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Selection of chitinolytic bacteria as biological control of *Colletotrichum capsici*

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ABSTRACT

Aims: The objectives of this study were to screen chitinolytic bacteria isolated from soil of Taman Nasional Bukit Duabelas, Jambi, Indonesia. Isolates were selected based on chitinolytic index and antagonism activity of *Colletotrichum capsici*. Chitinase enzyme from selected isolates was investigated for growth inhibition of *C. capsici*.
Methodology and results: Two chitinolytic bacteria were selected based on their ability to degrade colloidal chitin and inhibit of the growth of *C. capsici*. Those isolates were KAHN 15.12 and SAHA 12.12, identified as *Serratia marcescens* and *Bacillus thuringiensis* respectively based on 16S rRNA gene. The chitinase maximum specific activity of isolate KAHN 15.12 was 52.03 U/mg after 36 h of incubation and SAHA 12.12 was 45.67 U/mg after 24 h of incubation. The enzyme was precipitated by ammonium sulfate 40% and 60% respectively for KAHN 15.12 and SAHA 12.12. The precipitated chitinases were active over a broad range of pH (5 to 10) and temperature (20 to 80 °C). Enzymes were stable in optimum temperature for 180 min. The precipitated of chitinase KAHN 15.12 and SAHA 12.12 had five and two protein bands respectively on SDS-PAGE gel. Chitinases exhibited an antifungal activity against *C. capsici* at concentration of 60 ppm.
Conclusion, significance and impact of study: Isolates KAHN 15.12 and SAHA 12.12 were successfully selected by their ability to degrade colloidal chitin and inhibit the growth of *C. capsici*. The isolates had a broad range of pH and temperature, moreover relatively stable at the optimum temperature. Chitinase was effective as biological control for anthracnose caused by *C. capsici* in chilli.

Keywords: Bacillus thuringiensis SAHA 12.12, characterization, chitinase, *Colletotrichum capsici*, *Serratia marcescens* KAHN 15.12.

INTRODUCTION

Chitinolytic bacteria were able to degrade chitin by chitinase-producing bacteria. Among bacteria, only the chitinases from the genera *Bacillus*, *Aeromonas*, *Vibrio*, *Enterobacter*, *Serratia* and *Pseudomonas* (Thompson *et al.*, 2001) have been characterized. Chitin is homopolymer of β-1.4-N-asetil-D-glucosamine (GlcNAc) with broad spectrum distribution in biosphere after cellulose such as in shells of crustaceans, insect exoskeletons, and cell wall of fungi (22% to 44%) (Gohel *et al.*, 2006; Mukherjee and Sen, 2006).

Colletotrichum capsici is one of fungi that cause anthracnose in chilli plants (Amusa *et al.*, 2004). Symptoms of anthracnose can be found in the form of concentric spots with black spots on the surface of fruit and appearance of necrotic (Than *et al.*, 2008). Anthracnose is a problem of crop yield in most tropical and subtropical regions. Anthracnose infection was reported cause of yield losses of up to 50% and 100% in India, North America and tropical Africa (Amusa *et al.*, 2004), while in Indonesia losses of up to 10% to 80% in the rainy season and 2% to 35% in the dry season (Widodo, 2007).

Chitinolytic bacteria or chitinase enzyme have potential applications in the biological control of plant pathogenic fungi such as *C. capsici* because of their ability to degrade fungal cell wall (Singh *et al*., 1999; Asril *et al.*, 2014). Chitinases are divided into two categories according to their enzymatic function: endochitinase (EC3.2.1.14) and exochitinase (EC3.2.1.52) which constitute a complex of different degradation enzymes (Toharisman *et al.*, 2005). Biological control of plant pathogens provides an alternative for management of plant disease without the negative impact of chemical fungicides, such as the occurrences of environmental pollution, development resistance, and hazardous for organism (Chang *et al.*, 2003; Gohel *et al.*, 2006).

Some chitinolytic bacteria strains have been studied and characterized from different environments (Mukherjee and Sen, 2006; Ashwini and Srividya, 2014; Asril *et al.*, 2014). However, there were no reports yet about the selection chitinolytic bacteria and characterization of that chitinase which used as a biological control anthracnose

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by *C. capsici*. The research describes the selection chitinolytic bacteria and characterization of chitinase enzyme from selected isolate, with ability to improve its biological control of fungal pathogen *C. capsici*.

