

Chitinolytic Bacteria Isolated from Chili Rhizosphere: Chitinase Characterization and Application as Biocontrol for *Aphis gossypii*

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Chitin, a common constituent of insect exoskeleton, could be hydrolyzed by chitinase. This research was conducted to select rhizobacteria isolated from the rhizosphere of chili pepper that produced chitinase and to examine their chitinase activity in degrading chitin of the *Aphis gossypii*. A total of 25 rhizobacteria isolates formed a clear zone when grown on chitin agar. Three of them had the highest chitinolytic index and were identified as *Bacillus* sp. strain I.5, I.21, and II.14. The II.14 was chosen for characterization of chitinase activity. The isolate showed maximum chitinase activity at 48-h-incubation. Maximum temperature and pH of the chitinase activity were 55°C and 7.0, respectively. The cell culture and the enzyme crude extract of the above three isolates were tested against *A. gossypii* and the result was compared to the control through microscopic observation. Hydrolytic analysis showed that the enzyme crude extract of these isolates were able to degrade chitin of insect exoskeleton since the first 3-h-incubation. Meanwhile, the cell culture treatment on the chitin showed degrading activity after 12 h (*Bacillus* sp. strain I.21 and II.14), and 9 h (*Bacillus* sp. strain I.5). Chitin degradation of *A. gossypii* exoskeleton by enzyme crude extract was better than the cell culture treatment. Chitinases produced by *Bacillus* sp. strains I.5, I.21, and II.14 are potential as biocontrol agents for *A. gossypii*.

Key words: rhizobacteria, chitinase, biocontrol, *Aphis gossypii*

Aphis gossypii or melon aphid or cotton aphid was one of the leaf miners that attack many kinds of plants, including chili pepper. Chili peppers attacked by the aphids have wrinkled leaf, obstructed shoot, and damaged leaf tissue caused by the chili veinal mottle virus (CVMV) carried by adult aphids. Chili pepper yields may decrease up to 60% due to the attack of this virus (Wagiman *et al.* 2001). *A. gossypii* is the most effective vector for viruses; aside of CVMV, the aphid also takes part as a vector for *cucumber mosaic virus* (Garzo *et al.* 2003).

Insecticide have been used in high frequency to control pest, but the limitation in chemical control is increasing due to a rapid development of insecticide resistance and also the accumulation of chemical residue is difficult to degrade. Therefore, biological control has been chosen as an alternative to reduce chemical pesticides usage. Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as plant growth-promoting rhizobacteria (PGPR). The PGPR may become a promising biocontrol agent for plant diseases. Large scale application of PGPR to crop as inoculants will substantially reduce the use of chemical fertilizers and pesticides, which often pollute the environment (Bloembergen and Lugtenberg 2001).

Chitin is the most abundant polymer after cellulose. It is a common constituent of algae and fungal cell walls, insects, and nematodes. Insect's exoskeleton or cuticle is composed of chitin. The cuticle is divided into three layers, namely the cover layer, epicuticle, and procuticle (Merzendorfer and Zimoch 2003; Schwarz and Moussian 2007). Chitinases (EC 3.2.1.14) hydrolyse the glycosidic -1,4 bonds in chitin to its oligomer or monomer and are found in a broad range of

organisms, including bacteria, fungi, and higher plants, and play different roles in their origin (Lonhienne *et al.* 2001; Funkhouser and Aronson 2007).

The presence of chitinolytic bacteria in the crop rhizosphere soils is highly beneficial as they are able to suppress plant pathogenic fungi near the root zone and provide sustainable plant protection against root diseases. Saikia *et al.* (2005) reported the presence of PGPR *Pseudomonas* sp. that produced chitinase and -1,3-glucanase and showed ability in inhibiting the growth of *Fusarium oxysporum*.

Previous research by Mubarik *et al.* (2010) showed that the chitinase of *Bacillus* sp. strain I.5 and I.21 showed their ability to degrade exoskeleton chitin of the whitefly *Bemisia tabaci*. The objectives of this research are to screen potential PGPR from pepper rhizosphere that produce extracellular chitinase and to use the enzyme for controlling leaf miner such as *A. gossypii*.

MATERIALS AND METHODS

Bacteria and Test-Objects. Twenty five bacteria isolates from the rhizosphere of chili pepper (Damayanti *et al.* 2007) were collected at Microbiology Laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor (IPB) and IPB Culture Collection. *A. gossypii* were obtained from suffering plants around IPB.

