Biochemical and Functional Characterization of a Narrow-Spectrum Antibacterial Peptide from the Wild-type *Bacillus* sp.

THESIS

Submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

AJAY GHOSH CHALASANI

Under the Supervision of

UTPAL ROY, Ph.D.

&

Co-supervision of

ANGSHUMAN SARKAR, Ph.D.

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA
NOVEMBER 2017

Birla Institute of Technology and Science (BITS),

Pilani-K.K. Birla Goa Campus, Zuarinagar, Goa 403726, India

CERTIFICATE

This is to certify that the thesis entitled **"Biochemical and Functional Characterization of a Narrow-Spectrum Antibacterial Peptide from the Wild-type *Bacillus* sp."** submitted by **Ajay Ghosh Chalasani**, ID No. **2011PHXF0015G** for award of Ph.D. degree of the Institute embodies original work done by him under my supervision.

Utpal Roy

Supervisor

UTPAL ROY, Ph. D

Associate Professor
(Department of Biological Sciences)
Date: 08/3/2018

Co-supervisor

ANGSHUMAN SARKAR, Ph.D.

Associate Professor
(Department of Biological Sciences)
Date: 8/3/18

Dedicated to
Alexandra Elbakyan

ACKNOWLEDGEMENTS

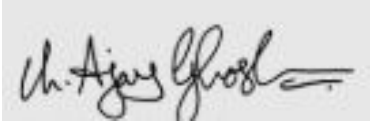
I express my deep sense of gratitude and obeisance to my Mentor and Supervisor **Dr. Utpal Roy**, Associate Professor at the Department of Biological Sciences, BITS-Pilani K.K Birla Goa Campus, for his extremely valuable guidance to carry out the thesis work towards the successful completion. It goes without saying that without his heartfelt endeavor, unstinting effort and constant persuasion this doctoral thesis could never ever have seen the daylight. It has been the greatest learning experience in my life. I would also like to acknowledge the co-supervisor Prof. Angshuman Sarkar, Associate Professor at the Department of Biological Sciences for his kind help.

I would like to register my heartfelt thanks to the Hon'ble Vice Chancellor Professor Souvik Bhattacharya, the campus Director Professor G. Raghurama, Hon'ble Ex-Vice Chancellor Prof. B.N. Jain, Prof. S.K. Verma, DEAN, RCD BITS-Pilani, Prof. P.K. Das campus Associate Dean ARD, Prof. Sunil Bhand Dean SRCD, BITS-Pilani K.K Birla Goa Campus, Prof. Judith Braganca, Head, Department of Biological Sciences, Prof. Meenal Kowshik, former HOD, DAC members Prof. Vijayashree Nayak and Prof. Anasuya Ganguly, Department of Biological Sciences, DRC Convener Dr. Utpal Roy and Prof. S. D. Manjare, former in-charge, RCED, BITS-Pilani K.K Birla Goa Campus, for all their kind support. I also express sincere thanks to the all faculty of the Department of Biological Sciences, BITS-Pilani Goa campus for their support during the study. A special thanks to Prof. Ramkrishna Sen, IIT Kharagpur and Dr. Gunaseelan, IIT Kharagpur for letting me use the facilities at Indian Institute of Technology (IIT) Kharagpur and helping me in standardizing the HPLC based purification. I am indebted to them. Special thanks, are also due to Dr. Nicholas Smarigiaaso and Dr. Denis Morsa, GIGA Proteomics facility, Belgium for helping the analysis of the peptide sequences.

My thanks, are also due to non-teaching staff Mrs. Kamna Upadhyay, Mr. Mahadeo Shetkar, Mr. Mahaling and Mr. Prathap for their constant help. Thanks are due to research scholars Chandra Sekhar Chanda, Kabilan Mani, Bhakti Salgaonkar, Shruti Balaji, Vidhya Prabhudesai, Sivaraman, Raeesh, Vilas, Kshipra, Gowdaman, Ketaki, Celisa, Archana, Priyanka, Mukund, Rachana, Manash, Divya, Nupur, Akriti, Chitra, Mansi, Om Prakash, Angela, Bharani, Rakesh, Ansie, Rehan, Gautam Krishnan, Gautam Baboo, Joseph, Jiss, Yogesh, Amal Raj, Mr. & Mrs. Saini, Venkatesan, Anu Roshni. I

would like to mention the support provided by my neighbors Saif, Ashu, Praveen, Dayananda, Shamanth, Bharath and Selvaganapathy during the course of my stay. I would take this opportunity to thank all the support staff of BITS Pilani, Goa campus for all the support without which it wouldn't have been possible to complete my work.

A special mention to my batch mates Geethanjali, Pallavee, Jigni, Ram, Rajesh, Meghanath and Monsoor who have been helpful numerous times. Thanks to Master students who worked with our group Vidhya, Riaz, Krittika, Kanika and Namitha for their help. Thanks to DBZ Boys Karthik, Ravi, Venky, Sandeep, Rajkumar, Manoj and Mallik. No words would be sufficient to say the support provided by my family away from family the 'BLD group' Ramya Ramachandran, Deepthi Das, Uday Kumar. Special thanks to Dear Dhirendra Singh for all the support. Last but not the least thanks won't be sufficient to the person who supported me emotionally all through my journey and is just a message away to call me anytime, my Dear friend Bhavani. Of course, this journey wouldn't have been possible without the constant support from all my family members and parents.



(Ajay Ghosh Chalasani)

ABSTRACT

The rapid globalization of multidrug resistant pathogens enforces an urgent need for the discovery of novel antimicrobial agents with narrow-inhibitory spectrum and selective toxicity to address the deficiencies of existing antimicrobial agents. Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. epidermidis* (MRSE) posed a serious challenge to the clinicians due to the ability of microbes to resist the antibiotic therapy (WHO, 2017).

S. aureus is frequently isolated from human skin and wounds as a common pathogen, while *Staphylococcal* biofilms and toxins evade the host immune system that lead to recurring or chronic infections delaying wound healing. The emergence of MRSA, a 'superbug' against the topical drugs, including daptomycin, mupirocin and fusidic acid is a significant public health challenge that requires therapeutic alternatives. Antimicrobial peptides (AMPs) are advantageous over conventional antibiotics because bacteria are less prone to develop drug-resistance due to the AMP's characteristic mode of action and the genes that encoded resistance against the AMPs are less prone to mutations **(Sang & Blecha 2008)**. On the other hand, limitations of natural AMPs for the treatment of invasive infections include host toxicity, protease degradability, serum binding, and loss of activity at alkaline pH or in presence of salts (at physiological concentration) that restrict their use in therapeutics. For utilizing naturally-derived AMPs, particularly for treatment of invasive infections, including host toxicity, protease degradation, serum binding, loss of antimicrobial activity in presence of salts (at physiological concentration) restrict the use of AMPs. In order to transform AMPs into novel therapeutic molecule these limitations need to be addressed. One way which is gaining momentum is the utilization of AMPs as topical antibacterial agents.

As a result of a systematic initial screening, a gram-positive bacterial isolate was recovered with a consistent antibacterial activity against *S. aureus* and *Micrococcus luteus*. Continuing this investigation further, the cell free supernatant obtained from the growth of the isolated *Bacillus* sp. showed significant antimicrobial activity against multidrug-resistant strains of *S. aureus, S. epidermidis*, *Streptococcus pyogenes* and *Enterococcus faecalis* when tested by cut-well agar assay. The test strain in the study was subsequently identified by a battery of biochemical tests, Vitek2 tests, MALDI-TOF

(Matrix-assisted Laser Desorption/Ionization-time of Flight) Biotyper and 16S rDNA sequencing. The MALDI biotyping identified the producer strain as *Bacillus subtilis* with a score of 1.905 which shows the high-level confidence of identification.

Based on the 16S rDNA sequence (945 base pairs), the bacterial strain was identified as *Bacillus atrophaeus* and assigned the GenBank accession number JX156420.1 by the NCBI. The phylogenetic tree analysis using Mega 5.0 has shown the URID 12.1 more closely related to *Bacillus subtilis* with boot strapping for 1000 replicates and displaying for 100.

The antimicrobial activity of the URID 12.1 was tested against the *S. aureus* MTCC 96 as the indicator strain and expressed as Arbitrary Units (AU) per milliliter. The production of the antimicrobial compound started at 8 h of inoculation and reached maximum by 40 h (26.0 mm); however, after 48 h, the antibacterial activity declined gradually. The maximum production of the antimicrobial compound was at the late logarithmic and early stationary phase. At 44 h the antimicrobial value was detected to be 3200 AU/mL.

Cell-free supernatant of the producer strain *B. subtilis* URID 12.1 was tested against 13 gram-positive bacteria, including MRSA, vancomycin resistant *S aureus* (VRSA), *Streptococcus pyogenes*, and Gram-negative bacteria along with *Candida* strains, wherein the antimicrobial activity was observed only against the gram-positive bacteria. It was hypothesized that the antimicrobial peptide (ASP–1) could be purified to homogeneity for its identification and evaluation of its antimicrobial efficacy and cytotoxic property. The objectives were focused on the identification and elucidation of the secondary structure of ASP–1, functional characterization of ASP–1 activity against the MRSA strains and other gram-positive bacteria by estimating the MIC values, evaluation of the anti-biofilm efficacy and cytotoxicity against the mammalian cell lines and human erythrocytes.

Subsequently a strong anti-methicillin resistant *Staphylococcus aureus* (MRSA) peptide designated as ASP-1 from *Bacillus subtilis* URID 12.1 strain active against cefoxitin and methicillin resistant clinical isolates was purified to homogeneity by solvent extraction, silica gel-based adsorption chromatography and reversed phase High

Performance Liquid Chromatography. The sequence of the purified ASP-1 by MALDI-TOF/MS and ESI-FTICR MS revealed it as acetylated Phe-Thr-Ala-Val-Dhb-Phe-Ile/Leu. The peptide was further analyzed by alkaline hydrolysis, ESI-Q-TOF-MS and ion mobility assay which detected the presence of lactone ring in the intact peptide and its cyclic nature subsequently revealing the peptide sequence of the linearized peptide as acPhe-Leu-Phe-Thr-Val-Ala-Dhb. Based on the molecular mass (804.5 Da), peptide sequence and amino acid composition, the ASP-1 was identified as lactone-ring containing peptide similar to TL-119, a poorly studied cyclic depsipeptide. The circular dichroism spectroscopy study revealed its predominantly random structure in aqueous solution and β-sheet conformation in methanol. The cyclic 7-mer peptide (with an unusual amino acid) ASP-1 exhibited random and β-sheet conformation in aqueous solution, heat-stability, resistance to proteolytic (tryptic) digestion, salt-tolerance (at physiological concentration) with strong anti-biofilm forming and antibiotic potential. This peptide exhibited strong anti-*Staphylococcal* activities against cefoxitin-resistant MRSA (11), MRSE (1), *S. aureus* isolate (1) and ATCC strains (5) with low MIC values as compared to the MIC values produced by oxacillin.

The investigation also revealed that ASP-1 possesses strong anti-MRSA activities against *mecA, IS256* and *ica*-positive MRSA clinical isolates at geometric mean MIC values of 26.5 µg/ml. The MIC values of the purified peptide against *S. aureus* and MRSA ranged from 1 to 64 µg/ml. At sub-MIC and 1X MIC concentrations, ASP-1 showed strong anti-biofilm forming characteristic. It is worth noting that ASP-1 at a concentration of 128 µg/ml did not show hemolytic activity, and less cytotoxicity was observed when tested against hepatic carcinoma (HepG2) and breast carcinoma (MCF-7) cell lines at the same concentration. Taken together, ASP-1 with its anti-MRSA and anti-biofilm forming prowess, non-hemolytic and less-cytotoxic properties was not reported earlier. These findings suggest that it may serve as a lead molecule for developing into an alternate topical antibacterial agent.

BRIEF CONTENTS

TABLE of CONTENTS

LIST of TABLES

LIST of FIGUERS

LIST of ABBREVIATIONS

ABC- ATP-Binding Cassette

ACME- Arginine Catabolic Mobile Element

AIDS- Acquired Immunodeficiency Syndrome

AMP- Antimicrobial peptide

arc- Arginine Catabolism

ATCC- American Type Culture Collection

AU- Arbitrary Unit

BCA- Bicinchoninic Acid

BHI- Brain Heart Infusion

BLIS- Bacteriocin-Like Inhibitory Substances

bp- base pair

BSA- Bovine Serum Albumin

CA- Community Acquired

CA-MHB- Cation Adjusted Muller-Hinton Broth

CCS- Collision Cross Section

CD- Circular Dichroism

CFS- Cell Free Supernatant

CFU- Colony Forming Units

CID- Collision Induced Dissociation

CL- Cardiolipin

CLSI- Clinical and Laboratory Standards Institute

CRA- Congo Red Agar

DMEM- Dulbecco's Modified Eagle's Medium

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic Acid

EC- Effective Concentration

ELISA- Enzyme-Linked Immunosorbent Assay

EPS- Extracellular Polymeric Substance

ESI-FTICR MS- Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

FBS- Fetal Bovine Serum

FDA- Food and Drug Administration

GM- Geometric Mean

GRAS- Generally Recognized as Safe

GRAVY- Grand average of hydropathy

HaCat- Human keratinocytes

HCCA- α-Cyano-4-Hydroxycinnamic Acid

HepG2- Human Liver Epithelial Carcinoma cells

HGT- Homologous Gene Transfer

IC- Inhibitory Concentration

ica- Intercellular Adhesion

IDSA- Infectious Diseases Society of America

LAB- Lactic Acid Bacteria

LR- Log10 Reduction

m/z- mass-to-charge

MALDI-TOF/MS- Matrix-Assisted Laser Desorption/Ionization-Time of Flight/ Mass Spectrometry

MBC- Minimum Bactericidal Concentration

MCF- Michigan Cancer Foundation

MDR- Multi Drug Resistant

MHB- Mueller-Hinton Broth

MIC- Minimum Inhibitory Concentration

MRSA- Methicillin-Resistant *Staphylococcus aureus*

MRSE- Methicillin-Resistant *Staphylococcus epidermidis*

MSSA- Methicillin-Sensitive *Staphylococcus aureus*

MTCC- Microbial Type Culture Collection

mTSB- modified Tryptone Soya Broth

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NADP- Nicotinamide Adenine Dinucleotide Phosphate

NCBI- National Center for Biotechnology Information

NCIM- National Collection of Industrial Microorganisms

NMR- Nuclear Magnetic Resonance

NRPSs- Non-Ribosomal Peptide Synthases

OD- Optical Density

PAGE- Polyacrylamide Gel Electrophoresis

PBP- Penicillin-Binding Protein

PBS- Phosphate Buffered Saline

PC- Phosphatidylcholine

PCR- Polymerase Chain Reaction

PE- Phosphatidylethanolamine

PG- Phosphatidylglycerol

PIA- Polysaccharide Intercellular Adhesin

PKs- Polyketides

PS- Phosphatidylserine

PSMαs- α-type Phenol-Soluble Modulins

PTS- phosphotransferase Systems

PVL- Panton-Valentine Leukocidin

RBCs- Red Blood Cells

rDNA- ribosomal Deoxyribonucleic Acid

RNA- Ribonucleic Acid

RP-HPLC- Reversed Phase High Performance Liquid Chromatography

rRNA- Ribosomal Ribonucleic acid

SCC- Staphylococcal Chromosome Cassette

SDS- Sodium Dodecyl Sulphate

SDS-PAGE - Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SM- Sphingomyelin

TFA- Trifluoroacetic Acid

TFE- Trifluoro Ethanol

TI- Therapeutic Index

TLC- Thin Layer Chromatography

TTC- Triphenyl Tetrazolium Chloride

UV- Ultra Violet

VISA- Vancomycin Intermediate *Staphylococcus aureus*

VRSA- Vancomycin Resistant *Staphylococcus aureus*

WHO- World Health Organisation

"What you see is that the most outstanding feature of life's history is a constant domination by bacteria"

-Stephen Jay Gould

1.1 INTRODUCTION:

Antimicrobial peptides (AMPs) are an important constituent of the immune system and are evolutionarily conserved in different classes of life ranging from prokaryotes to mammals **(Zasloff 2002; Boman 1995)**. The discovery and introduction of antibiotics for the treatment of human disease had played an important role in reducing the suffering and mortality. However, the excessive use of antibiotics has led to the development of resistance in the microorganisms. Resistance to antibiotics greatly reduces the efficiency of treatment and leads to a fatal outcome **(Andersson & Hughes 2010)**.

In terms of phylogenetics, *Bacillus* genus bacteria belong to the bacilli class of the phylum *Firmicutes*. Bacteria belonging to the genus *Bacillus* are generally rod shaped, aerobic, positive for Gram staining, catalase producing and endospore forming. They are omnipresent and distributed over a range of environments ranging from soil, vegetation, aquatic environments and gastrointestinal tracts of various animals **(Nicholson 2002)**. Their ability to survive and multiply in both favorable and harsh conditions is shown by their ability to produce endospores, diverse physiological properties and their growth requirements. Moreover, *Bacillus* species are nitrogen fixers, oxidizers and reducers of manganese, heterotrophic nitrifiers, facultative chemolithotrophs, denitrifiers, psychrophiles, iron precipitators, acidophiles, alkalophiles and thermophiles among others **(Priest 1993; Slepecky & Hemphill 2006)**. *Bacillus* species could colonize a variety of habitats due to their phenotypic and genotypic diversity **(Priest 1993; Slepecky & Hemphill 2006)** which allow them to exhibit diverse physiological properties such as ability to utilize substrates from plant, animal sources and also biofuels **(Lutz et al., 2006)**.

Bacillus is considered to be a potentially useful genus to investigate for antimicrobial activity since *Bacillus* species are capable of producing a large number of peptide antibiotics representing a variety of basic chemical structures. The production of

bacteriocins or bacteriocin-like substances has been described for *Bacillus subtilis, Bacillus cereus, Bacillus stearothermophilus, Bacillus megaterium* and other *Bacillus* species (**Tagg et al. 1976**).

Bacteria belonging to the *sensu lato* group of *Bacillus* produce antimicrobial compounds that include peptide and lipopeptide antibiotics in addition to bacteriocins **(Stein 2005)**. It has been mentioned earlier that cadres of the genus *Bacillus* are versatile and prolific producer of a wide arsenal of antimicrobial compounds. The natural properties of forming spores and producing antimicrobial substances are considered to possibly play an important role in the survival of *Bacillus* in hostile environments. Lantibiotics, a type of post-translationally modified peptides that are also widely distributed in different bacterial species constitute for most of the bacteriocins produced by *Bacillus*. Many of the bacteriocin producing strains show their activity against a narrow spectrum of bacteria **(Naclerio et al., 1993; Lee et al., 2001)**, however, there are some strains that produce broad spectrum bacteriocins that act against important pathogens such as *Streptococcus pyogenes* **(Cherif et al., 2001)** and *Listeria monocytogenes* **(Oscáriz & Pisabarro 2000)**.

1.1.1 Antimicrobial peptides produced by *Bacillus* Species:

In Nature, many Gram-positive bacteria are armed by the genome arsenal to produce a large variety of antimicrobial peptides. These diverse arrays of antimicrobial substances having differences in their chemical structures **(Gebhardt et al., 2002; Stein 2005)** enable them to subsist in the danger-infested ecological niches by wrestling against other highly competitive species, and creating a discernible benefit for their producers. Beneficial bacteria that can be potentially used for the intestinal disorder treatment include *Lactobacillus, Clostridium butyricum, Bifidobacterium, Bacillus subtilis, Lactobacillus sporogenes,* and *Bacillus polyfermenticus*. Live strains of *B. polyfermenticus*, in the form of active endospores have been used to treat long-term intestinal disorders as they find it easy to reach the intestine **(Lee et al., 2001)**.

Bacteriocins are bacterial antimicrobial peptides that are synthesized ribosomally. They are proteins or protein complexes showing antibacterial activity against species that are related closely to the producer strains **(Tagg et al., 1976)** and are produced by every

major lineage of bacteria **(Riley & Wertz, 2002 a, b)**. Even though, bacteriocins have target specificity towards bacteria related to their producers, there are many having wider spectrum **(Jack et al., 1995)**. The proteinaceous nature of the bacteriocins allow them to be degraded in the gastrointestinal tract, paving the way to use them as preservatives in the food industry **(Cleveland et al., 2001)**. Most of the currently researched bacteriocins are produced by lactic acid bacteria (LAB), and few of them are used in the food industry as bio preservatives and regarded as 'generally recognized as safe' (GRAS) **(O'sullivan et al., 2002)** and *Bacillus* derived bacteriocins have been safely used in the food industry **(Pedersen et al., 2002)**. Several other antimicrobial compounds that were not characterized completely were termed bacteriocin-like inhibitory substances (BLIS). When it comes to bacteriocins produced by *Bacillus* it is necessary to verify their ribosomal synthesis, since *Bacillus* are known to produce nonribosomally synthesized antimicrobial peptides such as fengycins, and iturins. Many bacteriocins from the genus *Bacillus* have been reported **(Tagg et al., 1976)** and among them the best characterized are subtilin produced by *B. subtilis* **(Jansen and Hirschmann 1944; Banerjee and Hansen 1988),** megacin produced by *B. megaterium* **(Von Tersch and Carlton 1983),** bacteriocins such as thuricin produced by *B. thuringiensis* **(Favret and Youston 1989; Paik et al., 1997)** and cereins produced by *B. cereus* **(Naclerio et al., 1993)**. Despite the importance of bacteriocins produced by *Bacillus* they were not extensively explored. Screening of a *Bacillus* producing a new bacteriocin is of major interest in the research of bacteriocins.

Bacteriocins and BLIS produced by the bacteria belonging to the genus *Bacillus* are considered to be the second most important next only to the LAB produced bacteriocins. However, till date no classification scheme has been developed despite the efforts to classify them along with LAB bacteriocins. This might probably due to non-availability of amino acid sequence information of these bacteriocins or lack of significant diversity among the bacilli produced antimicrobial peptides/proteins. Some of the antimicrobial peptides were included already reported groups or among the classes of bacteriocins from LAB, for example Lantibiotics a unique class of *Bacillus* AMPs were included in Class I of LAB bacteriocins **(Drider et al., 2006; Franz et al., 2007; Nes et al., 2007)**. Lantibiotics are synthesized ribosomally as precursor peptides before undergoing post translational modifications such as threonine and serine dehydration

followed by intramolecular addition to cysteine to form the Lantibiotics characteristic (β-methyl) lanthionine thioether bridges **(Chatterjee et al., 2005; Willey & van der Donk 2007; Bierbaum & Sahl 2009)**. Several bacteriocins/BLIS such as pediocin-like bacteriocins and the two-peptide bacteriocins produced by *Bacillus* are classified within LAB Class II bacteriocins **(Klaenhammer, 1993; Nes et al., 2007)**.

1.1.2 Classification of *Bacillus* Bacteriocins:

Some of the bacteriocins produced by the genus *Bacillus* belong to lantibiotics **(Willey & van der Donk 2007; Asaduzzaman & Sonomoto 2009; Bierbaum & Sahl 2009)**. Previously Lantibiotics were classified into two different types: type A consists positively charged and elongated Lantibiotics, while the noncharged and globular Lantibiotics were included in type B **(Jung & Sahl 1991)**. Based on the enzymes involved in the maturation, type A Lantibiotics were sub grouped into AI and AII **(Willey & van der Donk 2007)**. LanB and LanC enzymes were involved in the modification along with a serine protease LanP for processing of Class-A1 lantibiotics. Whereas only one modification enzyme LanM is involved in modifying Class-AII lantibiotics, and an N-terminal protease activity associated ABC-transporter LanT was involved in export and activation. In addition, there is a two component lantibiotic that consists of synergistically acting two post-translationally modified peptides which could be classified into a third Class **(Asaduzzaman & Sonomoto 2009)**. Nevertheless, these two components are modified by a single LanM type enzyme similar to subgroup AII. One of the subunits modifications resembles Mersacidin, which is a prototype of AII subgroup. There are several other lantibiotics such as paenibacillin or sublancin 168 which do not fit in any of the above-mentioned classes. With the ever-increasing number of lantibiotics, it has become a herculean task to build a unified classification platform and several classifications were proposed therefore **(Nes et al., 2007)**.

In the classification proposed by **Abriouel et al., 2010**, there were 3 Classes. The antimicrobial peptides that undergo post-translational modifications were included in Class I, which was further divided into four subclasses. Lantibiotics with modifications such as formation of lanthionine and β-methyl lanthionine residues were included in subclasses I.1-I.3, whereas the others with unique modifications were included in subclass I.4. Lantibiotics like subtilin, ericin S and ericin A with an elongated structure were

included in subclass I.1. Lantibiotics with type B globular structure such as mersacidin, and others like paenibacillin and sublancin 168 were included in subclass I.2. Two component lantibiotics such as lichenicidin and haloduracin were included in subclass I.3. While the unique cyclic peptide subtilosin A that contains sulfide bridges formed between cysteine residues, head-to-tail peptide bond and dehydrated amino acids were included in subclass I.4. The clustering of the published amino acid sequences by comparative analysis is in acceptance with the above classification **(Abriouel et al., 2010)**.

Ribosomally synthesized, heat and pH stable, nonmodified and linear peptides were included in Class II bacteriocins. This class was subdivided into three subclasses. Peptides with a conserved YGNGVXC motif near to N-terminus like pediocin were included in subclass II.1 along with *B. coagulans* I_4 that produced coagulin, *Paenibacillus polymyxa* strains (SRCAM 37, SRCAM 602, SRCAM 1580) and *Bacillus circulans* produced several bacteriocins of this subclass. Antimicrobial peptides like thurincin H, bacthuricin F4, thurincins S and 17 produced by *B. thuringiensis* strains, and *B. cereus* produced cerein MRX1 which has a conserved DWTXWSXL motif near the N-terminus were included in subclass II.2. A coherent cluster was observed on comparative analysis of amino acid sequences of these thuricin like peptides **(Abriouel et al., 2010)**. Other linear peptides like cereins 7A and 7B, and *B. licheniformis* produced lichenin were included in subclass II.3.

Large proteins (>30 kDa) such as *Bacillus megaterium* produced megacins A-216 and A-19213 with phospholipase activity were included in Class III. Several other antimicrobial polypeptides of larger size and intermediate size (10-30 kDa) produced by bacilli but lacking protein or gene sequences were not included in this classification, while they may be considered as BLIS. Table 1 summarizes the classification of antimicrobial peptides and bacteriocins produced by many species belonging to the genus *Bacillus*.

Table 1: Classification of *Bacillus* Antimicrobial Compounds (Abriouel et al., 2010)

<table>
<tr><th>Class I</th><th>Class II</th><th>Class III</th><th>NRPs and Polyketides (PKs)</th><th>Lipopeptides</th></tr>
<tr><td>Ribosomally produced and post translationally modified peptides (RiPPs)</td><td>Unmodified bacteriocins: small (less than 10 kDa), ribosomally synthesized, heat-stable, membrane-active linear peptides</td><td rowspan="5">Large (> 10 kDa) like megacins A-216 and A-19213 that has phospholipase activity</td><td rowspan="5">PKs represent a group of secondary metabolites, exhibiting structural and functional diversity. Possess a wealth of pharmacologically important activities, including antimicrobial, antifungal, antiparasitic, and antitumor properties</td><td rowspan="5">7-amino-acid surfactin containing lipopeptide antibiotics are produced by some Bacillus subtilis. Quorum-sensing control mechanisms are involved in surfactin synthesis. Large multienzyme complexes are involved in the synthesis of amphipathic lipopeptides with 1028 to 1084 Da</td></tr>
<tr><td>Subclass I: Lanthipeptide</td><td>Subclass I: Pediocin-like peptides; antilisterial peptides that have a YGNGVXC consensus motif; Coagulin produced by B. coagulans I₄ is a peptide of 44 residues with an amino acid sequence similar to that described for pediocins AcH and PA-1.</td></tr>
<tr><td>Subclass II: Head to tail cyclized peptides</td><td>Subclass II: Antimicrobial peptides with a conserved DWTXWSXL motif near the N-terminus</td></tr>
<tr><td>Subclass III: Sactipeptides</td><td rowspan="2">Subclass III: Other linear peptides like cereins 7A and 7B</td></tr>
<tr><td>Subclass IV: Linear azole containing peptides</td></tr>
</table>

Subclass V: Thiopeptides; Thiazolyl peptides are highly modified via either non-ribosomal or ribosomal assembly, with a 6- membered nitrogenous macrocycle being central of piperidine/pyridine/dehydropiperidine and including additional thiazoles and dehydrated amino acid residues. Example, Thiocillins, found in the producer *B. cereus* ATCC 14579, at least 10 and up to 13 of the 14 C-terminal residues undergo post translational modification to generate a set of 8 related antibiotics.				molecular mass
Subclass VI: Glycocins; Sublancin 168, produced by *B. subtilis* with a β-S-linked glucose moiety attached to cysteine22 and two disulfides				
Subclass VII: Lasso peptides: plantazolicin A and B produced by *B. amyloliquefaciens* and *B. methylotrophicus;* *LAPs* RiPPs with a distinguishing heterocyclic ring of oxazoles and thiazoles derived from serine/ threonine and cysteine by enzymatic cyclodehydration and dehydrogenation.				

1.2 REVIEW OF LITERATURE:

1.2.1 Antimicrobial Peptides:

Antimicrobial peptides are comparatively short (12-100) amino acids in length, amphiphilic in nature, positively charged and are produced as a first line of defense by a wide range of organisms **(Martin et al., 1995; Wang & Wang 2004)**. Cationic peptides have a variety of secondary structures and the sequence homology is very less in spite of having a common function. The most common among secondary structures are amphiphilic peptides with 2-4 β-strands, loop structures, amphipathic α-helices and extended structures **(Boman 1995; Hancock 1997)**. Bacterial AMPs were among the first to be isolated and characterized **(Mattick et al., 1947)**. They help in the survival by inhibiting the other bacteria present in the same niche and compete for the nutrients. Bacterial AMPs, commonly called bacteriocins produced by most of the bacteria are very competent when compared to the AMPs from eukaryotes **(Klaenhammer 1988; Riley 1998)**. All the cationic AMPs must interact with the cytoplasmic membrane irrespective of their actual target **(Hancock & Rozek 2002)**. Secondary structure of the peptide was mostly observed only when interacting with the membranes; e.g., the random structure of bovine neutrophil indolicidin in aqueous environment changes to boat-like confirmation when interacting with membranes **(Rozek et al., 2000)**.

1.2.2 Mechanism of Action of Antimicrobial Peptides:

The mechanism of membrane permeation and disruption by AMPs depends on several parameters like the sequence of amino acids, membrane lipids and more importantly the peptide concentration. All the AMPs interact with the target organism membranes and are broadly divided in to two classes: 1) membrane disruptive (barrel stave, carpet, toroidal and micellar aggregate models) and 2) non-membrane disruptive (intracellular targets) **(Powers & Hancock 2003)**. Bacterial membranes are negatively charged with lipids having phospholipid headgroups such as phosphatidylglycerol (PG), cardiolipin (CL), or phosphatidylserine (PS). However, mammalian membranes are enriched with zwitterionic phospholipids such as phosphatidylethanolamine (PE), sphingomyelin (SM) or phosphatidylcholine (PC) **(Yeaman & Yount 2003)**. Additionally, cholesterol which is present as a major constituent in the cellular membranes of the mammals reduces the AMPs activity either by lipid bilayer

stabilization or by direct interaction with the AMPs and neutralizing them **(Matsuzaki 1999)**.

1.2.2.1 Peptide insertion and membrane permeability:

1.2.2.1.1 Barrel-Stave Model:

In the 'barrel-stave model' **(Fig. 1)**, a central lumen is formed in the membrane by the arrangement of peptide helices into a bundle, like a barrel of staves composed of helical peptides **(Yang et al., 2001)**. The lipid core of the bilayer will be aligned by the hydrophobic peptide region and the interior region of the pore is formed by hydrophilic regions of the peptide. The peptide aggregation equilibria and the number of peptides in the aggregate is modulated by lipid compositional changes in the bilayer **(Cantor 2002)**. E.g.: alamethicin.

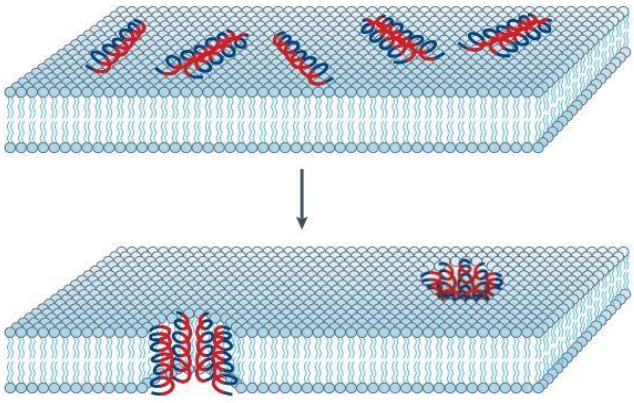


Figure 1: Antimicrobial peptide induced pore formation by barrel stave model. Red color indicated hydrophilic regions and blue the hydrophobic regions **(Brogden 2005)**.

1.2.2.1.2 Carpet Model:

In the 'carpet model' **(Fig. 2)** peptides accumulate parallel to the surface on the bilayer membrane **(Pouny et al., 1992)**. Peptides are electrostatically attracted on to the head groups of anionic phospholipids in the membrane to form a carpet like layer. Bilayer is thought to be disturbed by the surface oriented peptides at a high concentration, causing the formation of micelles **(Shai 1999; Ladokhin & White 2001)**. Toroidal transient holes in the membrane are formed when the peptide concentration reaches a critical threshold paving access for the additional peptides to the membrane. Finally, the bilayer curvature

is disrupted leading to membrane disintegration and forms micelles **(Oren & Shai 1998)**. E.g.: ovispirin

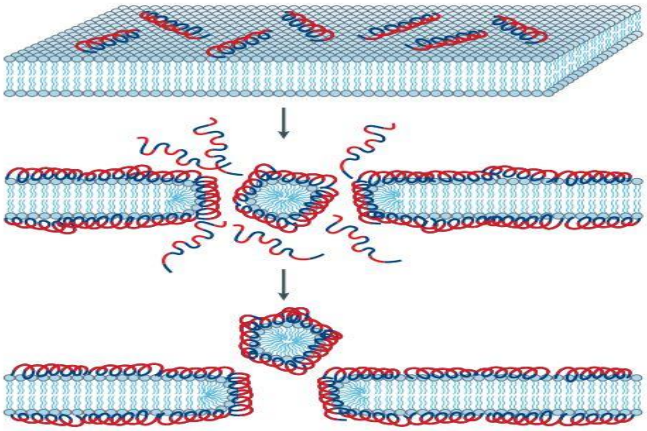


Figure 2: Antimicrobial peptide induced disruption of the membrane by carpet model (Brogden 2005).

1.2.2.1.3 Toroidal-Pore Model:

In the 'toroidal-pore model' **(Fig. 3)**, insertion of the peptide helices in the cell membrane causes the lipid monolayers to bend continuously along the pore in such a way that the inserted peptides and the lipid head groups line the water core. **(Matsuzaki et al., 1996)**. Association of the polar face of antimicrobial peptide with the lipids polar head group is observed in the formation of a toroidal pore. A continuous bend from top to bottom causing a toroidal hole is formed due to connection of two leaflets of the membrane by tilting of the lipids from the lamellar normal; The peptides and the lipid head groups line the pore, which screen and hide the charge on the cationic peptide **(Yang et al., 2001)**. E.g.: magainins, protegrins and melittin

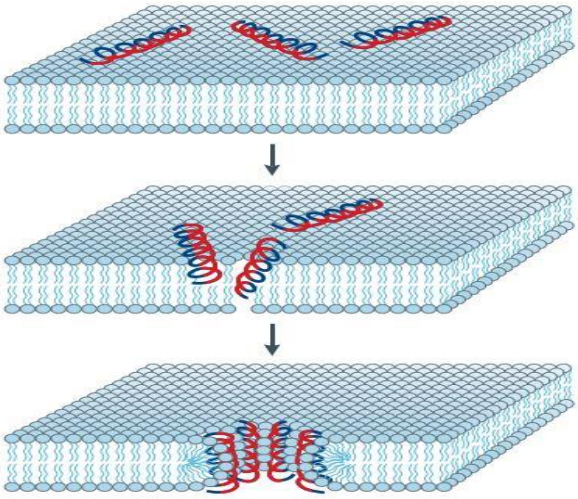


Figure 3: Antimicrobial peptide induced membrane disruption by toroidal model. (Brogden 2005).

1.2.2.2 Intracellular Killing:

The formation of ion channels, membrane rupturing and transmembrane pores lead to the eventual lysis of the microbial cells but this is not the only mechanism of killing microbial cells by antimicrobial peptides. External non-membrane targets like phospholipases and autolysins are activated by the antimicrobial peptides. An autolysin, N-acetylmuramoyl-L-alanine amidase, in *Staphylococcus simulans,* which generally is inhibited by the lipoteichoic and teichuronic acids present in the cell wall, is reactivated by Pep5, an antimicrobial cationic peptide **(Bierbaum & Sahl 1987)**. Ability to traverse the cytoplasmic membrane is an important aspect of the antimicrobial peptide required for the intracellular activity; exclusive mechanisms have been developed for the translocation such as endocytosis, lipid-raft-dependent macropinocytosis and permease/transporter-mediated mechanism. After entering the cytoplasm, the antimicrobial peptides alter the membrane septum formation, inhibit nucleic acid synthesis, cell-wall synthesis, enzymatic activity or protein synthesis **(Table 2, Fig. 4).**

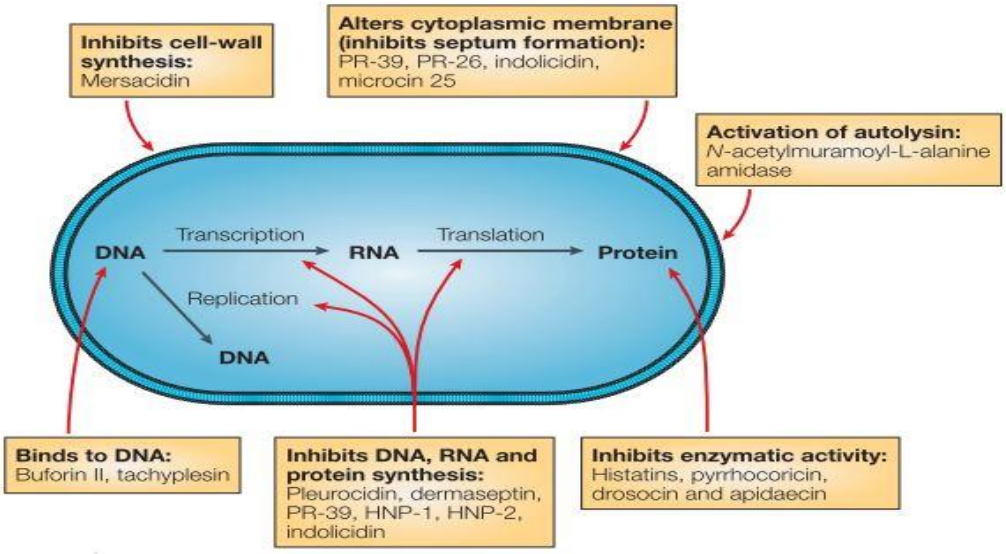


Figure 4: Mechanisms for the intracellular action of AMPs. *Escherichia coli* is used as a model organism in this figure **(Brogden 2005).**

Table 2: Mechanisms of intracellular killing by AMPs (Brogden 2005)

Modes of intracellular killing	Examples of peptides
Intracellular contents flocculation	Anionic peptides
Cytoplasmic membrane septum formation altercation	PR-39, PR-26, indolicidin and microcin25
Inhibits cell-wall synthesis	Mersacidin
Nucleic acids binding	Buforin II and tachyplesin
Nucleic-acid and protein synthesis inhibition	Pleurocidin, PR-39, HNP-1 dermaseptin, and indolicidin
Enzymatic activity inhibition	Histatins, drosocin, pyrrhocoricin and apidaecin

1.2.3 Antimicrobial Peptides from Genus *Bacillus*:

Antimicrobial peptides have a huge role to play in combating the resistance of microbes to antibiotics. Greater than 2000 AMPs have been identified till now from different sources like plants, animals, fungi and bacteria, and some have entered the clinical trials also **(Rotem & Mor 2009; Jenssen et al., 2006)**. Since AMPs are naturally part of the human innate immunity system, the chances of side effects and development of microbial resistance is less when compared to chemical antibiotics. AMPs act by

killing the microbes by pore formation in the membrane whereas the conventional antibiotics act by targeting the metabolic pathways thus making it difficult for the microbes to develop resistance **(Sang & Blecha 2008)**. The genus *Bacillus* is known to produce a wide variety of antimicrobial peptides and a good source in search for new antimicrobial compounds **(Bizani et al., 2005; Xie et al., 2009)**. Bacteria of the genus *Bacillus* are rod shaped, Gram-positive, endospore forming that are available in the soil and produce a variety of secondary metabolites which show a wide spectrum of antimicrobial activity **(Paik et al., 1997; Motta et al., 2008; Li et al., 2012)**. AMPs from *Bacillus* are either small peptides that are synthesized non-ribosomally by enzyme complexes or ribosomally synthesized peptides **(Marx et al., 2001; Li et al., 2012)**.

1.2.3.1 Nonribosomally Synthesized Antimicrobial Peptides:

The non-ribosomal synthesis of antimicrobial peptides in *Bacillus* involves a multi-step mechanism in which selection and condensation of amino acids is done by multienzyme thiotemplates. The process is mediated by nonribosomal peptide synthetases and involves multi subunit enzymes which are as large as 100 to 1600 kDa **(Stachelhaus & Marahiel 1995)**. These nonribosomally synthesized peptides can either be linear or cyclic and are formed from among 300 precursor molecules. These peptides also can contain branched cyclic structures with D-amino acids or L-amino acids, hydroxyl groups and can further be modified by heterocyclic ring formation, acetylation, N-methylation or glycosylation **(Hancock & Chapple 1999)**. A multifunctional enzyme complex gramicidin S synthetase catalyzes the synthesis of gramicidin S. A multifunctional thiotemplate mechanism is used in the synthesis of bacitracin A, a 12-amino-acid peptide antibiotic produced by *Bacillus licheniformis* **(Nakano & Zuber 1990)**. A mechanism different from the multienzyme thiotemplate is involved in the synthesis of a 7-amino-acid surfactin containing lipopeptide antibiotic by some *Bacillus subtilis*. Quorum-sensing control mechanisms are involved in surfactin synthesis along with the control of glucose and glutamine in the fermentation medium **(Nakano & Zuber 1990; Schallmey et al., 2004)**. Large multienzyme complexes are involved in the synthesis of amphipathic lipopeptides with 1028 to 1084 Da molecular mass **(Peypoux et al., 1999; Baindara et al., 2013)**.

A depsipeptide is an oligo or polypeptide that consists of one or more ester bonds along with peptide bonds. Cyclic depsipeptides that occur naturally, due to the diversity in their structure and high potency have attracted high attention in discovery of new antibiotics **(Bionda & Cudic 2011; Giuliani et al., 2007)**. These depsipeptides are biosynthesized nonribosomally and catalyzed by a complex of multifunctional enzymes called as non-ribosomal peptide synthases (NRPSs) **(Marahiel 2009; Sieber & Marahiel 2003)**. Among this class of naturally occurring products, daptomycin a cyclic lipodepsipeptide has been approved in Canada, USA and European Union for the treatment of soft tissue and complicated skin infections that are caused by gram-positive bacteria, that includes MRSA, *Enterococcus faecalis, Streptococcus agalactiae* and *Streptococcus pyogenes* **(Bionda & Cudic 2011; Woodford 2003; Kern 2006)**. Another cyclic depsipeptide telomycin with antibacterial activity was isolated from an unidentified *Streptomyces* fermentation broth **(Misiek et al., 1956)**.

