LC-MS/MS Profiling-based Metabolite Screening of Thermophilic Bacteria from Jordanian Hot Springs

Khalid Fandi, Muhanad Massadeh and Hartmut Laatsch

***Abstract*—** Thermophilic microorganisms are well-known producer of thermostable enzymes and bioactive compounds that have importance in biotechnological applications. This study was designed to screening and comparing the diversity of metabolites production using different culture media of thermophilic *Bacillus* species isolated from hot springs in Jordan. Therefore, a collection of thermophilic strains was isolated from Ma'en thermal springs. They had the ability to grow under high temperature condition (45-55 °C)**.** Crude extracts were obtained by organic solvent extraction of the culture broths. HPLC-MS analysis of fermentation extracts was performed to trace the number of secondary metabolites produced under various growth conditions. Three out of sixteen screened strains had the potential of producing biologically active metabolites. The crude extracts were subjected to silica gel, Sephadex LH-20, RP18 column chromatography and preparative PTLC for further purification. Several indole derivatives have been isolated from these selected strains namely 1-acetyl-β-carboline, tryptophol, and indole-3-carboxylic acid. Furthermore, adenosine, tyrosol, *p*-hydroxy-benzaldehyde, ferulic acid, uracil and 3-methyl uracil were also isolated. Additionally, the selected strains afforded four diketopiperazine derivatives, namely cyclo (Phe, Pro), cyclo (Leu, Pro), cyclo (Pro, Ile) and cyclo (Pro, Tyr). The structures of all isolated metabolites were confirmed by searching in AntiBase using ^{1}H NMR and MS data and comparison with the literature. The selected studied strains showed similarity in their metabolic profiles while their activities were varying from one to another. The identification of strains with antimicrobial activity suggests that the environment of thermal springs may represent an ecological niche, which harbors a largely untapped microbial diversity and a yet unexploited potential in the search for new secondary metabolites. The chemodiversity of thermophilic microorganisms is important for functional genomics, and the search for new compounds may deliver new biotechnology products.

***Keywords*—** LC-MS/MS, Metabolites, Thermophilic bacteria.

I. Introduction

THERMOPHILES are a group of organisms characterized by their ability to live and flourish in unusually harsh conditions of high temperatures. Over the past decade, thermophiles have generated great interest both from fundamental and applied science perspectives. From biotechnological point of view, thermophiles are of interest as sources of unique enzymes with unusual properties, as the active agents in high-temperature fermentation, in waste-treatment processes, and in mineral leaching [1]–[3].

The local resources of hot springs in Jordan are very rich in thermophilic microorganisms that are capable to produce thermostable enzymes and biological active compounds that have importance in industrial and biotechnological applications. Biodiversity and metabolic activities of prokaryotes live in extreme habitats, Thermophiles, which have not been fully examine yet in Jordan hot springs, is still not satisfactory. Therefore, this study was designed to investigate for the production of biochemical compounds of aerobic thermophilic bacteria using different culturing media as carbon sole sources.

II. Materials and Methods

A. Water sampling

Water samples were collected from different sites of the main thermal springs located in Ma'en in Jordan using 50 ml sterile thermal glass tubes. Samples were collected away from the margin, under 15 cm below the surface of springs, and transported directly to the laboratory for immediate culturing and isolation of thermophilic aerobic bacteria. The temperature and pH of the collected thermal waters ranged from 45°C to 60°C and 7.2 to 7.8 respectively.

B. Bacterial strain and growth conditions

Water samples from each tube were cultured in nutrient broth. 5 ml of each sample was cultured in 50 ml nutrient broth (1.5%peptone, 0.5%meat extract, 0.5%NaCl, 0.03%K2HPO4, pH7.2) and incubated at 50° C for 48 hours with 180 rpm agitation in a laboratory shaker. After then, a loopful of each culture was streaked on nutrient agar and incubated on the same conditions. Separate colonies from each plate were picked and grown on a new plate of nutrient agar to prepare pure culture on each plate. The process was repeated until pure cultures were obtained on each plate. Sixteen pure isolates were recovered and they were: M1a, M1b, M2a1, M2a2, M2b, M2c, M3a, M3c, M4a, M4b22a, M4b221, M4d22a, M4c, M5a, M5b1, M5b2.

