



Diversity of Culturable Bacterial Gut Microbiome of *Oryctes rhinoceros* Larvae and Their Potency as Source of Protease and Lipase Enzymes

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ABSTRACT

Oryctes rhinoceros is an important pest of various coconut and palm species. Studies on the bacterial gut microbiome of *O. rhinoceros* larvae and their potential use as producers of industrial enzymes are still limited. This study aims to examine the diversity and abundance of culturable bacterial microbiome in the gut of *O. rhinoceros* and their potential to produce protease and lipase. This study consisted of several stages, i.e., collection of *O. rhinoceros* larvae from coconut plantations; isolation of culturable bacteria from larval gut; protease and lipase enzyme activity assays; and partial characterization of culturable gut bacteria in terms of morphology, physiology, and biochemistry. There were seven bacteria that were isolated from the gut of *O. rhinoceros*, which were differentiated by their morphotype. The seven bacterial isolates also showed variations in physiological, and biochemical characteristics, and the larval guts were dominated by Gram-positive bacteria. The Shannon diversity index was 1.33, evenness was 0.682, richness was 7, and the abundance of each type of bacteria varied between 2×10^5 and 9.1×10^{10} . Bacteria UBOG1, UBOG3, and UBOG5 were able to produce protease, but all seven bacteria were unable to produce lipase. Thus, bacterial isolates UBOG1, UBOG3, and UBOG5 are potential to be used in industry.

INTRODUCTION

Insects are the most successful group of animals on earth because of their high diversity and ability to occupy various ecological niches. Throughout their lifetime, insects have intricate associations with microorganisms. Particularly, the gut of insects contains a greater variety of microorganisms than other organs, including archaea, bacteria, fungi, and protists. Microorganisms colonize the gut of insects through feeding where they contribute to various aspects of insect metabolism and physiology, such as digestion, nutrient acquisition, and defense against pathogens (Hosokawa & Fukatsu, 2020; Krishnan et al., 2014; Lemoine et al., 2020). Through the secretion of several lytic enzymes, gut microbiota enable the insect hosts to obtain rich cellulose and lignin nutrients from plant material (Gurung et al.,

2019). These microorganisms also increase the host insect's immunity to pathogen infections and environmental stress (Bashir et al., 2013; Lemoine et al., 2020; Mereghetti et al., 2017; Singh et al., 2021).

Comprehensive studies of the abundance and diversity of microorganisms in the insect gut are crucial in the field of insect physiology to understand in more detail the ecological role of symbiont microorganisms and their interactions with host insects (Lemoine et al., 2020; Singh et al., 2021). Likewise, microorganisms such as bacteria in the insect gut are also useful natural resources for beneficial enzymes such as protease and lipase that have industrial value (Banerjee et al., 2022). Additionally, several types of insect gut bacteria have been known to be able to degrade pesticide compounds; hence, they can be applied as bioremediator agents to overcome

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environmental pollution problems (Hadi *et al.*, 2021). However, most studies on the diversity and utilization of insect gut microorganisms still focus on certain taxa, especially grasshoppers, termites, and honey bees (Yun *et al.*, 2014).

In Indonesia, *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae) is an important pest of oil palm, coconut, and sago plants. *O. rhinoceros* is a phytophagous insect that lives on decayed tree trunks during its larval stage. The decayed trunks of oil palm and coconut tree are highly favored by *O. rhinoceros* larvae to be their nests because they contain the organic sources required for their growth and mating (Paudel *et al.*, 2023). Until now, only few studies have focused on the bacterial microbiome of the gut of *O. rhinoceros* larvae and their use in industry, particularly as producers of protease and lipase enzymes. Therefore, this study aims to examine the abundance and diversity of culturable bacterial microbiome in the gut of *O. rhinoceros* larvae and their potential to produce protease and lipase enzymes.

MATERIALS AND METHODS

Sampling of *O. rhinoceros* larvae

This study was conducted at the Plant Disease Laboratory, Department of Pests and Plant Diseases, Faculty of Agriculture, Universitas Brawijaya, from June to September 2022. *O. rhinoceros* larvae were collected using a purposive sampling method from three sites of coconut plantations in the area of Ngawi regency, East Java, Indonesia. At each location, 10 larvae were collected from decayed trunk piles at the surrounding damaged coconut plants.

