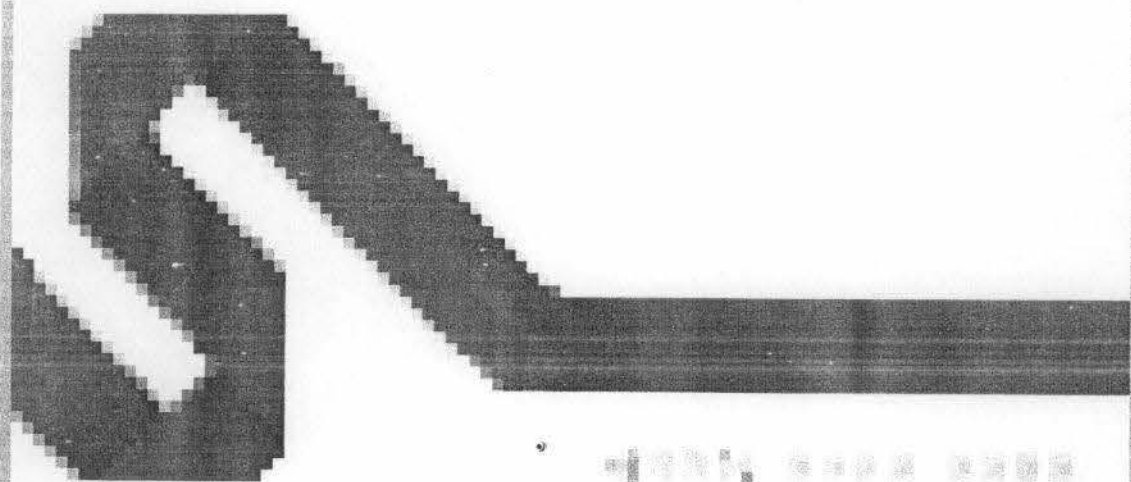


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Partial Purification of Bacterial Chitinase as Biocontrol of Leaf Blight Disease on Oil Palm

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ABSTRACT

Chitin is a major component of fungi cell wall, mycelia, stalks and spore which can be hydrolyzed by chitinase. This study was conducted to measure the ability of chitinase producing bacteria in degrading chitin of fungal pathogens such as *Curvularia affinis* and *Colletotrichum gloeosporioides*. These pathogens caused antrachnose, leaf blight and rotting on oil palm leaves. Chitinase producing bacteria, *Bacillus thuringiensis* SAHA 12.08 isolate were used in this study. SAHA 12.08 showed maximum chitinase with specific activity (7.896 U mg⁻¹ protein) at 60 h incubation. Maximum temperature and pH of chitinase activity were 35°C and 7.0, respectively. Chitinase was partially purified by 30% ammonium sulphate precipitation could increase 2.35 fold than the specific activity. The activity of partially purified chitinase was optimal at 45°C and 7.0, respectively. This chitinase was stable at optimum temperature for 180 min incubation. On SDS-PAGE analysis, the enzyme had molecular weight of 107, 102, 82, 63, 55, 46 and 44 kDa from zymogram analysis only 82 kDa protein band showed chitinase activity. *In vitro* and detached leaf bioassay showed that chitinase of SAHA 12.08 had antagonist activity and biocontrol efficacy to *C. affinis* and *C. gloeosporioides* in oil palm leaves.

Key words: Antagonistic activity, *Colletotrichum gloeosporioides*, *Curvularia affinis*, biocontrol efficacy, chitinolytic bacteria

INTRODUCTION

Chitin is a polysaccharide consisting of β -1,4-N-acetylglucosamine (GlcNAc) residues that are highly cross-linked by hydrogen bonds. Chitin is one of the most abundant renewable natural resource after cellulose. Chitin is widely distributed in nature as it is found in the cuticles of insects, shells of crustaceans, nematodes and cell walls of most fungi (Gohel *et al.*, 2006; Bhattacharya *et al.*, 2007; Aranaz *et al.*, 2009).

Curvularia affinis and *Colletotrichum gloeosporioides* are fungi causing leaf blight and leaf spots disease on oil palm nursery which decrease its economic value. In Indonesia the disease was still categorized as secondary diseases but in Thailand it seems to be serious disease in plant where 61.01% caused by *Curvularia* sp. and 22% caused by *Colletotrichum* sp. (Kittimorakul *et al.*, 2013) thus, the study about early prevention of this disease is required. Chitin contained in the cell walls, mycelium, stalks and spores of pathogenic fungi are able to degrade by chitinase (Peter, 2005).