C. Pre-Screening

The microbial isolates obtained from thermal springs were

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cultured in multiple 1 L Erlenmeyer flasks containing 250 ml fermentation medium. Five different media (modified at Laatsch's lab) were used to determine the most effective medium for mass cell production of thermophiles. The different media had its constituents (g/l) as following: (A: 10 meat extract, 4 yeast extract, 4 glucose; B: 20 soya fetter, 20 mannit; C: 10 glucose, 2 peptone 1 yeast extract 1 meat extract; D: 40 yeat, 5 glucose, 45 $CaCl_2$; E: 10 trypton, 5 yeast extract, 10 NaCl, 5 glucose). The pH of the media was adjusted to 7.8 before being autoclaved at 121°C for 15 min. The bacterium was grown for 3-5 days at 55°C in incubator shaker at 180 rpm. After which the entire fermentation broth were extracted with organic solvents. On evaporation, the extracts yield a crude mixture of microbial secondary metabolites and fats. Other screening methods make use of the chemical screening where more or less selective spray reagents are used to visualize the spots in the TLC.

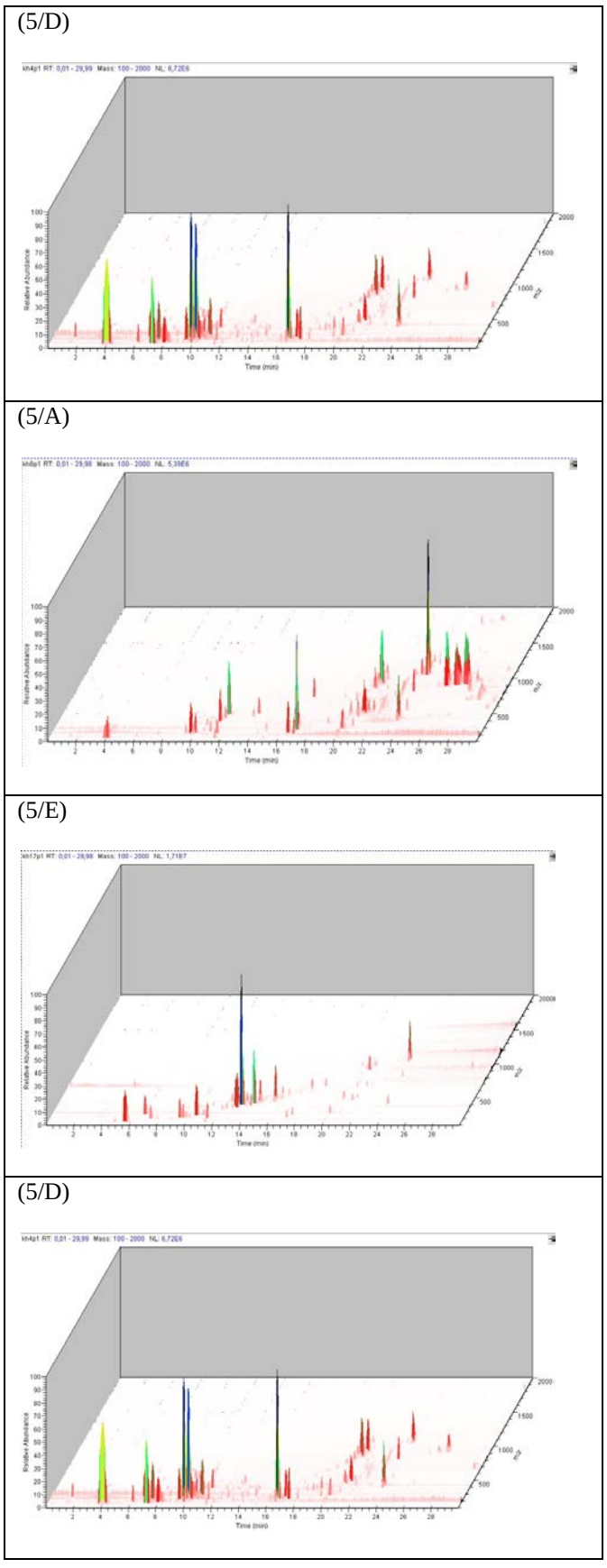
D. Upscaling, Purification of Active Principles and Structural Elucidation

