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Bioprospecting of Chitinolytic Diazotroph Rhizospheric Bacterial Isolated From *Mucuna bracteata* as Biocontrol Against *Ganoderma boninense*

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Abstract. *Ganoderma boninense* is an infectious phytopathogenic fungus of basal stem rot in oil palm with the need of significant biocontrol strategy or alternative. Bioprospecting of chitinolytic diazotroph bacteria from rhizosphere of Mucuna bracteata as Legume Crop Cover (LCC) in oil palm plantations is potential to investigated. The aims of this study were to obtain total population of diazotroph bacteria and chitinolytic diazotroph bacteria, to evaluate its antagonistic properties followed by identification of the isolate based on 16S rRNA gene encoding. Diazotroph bacteria were isolated from M. bracteata rhizosphere originating from three oil palm plantations (PTPN III, PTPN IV unit Adolina Medan, and Perkebunan rakyat Simalingkar), followed with chitinolytic assay, morphological characterization, antagonistic assay against G. boninense, lytic assay of *G. boninense* mycelium by using crude chitinase and molecular identification of potential isolate. Total population of diazotroph bacteria in M. bracteata rhizosphere ranged between 2.80–3.83 × 106 CFU/g. Screening of chitinolytic diazotrophs using colloidal chitin medium obtained 23 bacterial isolates and 14 of them were known as antagonists to *G. boninense*. Five isolates with the highest anti Ganoderma were DK17 (71,15%), DK10 (69,70%), DK07 (59,63%), DK21 (53,48%), and DK19 (52,0%). The highe1st lytic activity of crude chitinase to *G. boninense* mycelium was produced by DK17. Molecular identification of five potential isolates revealed different identities, e.g. *Enterobacter aerogenes* (DK07), *Mycobacterium senegalense* (DK10), *Bulkhoderia cepacia* (DK17), *Pseudomonas stutzeri* (DK19), and *Bulkhoderia cepacia* (DK21). Based on these results, chitinolytic diazotroph bacteria isolated from *M. bracteata* rhizosphere were able to control *G. boninense.*

Keyword: Chitinolytic diazotroph bacteria, Ganoderma boninense, Mucuna bracteata, and Biocontrol, Rhizosphere.

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1 Introduction

Palm oil is a plantation commodity that is well-known as a foreign exchange earner in Indonesia, which is around 300 trillion per year. Oil palm plantations are spread across various provinces in Indonesia. One of the provinces that has a fairly large oil palm plantation is North Sumatra. In 2015, oil palm plantations in North Sumatra covered an area of 1,445,725 ha with an oil production of 5.5 million tons [1]. Oil palm plantations in North Sumatra are old plantations that have existed since the Dutch era. The problem that is often faced by old plantations that have

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gone through more than three generations is the high attack of pathogenic fungi including *Ganodema boninense.* This *G.boninense* attack resulted in the death of many oil palm plants and resulted in a decrease in the productivity of oil palm, which indirectly resulted in a decrease in state income. The fungus *G. boninense* is a pathogenic fungus that causes stem rot in oil palm plantations (*Elaeis guineensis* Jacq.) and live in oil palm plant tissue latently without showing obvious symptoms. The plant will look sick if the plant has been heavily infected. Control efforts that have been carried out physically, chemically and biologically have not yielded maximum results. Biological control using bacterial or fungal biological control agents is still considered a control method that still gives hope because it is more environmentally friendly. Biological control agents that have been tested for their antagonistic ability against *G. boninense* include endophytic fungi [2], *Trichoderma* sp [3], Actinomycetes and other agents. The use of biological agents in the control of Ganoderma spp. generally by utilizing the antifungal ability and the production of lytic enzymes produced by biological agents [4]. The lytic enzyme that has received much attention from researchers is the chitinolytic enzyme. Chitinolytic enzymes are enzymes that can hydrolyze chitin by breaking the glycosidic bond of the chitin polymer. Chitin is a polymer of N-acetyl glucosamine which is also known as the main component of fungal cell walls. Several types of chitinolytic bacteria have been isolated and tested for their antagonistic ability against pathogenic fungi, namely Bacillus cereus [5;6] Enterobacter [7],Pseudomonas [8], but the use of several Biological agents that have been used so far have not been able to completely control *G. boninense* infection. Microorganisms that are expected as biological agents controlling *G. boninense* as well as assisting the availability of nutrients in plants that can be prospected are chitinolytic diazotrophic bacteria. This bioprospecting bacteria has two advantages, namely having properties as a nitrogen fixator thereby increasing soil fertility (almost all nitrogen-fixing bacteria are safe for plants), and having antifungal properties that can inhibit the growth of the fungus *G. boninense*. The use of bacteria that have both nitrogen-fixing properties and are able to act as biological controllers will provide high benefits, so that plants can be protected and soil conditions are more fertile. Chitinolytic bacteria can be isolated from various sources such as humus soil, rhizosphere and endophytes. In this study, the sources of chitinolytic bacteria isolates were from the rhizosphere and endophytic soil of *M. bracteata*. Mucuna is an intercrop that is widely planted in oil palm plantations as a legume cover crop. All groups of legumes have associations with diazotrophic bacteria, both symbiotic and non-symbiotic. Isolation of diazotrophic bacteria with chitinolytic ability from Mucuna plants and their antagonistic ability against *G. boninense* have not been carried out. The presence of chitinolytic diazotroph bacteria as a biological controller of *G. boninense* from the Mucuna plant is very promising for its potential use as a biological control agent. Mucuna plants have fast growth, and chitinolytic diazotrophic microorganisms found in the rhizosphere and endophytic areas of this plant can be used to control