Chitinase is a group of enzymes that can degrade chitin polymer with 2 stages. Endochitinase (EC 3.2.1.14) degrade the polymer into oligomers then degraded to monomers by exochitinase (β -N-acetylhexosaminidase (EC 3.2.1.52). Enzyme is found in a wide variety of organisms such as

bacteria (Liu *et al.*, 2010), actinomycetes (Gherbawy *et al.*, 2012), fungi (Lee *et al.*, 2009), insects (Bansode and Bajekal, 2006) and plants (Matsushima *et al.*, 2006). The use of chitinase some this decades is increasing along with the wide range application of this enzyme. One of them is used as a biocontrol against various types of fungal pathogens (De la Vega *et al.*, 2006) which is expected to reduce the use of synthetic fungicides. Those efforts are continued throughout the world wide to increase the production of chitinase from various bacterial isolates. The most species is often used as biocontrol agent from group *Bacillus*. Various chitinase producing *Bacillus* species have been reported among *B. cereus* (Huang *et al.*, 2005), *B. thuringiensis* (De la Vega *et al.*, 2006) and *B. licheniformis* (Kamil *et al.*, 2007). Some of the latest characteristics of bacterial chitinase has been widely reported (Bhattacharya *et al.*, 2007). However, there were still no reports about the purification and characterization of indigenous bacterial chitinase which used as a biocontrol agent of *C. affinis* and *C. gloeosporioides*. The objectives of the study were to evaluate partial purified and characterize extracellular chitinase of *Bacillus thuringiensis* SAHA 12.08 and determine its potency as biocontrol of *C. affinis* and *C. gloeosporioides*.

MATERIALS AND METHODS

Microorganism strain and growth conditions: *Bacillus thuringiensis* SAHA 12.08 was previously isolated from soil in Jambi, Indonesia (Haryanto, 2013). Culture was grown and maintained on solid medium chitin agar at 37°C. The fungal pathogens *C. affinis* and *C. gloeosporioides* are isolated from infected oil palm leaves (Haryanto, 2013; Purnamasari, 2013). The isolated fungi were grown on Potato Dextrose Agar (PDA) plates and incubated at 28°C for 4-6 days. The both of isolate are collected at IPB Culture Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Indonesia.

Modelling bacterial growth curve from isolates and chitinase production: Inoculation of 2-4 loopful of selected isolates into 50 mL nutrient broth (NB) supplemented with 0.3% chitin colloidal and incubated at 37°C for 15 h. In amount of 1% culture (10^8 cells mL⁻¹) was inoculated into 100 mL NB with colloidal chitin which used enzyme production medium. Each culture was incubated at 37°C, 120 rpm and collected every 6 until 72 h and their optical density were measured spectrophotometrically at 600 nm. The cultures were then centrifuged at 6000 rpm (centrifuge Hermle with rotor 220.97) for 20 min to obtain the crude extract of extracellular chitinase enzyme.

Measurement of chitinase activity and protein concentration: Chitinase activity was measured by Spindler (1997). The crude extract of extracellular enzyme was added to 0.3% colloidal chitin and 0.1 M phosphate buffer at 37°C, pH 7.0 and agitated at 120 rpm. The mixture was incubated at 30°C for 30 min. After centrifugation at 8400 g for 5 min, the filtrate was added to 750 µL distilled water and 1500 µL Schales reagent (K-Ferrisianida and 0.5 M Na₂CO₃) and the mixture was boiled at 100°C for 10 min. Enzyme activity was determined by measuring absorbance at 420 nm and using Glc-NaCl as a standard. Protein concentration was determined by the Bradford (1976) using bovine serum albumin as a standard.

Partial purification of chitinase: Partial purification was performed using ammonium sulphate. Crude enzyme obtained was precipitated with ammonium sulphate concentration 0-10% up to 60-70% (Scopes, 1994). Precipitates are stored at 4°C for 24 h and centrifuged at 6000 rpm (centrifuge Hermle with rotor 220.97) for 30 min at 4°C. The protein pellet was added to phosphate buffer (0.1 M) at pH 7. Suspension of the protein was stored at -10°C for further testing.

Electrophoresis and zymogram: The protein pellet was added by phosphate buffer (0.1 M) pH 7. Electrophoresis was carried out under denatured proteins (SDS-PAGE) and zymogram. Chitinase solution from crude enzyme and ammonium sulphate precipitation was viewed using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970) with 4% acrylamide concentration in the stacking gel and 10% acrylamide in the separation gel. After electrophoresis, gels were stained with coomassie brilliant blue G-250. The chitinase activities were also detected on gels by using SDS-PAGE and zymogram supplemented with 0.3% colloidal chitin.

Characterization of chitinase activity: The crude extract and partially purification was characterized at its optimum pH and temperature with its stability was determined at optimum pH and temperature. The optimum pH for chitinase activity was determined by measurements at different pH values (4.0-10.0) using colloidal chitin as a substrate under standard assay conditions. The buffers used were as follows: 0.1 M citrate buffer (pH 4.0-6.0), 0.1 M phosphate buffer (pH 7.0-8.0) and 0.1 M glycine-NaOH buffer (pH 9.0-10.0). The optimum temperature was determined by incubating the reaction mixtures at different range of temperature from 25-60°C with 5°C interval from the optimum pH, obtained from the previous experiment. The stability of chitinase was investigated by incubating the enzyme with optimum pH and optimum temperature for 180 min.

