

**PURIFICATION AND IDENTIFICATION OF ANTIMICROBIAL
PEPTIDES PRODUCED BY THERMOPHILIC BACTERIA ISOLATED
FROM HOT SPRINGS**

BY

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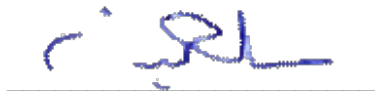
DEANSHIP OF GRADUATE STUDIES

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Dedication

This journey has been shaped by the love and support of my parents and siblings. Your patience, belief, and the warmth of your presence have carried me through every challenge.

I dedicate this work to you, with deepest gratitude and love.

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LIST OF ABBREVIATIONS

AMP	:	Antimicrobial Peptide
UPLC	:	Ultrahigh Performance Liquid Chromatography
DAD	:	Diode Array Detector
FTIR	:	Fourier-Transform Infrared Spectroscopy
NMR	:	Nuclear Magnetic Resonance
MS	:	Mass Spectrometry
RiPP	:	Ribosomally Synthesized and Post-translationally Modified Peptides
NRP	:	Non-Ribosomally Synthesized Peptide
LPS	:	Lipopolysaccharide
AME	:	Aminoglycoside-Modifying Enzymes
SMR	:	Small Multidrug Resistance
RND	:	Resistance-nodulation-Cell Division
ABC	:	ATP-Binding Cassette
MFS	:	Major Facilitator Superfamily
MATE	:	Multidrug and Toxic Compound Extrusion
ARG	:	Antibiotic Resistance Genes
AMR	:	Antimicrobial Resistance
CDC	:	Centers for Disease Control and Prevention
CRE	:	Carbapenem-Resistant Enterobacteriaceae
ESBL	:	Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae
MRSA	:	Methicillin-Resistant Staphylococcus Aureus
SDG	:	Sustainable Development Goal

NRSP	:	Non-Ribosomal Peptide Synthases
PTM	:	Posttranslational Modification
LTA	:	Lipoteichoic acid
ROS	:	Reactive Oxygen Species
LAB	:	Lactic Acid Bacteria
BCA	:	Biological Control Agents
GRAS	:	Generally Recognized as Safe
SPE	:	Solid Phase Extraction
SEC	:	Size Exclusion Chromatography
MALDI	:	Matrix-Assisted Laser Desorption/Ionization
ESI	:	Electrospray Ionization
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TOF	:	Time of Flight
CID	:	Collision-Induced Dissociation
COD	:	Collision-activate Dissociation
BGC	:	Biosynthetic Gene Cluster
BAGEL	:	Bacteriocin Genome Mining Tool
AntiSMASH	:	Antibiotic and Secondary Metabolite Analysis Shell
BLAST	:	Basic Local Alignment Search Tool
TT	:	Thermus medium
NA	:	Nutrient Agar
CFS	:	Cell Free Supernatant
ACE	:	Antimicrobial Crude Extract

ABSTRACT

Full Name : Latifat Abdulsalam
Thesis Title : Purification and Identification of Antimicrobial Peptides Produced by
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Every year, bacterial infections lead to over 7.7 million deaths, with 4.95 million of these caused by drug-resistant organisms. This growing resistance emphasizes the urgent need for a different approach to combat bacterial infections. Antimicrobial Peptides (AMPs) offer a promising solution due to their unique mechanisms of action. However, their clinical application is often limited by their instability under extreme physiological conditions like high temperatures. To address this limitation, this study focuses on extracting and characterizing AMPs from thermophilic bacteria, specifically *Bacillus licheniformis*, *Geobacillus stearothermophilus*, *Brevibacillus borstelensis* AK1, and *Anoxybacillus flavithermus* AK1 isolated from a hot spring in Saudi Arabia. These strains exhibited strong antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. The AMPs were purified by column chromatography using a column packed with C18 reverse-phase material. Their characterization was conducted using various spectrometric techniques, including UPLC, FTIR, NMR, Mass Spectrometry (MS), and *in silico* analysis. The compounds extracted from these selected isolates were confirmed to be proteinaceous, with molecular masses ranging from 500 to 1,930 Da. *In silico* genome analysis of *Bacillus borstelensis* AK1 and *Anoxybacillus flavithermus* AK1 identified gene clusters encoding ribosomally synthesized and post-translationally modified peptides (RiPPs), non-ribosomally synthesized peptides (NRPs), and a terpene. While some thermal sensitivity existed in the compounds produced, the Compounds remained stable and retained most of their activity after being subjected to a high temperature of up to 70 °C. These findings highlight thermophilic bacteria from Saudi Arabian hot springs as a valuable source of AMPs with applications in medicine, food preservation, and agriculture.

Keywords: Antimicrobial peptides, thermophilic bacteria, hot spring, antimicrobial resistance, bioactive compounds

ملخص الدراسة

الاسم الكامل: لطيفة عبدالسلام

عنوان الرسالة: تنقية وتحديد الببتيدات المضادة للميكروبات المنتجة من قبل بكتيريا محبة للحرارة معزولة من الينابيع الحارة

التخصص: علوم أحياء

تاريخ الحصول على الدرجة: مايو 2025

تتسبب العدوى البكتيرية سنوياً في أكثر من 7.7 مليون حالة وفاة، منها 4.95 مليون حالة ناتجة عن كائنات مقاومة للأدوية. وتسلب هذه المقاومة المتزايدة الضوء على الحاجة الماسة إلى اتباع نهج مختلف لمكافحة العدوى البكتيرية. تمثل الببتيدات المضادة للميكروبات (AMPs) حلاً واعداً نظراً لآليات عملها الفريدة. ومع ذلك، غالباً ما يحد من استخدامها السريري عدم استقرارها في الظروف الفسيولوجية القاسية، مثل درجات الحرارة المرتفعة. لمعالجة هذا التحدي، ركزت هذه الدراسة على استخلاص وتوصيف الببتيدات المضادة للميكروبات من بكتيريا محبة للحرارة، وهي على وجه التحديد *Bacillus licheniformis*، و *Anoxybacillus flavithermus* و *Geobacillus stearothermophilus*، و *Brevibacillus borstelensis* AK1 المعزولة من ينبوع حار في المملكة العربية السعودية. أظهرت هذه السلالات نشاطاً مضاداً قوياً ضد *Staphylococcus aureus* و *Escherichia coli*. تم تنقية الببتيدات باستخدام كروماتوغرافيا العمود باستخدام عمود مملوء بمادة C18 ذات الطور العكسي. وقد أُجري توصيفها باستخدام تقنيات طيفية متعددة، شملت UPLC، و FTIR، و NMR، ومطياف الكتلة (MS)، بالإضافة إلى التحليل الحاسوبي. وقد أثبت أن المركبات المستخلصة من هذه العزلات بروتينية بطبيعتها، وتتراوح أوزانها الجزيئية بين 500 و 1930 دالتون. كما كشف التحليل الجينومي الحاسوبي لكل من *Brevibacillus borstelensis* AK1 و *Anoxybacillus flavithermus* AK1 عن وجود تجمعات جينية ترمز لببتيدات مُصنَّعة ريبوسومياً وخضعت لتعديلات ما بعد الترجمة (RiPPs)، وأخرى غير مُصنَّعة ريبوسومياً (NRPs)، بالإضافة إلى مركب تيربين. على الرغم من وجود حساسية حرارية جزئية في بعض المركبات المنتجة، فقد احتفظت بفعاليتها بعد التعرض لدرجة حرارة مرتفعة تصل إلى 70 درجة مئوية. وتبرز هذه النتائج بكتيريا الينابيع الحارة في السعودية كمصدر غني للببتيدات المضادة للميكروبات ذات التطبيقات الواعدة في المجالات الطبية، وحفظ الأغذية، والزراعة.

الكلمات المفتاحية: الببتيدات المضادة للميكروبات، بكتيريا محبة للحرارة، ينبوع حار، مقاومة مضادات الميكروبات، مركبات نشطة بيولوجياً.

Chapter 1

Introduction

1.1 Antibiotics: The Past and Future

During the pre-antibiotic era, knowledge of microorganisms and infections was limited. Efforts to treat and prevent the spread of contagious diseases were largely ineffective, often leading to epidemics that claimed the lives of millions [1]. A typical example of catastrophic conditions in the pre-antibiotic era is the Justinian Plague (541–542 CE), which claimed nearly 100 million lives. Another significant event was the Black Death in the 14th century, which resulted in the deaths of over 50 million people in Europe. Additionally, a plague outbreak between 1895 and 1930 caused approximately 12 million deaths [2]. This could have been prevented if it were known that antibiotics, a chemical substance that inhibits the growth of or kills bacteria, could effectively treat these infections [3].

In 1928, the pre-antibiotic era came to an end thanks to Scottish bacteriologist Alexander Fleming, who accidentally discovered the first true antibiotic, penicillin [4]. This groundbreaking discovery, combined with the contributions of other pioneering scientists, ushered in the celebrated era of antibiotic discovery, spanning from the 1940s to the 1970s [5]. Since then, we have lived through what has been referred to as the golden age of miraculous drugs. However, this era is now ending. Today, we find ourselves on the brink of a post-antibiotic era, a time when simple infections could once again become deadly due to the alarming rise of antibiotic resistance [6].

1.2 Mechanisms of Antibiotic Resistance

Antibiotic resistance occurs when microorganisms can survive or grow despite treatment with antibiotic levels that are typically sufficient to inhibit or kill susceptible species. Resistance can be natural when bacteria inherently express resistance or activate resistance genes in response to antibiotic exposure [7]. Alternatively, antibiotic resistance can be acquired through several mechanisms, including the acquisition of genetic material via transformation, conjugation, or transduction, as well as through mutations in chromosomal DNA. Mechanisms of antibiotic resistance can be categorized into four main groups: (1) limitation of drug uptake; (2) modification

of drug targets; (3) inactivation of drugs; and (4) drug efflux. Additionally, other mechanisms such as biofilm formation and quorum sensing also contribute to antibiotic resistance (**Figure 1**) [8].

1.2.1 Drug uptake limitation

Gram-negative bacteria are less sensitive to several antibiotics than Gram-positive bacteria, primarily due to the lipopolysaccharide (LPS) layer in their outer membrane, which acts as an additional line of defense [6]. Gram-negative bacteria's outer membrane restricts the penetration of large molecules, such as glycopeptide antibiotics like vancomycin. Additionally, hydrophobic antibiotics, including tetracyclines, β -lactams, and certain fluoroquinolones, tend to be less effective due to the ability of these bacteria to modify the permeability of the outer membrane [9]. Furthermore, resistance to certain antibiotics is more likely when porin channels, which serve as gatekeepers allowing specific molecules to enter the cell, are expressed less frequently [10].

1.2.2 Drug target modification

Several types of antibiotics target specific proteins essential for bacterial survival. For instance, some antibiotics inhibit transpeptidases, which are essential for bacterial cell wall synthesis [11]. Antibiotics can impact ribosomes, which are crucial for protein synthesis, as well as DNA-dependent RNA polymerase, which is necessary for RNA synthesis. Others target topoisomerase and DNA gyrase, which are crucial for DNA replication [12]. Modification of bacterial targets is a common mechanism behind antibiotic resistance. For example, changes in topoisomerase IV and DNA gyrase contribute to bacterial resistance to fluoroquinolones, which inhibit nucleic acid synthesis. Similarly, the 16S rRNA is methylated by the enzyme erythromycin ribosome methylase (erm), which modifies the drug-binding sites of macrolide antibiotics like erythromycin. [13].

1.2.3 Drug inactivation

Bacteria can render antibiotics ineffective by producing enzymes that attach to various chemical groups of the drugs. This attachment prevents the antibiotics from binding to their specific targets within the bacterial cell. Some key enzymes responsible for inactivating antibiotics include beta-lactamases, which break down all β -lactam antibiotics; aminoglycoside-modifying enzymes (AMEs), which stop aminoglycoside antibiotics from reaching their targets; and chloramphenicol acetyltransferases, which modify chloramphenicol, making it ineffective [10], [14].

1.2.4 Drug efflux

Efflux pumps in the cytoplasmic membrane of bacteria help confer antibiotic resistance by expelling antibiotics from bacterial cells [15]. Efflux pumps are diverse and can be classified into several families. These include the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) family, the major facilitator superfamily (MFS), and the multidrug and toxic compound extrusion (MATE) family [2]. A common example of resistance mediated by efflux pumps is tetracycline resistance, where the Tet efflux pump from the MFS uses proton exchange as its energy source to expel tetracycline [16]. Another well-known example is resistance to macrolides, where the *mef* genes encode efflux pumps such as MacB, a member of the ABC family. MacB functions as a tripartite pump, actively extruding macrolide antibiotics from bacterial cells [17].

1.2.5 Biofilms and Quorum sensing

Another defense mechanism developed by bacteria to protect themselves from antibiotics is the formation of biofilms, which can increase resistance to antibiotic treatment by up to 1000-fold [18]. Because it can be challenging for both antimicrobial agents and the immune cells to penetrate biofilms, eliminating them can be quite difficult. Bacteria in biofilms have limited access to nutrients and oxygen, which slows their metabolism. As a result, they metabolize antimicrobials more slowly than necessary for effectiveness [19]. Additionally, bacteria can communicate through a mechanism known as quorum sensing. In this process, bacteria secrete signaling molecules called autoinducers, which help regulate resistance genes in response to antimicrobial stress detected by the bacterial community [20].

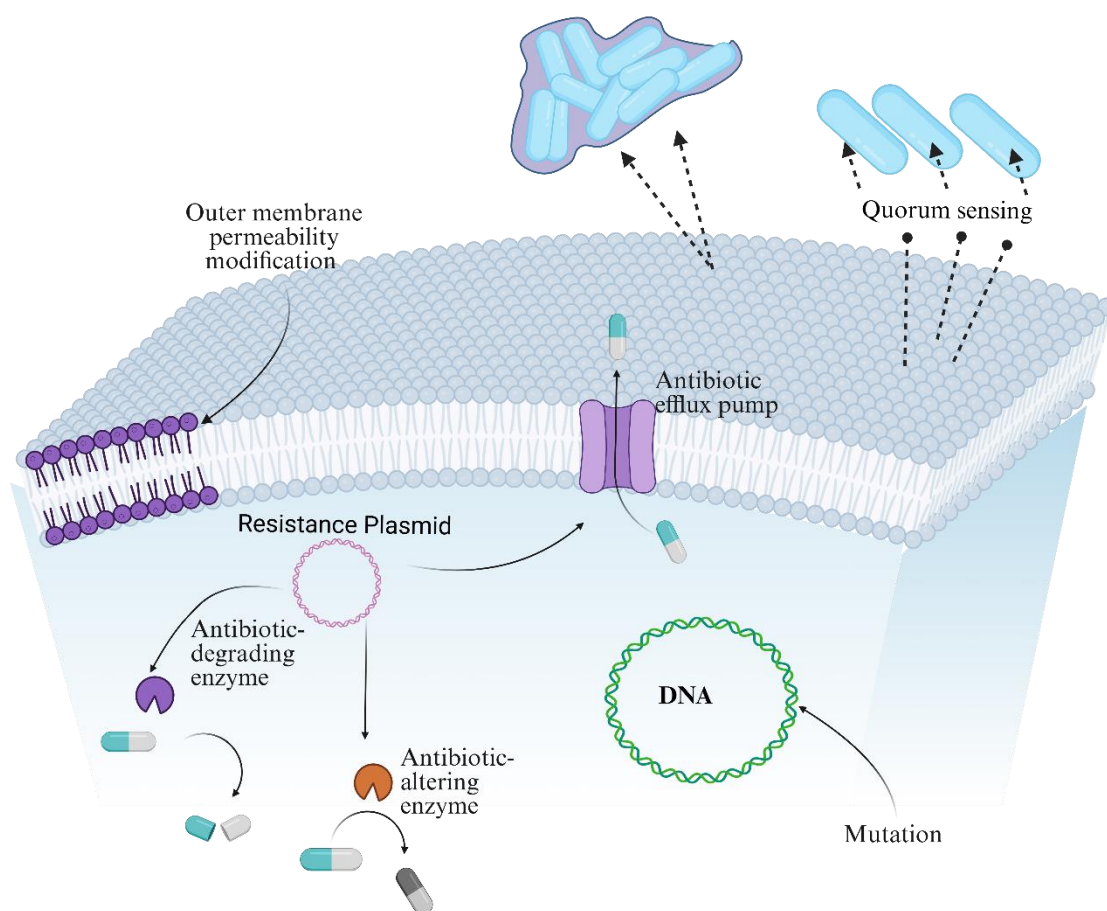


Figure 1. Mechanism of antibiotic resistance

Bacteria gain resistance through mutations, production of antibiotic degrading or inactivating enzymes, reduced permeability, target alterations, or the acquisition of resistance genes, making infection increasingly difficult to treat. This diagram was made with Biorender.com (assessed on 7th Feb 2025).

1.3 How Antibiotic Resistance Occurs

Antibiotic resistance is an unavoidable outcome of antibiotic use, but its emergence is significantly accelerated by its overuse and misuse worldwide. In some regions, over-the-counter sales and online availability provide unrestricted access to antibiotics for non-prescribers [21]. After consumption, antibiotics and their metabolites are excreted through urine and feces, entering wastewater systems. They can accumulate in sludge, which is often used as fertilizer on agricultural fields, or be discharged as runoff, contaminating surface water and wetlands [22] [23].

Antibiotics are extensively used for treatment and as growth promoters in livestock and poultry. These antibiotics and their byproducts are excreted in animal waste, which spreads into fields and groundwater. In fish farming, antibiotics are directly released into aquatic environments. Notably, wherever antibiotics are dispersed, resistant bacteria often follow the same pathways. This creates environments where antibiotics, antibiotic resistance genes (ARGs), resistant bacteria, and the natural bacterial flora may also take up these ARGs intermingle [24].

Humans can encounter these bacteria through several routes, specifically by drinking water from contaminated groundwater or surface water, swimming in marine environments connected to polluted surface water, and consuming crops that have been fertilized with contaminated sludge. The human microbiome may be exposed to these resistant bacteria's ARGs once they have entered the body (**Figure 2**) [25].

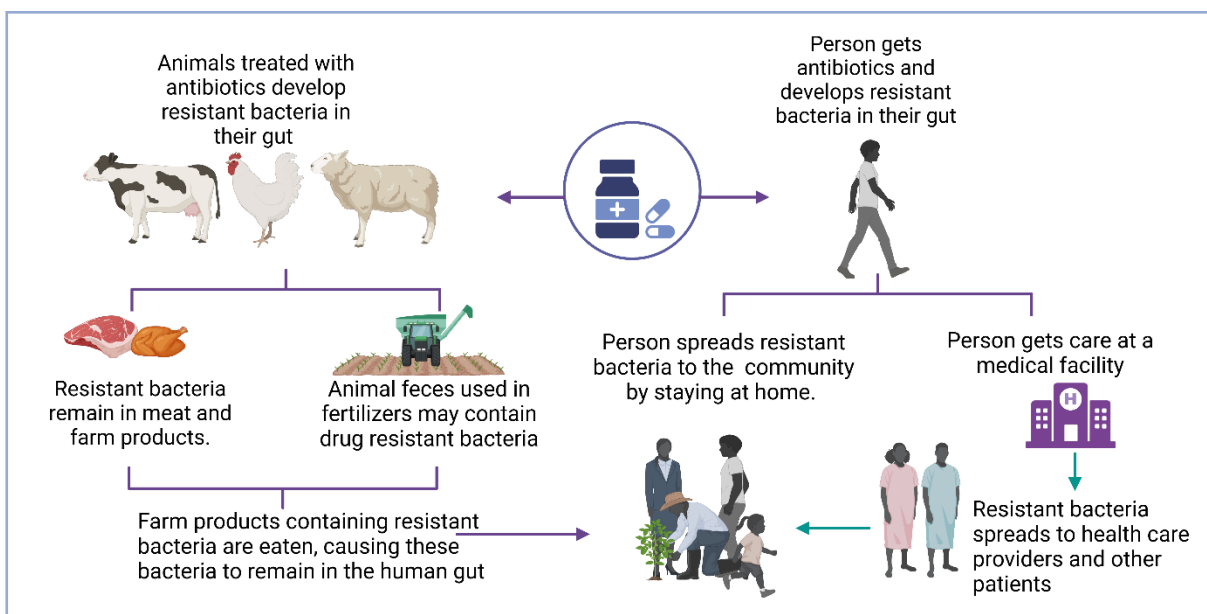


Figure 2. How antibiotic resistance spreads

ARGs can enter and re-emerge in humans through several pathways, as illustrated in this diagram. This diagram was made with Biorender.com (assessed on 7th Feb 2025). <https://app.biorender.com>

1.4 Implications of Antibiotic Resistance

Antimicrobial resistance is a significant threat to public health systems worldwide, impacting both developing countries and advanced economies [26]. Infections brought on by resistant bacteria in the healthcare system led to severe illnesses, extended hospital stays, greater medical bills, more expensive second-line medications, and treatment failures. The Centers for Disease Control and Prevention (CDC) estimates that antibiotic-resistant infections cause at least 23,000 deaths and more than two million illnesses in the US annually. Antibiotic-resistant bacterial infections affect all parts of the world and are on par with, if not worse than, those brought on by HIV and malaria [27].

It poses a serious threat to disease management across all age groups and hinders medical progress. For instance, neonatal sepsis is a major global health issue, ranking as the eighth most common cause of death among infants and children under five years old. Deaths resulting from sepsis are attributed to the inability of pathogens to respond to the antibiotics that are most readily available. Older individuals are more vulnerable to infections, especially healthcare-associated infections, due to factors such as immunosenescence, existing health conditions, and regular interactions with healthcare or assisted living facilities [28].

AMR also leads to extended treatment durations for infections, necessitating the use of costly medications for their management. The treatment of diseases like diabetes, cancer, asthma, and rheumatoid arthritis in individuals of all ages, as well as the execution of surgical procedures, is hindered by AMR [29]. AMR also threatens global health by worsening food insecurity and limiting our ability to treat sick livestock. The excessive use of antibiotics in agriculture, along with the release of active antimicrobials into the environment, contributes to the emergence of resistant strains and increases the risk of future pandemics [30][31] [29][32].

The economic burden of AMR is also profound, as it not only escalates healthcare costs but also reduces productivity. For instance, the CDC has stated that AMR adds approximately 20 billion dollars annually in direct healthcare costs in the US, which excludes approximately 35 billion dollars in productivity losses due to extended illnesses and hospitalizations. Without urgent and coordinated efforts to tackle this problem, it is estimated that by 2050, AMR could result in 10 million deaths annually and cause global economic losses of up to \$100 trillion[33]. Stakeholders

often overlook the growing burden of AMR, and the issue is further exacerbated by pharmaceutical companies' reduced efforts to develop new antimicrobial agents [34].

1.5 Antibiotic Resistance in Saudi Arabia

Antibiotic resistance is increasingly prevalent in Saudi Arabia, affecting both Gram-positive and Gram-negative bacteria. One major factor driving the rise of AMR is the substantial influx of expatriates and pilgrims [35]. Among the resistant pathogens reported in literature and surveillance studies in Saudi Arabia, carbapenem-resistant Enterobacteriaceae (CRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL) are the most encountered [36]. A study on the development and epidemiology of CRE in Saudi Arabia reported a carbapenem resistance prevalence of 22.23% [37]. In recent surveillance studies, Thabit et al. (2023) reported that MRSA constituted 32.5% of *S. aureus* isolates in Saudi Arabia [38], In 2024, Almutairi et al. found a high prevalence of MRSA among pediatric and maternal inpatients, measuring at 45.4% [39], Thabit et al. (2024) reported a surprisingly high rate of multidrug-resistant *Pseudomonas aeruginosa*, along with concerning rates of isolates with average susceptibility to antibiotics like carbapenems and colistin [40].

Data from various surveillance studies in Saudi Arabia highlights the serious threat posed by AMR in the country. If AMR continues to spread and resistant organisms proliferate, or if patients are unable to access the necessary medications for treatment, it will jeopardize Sustainable Development Goal 3 (SDG 3). This goal aims to ensure healthy lives and promote well-being for all ages, as well as to reduce newborn mortality to fewer than 12 deaths per 1,000 live births by 2030 [41], [42].

1.6 Nature as a source of novel antimicrobial products

The present state of antibiotic development is inadequate, and the future appears grim unless we supply our armamentarium with new weapons that these so-called resistant bacteria have not learned to resist, so we can rely on them as guardians. The first crucial stage in the hunt for new antibacterial chemicals is to look for unexplored sources, and one of the best treasures in the search for new alternatives is 'nature'[43]. The molecular variety found in nature is greater than what can be produced economically through chemical synthesis. Furthermore, natural compounds have already undergone prescreening and have evolutionarily been chosen for their biological

interactions and roles. New bioactive products are constantly being discovered in nature, and there are undoubtedly more products out there just waiting to be found [44].

Microbial secondary metabolites are crucial sources for the discovery of novel therapeutic compounds. Bacteria are suitable sources of antimicrobial agents due to their suitability for large-scale fermentation and the potential for incorporating synthetic biology for enhanced metabolite production [45]. Recent investigations have increasingly focused on extreme environments, such as drought conditions in deserts, high salinity in sea-ice brine, oil wells, and hot springs [46]. This is because the metabolites, especially enzymes produced under these extreme conditions, such as high pressure and temperature, are generally more stable both physically and chemically. These metabolites include a wide variety of compounds that are crucial in modern medicine, such as antimicrobials, antifungals, antitumor agents, antivirals, and antioxidants [47].

Thermophilic microorganisms are one of the most studied groups of extremophiles, noted for their adaptations to modern technologies. Besides their applications against pathogenic bacteria in the healthcare industry, they are particularly useful in industries that operate at elevated temperatures, such as the canned food and dairy sectors [48]. In addition to thriving in high temperatures, they also adapt to other physiological conditions like pH levels, varying salinity, different redox potentials, and can thrive in the presence of toxins [48].

1.7 Hot Springs as a Source of Antimicrobial Peptides-Producing Thermophilic Bacteria

Hot springs are natural springs where the water temperature is significantly higher than that of the surrounding air. In volcanic regions, shallow magma (molten rock) heats groundwater, which then flows to the surface as hot springs. In some cases, hot springs are formed when water is heated as it circulates deep underground. Groundwater can reach depths of a kilometer or more, where the Earth's crust naturally gets hotter around 30 °C (54 °F) for every kilometer within the first 10 km (6 miles) [46]. The heat in hot springs can also be produced by the radioactive decay of elements like potassium, thorium, uranium, and sulfur in the earth's crust and mantle or by friction generated along the margins of continental plates [49].

Hot springs exist worldwide and are often used for recreational purposes, such as spas, and for treating skin infections. For example, in Thailand, hot springs are widely regarded for their health benefits and disease-treating properties, making them popular destinations for relaxation and

wellness activities. A study by Duangjit *et al.* 2022 proved that water from Phusang hot spring of Thailand exerts an anti-inflammatory effect by modulating interleukin 6 (IL-6). Furthermore, combining the spring water with curcumin-loaded liposomes enhanced its antiaging effect. They found that this treatment was non-toxic to mouse and human cells and, hence, can find potential application in cosmetic products[50].

Hot springs provide ideal conditions for the growth of hyperthermophilic microorganisms due to their consistently high temperatures, abundant moisture, and unique chemical composition. Typically, hot springs maintain temperatures ranging between 43°C to 80°C and exhibit a slightly alkaline pH (6.7–9.5) [51],[52],[53].

To survive and adapt to extreme environments, thermophilic organisms have developed several adaptive mechanisms. One key adaptive feature is the alteration of the amino acid sequences in their proteins, resulting in the production of thermo-stable enzymes or proteins that remain functional at high temperatures. Factors enhancing the stability of these proteins include an increased number of hydrogen and covalent bonds that reinforce their structure, higher molecular weight, a more organized hydrophobic interior, shorter amino acid chains, a greater presence of saturated fatty acids, and the presence of disulfide bridges that further stabilize the proteins [54].

Thermophilic bacteria, like other organisms, produce antimicrobial agents as part of their strategy to compete for space and nutrients. Among these agents, AMPs have gathered attention in biomedical research. For example, Gramicidin extracted from *Bacillus brevis* in 1939 was the first-ever clinically applied AMP [55]. The clinical success of Gramicidin led to further discoveries of other AMPs with roles in the pharmaceutical and food industries, including those extracted from thermophilic bacteria [54], [56], [57],[58]. Examples of thermophilic AMPs include pallidocyclin from *Aeribacillus pallidus* PI8 [59], geobacillin 26 from *Geobacillus stearothermophilus* 15 [60], geobacillin 6 from *Parageobacillus thermoglucosidasius* [61], and pallidocin from *Aeribacillus pallidus* 8 [62]. However, research on AMPs produced by thermophilic bacteria remains scarce. Many of these antimicrobials are poorly described, and, crucially, their amino acid sequences are often not disclosed or characterized [61]. This highlights thermophilic bacteria as an untapped and valuable source of novel AMPs [61][63].

1.8 Antimicrobial peptides (AMP)

Antimicrobial peptides (AMPs) are short (<10-50 amino acids), naturally occurring proteins or peptides that are part of an organism's innate defense mechanisms [64]. Their structure and functionality enable them to activate unique mechanisms of action [65][66]. They demonstrate a wide range of biological activity against bacteria, viruses, fungi, and parasites, exhibiting high specificity and low toxicity [67]. They are also effective in preventing the formation of biofilms, demonstrate anticancer properties, and are capable of modulating immune response.

A key characteristic of AMPs is their amphiphilic nature and overall positive charge. Approximately 50% of the amino acid residues are hydrophobic, which is essential for their interaction with microbial membranes. Due to their low likelihood of causing resistance, AMPs are becoming prominent candidates for therapeutic applications [68].

Unlike conventional antibiotics, AMPs are generally safer and more effective. They are easily degradable in the environment and do not contribute to problems like pollution, bacterial resistance, or disruption of ecosystems. Compared to antibiotics that target specific sites within and outside the bacterial cell membrane (particularly sites prone to mutation), AMPs act on structurally conserved components of cell membranes. This means that bacteria would need to undergo prolonged mutations to fully recover their cell membrane structure, which significantly lowers the chances of developing resistance to AMPs [69]. AMPs can also disrupt the integrity of cell membranes and affect cell metabolism by interacting with the intracellular contents within the cells. Their multiple mechanism of action enhances their effectiveness and minimize the chances of bacteria developing resistance [69]. One distinctive feature of AMPs is their ability to differentiate between bacterial and mammalian cells. Zwitterionic lipids, such as phosphatidylcholine, lower the binding affinity of cationic AMPs to mammalian cell membranes. This reduces the potential toxicity of these peptides to human cells [70].

Different organisms, including plants, animals and microbes, produce AMPs for various specified purposes, for instance, bacteria synthesize AMPs to compete in nature for food, space, or their general survival; viruses synthesize AMPs to complete their life cycle, while higher organisms produce AMP as a host defense mechanism[55], [71]. Lysine, tryptophan, and arginine are the most

prevalent amino acids in AMPs, accounting for over half of their amino acid composition. In contrast, methionine, asparagine, glutamic acid, aspartic acid, threonine, and serine are among the least commonly found amino acids in AMPs. Currently, 3940 AMPs have been identified, as documented in the 2024 University of Nebraska database, reflecting their widespread occurrence and biological significance[72].

