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ORIGINAL ARTICLE

Characterization of antifungal activity of *Paenibacillus ehimensis* KWN38 against soilborne phytopathogenic fungi belonging to various taxonomic groups

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Abstract Soilborne fungal phytopathogens cause significant losses in many economically important crops and vegetables. The only way to control these devastating pathogens is by using higher doses of fungicides which not only increase the cost of production but also cause significant damage to the environment. Therefore alternate control measures are always looked for. In the present study, an antagonistic strain was isolated from the soil of the pepper fields around the seashore of Jellanamdo, South Korea and identified as *Paenibacillus ehimensis* KWN38 based on 16S rRNA sequencing. The strain showed high antifungal activity against six tested fungal pathogens belonging to various taxonomic groups on dual culture plates. Furthermore, the strain produced volatile antimicrobial compounds which had strong fungal growth inhibitory effect. The strain also showed high chitinase, cellulase, glucanase and protease activities. The hyphal morphologies of *Rhizoctonia solani* AG-1 (IA), *Fusarium oxysporum* f.sp. *lycopersici* and *Phytophthora capsici* were significantly destroyed by the crude enzymes and butanol extract from the culture supernatant and the affected hyphae showed abnormal bending, tip curling, and irregular branching. Hence, *Paenibacillus ehimensis* KWN38 is considered as a potential biocontrol agent of the soil-borne fungi causing plant diseases which is an important perspective of the present study.

Keywords Antagonism · Chitinase · β-1,3-glucanases · Hyphal inhibition, *Paenibacillus ehimensis* · Soilborne fungi

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Introduction

Fungal pathogens such as *Rhizoctonia solani*, *Fusarium oxysporum* and *Phytophthora capsici* cause severe plant diseases, limiting plant yields as well as the quality of the products. Moreover, they have wide host spectra, causing diseases in economically important agricultural crops worldwide (Agrios 2005). These fungal phytopathogens are difficult to control not only because of their wide host spectra, but also because of their soilborne nature (Summeral et al. 2003). To cope with this, chemical fungicides are generally used in higher doses. The synthetic fungicides applied to the soil or to the aerial portion of plants not only destroy the environment, but also contaminate the ground water bodies and may also enter the human food chains. Moreover, the poisonous methyl bromide used to fumigate the soil results in depletion of the ozone layer in the atmosphere. Therefore, alternate measures are required for the durable and environment-friendly control of the soilborne fungal phytopathogens.

The biological control offers a way to repress the fungal phytopathogens. The use of antagonistic microorganisms may not only control the plant pathogens more efficiently, but are also safer for the environment (Harman et al. 1993;

Blanco et al. 2007). Several bacterial genera including *Azotobacter; Bacillus*; *Lysobacter*; *Paenibacillus*; *Pseudomonas*; *Streptomyces* have so far been reported as plant growth promoting rhizobacteria (PGPR) (Park and Kloepper 2000; Zhang et al. 2003; Wachowska et al. 2004; Ryu et al. 2006; Herman et al. 2008). Moreover, different bacteria have been shown to control the variable plant diseases (Ko et al. 2009). Mechanisms involved in pathogen suppression by the antagonistic bacteria vary and may include direct parasitism, antibiotic production, substrate competition and induced systemic resistance in the plant host (Wisniewski et al. 1991; Van Loon et al. 1998; Moline et al. 1999; Dey et al. 2004). The cell wall-degrading enzymes, mostly known as chitinases, glucanases, and proteases are the major lytic enzymes that are secreted by different biocontrol agents (Harman et al. 2004). These enzymes attack the cell walls of phytopathogenic fungi, causing cell lysis and subsequent death (Castoria et al. 1997; Tseng et al. 2008). Moreover, the production of volatile antimicrobial compounds by the antagonistic bacteria and fungi has been well reported (Li et al. 2011).

The genus *Paenibacillus* was first suggested based on phylogenetic data from 16S rDNA sequences by Collins et al. (1994). Since then, numerous antagonistic strains belonging to this genus have been reported (Ding et al. 2011). Different *Paenibacillus* species are capable to produce several hydrolytic enzymes that play important roles in biocontrol of plant pathogens (Yang et al. 2004). Various antimicrobial substances produced by *Paenibacillus* spp. such as polymyxins by *Paenibacillus polymyxa*, pelgipeptins by *Paenibacillus elgii*, paenibacillin P and N by *Paenibacillus alvei* that affect a wide spectrum of microorganisms such as fungi, soil bacteria, plant pathogenic bacteria have been reported (Shoji et al. 1977; Anandaraj et al. 2009; Ding et al. 2011).

