

Full Length Research Paper

Chitinolytic activity of *Enterobacter* sp. KB3 antagonistic to *Rhizoctonia solani* and its role in the degradation of living fungal hyphae

P. Velusamy^{1*} and K. Y. Kim²

¹Department of Biotechnology, School of Bioengineering, SRM University, Chennai-603 203, India

²Division of Applied Bioscience and Biotechnology, Chonnam National University, Gwangju 500-757, Korea

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Chitinolytic bacterial strain KB3 was isolated from coastal soils of Buan area, Korea and tested for antagonistic activity against *Rhizoctonia solani*. Strain KB3 was identified as *Enterobacter* sp. based on morphological observation and 16S rRNA analysis. The strain exhibited a maximum chitinase production of 1.6 U/mL in colloidal chitin broth after 4 days of cultivation at 30°C. The molecular weight of the purified chitinase was estimated to be 46 kDa by 12% SDS-PAGE and zymogram. In vitro assays revealed that the purified chitinase inhibited activity of *R. solani* as identified by dual plate assay and microscopic methods. Hydrolysis products of the fungal cell wall by the purified enzymes of *Enterobacter* sp. KB3 were analyzed by high-pressure liquid chromatography (HPLC) and identified as oligosaccharides, which included monomers (GlcNAc), dimers (GlcNAc)₂, and trimers (GlcNAc)₃ using chitin oligomer standards. The purified chitinase obtained from *Enterobacter* sp. KB3 can be directly applied for suppressing growth of living fungal hyphae.

Keywords: Antifungal activity, Hydrolysis, *R. solani*, 16S rRNA, Zymogram.

INTRODUCTION

Chitin (C₈H₁₃O₅N)_n is an unbranched long chain polymer of glucose derivatives, composed of β-1,4 linked units of the amino sugar *N*-acetyl-D-glucosamine (NAGA). It is a major component of the cell walls of filamentous fungi, speculated to play a vital role in fungal defense against harm and pressures (Carlile and Watkinson, 1994). The interest in chitin degrading enzymes and their application in management of fungal pathogens are significant. Chitinases (EC 3.2.1.14) are a group of antifungal proteins that catalyze the hydrolytic cleavage of the β-1,4-glycoside bond present in the biopolymers of *N*-acetyl-D-glucosamine, mainly in chitin (Cohen and Chet, 1998). Many chitinases have been identified and characterized from various sources mainly from bacteria that can act as biological control of different phytopathogens (Oppenheim and Chet, 1992; Chernin et

al., 1995).

Biological control of pathogenic fungi provides an attractive and alternative management for fungal diseases without the negative impact of synthetic antifungal agents that can cause environmental pollution and may induce pathogen resistance (Haas and Défago, 2005). Several strains of bacteria, such as *Aeromonas caviae*, *Bacillus* sp., *Serratia plymuthica*, and *Enterobacter agglomerans* are well-known chitinolytic bacteria, which can be used as efficient biological control agents (Das et al., 2010). Among them, *Enterobacter* spp. have been described as being promising biocontrol agent for various diseases including *Pythium* induced damping-off and *Aphanomyces*-induced root rot of pea, *Botrytis*-induced gray mold of apple, and *Rhizoctonia solani*-induced root rot of cotton (Chernin et al., 1995).

Antagonistic bacteria are considered as an ideal biological control agents that mediate one or several mechanisms of disease suppression. A primary mechanism of pathogen inhibition is by production of antibiotics; however, lytic enzymes and others may also

*Corresponding author E-mail: vels@ktr.srmuniv.ac.in; Phone: +91-44-27455734, Fax: +91-44-27455721.

play a role. Among them, hyper parasitism relies on chitinase for degradation of the cell walls of pathogenic fungi (Chet et al., 1990). The soil borne *Enterobacter agglomerans* IC1270 has a broad spectrum of antifungal activity and secretes a number of chitinolytic enzymes including two *N*-acetyl- β -D-glucosaminidases and chitinase. Its biocontrol activity has been demonstrated with *Rhizoctonia solani* in cotton using Tn5 mutants deficient in chitinolytic activity (Chernin et al., 1995). Hence, chitinolytic enzyme might be considered as an important role in fungal disease management which was substantiated by several studies using chitinase, laminarinase, β -1,3-glucanase (Chernin et al., 1995; Hoster et al., 2005). It has frequently been reported that chitinase producing soil bacteria, in particular biocontrol strains, can lyse living fungal hyphae, thereby releasing substantial level of oligomers and other substances (Gooday, 1990; de Boer, et al., 2001; Van Nguyen et al., 2008). Different isolate of *Bacillus thuringiensis* produce chitinases that are able to hydrolyse chitin molecules into *N*-acetylglucosamine (GlcNAc) monomers (Thamthiankul et al., 2001) and chitobiose (Driss et al., 2005).

