ISOLATION AND SELECTION OF CHITINASE-PRODUCING BACTERIA WITH ANTIFUNGAL ACTIVITY AGAINST *FUSARIUM OXYSPORUM* FROM *LILIUM* RHIZOSPHERE SOIL

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Received: 03.07.2018 Published: 25.06.2020

ABSTRACT

Chitinases have many applications in various fields such as environment, agriculture, and medicine. Chitinolytic bacteria, which are found in various natural environments including the rhizosphere, soil, marine ecosystems, lakes, and chitinous waste, can be used for the degradation of chitin-containing wastes as well as in the production of chitinolytic enzymes with fungicidal activity against some fungal phytopathogens. This study consisted of isolating and screening chitinolytic bacteria based on a chitinase activity assay using the diffusion plate and colorimetric methods. Thirty-two chitinase-producing bacterial strains were isolated from *Lilium* rhizosphere soil. There were four strains, namely HP02, VRQ9, HM03, and GL10, which exhibited the highest chitinase activities, with values of 1.47, 1.44, 1.29, and 1.21 U/ml, respectively. These chitinase-producing bacterial strains were tested for antagonistic activity against the causal agent of bulb and stem rots of lilies, *Fusarium oxysporum*, and the results indicated antifungal activity of the four strains HP02, VRQ9, HM03, and GL10. The HP02 strain had the highest level of chitinase activity and was capable of being antagonistic to *Fusarium oxysporum*. Based on morphological and biochemical characteristics, 16S rRNA gene sequencing, and phylogenetic analysis of this sequence along with sequences from GenBank, HP02 was identified as *Bacillus subtilis*.

Keywords: Chitinase-producing bacteria, *Fusarium oxysporum*, rhizosphere.

Phân lập và tuyển chọn các chủng vi khuẩn sinh enzyme chitinase, đối kháng với nấm *Fusarium oxysporum* từ đất vùng rễ cây hoa lily

TÓM TẮT

Enzyme chitinase có tiềm năng ứng dụng trong nhiều lĩnh vực như môi trường, nông nghiệp và y học. Vi khuẩn sinh enzyme chitinase được tìm thấy trong môi trường tự nhiên (đất vùng rễ, nước biển) được ứng dụng để đối kháng với nấm gây bệnh thực vật và sản xuất enzyme chitinase phân huỷ các chất thải chứa chitin. Nghiên cứu đã tiến hành phân lập và tuyển chọn các chủng vi khuẩn có khả năng sinh enzyme chitinase có hoạt tính cao bằng phương pháp khuyếch tán đĩa thạch và phương pháp so màu với thuốc thử DNS (3,5-dinitrosalicylic acid). Từ đất vùng rễ cây hoa lily, 32 chủng vi khuẩn có khả năng sinh enzyme chitinase đã được phân lập. Trong đó, 4 chủng HP02, VRQ9, HM03 và GL10 có hoạt tính chitinase là cao nhất, lần lượt là 1,47; 1,44; 1,29; 1,21 U/ml. Các chủng sinh chitinase được thử đối kháng với nấm *Fusarium oxysporum* gây bệnh thối củ và thân ở cây hoa lily. Kết quả cho thấy rằng 4 chủng HP02, VRQ9, HM03 và GL10 đều có khả năng đối kháng với nấm. Chủng HP02 có hoạt tính chitinase cao nhất và có khả năng đối kháng nấm *Fusarium oxysporum*, được xác định là *Bacillus subtilis* dựa vào đặc điểm hình thái, hoá sinh, so sánh trình tự 16S rRNA với các trình tự trong ngân hàng gen và xây dựng cây phân loại.

Từ khoá: Vi khuẩn sinh chitinase, *Fusarium oxysporum*, vùng rễ.

1. INTRODUCTION

Chitinases are glycosyl hydrolases catalyzing the degradation of chitin, which is a homopolymer of beta-1,4-linked N-acetyl-D glucosamine residues. In nature, chitin is the second most abundant polysaccharide, next to cellulose. Chitinases have many applications

