Purification and characterization of antibacterial peptide MFAP9 with therapeutic potential from *Aspergillus fumigatus* BTMF9 isolated from marine sediment

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By

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This is to certify that the research work presented in the thesis entitled **"Purification and characterization of antibacterial peptide MFAP9 with therapeutic potential from *Aspergillus fumigatus* BTMF9 isolated from marine sediment"** is based on the original research work carried out by Ms. Rekha Mol K. R. under my supervision and guidance, in partial fulfilment of the requirement for the degree of Doctor of Philosophy, under the Faculty of Sciences of CochinUniversity of Science and Technology.

I certify that all the suggestions made by the doctoral committe during her presynopsis is included in the thesis, and that no part thereof has been presented for the award of any degree.

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This is to certify that the thesis entitled "**Purification and characterization of antibacterial peptide MFAP9 with therapeutic potential from *Aspergillus fumigatus* BTMF9 isolated from marine sediment**" is a record of bonafide research work done by Ms. Rekha Mol K.R under the guidance of Dr. Elyas K.K and my co-guidance in partial fulfilment of the requirement for the degree of Doctor of Philosophy, under the Faculty of Sciences of Cochin University of Science and Technology.

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DECLARATION

I hereby declare that the thesis entitled "**Purification and characterization of antibacterial peptide MFAP9 with therapeutic potential from *Aspergillus fumigatus* BTMF9 isolated from marine sediment"** is the authentic record of research work carried out by me at the Department of Biotechnology, Cochin University of Science and Technology for my doctoral degree, under the supervision and guidance of Dr. Elyas K K, Professor, Department of Biotechnology, Calicut University and that no part thereof has previously formed the basis for the award of any degree or diploma, associateship or other similar titles or recognition.

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Rekha Mol K.R

CONTENTS

LIST OF TABLES AND FIGURES

ABBREVIATIONS

%	-	Percentage
℃	-	Degree Celsius
A_{214}	-	Absorbance at 214 nm
A_{280}	-	Absorbance at 280nm
A_{600}	-	Absorbance at 600 nm
A_{414}	-	Absorbance at 414 nm
A_{570}	-	Absorbance at 570 nm
APS	-	Ammonium persulfate
ATP	-	Adenosine tri phosphate
AMP	-	Antimicrobial peptide
AU	-	Activity units
Aib	-	α- aminoisobutyric acid
AIDS	-	Acquired immunodeficiency syndrome
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
BSA	-	Bovine serum albumin
CFS	-	Cell free supernatant
CFU	-	Colony Forming Units
cm	-	Centimeter
CTAB	-	Cetyl trimethyl ammonium bromide
COX	-	Cycloxygenase enzyme
Da	-	Dalton
DMSO	-	Dimethyl sulphoxide

DPPH	-	2,2-diphenyl-1-picrylhydrazyl
DNA	-	Deoxyribonucleic acid
PMF	-	Peptide Mass Fingerprinting
dNTP	-	Deoxyribonucleotide triphosphate
DTT	-	Dithiothreitol
DMEM	-	Dulbecco's modified Eagles media
EDTA	-	Ethylene diamine tetra acetic acid
EtBr	-	Ethidium bromide
FBS	-	Fetal Bovine Serum
FDA	-	Food and Drug Administration
Fig	-	Figure
g	-	Grams
h	-	Hours
HCl	-	Hydrochloric acid
HPLC	-	High performance liquid chromatography
HDP	-	Host Defense Peptides
IEF	-	Isoelectric focusing
ie.	-	that is
IPG	-	Immobilised pH Gradient
kb	-	Kilobase
kDa	-	Kilo Dalton
L	-	Litre
LAB	-	Lactic acid bacteria
LPS	-	Lipopolysaccharide
Log	-	Logarithm
M	-	Molar

m	-	Metre
MALDI-TOF-		Matrix Assisted Laser Desorption Ionization- Time of Flight
mg	-	Milligram
MHB	-	Mueller Hinton broth
MIC	-	Minimum Inhibitory Concentration
MBC	-	Minimum Bactericidal Concentration
Min	-	Minutes
mL	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
MRSA	-	Methicillin resistant *Staphylococcus aureus*
MS	-	Mass spectrometry
N	-	Normality
NA	-	Nutrient agar
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NB	-	Nutrient broth
NCBI	-	National Center for Biotechnology Information
NCIM	-	National Collection of Industrial Microorganisms
NSAID-		Nonsteroidal Anti-Inflammatory Drugs
ng	-	Nanogram
Nm	-	Nanometer
No.	-	Number
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction

pH	-	Power of Hydrogen
pI	-	Isoelectric point
RNA	-	Ribonucleic acid
RP	-	Reverse phase
rpm	-	Revolutions per minute
rRNA	-	Ribosomal RNA
s	-	Seconds
SCD	-	Soyabean casein digest medium
SDS	-	Sodium dodecyl sulphate
SEM	-	Scanning Electron Microscopy
sp.	-	Species
TAE	-	Tris-acetate-EDTA
TD	-	Time to death
TE	-	Tris-EDTA
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine
TFA	-	Trifluroacetic acid
TSB	-	Tryptone soya broth
UF	-	Ultra filtration
UV	-	Ultraviolet
UV-VIS	-	Ultraviolet-Visible
V	-	Volts
v/v	-	Volume/volume
viz.	-	Namely
w/v	-	Weight/volume
ZB	-	Zobell marine broth
µg	-	Microgram

µL	-	Microlitre
µM	-	Micromole
µM	-	Micromolar
µm	-	Micrometer
A-	Ala-	Alanine
R-	Arg-	Arginine
N-	Asn-	Asparagine
D-	Asp-	Aspartic acid
C-	Cys-	Cysteine
E-	Glu-	Glutamic acid
Q-	Gln-	Glutamine
G-	Gly-	Glycine
H-	His-	Histidine
I-	Ile-	Isoleucine
L-	Leu-	Leucine
K-	Lys-	Lysine
M-	Met-	Methionine
F-	Phe-	Phenyl alanine
P-	Pro-	Proline
S-	Ser-	Serine
T-	Thr-	Threonine
W-	Trp-	Tryptophan
Y-	Tyr-	Tyrosine
V-	Val-	Valine
Na	-	Sodium
Ca	-	Calcium

Mg	-	Magnesium
Fe	-	Iron
Mn	-	Manganese
N	-	Nickel
Ba	-	Barium
Cd	-	Cadmium
Zn	-	Zinc
Cu	-	Copper
Al	-	Aluminium
Co	-	Cobalt

1. INTRODUCTION

Millions of people worldwide are increasingly affected by various infectious diseases caused by pathogenic bacteria and World Health Organization estimates revealed that nearly 50,000 people die each day throughout the world because of this reason only which accounts for one third of all deaths in the world (Chanda & Rakholiya, 2011). Antibiotics play a significant role in human history as they have saved billions of lives. The first discovered antibiotic was penicillin. Even though the discovery of antibiotics to fight against bacterial infections offered a blessing to mankind, development of antibiotic drug resistance created another threat that must be solved immediately. Many pathogenic microorganisms have unexpectedly developed resistance to various antibiotics. For example, methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecium* (VREF) (Enright *et al*., 2002).

Additionally, many pathogens such as *Mycobacterium tuberculosis* have revitalized and are posing a hazard to public health again. Meanwhile some new infectious diseases are also emerging, e.g., toxoplasmosis and cryptococcal meningitis, which are becoming prevalent (Morse, 1995). The currently available strongest antibiotics are becoming ineffective due to repetitive use and misuse by the agricultural and pharmaceutical industries. The evolution and rapid spread of drug resistant bacterial strains through hospitals pose another serious problem creating community acquired infections. Thus, there is an urgency to develop novel antibiotics effective against drug-resistant pathogenic bacterial strains.

Organisms use a variety of defense systems, including the production of antimicrobial proteins, lectins, secondary metabolites, membrane-interacting proteins,

and antifeedant proteins, to fight against microbial pathogen infections. These all come under innate immune responses as lower organisms do not have an adaptive immune system (Park *et al.*, 2008).

It is an important fact that since the last 20 years other than the derivatives of β-lactam and sulfonamide antibiotics which were discovered early in the 1930s, only a few new compounds have been approved for therapeutic use. Though there may be several economic reasons behind this, it also demonstrates the difficulty of obtaining novel compounds from existing antimicrobials (Butler & Buss, 2006). Many approved antimicrobials originate from natural products, so recent antimicrobial discovery has focused on natural sources as the starting material (Donadio *et al*., 2010; Butler & Cooper, 2011).

The natural environment is still the most important supply of novel drugs despite development of combinatorial chemistry, which quickly generated thousands of new chemicals (Zhang *et al*., 2010). Nowadays humans have recognized oceans as rich source of natural products with high therapeutic potential (Bhimba *et al*., 2012). Encouraged by the idea of "Drugs from the Sea", scientists have identified several bioactive compounds with novel structures from the rich marine bio-resource in the recent years (Marris, 2006). Marine derived fungi have an important share in them. Marine fungal strains have been isolated, screened, and identified to produce novel antimicrobial compounds belonging to the class of alkaloids, macrolides, terpenoids, peptide derivatives and other structure types (Saleem *et al.*, 2007). Fungal peptides/ proteins have extensive application in pharmaceutical industry. Screening of different fungi for the evaluation of their antimicrobial potential is essential for their applied use in drug manufacturing.

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Marine microorganisms have established to be an important source of pharmacologically active metabolites, and a rising number of marine- derived fungi have been reported to produce metabolites with unique structures and interesting Bioactivities (Rateb & Ebel, 2011). Moreover, in recent years marine microorganisms have become important in the study of novel microbial products exhibiting antimicrobial (Marderosian, 1969; Molinski, 2009), antiviral, antitumour as well as anticoagulant and cardiovascular properties. The genus *Aspergillus* (Moniliaceae), with more than 180 species, has attracted extensive attention as a rich source of bioactive compounds, some of which showed antifungal, antibacterial, antifouling, and cytotoxic activities (Wang *et al*., 2011; Sun *et al*., 2012; He *et al*., 2012). A standard clinical practice to use a combination of two or more antibiotics with different mechanisms of action may efficiently prevent the development of antibiotic resistance and improve the outcome of therapy. The synergistic antimicrobial activity obtained through combination therapy also facilitates expansion of the antimicrobial spectrum, prevention of the emergence of resistant organisms and minimized toxicity.

Antimicrobial peptides (AMPs) may be an important contributor in this context as they exhibit rapid killing often within minutes *in vitro*, and display a broad spectrum of activity against various targets of gram-positive and gram-negative bacteria, fungi, parasites, enveloped viruses, and tumor cells (Baker *et al*., 1997; Hancock & Scott, 2000; Fernandez-Lopez *et al*., 2001; Zasloff, 2002; Chen *et al*., 2009). Besides, their antimicrobial activity can be enhanced by the synergy between individual cationic peptides within the host and other host factor like lysozyme (Hancock & Scott, 2000). Hundreds of antimicrobial peptides have been isolated so far and most of these peptides share several common properties, irrespective of their

origin, spectrum of activity, and structure. They are generally composed of < 60 amino acid residues (mostly L-amino acids), possess net positive charge, amphipathic in nature, and mostly act on plasma membrane. The interaction of antimicrobial peptides with membranes alters the organization of the bilayer and makes it permeable, resulting in membrane depolarization (Matsuzaki *et al.*, 1997; Kourie & Shorthouse, 2000).

Proteins and peptides play vital roles in living body systems by controlling and coordinating inter and intra-cellular communications and cellular function. Further, peptides with low molecular weight have been known to be less allergenic than their native proteins, as indicated in the wide utilization of protein hydrolysates in the formulation of hypoallergenic infant foods (Høst & Halken, 2004). There is a large variety of physiological activities induced by bioactive peptides which are mainly determined by the type, number, sequence and properties of amino acids present in the peptide (Agyei & Danquah, 2011; Agyei & Danquah, 2012).

Structural modification (including library construction and screening) and immobilization of AMPs is relatively easy because AMPs are made up of amino acids. It is possible to make synthetic peptides by employing chemical synthesis or by using recombinant expression systems which are useful for modification of existing AMPs and for designing new synthetic AMPs. Such modifications have potential to change the targets of AMPs and also to improve the stability of AMPs against proteases (Bahar & Ren, 2013). Several antimicrobial proteins/peptides serve as the basis for the design of new synthetic proteins and analogs used as active ingredients of commercial biopesticides, medicines and food preservatives (Virágh *et al*., 2014).

Several structural and physico-chemical parameters can influence the potency and spectrum of activity of AMPs. These parameters include the size or length, residue arrangement, the propensity for the structure, the net charge (cationicity), hydrophobicity, amphipathicity, the angle subtended by each sector on a wheel projection, the sector depths, which are determined by the size of side-chains in the residues that form them (Yeaman & Yount, 2003), solubility and helicity (Bahar & Ren, 2013). These factors are closely related, so that modifications aimed at altering one can result in significant changes to one or more of the others. Understanding and controlling these interrelationships is the key to design novel peptides with increased potency and more directed activity.

