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## Antimicrobial Activity of *Geobacillus thermoleovorans* Ir1 Active Compounds against Pathogenic Bacteria

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### Abstract

It is necessary to follow new strategies for the isolation of bacteria with unique characteristics from novel sources during the search for new antimicrobial compounds. *Geobacillus thermoleovorans* strain Ir1 (JQ912239), a novel thermophilic bacterium, which was isolated from soil in Iraq (Basrah and Baghdad) showed a good ability to utilize hydrocarbon compounds. This study objective was to demonstrate *G. thermoleovorans* Ir1 potential antibacterial capabilities. The ability of the spore-forming bacterium *G. thermoleovorans* Ir1 to produce antimicrobial bioactive compounds that affects two pathogenic bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* was investigated. The zones of inhibition were 16 mm and 15mm respectively. Extracted bioactive compounds were identified using gas chromatography-mass spectrometry (GC-MS) analysis. The results showed that this strain inhibited the growth of the tested bacteria. The antimicrobial activity of the strain might be due to the combination between formamide and 2, 2-bis-(4-hydroxyphenyl)-butane compounds as prodrugs by comparing the gas chromatography-mass spectrometry analysis of the pure and mixed extracts.. Moreover, the filtrate of the thermophilic *G. thermoleovorans* Ir1 was examined for inhibition of biofilms formed by pathogenic bacteria, the results showed that the filtrate was able to inhibit the biofilms of *P. aeruginosa*, *S. aureus*, *B. subtilis*, *K. pneumonia*, and *E. coli*.. The results of this study are the first to record the production of these bioactive substances by the thermophilic *G. thermoleovorans* Ir1 at 65°C.

**Keywords:** *Geobacillus thermoleovorans*, Antibacterial activity, GC-MS analysis, thermophilic bacteria, bioactive compounds.

## النشاط الضد ميكروبي للمركبات النشطة لـ *Geobacillus thermoleovorans* Ir1 ضد البكتيريا المسببة للأمراض

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### الخلاصة

من الضروري اتباع استراتيجيات جديدة لعزل بكتيريا ذات مواصفات فريدة من مصادر جديدة خلال البحث عن مركبات جديدة مضادة للأحياء المجهرية. *Geobacillus thermoleovorans* Ir1 (JQ912239)

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بكتيريا جديدة محبة للحرارة ، تم عزلها من التربة العراقية (البصرة و بغداد) وأظهرت قدرة جيدة على استهلاك المركبات الهيدروكربونية. هدف الدراسة هو تحديد القدرات الكامنة لـ *G. thermoleovorans* Ir1 ضد البكتيريا. تم التحقق من قابلية البكتيريا *G. thermoleovorans* Ir1 المكونة للأبواغ لإنتاج مركبات فعالة حيويًا مؤثرة على اثنتين من البكتيريا المرضية *Pseudomonas aeruginosa* و *Staphylococcus aureus*، بمناطق تثبيط 16 و 15 ملم على التوالي. تم تشخيص مستخلص المركبات الفعالة حيويًا باستخدام تحليل كروماتوغرافيا الغاز-مطياف الكتلة (GC-MS)، وأظهرت النتائج أن هذه السلالة تثبط نمو البكتيريا التي تم اختبارها. فعالية السلالة ضد الأحياء المجهرية قد تكون ناتجة عن التأثير الجامع بين مركبات formamide and 2, 2-bis-(4-hydroxyphenyl)-butane كعقاقير أولية من خلال المقارنة بين تحليل كروماتوغرافيا الغاز-مطياف الكتلة للمستخلصين النقي والمختلط .. وهناك حاجة إلى تشخيص مركبات إضافية أخرى ذات تأثير مثبط. وأكثر من ذلك تم فحص التأثير المثبط لراشح *G. thermoleovorans* Ir1 المحبة للحرارة على تكوين الغشاء الحيوي للبكتيريا الممرضة وأظهرت النتائج قابلية الراشح على تثبيط الغشاء الحيوي لـ (*P. aeruginosa*, *S. aureus*, *B. subtilis*, *K. pneumonia*, *E. coli*) . أهداف هذه الدراسة هو تحديد القدرات المعنوية ضد البكتيريا لهذه البكتيريا. نتائج هذه الدراسة هي أول منشور ندركه يوثق إنتاج هذه المواد الفعالة حيويًا من *G. thermoleovorans* Ir1 المحبة للحرارة بدرجة 65 م°.

