

Diversity of Bioactive Metabolites Produced by Thermophilic *Bacillus* Strains Isolated from Jordanian Hot Springs

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Received: December 1, 2023; Revised: January 3, 2024; Accepted: January 14, 2024

Abstract

This study explores the metabolic diversity of thermophilic *Bacillus* species isolated from Jordanian hot springs. Sixteen strains from Ma'en thermal springs, exhibiting robust growth at elevated temperatures (45–55°C), were investigated for their potential in producing valuable bioactive compounds. Crude extracts, obtained through organic solvent extraction, underwent HPLC-MS analysis to reveal secondary metabolite spectra under various growth conditions. Strains M5a, M13a, and M1c displayed potential in producing biologically active metabolites. The purification process of the M5a strain extracts involved sequential techniques including silica gel, Sephadex LH-20, RP18 column chromatography, and preparative TLC. This process resulted in the isolation of diverse compounds, including indole derivatives (1-acetyl- β -carboline, indole-3-carboxylic acid, tryptophol), adenosine, tyrosol, p-hydroxy-benzaldehyde, ferulic acid, uracil, 3-methyl uracil, and the identification of four diketopiperazine derivatives (cyclo (Phe, Pro), cyclo (Pro, Ile), cyclo (Leu, Pro), and cyclo (Pro, Tyr)). Structural validation of these compounds was achieved through AntiBase, utilizing 1H NMR and MS data and literature comparisons. Despite similar metabolic profiles, strains exhibited varying activities, including antimicrobial potential. This study marks the first report on purified biochemical compounds from thermophilic bacteria, emphasizing untapped microbial diversity in thermal springs. Intriguing activities and distinct UV-absorbing bands on TLC suggest promising prospects for isolating novel, active metabolites. This research enhances our understanding of the biotechnological potential of thermophilic *Bacillus* strains, emphasizing the importance of exploring their chemodiversity for industrial applications and functional genomics.

Keywords: LC-MS/MS, Thermophilic bacteria, Metabolites diversity, Bacillus, hot springs

1. Introduction:

Extremophiles, a fascinating group of microorganisms, thrive and reproduce under extreme environmental conditions, such as high temperatures, extreme pH levels, elevated ion concentrations, and radioactivity. This unique adaptation involves the evolution of specialized biological components and metabolic pathways that enable survival and growth in challenging environments (Krishnaraj and Sani, 2017; Singh *et al.*, 2019). In recent years, the significant interest in extremophiles has been driven by their ability to catalyze chemical reactions under severe conditions, offering potential applications across various industries (Dumorne and Cordova, 2017; Geng *et al.*, 2018; van den Burg, 2003).

Among extremophiles, thermophilic microorganisms, flourishing in warm environments, play a pivotal role in the synthesis of fuels and chemicals. Their proficiency in operating under high-temperature conditions enhances product yields and mitigates contamination risks, making them particularly attractive for industrial applications. Additionally, thermophiles serve as rich sources of thermostable enzymes crucial for industrial processes (Böhme *et al.*, 2020; Rigoldi *et al.*, 2018). The temperature

spectrum for thermophilic microorganisms categorizes them into moderate thermophiles thriving at 50–60 °C, extreme thermophiles at 60–80 °C, and hyperthermophiles at 80–110 °C (Baker *et al.*, 2001). Extreme thermophiles, with their exceptional thermal stability and ability to grow at temperatures exceeding 80°C, present promising candidates for applications such as high-temperature fermentation, waste treatment, and mineral leaching (Brock, 1986; Kelly and Brown, 1993; Kelly *et al.*, 1994). Researchers find significant value in thermophiles due to their unique characteristics and potential applications in biotechnology.

In this context, the study of gene function on a genome-wide scale, known as functional genomics, has been instrumental in unraveling the molecular mechanisms underpinning the adaptation of thermophiles to extreme conditions. A significant outcome of functional genomics research on thermophilic microorganisms is the identification of heat shock proteins (HSPs) (Hartl *et al.*, 2011; Lindquist and Craig, 1988; Morimoto, 1998; Parsell and Lindquist 1993). These proteins play a critical role in preventing protein denaturation and aggregation, thereby shielding cells from the adverse effects of high temperatures (Kuczynska-Wisnik *et al.*, 2002).

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Delving into the realm of cellular stress responses in *Escherichia coli* (*E. coli*), Kuczynska-Wisnik *et al.* (2002) explored the protective function of the diminutive heat-shock proteins IbpA and IbpB, shedding light on how these proteins prevent the aggregation of endogenous proteins denatured in vivo during severe heat shock. This work significantly contributes to understanding the cellular response to extreme heat shock in *E. coli*, underscoring the crucial role of IbpA and IbpB as guardians against protein aggregation. In response to heat stress, additional genes implicated in protein folding, modification, and degradation have been found to be upregulated, marking another avenue of interest in functional genomics research on thermophiles. A crucial aspect of this research involves the exploration of the metabolic pathways enabling thermophilic microorganisms to thrive in high-temperature environments. The identification of genes involved in the production of compatible solutes, such as trehalose and proline, has provided insights into how thermophiles maintain cellular integrity and prevent protein denaturation under extreme conditions.

Continued progress in understanding the molecular mechanisms behind the adaptation of thermophilic microorganisms to high-temperature environments is facilitated by functional genomics. This ongoing exploration offers valuable insights into the potential applications of these unique organisms across disciplines like biotechnology and environmental science by identifying key genes and pathways involved in these processes.

