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Isolation and identification of *Bacillus velezensis* RTS-M11 and assessment of its antifungal activity

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Abstract

The selection of putative antagonists for the biological control of plant diseases usually involves collecting and screening large numbers of microbial isolates so as to increase the probability of discovering highly effective strains. Different strains of *Bacillus velezensis* produce secondary antifungal metabolites that could control plant diseases. The ability to form spores makes this bacterium an ideal candidate for biological control. Isolation, characterization, and identification of *B. velezensis* (native to Iran) from soil and its antifungal activity against *Fusarium* sp. have been reported in the present study. Eight out of 75 isolates showed antifungal activity against three main species of *Fusarium* under standard conditions. The morphological and biochemical characteristics, along with the 16S rRNA and *gyrB* genes sequences of the selected isolate, indicated that it belongs to the *B. velezensis* species. The results showed that sucrose as a carbon source and peptone as a nitrogen source in a culture medium at pH 7 and an agitation speed of 200 rpm led to the maximal growth rate and antifungal activity in the *B. velezensis* sp.RTS-M11 selected strain. This isolate seems potentially useful as a biological agent against a few *Fusarium* sp. but needs more study in the future.

1. Introduction

Plant pathogenic fungi are a major threat to crops and food production. One of the most important fungal diseases of plants is Fusarium head blight caused by the fungal pathogen *Fusarium* sp. (Haile et al., 2019). Contamination of plants with the *Fusarium* genus causes wilt, plantlets death, and yield and quality losses of products. *Fusarium* sp. is usually controlled using fungicides such as Orthocide and Metalaxyl-Mancozeb (Sardrood et al., 2018). However, their intensive use in conventional crop management has led to many problems, such as side effects on the environment and living beings and the emergence of pathogens resistant to chemicals, especially if the resistance is genetically-based (Khan & Ahmad, 2019). Therefore, biological control is now regarded as an alternative control tool.

In integrated pest management (IPM), biological control means the use of various organisms, especially bacteria and fungi, to directly or indirectly prevent the growth and development of plant pests by producing metabolites with

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antimicrobial properties (Day & Hong, 2019). Organisms that have naturally antagonistic effects on harmful microorganisms are able to inhibit plant pathogen activities (Munir et al., 2018). Biological control agents compete with pathogens for nutrients, inhibit pathogen growth by secreting antimicrobials, and reduce pathogen populations through parasitism. In addition, biological agents promote growth and boost the resistance of plants through synergism.

Aerobic Gram-negative bacteria, especially *Pseudomonas* spp., have been widely studied as biological control agents. However, despite *Pseudomonas* spp.'s favorable characteristics for biological control, their main limitation as a biocontrol agent is their inability to form spores. This limitation has diverted the focus of the research community toward *Bacillus* spp. Certain entophytic *Baciluus* spp. concomitantly promote plant growth and synthesize factors that suppress many plant pathogens, including *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Verticillium* sp., *Eutypa lata*, *Botrytis cinerea*, *Calonectria gracilis*, *Rhizoctonia solani* (Sethi & Mukherjee, 2018), and *Zymoseptoria tritici* (Platel et al. 2021) (both *in vitro* and *in vivo*). Members of the *Bacillus* genus are considered microbial agents for the production of a vast array of biologically active molecules with the potential ability to inhibit the growth of pathogenic fungi and bacteria. Among these antimicrobial compounds, cyclic lipopeptides (LPs), including surfactin, iturin, and fengycin, have been reported in strains identified as biocontrol agents (Phelan et al., 2019).

The advantage of using *Bacillus* strains in the biocontrol process is their ability to form endospores, which confers resistance to chemicals and physicals agents. *Bacillus velezensis* was isolated during a research program focused on discovering novel bacterial strains capable of synthesizing new lipopeptides with surfactant and/or antimicrobial activity. DNA-DNA hybridization showed less than 20% hybridization with other *Bacillus* species. Moreover, *B. velezensis* differed from the other species in phenotypic characteristics (Ruiz-Garcia et al., 2005).

The growth of microorganisms in a suitable medium plays an essential role in their biological activities. As the nutritional requirement of an organism is genetically predetermined, it is important to provide the appropriate carbon and nitrogen sources and also the proper environment for optimal activity. Production of bioactive products by microorganisms is not a fixed property and can thus be greatly increased or completely lost under different cultivation conditions and levels of nutrition availability. This is because antibiotic biosynthesis is a specific property of microorganisms that depends greatly on culture conditions. Optimized growth and antibiotic production can be achieved by manipulating the nutritional and physical parameters of the culture conditions. Hence, medium composition plays a vital role in the efficiency and economics of the ultimate product. Several cultivation parameters like pH, carbon and nitrogen sources, incubation time, and temperature also play major roles in the production of bioactive metabolites.