## Introduction

Secondary metabolites produced by both macro- and micro-organisms are what are known as natural antibacterial agents. In the history of medicine, natural ingredients have contributed most to the development of medications. Different plant, animal, fungal and bacterial species have developed various antimicrobials. Some of these antimicrobials have already been utilized as food bio preservatives and antibiotics in the food and pharmaceutical industries [1, 2]. The effectiveness of the existing antibiotics has been surpassed by the bacterial mechanisms of antibiotic resistance [3, 4]. Over 70% of pathogenic bacteria are resistant to the most common antibiotics on the market and some multi-drug resistant pathogens have mortality rates of up to 80% [5].

It has been established that biofilm-associated infections pose a serious challenge to modern medicine. Therefore, many preventative and control tactics like mechanical, physical and chemical procedures can be effectively used for preventing the formation of or eradicating the mature biofilm. A collection of microorganisms known as biofilms are found to be connected to both biotic and abiotic surfaces [6]. Different mechanisms play a role in the attachment and growth of biofilms which is a dynamic process. A key factor in the failure of antimicrobial therapy is the development of microbial biofilms. Biofilms essentially have four negative effects on health: they can cause chronic infections, antimicrobial treatment resistance to emerge, alter host immune responses and contaminate medical equipment [7].

Compared to plant and animal sources, bacteria and fungi offer opportunities for a competent scale-up of the study of natural products [8]. A large reservoir of possible novel medicinal compounds is found in bacterial and fungal metabolites [9, 10]. Forty-seven percent of all known bioactive natural compounds are derived from bacteria and fungi, and of these 84 % are antimicrobials [11].

Numerous substances produced by bacteria have antimicrobial effects on competing for bacterial strains and other organisms [12]. There are many proteinaceous and non-proteinaceous bacterial metabolites with antimicrobial activity such as bacteriocins, and lytic enzymes like bacterial amidases (proteinaceous one) and hydrogen peroxide, polyketides and organic acids (non- proteinaceous one) [13, 14].

Seeking new natural biologically active compounds and their characterization is one of the imperative tasks in modern biotechnology. Microorganisms are an important source of such compounds. Of special interest are antibiotic producers because the development of microbial pathogens resistant to common pharmaceuticals generate a need for new germicides [15].

New chemotherapeutics are always needed to treat emerging diseases and organisms that pose a serious hazard to public health. The demand for the discovery and development of novel and potent antimicrobial chemicals has increased due to the ongoing evolution of microbial pathogens and their development of antibiotic resistance [16]. There has been a great deal of interest in bacteria that can survive in harsh environments, such as thermophiles whose secondary metabolism has not yet been fully explored [17].

The most thoroughly investigated and approved source of secondary metabolites with a wide range of structural and functional properties for biotechnological applications is thermophilic bacteria [18]. The whole range of antibacterial chemicals produced by the *Geobacillus* species has not yet been fully studied, despite the genus's commercial and industrial interests [19].

The aim of current study was to detect the antimicrobial activity of bioactive compounds extracted from thermophilic *Geobacillus thermoleovorans* Ir1 (JQ912239) locally isolated [20] from oil-contaminated soil sites in Iraq. GC-MS analysis was used to confirm its ability.

## 2. Materials and Methods

### 2.1 Culture of *Geobacillus thermoleovorans* strain Ir1 (JQ912239)

*Geobacillus thermoleovorans* strain Ir1 (JQ912239) isolated from Iraqi soils was stored in silica gel. To activate the bacterium in culture media, 0.1 mg of bacterial isolate was inoculated into 500 ml of Lauria-broth (LB) medium supplemented with 0.1 ml MnCl<sub>2</sub> in a conical flask. The flasks were then placed in an incubator for 24-48 hours at 65°C and 150 rpm. After that, the bacterium was streaked on LB plates using a loop full and incubated overnight at 65°C. Using a sterile loop, single colonies were isolated and transferred into LB broth and incubated under the same conditions. The bacterium was stained with Gram +ve stain and the purity was checked by microscopic examination [20].

