RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2017.43.294

*ScienceAsia* 43 (2017): **294–301**

# Purification and characterization of an antifungal protein from *Bacillus subtilis* XL62 isolated in Vietnam

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*Received 8 May 2017*
*Accepted 6 Oct 2017*

**ABSTRACT**: *Bacillus subtilis* is considered as a potential antagonist for fungal control due to its ability to produce a wide range of antibiotics. This study determines the antifungal activity of the native *B. subtilis* XL62 and we purify, identify, and characterize the antifungal protein. The results show that the growth inhibitory activity to *Fusarium oxysporum* and *Rhizoctonia solani* of the crude supernatant of *B. subtilis* XL62 was proportional to the concentration. At the highest concentration (50%), the growth inhibition reached 90% and 100% for *F. oxysporum*, and *R. solani*, respectively. Furthermore, the crude supernatant of *B. subtilis* XL62 also inhibited the germination and growth of *R. solani*. The growth of sclerotia was almost completely inhibited at the concentration of 20% and the ability to germinate was lost at the concentration of 50%. Antifungal proteins were isolated from the crude bacterial supernatant using ammonium sulphate precipitation followed by passage over DEAE-cellulose and Biogel P100 columns. The purified protein had an apparent molecular mass of 22 kDa. Its antifungal activity was retained even at 100 °C, for 60 min and after treatment with proteinase K (0.5–2.5 μg/ml). The results of protein identification using a MALDI-TOF/TOF mass spectrometer suggest that the purified protein is indeed a chitin-binding protein.

**KEYWORDS**: *Fusarium oxysporum*, *Rhizoctonia solani*

## INTRODUCTION

Plant pathogenic fungi are among the most important biotic agent causing serious losses and damages to agriculture products. As a result, fungal diseases have major economic impacts in the agriculture industry. Chemical fungicides are so far the most popular choice for plant diseases. However, despite the effectiveness of the conventional chemical fungicides, concerns about the environmental contamination and human health risks associated with fungicide residues have been raised owing to their extremely high toxicity. Protection of crops against their fungal enemies using bacterial antagonists is, therefore, recently became an attractive alternative approach to facilitate sustainable agriculture in many countries. Three well-known and widely used bacterial biocontrol agents are *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. Among these, *Bacillus* spp. appears to be the most potential candidates. *Bacillus* spp. offer several advantages over other organisms since they can form endospores and can tolerate extreme pH, temperature, and osmotic conditions[1]. Furthermore, they produce a diverse array of antifungal compounds, which range from small peptides, such as iturin[2,3], surfactin[4,5], fengymycin, bacilysin[6], bacillomycin[7], mycosubtilin[8], and mycobacillin[9,10], to volatile metabolites[11,12].

To find out novel agro-ecological crop protection weapons, one hundred and forty-seven *Bacillus* strains isolated from trunks, leaves, roots, and soil from different provinces in Vietnam were screened for antifungal activity. Using the method of Weller[13], *B. subtilis* XL62 was selected for further study. This bacterial strain was identified based on its morphological, biochemical, and physiological characteristics, and by 16S rDNA analysis (GenBank by code No. FJ465166).

The present study was aimed at determining the antifungal activity of the crude supernatant from *B. subtilis* XL62 against *R. solani* and *F. oxysporum*, two soil phytopathogenic fungi that are widespread in Vietnam. The antifungal protein was purified and characterized with respect to thermal stability and proteolytic resistance to proteinase K.

## MATERIALS AND METHODS

### Microorganisms

The *F. oxysporum* and *R. solani* strains were provided by the Plant Diseases Division at the Institute of Plant Protection, Tu Liem, Hanoi. *B. subtilis* XL62 was supplied by the Department of Enzyme Biotechnology at the Institute of Biotechnology, Vietnam Academy of Science and Technology. *B. subtilis* XL62 was grown in NYD medium (pH 7.5), which comprised glucose (10 g/l), beef extract (8 g/l), and yeast extract (5 g/l).

### Chemicals

NaCl and D-glucose were purchased from Merck (Darmstadt, Germany). Minisart membranes and agar were purchased from Biotech (Vietnam). DEAE-cellulose, Biogel P100, Trisbase and Bovine serum albumin (BSA) were supplied Sigma-Aldrich (St. Louis, MO, USA). $NaHPO_4$, $Na_2HPO_4$, meat extract, and yeast extract were purchased from BioBasic Inc. (Ontario, Canada).

