STUDIES ON THE PRODUCTION OF ANTIMICROBIAL PEPTIDES OF MICROBIAL ORIGIN

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Certificate

The project work entitled "**Studies on the production of antimicrobial peptides of microbial origin**" submitted by **Ramita Taggar** for the degree of **Doctor of Philosophy**, has been carried out under the supervision of **Dr. Debendra Kumar Sahoo** and **Dr. Mani Shankar Bhattacharyya** at the CSIR-Institute of Microbial Technology, Chandigarh.

The research work is novel and has not been either partially or completely submitted for any other degree or diploma from any other institute or university.

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Dedicated to

My Family

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ABBREVIATIONS

°C	Degree Centigrade
ACN	Acetonitrile
AMPs	Antimicrobial Peptides
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATCC	American Type Culture Collection
CCD	Central Composite Design
CFU	Colony Forming Unit
CFW	Calcofluor white M2R (CFW)
CLSI	Clinical and laboratory standards institute
CLSM	Confocal laser scanning microscopy
CV	Crystal violet
Da	Dalton
DDW	Double Distilled Water
Dha	Dehydroalanine
Dhb	Dehdrobutyrine
$DiBAC_4$ (3)	Bis-(1, 3-Dibutylbarbituric Acid) Trimethine Oxonol
$DiSC_3$ (5)	3, 3-dipropylthiacarbocyanine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
EtBr	Ethidium bromide
H_2DCFDA	2', 7'-dichloro-dihydrofluoresceindiacetate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kD	Kilo dalton
m/z	Mass/Charge
MALDI-TOF:	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MIC	Minimum Inhibitory Concentration
MOI	Multiplicity of Infection
MRSA	Methicillin-resistant *Staphylococcus aureus*

MTCC	Microbial Type Culture Collection
MTT:	3-(4,5-dimethylthoazole-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
OD	Optical Density
PAE	Post antibiotic effect
PBD	Placket-Burman Desingn
PBS	Phosphate Buffer Saline
pH	Potentium hydrogenii
PI	Propidium iodide
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase - High-Pressure Liquid Chromatography
rpm	Revolution per minute
RPMI-1640	Roswell Park Memorial Institute
RNA	Ribonucleic Acid
RSM	Response Surface Methodology
SD	Standard deviation
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TEMED	N, N,N',N' tetramethylenediamine
Tricine SDS-PAGE	Tricine-sodiumdodecylsulfate polyacrylamide gel electrophoresis
UV-vis	Ultravoilet visible
VRE	Vancomycin resistant *Enterococci*
XTT	[2,3-bis(2-methoxy-4-nitro-5-sulfophenyl - 2H-tetrazolium-5 carboxynilide sodium salt]
β-Me	β-mercaptoethanol

SYMBOLS

α	Alpha
β	Beta
±	Plus-minus
µ	Micro
≤	Less than or equal to
≥	More than or equal to

UNITS OF MEASUREMENT

%	Percentage
×g	Centrifugal force equal to gravitational force
µg	Microgram
µl	Microlitre
µM	Micromolar
°C	Degrees Celsius
g	Gram(s)
h	Hour(s)
kDa	kilo Dalton
M	Molar
mg	Milligrams
min	Minutes
mL	Milliliter
mM	Millimolar
v/v	Volume/volume
w/v	Weight/volume

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INTRODUCTION

1. INTRODUCTION

In recent years increase in antibiotic resistance has emerged as one of the major threat in the global health scenario. This escalated the need to develop an alternate therapy to effectively and efficiently eradicate these drug-resistant bacteria (Shanks et al., 2012). Therefore, efforts have been directed towards the isolation of potential antimicrobial peptides (AMPs) from microbes isolated from different ecological niches to combat antibiotic-resistant pathogens (Malmsten, 2014). AMPs have the ability to modulate immune response of host, show slower drug resistance with antibiofilm activity and hence, being explored to replace the conventional antibiotics. AMPs show common including small size and linear or cyclic peptide structures, irrespective of their biological origin. Most of the AMPs are cationic (i.e., excess of lysine and arginine residues) and amphipathic in nature and are encoded naturally as "one gene peptide" (Ageitos et al., 2017; Hancock and Rozek, 2002) and kill or inhibits bacteria in the micro-molar concentration (μM), usually by some non-specific mechanism, and thus, it delays resistance development among the pathogenic microbes (Ageitos et al., 2017).

Lantibiotics, a class of antimicrobial peptides called Bacteriocins, exhibits a potential substitute for antibiotics. The members of class I linear lantibiotics are subtilin-like peptides and nisin, which are produced by several strains of *Bacillus subtilis* and *Lactococcus lactis*, respectively (Teng et al., 2012). AMPs also shows intense antimicrobial activity against multi-drug-resistant pathogens like Methicillin-Resistant *S. aureus* (MRSA) and Vancomycin-Resistant *enterococci* (VRE) (Fuchs et al., 2011; Piper et al., 2009). Moreover, the cationic AMP subtilin, produced by bacteria *B. subtilis* (ATCC 6633) has shown antimicrobial activity against Gram-positive bacteria by permeabilizing the cytoplasmic membrane of the target pathogen (Gross et al., 1973; Jack et al., 2013; Kordel et al., 1989). However, the bacteriocins from *Bacillus* species exhibit more powerful inhibitory activity than bacteriocins produced by a lactic-acid producing bacterium (LAB), e.g., enterocins possess bactericidal effect. Bacteriocins from Bacillaceae species such as geobacillin-I exhibits higher stability than nisin at various conditions (Garg et al., 2014; Garg et al., 2012). However, degradation at neutral pH and storage instability are few limitations of nisin (Garg et al., 2014; Sharma et al., 2011). Thus, the bacteriocins from Bacillaceae species can be a potential candidate than bacteriocins isolated from LAB and conventional antibiotics where multidrug resistance has become more prominent nowadays (Islam et al., 2012; van der Donk and Nair, 2014).

Natural sources conceal multiple species of bacteria which compete for nutrients and space. This leads to the synthesis and unceasing evolution of new AMPs. The urgency for novel antibiotics results in revisiting the natural resources from which these AMPs can be isolated. AMP's isolation from food is of great interest since food sources are generally rich in antioxidants, antimicrobial, and flavouring substances. Spices are widely used in many parts of the world because of their aroma and flavour. Few Indian spices are known to have antimicrobial activity. As the bacterial strains present in this niche of food ingredients are expected to have a meagre virulence capability, these sources were screened for isolating AMP producing organisms. Species from the *Allium* genus have attracted special attention, as they are being used for treating cardiovascular diseases, cancer, and other ailments.

Moreover, the father of ayurvedic medicine, Charak, has claimed the importance of garlic and onion in maintaining the fluidity of blood and strengthening the heart (Chutani and Bordia, 1981). *Allium cepa*, the common onion, contains many flavonoids, sulfur compounds, thiosulfinates, and fructooligosaccharides (FOS) (Slimestad et al., 2007). The phenolic content of onion is very high and has been found to have a beneficial effect against degenerative diseases (Griffiths et al., 2002). Previously, Cammue et al. (1995) have reported the isolation of Ace-AMP1 protein from the onion (*Allium cepa*) seeds (Cammue et al., 1995). The AMP showed immense antimicrobial activity against different pathogenic fungi and Gram-positive bacteria. Allicepin from the bulb of *Allium cepa* has also been reported to exhibit potent antifungal activity (Wang and Ng, 2004).

The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) have now become resistant to most antibiotics (Santajit and Indrawattana, 2016). *S. aureus* is a major causative pathogen of bacteremia, osteoarticular, nosocomial, pleuropulmonary, skin and soft tissue infections (SSTIs) in humans (DeLeo et al., 2010; Frank et al., 2010). Worldwide, *S. auresus* causes nearly 50% infections. The MRSA strains constitute a significant cause of mortality and morbidity has attracted greater medical attention. Thus, the resistance of this pathogen against various antibiotics has been developed (Guillamet and Kollef 2016; Mendoza and Tyring 2010) and poses a significant problem in *S. aureus* infections (Howlin et al., 2015; Koch et al., 2014). Moreover, chronic infections are also linked to the manner of biofilm growth of *S. aureus*, and is involved in the reoccurrence of many diseases linked to host tissues like bone and heart valves and causes diseases like osteomyelitis and endocarditis, and also from the biofilm on implanted devices like

pacemakers, prosthetic joints, etc. (Barrett and Atkins, 2014; Chatterjee et al., 2014; Kiedrowski and Horswill, 2011; Parsek and Singh, 2003).

Generally, *S. aureus* is considered an extracellular organism and survive within the phagocytic and non-phagocytic cells and are responsible for the frequent and relapsing nature of infection (Lemaire et al., 2007). The intracellular accumulation of bacteria complicates drug usage (Brinch et al., 2009); for example, oxacillin, moxifloxacin, and levofloxacin, etc., shows low intracellular penetration. Linezolid and gentamicin are affected by low cellular level of accumulation and are partially and inconsistently active, the acidic environment due to aminoglycosides and intra-lysosomal constituents binding (Van der Auwera et al., 1991). Furthermore, some antibiotics are used at a higher extracellular concentration to get a significant activity, which further causes drug resistance and side effects (Wang et al., 2018).

Various reports describe the potency of antimicrobial peptides that halts the of *S. aureus* growth. For instance, epidermin and aureocin inhibit the *S. aureus* strains in bovine mastitis. However, the combination therapy of aureocin 70 and A53 showed increased inhibitory potential against *S. aureus* strains (Varella Coelho et al., 2017). The tenth-century old remedy; Bald's Leechbook, a mixture of *Allium sativum* and *Allium cropleac*, has been reported recently to treat a clinical strain *S. aureus* in planktonic culture and its biofilm (Furner-Pardoe et al., 2020). There are large numbers of reports on work being carried out to curb the problem of drug resistance developed in *S. aureus* in response to vast arrays of antibiotics. Surprisingly, AMPs from food sources have shown promising efficacy in treating many infections. Considering the above reports, we planned our study for screening and isolation of an AMP from a food source (*Allium cepa*), purifying and characterizing and checking its antibacterial efficacy against the various *S. aureus* strains. Accordingly, the following objectives were framed.

- Screening of AMPs from potential isolates.
- Purification and characterization of AMPs from selected isolates.
- Exploration of the characterized AMP in food/pharmaceutical applications.
- Optimization of media components and cultural conditions for enhancing the production of characterized AMPs.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Antimicrobial peptides

Innate immunity system of almost all living organisms provides protection against microbial pathogens and has been reported about 2.6 billion years ago (Gordon et al., 2005). Production of natural antibiotic peptides of natural origin has emerged as an essential factor of innate immunity in plants and animals; also, the arsenal of the antimicrobial peptide is different from one species to another (Ganz, 2003a).

These are the short molecules produced by most living organisms which help them in competing with others for nutrients and biological habitat (Ageitos et al., 2017). This lead to the synthesis of more evolved AMPs with higher antimicrobial properties (Tobias et al., 2017). Moreover, AMPs are a part of multicellular organisms' immune systems and represent a biological weapon that evolved over millions of years due to an escalating arms race for survival among living organisms (Ageitos et al., 2017). Small cationic peptides are one of the important constituent of innate immune system with antimicrobial potential against bacteria, fungi, parasites, and few viruses (Brogden et al., 2003; Hancock and Lehrer, 1998).

Antimicrobial peptides have common characteristics, such as hydrophobic, cationic sequences within cyclic or linear structures, and small size (Boman, 1995a). These are short peptides encoded naturally as ‘one gene peptides’ and are well known from the past few decades (Hancock and Rozek, 2002). The ribosomally synthesized peptides are produced by all life forms, including bacteria, consisting of various post-translational modifications and different structural and functional diversities. The peptides which are synthesized non-ribosomally are mainly produced by bacteria and modified drastically, e.g., gramicidins, polymyxins, glycopeptides, etc. (Hancock and Chapple, 1999; Tajbakhsh et al., 2017). Till date, >5000 AMPs have been reported, and most of them are naturally found in both prokaryotes (bacteria) and eukaryotes (fungi, plants, protozoans, insects, and animals), and some are also synthesized (Leippe, 1999; Peters et al., 2010; Radek and Gallo, 2007; Zhao et al., 2013).

AMPs are produced in mammals within the granules of neutrophils and epithelial cells, including mucosal or skin secretions. Lysozymes, lactoferrin, and cathelicidins, etc., are produced in neutrophils with membrane permeability and bactericidal properties, mostly cationic in nature. On the other hand, epithelial cells produced β-defensins and other

microbicidal substances (Hancock and Chapple, 1999). Genetically most of the AMPs are encoded in clusters and co-expressed, resulting in multiple accumulations at a single site. As most AMPs are formed in an inactive form, they undergo proteolytic cleavage for activation. The regulation of AMPs depends on their expression and abundance of suitable proteases (Bals et al., 1998; Lai and Gallo, 2009). Sometimes AMPs are expressed in higher concentrations in an inactive state in granules and released near inflammation and infection sites. But in other cases, the expression was induced when there is a pathogen-associated molecular pattern (PAMPs) or cytokines response (Hancock and Diamond, 2000). Various animal proteins, like ribosomal protein S30, lactoferrin, cathepsin G, CAP18, pepsinogen C, transferrin, cystatin etc., show antibacterial activity due to its cationic property. Usually, these peptides show broad-spectrum activity, i.e., can neutralize or eradicate fungi, parasites, bacteria, viruses, and cancer cells. However, peptides are selective for only microbes instead of eukaryotic cells, e.g., indolicidin is a single 13 amino acid peptide that can kill bacteria, fungi, and even virus-like HIV (Hancock and Scott, 2000). AMPs are produced by animals upon contact of pathogens with their various parts e.g., skin, ear, eye, tongue, epithelial surfaces, etc. Neutrophils secrete azurophilic granules and have the tendency of killing through phagocytes (Hancock and Lehrer, 1998). Therefore, AMPs have a crucial role in inhibiting and spreading of many infections (Radek and Gallo, 2007; Schauber and Gallo, 2008; Zasloff, 2002).

2.2. History of antimicrobial peptides

Days back in 1939, Dubos discovered an antimicrobial peptide extracted from soil, *Bacillus brevis* strain. They found potent against a broad array of Gram-positive bacteria *in vivo* and *in vitro* (Dubos, 1939a, b). The antibacterial extract was named gramicidin that showed protection against *pneumococci* infection in mice (Dubos, 1939b; Hotchkiss and Dubos, 1940). However, gramicidin was reported as toxic with the intraperitoneal application but found useful for topical treatments of wounds and ulcers, indicating a therapeutic potential for clinical use and was the first AMP to be manufactured as antibiotics (Van Epps, 2006). Tryocidine was discovered in 1941 and found to be effective against both Gram-negative and Gram-positive bacteria (Dubos and Hotchkiss, 1941). However, tyrocidine showed its toxicity to human blood cells. Meanwhile, another AMP was extracted from wheat endosperm i.e., *Triticum aestivum*, founded to be efficacious against pathogenic bacteria and fungi such as *Pseudomonas solanacearum* and *Xanthomonas compestris* (Balls et al., 1942).

At present, this peptide was known as a member of the thionins family, widely distributed in the plant kingdom (Stec, 2006).

Defensin was the first reported as an animal originated antimicrobial peptide, isolated from rabbit leukocytes in 1956 (Hirsch, 1956) and was followed by bombinin from epithelia of *Bombina variegate* and lactoferrin from cow milk (Kiss and Michl, 1962). In the meantime, it was reported that lysosomes of human leukocytes contain AMPs (Zeya and Spitznagel, 1963). In 1981, Boman *et al.* injected bacteria into the silk moth pupae (*Hyalophora cecropia*) from which an inducible cationic antimicrobial peptide was isolated, i.e., P9A and P9B (Hultmark et al., 1980). Later, these peptides were sequenced, characterized, and named cecropins, and thereby reported as the first major α- helical AMPs (Steiner et al., 1981). A few years later, β-defensins and θ-defensins were respectively isolated and characterized from bovine granulocytes and leukocytes of rhesus monkeys and found to be different from α- defensins in terms of cysteine pairing (Tang et al., 1999). Anionic AMPs were first identified in the mid-90's by Brogden *et al.* in Xenopus laevis (Brogden et al., 1997). In the early 90s, the study reveals that lysozyme possesses antimicrobial activity that involves non-enzymatic mechanisms, i.e., similar to AMPs and justified as one of the first peptides to be discovered (Wiesner and Vilcinskas, 2010). Based on the available literature, AMPs constitutes the major defense system of organisms lacking adaptive immune system (Ganz, 2003b). After the deletion of the gene that encodes an AMP in Drosophila melanogaster, it will become susceptible to massive fungal infection (Lemaitre et al., 1996). As reported, AMPs have been explored in invertebrates, plants, and insects too (Barbosa Pelegrini et al., 2011; Bulet and Stocklin, 2005; Duran et al., 2011; Lee et al., 2012).

Moreover, most AMPs are produced in specific cells all the time; meanwhile, some AMPs are inducible. For instance, in the silk moth case as a model system, it was observed that AMP P9A and P9B would be induced in hemolymph by vaccination with *Enterobacter cloacae* (Hultmark et al., 1980). It is reported that, there is enhanced rate of transcription of mRNA for defensin in epithelial cells from different mice tissues after the infection with *Pseudomonas aeruginosa* (PAO1) (Bals et al., 1999). Many eukaryotic cells are also involved in production of AMP, such as lymph, gastrointestinal epithelial cells and genitourinary systems, lymphocytes, and phagocytes of the immune system (Ganz, 2003b; Niyonsaba et al., 2002a; Radek and Gallo, 2007). Apart from direct involvement in innate immunity, during infections, there has been an influence of AMPs on the host's inflammatory responses (Kindrachuk et al., 2013; Nijnik et al., 2012; Scott et al., 2000). Bacterial lipopolysaccharides

(LPS) have been reported as AMP inducer in mammals upon antibiotics treatment; HEK293 cells produce defensin after stimulation with LPS (Birchler et al., 2001; Hancock and Scott, 2000). AMPs like CAP18, CAP35 has been reported as inhibitor of LPS-induced cytokine released by macrophages, and a derivative of lactoferrins further reduces inflammatory responses. They do not have this type of regulation on the inflammatory response to the host immune system and LPS secretion compared to antibiotics. Which might cause an overreaction of the host immune system during antibiotic treatment and, in some cases, may even lead to sepsis (Brackett et al., 1997; Larrick et al., 1995; Loppnow et al., 1990; Zhang et al., 1999).

2.3. Classification of AMPs

AMPs are unique and varied classes of molecules. Till now, thousands of AMPs have been discovered, and the diversity of natural AMPs causes difficulty in their classification. These peptides can be classified into various groups based on amino acid composition, structures, biological properties, sources, and activity (Brogden, 2005; Huan et al., 2020).

2.3.1. Classification of AMPs based on sources

AMPs are derived from various sources like mammals, amphibians, insects, plants, and microorganisms and are attracted to a vast source. AMPs are found in mammals like humans, cattle, sheep, and other vertebrates. Cathelicidins and defensins are the prominent families of mammalian AMPs. Depending on the disulfide bond position, defensins can be divided into α, β, and θ (Rautenbach et al., 2016). Human host defense peptides (HDPs) show different expressions at every human growth stage while protecting them from various microbial infections. Such as cathelicidin LL-37, an AMP derived from newborn infant's skin, whereas β-defensin 2 is often expressed in elders instead of young ones (Gschwandtner et al., 2014). These AMPs can be produced by various body parts like skin, mouth, eyes, ears, respiratory tract, lung, intestine, etc. (Field, 2005).

On the other hand, amphibian derived antimicrobial peptides play an essential role in protecting them from pathogens, which are involved in declining the global amphibian population (Rollins-Smith, 2009). The primary source of amphibian AMPs is frogs such as magainin secreted from the skin of frog from genera Xenopous, Silurana, etc., under Pipidae family and are rich in antimicrobial peptides (Conlon and Mechkarska, 2014). Moreover, cancrin was also reported as the first AMP from sea amphibian, i.e., *Rana cancrivora.* However, AMPs derived from insects are mainly synthesized in their fat bodies and blood

cells and helps them for their strong adaptability for their survival (Vilcinskas, 2013). Cecropin, an AMP derived from insects, can be found in bees, guppy silkworm, and *Drosophila*. Cecropin A shows its activity against various inflammatory diseases and acts as an anticancer peptide (Dutta et al., 2019). Whereas, Jellein a peptide derived from bee royal jelly, shows its activity against various bacteria and fungi, and its lauric-acid conjugate shows its activity against *Leishmania major* (Zahedifard et al., 2020).

Compared to amphibians, mammals, and insects, microorganisms-derived AMPs have immense importance in the biotechnology field (Yazici et al., 2018) as the production of antibiotics at an industrial scale is carried out by using microorganisms. Gramicidin and nisin are the peptides initially isolated from the microbial sources in the early 90's i.e., *Lactococcus lactis* and *B. brevis,* respectively (Huan et al., 2020). The few reports also show the production of AMPs from yeast (Al-Sahlany et al., 2020; Cao et al., 2018). Presently bacteria and yeast are the more explored source of AMPs. Therefore, microorganisms have attracted toward themselves as a potential source of antimicrobial and among them bacteria is of utmost important source of AMPs (Wang et al., 2016).

2.3.2. Classification of AMPs based on activity

Based on the activity, AMPs can be divided into distinct categories according to the APD3 database and can be summarized as antiviral, antifungal, antibacterial, and antiparastitic etc. (Huan et al., 2020).

2.3.2.1. Antiviral peptides: Due to the emergence of serious virus infections such as foot and mouth disease, avian influenza virus, HIV are the long term treats to human life. Moreover, the recent outbreak i.e., COVID-19 causes a significant loss to human life and economic well being. Thus, it is necessary to solve the problem and antiviral peptides can emerge as a boon to these problems. The antiviral peptides show strong killing effect by (a) inhibiting virus attachment and virus cell membrane fusion, (b) can destroy the virus envelop, or (c) inhibits the virus replication (Jung et al., 2019). For example AMP Epi-1 intervenes in the inactivation of virus particle and can inhibit the virus causes foot-and-mouth disease (Huang et al., 2018). The most important example of anti-HIV peptides are defensins (α and β defensins). They have different modes of actions, LL-37, gramicidin, etc. The commercially available anti HIV drug is Fuzeon^{TM}, which inhibits the different stages in the virus's life cycle (Ashkenazi et al., 2011; Madanchi et al., 2020).

2.3.2.2. Antifungal peptides: There are many antifungal peptides which show activity against common pathogenic infections such as *Aspergillus* and *Candida albicans* in clinical medicine, whereas yeast and filamentous fungi, molds cause food and agriculture infections. Many natural and synthetic peptides show antifungal activity, for example, brevinin, ranatuerin, cecropins, which are naturally derived AMPs and exhibit antifungal activity. AurH1 is a synthetic peptide derived from aurein1.2 and inhibits *C. albicans* infection (Madanchi et al., 2020). *Lactobacillus plantarum* produce a total of 37 antifungal peptides including two chemically synthesized radish AMP with strong inhibitory effect against *Zygosaccharomyces rouxii* and *Z. bailii* (Muhialdin et al., 2020; Shwaiki et al., 2020).

2.3.2.3. Antibacterial peptides: In class of AMPs, antibacterial peptides accounts a large part and have broad range of inhibitory spectrum against many pathogenic bacteria such as *Acinetobacter baumannii*, VRE, MRSA in terms of clinical medicines, whereas *S. aureus*, *Listeria monocytogenes*, *Escherichia coli*, etc. as a food pathogens. Many natural and synthetic peptides like nisin, cecropins, and defensins show an excellent inhibition efficacy against Gram-positive and Gram-negative bacteria. Recently AMP P5 and P9 are designed based on *Aristicluthys nobilia* Interferon-I, can kill MRSA with low cytotoxicity (Li et al., 2019).

2.3.2.4. Antiparasitic and anticancer peptides: With increased resistance against parasitic drugs, the Antiparasitic peptides came up as a new treatment. These peptides are efficiently killed parasites which causes diseases like malaria and leishmaniasis (Mangoni et al., 2005; Rhaiem and Houimel, 2016). For example, cathelicidin, temporins-SHd shows its high efficacy against parasites; And Epi-1 is a synthetic peptide that inhibits *Trichomonas vaginalis* by rupturing its membrane (Abbassi et al., 2013; Neshani et al., 2019).

AMPs which show anticancer properties are indolocidin, puroindoline A, tritrpticin and its analogs, etc. These AMPs acts with a mechanism by (1) induction of necrosis or apoptosis in cancer cells, (2) inhibiting angiogenesis, (3) recruiting of immune cells (Arias et al., 2020; Ma et al., 2020; Wu et al., 2014). In anticancer peptides, the net charge and hydrophobicity play an essential role for better anticancer peptides.

Anti-inflammatory peptide reduces inflammatory mediator's secretion. Most of them suppress the anti-inflammatory signalling pathways (Gao et al., 2020; Meram and Wu, 2017). At the same time, anti-diabetic peptides modulate the G protein-coupled receptor kinase (GRK 2/3) or activate the glucagon-like peptide-1 (GLP-1) receptors (Graham et al., 2020; Marya et al., 2018).

2.3.2.5. Classification of AMPs based on amino acid composition and structure

AMPs can also have an array of activities, i.e., from bacterial killing to immune modulation; also prevent biofilm formation and having antiviral and anti-cancerous properties. However, the activity of AMPs depends on the amino acid sequence and structure so, it is necessary to consider both of these properties during the classification of AMPs. AMPs are classified into many different groups. One subgroup consists of anionic antimicrobial peptides, which are small i.e., ~700-800 Da, and are present in surfactant extract, airway epithelial cells, and bronchoalveolar lavage fluid (Brogden et al., 1998; Brogden et al., 1999). These AMPs are produced in millimolar (mM) concentration and required zinc as a cofactor for its potential activity and showed broad-spectrum antimicrobial activity. Further, these small anionic AMPs are rich in glutamic acid and aspartic acid (Brogden et al., 1996) and are similar to charge neutralizing pro-peptides of larger zymogens with antimicrobial activity synthesized alone (Brogden et al., 1997).

The second subgroup consists of cationic peptides, short in length, and lack of cysteine residues, mainly having hinge/kink in the middle (Gennaro and Zanetti, 2000; Tossi et al., 2000). For example, cecropin (A), andropin, moricin isolated from insects, cecropin P1from Ascaris nematodes (Andersson et al., 2003). Many of these peptides are in disordered form in the presence of aqueous solutions. Still, they become structured (partially or fully into α-helix form) when in contact with trifluoroethanol, surfactants/detergents, and above critical micellar concentration i.e., sodium dodecyl sulfate (SDS), phospholipid vesicle, liposomes (Johansson et al., 1998). The most studied human AMP in this group is LL-37, a member of the cathelicidins family. This AMP plays an essential role in the immunomodulatory and inflammation responses (Hancock et al., 2016). Magainins is also one of the explored human AMP, which is isolated from *Xenopus laevis*. This particular AMP was active against pathogenic bacteria, fungi, yeast, and viruses (Zairi et al., 2009). The structure of magainins and its functional relationship has been well studied and were first clinically tested but ultimately failed (Jenssen et al., 2006). On the other hand, the antibacterial activity against Gram-negative and Gram-positive bacteria also correlates with the amount of α-helicity present in the structure i.e., more is the content of α-helix, stronger the antibacterial activity (Park et al., 2000).

Some of the cationic peptides are rich in various other amino acids i.e., proline, glycine, arginine tryptophan, and histidine, and are considered a third subgroup. These peptides also lack cysteine residues and are linear, even though some can form extended coils (Gennaro

and Zanetti, 2000; Otvos, 2002), for example, abaecin i.e., a proline-rich peptide isolated from the honeybee, glycine containing peptide i.e., hymenoptaecin also from honeybee (Boman, 1995b). Tryptophan rich indolicidin AMP was isolated from neutrophils and having only 13 amino acids. The nuclear magnetic resonance (NMR) and circular dichroism (CD) structural studies reveal that indoloicidin forms a distinctive membrane-associated structure with the defined extended structure in the presence of micelles (Falla et al., 1996; Rozek et al., 2000). Another subgroup contains anionic and cationic peptide, which consists of cysteine-rich polypeptides that form disulfide bonds and stable β- sheets e.g. defensins, protegrins, and tachyplesins.

Disulfide bonds in defensins can provide a structural stability and reduces protease degradation (Cociancich et al., 1993; Dhople et al., 2006). In solution, β-sheet AMPs are more structurally stable and do not undergo significant structural changes while leaving an aqueous environment to a membrane environment (Lee et al., 2016). These AMPs can be further separated into subcategories based on single or multiple disulfide bonds (Haney et al., 2019); somewhat, the presence of these disulfide bridges in AMPs is necessary for its antimicrobial activity. For example, tachyplesins from horseshoe crabs (Kokryakov et al., 1993) and defensins have a potency of antiviral, antibacterial, antifungal, inflammation, and immune responses (Hancock et al., 2016). Approximate 55 α-defensins, 90 β-defensins, and 54 arthropod defensins isolated from humans, animals, and insects comprise different amino acid length and disulfide bridges (Ganz, 2002; Ganz et al., 1990; Schutte and McCray, 2002).

2.4. Common physiochemical characteristics of antimicrobial peptides

Even though AMPs are diverse groups of molecules in terms of structure, sequence, and sources, some properties are common in all AMPs, affecting the antimicrobial activity and specificity. These properties are net charge, size, sequence, solubility, and hydrophobicity, etc., which are discussed below.

2.4.1. Net charge: The net charge of antimicrobial peptides is the sum of all the charges of an ionizable peptide group, which varies from negative to positive. It is the main factor for the initial interaction with negatively charged cell membranes. The range of net positive charge in AMPs is +2 to +13 and is contained in a specific cationic domain. The cationic nature of AMPs associates the presence of lysine and arginine groups, and histidine residues are mostly involved (Gagnon et al., 2017; Hong et al., 2001; Jiang et al., 2008). The antimicrobial activity and hemolytic activity of AMPs can be altered to achieve its selective

killing of microbes with no or fewer effects on host cells by changing its net charge. For example, in magainin 2, the net charge increases from +3 to +5, and improves the efficacy against Gram-positive and Gram-negative bacteria.

In contrast, the hemolytic activity was high while increasing the charge from +6 to +7, and also it loses its antimicrobial activity (Dathe et al., 2001). On the other hand, in V13K, an increase in net positive charge from +8 to +9 results in higher hemolytic activity. A decrease in net charge is a loss of antimicrobial activity against *Pseudomonas aeruginosa* was observed (Jiang et al., 2008). The fact behind the loss of antimicrobial activity could be a strong interaction between the peptide and the phospholipid head group, which prevents the peptide's translocation into the inner leaflet of the membrane (Yeaman and Yount, 2003).

2.4.2. Confirmation and structure: Antimicrobial peptides can assume a variety of structures, which include secondary, structures i.e., α-helices, antiparallel β-sheets, and relaxed coils. Helicity is essential for determining the toxicity of eukaryotic cells (Huang et al., 2010). The helicity was reduced by adding D-amino acids into the primary sequence, which further showed the lower hemolytic effect, with antimicrobial effect (Oren and Shai, 1996). Papo *et al.* modified some α- helical peptides by replacing 35% of the L-amino acids with D- amino acids and found that this modification eliminated hemolytic activity (Papo et al., 2002) and also, this peptide was resistant to protease. Amino acids of the primary sequence in AMPs has correlation with the helicity, e.g., example, proline and glycine (Pace and Scholtz, 1998). For membrane insertion peptides should have tendency to change their confirmations (Jenssen et al., 2006).

2.4.3. Hydrophobicity: This property is the key feature for all-AMPs which enable water-soluble peptides to partition into the lipid bilayer membrane. And also, influence the selectivity and activity of the AMP molecules. The hydrophobicity of AMPs defines the percent of hydrophobic molecules present. Nearly 50% of amino acids present in the primary sequence of all-natural AMP are hydrophobic residues such as alanine, leucine, isoleucine, valine, methionine, tyrosine, phenylalanine and tryptophan (Tossi et al., 2000; Yeaman and Yount, 2003). In some cases, there is an increase in antimicrobial activity with increasing the hydrophobicity on the positively charged side below its threshold limit (Huang et al., 2010), despite a loss of antimicrobial activity while decreasing the hydrophobicity of AMPs (Lee et al., 2002). Membrane permeabilization is directly related to hydrophobicity and high hydrophobicity leads to cell toxicity and loss in its antimicrobial activity (Chen et al., 2007).

For example, in case of V13KL (a synthetic α- helical AMP) it was observed that there was an influence of hydrophobicity on its potency and hemolysis of human red blood cells (Chen et al., 2007). Further, the range of the targets can change while increasing the hydrophobicity of AMPs (Kustanovich et al., 2002; Zelezetsky et al., 2005). For example, magainin and its synthetic analogs with higher hydrophobicity showed antimicrobial activity against Gram-negative and Gram-positive bacteria (Dathe et al., 1997).

2.4.4. Amphipathicity: Another essential property of AMPs is amphipathicity, which provides its potential activity and interaction with microbial membranes. According to Vidal *et al.,* instead of hydrophobicity in AMPs, amphipathicity plays an essential role in binding to microbial membranes (Fernandez-Vidal et al., 2007) (Yeaman and Yount, 2003). The amphipathicity in α-helical AMPs, has lead to enhanced in its antimicrobial activity and a decreases RBC lysis (Yoshizawa et al., 1990). For example, in melittin related peptides, there is a decrease in RBC lysis while increasing the amphipathicity (Zhang et al., 2016). Moreover, in the case of indolicidin analogs, the antimicrobial activity was preserved even with an increase in charge; and also amphipathicity lowers the lysis of RBCs which increases the therapeutic index of peptides (Falla and Hancock, 1997; Smirnova et al., 2004). Meanwhile, protegrin-1 shows more amphipathicity than tachplesin-1 even though both are β-sheet peptides and ultimately lead to a two-fold increase in hemolysis without showing any major effect antimicrobial activity (Edwards et al., 2016).

2.4.5. Solubility: As the AMPs will act upon or enter via lipid membranes, their solubilization is needed in aqueous surroundings. If there is an aggregation of AMP molecules, it will lose its ability to interact with the cell membrane. For example, in the case of a hybrid synthetic peptide V681 which can form dimmers, substitution of a lysine residue on the non-polar side of this AMP prevented the dimerization and led to low hemolytic activity. These AMPs that can lose dimerization will become more useful for incorporating microbial membranes (Chen et al., 2005). Thus, it could be concluded that solubility is vital for structural optimization.

Therefore, the above-studied parameters showed a complicated relationship between net charge, solubility, hydrophobicity, and amphipathicity. These properties affect the activity of AMPs and showed the existence of some interactions between these peptides. However, any change in the primary sequence that can alter antimicrobial activity of AMPs against the target cells (Giangaspero et al., 2001).

2.5. Mode of action of antimicrobial peptides

As antimicrobial peptides are unique entities, their mode of action has been studied extensively. To facilitate AMPs as therapeutic agents, it is essential to understand its mode of action. Initially, only the membrane target was considered as the mode of action of AMPs. With time, the evidence has emerged, which showed that AMPs also have other specific molecular targets without any impact on the membrane (Ulm et al., 2012). As discussed above, it kills cells by damaging its membrane integrity while interacting with negatively charged membrane or by inhibiting proteins, targeting DNA and RNA synthesis, or via interaction with other intracellular targets. Earlier in the late 90s only cationic AMPs are known, but after discovering negatively charged AMPs, the concept of cationic AMPs was changed (Brogden et al., 1997). For example, maximin-H5 from frog skin and dermcidin from humans' sweat gland tissues were discovered as anionic peptides (Lai et al., 2002; Steffen et al., 2006). Usually, AMPs are only effective against one class of microorganisms i.e., bacteria and fungi (Hancock and Scott, 2000). But there are some exceptions, i.e., most AMPs show different modes of action against various microorganisms such as indolicidin active against bacteria, fungi, and even HIV (Robinson et al., 1998; Selsted et al., 1992). It shows antifungal activities by causing damage to cell membranes, and in the case of *E. coli,* it penetrates the cell membrane and inhibits the DNA synthesis. Inodlicidin as antiviral inhibits HIV integrase and acts as an anti-HIV peptide (Krajewski et al., 2004; Lee et al., 2003a; Subbalakshmi and Sitaram, 1998). In comparison, most of the AMPs have the same mode of action for different cell types. For example, PMAP-23 kills both fungi and bacteria by forming pores in the cell membrane (Lai et al., 2002; Park et al., 2004).

Bacterial membranes are associated with one-third of the total proteins, which have many critical functions i.e., active transport of nutrients, respiration, ATP generation, proton motive force, and intracellular communication (Zhang and Rock, 2009). Thus, AMPs can alter the function of these proteins even without lysis of the complete cells. So, the effect of the rapid killing of AMPs does not come from membrane disruption and the inhibition of these functional proteins. The modes of action of antimicrobial peptides are divided into two classes i.e., direct killing, and another is immune modulation as described in Fig. 1.

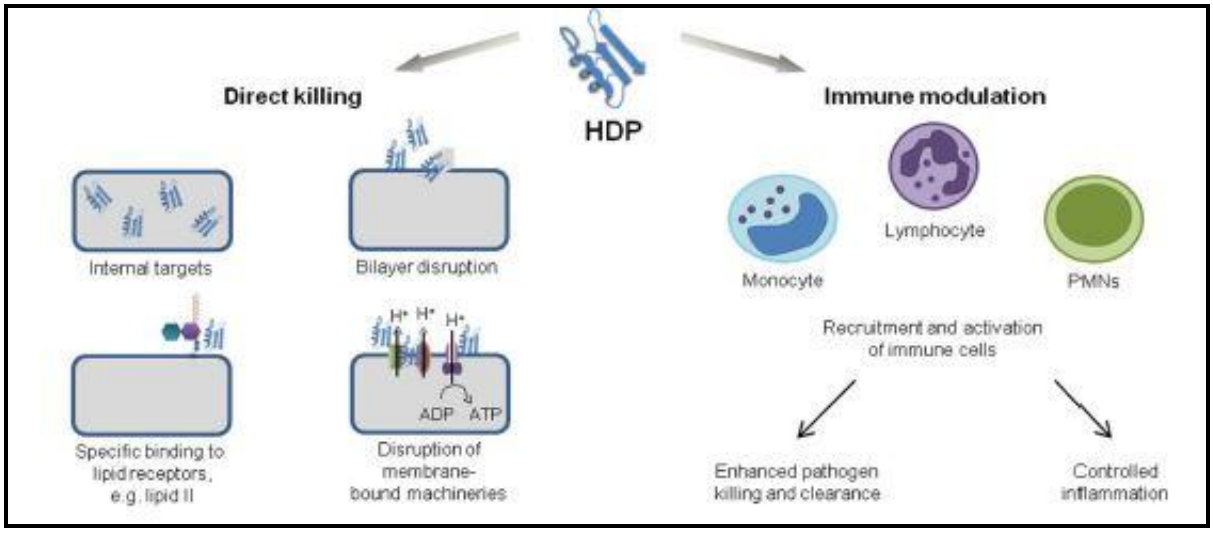


Figure 1. Antimicrobial peptides mode of action: Courtesy (Ulm et al., 2012) PMN: polymorphonuclear cells; ADP: adenosine diphosphate; ATP: adenosine triphosphate

2.5.1. Direct killing mechanism: The direct killing mode of action can be further divided into two target mechanisms i.e. membrane targeting and non-membrane targeting. In the case of membrane targeting, AMPs can interact via receptor-mediated or non-receptor mediated processes. Mostly AMPs produced by bacteria can interact with receptor-mediated pathways and are also active in the nanomolar range in vitro (Breukink et al., 1999). For example, nisin has two domains i.e., one domain binds with lipid II molecule (a membrane attached cell was precursor) with high affinity. The second region of the peptide depicts the membrane-embedded pore-forming domain (Fleury et al., 1996).

In a non-receptor mediated mechanism, AMPs are more abundant within this group and show broad-spectrum antimicrobial activity, which depends on its amino acid composition, length, and structure. These AMPs in micromolar concentrations target without any specific receptors; instead, it has more general target, i.e., the bacterial membrane (Shai, 2002; Yeaman and Yount, 2003). For antimicrobial activity, membrane interaction plays a significant key role as most AMPs target the bacterial membrane by disrupting the physical integrity of the lipid bilayer by membrane thinning, poring, and disrupting the physical barrier or act on the internal targets by translocating across the membrane (Hancock and Sahl, 2006). The primary reason for the interaction between AMPs and negatively charged bacterial membranes is developing the electrostatic forces (Ebenhan et al., 2014). The interaction of AMPs with Gram-positive bacteria involved only cytoplasmic membrane, which includes the association of teichoic and teichuronic acid of the cell wall with AMPs. In

the case of Gram-negative bacteria the dual complex membranes (outer and cytoplasmic) are involved i.e., lipopolysaccharide of outer membrane interacts with the peptides and disrupt the membrane self-promoted uptake mechanism. It suggested that the LPS have a greater affinity due to which the AMPs displace the cations and bind to LPS. After the divalent cations replacement, these AMPs become bulky on the surface, cause transient cracks and mobilize into the outer membrane, and permit the peptide passage further across the membrane (Yeaman and Yount, 2003).