Effect of chitinase on fungal pathogens by *in vitro* test: The effect of isolate SAHA 12.08 was tested against *C. affinis* and *C. gloeosporioides* on PDA. Antagonistic activity was tested by using cell culture, crude chitinase and partial purification enzyme of the strain with agar well diffusion method. Well containing 200 µL culture or crude chitinase was at distance of 3 cm from margin of PDA plate. Opposite the well, at a distance of at least 3 cm, suspected pathogenic fungi were placed. After incubation for 6 days at 27°C, inhibition of the pathogen's development was assessed with parameter. The percentage of inhibition of radial growth can be estimated by following equation:

$$\text{Inhibition of radial growth (\%)} = \frac{r_1 - r_2}{r_1} \times 100$$

where, r_1 is length of radial growth towards plate margin (3 cm) and r_2 is length of radial growth towards antagonistic (Fokkema, 1973).

Biocontrol assay by detached leaf test: The 60 h cell culture, crude chitinase and partial purification chitinase were tested to biocontrol effectiveness (He *et al.*, 2003). Oil palm leaf was washed using sterile aquadest. Treatment consists of 60 h cell culture, crude enzymes and partial purification enzyme. For negative control oil palm leaves were inoculated with fungi pathogens only, using *C. affinis* and *C. gloeosporioides*. The whole treatments were conducted four times. Each leaf was treated, cut up to size of 4×3 cm. Thereafter the leaf was dipped into treatments for 30 min then air dried. The leaves put and placed into a petri dish containing a wet filter paper to maintain the humidity. After 1-2 h, 50 µL of fungal pathogens at a concentration of 4×10^6 spores mL^{-1} to the one end of the surface of the leaves and then incubated for 7 days in the dark conditions at room temperature. Percentage of biocontrol efficacy of treatment (BC) and Disease Incidence (DI) calculated using the equation of Chanchaichavivat *et al.* (2007):

$$\text{BC (\%)} = \frac{T - A}{T} \times 100$$

and:

$$DI (\%) = \frac{A}{T} \times 100$$

Where:

BC = Biocontrol efficacy

T = No. of infected spots inoculated with pathogens only

A = No. of infected spots inoculated with antagonist and pathogens

DI = Disease incidence

Statistical analysis: Data was analysed using MS.Excel Software.

RESULTS

Growth curve and chitinase activity of isolate: The isolates were grown in NB was enriched by colloidal chitin (pH 7.0) at 37°C. The growth rate of SAHA 12.08 isolate was ascended from 0-24 h of incubation and then growth was relatively stable up to 60 h incubation and descended after 72 h incubation (Fig. 1). The chitinase was produced from 30 h incubation and relatively increased until 60 h incubation. The highest chitinase activity was found at the 60 h incubation, after 66 h of incubation the activity was not found (Fig. 1).

Partial purification of chitinase: The partial purification procedures of the chitinase secreted by the SAHA 12.08 isolate are summarized in Table 1. The results of precipitation using ammonium sulphate 0-10% up to 60-70% (w/v) showed that at concentration of 30% it was able to produce a

Table 1: Summary of chitinase partially purification produced by *B. thuringiensis* SAHA 12.08

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹ protein)	Purification (fold)	Yield (%)
Crude extract	19.6800	142.60	7.240	1.00	100
30% ammonium sulphate precipitation	0.0842	1.44	17.061	2.35	1.01

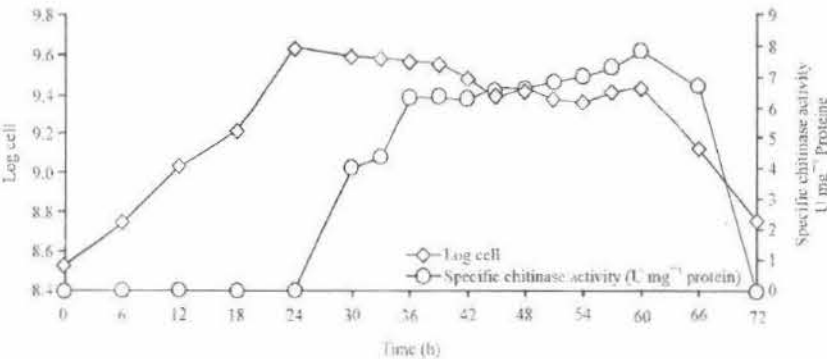


Fig. 1: Growth and chitinase activity of the *B. thuringiensis* SAHA 12.08 isolate on production media enriched with colloidal chitin

maximum chitinase specific activity 17.061 U mg^{-1} (Fig. 2) and increase the purity upto 2.35 fold. Furthermore, ammonium sulphate at 30% (w/v) was used for precipitation of protein in crude enzyme.