G. boninense inoculum in oil palm plantations. The ability of bacteria to fix nitrogen can help provide nutrients to plants.

2. Research Methods

2.1. Time and Place of Research

This research was conducted for 8 months at the Microbiology Laboratory, Genetics Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of North Sumatra, Macrogen Laboratory, South Korea.

2.2. Materials and tools

The materials used in this study were Potato Dextrose Agar (PDA), Ashy Mannitol Agar Media, Nutrient Agar (NA), Nutrient Broth (NB), primer, agarose, chitin colloidal media, Go Taq Greene and *G. boninense* mushroom. While the tools used in this study were petri dishes, test tubes, shakers, incubators, vortex, shakers, laminar air flow, autoclave, spectrophotometry, bunsen, erlenmeyer, measuring cup, tube rack, and oven.

2.3. Sampling Location

The rhizosphere soil of M. bracteata was obtained from three oil palm plantation locations in Medan and its surroundings, namely oil palm plantations in Simalingkar Medan, PTPN III and PTPN IV Adolina Medan. M. bracteata rhizosphere soil samples were taken randomly and composited from three points from each location. Soil was collected as much as 10 g at each location and stored in sterile plastic containers, then brought to the laboratory for isolation of chitinolytic diazotrophic bacteria.

2.4. Population Counting and Isolation of Diazotrophic Bacteria

Bacterial isolation was carried out using serial dilutions up to 10-6 10 g of soil was put into 90 ml of sterile distilled water and then homogenized with a shaker for 1 hour at a speed of 120 rpm. A total of 1 ml The suspension was then put into a test tube containing 9 ml of sterile distilled water and diluted serially to a dilution of 10-6. A total of 0.1 ml of suspension from three dilutions was put into each petri dish containing selective media for diazotroph bacteria, namely Ashby Mannitol Agar media. Petri dishes were incubated for 2-7 days at 28 °C. Bacterial growth in each petri was calculated to determine the total population of diazotrophic bacteria. Diazotrophic bacterial colonies that grew in Ashby's media were then used as suspension again by adding1 ml of sterile distilled water onto the surface of the medium was then homogenized. The bacterial suspension isolated in Ashby media was used as an inoculum to be spread on chitin agar media.

2.5. Chitinolytic Ability Selection

Selection of chitinolytic ability was carried out by inoculating bacterial suspension growing on Ashby's medium into Colloidal chitin medium with the composition (g/L) K_2HPO_4 0.7; KH_2PO_4 0.3; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.01; $ZnSO_4$ 0.001; $MnCl_2$ 0.001; 0.2% chitin colloidal and 2% agar. Bacterial cultures were incubated at room temperature for up to 48 hours. Bacterial colonies with a clear zone were counted and further purified only those with a clear zone. The purified chitinolytic bacterial isolates were re-confirmed their ability to grow on Ashby agar medium and then stocked in Ashby agar slanted media.