The 16S ribosomal RNA (16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes. Sequencing and analysis of the 16S rRNA gene is the most widely used method for identifying bacteria or for reconstructing phylogenies because (i) it is ubiquitous in almost all bacterial species, (ii) it is a highly conserved gene with a slow rate of evolution, and (iii) it's relatively large size (1500 bp) is suitable for informatics investigations (Patel, 2001). Another approach to constructing phylogenetic relationships is using protein-encoding gene sequences; for example, the *gyrB* gene encodes the subunit B protein of DNA gyrase, a type II DNA topoisomerase that is distributed universally among bacterial species (Wei et al., 2018). The rate of molecular evolution inferred from *gyrB* gene sequences is even faster than that inferred from the 16S rRNA gene sequences.

In the present study, *B. velezensis* was isolated from forest rhizospheric soil and identified by standard methods and direct sequencing of the *gyrB* and 16S rRNA genes. The effect of different cultural conditions on the growth and production of antifungal metabolites and their activity against *Fusarium solani, F. graminearum,* and *F. oxysporum* were also investigated. Additionally, the optimization of culture media, temperature, pH, and incubation time to achieve the highest antifungal activity were also reported.

2. Materials and methods

2.1. Soil sampling

Rhizospheric soil samples were collected from five forest parks located in fifteen regions of Tehran, Iran (Chitgar Forest Park, Sorkheh Hesar National Park, Koohsar Forest Park, Lavizan Forest Park, and Velayat Park). Soil sampling was carried out using a sterile spatula to transfer soil into 50 ml sterile falcon tubes. The samples were then transported aseptically at a low temperature (4°C) to the laboratory.

2.2 Microorganisms preparation

In this study, *B. velezensis* PTTC 1023 was used as bacterial control, and the three species of pathogenic fungi used were provided by the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST), including *F. solani* PTTC 5284, *F. graminearum*, and *F. oxysporum* PTTC 5115. Bacterial isolates were also isolated from the soil. For this purpose, the soil samples were sieved through a 2-mm-pore-size sieve. Then, soil suspensions were prepared by adding 5g of soil to 45 ml of sterile distilled water, then kept in an 80°C water bath for 30 min. Serial dilutions (10×) of the soil suspensions were made, and 1 ml aliquots of 10^{-1} to 10^{-10} dilutions were spread onto nutrient agar (NA, Merck) plates, which were then incubated at 35°C for 24 h. Colonies were sub-cultured three times on nutrient agar to obtain single pure colonies (Sengun et al., 2009).

2.3. Primary Bacterial identification

The isolates were identified by standards such as macroscopic and microscopic observations, Gram staining, catalase test, lecithin hydrolysis test, citrate utilization test, motility test, OF test, nitrate reduction test, and carbohydrate fermentation test using Berge's manual (Vos et al., 2011).

2.4. Antifungal activity assays

The *B. velezensis* isolates were tested for *in vitro* antagonistic activity against three pathogens using the standard well plate assay method on Sabouraud Dextrose Agar (SDA). The isolates of *B. velezensis* were tested for inhibiting the mycelial growth of three major fungal pathogens, *F. solani, F. graminearum,* and *F. oxysporum*. First, each of the fungi was cultured in Sabouraud Dextrose Broth, in which the turbidity of the suspension was adjusted with a spectrophotometer at 600 nm to obtain a 0.5 MaC-Farland turbidity standard (McFarland, 1907), and was cultivated in the middle of an SDA plate. Next, Four mm diameter wells were cut into pre-poured SDA plates using a sterile cork borer, and 100 µl of a 24 h culture supernatant of selected *Bacillus* isolates was added to each well. Then, the plates were incubated at 26 ± 2°C for 2 days and assayed the formation of the growth inhibition zones. Lastly, the isolates that showed strong inhibition of the growth of *F. graminearum*, *F. solani*, and *F. oxysporum* were selected for more studies.