Chitinase from precipitation method was analyzed by SDS-PAGE to obtain the number of protein bands and molecular weight. Zymogram was analyzed using 0.3% colloidal chitin as a substrate (Fig. 3). The result of SDS-PAGE using Coomassie Brilliant Blue G-250 (CBB G-250) showed at least seven bands (molecular) of SAHA 12.08 chitinase protein fraction of 30% ammonium sulphate with an estimated molecular weight viz 107, 102, 82, 63, 55, 46 and 44 kDa. Zymogram result using 0.1% Congo red showed one protein molecule which had chitinase activity with molecular weight of 82 kDa. It was calculated on the basis relative mobility of standard proteins.

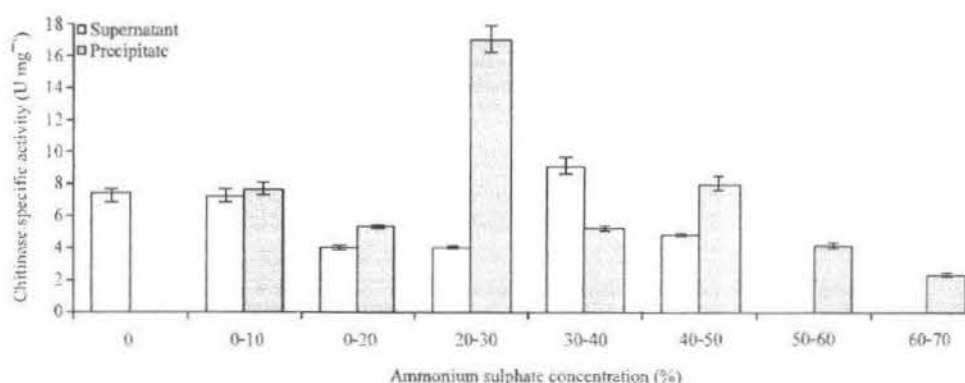


Fig. 2: Effect of ammonium sulphate concentration on precipitation of *B. thuringiensis* SAHA 12.08 chitinase. Bars represent standard error

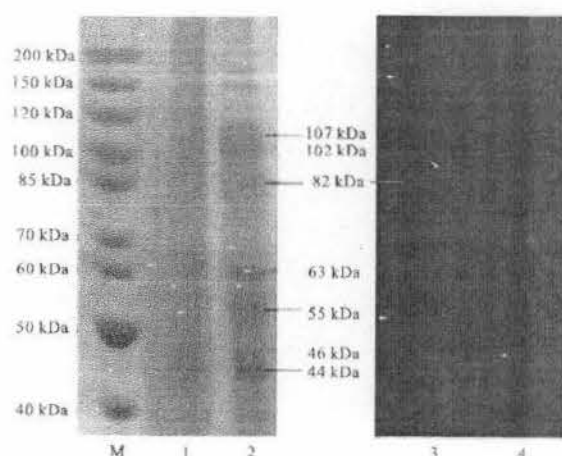


Fig. 3: SDS-PAGE and zymogram of chitinase SAHA 12.08. M: Molecular weight standards, 1: SDS PAGE crude enzyme, 2: SDS PAGE of 30% $(\text{NH}_4)_2\text{SO}_4$ precipitation, 3: Zymogram of 30% $(\text{NH}_4)_2\text{SO}_4$ precipitation, 4: Zymogram of crude enzyme

Characterization of chitinase activity: Maximum pH of the crude enzyme and partially purification enzyme isolate, SAHA 12.08 was maximum at pH 7.0 with the activity 0.697 and 0.721 U mL⁻¹, respectively (Fig. 4). Among the different temperature tested, the chitinase activity of crude enzyme was most active at 35°C being 0.722 U mL⁻¹ but maximum activity of partially purification of enzyme was observed at 25, 45 and 60°C being 0.722, 0.723 and 0.719 U mL⁻¹, respectively (Fig. 5). To determine its stability, the crude enzyme and partial purification enzyme was incubated at optimum temperature for a period of 3 h. Both chitinase were stable at maximum pH and temperature up to 180 min of incubation (Fig. 6).

Effect of chitinase on fungal pathogens by *in vitro* test: Inhibition of the growth of fungal pathogens i.e., *C. affinis* and *C. gloeosporioides* was observed *in vitro* by using 60 h cell culture, crude chitinase and partial purification chitinase on agar well-diffusion method (Fig. 7). Inhibition of those fungal pathogens by SAHA 12.08 isolate chitinase showed that it was able to inhibit *C. affinis* better than *C. gloeosporioides* after 5 days incubation. Partial purification chitinase could