### 2.2 Tested Organisms

*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Escherichia coli* from Al Yarmouk hospital, Baghdad

### 2.3 Specimens Collection

Specimens were collected from thirty patients suffering from skin infections and wounds with purulent discharge and those with severe burns who had visited Al Yarmouk hospital, Baghdad during 2020. Samples were collected using sterile, single-use cotton swabs and stored in test tubes with non-nutritional media which preserves organism viability without considerable proliferation (Stuart transport medium). Identification of the bacterial isolates was done by VITEK 2 system.

### 2.4 Agar Diffusion Assay for Antibacterial Production

Screening the ability of the thermophilic bacterial isolate to be antagonistic to the growth of *P. aeruginosa* and *S. aureus*, an agar well diffusion assay was used [21]. Tested colony of *P. aeruginosa* and *S. aureus* was grown to reach turbidity that is equal to 0.5 optical density of McFarland standard and spread on agar plates in a lawn form. In the agar plates wells were made by sterilized borer which were filled with the culture filtrate of the thermophilic bacterial

isolate and then incubated for 24 h at 37°C, after which the diameter of the zone of inhibition (in mm) was observed and measured for the activity of antimicrobial compounds.

### 2.5 Mixed Culture Fermentation of Bacterial Isolates

The thermophilic isolate was grown in a flask containing 100 ml of LB broth at 65°C. In separate flasks, *P. aeruginosa* and *S. aureus* were grown overnight at 37°C. After 24 h of incubation, 1 ml of each pathogenic bacteria was inoculated by thermophilic isolate for the induction of bioactive antimicrobial compound production and incubated for 24h at 65°C. The broth was then transferred for the extraction of secondary metabolites followed by an antimicrobial activity test [22].

### 2.6 Extraction of Metabolites from the Thermophilic Bacteria

The thermophilic *G. thermoleovorans* strain Ir1 was grown in 100 ml of LB broth at 65°C for one day under shaking conditions. Cultures were centrifuged at 6000 rpm for 10 min at 4°C and then keeping centrifugation ratio of 3:1, ethyl acetate was added to separate the bioactive compounds and subsequent filtration of the supernatant through 0.2 µm filter paper under aseptic conditions. The organic phase vacuum evaporated to dryness at 45°C.

### 2.7 GC–MS Analysis

Using GC-MS and a capillary column ZB-5MS (30m x 0.25mm, ID. 0.25m), bioactive chemicals in the ethyl acetate extracts were traced. The instrument carrier gas (He) flow rate was 1.61 ml/min and the electron in the ionization mode was adjusted to 70 eV using a split injection mode. The injector and detector had respective temperatures of 230°C and 280°C. The oven's temperature was first adjusted to 80°C and maintained there for 2 minutes. From there, it was raised to 200°C at a pace of 15°C per minute, and then to 230°C at a rate of 4°C per minute, and lastly to 280°C at a rate of 10°C per minute and maintained there for 2 minutes. The instrument's transmission line and ion source temperatures were set at 230°C and 260°C respectively. The injection volume was 1 l and there was no splitting of the injection during the process. The delay for the solvent was 4 minutes.

### 2.8 Preparation of the Thermophilic Filtrates:

The non-concentrated filtrate of *G. thermoleovorans* Ir1 was obtained by mixed culture fermentation of microbial strains (as mentioned above). Then the culture was centrifuged at 4°C by 6000 rpm for 15 min, and then its suspension was filtrated through 0.2 µm millipore filters. The concentrated filtrate was prepared by evaporating 100ml of the un-concentrated filtered suspension in the oven at 45°C to reduce the amount to 50ml to obtain the one-fold concentrated filtrate. The test was repeated with the one-fold concentrated filtrate to gain the two-fold concentrated filtrate (25ml), and so on for the three-fold concentrated filtrate (12.5ml) [23].