In the present work, we report a new strain *Enterobacter* sp. KB3 possessing strong chitinolytic activity, which exhibited an antagonism toward *R. solani*. The antifungal activity of the purified chitinase from *Enterobacter* sp. KB3 was also partially characterized.

MATERIALS AND METHODS

Screening of bacteria. Samples were collected from the coastal soil enriched with crab shells in Buan area, Korea. Soils were serially diluted with sterile water until a dilution of 10^6 colony forming units (CFU) g^{-1} of soils, inoculated on colloidal chitin agar (CCA) medium containing 0.5% colloidal chitin, 0.2% Na_2HPO_4 , 0.1% KH_2PO_4 , 0.05% NaCl, 0.1% NH_4Cl , 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.05% yeast extract and 2% agar, and incubated at 30°C for 3 days. Strains exhibiting a clear zone (degradation of chitin) around the colony were picked and further purified on the same medium. Of these, a strong chitinolytic (>8 mm in diameter of halos zone) strain was selected and successively examined for antifungal activity against *R. solani* (KACC 40117, Korean Agricultural Culture Collection, Suwon, Korea) grown on potato dextrose agar (PDA) medium containing 0.5% colloidal chitin at 30°C for 5 days.

Bacterial identification. To identify the bacterium, a polymerase chain reaction (PCR) was performed to amplify the 16S rRNA gene from the genomic DNA of strain KB3 as described earlier (Weisburg et al., 1991). The forward primer was 5'-TGGCTCAGAACGAACGCTGGCGGC-3', and the reverse primer was 5'CCCACTGCCTCCCGTAAGGAGT-

3'. The temperature cycle was at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min 30 s for 30 cycles and 5 min at 72°C for extension. The PCR product was cloned in a pGEM-T easy vector (Promega, Madison, WI, USA). The nucleotide sequence of the 16S rRNA gene was determined by an ABI Prism 377 DNA sequencer (PE Applied Biosystems, MA, USA) and compared with published 16S rRNA sequences using Blast search at Genbank data base of NCBI (Bethesda, MD, USA).

Chitinase assay. For determination of chitinase activities, strain KB3 was grown in CC broth at 30°C, and samples were taken at 1, 2, 3, 4, and 5 days. Each sample was centrifuged at $8,000\times g$ for 5 min and the supernatant was used for enzyme activities. Chitinase activity was assayed by measuring the amount of the reducing end group of *N*-acetyl-D-glucosamine (GlcNAc) from colloidal chitin (Lingappa and Lockwood, 1962). The assay mixture consisted of 0.05 mL of supernatant, 0.5 mL of 0.5% colloidal chitin, and 0.45 mL of 50 mM sodium acetate buffer [pH 5.0] at 37°C for 1 h. The reaction was terminated by the addition of 200 μ L of 1 N NaOH. After centrifugation at $10,000\times g$ for 5min, 750 μ L of supernatant was mixed with 1 mL of Schales' reagent (0.5 M sodium carbonate and 1.5 mM potassium ferricyanide) and then heated in boiling water for 15 min. The amount of chitinase produced was measured at 585 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). The activity was calculated from a standard curve based on known concentrations of GlcNAc. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μ mol of GlcNAc per h under the conditions of the study.