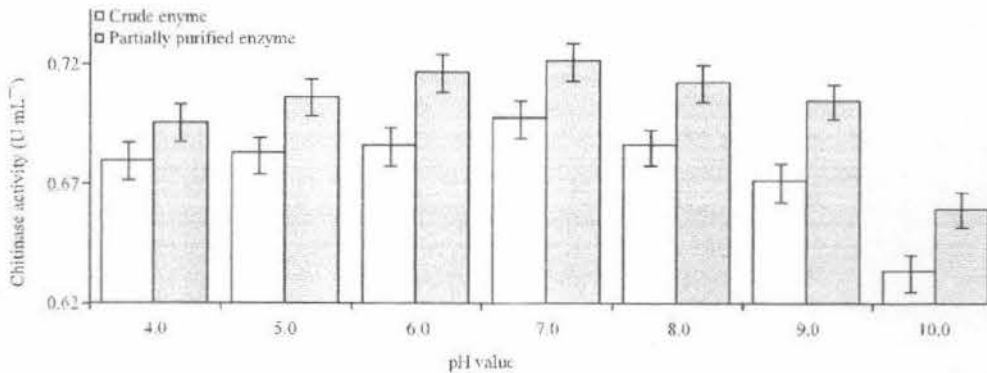


Fig. 4: Effect of pH values on chitinase activity of crude enzyme and partially purified enzyme of *B. thuringiensis* SAHA 12.08. The activity was measured at 37°C. Bars represent standard errors

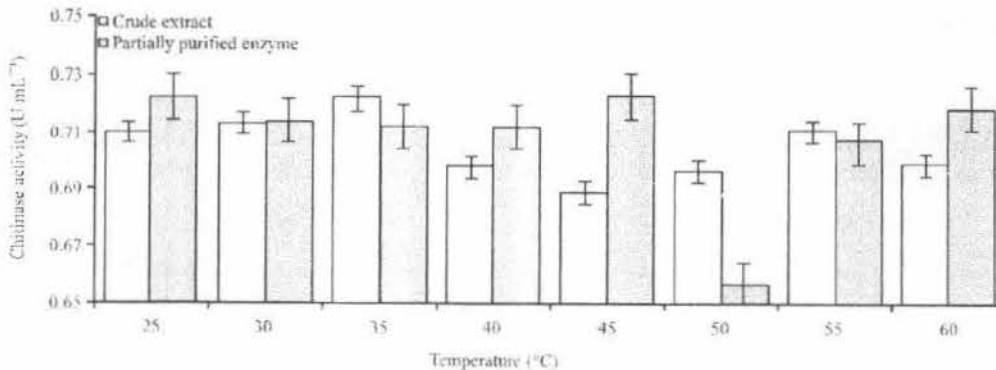


Fig. 5: Effect of temperature on chitinase activity of crude enzyme and partially purified enzyme of *B. thuringiensis* SAHA 12.08. The activity was measured at pH 7.0. Bars represent standard error

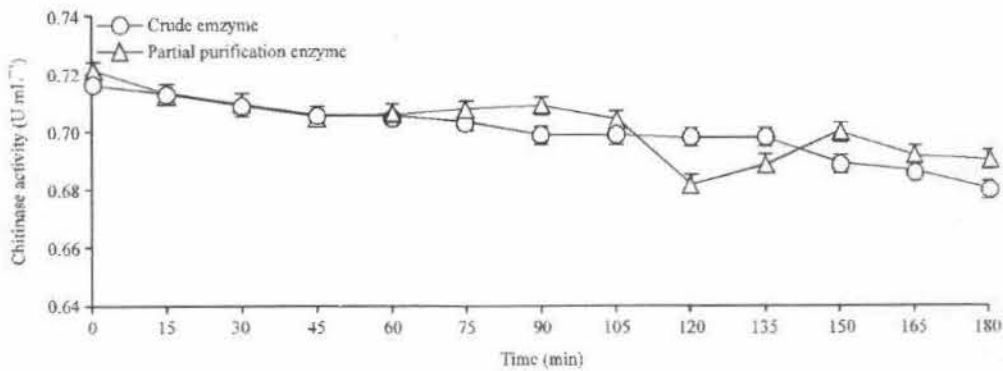


Fig. 6: Stability of crude chitinase and partially purified chitinase at optimum pH and temperature of *B. thuringiensis* SAHA 12.08. Bars represent standard error

