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Purification and characterization of a 1,3-β-D-glucanase from *Streptomyces torulosus* PCPOK-0324

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ABSTRACT

A hydrolytic enzyme designated as a 1,3-β-D-glucanase having an antifungal activity was purified and characterized from *Streptomyces torulosus* PCPOK-0324. Fungal growth inhibitors in the culture filtrates from an antagonistic *S. torulosus* PCPOK-0324 exhibited higher antifungal activity against the hyphal growth of *Phytophthora capsici* and *Rhizoctonia solani*. The 1,3-β-D-glucanase was purified by four chromatographic steps from culture supernatant. The molecular weight of the purified enzyme was estimated to be 31.5 kDa. The optimal pH and temperature were 7.5 and 50 °C. Both the purified enzyme and the antibiotic extract inhibited *R. solani* and *P. capsici* with minimal inhibitory concentration values of 12.50 and 6.25 mU ml^{-1} and 3.95 and 1.94 μg ml^{-1}, respectively. Our findings collectively show that the 1,3-β-D-glucanase in combination with the antibiotic extract have strong synergistic antifungal activity against the hyphal growth of both fungi causing root rot disease in pepper plants.

1. Introduction

Synthetic fungicides have long been used as active agents in reducing the incidence of plant diseases; however, their use is costly, can cause environmental pollution, and may induce pathogen resistance. Thus, considering the limitations of chemical fungicides, biological control affords a great deal of attention to develop an attractive or alternative supplement to pesticides for the management of plant diseases without the negative impact of chemical control. Further, biological control with natural substances has become an important approach to facilitating sustainable agriculture. The use of antagonistic species of *Streptomyces* as biocontrol agents has been widely studied due to their ability to produce several antimicrobial compounds, including hydrolytic enzyme such as a 1,3-β-D-glucanase that are known as one of the most potent enzymes for degrading fungal cell walls. 1,3-β-D-Glucanases (glucan endo-1,3-glucosidase, EC 3.2.1.39), a part of the defense of plants against pathogenic fungi, are highly regulated hydrolytic enzymes widely distributed in seed plants (Hong, Cheng, Huang, & Meng, 2002; Leelasuphakul, Sivanunsakul, & Phongpaichit, 2006; Nishimura et al., 2001). Root rots of pepper (*Capsicum annuum* L.) caused by *Phytophthora capsici* Leonian and *Rhizoctonia solani* Kuhn is a serious and economically important disease in most of the world's pepper growing areas (Ahmed, Ezziyyani, Pérez Sánchez, & Candela, 2003). This pathogen causes severe yield losses of the production of various vegetables (Cardinale, Ferraris, Valentino, & Tamietti, 2006; Hwang, Kim, & Kim, 1996; Ristaino & Johnston, 1999). Current disease control methods are primarily based on chemicals, however, exclusive reliance on the use of fungicide often cause problem in disease management (Jiang, Guo, Li, Qi, & Guo, 2006; Larkin & Fravel, 1999). In addition, it is hard to control this soil-born disease because the pathogens can survive in soil for several years (Jiang et al., 2006; Lamour & Hausbeck, 2001). Research reports about the biological control of *Phytophthora*-blight of pepper have described the isolation of many effective microorganisms, and their efficiencies have been tested in greenhouse or small field experiments (Chung, Hunag, Huang, & Jen, 2003; Guo, Tamanoi, & Novick 2001; Jiang et al., 2006). Thus, studying the role of hydrolytic enzymes in antifungal activity of *Streptomyces* spp. is of great interest and importance in biocontrol.

It is well known that *Streptomyces* is the largest genus of *Actinobacteria* and the type genus of the family *Streptomycetaceae*, producing over two-thirds of the clinically useful antibiotics of natural origin (e.g., neomycin, chloramphenicol). Actinomycetes of the genus *Streptomyces* are well known for their ability to suppress growth of fungal pathogens and they have been used extensively in the biological control (Fayad et al., 2001; Liu, Anderson, & Kinkel, 1995). Many antibiotics produced by actinomycetes have been used

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directly or have been assumed responsible for the potential biocontrol of the producing strain (Trejo-Estrada, Paszczynski, & Crawford, 1998). *Streptomyces* spp. are also known for their ability to produce fungal cell wall-degrading enzymes such as cellulases, hemicellulases, chitinases, and glucanases (Mitsutomi et al., 1998; Shekhar, Bhattacharya, Kumar, & Gupta, 2006). The role of these hydrolytic enzymes in antifungal activity and biocontrol ability of *Streptomyces* has been investigated (Chamberlain & Crawford, 2000; Joo, 2005). However, there are no reports on *Streptomyces torulosus* isolate as biological control agent for the *Phytophthora*-light disease and none has established their mode of action especially as being caused by a fungal cell wall degrading enzyme. This study presents the purification, characterization and application of 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324.

2. Materials and methods

2.1. Isolation and identification of glucanase-producing bacterium

The bacterium PCPOK-0324 was isolated from the rhizosphere of pepper plants. Rhizosphere soils closely associated with the roots were collected to a depth of 30 cm. Each bulk soil sample was collected approximately 2 m away from any plants and just below the root zone of any grasses growing on the surface. The soil samples were transported to the laboratory in ice-coolers and stored at 4 °C until analyze. Actinomycetes were cultured on yeast casamino acids extract and dextrose agar (YCED) which contained yeast extract (Difco; 0.3 g l^{-1}), casamino acid (Difco; 0.3 g l^{-1}), D-glucose (0.3 g l^{-1}), and agar (Difco; 18.0 g l^{-1}). The media were buffered with K_2HPO_4 (0.5 g l^{-1}) and adjusted to pH 7.2 prior to autoclaving. Cyclohexamide (100 μg ml^{-1}) was added to reduce fungal contamination. Plates were incubated at 25 °C for 14 days until sporulated actinomycetes colonies are visible. Isolates were purified and stored at 4 °C.

To select bacteria able to secret 1,3-β-D-glucanase, soil bacteria obtained from several locations were inoculated on agar plates containing pachyman (0.2%, w/v) and aniline blue (0.005%, w/v) on which the positive colony formed a clear halo around the colony (Mahasneh & Stewart, 1980). Out of 56 isolates, only 14 exhibited fungal antibiosis *in vitro* toward pepper pathogenic fungi, *P. capsici*, and *R. solani*. Because of broad-spectrum antifungal activity, the isolate tentatively named as PCPOK-0324 that formed the largest halo was selected for further study.

To identify this unknown isolate, the morphological, physiological and biochemical properties of unknown isolate were characterized as described previously (Choi, Kim, Kim, & Han, 2005). Further, 16S rRNA gene sequence was compared with the reference sequences available in Genbank database and Ribosomal Database Project-II (Klappenbach, Saxman, Cole, & Schmidt, 2001).