Bacterial Growth Media and Chitinolytic Bacteria Selection. Twenty five PGPR from the rhizosphere of chili pepper were grown on nutrient agar and incubated for 48 h at 37°C, pH 7.0. Each isolate was reinoculated to chitin agar (1% colloidal chitin, 0.1% MgSO₄·7H₂O, 0.02% K₂HPO₄, 0.1% yeast extract, and 1.5% agar) and incubated for 48 h at 37°C, pH 7.0. The isolates were screened based on chitinolytic index, defined as the ratio of clear zone to colony diameter on nutrient agar. The identification of bacterial isolate was carried

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out based on morphological structure, Gram staining, endospore staining, and catalase test.

Bacterial Growth Curve and Enzyme Production. A number of 10^8 cells mL^{-1} of selected isolate was inoculated to production media consisting of 0.3% colloidal chitin, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% K_2HPO_4 , and 0.1% yeast extract. The culture was incubated on a dry shaker at 37°C and 100 rpm. Cell cultures were collected every 12 h until 96 h and their density were measured spectrophotometrically at 600 nm. The cultures were then centrifuged at $8400 \times g$ for 10 min to obtain the crude extract of extracellular chitinase enzyme in supernatant.

Chitinase Activity and Protein Quantity Measurement. Chitinase activity was measured as described by Spindler (1997). A volume of 450 μL enzyme crude extract was added to 900 μL 0.3% colloidal chitin and 450 μL 0.1 M phosphate buffer. The mixture was incubated at 55°C for 30 min. Enzyme reaction was stopped at 100°C for 10 min. After centrifugation at $8400 \times g$ for 5 min, 600 μL filtrate was added to 1.5 mL distilled water and 3 mL Schales reagent, and the mixture was boiled at 100°C for 10 min. Enzyme activity was determined by measuring absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol reducing sugar (N-acetyl glucosamine) per min. Protein content was determined using as describe by Bradford (1976). Bovine serum albumin was used as a protein standard.

Chitinase Characterization. Enzyme characterization was done to define optimum pH and temperature of chitinase activity. Chitinase activity was measured at pH ranging from 4 to 10 using 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 effect of temperature on chitinase activity was measured at temperature ranging from 25 - 60°C using 5°C interval at optimum pH obtained from previous experiment. The data were based on duplicate experiments.

Chitinase Test to *Aphis gossypii*. Live aphids were selected based on their shape and size. Aphids with the same size were assumed as aphids of the same age and chitin thickness. The test were done by dropping 20 μL cells supernatant and 20 μL enzyme crude extract to each aphid at room temperature. Chitin hydrolysis was observed every 3 h until 24 h by comparing the thickness of chitin layer on each aphid (Mubarik *et al.* 2010). Treated aphid were first fixed with 70% ethanol for 24 h and then serially dehydrated for 10 min with 80, 95, and 100% ethanol. Clove oil was dropped onto the aphids for 15 min and cleaned with xylol. Each aphid was placed on the object glass, then covered and tightened with synthetic resin Entellan (a particularly rapid embedding agent). Chitin thickness was observed with light microscope at 40 times magnification.

RESULTS

Chitinolytic Index. A total of 25 PGPR isolates were obtained from the rhizosphere of chili pepper. They formed a clear zone when grown on chitin agar. It showed that they all produced extracellular chitinases that could hydrolyze

chitin in the media. Among the 25 chitinolytic bacteria, only 3 isolates produced 0.5 cm clearing zone, i.e. I.5, I.21, and II.14. Interestingly, the isolates also had both proteolytic and cellulolytic activities (Table 1). Chitinase activity and characterization of I.5 and I.21 had been reported by Mubarik *et al.* (2010). Therefore in this research only characterization of II.14 chitinase activity were described, and the isolate is a Gram-positive, endospore forming, rod shaped bacterium with catalase positive reaction. Based on the physical characteristics, the isolates are most likely belonging to *Bacillus* sp. (Holt *et al.* 1994).

Growth Curve and Chitinase Activity of II.14 Isolate.

The isolate was grown in liquid chitin media at pH 7.0 and 37°C . The growth rate of isolate II.14 ascended from 0-24 h incubation and was relatively stable for 24-84 h, and descended afterwards (Fig 1). The chitinase produced was detected since the 24th h and the highest chitinase activity were found at the 48th h, after 60 h the activity was not detected (Fig 1). The activity of the isolate was measured at 55°C pH 7.0.