such as a biocontrol agent of fungal diseases of plants in agriculture fields, the degradation of wastes containing chitin in environmental protection, the preparation of pharmaceutically important chitooligosaccharides and N-acetyl D-glucosamine in medicine, and the generation of protoplasts from fungi and yeasts (Neeraja *et al.,* 2010; Hamid *et al*., 2012). These enzymes can be found in a wide range of organisms such as bacteria, fungi, insects, animals, and plants (Hoster *et al.,* 2005). Some of the best known chitinase-producing bacteria belong to the *Aeromonas*, *Serratia, Streptomyces*, and *Bacillus* genera (Jabeen and Qazi, 2014). Chitinase-producing bacteria not only degrade chitin and utilize it as an energy source but also control plant diseases caused by various phytopathogenic fungi such as *Fusarium oxysporum* (the causal agent of bulb rot of lilies) (Lim *et al*., 2003), *Verticillium dahliae* (the causal agent of *Verticillium* wilt of cotton) (Li *et al*., 2013), *Phaeoisariopsis personata* (the causal agent of late leaf spot disease of groundnut) (Kishore *et al*., 2005a), *Aspergillus niger* (the causal agent of collar rot) (Kishore *et al*., 2005b), and *Pyricularia oryzae/Magnaporthe oryzae* (the causal agent of rice blast disease) (Nguyen *et al.,* 2013). Chitinases inhibit fungal growth by hydrolyzing the chitin present in the fungal cell wall (Hoster *et al*., 2005). Therefore, chitinase-producing bacteria have promise as replacements for the application of synthetic pesticides and fungicides (Veliz *et al.,* 2017).

Chitinolytic bacteria can be found in many different environments such as soil, marine ecosystems, lakes, or chitinous waste (Setia & Suharjono, 2015). In soil, bacteria show vast variation quantitatively and qualitatively across different collection sites and at various depths (Mukerji *et al*., 2006). Soil bacteria are the major source of chitinase production and could be used for bioconversion of chitinous waste into useful molecules and for applications in different areas such as medicine, biotechnology, and agriculture (Jabeen & Qazi, 2014). The rhizosphere region is the portion of the soil which is adjacent to the root system of a plant and is influenced by root exudates such as amino acids, vitamins, sugars, and tannins (Mukerji *et al*., 2006). It is the first line of defense for roots against attacks by pathogenic fungi (Chet *et al*., 1990). Therefore, microorganisms growing in the rhizosphere region have the potential to be used as biocontrol agents (Podile and Prakash, 1996). The rhizosphere is populated by different microorganisms including both beneficial and deleterious ones (Chet *et al.,* 1990), and there is a need to learn more about the beneficial rhizosphere microorganisms. Chitinolytic microorganisms, especially bacteria and actinomycetes, are heavily colonized in soil and the rhizosphere. In comparison with chitinolytic bacteria isolated from water and bottom sediments, chitinolytic bacteria isolated from soil have been shown to usually be more active (Brzezinska *et al*., 2014). Therefore, they could be more appropriate for agricultural use.

Lily is one of the most important bulbous plants worldwide for production as cut flowers, for potted plant purposes, and for landscape decoration. The genus *Lilium*, which is a perfect target for exploitation by the ornamental industry, includes more than 80 species with significant differences in flower shapes, colors, sizes, fragrances, and bulb morphologies (Nunez de Caceres Gonzalez *et al*., 2015). The soil-borne fungus *Fusarium oxysporum* is a serious threat to the bulb and flower production of members of the *Lilium* genus (Straathof & Van-Tuyl, 1994).

The objectives of this research were to isolate and determine potential bacteria from *Lilium* rhizosphere soil with high chitinase production, and to screen them for their antagonistic activities against *Fusarium oxysporum* for managing bulb and stem rots of lilies.

2. MATERIALS AND METHODS

2.1. Soil sampling

Seven rhizosphere soil samples were collected at different *Lilium* fields with the help of sterile spatulas in various locations in Ha

Noi, Hai Phong, Bac Ninh, and Ninh Binh. These samples were kept in plastic bags and brought to the laboratory for preservation at 4°C until isolation (Amin et al., 2015).

2.2. Isolation and screening of chitinase-producing bacterial strains

To isolate the bacteria, the soil samples were diluted by the serial dilution method. One gram of a *Lilium* rhizosphere soil sample was added into 10ml sterile water and mixed thoroughly by vortex. The initial solution (10^{-1}) was subsequently diluted to a 10^{-8} dilution. One-hundred microliters of the dilutions from 10^{-5} to 10^{-8} were spread on agar plates with Luria Bertani (LB) medium containing the following components: 10.0 g/l peptone; 5.0 g/l NaCl; 5.0 g/l yeast extract; and 20.0 g/l agar (pH 7.0). The plates were incubated at 37°C for 3 days to isolate single bacterial colonies. The obtained colonies were then purified by the three phase streak technique. The pure bacterial colonies were cultured in liquid LB media supplemented with colloidal chitin 1% (w/v) and incubated at 37°C for 48 hours on a rotary shaker (180 rpm). After incubation for 48 hours, the culture broth was centrifuged at 10,000 rpm for 10 min and the supernatants were collected for measurement of chitinolytic activities (Sadfi *et al.*, 2001).

The isolates were transferred to LB agar slants for storage at 4°C and sub-cultured once a month or maintained at -20°C in LB medium containing 30% glycerol for long-term storage (Daamin-Remadi *et al.*, 2006).