2.2. Chemicals

1,3-β-D-Glucans such as laminarin (from *Laminaria digitata*), curdlan (from *Alcaligenes faecalis*), zymosan A (from *Saccharomyces cerevisiae*) were purchased from Sigma–Aldrich (St. Louis, MO, USA), while pachyman was prepared from commercial fruiting bodies of the basidiomycete *Poria cocos*. Xylan, carboxymethylcellulose (CMC), and glucans with other types of glucosidic linkages, such as lichenan (1,3–1,4-β), cellulose (1,4-β), amylose (1,4-α), and β-cyclodextrin (1,4-α), were also purchased from Sigma–Aldrich (St. Louis, MO, USA). ISP mediums used for characterizing the *Streptomyces* species according to the International Streptomyces Project (ISP) were purchased from Difco Laboratories (Franklin Lakes, NJ, USA).

2.3. Fungal growth inhibition by culture filtrates

The culture filtrates of *S. torulosus* strain PCPOK-0324 were mixed (1:1) with double strength (2×) of melted sterile PDA and poured onto 9 cm diameter plates. Triplicate experiments were performed. A 0.5 cm agar plug of an actively growing fungal mycelium of *R. solani* and *P. capsici* cultures was placed on the center of the test agars. The fungal cultures were further incubated under moist conditions at 25 °C for 3 days to *R. solani* and 5 days to *P. capsici*. Radial growth of the fungal colony was measured and the percentage of mycelial growth inhibition was calculated according to the method of Gamliel, Katan, and Cohen (1989).

2.4. 1,3-β-D-Glucanase assay and protein determination

1,3-β-D-Glucanase activity was routinely determined by a colorimetric method (Ghose, 1987). The amount of reducing sugar released from laminarin was measured. The standard assay (0.5 ml) contained 10 μl of the crude enzyme solution and 90 μl of 5 mg ml^{-1} laminarin in 100 mM sodium acetate buffer at pH 5.5. After incubation at 37 °C with gentle shaking for 10 min, the reaction was quenched by boiling for 5 min and 200 μl of 1% dinitrosalicylate (DNS) and 200 μl of sodium acetate buffer was added into reaction mixture and boiled for another 5 min, then placed in ice bath. The optical absorption was measured at 540 nm. The reducing sugar released was calculated from a standard curve prepared with glucose. One unit of 1,3-β-D-glucanase activity was defined as the amount of enzyme that released 1 μmol of glucose equivalent per min under the standard assay conditions. All experiments were performed twice, with three replicates for each treatment. Protein concentrations were determined by the method of Bradford (1976) and bovine serum albumin was used as standard.

2.5. Enzyme purification

S. torulosus PCPOK-0324 was propagated at 30 °C on YCED and then, abundant sporulation was observed after 4 days of growth. The spores used for inoculation of glucanase production medium were collected from 10-day-old plates. For β-1,3-glucanse production, fungal strain PCPOK-0324 was grown in 2000 ml of medium containing lyophilized cells derived from *R. solani*. The lyophilized cell walls (10 g of dry weight) were suspended in 1800 ml of distilled water, autoclaved, and then combined with 200 ml of sterile minimum salt medium (per liter): $(NH_4)_2SO_4$, 10 g; K_2HPO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 100 mg. The culture was incubated for 12–15 h at 30 °C with 200 rpm.

To prepare the crude cell walls, cell walls were obtained from the mycelia of the fungus *R. solani* as described by Trejo-Estrada et al. (1998). The above fungal species was grown in potato dextrose broth, incubated at 30 °C, and then harvested after 72 h growth. The mycelium was filtered, homogenized in a Waring blender (31BL91, New Hartford, CT, USA), and thoroughly washed with distilled water. Microscopic analysis showed the presence of cell wall fragments. The preparation, at 45% solid, was autoclaved and then used in preparation of culture media.

After cultivation, proteins in the supernatant of the bacterial culture were precipitated from the supernatant with 60% saturation of an ammonium sulfate and centrifuged at 12,000 × *g* for 15 min. The pellet was dissolved in 50 mM Tris–HCl buffer, pH 7.5 (buffer A) and dialyzed in the same buffer. The crude 1,3-β-D-glucanase in preparation was loaded on ion exchange chromatography DEAE-Sephadex column (25 mm × 250 mm; Pharmacia Biotech., Piscataway, NJ, USA) equilibrated with 50 mM Tris–HCl, pH 7.5 (buffer A) and bound protein was eluted with 1 M NaCl in the same buffer. Following concentration to 25 ml on an Amicon PM-30 membrane (Millipore Co., Billerica, MA, USA) and

dialysis against 20 mM Tris–HCl, pH 7.2 (buffer B), the fraction was loaded onto a Phenyl-Superose HR 5/5 column (Pharmacia Biotech., Piscataway, NJ, USA). Bound protein was eluted by applying a linear 0.0–1.0 M NaCl gradient in buffer B. Finally, the 1,3-β-D-glucanase preparation was concentrated to 3 ml using an Amicon PM-30 membrane, dialyzed against 20 mM sodium acetate buffer, pH 5.0 (buffer C). Saturated $(NH_4)_2SO_4$ in buffer C was added to a final concentration of 1.7 M before applying the enzyme preparation onto a Mono Q HR 5/5 (Pharmacia Biotech., Piscataway, NJ, USA) equilibrated with 1.7 M $(NH_4)_2SO_4$ in buffer C. Bound protein was eluted with buffer C. The fractions with the 1,3-β-D-glucanase activity were pooled, and applied onto a prepacked Superose 12 Prep grad column (Amersham Bioscience, Buckinghamshire, UK). It was eluted at 3 ml h^{-1} dialyzed against buffer C, then against deinonized water, and freeze-dried. All steps were carried out at 4 °C.

2.6. Enzyme analysis and characterization

SDS-PAGE on 12% (w/v) polyacrylamide slab gels was carried out according to Laemmli (1970). Gels were visualized with silver staining using a silver staining kit following the instructor's manual (ElpisBio, Korea). Subsequently, the purified protein band was electroblotted onto a Millipore polyvinylidene fluoride membrane according to Cheng and Walker (1998) for N-terminal sequencing. Protein bands identified by silver stain solution were excised and subjected to Edman degradation using a Procise 491 Protein Sequencer (Applied Biosystems, CA, USA). To obtain an internal sequence of the protein, the 1,3-β-D-glucanase was digested with trypsin and the resulting peptide fragments were purified by HPLC. The N-terminal portion of a purified peptide fragment was sequenced. Analysis of the 1,3-β-D-glucanase amino acid sequence with other proteins was then done using the BlastX (http://www.ncbi.nlm.nih.gov/BLAST/).

The effect of temperature and pH on enzyme activity was determined. The standard conditions were used in the assay (temperatures between 20 and 90 °C for 30 min). The pH of the reaction mixture varied between 3 and 11 using 50 mM buffer (sodium acetate, pH 3.0–6.0; sodium phosphate pH 6.0–7.0; Tris–HCl, pH 7.0–9.0; and glycine buffer, pH 9.0–11.0).