The significant difference between the bacterial and mammalian cells is the cell membrane composition and architecture that assets the selectivity of AMPs target. Bacterial membranes are mainly composed of anionic phospholipid in large proportion, which are more susceptible to the interaction of peptides leading to membrane disruption compared to mammalian cells, contains phospholipids; however, these phospholipids are neutral and enriched in steroids. Further, due to the enrichment of negative charge in the bacterial membrane the transmembrane potential promotes the interaction with peptides. It leads to the electro-potential driving force for insertion and translocation of the AMPs. As the transmembrane potential of the bacterial cells is more negative than the mammalian cells which also contribute to more selectivity of AMPs towards bacteria (Fig. 2) (Matsuzaki, 2009).

Further, presence of cholesterol in higher amounts in the mammalian cell is responsible for the reduction of activity of AMPs by stabilizing the zwitterionic phospholipids bilayer (Zasloff, 2002). Apart from the electrostatic force and hydrophobicity, the AMP ligands bind to the phospholipid head and involve the bacterial membrane's disruption. Also, the anionic components of the cell membrane, i.e., LPS etc., serve as pseudo receptors and initiate the interaction between the pathogenic cell membrane and the AMPs (Ritchie and Humphreys, 1991). All these characteristics ensure a strong affinity of AMPs towards pathogens and help to disrupt the bacterial membrane. However, relatively weaker interaction in mammalian cell membranes results in the selectivity in a good sense even at high peptide concentrations (Ebenhan et al., 2014; Melo et al., 2009).

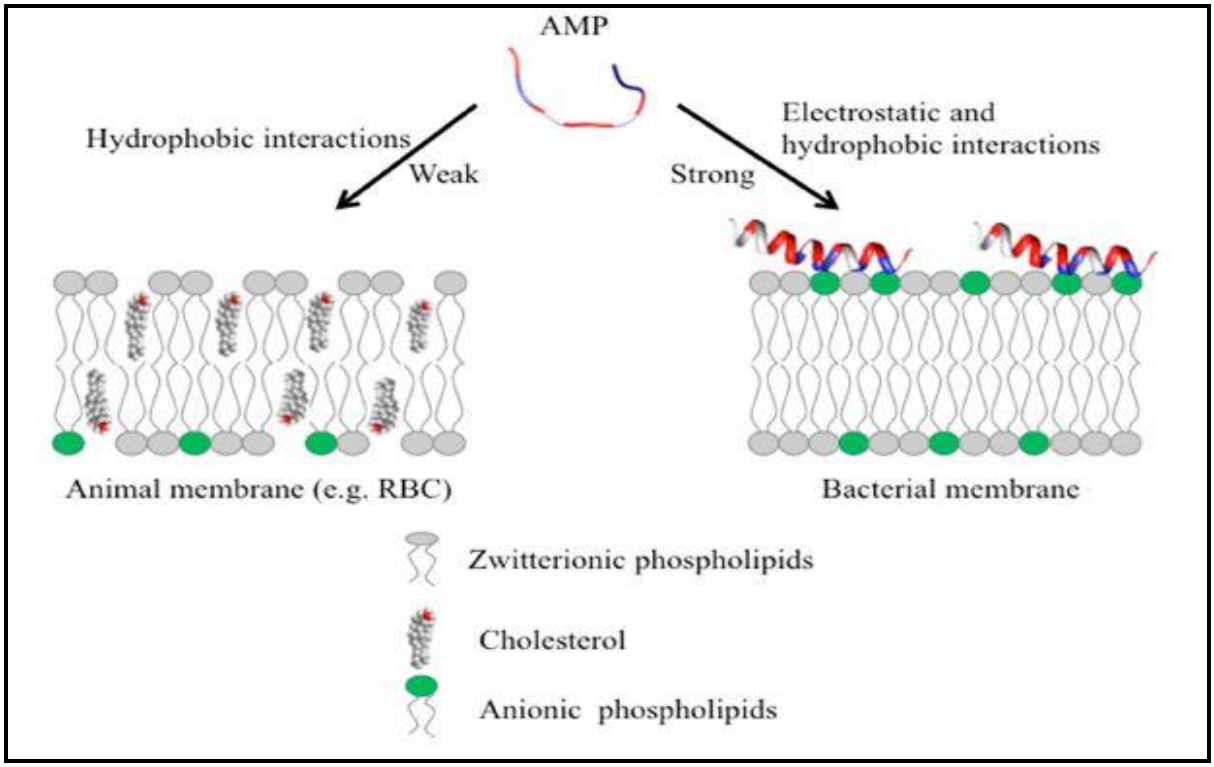


Figure 2. **Interaction of AMPs (cationic) with the cell membrane:** (left) animal membrane, (right) bacterial membrane. Courtesy (Kumar et al., 2018), RBC: red blood cell.

The membrane target mechanism can depend on lipids and its other properties instead of the charge to which AMPs are sensitive. And can later address the large difference in the lipid composition of the cell membrane of bacteria, fungi, and mammals. Main lipids include in the cell membrane are (1) glycerophospholipids (GPLs): sphingolipids, sterols, and lysolipids, (2) Phosphatidylethanolamine (PE), and (3) Cardiolipin. These are the most common anionic lipids in bacteria; however, Phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidic acids are the main GLPs in fungal cell membrane (Kumar et al., 2018; Li et al., 2017; Singh and Prasad, 2011). As compare to mammalian cells, cell membranes of fungi are more anionic and have higher PC content. However, ergosterol founds in lower eukaryotic plasma membrane-like fungi, in contrast to animals that contain cholesterol (Faruck et al., 2016). Thus, various AMPs take benefits of difference in the membrane components to venture their effect.

Membrane curvature, have been reported to play an important role (Drin and Antonny, 2010; Matsuzaki et al., 1998). In AMPs, the initial interactions mainly include ionic and hydrophobicity, through which it interacts with negatively charged membrane and insert into the membrane interior. The hydrophobic part of AMPs helps the molecule to insert into the

bacterial cell membrane. At a specific concentration AMPs starts self assemble on the surface of bacterial (Andersson et al., 2016; Epand et al., 2016; Madani et al., 2011). Two broad categories i.e., transmembrane pore and non-pore models have been used to decipher the mode of action. Further, transmembrane pore model can be subcategorized into the barrel-stave and toroidal pore model and are described as:

2.5.1.1. Toroidal Pore Model: This model is also known as the wormhole model, in this AMPs penetrates vertically in the cell membrane and further, bend to form a ring hole having diameter of 1-2 nm for example, magainin 2, arenicin, and lacticin Q (Matsuzaki et al., 1995, 1996). The cationic peptides like TC19, TC84 and BP2 compromise the cell membrane barrier by forming fluid domains (Omardien et al., 2018).

2.5.1.2. Barrel-Stave Model: After aggregation of AMPs with each other, penetrates the bilayer of the cell membrane in multimers form, and produces channels that results in cytoplasmic flow. Whereas, in severe cases AMPs can induces the cell membrane collapse and leads to the cell death (Lohner and Prossnigg, 2009). For example, Alamethicin uses this model and can perform pore-forming activity; however protegrin-1 can form the stable octameric β-barrels and tetrameric arcs in implicits explicits membranes by simulations (Lipkin and Lazaridis, 2015).

2.5.1.3. Carpet-Like model: In this model, AMPs are arranged parallel to the cell membrane; however the hydrophilic ends of the AMPs face towards the solution and whereas the hydrophobic ends towards the phospholipids bilayer. Then will cover the membrane surface similar to carpet model and destroy the cell membrane in a ‘detergent’- like manner (Oren and Shai, 1998). In this case the pore-forming mechanism requires a certain threshold and high concentration of AMP. For instance, catheter LL-37 manifests its activity via this mechanism; AMPs with β-sheet also play a role in this model (Corrêa et al., 2019; Shenkarev et al., 2011).

In classical models of membrane disruption, the peptides lying on the membrane and reach a concentration till threshold and embedded themselves to across the membrane and forms peptide-lined pores in the barrel stave model. In contrast, solubilize the membrane into micellar structure in case of carpet model or may form peptide and lipid lines pores in the toroidal pore model. In case of revised disordered toroidal pore model, the formation of pore is more stochastic and involves fewer peptides. The bilayer thickness can be affected by the presence of the peptides or membrane itself can be remodelled to form domains that are rich

in anionic lipids that surround the peptide. In case of more precise cases, there is an induction of non-bilayer intermediates in the membrane and peptide adsorption can be enhanced by targeting them to oxidized phospholipids. Therefore, the peptide may braced with small anions across the bilayer which results in efflux and their membrane potential can be dissipated without any other noticeable damage. Contrarily in the model of molecular electroporation, the peptide accumulation on the outer leaflet increases the membrane potential above threshold contribute the membrane transiently permeable to various molecules, includes the peptides themselves (Nguyen et al., 2011). Fig. 3 shows the diagrammatic view of the various models of AMPs targeting bacterial membrane.

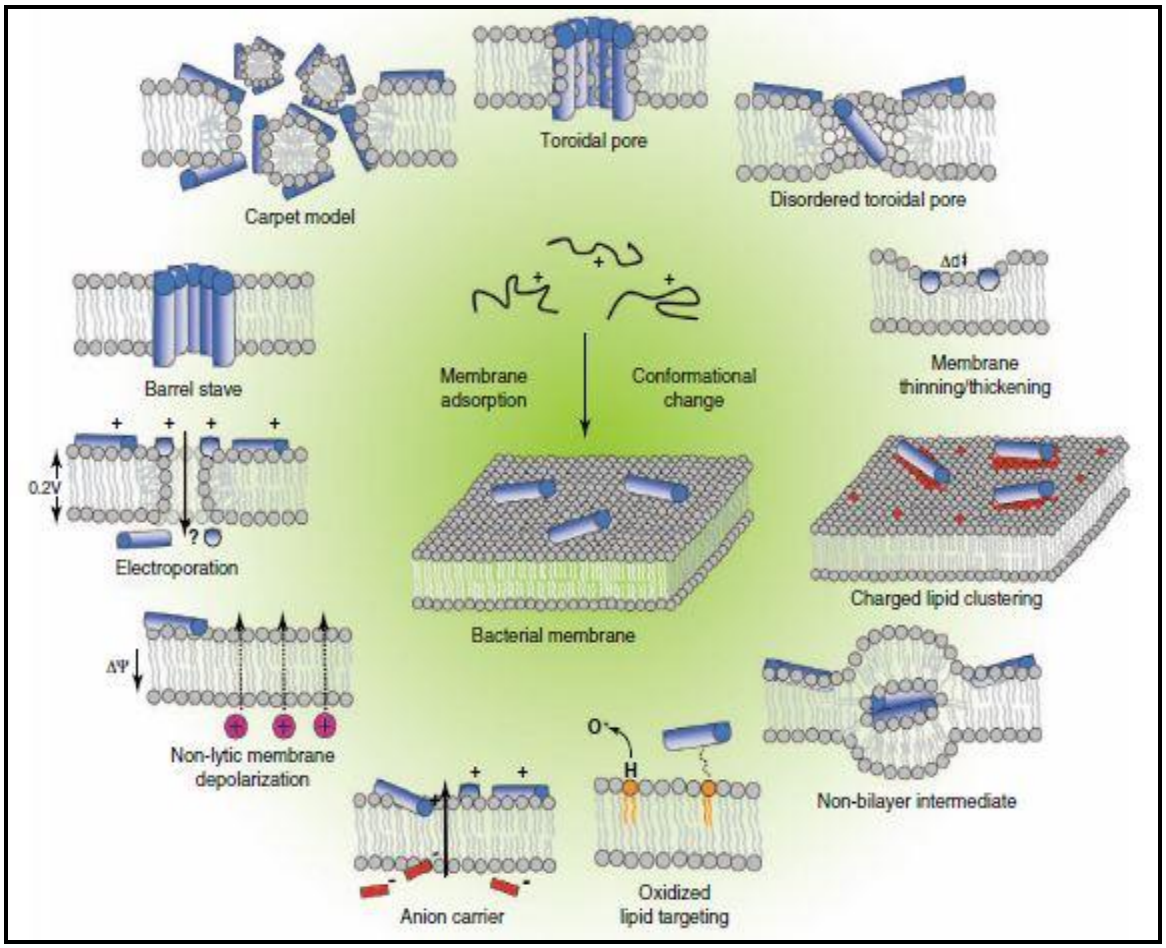


Figure 3. Mechanism of interaction of antimicrobial peptides (AMPs) with bacterial cell membrane (Nguyen et al., 2011).

In non-membrane targeting, AMPs can be divided into two categories i.e. peptides that target the bacterial cell wall and intracellular peptide targeting contents (Fig. 4). As compared to conventional antibiotics e.g., penicillin, antimicrobial peptides can also inhibit cell wall synthesis components. The highly preserved prime target molecule is lipid II, such as

defensins, which binds to negatively charged pyrophosphate sugar moiety of lipid II molecule. And thus, it promotes pore's formation and leads to disruption of the cell membrane (Malanovic and Lohner, 2016; Munch and Sahl, 2015). Initially, it was thought that the AMPs could not target the intracellular molecules. Studies which reveals that the α helical peptides i.e., cecropin, melittin, and magainin showed that all D version of these peptides are equivalent to all-natural L peptide, which supports the idea that the targets like proteins, DNA/RNA were not required for antimicrobial activity after confirming that AMPs can target the cell membrane (Epand and Vogel, 1999; Wade et al., 1990). Consequently, the studies reveal that other AMPs with all D and L amino acids did not show any equal activities. Further, it was established that several AMPs have intracellular targets and at minimal effective concentration, AMPs do not cause membrane permeabilization, however still show the bactericidal activity (Brogden, 2005; Vunnam et al., 1997). However, these AMPs first interact with the cytoplasmic membrane and then gradually accumulate intracellularly, blocking critical cellular processes. Novel mechanisms involved in intracellular targets are protein/nucleic acid synthesis inhibition and disruption of enzymatic/protein activity (Brogden, 2005). For example, buforin II AMP, derived from histone of frogs, get insert through the bacterial membrane without permeabilization and binds to the DNA and RNA of the *E. coli.* Indolicidin, human α, and β defensins are also targeted the bacterial intracellular components (Boman et al., 1993; Sharma and Nagaraj, 2015; Subbalakshmi and Sitaram, 1998).

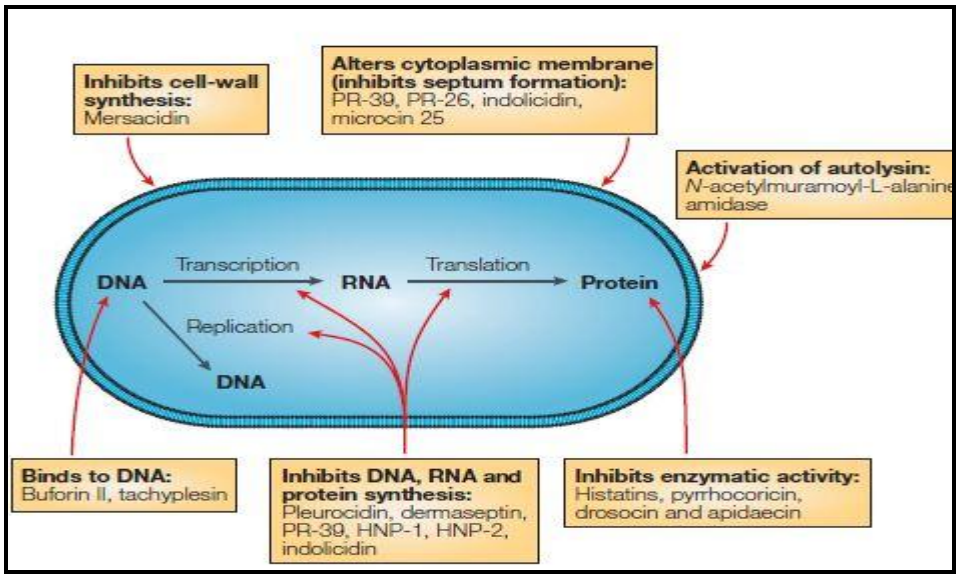


Figure 4. Intracellular target mechanism of antimicrobial peptide on the bacterial membrane. Courtesy (Brogden, 2005).

2.5.2. Immunomodulatory activities of AMPs

The fundamental property of AMPs over the pharmaceutical antibiotics is the ability to stimulate an immune response. Apart from killing pathogenic bacteria through AMPs, it also initiated immune cells recruitment, and in the meantime, it neutralizes an excessive inflammatory response at the site of infection. (Zhang and Gallo, 2016). Most AMPs could induce various immune responses like activation, attraction, and differentiation of white blood cells. It also reduces inflammation and stimulation of angiogenesis by lowering the pro-inflammatory chemokine expression and controls the expression of chemokine's and reactive oxygen species (ROS) (Afacan et al., 2012; Hancock et al., 2012; Hilchie et al., 2013; Lai and Gallo, 2009; Nijnik and Hancock, 2009). Cationic peptides showed a broad range of immunogenic properties like the expression of hundreds of genes in monocytes, epithelial cells, etc. Also included chemo-attraction of immune cells, induction of chemokine's, and promotion of angiogenesis and wound healing and infection resolution (Zborovskii et al., 1991). For example, human AMPs like LL-37 and β-defensins can attract immune cells such as mast cells, leukocytes, and dendritic cells (Garcia et al., 2001; Liu, 2001; Niyonsaba et al., 2002b). Apart from the interaction of AMPs with the innate immune system components, i.e., neutrophils and macrophages; involved in modulating the adaptive immune system such as T and B cells (Hilchie et al., 2013). Interestingly all these studies of AMPs work in various independent or multi-hit mechanisms of action makes AMPs an ideal candidate for future development (Zhang et al., 2000).

2.6. Bacterial Antimicrobial peptides

The antimicrobial peptide production is widespread among all forms of life, from multicellular organisms to bacterial cells. AMPs in higher organisms contribute the innate immunity and a part of the first line of defense against pathogenic organisms. Bacterial antimicrobial peptide benefits the individual species that may compete for the nutrients and some environmental habitat; and mediate the peptide killing of other bacteria (Mattick and Hirsch, 1947; Nissen-Meyer and Nes, 1997). AMPs from bacteria are known as bacteriocins; these peptides are highly heterogeneous groups of molecules similar to eukaryotic AMPs. It Shares common features such as size (20-50 amino acids), net charge, and the properties that facilitate the initial interactions with the negatively charged bacterial membrane. Moreover, there is some important difference between bacteriocins and eukaryotic AMPs, i.e., bacteriocins are generally very potent and show their activity in the pico to the nanomolar range.

In contrast, eukaryotic AMPs show activity at a micromolar concentration (Nissen-Meyer and Nes, 1997). Mostly bacteriocins show narrow-spectrum targets, i.e., active only against a few species/genera, closely related to the producers (Nissen-Meyer and Nes, 1997). On the other hand, eukaryotic AMPs are generally the least specific and target broad diversity of bacteria. Due to specificity and high potency, bacteriocins are considered promising antimicrobial agents for various applications, including food preservation and treating infectious diseases. The production of bacteriocins is ubiquitous in the bacterial world. Probably, the distribution of this trait has been facilitated by the fact that the genetic determinants are responsible for producing the bacteriocin, which is located on the mobile genetic elements i.e., conjugative plasmid or transposons (Jack et al., 1995). In nature, bacteriocins play an ecological role in providing the producer with an advantage in competition with other bacteria for common resources and playing an active role in defining the microbial composition in specific niches (Cotter et al., 2005c).

2.6.1. Classification of bacteriocins

Bacteriocins are composed of a heterogeneous group of peptides with variations in the structure, size, and action mechanism. In the case of Gram-negative bacteria, bacteriocins are mostly found in *E. coli* and other enterobacteria. These bacteriocins are mostly identified as microcins, small peptides or colicins, a large protein (Duquesne et al., 2007). Further, these microcins are grouped into two subparts i.e., class I microcins and class II microcins. The class I microcins are relatively small in size and undergoes extensive posttranslational modification, e.g., microcin C7 consists of only seven amino acid residues and microcin J25, which is 21 amino acid residue peptide (Novoa et al., 1986; Salomon and Farias, 1992). Another subcategory i.e., class II microcins, is larger and undergoes significantly less or no posttranslational modifications. This microcins class consists of microcin E492, H47, and colicin V (Havarstein et al., 1994; Lavina et al., 1990).

Bacteriocins produced from Gram-positive bacteria are divided into two categories i.e., lantibiotic and non-lantibiotic bacteriocins (Mattick and Hirsch, 1947). Lantibiotics are small peptides consists of ~19-38 amino acids and contain post-translational modifications along with thioether-based ring structures, i.e., lanthionine β-methyllanthionine (Wiedemann et al., 2006). Most of the lantibiotics may contain other unusual modified amino acids, for example, D-alanine in lactocin S (Skaugen et al., 1994). According to Cotter *et al.* (Cotter et al., 2005a), the lantibiotics are further classified into 11 subclasses based on the vast structural

variation. For example, subtilin, nisin, thuricin CD, and lactic 3147 (Bierbaum and Sahl, 2009; Cotter et al., 2005b), nisin is a well-characterized peptide from class I of lantibiotics. However, class II lantibiotics include small cationic peptides, i.e., ~25-60 amino acids. These peptides are heat stable, non modified antimicrobial peptides, but they lack disulfide bridges and cyclic peptides' circularization. The most common producers of class II lantibiotics are lactic acid bacteria (LAB) (Mattick and Hirsch, 1947). Further, this class was categorized into four subclasses (Cotter et al., 2005c). Class IIa contains N-terminal consensus sequences (YGNGVxCxxxxCxVxWxxA) where X is any amino acid and shows strong antilisterial activity, e.g., pediocin PA-1, enterocin A and P, etc. Class IIb comprises two-peptide bacteriocins, and its activity depends on the complementary action of two different peptides, for example, plantaricin EF and plantaricin JK. Whereas class IIc bacteriocins are cyclic and form head to tail fashion ring structure e.g., enterocin AS-48 and gyricin ML. And the last class i.e., IId bacteriocins, are linear non-pediocin like one peptide e.g., lactococcins A and enetrocin B (Cotter et al., 2005c; Kemperman et al., 2003). The bacteriocins classification was recently discovered; glycosylated bacteriocins include sublancin and glycocin, but this group has not been set. Meanwhile, sactibiotics are the new class of bacteriocins recently assigned and contain the modified peptides containing sulfur to α-carbon linkages (Oman et al., 2011; Rea et al., 2011). Hence, the alarming increase in antibiotic resistance became a severe threat to humans and animals. Bacteriocins are the most promising therapeutic tools with various interesting features i.e., activity in a narrow spectrum range, rapid killing, and high potency against various pathogenic organisms (Rossi et al., 2008).

2.6.2. Antimicrobial peptides from *Bacillus* group

Species of genus *Bacillus* are endospore-forming, rod-shaped, Gram-positive bacteria. Structurally this genus produces distinct secondary metabolites, which shows a good spectrum of antimicrobial activities (Cotter et al., 2005c; Motta et al., 2008; Paik et al., 1997; Sabate and Audisio, 2013) and are genotypically and phenotypically heterogeneous; exhibit various physiological properties, i.e., having the ability to degrade various substrates that are derived from plants and animal sources which includes cellulose, starch, proteins, hydrocarbons and biofuels (Priest, 1993), Slepecky & Hemphill 2006, Lutz et al., 2006). Moreover, some *Bacillus* species are heterotrophic nitrifiers, nitrogen fixers, denitrifiers, manganese reducers, oxidizers, acidophiles, facultative chemolithotrophs, psychrophiles, alkalophilic, and thermophiles, etc. These physiological properties of *Bacillus* species allow

colonization of a wide variety of ecological niches. The ability of immense ecological diversification was affected by the production of spores characterized by their remarkable ability of resistance and dormancy. It is also well known that strains of genus *Bacillus* produce a wide arsenal of antimicrobial compounds, including peptide and lipopeptides antibiotics and bacteriocins. Most of these bacteriocins are associated with lantibiotics, which are post-translationally modified peptides and widely distributed among different bacterial groups (Stein et al., 2005). Thus, properties like sporulation capacity and capability to produce antimicrobial compounds provide *Bacillus* strain good advantages in terms of their survival in various ecological habitats. The strain of *Bacillus* present in the food involved in spoilage is not always bad and could even be used for human and animal welfare. The metabolites produced by *Bacillus* species like peptide antibiotics, bacteriocins, glycopeptides, lipopeptides, and cyclic peptides (Baindara et al., 2013; Caldeira et al., 2008), also show antagonistic activities against different pathogenic organisms. For example, mersacidin, a lantibiotic tetracyclic peptide, was isolated from *Bacillus* species (Brotz et al., 1995; Chatterjee et al., 1992), showed activity against methicillin-resistant *Staphylococcus aureus* (MRSA), which was comparable to vancomycin and teicoplanin (Jenssen et al., 2006). So, AMPs from the genus *Bacillus* are a promising alternative to some conventional antibiotics, and their effective treatment combats a single-drug or multi-drug resistance pathogens.

2.6.2.1. *Bacillus subtilis*: The group of *B. subtilis* comprised of small vegetative cells (<1 µm wide) for which the strain *B. subtilis* subsp *subtilis* 168 is considered as a model organism (Barbe et al., 2009). These are mesophilic and neutrophilic and also can tolerate high pH conditions. More than 40 years ago, the four original species in this group are discovered i.e. *B. subtilis*, *Bacillus punilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* (Gordon et al., 1973; Priest et al., 1987). The strains of the *B. subtilis* group have the potential to produce a wide diversity of secondary metabolites and were recognized for decades. It was estimated that any strain of *B. subtilis* group has at least 4-5% of its genome allocated to antimicrobial compounds; which mainly includes antimicrobial peptides (AMPs) (Stein et al., 2002a). The structure of AMPs is usually cyclic, hydrophobic and consists of D-amino acid moieties or thioether bonds. The AMPs from this group are generally antifungal and antibacterial compounds (Stein, 2005). *B. subtilis* (ATCC 6633) produces several lantibiotics, two lipopeptides, surfactin, mycosubtilin, and iturin. Also, the strains of *Bacillus* produces other antimicrobial substances which are characterized to a lesser extent (Bierbaum et al., 1995; Paik et al., 1998) (Duitman et al., 1999). Due to the vast diversity of these AMPs, their

classification is complicated and based on several criteria i.e., biosynthetic process, sources, properties, biological functions, structure, the pattern of covalent bonding, or target specificity (Tagg et al., 1976; Wang et al., 2015).

2.6.2.3. Biosynthesis of *Bacillus* antimicrobial peptides

Based on the synthesis pathway, AMPs from genus *Bacillus* can be categorized into two subclasses i.e. ribosomally synthesized peptides and non-ribosomally synthesized peptides (Marx et al., 2001; Nakano and Zuber, 1990).

2.6.2.3a. Non-ribosomally synthesized peptides: Non-ribosomal synthesis of the peptide in bacteria is a multistep mechanism that serves as a template and biosynthetic machinery. It involves selecting and condensation of amino acid residues with a multienzyme thio template mechanism i.e., independent of ribosomes (Bushley and Turgeon, 2010). A non-ribosomal peptide synthetases enzyme mediates the mechanism of synthesis. The synthesis of these peptides, a large multi-subunit enzyme is ranging from 100 to <1600 kDa plays a significant role in this mechanism (Stachelhaus and Marahiel, 1995). More than 300 different precursors are being used from which these non- ribosomally peptides are assembled. The enzymes of non-ribosomal synthesis are incorporated into a specific manner i.e., one amino acid to the peptide backbone.

Further, the module can be divided into domains which catalyze each step in the synthesis of non-ribosomal peptides. Each module contains three domains: (A) adenylation domain, (PCP) peptidyl carrier protein, (T) thiolation domain, (C) condensation domain, involved in synthesis of non–ribosomal peptides. For example, the Fig. 5 presents a biosynthesis of surfactin was shown (Martínez-Núñez and y López, 2016). Usually, the modules' order is co-linear to the product peptide sequence (Finking and Marahiel, 2004; Hahn and Stachelhaus, 2004). The synthesis of non-ribosomal peptides results in N- terminal to C-terminal direction and mostly contains 3-15 residues of amino acids (Mootz et al., 2002). Structurally, these peptides are linear or cyclic, containing cyclic, branched structures with hydroxyl group, L, or D-amino acids. Further, these peptides are synthetically modified by the addition of tailoring enzymes after post-modifications i.e., acylation, glycosylation, N-, C- and O-methylation, heterocyclic ring formation, halogenations, etc., (Hancock and Chapple, 1999; Losey et al., 2001; Mootz et al., 2002; Schiffer et al., 1992). Peptides that are non-ribosomally synthesized from *Bacillus* genus are tyrocidine, gramicidin, surfactin, bacitracin, iturins, etc. (Chen et al., 2008; Stachelhaus and Marahiel, 1995). Moreover, antibiotic peptide

synthesis could occur through an alternative mechanism, for example, surfactin, a 7- amino acid residue lipopeptide isolated from *B. subtilis* synthesized differently from multienzyme thiotemplate mechanism (Nakano and Zuber, 1990). The regulation of surfactin biosynthesis includes the mechanism of controlled quorum sensing and the fermentation medium components, i.e., glucose and glutamine (Nakano and Zuber, 1990; Schallmey et al., 2004).

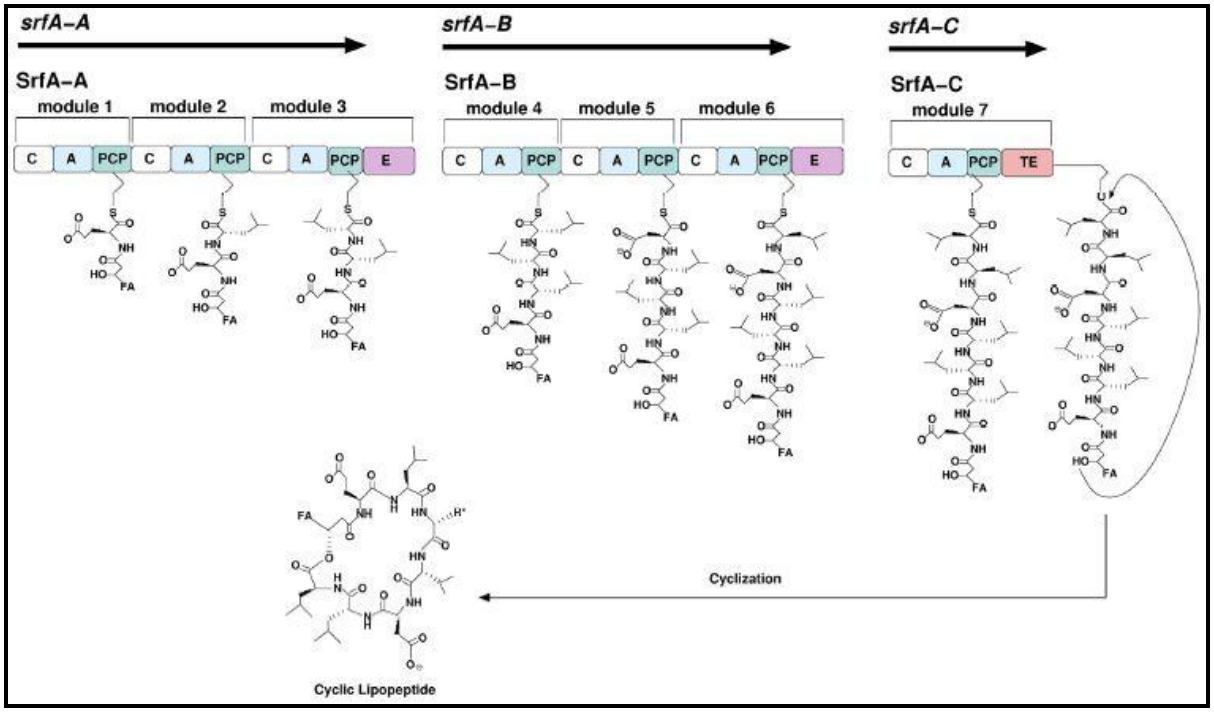


Figure 5. Structure of non-ribosomal peptide synthetase enzymes in the biosynthesis of surfactin: NRPS enzyme further divided into various modules by adding one amino acid each. There are three domains of each module: (A) adenylation domain, (PCP) peptidyl carrier protein, (T) thiolation domain, (C) condensation domain, (E) epimerization domain and (TE) thioesterase domain(Martínez-Núñez and y López, 2016).

2.6.2.3b. Ribosomally synthesized peptides: Ribosomally synthesized peptides are widely distributed in nature, consisting of 12 to 50 amino acid residues, and are typically cationic (Marx et al., 2001). Ribosomally synthesized AMPs are known as 'bacteriocins' and are active against other bacteria of the same species and other genera (Aunpad and Na-Bangchang, 2007; Papagianni, 2003). These peptides consist of heterogeneous groups i.e. amphiphilic and (or) hydrophobic AMPs (Oscariz et al., 1999). Furthermore, bacteriocins from *Bacillus* species shows a broader antimicrobial spectrum as compare to other lactic acid produced bacteriocins (Wang et al., 2014). Based on chemical structure, molecular mass, heat stability, enzymatic sensitivity, modified amino acids, and action mechanism, these

bacteriocins can be classified into various subclasses (Motta et al., 2008). In 1993, the bacteriocins were first classified by Klaenhamner and categorized into 4 subclasses: class I lantibiotics are modified amino acid lanthionine and small peptides (<5 kDa), whereas class II are small peptides consists of one or more disulfide bonds which are essential for their activity. The peptides of this class are <10 kDa and are heat stable membrane-active peptides. Class III consists of active sulfur- hydrogen group with a higher molecular mass i.e., <30 kDa, and class IV consists of complex proteins having one or more lipids or carbohydrate moieties (Klaenhammer, 1993). Further, the bacteriocins from the genus *Bacillus* were proposed by Abrioul *et al*. They were categorized into 3 subclasses. Post-translationally modified peptides are included in class I, whereas class II consists of non-modified peptides and class III contains larger proteins (Abriouel et al., 2011). However, class I will further subdivide into 4 subclasses and class II divided into 2 subclasses (Abriouel et al., 2011). In 2011, a group of Rea *et al*. updated Gram-positive bacteria's classification: class I includes modified peptides having lantibiotics and lanthipeptides, sactibiotics, and labyrinthopeptins. Class II non-modified peptides consist of pediocin like peptides, circular bacteriocins, 1 & 2-peptide bacteriocins, and linear non-pediocin like peptides. And lastly, class III consists of non-bacteriocin lytic peptides (Drider and Rebuffat, 2011). Lantibiotics and pediocins like peptides are further divided into four subclasses whereas sactibiotics, circular bacteriocins, and 2-peptide bacteriocins are divided into two subclasses (Drider and Rebuffat, 2011). Based on ribosomally synthesized bacteriocins Cotter and his colleagues in 2013 suggests a classification; in which class I consist of post-translational modified peptides consisting of lantibiotics, sactibiotics, and glycosins. However, class II i.e., unmodified and cyclic peptides are divided into 5 more subclasses (Cotter et al., 2013).

Lantibiotics are one of the best characterized AMPs; these are small peptides consisting of various amino acid residues, genetic determinants, and biosynthesis mechanisms (McAuliffe et al., 2001; Pattnaik et al., 2001). The premature peptides undergo post-translational modifications during maturation by adding unusual amino acids like lanthionine and methyllanthionine; the leader peptide undergoes proteolytic cleavage (Dischinger et al., 2009; Lawton et al., 2007). Typically mature lantibiotics contain one or more unusual dehydro-amino acids, which do not participate in the lanthionine bridges but may be useful in designing novel peptides (Paik et al., 1998). Various types of lantibiotics are well characterized i.e. Nisin A and Z, subtilin, epidermin, epicidin, and mersacidin (McAuliffe et al., 2001).

2.6.3. *Subtilin* is a single elongated type A-lantibiotic peptide that is cationic, pentacyclic, and a typical example of lantibiotic produced by B. *subtilis* has been widely studied in terms of its structure and genetic determinants (Gross et al., 1973; Guder et al., 2000; Sahl and Bierbaum, 1998). The genes which encode a subtilin peptide are precursor of 56 amino acid residues, and is further processed to yield 32 mature amino acid residues with the molecular mass of 3319.56 Da was revealed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI –TOF) (Banerjee and Hansen, 1988; Stein, 2008). The residues of serine, cysteine, and threonine are present in the precursor at a position which allows them to undergo a series of dehydration and cross-linking steps to yield a mature subtilin. It contains the unusual amino acids after maturation, i.e., lanthionine, β-methyllanthionine, D-alanine, dehydrobutyrine, and dehydroalanine. For an exported protein, the precursor peptide consists of a leader region that has unusual hydropathic characteristics. Its leader sequence shows 57% similarity with the leader sequence of nisin Z, whereas the pro region of subtilin and nisin Z shows 61% identity. For the biosynthetic gene cluster that encodes similar proteins, the subtilin shows high structural similarity with nisin (Siezen et al., 1996). The gene locus of the subtilin comprises the structural gene, i.e., spas, that encodes the subtilin's precursor and encodes proteins involved in the post-translational and modification of pre-subtilin precursors i.e., spaB which encodes dehydratase and spaC encodes cyclase. Further, the cluster also consists of genes responsible for the secretion of modified precursor, i.e., spaT, a transporter precursor, and the immunity gene (spaIFEG) against the associated bacteriocin. spaFEG helps in forming ABC transporter genes, which exports lantibiotic from the cytoplasmic membrane; on the other hand, spaI encodes for lipoprotein, which hampers the binding of bacteriocin to lipid II molecule (Koponen et al., 2004; Stein et al., 2005). The components i.e., spaB, spaT, and spaC are responsible for the modification and secretion of subtilin. It has been observed that they are a complex of multimeric proteins that interact with the subtilin's precursor (Kiesau et al., 1997; Siegers et al., 1996). The last processing step, which converts the pre-subtilin to mature subtilin incorporates non-dedicated and unspecific serine proteases, secreted by *B. subtilis* (Corvey et al., 2003). The subtilin production is regulated at the transcriptional level in a cell-density-dependent manner by quorum sensing. However, this autoregulatory module contains the subtilin lantibiotic itself as an auto-inducing agent (Kleerebezem et al., 2004; Klein et al., 1993; Stein et al., 2002b). Signal transduction is by the corresponding two-component regulatory system made of spaKR and the lantibiotic responsive; these three independent transcriptional promoters lead the *spaB, spaS,* and *spaI* (Kleerebezem et al., 2004; Klein et al., 1993; Stein et al., 2002b). When the extracellular

subtilin reaches its threshold concentration, it further activates the membrane located spaK, which auto-phosphorylates. However, this leads to the phosphorylation of the cytosolic spaR, which binds to the DNA motif in its active form and promotes the biosynthesis expression (*spas* and *spaBTC*) and immunity (*spaIFEG*) genes of subtilin. Further, the expression of SpaRK is controlled by the sporulation transcription factor i.e., SigH, and is repressed during the exponential phase of growth by the transition-state regulator i.e., AbrB (Fawcett et al., 2000). Hence, the production of the subtilin is linked to both i.e. cell density and sporulation. The subtilin production depends on the adaptive response to change in the environment, i.e., reducing the nutrient levels, which allows the cell to benefit optimally from the available resources. However, under starvation conditions, the subtilin is produced in a high quantity compared to less quantity when provided sufficient nutrients. The bactericidal activity of subtilin is via pore formation in the cytoplasmic membrane using cell wall precursors such as lipid II and undecaprenyl pyrophosphate. The hydrophobic carrier module for peptidoglycan monomer acts as a docking module and central constituents of the pore. The mode of action of subtilin is by the dissipation of transmembrane proton motive force, resulting in the formation of the pores. It shows bactericidal activity in lower concentration range against the pathogenic organisms (Bonev et al., 2004; Breukink et al., 2003; Parisot et al., 2008; Schuller et al., 1989). Subtilin B is a variant of subtiln isolated from *B. subtilis* ATCC 6633 and shows less antibacterial activity i.e., < 90%. This is because of post-translational succinylation of the amino group of the N- terminal tryptophan residues with the molecular mass of 3419.58 (Chan et al., 1993; Stein, 2008).

2.7. Purification of bacterial AMPs

It is essential to obtain AMPs in purified form to initiate its characterization studies. Purification of small peptides like bacteriocins is the most challenging step. Troubles encountered during purification of antimicrobial peptides are related to the propensity of such molecules associated with other properties like their hydrophobicity, amphipathicity, etc.

