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Evaluating the potential of gut bacteria in *Oryctes rhinoceros* larvae as biocontrol agents against *Ganoderma boninense* pathogen in oil palm plantations

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Abstract. *Surbakti RLB, Marheni, Bakti D. 2025. Evaluating the potential of gut bacteria in* Oryctes rhinoceros *larvae as biocontrol agents against* Ganoderma boninense *pathogen in oil palm plantations. Biodiversitas 26: 1414-1423.* Oil palm (*Elaeis guineensis*) is one of the important commodities in Indonesia. One of the plant pest and disease organisms that inhibit the growth of oil palm productivity is the *Ganoderma boninense* pathogen and the rhino pest insect (*Oryctes rhinoceros*). Insects have diverse and abundant microbes in their gut systems. Therefore, a comprehensive understanding of the function of gut bacteria in multispecies interactions may not only lead to the discovery of new resources for biocontrol but could also facilitate the development of new biopesticides. The larval stage of *O. rhinoceros* consumes empty oil palm fruit bunches to grow and develop into pupae. Various types of symbiont bacteria can be found in one type of insect. These bacteria are known to produce several hydrolase enzymes such as amylase, cellulase, protease, lipase, and chitinase. This study aimed to identify bacteria from the intestines of *O. rhinoceros* larvae and determine their potential to inhibit the growth of *G. boninense*, which causes stem rot disease in oil palm plants in vitro. This research was conducted at the Disease Laboratory, Faculty of Agriculture, University of Sumatera Utara, PT. Genetics Science Indonesia, Tangerang, between April - September 2024. The methods used include morphological identification of the isolate and physiological, biochemical, and molecular identification of the bacteria using 16s rRNA gene sequencing and PCR (Polymerase Chain Reaction), as well as antagonist tests and hydrolysis tests for chitinase, protease, and cellulase enzymes. In this study, two bacterial isolates capable of inhibiting the growth of *G. boninense* in vitro were identified; isolate BS1, identified as *Bacillus stercoris*, with an inhibition rate of 42.14%, and isolate BS2, identified as *Pseudomonas aeruginosa*, with an inhibition rate of 63.68%.

Keywords: Biochemistry, *Ganoderma boninense*, molecular, morphology, *O. rhinoceros* larvae, symbiont bacteria

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an imported commodity crop in Indonesia. Oil palm plantations in Indonesia include an area of thousands of hectares and can be found in almost all plantations in various provinces. Among the obstacles that often hinder oil palm productivity is the presence of the fungus *G. boninense*, which belongs to the Plant Pest Organism (PPO) category as a pathogen that causes Stem Base Rot (SBR) (Priwiratama et al. 2020). Pathogenic *Ganoderma* sp. attacks oil palm plants of all ages. Another pest is the *Oryctes rhinoceros* (Linnaeus, 1758) insect, which damages plants by eating empty oil palm fruit bunches (Moore et al. 2017). Evizal et al. (2020) found that in mature oil palm plantations (aged 21-24 years), the population of standing oil palms was only 54% in divisions with a high incidence of Stem Base Rot (SBR). Barcelos et al. (2015) reported a mortality rate of 3.7% per year, or the equivalent of 4 tree deaths per year in a standing population of 110 trees per hectare. The level of damage caused by *O. rhinoceros* to oil palm plants also varies. Studies have shown that attacks by *O. rhinoceros* in oil palm plantations can reduce fruit yields by up to 60% during the first harvest. Additionally, *O. rhinoceros* is responsible for 25% of all deaths of immature oil palm plants, significantly impacting the overall productivity and health of the plantation. In general, insects have diverse and abundant microbial populations in their intestinal digestive tracts. Some gut bacteria play a positive role in pest adaptation to host plants, including providing nutrition, aiding digestion, detoxification, and directing pest behavior. The symbiotic relationship between gut bacteria and insect pests contributes to the success and diversification of insects but also increases the difficulty of pest control. Interestingly, several studies have shown that the function of gut bacteria can change and be detrimental to the host insect when a third species participates in plant-pest interactions or due to other abiotic factors such as predators or parasites, host plants, and pathogens on insect pests. Therefore, a comprehensive understanding of the function of gut bacteria in multispecies interactions may not only reveal new resources for biocontrol but also facilitate the development of new biopesticides (Zhang et al. 2023).

The survival of *O. rhinoceros* larvae on empty oil palm fruit bunches lasted for 102.46 days, while on food with oil palm stems it reached 151.1 days (Marheni et al. 2021). Barbosa et al. (2020) state that these larvae contain cellulolytic bacteria in their hindgut. The process of food digestion occurs in the hindgut, where bacteria secrete cellulase enzymes to break down cellulose. Symbiont bacteria

found in the intestines of *O. rhinoceros* larvae include *Bacillus stratosphericus*, *Bacillus siamensis*, *Bacillus cereus*, *Haemophillus parainfluenzae*, *Achromobacter xylosoxidans*, and *Alcaligenes faecalis* (Marheni et al. 2021). Symbiotic bacteria are known to produce several hydrolase enzymes such as amylase, cellulase, protease, lipase, and chitinase, which are biopolymers that are distributed as constituents of the structural components of insect exoskeletons, as well as being found in fungal cell walls (22-40%) (Tariq et al. 2012). Several bacteria capable of producing this hydrolase enzyme have reportedly been isolated from the digestive tract of various animals (Cornwallis et al. 2023).

