereus, Haemophilus parainfluenzae, Achromobacter xylosoxidans, and Alcaligenes faecalis. The research also found several species of bacteria originating from the hindgut larvae O. rhinoceros.
The adults enter the terminal plant part and eat the soft tissues. When the attack reaches the growing point, the plant growth will ultimately stop and in most of cases, the plant will die. The insects mated and produced offsprings at the empty palm bunches and palm oil waste that were available surrounding the plantation area. (Bintang, Wibowo, & Harjaka, 2015).

Insect organ can serve as the harbor gut of various microorganisms, including from simple to complex bacterial communities. Several studies have indicated the important contributions of gut-associated microbes to the physiological attributes of the insect hosts and their life cycle. However, there are still less reports the diversity, physiology, and ecology of microorganisms associated with the guts of diverse groups of beetles that develop within the wood and bark of trees (Baharuddin, Patong, Ahmad, & La Nafie, 2014).

Fat body is composed of three cells including mycetocytes, which are cells that contain a number of microorganisms in the intestine and symbiosis with insects (Roma, Bueno, & Camargo-Mathias, 2010). The ability of microbes in the degradation process is because they have cellulase enzymes. Microbes in insect gut are symbiont microbes that are interconnected with other organisms in the process of lignocellulose degradation (Aini & Subekti, 2015).

In the digestive tract of insects, there are nonpathogenic microorganisms within the intestine, there are 105-109 prokaryotic cells that have been affiliated with twenty-six phyla. Insect microbiota is very important for normal growth and development and for about 65% of insects have symbiotic bacteria. Symbiotic relationships between bacteria and insects vary from mutualistic to commensal. Based on the role of the symbionts bacteria, intracellular symbionts in insects are classified as primary and secondary endosymbionts. Frond borer from the genus Oryctes is considered a very detrimental pest. The presence of endosymbionts in the genus Oryctes, which is an insect-bacterial association in this case will be useful to be elucidated (El-Sayed & Ibrahim, 2015).

In insects that digest wood components as nutrients are often assisted by microorganisms in various ways including the first acquisition of microbial-generated enzymes on digestible substrates, pre-digestion of the substrate by microorganisms before digestion, then enrichment of nutrients in the form of microbial cells and metabolites.

Identification of bacteria can be conducted using conventional and molecular methods (Gugliandolo, Lentini, Spanò, & Maugeri, 2011). The conventional method was carried out based on the typical phenotype, namely Gram staining, morphology and biochemical reactions. However, this conventional method of determining bacteria has several disadvantages. In the isolation of O. rhinoceros larvae from empty oil palm bunches, Bacillus stratospherichus was found. While whitin larvae from rotten palm trees, Bacillus siamensis was observed. (Sijabat, Marheni, & Bakti, 2018).

Bacteria have a variety of biochemical activities (growth and multiplication utilizing raw material (nutrients) obtained from the surrounding environment. Biochemical transformations can take place at inside and outside of bacteria regulated by biological catalysts known as enzymes. The original bacteria of the digestive tract have a mutual relationship with their host, which is using the host as their place of life. This study was aimed to determine the presence of symbiotic bacteria in the digestive tract of O. rhinoceros larvae that live in empty oil palm bunches.

**MATERIALS AND METHODS**

**Research Location and Specimen Preparation**

The 3rd instar larvae of O. rhinoceros were taken from the piles of empty oil palm bunches in smallholder oil palm in the Langkat regency, North Sumatera, Indonesia. Experiments were under laboratory conditions at the Faculty of Agriculture, University of Sumatera Utara (USU) from May to October 2018. This research was carried out using a descriptive method after field collections of larvae. Exploration of symbiotic bacteria of O. rhinoceros larvae was conducted several steps, i.e. isolation, growth induction, multiplication and biochemical evaluation.