1.2.3.2 Ribosomally Synthesized Antimicrobial Peptides:

Peptide antibiotics synthesized ribosomally are generally cationic in nature, contains 12-50 amino acid residues and are structurally very diverse **(Marx et al., 2001)**. Bacteriocins produced by bacteria are synthesized ribosomally and are generally active against the strains that are related closely to the producer strain **(Motta et al., 2008; Aunpad & Na-Bangchang 2007)**. Bacteriocins from *Bacillus* spp. have broader antimicrobial spectrum when compared to bacteriocins produced by lactic acid bacteria **(Wang et al., 2014a)**. Bacteriocins were first classified into four classes by **Klaenhammer (1993):** class I lantibiotics containing lanthionine, a modified amino acid and are small peptides (<5 kDa), class II cystibiotics are small, heat stable, and membrane-active peptides (<10 kDa) with disulfide bonds that are required for their activity, class III thiolbiotics are high molecular weight (<30 kDa) heat-labile proteins with an active -SH group, and class IV contains complex proteins with one or more carbohydrate or lipid moieties (**Klaenhammer 1993; Rea et al., 2011**).

Lantibiotics are one of the best characterized antimicrobial peptides. These are small and contain different unusual amino acids and are synthesized from different genetic determinants by a variety of mechanisms **(Paik et al., 1998; McAuliffe et al., 2001)**. Post-translational modifications happen in premature peptides by the introduction

of lanthionine and methyllanthione, unusual thioether amino acids along with the removal of leader peptide by proteolysis **(Lawton et al., 2007; Dischinger et al., 2009)**. Mature lantibiotics characteristically has one or more unusual dehydro residues which do not participate in lanthionine bridges may be helpful in designing new biomolecules **(Paik et al., 1998)**.

Subtilosin A, a ribosomally synthesized, post translationally modified bactericin peptide was isolated from a wild type *Bacillus subtilis* 168 and has a distinct structure indicating that it belongs to a unique class of bacteriocins **(Kawulka et al., 2003; Shelburne et al., 2007; Sutyak et al., 2008)**. This cyclic bacteriocin unlike other cationic membrane disrupting bacteriocins is anionic **(Huang et al., 2009)**. Tyrothricin is a complex mixture of antibiotics contains 28 different non-ribosomally produced tyrocidines and tryptocidins along with 9 linear gramicidins that are synthesized ribosomally was reported to be produced by *Brevibacillus brevis* **(Rautenbach et al., 2007)**.

1.2.3.3 Recently Identified Antimicrobial Peptides from *Bacillus* species:

The soil organism *Bacillus subtilis* is known to produce a variety of potential antibiotics that are synthesized both ribosomally and non-ribosomally **(Stein 2005)**. *B. subtilis* SC-8 (BSSC8), isolated from fermented soybean paste Cheonggukjang produces a lipopeptide like AMP BSAP-254 that is 3.4 kDA with likely 36 amino acids and lipid moieties **(Lee et al., 2010; Lee et al., 2011; Yeo et al., 2011)**. Another isolate *B. subtilis* subsp. *spizizenii* DSM15029^{T}, produces entianin an unsuccinylated AMP that differs at 3 amino acid positions from subtilin and shows activity against *S. aureus*, *Enterococcus faecalis* and other Gram-positive pathogens **(Fuchs et al., 2011)**.

B. cereus is a Gram-positive bacterium that is widely distributed in the environment and can be isolated from a variety of food items like cereals, dairy products, spices and meats. Different *B. cereus* strains produce a variety of AMPs including cerein 7, cerecidin, cerein 8A and cerein 8B **(Naclerio et al., 1993; Oscáriz et al., 1999; Wang et al., 2014a)**. Cerecidins, *Bacillus cereus* produced novel class of lantibiotics were recently identified (**Wang et al., 2014b)**. These antibiotics contains *cre* locus that has 7 structural genes and differs from those that are present in lantibiotics class II (**Wang et al., 2014b**).

Bacitracin, was the first peptide antibiotic derived from the *Bacillus licheniformis* that revealed good safety profile and has been extensively used in medical and veterinary applications **(He et al., 2006)**. An antimicrobial substance produced by *B. licheniformis* DSM 13 strain resembles lichenicidin, a 2-peptide lantibiotic and shows homology with mersacidin. It shows activity against MRSA and a range of Gram-positive bacteria, but it neither shows hemolysis nor activity against Gram-negative bacteria **(Dischinger et al., 2009)**.

Bacteriocins produced by *B. thuringiensis* have shown anticancer activities along with antifungal and antibiotic activity **(Cherif et al., 2003)**. Partial characterization of several bacteriocins from *B. thuringiensis* have been achieved such as, *B. thuringiensis* subsp. *thuringiensis* HD2 produced thuricin; *B. thuringiensis* B439 produced thuricin B439; *B. thuringiensis* subsp. *tochigiensis* HD868 produced chicin; and bacthuricin F4, produced by a local isolate **(Paik et al., 1997; Ahern et al., 2003; Kamoun et al., 2005; Cherif et al., 2008)**. Tolworthcin 524, produced by *B. thuringiensis* subsp. *tolworthi* is a bacteriocin that has high identity with thuricin 17 and hurincin H **(Pacheco-Cano et al., 2014)**.

Subtilosin isolated from *B. amyloliquefaciens* KATMIRA1933 strain has shown antiviral property and it's the first instance of this compound showing antiviral property **(Torres et al., 2013)**. A significant antimicrobial activity against Gram-positive, Gram-negative organisms and fungal strains was shown by a mixture of the nonribosomally synthesized surfactin, fengycin, pumilacidin and mojavensin produced by a marine water isolate *Bacillus mojavensis* A21 **(Ayed et al., 2014)**. A marine soil isolate *Bacillus sonorensis* MT93 produced a novel AMP sonorensin, belonging to the heterocyclonantracin subfamily of bacteriocins. It has a molecular mass of 6.27 kDa and shown activity against pathogens like *S. aureus* and *L. monocytogenes* **(Chopra et al., 2014)**.

1.2.3.4 *Bacillus* Species AMPs and their Potential Applications:

The *Bacillus* species have high industrial importance due to their safety record and fast growth rates to go along with their ability to secrete high quantities of proteins into the extracellular medium **(Schallmey et al., 2004; Benitez et al., 2010)**. Many *Bacillus* species were used as probiotics in animals and humans **(Cutting 2011)**. *B. pumilus*

WAPB4 produces pumilicin 4, a novel bacteriocin that inhibits Gram-positive bacteria including MRSA and VRE **(Aunpad & Na-Bangchang 2007)**. *Bacillus* species were reported to inhibit *Listeria monocytogenes*, an emerging pathogen that affects the children, pregnant women, elderly people and AIDS patients **(Sabaté & Audisio 2013)**.

Bacillus species AMPs have broad activity and rapidly kill various pathogens. However, further studies need to be carried out on their toxicity, mode of action and immunogenicity for their safe and effective use in humans. Cytotoxicity of these AMPs against mammalian cell lines and the hemolytic activity against red blood cells (RBCs) need to be thoroughly investigated before drawing any conclusions. Detailed studies leading to comprehensive understanding of the chemical properties, structure, mode of action and synthesis of these AMPs should be conducted as they have the potential to combat the problem of treating infections caused by multi-drug resistant pathogens.

1.2.4 Antimicrobial Resistance:

Acquiring of resistance against antimicrobial peptide by sensitive strain is formidably difficult when compared to that against conventional antibiotics. Resistance against AMPs requires profound changes in the structure of membrane **(Zasloff 2002)** due to greater diversity among AMPs. Nonetheless, resistance to AMPs is innately observed in certain pathogens because of the stable structural and functional properties or pathogenesis approaches. For example, prevention of peptide binding on the outer membrane by expressing less density of acidic lipids is observed in resistant species of the genera *Morganella* and *Serratia.* Secretion of proteases that can destroy the peptides by digestion is observed in species like *Porphyromonas gingivalis* **(Zasloff 2002)**. Elongation of capsule to evade the phagocytosis by opsonization is observed in some pathogenic bacteria. For example, highly anionic capsular exopolysaccharide is produced by virulent strains of *Pseudomonas aeruginosa* **(Friedrich et al., 1999)**.

Since 1930s with the clinical use of penicillin and sulphonamide antibiotics bacterial resistance started to develop. From then, there has been a steady rise in the resistance to almost all the classes and analogues of antibiotics that were marketed. This led to previously effective treatments being abandoned (example, penicillin against staphylococcal infections and ampicillin against cystitis treatment), which in turn led to

increase in mortality rate and severe infections due to treatment failure **(Ibrahim et al., 2000)**.

Penicillin was discovered in 1928 by Alexander Fleming, and in 1940, many years prior to the introduction of penicillin as a therapeutic, penicillinase was found from bacteria **(Abraham & Chain 1940)**. With the regular use of penicillin there was an increase in the number of bacteria capable of inactivating penicillin, and led to the studies of modifying penicillin chemically to avoid the inactivation by β-lactamases. Many of the microbial pathogens related with human epidemic diseases developed into multidrug-resistant (MDR) strains after the use of antibiotics **(Davies & Davies 2010)**.

Presently, *Staphylococcus aureus*, a Gram-positive organism is one of the most notorious superbugs. *S. aureus* is observed as a nasal commensal in almost 30% of the population and is commonly associated with skin infections. Even though *S. aureus* does not have the historical reputation of *M. tuberculosis*, in recent years, MDR *S. aureus* has cropped up as a major nosocomial infection **(Enright et al., 2002)**.The breakthrough discovery and introduction of methicillin as an antibiotic which is anti-resistant in 1959 were believed as a certain defense against the penicillinases, but within 3 years methicillin resistant *S. aureus* (MRSA) appeared leading inevitably to the development of multi antibiotic-resistant variants, and the abbreviation MRSA now signifies multidrug-resistant *S. aureus*. Lately MRSA, a hospital pathogen has acquired enhanced virulence and transmission characteristics and moved out becoming a community acquired (CA) pathogen. All the characteristics of MRSA were observed in CA-MRSA even though through the presence of different *mec* gene clusters and Panton-Valentine leukocidin, a cytotoxic pathogenic gene **(DeLeo & Chambers 2009)**.

1.2.5 Antibiotic Resistance Origins and Mechanisms:

1.2.5.1 Genetic Jugglery:

The β-lactamase enzymes encoding genes are most widely distributed around the world; modified catalysts having increasingly high spectra of resistance were formed due to the random mutations of the β-lactamase enzyme encoding genes **(Gniadkowski 2008)**. The archetypical plasmid-encoded β-lactamase, TEM, was observed in many related enzyme families, showing its adaptable nature. The β-lactamase genes are prehistoric **(Barlow & Hall 2002)** and have been found in remote and isolated

environments **(Allen et al., 2009)**, which suggests that new β-lactamases with modified substrate varieties are present in the environment. Macrolide antibiotics, like erythromycin and its descendants, were thought to solve the problem caused by the resistance to methicillin and are extensively used for treating infections caused by Gram-positive bacteria. Unsurprisingly, resistant strains developed by a variety of mechanisms are now distributed widely **(Roberts 2008)**. The macrolides act by binding the 50S ribosome subunit peptide exit tunnel at different sites. Resistance can be caused by changes to the RNA or the protein portions of the tunnel. Recently, resistance to antibiotics was observed due to rRNA modification at this site **(Long et al., 2006)** and this modification is becoming wide spread.

1.2.5.2 Inherent Resistance:

Inherent resistance refers to the bacterial genome containing the genes that can lead to resistance, i.e., proto- or quasi-resistance. Since the start of this century, the accessibility to methods such as genome wide mutagenesis and whole genome sequencing techniques had led to the identification of many potential/intrinsic genes within bacteria that can potentially cause evolution of resistant strains in the clinical conditions. For example, gene amplification is a routine genetic mechanism to acquire higher antibiotic resistance, markedly sulfonamide resistance **(Kashmiri & Hotchkiss 1975)** and trimethoprim **(Brochet et al., 2008)**. Use of saturation mutagenesis of bacterial genomes for phenotypic analyses of complete or partial gene knockout libraries allows for predicting definite mutant strains causing hypersensitivity responses to antibiotic. Overexpression of such wild-type genes is assumed to create resistant phenotypes. One such detailed study of Keio *E. coli* mutant gene library lead to identification of 140 different strains that were sensitive to different classes of antibiotics **(Tamae et al., 2008)**. In short, intracellular concentration of the antibiotic can be reduced by increasing the copies of the target genes **(Davies & Davies 2010)**.

1.2.5.3 Resistance Caused by Anthropogenic Activities:

The human activities play an important role in generating reservoirs for resistance against antibiotics in the environment. From the time of discovery, manufacturing and clinical use in 1940s, antibiotics have been released and extensively distributed into the environment creating selection and maintenance pressure for the resistant strains. Past

half century has witnessed the release of thousands of tons of antibiotics into the natural environment. The data reveals that very less quantity of antibiotics is contributed to the environment by the naturally occurring antibiotic producing strains **(Gottlieb 1976)**. The extensive use of antibiotics in aquaculture, domestic pets, agriculture, animal husbandry, toiletries, research and human therapy had led to release of antibiotics into the biosphere.

The disposal of toxic waste, disinfectants, biocides, and remains in manufacturing processes into the environment and the fact remains that most of these are non-biodegradable only worsen the issue. For example, the dumping of ciprofloxacin, more than 50 kg/day into the rivers by a pharmaceutical company in Hyderabad, India is a classic example of irresponsible disposal **(Fick et al., 2009)**. The study of genomic analysis in waste water treatment plants reveal that they are pools of resistant genes and resistant bacteria **(Schlüter et al., 2008; Szczepanowski et al., 2009)**. These genes are generally carried on transmissible plasmids and are ready source for the cause of resistance. Proper measures for the use and disposal of the antibiotics into the environment is important and immediate concern. Recent studies of the flora in the gut of people living in regions not accessed by modern civilization and inaccessible to treatment by antibiotics have revealed the existence of genes resistant to antibiotics and resistance-encoding integrons **(Bartoloni et al., 2009; Pallecchi et al., 2007)**.

1.2.5.4 Genetics of Antibiotic Resistance:

The presence and distribution of antibiotic-resistance in pathogens have inspired numerous studies of the genetic characteristics of the diverse phenomena accompanying with resistance development, like gene pickup, heterologous expression, Homologous Gene Transfer (HGT) and mutations **(Bushman 2001; Phillips & Funnell 2004; White et al., 2005)**. It has long been believed that acquisition of resistance causes energy loss to the bacteria and truly, many resistant mutants are growth limited in laboratory settings, leading to the perception that MDR organisms are unstable and short lived when there is no selection pressure **(Andersson 2006)**. Nevertheless, as often proved, laboratory settings do not replicate real-life circumstances.

1.2.5.5 Transmission of Resistance Genes:

The acquisition and promotion of the resistant genes in bacteria is essentially done by any of the accessory genetic elements. However, the most common method of resistant gene transfer is by plasmid mediated horizontal gene transfer (HGT) **(Norman et al., 2009)**. When compared to the laboratory conditions the frequency of conjugation in the environment is much higher in magnitude **(Sørensen et al., 2005)**. Spontaneous transfer occurs between the bacteria in the intestinal tracts of humans and animals **(Shoemaker et al., 2001)**. Human intestinal microbiome has been shown to inhabit different resistance genes in the recent studies **(Sommer et al., 2009)**. Horizontal gene transfer has been a constant phenomenon in the history of evolution. However, the development of antibiotic resistance and transfer in the last few years cannot be compared to the process that happened in the bacteria and other microorganisms over millions of years. Selection pressure created by the antibiotics is very much intense when compared to the natural evolution. Pathogens isolated in the pre-antibiotic era have been found to harbor plasmids, but the resistance genes were a rarity, consistent with the idea that plasmids with antibiotic resistant genes and multidrug resistant strains were evolved recently **(Datta & Hughes 1983)**. Higher level of resistance to the antibiotics is caused by the severe selection pressure created due to exposure of pathogens to higher concentrations of antibiotics over an extended period during therapeutic treatment. It is also important to note that resistant to antibiotics is also facilitated by use of antibiotics at sub inhibitory concentrations **(Davies et al., 2006)**.

1.2.6 Antibiotic Era – *Staphylococcus aureus*:

S. aureus by nature is susceptible to almost all the antibiotics but resistance is obtained by acquiring the resistant genes through horizontal gene transfer, mutations or by antibiotic selection. *S. aureus* has this ability to colonize healthy individuals without showing any symptoms. It is observed as a nasal carrier in almost 30% of human population **(Kluytmans et al., 1997; Gorwitz et al., 2008)** and they carry greater risk of infection. *S. aureus* is usually transmitted by direct contact with the infected individual, even though there is a role through contact of contaminated surfaces and objects **(Kazakova et al., 2005; Lowy 1998)**. Globally infections caused by *S. aureus* resistant to antibiotics have reached epidemic level **(Grundmann et al., 2006)**. The burden caused

especially by MRSA strains on health care and community settings is increasing in many countries. Emergence of community-associated MRSA (CA-MRSA) which causes soft tissue and skin infections is a main reason for the increase of burden in health care in United States **(Moran et al., 2006; Fridkin et al., 2005)**. In addition, CA-MRSA are increasingly becoming virulent, supported by the fact that there is an increase in the tissue destruction infections like necrotizing pneumonia and necrotizing fasciitis after the emergence of CA-MRSA **(Francis et al., 2005; Gonzalez et al., 2005; Kallen et al., 2009)**.

Initial surge of drug-resistance in *S. aureus* strains started in mid 1940s with the development of resistance against penicillin **(Barber & Rozwadowska-Dowzenko 1948)**. Penicillinase and enzyme that hydrolyses the β-lactam ring of the penicillin was produced by these resistant strains. Introduction of methicillin had led to the disappearance of an *S. aureus* clone phage type 80/81, which was responsible for most of the hospital and community infections caused by *S. aureus* **(Jevons & Parker 1964)**. However, the introduction of methicillin led to the second wave of resistance. Methicillin-resistant *S. aureus* for the first time was reported in 1961 **(Barber 1961; Jevons 1961)** however, *mecA* gene responsible for the resistance to methicillin was identified after two decades of that finding. MRSA isolate COL, belonging to MRSA clone 'archaic' was one of the early MRSA strains isolated **(Jevons 1961)**. Archaic MRSA were highly prevalent all-around Europe **(Crisóstomo et al., 2001)** till 1970s and were also reported in United States **(Barrett et al., 1968)**, however rest of the world remained unaffected. Interestingly, the second wave of resistance got ended by the disappearance of archaic MRSA due to reasons unknown from the European hospitals by 1980s.

Outbreaks due to MRSA strains infections marking the third wave of resistance were reported in several hospitals of United States in the late 1970s and these strains were widespread by mid 1980s, leading to MRSA epidemic in hospitals worldwide that lasts even today **(Crossley et al., 1979; Peacock et al., 1980)**. Even though its distribution is global, MRSA is still more prevalent in hospital settings. Vancomycin is the antibiotic to which MRSA are reliably susceptible, however the selective pressure caused due to its increased use led to the appearance of vancomycin-intermediate (VISA) **(Hiramatsu et al., 1997)** and vancomycin-resistant *S. aureus* (VRSA) strains, which need >16µg/ml *in vitro* **(Weigel et al., 2003)**. The most recent and the fourth wave of resistance in MRSA

involves its invasion into the communities. In early 1990s in Western Australia early cases of community associated MRSA infections were reported **(Udo et al., 1993)**. As suggest by the pulsed field gel electrophoresis patterns and their vulnerability against antibiotics other than β-lactams they were distinct from their contemporary clones present in the Australian hospitals. They might be remote or community strains, with *mecA* acquired by horizontal gene transfer mechanism. First documented cases of CA-MRSA infections in United States were reported from 1997 to 1999 in otherwise healthy children who had almost no risk of MRSA infections and all died due to irresistible infection, suggesting the highly virulent nature of CA-MRSA **(CDC 1999)**. The analysis of the genomes of CA-MRSA indicate that there is no close relation with hospital clones and they are also susceptible to many antibiotics to which resistance is observed in hospital strains.

Infections caused by CA-MRSA are lethal and the clinical outcomes are worse when compared to the infections caused by MRSA strains from hospital and community associated methicillin sensitive *S. aureus* strains indicating the more virulent nature of CA-MRSA, specifically USA300 which have been extensively studied then other strains **(Francis et al., 2005; Davis et al., 2007)**. The genome analysis of USA300 strain has shown it to be similar to early MRSA strain COL **(Diep et al., 2008)**. However, USA300 is observed to be more virulent then COL as indicated by the animal studies **(Voyich et al., 2005; Li et al., 2009)**. The difference in the genomes of USA300 and COL was mainly in their mobile genetic materials like plasmids, transposons, prophages and pathogenicity islands which were obtained by horizontal gene transfer. Virulence of USA300 could be due to presence of Prophages ΦSA2 and ΦSA3, which were absent in COL. Panton–Valentine leukocidin (PVL), encoded by *luk*S–PV and *luk*F–PV is present in prophage ΦSA2.

PVL is secreted by bacteria and is composed of two subunits, LukS-PV and LukF-PV **(Woodin 1960)**. The subunits specifically bind to unidentified receptors on the membrane of host leukocytes and form pores **(Meyer et al., 2009; Colin et al., 1994)**. PLV at high concentrations (200nM) causes cell death by lysis whereas at low concentration (5nM) they secrete leukotriene b4 and interleukin 8, mediators of inflammation, leading to partial activation of neutrophils a phenomenon called as priming and causes the exocytosis of neutrophil granules **(König et al., 1995; Genestier et al.,**

2005). The MRSA USA300, which is a major cause of severe skin and soft tissue infections in United States and phage type 80/81 penicillin resistant strains that were involved in disease outbreaks in 1950s are known to produce PVL. The emergence of USA300 as a predominant CA-MRSA over USA 400, even though both secrete PLV, suggests that there are other factors that are important for the emergence of CA-MRSA.

α-haemolysin also known as Hla or α-toxin, common among clinical isolates, is a pore forming toxin that causes destruction of a host cells and is observed to be lethal when injected in purified form in animal models **(Bhakdi & Tranum-Jensen 1991)**. Recent studies in mouse models by Bubeck Wardenburg *et al*. have shown that in USA300 and USA400 α-haemolysin is essential to cause lethal infection. The amount of α-haemolysin produced *in vitro* by these strains correlates to the resultant lung disease severity **(Wardenburg et al., 2007; Bhakdi & Tranum-Jensen 1991)**.

α-type phenol-soluble modulins (PSMαs) are a group of peptides newly discovered in *S. aureus* and are similar to *S. epidermidis* produced PSMs. USA300 and USA400 produce higher amounts of PSMs when compared to hospital-acquired MRSA strains **(Wang et al., 2007)**. PSMα peptides promote the *S. aureus* pathogenesis in humans by recruiting, activating and finally lysing the neutrophils. Virulence of USA300 and USA400 is greatly enhanced by PSMα peptides USA400 in mouse abscess and sepsis models **(Wang et al., 2007)**.

Arginine catabolic mobile element (ACME) is a DNA segment of 30.9 kb and it is unique to USA300 **(Diep et al., 2008)**. ACME is present in the vicinity of SCCmecIV mobile element and utilizes the recombinases encoded by SCC*mec* for its mobility. It has two genes associated with virulence, arginine deiminase pathway encoding arginine catabolism (*arc*) genes and oligopeptide permease encoding *opp*3 **(Coulter et al., 1998; Degnan et al., 1998)**. Decrease in the fitness of USA300 was noticed in rabbit bacteraemia model when only ACME is deleted but not SCC*mec* indicating its role in USA300's fitness and epidemic spread **(Diep et al., 2008)**.

Most of the β-lactam antibiotics are no longer effective against the staphylococcal infections, especially for soft tissue and skin infections. Most of the serious MRSA infections are treated by vancomycin, however, there is an increasing occurrence of non-susceptible strains **(Steinkraus et al., 2007; Wang et al., 2006)**. Clinical trials of

linezolid and daptomycin, have shown comparable results to standard therapy **(Shorr et al., 2005; Arbeit et al., 2004)**. Vancomycin derivatives telavancin, oritavancin and dalbavancin kills *S. aureus* rapidly *in vitro* in a dose dependent manner, however, its efficacy and effectiveness over vancomycin need to be determined. Judicious and careful use of the antibiotics that are currently available is necessary to prevent further development of resistant strains.

1.2.7 Steps to Tackle Antibiotic Resistance:

An increase in the out breaks of bacterial infections can cause a significant setback to the economy. An epidemic in Germany due to infections by *Escherichia coli* contaminated vegetables led to 50 deaths and affected 5000 other people. Luckily there was no antibiotic resistance involved in this case. There is an increase in the mortality rate in hospitals of New Delhi due to the wide spread of metallo β-lactamase-resistance gene (bla_{NDM-1}) and its association with other resistant strains. In 2007, in Europe the infections caused due to MDR bacteria were 4,00,000 of which 25,000 led to the death. The cost due to these infections towards the hospital expenditure and lose of productivity is more than €1.5 billion per year **(ECDC–EMEA., 2009)**. The health care cost due to the antibiotic resistant infections in US is $20 billion per year and an additional 8 million hospital days per year **(Roberts et al., 2009)**.

1.2.7.1 Priorities in Research to Control Resistance in Bacteria:

The bacteria that live in and on humans, animals and in the environment, harbor the resistance genes, but there is no proper information on the factors and conditions that cause the movement and selection of these genes between animals and humans. What are the critical stages in the environment that help to develop the antibiotic resistance in these microorganisms? By using this data can we predict the emergence of resistance mechanisms? How the modern technology be improved to diagnose the infections at an individual level? How can reliable city and world-wide data be established and maintained to ensure proper prescription of the antibiotics for treatment of the infections? To carry out the research to track and treat new threats caused by the infections, international funding is an absolute necessary. Physicians and scientists equipped with modern diagnostic approaches need to be trained **(Bush et al., 2011)**.

1.2.7.2 Educating Public:

Public education regarding the bacterial infections and the antibiotic resistance in microbes is very important. The beneficial role of the microbes and the important role they play in our wellbeing, the importance of the antibiotics and their proper use should be conveyed to the public from their schooling. The e-Bug program started in Europe is a very good example of educating the children about the use of antibiotics **(Lecky et al., 2011)**.

1.2.7.3 Sanitation, Public Health and Quality of Life:

In certain regions of the world a combination of issues such as lack of access to clean water, proper sanitation, no proper treatment of sewage and industrial effluent, excessive and unnecessary use of antibiotics, high population density creates an appropriate environment and conditions that help in selection and propagation of the resistant bacteria. The governments should be supported and made to invest in the development of proper sanitation facilities. And tighter laws should be enforced for prescription and sale of the antibiotics. These issues should be considered and treated at a worldwide level **(Lecky et al., 2011)**.

1.2.7.4 Development of New Antibiotics:

Antibiotic resistance and tackling the emergence of new resistant strains is an important issue for the last 50 years. There is a continuous need for discovery and development of new classes of antibiotics that are not affected by the existing resistance mechanisms. Given the costs that are involved in the development of new antibiotics along with the success rate and the time needed to invest before getting profits it's not justifiable for private pharmaceutical companies alone to invest. There is a need by the governments in the developed countries to provide necessary subsidiaries and reducing the regulatory barriers for the release of drug into the market. Public-private partnerships for the development of new antibiotics should be encouraged **(Lecky et al., 2011)**.

1.2.7.5 Use of Old Antibiotics:

The antibiotics which are discarded or rejected due to various reasons should be reinvestigated and be used where it is possible. Startups should be encouraged to use these antibiotics to generate effective combinations that can produce positive results in

the treatment. For example, daptomycin, which due to toxicity reasons was abandoned by a company, when used at a different dose has become an effective treatment for Gram-positive infections **(Tally & DeBruin 2000)**.

1.2.7.6 Regulation of Antibiotic Use:

The irregular use of antibiotics should be avoided. The use of antibiotics for other than therapeutic purpose such as in animal husbandry and fisheries should be stopped. At present, greater than 50% of antibiotics that are produced are used as a part of animal feed to promote the growth. This is an ongoing process from the past few decades despite the efforts from WHO and United Nations to prevent the non-prescription use. Rather than following good animal husbandry practices will prevent the unnecessary need to use the antibiotics. Strict laws should be enforced by the local governments regarding the sale of antibiotics without prescription **(Bush et al., 2011)**.

1.2.7.7 Antibiotic Alternatives:

As a high priority research, novel approaches or alternatives to the antibiotics for prevention of the infections by the bacteria should be encouraged. Alternate therapies such as antibacterial vaccines, adjuvants, probiotics should be investigated thoroughly **(Alekshun & Levy 2004)**. Deployment of antibacterial vaccines especially in the prevention of animal infections needs to be investigated. Gastrointestinal population studies have revealed a larger role for the bacteria in human health and disease. In view of this the use of probiotics as an alternative to antibiotics would become important (**Kau et al., 2011**).

The discovery and production of new antibiotics should be in parallel with the increasing requirement. The Infectious Diseases Society of America (IDSA) had set a goal to identify 10 new antibiotics by 2020 **(Policy 2010)**, however, this appears to be an uphill task. Till now the drug discovery from the natural products was focused from the sources that are readily available **(Payne et al., 2007)**, but there is a huge reservoir of antimicrobial compounds available in the unexplored places. For example, around 1000 different compounds were screened from marine microorganisms in 2008 **(Blunt et al., 2011)**. The cost to undertake the above measures is small when compared to the

economic impact and human lives that are at risk. Commitment from every individual and collaborations at every level are required at a worldwide scale to tackle the issue.

1.3 GAPS IN EXISTING RESEARCH:

Antimicrobial resistance is a major concern in treating and prevention of the ever-increasing infections caused by different classes of bacteria. High levels of resistance have been observed in bacteria associated with health-care and community acquired diseases. There has been a huge void in the past 30 years in the development of new classes of antibiotics **(Fig. 5)**. And there is an increase in the resistant strains. Over the past 30 years only 2 classes of antibiotics have been developed, the oxazolidinone linezolid and the cyclic lipopeptide daptomycin **(Hamad 2010; Hassan et al., 2012)**.

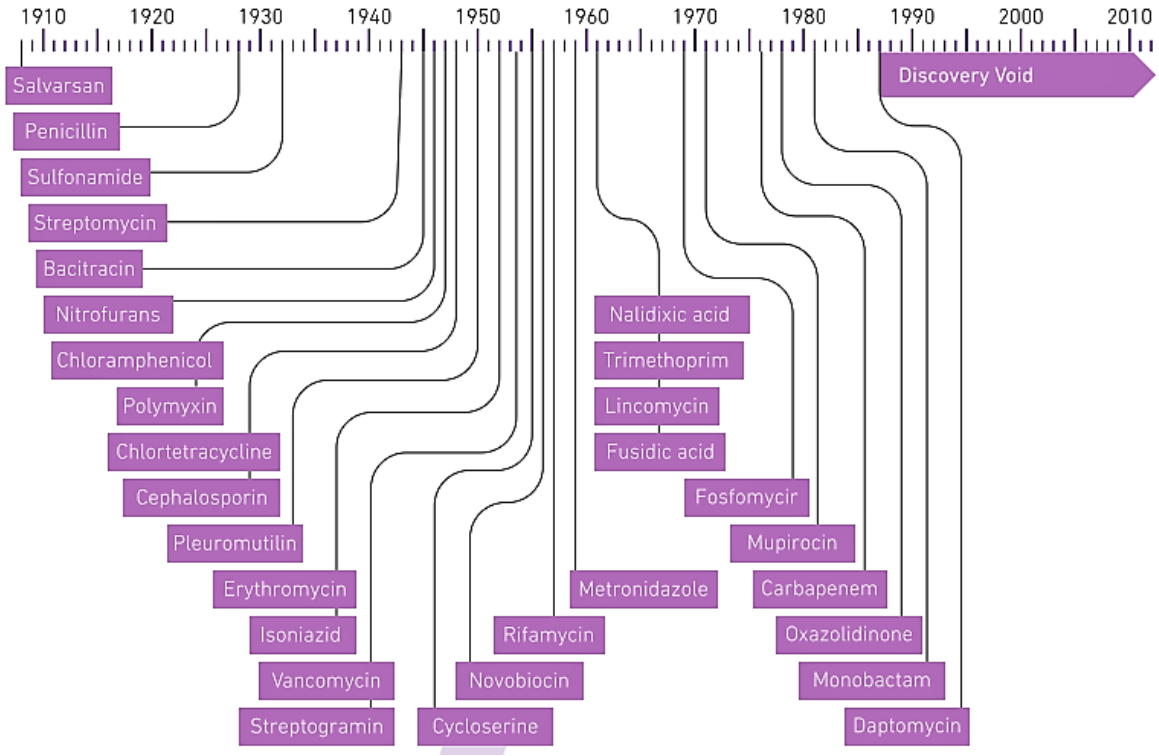


Figure 5: Timeline showing the discovery of Antibiotics and the void in discovery of new classes (Silver 2011)

S. aureus is cause of many infections, notably skin, soft tissue, blood stream, bone and also main cause of post-operative infections. Methicillin resistant *S. aureus* (MRSA) strains have acquired a novel *mec*A gene that codes for a penicillin binding protein. Initially MRSA were prevalent only in hospital settings, however in the past decade they

have spread and community acquired MRSA have been in rise in a number of countries. Hospital infections are caused by multi drug resistant MRSA strains for whom the last resort of treatment is vancomycin and teicoplanin, which has to be injected and needs careful monitoring to avoid the associated adverse side effects. Other treatment options include linezolid (1970s) and daptomycin (1980s), but they are also associated with the side effects which in turn will impact cost of treatment and is a huge burden especially in the developing and under developed countries **(WHO 2014)**.

The genus *Bacillus* is known to produce a variety of antimicrobial peptides and a good source in search for new antimicrobial compounds **(Bizani et al., 2005; Xie et al., 2009)**. A large number of peptide antibiotics having different basic chemical structures are produced by genus *Bacillus* **(Cladera-Olivera et al., 2004)**. A tetracyclic peptide mersacidin (1.8 kDa) belonging to lantibiotics group **(Chatterjee et al., 1992)** shows activity against MRSA that is comparable to vancomycin and teicoplanin. Cytotoxicity of the AMPs against eukaryotic cells and erythrocytes is one problem that's hindering the development as potential antibiotics. Very few *Bacillus* AMPs have been clinically tested and till now none has been approved by FDA for clinical use.

In view of the above challenges it was proposed to isolate a *Bacillus* strain that shows consistent antimicrobial activity against Gram-positive bacteria, purify the antimicrobial compound, test its antimicrobial potency against multidrug resistant strains, study its toxic effects on the mammalian cell lines and human erythrocytes and study its structural and biochemical characteristics using different techniques.

1.4 OBJECTIVES OF THE RESEARCH WORK:

The following objectives were undertaken for the current study:

1) To identify the promising wild-type *Bacillus* isolate up to species level and optimize the production of the antimicrobial compound.
2) To purify and biochemically characterize the antimicrobial peptide.
3) Functional characterization of the antimicrobial peptide.

2.1 INTRODUCTION:

Diseases caused by the infectious microbes are the second most common reason for death worldwide; therefore, it is very important to find new antibiotics to combat these infections **(Lam 2007; Pálffy et al., 2009)**. 1940s to 1960s is called as the golden era for the antibiotic research since a lot of antimicrobial agents for the treatment of infections were identified from natural sources **(Walsh 2003; Brown 2006; Tenover 2006)**. However, resistance has been developed by pathogenic bacteria to many available antimicrobials. Warning has been issued by World Health Organisation (WHO) that we are heading back to the pre-antibiotic era regarding treatment of multi drug resistant microbes **(Parisien et al., 2008)**. Novel antimicrobial agent's development is an absolute necessary to combat the increasing growth of antimicrobial resistance **(Coates et al., 2002; Walsh 2003; Clardy et al., 2006)**. Antimicrobial peptides (AMPs) are a promising alternative for the new generation of antibiotics **(Pálffy et al., 2009; Rotem and Mor 2009; Cotter et al., 2013)**. With an increase in the antibiotic resistant pathogens there is a need for discovery of novel antibiotics with different inhibitory mechanisms. These can be used in combination with other antibiotics to combat the infections caused by antibiotic resistant pathogens **(Hassan et al., 2012)**. *Bacillus* species derived antimicrobial peptides are promising candidates with a great potential to overcome the current antibiotic shortcomings **(Hassan et al., 2012)**.

In this study, the isolate URID 12.1 was isolated from soil by spot-on-lawn method and the culture was identified by 16s rRNA gene sequencing and compared to identifications based on biochemical tests, Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) MS Biotyping and Vitek2 compact Identification. The 16S rRNA gene sequencing is widely used for identification and detection of new bacterial species **(Wang et al., 1994)**.

2.2 MATERIALS AND METHODS:

2.2.1 Microbial Cultures, Media and Growth Conditions:

The wild type soil isolate (URID 12.1), the producer strain of the antimicrobial compound was grown in modified Tryptone Soya Broth (mTSB, with 0.5% yeast extract) at 37 ^{o}C under shaking conditions (110 rpm). All indicator bacterial strains including the

quality control strains *S. aureus* ATCC 29213 and *S. aureus* MTCC 737 (ATCC 6538P) and E. *faecalis* ATCC 29212, were sub cultured in Mueller-Hinton and Brain Heart Infusion (BHI) broths at 37 ^{o}C. The quality control strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were obtained from OncQuest Laboratory, Indore, whereas the *S. aureus* MTCC 737 (ATCC 6538P) was procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. All the cultures used as indicator strains (Tables 1, 4) were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh and National Collection of Industrial Microorganisms (NCIM), Pune and the clinical isolates from the Central Laboratory, OncQuest Laboratory, Indore, and *S. aureus* GMC from Goa Medical College, India. The cultures were also maintained as 20% glycerol stocks at −80◦C.

2.2.2 Screening for Antimicrobial Activity:

The spot-on-lawn method was used for the bioassay of antimicrobial activity. Bacterial strain *S. aureus* MTCC 737 was used as indicator initially for the screening purpose. Soil samples were serially diluted in sterile 0.85% saline and spread to obtain single colonies. The colonies were inoculated into fresh TSB and the supernatant was collected after 24–48 h incubation. Freshly grown overnight indicator strain was mixed with soft agar (0.7%) and the mixture was transferred to plates prepared with 1.8% agar. The supernatant was spotted; plates were incubated at 37 ^{o}C for 24–48 h and inspected for the zone of inhibition **(Ramachandran et al., 2014)**. The wild-type bacterial isolate showing consistently reproducible antibacterial activity against the indicator strain was designated as URID 12.1.

2.2.3 Identification of the Producer Strain:

2.2.3.1 Bio-Chemical Tests:

Morphological and biochemical tests were conducted in order to identify the wild-type bacterial isolate the cell free supernatant (CFS) of which inhibited *S. aureus* MTCC 737 and *Micrococcus luteus*. The wild-type isolate was identified by various biochemical tests such as Methyl Red and Voges-Proskauer tests, production of indole and catalase and different concentrations of NaCl according to Bergey's Manual of Determinative

Bacteriology **(Holt et al., 1994)**. Carbohydrate fermentation tests were done using KB009 Kit from HiMedia Pvt. Ltd., India.

2.2.3.2 Vitek2 compact Identification:

In Vitek2 compact based identification, the culture is freshly grown on TSB agar plate at 37 ^{o}C. Freshly grown culture was taken with a sterile swab and transferred to saline to make a suspension with McFarland turbidity range of 1.8-2.2. The culture is inoculated into reagent cards with 64 wells containing an individual test substrate. The substrates are used to measure different metabolic activities such as carbon source utilization, enzymatic activities, acidification, alkalization and resistance to inhibitory substances. BCL card is used for identification of Gram-positive spore-forming bacilli. The cards are incubated at 35.5 +/- 1^{o}C and removed every 15 mins and reactions readings are taken in an optical system **(Pincus 2006)**. The data are collected every 15 mins during the whole incubation period.

The test reactions are interpreted by a transmittance optical system using different wavelengths in visible spectrum. Either the turbidity or the coloured products formed due to substrate metabolism are measured every 15 mins. False readings due to bubble formation are avoided by using a special algorithm. The test reaction results appear as "+", "–", "(–)" or "(+)". Reactions that appear in parentheses are indicative of weak reactions that are too close to the test threshold. Various identification levels are assigned based on the numerical probability calculation. When a single choice is given a score of 96-99, 93-95, 89-92 and 85-88 are considered as excellent, very good, good and acceptable respectively. When there are 2-3 choices it is understood as low discrimination and can be identified by standard microbiology references.

2.2.3.3 MALDI-TOF (Matrix-assisted Laser Desorption/Ionization-Time of Flight) MS Biotyping:

Ethanol/Formic Acid extraction method:

About 5–10 mg of freshly grown cells was suspended in 300 µl of sterile distilled water in a micro centrifuge tube by repeated pipetting or vortexing. A series of three washes were given with 900µl absolute ethanol and the pellet is air dried for some time.

50 µl of 70% formic acid is added to the dried pellet and mixed by pipetting, equal volume of acetonitrile is added and mixed vigorously and centrifuged at 12000 rpm for 2 min. small pellet with residual matter and supernatant with extracted proteins are formed. One microliter from the supernatant was loaded on target and dried followed by addition of 1 µl of matrix solution and drying **(Cramer 2016)**. Oxidation and methylation of the sample may result in shift in the peaks of spectrum, therefore, to prevent this shifting, sample should be mixed with the matrix solution as soon as possible. The sample is analysed using MALDI-Biotyper Microflex, Bruker, Germany.

2.2.3.4 Polymerase Chain Reaction (PCR) Based Identification:

Genomic DNA of the isolate URID 12.1 was extracted from overnight grown culture by using the method after **(Neumann et al., 1992)**. The genomic DNA was used to perform the 16S rRNA gene sequencing PCR by using the primers "27F, fwd_seq: AGAGTTTGATCCTGGCTCAG, 1492R, rev_seq: GGTTACCTTGTTACGACTT" for 16S rDNA-based identification. The amplified product was sequenced and analysed using the NCBI BLAST. The phylogenetic tree was constructed using Mega 5.0 software **(Tamura et al., 2011)**. Databases (GenBank) were used for sequence similarity comparison with the 16S rDNA sequence obtained.

2.2.4 Growth and Production Kinetics:

The antimicrobial compound producing strain URID 12.1 was inoculated at a rate of 1% in to 100 ml of mTSB and incubated at 37 ^{o}C under shaking condition at 110 rpm. After every 4 h, 2 ml of sample was collected, and 1 ml was centrifuged at 12000 rpm for 20 min and the supernatant was used to test the antimicrobial activity against the indicator strain *S. aureus* MTCC 96 by cut-well agar diffusion assay and the zone of inhibition in mm was noted. Simultaneously the growth was also measured spectrophotometrically at OD 600 nm and the activity was expressed as Arbitrary Unit per millilitre (AU/mL); this value is the reciprocal of the highest two-fold dilution exhibiting a zone of inhibition and estimated using the formula $(2^n \times 1000)/V$ (µl), where n = highest two-fold dilution showing activity and V = volume used to test antimicrobial activity.

2.2.5 Antimicrobial Activity Spectrum:

The antimicrobial compound producing strain URID 12.1 was inoculated into 100 ml of mTSB and incubated at 37 ^{o}C for 44 h and the supernatant was collected by centrifugation at 12000 rpm for 20 min. The supernatant was tested for antimicrobial activity against the indicator strains **(Table 4)** by cut-well agar diffusion assay and the diameter of the zone of inhibition in millimetre was noted after 24–48 h of incubation.

2.3 RESULTS:

2.3.1 Identification of Producer strain:

2.3.1.1 Biochemical tests:

Initially the genus of producer isolate was preliminarily identified as *Bacillus* by a battery of biochemical tests. The results of the biochemical tests are summarized in **Table 3**. The organism was tested negative for Methyl Red, positive for Voges-Proskauer tests, production of indole is negative and catalase positive. The growth of the bacterial isolate at different concentrations of NaCl and the results of carbohydrate fermentation tests are summarized in **Table 3**. When observed under microscope rod shaped structures were observed **(Fig. 6)**. The results indicate that the wild-type bacteria belong to the genus *Bacillus*.

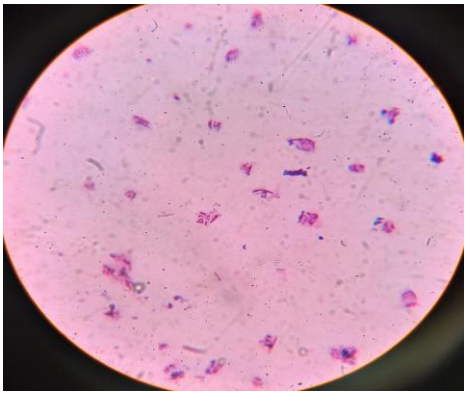


Figure 6: Microscopic view of the culture showing the gram staining and rod shape structures.

Table 3: Morphology, NaCl tolerance and Biochemical tests for culture identification.