Isolation of Culturable Bacterial Microbiome from Gut of *O. rhinoceros*

The larvae were surface sterilized with 70% alcohol and rinsed twice in sterile distilled water. Each larva was dissected aseptically using a sterile scalpel, and the entire gut was removed and put into a new sterile 15-ml corning tube. The larval gut was briefly surface sterilized with 70% alcohol, washed twice with sterile distilled water, and then transferred to a 1.5-ml tube containing 500 μ L of normal saline solution. The gut was then homogenized using a microtube pellet pestle, serially diluted, and cultured in nutrient agar (NA) medium. The bacterial culture was then incubated for 48 hours at room temperature. Bacterial colonies obtained

were differentiated, counted and collected based on morphological appearances (morphotype) including shape, color, margins, elevation, and texture. Each bacterial colony was purified by culturing each distinct single bacterial colony.

Partial Characterization of the Bacterial Isolates

The partial characterization of bacterial isolates includes physiological and biochemical assays to determine the genus of the collected bacterial isolates. Characterization was conducted according to the method of Schaad *et al.* (2001), which included a Gram determination (i.e., 3% KOH and Gram staining), endospore production, oxidative and fermentative assays, and growth on YDC medium.

Protease and Lipase Enzyme Activity Assay

The ability of bacteria to produce protease enzymes was tested in Skim Milk Agar (SMA) medium. One ose of bacterial cells was streaked on the surface of the SMA medium and then incubated for 48 hours. Bacteria that produce protease are indicated by the presence of a clear zone around the colony. Assays for bacteria's ability to produce the lipase enzyme were carried out by growing the bacteria for 48 hours in Tween 20 agar medium. Lipase-producing bacteria were indicated by an opalescent zone around the colony due to the precipitation of water-insoluble fatty acids from hydrolysed Tween.

Data Analysis

Based on the type of data, diversity index was adopted to run the analysis. The Shannon diversity index, evenness, and richness were chosen to be used. The Shannon diversity index, evenness, and richness were calculated using the web omni calculator <https://www.omnicalculator.com/ecology/shannon-index> which was based on the following formula:

$$H = -\sum[(p_i) \times \ln(p_i)] \dots\dots\dots 1)$$

Which H = Shannon diversity index; and p_i = proportion of individuals of i -th species in a whole community.

In addition, descriptive interpretation was also used to describe the morphological, physiological and chemical characteristic of each colony that found in this research. Descriptive tables and pictures were modified to describe the data clearly.

RESULTS AND DISCUSSION

Sampling Sites Description

O. rhinoceros larvae were obtained from litter around coconut plantations in the area of Ngawi Regency, East Java (Fig. 1). In several coconut plantation locations, there were piles of animal waste, organic waste, and decayed coconut trunks and remains, which were the preferred reproduction places for the *O. rhinoceros*. The average humidity in the pile where the larvae were found ranges from 86.4% to 88.3.3%, which is suitable for the growth of *O. rhinoceros* larvae. This result is in accordance with Xu *et al.* (2022) report that the optimum habitat humidity for the larval and pupal stages of *O. rhinoceros* is 85%–95%.

Diversity and the Abundance of Gut Bacteria Isolated from *O. rhinoceros*

Seven bacterial morphotypes were obtained after the isolation of culturable bacterial microbiomes from larval gut samples. The abundance of each bacterium varied between 2×10^5 – 9.1×10^{10} CFU/g (Table 1). The results of the calculation showed that the Shannon diversity index was 1.33, evenness was 0.682, richness (number of species) was 7 (Fig. 2), the total number of individuals was 2.45×10^{11} and the average population size was 3.5×10^{10} . These results indicated the diversity and high abundance of culturable bacteria in the gut of *O. rhinoceros* larvae. However, when compared with previous reports on the isolation of bacteria from other insect