MATERIALS AND METHODS

Selection of chitinolytic and antagonist isolates

Five chitinolytic bacteria used in this research were isolated from soil in Taman Nasional Bukit Duabelas, Jambi, Indonesia (Haryanto, 2013). Isolates were grown and maintained on chitin agar media (1% colloidal chitin, 0.1% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, 0.1% yeast extract, 1.5% bacto agar) at 37 °C. These chitinolytic bacteria were subjected to dual culture with five antagonist isolates and one fungal pathogen *C. capsici* on Potato Dextrose Agar (PDA) media at 28 °C. Isolates were selected for use in the current study based on their chitinolytic index and antagonism activity of *C. capsici*. Chitinolytic colonies were identified after incubation for 72 h, by the presence of a clear zone. Morphological characteristics of bacterial colony and Gram stain of the isolates were also observed.

Molecular identification of bacteria selected

The total genomic DNA of KAHN 15.12 and SAHA 12.12 was isolated with the DNA extraction kit and used as the template in PCR. Two oligonucleotide primers were used, a forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.*, 1998). The PCR was started with an initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min and post elongation at 72 °C for 7 min followed by 30 cycles. The PCR products were separated on a 1% (w/v) agarose gel of 80 V for 45 min. Visualization of DNA was carried out using UV transilluminator. The 16S rRNA sequence was analyzed via BLAST server of the National Center for Biotechnology Information (http:ncbi.nlm.nih.gov). A phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) programs, version 6.

Bacterial growth curve from isolate and chitinase production

Two milliliters of liquid culture (OD 0.6 to 0.8) was inoculated into 200 mL NB with 0.3% colloidal chitin as the substrate. Enzyme production medium was incubated on rotary shaker (120 rpm, at 37 °C) and collected every 3 h until 42 h. The optical density was measured spectrophotometrically at 600 nm. The cultures were then centrifuged at 6000 rpm for 30 min to obtain the crude extract of extracellular chitinase enzyme. The crude extracts were used for chitinase activity assay and antagonism activity.

Measurement of chitinase activity and protein determination

Chitinase activity was measured by Toharisman *et al.* (2005) with modification. For the measurement of chitinase activity, colloidal chitin was selected as the substrate. The enzyme solution (150 µL) was mixed with 300 µL of 0.3% (w/v) colloidal chitin dissolved in 150 µL of phosphate buffer (pH 7.0) and incubated at 60 °C for 30 min. The control was prepared by adding inactivated enzyme after incubation. The solution was then centrifuged at 8400 ×*g*, 4 °C for 5 min. The amount of reducing sugar was determined at 420 nm by a modified Schales reagent method (Spindler, 1997; Asril *et al*. 2014). One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1 µmol of reducing N-acetylglucosamine substrate per min. Protein was determined by measuring the absorbance at 595 nm. Protein was also determined by the Bradford method with Bovine Serum Albumin as the standard.

Ammonium sulfate precipitation and characterization of chitinase

Precipitation used serial concentrations of ammonium sulfate from 10% to 80% saturation. The crude extract and precipitate of ammonium sulfate was characterized with several pH and temperature range. The optimum pH for chitinase activity was determined by measurements at different pH values (4.0 to 10.0) using colloidal chitin as a substrate under standard assay conditions. The buffers were as follows: 0.1 M citrate buffer (pH 4.0 to 6.0), 0.1 M phosphate buffer (pH 7.0 to 8.0), and 0.1 M glycine-NaOH buffer (pH 9.0 to 10.0). The optimum temperature was determined by incubating the reaction mixtures at different range of temperature of 20 to 90 °C with 10 °C interval from the optimum pH obtained from the previous experiment. The stability of chitinase was investigated by incubating the enzyme with optimum temperature for 180 min.

SDS-PAGE and zymogram analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the standard method (Laemmli, 1970), using 10% separating gel and 4% stacking gel. The protein was stained by Coomassie brilliant blue R-250. Zymogram analysis was performed on a 10% separating gel containing 0.1% glycol chitin as the substrate. Electrophoresis was carried out with a current of 50 mA for 35 min. After electrophoresis, protein in the gel was renatured in 2.5% Triton X-100 at 37 °C. Next, the gel was submerged for 45 min in 0.1% Congo red dye, and then transferred to 1 M NaCl solution. The zone was visualized using a UV-transilluminator.