Chitinolytic ability test was carried out qualitatively. The purified bacterial isolates were grown on colloidal chitin medium by spotting them with a needle, then incubating at room temperature for 24 hours [4]. Bacteria with chitinolytic ability were characterized by the formation of a clear zone around the bacterial colony. The clear zone formed was measured with a caliper.

2.6. Antagonist Test Against Ganoderma boninense

Chitinolytic diazotrophic bacteria isolates were tested for their antagonistic ability against G. boninense. Bacteria were grown on Nutrient Agar (NA) medium for 24 hours, and then a suspension was made with an absorbance turbidity of 0.5 or equivalent to McFarland's solution (108 CFU/ml). 6 mm paper disc (Oxoid®) which has been 5 l of chitinolytic diazotroph bacteria was dropped, placed on the outer side of the *G. boninense* colony which was cultured for 3 days at a distance of 2 cm. The inhibition zone formed is calculated and the value of the antagonistic index is determined based on the formula [2] :

$$Antagonistic\ Index\ = \frac{R1-R2}{R1} x\ 100 \quad (1)$$

R1 : Radius of pathogenic fungus colony towards control

R2 : Radius of pathogenic fungus colony towards pathogenic bacteria

A total of 5 isolates with the highest chinolytic index were then selected for use in the next stage.

2.7. Production of Coarse Chitinase Enzymes

Chitinase enzyme production was carried out by inoculating 10 ml of bacterial suspension with a cell concentration of 108 CFU/mL (equivalent to McFarland's solution) into 100 ml of liquid chitin medium. The composition of the liquid chitin medium is the same as that of the chitin agar medium, only without the addition of agar and the colloidal chitin concentration used is 1%. Bacterial cultures were incubated at 37 C for 48 hours in a shaker incubator at a speed of 120 rpm. The culture liquid which is the crude enzyme is separated from the bacterial biomass by centrifugation at a speed of 11,000 rpm for 5 minutes. The supernatant formed is a crude enzyme which will then be used to test the growth inhibition and lysis of *G. boninense* mycelium.

2.8. Antifungal Ability Test and Chitinase Enzyme Lysis Test

The antifungal ability test of crude chitinase enzyme was determined based on the inhibition-elongation of the fungal mycelium of *G. boninense* upon contact with the chitinase enzyme. At the edge of the mycelium of the fungus *G. boninense* growing on PDA medium aged 72 hours, a paper disc that had been soaked in crude enzyme liquid was placed at a distance of 1 cm from the edge of the fungal colony. The control used paper discs soaked with sterile distilled water. Mycelium elongation inhibition *G. boninense* leading to a disc containing enzymes and aqudest (control) visually observed after 12 hours of incubation at room temperature. The ability of crude chitinase enzyme to lyse fungal cell walls was carried out by mixing crude enzyme and suspension of fungal mycelium *G. boninense*, and then observing the morphological changes of *G. boninense* mycelium that occurred. Initially, *G. boninense* mushrooms were grown on Potato Dextrose Broth (PDB) media for 15 days at room temperature. The mycelium was harvested by filtering it on Whatman filter paper number 1, then washed with sterile distilled water 3 times. The mycelium was blended for 1 minute at a slow speed, and then filtered again. The mycelium was washed again with sterile distilled water. Experiments to determine the lysis of fungal cell walls due to enzyme treatment of chitinolytic isolates were carried out by mixing each mycelium suspension and enzyme with the same volume. The mixture was incubated at room temperature for 2, 8, and 12 hours. As a control, fungal mycelium suspension was used without the addition of enzymes. Changes in the mycelium structure were observed under a microscope [4].