In the present study, we isolated a new strain of *Paenibacillus ehimensis* showing high chitinolytic activity. The antifungal potential of the strain against the different economically important soilborne fungal phytopathogens was evaluated. Moreover, the ability of the strain to produce antifungal compounds as well as cell wall–degrading enzymes in the culture media was demonstrated. The destructive effects of the crude enzymes as well as the bacterial extracts on the pathogenic fungal hyphae were also experimented in the present study.

Materials and methods

Isolation and identification of the antagonistic bacterial strain

Soil samples collected from the pepper fields around the seashore of Jellanamdo, Korea were serially diluted to 10^{-6} and inoculated on chitin agar medium (CM) containing chitin 0.2 %, Na_2HPO_4 0.2 %, KH_2PO_4 0.1 %, NaCl 0.05 %, NH_4Cl 0.1 %, $MgSO_4{\cdot}7H_2O$ 0.05 %, $CaCl_2{\cdot}2H_2O$ 0.05 %, yeast extract 0.01 %, and agar 2 % at pH7. After incubation at 30 °C for 7 days, colonies possessing strong clear zones were selected, and successively examined for antifungal activity against *Phytophthora capsici*, *Rhizoctonia cerealis, R. solani* AG-1 (IA), *Fusarium oxysporum* f.sp. *lycopersici, Colletotrichum caudatum,* and *Pythium aphanidermatum* on CP agar medium using dual culture technique (Yoon et al. 2012). The dual cultured plates were then incubated for 3 days for the two *Rhizoctonia* species and 7 days for the other fungal pathogens at 26 °C. Finally, the strain KWN38 showing the highest antifungal activity against all the tested fungal pathogens was selected for further study.

KWN38 was cryopreserved at −70 °C for further use (Yoon et al. 2012). KWN38 was identified at species level by 16S rRNA gene sequencing. The phylogenic tree was constructed by using the CLUSTAL-W program comparing its 16S rRNA sequences with the published ones at Gene Bank Data base of National Center for Biotechnology Information (NCBI; Bethesda, MD).

Preparation of the culture supernatant for antifungal assays

KWN38 was grown in liquid medium [g l^{-1}; crab shell powder 1.0, gelatin powder 1.0, complete fertilizer (% $N{:}P_2O_5{:}K_2O$; 21:17:17) 3.0, sucrose 3.0, yeast extract 0.03, $FeCl_3$ 0.03] for 7 days at 30 °C with shaking at 170 rpm. The culture broth was centrifuged at 7,000 *g* for 20 min and the supernatant collected was then filtered through Whatman filter paper No. 2.

Antifungal activity of butanol extract

The culture supernatant was acidified with 1 N HCl to pH3 and extracted with an equal volume of n-butanol. The n-butanol soluble organic fraction was concentrated by using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland) and dissolved in methanol to prepare a final concentration of 1 % stock solution. The stock solution was filter-sterilized to remove any existence of live or dead bacterial cells.

The in vitro antifungal activity of the extracted organic fraction was assessed by inoculating 8 mm Ø mycelial plugs taken from the freshly growing fungal cultures using cork borer, onto the centers of Petri plates containing PDA amended with 0, 250 or 500 ppm of n-butanol extracted organic fraction. The plates were incubated at 26 °C for 3 to 5 days. The percentage of growth inhibition was calculated using the following formula (Chilpa et al. 1997):

$$\% \text{ Inhibition} = (A - B)/A \times 100$$

(Where A and B denote the radial growths of the fungi in non-amended or amended culture media with the extracted organic fraction respectively)

The data were statistically analyzed using software SAS (ver. 9.2). The means were compared by least significant difference (LSD) test ($P<0.05$).

Furthermore, the direct inhibitory effect of the n-butanol extracted organic fraction on the fungal hyphae was studied using 24-well culture plates where each well measured as 18 mm Ø. *Rhizoctonia solani* AG-1(IA), *F. oxysporum* f.sp. *lycopersici* and *Phytophthora capsici* were cultured in potato dextrose broth at 30 °C for 3 days and 200 μl of each fungal culture was added to the different wells of the culture plate. The extracted organic fraction was then added to each well to make final concentrations 250 or 500 ppm in each well along with the non-treated controls where same volumes of methanol were added. The plates were then incubated at 26 °C for 2 days and observed under stereomicroscope using 100× magnifications. The experiments were repeated four times.

