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Original

Isolation, Screening and Identification of Native and New *Bacillus subtilis* with Strong Antifungal Compound against *Fusarium oxysporum*

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The genus *Fusarium* causes a wide range of infections in human, animals and herbs. The purpose of this research was to investigate and identify the native strains of *Bacillus subtilis* playing an inhibitory role against *Fusarium oxysporum* by producing surfactin. *B. subtilis* was isolated from the soil of various parks in Tehran-Iran, and identified by biochemical tests. Growth inhibition zone, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *B. subtilis* were determined. After purification of surfactin, quantitative and qualitative analysis of surfactin conducted using high performance liquid chromatography (HPLC). Finally, two selected native strains with the highest production rate of surfactin identified using PCR for 16S rRNA and phylogenetic tree was drawn. Sixty strains of *B. subtilis* were isolated from soil, after identification through phenotypical and biochemical tests, the antagonistic activity of 27 different strains against *F. oxysporum* by Agar well diffusion assay determined and the highest inhibition zone was 13.66 mm. Six strains showing the best inhibitory effect, were isolated and their metabolite were purified by methanol. MIC and MFC values of different strains were in the range of 0.5-1.6 and 1.6-2.6 mg/mL. Using HPLC, the purified surfactin content in *B. subtilis* was about 56.7 – 131.9 µg/mL. Based on the curves of the chromatogram, the preferred strains with the highest production of surfactin, by molecular identification, displayed high similarity to *B. subtilis*. We got a maximum amount of yellow and transparent surfactin from native strains. Furthermore, the selected bacteria can be good candidates for biological control of fungal pathogens.

Key words：*Bacillus subtilis* / *Fusarium oxysporum* / Antifungal agent / Surfactin.

INTRODUCTION

Infection by fungal pathogens can lead to diseases in animals and plants, and resulting in high costs and significant losses (Hussain & Khan, 2020). *Fusarium* species cause various of infections and diseases by generating toxins (Mibielli et al., 2020). Fusariosis is the second most opportunistic fungal infection after aspergillosis (Thornton, 2020). Keratitis, caused by *Fusarium* spp, is one of the major reasons of corneal infections among contact lens wearers in the developing world (Thornton, 2020). One example in plants is the white patch caused by *Fusarium* mold which is generally a very serious disease in both industrialized and developing countries (Maksimov & Khairullin, 2016). The *F. oxysporum*, as the main fungal plant pathogen, has scientific importance. Several species of *Fusarium* can cause *Fusarium* head blight (FHB) in the regions with

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high temperature and wet climate (Janssen, Mourits, Van Der Fels-Klerx, & Lansink, 2019). In recent years owing to resistance to pathogens less useful synthetic fungicide and biocontrol of fungi, by some microorganisms such as *Bacillus* spp. or their secretions, can serve as a practical and normally safe and effective alternative agent (Lastochkina et al., 2019).

The bacilli are able to produce a variety of metabolites, therefore these are suitable options for biological control and clinical purposes (Erjaee, Shekarforoush, Hosseinzadeh, Dehghani, & Winter, 2020). In terms of the production pathway, lipopeptides are synthesized through ribosomal and non-ribosomal pathways. The metabolites of the second class include cyclic lipopeptides (Sudarmono, Wibisana, Listriyani, & Sungkar, 2019). A well-known class of cyclic lipopeptides with powerful antimicrobial activities includes the fengycin, iturin and the surfactin compounds (Kaspar, Neubauer, & Gimpel, 2019). Many lipopeptides are chemically produced, but in recent years, more attention leads to biocontrol of plant pathogenic fungi using biosurfactants because of their advantages as renewable resources and their biodegradability (Mibielli et al., 2020; Willenbacher et al., 2014). Surfactin is a cyclic lipopeptide with a hydrophilic ring of seven aminoacids that stays in the soil solution and with a hydrophilic hydrocarbon which that can create pores in cell membranes and inhibit growth of pathogens (Selseleh-Zakeri, Akhavan-Sepahy, Khanafari, & Saadat-Mand, 2016). *Bacillus subtilis* is one of the major producers of antibiotics and it can synthesize several lipopeptides such as iturin, surfactin, fengycin and bacillomycin non ribosomally (Kinsella, Schulthess, Morris, & Stuart, 2009). It is the best candidate to biocontrol various fungal pathogens including *Fusarium* spp., *Pythium, Phytophtora, Rhizoctonia, Sclerotinia, Septoria and Verticillium* (Hussain & Khan, 2020). The *B. subtilis*, as a gram-positive bacterium, has several benefits over other bacteria. *B. subtilis* as a ubiquitous and spore forming bacterium, has a longer shelf life and can survive in extreme environmental conditions of temperature and dryness. It is also highly amenable to genetic manipulation. In addition, it is recommended by the US Food and Drug Administration (FDA) as one of the generally recognized as safe (GRAS) microorganisms (Su, Liu, Fang, & Zhang, 2020). Several strategies have been applied to control the causative pathogen, *F. oxysporum* such as soil solarization, fungicide seed treatment and biological control (Chen, Lin, Wang, Huang, & Li, 2019). In this study, the main aim is isolation and identification of the native *Bacillus* strains from soil samples and study their effects against *F. oxysporum* by producing surfactin.