1.8.1 Classification of Bacterial AMPs

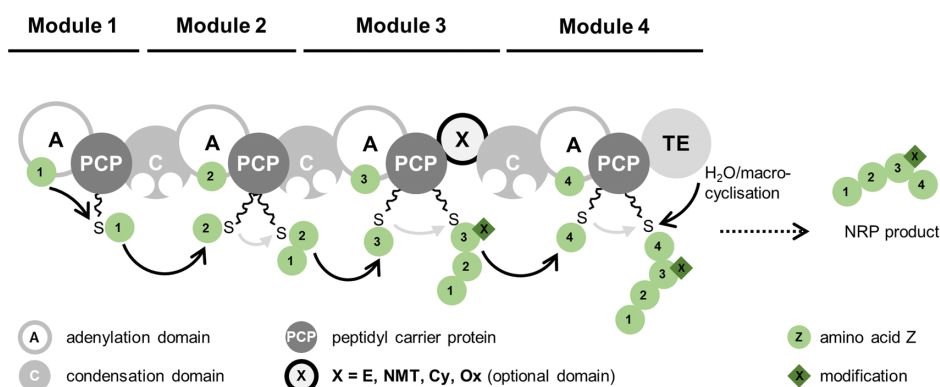
I. Synthesis

Most AMPs produced by bacteria are divided into two categories: RiPPs and NRPs [73]. NRPs are a broad family of natural peptides that frequently contain non-proteinogenic amino acids, α -hydroxy acids, D-amino acids, and fatty acids. Their composition gives them a vast structural diversity that complicates chemical synthesis as compared to polypeptides made via ribosomal synthesis[74]. Large multienzyme complexes called nonribosomal peptide synthetases (NRPS) are responsible for the synthesis of NRPs. These complexes consist of various biosynthetic modules that collaborate to construct the peptide chain in a manner similar to an assembly line. Typically, each module adds one amino acid building block to the final product [74].

NRPs synthesis starts with the adenylation (A) domain, which selects and activates a specific amino acid, whether proteinogenic or nonproteinogenic, using ATP to form an acyl-adenylate intermediate [75]. The intermediate product is then transferred to the peptidyl carrier protein (PCP), where it forms a covalent bond with the 4'-phosphopantetheine cofactors. Optional domains can introduce various modifications. For example, the epimerization (E domain) alters stereochemistry, the N-methylation (NMT domain) adds methyl groups, the cyclization (Cy domain) creates circular structures, and the oxidation (Ox domain) introduces oxygen-containing groups. The condensation (C) domain facilitates the formation of peptide bonds, leading to the elongation of the peptide chain. The final module includes either a thioesterase (TE) or reductase (Red) domain, which catalyzes the release of the peptide through hydrolysis, cyclization, or reduction. After the peptide is released, additional modifications such as glycosylation, acylation, halogenation, or hydroxylation can enhance the final natural product. It's important to note that nonribosomal peptide synthetase (NRPS) products are typically limited in size, usually consisting of fewer than 10 amino acids [74].

In contrast to NRPs, RiPP gene clusters encode precursor peptides that contain core regions where posttranslational modifications (PTMs) occur along with an N-terminal leader and/or C-terminal follower region that help to enhance binding affinity for various PTM enzymes. These regions are subsequently removed during biosynthesis. Maturation enzymes add PTMs to the core peptide before it is released through proteolytic cleavage. Unlike NRPs, which use the A domains of NRPS enzyme complexes as templates for synthesis, RiPPs use mRNA as their synthesis template [76].

A NRPS biosynthetic principle



B RiPP biosynthetic pathway

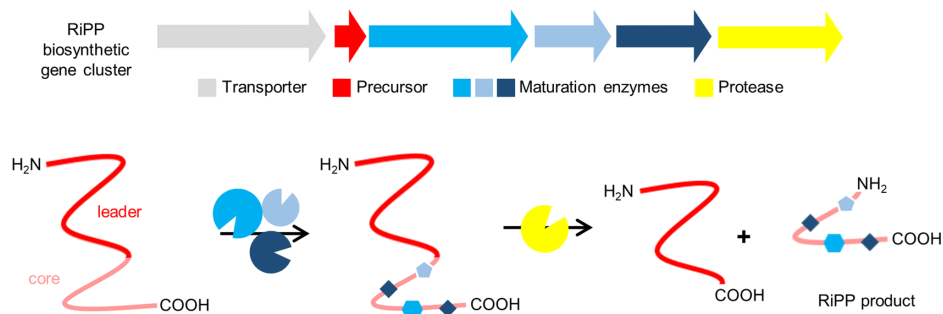


Figure 3. General overview of NRP and RiPP

(A) NRPSs are large enzyme complexes composed of repeated units known as modules. Each module plays a specific role in the process, including the selection, activation, and modification of functional groups, as well as the connection of particular amino acids. These modules work together in an assembly line-like fashion to create the final product. (B) Most precursor peptides for RiPPs consist of two main regions: a leader region and a core region. In some instances, there is also a follower peptide. The core peptide undergoes post-translational modifications to form the mature RiPP, which often requires the removal of the leader and/or follower peptides at some stage [77].

II. Structure

AMPs are classified into three distinct structural motifs which include β -sheet peptides (stabilized by disulfide bridge), α -helix peptides (which are the most encountered), and extended loops [78], [79]. Cyclic peptides, as well as those rich in disulfide bonds and lasso peptides, are being considered as the fourth and fifth groups [80]. Among these, the α -helical peptides make up the largest and most extensively studied [81]. α -helical AMPs lack cysteine residues in their structure, which prevents them from forming disulfide bonds. They are also abundant in glycine, alanine, lysine, and leucine. In aqueous solutions, they exist in a disordered state but transition into amphiphilic helical structures when interacting with membranes or a membrane-simulated environment. This structural transformation is essential for the peptide's activity as it accounts for approximately 50% of the energy needed for binding to the membrane. This promotes the initial interaction between the peptide and the bacterial membrane [82].

β -sheet peptides, like α -helical, are also rich in glycine, alanine, lysine, and leucine. However, In contrast, β -sheet peptides contain 2-4 disulfide bonds between at least two β -strands containing cysteine residues in the molecule. The presence of the disulfide bond aids in stabilizing the AMP structure, minimizes protease degradation, and promotes AMP penetration into the target cells. In comparison to α -helical AMPs, these groups of peptides do not undergo any significant structural changes when transitioning from an aqueous environment to the cell membrane environment.

The third conformational motif of AMP, i.e., peptides with extended structure are AMP rich in proline, glycine, and tyrosine in their sequence. They are also referred to as non- α/β peptides (those without α -helix or β -sheet structure) or loop AMP.

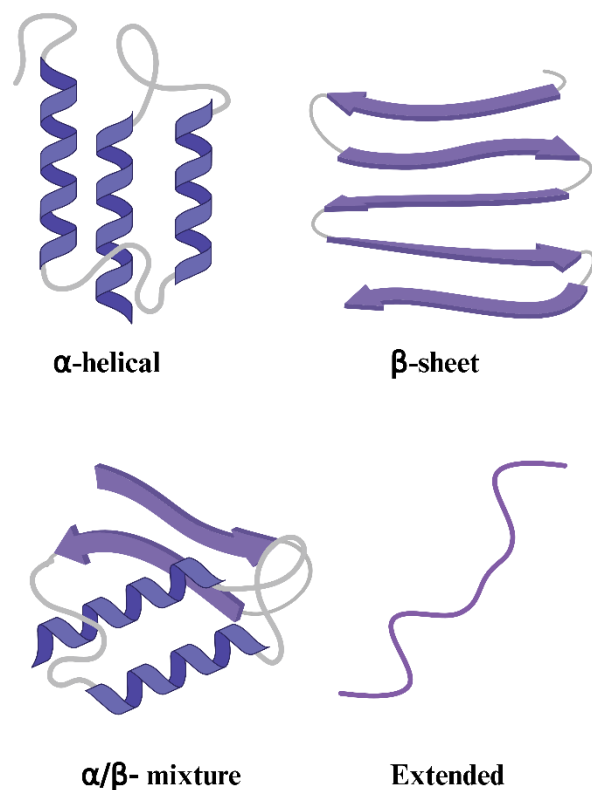


Figure 4. Examples of the structural types of AMPs

II. Antibacterial activity

The structure of AMPs significantly influences their antibacterial activity. For instance, AMPs that target bacterial cell membranes are usually alpha-helical and cationic, while those with intracellular targets are commonly beta sheets. AMPs can be classified as either non-membrane active (intracellularly active) or membrane-active based on their unique characteristics. Most membrane-active AMPs are amphipathic, harboring cationic and hydrophobic faces [81]. This characteristic enables the initial electrostatic interaction with the microbial cell membrane, which is negatively charged due to components like LPS and lipoteichoic acid (LTA). This interaction facilitates the mode of action. As positively charged AMP integrates with negatively charged bacterial membrane, these peptides create transmembrane holes causing the bacterial cell content to leak, ultimately resulting in cell death [83].

Four different models are used to describe how AMPs disrupt cell membranes. These include the Aggregate Model, where AMPs insert themselves into the cell membrane once they reach a certain concentration threshold, and the Barrel-Stave Model, where the AMPs are inserted vertically into the cell membrane, forming pore-like structures. The hydrophobic regions of the peptides align with the lipid core of the membrane, while the hydrophilic regions form the interior of the pores. In the Carpet Model, peptides spread across the membrane surface in a parallel orientation, leading to membrane disruption in a detergent-like manner. This can result in the formation of micelles. Additionally, the Toroidal Model describes a gradual curvature of the membrane that ultimately leads to disruption, with the peptides also covering the membrane surface in a parallel arrangement [84].

Non-membrane active AMPs, which target intracellular components, function by translocating across the cell membrane to interfere with normal cell function without damaging the cell membrane. These AMPs can exert their effect on different intracellular targets, including cell walls, nucleic acids, and proteins, in different ways. AMPs can also inhibit bacterial cell wall synthesis either by binding to lipid II, a precursor involved in cell wall synthesis located within the cytoplasm, or by inhibiting the synthesis of cell wall teichoic acid [85]. They can also exert their effect by binding to nucleic acids, disrupting their conformation, and interfering with the synthesis of DNA, RNA, and proteins. They can interfere with enzymes or protein activity in nucleic acid and protein-protein synthesis pathways, damage nucleic acid repair pathways, bind to the ribosome to inhibit protein synthesis, interfere with the proper folding and assembly of proteins, and finally block cell division and disrupt the cell cycle [86][87][88][89].

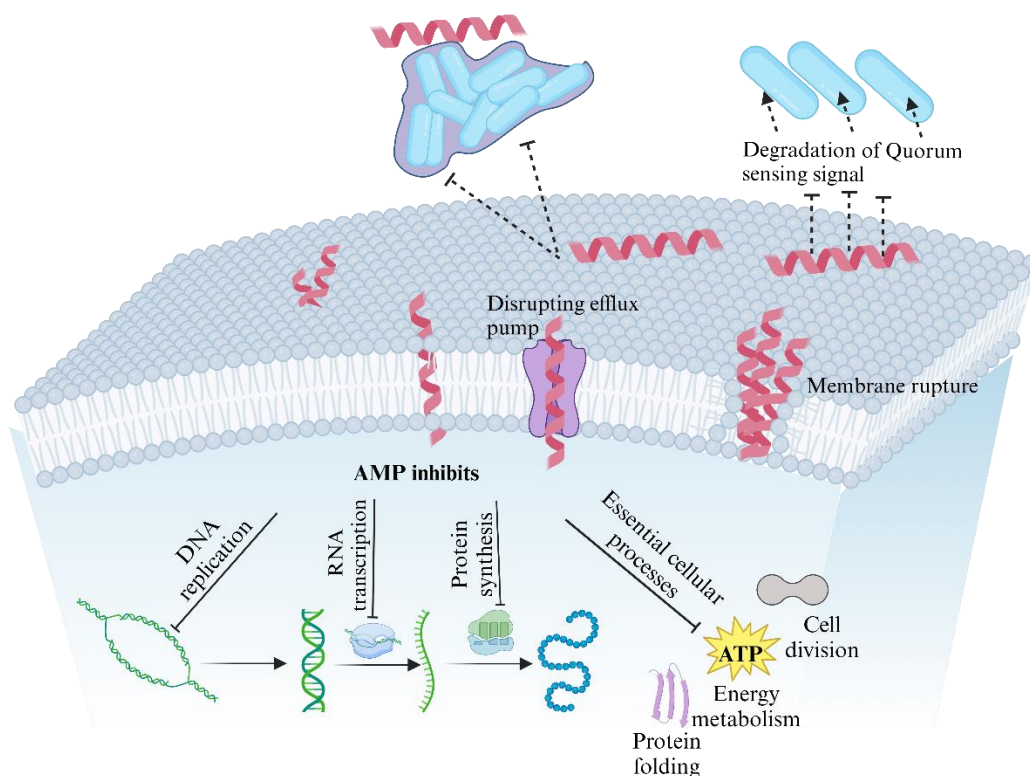


Figure 5. Antimicrobial Mechanism of AMPs

1.8.2 Potential Applications of Antimicrobial Peptides isolated from thermophilic bacteria

I. Medicine

While research on AMPs has primarily focused on their antimicrobial potential, an increasing number of studies have highlighted their diverse roles in cellular processes, including chemotaxis, wound healing, and antitumor activities. Traditional chemotherapeutics often lack specificity to tumor cells, which in turn can lead to severe side effects and the potential development of resistance in tumor cells. To address this issue, AMPs can selectively target the negatively charged membranes of cancer cells, leading to their disruption [90]. The selectivity of AMPs for tumor cells is attributed to the unique structural characteristics of tumor cell membranes compared to those of normal cells. Cancer cells have a net negative charge because they exhibit elevated levels

of anionic molecules, such as phosphatidylserine (PS) and O-glycosylated mucins, on their outer membrane leaflet. This negative charge significantly enhances the electrostatic interactions between AMPs and cancer cells. As a result, the binding affinity of certain AMPs to tumor cells can be ten times higher than that to normal cells, demonstrating their selectivity for cancer cells [91]. AMPs such as LL-37 and Piscidin-1 have the potential to inhibit cancer cell proliferation, induce apoptosis, and suppress tumor growth. LL-37 is known to cause DNA damage and cell cycle arrest by generating reactive oxygen species (ROS) [92]. Piscidin-1 is a naturally occurring cationic AMP derived from the mast cells of hybrid striped bass (*Morone saxatilis* × *M. chrysops*) that induces intrinsic apoptosis in cancer cells through a reactive ROS-mediated mechanism.[93]. As stability issues hinder the clinical application of AMPs, AMPs derived from thermophiles may offer greater potential as candidates for therapeutic use in cancer treatment [94], [95].

Several studies have highlighted the role of AMPs in promoting wound healing through various mechanisms, including immunomodulation to enhance macrophage recruitment and polarization, stimulation of cytokine production, facilitation of cell proliferation and migration, and sometimes, angiogenesis [96]. LL-37 regulates macrophage polarization, promoting wound healing by releasing anti-inflammatory cytokines [97]. Moreover, the AMPs Mastoparan-MO and Clavanin-A can activate receptor-signaling pathways that facilitate cell proliferation and migration and enhance re-epithelialization and wound healing [98].

The use of AMPs in wound treatment is often limited by their instability and susceptibility to physiological factors like oxidation, hydrolysis, and photolysis. Due to these challenges, the topical application of AMPs is recommended as the most effective route of administration for wound healing. This method allows for localized delivery, resulting in higher concentrations of the peptides at the site of action. However, even with topical application, AMPs remain vulnerable to degradation due to various conditions within the wound microenvironment, including alkaline pH levels, proteolysis, and the short residence time on the wound surface [99]. To effectively address stability issues and enhance wound healing, there is a need for new antimicrobial peptides (AMPs) that can withstand challenging conditions. Stability concerns hinder the clinical use of AMPs, but those derived from thermophilic organisms may offer greater potential as therapeutic candidates for cancer and wound treatment [94], [95].

II. Industries

Antimicrobial peptides from lactic acid bacteria (LAB) have garnered considerable attention for their potential applications in the food industry as natural preservatives or in packaging materials [100], [101], [102], [103], [104], [105]. For example, A recent study incorporated a LAB-derived AMP, α s2-casein151-181, into an antimicrobial edible coating/film to improve cheese's quality and shelf life. The AMP coating showed good stability in both salt and acidic solutions. It effectively inhibited the growth of psychotropic bacteria, yeast, and mold, and prevented the development of *E. coli* and *Bacillus subtilis*. This study highlighted the potential use of AMP as a barrier against spoilage bacteria during cheese storage. Additionally, the antimicrobial peptide KMY15, derived from *Lactiplantibacillus plantarum*, has also been identified as a potent preservative and antimicrobial agent [106].

Antimicrobial peptides derived from mesophilic bacteria may not have beneficial applications in the canned and dairy food industries where high temperatures are used and where spore-forming thermophiles present a challenge. Advances in research on thermophilic microorganisms and their peptides, especially those belonging to the class I and II bacteriocins, have opened new research avenues for their use at elevated temperatures. Thermostable AMPs such as geobacillin 26, geobacillin 6 and AMP SAT4 offer promising solutions for preventing contaminating microorganisms such as (*para*)*Geobacillus* sp., and *Aeribacillus pallidus* that can survive pasteurization and form biofilms within pipes of food processing equipment [60], [61], [107].

The cosmetic industry is one of the most rapidly developing sectors globally, with preservatives playing a crucial role in inhibiting the growth of spoilage-causing microbes. Commonly used preservatives include parabens, isothiazolinones, formaldehyde releasers, phenoxyethanol, and organic acids. These preservatives can cause contact dermatitis, disrupt skin microbiota balance, and are a common source of allergens. The synthetic AMP IK-16-1 has demonstrated a broad-spectrum activity against common microbes causing cosmetic spoilage. It also showed an excellent safety profile, with no cytotoxicity to keratinocytes and minimal hemolytic activity [108]. While research on the potential application of AMPs in the cosmetic industry remains limited, AMPs derived from thermophilic bacteria may hold promise as an effective preservative for cosmetic products.

III. Agricultural industry

In agriculture, Antimicrobial Peptides (AMPs) are used to improve the health of crops and livestock, fight various diseases, and enhance overall productivity. Research shows that AMPs can stimulate the growth of plants, alleviate the impacts of drought and saline conditions, and provide protection against a range of pathogens [109]. The AMP Bac 14B is a powerful exogenous growth enhancer. Research on traditional agronomic seed vigor parameters demonstrated that it improved the seedling vigor of tomatoes and muskmelons. Additionally, AMPs can be used as disinfectants against a wide variety of seed-borne and root diseases [110].

In aquaculture, more than 200 diseases are attributed to bacterial infections, leading to economic loss estimated at \$750-1000 million US dollars annually. Antibiotics are not only used to prevent diseases in aquaculture; they are also used as dietary supplements to promote the growth of aquatic organisms. Their improper or excessive use in fish feed has resulted in the emergence of antibiotic-resistant pathogens impacting fish health [111]. To address this, AMPs can be utilized as an alternative therapeutic in aquaculture and animal husbandry to bolster fish and livestock immunity, increase milk production in cows, and reduce the mortality rate in poultry and other farm animals [112].

AMPs can also be used as Biological control agents (BCAs) for managing plant diseases and insect pest damage in agriculture, forestry, and fruit tree production. BCAs are especially beneficial because they do not leave toxic residues in plants or the environment. Amicoumacin and thuricin 7 are examples of antimicrobial peptides (AMPs) that have been utilized against plant pathogens. Amicoumacin has shown herbicidal activity against duckweed in freshwater environments, while the AMP thuricin 17 plays a crucial role in alleviating plant stress [113].

1.9 Problem Statement

The world is currently in an alarming war with an invisible enemy, the so-called superbug or resistant bacteria. These bacteria are continually developing and evolving resistance mechanisms against existing antimicrobial drugs at an alarming pace. Without urgent effort to address this issue, it is predicted that by 2050, AMR is projected to cause 10 million deaths yearly and a global economic loss of \$100 trillion. This grim scenario will happen because common diseases that

were once manageable will become life-threatening as antibiotics lose their effectiveness over time.

Despite the looming threat, the clinical pipeline for novel antimicrobial drugs is severely limited. AMR often goes unnoticed by many stakeholders, and the issue is further exacerbated by the reduced effort of pharmaceutical companies to develop new therapeutic agents. To address this issue, researchers must find new effective weapons that can serve as a future guardian against antimicrobial-resistant bacteria.

Bacteria produce antimicrobial peptides, potent molecules with broad-spectrum activity to outcompete their rivals for nutrients and space. While Antimicrobial peptides offer promising alternatives to conventional antibiotics, most peptides, especially those derived from mesophiles, lose their activity when exposed to environmental conditions such as high temperatures, neutral to alkaline pH, and proteolytic enzymes. Their denaturation under these conditions limits their applications in clinics and industries. To overcome this situation, AMPs with resistant features may be suitable alternatives to antibiotics. One way forward in addressing this limitation is to explore new, untapped sources of peptides that have not been previously exploited, such as thermophilic bacteria found in hot springs. In recent years, thermophilic bacteria have garnered significant attention due to their biotechnological potential in enzyme and biofuel applications.

Saudi Arabia is rich with many hot springs, most of which are believed to possess therapeutic benefits. These hot springs offer an opportunity for discovering novel antimicrobial agents. Studies have proven that thermophilic bacteria from hot springs in Saudi Arabia are capable of producing stable biomolecules, particularly enzymes, under harsh conditions. These bacteria may also produce antimicrobial peptides, which may be relatively stable at high temperatures, varying pH, and proteolytic degradation. This exceptional stability of AMPs will render them important candidates for industrial processes, such as food processing at elevated temperatures, and application in therapeutics, including cancer treatments, wound healing, and combating infections. There is limited information on the diversity of thermophilic bacteria from Saudi Arabia's hot springs and their thermostable AMP. Given the immense potential of hot springs, we need to explore these unique environments to discover novel therapeutics that can help us win this battle against antimicrobial-resistant bacteria.

1.10 Objective

The objective of this study is to:

- Screen thermophilic bacteria previously isolated from hot springs for antimicrobial activity using *Staphylococcus aureus* and *Escherichia coli* as test organisms.
- Perform extractions using organic solvents to isolate bioactive fractions exhibiting antimicrobial properties.
- Analyze and characterize the stability of AMPs.
- Elucidate the structures of the bioactive compounds using various spectroscopic techniques such as FTIR, NMR, and LCMS.

Chapter 2

Literature Review

2.1 Introduction

To address the growing challenge of antimicrobial resistance, researchers have turned their attention to exploring extreme environments for novel solutions. The unique and harsh conditions of these environments, such as those found in hot springs, are loved by extremophiles (organisms adapted to extreme conditions). Organisms that are specially adapted to survive and thrive in extreme conditions have remarkable biochemical abilities. By leveraging the vast diversity of extremophiles, researchers can uncover novel resources to combat bacterial resistance. AMPs derived from thermophilic bacteria hold immense promise due to their diverse properties, including antimicrobial, anticancer, antioxidant, and immunomodulatory activities. These characteristics make them valuable for drug discovery, biotechnological, and industrial applications [114].

Hot springs are abundant in Saudi Arabia, however, the antimicrobial potentials of thermophilic bacteria dwelling in this unique ecological niche are yet to be fully explored. This study focuses on four strains; *Bacillus licheniformis*, *Brevibacillus borstelensis* AK1, *Anoxybacillus flavithermus*, and *Geobacillus stearothermophilus*, all of which were isolated from a hot spring in Saudi Arabia. Before delving into the thesis work, this review offers a summary of the potential strains and their ability to produce AMPs, as described in related studies.

2.2 *Bacillus* species

The genus *Bacillus* includes a diverse group of rod-shaped, Gram-positive, endospore-forming bacteria that have been the subject of scientific research for many years. Bacteria belonging to this genus can be isolated from environments, including soil, water, and the gastrointestinal tracts of many organisms [115]. Similar to lactic acid bacteria (LAB), the genus *Bacillus* consists of several species that hold Generally Recognized as Safe (GRAS) status from the United States Food and Drug Administration. These bacteria are highly valued for their excellent safety profile, rapid growth rates, and efficiency in secreting proteins into the extracellular medium, which allows for shorter fermentation cycles [116].

Bacillus sp. has diverse roles in various ecosystems. For example, they can promote crop growth (serve as rhizobacteria) through nitrogen fixation and phosphate solubilization and enhance crop yield [117], [118]. Some species, such as *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* (Bs), are widely used as an eco-friendly biopesticide, serving as an alternative to traditional chemical pesticides [119]. *Bacillus sp.* is essential in the industry for valuable metabolites, including enzymes and antibiotics [120]. In medicine and biotechnology, *Bacillus* genus can also be used as probiotics to enhance gut health, potentially offering protection against pathogens [121], [122].

Bacillus sp. is also known for their ability to produce AMPs, making them a promising source for discovering novel peptides. Different *Bacillus* species synthesize AMPs through either ribosomal or non-ribosomal pathways [116]. For example, bacitracin, a 12-amino-acid AMP, is synthesized non-ribosomally by certain strains of *Bacillus licheniformis* and *Bacillus subtilis*. Bacitracin is a significant AMP that effectively inhibits the growth of *Streptococcus pyogenes* and *Staphylococcus aureus* [123]. AMP pumilicin 4 is a ribosomally synthesized antimicrobial peptide produced by *Bacillus pumilus*. Pumilicin 4 demonstrates bactericidal activity against MRSA and vancomycin-resistant *Enterococcus faecalis* [124]. Other antimicrobial peptides produced by the genus *Bacillus* include lichenicidin from *Bacillus licheniformis* ATCC 14580 [125], ericin S from *Bacillus subtilis* A1/3 [126] and Bac14B produced by *Bacillus subtilis* strain 14B [110].

The investigations into the ability of thermophilic *Bacillus* to produce highly stable AMPs are limited. Therefore, to address the challenge of antimicrobial resistance, it is essential to explore the potential of thermophilic bacillus to produce AMPs.

2.3 *Geobacillus* species

The genus *Geobacillus*, previously classified as belonging to the genus *Bacillus* comprises a group of Gram-positive, rod-shaped, thermophilic spore-forming bacteria widely distributed in different habitats, including oilwells [127], hot springs [57], deserts [128], soil [129], and even in the atmosphere[130]. *Geobacilli* are either aerobic or facultative anaerobic. Their growth temperature is observed in the range between 37-75°C and pH in the range between 6.0-8.5. The widespread distribution of members of this genus reflects their high level of environmental adaptability [44]. These bacteria have attracted the attention of industries as metabolic factories for producing enzymes[131], [132], surfactants[133], biomass energy, and antimicrobial agents[134] [135]. Most

studies concerning this strain have focused on three main areas: hydrocarbon degradation, enzyme production, and synthesis of antibiotics.

Recent studies have shown promising antimicrobial potential in *Geobacillus* sp. One such study by Koniuchovaite *et al.* (2023) described the biosynthesis and characterization of a novel leaderless AMP named geobacillin-6, which was identified in the thermophilic bacterium *Parageobacillus thermoglucosidasius*. Geobacillin-6 exhibited narrow-spectrum antimicrobial activity against closely related thermophilic species. It demonstrated stability across a wide pH range (pH 3-11) and at high temperatures, retaining 100% of its activity even after being incubated at 95°C for 6 hours. Given the stability of geobacillin-6 under harsh environmental conditions and its effectiveness against thermophilic bacteria, it could be valuable in the food industry and biotechnological processes where contamination by thermophilic bacteria is a concern. [61].

More recently, AMPs from *Geobacillus thermoleovorans* and *G. stearothermophilus* were studied for their ability to prevent the growth of *Paenibacillus larvae*, which are the cause of American foulbrood (AFB), a serious and extremely contagious disease that affects honey bees. The findings of this investigation helped to provide light on AMPs that may be useful in suppressing *P. larvae*[136]. Growth inhibition of important bacteria by *Geobacillus* has also been demonstrated in other studies. Its potential as a source of new antimicrobials is highlighted in Table 1.

2.4 *Brevibacillus* species

The genus *Brevibacillus* was genetically reclassified from strains that were previously assigned to be *Bacillus brevis* in 1996, just like the genus *Geobacillus*. *Brevibacillus* sp. shares several traits with *Bacillus* sp. in terms of environment and phenotype. They are a group of rod-shaped, aerobic bacteria that are Gram-positive. Members of this genus have been employed as probiotics and as an abundant source of AMPs like gramicidin A and S, the first clinically applied AMP [137].

Unlike other thermophilic strains, the characterization of AMPs from *Brevibacillus* taxa has been limited. The first characterization of an AMP laterosporulin generated by *Brevibacillus* sp. Strain GI-9 was presented in a study conducted by Kumar et al. in 2012. Laterosporulin is a low molecular weight peptide (5.6kDa) that exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria. This peptide holds promise as a therapeutic or food preservation agent due to its stability across varying pH, temperature, and resistance to proteolytic enzyme [138].

Brevibacillus laterosporus has also been previously identified as a producer of AMPs. It secretes the antimicrobial peptide BL-A60 an eleven-amino-acid containing AMP with a molecular weight of 1602.05 Da. BL-A60 is active against *Pseudomonas solanacearum*, *Xanthomonas vesicatoria*, *Bacillus subtilis*, *Phytophthora capsici*, *Botrytis cinerea*, *Verticillium dahlia*, and *Fusarium oxysporum*. It remains stable across the pH range of 3 to 11 and after heating at 100°C for 15 minutes [139].

Additionally, Ning *et al.*, 2021 discovered multiple AMPs (designated as brevilaterin A-E) extracellularly produced by *B. laterosporus* S62-9. These AMPs shared similar primary structures and molecular weights, exhibiting broad antimicrobial activities against both bacteria and fungi. After 20 minutes of heating at 120°C, these AMPs maintained over 80% of their antibacterial activity and showed no discernible decline in activity throughout a pH range of 2–12 [140].

In addition to producing thermostable enzymes, the four thermophilic *Brevibacillus* species; *Brevibacillus thermoruber*, *Brevibacillus aydinogluensis*, *Brevibacillus borstelensis*, and *Brevibacillus limnophilus* may also be prospective microbial factories for the synthesis of antimicrobial peptides.

2.5 *Anoxybacillus* species

Anoxybacillus is a member of the phylum Firmicutes and family Bacillaceae. The genus *Anoxybacillus* was proposed in 2000 and is relatively new compared to the well-characterized genera *Bacillus* and *Geobacillus*. *Anoxybacillus flavithermus* (formerly known as *Bacillus flavithermus*) was the first species to be described under this genus. It was found in a hot spring in New Zealand and described as a facultative anaerobic, Gram-positive, endospore-forming microbe that could survive at temperatures between 37°C and 70°C. Although *Anoxybacillus* species are found around the world, they are mostly isolated from thermophilic habitats such as terrestrial hot springs [141].

Anoxybacillus species are known to produce thermo- and alkali-stable biotechnologically significant enzymes, such as the thermostable extracellular alkaline protease called SAPA, which *Anoxybacillus kamchatkensis* M1V produces [142] The thermostable alkaline xylanase, which is produced by *Anoxybacillus kamchatkensis* NASTPD13 and is essential for the breakdown of

hemicellulose [143] and the carboxylesterases EstD9, which is derived from *Anoxybacillus geothermalis* D9 and are lipolytic enzymes that hydrolyze and synthesize esters 9 [144].