2.5. Evaluation of culture conditions on antifungal activity

Bacilli can grow in a pH range between 3 and 9, but the optimal pH for the growth of many *Bacillus* species is around 7 (Hanim, 2017). The effect of different pH values on the growth and antifungal activity of the isolates was studied using a nutrient broth (NB) media with different pH values (5.5, 6,

6.5, 7, 7.5, and 8). Antifungal activity was assayed by determining the inhibition of fungal mycelial growth (Vincent, 1947). Similarly, the effect of different temperatures (25°C, 33°C, and 35°C) on antifungal activity was studied by using a nutrient broth (NB) medium to grow fungi at different temperatures. The antifungal activity was assayed by the well plate assay method (Hanim, 2017). The effect of three different shaking rates (150, 180, and 200 rpm) on the antifungal activity was studied, and the well plate assay method was used to determine antifungal activity of cells grown at the three different agitation rates (Hanim, 2017). The effect of different carbon (glucose, xylose, arabinose, sucrose, maltose, lactose, and starch) and nitrogen (peptone, yeast extract, and ammonium nitrate) sources on antifungal activity was studied, and their ability to inhibit the growth of fungi was tested by the well plate assay method (Hanim, 2017).

2.6. Molecular Identification of the target isolate

Pure culture of the target isolate was grown overnight in a nutrient broth medium, and total DNA was extracted from the bacterial cells by using the CinnaPure DNA kit for isolation of DNA from gram positive bacteria (Cat.NO: PR881614). A PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The universal primers 16SrDNAF (5´-AGAGTTTGATCCTGGCTCAG -3´) and 16SrDNAR (5´- AGGGAGGTGATCCAGCCG CA-3´) were used for the amplification of the 16S rDNA gene fragment. PCR amplification consisted of denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing of primers at 58°C for 1 min, and extension at 72°C for 90 s. The final extension was conducted at 72°C for 10 min (Kim & Chun, 2014, Li et al., 2016).

The *gyrB* gene was amplified using the primer pairs BS-F (5'-GAAGGCGGNACNCAYGAAG-3') and BS-R (5'-CTTCRTGNGTNCCGCCTT C-3') to more accurately identify the putative *B. velezensis* isolates. PCR amplification consisted of denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing of primers at 58°C for 1 min, and extension at 72°C for 90 s. The final extension was conducted at 72°C (Li et al., 2016).

The resulting amplicons were purified using the Qiagen gel extraction kit, and then the 16S rDNA- and *gyrB*-amplified PCR products (100 ng concentration) were sequenced through cycle extension in an ABI 373 DNA sequencer (Macrogen (Seoul, South Korea), Applied Biosystems, USA), following the manufacturer's instructions. The same primers were used for the sequencing reactions. The resulting sequences were aligned manually with representative sequences of *Bacillus* and related taxa obtained from the GenBank database.

DNA sequences were edited, and consensus sequences were obtained using the DNASTAR Lasergene software. The resulting sequences were then compared with those available in the GenBank database (National Centre for Biotechnology Information (NCBI); http://www.ncbi.nih.gov/) using the Basic Local Alignment Search Tool for nucleotide sequences (BLASTN). A phylogenetic tree based on the 16S rDNA gene sequences was inferred using the maximum likelihood method based on the Tamura-Nei model using the MEGA 10 software. The evolutionary distance matrices were generated according to Kimura’s 2-parameter distance model.

3. Results

3.1. Bacterial isolates identification and characterization

Based on macroscopic and microscopic observations, 75 bacterial isolates were identified as the endospore-forming gram positive *Bacillus*

genus. Additional biochemical tests confirmed that 44 isolates were *B. velezensis.*

3.2. Antifungal activity

The antifungal activities of the bacterial suspensions obtained from the 44 isolates were assayed on agar plates against *F. graminearum*, *F. solani*, and *F. oxysporum* using the agar well diffusion method (Fig 1). After 3 days of incubation, several isolates of *B. velezensis* demonstrated strong *in vitro* antifungal activity by reducing fungal growth and showed a 100% inhibition for mycelial growth when compared to the untreated pathogen control. Eight isolates that strongly inhibited the growth of *F. graminearum*, *F. solani*, and *F. oxysporum* were selected, and the clear zones were measured after 48 h using the coulis scale (Table 1).

