Isolation and Identification of chitinolytic bacteria as biocontrol agent of pathogenic fungi on gold silkworm cocoon *Cricula trifenestrata*

Melda Yunita¹, Nisa Rachmania Mubarik², Dedy Duryadi Solihin²

¹Postgraduate School of Bogor Agricultural University, IPB Dramaga, Bogor 16680, Indonesia.
²Department of Biology Study Program of Microbiology Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, IPB Dramaga, Bogor 16680, Indonesia.

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**ABSTRACT**

Aims: *Cricula trifenestrata* is one of natural insects which has not been domesticated yet, thus called as the wild silkworm. *C. trifenestrata* is known as a silk producer which has high economic and market value. However, the fungi attack on *C. trifenestrata* cocoon decreased quality and quantity of silk yarn. Chitinolytic bacteria have a high potential as biological control against pathogenic fungi. This research aimed to isolate, select, characterize, and identify chitinolytic bacteria as pathogenic fungi growth inhibitors on *C. trifenestrata* cocoon.

Methodology and results: Chitinolytic bacteria was isolated from the uninfected and infected cocoon while fungi was isolated from the uninfected cocoon. Inhibition test was conducted by Fokkema method and chitinase activity was measured by Spindler method. A total of 36 chitinolytic bacteria and 10 suspected pathogenic fungi isolates have been isolated. Fungal pathogenicity test showed that isolate CSAJ.2 was suspected as fungal pathogen. *In vitro* inhibition test indicated that chitinolytic bacteria isolate BSEP.3 could inhibit the growth of pathogenic fungi *C. trifenestrata* with percentage of inhibition 50%. Isolate BSEP.3 showed highest chitinase activity (5.11 U/mL) at the 15th h. It able to inhibit the growth of pathogenic fungi with percentage of inhibition of 47.5% and 46.25%, respectively.

Conclusion, significance and impact of study: Identification of bacteria targeted on 16S rRNA gene showed that isolate BSEP.3 had 98% identity with *Bacillus amyloliquefaciens* B5 while identification of fungi using ITS region of the rDNA showed that isolate CSAJ.2 had 100% identity with *Trichoderma virens* TV242. Chitinase crude extract was effective to be used as a biological control agent of T. virens CSAJ.2.

Keywords: *Cricula trifenestrata*, *Trichoderma virens*, chitinase

**INTRODUCTION**

*Cricula trifenestrata* is one of the natural insects as silk producer that have not been domesticated and known as the wild silkworm. *Cricula trifenestrata* salivate contains proteins as cocoon former. This cocoon is collected to be processed into silk yarn. Processed silk from *C. trifenestrata* cocoon has high economic and market value because of its typically golden yellow color. This is the difference between *C. trifenestrata* with other silkworm (Mondal *et al.*, 2007).

One of the known pathogen of cocoon silkworm is the fungi. The fungus attacks decreased quality and quantity of silk yarn. In India, cocoon crop failure reached 30-40% influenced due to attack of *Aspergillus* sp. Fungus cell wall composed of chitin complex becoming major target for antifungal agents, such as chemical fungicides. However, the negative impacts due to chemical fungicide usages can be an important reason to find a biological fungicide such as the potential chitinolytic bacterium which is able to inhibit or even kill pathogenic fungi (Kim *et al.*, 2004).

Chitinase is an enzyme that catalyzes degradation of chitin by cutting the glycosidic bond to become N-acetylglucosamine monomers. Chitinolytic bacteria has a high potential to be used as biological control against pathogenic fungi on *C. trifenestrata*, thus reduction of quality and quantity of gold silkworm can be avoided. Examples of bacteria which have been reported to possess chitinolytic activity are *Pseudomonas aeruginosa*, *Serratia marcescens*, *Vibrio furnissii*, *Bacillus circulans*, *B. cereus*, and *Streptomyces griseus* (Gohel *et al.*, 2006; Anitha and Rabeeth, 2010).