Characterization of Chitinase from II.14 Isolate.

Among the different temperature tested, maximum chitinase activity was observed at 55°C being 0.115 unit per mg protein. There were no chitinase activity lower than 30°C and between 30 - 50°C (Fig 2). Among different pH values tested, at pH 7.0 the enzyme showed maximum activity of 0.1 unit per mg protein. There was no chitinase activity at pHs lower than 5.0 and higher than 8.0 (Fig 3).

Table 1 Chitinolytic index of rhizobacteria isolates

Isolate	Colony diameter (cm)	Clear zone diameter (cm)	Chitinolytic index	Other hydrolytic index	
				Proteolytic index	Cellulolytic index
I.1	0.88	0.90	0.02	0.16	-
I.2	0.70	0.70	0.00	0.06	-
I.3	0.80	0.90	0.13	0.12	0.15
I.4	0.85	0.90	0.06	-	-
I.5	0.60	1.20	1.00	0.51	0.10
I.8	0.76	0.80	0.05	-	-
I.14	0.96	1.00	0.04	-	-
I.15	0.66	0.71	0.08	0.38	-
I.21	0.70	1.30	0.86	0.82	0.18
I.25	0.52	0.69	0.33	1.08	0.08
I.26	0.87	0.97	0.11	0.13	-
I.28	0.59	0.65	0.10	1.00	-
I.33	0.57	0.60	0.05	-	-
I.34	0.81	0.92	0.14	0.63	0.25
II.7	0.62	0.65	0.05	-	-
II.8	0.94	1.00	0.06	-	-
II.10	0.84	0.92	0.10	-	-
II.11	0.59	0.65	0.10	-	-
II.12	0.75	0.77	0.03	-	-
II.13	0.76	0.79	0.04	-	0.05
II.14	0.60	1.05	0.75	0.28	0.40
II.15	1.00	1.10	0.10	-	-
II.16	0.65	0.67	0.03	-	-
II.17	0.57	0.58	0.02	-	-
II.34	0.90	0.99	0.10	-	-

- no hydrolytic activity

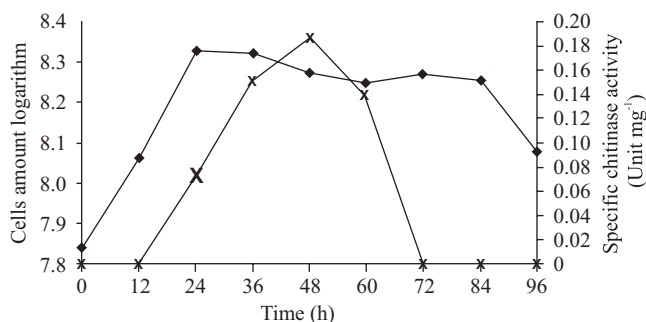


Fig 1 Growth and chitinase activity of II.14 isolate on production media enriched with colloidal chitin. ♦ Log of cell number; x specific chitinase activity.

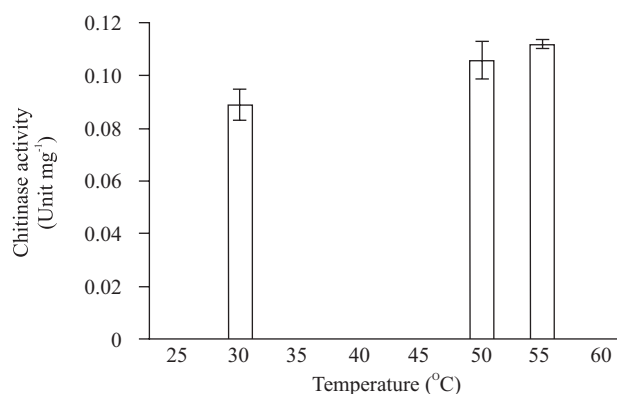


Fig2 Effect of temperature on chitinase activity of II.14 isolate.