Noteworthy among the rich sources of thermophilic bacteria are Jordan's hot springs, recognized for their capacity to produce thermostable enzymes and bioactive substances (Al-Daghistani *et al.*, 2021; Obeidat and Al-Shomali, 2023), which constitute a valuable reservoir of thermophilic bacteria. This study is dedicated to addressing the existing knowledge gaps concerning aerobic thermophilic bacteria derived from Jordan's hot springs. Despite their diverse metabolic processes, these bacteria remain relatively unexplored. Our investigation aims to elucidate the production of biochemical compounds by these bacteria when cultured using various media as the exclusive source of carbon. Through a comprehensive exploration of their metabolic capabilities, our study seeks to unveil the untapped biotechnological potential of thermophilic *Bacillus* strains and contribute to a broader comprehension of the roles of extremophiles in natural product discovery. Significantly, this work marks the inaugural report on the purified biochemical compounds profiled from thermophilic bacteria, emphasizing its novelty and potential impact on biotechnology.

2. Materials and Methods

2.1. Sampling of Thermal Springs Water:

Water samples were carefully gathered from diverse points within Ma'en's primary thermal springs in Jordan, utilizing sterile thermal glass tubes (50 ml). The collection points were strategically selected away from the peripheries, and samples were obtained from a depth of 15 cm below the spring's surface. Subsequently, the samples were promptly transported to the laboratory for immediate

culturing. The collected thermal water exhibited temperatures ranging from 45°C to 60°C and a pH between 7.2 to 7.8. This approach was implemented to ensure a representative sample of thermophilic aerobic bacteria.

2.2. Isolation and cultivation of thermal bacterial strains

The culturing of water samples from each tube was carried out in a nutrient broth (NB) medium. Specifically, 5 ml from each sample was introduced into 50 ml of nutrient broth containing 1.5% peptone, 0.5% NaCl, 0.5% meat extract, 0.03% K₂HPO₄, and adjusted to a pH of 7.2. The mixture was then incubated at 50°C for 48 hours with agitation at 180 rpm in a laboratory shaker. Subsequently, a loopful from each culture was streaked onto nutrient agar plate and subjected to the same incubation conditions. Pure cultures were established through successive transfers on new nutrient agar plates, resulting in sixteen isolates designated as M1a, M2a, M3a, M4a, M5a, M1b1, M13a, M1c, M2c, M3c, M3c1, M4d22a, M4c, M5a2, M5b1, and M5b2.

2.3. Cultivation and crude extract preparation for pre-screening

The sixteen selected isolates were cultured as subcultures on agar plates for 24 hours at 50°C. This was achieved using 1.5% nutrient agar, composed of beef extract (3 g/l), peptone (5 g/l), NaCl (8 g/l), and agar (15 g/l). Subsequently, the resulting subcultures were utilized to inoculate Erlenmeyer flasks (1000 mL) with a half loop of growth obtained from the colonies on agar plates. Each flask contained 250 mL of NB medium, with the pH adjusted to 7.8 prior to sterilization. These flasks were then incubated at 50°C on a linear shaker set to 180 rpm for 24 hours.

For the large-scale preparation of crude extracts for each strain, cultivation was carried out using 5 ml of culture obtained from the previous step. This culture was inoculated into four separate 250 mL Erlenmeyer flasks (a total volume of 1L) containing NB medium. Additionally, L-Valine and L-Lucine amino acids were added to the culture medium at concentrations of 0.3% and 0.01%, respectively. The incubation continued for 2 days on a linear shaker under the same aforementioned conditions. Following the harvesting of the dark brown culture, the broth underwent filtration over a celite bed using vacuum filtration with a Buchner funnel to separate the biomass from the liquid phase. After filtration, the biomass scraped from the top of the filter underwent ethyl acetate extraction three times (3 ×). Each extraction cycle occurred under ultrasonic irradiation (Ultraturrax: Janke & Munkel KG) for 10 min per cycle. Simultaneously, the liquid phase was extracted twice with ethyl acetate. Subsequently, the organic phases from all these extractions were combined and then concentrated by evaporation using a rotary evaporator (Rotavapor R152) to obtain the crude extracts. For quantification, the crude extract was suspended in 10% CH₂Cl₂, dried with air, and stored in a refrigerator at 4°C. These extracts underwent a comprehensive analysis, including chemical and biological screening, as well as HPLC-MS analysis.

2.4. Media optimization and production

Five distinct media, which were modified in Laatsch's laboratory, were employed to determine the most efficient

medium for the large-scale cultivation of thermophiles. These five different media, labeled as A, B, C, D, and E, consisted of the following component: **A:** yeast extract 4 g, malt extract 10 g, and glucose 4 g in 1 L of dH₂O, **B:** mannitol 20 g, soya bean fat 20 g, and in 1 L of dH₂O, **C:** peptone 2 g, glucose anhydrous 10 g, yeast extract 1 g, and meat extract 1 g in 1 L of dH₂O, **D:** yeast extract 40 g, CaCl₂ 45 g, glucose 5 g, and in 1 L of dH₂O, **E:** yeast extract 5 g, trypton 10 g, NaCl 10 g, and glucose 5 g in 1 L of dH₂O. The pH of each medium was adjusted to 7.8 before undergoing autoclaving at 121°C for 15 minutes. Before autoclaving at 121°C for 15 minutes, the pH of each medium was adjusted to 7.8. The bacterium was then cultivated in an incubator shaker at 55°C, with agitation at 180 rpm, for a period of 3-5 days.