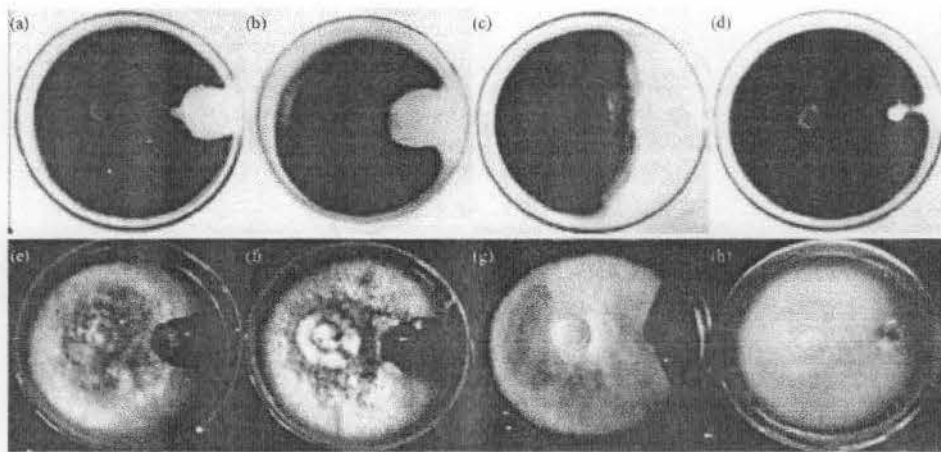


Fig. 7(a-h): Effectiveness of antagonist *B. thuringiensis* SAHA 12.08 against *C. affinis* and *C. gloeosporioides* in antagonist assay after 7 days incubation on PDA medium. For *C. Affinis* (a) 60 h cell culture, (b) Crude enzyme, (c) Partial purification enzyme, (d) Control. For *C. gloeosporioides* (e) 60 h cell culture cell, (f) Crude enzyme, (g) Partial purification enzyme and (h) Control of after 7 day incubation

inhibit the growth of *C. affinis* and *C. gloeosporioides* better than crude chitinase and 60 h cell culture for about 39.89 and 35.80%, respectively after 5 days inoculation. Observations for 9 days showed that culture cell 60 h, capable to inhibit *C. affinis* up to 9 days while the crude extract has the highest inhibition of fungal pathogens at the early inoculation and the activity tend to decrease until 9 days. The same result was also showed by partial purification enzyme, the ability to inhibit was decreased. However, it was still higher than the other treatments (Fig. 8a). Whereas, the inhibition of *C. gloeosporioides* among those three treatments showed the similar patterns in which there was a declining of the activity along the incubation period (Fig. 8b).

Biocontrol assay by detached leaf test: Biocontrol efficacy test used two treatments, as cell culture 60 h and crude enzyme. However, partial purification of enzyme was not done because the enzyme can destruct the tested leaves. Effective test of the biocontrol from

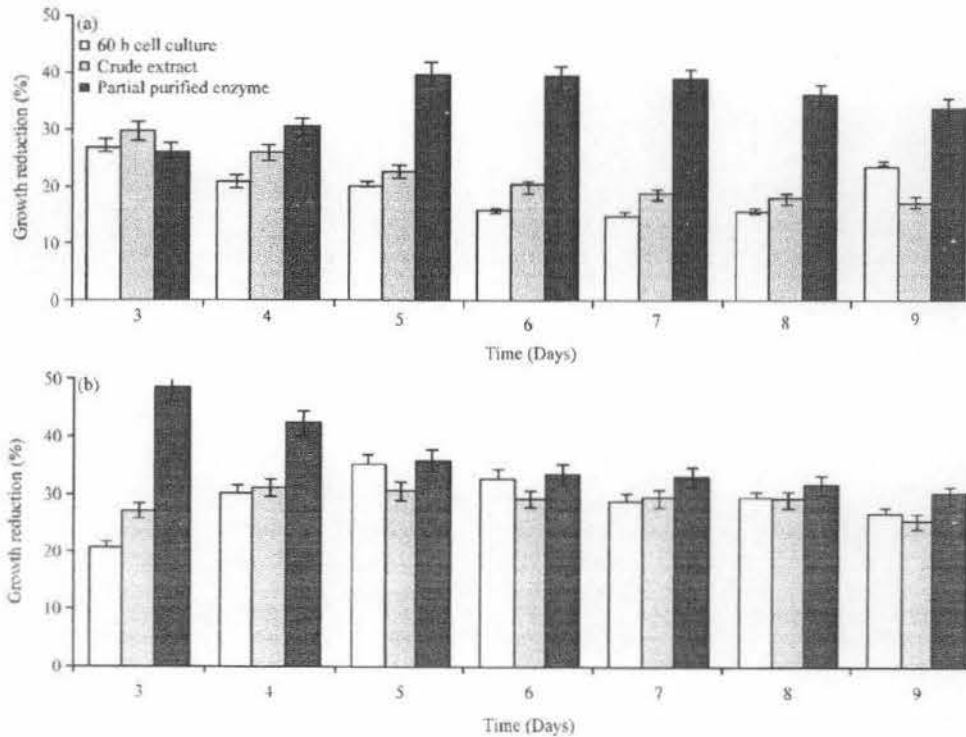


Fig. 8(a-b): Growth reduction of (a) *Cryptocoryne affinis* and (b) *Colletotrichum gloeosporioides* in the presence of *B. thuringiensis* SAHA 12.08 chitinases

