Research Article

Ozlem Oztopuz*, Nermin Sarigul, Fakhra Liaqat, Ro-Dong Park and Rengin Eltem

Chitinolytic *Bacillus subtilis* Ege-B-1.19 as a biocontrol agent against mycotoxigenic and phytopathogenic fungi

Mikotoksijenik ve fitopatojenik küflere karşı biyokontrol ajanı olarak kitinolitik *Bacillus subtilis* Ege-B-1.19

<https://doi.org/10.1515/tjb-2018-0144>

Received April 14, 2018; accepted July 17, 2018; previously published online October 18, 2018

Abstract

Background: Biological control of pathogenic fungi is a possible alternate to the chemical control, which is harmful to humans and environment. Soil-borne *Bacillus* strains can be potential biocontrol agents and a source of lytic enzymes.

Aim: This study aimed to examine biocontrol potential and lytic enzyme activities of a soil isolate *Bacillus subtilis* Ege-B-1.19.

Materials and methods: Strain was identified by biochemical and 16S rRNA gene analysis and its biocontrol activity was investigated against *Aspergillus niger* EGE-K-213, *Aspergillus foetidus* EGE-K-211, *Aspergillus ochraceus* EGE-K-217, *Fusarium solani* KCTC6328, *Rhizoctonia solani* KACC40111

and *Colletotrichum gloeosporioides* KACC40689. Chitinase, chitosanase, N-acetyl- β -hexosaminidase and protease activities of *B. subtilis* Ege-B-1.19 were also determined. Chitosanase was purified using Sephadex G-150 column and its molecular weight was determined by SDS-PAGE. Chitooligosaccharides production using chitosanase was carried out and analysed by TLC and HPLC.

Results: Results depicted that *B. subtilis* Ege-B-1.19 has shown inhibitory effects against all the test fungi. Chitinase, chitosanase, N-acetyl- β -hexosaminidase and protease activities were determined as 2.7 U mL⁻¹, 7.2 U mL⁻¹, 6.2 U mL⁻¹ and 12.2 U mL⁻¹, respectively. Molecular weight of purified chitosanase was 44 kDa. Chitosanase hydrolysed chitosan to glucosamine (GlcN), dimers (GlcN)₂ and trimers (GlcN)₃.

Conclusion: *Bacillus subtilis* Ege-B-1.19 can be anticipated as useful biocontrol agent and its chitosanase can be utilized for enzymatic synthesis of chitooligosaccharides.

Keywords: Vineyard soil; *Bacillus subtilis*; Chitosanase; Biocontrol; Chitooligosaccharides.

*Corresponding author: Ozlem Oztopuz, Department of Biophysics, Çanakkale Onsekiz Mart University, 17000 Çanakkale, Turkey, Tel.: +905325851112, Fax: +902862180393, e-mail: ozlemoztopuz@yahoo.com.tr.
<https://orcid.org/0000-0002-1373-6311>

Nermin Sarigul: Department of Molecular Biology and Genetic, Mehmet Akif Ersoy University, Burdur, Turkey

Fakhra Liaqat: Graduate School of Natural and Applied Sciences, Department of Biotechnology, Ege University, Izmir, Turkey

Ro-Dong Park: Glucosamine Saccharide Materials-National Research Laboratory, Chonnam National University, Gwangju, Korea

Rengin Eltem: Department of Bioengineering, Ege University, Izmir, Turkey

Öz

Giriş: Patojenik küflerin biyolojik kontrolü, insanlara ve çevreye zararlı kimyasal kontrole olası bir alternatiftir.

Amaç: Bu çalışmada toprak kaynaklı bir izolat olan *Bacillus subtilis* Ege-B-1.19'un litik enzim ve potansiyel biyokontrol aktivitesinin incelenmesi amaçlanmıştır.

Gereç ve Yöntem: Suş biyokimyasal ve 16S rRNA gen analizi ile tanımlandı ve *Aspergillus niger* EGE-K-213, *Aspergillus foetidus* EGE-K-211, *Aspergillus ochraceus*

EGE-K-217 ve *Fusarium solani* KCTC6328 *Rhizoctonia solani* KACC40111 ve *Colletotrichum gloeosporioides* KACC40689 karşı antifungal aktivitesi araştırıldı. *B. Subtilis* Ege-B-1.19'un kitinaz, kitosanaz, n-asetil- β -hekzosaminidaz ve proteaz aktiviteleri de ayrıca belirlenmiştir. Kitosanaz Sephadex G-150 kolonu kullanılarak saflaştırıldı ve molekül ağırlığı SDS-PAGE ile belirlendi. Kitosanaz kullanılarak kitosan-glikozilazların üretimi TLC ve HPLC ile gerçekleştirildi ve analiz edildi.

