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An extracellular antifungal chitinase from *Lecanicillium lecanii*: purification, properties, and application in biocontrol against plant pathogenic fungi

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Abstract: An extracellular antifungal chitinase from *L. lecanii* strain 43H was purified by ammonium sulfate precipitation and DEAE-Sephadex A-50 ion exchange chromatography; it showed a molecular mass of approximately 33 kDa with a specific activity of 167.5 U/mg protein and purification factor of 2.5. Optimum temperature and pH were observed at 40 °C and pH 6.0, respectively. This enzyme was stable at up to 40 °C and at pH 5.0–6.0. The kinetic constants K_m and V_{max} determined for the chitinase with colloidal chitin as substrate was 0.82 mg/mL and 4.51 U/mg, respectively. The presence of 5–15 mM tested metal ions led to activation of the chitinase activity with an increase of up to 126% except for Al^{3+}, Ag^{+}, and Hg^{2+}. Tween 80 (0.5%), Tween 20 (1%–2%), and Triton X-100 (1%) increased the enzyme activity by up to 25%, whereas higher concentration of 2% SDS completely inhibited the enzyme. The chitinase exhibited high resistance to organic solvents (methanol, acetone) and retained 89%–95% of its initial activity. The chitinase was found to inhibit the conidial germination and mycelial growth of plant pathogenic fungi. These results suggest that chitinase might be used in enzymatic reactions and as a potential fungicide against pathogens.

Key words: Antifungal activity, characterization, chitinase, *Lecanicillium lecanii*, purification

1. Introduction

Chitin, a homopolymer of N-acetyl glucosamine (GlcNAc) residues linked by β-1,4 bonds, is widely distributed in nature as a component of crustacean exoskeleton, insect outer shell, diatoms, fungal cell walls, and squid pens. It is the second most abundant biopolymer next to cellulose, as well as a constant source of renewable raw materials on earth (Tharanathan and Kittur, 2003). Two enzymes catalyzing hydrolysis of the chitin chain to its monomer by synergistic and consecutive action are endochitinases (EC 3.2.1.14), which randomly hydrolyze the β-1,4 glycosidic bonds of chitin, and N-acetylglucosaminidases (chitobiase, EC 3.2.1.30), which preferentially break lower chitooligomers to produce N-acetyl glucosamine (GlcNAc) monomers (Patil et al., 2002).

Chitinases play important roles in biological activities such as nutrient intake, morphological change, defense, and attack. Fish and squid can digest chitinous substances as food by using chitinases in the stomach and liver (Matsumiya and Mochizuki, 1996; Matsumiya et al., 1998), whereas insects and shellfish use chitinases to degrade chitinous substances in the exoskeleton during ecdysis (Kramer and Koga, 1986). In plants, chitinases serve to attack fungal pathogens that contain chitinous substances for self-defense (Singh et al., 2007). Chitinases have received considerable attention due to potential applications in the biocontrol of plant pathogenic fungi and insects (Patil et al., 2002) as a target for biopesticides. Chitinases play an important role in the virulence of many bacteria and fungi for insects and fungi by lysing their cell walls. In addition, chitinases inhibit spore germination and germ tube elongation of the phytopathogenic fungi (Mathivanan et al., 1998). Chitinases can be used widely in the production of (GlcNAc)n and GlcNAc, the formation of yeast and fungal spheroplast and protoplast, and the bioconversion of chitin waste to single cell protein for animal feed (Mathivanan et al., 1998).

Chitinases are produced by various microorganisms such as bacterial (Singh et al., 2008) and fungal antagonists of other fungal mycoparasistes, nematophagous and entomopathogenic fungi, and others (Rocha-Pino et al., 2011). Because of their wide range of biotechnological applications, chitinases have been purified and characterized.

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Lecanicillium lecanii (*Verticillium lecanii*) is an well-known entomophathogenic fungus that is used as an alternative to chemical pesticides, although its utility is limited due to the relatively low death rate of insects (St. Leger et al., 1991). To elucidate fungus pathogenesis of *L. lecanii* towards insects, implied genes and chitinases from *L. lecanii* have been isolated and characterized. The gene chi2 from *V. lecanii* (Zimmermann) Viegas (cDNA, 1269 bp) encoding a chitinase (423 aa) with a molecular mass of 45.95 kDa was analyzed (Lu et al., 2005). The purification of chitinase in *Verticillium cfr. lecanii* A3 was a glycoprotein with apparent molecular weight (MW) of 45 kDa (Fenice et al., 1998). The chitinase *L. lecanii* had a molecular mass of approximately 23 kDa (Mayorga-Reyes et al., 2012). Rocha-Pino et al (2011) reported chitinases with MW of 26, 32, and 51 kDa in solid-substrate culture and with MW of 10, 26, 32, and 45 kDa in submerged cultures of *L. lecanii* (Rocha-Pino et al., 2011). However the chitinase with MW of 32 kDa has not been characterized yet. Therefore, it would be of great importance to study thoroughly the physicochemical properties of the chitinase with MW of 32 kDa from *L. lecanii*. The present study describes the purification and characterization of an extracellular antifungal chitinase from *L. lecanii* 43H and the identification of its biocontrol potential against fungal pathogens.