The most common and prevalent species among the filamentous fungi are *Aspergillus* enduring interest with their biotechnological prospective in natural ecosystems and the human economy. In addition to the production of several useful extracellular enzymes and organic acids, these species also produces metabolites like antimicrobial peptides of high therapeutic value. Especially, marine fungi have the capacity to produce unique and unusual secondary metabolites most probably due to their adaptation to a very distinct set of environmental pressures (Bhadury *et al*., 2006).

The filamentous fungal strain *Aspergillus fumigatus* has been reported to be the producer of metabolites with broad range of biological activities (Son *et al*., 1996; Cui *et al*., 1997;). Even though *A. fumigatus* looks taxonomically homogeneous in terms of morphology, the intraspecies variability at the genomic level is very high (Debeaupuis *et al*., 1997) as evident in the report of genetic polymorphism of *A. fumigatus* isolates by Symoens *et al*. (2000). A general outline of the techniques used for the examination of the genetic variability of *A. fumigatus* suggested that

recombination to play an important role in *A. fumigatus* populations (Varga and Tóth, 2003).

The aim of the present study is the screening of fungal isolates from terrestrial and marine environment for antimicrobial peptides, and the production, purification and characterization and potential therapeutic applications of antimicrobial peptide from the selected *Aspergillus fumigatus* BTMF9 strain.

1.1 OBJECTIVES OF THE STUDY

Bioactive peptides represents a suitable candidates for a new era of pharmaceutical products, especially with the heightened concern of side effects and diverse physiological roles of peptides make them suitable candidates for the development of therapeutic agents in the discovery of new drugs. Filamentous fungal cells are major biofactories of the past, present and the future and advances in the production of many homologous and heterologous products consider them as an excellent choice for the isolation of high value products in many new application areas.

Thus the primary objectives of the study included

1. **Screening for antimicrobial peptides in fungi isolated from terrestrial and marine sources.**

2. **Identification of antimicrobial peptide producing fungus.**

3. **Optimization of culture conditions for maximum production of the peptide on lab scale by one-factor-at-a-time method.**

4. **Purification and characterization of active peptide.**

5. **Application studies.**

REVIEW OF LITERATURE

2.1 ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs) are endogenous antibiotics which are remarkably striking candidates as therapeutic agents due to their wide spectrum of antimicrobial activity and mechanism of action. These are short oligopeptides of five to hundred aminoacid residues. AMP has been recognized as early as 1939 in prokaryotic cells, when gramicidin was isolated from *Bacillus brevis* by Dubos (1941). It was found to be active against gram-positive bacteria. Some authors considered lysozyme, identified by Alexander Fleming in 1920 as a peptide with antimicrobial activity (Fleming, 1922). As the "Golden era of antibiotics" began with the advent of penicillin and streptomycin in 1943, a rapid loss of interest in the therapeutic potential of natural host antibiotics occurred. Rise of multidrug-resistant pathogens in the early 1960s, reawakened interest in the host defense molecules. The true origin of research into AMPs began with the studies that were conducted in the 1950s and 1960s.

Natural AMPs can be found in both prokaryotes (e.g., bacteria) and eukaryotes (e.g., protozoan, fungi, plants, insects, and animals). Particularly organisms that lack an adaptive immune system rely extremely on the action of AMPs as first line of defense (Patil *et al*., 2005). A major component of innate immunity against infection involves production of AMPs. Their production is either constitutive or induced, depending on a wide range of characteristics such as type of organism, cell type and peptide. For example, epithelial cells from different tissues of mice showed increased rate of mRNA transcription for defensin production after infection with *Pseudomonas aeruginosa* PAO1(Bals *et* al., 1999).

Antimicrobial peptides are highly diverse in their sequence and structures. They are generally amphipathic in nature due to their positive charges and

hydrophobic amino acid residues. These chemical properties of AMP permits the complex interaction with the membrane, major rearrangements of its structure may result from the formation of peptide-lipid specific interactions, the peptide translocation across the membrane and interaction with intracellular targets or the most common mechanism, a membranolytic effect. Such a characteristic mechanism of action enables AMPs to avoid the common resistance mechanisms observed for conventional antibiotics (Seo *et al*., 2012). Moreover, AMPs are also known to augment the activities of antibiotics through synergistic effects which enable the reduction in the dose of the individual drugs. For example, the combination of penicillin with pediocin and ampicillin with nisin Z exhibited activity against *Pseudomonas fluorescens* with 13- and 155-fold lower minimum inhibitory concentration (MIC) respectively, in comparison to antibiotics alone (Naghmouchi *et al*., 2012). Till date, more than 5000 AMPs with different sequence motifs have been isolated from a wide range of organisms including bacteria, fungi, plants and vertebrates. Some of them are chemically synthesized (Bahar & Ren, 2013).

The discovery of two classes of antimicrobial peptides, non-ribosomally synthesized (present in bacteria, lower eukaryotes and plants) and ribosomally synthesized peptides of wider distribution provided a new therapeutic strategy (Hancock & Chapple, 1999). Recent studies have shown that majority of these peptides are cationic and some non-cationic peptides expressed in many vertebrate, invertebrate and bacterial species (Lüders *et al*., 2003) act synergistically to improve immune responses. Antimicrobial activities reported for these molecules suggest that they have potential benefits in the treatment of cancer, viral or parasitic infections and many other applications. Despite these advantageous features of AMPs, there are still some challenges to their applications such as probable toxicity to humans, sensitivity to harsh environmental conditions (proteases, extreme pH, temperature etc) lack of

selectivity against specific strains, high production costs, folding problems, reduced activity when used for surface coating and bacterial resistance to some AMPs. AMPs target the lipopolysaccharide layer of cell membrane, which is unique in microorganisms. Presence of a high level of cholesterol and low anionic charge puts eukaryotic cells out of the target range of many AMPs (Jenssen *et al.*, 2006). Although it is expected that some antimicrobial peptides mainly utilize one mechanism to inhibit bacteria, a single peptide may entail multiple mechanisms, rendering it difficult for pathogens to develop resistance.

2.2 STRUCTURAL CLASSIFICATION

Antibacterial peptides can be grouped into five classes based on 3-D structures and motifs. An additional class may be added for the human defensins, which are circular peptides made from two genes with a novel biosynthesis. However, so far the scientific interest has been focused mainly on three of the classes: (i) linear peptides free of cysteines and often with an α-helical and amphipathic structure, (ii) peptides with three disulphide bonds giving peptides with a flat dimeric β-sheet structure and (iii) peptides with unusual bias in certain amino acids, such as proline, arginine, tryptophan or histidine (Boman, 2003).

Antimicrobial peptides exist in a wide variety of structural motifs. Notwithstanding the number and diversity of AMPs in nature, there are some key structural arrangements shared by most of them (Wang, 2009; Novković *et al.*, 2012; Gogoladze *et al.*, 2014), allowing the classification of most AMPs reported till date as one of the following three types based on their secondary structures: i) α-helix, ii) β-sheet, and iii) extended peptides (Figure 2.1). Among these structural groups, α-helix and structures are more common. The majority of these peptides are cationic and amphipathic but there are also hydrophobic α-helical peptides which are anionic and have antimicrobial activity. In addition, some β-sheet peptides have antimicrobial activity and even antimicrobial α-helical peptides

which have been modified into a β-structure retain part of their antimicrobial activity. There are some AMPs which are not folded into regular α-helix or β-sheet structures and are rich in Arg, Trp, Pro or Cys residues. One example of peptides rich in His residues is constituted by human salivary histatins, which show significant activity against parasites (Luque-Ortega *et al.*, 2008) or fungi such as *Candida albicans* and *Cryptococcus neoformans* (Tsai & Bobek, 1998). In addition, AMPs do exist with thio-ether rings, which are lipopeptides or which have macrocyclic Cystine knots. In spite of the structural diversity, a common feature of the cationic antimicrobial peptides is that, they all have an amphipathic structure which allows them to bind to the membrane interface. Indeed, most antimicrobial peptides interact with membranes and may be cytotoxic as a result of disturbance in the inner or outer bacterial membranes. Alternatively, a necessary but not sufficient property of these peptides is that it may be able to pass through the membrane to reach a target inside the cell. The interaction of these peptides with biological membranes is not just a function of the peptide but is also modulated by the lipid components of the membrane. It is not likely that this diverse group of peptides has a single mechanism of action, but interaction of the peptides with membranes is an important requirement for most, but not all, antimicrobial peptides (Epand & Vogel, 1999).

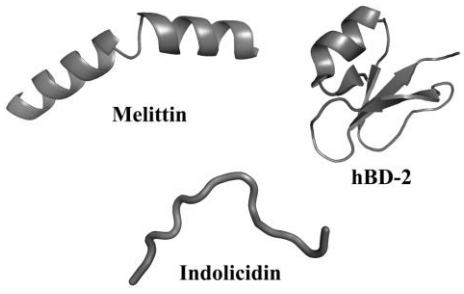

Fig: 2.1 Examples of secondary structures of three representative antimicrobial peptides (Galdiero *et al*., 2015).

Perhaps one of the larger and better studied classes of bacteriostatic peptides has members that form cationic amphipathic helices such as magainins, temporins and melittin (König *et al.,* 2015). However, there are also α-helical peptides that are hydrophobic or even slightly anionic. An example of a well-studied peptide is alamethicin (Sansom, 1993). It is hydrophobic and negatively charged cytotoxic peptide and has preferential activity against gram-positive bacteria and fungi. This helical peptide forms clusters of helices that traverse the bilayer and surround an aqueous pore which can transport ions. These unstructured peptides in aqueous solution folds into their α-helical configuration up on binding the bacterial membrane, where they are either adsorbed onto its surface or inserted into it. A direct correlation has been recognized between α-helical conformation and antibacterial activity (Park *et al.,* 2000). Most α-helical AMPs disrupt bacterial membranes either by forming barrel-like bundles, and these transmembrane clusters line amphipathic pores (barrel-stave model) or by forming carpet-like clusters of peptides including magainins and cecropins. Most of the biophysical studies performed on cecropins (cecropins A and B), which were isolated from the *Hyalophora cecropia* lysozyme in 1980. In mature cecropins, it was found that the polar and hydrophobic side chains at the N terminus were interspaced in a regular pattern (Boman, 1991); this amino acid distribution is expected to stabilize two fully ordered amphipathic α-helices interrupted by a hinge region containing a Gly-Pro sequence. Incorporation of the proline residue plays a critical role on the conformational flexibility of the peptide by breaking α-helix and that hinged region with proline is crucial for antibacterial potency and selectivity (Vermeer *et al*., 2012).

β –sheets are formed when a monomer aggregate or form a bend to allow an intramolecular anti-parallel β-structure to form. Many of the antimicrobial peptides which form a β-structure are able to do so because they are cyclic

peptides and hence there is less entropy loss, which results in the formation of a β-structure. In case of the tachyplesins, protegrins and lactoferrin the ring structure is formed either by disulfide bonds, or by cyclization of the peptide backbone, as in the case of gramicidin A (Wallace, 1984). β -hairpin peptides and defensins belong to β-sheet AMPs. Protegrins belongs to the β-hairpin peptides which form oligomeric transmembrane β-sheets (Tang & Hong, 2009). Defensins have been classified in to three subfamilies: α, β and θ. α- defensins are a type of defensins is found in mammals. Their cysteine residues are connected and show a structure of triple-stranded β-sheet stabilized by a conserved triple disulfide bridges array (Hadjicharalambous *et al.*, 2008). Six cysteine residues responsible for three intramolecular disulfide bonds combined in a specific pattern are recognized features of β-defensins. The third class, θ- defensins are macrocyclic octadeca peptides connected head to tail. These peptides are present in monkeys but absent in human. Defensins are able to act by various mechanisms other than membrane disruption including the inhibition of DNA synthesis and interfering with the metabolism turnover (Jarczak *et al.*, 2013). For example, even though tachyplesin from horseshoe crabs is generally identified as a membrane active peptide, it is also capable of binding to the minor groove of DNA, which may interfere with DNA–protein interactions (Brogden, 2005).

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There are several peptides synthesized by bacteria which contain small ring structures enclosed by a thio-ether bond. This group of peptides is called lantibiotics. Their structure and properties have been reviewed (Holz & Stahl, 1995). Lanthionine is composed of two alanine residues that are cross-linked on their β-carbon atoms by a thioether (monosulphide) linkage. Nisin is a polycyclic antibacterial peptide with 34 amino acid residues produced by several strains of *Lactococcus lactis*, which inhibits growth of a wide range of gram-positive bacteria and is commonly used as a food preservative. It contains several post-

translationally modified amino acid residues: (α,β)-di-dehydroalanine, (α,β)-8-didehydrobutyrine, *m*-lanthionine and (*2S,3S,6R*)-3- methyllanthionine (Twomey *et al*., 2002). Several AMPs have a cyclic peptide structure, with usually less than 15 peptide groups. Recently four macrocyclic end-to-end, 30 amino acid residue cyclic peptides from plants of the *Rubiaceae* family were found to possess potent antimicrobial activity. Four macrocyclic cystine-knot peptides of 29–31 residues like kalata, circulin A and B (CirA and CirB) and cyclopsychotride have been isolated from coffee plants (Tam *et al*., 1999). Certain antimicrobial peptides have unusual amino acid composition, having a sequence which is rich in one or more specific amino acids. For example, the peptide histatin, which is produced in parotid secretion with significant activity against different microorganisms such as bacteria or fungi, is extremely rich in His residues (Brewer & Lajoie, 2000).