S. No	Test	Result
	Morphology	
1	Colony Morphology	Irregular and Wrinkled
2	Colour	White
3	Gram staining/Shape	Positive/Rod
4	Spores	+
5	Pigment Production on Glutamate glycerol agar media	+
	NaCl	
6	2%	+
7	5%	+
8	7%	+
9	10%	+
	Biochemical tests	
10	Catalase	+
11	Indole	-
12	Production of mixed acid (MR test)	-
13	Acetoin production (VP test)	+
14	Lactose	-
15	Xylose	-
16	Maltose	+
17	Fructose	+
18	Dextrose	+
19	Galactose	-
20	Raffinose	-
21	Trehalose	+
22	Melibiose	-
23	Sucrose	+

24	L-Arabinose	-
25	Mannose	+
26	Inulin	+
27	Sodium gluconate	-
28	Glycerol	+
29	Salicin	+
30	Dulcitol	-
31	Inositol	-
32	Sorbitol	+
33	Mannitol	+
34	Adonitol	-
35	Arabitol	-
36	Erythritol	-
37	Methyl-D-glucoside	-
38	Rhamnose	-
39	Cellobiose	+
40	Melezitose	-
41	Methyl-D-mannoside	-
42	Xylitol	-
43	ONPG	-
44	Esculin hydrolysis	+
45	D-Arabinose	-
46	Citrate utilization	-
47	Malonate utilization	-
48	Sorbose	-

2.3.1.2 Vitek 2 Compact Identification:

The identification of the bacteria was further validated by Vitek 2 compact based identification that showed a confidence level of good identification with a percentage probability of 91% for *Bacillus subtilis/amyloliquefaciens/atrophaeus.* Based on carbon

source utilization, enzymatic activities, and antibiotic resistance pattern, 46 biochemical tests were conducted. The results of the biochemical tests were shown in **Table 4.**

Table 4: Results of biochemical tests by Vitek 2 compact identification

S. No	Test	Result
1	Beta-Xylosidase	+
2	L-Lysine-Arylamidase	-
3	L-Aspartate Arylamidase	-
4	Leucine Arylamidase	+
5	Phenylalanine Arylamidase	+
6	L-Proline Arylamidase	-
7	Beta-Galactosidase	**(-)**
8	L-Pyrrolidonyl- Arylamidase	+
9	Alpha-Galactosidase	+
10	Alanine Arylamidase	-
11	Tyrosine Arylamidase	+
12	Beta-N-Acetyl-Glucosaminidase	-
13	Ala-Phe-Pro Arylamidase	+
14	Cyclodextrin	-
15	D-Galactose	-
16	Glycogen	-
17	myo-Inositol	+
18	Methyl-A-D-Glucopyranoside acidification	+
19	Ellman	-
20	Methyl-D-Xyloside	-
21	Alpha-Mannosidase	-
22	Maltotriose	+
23	Glycine Arylamidase	-
24	D-Mannitol	+
25	D-Mannose	+
26	D-Melezitose	-
27	N-Acetyl-D-Glucosamine	-

28	Palatinose	+
29	L-Rhamnose	-
30	Beta-Glucosidase	+
31	Beta-Mannosidase	-
32	Phosphoryl Choline	-
33	Pyruvate	+
34	Alpha-Glucosidase	-
35	D-Tagatose	-
36	D-Trehalose	+
37	Inulin	+
38	D-Glucose	+
39	D-Ribose	+
40	Putrescine assimilation	-
41	Growth in 6.5% Nacl	+
42	Kanamycin Resistance	-
43	Oleandomycin Resistance	-
44	Esculin hydrolyse	+
45	Tetrazolium Red	+
46	Polymixin_B Resistance	-

2.3.1.3 MALDI-TOF MS Biotyping:

The MALDI Biotyper identification of bacteria utilizes the ribosomal protein fingerprint of the cell and relies on high abundance proteins. The MALDI biotyping identified the producer strain as *Bacillus subtilis* with a score of 1.905 which shows the high-level confidence of identification.

2.3.1.4 Polymerase Chain Reaction (PCR) Based Identification:

Based on the 16S rDNA sequence (945 base pairs), the bacterial strain URID 12.1 responsible for production of the bioactive metabolite was identified as *Bacillus atrophaeus* and assigned the GenBank accession number as JX156420.1 by the NCBI. The phylogenetic tree analysis using Mega 5.0 has shown the URID 12.1 more closely

related to *Bacillus subtilis* **(Fig. 7)** with bootstrapping for 1000 replicates and displaying for 100.

CAACAGTTGCGGGCTGGCCTTATAATTGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGC
GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAACCGGGGCTA
ATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGA
CCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGCGATGCGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTG
TTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAAACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCT
CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGG
AACTTGAGTGCAGAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC
ACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
GATTGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTG
CTGCAGCTAACGCATTTATGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTACAAAGCAACG

Sequence 1: 16S rDNA sequence of URID 12.1

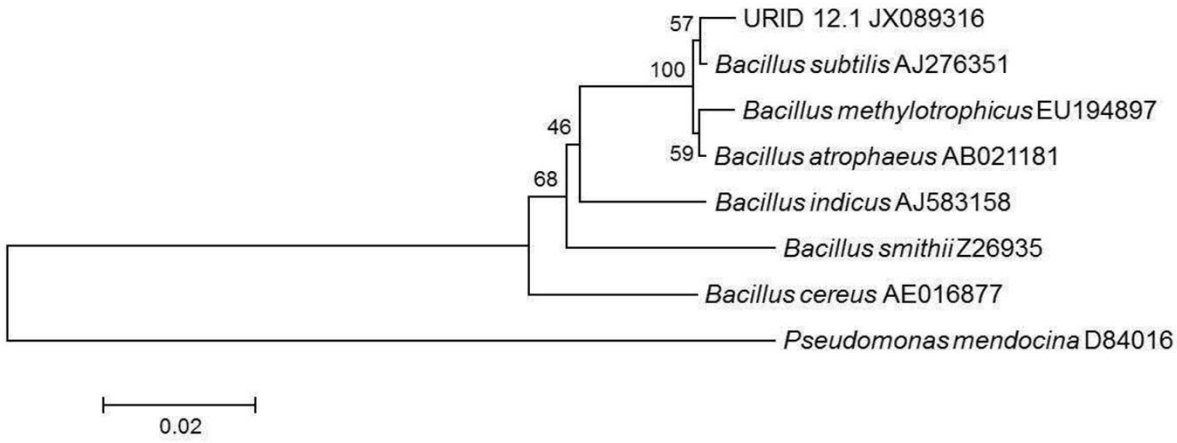


Figure 7: Phylogenetic tree showing the similarity of URID 12.1 among other *Bacillus* species. *Pseudomonas mendocina* was used as an outgroup.

2.3.2 Production Kinetics:

The antimicrobial activity of the URID 12.1 was tested against the *S. aureus* MTCC 96 as the indicator strain. The production of the antimicrobial compound started at 8 h of inoculation and reached maximum by 40 h (Zone of inhibition diameter, 26 mm); however, after 48 h, the activity declined gradually **(Fig. 8)**. The maximum production of

the antimicrobial compound was at the late logarithmic and early stationary phase. At 44 h, the antimicrobial value was detected to be 3200 AU/mL.

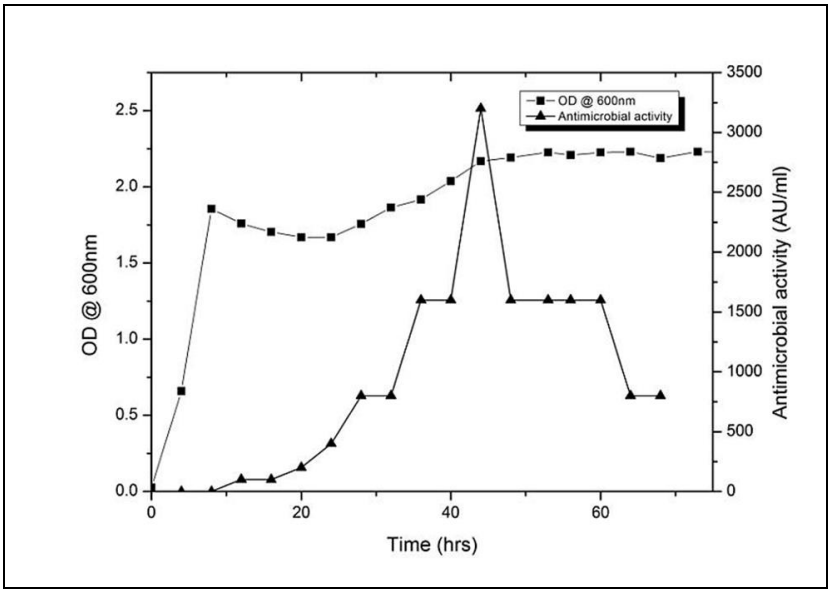


Figure 8: Growth and production kinetics of URID 12.1; the antimicrobial compound production is expressed as AU/mL.

2.3.3 Antimicrobial Activity Spectrum:

The sensitivity of different microbial strains to the antimicrobial compound produced by URID 12.1 was tested by cut well agar diffusion assay using the CFS at 44 h of growth **(Table 5)**. The activity was expressed as zone of inhibition in millimetres. No activity was observed against gram-negative strains.

Table 5: Activity of the antimicrobial compound against different bacterial strains expressed as zone of inhibition.

S.No.	Indicator Organisms	Antimicrobial Activity (zone of inhibition in mm)
1.	Methicillin and vancomycin resistant *Staphylococcus aureus* 4	25
2.	Methicillin-resistant *S. aureus* 2	22
3.	*S. aureus*-GMC*	23
4.	*Micrococcus luteus*	25
5.	Methicillin-resistant *Staphylococcus epidermidis* 3	26
6.	Vancomycin-resistant *Enterococcus faecium* 13	17
7.	*S. aureus* MTCC 96	26
8.	*S. aureus* MTCC 737	21
9.	*S. aureus* MTCC 5021	18
10.	*Streptococcus pyogenes* MTCC 442	22
11.	*S. pyogenes* MTCC 1928	16
12.	*S. pyogenes* NCIM 2608	18
13.	*Staphylococcus epidermidis* ATCC 12228	27
14.	*Escherichia coli* MTCC 723	-
15.	*Klebsiella pneumoniae*	-
16.	*Acinetobacter baumannii* MTCC 1425	-
17.	*Salmonella infantis* MTCC 1167	-

*GMC denotes Goa Medical College

2.4 DISCUSSION:

Identification of the soil isolate in the current study was done by using different techniques biochemical tests, Vitek 2 compact, MALDI bio typing and 16s rRNA gene sequencing. The culture was found positive for Gram staining, rod shaped, spore forming and have irregular colony shape which is the characteristic of *Bacillus subtilis*. Since this organism's natural habitat is soil microcosm which is the source of a wide variety of carbohydrates derived from microflora and fauna, it is expected that this organism would harbor the enzymes necessary for the utilization of the oligosaccharides and

polysaccharides from this wide variety of substrates. In our study, it was observed that the isolate URID 12.1 was able to utilize a variety of sugars such as maltose, fructose, dextrose, trehalose, sucrose, mannose, inulin and cellobiose. These carbohydrates are taken into the cells by ATP-binding cassette (ABC) transporters and phosphotransferase systems (PTS); there are about 77 supposed ABC transporters and a minimum of 16 PTS sugar transporters encoded in the genome of *B. subtilis* **(Kunst et al., 1997)**. Aerobically growing *Bacillus* species produce acid from carbohydrates by means of oxidation rather than fermentation and are generally known to produce less acid from carbohydrates and the small amount of acid produced is neutralized by the ammonia produced from peptones. This observation has correlation to the negative result for production of mixed acid (MR test). The culture has shown tolerance to grow in high salt concentration as evident from its growth at 10% NaCl and in 6.5% in Vitek 2 compact system. A single ABC-type putative Na^+ efflux system was observed in the genome of *B. subtilis* 168 **(Saier et al., 2002)** to support the observed growth of URID 12.1 at relatively high concentration of NaCl.

B. subtilis can utilize ammonium, amino acids, some purines, urea, and peptides as sole nitrogen sources. Three proteins GlnR, TnrA and CodY control the gene expression in response to nitrogen availability in *B. subtilis*. *sigL* regulon in this organism was found to contain σ^{54} homolog, believed to be present in gram-negative bacteria only that is involved in nitrogen and carbon source utilization **(Fisher and Débarbouillé, 2002)** and the test strain URID 12.1 showed positive for leucine, phenylalanine, tyrosine utilization and indicated the presence of Ala-Phe-Pro arylamidase in Vitek 2 compact analysis.

In the recent years soft ionization methods that can analyse biomacromolecules, such as MALDI-TOF-MS have emerged as important tools in protein analysis. It is of importance in analysing the complex biological systems because individual components can be resolved according to their mass-to-charge (m/z) values, allowing to gather the structural information. Characteristic profiles of the intact microorganisms have been recently demonstrated using the MALDI-TOF-MS **(Claydon et al., 1996)**. Cells are embedded in a matrix of a UV absorbing chemical and illuminated with a UV laser. The matrix volatises and carries with it any desorbed material from the outer layers of any

embedded cells. It is designed in such a way that ions are produced with single charge, that the m/z values are equal to the molecular masses. The region of interest is between 600 to 4000 Da and strongly reflects the composition of outer cell layers. Discrimination is obtained at all taxonomic levels from strain, to species, to genus **(Claydon et al., 1996)**. Long term reproducibility is obtained with the spectra and can be interpreted visually at species level with experience and is also critical for rapid identification by comparing the spectra for known reference strains in the database. In the present study, the producer strain had been identified as *B. subtilis* with a score of 1.905 showing a high level of confidence. As described previously by **Fernández-No et al., (2012)**, 16s rRNA gene couldn't differentiate the *B. cereus* from *B. thuringiensis* and *B. subtilis* from *B. amyloliquefaciens* due to the similarities that exist between the 16s rRNA gene nucleotides. However, MALDI-TOF analysis could differentiate these strains. Even in our current study Vitek 2 compact method identified the culture as *B. subtilis/B. amyloliquefaciens/B. atrophaeus* whereas based on MALDI-TOF the producer strain was identified as *subtilis*.

16S rRNA gene sequences are generally used for the classification of the bacterial species. The sequencing of this gene is used as a first tool of identification. 16S rRNA gene sequence which shows a similarity of less than 97% have DNA-DNA relatedness of less than 70% **(Stackebrandt and Goebel 1994)** and according to species delineation recommendations **(Stackebrandt et al., 2002)** those strains must be considered as belonging to different species. The sequences may or may not represent different species when the similarity of 16S rRNA gene is greater than 97% **(Stackebrandt and Goebel 1994)**. However, there is no clear consensus for classification at species-level using the phylogenetic data and care must be taken when interpreting the 16S rRNA gene sequences **(Forney et al., 2004)**. Based on the 16S rDNA sequence the culture in the current study was identified as *Bacillus atrophaeus* (JX089316.1) and the phylogenetic tree analysis using Mega 5.0 showed it to be closely related to *Bacillus subtilis*. Based on all the four analyses the strain URID 12.1 can be concluded as belonging to the genus *Bacillus* and the species *subtilis.*

3.1 INTRODUCTION:

Microorganisms compete among themselves for the nutrients and the limited space available in their natural microhabitats. In order to survive in this competitive environment, they have developed different strategies; one among them is the production of antimicrobial agents. Gram-positive bacteria especially *Bacillus* are now being increasingly investigated for the production of antimicrobial compounds **(Hassan et al., 2012)**. Generally, the antimicrobial peptides share a net positive charge which makes them fold into an amphiphilic conformation when they interact with the microbial membranes **(Drider et al., 2006)**. There is a very high interest in naturally produced antimicrobial compounds which do not show adverse side effects.

Complete purification of the antimicrobial compound is necessary to completely characterize the compound. For the elucidation of the biochemical structure of antimicrobial compound a pure, homogenous peptide is an absolute necessity. High Performance Liquid Chromatography (HPLC) is a very versatile and widely used technique for the purification of the peptides and proteins from natural sources. Matrix-assisted laser desorption/ionization (MALDI)–Time of Flight (TOF) has become an important tool for the determination of the molecular mass with precision and to know the impurities.

In the current study, the purification of the peptide was achieved by acid precipitation of cell free supernatant (CFS) followed by solvent extraction, adsorption chromatography and reversed phase HPLC. The secondary structure of the peptide is determined by CD spectroscopy and electrospray ionization Fourier Transform Ion Cyclotron Resonance mass spectrometry (ESI-FT-ICR-MS) was used to determine the peptide sequence.

Circular dichroism (CD) spectroscopy is an excellent technique for determining the secondary structure of proteins. CD is defined as the unequal absorption of left-handed and right-handed circularly polarized light. Since the spectra of proteins are very much dependent on their conformation, CD can be used to estimate the secondary structure of unknown proteins **(Greenfield 2006)**. The progress made in the development of ionization techniques for nonvolatile molecules has brought in a revolution in the mass spectrometry (MS). MALDI **(Hillenkamp et al., 1991)** and electrospray ionization (ESI)

(Whitehouse et al., 1989) techniques have made possible the intact molecular ions generation from peptides. Fourier transform (FT) ion cyclotron resonance (ICR) MS has developed into an important tool for the analysis of peptides **(Williams 1998)**.

3.2 Principles of the Instruments:

3.2.1 High Performance Liquid Chromatography (HPLC):

High performance liquid chromatography also called as high-pressure liquid chromatography is the largest and widely used separation technique. HPLC involves the injection of liquid sample into a tube tightly packed under pressure with small particles (3-5 µm diameter) called as stationary phase. The individual components of the sample move through the column with a liquid mobile phase delivered at high pressure by a pump. The components of the sample are separated from each other by the column packing with which the sample components interact by physical and/or chemical interactions. The separated components are measured at the end of the tube by a detector and the output is called as a liquid chromatogram.

The major components of the HPLC include the pump which force the liquid at a certain flow rate generally in the range of 1-2 mL/min. the pump can deliver the mobile in a constant composition called isocratic or in an increasing mobile phase composition called as gradient. Injector helps in introducing the liquid sample into the flow stream of the mobile phase. The column filled with the stationary phase is considered as the heart of the chromatograph. It separates the sample components by way of physical or chemical interactions. The small tightly packed components of the column cause the high back pressure and the pump pushes the mobile phase hard to pass through the column. The detector helps to measure the amount of the sample components coming out of column. Computer along with controlling all the modules of the instrument will receive the signal from the detector and determine the retention time, qualitative and quantitative analysis of the sample components. HPLC is used for several applications such as qualitative analysis, quantitative analysis, finding the traces and preparation of the pure components.

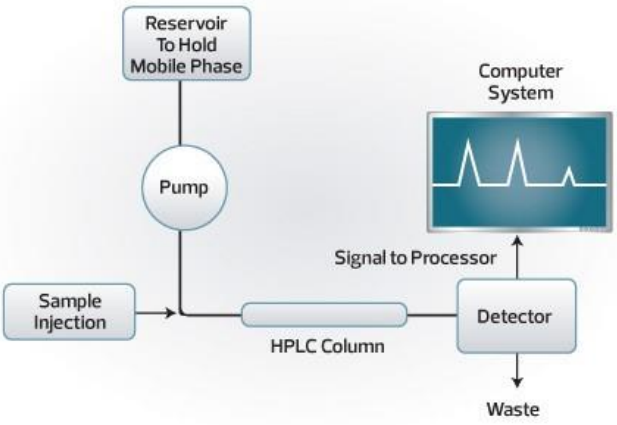


Figure 9: Schematic representation of HPLC

3.2.2 Matrix-assisted laser desorption/ionization (MALDI)–Time of Flight (TOF):

MALDI is an ionization technique that utilizes the matrix that can absorb energy from the laser to generate ions of large molecules with less fragmentation. The sample is mixed with matrix and allowed to dry on a sample support. Then the mixture is irradiated with a nanosecond laser beam such as UV laser at an energy generally in the range of 1×10^7–5×10^7 W/cm2. The energy from the laser causes decomposition in the structure of the crystal and creates a cloud of particles from which the electrical field is used to extract the ions. The laser energy might be converted to vibrational oscillation of the crystal molecules. After passing through the electric field, the ions drift through a path free of electric field and reach the detector (Fig. 6). The mass of the ions, mass-to-charge ratio (*m/z*) is generally calculated by measuring the time of flight of the ions. When the initial energies are same the large molecules have longer time of flight than the smaller ones. The TOFs calculated by using MALDI are generally in the range of microseconds **(Jurinke et al., 2004)**.

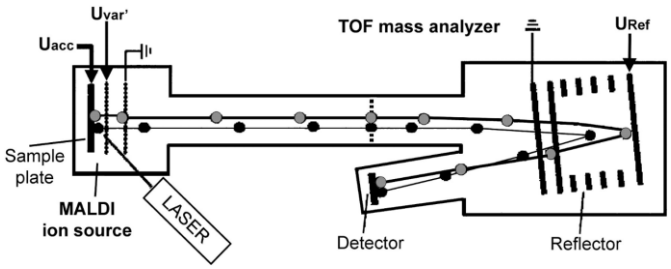


Figure 10: Schematic representation of MALDI-TOF mass analyzer

3.2.3 Circular Dichroism (CD) Spectroscopy:

Circular dichroism is used to calculate the difference in absorption between left hand polarized light and right hand polarized light caused due to the asymmetry in the structure. Time dependent magnetic and electric fields are associated with a beam of light. When polarized by passing through a suitable prism the electrical field, E, of the light oscillate sinusoidally in a single plane. The sinusoidal wave when visualized from the front can be seen as a result of two equal length vectors, one that rotates clockwise (E_R) and the other anticlockwise (E_L), which trace out as circles and have physical existence. The waves are out of phase by 90 degrees and can be separated by prisms using Pockel's effect **(Velluz et al., 1965)**. When the polarized light is encountered by asymmetrical molecules, they absorb the right and left handed polarized light to different extents and the two waves will have different refraction indices. As a result, rotation in the plane of light wave is observed and the vector that traces out on the addition of E_R and E_L will be elliptical and the light is called elliptically polarized **(Greenfield 2006)**.

CD can be used to determine proteins' secondary structure. When the chromophores present in the amides of protein polypeptide backbone are aligned in arrays, due to "exciton" interactions the optical transitions are split into multiples. As a result, each structural element has its characteristic CD spectra **(Fig. 11)**. For example, a positive band at 193 nm and negative band at 208 and 222 nm is observed for α-helical proteins **(Holzwarth & Doty 1965)** and β-helices have positive and negative bands at 195 and at 218 nm respectively **(Greenfield & Fasman 1969)**. However random coil proteins have negative bands at 195 nm and above 210 nm they have very low ellipticity **(Venyaminov et al., 1993)**.

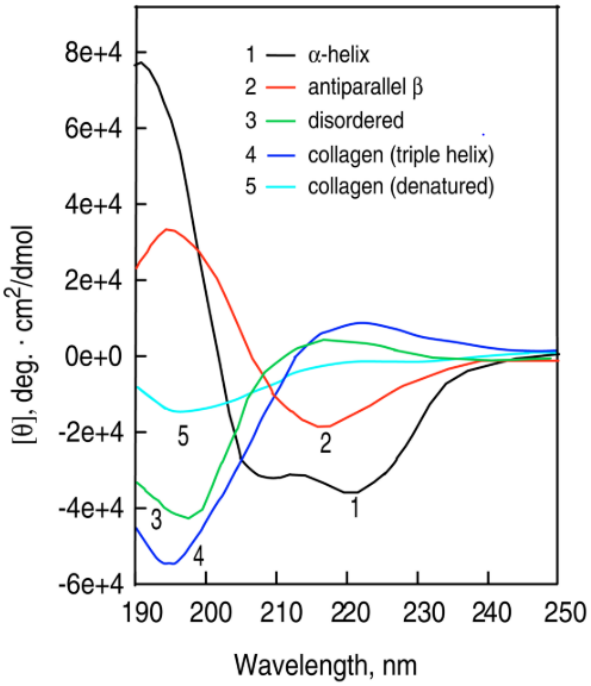


Figure 11: Circular dichroism (CD) spectra of polypeptides and proteins with representative secondary structures.

3.2.4 Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR-MS):

The introduction of sample is an important step in any mass spectral analysis, the sample need to be ionized before analysis. Introduction of volatile sample in to high vacuum region is relatively easy and there it can be ionized by an electron beam; however nonvolatile samples pose a unique challenge. Electrospray ionization is widely used for the introduction of nonvolatile samples. Production of multiple charges is one of the major advantages of ESI. It allows the ions to be analyzed based on their mass to charge (*m/z*) ratio, thus extending the mass range of the analyzer. A mass range of 0-2000 units is typical for a quadrupole mass analyzer, thus a singly charged largest molecule that can be analyzed is of 2000 Da. A 30 kDa molecule with 20 charges will have a *m/z* ratio of 1500 and can be easily analyzed by using the multi charge capability of ESI **(Hendrickson & Emmett 1999)**.

The process of ionization involves the flow of sample through a capillary that is held at a potential. The solution is dispersed as fine charged particles at the end of capillary due to the change in the potential. These particles are drawn into the source,

passed through a heated chamber by a gradient in pressure finally to the analyzer **(Fig. 12)**. The charged droplets are converted into ions due to the combination of pressure drop and increased temperature. The number of charges depends on multiple factors such as sample, the solvent used, pH and temperature **(Hendrickson & Emmett 1999)**.

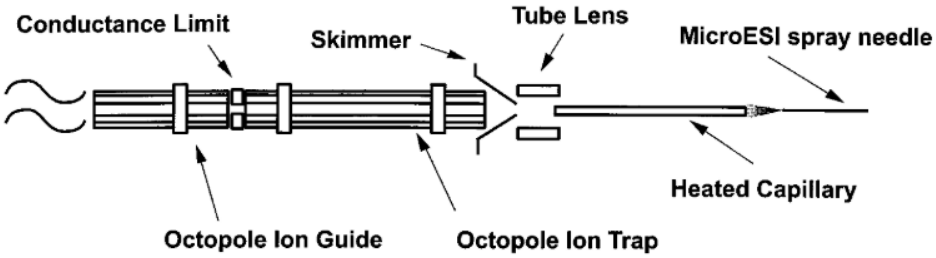


Figure 12: Schematic representation of ESI source and octopole trap (Freitas et al., 1999).

FT-ICR MS is based on the principle of motion of a charged particle in a spatially uniform magnetic field. An ion moving in a magnetic field is subjected to force, and if it maintains constant speed the path of the ion is bent into a circle by the magnetic field. The ICR frequency is independent of the velocity of the ion making it useful for mass measurement. The ICR frequency is inversely proportional to the mass to charge ratio, making it less sensitive as the *m/z* ratio of the ions increase. Thus, the multiple charging feature of ESI is significant when combined with FT-ICR MS.

3.3 MATERIALS AND METHODS:

3.3.1 Solubility in Organic Solvents:

Cell free supernatant (10 ml) of ASP-1 after 44 hours of growth was mixed with equal volume of organic solvents (chloroform, methanol, and n-butanol), stirred for 4 h and then centrifuged at 12000 rpm for 20 min. Both the soluble and insoluble fractions were completely evaporated at 55^{o}C and dissolved in 2 ml of sterile Milli Q water and tested for activity against the indicator strain methicillin and vancomycin-resistant *S. aureus* 4 by spot agar assay.

3.3.2 Effect of pH and Temperature:

To determine the effect of pH on the stability of the antimicrobial compound, cell free supernatant (CFS) was adjusted to a pH ranging from 1.0 to 14.0 by using 1 N HCl and 1 N NaOH and then incubated at 37^{o}C for 2 h. After incubation, the CFS was neutralized to pH 8.0 and tested for its activity against the indicator organisms (*S. epidermidis* ATCC 12228 and *S. aureus* MTCC 737). The combined effect of the pH and temperature on the activity of the antimicrobial activity was tested by adjusting the pH to different ranges and then incubating at 80^{o}C for 1 h, 100^{o}C for 30 min and autoclaving for 20 min. The untreated CFS was used as control.

3.3.3 Purification of the Antimicrobial Compound:

The purification of the antimicrobial compound from the cell free supernatant of URID 12.1 was achieved by a multistep process involving acid precipitation, solvent extraction, and adsorption chromatography on silica gel followed by Reversed Phase High Performance Liquid Chromatography (RP-HPLC).

3.3.3.1 Acid Precipitation:

URID 12.1 strain was grown at 37^{o}C, 110 rpm for 44 h in modified tryptone soya broth (mTSB, pH 7.4 ± 0.2) and the cell free supernatant was collected by centrifugation at 12000 rpm, 4^{o}C for 20 min. The pH of the CFS was adjusted to 2.0 by slowing adding 1 N HCl under stirring condition and kept under stirring for overnight in cold room **(Hernández et al., 2005; Ramachandran et al., 2014)**. The precipitate was collected by centrifugation at 12000 rpm, 4^{o}C for 20 min and dissolved in 20 mM sodium phosphate buffer pH 7.0.

3.3.3.2 Solvent Extraction:

The acid precipitate dissolved in buffer was mixed with equal volume of methanol and stirred for 3 h and then centrifuged at 12000 rpm, 4ºC for 20 min. The supernatant was collected and completely evaporated at 55ºC. The pellet was dissolved in methanol and the process was repeated **(Baindara et al., 2013)**. The residue was dissolved in buffer and extracted twice with equal volume of chloroform by stirring followed by centrifugation at 12000 rpm, 30ºC for 20 min. The fraction with chloroform was collected and completely evaporated at 55ºC.

3.3.3.3 Adsorption Chromatography:

The residue was dissolved in chloroform and silica gel 230-400 mesh was added and dried. The dried powder was added to the silica gel 230–400 mesh column equilibrated with chloroform. The sample was eluted with chloroform and methanol in the ratio 65:25 and the fractions were collected in 2 ml micro centrifuge tubes. The solvents were evaporated and the residue in each fraction was dissolved in 10 mM sodium phosphate buffer pH 7.0 and tested for the activity against the indicator strain. The fractions showing activity were pooled together.

3.3.3.4 Analytical Reversed-Phase High Performance Liquid Chromatography:

Pooled active fractions after adsorption chromatography were purified by reversed-phase high-performance liquid chromatography on a C18 column (Zorbax, 5 µm). Each run included loading a 50 µl sample to the column. HPLC-separation was performed by using acetonitrile (with 0.1% trifluoroacetic acid (TFA) and water gradient for 40 min. The gradient used was 0–50% acetonitrile for 18 min at a flow rate of 1ml/min, 50–63% from 18 to 26 min at 0.6 ml/min, 63–68% from 26 to 38 min at 0.4 ml/min and 68–95% from 38 to 40 min at 1 ml/min. Peaks eluting from the column were detected by the diode array detection system at 210 nm. Fractions from multiple runs were pooled and tested for the antimicrobial activity against the indicator strain by spot on lawn assay.

3.3.3.5 Semi-Preparative Scale purification on Reversed-Phase HPLC (RP-HPLC):

After analytical HPLC the purification has been scaled up to semi preparative scale. 200 µl of sample was injected into Eclipse XDB-C18 column (9.4 mm × 250 mm,

particle size 5 µm) connected to RP-HPLC system. The mobile phase water (A) with 0.1% trifluoroacetic acid (TFA) and Acetonitrile (B) with 0.1% TFA were used as solvent system. The gradient of solvent B used was as follows: 0-50% for 0-18 min at a flow rate of 1 ml/min, 50-63% from 18-26 min at 1 ml/min, 63-68% from 26-38 min at 1 ml/min, 68-95% from 38-40 min at 1 ml/min and 95% from 40-65 min at 2 ml/min. Peaks eluting from the column were detected by the diode array detection system at 214 nm. The fractions from multiple runs were pooled together and the solvents were evaporated using speed vacuum ssytem. The fractions were dissolved in sterile Milli Q water and tested for activity against indicator strains. The active fractions were further purified by analytical HPLC.

3.3.4 Determination of HPLC purified ASP-1 concentration:

The concentration of ASP-1 was measured by microplate procedure using Pierce^{TM} BCA protein assay kit. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by the protein in an alkaline medium with the highly sensitive colorimetric detection of the cuprous cation (Cu^{+1}) using bicinchoninic acid **(Smith et al., 1985)**. The ASP-1 was dissolved in sodium phosphate buffer pH-8.0. Dilutions of protein standards, Bovine Serum Albumin (BSA) in the range 0-2000 µg/ml were prepared by dissolving in the same buffer. BCA working reagent was prepared by dissolving reagent A and B in the ratio 50:1. 25 µl of each standard and ASP-1 sample was pipetted into microplate wells. 200 µl of working reagent was added into each well and mixed thoroughly. The plate was covered with lid and incubated at 37ºC for 30 min. The plate was cooled to room temperature and the absorbance was measured at 562 nm on a plate reader. Buffer mixed with BCA working reagent was used as blank. The average of blank was subtracted from the average of standards and ASP-1 sample measured at 562 nm. A standard curve was prepared by plotting each BSA standard vs. its concentration in µg/ml. The standard curve was used to determine the concentration of the ASP-1 sample.

3.3.5 Matrix Assisted Laser Desorption and Ionization–Time of Flight Mass Spectrometry:

The molecular mass and purity of the purified compound was determined by matrix-assisted laser desorption and ionization–time of flight (MALDI-TOF) mass spectrometry (UltrafleXtreme, Bruker Daltonics, Germany). An aliquot of 5 µL sample

was mixed with 5 µl matrix (2,5-dihydroxy benzoic acid in acetonitrile with 0.1% TFA). The sample was spotted onto the MALDI target and air dried. Mass spectrum was analysed in the range of 500–3500 Da.

3.3.6 Effect of Proteolytic Enzymes:

To determine the effect of proteolytic enzymes on the stability of the antimicrobial compound, partially purified antimicrobial compound after adsorption chromatography was incubated with trypsin at a final concentration of 10 mg/ml at 37ºC for 13 h and proteinase K, final concentration of 5 mg/ml at 55ºC for 3 h **(Shekh and Roy 2012)**. After incubation, the enzymes were inactivated by heating at 80ºC for 10 min and the antimicrobial activity was tested against the indicator strain. The untreated sample and the enzyme alone were used as positive and negative controls, respectively.

3.3.7 Effect of Surfactants:

To determine the effect of the surfactants on the stability and activity, partially purified antimicrobial compound after adsorption chromatography was incubated at 37 ºC for 5 h in the presence of surfactants namely Sodium dodecyl sulphate (SDS), Tween 20, Tween 80, and Triton X-100 at a final concentration of 1% (v/v) **(Kayalvizhi and Gunasekaran 2010; Ramachandran et al., 2014)** and activity was tested against the indicator strain. The untreated sample and the surfactants at the final concentration were used as positive and negative controls, respectively.

3.3.8 Effect of Metal Salts:

To determine the effect of the metals on the stability of the peptide, the partially purified antimicrobial compound obtained after adsorption chromatography was treated with metal salts ($MgSO_4$, $FeSO_4$, $MnCl_2$, $AgNO_3$, $ZnSO_4$, $CdCl_2$, $CuSO_4$ and $CaCl_2$) at a final concentration of 1 mg/ml and kept for incubation at 37ºC for 1 h **(Ramachandran et al., 2014)** before testing for activity against the indicator strain. Untreated sample and the metal salts at the final concentration were used as positive and negative controls, respectively **(Kayalvizhi & Gunasekaran 2010)**.

3.3.9 Polyacrylamide Gel Electrophoresis (PAGE) and Gel Overlay Assay:

3.3.9.1 Sodium Dodecyl Sulphate (SDS)-PAGE:

3.3.9.1.1 Reagents for SDS-Polyacrylamide Gel:

Acrylamide mix: 29% Acrylamide (W/V) + 1% Bisacrylamide (W/V)

Tris buffer: 1.5 M, pH-8.8 for resolving gel and 1 M, pH-6.8 for stacking gel

SDS: 10% (W/V)

Ammonium Per-Sulphate: 10% (W/V) freshly prepared

3.3.9.1.2 SDS-PAGE Running Buffer 1X:

Glycine: 1.44% (W/V)

Tris-HCl: 0.3% (W/V)

SDS: 0.1% (W/V)

3.3.9.1.3 SDS-PAGE Loading Dye 5X:

Tris buffer: 0.5 M, pH-6.8, 17.5% (V/V)

Glycerol: 45% (V/V)

SDS: 5% (W/V)

Bromophenol Blue: 0.2% (W/V)

β-mercaptoethanol: 12.5% (V/V)

3.3.9.1.4 SDS-PAGE Sample Preparation and Electrophoresis:

Partially purified antimicrobial compound was mixed with 5X loading buffer and boiled for 3 mins and loaded in duplicates into 15% SDS-PAGE and was electrophoresed at a constant voltage of 60 V in stacking gel and 90 V in resolving gel **(Laemmli & Favre 1973)**. Molecular marker in the range of 43 kDa to 3.5 kDa was also loaded in another lane. After electrophoresis, the gel was divided into two parts such that one part has marker and sample and the other part with only sample.

3.3.9.1.5 Washing the Gel to Remove SDS and Gel Overlay Assay:

After electrophoresis, the gel with only sample was fixed with 25% ethanol and 5-10% acetic acid for 30 mins by placing it on gel rocker followed by water for five mins. The fixed gel was washed thrice for 40 mins each with 0.1% Tween 80 to remove the SDS followed by two times with sterile distilled water for 20 mins. Then the gel was transferred into fresh Petri dish and washed with sterile distilled water till the foam is removed. Finally, the gel was transferred into a fresh plate and kept in a slant position and air dried to completely remove the water on the surface. The dried gel was transferred into fresh petri dish and Brain Heart Infusion media with 1% agar and 0.1% indicator strain was poured over the gel and incubated at 37ºC for 24 h **(Bhunia et al., 1987; Yamamoto et al., 2003)**.

3.3.9.2 Silver Staining:

The part of the gel with molecular marker and the sample containing antimicrobial compound was silver stained to observe the location of the bands and compare the active region after gel over lay assay.

3.3.9.2.1 Reagents:

3.3.9.2.1.1 Fixing Solution:

50% Methanol

10% Acetic Acid

0.05% Formalin

3.3.9.2.1.2 Wash Solution:

20% Ethanol

3.3.9.2.1.3 Sensitising Solution:

0.02% Sodium thiosulphate

3.3.9.2.1.4 Silver Nitrate Solution:

0.2% Silver Nitrate

0.076% Formalin

3.3.9.2.1.5 Developer Solution:

6% Sodium bicarbonate

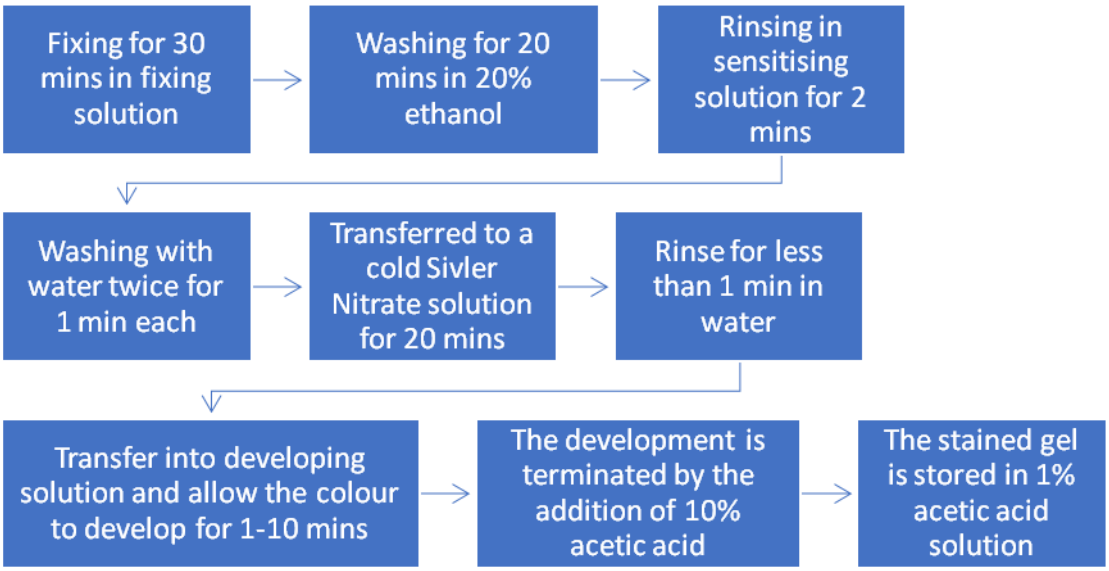
0.05% Formalin

0.0004 % Sodium thiosulphate

3.3.9.2.1.6 Stop Solution:

10% Acetic acid

3.3.9.2.2 Silver Staining Protocol:



3.3.9.3 Native PAGE:

3.3.9.3.1 Reagents for Native Polyacrylamide Gel:

Acrylamide mix: 29% Acrylamide (W/V) + 1% Bisacrylamide (W/V)

Tris buffer: 1.5 M, pH-8.8 for resolving gel and 1M, pH-6.8 for stacking gel

Ammonium Per-Sulphate: 10% (W/V) freshly prepared

3.3.9.3.2 Native PAGE Running Buffer 1X:

Glycine: 1.44% (W/V)

Tris-HCl: 0.3% (W/V)

3.3.9.3.3 Native PAGE Loading Dye 5X:

Tris buffer: 0.5 M, pH-6.8, 17.5% (V/V)

Glycerol: 45% (V/V)

Bromophenol Blue: 0.2% (W/V)

3.3.9.3.4 Native PAGE Sample Preparation and Electrophoresis:

Partially purified antimicrobial compound was mixed with 5X loading buffer and loaded in duplicates into 15% native PAGE and was electrophoresed at a constant voltage of 60 V in stacking gel and 90 V in resolving gel. Molecular marker in the range of 43 kDa to 3.5 kDa was also loaded in another lane. After electrophoresis, the gel was divided into two parts such that one part has marker and sample and the other part with only sample.

3.3.9.3.5 Gel over Lay Assay:

The part of the gel with only sample was fixed with 25% ethanol and 5-10% acetic acid for 30 mins followed by washing in water twice for 10 mins. The gel was transferred into a fresh petri dish and over laid with Brain Heart Infusion media with 1% agar and 0.1% indicator strain and incubated at 37^{o}C for 24 h.

3.3.10 Thin Layer Chromatography (TLC) and Bioautography Assay:

Partially purified biologically active antimicrobial compound after adsorption chromatography was spotted on to silica gel 60 plate (7 × 4 cm; layer thickness, 0.20 mm, Merck) and developed with chloroform-methanol-water (65:25:4) as mobile phase. One part of the TLC plate was sprayed with ninhydrin (0.2%) to detect the presence of amino acids. The other part was completely dried of solvents before placing in a fresh Petri dish; the molten BHI broth was pre-inoculated with 0.1% indicator strain along with Triphenyl tetrazolium chloride (TTC) dye, poured on TLC plate and incubated at 37^{o}C overnight **(Tabbene et al., 2009)**. The Rf value of the antimicrobial compound was estimated. The Rf of the detected spots is defined as the ratio between the distance travelled by the compound divided by the distance travelled by the solvent.

3.3.11 Amino Acid Composition Analysis of HPLC purified ASP-1:

An amino acid analysis of the purified antimicrobial peptide was done on HPLC by comparing the retention times with the standard amino acids. 100 µg of peptide was mixed with 6N hydrochloric acid (HCl) and heated for 24 h at 110^{o}C. After hydrolysis, the sample was derivatized by adding coupling reagent (Phenyl IsoThio Cyanate: Methanol: Triethyl amine: Milli-Q water in 1:7:1:1 respectively) and kept in thermomixer for 1 h at 45^{o}C. The mixture was then dried and reconstituted in 10 mM sodium acetate, pH 6.4 and injected for HPLC with C18 analytical 4.6 x 250 mm, 5 µm column at a flow rate of 1 ml/min. 10 mM sodium acetate, pH 6.4 and 60% acetonitrile in 10 mM sodium acetate, pH 6.4 were used as gradient system **(Dhillon et al., 2014).** The retention times were compared with the standards mixture of amino acids.

3.3.12 CD Spectroscopy for Secondary Structure Analysis:

The secondary structure of the purified antimicrobial peptide was analyzed by using CD. A Jasco (Japan) J-810 Spectro polarimeter was used to measure the circular dichroism spectra. The data were collected and analyzed by Jasco v1 software. 200 µg peptide was diluted in 400 µl of 100% solvents (water, methanol, Trifluoro ethanol (TFE)) and incubated at room temperature for 10 min before the CD measurement. CD analysis was performed at room temperature on a 1-mm-path-length, capped, square optical cell using a Jasco J-810 Spectro polarimeter. All CD Spectra were obtained at wavelengths of 190 to 260 nm at room temperature. Each spectrum was obtained from averaging three scans taken at a scan rate of 50 nm/min with a 2-nm spectral bandwidth. The peptide configuration was obtained by analyzing the data with spectra manager software, Jasco secondary structure estimation, version 1, CD reference spectra by **(Yang et al., 1986)**.