The antimicrobial and entomopathogenic potential of various bacterial species including *Bacillus* sp., *Pseudomonas* sp., *Streptomyces* sp., *Lysobacter* sp., and *Seratia* sp. against various plant disease pathogens been studied. *Bacillus* sp., in particular, has been shown to produce metabolites that facilitate survival, competition, specialized colonization, phytopathogenic antagonism, and entomopathogenic effects. Various *Bacillus* sp. have been reported to produce a wide variety of lipopeptides, polyketides, bacterial volatile compounds, and other environmental signaling compounds (Ajuna et al. 2021). Therefore, further investigation is needed to understand the antagonism system of the symbiont bacteria found in the intestines of *O. rhinoceros* larvae and to determine its potential as a biopesticide for the pathogen *G. boninense*, which causes stem rot disease in oil palm plants. Furthermore, current scientific information regarding the use of symbiotic bacteria found in the bodies of insect pests in Indonesia remains limited. Therefore, physiological, morphological, and biochemical identification was conducted along with molecular identification to determine the symbiont bacterial species capable of inhibiting the growth of *G. boninense* in vitro.

MATERIALS AND METHODS

Isolation of symbiotic bacteria from the gut of *O. rhinoceros* larvae

Samples of *O. rhinoceros* larvae were taken from empty oil palm bunches from smallholder plantations in the Perbaungan area, North Sumatra, Indonesia, and taken to the laboratory. *O. rhinoceros* larvae were surface sterilized with 70% alcohol and rinsed twice with sterile distilled water. Larvae were dissected aseptically using a sterile scalpel, and the hindgut was removed. The dilution process was carried out by inserting 1 g of sample into an Erlenmeyer tube containing 9 mL of sterile distilled water (a dilution of 10^{-1} was obtained). The Erlenmeyer tube was shaken using a shaker (speed 150 rpm, 30 minutes). The suspension was then diluted 10^{-1} to 10^{-8} before being homogenized with a vortex mixer and grown on agar nutrient media. The bacterial isolates were then incubated for 24 hours at room temperature (Batubara et al. 2021).

Identification of bacterial

Identification was conducted through morphological observations and biochemical tests. The morphology of bacteria recovered from the intestines of *O. rhinoceros* larvae was examined both macroscopically and microscopically. Macroscopic observation of bacterial isolation included the shape, color, size, edges, and elevation. The single colonies obtained during purification were then separated and grown again on new media and their growth was observed. If the colony is pure, it is indicated by the similarity in shape and color of the bacterial isolate. The bacteria that were isolated had different colony morphology characteristics. Morphological characterization of bacterial colonies was carried out using the book "Microbiology: A Laboratory Manual" (Cappuccino and Welsh 2017). Bacterial cell shape and Gram properties are two examples of microscopic morphological traits. Microscopic identification of bacteria using Gram staining is a way to identify between two broad groups of bacteria, Gram-positive and Gram-negative, as well as examine the structure of bacterial cells under a microscope. In the 3% KOH test, the test is considered positive if the string is visible within the first 30 seconds after mixing in the KOH solution, meaning the bacteria are Gram-negative. On the other hand, if the addition of the KOH solution does not produce mucus or a negative reaction, it means the bacteria are Gram-positive.

Biochemical identification

Biochemical identification of symbiont bacteria aims to characterize the chemical properties of symbiont bacteria in the intestines of *O. rhinoceros* larvae, including through catalase test, carbohydrate fermentation test, starch hydrolysis test, gelatin hydrolysis test, and Simonns' Citrate Agar (SCA) test. Observations of these properties help in classifying bacterial genera, and biochemical identification work begins with Bacterial Isolation. Bacterial samples were taken from the intestines of *O. rhinoceros* larvae, then isolated using nutrient agar media and incubated at 37°C for 24 hours.

Catalase test: Bacteria were tested by adding H_2O_2. Bubble formation indicates a positive result. Carbohydrate Fermentation Test: Bacteria are inoculated into a fermentation medium containing a pH indicator. A change in color indicates fermentation. Starch Hydrolysis Test: Bacteria are grown on a starch agar medium. After incubation, an iodine solution is added to see a clear zone as an indication of hydrolysis. Gelatin Hydrolysis Test: Bacteria were inoculated onto a gelatin medium and incubated. Thawing of the medium indicated a positive result. Simmon's Citrate Agar (SCA) test: Bacteria were grown on an SCA medium. Green to blue color change indicates the ability to utilize citrate.

Antagonism test

A dual culture method on Potato Dextrose Agar (PDA) media, which was prepared according to a Completely Randomized Design (CRD) was used in this research. This study specifically selected two isolates of symbiont bacteria from the intestines of *O. rhinoceros* larvae based on their potential antagonistic properties. In addition, one medium was injected with *G. boninense* without symbiotic bacteria as a control. The *G. boninense* fungus was cut using a sterile cork borer measuring 10 mm. Samples were then placed in a 9 cm Petri dish at a distance of 3 cm from the symbiont bacteria in PDA media. After three days of

incubation, the symbiotic bacteria used in the treatment were stored at room temperature (Figure 1).

The 3 treatments used in this test are as follows: B0: *G. boninense* (Control); BS1: *G. boninense* >< symbiont bacteria 1; BS2: *G. boninense* >< symbiont bacteria 2. These 3 treatments were repeated 6 times to obtain 20 experimental units.

The diameter of the incubated *G. boninense* colonies was measured every day (referred to as Day After Inoculation (DAI)) from the fourth day after bacterial inoculation to the tenth day after inoculation to monitor growth. Observations were made by measuring the diameter of the colony on two axes, namely the horizontal axis (d1) and the vertical axis (d2), in a Petri dish. Average growth was calculated based on measurements of both axes to produce average daily growth values for *G. boninense* according to the scheme in Figure 2. Average value of fungal growth:

$$D = \frac{d1 + d2}{2}$$

Where:
D : Diameter of *G. boninense*
D1 : Horizontal diameter of *G. boninense* colonies
D2 : Vertical diameter of *G. boninense* colonies

Calculation of the percentage of inhibition against *G. boninense* is carried out using the formula:

$$Percentage\ of\ resistance = \frac{R1 - R2}{R1}\ 100\%$$

Where:
P : Percentage of resistance
R1 : Diameter B0 (*G. boninense*) control
R2 : Treatment diameter

Živković et al. (2010) quantify the ability of bacteria to inhibit plant pathogenic fungi by the percentage of inhibitory power in the categories 0-4 (0 = no inhibition, 1 = 1-25% (low), 2 = 26-50% (medium/average), 3 = 51-75% (high), and 4 = 76-100% (very high). Once the percentage of inhibitory power is known, macroscopic and microscopic observations of the bacterial inhibitory power against *G. boninense* are needed.