**Isolation and Biochemical Test**

Third instar larvae from the OPEFB were collected and their hind guts were mashed up. After that, a series of dilutions were carried out. The diluting processes were conducted by taking 1 g sample into a test tube containing 9 ml of distilled water (10⁻¹ dilution was obtained). The dilution series were employed to get the concentration of 10⁻⁵. For about 1 ml solution from 10⁻⁴ and 10⁻⁵ dilution were taken and put into a petri dish containing NA. The solution
was flattened evenly and then incubated with an inverted position for 24-48 hours at the temperature of 37ºC. After 48 hours of incubation, bacterial isolation was following several steps of quadrant method to obtain the pure isolate. The bacteria which had been grown and propagated served as the biochemical testing materials in determining the biochemical properties and bacteria identification. The simple biochemical tests incorporated into TSIA tests (Triple Sugar Iron), SCA (Simmons Citrate Agar), SA, Gelatin, and Catalase.

**Identification of Bacteria**

The order of 16S rDNA was determined based on the Bank Gen database by applying the BLAST program at the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/). The sequences obtained from sequencing results were subsequently combined and analyzed with the BLAST program.

**Phylogenetic Analysis**

The PCR results were visualized on 1% agarose gel and stained with ethidium bromide. DNA amplification was subsequently purified and sequenced to find the bases of DNA sequences. Afterward, the sequence data was compared with GenBank data from The National Center database for Biotechnology Information (NCBI) ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)), using the Basic Local Alignment Search Tool (BLAST) program.

**RESULTS AND DISCUSSION**

**Gram Test**

From six bacterial isolates, there were three isolates identified as bluish purple positive-gram and the other three were categorized as red negative-gram bacteria. The color differences in gram-positive and gram-negative bacteria indicated that there were differences in cell wall structures between the two types of bacteria. Gram-positive bacteria have cell wall structures with thick peptidoglycan content while gram-negative bacteria are attributed to cell wall structures with high lipid content (Fitri & Yasmin, 2011). Based on staining test purple color was formed on the bacterial cell walls because the bacteria had lower lipid content so that the bacterial cell walls would be hydrated more easily due to treatment using alcohol. Morphological observations on 6 isolates indicated that the bacteria possess quite large, round, and white colonies, convex surfaces, flat edges and spreading growths.

The bacteria which are gram-positive and rod-shaped are considered as Bacillus group. In addition to the morphological observations, physiological observations also were included to the biochemical tests of 6 isolates (Table 1).

**Table 1. Gram staining and biochemical tests**

<table>
<thead>
<tr>
<th>Isolates Code</th>
<th>Gram</th>
<th>SA</th>
<th>Gelatin</th>
<th>SCA</th>
<th>TSIA</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>B2</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>B3</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>B4</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>B5</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>B6</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Remarks: (+) = Positive, (-) = Negative

The result of the starch hydrolysis test was obtained by giving a few drops of iodine solution on the bacteria isolate. The color change indicated the presence of starch. If the starch on the medium cannot be changed into simple molecules by the bacteria, the color will turn into purple or blackish blue because iodine leads to the bending of the straight structure of the starch which is visible as blue. Positive gelatin test was indicated by gelatin medium which remained liquid after being set in the refrigerator for ± 30 minutes. The test pointed out that isolates could hydrolyze gelatin or proteins in large molecules into polypeptides and amino acids using extracellular proteolytic enzymes in the form of gelatinase and protease.

The bacteria are able to grow using citrate as the only source of carbon. There will be a change into blue due to the increase of media pH. Characterized by a yellow top and yellow underside, the TSIA test demonstrated that lactose and sucrose could be fermented. If the upper part is red and the bottom is yellow, only glucose that can be fermented by bacteria. The test result was based on the capability of the six isolates of bacteria to produce catalase enzyme. With respect to Lay’s suggestion, the catalase test was carried out to determine the presence of the catalase enzyme in bacterial isolates. Catalase is an enzyme that can catalyze the decomposition of hydrogen peroxide (H₂O₂) into H₂O and O₂. This test is very critical to determine the ability of bacteria to produce O₂ by deciding hydrogen peroxide (H₂O₂) to defend themselves.
The results demonstrated that there were 6 bacterial isolates and identified molecularly, for species analysis using sequencing of 16S rDNA bacterial species, namely (B1) *Bacillus stratosphericus*, (B2) *Bacillus siamensis*, (B3) *Bacillus cereus*, (B4) *Haemophilus parainfluenzae*, (B5) *Achromobacter xylosoxidans*, and (B6) *Alcaligenes faecalis* (Fig. 1). Gram-positive bacteria were marked in purple showing that the bacteria were able to bind crystalline violets, while gram-negative bacteria were marked with pink indicating that the bacteria could not bind violet crystal colors and were only colored by safranin. Gram stain makes it easy to microscopic examination to observe bacterial cell shape so that it is easy to identify by knowing the reaction of bacterial cell walls through a series of staining. Gram staining reactions are based on differences in the chemical composition of bacterial cell walls. Gram positive cells have a thick peptidoglycan, while the peptidoglycan layer in gram negative cells is much thinner (Cappuccino & Sherman, 2014; Setiawan, Sulistyanto, & Senjarini, 2017).