2.9. Identification of Bacteria Based on the Gene Encoding 16S rRNA

Bacterial cultures grown on NB media for 24 hours were added 100 l into a 1.5 ml eppendorf tube. Then the cell suspension was frozen at -10 °C until the solution crystallized and thawed at 95°C for 10 minutes. The cycle was repeated 7 times for efficient cell breakdown. Amplification was carried out using a polymerase chain reaction (PCR) machine using universal prokaryote primers, namely 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3) and 387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al., 1998). The PCR reaction mixture (PCR mix) was made in a volume of 25 l with the constituent components, including Master Mix Go Tag Green 12, 5 µl, forward primer (10 pmol) 1 µl, reverse primer 10 (pmol) 1 µl, DNA template 2 µl, Nuclease Free Water 8, 5 µl. The PCR machine was programmed and run under the following conditions: predenaturation 94 °C for 2 minutes, denaturation 92 °C for 30 seconds, annealing 55 °C for 30 seconds, or primer elongation 72 °C for 1 minutes, and post-PCR 72 °C for 5 minutes. The PCR process was carried out for 40 cycles. The amplified DNA profile was observed on 1% agarose gel electrophoresis (1 g agarose in 100 ml TAE 1x), with ethidium bromide (EtBr) dye. DNA bands will glow after irradiating with UV light on a UV-transilluminator. The electrophoresis apparatus was run. DNA purity was checked using a nanophotometer with ddH2O as a blank. The DNA from the PCR was then sent to Macrogen, South Korea for sequencing. The obtained sequences were then matched with the sequence database on GeneBank at The National

Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool nucleotide (BLASTn) program. Several reference sequences that have a high degree of similarity are taken to be further aligned using the Muscle feature in the Mega7.0 program. The phylogenetic tree was then constructed using the Neighbor-joining (NJ) method with 1000 bootstrap.

3. Result and Discussion

3.1. Population Counting and Isolation of Diazotrophic Bacteria

Diazotrophic bacteria isolated from the rhizosphere of M. bracteata from three locations of oil palm plantations in Medan and surrounding areas have varying numbers. The population of diazotroph bacteria isolates M. bracteata in two locations of government-owned plantations (PTPN III and IV) were more numerous than those on oil palm plantations in Simalingkar people's oil palm (Table 1)

Table 1 . Populations of diazotroph bacteria isolate rhizosphere M. bracteata from three oil palm plantation sites in Medan and surrounding areas

No.	Location	Coordinate	Population of diazotrophic bacteria (CFU/g)
1.	People's Plantation Simalingkar	LU 3^{o} 5’ 9”N BT 98^{o}37’ ” E	2,8 x 10^{6}
2.	PTPN III	LU 3^{o} 38’ 0”N BT 98^{o} 38’ 4”E	3,83 x 10^{6}
3.	PTPN IV Adolina	LU 3^{o}34’ 0” N BT 98^{o}56’59”E	3,27 x 10^{6}

The difference in the number of diazotrophic bacterial populations from the three rhizosphere sites of Mucuna plants is likely due to differences in soil environmental conditions and the lifespan of Mucuna plants. Mucuna plants on PTPN land have aged quite old, so there are many bacteria found in the rhizosphere area. The number of diazotroph bacteria from mucuna rhizosphere in Simalingkar People's Plantation has the least amount, and is thought to be due to Mucuna's young age and small number. Old plants have a wider rooting zone than young plants. The plant rooting area is one of the parts of the plant that has a high population of microorganisms [9]

The high population of diazotrophic bacteria in the rhizosphere of M. bracteata is likely also influenced by plantation age factors. The high population in PTPN III is suspected because this plantation has had a long enough age so that the level of organic matter in the area is already

high. The amount of organic matter and the age of the soil can increase the presence of microorganisms and instead affect each other[10]. Aggregation of the number of bacteria in the soil can be known by the number and condition of bacteria, the more bacteria, the aggregation and soil conditions the better and fertile and vice versa. Basically, the legume plant group in the rooting system is enveloped by a very diverse group of diazotrophic bacteria. So that the number of diazotrophic bacteria from the three places has a fairly high density.

3.2. Chitinolytic Diazotroph Bacteria Selection

The results of the selection of the chitinolytic ability of diazotrophic bacteria isolates from rhizosphere M. bracteata were obtained as many as 23 isolates. Most of the chitinolytic diazotrophic bacteria came from the rhizosphere of M. bracteata in PTPN III land, and the least from PTPN IV Adolina Medan (Table 2). Chitinolytic bacteria were found in higher numbers in PTPN III, probably because this garden was older than the other two gardens.

Table 2 Total chitinolytic bacteria from diazotroph bacteria isolate rhizosphere M. bracteata.