Mostly bacteriocins are secreted extracellularly in the growth medium; thus, bacteriocins in crude form consist of undesirable residual medium components that could be toxic/ undesirable in various applications. For this, different strategies were used for purification of peptides, to start with a step to concentrate peptides from the culture medium by different processes i.e., pH-dependent adsorption of peptides on producer cells or by heat killing the producer cells, rice hull ash, diatomite calcium silicate, silicic acid precipitation, ammonium sulphate precipitation or organic solvent extraction (Carolissen-Mackay et al., 1997;

Coventry et al., 1996; Venema et al., 1997). These strategies are only used to reduce the working volume but do not provide purity of peptides. However, several strategies are reported for the purification and recovery of bacteriocins. But three strategies are mainly used for the recovery and the purification of bacteriocins i.e., conventional multistep method, single-step bed adsorption, and simple three-step method (Piva and Headon, 1994). Using a conventional method, peptides yield is low, maybe due to multiple steps involved in this method, i.e., ammonium precipitation, ion exchange, hydrophobic interaction, gel filtration, and reverse-phase high-pressure liquid chromatography. Due to this multi-step method, one of the significant problems is less recovery of the purified bacteriocins (Piva and Headon, 1994). Many three-step protocols have been developed for the purification of bacteriocins from complex media with high recovery rate i.e., (1) ammonium sulphate precipitation/ hydrophobic interactions on resins like Diaion HP-20, (2) ion-exchange chromatography and (3) reverse-phase high-pressure liquid chromatography (Ghrairi et al., 2008; Singh et al., 2012).

Ammonium sulphate precipitation is commonly used as a primary concentration step, concentrating the peptides from the cell-free supernatant for volume reduction. It also increases the specific activity against the target organisms (Muriana and Klaenhammer, 1991).This salting-out method is commonly used due to high solubility and low-cost (Pingitore, 2007). Ammonium precipitation works by reducing the repulsion of similar molecules that can aggregate and separates the proteins from a medium by changing solubility in the presence of high salt concentration (Moore and Kery, 2009). Several factors need to be considered while choosing the saturation concentrations i.e., the initial volume of bacteriocins, desired volume after the procedure, the mass of the bacteriocin, absorptivity, and time requirement for the concentration, and desalting of bacteriocin (Pohl, 1990). Utilization of hydrophobic interaction chromatography that have resins to extract selective peptides e.g., HP-20, amberlite XAD-16, which results in high recovery of peptides as an alternative to the ammonium sulphate precipitation method (Lee et al., 2003b). Precipitations of peptides are also carried out by bringing down the dielectric constant of the peptides with organic solvents. The organic solvent precipitation must be carried out at a low temperature. A considerable amount of heat is released when solvents are added to the water and sometimes becomes a significant cause of peptide degradation (Jamuna and Jeevaratnam, 2004). For example, *Lactobacillus plantarum* ZRX03 produces bacteriocin from ethyl acetate, pediocin from Pediococcus acidilactici was partially purified by cold acetone (Jamuna and Jeevaratnam, 2004; Lei et al., 2020). Later, various steps are involved in the

purification i.e., size exclusion chromatography, ion-exchange chromatography, and reverse-phase high-pressure liquid chromatography, to polish the isolated product (Stern et al., 2006).

Also, bacteriocins low productive yield due to the variations in the physicochemical properties of bacteriocins, mostly their net charge (Tahiri et al., 2004). To overcome the trouble of low yield of peptides, techniques such as cation-exchange chromatography have been developed, based on the exploitation of hydrophobic C-terminal domains of the bacteriocins (Berjeaud and Cenatiempo, 2004). This chromatography technique is usually applied in bacteriocins purification because most of them are positively charged and near-neutral pH (Pingitore et al., 2007). For example, a 7-fold improvement was observed during the purification of pediocin PA-1/Ach following cation exchange chromatography (Gaussier et al., 2002). Due to bacteriocins amphiphilic properties, hydrophobic chromatography has been used to separate the highly hydrophobic bacteriocins (Borzenkov et al., 2014). Gel filtration chromatography could be included in the purification step and is generally used for the final polishing step (Tan et al., 2015). However, at the end of the purification RP-HPLC is usually used to eliminate any contaminants (Aran et al., 2015; Simha et al., 2012). These chromatographic techniques were suitable for the bacteriocins, as they are stable in organic solvents which are used as a mobile phase and high pressure was used in the HPLC chromatography (Pingitore et al., 2007). But the low yield was also correlated with these methods because of the large number of procedures are involved (Parada et al., 2007). The desalting technique or dialysis technique was used after cation exchange chromatography to reduce the ionic strength of the bacteriocin containing solution; it may also be reduced by diluting the bacteriocin solution with distilled water or suitable buffer. The difference in some bacteriocins relative thermostability, the partial purification is achieved by heat treatments. For example, a negligible contamination fraction in the crude colicin produced by *Shigella sonnei* P9 can be destroyed by pasteurization at 65o for 20 min, leaving it pure with respect to colicin E2 (Mayr-Harting et al., 1972).

The two-step purification method is a fast and time-saving protocol. The bacteriocins like pediocins and other cationic antimicrobial peptides are purified by directly applying the complex medium culture onto cation exchange chromatography followed by a low-pressure reverse phase chromatography (Uteng et al., 2002) yielding more than 90% purity. This method was quick and easy compared to three-step purification giving an 80% recovery of antimicrobial peptides (Uteng et al., 2002). Also, nisin was extracted by the two-step method via PEG/Salt aqueous phase. The PEG/Salt two-phase system has a difference in hydrophobicity between them as nisin is a hydrophobic polypeptide and was portioned to

PEG-rich top phase. This method was reported to be an economical approach in recovering nisin at a large scale (Li et al., 2001). The medium generally contains the complex nutrients for the optimal production of antimicrobial peptides, but a high amount of the peptides might obstruct the process of purification (Carolissen-Mackay et al., 1997). An ideal procedure for peptide production should be the one that is appropriate for large-scale purification, leading to yields of peptide/bacteriocins higher than 50% and purity around 90% (Schöbitz et al., 2006). For the higher yield of peptides, there is a need to reduce the overall number of purification steps, which also becomes more cost-effective. The group of Cheigh *et al.* has developed a one-step purification method, in which they employed bed ion-exchange chromatography for the purification of nisin Z from *Lactobacillus lactis* subp. Lactis A164. The purification from the unclarified culture broth of strain A164 resulted in a 90% nisin yield (Cheigh et al., 2004). An overall reduction in the total number of purification steps resulted in higher product yield and cost-effectiveness.

The techniques for the purification of bacteriocins employed by other researchers could be summarized as (i) anion exchange chromatography and reverse-phase HPLC, (ii) Ammonium sulfate precipitation (80%) followed by cation exchange chromatography (SP-sepharose fast flow cation column), hydrophobic interaction chromatography (phenyl-sepharose) and C2/C18 reverse-phase chromatography, (iii) Ammonium sulfate precipitation (55%) followed by hydrophobic interaction (C18) and cation exchange chromatography (iv) ammonium sulfate precipitation (40%) followed by cation exchange SP-sepharose (Todorov et al., 2000).

2.8. Techniques used in antimicrobial peptide studies and their action mechanism

2.8.1. Tricine SDS-PAGE: It has been observed that small proteins and peptides of molecular weight less than 14kDa cannot be separated by conventional SDS-PAGE using the Laemnli buffer system. The SDS-protein complexes formed by the small polypeptides having similar properties such as size and charge could move together and not separate on electrophoresis (Schagger and von Jagow, 1987). Tricine SDS-PAGE is generally employed to separate proteins in the molecular weight range to 1-100 KDa and is preferred for resolving the proteins with molecular weight less than 30 kDa. It is tough to visualize smaller peptides using SDS-PAGE, and tris-tricine provides improved resolution. In this protocol, the buffer's molarity is increased and tricine is used as an alternative to glycine as tailing ion. Tricine has a more negative charge than glycine; thus it permits to move at a faster rate. Meanwhile, its high ionic strength results in more ion movement and less protein movement, allows low molecular weight proteins to separate in lower percent acrylamide gels (Schagger, 2006).

2.8.2. Microscopic techniques

Techniques used for the assessment of antimicrobial peptides mechanisms include microscopic imagining. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are the conventional methods, which are used to visualize the effect of AMPs on the bacterial membrane structure. This cryo-preservation method used to visualize the morphological changes in the bacterial membrane structure in its native condition. And also, tells the perspective to understand the mode of action of AMPs at the molecular level. SEM microscopy gives high-resolution images to observe the protuberance related to the local destabilization of the bacterial cell envelop caused by AMPs. The global damage of the cell can be visualized as a generalized disturbance on cell morphology (Torrent et al., 2010). Fibrous material i.e., probably arising due to the leakage of the cell content and cell debris, are scattered around the bacteria while treating with AMPs (Yenugu et al., 2006). In TEM microscopy, with the simple negative staining of bacterial cells were visualize the effect of AMPs at the ultrastructural level; which reports the evidence of the mechanism of membrane distruption (Hammer et al., 2010). The ultrathin sections were obtained by conventional steps, namely fixation with aldehydes, post-fixation with osmium tetraoxides followed by dehydration, and further, allow the observation of membrane and cytoplasmic alterations (Torrent et al., 2009).

Moreover, confocal microscopy is also of great interest to be used in the studies of antimicrobial peptides. This microscopy helps to analyze the characteristics properties of each drug. The cell integrity and overall morphology and the population of the cells can be assessed via confocal microscopy wit the help of live-dead staining. It is also possible to assess the kinetic effect of AMPs on the bacterial membrane and also in meantime analyze the agglutination and other population behavior. SYTO 9 and Propidium iodide (PI) is the fluorescent staining dyes that assess the viability of the cells (Boulos et al., 1999). The dye SYTO 9 stains the DNA of the bacteria, whether the cell membrane is intact or damaged. Whereas, PI stains only damaged bacterial membrane and also displaces SYTO 9 when both are present in the same solution. The bacterial intact membrane stains green whereas the damaged cells stain red when both dyes are stained with an appropriate amount. However, these dyes are complement with the fluorescence microscopy as well as flow cytometry for the analysis of bacterial cell viability after treating with AMPs (Robertson et al., 2019; Rosenberg et al., 2019; Torrent et al., 2010). Thus, in conclusion, microscopic techniques are powerful techniques that can facilitate the mode of action of antimicrobial peptides. These techniques have a vast prospect for visualizing the molecular structure and functioning at

cellular content. While imaging techniques at ultrastructural levels provides the knowledge of AMPs structure and their effect at the cellular level at its native stage.

2.8.3. Microscopic analysis of membrane potential

In bacterial and eukaryotic cells, membrane potential plays a most important role. The effects of membrane potential on the action of antimicrobial peptides can be examined using cells and lipid vesicles (Moghal et al., 2020). Dissipation of membrane potential can be induced by ion-conducting membrane pores, by increasing membrane permeability or by acting as an ion carrier (Brogden, 2005; Wimley and Hristova, 2011; Yeaman and Yount, 2003). To rule out the membrane potential depolarization effect, a rapid method is provided i.e., a dye-based technique; which is used when there is extensive interference with the fluorescence is observed. to measure the membrane potential, a voltage-sensitive dye i.e., Dipropylthiadicarbocyanine iodide, $DISC_3$ (5) and Bis-(1, 3-Dibutylbarbituric Acid) Trimethine Oxonol, $DiBAC_4$ (3). $DISC_3$ (5) is a cationic membrane-potential dye. Due to ti its charged nature, it interacts with the sufficient hydrophobicity which further penetrates lipid bilayers and allows the dye to enter and accumulate into a polarized cell until a Nernstain equilibrium is achieved (Bashford, 1981; Waggoner, 1976). Due to the strong accumulation in the energized cells were results in the quenching of the fluorescence of the cell suspension. There is a rapid release of dye in the medium during depolarization of the cell which results in the dequenching of the fluorescence (Shapiro, 1994; Singh and Nicholls, 1985). On the other side $DiBAC_4$ (3) is an anionic dye that enters into the cell and the fluorescence was measured when membrane potential was changed. In the case of hyperpolarization, the fluorescence of $DiBAC_4$ (3) decreases unlike depolarization of cell membranes where fluorescence increases (Adams and Levin 2012; Yamada et al. 2001).

2.9. Applications of Antimicrobial Peptides

2.9.1. Antibiofilm properties of antimicrobial peptides

Bacteria in nature generally dwell a complex and dynamic surface-associated community called a biofilm. These are sessile microbial consortia which establish in a three- dimensional structure (Costerton et al., 1999). These communities of microbes adhere to various surfaces and confined in a self-produced extracellular matrix (Monroe, 2007). It might forms on living or non-living surfaces and can prevail in natural, industrial, and hospital settings. Bacterial cells dwelling in a biofilm are physiologically diverse from the planktonic cells of the same bacteria and are entrenched within the matrix of extracellular polymeric substance (EPS) (Hall-Stoodley et al., 2004). And also, there is a rapid increase in antibiotic resistance by up

to 1000 folds in biofilms (Stewart and Costerton, 2001). Less than 0.1% of the total microbial population has been estimated that it is in a planktonic mode (single bacterial cell population), but the majority of bacteria are organized in a complex structure i.e., biofilms. More than 80% of all the bacterial infections are caused by microorganisms in a biofilm mode growth (Evans and Bolz, 2019). There are numerous reasons which explain the unique phenotypic characteristics of bacteria within biofilm: (i) Slow or partial penetration of antibiotics due to the presence of matrix as a barrier, (ii) existence of altered micro-environment within the biofilm which includes localized anaerobic and acidosis, (iii) stimulation of a resistant phenotype due to distinctive modes of gene expression and (iv) presence of an increased population of persister cells in biofilms that are metabolically dormant cells but resistance to antibiotics (Walters et al., 2003).

Gram-positive *S. aureus* is a ubiquitous bacterial species. In humans, the inhabitant of *S. aureus* is the anterior nostrils. Approximately 20%-25% become persistently colonized in humans and 75-80% is in steady-state and never colonized in humans (Dall'Antonia et al., 2005; Kluytmans et al., 1997). It was observed that there is a strong causal relation between *S. aureus* nasal carriage and also increases the risk of nosocomial infection. Nasal carriage provides a space for *S. aureus* and spread to other parts of the body. Once it transmitted to the circulatory system through an epithelial opening the planktonic growth and up-regulation of adherence factors occur (Beenken et al., 2004; Fitzpatrick et al., 2005). Invade staphylococci are removed by the host innate immune response or may be attached to the host extracellular matrix proteins and form a biofilm.

A multilayered biofilm produced by *S. aureus* which embedded within the glycocalyx or slime layer with the heterogeneous expression of proteins. Earlier it was described that the solid component of glycocalyx is composed of ~ 80 % of teichoic acid, staphylococcal and host proteins (Hussain et al., 1993). Later, a specific polysaccharide antigen called polysaccharide intercellular antigen (PIA) was isolated. The PIA was composed of 80-85% β-1, 6 linked N-acetyl glucosamine residues, and 15-20% anionic fraction of N-acetylated D-glucosaminyl residues which contains phosphate and ester-linked succinate (Mack et al., 1996).

The maturation of biofilm is associated with EPS production with the maturation of biofilm and allows the stability to it. The dispersion, the last phase corresponds to the release of cells that colonize a new site. The micro environmental conditions like oxygen level, pH, etc. are changed by mature biofilms and also weaken the effect of antibiotics. Mature biofilms also create the condition which induces the state of dormant and non-dividing cells known as

persisters, it has also played a crucial role in resistance. EPS produces an appropriate environment for the bacteria i.e., it favours cell to cell adhesion and cell to the surface; also forms a barrier interfere within the activity of antimicrobial agents and decreases its biological activity. Biofilm cells can communicate through the secretion of molecules which are auto-inducers and control the quorum sensing (QS). A drug which exhibits anti-biofilm properties are not available, many studies are devoted to designing AMPs that can display a combination of these features (de la Fuente-Nunez et al., 2014; Haisma et al., 2014).

Widely distributed antimicrobial peptides are a promising class of compounds, part of the innate immunity of organisms, and also a potential alternative to conventional antibiotics to prevent infections from pathogenic organisms (Ebbensgaard et al., 2015; Galdiero et al., 2015; Pasupuleti et al., 2012; Yeaman and Yount, 2003). As we know most of the AMPs are positively charged, amphiphilic molecules that kill bacteria via membrane disruption or pore formation and also targets intracellularly such as inhibiting proteins, cell wall, or enzyme synthesis (Pletzer and Hancock, 2016). Various studies reveal that AMPs are active against multidrug-resistant bacteria as well as fungi i.e., not only by disrupting the membrane due to change in the membrane potential but also interfere with the metabolic processes by inhibiting cell wall synthesis, nucleic acid synthesis or protein biosynthesis. Thus, these properties of AMPs render it more difficult for bacteria to develop a resistance against it (Seal et al., 2018). Moreover, positive charges on AMPs are essential for their activity and also represent an obstruction to AMPs penetration into biofilms. Because the polysaccharide of biofilm is negatively charged this traps the positively charged AMPs and masks its activity against bacterial biofilm (Yasir et al., 2018). Some studies show that how AMPs and their modified sequence obtain the novel antibiofilm agents with increased activity and proteolytic stability. LL-37 shows its activity against *S. aureus* and prevents against the biofilm formation. Apart from its potency against bacteria, its usage is limited because of cytotoxicity against mammalian cells and also it is easily cleaved by endogenous enzymes that are present in the gut i.e., trypsin and pepsin. Meanwhile, LL-37 also cleaved by enzymes secreted by *S. aureus* such as aureolysin and V8 protease (Batoni et al., 2011; Scudiero et al., 2010; Zapotoczna et al., 2017; Zhu et al., 2013). Recently, a membrane-penetrating peptide i.e., gH625 and its analog gH625-GCGKKKK were both active against planktonic cell and biofilms of *S.aureus*, *Candida tropicalis,* etc. The modified analogue shows stronger eradication of bacteria at a concentration lower than that of its minimal inhibition concentration on planktonic cells (de Alteriis et al., 2018). The three bacteriocins i.e., nisin A, nukacin ISK-1, and lacticin Q, destroys the membrane potential of biofilm of *S. aureus* and

its MRSA strains and cause releases ATP from the cells (Okuda et al., 2013). RN9 (5-17P22-36), an engineered peptide derived from cationic proteins of eosinophil granules kills bacteria through membrane disruption (Acharya and Ackerman, 2014; Pulido et al., 2016; Venge et al., 1999). AMPs can also target the extracellular matrix of bacterial biofilms such as peptide PI degrades the EPS which is produced by *Streptococcus mutans* leads to the reduction of biofilm formed on polystyrene/ saliva-coated hydroxyapatite (Ansari et al., 2017).

The mechanism of degradation of biofilm is not yet clear, the fast destruction of biofilm embedded cells indicated by the membrane disruption. There are various mechanisms of inhibition of biofilm and degradation by AMPs and are listed below and Fig. 6 (Haney et al., 2017; Sun et al., 2017).

1. Degradation/disruption of the membrane potential of biofilm embedded cells
2. Interrupting the bacterial cell signaling system
3. Polysaccharide and biofilm matrix degradation
4. Alarmone system inhibition to avoid the bacterial stringent response
5. Genes responsible for the formation of biofilm and transportation of binding proteins are down-regulated by the AMPs

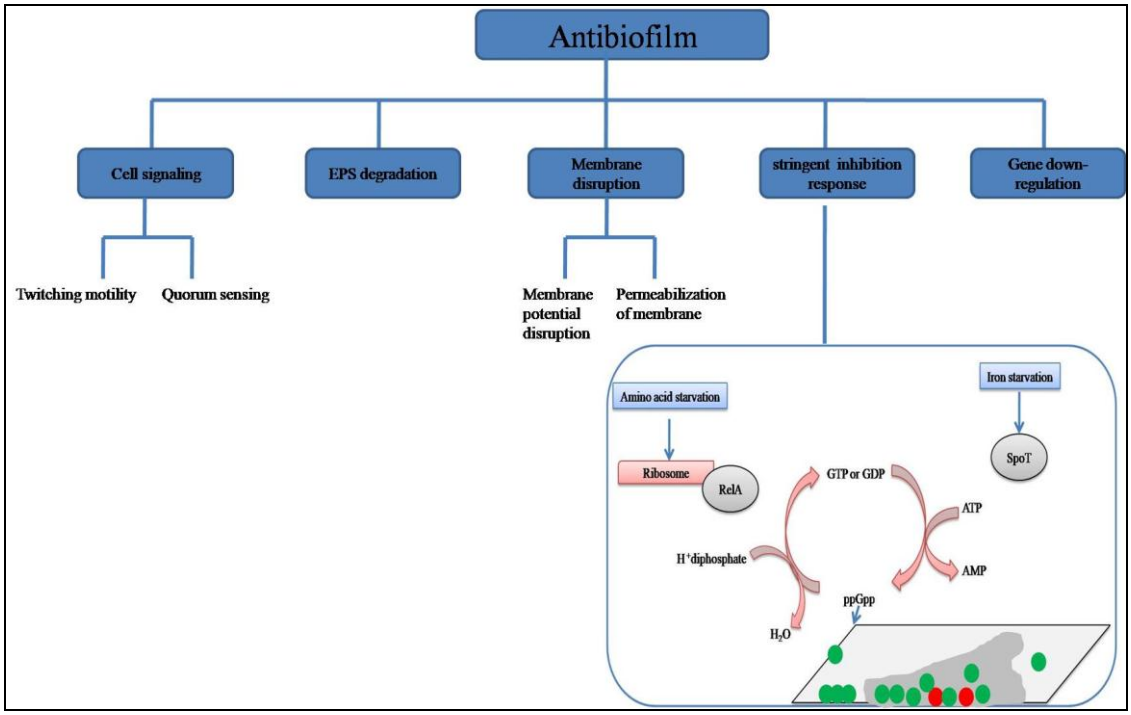


Figure 6. Mechanism of action of AMPs against biofilm: The mechanisms of stringent response inhibition were in more detail. The alarmone leads to the formation of biofilms. The biofilm contains live cells (green) and dead cells (red) as well as EPS (grey).

2.10. Optimization of media and process parameters

The use of microorganisms and numerous important compounds which have applications in pharmaceutical, chemical, and food industries; the fermentation technology is widely used for their production and to meet the economic necessity (Singh et al., 2016). By using the fermentation process the viable yield of products can be increased and also it reduces the effectiveness of cost to meet the market demand (Singh et al., 2016). Many microorganisms have been reported for the production of primary metabolites (amino acids, proteins, nucleotides, lipids, etc.) and secondary metabolites (antibacterial, antifungal, etc.) which are widely used in the various industries at large scale (Demain, 2000). Currently, there are limited known facts about the role of factors and their levels in controlling the metabolite production by various strains. The requirement of nutrients for the productivity of secondary metabolites (AMPs etc.) differs from strain to strain. The determination of microbial nature and its metabolic activity depends on the quality and the quantity of the available nutrients (Venegas-Ortega et al., 2019). One of the most important phenomena for the production of metabolites at large scale is the optimization of media where medium components or different parameters can be varied in terms of concentration or replaced for higher productivity and desirable growth of the microorganisms. In the case of microbial product estimation of growth parameters i.e., pH, temperature, and medium components (carbon, nitrogen, etc.) are more relevant for optimizing the fermentation conditions for maximum product yield. (Juarez Tomas et al., 2002). For the production of antimicrobial peptides, the most important aspect is its production and purification as well. In the complex media, the production of AMPs is very low and also interferes with its purification. For that, optimization of the media is required which can depend on various factors and also on specific strains (Suganthi and Mohanasrinivasan, 2015). There is an intense effect of bacterial growth on the production of the active antimicrobial peptide, mainly during exponential phase under favourable conditions i.e., the production increases with the high cell densities but in many cases, the high cell densities are not responsible for the high production of AMPs. So it is important to check the numerous growth conditions which affect the production of AMPs. Hence optimization of the media has a major significant effect on production and enhancing the yield of AMPs at its maximum concentration in an economical manner (Lenzi et al., 1986; Mortvedt-Abildgaa et al., 1995).

Few methods are reported through which the optimization of fermentation media can be done i.e., classical method and statistical method.

2.10.1. The classical method for media optimization

The technique used in the classical media optimization method is one-variable at a time (OVAT), which is used to obtain the maximum production in the fermentation process. Instead of checking multiple factors, this technique is used to design by using one factor at a time by keeping another variable constant (Abou-Taleb and Galal, 2018). This technique is one of the most preferred choices for designing the medium composition used in initial stages in various fields (Gonzalez et al., 1995). But the major disadvantage of this technique is in finding the difficulty in calculating the interactions between the various variables/factors and is inappropriate for several optimizing factors (Gupte and Kulkarni, 2003; Wang and Liu, 2008). And also OVAT is a laborious and time-consuming method which often leads to an incomplete understanding of system behaviour and resulting in the insufficient anticipating the ability of factors and also confuse the data (Ibrahim and Elkhidir, 2011).

2.10.2. Statistical method for media optimization

To optimizing the medium condition one of the most effective strategies is the statistical design method which has found new techniques and dimensions for the improvement of the process efficiency and reduces the processing time as well as labour cost as compared to the classical method (Li et al., 2007). As we know the microbial process contains natural variations in a large amount. The systems integrated with microbial reactions are composite and these systems are affected by various factors on its different parts (Elibol, 2004). In this method, a statistical equation is derived by using the effect of various factors and their interactions and also reduces the amount of required experiment for process optimization (Al Mamun et al., 2017).

2.10.3. Plackett Burman Design (PBD)

It is a widely used statistical method to evaluate relatively important different variables or medium components for a particular production and also screen all the non-contributing factors and eliminate from the medium components for the production of microbial metabolite e.g., antibiotic or other cellular metabolites. While using the PBD method the no. of experiments decreases excessively (Adinarayana and Ellaiah, 2002; Ganesan et al., 2017). Thus, this design is efficient in screening the main effects of interest by neglecting another interacting factor while comparing it with major important effects of factors. This is a statistical method where ‘n’ variables are studied with ‘n+1’ experimental runs (Naveena et al., 2005). There are two types of variables, each represented in high (+) and low (-) levels.

Further, these are '*real variables'* (concentration changes during the experimental runs and another is *'dummy variables'* (concentration remains constant during experimental runs) as well as used to calculate the error (Ekpenyong et al., 2017). Moreover, during the present studies, the optimization of media for the production of antimicrobial peptide from various microbial species, the PBD method found to be more efficient to identify the most effective components in the media for obtaining the maximum yield (Ghribi et al., 2012; Saraniya and Jeevaratnam, 2014). But there are some drawbacks while using the PBD method which is correlated with its efficacy. This method is used only when there are no interactions with the variables, else the variables which are to be analyzed will be masked by another variable and it fails to decode the effect of one variable depends on another variable. Usually, the PBD method is known to only screen the designs to help out only non-contributing variables from that of contributing variables for the production of higher-yield products.

2.10.4. Response Surface Methodology (RSM)

This study is a more efficient and rousted method with the mathematical approach also includes the statistical design of experiments with multiple regression analyses, for searching for the better formulation of variables and optimizing the fermentation process. Thus, this technique is more significant for the determination of the interactions between the dependent and independent variables, which also reduces the number of experimental runs (Dinarvand et al., 2013; Khare et al., 2015). For example, in the case of secondary metabolite production, the use of this methodology is more efficient which simplifies the purification and isolation of the product with high yield and good quality (Yang et al., 2017). In 1951, Box and Wilson described a new method i.e., Central Composite Design (CCD) which is widely used in the RSM design (Box and Wilson, 1951). The number of experiments used in this design is to develop a quadratic model for the response of the variables. Through which the direct and interactions of effective variables can be calculated (Aghdasinia et al., 2016). The CCD design consists of three different kinds of experimental set up i.e. **1. Factorial design**: in which the studied factors has two levels (+) and (-). **2. Centre point:** in this, each factor has its median value in experimental runs. This point replicates frequently to improve the accuracy of the design of the experiment. **3. Star point:** in this, except for the one factor the experimental runs are identical to a centre point. The value will be taken as a median of low and high of the two factorial levels. In this design, the number of star points is double the number of factors used (Singh et al., 2016).

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. EQUIPMENT USED

Device	Model	Company
Centrifuge	1. 810 R 2. 5814 3. 6K15	Eppendorf, Germany Sigma, USA
DNA quantifier	Nano Drop ND-1000	Thermo Fischer Scientific, USA
Dry Bath Genei	SLM _DB-120	Bangalore , India
Thermomixer C		Eppendorf, Germany
Scanning Electron Microscope	EVOb40 Zeiss	GmbH, Germany
Horizontal Gel Electrophoresis system	Genei	Genei, India
Magnetic stirrer	1 MLH	Remi Equipment, India
DNA sequencer	ABI 310 genetic analyzer	Applied biosystems, USA
microfuge	Centrifuge 5424	Eppendorf, Germany
Multi-Temp Water Circulating Bath	HMT300	Heto
PCR Machine	Mastercycler nexus gradient	Eppendorf, Germany
pH Meter	Mettler toledo	Mettle toledo , USA
Plate reader	Powersave 340	Biotek, India
Power pack	EV261	Consort, UK
SDS-PAGE Gel Electrophoresis System	Z37,240-4	Sigma chemical Co, USA
Sonicator	S-4000 Misonix	USA
Spectrophotometer double beam	Cary 100 Bio	Varian, USA
Spectrophotometer single beam	UV-2550	Shimadzu, Japan
Spectrophotometer	Kinetic UV-2550	Eppendorf, Germany
UV transilluminator	Benchtop 3UV	UVP, USA
Purification system	AKTA Pure	GE, USA
Vortex Mixer	Vortex Genei 2	Bangalore Genei, India
Water Bath	C76 Shaker	NBS,NJ, USA
Lyophilizer	Free ZONE$^{6\ Plus}$	Labcono, Kansas City, USA
Weighing Balance	B502 (1g-510g) Ms104S (10mg 120mg)	Mettler Toledo, USA
HPLC	Shimadzu	Japan
Flow Cytometer	Accuri C6	Becton Dickson, USA
Rotavapor R-300	Buchi	Switzerland
Mass Spectrometer	Voyager DESTR	Applied Biosystems, USA
Incubator	INNOVA 42	NBS, NJ, USA
Laminar Air Flow	1300 Series, A2	Thermo Scientific, USA
Gel Documentation Unit	G: BOX	Syngene, USA
Transmission electron microscope (TEM)	JEM- 2100CR	USA
Freezer (-20^{o}C)	Vestfrost	Vest frost Solutions, Denmark
Freezer (-80^{o}C)	U535	NBS, NJ, USA

3.2 MATERIALS

The different reagents used during the work are listed here: Routine chemicals were of analytical grade, procured from Sigma-Aldrich Co. (St. Louis, MO, USA), USB, Merck, HiMedia. India.

Molecular Biology reagents and kits were procured from Axygen, USA; Qiagen, USA; New England Biolabs (NEB), UK and Fermentas, USA. The list includes:

1. Bacterial genomic DNA Mini Prep kit (AXYPREP, Axygen, promega)
2. Plasmid Mini-prep kit (QiA®Prep, Qiagen)
3. 6X Gel loading Dye (NEB)
4. 1kb and 100bp DNA ladder (NEB)

Proteins and enzymes analysis reagents were obtained from Sigma- Aldrich (St. Louis, MO, USA). Chemicals used were.

- **Tricine SDS-PAGE**- acrylamide, N, N'-methylene bis-acrylamide, Ammonium persulphate (APS), N,N, N', N'- Tertamethylenediamine (TEMED), Trizma Base, Sodium Dodecyl sulphate (SDS), Coomassie Brilliant Blue R-250, Tricine.
- **BCA Reagent Assay kit**- BCA reagent was prepared according to QuantiPro™ BCA assay kit. 25 part of QA and 25 Part QC were mixed to one part of 4% copper (II) sulphate penthydrate) giving a light green solution
- Polypeptide SDS-PAGE molecular standard was obtained from Bio-Rad (USA).
- Low molecular weight SDS-PAGE marker, SP-Sepharose fast flow matrix, Sephadex-G10 (PD-10 Desalting Columns were obtained from GE health care (UK).
- Sun Fire® C18 OBD™ Prep Column, 100 Å, 5 µm, 10 mx250mm semi prep, Water Corporation, USA.
- Diaion HP-20 (Supelco) resins were obtained from Sigma-Aldrich (St. Louis, MO, USA).
- Microtitter plates were procured from Eppendrof (USA scientific, Inc., New Brunswick Scientific, Germany).
- Silica gel plates were procured from Merck, Darmstadt, Germany
- SunFire® C18 OBD™ Prep Column, 100Å, 5µm, 10 mm × 250 mm, part no.186008155 was procured from Water Corporation, USA
- Kinetex® 5µm F5 100Å, LC Column 250x 4.6 mm part no. 00G-4724-F0 was procured from Phenomenex, India Pvt. Ltd.
- SP-Sepharose Fast Flow, Code No. 17-0729-01 was procured from Pharmacia, Uppsala, Sweden

3.2.1 MEDIA COMPONENTS

Luria Broth (LB) (g/L)	
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00
pH	7.00±0.2

For making plates, agar 15g/L was added to the LB broth

Tryptone Soya Broth (TSB) (g/L)	
Pancreatic digest of casein	10.00
Papaic digest of soya bean meal	5.00
Sodium chloride	10.00
pH	7.30±0.2

For making plates, agar 15g/L was added to the TSB broth

Zobell's Marine Broth (ZMB) (g/L)	
Peptic digest of animal tissue	5.00
Yeast extract	1.00
Ferric citrate	0.10
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.24
Calcium chloride	1.80
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Sodium fluorate	0.002
pH	7.6 0±0.2

For making plates, agar 15g/L was added to the ZMB broth

Brain Heart Infusion Broth (BHI) (g/L)	
Calf brain, infusion from	200.00
Beef heart, infusion from	250.00
Proteose peptone	10.00
Dextrose	2.00
Sodium chloride	5.00
Disodium phosphate	2.50
pH	7.40±0.2

For making plates, agar 15g/L was added to the BHI broth

Mueller Hinton Broth (MHB) (g/L)	
Beef infusion	300.00
Casein acid hydrolysate	17.50
Starch	1.50
pH	7.40±0.2

For making plates, agar 15g/L was added to the MHB broth

Nutrient Agar (NA) (g/L)	
Peptone	10.00
Beef extract	10.00
Sodium chloride	5.00
pH	7.40±0.2

For making plates, agar 15g/L was added to the NA broth

3.2.2. Stock buffers for agarose gel electrophoresis

50X TAE (Tris-Acetate EDTA) buffer

Tris Base	242.00g
Glacial acetic acid	57.00ml
0.5M Ethylenediaminetetraacetic acid (EDTA) (pH 8.0)	100 ml

Double distilled water was added to the volume of 1L. For gel electrophoresis the buffer was further diluted to 1X

6X DNA loading buffer

Bromophenol blue	0.25% (w/v)
Xylene Cynol FF	0.25% (w/v)
Sucrose	40.0% (w/v)
Tris-EDTA buffer (pH 8.0)	Upto 10 ml

Ethidium bromide stock solution (1% w/v)

Ethidium bromide	0.10g
Double distilled water (DDW)	10 ml

Stock solution was stored in amber tube at 4^{o}C for further used.

3.2.3. Buffer and solutions for detection and analysis of proteins

Buffers for Tricine SDS-PAGE: all solutions were prepared in DDW

Polyacrylamide mix solution

Acrylamide	48%
N,N,N',N'-methylene-bis-acrylamide	1.5%

Solution was stored in amber bottle

Gel loading buffer (4X)

Tris-HCL (pH 7.0)	150 mM
SDS	12.0%
Glycerol	30.0%
β-Mercaptoethanol	6.0%
Coomassie Brilliant Blue	0.05%

Tricine electrophoresis buffers

3X Gel Buffer (3M Tris, 0.3% SDS, pH 8.45)	
Tris	36.32g
SDS	0.30g

Cathode buffer (100mM Tris, 100mM Tricine, 0.1% SDS, pH 8.25)	
Tris	12.11g
Tricine	17.92g
SDS	1.0g

Volume make up to 1000ml with DDW

Anode buffer (100mM Tris-HCL, pH 8.9)	
Tris	12.11g

Volume make up to 1000ml with DDW

Tricine SDS-PAGE composition

Components	16% Resolving gel (10ml)	10% Resolving gel (10ml)	4% Stacking gel (4ml)
Double distilled water	2.33 ml	3.7 ml	2.7 ml
Acrylamide mix	3.33 ml	2 ml	0.33 ml
Gel buffer (3X)	3.33 ml	3.33 ml	1.0 ml
Glycerol (100%)	1.0 ml	1 ml	-
APS (10%)	0.033 ml	0.05 ml	0.05 ml
TEMED	0.006 ml	0.005 ml	0.003 ml

Fixing solution

Acetic acid	10.0% (v/v)
Methanol	50.00% (v/v)
DDW	40.0 % (v/v)

Staining solution

Acetic acid	10.0% (v/v)
DDW	90.0% (v/v)
Coomassie brilliant blue R-250	0.25% (w/v)

Destaining solution

Acetic acid	10% (v/v)
DDW	90.0% (v/v)

3.2.4. Indicator organisms

Listeria monocytogenes (MTCC 839), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MTCC 1430), *Klebsiella pneumonia* (MTCC 816), and *Candida albicans* (MTCC 3017), *Staphylococcus epidermidis* (MTCC 3382^{T}), *Staphylococcus lentus* (MTCC 2292^{T}), *Staphylococcus gallinarum* (MTCC 2992^{T}), *Staphylococcus haemolyticus* (MTCC 3383^{T}), *Staphylococcus chromogenes* (MTCC 3545^{T}), *Staphylococcus lugdunensis* (MTCC 3614^{T}), *Staphylococcus scuiri* (MTCC 6154^{T}), *Staphylococcus warneri* (MTCC 4436^{T}), *Staphylococcus intermidus* (MTCC 6152^{T}).

3.2.5. Methiciln-Resistant *S.aureus* strains (MRSA)

Staphylococcus aureus (ATCC 33591) (MRSA), *Staphylococcus aureus* (ATCC 43300) (MRSA), MRSA 831, MRSA 839, MRSA 1, MRSA 2, MRSA 3, MRSA 4.

3.2.6. Cell lines

The eukaryotic cell lines were procured from ATCC; L929 (CCL-1), RAW264.7 (TIB-71), and HEK- 293T (CRL-3216).

3.2.7. XTT preparation: 0.5 gm/L stock solution of XTT tetrazolium salt (Sigma X4626-100mg) in PBS was filter sterilized through 0.22µm pore size filter and stored in aliquots at −80°C.

3.2.8. Menadione preparation: (MW- 172.18g/mol), 10 mM stock in 100% acetone, final working concentration 1µM.

Just prior assay, an aliquot was thawed and 1µM final concentration of freshly prepared menadione (Sigma) was added to the XTT solution.

3.2.9. Tricine SDS-PAGE electrophoresis

The purity of the active fraction after HPLC purification was checked using 16 % Tricine SDS-PAGE (Schagger, 2006). The ratio of 4:1, purified sample was and sample buffer [150 mM Tris/HCl, pH 7.0, 5% (w/v) SDS, 6% (v/v) β-mercaptoethanol, 30% (v/v) glycerol and 0.01% (w/v) coomassie blue G-250)] were mixed. Later, the mixture was heated at 40^{o}C for 30 min and further separated by 16% tricine SDS-PAGE gel electrophoresis at 25mA per gel (2449095, 17406207). After the electrophoresis, the gel was fixed with 50% (v/v) methanol and 10% (v/v) acetic acid for 60 min. The molecular mass of peptide band, visualized after staining with Coomassie serva blue G-250 dye followed by destaining with 10% acetic acid, was determined by comparing the band with bands of molecular weights standards (Bio-Rad) on SDS-PAGE gels.

3.2.10. Isolation of Genomic DNA from bacteria Ba49

For Genomic DNA isolation from bacteria, the cells were grown overnight in the LB media at 30^{o}C. Further, the cells were harvest from a culture by centrifugation in a microcentrifuge tube. Later, the supernatant was discarded, and approximately up to 40 mg of wet weight microbial cell culture pellets were taken. Transfer the cell suspension into the NuleoSpin® Bead Tube Type B. add 40 µl buffer MG, then add 10µl Liquid Proteinase K and vortex till 10 to 12 min. Centrifuge the NuleoSpin® Bead Tube for the 30s at 11,000g to clean the lid.

Add 600 µl Buffer MG and vortex for the 30 s and centrifuge for 30 s at 11,000 g. Later, the supernatant (~500 µl -600 µl) was to the NuleoSpin® Microbial DNA column and then placed in a 2ml collection tube. Centrifuge for the 30 s at 11,000 g and discard the collection tube with flow through. Put column into a fresh Collection Tube (2ml). Add 500 µl Buffer BW and centrifuge for the 30 s at 11,000 g. Discard the flow-through. Then add 500 µl of Buffer B5 to the column for the 30 s at 11,000 g. Discard the flow-through and place the column back into the collection tube. Dry the silica membrane by centrifugation for the 30 s at 11,000 g. Place the NuleoSpin® Microbial DNA column into a 1.5 ml nuclease-free tube and add 50 µl Buffer BE/autoclaved water onto the column. Incubate at room temperature for 1 min, centrifuge 1 min at 11,000 g and 0.8% (w/v) agarose gel was run to confirm the isolation of genomic DNA.