### 2.9 Detection of Antimicrobial Activity of Thermophilic Filtrates on Biofilm Production by Microtiter Plate Assay:

The ability of the bacterial filtrates to inhibit biofilm formation (prevention of initial cell attachment) was detected by the biofilm inhibition assay. Briefly, 100 µl pathogenic microorganisms (*P. aeruginosa*, *S. aureus*, *B.subtillus*, *K. pneumonia*, and *E. coli*) were added to individual 96-well microtiter plates with flat bottoms and incubated for 4 h at 37°C without shaking. To achieve a final concentration of 7.81 mg/mL, 100 l (1 mg/ml) filtrate was put in eight repetitions into the wells of 96-well microtiter plates. The plates were then withdrawn from the incubator and incubated for a further 24 h at 37°C without being stirred. Muller Hinton media served as the negative control, while DMSO served as the positive control. The biomass

was measured utilizing a modified crystal violet staining technique [24]. The 96-well microtiter plates underwent a quick test, five sterile distilled water washes, air drying, and a 45-minute oven drying at 60°C. Following a 15-minute incubation period at room temperature during which the wells were dyed with 100  $\mu$ l of 1% crystal violet, the plates underwent three washes with sterile distilled water to remove any remaining stains. Biofilms were now visible as purple rings at the side of the wells. After de-staining the wells with 125  $\mu$ l of ethanol, the semi-quantitative evaluation of biofilm formation was carried out. A 100- $\mu$ l aliquot of the dye-staining solution was transferred to a fresh, sterile plate, and a microplate reader was used to detect the absorbance at 570 nm. Samples average absorbance and the percentage of biofilm inhibition were calculated using the equation below:

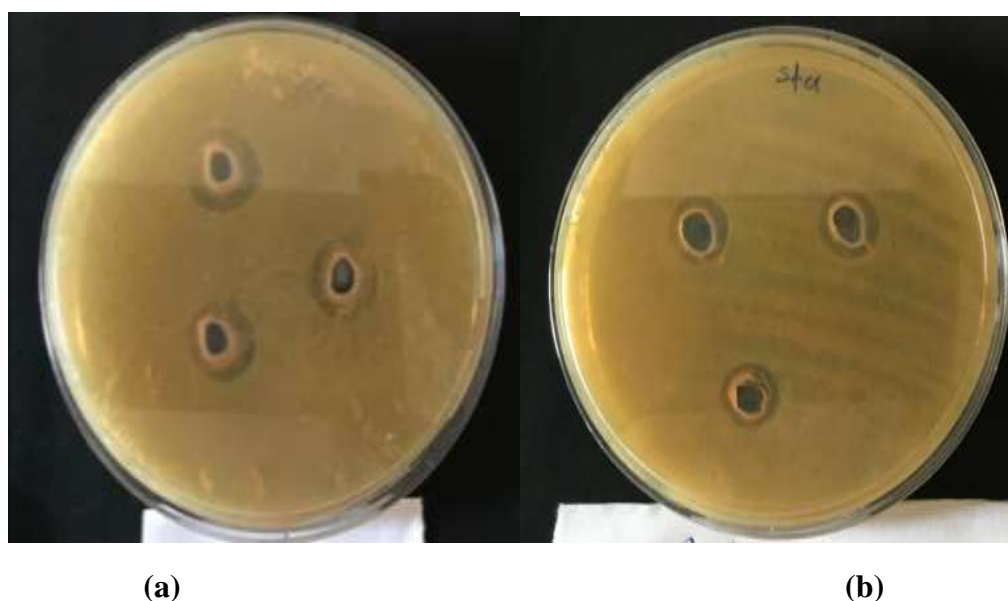
• Inhibition percentage (%) =  $\frac{\text{Negative control OD} - \text{Experimental OD}}{\text{Negative control OD}} \times 100$

It would have been interesting to use the crystal violet assay to see if the cells in the biofilm were still viable, even if it was not mentioned in the protocol we followed.

### 3. Results and Discussion

#### 3.1 Antibacterial Production by *G. thermoleovorans* Ir1:

Samples collected from batch fermentation were evaluated by an agar diffusion assay, using *P. aeruginosa* and *S. aureus* as test organisms. Antimicrobial activity was determined by measuring the zone of inhibition. It was observed that *G. thermoleovorans* Ir1 bioactive filtrate exerted activity against *P. aeruginosa* and *S. aureus* measured as a zone of inhibition of 16mm and 15mm respectively (Figure 1).



**Figure 1:** The antimicrobial activity of the thermophilic bacterium filtrate (50 $\mu$ L) against multidrug-resistant bacteria. (a) *Pseudomonas aeruginosa* and (b) *Staphylococcus aureus*.