Bulgular: Elde edilen sonuçlar *B. subtilis* Ege-B-1.19'un tüm test küflerine karşı inhibitör etki gösterdiğini ortaya koymuştur. Kitinaz, kitosanaz, n-asetil- β -hekzosaminidaz ve proteaz aktiviteleri sırasıyla 2,7 U mL⁻¹, 7,2 U mL⁻¹, 6,2 U mL⁻¹ ve 12,2 U mL⁻¹ olarak belirlendi. Saflaştırılmış kitosanazın moleküler ağırlığı 44 kDa idi. Kitosanaz kitosanı glukozamin (GlcN), dimerlere (GlcN)₂ and trimerlere (GlcN)₃ kadar hidroliz etti.

Sonuç: *Bacillus subtilis* Ege-B-1.19 faydalı bir biyo-kontrol ajanı olarak öngörülebilir ve kitosanaz kitooligosakkaridlerin enzimatik sentezi için kullanılabilir.

Anahtar kelimeler: Üzüm Bağı toprağı; *Bacillus subtilis*; Kitosanaz; Biyokontrol; Kitooligosakkaridler.

Introduction

In the absence of effective chemical control methods and to reduce environmental pollution, the biological control of plant pathogens is currently considered a key practice in sustainable agriculture. *Bacillus*, *Pae-nibacillus*, *Pseudomonas* and *Trichoderma* species all express good antagonistic activities under controlled laboratory or greenhouse conditions. Thus, antagonistic microorganisms play a very important role in biological control [1]. In the past 30 years, there have been extensive research activities to explore and develop strategies based on microbial antagonists to biologically control postharvest pathogens [2]. The bacterial biocontrol mechanisms for plant protection against fungal diseases include systemic resistance, parasitism and competition for nutrients. Bacteria present in soil are also known to produce a number of enzymes and antimicrobial compounds [3].

Bacillus species are the most studied antagonist among the soil born bacteria [4]. They have been employed in biocontrol [5, 6] and bioremediation [7]. *Bacillus* species were found to inhibit a number of fungi under in vitro conditions [8, 9]. *Bacillus* strains can secrete various secondary metabolites, which inhibit the pathogenic fungi. It is also known that *Bacillus* strains

are able to produce various hydrolytic enzymes, including chitosanases, chitinases, N-acetyl- β -hexoaminidase, proteases and laminarinases in order to inhibit the growth of pathogenic fungi by degrading their cell wall [8, 10, 11]. Among these hydrolytic enzymes chitosanase (EC 3.2.1.132) are important enzymes not only for the bio-control of pathogenic fungi, but also with reference to their ability to produce chitoooligosaccharides (COS): a degradative product of chitosan. Chitosan and its derivatives have shown various antibacterial, antifungal, anti-tumor and antioxidant activities [12] which made them important to be used in several industries including agriculture, food, cosmetics, biocontrol, and wastewater treatment. However, the difficult dissolution of chitosan limits its use in several industries including food and biomedical applications [13]. Unlike chitosan, chitosan oligosaccharides and short chain D-glucosamine units are readily soluble in water. Chemical and enzymatic ways are generally used to synthesize chitosan oligosaccharide, however, chemical methods have some disadvantages such as difficult control of chemical reactions, formation of various secondary compounds and use of harmful chemicals [12, 13]. Enzymatic methods including microbial chitosanases showed excellent performances in COS production [14–16].

With the aim to discover potential biocontrol agents, this study was designed to isolate chitinolytic *Bacillus* strains from vineyard soil. *Bacillus subtilis* Ege-B-1.19 having the highest chitinolytic activity was identified and examined for its in vitro antagonistic activity against phytopathogenic and mycotoxigenic fungi. Furthermore, partial purification of chitosanase from *B. subtilis* Ege-B-1.19 and enzymatic production and quantitative analysis of COS were also the objectives of this study.

Materials and methods

Chitin and chitosan with various DA were purchased from Taehoon Bio (Seoul, Korea). Chitoooligosaccharides, (GlcN)_n (n=2–7), were purchased from Wako Chemicals (Osaka, Japan). N-acetyl-D-glucosamine, D-glucosamine, glycol chitosan, p-nitrophenyl-N-acetyl-b-D-glucosaminide (p-NP-GlcNAc) and azocasein were purchased from Sigma. Plate count agar (PCA), LB-broth and nutrient agar (NA) were purchased from Merck, Germany. *Aspergillus niger* EGE-K-213, *A. foetidus* EGE-K-211, *A. ochraceus* EGE-K-217 were obtained from Mold Culture Collection, Ege University, Izmir, Turkey. *Fusarium solani* KCTC 6328, *Rhizoctonia solani* KACC 40111 and *Colletotrichum*

gloeosporioides KACC 40689 strains were kindly provided by Materials-National Research Laboratory, Chonnam National University, South Korea.