2. Materials and methods

2.1. Chemicals

Chitin was purchased from HiMedia Laboratories (Mumbai, India) and colloidal chitin was prepared by the Jeuniaux method (1966). DNS (3,5-dinitrosalicylic acid) was obtained from Fluka (Sigma Aldrich Co., St. Louis, MO, USA). DEAE Sephadex A-50 for ion exchange chromatography was supplied by Phamarcia Co. (GE Healthcare), SDS by Sigma Aldrich Co. (St. Louis, MO, USA), Tween 20 and Tween 80 by Bio Basic Inc. (New York, NY, USA), and Triton X-100, Triton X-114, and EDTA by Merck (Darmstadt, Germany). All other chemicals were of analytical grade unless otherwise stated.

2.2. Microorganism and culture conditions

The filamentous fungus *L. lecanii* 43H from the Laboratory of Functional Biocompounds, Institute of Biotechnology, VAST, was grown in 250-mL Erlenmeyer flasks containing 50 mL of the optimal medium with the following compositions (w/v): 0.75% colloidal chitin, 0.1% peptone, 0.1% yeast extract, 0.1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.5% $(NH_2)_2SO_4$, pH 8.0. The inoculated flasks were incubated for 6 days at 28 °C on a rotary shaker at 200 rpm. The culture broth was used immediately for chitinase purification.

2.3. Estimation of chitinase activity

The chitinase activity was determined by measuring the increase in concentration of reducing sugar formed by enzymatic hydrolysis of colloidal chitin. A mixture of 100 µL of the crude or purified chitinase (6 µg for each reaction) was incubated with 300 µL of 0.5% (w/v) colloidal chitin in 50 mM Tris-HCl pH 7.0 at 40 °C for 2 h with an agitation of 150 rpm on a thermostat (Esco Micro Pte Ltd, Singapore). To arrest the reducing sugar released in the reaction mixture, 1 mL of DNS (Miller, 1959) was added and boiled for 10 min. The tubes were cooled down to room temperature and centrifuged at 10,000 rpm for 10 min. The reducing sugars were determined by measuring the absorbance at 540 nm by using N-acetyl glucosamine as a standard. One unit (U) of the chitinase activity was defined as the amount of enzyme that released 1 µmol of N-acetyl glucosamine per hour under the standard assay conditions. All measurements were carried out in triplicate and the mean values were obtained.

2.4. SDS-PAGE and protein concentration

The homogeneity and molecular mass of chitinase was determined by 12.5% SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970), using Biometra equipment (Göttingen, Germany). Proteins were visualized by staining with 0.1% (w/v) silver nitrate. Protein concentrations were measured by the method of Bradford with bovine serum albumin as a standard (Bradford, 1976).

2.5. Purification of chitinase

The culture was centrifuged at 12,000 rpm for 10 min. One hundred milliliters of crude enzyme extract (1 unit) was precipitated with 65% (w/v) ammonium sulfate. The precipitate was collected by centrifugation at 12,000 rpm for 15 min, and resuspended with 6 mL of 20 mM phosphate buffer at pH 7.0. The precipitate was dialyzed against the same buffer overnight at 4 °C and the dialysate (9 mL) was applied to a DEAE-Sephadex ion exchange column (2.6 × 6 cm) and washed with 150 mL of 50 mM Tris-HCl buffer pH 7.0 at a flow rate of 30 mL/h. The proteins were eluted with 50 mM Tris-HCl buffer pH 7.0 containing stepwise gradient 0–1.0 M NaCl at a flow rate of 24 mL/h until $OD_{280} < 0.01$. The eluate was collected with 1.5 mL per fraction. The fractions showing high chitinase activity were pooled and used as purified enzyme for characterization. All purification steps were carried out at 4 °C unless otherwise specified.

2.6. Kinetic parameters

The Michaelis–Menten kinetic parameters (K_m and V_{max}) were determined against 2–20 mg/mL of colloidal chitin as substrate using Lineweaver–Burk plots.

