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# Assessment of Antimicrobial Effect of Soil Bacillus sp. Isolates against Select Pathogenic Bacteria

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## Abstract:

**Background:** Soil Bacillus sp. provides good resources for the isolation of therapeutically important products that fight against bacteria that cause infections**,** and a variety of bacteria have acquired antibacterial resistance against commercial antibiotics; therefore, it was necessary to find substances that avoid the formation of resistance against these antibiotics. Examination of the effectiveness of secondary substances produced by Bacillus sp. against *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia Coli*, *Acetobacter aceti*, *Acetobacter baumannii*, and *Pseudomonas aeruginosa*. **Materials and Methods:** Bacillus sp. were collected from different sites. After isolation and identification of *Bacillus sp*. the primary screening was observed against five pathogenic bacteria, then the Bacillus sp. was fermented to observe the secondary metabolite antimicrobial effect on these bacteria. Gas chromatography analysis for secondary metabolites was done to detect the components of this substance. **Results:** Evidence of antimicrobial effectiveness against E. coli was noted. Absence of antimicrobial effects was detected on other pathogenic bacteria during the initial screening phase. The antibacterial activity of the secondary metabolite was observed against all test pathogens. Notably, *Klebsiella pneumoniae*, a gram-negative pathogen, was more sensitive than other pathogenic bacteria in this research. Additionally, *Staphylococcus aureus*, a gram-positive pathogen, was also affected. **Conclusion:** The secondary metabolites from Bacillus sp. demonstrated potential as effective antibacterial agents against select pathogenic bacteria.

**Keywords:** Bacillus sp., Fermentation, Pathogenic bacteria

## Introduction

Soil Bacillus sp. provides good resources for the isolation of therapeutically key products. It's an important genus [1]. Bacillus sp. is widely distributed in the soil, which is its primary habitat. They are generally found in vegetables, decaying organic matter, water, dust, and some sp. are normal flora [2]. Bacillus sp. is a gram-positive, rod-shaped bacterium. *Bacillus* has endospores and can persist in this dormant state for years. Many sp. of *Bacillus* can yield copious amounts of enzymes, which are used in several industries, such as

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producing alpha amylase, which is used in starch hydrolysis, or the protease subtilisin used in detergents [3]. Antibiotics play a crucial role in combating infectious bacteria, yet antimicrobial treatments can lead to significant human health issues due to associated side effects. A variety of bacteria have acquired antibacterial resistance in the last two decades [4].

However, the antibiotic often leads to dysbiosis of microbiota and multi-bacterial infection. Also, in some cases, antibiotics may cause the progression of MDR bacteria. Particularly, sublethal concentrations of antibiotics improve the production of virulence factors to improve the bacteria's persistence [5]. Although the emergence of resistance to antibacterial is a natural phenomenon, the substance of antibiotic use, and to conquer the AMR emerges and extends globally, several novel antibacterial approaches are in improvement, including using secondary metabolites of some naturally occurring bacteria to overcome the phenomenon of antibiotic dwarfism [6]. Secondary metabolites intensely affect bacterial physiology, stress responses, and metabolism. Increasing evidence proposes that these products can modify bacterial susceptibility to used antibiotics [7]. The secondary-metabolite produced is a compound produced from the secondary metabolic process, which can generally be in the form of enzymes, antibiotics, toxins, growth regulators, and insecticides [8]. Low pH with fermentation and high concentrations of acids, these conditions can inhibit the growth of many bacteria [9]. Our comprehension of the biological functions of secondary metabolites remains restricted, especially in light of their structural variety and pharmaceutical significance. Therefore, it was necessary to find solutions to these problems, and one of these solutions is to find substances that work against bacteria and to avoid the formation of resistance against these antibiotics.