pests, the bacterial morphotypes obtained in this study were lower. This is because in this study we only used one type of bacterial culture medium, namely NA medium. Standard agar media such as Nutrient Agar, Luria Bertani Agar, or Tryptic Soy Agar were considered less in providing a dynamic gut physicochemical environment specifically for each type of insect, such as variability in carbon or sugar sources, oxygen availability, as well as various redox, pH, and chemical gradients for the growth of all different bacteria (Riesenfeld *et al.*, 2004). Several reports succeeded in isolating 61 bacterial isolates from the longhorned beetle *Anoplophora chinensis* (Forster) (Coleoptera: Cerambycidae) (Rizzi *et al.*, 2013) and more than 120 bacterial isolates in the intestines of insecticide-resistant *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) (Almeida *et al.*, 2017).

Table 1. The abundance of gut bacteria isolated from *O. rhinoceros* larvae

Bacterial isolate	Abundance (CFU/g)
UBOG1	1.82×10^7
UBOG2	2.0×10^5
UBOG3	6.0×10^5
UBOG4	6.8×10^{10}
UBOG5	9.1×10^{10}
UBOG6	3.4×10^{10}
UBOG7	5.2×10^{10}



Fig. 1. *O. rhinoceros* larvae obtained from Ngawi regency

The diversity and abundance of bacteria in the insect gut can be influenced by several factors, such as food, host, habitat, and the developmental stage of the insect (Jang & Kikuchi, 2020; Yun et al., 2014). *O. rhinoceros* are phytophagous insects whose habitat is piles of organic material and soil. It has been widely known that organic piles and soil contain a high diversity and abundance of bacteria; hence, they can increase the diversity and abundance of bacteria in the insect larval gut.

The Activity of Protease and Lipase Enzymes of *O. rhinoceros* Larval Gut Bacteria

The results of the qualitative assay showed that bacterial isolates UBOG1, UBOG3, and UBOG5 were able to produce protease enzymes, whereas all the bacterial isolates tested were unable to produce lipase enzymes (Table 2). A protease enzyme functions to hydrolyze peptide bonds into oligopeptides and amino acids (Deu et al., 2012). Protease-producing bacteria showed the ability to develop clear zones surrounding the bacterial colonies when grown on SMA medium (Fig. 3). The clear zone formed indicates that the proteins contained in skim milk have been hydrolyzed by protease enzymes produced by bacteria into simple compounds, namely peptides and amino acids, which are dissolved in the medium (Masi et al., 2021). The larger the clear zone produced by a bacterial isolate, the higher the bacteria's ability to produce protease enzymes.

All living things contain proteolytic enzymes, which promote cell growth and differentiation.

The hydrolytic enzymes known as proteases serve as biological catalysts for the breakdown of proteins into smaller peptides and amino acids. Alkaline protease enzymes can be produced continuously and consistently by microorganisms such as bacteria at a reasonable cost (Sharma et al., 2019). Thus, the protease-producing bacteria from the gut of *O. rhinoceros* larvae obtained in this study have the potential to be developed as a source of protease enzymes that are useful in industry. Other than the food industry, proteases are used in detergents, waste management, agriculture, animal husbandry, cosmetics, and pharmaceuticals. These enzymes not only have economic and commercial worth, but they also provide a sustainable and safer solution for the environment (Solanki et al., 2021).

Table 2. Protease and lipase enzyme activity of gut bacteria from *O. rhinoceros*

Bacterial isolate	Protease	Lipase
UBOG1	+	-
UBOG2	-	-
UBOG3	+	-
UBOG4	-	-
UBOG5	+	-
UBOG6	-	-
UBOG7	-	-