Soil sampling and analysis

Soil samples were obtained as composites from five different sampling points of a vineyard in Manisa province, Turkey (38° 40'N and 27° 03'E and 39° 08'N and 27° 37'E). At each sampling point, firstly, surface material was dug to a depth of 2–3 cm. Soil samples were taken using sterile equipments at a depth of 25–30 cm for chemical analysis and isolation of potential biocontrol agents. The sample soils were analyzed in TARIS R&D Center, Soil Laboratory, Bornova, Izmir for water content, pH, salt (%), carbonate (%), texture, clay (%), sand (%), nitrate-N (%), total phosphorus and potassium.

Isolation of endospore forming bacteria and screening of chitosanolytic activity

Soil samples were suspended in sterile distilled water, shaken vigorously and incubated on a water bath (100 rpm, 80°C) for 30 min to kill all vegetative microbial cells. After incubation, the soil samples were serially diluted (10-fold), plated on NA medium and incubated at 30°C for 2 days and spore forming bacteria were isolated. Colonies arising from the isolates survived after heat shock were transferred to a maintenance medium. The isolates were stored in glycerol at –80°C [17, 18]. Screening of chitosanase production was carried out using chitosanase detection agar (CDA) containing 1% soluble chitosan, 0.13% sodium phosphate, 0.3% potassium phosphate, 0.05% sodium chloride, 0.1% ammonium chloride, 0.024% magnesium sulfate, 0.001% calcium chloride and 2% agar (pH 6.5). Isolates were inoculated on the plates, incubated at 30°C for 5 days and colonies surrounded by clear zones were isolated [8]. The isolates selected through the screening procedure were then cultured in LB-broth containing 0.2% swollen chitin and 0.2% colloidal chitosan and incubated at 30°C and 200 rpm for 2 days for the measurement of lytic enzyme activities.

Identification of the bacterial isolate

Isolate Ege-B-1.19, showing highest chitosanolytic activity was selected and performed Phenotypic identification test by using BBL Chrystal Gram Positive ID System Test Kit in

accordance with the manufacturer's instructions. Results were interpreted according to BBL™ Crystal™ Identification System. For molecular identification, DNA isolation were performed using the UNIQ-10 Spin Column Genomic DNA Minipreps Kit (SK201&202) by the manufacturer's instructions. Bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal eubacteria primers 8F, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R, 5'-GGTTACCTTGTACGACT-3'. Partial 16S rDNA raw sequence data were imported into the BioEdit Sequence Alignment Editor and a contiguous consensus sequence was generated. Using blast against GenBank database (EU099356), the nearest relatives of these consensus sequence were identified. Data obtained after sequencing were submitted in the NCBI GenBank database to attain accession numbers.

Measurement of lytic enzyme activities

Chitinase activity was detected by measuring the amount of reducing sugar released from swollen chitin by the Schale's method using a standard curve for N-acetylglucosamine (GlcNAc) [19]. The assay was performed by mixing 0.9 mL of 1% swollen chitin (pH 6.0) and 0.1 mL of fermentation broth. After incubation for 2 h, at 37°C, chitin hydrolysis reaction was terminated by adding 0.2 mL NaOH (1 N). One millilitre Schale's reagent was then added and the reaction mixture was kept in boiling water for 15 min followed by chilling on ice. The amount of reducing sugars released in the supernatant was measured spectrophotometrically at 420 nm. One unit of chitinase activity was defined as the amount of enzyme required to produce 1 µmol GlcNAc per hour [19].

Chitosanase activity was measured by DNS method using a standard curve of D-glucosamine (GlcN) [20]. The assay was performed by mixing 0.9 mL of 1% soluble chitosan (pH 6.0) and 0.1 mL of fermentation broth. After incubation for 30 min, at 37°C, hydrolysis reaction was terminated by adding 0.2 mL NaOH (1 N) and 1.5 mL DNS reagent. The reaction mixture was kept in boiling water for 5 min followed by chilling on ice. The amount of reducing sugars released in the supernatant was measured spectrophotometrically at 540 nm. One unit of the enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars per min at 37°C [20].

N-acetyl-β-hexosaminidase activity was assayed by using p-nitrophenyl-N-acetyl-b-D-glucosaminide (p-NP-GlcNAc) as substrates. The reaction mixture containing 50 µL of

5 mM p-nitrophenyl-N-acetylglucosaminide, 50 μ L of crude enzyme, and 100 μ L of 50 mM McIlvaine buffer (pH 5.0) was incubated at 45°C for 15 min. The reaction was stopped by adding 1 mL of 0.2 M Na_2CO_3 and the amount of p-nitrophenol released was determined by measuring the absorbance at 405 nm. One unit of β -N-acetylglucosaminidase was defined as the amount of enzyme required to release 1 μ mol of p-nitrophenol per min [21].