## Subjects and Methods

### Study design

Bacillus sp. were collected from different sites. After isolation and identification of Bacillus sp. the primary screening was observed against five pathogenic bacteria (*Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia Coli*, *Acetobacter aceti*, *Acetobacter baumannii*, *Pseudomonas aeruginosa)*, then the Bacillus sp. was fermented to observe the secondary metabolite antimicrobial effect on these bacteria. Gas chromatography analysis for secondary metabolites was done to detect the components of this substance**.**

### Exclusion and inclusion criteria

Bacillus strains isolated from soil environments are included. The secondary metabolites of Bacillus sp. are included in the study. The study includes consistent methods for Bacillus sp., secondary metabolite extraction, and antimicrobial testing.

Bacillus sp. isolated from sources other than soil is excluded. Pathogenic bacteria not relevant to the research objectives are excluded. Unreliable methods for Bacillus isolation, metabolite extraction, or antimicrobial testing are excluded.

### Soil sampling

A soil sample was collected from different niche habitats of Ramadi and Falluja city, Iraq. Soil samples were taken from various places in the cities mentioned above. Samples were collected from the soil by a sterile corer. For each location, the corer was sterilized with alcohol before taking a sample. 10 cm for each collection was made below the surface of the ground. Sterile bags were used to store and transport the samples, and then immediately transported to the lab. The soil samples were dried for an hour at 70 °C, sieved, and smashed before being used for the isolation purpose [10].

### Bacterial isolates

Bacillus sp. isolates were obtained from soil from three sites in Ramadi city center. Ten grams of each sample were suspended in sterile distilled water with

phosphate buffer. A water bath was used in heat treatment to perform at 70 °C for 60 min with agitation. 10-fold dilutions of the samples were prepared using phosphate buffer. The sample from each dilution was cultured on a nutrient agar, which contains cycloheximide (100 $\mu g\, mL^{-1}$) to evade fungal growth, then incubated at 37 °C for 24 h [11].

### Identification of Bacillus species: Biochemically and morphologically Characterization

The colonies of Bacillus were identified based on morphology and then Gram stain. Later identification tests involved starch hydrolysis, gelatin hydrolysis, hemolysis, Voges–Proskauer test, citrate test, and temperature at different degrees and growth pH [12].

### Pathogenic organisms:

The organisms were obtained from AlRamadi Teaching Hospital (Ramadi, Iraq). The selected human pathogenic bacteria used in the antimicrobial study were *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia Coli*, *Acetobacter aceti*, *Acetobacter baumannii*, and *Pseudomonas aeruginosa*.

### Primary Screening for antimicrobial activity of Bacillus sp. isolates:

The screening method for the activity of Bacillus sp. of pure isolates was determined by the cross-streak method on Mueller-Hinton Agar. Then, by agar well diffusion method with crude extract of ethyl acetate after secondary metabolite extraction [13].

### Secondary Screening Processes (Fermentation):

The International Streptomyces Project (ISP-2) medium, malt, and yeast extract were used in this research; the samples were fermented using shaking in an incubator at 150 rpm for a week under 28 $^{o}$C figure (Figure 2) [13].

### Extraction of Extracellular Bacillus sp. Metabolites

Fermentation was done to evaluate the bioactive compound. 50 ml of ISP1 broth was inoculated with Bacillus sp. isolates in a conical flask, then incubated at 30°C for a week at 150 rpm. Next, fermentation, the metabolites were separated from the bacteria by centrifugation for 10 min to eliminate cells and debris, and then harvested from the broth that fermented. This broth was added to ethyl acetate to achieve the best extraction of antimicrobial metabolites. it evaporated dry residues were again dissolved in DMSO and lyophilized [14].

### Analysis of Gas Chromatography-Mass Spectrometry

The Secondary metabolites of Bacillus sp. were identified using gas chromatography-mass spectroscopy at the Ministry of Science and Technology (Baghdad, Iraq) [15].

## Results

The soil-derived isolates of Bacillus sp. (n = 5) on the nutrient agar plate with cycloheximide were observed to be gray-white, opaque, round, flat, medium-sized, drying, and beta-hemolytic.

**Primary Screening of Antimicrobial Activity of Bacillus sp. Isolates:**

Bacillus sp. activity was evaluated by measuring the inhibition zone around the Bacillus sp. colonies. Production of antimicrobial substances against *E. coli* was observed. No antimicrobial activity was observed against other pathogenic bacteria (Figure 1). The secondary metabolites of the five samples were similar in their effect on pathogenic bacteria.