Remarks: (+) = produced enzyme; (-) = did not produce enzyme

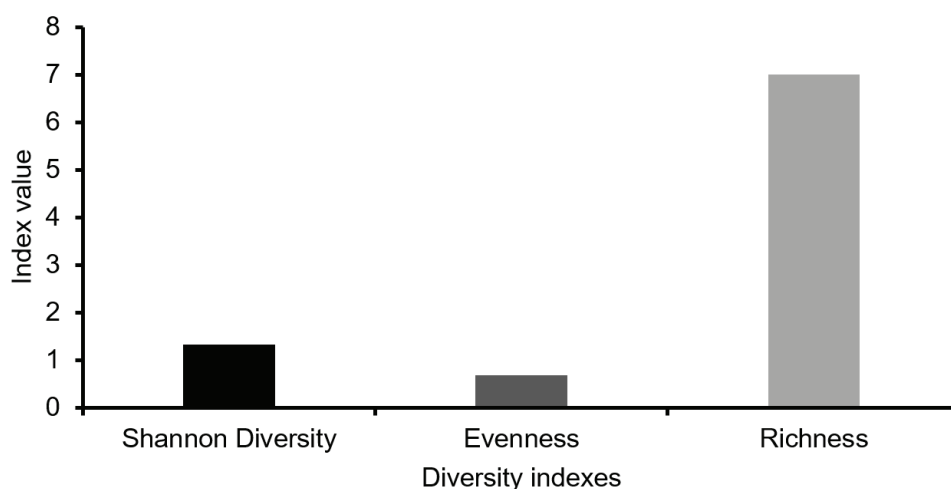


Fig. 2. Shannon diversity index, evenness, and richness of culturable bacterial gut microbiome of *O. rhinoceros* larvae

Colony and Cell Morphology of Gut Bacteria of *O. rhinoceros*

Each of the seven *O. rhinoceros* gut bacteria has different colony characteristics, including shape, elevation, color, and margin. However, the cell type is all the same, i.e., bacilli. UBOG1 colonies on NA medium showed a milky white color, a round shape, entire margins, and flat elevations. UBOG2 colonies showed a red color, a round shape, an entire margin, and a convex elevation. UBOG3 colonies showed a milky white

color, an irregular shape, undulate margins, and flat elevations. UBOG4 colonies showed a yellow color, an irregular shape, an entire margin, and flat elevations. UBOG5 showed a milky white color, a round shape, an entire margin, and a flat elevation. UBOG6 colonies showed a white color, a round shape, an entire margin, and a flat elevation. UBOG7 colonies showed milky white color, round shape, entire margin, flat elevation, and bacillary cell shape (Table 3; Fig. 4; Fig. 5).



Fig. 3. Clear zone surrounding colonies of bacterial isolates grown on SMA medium: a) UBOG1, b) UBOG3, c) UBOG5, d) UBOG2

Table 3. Morphological characteristics of gut bacteria isolated from *O. rhinoceros* larvae

Bacterial Isolate	Color	Shape	Margin	Elevation	Cell Morphology
UBOG1	Milky white	Round	Entire	Flat	Bacilli
UBOG2	Red	Round	Entire	Convex	Bacilli
UBOG3	Milky white	Irregular	Undulate	Flat	Bacilli
UBOG4	Yellow	Irregular	Entire	Flat	Bacilli
UBOG5	Milky white	Round	Entire	Flat	Bacilli
UBOG6	White	Round	Entire	Flat	Bacilli
UBOG7	Milky white	Round	Entire	Flat	Bacilli

