

**SCREENING AND PRODUCTION OF ANTIMICROBIAL AGENTS
FROM THERMOPHILIC BACTERIA ISOLATED FROM HOT
SPRINGS OF SAUDI ARABIA**

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BY

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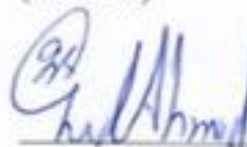
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Dedication

This work is dedicated to my dear mother and father for their endless support throughout my life until they made me the person I am today, words cannot express my worm feelings and appreciation to the constant love, support, and encouragement they generously and continuously offered me throughout my life. I thank my wife Anfal for her continuous support throughout my master journey which has made it a lot easier for me.

I dedicate my thesis to my great country Saudi Arabia for providing me many opportunities that shaped my current knowledge.

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LIST OF ABBREVIATIONS

AMAs: Antimicrobial Agents

TB: Thermophilic bacteria

CFE: Cell Free Extract

ABSTRACT

Full Name: Abdulkareem Mohammed Khaled Almazied

Thesis Title: Screening and production of Antimicrobial Agents from Thermophilic Bacteria Isolated from Hot Springs of Saudi Arabia

Major Field: Life Science Department

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Finding a solution to bacterial resistance to antibiotics is extremely important now a days. Four strains of thermophilic bacteria that were isolated from hot springs of Saudi Arabia are used in this study. These strains are *Brevibacillus borstelensis* AK1 (strain 43), *Bacillus licheniformis* (strain 13), *Geobacillus stearothermophilus* (strain 2) and *Anoxybacillus flavithermus* AK1 (strain 38). These strains were incubated and grown on TT media at 50°C for 24 hours. The aim of this study is to detect potential antimicrobial agents (AMAs) and elucidate their structure using layer chromatography (TLC), Gas chromatography mass spectrometry (GCMS) and Liquid chromatography–mass spectrometry (LC-MS). It was found that all strains have caused inhibition against selected test organisms, where *Anoxybacillus flavithermus* AK1 have caused the highest inhibition against *Staphylococcus aureus*. GCMS analysis for *Brevibacillus borstelensis* AK1 cell free extract (CFE) showed that it contains 2,3-Butandiol as a major secondary metabolite which is proven to have antimicrobial abilities.

Keywords: thermophilic bacteria, antimicrobial agents

ملخص الرسالة

الإسم الكامل: عبدالكريم محمد خالد المزيد

عنوان الرسالة: التحري عن وإنتاج مضادات حيوية من أنواع البكتيريا المحبة للحرارة والتي تم استخلاصها من
الينابيع الساخنة في المملكة العربية السعودية

التخصص: العلوم الحياتية

تاريخ الدرجة العلمية: 2020 / 12

إيجاد حل لمقاومة البكتيريا للمضادات الحيوية أصبح ذو أهمية مطلقة في وقتنا الحالي. أربع سلالات من بكتيريا
تم استخلاصها من الينابيع الحارة في جنوب السعودية لأجراء هذا البحث. هذه السلالات هي سلالة رقم ٤٣، سلالة
رقم ١٣، سلالة رقم ٢ وسلالة رقم ٣٨. تم تحضين هذه السلالات ونموها في وسط التي تي عند درجة حرارة ٥٠
مئوية ولمدة ٢٤ ساعة. الهدف من هذا البحث هو إيجاد مضادات بكتيرية محتملة وتحديد تركيبها الكيميائي
باستخدام تي ال سي، الجي سي ام اس وال سي ام اس. تم إيجاد ان جميع هذه السلالات تسببت في تثبيط نمو
الكائنات التي تم استخدامها. سلالة رقم ٣٨ تسببت في اعلى تثبيط ضد بكتيريا الستافيلوكوكيس اوريوس. تحاليل
الجي سي ام اس لمستخلص سلالة ٤٣ الخالي من الخلايا اظهر انه يحتوي على مركب ٢،٣- بيوتاندايول والذي تم
اثبات بقابليته على تثبيط نمو البكتيريا.

CHAPTER 1

INTRODUCTION

The growing demand for antibiotics has caused increased resistance to many drugs, which has driven the need to find an alternative antimicrobial agent. As an illustration, according to the Centers for Disease Control and Prevention (CDC), more than 2.8 million antibiotic-resistant infections occur every year in the US alone. Thus, 35 thousand people die yearly. Fight for survival compelled organisms to develop different defense strategies to outlive competitors. A strategy that has been developed by thermophiles is the production of antimicrobial agents to put an end to competitors' annoyance (Khalil, 2006). These agents have future potential for clinical use (Jun and Lichun, 2019). These potential antimicrobial substances will help target antibiotic-resistant bacteria and reduce the cost of many treatments, which have increased because bacteria acquire resistance to drugs. According to the CDC (Centers for Disease Control), due to the drug resistant bacteria causing tuberculosis (TB), the average cost of TB treatment has vastly increased from an average cost of \$18,000 to nearly \$513,000.

Furthermore, drug-resistant microbes are a very concerning problem in hospitals with acquired infections, which leaves physicians in a difficult position with limited therapeutic options (cornejo-juárez, 2015). The resistant to drugs can also occur due to the misuse of antibiotic where the patient take drugs in a manner or dose other than what was prescribed by his or her medical health provider (U.S. Department of Health

and Human Services,2022). With the search for new drugs to which bacteria have not become resistant, a search for new targeting methods should be developed. Ecological competitions have provided us with a promising new method of bacterial targeting from extremophile secretions.

Thermophilic bacteria extracted from hot and saline water lakes display distinctive characteristics that enable them to survive in such extreme environment. Thermophilic bacteria are a source of valuable industrially important metabolites such as thermostable enzymes. A study on Jordanian hot springs thermophiles has proven valuable thermostable enzymes such as amylase, protease, and cellulose (Al Daghistani,2017). One of these is the secretion of antimicrobial peptides, which makes them a potential source to target bacteria (Jorgensen, 2003). Although various naturally produced AMAs have been extracted, most of them cannot be clinically used due to their toxic activities. Of the small group that has not recorded any toxic activities and can be used in clinical therapy is an example of nontoxic antimicrobial producer bacterial species such as penicillin, streptomycin, and bacillus (brock, 1986).

These antimicrobial agents are investigated for their potential use in various industries. Thus, large numbers of extremophiles have been isolated since 1970s. Some of the peptides extracted from thermophilic bacteria have shown great antimicrobial activity against gram-positive bacteria, such as meracidin (Khalil, 2006). These antimicrobial peptides have a secondary metabolite with cyclic hydrophobic structures and compounds such as d-amino acids, which exhibit more resistance to proteolytic degradation (Mikulík, 2017). Only 10% of microorganisms have been explored and

isolated from Extreme habitats. Highlighting their importance will encourage the scientific community to uncover the golden potentials of this niche (Romano, 2005).

Studies that have been conducted on hot springs in Saudi Arabia suggests the presence of AMAs on different types of isolates that present a promising opportunity for industrial and pharmaceutical uses. A 2018 study on the hot springs of Jizan Saudi Arabia have investigated more than 84 bacterial isolates, 50 of which have shown antimicrobial effects against one or more human pathogens. 4 of those isolates have revealed highest antimicrobial activity against human pathogens, showing wide range of inhibitory mechanisms. These isolates exhibit antimicrobial activity by inhibiting the growth of all tested human pathogens. These isolates when tested with gas chromatography have shown many industrially and pharmaceutically important secondary metabolites such as cyclohexyl acrylate, imiloxan, tabtoxine- β -lactam, nicotiny alcohol, mephenesin, etomidate and L-menthyl lactate. *Candida albican* and *Proteus mirabilis* are known to exhibits resistance against many AMAs but have failed to do the same with these isolates which again shows great potential and value of hot springs in southern Saudi (Alrumman,2018)

A study conducted in 2011 to isolate TB strains that produces thermos stable enzymes such as amylase, cellulase and lipase. These enzymes have degradations capability that could be used in many industries. The harsh environments of these TB have encouraged many studies to dig deep into their physiology to study their biological products (Amjad,2011). Hot springs have never been extensively exploited for the existence of such organisms in regard to their AMAs (jain & Pednekar, 2011)

1.2 Statement of the problem

With increasing number of microbial drug-resistance cases, the need for new methods to eliminate pathogens has become a necessity. Therefore, thermophilic bacterial antimicrobial agents may be the solution for this problem. This study aims to screen and identify AMAs from thermophilic bacteria extracted from hot springs of Saudi Arabia.

1.3 Study Significance

Antimicrobial agents from TB show great future potential as a new method to target microbial pathogens, which will provide a cure for many diseases and reduce the cost of treatment of drug-resistant pathogens. To achieve that goal, screening and characterization of AMAs from TB constitute a huge step toward that goal.

1.4 Study Objectives

There are two objectives of this work:

- 1- Detection of potential antimicrobial agents from thermophilic bacterial strains isolated from hot springs in Saudi Arabia.
- 2- Elucidate the structures of bioactive compounds using various spectroscopic techniques such as Thin-layer chromatography (TLC), Gas chromatography–mass spectrometry (GCMS) and Liquid chromatography–mass spectrometry (LC-MS)

CHAPTER 2

LITERATURE REVIEW

Thermophiles are organisms that love heat that is required it for their optimal growing conditions. Different strains of thermophiles have different optimum conditions starting over 45°C, where many studies have supported that anything above is considered a thermophilic condition (LaPara and Allenan, 1999). They live in different ecological niches, such as hot springs, hydrothermal vents, and deep seas (Panda, 2019).