Effect of chitinase on fungal pathogen

The effect of chitinase from selected isolate was tested against *C. capsici* on PDA. Antagonistic activities were

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tested using crude chitinase and precipitate enzyme with concentration of 20 ppm, 40 ppm and 60 ppm. After incubation for 7 days at room temperature, inhibition of the pathogen's growth was assessed by the percentage of inhibition [θ control - θ treatment/ θ control × 100].

RESULTS

Selection of chitinolytic and antagonist isolates

Isolates were selected for use in the current study based on their chitinolytic index and antagonism activity of *C. capsici*. There were five isolates performing a clear zone around their colony on chitin agar and the inhibition of the growth against *C. capsici* (Table 1). The results showed one isolates of the highest chitinolytic index, KAHN 15.12 and one isolate of the highest antagonistic activity, SAHA 12.12. Isolate KAHN 15.12 had the greatest chitinolytic index, but the ability of inhibition of *C. capsici* was much smaller only 25% compared to SAHA 12.12 i.e. 37.5%. Isolate KAHN 15.12 was a Gram negative bacterium with, round and red colony, a short rod shape while isolate SAHA 12.12 was a Gram positive bacterium, round and white colony, rod-shape, and endospore forming (Figure 1).

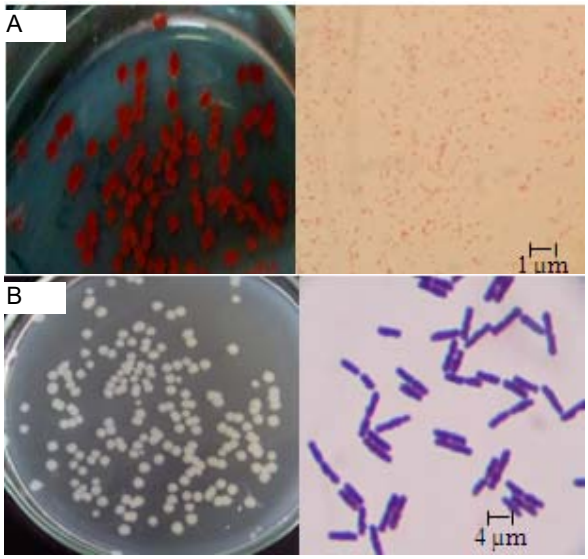

Figure 1: The morphology of colony in nutrient agar (NA) media and Gram stain of isolate A, KAHN 15.12 and B, SAHA 12.

Table 1: Properties of 5 bacterial isolates from soil in Taman Nasional Bukit Duabelas, Jambi, Indonesia.

Isolates	Chitinolytic index	Inhibition of *C. capsici* (%)	Colony		Cell shape	Gram stain
			Shape	Color		
SAHA 12.14	0.25	29	Round	White	Rod	Positive
SAHA 12.12	0.22	37.5	Round	White	Rod	Positive
KAHN 15.12	1.5	25	Round	Red	Short-rod	Negative
SAHA 5.8	0.22	25	Round	White	Rod	Negative
KAHA 7.02	0.38	21	Round	White	Rod	Positive

Molecular identification of bacteria selected

Isolate KAHN 15.12 was previously identified was *Serratia marcescens*. Molecular identification of the isolate was performed based on 16S rRNA gene sequence. The results showed that isolate SAHA 12.12 produced an amplicon size of 1300 bp. DNA sequence analysis of the PCR product using the BLASTN showed that isolate SAHA 12.12 had the closest similarity to *Bacillus thuringiensis* NBRC 101 235 strain with similarities of 99%. Phylogenetic analysis of the *B. thuringiensis* revealed that the closest relatives of the isolate were *Bacillus mycoides*, *B. cereus* and *B. anthracis* (Figure 2). *Serratia marcescens* KAHN 15.12 and *Bacillus thuringiensis* SAHA 12.12 were identified as the chitinolytic bacteria. Various reports had reported about the bacteria that were able to produce the chitinase enzyme, among others *Aeromonas*, *Vibrio*, *Streptomyces*, *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Serratia* (Thompson *et al.*, 2001).