Preparation of bacterial crude enzymes and their inhibitory effect on fungal hyphae

The protein precipitation from the culture supernatant was done by the salting-out method (Jakoby 1971). While stirring the culture supernatant, the known amount of saturated ammonium sulfate solution (four times of culture supernatant; V/V) was added drop-wise. Precipitated crude enzymes were collected by centrifugation at 7,000 *g* for 20 min and the pellet was dissolved in a minimal amount of 0.05 M potassium phosphate buffer (PPB; pH6.0). The crude enzyme solution was then dialyzed extensively at 4 °C against PPB. The buffer from the dialysis tubing was removed by using polyethylene glycol. The crude enzyme was then dissolved in a small amount of PPB. The concentration of crude enzyme was determined using Bradford method (Bradford 1976).

The fungal hyphal growth inhibition assays were performed in 24-well culture plates as explained above. Each fungal phytopathogen was cultured in sterilized potato dextrose broth in 250 ml Erlenmeyer flask at 30 °C and 170 rpm for 5 days. Hyphal culture and crude enzymes were added to 24-well culture plates with final concentrations 500 and 1,000 $\mu g ml^{-1}$. The same volumes of sterilized buffer were added to control wells. The plates were then incubated at 26 °C for 2 days and the interaction effect was observed under stereomicroscope using 100× magnifications. The experiments were replicated four times.

Lytic enzyme assays

To examine chitinase activity the bacterial strain was cultured in chitin medium (%; w/v; colloidal chitin 0.2; Na_2HPO_4 0.2; KH_2PO_4 0.1; NaCl 0.05; NH_4Cl 0.1; $MgSO_4{\cdot}7H_2O$ 0.05; $CaCl_2{\cdot}2H_2O$ 0.05; KNO_3 0.05; yeast extract 0.01) at pH7.0. To test β-1,3-glucanase and cellulase activities the colloidal chitin of this medium was substituted with crab shell powder (0.1 %; w/v). Similarly, the bacterial strain was cultured in protease production medium (tryptone 1.0; $(NH_4)_2SO_4$ 0.1; KH_2PO_4 0.05; $MgSO_4$ 0.03; $CaCl_2$ 0.1; NaCl 0.1; glycerol 0. 63; %; w/v) at pH7.0 to determine the protease activity. The bacterial culture flasks were incubated at 30 °C and 170 rpm. To examine the enzyme activities the bacterial supernatant was collected daily. For the chitinase activity, assays were performed according to Lingappa and Lockwood (1962). For the β-1,3-glucanase and cellulase activities, assays were performed by methods of Yedidia et al. (2000). Moreover, the protease activity assay was performed by following the Sigma universal protease activity assay protocol (Sigma Aldrich).

Antifungal volatile inhibition assays

The antifungal volatile compounds assays were performed following the method used by Raza et al. (2009) with some modifications. Bacterial strain was cultured by spreading 50 μl of bacterial culture on LB plate and incubated at 28 °C for 48 h. Mycelial plug (5 mm) taken from the actively growing culture of *R. solani* AG-1(IA), *F. oxysporum* f.sp. *lycopersici* or *Phytophthora capsici* was placed in the centre of another Petri dish containing PDA. The dishes containing the mycelial plugs were inverted over the bacterial plates by replacing their covers and sealed together face to face with parafilm followed by incubation at 26 °C. The diameter of fungal mycelium was measured every 6 h for *R. solani* AG1 (IA) and 24 h interval for *F. oxysporum* f.sp. *lycopersici* and *Phytophthora capsici*. The fungal plates with covers replaced by LB plates containing no bacterial cultures were used as controls. The data were analyzed by the student's *t*-test using Microsoft excel 2007 program ($p=0.05$).

Results

Identification and antagonistic activity of the isolated bacterial strain

The selected bacterial strain (KWN38) could completely inhibit all the tested fungal pathogens at a distance of 18–25 mm (Table 1). The dual culture plates kept in the incubator for further 10 days showed that none of the fungal pathogens could overwhelm the bacterial colony and the inhibition was persistent (Fig. 1). The 16 s rRNA sequence of the strain showed 100 % similarity with *Paenibacillus ehimensis* NR-025666 in NCBI database (Fig. 2). The sequence is submitted to NCBI (accession # JN050969).