Macroscopic and microscopic observations of hyphal damage to *G. boninense* by bacteria

Macroscopic observations were carried out visually by displaying the isolate on a Petri dish, while microscopic observations were carried out by observing the hyphae of *G. boninense* in the control colonies, which appeared to be normal, and in the treatment of symbiont bacteria, which had suffered damage such as bent hyphae tips, broken hyphae, split hyphae, branched hyphae, lysed hyphae and stunted hyphae.

Molecular identification of DNA 16s rRNA

Molecular identification utilizing 16S rRNA gene analysis is an important method in characterizing and identifying bacteria at the species level. These processes entail isolating bacterial DNA, amplification of the 16S rRNA gene using PCR methods, and sequencing analysis. Identification was carried out by sending samples of symbiont bacterial isolates to the Testing Laboratory, PT Genetics Science Indonesia, Tangerang City, Banten, Indonesia, for sequencing services. Analysis of the obtained sequences was then matched with data from the GenBank database to reliably identify bacterial species.

Construction of a phylogenetic tree

The DNA sequence results of the *O. rhinoceros* larval symbiotic bacterium were utilized to provide data that can be compared to other species with Genbank accession numbers. Similar sequencing data acquired from Genbank was then aligned with the CLUSTAW program. Phylogenetic trees were generated using MEGA XI software and the Neighbor-Joining Method.

Chitinase activity

Following Gonfa et al. (2023), chitinase activity was measured via the paper disk diffusion method, using two bacterial isolates grown and incubated in chitin agar media with the composition (0.5 g colloidal chitin, 0.1 g K_2HPO_4, 0.01 g $MgSO_4.7H_2O$, 0.05 g yeast extract, 0.1 g peptone, 0.1 g bactotryptone, 0.5 g NaCl, 0.1 g $(NH_4)2SO_4$, 1 g agar, 100 mL distilled water). Next, 2 loops of symbiont bacterial isolates were then diluted in 100 µL of distilled water and vortexed before being poured into a new Petri dish. Meanwhile, the required amount of paper discs with circular holes with a diameter of 0.6 mm were printed.

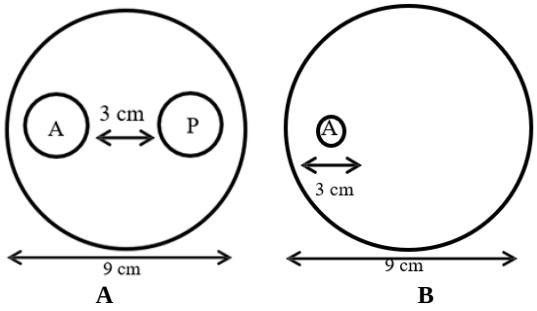

Figure 1. A. Scheme for placing the inoculum for the dual culture test of *G. boninense* and symbiotic bacteria; B. *G. boninense* inoculum placement scheme (Control). Note: A: *G. boninense*; P: Bacteria

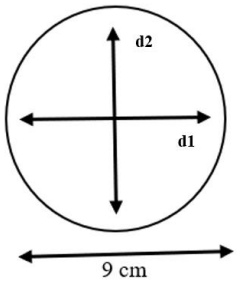

Figure 2. Diameter measurements of *G. boninense*. d1: Horizontal diameter of the Petri dish; d2: Vertical diameter of the Petri dish

These were then sterilized (autoclaved) and soaked in two isolate bacterial solutions that have been provided in different containers. After the formation of a clear zone, 0.1% Congo Red was added for 15 minutes before being then rinsed away using distilled water. The formation of a clear zone indicates the chitinolytic ability of the symbiont bacterial isolate. The clear zone formed is measured using a caliper. The formation of a lysis zone around the colony indicates chitinase enzyme activity. The larger the lysis zone formed, the greater the chitinase enzyme activity of the test bacteria. The Chitinolytic Index (CI) is considered high if the isolate has a CI value ≥2, while the CI is considered low if the value is <2. The Chitinolytic Index (CI) is calculated using the following formula:

$$CI = \frac{diameter\ of\ the\ clear\ zone\ formed}{bacterial\ colony\ diameter}$$

Where CI is Chitinolytic Index.

Protease activity

Preparation of Skim Milk Agar (SMA) media, as much as 2 g and added 100 mL distilled water. The media was sterilized using an autoclave at 121°C for ±15 minutes; after a while, the media was poured into petri dishes. Qualitative tests are used to determine bacterial isolates that have protease enzyme activity. Bacterial colonies were taken using a sterile ose needle, then planted on SMA media aseptically by bottling. Then incubated at room temperature for 7×24 hours. Protease enzyme activity can be seen by the clear zone formed on skim milk agar media. Furthermore, bacterial colonies were observed, and the growth and clear zone were measured using a bar. A qualitative protease enzyme activity test was carried out by calculating the Proteolytic Index on Skim Milk Agar media (Hengkengbala et al. 2021).

Calculation of the Proteolytic Index (PI) is the ratio of the diameter of the clear zone to the diameter of the bacterial colony using the following formula:

$$PI = \frac{Clear\ zone\ diameter}{Colony\ diameter}$$

Where PI is Proteolytic Index.