Additionally, members of the genus *Anoxybacillus* generate immunomodulators that improve the function of the immune system and boost defenses against infectious diseases. For instance, A study found that *Anoxybacillus kamchatkensis* XA's secondary metabolites contained the cyclic dipeptides cyclo (Gly-L-Pro) and cyclo (L-Ala-4-hydroxyl-L-Pro), which showed a strong stimulatory effect on several innate immune mechanisms, such as phagocytic activity, elevated serum superoxide dismutase (SOD) activity, elevated serum lysozyme levels, and enhanced serum bactericidal activity in common carp [145].

Anoxybacillus species are a potential source for isolating bioactive compounds and are a useful asset in environmental restoration operations because they are not harmful. It is important to note that, despite the genus *Anoxybacillus*' potential for several biotechnological uses, there is presently no evidence on its capacity to create antimicrobial peptides.

Table 1. Summary of Bacterial Strains Related to This Study and Their Associated Antimicrobial Peptides

Antimicrobial peptide	Producer	Molecular weight	Spectrum	Stability	Sensitivity	Reference
Lichenicidin	<i>Bacillus licheniformis</i> ATCC 14580	3.2kDa	Broad spectrum activity	Heat stable	<i>Listeria monocytogenes</i> , methicillin-resistant <i>S. aureus</i> (MRSA), and vancomycin-resistant <i>Enterococcus</i> (VRE) strains	[125]
Ericin S	<i>Bacillus Subtilis</i> A1/3	3.4kDa	Broad spectrum activity	Thermo-labile	<i>B. amyloliquefaciens</i> ATCC 15841, <i>B. brevis</i> ATCC 7577, <i>B. cereus</i> ATCC 14579, <i>B. firmus</i> ATCC 14575, <i>B. polymyxa</i> ATCC 842, <i>B. sphaericus</i> ATCC 14577, <i>B. subtilis</i> (natto) DSM 1088, <i>B. subtilis</i> DSM 3256, <i>B. subtilis</i> DSM 3258, <i>L. lactis</i> IL-1403	[126]
Bac 14B	<i>Bacillus subtilis</i> 14B	21kDa	Broad spectrum activity	Heat stable	<i>Micrococcus luteus</i> LB 14110, <i>S. aureus</i> ATCC 6538, <i>B. subtilis</i> ATCC 6633, <i>B. cereus</i> ATCC 4464, <i>B. licheniformis</i> , <i>E. coli</i> ATCC 8739 and others.	[110]
Bacillocin 490	<i>Bacillus licheniformis</i>	2kDa	Narrow spectrum activity	Heat stable	<i>B. licheniformis</i> 5 A2, <i>Listeria innocua</i> , <i>S. epidermidis</i> , <i>B. subtilis</i> AZ56, <i>B. anthracis</i> 7700, <i>B. cereus</i> 6A2, <i>B. stearothermophilus</i> 9A19, <i>B. smithii</i> PRO/S	[146]
Pumilicin 4	<i>Bacillus pumilus</i>	1.9kDa	Narrow antimicrobial spectrum		MRSA and VRE	[124]

Geobacillin 6	<i>Parageobacillus thermoglucosidasius</i> DSM 2542	5.5 kDa	Narrow antibacterial spectrum	Heat stable	<i>Aeribacillus pallidus</i> DSM 3670T, <i>Anoxybacillus tepidamans</i> DSM 16325T, <i>P. toebii</i> DSM 14590T, <i>B. zanthoxyli</i> CH07 and various <i>Geobacillus</i> spp	[61]
Geobacillin I	<i>Geo-bacillus thermodenitrificans</i> NG80-2	3.3 kDa	Active against a wide range of Gram positive bacteria	Stable temperature 37°C and 60°C	<i>S. dysgalactiae</i> , <i>B. anthracis</i> Sterne 7702, MRSA, <i>B. subtilis</i> ATCC 6633, and <i>Lactococcus lactis</i> HP	[147]
AMP GT-1326	<i>Geobacillus thermoleovorans</i>	32 kDa	Narrow antibacterial spectrum	Unstable (instability index of 50)	<i>Paenibacillus larvae</i>	[136]
AMP GS-136	<i>Geobacillus stearothermophilus</i>	32 kDa	Narrow antibacterial spectrum	Unstable (instability index of 50)	<i>Paenibacillus larvae</i>	[136]
Geobacillin 26	<i>Geobacillus stearothermophilus</i> 15	24.5 kDa	Narrow spectrum	Thermo-labile	<i>G. stearothermophilus</i> , <i>G. toebii</i> and <i>Parageobacillus thermoglucosidasius</i>	[60]
Toebicin 218	<i>Geobacillus toebii</i> HBB-218	5.5kDa	Active against a wide range of Gram positive bacteria	Heat stable	Active against a wide range of bacteria, including <i>E. faecalis</i> , <i>M. luteus</i> , and more active against thermophilic bacterial isolates.	[129]
Tostadin	<i>Brevibacillus brevis</i> XDH	1.1kDa	Narrow spectrum	NA	<i>E. coli</i> and <i>S. aureus</i>	[148]
BL-A60	<i>Brevibacillus laterosporus</i> strain A60	1.6kDa	Broad spectrum	Heat stable	<i>Pseudomonas solanacearum</i> , <i>Xanthomonas Vesicatoria</i> , <i>B. subtilis</i> , <i>P. capsici</i> , <i>B. cinerea</i> , <i>Verticillium dahliae</i>	[139]

					and <i>Fusarium omysporum</i>	
Brevicillin	<i>Brevibacillus sp.</i>	-	Narrow spectrum	Heat stable	<i>S. aureus</i> , <i>E. coli</i> , and <i>Candida albicans</i>	[149]
Laterosporulin	<i>Brevibacillus sp.</i> <i>Strain GI-9</i>	5.6 kDa	Active against a wide range of Gram-positive and Gram-negative bacteria	Heat stable	Active against a wide range of Gram-positive and Gram-negative bacteria including <i>B. subtilis</i> and <i>S.aureus</i>	[138]
Brevilaterin (A-E)	<i>Brevibacillus laterosporus</i> S62-9	1.5-1.6kDa	Broad spectrum	Heat stable	<i>S. aureus</i> , <i>E. coli</i> , <i>Botryosphaeria dothidea</i> , <i>Listeria innocua</i> , <i>Phytophthora capsica</i>	[140]

2.6 Hot spring selected for this study

Saudi Arabia is rich with many hot springs, most of which are in the western part of the country. There are ten geothermal springs available in Saudi Arabia. These sites serve as prominent therapeutic and tourist destinations within the region. These hot springs host a diverse array of thermophilic bacteria and archaea, including *Streptomyces* sp.[150], *Brevibacillus linens*, *Bacillus subtilis* [151], *Bacillus licheniformis*[152] and many more. Studies have revealed that these thermophiles have the potential to produce bioactive compounds, including biosurfactants, enzymes, and antimicrobial agents.

For instance, Alqahtani *et al.*, 2023 studied the potential of thermophilic *Bacillus* to produce antimicrobial agents with bactericidal properties against various Gram-positive bacteria. They identified four compounds, isovaleric acid, N-acetyltryptamine, ethyl-4-ethoxybenzoate, and phenylacetic acid from the bacterial supernatant, which were active against all strains tested in the study [153]. Similarly, Alrumman *et al.* (2019) isolated four thermophilic *Bacillus* strains confirmed to be *Bacillus sonorensis*, *Brevibacillus borstelensis*, *Bacillus thermocopriae*, and

Brevibacillus parabrevis. GC-MS analysis of the bacterial extracts detected major secondary metabolites, including imiloxan, cyclohexyl acrylate, tabtoxinine- β -lactam, L-menthyl lactate, nicotiny alcohol, (3-aminopropyl) dibutylborane, mephenesin, etomidate, 1-dotriacontanol, and filbertone, all of which possessed antimicrobial activity[154].

Thermophilic bacterial isolates from Jazan's hot springs are the subject of our investigation. The water temperature of this hot spring varies from 41°C to 96°C. It is believed that bathing or drinking hot water from the spring helps in treating several health and skin issues like eczema, psoriasis, joint problems, and other chronic diseases[155], [156]. Thermophilic bacteria isolated from these hot springs have been studied for their ability to produce different antimicrobial, biodegrading agents, enzymes, and surfactants. However, there are no studies on the antimicrobial peptide-producing potentials of these thermophiles. Exploiting bacteria from this hot spring may bring us the solution we have been longing for to eradicate the global challenge of antimicrobial resistance.

2.7 Antimicrobial peptide extraction, clean up, and Purification

2.7.1 Extraction

The extraction of antimicrobial peptides is a complex process. The extraction method depends on several factors, including the source of the starting material, the cellular location of the target peptide, and the intended downstream applications. Additionally, it is crucial to preserve peptide activity and functionality while minimizing background interference during the extraction process [157]. To obtain AMPs from bacteria, the extraction method depends on whether the peptides are released into the external environment or retained within the bacterial cells. In such cases, AMPs can be obtained using extracellular extraction techniques or intracellular extraction methods [158].

Extracellular extraction techniques

Extracellular extraction involves cultivating bacteria in a suitable liquid medium under optimized conditions to maximize antimicrobial peptide (AMP) production. After the bacteria are grown, the culture is centrifuged to separate the cell-free supernatant, which contains the AMP. The AMP is

then extracted from the supernatant using various solvents depending on the physicochemical properties of the peptides and their desired applications [159]. Organic solvents, including methanol, ethanol, and ethyl acetate are commonly employed for the selective isolation of specific peptide groups. This approach is cost-effective and relatively easy to implement. However, it is characterized by co-extraction of low molecular weight compounds, necessitating additional fractionation steps to isolate the target AMPs. Furthermore, the overall yield of peptides obtained via this method is often limited, which may pose challenges in large-scale applications[160]. Ammonium sulfate is commonly used to precipitate antimicrobial peptides from bacterial supernatants. This method relies on the principle that protein solubility decreases at high salt concentrations. Additionally, salts that reduce protein solubility often enhance the stability of the protein's native conformation [161].

Intracellular extraction technique:

For the Intracellular extraction of AMPs from bacteria, the cell pellet is first obtained and lysed either by mechanical disruption via sonication or lysed using gentle buffers such as phosphate buffers at pH ranges between 5.4-7.5 organic solvents like isopropyl alcohol [158].

2.7.2 Antimicrobial peptide cleanup

Salts and detergents employed in protein extraction formulations may interfere with downstream analysis or negatively impact the stability or function of proteins. Consequently, after solvent extraction or cell lysis, these pollutants may need to be eliminated or reduced [162]. Different methods can be used for protein cleanup. Protein cleanup can be accomplished in a variety of ways. These consist of concentration, desalting, and dialysis. The most popular separation method, dialysis, uses a semipermeable membrane to selectively diffuse tiny, undesirable molecules out of proteins in solution. While low molecular weight contaminants can be eliminated during several buffer exchanges, proteins larger than the membrane pores are retained on the sample side of the membrane [157].

2.7.3 Antimicrobial Peptide Concentration and Purification

Following extraction, a protein of interest is separated from other proteins and components in a crude cell lysate or other material using a variety of techniques. Solid-phase extraction (SPE) and

HPLC are two popular methods for partial or one-step purification.

Solid phase extraction

SPE is considered a rapid and selective sample preparation method used in the laboratory to separate analytes of interest from complex matrices prior to chromatographic analysis (for example High-performance liquid chromatography) [163]. In an idealized SPE procedure, the analyte is trapped on a solid phase, interferences that are less attracted to the sorbent are washed off, and the analyte or analytes are eluted, leaving interferences that are more attracted to the sorbent [164]. SPE based on hydrophobic phases is a practical, effective, and quick method for extracting and concentrating peptides. With trifluoroacetic acid present, it enables a successful peptide fractionation utilizing eluents that have a rising gradient of acetonitrile. There are variations among sorbents in terms of selectivity. C18 sorbent consists of silica particles with long hydrophobic alkyl chains (18 carbon atoms). The hydrophobicity of antimicrobial peptides allows effective binding to the C18 sorbent through van der Waals forces, while polar contaminants, such as salts, sugars, or nucleic acids, are washed off during the process. Only extremely hydrophobic peptides can be absorbed by CN sorbents. Hydrophobic and non-polar peptides can be isolated and fractionated using PH, CH, C8, and C2 sorbents, but typically not very polar peptides [165].

High-performance Liquid Chromatography (HPLC)

The chromatographic technique known as HPLC is used to analyze any substance that is soluble in a liquid (mobile phase). This technique is frequently utilized in food, pharmaceutical and other industries to separate and purify macromolecules or quantify ions and small molecules. Even while HPLC is most frequently utilized as an analytical method, it can also be applied in preparatory settings. HPLC has numerous benefits over conventional low-pressure column liquid chromatography. The primary parts of an HPLC system includes an injector, pump, column, detector, and data system [166].

The injector inserts the sample into the mobile phase for insertion onto the column, while the pump transports the mobile phase in a regulated, precise, and accurate way, usually at a flow rate of 0.4-1 ml/min. A gradient elution system is usually used to mix the mobile phase from two or more reservoirs to change its concentration throughout the run. This can be done using low-pressure mixing, which involves mixing the components of the mobile phase before they enter the high-

pressure pump, or high-pressure mixing, which is achieved using two or more separate, programmable pumps [166].

An HPLC column is typically made of a stainless-steel tubing that can be connected to the injector and detector systems via terminators. Glass, titanium, fused silica, and polyether ketone (PEEK) resin are also used to make columns. The analytical HPLC columns that are most frequently used have an internal diameter of 4.6 or 5 mm and are 10, 15, or 25 cm long. Fast separations are becoming more and more common with short (3 cm) columns filled with $\leq 3\text{-}\mu\text{m}$ particles. The majority of packing material for HPLC columns functions as both the stationary phase and the support matrix. In most cases, the packing material used in HPLC columns serves as both a support matrix and a stationary phase. High-quality packing materials should possess good chemical stability and mechanical strength to endure the high pressure generated during use. Additionally, they should have a well-defined particle size with a narrow size distribution. Two materials that meet these criteria are porous silica and synthetic organic resins [166]. Silica-based packings with covalently bonded octyl (C8) or octadecyl (C18) groups are preferred for peptide separation. Peptides are eluted from these hydrophobic stationary phases according to their increasing overall hydrophobicity [167].

The three main HPLC modes used for peptide separations include the size-exclusion HPLC, which makes use of variations in peptide size, reverse phase HPLC (RP-HPLC), which makes use of peptide hydrophobicity, and ion-exchange chromatography, which makes use of peptide net charge [167]. RP-HPLC is the most used mode for peptide separation as its speed and efficiency are better than those of other HPLC modes. Furthermore, it is perfect for both preparative and analytical separations due to the availability of volatile mobile phases like aqueous trifluoroacetic acid [TFA]/acetonitrile [CH₃CN] systems. Most researchers tend to perform HPLC at pH levels below 3.0. When conducting preparative purification of peptides or using RP-HPLC as a final desalting and purification method in a multistep procedure, such mobile phase volatility is particularly beneficial [168].

The most commonly used detector system for HPLC analysis is the UV-Vis absorption detector, which measures the amount of radiation absorbed by compounds containing chromophores. These detectors include a diode array, a variable wavelength, and a fixed wavelength spectrophotometer.

Peptide bonds offer a practical method of detection (usually at 210–220 nm) by strongly absorbing light in the far ultraviolet (UV) (<220 nm). Moreover, tryptophan, phenylalanine, and tyrosine's aromatic sidechains absorb light in the UV region of 250–290 nm [166].

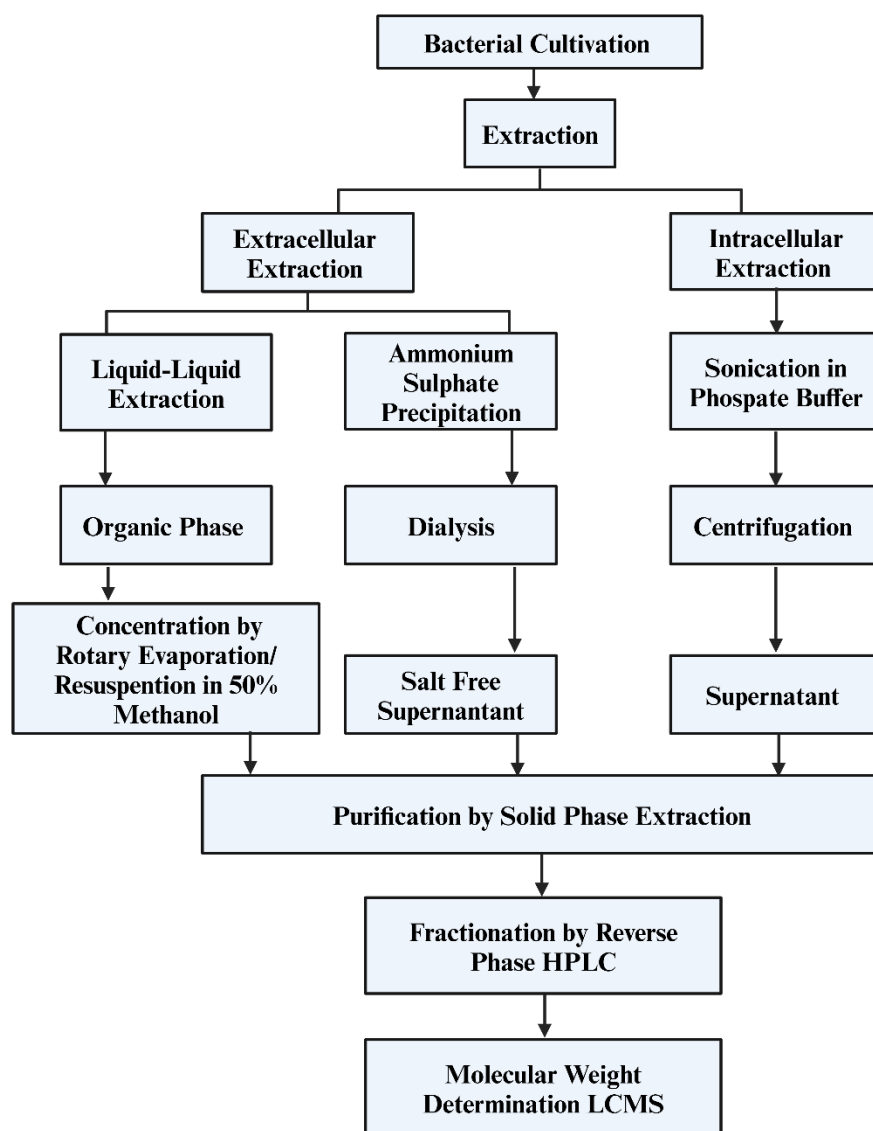


Figure 6. Typical Procedure for Isolation and Purification of AMPs

2.8 Characterization of Antimicrobial Peptides using Spectroscopic Techniques

2.8.1 Fourier Transform Infrared (IR) Spectroscopy (FTIR)

Many chemical and inorganic substances, including proteins and peptides, can be structurally characterized using FTIR [169]. FTIR works by exposing a sample to infrared radiation and determining which wavelengths of the infrared spectrum are absorbed by the sample. Details like the chemical characteristics of a molecule's functional groups, structural conformations, and H-bonding patterns can be gleaned from the infrared spectrum because different structural and functional groups of biomolecules have distinct chemical compositions and are absorbed at a particular frequency [170]. Nine distinct IR absorption bands are produced by a polypeptide chain, including amides A, B, and I–VII (**Table 2**). Of these, the amide I and amide II modes have been the subject of the most in-depth analysis due to their remarkable sensitivity to the secondary structure (**Figure 8**).

Table 2. Characteristic Infrared Bands of Peptide Linkage

Designation	Approximate wavenumber (cm ⁻¹)	Description
Amide A	3300-3270	NH stretching
Amide B	3030-3100	NH stretching, NH bending
Amide I	1600-1700	C=O stretching
Amide II	1480-1600	CN stretching
Amide III	1400-1200	CN stretching
Amide IV	625-767	OCN Bending
Amide V	640-800	Out-of-plane NH bending
Amide VI	537-606	Out-of-plane C=O bending
Amide VII	200	Skeleton torsion

Data extracted from Jilie Kong and Shaoning [171]

The amide modes are delocalized vibrational modes that are influenced by different amide group chemical connections. The most helpful absorbance band for determining the structural makeup of proteins and peptides is by far the amide I mode. It is produced mostly by the C=O stretching vibration, together with CN and CCN out-of-plane bending vibrations, and to a lesser extent by the NH in-plane bending mode. It takes place between 1,700 and 1,600 cm⁻¹. The resultant amide I oscillators are connected to one another through Covalent bonds, H-bonding, and space. These methods vary depending on the secondary structure [171].

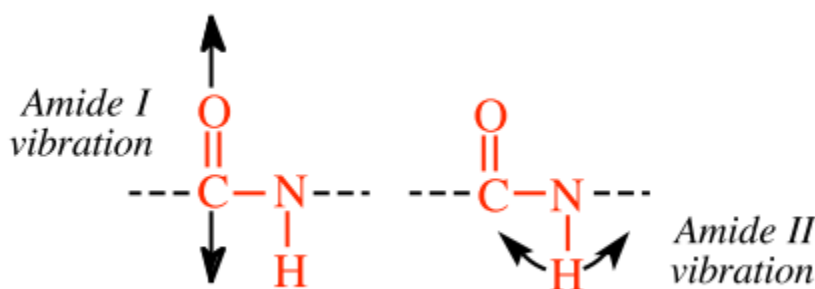


Figure 7. The Amide I and Amide II Bands in the Infrared Spectra of Proteins and Polypeptides. Carbonyl stretching vibrations are responsible for the Amide I band. While NH bending vibrations are responsible for the Amide II band [172].

Each kind of secondary structure in the amide I region ($1700\text{--}1600\text{ cm}^{-1}$) results in a somewhat different C=O stretching frequency because of its distinct hydrogen bonding pattern and molecular geometry. The Amide I band's shifts are small in relation to the band's inherent width, which makes it challenging to analyze the band for secondary structure [171]. For each sort of secondary structure, there is just one large lumpy peak seen rather than several well-resolved peaks. Proteins or polypeptides' observed amide I band contours are made up of overlapping component bands that depict random structures, α -helices, β -sheets, and twists (**Table 3**). To improve the apparent resolution of the Amide I band and enable estimations of the secondary structure content, mathematical techniques such as the Fourier self-deconvolution method (curve fitting), which applies the Gaussian or Lorentzian line shapes, or a linear combination of the two, are used. The second derivative spectra, whose downward peaks identify the amide I components, can be used to determine the quantity and positions of amide I band components. But for clarity, the inverted second derivative spectra are frequently employed [173].

Table 3. Characteristic Amide I Wavenumber of Various Secondary Structures in H₂O

Secondary Structure	Wavenumber (cm ⁻¹)
α -Helix	1,658-1,647
α_{11} -Helix	1,666-1,658
α -Helical coiled coil	1,640-1,630
3_{10} -Helix	1,665-1,655
3_1 -Helix	1,639
β -Helix	1,640-1,630
Parallel β -sheet	1,638-1,632
Antiparallel β -sheet	1,638-1,632 (strong) and 1,695-1,675 (weak)
Intermolecular (aggregated) β -sheet	1,627-1,615
α -Pleated Sheet	1,653-1,650
β -Turns	1,685-1,655
γ -Turns	1,690-1,650
Irregular (“unordered”) structure	1,660-1,652

Data extracted from Suren A. Tatulian [173]

2.8.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

One effective technique used for determining the structure of biomolecules like peptides is NMR spectroscopy. It is also a helpful technique for chemically characterizing a material. Fortunately, the primary components of biomolecules—hydrogen, nitrogen, carbon, phosphorus, and oxygen—have at least one stable, NMR-active isotope. It is based on the measurement of nuclei with net spin. Most literature on NMR of biomolecules has been concerned with one-dimensional NMR, but with advancements, more complex multidimensional experiments are being conducted [174]. In the fundamental one-dimensional (1D) pulsed NMR experiment, the sample is placed in a powerful magnetic field, radiofrequency radiation is pulsed through it, and the system's reaction is recorded.

One-dimensional NMR, such as the ^1H NMR, gives information about the number of hydrogens within a compound, their connection, and their chemical environment (chemical shifts). It is measured in parts per million (ppm) and ranges from 0-15 ppm [175]. Protons in different chemical environments (i.e., near electronegative atoms or within functional groups) appear at different positions on the NMR. The chemical changes are influenced by the secondary structures of proteins and peptides. Because amide and $\text{C}\alpha$ proton resonances have unique chemical shift patterns, it is possible to identify secondary structures from the ^1H spectrum [176].

The hydrogen atom may be affected by shielding or deshielding. Deshielding occurs when electron density around a nucleus is reduced, exposing it more to the external magnetic field. Electronegative atoms such as oxygen, nitrogen, and halides withdraw electron density, causing deshielding. Deshielded nucleus appears downfield in the spectrum. Meanwhile, shielding occurs when the electron density around a nucleus reduces the strength of the external magnetic field. Because the nucleus requires a lower radiofrequency, it appears at a lower chemical shift [177]

^{13}C is are commonly used isotope in the investigation of peptide and protein structure. They have a large chemical shift ranging from 0-220 ppm. Deshielded carbons are detected at greater ppm, while shielded carbons are found at lower chemical shift values, like the proton NMR. The ^{13}C signals help in identifying certain classes or symmetries in molecules. Additional characteristics such as the existence of aromatic systems, double or triple bonds, or $\text{C}=\text{O}$ groups, can also be recognized (**Figure 9**) [178].

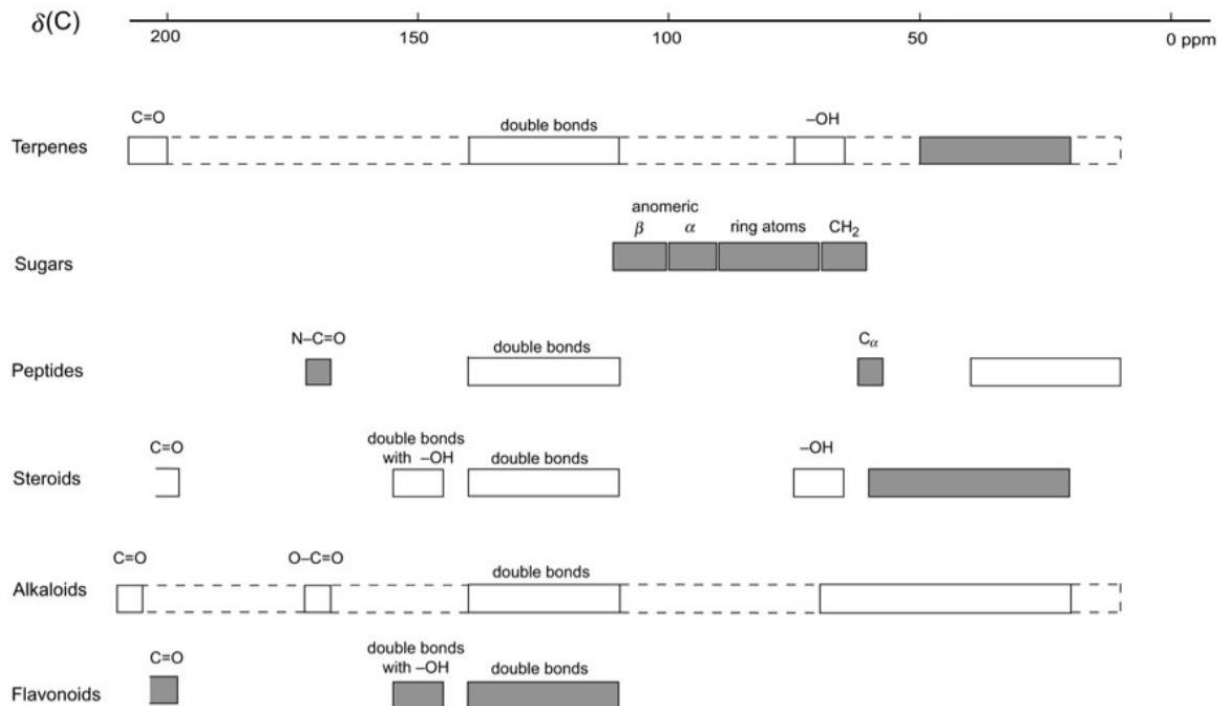


Figure 8. Typical ^{13}C NMR Chemical Shift Ranges for Different Natural Product Classes.

Areas with filled bars are those where signals are constantly detected; unfilled bars indicate areas where extra signals are frequently encountered [179].

2.8.3 Mass Spectrometry (MS)

Mass spectrometry is a highly sensitive and precise technique for determining the molecular masses of various molecules, including peptides, even in trace amounts. The mass of the peptide can be precisely determined using methods like matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) mass spectrometry (MS) following the detection of an active AMP with antimicrobial properties and confirmation of its proteinaceous nature through separation techniques like gel electrophoresis (SDS-PAGE) or reversed-phase chromatography [180]. Three basic parts are shared by all mass spectrometers: an ion source, a mass analyzer, and an ion detector. The ion source ionizes the analyte and transfers it to the gas phase, allowing it to enter the mass spectrometer. The mass analyzer then separates the generated ions based on their mass-to-charge ratio (m/z). Finally, the ion detector records the signals, which are processed by a computer for analysis. Ionization occurs through mechanisms such as electron capture, electron ejection, protonation, deprotonation, or cationization, leading to the formation of charged analyte ions.

To determine the peptide sequence, tandem mass spectrometry (MS/MS) is used, which fragments the peptide and analyzes the resulting ions. Ion traps (IT), triple quadrupole (QqQ), hybrid quadrupole/time-of-flight (QqTOF), and time-of-flight/time-of-flight (TOF/TOF) analyzers are essential parts of MS/MS equipment, and they all provide great sensitivity and accuracy for peptide identification. Because of their great sensitivity and mass accuracy, hybrid instruments offer particularly good access to the peptide sequences. MS/MS fragments peptides by breaking peptide bonds through collision-induced dissociation (CID), also known as collision-activated dissociation (CAD). This process generates b-ions (N-terminal fragments) and y-ions (C-terminal fragments), allowing researchers to reconstruct the peptide sequence by analyzing the fragment patterns[180].

One limitation of peptide sequencing via fragmentation is that if the peptides are not found in a database, researchers must perform de novo sequencing, which is generally limited to peptides 10–25 amino acids long. Larger peptides must be enzymatically digested into smaller fragments before analysis. Additionally, this method can be inaccurate due to challenges such as isobaric amino acids (e.g., leucine and isoleucine, which have the same mass) or overlapping ion series, making spectral interpretation more complex. To verify the correctness of the peptide sequence, the MS/MS spectra of the synthetically produced peptide are compared with the MS/MS spectra of the isolated peptide. If the spectra match, the sequence is considered valid [181].

2.9 Bioinformatics Analysis

The traditional method of detecting AMP has involved assessing its biological activity using chemical isolation, substantial purification procedures, and comprehensive testing of the producer strain for its antimicrobial activity. However, this method is particularly costly in terms of resources, expertise, and time. Additionally, experimental condition constraints may generate false results, thereby reducing the accuracy of screening [182]. A complementary method that can help to determine the genetic source of an observed antimicrobial activity and, consequently, identify the related chemical structure of the AMPs is by utilizing genome mining web servers to search genomes for secondary/specialized metabolites (SM) biosynthetic gene clusters (BGCs) [183].

