Malaysian Journal of Microbiology, Vol 12(1) 2016, pp. 69-75

Malaysian Journal of Microbiology
Published by Malaysian Society of Microbiology
(In SCOPUS since 2011)

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

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Received 6 July 2015; Received in revised form 8 August 2015; Accepted 8 August 2015

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 CSAJ.2 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

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

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 uninfected 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% $MgSO_4 \cdot 7H_2O$, 0.02% K_2HPO_4, 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 $[100\% \times (r_1-r_2)/r_1]$, 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% (10^8 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^{TM}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^R DNA Mini Kit (Qiagen, U.S) following manufacturer's instructions. Concentration and purity of extracted DNA

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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 10^3 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.

Tabel 1: Chitinolytic bacteria isolates based on Chitinolytic Index (CI).

Code of isolates	CI	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.28	0,69
BSEP.2	1,77	BSEP.11	0,54	BSEP.20	0,74	BSEP.29	0,29
BSEP.3	1,73	BSEP.12	0,41	BSEP.21	0,96	BSEP.30	0,51
BSEP.4	0,57	BSEP.13	0,87	BSEP.22	0,88	BSEP.31	0,38
BSEP.5	1,69	BSEP.14	0,76	BSEP.23	0,55	BSEP.32	0,34
BSEP.6	1,42	BSEP.15	0,43	BSEP.24	0,85	BSEP.33	0,42
BSEP.7	1,36	BSEP.16	0,68	BSEP.25	1,24	BSEP.34	0,21
BSEP.8	1,13	BSEP.17	1,37	BSEP.26	0,29	BSEP.35	0,13
BSEP.9	0,9	BSEP.18	0,84	BSEP.27	0,47	BSEP.36	0,26

Isolation of pathogenic fungi and patogenicity 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.

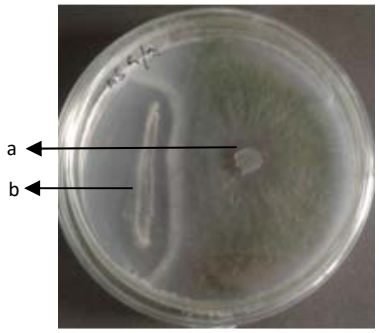

Figure 1: Inhibition activity of chitinolytic bacteria isolate BSEP.3 (a) against pathogenic fungi isolate CSAJ.2; (b) on PDA media.

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

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obtained during the early stationary phase after 16 h of incubation (Figure 2).

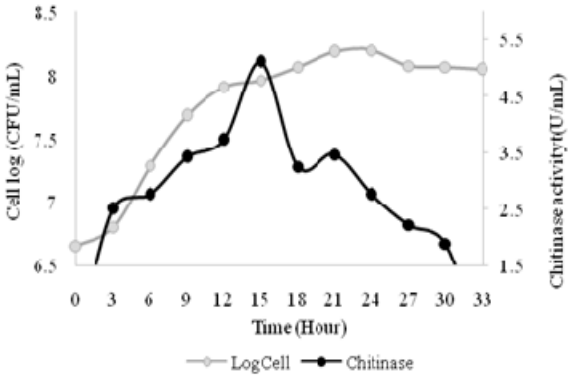


Figure 2: Growth curve and chitinase production of chitinolytic bacteria isolate BSEP.3.

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 occured due to impact of chitinase (Figure 3b).

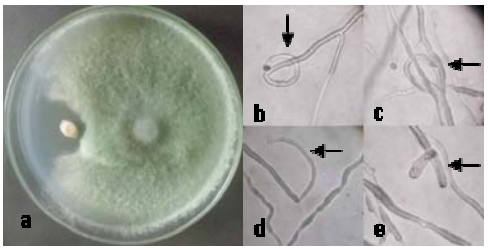


Figure 3: (a) Inhibition activity of chitinase crude extract on PDA media and microscopic observations; (b) roll hyphae at the tip of hyphae; (c) circular hyphae at the middle of hyphae; (d) bend hyphae; (e) stunted and lysis hyphae.

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*.

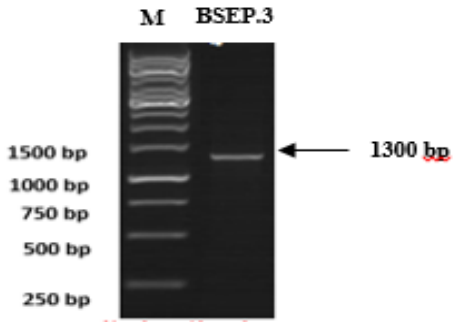


Figure 4: DNA Visualization of 16S rRNA gene of chitinolytic bacteria isolate BSEP.3.