Purification of chitinase. Strain KB3 was cultured in a 2000 mL Erlenmeyer flask containing 1000 mL of CC broth at 30°C for 4 days in a shaking incubator (180 rpm). After centrifugation of the broth culture at $8,000\times g$ for 30 min, ammonium sulfate was added to the supernatant at 50% saturation, and the mixture was left overnight at 4°C. The precipitate was centrifuged at $12,000\times g$ for 30 min and the pellet was resuspended in 50 mM Tris-HCl buffer [pH 8.0], and dialyzed against the same buffer for overnight. The dialyzate was loaded onto a DEAE-Sepharose column (2.4 x 45 cm; Amersham, Uppsala, Sweden), which was previously equilibrated with Tris-HCl at pH 8.0. Elution with a linear gradient of 0.0 to 1.0 M NaCl resulted in a single peak of chitinase activity. The peak that exhibited a higher chitinase activity was pooled to concentrate and polished using a Sepharose G-75 column (1.5 x 25 cm; Sigma Chemical Co., St. Louis, MO, USA), which had been equilibrated with Tris-HCl at pH 8.0. All the fractions were collected separately and absorbance was read at 280 nm in a spectrophotometer. Each purification step was performed in 4°C.

Determination of protein concentration. The active fraction was concentrated by lyophilization, and the

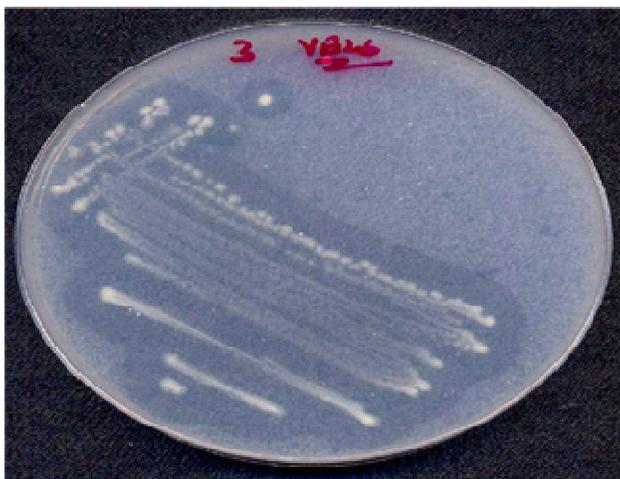


Figure 1: Growth of *Enterobacter* sp. KB3 on colloidal chitin agar (CCA) medium at 30°C for 3 days.

concentration of protein was determined by the method of Bradford (1976) using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

Electrophoresis. The purified enzyme was subjected to electrophoresis in 12 % SDS-PAGE (Bio-Rad Mini Protein II Apparatus, CA, USA), according to the method described by Laemmli (1970). The gel was stained with the Coomassie brilliant blue R-250 and destained. On the other hand, chitinase activity of the gel was performed by a standard method (Trudel and Asselin, 1989) to obtain a zymogram on SDS-PAGE containing 0.01% glycol chitin (Sigma, Chemical Co, St. Louis, MO, USA). After electrophoresis, the gel was incubated at 37°C for 2 h with 100 mM sodium acetate buffer [pH 5.0] containing 1% Triton X-100 and 1% skim milk (v/v). Subsequently, the gel was immersed in 500 mM Tris-HCl [pH 8.9] solution containing 0.01% Calcofluor white M2R (Sigma, Chemical Co, St. Louis, MO, USA) for 5 min and examined for the active bands on a UV transilluminator.

Antifungal activity of chitinase. The purified chitinase was assayed for antifungal activity against *R. solani* by well diffusion assay on a PDA plate. A fungal plug (6 mm diameter) was removed from the 5 day old culture. The plugs were transferred onto the center of the PDA plates, which had been loaded with chitinase in the right well and the left well was loaded with the same volume of buffer. The plates were incubated for 5 days at 30°C and were monitored for a zone of inhibition around the well.

Microscopic observations. Antifungal effect of chitinase was also observed by a light microscope (Nikon, Tokyo, Japan). Two milligrams of hyphae of the *R. solani* suspension with purified chitinase (final concentrations of 75 U/mL) in 50 mM of sodium acetate buffer [pH 6.0] were added into a 12 well plates (12 mm, Corning, NY,

USA). A mixture of hyphae suspension and buffer was treated as control. In order to determine the deformation of hyphae, the experiment was carried out at varying conditions such as pH, temperatures, incubation time, and different buffers.