2.7. Temperature and pH optimum

The optimum pH and temperature of chitinase were determined by measuring the activity as described above, using 20 mM potassium acetate buffer (pH 3.5–6.0) and potassium phosphate buffer (pH 6.0–8.0) at 40 °C, and 20 mM potassium phosphate buffer (pH 7.0) for the range of 20 to 70 °C.

2.8. Temperature and pH stability

For the determination of temperature and stability, the purified enzyme, 6 µg for each reaction, was preincubated at different temperatures, ranging from 30 to 40 °C and pH 6.5 for 0–84 h, and at pH range 4.0 to 8.0 (20 mM potassium acetate buffer pH 4.0–6.0 and potassium phosphate buffer 7.0–8.0) and 40 °C for 0–48 h. The residual activity was then determined.

2.9. Effect of metal ions, detergents, and organic solvents

The purified chitinase, 6 µg for each reaction, was preincubated in the presence of 5–15 mM of various ions (Ag^{1+}, Al^{3+}, Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, K^{1+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) and EDTA, or in the presence of 0.5%–2.0% (w/v) of various detergents (SDS; Tween 20, Tween 80, Triton X-114, and Triton X-100), or in the presence of 10%–30% (v/v) of various solvents (methanol, ethanol, isopropanol, n-butanol, and acetone) at 40 °C for 2 h. The residual activity was then determined.

2.10. Enzymatic product analysis

For enzymatic hydrolysis of colloidal chitin, 600 µL of 0.5% (w/v) colloidal chitin as substrate prepared in 50 mM potassium acetate buffer (pH 6.0) was incubated with 100 µL of 6 µg purified chitinase at 40 °C for 96 h. Products were detected by TLC (20 × 20 cm layer of silica gel 60, Merck, Darmstadt, Germany), performed with a mobile phase of n-butanol/acetic acid/water (2:1:1, v/v/v). Carbohydrate products were visualized by spraying the TLC plate with 10% (v/v) sulfuric acid in ethanol and incubating at 120 °C for 15 min. N-acetyl glucosamine was used as the standard.

2.11. Inhibition of fungal growth by chitinase from *L. lecanii* 43H

To assess the hydrolytic activity of chitinase from *L. lecanii* 43H toward fungal mycelia, the reaction mixture (1 mL) containing 2 mg/mL of *Fusarium oxysporum* and *Rhizoctonia solani* mycelia and 200 µL of crude enzyme (or 200 µL of water for the control) was incubated at 40 °C for 24 h. To arrest the reducing sugar released in the reaction mixture, 1 mL of DNS (Miller, 1959) was added and boiled for 10 min. The tubes were cooled down to room temperature and centrifuged at 10,000 rpm for 10 min.

2.12. Determination of antifungal activity of chitinase

In order to determine the antifungal activity of chitinase, 100 mL of spores (10^8/mL) of *F. oxysporum* and *R. solani* was brushed on 0.5-cm-thick PDA plates. The PDA plates were perforated with 0.5-cm-diameter holes and supplemented with 100 µL of crude enzyme solution (3 U/mL) into each hole, or with water for the control. The inhibition activity of chitinase against fungal growth was observed after incubation at 30 °C for 4 days.

3. Results

3.1. Chitinase purification

Chitinase production by *L. lecanii* 43H in the culture medium was 0.528 U/mL (66 U/mg protein) after growth for 144 h. Protein from culture supernatant was precipitated with ammonium sulfate, dialyzed, and applied to DEAE-Sephadex A-50 ion exchange chromatography. Through precipitation by ammonium sulfate, the chitinase gained a specific activity of 92.11 U/mg proteins with a purification factor of 1.4 and a recovery of 15.7% (Table 1). The chitinase through the DEAE-Sephadex A-50 ion exchange chromatography was purified with a factor of 2.5, recovery of 1.9%, and specific activity of 167.5 U/mg (Table 1). The purified chitinase showed a single protein band of approximately 33 kDa on SDS-PAGE (Figure 1A, lanes 5–9).

The activity of crude chitinase and fractions were determined on agar plate containing 0.5% colloidal chitin. The result showed that a white loop of hydrolyzed chitin appeared on the walls after staining with lugol on agar plate (Figure 1B). Therefore, we have purified chitinase from *L. lecanii* 43H.

3.2. Temperature and pH optimum

The optimum temperature and pH for chitinase activity were 40 °C and pH 6. The chitinase activity increased gradually from 28% (185.1 U/mg) at 20 °C to the maximum (663.9 U/mg) at 40 °C, decreased steeply to 16%

Table 1. Purification steps of chitinase from crude enzyme by *L. lecanii* 43H.