2.3. Enzyme assay

2.3.1. Preparation of colloidal chitin

The colloidal chitin was prepared by using 1 g of chitin powder added to H_3PO_4 85% and kept for 24 hours at 4°C. The colloidal chitin prepared for each of the samples was washed several times with a large volume of distilled water to adjust the pH to 7.0 (Alam & Mathur, 2014).

2.3.2. Detection of chitinolytic activity on plates

Chitinolytic activities of the bacterial strains were determined by the diffusion plate method (Dhanasekaran *et al.*, 2012). The sterilized chitin agar medium containing 1.0g colloidal chitin, 2.0g agar, and 100 ml phosphate buffer (pH 7.0) was prepared in Petri dishes. Four wells were made in each plate using a sterile well cutter. Fifty microliters of each chitinase-containing supernatant of the different strains was added into each well in the plates. The plates were placed in a refrigerator at 4°C for 2 hours and then incubated at 30°C for 24 hours. After incubation, Lugol solution was added to the plates for observing and recording the clear zone around the wells, indicating that the hydrolysis of chitin had occurred. Each assay was carried out three times and mean values were calculated.

Chitinase activity was identified by the following formula:

$K = D - d$,

where, K is the chitinase activity, D is the diameter of the clear zone, and d is the diameter of the well (d = 7mm).

2.3.4. Determination of chitinolytic activity based on colorimetric method

The chitinolytic activity of each isolate was determined by the estimation of released reducing sugars from the chitin. The mixture of 1ml of cell free supernatant and 1ml of 1% colloidal chitin suspension was incubated at 40°C for 1 hour. The reaction was terminated by adding 1ml 1N NaOH and boiling the mixture at 100°C for 5 min. After centrifugation of the reaction mixture at 10,000 rpm for 10 min, 1 ml of solution containing reducing sugars (N-acetylglucosamine, NAG) was added by 1ml DNS and heated at 100°C for 10 min in a boiling water bath. After cooling to room temperature, the solution was measured for OD at λ = 535nm. The amount of reducing sugars released was calculated from the standard curve for NAG (Sadfi *et al.*, 2001; Jabeen & Qazi,

2014). The experiment was performed in triplicate and results represented mean values.

2.4. Antifungal assay

For the detection of antifungal activity of the chitinase-producing bacteria, the inhibition zone assay was carried out. The mycelium of the phytopathogenic fungus *Fusarium oxysporum* was inoculated in the center of agar plates containing potato dextrose agar (PDA) medium (20.0 g potato, 20.0g D-glucose, 20.0g agar, and 1.0 liter distilled water, pH 7.0) and incubated at 30°C for three days. This fungus was provided by the Department of Microbial Biotechnology, Faculty of Biotechnology, Vietnam National University of Agriculture. When the radial diameter of the fungal colonies was about 2cm, 50µl of the supernatants from the four strains with high chitinolytic activities were loaded in wells located equidistantly around the center of the plates and then incubated at 30°C for three days. The diameters of the inhibition zones were measured. The experiment was repeated three times (Et-Katatny *et al.*, 2000).

2.5. Morphological and biochemical analysis

The bacterial strain that had the highest chitinolytic activity and was capable of inhibiting the growth of the phytopathogenic fungus *Fusarim oxysproum* was selected and characterized further through tests, namely, the Gram stain, catalase, motility, citrate, sugar utilization, and Voges-Proskauer (VP) tests, according to the methods described by Barrow & Feltham (2004).

2.6. Identification of isolates

Identification of the isolated bacteria was carried out by 16S rRNA sequence analysis. The 16S rRNA genes of the isolated bacteria were amplified by PCR using the forward primer 27F (5'-AGATTTGATCCTGGCTCAG-3') and the reverse primer 1492R (5'-GGTTACCTT GTTACGACTT-3'). The final volume for the PCR amplification was 25µl including 1µl primer 27F, 1µl primer 1492R, 2µl DNA template, 8.5µl H_2O, and 12.5µl Mastermix 2X. The program for PCR amplification was performed using a Bio-Rad Thermal Cycler C1000 as follows: an initial denaturation at 94°C (3 min) followed by 30 cycles of denaturation at 94°C (1 min), annealing at 57°C (2 min), and extension at 72°C (2 min), and a final extension at 72°C (4 min). The PCR products were visualized using gel electrophoresis on 1% agarose. Then, 5µl of PCR products were sent to 1st BASE (Singapore) for DNA sequencing in an automated DNA sequencer. The sequence was compared to the GenBank database available at NCBI using the BLAST search engine for recognition of the highest percent similarity with the described strains and aligned through the ClustalW Multiple Alignment tool. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 6.0 with 1000 bootstraps (Hoster *et al.*, 2005; Setia and Suharjono, 2015).