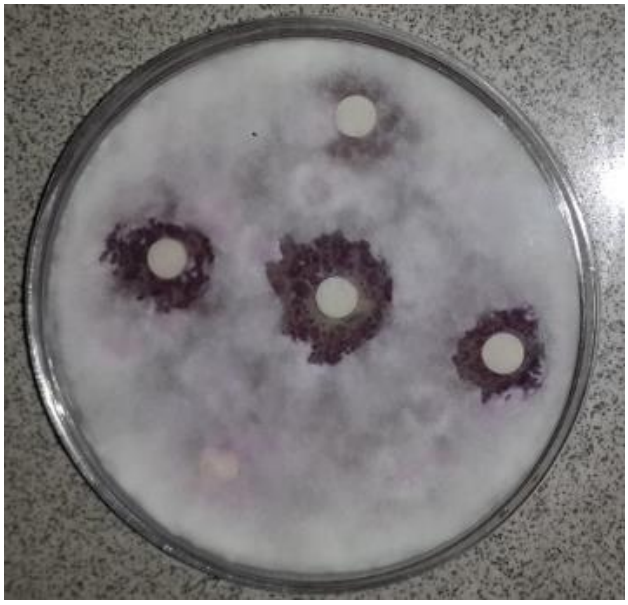

Figure 1. Antifungal activity assay of *B. velezensis* isolates using the agar well-diffusion method.

Table 1. Antifungal activity of eight *B. veleziensis* isolates against pathogens

Bacterial isolates	**Growth inhibition halo diameter of pathogen (mm)**		
	F. solani	*F. graminearum*	*F. oxysporrum*
PTTC 1023	7.3	7.6	9
B_1	8.6	15	12.6
B_2	11.3	9	8.3
B_3	8	5.3	5
B_4	11.6	11	11
B_7	15.6	16.3	8.6
B_8	13	10	11.6
B_9	9.3	10	9
B_{28}	14.3	16.6	10.3

3.3. Optimization of growth conditions

Optimization of growth conditions is required for maximum antifungal activity based on several variables and their effects on the inhibition of fungal growth. Therefore, different parameters were examined for this purpose.

3.4. Temperature optimization

The internal temperature of microorganisms is strongly dependent on their environment. So, the antifungal activity against fungi was observed at different temperatures of 25°C, 33°C, and 35°C in nutrient broth (NB) media. The results showed that 33°C is the best temperature for antifungal activity. The highest antifungal activity was related to the B28 strain (Table 2).

Table 2. Antifungal activity at different temperatures

Bacterial isolates	*F. solani*			*F. graminearum*			*F. oxysporrum*		
	Temperature˚C								
	25	**33**	**35**	**25**	**33**	**35**	**25**	**33**	**35**
PTTC 1023	6.5	7.5	7.3	6.2	7.5	7.4	8.2	9.1	9
B_1	6.9	8.6	8.3	13.8	15.3	15.1	10.8	12.6	12.3
B_2	10	11.5	11.3	8.1	8.9	8.3	7.6	8.3	8
B_3	6.8	8	7.8	4.8	5.5	5.2	5	5.3	5.1
B_4	10.8	11.5	11.3	10.1	11	10.8	10.3	11	11
B_7	8	10	11.3	14.8	16.4	16.2	6.9	8.7	8.6
B_8	12.4	13.1	13	9.5	10.2	10	10.4	11.7	11.1
B_9	8.4	9.5	9.1	9.9	10.7	10.7	7.5	9	9
B_{28}	13.5	**14.2**	14.2	14.4	**16.5**	16.1	8.2	**10.3**	10.1

3.5. pH optimization of antifungal activity

Bacilli can grow in a pH range between 3 and 9, but the optimal pH of the bacilli growth is around 7. Therefore, pH values of 5.5 to 8 for the culture media was evaluated on the growth and antifungal metabolites production of the selected isolates. According to the results, the optimal pH for suppression of fungal growth were 7-7.5 (Table 3).

Table 3. Efficiency of different pH values on antifungal activity of the selected isolates