BAGEL (Bacteriocin Genome mining tool) (<http://bagel4.molgenrug.nl/>), is an AMP web mining server that helps identify RiPPs and their biosynthetic gene clusters. It utilizes a knowledge database founded on the observation that accessory genes, which encode proteins necessary for

processing, modification, transport, regulation, and immunity, are often located near AMP genes. To use BAGEL, FASTA nucleotide sequences are required as input [184].

To identify an AMP gene cluster, the software first conducts a six-frame translation. Next, it screens hypothetical proteins for the presence of motifs associated with modification enzymes specific to the defined RiPP classes. Based on the results from this second step, the software then identifies regions of interest within the genome. Finally, these areas are thoroughly examined in the last stage using GLIMMER [185] and annotated using the UniRef database and BLAST [186]. The core peptide is identified based on predicted qualities for peptides of the specified class or through homology to known core peptides [187].

Currently, AntiSMASH (Antibiotic and Secondary Metabolite Analysis Shell) (<https://antismash.secondarymetabolites.org/>) is the most popular tool for identifying and characterizing biosynthetic gene clusters in bacteria, fungi, and archaea[188]. Apart from its application in RiPP discovery, it facilitates the mining of chemicals that are not ribosomally encoded. Compared to BAGEL, AntiSMASH supports prokaryotic and eukaryotic DNA input and generates a more comprehensive output [187].

Chapter 3

Materials and methods

3.1. Growth media and cultivation conditions

To cultivate the thermophilic isolates, Thermus (TT) medium (ATCC medium 697) containing 8g/L tryptone, 14g/L agar, 4g/L yeast extract, and 2g/L sodium chloride at a final pH of 7.0 was used. For the batch production of antimicrobials (AMP) from thermophilic bacteria, Thermus broth (without agar) maintained at a final pH of 7.0 was used. For routine cultivation of test strains (*Staphylococcus aureus* as Gram-positive and *Escherichia coli* as Gram-negative bacteria) Scharlau® nutrient broth (NB) (containing 2g/L yeast extract, 1.0g/L meat extract, 5g/L peptone, and 5g/L sodium chloride at a final pH 7.5) and Microgen® nutrient agar (NA) medium (containing 5g/L peptic digest of animal tissue, 5g/L of sodium chloride, 1.5g/L beef extract, 1.5g/L yeast extract, and 15g/L of bacteriological agar at a final pH of 7.5) were used. Test stains were cultivated aerobically at 37°C, while thermophiles were cultivated at 50°C with aeration. To preserve ideal humidity and keep the medium from drying out, thermophilic bacteria cultivated on agar plates were enclosed in plastic bags. All bacterial strains utilized in this study were obtained from the Bioengineering Laboratory at KFUPM.

3.2 Preliminary Screening of Thermophilic Bacterial Strains for Antimicrobial Activity

3.2.1 Test Strain Inoculums Preparation

To evaluate the antimicrobial activity of the thermophilic bacterial isolates, stock cultures of the test strains were streaked onto the surface of NA plates and incubated at 37°C for 24 hours aerobically. Colonies of each respective strain were suspended in sterile saline solution (0.85% v/w NaCl) and used to seed pre-warmed NA at approximately 50°C, adjusted to an optical density of approximately 0.125 [189].

3.2.2 Agar-deferred Spot Method

Thirty-nine thermophilic bacterial strains, previously isolated from the hot spring, “Al-Ain Alhara” located in the southeastern region of Jazan City, Saudi Arabia (16°56'N, 43°15'E), were evaluated for their potential to produce antimicrobial agents by employing the agar-deferred spot

assay. A single colony from an overnight culture of each thermophilic isolate was spot inoculated onto the center of a freshly prepared Thermus (TT) agar plate and incubated at 50°C for 24 hours. Following incubation, the plates were overlaid with prewarmed NA seeded with the test strain and incubated at 37°C for 18-24 hours. The antimicrobial activity of each strain was assessed by recording the presence or absence of inhibition zones.

3.3 Sensitivity of Antimicrobial Substance to Proteolysis by Proteinase K

To confirm that the antimicrobial agents produced by the thermophilic bacterial strains are protein-based, Strains demonstrating activity against *S. aureus* and *E. coli* were selected for further analysis. These strains were spot inoculated onto freshly prepared agar plates and incubated overnight at 50°C. The resulting cells were treated with 3 µL of proteinase K solution (2 mg/mL) and incubated at 37 °C for 2 hours. After incubation, prewarmed agar seeded with the test strain was poured over the plates, which were subsequently incubated overnight at 37°C. Thermophilic bacteria not treated with proteinase K were used as a control.

The zone of inhibition around the spotted thermophilic bacterial strains was recorded. For further study, four strains (*Geobacillus stearothermophilus* (S9), *Bacillus licheniformis* (S12), *Anoxybacillus flavithermus* AK1 (S13) and *Brevibacillus borstelensis* AK1 (S43)) exhibiting the highest inhibition against both test organisms, but no inhibitory activity after treatment with proteinase K were selected.

3.4 Physiological Characterization of Selected Thermophilic Strains

The physiological characteristics of the selected antimicrobial-producing strains (*Geobacillus stearothermophilus* (S9), *Bacillus licheniformis* (S12), *Anoxybacillus flavithermus* AK1 (S13), and *Brevibacillus borstelensis* AK1 (S43)) were evaluated to determine their growth dependence on varying environmental conditions. These conditions included pH levels ranging from 5 to 10, temperatures from 35°C to 70°C, and NaCl concentrations between 0% and 0.8%.

3.5 Growth Kinetics and Crude Antimicrobial Metabolite Production by Selected Thermophilic Strains

A single colony from each of the four selected antimicrobial-producing bacterial strains was inoculated into separate 50 mL centrifuge tubes containing 10 mL of standard TT broth (supplemented with 2% (w/v) NaCl at pH 7.0). These cultures were incubated in a shaker at 100

rpm and 50°C until they reached a final optical density OD_{600nm} of 0.1 (8×10^7 cells/mL). Subsequently, 2 mL of each culture was transferred into separate 500 mL Erlenmeyer flasks containing 200 mL of TT broth, and the flasks were incubated under the same conditions.

Growth curves of the selected strains were plotted based on OD_{600nm} values. Briefly, the absorbance of 1 mL liquid culture was measured every two hours over a 2–24-hour period. The cultures were centrifuged at 6000 rpm for 15 minutes at 4°C to obtain cell-free supernatants. These supernatants were then tested for antimicrobial activity against the test strains using the agar well diffusion method. Zones of inhibition were observed on all plates. Additionally, supernatants collected after extended incubation periods of 48 and 72 hours were also assessed for antimicrobial activity.

3.6 Production of the Antimicrobial Peptide by Cell immobilization Technique

Cell immobilization technique was carried based on the method described by Alkhalili *et al.*, 2016 with minor modification. Cell pellet harvested from culture broth were suspended into 10mL of fresh sterile TT broth and mixed with 20ml of 3% (w/v) prewarmed agar at approximately 50°C. The mixture was collected using sterile syringe and added dropwise onto 10ml of sunflower oil to form agar beads with diameter of approximately 5mm. The produced beads were properly rinsed in sterile distilled water and transferred into a 500 mL Erlenmeyer flask containing 200mL of sterile TT broth. This was followed by incubating in an incubator shaker at 50°C and 100rpm for 24hours. After incubation, the broth was carefully poured into sterile centrifuge tubes to separate them from the beads. The broth for each sample was centrifuged at 6000rpm for 30 minutes at 4°C. The cell free supernatants (CFS) obtained after centrifugation were tested for antibacterial activity using agar well diffusion assay (**Figure 10**).

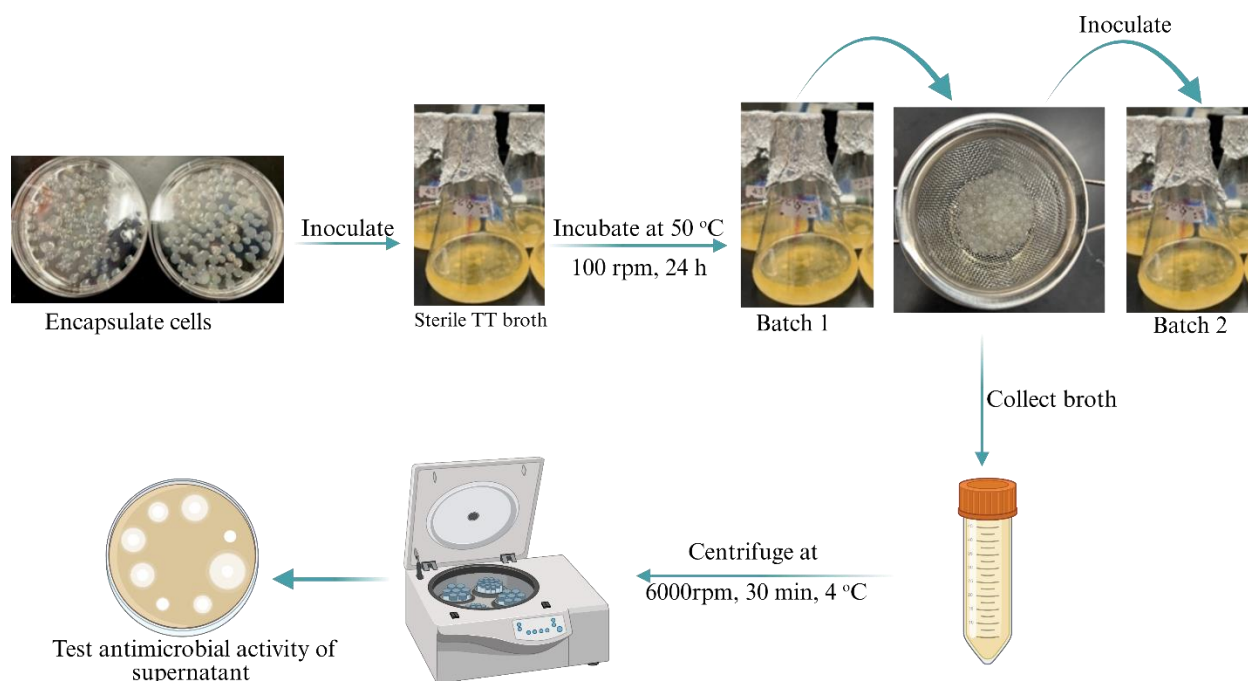


Figure 9. Schematic Illustration of the Steps Involved in the Sequential Batch Cultivation of Immobilized Cells to Produce an Antimicrobial Substance

3.7 Evaluation of Antimicrobial Activity Using Culture Broth Containing Selected Thermophilic Strains

The inhibitory activity of the selected thermophilic strains was evaluated using the agar well diffusion method. A single colony of test strain *S. aureus* was grown overnight at 37°C (100 rpm) in 10 mL NB. 500µl of the culture was inoculated into 500 ml molten NA (at approximately 50°C) and poured into sterile plates. After agar solidification, wells of approximately 8 mm in diameter were created. Next, 10 mL of TT broth containing thermophilic strains was added to the wells to determine if antimicrobial activity occurred only in the presence of competing strains. The cell-free supernatant of thermophilic strains and sterile TT broth were used as a control. After an incubation period of 18 hours, plates were analyzed for the inhibition zone.

3.8 Production of Antimicrobial Peptides by the Intracellular Extraction Technique.

Freshly obtained S9, S12, S13, and S43 cell pellets were collected and suspended in 25 mL of 20 mM phosphate buffer (pH 6.8). Cells were disrupted via sonication on ice for 5 minutes (at 2s on, 7s off, 20% amplitude) using a Q55 ultrasonic homogenizer. The cell lysate from each strain was

centrifuged at 6000 rpm for 20 min at 4°C to remove the cell debris and obtain a CFS. The antimicrobial activity of the CFS was confirmed by agar well diffusion assay. To selectively recover AMPs, the CFS was extracted thrice with an equal volume of ethyl acetate. After separation, the organic phase was concentrated using a rotary evaporator. Concentrated extracts were redissolved in 5 mL of deionized water and referred to as antimicrobial purified extract (APE) (**Figure 13**). The antimicrobial activity of the APE was confirmed by agar well diffusion assay and turbidimetric assay as explained in section 3.9, with *S. aureus* as the test strain. The APE was also utilized for further analysis.

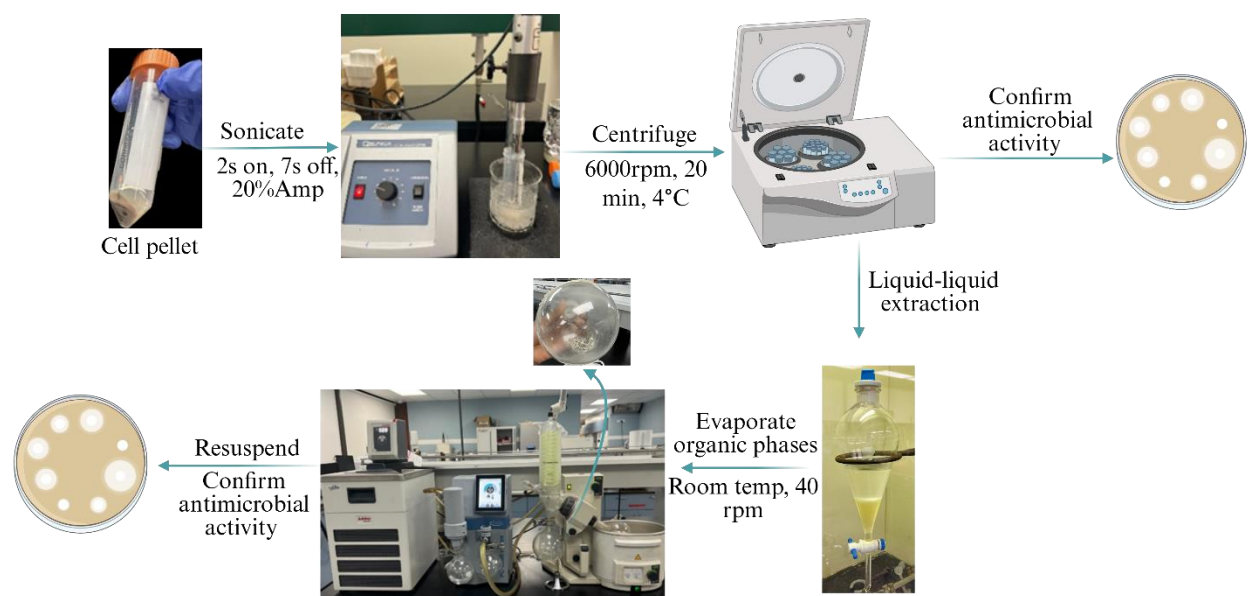


Figure 10. Schematic illustration describing the production of antimicrobial peptides by Intracellular extraction technique.

3.9 Turbidimetric assay

To calculate the percentage inhibition of microbial growth caused by APE, 100µL of APE from S9, S12, S13, and S43 were added into separate tubes containing 500µL of *S. aureus* at OD₆₀₀ 0.5. Samples containing 100µL TT broth instead of APE were used as controls. The samples were incubated in a shaker (100 rpm, 37 °C) for 20 h. Absorbance OD₆₀₀ of the samples was measured after incubation. Percentage Inhibition was calculated as $((OD_{Control} - OD_{Control}) \div OD_{Control}) \times 100$. All experiments were conducted in triplicate.

3.10 Stability of Crude Antimicrobial Extract to Temperature

To analyze the stability of the APE, aliquots of the crude extract were exposed to different temperatures, specifically 40, 50, 60, and 70 °C for 30 min. The APE that received no temperature treatment was used as a control. After treatment with the desired temperature, the APE was cooled to room temperature and tested for inhibitory activity against *S. aureus* and *E. coli* by turbidometry assay.

3.11 Characterization of Antimicrobial Compound

3.11.1 Spectrophotometric Analysis by Ultra-High-Performance Liquid Chromatography (UPLC)

The presence of peptides in the APE was confirmed using UPLC (waters® ACQUITY UPLC® H-Class) system equipped with a photodiode array detector (PDA). Five microliters of pre-concentrated APE from S9, S12, S13, and S43 were loaded into the C18 column (1.7µm, 2.1 × 150mm). The mobile phase was an isocratic system composed of 50% solvent A (ultrapure water containing 0.1% TFA (v/v)) and 50% solvent B (acetonitrile containing 0.1% TFA (v/v)) over 15 minutes and a flow rate of 0.08 mL/min. Peaks were detected at wavelengths 210-400nm.

3.11.2 Fourier Transform Infrared (FTIR) Spectroscopy

A disk-shaped pellet was prepared by pressing 500 mg of KBr. The APE containing AMP was then applied to the pellet's surface and allowed to absorb. A KBr disk absorbed with 50% methanol served as the blank for analysis, with its spectrum subtracted from the background. FTIR was carried out using a ThermoScientific® Nicolet Is10 FT-IR spectrometer in the range of 4000-400 cm⁻¹ at 4 cm⁻¹ resolution, using deuterated triglycine sulfate (DTGS) as a detector. A total scan of 120 spectra was recorded and averaged. Spectral processing and secondary structure determination were carried out by analyzing the band intensities in the second derivative of the amide I spectra.

3.11.3 Nuclear magnetic resonance (NMR)

For each of the four APE samples obtained from the thermophilic strain, 70 µl APE was dissolved in 630 µl of deuterated DMSO. The sample was transferred into a clean, air-bubble-free NMR tube. Proton (¹H) and carbon (¹³C) NMR spectra were collected on a JEOL high-performance 600

MHz spectrometer. The NMR data were processed using Joel Delta NMR processing and control software v5.3.3.

3.12 Peptide Fractionation

Preconcentrated APE obtained from selected bacteria were all fractionated by column chromatography at room temperature using a 25ml chromatography column packed with 10g C18, equilibrated at room temperature with 10ml of 75:25 solvent A (ultrapure water containing 0.1% TFA (v/v)) and solvent B (acetonitrile containing 0.1% TFA (v/v)). Elution was done under an isocratic gradient of 50% A and 50% B, followed by a final elution with 100% solvent B. Seven fractions were collected manually from each sample. The fractions were lyophilized, reconstituted in deionized water, and used in an antimicrobial activity assay. Fractions with antimicrobial activity against *S. aureus* were resubmitted for a second fractionation by UPLC to determine their purity.

3.13 LC-ESI-MS Analysis of active Peptide fractions

Ten microliters of active antimicrobial fractions were analyzed in a PAL HPLC-tx System equipped with a C18 analytical column (75mm I.D. × 100 mm; ACQUA 5 mm, Phenomenex Inc.) at a flow rate of 0.8 mL/min. the system was coupled to a mass spectrometer fitted with an ESI source in the positive ion mode. Spray voltage was at 2kV, capillary temperature was set at 200°C. A Fourier Transform MS analyzer operating in full scan mode was used to obtain MS spectra in the 50–2000 m/z range and a resolution of 60,000 at 400 m/z.

3.14 In Silico Identification of Bioactive Gene Clusters in *Brevibacillus borstelensis* AK1 and *Anoxybacillus flavithermus* AK1

The discovery of bacteriocin gene clusters of antimicrobial compounds in *Brevibacillus borstelensis* AK1 (APBN01000001.1) and *Anoxybacillus flavithermus* (AK1 APCD01000001 to APCD01000050) was carried out using BAGEL4. The DNA nucleotide sequence was uploaded in FASTA format as input. Two distinct methods were used to evaluate the input DNA sequences in parallel: mining based on the bacteriocin or RiPP gene and mining directly by Glimmer using a precursor (structural gene). The output was visualized on an HTML page, displaying classified putative bacteriocins, gene cluster graphics, ORF annotations, and detailed bacteriocin information, including BLAST hits and pI values. A detailed prediction of the gene clusters of

NRPs, polyketides, and other antimicrobials was carried out using antiSMASH v.7.0 web server. The Refseq accession number was used as the input in the case of *Brevibacillus borstelensis* AK1, while 50 FASTA sequence fragments for *Anoxybacillus flavithermus* AK1 were used as an input. The predicted core structure, gene cluster description, annotation, percentage of gene homology with known gene clusters, and published genome sequence were all acquired, along with the NRPS/PKS domains necessary for the manufacture of the PKSs and NRPSs' core chemical structure.

Chapter 4

Thesis Results

4.1 Antimicrobial Activity Assessment of Thermophilic Bacterial Strains

Thirty-nine thermophilic bacterial strains were obtained from the Bioengineering Laboratory at KFUPM. The ability of these strains to inhibit the growth of *S. aureus* and *E. coli* was confirmed using agar-deferred spot assay. Out of the strains tested, 25 strains inhibited the growth of *E. coli*, while 24 strains inhibited the growth of *S. aureus* (**Table 4**). The inhibition zone of the thermophiles developed against *S. aureus* was more prominent than that developed against *E. coli* (**Figure 11(a)-(d)**). The antimicrobial activity disappeared after treatment with proteinase K, indicating the proteinaceous nature of the secreted antimicrobial agent (**Figure 11(e)-(h)**). Based on the result obtained, four highly promising isolates S9 (*Geobacillus stearothermophilus*), S12 (*Bacillus Licheniformis*), S13 (*Anoxybacillus flavithermus* AK1), and S43 (*Brevibacillus borstelensis* AK1), were selected for further analysis.

Table 4. Antimicrobial Activity of Selected Thermophilic Bacteria Isolated Against Indicator Bacterial Strains

Thermophilic bacterial number	Strain	Zone of inhibitions shown by thermophilic bacterial isolates against test strains (mm)	Zone of inhibitions shown by thermophilic bacterial isolates against test strains after treatment with proteinase K (mm)	
			<i>E. coli</i>	<i>S. aureus</i>
2		14.00	18.00	2.00
3		15.00	16.00	0.00
4		-	-	-
5		-	-	-
6		14.00	17.00	1.00
7		15.00	17.00	2.00
*9		14.00	20.00	0.00
10		-	-	-
11		15.00	15.00	0.00
*12		20.00	13.00	2.00
*13		16.00	20.00	1.00
14		17.00	23.00	2.00
16		-	-	-
17		10.00	13.00	0.00
18		15.00	18.00	2.00
20		-	-	-
22		13.00	26	5.00
23		-	-	-
30		13.00	20.00	3.00
31		-	10.00	-
34		15.00	20.00	0.00
35		16.00	-	-
37		-	-	-
38		11.00	15.00	2.00
40		20.00	-	-
41		13.00	13.00	2.00
42		-	-	-
*43		16.00	15.00	0.00
45		-	12.00	-
47		-	-	-
49		-	-	-
51		-	-	-
52		20.00	15.00	5.00
54		14.00	15.00	0.00
55		15.00	20.00	6.00
56		15.00	20.00	1.00
57		-	-	-
58		22.00	-	-
59		13.00	22.00	0.00

Zone of inhibition ≤ 9 = absence of antimicrobial activity

Zone of inhibition > 9 = presence of antimicrobial activity

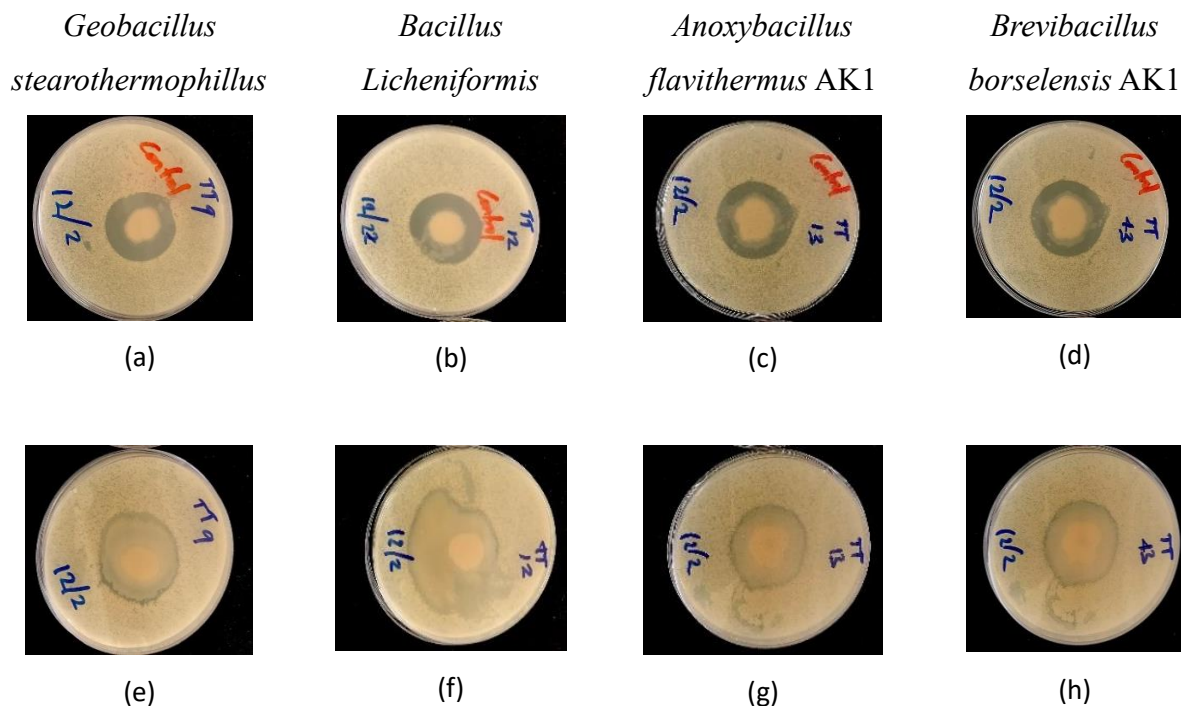


Figure 11. Antimicrobial activity of the selected thermophilic strains against *Staphylococcus aureus* (a)- (d) Before treatment with proteinase K and (e)-(h) After treatment with proteinase K.

4.2 Evaluation of Physiological Characteristics of Promising Antimicrobial-Producing Strains

Physiological characteristics of the selected strains (S9, S12, S13, and S43) were investigated to determine their optimum growth conditions, including temperature, pH, and tolerance to different NaCl concentrations. The optimal growth temperature for all strains was identified as 50°C. Among the selected strains, S12 exhibited the most pronounced growth at its optimal temperature, surpassing the others in biomass yield. Strain S9 displayed consistent growth with no significant variations across range of temperatures 45°C to 55°C. Conversely, minimal growth was observed for all strains 35°C, 40°C and 60°C (**Figure 12a**). All strains demonstrated optimal growth at a neutral pH of 7. However, they exhibited a broad tolerance for pH 5,6 and 8. Strain S43 could grow in alkaline conditions (pH 9 and 10), suggesting its tolerance to alkaline environment as well (**Figure 12b**). All strains were able to grow in salt-free media. However, the

highest growth rate for all strains was observed at 2% NaCl concentration. All strains also demonstrated tolerance to NaCl concentrations ranging from 0.4% to 1% NaCl(w/v) (**Figure 12c**).

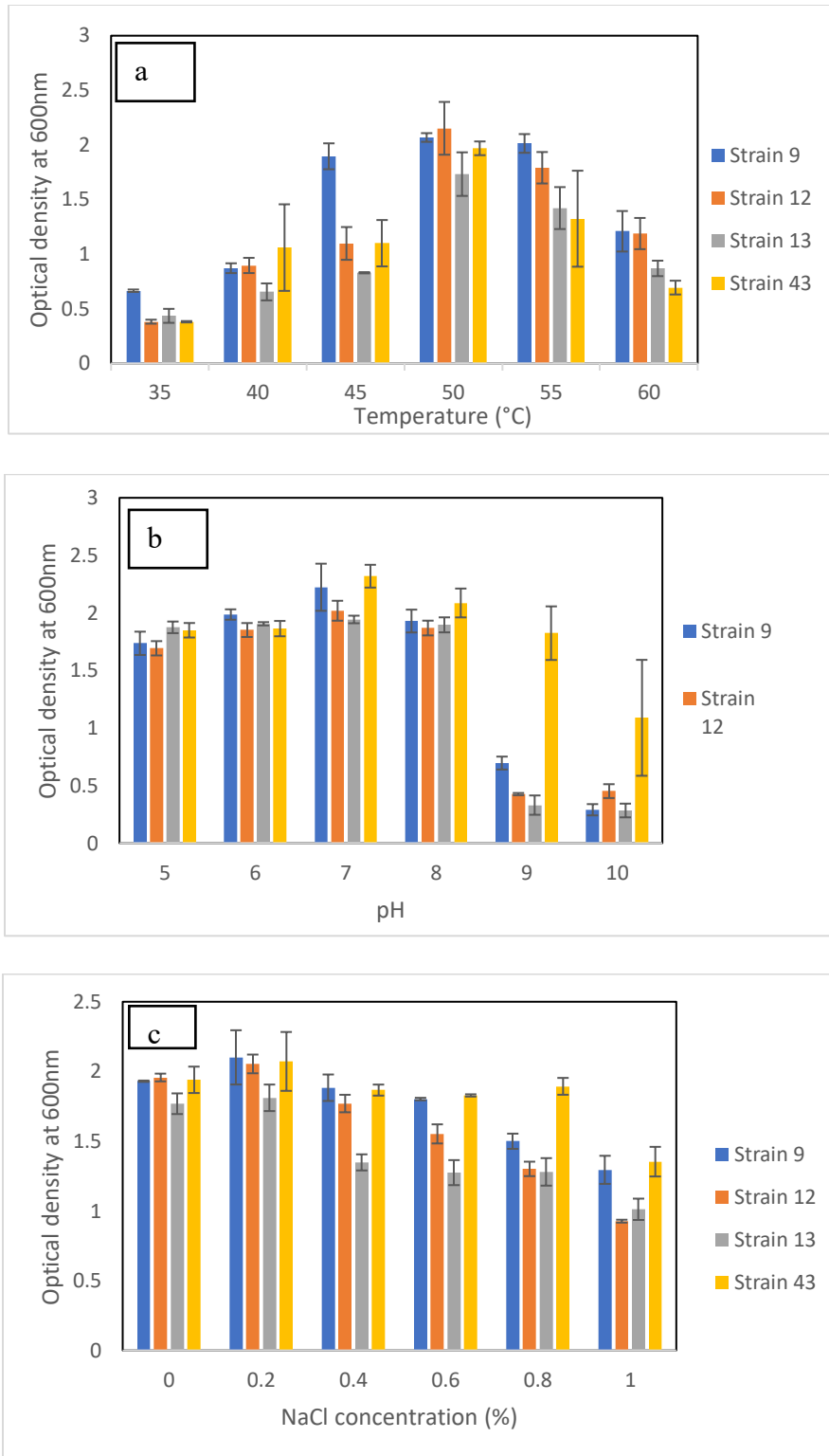


Figure 12. Optimal growth (a) Temperature, (b) pH, and (c) NaCl tolerance of the four selected strains (S9, S12, S13, and S43)

4.3 Growth Kinetics of Selected Thermophilic Strains and Their Antimicrobial Activity in Liquid Medium

Following the physiological characterization of the thermophilic strains, their growth kinetics and antimicrobial activity over time were simultaneously examined (**Figure 13**). Analysis of their growth curves revealed that all strains followed the typical bacterial growth pattern, including a lag phase, exponential (log) phase, stationary phase, and death phase. The exponential phase began approximately 4 hours after inoculation for all strains. The stationary phase was reached at around 20 hours for all strains.