2.7. Preparation and antifungal activity of antibiotic substance

The cell-free supernatants of 5-day-old cultures of *S. torulosus* PCPOK-0324 in minimum salt medium containing 0.5% of lyophilized cells derived from *R. solani*, at pH 6.0 were adjusted to a pH of 2.5 with 12 M HCl. The precipitate was collected by centrifugation at 15,000 × *g* for 20 min (McKeen, Reilly, & Pusey, 1986). Partial purification was performed by extracting the sediment three times with 80% ethanol and dried by rotary evaporation. The pale brownish substances were dissolved in 80% ethanol and kept at 4 °C. The inhibitory activity of the antibiotic substances on the hyphal growth of *P. capsici* and *R. solani* as (1.0–0.007 mg ml^{-1}) was tested, and minimum inhibitory concentrations (MICs) and IC_{50} values were determined. MIC for individual antifungal activity as well as for binary mixtures was defined as the minimal tested concentration that inhibited *R. solani* and *P. capsici* growth.

Assays were performed under sterile conditions. The test was carried out by the method described by Lorito, Peterbauer, Hayes, and Harman (1994) with slight modification and Schirmböck et al. (1994). Test suspensions with 1000–3000 spores per well were transferred to sterile flat-bottom ELISA plates and incubated at 25 °C. After 22–30 h, the plates were observed under an inverted microscope. The percentage of conidia germinating was determined as the percentage of the first 100 spores randomly found in the well. In addition, the lengths of 20 germ tubes were measured and averaged. All experiments were performed twice, with three replicates for each treatment.

2.8. Assays for antifungal activity of the enzyme and antibiotic substances

The synergistic antifungal activity against *P. capsici* and *R. solani* of the mixtures of the 1,3-β-D-glucanase enzyme and antibiotics prepared from the culture filtrate of *S. torulosus* PCPOK-0324 was determined using the modified checkerboard method (Lorian, 1996). The choice of the concentrations of the initial enzyme and antibiotic solutions used for this assay was 20 times higher than that of their MIC values with each fungus. This was 250.0 and 125.0 mU ml^{-1} for the enzyme and 62.6 and 31.2 μg ml^{-1} for the antibiotic. Enzyme was serially diluted (1:2, 1:4, 1:8) with 50 μl of 50 mM Tris–HCl buffer (pH 7.5), and antibiotics diluted with 80% 50 μl of ethanol were mixed thoroughly with 900 μl of 50 mM Tris–HCl buffer, then 100 μl was dropped into the 6 mm-diameter paper disc. A mixture of equal volumes of Tris–HCl buffer and 80% ethanol was used as control. All experiments were performed twice, with three replicates for each treatment. The inhibition values obtained for the two experiments were determined, and standard deviations were calculated from these data. Further, MIC and IC_{50} values were calculated (Picman, Schneidera, & Gershenzon, 1990). To determine the 50% effective dose, the dose–response curves were subjected to regression analysis by using a binomial regression of the third order, with R^2 ranging between 0.95 and 0.99.

The combination data of 1,3-β-D-glucanase and antibiotic extract against *R. solani* and *P. capsici* was obtained by a broth checkerboard method (Li & Rinaldi, 1999). A series of twofold dilutions of one compound were tested in combination with the other compound. The result of the checkerboard test was expressed as the fractional inhibitory concentration (FIC) index. MICs were transformed into fractional inhibitory concentration (FIC) as suggested by Davidson and Parish (1989).

$$FIC_A = \frac{\text{MIC of A in presence of B}}{\text{MIC of A}} \quad (1)$$

$$FIC_B = \frac{\text{MIC of B in presence of A}}{\text{MIC of B}} \quad (2)$$

Fractional inhibitory concentration index (FIC_{index}) was then calculated from FIC values for each antimicrobial as follows:

$$FIC_{index} = FIC_A + FIC_B \quad (3)$$

The interaction was defined as synergistic if the MIC of enzyme/antibiotic was at least fourfold lower when the enzyme/antibiotic was used in combination than when it was used alone. In this method, synergism is defined as an FIC_{index} of $\leq$0.5; additive as an FIC_{index} of 0.5–1.0; and antagonism as an FIC_{index} of >1.0. The lowest FIC_{index} from each checkerboard was recorded.

3. Results and discussion

3.1. Isolation of bacterial new strain

Numerous microorganisms with antifungal activities and their active factors such as chitinolytic enzymes and beta-glucanases have been characterized. Consequently, the antifungal mechanisms by which these microorganisms inhibit the growth of potentially pathogenic fungi have been demonstrated (Hong et al., 2002; Leelasuphakul et al., 2006; Nishimura et al., 2001), although many questions remain to be addressed. Recently, biochemical research on plant disease biocontrol focused on three primary objectives: (1) selection of microorganisms with antifungal activities; (2) isolation and characterization of the specific fungicidal factors such

as antibiotics, β-glucanases and chitinolytic enzymes; (3) determination of the operative mechanisms of these antifungal agents. Therefore, screening of a new antagonistic species of *Streptomyces* as biocontrol agents producing several antimicrobial compounds including hydrolytic enzyme like 1,3-β-D-glucanases known as one of the most potent enzymes for degrading fungal cell walls (Hong et al., 2002; Leelasuphakul et al., 2006; Nishimura et al., 2001) is important step to evaluate and understand their biological activity.

We recently isolated a new strain named as *S. torulosus* PCPOK-0324 based on 16S rRNA sequence analysis. The optimal growing conditions for this bacterium were pH 7.0 and temperature 40 °C. The bacterium showed typical characteristics of *Streptomyces*, such as aggregation of mycelium in broth medium and formation of hard colony surfaces on agar plates. The isolate grew on ISP media 1–7, forming an olive brown pigment on ISP 6 and 7, respectively. The isolate was Gram-positive, and whole-cell hydrolysate had positive LL-diaminopimelic acid (LL-DAP) activity and consequently was classified to have cell wall type I (data not shown). All biochemical characteristics were consistent with that of the genus *Streptomyces*. Specifically, the aerial mycelium observed growing on ISP 1 began as yellow but progressed to black, white, and then gray according to incubation time, which is a characteristic feature of *S. torulosus*. The comparison of 16S rRNA gene sequence of a new strain *S. torulosus* with the reference sequences is available in Genbank database and Ribosomal Database Project-II. Although the 16S rRNA sequence of the isolate strain was most similar to that of *S. torulosus* LGM 20305T (100%), we identified our isolate as *S. torulosus* PCPOK-0324 due to differences of biochemical properties of the new strain.

3.2. Antifungal activity and heat stability of S. torulosus and culture filtrates of growing S. torulosus

S. torulosus strain PCPOK-0324 inhibited the mycelial growth of *P. capsici* and *R. solani* on a PDA plates as showed by the falcate formed fungal growth in the region of *S. torulosus* growth. The antifungal compounds in cell free culture fluids increased with the age of cultures from 1 to 7 days (Fig. 1 and Table 1). The fungal growth was inhibited by 60% at the 3-day-incubation. The antifungal compounds released from *S. torulosus* PCPOK-0324 in culture filtrates were found to be heat stable as the autoclaving of culture filtrates for 15 min mostly inhibited mycelial growth of the both fungi. The culture filtrates of *R. solani* sterilized by filtration were always more effective than those of the autoclaved culture filtrates. In the 5-day-old culture, the hyphal growth was dramatically reduced from 100 to 85% comparing with those of the control. While the cells of *P. capsici* were very sensitive to the antifungal compound in the cell broth of the strain PCPOK-0324, no differences were observed after 3 days of incubation between the autoclaved and non-autoclaved culture filtrate samples. The culture filtrates sterilized by filtration were better inhibitors of fungal growth than those of the culture filtrates sterilized by autoclave.

