Journal of Bio-Molecule Research and Engineering
Vol. 2 | No. 2 |32 - 43| 2023
e-ISSN: 2962-343X
http://www.e-journal.unair.ac.id/biome

Hydrolytic Enzyme Producing Bacteria From The Gut Of *Oryctes rhinoceros* Larvae Inhabiting Household Waste Composer

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ARTICLE INFO
Article history
Received 20th Sep 2023
Accepted 27th Nov 2023
Keywords:
Bacillus
gut bacteria
hydrolytic enzymes
Oryctes rhinoceros
solid waste

ABSTRACT

Oryctes rhinoceros which has been known as a pest on coconut trees, has a relatively shorter adult phase than the larval stage. The larval stage of this organism lives in piles of organic matter, including in piles of household waste. The ability of larvae to utilize organic matter in their environment is inseparable from the role of potential microbes that produce enzymes associated with their gut. This study aimed to isolate, screen, and identify the potential bacteria in producing hydrolytic enzymes from gut of *O. rhinoceros* larvae. The third instar larvae were dissected aseptically to obtain gut bacterial isolates. The isolates were tested for their ability to produce amylase, cellulose, lipase, and protease using agar plate containing specific substrates. There were eight different isolates, and three of them had the ability to produce all four enzymes. The three isolates were identified as *Bacillus*. These results can be used as a reference for handling household organic waste.

1. Introduction

Oryctes rhinoceros is an insect which classified to the Ordo Coleoptera, Family Scarabaeidae, subfamily Dynastinae [1]. As an indigenous species, O. rhinoceros is widely dispersed throughout tropical and subtropical southeast Asia, from India and Pakistan to Indochina, and eastward to Ikonawa, the Philippines, and Indonesia [2], [3]. Its main routes of

http://dx.doi.org/ 10.20473/jbiome.v2i2.67564

transmission are caused by human-transported host materials, floating logs carried by ocean currents, and the conveyance of warfare equipment [4].

O. rhinoceros gnaws the apices of coconut trees, which harms the growing points and impeding coconut growth [5]. This species is regarded as a pest in its imago stage, nevertheless, it predominantly exists in the larval stage, which lasts between 80 and 200 days. The first instar lasts 10-21 days, the second instar lasts 12-21 days, and the third instar lasts 60-125 days [6], [7].

O. rhinoceros larvae inhabit and mature in breeding locations where eggs are deposited, such as decaying coconut plant remnants, tree stumps, compost piles, manure heaps, sawdust, and refuse dumps [6], [7]. The larvae of *O. rhinoceros* inhabit accumulations of decomposed organic material and consume the remnants as sustenance [8]. The larvae's decomposition capability necessitates hydrolytic enzymes to degrade organic materials.

Hydrolytic enzymes include cellulases, cellobiases, xylanases, and amylase, which function to convert carbohydrates into sugars, proteases to hydrolyze proteins into amino acids, and lipases to degrade lipids into glycerol and long-chain fatty acids (LCFA) [9]. The hydrolytic enzymes in *O. rhinoceros* larvae were derived from the symbiosis with hydrolytic bacteria in their digestive organs. Bacteria in the digestive organs of the larvae form a symbiotic relationship of mutualism with the larvae, where the bacteria use the digestive organs of the larvae as a place to live, and the larvae take advantage of the presence of bacteria to help digest food [10].

Several studies have revealed the presence of associated bacteria in the gastrointestinal tract of *O. rhinoceros* larvae. Microbial isolates from the digestive tract of *O. rhinoceros* larvae, obtained from rice stalk composting fields, yielded cellulolytic, xylanolytic, and mannanolytic bacteria, which were dominated by the genus *Bacillus* [8]. Furthermore, cellulolytic and lignolytic bacteria from the genera *Proteus* sp., *Bacillus* sp., *Ochrobactrum* sp., *Erwinia* sp., *Aeromonas* sp., *Citrobacter* sp., and *Pseudomonas* sp. were successfully isolated from the digestive tract of *O. rhinoceros* larvae obtained from the PTPN V Sei Plantation Sei Galuh area [11]. This study demonstrated that bacteria exhibited degrading activity on the predominant organic compound components present in the larval habitat, specifically amylase, cellulose, protease and lipase.