Three selected strains out of sixteen screened strains have potential of producing biologically active metabolite were scaled up by 20-50 L fermentation containing medium D. The crude extracts were subjected to silica gel, sephadex LH-20, RP18 column chromatography and preparative PTLC for further purification step. All fractions were monitored by thin layer chromatography and 1H-NMR spectroscopy in order to isolate the major metabolites in the crude extracts. The structure elucidation of secondary metabolites were carried out by a combination of spectroscopic techniques and chemical conversions, particularly by 1 and 2D high field NMR spectroscopy (up to 600 MHz).

III. Results and Discussion

A. LC-MS/MS Screening of Thermophilic Metabolites

Of the 5 different mediums for optimum thermophilic bacteria growth, the $CaCl_2$ medium (D) resulted in the highest yield. Three out of sixteen screened strains had the potential of producing biologically active metabolites. HPLC-MS analysis of fermentation extracts obtained from these three strains (5, 8 and 13) showed significant differences profiles of secondary metabolites produced under various growth conditions (Fig. 1). Interestingly, the three 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* sp. against gram positive bacteria [4]. The bacillus species are widely recognized as a rich source of antimicrobial compounds with a number of anti microbial compounds being reported from them [5]. But thermophilic bacillus strains are yet to be exploited properly for novel and stable antimicrobial compounds [6]. Therefore, these strains were scaled up and subjected for further purification of the biological active compounds.



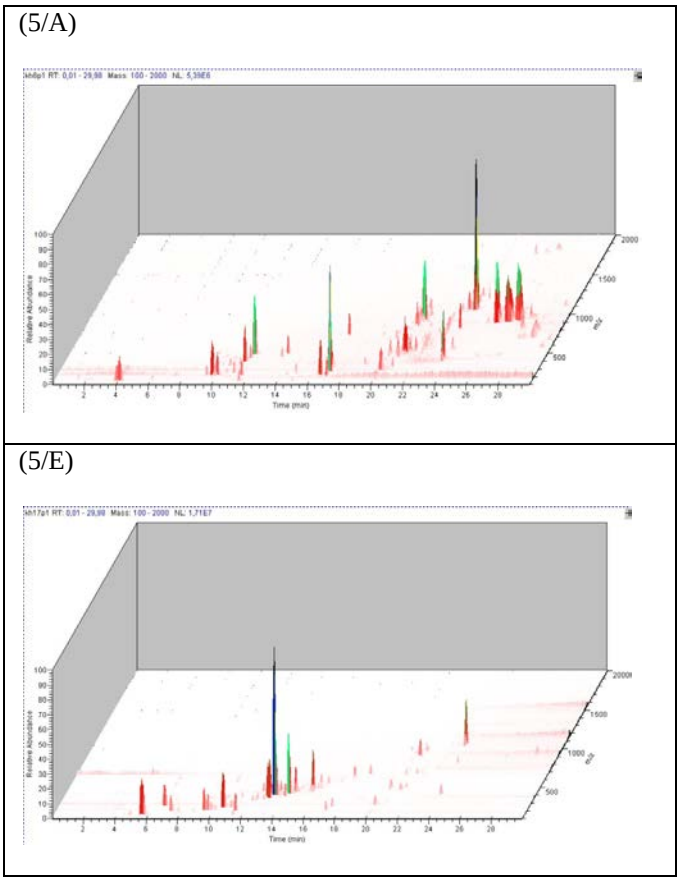


Fig.1 LC-MS/MS screening profile metabolites production by thermophilic bacteria using different growth media. Strains indicated with number with the media.

