Characterization of a Chitinase Gene Exhibiting Antifungal Activity from a Biocontrol Bacterium *Bacillus licheniformis* N1

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A biocontrol bacterium *Bacillus licheniformis* N1 grown in nutrient broth showed no chitinolytic activity, while its genome contains a gene which encodes a chitinase. The gene for chitinase from *B. licheniformis* N1 was amplified by PCR and the deduced amino acid sequence analysis revealed that the chitinase exhibited over 95% identity with chitinases from other *B. licheniformis* strains. *Escherichia coli* cells carrying the recombinant plasmid displayed chitinase activity as revealed by the formation of a clear zone on chitin containing media, indicating that the gene could be expressed in *E. coli* cells. Chitinase gene expression in *B. licheniformis* N1 was not detected by RT-PCR analysis. The protein was over-expressed in *E. coli* BL21 (DE3) as a glutathione S-transferase fusion protein. The protein could also be produced in *B. subtilis* 168 strain carrying the chitinase gene of N1 strain. The crude protein extract from *E. coli* BL21 carrying GST fusion protein or culture supernatant of *B. subtilis* carrying the chitinase gene exhibited enzyme activity by hydrolyzing chitin analogs, 4-methylumbelliferyl- β -D-N,N'-diacetylchitobioside and 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotrioside. These results indicated that even though the chitinase gene is not expressed in the N1 strain, the coding region is functional and encodes an active chitinase enzyme. Furthermore, *B. subtilis* 168 transformants expressing the chitinase gene exhibited antifungal activity against *Fulvia fulva* by suppressing spore germination. Our results suggest that the proper engineering of the expression of the indigenous chitinase gene, which will lead to its expression in the biocontrol strain *B. licheniformis* N1, may further enhance its biocontrol activity.

Keywords : antifungal activity, *Bacillus licheniformis*, chitinase, *Fulvia fulva*

Biological control of plant diseases has received much interest since it is an environment friendly method for controlling various plant diseases (Fravel, 2005; Haas and Défago, 2005). Many antagonistic microorganisms have been selected as potential biocontrol agents and have been developed as biofungicides to control plant diseases (Emmert and Handelsman, 1999; Fravel et al., 1998). Suppression of plant fungal diseases by the biocontrol bacteria is mostly dependent on several bacterial traits, such as the production of various antimicrobial compounds and fungal cell-wall degrading enzymes (Compant et al., 2005; Ongena and Jacques, 2008). Niche exclusion, nutrient competition and defense induction are also important bacterial traits that are responsible for the successful plant disease control (Handelsman and Stabb, 1996; Haas and Défago, 2005; Park et al., 2007; Whipps, 2001).

An important bacterial trait for biological control of plant disease is the production of chitinase which has been shown to play an important role in the suppression of fungal growth (Dahiya et al., 2006; Singh et al., 1999). Chitin is an insoluble linear β -1,4-linked polymer of *N*-acetylglucosamine and a major constituent of the cell wall of fungi (Bartnicki-Garcia, 1968). Chitin hydrolysis is mostly dependent on chitinase production from various organisms such as plant, fungi, and bacteria. Plant chitinases have been reported to function in self-defense against pathogenic fungi (Roberts and Selitrennikoff, 1988; Selitrennikoff, 2001), while yeast and fungal chitinases are required for cell division and elongation during growth (Adams, 2004). Bacterial chitinases play an important role in digestion and utilization of chitin as a nutrient. Chitinases are classified into families 18 and 19 of the glycosyl hydrolases based on the amino acid sequence similarities of their catalytic domains (Cohen-Kupiec and Chet, 1998). Plant chitinases and some of the bacterial chitinases belong to family 19. These chitinases have antifungal activity and function as endo-chitinases (Robert and Selitrennikoff, 1988; Watanabe et al., 1999). On the other hand, most of the bacterial chitinases are classified into family 18 of the glycosyl

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hydrolases (Henrissat and Bairoch 1993), except for some chitinases from *Streptomyces* spp. which belong to family 19 (Watanabe et al., 1999). The chitinases in the family 18 show relatively weak antifungal activity in comparison to chitinases belonging to family 19 of the glycosyl hydrolases (Dahiya et al., 2006).