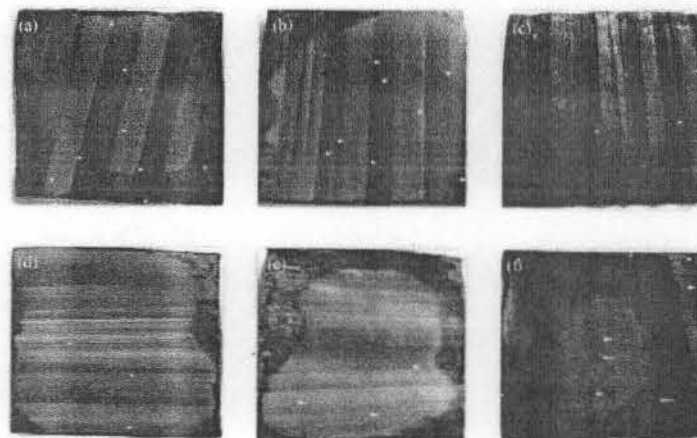


Fig. 9(a-f): Effectiveness of antagonist *B. thuringiensis* SAHA 12.08 against leaf blight disease caused by *C. affinis* and *C. gloeosporioides* in bioassay using oil palm leaves as for *C. affinis*, (a) 60 h cell culture, (b) Crude enzyme, (c) Control and for *C. gloeosporioides*, (d) 60 h cell, (e) Crude enzyme and (f) Control

each treatments showed that the culture cell 60 h and the crude chitinase have the ability to inhibit the leaf blight caused by *C. affinis* and *C. gloeosporioides* (Fig. 9). The crude enzyme could reduce

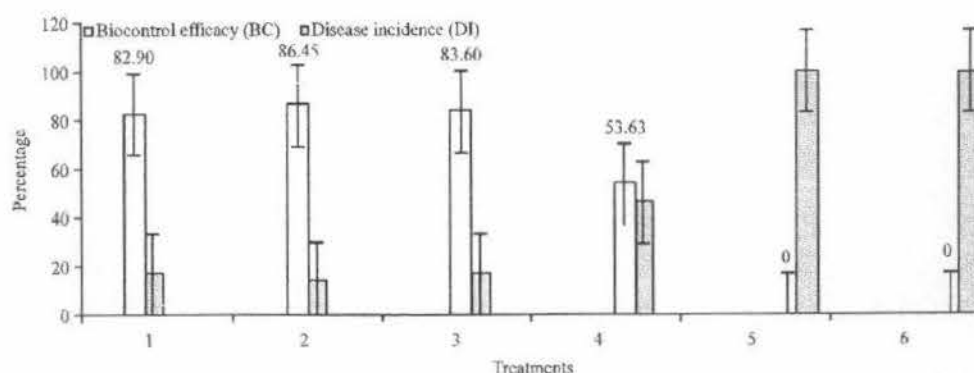


Fig. 10: Effectiveness of the treatments *B. thuringiensis* SAHA 12.08 in reduction of disease incidence in *C. affinis* and *C. gloeosporioides*. 1: 60 h cell culture cell + *C. affinis*, 2: Crude enzyme + *C. affinis*, 3: 60-h cell culture + *C. gloeosporioides*, 4: Crude enzyme + *C. gloeosporioides*, 5: Control of *C. affinis* and 6: Control of *C. gloeosporioides* after 5 days incubation

leaf blight caused by *C. affinis* better than the cell culture 60 h with the effectiveness value of biocontrol for about 86.45% or with disease incidence for about 13.5%. Whereas, the reduction of leaf blight caused by *C. gloeosporioides* showed by using 60 h cell culture was better than crude enzyme for about 83.6% or with disease incidence for about 16.36% (Fig. 10). The results indicated that there was correlation between the activity of *in vitro* antagonist and biocontrol efficacy by detached leaf assay test.

DISCUSSION

Isolate of *B. thuringiensis* SAHA 12.08 produced extracellular chitinase which are capable to hydrolyze substrates such as colloidal chitin. The specific activity of the enzyme began to find at the stationary phase when the number of cell tend to decrease. The highest specific activity of the enzyme occurred in the 60 h of incubation. The highest enzyme production was also previously reported by Narayana and Vijayalakshmi (2009), the maximum chitinase production of *Streptomyces* sp. ANU 6277 observed at 60 h of incubation and tended to decrease due to the increasing of incubation period. Almost all chitinase from bacteria has shown maximum activity at 30-96 h of incubation (Zhu *et al.*, 2007; Faramarzi *et al.*, 2009; Nurdebyandaru *et al.*, 2010).

Crude extracts of enzyme obtained from the highest enzyme production could be precipitated with ammonium sulphate 30% (w/v) and capable to increase the purity of 2.35 fold. The percentage saturation of ammonium sulphate for each chitinase from different isolates was not the same. The percentage saturation of ammonium sulphate for each chitinase from different isolates was not same. The saturation level of precipitation are ranged from 30-85% (Zhang *et al.*, 2001; Kim *et al.*, 2003; Rabeeth *et al.*, 2011). Previous studies reported that chitinase from *B. circulans* WL-12 was capable to precipitate with 46% ammonium sulphate (Watanabe *et al.*, 1994). The molecular weight of the protein was found to be about 107, 102, 82, 63, 55, 46 and 44 kDa by 10% SDS PAGE and showed a single band 82 kDa on 10% zymogram analysis indicating the chitinase activity. The molecular weight of chitinase of *Bacillus* sp. i.e., *B. thuringiensis* was analyzed by SDS-PAGE, had more than one protein molecule in the range of 32-125 kDa (De la Vega *et al.*, 2006; Barboza-Corona *et al.*, 2008; Liu *et al.*, 2010; Kuzu *et al.*, 2012).