B. Isolation and Purification of Chemical Compounds

The study of the chemical composition of the bacterial extracts of three selected strains of thermophiles which is belong to the genus *Bacillus* from thermal springs in Jordan led to isolation and identification of several indole and indole derivative compounds such as: 1-acetyl-β-carboline (**1**), tryptophol (**2**), and indole-3-carboxylic acid **(3)** adenosine (**4**), tyrosol (**5**), p-hydroxy-benzaldehyde (**6**), ferulic acid (**7**), uracil (**8**) and 3-methyl uracil (**9**). Additionally, the selected strains afforded four diketopiperazine derivatives isolated namely cyclo (Phe, Pro) (**10**), cyclo (Leu, Pro) (**11**), cyclo (Pro, Ile) (**12**) and cyclo (Pro, Tyr) (**13**) (Fig. 2). The structures of the isolated compounds were confirmed by searching in AntiBase [7]. Their structures were determined using the spectroscopic information such as ^{1}H NMR technique as well as HR-ESI-MS and mass spectrometry and comparing with the literature data. Previous reports confirmed that compound **1** exhibited antitumor activity, while compound **5** and other indole derivatives possess antibacterial and antifungal activities [8], [9]. Moreover, compound **7** has been reported recently as a strong agent in prevention of Hypercholesterolemia [10].