Hydrolysis of fungal hyphae. To determine the hydrolysis of *R. solani* by purified chitinase (as described in the section on microscope observations) after 24 h, the reaction was stopped by addition of 200 μ L 1 M NaOH. The reaction product was centrifuged at 6,000 \times g for 30 min, and the supernatant was passed through a 0.22 μ m membrane filter (Nalgene, Rochester, NY, USA). Finally, the enzyme hydrolysate was analyzed by high-performance liquid chromatography (HPLC). The HPLC was performed with acetonitrile:water (70:30, v/v) as the mobile phase, at the flow rate of 1 mL/min and detected at 210 nm with NH2P-50 4E column (Shodex, Tokyo, Japan) as described previously (Kuk et al., 2005). The retention times for the peaks obtained in the crude samples of hydrolytic products were compared with the chitin oligomer standard.

RESULTS

Identification of chitinolytic bacteria with antagonism against *R. solani*. A total of 35 samples were obtained from the coastal soil of Buan region, Korea. In our pilot scale screening, various microbial colonies were able to degrade chitin on CCA medium. Among these, a bacterial isolate that exhibited the maximum halo zone around the colony (Figure 1) was designated as strain KB3. Subsequent antimicrobial activity was examined through laboratory dual plate assays using various



Figure 2: Inhibition of the growth of *R. solani* by *Enterobacter* sp. KB3 on potato dextrose agar (PDA) medium containing 0.5% of colloidal chitin at 30 °C for 5 days.

Table 1: Summary of the steps in chitinase purification from one liter of *Enterobacter* sp. KB3.

Purification step	Total Protein (mg)	Total Activity (U)	Specific activity (U.mg ⁻¹)	Purification (n-fold)	Yield (%)
Culture filtrate	74.2	981	24.1	1.0	100
NH ₄ (SO ₄) ₂ precipitate	31.6	722	52.6	2.2	70
DEAE cellulose	5.7	359	183	7.6	24
Sephadex G-100	1.3	126	230	9.6	11

phytopathogens. Interestingly, strain KB3 exhibited a strong antifungal activity against *R. solani* (Figure 2).

Identification of bacterium. From biochemical and morphologic observation, strain KB3 was found to be a Gram-negative, rod-shaped bacterium with permissive temperature ranging between 25-37 °C with an optimum at 30 °C. The 16S rRNA gene was amplified using universal primers, cloned, sequenced, and analyzed. Alignment of this sequence (1452 bp) through matching with reported 16S rRNA gene sequences in the Genbank by BLASTn program showed high similarity (98-100%) to *Enterobacter* sp. On the basis of these results, strain KB3 was identified as a member of *Enterobacter* sp., and designated as *Enterobacter* sp. KB3 (GenBank Accession No. EU741245).

Determination of chitinolytic activity. Extracellular chitinase production by the strain KB3 in CC broth was determined spectrophotometrically. At 24 h intervals, aliquots of cell cultures were taken, and the chitinase activity was determined. The results from culture filtrate

of strain KB3 exhibited maximum chitinase of 1.6 U/mL after 4 days of cultivation and gradually decreased thereafter (Figure 3).

Purification of chitinase. The chitinase was purified from culture broth of strain KB3 by ammonium sulphate precipitation followed by DEAE-Sepharose (anion exchange), and Sepharose G-75 (gel filtration). After ammonium sulphate precipitation, the concentrated supernatant exhibited chitinase activity. The anion exchange chromatography yielded a single peak that could be detected at 280 nm. The peak with chitinase activity was further purified by gel filtration chromatography and results were summarized in Table 1. The data indicate that chitinase produced by strain KB3 was purified to 9.6-fold with an overall yield and specific activity of 11% and 230.5 units/mg, respectively. The final amount of chitinase obtained was 1.3 mg from 1 liter of broth culture.

Molecular weight determination of purified chitinase. The molecular weight of purified chitinase was

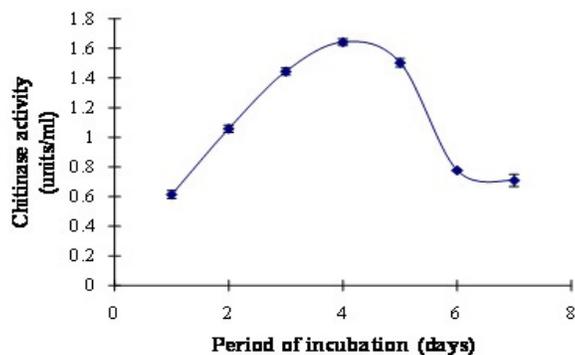


Figure 3: Determination of chitinolytic activity by *Enterobacter* sp. KB3 in CCA medium at 30°C for 7 days. Mean values were 3 replicates. Bars represent standard error