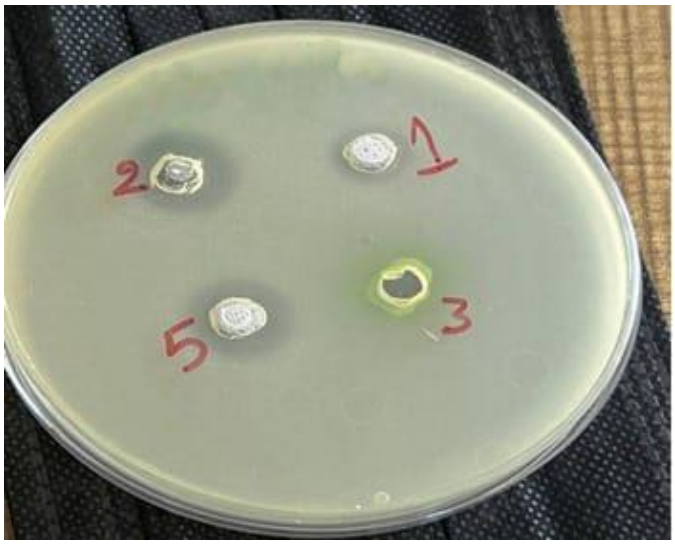


Figure (1): Antibacterial activities of primary metabolites were tested for *E. coli* no.. 2 and 5.

**Secondary Screening Processes (Fermentation)**:
Fermentation was done by using malt and yeast extract to test its ability to produce dyes, table (1), figure (2).

**Table 1: Bacillus sp. isolates from soil**

| Isolate | City | Color of fermentation |
|---|---|---|
| 1 | Ramadi city | Green dye |
| 2 | Ramadi city | Green dye |
| 3 | Ramadi city | Green dye |
| 4 | Falluja city | Yellow dye |

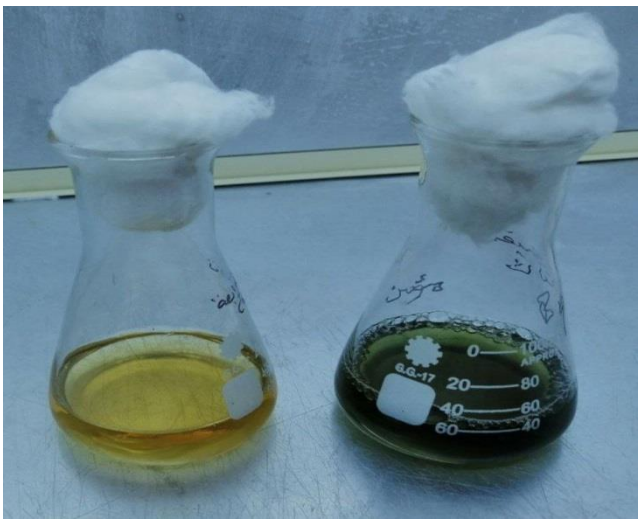

**Figure 2: Production of secondary metabolites using fermentation**

Antibacterial activities of secondary metabolites were tested for four isolates, as in Table 1, against *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia Coli, Acetobacter baumannii,* and *Pseudomonas aeruginosa* (Figure 3). The antibacterial activity of four isolates was observed against all test pathogens. One gram negative was more sensitive than other pathogenic bacteria in this research, which was *Klebsiella pneumoniae*, and a gram-positive pathogenic bacterium, which was *Staphylococcus aureus,* and the isolate showed moderate activity against *Escherichia Coli*. No antimicrobial activity was recorded against *Acetobacter baumannii* and *Pseudomonas aeruginosa*.