MATERIALS AND METHODS

Collection and preparation of samples from soil

Soil samples were collected from some parks in five regions of north, south, east, west and central Tehran from the depth of 100-150 mm near the root of apparently healthy plants with a sterilized spatula. The samples were quickly transferred to the laboratory. Soil samples were sieved through a mesh screen (2 mm pore size), and 5 g of soil was dissolved in 45 mL of sterile distilled water in a 250 mL of Erlenmeyer flask. The contents were heated at 80℃ for 30 min and after cooling the flasks, the soil samples were tenfold diluted serially in a phosphate-buffered saline (10^{-1} to 10^{-8}) solution. Then, 1 mL of each dilution was transferred to a sterile plate and 20 mL of nutrient agar (NA) medium was added to each plate and left to solidify. The contents of the plate, under aerobic conditions, were incubated at 35℃ for 24 h (Mizumoto, Hirai, & Shoda, 2007).

Isolation of *B. subtilis* by chemical analysis

After the appearance of bacterial colonies, the samples were evaluated in terms of cell morphology, appearance, consistency, color and shape of colonies and biochemical properties (Tashakor, Hosseinzadehdehkordi, Emruzi, & Gholami, 2017). Gram-positive and spore forming bacteria, isolated by Gram and malachite green staining, were identified by biochemical tests including catalase, lecithin hydrolysis, citrate, SIM, nitrate reduction, opacity factor test and fermentation of glucose, arabinose, mannitol and xylose (Perez et al., 2017).

Microorganism and culture media

The positive and negative samples were also compare to control. The positive strain of *B. subtilis* PTCC 1023 was prepared by IROST, the center of the microbial collection of industrial bacteria in Iran (de Faria et al., 2011). All isolated samples were collected in sterile microtubes using a phosphate buffer which contained 20% (v/v) glycerol and stored in -80℃ (Wei, Wang, Chen, & Chen, 2010). To investigate the antifungal effect, *F. oxysporum* PTCC 5115 was obtained from the center of the microbial collection of industrial bacteria in Iran and it was cultured on a sabouraud dextrose agar (SDA), incubated for 72 h at 25℃. The isolated strains of bacteria were purified on NA plates (Ongena & Jacques, 2008).

Determination of antifungal activity

The ability to produce antifungal metabolites of isolated *Bacillus* strains was investigated using an Agar well diffusion assay (Jasim, Sreelakshmi, Mathew, & Radhakrishnan, 2016). First bacterial inoculum was

prepared. For this purpose direct colony suspension method was used in preparing the inoculum. Three to five morphologically similar colonies from fresh NA plates were transferred with a loop into about 5 mL of normal saline in a capped test tube and vortex. The suspension formed was adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard ($BaSO_4$ prepared spectrophotometrically) to give an approximate 1.5×10^8 CFU/mL. Fungal strains were freshly subcultured on sterile Sabouraud Dextrose Agar and incubated at 30℃ for 2-5 d. The resultant cells were washed into sterile normal saline and the turbidity adjusted to a 0.5 McFarland standard equivalent. Under sterile conditions, two wells with a diameter of 7 mm were made in SDA on the plate. A fungal suspension was spread on medium and in each well, 50 µL of bacterial suspension was inoculated and incubated 72 h in 25℃. Then the inhibition zone was measured with an antibiogram ruler (Selseleh-Zakeri et al., 2016). According to the results, antifungal metabolite (surfactin) was extracted from strains with the strongest activity. All experiments for all selected strains were repeated three times.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC was defined using broth micro-dilution method. Different concentrations from each bacterial suspension were prepared (0.125, 0.5, 1, 2, 4, 6, 8, 10, 20, 40, and 80 mg/mL) using inoculum prepared in the previous step and phosphate-buffered saline. Each well (96 well micro plate) was complemented with 20 µL of fungal suspension (0.5 McFarland standard), 80 µL of sabouraud dextrose broth (SDB) and 100 µL of bacterial suspension. The well containing without fungal suspension was the negative control and positive control did not have bacterial suspension. Microplates were incubated for 3 d at 25℃. The smallest concentration of bacterial suspension that prevented the fungal growth is defined as MIC, and MFC was determined by incubating the SDA plates contain 10 µL from each well, where there was no visible growth of fungi at 25℃ for 5 d (Mefteh et al., 2017).