Bacillus licheniformis N1 strain has shown to be an effective biocontrol agent against several plant diseases and microbial formulations of this strain have been developed to control some plant diseases (Kim et al., 2007; Lee et al., 2006). *B. licheniformis* N1 produces antifungal compounds that play a role in inhibiting growth of plant pathogenic fungi. The strain produces several hydrolytic enzymes but not chitinolytic enzymes. While the N1 strain does not exhibit chitinase activity, recent whole genome analysis of *B. licheniformis* strains has revealed the presence of a chitinase gene, suggesting that the chitinase gene might be present in the genome of N1 strain (Rey et al., 2004; Veith et al., 2004). Microorganisms, which produce chitinases, have been reported as biological control agents against several plant pathogenic fungi (Chernin et al., 1995; Kobayashi et al., 2002). Therefore, the proper expression of the indigenous chitinase gene in the N1 strain would further enhance its biological control efficacy.

In the present study, we isolated a chitinase gene from *B. licheniformis* N1 which is deficient in chitinolytic activity under a laboratory culture condition. The chitinase gene of *B. licheniformis* N1 was characterized and expressed in *Bacillus subtilis* 168 and was found to exhibit antifungal

activity against *Fulvia fulva* by inhibiting spore germination.

Materials and Methods

Microorganisms, plasmids and culture conditions.

Escherichia coli cultures were routinely grown at 37°C on Luria-Bertani (LB) agar or in LB broth (Sambrook et al., 1989) supplemented with the appropriate antibiotics. *Bacillus* strains were routinely grown at 30°C on nutrient agar (NA) or in nutrient broth (NB). All *Bacillus* transformants were grown on NA or NB supplemented with appropriate antibiotics. The following antibiotic concentrations were used for *E. coli* and *Bacillus* strains (Table 1): chloramphenicol, 25 µg/ml; ampicillin, 100 µg/ml; kanamycin, 25 µg/ml; and tetracycline, 15 µg/ml. When necessary, 0.5% of colloidal chitin was supplemented in LB or NB agar to test the hydrolysis of colloidal chitin by chitinase producing bacterial strains. Colloidal chitin was prepared by the method of Rodriguez-Kabana et al. (1983) by partial hydrolysis of crustacean chitin with 10 N HCl for 1.5 hr at room temperature. The colloidal chitin was then washed with large volumes of tap water several times to give a final pH value between 6.0 and 6.5.

General DNA manipulation. Plasmid preparation, restriction endonuclease digestion, DNA ligation, plasmid DNA transformation, agarose gel electrophoresis, and other standard recombinant DNA techniques were carried out by standard methods described by Sambrook et al. (1989).

Table 1. Bacterial strains and plasmids used in this work

Strains or plasmids	Relevant characteristics ^a	Source or reference
<i>E. coli</i> strains		
DH5α	F <i>recA1</i> Δ(<i>lacZYA-argF</i>) U169 <i>hsdR17 thi-1 gyra66 supE44 endA1 relA1</i> Φ80Δ(<i>lacZ</i>) M15	Sambrook et al., 1989
Top10F'	F{ <i>lacI</i> ^q Tn10(Tc ^R)} <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 deoR recA1 araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 mupG</i>	Invitrogen, USA
BL21(DE3)	F <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Invitrogen, USA
<i>B. subtilis</i> 168	<i>trpC2</i> (X-ray mutagenesis of <i>B. subtilis</i> Marburg)	Burkholder and Giles, 1947
<i>B. licheniformis</i> N1	Biocontrol strain	Lee et al., 2006
Plasmids		
pGEM-T Easy	Ap ^r ; TA cloning vector	Promega, USA
pWH1520	Tc ^r ; expression plasmid for <i>B. megaterium</i>	Mo Bi Tec, Germany
pET-42a	Km ^r ; Over-expression plasmid for <i>E. coli</i> BL21(DE3)	Novagen, Germany
pN1-Chi	PCR amplified N1 chitinase gene with Chi-F and Chi-R primers and cloned into pGEM-T Easy	This study
pET-42-Chi	1.8 kbp <i>HindIII</i> - <i>NotI</i> fragment from pN1-Chi, cloned into <i>HindIII</i> - <i>NotI</i> site of pET-42a	This study
pBex WH1-4	PCR amplified N1 chitinase gene with Bex-F1 and Bex-R1 primers, digested with <i>Spe</i> I and <i>Bam</i> HI, and cloned into pWH1520	This study

^aStr^r, chromosomal streptomycin resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

DNA sequencing and primer synthesis were performed commercially at the DNA sequencing facility of GenoTech Corp. (Daejeon, Korea). DNA sequences were analyzed with a BLAST program provided by the National Center for Biotechnology Information.

Isolation of chitinase gene from *Bacillus licheniformis*.