Peptaibiotics represent a constantly growing group of peptide antibiotics and are defined as linear or cyclic polypeptide antibiotics of 4–21 amino acid residues have molecular weight between 500 and 2200 Da. These are characterized by a high α-aminoisobutyric acid (Aib) content with the presence of other non-proteinogenic amino- or lipoamino acids, an acylated N-terminus, and a C-terminal residue (if linear) mostly consisting of a free or acetylated amide-bonded 2-amino alcohol. The subgroup of Aib-containing peptides carrying a C-terminal 2-amino alcohol residue is referred to as peptaibols (Rebuffat *et al*., 1991). These residues are conformationally restricted and favor the formation of α-helix. These specific peptides have structural properties that induce spontaneous formation of voltage-gated channels in membrane lipid bilayer, and have been found to have antibacterial, antifungal, and innumerable biological activities. It also occurs in the case of the short antimicrobial peptide trichogin (Epand *et al*., 1999). In addition, these peptides are lipopeptides which are acylated at the N-

terminus, thus favoring their partitioning into membranes. The most widely known peptaibol is alamethicin (Leitgeb *et al*., 2007).

2.3 MODE OF ACTION

Although the exact mechanism of action of AMPs remains a matter of controversy, there is a consensus that AMPs kill cells by disrupting membrane integrity (via interaction with negatively charged cell membrane), by inhibiting proteins, DNA and RNA synthesis, or by interacting with certain intracellular targets. The properties of selectivity and the amphipathic structural arrangement of the peptides are believed to play an important role in the anti-microbial mechanism.

2.3.1 Membrane Active AMPs

The outer surface of gram-negative bacteria contains lipopolysaccharides while gram-positive bacteria holds acidic polysaccharides (teichoic acids), giving the surface of bacteria a negative charge. Furthermore, the phospholipids composing the cytoplasmic membrane of gram-negative bacteria and the single membrane of gram-positive bacteria are negatively charged. In contrast, the outer leaflet of a normal mammalian cell is composed predominantly of zwitterionic phosphatidylcholine and sphingomyelin phospholipids (Devaux, 1991; Dolis *et al*., 1997). The charge on cell membrane due to the presence of phospholipids head group and charge distribution on peptide appears to play an important role in the peptide membrane interactions. The study involved the replacement of positively charged arginine or hydrophobic tryptophan residues of lactoferricin with alanine causes significant reduction in antibacterial activity. This result suggests significance of positively charged amino acids and hydrophobic amino acids to interact with bacterial membrane (Strom *et al*., 2002). The accumulating evidence suggests that the usually highly basic antibacterial or self defense

peptides can recognize the acidic phospholipids exposed on the surface of the bacterial membrane (Reddy *et al*., 2004).

Usually an AMP is only effective against one class of microorganisms for example bacteria or fungi (Hancock & Scott, 2000), exceptions are there and some AMPs are identified with different modes of action against different types of microorganisms. For example, indolicidin can act against bacteria, fungi, and HIV (Selsted *et al*., 1992; Robinson *et* al., 1998). It kills *E. coli* by inhibiting DNA synthesis after penetrating into the cells (Subbalakshmi & Sitaram, 1998). However, it exhibits antifungal activities by causing damages to cell membrane (Lee *et al*., 2003) and anti-HIV activities by inhibiting HIV-integrase (Krajewski *et al*., 2004). In some other cases AMPs apply same mode of action on different cell types as in the given example, PMAP-23 can kill both fungi and parasites through pore formation in their cell membranes (Lee *et al*., 2002; Park *et al*., 2004). One third of the total proteins of a bacterial cell are the membrane proteins and they have many functions that are critical to the cell including active transport of nutrients, respiration, proton motive force, ATP generation, and intercellular communication (Zhang & Rock, 2009). The treatment with AMP also alters the function of these proteins with out complete lysis of cell. For that reason, AMPs' rapid killing effect occurs not only by membrane disruption but also due to inhibition of these functional proteins.

Lipopolysaccharides (LPS) are major components of the outer leaflet of the outer membrane in gram-negative bacteria. Structurally LPSs consist of an O-specific chain that is highly variable in unrelated bacterial strains, a core oligosaccharide, and lipid A (Gutsmann *et al*., 2005). LPSs are essential for bacterial growth, viability and also in virulance, but septic shock occur when macrophages stimulated by LPS induce the release of pro-inflammatory cytokines (TNF-α, IL1 and IL6) into the blood. Antimicrobial peptides generally form complex with LPS by binding through electrostatic interactions between their

basic amino acids (Lysine and Arginine) and head groups of LPS and hydrophobic interactions between the hydrophobic amino acids of the peptide and fatty acyl chains of LPS further stabilize this complex (Li *et al*., 2006; Bhattacharjya *et al*., 2007). The action of polymyxins, which are pentabasic decapeptide antibiotics discovered in *Bacillus polymyxa* (Storm *et al*., 1977), occurs via binding to lipid A of LPS and permeabilization of the outer membrane, is limited to gram-negative bacteria (Mogi & Kita, 2009). Sushi peptides, which are derivatives of Factor C (LPS-sensitive serine protease of the horseshoe crab coagulation cascade), disrupt LPS aggregates through detergent-like action and also have LPS-neutralizing capacity (Li *et al*., 2004; Ding *et al*., 2008).

Gram-positive bacteria possess peptidoglycan cell walls outside the plasma membrane, formed of polymers of sugars and amino acids (Glaser, 1973). Inhibition of the production of peptidoglycan leads to resistance against β-lactam antibiotics such as penicillin due to the presence of penicillin-binding proteins or transpeptidases (Di Guilmi *et al*., 2002; Wilke *et al*., 2005). For example, there is an existence of the penicillin-binding protein 2a (PBP2a) in MRSA, while it is absent in susceptible *S. aureus* (Brakstad & Maeland, 1997; Berger-Bächi & Rohrer, 2002). Antibacterial peptides with unusual amino acids, which are known as lantibiotics exert antibacterial action through the interaction with cell wall components. These compounds are produced by gram-positive bacteria and exert potent inhibitory action against a wide-spectrum of bacteria. These compounds are classified as either type-A or type-B based on its mode of action. Type-A lantibiotics cause damage to the bacterial membrane and Type-B compounds inhibit the production of enzymes (Bierbaum & Sahl, 2009). The most well-known lantibiotic nisin isolated from *Lactococcus latis* (Mattick *et al*., 1947) was initially discovered as complex formers with lipid I and lipid II, and then inhibits cell wall biosynthesis (Hasper *et al*., 2006). But recently, it was shown that nisin can produce short-lived pores that cause the cytoplasmic membrane to be

permeable (Bierbaum & Sahl, 2009). The solid-state NMR studies of piscidins exposed peptide tilting to achieve an optimal interaction and both peptide sequence and lipid composition influence the extent of tilting. The glycine at position 13 may be important for peptide plasticity (Perrin *et al*., 2014). Structural determination of gramicidin and alamethicin yielded evidence for formation of channel or pore (Fox & Richards, 1982; Kovacs *et al*., 1999). Recently, the crystallographic structural analysis of human dermcidin implies another probable channel (Burian & Schittek, 2015; Song *et al*., 2013). It is proposed that C-type lectin form a pore in bacterial membranes on the basis of combined structural determination by X-ray diffraction and with electron microscopy data (Mukherjee, *et al*., 2014). Some other studies revealed that peptides like RTD-2 and lantibiotic Pep5 did not bind to lipid II. Rather, it induced the release of peptidoglycan lytic enzymes (or autolysins) by *S. aureus* after interacting with bacterial membranes in the presence of glucose (Wilmes *et al*., 2014).

Mode of action of antimicrobial peptides in the cytoplasmic membrane is considered to be more important than other targets. Even though the exact mechanisms of antimicrobial peptides are not completely understood, their performance might cause the efflux of intracellular materials by disrupting the cytoplasmic membrane via pore formation either through a Barrel-Stave or a toroidal pore mechanism, or through a nonpore carpet-like mechanism (Figure 2.2).

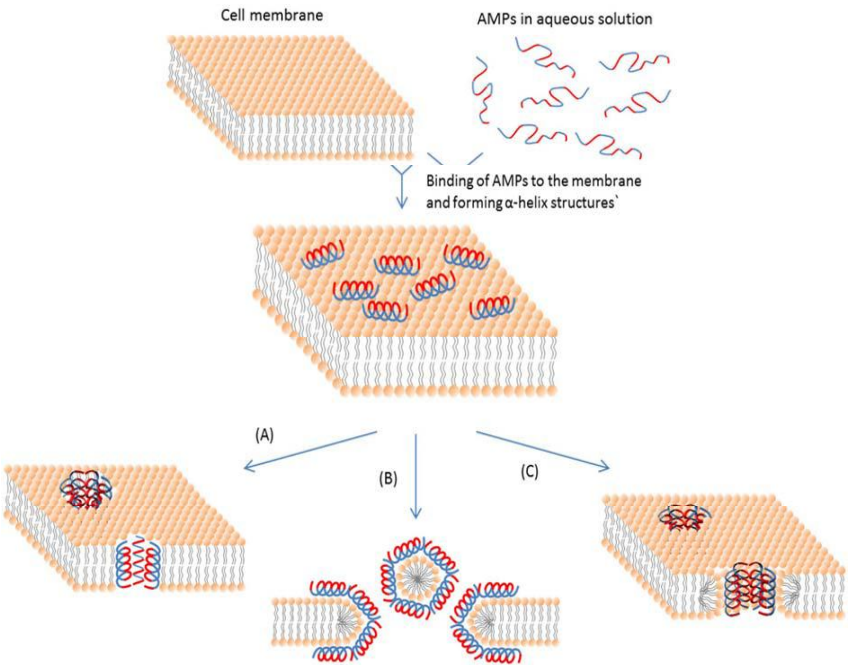


Fig: 2.2 Schematic representations of different mechanisms of action of membrane-active AMPs. (A) Barrel-Stave model, (B) Carpet model and (C) Toroidal pore model. The blue color represents the hydrophobic portions of AMPs, while the red color represents the hydrophilic parts of the AMPs (Bahar & Ren, 2013).

In the Barrel-Stave model (Figure 2.2A), a variable number of transmebrane channel-forming peptides are positioned in a barrel-like ring around an aqueous pore. Generally, the peptide, which is most likely in monomeric form, must associate with the surface of the membrane prior to insertion, and their hydrophobic surfaces interact with the lipid core of the membrane and their hydrophilic surfaces point inward producing an aqueous pore. When the bound peptides attain a threshold concentration, peptide monomers self-aggregate and are inserted deeper into the hydrophobic membrane core. The hydrophobic faces of the peptides then align and face the hydrophobic lipid core region, whereas their

hydrophilic faces form the interior region of a water-filled pore (Mihajlovic &Lazaridis, 2010; Ramamoorthy *et al*., 2010). This type of transmembrane pore is induced by alamethicin and ceratotoxin (Cantor, 2002; Bessin *et al*., 2004).

The carpet model was proposed first time to describe the mode of action of dermaseptin. In this model (Figure 2.2B), antimicrobial peptides accumulate on the membrane surface for cover it in a carpet like manner at numerous sites by electrostatical interaction with the anionic phospholipid head groups. When a threshold peptide concentration is reached, membrane disruption occurs in a detergent-like manner that the peptides are not inserted into the hydrophobic core of the membrane nor do they form pore by assembling their hydrophilic surfaces facing each other (Pouny *et al.*, 1992). The membrane disruptive mechanism of cecropin P1 and caerin 1.1 was explained by this model (Gazit *et al*., 1996; Wong *et al*., 1997).

A toroidal pore model (Figure 2.2C) has been suggested to explain the mode of action of magainin, cathelicidin and HPA3 (Tamba & Yamazaki, 2009; Henzler Wildman *et al*., 2003; Lee *et al*., 2010). The aggregated peptides either prior or after binding with the membrane surfaces induce membrane depolarization or form toroidal shaped transmembrane pores with micellar formation that leads to cell death (Brogden, 2005). Compared to Barrel-Stave model, the antimicrobial peptides are associated onto the lipid head groups even when they are perpendicularly inserted into the lipid bilayer (Ramamoorthy *et al*., 2010). Recently, Han *et al.,* 2009, directly observed magainin action on artificial vesicles using cryo-transmission electron microscopy (TEM) and proposed that magainin-induced pores in lipid vesicles possess a mean diameter of approximately 8 nm. Studies of other groups verified that melittin formed a pore via the toroidal mechanism, though it was dependent on the lipid material properties and peptide concentrations (Allende *et al*., 2005).

