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Heat-shock Technique for Isolation of Soil *Bacillus* Species with Potential of Antibiotics Secretion in Saudi Arabia

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

The present research aimed to isolate soil *Bacillus spp.* from Al-Ahsa Province, Kingdom of Saudi Arabia, and test their potential of antibiotics production. Out of 21 soil samples, 34 bacterial isolates were obtained and following identification tests, 26 bacterial isolates in an isolation rate of 76.5% were identified as *Bacillus spp* by conventional methods and commercial automated identification system. Identified species were *Bacillus subtilis* (7 strains), *B. polymyxa* (4 strains), *B. licheniformis* (4 strains), *B. cereus* (3 strains), *B. mycoides* (3 strains), *B. pumilus* (1 strains), and *Bacillus spp.* (4 strains). Disk diffusion test was employed to test the metabolites in sterilized cell free supernatant of isolates against some selected pathogenic bacterial species. The test indicated that *B. polymyxa, B. licheniformis, B. cereus, B. mycoides* and to a lesser degree *B. subtilis* produced promising anti-microbial effects on tested bacterial species comparable to standard antibiotic disk.
The study suggests that some of *Bacillus spp* from the study area have potential to produce antibiotics that may be used to control microbial growth.

Keywords: Antibiotics; soil; heat treatment; Bacillus species; gram-positive; Saudi Arabia.

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1. INTRODUCTION

Bacillus species are Gram-positive aerobic or facultative anaerobic, rod shaped, catalase positive, motile with peritrichous flagella, spore-forming bacteria which are widely distributed in nature all over the World. Some species may be involved in human and animal disease. They exhibit a wide range of physiologic capabilities that allow the species to live in every environment competing favourably with other organisms co-existing therein. Such capabilities are brought about by forming resistant spores that withstand adverse environmental conditions and production of metabolites with various biochemical activities [1].

Classical methods of screening soil to obtain suitable organisms for a potential biological process, are time consuming, expensive and results may be unpromising. If a desired characteristic which gives the organism a selective advantage has already been recognized, a screen might be designed including this characteristic as a selective factor. The isolation may begin with pretreatment with suitable substances to favour the survival of the preferred organism. This is followed by growth on selective or non selective media and often associated with enrichment [2]. Thus screening may be defined as the use of highly selective procedures to allow the detection and isolation of only those microorganisms of interest from among a large microbial population. Bacilli compete favourably with other organisms mainly due to the ability to form spores that are heat stable. Soil is a suitable ecosystem for a variety of microorganisms making selective isolation of a particular organism a must. Heat treatment of soil specimens may lead to inhibition of mesophilic non-spore-forming bacteria to favour *Bacillus spp.* selection [3].

The primary aims of modern identification systems are that they reduce identification time, increase throughput of samples and achieve these within a highly automated, reliable and flexible piece of laboratory equipment [4]; they must have accurate and robust databases, derived from satisfactory classifications of all included organisms.

The VITEK 2 Automated System (bioMérieux, Marcy L'Etoile, France) is one of the most widely used instruments in clinical microbiology laboratories for the identification of Gram positive and negative bacteria up to species level. In 2004, the company launched an enhanced Bacillus spp. identification card (BCL). The BCL card contains 46 test substrates based on carbon source utilization, enzymatic activities, inhibition of growth by 6·5% sodium chloride and resistance to the antibiotics oleandomycin, kanamycin and polymyxin B. Identifications are made after 14 h incubation [5].

After the discovery of penicillin and other antimicrobial agents by Fleming in 1928 [6], antibiotics have been recognized as a useful means of microbial growth control [7]. Since then, there has been continuous search for more effective antibiotics that can fight the emerging drug resistance trend among microorganisms worldwide [8]. The number of multi-resistant bacterial strains as etiological agents of infectious diseases has increased at an alarming rate, challenging physicians to find effective therapy that ensures effective result [9]. Resistance to antibiotics has resulted in an increase in morbidity and mortality from treatment failures [10] and leads to the steady decline of available effective antibiotics [7]. The increases in antibiotics resistant may be due to inappropriate use and inadequacies of the manufacturing process [11]. The production of secondary metabolites with antibiotic properties is a common characteristic of *Bacillus spp*. [12].

Thorough search of the literature revealed that there were no investigations on anti-microbial agents from soil bacilli in the Kingdom of Saudi Arabia (KSA). Therefore, the present investigation aim was to isolate and identify Bacillus species from soil of Al-Ahsa Province, KSA, with potential of antibiotic production.

2. MATERIALS AND METHODS

2.1 Collection of Soil Samples

Upper soil samples to a depth of 10 – 15 centimeters were obtained from different regions of Al-Ahsa town. A total of 21 soil samples were collected during the period April 2015 till June 2015 between the hours of 7:00 am and 11:00 am in the morning. The samples (approximately 10 g each) were collected using some clean, dry and sterile polythene bag along with sterile spatula. All the samples were transferred to the laboratory under sterile conditions and were analyzed in the Bacteriology laboratory, College of Veterinary Medicine, King Faisal University, KSA.

