

Isolation and identification of *Bacillus subtilis* and its potential in biocontrol of phytopathogenic fungi

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Abstract. In the work, based on morphological, cultural, biochemical properties and on the basis of the 16S rRNA gene, a local strain of bacteria *Bacillus subtilis* TM was isolated from saline soils of the Fergana region of Uzbekistan. The strain was registered with NCBI under the number: OR944512.1. It was revealed that the strain *Bacillus subtilis* TM exhibits high antagonistic activity against the phytopathogenic fungi *Fusarium verticillioides*, *Fusarium solani*, *Aspergillus orchaceus*, *Alternaria alternata*, *Fusarium culmorum*, *Alternaria tenuissima* with an inhibition zone of 40-60 mm. At the same time, the *B. subtilis* TM strain showed moderate antagonistic activity against *Fusarium oxysporium*, *Penicillium chrysogenum* (with an inhibition zone of 22-23 mm) and relatively less antagonistic activity against *Cladosporium sp* (with an inhibition zone of 12 mm).

1 Introduction

Over the past few years, there has been a food safety and quality crisis around the world. Crop seeds are most vulnerable to contamination by *Aspergillus*, *Penicillium* and *Fusarium* species during harvest, storage and/or transport, resulting in approximately 1 billion tons of food lost annually. Among contaminating microorganisms, *A. flavus* is the main virulent fungus that contaminates corn, peanuts, and cotton seeds with aflatoxins. The Food and Agriculture Organization of the United Nations reported (FAO) that about 25% of the world's grains are affected by mycotoxins, mainly caused by *A. flavus* infections [1].

Fusarium oxysporum is a soil-borne plant pathogen that can persist in soil for long periods of time and is difficult to control. *Fusarium* species limit plant development and crop yields, resulting in huge financial losses in agriculture. *Fusarium* produces a carcinogenic mycotoxin that has serious implications for human health and food safety. Its phytotoxicity is considered a factor in the development and severity of plant diseases. For example, *Fusarium oxysporum* can cause Fusarium leaf wilt, limiting global cucumber production [2].

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Alternaria alternata is a plant pathogen with a wide host range and causes black spot in many fruits, vegetables and grains worldwide. *A. alternata* spores can destroy grains, fruits, food and nutritive parts of plants and ultimately reduce their nutritional value by releasing several toxic metabolites. Tobacco brown spot, caused by *A. alternata*, is one of the most serious fungal diseases in the tobacco industry [3]. This disease has been reported worldwide to be commonly caused by *Alternaria* species and its symptoms range from patchy to oval dark brown necrotic lesions on the leaf surface. With severe infection, lesions appear and the leaves dry out, which leads to a significant reduction in yield [4].

Frequently used fungicides such as sulfur dioxide, imidazoles and pyrrolidines have many disadvantages such as chemical toxicity, residues in food and fungal resistance [5]. Also, chemical agents are highly polluting to the environment, leading to food security problems, high costs and creating health problems for other organisms. After all, these chemicals are easily disposed of and are highly effective; they cause significant pollution of water bodies, more precisely ground and soil water bodies. In addition, they accumulate as unwanted chemical residues in the food chain [6].

As a result, it is necessary to develop new types of biofungicides based on microorganisms and products, known as biopesticides, which are environmentally friendly and do not have resistance to pathogens [7]. Over the years, interest in the biological control of phytopathogens has increased significantly due to the need to introduce more environmentally friendly alternatives to the massive use of chemical pesticides [8]. Unlike chemical fungicides, biological control agents provide an environmentally friendly and sustainable alternative strategy that can significantly suppress the activity of soil pathogens and increase plant resistance to pathogens [9]. Among these promising biological control agents, *Bacillus spp.* produces stress-resistant spores and exhibits resistance to extreme conditions, rapid reproduction and strong colonizing ability. It is fermentable and culturable, making it ideal for use as a biological control bacterium. Bacilli can also produce various antagonistic proteins, enzymes and other antifungal substances (lipopeptides, polyketides, peptides and antibiotics), thereby inhibiting the growth of phytopathogens and causing plant resistance [9].

Bacillus species are Gram-positive, spore-forming bacteria and are widely distributed in the environment. Members of *Bacillus* such as *B. subtilis*, *B. amyloliquefaciens* and *B. velezensis* have been proven to have a great influence on plant growth and exhibit antagonistic effects on various pathogens in wheat, cucumber, lettuce and tobacco. One of the important characteristics of *Bacillus spp.* is that they produce broad-spectrum antibiotics such as surfactin, iturin and fengycin. In recent years, the iturin family has received more attention due to its broad spectrum antifungal activity, low toxicity and allergenicity. Iturins have been shown to inhibit the growth of various plant pathogenic fungi, such as *Colletotrichum dematium*, *Penicillium digitatum*, *Bipolaris maydis* and *Fusarium graminearum*. It has been reported that a sterile filtrate containing iturin produced by *Bacillus* still retains its antagonistic ability against *Penicillium* after treatment with ultraviolet rays, sunlight, evaporation, high temperature and extreme pH, indicating that iturin has good stability and is not sensitive to environmental changes [3]. A dense community of beneficial microbes forms a barrier and stimulates the production of secondary metabolites, which reduces the population of harmful microorganisms and promotes plant growth [10]. In addition to the protective effect, many strains of microorganisms also have properties that stimulate plant growth [8]. Based on these data, the goal of our work was to study the antagonistic properties of *B. subtilis* against phytopathogenic fungi.

