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Antagonistic activity of cellulose-degrading bacteria isolated from soil and bovine waste against some phytopathogenic fungi

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ABSTRACT

Cellulolytic bacteria can be found in soil and ruminal wastes. The present study was conducted to isolate and identify cellulose degrading bacteria from forest soil and bovine waste and their screening for potential antifungal activity. The cellulolytic bacteria were isolated from the samples by serial dilution method on modified Czapeck (CMC) agar and following Congo red assay. Seventeen isolates were selected on the basis of cellulolytic activity through Congo red assay. The antifungal activity of these isolates was also determined against different phytopathogenic fungi including *Alternaria*, *Cladosporium*, *Verticillium*, *Fusarium*, *Mucor* and *Rhizopus*. The isolates were identified using standard biochemical tests according to Bergey's manual. Among the 17 isolates, 11 to *Bacillus* spp., 2 to *Pseudomonas* spp., 1 to *Citrobacter* spp., 3 to *Staphylococcus* spp. belonged. The antifungal activity against the target phytopathogens was shown by the 2 isolates of *Bacillus*. Isolate *Bacillus* spp. R7 from the bovine waste showed high activity against *Alternaria* by giving a zone of inhibition of 16 mm while isolate R1 from the forest soil showed antifungal activity against *Fusarium* by giving a clear zone of 13 mm. Based on the results of sequencing isolates R7 and R1 were most similar (more than 90% identity) to *Bacillus licheniformis* and *Bacillus Subtilis* strains, respectively. The results of the present study show that most cellulolytic bacteria isolated from soil and bovine waste belonged to the *Bacillus* that some of them had antifungal activity, so they are promising for biocontrol of phythopathogens and it is possible to use them as an effective strategy to manage plant diseases and protect the environment.

1. Introduction

Plant fungal diseases are one of the most important topics in agriculture and food production in the world. The decline in crop losses due to plant diseases it is estimated to be 25% and 50% in the West and developing countries, respectively, which one-third of these lossess are related to fungal diseases. The primary approach for control of fruits rotting fungi and other agricultural products is use of chemical fungicides. However, some of fungicides are not suitable for the treatment of these products and have been removed from use due to potential toxic effects on the environment and non-target organisms and possible carcinogenicity of some chemicals. In addition, due to the lack of public acceptance of these pesticides, resistance of some fungal pathogens and the high cost of producing new chemicals

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need further research to find suitable alternative methods such as biological control (Heydari and Pessarakli, 2010).

According to cellulose is a component in fungi cell wall and some microorganisms have been observed with cellulolytic activates, these microorganisms as a biocontrol for phythopathogenes have received attention of many researchers. Bacteria belonging to the genera *Clostridium*, *Cellulomonas*, *Cellulosimicrobium*, *Thermomonospora*, *Bacillus*, *Ruminococcus*, *Erwinia*, *Alteromonas*, *Bacteriodes*, *Acetovibrio*, *Streptomyces*, *Microbispora*, *Fibrobacter*, and *Paenibacillus* have been reported to produce cellulases (Liang et al., 2014, Seo et al., 2013). Therefore, it seems they could be a good candidate for biocontrol of phytopathogenic fungi. Many cellulolytic *Bacillus* species have been isolated from compost, swine waste and hot springs (Rastogi et al., 2010, Mawadza et al., 2000, Liang et al., 2009). In this study we aimed to isolate and identify cellulolytic bacteria from forest soil and bovine waste as a rich source for screening cellulolytic bacteria with antifungal activity against some phytopathogenic fungi.

2. Materials and Methods

2.1. Sample Collection

Ten gram forest soil and bovine waste were transferred to sterile zip lock bag. After collection all the samples were properly sealed, labeled and sent to laboratory of Gorgan Branch of Islamic Azad University, kept at 4°C (Ahmad et al., 2013).

2.2. Screening, Isolation and Purification

To screen and isolate cellulolytic bacteria, serial dilution and direct plating method was used on modified Czapeck agar medium (CMC medium). The sample was diluted up to 10^{-5} and then 1 mL of this concentration was spread on CMC agar plates. After incubation at 37°C for 2 days, the plates were flooded with 0.2 % (w/v) Congo red dye for 20 min (Congo red assay). Dye was discarded and the plates were flooded with 1 M NaCl for 20 min. Colonies were marked and subcultured on CMC agar (Ponnambalam et al., 2011).