3.3. Enzyme production

1,3-β-D-glucanase was routinely produced on a complex medium containing 0.5% suspension of lyophilized *R. solani* cells as main nutrient source (Kaur, Munshi, Singh, & Koch, 2005). The enzyme was produced in low levels when *S. torulosus* PCPOK-0324 was cultivated on minimal medium with glucosidic linkages such as lichenan, cellulose, amylose and β-cyclodextrin. In addition, the enzyme was weakly formed on media including cell wall derived from *A. faecalis* and *S. cerevisiae*. By contrast, glucanase was greatly induced by the addition of *R. solani* cell walls as carbon source into the culture. Consequently, the enzymatic activity

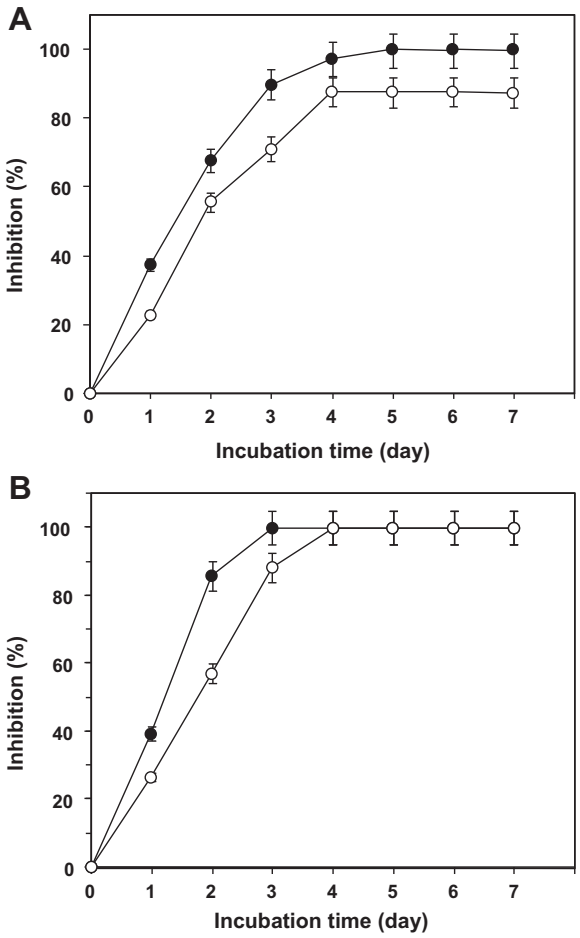

Fig. 1. The relationship between percentages of hyphal inhibition and age of cultures of *S. torulosus* strain PCPOK-0324 against (A) *R. solani* and (B) *P. capsici*. Symbols are (●) filter sterilized culture filtrates and (○) autoclaved culture filtrates.

of glucanase significantly increased in the culture filtrates supplemented with various concentrations of fungal crude cell walls. The optimal amount of added fungal cell walls was 0.5% dry weight of newly cultured *R. solani* cells which were autoclaved (data not shown). This is higher than the 0.1–0.3% of chitin (Leelasuphakul et al., 2006; Tweddell, Jabaji-Hare, & Charest, 1994). The 1,3-β-D-glucanase activity increased rapidly from day 1 to 6 and remained constant until day 7. The increase of 1,3-β-D-glucanase production preceded the increase in β-1,6-glucanase which reached the maximum activity at day 3 (data not shown). Rapid increase in the production of extracellular glucanase during bacterial cell growth showed the fungal inhibitory activity in the culture filtrates during growth.

The culture broth of *S. torulosus* PCPOK-0324 contains extracellular enzymes with antifungal activity and antibiotic materials that inhibits the mycelial growth of both pepper pathogens *R. solani* and *P. capsici*. The antibiotic showed no direct change in antifungal activity after autoclaving and hence it was heat stable. These results indicate that some heat sable antifungal compounds were partly involved in the observed fungal growth inhibition in combination with 1,3-β-D-glucanase. Various studies reported that extracellular lytic enzymes and antibiotics from antagonistic bacteria such as *Bacillus subtillis* (Leelasuphakul et al., 2006), *Pseudomonas aeruginosa* (Wang, Yieh, & Shih, 1999) and *S. violascens* (ElTarabily et al., 1996) are potential biocontrol agents which are acting alone or synergistically during the degradation of fungal cell walls.

Table 1
Inhibitory effect of cell free culture filtrates of *S. torulosus* PCPOK-0324 on hyphal growth.

Incubation time (day)	Radius of *R. solani* culture ± S.D. (mm) (% inhibition)		Radius of *P. capsici* culture ± S.D. (mm) (% inhibition)	
	Autoclaved culture filtrate	Filter sterilized culture filtrate	Autoclaved culture filtrate	Filter sterilized culture filtrate
Control	48.6 ± 2.3	48.6 ± 2.3	38.4 ± 1.8	38.4 ± 1.8
1	38.3 ± 1.2 (22.8)	31.4 ± 1.9 (37.3)	28.8 ± 1.4 (26.7)	23.2 ± 1.7 (39.5)
2	22.9 ± 1.9 (55.7)	17.2 ± 2.9 (67.7)	17.9 ± 0.7 (57.1)	7.6 ± 0.4 (85.8)
3	15.8 ± 1.8 (71.1)	7.1 ± 0.9 (90.0)	6.7 ± 0.3 (88.3)	2.5 ± 0 (100)
4	7.7 ± 0.9 (87.7)	3.7 ± 0.2 (97.4)	2.5 ± 0 (100)	2.5 ± 0 (100)
5	7.7 ± 0.9 (87.7)	2.5 ± 0 (100)	2.5 ± 0 (100)	2.5 ± 0 (100)
6	7.7 ± 0.9 (87.7)	2.5 ± 0 (100)	2.5 ± 0 (100)	2.5 ± 0 (100)
7	7.7 ± 0.9 (87.7)	2.5 ± 0 (100)	2.5 ± 0 (100)	2.5 ± 0 (100)

Radius of fungal inoculum size is 2.5 mm and three replicates were performed.

Table 2
Purification of 1,3-β-D-glucanase from culture filtrates of *S. torulosus* PCPOK-0324.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	751.3	1282.1	1.71	100.0	1
60% $(NH_4)_2SO_4$	412.9	784.5	1.90	61.2	1.11
DEAE-Sepharose	62.8	580.8	9.25	45.3	5.41
Pheny1-Sepharose HR 5/5	16.3	393.6	24.15	30.7	14.12
Mono Q HR 5/5	5.8	289.7	49.95	22.6	29.21
Superose 12	1.7	125.6	73.88	9.8	43.21

Table 3
Aligmentation of the internal amino acid sequences of the 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324 by Clustal W with other bacterial 1,3-β-D-glucanases sequences. The amino acids (R, KLP, G, and LWP) of conservation are highlighted and shadowed.