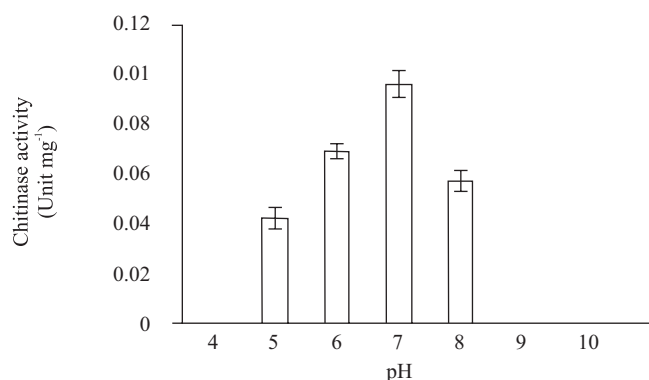


Fig 3 Effect of pH on chitinase activity of II.14 isolate.

Aphid Chitin Hydrolysis. Chitinases from I.5, I.21, and II.14 isolates were used to observe their activities on the aphids' exoskeleton. Microscopic observation showed that the aphid exoskeleton were thinner in the presence of enzymes. This was even observed at 3 h after treatments. Treatment with the free cell supernatant made the exoskeleton thinner 12 h (I.21 and II.14) and 9 h (I.5) after treatment. The thickness of aphids' exoskeleton after treatments with chitinase or the cell culture were significantly different compared to the control (Fig 4).

DISCUSSION

Shanmugaiah *et al.* (2008) reported that among the 39 chitinolytic bacteria isolated from rice rhizosphere, only 11 isolates produced clearing zone over 0.5 cm on the chitin colloidal agar. Chitinase produced by *B. laterosporus* showed the highest clear zone of 1.1 cm around the colony in chitin colloidal agar. In this research, among the 25 chitinolytic bacteria, only 3 isolates produced clearing zone over 1.05 cm with chitinolytic index over 0.5, i.e. I.5, I.21, and II.14. All isolates most likely belong to *Bacillus* sp. (Holt *et al.* 1994). Chitinase production was reported in different species of *Bacillus* such as *B. amyloliquefaciens* (Sabry 1992), *B. cereus* (Huang *et al.* 2005), *B. circulans* (Chen *et al.* 2004), *B. licheniformis* (Waldeck *et al.* 2006), *B. megaterium* (Sabry 1992), *B. stearothermophilus* (Sakai *et al.* 1994), *B. subtilis* (Wang *et al.* 2006), *B. thuringiensis* sub sp. *aizawai* (Vega *et al.* 2006), and *B. thuringiensis* sub sp. *kurstaki* (Driss *et al.* 2005).

Previous reports have shown that PGPR *Bacillus* sp. strain I.5 and I.21 demonstrated their ability to degrade exoskeleton chitin of whitefly *Bemisia tabaci* (Mubarik *et al.* 2010). However, this is the first report on the production of chitinase by *Bacillus* sp. strain II.14 and its application to degrade exoskeleton of aphid *A. gossypii*. Cell culture and chitinase of I.5 and I.21 isolates were used as comparison.

II.14 isolate secreted chitinase that were induced by the presence of substrates such as colloidal chitin. The highest specific activity was observed at the stationary phase, when the cell density began to decrease after 48 h incubation. From this time on, the activity started to decrease and no activity was detected after 72 h until the end of observation

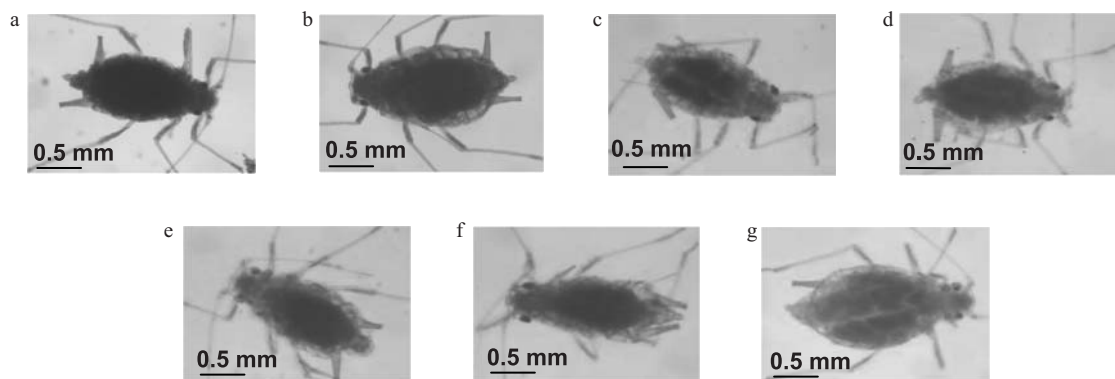


Fig 4 Aphids without treatments or control (a), cell culture treatment (b-d), enzyme treatment (e-g). Cell culture treatment of I.5 isolate at 9 h after treatment (b), I.21 isolate at 12 h (c), II.14 isolate at 12 (d). Enzyme treatment isolated from I.5 isolate at 3 h after treatment (e), I.21 isolate at 3 h (f), II.14 isolate at 3 h (g). Microscope observation was conducted with 40 times magnification.