Bacterial growth curve from isolate and chitinase production

Two isolates were measured its growth curve for 42 h in 3 h interval. The growth of isolate KAHN 15.12 was relatively stable from 9 h to 42 h and SAHA 12.12 was descended after 33 h incubation. The chitinase of isolate KAHN 15.12 showed maximum specific activity of 52.03 U/mg after 36 h incubation and *B. thuringiensis* SAHA 12.12 of 45.67 U/mg after 24 h incubation (Figure 3).

Ammonium sulfate precipitation of chitinase

The results showed that isolate KAHN 15.12 at concentration 40% was able to produce maximum specific chitinase activity 34.84 U/mg and increased 1.92 fold higher than the specific activity of crude enzyme with a yield of 1.47%. Isolate SAHA 12.12 precipitated in 60% ammonium sulfate showed chitinase activity 98.61 U/mg and increased up to 3.14 fold with a yield of 1.47% (Table 2).

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Characterization of chitinase

Chitinase activities were characterized by pH, temperature, and stability of the enzyme. The crude and precipitated chitinase KAHN 15.12 showed good activity between temperature of 20 to 80 °C, with optimum activity at 60 °C. The chitinase also maintained good activity in a range of pH from 4.0 to 10 with optimum activity at pH 7.0 and pH 6.0. Crude enzyme and precipitate SAHA 12.12 showed optimum activity at pH 8.0 with optimum temperature at 50 °C, and then the optimum temperature changed to 40 °C after the ammonium sulfate precipitation of the enzyme (Figure 4). Both chitinases were relatively stable at maximum temperature up to 180 min of incubation.

SDS-PAGE and zymogram analysis

Analysis of concentrated enzyme from crude chitinase and precipitated enzyme of KAHN 15.12 and SAHA 12.12 by SDS-PAGE showed multi bands of chitinase protein (Figure 5). The protein bands of KAHN 15.12 were estimated 109.13 kDa, 75.99 kDa, 61.79 kDa, 52.91 kDa and 43.03 kDa, respectively. The protein bands isolate SAHA 12.12 were detected at 80.02 kDa and 68.51 kDa. Zymogram result showed one protein molecular which had activity chitinase with molecular weight of 52.91 kDa of KAHN 15.12 and 68.51 kDa of SAHA 12.12.

Effect of chitinase on fungal pathogen

The antagonistic activity was performed via dual culture. Antagonistic activity of precipitated enzyme KAHN 15.12 showed that the highest inhibition of 46.67% occurred in 5 days and precipitated chitinase SAHA 12.12 of 48.72% occurred in 6 days (Figure 6). The highest concentration to inhibit the growth of fungal pathogen in both isolates was 60 ppm. Precipitated chitinase could inhibit the growth of *C. capsici* better than crude chitinase. Inhibition of *C. capsici* showed that crude extract had a very low activity of 40 ppm and 60 ppm, however concentration 20 ppm showed no activity to inhibit *C. capsici*.

DISCUSSION

Two isolates were selected by chitinolytic index and antagonistic activity against fungal pathogens *Colletotrichum capsici*. Those isolates were KAHN 15.12 and SAHA 12.12. A clear zone of hydrolytic indicated chitin degradation by chitinase-producing bacteria (Han *et al.*, 2014). Incompatibility of chitinolytic index with the percentage inhibition of fungal pathogens proved that the two are not interconnected. High chitinase activity was not always correlated with ability to inhibit the growth of pathogenic fungi. This could be due to difference of substrate structure and presence of other metabolites antifungi which can increase percentage of inhibition. The structure of chitin in cell wall of fungi was more complex than colloidal chitin used as a carbon source in chitinase activity assay, so that colloidal chitin used as the substrate could be more easily hydrolyzed than the chitin found in the cell wall of fungi.

The results of cell growth showed that isolates started exponential phase after 6 to 12 hours incubation and that was not followed by an increase in the production of the enzyme chitinase. The highest specific activity was observed at the stationary phase. Similar results had been reported in chitinase from *Serratia* sp. and *Bacillus* sp. (Asril *et al.*, 2014; Han *et al.*, 2014). Chitinase activities had increased in the stationary phase due to the decrease of nutrients in the media, so chitinase was secreted in high quantities. The bacteria produced extracellular chitinase enzymes to degrade colloidal chitin in the media that used as a source of carbon and nitrogen. Results of colloidal chitin degradation by the enzyme chitinase into the monomer form of GlcNAc to be used by the bacteria to metabolism and its metabolites were used for growth of bacteria (Thompson *et al.*, 2001).