Organism	N-terminal sequence	Reference
Streptomyces torulosus PCPOK-0324	1 **R**I**KLP**K**G**ER**LWP** 12	This study
Pyrococcus furiosus DSM 3638	140 **R**I**KLP**K**G**KG**LWP** 151	Robb et al. (2001)
Bacillus circulans	117 **R**I**KLP**S**G**QG**LWP** 128	Asano et al. (2002)
Laccaria bicolor S238N-H82	273 **RA KLP**R**G**DW**LWP** 284	Martin et al. (2008)
Paenibacillus sp. CCRC 17245	633 SI**KLP**E**G**EG**LWP** 643	Hong and Meng (2003)

Table 4
MICs and IC_{50} of antibiotic extract and 1,3-β-D-glucanase from culture filtrates of *S. torulosus* PCPOK-0324 against of *R. solani* and *P. capsici*.

Antifungal substance	Fungus	MIC[a]	IC_{50}[a]	Regression equation	R^2
Antibiotic extract	*R. solani*	3.95	75.38	$y = 0.5661x + 7.3282$	0.9727
	P. capsici	1.94	57.70	$y = 0.7476x + 6.8663$	0.9636
β-1,3-Glucanase	*R. solani*	12.50	172.85	$y = 0.2263x + 10.884$	0.9587
	P. capsici	6.25	121.89	$y = 0.3163x + 11.447$	0.9626

[a] Units of MIC and IC_{50} of antibiotic extract = μg ml^{-1}, and 1,3-β-D-glutamate = mU ml^{-1}.

3.4. Purification of β-1,3-glucanase

A summary of the enzyme purification is given in Table 2. The 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324 was purified by fractionation with ammonium sulfate and four chromatographic steps. The 6-day culture filtrates of the *S. torulosus* PCPOK-0324 having a high level of the 1,3-β-D-glucanase activity was precipitated by ammonium sulfate. The pellet was collected and dialyzed. The dialysate was passed through a DEAE-Sepharose column to eliminate other proteins (data not shown). The fractions with glucanase activity were pooled, concentrated, and showed 5.41-fold concentration. Further, the purification of the enzyme was performed on a Pheny1-Sepharose HR 5/5 column. Two peaks were noticed (Fig. 2A), and the fractions in second peak were selected for the next purification steps. Chromatographic separation on Mono Q HR 5/5 and gel-filtration columns (Fig. 2B and C) allowed complete removal of other contaminating proteins. At the end of the steps, 1,3-β-D-glucanase was purified approximately 43.21-fold with a yield 9.8% having to a specific activity of 73.88 U mg^{-1} of protein (Table 2).

The molecular weight of the 1,3-β-D-glucanase of *S. torulosus* PCPOK-0324 was estimated to be 31.5 kDa by SDS-PAGE (Fig. 3) and 32.1 kDa by gel filtration on Superose 12 (data not shown), suggesting that the enzyme may be a monomeric protein. Internal sequence of the protein showed that the sequence of the first 12 residues of the 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324 was RIKLPKGERLWP. A BlastX search did not detected significant homology of these sequences with known proteins. Although this region does not appear to be among known 1,3-β-D-glucanases, the internal amino acid sequence of *S. torulosus* PCPOK-0324 1,3-β-D-glucanases had some amino acid conservation with that of *Pyrococcus furiosus* (Table 3).

The 1,3-β-D-glucanase cleaves the 1,3-β-bonds found in a variety of β-glucans of the two fungi, *R. solani* and *P. capsici* causing root rot disease in pepper plants (Stone & Clarke, 1992). These fungal cell walls are generally composed of a linear cellulose-like 1,4-β-glucan and a highly branched 1,3-β-glucan with branches starting with 1,6-β-links (Bartnicki-Garcia & Wang, 1983; Fayad et al., 2001). Although the 1,3-β-D-glucanase played an important role in the suppression of fungal growth, it cannot be absolutely related to the

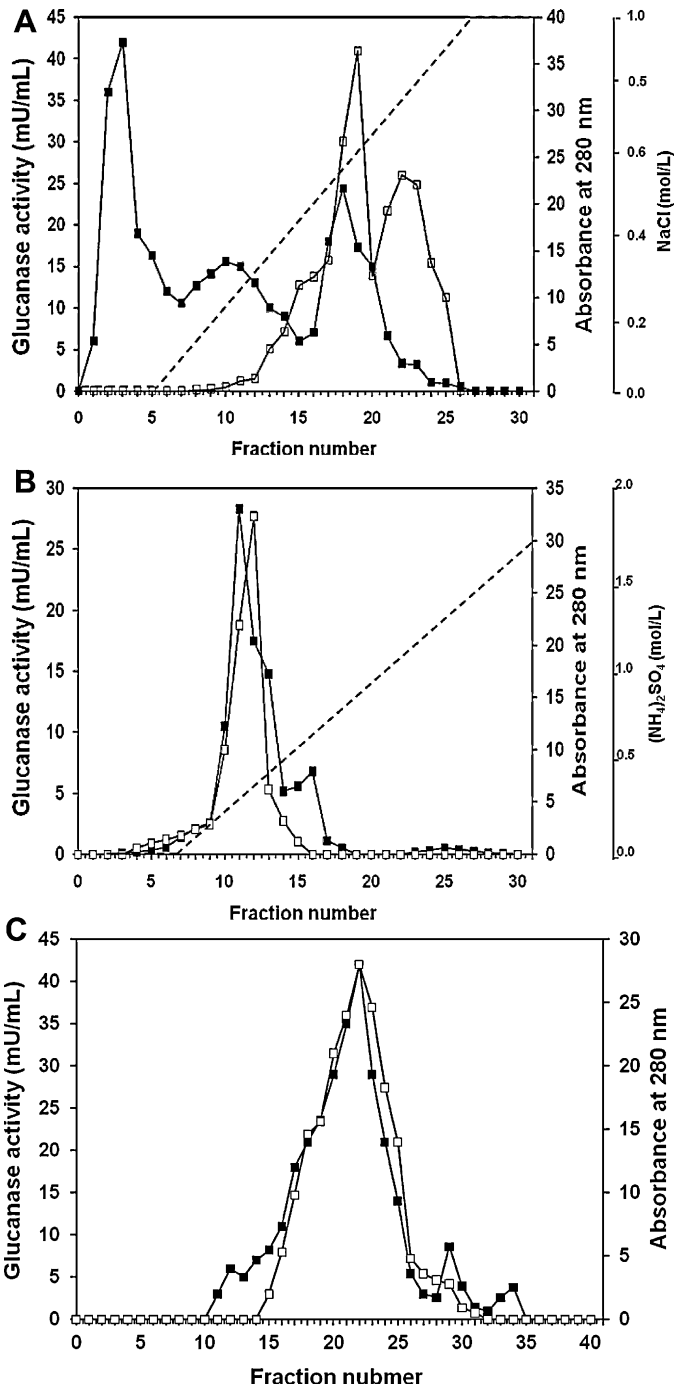

Fig. 2. Enzymatic activity at the different NaCl and $(NH_4)_2SO_4$ (- - -) during each of the three chromatographic steps of the purification procedure of 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324. (A) Phenyl-Superose HR 5/5 chromatography; (B) Mono Q HR 5/5 chromatography; (C) Superose 12 chromatography. Protein concentration (■) were monitored by absorbance at 280 nm and the 1,3-β-D-glucanase activity (mU ml^{-1}; □) was measured by the reducing sugar released from laminarin using DNS method at a wavelength of 540 nm.