The discovery of 17 chitinolytic bacteria isolates and 4 chitinase from Novitasari (2013) has narrowed down the spectrum that only could inhibit *Scopulariopsis* sp. origin from *C. trifenestrata* cocoon. In addition, the previous research did not perform the molecular identification of chitinolytic bacteria. Therefore, it is necessary to repeat this research in order to obtain the potential chitinolytic...
bacteria which have broad spectrum in inhibiting a variety of pathogenic fungi and to study its molecular identification. This research aimed to isolate, select, characterize, and identify chitinolytic bacteria as pathogenic fungi growth inhibitors on C. trifenestrata cocoon.

MATERIALS AND METHODS

Source of organism

Cricula trifenestrata silkworm cocoon was obtained from avocados plantation in Babakan Lebak Village, Bogor, West Java, Indonesia (6.55733°SL and 106.73799°EL).

Isolation and selection of chitinolytic bacteria

Sample originated from C. trifenestrata cocoon which are unininfected pupae and infected pupae. Cocoon were treated into suspension. A total of 3 g of each cocoon were put in 100 mL Nutrient Broth (NB) (Merck, U.S) containing 0.5% colloidal chitin in 500 mL Erlenmeyer flask, then the suspensions were incubated in the incubator shaker at room temperature for 24 h. Chitinolytic bacteria isolation was initiated by serial dilution from 10⁻¹ to 10⁻⁸ in 0.85% NaCl (Merck, U.S). The suspensions were poured on chitin agar media consisted of 0.1% MgSO₄·7H₂O, 0.02% K₂HPO₄, 0.1% yeast extract, and 1.5% agar and incubated for 24 h at 37 °C. The quadrant streak was done on chitin media aimed to collect pure isolates. Isolates were selected based on Chitinolytic Index (CI).

Isolation of pathogenic fungi and pathogenicity test

Isolation of pathogenic fungi was conducted using Lim method (Lim et al., 2002) with several modifications. Isolation was initiated by cutting infected cocoon into four parts. All cocoon pieces were soaked in a mixture of 2 mL of 0.8% chloroxilenol and 100 mL of sterile water and then placed on Potato Dextrose Agar (PDA) (Merck, U.S) media supplemented with chloramphenicol (500 mg/L). After that, the cultures were incubated for five days at 25 °C. Colonies of fungi which grew were transferred to the new PDA media to obtain pure cultures. Fungal pathogenicity test was done by making a suspension of the fungus spores and then dropped into uninfected cocoon in a sterile petri dish, then incubated for five days at 25 °C. Colonies of fungus that grow on the cocoon and cause symptoms of pathogenicity such as the yellow colour of cocoon became darker, mushy, and stench were isolated again on the new PDA.

Screening of chitinolytic bacteria through inhibition test against pathogenic fungi

A total of 36 chitinolytic bacterial isolates were inscribed along 4 cm on the edge of PDA media and pieces of pathogenic fungi (Φ 1 cm) were put in the middle of PDA media at the same time. Sterile distilled water was used as a control. The percentage inhibition of pathogenic fungi was measured by using the formula \( \left[ 100\% \times \left( \frac{r_1 - r_2}{r_1} \right) \right] \), where \( r_1 \) is the long of the mycelium growth toward edge of petri dish (3 cm) and \( r_2 \) is long of the mycelium growth toward bacterial isolates (Fokkema, 1973).

Determination of growth curve and production of chitinase crude extract of selected isolate

Two loops of bacterial isolates were inoculated into nutrient broth (NB) supplemented with 0.3% colloidal chitin. The culture was incubated on rotary shaker with 120 rpm for 32 h at 37 °C. Furthermore, 1% (10⁶ cells/mL) of inoculum was inoculated into new media. Every 3 h, cell culture was taken and cell density was measured at wavelength of 600 nm. This process was carried out for 24 h. The same cell culture was centrifuged for 10 min at speed of 10,000 rpm at 4 °C. Supernatant consisted of chitinase crude extract was measured using the Spindler method (Spindler, 1997). One unit of enzyme activity is defined as amount of enzyme that produces 1 mol of N-acetyl glucosamine per minute. The protein content was measured by Bradford method (Bradford, 1976). The protein content is calculated based on a standard curve of Bovin Serum Albumin (BSA).