Steps	Total protein (µg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme	800	52.8	66	1	100
65% $(NH_4)_2SO_4$	90	8.29	92.11	1.4	15.7
DEAE-Sephadex	6	1.005	167.5	2.5	1.9

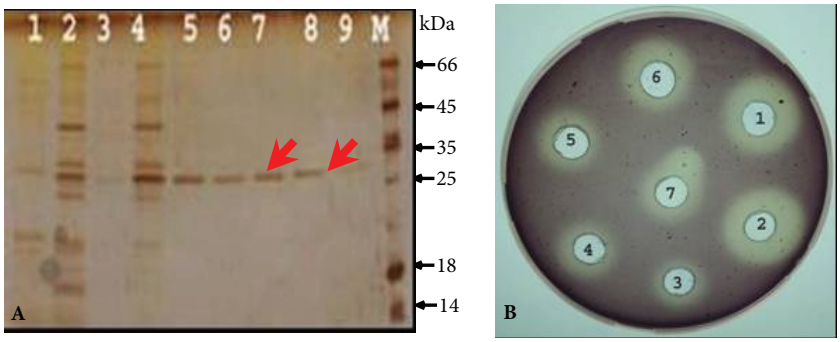

Figure 1. SDS-PAGE of the purifed chitinase from *L. lecanii* 43H through DEAE-Sephadex A-50 (A) and the chitinase activity staining with lugol on agar plate (B).
Lane 1: the crude enzyme; lane 2, 4: precipitated by ammonium sulfate; lane M: Molecular mass marker; lane 3, 5–9: fractions from DEAE-Sephadex A-50 column.

(107.5 U/mg) at 55 °C, and then gradually to 64.5 U/mg (10%) at 70 °C (Figure 2). This enzyme works at a broad pH range of 5.0 to 8.0. The activity showed no change from 11% (61.7 U/mg) at pH 3.5 to 11.2% (63.5 U/mg) at pH 4.5, increased steeply to 84% (476.3 U/mg) at pH 5.5, reached the maximum of 100% (568.2 U/mg) at pH 6.0, and then decreased gradually to 83% (436.4 U/mg) at pH 7.5 (Figure 3).

3.3. Temperature and pH stability

Chitinase from *L. lecanii* 43H was stable at temperature 30, 37, and 40 °C after incubation up to 8 h and the enzyme retained over 80% of its initial activity. The chitinase activity dramatically decreased in the time interval between 8 and 12 h and then gradually decreased in the interval between 12 h and 84 h (Figure 4). The enzyme was stable at pH 5.0 to 6.0 after incubation up to 12 h and retained over 65% of its original activity. At lower (≤4.0) or higher pH (≥7.0), the chitinase activity decreased dramatically just in the time interval 0 to 1 h to approximately 40% of its initial activity, and then gradually in the time interval between 1 and 48 h (Figure 5).

3.4. Effects of metal ions and EDTA on chitinase activity

The addition of EDTA and tested metal ions for Ag^{+}, Hg^{2+}, and Al^{3+} at the concentration of 5 to 15 mM in general increased the chitinase activity by up to 126%. Zn^{2+}especially activated the enzyme with an increase of 44% to 126% in chitinase activity. The addition of Ag^{+}, Hg^{2+}, and Al^{3+} reduced the enzyme activity to half of its original activity (Table 2).

3.5. Effects of detergents on chitinase activity

The effect of ionic (SDS) and nonionic detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114) currently used for denaturing of glycoproteins was tested on chitinase activity. The addition of 0.5% (w/v) of Tween 80, 1.0%–2.0% (w/v) of Tween 20, and 1% (w/v) of Triton X100 increased the enzyme activity by up to 25%. The addition of 0.5%–1.5% (w/v) of SDS reduced the activity to one third, and at the higher concentration of 2.0% (w/v) completely inhibited it (Figure 6).

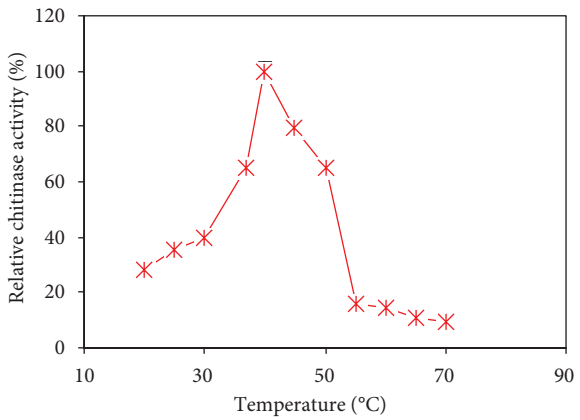

Figure 2. Optimum temperature of chitinase from *L. lecanii* 43H.