degradation of fungal cells as several authors have reported that β-glucanase activity produced by other *Streptomyces* species consist of various isoenzymes with different molecular weights. For instances, *S. sioyaensis* of 29.4 and 44.6 kDa (Hong et al., 2002), *S. matensis* of 26 and 40 kDa (Nishimura et al., 2001), *Streptomyces* sp. EF-14 of 65 kDa (Fayad et al., 2001), and *Cellulosimicrobium cellulans* of 40.8, 28.6 and 54.5 kDa (Ferrer, 2006) were reported, respectively.

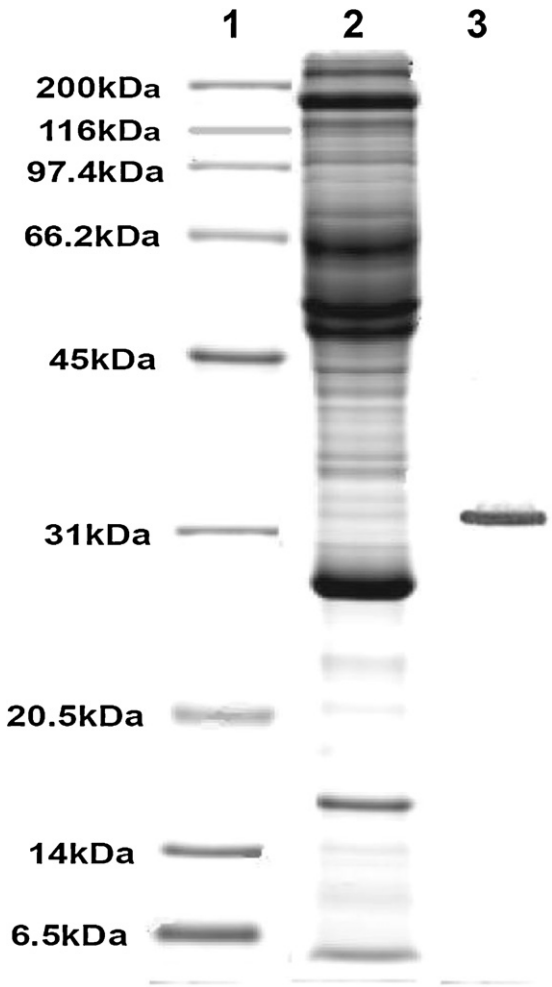

Fig. 3. SDS-PAGE of 1,3-β-D-glucanase purified from *S. torulosus* PCPOK-0324 filtrate: lane 1, molecular mass size markers; lane 2, 30 μg crude protein; lane 3, 5 μg of purified glucanase, showing a major band at M_r 31.5 kDa.

3.5. Effect of pH and temperature on the activity and stability of β-1,3-D-glucanase

The effects of pH and temperature on the activity of the 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324 were determined, respectively. The purified 1,3-β-D-glucanase enzyme was active in the buffer pH ranges 6.5–9.5 with the maximum activity at pH 7.5, while many other 1,3-β-D-glucanases are active at an acidic pH. Similar pH values were reported for 1,3-β-D-glucanases from other bacteria (Hakamada et al., 2002; Leelasuphakul et al., 2006; Miyanishi, Hamada, Kobayashi, Imada, & Watanabe, 2003; Ohara et al., 2000). The activity of the enzyme remaining after incubation at different temperature (20–90 °C) for 30 min was evaluated and the activity was stable up to 50 °C above which a rapid decrease in the activity was observed (data not shown). The β-1,3-D-glucanases enzyme retained almost 100% activity at temperature between 20 and 45 °C. Moreover, the 1,3-β-D-glucanase was thermostable at 50 °C, and retained 50% activity after 30 min incubation at 60 °C. The half-lives at 40 °C, 50 °C, 60 °C, and 70 °C were 59 min, 43.5 min, 30 min, and 5 min, respectively (Fig. 4). Therefore, the 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324 exhibited thermostability at temperature up to 60 °C and reduced the thermostability at temperature higher than 60 °C. The other bacterial 1,3-β-D-glucanase showed the highest activity at the optimal temperature between 45 and 55 °C (Hakamada et al., 2002; Miyanishi et al., 2003), although they became more stable at 60 °C. In this study, the purified enzyme

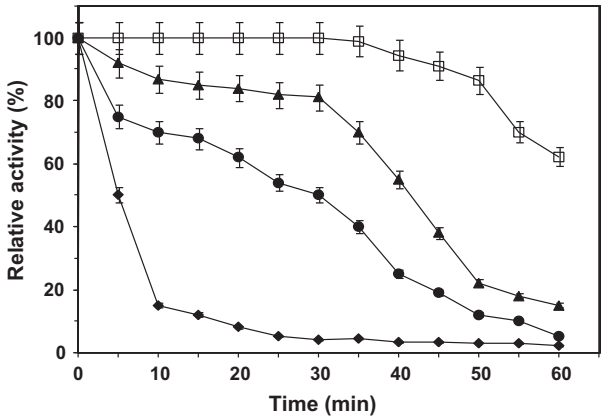

Fig. 4. Kinetics of the purified 1,3-β-D-glucanase from *S. torulosus* PCPOK-0324. Activity was estimated as a percentage of the maximum. The maximum activity was defined as 100%. The specific activity of the enzyme at 100% was 44.2 ± 2.6 U mg^{-1}. Symbols are (□) 40 °C, (▲) 50 °C, (●), 60 °C and (♦) 70 °C.

was stable up to 50 °C, which is similar to other 1,3-β-D-glucanase, but this enzyme has shown higher activity than those of the others below 40 °C (Leelasuphakul et al., 2006; Ohara et al., 2000).

3.6. Antifungal activities of 1,3-β-D-glucanase and antibiotic extract from S. torulosus PCPOK-0324

Cell culture filtrate containing potent antibiotics from *S. torulosus* PCPOK-0324 grown in 2 l of minimum salt medium, which supplemented with 0.5% of lyophilized cells derived from *R. solani* was prepared. The 285 mg of pale brown extract was collected, suspended in 80% ethanol, and kept at 4 °C. The antibiotic compounds extracted from the cell culture filtrates were involved in inhibition of fungal growth of both *R. solani* and *P. capsici* according to their MICs and IC_{50}. MICs of the antibiotic extract against *R. solani* and *P. capsici* were 3.95 and 1.94 μg ml^{-1}, respectively (Table 4). No significant effect on the inhibition of mycelial growth of both fungi by the antibiotic extract was observed; although the different effect was observed after the first diluted concentration of the MIC was treated on the two fungi, *R. solani*, and *P. capsici* (data not shown). The similar phenomena were observed in the purified 1,3-β-D-glucanase against *R. solani* and *P. capsici* and the activities were 12.50 and 6.25 mU ml^{-1}.