In the disordered toroidal pore, lipid molecules are still curved inwards, but pore formation is more stochastic. There is only one or two peptides located near the center of the water-permeable pore, with more peptides placed at the mouth of the pore on the external leaflet. As they translocate to the interior of the cell, the peptides remain mostly parallel to the bilayer plane as only a small tilt is observed. Moreover, the peptides undergo partial unfolding during translocation and their α-helicity is not always maintained. In other molecular dynamics (MD) simulations, similar tilted orientations for α- helical peptides were observed and the concept of imperfect amphipathicity was suggested (Mihajlovic & Lazaridis, 2010). This refers to the occurrence of some polar or charged residues in the hydrophobic face of an AMP to pull lipid head groups into the membrane interior facilitating pore formation. Some AMPs enhance their activity by targeting oxidized lipids on membrane. The α-helical AMPs temporin B and L intercalate more efficiently into membranes containing an oxidized phosphatidylcholine lipid by employing schiff base formation between the peptide amino groups and lipid aldehyde groups (Mattila *et al*., 2008). An increase in bacterial membrane susceptibility to AMPs resulted when lipid oxidation occurred with the release of reactive oxygen species during phagocytosis.

Some bacteriocins find receptors as the target. It was found that lactococcin G and enterocin 1071 (two-chain bacteriocins) used UppP as the receptor, UppP is an enzyme involved in cell wall synthesis (Kjos *et al*., 2014). While garvicin ML recognizes a maltose ABC transporter and another peptide LsbB uses metallopeptidase as the targets (Cotter, 2014). Such findings enrich our view on the mechanisms of action of antimicrobial peptides, despite open new opportunities for antimicrobial development. In 2014, Wenzel *et al*. (2014) illustrated model arginine and tryptophan-rich peptide RWRWRW-NH2 (C-terminal amidation) with multiple mode of action. The peptide is primarily membrane targeting (e.g., D and L-forms have same activity) and the authors

found that multiple surface proteins could be delocalized by the peptide. For example, the replacement of cytochrome C hinders bacterial energy metabolism and delocalization of MinD interferes with bacterial replication respectively. Another surface protein, MurG, can also be delocalized, leading to impaired bacterial cell wall synthesis (Wenzel *et al*., 2014).

Another interesting membrane disturbing effect induced by some α-helical AMPs is that of lipid segregation, where peptide-induced clustering of anionic lipids in bacterial membranes causes slow leakage of intracellular contents and/or membrane depolarization (Epand & Epand 2011). On the other hand, the phase boundary effects between domains of differently charged lipids may compromise the overall membrane stability. Moreover, many bacterial membranes are mostly composed of phosphatidylglycerol and phosphatidylethanolamine lipids, where the latter does not like to form stable bilayers after peptide-induced alterations. The peptide clavanin A rich in Phe-, His- and Gly- amino acids has a distinctive dual pH-dependent mode of action (van Kan *et al*., 2002). At neutral pH, clavanin A permeabilizes membranes like regular α- helical AMPs where as at slightly acidic pH, it becomes membrane disruptive peptide. Interestingly, this does not occur via lipid interactions, but through inhibitory interactions with proteins involved in maintaining the pH gradient across the membrane.

2.3.2 Intracellularly Active AMPs

In early AMP studies, permeabilization of bacterial cell membrane by AMP was considered as the primary mechanism of antimicrobial activity. But, it was suggested that high concentrations of AMPs should be used so that they can kill microorganisms by disrupting the membrane with sufficient channels and pores (Cudic & Otvos, 2002). However, some AMPs were found to start membrane permeabilization at concentrations lower than their MICs. The finding that some AMPs can kill their target cells without causing membrane

permeabilization suggests the possibility of other mechanisms of killing. Recently, intracellularly active AMPs have been shown to interact with intracellular targets (Mookherjee *et al.*, 2009). For example indolicin was shown to bind to DNA with a preferred sequence and also found that the WW motif is essential for DNA binding. They provided high-resolution structural information for its interaction with duplex DNA (Ghosh *et al.*, 2014). Some AMPs are able to inhibit both DNA and protein synthesis as it is clear in an example of a peptide PR-39 from pig intestines, which kills bacteria in a non-lytic process by acting like a proteolytic agent and stopping protein and DNA synthesis (Boman *et al*., 1993). Also, some human immune system derived AMPs such as tPMP-1 and aHNP-1 inhibit DNA and protein synthesis within an hour after their entry in to the cells (Xiong *et al*., 1999). It is suggested that apidaecin is actively transported with a transporter protein and then it blocks protein synthesis with a series of molecular interactions with different targets (Castle *et al*., 1999). Recently published papers report that insect-derived proline-rich apidaecins and oncocins inhibited bacterial protein translation at the 70S ribosome (Krizsan *et al*., 2014). Interestingly, Mardirossian *et al*. (2014) also observed that proline-rich peptide corresponding to N-terminal 35 residues of bovine cathelicidin Bac7 could accumulate within *E. coli* to a high concentration of 340 μM and inhibits protein synthesis by targeting ribosomal proteins. A ribosomally synthesized antimicrobial peptide Microcin B17from *Enterbacteriaceae* is believed to inhibit DNA replication by targeting DNA gyrase (Vizan *et al*., 1991). Proline-rich antibacterial peptides such as pyrrhocoricin (Kragol *et al*., 2002) and drosocin (Otvos *et al*., 2000) have been shown to kill bacterial species by binding to the multi-helical lid region of the bacterial DnaK heat shock protein, which plays crucial role in the initiation of chromosomal DNA replication. Some AMPs can also inhibit proteases of microbes. For example, histatin 5 inhibits a protease from *Bacteriocides gingivalis* causing the periodontal tissue destruction (Nishikata *et al*., 1991) and eNAP-2 has anti-protease activities

against microbial serin proteases (Couto *et al.*, 1993). Interestingly, there are some intracellular AMPs which can only kill cells at certain growth stages. For example, diptericin is only effective against actively growing bacterial cells, suggesting it may interact with certain specific metabolic pathways during bacterial growth (Ishikawa *et al.*, 1992).

Among these intracellularly active AMPs, some of them have multiple targets. For example seminal plasmin inhibits RNA polymerase, prevent RNA synthesis completely at concentrations lower than many other antibacterial agents and also able to activate an autolysin protein inside the target cells leading to autolysis (Bahar & Ren, 2013). The finding that AMPs can inhibit intracellular pathways suggests that there might be mechanisms of cellular uptake of AMPs. Two such mechanisms have been reported: direct penetration and endocystosis (Madani *et al.*, 2011). It was proposed that cellular uptake of AMPs can take place through endocytosis, which includes macropinocytosis and receptor mediated endocytosis (Jones, 2007). The energy independent uptake route of AMPs occurs through macro pinocytosis. Macropinosomes are vesicles formed by inward folds of the plasma membrane of the target cells along with the peptide. Subsequently, the AMPs within the vesicles get released into the cytoplasm and exert their antimicrobial action (Brogden, 2005). In receptor mediated endocytosis, a part of the membrane is coated with clathrin or caveolin proteins followed by pit formation. Later, these pits bud from the membrane to inner side of the cell and form vesicles (Mayor & Pagano, 2007).

2.4 RESISTANCE TO ANTIMICROBIAL PEPTIDES

The acquisition of resistance to AMPs is very rare, compared to conventional antibiotics, for the reason that mainly the mechanism of action involves direct disruption of cellular components, including the microbial membrane and DNA. The development of resistance by gene mutation to such a

microbicidal mechanism of action is difficult, even if microorganisms can coordinate to outwit the attack by AMPs to some degree (Nizet, 2006). The major mechanism appears to alter cell envelope charge and composition. Elucidation of the basis of bacterial resistance may be helpful for the design of more potent antibiotics. Specifically, there are mainly two different types of resistance mechanisms against AMPs: constitutive resistance and inducible resistance. The constitutive resistance mechanisms include electrostatic shielding, changes in membrane potential during different stages of cell growth and biofilm formation (Yeaman & Yount, 2003). The inducible resistance mechanisms include substitution, modification and acylation of the membrane molecules (Lewis *et al.*, , 2009), activation of some proteolytic enzymes, efflux pumps and modifications of intracellular targets (Guina *et al.*, 2000; Del Castillo *et al.*, 2001). For example the activity of some AMPs against *S. aureus* can be inhibited by adhesin molecules on the cell surface of this bacterium (Vuong *et al.*, 2004). Adhesin forms a repulsive barrier against positively charged AMPs because it is a positively charged polymer. Additionally, an ATP-binding cassette (ABC) transporter coupled with an adjacent two-component system (TCS) also constitutes a resistance element against antimicrobial peptides (Gebhard *et al.*, 2014; Dintner *et al.*, 2014). The toxic effect of cationic antimicrobial peptides can be reduced by the well-established major sensing and resistance five-component system GraXSR-VraFG of *S. aureus* (Falord *et al.*, 2012) by upregulating genes such as *mprF* and *dltABCD*.

Modifications of bacterial LPS provide a general mechanism that confers resistance to cationic antimicrobial peptides in the case of gram-negative bacteria (Chen & Groisman 2013). *Salmonella typhimurium* possess a membrane bound lipid A modification system, which defends themselves against AMPs. The PhoPQ two-component system regulates peptide resistance, bacterial lipid A remodeling, and intracellular survival within acidified phagosomes. Further the

PhoPQ system was found to regulate acidic glycerophospholipid content in the outer membrane (Dalebroux *et al.*, 2014). Although bacteria have diverse mechanisms for resistance to AMPs, it is encouraging to become aware that the general lipid bilayer structure of bacterial membranes makes it tough to develop a complete resistance against AMPs. Moreover, the resistance against AMPs reported to date is not as strong as those against antibiotics and it covers only a limited number of AMPs.

2.5 IMPROVEMENT OF AMPs FOR CLINICAL USE

Despite the fact that AMPs have a great potential for therapeutic application as drug candidates, there are some limitations due to their peptidic nature. They are susceptible to inactivation by several conditions (pH, presence of salts and divalent cations, etc.) and action of proteases or other plasma components (Lee *et al*., 1997; Rotem & Mor, 2009). Additionally, few of them are potentially cytotoxic to human cells, producing in some cases allergies and present high manufacturing costs at large scale. Thus, the development of novel AMPs which are potent against microbes but non-toxic to host cells represents an ultimate goal of many research groups (Bradshaw, 2003; Hancock *et al.*, 2006).

Many strategies like: chemical modification of terminal ends of peptides (Danial *et al*., 2012), development of analogues containing unnatural amino acids (Papo *et al*., 2002; Hicks *et al*., 2007), shortening of the native sequence (Park and Hahm, 2012), modifications of their amphipathic balance (Kang *et al.*, 2009) etc. may be applied to overcome these limitations. By now, many novel strategies are being used which have allowed improvement of peptide stability and decrease of cytotoxicity. Combinatorial chemistry and highthroughput screening are also effective tools which may enable the identification of families of peptides, with broadspectrum antimicrobial activity.

Although AMPs bind to bacterial surfaces via electrostatic interactions, some types of AMPs can directly interact with host cells and lyses them. The ratio of antimicrobial activity to hemolytic activity is defined as the therapeutic index (TI), and a high therapeutic index is necessary for avoiding hemolysis of host cells. To solve the issue of hemolysis, it is important to use non-hemolytic AMPs as seed compounds. It would also be beneficial to optimize the peptide sequences to decrease hemolytic activity. Many naturally occurring AMPs are amidated at the *C* terminus; amidated peptides exhibit higher antimicrobial activity but are also more hemolytic. Strandberg *et al*. (2007) reported that *C*-terminal deamidation of AMPs reduces undesired hemolytic activity while maintaining antimicrobial effects.

AMPs need electrostatic interaction with microbial membranes to form secondary structures. This step is salt sensitive and often causes problems in clinical application. Human body fluids containing high salt concentrations deactivate many AMPs; therefore, it is necessary to develop salt-insensitive AMPs. Stabilization of the secondary structure of AMPs could confer salt insensitivity. Helix capping motifs, replacing tryptophan and histidine with the bulky β-naphthylalanine and β-(4, 4'-biphenyl) alanine increased salt resistance of the AMP.

Though large quantities of AMPs are required for clinical trials, the cost of production of AMPs is very high compared to that of conventional antibiotics. Therefore, a suitable production method is essential to develop AMPs as novel pharmaceuticals. Furthermore, heterologous production of AMPs in prokaryotic systems tends to be difficult because AMPs are toxic to prokaryotic cells. In this section, we have reviewed several strategies to reduce the toxicity for host cells and obtain large amounts of AMPs. Fusion expression using solubility-enhancing carriers is the most popular strategy to obtain a high yield of AMPs. Another production strategy is the use of aggregation-promoting carriers. The production

of insoluble AMPs could mask their toxic activity and protect them from degradation by host proteases. Furthermore, expression of insoluble AMPs enables their quick purification using a simple centrifugation procedure (Aoki & Ueda, 2013).