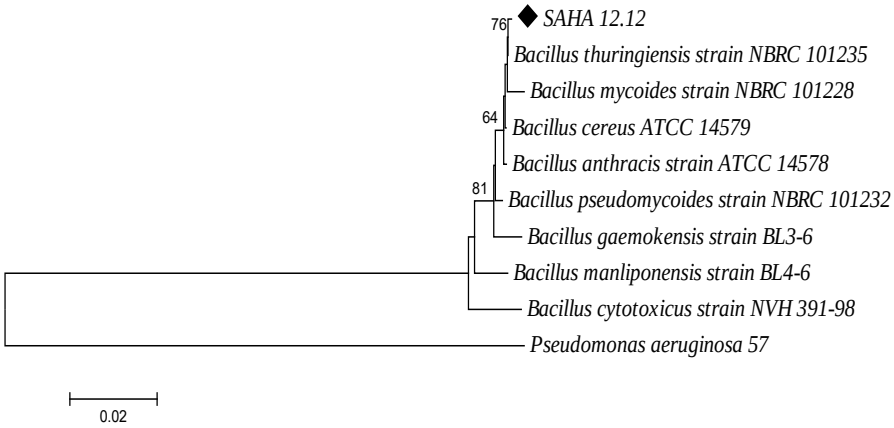

Figure 2: Phylogenetic tree for the SAHA 12.12 isolate showing similarities with *Bacillus thuringiensis*.

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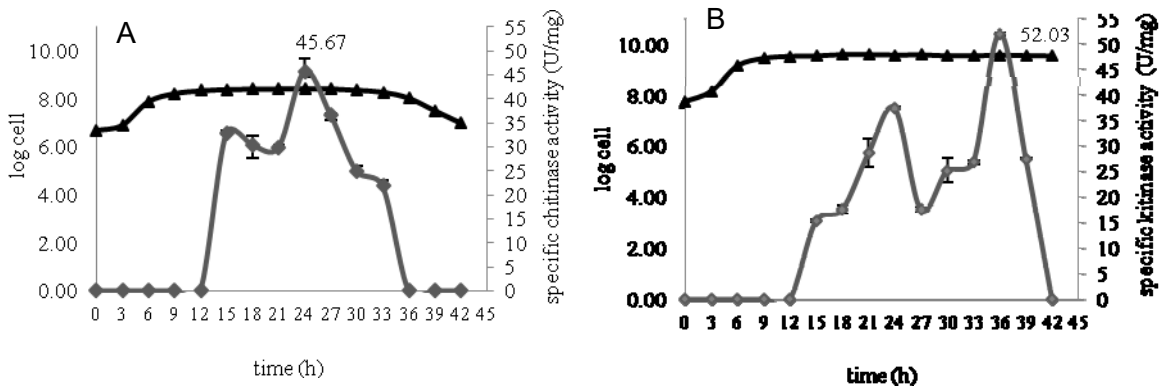

Figure 3: Growth and chitinase activity on production media enriched with colloidal chitin 0.3% of isolate A, KAHN 15.12 and B, SAHA 12.12 ▲, log cell; ♦, specific chitinase activity (U/mg).