The comparable effects on the growth of the both fungi as had been shown by their MICs were detected in the IC_{50} of the enzyme and antibiotic extract. The FIC_{index} calculated from the sum of FIC of the enzyme and FIC of the antibiotic extract is lower than 0.5. In the presence of antibiotic extract, the 1,3-β-D-glucanase sensitivity of both fungal strain, *R. solani* and *P. capsici*, was increased. In the case of *R. solani*, the addition of 0.65 μg ml^{-1} antibiotic extract reduced the 1,3-β-D-glucanase activity as MIC from 12.5 mU ml^{-1} to 3.75 mU ml^{-1} while in the *P. capsici*, the same level of antibiotic extract decreased the 1,3-β-D-glucanase activity as MIC from 6.5 mU ml^{-1} to 1.56 mU ml^{-1}. Therefore, the results clearly show that in the two fungal strains the combination of the two antifungal agents created an effect greater than would be expected based on their individual contribution.

The combined mixture of the enzyme and the antibiotic compound extracted from the culture filtrates of *B. subtilis* inhibit the growth of *Pyricularia grisea* and *R. solani* with similar results (Leelasuphakul et al., 2006). Moreover, the chitinase derived from *Gliocladium virens* and gliotoxin are known as antifungal agents that showed synergistic activity against mycelial growth of phytopathogenic fungi (Di Pietro, Lorito, Hayes, Broadway, & Harman, 1993). Investigating the ability of *S. torulosus* PCPOK-0324 to produce fungal inhibitors against these two pepper-pathogenic fungi *in vivo* and to regulate their growth will be advantageous to improve the crop yield. It seems that the antifungal metabolites produced by *S. torulosus* PCPOK-0324 *in vivo* are vary important for the biocontrol mechanisms of this strain. They could have been well active as key fungicides to control root rot disease caused by *P. capsici* and *R. solani* in pepper plants. Further studies are needed to understand the mechanisms underlying the observed synergistic interaction and are warranted in order to develop new management strategies for control of root rot disease occurred by *P. capsici* and *R. solani* in pepper plants.

4. Conclusion

The present results highlight the possibilities for using 1,3-β-D-glucanase and antibiotic in the culture broth of *S. torulosus* PCPOK-0324 in fungal management. Although many questions remain to be addressed before its application in the agricultural field, *S. torulosus* PCPOK-0324 appears to have potential as a biocontrol agent for plant pathogens toward *P. capsici* and *R. solani*. It produced a powerful fungicidal 1,3-β-D-glucanase and antibiotic that were not only effective in inhibiting the growth of plant pathogens but was also unique in its stability at high temperature and some broad range of pH. Within a framework of fungicidal in combination with biological control, 1,3-β-D-glucanase and antibiotic from *S. torulosus* PCPOK-0324 could be considered as compatible tools in the development of fungicidal agents.

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References

Ahmed, A. S., Ezziyyani, M., Pérez Sánchez, C. & Candela, M. E. (2003). Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. *European Journal of Plant Pathology*, *109*, 633–637.

Asano, T., Taki, J., Yamamoto, M. & Aono, R. (2002). Cloning and Structural Analysis of bglM Gene Coding for the Fungal Cell Wall-lytic β-1,3-Glucan-hydrolase BglM of Bacillus circulans IAM1165. *Bioscience, Biotechnology, and Biochemistry*, *66*, 1246–1255.

Bartnicki-Garcia, S. & Wang, M. C. (1983). *Biochemical aspects of morphologenesis in Phytophthora*. St. Paul, MN, USA: APP.

Bradford, M. M. (1976). A rapid and sensitive method for the guntitation on microgram quntities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, *72*, 248–254.

Cardinale, F., Ferraris, L., Valentino, D. & Tamietti, G. (2006). Induction of systemic resistance by a hypovirulent *Rhizoctonia solani* isolate in tomato. *Physiological and Molecular Plant Pathology*, *69*, 160–171.

Chamberlain, K. & Crawford, D. L. (2000). Thatch biodegradation and antifungal activities of two lignocellulolytic *Streptomyces* strains in laboratory cultures and in golf green turfgrass. *Canadian Journal of Microbiology*, *46*, 550–558.

Cheng, H. P. & Walker, G. C. (1998). Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. *Journal of Bacteriology*, *180*, 20–26.

Choi, H. J., Kim, B. H., Kim, J. D. & Han, M. S. (2005). *Streptomyces* neyagawaensis as a control for the hazardous biomass of *Microcystis aeruginosa* (*Cyanobacteria*) in eutrophic freshwaters. *Biological Control*, *33*, 335–343.

Chung, W. C., Hunag, J. W., Huang, H. C. & Jen, J. F. (2003). Control, by Brassica-seed pomace combined with *Pseudomonas boreopolis*, of damping-off of watermelon caused by *Pythium* sp. *Canadian Journal of Plant Pathology*, *25*, 285–294.

Davidson, P. M. & Parish, M. E. (1989). Methods for testing the efficacy of food antimicrobials. *Food Technology*, *43*, 148–155.

Di Pietro, A., Lorito, M., Hayes, C. K., Broadway, R. M. & Harman, G. E. (1993). Endochitinase from *Gliocladium virens*: Isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. *Phytopathology*, *83*, 308–313.

ElTarabily, K. A., Sykes, M. L., Kurtbke, I. D., Hardy, G. E. St. J., Barbosa, A. M. & Dekker, R. F. H. (1996). Synergistic effects of a cellulase-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* on the suppression of *Phytophthora cinnamomi* root rot of *Banksia grandis*. *Canadian Journal of Botany*, *74*, 618–624.

Fayad, K. P., Simao-Beaunoir, A. M., Gauthier, A., Leclerc, C., Mamady, H., Beaulicu, C., et al. (2001). Purification and properties of a beta-1,6-glucanase from *Streptomyces* sp. EF-14, an actinomycete antagonistic to *Phytophthora* spp. *Applied Microbiology and Biotechnology*, *57*, 117–123.

Ferrer, P. (2006). Revisiting the *Cellulosimicrobium cellulans* yeast-lytic beta-1,3-glucanases toolbox: A review. *Microbial Cell Factories*, *5*, 1–8.

Gamliel, A., Katan, J. & Cohen, E. (1989). Toxicity of chloronitrobenzenes to *Fusarium oxysporum* and *Rhizoctonia solani* as related to their structures. *Phytoparasitica*, *17*, 101–106.

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure Applied Chemistry*, *59*, 257–268.

Guo, W., Tamanoi, F. & Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. *Nature Cell Biology*, *3*, 353–360.