3.2.11. Agarose gel electrophoresis

DNA fragments were separated by running DNA on 0.8% (w/v) agarose gel having ethidium bromide (10 mg/ml). For visualization of DNA, a UV transilluminator was used. DNA samples were then mixed with the DNA loading dye and further, loaded on the agarose gel. Electrophoresis was carried out in 1X TAE buffer at 90 Volts. DNA marker (1Kb DNA ladder, NEB) was run parallel, to estimate the sample size of the DNA fragments from their relative mobility.

3.3. Methods

3.3.1. Isolation of bacteria and antimicrobial screening

The outer layer of *Allium cepa* (locally purchased) was removed aseptically and the inner part was crushed in sterile 1xphosphate buffer saline (PBS) to make a homogenous paste (~ 5g in 10ml PBS). The homogenate was further serially diluted (10^{-1} - 10^{-10}) in 1x PBS and plated on different sterile media i.e., Tryptone Soya Agar (TSA), Nutrient agar (NA), Zobell Marine agar (ZMA), Luria Broth (LB) agar, and Brain Heart Infusion agar (BHIA), all procured from HiMedia, India. The plates were incubated at different temperatures i.e. 25^{o}C, 30^{o}C, and 37^{o}C for 7 days. Colonies on the plate which inhibited the growth of nearby colonies were picked and re-streaked on sterile fresh media to get the pure colonies. These pure colonies were preserved in 20 % (v/v) glycerol stock at -80^{o}C. More than 50 isolates were screened for the antimicrobial activity against various indicator strains i.e., *Listeria monocytogenes* (MTCC 839), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MTCC 1430), *Klebsiella pneumonia* (MTCC 816), and *Candida albicans* (MTCC 3017). Those isolates which showed potential activity against *S. aureus* (ATCC 25923 and MTCC 1430) were further grown in 250

ml flask containing 50 ml of sterile BHI medium at 30^{o}C and under the shaking condition (150 rpm). In the meantime, a 1ml sample was withdrawn at a different time point (24 h and 36 h) and centrifuged (10,000 rpm, 10 min, 4^{o}C) to make cell-free supernatant. These cell-free supernatants were used to determine antibacterial activity by agar well diffusion assay (Kimura et al., 1998). 100 µl aliquot of cell-free supernatant was applied to bored wells (6 mm) on Muller Hinton agar (MHA) plates having 10^{6} CFU/ml of indicator strain according to guidelines of the Clinical and Laboratory Standards Institute (CLSI). The plates were incubated overnight at 37^{o}C and the activity was determined by measuring the zone of inhibition of test organisms around the wells.

3.3.2. Extraction of liquid cultures

The cell-free broth (50 ml) of selected isolates which shows the activity against indicator strains were mixed with 1% of Diaion HP-20 (Supelco) resins and were shaken at 30^{o}C and 150 rpm for 2.5 h. The resins were washed with water and then were eluted with 100% methanol. Methanol was evaporated using Rotavapor (BUCHI R-300) and the activity of the crude extract was checked against various indicator strains for further screening. Further, studies were performed with the Ba49 isolate based on preliminary findings, and the isolate was deposited in Microbial Type Culture Collection (MTCC) repository, CSIR- IMTECH, Chandigarh, India under accession number MTCC 13006.

3.3.3. Whole-genome sequencing

The strain Ba49 was grown in the BHI medium overnight and the genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega, USA). For whole-genome sequencing, Illumina *De novo* sequencer (Illumina HiSeq 2500) was used. The sequencing reads of strain Ba49 were error-corrected and *de novo* assembled using SPA des v 3.13.0 (Bankevich et al., 2012). Genome statistics and coverage were estimated using QUAST v 5.0.2 (Gurevich et al., 2013) and BBMap v 38.42 (Bushnell, 2014). The assembled genome was then submitted to NCBI and annotated using the PGAAP pipeline. For identification of the biosynthetic gene cluster, the complete genome assembly further uploaded on antiSMASH (version 3.0) (Weber et al., 2015).

3.3.4. Determination of the antimicrobial activity of Ba49

3.3.4.1. Effect of different media on antimicrobial peptide production: The AMP producer strain Ba49 was grown in six different types of media broth and tested against *S. aureus* to compare the effectiveness of different culture media on peptide production. The media used i.e. Luria Broth (LB), Nutrient broth (NB), Zobell Marine Broth (ZMB),

Tryptone Soya Broth (TSB), Reasoner's Broth (R-2A) and Brain Heart Infusion (BHI). The samples were then aliquot at the intervals of 24 h and 32 h. Later the fermented broth was centrifuged at 10,000 g for 10-20 min and the cell-free supernatant was further checked for its antimicrobial activity by well diffusion assay conducting by seeding *S. aureus* on MHB agar plate. The plates were further incubated overnight at 37^{o}C and the activity was determined by measuring the zone of inhibition around the wells against test organism.

3.3.4.2. Effect of different physiological parameters on antimicrobial peptide production: After screening the best producing medium conditions for the antimicrobial peptide production from strain Ba49 was further grown at different conditions of temperature, agitation, and pH to test against *S.aureus* to compare the effectiveness of different physiological conditions on peptide production. Different temperatures were used for the production of peptide i.e. 25^{o}C, 30^{o}C, 37^{o}C, and 45^{o}C. Later, after optimizing the incubation condition the agitation rate (50 to 300 rpm) followed by the effect of various pH ranges i.e. from 4.5 to 10.5 was used to check the effectiveness of the production of the peptide from strain Ba49. The samples were then aliquot at the intervals of 24 h and 32 h. Later the fermented broths were centrifuged at 10,000 rpm for 10-20 min. The cell-free supernatant each of the above-mentioned conditions was then assayed for antimicrobial activity by measuring the zone of inhibition (mm) against the *S.aureus* test organism.

3.3.5. Comparison of antimicrobial peptide condition at the different growth phase

The growth curve of strain Ba49 was performed to examine the production of the antimicrobial compound at different stages of growth. The samples were withdrawn at regular intervals of time. The activity of cell-free supernatant was determined by using the agar well diffusion method, by measuring the zone of inhibition (mm) around the wells against test organism *S. aureus*. The assay was done in triplicates with at least two independent repeats.

3.3.6. Purification of antimicrobial compound from strain Ba49

Ba49 strain was grown, and a single colony was inoculated into a 250 mL flask containing 50 mL of sterile ZMB broth and incubated at 25°C and 150 rpm for 24 h in an orbital shaker. A flask (1 L) containing 500 mL sterile ZMB (4) medium as listed in table S1was inoculated with 10 ml of pre-culture and incubated at 25°C and 150 rpm for 32 h (as optimized in the growth curve, Fig. 3). Subsequently, the cells were separated by centrifugation (30 min, 8000 rpm, 4°C). The cell-free supernatant was allowed to bind to activated Diaion HP-20 (Supelco) resin (1% w/v) taken in a 1 L flask and incubated for 2.5 h at 25°C and 150 rpm in

an incubator. The resin was washed with double distilled water to remove extra-medium components and salts followed by elution with the combination of methanol and isopropanol (4:1). Elute was then concentrated by solvent evaporation using Rotavapor R-300 (Buchi, Switzerland) and reconstituted in double-distilled water. The crude extract was partially purified using cation-exchange chromatography in a manually packed SP Sepharose column pre-equilibrated with 10 mM ammonium acetate buffer pH 5.0 at a flow rate of 1 mL/min and connected to an ÄKTA Explorer chromatography system of GE Healthcare (USA), followed by linear gradient elution in the same buffer with 0 to 1.0 M sodium chloride. Active fractions were pooled, concentrated by lyophilization and further loaded onto a Sephadex G10 (PD-10 Desalting Columns, GE Healthcare, USA), which was pre-equilibrated with 5 mM ammonium acetate buffer (pH 5.0). The elution was carried out using the same buffer, and the active fractions were pooled and further processed using reverse-phase high-pressure liquid chromatography (RP-HPLC) (SunFire® C18 OBD™ Prep Column, 100Å, 5µm, 10 mm × 250 mm, Water Corporation, USA). The mobile phase consisted of solvent A (5 mM ammonium acetate with 0.05% (w/v) sodium 1-octane sulfonate (SOS), pH 5.0) and solvent B (22% (v/v) acetonitrile, ACN). The column was pre-equilibrated with 22 % Solvent B and Solvent A. The gradient elution i.e., 22 % to 80% of solvent B over of 57.1 min and reverse 80% to 22% of solvent B in 7 min at flow rate of 3 mL/min was used in HPLC. The absorbance was monitored at 220 nm using Shimadzu, SPD- 10A UV-VIS detector. Fractions were collected and then a vacuum concentrator was used to evaporator ACN. The concentrated sample was further re-injected onto Kinetex® 5µm F5, 100Å, Phenomenex HPLC column for removal of SOS, pre-equilibrated with 20% ACN/5 mM ammonium acetate (pH 5.0) and the concentration of ACN in the eluted solvent was raised from 20% (v/v) to 80% (v/v) over 47.1 min using a linear gradient at a flow rate of 1mL/min. The absorbance was monitored at 220 nm and 254 nm by Shimadzu, SPD-10A UV-VIS detector (data not shown). The purified compound was further concentrated and then used to test its antimicrobial activity against *S. aureus (*ATCC 25923).

3.3.7. In-gel activity assay for determination of peptide activity

Tricine sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (Tricine SDS-PAGE, 16 % (w/v)) of HPLC peptide-Ba49 was performed (Schagger, 2006). Peptide-Ba49 was applied onto the SDS-PAGE gels in duplicates. Following electrophoresis, one part of the gel was stained with the Coomassie serva blue G-250 dye along with the low molecular weight (LMW) marker (Bio-Rad) for the visualization of the bands. The second part of the gel was

used for *in-situ* detection of the antimicrobial activity upon fixing it in a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 15 min and repeated washing with sterile distilled water for 60 min followed by placing it on 0.8% MHA plate seeded with 10^6 CFU/mL of *S. aureus* (ATCC 25923). The plate was incubated overnight at 37^{o}C to observe the zone of inhibition (Bizani et al., 2005).

3.3.8. Mass spectrometry and peptide characterization

The pure peptide was subjected to MALDI-MS. α-Cyano-4-hydroxycinnamic acid was used as the matrix for sample preparation for MALDI-MS analysis. The mass spectra, in positive-ion mode with a spectral range of 400– 4000 m/z, were recorded on the MALDI-TOF spectrometer (Brucker's ultraflex TOF/TOF). Later, the peak was subjected to electron spray ionization (ESI-MS/MS) analysis for the determination of the monoisotopic mass and the pattern of fragmentation. Upon fragmentation the generated ions were manually assigned and the sequence was compared with antiSMASH results.

3.3.9. Effect of extrinsic factors on antimicrobial peptide activity

The sensitivity of purified antimicrobial compounds towards various physiological factors like temperature, pH, hydrolytic enzymes, organic solvents, surfactants, and reducing agents was studied.

3.3.9.1. Effect of temperature: The sample of the purified antimicrobial compound was incubated at different temperatures i.e. 45°C, 60°C, 75°C, and 100°C for 30 min and at 121°C for 20 min. After the treatment, the activity of the purified compound was determined by agar well diffusion assay (Kimura et al. 1998) against strains of *S. aureus*. The antimicrobial activity was measured by the zone of inhibition (mm) and comparing it with the untreated one for the loss of antibacterial activity as stated. The assay was done in triplicates with at least two independent repeats.

3.3.9.2. Effect of pH: Similarly, the stability of the purified AMP in different pH buffering conditions was investigated. The pH buffer range was used from 5-10, samples were mixed with different pH buffers and incubated at 37°C for 2 h. Then the potency of peptide activity against *S. aureus* was measured by the zone of inhibition (mm) and comparing it with the untreated one for the loss of antibacterial activity as stated. The assay was done in triplicates with at least two independent repeats.

3.3.9.3. Influence of enzymes on peptide activity: In another set of experiments, the purified antimicrobial compound was treated with various enzymes, at the final concentration of 2 mg/ml. Further, the enzyme-treated samples were treated at 80^{o}C for 5 min to inactivate

the added enzymes before the analysis of antimicrobial activity (Singh et al., 2014). The potency of the treated peptide was checked against *S. aureus*, measured by the zone of inhibition (mm), and comparing it with the untreated one for the loss of antibacterial activity as stated. The assay was done in triplicates with at least two independent repeats.

3.3.9.4. Effect of organic solvent: To study the effects of organic solvents, the purified antimicrobial compound was incubated with various organic solvents, at a final concentration of 10% (v/v), along with appropriate controls, at room temperature for 1 h. After incubation, the solvents were removed by vacuum concentration at 45°C for 1 h, and then residual antimicrobial activity was determined. Zone of inhibition (mm) and comparing it with the untreated one for the loss of antibacterial activity as stated. The assay was done in triplicates with at least two independent repeats.

3.3.9.5. Effect of surfactants and reducing agents: The effects of surfactants and reducing agents were studied by the addition of the reagents to the purified AMP. Surfactants that were used are SDS, Tween 80, Triton X-100, and urea, which are added to the purified peptide in a final concentration of 1% (v/v) and incubated at 25^{o}C for 2 h. surfactants at 1% (v/v) in water were used as controls and were filter sterilized before use. In another set of experiments, the purified peptide was mixed with the reducing agents i.e. DTT and β-mercaptoethanol to a final concentration of 10 mmol/l and 10% (v/v) respectively. Further, the mixture was incubated for 2 h at room temperature (25^{o}C). Reducing agents (DTT and β-mercaptoethanol) at the same final concentration in water was used as control. The samples after each of the above-mentioned treatments were then assayed for antimicrobial activity by measuring the zone of inhibition (mm) and comparing it with the untreated one for the loss of antibacterial activity as stated. All these assays were done in triplicates with at least two independent repeats.

3.3.9.6. Influence of different metal ions on peptide potency: The effects of various metal ions were studied by the addition of metal ions at a final concentration of 10mM to the purified AMP. The potency of the peptide against *S. aureus* was examined by treating with $CaCl_2$, $CuSO_4$, $CoCl_2$, $FeSO_4$, $MnCl_2$, $NiSO_4$, and $ZnSO_4$. The untreated purified peptide was used as the positive control and metal salts at the same concentrations were used as negative controls. All samples were incubated at room temperature for 2 h. Antimicrobial activity was assayed by agar well diffusion assay, and the zone of inhibition (mm) was measured by comparing it with the untreated one for the loss of antibacterial activity as stated. All these assays were done in triplicates with at least two independent repeats.

3.3.10. Assessment of antibacterial activity of peptide-Ba49 against *S. aureus*

3.3.10.1. Determination of minimum inhibitory concentration (MIC): The Minimum inhibitory concentrations (MICs) of the antimicrobial compound were determined by using a micro-dilution broth method in 96-well micro-titer plates according to guidelines of the Clinical and Laboratory Standards Institute (CLSI). The test strains were grown in Mueller Hinton Broth (MHB, HiMedia) to its exponential phase in optimal conditions (up to 1 O.D at 600 nm), and further serially diluted to reach a final bacterial cell count of about ~ 10^5 CFU/ml. The test was performed in triplicate. To each well of the microtiter plate, 100 µl of fresh MHB medium was added. 100 µl of the purified antibacterial peptide was added to column 1 of the same micro-titer plate. The contents of well 1 were mixed and 100 µl of this mixture was transferred to the well 2. This made the two-fold dilution of column 1. This procedure was repeated down to column 8. 100 µl of the indicator strains were dispensed in all the above-mentioned wells i.e. from 1 to 8. The micro- titter plate was incubated at 37^{o}C for 16 h. After the incubation, XTT (sodium3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (Sigma Aldrich, USA) was added to each well to determine the viability of the cell at the lowest concentration and considered as the MIC of that particular strain (Yi et al., 2018) (Yi et al., 2018).

3.3.10.2. Time-dependent killing kinetics: The bactericidal potential of the purified AMP was evaluated based on colony counts obtained at different time points. The assay was performed in triplicates by incubating~ 10^5 cells/ml of *S.aureus* (ATCC 25923) with 2xMIC (8µM) concentration of peptide in MHB media. Aliquots were collected at fixed time intervals, diluted appropriately in sterile MHB broth, and plated on MHA plate. CFU was counted after 24 h of incubation of MHA plates at 37^{o}C (Jangra et al., 2019). The test was carried out in triplicates with two independent repeats.

3.3.10.3. Post antibiotic studies: *S. aureus* cells (~10^5 CFU/ml) were treated with different concentrations of peptide-Ba49 i.e., 1xMIC (4µM) and 2xMIC (8µM) in MHB medium. Later the cells were incubated at 37^{o}C at 150 rpm. Post 2h and 4h incubation, the samples were centrifuged and further diluted in the ratio of 1:100 to minimize the effect of the peptide in fresh MHB medium and transfer to 96 well plate. The cell growth kinetics was studied by incubating the cells at 37^{o}C and low shaking in infinite M plex TECAN and estimating cell $O.D_{600}$ upto 12 h with an interval of 30 min. The bacterial cells without any treatment were taken as positive control and processed similarly. The experiments were carried out in duplicates with measurement of cell OD_{600} nm in triplicates.

3.3.11. Mechanism of action studies of peptide-Ba49 against *S. aureus*

3.3.11.1. Reactive oxygen species assay (ROS assay): The production of reactive oxygen species by *S. aureus* (ATCC 25923) after the treatment with peptide-Ba49 was investigated by using sensitive probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent dye (Sigma, Aldrich, USA) which can detect a broad range of ROS including nitric oxides and hydrogen peroxides (Arakha et al., 2015). For this $5x10^4$ CFU/ml cells were treated with different concentrations of peptide-Ba49 corresponds to 1xMIC and 2xMIC for the sub-lethal stage i.e., 2 h. Later, the cells were pelleted down and washed with 1xPBS and transfer to 96 wells plate. Further, the cells were incubated with H_2DCFDA at a final concentration of 5 µM at 37^{o}C for 1 h. Untreated cells were taken as control whereas polymyxin B was taken as a positive control, and the fluorescence was measured at excitation and emission of 485 nm and 525 nm. The experiment was done in triplicates, each with three individual repeats.

3.3.11.2. Cytoplasmic membrane disruption assay: By measuring the release of membrane potential-sensitive dye i.e., 3, 3-dipropylthiacarbocyanine [$DiSC_3$ (5)], the effect of peptide-Ba49 on S. aureus (ATCC 25923) membrane was evaluated as described 31363941 with some modifications. Briefly, the strain *S. aureus* (ATCC 25923) were grown overnight into MHB medium at 37^{o}C. Later, the cells were washed with 5 mM HEPES buffer containing 20 mM glucose and followed by resuspension of cells to O.D 600 (nm) of 0.05 in 5mM HEPES buffer, 20mM glucose and 100 mM KCl. Further, the $DiSC_3$ (5) was added at a final concentration of 0.4 µM to each well of 96 wells plate and incubated for 1h. The mixture was stand till the fluorescence was decreased and stable, and then different concentrations of peptide-Ba49 i.e. 1xMIC (4 µM) and 2xMIC (8 µM) were added to each well. The cells without any treatment were taken as control whereas polymyxin B (30 µg/ml) was taken as the positive control. The fluorescence was measured at excitation and emission of 622 nm and 670 nm, respectively, after the incubation of 1 h. The experiments were done in triplicates with two independent repeats.

3.3.11.3. Fluorometric measurement of membrane potential using $DiBAC_4$ (3): The effect of purified AMP on the membrane potential of S.aureus was investigated using voltage-sensitive dye i.e. $DiBAC_4$ (3) (Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol) (Invitrogen) (Te Winkel et al. 2016). S. aureus cells (1×107 CFU/ml), grown in NB medium, were treated with 2xMIC (4 µM) and 4xMIC (8 µM) of purified AMP for 4 h at 37^{o}C in an incubator. Then these cells were washed with 1xPBS (pH 7.4) and centrifuged at 4,000 rpm for 5 min. The pellet was resuspended in 1 ml of 1xPBS ($1×10^7$ CFU/ml) followed by

addition of 1 µl from the 1 mM $DiBAC_4$ (3) stock to get a final concentration of 1 µM and mixed thoroughly. Then, 200 µl of the cell sample was added to each well of 96 well plates followed by incubation for 30 min in dark at 37^{o}C. Fluorescence of the mixture was measured spectrophotometrically at an excitation wavelength of 516 nm and an emission wavelength of 490 nm. Polymyxin B treated *S. aureus* cells were taken as a positive control. The experiment was carried in triplicates with two independent repeats.

3.3.11.4. Membrane permeabilization assay: Membrane specific activity of purified AMPs was determined by using flow cytometry. Propidium iodide (PI) was used to label the bacterial cells after the treatment with the purified AMP. PI, the most commonly used dye for dead cell detection, contains a positive charge. Due to its divalent nature, it usually gets excluded from the cells, and therefore it can only enter the permeabilized cytoplasmic membrane (Yasir et al. 2019). 1×10^7 CFU/ml *S .aureus* (MTCC 1430) cells were treated with 2xMIC (4 µM) of purified AMP followed by incubation at 37^{o}C for 4 h. Then these *S. aureus* cells were withdrawn at different time points (0 min, 120 min, and 240 min) and centrifuged at 10,000 rpm for 15 min. Then these cells were incubated with PI (1 mg/ml in 1xPBS) for 15 min in dark at room temperature. Untreated bacterial cells were taken as a negative control and 1% Triton X 100 treated cells were taken as a positive control. Samples were acquired and analyzed on Accuri C6 Flow Cytometer (Becton Dickinson, San Jose, Ca, USA).

3.3.11.5. Scanning electron microscopy (SEM): Scanning electron microscopy was used to examine the structural changes of purified peptide-Ba49 treated *S.aureus* (MTCC 1430). *S. aureus* ($1x10^7$ CFU /ml) in the NB medium was mixed with purified AMP of 2xMIC (4 µM) and incubated at 37^{o}C for 240 min. Cells not treated with the purified AMP was taken as a control. Both AMP treated and control (untreated) *S.aureus* cells were centrifuged at 5,000 rpm for 10 min at 4^{o}C and washed twice with 1xPBS (0.1 M, pH 7.4). Poly (L-lysine) coated coverslip was used for cell attachment. Cells were then fixed on these coated coverslips with Karnovsky's fixative for 2 h at 4^{o}C followed by twice washing with 1xPBS. Then these cells were dehydrated with gradients of ethanol (30, 50, 70, 90, and 100%) each for 30 min (David et al., 1973). Then, the cells were air-dried, coated with platinum, and observed under the SM-IT 300 LV scanning electron microscope (JEOL, Tokyo Japan).

3.3.11.6. Transmission electron microscopy (TEM): Transmission electron microscopy was used to examine the intracellular changes in AMP (purified) treated *S. aureus* cells. *S. aureus* cells (2×10^6 CFU/ml) was mixed with purified AMP of 2xMIC (4 µM) for 240 min, followed by washing twice with 1xPBS (0.1 M, pH 7.4) to remove the excess medium

components. Then the cells were resuspended in 1xPBS (0.1 M, pH 7.4), placed on carbon-coated grids, and viewed under JEOL 2100 transmission electron microscope (Malhotra et al., 2017).

3.3.12. Application of antimicrobial peptide from strain Ba49

3.3.12.1. Measurement of biofilm inhibition assays

3.3.12.1a. Biofilm mass assay determination: The crystal violet method was used for staining the biofilm mass, as described by Toole *et al*. (O'Toole, 2011), with modifications. Later, the remaining methanol was aspired and the plate was air-dried for 15-20 min. CV at 0.1% concentration was added to the plates and the plates were incubated for 15 min at room temperature. This was followed by washing the plates twice with distilled water to remove extra stain and proper drying of the plates. Later, a suspension of biofilm was prepared by adding 30% acetic acid and left for 30 min at room temperature to dissolve the stain properly. The absorbance was measured at 595 nm and the values obtained were considered as adhere biofilm index to the surface of the well and the extracellular mass-produced by them. The percentage of biofilm was calculated by the formula described previously (Shikha et al., 2020):

$$\% \text{ Biofilm} = (OD_{595} \text{ of treated cells}/OD_{595} \text{ of untreated cells}) * 100$$

3.3.12.1b. XTT reduction assay: As the CV is a basic dye, which stains negatively charged surface molecules and extracellular polysaccharide matrix. CV stains a viable and non-viable cell, which estimates the only mass of the biofilm, but not its metabolic activity (Pitts et al., 2003; Scudiero et al., 1988). Further, to estimate the viability of biofilm, XTT sodium3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (Sigma Aldrich, USA) was used. In this method, the *S. aureus* strains (ATCC 25923) were grown overnight at 37^{o}C in rotary orbital shaker incubator. Further, the cells were diluted to 1:100 ratios in the fresh L.B. medium + 1% (w/v) sucrose. Then 100 µl of diluted culture was poured into each well of 96-well plate with different concentrations of a peptide and untreated cells were taken as control. This was followed by incubation of the plates at 37^{o}C for 24 h, removal of the media by gentle decantation and washing of the wells twice with 1xPBS (pH 7.2). Later, the aspired cells were dissolved in 100 µl 1xPBS and 30 µl % glucose-containing XTT-menadione solution and incubated in dark at 37^{o}C for 3 h. ELISA plate reader was used to measure plate OD at 490 nm which reflected the viability of the cells present in the biofilm (Shikha et al., 2020). The percentage of viability was measured by the following formula:

$$\% \text{ Biofilm} = (OD_{490} \text{ of treated cells}/OD_{490} \text{ of untreated cells}) * 100$$

3.3.12.2. Biofilm formation studies: To investigate the effect of the purified peptide-Ba49 on biofilm formation was studied in the 96-well microtiter plate. A method was used for further evaluation i.e. crystal violet (CV) staining and metabolism-based tetrazolium dye i.e. XTT. The strains of *S. aureus* (ATCC 25923) were grown overnight in LB medium at 37^{o}C to form a biofilm. Later, the culture was diluted to 1:100 in fresh LB + 1% sucrose, under stress condition. 100 µl of diluted culture was poured into each well in 96 wells plate containing different peptide; untreated cells were taken as control and incubated at 37^{o}C for 24 h. After incubation, the media was decanted and the wells were washed twice with 1xPBS (pH 7.2) to remove the planktonic cells (Shikha et al., 2020). Further, the formation of biofilm was measured by the CV and XTT method, as described before.

3.3.12.3. Biofilm maturation studies: To determine the effect of peptide-Ba49 on eradicating the mature biofilm, the strain of *S. aureus* (ATCC 25923) was taken. For the formation of mature biofilm, the *S. aureus* (ATCC 25923) strains were grown overnight at 37^{o}C in orbital shaker and further diluted to ratio of 1:100 in fresh LB (1% sucrose) medium. Later, 100 µl of diluted cultures were seeded into 96 wells plate before treatment for 24 h at 37^{o}C. After incubation, the media was removed and washed twice with 1xPBS to remove the planktonic cells, then treated mature biofilm with different concentrations of peptide (4 µM, 8 µM, 16 µM, and 32 µM). Biofilm with polymyxin B (30 µg/ml) biofilm without any treatment was taken as negative control and incubated for 24 h at 37^{o}C. After incubation, gently decant the media and wash the wells twice with 1xPBS (pH 7.2) to remove the planktonic cells (Shikha et al., 2020). Further, the formation of biofilm was measured by the CV and XTT method as described before. All the experiments were carried out in triplicates and the mean value was expressed in terms of percent biofilm formed which was normalized with untreated biofilm.

$$\% \text{ Biofilm} = (OD_{490} \text{ of treated cells}/OD_{490} \text{ of untreated cells})^* 100$$

3.3.12.4. Confocal laser scanning microscopy (CLSM) of *S.aureus* biofilm: CLSM qualitatively analyzed the effect of peptide-Ba49 on *S. aureus* (ATCC 25923) biofilm formation. Using a modified method (Haque et al., 2016) the *S. aureus* biofilm was formed on autoclaved glass cover slip (Blue star, India) in 12-well cell culture plates (Eppendorf). The cover slips were placed in wells of microtiter plates and the biofilm formation of *S. aureus* (ATCC 25923) was carried out by growing the cells overnight in LB medium at 37^{o}C in an orbital shaker incubator. Later, the culture was diluted to 1:100 in fresh LB + 1% (w/v) sucrose under stress condition. 1000 µl of diluted culture was poured into each well in 12

well-plates containing coverslips and treated with 16 µM of peptide-Ba49. Polymyxin B treated cells, as a positive control and untreated cells as negative control were further incubated at 37^{o}C for 24 h. After incubation, the media was gently decanted and he wells were twice washed with 1xPBS (pH 7.2) to remove the planktonic cells.

Three fluorescent dyes were used i.e., SYTO 9 and PI (LIVE/DEAD® BacLight™ Bacterial Viability Kit, Thermo Fisher Scientific, India) stains live and Calcofluor white M2R (CFW) (Sigma St. Louis, MO, United States). Working solutions were prepared in 1xPBS with concentrations of 5 µM, 30 µM, and 0.15 µM, respectively. The coverslips were further stained with 500 µl working solution of SYTO 9 and PI for 15 min in the dark, followed by staining with 500 µl of working solution of CFW for 3 min in dark. Later, the coverslips were washed with sterile distilled water to remove the extra stain and placed on the glass slide with a drop of mounting oil in between to avoid the dryness and seal the coverslips to prevent any air contact. The stained biofilms were observed under CLSM with 40X numerical–aperture (NA). The biofilms were excited by 488 nm and 543 nm light and emitted at 495 nm to 535 nm (green color) for viable bacteria and 580 nm to 700 nm (red color) for dead bacteria. The EPS of biofilm was excited by 405 nm light and emitted at 413 nm to 480 nm (blue color) (Hou et al., 2018).

3.3.12.5. Mammalian cytotoxicity assay: *In vitro* efficacy of purified AMP on the viability of eukaryotic cells was determined by using the colorimetric method i.e. MTT [3-(4, 5-dimethylthoazole-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma Aldrich (Mosmann, 1983). The eukaryotic cell lines were procured from ATCC; L929 (CCL-1), RAW264.7 (TIB-71), and HEK- 293T (CRL-3216). These cells were seeded in 96 wells plate at a density of 1×10^{4} cells per well and kept overnight in 5% CO_2 incubator at 37^{o}C. After 24 h of incubation, these cells were treated with different concentrations of purified AMP (0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µM) for 24 h at 37^{o}C in a humidified CO_2 incubator. Then, 20 µl of MTT stock solution (5 mg/ml dissolved in DMSO) was added to each well and the plate was incubated in dark at 37^{o}C for 3 h. This was followed by the addition of a stop solution containing 50% (v/v) DMF and 20% (w/v) SDS to each well to dissolve the formazan crystals for 1 h followed by measurement of the absorbance at 570 nm. The assay was conducted in triplicates with two independent repeats. The percentage of cell viability was calculated as described earlier (Bansal et al., 2018) :

$$\frac{\text{Absorbance}_{\text{treated}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}} \text{X}\,100$$

3.3.12.6. Intracellular efficacy of peptide-Ba49 in macrophage cells: For Determining the intracellular activity of peptide-Ba49, the RAW 264.7 macrophages ($5x10^5$cells/ml) were seeded into 6 well plates and incubated for 24 h in RPMI-1640 and 10% FBS (without antibiotic) at 37^{o}C with 5% CO_2. Later, the macrophages were infected with 1:10 multiplicity of infection (MOI) i.e., 5x 10^6 CFU/ml cells of S. *aureus* (ATCC 259223) in RPMI-1640 with 10% FBS (without antibiotic) for 2h. After infection, to remove the effect of extracellular bacteria, gentamicin (50 µg/ml) was added and the plates were incubated for 30min. Further, the RAW 264.7 macrophages were washed twice with 1xPBS, followed by the treatment with different concentrations of peptide-Ba49, which corresponded to 1xMIC and 2xMIC. Later, the cells were washed with 1xPBS and then lysed with 0.1% saponin. The numbers of intracellular bacteria were measured at 12 h and 24 h by colony counting. The experiment was carried out in triplicates with two individual repeats.

3.3.12.7. Cell migration assay of peptide-Ba49: The wound healing capacity of purified peptide was carried out by performing in-vitro cell migration studies of L929 cells (Balekar et al., 2012). Bacterial cells at density of $5x10^5$ cells/well were seeded into 6 wells plate containing RPMI-1640 culture medium supplemented with 10% FBS and incubated overnight at 37^{o}C in a humidified CO_2 incubator (5% v/v) to allow the cells to adhere on the surface. After incubation, the media was decanted. The adherent layer was scratched with the help of a sterile yellow tip and followed by the removal of cellular debris by washing with 1xPBS (pH 7.2). Later, the fresh RPMI-1640 with 10% FBS supplement medium, having different MIC concentrations of purified peptide-Ba49, was added. Nisin and RPMI-1640 medium (without any AMP) were taken as a control. Further, the cells were incubated at 37^{o}C in a humidified CO_2 incubator (5% v/v). The scratch area images were captured using a 40x magnification microscope at different intervals of time, i.e., from 0 h, 12 h, 24 h, and 36 h.

3.3.13. Preliminary media optimization studies

The statistical analysis was performed for optimizing medium conditions for the production of antimicrobial peptide from strain Ba49 by using design expert statistical software version 10.0 (Stat- Ease Inc, Minneapolis, MN).

3.3.13.1. Optimization of production medium by Plackett Burman experimental design (PBD)

For optimization, the primary step is to determine the important ingredients of the culture medium. To evaluate the relative importance of various nutrients for antimicrobial peptide production primarily PB design was used. By using this design the important variables that affect the peptide- Ba49 production was used to screen in relative of few experiments as

compared to the one factor at a time technique. 11 medium components i.e. (A) yeast extract, (B) beef extract, (C) peptone, (D) NaCl, (E) $MgCl_2$, (F) KCl, (G) $NaHCO_3$, (H) ferric (III) citrate, (I) Na_2SO_4, (J) $CaCl_2$, and (K) glycerol were taken, were expected to influence the peptide production. Each of the 11 factors was determined in two levels: low (-1) and high (+1) levels which were based on the Plackett Burman matrix design, which is a fraction of two-level factorial design and allows the investigation of 'n-1' variable in at least "n" experiments. The lower and higher levels of each variable and the design matrix are shown in Table 1 and 2. All experiments were carried out at 25^{o}C for 32 h of the fermentation period.

3.3.13.2. Central Composite Design (CCD)

Later, the identification of the components which affect the peptide-Ba49 yield significantly, a CCD method was used to optimize the major variables (Yeast, $NaHCO_3$, ferric (III) citrate, NaCl, $MgCl_2$ and glycerol) which were selected through PB design. A factorial, central composite design (CCD) for three factors with the replicates at the center point and star points was used for the optimization of fermentation medium. All experiments were carried out at 25^{o}C for 32 h of the fermentation period. The total 30 experimental trials were obtained in a CCD design which includes 16 trials of factorial design, 8 trials of axial points (two for each variable) and 6 trials for replication of central points (Table 3).

ANOVA was used to estimate the statistical parameters for optimizing the fermentation medium. The relationship between the four variables was determined by fitting the second polynomial equation to the peptide response obtained from 30 experiments.

3.3.14. Statistical analysis

All data was statistically analyzed using one–way analysis of variance (ANOVA) with Dunnett's multiple comparison tests unless otherwise mentioned using Graph Pad Prism 7 software. $^{*}p < 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, $^{****}p< 0.0001$, $^{*****}p< 0.00001$, $p< 0.05$ were considered significant and $p>0.05$ were considered as non-significant (ns).

Table1. Levels of variables (g/L) tested in the Plackett-Burman design for optimising media components for peptide production

Variables		**Levels of variables**	
		-1	+1
A	Yeast extract	1	5
B	Beef extract	1	5
C	Peptone	1	5
D	NaCl	5	20
E	$MgCl_2$	5	15
F	KCl	1	5
G	$NaHCO_3$	0.5	5
H	Fe(III) citrate	0.1	0.5
I	Na_2SO_4	0.1	0.5
J	$CaCl_2$	1	5
K	Glycerol	1	5

Table 2: The matrix of Plackett-Burman design (PBD) along with predicted values

	Factor A	**Factor B**	**Factor C**	**Factor D**	**Factor E**	**Factor F**	**Factor G**	**Factor H**	**Factor I**	**Factor J**	**Factor K**	**Actual Value**
Run	Yeast extract	Beef extract	Peptone	NaCl	$MgCl_2$	KCl	$NaHCO_3$	Fe(III) citrate	Na_2SO_4	$CaCl_2$	Glycerol	Activity (mm)
1	5.00	1.00	1.00	5.00	15.00	5.00	0.50	0.10	5.00	5.00	1.00	13.2
2	5.00	5.00	1.00	20.00	15.00	0.50	0.50	0.10	1.00	1.00	5.00	0
3	**5.00**	**5.00**	**5.00**	**5.00**	**15.00**	**5.00**	**0.10**	**0.50**	**1.00**	**1.00**	**1.00**	**17.33**
4	5.00	5.00	1.00	20.00	5.00	0.50	0.10	0.50	5.00	5.00	1.00	14
5	1.00	1.00	1.00	5.00	5.00	0.50	0.10	0.10	1.00	1.00	1.00	14
6	5.00	1.00	5.00	20.00	5.00	5.00	0.10	0.10	1.00	5.00	5.00	0
7	1.00	5.00	5.00	20.00	5.00	5.00	0.50	0.10	5.00	1.00	1.00	13
8	1.00	1.00	5.00	20.00	15.00	0.50	0.50	0.50	1.00	5.00	1.00	13.33
9	1.00	1.00	1.00	20.00	15.00	5.00	0.10	0.50	5.00	1.00	5.00	0
10	1.00	5.00	1.00	5.00	5.00	5.00	0.50	0.50	1.00	5.00	5.00	12.6
11	1.00	5.00	5.00	5.00	15.00	0.50	0.10	0.10	5.00	5.00	5.00	0
12	5.00	1.00	5.00	5.00	5.00	0.50	0.50	0.50	5.00	1.00	5.00	12

Table 3: The experimental design and results of Central Composite Design (CCD) for optimization of fermentation medium

		Factor A	**Factor B**	**Factor C**	**Factor D**	**Actual value**
Block	Run	Glycerol g/L	Fe(III) citrate g/L	NaCl g/L	$MgCl_2$ g/L	Activity (mm)
Block 1	1	0.5	0.25	7.5	22.5	10
Block 1	2	1.5	0.75	2.5	7.5	18
Block 1	3	1	0.5	5	15	15
Block 1	4	0.5	0.25	2.5	22.5	12.5
Block 1	5	0.5	0.25	2.5	7.5	18
Block 1	6	0.5	0.75	7.5	7.5	18
Block 1	7	1	0.5	5	15	12.5
Block 1	8	0.5	0.75	7.5	22.5	10
Block 1	9	0.5	0.75	2.5	7.5	19
Block 1	10	1.5	0.25	7.5	7.5	16.5
Block 1	11	1.5	0.75	2.5	22.5	10
Block 1	12	1.5	0.25	2.5	7.5	18
Block 1	13	1.5	0.25	2.5	22.5	10
Block 1	14	1	0.5	5	15	13
Block 1	15	1.5	0.75	7.5	22.5	0
Block 1	16	0.5	0.75	2.5	22.5	14
Block 1	17	1	0.5	5	15	12
Block 1	18	1.5	0.75	7.5	7.5	14
Block 1	**19**	**0.5**	**0.25**	**7.5**	**7.5**	**20**
Block 1	20	1.5	0.25	7.5	22.5	0
Block 2	21	1	0	5	15	0
Block 2	22	1	0.5	5	30	0
Block 2	23	1	0.5	0	15	5
Block 2	24	1	0.5	10	15	0
Block 2	25	1	1	5	15	0
Block 2	26	2	0.5	5	15	0
Block 2	27	1	0.5	5	15	0
Block 2	28	1	0.5	5	15	0
Block 2	29	0	0.5	5	15	10
Block 2	30	1	0.5	5	0	14

RESULTS & DISCUSSION

4. RESULTS AND DISCUSSION

4.1. RESULTS

4.1.1. Isolation of bacteria and antimicrobial

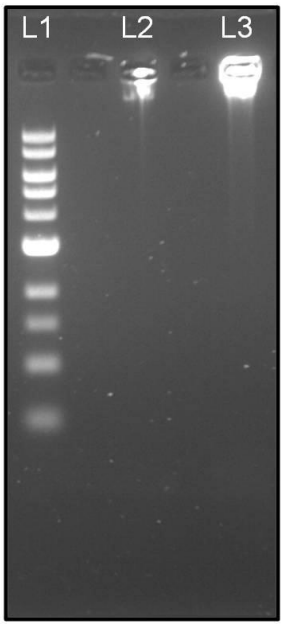
With increase in antibiotic resistance strains, natural sources have attracted a lot of attention to curb this drug resistance menace. In the present study, fermentation extracts of more than 50 isolates from onion extract were screened for their antimicrobial activity against various indicator strains *Listeria monocytogenes* (MTCC 839), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MTCC 1430), *Klebsiella pneumonia* (MTCC 816), and *Candida albicans* (MTCC 3017) using agar well diffusion method. The inhibitory activity of these isolates was detected against at least one indicator strain and mostly against the Gram-positive bacteria. The antimicrobials spectra are restricted to Gram-positive bacteria as suggested; the antimicrobial compounds are related to Gram-positive bacteria's specific feature. It is a well-known fact that the activity of AMPs produced by Gram-positive bacteria is restricted to other Gram-positive bacteria (Riley and Wertz, 2002).