In recent years various nanotechnological solutions have been optimized to improve antimicrobial activity. Nanotechnology is the creation of systems or materials on the nanometer scale, be able to exploit the high surface area to volume ratio and the intrinsic unique physico-chemical-properties to generate new classes of antimicrobial agents. Lipids, liposomes, polymers, micelles, nanocapsules and other colloidal drug delivery systems of dimensions up to a few hundred nanometers can be loaded with AMPs to deliver AMPs to infected sites. In this case, the result obtained can be in high fold due to the increased concentration to the site of the infection at lower doses, and the intracellular delivery.

2.6 SOURCE OF AMPs

So far more than 5000 different AMPs have been identified in various organisms ranging from prokaryotes, insects, plants and animals including humans (Table 2.1). Two major methods employed for peptide discovery were: a combination of chromatographic approaches and genomic and proteomic approaches. The proteomic approach has the prospective of identifying a large number of peptides. In 2014, 104 new antimicrobial peptides having known sequences were registered in the APD database (Wang & Wang, 2004; Wang *et al.*, 2009) and is comparable to those annual total reports of peptides (over 100) collected into the APD since 2000 (Wang, 2014). Unique peptides from various life kingdoms are emphasized in the following sections prove that it is likely to identify novel antimicrobial peptides from unexplored organisms.

Table 2.1 Various sources of AMPs (Pushpanathan, *et al*., 2013)

Source	AMPs
Insect	Cecropin A, Sarotoxin IA, ponericin G2, ceratotoxin, stomoxyn, spinigerenin, thanatin, heliomicin, Alo3, sapecin, defensin A, smD1, gallerimycin, termicin, royalisin, drosomycin, drosocin, metchnikowinapidaecin IA, abaecin, formaecin, lebocin, pyrrhocoricin, melittin, attacins, coleoptericin,diptericin
Amphibians	Japonicin-1 & 2, nigrocin 1 & 2, brevinin-20a, temporin-1Od, tigerin-1, pseudin-2, maximin-1, distinctin
Echinoderms	Strongylocins, centrocins, betathymosins, filamin A
Crustaceans	Callinectin, astacidin 2, armadillidin, homarin, scygonadin, penaeidin, crustin, hyastatin, arasin, stylicin, hemocyanin derived peptides
Plants	Thionins, plant defensins, lipid transfer proteins
Mammals	Defensin, histatin, LL-37, indolicidin, protegrin, lactoferricin
Bacteria	Iturin, bacillomycin, syringomycin, syringostatins, syringotoxins, nikkomycins
Fungi	Echinocandins, aculeacins, mulundocandins, FK463, aureobasidin, leucinostatins, helioferins
F ishes	Pardaxins, misgurin, pleurocidins, parasin, oncorhyncin II and III, chrysophsin and HFIAP

Microbes have an extraordinary array of defense systems including antimicrobial peptides like bacteriocins. The most promising antimicrobial peptides are produced by lactic acid bacteria (LAB) and most of them are non-toxic to eukaryotic cells and generally recognized as safe substances (GRAS), being active in the nanomolar range (Cotter *et al*., 2005; Peschel & Sahl, 2006). Gram-negative bacteria frequently produce relatively large HDPs (often >50 residues) and gram-positive organisms produce a broad range of smaller peptides (20–40 amino acids) that share many chemical features (e.g., amphipathicity,

cationicity) with HDPs from eukaryotes. Many of these HDPs are synthesized as precursors with anionic pro-peptide sequences that attenuate these microbicidal agents until they are compartmentalized or deployed. One of the most well characterized groups of gram-positive and ribosomally synthesized antimicrobial peptides are the class I bacteriocins known as lantibiotics (Yeaman & Yount 2003). A novel 5.6-kDa bacteriocin laterosporulin effective against a wide range of gram-positive and gram-negative bacteria, was purified from the culture of *Brevibacillus sp*. strain GI-9 grown under optimal conditions (Singh *et al*., 2012). Baceridin represents a non-ribosomally synthesized circular peptide with only six amino acids (50% D-amino acids) from a plant-associated *Bacillus* strain (Niggemann *et al*., 2014). This peptide has the ability to inhibit cell cycle progression and causes apoptosis in cancer cells independent of p53. Lassomycin was found to act against *Mycobacterium tuberculosis* by binding to ATP-dependent protease ClpC1P1P2 (Gavrish *et al*., 2014). Another peptide, sonorensin was obtained from a marine bacterium *Bacillus sonorensis* MT93 having broad activity spectrum against both Gram-positive and Gram-negative bacteria (Chopra *et al*., 2014).

Several AMPs have been discovered from various insects. Since the discovery of inducible AMPs in the moth *Hyalophora cecropia* more than 150 such peptides have been identified. These peptides are called cecropins (Moore *et al*., 1996). They are 3- 4 kDa linear amphipathic peptides and active against protozoa, metazoan parasites, bacteria and fungi. Insect defensins were initially isolated from cell cultures of the *Sarcophaga peregrine* and from challenged larvae of *Phormia terranovae*. Since then, a large number of insect defensins have been characterized from various species.

Apart from insects, other invertebrates from various phyla provide rich sources of antimicrobial peptides. Tachyplesins are 17-18 amino acid long peptides isolated from the haemocytes of the crab, *Tachypleus tridentatus*

identified by Nakamura, (1988). Tachyplesin inhibits the growth of gram-negative, gram-positive bacteria, marine bivalve pathogens *Bonamia ostreae*, *Perkinsus marinus*, and *Vibrio* P1 (Morvan *et al*., 1997). In 1972, bactericidal activities were observed in plasma hepatopancreas of the lobster *Homarus americanus* (Stewart & Zwicker1972). The clavanins, a family of four α -helical, amphipathic histidine-rich antimicrobial peptides that contain 23 amino acids and exhibit C-terminal amidation was isolated from the hemocytes of the ascidian *Styela clava*. The presence of antimicrobial activity in *Mollusca* has been studied from the mucus of the giant snail *Achatina fulica*, from the egg mass and purple fluid of the sea hare *Aplysia kurodai*, and from the body wall of the sea hare *Dolabella auricularia (*Tincu & Taylor, 2004*)*.

Defensins were first discovered in rabbit and guinea pig granulocytes as small cationic antimicrobial peptides. Now known as β defensins, these are small peptides with 29–35 residues with three intramolecular disulfide linkages. Humans are known to have six β-defensins viz. four human neutrophil peptides (HNP 1–4) expressed by granulocytesand certain lymphocytes (Reddy *et al*., 2004). Cathelicidins (Propeptides) occur in the neutrophils of cows, pigs, sheep, rabbits, mice and humans (Ganz & Lehrer, 1998). Recently in 2014, it was discovered that some known human peptides or proteins were demonstrated to be antimicrobial. These peptides comprise human α-defensin 6 (HD-6), β-defensin 120 (DEFB120), chemokine CCL24 (eotaxin-2), CCL26 (eotaxin-3) and human ribonuclease 6 (RNase 6). HD-6 is active against *Bifidobacterium adolescentis* (Schroeder *et al*., 2015), while recombinant DEFB120 is active against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* (Liu *et al*., 2014). Another class of known chemokines such as eotaxin-1 (CCL11), eotaxin-2, and eotaxin-3 are also active against the airway pathogens *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* (Gela *et al*., 2015). Besides, human RNase 6 is inducible and shows activity against uropathogens,

highlighting its defense role in the urinary tract (Becknell *et al.*, 2015). These characterized members further expand the known reservoir of human host defense peptides and proteins (Wang, 2014).

Like AMPs of animal origin, the molecular diversity of AMPs from plants is striking (Padovan *et al.*, 2010). Plant AMPs were assigned to different classes according to their tertiary structures they are: thionins, defensins, and lipid transfer proteins. The first AMP isolated from plants was a thionin, from the endosperm of wheat (Balls *et al.*, 1942). The protein moiety of a proteolipid was later shown to be a mixture of two forms of purothionins A and B (Nimmo *et al*., 1968). Additional thionins were isolated including hordothioninsA and B from barley endosperm, viscotoxins and phoratoxins from mistletoe species. Hispidalin is an antimicrobial peptide from winter melon *Benincasa hispida* (Sharma *et al*., 2014).

2.7 FUNGI AS A SOURCE OF AMPs

Peptides of fungal origin have involved special attention due to their multifarious applications in the pharmaceutical and food industries. Filamentous fungi encompass an important class of organisms of significant commercial relevance, even though they are less attended than their lower eukaryotic relatives, such as yeasts. For example, in the biotechnology industry, filamentous fungi are used to produce a wide variety of products ranging from human therapeutics (e.g. antibacterial agents) to specialty chemicals (e.g. commercial enzymes, organic acids), which together represent billions of dollars in annual sales. Filamentous fungi are valuable sources of small antifungal peptides.

Trichorzianines- A represents nine membrane-active peptaibols were isolated from *Trichoderma harzianum*. They are characterized by an acetylated N-terminal amino acid, and L-tryptophanol or L-phenylalaninol at the C-terminal. They also possess seven to eight alpha-aminoisobutyric acid residues and isovaline residue in the D-configuration (Hajji *et al.*, 1987). Two nonadecapeptide

peptaibols, tricholongins BI and BII having both antifungal and antibacterial activities were isolated from *Trichoderma longibrachiatum* cultures. They have mostly hydrophobic L-amino acids, eight to nine 2-aminoisobutyric acid residues, an acetylated N-terminal residue, and a C-terminal leucinol. Both peptides adopt a similar alpha-helical conformation with a hinge around Pro13 resulting from two 3(10) bonds suggest that the N-terminus contains mixed alpha/3(10) bonds (Rebuffat *et al*., 1991). Another four nonadecapeptide peptaibols with antibacterial and antifungal activities, designated as chrysospermins A, B, C, and D from *Apiocrea chrysosperma* mycelia and Ampullosporin was isolated from *Sepedonium ampullosporum* mycelia with narrow-spectrum antibacterial and antifungal activities (Dornberger *et al*., 1995; Ritzau *et al*., 1997). Two linear 19-amino-acid peptaibols, septocylindrin A and septocylindrin B, with a modified phenylalanine C-terminus related to the well known membrane-channel-forming peptaibol alamethicin, were isolated from an isolate *Septocylindrium* sp. The 18th residue in septocylindrin A was Glu where as in septocylindrin B, the residue was Gln (Summers *et al*., 2007). A mixture of peptaibols, 20-residue antifungal peptides with N-terminal acetylation and C-terminal phenylalaninol, was present in liquid culture of a cork oak endophytic strain of *Trichoderma citrinoviride* showed remarkable toxicity in a brine shrimp bioassay (Maddau *et al*., 2009). Trichogin GA IV from the fungus *Trichoderma longibrachiatum* is a lipopeptaibol with antibacterial activity towards gram-positive bacteria, especially methicillin-resistant *S. aureus*. Also it was found to be protease-stable with attenuated hemolytic activity. It exhibits an amphiphilic character by folding in a mixed 3(10)-/α-helical conformation, probably accounting for its ability to perturb bacterial membranes and to induce cell death (De Zotti *et al*., 2012). There is a synergism between the antifungal actions of the enzymes and peptaibols. For example, peptaibols trichorzianines A1 and B1, chitobiohydrolase, endochitinase, and beta-1, 3-glucanase activities were found in culture supernatants of

Trichoderma harzianum mycelia cultivated on glucose as the only carbon source and transferred to fresh medium with *Botrytis cinerea* cell walls. Subsequently the culture supernatants hinder spore germination and hyphal elongation of *B. cinerea* where as culture supernatants from medium in the absence of *B. cinerea* cell walls did not show hydrolase activities, synthesis of peptaibol-like compounds, or suppression of fungal growth (Schirmbock *et al.*, 1994).

Isarfelin, a cyclic peptide with antifungal activity towards the fungi *Rhizoctonia solani* and *Sclerotinia sclerotiorum* and insecticidal activity toward *Leucania separata* but lacking antibacterial activity, was isolated from *Isaria felina* mycelia (Guo *et al*., 2005). The saprophytic fungus *Ulocladium atrum Preuss* produces a cyclopeptolide characterized by an abundance of N-methylated amino acids and potent antifungal activity against *Botrytis cinerea* and a modest activity against *Alternaria alternata* and *Magnaporthe grisea* (Yun *et al*., 2007). Eujavanicin A is a cyclic depsipeptide, which suppresses the growth of filamentous fungus *Aspergillus fumigatus*, was isolated from *Eupenicillium javanicum* (Nakadate *et al*., 2008). The filamentous soil fungus *Coleophoma empetri* F-11899 produces a water-soluble echinocandin type l antifungal lipopeptide FR901379, the original source of micafungin used in treatment of deep-seated mycoses. The mechanism by which FR901379 act against *Aspergillus* and *Candida* species was found to be the inhibition of 1, 3-β-glucan and hence fungal cell wall synthesis (Yamada *et al*., 2009). A cyclic pentadepsipeptide designated as alternaramide, with slight antibacterial activity toward *Bacillus subtilis* and *Staphylococcus aureus*, has been isolated from an ethyl acetate extract of the fungus *Alternaria* sp. SF-5016 (Kim *et al*., 2009). A novel cyclodepsipeptide, cordycommunin, with lethal activity towards *Mycobacterium tuberculosis* H37Ra and weak cytotoxic to KB cells, was isolated from the entomopathogenic fungus *Ophiocordyceps communis* (Haritakun *et al*., 2010).