Bacterial isolates	*F. solani*						*F. graminearum*						*F. oxysporrum*					
	pH																	
	5.5	**6**	**6.5**	**7**	**7.5**	**8**	**5.5**	**6**	**6.5**	**7**	**7.5**	**8**	**5.5**	**6**	**6.5**	**7**	**7.5**	**8**
PTTC 1023	6	6.7	7.1	7.5	7.3	6.1	6.2	6.5	7.1	7.5	7	6.2	7.2	8.4	9	9	8.8	8.3
B_1	7	7.2	8.3	8.8	8.5	7.5	13.5	14.2	14.8	15.4	15.1	14.5	10.8	11.1	11.8	12.5	12.1	11.5
B_2	10	10.8	11.3	11.6	11.5	10.3	7.3	7.8	8.2	8.9	8.5	8	6.8	7.4	7.5	8.6	8.1	7.3
B_3	6.8	7.1	7.8	8	7.9	7.2	4.2	5	5.1	5.5	5.3	4.8	4.3	4.2	4.5	5.1	4.9	4.1
B_4	10.8	11	11.1	11.5	11.3	10.5	9.2	10.3	10.5	11	10.7	10.3	10.3	10.6	11	11.5	11.2	10.8
B_7	9	9.5	9.5	10.1	9.7	9.2	13.9	15.2	15.8	16.2	15.9	15	6.9	7.9	8.3	8.5	8.3	7.6
B_8	12	12.6	12.5	13	12.7	12.2	5.9	9	9.2	10	9.5	8.8	10.1	10.4	11.1	11.7	11.5	10.9
B_9	7.3	8.4	8.7	9.4	9.1	8.5	8.4	9	10.5	10.9	10.5	9.3	8	8.8	9	9.2	9	8.4
B_{28}	13	13.5	14	**14.5**	14.3	13.9	15.3	16	16.1	**16.5**	**16.5**	15.9	9	9.1	9.5	**10**	**10**	9.6

3.6. Optimization of carbon and nitrogen sources

The growth response of eight *B. velezensis* isolates were studied in the presence of seven different carbon sources, including glucose, xylose, sucrose, maltose, arabinose, lactose, and starch. The results showed that the effect of the different carbon sources on antifungal activity was ranked in the following order: sucrose> glucose> maltose> arabinose> xylose> starch> lactose (Table 4).

Table 4. Effect of carbon sources on antifungal activity of selected isolates (measured clear zones)

Bacterial isolates	***F. solani***						
	Carbon sources						
	Glucose	Xylose	Arabinose	Sucrose	Maltose	Lactose	Starch
PTTC 1023	7.5	6.7	6.8	8.2	7.1	5.2	6.5
B_1	8.7	7.6	8.1	9.5	8.5	5.9	7.2
B_2	10.5	11.2	10.8	11.6	11	8.3	9.9
B_3	7.9	6.8	7.1	8.4	7.6	5.1	5.9
B_4	11.3	10.3	10.5	11.8	11.1	8.6	9.8
B_7	10.2	9	9.2	10.4	9.7	7.6	8.2
B_8	12.6	11.8	12	13	12.5	10.5	11.1
B_9	9.4	8.9	8.9	9.8	9.3	8	8.4
B_{28}	14.5	13.5	13.8	**14.9**	14.2	12.4	13
Bacterial isolates	***F. graminearum***						
	Carbon sources						
	Glucose	Xylose	Arabinose	Sucrose	Maltose	Lactose	Starch
PTTC 1023	7.3	6.3	6.5	7.8	6.9	5.1	5.8
B_1	15.4	14.3	14.7	15.9	15	13.2	13.8
B_2	8.8	7.5	7.9	9.2	8.5	6	6.8
B_3	5.6	4.8	5.1	6.1	5.3	3.9	4.5
B_4	11.1	10.1	10.5	11.5	10.7	8.7	9.4
B_7	16.2	15	15.3	16.5	15.9	12.4	4.2
B_8	9.8	8.6	9	10.4	9.5	7.4	8.1
B_9	10.8	9.2	9.5	11.1	10.3	7.5	8.6
B_{28}	16.5	14.8	15.2	**16.9**	15.8	13	13.9
Bacterial isolates	***F. oxysporrum***						
	Carbon sources						
	Glucose	Xylose	Arabinose	Sucrose	Maltose	Lactose	Starch
PTTC 1023	9	7.8	8.1	9.2	8.5	6.1	6.7
B_1	12.5	11	11.3	12.5	12.1	9.7	10.6
B_2	8.4	7.2	7.5	8.8	8.1	5.8	6.4
B_3	5	3.8	4.2	5.1	4.3	2.9	3.2
B_4	11.5	10.5	10.8	11.9	11	9.3	9.9
B_7	8.3	7.5	7.5	8.5	7.8	6.4	7.2
B_8	11.9	10.3	10.5	12	11.2	9.1	9.6
B_9	9	8.4	8.2	9.6	9	6.5	7.6
B_{28}	9.5	8.5	8.6	**10.1**	9.2	7	7.8

The effect of ammonium nitrate, yeast extract, and peptone as the sole sources of nitrogen was also investigated. The results showed that peptone was the most favorable nitrogen source, providing the maximum antifungal activity for the eight bacterial isolates (Table 5).