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of chitinase-producing bacterial strains

From seven *Lilium* rhizosphere soil samples, a total of 61 bacterial strains were isolated. The results consisted of 42 strains from Ha Noi (accounting for the highest percentage of 68.9 %), 4 strains from Hai Phong (6.6%), 3 strains from Bac Ninh (4.9%), and 12 strains from Ninh Binh (19.6%). These strains were labeled as follows: GL01 - GL16; BT01 - BT13; HM01 - HM03; VRQ1 - VRQ10; HP01 - HP04; BN01 - BN03; and NB1 - NB12.

The colonies of the isolated bacterial strains exhibited different morphological characteristics in their size, shape, surface, margins, elevation, color, and texture. Their sizes ranged from 1 to 5mm. The colonies of the isolates were circular or irregular in shape, had entire or undulate margins, were raised or flat in elevation, appeared yellowish, orange, white or cream in color, and appeared dry and

wrinkled or shiny and smooth in texture. Most of them had a circular shape, flat elevation, and dry texture. Using a light microscope, the cellular morphologies of these isolated strains were rod-shaped, occurring in singles or pairs.

All of the isolated strains were screened for chitinase activity based on their zone of clearance on colloidal chitin agar plates as described above. Out of the 61 different isolates tested, 31 bacterial strains were identified as chitinase producers based on their clear zone production, accounting for 52.2%. The zones of clearance surrounding the wells indicated chitinase activity capable of breaking down the chitin compound in the medium. These results showed that *Lilium* rhizosphere soil was quite abundant in the number of chitinase-producing bacteria. The chitinase activities of some of the isolated bacterial strains are shown in Table 1 and Figure 1.

As described in Table 1, only four bacterial isolates (HP02, VRQ9, GL10, and HM03) were capable of producing chitinase with high chitinolytic activity (k >20mm), and had zones of 21.17, 20.83, 20.50, and 20.12mm, respectively. These four strains were the best producers of chitinase, and were chosen for determining their exact chitinase activities according to the colorimetric method.

Table 1. Screening of bacterial isolates for chitinase activities

No.	Strain	Chitinase activity (k, mm)	No.	Strain	Chitinase activity (k, mm)
1	GL3	15.33 ± 0.58	10	VRQ6	13.83 ± 0.76
2	GL4	17.17 ± 0.76	11	VRQ9	20.83 ± 0.29
3	GL10	20.50 ± 0. 50	12	HP02	21.17 ± 0.29
4	GL11	7.83 ± 0.29	13	HP03	5.17 ± 0.29
5	BT02	16.33 ± 0.58	14	BN03	16.50 ± 0.50
6	BT13	15.67 ± 0.76	15	NB1	16.12 ± 0.58
7	HM03	20.12 ± 0.29	16	NB2	15.83 ± 0.29
8	VRQ3	13.67 ± 0.58	17	NB3	15.50 ± 0.50
9	VRQ5	13.67 ± 0.58	18	NB9	15.33 ± 0.58

Notes: k <15mm: weak chitinase activity; 15mm≤ k <20mm: medium chitinase activity; 20mm≤ k <25mm: strong chitinase activity; k ≥25mm: very strong chitinase activity.

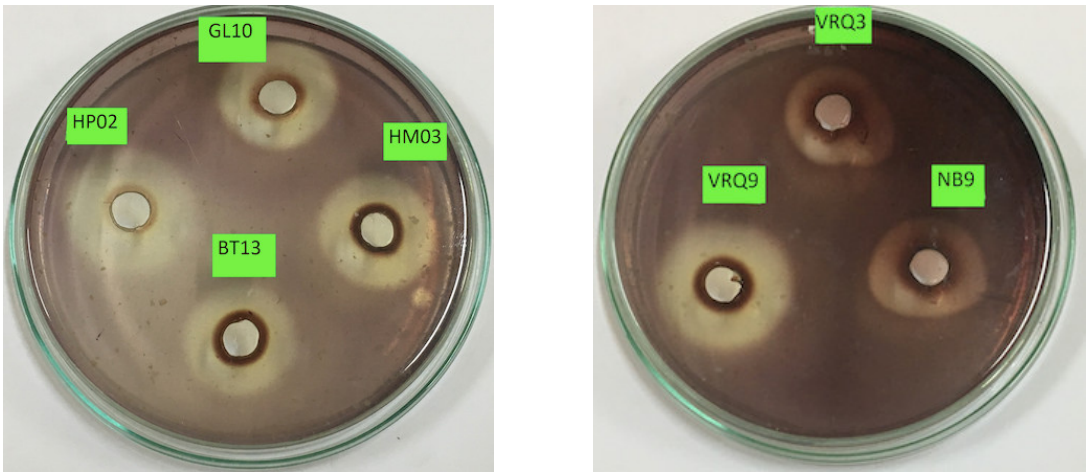


Figure 1. Chitinase activities of a selction of the isolated strains based on their clear zones around the wells after 2 days incubation