3.3.13 MALDI-TOF-MS Analysis:

The antimicrobial compound was analyzed by matrix assisted laser desorption-ionization mass spectrometry performed on Bruker Daltonics UltrafleXtreme Time-of-Flight/Time-of-Flight (TOF/TOF) mass spectrometer in positive ion mode and reflectron. External calibration was done with peptide calibrant mixture from Bruker. 10 mg/ml α-

Cyano-4-Hydroxycinnamic acid (HCCA) solubilized in 50% acetonitrile in water, 0.1% TFA was used as matrix. Stock solution of sample was prepared by dissolving in Milli-Q water at 0.33 µg/ml and then diluting it 100X. Sample-matrix mixture was prepared by mixing 1 µl of sample with 1 µl of matrix and allowed to dry on the plate **(Torres et al., 2015).**

3.3.14 Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI FTICR-MS):

ESI FTICR-MS analysis was carried on Bruker Solari X ESI FTI-CR mass spectrometer in positive ion mode. External calibration was done over a range of m/z 50 to 1500 using H_3PO_4 adducts and mean residual error obtained was <2 ppm. The sample was analyzed at a concentration of 36 µM in 50% acetonitrile with 0.1% final concentration of formic acid. Analysis of spectra and elemental compositions were generated using the Bruker Data Analysis Software vs 4.0.

The MS, MS/MS and the ion mobility analysis were performed on the potassium hydroxide (KOH) treated peptide. The peptide was incubated in the presence of KOH for 2 h at 37 °C and neutralized by TFA. Both the untreated and the treated samples were analyzed by Electrospray Ionization Quadrupole Time of Flight (ESI-Q-TOF) at a protein concentration of 75 µM, 50% acetonitrile, 50% MilliQ-water and 0.1% TFA.

3.4 RESULTS:

3.4.1 Effect of pH and Temperature:

The effect of pH on the stability of the antimicrobial compound was tested by adjusting the pH of the cell free supernatant from 1-14 by using 1N HCl and 1N NaOH. The antimicrobial compound was stable over a wide pH range from 1 to 10, and at pH 12.0 there was a slight reduction in the activity whereas a significant reduction in the activity at pH 14.0 was recorded. The antimicrobial compound was also stable at different temperatures approximately 80% activity was retained after incubation at 80^{o}C for 1 h, 75% at 100^{o}C for 30 min and 60% at 121^{o}C for 20 min. At acidic pH, there is a slight reduction in the activity at higher temperatures (100 and 121◦C) whereas at alkaline pH there was significant reduction in the activity with complete loss of activity at pH 12.0 after autoclaving **(Fig. 13)**.

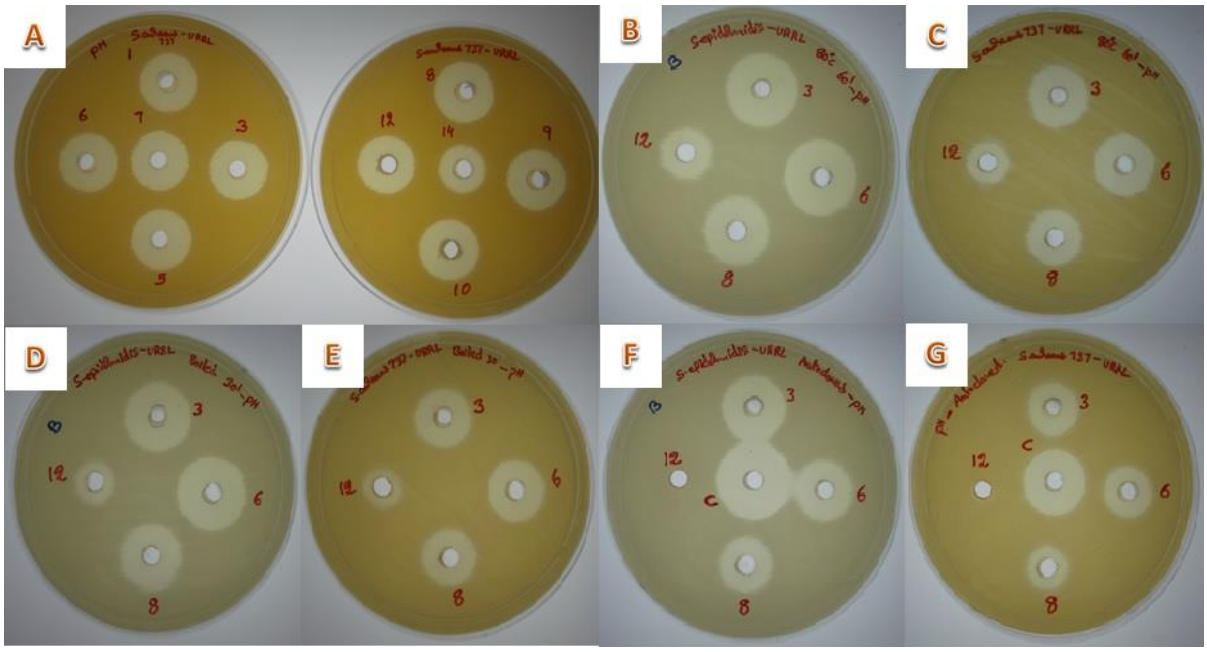


Figure 13: A) Effect of pH on the antimicrobial activity of peptide. B) & C) Effect of incubation at 80^{o}C for 60 min. D) & E) Effect of boiling for 30 mins on the activity of peptide. F) & G) Effect of autoclaving on the activity of peptide.

3.4.2 Solubility in Organic Solvents, Surfactants and Metal Salts:

The effect of the organic solvents, surfactants and metal salts on the stability of the antimicrobial peptide was tested. The antimicrobial activity of the peptide was not affected by the organic solvents (50% v/v) and surfactants (1% v/v) indicating the hydrophobic nature of the peptide. The biological activity of the compound was not affected in the presence of metal salts **(Fig. 14)**. The results were shown in **(Table 6)**.

Table 6: Effect of organic solvents, surfactants and metal salts on the stability of the antimicrobial compound.

Treatment	Concentration	Activity
Surfactants (Tween 20, Tween 80 and Triton X-100)	1% (v/v)	+
Organic solvents (chloroform, methanol, n-butanol)	50% (v/v)	+
Metal salts ($MgSO_4$, $FeSO_4$, $MnCl_2$, $AgNO_3$, $ZnSO_4$, $CdCl_2$, $CuSO_4$ and $CaCl_2$)	1 mg/mL	+

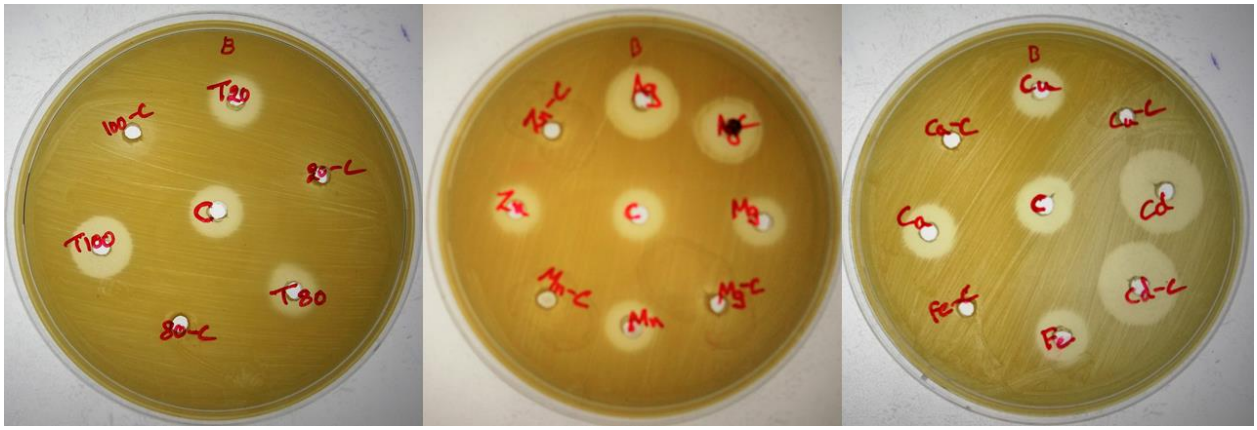


Figure 14: Effect of surfactants and metal salts on the antimicrobial activity of the peptide. C- partially purified peptide. Surfactants and metal salts alone acted as negative controls. Activity was increased in the presence of Ag and Cd as they alone showed toxic effect on the bacterial cells.

3.4.3 Effects of Proteolytic Enzymes:

Effects of proteolytic enzymes (Trypsin 10 mg/ml, Proteinase K 5 mg/ml) was tested on the semi purified compound after adsorption chromatography. There was no effect on the antimicrobial activity of the peptide showing it to be resistant to trypsin **(Fig. 15)** and proteinase K digestion. This could have been due to the modifications in the amino acids. The enzymes alone did not show any antimicrobial activity.

Figure 15: Effect of trypsin on the antibacterial activity of the peptide. As observed in the figure, the activity was completely retained after 13 h of incubation at 37 ºC in the presence of trypsin 10 mg/ml.

S+E = Sample + Enzyme, S+B = Sample + Buffer, E+B = Enzyme + Buffer

3.4.4 Purification of the Antimicrobial Compound:

After acid precipitation and solvent extraction, adsorption chromatography was performed using silica gel 230–400 mesh equilibrated with chloroform. Fractions were collected as 2 ml aliquots and tested for activity after evaporation of solvents. The active fractions **(Fig. 16)** were pooled together. Active fractions were further purified by RP-HPLC on a C18 column; the eluting active fraction showed a retention time of 30.5 min **(Fig. 17,** peak circled in black), indicating its non-polar nature. After Adsorption chromatography, the peptide was also purified by RP-HPLC on a semi preparative scale and the peaks were collected and tested for antibacterial activity. The active peak (circled in black) was subjected to rechromatography using analytical column and a single peak was observed **(Fig. 18)**. The concentration of the protein was calculated by using the standard curve obtained from plotting the BSA standard versus its concentration in µg/ml. By using the formula y=0.002x + 0.036 the concentration of ASP-1 peptide was calculated **(Standard curve 1)**. The molecular mass of the peptide was confirmed as 804.4 Da by MALDI-TOF with detection of sodium and potassium adducts **(Fig. 19)**.

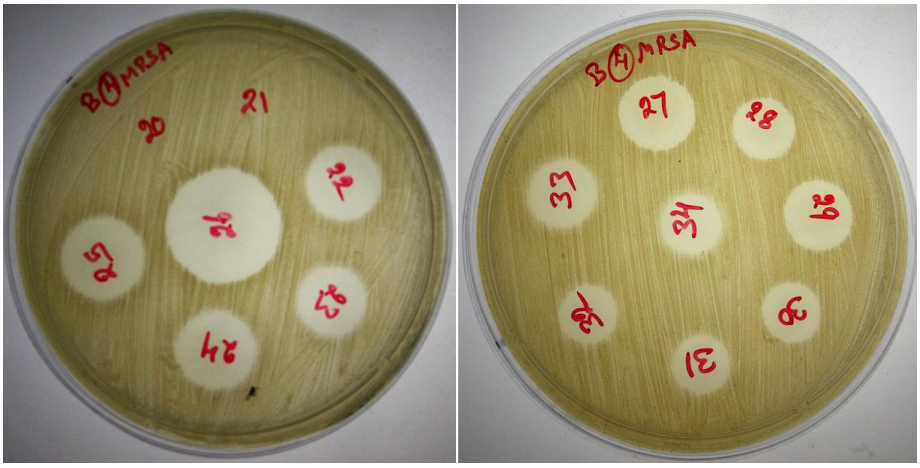


Figure 16: Fractions showing antibacterial activity after adsorption chromatography.

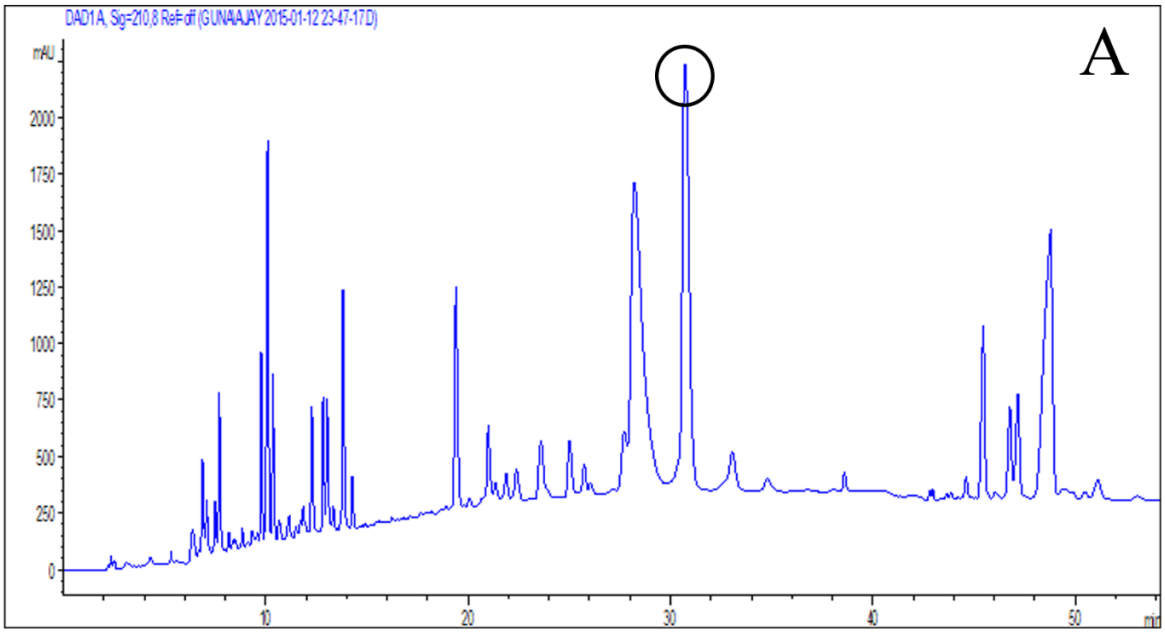


Figure 17: RP-HPLC chromatogram showing the active peak (circled in black) with a retention time of 30.5 min. Individual peaks were collected and tested for antimicrobial activity after evaporating the solvents and dissolving in MilliQ water.

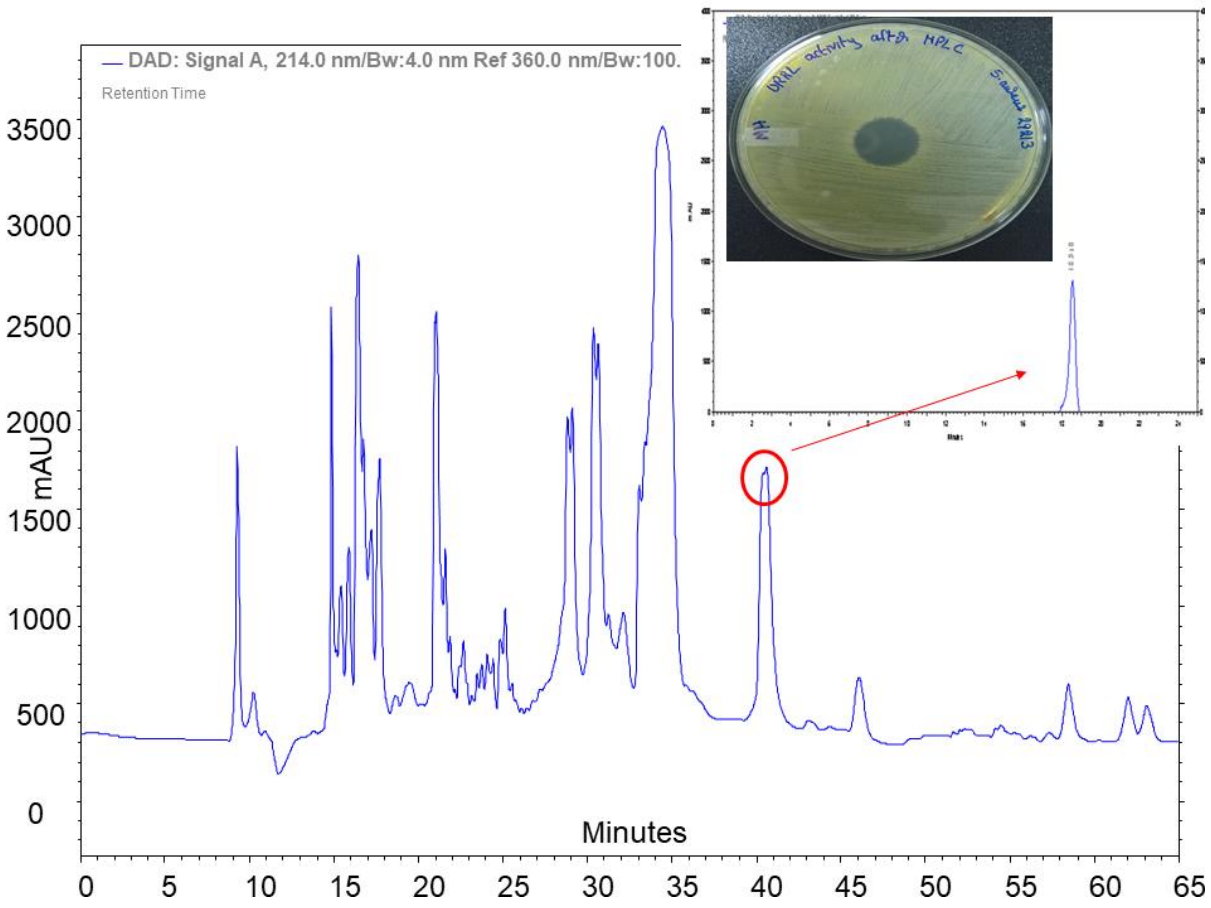
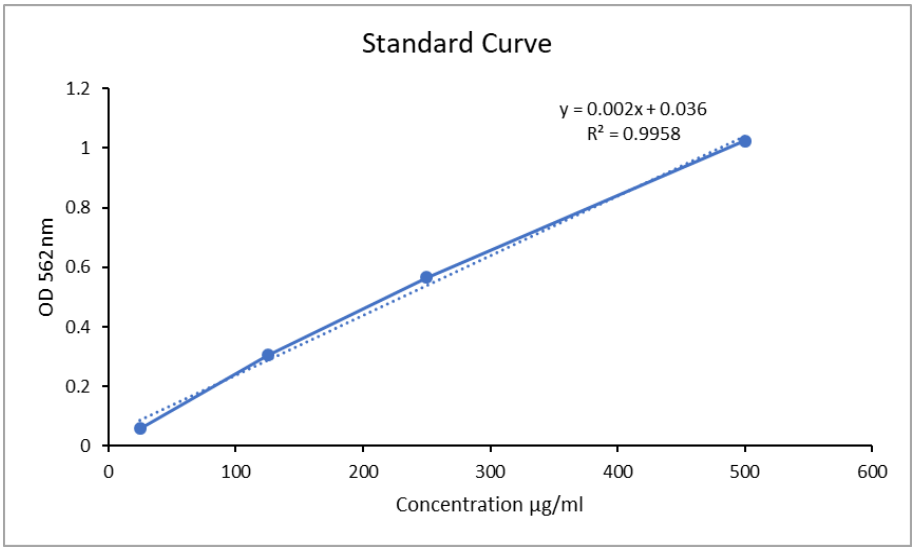


Figure 18: Semi-Preparative HPLC chromatogram showing the active peak (circled in red). Activity was observed as the zone of inhibition against the *S. aureus* ATCC 29213 strain and purity was checked by analytical HPLC (inset).



Standard Curve 1: Standard curve for measuring the peptide concentration by BCA method.

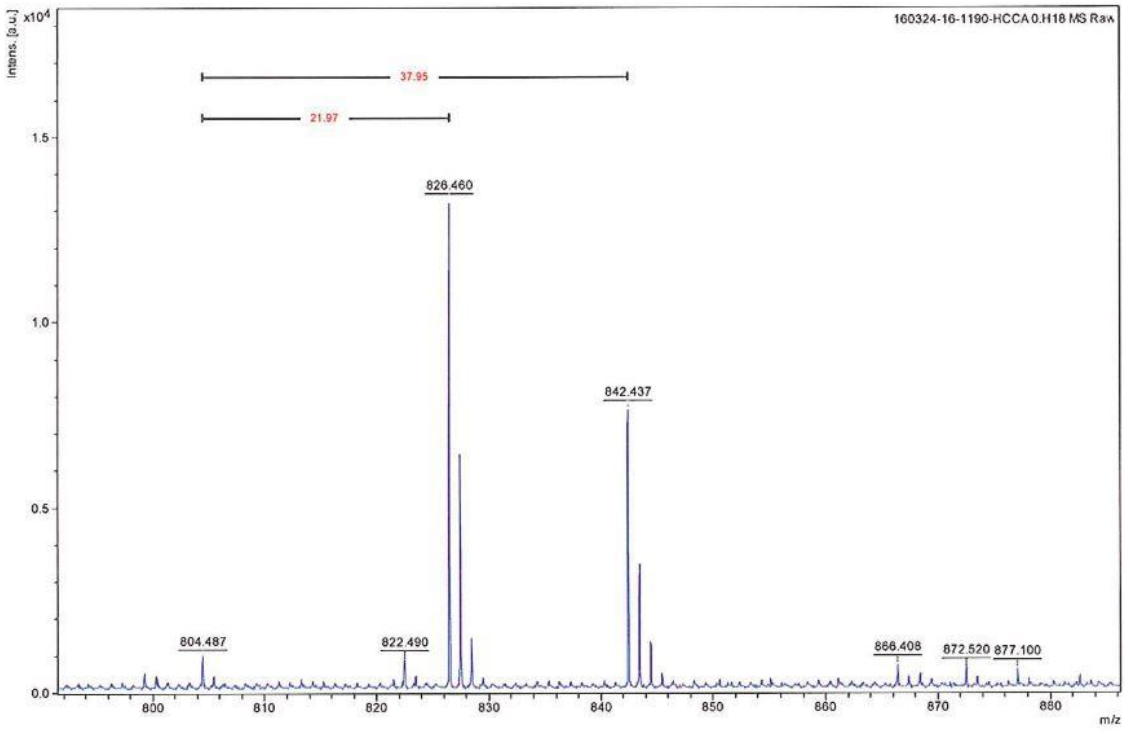


Figure 19: MALDI-TOF spectrum showing the molecular mass of antimicrobial compound as 804.4 Da along with its sodium and potassium adducts at 826.4 Da and 842.4 Da respectively.

3.4.5 Polyacrylamide Gel Electrophoresis (PAGE) and Gel Overlay Assay:

After SDS-PAGE one part of gel was used for staining and the other for gel over lay assay. The band less than 3.0 kDa has shown antimicrobial activity **(Fig. 20 A, B)** against the indicator strain *S. aureus* 737. In the Native PAGE, the activity was observed at the top of the resolving gel indicating either the net positive charge of the antimicrobial compound or aggregation in its native form **(Fig. 20 C)** not allowing the peptide to migrate further.

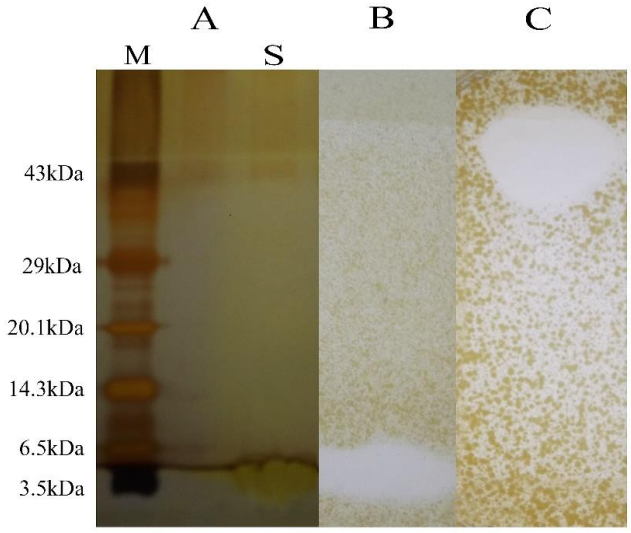


Figure 20: A) SDS-PAGE with molecular marker (lane M) and sample (lane S). B) Antimicrobial activity shown as zone of inhibition after SDS-PAGE and zymogram. C) Antimicrobial activity as indicated by zymogram after native PAGE.

3.4.6 Thin Layer Chromatography (TLC) and Bioautography Assay:

After thin layer chromatography, the plate was air dried to remove the solvents and one part of the TLC plate was used for bioautography assay and showed clear zone of inhibition **(Fig. 21 A, B)**. The region corresponding to the antimicrobial compound when sprayed with 0.2% ninhydrin showed negative result. This might be due to blocking of the N-terminal end by modification or because of the cyclic nature of peptide. The Rf value of the spot was 0.8 **(Fig. 21 A, B)**, the centre of zone of inhibition was taken as the distance travelled by the solute.

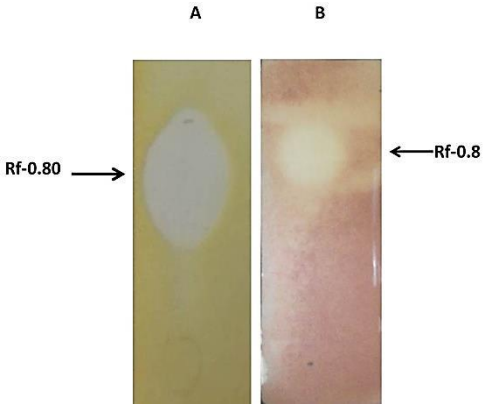


Figure 21: A) Bioautography assay after TLC showing zone of inhibition, against *S. aureus* MTCC 737. B) Bioautography assay after TLC (using the TTC dye) showing antimicrobial activity against methicillin and vancomycin-resistant *Staphylococcus aureus* 4 showing the Rf value 0.80.

3.4.7 Amino Acid Analysis on HPLC:

The amino acid composition of the HPLC-purified antimicrobial peptide is shown in **Table 7**. The amino acid composition analysis indicates the detection of hydrophobic amino acids Alanine, Valine, Phenylalanine, and neutral amino acids Asparagine and Serine besides containing unusual amino acids β-amino butyric acid and dipeptide carnosine (β-alanyl histidine) and 3-methyl histidine.

Table 7: Amino acid composition of antimicrobial compound estimated using HPLC after acid hydrolysis.

S. No	Amino Acids	µg/ml of sample
1.	Phosphoserine	0.614506351
2.	Aspartic acid	2.212929573
3.	Glutamic acid	1.229951541
4.	OH Proline	2.18732102
5.	Glycine	0.01803235
6.	Asparagine	3.195839903
7.	Alanine	1.529281496
8.	β-amino butyric acid	0.656590109
9.	Carnosine	1.294775637
10.	3-Methyl histidine	1.305791827
11.	Valine	2.255160536
12.	Cysteine	5.086563474
13	Phenylalanine	0.780441898

3.4.8 CD Spectroscopy - Secondary Structure Analysis:

Bioactive peptides might adopt specific peptide backbone-folding patterns (secondary structure) that display a characteristic CD spectrum **(Berova & Nakanishi 2000; Sreerama et al., 2000).** Ratios of Helix, Beta sheet, Turn, Random coil were obtained by secondary structure estimation. CD spectrometry **(Fig: 22A)** showed that peptide present in water as unordered (random) structures, which had a strong negative ellipticity near 200 nm. The CD spectrum of purified peptide in methanol and TFE demonstrated a negative ellipticity near 205 nm, typical of that seen for a type I β-structure **(Fig: 22 B & C)**. In TFE, the native peptide exhibited a beta structure (42.4%) and random structure (49.8%). In the presence of methanol, a β-sheet structure was dominant (58.6%) and less dominant was the random structure (41.4%).

From the CD spectra, it was observed that the antimicrobial peptide has a largely random coil conformation (43.5%) in water **(Fig: 22A)**. The concerned peptide also displayed the β- structure in water indicating its amphipathicity in aqueous solution. No helical content was found either in methanol or TFE (a membrane-mimicking solvent). However, in the presence of methanol, there was a high percentage (58.6%) of β-structure. The CD spectrum in TFE showed a beta-conformation of 42.4 % and beta-turn of 7.7%. The peptide exists as beta sheets and random coils with some turns. The helix structure is completely absent. The structures in water and TFE are more related.

Table 8: Percentage of Helix, Beta, Turn and Random structures of antimicrobial peptide observed in water, methanol and TFE.

	Water	Methanol	TFE
Helix	0	0	0%
Beta	48.5%	58.6%	42.4%
Turn	8.5%	0%	7.7%
Random	43%	41.4%	49.8%

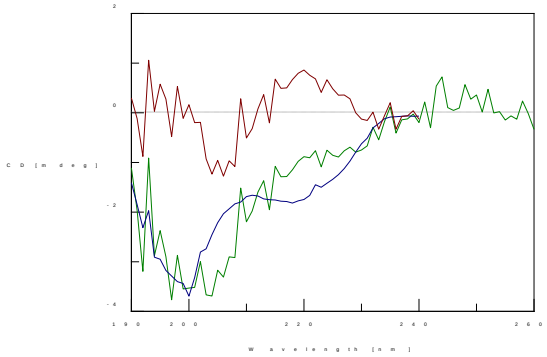


Figure 22A: Secondary structure estimation in water.

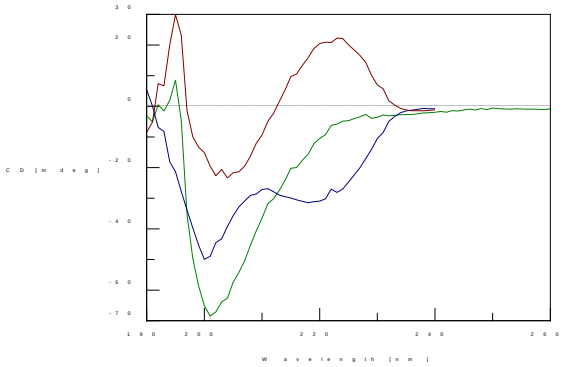


Figure 22B: Secondary structure estimation in methanol.

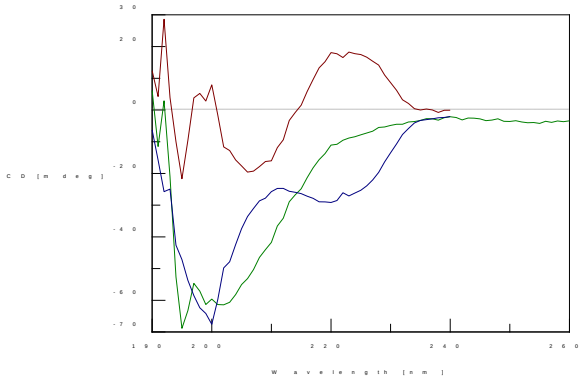


Figure 22C: Secondary structure estimation in TFE.

3.4.9 MALDI-TOF-MS Analysis:

HPLC-purified antimicrobial peptide was analyzed by MALDI-TOF MS. The raw spectrum has shown peaks in the range of 700 to 900 m/z. On zoom a small peak was observed at m/z 804.5 and its sodium and potassium adducts were observed at m/z 826.5 and 842.4 respectively **(Fig. 19)**. MS/MS spectrum of the peak at m/z 804.5 **(Fig. 23A & B)** was obtained by fragmentation using high energy collision induced dissociation (CID). **Fig. 24** shows the MS/MS spectrum of the ion detected at m/z 804.5. Based on the spectrum obtained, two potential sequences were deduced, Thr-Ala-Val-Dhb-Phe and Ala-Dhb-Val-Thr-Phe where Dhb is Dehydroamino-2-butyric acid (**Fig. 24**). Another potential partial sequence Phe-Ile/Leu was found as indicated by the peaks at m/z 86 and 120 corresponding to immonium ions of isoleucine or leucine and phenylalanine respectively. When analyzing the MS/MS spectrum obtained for ion at 826 m/z **(Fig. 25)** another potential sequence was deduced which is Glu-Ala- Pro-Abu-Phe with Abu = 2-amino-butyric acid.

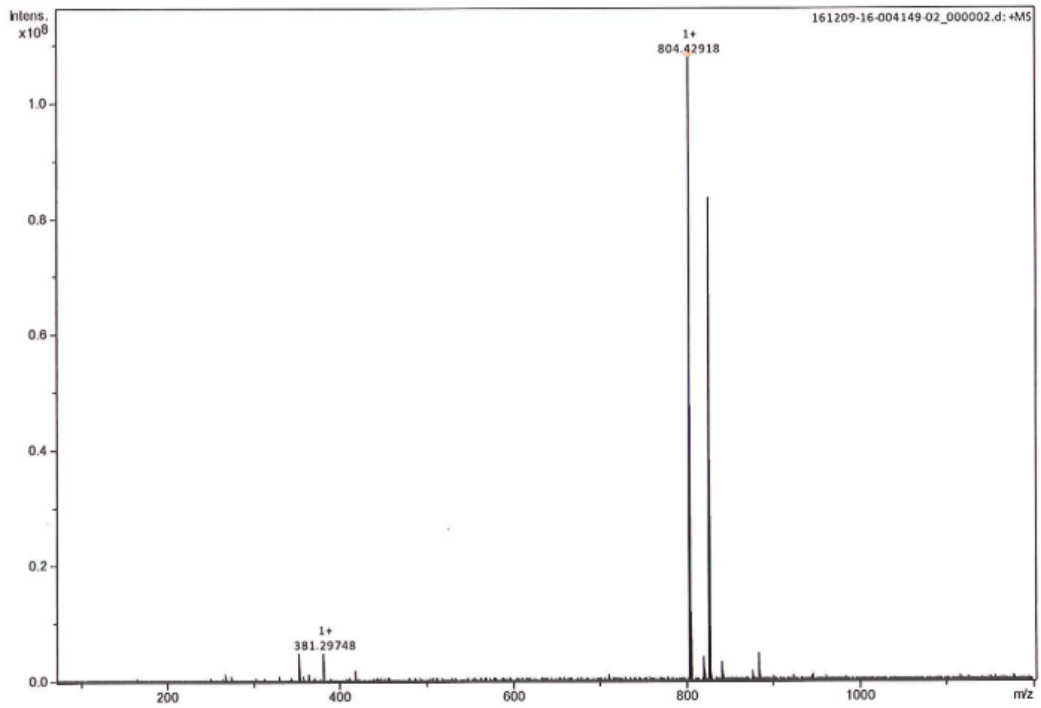


Figure 23A: Raw spectrum showing the molecular weight of ASP-1 as 804.4 Da

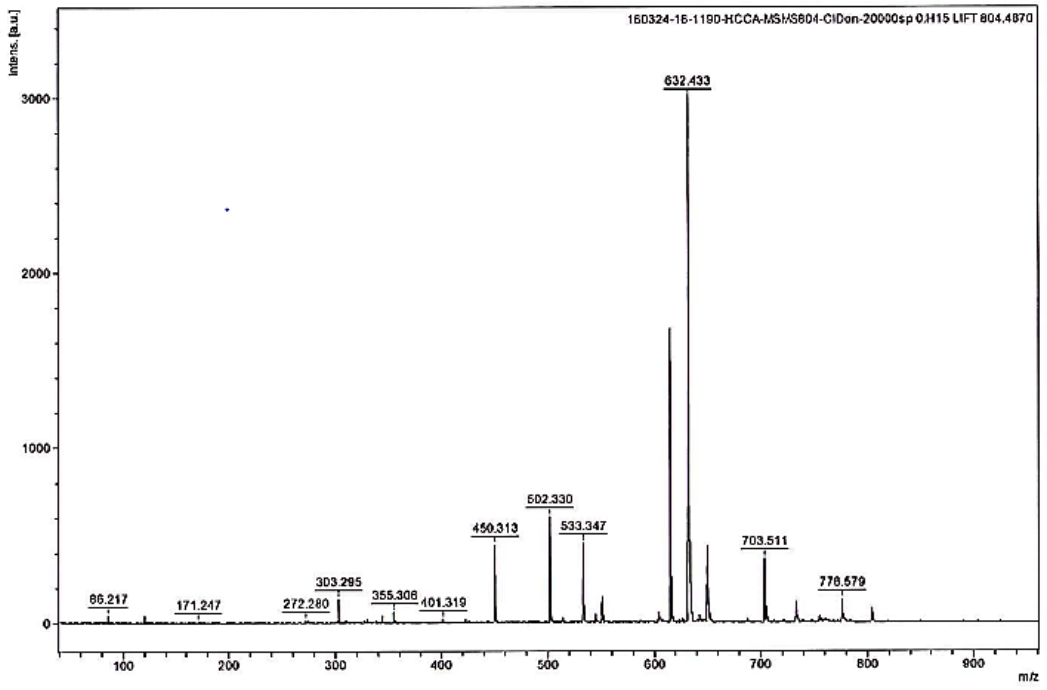


Figure 23B: MS-MS spectrum of peak at 804 m/z obtained by fragmentation using high energy collision induced dissociation.

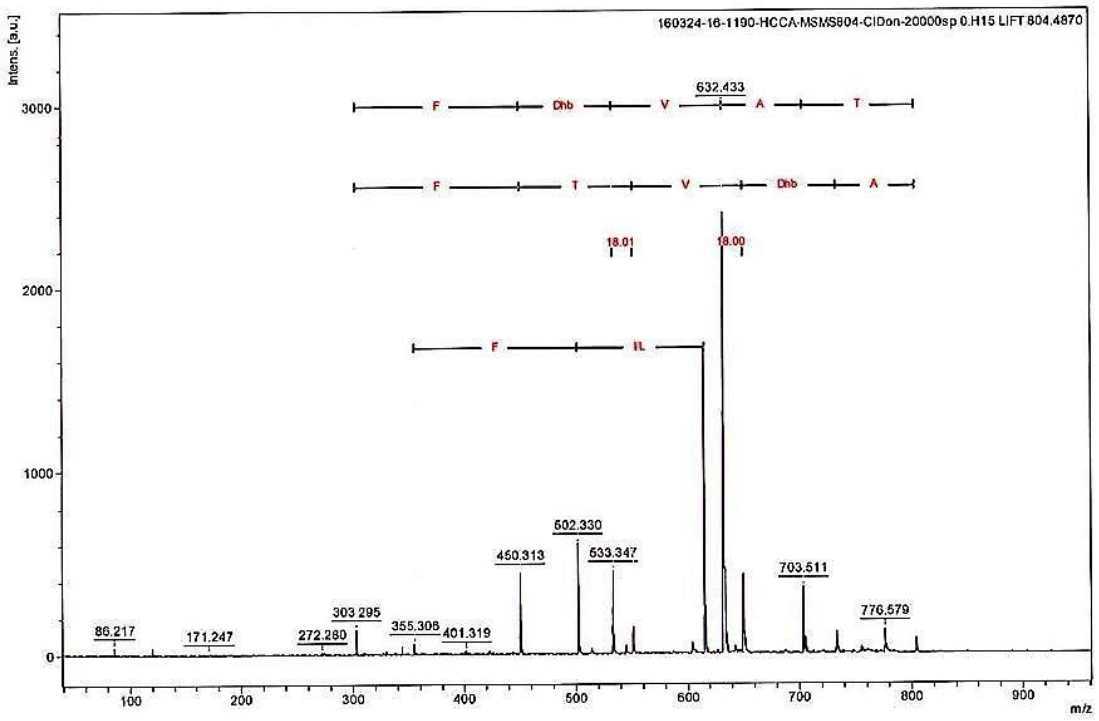


Figure 24: Annotated spectrum of the fragmented peak at 804 m/z.

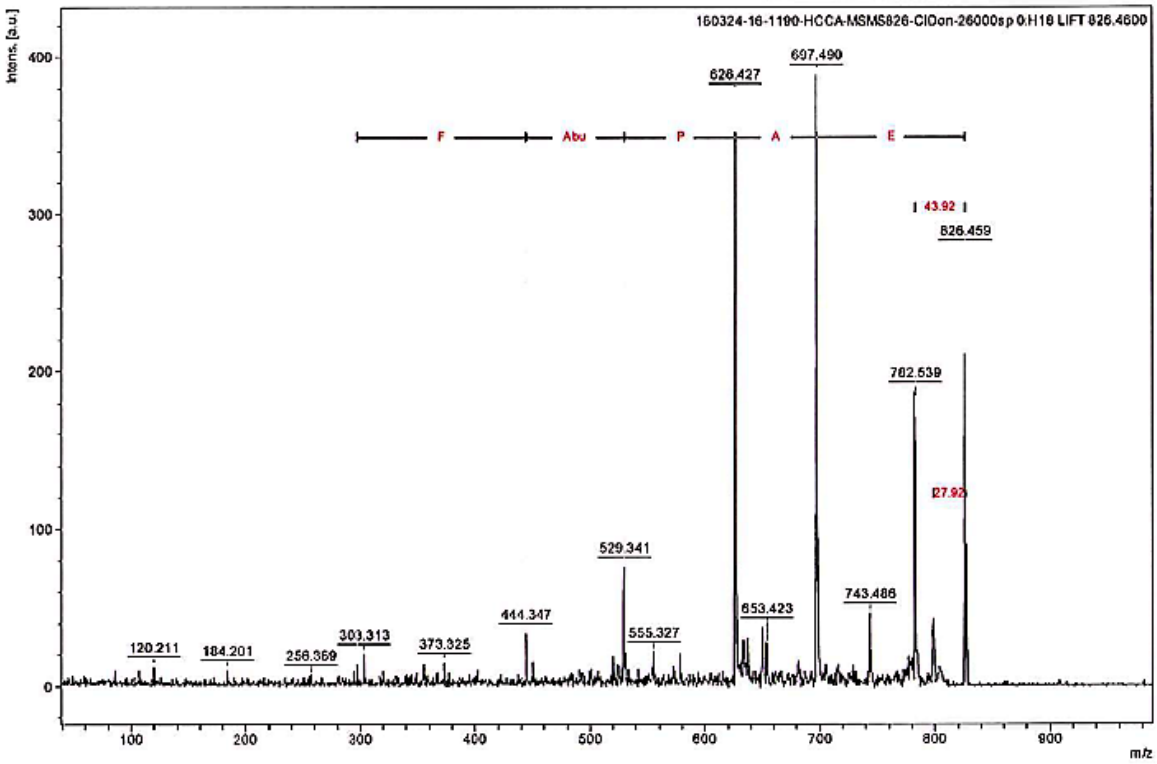


Figure 25: MS-MS spectrum of peak at 826 m/z obtained by fragmentation using high energy collision induced dissociation. A potential partial sequence E-A-P-Abu-F, Abu=2 amino butyric acid was deduced.

3.4.10 Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI FTICR-MS):

Analysis of the HPLC purified peptide by ESI FTICR-MS showed two intense peaks around 800 m/z. On zoom it has shown sodium and potassium adducts along with the ammonium adduct of ion at 804 m/z **(Fig. 26)**. MS/MS (CID) of 804.43 m/z was performed using an acceleration voltage of 20 *V*. From the recorded spectrum, five "y" type ions were detected that corresponded to the parent ions and the sequence was deduced as Thr-Ala-Val-Dhb-Phe. Another small sequence, Phe-Ile/Leu, corresponded to an ion at 355.19 m/z in agreement with the 'b' type ion containing Thr-Ala-Val-Dhb was observed **(Fig. 27)**. The longest potential sequence identified was Thr-Ala-Val-Dhb-Phe-Ile/Leu; based on the sequence, the remaining part of the compound would be $C_{11}H_{11}NO_2$ corresponded to an acetylated phenylalanine ($C_{11}H_{13}NO_3$). With the acetylated-Phe, the sequence would be Ac-Phe-Thr-Ala-Val-Dhb-Phe-Ile/Leu and the observed raw formula $C_{42}H_{57}N_7O_9$ with delta -0.16 ppm. The delta H_2O corresponds to loss of water molecule. Another potential sequence was obtained when the spectrum from 826.4 m/z was analyzed using the MALDI-TOF-MS/MS.