**Gas chromatography analysis**

The GC-MS analysis of green Secondary metabolites of Bacillus **sp.** is presented in Figure 4. Ten major compounds were identified, comprising 86.448% of the Secondary metabolites. These compounds were identified as Oleic Acid (20.2%), 9-Octadecenoic acid (15.1%), beta-Acetoxy-bisnor (11.957%), Thymol Phenol (9.351%), Hexadecanoic acid (8.89%), N-acetyltryptamine (7.87%), Benzene (4.13%), Methyl stearate Heptadecanoic acid (3.81%), Benzene (2.33%), and Indole (2.81%) figure(4).

The GC/MS analysis of the yellow Secondary metabolites of **Bacillus sp.** is presented in Figure 5. Eight major compounds were identified, comprising 78.71% of the Secondary metabolites. These compounds were identified as Pyran (5.33%), Trifluoroacetylimidazole (6.58%), N-acetyltryptamine (21.78%), Dibutyl phthalate Phthalic acid (5.89%), Methyl (5.29%), 9-Octadecenoic acid (18.9%), Methyl stearate (9.89%), and Pyrrole (5.05%).

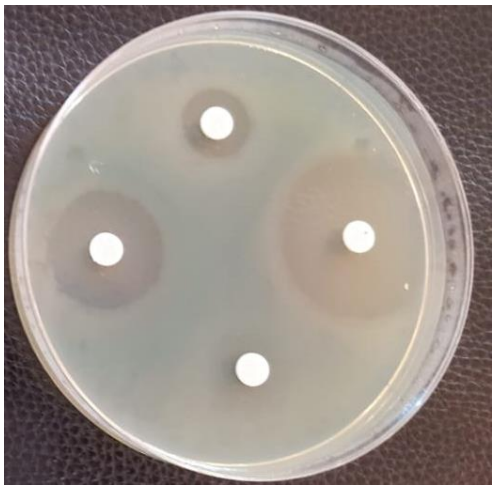

**Figure (3): Antibacterial activities of secondary metabolites were tested for *Staphylococcus aureus* and *Klebsiella pneumoniae***

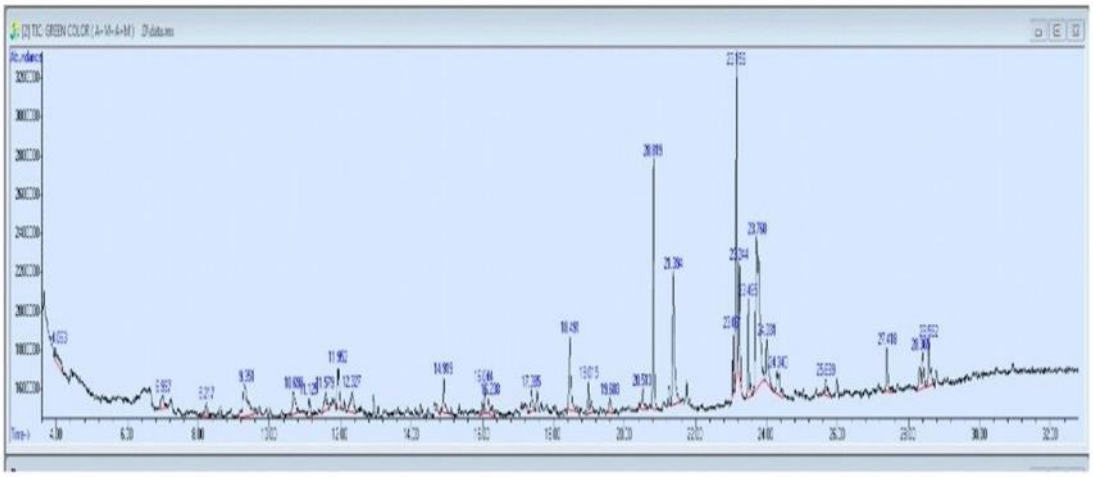

Figure (4): GC-MS spectrometry of the green secondary metabolites of Bacillus sp.