2 Materials and methods

2.1 Sample collection

Samples were collected from moderately saline soils in the Fergana region of the Republic. A soil sample (5–30 cm) was taken from the top surface of the soil with a sterile spoon; after collection, the samples were placed in sterile tubes and carefully transported to the microbiological laboratory [11].

2.2 Bacterial isolation

A serial dilution technique was used to isolate bacteria from soil samples. Soil samples were weighed 1 g, 10 ml of distilled water was added and shaken for 10–20 min until the mixture was homogeneous and a bacterial suspension of 10^{-1} was obtained. The suspension (10^{-1}) was then used to prepare serial dilutions of 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} for further experiments. From a serially diluted soil sample, 100 μ l was mixed with warm nutrient agar medium and poured into Petri dishes. After this, the petri dishes were incubated at 28–30°C. After 48 hours, various bacterial colonies formed on the dishes. Individual colonies were collected using sterile loops and spread onto fresh nutrient agar plates to obtain pure cultures. The pure culture was stored and used to test antibacterial activity against phytopathogenic fungi [11–12].

2.3 Screening of antagonistic activity of isolated isolates against phytopathogenic fungi

A total of 16 bacterial colonies were isolated from moderately saline soil samples in the Fergana region. Screening of soil bacteria for antagonistic activity was carried out in vitro against such phytopathogenic fungi as *Fusarium oxysporium*, *Aspergillus flavus*, *Fusarium verticillioides*, *Penicillium chrysogenum*, *Alternaria alternata*, *Alternaria tenuissima*, *Fusarium solani*, *Fusarium culmorum*, *Aspergillus orchaceus*, *Cladosporium sp.*, which are stored in the collection of the laboratory of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan. The well-diffusion method was used in the work. 20 ml of agar medium was added to a Petri dish. A well was made in the center 2/3 the thickness of the agar layer. After this, the lawn was seeded with phytopathogenic fungi, and cultural liquid was added to the hole. In the experiment, the culture was grown in two media: meat peptone broth and a molasses-based medium. Each experiment was carried out in triplicate. Incubation was carried out at 30°C for 3–7 days, after which the zone of inhibition of the growth of phytopathogenic fungi was measured and the dishes were photographed. Fungal growth inhibition zones were measured in millimeters. The larger the fungal growth inhibition zone, the higher the fungicidal activity of the crop. The zone of inhibition of fungal growth was considered to be the zone of complete suppression of colony growth [13].

2.4 Morphological and biochemical properties of bacteria

Morphological and biochemical characterization of bacteria that showed antibacterial activity was carried out using standard methods described in Bergey's Manual of Determinative Bacteriology [12]. Bacteria grown for 24 hours in nutrient broth were used for Gram staining and biochemical characteristics were studied.

2.5 Molecular identification of bacteria

First, a nutrient broth was prepared in which a bacterial culture was then grown at a temperature of 30°C for 12 hours. Next, bacterial DNA was isolated from the bacterial culture using the RIBO-prep reagent kit (InterLabServis, Russia). DNA extraction was performed according to the manufacturer's protocol. DNA samples were stored at -20°C. The 16S rRNA gene was amplified using the PCR method. The 16S rRNA gene was selected for molecular genetic identification of bacterial cultures.

The total volume of the amplification reaction was 20 µl; a ready-made lyophilized PCR kit was used (Isogene, Russia). Primers (5 pmol/µl) were added at 2 µl, free nucleotides at 2.5 µl, and DNA at 2 µl at 20 ng/µl. Enzymes and free nucleotides were in a ready-made kit and were placed separately in each tube in lyophilized form. Then 10 µl of buffer and up to 20 µl of distilled water were added. The reactions were carried out as follows: initial denaturation 95°C for 5 min, 95°C for 20 s, 35 cycles, 57°C for 20 s, 72°C for 40 s and final denaturation 72°C for 3 min. The PCR product was analyzed by electrophoresis in a 2% agarose gel. The purified PCR product was subjected to a sequencing reaction using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). The sequence product was purified using the BigDye XTerminator Purification Kit and loaded into the sequencer. Sequencing reactions and sequencing product purification procedures were performed according to the kit instructions. The resulting sequence product was processed in Codon Code Aligner software and compared (BLAST) with the NCBI database. The obtained sequence of the strain was entered into the international NCBI database and registered under the corresponding number (GenBank: OR944512.1) [14].