The highest activity of both enzymes was at pH 7.0. Chitinase isolates, both crude enzyme and partial enzyme showed a wide range pH from 4-10. Wang and Chang (1997) suggested that the optimum chitinase of most bacteria are varieties from acidic pH to alkaline. The highest activity of both enzymes was at pH 7.0. The same results also reported that chitinase of *B. thuringiensis* (Gomaa, 2012) and *Bacillus* sp. (Mubarik *et al.*, 2010) had the highest activity at pH 7.0. The enzyme activity was determined by the presence of donor protons and donor acceptors on the degree of ionization, obtained at pH optimum pH (Wilson and Walker, 2005). Proton donors and acceptors contained many degrees of ionization on the sites of catalytic enzyme to support optimum conditions, moreover, the pH also affects the solubility of the substrate. Extreme pH caused changes in the structure of the enzyme and then decrease the effectiveness and efficiency of enzyme activity (Farabee, 2001).

Crude enzyme showed the highest activity at 35°C. While the partial enzyme showed the highest range activity at a temperature of 25, 45 and 60°C. Most of the chitinase of bacteria have maximum activity at a wide temperature. Various reports indicated that the maximum activity was at 30-75°C (Frandberg and Schurer, 1994; Sakai *et al.*, 1998; Barboza-Corona *et al.*, 2008; Mubarik *et al.*, 2010; Thiagarajan *et al.*, 2011; Haggag and Abdallh, 2012; Margino *et al.*, 2012). Both of crude chitinase and partial chitinase stable at optimum temperature for 180 min of incubation. Kuzu *et al.* (2012) also reported the same thing on *B. thuringiensis* ssp. *kusrtaki* HBK-51 which remains stable and active for 3 h at 30-120°C.

Chitinase enzyme is currently used in biological control of fungal pathogens because of its ability to degrade chitin in fungal cell walls (Ulhoa and Peberdy, 1991). This study was conducted to analyze the ability of 60 h cell culture, crude enzyme and partial enzyme of *B. thuringiensis* SAHA 12.08 isolate to inhibit the growth of leaf blight pathogens (*C. affinis* and *C. gloeosporioides*) by *in vitro* test. The three chitinase treatments were capable to inhibit the growth of both pathogenic fungi *in vitro*. Inhibition by partial chitinase was better than crude enzyme and 60 h cell culture against these fungal pathogens. Those results were probably caused by the length of time needed by the cell culture to produce chitinase while partial chitinase and crude enzyme were capable to hydrolyze chitin found in fungal cell walls directly. Differences among the ability of inhibition against fungal pathogens were caused by the concentration of hydrolytic enzyme or secondary metabolic compounds found in partial enzyme or crude enzyme (Prapagdee *et al.*, 2008).

Effect of treatment of chitinase against leaves infected of fungal pathogens by detached leaf test has been conducted and the results are presented in Fig. 10. Crude enzyme has inhibitory against *C. affinis*, better than 60 h cell culture. However, different things are shown against *C. gloeosporioides* 60 h cell culture capable of inhibiting better than crude enzyme. The results indicated that there was correlation between the activity of *in vitro* antagonist and biocontrol efficacy by *in vivo* test. The results caused by the synergism of action of biocontrol mechanisms of cell culture such as antibiotic production and induction of plant resistance with genes activation such as chitinase, β 1, 3-glucanase, peroxydases and phenylalanine ammonia lyase (Chang *et al.*, 2007). Although there are no reports on oil palm leaf blight biocontrol using chitinase from *Bacillus* sp. Several *Bacillus* species have been widely used as biocontrol agents pathogenic fungi both of cells, crude enzyme and partial chitinase including the germination of seeds protected with *B. licheniformis* and *B. thuringiensis* for seed infested with *A. niger* (Gomaa, 2012) and chitinase of *B. thuringiensis* ssp. *aizawai* able to protect of bean seeds infested with six phytopathogenic fungi (De la Vega *et al.*, 2006).

CONCLUSION

Bacillus thuringiensis SAHA 12.08 produced chitinase at 60 h incubation. This chitinase was purified 2.35 fold with 30% ammonium sulphate. Crude chitinase and partially chitinase had maximum pH at 7.0, 35 and 45°C, respectively with molecular weight of 107, 102, 82, 63, 55, 46 and 44 kDa on SDS-PAGE analysis and 82 kDa on zymogram. The both of chitinase were stable at optimum temperature and pH for 180 min. This chitinase could better inhibit *C. affinis* than *C. gloeosporioides* in vitro and detached leaf assay and has potential application as biocontrol agents for *C. affinis* and *C. gloeosporioides*.

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