# Antimicrobial activities of some thermophiles isolated from Jordan hot springs

Khalid Fandi, Nayef Al-Muaikel, and Fouad Al-Momani

***Abstract*****—**The main object of this study was to screen thermophilic bacterial strains with selective antimicrobial potential from Ma'en thermal springs in Jordan. Therefore, a collection of aerobic thermophilic strains of the genus *Bacillus* was isolated using different culturing media as carbon sole sources. In a biological screening the crude extracts obtained from the culture broths by organic solvent of selected strains were analysed for their antimicrobial activity against a set of organisms including Gram-negative, Gram-positive bacteria, fungi and microalgae using agar well diffusion assay. Additionally, a cytotoxicity test was performed by means of the brine shrimp microwell cytotoxicity assay. In a chemical screening each of the crude extracts was analysed by TLC using various staining reagents. The results depicted an impressive chemical diversity of crude extracts produced by these strains. The extracts of Cell Free Supernatant (CFS) of three thermophilic bacterial strains D5, D8 and C1 were found to inhibit the growth of test organisms. The strain C1 showed maximum inhibition against fungal plant pathogens. Therefore, the working on these strains could be promising to isolate active new metbolites in the future.

***Keywords*****—**Thermophilic bacteria. Antimicrobial activity, TLC

## I. Introduction

PRODUCTION of antimicrobial compounds seems to be a general phenomenon for most bacteria. The prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. In recent years, identifying new ecological niches like thermophiles, insects, endosymbionts are providing access to new organisms and novel bioactive chemicals [1].

Antibiotics, in one form or another, have been in use for centuries. The vast majority of novel antibiotics have been detected by screening of "wild isolates" obtained from soil and other natural habitats. Antibiotics, as secondary metabolites, are generally produced by multi-step biosynthetic pathways starting from intermediates of primary metabolism to specific moieties. The genus of *Bacillus* species produce a large number of biological compounds active against bacteria, fungi, protozoa and viruses with various chemical properties [2]. In spite of the great attention to microorganisms living under extreme environmental conditions, including thermophiles and numerous studies of their physiology, genetics, and biochemistry [3], the secondary metabolism of thermophilic microorganisms is poorly understood. Antimicrobial secondary metabolites occur in some species of thermophilic actinomycetes, but thermophilic *bacillus* strains are yet to be exploited properly for novel and stable antimicrobial compounds, including peptides [4], [5]. The objective of the present study was therefore to isolate and characterize thermophilic bacterial strains from local recourses of hot springs in Jordan and screen for their antimicrobial activity against selected bacteria and fungi to test effects of different culture conditions and media as a carbon source on the production of active metabolites of selected strains.

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## II. Materials and Methods

### *A. Bacterial isolation*

Water samples were collected from five main thermal springs located in Zara and Main areas in Jordan using 200 ml sterile thermal glass containers. The samples were collected under 30 cm below the surface, away from the margin in order to be representative samples. Thermophilic bacteria were isolated and purified using the streaking method on thermus agar containing 0.5% NaCl, 1.5% peptone, 0.5% beef extract, 0.2% yeast extract and 2% agar, the pH of the medium was adjusted to 7.2 before autoclaving plates were incubated at 55°C for 48 hours, and purity of the colonies were checked microscopically.

### *B. Cultivation and extractiont*

Cultivation was carried out using Erlenmeyer flasks (1 L) containing 250 mL medium. Five different media (modified at Laatsch's lab) were used to determine the most effective medium for mass cell production of thermophiles. Each flask was inoculated with a small piece of agar slants of the strain. The flasks were incubated at 55°C on a linear shaker at 180 rpm for 3-5 days. After filtration, the cells were extracted with ethyl acetate under ultrasonic irradiation. The water phase was twice extracted with ethyl acetate. The organic phases were combined and evaporated to dryness under vacuum. For quantification, the crude extract was brought to 1 mL with 3:2 chloroform/methanol and stored in a refrigerator at –20°C.

### *C. Antimicrobial activity test*

The antimicrobial activity was determined by disk diffusion bioassays against a set of test organisms including Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis)*, fungi (*Candida albicans, Mucor miehei Rhizoctonia solani Pythium ultimum* and *Aphanomyces cochli-oides*. The test plates for bacteria were prepared by pouring 14 ml of L-agar

[6] as base layer; after solidifying, this was overlaid with 4 ml of the inoculated seed layer. The test plates for fungi were prepared by pouring 14 ml of M2 medium as base layer. After solidifying, this was overlaid with 4 ml of the agar inoculated with fungal spores as seed layer. Paper disks with diameter of 9 mm were impregnated with 40 µl of crude extract solution (crude extracts 1 µg/µl dissolved in CH2Cl2/MeOH 1:1); the disks were dried under sterile conditions and placed on the surface of test plates. The bacterial and fungal test plates were incubated at 37°C for 16–24 hours, while the algal test plates were incubated at room temperature in artificial daylight for 96 hours. After incubation, the diameter of inhibition zones was measured in mm.