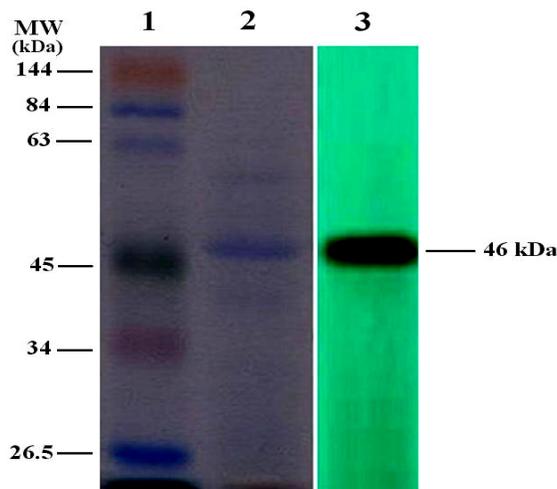


Figure 4: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram of purified chitinase from culture supernatant of *Enterobacter* sp. KB3

Lanes 1 and 2 were obtained from 12% SDS-PAGE of Coomassie brilliant blue R-250 where lane 1 is molecular weight (MW) of standard markers in kilodaltons, and lane 2 is purified chitinase sample. Lane 3 indicates zymogram demonstrated by copolymerizing 0.1% of glycol chitin in 12% SDS-PAGE. The chitinase activity was detected by visualization under UV light

determined by gel electrophoresis using a standard marker (iNtRON Biotech, Gyeonggi-Do, Korea). The purified chitinase of strain KB3 revealed a single band on 12% SDS-PAGE with an estimate of 46 kDa. Further, a chitinolytic activity of reported band was confirmed by zymogram (Figure 4).

Chitinase production and *R. solani* suppression by strain KB3. The inhibitory effect on the growth of hyphae of *R. solani* was investigated under different concentrations of purified chitinase by a well diffusion

assay on a PDA plate and a concentration of 50 μ L (75 U/mL) showed great inhibition. However, in the control well, the same volume of sodium acetate buffer could not inhibit the pathogen (Figure 5). Subsequently, the purified chitinase was further assayed for antimicrobial activity against various microorganisms. As listed in the Table 2, the percentage of inhibitory effect of chitinase against *F. oxysporum* was recorded to be approximately 50% and the remaining microorganisms were not significant.

Microscopic analysis. On further incubation with



Figure 5: Antifungal activity of chitinase against *R. solani* by well diffusion assay on potato dextrose agar (PDA) at 30°C for 5 days. Right well was loaded with chitinase and the left well loaded with same volume of buffer

Table 2: Inhibitory effects of chitinase against various microorganisms by well diffusion assay on potato dextrose agar (PDA) plate at 30 °C for 5 days.

Name of organisms	Inhibition ratio*
Bacteria	
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i> KACC 10057	-
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> KACC 10378	+
Fungi	
<i>Colletotrichum gloeosporioides</i> KACC 40689	ND
<i>Chaetomium globosum</i> KACC 40863	±
<i>Didymella bryoniae</i> KACC 40900	++
<i>Botrytis cinerea</i> KACC 40573	+
<i>Fusarium oxysporum</i> f. sp. <i>cucumerinum</i> KACC 40527	+++

*Antimicrobial effect of purified chitinase 50 µL (75 U/mL) was assayed by well diffusion assay on a PDA plate from Hoster et al. (2005). The percentage of inhibition of growth was calculated from the mean values as: % Inhibition = (A-B)/A x 100, where A = microorganism growth in control, and B = microorganism growth in purified chitinase. The inhibition was reported as (ND) not detected when growth inhibition was below 5%, (-) between 5% and 15%, (±) between 15% and 25%, (+) between 25% and 35%, (++) between 35% and 50%, and (+++) 50%. The experiment was done in triplicates

chitinase, morphological changes of the fungal hyphae were examined microscopically. As shown in Figure 6, the morphology of hyphae appeared as fragmented and distorted in the wells treated with the chitinase, whereas the hyphae from the control were normal and intact without any significant distortion.