2.2 Inoculation

Four grams of sample was suspended in 96 ml of sterile distilled water and shaken vigorously for 2 min. The samples were heated at 60℃ for 60 min in a water bath. Then the soil suspensions were kept on bench surface at room temperature, for two hours, for soil particles to settle. A measured volume (100 µl) of each soil samples was plated on nutrient agar (NA) medium using streak method of inoculation. The plates were incubated at 30℃ for 24-48 h. Colonies with different morphological appearance were sub-cultured onto fresh NA for the purpose of identification.

2.3 Presumptive Identification of the Bacillus Isolates

The isolates were identified using Gram staining, catalase test, motility, starch hydrolysis, voges proskauer, citrate utilizations tests, endospore staining, and haemolysis test. The isolates were subjected to confirmatory test to the genus level. It was achieved by plating the isolates on egg-yolk agar and mannitol salt agar respectively. The colonies that failed to grow were regarded as non bacillus species.

Confirmation of the isolates identification to the species level was done by the VITEK 2 Automated System.

2.4 Antibiotic Production Test

Each isolated Bacillus spp. was cultured under aerobic condition in nutrient broth for 72 hours at 30℃ in shaking incubator at 125 rpm. After incubation, sample was centrifuged for 15 min at 10,000 rpm by bench top centrifuge to get cell free supernatant. The cell free supernatant was sterilized using 0.22 µ membrane filter and then put into sterile Eppendorf tubes. Six mm sterile filter paper disks were dipped in each of the sterile supernatant in Eppendorf tubes and dried in a vacuum drier for 10 minutes. Agar disk diffusion assay was used to check the production of antimicrobial compounds according to guidelines given by the Clinical and Laboratory Standards Institute [13]. The following test organisms were used to assess the production of antibacterial compounds: *Staphyloccous aureus* (strain SA 011), *Corynebacterium* species (Strain Co 515), *Escherichia coli* (strain EC 114), *Pseudoymonas aeruginosa* (strain 16009). All the test organisms were isolated in our laboratory from clinical specimens referred from the Veterinary Teaching Hospital, King Faisal University except strain EC 114 which was isolated from a case of urinary tract infection in man. Test organisms were selected on basis of pathogenicity and antibiogram testing in routine laboratory diagnostic procedures. Standard antibiotic disk of Erythromycin (E; 15 mg, Oxoid) was used as control.

3. RESULTS

Out of 21 soil samples, 34 bacterial isolates were obtained and following identification tests, 26 isolates were identified as Bacillus spp. Identified species were *Bacillus subtilis* (7 strains), *B. polymyxa* (4 strains), *B. licheniformis* (4 strains), B. *cereus* (3 strains), *B. mycoides* (3 strains), *B. pumilus* (1 strains), and *Bacillus spp.* (4 strains), (Table 1).

Table 1. Soil Bacillus species and frequencies of recovery from Al-Ahsa Province, KSA

Isolated species	Number of strains	Percent of isolation
Bacillus subtilis	7	26.93
B. polymyxa	4	15.38
B. licheniformis	4	15.38
B. cereus	3	11.54
B. mycoides	3	11.54
B. pumilus	1	3.85
Bacillus spp.	4	15.38

Table 2. Antibiotics activity of isolated soil *Bacillus spp.* strains against gram positive and negative test bacterial species from Al-Ahsa Province, KSA

***Bacillus* species**	**I.Z. *Staph aureus***	**I.Z. *Corynebacterium***	**I.Z. *E. coli***	**I.Z. *P. aeruginosa***
B. subtilis	17	14	18	7
B. polymyxa	21	19	12	10
B. licheniformis	6	9	26	18
B. cereus	22	16	23	16
B. mycoides	24	12	8	4
B. pumilus	8	7	5	3
Bacillus spp	11	8	17	12

Key: I.Z.= Inhibition zone in mm

Results of antibiotic production by the isolated Bacillus strains are displayed, as average diameter of inhibition zone produced by the strains against test organisms, on Table 2. Anti-microbial sensitivity of E against test organisms is shown on Table 3.

Table 3. Anti-microbial reaction of a standard control erythromycin disk against test organisms

Test organisms	I.Z. by control antibiotic erythromycin
Staph aureus	19
Corynebacterium spp.	18
E coli	28
P aeruginosa	12

Key: I.Z.= Inhibition zone in mm. Erythromycin, 15 mg, (Oxoid)

4. DISCUSSION

Bacillus spp. are known to inhabit soil, because the organisms are known to withstand both high and low temperature condition. This special feature presented by *Bacillus spp* gives the organisms advantage over other bacterial species co-existing in the environment [14]. Bacilli have been used widely in industrial applications, particularly production of antibiotics for medical and veterinary use [15]. The main objective of the present investigation was to bio-prospect for thermophilic bacilli from soil of Al-Ahsa Province, KSA and to test their ability to produce anti-microbial agents.