2.5. Chemical screening

For the chemical screening process, a combination of TLC with spraying reagents and HPLC-MS methods was employed.

2.5.1. Thin layer chromatography (TLC)

The crude extracts obtained from the selected strains underwent Thin-Layer Chromatography (TLC) analysis to examine the nature of secondary metabolites produced by them. In this procedure, a small sample drop was accurately deposited onto the TLC plate using a capillary and allowed to dry. Each extract was applied to two TLC plates. To ensure an appropriate sample quantity (ranging from 2 to 5 micrograms) on the plate, this spotting process was repeated by overlaying additional drops onto the initial spot. Subsequently, the TLC plates were developed using a solvent system comprising CH₂Cl₂ and 5% MeOH. After development, the zones on the plates were observed under ultraviolet (UV) light at both 254 nm and 366 nm wavelengths. Components exhibiting UV absorption or fluorescence were identified and marked accordingly. Following UV visualization, one plate underwent staining with anisaldehyde/H₂SO₄ spraying reagent (anisaldehyde, methanol, H₂SO₄, acetic acid), while the second plate was sprayed with Ehrlich's reagent (HCl 37%, 4-dimethylamino benzaldehyde, MeOH) to detect different colored bands of compounds in the extracts, specifically designed for the detection of indoles. This treatment served to further pinpoint areas of interest. The resulting colored bands, resembling a metabolic fingerprint, resulted from reactions between the spray reagent and the metabolites. These bands were carefully marked and systematically documented through scanning procedures.

2.5.2. High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

The preparation of samples holds paramount importance when employing a High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) method for the analysis of biological samples. It necessitates the establishment of fundamental principles encompassing robustness and uniformity, which are prerequisites for any analytical assay. Typically, a concentration range spanning from 1 to 5,000 ng/ml is requisite for this purpose. Methanol/water was chosen as the solvent for this procedure, with methanol (LiChrosolv hypergrade for liquid chromatography) procured from Merck.

2.6. Extraction and purification of the active compounds

Selected strains were expanded through fermentation on a scale of 15-30 liters, and crude extracts were processed through chromatography and preparative thin-layer chromatography (PTLC) for additional purification steps.

2.6.1. Large-Scale Fermentation and crude extraction

A 30-liter fermentation of thermophilic *Bacillus* strain Ma5 was conducted by employing two sets of 60 Erlenmeyer flasks (1 L each), with each flask containing 250 mL of medium D. This fermentation was carried out at pH 7.8, using a linear incubator shaker set at 180 rpm and 55°C for a duration of 5 days. After harvesting the culture, the broth was filtered over celite using a filter press (Schenk Niro 212 B40) to separate the biomass from the liquid phase. The water phase was extracted by adsorption on Amberlite XAD-16 resin (obtained from Rohm and Haas, Frankfurt, Germany) in a large-size glass column (1.2 m x 10 cm). After washing with distilled water (2 L) to remove salts and sugars, the column was eluted with methanol. The methanol extract was concentrated in vacuo at 40°C to the aqueous residue. The remainder was re-extracted by ethyl acetate (3-4 times). Meanwhile, biomass was extracted using ethyl acetate (3 ×) followed by acetone (2 ×) under ultrasonic irradiation and mixed for 15 min each time. The acetone extract was concentrated in vacuo and the obtained aqueous residue was again extracted with ethyl acetate (2 ×). The ethyl acetate extracts obtained from both the filtrate and biomass, which contained bioactive metabolites, were combined and dried using a rotary evaporator (Rotavapor R152). This process yielded 6.94 grams of crude extracts in the form of dark brown residues.

2.6.2. Purification of the active compounds and structural elucidation

The crude extract (6.94 g) underwent fractionation through flash chromatography on silica gel column (1.5 × 50 cm) packed with 30 g of silica gel (Kieselgel 60, 230 ~ 400 mesh, Merck, Germany). Fractionation occurred by elution with a CH₂Cl₂: MeOH solvent mixture using a gradient of concentrations (1, 3, 5, 10, 20, 40, and 50%, vol/vol). The resulting fractions were monitored by TLC (CH₂Cl₂/MeOH 19:1, spraying with anisaldehyde/H₂SO₄ acid reagent and warming). Fractions with similar profiles in analytical high-performance liquid chromatography (HPLC) and TLC were combined, yielding four primary fractions labeled as I, II, III, and IV. The first fraction I underwent re-fractionated on silica gel column chromatography (1.2 × 30 cm, packed with 15 g of silica gel, CH₂Cl₂-MeOH), followed by purification on Sephadex LH-20 (column 5 × 40 cm, CH₂Cl₂ -40% MeOH). Fraction III underwent further purification through silica gel column chromatography, Sephadex LH-20 in a column (86 × 2.0 cm) eluted with CH₂Cl₂: 40% MeOH, and Preparative TLC (PTLC). The section of the PTLC plate containing a fraction was scraped, dissolved in methanol. The mixture was then centrifuged to separate from silica, and the obtained supernatant was further subjected to filtration. Following filtration, the solvent evaporated. The resulting precipitate was dried and then washed with a mixture of cyclohexane /CH₂Cl₂. Subsequently, it was dissolved in CH₂Cl₂ with 10%