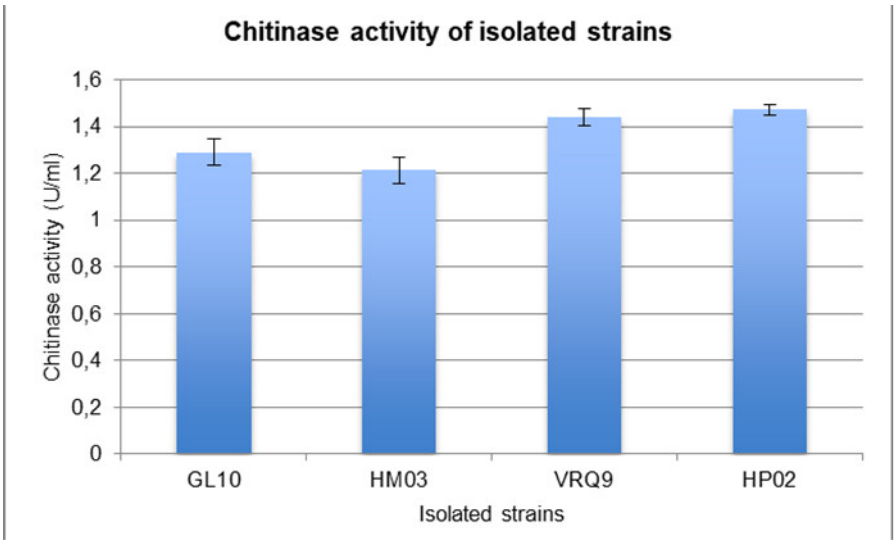


Figure 2. Chitinase activities of the four selected bacterial strains based on measurements of the release of reducing sugars (NAG)

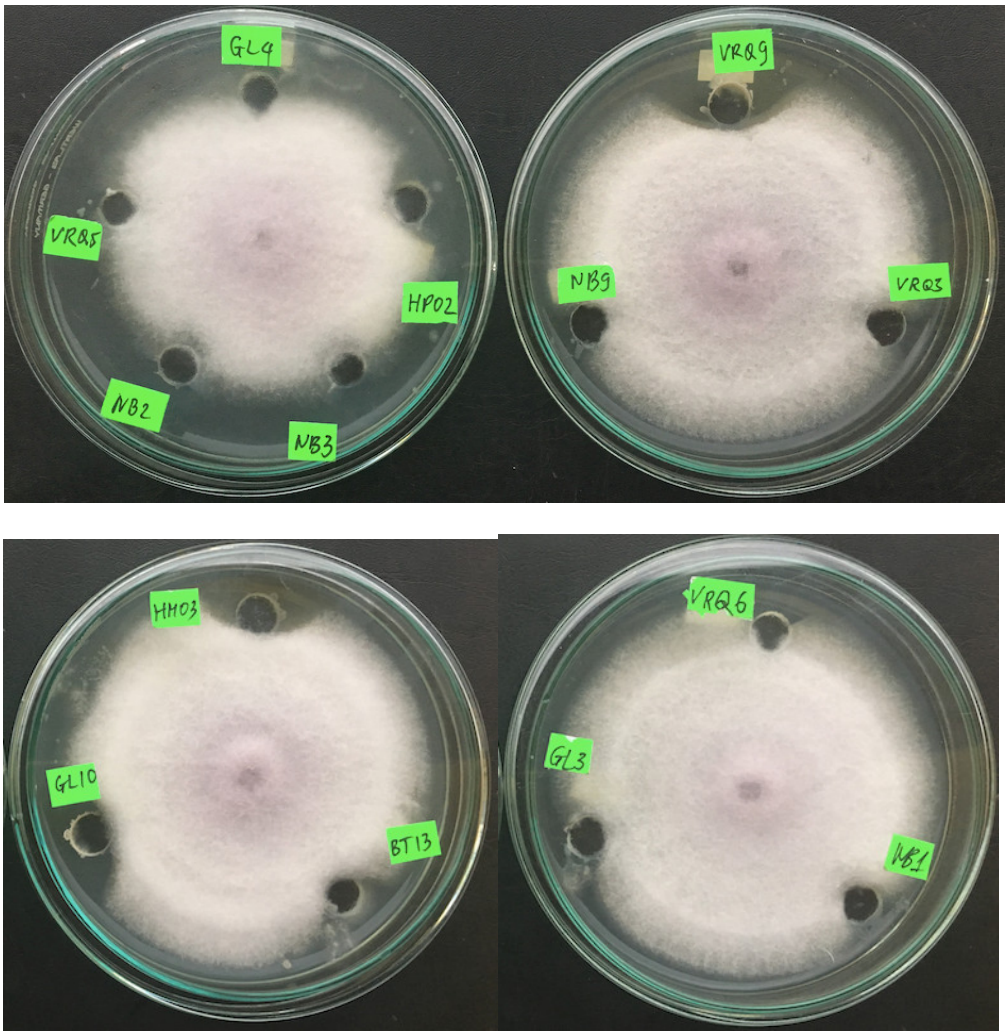


Figure 3. Antagonistic effects of some isolated bacterial strains against *Fusarium oxysporum*

3.2. Chitinase activity of the four selected strains based on the colorimetric method

The chitinase enzymes secreted by the four isolated bacterial strains were evaluated using the colorimetric assay and their chitinase activities are illustrated in Figure 2.