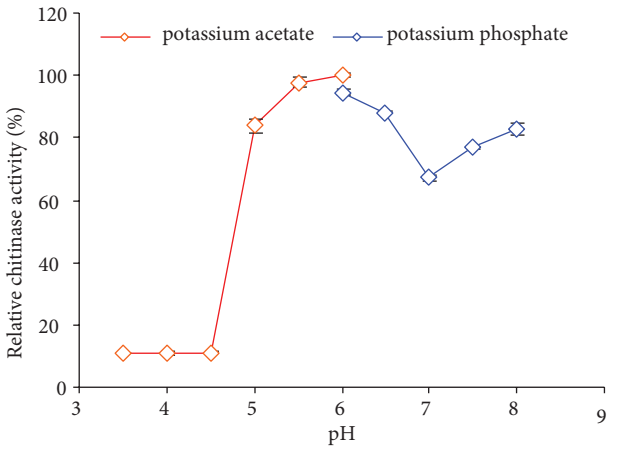

Figure 3. Optimum pH of chitinase from *L. lecanii* 43H.

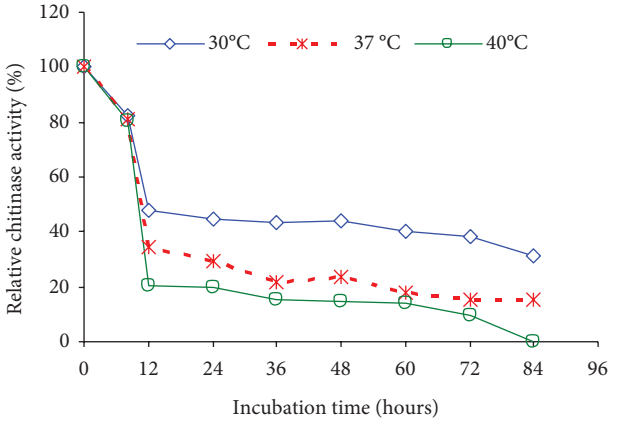

Figure 4. Temperature stability of chitinase from *L. lecanii* 43H.

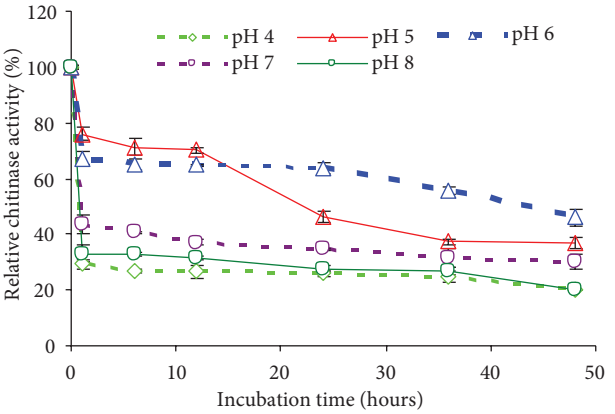

Figure 5. pH stability of chitinase from *L. lecanii* 43H.

Table 2. Effects of metal ions on chitinase activity from *L. lecanii* 43H.

Metal ions	Residual activity (%) at the concentration of (mM)		
	5	10	15
Ag^{+}	72 ± 2.3	56 ± 0.0	58 ± 1.4
Al^{3+}	62 ± 1.6	67 ± 4.0	56 ± 0.0
Ba^{2+}	113 ± 0.1	122 ± 4.6	156 ± 18.7
Ca^{2+}	115 ± 2.5	98 ± 4.8	166 ± 6.1
Cu^{2+}	130 ± 0.7	142 ± 8.7	156 ± 14.1
Fe^{2+}	131 ± 7.4	128 ± 2.6	71 ± 0.1
Hg^{2+}	56 ± 0.0	56 ± 0.0	56 ± 0.0
K^{+}	107 ± 6.8	102 ± 5.9	128 ± 1.3
Mg^{2+}	130 ± 0.3	123 ± 6.8	101 ± 1.3
Mn^{2+}	98 ± 8.2	113 ± 2.7	56 ± 0.0
Ni^{+}	135 ± 3.6	128 ± 10.2	157 ± 8.9
Zn^{2+}	144 ± 3.7	192 ± 5.6	226 ± 8.5
EDTA	156 ± 6.2	122 ± 6.5	101 ± 4.5

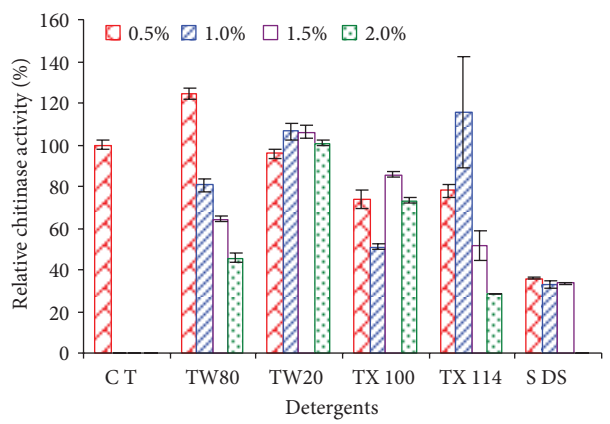

Figure 6. Effects of detergents on chitinase from *L. lecanii* 43H.