Thermophilic bacteria have acquired many features over the course of evolution that enable them to survive and prosper in such extremes. As a result of their distinctive survival properties, thermophiles have thermostable enzymes and high metabolism, which results in a lower rate of growth but high product yields (Mehta, 2016). These extremely tolerant biological features have given birth to many useful enzymes with wide industrial and clinical applications (such as protease and amylase) (Khalil, 2011). Applications of thermophilic bacteria extend to various other applications, such as biodegradation of crude oil, agriculture, and cancer treatments (Mehta, 2016).

In the early 1960s, microbiologists Thomas Brock and Hudson isolated the first species of thermophilic bacteria from hot springs in Yellowstone National Park (Földesi, 2018). An article published in 1967 by Brock said that “bacteria are able to grow and to isolate pink filamentous microbe, at a temperature of 70-74 °C” (Földesi, 2018).

Antibiotics are pharmaceuticals that are used to target bacterial infections. In the 19th century, a German physician named Paul Ehrlich concluded that it might be possible to create a chemical substance that could target bacteria without killing cells. He based his conclusion on the fact that in the laboratory, some dyes have penetrated some bacteria but cannot penetrate others. In 1909, the first antibiotic was discovered by Paul and called “chemotherapy”, which treated syphilis. Later, the discovery of many antibiotics were reported (Microbiology Society).

With the increased use of antibiotics, cases of resistance to antibiotics was increased. According to the World Health Organization (WHO), in 2000–2015, there was a nearly 26% increase in antibiotic use. Therefore, there was a more than 90% increase in antibiotics that are only used in cases with microbial resistance. According to the WHO One of the most likely scenarios for microbial drug resistance is the misuse of those antibiotics, which makes them rapidly evolve, resistance genes many passed to the next generation, which will decrease the effectiveness of targeted antibiotics (Stated by Sarah Moore, News Medical).

For resistance to develop in a certain strain of bacteria, first, there should be an antibiotic that can eliminate most of this strain in a culture. Second, at least one cell of bacteria will have the appropriate gene to not be targeted by those eliminating antibiotics, which will make this bacterium divide and create propose generations of bacteria with the appropriate survival gene to resist the antibiotics; thus, a different antibiotic must be made to fight that developed resistant bacteria (Levi and Marshall, 2004).

Resistance to antibiotics can occur either naturally, where a bacterium will naturally have the required genes to survive against a particular antibiotic, or via exposure to a specific antibiotic. Therefore, resistance can be acquired or innate. Bacteria can vertically or horizontally exchange genetic information, where they can spread survival genes fast. Hence, microbes can acquire resistance by accepting an antimicrobial gene from a neighboring bacterium or by mutation. These antibiotic-producing bacteria differ in the types and quantities of antibiotics produced. Some of these naturally produced antibiotics may have promising pharmaceutical potential. The laboratories worldwide are conducting studies to identify antibiotic-producing bacteria and identify the optimum protocol to obtain highest yields of these antibiotics while minimizing the cost of production.

Since discovery of the first antibiotic to treat human diseases, i.e., penicillin, many more antibiotics have been discovered and applied to treat human diseases. The only problem is that the antimicrobial ability of drugs does not last forever eventually, bacteria will develop resistance mechanisms to stop these drugs. Antimicrobial peptides (AMPs) produced from thermophilic bacteria were discovered in 1980s; since then, they have shown enormous potential in targeting microbes. AMP mechanisms to target microbes differ from those of antibiotics and hence can eliminate even drug-resistant bacteria. AMPs are macromolecules that naturally exist in many organisms, such as bacteria, animals, plants, and fungi. Among many isolated and identified AMPs, cationic ones carry a larger antimicrobial role (Jun and Lichun, 2019).

AMPs are among antimicrobial agents that are currently used industrially, including vancomycin and teicoplanin. The production of AMPs has been linked to many bacilli bacterial species, including *B. subtilis* and *B. cereus*. In bacillus AMPs, many chemical structures are associated with the peptide structure, one of which is bacteriocins (Teixeira, 2013).

Naturally, produced antimicrobial peptides (AMPs), still carry some disadvantages that must be considered when applying AMPs in clinical use. Naturally, produced AMPs are not stable and may cause toxic side effects due to their hemolytic activities. To include them in therapeutic use, a synthetic type of AMP that mimics natural AMPs and overcomes their disadvantages must be developed. AMP genes are controlled by external factors (Jun and Lichun, 2019).

Antimicrobial peptides (AMPs) exceed the efficiency of regular antibiotics in many ways. Regular antibiotics target bacteria in a specific pathway to disable their pathogenic activity and cause their death. The problem is that constant use of antibiotics may lead to mutations in bacteria, where resistance to those antibiotics will be developed. Resistance to antibiotics has often been reported and is considered a major health issue worldwide. Cationic AMPs target bacteria in a different manner: they neutralize the charge of the cell membrane of bacteria and penetrate the bacterial cell to finally cause it to die. This method is considered more effective than regular antibiotics because it reduces the ability of bacteria to develop resistance. Moreover, AMPs have a broad spectrum of antimicrobial activities, unlike antibiotics. AMPs have a strong antimicrobial potential and a synergistic effect with regular antibiotics to neutralize endotoxins. Daptomy is one of the approved AMPs in medicine and currently used to

treat skin infections. Daptomy peptides have shown a strong bacterial inhibitory ability and can even act on cancer cells to inhibit them. Many studies have shown the strong inhibition abilities of cationic AMPs to cancer cells by inserting themselves into the membrane of cancer cells and forming pores across the cells, which eventually leads to their death (Jun and Lichun, 2019).

To date, thousands of different AMPs have been discovered and classified based on their amino acid components, structure, and physiological function. AMPs are classified into two types: linear molecules with no cystine and cysteine polypeptides that form disulfide bridges (Jun and Lichun, 2019). The mechanism of action of antimicrobial peptides (AMPs) involves penetrating the membrane of gram-positive and gram-negative bacteria and disrupting the physiological function of the cell, leading to its death. However, some AMPs target bacteria by aiding the immune response by participating in it. These types of AMPs modulate the immune response in many ways, including strengthening the adaptive immunity, inducing the production of cytokines, and recruiting macrophages. By doing so, they enhance the body's antimicrobial ability without directly eliminating the bacteria. Moreover, cationic AMPs show antifungal abilities by attacking organelles such as mitochondria and resulting in their death. Among the many hypothesized mechanisms of action of AMPs is their ability to show their antimicrobial activities through attacking DNA and RNA to inhibit some protein synthesis and cell walls (Jun and Lichun, 2019).

There are antimicrobial peptides (AMPs) in many organisms, including humans, which act as a part of the innate immunity and are among the first lines of defense. AMPs play critical roles in the innate immunity of many organisms, and their

immunological activities include wound healing, chemokine production and phagocytosis enhancement (Jun and Lichun, 2019).

Thermophiles are of many beneficial uses in many industries such as pharmaceuticals, chemical and agricultural. Living at a very extreme condition gives TB unique characteristics such as producing AMPs, of those unique characteristics are the enzymes they produce. Thermophile Taq polymerase is one of the main enzymes used in Polymerase Chain Reaction (PCR) in the extension step. PCR is one of the greatest revolutionary inventions in the health industries where it allows us to amplify small quantities of DNA into billions and that is extremely helpful when it comes to the diagnosis of certain diseases and many other uses (PCR,2022)

CHAPTER 3

MATERIALS AND METHODES

3.1 Bacterial strains used:

The thermophilic strains used in this study are [*Brevibacillus borstelensis* AK1 (strain 43), *Bacillus licheniformis* (strain 13), *Geobacillus stearothermophilus* (strain 2) and *Anoxybacillus flavithermus* AK1(strain 38)] (khalil,2017).

3.2 bacterial culturing:

The bacterial strains are cultured using streak plate methods in TT media and incubated at 50°C for 24 hours. a stock culture for each strain was prepared.

3.3 Preliminary detection of antimicrobial agents:

The selected thermophilic strains were streaked and grown in the middle of TT plates and incubated for 24 hours at 50°C. Liquid Suspension of *Staphylococcus aureus* (Gram positive bacteria) and *Pseudomonas aeruginosa* (Gram negative bacteria) were flooded on the top of TT palates that contains the grown Thermophiles, and then incubated at 37 °C for 24 hrs. (Fig. 5 and Fig. 6).

3.4 Extraction of bacterial culture supernatant:

Isolated thermophilic bacteria showing antimicrobial features were grown in 100 ml of tetrathionate (TT) broth, which is a selective medium to grow thermophilic bacteria, using 250-ml flask, grown overnight at 50°C. Cells were pelleted by

centrifugation at 5000 rpm for 10 minutes. Then, the supernatant (cell free) (50 ml) was extracted with 100 ml (50×2) of petroleum ether. After that, the lower aqueous phase was further extracted with 100 ml (50×2) of chloroform. The last step was the addition of 100 ml (50×2) of ethyl acetate using an aspirating funnel and then the rest of the experiment were carried out using the lower aqueous phase of the last extraction. To remove trace amounts of water from the organic extract, 10g of anhydrous sodium sulfate was used. TT liquid media will be similarly extracted as the control.