Among the four strains, the HP02 strain gave the highest level of chitinase activity, with a maximum enzyme production of 1.47 ± 0.02 (U/ml) after 2 days incubation, while HM03 exhibited the lowest chitinolytic activity with 1.21 ± 0.06 (U/ml). Thus, the HP02 strain was selected for morphological and biochemical characterization and molecular identification based on 16S rRNA gene sequencing.

Trachuck *et al.* (1996) reported the chitinase activities of different segregant-type colonies, namely, rough (R), smooth (S), and mucous (M) colonies of *Bacillus licheniformis* B-6839. The authors observed that these variants (R, S, M) showed marked differences in chitinase activity levels, measuring 0.8, 1.3, and 1.7 U/ml, respectively. In 2007, Kamil *et al.* isolated four isolates (MS1, MS2, MS3, and MS4), which were the most potent chitinolytic bacteria from Egyptian rhizosphere soil samples of three different plants (maize, wheat, and rice). The MS3 strain, which was identified as *Bacillus licheniformis*, produced the highest level of chitinase enzymes (1.27 U/ml). However, the chitinolytic activity of the MS3 strain was still lower than that of the HP02, VRQ9, and GL10 strains, which recorded 1.47, 1.44, and 1.29 U/ml, respectively. Due to the high levels of chitinase production, these strains are considered to be potential biocontrol agents of plant diseases (Chang *et al.*, 2003; Kamil *et al.*, 2007) and were chosen for testing their antifungal activities against *Fusarium oxysporum*.

3.3. Antifungal activity of isolated strains

Screening tests for antagonism of the isolated strains against *Fusarium oxysporum* were carried out as described above. The results of the inhibitory activities against *Fusarium oxysporum* are shown in Figure 3.

It was apparent that four strains (HP02, VRQ9, GL10, and HM03) had inhibitory activities against this phytopathogenic fungus. The radial growth of the plant pathogenic fungus (*Fusarium oxysporum*) was restricted and inhibition zones around these bacterial strains were observed. These observations might be linked to the production of chitinase or other antibiotics. Bacterial chitinases can effectively hydrolyze the cell walls of phytophathogenic fungi due to the disruption of the glycosidic bonds in the chitin which serve as a fibrous-strengthening element in the fungal cell wall. As can be seen from Figure 3, the four isolates (HP02, VRQ9, GL10, and HM03) had high chitinolytic activities resulting in significantly higher antifungal activities than all the other isolated strains tested. Therefore, they were determined to have the potential efficacy for application as biocontrol agents. The biocontrol activity against plant pathogens of rhizobacteria is not only because they produce hydrolytic enzymes (chitinase and glucanase) but also because they synthesize other antimicrobial compounds such as siderophores and antibiotics (Ahmad *et al.*, 2017). Interestingly, although the HP02 strain had the highest chitinase activity, the antifungal activity of this strain was lower than that of the isolated VRQ9 strain.

Agrobacterium, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Cellulomonas*, *Enterobacter*, *Pseudomonas*, and *Serratia* are genera that are ideal for biocontrol (Podile and Prakash, 1996). *Pseudomonas* and *Bacillus* species living in the rhizosphere have been used to control diseases caused by a variety of plant pathogens including *Fusarium* spp. (Nam *et al.*, 2009). Nam *et al.* indicated that *Bacillus subtilis* BS87 and RK1 were capable of suppressing the mycelial growth of *Fusarium oxysporum* f. sp. *fragariae* (the causal agent of *Fusarium* wilt in strawberry). Strain RK1 exhibited the highest level of fungal growth inhibition. Bacilli are the most popular bacterial biocontrol agents