Seeking a new source of antimicrobial substances is a challenging endeavor that requires a variety of approaches. Although the risk of re-isolating existing compounds exists when natural compounds are obtained through nature screening, this approach has led to the discovery of a variety of unique natural antibacterial compounds from various organisms [25]. Extremophiles production of antimicrobials is not surprising given that they compete with other species for nutrition and habitat to inhabit; extremophile-produced antimicrobials have received increased attention [26]. In nature, there are undoubtedly other unique products that are just waiting to be discovered [27].



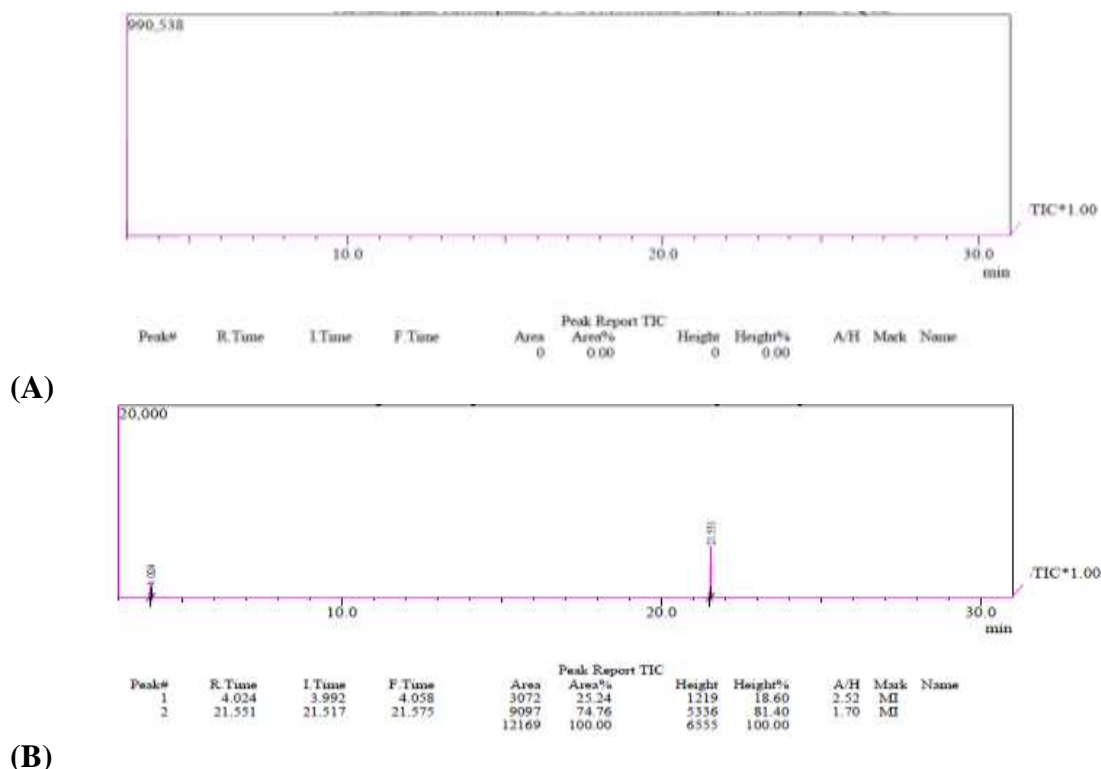
### 3.2 GC–MS Analysis to Confirm the Presence of Bioactive Compounds:

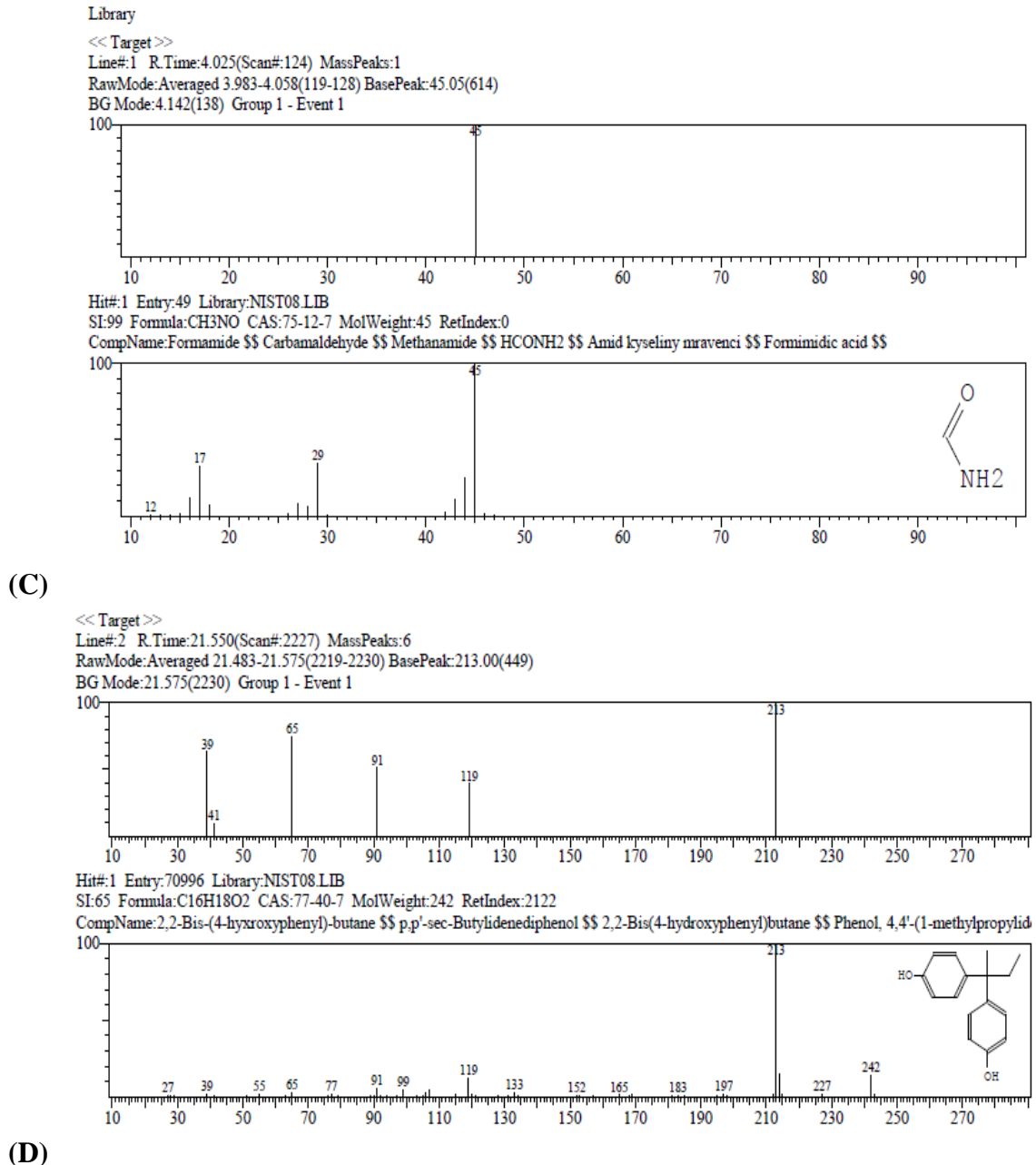
GC–MS analysis was carried out in the Ministry of Industry and Minerals the ethyl acetate extract chemical makeup is clarified in (Figure 2), which shows the presence of different chemicals with varying abundances and retention times, formamide and 2,2-bis-(4-hydroxyphenyl)-butane.

A significant supply of fresh antibacterial compounds continues to come from natural chemicals created by soil microbes. Particular attention should be paid to the *Geobacillus* and *Parageobacillus* genera of bacteria in this situation. Although these microbes are of great importance in the commercial and industrial fields, little is known about the full range of antibacterial chemicals produced by the *Geobacillus* and *Parageobacillus* species [19].

Recently, *Geobacillus* has come under scrutiny as a potential source of therapeutically effective thermostable L-asparaginase [28]. By creating a variety of volatile chemical compounds, *Geobacillus* sp. (M-7) is expected to have a distinct strategy for fending off rival bacteria (benzaldehyde; acetic acid; butanal, 3-methyl-; butanoic acid, 2-methyl-; butanoic acid; propanoic acid, 2-methyl- and benzeneacetaldehyde). The *Geobacillus* strain are able to stop *Geotrichum candidum*, *Verticillium dahliae*, *Botrytis cinerea* and *Aspergillus fumigatus* from growing after 48 h and to kill them after 72 h [29].