The gene encoding chitinase from *B. licheniformis* N1 was amplified using primers that were designed based on the DNA sequence information available for the gene encoding chitinase Chi68 from *B. licheniformis* TP-1 (Tantimavanich et al., 1998). Two primers, Chi-F (5'-TCAATCTCATTATGTAAGCGTTT-3') and Chi-R (5'-AAATCTCTCTTTATCGTTTCTA-3') were commercially synthesized by GenoTech Corp. (Daejeon). The PCR amplification was carried out under the following conditions: 94°C for 3 min initial denaturation; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 5 min. The reaction mixture (final volume, 50 µl) contained 1 µl DNA (approximately 100–200 ng), 1X reaction buffer with 2 mM MgCl₂, each dNTP at a concentration of 200 µM, each primer at a concentration of 1 µM and 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). The amplified product was cloned into the pGEM-T Easy vector (Promega).

Reverse transcription PCR (RT-PCR). Total RNA was isolated from *B. licheniformis* N1, *B. subtilis* 168 containing pWH1520 and *B. subtilis* 168 containing pBex WH1-4. The bacterial cells were grown in the absence and presence of 0.5% colloidal chitin. Total RNA was isolated by using Easy-spin™ Total RNA Extraction kit (Intron Biotechnology Corp., Korea). RT-reaction and subsequent PCR was performed as described by Sambrook et al. (1989). A chitinase gene specific primer, RTR2 (5'-TATGGAGTTCTCCATCGGCGATCCTT-3'), was used for the first reverse transcription reaction to synthesize the first cDNA strand and two gene specific primers, RTF1 (5'-TAGTGCTTGGAAACACCGTTCTACGG-3') and Chi-R2 (5'-GTTTGCTCCGCTGCTCGGCGCAT-3'), were used for the subsequent PCR reaction.

Detection of chitinase activity. Chitin hydrolysis activity was analyzed based on the colloidal chitin hydrolysis assay and fluorogenic assay using fluorogenic chitin analogues. Chitin hydrolysis assay was performed using NA or LB agar plate supplemented with 0.5% colloidal chitin to detect clear zone formation around bacterial colonies. The chitinase activity was determined using the following fluorogenic substrate analogs: 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (MUF-NAG), 4-methylumbelliferyl-β-D-*N,N'*-diacetylchitobioside (MUF-diNAG), and 4-methylumbel-

liferyl-β-D-*N,N',N''*-triacylchitotrioside (MUF-triNAG). The substrates were purchased from Sigma-Aldrich, St. Louis, USA. The substrates were dissolved in dimethyl sulfoxide and added at the final concentration of 100 µM to the reaction mixture containing 200 mM of sodium phosphate buffer (pH 7.0) and enzyme solution. The reaction mixture was incubated at 37°C and terminated by the addition of sodium carbonate (0.6 M). Formation of MUF fluorescence was observed under UV light (365 nm).

Expression of N1 chitinase gene in *Escherichia coli* BL21(DE3).

The chitinase gene of *B. licheniformis* N1 was cloned into an expression vector, pET-42a (Novagen Corp., Germany) to produce the chitinase as a glutathione-S-transferase (GST) fusion protein. The chitinase gene was amplified using the following two primers: Hind chi F (5'-AAAGCTTTGGATGAAAAGGAG-3': underlined sequence corresponds to *Hind*III restriction site) and Chi-R (5'-AAATCTCTCTTTATCGTTTCTA-3'). Amplified PCR product was first cloned into pGEM-T Easy vector. The recombinant plasmid was digested with *Not*I and *Hind*III and the chitinase gene was subsequently cloned into pET-42a to generate pET-42-Chi. *E. coli* BL21 (DE3) cells carrying pET42-Chi were grown in LB broth at 37°C until absorbance at 600 nm reached 0.6–0.8. The chitinase gene was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside to the bacterial culture. The expressed protein was analysed by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The gel was then stained with Coomassie blue R250 and destained as described previously (Lane 1978).