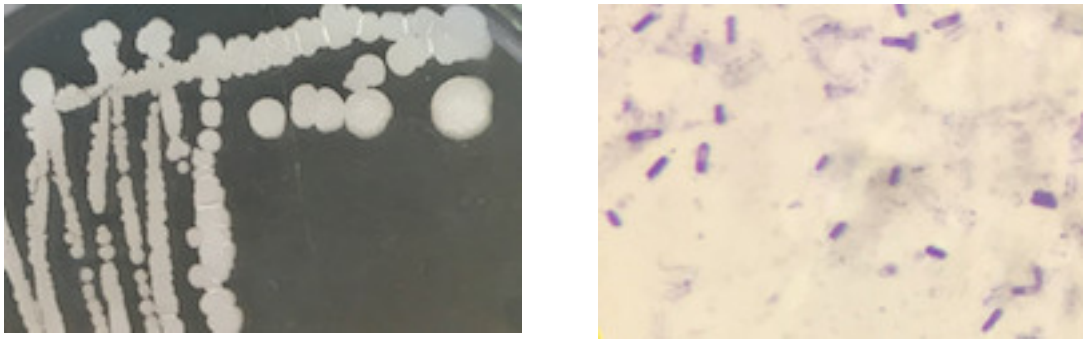
because they are able to form endospores that are tolerant to heat, desiccation, and organic solvents. This is an advantage over some root colonizing bacteria. Besides *Fussarium* spp., *Bacillus subtilis* has also been shown to be inhibitory to other fungal pathogens such as *Aspergillus niger* (Podile & Prakash, 1996). The results obtained by Kamil (2017) indicated that *Bacillus licheniformis* strain MS3 was the most active species leading to the suppressed growth of various tested pathogenic fungi, namely, *Rhizoctonia solani*, *Macrophomina phasiolina*, *Fusarium culmorum*, *Alternaria alternate*, and *Sclerotium rolfsii*. The three bacterial strains HP02, VRQ9, and GL10, which had higher levels of chitinase activities than that of MS3, might be resistant to *Fusarium oxysporum*. Therefore, they might have the potential to have antagonistic activity against other fungal pathogens. Kishore *et al.* (2005b) indicated that *Pseudomonas* sp. GRS175 and *Pseudomonas aeruginoase* GPS 21 were highly inhibitory against fungal pathogens of groundnut. *Pseudomonas fluorescens* MM-B16 had protective activities against *Phytophthora* disease of pepper and anthracnose of cucumber (Lee *et al.*, 2003).

Pseudomonas and *Bacillus* are also important members of plant-growth promoting bacteria (PGPB), which induce plants to tolerate environmental stresses and can be used as biofertilizers (Ahmad *et al.,* 2017). Mishara *et al.* (2012) observed that the *Bacillus subtilis* strain WR-W2, which has high similarity with the HP02 strain (99%), was found to be capable of synthesizing siderophore and indole acetic acid (IAA). *Bacillus amyloliquefaciens* FZB42, which was isolated from the rhizosphere of *Beta vulgaris* (sugar beet), *Bacillus amyloliquefaciens* NJN-6 (from the banana rhizosphere), and *Bacillus subtilis* HJ5 (from the cotton rhizosphere) had plant growth promoting activity and disease suppression based on their abilities for plant root colonization and antibiotic production (Li *et al.*, 2013; Chowdhury *et al.*, 2015).

3.4. Morphological and biochemical characteristics of HP02

The morphological and biochemical characteristics of HP02 strain are shown in Figure 4 and Table 2.

HP02 was found to be Gram positive, with spore-forming rod bacterium, and formed colonies that were 3-5 mm in size with circular form, undulate margins, flat elevation, dry in texture, and cream in color. Cells of this strain were short rod-shaped, occurring in singles or in pairs. Because the endospores strongly resisted the stain dyes, they appeared as non-staining entities in the Gram stain. As indicated in Figure 4(b), endospores were extremely refractive. The isolated strain HP02 was catalase-positive, aerobic, and motile, and had distinct fermentation profiles for the carbon sources maltose, xylose, lactose, mannitol, and glucose.



(a) (b)

Note: (a) Colony of the HP02 strain; (b) Cellular morphology of the HP02 strain (magnification x 1000).

Figure 4. Morphological characteristics of the HP02 strain

Table 2. Morphological and biochemical tests for the identification of the HP02 strain

Tests		Morphological/Biochemical characterization
Gram staining		+
Shape		Rod
Spore formation		+
Motility		+
Catalase		+
Citrate utilization		+
Voges-Proskauer (VP)		+
Fermentation for different carbon sources	Maltose	+
	Xylose	+
	Lactose	+
	Mannitol	+
	Glucose	+

Based on the observed phenotypic and biochemical characteristics, the HP02 strain was grouped into the genus *Bacillus* according to the description in *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994).