Protease activity was measured using 450 μ L of 1% azocasein in 0.1 M phosphate buffer (pH 7.0) and 50 μ L enzyme solution. Reaction mixture was incubated at 50°C for 30 min. Reaction was terminated by adding 250 μ L of 25% (w/v) trichloroacetic acid. Mixture was centrifuged at 10,000 rpm and 600 μ L of supernatant was mixed with 600 μ L of 1 N NaOH. Enzyme activity was measured at 440 nm and 1 U was defined as the amount that increased absorbance to 0.01 at 440 nm per min [22].

In vitro antifungal activity assay

Isolate Ege-B-1.19 was tested for antifungal activity against *A. niger* EGE-K-213, *A. foetidus* EGE-K-211, *A. ochraceus* EGE-K-217, *F. solani* KCTC 6328, *R. solani* KACC 40111 and *C. gloeosporioides* KACC 40689. Plugs of 6 mm in diameter were cut from the outer edges of the colony from the test fungi and placed in the center of PCA plates. Fermentation broth of isolate Ege-B-1.19 was filtered through 0.22 μ m pore size membrane filters and used to prepare filter paper discs. Fifty microliter of filtered fermentation broth was dispensed on sterilized filter paper discs (9 mm) and discs were placed on the PCA plates around the fungal plugs. Fifty microliter of sterilized water was used as a control. The plates were incubated for 10 days at 28°C and the ability of the isolate Ege-B-1.19 to inhibit test fungi was observed daily.

Hydrolysis of chitosan by chitosanases to produce chitosan oligomers

For the hydrolysis of chitosan to chitosan oligosaccharides, the reaction mixture containing 1 mL of 1% soluble chitosan, 1 mL of 100 mM sodium acetate buffer (pH 5.5), and 20 μ L of culture supernatant was incubated at 37°C for 30 min and the reaction was stopped by immersing the tubes in boiling water for few minutes.

Investigation of chitosan oligomers by analytical methods

Chitosan oligosaccharides were analyzed by TLC on Silica gel 60 (Kieselgel 60 F254; Merck). A solvent system of *n*-propanol:ethylacetate:ammonia solution:water (6:3:3:1) was used and sugar spots on the plates were visualized by spraying 0.2% ninhydrin into 99% ethyl-alcohol. For the quantitative analysis of chitosan oligosaccharides, HPLC analysis was carried out with a Carbohydrate Analysis column (300 $\times$ 3.9 mm) using a solvent system of acetonitrile and water (68:32) at a flow rate of 1.0 mL min⁻¹ with a refractive index detector [20].

Purification of chitosanase from the isolate Ege-B-1.19

The isolate Ege-B-1.19 was grown in LB-CC broth at 30°C, 200 rpm for 48 h. The cells were removed by centrifugation at 6000 g for 20 min. The supernatant was fractionated by addition of 85% saturation of $(\text{NH}_4)_2\text{SO}_4$ and precipitates were collected by centrifugation. The precipitates were dissolved in appropriate volume (2 mL) of 50 mM sodium acetate buffer at pH 5.5 followed by overnight dialysis against the same buffer. After dialysis, the enzyme was concentrated with polyethylene glycol (PEG) and eluted on Sephadex G-150 gel column (2.6 $\times$ 40 cm) previously equilibrated with the same buffer. The fractions were collected and chitosanase enzyme activity and protein amount were measured [23].

SDS-PAGE for the detection of molecular weight and chitosanase activity

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% polyacrylamide gel containing 0.01% soluble chitosan as a substrate for chitosanase activity. After SDS-PAGE, the gel was incubated at 37°C for 2 h with gentle shaking for renaturation in 100 mM sodium acetate buffer (pH 5.0) containing 1% purified Triton X-100 and 1% skim milk. The gel was washed with distilled water and incubated for another 1 h at 37°C in 100 mM sodium acetate buffer (pH 5.0) with 1% purified Triton X-100. After renaturation, the gel was transferred to Tris-HCl buffer (0.5 M, pH 9.0) containing 0.01% (w/v) Calcofluor white M2R stain, and incubated for 7 min followed by several washes. Chitosanase activity on the gel was visualized under UV light [23].

Results

Characterization of the soil sample

The vineyard soil sample was collected from center of Manisa in the summer season (2004–2005). Table 1 summarizes the properties of soil samples.

Isolation of endospore forming bacteria and screening of chitosanolytic activity

A total of 28 endospore forming isolates were obtained from the vineyard soil samples and 13 isolates have shown chitosanolytic activity on CDA plates. Isolate Ege-B-1.19

represented the best result by showing the largest clear zone, and therefore, it was selected for further studies.