Cellulose activity

The rough cellulase activity test in this study followed the procedure of Syukri et al. (2024). 0.5% CMC media was prepared and then poured into a petri dish; the petri dish was divided into 3 equal parts. Purified cellulolytic bacterial isolates were taken and photographed on each part of the dish using a toothpick and then incubated. Clear zones were observed on CMC media by pouring 0.1% congo red solution, allowed to stand for 15 minutes, rinsed with 0.3 M NaCl, and allowed to stand again for 15 minutes; then, the liquid was discarded.

The diameter of the bacterial colony and the diameter of the clear zone formed were measured using a caliper. The diameter of the clear zone and the diameter of the bacterial colonies were observed and measured on day seven after incubation. After the observation data is obtained, the bacterial cellulolytic index is calculated using the following equation:

$$CI: \frac{clear\ zone\ diameter - bacterial\ colony\ diameter}{bacterial\ colony\ diameter}$$

Where CI is Cellulotic Index.

RESULTS AND DISCUSSION

Identification of bacterial symbionts of *O. rhinoceros* larvae

In Figure 3, macroscopically, BS1 bacteria have a round colony with rhizoid edges (pseudo-root structures like fine threads) and convex elevations, which is white, while BS2, which has an irregular colony shape, is cloudy white. Table 1 shows that the two bacteria share one common characteristic, namely convex edges.

In Table 2, the results of the Gram staining and 3% KOH test have consistent results, whereby BS1 was Gram-positive and BS2 was Gram negative (Figure 4). The difference in color is because the components that make up the cell walls of Gram-positive bacteria and Gram-negative bacteria are different. Gram-positive bacteria can retain the main paint containing crystal violet because their cell walls contain thick peptidoglycan. Gram-negative bacteria cannot maintain the main paint color because their cell walls contain a lipoprotein layer, which will dissolve when washed with ethanol due to the thin peptidoglycan layer (Wulandari and Purwaningsih 2019). This principle of peptidoglycan is a reference for 3% KOH solution in penetrating bacterial cell walls. The results of the same test show that BS1 bacteria have a negative reaction with KOH, indicating that BS1 bacteria are Gram-positive. In contrast, BS2 bacteria have a positive reaction with KOH, which indicates that BS2 bacteria are Gram-negative. In Figure 4, it can be seen the bacterial cells of both BS1 and BS2 are rod-shaped.

Biochemical test

Classification of bacterial isolates into genera was conducted based on the characteristics of colony morphology, cell morphology, and biochemical test results, with reference to Bergey's Manual of Determinative Bacteriology. The BS1 bacterial isolate belongs to the *Bacillus* genus of the Bacillaceae family, as shown in the identification results. Meanwhile, the results of biochemical tests on BS2 bacterial isolates show that these bacteria are similar to groups of bacteria from the *Pseudomonas* genus of the Pseudomonadaceae family.

Table 1. Macroscopic characteristics of potential symbiont bacteria

Isolate code	Characteristics of bacterial colonies			
	Shape	Colony Color	Margin	Elevation
BS1	Circular	White	Rhizoid	Convex
BS2	Irregular	White	Entire	Convex

Notes: BS1: Symbiont isolates 1; and BS2: Symbiont isolates 2

Table 2. Microscopic (physiological) symbiont bacteria

Isolate code	Coloration (Gram)	KOH 3% (Gram)	Cell shape
BS 1	+	+	Rod-shaped
BS 2	-	-	Rod-shaped

Table 3. Biochemical test results for symbiont bacteria

Isolate code	Biochemical test				
	Catalase	Simmons Citrate Agar	Triple Sugar Iron Agar	Gelatin	Starch Hydrolysis
BS 1	+	+	K/A	+	+
BS 2	+	+	K/K	+	-

Notes: BS1: Symbiont isolates 1; and BS2: Symbiont isolates 2

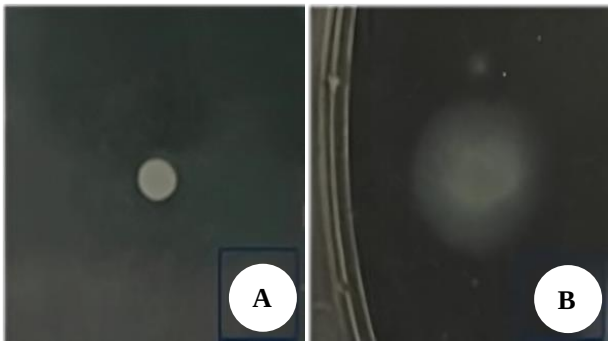

Figure 3. A single colony of symbiont bacteria isolated from the gut of *O. rhinoceros* larvae; A. Single colony of symbiont isolates 1 (BS1); B. Single colony of symbiont isolates 2 (BS2)

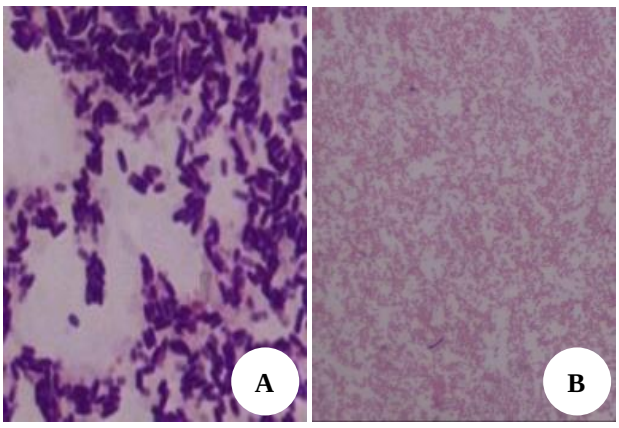

Figure 4. Visualization of Gram staining of intestinal symbiont bacteria of *O. rhinoceros* larvae at 100× magnification. A. Symbiont isolate 1 (BS1); B. Symbiont isolate 2 (BS2)

The catalase test findings (Table 3) demonstrated the positive results that the BS1 and BS2 bacterial isolates produced air bubbles. This is consistent with previous research indicating that air bubbles are a sign of a positive reaction in bacterial isolates containing catalase (Khatoon et al. 2022). This shows that these bacteria produce the enzyme catalase, which allows them to break down the H_2O_2 produced during respiration into water and oxygen, which is visible from the appearance of bubbles.