Inhibitory activity of liquid culture and chitinase crude extract were tested again by paper disk method. Paper discs were soaked in 100 mL of liquid culture and chitinase crude extract and placed 4 cm from the edge of PDA media and the pieces of pathogenic fungi (Φ 1 cm) in the middle of PDA at the same time (Fokkema, 1973).

Identification of chitinolytic bacteria

The selected chitinolytic bacteria isolate was characterized based on morphological characteristics, Gram staining, biochemical identification using Kit Analytical Profile Index (API) 50 CHB. Molecular identification based on 16S rRNA gene was conducted by growing selected bacterial isolate on Luria Bertani media (LB) for 24 h. DNA extraction was conducted by following the procedures of Presto™Mini gDNA Bacteria Kit (Geneaid, Japan). Concentration and purity of extracted DNA were measured by Nano Drop 2000 spectrophotometer. The extracted DNA was amplified by polymerase chain reaction (PCR) with primers 67F (5'-CAG GCC CAC ATG TAA CAA GTC-3') and 1387R (5'-GGG CGG WGT CAA GGC GTA-3') (Marchesi et al., 1998). Amplification of 16S rRNA gene resulted in PCR product with size of 1300 bp. Sequences were aligned in GenBank using BLASTN program and construction of phylogenetic tree was performed by MEGA 5.05 program.

Identification of pathogenic fungi

The selected pathogenic fungi from pathogenicity test was identified morphologically as described by Barnett and Hunter (1987). DNA extraction was done by QIAmp™DNA Mini Kit (Qiagen, U.S) following manufacturer’s instructions. Concentration and purity of extracted DNA...
were measured using Nano Drop 2000 spectrophotometer. The extracted DNA was amplified using PCR with primers ITS5R (5'-AAA GGA AGT AGT CGT AAC AAG G-3') and ITS4F (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990). Sequences were aligned in GenBank using BLASTN program and construction of phylogenetic tree was done by MEGA 5.05 program.

**Application of chitinase crude extract on uninfected pupae (in vivo)**

The application was initiated by dropping spores of pathogenic fungi with total of density 10^5 spores/mL on the uninfected cocoon, and then incubated until cocoon was attacked by pathogenic fungi. A total of 1000 mL of chitinase crude extract were dropped into infected cocoon followed by incubation for 8 days.

**RESULTS**

**Isolation and selection of chitinolytic bacteria**

Isolation of chitinolytic bacteria from uninfected and infected cocoon around the village of Babakan Lebak, Bogor, West Java, have produced 36 isolates (Table 1). Chitinolytic bacteria were selected based on Chitinolytic Index (CI) at various range of 0.21 to 1.81.

| Code of isolates | CI     | Code of isolates | CI     | Code of isolates | CI     |
|------------------|--------|------------------|--------|------------------|--------|
| BSEP.1           | 1.81   | BSEP.10          | 0.87   | BSEP.19          | 0.6    |
| BSEP.2           | 1.77   | BSEP.11          | 0.54   | BSEP.20          | 0.74   |
| BSEP.3           | 1.73   | BSEP.12          | 0.44   | BSEP.21          | 0.96   |
| BSEP.4           | 0.57   | BSEP.13          | 0.87   | BSEP.22          | 0.88   |
| BSEP.5           | 1.62   | BSEP.14          | 0.76   | BSEP.23          | 0.55   |
| BSEP.6           | 1.42   | BSEP.15          | 0.43   | BSEP.24          | 0.85   |
| BSEP.7           | 1.36   | BSEP.16          | 0.68   | BSEP.25          | 1.24   |
| BSEP.8           | 1.13   | BSEP.17          | 1.37   | BSEP.26          | 0.29   |
| BSEP.9           | 0.9    | BSEP.18          | 0.84   | BSEP.27          | 0.47   |

**Isolation of pathogenic fungi and pathogenicity test**

Ten pathogenic fungi (CSAJ.1 – CSAJ.10) were isolated from infected cocoon. Fungal pathogenicity test was done to study the level of pathogenicity that has been caused by pathogenic fungi on uninfected cocoon. The pathogenicity test showed that only 1 coded fungi isolate CSAJ.2 has successfully grown on the uninfected cocoon and caused the darker yellow on cocoon. This indicated that CSAJ.2 was able to attack the cocoon. Isolate CSAJ.2 was suspected as pathogenic fungi. This isolate was isolated and purified again on PDA media. CSAJ.2 isolate was used as a pathogenic fungus in the subsequent tests.