*D. Cytotoxicity assay*

Toxicity assay was carried out using brine shrimp mortality test according to the Meyer method with slight modifications [7]. The eggs of *Artemia salina* were hatched in a beaker filled with artificial seawater. Seawater (195 µL) containing 30–40 nauplii was added to each well of a 24- well plate. A 5-µL solution of the crude extract dissolved in dimethyl sulfoxide (DMSO) with the respective concentration of 18 mg/mL, 3 mg/mL, 0.6 mg/mL, 125 µg/mL, and 25 µg/mL was inoculated to each well. Actinomycin D (5 µL) was used as a positive control. DMSO (5 µL) was applied as a negative control. After incubation for 24 hours and 48 hours, the mortality of brine shrimps was calculated.

*E. Thin layer chromatography (TLC)*

The crude extracts obtained from the selected strains were analysed by TLC. In this method a small drop of a sample was spotted onto the TLC plate with a capillary and dried; the spotting process was repeated by superimposing more drops on the original spot for obtaining appropriate quantity (2–5 µg) of the sample on the plate. The TLC plates were developed with a CH2Cl2/5% MeOH solvent system. Zones on the developed plates were visualized under u.v. light (at 254 nm and 366 nm) and the components showing u.v. absorption or fluorescence were marked. Later the TLC plates were sprayed with anisaldehyde/sulfuric acid or Ehrlich's reagent (detection of indoles) for the further localization of interesting zones. The colored bands (metabolic finger-print) produced by the reaction between spray reagent and the metabolites were marked and documented by scanning.

TABLE I
RESULTS OF BIOLOGICAL SCREENING (ANTIMICROBIAL ACTIVITY AND CYTOTOXICITY TESTS

| Thermophiles strains | Antimicrobial activity test organisms zone of inhibition (mm) | | | | | | | Cytotoxicity test organism (% age mortality) |
|---|---|---|---|---|---|---|---|---|
| | *B. subtilis* | *S. aureus* | *M. miehei* | *C. albicans* | *R. solani* | *P. ultimu* | *A. cochli* | *Artemia salina* |
| A5 | - | - | - | - | - | - | - | 15.7 |
| B5 | - | - | - | - | - | - | - | 14.5 |
| C1 | 12 | 13 | 11 | 13 | ++ | + | + | 36.8 |
| C2 | 11 | 11 | - | 15 | - | - | - | 3.7 |
| C3 | 11 | 12 | - | 14 | - | - | - | 11.4 |
| C5 | - | - | - | 12 | - | - | - | 18.6 |
| D5 | - | 12 | - | 16 | ++ | + | - | 9.4 |
| D8 | 13 | 16 | - | 14 | ++ | - | + | 4.6 |
| D13 | 13 | 14 | - | 12 | - | - | - | 6.7 |
| F5 | - | - | - | - | - | - | - | 15.2 |

(++) = Major and (+) = Minor zone of inhibition around the disc, (-) = No activity against the test organism was observed

## III. RESULTS AND DISCUSSION

*A. Antimicrobial activity*

In all ten thermophiles strains were selected from the isolates and screened for their antimicrobial activity against gram-positive bacteria and fungal pathogens. The strains were found to be active against a variety of indicator microorganisms in biological screening. As seen in Table 1, the strains C1, D5, and D8 exhibited antifungal activity against *Candida albicans* and *Rhizoctonia solani*, however activity against *Mucor miehei* was exhibited only by C1 strains. Minor activity was exhibited by D5 and C1 against *Pythium ultimum* and by D8 and C1 against *Aphanomyces cochlioides*. Almost six strains were found to be active against Gram-positive tested Bacteria (*Staphylococcus aureu* and *Bacillus subtilis*).

Interestingly, the five strains which showed promising antimicrobial activity were specific against gram positive bacteria. This is also in concurrence to other reports suggesting better antagonistic activity of *Bacillus* species against gram positive bacteria [8]. The *bacillus* species are widely recognized as a rich source of antimicrobial compounds with a number of anti microbial compounds being reported from them [9]. But thermophilic *bacillus* strains are yet to be exploited properly for novel and stable antimicrobial compounds [10]. Biological screening results proved the strains as potent producers of bioactive secondary metabolites

and clearly suggested them as a good source of interesting antibacterial and antifungal compounds. Therefore, these strains were scaled up and subjected for further purification of the biological active compounds (data not shown).

*B. Cytotoxicity assay*

Results of the cytotoxic activities of the crude bacterial extracts of the Thermophilic *bacillus* is shown in Table 1. Among the 10 strains exanimated they showed variable degree of cytotoxicity against *Artemia salina*. The minimum cytotoxicity was observed in case of isolates C2, D8, D5 and D13, while the isolates C1 crude extract exhibited 36.8% mortality of the larvae of *Artimia salina* in microwell cytotoxicity assay (Table 1).