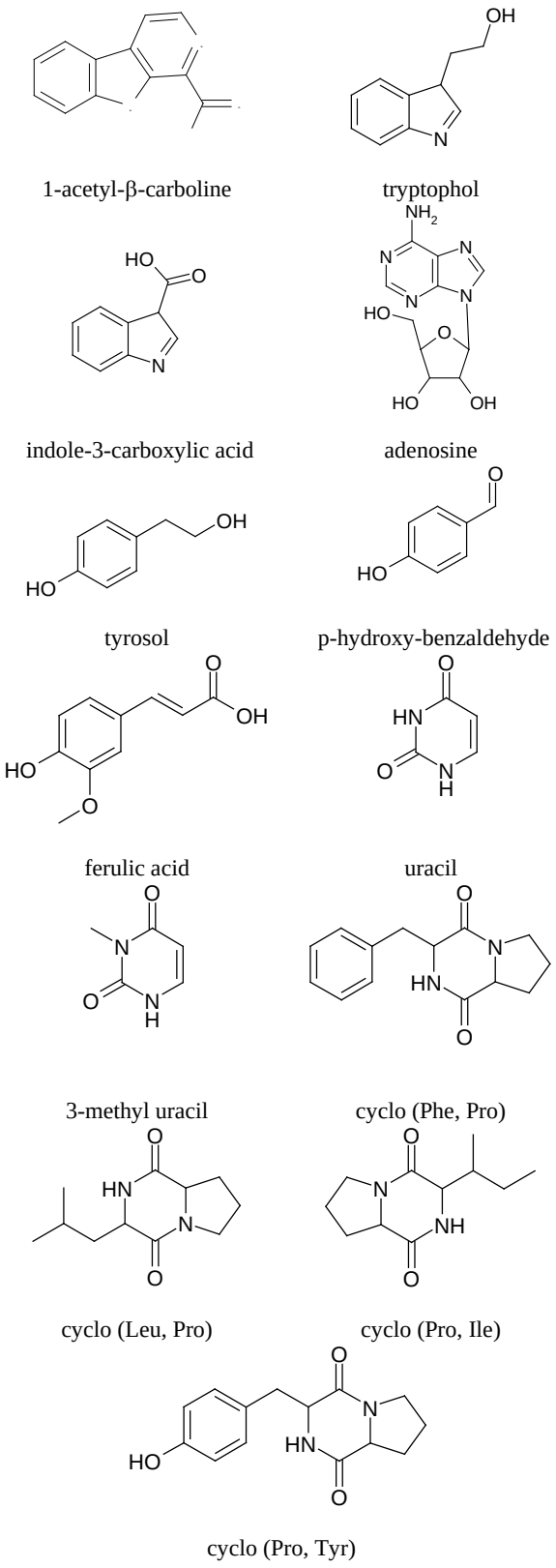


Fig.2 Chemical structure of different metabolic compounds isolated from thermophilic *Bacillus* species of Jordan hot springs

IV. Conclusions

Although selected aerobic thermophilic strains of *Bacillus* showed similarity in their metabolic profiles, their activity were varies from one to another. This was the first report on chemical screening from thermophilic bacteria and other strains showed interesting activities as well as different UV absorbing bands on TLC. The recovery of strains with antimicrobial activity suggests that thermal spring's environment may represent an ecological niche, which harbors a largely uncharacterized microbial diversity and a yet unexploited potential in the search for new secondary metabolites. The chemodiversity of thermophilic microorganisms is important for functional genomics, and the search for new compounds may deliver new biotechnology products. Therefore, the working on these strains could be promising to isolate active new metabolites in the future

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References

[1] T. D. Brock, *The Thermophiles: General, molecular, and applied microbiology*. New York: John Wiley & Sons, 1986

[2] R. M Kelly, and S. H. Brown, "Enzymes from high temperature microorganisms" *Curr. Opin. Biotech*., vol. 4, pp. 188-193, 1993.

[3] R. M. Kelly, T. L. Peeples, S. B. Halio, K. R. Rinker, and G. D. Duffaud, "Extremely thermophilic microorganisms: metabolic strategies, genetic characteristics and biotechnological potential," *An. New York Ac. Sci*. vol. 745, pp. 409-425, 1994.

[4] M., H Yilmaz,. Soran and Y. Beyatli, "Antimicrobial activities of some *Bacillus* sp. Strains isolated from the soil," *Microbiol. Res*., vol. 161, pp. 127-131, 2006.

[5] K. Nagai, N. Kamigiri, K. Arao, Y. Suzumura M., Kawano, and H. Yamaoka, "YM-266183 and YM-266184, novel thiopeptide antibiotics produced by *Bacillus cereus* isolated from marine sponge," *J. Antibiotics*, vol. 56, pp. 123-128, 2003.

[6] K. Gebhardt, J. Schimana, J. Muller, H. P. Fielder and H.G. Kallenborn, "Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods," *FEMS. Microbiol. Lett.*, vol. 217, pp. 199-205, 2002.

[7] H. Laatsch, *A Natural Product Database for Rapid Structure Determination*, *AntiBase*, Wiley VCH, Weinheim, 2009. http://wwwuser.gwdg.de/~ucoc/laatsch/antibase.htm

[8] P. Valentina, K. Ilango, K. Yamuna, D. Purushothaman, A. Samyuktha Rani, "QSAR analysis on β - Carboline as antitumor agent," *Journal of Young Pharmacists*, vol. 1, pp. 77-81, 2009.

[9] I. Erdogan, B. Sener, and T. Hega, "Tryptophol, A plant auxin isolated from marine sponge *Ircinia spinulosa*," *Biochemical Systematic and Ecology*, vol. 28, pp. 793-794, 2000.

[10] E.Y. Kwon, Y. Y. Cho, G. M. Do, H. J. Kim, S. M. Jeon, Y. B. Park, M. K. Lee, T. S. Min, and M. S. Choi, "Actions of Ferulic Acid and Vitamin E on Prevention of Hypercholesterolemia and Atherogenic Lesion Formation in Apolipoprotein E-Deficient Mice," *Journal of Medicinal Food*, vol. 12, no. 5, pp. 996-1003, 2009.