Table 1 The inhibition distance between the restricted hyphal colonies of pathogenic fungi and *Paenibacillus ehimensis* strain KWN38 on dual culture plates after 3 days of incubation for *Rhizoctonia* species and after 5 days of incubation for the rest of fungal pathogens at 26 °C

Fungal pathogen	Strain identification	Inhibition distance (mm)
Phytophthora capsici	KACC[b] 40483	24.0±2.0[a]
Rhizoctonia cerealis	KACC 40153	25.3±1.5
R. solani AG-1 (IA)	KACC 40101	22.0±2.0
Fusarium oxysporum f.sp. *lycopersici*	KACC 40037	20.0±2.6
Colletotrichum caudatum	KACC 41028	18.7±2.1
Pythium aphanidermatum	KACC 40156	20.0±1.0

[a] Each value is mean from three replicate (±SD)

[b] Korean Agricultural Culture Collection

Antifungal activity of the butanol extracted fraction of the bacterial extract

The butanol extracted fraction of organic compounds showed strong inhibition (>50 %) against the different pathogens (Fig. 3). Generally higher concentration of the fraction showed the higher inhibition and vice versa. The hyphal morphologies of all three fungal species were highly affected by the butanol extracted organic fraction (250 and 500 ppm; Fig. 4). Those hyphae lost their normal growth and branching patterns showing wrinkled and abnormal bendings. The destruction was quite significant in *R. solani* where the air bubbles were trapped in the hyphae (Fig. 4b, c) whereas the normal growth of hyphae was observed in control treatment. Similarly, 500 ppm butanol extract had very severe impact on the appearance of *F. oxysporum* which spun itself and the hyphae became somewhat flattened (Fig. 4f). In addition, the hyphae of *Phytophthora capsici* were also affected by 250 ppm butanol extract and the hyphal tips curled and deformed. Furthermore, the higher concentration of butanol extract caused irregular bendings in hyphae.

Fig. 1 Dual culture plate: *Paenibacillus ehimensis* KWN38 (on left) against *Rhizoctonia cerealis* (on right)

Antifungal activity of the crude enzymes produced by the bacterial strain

After 48 h, the morphology of the fungal hyphae was significantly altered by treating with the crude enzymes (Fig. 5). The deformed hyphae were twisted and lost the normal branching pattern as pointed with arrows. Both concentrations of crude enzymes (500 and 1,000 $\mu g ml^{-1}$) destroyed the normal hyphal growth of all three fungal pathogens. Generally, the destruction level increased with increasing concentration. *Fusarium oxysporum* f.sp. *lycopersici* was the most susceptible to higher enzyme concentration (500 $\mu g ml^{-1}$) and the destruction of its hyphae was quite obvious compared to others appearance.

Lytic enzyme activity by the bacterial strain

KWN38 produced all the four enzymes tested in the present study (Fig. 6). Briefly, there was a gradual increase in the chitinase activity from 1 day after incubation (DAI) to 3 DAI and reached to more or less plateau level at 4 and 5 DAI (Fig. 6a). The highest activity of 2.37 unit $ml^{-1}h^{-1}$ was observed 5 DAI and it declined thereafter (Fig. 6b). Similarly, the cellulase activity also increased up till 3 and 4 DAI and then declined. However, in case of β-1,3-glucanase activity, the increase was lagging up till 3 DAI and then became exponential at 4 and 5 DAI followed by a decline (Fig. 6c). In case of protease activity, there was a gradual increase until 8 DAI and then it declined (Fig. 6d).

Volatile inhibition of the pathogenic fungi

KWN38 could produce antifungal volatile compounds which inhibited the exposed fungal colonies (Fig. 7). The colony diameters were significantly reduced even from the first day of observation in case of *R. solani* AG1-IA (Fig. 7a). Nonetheless, the growth inhibition of the colonies was also significant in case of *Phytophthora capsici* visible from 48 h onwards (Fig. 7b). Similar results were observed in case of *F. oxysporum* f.sp. *lycopersici*, the growth of which was significantly inhibited after 48 h by the volatiles produced by KWN38.

Discussion

In the present study, we isolated a new, highly antagonistic bacterial strain identified as *Paenibacillus ehimensis* that could completely inhibit the different soilborne phytopathogenic fungi, although the fungi which were challenged