3.5 Test for Antimicrobial Agents Using Minimum Inhibitory Concentration (MIC)

The aqueous phase of the extract was tested against *staphylococcus aureus* and *pseudomonas aeruginosa*. Six tubes were prepared with 5 ml of TT media and 0.1 ml of these organisms. Extracts from thermophiles *Brevibacillus borstelensis* and *Geobacillus stearothermophilus* was added to each tube with varying concentrations starting from 0.1 mg of extract to 0.6 mg. A control tube containing only TT media plus test organisms was also prepared.

3.6 Evaporation by Rotary Evaporator:

Evaporation of the aqueous phases was done by rotary evaporator; the role of this evaporator is to evaporate all liquid in the substance. The aim of using this machine in the experiment is the gentle removal of excess liquid in the sample to be left out with crystals that is expected to have the AMP that will be further analyzed using other equipment.



Figure 1: Rotary evaporator.

3.7 Thin-layer chromatography:

Thin-layer chromatography (TLC) was conducted to determine the purity of the antimicrobial agents. The crystals from the evaporation were used in this part of the experiment. Silica gel plates (Merck60, F254, 0.1 mm thick, 20 mm×10 cm) was used to perform TLC. Forty microliters of the aqueous residues of each extract were spotted on silica gel plates. The same amount of the extracted TT liquid media was used as a

control. The mobile phase was composed of 20% methanol:80% chloroform. The aqueous residues of each extract were detected by UV light (Khalil, 2002), and R_f values were calculated as the ratio between the distance traveled by inhibitory bands (observed under UV light) and the distance traveled by the solvent (mobile phase). TLC is a fast method used to separate components of a mixtures. It is mainly used for its simplicity and fast molecules identifications. The principle of TLC comes from the different affinity of each molecule between the stationary and mobile phase. The stationary phase is an adsorbent such as silica and the mobile phase is the mixtures of multiple components. The mobile phase will migrate through capillary properties to the top of the silica plate and each component of a mixture will have a different travelled distance where the compounds of a mixture will be estimated based on the distance travelled for each (Kagan,2017).

In this experiment, different solvents (hexane, DCM, ethyl acetate, methanol, and Acetonitrile) were tested for the solubility of the different TB samples. The samples were only soluble in water. The samples were applied on TLC to get an insight into the composition of samples. The spots on the TLC were not UV active. To further study, different stains were prepared as the TLC analysis was not conclusive under UV light. The staining steps also did not give any conclusive results. The stains prepared were KMnO₄, FeCl₃, Sulfanilic acid and applied on the TLC.

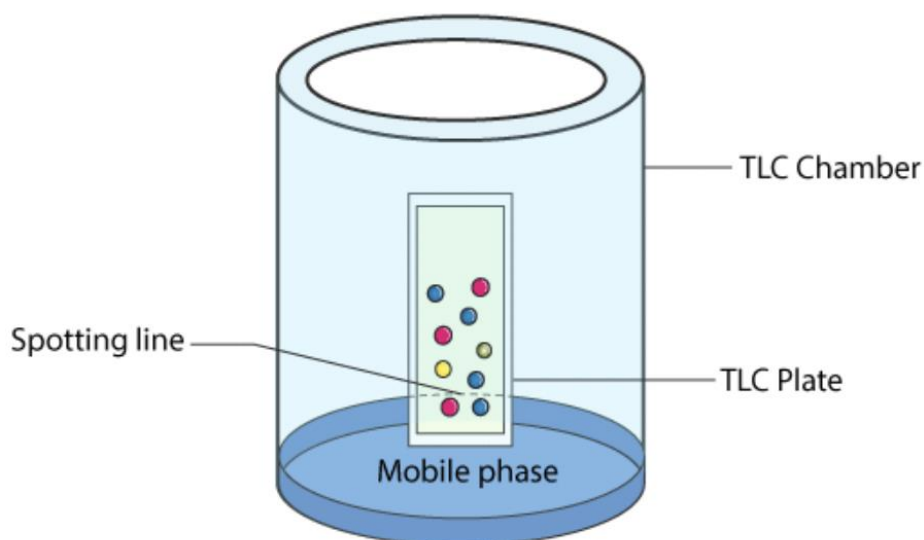


Diagram of Thin Layer Chromatography

Figure 2: Thin Layer Chromatography.

3.8 Gas chromatography mass spectrometry (GC/MS)

GC/MS is a device in which mixture of a sample are separated, identified, and quantified. Only volatile and thermally stable mixtures are used in GCMS. In this experiment the cell free extract obtained from step 3.4 were turned into gas and used in this device. In order to detect the composition of the extract mixture, an experiment of 3 steps were performed using GCMS and *Brevibacillus borstelensis* CFE. In each extraction step 5 ml of both aquas and organic phase was collected and numbered. A control sample was also taken where pure TT media was extracted and each extraction step was numbered and measured using GCMS.

Samples analyzed according to EPA Method 8260 by Gas Chromatography Mass Spectrometry manufacture by Thermo Scientific, Trace GC Ultra ISQ equipped with TriPlus HS head space analyzer. 1mL of liquid sample incubated in agitator for 5 minutes at 90°C then 1 mL from the head space pulled by syringe and injected (head space syringe heated at 120°C) into capillary column Cat. 10920 manufactured by

RESTEK Rtx-502.2, 60m, 0.32mmID 1.8 μ m film thickness. The GC programmed at initial temperature 40°C with initial holding time 2 minutes then raise to 165°C at rate of 5°C/min then raised to 250°C at rate of 30°C/min and holding time of 5 minutes. The total run time on GC is 35 minutes. The Mass Spectrometer analyzes using SIM mode. The calibration solution for MTBE was prepared using MTBE 1L purchased from Sigma-Aldrich Cat. 20256.



Figure 3: Gas Chromatography Mass Spectrometry.

3.9 Liquid Chromatography Mass Spectrometry (LC-MS)

The LC-MS analysis of control and *Brevibacillus borstelensis* cell free extract was achieved using Agilent LCMS-6120B coupled with Diode-Array Detector (DAD) and using Poroshell 120 EC-C18 (4.6 $\times$ 150 mm, 4 μ m) column with a flow rate of 1.0 mL/min, and an injection volume of 20 μ L of the sample. The temperature of the

column oven was kept at 25 °C. An isocratic separation method was used holding a mobile phase consisting of 0.3% formic acid in water (solution A, 86%) and acetonitrile with 0.3% formic acid (solution B, 14%) for 60 minutes. The following parameters used for the mass spectrometer: positive ion mode; peak width, 0.100 min; cycle time, 1.06 sec/cycle; drying gas flow, 12.0 L/min; heated capillary temp., 350 °C; capillary voltage, 3000 V; nebulizer pressure, 35 psi.



Figure 4: Liquid Chromatography-Mass Spectrometry .

CHAPTER 4

RESULTS

4.1 Preliminary detection of antimicrobial agents:

As indicated in Figure 5 and Figure. 6, next page, a preliminary detection of antimicrobial agents was recorded. Inhibition zones were measured for each strain and results of each were determined in millimeter (mm), 2mm or more is considered as a positive result (Aktypis *et al.*, 1998). The tested thermophiles have showed different degrees of sensitivity. *Geobacillus stearothermophilus* (strain 2) when tested against *pseudomonas aeruginosa* had an inhibition zone of 34 mm. while *Bacillus licheniformis* (13) had an inhibition zone of 28mm (Table 1)

Anoxybacillus flavithermus AK1 (strain 38) when tested against *Staphylococcus aureus* had an inhibition zone of 40 mm, while *Brevibacillus borstelensis* (strain 43) when tested against *staphylococcus*, had an inhibition zone of 35mm.

The highest inhibition zone measured was for *Anoxybacillus flavithermus AK1* against *staphylococcus aureus* where the recorded inhibition was 40mm. On the other hand, the lowest recorded inhibition zone was 28mm for *Bacillus licheniformis* against *pseudomonas*. This is shown in Figure 5 and 6. And Table 1.

Table 1: shows the TB antimicrobial activity against test organisms

TB	Inhibition	The Type of Bacteria Inhibited	Inhibition Zone(mm)	Figure
<i>Anoxybacillus flavithermus</i> AK1	Yes	<i>Staphylococcus aureus</i>	40mm	5
<i>Brevibacillus borstelensis</i> AK1	Yes	<i>Staphylococcus aureus</i>	35mm	5
<i>Geobacillus stearothermophilus</i>	Yes	<i>Pseudomonas aeruginosa</i>	34mm	6
<i>Bacillus licheniformis</i>	Yes	<i>Pseudomonas aeruginosa</i>	28mm	6

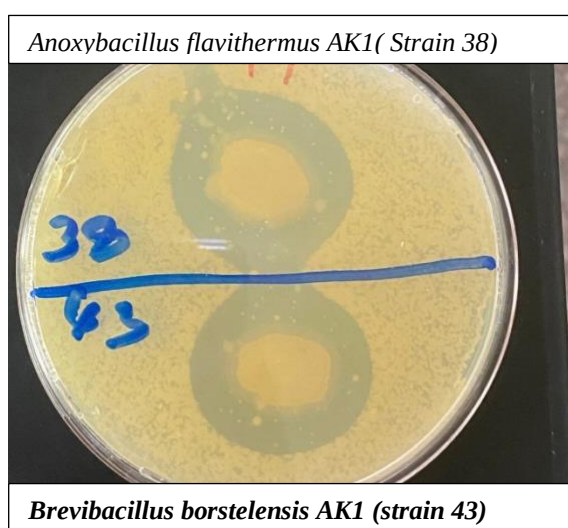


Figure 5: showing antimicrobial activity of TB against staphylococcus.