Extraction of surfactin

In order to extract the metabolite, 250 mL flasks containing 100 mL of nutrient broth (NB) medium, with 2% bacterial cultures, were incubated at 35℃ and 200 $\times g$ for 5 d (Wang, Zhang, Zhou, Liu, & Chen, 2015). Then, surfactin was extracted from the flask by acid precipitation. 50 mL of the sample was poured into the falcon and centrifuged at $8000\times g$ at 4℃ for 25 min. The pH of the supernatant was adjusted to 2 with 6 M HCl and acidic supernatant was again centrifuged in Falcon 50 mL conical centrifuge tubes at $1000\times g$ at 4℃ for 15min. The sediment collected on the Falcon tube wall contained antifungal lipopeptide. After drying the precipitant at 30℃ for 24 h, the dried surfactin dissolved in methanol, and again the solution centrifuged at $2000\times g$ at 4℃ for 10 min. The supernatant of yellow and transparent methanolic extract of antifungal metabolite was then prepared in 1.5 mL microtubes for high performance liquid chromatography (HPLC) (Vedaraman & Venkatesh, 2011).

Quantitative and qualitative analysis of surfactin by HPLC

The pure surfactin, purchased from Sigma-Aldrich (Germany,) was used as a control. 20 µL of methanolic solution of the control injected into HPLC apparatus. The Zorbax c-18 column and 100% methanol solution considered as constant phase and mobile phase, respectively. The product at a flow rate of 1 mL/min was monitored at a wavelength of 240 nm for 20 min, and the results were recorded in the chromatogram curve after injecting unknown samples (Chen et al., 2019). According to the results of HPLC, two samples with the highest production rate were investigated for detection of 16S rRNA (Alonso & Martin, 2016). The quantitative content of surfactin with external standards was measured.

Molecular identification of native bacteria

The isolates were cultured at 35℃ for 24 h and DNA extraction was performed using the DNeasy Blood and Tissue Handbook kit (Qiagen Co.) under the manufacturer's instructions. The PCR was used to amplify 16S rRNA gene using primers 27f: (5′-GAGTTTGATCCTGG CTCAG-3′) and 1541r: (5′-AAGGAGGTGATCCAGCC GCA-3′) (Jasim et al., 2016). The reaction steps in thermal cycler were as follows: initial denaturation at 95℃ for 3 min, denaturation in 93℃ for 45 s, annealing at 58℃ for 60 s and an extension at 72℃ for 90 s. DNA concentration was measured at a wavelength of 260 nm. The PCR purification was performed using Qiaquick PCR purification, followed by gel electrophoresis (1% agarose gels). The 16S rRNA gene sequences were compared with the standard sequence of *B. subtilis* in the Gene bank. The phylogenetic tree was rooted to *B. subtilis* using the Neighbor-joining algorithm present in MEGA version 7.0. and the support of nodes was estimated using 1000 bootstrap replicates (de Faria et al., 2011).

Statistical analysis

In this experiment growth inhibition zone, MIC, MFC and surfactin was performed with three repeat and

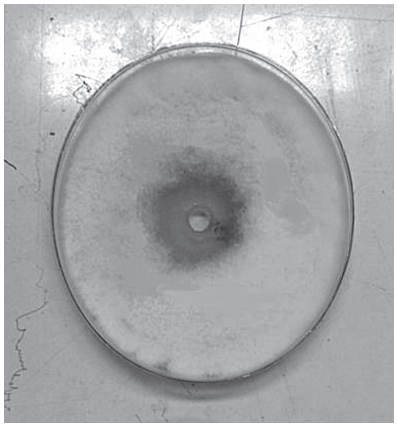

FIG. 1. Inhibition of radial mycelial growth of *F. oxysporum* by *B. subtilis* strains (Y3; Yas Forest Park) isolated from soil on SDA at 25℃ for 72 h. 50 µL of bacterial supernatant was added into each well.

statistical test of them was done using ANOVA test and SPSS (16.0 ver) software.