MeOH to obtain the purified fraction. Fraction II was re-chromatographed on a Sephadex LH-20 column (5 × 40 cm) using H₂O-MeOH mixtures for elution, resulting in three major sub-fractions. Sub-Fraction 1 was identified as fat using TLC and spraying reagents. Major Sub-fraction 2 (1 g) underwent re-chromatography on a Sephadex LH-20 column (2 × 20 cm), eluted with H₂O-MeOH mixtures, and further purified on an RP-18 column (2 × 20 cm) with elution using 10 to 50% MeOH, resulting in a white precipitate. Fraction IV was also purified on Sephadex LH-20 (MeOH), followed by RP-18 silica gel (MeOH/H₂O gradient 10 to 50% MeOH). The isolated pure compounds derived from distinct chromatographic fractions of the filtrate extract were characterized by PTLC and size exclusion chromatography. Their structures were elucidated through the analysis of 1 and 2D high field NMR spectroscopy (up to 600 MHz), MS, UV, and IR data, and were further validated by comparing them with information from the AntiBase database (Laatsch, 2009).

3. Results and Discussion

3.1. TLC Analysis and Optimization of Growth Medium

The investigation focused on analyzing extracts derived from the crude extract of three thermophilic *Bacillus* strains, meticulously chosen from a pool of sixteen isolates obtained from Jordan hot springs. These strains, cultivated under diverse growth conditions, revealed substantial differences in the profiles of biochemical compounds, as indicated by TLC plates. The TLC chromatogram profiles exhibited notable variations in metabolite production among the selected strains (Fig. 1 and Fig. 2). Remarkably, CaCl₂ medium (D) emerged as the most favorable for metabolite production, demonstrating superior results compared to the other four growth media tested (Fig. 1). The observed variations in TLC profiles suggest a dependency of metabolite production on the growth conditions and media composition. CaCl₂ medium (D)

□

was identified as a superior medium for metabolite production, aligning with the findings from the TLC results. Significantly, three out of the sixteen screened strains, namely M5a, M13a, and M1c, demonstrated substantial potential for production biologically active metabolites (Fig. 2). Notably, these three strains exhibited noteworthy antimicrobial activity, particularly against gram-positive bacteria, aligning with previous findings highlighting the heightened antagonistic activity of *Bacillus* species against this bacterial group (Gomez-Escribano and Bibb, 2011). This specificity against gram-positive bacteria is consistent with reports indicating superior antagonistic activity of *Bacillus* sp. against this bacterial group (Jia *et al.*, 2020; Yilmaz *et al.*, 2006). While *Bacillus* species are widely recognized as rich sources of antimicrobial compounds, with numerous reports documenting their antimicrobial potential (Nagai *et al.*, 2003), this study underscores that thermophilic *Bacillus* strains, especially those obtained from Jordan hot springs, remain relatively unexplored for the discovery of novel and stable antimicrobial compounds (Gebhardt *et al.*, 2002). The untapped potential was further highlighted as recent studies continue to unveil the isolation of novel antimicrobial agents from the *Bacillus* genus (Davies, 2013; Nielsen *et al.*, 2017). Nevertheless, our investigation positions thermophilic *Bacillus* strains, particularly those obtained from Jordan hot springs, as promising resources for the discovery of novel and stable antimicrobial compounds, showcasing their potential for diverse biotechnological applications (Xiao *et al.*, 2021). Consequently, these strains were scaled up and subjected to further characterization and purification of the biologically active compounds, paving the way for future exploration and potential applications in various fields. The intriguing activities and clearly identifiable UV-absorbing bands on TLC indicate promising avenues for future efforts in isolating novel and active metabolites from these strains.

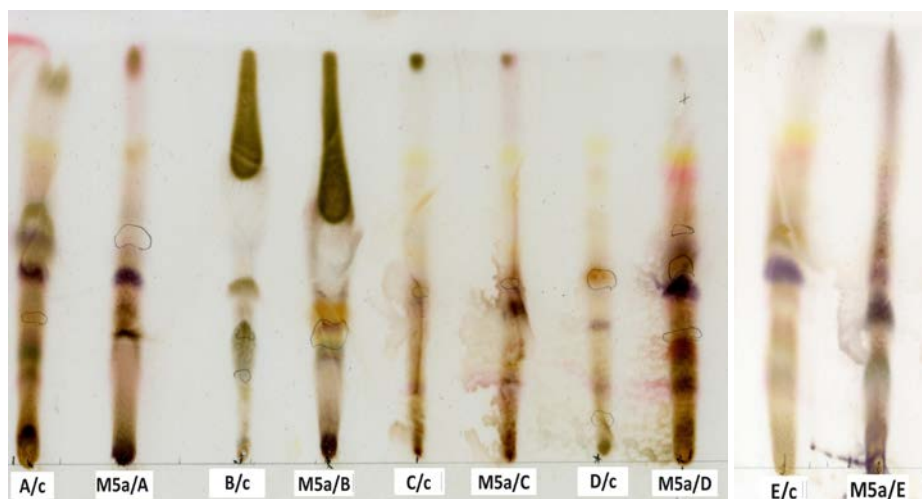


Figure 1. TLC chromatogram profile depicting the production of metabolites from the thermophilic bacillus strain (M5a) in various growth media (A, B, C, D and E). The control samples for each corresponding medium are indicated as A/c, B/c, C/c, D/c, and E/c.