Table 5. Effect of different nitrogen sources on antifungal activity of selected isolates (measured clear zones)

Bacterial isolates	*F. solani*			*F. graminearum*			*F. oxysporrum*		
	Nitrogen sources								
	Peptone	Yeast extract	Ammonium nitrate	Peptone	Yeast extract	Ammonium nitrate	Peptone	Yeast extract	Ammonium nitrate
PTTC 1023	8.5	8.2	5.3	7.9	7.6	5.4	9.5	9.4	7.3
B_1	9.8	9.5	5	16.4	15.9	13.8	12.8	12.5	9.9
B_2	11.6	11.3	8.6	9.5	9.2	6.8	9.3	8.8	7.5
B_3	8.6	8.4	5.6	6.3	6.1	4.2	5.5	5.1	4.2
B_4	12	11.6	9.1	11.7	11.3	9.2	12.8	12	10.3
B_7	10.5	10.5	7.3	16.5	16.4	14.6	9.1	8.7	7.5
B_8	13.4	12.8	9.4	11.1	10.5	8.8	12.5	11.9	9.2
B_9	9.8	9.7	5.1	11.3	10.9	8.9	9.9	9.5	7.3
B_{28}	15.3	15	11.8	17.3	16.5	14.2	10.5	10	8.1

3.7. Optimization of shaker agitation rate

Bacterial cells were shaken at 150, 200, and 250 rpm to find the best shaking rate (revolutions per minute (rpm)) for the highest antifungal activity. Maximum antifungal activity was obtained at 200 rpm (Table 6).

3.8. Molecular identification

The amplified 16S rDNA and *gyrB* genes of the selected isolate were first sequenced by the Sanger sequencing method. Then, sequences of the 16S rDNA and *gyrB* genes from different isolates were compared using the Basical Local Alignment Search Tools (BLAST) available at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As a results, the 16S rDNA sequences of the strain had 99.87% similarity to *B. velezensis* FZB42. Finally, the assembled sequence for the 16S rDNA fragment belonging to the RTS-M11 strain was submitted to the NCBI database (https://www.ncbi.nlm.nih.gov/) under accession number MH628438.1.

The phylogenetic trees constructed from the 16S rDNA gene sequences of different species of *Bacillus*, including the *B. velezensis* RTS-M11 from this study, are shown in Fig. 2. The phylogenetic tree based on the 16S rDNA gene sequences clearly delineated four distinct clusters: cluster 1 contained strains of *B. atrophaeous, B. amyloliquefaciens, B. ciamensis, and B. nakamuria*; cluster 2 contained strains of *B. vallismortis*; cluster 3 contained the *B. velezensis* strains, including the RTS-M11 strain and *B. nematocida;* and cluster 4 contained *B. subtilis, B. mojavensis, B. halotolerans*, and *B. tequilensis* strains. *B. velezensis* RTS-M11 shared 99.86-99.87% similarity with other *B. velezensis* strains, 99.73% similarity with *B. vallismortis*, and 98.42-99.8% similarity with *B. subtilis*. Moreover, *B. velezensis*, including the RTS-M11 strain, shared 95.47-99.54% similarity with other *B. velezensis* strains present in the phylogenetic tree, based on 16S rDNA gene sequences.

Table 6. Effect of shaker agitation rate on antifungal activity of selected isolates (measured clear zones)

Bacterial isolates	*F. solani*			*F. graminearum*			*F. oxysporrum*		
	Revolutions per minute (RPM)								
	150	**200**	**250**	**150**	**200**	**250**	**150**	**200**	**250**
PTTC 1023	8.7	10.6	7.6	8.8	9.1	8.1	10.8	11.4	10.1
B_1	10.8	11.3	9.9	18.1	19	17.2	13.8	15.1	12.6
B_2	12.7	13.3	12.3	9.1	10.5	8.6	9.8	11.3	9.2
B_3	9.8	10.2	9.1	6.9	8.1	6.8	6.8	7.6	5.8
B_4	13.3	14.6	13.1	12.6	13.5	12.3	13.8	15	13.9
B_7	11.1	11.8	10.5	17.3	18.6	16.9	10.8	11.6	10.2
B_8	14.4	15.3	13.6	11.5	12.9	11.3	13.9	15.2	13.3
B_9	8.6	10.8	7.3	12.2	13.5	11.6	9.9	10.7	9.7
B_{28}	17.6	**18.1**	16.3	18.8	**20.1**	17.9	11.2	**12**	10.8