### Antifungal activity of the crude bacterial supernatant determination

The crude supernatant from *B. subtilis* XL62 was obtained by centrifuging the bacterial culture at 11 000*g* for 10 min. The antifungal activity against *F. oxysporum* and *R. solani* of this crude supernatant was determined using the method of Huber[14]. Different amounts of the crude supernatant were added to potato dextrose agar (PDA) at 55 °C, mixed well, and poured into Petri dishes (90 × 15 mm). The final concentration of the crude supernatant was 5%, 10%, 20%, or 50%. Finally, an *R. solani* sclerotia or a mycelial plug (0.5 cm diameter) from 4-day-old cultures of *F. oxysporum* or *R. solani* was placed in the centre of the PDA plates and incubated at 30 °C for 5 days. The PDA plates without crude bacterial supernatant were used as negative controls. The growth of *F. oxysporum* and *R. solani* was determined by measuring colony diameter (in mm).

The ability of the crude supernatants to inhibit fungal growth was calculated as $I = (S_{\text{control}} - S_{\text{samples}})/S_{\text{control}}$, where $S_{\text{control}}$ is the area of the non-supernatant-containing PDA plates occupied by colonies and $S_{\text{samples}}$ is the area of the supernatant-containing PDA plates occupied by colonies. All the tests were conducted in triplicate.

### Antifungal protein purification

The bacterial culture was centrifuged at 11 000*g* for 10 min. Ammonium sulphate (30–70% saturation) was then slowly added to 200 ml of crude protein solution with constant stirring. The mixture was left overnight and then centrifuged at 12 500 rpm for 10 min. The supernatant was then carefully decanted, and the precipitate was re-dissolved in 0.02 M phosphate buffer (pH 6.8) and dialysed to remove the salt. The dialysed fraction (6 ml) was then applied to a DEAE-cellulose column (2.6 × 6 cm) pre-equilibrated with 0.02 M phosphate buffer (pH 6.8) at a flow rate of 25 ml/h until the OD 280 nm was $< 0.01$. The column was then eluted with 0.02 M phosphate buffer (pH 6.8) containing 1 M NaCl. The eluted fractions of 1.5 ml were collected (27 fractions). The antifungal activity of each purified fraction was tested against *F. oxysporum* and *R. solani*. Fractions with strong antifungal activity were continuously purified on a Biogel P100 column. The column was then eluted with 0.02 M phosphate buffer (pH 6.8) at a flow rate of 25 ml/h and fractions of 1.5 ml were collected. The antifungal activity of the purified fractions was once again tested against the two above pathogenic fungi. All purification steps were carried out at 4 °C unless specified otherwise.

### Gel electrophoresis and protein concentration measurement

The purified antifungal protein isolated from *B. subtilis* XL62 was examined in 12% (w/v) SDS polyacrylamide gels[15] using Biometra equipment (Göttingen, Germany). Separated proteins were stained with 0.1% (w/v) $AgNO_3$ [16]. Protein concentrations were determined using the Bradford method, with BSA as the standard[17].

### Determination of antifungal activity of purified protein

The antifungal activity of the purified protein was determined using the agar-disk diffusion method. At first, a mycelial plug from 4-day-old cultures of *F. oxysporum* or *R. solani* was placed in the centre of each 0.1% ampicillin containing PDA plate. Next, a sterile filter disk with a diameter of 0.5 cm (Whatman paper No. 3) soaked with 15 µl of purified protein was placed on the surface of the plates. Negative control disks were soaked with 15 µl of 20 mM $Na_3PO_4$ buffer (pH 6.8). These agar plates were then incubated at 30 °C for 3–5 days (Incubator, Sanyo, Japan) and the diameters of inhibition zones around the disks were measured. All the tests were conducted in triplicate.

To test for purification and characterization of the antifungal protein, ampicillin (0.1%) was added

to Petri plates (90 × 15 mm) containing 10 ml of PDA, and filter papers (0.5 cm in diameter) presoaked with 15 µl of the purified protein were placed on the surface. The agar plates were then incubated at 30 °C for 3–5 days (Incubator, Sanyo, Japan). Filter papers soaked in 0.02 M phosphate buffer (pH 6.8) were used as the control.