3.6. Effects of organic solvents on chitinase activity

Organic solvents are used for solubilizing hydrophobic substrates in enzymatic reactions; thus we have tested effects of various organic solvents. The addition of methanol and acetone at the concentration of 10%–30% showed a slight change of up to 16% of its initial activity. The addition of isopropanol, ethanol, and butanol decreased strongly the chitinase activity. The enzyme retained only 37%–42% of its original activity (Figure 7) when it was treated with butanol.

3.7. Kinetic parameters

The K_m and V_{max} obtained for the purified chitinase from *L. lecanii* 43H were 0.82 mg/mL and 4.51 U/mg protein with colloidal chitin as substrate, respectively.

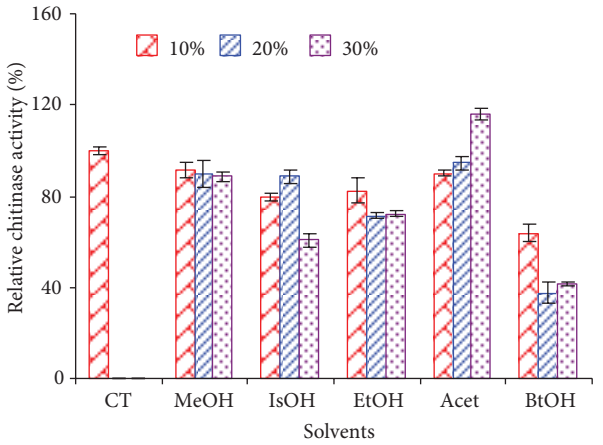

Figure 7. Effects of organic solvents on chitinase from *L. lecanii* 43H.

3.8. Hydrolysis products

The major hydrolysis product of colloidal chitin by the purified chitinase from *L. lecanii* 43H was N-acetyl glucosamine, detected on TLC (Figure 8).

3.9. Application in biocontrol against plant fungal pathogens

Chitinase from *L. lecanii* 43H strain exhibited inhibition of mycelial growth of plant pathologic fungi *F. oxysporum* and *R. solani* (Figure 9). The diameters of the inhibition cycles of these 2 strains were 0.54 and 0.44 cm, respectively, whereas the negative control hole (water) showed no inhibition cycle. Observations under the microscope (160×) revealed that growth inhibition by chitinase was accompanied by mycelial digestion and cell lysis, whereas mycelia treated with water maintained its shape (Figure 10). The amount of sugar N-acetyl glucosamine released by the treated mycelia of the plant pathologic fungi *F. oxysporum* and *R. solani* were 11.07 µg/mL and 9.18 µg/mL, respectively.

Figure 8. TLC analyses of hydrolysis products. Lane 1: purified chitinase, lane 2: hydrolysis products of colloidal chitin after 96 h, lane 3: 0.5% (w/v) of colloidal chitin, lane 4: N-acetyl glucosamine standard.

4. Discussion

Based on molecular mass, chitinases from *Lecanicillium* (*Verticillium*) can be divided into 4 groups: Group I with MW of ≤26 kDa from *L. lecanii* (Rocha-Pino et al., 2011; Mayorga-Reyes et al., 2012), *L. fungicola* (Ramirez-Coutino et al., 2006; Ramirez-Coutino et al., 2010), and *L. muscarium* (Barghini et al., 2013); Group II with MW of 32–33 kDa from *L. lacanii* (Liu et al., 2003; Rocha-Pino et al., 2011), *L. fungicola* (Ramirez-Coutino et al., 2006); Group III with MW of 40–50 kDa from *L. lecanii* (Fenice et al., 1998; Liu et al., 2003; Lu et al., 2005; Rocha-Pino et al., 2011), *V. chlamydosporium* and *V. suchlasporium* (Tikhonov et al., 2002), *V. fungicola* (Ramirez-Coutino et al., 2010), *L. psalliotae* (Gan et al., 2007); and Group IV with MW of ≥55 kDa from *L. muscarium* (Barghini et al., 2013), *L. fungicola* (Ramirez-Coutino et al., 2006; Ramirez-Coutino et al., 2010). Our chitinase belongs to Group II with MW of 32–33 kDa, and. according to Rocha-Pino et al. (2011), it could be an exochitinase.