No.	Location	Total Bacteria	Isolate Code
1.	People's Plantation Simalingkar	8	DK01, DK02. DK03. DK04, DK05,
2.	PTPN III Medan	10	DK09, DK10, DK11, DK12, DK13, DK14, DK15, DK16, DK17, DK18,
3.	PTPN IV Adolina	5	DK19, DK20, DK21, DK22, DK23
	Total		23 isolate

All bacterial isolates with chitinolytic ability grow on the chitin medium in order to form a clear zone around the perimeter of the colony. The clear zone formed indicates that the bacteria produce the enzyme chitinse which converts the chitin found around the colony into dissolved. Chitin polymers that were previously suspended in agar mediums, become dissolved after hydrolysis [2]. The large size of the clear zone diameter produced by the 23 diazotrophic bacterial isolates shows the magnitude of the ability of these bacteria to hydrolyze chitin. All isolates that have grown on the colloidal medium of chitin exhibit different chitinolytic abilities.

3.3. Characteristics of Bacteria and Colony Form

Colony morphological characteristics of 23 isolates of selected chitinolytic diazotroph bacteria showed varied shapes. Colony morphology of diazotrophic bacteria was dominated by round colonies, with white colonies, flat edges and flat elevations. The differences in each of these bacterial colony morphological characters may indicate different species. (table 3) Form the same

colony also does not necessarily indicate the same type of bacteria, this is because bacteria have many similarities between species, especially those that are still closely related

Table 3 Morphological Characteristics of Chitinolytic Diazotroph Bacteria isolate rhizosphere M. bracteata

No.	Isolate code	Colony morphology on NA media			
		Color	Edge	Elevation	Shape
1.	DK01	Round	Flat	Flat	White
2.	DK02	Round	Wavy	Convex	White
3.	DK03	Round	Wavy	Flat	Light blue
4.	DK04	Round	Flat	Flat	White
5.	DK05	Round	Flat	Convex	White
6.	DK06	Round	Flat	Convex	White
7.	DK07	Round	Flat	Flat	White
8.	DK08	Round	Flat	Flat	White
9.	DK09	Round	Flat	Flat	Yellowish white
10.	DK10	Irregular	Flat	Flat	White
11.	DK11	Irregular	Jagged	Flat	White
12.	DK12	Round	Jagged	Flat	White
13.	DK13	Round	Flat	Convex	White
14.	DK14	Round	Flat	Flat	White
15.	DK15	Irregular	Flat	Flat	White
16.	DK16	Round	Flat	Flat	White
17.	DK17	Round	Flat	Flat	White
18.	DK18	Round	Flat	Flat	White
19.	DK19	Round	Flat	Flat	White
20.	DK20	Round	Flat	Flat	White
21.	DK21	Round	Flat	Flat	White
22.	DK22	Round	Flat	Convex	White
23.	DK23	Round	Flat	Flat	White

3.4. Antagonist Test Against Ganoderma boninense

Antagonistic test of chitinolytic diazotroph bacteria in vitro against G. *boninense* showed different antagonistic abilities. A total of 23 were tested against G. *boninense*, there were 14 isolates of bacteria that had the potential to inhibit the growth of G. *boninense* mycelium (Figure 1). The antagonistic ability of the chitinolytic diazotroph bacteria varied with the antagonistic index ranging from 14.28 to 71.15%. The bacteria with the greatest inhibitory ability was shown by isolate DK17 (71.15%) and the lowest by isolate DK12(14.28%).