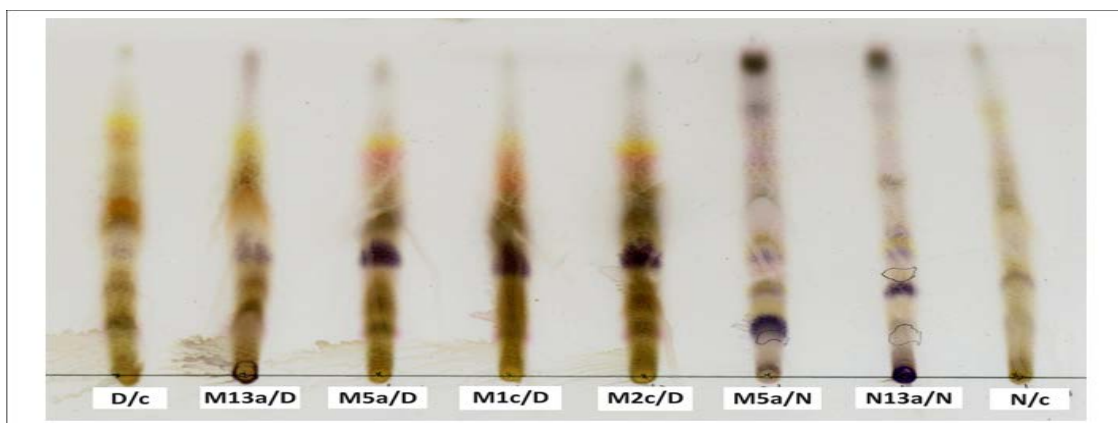


Figure 2. A comparison of the TLC chromatogram profiles illustrating the production of metabolites from various thermophilic *Bacillus* strains (M5a, M13a, M1c, and M2c). The strains and their corresponding media are provided, along with controls (D/c for CaCl₂ medium and N/c for nutrient broth).

3.2. LC-MS/MS Analysis: Revealing the Complexity and Diversity of Metabolites in Thermophilic *Bacillus* Strains

Expanding upon the insights garnered from TLC analysis and the optimization of growth media, LC-MS/MS analysis was employed to provide a more comprehensive exploration of the metabolites produced by thermophilic *Bacillus* strains. This in-depth analysis included the fermentation extracts of selected strains (M5a, M13a, and M1c) as well as additional strains that exhibited variation in metabolite production. This advanced analytical approach provided a deeper understanding of the metabolites produced under varying growth conditions, revealing a rich tapestry of secondary metabolites with distinct profiles (Fig. 3). The LC-MS/MS analysis unveiled diverse profiles of secondary metabolites across the selected strains, shedding light on the intricacies of their metabolic pathways under varying growth conditions (Fig. 3). The results unveiled a diverse array of secondary metabolite profiles influenced by varying growth conditions. Notably, CaCl₂ medium (D) emerged as the most favorable for metabolite production, showcasing the highest yields when compared to the other four tested growth media. This is exemplified by the distinct profile spectrum of the selected strain M5a under different growth conditions (Fig. 4). The LC-MS/MS analysis played a pivotal role in this exploration, providing a deeper understanding of the metabolites (Xiao *et al.*, 2012). The meticulous sample preparation, utilizing methanol/water as the solvent, ensured robust and uniform results. The chosen concentration range of 1 to 5,000 ng/ml optimized sensitivity and allowed for the detection of a broad range of metabolites (Chhonker *et al.*, 2023). The utilization of HPLC-MS unraveled a plethora of metabolites, accentuating the complexity and diversity of secondary metabolite production within thermophilic bacteria. The observed diversity in LC-MS/MS profiles among the selected strains further underscores the vast potential of thermophilic bacteria to produce a wide array of biologically active secondary metabolites. These variations in metabolite profiles serve as a testament to the significance of optimizing growth conditions for the enhanced production of specific metabolites, adding a layer of intricacy to the metabolic capabilities of thermophilic strains. Such endeavors will unravel the potential applications of these compounds in pharmaceuticals and other industries. The comprehensive

analytical foundation laid by TLC and LC-MS/MS serves as a cornerstone for understanding the metabolic intricacies of these thermophilic strains, propelling us forward into further exploration within the realm of bioactive compound discovery.