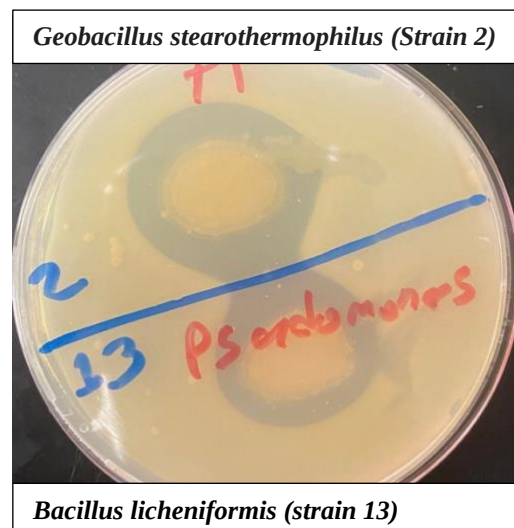


Figure 6: showing antimicrobial activity of TB against Pseudomonas.

4.2 Test for Antimicrobial Agents Using Minimum Inhibitory Concentration (MIC)

The results were recorded by measuring the growth of each tube where a positive result is when there is no growth. When there is no growth in a tube it means that the antimicrobial agents in the thermophiles extract have inhibited the growth of the test organisms and therefore a positive result. In *Geobacillus stearothermophilus* the complete inhibition of *pseudomonas aeruginosa* has occurred in tube 2 that contains 0.2

mg of *Geobacillus stearothermophilus*'s extract. In *Brevibacillus borstelensis* the complete inhibition of *staphylococcus aureus* has occurred in tube 3 containing 0.3 mg of *Brevibacillus borstelensis*'s extract. The tests organisms have shown different degrees of sensitivity to the thermophile's extracts. The extract of *Geobacillus stearothermophilus* has shown a stronger inhibition activity than the extract of thermophile *Brevibacillus borstelensis* as shown in figure 7 and 8

Table 2: showing antimicrobial activity of TB against test organisms.

TB	Inhibition	The Type of Bacteria Inhibited	Inhibition Started at (mg/ml)	Figure
<i>Geobacillus stearothermophilus</i>	Yes	<i>Pseudomonas</i>	0.3 mg/ml	7
<i>Brevibacillus borstelensis</i>	Yes	<i>Staphylococcus</i>	0.2 mg/ml	8

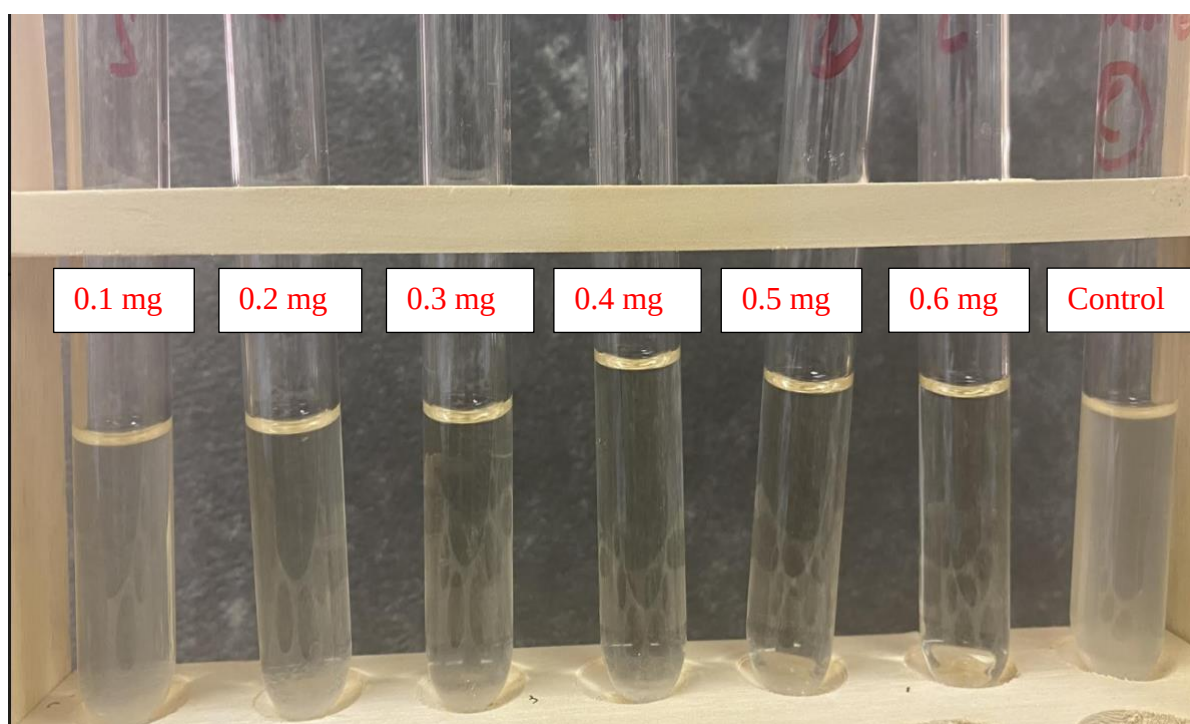


Figure 7: Antimicrobial activity of *Geobacillus stearothermophilus* against *pseudomonas*, as seen the complete inhibition started at concentration 0.3mg.

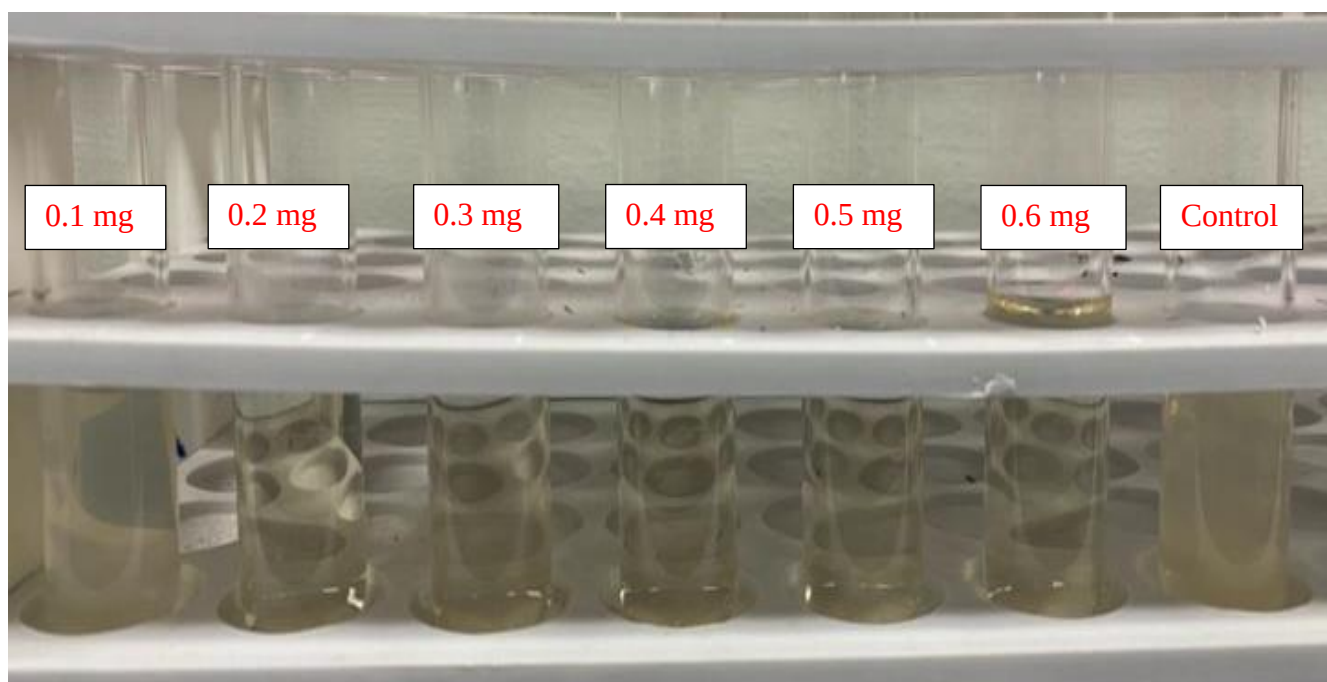


Figure 8: Antimicrobial activity for *Brevibacillus borstelensis* against staphylococcus, complete inhibition started at concentration 0.2 mg of extract.

4.3 Thin layer chromatography (TLC)

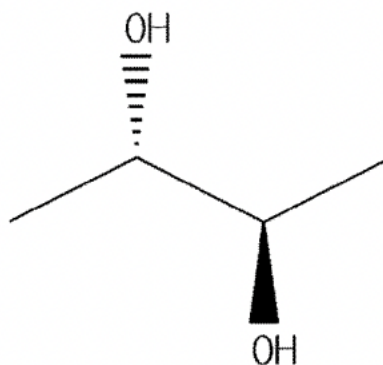
TLC results and water solubility tests suggests that the tested samples are highly polar and have a high-water solubility.