Based on our knowledge, various studies on the potential of microbes associated with *O. rhinoceros* larvae have been carried out using larvae that live in piles of organic matter from plants. In contrast, these larvae can also be found in piles of other organic materials, such as household organic waste. In addition, research on microbes associated with *O. rhinoceros* larvae is mostly about testing the potential to produce enzymes that play a role in degrading the components of organic matter that makeup plant cell wall. The potential test of bacteria isolated from the gut larvae in producing other hydrolytic enzymes has not been widely carried out.

2. Materials and methods

2.1 Organism and morphological characterization

The *O. rhinoceros* larvae and the compost were collected in the large glass box from organic waste composter in Kedinding, Surabaya (7˚13'13"S 112˚46'32"E) then transported to the microbiology laboratory, Universitas Airlangga. The compost condition was maintained in the stable condition (temperature: 26-32˚ C, pH: 6.2-6.9, and humidity: 50-80%) for up to seven days before identification and dissection. The 3rd instar larvae were hand-collected and anesthetized using chloroform. Subsequently, they were stored in the glass jar containing 70% ethanol until the whole specimen was submerged. Identification of the larval samples was also carried out to ensure that the larvae were *O. rhinoceros*.

2.2 Dissection and isolation of bacteria

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Specimens with similar characteristics as those characterized were taken and then anesthetized using chloroform and put in the dissecting table. The digestive tract was dissected, then the midgut and hindgut were taken, and the surface was sterilized using ethanol then rinsed using sterile distilled water. Sample was grounded aseptically and dissolved in physiological saline solution. The sample was then serially diluted. One mL of the last three dilutions was grown on nutrient agar (Oxoid) by pour plate. The cultures were incubated aerobically for 24 hours at room temperature. All single colonies were rejuvenated for macroscopic and microscopic characterization.

2.3 Screening for hydrolytic enzyme-producing bacteria

To screen amylolytic, cellulolytic, proteolytic, and lipolytic activity, isolate colonies were grown on specific agar media by adding 2% starch (w/v), 2% carboxymethyl cellulose (CMC) (w/v), 3% skim milk (v/v), and 1% glycerol (v/v) respectively [12]. After 24 hours the clear zone was observed and measured using a caliper. Clear zone was clarified by flooding the agar plate with 2% iodine for amylase, rhodamine for lipase, congo red then destained with 1% NaCl for cellulase, and direct visualization for protease without adding any reagent. The results of the clear zone measurements were then calculated using the formula [13]:

$$hydrolytic\ activity\ index = \frac{bacteria\ colony\ zones\ diameter\ (mm)}{bacteria\ colony\ diameter\ (mm)}$$

3. Results and discussion

3.1 Morphological character of *Oryctes rhinoceros* larvae

Confirmation of the larval status of *O. rhinoceros* was carried out to ensure that the larvae used in this study were *O. rhinoceros* beetles by observing morphological characters. Based on the key of Beaudoin-Ollivier et al. (2000) larvae were identified as *O. rhinoceros* [14]. The larval sample used in this study is shown in Fig 1 (a).

Beaudoin-Ollivier et al. (2000) reported that *O. rhinoceros* move on their side when put on a flat surface and uncurl [14]. Their head capsule is dark-moderate brown, capsule size is 10.6 - 11.4 mm, Thoracic spiracles are 1-85-2-23 mm long, 1-25-1-53 mm wide, body length 44-49 mm and 20-23 mm wide. *O. rhinoceros* have one long seta on the first thoracic segment and 3-8 medium-length setae, no longer than the width of the sclerite, and also a specific ring on the last ventral abdominal segment [15]. Some larvae were reared to adults, and confirmed also being *O. rhinoceros* (Fig. 1 (b).