### Thermal stability and proteolytic resistance to proteinase K of antifungal protein

The thermal stability of the antifungal protein was determined by incubating the purified protein (20 µl) at 80 °C for 5–60 min or at 100 °C for 60 min. Antifungal activity was then determined as described above. The proteolytic resistance to proteinase K of the antifungal protein was determined by incubating the purified protein (20 µl) with 0.5–2.5 µg/µl proteinase K at 37 °C for 60 min prior to the measurement of the antifungal activity.

### Protein identification

The purified antifungal protein was characterized using MALDI-TOF MS. The predicted protein was trypsin digested and peptides extracted according to standard techniques[18]. Peptides were analysed by MALDI-TOF/TOF mass spectrometer using a 5800 Proteomics Analyser (AB Sciex). Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with MSPnr100 Database. Peptide fragments showing ion scores $> 59$ were identified uniquely or high similarly ($p < 0.01$).

### Statistical analysis

All measurements were carried out in triplicate. The means were presented as averages of the experiments.

## RESULTS

### Antifungal activity of the crude supernatant from *B. subtilis* XL62

Five different concentrations of crude supernatant (0%, 5%, 10%, 20%, and 50%) of *B. subtilis* XL62 were tested for their antifungal activity against *F. oxysporum* and *R. solani*. using the method of Huber as described above.

The results (Table 1, Table 2) show that the crude supernatant of *B. subtilis* XL62 has a significant antifungal activity on both fungal pathogens. At the concentration of 5% of crude supernatant of *B. subtilis* XL62, the growth inhibition reached about 62% and 22% for *F. oxysporum* and *R. solani*, respectively. The antifungal activity of the crude supernatant of *B. subtilis* XL62 was proportional to the concentration. At a concentration of 5%, the crude supernatant inhibited the growth of *R. solani* by only about 20%, but at a concentration of 10% this inhibitory activity increased up to 65% and reach 94% at a concentration of 20%. The increase in inhibitory activity against *R. solani* occurred over a narrow range of concentrations, but over a little wider range for *F. oxysporum*. At the highest concentration tested (50%), the growth inhibition reached 100% for *R. solani* but only about 89% for *F. oxysporum*. A similar effect was noted when the effect of the crude supernatant on the germination and growth of *R. solani* sclerotia was examined (Table 3).

**Table 1** Effect of a crude supernatant (c.s.) from *B. subtilis* XL62 on the growth of *F. oxysporum*.

| Conc. of c.s. (%) | Fung. growth* (⌀: cm) | Inhibitory activity (%) | Colony morphology |
|---|---|---|---|
| 0 | 7.8 ± 0.3 | 0.0 | CY†, light purple |
| 5 | 4.8 ± 0.1 | 62.1 | CY†, more purple |
| 10 | 4.5 ± 0.1 | 66.7 | CY†, more purple |
| 20 | 3.8 ± 0.2 | 76.3 | CY†, dark purple |
| 50 | 2.5 ± 0.1 | 89.7 | CY†, dark purple |

* After 120 h; †cotton yarn.

**Table 2** Effect of a crude supernatant (c.s.) from *B. subtilis* XL62 on the growth of *R. solani* mycelia.

| Conc. of c.s. (%) | Fung. growth* (⌀: cm) | Inhibitory activity (%) | Colony morphology |
|---|---|---|---|
| 0 | 8.5 ± 0.2 | 0.0 | CY†, white |
| 5 | 7.5 ± 0.2 | 22.2 | CY†, white |
| 10 | 5.0 ± 0.3 | 65.4 | CY†, white |
| 20 | 2.0 ± 0.1 | 94.5 | CY†, white |
| 50 | ⩽ 0.50 ± 0.01 | 100.0 | Withered |

*After 72 h; †cotton yarn.

**Table 3** Effect of a crude supernatant (c.s.) from *B. subtilis* XL62 on the growth of *R. solani* sclerotia.

| Conc. of c.s. (%) | Fung. growth* (⌀: cm) | % versus antag. vol.‡ | Mycelia morphology |
|---|---|---|---|
| 0 | 8.5 ± 0.2 | 0.0 | CY†, white |
| 5 | 5.1 ± 0.2 | 64.0 | CY†, white |
| 10 | 2.0 ± 0.1 | 94.5 | CY†, white |
| 20 | 1.0 ± 0.1 | 98.6 | CY†, white |
| 50 | ⩽ 0.10 ± 0.01 | 100.0 | Withered |

* After 72 h; ‡antagonist volume; †cotton yarn.