4.4 Gas chromatography mass spectrometry (GC/MS)

GCMS was performed on the free cell extract of *Brevibacillus borstelensis* that contains the antimicrobial agents. where it was turned into its gas phase and then injected into the GCMS device. The major agents found in this part is 2,3-butanediol. It was performed on the free cell extract of *Brevibacillus borstelensis* where many secondary metabolites are found in the library as shown in Figure 14. The major secondary metabolites detected was 2,3 butanediol which is found to have antiseptic capabilities when used in small amounts and can have great inhibition effects against

skin (infection) disease that affected by bacteria and molds (Heung soo,2017). The molecular mass of 2,3-butanediol is 90.21g/mol, its chemical formula is $C_4H_{10}O_2$ and has three different stereoisomers. The study has found that the form meso-2,3-butandiol has the highest antiseptic ability while having less skin irritation. When the amount of meso-2,3-butandiol is controlled it can have an effective antiseptic ability without the addition of any antiseptic agents. Meso-2,3-butandiol is a very good candidate for cosmetic use because it has a high inhibition ability for skin pathogens while causing less irritation to skin as well as having a very good affinity to other cosmetic components (Heung soo,2017).

[Chemical Formula 1]



meso-2,3-butanediol

The antimicrobial activity of the hydroxy position and the different stereotypic orientation of the diols such as 1,3 butanediol were measures and compared. The experiment has been conducted using a test organism such as *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231), and *Aspergillus niger* (ATCC 16404). Yeast and Fungai were used as well. Substances were added to a Tryptic Soy broth (is an enrichment media used for the sterility test) and Sabouraud Dextrose broth (is a

selective media for fungi) at a concentration of 0.01 to 10%, cultures were in a liquid phase and bacteria, molds, and yeasts were inoculated then cultured at 25, 32C for 24 to 72 hours and the results were measured in Minimum Inhibitory Concentration (MIC) as shown in Table 3 (Heung soo, 2015). In Table 3 different diols antimicrobial abilities were tested against test organisms, As shown the Meso-2,3-butanediol has a very low MIC compared to the other stereotypic isomers which means that a low concentration of it has caused an inhibition. MIC is defined as the lowest concentration of antimicrobial that will cause a visible growth inhibition of a microorganism after an overnight incubation, it is measured by testing different test organisms against one antimicrobial compound in a multi wellled plate where the concentration of the antimicrobial agent will be gradually decreased until a visible growth is observed, this point is then called MIC, the lower the MIC concentration the better the antimicrobial agent used is (JD,A). MIC is explained in Figure 9

Table 3: shows the antimicrobial abilities of different diols against selected test organisms.

Test Material	E. coli	P. aeruginosa	S. aureus	C. albicans	A. niger
1,3-butanediol	>10	>10	>10	>10	>10
1,2-butanediol	5	2.5	1.25	5	2.5
S, S-2,3-butanediol	5	5	2.5	5	1.25
R, R-2,3-butanediol	2.5	5	10	5	5
Meso-2,3-butanediol	1.25	2.5	5	2.5	1.25
1,2-hexanediol	1.25	1.25	2.5	1.25	0.0625

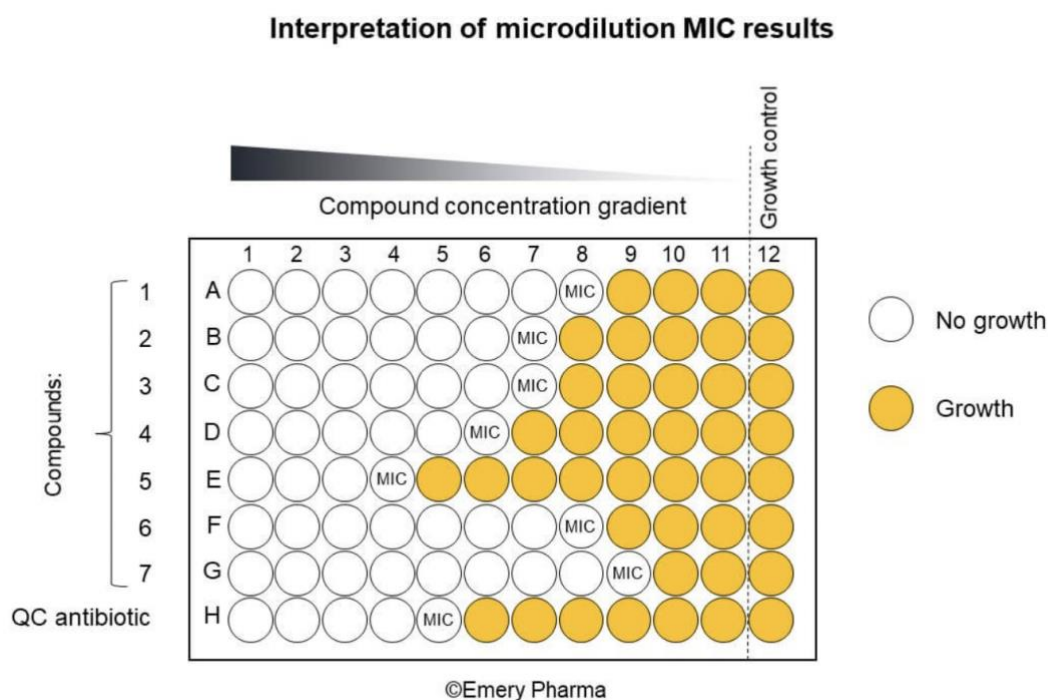


Figure 9: The method of MIC.

A study that was published in 1998, has also shown an antimicrobial effect of the diol derivatives such as 1,3-butanediol and 1,2-pentanediol when was tested against six types of test organisms using isothermal calorimeter (Which is a technique to measure biological interactions). The study has concluded that the 1,3-butanediol and 1,2-pentanediol has a significant antimicrobial capability with an MIC value of (1.72 for 1,2-butandiol and 4.34 for 1,3-butandiol) even though it is mainly used in cosmetics as 2,3butandiol (Antoce,1998). In a study that was published in 2016. It was found that the compound 2,3-butandiol that is produced by root-associated bacteria can increase plants fitness by increasing their resistance. Bacterial Volatile Compounds (BVCs) such as alcohols, acetone and 2,3-butandiol that are produced naturally in some species of root-associated bacteria has been found to play a role in plants defense (Yi, H.-S,2016).

Another important metabolite that was detected in *Brevibacillus borstelensis* extract but in a small concentration is isopropyl alcohol which is an organic compound that has various applications, one of which is having an antiseptic capability as well as other industrial uses. isopropanol is known to have a strong antiseptic activity against spores (Aboualizadeh,2017).

These secondary metabolites discovered suggests the volubility of hot springs of Jizan Saudi Arabia that has these unique thermophiles which can serve as a source of bioactive materials that support industrial and pharmaceutical fields.

This experiment started with the first extraction step; 100 ml of the CFE of *Brevibacillus borstelensis* was mixed with 200 ml of petroleum ether. 5 ml of the upper organic phase and 5 ml of the lower aqueous phase was collected and numbered 7 and 8 while the control of the same step of the extraction experiment using only pure TT media with no grown cells was also conducted and numbered 1 and 2. GCMS analysis conducted to sample 1 (organic phase of the control) and sample 7 (organic phase of *Brevibacillus borstelensis* CFE), the results shown in Figure 10 shows that both graphs are similar as expected since it was hypothesizes that the AMAs will be found in the aqueous phase only.