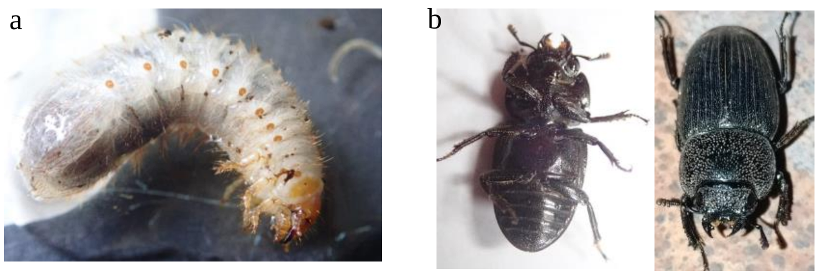


Fig. 1. *Oryctes rhinoceros* (a) Larvae, (b) Adult.

3.2 Isolation of Gut Bacteria

In this study, eight bacterial were isolated from the gut of *O. rhinoceros* larvae. All isolated bacteria had various macroscopic and microscopic characters. The characteristics of the eight microbial isolates are shown in Table 1.

Table 1. Morphological characters of gut bacteria isolated from *Oryctes rhinoceros* larvae

No	Colony Characters							Cell Characters	
	Isolates	**Color**	**Size**	**Form**	**Edge**	**Elevation**	**Consistency**	**Form**	**Gram Staining**
1	EL 1	White	Large	Filamentous	Filiform	Flat	Opaque	Rod	+
2	EL 2	White	Large	Irregular	Lobate	Flat	Opaque	Rod	+
3	EL 3	White	Moderate	Circular	Curled	Raised	Opaque	Rod	+
4	EL 4	White	Moderate	Circular	Entire	Flat	Translucent	Rod	+
5	EL 5	Whitebone	Small	Circular	Entire	Raised	Opaque	Rod	+
6	EL 6	White	Small	Irregular	Lobate	Flat	Transparent	Rod	+
7	EL 7	White	Small	Circular	Entire	Flat	Opaque	Coccus	+
8	EL 8	White	Moderate	Irregular	Lobate	Flat	Translucent	Rod	+

The macroscopic characters of the eight isolates were observed based on color, size, form, edge, elevation, and consistency, and the results obtained were quite varied. According to Volk (1998) and Dini et al. (2018) differences in the growth of each bacterial isolate in Petri dish media to indicate that all isolates came from various types of bacteria, where the morphological characteristics of colony bacteria and pure cultures can be used as a basis for identification of the type of microorganisms [11], [16]. While, the microscopic characters observed were cell shape and Gram staining. According to Rostinawati (2008) Gram staining is used to determine the morphology of bacterial cells and to distinguish Gram-positive and Gram-negative bacteria [17]. Based on the results of microscopic observations, seven isolates had rod shape, namely EL 1, EL 2, EL 3, EL 4, EL 5, EL 6, EL 8, and one isolate was coccus, namely EL 7.

The results of Gram staining of eight isolates showed that all isolates were Gram positive bacteria. This is the same as the research of Sari et al. (2016) who isolated bacteria from the gut of *O. rhinoceros* and found that from 11 isolates there were 10 Gram-positive isolates from the *Bacillus* group and 1 Gram-negative isolate from the *Citrobacter* group [8]. The variation of bacteria in the gut of *O. rhinoceros* is related to the type of food that insects eat from their environment. The composition of bacteria in the gut of insects is also related to the type of feed [18]. Digestion and absorption of food takes place in the digestive tract that divided into three parts: foregut, midgut, and hindgut, the process of digestion of food takes place in the hindgut, in this section there are number of bacteria that secrete cellulase enzymes to break down cellulose [19], [20]. The presence of various bacteria in the gut of *O. rhinoceros* is a reciprocal relationship between bacteria and their hosts. According to Pelczar & Chan (1986), the gastrointestinal bacteria have mutual relations with their host, which is utilizing host as their place of life. The benefit to host is that bacteria feed on waste or use waste materials, many bacteria living in the intestine can synthesize vitamins, secrete enzymes, and aid digestion of nutrients, and the presence of these bacteria tends to suppress the growth of pathogenic bacteria that can protect host against disease and stimulate immune function its host [21].