The publication of plectasin from saprophytic ascomycete *Pseudoplectania nigrella* in 2005 resulted in the addition of fungi to the list of defensin producing organisms, as the last biological kingdom (Mygind *et al.*, 2005) and is strongly active against Gram- positive bacteria such as *Streptococcus* sp. and *Staphylococcus* sp. This led to the assumption that there is a common ancestor of plant, fungal, and invertebrate defensins, further supported by the finding of defensin-like peptides in bacteria (Zhu, 2007). Due to a high efficacy of plectasin *in vivo* against pneumococcal infections in mice, efforts have been done to promote to a commercial drug and entered clinical phase 1 trials. Though it was considered as one of the most promising drug candidates tested, due to commercial and scientific reasons Novozymes and Sanofi-Aventis, both companies involved in the development, decided to quite the studies (Fox, 2013). Following the discovery of plectasin, plectasin-related peptides in fungi have also been reported using bioinformatics approaches (Zhu, 2008). Zhu *et al.* (2012) described 17 fungal defensin-like peptide (DLP) genes from fungal genomes of members mainly from *Ascomycota (*Pezizomycotina) except labisin in *Basidiomycota* (Agaricomycotina) and the detailed characterization of micasin from the dermatophytic fungus *Microsporum canis* using the synthetic peptide. Defensin like proteins with antifungal activity have been isolated and investigated from other ascomycetous fungal species includes *Penicillium chrysogenum*, *P. nalgiovense*, *Aspergillus giganteus* and *A. niger*, further more one similar protein with an antibacterial effect only against some gram-positive bacteria has been found in a zygomycetous *Rhizopus microsporus* var. *oligosporus* (Galgóczy *et al*., 2010). A thermostable peptide with a molecular mass of 5773-Da was obtained from an indigenous fungal strain (VR) of *Aspergillus clavatus* by ultrafiltration followed by reverse phase-HPLC on a C18 column. It inhibits mycelial growth of *Alternaria solani*, *Aspergillus niger, Botrytis cinerea*, *Fusarium oxysporum*, and *F. solani*, but no effect towards yeasts and bacteria (Skouri-Gargouri & Gargouri,

2008). Another *Aspergillus clavatus* ES1 produced a basic 6.0-kDa cysteine-rich thermostable and protease-sensitive antimicrobial peptide designated as AcAMP, possess antifungal and antibacterial activities relevant in biocontrol of plant diseases and food preservation (Hajji *et al*., 2010). A new tetrapeptide D-Phe-L-Val-D-Val-L-Tyr, from a *Penicillium canescens* culture collected from beehive pollen showed antifungal activity against the soybean pathogen *Fusarium virguli-forme* (Bertinetti *et al*., 2009). Penicillium antifungal protein (PAF) from *Penicillium chrysogenum* is a small cationic protein non- toxic to mammalian cells and is thus a remedy candidate for treating the usually fatal *Aspergillus* infections in humans. Its highly conserved and positively charged lysine-rich surface region contributes to the augmented toxicity (Batta *et al*., 2009). *Aspergillus giganteus* secreted a basic, low-molecular-weight antifungal protein as an inactive precursor with a six amino-acid extension at the NH_2-terminal displayed antifungal activity both *in vitro* and *in vivo* (Lopez-Garcia *et al*., 2010). The culture supernatant of *Saccharomyces cerevisiae* CCMI 885 contained antimicrobial peptides with fungistatic effect against *Hanseniaspora guilliermondii, Kluyveromyces thermotolerans,* and *Torulaspora delbrueckii,* and fungicidal activity against *Kluyveromyces marxianus* (Albergaria *et al*., 2010). A 6.6-kDa basic and cysteine-rich antifungal peptide, with homology to similar proteins from *Aspergillus clavatus, A. giganteus, A. niger,* and *Penicillium chrysogenum* was isolated from the culture supernatant of the mold, *Neosartorya fischeri* (anamorf: *Aspergillus fischerianu*s). It is protease resistant and stable within broad pH and temperature ranges (Kovács *et al*., 2011).

Aspergillus giganteus produces a cationic cysteine-rich defensin-like antifungal protein AFPNN5353, which suppresses the growth of filamentous ascomycetes, such as human and plant pathogens and the model organisms *Aspergillus nidulans* and *A. niger* (Binder *et al*., 2011). Fermentation extracts of *Cordyceps heteropoda* (ARSEF #1880) isolated from an *Australian cicada*

yielded two major peptides, cicadapeptins I and I, display antibacterial activity and slight antifungal activity. The cicadapeptins are N-terminal acylated by *n*-decanoic acid and C-terminal amidated by 1, 2-diamino-4-methylpentane (Krasnoff *et al*., 2005). A 44-kDa dimeric antibacterial protein from the wild mushroom *Clitocybe sinopica* exhibits activity against *Agrobacterium rhizogenes, A. tumefaciens, A.vitis, Xanthomonas malvacearum* and *X. oryzae* but not against *Erwinia herbicola, Escherichia coli, Pseudomonas batatae* and *Staphylococcus aureus* (Zheng *et al*., 2010). Also antifungal peptides and proteins have been isolated from mushrooms can be designated as agrocybin, alveolarin, eryngin, ganodermin, hypsin, lyophylin, Lyophyllum antifungal protein, *Hypsizygus marmoreus* antifungal protein, lentin and pleurostrin with an IC50 value at micromolar concentrations (Suzuki *et al*., 2011).

Even though the soil organisms have been greatly exploited for their antibiotics, the skein of intricate factors capable of destructing microbial organisms in marine environments has yet to be completely unravelled. Marine derived fungi have been rich sources of structurally novel and biologically active secondary metabolites which have been striking as vital sources for new chemicals in drug discovery (Xia *et al*., 2007; Li *et al*., 2009). The first report of a bioactive natural product from a marine derived fungus dates back to the identification of cephalosporin C in *Acremonium chrysogenum* in the 1940s, which became the source of the parent compound of modern cephalosporin antibiotics now indispensible for the treatment of numerous bacterial infections (Proksch *et al*., 2008). Ecology refers to the relationship between the organism and its environment. Possibly its spores are washed into the soil from the surface (Burges, 1958), making it a "soil invader" (Garrett, 1955). Alternatively it may be a "soil inhabitant" of specialized substrates. Many biologically active metabolites have been identified and isolated from marine-derived fungi and they have become important sources of new bioactive molecules (Lam, 2007). Numerous marine-

derived fungi actually belong to genera well known in the terrestrial environment, such as *Aspergillus, Penicillium, Cladosporium, Phoma,* and *Fusarium* (Holler *et al*., 2000). A large number of novel compounds, including, alkaloids, peptides, polyketides, terpenes, sterols, and cerebroside analogues have been isolated from this genus and many of these substances exhibit interesting biological properties. On malt agar two new alkaloids, fumigaclavine A and 13 and festuclavine were obtained from a 60 day old culture (Spilsbury & Wilkinson, 1961). Marine-derived fungi continue to play pivotal roles in the developments of antibacterial drugs. Two new cyclic hexapeptides containing both anthranilic acid and dehyroamino acid units, sclerotides A and B, were isolated from the marine-derived halotolerant *A. sclerotiorum*, which was cultured in a nutrient-limited hypersaline medium (Zheng *et al*., 2009). A new tyrosine-derived metabolite, aspergillusol A, was isolated from the marine-derived fungus *A. aculeatus*, and selectively inhibited α-glucosidase from the bacteria (Ingavat *et al*., 2009). Azonazine (107), a unique hexacyclic dipeptide, was isolated from a sediment derived strain (Wu *et al*., 2010). Two cytotoxic lipopeptides, fellutamides C and F were isolated from the sponge-derived strain *A.versicolor* (Lee *et al*., 2010). A known compound N-acetyl tyramine was isolated from marine sediment derived *A. fumigatus* and found to be cytotoxic to K562 cells (IC50, 17.4 μM) (Zhao *et al*., 2010). A cyclic tetrapeptide asperterrestide A was isolated from fermentation broth of a marine-derived fungal strain *Aspergillus terreus* SCSGAF0162 (He *et al*., 2013).

2.7.1 GENUS *ASPERGILLUS*

Micheli in 1729 noticed that the pattern of the conidial head of *Aspergillus*, resembled an aspergillum (a brush or perforated globe used for sprinkling holy water) with its spore heads radiating from a central structure. For that reason, he named the genus as *Aspergillus*. *Aspergillus* is aerobic mold

species, which are found in almost all oxygen rich environments. Many species of *Aspergillus* demonstrate oligotrophy; that is, they are capable of growing in nutrient depleted environments or environments completely lacking key nutrients. In addition to producing numerous useful extracellular enzymes and organic acids, these molds also produce secondary metabolites important in biotechnology (Bennett, 2009). Several *Aspergillus* secondary metabolites have major economic importance of which the statins and their derivatives are most beneficial (Tobert, 2003). These cholesterol lowering drugs are now among the mostly widely used medicines like the first identified statin, mevastatin from *Penicillium citrinum*, was discovered by Endo and colleagues. The first statin approved for human use was lovastatin, a secondary metabolite discovered from *Aspergillus terreus* (Alberts, 1998). The statins are merely a family of useful, biologically-active secondary metabolites isolated from *Aspergillus*. Other compounds with pharmacological relevance include cholecystokinin and neurokinin antagonists, ion channel ligands, antifungal drugs and hosts of other compounds. Despite the fact that some *Aspergillus* species are able to cause diseases in humans and animals, some species is very important in commercial microbial fermentations.

The classification and identification of Aspergillus has been based on phenotypic characters but in the last decades it was strongly dependent on molecular and chemotaxonomic characterization. Morphological identification of *Aspergillus* mostly correspond to the protocols of Raper & Fennell (1965), Klich, (2002), Pitt & Hocking (2009) and Samson *et al*. (2010), where as molecular tools for phylogenetic species recognition, are increasingly being used with the internal transcribed spacers of the nrDNA (ITS) and accepted as the official DNA barcode for fungi (Schoch et al. 2012). The initial studies on the phylogeny of *Aspergillus* and related genera often used a limited number of strains and phylogenetic markers. The possible monophyly of *Penicillium* was studied using ITS and 18S rDNA sequences with a dataset of 17 strains (Berbee *et al*., 1995) and observed

that *Eupenicillium javanicum* (= *Penicillium javanicum*), *Monascus purpureus*, *Neosartorya fischeri* (= *A. fischeri*), *Eurotium rubrum* (= *A. ruber*) and *A. fumigatus* form a well-supported clade (98 % bootstrap value, bs) indicating the close relationship among these species. Moreover, *A. ruber, A. fumigatus* and *A. fischeri* were placed together on a branch with moderate statistical support (77 % bs), demonstrating that *Aspergillus* is monophyletic. When the phylogenetic study was done based on 18S rDNA data, similar results were obtained as *E. rubrum, N. fischeri and A. fumigatus* also formed a well-supported clade (99 % bs), distinct from *Penicillium* and *Monascus* (Ogawa et al., 1997). Tamura et al. (2000) also determined the relationships within *Aspergillus* using 18S rDNA. Based on the recent 25-gene phylogeny, *Penicillium* and *Aspergillus* are divided in to two dissimilar well supported genera, strongly suggesting the monophyly of both genera (Houbraken *et al*. 2014).

2.7.2 *ASPERGILLUS FUMIGATUS*

Fresenius in 1863 first described about *Aspergillus fumigatus*. These molds regularly inhabit the soils and the cultures can be obtained from any samples which are contaminated. Thom & Raper, (1945) regard it as "an extremely cosmopolitan mold which occurs with particular frequency in soil containing appreciable organic materials, upon vegetable matter undergoing slow decomposition and upon imperfectly dried, stored grains". Colonies of some strains strictly velvety, in others with varying amounts of tufted aerial mycelium up to felted floccose forms, green to dark green, becoming almost black in age, spreading. Conidiophores short, usually densely crowded, up to 300 µm long by 2-8 µm in diameter, frequently more or less green colored, especially in the upper part, arising directly from submerged hyphae or as branches from aerial hyphae, septate or un-septate, gradually enlarged upward, with apical flask-shaped vesicles up to 20-30 µm in diameter, fertile only on the upper half, bearing sterigmata in

one series, usually about 6-8 µm, crowded, closely packed with axis parallel to axis of the stalk; chains of conidia form solid columns up to 400 µm by 50 µm, but usually much shorter; conidia dark green in mass, globose, 2-3.5 µm mostly 2.5-3 µm in diameter.