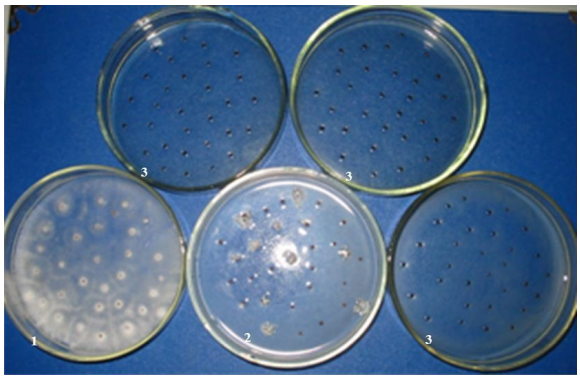


**Fig. 1** Testing of the long-term effect of the supernatant on the germination of *R. solani* sclerotia (1–3: sample *R. solani* cultured on PDA medium containing 5%, 10% and 50% crude supernatant and re-cultured the mycelia on PDA plates without crude supernatant.

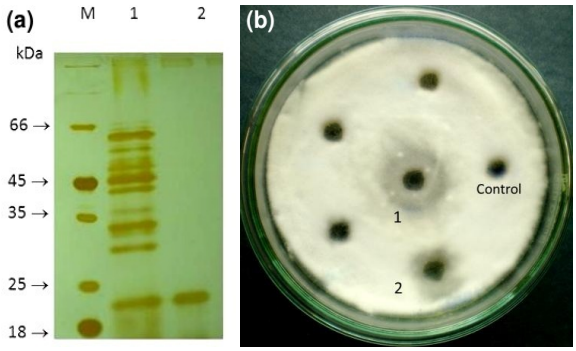


**Fig. 2** (a) Protein profile on SDS-PAGE of the crude supernatant (Lane 1), fractions from peak 1 after passage through the Biogel P100 column (Lane 2), M: molecular mass of standard proteins (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA); (b) anti-*F. oxysporum* activity of the purified protein (control: 20 mM $Na_3PO_4$ buffer, pH 6.8; 1: the crude supernatant; 2: the purified protein from peak 1).

When grown on PDA medium containing 5%, 10%, or 20% crude supernatant, *R. solani* sclerotia were still able to germinate (although growth was strongly inhibited). The growth of sclerotia was inhibited by more than 60% at a supernatant concentration of 5% and by over 90% at a concentration of 10%. Growth of sclerotia was almost completely inhibited at a concentration of 20%. These results suggest that the inhibitory effect of the crude supernatant against *R. solani* may depend on the age of the mycelium. In particular, this fungal strain lost the ability to germinate at a supernatant concentration of 50%.

To test the long-term effect of the supernatant, we took *R. solani* cultured on PDA medium containing 5%, 10% and 50% crude supernatant and re-cultured the mycelia on PDA plates without crude supernatant. The results showed that *R. solani* was unable to restore growth. Hence the crude supernatant of *B. subtilis* XL62 at a concentration of 50% was able to prevent the growth of *R. solani* fibres (Fig. 1).

The crude supernatant also showed different effects on colony morphology. On PDA plates, the amount of synthetic pigment produced by *F. oxysporum* increased in line with the increase in supernatant concentration (Table 1). However, the inhibitory effect on mycelia of *R. solani* was only observed at a concentration of 50%. At this concentration, the normal mycelium morphology of *R. solani* was lost.

## Purification and characterization of antifungal protein

After 120 h of cultivation, the crude supernatant of *B. subtilis* XL62 was purified by addition of 30 −70% ammonium sulphate, followed by passage over a DEAE-cellulose column. Three range of fractions containing high protein concentration were observed (corresponding to peaks 1, 2, and 3). These fractions were tested for antifungal activity against *F. oxysporum* and all were positive (data not shown). Fractions 5–11 from peak 1 were then mixed and passed over a Biogel P100 column. The purity and relative molecular mass of the proteins were assessed by 12% polyacrylamide gel electrophoresis with Fermentas protein standards, followed by silver staining. A protein of ∼22 kDa was observed (Fig. 2a) and the yield of the purification process was 12% (Table 4), and exhibited antifungal activity for *F. oxysporum* (Fig. 2b, Fig. 3).