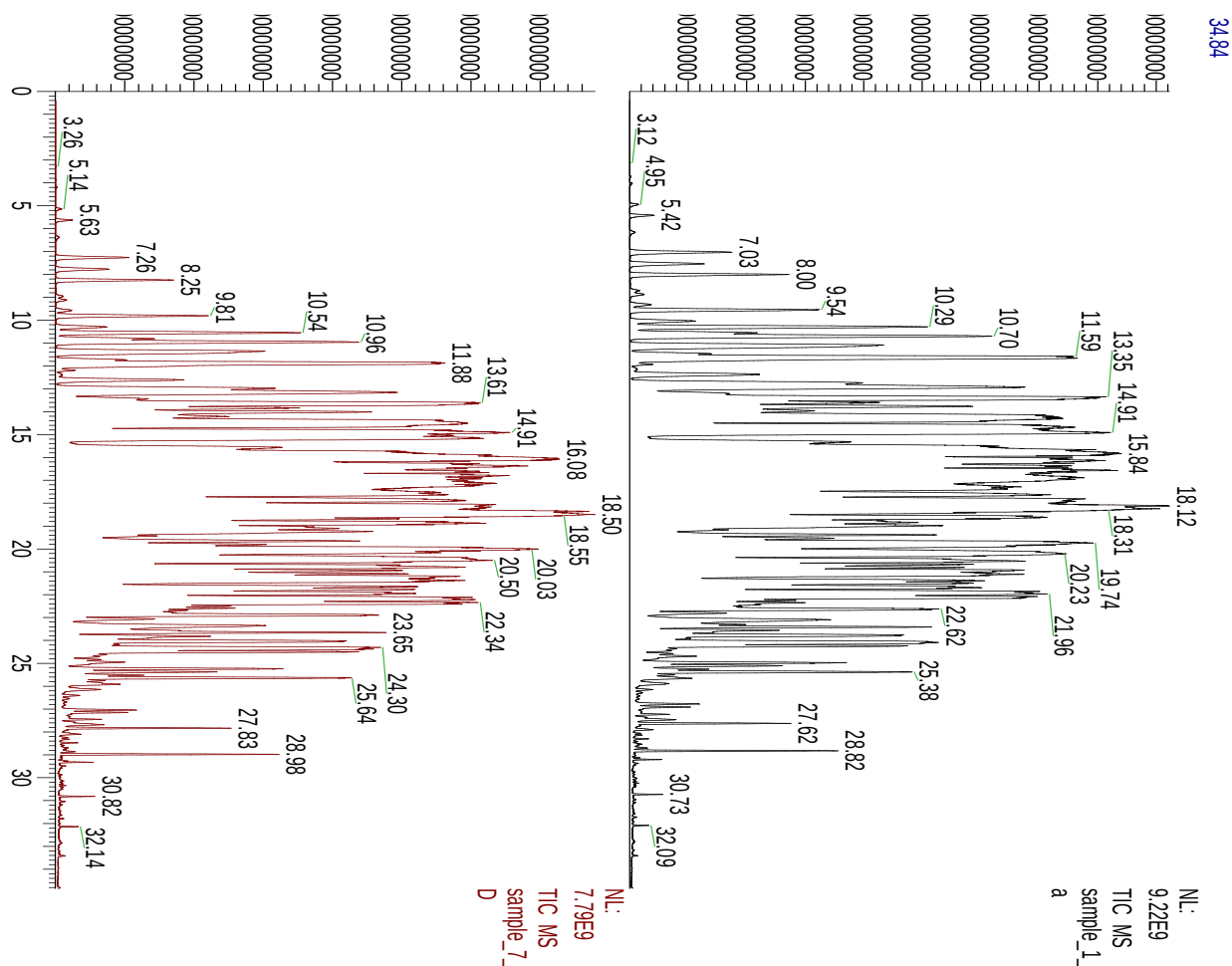


Figure 10: chromatograms of the organic phase of the first extraction step for sample 1 and 7.

The second step of the extract is performed on the aqueous phase of the last step using 200 ml of chlorophorm where in this step the lower phase the organic phase and the upper phase is the aqueous phase, 5 ml of both phases was collected and numbered 3 and 4 for the control and 9 and 10 for the *Brevibacillus borstelensis* AK1 cell free extract. GCMS analysis was conducted on sample 3 (the control) and sample 9 (*Brevibacillus borstelensis* CFE) both from organic phase. As shown in Figure 11 Both

chromatograms show similar results as expected since it was hypothesized that the antimicrobial activity will be in the aqueous phase.

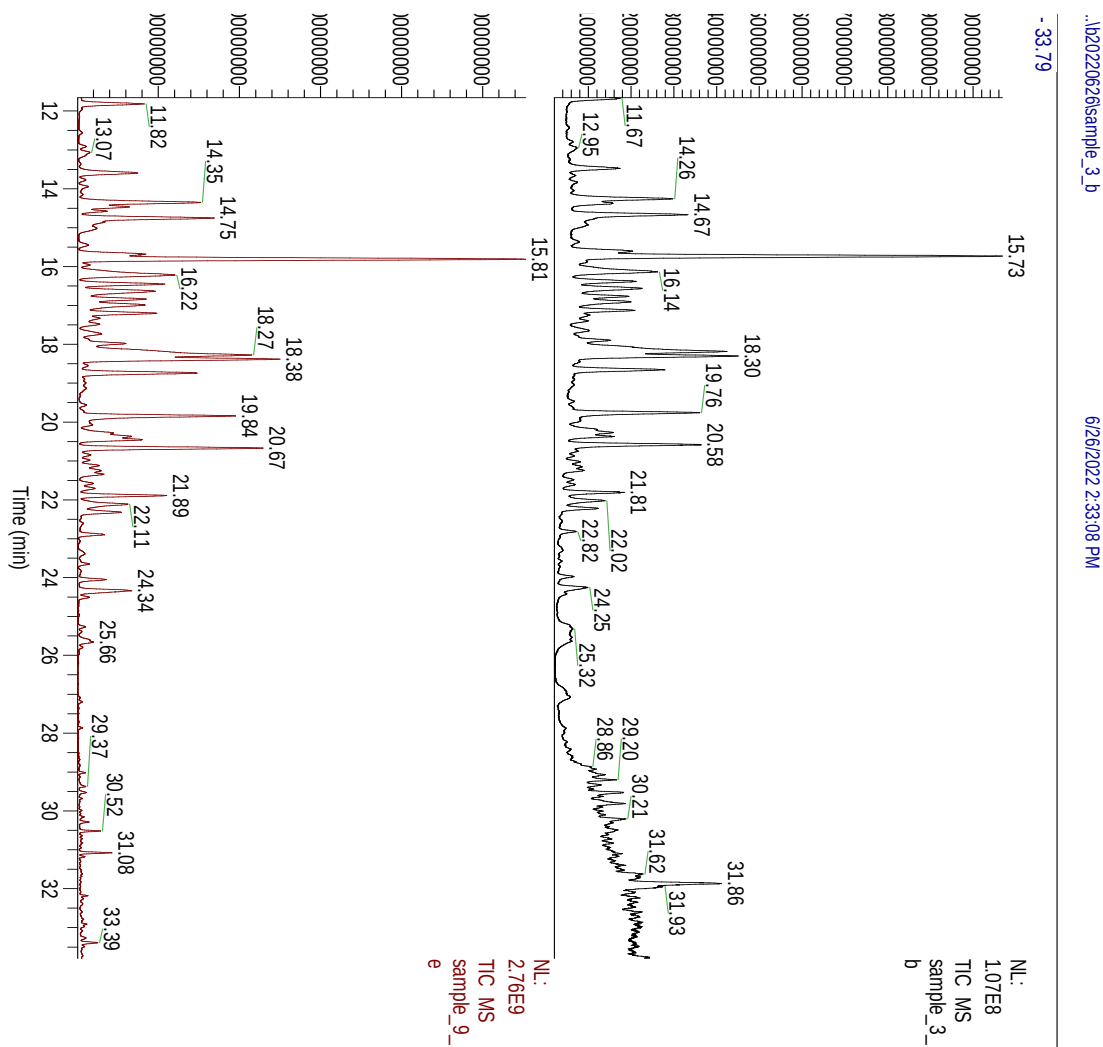


Figure 11: chromatograms of the organic phase of the 2nd extraction step of sample 3 and 9.

The final step of the extraction was conducted on the aqueous phase of the second extraction step where it was mixed with 200 ml of ethyl acetate. In this last step, the upper phase is the organic phase, and the lower is the aqueous phase. 5 ml of both was

collected and numbered 5 and 6 for the control and 11 and 12 for the *Brevibacillus borstelensis* CFE.

GCMS was conducted on sample 6 (the aqueous phase of the control) and sample 12 (the aqueous phase *Brevibacillus borstelensis* CFE). Figure 12 shows that the aqueous phase of *Brevibacillus borstelensis* CFE has a different graph when compared to the control which means that it has different compounds in it. As it was hypothesized that the

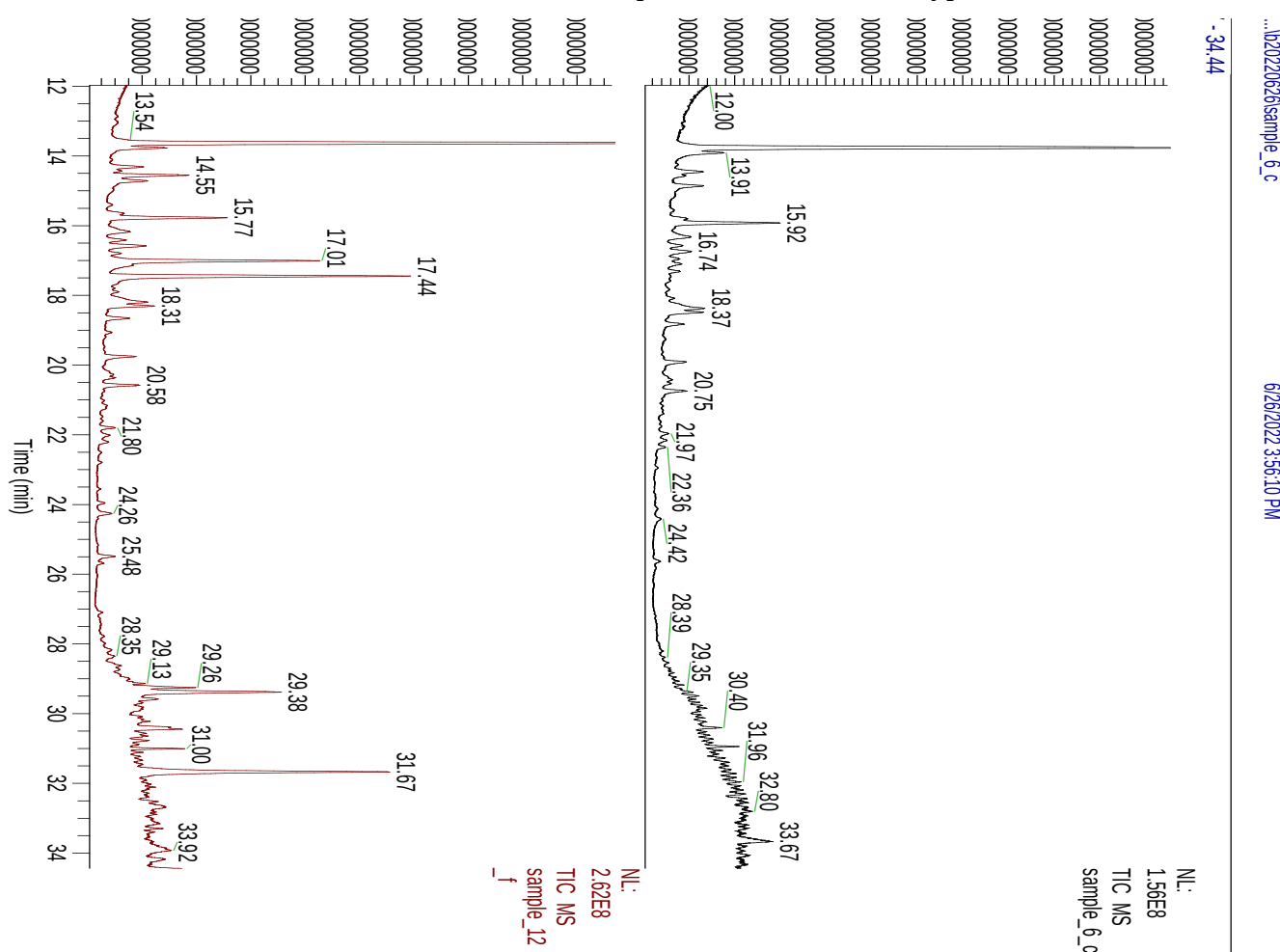


Figure 12: chromatogram of the aqueous phase of sample 6 and 12..