After KOH hydrolysis, there is a gain of 18 Da indicating the presence of a lactone ring in the intact peptide. The MS/MS spectrum obtained on the linearized peptide after KOH hydrolysis paved the way for easier interpretation in comparison to the spectrum that was obtained from the cyclic peptide; the presence of a lactone ring might have induced specific fragmentation pattern, thereby leading to a potential bias for the identification. As per the fragmentation results, the amino acid sequence was confirmed as Ac-Phe-Leu-Phe-Thr-Val-Ala-Dhb in the linearized peptide and the presence of *b5*, *b5*-$H_2O$and *y2* ions **(Fig. 28 A & B)** unambiguously validated the detection of V-A in the sequence indicating that this antimicrobial peptide might belong to the class of lactone ring-containing peptide antibiotics that includes TL-119 **(Nakagawa et al., 1975)**.

An ion mobility experiment was performed to further investigate the cyclic structure of the compound. Collision Cross Section (CCS) may be considered as the rotationally averaged 2-dimensional projection of an ion's 3D structure. In the ion mobility assay, the linearized peptide displayed a CCS of 216 Å² while the intact peptide

appeared more compact at 210 Å^2 **(Fig. 28 C & D)**. This is compatible with the presence of a lactone ring since the later induced structural compaction in the intact peptide.

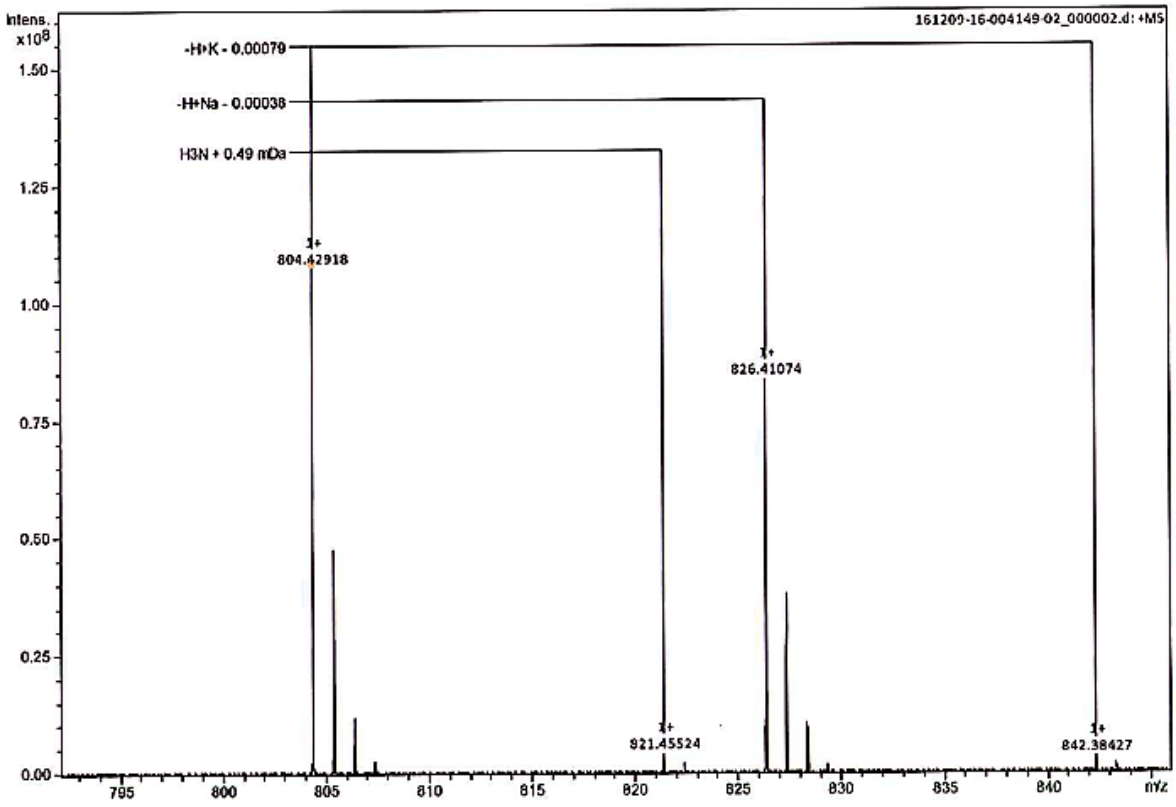


Figure 26: Spectra of the sample showing ion at 804 m/z along with ammonium, potassium and sodium adducts at 821, 826 and 842 m/z respectively.

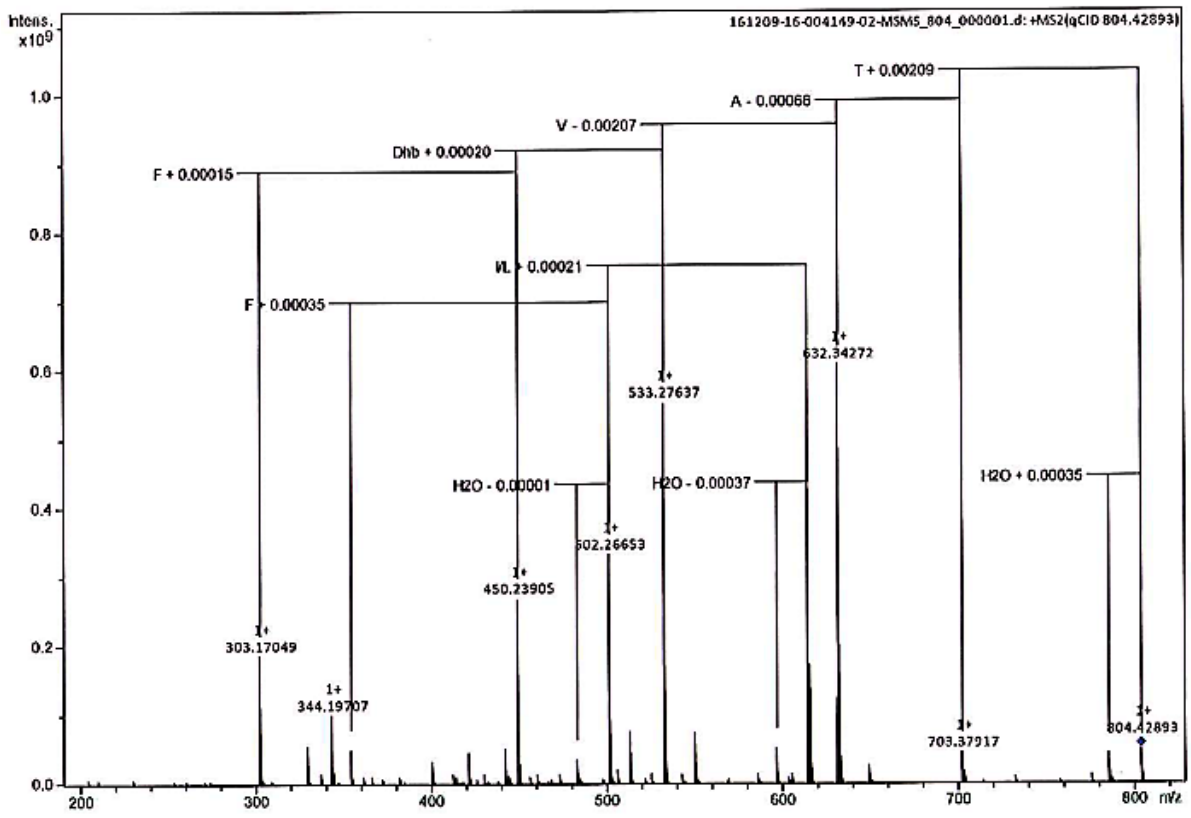


Figure 27: Annotated spectrum of the fragmented ion at 804 m/z yielded a potential sequence Ac-Phe-Thr-Ala-Val-Dhb-Phe-Ile/Leu.

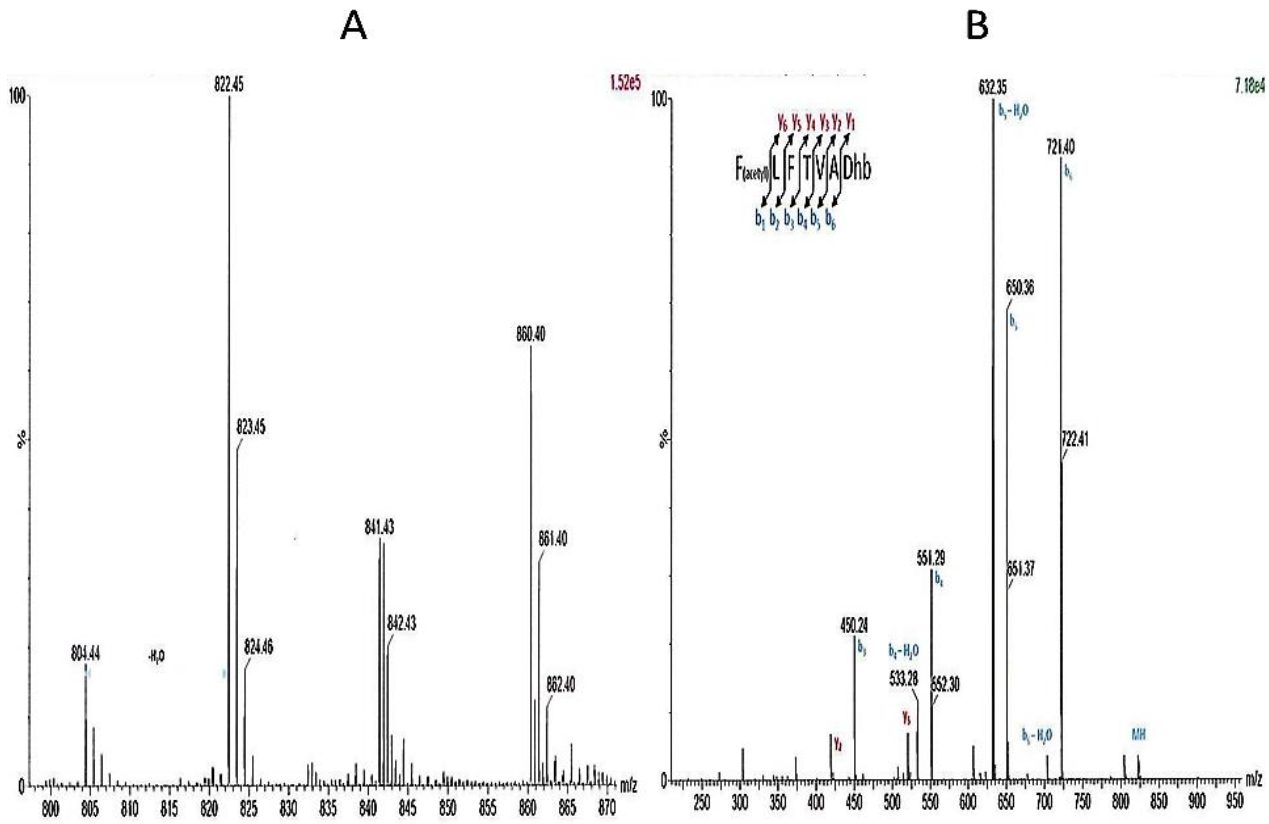


Figure 28: A) A mass gain of 18 Da was observed after KOH hydrolysis indicating the presence of a lactone ring in the intact peptide. B) MS/MS spectrum of the linearized peptide identified the sequence as Ac-Phe-Leu-Phe-Thr-Val-Ala-Dhb which resembles TL-119: Ac-Phe-Leu-Phe-Thr-Val-Ala-Abu.

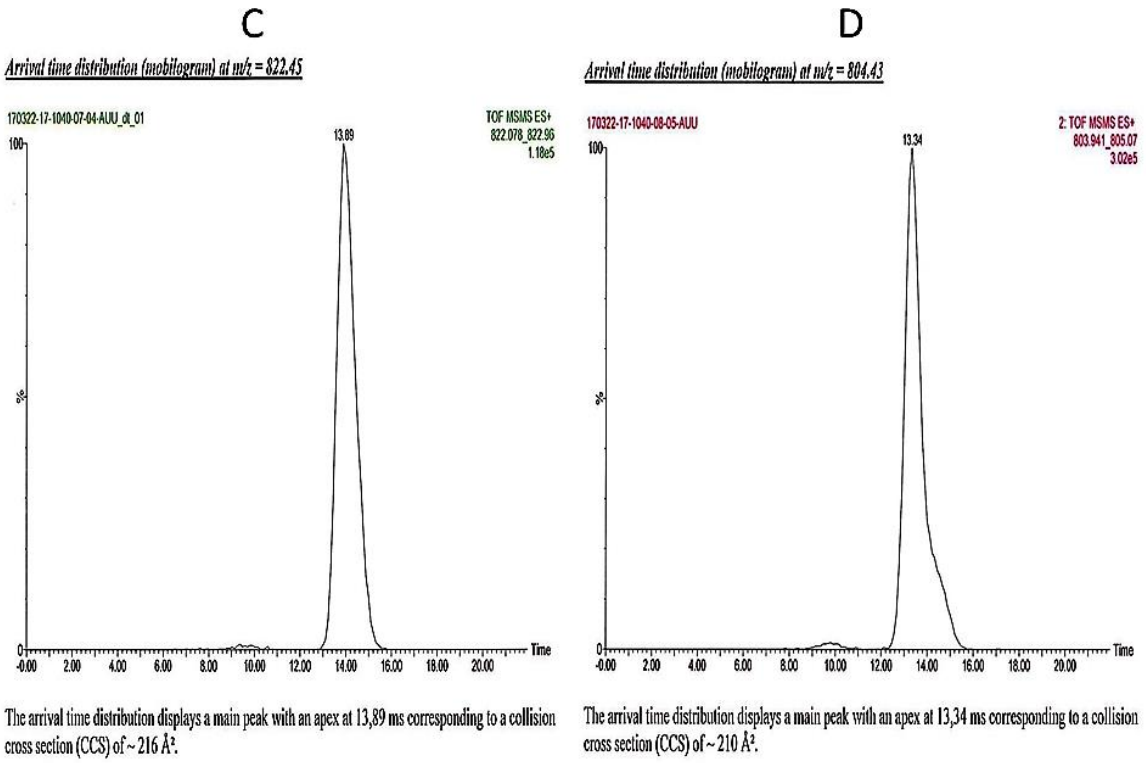


Figure 28: C& D) Ion mobility experiments show a Collision Cross Section of 216 Å² and 210 Å² for the linearized and intact peptide, respectively.

3.5 DISCUSSION:

The semi-purified antimicrobial substance was subjected to various types of physico-chemical tests. After heat, acid and alkaline treatment, the inhibition zones produced by the partially purified antimicrobial compound remained almost the same **(Fig. 13)**. This observation indicated that antimicrobial compound was very stable. Similar type of observations was reported in several cases, for example, Bacillocin Bb produced by the *Brevibacillus brevis,* from soil **(Saleem et al., 2009; Faheem et al., 2007)** is a bacteriocin like inhibitory substance (BLIS) that is stable within the pH range of 1.0–9.0, resistant to heat (100 °C for 30 min), as well as detergents and organic solvents. The adsorption chromatography- purified antimicrobial substance in the present study also demonstrated resistance to surfactants and organic solvents **(Table 6)**.

The antimicrobial spectrum, pH-tolerance, thermal stability and resistance to proteolytic enzymes displayed by the antimicrobial compound produced by URID 12.1 resemble those of several other *Bacillus*-derived antibacterial peptides, such as bacillocin Bb **(Faheem et al., 2007)**, polyfermenticin SCD **(Lee et al., 2001)**, pumilicin **(Aunpad and Na-Bangchan 2007)**, brevicin AF01 **(Guo et al., 2012)**. It was reported recently that the crude extract of *Paenibacillus* OSY-SE was resistant to heat and changes in pH; most of its antimicrobial activity was retained after holding at 80°C for 24 h, autoclaving at 121°C for 5 min, and exposure to pH 3.0, 5.0, and 9.0. Paenibacterin was resistant to treatment with trypsin but not the other proteolytic enzymes like pronase **(Guo et al., 2012)**. A comparison of ASP-1 with other antimicrobial compounds from *Bacillus subtilis* was shown in **Table 10**.

To explore the structural characteristics of the antimicrobial peptide, CD spectroscopy was performed. From the CD spectra, it was observed that the peptide has a largely random coil conformation (43.5%) under aqueous conditions. The concerned peptide also displayed the β- structure in water indicating its amphipathicity in aqueous solution. No helical content was found either in methanol or TFE (a membrane-mimicking solvent). However, in the presence of methanol, there was a high percentage (58.6%) of β-structure. The CD spectrum in TFE showed a beta-conformation of 42.4 % and beta-turn of 7.7%. This shows a conformational transition from a random coil in aqueous solution to a β-conformation in hydrophobic environments such as methanol. An eighteen-residue

synthetic peptide $(KIGAKI)_3$-NH_2 designed by **Blazyk et al. (2001)** formed β-sheet structures when bound to lipids as was evident by CD spectroscopy and Fourier transform infrared that showed improved antimicrobial activity when compared to amphipathic α-helix forming peptides **(Blazyk et al., 2001)**. It was reported in an erstwhile study, the active amphipathic β-sheet peptides were less hemolytic and appear to be more selective than the amphipathic α-helical peptides **(Jin et al., 2005)**. Hemolytic results obtained in the present study support the findings and suggest that antimicrobial peptides with an amphipathic β-sheet may be sufficiently potent and selective for clinical development. The observation of the β-sheet might be explained possibly in the light of higher than optimum concentration of the peptide used in the CD study in the presence of membrane-mimicking solvents TFE-water and methanol-water (**Wimley et al., 1998; Thundimadathil et al., 2006**), which might have resulted in self-aggregation or self-association (due to hydrophobicity) of the monomers of this small peptide.

The molecular mass derived from intact peptide as shown in the **Fig 23A** and **Fig 26** was found to be less than the observed molecular mass by 18 Da. Therefore, it was hypothesized that this mass difference of 18 Da might be due to the cyclization of the structure in the peptide. A second hypothesis was also drawn with the linear peptide having the possible following sequence: Ac-Phe-Leu-Phe-Dhb-V-A-Dhb. This would be supported by the peak assignment presented in the **Fig 29**.

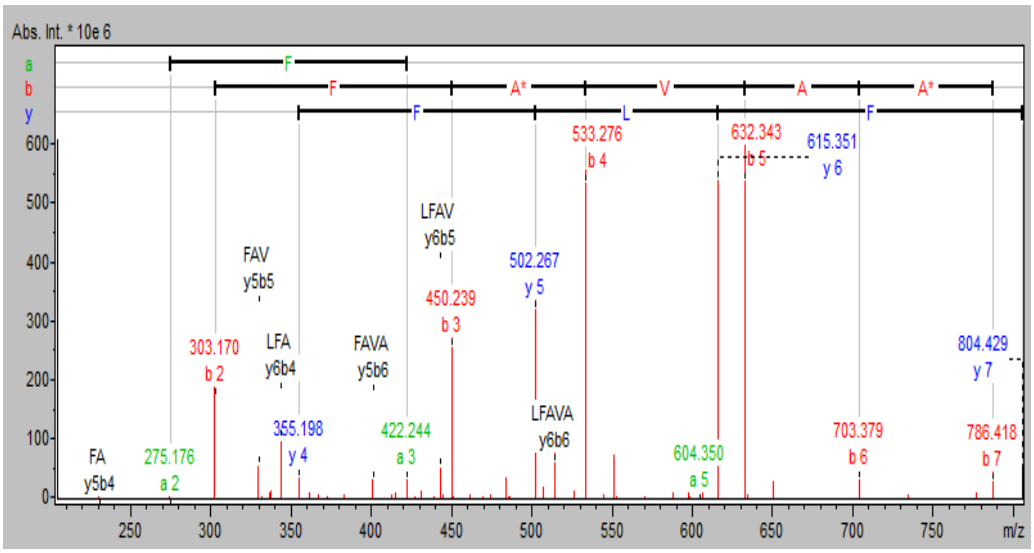


Figure 29. The hypothesis from the figure led to the sequence Acetyl-Phe-Leu/Ile-Phe-Dhb-V-A-Dhb. Dhb has appeared as either A or A*.

To distinguish both the hypotheses, an open-ring reaction by mild alkaline hydrolysis was performed to hydrolyze the macro lactone ring. Therefore, if the mass is increased by 18 Da, it would indicate the presence of a cyclic structure and the linearized peptide could then be sequenced. If the mass remains the same, it would be a strong argument in favor of the second hypothesis.

ESI-FTICR data analysis revealed the longest potential sequence from the mass spectrum as acetylated-Phe-Thr-Val-Ala-Dhb-Phe-Ile/Leu **(Fig. 27)**. However, the MS/MS spectrum obtained on the linearized peptide after KOH hydrolysis enabled the easier interpretation in comparison to the spectrum (**Fig. 28 B**) that was obtained from the cyclic peptide. The ion mobility assay facilitated the structural elucidation of the compound to corroborate its cyclicity in the intact peptide (**Fig. 28 C & D**). **Guo et al. (2012)** while working on a broad-spectrum peptide antibiotic from *Paenibacillus* sp. also performed a mild alkaline hydrolysis (an open-ring reaction) to open a potential lactone linkage; in their investigation, the mass difference (after MALDI-TOF mass spectrometry) between the compound exposed to KOH hydrolysis and the intact peptide was 18 Da revealed the presence of a lactone ring in the later. The collision cross section (CCS) might be considered as the rotationally averaged 2-D projection of an ion's 3D structure. It, therefore, has surface unities and provides an idea of the ion's shape and size. The ion mobility experiment revealed that the CCS of the KOH-untreated peptide was more compact (210 $Å^2$) than that of the KOH-hydrolyzed peptide (216 $Å^2$) **(Fig. 28 C & D)**. Such an observation further proves the presence of a lactone ring because of the latter's tendency of inducing structural compaction in the intact peptide. In contrast to the MS/MS spectrum **(Fig. 27)** obtained with the non-hydrolyzed peptide, the peaks associated with *b* fragment ions, found to be located close to the C-terminal (b_6, b_5 and b_4), were more intense **(Fig. 28 B)**. Such an observation further supports the hypothesis of the presence of a lactone ring at the C-terminal extremity of Dhb in the non-hydrolyzed peptide preventing the fragmentation of the peptide. The deduced sequence of ASP–1 **(Fig. 28B)** indicates that it resembles TL-119, a cyclic depsipeptide that is produced by *B. subtilis* strain **(Nakagawa & Nakazawa 1975)**. Kitajima et al., reported in their experiments that an α-aminobutyric acid (Aba) was also detected in the acid hydrolysate of the hydrogenated material of natural TL-119 **(Kitajima et al., 1990)**.

The sequence of the peptide was analysed using ProtParam from the bioinformatics resource tool ExPASy. The unusual amino acid Dhb was excluded from the analysis. The Isoelectric point (pI) of the peptide was estimated to be 5.52 with an instability index of 8.33 indicating the stable nature of the peptide, which was also evident from the retention of biological activity after exposure to autoclaving and various pH conditions. The grand average of hydropathy (GRAVY) number of the peptide which is a measure of its hydrophobicity or hydrophilicity was calculated to be 2.567 indicating the highly hydrophobic nature of the peptide **(Kyte & Doolittle 1982)** which can be corroborated from its extractability by organic solvent and its retention time during the RP-HPLC.

Table 10: Comparison of ASP-1 with antimicrobial compounds from *Bacillus subtilis*

S.No.	Name	Producer	Target	Sequence	Special features	Reference
1.	ASP-1	Bacillus subtilis	Gram-positive	acPhe-Leu-Phe-Thr-Val-Ala-Dhb	Active over wide pH, temperatures	**Chalasani et al., 2015, 2017**
2.	Subpeptin JM4-B	Bacillus subtilis	Gram-positive and negative	XXKEIXHIFHDN	Active over wide pH and temperatures	**Wu et al., 2005**
3.	Subtilosin-A	Bacillus subtilis	Gram-positive	MKKAVIVENKG CATCSIGAACLV DGPIPDFEIAGAT GLFG LWG	Cyclic and thermally stable	**BABASA KI et al., 1985**
4.	Sublancin 168	Bacillus subtilis	Gram-positive	MEKLFKEVKLEE LENQKGSGLGKA QCAALWLQCASG GTIGCGGGAVAC QNYRQFCR	Stable at low and high pH	**Paik et al., 1998**
5.	Subtilin	Bacillus subtilis	Gram-positive, few Gram negative and fungi	MSKFDDFDLDV VKVSKQDSKITP QWKSESLCTPGC VTGALQTCFLQT LTCNCKISK		**Salle & Jann 1945**

4.1 INTRODUCTION:

WHO classified seven bacteria of international concern considering their identification and resistance out of which *S. aureus*: resistant to β-lactam antibacterial drugs was one of the major candidates. Methicillin-resistant *S. aureus* (MRSA) is an *S. aureus* that turned resistant to β-lactam antibiotics by acquiring *mec*A gene on its chromosomal DNA **(Hiramatsu, 1995)**. *mecA* gene, that encodes for the low-affinity penicillin-binding protein (PBP) PBP2a is used to determine the methicillin resistance **(Beck et al., 1986)**. *mecA* gene is part of a 21 to 60-kb mobile genetic element staphylococcal chromosome cassette *mec* (SCC*mec*), that also consists of genes encoding for resistance to non-β-lactam antibiotics such as Tn*554*, pUB110, and pT181 **(Ito & Hiramatsu 1998)**. DNA microarray technology has been used to determine at least five divergent lineages for the appearance of *mecA* in *S. aureus*, indicating the role of horizontal gene transfer in the evolution of MRSA **(Fitzgerald et al., 2001)**. Transfer of *mecA in vivo* from *S. epidermidis* to *S. aureus* was observed recently, suggesting the frequent transfer of *mecA* to MSSA **(Wielders et al., 2001)**. Generally, four PBPs are produced by *S. aureus* **(Georgopapadakou et al., 1986)**, that are anchored on the cytoplasmic membrane and enables the assembly and regulation of cell wall biosynthesis in the later stages **(Bush & Mobashery 1998; Goffin & Ghuysen 2002)**. These four PBPs are targets for the modification by β-lactam antibiotics, leading to the death of the bacteria. However, PBP2a is resistant to the action of all the available β-lactam antibiotics and can take over the functions of the four typical PBPs of *S. aureus* causing the resistance against β-lactam antibiotics. Of note the 'gold standard' used to identify MRSA is amplification of the *mec*A gene **(Predari et al., 1991)**.

Transposition of IS256 in *S. aureus* has been described to play an important role in antibiotic resistance and biofilm formation. **Kozitskaya et al. (2004)** described the correlation between IS256 and the resistance toward oxacillin and gentamicin. The insertion element IS256 more frequently occurs in multi-resistant and *ica*-positive isolates. Moreover, detection of IS256 was found to be associated with biofilm formation and the presence of the *ica*ADBC operon as well as with gentamicin and oxacillin resistance in the clinical strains, suggesting that IS256 is a characteristic element in the genome of multi drug-resistant nosocomial isolates that might be involved in the

flexibility and adaptation of the genome in clinical isolates. **Kleinert et al. (2017)** reported the important role played by IS256 in conferring intermediate resistance to vancomycin; they concluded that the presence of this mobile element can ably promote the genomic flexibility enabling the adaptation to antibiotic stress **(Kleinert et al., 2017)**. *S. aureus* and *S. epidermidis* strains are well known to produce biofilms that enable them to survive in hostile environments. A biofilm is an aggregate of microorganisms in which cells are embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhering to each other and/or to a surface.

S. aureus produces biofilm which helps it to survive in the extreme conditions within the host, and cause chronic or persistent infections. Several diseases such as osteomyelitis, septicemia and endocarditis are found to be associated with biofilm forming *S. aureus* **(Götz 2002; Kloos & Bannerman 1994)**. Biofilm formation in *Staphylococci* occurs in two steps. That involves bacterial attachment to the surface followed by accumulation of bacteria by using biofilm development polysaccharide intercellular adhesin (PIA). The aggregation of the bacterial cells and the formation of biofilm are mediated by chromosomal *ica* gene locus products, comprising four intercellular adhesion genes (*icaA, icaB, icaC* and *icaD*) along with a regulator gene (*icaR*) **(Mack et al., 1996; Conlon et al., 2002)**. PIA, a partially *N*-acetylated linear β-1–6-linked glucosaminoglycan backbone substituted with different side groups production is influenced by these genes. PIA in involved in the biofilm formation by acting as an intercellular adhesin on glass and other hydrophilic surfaces **(Heilmann et al., 1996)**. The sequence of *ica* products between *S. epidermidis* and *S. aureus* revealed the identity of 59% to 78% **(Cramton et al., 1999)**. Changing environmental conditions such as anaerobiosis, ethanol stress, high temperature, osmolarity along with the sub inhibitory concentrations of certain antibiotics influence the amount of biofilm production **(Rachid et al., 2000)**.

The study conducted by **Heilmann et al.,** has shown that *icaA* alone can produce a low *N*-acetylglucosaminyltransferase activity, however, the co-expression of *icaA* along with *icaD* has increased the activity significantly and *icaC* is essential for long-chain PIA synthesis. *In vitro* synthesis of PIA intermediates or *in vivo* biosynthesis of PIA was not affected by the antibiotics tunicamycin and bacitracin, suggesting no involvement of

undecaprenyl phosphate carrier. Besides this, a high level of association between the presence of *ica* genes that encode biofilm polysaccharide as well as of mobile element insertion sequence IS256 and multiple-resistance to all the antibiotics (oxacillin, penicillin, vancomycin, teicoplanin, gentamicin, erythromycin, clindamycin, chloramphenicol, and ciprofloxacin) was reported **(Montanaro et al., 2007)**.

Biofilms are formed due to various reasons including cellular recognition of specific or non-specific attachment sites on a surface, exposure to sub-inhibitory concentrations of antibiotics and nutritional cues. Staphylococcal biofilms can escape the immune system of the host, causing recurring/chronic infections, inflammation, and inhibiting the wound healing process **(Wolcott et al., 2010)**. One of the major issues pertaining to use of antimicrobial peptides is their toxic effect on the host. It is very important to test the hemolytic and cytotoxic effect of the AMPs on the host system.

It is very important to determine the minimum inhibitory concentration (MIC) of drugs against the infecting organism to identify the correct drug to be used for the infection **(Andrews 2001)**. Generally, the first step in a drug industry is to screen the drugs for MICs against the bacteria as the MICs are the base for taking the drug to the preclinical trails **(O'Neill & Chopra 2004)**. Time-Kill kinetic study is determining the rate at which an antimicrobial compound kills the microorganisms as a function of survival data of the microorganisms collected at enough exposure points in a way that a graph can be constructed showing the decline in the microbial population to a point of extinction.

In view of the above the presence of *mecA*, IS256 and *ica*A and *ica*D genetic elements were determined in few of the test organisms used in the present study. The minimum inhibitory concentrations of the peptide ASP-1 were determined against methicillin-resistant *S. aureus* strains and other gram-positive bacteria along with evaluating the antibiofilm forming prowess of the peptide. The cytotoxic effect of the peptide was tested on the human erythrocytes and mammalian cell lines.

4.2 MATERIALS AND METHODS:

4.2.1 Characterization of Clinical Isolates:

4.2.1.1 PCR-based Detection of *mecA* Genes:

The cultures were tested for methicillin resistance by broth microdilution using oxacillin, a surrogate for methicillin. isolates showing a MIC greater than 0.5 µg/ml were screened for the presence of *mec*A gene by PCR using the forward primer 5'-AAAATCGATGGTAAAGGTTGGC-3' and reverse primer 5'-AGTTCTGCAGTACCGGATTTGC-3'. The genomic DNA was isolated using the Wizard Genomic DNA purification kit from Promega. By using 100 ng of DNA, 50 pmol of each primer, EmeraldAmp PCR Master Mix（2X) by Takara Bio Inc., DNA amplification was carried out for 30 cycles in 25 µl of reaction mixture as follows: initial denaturation at 94ºC for 3 min, denaturation of 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 45 s with a final extension at 72°C for 7 min **(Murakami et al., 1991).** Template DNA from *S. aureus* ATCC29213 was used as a negative control. 8 µl of PCR product was analyzed by 1.5% agarose gel electrophoresis. The PCR products showing expected size formation were subsequently bidirectionally sequenced.

4.2.1.2 PCR-based Detection of IS256 Genes:

IS256 genes are found in many MRSA and MRSEs as revealed by numerous reports available to establish the fact that the presence of this genetic element in the genome of methicillin-resistant *S. aureus* and *S. epidermidis* are responsible for teicoplanin, aminoglycoside and methicillin resistance. The organisms that were positive for the *mec*A gene were tested for the presence of IS256 by PCR using the forward primer 5′-GCGCAGCTTTACGACACGTGTAGGC-3' and reverse primer 5′-CATGAAGCCGATAATTTCACGGTCGCC-3'. The genomic DNA was isolated using the Wizard Genomic DNA purification kit from Promega. By using 100 ng of DNA, 50 pmol of each primer, EmeraldAmp PCR Master Mix（2X) by Takara Bio Inc, DNA amplification was carried out for 30 cycles in 25 µl of reaction mixture as follows: initial denaturation at 94ºC for 3 min, denaturation of 94°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 45 s with a final extension at 72°C for 7 min **(Petrelli et al., 2006).** *S. aureus* ATCC29213 was used as a negative control. 8 µl of PCR product was

analyzed by 1.5% agarose gel electrophoresis. The PCR products showing expected size formation were subsequently bidirectionally sequenced.

4.2.2 Determination of Minimum Inhibitory Concentration (MIC) of Antimicrobial Peptide Against Different Strains:

The minimum inhibitory concentration (the lowest concentration that prevents the visible growth of microorganisms in a broth dilution or agar susceptibility test) of the peptide against different strains was detected by following the guidelines from Clinical and Laboratory Standards Institute (CLSI). The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) is an interdisciplinary, nonprofit, international, standards-developing and educational organization that promotes the development and use of voluntary consensus standards and guidelines within the healthcare community. It is recognized worldwide for the application of its unique consensus process in the development of standards and guidelines for patient testing and related healthcare issues.

Staphylococci resistant to oxacillin are resistant to all β-lactam antibiotics that are currently available. Thus, testing of susceptibility or resistance to penicillin and oxacillin can be used to deduce the resistance to a wide variety of β-lactam antibiotics. Either agar or broth dilution method is used to quantitatively measure the antimicrobial activity of an antimicrobial agent in vitro against a given bacterial isolate. A series of tubes or plates is prepared to perform the test with a broth or agar medium, to which antimicrobial agents are added at various concentrations. After that, standardized suspension of the test organisms is added to the tubes or plates and incubated at 35 ± 2°C. After incubation, the tests are examined to determine the MIC. The result is influenced significantly by methodology that should be controlled carefully to achieve reproducible results (intralaboratory and interlaboratory).

4.2.2.1 Weighing Standard Antimicrobial Powders:

Generally, the assay units of the antimicrobial agents are different from the actual weight of the powder and generally differ from the production lot also. Thus, standardization of the antimicrobial solutions in the laboratory is very important. The potency of the antimicrobial agent given by the manufacturer is considered for preparation of the stock antibiotics. The stock solution of oxacillin was prepared by

weighing the oxacillin powder and then dissolving it in required quantity of the diluent calculated by using the formula

$$Volume\ (mL) = \frac{Weight\ (mg)\ x\ Potency\ (\mu g/mg)}{Concentration\ (\mu g/mL}$$

4.2.2.2 Preparing and Storing Stock Solutions of Antibiotics:

The desired quantity of water is added to the oxacillin powder and dissolved completely, the homogenous solution is filter sterilized by using 0.22 µm syringe filter. After filtration, the solution was aliquoted into 2 ml micro centrifuge tubes and stored at -80^{o}C. When necessary the aliquots were taken and immediately used after thawing them. Any significant deterioration in the antimicrobial agent potency is determined by the results of susceptibility testing using quality control strain *S. aureus* ATCC 29213.

4.2.2.3 Preparation of Media, Cation-Adjusted Muller-Hinton Broth (CA-MHB):

Despite the availability of various microbiological media, CLSI recommends the use of Mueller-Hinton (MH) medium due to its acceptable batch-to-batch reproducibility for susceptibility testing, presence of low inhibitors affecting sulfonamide, trimethoprim, and tetracycline susceptibility test results and its support to most pathogens satisfactory growth. The MHB was adjusted with 10 mg/ml stock solutions of $MgCl_2.6H_2O$ and $CaCl_2.2H_2O$. The stock solutions were prepared by adding 8.36 g of $MgCl_2.6H_2O$ and 3.68 g of $CaCl_2.2H_2O$ in 100 ml of Milli Q water. CA-MHB was prepared by adjusting the final concentration of Mg^{2+} and Ca^{2+} to 10-12.5 µg/ml and 20-25 µg/ml respectively in MHB. BHI media was used to determine the MIC for some non-*aureus* strains.

4.2.2.4 Broth Microdilution:

Broth microdilution was performed in sterile, plastic microdilution 96 well plates. The wells (except for 1st column) were dispensed with 100 µl of CAMHB and to the 1st well oxacillin was added such that the final concentration was as desired in 200 µl and volume adjusted with CAMHB to 200 µl. The antibiotic was diluted by 2-fold dilution into the subsequent wells till the 10th column. The last two columns were left with just the CAMHB without antibiotic. In case, of the peptide it was lyophilized and dissolved in

CAMHB and diluted by two-fold dilution. The oxacillin was tested in range of 1024 - 0.125 µg/ml and ASP-1 in range of 512 – 0.125 µg/ml.

4.2.2.5 Inoculum Preparation by Direct Colony Suspension Method:

Direct colony suspension is the most convenient method of inoculum preparation and can be used with most organisms. The culture was streaked on agar plates and incubated for 18-24 h and from there isolated colonies were picked and suspended into saline. The suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland standard (0.1 OD at 625 nm) by using a spectrophotometer in sterile plastic cuvettes. This suspension consists of approximately 1-2 x 10^8 colony forming units (CFU)/ml.

4.2.2.6 Determination of Minimum Inhibitory Concentration (MIC):

Within 15 min of preparation the adjusted inoculum was diluted 1:20 to yield 5 x 10^6 CFU/ml and from there 10 µl was inoculated into each well containing 100 µl of media to obtain a final concentration of 5 x 10^5 CFU/ml. The culture was added from columns 1-11 of which the 11^{th} act as a positive control for growth and the 12^{th} column was left without inoculating the organism and it serves a negative control to test for contamination. Resazurin dye was added to each well at a final concentration of 0.02%, as an indicator of growth. The plates were incubated at 37ºC for 18-24 h in an incubator and the plates were wrapped in aluminum foil to prevent the evaporation and exposure of dye to light. The MIC is the lowest concentration of the oxacillin or the peptide at which there is no visible growth as indicated by the change in color of the dye from blue to pink. For antibacterial susceptibility testing, the quality control strain *S. aureus* ATCC 29213 and antibiotic oxacillin were used.

4.2.2.7 Determination of Minimum Bactericidal Concentration (MBC):

Minimum bactericidal concentration is the lowest concentration of the antimicrobial agent of the peptide that results in the complete death of the microorganisms. This was calculated by taking 10 µl from the wells after incubation and streaking on to the agar plates without the antimicrobial agent and the plates were incubated at 37ºC for 24 h. The concentration at which there was no formation of the colonies was considered as the MBC of the compound.

4.2.3 Time-Kill Kinetics:

This method measures the change in the population of the aerobic microorganisms within a specific sampling time when tested against a particular antimicrobial agent *in vitro*. For this study, Cation-adjusted Muller Hinton broth was used for the growth of the test organism.

4.2.3.1 Inoculum Preparation:

Freshly grown single colonies of 18-24 h old *S. aureus* ATCC 29213 and MRSA 15 were picked and suspended into sterile 0.85% saline. The suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland standard (0.1 OD at 625 nm) by using a spectrophotometer in sterile plastic cuvettes. This suspension consisted of approximately 1-2 x 10^8 colony forming units (CFU)/ml.

4.2.3.2 Inoculation and Determination of Microbial Population:

The suspension was diluted and added at final concentrations of approximately 5 x 10^5 CFU/ml and 5 x 10^4 CFU/ml of *S. aureus* ATCC 29213 and MRSA 15 respectively to the CAMHB with 1X and 5X concentrations of the antimicrobial peptide. The tubes were incubated at 37ºC. After every two hours, 5 µl of aliquots was taken and spread on Muller- Hinton agar plates undiluted and in serial dilution. The plates were incubated at 37ºC for 24 h. After incubation, the numbers of colonies were counted, and the results were recorded. The plates exhibiting colonies ranging from 30-300 were considered for counting. The tube without the antimicrobial peptide was used as control. The experiment was conducted in duplicates.

4.2.3.3 Calculating Microbial log_{10} Reduction and Percentage Reduction:

The initial microbial population of the control was transformed into log_{10} scale. The surviving microbial population in the tube with antimicrobial peptide was also transformed into log_{10} scale. Each measured value of the duplicate was first transformed into log_{10} and then mean and the variance associated were determined.

The log reduction was calculated by using the formula given below

Log10 reduction (LR) = mean log10 (measured initial microbial population) - mean log10 (surviving microbial population)

Standard error of the mean was calculated by using the formula given below

Standard error = square root [(variance of the log_{10} measured initial microbial population/ number of replicates) + (variance of the log_{10} surviving microbial population/number of replicates)]

Percentage reduction was calculated using the formula given below

Percent reduction (%) = 100 X (1 - 10^{LR})

4.2.4 Screening of Biofilm Forming Strains:

4.2.4.1 Spectrophotometric Biofilm Screening Assay:

A total of 17 bacterial isolates were screened for the formation of biofilm in 96 well polystyrene plates. Cultures at a rate of 10^6 cells/ml calculated by adjusting the OD to 0.5 McFarland standard were inoculated in Tryptic Soy Broth (TSB) supplemented with 0.25% of glucose. After incubation at 37ºC for 24 h the media was removed, and the wells were washed twice with 0.85% saline. The plates were air dried and the surface adsorbed cells were stained with 0.1% safranin. After five mins, the wells were washed twice with saline to remove the excess unbound safranin. The plates were air dried and methanol was added to the wells to dissolve the safranin and the absorbance was measured at 490 nm in Micro-ELISA plate reader **(Heilmann et al., 1996)**. The wells with only TSB lacking cells served as blank and the absorbance value of blank was subtracted from experimental readings. *S. epidermidis* ATCC12228 was used as a negative control.

4.2.4.2 Congo Red Agar (CRA) Assay:

The cultures which were positive for biofilm formation by spectrophotometric assay were tested by Congo red agar assay. The medium was composed of Brain Heart Infusion (BHI) broth, 5% sucrose, 1.7% agar-agar and 0.08% Congo red. Congo red was prepared as a concentrated solution, autoclaved separately from the other constituents of the medium and added after the medium was cooled to 55ºC **(Freeman et al., 1989)**. The cultures were streaked on to the plates and incubated at 37ºC for 24 hrs.

4.2.4.3 Detection of *ica* Genes by PCR:

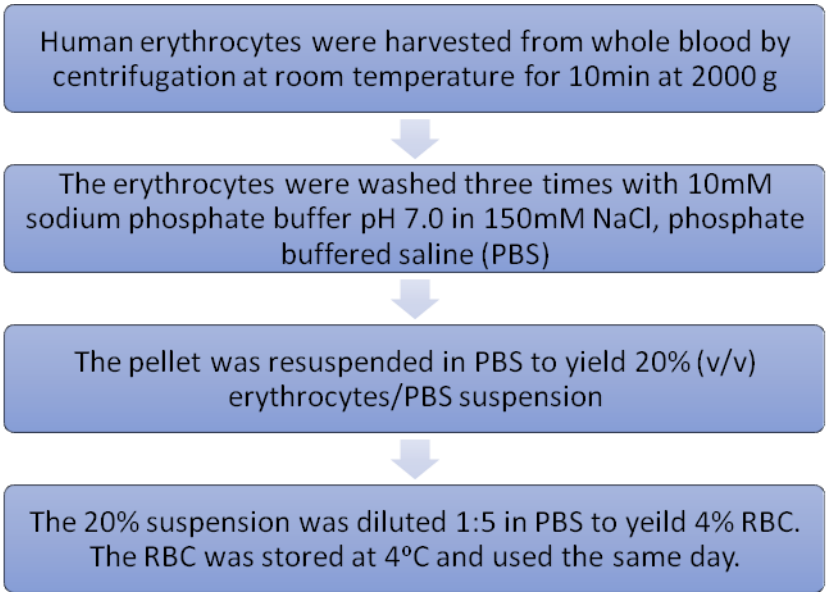
The presence of the *ica*A and *ica*D genes was detected by PCR based method. For the detection of *ica*A gene forward primer 5′-TCTCTTGCAGGAGCAATCAA and reverse primer 5′-TCAGGCACTAACATCCAGCA were used. For *ica*D gene forward primer 5′-ATGGTCAAGCCCAGACAGAG and reverse primer 5′-CGTGTTTTCAACATTTAATGCAA were used. The genomic DNA was isolated using the Wizard Genomic DNA purification kit from Promega. The PCR reaction was 25 µl consisting of 100 ng genomic DNA, 50 pmol of each primer, Emerald Amp PCR Master Mix（2X) by Takara Bio Inc, DNA amplification of both the icaA and icaD genes was carried out for 30 cycles as follows: initial denaturation at 94^{o}C for 3 min, denaturation of 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 30 s with a final extension at 72°C for 7 min **(Arciola et al., 2001)**. *S. epidermidis* ATCC 12228 has been used as a negative control. 8 µl of PCR product was analyzed by 1.5% agarose gel electrophoresis.