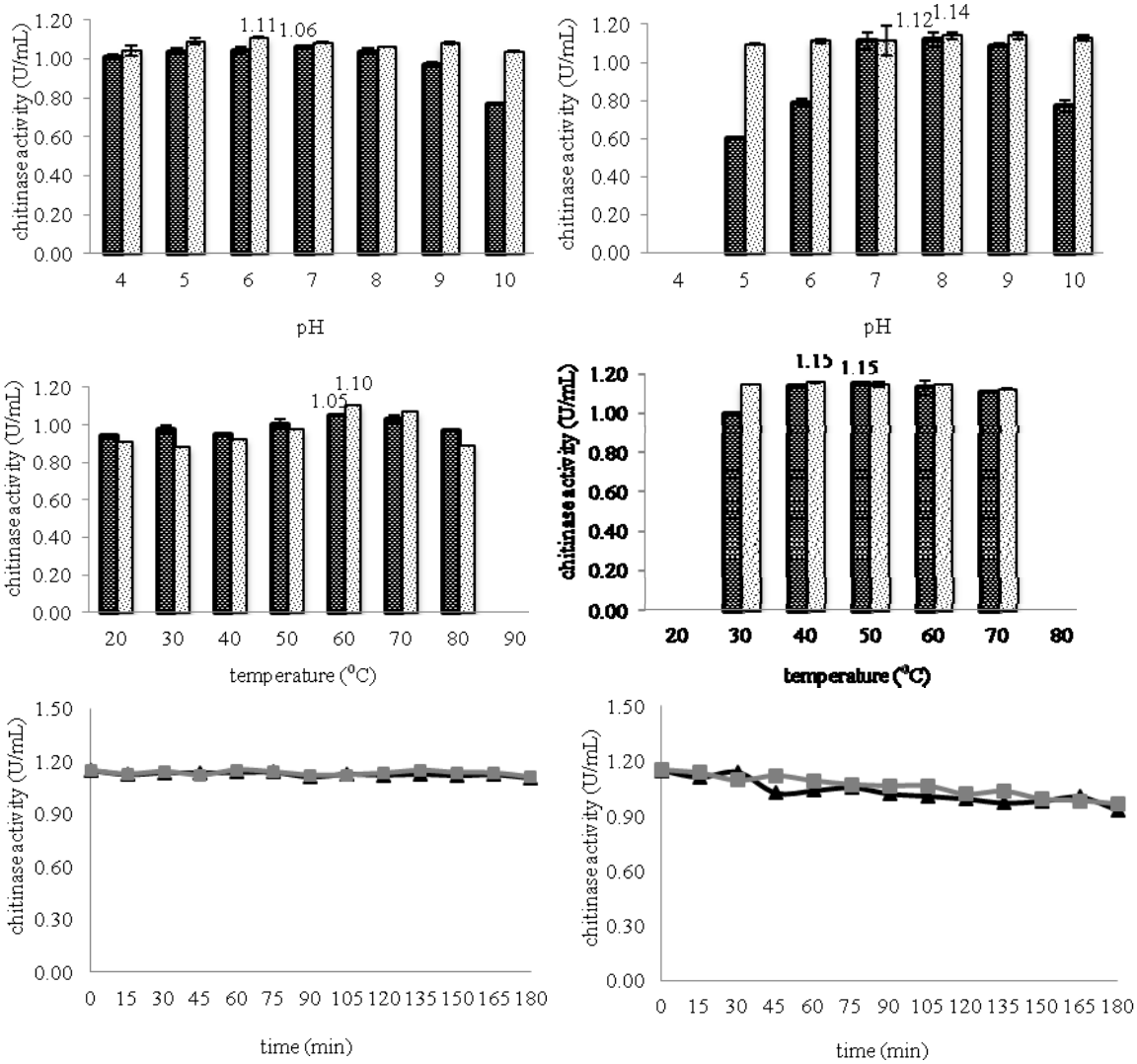

Figure 4: Crude and precipitated chitinase of isolate KAHN 15.12 (left) and SAHA 12.12 (right). Effect of pH (top), temperature (middle), and stability at optimum pH and temperature for 180 min (bottom) ■, crude enzyme; □, precipitated enzyme.

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

Table 2: Summary of chitinase ammonium sulfate precipitation produced by KAHN 15.12 and SAHA 12.12.

Isolate code	Steps	Sample volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
KAHN 15.12	Crude enzyme	100	5.9	107	18.10	1	100
	40% ammonium sulfate precipitation	1.5	0.045	1.575	34.84	1.92	1.47
SAHA 12.12	Crude enzyme	100	3.7	115	31.42	1	100
	60% ammonium sulfate precipitation	1.5	0.018	1.695	98.61	3.14	1.47