2.3. Congo Red Assay for Cellulolytic Activity of Bacterial Isolates

The bacterial isolates were cultured on CMC agar plates in circular batches and were flooded with Congo red dye. The clear zones around the culture batches were measured for cellulolytic activity. The isolates with the highest zone diameter were selected and purified (Ghose, 1987).

2.4. Identification of Bacterial Isolates

Bacterial isolates were identified by using standard identification tests given in Bergey's Manual of Determinative Bacteriology (Bergey's Manual of Determinative Bacteriology, 2000).

2.5. Detection of Antifungal Activity

Antifungal activity of these cellulolytic isolates was determined by agar well diffusion method against some phytopathogenic fungi including *Alternaria*, *Cladosporium*, *Verticillium*, *Fusarium*, *Mucor* and *Rhizopus*. With a sterile cotton swab a diameter of 5 mm was cultured from the margin of the five-day mycelium culture on Potato Dextrose Agar (PDA) plates. The plates were allowed to dry for 15 min. Wells of 0.5 cm were punched on the media with the a sterile borer. The isolated colonies were inoculated in CMC broth and incubated for 48 hours at 37°C. Subsequently, the CMC broth was centrifuged at 10 000×g for 15 minutes to obtain cell-free supernatants (CFSs). The CFS was sterilized by passage through 0.45 µm Millipore filters. One hundred µL of the supernatant and Fluconazole (concentration 1mg/mL) was poured to the each well. Sterile distilled water was used as a negative control. After incubation the plates at 25-27 °C for 5 days, zones of inhibition for the supernatant and positive control were measured. The experiment was repeated three times (Delahaye et al., 2009).

2.6. Molecular Identification of cellulolytic bacteria

The bacterial isolate was cultured in nutrient broth overnight. Then, the genomic DNA of isolate overnight culture was extracted using Geno Plus TM Genomic DNA Extraction Miniprep System (Viogene, China). The

qualification and quantity of the extracted DNA was determined by agarose gel electrophoresis (1%). The genomic DNA was used as a template for 16S rDNA gene amplification using consensus primers. To do PCR, 2X Master Mix (Thermo scientific, USA) was applied. The reaction mixture was prepared by adding 1 µl of each primer (20 pmol), 5 µl of DNA template and 19 µl of double-distilled water (DDW). The PCR amplification was performed with 30 cycles of denaturation at 94˚C for 30 s, annealing at 56˚C for 30s, and extension at 72˚C for 30 s. The initial denaturation and final extension were 94˚C for 3min and 72˚C for 10min respectively. The primers used in this study are summarized in Table 1. The 16S rDNA genes were successfully amplified with consensus primers and anticipated fragments with 1500 bp were observed on 1% agarose gel. Then, the purified PCR products were sent to Macrogen (South Korea) for sequencing. The obtained nucleotide sequences were searched for homology in the NCBI nucleotide database using BLAST tool. The 16S rDNA gene sequences were aligned against the reference nucleotide sequences retrieved from GenBank (Ebrahimi and Ahani Azari, 2016).

Table 1: Primers used in this study (Weisburg et al., 1991).

Primer name	Primer sequence	PCR product
16S-F	5'-AGAGTTTGATCCTGGCTCAG 3'	1500bp
16S-R	5'-ACGGCTACCTTGTTACGACTT3'	

3. Results

Twentyseven cellulolytic bacteria were isolated from the samples which by further subculturing on Czapeck modified agar (CMC) 17 pure isolates were obtained. All the 17 bacterial isolates showed clear zones around colonies. The bacterial isolates were Gram-positive rods and cocci and Gram-negative rods. Out of the 17 isolates, 11 and 3 isolates were considered to be *Bacillus* and *Staphylococcus* species, respectively. The rest 3 isolates were belonged to *Pseudomonas* and *Citrobacter* species. Among these isolates, only two isolates showed activity against each of the target fungal pathogens. Against *Alternaria*, *Bacillus* spp. R7 from bovine waste showed high activity by forming a zone of inhibition of 16 mm. While for *Fusarium*, *Bacillus* spp. R1 from forest soil showed antifungal activity by forming a clear zone of 13 mm.

Based on the results of sequencing isolates R7 and R1 were most similar (more than 90% identity) to *Bacillus licheniformis* and *Bacillus Subtilis* strains, respectively.