Further, the selected isolates were then grown in 50 ml of BHI broth and incubated at 30^{o}C, 150 rpm for 36 h. The cell-free broths of these isolates were then mixed individually with 1% of Diaion HP-20 (Supelco) followed by methanol extraction. The crude extracts were further used to check their antimicrobial activity against the test organisms. However, as discussed earlier, our study is focused on antibacterial compound isolated and purified from natural microbial sources having activity against *S. aureus*. Accordingly, the isolates were screened, which shows potent activity against *S. aureus*. Among them, strain Ba49 showed promising and consistent antimicrobial activity against *S. aureus* (MTCC 1430 and ATCC 25923).

4.1.2. Identification of strain Ba49

The isolate Ba49 was identified as *Bacillus subtilis* subsp. *spizizenii* based on *de novo* whole-genome assembly (NCBI GenBank accession number VOOM00000000) (Fig. 7) and was deposited in Microbial Type Culture Collection (MTCC) repository, IMTECH, Chandigarh, India with accession number MTCC 13006.

A



B

Strain	*Bacillus subtilis* subsp. *spizizenii* strain Ba49
Accession number	VOOM00000000
# contigs	42
Genome size	4001118 BP
GC	43.86%
N50	269960
Coverage	315X

Figure 7. Identification of strain Ba49: (A) Representing the DNA gel pictogram of Ba49 strain, (B) *De novo* analysis of whole genome assembly. L1: 1kb DNA ladder; L2 and L3: Ba49 DNA.

4.1.3. Effect of physiological parameters on antimicrobial production from strain Ba49

Usually, the AMPs are produced using complex mediums, including BHI, TSB, NB, LB, etc. However, their production can be affected by other culture conditions like pH, incubation, temperature, and phase of microbial growth (Yang et al., 2018). In our study, the production of antimicrobial compound from strain Ba49, we initially used parameters such as, i.e., different media, temperature, pH, and agitation rate (rpm). Initially, the strain Ba49 was grown in flask using different media and incubated in a rotary incubator shaker for 36 h at 30^{o}C, and 150 rpm. Based on the zone of inhibition, the activity was checked against *S. aureus,* and on comparing different media, we founded that ZMB showed better production of antimicrobial compounds from this strain (Fig. 8A).

Generally, it has been suggested that fermentation temperature should be lower than that of the optimal temperature, i.e., 29-40^{o}C favours higher production of bacteriocins (Fickers et al., 2008). Further, the influence of fermentation temperature on the production of antimicrobial compounds from strain Ba49 was studied. Upon checking the activity against *S. aureus* (based on the zone of inhibition); 25^{o}C was found to be the best fermentation temperature for higher production of antimicrobial compound from this strain (Fig. 8B). For producer strain to produce a high amount of antimicrobial compound, it is required to supply a sufficient agitation and aeration rate. In the submerged fermentation technique, the producer strains were heterologously mixed with media components by agitation, and other mixing

means that eventually increased the substrate contact and mass transfer rate (Lajis, 2020). In the present study, the best agitation rate for producing antimicrobial compound from strain Ba49 is 100 rpm at flask level (Fig. 8C).

The growth and production of antimicrobial compound by strain Ba49 were also affected by the initial fermentation pH. It has been reported that, to support the producer strain, the initial pH value is essential to provide optimal growth pH conditions (Cladera-Olivera et al., 2004a; Kayalvizhi and Gunasekaran, 2010). In our study, the studies on the effects of initial pH showed higher production of antimicrobial compound from this strain at pH 6.5 based on the zone of inhibition against *S. aureus* (Fig. 8D).

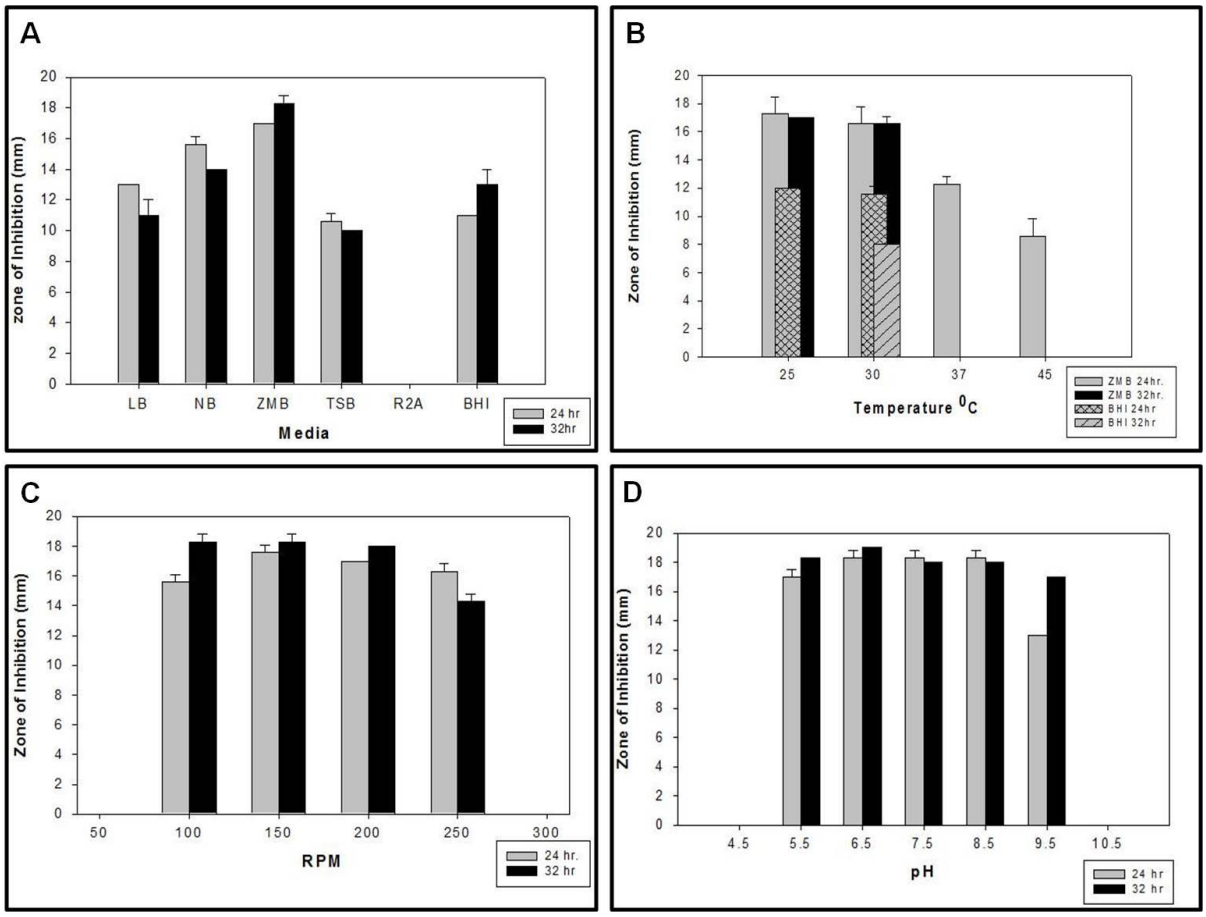


Figure 8. Effect of physiological parameters on antibacterial production from strain Ba49: Effects of (A) Media and (B) temperature on the production of the antibacterial compound from strain Ba49 in terms of zone of inhibition (mm). The production was higher in ZMB medium at 32 h of fermentation as compared to other media and temperature of 25^{o}C yielded highest production of antibacterial in ZMB media: (C) and (D) represents, the effects of agitation (rpm) and pH on the production of antimicrobial compound. An agitation rate of, 100 rpm and pH 6.5 resulted in maximum productivity of antimicrobial compound at 32 h of fermentation (estimated against *S. aureus*). Results were presented as mean ± SD of two independent experiments carried out in triplicates.

4.1.4. Antibacterial compound production by strain Ba49 at different growth phase

During the growth of strain Ba49, the cell-free broth was collected at different time intervals and used to perform antimicrobial activity assay against *S. aureus*. The growth curve of strain Ba49 (Fig. 9) revealed that antibacterial compound production was initiated after 14 h of its growth. Also, it was founded that, there was a significant increase in antibacterial compound production till 34 h and it followed a decline in antibacterial activity (as measured by the zone of inhibition against the indicator strain). This result indicated that the strain Ba49 produced the maximum extracellular antimicrobial compound at 32 h in ZMB medium, and was followed by declining, with decline in growth of the producer strain.

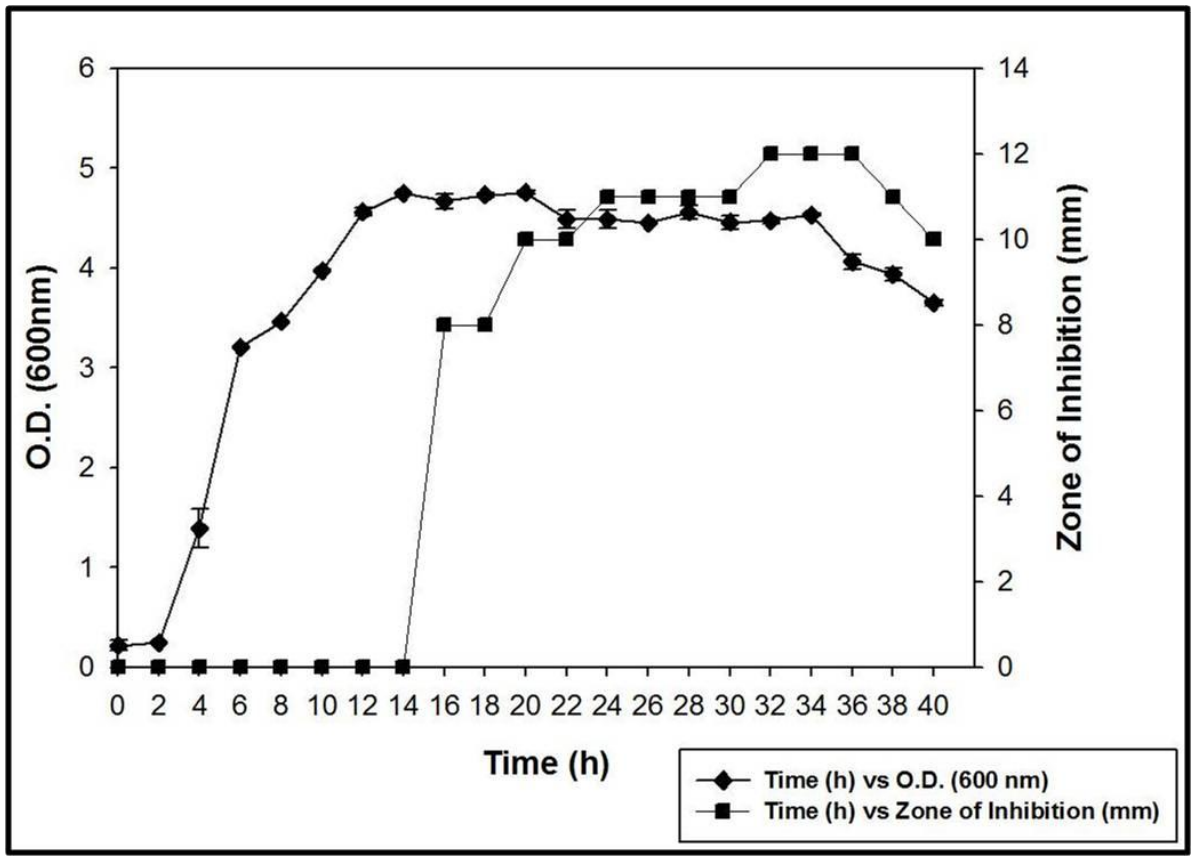


Figure 9. Antibacterial compound production by strain Ba49 at different phases of growth: Antibacterial activity of cell-free supernatant, collected at different time intervals was assayed by using *S. aureus* (in terms of zone of inhibition). The maximum production of the antibacterial compound was observed at the mid stationary phase (32 h).

4.1.5. Purification of the antibacterial compound from strain Ba49

As above mentioned strain Ba49 produced maximum extracellular antibacterial compound at 32 h in ZMB medium. Thus, it was essential to obtain the antimicrobial compound in a purified form. Following ammonium sulphate precipitation, use of various chromatographic techniques, including hydrophobic interaction chromatography, affinity chromatography, and gel permeation chromatography for purification of antibacterial compounds, has been reported (Maldonado et al., 2003; Mortvedt et al., 1991; Pingitore et al., 2007). Nowadays, hydrophobic or ionic resins, i.e., Amberlite XAD-16 or HP-20 diaions, are used to initiate separation of antibacterial compounds in purification strategies due to the limitations of the ammonium sulphate precipitation in precipitating all proteins. In the present study, the cell-free broth was extracted using Diaion HP-20 resin and this was followed by a series of chromatographic techniques, i.e., ion-exchange chromatography, SP- resins was used (Fig. 10A) followed by Sephadex G-10 for desalting. The fractions corresponding to the peak of the chromatogram exhibited antibacterial activity against *S.aureus*. This separation of compound Ba49 by Diaion HP-20 resins indicated that the compound to be of hydrophobic in nature (Chopra et al., 2014). Further, the ion exchange extract was purified on semi-preparative reverse-phase RP-HPLC (Fig. 10B), and the purified peak was found to be active against indicator strain i.e., *S. aureus*. Further to check the purity if the peptide, it was re-injected to RP-HPLC column (Fig. 10C) followed by desalting of peptide in column kinetix F5 (Fig. 10D).

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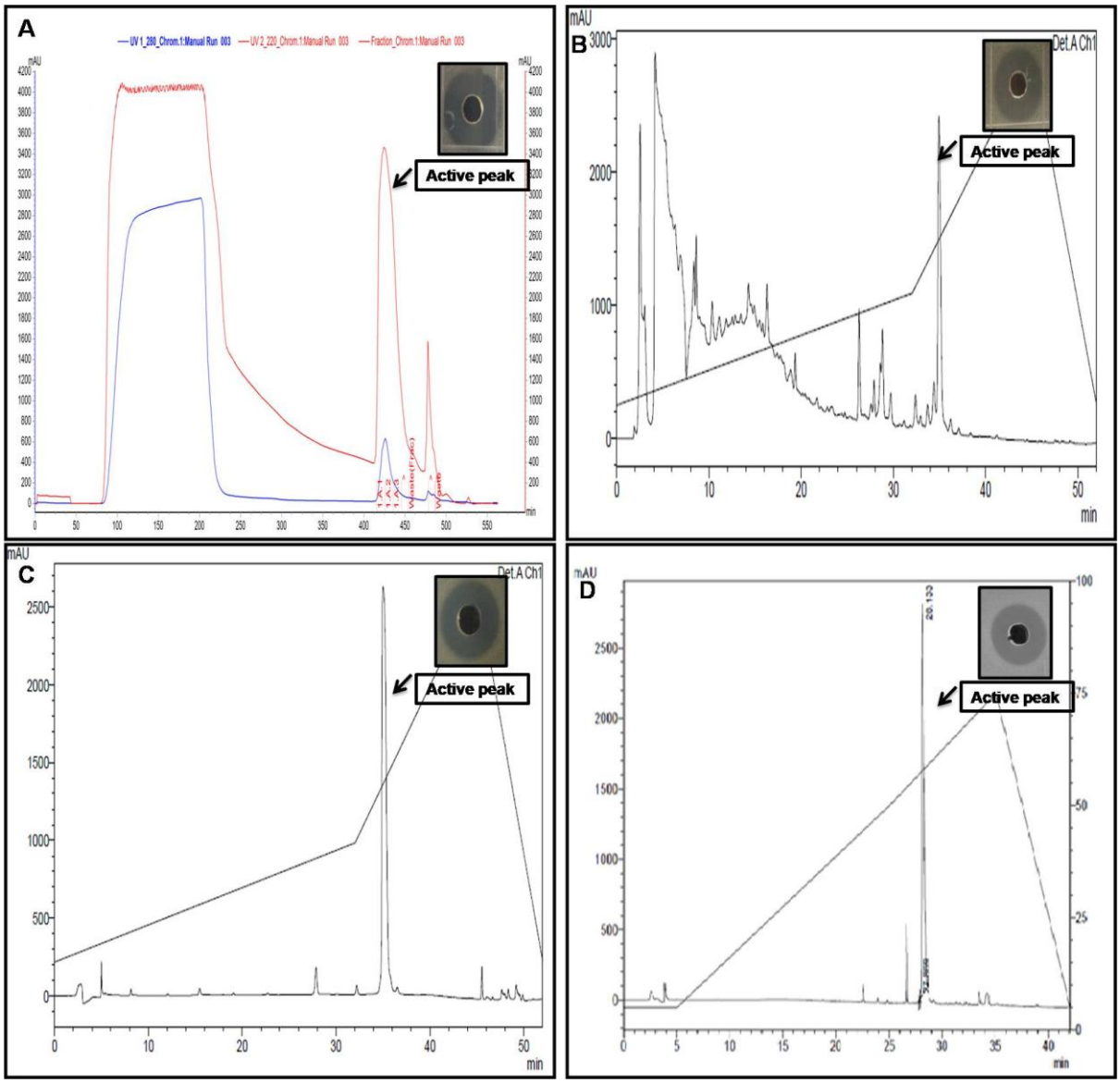


Figure 10. Purification profile of peptide-Ba49: (A) SP-Sepharose chromatogram of Diaion HP-20 crude, the arrow indicated active peak (inset shows the antibacterial activity of SP-Sepharose chromatography purified peptide-Ba49 against *S. aureus* by agar well diffusion assay); **(**B) RP-HPLC chromatogram of purified cation exchange active peptide-Ba49 fraction from SP-Sepharose chromatography step using SunFire® C18 OBD™ Prep Column (inset shows the antibacterial activity of HPLC purified AMP against *S. aureus*); (C) RP-HPLC analysis of an active HPLC purified peptide-Ba49 fraction re-injected into SunFire® C18 OBD™ Prep Column for confirming its purity (inset shows the antibacterial activity of samples collected from re-injected peak against *S. aureus*); (D) RP-HPLC chromatogram of purified peptide Ba-49 on kinetex F5 column for desalting.

4.1.6. In-gel activity assay to determine peptide activity

The zymographic study (Fig. 11B) had also revealed the antibacterial effect of peptide-Ba49 on test strain i.e., *S. aureus* (ATCC 25923), which was corresponding to the same band in the gel stained with Coomassie Blue (Fig. 11A).

4.1.7. Mass spectrometry and peptide characterization

The purified compound was subjected to mass spectroscopic analysis (Fig. 12A), which showed an m/z value of 3319.3303 $(M+H)^+$, similar to the molecular weight of this peptide determined using Tricine SDS-PAGE analysis (Fig. 11A).

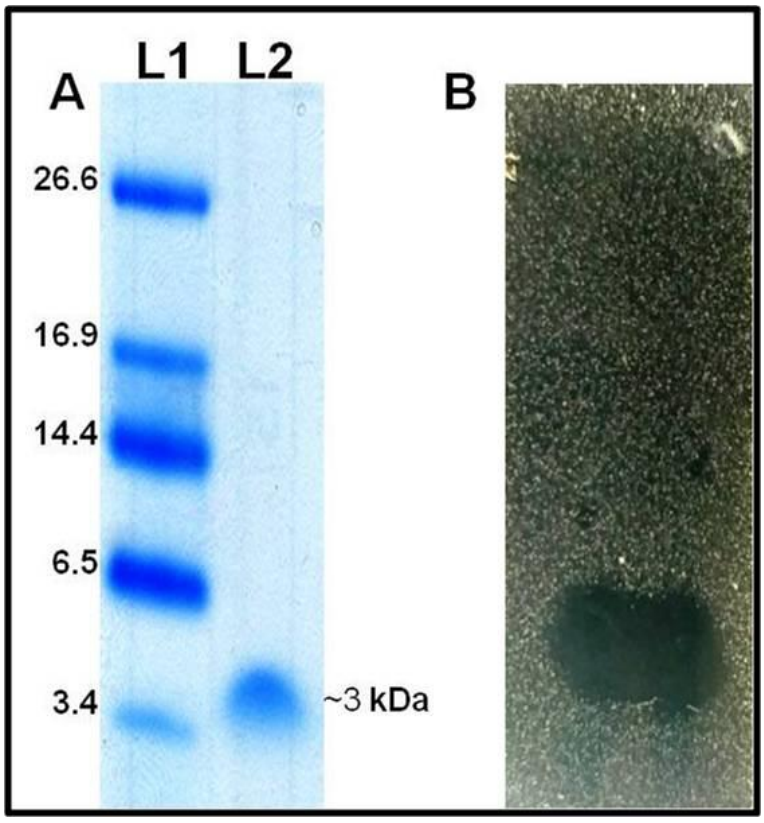


Figure 11: Tricine–SDS PAGE analysis of purified peptide-Ba49 and in-gel activity of purified band against *S. aureus* (ATCC 25923). (A) Shows a single band of purified peptide (L2) along with low molecular weight (LMW) marker (L1). (B) overlaid gel on 0.8% soft agar (MHA) bioassays of purified peptide-Ba49 against *S. aureus* (MTCC 25923).

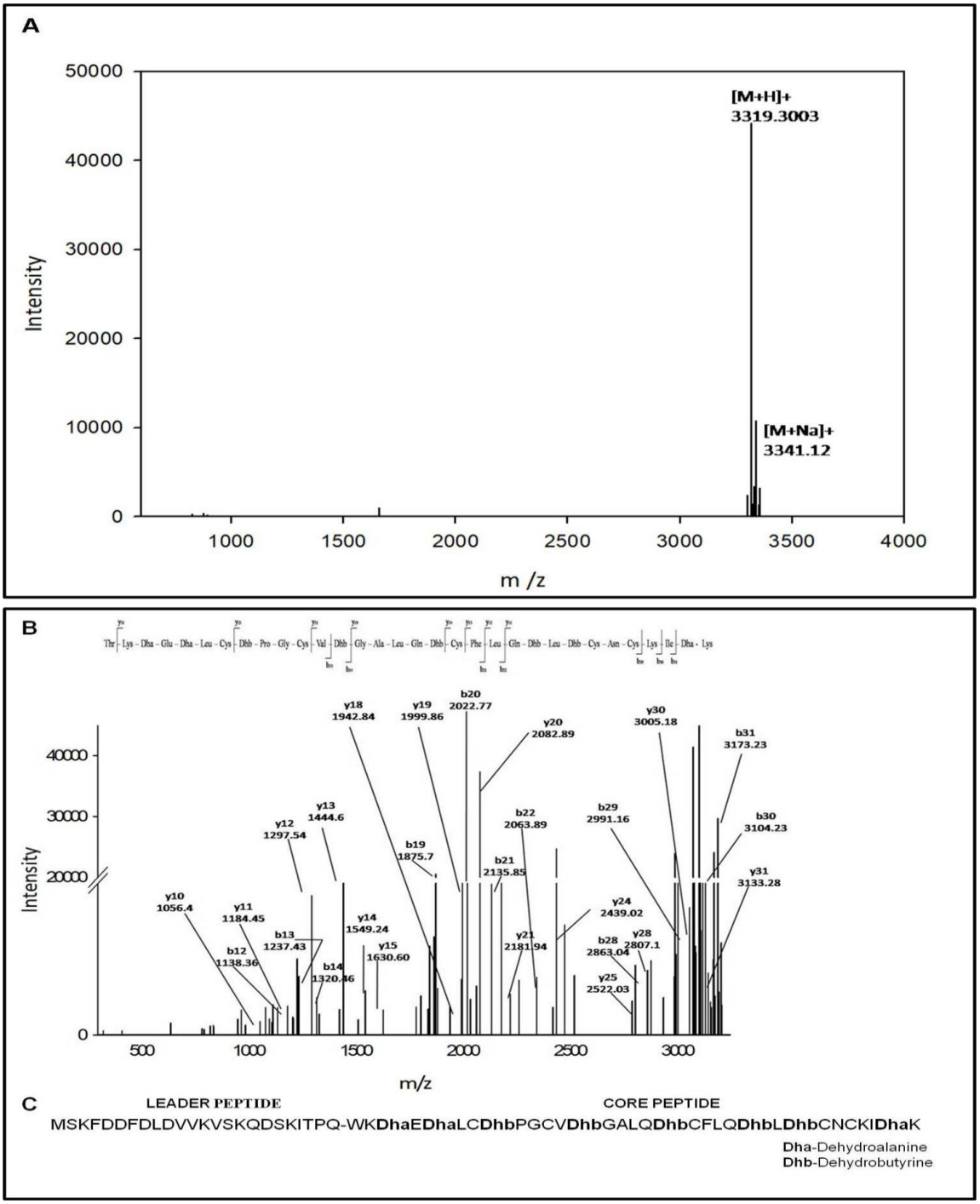


Figure 12. Molecular mass and *de novo* amino acid sequence of the purified peptide-Ba49: (A) MALDI-TOF mass spectra of RP- HPLC purified peptide-Ba49 sample showing an m/z ratio corresponding to the monoisotopic peak of 3319.3003 $[M+H]^+$ along with sodium adduct ion; (B) The partial amino acid sequence was shown by *de novo* sequencing and MS/MS spectrum at N- terminus. The b and y-ions were observed and assigned manually upon fragmentation, corresponding to the partial amino acid sequence shown in the figure (Dha-didehydroalanine; Dhb-didehydrobutyrine); (C) Whole-genome analysis yielding the sequence of peptide-Ba49 along with the modified sequence encoded by the biosynthetic gene. The sequence was the same as that with the partial *de novo* sequence obtained from the MS/MS spectrum.

4.1.8. Biosynthetic gene cluster analysis

Further, the draft genome was uploaded on antiSMASH software to find the antimicrobial peptide's biosynthetic gene cluster. Out of thirteen contigs (Fig. 13A and B), a single gene cluster of lanthipeptide displayed the highest similarity with subtilin gene cluster from strain *B. subtilis* subsp. *spizizenii* (ATCC 6633) (Fig. 13D; Fig. 14A and B). In tandem mass spectrometry, limited fragmentation was observed (Fig. 12B). The *De-Novo* sequencing yielded the partial amino acid sequence, which matched precisely with subtilin (Fig. 13C). The molecular weight and primary sequence of peptide-Ba49 obtained from the gene cluster was identical to subtilin. Based on mass spectometery data, amino acid composition, and analysis of the biosynthetic gene cluster of Ba49, we concluded that peptide-Ba49 is subtilin.

It is well known that the cationic antimicrobial peptide subtilin produced by bacteria *Bacillus subtilis* strain (ATCC 6633) shows a good range of antimicrobial activity against gram-positive bacteria by permeabilizing the cytoplasmic membrane of the target bacteria (Gross et al., 1973; Jack et al., 2013; Kordel et al., 1989). A fascinating observation with this isolate was that it only inhibits the growth of *S. aureus* strains. But as reported, other subtilin isolated from *Bacillus subtilis* strain (ATCC 6633) shows activity against various Gram-positive strains (Klein and Entian, 1994).

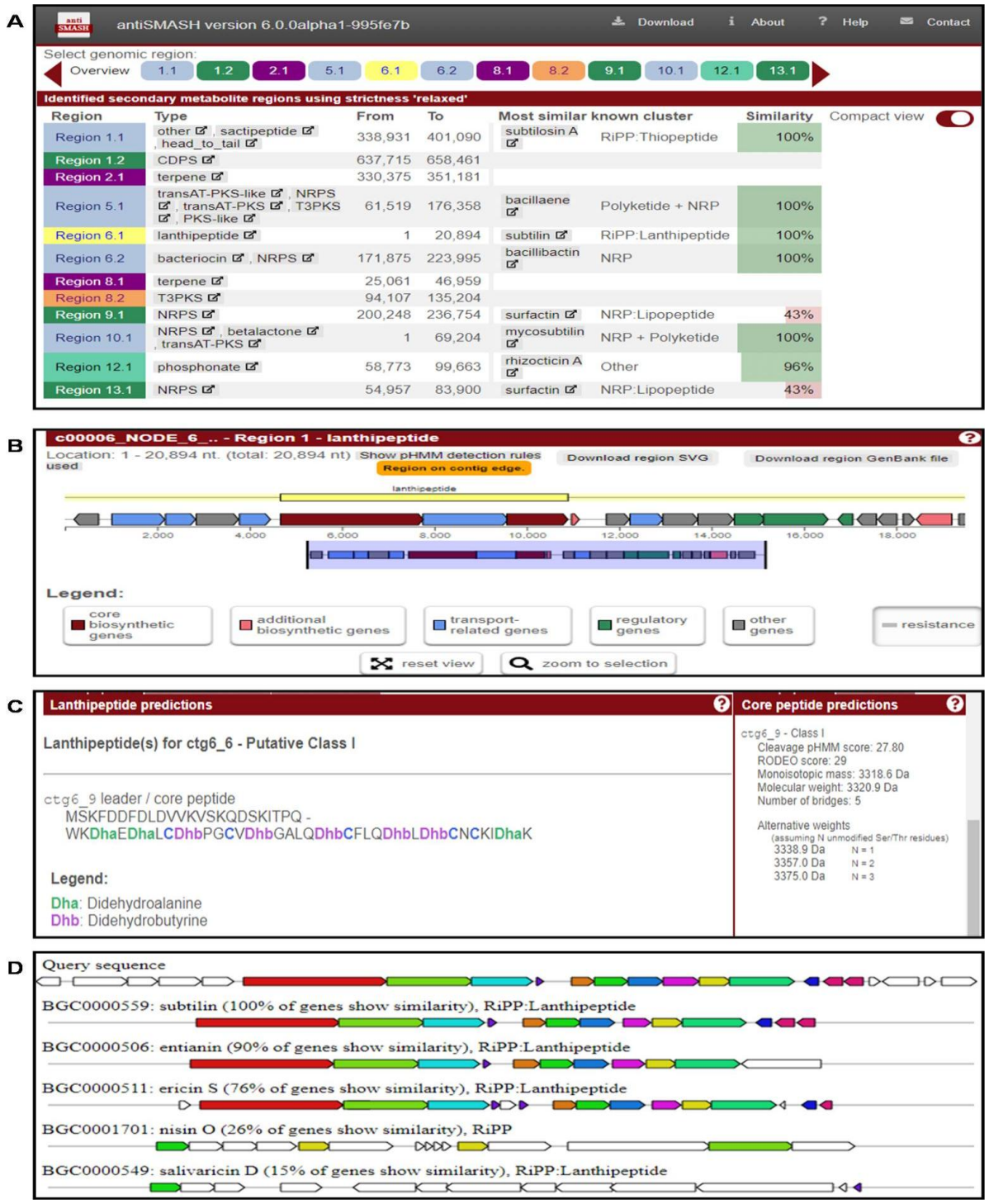
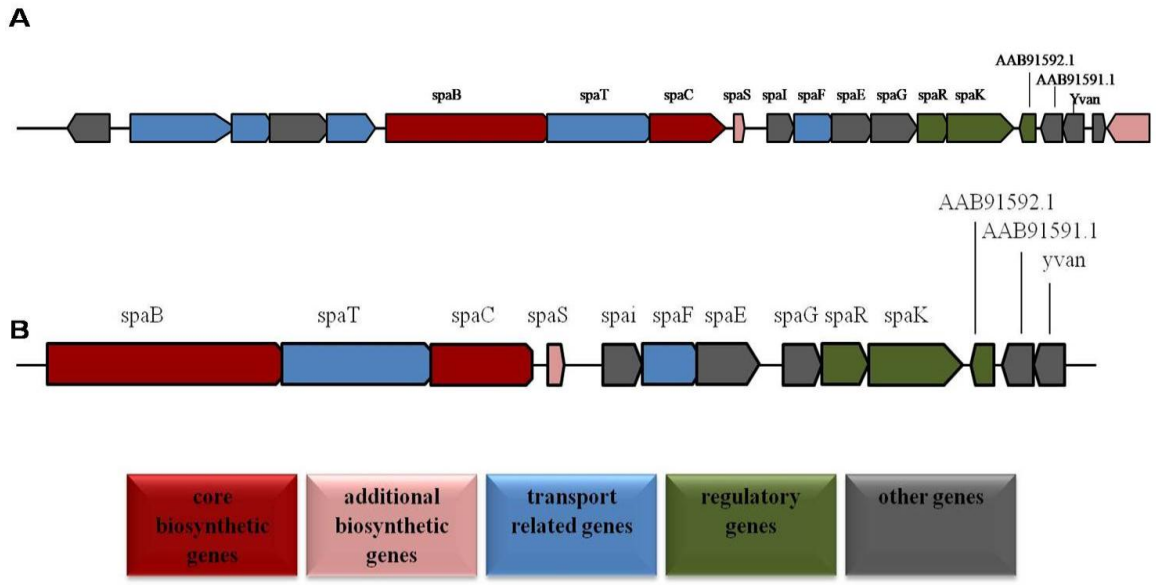


Figure 13. **Putative antiSMASH analysis of whole-genome of strains Ba49:** (A) secondary metabolite clusters predicted in the genome region. (B) And (C) The subtilin cluster identified in contig_6 using antiSMASH and a putative sequence of lanthipeptide; (D) Homologous gene cluster identified and percentage similarity with the identified cluster.



C

Cluster genes	spa B	spa T	spa C	spa S	spa I	spa F	spa E	spa G	spa R	spa K	Y van
Genome location of ATCC6633	495-3587	3578-5422	5395-6720	6795-6965	7548-8045	8032-8775	8772-9527	9688-10299	10317-10979	10970-12349	13424-13831
Genome location of Ba49	4669-7761	7752-9596	9569-10894	10969-11139	11725-12222	12236-12952	12949-13704	13706-14476	14494-15156	15129-16526	17601-18017
% Identity	100	100	99	100	100	100	100	99	100	98	100

Figure 14. Prediction of a biosynthetic gene cluster of Peptide-Ba49: Comparative analyses of lanthipeptide biosynthetic gene cluster of strain Ba49 (A) its identical gene from strain ATCC 6633; (B) The subtilin gene cluster (spa), spa BTC involved in the post-translational modifications and transport of the subtilin, spa S indicates the pre peptide encoding gene, spaIFEG, similar to the immunity gene, and spa RK is a two-component regulatory system; (C) A comparative analysis of open reading frames (ORFs) of the subtilin gene cluster in Ba49 strain with subtilin gene cluster in ATCC 6633.

4.1.9. Effect of extrinsic factors temperature, pH, and enzymes on antimicrobial peptide activity

The effect of various physicochemical parameters such as temperature, pH, and enzymatic hydrolysis on the efficacy of peptide-Ba49 (characterized as subtilin) against *S. aureus* (ATCC 25923) was studied. The purified peptide obtained as elute following HPLC purification (without adding any buffer) were taken as untreated sample (control). Peptide-Ba49 was found to be stable at high temperatures (up to 121^{o}C) and in a wide range of pH (pH 5-10). In terms of thermostability, the antibacterial activity of peptide-Ba49 remained unaffected (100%) in the range of 45^{o}C to 75^{o}C for 30 min. Furthermore, at 100^{o}C (for 30

min) and upon autoclaving (at 121^{o}C for 20 min), the peptide retained 92% and 94% of its antimicrobial activity against *S. aureus* (ATCC 25923), respectively (Fig. 15A). Upon treatment in a range of pH buffers, the peptide-Ba49 showed its stability in the pH range of 5-10 (Fig. 15B). The effect of hydrolyzing enzymes on purified peptide-Ba49 showed complete loss of its antimicrobial activity after treatment with pepsin, protease E, proteinase K, trypsin, and chymotrypsin as compared to an un-treated peptide (control) (Table 4). However, the antimicrobial activity was not affected by lysozyme, RNase, lipase, catalase, and α-amylase, suggesting that the purified AMP was proteinaceous in nature and was not a lipidated peptide.

4.1.10. Effect of metal ions, reducing agents and surfactants on peptide activity

The effects of metals on peptide-Ba49 were studied, as metal ions are known to affect the antimicrobial activity of AMPs (Łoboda et al., 2018; Walkenhorst et al., 2014; Zasloff, 1987). The antimicrobial activity of purified peptide-Ba49 decreased significantly ($p<$ 0.0001) upon treatment with $FeSO_4$ (at 10 mM) at 37^{o}C, and it showed ~ 50% of its activity as compared to other metal ions (Fig. 15C). Purified peptide-Ba49 not treated with any metal ion was taken as a control. The effect of various organic solvents, surfactants, and reducing agents on the peptide was examined separately. It was found that purified peptide-Ba49 retained more than 80% of its antimicrobial activity upon treating with various organic solvents. The effect of these organic solvents on peptide-Ba49 was shown in Table 5. The investigations on the effect of surfactants on antimicrobial activity of peptide-Ba49 showed this peptide retaining more than 80% of its antimicrobial activity after treatment with surfactants (Triton X100, Tween 80, and SDS). Even upon treatment with reducing agents (DTT, β-mercaptoethanol, and urea), this peptide retained more than 80% of its activity. Purified peptide-Ba49 without organic solvents, surfactants, and reducing agents were taken as a control for respective assays. These results revealed the hydrophobic nature of the purified peptide-Ba49 (Chalasani et al., 2015). In the available literature, subtilin L-Q11 was reported to retain 97.9, 97.2, and 95.9% of its activities after 3 h of treatment by 1% (V/V) of Tween-20, Tween 80, and Urea, respectively (Qin et al., 2019).

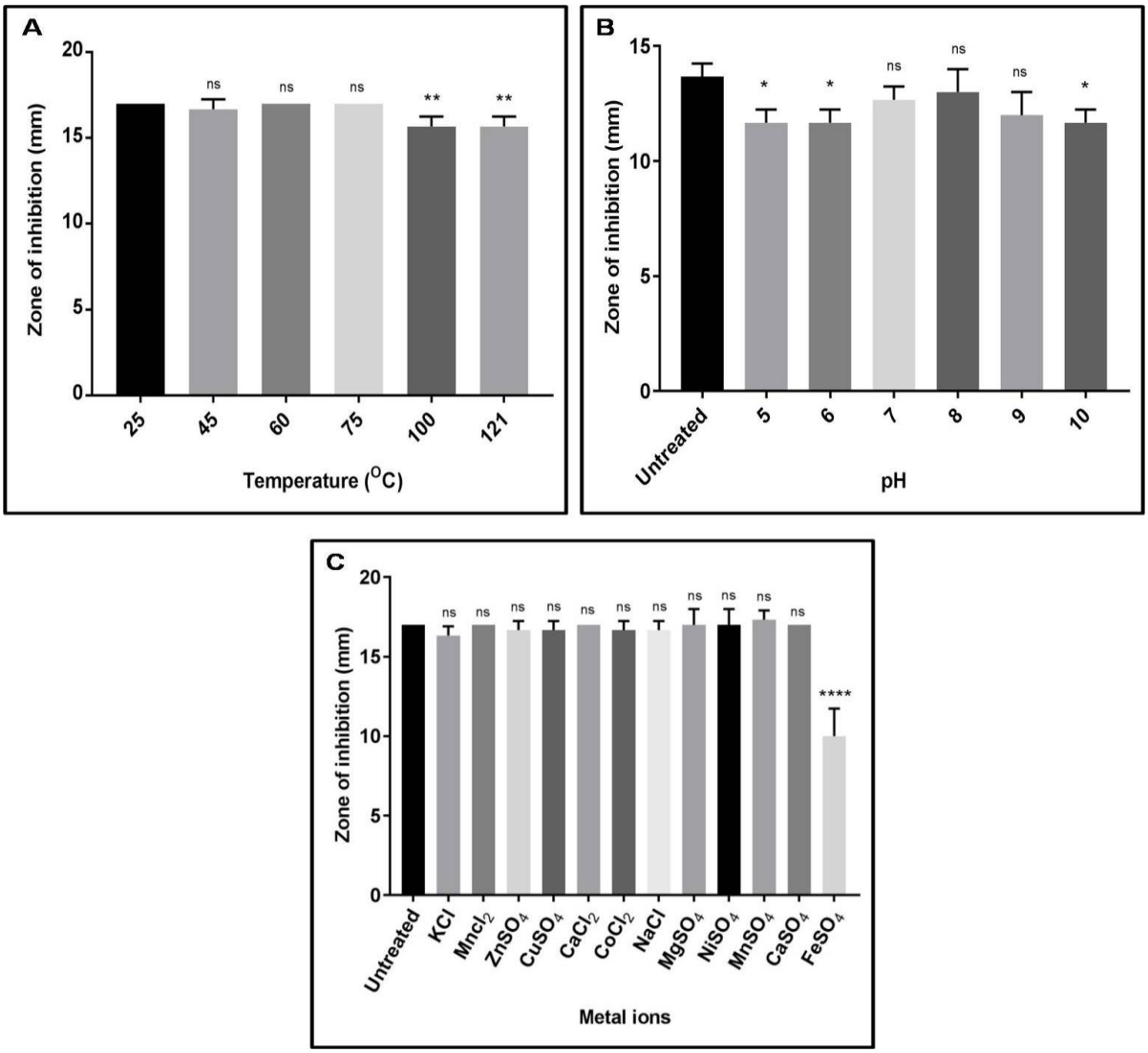


Figure 15. Effect of extrinsic factors on antibacterial peptide-Ba49 activity: (A) Temperature and (B) pH on the antibacterial activity of purified AMP produced by strain Ba49 in terms of zone of inhibition (mm). There was no significant effect of pH in the range of pH 7 to pH 9 and temperature at 45^{o}C, 60^{o}C, and 75^{o}C on purified AMP's antibacterial activity compared to untreated. A significant reduction in purified AMP antibacterial activity was observed at pH 5, 6, and 10 and temperature at 100^{o}C and 121^{o}C; **(**C**)** There was no significant reduction in purified AMP's antibacterial activity from strain Ba49 on treated with metal ions. $FeSO_4$ treated purified peptide-Ba49 significantly lost ~ 50% of its antibacterial activity as compared to untreated AMP. Results were presented as mean ± SD of two independent experiments done in triplicates. $^{****}p < 0.0001$, $p < 0.05$ were considered significant and 'ns' was considered as non-significant

Table 4: Effect of various enzymes on the antibacterial efficacy of purified peptide-Ba49

Enzyme	Activity (mm)*
Control	17±0
Pepsin	0
Protease E	0
Proteinase k	0
Chymotrypsin	0
Trypsin	0
Lysozyme	17±0
RNase	17±0
Lipase	16±0
Catalase	17±0
α-amylase	17±0

'*' Value represents the mean ± SD. Results were presented as mean ± SD of two independent experiments done in triplicates.