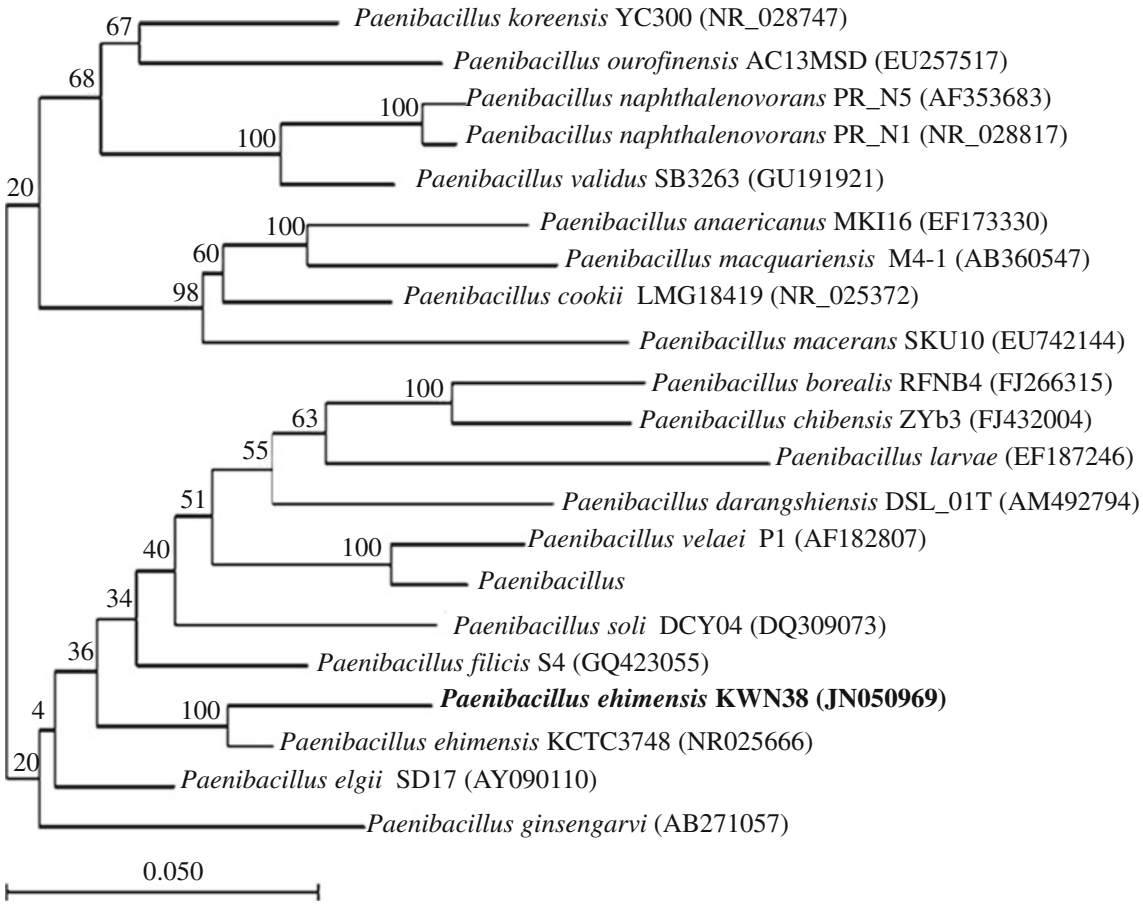

Fig. 2 Neighbor-joining tree showing the position of *Paenibacillus ehimensis* KWN38 (*bold*, accession No. JN050969) compared to related species in a 16S rRNA gene tree. Numbers at nodes indicate levels of bootstrap support (%) based on 1000 resampled datasets. The scale bar at the bottom indicates genetic distance units based on Nei's genetic distance

belonged to various taxonomic groups. The growth inhibition of fungal plant pathogens by a number of *Paenibacillus* species has been previously reported, however, there are very few studies about the antifungal activities of *Paenibacillus ehimensis* and its inhibitory mechanisms against fungal plant pathogens (Shoji et al. 1977; Lee et al. 2008; Anandaraj et al. 2009; Ding et al. 2011). Our study further reinforces the findings of Aktuganov et al. (2008) who reported that *Paenibacillus ehimensis* IB-X-b had pronounced antifungal activity against a wide range of fungi. Hence *Paenibacillus ehimensis* KWN38 inhibited all the different strains of fungi tested in the present study belonging to three wide taxonomic classes i.e. Ascomycetes (*F. oxysporum* and *C. caudatum*), Basidiomycetes (*Rhizoctonia* species), and Oomycetes (*Phytophthora capsici* and *Pythium aphanidermatum*).

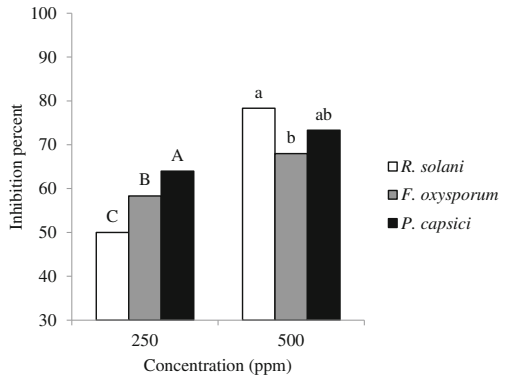

Fig. 3 The growth inhibition of *Rhizoctonia solani*, *Fusarium oxysporum*, and *Phytophthora capsici* by different concentrations of butanol extract from the culture supernatant of *Paenibacillus ehimensis* KWN38. Different letters at each concentration of butanol extract were significantly different with each other at $p<0.05$ level of LSD test