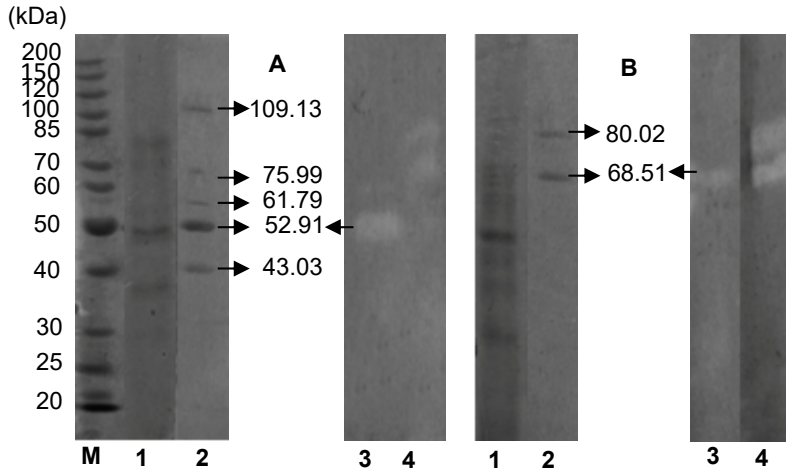

Figure 5: SDS PAGE and zymogram of chitinase *S. marcescens* KAHN 15.12 and *B. thuringiensis* SAHA 12.12. M, Marker; A, KAHN 15.12; B, SAHA 12.12. 1, SDS-PAGE of crude enzyme; 2, SDS-PAGE of precipitated enzyme 3, Zymogram of precipitated enzyme; 4, Zymogram of crude enzyme.

Chitinase activities were characterized by pH, temperature and stability of the enzyme. The optimum pH and temperature of precipitated enzyme KAHN 15.12 were determined to be pH 7.0 and 60 °C. The optimum temperature KAHN 15.12 was comparable to the termostable chitinase, such as Chit62 from *S. marcescens* B4A optimum temperature at 55 °C (Babashpour *et al.*, 2012). Precipitated enzyme SAHA 12.12 showed optimum activity at pH 8 with the optimum temperature at 50 °C, which then changed the optimum temperature to 40 °C after the ammonium sulfate precipitation the enzyme (Figure 4). Both chitinase were stable at maximum pH and temperature up to 180 min of incubation. Differences in the characteristics of temperature and pH on the crude extract and precipitated enzyme results might be due to changes in the structure of proteins after ammonium sulfate precipitation. Increased temperature would lead to increase activity of the enzyme, but after the optimum temperature had reached, temperature rise would lead to decrease enzyme activity. This was due to the protein denaturation of the enzymes that could damage enzyme and change its active site structure. Similar results had been reported in chitinase from *S. marcescens* NK1 which had a pH range from acidic to alkaline with pH optimum at pH 6 and 40 °C to 60 °C (Nawani and Kapadnis, 2001) while for *Bacillus* had a pH range of 5 to 8 and 40 °C to 80 °C (Chang *et al.*, 2003).

The molecular weight of crude chitinase and precipitated chitinase was analyzed by SDS PAGE. The basic principle of this method was the movement of protein molecules in the media that charged by electricity. In addition to the analysis the molecular weight of the protein indicated the level of purity of the produced protein. SDS-PAGE analysis of the crude chitinase and precipitated chitinase from *S. marcescens* KAHN 15.12 and *B. thuringiensis* SAHA 12.12 showed protein molecular weight in the range between 40 kDa to 109 kDa. Crude chitinase produced more bands proteins than precipitated chitinase. Differences in the number of protein bands on SDS PAGE analysis indicated that purity of each protein was different. It was reported that 62, 58, 54, 52, and 35 kDa chitinase proteins exist in the culture of *S. marcescens* (Gal *et al.*, 2002; Babashpour *et al.*, 2012), while *B. thuringiensis* have molecular weight of the chitinase of 130, 70, 65, 66, 60, 47, and 32 kDa (Avelizapa *et al.*, 1999; Thamthiankul *et al.*, 2001). Zymogram analysis showed a single band of precipitated chitinase from 2 isolates, which was indicative of chitinolitic activity.