Thermal shock technique has been used for isolation of *Bacillus spp*. in the present study. Non-spore-forming microorganisms were minimized by heat treatment at 60℃ for 60 min thus avoiding the problem inherent in enrichment culture. A total of 34 bacterial isolates were recovered from the soil samples; 26 of which were confirmed as Bacillus spp. in an isolation rate of 76.5%. This high recovery rate of *Bacillus spp*. proved the method to be rapid and efficient in isolation of Gram positive spore-forming bacilli and in suppression of other microbial organisms. Other investigators reported identification of 30 Bacillus spp. out of 40 bacterial isolates after heat treatment of soil [16]. Furthermore, a number of soil bacilli in Malaysian soil exhibited quorum quenching activity was isolated using heat treatment of soil which was demonstrated to be rapid and efficient [17]. Microscopically, *Bacillus spp*. are large rods with spores of different size and shape. Cultural characteristics displayed by bacteria on agar media, are used as an auxiliary means to identify bacteria because of their different and specific growth patterns [18]. Confirmation of the isolates to the species level, in the present study, using the automated VITEK2 system was easy, quick and reliable. In a previous study, [5] reported that the VITEK2 BCL card provides a major advance in the reliable identification of Bacillus species and members of related genera.

Using morphological, cultural and biochemical methods, 26 *Bacillus spp*. were isolated and confirmed with *B subtilis* as the most frequent strains, as shown on Table 1. Other workers reported isolation of similar species from soil samples elsewhere [19,20].

Antibiotics production, in the present study, revealed that all the isolated strains produced inhibitory effects at different rates on test bacterial species (Table 2). Antibacterial effects observed could be attributed also to bacteriocins. The method described to isolate the antibacterial agents does not rule out this possibility. Bacteriocins usually show a high degree of specificity against target bacterial species, although some have a broad spectrum of activity [21]. Bacteriocin Type A kills Gram-positive target cells by forming voltage-dependent pores into the cytoplasmic membrane. This group includes subtilin produced by *B. subtilis* ATCC 6633 which inhibits a broad range of Gram-positive bacteria including other species of *Bacillus* [22]. For instance, *P aeruginosa* showed resistance to anti-microbial agents produced by all strains together with the E control disk. *B. subtilis* and *B. polymyxa* produced similar effects on Gram positive and negative bacteria. Their effect on Gram positive is comparable to E control, while on Gram negative E is better. This is in agreement with [16] who documented that Bacillus strains had greater effects on Gram-positive bacteria than on Gram-negative bacteria. In contradiction, it was reported that the antibiotic bacitracin produced by Bacillus sp. inhibits *E. coli* and *S. aureus* [23]. Generally, most isolated strains, in the present study, produced I.Z. between 17 – 24 mm against *S. aureus.* Other workers documented that Bacillus spp. maximum activity against *S. aureus* was I.Z. of 18 mm [24]. In the present study, *B. licheniformis* produced narrow I.Z. on all tested organisms except *E. coli,* in which it produced I.Z. of 26 mm diameter similar to I.Z. produced by the control E (28 mm). *B. cereus* produced I.Z. of 22 and 23 mm against

S. aureus and *E. coli* respectively; and I.Z. of 16 mm against *Corynebacterium spp* and *P. aeruginosa* both. [25] isolated and identified a bacteriocin-producing strain of *B. cereus* from a soil sample that was active against most Gram-positive but not Gram-negative bacteria. Another study reported that *B. cereus* M15 has inhibitory effect against both Gram-positive and Gram-negative bacteria [19]. Discrepancy between results of the present study and other studies may be explained by differences in strains used, culturing and isolation techniques and methods of obtaining anti-microbial agents.

The present study is a preliminary survey on soil bacilli in the study area, yet it has revealed the potential of Bacillus strains on anti-microbial agents' production. This opens the door for further research on isolation, purification and identification of the metabolites to determine possibility of using in medical treatment.

5. CONCLUSION

In conclusion, analysis of soil samples from Al-Ahsa Province, KSA, using heat shock technique led to isolation of 26 *Bacillus spp*. strains. The technique proved to be easy, quick and efficient for the purpose. These were *Bacillus subtilis* (7 strains), *B. polymyxa* (4 strains), *B. licheniformis* (4 strains), B. *cereus* (3 strains), *B. mycoides* (3 strains), *B. pumilus* (1 strain), and *Bacillus spp*. (4 strains). Test for production of anti-microbial agents against test organisms, revealed promising ability comparable with the standard antibiotic disk erythromycin. Further work is needed for purification, chemical characterization and testing metabolites from the soil strains.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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