3 Results and Discussion

3.1 Isolation and screening of a strain with high antagonistic activity against phytopathogenic fungi

Among 16 samples of bacteria isolated in pure culture, 1 strain was selected, which showed high antagonistic activity against phytopathogenic fungi. This strain was selected for the next stage. According to the results of the study, this isolate showed high antagonistic activity against *Fusarium verticillioides*, *Fusarium solani*, *Aspergillus orchaceus*, *Alternaria alternata*, *Fusarium culmorum*, *Alternaria tenuissima*, with an inhibition zone of 40-60 mm (Table 1, Figure 1).

Table 1. Diameter of the zone of inhibition of bacteria *B. subtilis* TM in relation to phytopathogenic fungi.

Phytopathogenic fungi	Diameter of growth zone of inhibition, mm
<i>Fusarium verticillioides</i>	60
<i>Fusarium solani</i>	60
<i>Aspergillus orchaceus</i>	50
<i>Fusarium culmorum</i>	45
<i>Alternaria alternata</i>	40
<i>Alternaria tenuissima</i>	41
<i>Penicillium chrysogenum</i>	23
<i>Fusarium oxysporium</i>	22
<i>Cladosporium sp.</i>	12
<i>Aspergillus flavus</i>	0

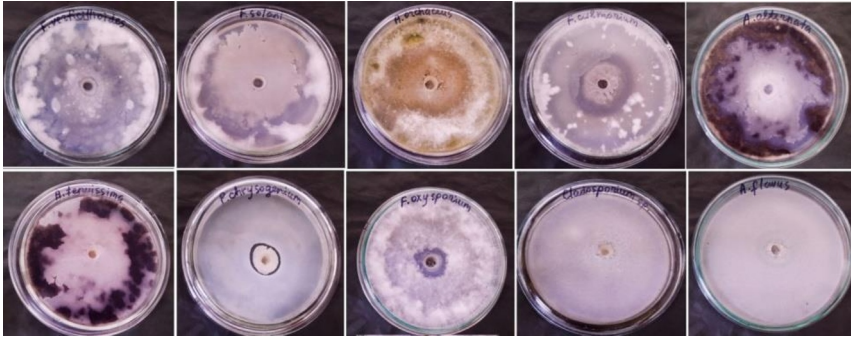


Fig. 1. Zones of inhibition of phytopathogenic fungi when exposed to bacteria *B. subtilis* TM.

At the same time, the *B. subtilis* TM strain showed moderate antagonistic activity against *Fusarium oxysporium*, *Penicillium chrysogenum* (with an inhibition zone of 22-23 mm) and relatively less antagonistic activity against *Cladosporium* sp (with an inhibition zone of 12 mm). However, it did not show antagonistic activity against *Aspergillus flavus*.

3.2 Study of morphological and biochemical properties

To study the morphological and biochemical properties, the *B. subtilis* TM culture was grown on 2% meat peptone agar (MPA) for 1-3 days. Next, the features of the colonies were studied in Petri dishes, and the morphology of the cells under a microscope.

Milky-white velvety colonies with uneven wavy edges and a viscous consistency were observed on the surface of the MPA (Figure 2). Gram-positive rods with a diameter of 0.5–0.7 μm and a length of 3.5–5.0 μm .



Fig. 2. Morphology of colonies and cells of *Bacillus subtilis* TM after 24 hours of incubation.

A study of the biochemical properties of the studied strains showed that the bacteria ferment sucrose, glucose, mannitol, fructose and do not ferment lactose, maltose, rhamnose, and galactose.

3.3 Strain identification

MALDI-TOF MS analysis was performed and based on the results obtained, this strain was tentatively classified as a *Bacillus* species. Next, for more accurate identification, the nucleotide sequence of the 16S rRNA gene of *Bacillus subtilis* TM was determined. The results showed 99.6% similarity with the species *B. subtilis*. The DNA sequences were then BLAST tested against the NCBI (National Center for Biotechnology Information) nucleotide database. The strain was registered with NCBI under the number: OR944512.1.

4 Conclusion

The *Bacillus subtilis* TM strain isolated from saline soils of the Fergana region showed antagonistic activity against the studied phytopathogenic fungi *Fusarium oxysporium*, *Fusarium verticillioides*, *Penicillium chrysogenum*, *Alternaria alternata*, *Alternaria tenuissima*, *Fusarium solani*, *Fusarium culmorum*, *Aspergillus orchaceus*, *Cladosporium sp.*, except *Aspergillus flavus*. Thus, in the future, the studied strain of *Bacillus subtilis* TM can be used to suppress fungal diseases of plants.

Thus, the studied bacterial strain *B. subtilis* TM can be used in various biological products as a biological control agent for a number of pathogenic fungi that cause diseases of agricultural crops.

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