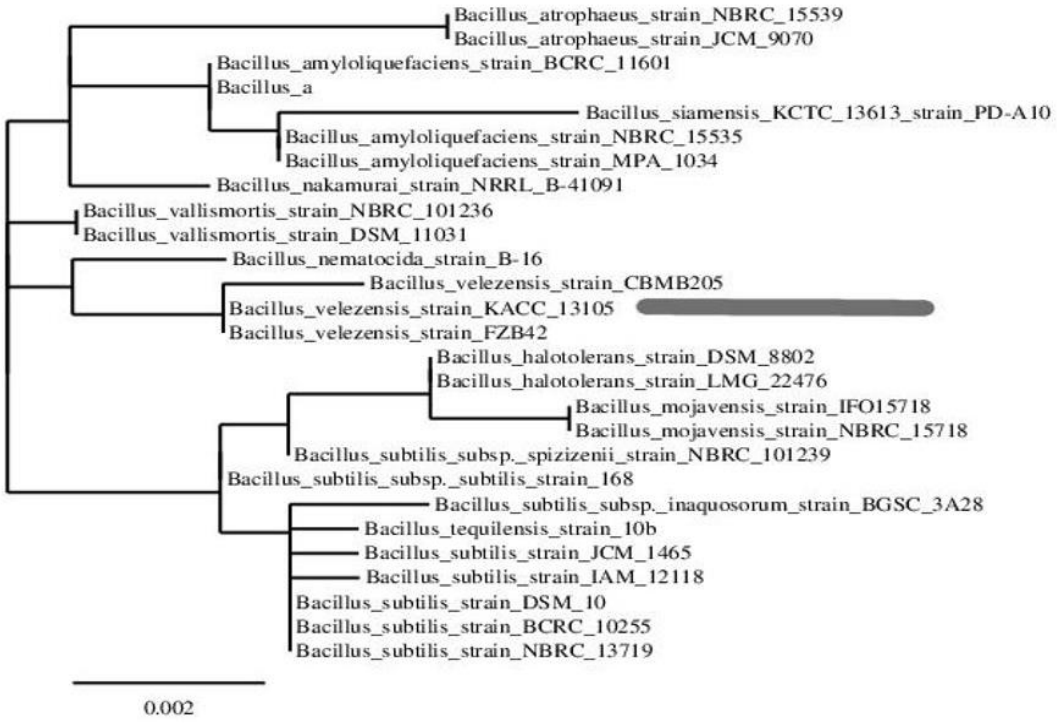

Figure 2. Phylogenetic trees of *B. velezensis* M11-RTS (KACC_13105) based on 16S rDNA. The ClustalW method was used for multiple sequence alignment and the trees were constructed using the neighbor-joining method. Genetic distances were computed by Kimura's two-parameter model.

4. Discussion

The selection of putative antagonists for the biological control of plant diseases usually involves collecting and screening large numbers of microbial isolates to increase the probability of discovering highly effective strains. Therefore, in the present study, the rhizosphere soil of forest plants in Tehran was collected and used for the isolation, identification, and analysis of antifungal activity of the resulting *B. velezensis* strains as biocontrol agents are known to improve the growth and productivity of crops. From a collection of 171 colonies obtained from the rhizospheric soil, we found 75 *Bacillus*-like colonies, eight of which were effective in biological control.

The use of biological control to manage agricultural pests and diseases is an effective

alternative to pesticides. Chemical pesticides that accumulate in plants can be lethal to humans, and beneficial organisms present in the soil may develop resistance. Many studies have reported the prevalence of different strains of *Bacillus* in the rhizosphere. The spore-forming *Bacillus* group is predominantly present in the rhizosphere of healthy chickpea plants. *Bacillus* species have the ability to form endospores and synthesize a vast number of metabolites and, with the exception of toxin-producing *B. anthracis* and *B. cereus*, they are often considered beneficial and safe for plants and the ecological environment (Phelan, 2019). These properties of the *Bacillus* species make them good biocontrol agents and suitable alternatives to chemical fungicides. *Bacillus* species, especially *B. subtilis* and *B. amyloliquefaciens*, play a prominent role in protecting plants from pathogens and promoting plant growth based on their capacity to colonize plant roots (Fira et al., 2018). In a previous study, 905 strains of *B. subtilis* were isolated from the rhizosphere of an avocado plant in Spain. Four strains showed significant antifungal activity against *Rosellina necartix* and *F. oxysporum* (Cazorla et al., 2007). From 205 *Bacillus* spp. isolated from the soil, 23 strains showed antagonistic activity against *Penicillum digitatum*, and nine strains demonstrated more than 80% antifungal activity (Leelasuphakul et al., 2008).
In one study, 69 strains of *B. subtilis* were isolated from the salty soil of north Tunisia; one strain designated SRT46 showed high antifungal activity of 82.85% against *F. solani* (Rebib et al., 2012). The effectiveness of *B. methylotrophicus* in reducing root-knot-disease was also shown in another study (Zhou et al., 2016). Antagonistic *Bacillus* strains might be useful in formulating new inoculants, offering an alternative environmental-friendly biological control for plant diseases.