Hydrolysis of fungal hyphae. To investigate the hydrolytic effect of chitinase, samples of treated hyphae of *R. solani* were analyzed by HPLC revealed various products of chitin oligosaccharides, among which

monomers (GlcNAc), dimers (GlcNAc)₂, and trimers (GlcNAc)₃ were identified by the retention time via HPLC using chitin oligomer standards (Figure 7). However, there was no detectable number of peaks found in control.

DISCUSSION

In recent years, much attention has been given to the antagonistic bacteria that can be used for biological

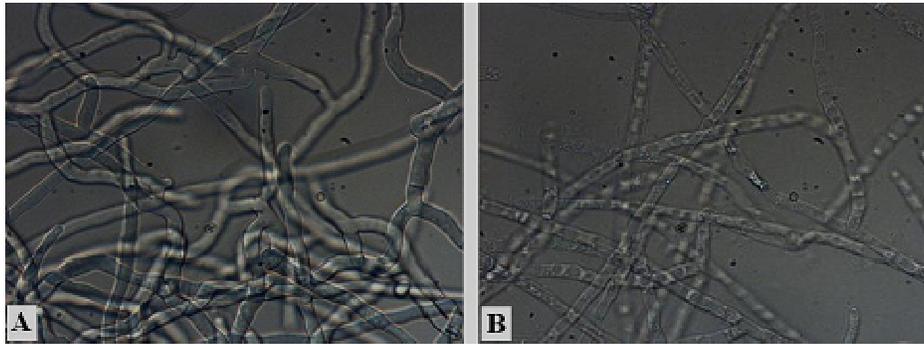


Figure 6: Morphological study of the hyphae of *R. solani* in sodium acetate buffer supplemented with chitinase at a concentration 75 μM and incubated at 45°C for 48 h. (A) Control: hyphae of *R. solani* + buffer, (B) Treatment: hyphae of *R. solani* + purified chitinase.

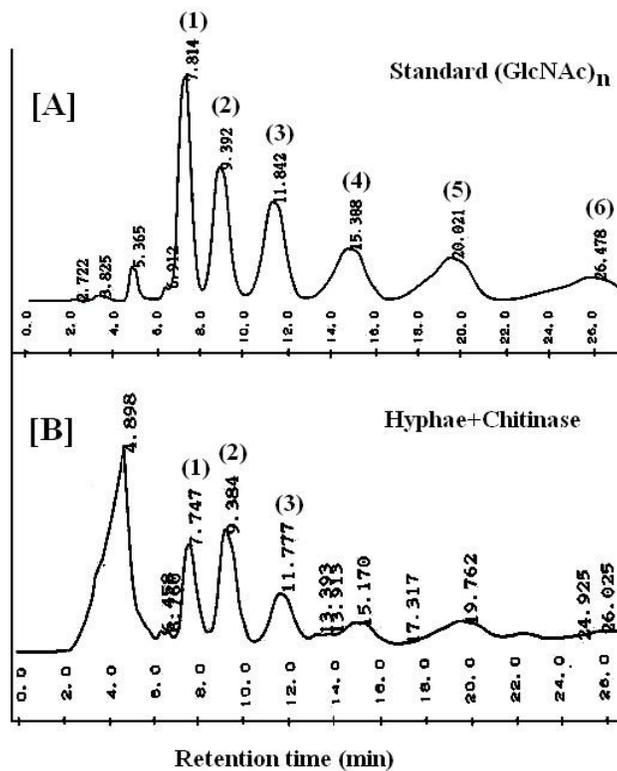


Figure 7: HPLC of hydrolysis products from the hyphae of *R. solani* and chitinase (75 μM) mixture after 24 h of incubation at 45°C.

[A] Chitin oligomer standard (GlcNAc)_n, [B] Hydrolytic products of crude sample obtained from hyphae of *R. solani* + purified chitinase. Peak numbers are referred as 1- monomer (GlcNAc); 2- dimer (GlcNAc)₂; 3- trimer (GlcNAc)₃; 4- tetramer (GlcNAc)₄; 5- pentamer (GlcNAc)₅; and 6- hexamer (GlcNAc)₆.

control of pathogenic fungi. Several studies support the important role of chitinolytic bacteria as promising biological control agents for various phytopathogens (Chernin et al., 1997; Hoster et al., 2005). *Enterobacter*

sp. has been considered as ideal biological control agent and has been widely evaluated for the production of chitinases (Chernin et al., 1995; Park et al., 1997). *R. solani* is one of the most important fungal pathogens that

cause leaf rot, root rot and damping-off of cabbage. However, there is no detailed study on antifungal activity of chitinase producing *Enterobacter* species against *R. solani*. In this study, purification and characterization of the chitinase, produced by *Enterobacter* sp. KB3 in solid or liquid culture media containing 0.5% colloidal chitin and their efficacy in biological control of *R. solani* in vitro, has been described.