RESULTS

Identification of *B. subtilis* strains

Based on chemical characterization, *B. subtilis* strains are aerobic mesophilic heterotrophic microorganisms, they are able to produce endospores resistant to 80℃. Furthermore, theses strains can formed colonies with a diameter of 1-3 mm, contained white, circular, erose margin, flat, umbonate, dry and rough features. Out of all samples taken at the beginning of the study, 60 samples approved in the first stage. Twenty-seven bacilli isolates (gram-positive, red vegetative cells containing green endospores in malachite green staining) selected. Following biochemical examinations, catalase (+), lecithinase (-), citrate (+), reductase (+), glucose (+), arabinose (+), mannitol (+), xylose (+), motile and aerobic strains were identified as *B. subtilis*.

Determination of antifungal activity

The selected *B. subtilis* strains, showed antagonistic activities towards the *F. oxysporum* by Agar well diffusion assay (Fig. 1). Results indicated that 6 strains (M2; Mellat Park, V1; Velayat Park, Y1; Yas Forest Park, Y3; Yas Forest Park, Y4; Yas Forest Park, Y5; Yas Forest Park) had the largest inhibition zones and produced the most antifungal metabolites (Fig. 2a).

Determination of MIC and MFC

The MIC and MFC values different strains against *F. oxysporum* were in the range of 0.50-1.6 and 0.83-2.6 mg/mL for *Bacillus* spp. The MIC and MFC values determined against *F. oxysporum* in M_2 strain were 0.5 and 0.83 mg/mL respectively, which smallest content and highest content observed in Y_5 strain about 1.6 and 2.6 mg/mL. The MIC and MFC in V_1, V_3 and Y_3 strains were in range of 0.66-0.83 and 1.3-1.6 mg/mL and these strains had insignificant different and antagonistic activities (Fig. 2b).

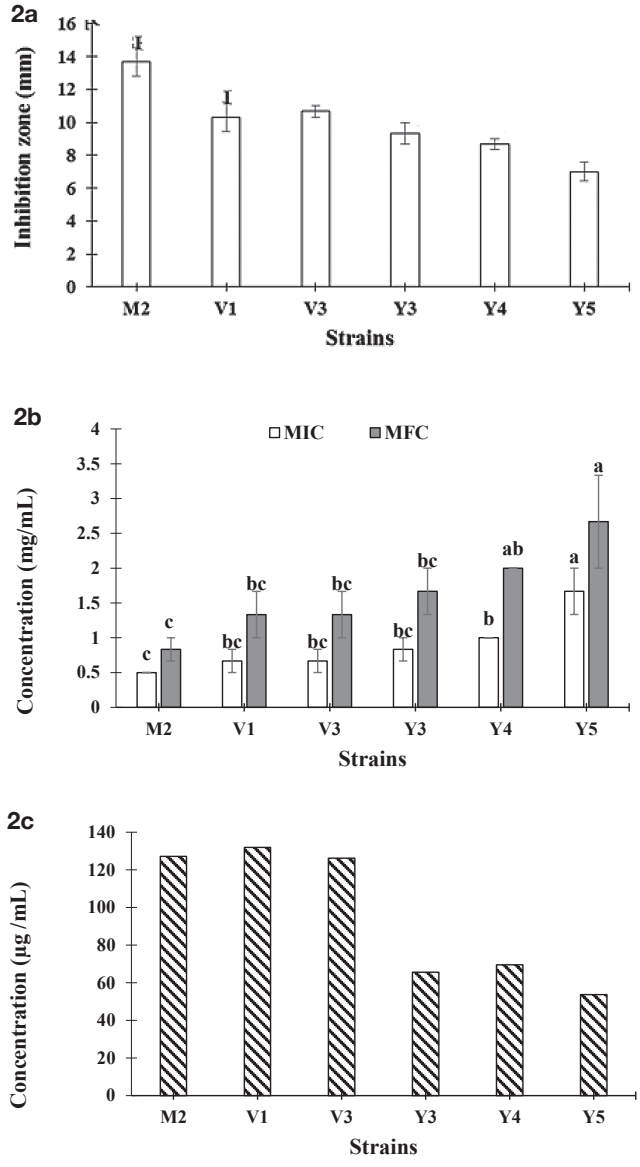

FIG. 2. (a) Inhibition zone of six isolates of *B. subtilis* from soil. (b) Result of MIC, and MFC against *F. oxysporum*. (c) and surfactin content. Data represent the mean ± standard error and different letters in each column indicate a significant difference