Table 5: Effect of organic solvents, surfactants and reducing agents on antibacterial efficacy of purified peptide-Ba49

Treatment		Activity(mm)*
	Control	19±0
Organic solvents (50% v/v)	Methanol	15.6 ±0.57
	Ethanol	16±0
	Isopropanol	17±0
	Acetone	18±0
	Ethyl acetate	17±0
	Chloroform	16±0
	Acetonitrile	19±0
	Butanol	16±0
Surfactants (1% w/v)	Triton X 100	18.3±0.57
	Tween 80	17±0
	SDS	15±1
Reducing agents	DTT (10mmol l^{-1})	14.6±0.57
	β-mercaptoethanol (10% v/v)	11.6±0.57
	Urea (1%)	16.6±0.57
	Urea (1M)	16.3±0.57

'*' Value represents the mean ± SD. Results were presented as mean ± SD of two independent experiments done in triplicates

4.1.11. Assessment of antibacterial activity of peptide-Ba49 against *S. aureus*

4.1.11.1. Determination of minimum inhibitory concentration (MIC)

MIC of the purified peptide-Ba49 and nisin against different *Staphylococcus* genus strains were determined using a two-fold serial dilution method in 96-well plates. Though MIC values of the peptide varied from 0.5 μM to >16 μM against different strains of *Staphylococcus* (summarized in Table 6), intriguingly, the compound inhibited most of the MRSA at 2–8 μM concentration (Table 7). It may be noted that peptide-Ba49 exhibited superior activity than nisin against some of these strains.

4.1.11.2. Time kill kinetics

A time-kill kinetic assay was performed to evaluate the time-dependent profile of killing the target *S. aureus* (ATCC 25923) in suspension. A moderate decline in the $\log_{10}$ (CFU/mL) of *S. aureus* till 120 min was followed by a sharp decline in cell viability up to 240 min (Fig. 16), suggesting the bactericidal potential of the purified peptide-Ba49.

4.1.11.3. Post antibiotic studies

To determine the retention and accumulation of peptide-Ba49 in *S. aureus* for its prolonged post-antibiotic effect (PAE). After exposing *S. aureus* cells with 1xMIC and 2xMIC of peptide-Ba49 for 2 h and 4 h, it was observed that the PAE was significantly prolonged at 2x MIC. Exposure of 2 h and 4 h at 2xMIC halted the bacteria up to 10.5 h, and at post 4h incubation with 2xMIC, the bacterial growth was observed to be more hampered with prolonged PAE effect (Fig. 17A and B). The result suggests that the PAE degree is related to the degree of cellular damage done by the antibiotic to the bacterial cell. Higher the cell damage, higher will be the repairing time (Craig, 1991).

Table 6: Minimum inhibitory concentration (MIC) values of purified antibacterial peptide-Ba49 against different *Staphylococcus* strains

S.No.	Strain Number	Strain Name	MIC (µM) of Ba49 Peptide	MIC (µM) of Nisin
1.	MTCC 3382^T	*Staphylococcus epidermidis*	16	8
2.	MTCC 2292^T	*Staphylococcus lentus*	2	2
3.	MTCC 2992^T	*Staphylococcus gallinarum*	16	4
4.	MTCC 3383^T	*Staphylococcus haemolyticus*	>16	<0.25
5.	MTCC 3545^T	*Staphylococcus chromogenes*	>16	<0.25
6.	MTCC 3614^T	*Staphylococcus lugdunensis*	16	8
7.	MTCC 6154^T	*Staphylococcus scuiri*	>16	2
8.	MTCC 4436^T	*Staphylococcus warneri*	>16	<0.25
9.	MTCC 6152^T	*Staphylococcus intermidus*	0.5	1
10.	MTCC 1430^T	*Staphylococcus aureus*	2	4
11.	ATCC 25923	*Staphylococcus aureus*	4	4

Table 7: Minimum inhibitory concentration (MIC) of the purified peptide-Ba49 against MRSA isolates

Sr. No.	Strain		MIC (µM) of Ba49 Peptide	MIC (µM) of Nisin
1	*Staphylococcus aureus* ATCC 33591 (MRSA)		2	8
2	*Staphylococcus aureus* ATCC 43300 (MRSA)		2	8
3	MRSA 831	Clinical isolates	16	>32
4	MRSA 839		16	8
5	MRSA 1		2	4
6	MRSA 2		2	8
7	MRSA 3		8	8
8	MRSA 4		2	8

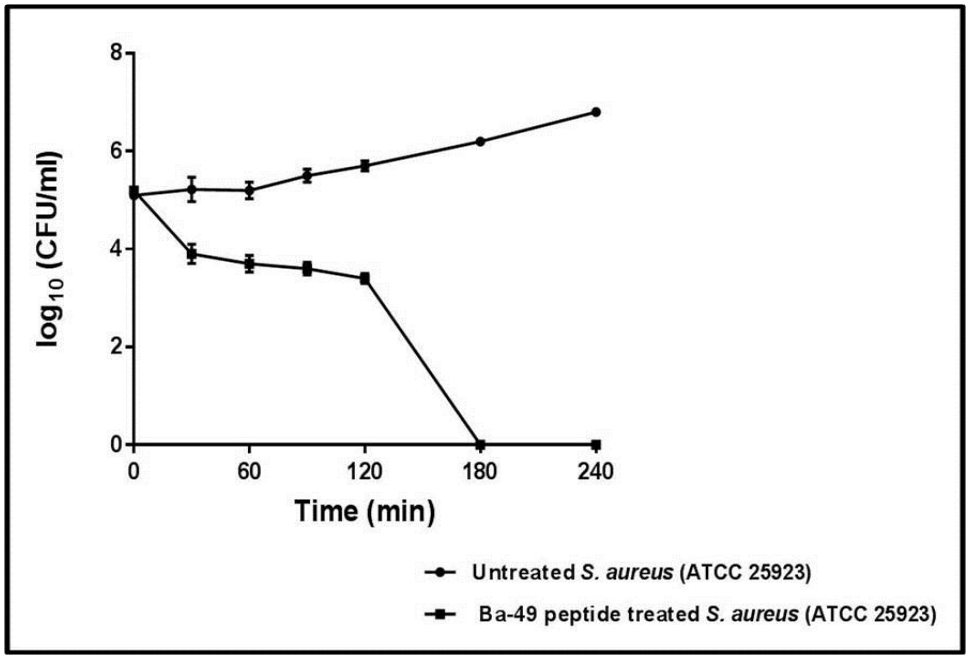


Figure 16. Time kill kinetics of purified peptide-Ba49 against *S. aureus* (ATCC 25923): Time kill kinetics of the purified peptide-Ba49 was assessed at 2xMIC (8µM). The time profile shows AMP triggered cell lysis of *S. aureus* (MTCC 25923) at different time intervals. Results were presented as mean ± SD of two independent experiments done in triplicates (error bars indicate the mean ± SD.

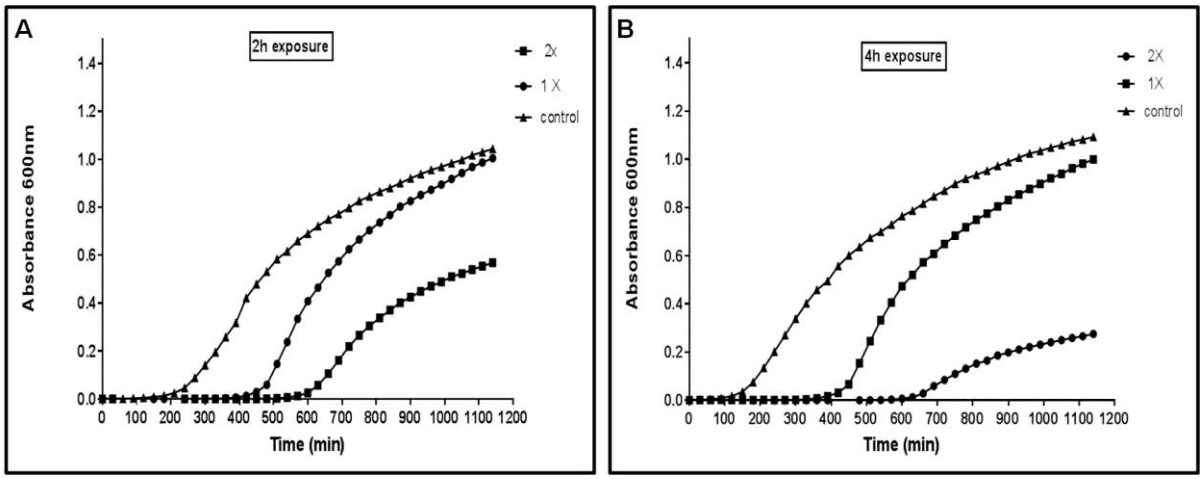


Figure 17. Post antibiotic effect of peptide-Ba49: (A) Post 2 h and (B) Post 4 h of exposure. The bacteria showed suppressed survival for approximately 10 h post 2 h and 4 h exposure at a concentration of 2xMIC. In contrast, the bacterial repair was reduced at post 4 h exposure at a concentration of 2xMIC.

4.1.12. Mechanism of action studies of peptide-Ba49 against *S. aureus*

In the healthy untreated bacterial cell, ROS production is a natural side effect of aerobic respiration. The bacteria can produce enzymes like catalase and superoxide dismutase to prevent damage, which further detoxifes the ROS (Gasser et al., 2016; Greenberg and Berger, 1989). For determining the effect of the peptide on the enhancement of ROS production, *S. aureus* was treated with different concentrations of peptide in the presence of DCFH-DA, an unspecific probe for ROS. It showed that the ROS production was enhanced when treated with 2xMIC (8µM) of peptide than control; whereas, at 1xMIC, ROS production was low (Fig. 18A). This suggested that by increasing the concentration of peptide, there is an enhancement of ROS production, which indirectly affected the growth of *S. aureus* (ATCC 25923).

However, if the bacterial cells fail to minimize the excess of intracellular ROS production, membrane depolarization occurs, which ultimately leads to cell death (Yadav et al., 2020; Zhou et al., 2020). The same phenomenon was observed in our study; so, to check the change in membrane potential of *S. aureus* in the presence of peptide-Ba49 a voltage-sensitive fluorescent cationic probe 3, 3-dipropylthiacarbocyanine [$DiSC_3$ (5)] localization was studied. Due to its cationic nature it accumulates on the polarized membranes, which results in self-quenching of fluorescence. However, during membrane depolarization, de-quenching of fluorescence dye occurred (Boix-Lemonche et al., 2020). After the addition of peptide-Ba49 for 1 h, an increase in fluorescence was observed compared to that untreated one. Increase fluorescence was treated with peptide-Ba49 at 1xMIC and 2xMIC results in depolarization of the cytoplasmic membrane of the *S. aureus* (Fig. 18B).

Further, the alteration in membrane potential was evaluated using the fluorescent voltage-sensitive dye $DiBAC_4$ (3), which senses the change in charge present on the cytoplasmic membrane. Fig. 18C represents the change in *S. aureus* cells fluorescence treated with purified peptide-Ba49 at different concentrations, i.e., 0xMIC, 2xMIC (4 µM), and 4xMIC (8 µM). Polymyxin B was used as a positive control. Upon treating the *S. aureus* cells with 2xMIC (4 µM) of the peptide, *S. aureus* cells inner leaflet became more positive (depolarization). Hence, there was a significant ($p < 0.001$) increase in fluorescence. However, on enhancing the concentration of purified peptide-Ba49, i.e., 4xMIC (8 µM), there was a significant decrease ($p< 0.01$) in the fluorescence, which could be due to leakage of net

positive charge from inside to outside, thereby making the inner leaflet of *S. aureus* cells more negative (hyperpolarization) (Adams and Levin, 2012).

Further, the bacterial cell viability in terms of its membrane integrity, *S. aureus* cells were stained with propidium iodide (PI) dye, which stains the nucleic acid of cells with damaged membrane. The cells were treated with the purified AMP for 2 h and 4 h was later assessed by PI uptake. It could be seen from Fig.19 that 96.8%, 42.7%, and 54% of *S. aureus* cells remained viable in case of untreated cells (control), cells treated with purified peptide-Ba49 (for 4 h), and cells treated with nisin (for 4 h), respectively indicating loss of *S. aureus* membrane integrity on treated with purified peptide-Ba49. The detailed information on morphological changes was acquired from scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM study revealed the smooth surface in untreated *S. aureus* cells (Fig. 20A and B). However, upon treatment with the peptide-Ba49 (Fig. 20C and D), some morphological alterations were observed, like membrane disruption and pore like formation.

The electron density images of transmission electron microscopy (TEM) (Fig. 21C and D) indicated a drastic impact of peptide-Ba49 on *S. aureus* cells as compared to untreated cells (Fig. 21 A, B). It was founded that untreated cells have a uniform shape with intact cell walls, whereas the cells treated with purified peptide-Ba49 lost their uniformity because of rupturing. These images also showed that the cytoplasmic contents came out of the cells following peptide-Ba49 treatment compared to untreated cells.

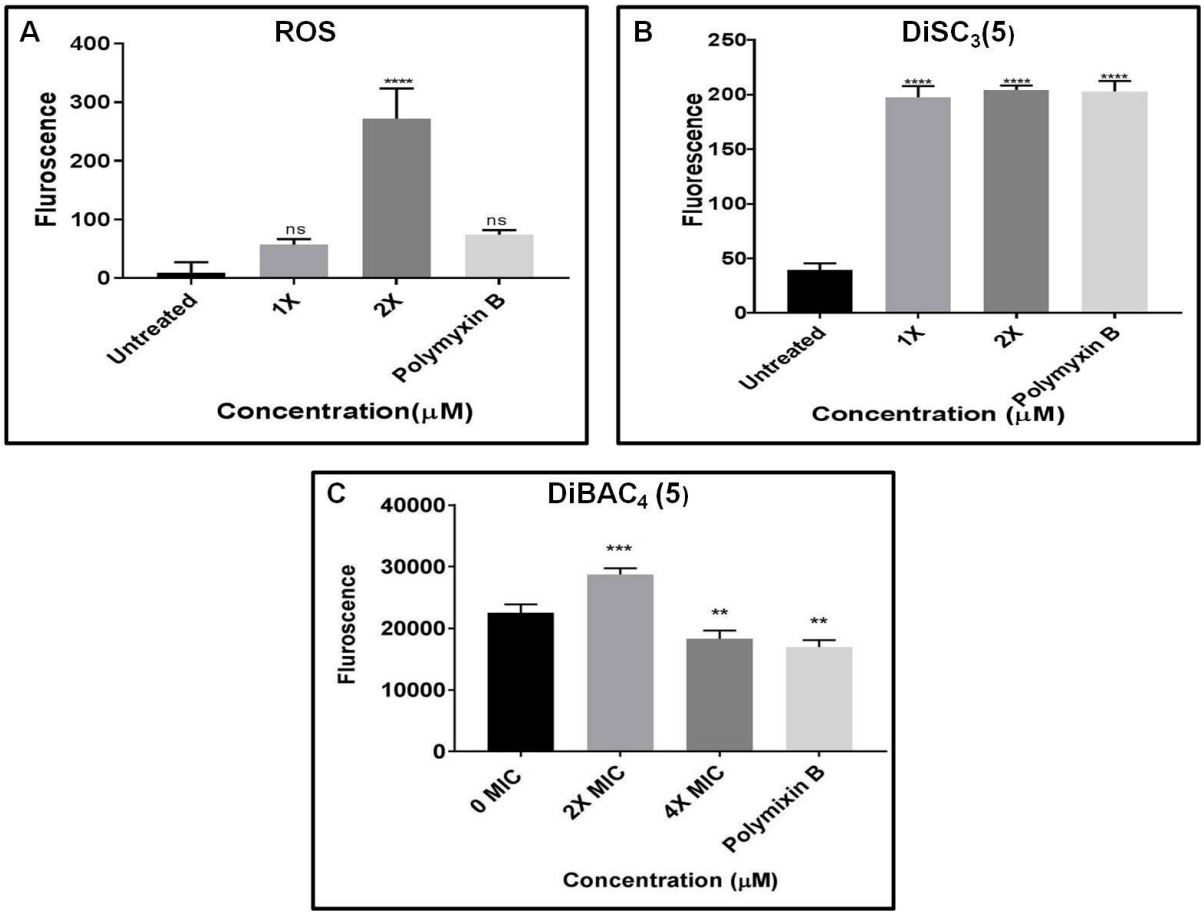


Figure 18. Deciphering the mechanism of action of peptide-Ba49 on *S. aureus* (ATCC 25923) cell membrane: (A) The different concentration of peptide-Ba49 was used to treat *S. aureus* cells and then stained with ROS (H_2DCFDA). The histogram presents fluorescence normalized with untreated cells by H_2DCFDA dye. No significant difference was observed between 1xMIC and ploymixin B, but a significant difference was observed in 2xMIC compared to untreated; (B) Cell membrane permeability of *S. aureus* was evaluated with the release of voltage-sensitive dye $DiSC_3$-(5) during the peptide-Ba49 treatment. Fluorescence was measured spectroscopically at 622 nm to 670 nm excitation and emission wavelengths. *S. aureus* cells were treated with 1xMIC (4 μM) and 2xMIC (8 μM) MIC with peptide- Ba49. A significantly increased fluorescence signal was observed peptide treated cells as compared to untreated cells. Polymyxin B treated cells were taken as a positive control; (C) *S. aureus* cells were treated with 2xMIC (4 μM) and 4xMIC (8 μM) MIC of purified peptide-Ba49. Following treatment of *S. aureus* cells (1×10^7 CFU/mL) with 2xMIC (4 μM) and 4xMIC (8 μM) of purified peptide for 4 h at 37^{o}C, $DiBAC_4$ (3) was added to a final concentration of 1μM and fluorescence of the mixture was monitored (excitation, 516 nm and emission, 490 nm). The increased fluorescence signal was observed in peptide-Ba49 treated cells as compared to untreated cells. Polymyxin B treated cells were taken as a positive control. Results were presented as mean ± SD of two independent experiments done in triplicates. $^{**}p< 0.01$, $^{***}p< 0.001$, $^{****}p < 0.001$.$p< 0.05$ were considered significant.

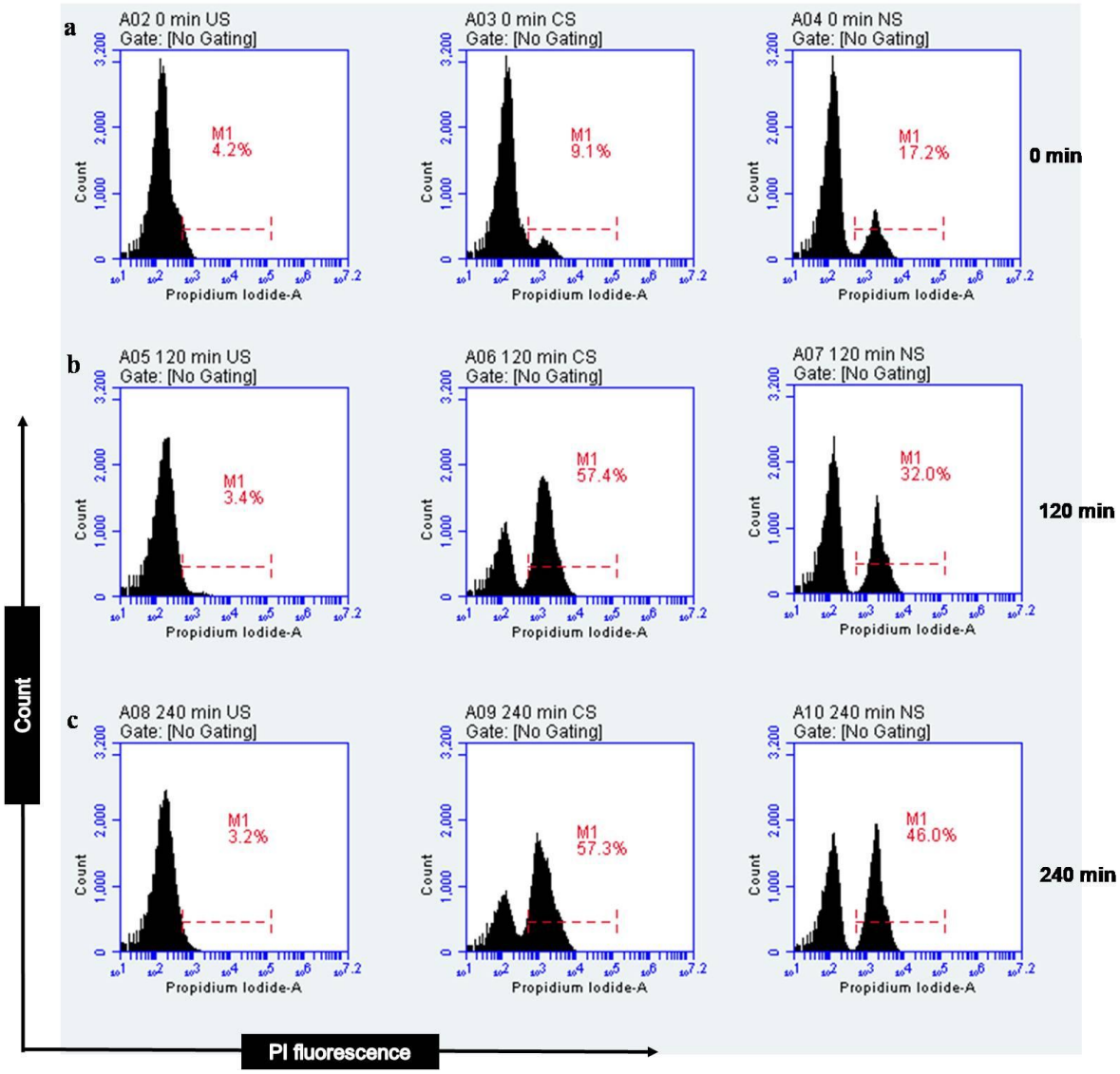


Figure 19. Flow cytometry analysis of *S. aureus* (MTCC 1430) cells treated with purified peptide-Ba49: The effect of treatment of purified AMP at 2xMIC (4 µM) on *S. aureus* cells' viability was determined by the Propidium iodide (PI) uptake assay at 0, 120, and 240 min. Untreated cells, AMP treated cells, and nisin treated cells were marked as US, CS, and NS, respectively. Rows a, b and c represent data for untreated cells, peptide-Ba49 treated cells 0 h, 120 h, and 240 h, respectively, whereas the columns represent untreated, peptide-Ba49 treated, and nisin treated cells of *S. aureus* (ATCC 252923). The number in the inset of flow cytometry histogram plots depicts the percentage of PI-positive cells.

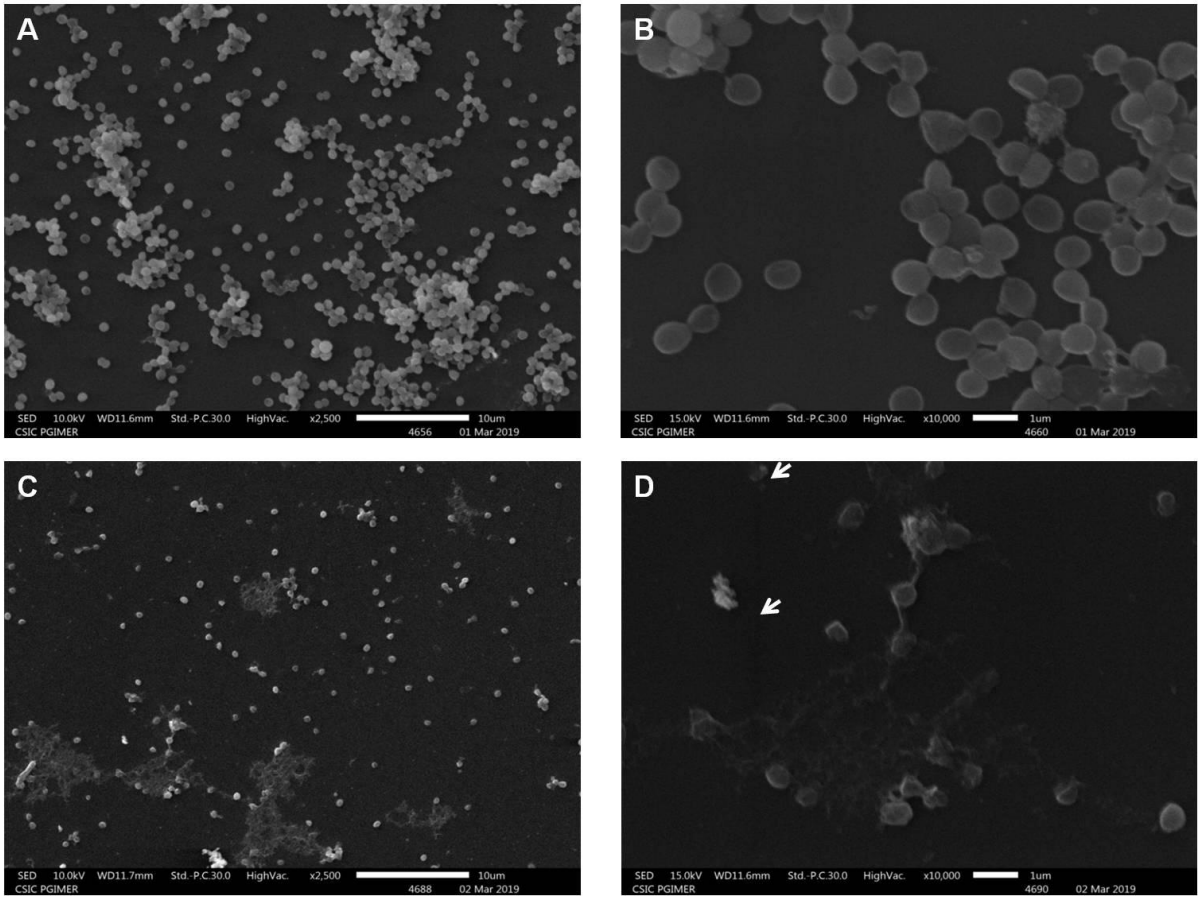


Figure 20: Scanning electron microscopy of *S. aureus* cells treated with purified peptide-Ba49. (A-B) Untreated *S. aureus* cells from the control group at 2.5K and 10K resolution; (C-D) Purified peptide treated *S. aureus* cells at 2.5K and 10K resolution. A white arrow shows ruptured cells after peptide treatment for 240 min.

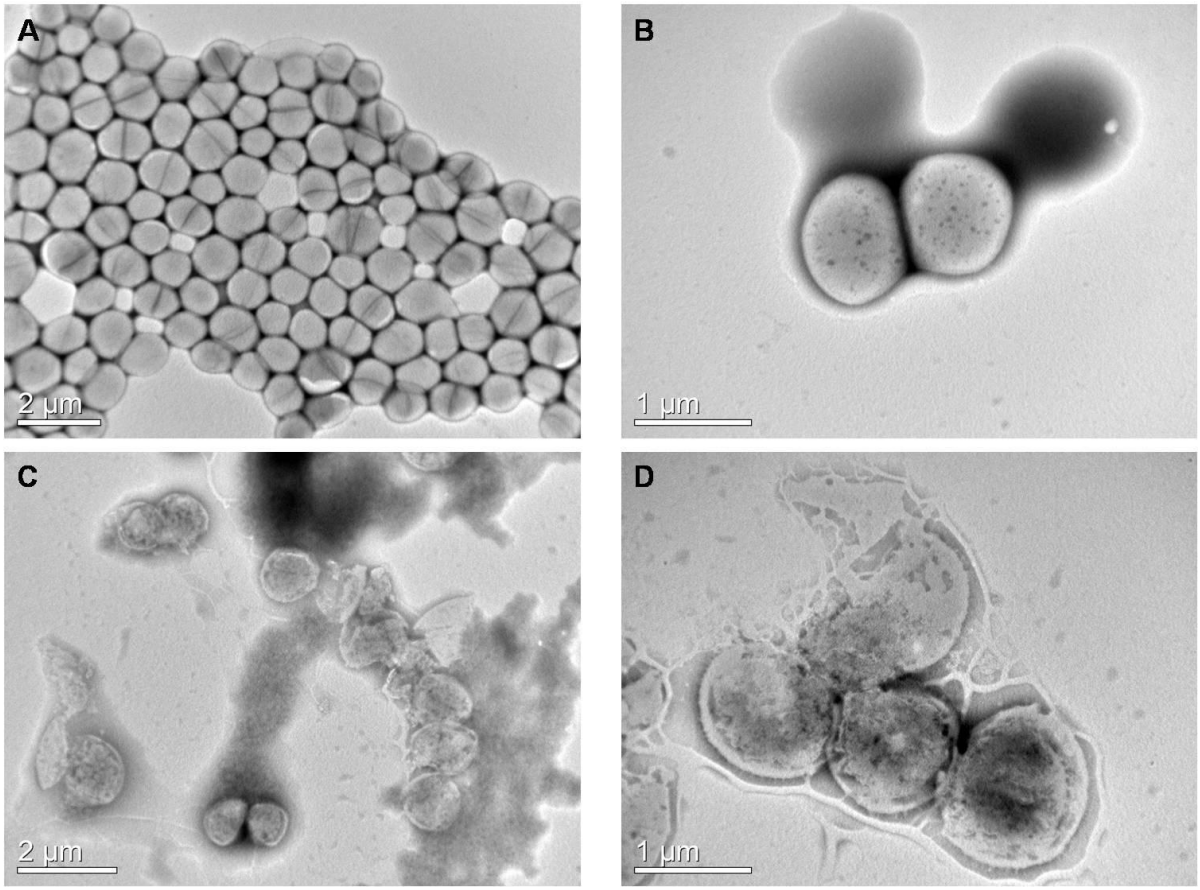


Figure 21: Transmission electron microscopy of *S. aureus* cells treated with peptide-Ba49. (A and B) Untreated *S. aureus* cells from the control group; (C and D) *S. aureus* cells treated with 2xMIC (4 µM) of purified peptide-Ba49. Purified peptide treated cells showed disrupted cell membrane with biomass leakage.

4.1.13. Application of antimicrobial peptide from strain Ba49

4.1.13.1. Biofilm- prevention studies

4.1.13.1a. Effect of the peptide on biofilm formation

The effect of different concentrations of peptide-Ba49 on *S. aureus* (ATCC 25923 and MTCC 1430) biofilm formation was studied. In this case, the OD_{595} was plotted in terms of percentage. Investigations on the effects of different concentrations (0, 2, 4, 8 and 16 µM) of peptide-Ba49 on prevention of biofilm formation showed peptide concentrations of 8 µM and 16 µM inhibiting nearly 90% of biofilm formation (Fig. 22A and C). However, on performing the biofilm metabolic activity by XTT assay, it showed that at peptide concentration of 8 µM, < 50 % biofilm was metabolically inactive while peptide concentration of 16 µM almost completely inhibited biofilm formation as compared to untreated biofilms (Fig. 22 B and D).

4.1.13.1b. Confocal laser scanning microscopy (CLSM) of *S.aureus* biofilm

By using confocal imaging, it could be observed that the peptide treated *S. aureus* biofilms maintained its overall structure compared to the untreated one. The viable cells within the peptide treated biofilm were less as compared to the untreated ones. Because AMPs generally interact with the cytoplasmic membrane and causes membrane rupturing, leading to cell lysis (Bessa et al., 2019). Thus, in peptide-treated biofilm, the red (nonviable) cells are more than the untreated ones (Fig. 23A and B).

Extracellular polymeric substance (EPS) is an essential substance for cellular encasement and community function in different stages (Miao et al., 2019). Further, to confirm the prevention of *S. aureus* biofilm, a Calcofluor white M2R dye that stains EPS was used. The blue signal reduction was observed in peptide treated biofilm compared to that untreated one (Fig. 24A and B), indicates that the peptide-Ba49 reduced the biofilm biomass by rupturing the bacterial cell membrane.

4.1.13.1c. Effect of the peptide on preformed biofilm

The biofilm cells are < 1000 times resistant to planktonic cells, though it may vary from organism to organism. Various factors are responsible for biofilm resistance, such as growth rate, temperature, nutritional value, pH, etc. An essential biofilm resistance factor is the slower diffusion of drugs (Shikha et al., 2020). In the present study, a mature *S. aureus* biofilm was treated with different peptide concentrations to observe the reductive effect, followed by an additional incubation of 24 h. In the case of preformed biofilm assay, the number of treated bacteria was increased to 10-fold compared to inhibition for biofilm formation; thus, a higher concentration of peptide was exposed, i.e. (8, 16, 32 and 64 µM) in this assay. As shown in Fig. 25A and C, after treating with peptide-Ba49 (16 µM to 64 µM), there was a significant reduction in preformed biofilm biomass at $OD_{595.}$ Based on the colorimetric reduction assay of tetrazolium salt (XTT), ~80% of biofilm metabolic activity was lost at 64 µM of peptide concentration (Fig. 25 B and D).

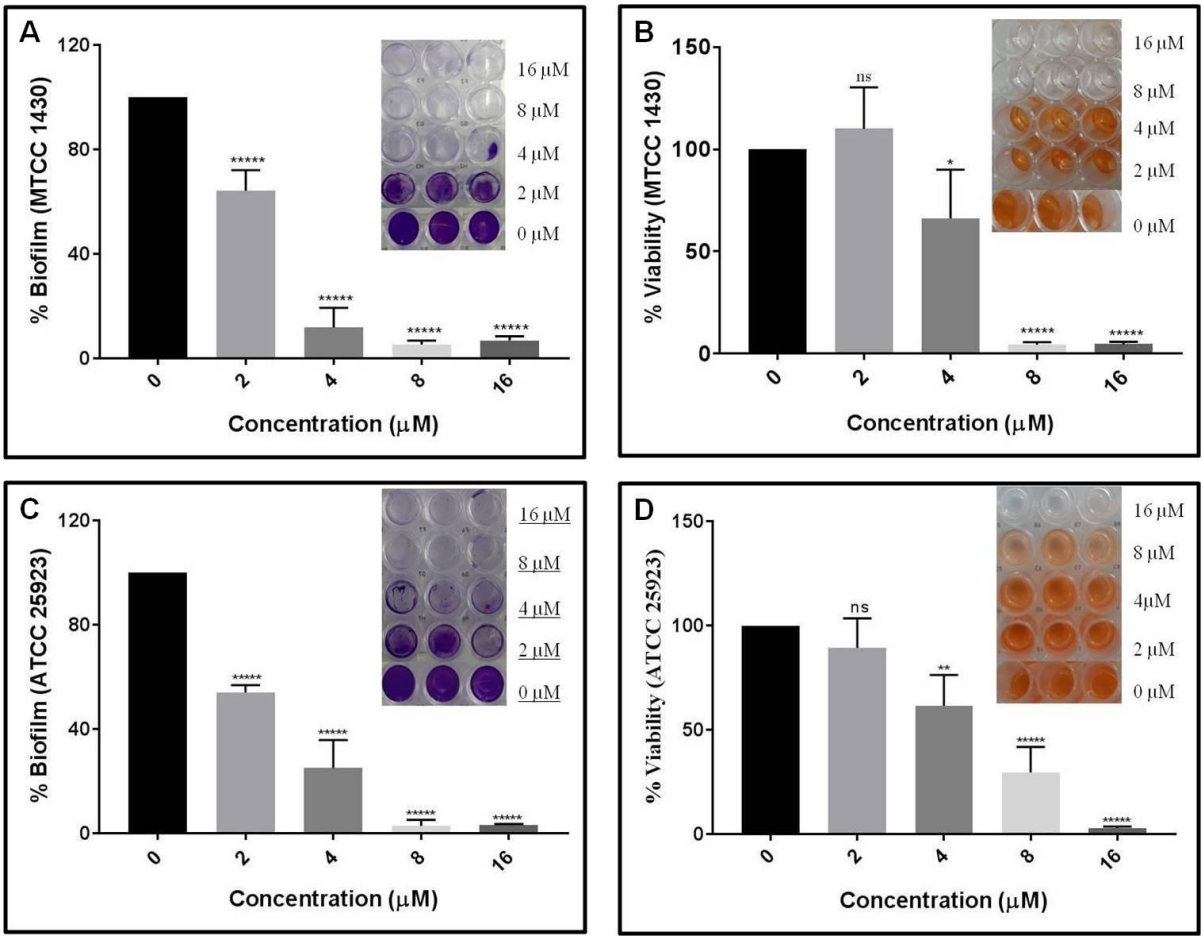


Figure 22. Inhibition of *S. aureus* (ATCC 25923 and MTCC 1430) biofilm formation by peptide-Ba49: (A) and (C) depicts *S.aureus* biofilm biomass as measured by using crystal violet assay; (B) and (D) represents cell viability as measured by using XTT assay. One-way ANOVA followed by Dunnett's test for multiple comparisons, N=2 independent experiments with triplicates. $^{**}p< 0.01$, ***** $p< 0.00001$ and $p>0.05$ were considered as non-significant (ns).

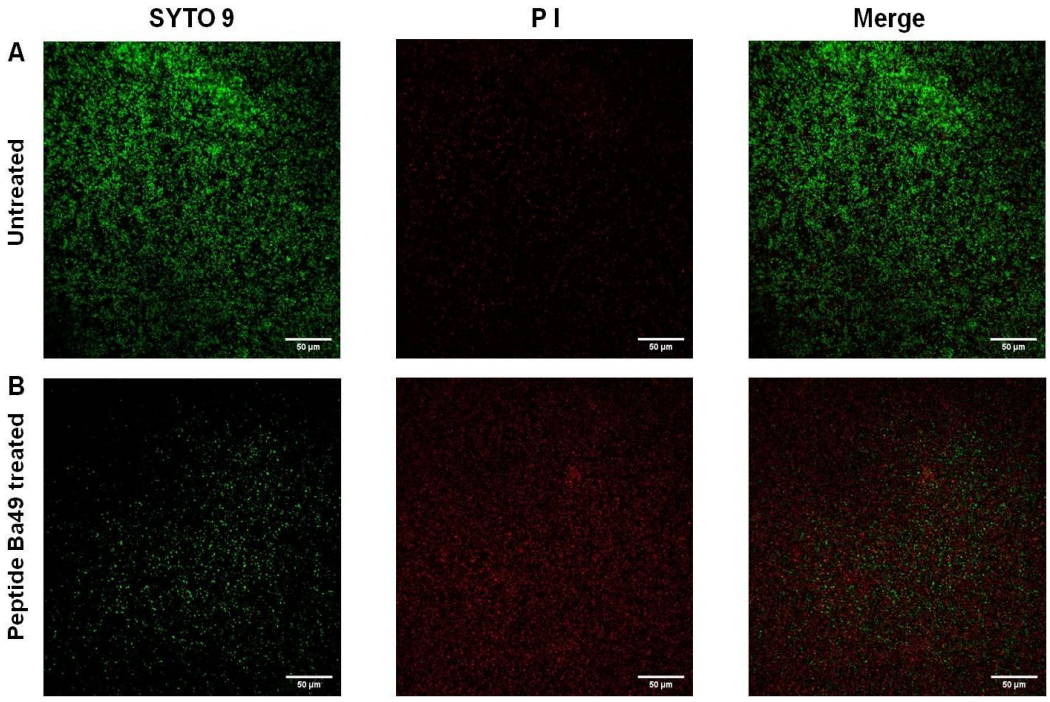


Figure 23. Confocal imaging of *S. aureus* (ATCC 25923) biofilm (left-green channel (live), middle-red channel (dead), and right-merged channel): (A) untreated *S. aureus* biofilm and (B) peptide-Ba49 treated *S. aureus* biofilm. SYTO 9 excitation, 485 nm and emission, 498 nm, stains the live cells, where as PI excitation, 535 nm and emission, 617 nm stains the dead cell. Images are representative of two independent experiments.