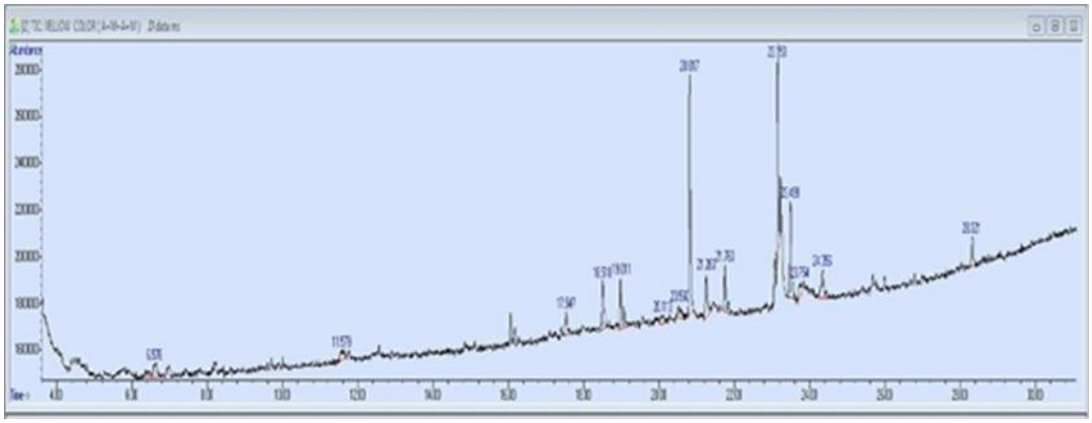

Figure 5: Gas chromatography-mass spectrometry analysis of the yellow secondary metabolites of Bacillus sp.

## Discussion

The emergence of drug-resistant pathogens has evolved into a critical global health challenge within the realm of infectious diseases. Bacillus sp. has been studied in several environments and extreme habitats in various areas in the world the recent years. There are no reports regarding the Bacillus sp. isolated from Anbar governorate (Iraq). Consequently, the Bacillus sp. has been isolated from this unexplored area to find new species that may help to find substances against some pathogenic bacteria. Bacillus sp. can produce a wide variety of metabolites, such as inorganic and organic acids, enzymes, biopigments, and hydrogen sulphide [16].

The results were in line with Alqahtani *et al*., 2023, [17] which proved that there is antibiotic activity against some pathogenic bacteria, such as *E. coli* and *Kl. pneumonia*. Some compounds have antibiotic activity that is produced by these bacteria [17]. One of these compounds is Oleic Acid; GC-MS analysis showed there is 20% of the compounds are Oleic

acid. Dilika *et al*., 2000, [18] showed there is antibiotic activity against gram-negative and gram-positive bacteria (*Staph. aureus*). This could be because of the generation of various forms of antimicrobial substances. Bacillus sp. encoded high genetic capacity for antimicrobial compound production, polyketides, cyclic lipopeptides, and others [19].

Also, N-acetyltryptamine has antibiotic activity against pathogenic bacteria, as suggested by Ramlawi et al., 2021, [20]. *Bacillus* isolates obtained from soil can produce both peptides and non-peptide metabolites that can inhibit a broad range of pathogenic bacteria. the bacteria themselves or their metabolites could be used as biocontrol to decrease the pathogenic bacteria found in soil. Moreover, if the compounds are new and safe, they may be good for the development of new drugs [21].

## Conclusion

The research implies that soil-collected bacteria from unexplored regions have the capability to generate diverse compounds with potential multi-purpose benefits; one of these purposes is used as an antimicrobial agent. Such compounds may exhibit reduced side effects compared to standard antibiotics and potentially offer enhanced efficacy.

## Author contribution:

This research was divided into isolation, identification, and extraction of secondary metabolites, which was done by A.K. Al-Qaysi, H.R.S. Al-Alwani, and S.A. Al-Meani. M.M. Ahmed was to identify the chemical compounds in the bacterial extract using GC mass spectrometry**.**

**Ethical statement:** Ethical approval obtained from the University of Anbar, Ramadi, Iraq (approval number 112, October 29, 2024).

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**Availability of data and materials:** not available.

**Competing interests:** The authors have no conflicts of interest to declare.

**Consent for publication:** We announce our consent to publish the information included in the research.

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