Although the selected thermophilic isolates grown on solid TT agar medium exhibited strong antimicrobial activity against the test strains, their culture cell-free supernatants showed no detectable antimicrobial activity when the thermophiles were grown in TT liquid medium across all time points tested (2–24, 48, and 72 hours) (**Figure 14 e-h**). However, clear inhibition zones were observed when the culture broth (containing both cells and supernatant) collected 20 hours after incubation was co-cultured with the test strains (positive control) (**Figure 14 a-d**).

The absence of antimicrobial activity in the liquid medium led to the formulation of two hypotheses: (A) the production of antimicrobial agents is enhanced in a solid-state medium, as consistent with findings from previous studies [189], or (B) the thermophilic strains secrete antimicrobial agents only in response to the presence of competing microorganisms[190].

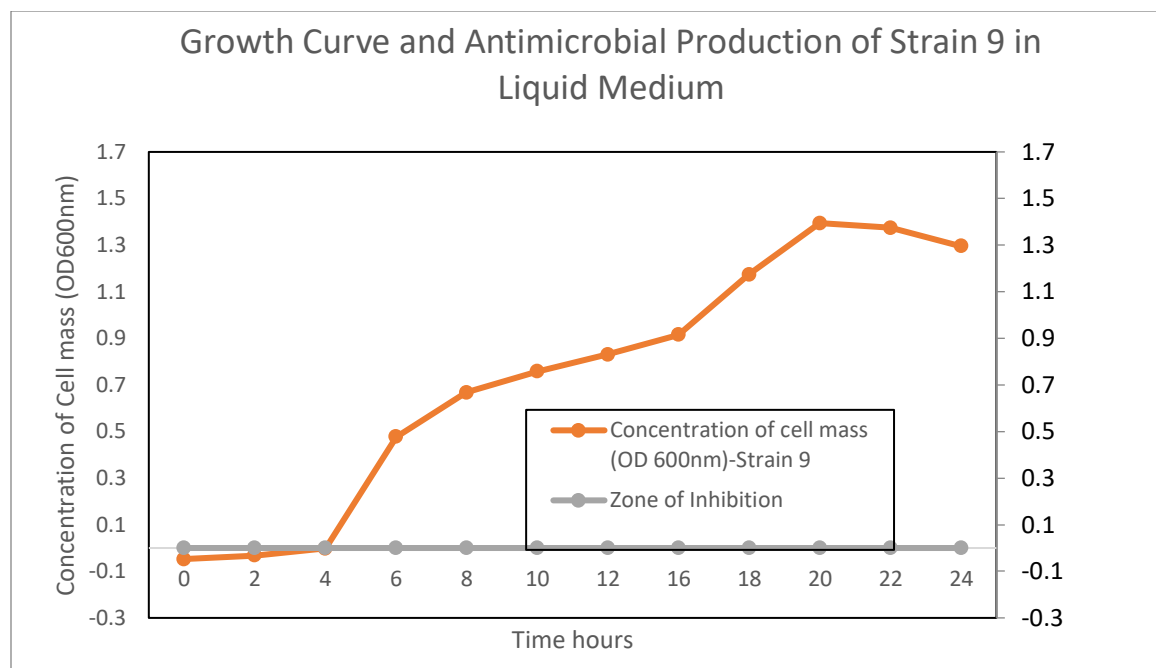


Figure 13. Growth kinetics and antimicrobial production of the strain 9. No antimicrobial activity was detected at all growth phases.

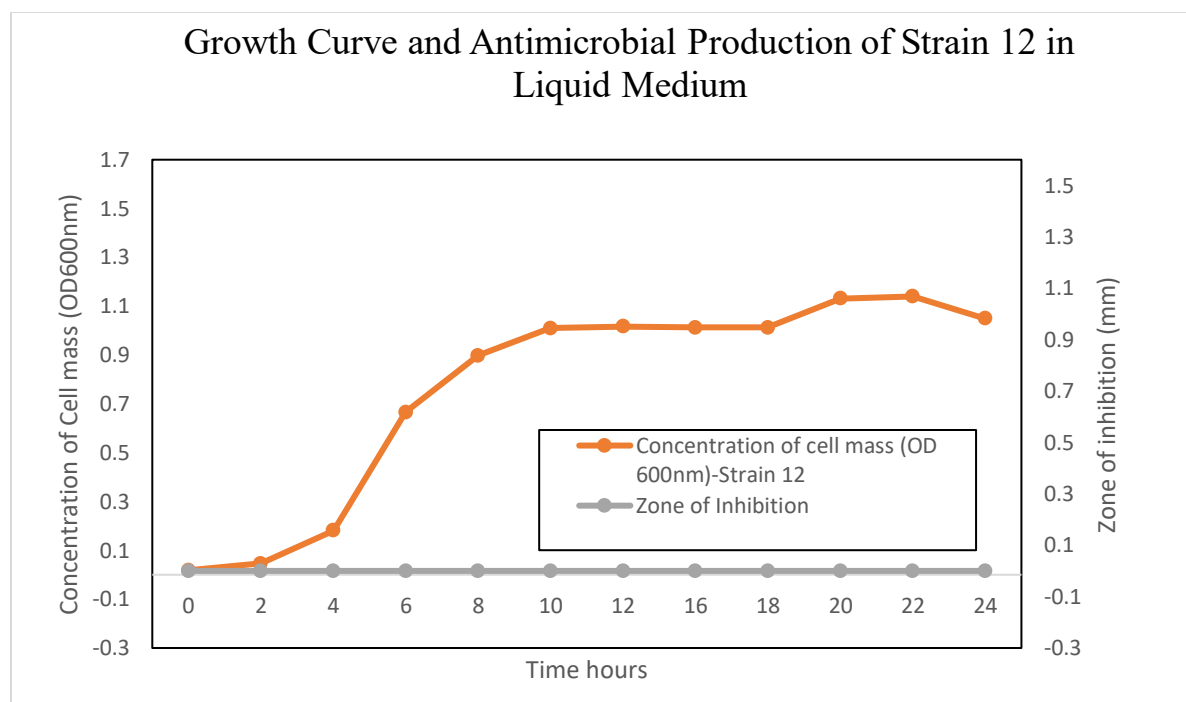


Figure 14. Growth kinetics and antimicrobial production of the strain 12. No antimicrobial activity was detected at all growth phases.

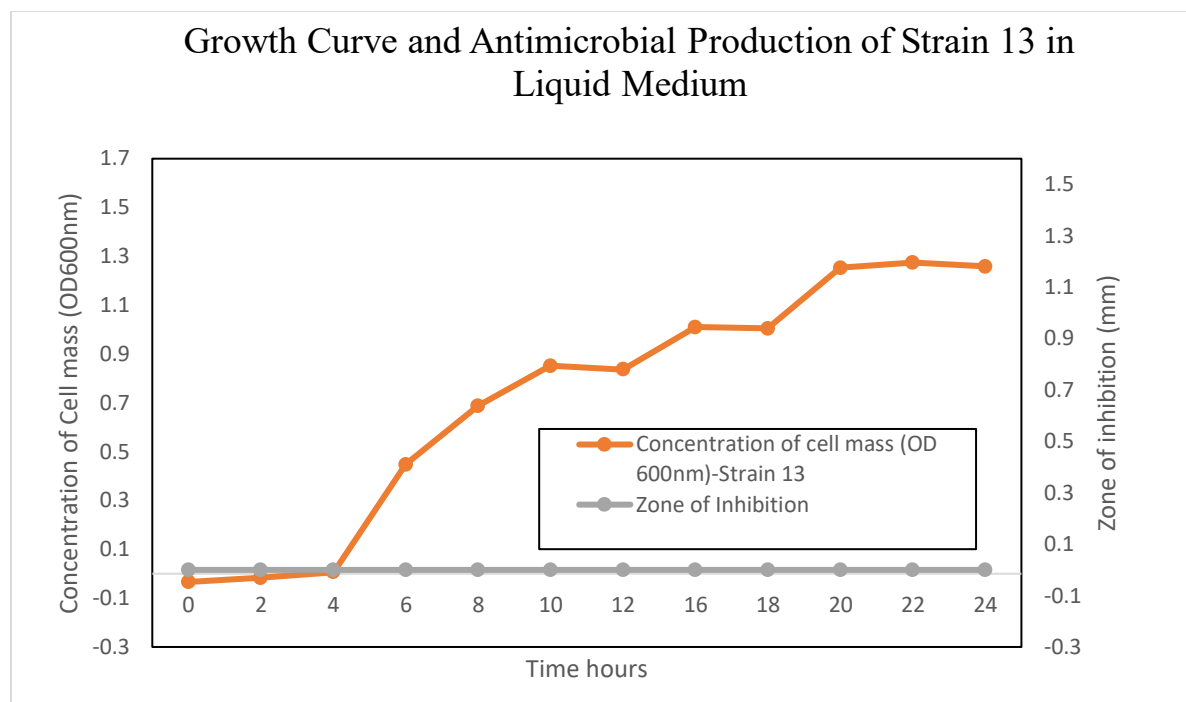


Figure 15. Growth kinetics and antimicrobial production of the strain 13. No antimicrobial activity was detected at all growth phases.

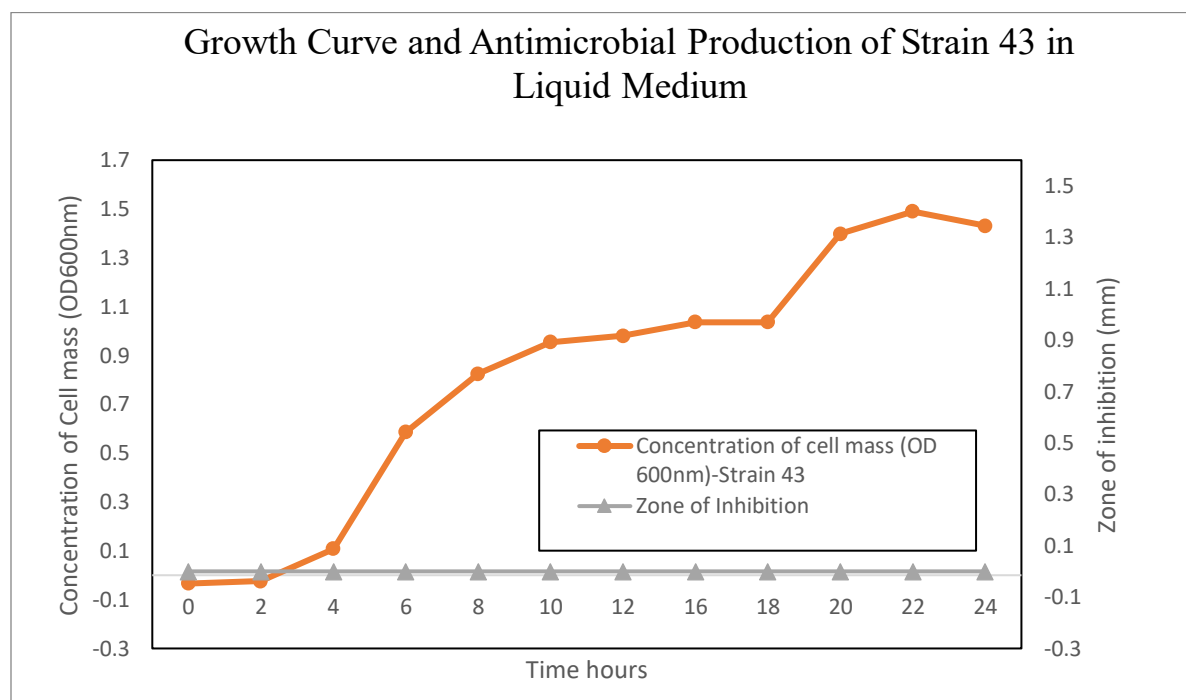


Figure 16. Growth kinetics and antimicrobial production of the strain 43. No antimicrobial activity was detected at all growth phases.

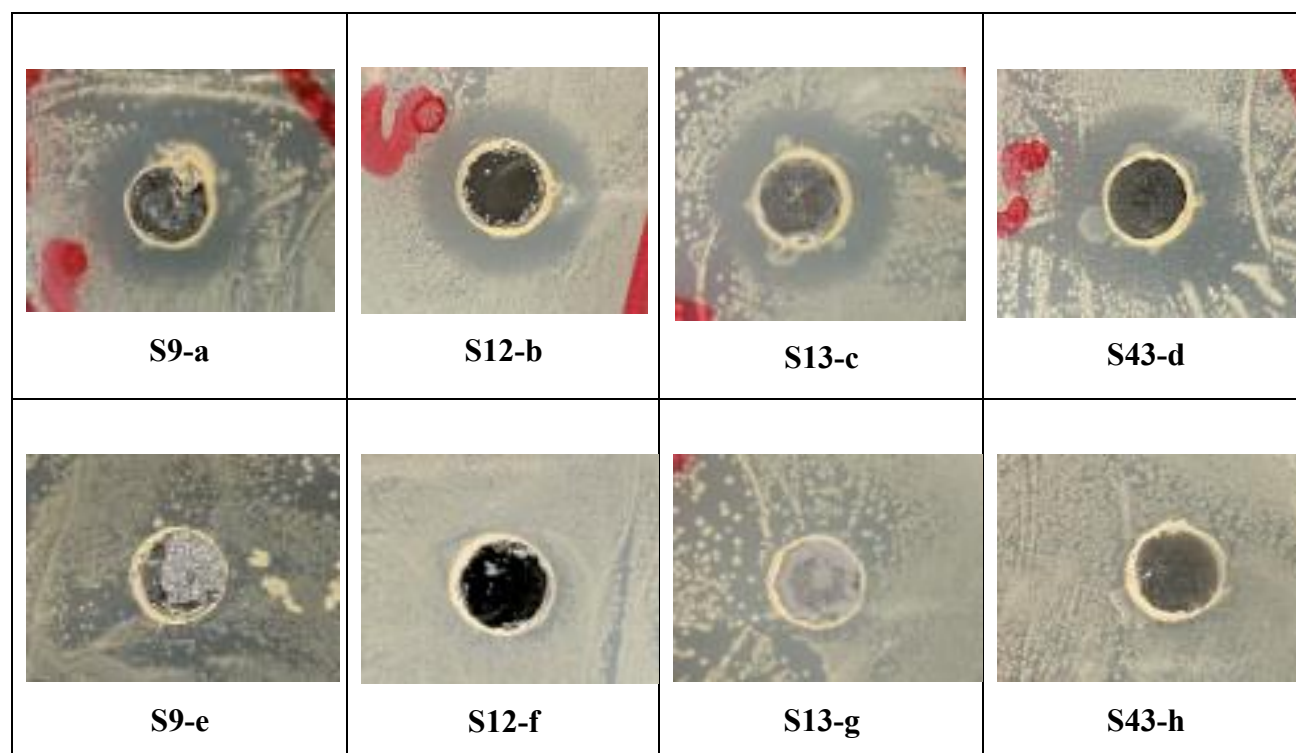


Figure 17. Growth kinetics and antimicrobial production. No antimicrobial activity was detected at any growth phase. Clear inhibition zones were observed when the culture broth (containing both cells and supernatant) collected after 20 hours of incubation was co-cultured with *S. aureus* (S9-a, S12-b, S13-c, S43-d). No detectable antimicrobial activity when treated with CFS (S9-e, S12-f, S13-g, S43-h).

4.4 Production of the Antimicrobial Peptide by Cell immobilization Technique

To verify whether antimicrobial metabolites are produced exclusively on a solid-state medium, cell immobilization technique was employed. No antimicrobial activity was produced in the first and second day of incubation. Although, activity was confirmed on the third day (**Figure 18**). This disappeared during the 4th cycle of incubation. The inability of the immobilized cells to produce antibacterial activity in the first and second cycle may be attributed to the small pore size of agar which may have restricted effective nutrient diffusion to the immobilized cells. The disappearance of antimicrobial activity in the 4th cycle may be due to the instability of agar under high temperatures. Agar melts at temperatures over 45°C. Incubation at 50°C may compromise the structural integrity of the immobilized matrix and lead to cellular leakage. Similar findings have

been reported in studies utilizing agar or gelatin (a gelling agent with properties similar to agar) to produce antimicrobial agents [191][192][193].

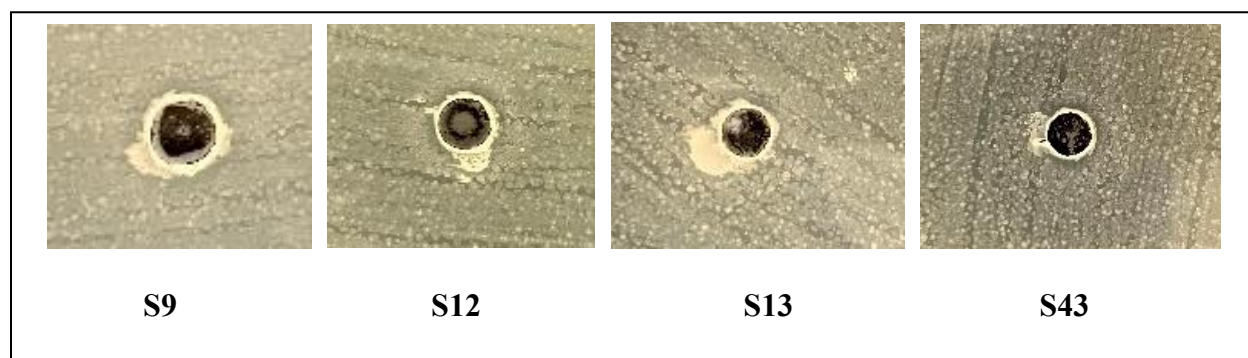


Figure 18. Antimicrobial activity of CFS obtained from broth culture of thermophilic strains (Cycle 3)

4.5 Production of Antimicrobial Peptides by Intracellular Extraction Technique

Due to the thermophilic strains' inability to efficiently produce antimicrobial agents on solid-state media, an alternative approach was adopted. In this method, antimicrobial crude extract was obtained from bacterial whole cells, following the procedure described by Kita *et al.*, 2023 and Alkotaini *et al.*, 2014 with slight modifications. The antimicrobial activity of the extracts was evaluated through agar well diffusion to assess the zone of inhibition. Because intracellular extract may contain other impurities that are not peptides, the extract was further purified by liquid-liquid extraction using ethyl acetate. The antimicrobial activity of the purified extract (APE) was confirmed by agar well diffusion and turbidimetry assay to quantify the percentage inhibition, using *S. aureus* as the test strain. The concentrated extracts obtained from all the selected strains effectively inhibited the growth of *S. aureus* (**Figure 19**).

The turbidimetric assay revealed that strain S43 exhibited the highest percentage inhibition against *S. aureus* (91.50%), while strain S12 demonstrated the lowest inhibition (66.12%) under the same conditions. The percentage inhibition values of the APEs against *S. aureus* are summarized in **Table 5**. Furthermore, S43 maintains the highest inhibition across all temperatures tested (above 84% even at 70°C), indicating the highest thermostability. S13 also retains moderate inhibition but

shows a decline at higher temperatures, suggesting it is less thermostable than S43. S9 and S12 show a more significant drop in inhibition, especially at 60°C and 70°C, indicating lower thermostability (**Table 6**).

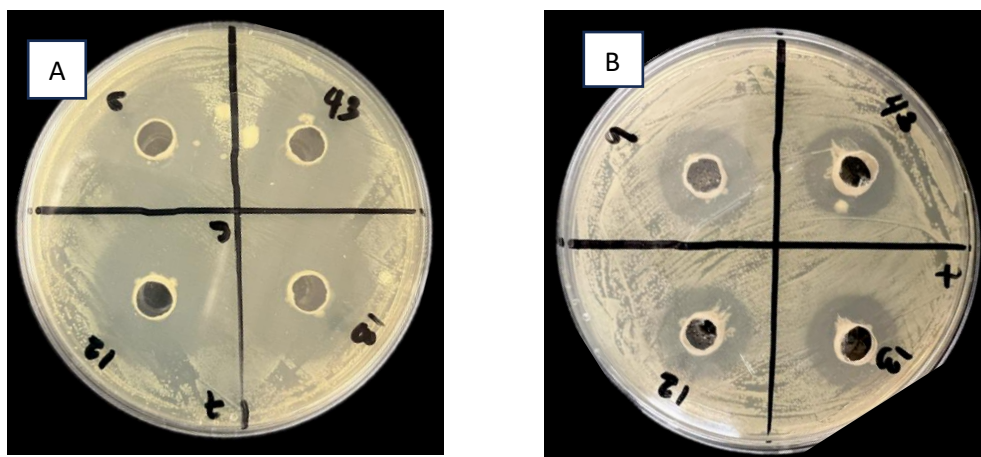


Figure 19. Inhibitory activity of thermophilic bacterial strains against *S. aureus* by agar well diffusion assay (A) Crude intracellular extract. (B) Extract after purification by liquid-liquid extraction.

Table 5. Inhibitory activity of the antimicrobial crude extract against *S. aureus* by turbidimetry assay.

Thermophilic strains	OD _{Control}	OD _{treated sample}	Inhibition (%) against <i>Staphylococcus aureus</i>
S9	1.312	0.259	80.259
S12	1.098	0.4612	57.996
S13	1.073	0.3319	69.068
S43	1.122	0.0933	91.684

%Inhibition was calculated $((OD_{Control} - OD_{treated sample}) \div OD_{Control}) \times 100$.

Table 6. Effect of Temperature on the activity of ACE against *S. aureus*

Temperature Effect	Inhibition%							
Temperature	S9		S12		S13		S43	
	OD _{treated} sample	Inhibition (%)	OD _{treated} sample	Inhibition (%)	OD _{treated} sample	Inhibition (%)	OD _{treated} sample	Inhibition (%)
40°C	0.3588	72.652	0.5106	53.497	0.3627	66.198	0.1064	90.517
50°C	0.5310	59.520	0.5962	45.701	0.3692	65.592	0.1253	88.832
60°C	0.5749	56.181	0.6349	42.177	0.5226	51.295	0.1394	87.576
70°C	0.5894	55.076	0.9482	13.643	0.6420	40.168	0.1701	84.840

4.6 Purification and Characterization of AMP

4.6.1 UV Spectrophotometric analysis by UPLC

The absorbance spectra of preconcentrated APE from the selected strains (S9, S12, S13, and S43) were measured across the wavelength range of 210–400 nm. Distinct peaks were observed for each strain: for strain S9, prominent peaks were found with retention times ranging from 4.242 to 7.333 minutes and wavelength ranges from 212.5 to 297.7 nm. For strain S12, peaks were detected with retention times ranging from 4.235 to 7.152 minutes and wavelength ranges from 212.5 to 297.7 nm. Similarly, strain S13 exhibited distinct peaks with retention times ranging from 4.267 to 6.909 minutes and wavelength ranges from 212.5 to 296.6 nm. Finally, strain S43 displayed prominent peaks with retention times ranging from 4.304 to 6.916 minutes and wavelength ranges from 217.2 to 296.6 nm. The specific characteristics of each peak, including their retention times and absorbance spectra, are summarized in **Table 11**.

For all samples, no appreciable peaks were detected between 300-400nm (**Figures 36, 38, 40 and 42**). The ultraviolet spectrum of the ACE obtained from all strains suggests that all samples contained peptides [194].

4.6.2 Functional group analysis

Fourier transform-infrared (FTIR) spectrum was recorded for the preconcentrated ACE isolated from S9, S12, S13, and S43. Analysis of the spectrum offered more evidence that the crude extract contained peptides. Prominent peaks consistent with stretching and bending vibrations of N-H,

C=O, C-H, and C-N were observed (**Figure 20**). The Characteristic absorption bands displayed by ACE from each strain are summarized in **Table 7**.

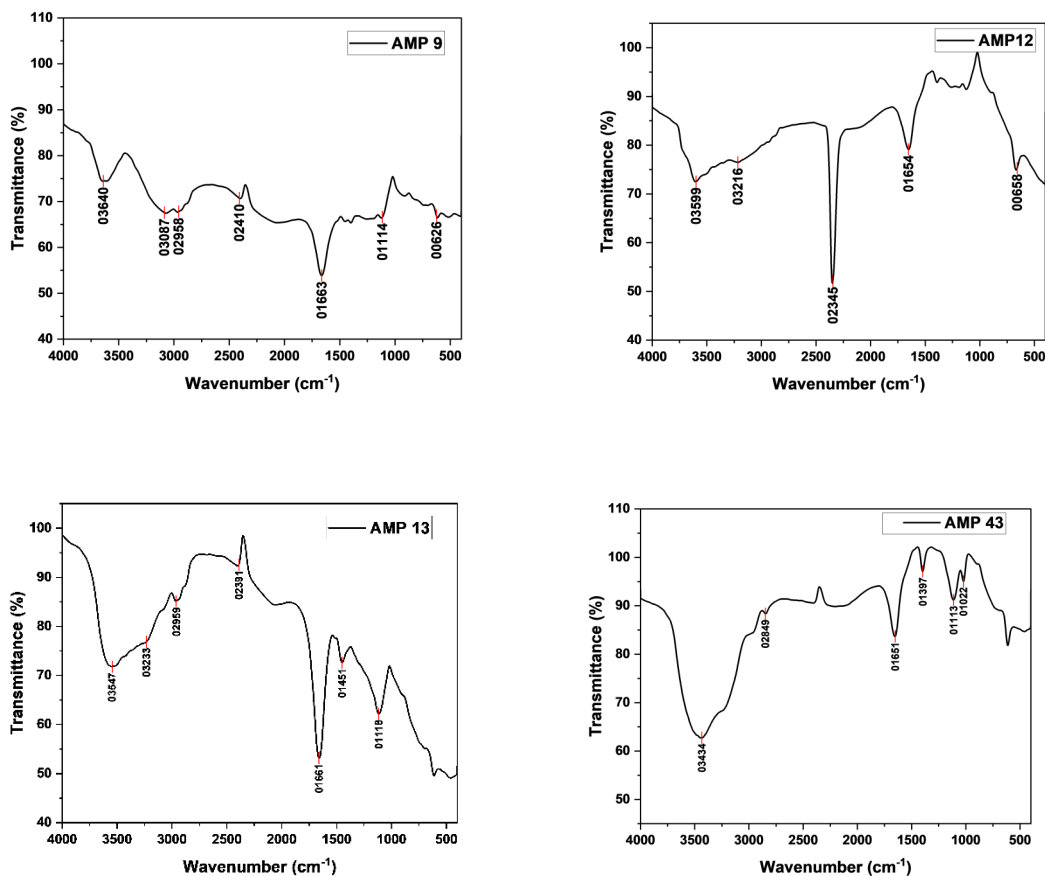


Figure 20. FTIR spectrum of peptide extracts from S9, S12, S13, and S43.

Amide A and B bands span 3100-3500cm⁻¹, Amine I band is from 1600 to 1700 cm⁻¹, Amine II band is from 1480-1600 cm⁻¹, and the region from 500 to 1300 cm⁻¹ represents Amides III-VI bands.

Table 7. Characteristic absorption bands and their possible functional groups

FTIR Peaks (cm ⁻¹) from Strain 9	Possible Assigned Functional Groups
3087	NH stretching (N-H stretching)
2958	Aliphatic chain (C-H stretching)
1663	C=O stretching (Amide I)
626	Out of plane NH bending (Amide V)
FTIR Peaks (cm ⁻¹) from Strain 12	Possible Assigned Functional Groups
3599	NH stretching
3216	NH stretching (Amide A)
1654	C=O stretching
658	Out of plane NH bending (Amide V)
FTIR Peaks (cm ⁻¹) from Strain 13	Possible Assigned Functional Groups
3547	NH stretching
3233	NH stretching
2958	Aliphatic chain (C-H stretching)
1661	C=O Stretching (Amide I)
1451	CN stretching (Amide III)
FTIR Peaks (cm ⁻¹) from Strain 43	Possible Assigned Functional Groups
3434	NH stretching
2849	Aliphatic chain (C-H stretching)
1651	C=O Stretching (Amide I)
1397	CN Stretching

4.6.3 NMR spectroscopy of Crude AMP

The proteinaceous nature of the preconcentrated ACE was analyzed using ¹H and ¹³C NMR spectroscopy. The ¹H NMR spectra from all samples exhibited signals corresponding to amide protons (6.5–9.5 ppm), aliphatic groups (1.0–2.0 ppm, attributed to A-CH₃ and C-CH₂-C groups), and alkyl amine resonances (3.0–4.0 ppm, RNH₂), suggesting the polypeptide nature of the ACE. Key regions indicative of amide protons were partially obscured due to the low concentration of the extracted material used for analysis. However, amplification of these regions revealed weak peaks representing amide protons.

Additionally, ¹³C NMR spectra demonstrated carbonyl signals between 169–173 ppm in ACE samples obtained from strains S9, S12, and S13, further supporting the presence of amino acid residues in all analyzed samples (**Figures 21-24**). Due to the presence of OH in our samples, DMSO peaks appeared at 3.33ppm in all samples.