The purified protein was then characterized by MALDI-TOF mass spectrometry. Peptide fragments showing ion scores above 59 identified uniquely

**Table 4** The yield of antifungal protein purification from *B. subtilis* XL62.

| Purification steps | Total protein (mg) | Yield (%) |
|---|---|---|
| Precipitated protein | 3.52 | 100 |
| DEAE-cellulose | 1.44 | 40.9 |
| Biogel P100 | 0.42 | 11.9 |

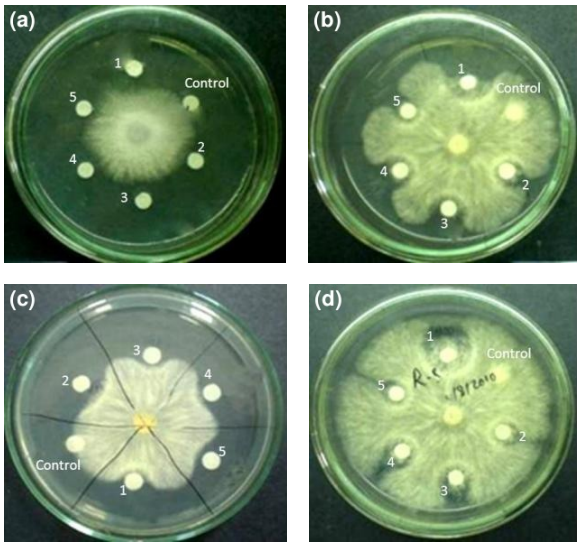


**Fig. 3** Antifungal activity against *F. oxysporum* and *R. solani* of the purified protein. The growth inhibition of (b,d) *R. solani* and (a,c) *F. oxysporum* by the purified protein after (a,b) 3 days or (c,d) 5 days of exposure. Control: 15 µl of 20 mM $Na_3PO_4$ buffer, pH 6.8; 1: crude supernatant; 2–5: fractions after passage through the Biogel P 100 column.

**Table 5** Peptides exceeding the identity threshold.

| Q.[a] | Obs.[b] | P.M.[c] | S.[d] | Peptide sequence |
|---|---|---|---|---|
| 151 | 450.234 | 898.451 | 62 | ADTNLTHK |
| 223 | 521.268 | 1040.504 | 67 | GFPAAGPPDGR |
| 312 | 587.820 | 1173.614 | 64 | QTLGWTAAAQK |
| 491 | 795.401 | 1588.773 | 97 | YGSVIDNPQSVEGPK |
| 492 | 795.401 | 1588.773 | 99 | YGSVIDNPQSVEGPK |
| 522 | 825.416 | 1648.806 | 146 | IASANGGSGQIDFGLDK |
| 527 | 830.900 | 1659.774 | 106 | DEFELIGTVNHDGSK |
| 669 | 881.102 | 2640.282 | 141 | IASANGGSGQIDF GLDKQTADYWVK |

[a] Query; [b]observed; [c]peptide mass; [d]score.

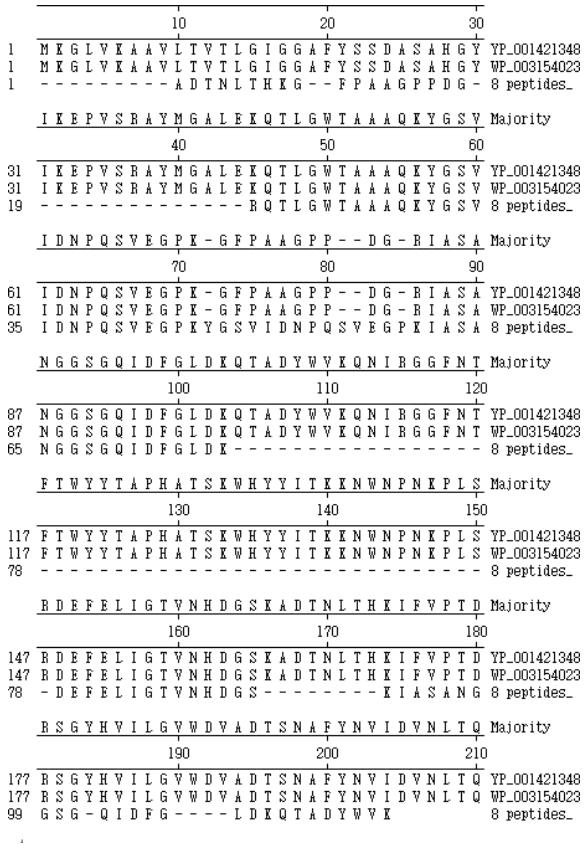


**Fig. 4** Alignment of eight neutral identified peptides (8 peptides) with chitin-binding protein from *Bacillus amyloliquefaciens* FZB42 (YP_001 421 348) and chitin-binding protein from *Bacillus* (WP_003 154 023).