Identification of Ege-B-1.19 isolate

Ege-B-1.19 isolate was a Gram-positive, rod-shaped and endospore forming bacterium. The cells were 0.8–1.0 μm wide and 2.0–3.0 μm long. Oval shaped, 0.7–0.8 μm wide and 1.0–1.5 μm long spores were produced by this isolate as observed by OLYMPUS (CX-31) model digital camera with microscope (10X40). Ege-B-1.19 isolate was identified as *B. subtilis* by the BBL™ Crystal™ Identification System which was further confirmed by 16S rRNA gene sequence. Sequence was submitted to GenBank and accession number (EU099356) was obtained. Phylogenetic tree was constructed with MEGA 5.0 by using the Neighbor-joining method with 1000 bootstrap replications (Figure 1).

Table 1: Characteristics of vineyard soil samples.

Analysis	Results
Moisture (%)	7.5
pH	7.88
Salt (%)	0.03
Carbonate (%)	2.845
Texture	Silty-loamy
Clay (%)	20
Sand (%)	20
Nitrogen (%)	0.091
Phosphorus (ppm)	0.9
Potassium (ppm)	280

Lytic enzymatic activities

Activities of four different lytic enzymes including chitinase, chitosanase, N-acetyl- β -hexosaminidase and protease activities were measured from the culture supernatant of *B. subtilis* Ege-B-1.19. Chitinase, chitosanase, N-acetyl- β -hexosaminidase and protease activities were found as 2.7 U mL⁻¹, 7.2 U mL⁻¹, 6.2 U mL⁻¹ and 12.2 U mL⁻¹, respectively.

Figure 1: Phylogenetic tree based on 16S rRNA sequences showing the positions of strain *Bacillus subtilis* Ege-B-1.19, and representatives of *Bacillus* species.

The scale bar represents 0.01 substitutions per nucleotide position.

Detection of antifungal activity

Culture supernatant of *B. subtilis* Ege-B-1.19 showed a very high inhibitory effect (++++) against *F. solani* KCTC 6328, *C. gloeosporioides* KACC 40689 and *A. ochraceus* EGE-K-217, high inhibitory effect (+++) against *A. niger* EGE-K-213 and *A. foetidus* EGE-K-211, while a moderate inhibitory effect (++) was observed against *R. solani* KACC 40111 by in vitro antifungal activity assay (Table 2, Figure 2).

Chitosan hydrolysis and analysis of chitosan oligomers

Crude chitosanase was used to hydrolyse soluble chitosan for the synthesis of COS. TLC results showed that the chitosanase had released chitosan oligosaccharides from chitosan, the hydrolysate was primarily containing GlcN, small amount of dimers (GlcN)₂ and trimers (GlcN)₃. The results suggested that chitosanase from *B. subtilis* Ege-B-1.19 showed exo- and endo-type cleavage. These results

were also confirmed by the HPLC analysis results, and quantitative analysis was done using a standard curve (Figure 3). Quantitative analysis showed that chitosan hydrolysate was containing 50.6 mg of GlcN, 5.2 mg of (GlcN)₂ and 8.2 mg of (GlcN)₃.

Partial purification of chitosanase from *B. subtilis* Ege-B-1.19

An extracellular chitosanase was partial purified from the culture supernatant (1500 mL) of *B. subtilis* Ege-B-1.19 by Sephadex G-150. As shown in Figure 4, two chitosanase peaks of F1 (major fraction) and F2 (minor fraction) were separated. Specific chitosanase activity of F1 fraction was 4.7 U mg⁻¹ and of F2 fraction was 33.7 U mg⁻¹. The purification steps were summarized in Table 3. There was 0.3 fold increase in specific activity of the F1 fraction of chitosanase as compared to the fermented broth, while purification fold of F2 fractions was 2.2. The protein obtained from F1 and F2 fractions were subjected to SDS-PAGE and chitosanase enzyme activity was detected by Calcofluor white M2R staining. More than 10 bands of different molecular weights were observed in F1 fraction, while no chitosanase enzyme activity was detected. On the other hand, only one sharp band of chitosanase was observed in F2 fraction (44 kDa) and chitosanase activity was also detected (Figure 5).

Table 2: Antifungal activity of *Bacillus subtilis* Ege-B-1.19 against different fungi.

Test fungi	Antifungal spectrum of <i>B. subtilis</i> Ege-B-1.19
<i>Fusarium solani</i> KCTC 6328 ^a	++++
<i>Colletotrichum gloeosporioides</i> ^a KACC 40689	++++
<i>Rhizoctonia solani</i> KACC 40111 ^a	++
<i>Aspergillus niger</i> EGE-K-213 ^b	+++
<i>Aspergillus foetidus</i> EGE-K-211 ^b	+++
<i>Aspergillus ochraceus</i> EGE-K-217 ^b	++++

Inhibition strength: +, ++, +++, ++++; very high, high, moderate.

^aPhytopathogenic fungi. ^bMycotoxigenic fungi.