A variety of mechanisms have so far been reported to contribute towards the biocontrol activity of microbes (Harman 2006). It is well known that cell wall–degrading enzymes, such as chitinases, β-1,3-glucanases, cellulases and proteases, are involved in the antagonistic activity of the biological control agents against phytopathogenic fungi (Ordentlich et al. 1988; Shapira et al. 1989; Harman et al. 1993). These enzymes are important for the cell lysis and the subsequent cell death (Tseng et al. 2008). In general, the bacterial chitinases are shown to play a role in the digestion of chitin for its utilization as a carbon and energy source and recylcling chitin in nature (Chitnis et al. 2001). The resistivity

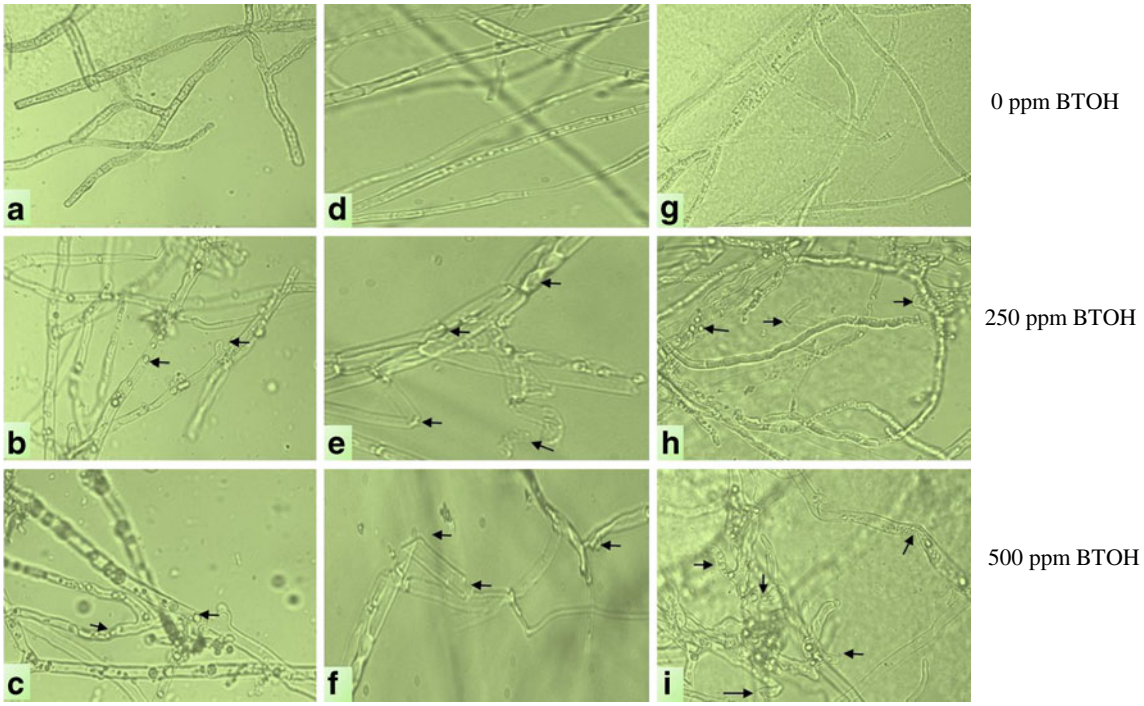

Fig. 4 Destruction of fungal hyphae by different concentrations of the butanol extract from the culture of *Paenibacillus ehimensis* KWN38