The present study collected the strain that exhibited the strongest antifungal characteristics against *F. solani*, *F. graminearum*, and *F. oxysporum* from the Koohsar Forest Park in Tehran. The selected isolate was identified as *B. velezensis* using molecular techniques. The new strain was deposited in Iran's Persian Type Culture Collection (PTCC), and the 16S rDNA sequence was registered in the Genbank under accession no. MH628438.

Growth patterns are highly sensitive to conditions including temperature, pH, oxygen and nutrient concentrations, growth medium content, and the number of bacterial cells used for seeding cultures. Two parameters, proper temperature and aeration, can be controlled by orbital shakers (Bates et al., 2016). For the *B. velezensis* in this study, maximal growth was obtained using sucrose as the carbon source, peptone as the nitrogen source, a medium pH of 7, and an agitation rate of 200 rpm. In a study, the highest *B. subtilis* biomass was achieved when using sucrose and xylose (Kumari and Khanna, 2014). High levels of levan and exo-polymeric substances of *B. subtilis* have been observed in sucrose-containing mediums (Shih et al., 2005). Also, peptone has been used as a nitrogen source for the production of α-amylase by *B. subtilis* (Aiyer, 2004). Organic nitrogen sources such as peptone are also better than the inorganic sources for amylase production by *B. licheniformis* (Lal et al., 2016). The optimal pH for growth of this bacterium is around 7, and antibiotics produced by *B. subtilis* are only active at pH values of 5.6 and above (Moita et al., 2005). 16S rDNA analysis is a reliable method in the field of microbiology; it is used to explore microbial diversity and identify new strains. In this study, a 1500 bp fragment of the 16S rDNA gene was sequenced and used to identify isolated bacterial strains. Subsequently, a 16S rRNA gene sequence-based phylogenetic tree was constructed, showing high sequence homology (>99%) between the studied isolate and other *B. velezensis* strains. In a previous study, a bacterium isolated from rice rhizosphere soil was identified using 16S rDNA sequencing and phylogenetically classified as a novel *B. methylotrophicus* species of the genus *Bacillus* (Grover & Dadarwal, 1997). The latter sequence was very similar to the 16S rDNA sequence of this study. In another research, the association of bacteria with chickpea was studied, and the results showed that out of 150 isolates, 40 isolates belonged to the genus *Bacillus*, according

to the physiological, morphological, and biochemical characteristics outlined in Bergey's *Manual of Systemic Bacteriology* (Kumari and Khanna, 2014). In the past 20 years, sequencing of the gene coding 16S ribosomal RNA (rRNA) has become the most valuable tool for the identification of bacteria. However, other genes can also be used for PCR-based strain identification and have been successfully employed to differentiate between bacterial species.

It is broadly accepted that the identification of bacteria at the species or strain level based on physiological and biochemical features is very ambiguous, complicated, and unreliable. Even the 16S rDNA sequence analysis, which is considered to be rapid and reliable, might yield confusing results. Therefore, the use of multiple strategies is necessary to identify bacterial species or strains. Besides the 16S rDNA sequence analysis, the *gyrB* gene may be useful for the identification and phylogenetic analysis of members of the *Bacillus* group at the species and subspecies level, with some exceptions (Mercier & Lindow, 1996). Our analysis of the *gyrB* sequence confirmed the results obtained from the 16S rRNA gene sequence analysis. *gyrB* gene sequence analysis has been used in studies of *Salmonella*, *Shigella*, *Escherichia coli* (Fukushima et al., 2002), and the *Bacillus anthracis–cereus–thuringiensis* group (La Duc et al., 2004). Comparative analysis of 16S rDNA and *gyrB* sequences demonstrated excellent correlation for identifying bacteria belonging to *B. velezensis*.

Conflict of Interest

The authors declare that there is no conflict of interests.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors.

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