Expression of N1 chitinase gene in *Bacillus subtilis*. In order to express the chitinase gene in *B. subtilis*, the chitinase gene of *B. licheniformis* N1 was cloned into an expression vector, pWH1520 (Mo Bi Tec., Germany). The chitinase gene was amplified using the following two primers: Bex-F1 (5'-GGACTAGTCCCTTGTGACTTCTGTGC-3': underlined sequence for *Spe*I restriction site) and Bex-R1 (5'-CCGGATCCCTATTTCGACGCTCCGACCAG-3': underlined sequence for *Bam*HI restriction site). Amplified PCR product was digested with *Spe*I and *Bam*HI, and subsequently cloned into pWH1520 to generate pBexWH 1-4. To introduce pBexWH 1-4 into *B. subtilis* 168 strain, electro-competent cells were prepared by high-osmolarity protocol as described previously (Xue et al., 1999). Electroporation was conducted with purified plasmid DNA (pBexWH 1-4) and electro-competent cell using MicroPulser (Bio-Rad, USA). Electroporation was performed using 0.1 cm disposable cuvettes under the following parameters: field strength, 16 kV/cm; capacitance, 20 µF; resistance, 200 Ω; resulting in a time constant of 4.5–5.0 msec.

Antifungal activity assay of N1 chitinase *in vitro*. Antifungal activity of *B. licheniformis* N1 chitinase was investigated using non-antagonistic bacterial strain *B. subtilis* 168 which is also defective in chitinase activity and antifungal activity. The fungal pathogen *Fulvia fulva* that causes tomato leaf mold was previously isolated from a diseased tomato plant. The *F. fulva* strain TF13 was routinely grown on potato dextrose agar (PDA) at 25°C. The conidia suspension (1×10^5 conidia/ml) of *F. fulva* was prepared in sterile water from fungal culture grown on PDA media. *B. subtilis* strain 168 transformed with pBexWH1-4 was grown on nutrient broth containing tetracycline in the presence or absence of xylose at 30°C for 24 hr. *B. subtilis* strain 168 transformed with pWH1520 was used as a control. Bacterial culture supernatant containing chitinase enzyme was directly used to investigate the inhibition of conidia germination. Two hundred microliter of cell-free supernatant was mixed with 10 μ l of conidia suspension (1×10^3 conidia) of *F. fulva* and incubated at 25°C overnight. Germination of the fungal spores was observed under the light microscope and germination spores were counted using hemacytometer. The experiment was performed in triplicate.

Nucleotide sequence accession number. The nucleotide sequence of the chitinase gene of *B. licheniformis* N1 has been deposited in the GenBank database under accession number GQ375847.

Results and Discussion

Cloning of a gene encoding chitinase from *Bacillus licheniformis* N1. Chitinase gene was amplified from *B. licheniformis* N1 and cloned into pGEM-T Easy to generate plasmid pN1-Chi. *E. coli* TOP10 cells containing pN1-Chi exhibited a clear zone formation around colonies on LB media containing colloidal chitin (data not shown), indicating that the gene from *B. licheniformis* N1 could be expressed in *E. coli*. The cloned DNA insert (1,794 bp) in pN1-Chi was completely sequenced. Deduced amino acid sequence analysis of N1 chitinase revealed an open reading frame encoding a 597 amino acid protein. Sequence analysis indicated that the N1 chitinase was highly similar to the chitinase Chi68 from *B. licheniformis* TP-1 (Tantimavanich et al., 1998) and exhibited 98% identity. Deduced amino acid sequence analysis of N1 chitinase showed the presence

Fig. 1. Alignment of the catalytic domain (A) and chitin binding domain (B) of N1 chitinase with bacterial and fungal chitinases. Conserved residues are shown in dark shade. Four arrows indicate conserved residues found in N1 chitinase as well as in other bacterial and fungal chitinases. Asterisk (*) indicates a residue which is necessary for chitinase activity, and conserved aromatic residues are underlined. The putative catalytic domain of *B. licheniformis* N1 was compared with the catalytic domains of *Aphanocladium album* (GenBank Accession no. X64104), *B. subtilis* (AF069131), *B. circulans* (A1 M57601), *B. licheniformis* TP-1 (U71214), *B. circulans* No. 4.1 (AF154827), *B. thuringiensis* (U89796), *Chironomus tentans* (Y13234), *Clostridium thermocellum* (Z68924), *Kurthia zopfii* (D63702), *Metarhizium anisopliae* (AF027498), *M. flavoviride* (AJ243014), *Trichoderma hamatum* (U88560), *B. circulans chiD* (D10594), *Pseudoalteromonas* sp. *chiC* (AF007896), *Streptomyces griseus chiC* (AB009289), *Aeromonas* sp. 10S-24 (D31818).

of conserved domains (Fig. 1), including a catalytic domain spanning amino acids 44-433 and a chitin binding domain spanning amino acids 551-580. Even though the chitinase of N1 strain was found to be similar to the chitinase Chi68 of TP-1 strain, there were some variations in the C-terminal region of the enzyme (data not shown). Overall, based on the deduced amino acid sequence analysis, the N1 chitinase was found to exhibit similarity with chitinases belonging to family 18 of the glycosyl hydrolase.