antimicrobial activity will be in the aqueous phase of the CFE of TB. looking at the chromatogram of sample 12 there is a peak at 17.44 minutes which is found that it contains multiple secondary metabolites that are of value to pharmaceuticals and other

industrial fields. 2,3-butanediol, the most concentrated compound in the GCMS library has been proven to have antiseptic capabilities.

The peak at 17.44 minute of sample 12 is shown in figure 13.

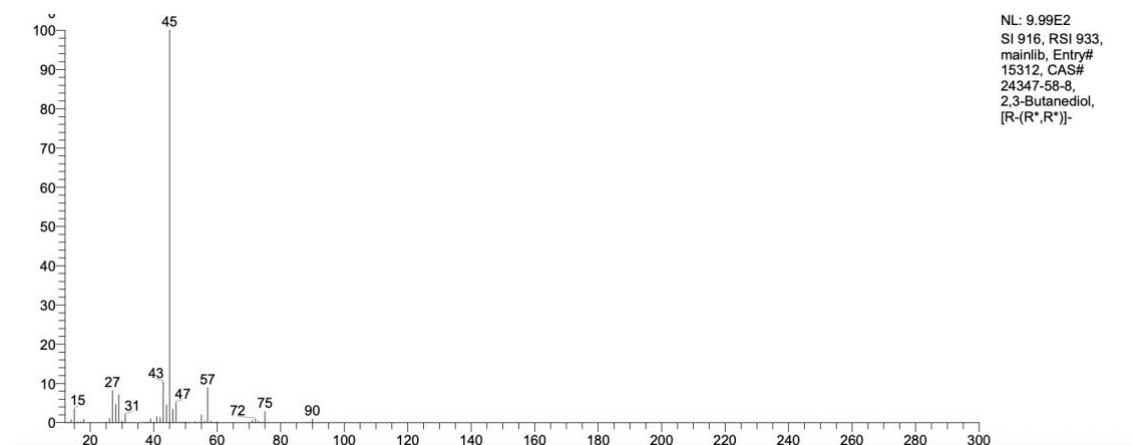


Figure 13: The peak at 17.44 minutes in sample 12.

In figure 14 the library the peak at 17.44 minutes of sample 12 is shown and as noticed the major secondary metabolite is 2,3-butandiol.

Hit	SI	RSI	Prob	Name	Library Name
1	911	936	40.21	2,3-Butanediol, [R-(R*,R*)]-	mainlib
2	900	931	27.58	2,3-Butanediol, [S-(R*,R*)]-	mainlib
3	899	941	26.51	2,3-Butanediol	replib
4	880	903	26.51	2,3-Butanediol	mainlib
5	838	863	26.51	2,3-Butanediol	replib
6	772	796	1.15	2-Propanol, 1-methoxy-	mainlib
7	758	759	0.72	2-Butanol, 3-chloro-, (R*,R*)-	mainlib
8	757	777	1.15	2-Propanol, 1-methoxy-	replib
9	756	777	1.15	2-Propanol, 1-methoxy-	replib
10	747	894	0.49	DL-2,3-Butanediol	mainlib
11	743	751	0.41	2-Butanol, 3-chloro-, (R*,S*)-	mainlib
12	741	756	0.38	Propylene Glycol	replib
13	730	887	0.26	Isopropyl Alcohol	mainlib
14	728	758	0.24	2-Octanol, (R)-	replib
15	725	755	0.21	2-Octanol, (S)-	replib
16	717	736	0.16	Propanoic acid, 2-hydroxy-, ethyl ester, (S)-	replib
17	712	754	0.13	(S)-(+)-1,2-Propanediol	replib
18	712	733	0.13	2-Butanol, 3-chloro-	mainlib
19	711	796	0.12	2-Butanol, 3-methyl-	replib
20	709	739	0.11	(S)-2-Hydroxypropanoic acid	mainlib
21	708	798	0.11	Propanoic acid, 2-hydroxy-, ethyl ester	replib
22	705	713	0.09	1,3-Butanediol	replib
23	701	711	0.08	1,3-Butanediol, (S)-	mainlib
24	699	707	0.07	1,3-Butanediol, (R)-	mainlib
25	698	733	0.38	Propylene Glycol	mainlib
26	698	706	0.07	Propanoic acid, 2-hydroxy-	replib
27	696	699	0.06	2-Heptanol	replib
28	695	734	0.06	(R)-(-)-3-Methyl-2-butanol	mainlib
29	695	723	0.21	2-Octanol, (S)-	mainlib
30	690	720	0.05	2-Pentanol, 3-methyl-	replib
31	688	716	0.11	Propanoic acid, 2-hydroxy-, ethyl ester	mainlib
32	685	719	0.04	2-Heptanol, (S)-	mainlib
33	684	723	0.04	(R)-(-)-2-Pentanol	replib
34	683	708	0.16	Propanoic acid, 2-hydroxy-, ethyl ester, (S)-	mainlib
35	680	707	0.03	3-Ethyl-2-pentanol	replib
36	679	703	0.03	(+)-5-Methyl-2-hexanol	mainlib
37	678	753	0.26	Isopropyl Alcohol	replib
38	676	715	0.02	(S)-(+)-2-Pentanol	mainlib
39	675	698	0.02	2-Heptanol, 6-methyl-	mainlib
40	672	704	0.12	2-Butanol, 3-methyl-	mainlib
41	672	694	0.02	2-Decanol	replib
42	671	712	0.38	Propylene Glycol	replib
43	670	698	0.05	2-Pentanol, 3-methyl-	mainlib

Figure 14: Library of possible compounds for the peak at 17.44 minutes of sample 12.

4.5 Liquid Chromatography Mass Spectrometry (LC-MS)

LC-MS was conducted on *Brevibacillus borstelensis* cell free extract (sample 12) in order to further characterized the sample chemical composition. LCMS was conducted to support what was found on GCMS. The results of LCMS have shown that the *Brevibacillus borstelensis* CFE has different retention time than the control (the control is purely extracted TT media) which can be due to the difference composition of each. LCMS results were not conclusive and further work needs to be conducted to accurately elucidate and confirm the composition of *Brevibacillus borstelensis* extract.

It was noticed that the system was very effective in separating compounds. It was noticed by following the area percent of separated sample compared to the control, that the extract of *Brevibacillus borstelensis* has an effect on some of the compound while some has not been affected. Further work is needed to separate the obtained compounds using classical chromatography followed by spectral characterization of the isolated compounds using NMR technique in order to fully elucidate the structure of the separated compounds.

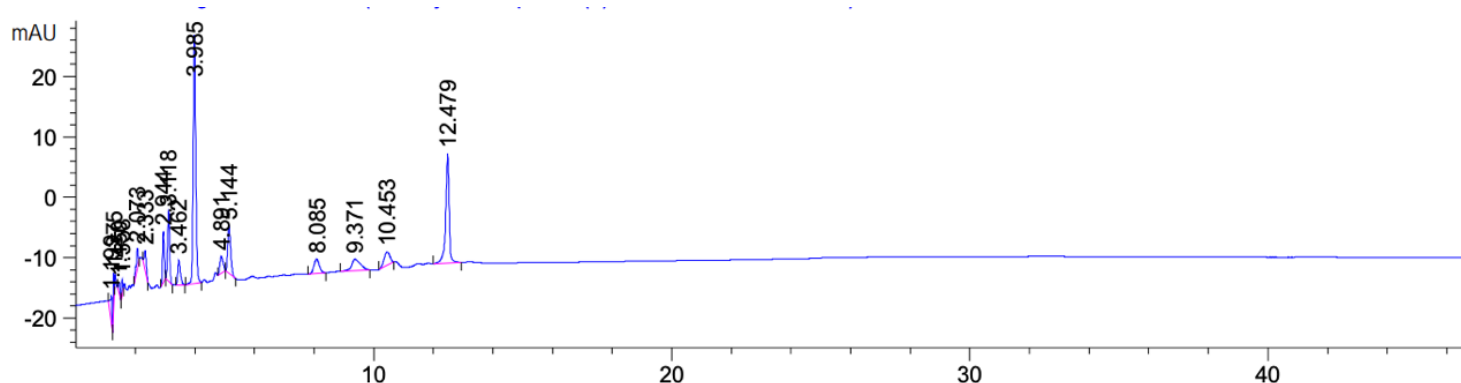


Figure 15: UV detection of the control using Liquid chromatography.