4. Discussion

Considering that Golestan province is one of the important agricultural hubs in Iran in order to reduce the use of plant pesticides that are used to control pathogenic fungi in this region, in this research we aimed to isolate and identify cellulolytic bacteria with antifungal activity against some phytopathogenic fungi. Out of 17 isolates with cellulolytic activity, only 2 isolates belonging to *Bacillus* genus showed antifungal activity against *Alternaria* and *Fusarium* which after sequencing of 16S rRNA gene, it was found that these isolates were more than 90% similar to *Bacillus licheniformis* and *Bacillus Subtilis* strains.

In consistent with our study, Lynd et al. reported both *Bacillus* and *Pseudomonas* species are capable of cellulases production (Lynd et al., 2002). Rastogi et al. isolated and characterized cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. The cellulose-degrading bacteria belonged to the genera *Brevibacillus*, *Paenibacillus*, *Bacillus*, and *Geobacillus* (Rastogi et al., 2009). In a study in Pakistan, Ahmad et al. isolated and identified cellulose degrading bacteria from municipal waste. Among the isolates, *Staphylococcus* spp. and some *Bacillus* species exhibited antifungal activity against the *Aspergillus niger* and *Candida albicans* (Ahmad et al., 2013).

Table 2. Mean diameter of growth inhibition zones (mm) caused by supernatant of cellulolytic isolates

Sample type	Isolates	*Alternaria*	*Fusarium*	*Cladosporium*	*Mucor*	*Rhizopus*	*Verticillium*
Soil	*Bacillus* spp. R1	0	13	0	0	0	0
Soil	*Bacillus* spp. R2	0	0	0	0	0	0
Soil	*Bacillus* spp. R3	0	0	0	0	0	0
Soil	*Bacillus* spp. R4	0	0	0	0	0	0
Soil	*Bacillus* spp. R5	0	0	0	0	0	0
Soil	*Bacillus* spp. R6	0	0	0	0	0	0
Bovine waste	*Bacillus* spp. R7	16	0	0	0	0	0
Bovine waste	*Bacillus* spp. R8	0	0	0	0	0	0
Bovine waste	*Bacillus* spp. R9	0	0	0	0	0	0
Bovine waste	*Bacillus* spp. R10	0	0	0	0	0	0
Bovine waste	*Bacillus* spp. R11	0	0	0	0	0	0
Soil	*Pseudomonas* spp. R1	0	0	0	0	0	0
Soil	*Pseudomonas* spp. R2	0	0	0	0	0	0
Soil	*Staphylococcus* spp. R1	0	0	0	0	0	0
Soil	*Staphylococcus* spp. R2	0	0	0	0	0	0
Soil	*Staphylococcus* spp. R3	0	0	0	0	0	0
Soil	*Citrobacter* spp.	0	0	0	0	0	0

In Pakistan, Qazi et al. isolated 6 strains of *Bacillus cereus* exhibiting antifungal activity against *Saccharomyces cerevisiae* and *Candida albicans* (Qazi et al., 2009). Silva et al. isolated cellulolytic bacteria from typical fruit of Cerrado in Minas Gerais State, Brazil. After sequencing the bacterial isolates with an antagonistic effect were identified as *Enterobacter aerogenes*, *Lysinibacillus fusiformis*, *Klebsiella oxyt*oca and *Hafnia alvei* (Silva et al., 2015). Petatán-Sagahón et al. isolated bacteria with antifungal activity against the phytopathogenic fungi *Stenocarpella maydis* and *Stenocarpella macrospora*. These bacteria were identified as *Bacillus subtilis*, *Pseudomonas* spp., *Pseudomonas fluorescens*, and *Pantoea agglomerans* (Petatán-Sagahón et al., 2011). In a study by Seo et al., *Bacillus licheniformis* JK7 with cellulolytic and xylanolytic activity was isolated from the rumen of a native korean goat (Seo et al., 2013). In another study *B. licheniformis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus laterosporus* and three isolates resemble to *Bacillus pumilus* were

isolate from the rumen contents of animals fed on hay (Williams and Withers, 1983). The results of two latter studies about presence of *B. licheniformis* in the rumen contents of animals is in agreement with the finding of the present study as a cellulolytic bacterium. In Korea, Han et al. isolated *Bacillus* spp. strains with antagonistic activity towards anthracnose pathogens *Colletotrichum acutatum* and *C. gloeosporioides* (Han et al., 2015). The present study and other investigations conducted by other researchers suggest that most cellulolytic bacteria isolated from soil and bovine waste belonged to the *Bacillus* and they may also have a potential to produce antifungal metabolites against phythopathogenic fungal species and used as a biological control of plant diseases.

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