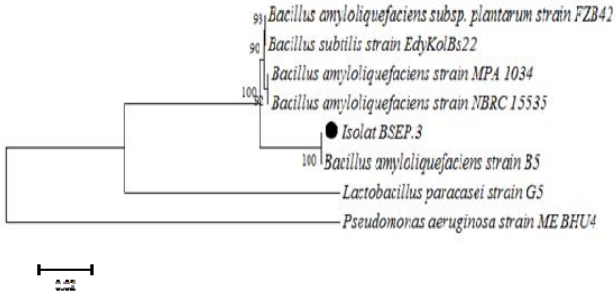


Figure 5: Construction of phylogenetic tree of chitinolytic bacteriaisolate BSEP.3.

On the other hand, amplification of 16S rRNA gene on 1% agarose gel produced DNA size of 1300 bp (Figure 4). Sequence analysis of gene encoding 16S rRNA in the GenBank (BLASTN) also demonstrated that isolate BSEP.3 had 98% identity with *B. amyloliquefaciens* strain B5 (KM384034.1). The relationship of isolate BSEP.3 with other chitinolytic bacteria was shown in Figure 5.

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.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

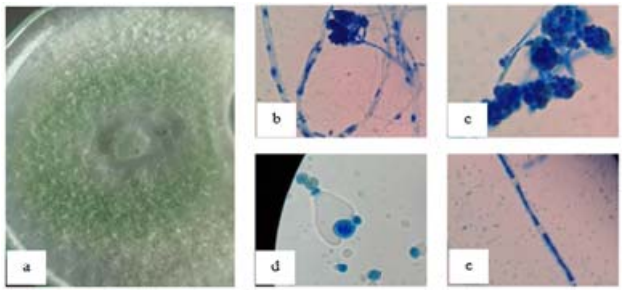


Figure 6: Morphology and anatomy of pathogenic fungi isolate CSAJ.2 (a) green circle of isolate CSAJ.2 on Petri disk; (b) conidiophore; (c) conidia; (d) chlamidyspore; (e) hyphae.

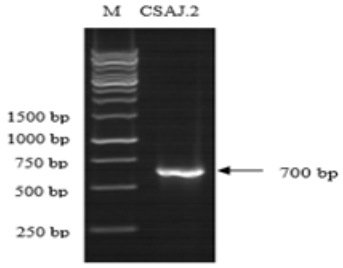


Figure 7: DNA visualization of ITS region of the rDNA of pathogenic fungi isolate CSAJ.2.

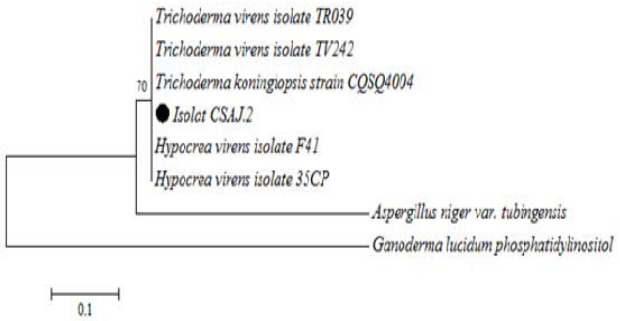
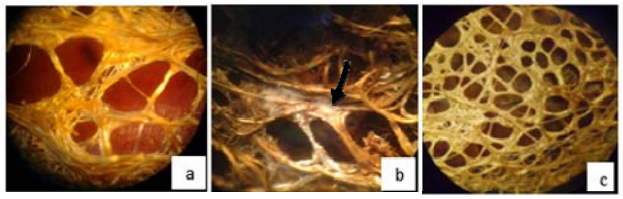


Figure 8: Construction of phylogenetic tree of pathogenic.



fungi isolate CSAJ.2.

Figure 9: (a) Uninfected cocoon (control); (b) infected cocoonby *Trichoderma virens* CSAJ.2; (c) applied cocoon by chitinase crude extract of *Bacillus amyloliquefaciens* BSEP.3.

DISCUSSION

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 spesific 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 occured 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. amyloliquefaciens* B5 and had a close relationship with *B. subtilis. Bacillus amyloliquefaciens* 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-1,3glucanase activity produced by *Bacillus* sp. could degrade the cell wall of the cell wall of *Trichoderma* sp. In addition, *B. amyloliquefaciens* has been widely reported as a biocontrol agent against *Colletotrichum lagenarium*,

ISSN (print): 1823-8262, ISSN (online): 2231-7538

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. trifenestrata* which can reduce the quality and quantity of gold silkworm *C. trifenstrata*. 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. trifenestrata*. Isolation of chitinolytic bacteria as antagonistic bacteria was the first step in biological control procesess. 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. trifenestrata* so that the qualities and quantities of cocoon can be maintained.

ACKNOWLEDGEMENT

This research was supported by the Director General of Higher Education Republic of Indonesia in 2013/2014.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538