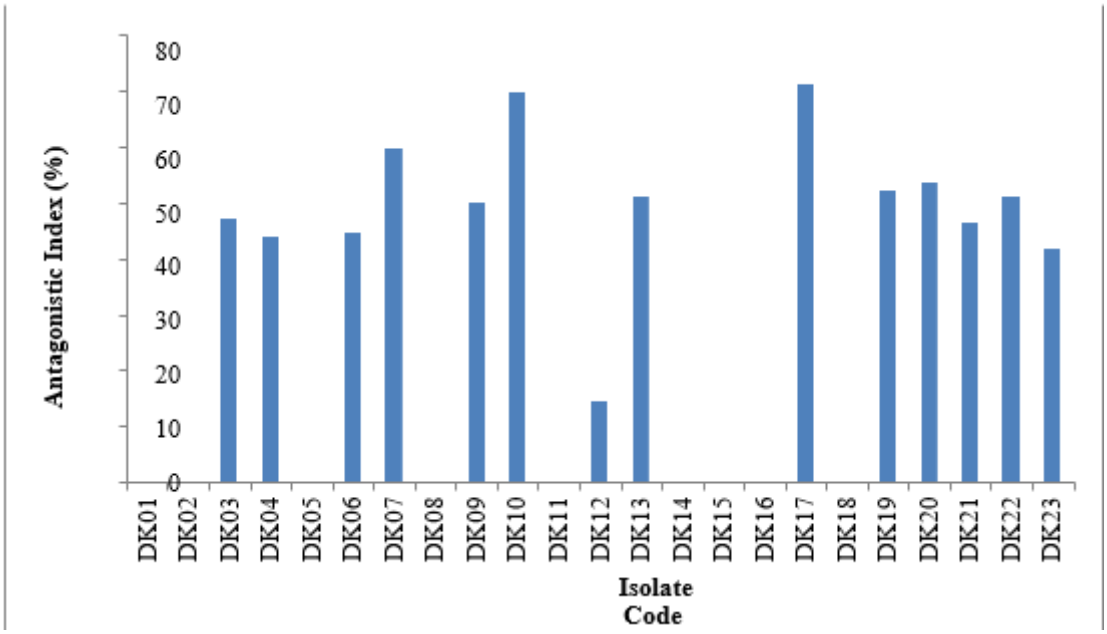

Figure 1 Antagonistic index of chitinolytic diazotrophic bacteria isolating rhizosphere *M.bracteata* against *G.boninense*

3.5. Antifungal Ability Test and Chitinase Enzyme Lysis Test

The ability of 14 isolates that were able to inhibit the growth of the mycelium of G. *boninense* was tested for the enzyme ability and the isolates showed changes in different mycelium. Changes in the structure of the mycelium were first observedis a lysis process in the mycelium of the fungus G. *Boninense*

Table 4. Description of morphology and condition of mycelium *G. boninense* after treatment of enzyme administration and isolation of chitinolytic diazotroph bacteria

Isolate Code	Mycelium morphology	
	Bacterial	Isolate Enzymes
DK03	Mycelium is thin	Mycelium is thin and little Frown
DK04	No changes to mycelium	Bent mycelium
DK06	Mycelium is thin	No changes to mycelium
DK07	Mycelium thins and dries	Lysed mycelium
DK09	No changes to mycelium	No changes to mycelium
DK10	Bent mycelium	Mycelium is bent and shriveled
DK12	No changes to mycelium	No changes to mycelium
DK17	Lysed mycelium	Lysed mycelium
DK19	Lysed mycelium	Lysed mycelium
DK20	Mycelium is thin	Mycelium is thin

DK21	Mycelium is thin	Mycelium is thin
DK22	Mycelium is thin	Lysed mycelium
DK23	No changes to mycelium	Mycelium is thin

Symptoms and morphology of the mycelium of G. boninense after being treated with enzymes and bacterial isolates showed different characteristics. The enzymes from isolates DK03, DK06, DK07, DK20, DK21, and DK22 showed mycelium depletion. The ability of enzymes to lyse mycelium was only shown by DK17 and DK19, while isolates DK04, DK09, DK12, and DK23 were not able to degrade the mycelium of G. boninense. The direct administration of isolates into the mycelium media of G. boninense showed different changes. isolates DK03 DK10, DK20, DK21, and DK23 showed thinning, bent, and shriveled mycelium. Isolates capable of lysing the mycelium of G. boninense DK07, DK17, DK19, and DK22. Isolates that were unable to degrade mycelium were shown by isolates DK06 and DK12.

3.6. Identification of Bacteria Based on the Gene Encoding 16S rRNA

The identification results based on the 16S rRNA encoding gene showed that DK07 was similar to *Enterobacter aerogenes*, DK10 was similar to *Mycobacterium senegalense*, DK17 was similar to *Bulkhoderia cepaci*, DK19 was similar to *Pseudomonas stutzeri*, and DK21 are similar *to Bulkhoderia cepacian* (Figure 2)