Chitinases belonging to family 19 of the glycosyl hydrolases are well known for exhibiting antifungal activity, such as some bacterial chitinases from *Streptomyces* spp. and plant chitinases involved in plant defense response (Leah et al., 1991; Ohno et al., 1996; Watanabe et al., 1999). On the other hand, most of the bacterial chitinases are classified into family 18 of the glycosyl hydrolases (Henrissat and Bairoch 1993) and we found that the N1 strain chitinase showed higher similarity with the family 18 chitinases of the glycosyl hydrolases. However, the role of chitinases in the family 18 is not clear regarding their ability to exhibit antifungal activity (Cohen-Kupiec and Chet, 1998). In the present study, the chitinase from N1 strain was examined for its ability to exhibit antifungal activity against plant pathogenic fungi *F. fulva*.

Expression of the chitinase gene in *E. coli*. *B. licheniformis* N1 was defective in chitin hydrolysis activity as analyzed by colloidal chitin hydrolysis assay and fluorogenic assay (data not shown). However, our result has shown that a chitinase gene is present in the genome of N1 strain. Moreover, genome sequence analysis of two industrial strains of *B. licheniformis* (strain DSM13 and strain ATCC 14580) has also revealed the presence of a chitinase gene (Rey et al., 2004; Veith et al., 2004). The absence of chitinolytic activity but the presence of chitinase gene in *B. licheniformis* N1 strain raises a question regarding the functionality of the gene. Therefore, to address this question, we cloned the chitinase gene in an expression vector, pET-42a to generate pET-42-Chi and over expressed the N1 chitinase as a GST fusion protein in *E. coli* BL21 (DE3). SDS-PAGE analysis indicated that the expected fusion protein was successfully expressed in *E. coli* cells (data not shown). When *E. coli* crude extract obtained after ultrasonication was tested for the chitinase activity using fluorogenic analogues of chitin, the hydrolysis of MUF-diNAG and MUF-triNAG was apparent resulting in the production of fluorescent MUF (Fig. 2A). In contrast, crude extract from *E. coli* cells with pET-42a did not show any chitinase activity. This result indicated that the coding region of chitinase gene is functional and encodes an active chitinase enzyme.

Expression of the chitinase gene in *B. subtilis*. The

Fig. 2. Detection of chitinase activity using fluorescent analogues of chitin. (A) Chitinase activity observed using crude extract of *E. coli* BL21 (DE3) cells carrying pET-42-Chi, (B) Chitinase activity observed using *B. subtilis* 168 cultures carrying pBex WH1-4 (lane 1 and 2), *B. subtilis* 168 cultures carrying pWH1520 (lane 3), and *B. subtilis* 168 cultures carrying pBex WH1-4 in the absence of substrates (lane 4). Xylose (0.5%) was supplemented as an inducer (lane 2 and 3).

chitinase gene from *B. licheniformis* N1 was also cloned in a *Bacillus-E. coli* shuttle vector, pWH1520, generating pBex WH1-4. Since pWH1520 and pBex WH1-4 include a strong promoter which is activated in the presence of xylose, the chitinase from N1 strain will be produced in *B. subtilis* 168 strain carrying pBex WH1-4 upon addition of xylose to the bacterial culture. Our study showed that the culture supernatant of *B. subtilis* 168 containing pBex WH1-4 hydrolyzed MUF-diNAG and MUF-triNAG within 30 min (Fig. 2B), indicating that the chitinase enzyme was produced and excreted in *B. subtilis* 168 strain. In the absence of xylose, the enzyme was produced at a very low level and only slightly hydrolyzed MUF-triNAG, when the reaction time was extended upto 24 hr (data not shown). As expected, the culture supernatant from *B. subtilis* 168 strain carrying pWH1520 did not exhibit any chitinase activity (Fig 2B). Together, our result suggested that the chitinase gene is functional and is expressed in *E. coli* and *B. subtilis* 168 strain but it is not expressed in *B. licheniformis* N1.