HPLC analysis

The results of identification of surfactin composition using HPLC chromatogram are shown in Fig. 3. This composition produced by Sigma-Aldrich as standard,

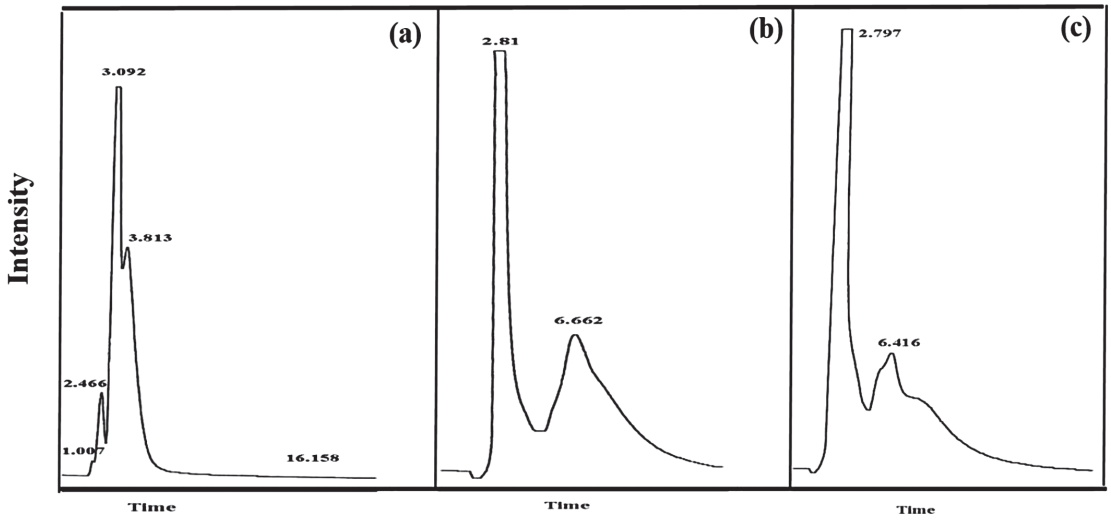

FIG. 3. HPLC chromatogram of Sigma-Aldrich surfactin as standard (a), surfactin extracted from *B. subtilis* PTCC 1023 as reference strain (b) and surfactin produced by native *B. subtilis* strain isolated from soil (M2; Mellat Park) (c). Column Zorbax C-18 eluted with 100% methanol at 1 mL/min as mobile phase. Peaks showed various isoforms of surfactin.

shows peaks in two time intervals (3.09 and 3.8 min). Output HPLC chromatogram related to surfactin extracted from *B. subtilis* PTCC 1023 as reference strain revealed, in this strain, surfactin in 2.81 and 6.6-recorded peaks and has about 103 µg/mL surfactin. Result related to six native strains also have two peaks in 2.8 and 6.4 min this indicates the production of this compound in these strains (Fig. 3).

The results quantification showed the amount of surfactin in the six studied strains using external standards, surfactin content in *B. subtilis* about 56.7 – 131.9 µg/mL. Highest surfactin contents observed for M_2, V_1 and Y_1 strains and least content related to Y_5 strain (Fig. 2c).

Molecular Identification

Based on result output HPLC chromatogram, two strains of *B. subtilis*, which showed the highest production of surfactin (M_2 and V_1), were identified by 16S rRNA method. Concentration of DNA at 260 nm was shown to be 4.5 ng/µl. By purifying the PCR and its electrophoresis products, the presence of the 1500 bp fragment was confirmed and the results showed that the selected bacteria had a strong similarity to *B. subtilis* (Fig. 4).

The phylogenetic 16SrRNA tree also confirmed this similarity, and the fact that this bacterium is located in a distinct branch of this phylogenetic tree shows that this strain is novel in its genus, and the results of molecular analysis confirmed the same biochemical results (Fig. 5).