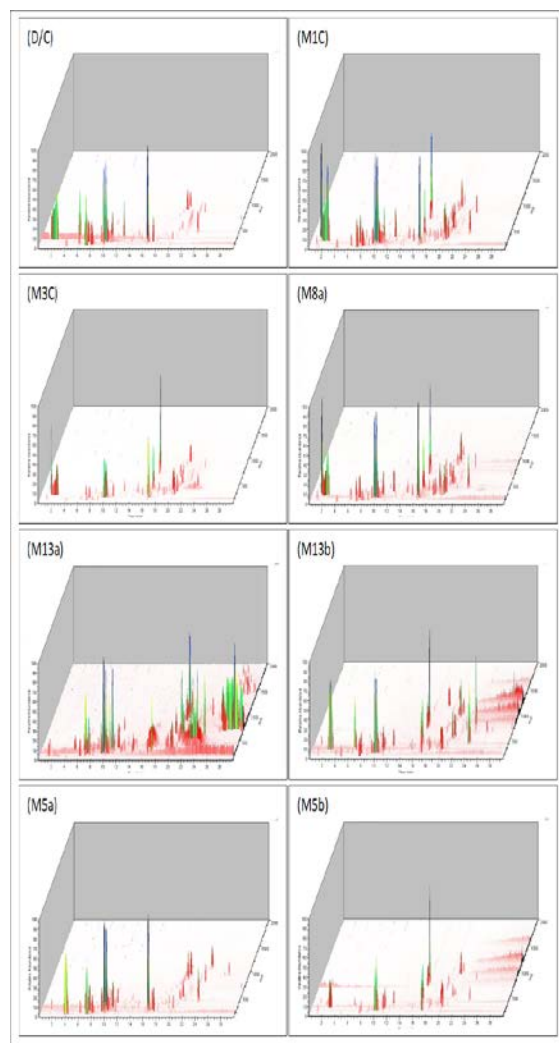


Figure 3. LC-MS/MS screening profile of metabolite production by different strains of thermophilic bacteria under growth media D, as derived from crude extracts. Strains are labeled with the number, and D/C refers to the control group.

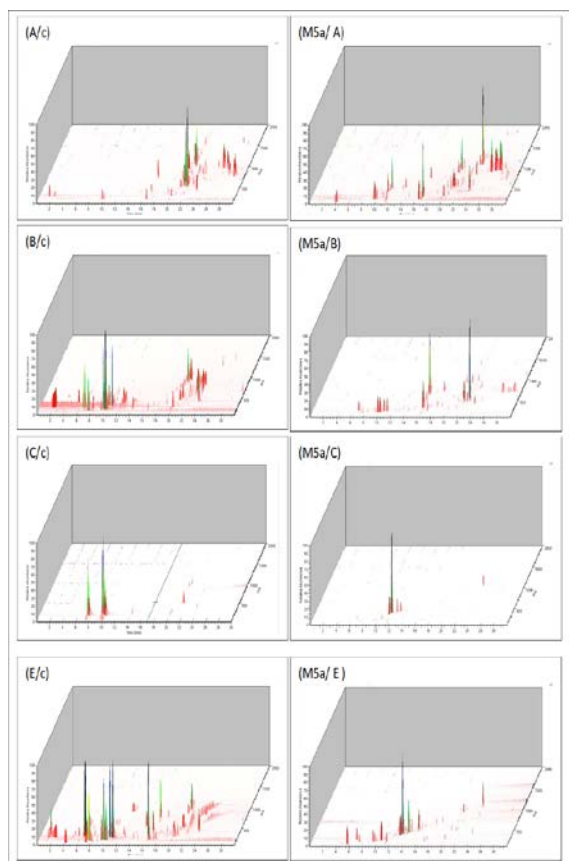


Figure 4. LC-MS/MS screening profile of metabolite production by the thermophilic *Bacillus* strain M5a under various growth media conditions (A, B, C, and E). The strain is denoted along with the respective media, and controls (C) are also specified with their corresponding media.

3.3. Purification and structure elucidation of the active compounds

Bulk fermentation, crucial for large-scale production, was conducted utilizing CaCl_2 medium (D), previously identified as the optimal medium for metabolite production. Following the fermentation process, cell separation and extraction were meticulously carried out using three distinct solvents: methanol, ethyl acetate, and acetone as illustrated in Fig. 5. The distinct TLC chromatogram profiles facilitated a clear differentiation of metabolites extracted using these solvents, revealing their efficiency in extracting diverse components from the M5a strain (Fig. 5). The amalgamation of these extracts yielded a total of 6.94 g of crude extract obtained from both the cell mass and the liquid phase. Subsequently, the components of the crude extract underwent purification through Flash chromatography on a silica gel column. TLC profiles were instrumental in guiding the purification process, leading to the isolation of four primary fractions (Fig. 6). Each of the distinct fractions (Fraction I, Fraction II, Fraction III, and Fraction IV) encompassed unique components with potential bioactive properties. The subsequent TLC chromatogram, generated from the four primary fractions obtained through flash chromatography on a silica gel column (Fig. 6), unequivocally illustrated the efficacy of the purification process. Each fraction underwent meticulous collection and further analysis. Fraction II underwent re-chromatography on a Sephadex LH-20 column, resulting in the generation of three major

