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Isolation and Identification of *Bacillus* Species From Soil and Evaluation of Their Antibacterial Properties

Mansour Amin [1]; Zeinab Rakhisi [2,*]; Amanollah Zarei Ahmady [3]

[1]Health Research Institute, Infectious and Tropical Diseases Research Center, Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran
[2]School of Basic Sciences, Islamic Azad University, Fars Science and Research Branch, Shiraz, IR Iran
[3]Nanotechnology Research Center, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran

*Corresponding author: Zeinab Rakhisi, School of Basic Sciences, Islamic Azad University, Fars Science and Research Branch, Shiraz, IR Iran. Tel: +98-9166319380, E-mail: zeinabrakhisi@yahoo.com

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Background: *Bacillus* species are the predominant soil bacteria because of their resistant-endospore formation and production of essential antibiotics such as bacitracin.
Objectives: The aim of this study was to isolate *Bacillus* spp. from riverside soil and investigate their antimicrobial characteristics against some pathogenic bacteria.
Materials and Methods: Fifty soil samples were collected from different sites of Bahmanshir riverside in Abadan city, Iran, and analyzed for the presence of *Bacillus* species. The media used in this research were nutrient broth and agar. *Bacillus* species were identified by their phenotypic and biochemical characteristics. The antimicrobial effects of *Bacillus* extract against the target bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae* and *Corynebacterium diphtheriae* were examined.
Results: The identified *Bacillus* species included *B. cereus* (86.6%), *B. subtilis* (6.6%), *B. thuringiensis* (3.3%), and *B. pumilus* (3.3%). Evaluation of the antimicrobial activity of the extracted compounds was carried out against five different bacteria. Antibiotic production tests indicated that two *Bacillus* strains belong to *B. cereus*, which showed antimicrobial properties. The minimum inhibitory concentrations (MICs) of these compounds ranged between 8.34-33.34 mg/mL for the target bacteria.
Conclusions: This study indicated that some *Bacillus* species have the potential to produce antimicrobial compounds which can be used to control microbial infections.

Keywords: Soil; Bacteria; Anti-Infective Agents; *Bacillus*

1. Background

Bacillus species are Gram-positive, endospore-forming, chemoheterotrophic rod-shaped bacteria which are usually motile with peritrichous flagella; they are erobic or facultative anaerobic and catalase positive (1). Members of the *Bacillus* genus are generally found in soil and represent a wide range of physiological abilities, allowing the organism to grow in every environment and compete desirably with other organisms within the environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms (2).

Many *Bacillus* species are of remarkable importance because they construct antibiotics (1). The potential of *Bacillus* species to synthesize a wide variety of metabolites with antimicrobial activity has been widely used in medicine and pharmaceutical industry; one of its abilities is to control various diseases in animals, humans and plants when applied as a biological control agent (3-5). Owing to the fact that *Bacillus* species have constructed antibiotics in the soluble protein structure and that these antibiotics have been inexpensive and more effective in studies accomplished to date, these microorganisms are desirable for commercial production (6, 7). In recent years, many investigations have utilized the antimicrobial properties of *Bacillus* strains (8-12). In a study, Al-Ajlani determined that 54 of the 118 *Bacillus* strains isolated from soil samples demonstrated antagonistic activities against at least two or more strain from a panel of pathogenic and nonpathogenic microorganisms (13).

2. Objectives

This study identified *Bacillus* strains isolated form different regions of Bahmanshir riverside in Abadan city, Iran, and examines them with respect to their antimicrobial properties.

3. Materials and Methods

3.1. Collection and Preparation of Soil Sample

Fifty Soil sample from September 2013 till March 2014

were collected from different regions of Bahmanshir riverside. The samples (approximately 4 g each) were collected using some clean, dry and sterile polythene bag along with sterile spatula. All the samples were transferred to lab; under sterile conditions, 1 g of each soil samples was added to 5 mL of nutrient broth and incubated at 35°C for 24 hours.