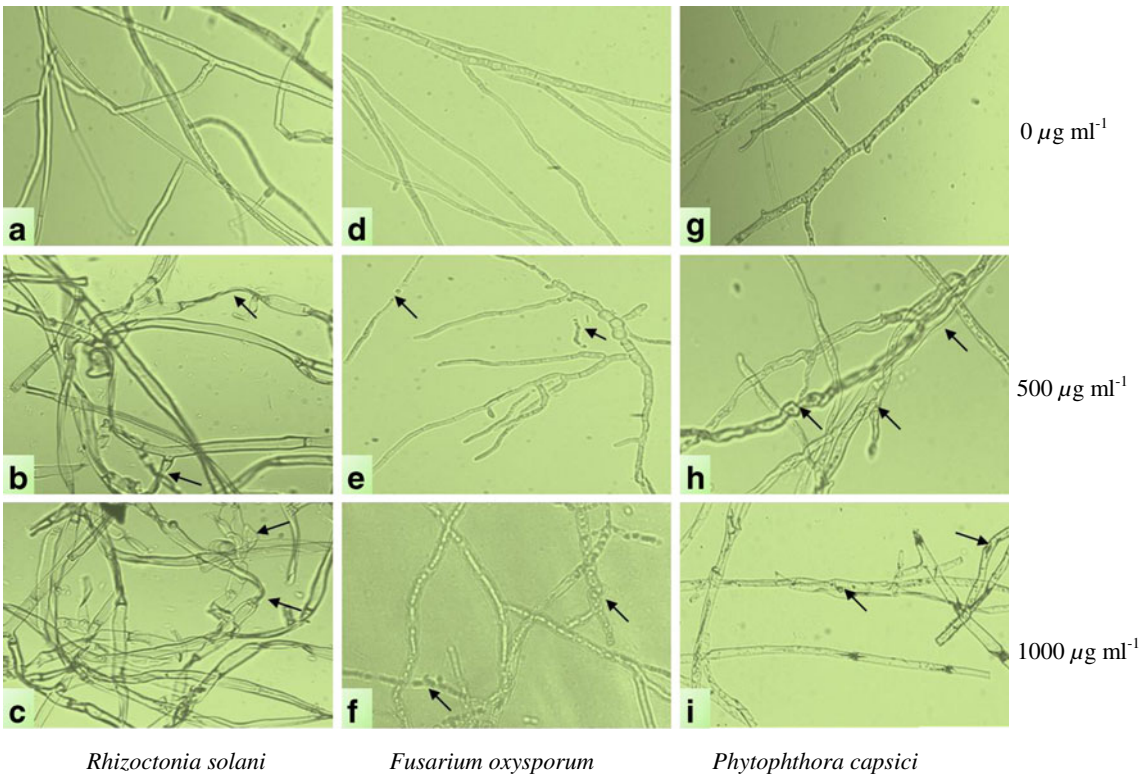

Fig. 5 Destruction of hyphae by different concentrations of the crude enzymes produced by *Paenibacillus ehimensis* KWN38

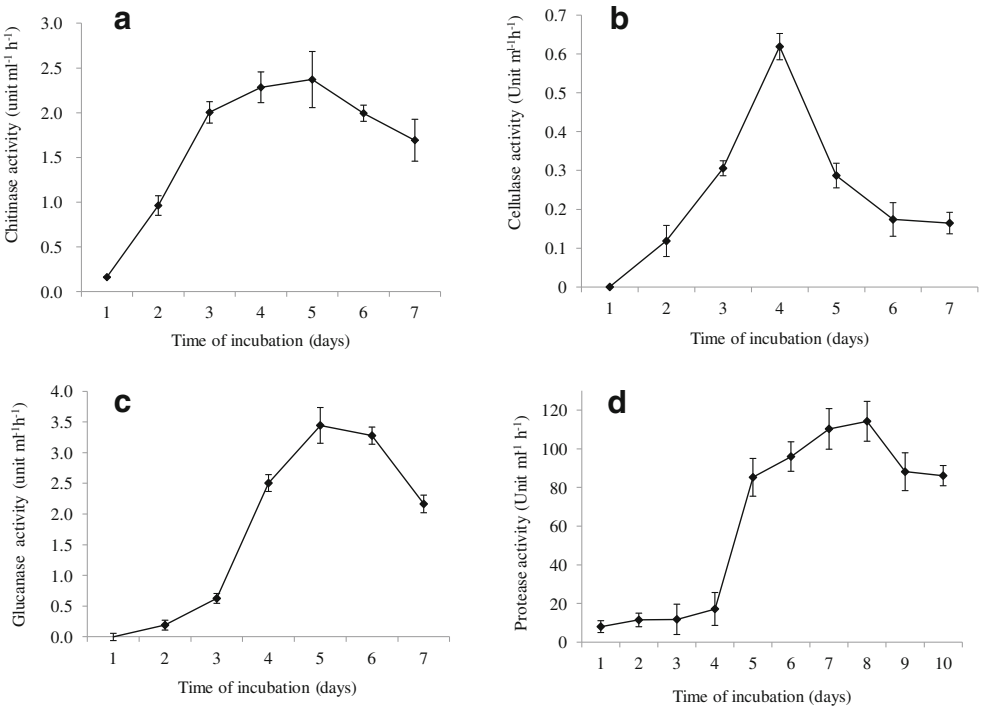

Fig. 6 The changes in chitinase (**a**); cellulase (**b**); β-1,3-glucanase (**c**) and protease (**d**) activities versus incubation time in different culture media grown with *Paenibacillus ehimensis* KWN38 and incubated at 30 °C and 170 rpm. Error bars represent standard deviation from three replicates