(96 h). It was estimated that after 72 h, the bacterial culture were already at the end of stationary phase and in the beginning of death phase. The enzymes were no longer produced and toxic metabolites, such as antibiotic, were detected in stationary phase.

Chitinase activity was measured using the method of Spindler (1997). This method defines the amount of N-acetyl glucosamine formed following hydrolysis, which is then mixed with Schales reagent [0.05% $K_3Fe_3(CN)_6$ and 5.3% Na_2CO_3] and measured with spectrophotometer. Low chitinase activity shows that there are a high amount of unhydrolyzed chitins and little amount of released N-acetyl glucosamine. Chitinase of II.14 had the highest activity at 55°C. Chitinase activity of I.21 and I.5 were also measured at 55°C (Mubarik *et al.* 2010). Chitinase of II.14 also showed activity at 30°C and 50°C, but not as high as at 55°C. There was no chitinase activity at 30-50°C. We assumed there were more than one chitinase molecules in the crude enzymes that had optimum activity at 30 and 55°C. Chitinases have been isolated from a variety of bacteria, including *Bacillus* spp. and some of them were reported to produce multiple forms of chitinases with different molecular masses (Sanmugaiah *et al.* 2008).

Enzyme activity was also affected by pH. Chitinase of isolate II.14 showed activity at pH 5.0-8.0. The highest activity was observed at pH 7.0. Bacterial chitinase generally shows optimum activity at low pH (Ueda and Arai 1992; Evvyernie *et al.* 2000; Natsir 2000), but some also reported optimum activity at neutral pH (Wang and Chang 1997; Purwani 2004).

Chitin hydrolyzation test against *Bemisia tabaci* showed that cell cultures and chitinase crude extracts of isolate I.5 and I.21 were able to hydrolyse the insect's exoskeleton (Mubarik *et al.* 2010). This research analysed the ability of cell culture and chitinases of I.5, I.21, and II.14 isolate to degrade the exoskeleton of leaf miner *A. gossypii*. Damage level was caused by the treatment were observed at every 3 h for 24 h. Crude extracts worked better than the cell cultures in degrading chitin. Microscopic observation showed that aphids cuticle were hydrolyzed at 3 h after treatment. Cell culture treatment showed that the hydrolysis started after 12 h (I.21 and II.14) and after 9 h (I.5) after treatment. It showed that chitinase were faster than the cell cultures in hydrolyzing the cuticles. In cell cultures treatment, more time is needed by the cells to produce chitinase, while the chitinase treatment made it possible to directly hydrolyse the chitin in exoskeleton. Dark exoskeletons were degraded and became more transparent, therefore objects with higher transparency showed a higher level of hydrolyzed chitin.

Chitinases-encoding gene may be cloned into high economy plant. Expression of the chitinase genes on tomato plants decreased the growth of Colorado potato beetle (Lawrence and Novak 2006). Koga (2005) reported that transgenic strawberry plants carrying chitinase gene were resistant to pathogenic fungi. Chitinase genes transformation on papaya increased its resistancy to red spiders (McCafferty *et al.* 2006). However, the general attitude of Indonesians toward transgenic plants is still not positive (Abbas 2003).

I.5, I.21, and II.14 isolates showed their ability to degrade chitin of *A. gossypii*. Chitin degradation with enzyme

treatment was better than the cell culture treatment. Chitinase produced by the three selected isolates were potential to be improved and used as a biocontrol agent for insect such as aphid *A. gossypii*. Application of chitinase may be done by spraying the enzyme directly to the plants. Leaves and fruits of chitinase-sprayed-strawberry showed no presence of any insects or pathogenic fungi (Koga 2005). Combination between chitinase and σ -toxin of *B. thuringiensis* were found more effective in killing pest insects (Patil *et al.* 2000). Spreading chitin surrounding the plant is also an alternative, as this will induce chitinolytic rhizosphere bacteria to secrete chitinase (Metcalfe *et al.* 2002).

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