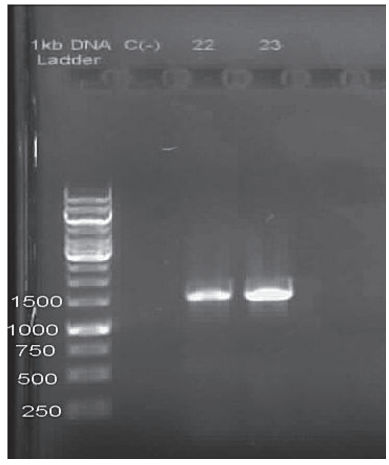

FIG. 4. Polymerase chain reaction product using specific primers for *B. subtilis* isolated from soil was loaded in a 1% agarose gel. Lane 1. 1kbp DNA ladder. Lane 2. Negative control. Lane 3 and 4. Genome PCR of *B. subtilis* strains (M_2 and V_1) with high activity selected from soil (1500bp).

DISCUSSION

Fungal diseases are currently one of the most common reasons of major damages in agricultural yields (Thornton, 2020). On average, one out of every eight plants will not be productive due to fungal disease (Vedaraman & Venkatesh, 2011). Because of special attention to environment and the resistance of some pathogen to chemical fungicides and environmental pollution by them, scientists have tried to use natural soil organisms to protect plants (Meena, Sharma,

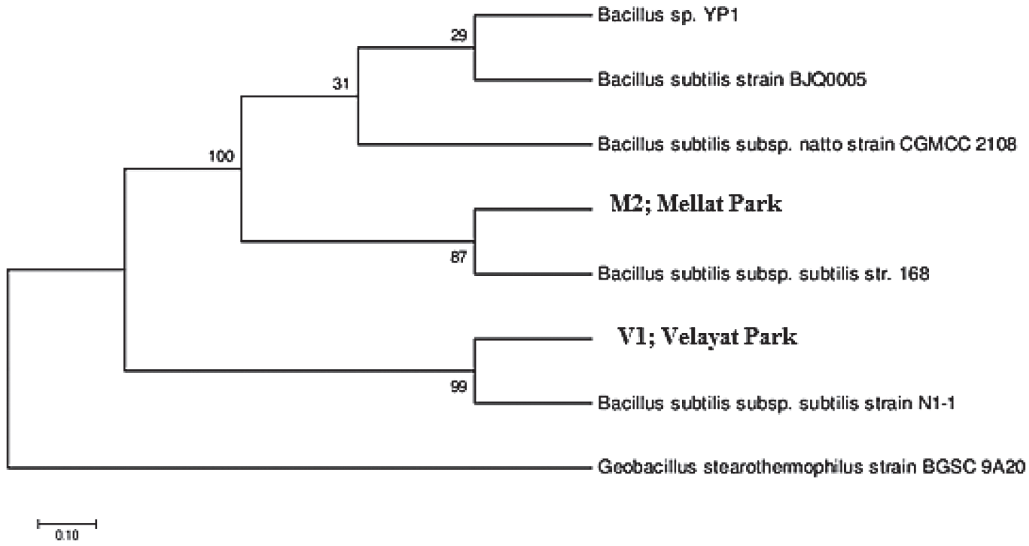

FIG. 5. Phylogenetic tree of native *B. subtilis* with Neighbor- joining method using 1000 bootstrap replicates. Two strains of M2; Mellat Park and V1; Velayat Park (shown in bold) with the highest amount of surfactin production were used to draw the phylogeny tree.

Kumar, & Kanwar, 2020).

In recent years, the use of microbial antagonists as a biological control agent against fungal diseases has become a hopeful solution for many countries around the world (Nega 2014; Al-Fadhal, AL-Abedy, and Alkhafije 2019). Soil and non-pathogenic microbes with antagonistic properties to prevent pathogenic fungi are the best alternative to chemical fungicides and are additionally one of the most significant reasons affecting the creation of sustainable nature (He, Zhu, Huang, Hsiang, & Zheng, 2019). *Bacillus* species, notably *B. subtilis*, has a high efficiency in the control of pests (Jasim et al., 2016). In Vietnam, experiments were performed on different strains of *Bacillus*, *B. subtilis* XL62 was selected for its antifungal activity against *F. oxysporum* and *R. solani*, as the most important food crops contaminants (Doa, Lea, Nguyena, Nguyena, & Daoc, 2017). Due to a number of specific features such as the production of antifungal antibiotics, hydrolytic enzymes and the ability to produce persistent spores, *B. subtilis* can be considered as a biological control agent with high efficiency in the management of pests (Goswami & Deka, 2019). The use of *B. subtilis* seems to be an advantageous approach to manage pests by combining biological, physical and chemical methods, because it does not entail any economic or environmental hazards (Mihalache et al., 2018).