3.2. Isolation of Bacillus spp.

After the incubation period, 0.1 mL of the supernatant of each tube containing suspension of soil and culture media were inoculated in nutrient agar plates by streaking at 30°C for 24 hours. After that, the plates were examined and the suspected colonies were stained by Gram staining method. The Gram-positive, rod-shaped, spore forming bacilli were selected for additional identification tests. Subsequent identification tests including susceptibility test to penicillin, citrate hydrolysis, motility, Voges-Proskauer VP, Indole production, catalase, nitrate reduction, and production of H_2S were performed.

3.3. Antimicrobial Compound Extraction From Bacillus spp.

Each isolate was cultured in tryptic soy broth (TSB) (Merck, Germany) medium and incubated at 30°C for 48 hours; then, the antimicrobial compound was extracted using three methods.

Method 1: after incubation, a part of the culture medium was directly mixed with ethyl acetate (50:50) and then stirred using a magnetic stirrer for six hours. The upper organic layer was separated using a separating funnel and centrifuged at 5000 rpm for 10 minutes. The ethyl acetate layer was then removed and transferred into a clean flask. The extract was pooled and dried in a rotary evaporator (Heidolph, Germany) at 50°C. The yield from the extract was dissolved in ethanol for antimicrobial susceptibility testing.

Method 2: the second part of the medium was shocked by boiling in water for five minutes and then putting in cold water for five minutes. Afterwards, the extraction was followed by adding ethyl acetate similar to the first method.

Method 3: the third part of the culture medium including bacteria was stressed using an ultrasonic devise for three minutes (160 W); then, similar to the first method, extraction of the antimicrobial compound was performed.

3.4. Antimicrobial Activity of Isolated Bacillus spp.

Three kinds of obtained extracts using disc diffusion method were tested against pathogenic bacteria including *Staphylococcus aureus* (PTCC 1112), *Shigella dysenteriae* (PTCC 1188), *Escherichia coli* (PTCC 1396), *salmonella typhi* (PTCC 1609), and *Corynebacterium diphtheriae* (ATCC 27010) (14). The minimum inhibitory concentration (MIC) of *Bacillus* spp. extracts (the ones showing antimicrobial activity) was determined against pathogenic bacteria base on modified E. test method (AB Biodisk Solna, Sweden). The microbial suspensions of freshly grown cultures were prepared in sterile saline and adjusted to a density of 106 cell mL^{-1}, corresponding to 68 to 82% transmittance at 530 nm. The plate of Mueller Hinton Agar, Hi Media (MHA) was inoculated by dipping a sterile cotton swab into the cell suspension and streaking it across the agar surface in three directions. The plates were dried at ambient temperature for 15 minutes before applying the discs. Eight sterile discs (6 mm) were put on the agar surface in a line. The bacterial extract was serially diluted in methanol and 10 μL of each dilution was separately used to impregnate the discs. The plates were incubated for 18 hours at 37°C. The MIC values were read as the antimicrobial concentrations at the points where dense colonial growth intersected the discs. The test was performed in quadruplicate for each culture (15).

4. Results

This study was on the basis of morphological and biochemical characteristics and conventional techniques according to Bergey's manual of determinative bacteriology (16, 17). Among 50 soil samples, only 30 strains of *Bacillus* spp. were isolated. These bacteria were classified in four spices including *B. cereus* (86.6%), *B. subtilis* (6.6%), *B. thuringiensis* (3.3%) and *B. pumilus* (3.3%).

Two strains of *B. cereus* out of 15 extracted strains showed antimicrobial activity. The MICs obtained on the basis of modified E. test method ranged from 8.34 to 33.34 mg/mL against the target bacteria (Figure 1 and Table 1).