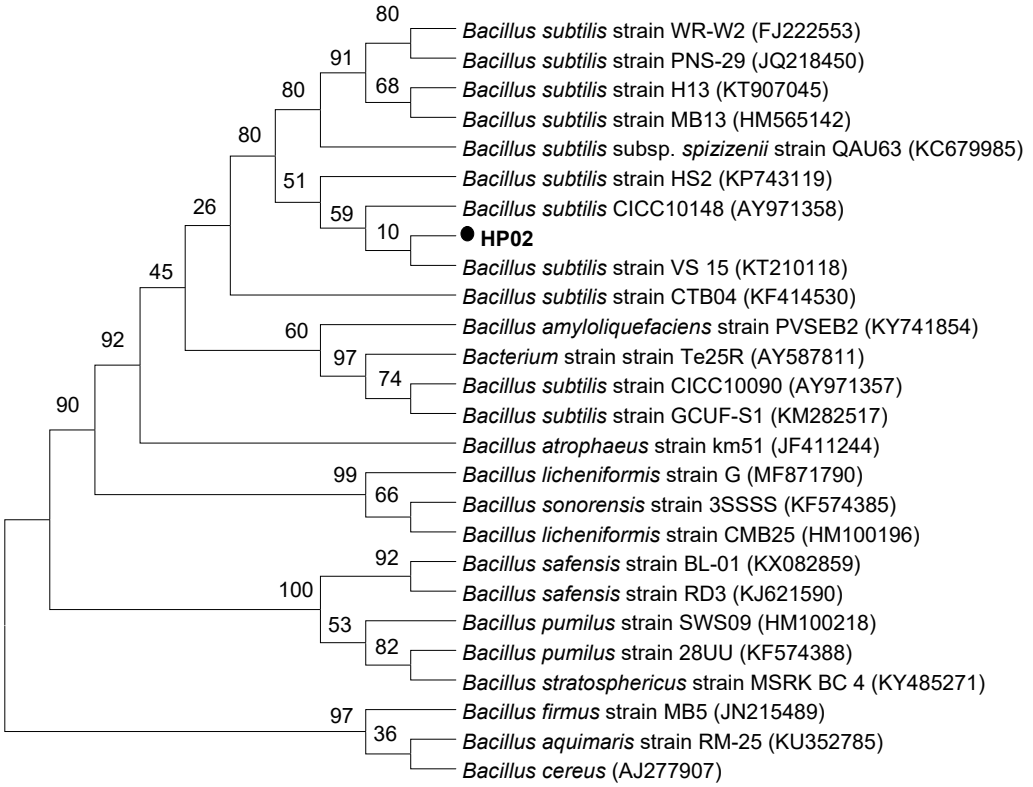
3.5. Phylogenetic analysis of 16S rRNA sequences

Phylogenetic analysis of the 16S rRNA gene sequence using MEGA6 revealed that the isolated HP02 strain was *Bacillus subtilis* (Figure 5). The sequence of the HP02 strain and closely related sequences were analyzed using the neighbor-joining method. The GenBank accession number of each isolate is given in the parentheses. Bootstrap values based on 1000 replicates are shown next to the branches. This strain showed high sequence similarity (99%) to *Bacillus subtilis* strain WR-W2 (FJ222553), and 98% to the closest known species in the Genbank database including *Bacillus subtilis* strain VS15 (KT210118), *Bacillus subtilis* strain CICC10090 (AY971357), and *Bacillus subtilis* strain PNS-29 (JQ218450). The phylogenetic tree showed that the HP02 strain is located in the same branch with *Bacillus subtilis* strain VS15 with a bootstrap value of 100.

Sequencing using the 16S rRNA gene is a good method for resolving problematic phenotypic identifications and placing isolated bacteria in their right taxonomic position (Gomaa and Momtaz, 2006). The HP02 strain, which was identified as *Bacillus subtilis*, was a chitinolytic bacterial strain. Members of the genus *Bacillus* including *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus subtilis, Bacillus licheniformis, Bacillus laterosporus, Bacillus magaterium, Bacillus pabuli*, and *Bacillus thuringiensis* have been reported to secrete a number of degradative enzymes such as chitinase (Shanmugaiah *et al.*, 2008; Karunya *et al.*, 2011). The results of this study also confirm the same results.

4. CONCLUSIONS

Chitinases have many applications in different fields such as waste management, pest and pathogen control in agriculture, the generation of fungal protoplasts, and human health care. The present study indicated that four bacterial strains (HP02, VRQ9, HM03, and GL10) isolated from *Lilium* rhizosphere soil not only produced chitinase with high chitinolytic activity but also were capable of controlling *Fusarium oxysporum* (the causal agent of bulb and stem rots of lilies). Among them, the HP02 strain showed the highest chitinolytic activity. Based on morphological and biochemical characteristics, and 16S rRNA gene sequencing, HP02 was identified as *Bacillus subtilis*. This isolated strain is considered as a potential chitinase producer and as a biological control agent for the phytopathogenic fungus *Fusarium oxysporum*.



Note: The tree was constructed by the neighbor-joining method using MEGA6 software. Nucleotide sequence accession numbers are indicated in the parentheses.

Figure 5. Phylogenetic tree representing the HP02 strain under study compared to selected bacteria from the GenBank database

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Microbial Biotechnology, Faculty of Biotechnology for providing us with the phytopathogenic fungus *Fusarium oxysporum*. We are thankful for financial support from the Belgo-Vietnamese Project.

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