Table 3 shows that isolates of the symbiont bacteria BS1 and BS2 showed positive results (+), indicated by a change in the color of the media from green to blue, which indicates the ability of these bacteria to use citrate as a carbon and energy source. In Gebreyohans et al. (2024), the citrate utilization test was carried out to determine the ability of bacterial isolates to utilize citrate as the only carbon source. Growth in the medium was accompanied by an increase in pH and a change in the medium from its initial green color to dark blue.

Observation of the carbohydrate fermentation test, test results in Table 3 demonstrated that the BS1 and BS2 bacteria at the top (slant) and bottom do not change color, so the code is K/K (alkaline/alkaline), but BS2 produces H_2S gas. In Gebreyohans et al. (2024), the TSIA test is designed to distinguish several types of bacteria that are able to break down carbohydrates into acidic glucose so that they can be differentiated from other bacteria that are non-fermenters.

In the gelatin test in Table 3, two isolates of the intestinal symbiont bacteria of *O. rhinoceros* larvae, namely BS1 and BS2, were positive, with the gelatin medium remaining liquid after being placed in the refrigerator for 30 minutes. As highlighted by Mortezaei et al. (2021), the gelatin testing process is instrumental in understanding the enzymatic mechanism whereby the gelatinase enzyme, produced by bacteria, plays a crucial role in hydrolyzing gelatin (collagen protein) into amino acids. This enzyme's role in microbial identification is significant, as it aids in categorizing the microbial protein degradation capabilities.

Based on Table 3, the starch hydrolysis test on the two bacterial isolates using SA (Starch Agar) media showed positive results for BS1, as indicated by the formation of a clear zone after adding iodine solution, and negative results for BS2. This indicates that BS1 bacteria are able to hydrolyze starch by producing the amylase enzyme. According to Miłek and Lamkiewicz (2022), bacteria that produce extracellular amylase enzymes are capable of hydrolyzing starch, including amylose and amylopectin, in Starch Agar media.

Molecular identification of bacterial symbionts

Molecular identification was conducted on two isolates of the gut symbiont bacteria of *O. rhinoceros* larvae, which are known to inhibit the growth of *G. boninense* in vitro. Table 4 presents the PCR amplification results of two bacterial isolates consisting of the bacterial primer code and amplicon size (base pairs).

Figure 5 presents the results of amplification of the 16S-rRNA gene on gel electrophoresis. The primary code from BS1, namely G-3341-1, has a base pair length of 1400 bp, while the primary code from BS2, namely G-3434-1, has a base pair length of 1399 bp.

The results of sequencing analysis in Table 4 using the BLAST (Basic Local Alignment Search Tools) feature on NCBI show that the 16S rRNA gene sequence in isolate BS1 has a similarity value of 100% with *Bacillus stercoris* strain M-5 in access code PP972774.1., max score 2586

and e-value 0.0. Furthermore, a similarity value of 100% with *Bacillus stercoris* strain V6312 in access code PP962875.1 has a max score of 2586 and an E-value of 0.0. Meanwhile, the bacterial isolate BS2 has a similarity value of 99.86% with *Pseudomonas aeruginosa* strain SE5430 in access code CP054791.1, having a max score of 2573 and an e-value of 0.0. Furthermore, the similarity value is 99.86% with *Pseudomonas aeruginosa* strain F059 in access code CP115220.1 with a max score of 2573 and an E-value of 0.0.

The results of the phylogenetic tree analysis in Figure 6 show that the DNA alignment of isolates BS1 and BS2 forms different branch groups. The BS2 isolate is in the same node as *Pseudomonas aeruginosa* strain SE5430, where both are closely related to *Pseudomonas aeruginosa* strain F059. Meanwhile, the BS1 bacterial isolate was in the same node as *Bacillus stercoris* strain M-5, both of which are closely related to *Bacillus stercoris* strain V6312. The same branching point indicates that the two samples are closely related or have the same evolutionary line.

On phylogenetic and genetic sequence analysis (16S rRNA gene), *Streptomyces* and *Bacillus* have a closer common ancestor (because both are Gram-positive bacteria) than *Pseudomonas* (Gram-negative bacteria). *Streptomyces* also exhibits more primitive traits and structure. This suggests that using Streptomyces as an outgroup will give useful differentiation when evaluating phylogenetic connections and can aid in understanding the traits of the common ancestor of *Bacillus* and *Pseudomonas*.

Macroscopic observation of *G boninense* isolates

Macroscopic observation of *G. boninense* isolates on PDA media (Figure 7) shows the appearance of white fungal mycelium on the top and bottom, without exudate, the surface is quite bumpy, has concentric circles, the mycelium is dense and looks smooth. In line with Hamzah et al. (2021), the *Ganoderma* isolates with the code Gan1 have a white mycelium surface, a wavy surface, and concentric circles.

Antagonism test

Antagonism test using the dual culture method on PDA media where the two bacterial isolates were able to inhibit the growth of *G. boninense* fungal hyphae with isolate codes BS1 (*B. stercoris*) and BS2 (*P. aeruginosa*).