Discussion

Vineyard soil sample from Manisa province (38° 30'N 27° 42'E). Manisa province has mainly 20% clay, 20% sand, 20% sandy-clay, 14% loamy-clay, 9% clay type of soil with a very high organic matter content. Soil texture,

Figure 2: Antifungal activity of *B. subtilis* Ege-B-1.19 against *Fusarium solani* KCTC 6328. (A) Sterilized water (control) (50 µL). (B) Filtered fermentation broth of *B. subtilis* Ege-B-1.19 (50 µL).

Figure 3: HPLC chromatograms of the chitooligosaccharide standards (A) and chitooligosaccharides produced by enzymatic hydrolysis of chitosan (B).

Figure 4: Sephadex G-150 column chromatography.

mineralogy, nutrient contents, pH, and organic matter is proposed as the major driver affecting the bacterial community composition [24]. *Bacillus* species are the most studied antagonist among the soil borne bacteria [4].

Figure 5: SDS-PAGE and activity staining of chitosanase. S, molecular weights marker (kDa); 1, crude enzyme; F1, the first highest fraction in purification step; F2, the second highest fraction in purification step; F2, activity staining of purified chitosanase enzyme (44 kDa). Fifty microgram sample of protein was loaded in each lane.

Since *Bacillus* species have the ability to produce spores, therefore, it is advantageous to use them for biocontrol as compared to Gram-negative and non-spore forming bacteria. Endospores are particularly suitable to be used as bio-control agents, because spores can stay alive for a longer period of time in highly variable environmental conditions (nutrient levels, temperature and water activity). Also many *Bacillus* spp. are known to produce antifungal antibiotics and extracellular hydrolytic enzymes.

In the present study, *B. subtilis* Ege-B-1.19 which was able to secrete several hydrolytic enzymes, including chitinase, chitosanase, N-acetyl- β -hexosaminidase and protease, suppressed the growth of *A. niger* EGE-K-213, *A. foetidus* EGE-K-211, *A. ochraceus* EGE-K-217, *F. solani* KCTC

Table 3: Purification of chitosanase from *B. subtilis* Ege-B-1.19.

Purification step	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Purification folds
Crude enzyme	1500	3607	233	15.5	100	1
AS	140	1085	124	8.75	30	0.57
PEG	10	912	118	7.7	25	0.5
Sephadex G-150						
F1	35	260	55	4.7	7	0.3
F2	40	143	4	33.7	3	2.2

An extracellular chitosanase was purified from the culture supernatant (1500 mL) of *B. subtilis* Ege-B-1.19 by Sephadex G-150. The total enzyme activity is calculated per purification step. Specific enzyme activity is calculated per mg of protein, determined with Bradford. AS, ammonium sulphate precipitation; PEG, polyethylene glycol (PEG) concentration; F1, major fraction in purification step; F2, minor fraction in purification step.

6328, *R. solani* KACC 40111 and *C. gloeosporioides* KACC 40689. Previously, inhibitory effects of *B. subtilis* against pathogenic fungi have been demonstrated for *A. niger* [25], *Fusarium verticillioides* [26], *Aspergillus flavus* [27], *Penicillium digitatum* [28] and *Penicillium digitatum* Sacc [29]. These findings suggest the effectiveness of *B. subtilis* as biocontrol agent against pathogenic fungi and our present study has also demonstrated the inhibitory effect of *B. subtilis* against various phytopathogenic and mycotoxigenic fungi.

Chitosanases are hydrolase class enzymes produced by many microorganisms including bacteria [20, 30, 31] and fungi [32, 33]. Chitosanase enzyme of *B. subtilis* Ege-B-1.19 partially purified in this study has a molecular weight of 44 kDa, which is similar to *Bacillus* sp. 739 (46 kDa) [34], *Bacillus* sp. P16 (45 kDa) [20], *B. cereus* S1 (45 kDa) [35] and *Bacillus* sp. strain KFB-C108 (48 kDa) [36].

COS, being recognized as low molecular weight and water soluble chitosans, have much greater demand in many fields than that of precursor molecule. COS possess a wide range of biological activities and have numerous promising applications in multiple fields such as medicine, cosmetics, food and agriculture [12]. Chitosanase have the ability to split chitosan into oligosaccharides ranging from dimer to octamers. Many chitosanases from *Bacillus* strains have shown endo-type cleavage [37–39], whereas some chitosanases from fungi such as *Aspergillus fumigatus* KH-94, *Aspergillus oryzae* IAM2660 and some bacteria such as *Nocardia orientalis* IFO 12806, *Enterobacter* sp. strain G-1 have exo-splitting activity that hydrolyse chitosan to glucosamine [37, 40]. In the present study, the catalytic pattern of *B. subtilis* Ege-B-1.19 chitosanase was examined by using soluble chitosan. TLC analysis showed that the chitosanase had released chitosan oligosaccharides, mainly GlcN, GlcN₂, and GlcN₃ from soluble chitosan (Figure 3). Although primary products from chitosan by plenty of chitosanases were di-, tri- and tetra-chitooligosaccharides, *B. subtilis* Ege-B-1.19 chitosanase hydrolysed chitosan to di- and tri-chitooligosaccharides along with the production of high amount of glucosamine. These results suggested that the mode of action of chitosanase produced by *B. subtilis* Ege-B-1.19 is both exo- and endo type.