3.3 The Ability of Bacteria to Produce Hydrolytic Enzymes

All bacterial isolates from gut larvae have been screened for their potential to produce hydrolytic enzymes, such as amylase, cellulase, protease, and lipase. The results of bacterial screening in producing hydrolytic enzymes are shown in Table 2.

Based on Table 2, it can be seen that all isolates that were isolated had the ability to produce at least one of the four hydrolysis enzymes. EL1, EL6, and EL8 isolates had protease and cellulase abilities, while EL7 isolates only had protease abilities. The isolates EL2, EL3, EL4, and EL5 had multienzyme potential that was able to produce all the enzymes tested in this study, there are amylase, cellulase, protease, and lipase. The best amylolytic and cellulolytic activities were obtained by EL3 isolates of 2.392 ± 0.234 and 2.116 ± 0.195, respectively. Meanwhile, the highest proteolytic activity was produced by EL6 of 3,950 ± 0.385.

Table 2. Screening result of amylolytic, cellulolytic, proteolytic, and lipolytic activity from the gut bacteria of *Oryctes rhinoceros* larvae

Code of Isolates	Clear zone diameter			
	Starch medium	CMC medium	Skim milk medium	Olive oil medium
EL1	0.000 ± 0.000	0.000 ± 0.000	1.121 ± 0.081	+++
EL2	1.677 ± 0.089	1.064 ± 0.103	1.964 ± 1.053	++
EL3	2.392 ± 0.234	2.116 ± 0.195	2.405 ± 0.421	+++
EL4	1.906 ± 0.047	1.722 ± 0.156	2.597 ± 0.161	++
EL5	1.783 ± 0.144	1.102 ± 0.049	1.909 ± 0.187	+++
EL6	0.000 ± 0.000	0.000 ± 0.000	3.950 ± 0.385	+
EL7	0.000 ± 0.000	0.000 ± 0.000	3.085 ± 0.093	-
EL8	0.000 ± 0.000	0.000 ± 0.000	2.241 ± 0.076	+++

(-) not detected, (+) fluorescent colony, (++) fluorescent zone, (+++) fluorescent colony and zone

Bacteria in symbiosis with animals in the digestive tract play a role in helping the host's digestive process [22]. In this study, larvae were obtained from household composters which had varied compositions of organic matter. Organic materials contained in household waste composters can contain starch, cellulose, protein, and lipids from garden waste and food waste. The presence of bacteria that have the ability to produce amylase, cellulase, lipase, and protease hydrolysis enzymes in the larvae's gut means that the digestive process of larval food is assisted by bacteria associated in their gut.

Based on these results, it is known that EL3 isolate has the potential as a decomposer agent for agricultural and plant wastes where the main component is lignocellulose. These results are in line with research by Dini et al., (2018) which showed that bacteria isolated from the gut of *O. rhinoceros* larvae had different abilities in degrading lignin. This can be seen from the size of the clear zone formed on the test media and the index value of the lignolytic index. Isolates with code ORL 6 had the highest lignolytic index value of 3.52, followed by ORL 17 with a value of 3.36 and ORL 19 with a value of 2.4 [11]. Based on their multienzyme ability, the three best isolates were isolates EL3, EL4 EL5. Ni'matuzahroh et al. (2021) was also found that endosymbiont bacteria isolated from gut of *Cylindroiulus* sp. had the ability of hydrolytic amylase, cellulase, protease, and lipase multienzymes, namely EKG1, EKG4, and EKG5 [12].