**Fig. 1.** Gram staining bacterial from hindgut larvae. (a) *Bacillus stratosphericus* (B1), (b) *Bacillus siamensis* (B2), (c) *Bacillus cereus* (B3), (d) *Haemophilus parainfluenzae* (B4), (e) *Achromobacter xylosoxidans* (B5) and (f) *Alcaligenes faecalis* (B6)

**Identification Results of Bacterial Species Based on 16S rRNA**

The homology of the 16S rRNA encoding gene sequences from the 6 isolates was traced to GenBank through the BLAST program. The 16S rRNA sequences obtained from the BLAST program were stored in the FASTA format and reprocessed using ClustalW and MEGA5 to obtain multiple sequence alignment (MSA) and saved in MEGA format (Table 2). The phylogenetic tree was designed by assigning a comparison of 16S rRNA sequences from other bacteria in the BLAST database tracking program, visualized by the ClustalW program. The formation of phylogenetic trees was conducted using the MEGA06 program with Neighbor-Joining Tree (Fig. 2).

Identification of B6 isolates had a compatibility of up to 98% against *Alcaligenes faecalis*. This bacterium is aerobic, gram-negative and pigmented rod-shape. The characteristic distinguishes it from *Pseudomonas*. This species is motile with one or more flagella. The bacteria from the *Achromobacter* family do not encapsulate and form spores, but grow...
slowly (Kavuncuoglu et al., 2010). Identification of bacteria employed the 16S rRNA to determine the composition of nucleotides because all species had different arrangements or sequences from each nucleotide chain (Thiamin, Guanine, Adenine, Cytosine) so that the nucleotide arrangement could be seen or identified species or name of an organism. The results demonstrated that there were 6 bacterial isolates and identified molecularly, namely *B. stratosphericus*, *B. siamensis*, *B. cereus*, *H. parainfluenzae*, *A. xylosoxidans*, and *A. faecalis*.

*B. stratospheric* can be found at altitude in the stratosphere and is able to survive in unfavorable environmental conditions and nutrients. Hosseini-Abari, Emtiazi, Lee, Kim, & Kim (2014) stated that strain of *B. stratosphericus* has been originally isolated from cryogenic tubes of atmospheric air sample. However, atmospheric cycling processes can establish its presence on earth. The vegetative cells of this bacterium have been found to have several specific properties such as the ability of catalase production, resistance to UV radiation, and potential to produce electricity. The properties of the *B. stratosphericus* spores are still unknown. Herein, some properties of the spores were figured out, and they were introduced as an efficient tool for silver nanoparticle synthesis (AgNPs). Our results revealed that vegetative cells of this strain were not able to synthesize silver nanoparticles; however, the spores are represented this ability during a short time.

*B. stratospheric* resistance in adverse conditions incorporates resistance to UV radiation and NaCl with the concentration up to 17.5% will not damage the cell. These bacteria can grow at temperatures between 8-37°C and at pH 6-10. *B. stratospheric* is highly tolerant to Fe, Co, Ni, and Cu ions, and is quite tolerant to Zn (Bindu, Silpa, Rajesh, & Reddy, 2013). Dunlap, Kim, Kwon, & Rooney (2015) reported that the substrate supplies the carbon and triglycerides source is produced from the lipase enzyme which is compatible with the bio detergent from *B. Stratospheric* using coconut dregs. Likewise, Marheni (2019) also states that *B. stratospheric* can be used to increase the composting of oil palm empty bunches plus other bioactivators.