Genus *Fusarium* causes wilting, drying and rotting of seeds, roots, stems and seedling death (Janssen et al. 2019; Al-Fadhal, AL-Abedy, and Alkhafije 2019). We hypothesized by identifying the appropriate strains of antibiotic surfactin producers and their effective control of the pathogenic fungi, suitable strategies will be identified to reduce the diseases of plants, by safe biological agents without environmental hazard (Jasim et al., 2016).

According to studies *B. subtilis* has an important antifungal activity on fungal pathogens. The antifungal activity of selected strains was evaluated using the standard strain PTCC 1023 and the highest inhibition zone was 13.66 mm. Similar results with the antagonistic properties of *B. subtilis* strains against pathogenic fungi, using an Agar well diffusion assay, can be found in a previous study by Yu et al. (2002). Also the antifungal activity of *B. amilolicofasian* in the suppression of diseases caused by pathogenic *R. solani*, was examined, and the mean diameter of the fungal growth inhibition zone was 25 mm in the presence of bacterial suspension (Yu, Sinclair, & Hartman, 2002). Additionally our result indicated, the MIC and MFC values of different strains against *F. oxysporum* were in the range of 0.5-1.6 and 0.83-2.6 mg/mL for *Bacillus* spp. Results of this paper are in agreement with those of Thi et al. (2017). They reported the antifungal activity of *B. subtilis* XL62 and RMB7 that inhibited the growth of nine fungal phytopathogens more than 70% in vitro (Ali et al., 2020). Moreover a study by Cazorla et al. (2007) shows that in the 905 strains isolated from the rhizosphere of avocado trees in Spain, four ones were identified as *B. subtilis* and showed significant antifungal activity against *F. oxysporum* and *R. necartix*. Furthermore Rebib et al. (2012) examined 69 strains of *B. subtilis* isolated from saline soils in northern Tunisia and tested them for their properties against different species of *Fusarium* and

recognized the strain *B. subtilis* SR146 as beneficial. The *B. subtilis* SR146 exhibited potent antagonistic activity up to 82%, against *F. culmorum*, but it did not show a significant inhibitory effect on *F. solani*. To find the component responsible for the antifungal properties of *B. subtilis*, the metabolites were extracted from six strains, and the results were obtained of HPLC and chromatograms of selected indigenous strains from soil. Result related to surfactin showed, this compound produced by all selected strains, isolated with antifungal ability, were able to produce surfactin. Its concentration in these strains in range 56.7 – 131.9 µg/mL and strains that produced higher surfactin levels showed lower MIC and MFC content. Reports the production of two major antibiotics of surfactin and iturinA in *B. subtilis* QST713, isolated from the cucumber rhizosphere. These antibiotics were isolated using HPLC. It was concluded that this strain has the ability to produce both antifungal lipopeptides, iturin A and surfactin, as observed on chromatograms obtained from the sample (Kinsella et al., 2009). As a result, the soil was determined to be a suitable source for isolating biological control agents such as *B. subtilis* (He et al., 2019). Molecular features of the best strains were investigated with 16S rRNA and their genetic correlation with standard *B. subtilis* (1500 kDa) was confirmed (de Faria et al., 2011). Also Liu et al., (2007) extracted bacisubin from *B. subtilis* B916 (41.9 kDa) which inhibits *F. oxysporum* and *R. solani* growth.

We hypothesized different strains of *B. subtilis* collecteded from different areas of soil has strong fungal activity on *F. oxysporum* and the strains with more production of surfactin, had minimum MIC and MFC for control of *F. oxysporum*. Therefore this metabolite can be used for biological control of fungi using native strains in each region.

CONCLUSION

Biological metabolites are an effective alternative to synthetic samples and the importance of this research is the production of surfactin as a biological fungicide. Native strains of *B. subtilis* with capacity of production of surfactin can be used as a suitable biocontrol. Therefore, this strains could be a promising candidate to be developed as commercial biofertilizer and biocontrol formulation in sustainable agriculture.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

HUMAN AND ANIMAL RIGHTS

This article does not contain any studies involving human or animal subjects performed by any of the authors.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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