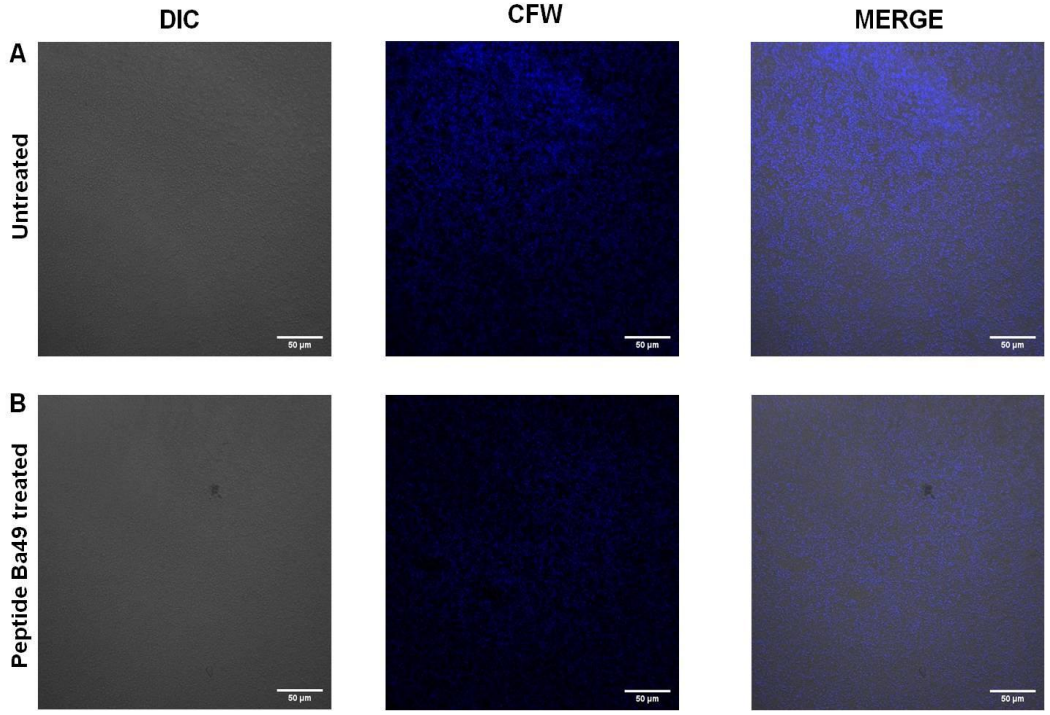


Figure 24. Confocal imaging of Extracellular polymeric substance EPS during *S. aureus* (ATCC 25923) biofilm formation stained with CFW dye (left-DIC channel, middle-CFW channel, and right-merged channel): (A) untreated *S. aureus* biofilm and (B) peptide-Ba49 treated *S. aureus* biofilm. CFW excitation, 413 nm and emission, 480 nm, stains EPS of biofilm. Images are representative of two independent experiments.

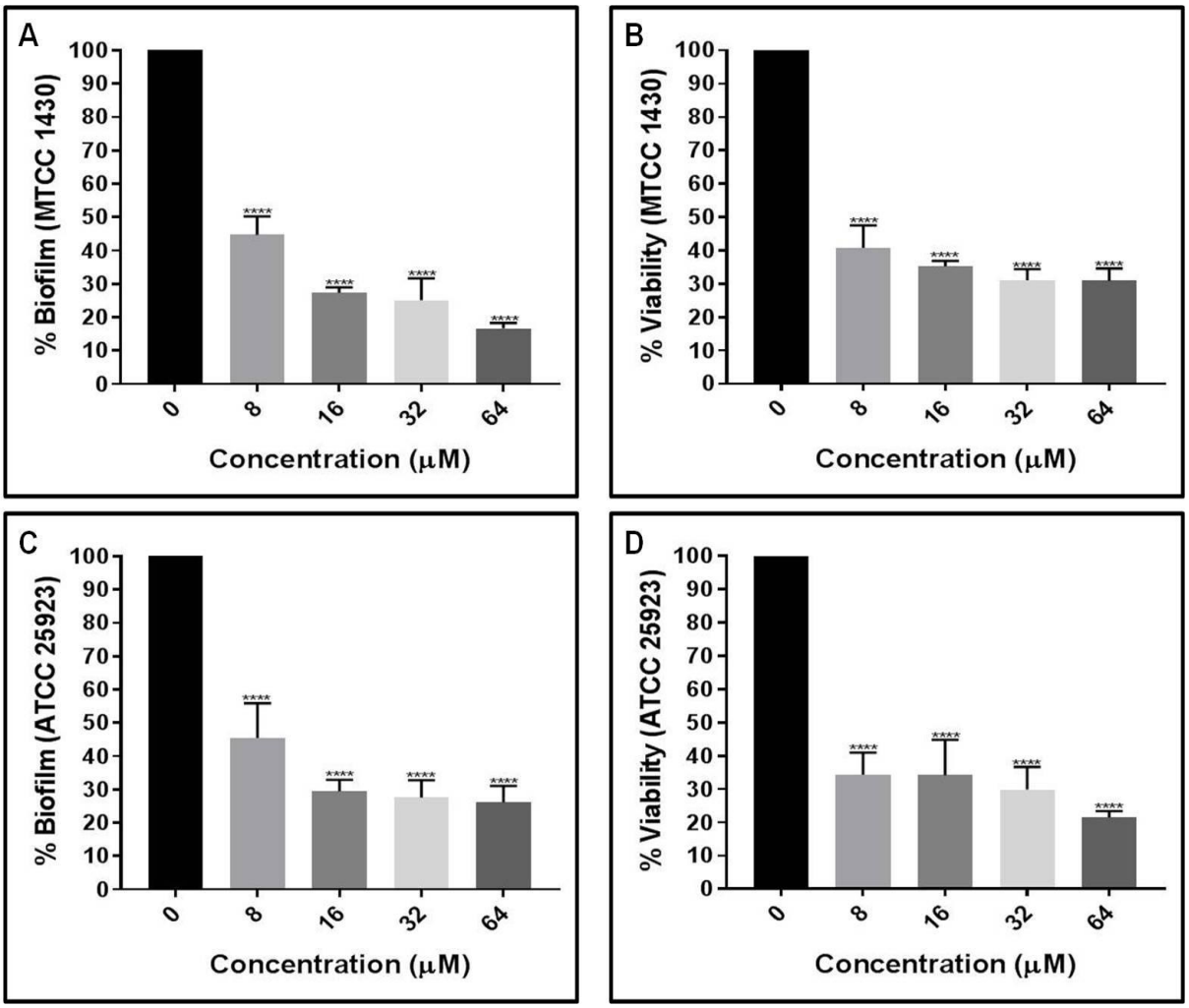


Figure 25. Prevention and inhibition of preformed biofilm of *S. aureus* (ATCC 25923 and MTCC 1430) after peptide-Ba49 treatment: (A) Represents preformed *S. aureus* biofilm biomass after peptide-Ba49 treatment and measured using crystal violet assay; (B) Cell viability was measured by using XTT assay. One-way ANOVA followed by Dunnett's test for multiple comparisons, N=2 independent experiments with triplicates, $^{****}p < 0.0001$.

4.1.13.2. Mammalian Cytotoxicity

The cytotoxic effect of the purified peptide-Ba49 on three different mammalian cell lines, i.e., HEK 293T, RAW 264.7, and L929, was investigated by MTT assay. MTT assay, based on reducing a yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells (Mosmann, 1983), measures the cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. The cell viability was elucidated through the detection of cellular oxidative metabolism. Fig. 26 shows the survival rate of mammalian cells, indicating a low cytotoxic effect in the range of MIC of the purified peptide-Ba49 on mammalian cells compared to the untreated cells (0 μM).

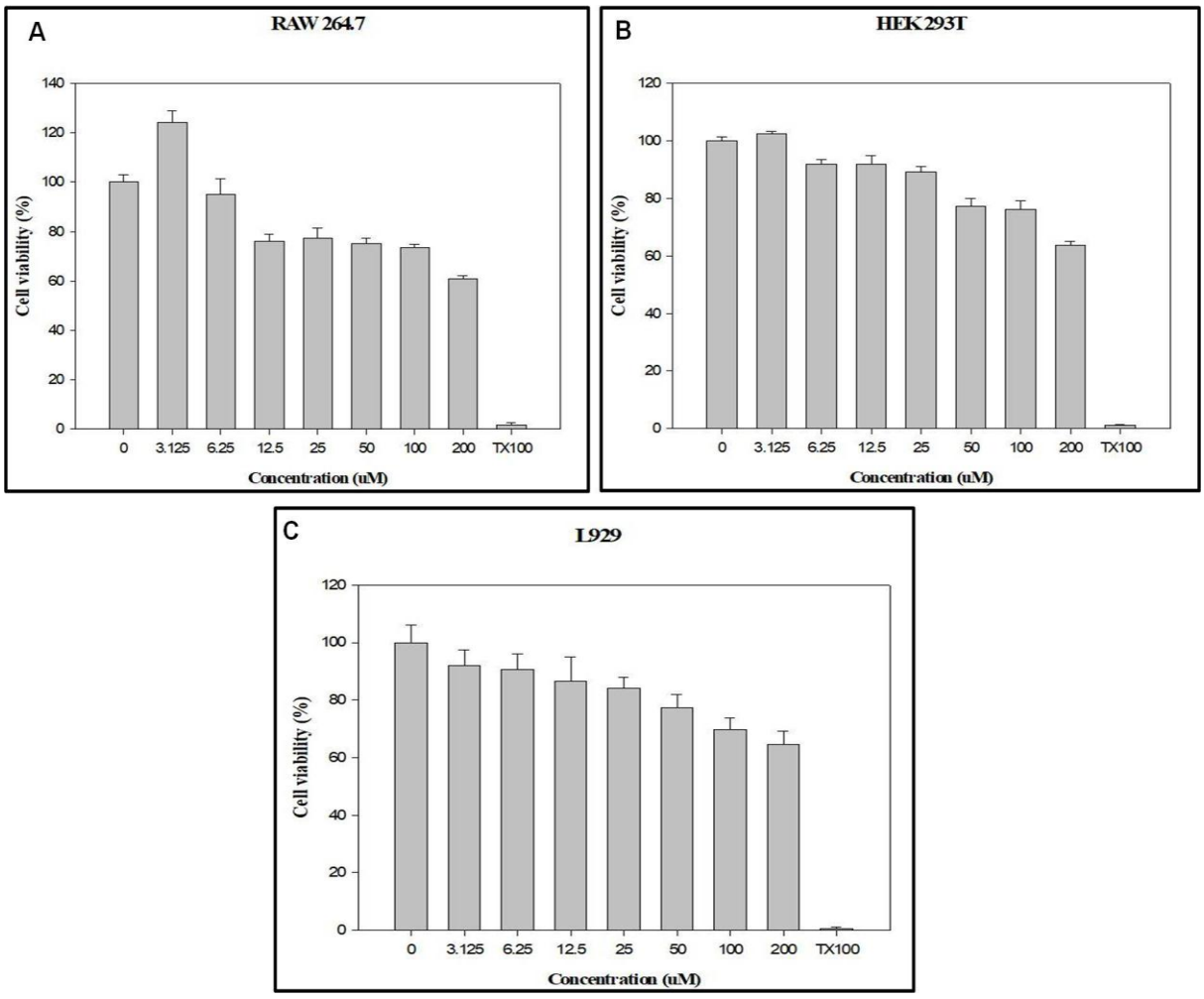


Figure 26. Cytotoxic effect of the purified peptide-Ba49 on HEK 293T, RAW 264.7, and L929 cell line: Following incubation of these cells for 24 h at 37°C in a humidified CO_2 incubator, the cells were treated with purified peptide-Ba49 at different concentrations (3.12, 6.25, 12.5, 25, 50, 100, and 200 μM) for 24 h. Triton X-100 (0.1%) was taken as a positive control. The addition of MTT followed by a stop solution, and absorbance was taken at 570 nm. Results were expressed as Mean ± SD in terms of percent viability. Data is representative of two independent experiments done in triplicates.

4.1.13.3. Cell migration assay

Cell migration is a rate-limiting factor event in the wound healing process (Cappiello et al., 2018). A scratch assay was used to observe the cell migration capacity of the murine fibroblast cells (L929) under the stimulation of peptide-Ba49. It was observed that after the stimulation of L929 cells with purified peptide-Ba49, the cell migration capacity was enhanced and hence covers the scratched area by 50% at 24 h time point. Moreover, at 36h, the scratch was almost completely full compared to that of untreated scratch (Fig. 27).

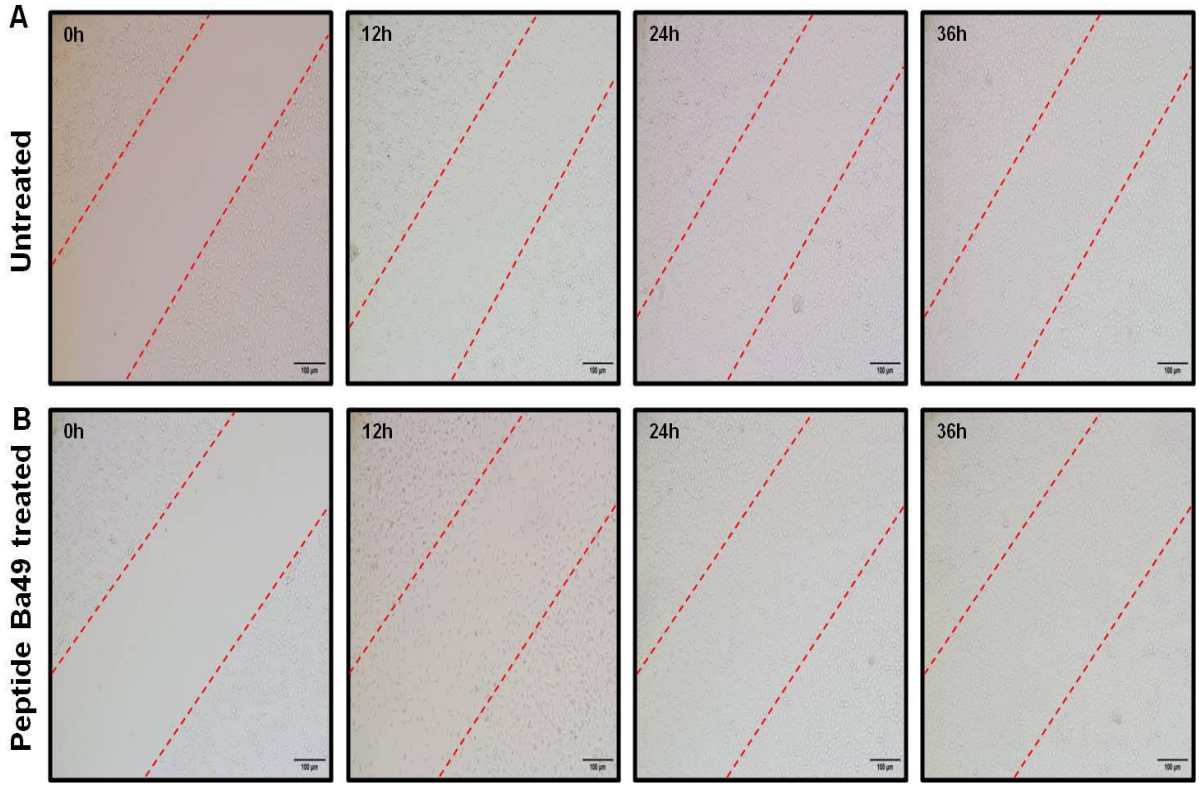


Figure 27. Cell migration assay: To study the cell migration, fibroblast cells L929 were treated with peptide-Ba49 and observed under microscope till 36 h (A) untreated L929 cells (B) peptide-Ba49 treated L929. With each time points (0, 12, 24, and 36 h) the peptide-Ba49 treated cells migrates fastly and covers the scratch area as compared to untreated cells

4.1.13.4. Intracellular activity of peptide-Ba49 against *S. aureus* in macrophage cell line

The intracellular activity of peptide-Ba49 was tested by infecting the macrophage cell line (RAW 264.7) with *S. aureus* (ATCC 25923). Further, the infected macrophages were treated with two different peptide concentrations at their MIC values, i.e., 1xMIC (4 µM) and 2xMIC (8 µM) for 12 h and 24 h. After 24 h, a sharp decline in intracellular bacterial burden was observed (Fig.28) in peptide treated *S.aureus* macrophages at 2xMIC compared to the untreated macrophages. These results suggested that the peptide-Ba49 also exhibited

intracellular killing potential against *S. aureus* (ATCC 25923) in the macrophage cell line, i.e., RAW 264.7.

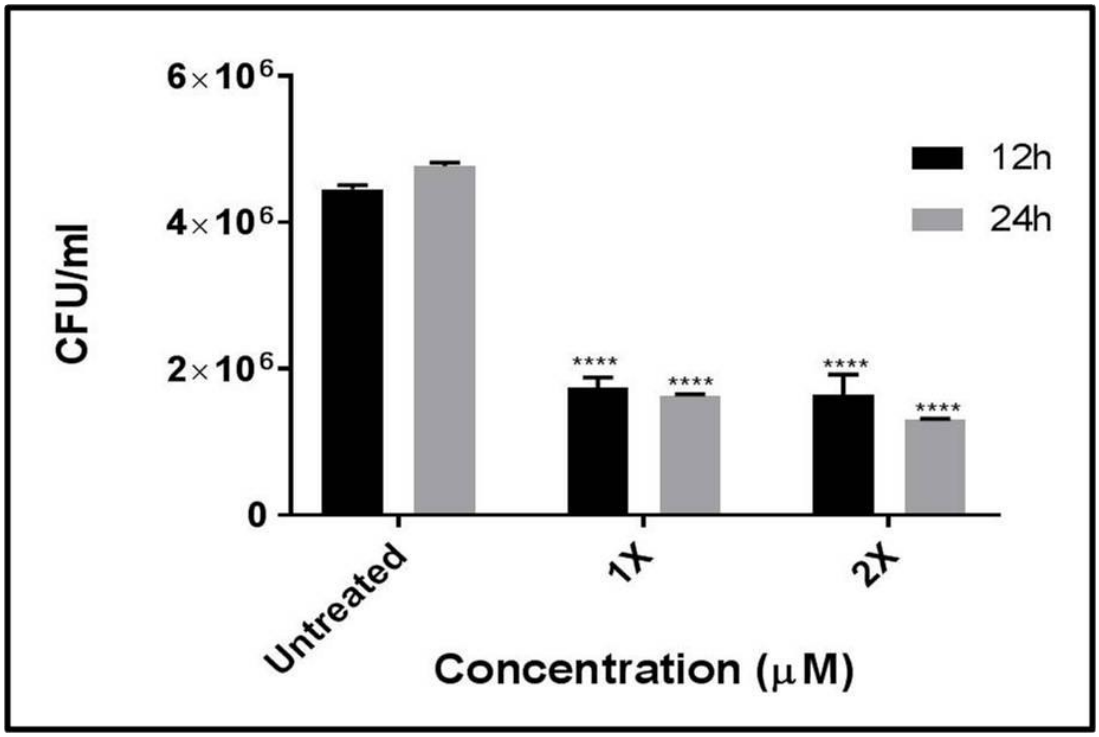


Figure 28. Intracellular activity of peptide-Ba49 against *S. aureus* (ATCC 25923) infected RAW 264.7 cells: The ordinate showed log CFU/ml *S.aureus* (ATCC 25923) cell reduction of broth in RAW 264.7 cells after 24 h of incubation with the peptide-Ba49 compared to the untreated one. Values represent the Mean ± standard errors of two independent experiments performed in triplicates, $^{****}p< 0.0001$.

4.1.14. Preliminary media optimization studies

4.1.14.1. Placket-Burman experimental design (PBD)

The influence of eleven variables namely (A) yeast extract, (B) beef extract, (C) peptone, (D) NaCl, (E) $MgCl_2$, (F) KCl, (G) $NaHCO_3$, (H) ferric (III) citrate, (I) Na_2SO_4, (J) $CaCl_2$, and (K) glycerol on the production of antimicrobial peptide production from strain Ba49 was explored in 11 trials by using Placket-Burman experimental design (PBD) as presented in Table 8. The corresponding response for peptide production showed variations in the range of 0-17 mm (zone of inhibition) of peptide activity in 12 trials. The most effective factors observed for the peptide production were found to be glycerol, ferric (III) citrate, NaCl, $MgCl_2$, and selected for further optimization (Fig. 29). According to the regression analysis results of Placket-Burman design using design expert statistical software version 10.0, a first-order model could be attained as shown:

$$Y_1 = +2.46 - 0.63*D - 0.55*E + 0.63*H - 1.29*K \ldots\ldots (1)$$

Where Y_1 is the peptide activity (zone of inhibition mm), D, E, H, and K are the variables as mentioned before.

The goodness of fit of model could be checked by coefficient of determination (R^2), which describes how well the set of observations fit in model summarizing the variations between the observed values and the expected values as mentioned in the design. The value of R^2 should be between 0 and 1. If the value of R^2 is closer to 1.0, then the model predicts a more robust model, and it's better to analyze the response. The linear regression model of the peptide production was evaluated by the coefficient of the determination, i.e., $R^{2,}$ which was 0.9081 and indicated that the model could explain 90.81% of the variability of the response. The high value of the adjusted determination coefficient (adjusted $R^2 = 0.8556$) also indicated that the model was significant (Table 9). These measures suggested that the accuracy and the general ability of the polynomial model were good, and the analysis of the response trends using the model was reasonable. The variable with the confidence level was 95%, and considered as a significant model.

In the present study, it was clear from the above equation (1) that (D) NaCl, (E) $MgCl_{2,}$ (H) ferric (III) citrate, and (L) glycerol were the significant factors with a confidence level of 95%. Among these parameters (K) glycerol, (D) NaCl, and (E) $MgCl_2$ showed positive effects at a low (-) level, whereas (H) ferric (III) citrate had a positive effect at a high (+) level but at a low level. However, at low level ferric (III) citrate produced a negative effect on the peptide production (Fig. 30).

4.1.14.2 Central Composite Design (CCD)

The response surface methodology has been reported for the media optimization of the microbial fermentation medium conditions. In this response, effect of one component on the other present in the medium has been investigated and determined the significance of the one component on the formation of desired products. In biochemical processes, the interaction between the parameters would be critical in synergism and antagonism. The equation obtained from this model will easily clarify the effect of binary combinations of the independent variables.

The RSM based on the central composite design was employed to determine the four significant variables at optimal levels, i.e., NaCl, $MgCl_{2,}$ ferric (III) citrate, and glycerol. The respective low and high levels of variables and the experimental results design matrix were

presented in Table 10. ANOVA evaluated the model acceptability and the peptide production fitness, and the regression coefficient calculations presented in Table 11. The Fischer variance value (F-value), which statistically measures the wellness of the factors which describes the variation in data about its mean and can be calculated from an ANOVA. The high F- value and the very low probability ($p > F = 0.0001$) indicated that the peptide production model is in good agreement with the expected and the predicted values. Predicted values and actual values were close to each other, as shown in Table 10; the value of coefficient of determination (R^2) value of 0.9568 indicated that the model could explain 95.68% of the variability in the response. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9137$) was also found to be high. This indicated that 91.37 % variability in the response attributed to the independent variables, and this model could not explain the variability only about 8.63 %. Adequate precision (AP) compares the predicted value range at the design point. A ratio greater than 4 indicates the model is adequate and can be used to navigate CCD design (Moradi et al., 2016). In this study, the AP value was determined to be 19.302, which was greater than 4, and this design was considered an adequate model. The degree of precision indicated by the coefficient of variation (CV) with which the treatments are to be compared; the higher the value of CV indicates the least reliability of experiments and vice versa (Zhou et al., 2017). In this study, the CV was found to be 16.43%, which was lower as it indicating the experiment's reliability. The *F* value (2.31) of lack of fit was lower than the tabulated *F* value (22.17). Being lower than that of pure error i.e., 5.19, it was not significant and indicated the model's acceptability.

In the ANOVA analysis of the peptide production, except for interaction terms AB, BC and BD, the linear and quadratic i.e., B and C^2, the other interactions (AC, AD, and CD), all other liner terms (A, D and C) and quadratic (A^2, B^2, and D^2) terms were statistically significant ($p< 0.005$) as present in Table 8. The multiple regression analysis was used for analysis, and the second-order equation was derived for the optimization of medium composition for peptide production i:

$$Y_2 = +7.06-2.29*A-0.083*B-1.71*C-4.29*D-0.19*AB-1.25*AC-1.12*AD-0.44*BC+0.31*BD-1.37*CD+0.62*A^2-0.63*B^2+0.000*C^2+1.13*D^2 \ldots\ldots\ldots\ldots (2)$$

Where, Y^2 is the peptide activity, (A) glycerol g/L, (B) Fe (III) citrate g/L, (C) NaCl g/L, and (D) $MgCl_2$ g/L.

Contour plots of RSM represent a function of two factors simultaneously, holding all other factors at the fixed levels which help in understanding both the primary and interaction effects of these two factors. The 3D response surface plots described by the regression model were drawn to illustrate each independent variable's interactive effects on peptide activity. The response surface was plotted between NaCl and glycerol (Fig. 31A), presented the interaction between two of them, and positively affected the peptide production when Fe (III) citrate and $MgCl_2$ were fixed at 0.25 g/L and 7.5g/ L, respectively. Fig. 31B suggested that the interaction between $MgCl_2$ and glycerol was also negatively affected when Fe (III) citrate and NaCl at 0.25g/L and 7.5g/L were kept constant, respectively. In the Fig. 31C, the interaction between $MgCl_2$ and NaCl shows a positive response by keeping glycerol and Fe (III) citrate constant at 0.25g/L and 7.5g/L, respectively. The peptide activity increased with an increased in NaCl concentration, and NaCl concentration could be further increased according to the model. Whereas, in the case of $MgCl_{2,}$ ferric (III) citrate, and glycerol, peptide activity was sensitive to these factors, with a decrease in concentrations of three above factors, i.e., $MgCl_{2,}$ ferric (III) citrate, and glycerol, the activity was observed to be increased.

Based on the preliminary results of the optimized fermentation medium, peptide activity from strain Ba49 i.e., *Bacillus subtilis* subsp. *Spizizenii* against *S. aureus* was increased by 1.3 fold with the addition of NaCl, $MgCl_{2,}$ ferric (III) citrate, and glycerol.

On the basis of result obtained from PBD and RSM, optimized fermentation medium was formulated as (g/l): yeast extract 5.0 g/l, beef extract 5.0 g/l, peptone 5.0 g/l, KCl 5.0 g/l, $NaHCO_3$ 0.1 g/l, Fe (III) citrate 0.25 g/l, Na_2SO_4 1.0 g/l, $CaCl_2$ 1.0 g/l, glycerol 0.5 g/l, NaCl 7.5 g/l, $MgaCl_2$ 7.5 g/l. yielding 1.3 fold increase of peptide activity.

Table 8: List of variables with high and low values chosen for PBD experimental design

	Factor A	Factor B	Factor C	Factor D	Factor E	Factor F	Factor G	Factor H	Factor I	Factor J	Factor K	Actual Value	Predicted Value
Run	Yeast extract	Beef extract	Peptone	NaCl	$MgCl_2$	KCl	$NaHCO_3$	Fe(III) citrate	Na_2SO_4	$CaCl_2$	Glycerol	Activity (mm)	Activity (mm)
1	5.00	1.00	1.00	5.00	15.00	5.00	0.50	0.10	5.00	5.00	1.00	13.2	12.31
2	5.00	5.00	1.00	20.00	15.00	0.50	0.50	0.10	1.00	1.00	5.00	0	-2.53
3	**5.00**	**5.00**	**5.00**	**5.00**	**15.00**	**5.00**	**0.10**	**0.50**	**1.00**	**1.00**	**1.00**	**17.33**	**17.15**
4	5.00	5.00	1.00	20.00	5.00	0.50	0.10	0.50	5.00	5.00	1.00	14	15.98
5	1.00	1.00	1.00	5.00	5.00	0.50	0.10	0.10	1.00	1.00	1.00	14	15.93
6	5.00	1.00	5.00	20.00	5.00	5.00	0.10	0.10	1.00	5.00	5.00	0	1.09
7	1.00	5.00	5.00	20.00	5.00	5.00	0.50	0.10	5.00	1.00	1.00	13	11.13
8	1.00	1.00	5.00	20.00	15.00	0.50	0.50	0.50	1.00	5.00	1.00	13.33	12.35
9	1.00	1.00	1.00	20.00	15.00	5.00	0.10	0.50	5.00	1.00	5.00	0	2.31
10	1.00	5.00	1.00	5.00	5.00	5.00	0.50	0.50	1.00	5.00	5.00	12.6	10.73
11	1.00	5.00	5.00	5.00	15.00	0.50	0.10	0.10	5.00	5.00	5.00	0	2.27
12	5.00	1.00	5.00	5.00	5.00	0.50	0.50	0.50	5.00	1.00	5.00	12	10.73

Table 9: Details of PBD ANOVA model predict by the software

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	33.32	4	8.33	17.30	0.0010	significant
D-NaCl	4.75	1	4.75	9.87	0.0163	
E-MgCl2	3.69	1	3.69	7.67	0.0277	
H-Fe(III) citrate	4.80	1	4.80	9.97	0.0160	
L-Glycerol	20.08	1	20.08	41.69	0.0003	
Residual	3.37	7	0.48			
Cor Total	36.69	11				

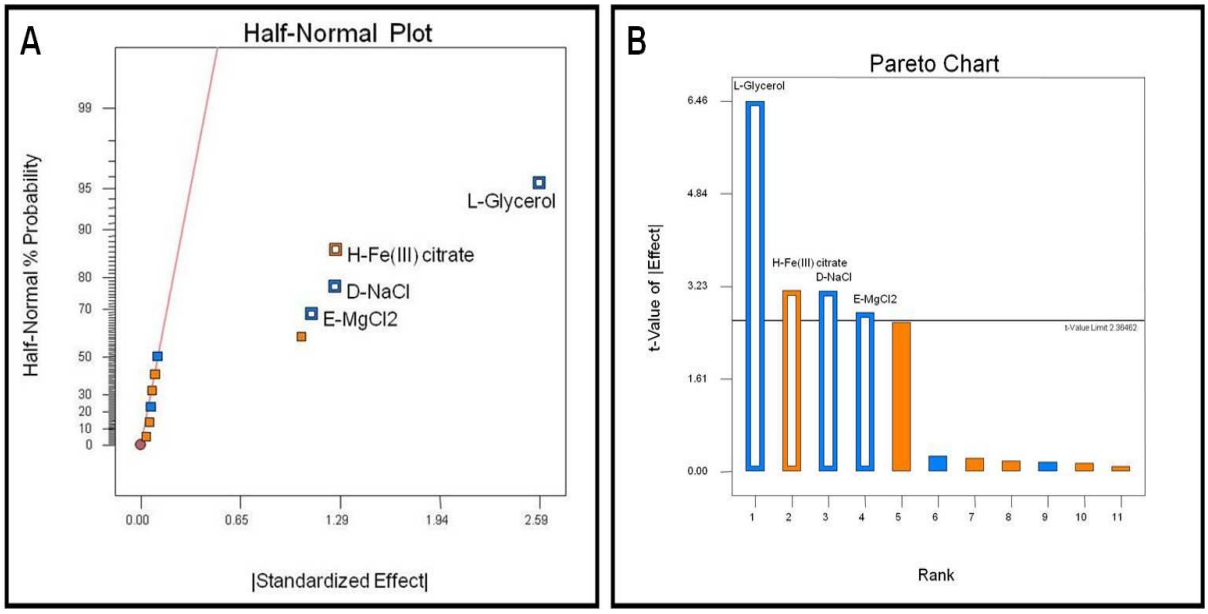


Figure 29. Effect of different variables chosen in PBD on peptide-Ba49 production: (A) In half-normal plot Fe (III) citrate showed the positive response in producing an antibacterial peptide. Glycerol, $MgCl_2$, and NaCl were found to be significantly negative responses; (B) representing a Pareto chart showing the t-value of different components effects, the bar with the orange outline showed a positive effect on compound production. In contrast, the blue outline bars are the components that showed a negative effect on the production of an antibacterial peptide. More is the t-value of a component; more significantly showed the effect on the production.

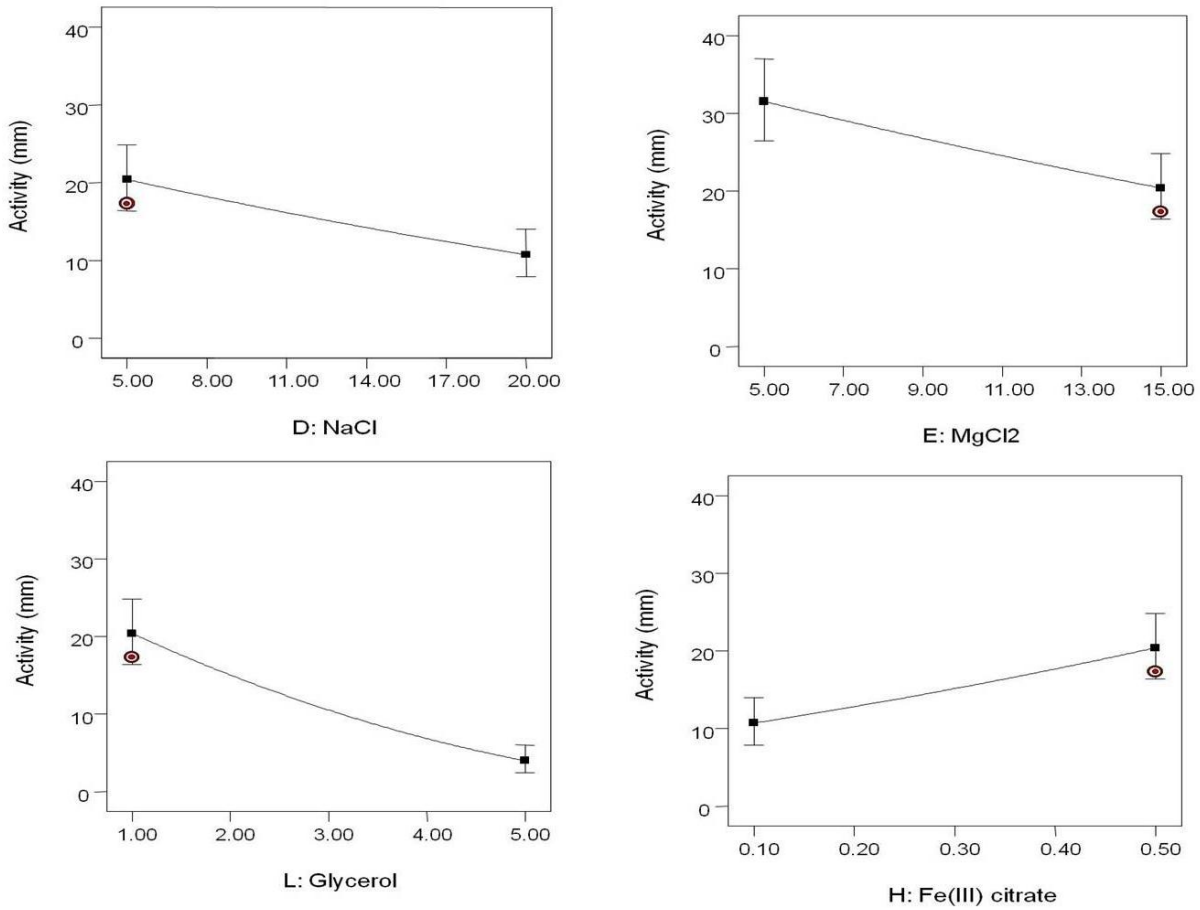


Figure 30. Effect of NaCl, Fe(III) citrate, Glycerol and $MgCl_2$ on peptide-Ba49 production: As the concentration of NaCl and Glycerol increases, antibacterial activity of peptide-Ba49 decreases. On the other side with increase in concentration of $MgCl_2$ and Fe (III) citrate, there was increase in antibacterial activity of peptide-Ba49.

Table 10: List of variables with high and low values chosen for RSM experiment design

		Factor A	Factor B	Factor C	Factor D	Actual value	Predicted value
Block	Run	Glycerol g/l	Fe(III) citrate g/l	NaCl g/l	$MgCl_2$ g/l	Activity (mm)	Activity (mm)
Block 1	1	0.5	0.25	7.5	22.5	10	10.56
Block 1	2	1.5	0.75	2.5	7.5	18	17.81
Block 1	3	1	0.5	5	15	15	12.13
Block 1	4	0.5	0.25	2.5	22.5	12.5	13.35
Block 1	5	0.5	0.25	2.5	7.5	18	17.56
Block 1	6	0.5	0.75	7.5	7.5	18	18.98
Block 1	7	1	0.5	5	15	12.5	12.13
Block 1	8	0.5	0.75	7.5	22.5	10	10.52
Block 1	9	0.5	0.75	2.5	7.5	19	18.02
Block 1	10	1.5	0.25	7.5	7.5	16.5	15.81
Block 1	11	1.5	0.75	2.5	22.5	10	10.35
Block 1	12	1.5	0.25	2.5	7.5	18	18.10
Block 1	13	1.5	0.25	2.5	22.5	10	9.40
Block 1	14	1	0.5	5	15	13	12.13
Block 1	15	1.5	0.75	7.5	22.5	0	0.81
Block 1	16	0.5	0.75	2.5	22.5	14	15.06
Block 1	17	1	0.5	5	15	12	12.13
Block 1	18	1.5	0.75	7.5	7.5	14	13.77
Block 1	**19**	**0.5**	**0.25**	**7.5**	**7.5**	**20**	**20.27**
Block 1	20	1.5	0.25	7.5	22.5	0	1.60
Block 2	21	1	0	5	15	0	-0.33
Block 2	22	1	0.5	5	30	0	-2.08
Block 2	23	1	0.5	0	15	5	5.42
Block 2	24	1	0.5	10	15	0	-1.42
Block 2	25	1	1	5	15	0	-0.67
Block 2	26	2	0.5	5	15	0	-0.083
Block 2	27	1	0.5	5	15	0	2.00
Block 2	28	1	0.5	5	15	0	2.00
Block 2	29	0	0.5	5	15	10	9.08
Block 2	30	1	0.5	5	0	14	15.08

Table 11: ANOVA for response surface quadratic model predicted by the software

Source	Sum of Squares	df	Mean Square	F Value	P-value (Prob> F)	
Block	683.44	1	683.44			
Model	780.43	14	55.74	22.17	< 0.0001	significant
A-Glycerol	126.04	1	126.04	50.12	< 0.0001	
B-Fe(III) citrate	0.17	1	0.17	0.066	0.8006	
C-NaCl	70.04	1	70.04	27.85	0.0001	
D-MgCl2	442.04	1	442.04	175.77	< 0.0001	
AB	0.56	1	0.56	0.22	0.6435	
AC	25.00	1	25.00	9.94	0.0071	
AD	20.25	1	20.25	8.05	0.0132	
BC	3.06	1	3.06	1.22	0.2884	
BD	1.56	1	1.56	0.62	0.4437	
CD	30.25	1	30.25	12.03	0.0038	
A^2	10.71	1	10.71	4.26	0.0581	
B^2	10.71	1	10.71	4.26	0.0581	
C^2	0.000	1	0.000	0.000	1.0000	
D^2	34.71	1	34.71	13.80	0.0023	
Residual	35.21	14	2.51			
Lack of Fit	30.02	10	3.00	2.31	0.2173	not significant
Pure Error	5.19	4	1.30			
Cor Total	1499.07	29				

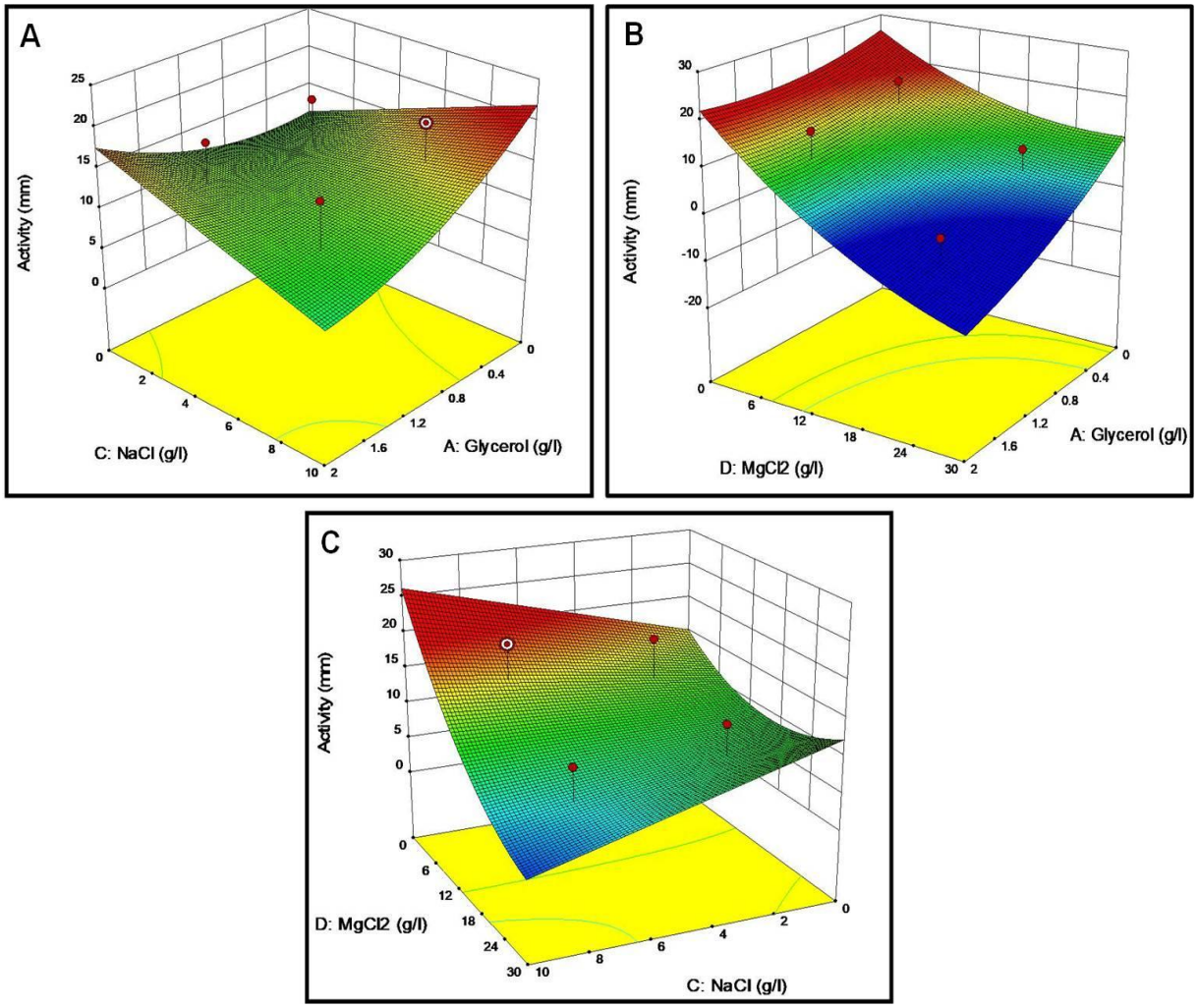


Figure 31: **3-D surface plot showing interaction between two factors:** (A) NaCl and Glycerol, (B) $MgCl_2$ and Glycerol, (C) $MgCl_2$ and NaCl.