The purification factor for chitinase from *L. lecanii* 43H was lower than those of *M. anisopliae* (4.98) and *G. virens* (105) and as high as that of chitinase from *T. hariuzaum* (1.3). The purification yield of chitinase from *L. lecanii* 43H was lowest (2.5%) among those reported (St. Leger et al., 1991; di Pietro et al., 1993; Harma et al., 1993). The specific activity of chitinase in this study (167.5 U/mg) was higher than that (1.1–2.3 U/mg) of *T. harizum* and *M. anisopliae,* but lower than that of *G. virens* (1018.5 U/mg).

The optimum pH and temperature were observed at pH range of 4.0–6.0 and temperature range of 40–60 °C for most fungal chitinases such as from *Lecanicillum* species (Tikhonov et al., 2002; Barghini et al., 2013), *Talaromyces flavus* (Duo-Chuan et al., 2005), *Gliocladium catenulatum* (Ma et al., 2012), *M. anisopliae* (Kang et al., 1999), and *Aspergillus fumigatus* (Xia et al., 2009).

Similarly, most fungal chitinases show temperature stability up to 40 °C and pH stability of 4.0–6.0, and retained over 80% of their original activity after incubation for a certain time period. The chitinase from *Fusarium chlamydosporum* was stable at temperature up to 40 °C and pH range of 4.0–6.0 (Mathivanan et al., 1998). *A. fumigatus* chitinase exhibited thermostability at 45 °C and pH stability at 4.0–7.5 (Xia et al., 2009). The chitinase from *G. catenulatum* showed a pH stability of 4.0–5.0 and thermostability of 20–40 °C after incubation for 20 min (Ma et al., 2012).

For many microbial chitinases the addition of the metal ion Ca^{2+} enhances their activity. Such is the case for

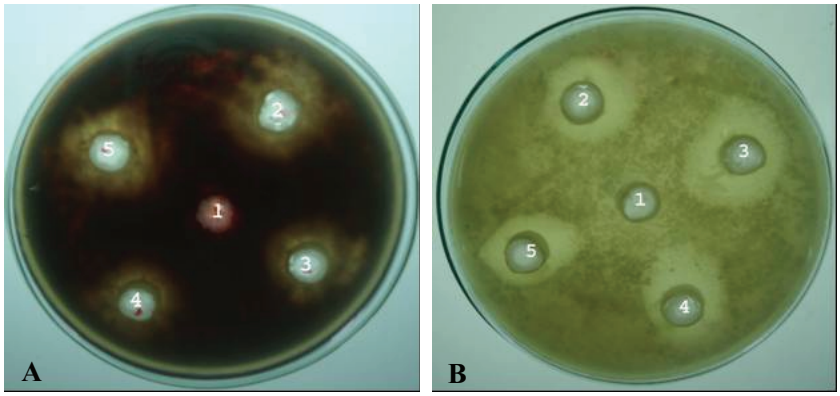

Figure 9. Inhibition of mycelial growth of plant pathogenetic fungi *F. oxysporum* (A) and *R. solani* (B) by chitinase from *L. lecanii* 43H. Lane 1: water, lane 2–5: chitinase.

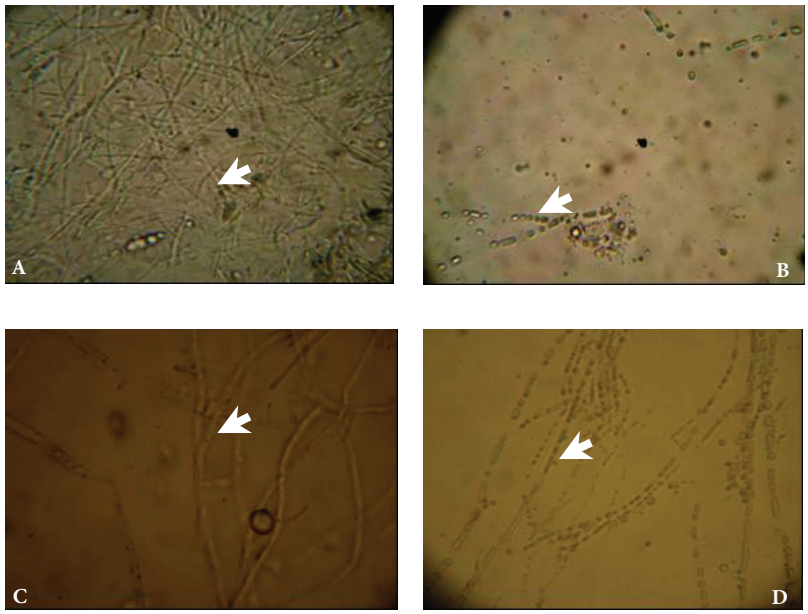

Figure 10. *R. solani* (A, B) and *F. oxysporum* (C, D) mycelia treated with water as negative control (A, C) and with chitinase from *L. lecanii* 43H (B, D).