Additionally, it was revealed that the thermophilic bacterial species, *Geobacillus* sp. LEMMJ02, was recovered on Deception Island from an Antarctic volcano sediments. The existence of genes linked to the synthesis of secondary metabolites with antibacterial characteristics was discovered by observing *Geobacillus* sp. LEMMJ02 genome. The strain probably generates bacteriocins, terpenes, and phengycin (an antifungal lipopeptide) [30].

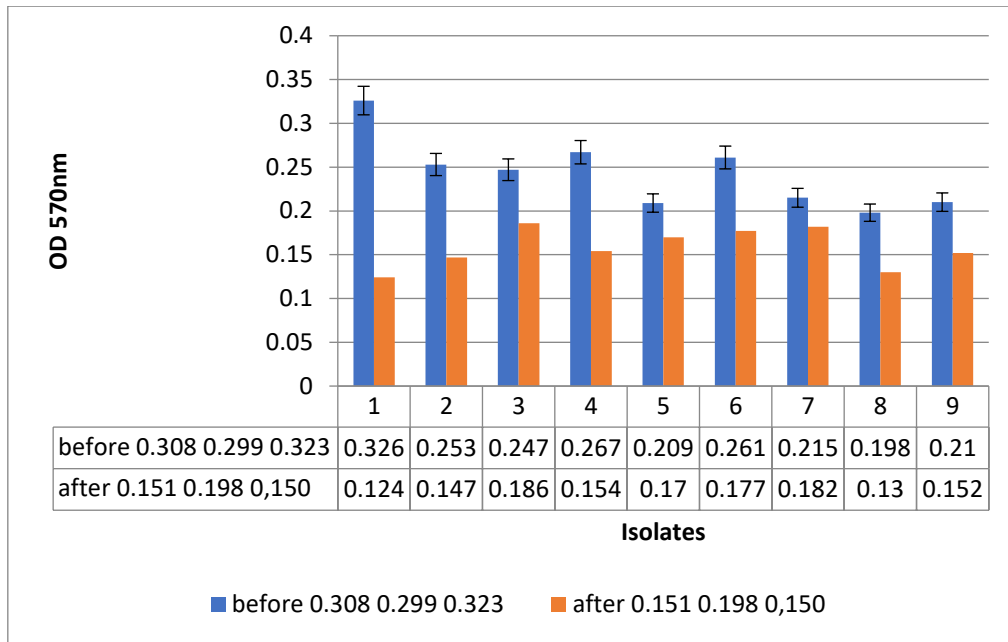




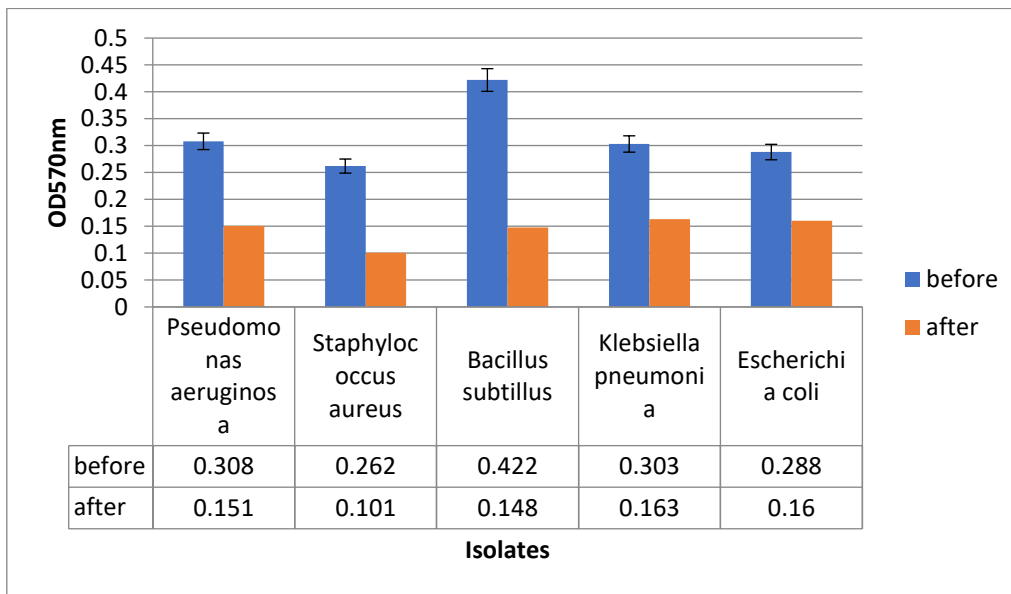
**Figure 2:** GC–MS spectrum of ethyl extract of *Geobacillus thermoleovorans* strain Ir1 (JQ912239) under static condition, PH 7, 65 °C, and 24 h. (A) before treatment, (B) after treatment, (C) line 1 formamide, (D) line 22, 2-bis-(4-hydroxyphenyl)-butane.