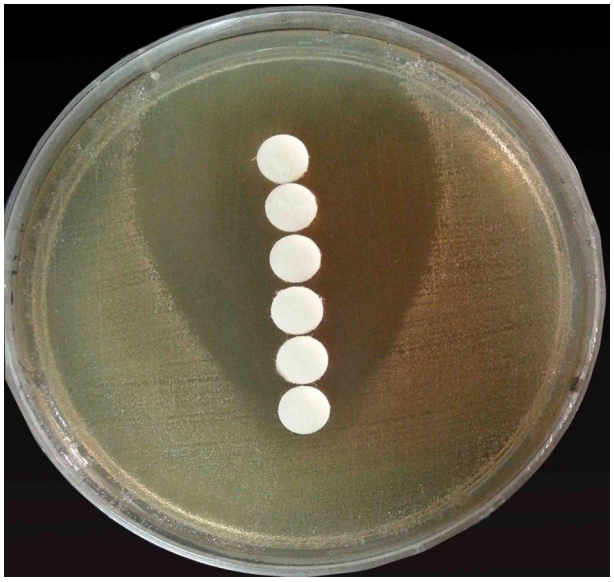

Figure 1. E Test Representing Minimum Inhibitory Concentration of Antimicrobial Extract Against *Shigella dysenteriae*

Table 1. Minimum Inhibitory Concentrations of Antimicrobial Substance Extracted From two *B. cereus* Species Isolated From Soil Against the Target Bacteria (mg/mL)

Number of *B. cereus* **Strains**	*S. aureus*	*E. coli*	*S. typhi*	*C. diphtheria*	*S. dysenteriae*
3-7	20.67	16.34	16.67	25.00	8.34
5-7	33.34	33.34	20.84	33.34	10.41

5. Discussion

Screening for new antibiotics from natural sources is becoming increasingly important for the pharmaceutical industry (18), as pathogenic bacteria are significantly becoming resistant to generally used curative agents (19).

Antibiotic production is a feature of several kinds of soil bacteria and fungi and may represent a survival mechanism where organisms can eliminate competition and colonize a niche (20).

The present study was carried out to evaluate the production of antibiotic from newly isolated *Bacillus* specie from soil. The obtained results showed that two isolated strains of *B. cereus* have the potential for producing antimicrobial substances. We partially purified these compounds using ethyl acetate. The MIC values are scientific and significant factors for evaluating the potential of these antimicrobial substances. This method also could compare natural obtained antimicrobials with commercial antibiotics. The researchers mostly use well or disc diffusion methods for evaluating the activity of antimicrobial substances. However, this cannot be a good method for comparing the antimicrobial activities of compounds. Antimicrobial compounds in our study showed inhibitory effects against some Gram-negative and Gram-positive organisms.

Al-Ajlani et al. (13) in his research discovered the production of antibacterial substances by *Bacillus* sp. Prescott et al. (21) reported that the bacitracin produced by *Bacillus* sp. inhibits *E. coli* and *S. aureus,* which confirmed the results of our study. However, Oscariz et al. (22) reported that *B. cereus* strain isolated from soil was active against most Gram-positive, but not Gram-negative bacteria. Aslim et al. (23) demonstrated that *Bacillus* strains had greater effects on Gram-positive bacteria than on Gram-negative bacteria. In a study by Basurto-Cadena and his team, *B. subtilis* isolated from soil showed inhibitory activity in vitro and under greenhouse conditions against several fungi with economic importance including *Verticillium* sp. and the agent of "Secadera disease" and also showed that it produces extracellular compounds such as proteases and bacteriocin-like inhibitors which could be implicated in antagonistic activities against fungi and food-borne pathogenic bacteria (24). In all of these studies, the inhibition zone around the discs was measured, whereas in our study the MIC values of antimicrobial compounds were measured. It is concluded that *Bacillus* spp. isolated during the course of this study from the soil samples possessed antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria.

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Authors' Contributions

Study supervision: Mansour Amin; Drafting of the manuscript: Zeinab Rakhisi; Analysis and interpretation of data: Amanollah Zarei Ahmady.

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