Hakamada, Y., Endo, K., Takizawa, S., Kobayashi, T., Shirai, T., Yamane, T., et al. (2002). Enzymatic properties, crystallization, and deduced amino acid sequence of an alkaline endoglucanase from *Bacillus circulans*. *Biochimica et Biophysica Acta*, *1570*, 174–180.

Hong, T. Y., Cheng, C. W., Huang, J. W. & Meng, M. (2002). Isolation and biochemical characterization of an endo-1,3-beta-glucanase from *Streptomyces sioyaensis* containing a C-terminal family 6 carbohydrate-binding module that binds to 1,3-beta-glucan. *Microbiology*, *148*, 1151–1159.

Hong, T. Y. & Meng, M. (2003). Biochemical characterization and antifungal activity of an endo-1,3-b-glucanase of Paenibacillus sp. Isolated from garden soil. *Applied Microbiology and Biotechnology*, *61*, 472–478.

Hwang, B. K., Kim, Y. J. & Kim, C. H. (1996). Differential interactions of *Phytophthora capsici* isolates with pepper genotypes at various plant growth stages. *European Journal of Plant Pathology*, *102*, 311–316.

Jiang, Z. Q., Guo, Y. H., Li, S. M., Qi, H. Y. & Guo, J. H. (2006). Evaluation of biocontrol efficiency of different *Bacillus* preparations and field application methods against Phytophthora-blight of bell pepper. *Biological Control*, *36*, 216–223.

Joo, G. J. (2005). Purification and characterization of an extracellular chitinase from the antifungal biocontrol agent *Streptomyces halstedii*. *Biotechnology Letters*, *27*, 1483–1486.

Kaur, J., Munshi, G. D., Singh, R. S. & Koch, E. (2005). Effect of carbon source on production of lytic enzymes by the sclerotial parasites *Trichoderma atroviride* and *Coniothyrium minitans*. *Journal of Phytopathology*, *153*, 274–279.

Klappenbach, J. A., Saxman, P. R., Cole, J. R. & Schmidt, T. M. (2001). rrndb: The ribosomal RNA operon copy number database. *Nucleic Acids Research*, *29*, 181–184.

Laemmli, U. K. (1970). Cleavage of structural protein during assembly of the head of bacteria phage T4. *Nature*, *227*, 680–685.

Lamour, K. H. & Hausbeck, M. K. (2001). Investigating the spatiotemporal genetic structure of *Phytophthora capsici* in Michigan. *Phytopathology*, *91*, 973–980.

Larkin, R. P. & Fravel, D. R. (1999). Mechanisms of action and dose–response relationships governing biological control of fusarium wilt of tomato by nonpathogenic *Fusarium* spp. *Phytopathology*, *89*, 1152–1161.

Leelasuphakul, W., Sivanunsakul, P. & Phongpaichit, S. (2006). Purification, characterization and synergistic activity of beta-1,3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. *Enzyme and Microbial Technology*, *38*, 990–997.

Li, R. K. & Rinaldi, M. G. (1999). In vitro antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. *Antimicrobial Agents and Chemotherapy*, *43*, 1401–1405.

Liu, D., Anderson, N. A. & Kinkel, L. L. (1995). Biological-control of potato scab in the field with antagonistic *Streptomyces scabies*. *Phytopathology*, *85*, 827–831.

Lorian, V. (1996). *Antibiotics in laboratory medicine*. Williams Wilkins: Baltimore.

Lorito, M., Peterbauer, C., Hayes, C. K. & Harman, G. E. (1994). Synergistic Interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. *Microbiology*, *140*, 623–629.

Mahasneh, A. M. & Stewart, D. J. (1980). A medium for detecting 1,3-β-D-glucanaseactivity in bacteria. *Journal of Applied Microbiology*, *48*, 457–458.

Martin, F., Aerts, A., Ahren, D., Brun, A., Danchin, E. G., Duchaussoy, F., et al. (2008). The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. *Nature*, *452*, 88–92.

McKeen, C. D., Reilly, C. C. & Pusey, P. L. (1986). Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. *Ecology and Epidemiology*, *76*, 136–139.

Mitsutomi, M., Isono, M., Uchiyama, A., Nikaidou, N., Ikegami, T. & Watanabe, T. (1998). Chitosanase activity of the enzyme previously reported as beta-1,3-1,4-glucanase from *Bacillus circulans* WL-12. *Bioscience Biotechnology and Biochemistry*, *62*, 2107–2114.

Miyanishi, N., Hamada, N., Kobayashi, T., Imada, C. & Watanabe, E. (2003). Purification and characterization of a novel extracellular beta-1,3-glucanase produced by *Bacillus clausii* NM-1 isolated from ezo abalone *Haliotis discus hannai*. *Journal of Bioscience and Bioengineering*, *95*, 45–51.

Nishimura, T., Bignona, C., Alloucha, J., Czjzeka, M., Darbona, H., Watanabeb, T., et al. (2001). Streptomyces matensis laminaripentaose hydrolase is an 'inverting' beta-1,3-glucanase. *FEBS Letters*, *499*, 187–190.

Ohara, H., Noguchi, J., Karita, S., Kimura, T., Sakka, K. & Ohmiya, K. (2000). Sequence of egV and properties of EgV, a *Ruminococcus albus* endoglucanase containing a dockerin domain. *Bioscience Biotechnology and Biochemistry*, *64*, 80–88.

Picman, A. K., Schneidera, E. F. & Gershenzon, J. (1990). Antifungal activities of sunflower terpenoids. *Biochemical Systematics and Ecology*, *18*, 325–328.

Ristaino, J. B. & Johnston, S. A. (1999). Ecologically based approaches to management of *Phytophthora* blight on bell pepper. *Plant Disease*, *83*, 1080–1089.

Robb, F., Maeder, t., Brown, D. L., DiRuggiero, J. R., Stump, J., Yeh, M. D., et al. (2001). Genomic sequence of hyperthermophile, Pyrococcus furiosus: Implications for physiology and enzymology. *Methods in Enzymology*, *330*, 134–157.

Schirmböck, M., Lorito, M., Wang, Y. L., Hayes, C. K., Arisan-Atac, I., Scala, F., et al. (1994). Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Applied and Environmental Microbiology*, *60*, 4364–4370.

Shekhar, N., Bhattacharya, D., Kumar, D. & Gupta, R. K. (2006). Biocontrol of wood-rotting fungi with *Streptomyces violaceusniger* XL-2. *Canadian Journal of Microbiology*, *52*, 805–808.

Stone, B. A. & Clarke, A. E. (1992). *Chemistry and biology of (1,3)-beta-glucanase*. Bundoora, Australia: La Trobe University Press.

Trejo-Estrada, S. R., Paszczynski, A. & Crawford, D. L. (1998). Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. *Journal of Industrial Microbiology & Biotechnology*, *21*, 81–90.

Tweddell, R. J., Jabaji-Hare, S. H. & Charest, P. M. (1994). Production of chitinases and beta-1,3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. *Applied and Environmental Microbiology*, *60*, 489–495.

Wang, S. L., Yieh, T. C. & Shih, I. L. (1999). Production of antifungal compounds by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as a carbon source. *Enzyme and Microbial Technology*, *25*, 142–148.