Aspergillus fumigatus is a common environmental isolate and a true saprobic fungus which turns pathogenic only when the human defense reactions get very weakened, for example, in the terminal stage of AIDS infection or as a result of heavy immunosuppressive therapies. *A. fumigatus* appears taxonomically homogeneous when considering morphology. On the contrary, intraspecies variability at the genomic level appears to be very high (Debeaupuis *et al*., 1997). Nevertheless, a number of bioactive compounds such as dioxopiperazine, alkaloids, dibenzofurans, and indole diketopiperazine have been isolated from *Aspergillus fumigatus* (Li *et al*., 2012*)*. Six new prenylated indole diketopiperazine alkaloids, spirotryprostatines C-E, two fumitremorgine B derivatives, and 13-oxoverruculogen, were isolated from the holothurian-derived strain *A. fumigatus*. These compounds were evaluated for their cytotoxicities (Afiyatullov *et al*., 2005; Wang *et al*., 2008). Diketopiperazine alkaloids 65–67, which were isolated from a mud-derived strain of *A. fumigatus*, exhibited significant cell growth-inhibitory activities against the U937 cell line, with IC_{50} values of 1.8, 0.2, and 0.5 µM, respectively (Wang *et al*., 2012). Strains of *A. fumigatus* produced fumagillin, fumitremorgins, fumiquinazolins, gliotoxin, pseurotins, trypacidin and verrucologen. Pyripyropens, tryptoquivalins and tryptoquivalons are produced by *A. fumigatus, A.lentulus* and *A. novofumigatus*. *A. fumigatiaffinis* and *A. novofumigatus* shared an unknown compound with a characteristic UV spectrum and cycloechinuline (Hong *et al*., 2005). Most *A. fumigatus* strains produced fumagillin, fumigaclavines, fumitremorgin A, B, C and TR-2, fumiquinazolins, gliotoxin, helvolic acid, pseurotins, pyripyropens, trypacidin, tryptoquivalins, tryptoquivalons and verrucologen consistent with

literature data (Cole & Cox, 1981). Successful therapeutic use of fumagillin has been reported for topical application in cases of microsporidial keratoconjunctivitis in humans (Grossnikiaus *et al.*, 1993).

2.8 OPTIMIZATION OF CULTURE CONDITIONS FOR MAXIMUM PRODUCTION

To find novel compounds with promising bioactivities, many high-cost methods such as high-throughput screening of different biological sources have been employed (Grabley &Thiericke, 1999; Maier *et al.*, 1999). Alternatively, an effective screening process can be achieved through systematic manipulation of culture conditions for a small number of promising organisms. As a part of optimization of the production medium, the influence of physical and chemical parameters on the production needs to be analyzed (Zhang *et al.*, 1996). In fact, culture conditions have a major impact on the growth of microbes and the production of microbial products. As far as culture conditions are concerned, there is usually a dilemma between achieving maximal growth rates and maximal yields because conditions that allow fast cell growth could be unfavorable to metabolite production (Audhya and Russell, 1974; Frisvad and Samson, 1991). The yield of bioactive compounds can sometimes be substantially increased by the optimization of physical (temperature, salinity, pH value, and light) (Masuma *et al.*, 2001) and chemical factors (media components, precursors, and inhibitors) for the growth of microbes (Calvo *et al*., 2002; Llorens *et al.*, 2004).

Influence of culture conditions like medium culture, pH, temperature and time of fermentation for the production of antimicrobial metabolites by *Aspergillus fumigatus* was studied by Furtado *et al.* (2005). The effects of temperature were studied by growing fungus *Arthrinium* in GPY medium at 15, 20, 25, 30, and 35°C in the salinity of 34 ppt (Miao *et al.*, 2006). The effects of salinity on mycelial growth and bioactivity have been studied by growing fungal

cultures in in GPY media made of three different salinities, 0, 17, and 34 ppt at 25℃. The effects of pH were studied by growing the fungal cultures in GPY media at seven different pH values ranging from 3.5 to 9.0. The medium was adjusted to acid and alkaline pH by the addition of 50 mM citrate phosphate buffer (pH 3.5 to 6.5) or 50 mM Tris–HCl buffer (pH 7.5 to 9.0), respectively. The final pH values of the medium (mean of three replicates) were 3.68±0.02, 4.35±0.01, 5.40±0.01, 6.27±0.01, 7.41±0.03, 8.24±0.01, and 8.68±0.02 (Miao *et al*., 2006).

The basal medium was inoculated and incubated at different temperatures ranging from 20 to 45^{o}C. To study the effect of pH of the culture medium, the basal medium was adjusted with different levels of acidic and alkaline pH. For the effect of shaking condition culture flasks were incubated at 28^{o}C in a gyratory shaker, 3.3 Hz, at 200 rpm. The effect of prolongation of incubation for maximum growth and antibiotic production was observed up to 15 days of incubation. In addition, to determine the effect of the size of inocula flasks were inoculated separately with spores ranging from $2x10^{5}$ to $2x10^{9}$ spores per milliliter (Thakur *et al*., 2009).

For optimization of production media of the shiitake mushroom *Lentinula edodes* (Berk.) Pegler, different factors like agitation, temperature, and initial pH were analyzed (Hiroko Hassegawa *et al*., 2005). The effects of nutrient concentrations on mycelia growth and bioactivity of *Arthrinium* were examined by adjusting the concentrations of glucose, peptone and yeast extract in media Miao *et al.,* 2006)Various carbon and nitrogen sources may influence the growth and productivity because the capability to use these resources may vary from one organism to other. Glucose, glycerol, mannitol, lactose, sodium citrate, sodium acetate, sucrose, galactose, xylose, starch, arabinose, fructose, maltose and ribose as carbon sources and ammonium chloride, ammonium sulphate, sodium nitrate, arginine, potassium nitrate, asparagine, threonine, glutamic acid, glycine, tyrosine, valine and aspartic acid were used as nitrogen sources, which were supplemented

separately into the basal medium at 1% concentration (w/v) for Streptomyces (Thakur *et al*., 2009).

Similarly in the production of exAP-AO17 by *Aspergillus oryzae* in various media, all liquid cultures were incubated in shaken 500 mL flasks containing 100 mL of medium at 28^{o}C and 150 rpm. *A. oryzae* was cultivated in media containing different carbon sources, nitrogen sources, and ambient pH. Mycelia were harvested after incubation for 72 h in a shaking incubator (150 rpm), and the filtered supernatants were examined on SDS-PAGE gel (Park *et al*., 2008). Upon studying the effect of medium supplementation with divalent metal cations, it is recognized that metals are necessary for the normal metabolism of microorganisms as microelements or trace elements, in addition for the production of secondary metabolites (Halonen *et al.*, 2005).

A major difficulty in peptide or protein research and applications is obtaining accurate quantification using bioassays which are based on the measurement of the inhibition produced in a sensitive microorganism (Turcotte *et al*., 2004). Although various other methods have also been suggested such as ELISA (Suarez *et al.*, 1996; Bouksaim *et al*., 1999) ATP-bioluminometry (Waites & Ogden, 1987), radiometry (Culter *et al*., 1989), conductance measurements (Giraffa *et al*., 1990) or even sophisticated bioassays based on self-induction of the *nis* promoter and bioluminescence (Walstrom & Saris,1999), growth inhibition techniques are still the most commonly used technique for quantitative estimation in everyday trials. The other methods didn't get wide acceptance due to the requirements for dedicated equipment, supplies, and skills and moreover, the results produced by such methods cannot necessarily be correlated with antimicrobial activity.

Based on the multiple procedures for determining growth inhibition which were described in the literatures, it was apparent that the most commonly used method was agar diffusion assay regardless of its inconveniences

and limitations like diffusion-related difficulties of the active substance. The need to eradicate diffusion-related problems associated with the agar techniques, Reeves, (1965) introduced another liquid medium method in a study with colicins. Since then, applications of turbidometric assays can be found in a number of reports which show large variability regarding extraction, general experimental conditions and definition of the activity unit (Papagianni *et al*., 2006).

2.9 PURIFICATION OF AMPs

Purification to homogeneity is an essential requirement for further characterization and property studies of any peptide or protein. There are several reports on purification of antimicrobial and other bioactive peptides from plants, animals and microbes. Antimicrobial proteins can be purified using basic protein purification methods. Frequently utilizing protein purification methods include ammonium sulfate precipitation, ion exchange chromatography, affinity chromatography, gel filtration chromatography and RP-HPLC.

The antifungal peptide AcAFP was purified at homogeneity after three-step purification procedure involved heat treatment, ultrafiltration and RP-HPLC on C18 column (Skouri-Gargouri & Gargouri, 2008). Some studies involve the isolation procedures like ethanol extraction, adsorption on YPR II macropore adsorption resin, ethyl acetate extraction, petroleum ether precipitation, and recrystallization from ethyl acetate (Guo *et al*., 2005). Some antifungal proteins were isolated and purified using ion exchange chromatography on CM-sepharose. Fractions showing antifungal activity were further purified using ultra sphere C18 reversed-phase high-performance liquid chromatography (RP-HPLC) (4.6 mm × 250 mm), which was previously equilibrated with 0.1% (v/v) trifluoroacetic acid in HPLC-grade water containing 5% acetonitrile. Proteins were eluted with a linear gradient of increasing concentration (2%/min) of acetonitrile containing

0.1% (v/v) trifluoroacetic acid (flow rate, 1 mL/min) (Park *et al.,* 2008). C18 columns are used for HPLC of antimicrobial peptides (Al-Bayati., 2009).

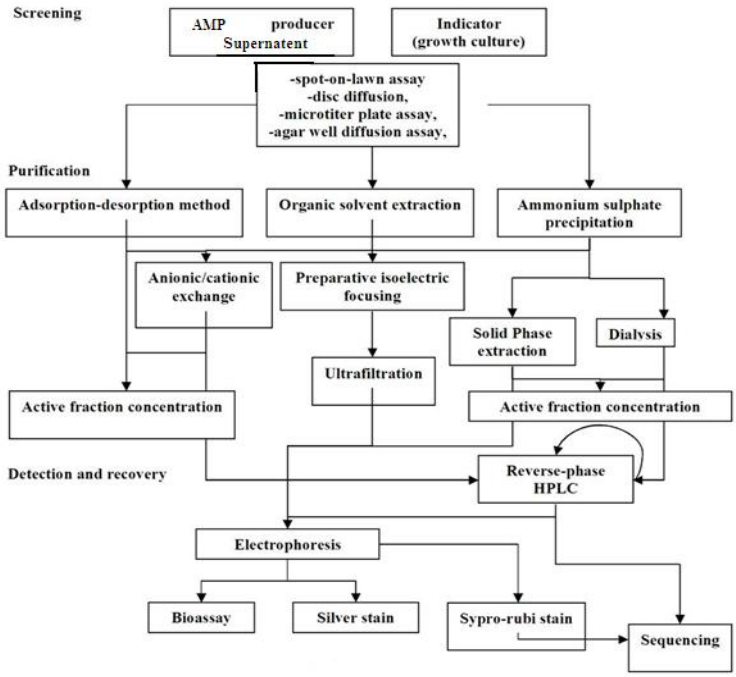


Fig: 2.3 Steps involved in purification of AMPs

2.10 CHARACTERIZATION of AMPs

Homogeneity of the protein preparation is confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid composition analysis as well as by its spectroscopic features (Lacadena *et al*., 1995). The filtered supernatant of *Aspergillus oryzae* containing AP-AO17 was examined on SDS-PAGE. The protein band was identified by staining with Coomassie brilliant blue followed by destaining with 10% acetic acid and further the peptide band can be excised for electroelution for further processing (Park *et al*., 2008). Implication of either SDS-PAGE method by Laemmli (1970), or that modified method by

Schagger & von Jagow (1987) that utilizes the tricine buffer, mainly suitable for the separation of low molecular weight peptides can be used to analyze the partial or purified peptide extracts. After electrophoresis, the peptides can be stained with silver-stain or with the luminescent stain SYPRO-Ruby (Molecular Probes-Invitrogen) or, another recently introduced fluorescent dye, Lumitein (Pingitore *et al*., 2007) other than Coomassie blue stainining.

Inorder to confirm the identity of a peptide, the bioassay method is beneficial to detect the antimicrobial compound in the gel. For that the SDS-PAGE gel is placed on a base agar plate followed by overlaying with soft agar containing the sensitive strain. After appropriate incubation, a clear zone of inhibition will be observed at the site of active peptide. It is very essential to wash the gel before bioassay to remove SDS content inorder to avoid inhibition of the test strain by the detergent itself. So that SDS removal from the gel is possible by pre-treatment with tween 80 (0.5%) and subsequent washes in water as described by Bhunia & Johnson, (1992).

As a part of characterization of purified peptide, molecular mass can be determined by SDS-PAGE and is further affirmed by employing MALDI-TOF mass spectrometry. Peptide mass fingerprinting (PMF) is a method of identifying unknown proteins or peptides by hydrolyzing them with specific proteases (e.g. trypsin) to generate peptides and the masses of protease treatment generated peptides are determined by mass spectrometry (usually MALDI-TOF). Identification is achieved through comparison of obtained masses with available peptide masses from a protein sequence database.