sub-fractions. These sub-fractions were subsequently subjected to further purification on an RP-18 column, leading to the isolation and identification of various indole and indole derivative compounds, including 1-acetyl- β -carboline (1), indole-3-carboxylic acid (2) tryptophol (3), adenosine (4), and tyrosol (5). Fraction IV underwent purification on Sephadex LH-20, followed by RP-18 silica gel, yielding p-hydroxy-benzaldehyde (6) and ferulic acid (7) (refer to Table 1). The subsequent purification steps for Fraction I involved re-fractionation and purification on Sephadex LH-20, employing additional chromatographic techniques. Fractions underwent re-fractionation on a silica gel column, preparative TLC, and purification on a Sephadex LH-20 column, resulting in the isolation of uracil (8) and 3-methyl uracil (9) (refer to Table 1). Fraction III underwent a rigorous purification process, including silica gel column chromatography, Sephadex LH-20, and preparative TLC. This comprehensive methodology highlights the meticulous approach utilized in the isolation and purification of active compounds, resulting in the identification of four diketopiperazine derivatives: cyclo (Phe, Pro) (10), cyclo (Leu, Pro) (11), cyclo (Pro, Ile) (12), and cyclo (Pro, Tyr) (13). The chemical structures of the isolated compounds were rigorously validated through comparisons with the AntiBase database (Laatsch, 2009), confirming their identities. The determination of these structures involved comprehensive spectroscopic analyses, including ^1H NMR, HR-ESI-MS, and mass spectrometry, aligning with literature data (Table 1). The isolation of various bioactive compounds from thermophilic *Bacillus* strains, particularly those from Jordanian thermal springs, signifies a significant discovery. These compounds, encompassing indoles, their derivatives, and diketopiperazines, present diverse biological activities with potential applications (Table 1). The identification of indole and indole derivative compounds, such as 1-acetyl- β -carboline, tryptophol, and indole-3-carboxylic acid, is noteworthy. Indole derivatives have garnered significant attention due to their wide-ranging biological activities and anticancer (Kaushik *et al.*, 2013; Kumar & Ritika, 2020; Salerno *et al.*, 2023). For instance, 1-acetyl- β -carboline (compound 1) has been reported to exhibit antitumor activity antioxidant, and neuroprotective properties (Ayipo *et al.*, 2021; Moura *et al.*, 2007; Venkataramana *et al.*, 2018), suggesting potential implications for cancer research and therapy. Additionally, the isolation of indole-3-carboxylic acid (2) and tryptophol (3) highlights the potential involvement of these strains in neurotransmission, sleep regulation, and plant growth regulation, as indole derivatives are known to play critical roles in these processes (Chen *et al.*, 2020; Fernstrom, 2013). Moreover, adenosine (4) is of interest due to its involvement in various physiological processes, such as neurotransmission and vasodilation, with pharmaceutical applications in cardiovascular medicine (Fredholm *et al.*, 2001). Tyrosol (5) with antioxidant, anti-inflammatory, and cardioprotective properties, may contribute to the health benefits associated with the Mediterranean diet (Visioli *et al.*, 2002). Other indole derivatives like p-hydroxy-benzaldehyde (6) have exhibited antibacterial and antifungal properties (Cushnie and Lamb, 2011; Erdogan *et al.*, 2000; Santos *et al.*, 2018; Valentina *et al.*, 2009). This suggests that these thermophilic *Bacillus* strains may

produce compounds with potential antimicrobial applications, which is highly relevant in the context of antibiotic resistance. Compound 7, ferulic acid exhibits antioxidant, anti-inflammatory properties and is known for its strong cholesterol-lowering effects (Bumrungpert *et al.*, 2018; Srinivasan *et al.*, 2007; Thapliyal *et al.*, 2021), aligning with the interest in natural compounds for managing hypercholesterolemia and related health concerns (Ghaisas *et al.*, 2014; Luo *et al.*, 2022; Kwon *et al.*, 2009). The identification of uracil, and 3-methyl uracil further enriches the repertoire of isolated compounds, plying fundamental roles in genetics DNA replication, and their analogs offering potential applications in chemotherapy and antiviral drugs (Jordheim *et al.*, 2013; Ladner 2001; Seley-Radtke and Yates 2018). Moreover, the isolation of diketopiperazine derivatives, including cyclo (Phe, Pro), cyclo (Pro, Ile), cyclo (Leu, Pro), and cyclo (Pro, Tyr), highlights the structural diversity of these thermophilic *Bacillus* strains. The antibacterial, antiviral, and antifungal properties of diketopiperazine derivatives have been well-documented (Kumar *et al.*, 2012; Ren *et al.*, 2022; and Song *et al.*, 2021). Our study is consistent with these reports, as the bacterial strains that produced these compounds demonstrated promising antimicrobial activity, particularly against gram-positive bacteria. This is in line with previous findings that suggest *Bacillus* species, including thermophilic strains, often exhibit strong antagonistic activity against gram-positive bacteria (Nishanth *et al.*, 2012). Certain diketopiperazine derivatives have been investigated for their cytotoxic effects on cancer cells, indicating potential antitumor activity. While our study did not directly assess the antitumor properties of these compounds, it is noteworthy that cyclo (Pro, Tyr) (13) was among the derivatives we isolated. This compound has been previously studied for its antitumor potential (Ding *et al.*, 2020; Karanam and Arumugam, 2020). Additionally, the antioxidative properties of diketopiperazines align with our findings, as these compounds may help protect cells from oxidative damage. Oxidative stress is implicated in various diseases, and the antioxidant activity of diketopiperazine derivatives could have potential health benefits. Our research adds to the expanding body of evidence supporting the biological activities of diketopiperazine derivatives. While further research and specific assays are needed to fully harness their therapeutic potential, our findings underscore the biotechnological and pharmaceutical promise of thermophilic *Bacillus* strains as sources of diverse and bioactive metabolites. This study highlights the rich potential of thermophilic *Bacillus* strains as sources of diverse biologically active compounds. The identification of known bioactive compounds, coupled with the discovery of structurally unique molecules, opens avenues for further research and development in biotechnology and pharmaceuticals. Future explorations could strategically focus on delving deeper into the specific applications of these compounds, optimizing growth conditions for enhanced metabolite production, and exploring potential

synergies among different compounds. Consequently, our study positions thermophilic *Bacillus* strains as invaluable resources for ongoing investigations in the realm of natural product discovery. The untapped potential of thermophilic *Bacillus* strains emphasizes the importance of further exploration to harness novel compounds for various industrial and medical purposes, contributing to our growing understanding of extremophiles roles in producing bioactive molecules with diverse functions in different ecosystems and industries.