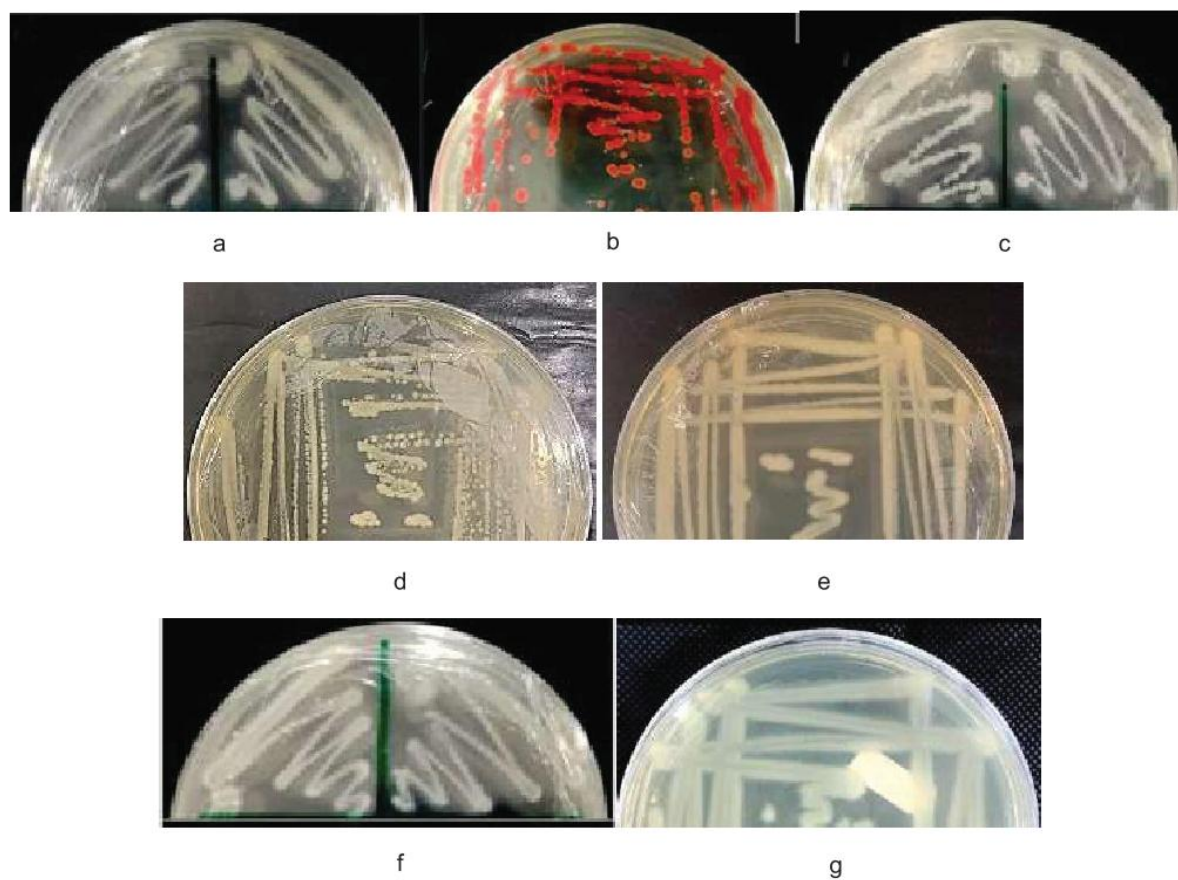


Fig. 4. Colony morphology of gut bacteria of *O. rhinoceros* grown on NA medium: a) UBOG1, b) UBOG2, c) UBOG3, d) UBOG4, e) UBOG5, f) UBOG6, g) UBOG7