4.2.5 Determination of Anti-Biofilm Forming Activity of ASP-1:

The organism's positive for *ica*A and *ica*D genes were used to test the antibiofilm forming effects of the purified antimicrobial peptide. The compound at a concentration of 1X, 0.5X and 0.25X MICs was tested against all the *ica*A and *ica*D positive strains. *S. epidermidis* ATCC 12228 was used as a negative control. TSB broth supplemented with 0.25% of glucose with compound at different concentrations was inoculated with 10^6 cells/ml of test culture calculated by adjusting the OD to 0.5 McFarland standard. The plates were incubated at 37^{o}C for 24h and washed two times with 0.85% saline followed by addition of 100 µl 0.1% safranin. After five mins, safranin was removed from the wells and the wells were washed with saline to remove the unbound safranin. After air drying the plates 200 µl of methanol was added to the wells and thoroughly mixed by shaking. The absorbance was measured at 490 nm in Micro-ELISA plate reader **(Heilmann et al., 1996)**. Culture inoculated into media without the antimicrobial compound acted as a control and only media without culture served as a blank. The OD of the blank was subtracted from the experimental readings.

4.2.6 Hemolytic Assay:

4.2.6.1 Isolation of Human Erythrocytes (RBC) from Whole Blood:



4.2.6.2 Hemolysis and Percentage Hemolysis Calculation:

Equal volume of the antimicrobial compound in same buffer was added to 4% red blood cells (RBC) at different concentrations ranging from 256-16 µg/ml and incubated at 37°C for 1 h. Erythrocytes in PBS suspension with buffer alone and 1% (v/v) Tween 20 were used as negative and positive controls, respectively. The dilutions were centrifuged at room temperature for five mins at 1500 g and the OD of the supernatant was measured at 450 nm on Micro-ELISA plate reader. Percentage hemolysis was calculated using the formula

$$\frac{(A450\ of\ peptide\ treated\ sample\ -\ A450\ of\ buffer\ treated\ sample)\ \times\ 100}{(A450\ of\ Tween\ 20\ treated\ sample\ -\ A450\ of\ buffer\ treated\ sample)}$$

4.2.7 *In vitro* Cytotoxicity Assay:

The antimicrobial compound was assayed at different concentrations (1-256 µg/ml) against human liver epithelial carcinoma cells (HepG2) and Michigan Cancer Foundation (MCF-7) cell lines to determine the potential toxic effect by MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in living cells is reduced into insoluble formazan, that has purple color. The reduction of the tetrazolium

dye is dependent on the NAD(P)H-dependent oxidoreductase enzymes and so depends on the cellular metabolic activity due to NAD(P)H flux. The formation of formazan crystals is proportional to the number of living cells and so can be used as a measure for cell viability **(Mosmann 1983)**. MTT assays are generally performed in dark since the MTT reagent is light sensitive. The HepG2 and MCF-7 cells were seeded at a density of $1x10^5$ cells per well in a 96 well tissue culture plate in 100 µl Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37^oC in a 5% CO_2 atmosphere for 24 h. After 24 h, 100 µl of media with the antimicrobial compound was added and incubated for another 24 h. Untreated cells were used as negative control. After incubation 20 µl of 5 mg/ml MTT was added to each well and further incubated for 4 h **(Mohamed et al., 2014)**. The incubation was followed by removal of the contents of the wells and dissolution of the formazan crystals in 100 µl/well of dimethyl sulfoxide solution (DMSO) and absorbance taken at 570 nm on Micro-ELISA plate reader. Cell viability was expressed as percentage of absorbance in comparison to negative control, untreated cells.

4.3 RESULTS:

4.3.1 PCR-based Detection of *mec*A Genes:

In *S. aureus* and *S. epidermidis*, *mec*A genes belonging to the SCCmec operons are responsible for encoding penicillin binding protein 2 responsible for methicillin resistance. The presences of *mec*A genes were confirmed by PCR amplification. The PCR products resulted from *mec*A primers corresponded to a size of 533 bp for all the MRSA clinical isolates tested; however, no PCR amplification was observed when template DNA from the *S. aureus* ATCC 29213 strain was used as negative control. Samples showing visible 533 bp band on agarose gel **(Fig. 30)** were assigned as *mec*A positive. The nucleotide sequences of amplified *mec*A genes had similarity with the existing *mec*A genes sequences available in NCBI database **(Fig. 31, 32)**.

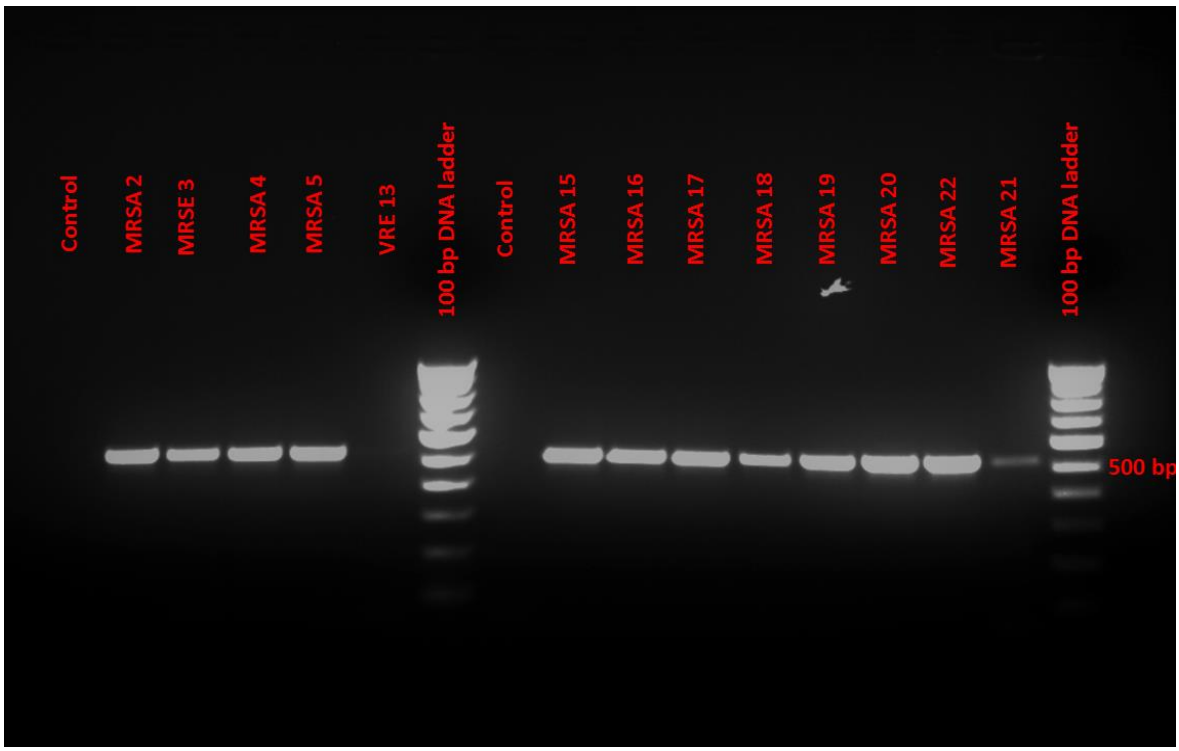


Figure 30: Agarose gel electrophoresis showing PCR amplification of the *mec*A genes in clinical isolates.

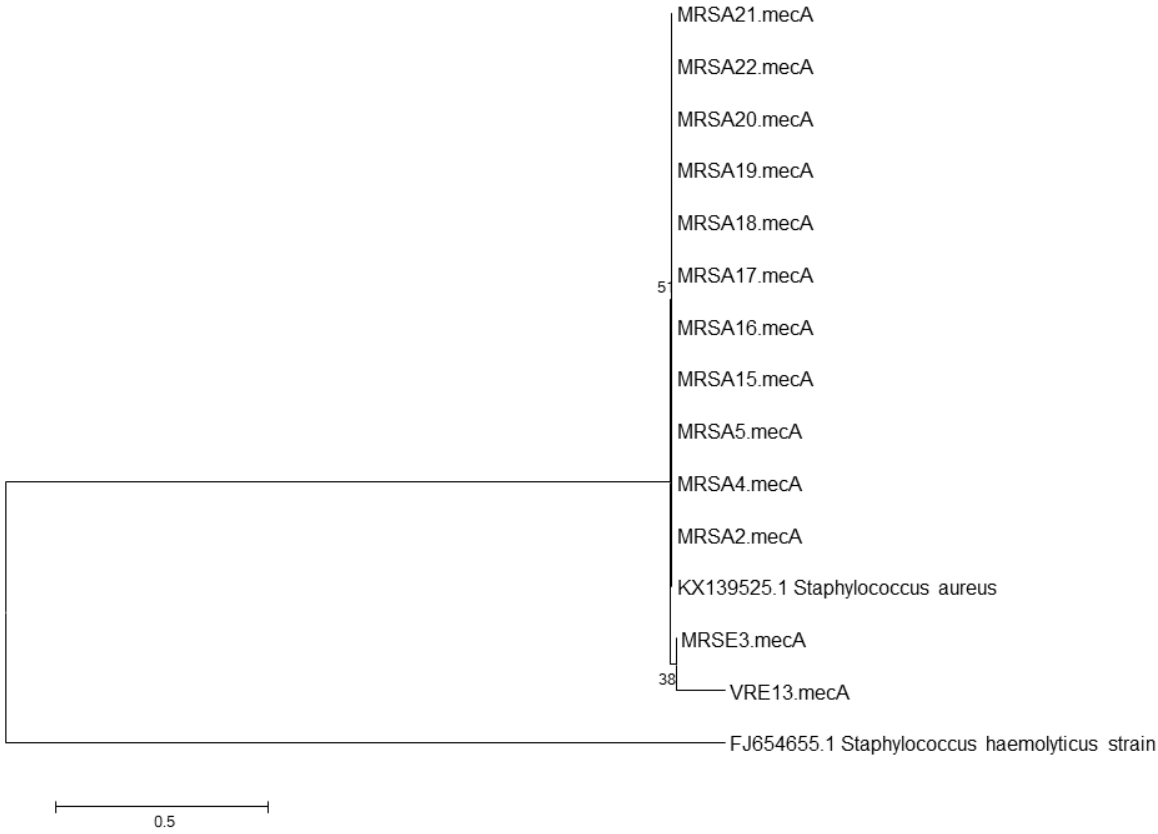
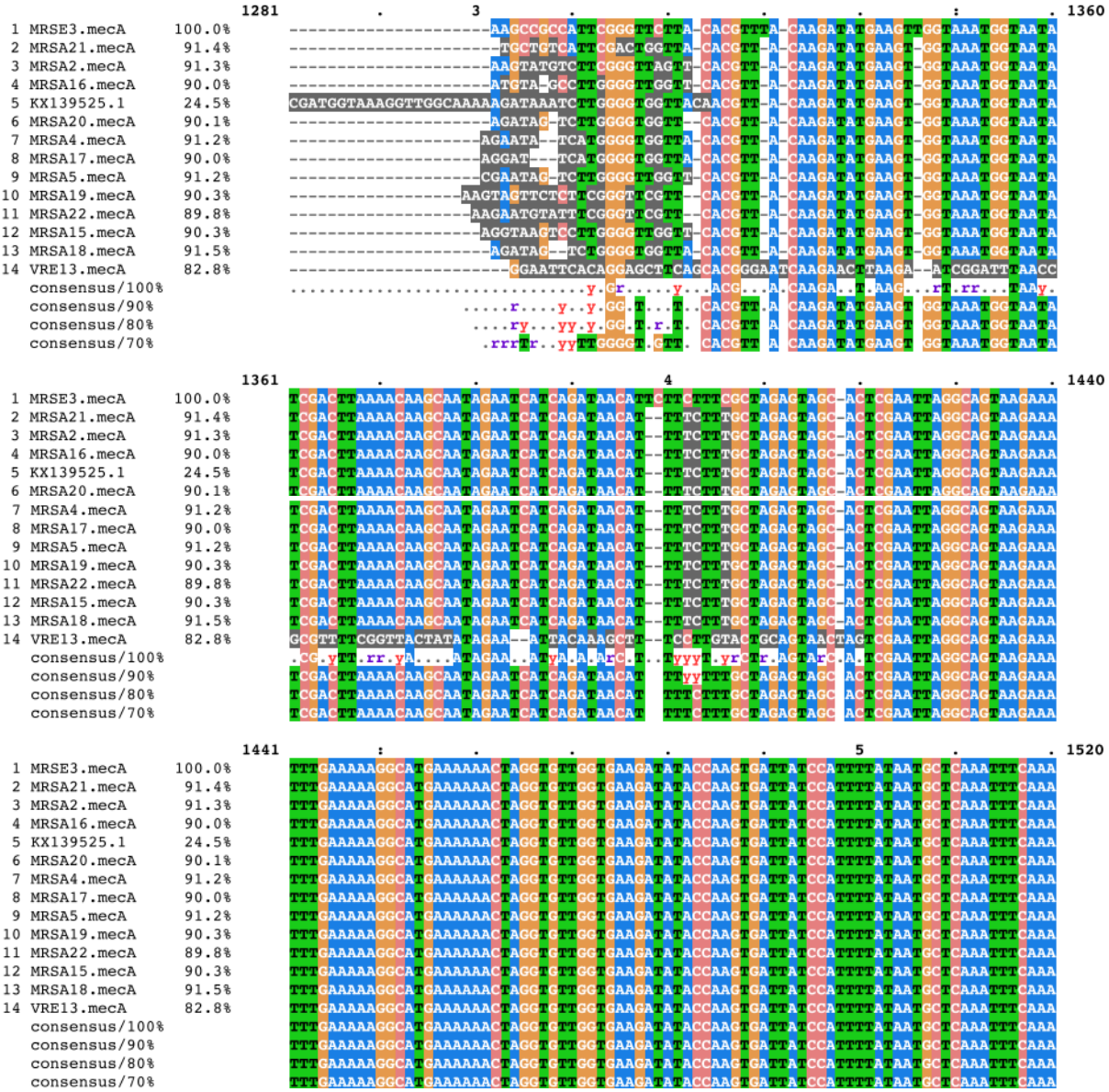


Figure 31: Phylogenetic tree showing close relation between the *mec*A gene from MRSA clinical isolates and *S. aureus* 265 *mec*A gene. *Staphylococcus haemolyticus* was used as an outgroup.


1281 1360
1 MRSE3.mecA 100.0%
2 MRSA21.mecA 91.4%
3 MRSA2.mecA 91.3%
4 MRSA16.mecA 90.0%
5 KX139525.1 24.5%
6 MRSA20.mecA 90.1%
7 MRSA4.mecA 91.2%
8 MRSA17.mecA 90.0%
9 MRSA5.mecA 91.2%
10 MRSA19.mecA 90.3%
11 MRSA22.mecA 89.8%
12 MRSA15.mecA 90.3%
13 MRSA18.mecA 91.5%
14 VRE13.mecA 82.8%
consensus/100%
consensus/90%
consensus/80%
consensus/70%
1361 1440
1441 1520

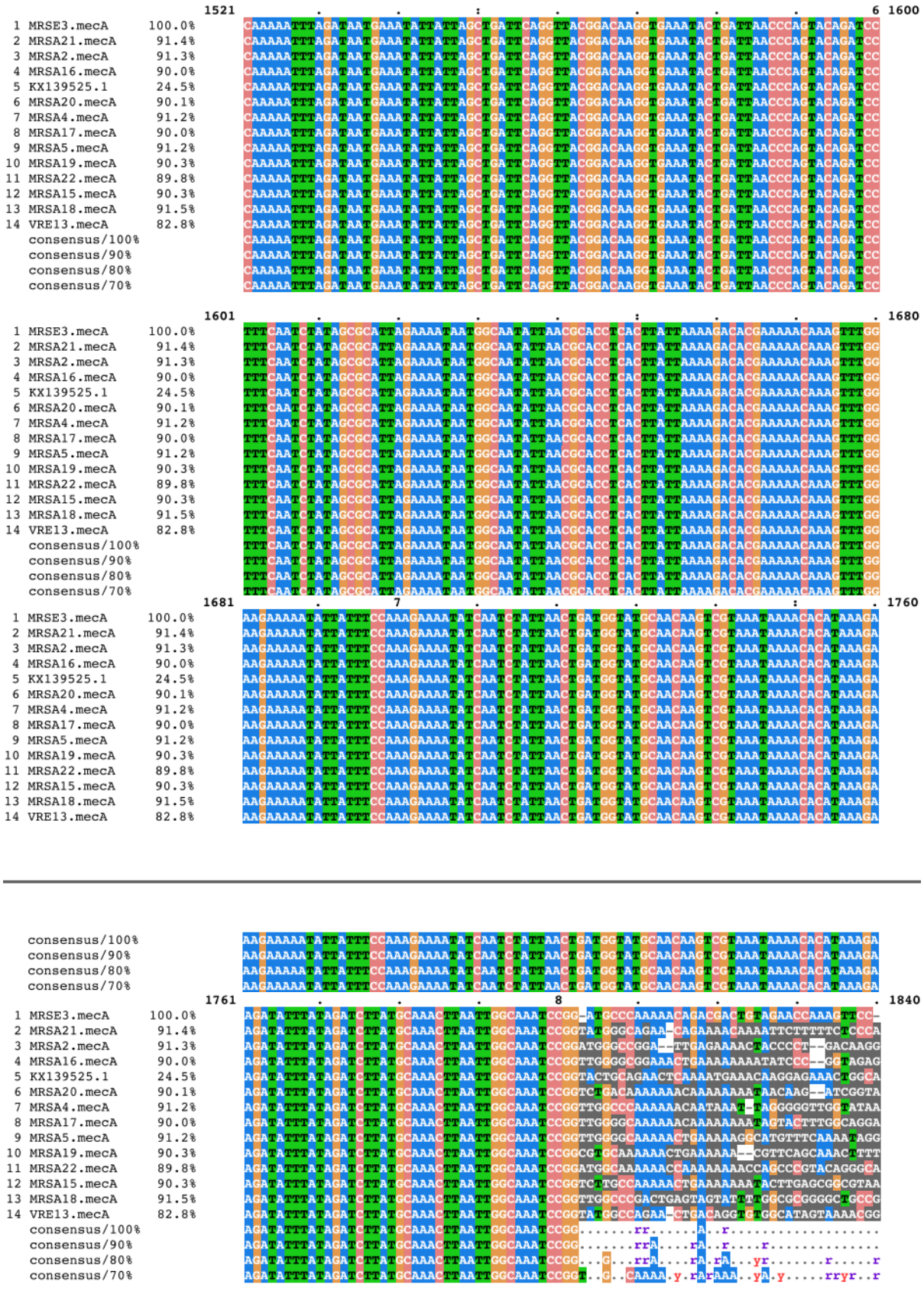


Figure 32: Multiple sequence alignment showing similarity of *mec*A genes with the sequence available in NCBI database.

4.3.2 PCR-based Detection of IS256 Genes:

The presences of IS256 genes in the staphylococcal strains use in the present study were confirmed by PCR amplifications. The PCR products resulted from the amplification corresponded to a size of 408 bp for all the MRSA clinical isolates tested; however, no observable band after PCR amplification was observed when template DNA from the *S. aureus* ATCC 29213 strain was used as a negative control **(Fig. 33)**. All the PCR amplicons resulted from the DNA templates of clinical isolates were subsequently sequenced using the appropriate set of primers used for IS256 gene amplification. The nucleotide sequences of amplified IS256 genes showed similarity with the existing IS256 genes sequences available in NCBI database **(Fig. 34, 35)**.

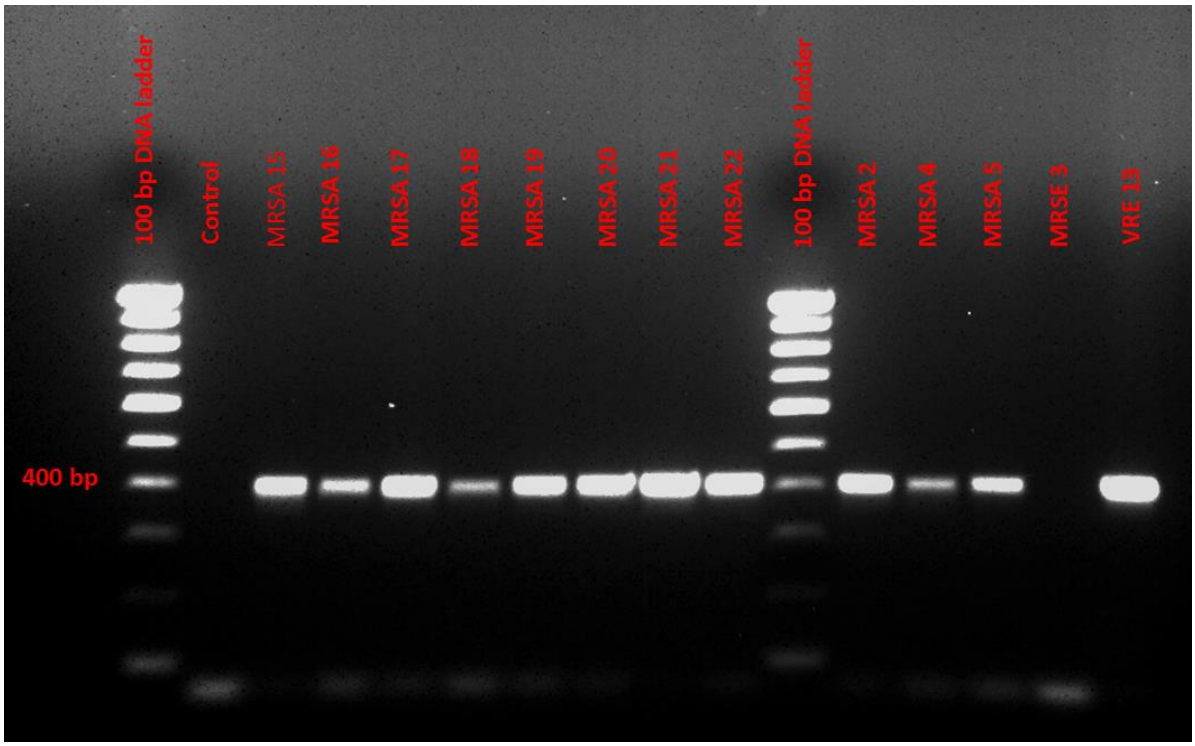
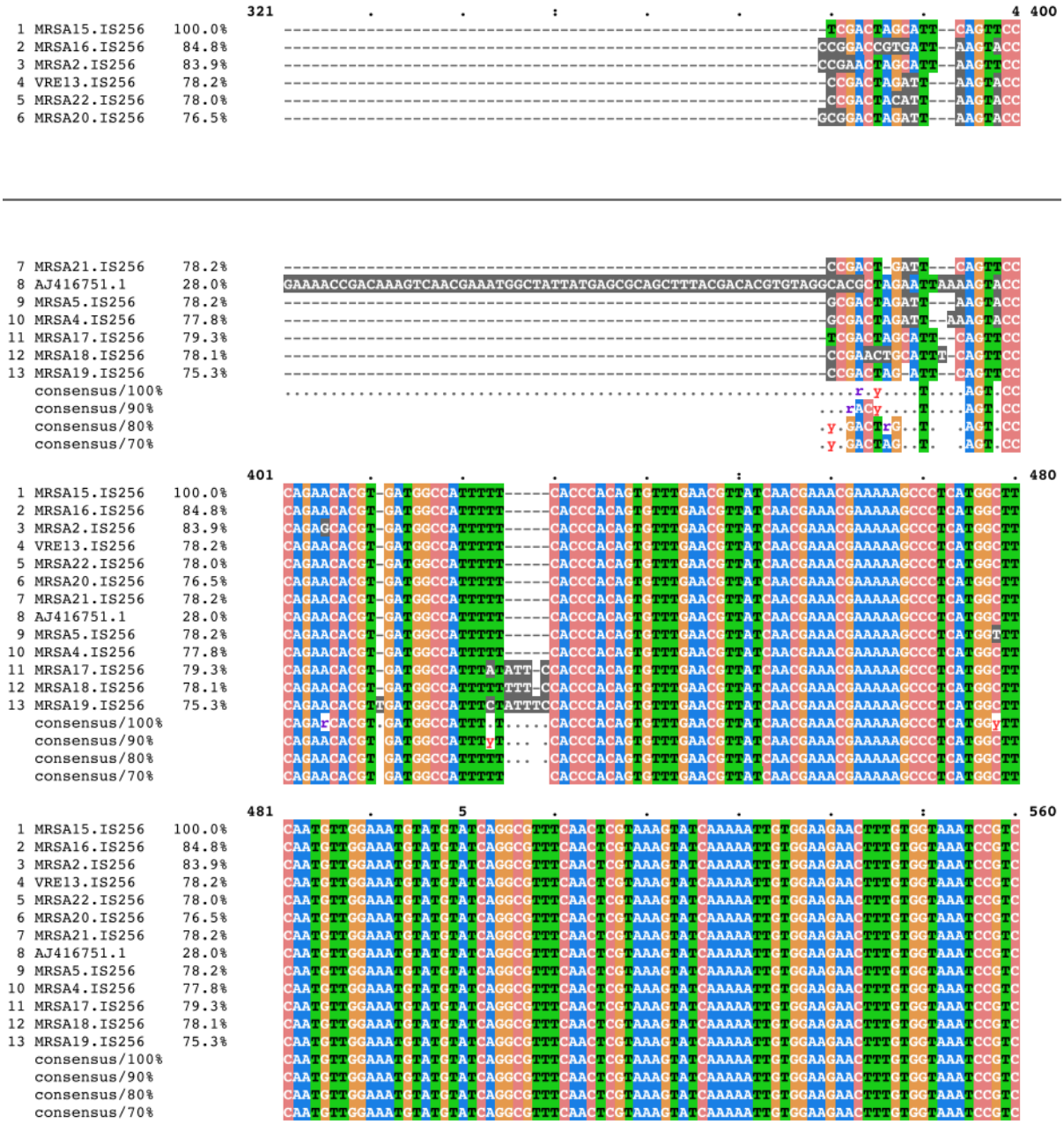


Figure 33: Agarose gel electrophoresis showing PCR amplification of the IS256 genes in clinical staphylococcal isolates.


321 . . : 4 400
1 MRSA15.IS256 100.0%
2 MRSA16.IS256 84.8%
3 MRSA2.IS256 83.9%
4 VRE13.IS256 78.2%
5 MRSA22.IS256 78.0%
6 MRSA20.IS256 76.5%
7 MRSA21.IS256 78.2%
8 AJ416751.1 28.0%
9 MRSA5.IS256 78.2%
10 MRSA4.IS256 77.8%
11 MRSA17.IS256 79.3%
12 MRSA18.IS256 78.1%
13 MRSA19.IS256 75.3%
consensus/100%
consensus/90%
consensus/80%
consensus/70%
401 . . . . : . . . 480
481 . 5 . . . . : . 560

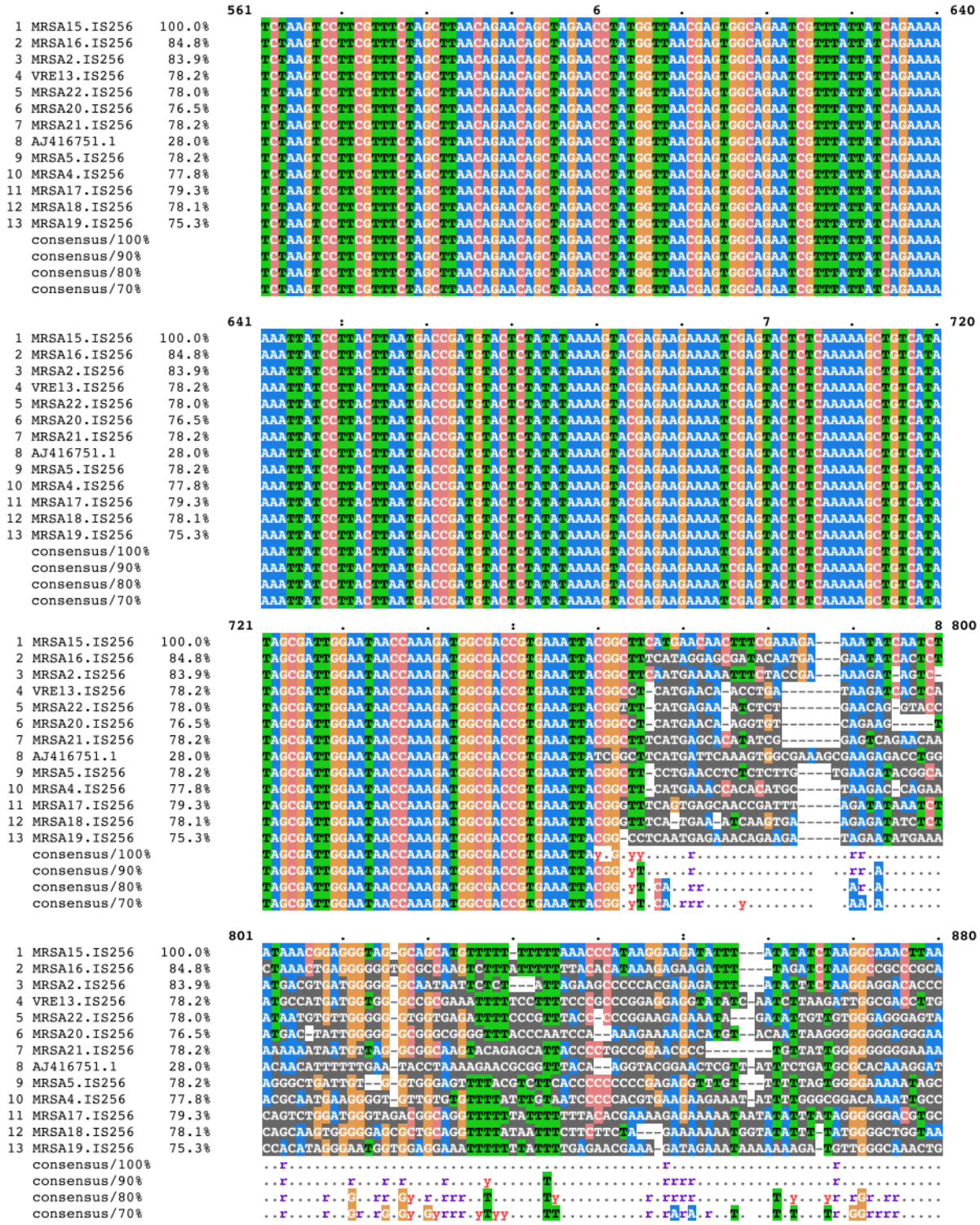


Figure 34: Multiple sequence alignment showing similarity of IS256 genes with the sequence available in NCBI database.

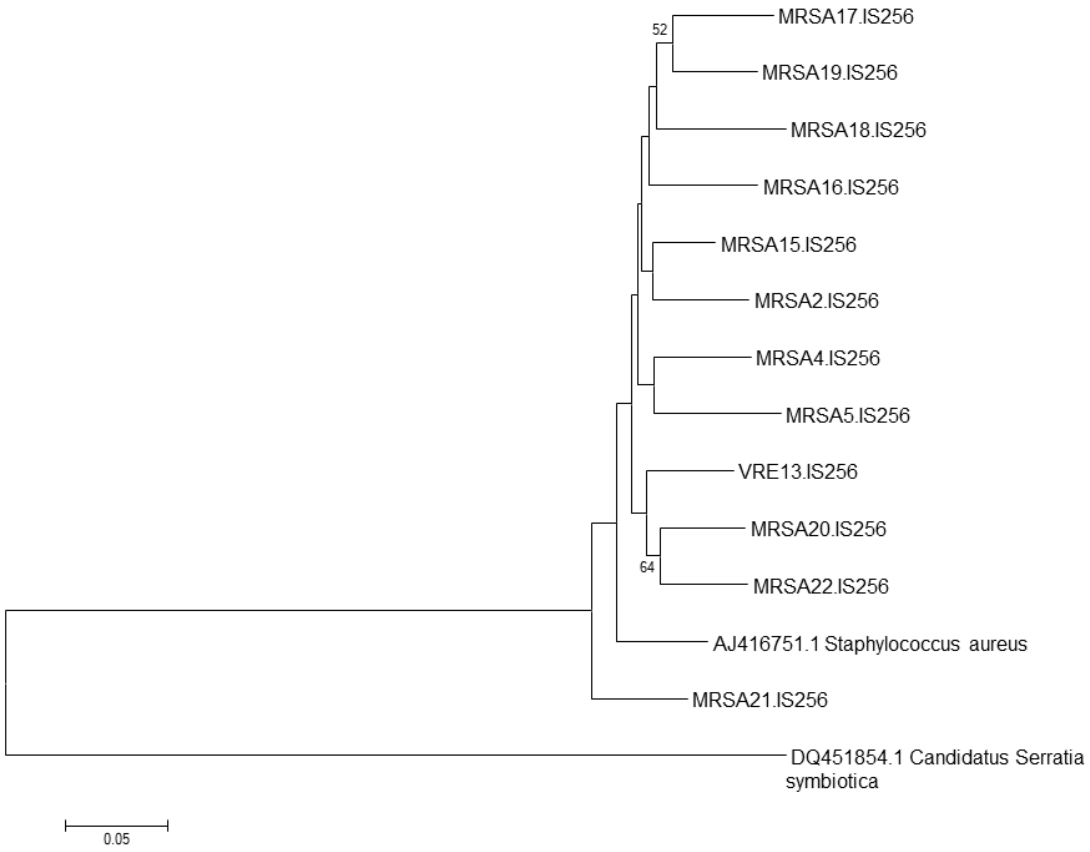


Figure 35: Phylogenetic tree showing close relation between the IS256 gene from MRSA clinical isolates and *S. aureus* tnp (IS256) gene. S*erratia symbiotica* was used as an outgroup.

4.3.3 Minimum Inhibitory and Bactericidal Concentration:

MICs of the purified antimicrobial peptide were tested against different bacterial strains by 2-fold micro broth dilution method in 96-well plates. The MICs were in the range of 0.3-512 µg/ml. The MICs were mostly <64 µg/ml even in case of highly oxacillin resistant clinical strains **(Table 10)**. The MBC values of the peptide were mostly in 4-fold range of the MICs. The antimicrobial peptide is predominantly active against *S. aureus* strains as indicated by the low MIC values when compared to other species of bacteria. The peptide had showed significant activity against clinical MRSA strains.

Table 10: Minimum inhibitory and bactericidal concentrations of the antimicrobial peptide ASP-1 against different gram-positive bacteria compared to oxacillin.

S. No	Indicator strain	MIC (µg/ml) Oxacillin	MIC (µg/ml)	MBC
1	*S. aureus* ATCC 29213	0.25	8	16
2	*S. aureus* MTCC 737	0.25	4	8
3	*S. aureus* GMC*	<4	1	4
4	*S. aureus* NCIM 2901	0.25	2	8
5	*S. aureus* NCIM 5021	0.5	16	>64
6	*S. aureus* NCIM 2127	0.25	8	32
7	MRSA 2	>4	8	64
8	Mupirocin-resistant MRSA 4	32	8	16
9	MRSA 5	8	16	64
10	MRSA 15	>1024	16	16
11	MRSA 16	32	32	>64
12	MRSA 17	512	32	256
13	MRSA 18	128	32	>64
14	MRSA 19	>1024	64	256
15	MRSA 20	>1024	8	32
16	MRSA 21	>1024	512	>512
17	MRSA 22	256	32	128
18	*S. epidermidis* ATCC 12228*		2	4
19	Methicillin resistant *S. epidermidis* 3		2	16
20	*S. pyogenes* NCIM 2608*		120	120
21	*S. pyogenes* MTCC 1928*		60	>60
22	*S. pyogenes*	0.125	16	>64
23	*Enterococcus faecalis* ATCC 51299	16	>64	>64
24	*E. faecalis* ATCC 29212	16	64	>64
25	*Streptococcus faecalis* ATCC14506	16	>64	>64
26	*E. faecium* NCIM 5443	1	>64	>64
27	Vancomycin resistant *E. faecium* 13*		128	128
28	*M. luteus* MTCC 2170*		0.3	15

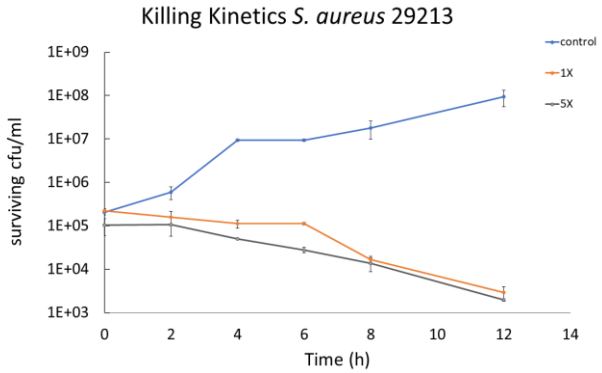
29	*Bacillus* wild-type soil isolate*		30	120

*MIC determined in BHI broth

4.3.4 Time-Kill Kinetics:

To determine the killing kinetics of the peptide *S. aureus* 29213 was exposed to 1X and 5X MIC of the peptide. As shown in **Fig.36A** there was 1.88 $\log_{10}$ and 2.01 $\log_{10}$ reduction after 12 h at 1X and 5X MICs respectively for *S. aureus* 29213. There was a reduction of 98.68% and 99.02% at 1X and 5X MICs respectively for *S. aureus* 29213. However, in case of MRSA 15 which was found highly oxacillin-resistant (>1024 µg/ml), only bacteriostatic effect after 8 hours was observed. There was neither significant reduction nor increase in the number of cells at both 5X and 1X MICs. These results indicate that the ASP-1 may be used in synergy with the existing potential antibiotics for the treatment of MRSA infections.

A



B

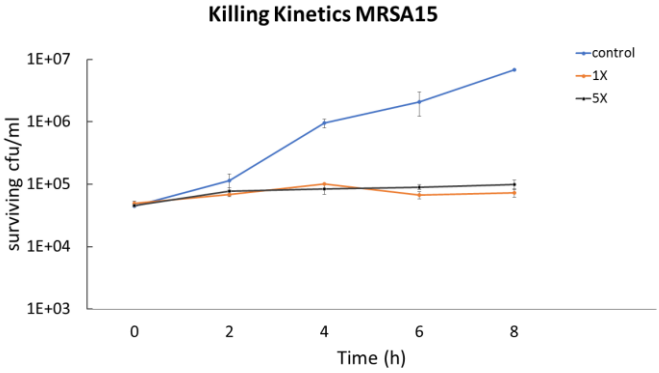


Figure 36: A) Killing kinetics of the peptide at 1X and 5X MIC against *S. aureus* ATCC29213. B) Killing kinetics of the peptide at 1X and 5X MIC against MRSA 15.

4.3.5 Screening of Biofilm Forming Strains:

4.3.5.1 Spectrophotometric Biofilm Screening Assay and Congo Red Agar Assay:

After spectrophotometrically determining the biofilm formation in comparison to the negative biofilm forming strain *S. epidermidis* ATCC12228 eight strains have been identified as biofilm forming. *S. epidermidis* ATCC12228 has shown an optical density (OD) of 0.07 at 490 nm. The strains showing an OD of above 0.5 were considered as biofilm forming **(Fig. 37)**. The 8 strains considered as biofilm forming were screened for the presence of *ica* genes by PCR and have shown black to dark black colonies on Congo red agar plates **(Fig. 38)**.

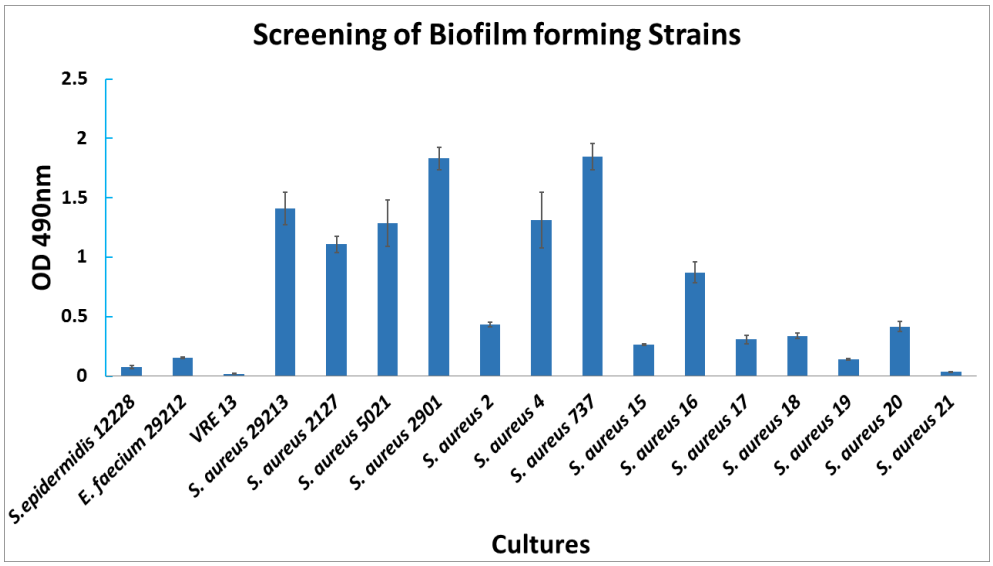


Figure 37: Screening of biofilm-forming strains by comparing spectrophotometric readings with those of *S. epidermidis* ATCC12228. VRE stands for vancomycin-resistant *Enterococcus faecium*. Quantitative assays of biofilm-formation were performed in a 96-well polystyrene microtiter plate by staining the attached cells with safranin.

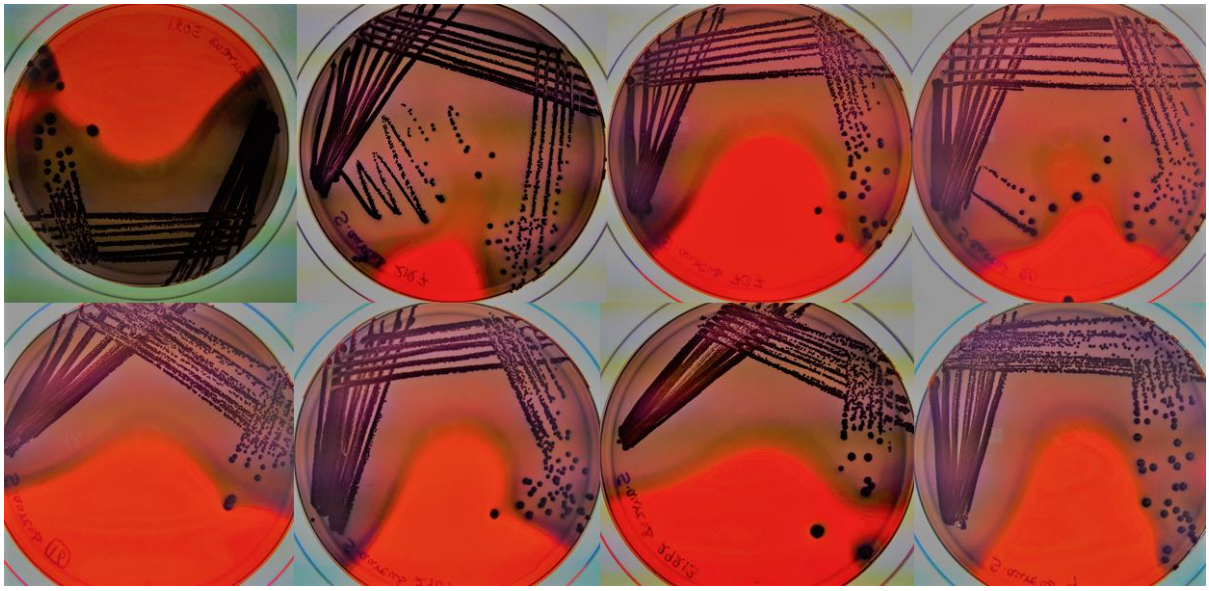


Figure 38: Biofilm forming cultures showing black to dark black colonies on Congo red agar plates.