### 3.4 Detection of Antimicrobial Activity of Thermophilic Filtrates on Biofilm Production by Microtiter Plate Assay:

*Geobacillus thermoleovorans* strain Ir1 (JQ912239) three-fold concentrated filtrate was found to have an inhibitory effect on biofilms formed by the pathogenic strains (*P. aeruginosa*, *S. aureus*, *B. subtilis*, *K. pneumonia*, *E. coli*). Figure 3 shows the OD of biofilm formation in the microtiter plate by nine isolates of pathogenic *P. aeruginosa* before and after treatment with the thermophilic filtrate (0.308, 0.299, 0.323, 0.326, 0.253, 0.247, 0.267, 0.209, 0.261), (0.151, 0.198, 0.150, 0.124, 0.147, 0.186, 0.154, 0.170, 0.177 respectively). While Figure 4 shows the OD of biofilm formation in microtiter plates by the other pathogenic isolates before and after treatment.



**Figure 3:** The detection of antimicrobial activity of thermophilic bacteria filtrate in micro-titter plates against biofilm formation by nine isolates of *Pseudomonas aeruginosa* before and after treatment. The differences were analyzed by performing t-test and a p-value of <0.05 was considered significant.



**Figure 4:** The detection of antimicrobial activity of thermophilic bacteria filtrate in microtiter plates against biofilm formation by (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *B. subtilis*, *K. pneumonia*, *E. coli*) before and after treatment. The differences were analyzed by performing t-test, and a p-value of <0.05 was considered significant.

Promising reports arise about *Geobacillus* antibacterial uses. *Geobacillus* sp. strain ZGt-1, discovered in Jordan, was shown by Alkhalili *et al.* to have antibacterial activity against *Geobacillus stearothermophilus* *Salmonella typhimurium*, and *Bacillus subtilis* [31]. Furthermore, Pokusaeva and associates showed that they were able to identify and partially purify the bacteriocins (proteins ribosomally generated that suppress other bacterial species or strains) produced by the oil well isolated bacterial strains (*G. stearothermophilus*) in Lithuania.



These studies might point to a variety of novel, beneficial uses for the *Parageobacillus* and *Geobacillus* genera [32].

A distinctive antibiotic pigment similar to cyanoxanthomycin is produced by *Geobacillus* sp. Iso5. With respect to specific Gram-negative and -positive bacteria, such as *E. coli* (MTCC 1698), *B. subtilis* (MTCC 3053), *Streptococcus* sp. (MTCC 9724), *Staphylococcus aureus* (MTCC 6908), and *Pseudomonas aeruginosa* (MTCC 6458), this fluorescent pigment has strong antibacterial activity [33].

The soil-contaminated oil in Iraq contains bacterial communities that are capable of enduring challenging circumstances. These microbes appear to have gained the ability to adapt to their environment by producing bioactive chemicals. Additionally, since they are only effective against a specific class of infections, research on narrow-spectrum antibiotics might be more beneficial. With the rapid development of real-time diagnostics which may soon allow for earlier disease diagnosis, this strategy will become more logical. The development of clinically important resistant pathogens would be slowed by the availability of narrow-spectrum antibiotics which would prevent the overuse of broad-spectrum antibiotics [34].

#### 4. Conclusion

In conclusion, *Geobacillus thermoleovorans* strain Ir1 (JQ912239) exhibited antimicrobial activity against all tested human pathogens, and GC-MS analysis confirmed the production of antimicrobial compounds. The results revealed that this strain could act as a source of components with broad-spectrum activity against pathogenic microbes.

#### 5. Acknowledgment

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#### 7. Conflict of Interest

The authors declare that they have no conflict of interest.

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