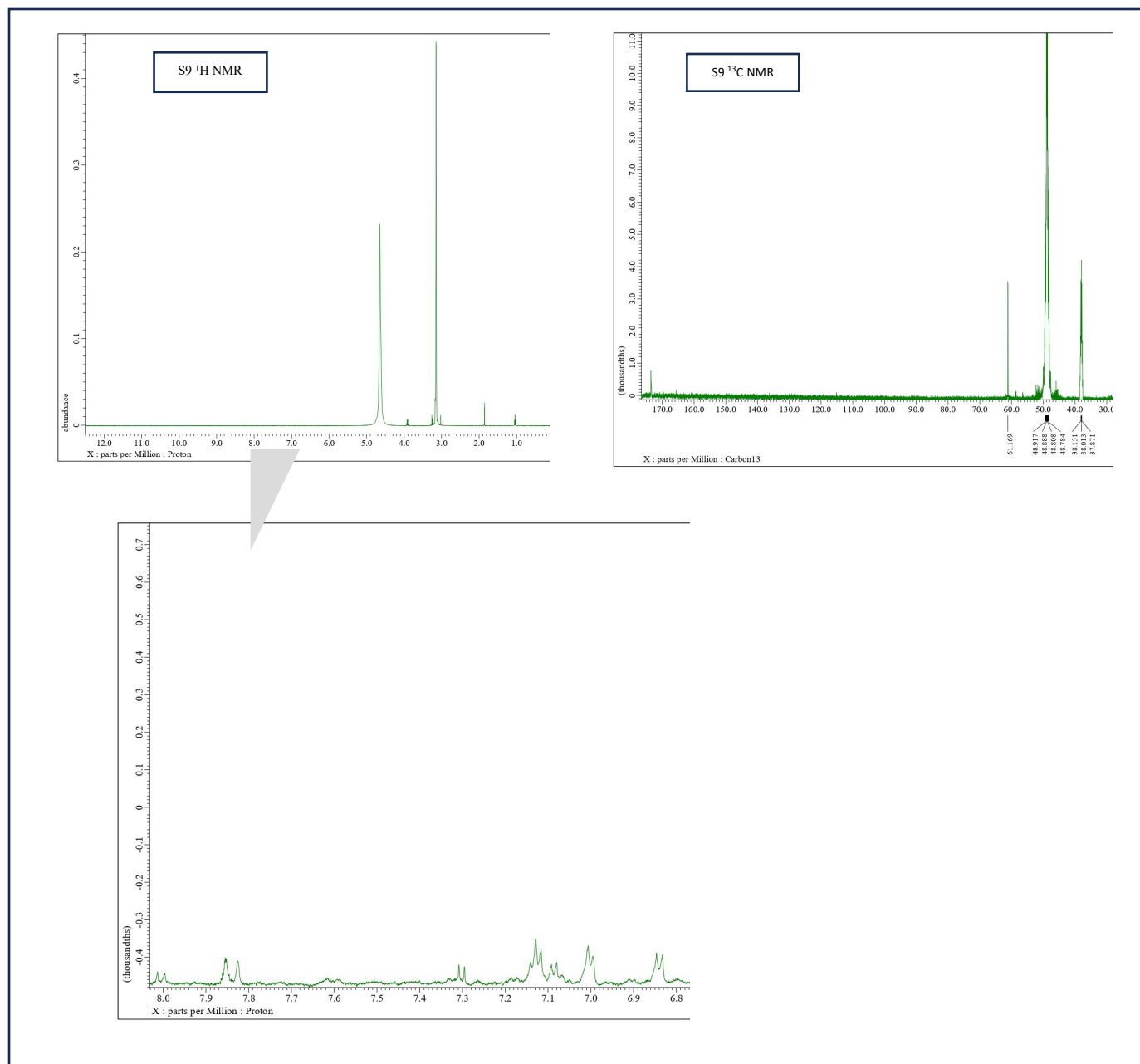


Figure 21. a) ^1H NMR b) ^{13}C spectra of pre-concentrated ACE produced by *Geobacillus stearothermophilus* c) Amplified ^1H spectra around the 8-7 region

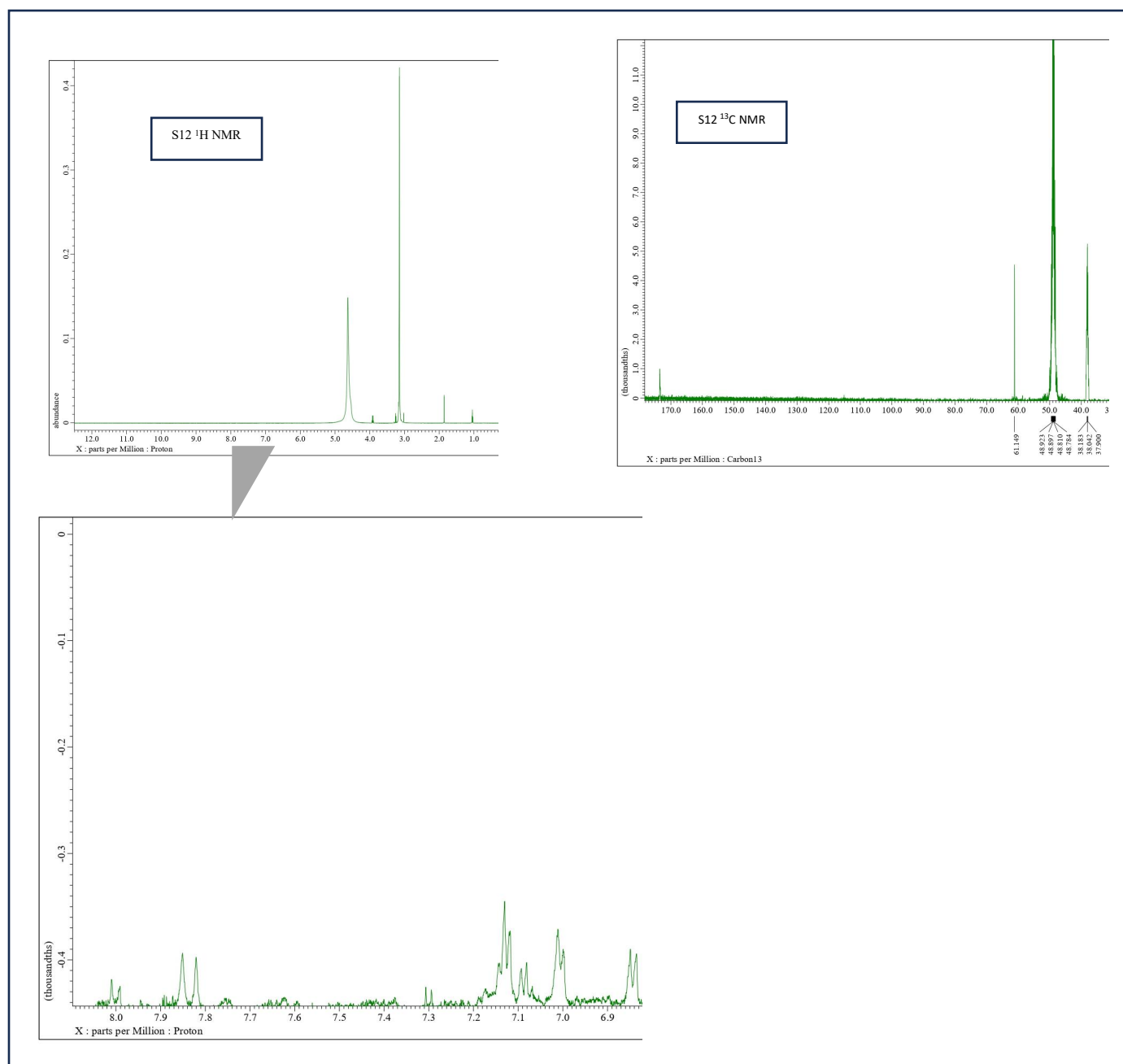


Figure 22. a) ^1H NMR b) ^{13}C spectra of preconcentrated ACE produced by *Bacillus licheniformis* c) Amplified ^1H spectra around the 8-7 region

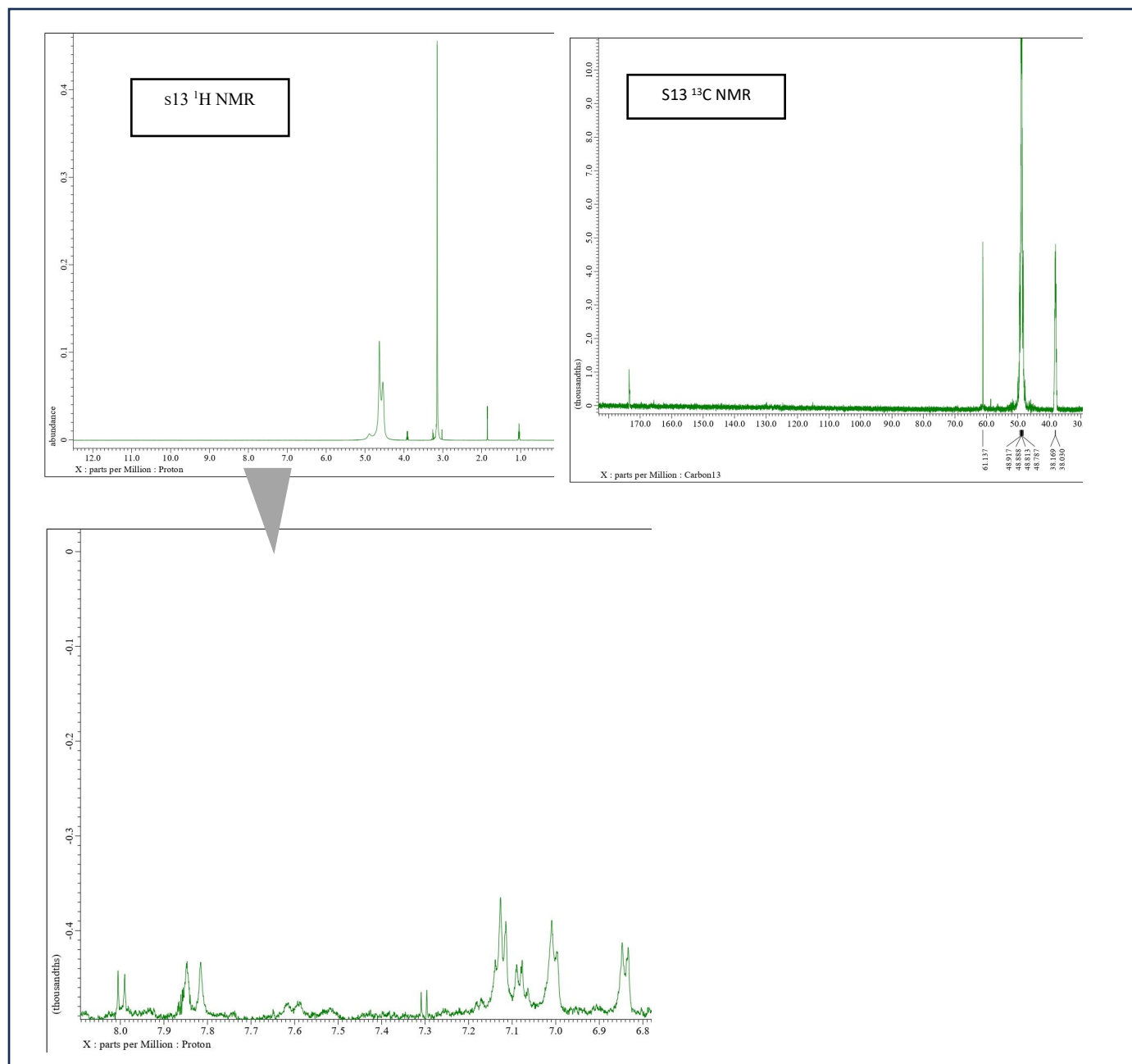


Figure 23. a) ^1H NMR b) ^{13}C spectra of preconcentrated ACE produced by *Anoxybacillus flavithermus* AK1 c) Amplified ^1H spectra around the 8-7 region

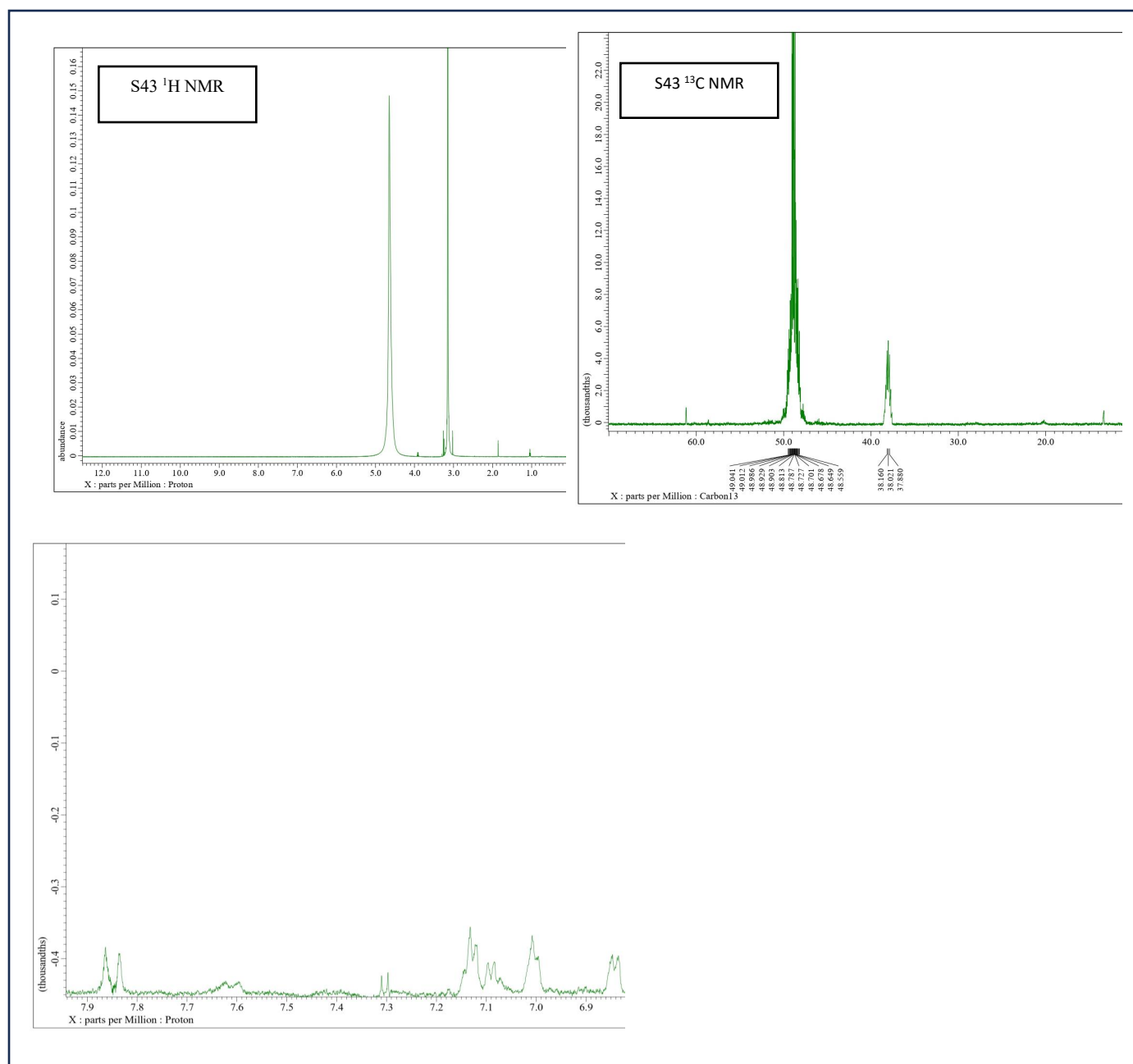


Figure 24. a) ^1H NMR b) ^{13}C spectra of preconcentrated ACE produced by *Brevibacillus borstelensis* AK1 c) Amplified ^1H spectra around the 8-7 region

4.6.4 Mass Spectrometry Analysis of active Peptide fractions

Antimicrobial crude extracts from selected strains were further fractionated by liquid chromatography. The fractions eluted from each ACE were tested against *S. aureus* to identify those with antimicrobial activity. Fractions identified as having potential antimicrobial activity against *S. aureus* were carried out for a second UPLC to verify their homogeneity. The Fractions 9-1, 9-2, 9-3, 12-1, 12-2, 12-C, 13-1, 13-2, 13-3, 43-1, 43-2, 43-3 which were confirmed to be homogenous were analyzed using ESI-MS. The ESI-MS spectrum from the selected Samples revealed a range of molecular ions from m/z 1642 - 1930 for 9-1, 500 - 1910 for 9-2, 500 - 1805 m/z for 9-3, 500 - 1405 for 12-1, 600 - 1708 for 12-2 and 12-3, 400 - 2000 m/z for 13-1, 100 - 1902 m/z for 13-2, 300 - 1800 m/z for 13-3, 600 - 1797 for 43-1, 300 - 1705 for 43-2, and 500 - 1810 m/z for 43-3 (**Figure 25-32**). The presence of several species in this range could suggest a mixture of peptides, some of which may have different lengths or modifications.

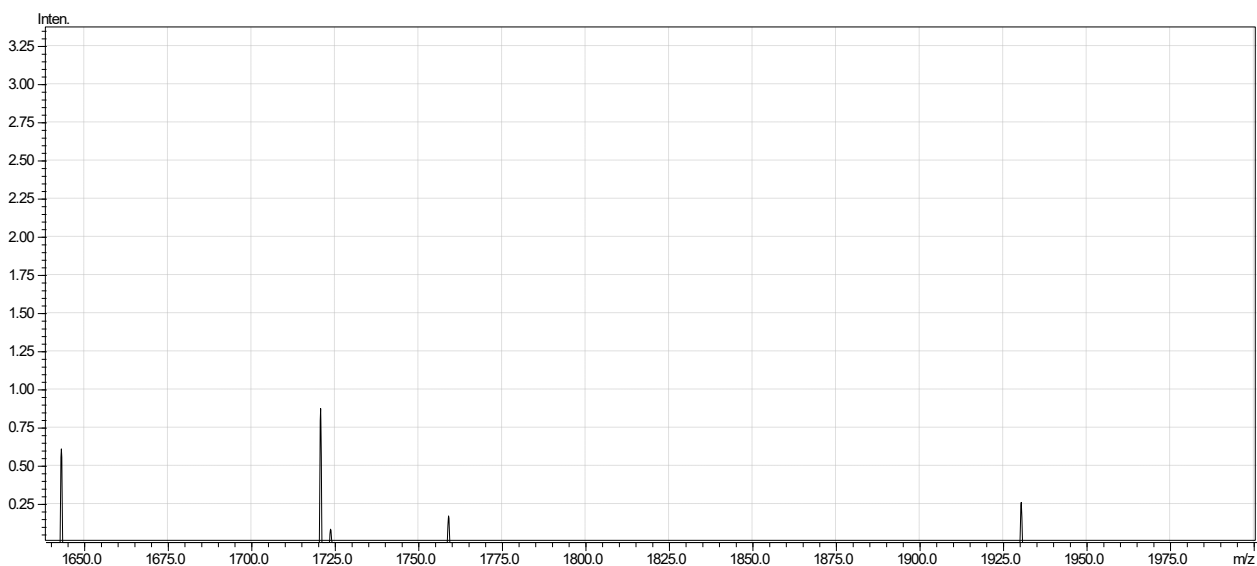


Figure 25. Full MS spectrum of the active fraction (9-1n). The obtained spectrum contains ions ranging between 500 and 1840 m/z (mass/charge ratio).

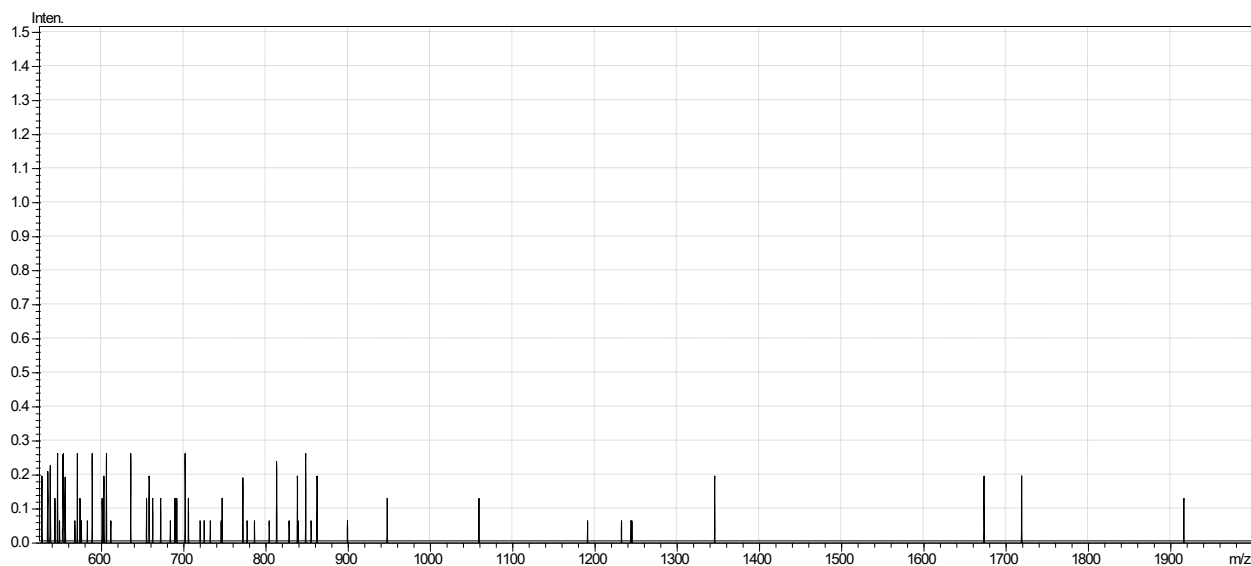


Figure 26. Full MS spectrum of the active fraction (9-2n). Obtained spectrum contains ions ranging between 500-1840 m/z (mass/charge ratio).

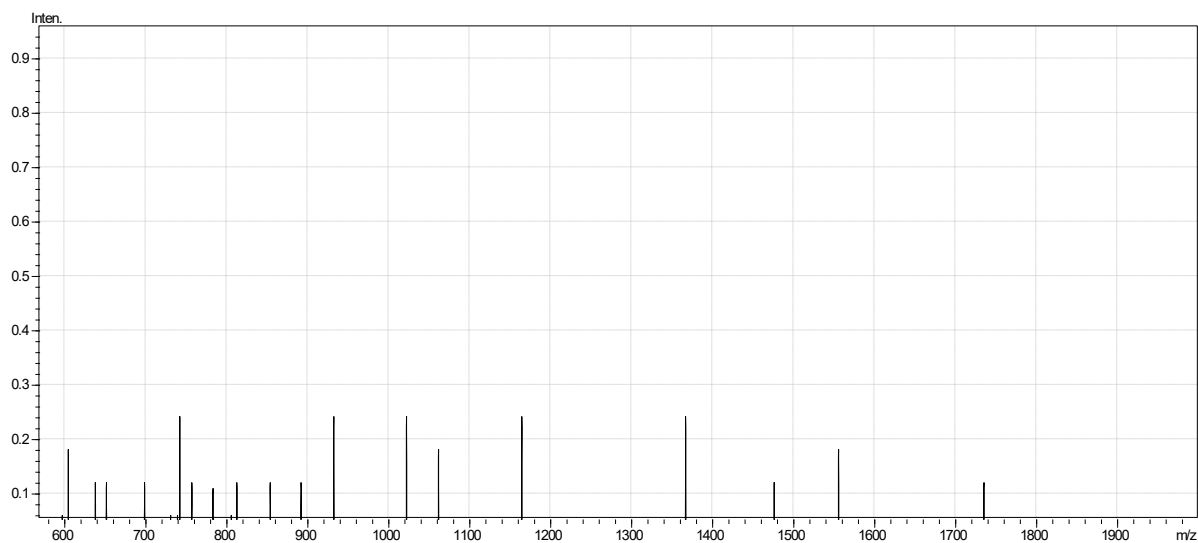


Figure 27. Full MS spectrum of the active fraction (12-1n). The obtained spectrum contains ions ranging between 500 and 1840 m/z (mass/charge ratio).

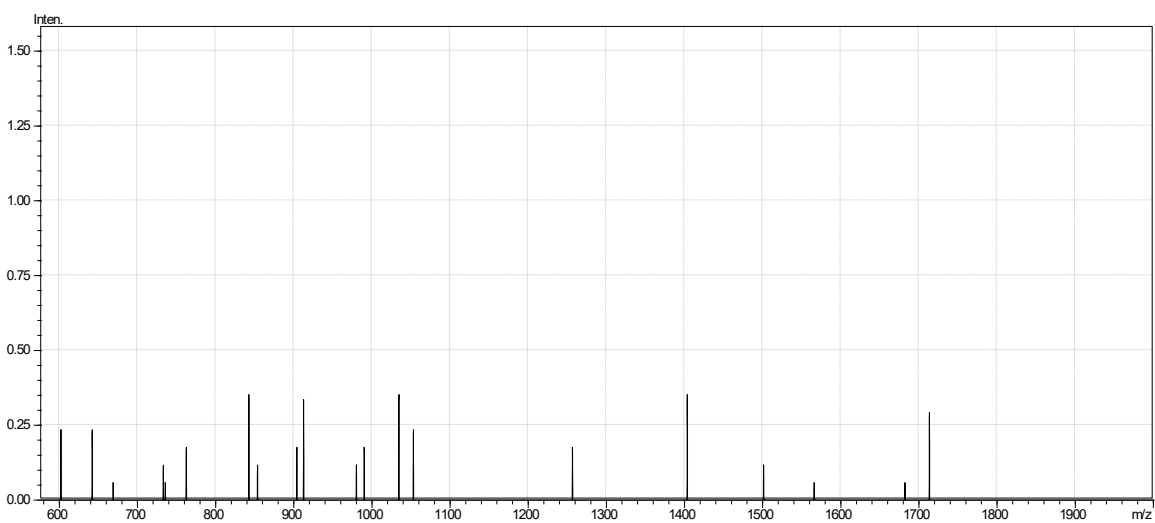


Figure 28. Full MS spectrum of the active fraction (12-En). The obtained spectrum contains ions ranging between 500 and 1840 m/z (mass/charge ratio).

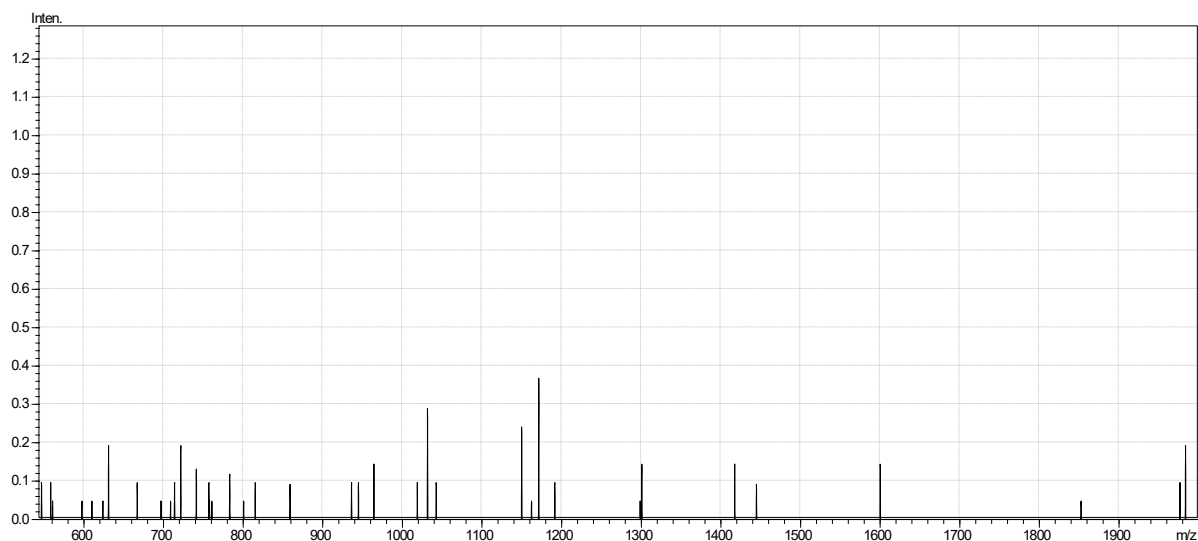


Figure 29. Full MS spectrum of the active fraction (13-1n). The obtained spectrum contains ions ranging between 500 and 1840 m/z (mass/charge ratio).

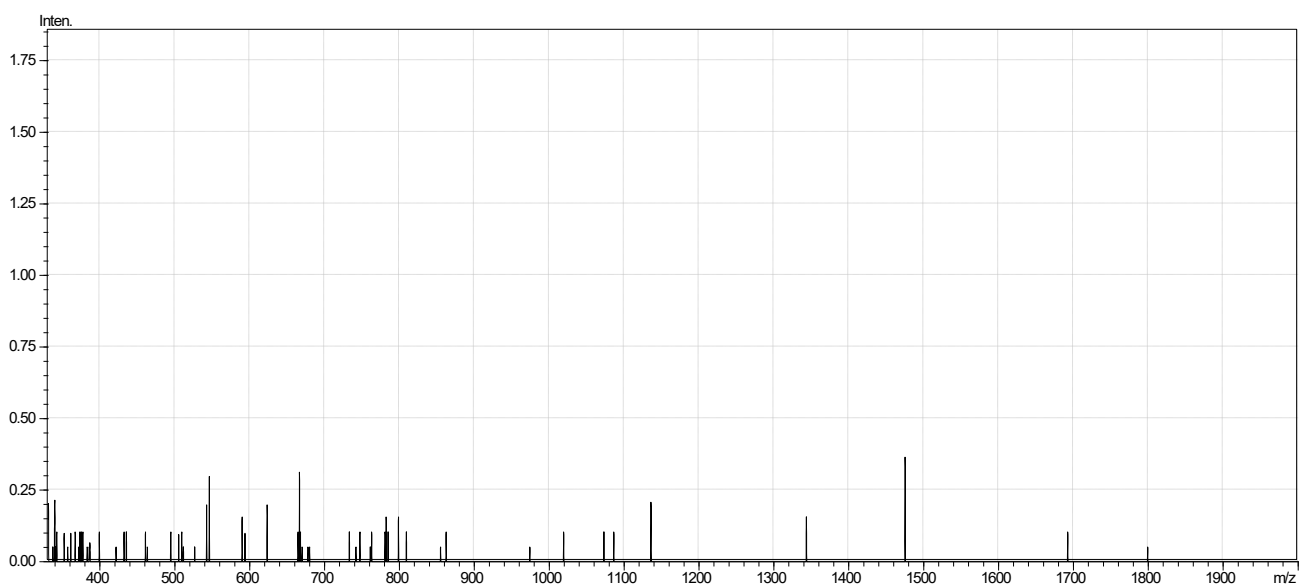


Figure 30. Full MS spectrum of the active fraction (13-2n). The obtained spectrum contains ions ranging between 500 and 1840 m/z (mass/charge ratio).

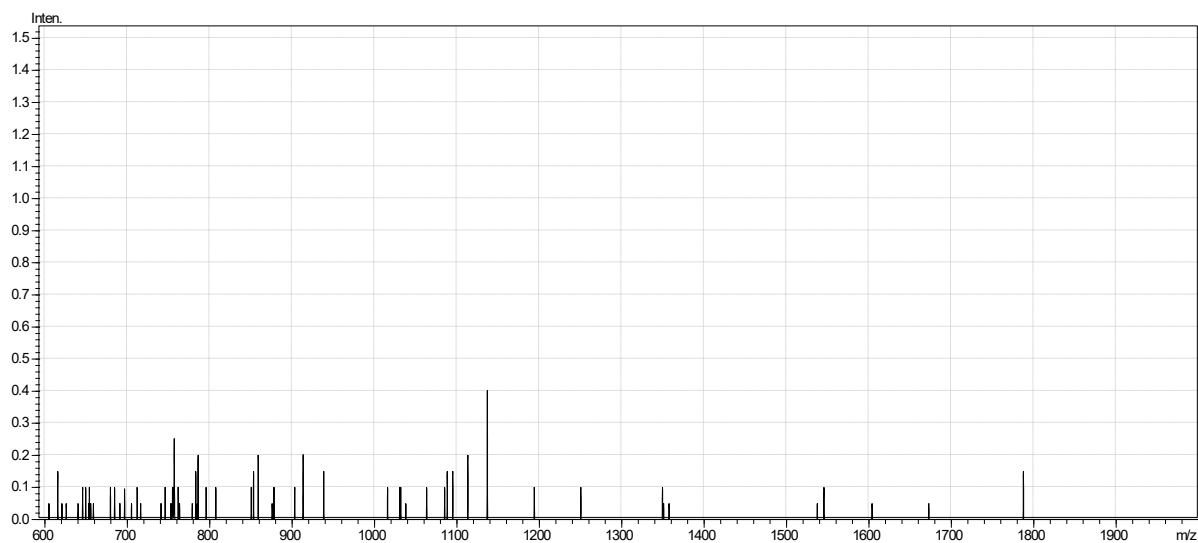


Figure 31 Full MS spectrum of the active fraction (43-2n). Obtained spectrum contains ions ranging between 300-1720 m/z (mass/charge ratio).