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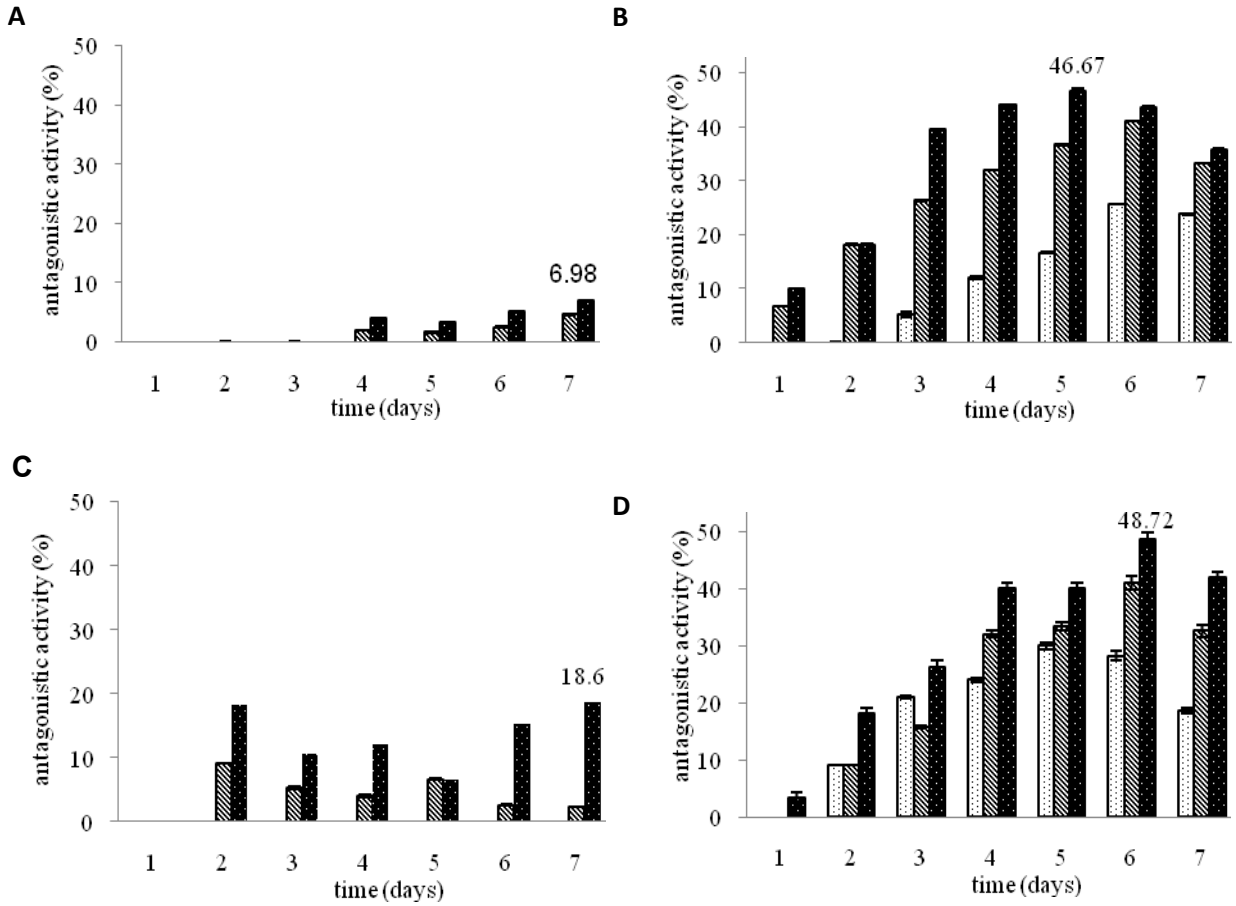

Figure 6: The percentage inhibition of chitinase. A, KAHN 15.12 crude enzyme; B, KAHN 15.12 precipitated enzyme; C, SAHA 12.12 crude enzyme; D, SAHA 12.12 precipitated enzyme against *Colletotrichum capsici* on PDA after 7 days of incubation. ⬚, 20 ppm; ▨, 40 ppm; ■, 60 ppm

The antagonistic activities showed that the crude chitinase has a very low inhibition at various concentrations, but using precipitated chitinase the percentage of *C. capsici* inhibition was increased at concentrations of 60 ppm (Figure 6). Chitinase did not show significant antagonism against *C. capsici* because the chitin compositions of the cell wall of fungi were mismatch with the chitinase of bacteria and species specificity (Liu *et al*., 2010). The cell wall of fungi in general were not only composed of chitin but also composed by other polysaccharides, ex. ß-1.3 glucan (Adams, 2004; Gohel *et al.*, 2006). Difference in chitinolytic ability must result from the subsite structure in the binding cleft (Sasaki *et al.*, 2002; Zarei *et al.*, 2011). The selection of antagonistic organisms was the first step in biological control. These observations and further studies will help to develop the *S. marcescens* KAHN 15.12 and *B. thuringiensis* SAHA 12.12 isolates as a biological control agent against *C. capsici* causing antrachnose in chilli.

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