Table 5 displays data on the increase in diameter of the *G. boninense* fungus from day four (DAI) to ten. This data, obtained from the control sample that was grown in a petri dish without bacterial inhibition, was instrumental in evaluating the inhibitory effect of the other two symbiont bacteria isolates. The treatment with *B. stercoris* bacteria exhibited a level of inhibition, with the fungal isolate measuring 52.06 mm on day 10 of DAI. The *P. aeruginosa* treatment, in contrast, recorded the least growth at 32.68 mm, indicating its strong antagonistic properties that were sustained throughout the observation period.

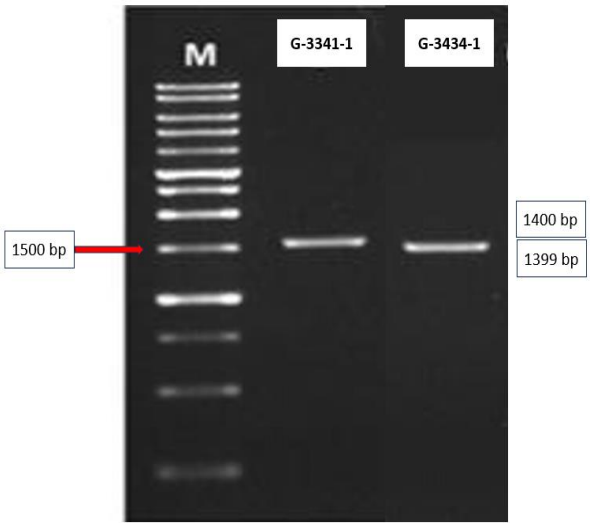

Figure 5. Visualization of electrophoresis results of PCR amplification products of Symbiont Bacterial DNA samples; M: Marker 1 kb ladder DNA, G-3341-1 (BS1), G-3434-1 (BS2)

Table 4. BLAST results of bacteria Percentile

Species	Sequencing results	Max score	Query cover	Percentile	Accession number
BS1	*Bacillus stercoris* strain M-5	2586	100%	100%	PP972774.1
	Bacillus stercoris strain V6312	2586	100%	100%	PP962875.1
BS2	*Pseudomonas aeruginosa* strain SE5430	2573	100%	99.86%	CP054791.1
	Pseudomonas aeruginosa strain F059	2573	100%	99.86%	CP115220.1

Table 5. Diameter growth of *G. boninense* culture in PDA

Isolate code	Days After Inoculation (DAI)						
	4	5	6	7	8	9	10
Control	35.36^{c}	44.90^{c}	59.40^{c}	71.50^{a}	79.02^{a}	87.15^{a}	90.00^{a}
B. stercoris	32.33^{b}	39.94^{b}	45.30^{b}	48.23^{b}	51.48^{b}	52.41^{b}	52.06^{b}
P. aeruginosa	23.10^{a}	25.43^{a}	34.36^{a}	37.79^{c}	38.38^{c}	38.96^{c}	32.68^{c}

Table 6. Percentage of inhibitory power

Isolate code	Days After Inoculation (DAI)						
	4	**5**	**6**	**7**	**8**	**9**	**10**
Control	-	-	-	-	-	-	-
B. stercoris	8.63[b]	12.34[b]	23.56[b]	32.24[b]	34.84[e]	39.82[b]	42.14[b]
P. aeruginosa	34.67[a]	43.32[a]	41.97[a]	46.87[a]	51.38[a]	56.26[a]	63.68[a]

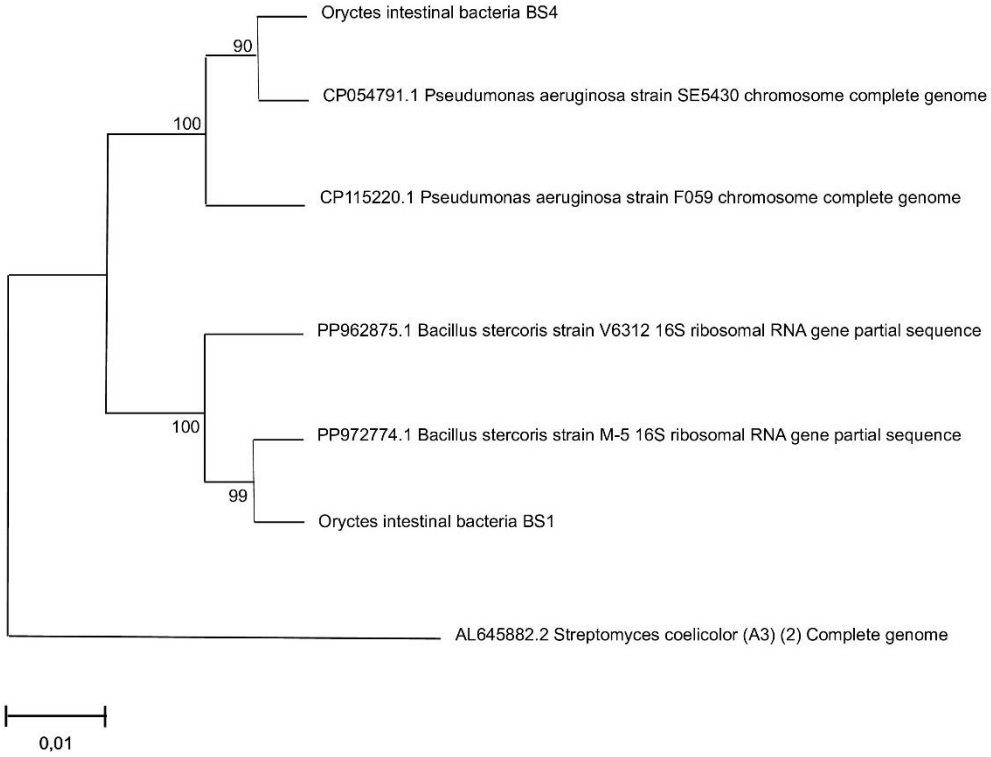

Figure 6. Phylogenetics of symbiont bacterial isolates from the intestines of *O. rhinoceros* larvae based on the *Neighbor-Joining* 1000 bootstrap method