*B. siamensis* is a gram-positive, facultative anaerobic, rod-shaped and movable bacterium. Its colony on media is creamy white and grows at 37°C with 6-7 pH (Chen et al., 2016). *B. siamensis* is a bio-control bacterium producing lipopeptides which can potentially reduce the use of pesticides in agricultural areas and inhibit the development of *Fusarium oxysporum* fungi.

*Bacillus cereus* is a gram-positive rod shaped. This bacterium is a mesophilic organism which can construct at temperatures of 30-35°C. The bacterium produce amylase enzymes and proteases, which can break down proteins and clot milk. In stressful situations such as the bad environment and the unsupported food availability, the bacterium will experience sporulation. The produced spores will be able to change back into vegetative cells, influenced by temperature, oxygen and Nitrogen. *Bacillus* spp. has antagonistic ability in the form of antibiotics against fungi (Abidin, Aini, & Abadi, 2015). Setiawan, Sulistyanto, & Senjarini (2017) reported *B. cereus* is one of the agent pathogens that have great potential for used as a biological controller. Bacteria has a specific host, no harmful to natural enemies of pests and other non-target objects, easy biodegradable by the environment and can increase its pathogenicity by genetical manipulation.

### Table 2. Blast results of symbiotic bacteria from hindgut larvae of *O. rhinoceros*

<table>
<thead>
<tr>
<th>Species</th>
<th>Max Score</th>
<th>Query Cover</th>
<th>Percent Identity</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus siamensis</em></td>
<td>2303</td>
<td>100%</td>
<td>99%</td>
<td>KY643639.1</td>
</tr>
<tr>
<td><em>Haemophilus parainfluenzae</em></td>
<td>2122</td>
<td>100%</td>
<td>97%</td>
<td>NR 118143.1</td>
</tr>
<tr>
<td><em>Bacillus stratosphericus</em></td>
<td>2041</td>
<td>100%</td>
<td>99%</td>
<td>NR 042336.1</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>1707</td>
<td>100%</td>
<td>99%</td>
<td>KY 628813.1</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em></td>
<td>917</td>
<td>89%</td>
<td>88%</td>
<td>NR 113606.1</td>
</tr>
<tr>
<td><em>Achromobacter xylosoxidans</em></td>
<td>2056</td>
<td>100%</td>
<td>96%</td>
<td>NR 044925.1</td>
</tr>
</tbody>
</table>
Fig. 2. Phylogenetic tree of the larval symbiont gene with CLUSTAL W and 1000 replications and GenBank
Morphology of *Haemophilus* sp. is pleomorphic and sometimes looks like a small stem, a cocccobacilli or a short filament. This bacterium is gram-negative and motile. The habitat of *Haemophilus* sp. is in facultative anaerobic atmosphere. *H. influenzae* is difficult to be grown and considered as fastidious bacterium. The bacteria growth requires nutrition and special environment. In biogeochemical cycles in waters, heterotrophic bacterium has a role as decomposers and is able to remineralize organic materials into simple inorganic components returned to the soil and atmosphere as nutrient.

**CONCLUSION**

The isolation and identification of symbiotic bacteria from *O. rhinoceros* larvae in the OPEFB revealed species, namely *B. stratosphericus*, *B. siamensis*, *B. cereus*, *H. parainfluenzae*, *A. xylosidans* and *A. faecalis*. Biochemical testing of catalase pointed out that all the symbionic bacteria produced catalase enzyme, extracellular proteolytic enzymes in the form of gelatine and protease produced by *B. stratosphericus*, *B. siamensis*, *B. cereus* and *A. faecalis* through biochemical gelatin testing. Lactose and glucose fragmentation through biochemical testing of TSIA was found in *A. faecalis*, and only *B. cereus* possessed the ability to hydrolyze starch through SA biochemical tests.

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**REFERENCES**


Marheni et al.: Exploration of Symbiotic Bacteria