During screening of new isolates for drug discovery, many potentially interesting microorganisms might be excluded due to inadequate techniques, too high selectivities or just because of missing tests [11]. We decided therefore, to use a so-called horizontal screening, i.e., a combination of tests with low selectivity covering a broad range of antibacterial, antifungal, and cytotoxic activities [12]. Additionally we utilized the chemical screening approach to establish similarities or differences between the secondary metabolite patterns of our isolates assuming that each strain produces a specific metabolic fingerprint when it is grown under the same culture conditions. The result indicates that the crude extract of Themophilic *bacillus* of the selected strains has no toxicity to the aquatic ecosystem. Thus, these strain can be applied as a biocontrol agent

*C. TLC screening of thermophilic chemical compounds*

TLC was performed on the extracts derived from the crude extract obtained by ethyl acetate of selected thermophilic strains grown with different medias. Variations of the TLC plates profile showed that significant differences of biochemical compounds produced under various growth conditions obtained from different strains (Fig. 1). In chemical screening, the pattern of colored bands after treatment of TLC plates with staining reagents (anisaldehyde/H2SO4 and Ehrlich's reagent) is visible in Fig. 1a and Fig. 1b respectively. The constituents of each of the crude extracts produced different colors including dark brown, blue, pink and yellow depending on the staining reagent. The highest variety of colored bands was observed in crude extracts of strains C1, C5, D5, D8 and D13). Biochemical compounds could not be accurately identified by TLC analysis alone so these spots were scraped from TLC plates, eluted with ethyl acetate, and subjected to GC/MS analysis (data not shown).

Bacterial strain isolated from unusual extreme habitat were often reported to produce antimicrobial compound [13], [14]. Production of bioactive compounds seems to be the adaptation technique developed by these microbes to survive in such environment. The limited spectrum antimicrobial activity of these thermophiles further illustrates this survival strategy. The results implied that some thermophilic *bacillus species* could probably release various antibiotic compounds to provide themselves the survival competition superiority. In addition, the TLC screening profile demonstrated that

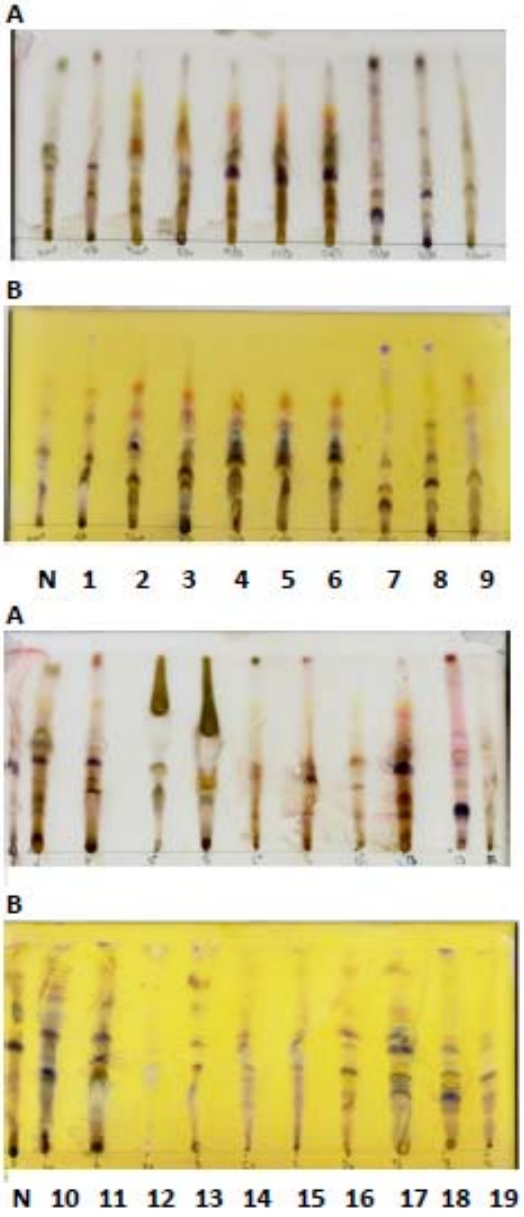


Fig.1 Chemical screening using TLC detection. TLC plates A after treatment with anisaldehyde/H2SO4 sol., B after treatment with Ehrlich's reagent. Numbers 1–19: Crude extracts of thermophilic *Bacillus* strains.

different species could produce different antimicrobial metabolites, and some had more than one antimicrobial substance.

## IV. Conclusions

In this study, five thermophilic bacterial strains showing antimicrobial activity were isolated from Jordan thermal springs. The results suggest that thermophilic *Bacillus* strains may be a good producer of antibiotics, stable at higher temperature and having antimicrobial activity against Gram-positive bacteria. Regarding their metabolic fingerprint visualized by the chemical screening including TLC it is possible to select the talented organisms from this collection, which may produce rare or a large variety of structurally

diverse secondary metabolites. Moreover the spectrum of antimicrobial activity observed in biological screening depicts that these thermophiles strains could act as a potential source of the compounds with broad spectrum activity, for controlling bacterial and fungal pathogens.

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