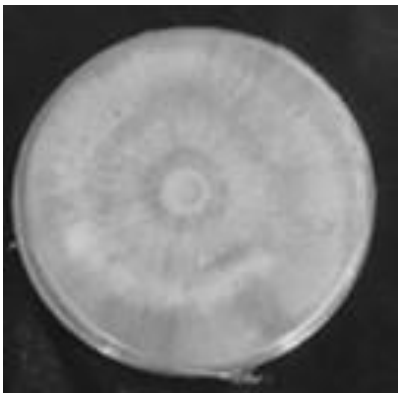

Figure 7. Characteristics of *G. boninense* isolates on PDA media

The inhibition test against *G. boninense* was performed using a PDA media with two isolates of bacteria obtained from the intestines of *O. rhinoceros* larvae. The proportion of inhibition by bacteria against *G. boninense* on day ten after inoculation is presented in Table 6, where *P. aeruginosa* showed the highest inhibitory activity with a percentage of 63.68%, followed by *B. stercoris* with a percentage of 42.14%. The higher the percentage of inhibition, the greater the ability of the symbiont bacteria to inhibit the growth of *G. boninense*. Trung et al. (2020) show that *P. aeruginosa* bacterial isolates are capable of producing highly effective antifungal compounds with the potential to act as biofungicides. In addition, Kurniawan et al. (2018) found that the bacterial genus *Bacillus* sp. and *Pseudomonas* sp. are able to inhibit the growth of fungal mycelium that attacks blueberries. Macroscopic observation of *G. boninense* isolates on PDA media (Figure 7) shows the appearance of fungal mycelium that is inhibited in growth caused by symbiont bacteria.

BS1 and BS2 bacterial isolates exhibited antagonistic activity, as evidenced by their ability to generate compounds that impede the growth of *G. boninense*. The antibiotic compounds produced by these bacteria also play a role in causing hyphal growth abnormalities. This finding is supported by Elfina et al. (2024), who state that antibiotic compounds can cause hyphae damage, such as shrinkage, lysis, or malformation, through the degradation of fungal cell walls, thereby disrupting their growth.

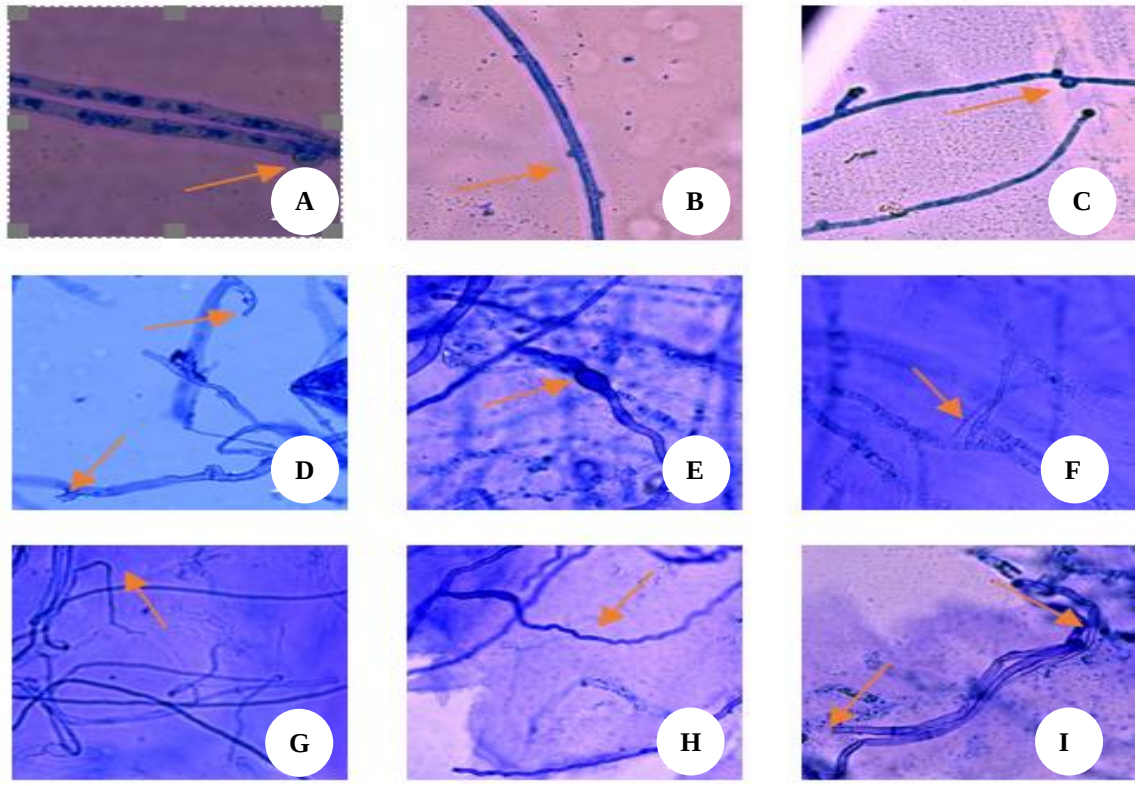

Figure 8. *G. boninense* fungus microscope on treatments; A, B, C: Control; D, E, F. *B. stercoris*; and G, H, I: *P. aeruginosa*. A, B, C. Normal hyphae; D. Shortened; E. Swollen hyphae; F. Lysis; G. Bent; H. Shriveled; I. Dead and lysed hyphae. 100× microscope magnification