of a fungal pathogen also varies with its cell wall composition. Of course, the cell walls of the fungi belonging to different classes are variable. The filamentous fungi generally contain a large fraction of alkali-soluble glucans and chitin which is different than oomycetous fungal cell walls (Schoffelmeer et al. 1999; Theis and Stahl 2004). Therefore, in pathogen control aspect, there are many reports about the inhibition of *F. oxysporum* and *R. solani* by the purified chitinase (Kim et al. 2003; Jankiewicz et al. 2012). On the other hand, the cell walls of oomycetes consists predominantly of (1–3)-β -D-glucans, (1–6)-β-D-glucans, and cellulose (Bartnicki-García 1968). Moreover, the protein level in the walls of most fungi is as high as 11 % (Meijer et al. 2006). *Paenibacillus ehimensis* KWN38 showed a wide range of enzymatic activity in the present study. It could produce chitinases, cellulases, glucanases and proteases; this may be why it was equally effective against the diverse fungal pathogens tested in the present study with variable cell wall compositions. Moreover, the present study also demonstrated the ability of the crude enzymes produced by *Paenibacillus ehimensis* KWN38 to destroy the fungal cell walls.

Along with the CWDEs, the production of antifungal compounds such as fusaricidins produced by *Paenibacillus polymyxa,* as well as peptide-nature antifungal metabolite(s) produced by *Paenibacillus lentimorbus* WJ5, isolated from the butanol extracted fraction of organic compounds from the culture supernatant, have been well reported (Lee et al. 2008; Raza et al. 2009). This may be why the butanol extracted antifungal metabolites from the culture supernatant showed high antifungal activity against all the fungal pathogens tested in the present study. Moreover, the volatiles produced by KWN38 could restrict the growth of the tested pathogenic fungi. This is in accordance with the previous findings where various volatile compounds produced by antagonistic fungi and bacteria showed the antifungal activities (Fernando et al. 2005; Kai et al. 2006).

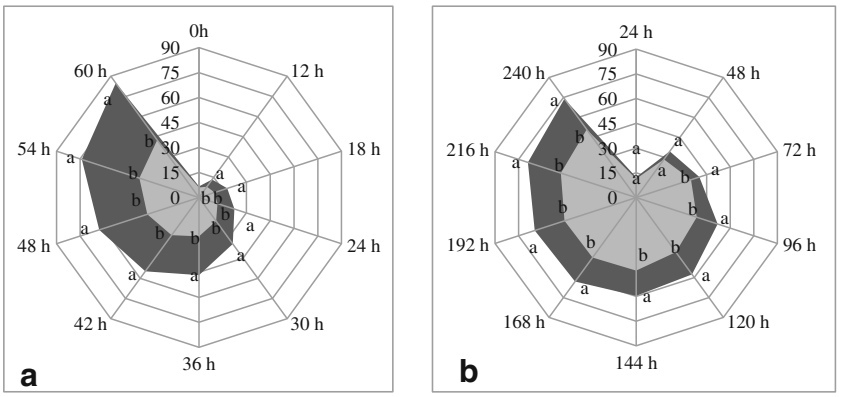

Fig. 7 The inhibition patterns of *Rhizoctonia solani* (**a**); and *Phytophthora capsici* (**b**) by the volatile compounds (*inner shaded area*) and each control (*outer shaded area*) in terms of colony diameter. The unit for all axes in chart is millimeter. Different letters within the same axis in the spider chart are significantly different each other at $P<0.05$ level of *t*-test

Furthermore, the volatile compounds produced by KWN38 reduced the pigment production in *F. oxysporum* f.sp. *lycopersici*. This finding is in agreement with Zhao et al. (2011), who reported that the citronellol microbial volatile compound completely prevented the pigment production of *F. oxysporum*. Some pigments including melanin may be related to their virulence towards the plant hosts and without these pigments, the fungi may lose their pathogenicity (Bassam et al. 2002). Furthermore, there is possibility of using the volatiles produced by the antagonist for the post harvest disease control of fruits, vegetables and grains.

In conclusion, *Paenibacillus ehimensis* KWN38 is a highly antagonistic antifungal bacterial strain isolated in the present study. Briefly, it is able to produce versatile lytic enzymes and antifungal compounds that make it able to control diverse soilborne fungal plant pathogens. This strain represents a potential biocontrol agent for soilborne fungi that cause plant diseases, which is an important perspective of the present study.

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