Bacillus subtilis Ege-B-1.19 could be a potential biocontrol agent biocontrol agent for vineyard uses. More investigations are necessary to increase the effectiveness of the bacterium before applying the bacterium to the vineyards. The chitosanase of *B. subtilis* Ege-B-1.19 hydrolysed chitosan into GlcN as well as oligomer, therefore, this chitosanase has shown both exo- and endo type hydrolysis and could be a preferable candidate for many biotechnological application.

Acknowledgements: This work was supported by the Research Project fund of TUBITAK (104O339).

Conflict of interest: The authors declare that they have no conflict of interest.

References

- Lin Y, Du D, Si C, Zhao Q, Li Z, Li P. Potential biocontrol *Bacillus* sp. strains isolated by an improved method from vinegar waste compost exhibit antibiosis against fungal pathogens and promote growth of cucumbers. *Biol Control* 2014;71:7–15.
- Spadaro D, Droby S. Development of biocontrol products for postharvest diseases of fruit: the importance of elucidating the mechanisms of action of yeast antagonists. *Trends Food Sci Technol* 2016;47:39–49.
- Ciancio A, Pieterse CM, Mercado-Blanco J. Harnessing useful rhizosphere microorganisms for pathogen and pest biocontrol. *Front Microbiol* 2016;7:1620.
- Zhang X, Zhou Y, Li Y, Fu X, Wang Q. Screening and characterization of endophytic *Bacillus* for biocontrol of grapevine downy mildew. *Crop Protect* 2017;96:173–9.
- Joshi S, Bharucha C, Desai AJ. Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B. *Bioresour Technol* 2008;99:4603–8.
- Yokota K, Yatsuda M, Miwa E, Higuchi K. Comparative study on sample preparation methods for the HPLC quantification of iturin from culture supernatant of an antagonistic *Bacillus* strain. *J. ISSAAS* 2012;18:70–5.
- Kathiravan MN, Karthick R, Muthukumar K. Ex situ bioremediation of Cr (VI) contaminated soil by *Bacillus* sp.: batch and continuous studies. *Chem Eng J* 2011;169:107–5.
- Chang W-T, Chen Y-C, Jao C-L. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. *Bioresour Technol* 2007;98:1224–30.
- Velho R, Medina L, Segalin J, Brandelli A. Production of lipopeptides among *Bacillus* strains showing growth inhibition of phytopathogenic fungi. *Folia Microbiol (Praha)* 2011;56:297.
- Helistö P, Aktuganov G, Galimzianova N, Melentjev A, Korpela T. Lytic enzyme complex of an antagonistic *Bacillus* sp. Xb: isolation and purification of components. *J Chromatogr B: Biomed Sci Appl* 2001;758:197–205.
- Gao X-A, Ju W-T, Jung W-J, Park R-D. Purification and characterization of chitosanase from *Bacillus cereus* D-11. *Carbohydr Polym* 2008;72:513–20.
- Liaqat F, Eltem R. Chitooligosaccharides and their biological activities: a comprehensive review. *Carbohydr Polym* 2017;184:243–59.
- Kim S, Je J. Continuous production of chitooligosaccharides by enzymatic hydrolysis. Chitin, chitosan, oligosaccharides and their derivatives: biological activities and applications. Boca Raton: CRC Press, 2010:47–51.
- Kim S-K, Rajapakse N. Enzymatic production and biological activities of chitosan oligosaccharides (COS): a review. *Carbohydr Polym* 2005;62:357–68.
- Lin Y-W, Hsiao Y-C, Chiang B-H. Production of high degree polymerized chitooligosaccharides in a membrane reactor