4.3.5.2 Detection of *ica* Genes by PCR:

In *S. aureus* and *S. epidermidis*, *ica* genes belonging to the ica operons are responsible for producing polysaccharide intracellular adhesion (PIA) an important component of biofilm. The presence of *ica* genes were confirmed by using *icaA* and *ica*D primers. The PCR products resulted from the *icaA* and *icaD* pairs of primers corresponded to the size of 188 and 198 bp respectively on 1.5% agarose gel for all the isolates tested in the present study **(Fig. 39 A and B)**; however, no PCR product was formed when template DNA from the *S. epidermidis* ATCC 12228 was used as negative control strain.

A **B**

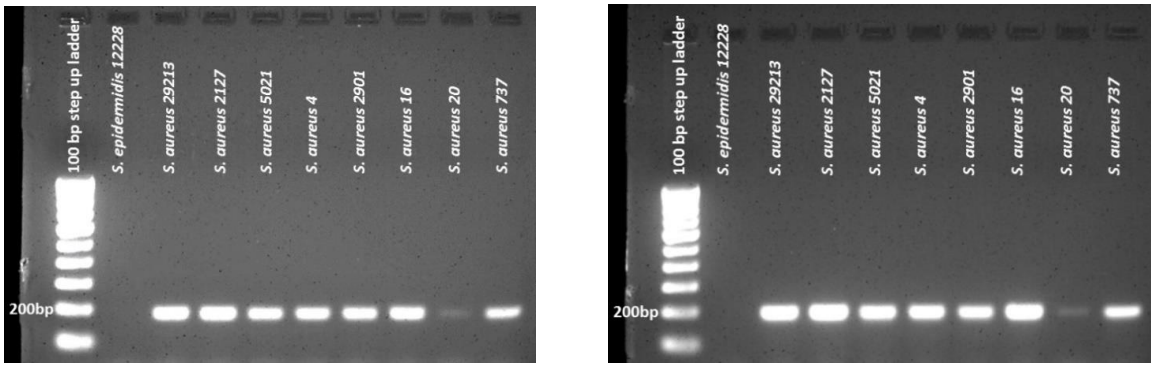


Figure 39: A & B) Agarose gel electrophoresis showing the PCR amplification of *ica*A and *ica*D genes respectively

4.3.6 Determination of Anti-Biofilm Forming Activity of ASP-1:

Purified peptide has shown a complete inhibition of biofilm formation at 1X MIC concentration against eight biofilm forming staphylococcal strains **(Fig. 40)**. Except for the MRSA 20, even 0.5X concentration of MIC have shown the inhibition of biofilm formation. 0.25X concentration of MIC has shown the reduction in the biofilm formation. These results indicate that the peptide has potential to act as inhibitor of biofilm formation.

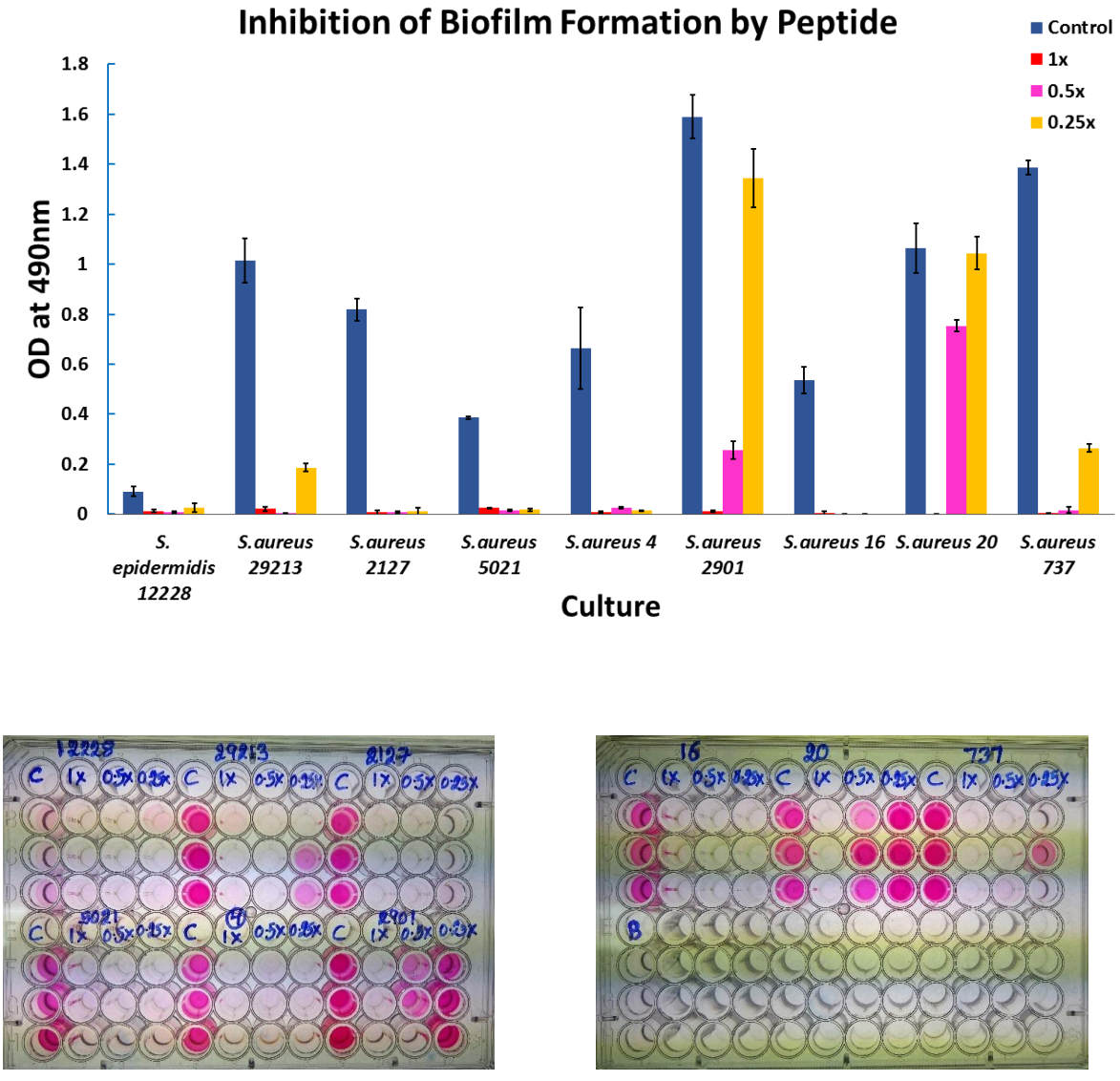


Figure 40: Inhibition of biofilm-formation by ASP-1 peptide at 1X and 0.5X concentration of MIC and reduction at 0.25X concentration. A quantitative assay of biofilm was performed in 96-well polystyrene microtiter plates by staining the attached cells using safranin.

4.3.7 Hemolytic Assay and *in vitro* Cytotoxicity Study:

To evaluate the safety of the peptide toward mammalian cells, hemolytic and cytotoxic effect at different concentration of peptide was evaluated using human RBC and mammalian cell lines respectively. As indicated **(Fig. 41)** in the hemolytic study, a mere ~6% hemolysis at 128 µg/ml concentration of the peptide and 31.5% hemolysis at 256 µg/ml was observed. The breast cancer (MCF-7) and liver cancer (HepG2) cell lines were used to test the cytotoxicity of the peptide by MTT assay. As depicted in **Fig. 42**, the antimicrobial peptide ASP-1 was not toxic to the mammalian cell line MCF 7 and HepG2 tested up to concentrations of 128 µg/ml. The purified compound was found to exhibit cytotoxicity at high concentration (256 µg/ml) with IC_{50} = 192 µg/ml against the HepG2 cell line. While for MCF-7, the IC_{50} observed was 280 µg/ml.

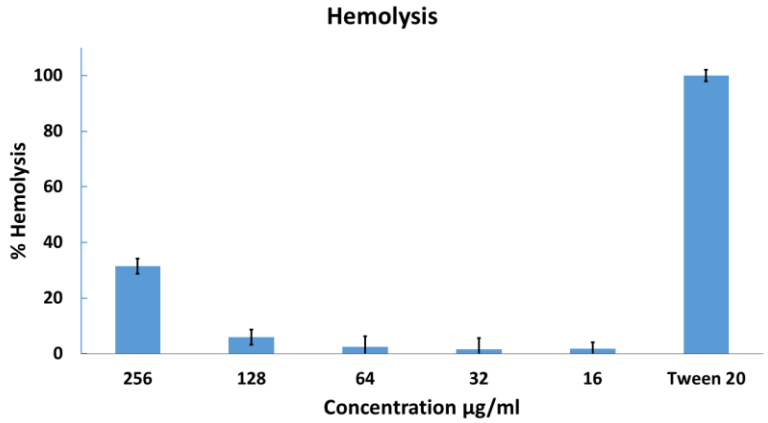


Figure 41: Hemolysis assay showing the percentage of hemolysis caused by the peptide at different concentrations.

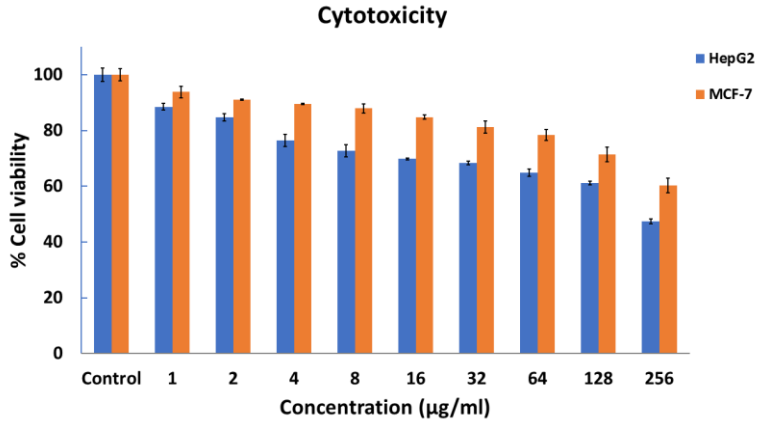


Figure 42: Cytotoxicity assay showing the percentage cell survival of HepG2 and MCF-7 cells after treatment with peptide at different concentrations compared to untreated cells.

4.4 DISCUSSION:

Antibiotic resistance is rapidly evolving towards most known antibiotics and the development of alternative antibiotics is lagging far behind to combat serious infectious diseases. MRSA strains often causing nosocomial infections develop resistance to antimicrobial compounds by modifying its cell surface teichoic acid with D-alanine and show increased MIC **(Peschel et al., 2001; Peschel 2002)**. High prevalence of mupirocin resistance in *S. aureus* isolates from a pediatric population as a result of mupirocin exposure in New York City area was reported underlining the importance of antimicrobial substance effective against mupirocin-resistant *S. aureus* **(Antonov et al., 2015)**. In the present study, a total of 29 organisms were tested for the minimum inhibitory concentrations. A mean MIC of 16.7 µg/ml was observed against all the organisms tested in our study. A mean MIC of 26.5 µg/ml was observed against the MRSA strains by the peptide whereas oxacillin has shown a mean MIC of 145 µg/ml indicating the effectiveness of our peptide against MRSA strains **(Table 11)**. For 9 MRSA strains tested in our study the MIC values are in the range of 8-32 µg/ml this is in the same range as the MICs of unsuccinylated entianin (4 to 16 µg/ml) tested against *S. aureus* and *E. faecalis* strains **(Fuchs et al., 2011)**.

The MIC against MRSA strain 2, resistant to amoxicillin/clavulanate, high level gentamicin (500 µg/ml), clindamycin, ciprofloxacin and erythromycin, was 8 µg/ml. Recently, Mohamed et al., reported the strong anti-*Staphylococcal* activities of short synthetic peptides (WR12 and D-IK8) that showed MIC values between the range of 1–16 µM. In a separate study, the MIC values of pexiganan against methicillin-sensitive *S. aureus* (MSSA) and MRSA strains were determined in the range of 16 to 32 µg/ml **(Flamm et al., 2015)**. An anti-MRSA bioactive compound, which was isolated from the halophilic *Bacillus*, showed an MIC value of 62.5 µg/mL against an MRSA strain **(Jeyanthi & Velusamy 2016)**. The peptide has shown an MIC of 2 µg/ml against *S. epidermidis* ATCC 12228 and methicillin resistant *S. epidermidis* 3 which was similar to the MIC (2-4 µg/ml) shown by Epidermicin against *S. epidermidis* MRSA isolates **(Sandiford & Upton 2012)**. Against *S. pyogenes* the peptide has shown an MIC ranging from 16-120 µg/ml. The MIC against *Micrococcus luteus* was 0.3 µg/ml which was similar to an MIC of 0.12 µM shown by ixodidin, an antimicrobial peptide from the hemocytes of the cattle tick *Boophilus microplus* **(Fogaça et al., 2006)**. In the study

conducted by Kitajima et al. (1990), TL-119 showed an MIC of 1.6 µg/ml against *S. aureus* FAD 209 JC-1 whereas ASP-1 the sequence of which resembles TL-119 showed a MIC of 2 and 1 µg/ml against *S. aureus* NCIM 2901 and *S. aureus* GMC respectively.

Gad et al. (2009) in a clinical survey on the biofilm producing *S. aureus* and *S. epidermidis* present on the medical device cardiac stent confirmed that all of the biofilm forming strains harbored the *icaA* and *icaD* genes **(Gad et al., 2009)**. The *Staphylococcal* isolates used in the present study are also observed to possess *icaA* and *icaD* genes. In the clinical settings, biofilm-associated infections are difficult to treat due to their adaptive resistance to most of the antibiotics and consequent recalcitrance to treatment with the conventional antibiotics **(Mataraci & Dosler 2012)**. In the purview of this, the evaluation of the anti-biofilm potential of ASP–1 was carried out in the present study. The peptide has significantly inhibited the formation of biofilms, especially at 1X MIC and sub-MIC concentrations in agreement with the findings of **Mataraci and Dosler**. They found that antimicrobial cationic peptides alone or in supplementation of antibiotics can inhibit the attachment of bacteria at 1/10 MIC and the biofilm formation at 1X MIC. The authors concluded that the AMPs seemed to be good candidates for further investigations in the treatment of MRSA biofilms, alone or in combination with antibiotics **(Mataraci & Dosler 2012)**. It has also been reported that the peptide 1018 at very low concentration (2.5 µg/ml) demonstrates strong anti-biofilm potential against MRSA isolates **(de la Fuente-Núñez et al., 2014)**. LL-37, a human cationic host defense peptide prevented both the attachment action and development of biofilms by *Staphylococcus epidermidis*, being commensal in human skin and mucous membrane and has shown 40% reduction in the biofilm formation against *S. aureus* at 10 µg/ml **(Dean et al., 2011)**.

One of the major problems associated with the use of antimicrobial peptides is the toxicity on the host cells. It is desirable for the peptide to have a high therapeutic index (TI) as it indicates the effectiveness of the peptide against the microbes at the same time its low toxicity towards the host cells **(Aoki & Ueda 2013)**. In the present study, the antimicrobial peptide has shown much less than 50% reduction in the viability of cells at high concentration (128 µg/mL) (**Fig. 42**), which corresponds to 7.5 times of the geometric mean MIC of peptide for all the indicator strains that were used in this study. Concomitantly, the same concentration of the peptide showed negligible (~6%) hemolysis

(Fig. 41). The ASP-1 peptide also demonstrated a high TI as there was significantly low toxicity towards the mammalian cells (MCF–7 and HepG2) at concentrations that were several times higher than the bactericidal concentrations **(Table 11)**. The TI for MCF-7 was 16.7, and for HepG2 it was 11.4. The low toxicity of antimicrobial peptides against mammalian cells when compared to their antimicrobial potency suggests their selective actions against the negatively charged bacterial membranes compared to the zwitterionic mammalian membranes **(Mohamed et al., 2016)**. The half maximal effective concentrations (EC_{50}) of 128 and > 256 μM were observed for the peptides WR12 and D-IK8 respectively when tested against human keratinocytes (HaCat cells). These values correlate to 64 and > 32-fold of the MIC_{50} for WR12 and D-IK8, respectively **(Mohamed et al., 2016)**. These results suggest the therapeutic potential of the ASP-1 along with the safety aspect of the peptide as demonstrated by less toxicity on the host cells. Further characterization and pre-clinical safety/toxicity studies in appropriate animal models is required.

Table 11: Cytotoxicity and Therapeutic Index of ASP-1

GM MIC[a] (all strains)	**GM MIC (MRSA)**	**GMO MIC (MRSA)**	**IC_{50}[c] HepG2**	**IC_{50} MCF-7**	**TI[d] HepG2 (all strains)**	**TI HepG2 (MRSA)**	**TI MCF-7 (all strains)**	**TI MCF-7 (MRSA)**
16.77	26.48	145.19	192	280	11.44	7.25	16.69	10.57

[a] GM, geometric mean of the MICs of ASP-1 against indicator strains.
[b] GMO, geometric mean of the MICs of oxacillin against MRSA strains.
[c] IC_{50} (Inhibitory Concentration 50), peptide concentration that inhibits 50% of cell line HepG2 or MCF-7.
[d] TI (Therapeutic index), the ratio of the IC_{50} over the geometric mean MIC value.
MRSA- Methicillin-Resistant *S. aureus*

SUMMARY OF RESULTS:

A soil isolate showing antimicrobial activity has been isolated. Based on the biochemical tests, colony morphology it was identified as genus *Bacillus*. Vitek 2 compact identification have shown it to be one among *Bacillus subtilis/ amyloliquefaciens/ atrophaeus* with a confidence level of good identification, 91% probability. However, MALDI-TOF Bio typing identified the culture as *Bacillus subtilis* with a high-level confidence score of 1.905. 16S rDNA amplification identified the isolate as *Bacillus atrophaeus* and assigned the GenBank accession number as JX156420.1 by the NCBI. The phylogenetic tree analysis using Mega 5.0 has shown the URID 12.1 more closely related to *Bacillus subtilis*. The optimum antimicrobial peptide production was observed at the end of logarithmic and early stationary phase. The antimicrobial activity of the cell free supernatant was tested against Gram positive, negative bacteria and *Candida* strains by cut well agar assay. However, the peptide was active against only Gram-positive bacteria.

The antimicrobial peptide, ASP-1 was stable over a range of pH values ranging from 1-14 with a reduction in the activity at high alkaline pH. It was also stable at high temperatures with more than 50% activity after autoclaving for 20 mins at 121^{o}C and 15 lbs pressure. However, there is complete loss of activity at pH-12 after autoclaving. The activity of ASP-1 was not affected by organic solvents (50% v/v) and surfactants (1% v/v) indicating the hydrophobic nature of the peptide. The antimicrobial compound was resistant to the action of proteolytic enzymes, Trypsin 10 mg/ml and Proteinase K 5 mg/ml. Gel overlay assay after SDS PAGE has shown activity in the region of a band less than 3 kDa indicating the low molecular weight of ASP-1. Whereas, in native PAGE the activity was observed at the top of the resolving gel indicating may be the net positive charge or aggregation of the peptide in its native form. Ninhydrin test after TLC have shown negative results.

ASP-1 was purified to homogeneity by a series of steps involving acid precipitation of the cell free supernatant, followed by solvent extraction, adsorption chromatography and RP-HPLC. The purity of the peptide was confirmed by analytical HPLC and MALDI-TOF. The molecular weight of ASP-1 was identified by MALDI-TOF as 804.4 Da along with it its sodium and potassium adduct at 826.4 Da and 842.4 Da

respectively. Secondary structure analysis using circular dichroism spectroscopy has shown that ASP-1 exists as random coils and β-sheets in the aqueous solution with an increase in the β-sheets in methanol, however, the helix structure is completely absent. The sequence of ASP-1 by MALDI-TOF/MS and ESI-FTICR MS revealed it as acetylated Phe-Thr-Ala-Val-Dhb-Phe-Ile/Leu. The peptide was further analyzed by alkaline hydrolysis, ESI-Q-TOF-MS and ion mobility assay which detected the presence of lactone ring and its cyclic nature in the intact peptide, subsequently revealing the sequence as acetylated-Phe-Thr-Val-Ala-Dhb-Phe-Ile/Leu. Based on the molecular mass (804.5 Da), peptide sequence and amino acid composition, the ASP-1 was identified as lactone-ring containing peptide similar to TL-119, a poorly studied cyclic depsipeptide.

Genetic analysis of the clinical isolates has shown that they have *mec*A gene, responsible for methicillin resistance and IS256 transposon which plays an important role in antibiotic resistance and biofilm formation. After screening 16 strains for biofilm formation against the negative strain *S. epidermidis* ATCC12228, 8 strains were identified as biofilm forming and tested for the antibiofilm forming activity of ASP-1. All the biofilm forming strains were tested positive for the presence of *icaA* and *icaD* genes and have shown black to dark black colonies when grown on Congo red agar plates. ASP-1 has completely inhibited the biofilm formation at 1X and 0.5X MIC concentrations and reduction in the biofilm formation was observed at 0.25X concentration.

MIC of the ASP-1 was determined against 29 Gram positive bacteria with a geometric mean MIC of 16.7 µg/ml. ASP-1 has shown a geometric mean MIC of 26.5 µg/ml against methicillin-resistant *S. aureus* strains. The MICs and MBCs are generally in the ratio of 1:4. Time-kill kinetics against *S. aureus* 29213 at 1X and 5X concentration of MIC have shown 1.88 $\log_{10}$ and 2.01 $\log_{10}$ reduction respectively after 12 h. There was a reduction of 98.68% and 99.02% of initial inoculum at 1X and 5X MICs respectively. Negligible hemolysis (6%) of human erythrocytes was observed at 128 µg/ml concentration of ASP-1 and 31.5% at 256 µg/ml. ASP-1 was not toxic to the mammalian cell line MCF 7 and HepG2 up to concentrations of 128 µg/ml with an IC_{50} of 192 µg/ml against the HepG2 and 280 µg/ml against MCF-7. The peptide also has a high (>10) therapeutic index.

CONCLUSIONS:

Some of the conclusions that can be drawn from the current study are as follows:

1) The ASP-1 has shown narrow spectrum activity, only against Gram-positive bacteria so the chances of developing resistance and the spread of resistance to other bacteria are very less.
2) In comparison to oxacillin, ASP-1 showed good antimicrobial activity against methicillin/mupirocin/oxacillin-resistant *S. aureus* strains as compared to methicillin- sensitive strains; this observation indicates that the mechanism of action of this antimicrobial peptide may be different from the existing antibiotics or antibacterial peptides.
3) ASP-1 exhibited stability over a wide range of pH and temperatures; its antibiofilm forming activity, non-haemolytic and less cytotoxic properties at its respective MIC values have indicated that ASP-1 could be a potential candidate for future clinical application as topical agent.
4) Based on the molecular mass, amino acid composition analysis and the sequence, it was identified as a novel antibacterial peptide.

FUTURE SCOPE OF WORK

1. To study the molecular mechanism of action of ASP-1.
2. Further structural characterization by NMR studies.
3. To study the in *vivo* antibacterial activity of the ASP-1 using mouse thigh infection model and perform skin irritation study for dermal safety aspect.
4. To study the pharmacokinetic and pharmacodynamic properties of the purified ASP-1.
5. Synthesis of the synthetic peptide based on the peptide sequence of ASP-1 and testing its activity along with modifying the position of amino acids to observe whether it can increase the stability and antibacterial spectrum and potential.

Abraham, E. P., & Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature*, *146*(3713), 837.

Abriouel, H., Franz, C. M., Omar, N. B., & Gálvez, A. (2010). Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiology Reviews*, *35*(1), 201-232.

Ahern, M., Verschueren, S., & Van Sinderen, D. (2003). Isolation and characterisation of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. *FEMS Microbiology Letters*, *220*(1), 127-131.

Alekshun, M. N., & Levy, S. B. (2004). Targeting virulence to prevent infection: to kill or not to kill? *Drug Discovery Today: Therapeutic Strategies*, *1*(4), 483-489.

Allen, H. K., Moe, L. A., Rodbumrer, J., Gaarder, A., & Handelsman, J. (2009). Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil. *The ISME journal*, *3*(2), 243-251.

Andersson, D. I. (2006). The biological cost of mutational antibiotic resistance: any practical conclusions? *Current Opinion in Microbiology*, *9*(5), 461-465.

Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology*, *8*(4), 260-271.

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48*(suppl 1), 5-16.

Antonov, N. K., Garzon, M. C., Morel, K. D., Whittier, S., Planet, P. J., & Lauren, C. T. (2015). High prevalence of mupirocin resistance in *Staphylococcus aureus* isolates from a pediatric population. *Antimicrobial Agents and Chemotherapy*, *59*(6), 3350-3356.

Aoki, W., & Ueda, M. (2013). Characterization of antimicrobial peptides toward the development of novel antibiotics. *Pharmaceuticals*, *6*(8), 1055-1081.

Arbeit, R. D., Maki, D., Tally, F. P., Campanaro, E., & Eisenstein, B. I. (2004). The safety and efficacy of daptomycin for the treatment of complicated skin and skin-structure infections. *Clinical Infectious Diseases*, *38*(12), 1673-1681.

Arciola, C. R., Baldassarri, L., & Montanaro, L. (2001). Presence of icaA and icaDGenes and slime production in a collection of Staphylococcal strains from catheter-associated infections. *Journal of Clinical Microbiology*, *39*(6), 2151-2156.

Asaduzzaman, S. M., & Sonomoto, K. (2009). Lantibiotics: diverse activities and unique modes of action. *Journal of Bioscience and Bioengineering*, *107*(5), 475-487.

Aunpad, R., & Na-Bangchang, K. (2007). Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4. *Current Microbiology*, *55*(4), 308-313.

Ayed, H. B., Hmidet, N., Béchet, M., Chollet, M., Chataigné, G., Leclère, V., Jacques, P., & Nasri, M. (2014). Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. *Process Biochemistry*, *49*(10), 1699-1707.

Babasaki, K., Takao, T., Shimonishi, Y., & Kurahashi, K. (1985). Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. *The Journal of Biochemistry*, *98*(3), 585-603.

Baindara, P., Mandal, S. M., Chawla, N., Singh, P. K., Pinnaka, A. K., & Korpole, S. (2013). Characterization of two antimicrobial peptides produced by a halotolerant *Bacillus subtilis* strain SK. DU. 4 isolated from a rhizosphere soil sample. *AMB Express*, *3*(1), 2.

Banerjee, S., & Hansen, J. N. (1988). Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. *Journal of Biological Chemistry*, *263*(19), 9508-9514.

Barber, M. (1961). Methicillin-resistant *staphylococci*. *Journal of Clinical Pathology*, *14*(4), 385.

Barber, M., & Rozwadowska-Dowzenko, M. (1948). Infection by penicillin-resistant *staphylococci*. *The Lancet*, *252*(6530), 641-644.

Barlow, M., & Hall, B. G. (2002). Phylogenetic analysis shows tha-t the OXA b-lactamase genes have been on plasmids for millions of years. *Journal of Molecular Evolution*, *55*(3), 314-321.

Barrett, F. F., McGehee Jr, R. F., & Finland, M. (1968). Methicillin-resistant *Staphylococcus aureus* at Boston City Hospital: bacteriologic and epidemiologic observations. *New England Journal of Medicine*, *279*(9), 441-448.

Bartoloni, A., Pallecchi, L., Rodríguez, H., Fernandez, C., Mantella, A., Bartalesi, F., Strohmeyer, M., Kristiansson, C., Gotuzzo, E., Paradisi, F., & R9*-kjm nh`1 ossolini, G. M. (2009). Antibiotic resistance in a very remote Amazonas community. *International Journal of Antimicrobial Agents*, *33*(2), 125-129.

Beck, W. D., Berger-Bächi, B., & Kayser, F. H. (1986). Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of mec-specific DNA. *Journal of Bacteriology*, *165*(2), 373-378.

Benitez, L. B., Velho, R. V., Lisboa, M. P., da Costa Medina, L. F., & Brandelli, A. (2010). Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM5006. *The Journal of Microbiology*, *48*(6), 791-797.

Berova, N., & Nakanishi, K. (2000). *Circular dichroism: principles and applications*. John Wiley & Sons.

Bhakdi, S., & Tranum-Jensen, J. (1991). Alpha-toxin of *Staphylococcus aureus*. *Microbiological Reviews*, *55*(4), 733-751.

Bhunia, A. K., Johnson, M. C., & Ray, B. (1987). Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Industrial Microbiology & Biotechnology*, *2*(5), 319-322.

Bierbaum, G., & Sahl, H. G. (1987). Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. *Journal of Bacteriology*, *169*(12), 5452-5458.

Bierbaum, G., & Sahl, H. G. (2009). Lantibiotics: mode of action, biosynthesis and bioengineering. *Current Pharmaceutical Biotechnology*, *10*(1), 2-18.

Bierbaum, G., Brötz, H., Koller, K. P., & Sahl, H. G. (1995). Cloning, sequencing and production of the lantibiotic mersacidin. *FEMS Microbiology Letters*, *127*(1-2), 121-126.

Bionda, N., & Cudic, P. (2011). Cyclic lipodepsipeptides in novel antimicrobial drug discovery. *Croatica Chemica Acta*, *84*(2), 315-329.

Bizani, D., Motta, A. S., Morrissy, J. A., Terra, R., Souto, A. A., & Brandelli, A. (2005). Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus*. *International Microbiology*, *8*(2), 125-131.

Blazyk, J., Wiegand, R., Klein, J., Hammer, J., Epand, R. M., Epand, R. F., Maloy, W.L., & Kari, U. P. (2001). A novel linear amphipathic β-sheet cationic antimicrobial peptide with enhanced selectivity for bacterial lipids. *Journal of Biological Chemistry*, *276*(30), 27899-27906.

Blunt, J.W., Copp, B.R., Munro, M.H., Northcote, P.T., Prinsep, M.R. (2011). Marine natural products. *Nat. Prod. Rep*, *28*, 196–268.

Boman, H. G. (1995). Peptide antibiotics and their role in innate immunity. *Annual Review of Immunology*, *13*(1), 61-92.

Boucher, Y., Labbate, M., Koenig, J. E., & Stokes, H. W. (2007). Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends in Microbiology*, *15*(7), 301-309.

Brochet, M., Couvé, E., Zouine, M., Poyart, C., & Glaser, P. (2008). A naturally occurring gene amplification leading to sulfonamide and trimethoprim resistance in *Streptococcus agalactiae*. *Journal of Bacteriology*, *190*(2), 672-680.

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology*, *3*(3), 238-250.

Brown, E. D. (2006). Microbiology: antibiotic stops 'ping-pong'match. *Nature*, *441*(7091), 293-294.

Bush, K., & Mobashery, S. (1998). How β-lactamases have driven pharmaceutical drug discovery. In *Resolving the Antibiotic Paradox* (pp. 71-98). Springer US.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G.A., Kishony, R., Kreiswirth, B.N., Kutter, E., & Lerner, S. A. (2011). Tackling antibiotic resistance. *Nature Reviews Microbiology*, *9*(12), 894-896.

Bushman, F. (2001). Lateral DNA transfer: mechanisms and consequences. *Cold Spring Harbor Laboratory Press*.

Cantor, R. S. (2002). Size distribution of barrel-stave aggregates of membrane peptides: influence of the bilayer lateral pressure profile. *Biophysical Journal*, *82*(5), 2520-2525.

Centers for Disease Control and Prevention (CDC. 1999). Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*—Minnesota and North Dakota, 1997-1999. *MMWR. Morbidity and mortality weekly report*, *48*(32), 707.

Chatterjee, C., Paul, M., Xie, L., & van der Donk, W. A. (2005). Biosynthesis and mode of action of lantibiotics. *Chemical Reviews*, *105*(2), 633-684.

Chatterjee, S., Chatterjee, S., Lad, S. J., Phansalkar, M. S., Rupp, R. H., Ganguli, B. N., Fehlhaber, H.W., & Kogler, H. (1992). Mersacidin, a new antibiotic from *Bacillus*. *The Journal of Antibiotics*, *45*(6), 832-838.

Cherif, A., Chehimi, S., Limem, F., Hansen, B. M., Hendriksen, N. B., Daffonchio, D., & Boudabous, A. (2003). Detection and characterization of the novel bacteriocin entomocin 9, and safety evaluation of its producer, *Bacillus thuringiensis* ssp. *entomocidus* HD9. *Journal of Applied Microbiology*, *95*(5), 990-1000.

Cherif, A., Ouzari, H., Daffonchio, D., Cherif, H., Ben Slama, K., Hassen, A., Jaoua, S., & Boudabous, A. (2001). Thuricin 7: a novel bacteriocin produced by *Bacillus thuringiensis* BMG1. 7, a new strain isolated from soil. *Letters in Applied Microbiology*, *32*(4), 243-247.

Cherif, A., Rezgui, W., Raddadi, N., Daffonchio, D., & Boudabous, A. (2008). Characterization and partial purification of entomocin 110, a newly identified bacteriocin from *Bacillus thuringiensis* subsp. *entomocidus* HD110. *Microbiological Research*, *163*(6), 684-692.

Chopra, L., Singh, G., Choudhary, V., & Sahoo, D. K. (2014). Sonorensin: an antimicrobial peptide, belonging to the heterocycloanthracin subfamily of bacteriocins, from a new marine isolate, *Bacillus sonorensis* MT93. *Applied and Environmental Microbiology*, *80*(10), 2981-2990.

Cladera-Olivera, F., Caron, G. R., & Brandelli, A. (2004). Bacteriocin-like substance production by *Bacillus licheniformis* strain P40. *Letters in Applied Microbiology*, *38*(4), 251-256.

Clardy, J., Fischbach, M. A., & Walsh, C. T. (2006). New antibiotics from bacterial natural products. *Nature Biotechnology*, *24*(12), 1541-1550.

Claydon, M. A., Davey, S. N., Edwards-Jones, V., & Gordon, D. B. (1996). The rapid identification of intact microorganisms using mass spectrometry. *Nature Biotechnology*, *14*(11), 1584-1586.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1-20.

Coates, A., Hu, Y., Bax, R., & Page, C. (2002). The future challenges facing the development of new antimicrobial drugs. *Nature Reviews Drug Discovery*, *1*(11), 895-910.

Colin, D. A., Mazurier, I., Sire, S., & Finck-Barbancon, V. (1994). Interaction of the two components of leukocidin from *Staphylococcus aureus* with human polymorphonuclear leukocyte membranes: sequential binding and subsequent activation. *Infection and Immunity*, *62*(8), 3184-3188.

Conlon, K. M., Humphreys, H., & O'Gara, J. P. (2002). icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in *Staphylococcus epidermidis*. *Journal of Bacteriology*, *184*(16), 4400-4408.

Cotter, P. D., Ross, R. P., & Hill, C. (2013). Bacteriocins-a viable alternative to antibiotics? *Nature Reviews Microbiology*, *11*(2), 95-105.

Coulter, S. N., Schwan, W. R., Ng, E. Y., Langhorne, M. H., Ritchie, H. D., Westbrock-Wadman, S., Hufnagle, W.O., Folger, K.R., Bayer, A.S., & Stover, C. K. (1998). *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Molecular Microbiology*, *30*(2), 393-404.

Cramer, R. (2016). *Advances in MALDI and laser-induced soft ionization mass spectrometry*. Springer.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W., & Götz, F. (1999). The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infection and Immunity*, *67*(10), 5427-5433.

Crisóstomo, M. I., Westh, H., Tomasz, A., Chung, M., Oliveira, D. C., & de Lencastre, H. (2001). The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and-resistant isolates and contemporary epidemic clones. *Proceedings of the National Academy of Sciences*, *98*(17), 9865-9870.

Crossley, K., Landesman, B., & Zaske, D. (1979). An outbreak of infections caused by strains of *Staphylococcus aureus* resistant to methicillin and aminoglycosides. II. Epidemiologic studies. *Journal of Infectious Diseases*, *139*(3), 280-287.

Cutting, S. M. (2011). Bacillus probiotics. *Food Microbiology*, *28*(2), 214-220.

Datta, N., & Hughes, V. M. (1983). Plasmids of the same Inc groups in *Enterobacteria* before and after the medical use of antibiotics. *Nature*, *306*(5943), 616-617.

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*, *74*(3), 417-433.

Davies, J., Spiegelman, G. B., & Yim, G. (2006). The world of sub-inhibitory antibiotic concentrations. *Current Opinion in Microbiology*, *9*(5), 445-453.

Davis, S. L., Perri, M. B., Donabedian, S. M., Manierski, C., Singh, A., Vager, D., Haque, N.Z., Speirs, K., Muder, R.R., Robinson-Dunn, B., & Hayden, M. K. (2007). Epidemiology and outcomes of community-associated methicillin-resistant *Staphylococcus aureus* infection. *Journal of Clinical Microbiology*, *45*(6), 1705-1711.

de la Fuente-Núñez, C., Reffuveille, F., Haney, E. F., Straus, S. K., & Hancock, R. E. (2014). Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathogens*, *10*(5), e1004152.

Dean, S. N., Bishop, B. M., & van Hoek, M. L. (2011). Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphylococcus aureus*. *BMC Microbiology*, *11*(1), 114.

Degnan, B. A., Palmer, J. M., Robson, T., Jones, C. E. D., Fischer, M., Glanville, M., Mellor, G.D., Diamond, A.G., Kehoe, M.A., & Goodacre, J. A. (1998). Inhibition of human peripheral blood mononuclear cell proliferation by *Streptococcus pyogenes* cell extract is associated with arginine deiminase activity. *Infection and Immunity*, *66*(7), 3050-3058.

DeLeo, F. R., & Chambers, H. F. (2009). Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *The Journal of Clinical Investigation*, *119*(9), 2464-2474.

Dhillon, M. K., Kumar, S., & Gujar, G. T. (2014). A common HPLC-PDA method for amino acid analysis in insects and plants. *Indian Journal of Experimental Biology*, *52*, 73-79.

Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H.A., Mongodin, E.F., & Sensabaugh, G. F. (2006). Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *The Lancet*, *367*(9512), 731-739.

Diep, B. A., Stone, G. G., Basuino, L., Graber, C. J., Miller, A., des Etages, S. A., Jones, A., Palazzolo-Ballance, A.M., Perdreau-Remington, F., Sensabaugh, G.F., & DeLeo, F. R. (2008). The arginine catabolic mobile element and *staphylococcal* chromosomal cassette mec linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant Staphylococcus aureus. *Journal of Infectious Diseases*, *197*(11), 1523-1530.

Dischinger, J., Josten, M., Szekat, C., Sahl, H. G., & Bierbaum, G. (2009). Production of the novel two-peptide lantibiotic lichenicidin by *Bacillus licheniformis* DSM 13. *PLoS One*, *4*(8), e6788.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. M., & Prévost, H. (2006). The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, *70*(2), 564-582.

ECDC–EMEA. (2009). European Centre for Disease Prevention and Control (ECDC) & European Medicines Agency (EMEA). Joint technical report: the bacterial challenge. Time to react (ECDC–EMEA, Stockholm, 2009).

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences, USA*, *99*(11), 7687-7692.

Faheem, F., Saeed, S. A. D. I. A., & Rasool, S. A. (2007). Studies on brevicin AF01: a bacteriocin like inhibitory substance active against methicillin resistant *Staphylococcus aureus*. *Pakistan Journal of Botany*, *39*(4), 1293.

Favret, M. E., & Yousten, A. A. (1989). Thuricin: the bacteriocin produced by *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, *53*(2), 206-216.

Fernández-No, I. C., Böhme, K., Díaz-Bao, M., Barros-Velázquez, J., & Calo-Mata, P. (2012). Identification and characterisation of *Bacillus* strains by MALDI-TOF mass fingerprinting and genomic analysis. *Microbes in Applied Research: Current Advances and Challenges*, 273-277.

Fick, J., Söderström, H., Lindberg, R. H., Phan, C., Tysklind, M., & Larsson, D. G. (2009). Contamination of surface, ground, and drinking water from pharmaceutical production. *Environmental Toxicology and Chemistry*, *28*(12), 2522-2527.

Fisher, S. H., & Débarbouillé, M. (2002). Nitrogen source utilization and its regulation. In *Bacillus subtilis and its Closest Relatives* (pp. 181-191). American Society of Microbiology.

Fitzgerald, J. R., Sturdevant, D. E., Mackie, S. M., Gill, S. R., & Musser, J. M. (2001). Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proceedings of the National Academy of Sciences, USA*, *98*(15), 8821-8826.

Flamm, R. K., Rhomberg, P. R., Simpson, K. M., Farrell, D. J., Sader, H. S., & Jones, R. N. (2015). In vitro spectrum of pexiganan activity when tested against pathogens from diabetic foot infections and with selected resistance mechanisms. *Antimicrobial Agents and Chemotherapy*, *59*(3), 1751-1754.

Fogaça, A. C., Almeida, I. C., Eberlin, M. N., Tanaka, A. S., Bulet, P., & Daffre, S. (2006). Ixodidin, a novel antimicrobial peptide from the hemocytes of the cattle

tick *Boophilus microplus* with inhibitory activity against serine proteinases. *Peptides*, *27*(4), 667-674.

Forney, L. J., Zhou, X., & Brown, C. J. (2004). Molecular microbial ecology: land of the one-eyed king. *Current Opinion in Microbiology*, *7*(3), 210-220.

Francis, J. S., Doherty, M. C., Lopatin, U., Johnston, C. P., Sinha, G., Ross, T., Cai, M., Hansel, N.N., Perl, T., Ticehurst, J.R., & Carroll, K. (2005). Severe community-onset pneumonia in healthy adults caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine leukocidin genes. *Clinical Infectious Diseases*, *40*(1), 100-107.

Franz, C. M., Van Belkum, M. J., Holzapfel, W. H., Abriouel, H., & Gálvez, A. (2007). Diversity of *enterococcal* bacteriocins and their grouping in a new classification scheme. *FEMS Microbiology Reviews*, *31*(3), 293-310.

Freeman, D. J., Falkiner, F. R., & Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology*, *42*(8), 872-874.

Freitas, M. A., Hendrickson, C. L., Emmett, M. R., & Marshall, A. G. (1999). Gas-phase bovine ubiquitin cation conformations resolved by gas-phase hydrogen/deuterium exchange rate and extent. *International Journal of Mass Spectrometry*, *185*, 565-575.

Fridkin, S. K., Hageman, J. C., Morrison, M., Sanza, L. T., Como-Sabetti, K., Jernigan, J. A., Harriman, K., Harrison, L.H., Lynfield, R., & Farley, M. M. (2005). Methicillin-resistant *Staphylococcus aureus* disease in three communities. *New England Journal of Medicine*, *352*(14), 1436-1444.

Friedrich, C., Scott, M. G., Karunaratne, N., Yan, H., & Hancock, R. E. (1999). Salt-resistant alpha-helical cationic antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*, *43*(7), 1542-1548.

Fuchs, S. W., Jaskolla, T. W., Bochmann, S., Kötter, P., Wichelhaus, T., Karas, M., Stein, T., & Entian, K. D. (2011). Entianin, a novel subtilin-like lantibiotic from *Bacillus subtilis* subsp. *spizizenii* DSM 15029T with high antimicrobial activity. *Applied and Environmental Microbiology*, *77*(5), 1698-1707.

Funnell, B. E., & Phillips, G. J. (Eds.). (2004). *Plasmid Biology* (Vol. 672). Washington, DC: ASM press.

Gad, G. F. M., El-Feky, M. A., El-Rehewy, M. S., Hassan, M. A., Abolella, H., & El-Baky, R. M. A. (2009). Detection of icaA, icaD genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. *The Journal of Infection in Developing Countries*, *3*(05), 342-351.

Gebhardt, K., Schimana, J., Müller, J., Fiedler, H. P., Kallenborn, H. G., Holzenkämpfer, M., Krastel, P., Zeeck, A., Vater, J., Höltzel, A., & Schmid, D. G. (2002). Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. *FEMS Microbiology Letters*, *217*(2), 199-205.

