In vitro Test of Rhizosphere Chitinolytic Bacteria as a Biocontrol for *Ganoderma boninense*

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Abstract

G. boninense is a fungal pathogen that causes basal stem rot in oil palm (*Elaeis guineensis*). Chitinolytic bacteria are abundant in soil and well known as a natural biocontrol of fungal pathogen. The objective of this research is to obtain rhizosphere chitinolytic bacteria as a candidate of biocontrol agent for *G. boninense*. Nineteen indigenous bacteria from Padang Halaban Estate, North Sumatera, were tested for their antagonistic properties against *G. boninense* by *in vitro* dual culture on PDA plates. Their chitinolytic activities were tested using colloidal chitin as the substrate. Chitinase activity was determined colorimetrically by detecting the amount of N-acetylglucosamine released from colloidal chitin substrate. Crude enzyme secreted by the bacteria were characterized by SDS-PAGE with chitinase from *Trichoderma* as a control. Six of the isolates have an inhibition activity against *G. boninense* growth with B3.4 showed the highest percentage inhibition ratio growth or PIRG (58.75%). Inhibition of *G. boninense* growth *in vitro* might due to a competition for nutrients or other antifungal compound from certain bacteria which diffuses in the agar. B3.2 showed the highest activity of chitinase (10.44 U/mL), but B3.4 has lower activity of chitinase (7.08 U/mL). It can be assumed from the result that PIRG does not have correlation with the activity of chitinase. The results of enzyme characterization showed that B3.3, B3.2 and B3.4 has a molecular mass of 37kDA, which is a similar molecular mass with chitinase from *Trichoderma viridae*

Keywords: rhizosphere bacteria, Ganoderma boninense*,* Elaeis guineensis*, chitinase*

Introduction

Basal stem rot disease (BSR) caused by *Ganoderma boninense* is currently a major disease in oil palm plantations (Darmono, 1998). The disease can destroy up to 80% of the stand palms by the time when the palms are halfway through their normal economic life span (Idris, 2003). *Ganoderma boninense* is a saprophytic fungus activated by favorable conditions to behave parasitically (Statmets, 2004). Like all fungi, it has no chlorophyll and thus, lack photosynthetic capability. Instead of manufacturing their own food, fungi absorb nutrients from either living or dead host tissue (Haniff, 2005). the fungus typically attack already weakened oil palm as *Ganoderma* seldom seriously infects undamaged trees (Paterson, 2007). The effects of *Ganoderma* infection on productivity decline in palm crops have been concerned since replanting of oil palm land was began in South-East Asia, especially in Malaysia and Indonesia (Turner, 2003).

BSR has been an endemic disease found in PT. SMART Tbk. oil palm plantation especially in Padang Halaban, North Sumatera, Indonesia. Biological control of pathogenic fungi provides an attractive and alternative management for fungal diseases without the negative impact of synthetic antifungal agents that can cause environmental pollution and may induce pathogen resistance (Haas & Défago, 2005). Plant disease control by chitinolytic bacteria has long been reported (Sneh, 1981). Several strains of bacteria, such as *Aeromonas caviae*, *Bacillus sp*., *Serratia plymuthica*, and *Enterobacter agglomerans* are well-known chitinolytic bacteria (Das *et al*., 2010). Some *Trichoderma* sp. have been described as biological control agents against fungal pathogens and widely used as biocontrol because of its mycoparasitic activity (De La Cruz, 1992). Chitinolytic

enzymes have been considered as an important factor to control soilborne pathogens because of their ability to degrade fungal cell walls, of which a major component is chitin (Chet, 1987).

Chitin is widely available in the soil derived from the decay and degradation of dead cells, so that some bacteria that live on the ground are good in degrading chitin (Suryanto *et al*., 2005). The aim of this study was to isolate chitinolytic bacteria from rhizosphere soils in Padang Halaban estate and to screen their antagonistic activity against *G. boninense*.

Materials and Methods

Isolation of bacteria

Rhizospheric soil were collected near the root of the uninfected plant in endemic site of *G. boninense*. Suspension were made by adding 10 g of soil to 100 mL sterile basic salt solution (0.85% NaCl). Ten fold serial dilutions of these suspensions were made and plated on Nutrient Agar (NA). The colony were selected and purified with 4-way streak method on NA.

Dual-culture technique

Dual culture were made to obtain the percentage inhibition of radial growth (PIRG) of *G. boninense* (Bivi, 2010). Potato Dextrose Agar (PDA) was poured onto 9 cm diameter Petri dishes. Isolated bacteria was streaked into the PDA plate 2.5 cm from the edge of Petri dish. A 5 mm agar disc cut from the side of an actively growing pure culture of 5-day old *G. boninense* placed 2.5 cm from the edge at the opposite side of the same Petri dish. For the control plate, only *G. boninense* was placed in a similar manner without bacteria on a fresh Petri dish. The experiment was made in three replication each bacteria. The plates were incubated at 28 ^{o}C for five days. Results revealed as mean colony growth of the causal pathogen in the presence of the bacteria and its growth on the control plate (without the bacteria). The outcome of two readings was calculated into the formula for the PIRG as below :

$$\% \text{ PIRG} = \frac{R1 - R2}{R1} \times 100$$

Where, PIRG = percentage inhibition of radial growth; R1 = radial growth of *G. boninense* in the absence of bacteria (control); R2 = radial growth of *G. boninense* in the presence of bacteria.