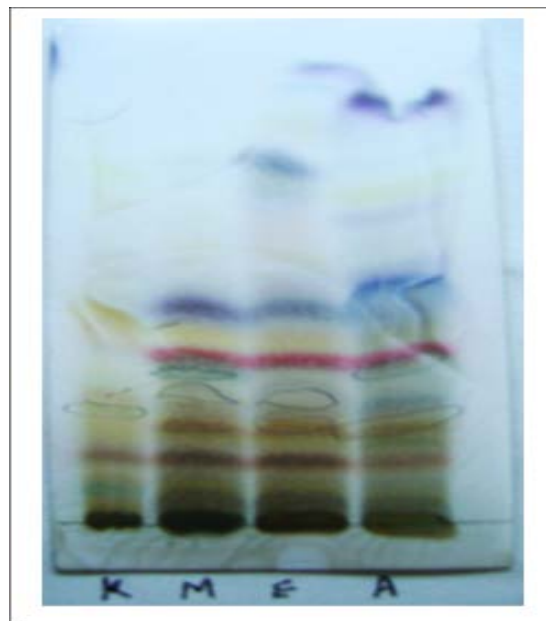
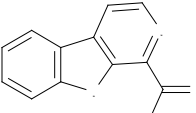
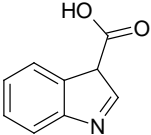
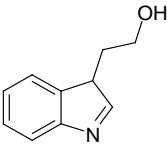
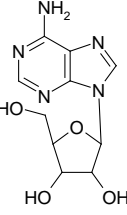
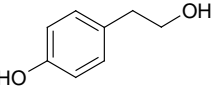
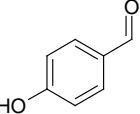
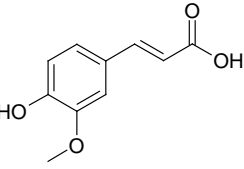
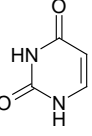
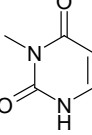


Figure 5: TLC chromatogram of metabolites from the M5a strain extracted using different solvents (K: control, M: methanol, E: ethyl acetate, A: acetone)

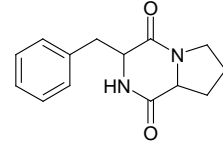
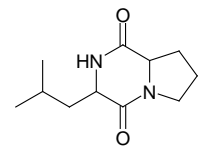
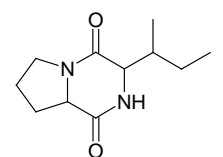
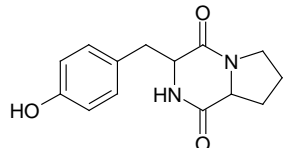


Figure 6: TLC chromatogram of the four primary fractions derived from the M5a strain through flash chromatography on a silica gel column (A: Fraction I, B: Fraction II, C: Fraction III, D: Fraction IV).

Table 1: Chemical structures composition of various metabolic

Metabolite	Compound	Chemical structure	Biological activity
Indole	1-acetyl- β -carboline (1)		Exhibited antitumor & antioxidant activity,
	Indole-3-carboxylic acid (2)		Plant growth regulation
	Tryptophol (3)		Play critical roles in neurotransmission and mood regulation
Indole derivatives	Adenosine (4)		Associated with antibacterial and antifungal activities
	Tyrosol (5)		Possess antibacterial and antifungal activities
	p-hydroxy-benzaldehyde (6)		Boost antioxidant activity and wound healing possess strong cholesterol-lowering effects
	Ferulic acid (7)		Strong agent in prevention of Hypercholesterolemia exhibits antioxidant, anti-inflammatory, and potential anticancer properties
	Uracil (8)		Uracil analogs are used in chemotherapy
	3-methyl uracil (Thymine) (9)		Thymine analogs are used in chemotherapy and antiviral drugs

compounds extracted from the Thermophilic *Bacillus* species of M5a strain.

Diketopiperazine derivatives	Cyclo (Phe, Pro) (10)	
	Cyclo (Leu, Pro) (11)	 antimicrobial activity antibacterial and antifungal activity
	Cyclo (Pro, Ile) (12)	
	Cyclo (Pro, Tyr) (13)	 antitumor activity

4. Conclusions

This study delved into the bioactive potential of thermophilic *Bacillus* strains isolated from Jordanian hot springs, employing a holistic approach to metabolite analysis. TLC analysis and optimization of growth media revealed substantial variations in biochemical profiles, with CaCl₂ medium (D) identified as optimal for metabolite production. LC-MS/MS analysis provided a comprehensive exploration of secondary metabolites, showcasing the complexity and diversity influenced by varying growth conditions. The subsequent purification process yielded bioactive compounds with diverse properties, including antitumor, antioxidant, antimicrobial, and cholesterol-lowering effects. This research emphasizes the untapped potential of thermophilic *Bacillus* strains as prolific sources of diverse and bioactive metabolites. The identification of known compounds, coupled with the discovery of unique molecules, adds depth to our understanding of these strains' capabilities. Future research directions may focus on exploring specific applications, optimizing growth conditions, and investigating potential synergies between different compounds. In summary, this study positions thermophilic *Bacillus* strains as valuable resources for continued exploration in the realm of natural product discovery, contributing to our understanding of their untapped biotechnological and pharmaceutical potential.

Acknowledgements

Funding for this project was provided by Al-Hussein bin Talal University and The Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) at the University of Göttingen, Germany.

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