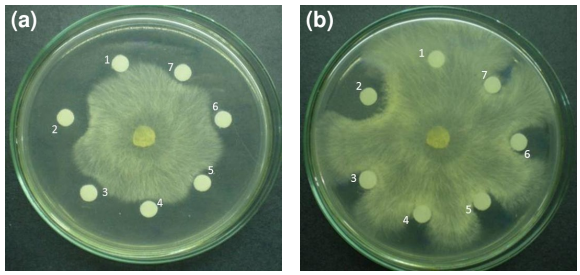


**Fig. 5** Effects of temperature on the antifungal activity of the purified protein against (a) *F. oxysporum* and (b) *R. solani*. 1: control (20 µl of 20 mM $Na_3PO_4$ buffer, pH 6.8); 2–6: samples treated at 80 °C for 5–60 min; 7: sample treated at 100 °C for 60 min.

or high similarly with $p < 0.01$. These peptides were 100% identical to corresponding fragments of the chitin-binding protein (Table 5; Fig. 4). Chitin binding protein from *Bacillus* (Accession number: WP_003154023) has 206 acid amine containing chitin-bind—3 region such as "Chitin binding domain; pfam03067″ from acid amine position 28–202 with a relative molecular mass of 22 230 Da.

### Characterization of purified antifungal protein

The effect of temperature on the purified antifungal protein was studied at 80 °C for 5–60 min and at 100 °C for 60 min. The results show that the antifungal protein retained activity when heated to 80 °C and even to 100 °C for 60 min (Fig. 5a and 5b).

Proteinase K (EC 3.4.21.64) is a broad-spectrum

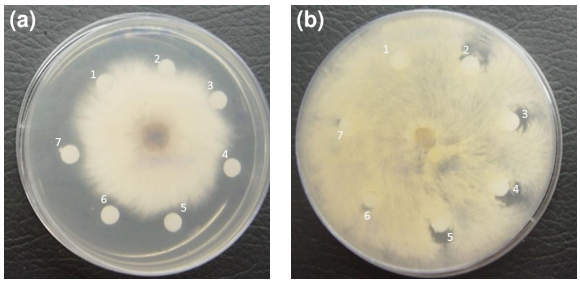


**Fig. 6** Effect of proteinase K on antifungal activity of the purified protein against (a) *F. oxysporum* and (b) *R. solani*. 1: 20 mM $Na_3PO_4$ buffer, pH 6.8; 2–5: samples treated with proteinase K (0.5, 1.0, 2.0, and 2.5 μg, respectively); 6: proteinase K 0.5 μg; 7: sample not treated with proteinase K.

serine protease commonly used in molecular biology to digest proteins. Here, we incubated the purified protein with proteinase K at final concentrations between 0.5 and 2.5 μg/ml at 37 °C for 60 min. The data showed that proteinase K did not inhibit antifungal activity of the purified protein (Fig. 6a and 6b).

## DISCUSSION

Fungal diseases are one of the most popular causes of major losses suffered by agricultural crops today. In fact, one in every eight crop plants, on average, will fail to yield because of fungal disease[19]. Using bacterial antagonist as a biological control agent against plant fungal pathogen has recently become a promising solution for the agriculture of many countries in the world.

From more than one hundred *Bacillus* strains isolated from different samples in Vietnam, *B. subtilis* XL62 was chosen to be examined for its antifungal activity against *F. oxysporum* and *R. solani*, two fungi that infect many important food crops. The results show that the crude supernatant of *B. subtilis* XL62 has a significant antifungal activity on both fungal pathogens. Our results are in agreement with those of Islam et al who reported the antifungal activity of an ethyl acetate extract (2 mg/well) of *B. subtilis* C9, which inhibited the growth of *R. solani* AG2-2(IV), *R. solani* AG1-1(A), *R. solani* AG-4, and *F. oxysporum* mycelia by 88%, 32%, 72%, and 72%, respectively[1]. In addition, Ali et al showed that a culture filtrate from *Bacillus* sp. RMB7 inhibited the growth of nine fungal phytopathogens by > 70% in vitro[20]. *B. subtilis* B29 shows antifungal activity against *F. oxysporum*, *R. solani*, *F. moniliforme*, and *S. sclerotiorum*, although *F. oxysporum* and *R. solani* showed the greatest sensitivity[21].