3.4 Identification of Hydrolytic Bacteria

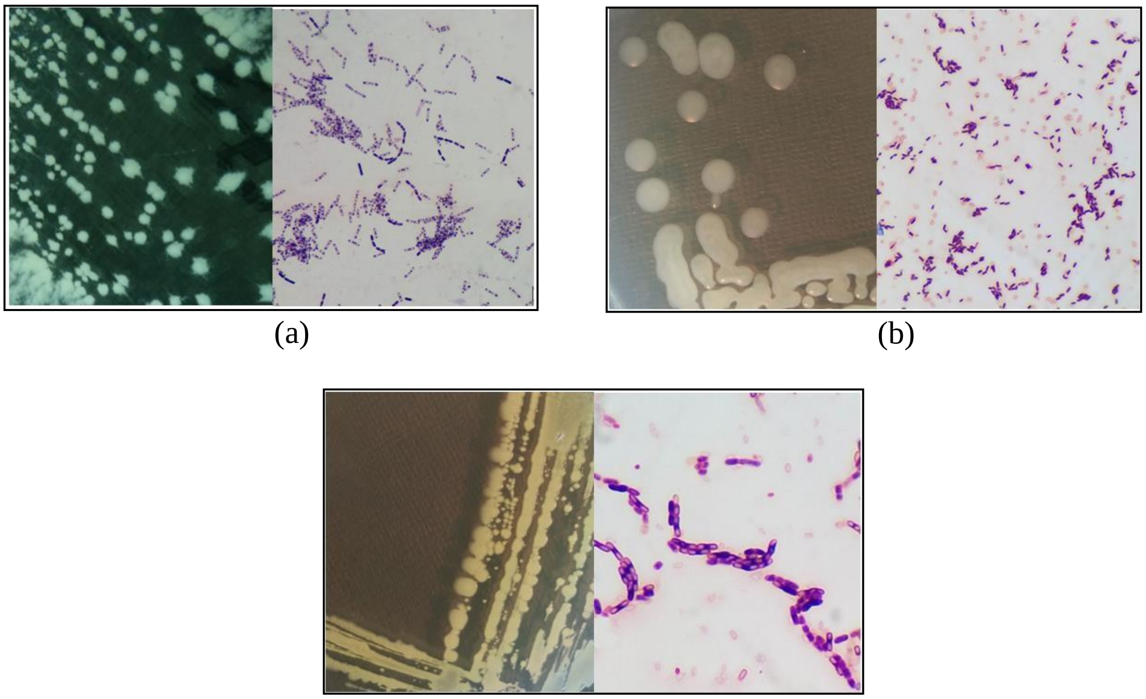
Three isolates with the best multienzyme hydrolysis ability, namely EL3, EL4, and EL5 were identified. The identification was carried out based on macroscopic and microscopic characters (Fig. 2), as well as physiological characters through biochemical tests. Physiological characters of the three isolates are shown in Table 3.

Table 3. Physiological characters of EL 3, EL 4, dan EL 5

Characteristics	Code of Isolates			Characteristics	Code of Isolates		
	EL3	EL4	EL5		EL3	EL4	EL5
Oxidase	+	+	+	TDA	-	-	-
Motility	+	-	-	Gelatin	+	+	+
Nitrate	+	+	-	Malonate	-	-	-
Lysine	-	-	-	Inositol	-	-	-
Ornithine	-	-	-	Sorbitol	-	-	-
H_2S	-	-	-	Rhamnose	-	-	-
Glucose	-	-	-	Sucrose	-	-	-
Mannitol	-	-	-	Lactose	-	-	-
Xylose	-	-	-	Arabinose	-	-	-

ONPG	-	-	+	Adonitol	-	-	-
Indole	-	-	-	Raffinose	-	-	-
Urease	-	-	-	Salicin	-	-	-
VP	-	+	-	Catalase	+	+	+
Citrate	-	-	-	Arginine	-	+	+

The macroscopic, microscopic, and physiological characters of the three isolates were then matched with the reference isolate characters in Bergey's Manual of Determinative Bacteriology to determine the species of the three best isolates.