The results from culture filtrate of strain KB3 produced a maximum activity of chitinase after 4th day of cultivation (Figure 3). As shown in Table 1, the chitinase was purified to 9.6-fold with a recovery of 11% and specific activity of 230.5 units/mg protein from culture broth of the strain KB3. The molecular mass of the purified chitinase was estimated as 46 kDa on 12% SDS-PAGE and this was confirmed by zymogram (Figure 4). The molecular weights of bacterial chitinases ranged from 20,000 to 120,000, with little consistency. Different molecular masses have been reported for other bacterial chitinases (Ueda et al., 1995; Park et al., 1997). The optimum temperature for the chitinase activity of the strain KB3 was determined to be 45°C and was stable from 30 to 55°C. In addition, purified chitinase exhibited an optimum activity at a pH of 6.0 and was stable from pH 4.5 to 7.5 with the retention of more than 80% of enzyme activity (data not shown). Several workers have reported that the bacterial chitinases were functional in broad ranges of pH (4.0-8.0) with an optima of pH 4.0 for *Aeromonas* sp. 10S-24 (Ueda et al., 1995), pH 5.5 for *Bacillus* sp. WY22 (Woo and Park, 2003), and pH 6.0 for *Enterobacter* sp. G-1 (Park et al., 1997). The optimum temperature and activity of chitinases from *Piromyces communis* was 60°C and has stability from 40 to 60°C (Sakurada et al., 1996), and that of *Aeromonas* sp. GJ-18 was 45°C and has stability from 35 to 55°C (Kuk et al., 2005).

The results presented here extend with antibiosis as the mechanism of antagonism against *R. solani* by strain KB3 through mediation of chitinase production. The purified chitinase from the strain KB3 exhibited antifungal activity against *R. solani*, identified by well diffusion and microscopic assays (Figures 5 and 6). Several studies have demonstrated that chitinases of potential biocontrol strains can cause deformation of fungal hyphae and result in inhibition of growth of test fungi (Hoster et al., 2005). A strong correlation was observed between the chitinolytic potential of different bacterial strains and in vitro lysis of fungal mycelium (Renwick et al., 1991), whereas such a correlation was absent in the chitinolytic bacteria isolated from cereal grain (Frandsberg and Schnwer, 1998). In this report, correlation between chitinase production and antifungal activity has been observed.

Chitin is a major constituent of the cell walls of filamentous fungi. Several attempts have been made to exploit the biological control of *R. solani* by chitinase

producing microorganisms (Benhamou et al., 1993). Hydrolysis of fungal hyphae by chitinase would result in the release of different forms of GlcNAc. As shown in Figure 7, various products of chitin oligosaccharides such as monomers (GlcNAc), dimers (GlcNAc)₂, and trimers (GlcNAc)₃ were identified by HPLC. Previous study reported that chitinase ChiA71 from *Bacillus thuringiensis* sub sp. *pakistani* hydrolyses colloidal chitin completely to GlcNAc monomers after incubation for 24 h (Thamthiankul et al., 2001). More recently, Van Nguyen et al. (2008) suggested that chitinases from *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9 could release different oligosaccharides after hydrolysis from the hyphae of *Fusarium solani*.

From the results, we draw a positive correlation between the production of chitinase and suppression of the growth of *R. solani*. However, it is necessary to study the secretion of other lytic enzymes as well, especially cellulase, β-1,3-glucanase, and laminarinase, as chitinase may combine with other lytic enzymes to exhibit synergism, and result in high levels of antifungal activity. Therefore, we suggest that *Enterobacter* sp. KB3 may be an optimal candidate for use as a biocontrol agent of leaf rot, root rot and damping-off in cabbage, but further studies are needed to evaluate this possibility.

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