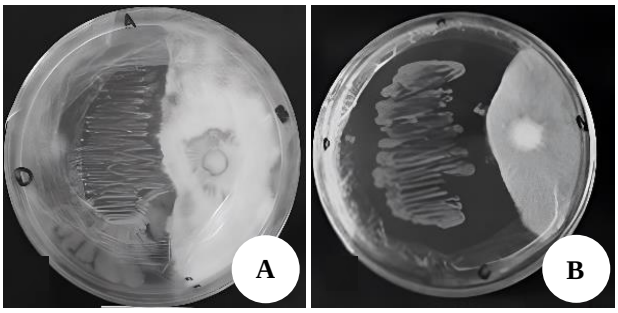

Figure 7. Bacterial antagonist test on 10 HIS: A. *B. stercoris*; and B. *P. aeruginosa*

In Figure 8, the macroscopic and microscopic characteristics of *G. boninense* after application show that the hyphae of control treatment *G. boninense* (A, B, and C) look normal at the microscopic level. Meanwhile, the treated isolates of *B. stercoris* bacteria (D, E, and F) showed the growth of *G. boninense* hyphae, which were shortened, swollen, and lysed, respectively. Meanwhile, in the treated isolates of *P. aeruginosa* bacteria (G, H, and I), the hyphae were bent, shriveled, and lysed, respectively. This phenomenon occurs due to the presence of compounds that inhibit fungal growth. The ability of bacteria to produce secondary metabolites can inhibit the synthesis of pathogen cell walls, resulting in hyphae shortening. In addition, it is known that the ability of *Bacillus* sp. to produce hydrolytic enzymes such as chitinase, protease, and cellulase also plays an important role in influencing the development of fungal hyphae (Elfina et al. 2024). The synergistic effect of these three enzymes not only impairs the fungal cell wall structure but also has the potential to cause hyphal swelling and deformation, as demonstrated in the treated bacterial isolates. Thus, the ability of bacteria to produce these enzymes can have a substantial impact on suppressing *G. boninense* and increasing the bacteria's potential as biocontrol agents in agriculture.

The potential of bacteria *O. rhinoceros* in inhibiting the growth of *G. boninense*

Some microorganisms can produce enzymes that function to inhibit the growth of pathogens. The enzymes produced by these microorganisms include chitinase enzymes, cellulase enzymes, and protease enzymes (Wang et al. 2022). Based on the hydrolysis activity of bacterial chitinase in Table 7, the data displayed are colony diameter, clear zone diameter, and chitinolytic index. The difference in ability to utilize these nutrients is related to the incubation period required to break down the chitin compounds from each different isolate. The formation of the clear zone began 48 hours after inoculation, then on day 7, or at 168 hours, the clear zone was measured. This shows that the isolate requires a period to hydrolyze chitin. The *P. aeruginosa* bacterial isolate had a higher chitinolytic index value of 2.99, while the *B. stercoris* bacterial isolate displayed an index value of 2.73.

Table 7. Chitinase activity

Isolate code	Colony diameter (mm)	Clear zone diameter (mm)	Chitinolytic Index (CI)	Description
B. stercoris	16.80	45.90	2.73	High
P. aeruginosa	14.50	43.30	2.99	High

Table 8. Proteolytic activity

Isolate code	Clear zone diameter (mm)	Colony diameter (mm)	Proteolytic Index (PI)	Description
B. stercoris	16.4	40.4	2.46	Medium
P. aeruginosa	28.4	81.3	2.86	Medium

Table 9. Cellulase activity

Isolate code	Clear zone diameter (mm)	Colony diameter (mm)	Cellulosic Index (CI)	Description
B. stercoris	87.40	37.10	1.36	Medium
P. aeruginosa	89.00	30.80	1.89	Medium

Table 8 shows the proteolytic activity of bacteria with the formation of a clear zone around bacterial colonies growing on Skim Milk Agar (SMA) media. The findings of the proteolytic activity test on the bacterial isolates after 7x24 incubation at room temperature revealed that both bacterial isolates were proteolytic. The presence of a clear zone around the colony indicates positive results. Protease-producing bacteria have the ability to break down proteins, including those found in the cell walls of pathogenic fungi such as *Ganoderma* so that they can inhibit the growth of mycelium. Antagonism tests were carried out to evaluate the impact of protease-producing bacteria on the growth of *Ganoderma*, both through in vitro methods, which can provide a further understanding of its potential in biological control. Overall, protease-producing bacteria showed a significant relationship with the antagonism test against fungi (Lee et al. 2023).

The ability of bacteria to hydrolyze cellulose is expressed in the form of the Cellulosic Activity Index (IAS). The IAS value calculation is based on the diameter of the clear zone formed and the diameter of the bacterial isolate colony. In Table 9, both the *B. stercoris* and *P. aeruginosa* isolates fall in the medium category with cellulosic indices of 1.36 and 1.89, respectively, which means that the isolates show cellulase activity.

The use of cellulase-producing microbes can act as direct competitors for plant pathogens. Cellulolytic microbes can compete directly with pathogens such as *G. boninense* in terms of access to cellulose nutrients. This competition limits the use of cellulose by *G. boninense* in the roots and base of oil palm stems, thereby preventing infection and spread of the pathogen. Other research (Lee et al. 2023) even shows that the properties of cellulase from bacteria can become antagonistic as it is able to degrade cell membranes and fungal cell walls.

In conclusion, our research has revealed promising results. The antagonism test of symbiont bacteria to *G. boninense* showed that *P. aeruginosa*, bacterial isolate BS2, exhibited the highest inhibitory power at 63.68%. This underscores the potential of *P. aeruginosa* as a potent antagonistic agent. The positive results of the hydrolysis activity test of chitinase enzymes, protease enzymes, and cellulose enzymes are positive, with the highest different index results in *P. aeruginosa* bacteria, further supporting this potential. Looking ahead, we are excited about the prospect of utilizing *P. aeruginosa* and other bacteria as antagonistic agents in plant diseases, with plans for future nursery field trials.

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