To find out the element responsible for the antifungal activity of *B. subtilis* XL62, the crude supernatant of *B. subtilis* XL62 was precipitated by addition of 30–70% ammonium sulphate, and then was continuously purified using ion exchange chromatography on DEAE-cellulose and Biogel P100 chromatography. These methods are similar to those used by other studies[22,23]. Indeed, ion exchange chromatography on DEAE-cellulose, followed by Biogel P100 chromatography, has been used to extract other proteins with antifungal activity[24,25]. In our study, the purified protein has activity against both *F. oxysporum* and *R. solani* (Fig. 3a and 3b) and a molecular weight of ∼22 kDa. The molecular weight of this antifungal protein was different from that of other antifungal proteins purified from *B. subtilis*. Liu et al extracted bacisubin from *B. subtilis* B916, which has a molecular weight of 41.9 kDa and exhibits antifungal activity against *F. oxysporum* and *R. solani*[25]. Li et al extracted protein B29I from *B. subtilis* strain B29, which has a molecular weight of 42.9 kDa and shows antifungal activity[21]. Tan et al purified antifungal protein with molecular weight of 38 kDa from *B. subtilis* B25[26]. The difference in antifungal peptides produced by different strains of *B. subtilis* may be due to the fact that biosynthesis of antibiotics from microorganisms is often regulated by nutritional and environmental factors[1]. Using MALDI-TOF mass spectrometry to characterize the purified protein, we found that it is a chitin-binding protein. This result was in agreement with other reports that chitin-binding proteins play an important role in the protection of plants against pathogen infection[27,28]. The mechanisms against fungal attack of chitin-binding proteins was supposed to take part in degrading chitin and/or adhering to the chitinous surfaces of the host cells[29,30].

Because *Bacillus*-derived compounds are the predominant class of antibiotics[31], several studies examined the effects of heat, pH, and proteolytic enzymes (proteinase K and trypsin) on the activity of antifungal compounds produced by *Bacillus* sp. In the present study, the thermal stability and the proteinase K resistance of the purified protein were also examined. The data showed that proteinase K did not inhibit antifungal activity (Fig. 6a and 6b). Our result was in agreement with some previous studies which reported that the antifungal compound was resistant to several proteolytic enzymes, including proteinase E, proteinase K, and chymotrypsin[32]. Balouiri et al[33] showed that the anti-candida activity of bioactive compounds pro-

duced by *Bacillus* spp. was not affected by trypsin or proteinase K. In the presence of proteinase K, the inhibition zone reached $21.3 \pm 0.6$ mm, compared with $23.3 \pm 0.6$ mm for the positive control[33]. The protease resistance ability of antifungal protein may be because they are hydrolysed into smaller peptides, which retain antifungal activity.

The purified protein is also quite thermostable as it retained activity when heated to 80 °C and even to 100 °C for 60 min. This result was in agreement with previous studies such as that of Zhang et al. The purified novel protein, BTL, from *Bacillus* strain BTL2 from tobacco stems was also thermostable, retaining almost 100% activity when heated to 100 °C for 15 min[34]. Chitarra et al reported that an antifungal compound produced by *B. subtilis* YM 10-20 was heat stable. The protein inhibited the growth of *P. roqueforti* after heating to 70 °C or 100 °C for 1 h[32]. The abovementioned result of MALDI-TOF, which indicated that the purified protein TOF was chitin-binding protein, may give us logical explanation for this thermal stability.

In conclusion, this study evaluated that the crude bacterial supernatant from *B. subtilis* XL62 has a significant antifungal activity against *F. oxysporum* and *R. solani*, two harmful pathogenic fungi. In addition, we successfully purified an antifungal protein from this supernatant. These results help to open the door for the *B. subtilis* XL62 to become a potential environmentally friendly agent to control plant fungal diseases.

*Acknowledgements*: This study was supported by the National Foundation for Science and Technology Development Vietnam (Nafosted), project 106.05-2012.35: "Screening, extraction, purification and structure analysis of some novel antagonistic compounds (or derivatives) against *Fusarium* and *Rhizoctonia* from *Bacillus*, *Burkholderia*, *Pseudomonas* and *Serratia* isolates in Vietnam", 2013–2015.

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