(a) (b)

(c)

Fig. 2. Characters of colony and cells of EL3 (a), EL4 (b), and EL5 (c).

The identification results showed that the EL3 isolate had similarities with three bacterial species, namely *Bacillus sphaericus, Bacillus badius,* and *Bacillus pantotheticus* with a percentage of 81.48%. The EL4 isolate had similarities to *Bacillus anthracis, Bacillus mycoides, Bacillus pantotheticus,* and *Bacillus sphaericus* with a percentage of 77.78%, while the EL5 isolate had similarities to two bacterial species, namely *Bacillus badius* and *Bacillus pantotheticus* with a percentage of 77.78 %.

The three isolates with the best enzymatic activity were identified as the genus *Bacillus*. The same result was shown by Sari et al. (2016) who succeeded in isolating 10 isolates of cellulolytic and hemicellulolytic bacteria from the gut of *O. rhinoceros* larvae, most of the isolates were identified as the genus *Bacillus* [8]. Dini et al. (2018) in their research succeeded in isolating 24 pure isolates with 9 isolates showing cellulolytic and lignolytic abilities, 2 of the 9 potential isolates belonged to the genus *Bacillus* [11]. Research on microbes from the gastrointestinal tract of *O. rhinoceros* has also been carried out using culturing or culturing-independent methods by Shelomia and Chen (2020) and obtained 12 microbes that can grow on CMC media, some of which are *Bacillus cereus, Bacillus proteolyticus, Lysinibacillus fusiformis* (syn). *Lysinibacillus sphaericus, Bacillus fusiformis, Bacillus sphaericus*) with the dominant bacteria being *Bacillus cereus* [23]. Meanwhile, based on culturing-independent methods, species were identified as *Paracoccus, Citrobacter koseri, Lysinibacillus* with the dominant species being *Paracoccus* and *Citrobacter koseri.*

In addition to the larvae of *O. rhinoceros*, the genus *Bacillus* which has the potential to produce hydrolytic enzymes has also been isolated from the digestive tract of other animals that live in organic waste composters. Previous research showed that the best bacterial isolates were seen from their ability to produce four hydrolytic enzymes, namely amylase, cellulase, protease and lipase isolated from the gut of *Pycnoscelus surinamensis* and *Cylindroiulus sp*. also belongs to the genus *Bacillus* [12], [24]. The genus *Bacillus* is a group of cosmopolitan bacteria that are found in various habitats, including living inthe intestines of other animals and in association with these animals. König (2006) states that *Bacillus* is found in the intestines of various soil invertebrates, such as termites, earthworms, springtails, isopods, and millipedes [25].

4. Conclusions

In the gut of *Oryctes rhinoceros* larvae found eight bacterial isolates producing hydrolytic enzymes (amylase, protease, cellulase, and lipase) with varying ability to produce enzymes. The best three isolates producing multi-enzyme hydrolases were EL 3, EL 4, and EL5, which identified as *Bacillus*.The presence of this bacteria is thought to be in symbiosis with *O. rhinoceros* larvae in digesting organic matter. Isolates that produce hydrolysis enzymes obtained from larvae are bioresources of hydrolytic enzymes and have potency to be used in decomposition of organic waste.

Acknowledgements

This research was funded by Direktorat Riset dan Pengabdian Masyarakat, Deputi Bidang Penguatan Riset dan Pengembangan Kementerian Riset dan Teknologi/ Badan Riset dan Inovasi Nasional for funding this research through the Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) scheme, year 2021, with contract number 472/UN3.15/PT/2021. The authors are also thanks to students of Biology Department, Universitas Airlangga for their assistance and support.

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