- using purified chitosanase from *Bacillus cereus*. Food Res Int 2009;42:1355–61.
16. Waghmare SR, Ghosh JS. Chitobiose production by using a novel thermostable chitinase from *Bacillus licheniformis* strain JS isolated from a mushroom bed. Carbohydr Res 2010;345:2630–5.
 17. Travers RS, Martin PA, Reichelderfer CF. Selective process for efficient isolation of soil *Bacillus* spp. Appl Environ Microbiol 1987;53:1263–6.
 18. Nicholson W, Setlow P. Molecular biological methods for *Bacillus*. West Sussex: John Wiley & Sons, LTD, 1990:391–451.
 19. Imoto T, Yagishita K. A simple activity measurement of lysozyme. Agric Biol Chem 1971;35:1154–6.
 20. Jo Y-Y, Jo K-J, Jin Y-L, Jung W-J, Kuk J-H, Kim K-Y, et al. Characterization of endochitosanases-producing *Bacillus cereus* P16. J Microbiol Biotechnol 2003;13:960–8.
 21. Shibata H, Yagi T. Rate assay of N-acetyl- β -D-hexosaminidase with 4-nitrophenyl N-acetyl- β -D-glucosaminide as an artificial substrate. Clin Chim Acta 1996;251:53–64.
 22. Chun D-S, Kang D-K, Kim H-K. Isolation and enzyme production of a neutral protease-producing strain, *Bacillus* sp. DS-1. Korean J Microbiol Biotechnol 2002;30:346–51.
 23. Jung W-J, Kuk J-H, Kim K-Y, Jung K-C, Park R-D. Purification and characterization of exo- β -D-glucosaminidase from *Aspergillus fumigatus* S-26. Protein Expr Purif 2006;45:125–31.
 24. Sun D, Meng J, Liang H, Yang E, Huang Y, Chen W, et al. Effect of volatile organic compounds absorbed to fresh biochar on survival of *Bacillus mucilaginosus* and structure of soil microbial communities. J Soils Sed 2015;15:271–81.
 25. Podile A, Prakash A. Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1. Can J Microbiol 1996;42:533–8.
 26. Cavaglieri L, Orlando J, Rodriguez M, Chulze S, Etcheverry M. Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro and at the maize root level. Res Microbiol 2005;156:748–54.
 27. Zhang T, Shi Z-Q, Hu L-B, Cheng L-G, Wang F. Antifungal compounds from *Bacillus subtilis* B-FS06 inhibiting the growth of *Aspergillus flavus*. World J Microbiol Biotechnol 2008;24:783.
 28. Leelasuphakul W, Hemmanee P, Chuenchitt S. Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. Postharvest Biol Technol 2008;48:113–21.
 29. Waewthongrak W, Pisuchpen S, Leelasuphakul W. Effect of *Bacillus subtilis* and chitosan applications on green mold (*Penicillium digitatum* Sacc.) decay in citrus fruit. Postharvest Biol Technol 2015;99:44–9.
 30. Kang L-X, Chen X-M, Fu L, Ma L-X. Recombinant expression of chitosanase from *Bacillus subtilis* HD145 in *Pichia pastoris*. Carbohydr Res 2012;352:37–43.
 31. Wang S-L, Liang T-W, Yen Y-H. Bioconversion of chitin-containing wastes for the production of enzymes and bioactive materials. Carbohydr Polym 2011;84:732–42.
 32. Cheng CY, Li YK. An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. Biotechnol Appl Biochem 2000;32:197–203.
 33. Zhang X-Y, Dai A-L, Zhang X-K, Kuroiwa K, Kodaira R, Shimosaka M, et al. Purification and characterization of chitosanase and exo- β -D-glucosaminidase from a koji mold, *Aspergillus oryzae* IAM2660. Biosci Biotechnol Biochem 2000;64:1896–902.
 34. Aktuganov G, Shirokov A, Melent'ev A. Isolation and characterization of chitosanase from the strain *Bacillus* sp. 739. Appl Biochem Microbiol 2003;39:469–74.
 35. Kurakake M, Yo-u S, Nakagawa K, Sugihara M, Komaki T. Properties of chitosanase from *Bacillus cereus* S1. Curr Microbiol 2000;40:6–9.
 36. Yoon HG, Ha SC, Lim YH, Cho HY. New thermostable Chitosanase from *Bacillus* sp. J Microbiol Biotechnol 1998;8:449–54.
 37. Choi YJ, Kim EJ, Piao Z, Yun YC, Shin YC. Purification and characterization of chitosanase from *Bacillus* sp. strain KCTC 0377BP and its application for the production of chitosan oligosaccharides. Appl Environ Microbiol 2004;70:4522–31.
 38. Omumasaba CA, Yoshida N, Sekiguchi Y, Kariya K, Ogawa K. Purification and some properties of a novel chitosanase from *Bacillus subtilis* KH1. J Gen Appl Microbiol 2000; 46:19–27.
 39. Lee Y-S, Yoo J-S, Chung S-Y, Lee Y-C, Cho Y-S, Choi Y-L. Cloning, purification, and characterization of chitosanase from *Bacillus* sp. DAU101. Appl Microbiol Biotechnol 2006;73:113.
 40. Kim S-Y, Shon D-H, Lee K-H. Purification and characteristics of two types of chitosanases from *Aspergillus fumigatus* KH-94. J Microbiol Biotechnol 1998;8:568–74.