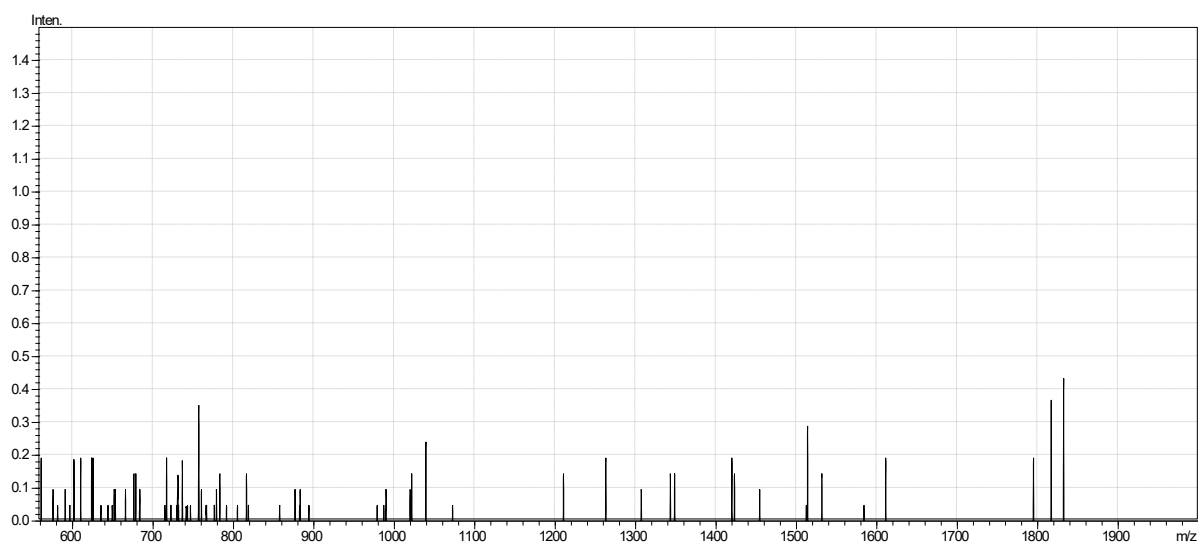


Figure 32. Full MS spectrum of the active fraction (43-3n). The obtained spectrum contains ions ranging between 500 and 1840 m/z (mass/charge ratio).

4.7 In Silico Identification of Bioactive Gene Clusters in *Brevibacillus* and *Anoxybacillus*: Insights into Potential Antimicrobial Compounds

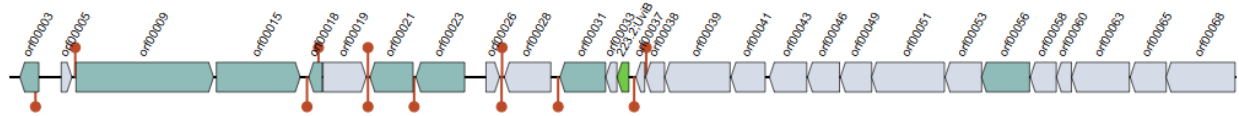
To reveal new antimicrobial peptides encoded in thermophilic bacteria, the NCBI-available genomes from *Brevibacillus borstelensis* AK1 and *Anoxybacillus flavithermus* AK1 were analyzed using the BAGEL4 web tool. (**Table 8**). Genome analysis revealed that the genomic DNA of *Brevibacillus borstelensis* AK1 contained a putative gene cluster encoding a holin-like peptide, specifically a BhIA/UviB family holin-like peptide (**Figure 33(Ia)**). BLAST analysis and alignment of the BhIA/UviB peptide revealed that its sequence exhibited 40.58% similarity to UviB from *Bacillus thuringiensis* serovar *israelensis* ATCC35 strains. The gene cluster contained several proteins likely involved in processing the BhIA/UviB peptide. These include the peptide-processing proteins Family UPF0145, an uncharacterized FasG protein, and a phage-like element PBSX protein Xkdt. Regulatory proteins Competence protein ComM, SOS response-associated peptidase (STAP), and phospholipase D, protein involved in transportation (ATP-binding cassette (ABC) transporter), and self-immunity (Ktr system potassium uptake protein D) (**Table 9**).

The genomic data for *Anoxybacillus flavithermus* AK1, available on NCBI, were fragmented into 50 sequences. A comprehensive analysis of these fragments using BAGEL4 identified two putative bacteriocin clusters (**Figure 33 (IIa-b)**) that may be functionally related. These gene clusters included a gene encoding a 5 kDa thurincin H (thuricin 17), a subclass IId bacteriocin with 63.16% similarity to thuricin 17 from *Bacillus cereus* and *Bacillus thuringiensis* [184]. The biosynthetic gene clusters of thurincin H consisted of genes putatively involved in peptide processing, regulation, modification, exportation, and producer self-immunity (**Table 10**), as previously elucidated in the study by Lee et al., 2009.[195].

Table 8. Detected bacteriocins and secondary metabolites in *Brevibacillus borstelensis* AK1 and *Anoxybacillus flavithermus*.

Genome Location			Group	AMP/Secondary metabolite	Amino acid Similarity (%)	Calculated molecular mass	Sensitive strain	Potential application	Reference
Start	Finish								
Brevibacillus borstelensis Strain AK									
1	278796	298985	Holin-like peptides	BhlA/UviB family holin-like peptide	40.58 %	8279Da	Methicillin-resistant Staphylococcus aureus (MRSA)	Bio drug for efficient control of multidrug resistant bacterial infection	[196]
2	90,695	142,849	NRP	Napsamycin/mureidomycin	10%	855Da/841Da	Pseudomonas sp.	Novel anti Pseudomonas agent	[197], [198]
3	19,975	79,138	NRP	Cusperin A/B	18%	561.29Da/547.27Da	NA	Potential antitumorigenic agent	[199]
Anoxybacillus flavithermus Strain AK1									
4	11496	12687	Subclass II Bacteriocin (Sactipeptide)	Thurincin H/Thuricin 17	63%	3.14kDa/3.16kDa	B. cereus, Clostridium perfringens, Listeria monocytogenes, and Staphylococcus aureus	Bio preservative in foods and sustainable Agriculture.	[200]
5	11,169	33,468	Lipopeptide; Betalactone	Fengycin	46%	1436-1493Da	Fungi: F. oxysporium, F. avenaceum and Mucor sp., A. alternata	Antifungal agent, biocontrol of fruit diseases.	[201],[202]
6	106,862	127,713	Terpene: siderophore	Carotenoid	83%		Salmonella colorless, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes and toxigenic fungi	Antioxidant	

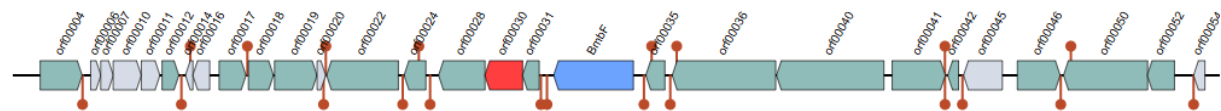
(Ia) Bh1A/UviB family holin-like peptide



(IIa) Thurincin



(IIb)



Legend

- No function determined
- Blast hit with UniRef90
- Core Peptide
- Modification
- Immunity / Transport

Figure 33. (1a-2b) represents contigs containing putative bacteriocin (illustrated in green arrow) and other genetic elements (other colors) detected by the BAGEL4 program.

Table 9. Blastp results of the proteins coded by UviB biosynthetic gene cluster of *B. borstelensis* AK1. Biosynthetic

Name	Product/proposed function	Homologue (accession, organism)	Specie match
orf00003	UPF0145 protein GK1405 YbjQ family protein	OS=Geobacillus kaustophilus (strain HTA426) OX=235909 GN=GK1405 PE=3 SV=1	82.69%
orf00009	Uncharacterized protein YhgE	OS=Bacillus subtilis (strain 168) OX=224308 GN=yhgE PE=4 SV=2	28.44%
orf00015	Ktr system potassium uptake protein D	OS=Bacillus subtilis (strain 168) OX=224308 GN=ktrD PE=3 SV=1	50.68%
orf00018	Competence protein ComM	OS=Haemophilus influenzae (strain ATCC 51907 / DSM 11121 / KW20 / Rd) OX=71421 GN=comM PE=1 SV=1	49.27%
orf00021	SOS response-associated protein YoqW	OS=Bacillus subtilis (strain 168) OX=224308 GN=yoqW PE=3 SV=1	56.44%
orf00023	Phospholipase D	OS=Rickettsia conorii (strain ATCC VR-613 / Malish 7) OX=272944 GN=pld PE=1 SV=1	30.70%
orf00031	Uncharacterized protein FadG	OS=Bacillus subtilis (strain 168) OX=224308 GN=fadG PE=2 SV=2	30.38%
223.2;UviB	223.2;UviB	OS= Brevibacillus borstelensis WP_003385926.1	100%
orf00056	Phage-like element PBSX protein XkdT	OS=Bacillus subtilis (strain 168) OX=224308 GN=xkdT PE=3 SV=1	23.16%

OS = Organism, OX = Taxonomic identifier, GN = Gene name, PE = Protein existence level, SV = Sequence version

Table 10. Blastp results of the proteins coded by Thurincin biosynthetic gene cluster of *A. flavithermus* AK1. Biosynthetic

Name	Product/proposed function	Homologue (accession, organism)	Specie match
orf00006	GMP synthase [glutamine-hydrolyzing]	OS=Geobacillus kaustophilus (strain HTA426) OX=235909 GN=guaA PE=3 SV=1	89.53%
orf00012	Putative transposase MJ0751	OS=Methanocaldococcus jannaschii (strain ATCC 43067 / DSM 2661 / JAL-1 / JCM 10045 / NBRC 100440) OX=243232 GN=MJ0751 PE=3 SV=1	27.35%
orf00025	Probable cadmium-transporting ATPase	OS=Bacillus pseudofirmus (strain OF4) OX=398511 GN=cadA PE=3 SV=2	67.72%
orf00038	Uncharacterized protein y4hP	OS=Sinorhizobium fredii (strain NBRC 101917 / NGR234) OX=394 GN=NGR_a03340 PE=3 SV=2	26.20%
orf00043	Insertion element IS2A uncharacterized 48.2 kDa protein	OS=Escherichia coli OX=562 PE=3 SV=2	29.90%
136.1;Thurincin_H_(thurincin17)	136.1;Thurincin_H_(thurincin17)	Bacillus thuringiensis/ WP_309568072.1	63%
orf00004	CRP-like cAMP-activated global transcriptional regulator	OS=Mycobacterium tuberculosis (strain ATCC 25618 / H37Rv) OX=83332 GN=crp PE=1 SV=1	24.48%
orf00012	Fe-S protein maturation auxiliary factor YitW	OS=Bacillus subtilis (strain 168) OX=224308 GN=yitW PE=3 SV=2	51.08%
orf00017	HTH-type transcriptional regulator MhqR	OS=Bacillus subtilis (strain 168) OX=224308 GN=mhqR PE=2 SV=1	53.62%
orf00018	Putative oxidoreductase MhqP	OS=Bacillus subtilis (strain 168) OX=224308 GN=mhqP PE=2 SV=1	56.25%
orf00019	Quercetin 2,3-dioxygenase	OS=Escherichia coli (strain K12) OX=83333 GN=yhhW PE=1 SV=1	41.88%
orf00022	Uncharacterized MFS-type transporter YfnC	OS=Bacillus subtilis (strain 168) OX=224308 GN=yfnC PE=3 SV=1	53.65%

orf00024	Uncharacterized protein YetH	OS=Bacillus subtilis (strain 168) OX=224308 GN=yetH PE=4 SV=1	72.26%
orf00028	Uncharacterized membrane protein YhfC	OS=Bacillus subtilis (strain 168) OX=224308 GN=yhfC PE=4 SV=1	35.87%
orf00030	Immunity protein SdpI	OS=Bacillus subtilis (strain 168) OX=224308 GN=sdpI PE=1 SV=1	26.92%
orf00031	Transcriptional repressor SdpR	OS=Bacillus subtilis (strain 168) OX=224308 GN=sdpR PE=2 SV=1	62.92%
BmbF	Anaerobic sulfatase-maturing enzyme homolog YdeM	OS=Escherichia coli (strain K12) OX=83333 GN=ydeM PE=3 SV=2	23.78%
orf00035	UPF0145 protein Aflv_1588	OS=Anoxybacillus flavithermus (strain DSM 21510 / WK1) OX=491915 GN=Aflv_1588 PE=3 SV=1	99.05%
orf00036	Sulfite reductase [NADPH] hemoprotein beta-component	OS=Anoxybacillus flavithermus (strain DSM 21510 / WK1) OX=491915 GN=cysI PE=3 SV=1	94.75%
orf00040	Sulfite reductase [NADPH] flavoprotein alpha-component	OS=Bacillus subtilis (strain 168) OX=224308 GN=cysJ PE=1 SV=1	63.98%
orf00041	HTH-type transcriptional regulator CysL	OS=Bacillus subtilis (strain 168) OX=224308 GN=cysL PE=1 SV=1	56.60%
orf00042	Small, acid-soluble spore protein H 2	OS=Geobacillus thermodenitrificans (strain NG80-2) OX=420246 GN=sspH2 PE=3 SV=2	64.41%
orf00046	NAD-dependent protein deacetylase 1	OS=Geobacillus kaustophilus (strain HTA426) OX=235909 GN=cobB1 PE=3 SV=1	63.83%
orf00050	UPF0699 transmembrane protein YdbT	OS=Bacillus subtilis (strain 168) OX=224308 GN=ydbT PE=3 SV=1	30.33%

orf00052	UPF0699 transmembrane protein YdbS	OS=Bacillus subtilis (strain 168) OX=224308 GN=ydbS PE=3 SV=1	32.90%
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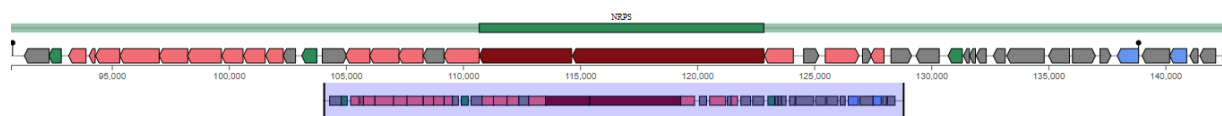
OS = Organism, OX = Taxonomic identifier, GN = Gene name, PE = Protein existence level, SV = Sequence version

In addition to ribosomally synthesized AMPs, several biosynthetic gene clusters involved in the synthesis of bioactive peptide-polyketides and other important metabolites, mediated by modularly organized mega-enzymes, were identified using antiSMASH. In the genome of *Brevibacillus borstelensis* AK1, analysis identified two gene clusters encoding non-ribosomal peptide synthetases (NRPSs). These NRPS gene clusters exhibited 10% and 18% similarity to the napsamycin/mureidomycin gene cluster from *Streptomyces* sp. DSM 5940 and the cusperin gene cluster from *Cyanobacterium Cuspidothrix issatschenkoi*, respectively (**Figure 34 (a and b)**).

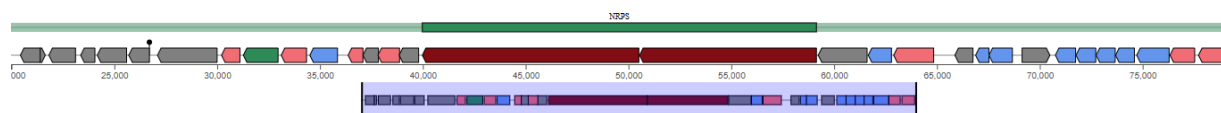
The napsamycin/mureidomycin gene cluster consisted of 42 genes involved in regulation, transport, and biosynthesis. It contained two NRPS genes encoding 12 modules, including adenylation (A) domains, peptide carrier protein (CP) domains, condensation (C) domains, a thioesterase (T) domain, and an iterative ketosynthase (KS) domain. These modules assemble a peptide predicted to contain serine along with three unidentified amino acids (**Figure 35a**). Similarly, the cusperin gene cluster comprised 26 hypothetical genes, including two NRPS genes with 18 domains responsible for assembling the core structure. The assembled peptide was predicted to consist of (NH₂-Thr-Ala) + (Pro-Val-Asn) (**Figure 35b**).

In the genome of *Anoxybacillus* sp., a gene cluster encoding NRPS/betalactone and a terpene was identified. The betalactone cluster showed 46% similarity to the fengycin gene cluster from *Bacillus amyloliquefaciens* strain FZB42, while the terpene cluster exhibited 83% similarity to the carotenoid gene cluster from *Halobacillus halophilus* DSM 2266 (**Figure 34c and d**).

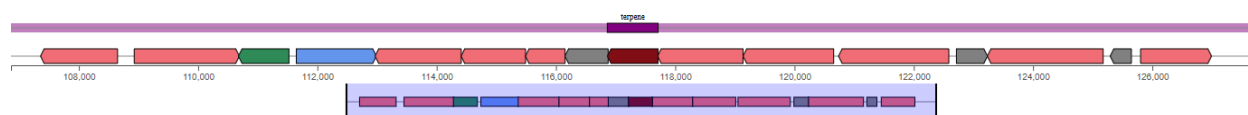
(a) Napsamycin



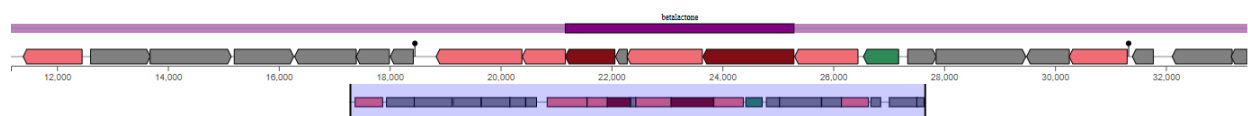
(b) Cusperin



(c) Carotenoid



(d) Fengycin

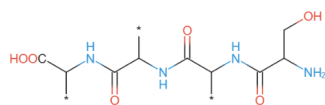


Legend:



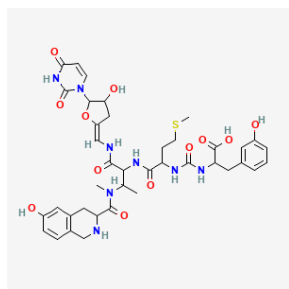
Figure 34. Gene arrangements of the biosynthetic gene clusters generated by antiSMASH.

(a)



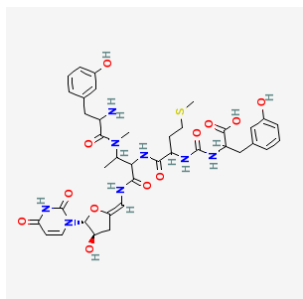
Predicted core structure of Napsamycin.

(b)



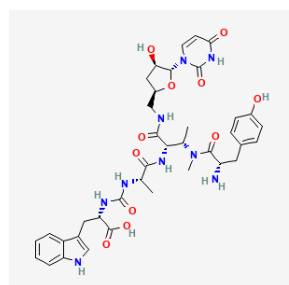
Napsamycin A

(c)



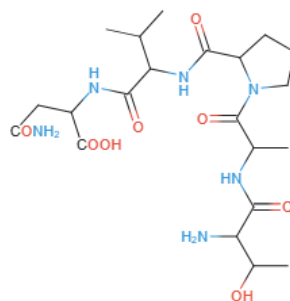
Mureidomycin A

(d)



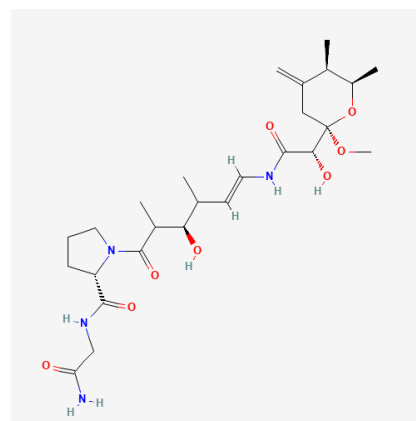
Pacidamycin

(e)



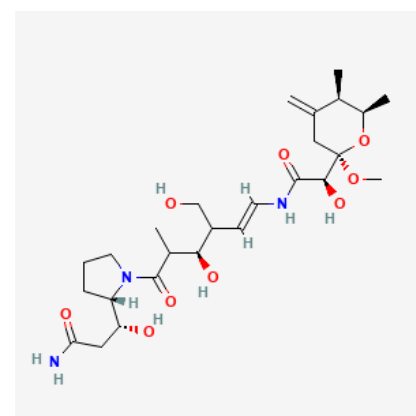
Predicted core structure of Cusperin

(f)



Cusperin

(g)



Nosperin

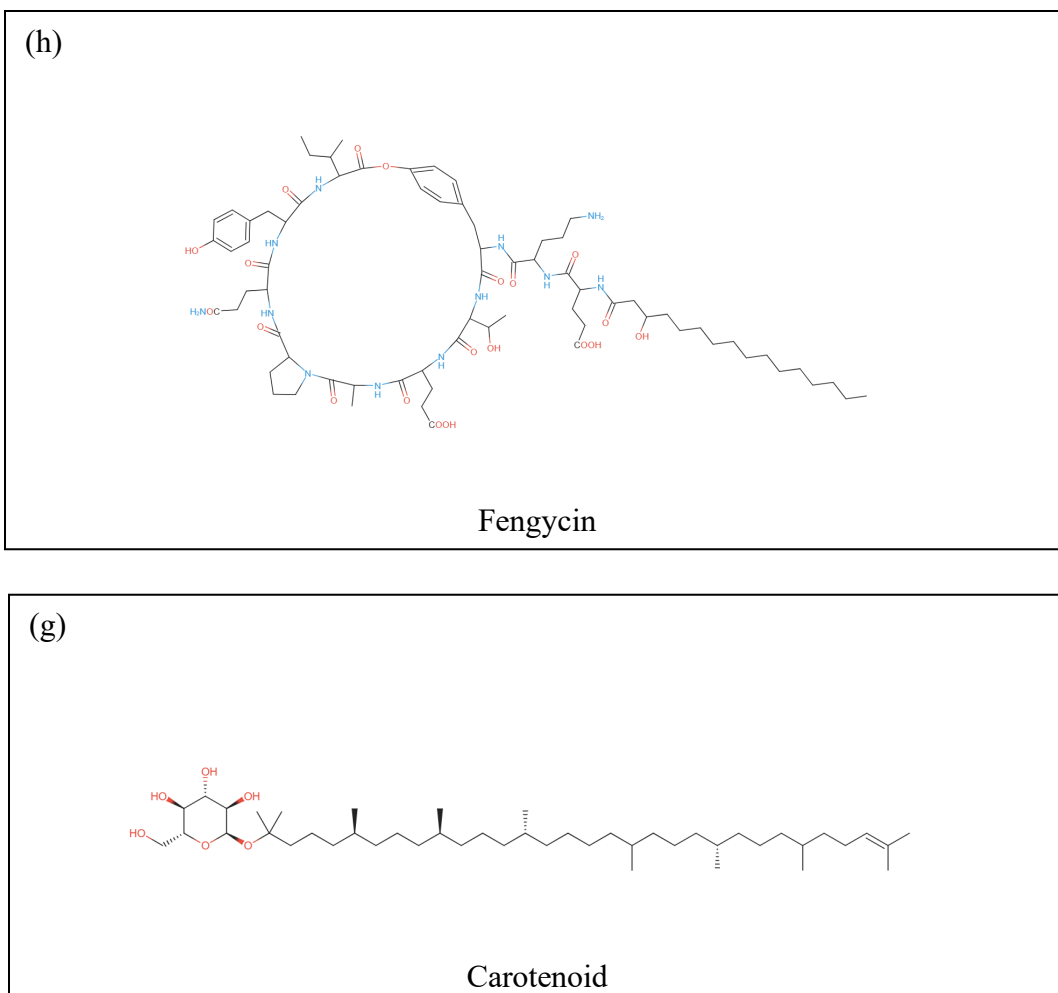


Figure 35. Structures of the non-ribosomally synthesized AMPs (a) Core structure of Napsamycin predicted by antiSMASH, (b) Napsamycin A (c) Mureidomycin A (d) Pacidamycin (e) Core structure of Cusperin predicted by antiSMASH (f) Cusperin (g) Nosperin (h) Fengycin (g) Carotenoid

CHAPTER 5

Discussion

This study focused on the purification and characterization of antimicrobial peptides extracted from thermophilic bacteria isolated from a hot spring in Saudi Arabia. The antibacterial activity of thermophilic strains against *S. aureus* and *E. coli* was evaluated. The inhibition observed against *S. aureus* was significantly more pronounced compared to that against *E. coli*. Differences in the cell wall architectures of Gram-positive and Gram-negative bacteria account for the disparity in susceptibility between them. Gram-positive bacteria have a porous peptidoglycan mesh that is 40–80 nm thick and exposed to the environment; AMPs can readily pass through this mesh [203]. Additionally, this peptidoglycan layer is rich in negatively charged teichoic acids that readily interact with cationic AMPs. In contrast, Gram-negative bacteria possess an extra protective outer membrane composed of phospholipids, LPS, and anionic lipids such as phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin [20]. Moreover, the outer membrane of Gram-negative bacteria is reinforced by noncovalent interactions between lipid A molecules and divalent cations, such as magnesium (Mg^{2+}) and calcium (Ca^{2+}). This strong, polarized network forms a defensive barrier that prevents the entry of harmful substances, including AMPs [204].

Another critical factor that may have led to the reduced susceptibility of *E. coli* to AMP is their ability to produce proteases such as the endopeptidase PgtE, which specifically degrade AMPs, undermining their antimicrobial efficacy [205]. Furthermore, *E. coli* secretes capsular polysaccharides (CPS), forming a protective outer layer that shields the cell from external threats [206]. With over 70 chemically and serologically distinct CPS variants [207], free CPS molecules in the environment can trap AMPs, preventing them from reaching their membrane targets [208].

Exposure to AMPs also triggers sensory systems in *E. coli* that activate genes encoding enzymes to modify the bacterial surface. For instance, the addition of aminoarabinose (L-Ara4N) to the lipid A component of the outer membrane reduces the affinity of cationic AMPs for the membrane and stabilizes the outer membrane against AMP-induced perturbation [209]. Additionally, the presence of porins, such as OmpF, in the outer membrane can serve as gateways for AMPs to enter the cell. However, blocking these proteins can diminish AMP activity, as observed in *E. coli* where the inhibition of OmpF significantly reduced the efficacy of colicin, an AMP [210]. Another contributing factor is the thermophilic origin of the AMPs. AMPs derived from thermophilic

bacteria may exhibit enhanced efficacy against closely related thermophilic strains rather than mesophilic organisms like *E. coli*. This specificity was demonstrated in a study by Koniuchovaitė (2023), where AMPs from thermophilic bacteria showed activity against thermophilic targets but limited or no activity against mesophilic bacteria, including *E. coli*.

The growth characteristics of the thermophilic isolates in this study were consistent with findings reported by Amjad Khalil (2011), albeit with some notable differences. All four isolates demonstrated optimal growth at temperatures ranging from 50°C to 55°C, with minimal growth at lower temperatures (34°C to 40°C) and at 60°C. This is in line with Khalil's observations, where thermophilic isolates exhibited optimal growth between 50°C and 65°C. Regarding pH preferences, the isolates in this study showed a preference for acidic conditions, with limited growth at pH 9 to 10. In contrast, Khalil's isolates were classified as alkali tolerant or alkalophilic, growing optimally at pH 7 to 9 but exhibiting reduced cellular yields at pH 6. Interestingly, strain S43 in this study was the only isolate tolerant of alkaline pH. The isolates in this study grew well in standard TT medium containing 0.2% (w/v) NaCl and tolerated up to 1% (w/v) NaCl. However, Khalil's isolates demonstrated a broader salt tolerance, growing in concentrations of up to 10% (w/v) NaCl with optimal growth at 0.25% (w/v) NaCl.

The lack of antimicrobial activity in culture supernatants from liquid-grown thermophilic isolates aligns with prior findings by Alkhalili et al. (2016), who reported enhanced antimicrobial production by these strains when grown on solid-state media. This phenomenon is consistent with the natural behavior of microorganisms, which predominantly exist as biofilms (structured communities of microorganisms embedded within a self-producing extracellular matrix) in nature. Biofilms engage in complex metabolic interactions, enhancing their stability and production of secondary metabolites, including antimicrobial agents [211]. In contrast, free-living cells in liquid cultures tend to exhibit lower stability and vulnerability to environmental stresses, potentially explaining their reduced antimicrobial production. Qi et al. (2024) further demonstrated that direct contact with competing strains often triggers antimicrobial production. This was proven when culture broth-containing cells were co-cultured with *S. aureus*.

To address this limitation, immobilization techniques were explored to assess their effect on antimicrobial peptide (AMP) production. Cell immobilization is the process of confining live microbial cells to a specific area of space (a carrier) to restrict their ability to freely migrate into

the liquid medium [212]. In biological processes, the immobilization of microbial cells can happen naturally or via an artificial procedure. Entrapment, encapsulation, and crosslinking are the techniques used to achieve immobilization utilizing a high molecular hydrophilic polymeric gel, such as alginate, agarose, polyacrylamide, polyester, carrageenan, and polystyrene [213]. This technique offers several advantages such as increased productivity, continuous use, protection from ambient acidity and shear stress, and most importantly, relief of feedback inhibition that cells encounter from their metabolic product[214].

Immobilized microbial consortia have been extensively used in waste management applications such as organic matter degradation, nitrification, and hydrogen sulfide mitigation in aquaculture ponds [215][216] as well as in the production of biomolecule [217]. In this study, thermophilic isolates were immobilized using agar beads to evaluate AMP production. Initial results showed no detectable antimicrobial activity during the first two days of incubation. On the third day, activity was observed, albeit at low levels, which disappeared entirely by the fourth day. This limited success could be attributed to the compact pore size of agar beads, which may restrict nutrient diffusion to the entrapped cells, and the structural fragility of agar, which allowed cells to escape into the surrounding medium, reducing the efficacy of immobilization. Agar's high melting point further complicates its application for large-scale AMP production at elevated temperatures [203](972-976, n.d.).

The challenges observed in this study are consistent with findings from Trabelsi et al. (2013), who reported enhanced antimicrobial activity of *Lactobacillus plantarum* TN9 when chitosan was used as a coating material for immobilization. In contrast, no activity was observed when gelatin, a material with properties similar to agar, was used, due to bacterial leakage from the matrix [191]. A similar issue of bacterial leakage was noted in our study. These findings underscore the need for improved immobilization materials and methods to optimize antimicrobial peptide production.

Due to the challenges associated with extracting antimicrobial peptides through cell immobilization, APE was directly obtained from the thermophilic isolates. These extracts displayed inhibition against *Staphylococcus aureus*; however, the inhibition zones were notably less prominent compared to those observed when the thermophilic bacteria were co-cultured with competing strains. This discrepancy can be attributed to the natural regulatory mechanisms of bacteria. In competitive environments, bacteria prioritize energy allocation toward the production

and secretion of antimicrobial compounds to outcompete rival strains. This heightened production is often triggered by direct interactions or environmental signals associated with the presence of competitors. Conversely, in the absence of such stimuli, bacteria may downregulate antimicrobial synthesis, opting to conserve energy and store these compounds intracellularly until needed.