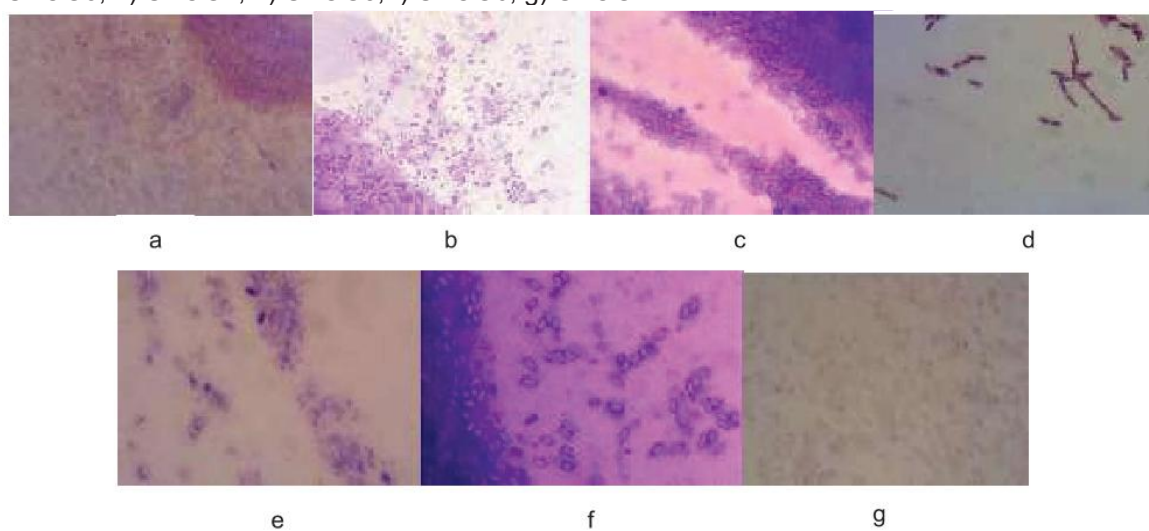


Fig. 5. Cell morphology of *O. rhinoceros* gut bacteria: a) UBOG1, b) UBOG2, c) UBOG3, d) UBOG4, e) UBOG5, f) UBOG6, g) UBOG7

Table 4. Physiological and biochemical characterization of the gut bacteria of *O. rhinoceros* larvae

Isolate	Gram Staining	KOH assay	Endospore staining	Oxidative Fermentative	Grow on YDC	Genus
UBOG1	-	-	ND	Oxidative	-	ND
UBOG2	-	-	ND	Oxidative	ND	<i>Serratia</i>
UBOG3	+	+	+	Oxidative	ND	<i>Bacillus</i>
UBOG4	+	+	+	Oxidative	ND	<i>Bacillus</i>
UBOG5	+	+	+	Oxidative	ND	<i>Bacillus</i>
UBOG6	+	+	+	Oxidative	ND	<i>Bacillus</i>
UBOG7	-	-	ND	Oxidative	-	ND

Remarks: (+) positive reaction, (-) negative reaction; ND = not defined

Physiological and Biochemical Characteristics

Based on the results of the Gram staining and KOH assay, four gut bacteria were Gram-positive and produced endospores, namely UBOG3, UBOG4, UBOG5, and UBOG6. In contrast, the bacterial isolates of UBOG1, UBOG2, and UBOG7 are classified as Gram-negative. The oxidative-fermentative (OF) assay showed that all bacterial isolates were oxidative (Table 4). The growth assay on YDC medium showed that colonies of UBOG1 and UBOG6 were white in color. These results suggest that UBOG2 belongs to the genus *Serratia* because the morphology of the bacterial colonies is red, Gram-negative, and oxidative. Whereas UBOG3, UBOG4, UBOG5, and UBOG6 are thought to belong to the genus *Bacillus* and are Gram-positive, oxidative, and produce endospores. However, the genus of UBOG1 and UBOG7 cannot be determined because they require further biochemical characteristics. Further characterization, both biochemically and molecularly, needs to be carried out to determine the species of the *O. rhinoceros* gut bacteria isolates. From these results, it can be suggested that Gram-positive bacteria from the genus *Bacillus* are more dominantly found in the gut of *O. rhinoceros*. This is in line with reports from several studies, such as Idris *et al.* (2021), who found the Gram-positive bacteria *B. cereus* from *O. rhinoceros* larvae capable of producing chitinase. Another report showed that some of the bacteria found in the gut of *O. rhinoceros* were predominantly from the genus *Bacillus*, which includes *B. stratosphericus*, *B. siamensis*, and *B. cereus* (Marheni *et al.*, 2021).

CONCLUSION

The bacteria that were successfully isolated from the gut of *O. rhinoceros* larvae were seven bacterial isolates, namely UBOG1, UBOG2, UBOG3, UBOG4, UBOG5, UBOG6, and UBOG7, which were differentiated based on morphotype. The seven bacterial isolates showed variations in morphology, physiology, and biochemistry, and the types found in the gut were dominated by Gram-positive. Further analysis showed that the Shannon diversity index was 1.33, evenness was 0.682, richness was 7, and the abundance of each type of bacteria varied between 2×10^5 and 9.1×10^{10} . Bacteria UBOG1, UBOG3, and UBOG5 were able to produce protease enzymes, but all seven bacteria were unable to produce lipase enzymes. Thus, bacterial isolates UBOG1, UBOG3, and UBOG5 have the potential to be used in industry.

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