4.2. DISCUSSION

With the emergence of antibiotic resistance in bacterial strains against the existing antibiotics has become a major global threat to public health. It underscores the need to develop either novel antibiotics or alternative therapies to conventional antibiotics. AMPs could be a better alternative to these conventional antibiotics as they kill bacteria by disrupting membrane integrity and are less prone in inducing resistance against them (Sang and Blecha, 2008). AMPs have been isolated from various sources such as humans, animals, plants, bacteria, fungi, etc. To date, the antimicrobial peptide database has catalogued more than 2,600 natural antimicrobial peptides up to date, including those annotated as immunomodulatory (Mookherjee et al. 2020). Moreover, natural sources are the most exploited sources for the isolation of AMPs as about 60% of approved drugs are derived from natural habitats (Rahman et al., 2017). The genus *Bacillus* is a well-known producer of AMPs and has been studied as the most promising host for screening new growth inhibitory substances (Sumi et al., 2015).

Herein, an antibacterial peptide produced by strain Ba49 (MTCC 13006), isolated from *Allium cepa*, was studied. It is vital to obtain an AMP in purified form to initiate its characterization studies, and this is mostly achieved using various chromatographic techniques (Maldonado et al., 2003). The downstream processing protocol consisting of adsorption of the peptide in Diaion HP-20 resins was followed by a three-step procedure consisting of ion-exchange chromatography (SP-Sepharose), desalting (Sephadex- G10), and RP-HPLC for the purification of this antibacterial peptide. The purified peptide showed a single band on Tricine–SDS–PAGE. Further, the molecular mass of this purified peptide was determined by MALDI-TOF as a single peak of 3319.2 Da. These results indicated that the above-said protocol to be capable of producing highly purified peptide from Ba49 fermentation broth. Whole-genome analysis of isolate Ba49 identified the organism as a *B. subtilis* subps. *spizizenii*. It is well known that strains of genus *Bacillus* produce a wide arsenal of antimicrobial compounds, which are small microbial peptides synthesized ribosomally and non-ribosomally (Abriouel et al., 2011; Marx et al., 2001). Additionally, the antiSMASH database genome analysis located a lanthipeptide gene cluster identical to the subtilin gene cluster. *De-novo* amino acid sequencing, molecular weight, and biosynthetic gene cluster together confirmed peptide-Ba49 as subtilin.

Moreover, utilization of bio-manufacturing process using biological system is to produce AMPs in favorable fermentation conditions (different media, temperature, pH, and agitation). Media compositions play an important role in producing antibacterial compounds from various species. Generally, non-specific media (BHI, MRS, LB, NB, and TSB, etc.) are used to produce antibacterial compounds due to their abundant source of nutrients, nitrogen, carbon, minerals, and vitamins. For instance, BHI, TSB, MRS was used to produce AMPs from strain *B. subtilis* R75, *Bacillus cereus*, *Bacillus coagulans*, etc., (Lajis, 2020). In the case of strain Ba49, the ZMB medium showed good production of antimicrobial compound.

Also, the optimum temperature will allow rapid cell proliferation and increase the production of essential enzymes and proteins, which further catalyzing the biosynthesis or modification of biologically active bacteriocins (Fickers et al., 2008). In our case of strain Ba49, 25^{o}C was found to be an optimum temperature for the growth of strain Ba49 and antibacterial peptide production. Previously, cerein 8A from *B. cereus* was reported to be produced in good quantity in the temperature range of 22^{o}C and 34^{o}C (Ayed et al., 2015; Huang et al., 2016). The initial fermentation pH is known to affect the growth and AMP production. However, generally, pH is not controlled throughout the fermentation time in case of antibacterial peptide production. In the case of strain Ba49 the pH of fermentation broth was observed to be 6.5, which was almost neutral pH. It has been reported in the literature that a very high or low pH milieu is to be unsuitable for the bacterial growth and production of the AMPs. This might be due to the neutrophilic behavior of the producer strains. For example, *B. licheniformis* produces bacteriocins in the pH range of 6.5-7.5 (Cladera-Olivera et al., 2004b; Kayalvizhi and Gunasekaran, 2010).

An effective system of aeration and agitation is also essential to supply sufficient oxygen and nutrients for producer strain to produce high amounts of antimicrobial compounds. Almost all studies use agitation to increase the antimicrobial compound's production due to the aerobic or facultative anaerobic nature of the producer strains. In case of strain Ba49, low rate of agitation (100 rpm) was found to support higher production of antibacterial compound. There are some reports which also show low agitation rates favoring the higher production of antimicrobial peptides. For instance, *B. licheniformis* produced a higher bacteriocin P40 at a relatively low agitation rate of 125 rpm; similarly, entianin from *B. subtilis* was reported to be produced at 150 rpm (Mathur et al., 2017; Rea et al., 2010).

The purified peptide-Ba49 also showed excellent bactericidal and biophysical characteristics. The cell-free supernatant and purified peptide inhibited the growth of different *Staphylococcus* strains, including MRSA strains. However, this peptide's antibacterial activity was susceptible to complete degradation by human digestive proteases (pepsin, protease, proteinase K, trypsin, and chymotrypsin). AMPs that are degraded easily in the human digestive system have also been reported to be safe with no harmful effects (Hu et al., 2013). Further, the antibacterial activity was not affected by lysozyme, RNase, lipase, catalase, and α-amylase, suggesting that the purified AMP was proteinaceous in nature and not a lipidated peptide. In comparison to conventional antibiotics, AMPs are more stable at high temperature and in extreme pH environment. This physiochemical stability of AMPs is directly related to their diverse structure and level of post-translational modifications, which ultimately endows them with strong antibacterial activity (Hols et al., 2019; Meade et al., 2020). Peptide-Ba49 retained its activity at high temperature (up to 121^{o}C) and a wide range of pH (pH 5- pH 10). In terms of thermostability, peptide-Ba49 retained 94% of its antimicrobial activity (against *S. aureus*, ATCC 25923) after autoclaving (at 121^{o}C for 20 min) as compared to other antimicrobial peptides isolated from different microbial sources. It has been founded that, the two reported subtilins, subtilin KU43, and subtilin L-Q11, retained 25% (for 30 min) and 54% (for 30 min) of its antimicrobial activity against *S. aureus* following heat treatment of 100^{o}C. Upon autoclaving (121^{o}C), subtilin KU43 lost all its activity within 15 min whereas subtilin L-Q11 retained more than 54% of its antimicrobial activity even after 20 min (Al-Sahlany et al., 2020; Kim et al., 2012; Qin et al., 2019). Investigations on the effect of pH on antimicrobial activity of peptide showed peptide-Ba49 retaining more than 90% of its antimicrobial activity in the pH range of 5.0-10. In contrast, subtilin L-Q11 retaining about 90% of its antimicrobial activity in pH range 2 to 7 (Qin et al., 2019), and subtilin KU43 remaining stable in a pH range of 3-9 for 4 h (Kim et al., 2012). More importantly, the presence of peptide backbones in AMPs, which otherwise is not present in the chemically synthesized antibiotics, makes these AMPs easily prone to the action of proteases. And hence have a short biological half-life in the natural host or system. Because of this, the chance of the development of drug resistance against these AMPs is significantly low (Hols et al., 2019).The activity of the peptide-Ba49 largely remained unaffected after treatments with various solvents, surfactants, and reducing agents, indicating its stability at different processing conditions. The results also demonstrated the hydrophobic nature of the peptide-Ba49, thus making it stable (Chalasani et al., 2015; Sharma et al., 2018). However, the activity of peptide-Ba49 was partially reduced after β- mercaptoethanol

treatment, which indicated the presence of free cysteine moieties in its structure. This peptide's antimicrobial potential was not hampered with being treated with catalase and α-amylase, indicating the omission of any carbohydrate and peroxide moieties in it (Elayaraja et al., 2014). The objective of analyzing the effect of metal ions on peptide-Ba49 was to check the influence of metal ions on its antimicrobial activity (Łoboda et al. 2018; Walkenhorst et al. 2014; Zasloff 1987). In our study, $FeSO_4$ significantly reduced the antibacterial activity of purified peptide against *S. aureus* (ATCC 25923), which could be due to the siderophores produced by the bacterium having a higher affinity for iron than peptide (Silva et al., 2009). Bacteremia, SSTIs, and nosocomial infections are increasing alarmingly day by day and have infected about 80 – 90% world population (Dayan et al., 2016; Peterson and Schora, 2016). The species of *Staypholococcus* and its MRSA strains are the causative agents behind these infections. In this study, peptide-Ba49 strongly inhibited the growth of *S. aureus* and its pathogenic and clinical strains at lower MIC values ranging from 0.5 μM – 16 μM as compared to that of nisin (Table 3 and 4).

The post-antibiotic effect is defined as the suppression period of bacterial growth, which persists after a limited exposure of organisms to antibacterial (Nedbalcova et al., 2019) and also the degree of PAE related to the degree of cellular damage done by the antibiotic to bacterial cell (Haukland and Vorland, 2001). In this study, the PAE for peptide-Ba49 was significant on *S. aureus* after 4 h of exposure at 2xMIC concentration. This might indicate that the cells damage is immense or takes a long time to repair, i.e., ~10 h.

AMPs from the *Bacillus* species show different kinds of modes of action, and one of the essential modes of killing a microorganism is by disrupting the integrity of the membrane (Perez et al., 2014). The cationic AMPs are also known to destroy the target organism by rupturing its membrane through pore formation (Sumi et al., 2015). Further, the mechanistic insight of peptide-Ba49, we observed the enhanced intracellular ROS generation in of *S. aureus* upon treated it with peptide-Ba49 as measured using ROS sensitive probe, i.e., H_2DCFDA. This suggested that intracellular ROS production enhances the cells oxidative environment, altering the cell membrane's resting potential.

Moreover, oxidative stress plays a crucial role in altering the bacterial membrane permeability and can damage cell membranes (Shaikh et al., 2019). Further, a voltage-sensitive $DiSC_3$ (5) probe was used to evaluate the change in *S. aureus* membrane potential following peptide treatment. We observed an increase in fluorescence indicated the change in membrane potential after the peptide-Ba49 treatment.

In addition the disruption of membrane integrity was also confirmed by using fluorescent voltage-sensitive dye, $DiBAC_4$ (3), which measured the resting membrane potential of *S. aureus* after treating with the purified AMP. $DiBAC_4$ (3) is an anionic dye that enters into the cell, and any change in membrane potential increases the fluorescence. Though the fluorescence of $DiBAC_4$ (3) increases in case of depolarization of cell membranes, it decreases under hyperpolarization of the cell membrane (Adams and Levin, 2012; Yamada et al., 2001). Similar results were observed in the present case, i.e., following treatment of *S. aureus* with 4-fold MIC of purified peptide-Ba49, the fluorescence signal was decreased, confirming the hyperpolarization of the cell. It could be concluded that the bacteria failed to minimize the abundance of intracellular ROS and, at the same time, imposed depolarization, which ultimately could have led to cell death (Yadav et al., 2020; Zhou et al., 2020). The phenomenon was further confirmed by flow cytometry using PI staining.

SEM and TEM analysis were carried out to study the morphological changes in *S. aureus* cells after peptide-Ba49 treatment. SEM studies showed the damage of the cell membrane following treatment of *S. aureus* cells with the peptide-Ba49 and in case of TEM studies, it was visualized that killing of the cells was due to disruption in membrane integrity. Based on these results, it could be concluded that peptide-Ba49 displaying its cationic nature ruptured the cell membrane *S. aureus*.

Biofilm infections caused by *S. aureus* are of a primary concern compared to planktonic cell infections as it commonly leads to resistance against conventional antibiotics (Savage et al., 2013), (Koch et al., 2014), (Olsen, 2015). An infection associated with biofilm requires high drug concentrations and it is extremely difficult to treat such infections (McConoughey et al., 2014), (Olsen, 2015), (Koch et al., 2014), (Liu et al., 2015). The peptide-Ba49 was studied for the disruption of *S.aureus* biofilm formation at a concentration of 8 µM and 16 µM within 24 h. a significant reduction of the bacterial cells metabolic activity in biofilm was observed at a concentration of 16 µM of peptide-Ba49. Moreover, the Confocal imaging using PI and SYTO-9 showed the inhibition of biofilm formation treated with peptide-Ba49 compared to the control. The biofilm inhibition was further confirmed by EPS production during biofilm formation, indicated that fewer EPS were produced in the presence of peptide than with control, indicated that the inhibition of *S.aureus* biofilm in the presence of peptide-Ba49. Additionally, the peptide- Ba49 killed bacterial cells in mature biofilm of *S. aureus* (ATCC 25923 and MTCC 1430) after 24 h of treatment and significantly reduced the mature biofilm biomass and metabolic activity at 64 µM. This result signified that the peptide-Ba49 could

develop as an antimicrobial agent for treating *S. aureus* planktonic as well as biofilm-associated infections.

The toxicity studies on mammalian cells are necessary for predicting the toxicological effect of AMPs on humans. *In vitro* toxicity studies against cell line (L929, HEK 293T, and RAW 264.7) confirmed the biosafety of this antimicrobial peptide-Ba49. Intriguingly, we observed an L929 cell line treated with purified peptide showing >80% viability in the MIC range (4 μM and 8 μM) compared to untreated cells showing ~ 100% viability. Recently, AMPs from a *Bacillus* sp. were reported to show 48% and 91% cell viability on HEK293 and HT29 cells, respectively (Sharma et al., 2018). These variable effects shown by cationic antimicrobial peptides on mammalian cell lines might be because of factors such as different preparation strategies, duration of exposure of cells to these AMPs, etc. The cell surface hydrophobicity also got affected by the peptide's binding, leading to less peptide binding. The resultant variation in fluorescence indicated a change in the cell cytotoxicity though this variation mechanism is still unknown (Laverty and Gilmore, 2014).

Further, *S. aureus* can penetrate and survive within the host cell and causes chronic infections. Due to the difficulty in antibiotic passaging through cellular membranes, it becomes more challenging and difficult to treat the infection at the intracellular stage. So, in addition to the extracellular activity of the antimicrobial peptide, it is also required to have intracellular activity. Peptide-Ba49 was founded to reduce the intracellular bacterial burden at 2xMIC after 24 h in *S. aureus* (ATCC 25923) infected RAW 264.7 macrophages.

In the wound-healing phase, cell proliferation and migration are two essential features and *in vitro* scratch assay is a form to mimic a wound and evaluate cell migration rate. Due to the disruption of cell monolayer, it losses cell-cell interaction, increasing concentration of growth factors and cytokines at the edge of the wound, which further initiates cell migration and proliferation (Liang et al., 2007), (Pitz Hda et al., 2016). Interestingly, peptide-Ba49 prompted the L929 fibroblast cell proliferation at 2xMIC, a positive event for the wound healing process, as fibroblast cells are essential cells because of their involvement in wound contraction and ECM production (Balekar et al., 2012).

Media optimization is a process that utilizes a biological system to produce antimicrobial compounds in a large-scale quantity at an economical cost. There are various ways to optimize the media processes, as discussed earlier, i.e., is one variable at a time (OVAT). Another approach is the statistical approach used to analyze the factors that influence the

productivity of antimicrobial compounds. However, the statistical approaches which have been reported are Placket-Burman design (PBD), Response Surface Methodology (RSM), and Artificial Neural Network (ANN). There are varieties of factors used to regulate the biosynthesis and production of antimicrobial compounds in various organisms that have been identified and extensively used, i.e., media composition, pH, temperature, agitation speed, etc.

However, for strain Ba49, the statistical approaches are being used PBD followed by RSM. Initially, 11 variables are chosen to screen the influence of individual factors that enhances antibacterial production. However, from the PBD statistical approach, factors such as glycerol, ferric (III) citrate, NaCl, and $MgCl_2$ show a positive or negative effect on peptide production- Ba49. Later, by keeping other factors constant, these four factors were chosen for designing RSM to check their synergistic influence on the production of peptide-Ba49. So, from the RSM approach, the factor NaCl shows positive influence, i.e., with an increase in NaCl concentration, the activity of peptide-Ba49 also increases against *S.aureus* strains. There are reports which showed that the presence of Na^+ stimulates the production of the bioactive compound; for example, the production of elgicin from strain *Paenibacillus elgii* B69 enhances in the presence of NaCl (22443157). Although, there was a decrease in the peptide production from strain Ba49 was observed in glycerol, ferric (III) citrate and $MgCl_2$. For instance, in bacteriocin MKU3 from strain *B. licheniformis*, there was a decrease in activity in the presence of Mg^+ ions (Kayalvizhi and Gunasekaran, 2010). Some reports show Fe3+ productivity gets stimulated (Teng et al., 2012), but in the case of strain Ba49, it shows the opposite effect. However, glycerol is an essential intermediate in metabolic pathways that regulates the organism's metabolism (Yi et al., 2013). In strain Ba49, at 0.5% (v/v) of glycerol promotes peptide production, but with increased glycerol concentration, the production was declined. It is also reported in the case of Lac-B23 production from strain *Lactobacillus paracasei* J23 was enhanced by adding 1% (v/v) of glycerol. Similar results are reported in bacteriocin ST12BZ production; the productivity increases at 1.0g/L but at higher concentration, i.e., up to 50 g/L, the decline in production was observed (Todorov and Dicks, 2007; Yi et al., 2013). Thus, it concluded that the glycerol might be a positive inducer, which enhanced the production of a peptide from strain Ba49 instead of involving in cell growth.

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Microbial infections substantially contribute to the global mortality trend, and antibiotics can treat most bacterial infections. An increase in the emergence of antibacterial resistance reduces the effectiveness of conventional antibiotics. The red-alert pathogens intimidate the use of most front-line antibiotics and have been involved in severe bloodstream, urinary tract, respiratory tract infections. Moreover, the designs of antimicrobials are less susceptible to evolutionary resistance mechanism than conventional antibiotics. Despite of being known the capability to elicit the mechanism of bacterial resistance, antimicrobials are at the forefront of replacing conventional antibiotics.

Antimicrobial peptides (AMPs) are a diverse group of small bioactive molecules and a part of the body's first line of defense to inactivate the pathogens. They act by disrupting the bacterial membranes, help in modulating the immune response and regulate inflammation. These AMPs kill or inhibit the bacteria by non-specific mechanisms; therefore, resistance to antimicrobials is rarely developed. Furthermore, they can kill antibiotic-resistant bacteria; include insidious microbes like *Acinetobacter baumannii* and Methicillin-resistant *Staphylococcus aureus*. Based on the results obtained so far, antimicrobials peptides represent the novel alternative to conventional antibiotics in controlling human pathogens in the near future.

There are various sources from which an AMP can be isolated, such as humans, animals, plants, bacteria, fungi, etc. Moreover, the natural sources are the most exploited source for the isolation of AMPs. Furthermore, the genus *Bacillus* is an arsenal of antimicrobials and is a promising host for screening new bioactive compounds.

In this study, More than 50 bacterial isolates from fermentation extracts were screened for antimicrobial activity using the agar well diffusion method. Among them, strain Ba49 showed promising and consistent antimicrobial activity against *S. aureus* (MTCC 1430 and ATCC 25923). The isolate was identified as *Bacillus subtilis* subsp. *spizizenii* based on *de novo* whole-genome assembly (NCBI GenBank accession number VOOM00000000) and was deposited in Microbial Type Culture Collection (MTCC) repository, IMTECH, Chandigarh, India with accession number MTCC 13006. Further, the Influence of various physiological parameters on antimicrobial production by strain Ba49 in the fermentation process at the flask level was examined which showed higher production in ZMB medium at 25^{o}C, 100 rpm, and pH of 6.5. Later, the production of antibacterial compound was studied at different growth phases which showed the yield of the maximum extracellular antimicrobial

compound at 32 h in ZMB medium. This was followed by its extraction using Diaion HP-20 resin and purification using a series of chromatographic techniques, i.e., ion-exchange chromatography and RP-HPLC. The purified peptide-Ba49 was subjected to mass spectroscopic analysis, which showed an m/z value of 3319.3303 $(M+H)^+$, similar to the molecular weight of peptide determined using Tricine SDS-PAGE analysis. The zymographic study had also revealed the inhibitory effect of peptide-Ba49 on the test strain (ATCC 25923) which corresponded to the same band in the gel stained with Coomassie Blue.

The proteinaceous nature of the peptide-Ba49 was established by its sensitivity to various proteolytic enzymes i.e., pepsin, protease E, proteinase K, trypsin, chymotrypsin. In contrast, lysozyme, RNase, lipase, catalase, and α-amylase did not affect the activity of peptide-Ba49 suggesting that a peptide is only essential for antibacterial activity. Peptide-Ba49 was found to be stable at high temperatures up to 121^{o}C for 30 min and upon autoclaving (at 121^{o}C for 20 min), the peptide retained 94% of its antimicrobial activity against *S. aureus* (ATCC 25923). Its activity also remained stable even in a wide range of pH (pH5-10).

Peptide-Ba49 was found to be stable in the presence of organic solvents like methanol, ethanol, isopropanol, acetone, ethyl acetate, chloroform, acetonitrile, and butanol. The investigations on the effect of surfactants on antimicrobial activity of peptide-Ba49 showed this peptide retaining more than 80% of its antimicrobial activity after treatment with surfactants (Triton X100, Tween 80, and SDS). Even upon treatment with reducing agents (DTT, β-mercaptoethanol, and urea), this peptide retained more than 80% of its activity. This result depicted the hydrophobic nature of the peptide-Ba49 and indicates the omission of any carbohydrate and peroxide moieties in it. Studies on the effects of metal ions on peptide-Ba49 showed its activity to be stable at 20 mM concentration of various metal ions. Through a decrease in the activity of peptide-Ba49 by 50% against *S. aureus* was observed during the treatment with $FeSO_4$, which could be due to the production of siderophores by bacterium having a higher affinity for iron than a peptide.

The antibacterial activity of peptide-Ba49 revealed that it strongly inhibiting the growth of different *Staphylococcus* species and its pathogenic strains at lower MIC values in the range of 0.5 μM – 16 μM as compared to nisin. Furthermore, time-dependent killing kinetics of target of *S.aureus* (ATCC 25923) revealed the loss of cell viability within 240 min., suggesting the peptide's fast bactericidal potential. The post-antibiotic study (PAE) demonstrated prolonged effect after exposing the *S.aureus* (ATCC 25923) for 4 h at 2xMIC. This might have indicated that the cell damage was immense and took longer time to repair, i.e., ~10 h.

Studies on the probable mode of action of peptide-Ba49 against *S. aureus* revealed that it's bactericidal action of killing of the bacteria by disrupting the cell membrane. The peptide initially increased the ROS production and, further depolarized the target cell membrane leading to cell death. The membrane depolarization was depicted with the help of $DiSC_3$ (5); later, the hyperpolarization was observed at a higher concentration, 4xMIC (8 µM), with the help of $DiBAC_4$ (3). Evaluation of bacterial cell viability in terms of its membrane integrity by staining *S. aureus* cells with Propidium iodide (PI) showed that the dye intercalating with the impaired nucleic acid of the cells. The cell viability following membrane disruption and permeabilization of *S. aureus* cells treated with the purified peptide-Ba49 assessed by PI uptake indicated the loss of *S. aureus* membrane integrity on treatment with peptide. The morphological changes were observed through scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM study revealed smooth surface in untreated *S. aureus* cells, whereas upon treatment with the peptide-Ba49, morphological alterations such as damaging the cells envelope and destruction were apparent. The transmission electron microscopy (TEM) visualized the drastic impact of peptide-Ba49 on the *S. aureus* cell compared to untreated cells. It was found that untreated cells had a uniform shape with intact cell walls. Whereas, the cells treated with purified peptide-Ba49 lost their uniformity because of rupturing. These images also showed that the cytoplasmic contents came out of the cells following peptide-Ba49 treatment compared to untreated cells.

The characteristics resistance offered by biofilm-associated communities of microorganisms leading to their continued survival is a significant challenge; thus, biofilm has been attributed to severe illness. Though many antimicrobial peptides are only effective against planktonic cells, only a few of them showed antibiofilm activities. On examining the effect of peptide-Ba49 on *S. aureus* biofilm, a significantly reduced biofilm formation was observed at a lower concentration. Confocal imaging analysis using PI and SYTO 9, revealed the inhibition of bacterial cells in biofilm which was indicated by the minimum formation of EPS. Moreover, the peptide can inhibit the mature *S. aureus* biofilm at a concentration of 64 µM. These results signified that peptide-Ba49 could be developed as an antimicrobial agent for treating *S. aureus* planktonic as well as biofilm-associated *S. aureus* infections.

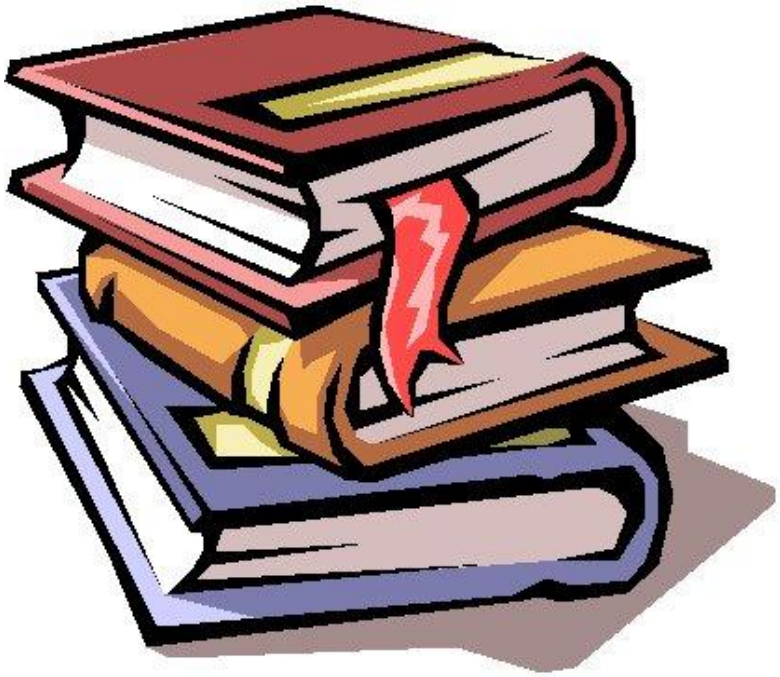
Furthermore, *S. aureus* can penetrate and survive within the host cell and cause chronic infections. Due to the difficulty in antibiotic passaging through cellular membranes, it becomes more challenging and difficult to treat the infection at the intracellular stage. So, in addition to the extracellular activity of the antimicrobial peptide, it also required intracellular

activity. The intracellular activity of peptide-Ba49 showed that the peptide reducing the bacterial burden in *S. aureus* (ATCC 25923) infected RAW 264.7 macrophages upon treatment at 2xMIC for 24 h. Interestingly, peptide-Ba49 prompted the L929 fibroblast cell proliferation at 2xMIC, a positive event for the wound healing process as fibroblast cells are essential cells because of their involvement in wound contraction and ECM production.

Process optimization is a critical step to ensure high productivity. The improvement in the production of microbial metabolites in the fermentation process is often achieved by studying physical and nutritional parameters. Optimization of the nutritional parameter is one of the most effective approaches. Optimization of Medium formulation can be obtained using either a conventional or statistical method or both. Herein, fermentation conditions of strain Ba49 was optimized using the statistical approaches PBD followed by RSM.

PBD statistical approach showed the factors such as glycerol, ferric (III) citrate, NaCl, and $MgCl_2$ to be affecting peptide-Ba49 production. Later, by keeping other factors constant, these four factors were chosen for designing RSM to check their synergistic influence on the production of peptide-Ba49. RSM studies showed the factor NaCl to be having positive effect, i.e., with an increase in NaCl concentration, the activity of peptide-Ba49 also increased. However, declines in the peptide production by strain Ba49 was observed by increasing ferric (III) citrate and $MgCl_2$ concentrations. Further, though, 0.5% (v/v) of glycerol promoted peptide production, the production was declined with increased glycerol concentration. Thus, it could be concluded that the glycerol might be a positive inducer which enhanced the peptide production from strain Ba49 instead of involving in cell growth.

In conclusion, the antimicrobial peptide-Ba49 produced by strain *B. subtilis subsp. spizizenii* Ba49 (MTCC 13006), isolated from *Allium cepa*, was successfully purified and characterized as a potential antibacterial peptide that killed the pathogenic and a clinical isolate of *S. aureus* strains at a very low MIC. Moreover, this peptide was highly stable at high temperatures and can resist various physicochemical conditions. Also, this peptide was found to be effective not only against *S. aureus* planktonic cells but also showed pre- and post-antibiofilm activity at low concentrations. The post-antibiotic effect was also prolonged following 4h of incubation. It also showed a potential intracellular killing activity at low concentration with immense wound healing capacity. It shows that the peptide-Ba49 has the tendency to replace the conventional antibiotics as a promising future therapeutic agent and will also help in reducing the development of drug resistance in pathogens.



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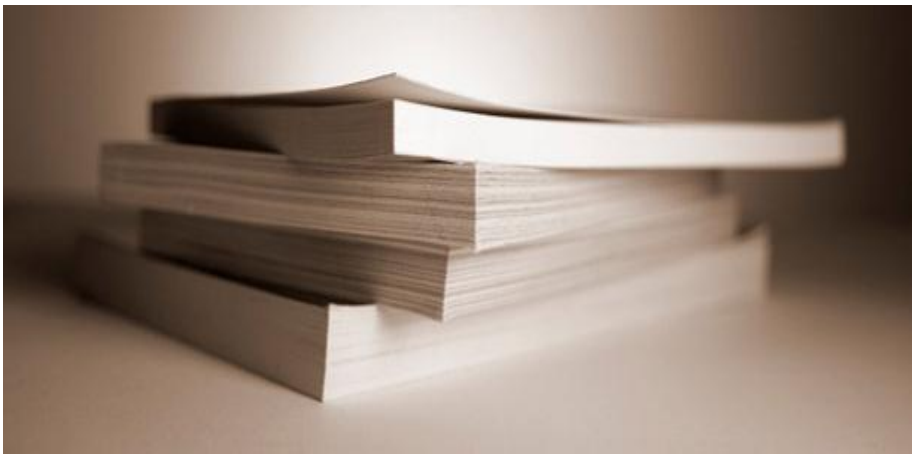

PUBLICATIONS

PUBLICATIONS

1. **Taggar R**, Jangra M, Dwivedi A, Bansal K, Patil PB, Bhattacharyya MS, Nandanwar H, Debendra K. Sahoo DK. Bacteriocin isolated from the natural inhabitant of *Allium cepa* against *Staphylococcus aureus*. World J Microbiol Biotechnol. 2020 Dec.
2. **Taggar R**, Singh S, Bhalla V, Bhattacharyya MS, Sahoo DK. Deciphering the antibacterial role of peptide from *Bacillus subtilis* subsp. *spizizenii* (Ba49) against *Staphylococcus aureus*. (In preparation).

Book Chapters

1. Chopra L, Singh G, **Taggar R**, Kumar R, Sahoo DK. Protein Nutraceuticals from Marine Microbes. Marine Microorganisms, Taylor and Francis, Chapter 5: 76-92. 2016.
2. Chopra L, Singh G, **Taggar R**, Dwivedi A, Nandal J, Kumar P, Sahoo DK. Antimicrobial Peptides from Bacterial Origin: Potential Alternative to Conventional Antibiotics. High Value Fermentation Products: Human Health, I. Wiley, Chapter 8: 193-203. 2019.

Bacteriocin isolated from the natural inhabitant of *Allium cepa* against *Staphylococcus aureus*

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Abstract

Extensive usage of antibiotics has led to the emergence of drug-resistant strains of pathogens and hence, there is an urgent need for alternative antimicrobial agents. Antimicrobial Peptides (AMPs) of bacterial origin have shown the potential to replace some conventional antibiotics. In the present study, an AMP was isolated from *Bacillus subtilis* subsp. *spizizenii* strain Ba49 present on the *Allium cepa*, the common onion and named as peptide-Ba49. The isolated AMP was purified and characterized. The purified peptide-Ba49, having a molecular weight of ~3.3 kDa as determined using mass spectroscopy, was stable up to 121^{o}C and in the pH range of 5-10. Its interaction with protein degrading enzymes confirmed the peptide nature of the molecule. The peptide exhibited low minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and its (Methicillin-resistant *Staphylococcus aureus*) MRSA strains (MIC, 2–16 µm/mL). Further, time kill kinetic assay was performed and analysis of the results of membrane depolarization and permeabilization assays (TEM, $DiBAC_4$ (3) and PI) suggested peptide-Ba49 to be acting through the change in membrane potential leading to disruption of *S. aureus* membrane. Additionally, cytotoxicity studies of peptide-Ba49, carried out using three mammalian cell lines viz. HEK 293T, RAW 264.7, and L929, showed limited cytotoxicity on these cell lines at a concentration much higher than its MIC values. All these studies suggested that the AMP isolated from strain Ba49 (peptide-Ba49) has the potential to be an alternative to antibiotics in terms of eradicating the pathogenic as well as drug-resistant microorganisms.

Keywords: Antimicrobial peptide (AMPs), *Staphylococcus aureus*, MRSA, Propidium iodide, $DiBAC_4$ (3)

Key points

1. *Bacillus subtilis* subsp. *spizizenii* (Ba49) isolated from *Allium cepa* was founded to be a potent source of AMP.
2. Characterized AMP was active against *S. aureus* and MRSA at low MIC.
3. AMP from strain Ba49 acts through membrane disintegration with no cytotoxicity in mammalian cell-lines.

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We are pleased to inform you that your manuscript, "Bacteriocin isolated from the natural inhabitant of Allium cepa against Staphylococcus aureus", has been accepted for publication in World Journal of Microbiology and Biotechnology.

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Our flexible approach during the COVID-19 pandemic

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CHAPTER 5

Protein Nutraceuticals from Marine Microbes

Lipsy Chopra, Gurdeep Singh, Ramita Taggar, Raj Kumar, and Debendra K. Sahoo

CONTENTS

5.1 INTRODUCTION

The term "nutraceuticals," coined from "nutrients" and "pharmaceuticals" by Stephen DeFelici in 1989, is frequently used interchangeably with "functional foods" even though there is a slight disparity between the two. Nutraceuticals are "naturally derived bioactive compounds that are found in foods, dietary supplements and herbal products, and have health promoting, disease preventing, or medicinal properties" (Pandey et al., 2011). Functional foods consist of an ingredient that provides a health-promoting property in addition to its usual nutritional value, for example, probiotic yogurts. When the food is cooked or prepared using "scientific intelligence" with or without understanding of how or why it is being used, the food is called "functional food" (FAO Report, 2007). A functional food that facilitates the prevention or treatment of disorders or diseases other than anemia is called a "nutraceutical." Functional foods supply the required amount of carbohydrates, proteins, vitamins, fats, and so on, needed for healthy survival (FAO Report, 2007). The use of functional foods is becoming an area of growth for the food industry due to the side effects of drugs and negative impact of supplements on human health. Functional foods are one of the largest growing markets in Japan and are defined as regular food derived from naturally occurring ingredients to be consumed as a part of the diet and not in the form of supplements (i.e., in the form of tablets and capsules). However, a thorough investigation of the characteristics and biological activity of functional foods and nutraceuticals, such as their therapeutic or disease-preventing efficacy, proper dosage, and possible adverse effects, is necessary. Significant prospective research in this field includes the following (Jackson and Paliyath, 2011):

1. Identification, quantification, and standardization of promising bioactive components in functional foods
2. Investigation on the effects of functional foods and nutraceuticals on human health
3. Development of strategies to enhance the levels of these compounds in raw and processed foods
4. Establishment of proper dosage and delivery systems
5. Studies on bioavailability and metabolism of functional foods and nutraceuticals
6. Studies on technical and safety issues that have a bearing on Food and Drug Administration (FDA) regulations and health claim evaluations
7. Examination of regulatory issues
8. Research on the stability of the functional foods and nutraceuticals after processing
9. Interaction of functional foods and nutraceuticals with drugs and other functional foods and nutraceuticals

5.2 MARINE ENVIRONMENT: THE WIDEST FRONT OF BIOACTIVE COMPOUNDS

The ocean is the mother of life and it is presumed that the most primeval forms of life originated from this "primordial soup" (Bhatnagar and Kim, 2010). Oceans are regarded as rich in organic compounds that are favorable for the evolution and growth of life. The marine environment covers a broad range of abiotic conditions such as temperature (from below freezing temperature in Antarctic water to about 350°C in deep hydrothermal vents), pressure (1–1000 atm), and nutrients (from oligotrophic to eutrophic), and it has

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Antimicrobial Peptides from Bacterial Origin: Potential Alternative to Conventional Antibiotics

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Abstract

Antimicrobial resistance (AMR) is a global public health threat in which microorganisms have generated the capacity to tolerate or withstand the impact of antimicrobials. In recent years, studies of antimicrobial peptides (AMPs), biologically active oligopeptides having bioactivity against broad spectrum of microorganism (from viruses to protozoans), have opened a new field of research and widened the application of AMPs which could also be a potential alternative to conventional antibiotics to combat the multiple drug resistant (MDR) pathogens in future. AMPs produced by bacteria are of two types (i) ribosomally synthesized i.e., bacteriocins, (ii) non-ribosomally synthesized. The non-ribosomally synthesized AMPs kill the bacteria by damaging its membrane, which leads to cell death. The ribosomally synthesised bacteriocins are mostly produced by gram-positive and gram-negative bacteria and have antimicrobial activities against similar or closely related bacterial strain(s). This chapter summarizes the current information on classification, mode of action and potential applications of AMPs including those in food and pharmaceutical industries.

Keywords: Antimicrobials; bacteriocins; bio-preservative; anti-biofilm; therapeutics

8.1 Introduction

Bacteriocins

The study of a few selected bacteriocins in-depth has created a new research field that further broadened the applications of these antimicrobial peptides. The majority of bacteriocins are from gram-positive and gram-negative bacteria which possess the antimicrobial activities [1] e.g., nisin in *Lactococcus lactis,* Colicin in *Escherichia coli*. These are proteinaceous, low molecular weight cationic or amphipathic molecules that restrain the growth of other bacteria; these are phenomenologically equivalent to killing factors produced by paramecium and yeast, defensins of mammals, techyplesins of crabs, cecropins of insects and thionins of plants. These all are structurally, functionally, and ecologically diverse.

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