Genestier, A. L., Michallet, M. C., Prévost, G., Bellot, G., Chalabreysse, L., Peyrol, S., Thivolet, F., Etienne, J., Lina, G., Vallette, F.M., & Vandenesch, F. (2005). *Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. *The Journal of Clinical Investigation*, *115*(11), 3117-3127.

Georgopapadakou, N. H., Dix, B. A., & Mauriz, Y. R. (1986). Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *29*(2), 333-336.

Gillings, M. R., Krishnan, S., Worden, P. J., & Hardwick, S. A. (2008). Recovery of diverse genes for class 1 integron-integrases from environmental DNA samples. *FEMS Microbiology Letters*, *287*(1), 56-62.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., & Stokes, H. W. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *Journal of Bacteriology*, *190*(14), 5095-5100.

Giuliani, A., Pirri, G., & Nicoletto, S. (2007). Antimicrobial peptides: an overview of a promising class of therapeutics. *Open Life Sciences*, *2*(1), 1-33.

Gniadkowski, M. (2008). Evolution of extended-spectrum β-lactamases by mutation. *Clinical Microbiology and Infection*, *14*(s1), 11-32.

Goffin, C., & Ghuysen, J. M. (2002). Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins versus lack of efficiency of penicillin as therapeutic agent. *Microbiology and Molecular Biology Reviews*, *66*(4), 702-738.

Gonzalez, B. E., Hulten, K. G., Dishop, M. K., Lamberth, L. B., Hammerman, W. A., Mason, E. O., & Kaplan, S. L. (2005). Pulmonary manifestations in children with invasive community-acquired *Staphylococcus aureus* infection. *Clinical Infectious Diseases*, *41*(5), 583-590.

Gorwitz, R. J., Kruszon-Moran, D., McAllister, S. K., McQuillan, G., McDougal, L. K., Fosheim, G. E., Jensen, B.J., Killgore, G., Tenover, F.C., & Kuehnert, M. J. (2008). Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001–2004. *Journal of Infectious Diseases*, *197*(9), 1226-1234.

Gottlieb, D. (1976). The production and role of antibiotics in soil. *The Journal of Antibiotics*, *29*(10), 987-1000.

Götz, F. (2002). Staphylococcus and biofilms. *Molecular Microbiology*, *43*(6), 1367-1378.

Greenfield, N. J. (2006). Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols*, *1*(6), 2876-2890.

Greenfield, N. J., & Fasman, G. D. (1969). Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry*, *8*(10), 4108-4116.

Grundmann, H., Aires-de-Sousa, M., Boyce, J., & Tiemersma, E. (2006). Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. *The Lancet*, *368*(9538), 874-885.

Guo, Y., Huang, E., Yuan, C., Zhang, L., & Yousef, A. E. (2012). Isolation of a *Paenibacillus* sp. strain and structural elucidation of its broad-spectrum lipopeptide antibiotic. *Applied and Environmental Microbiology*, *78*(9), 3156-3165.

Hamad, B. (2010). The antibiotics market. *Nature Reviews Drug Discovery*, *9*(9), 675-676.

Hancock, R. E. (1997). Peptide antibiotics. *The Lancet*, *349*(9049), 418-422.

Hancock, R. E., & Chapple, D. S. (1999). Peptide antibiotics. *Antimicrobial Agents and Chemotherapy*, *43*(6), 1317-1323.

Hancock, R. E., & Rozek, A. (2002). Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiology Letters*, *206*(2), 143-149.

Hassan, M., Kjos, M., Nes, I. F., Diep, D. B., & Lotfipour, F. (2012). Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. *Journal of Applied Microbiology*, *113*(4), 723-736.

He, L., Chen, W., & Liu, Y. (2006). Production and partial characterization of bacteriocin-like pepitdes by *Bacillus licheniformis* ZJU12. *Microbiological Research*, *161*(4), 321-326.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., & Götz, F. (1996). Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Molecular Microbiology*, *20*(5), 1083-1091.

Hendrickson, C. L., & Emmett, M. R. (1999). Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Annual Review of Physical Chemistry*, *50*(1), 517-536.

Hernández, D., Cardell, E., & Zarate, V. (2005). Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *Journal of Applied Microbiology*, *99*(1), 77-84.

Hillenkamp, F., Karas, M., Beavis, R. C., & Chait, B. T. (1991). Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Analytical Chemistry*, *63*(24), 1193A-1203A.

Hiramatsu, K. (1995). Molecular evolution of MRSA. *Microbiology and Immunology*, *39*(8), 531-543.

Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y., & Kobayashi, I. (1997). Dissemination in Japanese hospitals of strains of

Staphylococcus aureus heterogeneously resistant to vancomycin. *The Lancet*, *350*(9092), 1670-1673.

Holt, J. G. K., Sneath, N. R., Staley, P. H., Williams, J. T., & Stanley, T. (1994). *Bergey's Manual of Determinative Bacteriology* (No. QR81 S6 1994).

Holzwarth, G., & Doty, P. (1965). The ultraviolet circular dichroism of polypeptides1. *Journal of the American Chemical Society*, *87*(2), 218-228.

Huang, T., Geng, H., Miyyapuram, V. R., Sit, C. S., Vederas, J. C., & Nakano, M. M. (2009). Isolation of a variant of subtilosin A with hemolytic activity. *Journal of Bacteriology*, *191*(18), 5690-5696.

HYUN-DONG, P., Lee, N. K., Lee, K. H., Hwang, Y. I., & Pan, J. G. (2000). Identification and partial characterization of cerein BS229, a bacteriocin produced by *Bacillus cereus* BS229. *Journal of Microbiology and Biotechnology*, *10*(2), 195-200.

Ibrahim, E. H., Sherman, G., Ward, S., Fraser, V. J., & Kollef, M. H. (2000). The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. *Chest Journal*, *118*(1), 146-155.

Ito, T., & Hiramatsu, K. (1998). Acquisition of methicillin resistance and progression of multiantibiotic resistance in methicillin-resistant *Staphylococcus aureus*. *Yonsei Medical Journal*, *39*(6), 526-533.

Jack, R. W., Tagg, J. R., & Ray, B. (1995). Bacteriocins of Gram-positive bacteria. *Microbiological Reviews*, *59*(2), 171-200.

Jansen, E. F., & Hirschmann, D. J. (1944). Subtilin, an antibacterial product of *Bacillus subtilis*: culturing conditions and properties. *Archives of Biochemistry*, *4*(297), 37-58.

Jenssen, H., Hamill, P., & Hancock, R. E. (2006). Peptide antimicrobial agents. *Clinical Microbiology Reviews*, *19*(3), 491-511.

Jevons, M. P. (1961). "Celbenin"-resistant *staphylococci*. *British Medical Journal*, *1*(5219), 124.

Jevons, M. P., & Parker, M. T. (1964). The evolution of new hospital strains of *Staphylococcus aureus*. *Journal of Clinical Pathology*, *17*(3), 243-250.

Jeyanthi, V., & Velusamy, P. (2016). Anti-methicillin resistant *Staphylococcus aureus* compound isolation from halophilic *Bacillus amyloliquefaciens* MHB1 and determination of its mode of action using electron microscope and flow cytometry analysis. *Indian Journal of Microbiology*, *56*(2), 148-157.

Jin, Y., Hammer, J., Pate, M., Zhang, Y., Zhu, F., Zmuda, E., & Blazyk, J. (2005). Antimicrobial activities and structures of two linear cationic peptide families with various amphipathic β-sheet and α-helical potentials. *Antimicrobial Agents and Chemotherapy*, *49*(12), 4957-4964.

Jung, G., & Sahl, H. G. (1991). Lantibiotics: a survey. *Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands*, 1-34.

Jurinke, C., Oeth, P., & van den Boom, D. (2004). MALDI-TOF mass spectrometry. *Molecular Biotechnology*, *26*(2), 147-163.

Kallen, A. J., Brunkard, J., Moore, Z., Budge, P., Arnold, K. E., Fosheim, G., Finelli, L., Beekmann, S.E., Polgreen, P.M., Gorwitz, R., & Hageman, J. (2009). *Staphylococcus aureus* community-acquired pneumonia during the 2006 to 2007 influenza season. *Annals of Emergency Medicine*, *53*(3), 358-365.

Kamoun, F., Mejdoub, H., Aouissaoui, H., Reinbolt, J., Hammami, A., & Jaoua, S. (2005). Purification, amino acid sequence and characterization of Bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis*. *Journal of Applied Microbiology*, *98*(4), 881-888.

Kashmiri, S. V. S., & Hotchkiss, R. D. (1975). Evidence of tandem duplication of genes in a merodiploid region of pneumococcal mutants resistant to sulfonamide. *Genetics*, *81*(1), 21-31.

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., & Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature*, *474*(7351), 327-336.

Kawulka, K., Sprules, T., McKay, R. T., Mercier, P., Diaper, C. M., Zuber, P., & Vederas, J. C. (2003). Structure of Subtilosin A, an Antimicrobial Peptide from

Bacillus subtilis with Unusual Posttranslational Modifications Linking Cysteine Sulfurs to α-Carbons of Phenylalanine and Threonine. *Journal of the American Chemical Society*, *125*(16), 4726-4727.

Kayalvizhi, N., & Gunasekaran, P. (2010). Purification and characterization of a novel broad-spectrum bacteriocin from *Bacillus licheniformis* MKU3. *Biotechnology and Bioprocess Engineering*, *15*(2), 365-370.

Kazakova, S. V., Hageman, J. C., Matava, M., Srinivasan, A., Phelan, L., Garfinkel, B., Boo, T., McAllister, S., Anderson, J., Jensen, B., & Dodson, D. (2005). A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. *New England Journal of Medicine*, *352*(5), 468-475.

Kern, W. V. (2006). Daptomycin: first in a new class of antibiotics for complicated skin and soft-tissue infections. *International Journal of Clinical Practice*, *60*(3), 370-378.

Kitajima, Y., Waki, M., Shoji, J. I., Ueno, T., & Izumiya, N. (1990). Revised structure of the peptide lactone antibiotic, TL-119 and/or A-3302-B. *FEBS Letters*, *270*(1-2), 139-142.

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie*, *70*(3), 337-349.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, *12*(1-3), 39-85.

Kleinert, F., Kallies, R., Hort, M., Zweynert, A., Szekat, C., Nagel, M., & Bierbaum, G. (2017). Influence of IS256 on genome variability and formation of SCV in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, AAC-00144.

Kloos, W. E., & Bannerman, T. L. (1994). Update on clinical significance of coagulase-negative staphylococci. *Clinical Microbiology Reviews*, *7*(1), 117-140.

Kluytmans, J., Van Belkum, A., & Verbrugh, H. (1997). Nasal carriage of *Staphylococcus aureus:* epidemiology, underlying mechanisms, and associated risks. *Clinical Microbiology Reviews*, *10*(3), 505-520.

König, B., Prevost, G., Piemont, Y., & König, W. (1995). Effects of *Staphylococcus aureus* leukocidins on inflammatory mediator release from human granulocytes. *Journal of Infectious Diseases*, *171*(3), 607-613.

Kozitskaya, S., Cho, S. H., Dietrich, K., Marre, R., Naber, K., & Ziebuhr, W. (2004). The bacterial insertion sequence element IS256 occurs preferentially in nosocomial *Staphylococcus epidermidis* isolates: association with biofilm formation and resistance to aminoglycosides. *Infection and Immunity*, *72*(2), 1210-1215.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G. O., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., & Borriss, R. (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature*, *390*(6657), 249-256.

Kyte, J., & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*, *157*(1), 105-132.

Ladokhin, A. S., & White, S. H. (2001). 'Detergent-like' permeabilization of anionic lipid vesicles by melittin. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1514*(2), 253-260.

Laemmli, U. K., & Favre, M. (1973). Maturation of the head of bacteriophage T4: I. DNA packaging events. *Journal of Molecular Biology*, *80*(4), 575IN1593-592IN4599.

Lam, K. S. (2007). New aspects of natural products in drug discovery. *Trends in Microbiology*, *15*(6), 279-289.

Lawton, E. M., Cotter, P. D., Hill, C., & Ross, R. P. (2007). Identification of a novel two-peptide lantibiotic, haloduracin, produced by the alkaliphile *Bacillus halodurans* C-125. *FEMS Microbiology Letters*, *267*(1), 64-71.

Lecky, D. M., McNulty, C. A., Adriaenssens, N., Herotová, T. K., Holt, J., Kostkova, P., Merakou, K., Koncan, R., Olczak-Pienkowska, A., Avô, A.B., & Campos, J. (2011). Development of an educational resource on microbes, hygiene and prudent antibiotic use for junior and senior school children. *Journal of Antimicrobial Chemotherapy*, *66*(suppl 5), v23-v31.

Lee, K. H., Jun, K. D., Kim, W. S., & Paik, H. D. (2001). Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. *Letters in Applied Microbiology*, *32*(3), 146-151.

Lee, N. K., Yeo, I. C., Park, J. W., & Hahm, Y. T. (2011). Growth inhibition and induction of stress protein, GroEL, of *Bacillus cereus* exposed to antibacterial peptide isolated from *Bacillus subtilis* SC-8. *Applied Biochemistry and Biotechnology*, *165*(1), 235-242.

Lee, N. K., Yeo, I. C., Park, J. W., Kang, B. S., & Hahm, Y. T. (2010). Isolation and characterization of a novel analyte from *Bacillus subtilis* SC-8 antagonistic to *Bacillus cereus*. *Journal of Bioscience and Bioengineering*, *110*(3), 298-303.

Li, G., Liu, B., Shang, Y., Yu, Z., & Zhang, R. (2012). Novel activity evaluation and subsequent partial purification of antimicrobial peptides produced by *Bacillus subtilis* LFB112. *Annals of Microbiology*, *62*(2), 667-674.

Li, M., Diep, B. A., Villaruz, A. E., Braughton, K. R., Jiang, X., DeLeo, F. R., Chambers, H.F., Lu, Y., & Otto, M. (2009). Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences*, *106*(14), 5883-5888.

Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S., & Vester, B. (2006). The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. *Antimicrobial Agents and Chemotherapy*, *50*(7), 2500-2505.

Lowy, F. D. (1998). *Staphylococcus aureus* infections. *New England Journal of Medicine*, *339*(8), 520-532.

Lutz, G., Chavarría, M., Arias, M. L., & Mata-Segreda, J. F. (2006). Microbial degradation of palm (*Elaeis guineensis*) biodiesel. *Revista de Biología Tropical*, *54*(1), 59-63.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., & Laufs, R. (1996). The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1, 6-linked glucosaminoglycan: purification and structural analysis. *Journal of Bacteriology*, *178*(1), 175-183.

Marahiel, M. A. (2009). Working outside the protein-synthesis rules: insights into non-ribosomal peptide synthesis. *Journal of Peptide Science*, *15*(12), 799-807.

Martin, E., Ganz, T., & Lehrer, R. I. (1995). Defensins and other endogenous peptide antibiotics of vertebrates. *Journal of Leukocyte Biology*, *58*(2), 128-136.

Marx, R., Stein, T., Entian, K. D., & Glaser, S. J. (2001). Structure of the *Bacillus subtilis* peptide antibiotic subtilosin A determined by 1H-NMR and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Protein Chemistry*, *20*(6), 501-506.

Mataraci, E., & Dosler, S. (2012). In vitro activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant *Staphylococcus aureus* biofilms. *Antimicrobial Agents and Chemotherapy*, *56*(12), 6366-6371.

Matsuzaki, K. (1999). Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1462*(1), 1-10.

Matsuzaki, K., Murase, O., Fujii, N., & Miyajima, K. (1996). An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry*, *35*(35), 11361-11368.

Mattick, A. T. R., Hirsch, A., & Berridge, N. J. (1947). Further observations on an inhibitory substance (nisin) from lactic *streptococci*. *The Lancet*, *250*(6462), 5-8.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nature Reviews Microbiology*, *4*(8), 608-620.

McAuliffe, O., Ross, R. P., & Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*(3), 285-308.

Meyer, F., Girardot, R., Piémont, Y., Prévost, G., & Colin, D. A. (2009). Analysis of the specificity of Panton-Valentine leucocidin and gamma-hemolysin F component binding. *Infection and Immunity*, *77*(1), 266-273.

Misiek, M., Fardig, O. B., Gourevitch, A., Johnson, D. L., Hooper, I. R., & Lein, J. (1956). Telomycin, a new antibiotic. *Antibiotics Annual*, *5*, 852-855.

Mohamed, M. F., Abdelkhalek, A., & Seleem, M. N. (2016). Evaluation of short synthetic antimicrobial peptides for treatment of drug-resistant and intracellular *Staphylococcus aureus*. *Scientific Reports*, *6*, 29707.

Mohamed, M. F., Hammac, G. K., Guptill, L., & Seleem, M. N. (2014). Antibacterial activity of novel cationic peptides against clinical isolates of multi-drug resistant *Staphylococcus pseudintermedius* from infected dogs. *PloS One*, *9*(12), e116259.

Montanaro, L., Campoccia, D., Pirini, V., Ravaioli, S., Otto, M. & Arciola, C. R. (2007), Antibiotic multiresistance strictly associated with IS*256* and *ica* genes in *Staphylococcus epidermidis* strains from implant orthopedic infections. *Journal of Biomedical Materials Research*, 83A: 813–818.

Moran, G. J., Krishnadasan, A., Gorwitz, R. J., Fosheim, G. E., McDougal, L. K., Carey, R. B., & Talan, D. A. (2006). Methicillin-resistant *S. aureus* infections among patients in the emergency department. *New England Journal of Medicine*, *355*(7), 666-674.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1-2), 55-63.

Motta, A. S., Flores, F. S., Souto, A. A., & Brandelli, A. (2008). Antibacterial activity of a bacteriocin-like substance produced by *Bacillus* sp. P34 that targets the bacterial cell envelope. *Antonie van Leeuwenhoek*, *93*(3), 275-284.

Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., & Watanabe, S. (1991) Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. *Journal of Clinical Microbiology*, 29:2240–4.

Naclerio, G., Ricca, E., Sacco, M., & De Felice, M. (1993). Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. *Applied and Environmental Microbiology*, *59*(12), 4313-4316.

Nakagawa, Y., Nakazawa, T., & SHOJ, J. I. (1975). On the structure of a new antibiotic TL-119. Studies on antibiotics from the genus *Bacillus*. VI. *The Journal of Antibiotics*, *28*(12), 1004-1005.

Nakano, M. M., & Zuber, P. (1990). Molecular biology of antibiotic production in *Bacillus*. *Critical Reviews in Biotechnology*, *10*(3), 223-240.

Nes, I. F., Yoon, S., & Diep, D. B. (2007). Ribosomally synthesiszed antimicrobial peptides (bacteriocins) in lactic acid bacteria: a review. *Food Science and Biotechnology*, *16*(5), 675.

Neumann, B., Pospiech, A., & Schairer, H. U. (1992). Rapid isolation of genomic DNA from gram-negative bacteria. *Trends in Genetics*, *8*(10), 332-333.

Nicholson, W. L. (2002). Roles of *Bacillus* endospores in the environment. *Cellular and Molecular Life Sciences*, *59*(3), 410-416.

Norman, A., Hansen, L. H., & Sørensen, S. J. (2009). Conjugative plasmids: vessels of the communal gene pool. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *364*(1527), 2275-2289.

O'Neill, A. J., & Chopra, I. (2004). Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opinion on Investigational Drugs*, *13*(8), 1045-1063.

O'sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5), 593-604.

Oren, Z., & Shai, Y. (1998). Mode of action of linear amphipathic α-helical antimicrobial peptides. *Peptide Science*, *47*(6), 451-463.

Oscáriz, J. C., & Pisabarro, A. G. (2000). Characterization and mechanism of action of cerein 7, a bacteriocin produced by *Bacillus cereus* Bc7. *Journal of Applied Microbiology*, *89*(2), 361-369.

Oscáriz, J. C., Lasa, I., & Pisabarro, A. G. (1999). Detection and characterization of cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. *FEMS Microbiology Letters*, *178*(2), 337-341.

Pacheco-Cano, R. D., Norma, M., Salcedo-Hernández, R., León-Galván, M. F., Bideshi, D. K., Hernández-Guzmán, G., & Barboza-Corona, J. E. (2014). Characterization, N-terminal sequencing and classification of Tolworthcin 524: a bacteriocin

produced by *Bacillus thuringiensis* subsp. *tolworthi*. *Microbiological Research*, *169*(12), 948-953.

Paik, H. D., Bae, S. S., Park, S. H., & Pan, J. G. (1997). Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp *tochigiensis*. *Journal of Industrial Microbiology & Biotechnology*, *19*(4), 294-298.

Paik, S. H., Chakicherla, A., & Hansen, J. N. (1998). Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *Journal of Biological Chemistry*, *273*(36), 23134-23142.

Paik, S. H., Chakicherla, A., & Hansen, J. N. (1998). Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *Journal of Biological Chemistry*, *273*(36), 23134-23142.

Pálffy, R., Gardlík, R., Behuliak, M., Kadasi, L., Turna, J., & Celec, P. (2009). On the physiology and pathophysiology of antimicrobial peptides. *Moecularl Medicine*, *15*(1-2), 51-9.

Pallecchi, L., Lucchetti, C., Bartoloni, A., Bartalesi, F., Mantella, A., Gamboa, H., Carattoli, A., Paradisi, F., & Rossolini, G. M. (2007). Population structure and resistance genes in antibiotic-resistant bacteria from a remote community with minimal antibiotic exposure. *Antimicrobial Agents and Chemotherapy*, *51*(4), 1179-1184.

Pallecchi, L., Riccobono, E., Mantella, A., Bartalesi, F., Sennati, S., Gamboa, H., Gotuzzo, E., Bartoloni, A., & Rossolini, G. M. (2009). High prevalence of qnr genes in commensal enterobacteria from healthy children in Peru and Bolivia. *Antimicrobial Agents and Chemotherapy*, *53*(6), 2632-2635.

Parisien, A., Allain, B., Zhang, J., Mandeville, R., & Lan, C. Q. (2008). Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *Journal of Applied Microbiology*, *104*(1), 1-13.

Payne, D. J., Gwynn, M. N., Holmes, D. J., & Pompliano, D. L. (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews Drug Discovery*, *6*(1), 29-40.

Peacock, J. E., Marsik, F. J., & Wenzel, R. P. (1980). Methicillin-resistant *Staphylococcus aureus*: introduction and spread within a hospital. *Annals of Internal Medicine*, *93*(4), 526-532.

Pedersen, P. B., Bjørnvad, M. E., Rasmussen, M. D., & Petersen, J. N. (2002). Cytotoxic potential of industrial strains of *Bacillus* sp. *Regulatory Toxicology and Pharmacology*, *36*(2), 155-161.

Peschel, A. (2002). How do bacteria resist human antimicrobial peptides? *Trends in Microbiology*, *10*(4), 179-186.

Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W.F., Jung, G., Tarkowski, A., & Van Kessel, K. P. (2001). *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *Journal of Experimental Medicine*, *193*(9), 1067-1076.

Petrelli, D., Zampaloni, C., D'ercole, S., Prenna, M., Ballarini, P., Ripa, S., & Vitali, L. A. (2006). Analysis of different genetic traits and their association with biofilm formation in *Staphylococcus epidermidis* isolates from central venous catheter infections. *European Journal of Clinical Microbiology and Infectious Diseases*, *25*(12), 773-781.

Peypoux, F., Bonmatin, J. M., & Wallach, J. (1999). Recent trends in the biochemistry of surfactin. *Applied Microbiology and Biotechnology*, *51*(5), 553-563.

Pincus, D. H. (2006). Microbial identification using the bioMerieux VITEK® 2 System. *Encyclopedia of Rapid Microbiological Methods. Bethesda, MD: Parenteral Drug Association*.

Policy, I. P. (2010). The 10x20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clinical Infectious Diseases*, *50*, 1081-1083.

Pouny, Y., Rapaport, D., Mor, A., Nicolas, P., & Shai, Y. (1992). Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. *Biochemistry*, *31*(49), 12416-12423.

Powers, J. P. S., & Hancock, R. E. (2003). The relationship between peptide structure and antibacterial activity. *Peptides*, *24*(11), 1681-1691.

Predari, S. C., Ligozzi, M., & Fontana, R. (1991). Genotypic identification of methicillin-resistant coagulase-negative staphylococci by polymerase chain reaction. *Antimicrobial Agents and Chemotherapy*, *35*(12), 2568-2573.

Priest, F. G. (1993). Systematics and ecology of Bacillus. In *Bacillus subtilis and other Gram-positive bacteria* (pp. 3-16). *American Society of Microbiology*.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J., & Ziebuhr, W. (2000). Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy*, *44*(12), 3357-3363.

Ramachandran, R., Chalasani, A. G., Lal, R., & Roy, U. (2014). A broad-spectrum antimicrobial activity of *Bacillus subtilis* RLID 12.1. *The Scientific World Journal*, *2014*.

Rautenbach, M., Vlok, N. M., Stander, M., & Hoppe, H. C. (2007). Inhibition of malaria parasite blood stages by tyrocidines, membrane-active cyclic peptide antibiotics from *Bacillus brevis*. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1768*(6), 1488-1497.

Rea, M. C., Ross, R. P., Cotter, P. D., & Hill, C. (2011). Classification of bacteriocins from Gram-positive bacteria. In *Prokaryotic antimicrobial peptides* (pp. 29-53). Springer New York.

REFERENCES:

Riley, M. A. (1998). Molecular mechanisms of bacteriocin evolution. *Annual Review of Genetics*, *32*(1), 255-278.

Riley, M. A., & Wertz, J. E. (2002a). Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie*, *84*(5), 357-364.

Riley, M. A., & Wertz, J. E. (2002b). Bacteriocins: evolution, ecology, and application. *Annual Reviews in Microbiology*, *56*(1), 117-137.

Roberts, M. C. (2008). Update on macrolide–lincosamide–streptogramin, ketolide, and oxazolidinone resistance genes. *FEMS Microbiology Letters*, *282*(2), 147-159.

Roberts, R. R., Hota, B., Ahmad, I., Scott, R. D., Foster, S. D., Abbasi, F., Schabowski, S., Kampe, L.M., Ciavarella, G.G., Supino, M., & Naples, J. (2009). Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. *Clinical Infectious Diseases*, *49*(8), 1175-1184.

Rotem, S., & Mor, A. (2009). Antimicrobial peptide mimics for improved therapeutic properties. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1788*(8), 1582-1592.

Rozek, A., Friedrich, C. L., & Hancock, R. E. (2000). Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. *Biochemistry*, *39*(51), 15765-15774.

Sabaté, D. C., & Audisio, M. C. (2013). Inhibitory activity of surfactin, produced by different *Bacillus subtilis* subsp. *subtilis* strains, against *Listeria monocytogenes* sensitive and bacteriocin-resistant strains. *Microbiological Research*, *168*(3), 125-129.

Saier, M. H., Goldman, S. R., Maile, R. R., Moreno, M. S., Weyler, W., Yang, N., & Paulsen, I. T. (2002). Overall transport capabilities of *Bacillus subtilis*. In *Bacillus subtilis and Its Closest Relatives* (pp. 113-128). American Society of Microbiology.

Saleem, F., Ahmad, S., Yaqoob, Z., & Rasool, S. A. (2009). Comparative study of two bacteriocins produced by representative indigenous soil bacteria. *Pakistan Journal of Pharmaceutical Sciences*, *22*(3).

Salle, A. J., & Jann, G. J. (1945). Subtilin-An Antibiotic Produced by *Bacillus subtilis.* I. Action on Various Organisms. *Proceedings of the Society for Experimental Biology and Medicine*, *60*(1), 60-64.

Sandiford, S., & Upton, M. (2012). Identification, characterization, and recombinant expression of epidermicin NI01, a novel unmodified bacteriocin produced by *Staphylococcus epidermidis* that displays potent activity against staphylococci. *Antimicrobial Agents and Chemotherapy*, *56*(3), 1539-1547.

Sang, Y., & Blecha, F. (2008). Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. *Animal Health Research Reviews*, *9*(02), 227-235.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology*, *50*(1), 1-17.

Schlüter, A., Krause, L., Szczepanowski, R., Goesmann, A., & Pühler, A. (2008). Genetic diversity and composition of a plasmid metagenome from a waste water treatment plant. *Journal of Biotechnology*, *136*(1), 65-76.

Shai, Y. (1999). Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1462*(1), 55-70.

Shekh, R. M., & Roy, U. (2012). Biochemical characterization of an anti-Candida factor produced by *Enterococcus faecalis*. *BMC Microbiology*, *12*(1), 132.

Shelburne, C. E., An, F. Y., Dholpe, V., Ramamoorthy, A., Lopatin, D. E., & Lantz, M. S. (2007). The spectrum of antimicrobial activity of the bacteriocin subtilosin A. *Journal of Antimicrobial Chemotherapy*, *59*(2), 297-300.

Shoemaker, N. B., Vlamakis, H., Hayes, K., & Salyers, A. A. (2001). Evidence for Extensive Resistance Gene Transfer among *Bacteroides* spp. and among *Bacteroides* and Other Genera in the Human Colon. *Applied and Environmental Microbiology*, *67*(2), 561-568.

Shorr, A. F., Kunkel, M. J., & Kollef, M. (2005). Linezolid versus vancomycin for *Staphylococcus aureus* bacteraemia: pooled analysis of randomized studies. *Journal of Antimicrobial Chemotherapy*, *56*(5), 923-929.

Sieber, S. A., & Marahiel, M. A. (2003). Learning from nature's drug factories: nonribosomal synthesis of macrocyclic peptides. *Journal of Bacteriology*, *185*(24), 7036-7043.

Silver, L. L. (2011). Challenges of antibacterial discovery. *Clinical Microbiology Reviews*, *24*(1), 71-109.

Slepecky, R. A., & Hemphill, H. E. (2006). The genus *Bacillus*—nonmedical. In *The Prokaryotes* (pp. 530-562). Springer US.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M., Fujimoto, E.K., Goeke, N.M., Olson, B.J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*(1), 76-85.

Sommer, M. O., Dantas, G., & Church, G. M. (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science*, *325*(5944), 1128-1131.

Sørensen, S. J., Bailey, M., Hansen, L. H., Kroer, N., & Wuertz, S. (2005). Studying plasmid horizontal transfer *in situ*: a critical review. *Nature Reviews Microbiology*, *3*(9), 700-710.

Sreerama, N., Venyaminov, S. Y., & Woody, R. W. (2000). Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis. *Analytical Biochemistry*, *287*(2), 243-251.

Stachelhaus, T., & Marahiel, M. A. (1995). Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. *FEMS Microbiology Letters*, *125*(1), 3-14.

Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, *44*(4), 846-849.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H.G., & Vauterin, L. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, *52*(3), 1043-1047.

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology*, *56*(4), 845-857.

Steinkraus, G., White, R., & Friedrich, L. (2007). Vancomycin MIC creep in non-vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-susceptible clinical methicillin-resistant *S. aureus* (MRSA) blood isolates from 2001–05. *Journal of Antimicrobial Chemotherapy*, *60*(4), 788-794.

Sutyak, K. E., Wirawan, R. E., Aroutcheva, A. A., & Chikindas, M. L. (2008). Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. *Journal of Applied Microbiology*, *104*(4), 1067-1074.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gützkow, T., Eichler, W., Pühler, A., & Schlüter, A. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology*, *155*(7), 2306-2319.

Tabbene, O., Slimene, I. B., Bouabdallah, F., Mangoni, M. L., Urdaci, M. C., & Limam, F. (2009). Production of anti-methicillin-resistant *Staphylococcus* activity from *Bacillus subtilis* sp. strain B38 newly isolated from soil. *Applied Biochemistry and Biotechnology*, *157*(3), 407-419.

Tagg, J. R., Dajani, A. S., & Wannamaker, L. W. (1976). Bacteriocins of Gram-positive bacteria. *Bacteriological Reviews*, *40*(3), 722.

Tally, F. P., & DeBruin, M. F. (2000). Development of daptomycin for Gram-positive infections. *Journal of Antimicrobial Chemotherapy*, *46*(4), 523-526.

Tamae, C., Liu, A., Kim, K., Sitz, D., Hong, J., Becket, E., Bui, A., Solaimani, P., Tran, K.P., Yang, H., & Miller, J. H. (2008). Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*. *Journal of Bacteriology*, *190*(17), 5981-5988.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood,

evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*(10), 2731-2739.

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *The American Journal of Medicine*, *119*(6), S3-S10.

Thundimadathil, J., Roeske, R. W., & Guo, L. (2006). Effect of membrane mimicking environment on the conformation of a pore-forming (xSxG) 6 peptide. *Peptide Science*, *84*(3), 317-328.

Torres, M. J., Petroselli, G., Daz, M., Erra-Balsells, R., & Audisio, M. C. (2015). *Bacillus subtilis* subsp. *subtilis* CBMDC3f with antimicrobial activity against Gram-positive foodborne pathogenic bacteria: UV-MALDI-TOF MS analysis of its bioactive compounds. *World Journal of Microbiology and Biotechnology*, *31*(6), 929-940.

Torres, N. I., Noll, K. S., Xu, S., Li, J., Huang, Q., Sinko, P. J., Wachsman, M.B., & Chikindas, M. L. (2013). Safety, formulation and in vitro antiviral activity of the antimicrobial peptide subtilosin against *Herpes simplex* virus type 1. *Probiotics and Antimicrobial Proteins*, *5*(1), 26-35.

Udo, E. E., Pearman, J. W., & Grubb, W. B. (1993). Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. *Journal of Hospital Infection*, *25*(2), 97-108.

Velluz, L., Le Grand, M., & Grosjean, M. (1965). Optical circular dichroism: principles, measurements, and applications. Verlag Chemie.

Venyaminov, S. Y., Baikalov, I. A., Shen, Z. M., Wu, C. S. C., & Yang, J. T. (1993). Circular dichroic analysis of denatured proteins: inclusion of denatured proteins in the reference set. *Analytical Biochemistry*, *214*(1), 17-24.

Von Tersch, M. A., & Carlton, B. C. (1983). Bacteriocin from *Bacillus megaterium* ATCC 19213: comparative studies with megacin A-216. *Journal of Bacteriology*, *155*(2), 866-871.

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., Long, R.D., Dorward, D.W., Gardner, D.J., Kreiswirth, B.N., & Musser, J. M. (2005). Insights into mechanisms used by *Staphylococcus aureus* to

avoid destruction by human neutrophils. *The Journal of Immunology*, *175*(6), 3907-3919.

Walsh, C. (2003). Where will new antibiotics come from? *Nature Reviews Microbiology*, *1*(1), 65-70.

Wang, G., Hindler, J. F., Ward, K. W., & Bruckner, D. A. (2006). Increased vancomycin MICs for *Staphylococcus aureus* clinical isolates from a university hospital during a 5-year period. *Journal of Clinical Microbiology*, *44*(11), 3883-3886.

Wang, G., Manns, D. C., Churey, J. J., & Worobo, R. W. (2014a). Development of a homologous expression system for and systematic site-directed mutagenesis analysis of thurincin H, a bacteriocin produced by *Bacillus thuringiensis* SF361. *Applied and Environmental Microbiology*, *80*(12), 3576-3584.

Wang, J., Zhang, L., Teng, K., Sun, S., Sun, Z., & Zhong, J. (2014b). Cerecidins, novel lantibiotics from *Bacillus cereus* with potent antimicrobial activity. *Applied and Environmental Microbiology*, *80*(8), 2633-2643.

Wang, R. F., Cao, W. W., Franklin, W., Campbell, W., & Cerniglia, C. E. (1994). A 16S rDNA-based PCR method for rapid and specific detection of *Clostridium perfringens* in food. *Molecular and Cellular Probes*, *8*(2), 131-137.

Wang, R., Braughton, K. R., Kretschmer, D., Bach, T. H. L., Queck, S. Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A., & DeLeo, F. R. (2007). Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nature Medicine*, *13*(12), 1510-1514.

Wang, Z., & Wang, G. (2004). APD: the antimicrobial peptide database. *Nucleic Acids Research*, *32*(suppl 1), D590-D592.

Wardenburg, J. B., Bae, T., Otto, M., DeLeo, F. R., & Schneewind, O. (2007). Poring over pores: α-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nature Medicine*, *13*(12), 1405-1406.

Weigel, L. M., Clewell, D. B., Gill, S. R., Clark, N. C., McDougal, L. K., Flannagan, S. E., Kolonay, J.F., Shetty, J., Killgore, G.E., & Tenover, F. C. (2003). Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science*, *302*(5650), 1569-1571.

White, D. G., Alekshun, M. N., McDermott, P. F., & Levy, S. B. (2005). Frontiers in antimicrobial resistance: a tribute to Stuart B. *Levy. Washington, DC, American Society for Microbiology*.

Whitehouse, C. M., Dreyer, R. N., Yamashita, M., & Fenn, J. B. (1989). Electrospray ionization for mass-spectrometry of large biomolecules. *Science*, *246*(4926), 64-71.

WHO. (2014). Antimicrobial resistance: global report on surveillance 2014. *World Health Organization*, ISBN: 978 92 4 156474 8.

Wielders, C. L. C., Vriens, M. R., Brisse, S., de Graaf-Miltenburg, L. A. M., Troelstra, A., Fleer, A., Schmitz, F.J., Verhoef, J., & Fluit, A. C. (2001). Evidence for in-vivo transfer of mecA DNA between strains of *Staphylococcus aureus*. *The Lancet*, *357*(9269), 1674-1675.

Willey, J. M., & Van Der Donk, W. A. (2007). Lantibiotics: peptides of diverse structure and function. *Annual Review of Microbiology*, *61*, 477-501.

Williams, E. R. (1998). Tandem FTMS of Large Biomolecules. *Analytical Chemistry*, *70*(5), 179A-185A.

Wimley, W. C., Hristova, K., Ladokhin, A. S., Silvestro, L., Axelsen, P. H., & White, S. H. (1998). Folding of β-sheet membrane proteins: a hydrophobic hexapeptide model. *Journal of Molecular Biology*, *277*(5), 1091-1110.

Wolcott, R. D., Rhoads, D. D., Bennett, M. E., Wolcott, B. M., Gogokhia, L., Costerton, J. W., & Dowd, S. E. (2010). Chronic wounds and the medical biofilm paradigm. *Journal of Wound Care*, *19*(2), 45-6.

Woodford, N. (2003). Novel agents for the treatment of resistant Gram-positive infections. *Expert Opinion on Investigational Drugs*, *12*(2), 117-137.

Woodin, A. M. (1960). Purification of the two components of leucocidin from *Staphylococcus aureus*. *Biochemical Journal*, *75*(1), 158.

Wu, S., Jia, S., Sun, D., Chen, M., Chen, X., Zhong, J., & Huan, L. (2005). Purification and characterization of two novel antimicrobial peptides subpeptin JM4-A and subpeptin JM4-B produced by *Bacillus subtilis* JM4. *Current Microbiology*, *51*(5), 292-296.

Xie, J., Zhang, R., Shang, C., & Guo, Y. (2009). Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens. *African Journal of Biotechnology*, *8*(20).

Yamamoto, Y., Togawa, Y., Shimosaka, M., & Okazaki, M. (2003). Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. *Applied and Environmental Microbiology*, *69*(10), 5746-5753.

Yang, J. T., Wu, C. S. C., & Martinez, H. M. (1986). Calculation of protein conformation from circular dichroism. *Methods in Enzymology*, *130*, 208-269.

Yang, L., Harroun, T. A., Weiss, T. M., Ding, L., & Huang, H. W. (2001). Barrel-stave model or toroidal model? A case study on melittin pores. *Biophysical Journal*, *81*(3), 1475-1485.

Yeaman, M. R., & Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacological Reviews*, *55*(1), 27-55.

Yeo, I. C., Lee, N. K., Cha, C. J., & Hahm, Y. T. (2011). Narrow antagonistic activity of antimicrobial peptide from *Bacillus subtilis* SCK-2 against *Bacillus cereus*. *Journal of Bioscience and Bioengineering*, *112*(4), 338-344.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, *415*(6870), 389-395.

List of publications in Science Citation Indexed (SCI) journals related to this Ph.D. thesis:

1. **Chalasani Ajay Ghosh**., Sushma Nema, and Utpal Roy (2017). Purification and Characterization of a Novel Anti-Staphylococcal Peptide (ASP-1) from *Bacillus* sp. URID 12.1. ***International Journal of Antimicrobial Agents***. **(*In press*),** doi.org/10.1016/j.ijantimicag.2017.08.030. Impact factor: **4.307**

2. **Chalasani Ajay Ghosh**., Gunaseelan Dhanarajan, Sushma Nema, Ramkrishna Sen, and Utpal Roy (2015). "An Antimicrobial Metabolite from *Bacillus* sp.: Significant Activity Against Pathogenic Bacteria Including Multidrug-Resistant Clinical Strains." ***Frontiers in Microbiology***, 6:1335. doi:10.3389/fmicb.2015.01335. PMCID: PMC4678185. Impact factor: **4.076**

Conferences attended and posters presented:

1. **Ajay Ghosh Chalasani** and Utpal Roy (2016), '*An antimicrobial compound produced by B. subtilis that has the therapeutic potential during the atopic dermatitis in altering the skin microbiome'* in 19th ADNAT Convention, International Symposium on Microbiome in Health and Disease at National Institute of Animal Nutrition and Physiology, Bangalore on February 23-25, 2016.

2. **Ajay Ghosh Chalasani** and Utpal Roy (2013), '*Antimicrobial activity of a wild-type soil Bacillus isolate against gram-positive bacteria*' in 54th Annual Conference of Association of Microbiologists of India, International Symposium on Frontier Discoveries and innovations in Microbiology and its Interdisciplinary Relevance (FDMIR) at Maharshi Dayanand University, Rohtak, Haryana, India on November 17-20, 2013.

Other publication:

1. Roy Utpal, **Ajay Ghosh Chalasani**, and M. Raeesh Shekh (2013) "The anti-Candida activity by Ancillary Proteins of an *Enterococcus faecium* strain." ***Frontiers in Microbiology***, 6:339. doi: 10.3389/fmicb.2015.00339. PMCID: PMC4424852. Impact factor: **4.076**

Poster accepted:

Ajay Ghosh Chalasani, Raeesh Shekh and **Utpal Roy** (2017), 'Anti-*Candida* Activity produced by *Enterococcus faecium* and the relevance of the LysM domain' in 58th Annual Conference of Association of Microbiologists of India (AMI-2017) & International Symposium on "Microbes for Sustainable Development**:** Scopes and Applications" at Babasaheb Bhimrao Ambedkar University, Lucknow, India November 16-19, 2017.

Brief Biography of the Candidate

Name	Mr. Ajay Ghosh Chalasani
Education	M.Sc. (Biotechnology), Jaya College of Arts & Science, Chennai, Tamil Nadu (2009)
	B.Sc. (Biotechnology), Andhra Loyola College, Vijayawada, Andhra Pradesh (2007)
Email	ajayghosh.chalasani@gmail.com

Work Experience:

Mr. Ajay Ghosh Chalasani joined BITS Pilani K K Birla Goa campus as a research scholar in department of Biological Sciences. He has worked as a project assistant in University Grants Commission (UGC) sponsored project entitled "Biochemical and genetic characterization of antimicrobial (Candidacidal) Peptides Produced by the strain *E. faecalis* APR 210" (39-205/2010 (SR)). He has worked in the purification and characterization of an antimicrobial peptide showing activity against gram positive bacteria from a *Bacillus* soil isolate. He has published two research articles as first author and co-author of two other publications. He has presented his work as posters in two conferences. He has worked as Junior Research Associate in Excelra Knowledge Solutions Pvt. Ltd. (formerly GVK Informatics) for a period of 13 months from Dec 2009 to Jan 2011.

Brief Biography of the Supervisor

Dr. Utpal Roy, Ph.D., is Associate Professor of Biological Sciences, former Head and former-DRC Chairman and presently DRC convener at Department of Biological Sciences, BITS-Pilani KK Birla Goa campus obtained his Ph.D. degree in Microbiology from National Dairy Research Institute, ICAR, Karnal. He has been teaching various courses of Microbiology, Molecular Biology and Biotechnology to undergraduate and graduate students for more than 18 years. Most of his research has been on various antimicrobials and their purification and characterization. As a Principle Investigator, he has handled multiple research projects of CSIR, DST, UGC and DBT. He has published forty research papers in international and national journals of repute apart from contributing papers in various national and international symposia. He has also published a book entitled “A Handbook of Genetic Engineering”. He has supervised several Ph. D. students, and he has collaborations in India and abroad.