**Screening of chitinolytic bacteria through inhibition test against pathogenic fungi**

Inhibitory test showed that among all of isolates, isolate BSEP.3 was the only one had successfully inhibited the growth of pathogenic fungi which was signed by the formation of inhibition zone around pathogenic fungi with a percentage inhibition of 50% after 5 days incubation (Figure 1). Percentage inhibition indicated that a presence of chitin degradation on the cell wall of fungi by chitinase-producing bacteria.

**Figure 1:** Inhibition activity of chitinolytic bacteria isolate BSEP.3 (a) against pathogenic fungi isolate CSAJ.2; (b) on PDA media.

**Determination of growth curve and production of chitinase crude extract of selected isolate**

Growth curve was performed to determine the optimum time of chitinase production by chitinolytic bacteria isolate BSEP.3. Measurement of chitinase showed that chitinase activity increased when the incubation time was prolonged. The highest chitinase activity (5.11 U/mL) was determined after 5 days of incubation.
obtained during the early stationary phase after 16 h of incubation (Figure 2).

Figure 2: Growth curve and chitinase production of chitinolytic bacteria isolate BSEP.3.

In vitro inhibitory test showed that the growth of the fungi was inhibited by chitinase crude extract with percentage of inhibition 46.25% (Figure 3a). Microscopic observations showed that hyphae growth was inhibited as abnormal hyphae such as roll and circular hyphae, bend hyphae, stunted hyphae and lysis hyphae which occurred due to impact of chitinase (Figure 3b).

Figure 3: (a) Inhibition activity of chitinase crude extract on PDA media and microscopic observations; (b) roll hyphae at the tip of hyphae; (c) circular hyphae at the middle of hyphae; (d) bend hyphae; (e) stunted and lysis hyphae.

Identification of bacteria

Morphological characterization of chitinolytic bacteria isolate BSEP.3 showed that it is a Gram positive bacterium in rod shape, colony color is milky white, shape of colony is wrinkled, edge of colonies is wavy, and elevation of colony is hill. Biochemical identification of chitinolytic bacteria isolate BSEP.3 showed that isolate BSEP.3 had 96.6% similarity with Bacillus amyloliquefaciens.

Identification of pathogenic fungi

Pathogenic fungi isolates CSAJ.2 was identified as Trichoderma sp. based on morphology and anatomy characteristic using identification book of Barnett and Hunter (1987) (Figure 7). Pathogenic fungi isolates CSAJ.2 was also identified molecularly based on the internal transcribed spacer (ITS) region of the rDNA. Visualization of ITS region of the rDNA on 1% agarose gel produced DNA size of 700 bp (Figure 6). Sequence analysis of ITS region of the rDNA in the GenBank demonstrated that isolate CSAJ.2 had 100% identity with Trichoderma virens TV242 (KP263703.1). The relationship of isolate CSAJ.2 with other bacteria was shown in Figure 8.
A total of 36 bacterial isolates were able to grow and form a clear zone in chitin agar media. The existence of clear zone around the colony is related to the ability of these bacteria to decompose colloidal chitin as substrate that contained in the media as a carbon source for their growth (Liu et al., 2011). Chitinolytic bacteria BSEP.3 was the only one able to inhibit the growth of pathogenic fungi mycelium compared to other isolates.