Chitinase assay

Chitinase activity was determined colorimetrically by detecting the amount of *N*-acetylglucosamine (GlcNAc) released from a colloidal chitin substrate (Reissig, 1955). Selected isolates were inoculated into 100 ml of 10% chitin medium and incubated at 37 ^{o}C on rotary shaker 120 rpm for 5 days. Flask were removed every 24 hours and pipetted 10 mL from each mixture were filtered through filter paper. The filtrate as a crude enzyme were pipetted 1.5 ml into 0.75 ml phosphate buffer and 1.5 ml of 0.3% coloidal chitin in 15 ml Corning centrifuge tube. The reaction mixture were incubated in 37 ^{o}C for 30 min, and centrifuge the mixture to separate the product from the remaining substrate. Pipetted 2.5 ml supernatant into 5 ml Schales reagent and 2.5 ml distilled water. The mixture was boiled for 10 min to stop the reaction of the remain enzyme. The reaction was measured with spectrophotometer at 420 nm. *N*-acetylglucosamine was used as a standard. One unit of chitinase acivity was defined as the amount of enzyme that released 1 µmol GlcNAc or its equivalent from colloidal chitin in 1 min (Zilda, 2006).

Protein characterization

Crude enzyme secreted by the bacteria were characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 12 % separator gel and 4% stacking gel. Crude supernatants protein were mixed with 2x sample buffer (1:1), boiled for 4 min, then load. The

gel were stained with 0,1% Coomassie brilliant blue R-250, and destained with 10% acetic acid and 20% methanol.

Results and Discussion

There are 19 indigenous bacteria isolated from rhizosphere soil in Padang Halaban. Screening of biocontrol bacteria was carried out by dual culture. It showed that six of them have an inhibitory activity against *G. boninense* growth (Table.1). B3.4 showed the highest PIRG of *G. boninense* amongs other isolates, it showed 58.75% and the B3.3 is the lowest (12.20%). The inhibition of *G. boninense* growth might due to the presence of chitinase or other anti-fungal properties produced by the certain bacteria. Many publications have reported that soil bacterias are capable in producing anti-fungal metabolites properties that inhibited mycelia growth of phytopathogen fungi (Fernando & Linderman,1994; Sisler, 1969; Yiu-Kwok *et al*., 2003). Many of the anti-fungal properties affect the physiological activities of pathogenic fungi (Vesperman, 2007).

Table 1. Antagonistic potential of soil bacteria in dual culture test

Bacterial isolates	PIRG (%)
B3.3	12.20 d
B14.1	35.56 b
B3.1.1	22.22 c
B3.4	58.75 a
B11.2	31.11 b
B3.2	16.67 d

Mean in the same coloumn with different alphabet(s) are significantly different (P<0.05) according to DMRT

Bacteria B3.2 showed the highest activity of chitinase (10.44 U/mL) and the lowest was B14.1 (7.08 U/mL) on day four of incubation. The result revealed that PIRG has no correlation with chitinase activity. Bacteria with the highest PIRG (B3.4) showed less chitinase activity than bacteria with lowest PIRG (B3.3). This result is in accordance to Aktuganov (2003) statement that there is no correlation between PIRG and the ability of bacteria in producing chitinase. It can be assumed that other anti-fungal property was responsible for the inhibitory effect in dual-culture of B14.1.

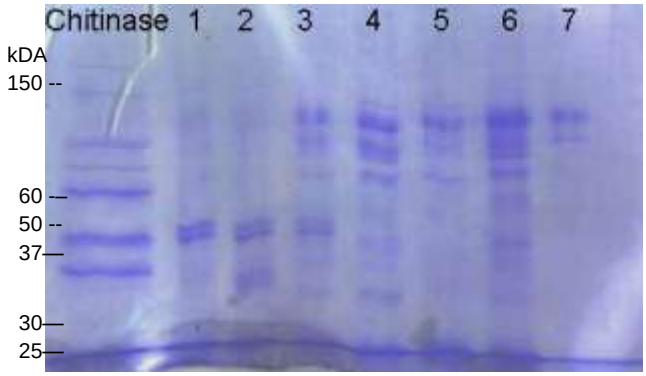
Table 2. Chitinase activity enzyme from isolated bacteria

Bacterial isolates	Enzyme activity (U/mL)	Incubation time (Days)
B3.2	10.44 a	4
B3.3	8.43 b	4
B3.4	9.94 a	4
B14.1	7.08 c	4
B3.1.1	8.37 b	4
B3.2.2	8.31 b	4

Mean in the same coloumn with different alphabet(s) are significantly different (P<0.05) according to DMRT.

Crude supernatan protein secreted by six bacterial isolates were characterized. Chitinase from *Trichoderma viridae* were used as a control. The results revealed that the six isolates have different banding pattern as shown in Figure 1. Chitinase from *Trichoderma* has a molecular mass of 37 kDA (De Marco, 200), and most of chitinases have molecular mass of between 28 kDA - 43 kDA (Nielsen, 1997). Result showed that B3.2, B3.3, and 3.4 have a similar band between 30 kDA – 40 kDA, in a range with most of chitinases molecular mass. The three other isolates showed a

similar band of 50 kDA. Singh *et al,* (1998) reported that chitinase from *Paenibacillus* sp. has molecular mass around 50 kDA.



First lane represent the control of chitinase from *Trichoderma viridae*; Lane 1, B3.2; Lane 2, B3.3; Lane 3, B3.4; Lane 4, B14.1; Lane 5, B3.1.1; Lane 6, B3.2.2; Lane 7, negative control.

Figure 1. SDS-PAGE analysis of total supernatan protein of isolates, stained with comassie brilliant blue.

The result showed that B3.2 was the best rhizosphere chitinolytic bacteria. The most promising bacteria is B3.4. It is because bacteria with more 50% PIRG can be used as bio control (Bivi, 2010). Synergism between chitinolytic and antagonistic bacteria could enhance the role of bacteria as biocontrol pathogen. Combination of these two bacteria can be use as a biocontrol candidate.

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