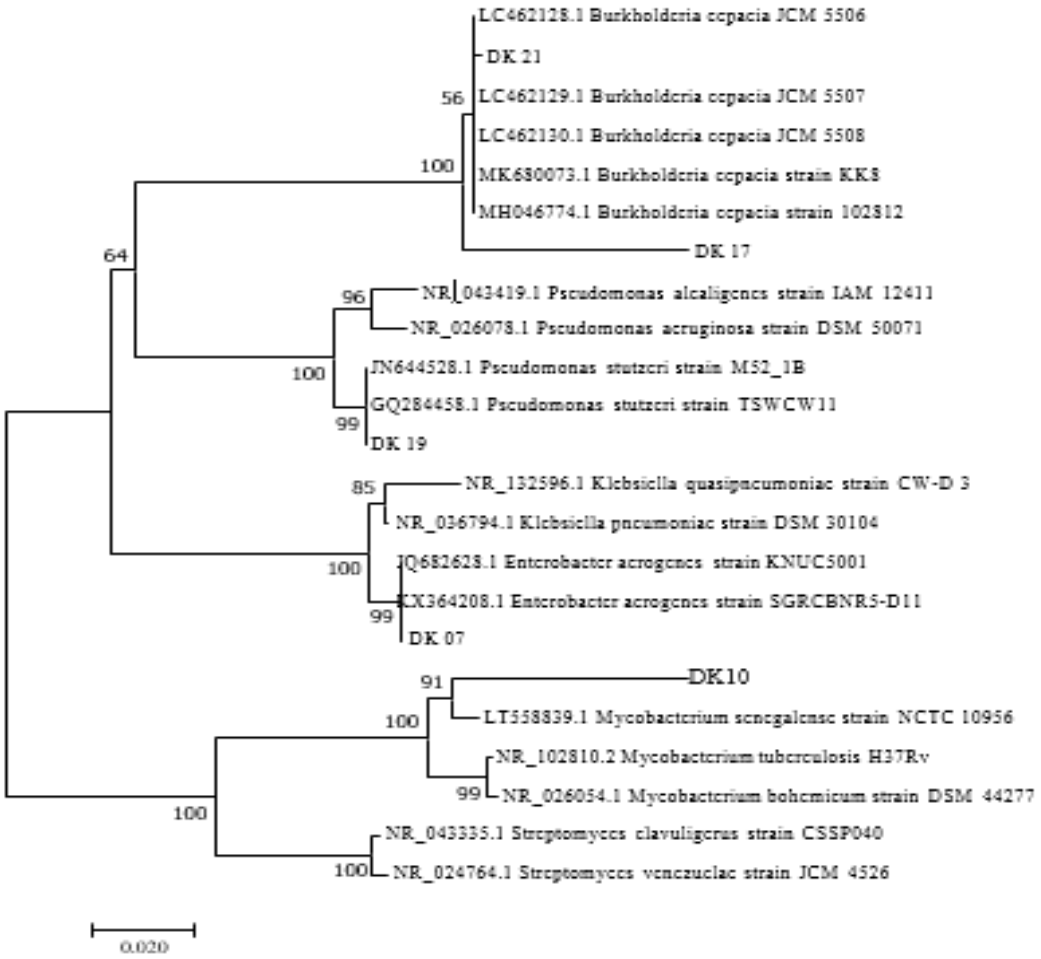

Figure 2 Phylogenetic analysis of chitinolytic diazotrophic isolates based on the Neighbor joining method (bootstrap 1000 ×) using the MEGA v.7.0 program.

Mycobacterium senegalense bacteria is a group that causes various diseases in the population of Senegal, but as a biocontrol agent has never been found [11] Enterobacter aerogenes are disease-causing bacteria that are often used for testing various antibiotic ingredients [12]. Bulkhoderia cepacia bacteria have so far had the ability to inhibit various fungi, among the fungus *Colletotrichum gloeosporioides* [13]. Pseudomonas stutzeri isolates are disease-causing bacteria in humans, but the Pseudomonas group is known to have a role as nitrogen binders in the environment and some species have symbiosis with various legume groups [14].

4. Conclusion

The conclusions of this research are:

a. A total of 23 chitinolytic diazotrophic bacterial isolates were obtained from the rhizosphere. mucuna plants from 3 oil palm plantation sites, and 14 isolates Among them are inhibiting the growth of *G.boninense*.

b. Chitinolytic diazotroph bacteria isolate DK 17 with the best ability inhibits and lyses the cell wall of fungi through enzymatic mechanisms and the isolate.

c. Molecular identification results of 5 selected isolates with ability The highest obtained DK07 has a similarity of *Enterobacter aerogenes*, DK10 is similar to *Mycobacterium senegalense*, DK17 is similar to *Bulkhoderia cepacia*, DK19 is similar to *Pseudomonas stutzeri*, and DK21 Similar to *Bulkhoderia cepacia*.

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