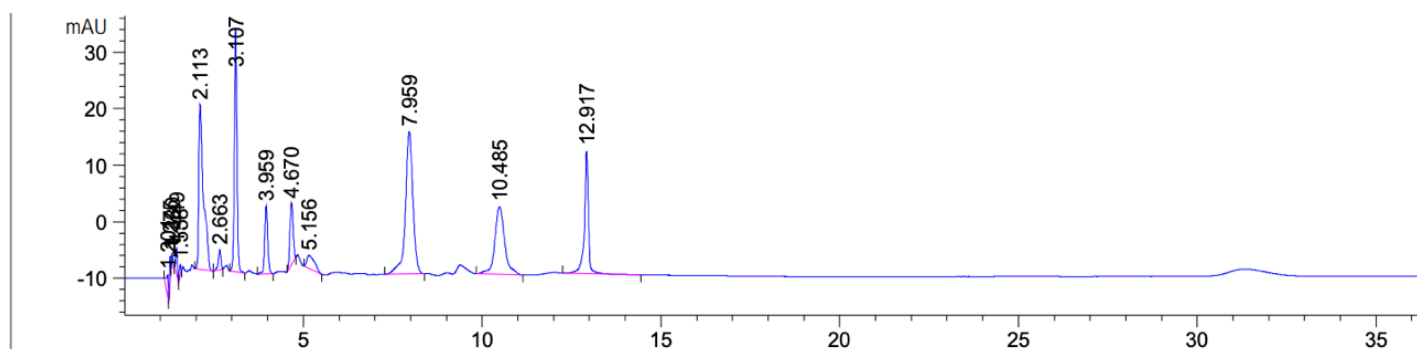


Figure 16: UV detection of *Brevibacillus borstelensis* extract.

It is noticed that there is a slight difference in retention time between *Brevibacillus borstelensis* extract (figure 15) and the control (figure16) which can be due to the compound difference of each sample.

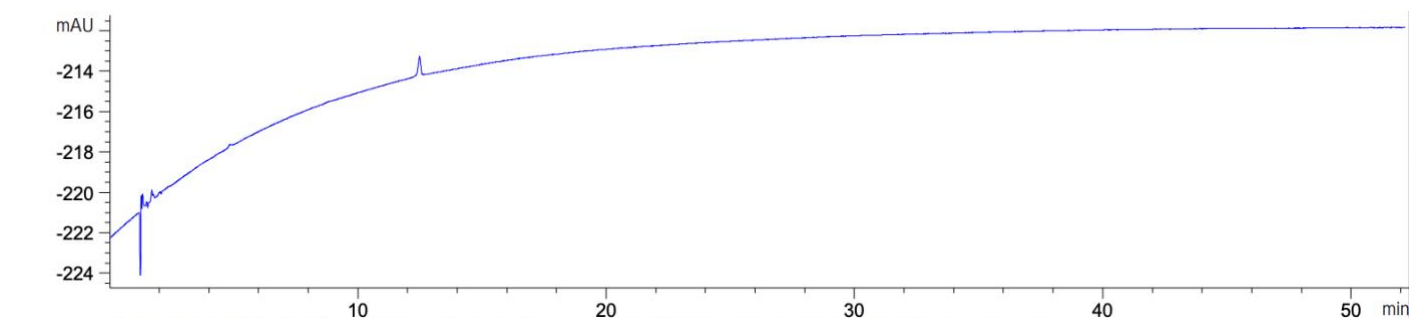


Figure 17: LC-UV chromatogram of the sample *Brevibacillus borstelensis* extract at wavelength 380 nm.

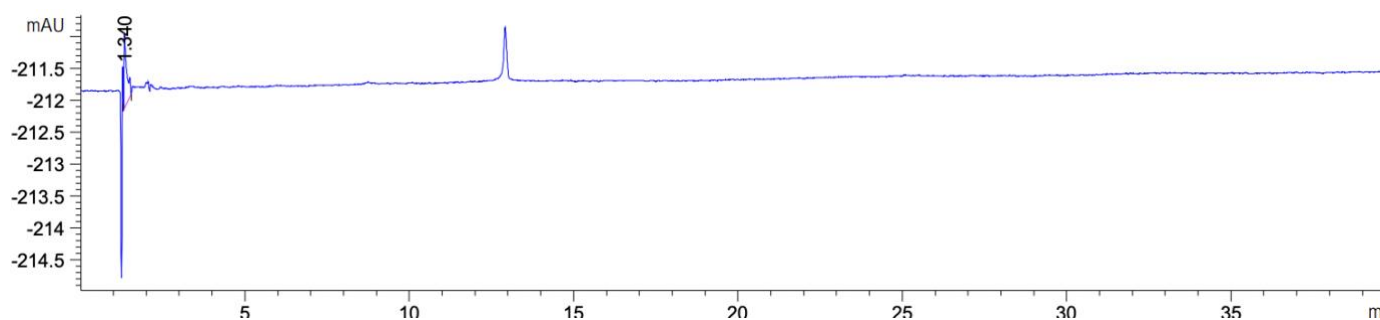


Figure 18: LC-UV chromatogram of the sample control at wavelength 380 nm.

It can be also seen from the LC-UV chromatograms of the control and *Brevibacillus borstelensis* extract at a wavelength 380 nm that some compounds showed signals at different retention time which indicate a color-nature of the compounds. In *Brevibacillus borstelensis* extract (figure 17) a signal is showing at retention time around 12 while in the control sample (figure 18) a signal is showing at retention time around 14 indicating colored compounds in each sample.

CHAPTER 5

DISSCUSSION

Thermophilic bacteria have a wide spectrum of applications due to the thermostable products they produce (Gomes,1970). The main aim of this research was to study thermophiles economic benefits in the pharmaceutical fields where these TB were extracted from the hot springs of Jizan, Saudi Arabia. This research has focused on two things, the first is the AMAs that are produced by thermophiles and their potential applications against bacterial pathogens and the second is the identification of these AMAs. The results obtained have shown the antimicrobial activity of thermophiles extract against test organisms as shown in figure 5 and 6. It was found that four of the isolated TB have shown antimicrobial activity against test organisms where thermophile *Anoxybacillus flavithermus AK1* has caused the biggest inhibition zone of 40 mm against *staphylococcus aureus* (figure 5&6).

Thin layer chromatography (TLC) has shown that the provided samples were only soluble in water which means that they are highly polar (polar compounds are the compounds that held together by polar covenant bonds) (admin,2022). 2,3-Butandiol is also highly polar and has a high-water solubility. The samples spots on the TLC were not visible due to their UV inactivity which can be due to some of the compounds in mixture were not UV active which has killed the activity of other UV active compounds.

GCMS was performed on *Brevibacillus borstelensis AK1* extract. It has been found that the main secondary metabolite on the sample was 2,3-butandiol (figure 14) which has been found that it has antimicrobial capabilities and many other industrial

applications (Heung soo,2017). Another secondary metabolite that was found is isopropyl alcohol which also has antimicrobial capabilities (Abuualizadeh,2017). This study has found that isolated TB has the ability to produce AMAs as well as other industrial important metabolites.

When LC-MS was conducted on *Brevibacillus borstelensis* extract it was noticed that there is difference in UV detection and separation between *Brevibacillus borstelensis* extract and the control (figure 15 & 16) which can be due to the differences in compounds each sample has which corresponds to what was hypothesized in this work.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Thermophiles are a valuable organism of a wide range of applications in many industries. This work has focused on an important aspect of TB importance in the clinical and other industries. This research has conducted two experiments to show antimicrobial activity of isolated TB. In the first experiment, four isolates, *Anoxybacillus flavithermus* AK1, *Bacillus licheniformis*, *Geobacillus stearothermophilus* and *Brevibacillus borstelensis* AK, were inoculated in the midpoint of a TT plate and grown at 50°C for 24 hours, the next day selected test organisms, *Staphylococcus aureus* and *pseudomonas*, were poured into the plates and grown again at 37°C for 24 hours. It was concluded that all four of these isolates showed antimicrobial activity against the selected test organisms where *Anoxybacillus flavithermus* AK1 had the most antimicrobial activity of 40 mm against *staphylococcus aureus* as shown in Figure 5. The second experiment was preparing a set of tubes to perform a serial dilution of the TB extract starting from 0.1mm to 0.6mm against test organisms. It was concluded that only two isolates have shown antimicrobial activity against test organisms where *Geobacillus stearothermophilus* has shown the most inhibitions activity where it started the killing of test organisms at concentration of 0.2ml of the *Geobacillus stearothermophilus* extract against *pseudomonas aeruginosa* as shown in Figure 8.

GCMS library of *Brevibacillus borstelensis* AK1 extract has shown that one of the compounds it has is 2,3-butanediol which has been proven to show antimicrobial activity. LC-MS has shown that *Brevibacillus borstelensis* extract and the control UV analysis has different retention times on some of the compounds due to the different contents of each.

The results of TLC, correspond to what was found in *Brevibacillus borstelensis* AK1 CFE in GCMS where the library suggests the presence of a major secondary metabolite (2,3-butanediol) which is also highly polar and has a high-water solubility.

Further work needs to be conducted on these isolates to fully elucidate the chemical structures of the compounds that have caused these isolates to exhibit antimicrobial activities.

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