We further tested the chitinase gene expression at the transcriptional level in *B. licheniformis* N1 strain. An expected 459-bp of RT-PCR product was obtained from *B. subtilis* 168 strain carrying pBex WH1-4 strain upon xylose induction (Fig. 3). The expected transcript was not detected from *B. subtilis* 168 or *B. subtilis* carrying pWH1520 (Fig. 3B), confirming that the introduced chitinase gene from N1 strain was indeed expressed in *B. subtilis*. However, the chitinase gene is not transcribed from the N1 strain because the expected RT-PCR product was not detected in the presence or absence of colloidal chitin (Fig. 3A). This result explains the deficiency in chitinolytic activity of *B. licheni-*

Fig. 3. RT-PCR analysis of chitinase gene expression. Lane 1, *B. licheniformis* N1 grown in the absence of chitin; lane 2, *B. licheniformis* N1 grown in the presence of chitin; lane 3, *B. subtilis* 168 strain carrying pBex WH1-4; lane 4, *B. subtilis* 168; lane 5, *B. subtilis* 168 carrying pBex WH1-4; lane 6, *B. subtilis* 168 carrying pWH1520.

formis N1 strain.

It is likely that the chitinase gene of *B. licheniformis* N1 strain is not expressed under laboratory culture conditions. The gene expression might be tightly regulated in the N1 strain, or the upstream region of the chitinase gene may contain unexpected mutations, which might inactivate promoter activity in this organism. Similar result was previously reported from other strain of *B. licheniformis* which is chitinase-deficient, although the genome was found to contain a chitinase gene (Waldeck et al., 2005). Further investigation of the promoter region is necessary to confirm this possibility. If we are able to express the chitinase gene in *B. licheniformis* N1 strain, it would further improve the biocontrol activity of this organism. The biocontrol activity by N1 strain has been already proven against some plant diseases by using bacterial cells and formulations of this strain (Kim et al., 2007; Lee et al., 2006). The N1 strain produces antifungal compounds that are able to inhibit the growth of plant pathogenic fungi (Lee et al., 2006) and the simultaneous production of chitinase exhibiting antifungal activity would certainly enhance the biocontrol activity of this bacterium.

Inhibition of spore germination of *Fulvia fulva* by chitinase from *B. licheniformis*. Antifungal activity of the chitinase of N1 strain against *F. fulva* was investigated on PDA medium and in aqueous solution. Since *B. licheniformis* N1 produces strong antifungal compounds, the potential of chitinase enzyme to inhibit fungal growth cannot be tested in this strain. Therefore, antifungal activity of the chitinase was analyzed using *B. subtilis* 168 with pBex WH1-4. *B. subtilis* 168 carrying either pBex WH1-4 or pWH1520 was not able to inhibit fungal mycelium growth on PDA plate (data not shown). However, *B. subtilis* 168 carrying pBex WH1-4 inhibited spore germination in aqueous solution. Culture supernatant of *B. subtilis* 168 carrying pBex WH1-4 suppressed the fungal spore germination (Fig. 4). After 6 - 12 hr incubation, the fungal spore germination in culture supernatant of *B. subtilis* 168 carrying pBex WH1-4 decreased to almost 50% in comparison

Fig. 4. Graph depicting the rate of germination (%) of fungal spores of *F. fulva* treated with the supernatant of *B. subtilis* 168 carrying pWH1520 or *B. subtilis* 168 carrying pBexWH1-4 or with a fresh medium containing tetracycline. Germination rate was measured by counting the number of fungal spores that germinated versus total number of spores, using hemacytometer under light microscope over time.

with other samples. The addition of tetracycline to the bacterial cell culture had no effect on fungal spore germination. Furthermore, the addition of conidia suspension of *F. fulva* to the supernatant of *B. subtilis* carrying pWH1520 showed no effect on fungal spore germination. This result demonstrates that the N1 chitinase when expressed in *B. subtilis* 168 has an adverse effect on *F. fulva* spore germination to contribute on biocontrol activity. In fact, there were a number of reports that chitinase production in biocontrol microorganisms contributes to the biological control activity against plant diseases (Lorito et al., 1993; Melent'ev et al., 2001). Compared to several chitinases in family 19 of the glycosyl hydrolases showing antifungal activity by inhibiting fungal mycelia growth, N1 chitinase exhibited only fungal spore germination. Therefore, it should be further investigated how the N1 chitinase will contribute on biocontrol activity when properly expressed.

In the present study, we have demonstrated that the chitinase gene isolated from *B. licheniformis* N1 is functional in *E. coli* and *B. subtilis* 168 strain, but is silent in the N1 strain. We also showed that the chitinase enzyme produced in *B. subtilis* 168 exhibited antifungal activity against *F. fulva* by suppressing spore germination. Restoration of the chitinase gene expression in *B. licheniformis* N1 may improve its biocontrol activity.

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