Bacteria have ability to inhibit pathogenic fungi in a variety of ways, including producing a variety of compounds that can inhibit the growth of fungi such as antibiotics, lytic enzymes and induction of plant resistance by activation of genes such as chitinase, β-1,3-glucanase, peroxidase and phenilalanine ammonia lyase (Chang et al., 2007). Chitinase is majored in biological control against pathogenic fungi because of its ability to degrade chitin in fungi’s cell wall (Chang et al., 2007).

Measurement of chitinase showed that isolate BSEP.3 started logarithm phase at 3 to 12 h of incubation time and not followed by an increase of chitinase production. The highest specific chitinase activity was observed at the stationary phase. The similar result had been reported from Serratia sp. and Bacillus sp. (Han et al., 2014). Chitinase activity had increased in the stationary phase due to the decrease of nutrients in the media, so that chitinase would be secreted in the high number. Chitinolytic bacteria isolate BSEP.3 produced extracellular chitinase to degrade colloidal chitin in the media into monomer-GlcNAc-form. This substrate can be used as carbon and nitrogen source for growth of bacteria (Thompson et al., 2001).

Inhibitory test showed that the growth of pathogenic fungi isolate CSAJ.2 was inhibited by liquid culture and chitinase crude extract. Inhibition zone occurred because lysis in the cell wall of fungal hyphae so protoplasm becomes leaky and disrupted physiological processes in the cell. Inhibition of fungi through chitinolytic system is able to produce antifungal compounds chitin-binding protein, which it will bind the new α chitin (nascent chitin) strongly on the tip of hyphae that will affect to exocytosis mechanism in the cell wall formation so the formation of cell walls would be obstructed. This phenomenon was evidenced by microscopic observations that showed the main effect of chitinase is to degrade fungi cell wall. It is also the main function of chitinase, which is one of the most important antifungal protein produced by Bacillus sp. (Liu et al., 2011).

Chitinolytic bacteria isolate BSEP.3 was identified as B. amyloliquiefaciens B5 and had a close relationship with B. subtilis. Bacillus amyloliquiefaciens is known as biological control agents of various pathogenic fungi, including Trichoderma sp. This is consistent with the research report of Kucuk and Kivanc (2004) which reported that chitinase and β-1,3 glucanase activity produced by Bacillus sp. could degrade the cell wall of Trichoderma sp. In addition, B. amyloliquiefaciens has been widely reported as a biocontrol agent against Colletotrichum lagenarium,
Sclerotinia sclerotiorum, Rhizoctonia solanacearum, Rhizopus stolonifer, Botrytis cinerea, and Penicillium expansum (Kim and Chung, 2004; Abdullah et al., 2008; Arrebola et al., 2010). The other Bacillus spp. also produces antibiotic compounds, including lipopeptide, and peptides. Bacillus sp. also has a heat and drought resistant endospore, and thus it made Bacillus sp. could colonize in the extreme condition such as in the cocoon microenvironment to prevent pathogenic fungi attacks.

Pathogenic fungi isolate CSAJ.2 was identified as Trichoderma virens, both morphologically and molecularly. Trichoderma virens is known as soil fungus that has ability to live fast and a high competitive ability as hyperparasites so it is commonly used as biological control against other pathogenic fungi (Pozo et al., 2004). However, in this study T. virens CSAJ.2 acts as a pathogen for gold silkworm cocoon of C. trifrenestrata which can reduce the quality and quantity of gold silkworm C. trifrenestrata. In another study, Trichoderma sp. also suspected pave the way for pathogenic bacteria to improve diseases on tomato plants (Lange and Smart, 2005).

According to these results, chitinase crude extract was effective in controlling the growth of pathogenic fungi CSAJ.2 on gold silkworm cocoon of C. trifrenestrata. Isolation of chitinolytic bacteria as antagonistic bacteria was the first step in biological control processess. In this study, chitinase could control the growth of pathogenic fungi both preventively and curatively so that, it could be used as a biocontrol agent of T. virens CSAJ.2. The further studies will help in developing Bacillus amyloliquefaciens CSAJ.2 as a biocontrol agent against variety of pathogenic fungi on cocoon C. trifrenestrata so that the qualities and quantities of cocoon can be maintained.

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