chitinase from *L. lecanii* 43H (this study), *G. catenulatum* HL-11 (Ma et al., 2012), *Micrococcus* sp. AG84 (Annamalai et al., 2010), *Alteromonas* sp. strain O-7 (Tsujibo et al., 1992), and *Enterobacter* sp. NRG4 (Dahiya et al., 2005). In contrast to Ca^{2+}, the addition of the metal ions Ag^{+} and Cu^{2+} exhibited strong inhibitory effects on chitinases from *G. catenulatum* HL-11 (Ma et al., 2012), *Micrococcus* sp. AG84 (Annamalai et al., 2010), *F. chlamydosporum* (Mathivanan et al., 1998), and *Enterobacter* sp. NRG4 (Dahiya et al., 2005), with the exception of Cu^{2+} in the current study, which stimulated the chitinase from *L. lecanii* 43H.

The detergents including Tween 20, Tween 80, and Triton X-114 stimulated the chitinase activity of *L. lecanii* 43H, whereas Triton X-100 was found to activate the chitinase from *Bacillus* sp. BG-11 (Bhushan and Hoondal, 1998). The chitinase produced by *Bacillus licheniformis* MB-2 was resistant to denaturation by urea, Tween 20, and Triton X-100, but unstable toward dimethyl sulfoxide and polyethylene glycol (Toharisman et al., 2005). In contrast, the chitinase from *L. lecanii* 43H showed high resistance to acetone, methanol, ethanol, and isopropanol.

The K_m value (0.82 mg/mL) was lower than that obtained for the chitinase from *G. catenulatum* HL11 (2.832 mg/mL) (Ma et al., 2012) and from *Enterobacter* sp. NRG4 (1.41 mg/mL) (Dahiya et al., 2005), but higher than those (0.3 and 0.5 mg/mL) obtained for the chitinases from *T. harzianum* CECT 2413 (de la Cruz et al., 1992). The V_{max} value (4.51 U/mg protein) obtained for the chitinase from *L. lecanii* 43H was lower than those obtained for the

chitinases from *Enterobacter* sp. NRG4 (74.07 μmol/h/μg protein) and *T. harziunum* CECT 2413 (3.6 and 5.2 μg/min/mg) when colloidal chitin was used as substrate.

The major hydrolysis products of colloidal chitin by the chitinase from *L. lecanii* 43H were identified as N-acetyl glucosamine on TLC without oligomer of N-acetyl glucosamine. In contrast, the *Aeromonas* chitinolytic enzymes hydrolyzed colloidal chitin predominantly to N,N-diacetyl-chitobiose and, to a much lesser extent, the mono-, tri-, and tetramer of N-acetyl glucosamine, and the enzymes were suggested to be endochitinases (Huang et al., 1996). Our chitinase belongs to Group II with MW of 32–33 kDa, and, according to Rocha-Pino et al. (2011), it could be an exochitinase. Thus, both results suggest that the chitinase from *L. lecanii* 43H might be an exochitinase.

The chitinase from *L. lecanii* 43H showed antifungal activity against plant pathogenic fungi including *F. oxysporum* and *R. solani*, in agreement with other studies. The chitinase from *G. catenulatum* HL-1-1 inhibited the hyphal growth, conidial germination, and sclerotial germination of 10 pathogenic fungi including *F. oxysporum* and *R. solani* (Ma et al., 2012). The purified chitinase from *F. chlamydosporum* inhibited the germination of uredospore of *Puccinia arachidis* and also lysed the walls of uredospore and germ tubes (Mathivanan et al., 1998). The purified endochitinase of *Trichoderma* had activity against *Sclerotium rolfsii* (El-Katatny et al., 2005). The chitinase from *Serratia marcescens* MO-1 had high activity and also inhibited the development of some fungi such as *Aspergillus niger*, *Rhizopus oryzae*, *F. oxysporum*, *T. harzianum*, and *Alternaria citri* (Okay et al., 2013)

In the current study, we purified and studied thoroughly the physiochemical properties of chitinase from the entomopathogenic fungus *L. lecanii* 43H. The results suggest that this chitinase might be an exochitinase and it has high potential for biotechnological application in biocontrol of plant pathogenic fungi.

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