The stability of antimicrobial agents under elevated temperatures is critical in determining their potential applications in various industries. The thermostability of antimicrobial activity was evaluated S9, S12, S13, and S43 at temperatures ranging from 40°C to 70°C. Among all tested samples, S43 exhibited the highest thermostability, maintaining an inhibition percentage above 84% across all temperature conditions. Even at 70°C, S43 retained 84.51% inhibition, indicating its strong resistance to heat-induced degradation. This suggests that the active antimicrobial components in S43 remain stable and functional at high temperatures. Its stability aligns with the general characteristics of antimicrobial peptides (AMPs) derived from thermophilic bacteria, which often display enhanced stability due to their adaptation to high-temperature environments, hence making it a promising candidate for applications requiring thermal stability [60], [61]. In contrast, S12 showed the least thermostability, with inhibition dropping sharply from 55.47% at 40°C to only 13.61% at 70°C. This significant decline suggests that the antimicrobial agents in S12 are highly sensitive to heat, leading to a rapid loss of activity. Similarly, S9 and S13 demonstrated moderate thermostability, with inhibition percentages decreasing as temperature increased. While S13 showed its highest inhibition (69.76%) at 50°C, its activity declined at higher temperatures, indicating partial thermal resistance but not as stable as S43. The reduced thermostability of S12's antimicrobial agents may result from a higher proportion of labile components in its composition or structural features that make it more susceptible to thermal degradation.

The ACE obtained from each strain were characterized using various spectroscopic techniques including UV spectrophotometry, FTIR and NMR. UV spectrophotometry was carried out using a UPLC system coupled to a diode array detector. At a wavelength of 200–300 nm, the physical characteristics of amino acid residues and peptide bonds can be identified. Particularly, residues of aromatic amino acids, including phenylalanine, tryptophan, and tyrosine, show two peaks: one at lower wavelengths, about 215-230 nm, and another around 250-280 nm. Furthermore, peptide bonds and cysteine residues absorb mid-UV radiation, primarily below 260 nm [218][219]. The

APEs in this study exhibited intense absorption peaks within 210-298nm, which are attributed to the presence of amino acids and peptide bonds [220]. This result suggests that ACE extracted from each thermophilic strain is proteinaceous.

The FTIR spectrum of the APE extracted from the studied strains was consistent with reported vibration spectra of peptides [107] [221]. The peak areas 3087 cm^{-1} for S9, 3216 cm^{-1} for S12, 3233 cm^{-1} for S13, and 3434 cm^{-1} for S43 were found to be within the range of $3600\text{--}3100\text{ cm}^{-1}$. This is attributable to the fact that carbon-containing compounds with amino groups have stretching vibrations of C-H and N-H moieties. This result indicates that the molecules present in the APEs feature N-H functional group [222]. The peaks formed by C-H stretching (2958 cm^{-1} for S9, 2958 cm^{-1} for S13, and 2849 cm^{-1} for S43) indicate the presence of an aliphatic chain [194]. Amphiphiles of amino acids were also identified due to the presence of C=O or amide I absorption peaks which falls within the range of $1700\text{--}1600$ (1663 cm^{-1} for S9, 1654 cm^{-1} for S12, 1661 cm^{-1} for S13, and 1651 cm^{-1} for S43). In addition, the signals 1451 cm^{-1} for S13 and 1397 cm^{-1} for S43 were attributed to C-N stretching vibration. Peaks corresponding to the amide V regions (612 cm^{-1} for S9 and 658 cm^{-1} for S12) can also be seen in the spectrum. Based on the presence of strong amide I absorption in all samples, it can be speculated that the extracts are rich in α -helical peptides [171]. Similarly, ^1H NMR showed signals for alkyl amine (3-4 ppm), aliphatic groups (1-2 ppm), and amide proton (7-10), suggesting the polypeptide nature of the preconcentrated APE. ^{13}C NMR also showed a signal of amide compounds. These results agreed with the studies of Ahire et al (2020) and Al-Thubiani et al (2018).

In the absence of a semipreparative column for reverse-phase HPLC, we developed an alternative separation method that replicates its principles by leveraging hydrophobic interactions. To analyze the resulting fractions, UHPLC was employed. The antimicrobial activity of the collected fractions was notably lower than that of the crude sample. This reduction in activity may be attributed to the separation process, where each fraction contained only a subset of the original components, potentially decreasing their concentration. Additionally, some antimicrobial peptides may have degraded due to exposure to solvents, pressure, or temperature fluctuations during fractionation. Furthermore, bioactive molecules might have irreversibly bound to the column material, leading to incomplete recovery and a further decline in antimicrobial activity.

Different molecular masses ranging between 500-2000 Da were assigned to fractions displaying antimicrobial activity. Due to the appearance of multiple fragments in the MS spectra, it was conceivable that other components present in very low concentrations, may not have been detected by UHPLC. These undetected compounds could have been eluted within the valleys of other chromatographic peaks, making their identification challenging. Our original goal was to use MS/MS analysis to ascertain the peptide sequence. However, we were unable to carry out this technique because the necessary analytical resources and equipment were unavailable. As an alternative, we employed bioinformatics-based sequence prediction for two bacterial strains used in this study, *Brevibacillus borstelensis* AK1 and *Anoxybacillus flavithermus* AK1, leveraging their publicly available whole genome sequences from the NCBI database.

The whole genome sequence analysis of *B. borstelensis* AK1 and *A. flavithermus* using BAGEL4 revealed biosynthetic gene clusters encoding RiPPs, specifically BhIA/UviB holin-like peptide and thurincin H/thuricin 17 respectively.

BhIA/UviB holin-like peptides are AMPs that have been described to have similar function to the phage-encoded holin, which is a hydrophobic protein that inserts and accumulates in the bacterial cytoplasmic membrane (CM) [223]. Holins are known to produce large membrane defects that allow endolysins to cross prokaryotic cytoplasmic membranes. The proton motive force (PMF) eventually collapses as a result of this disruption, causing immediate cell death, a phenomenon referred to as the "holin lethal effect." [224]. Although the specific functions of BhIA/UviB holin-like peptide ORFs have not yet been identified in bacillus and thermophilic strains, studies have shown that *Bacillus*'s bacteriocin-related holin-like peptide BhIA exhibits antibacterial action by rupturing cell membranes of Gram-positive bacteria, such as MRSA and *Micrococcus luteus* [225] [196], [226].

Thurincin H is a lantibiotic bacteriocin that are especially efficient against *Listeria monocytogenes*, *Micrococcus*, and various Gram-positive foodborne pathogens or spoilage microorganisms commonly found in dairy products [227]. Additionally, Thurincin H has been shown to inhibit multiple antibiotic-resistant strains of *Staphylococcus aureus* [200]. Thuricin 17 is a peptide bacteriocin that shares structural similarities with Thurincin H. It exhibits either bactericidal or bacteriostatic activity against a wide range of prokaryotes, effectively eliminating competition within its ecological niche.

In this study, *Brevibacillus borstelensis* and *Anoxybacillus* exhibited a strong inhibitory effect against *Staphylococcus aureus*. This antimicrobial activity may be attributed to the holin-like peptide BhlA/UviB and thurincin H/thuricin 17, both identified in their genomes. Since RiPPs are typically expressed under stressful conditions, especially in the presence of competing microbes, and are secreted upon expression, it is likely that these peptides were not produced when the thermophiles were grown in liquid medium under their optimal growth conditions. This could explain the absence of antimicrobial activity when the thermophiles were cultured in a liquid medium without microbial competition.

In addition to the identified RiPPs, NRPs such as napsamycin/mureidomycin and cusperin were detected in the genome of *Brevibacillus borstelensis* Strain AK, while fengycin and a terpenoid (carotenoid) biosynthetic cluster were identified in the genome of *Anoxybacillus flavithermus* Strain AK1. The masses observed in the MS analysis of active fractions obtained from both *Brevibacillus borstelensis* Strain AK and *Anoxybacillus flavithermus* Strain AK1 matched the expected masses of these compounds, suggesting that the active antimicrobial peptides extracted in this study were predominantly non-ribosomally synthesized AMPs.

Bacterial translocase I, a crucial enzyme in the manufacture of peptidoglycans, is known to be inhibited by napsamycins (NPSs), which are uridylpeptide antibiotics. They share a common structural framework with mureidomycins (MRDs), pacidamycins (PCDs), and the newly discovered sansanmycins (**Figure 32**) [172]. Uridylpeptide antibiotics have been shown in numerous studies to target the formation of peptidoglycans [228].

Cusperin is a bioactive compound belonging to the pederin family. Its chemical structure closely resembles nosperin, recently identified in the lichen cyanobiont *Nostoc*. They both share a tetrahydropyran moiety and a significant portion of the linear backbone. While pederin is a potent toxin known to cause severe skin lesions in humans, it also exhibits strong anticancer properties, making it of interest to the pharmaceutical industry. Interestingly, cusperin lacks the cytotoxic activity observed in other pederin family members. However, its potential as an anti-tumor agent remains an area for further investigation [199].

Fengycin is a physiologically active lipopeptide with structural similarity to surfactin. It exhibits 40-fold less hemolytic activity than surfactin and is known for its antifungal activity against filamentous fungi [201]. Fengycin has been shown to have a strong inhibitory impact on the fungi that cause postharvest foot rot, *Alternaria alternata* and *Rhizopus stolonifera* [202]. As a result, it might be useful in the food sector. Additionally, it has the potential to be an anticancer drug due to its capacity to stop the proliferation of cancer cells [229][230]. However, the antifungal activity of *A. flavithermus* was not examined in this study.

The organic, lipophilic, naturally occurring terpenoid compounds known as carotenoids can have a spectrum of colors from yellow to red because of the oxygen atoms and conjugated bonds that are present in their molecules. Usually isolated from fruits and flowers, they have also been found in other areas of life, including as fungus, bacteria, and archaea. Not only do they contain vitamin A, but they also have antibacterial, anticancer, and antioxidant properties.[231]. Several studies have assessed the antibacterial activity of carotenoid in *Bacillus sp.*, *Salmonella sp.*, *Pseudomonas sp.* and *E. coli* [232]. Carotenoid may have contributed to *A. flavithermus's* inhibitory action against *E. coli* and may be the cause of its distinctive yellow color.

Chapter 6

Conclusion and Recommendations

6.1 Conclusion

This study highlights the isolation and characterization of antimicrobial peptides from thermophilic bacteria, identifying *Geobacillus stearothermophilus*, *Bacillus licheniformis*, *Anoxybacillus flavithermus* AK1, and *Brevibacillus borstelensis* AK1 as the most promising strains due to their strong inhibitory activity against *S. aureus* and *E. coli*. The proteinaceous nature of the active compounds was confirmed through proteinase K treatment and spectroscopic analyses (UPLC, FTIR and NMR). Molecular mass analysis suggested that the active compounds are primarily NRPs as their molecular masses ranged between 500-2000Da.

Due to the availability of the whole genome sequence of *Anoxybacillus flavithermus* AK1 and *Brevibacillus borstelensis* AK1, *in silico* analysis was carried out to supplement the results obtained from the wet lab. *In-silico* analysis is an effective approach for characterizing AMPs because it saves time and reduces the effort needed to screen potential producers compared to traditional wet-lab methods. Additionally, this approach eliminates any biases that might arise from the specific laboratory conditions used during the cultivation of the possible producers. The *in-silico* analysis revealed that *Anoxybacillus flavithermus* AK1 and *Brevibacillus borstelensis* AK1 possess genes encoding RiPPs, such as BhlA/UviB holin-like peptide and Thurincin H/Thuricin 17, as well as NRPs and terpenoids, including Napsamycin/Mureidomycin, Cusperin A/B, Fengycin, and Carotenoid.

These findings underscore thermophilic bacteria as a valuable yet underexplored source of novel antimicrobial agents, particularly antimicrobial peptides. These agents have potential applications as biopharmaceuticals for combating multidrug-resistant bacterial infections, biopreservatives in food, sustainable agriculture solutions, and antifungal, antioxidant, and anticancer agents. Furthermore, the identification and characterization of AMPs from thermophilic bacteria contribute to our understanding of the diversity of AMPs by thermophilic bacteria.

6.2 Recommendation

Further comprehensive studies are required to fully characterize the antimicrobial peptides (AMPs) produced by the selected thermophilic bacteria. In particular, detailed tandem mass spectrometry (MS/MS) analysis needs be conducted to determine the specific molecular masses and amino acid sequences of the peptides extracted in this study. Additionally, targeted proteomics and peptide analysis should be employed to identify any novel or unexpected peptides that may be present in the samples.

In-silico analysis has identified potential AMP gene clusters in two of the strains studied; however, this does not guarantee that these AMPs will be expressed in vitro or confirm their antimicrobial activity. Therefore, it is essential to follow up the in-silico analysis with experimental validation to ensure the production of these AMPs and to evaluate their antimicrobial efficacy against various test microorganisms of interest. It is also crucial to obtain the whole genome sequences of *Geobacillus stearothermophilus* and *Bacillus licheniformis* to uncover additional putative AMP gene clusters they possess.

Expanding research on AMPs from other thermophilic strains holds significant potential for discovering new antimicrobial compounds against antibiotic-resistant pathogens. Future studies should focus on expanding the repertoire of characterized AMPs, assessing their stability and efficacy, and exploring their therapeutic applications in medicine, food preservation, and sustainable agriculture.

APPENDIX

Table 11. Characterization of antimicrobial crude extract by UV Spectrophotometric analysis

		Retention	Area	% Area	Height	Wavelength
ACE from S9		time				
1		4.302	18292625	21.84	569457	260.9
2		5.521	52517749	62.72	2010730	219.5-277.5
3		6.385	2380396	2.84	176071	223.1-283.5
4		7.333	10075445	12.03	410927	212.5-290.6
5		4.242	27894687	53.63	919100	260.9
6		5.503	12613828	24.25	478132	224.3-277.5
7		6.386	1845301	3.55	145318	223.1-283.5
8		7.147	7875111	15.14	526965	243.1-297.7
ACE from S12						
1		4.337	17964311	19.92	545995	260.9
2		5.520	57605265	63.87	1946883	221.9-277.5
3		6.367	2600298	2.88	102636	223.1-283.5
4		7.152	12020692	13.33	514177	212.5-297.7
5		4.235	24206601	48.51	824796	262.1
6		5.517	9008090	18.05	366632	224.3-276.3
7		6.138	3040716	6.09	135283	214.8-297.7
8		7.135	12943320	25.94	886916	212.5-297.7
ACE from S13						
1		4.341	17201664	19.88	536502	259.7
2		5.474	56007608	64.73	1616270	218.4-277.5
3		6.909	13319605	15.39	685379	212.5-296.6
4		4.267	25217494	49.74	826554	260.9
5		5.525	8496882	16.76	277486	277.5
6		6.898	16981448	33.50	1161141	212.5-296.6
ACE from S43						
1		4.358	14936953	20.60	498008	258.5
2		5.460	48589082	67.02	1462228	217.2-277.5
3		6.916	8972818	12.38	351087	243.1-296.6
4		4.304	22219299	61.14	768881	259.7
5		5.512	6353846	17.48	221643	217.2-278.7

6		6.876	7770171	21.38	525978	243.1-296.6
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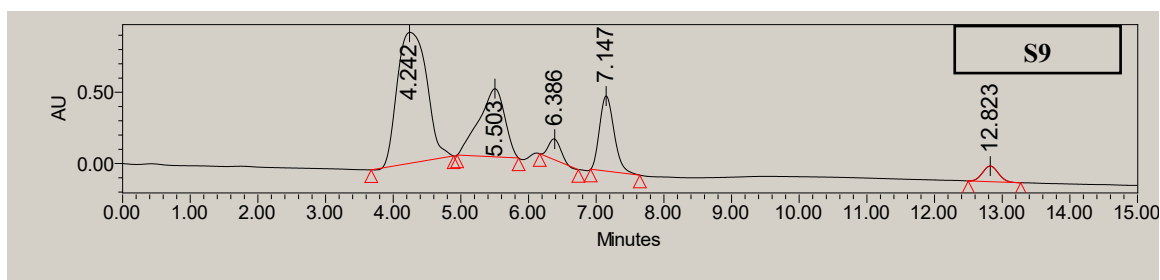


Figure 36. Ultra high-performance liquid chromatography profile of *Geobacillus stearothermophilus* APE before fractionation by column chromatography. Fractions were eluted on a waters® ACQUITY UPLC® H-Class C18 column (1.7 μ m, 2.1 $\times$ 150mm), under an isocratic gradient 50-50% ACN in acidified water for 15 min, at a flow rate of 0.8 μ L/min, monitored at wavelengths 210-400nm. system equipped with PDA).

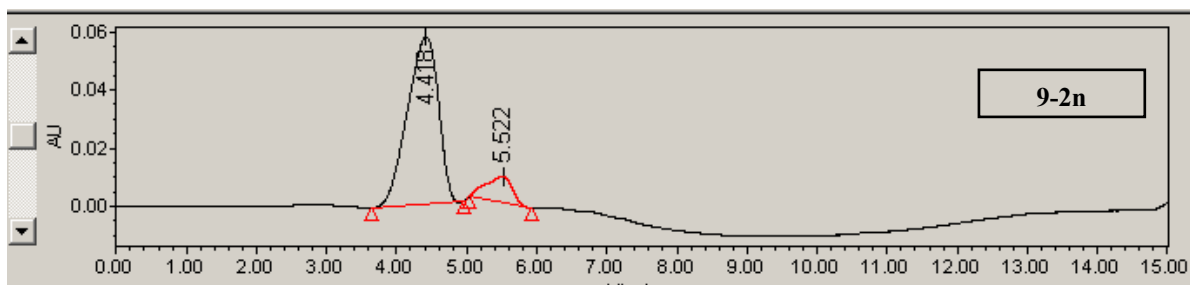
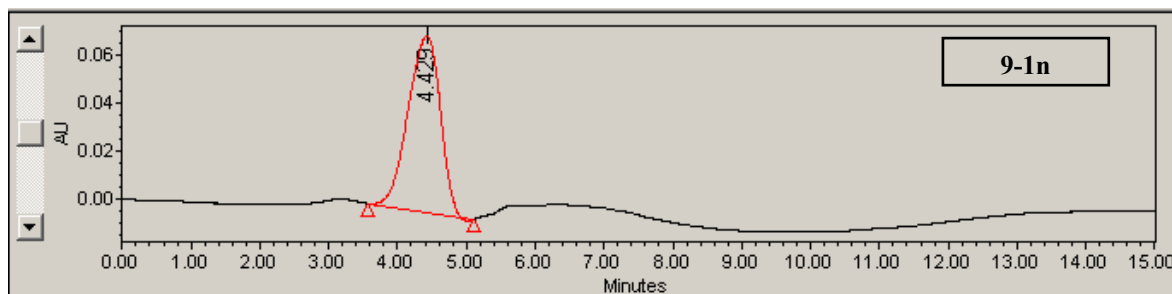


Figure 37. Ultra high-performance liquid chromatography profile of *Geobacillus stearothermophilus* active fractions (12-E-n and 12-1-n) obtained after second fractionation by column chromatography.

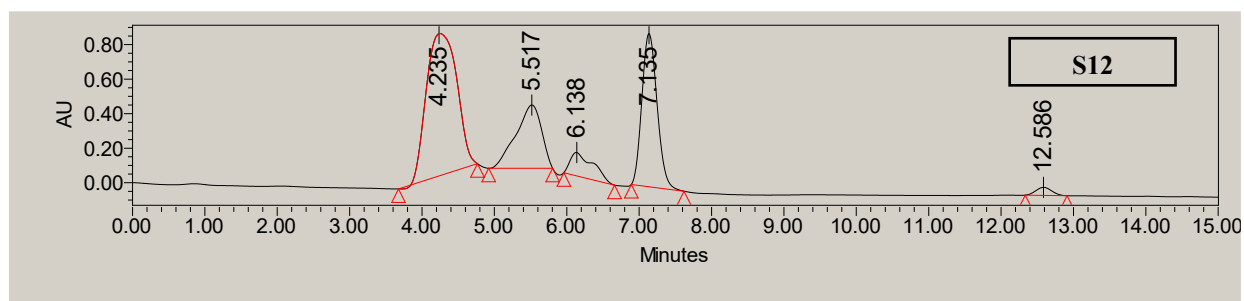


Figure 38. Ultra high-performance liquid chromatography profile of *Bacillus licheniformis* APE before fractionation by column chromatography.

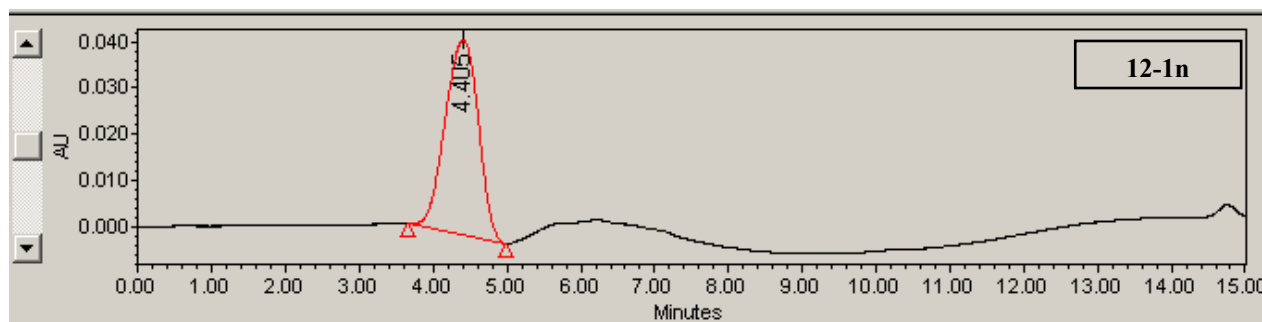
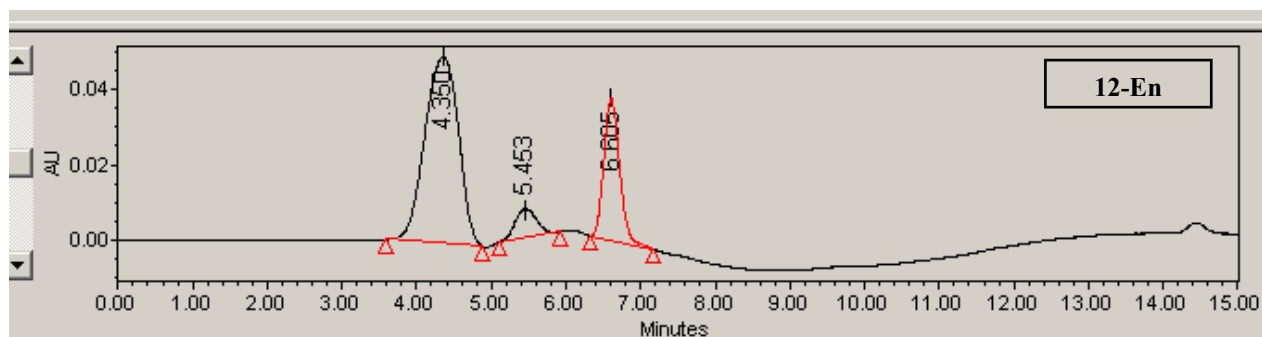


Figure 39. Ultra high-performance liquid chromatography profile of *Bacillus licheniformis* active fractions (12-E-n and 12-1-n) obtained after second fractionation by column chromatography.

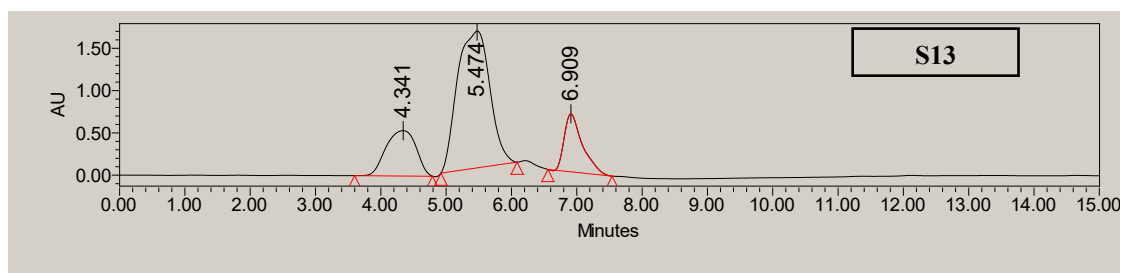


Figure 40. Ultra high-performance liquid chromatography profile of *Anoxybacillus flavithermus* AK1 APE before fractionation by column chromatography.

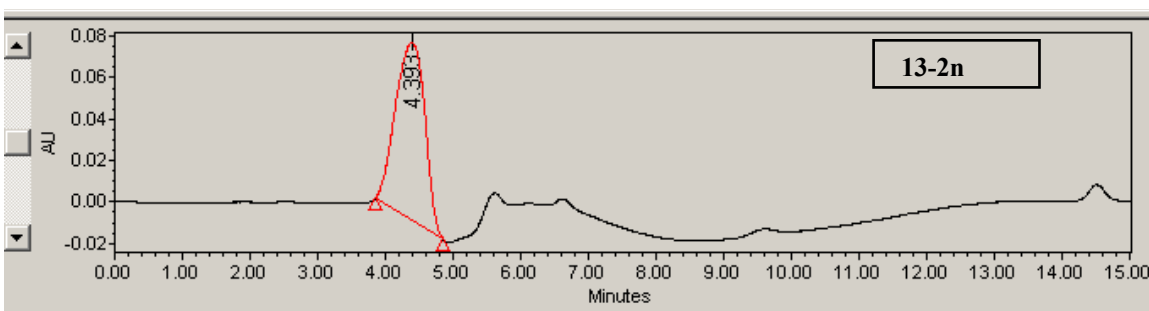
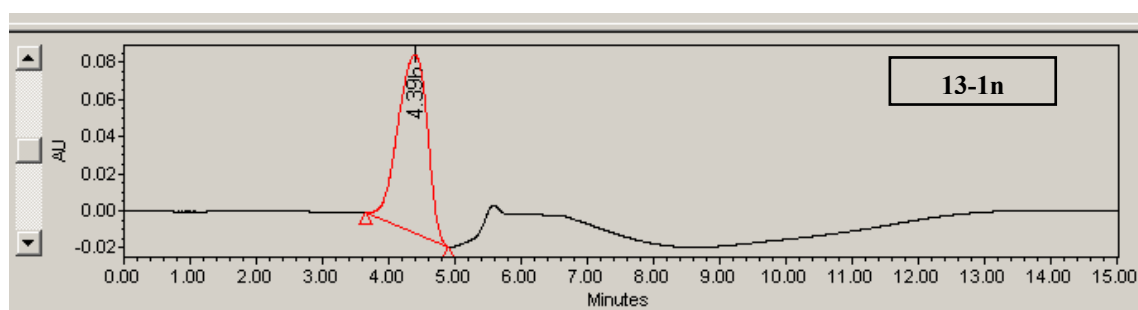


Figure 41. Ultra high-performance liquid chromatography profile of *Anoxybacillus flavithermus* AK1 active fractions (13-1-n and 13-2-n) obtained after second fractionation by column chromatography.

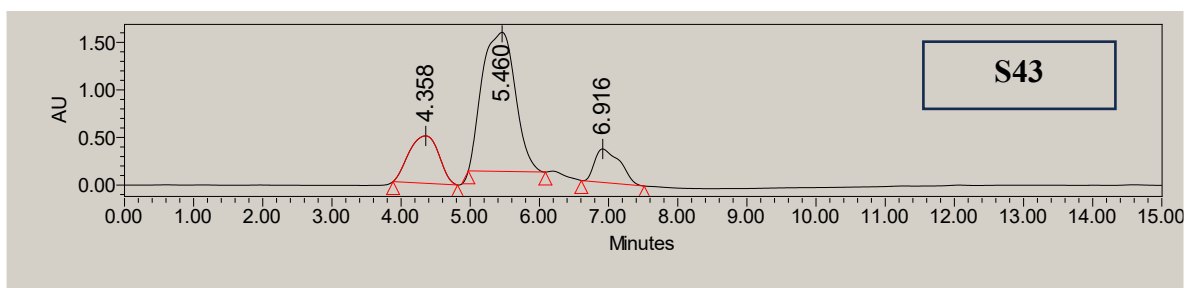


Figure 42. Ultra high-performance liquid chromatography profile of *Brevibacillus borstelensis* AK1 APE before fractionation by column chromatography.

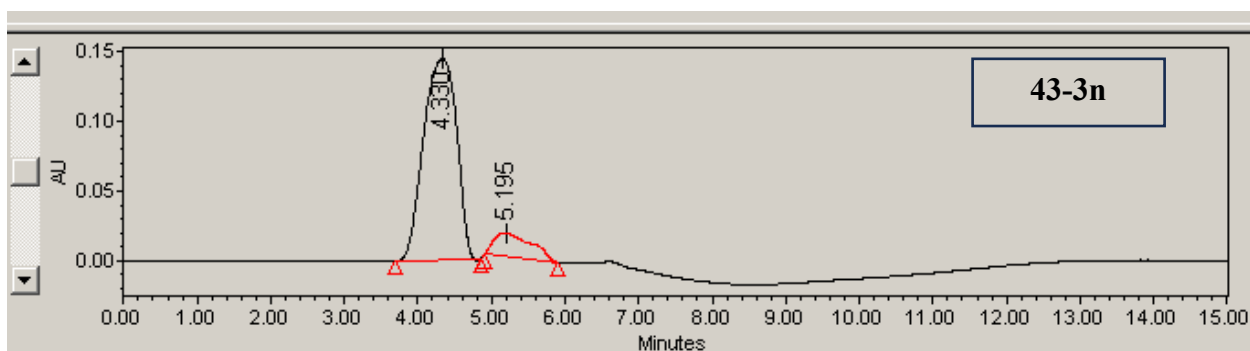
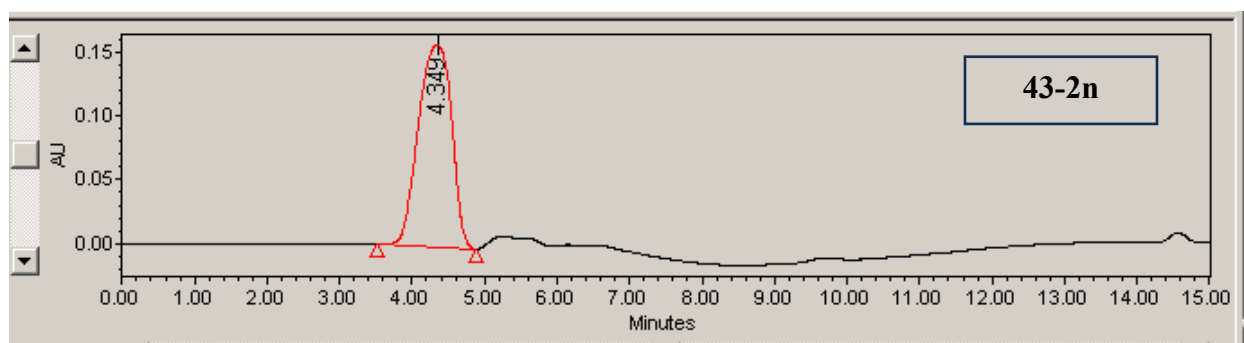


Figure 43. Ultra high-performance liquid chromatography profile of *Brevibacillus borstelensis* AK1 active fractions (43-2n and 43-3n) obtained after second fractionation by column chromatography.

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Publication

1. Abdulsalam, L., Mordecai, J., & Ahmad, I. (2025). Non-viral gene therapy for Leber's congenital amaurosis: progress and possibilities. *Nanomedicine (London, England)*, 20(3), 291–304. <https://doi.org/10.1080/17435889.2024.2443387>
2. Mahmoud, E. H. M., Abdulsalam, L., Moustafa, E. H., Alhanbali, I. A., Ullah, S., & Ahmad, I. (2025). Anti-Protozoan Applications of the Biogenic Nanoparticles and Their Mechanism of Action. In *Expanding Nanobiotechnology: Applications and Commercialization* (pp. 241-284). CRC Press.
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