Isoelectric focusing is an electrophoretic method used to determine the isoelectric point (pI) of a peptide/protein. The isoelectric point (pI) is the pH at which a particular molecule or surface carries no net electrical charge (exists as Zwitter ion). Peptides are amphoteric molecules contain both positive and negative charges depending on the functional groups present in it. The net charge

on the molecule is affected by pH of their surrounding environment and can become more positively or negatively charged due to the loss or gain of protons (H^+). At a pH below their pI, peptides carry a net positive charge and they carry a net negative charge when the pH is above their pI. Some of the reported antimicrobial proteins from various filamentous fungi have a pI value greater than 7 (Kobayashi *et al* ., 1992; Lee *et al*., 1999; Wnendt *et al*., 1994; Geisen, 2000; Marx *et al*., 2008; Marx, 2004).

An efficient peptide that can be used for disease treatment should be non-cytotoxic and non-hemolytic (Ryu *et al.*, 2011), thus studies against mammalian cells like human red blood cells (hRBCs) can be used for the cytotoxicity assessment. The cytotoxicities of NRC-16 and melittin were tested against hRBCs (human red blood cells) and the hemolytic activities of them are entirely different because the NRC-16 is inactive till the concentration of 150 μM, whereas melittin showed highest hemolytic activity even at 10 μM concentration (Gopal *et al*., 2013). This is evident that compared to melittin, NRC-16 has antimicrobial activity without a high degree of hemolysis. The presence of optimal balance between cationicity and hydrophobicity, which are key features needed for antimicrobial activity without hemolysis (Javadpour *et al*., 1996; Fernandez-Lopez *et al*., 2001)

2.10.1 Effect of various physico-chemical parameters on peptide stability

If a protein or peptide needs to be applied in any field, the stability of that protein to various physical and chemical parameters should be studied appropriately. Various factors may influence the stability and activity of the proteins such as temperature, pH, salts, metal ions, various oxidizing agents, reducing agents, detergents, amino acid modifiers etc. Other than the effect of these factors on the stability of the product, these may also have influence on the production of the specific protein or peptide.

2.10.2 Effect of pH, temperature and proteolytic enzymes on peptide stability

The proteinaceous antimicrobial compounds get denatured and consequently they lose their activity partially or completely in strong acidic or alkaline conditions. The stability of AFP and PAF against extreme conditions needs to be studied to prove stability of molecule in protecting experiments in common environmental conditions. The particular characteristic of molecules is accounted for their tertiary structures, which are stabilised by disulfide bridges (Vila *et al*., 2001; Moreno *et al*., 2003; Batta *et al*., 2009). AFP exhibits pertinent resistance to proteolysis and a remarkably high denaturation temperature, moreover it is relatively stable in a wide pH ranging from 2 to 12.

Lacadena *et al*. (1995) demonstrated that AFP was not degradated by SV-8 protease, trypsin, pepsin, or hermolysin under nondenaturing conditions and at 37 ^{o}C, although very extreme conditions were used in some cases. Only pronase and proteinase K treatment resulted partial degradation, but peptides remained bounding by the disulfide bridges (Vila *et al*., 2001). In an earlier study PAF proved to be stable over the pH range 1.5-11. A decrease of ~20 % antifungal activity of the protein was occured after treatment with 60-80^{o}C temperatures for 10-60 min. Significant reduction of the protein activity corresponded to the exposure time was observed in extreme temperature conditions (60 min at 95-100 ^{o}C). Protein digestions with Pepsin, proteinase K, pronase for 3-9 h did not have an effect on the activity of PAF, however long exposure to pronase and proteinase K (12 and 24 h) reduced the protein activity significantly. This inactivation was accompanied by protein degradation (Batta *et al*., 2009).

2.10.3 Effect of surfactants (detergents) on peptide

Detergents (cationic, anionic, zwitterionic and non-ionic) are used for solubilizing proteins from lipid membranes and also for improving the solubility of certain proteins without compromising the native structure of proteins

(Cardamone *et al*., 1994). Triton X-100, Tween 20 and Tween 80 are nonionic polyoxyethylene detergents and the major interaction with proteins is hydrophobic (Palacios *et al.*, 2014). Sodium dodecyl sulfate (SDS or NaDS) and CTAB (Cetrimonium bromide or Hexadecyltrimethylammonium bromide) are anionic and cationic detergents respectively. The antagonistic activity of plantaricin OL15 produced by *Lactobacillus plantarum* OL15 was greatly reduced when treated with SDS whereas triton X-100 and tween 20 completely inhibited the activity (Mourad *et al.*, 2005). It was observed in another study that tween 80 and SDS increased the activity while triton X-100 lowered the activity of plantarcin SR18 produced by *Lactobacillus plantarum* SR18 (El-Shouny, 2013).

2.10.4 Effect of reducing agents, oxidizing agents and metal ions

Proteins get oxidized when they are exposed to oxidizing agents like hydrogen peroxide (H_2O_2), periodate, dimethyl sulfoxide, chloramine-T, *N*-chlorosuccinamide and *in vivo* in response to oxidants released by neutrophils (*e.g.* superoxide, hydroxyl radical) (Brot & Weissbach, 1983; Vogt, 1995). Oxidation of methionine residues can cause a decline in the biological activity of the protein as observed that the specificity pattern of chymotrypsin was altered significantly upon oxidation of methionine to its sulfoxide (Weiner *et al*., 1966). Reducing agents which are able to act up on any disulfide bonds to split it apart include β-mercaptoethanol, dithiothreitol (DTT) etc. Because of their ability to disrupt the structure of proteins, reducing agents can be used in the analysis of proteins, for instance, to ensure monomeric, dimeric or higher order oligomeric nature of proteins.

2.10.5 Effect of metal ions on stability of peptide

Because the interaction of peptide and the bacterial membrane was the critical step for the destruction of bacteria, the presence of cations could prevent

the peptides from interacting with the membrane and subsequently hinder the capability of peptides to kill bacteria. In higher salt concentrations, it has been established that reduction of the available head group area for the lipids leads to tight packing of the lipids, which might indicate that the lipid bilayer with low salt concentrations facilitates partitioning or folding of peptide (Kandasamy & Larson, 2006). Many cationic antimicrobial peptides including β-defensins and the α-defensin HD-5 (Hoover *et al*., 2003; Tomita *et al*., 2000), lactoferricin B (Bellamy *et al*., 1992), histain 5 (Helmerhorst *et al*., 1999), human cathelicidin LL-37 (Cox *et al*., 2003), protegrins (Miyasaki *et al*., 1998) and pleurocidin (Cole *et al*., 2000) are salt sensitive, displayed reduction or lose their antimicrobial activity at high salt concentrations. The antibacterial efficacy of lactoferricin B was reduced when treated with Na^{+}, K^{+}, Mg^{2+} or Ca^{2+} ions, or in the presence of various buffer salts. Moreover heavy metal salts act to denature proteins in the same manner as that of acids and bases. As salts are ionic in nature they disrupt salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt.

2.10.6 Effect of chemical amino acid modifiers on stability of peptide

Identification of specific amino acids involved in the biological activity of proteins elucidates the relationship between its structure and the role played by amino acid side-chains in its activity. The use of amino acid side chain reactive reagents is a comparatively simpler approach rather than single crystal X-ray diffraction method and site directed mutagenesis (Gote *et al*., 2007) to know the reactive groups involved in the interaction. The chemicals like *N*-Bromosuccinanmide (NBS), phenylmethylsulfonyl fIuoride (PMSF), diethylpyrocarbonate (DEPC), trinitrobenzene-sulfonic acid (TNBS), and iodoacetamide are usually employed for this purpose. Cysteine and glutamic/aspartic acid modifications resulted in suppression of the anti-fungal

activity of CPI (Joshi *et al*., 1999). Following chemical modification of arginine, tyrosine, cysteine, aspartate, glutamate, lysine and tryptophan residues of purified *Ganoderma lucidum* lectin, it was observed that there is an involvement of two lysine and one tryptophan residues per monomer of lectin for its sugar binding activity.

2.11 APPLICATIONS OF AMPs

Almost all AMPs show direct bactericidal or bacteriostatic functions against several gram-negative and gram-positive strains. The serious problems caused by drug resistant bacteria have created an urgent need for the development of alternative therapeutics. In this scenario, AMPs can be considered as promising antimicrobial agents for producing new generation antibiotics. AMPs are attractive targets as novel antibiotics because of their broad-spectrum activity, including drug-resistant bacteria. Since the isolation of magainins from frog skin in 1987, there have been many attempts to develop antibiotics from the natural AMPs. However, in spite of the efforts over more than two decades, there is no AMP agent currently approved by Food and Drug Administration. They target bacterial cell membranes and cause disintegration of the lipid bilayer structure. The majority of these AMPs are amphipathic with both hydrophilic and hydrophobic domains. This structure provides AMPs the capability to bind to lipid components (hydrophobic region) and phospholipid groups (hydrophilic region). A few of the AMPs kill bacteria by inhibiting some important pathways inside the cell such as DNA replication and protein synthesis. For example, buforin II can diffuse into cells and bind to DNA and RNA without damaging the cell membrane (Uyterhoeven *et al*., 2008).

2.11.1 Biofilm inhibitory peptides

Biofilms are becoming a widespread problem in hospitals and healthcare facilities. Indeed, the United States National Institutes of Health found that the main reason behind 80% of chronic infections is microbial biofilm (Monroe, 2007). Moreover, many studies have established that biofilms are associated with dental plaque (Hojo *et al*., 2009; Paju &Scannapieco, 2007), endocarditis (Palmer, 2006), lung infection (Wagner & Iglewski, 2008; Lau *et al*., 2005), and infection through medical devices (Khardori & Yassien, 1995). A biofilm is a complex consortium of microorganisms existing as communities that exhibit a wide range of physical, metabolic and molecular interactions. These interactions facilitate the attachment, growth and survival of species as a biofilm layer to develop and persist in what often appear to be hostile environments such as the oral cavity. This particular community life-style provides prospective benefits to the resident microorganisms, including a broader habitat range for growth, increased metabolic diversity and efficiency, and enhanced resistance to environmental stress, antimicrobial agents and host defenses etc (Marsh, 2004). Antimicrobial peptides are believed to have the potential for use as anti-biofilm agents attributable to their different mechanisms, which include membrane-disrupting action, functional inhibition of proteins, binding with DNA, and detoxification of polysaccharides, lipopolysaccharide and lipoteichoic acid (Sutherland, 2001). For example, certain antimicrobial peptides can be transferred in biofilm EPS through holes or pores formed in the lipid component of the EPS, while others can disperse biofilms. Recently, certain antimicrobial peptides have been found to possess antibiofilm activity that is independent of their activity against planktonic bacteria. For example, a poorly active antimicrobial human LL-37 prevents biofilm formation in *Pseudomonas baeruginosa* (Overhage *et al.*, 2008). Nevertheless, the anti-biofilm activities of AMPs are not absolutely understood, a small number of studies have come up with possible explanations including matrix disruption;

binding of DNA; and altering the expression of biofilm-related genes, such as the production of pili and rhamnolipid, quorum sensing systems, and flagella assembly, and their dual capacity to act on both the cytoplasmic membrane and intracellular targets (Bahar & Ren, 2013; Jorge *et al*., 2012; Hancock & Sahl, 2006).

2.11.2 Peptides in cancer therapy

Even though incredible efforts have been put into the development of new treatments, cancer remains the major cause of death. Chemotherapies are still the principal treatment for cancer in the advanced or metastatic stages despite their severe side effects to normal cells and tissues, and the easy development of multi-drug resistances (Siegel *et al*., 2014). Therefore, the development of new cancer drugs with low toxicity to normal cells and a new mode of mechanism that can circumvent multi-drug resistance may give a new direction for anticancer therapy. The outer membranes of cancer cells have been reported to possess more negative charge than normal cells due to the presence of molecules like phosphatidylserines, negative glycoproteins, and glycosaminoglicans (Giuliani *et al*., 2007; Raz *et al*., 1980; Utsugi *et al*., 1991). Cationic antimicrobial peptides due to their cationic and amphipathic features, able to bind cancer cells by electrostatic interactions, and hence direct towards cytotoxicity of cancer cells with either necrosis or apoptosis phenotype (Buri *et al*., 2013; Gaspar *et al*., 2013; Hu *et al*., 2011; Papo *et al*., 2006; Sinthuvanich *et al*., 2012; Ting *et al*., 2014). Although many cationic antimicrobial peptides are discovered to have effective anticancer activity with better solubility and lower cost, there are several challenges, such as salt sensitivity in physiological conditions, high toxicity to normal cells, and susceptibility to proteolytic digestion, constraining their further applications (Wu *et al*., 2014). Several methods have been reported to surmount these problems, including D-form amino acid substitution, fusion with functional

peptides, and conjugation with chemotherapeutic agents (Chu *et al*., 2015). Cecropin A and B are able to lyse different types of human cancer cells at peptide concentrations that are not damaging to normal eukaryotic cells. Additionally, the combination of cecropin A and the conventional chemotherapeutic agents such as 5-fluorouracil and cytarabine, at certain doses, shows a synergistic cytotoxic effect on CCRF-SB human lymphoblastic leukemia cells (Hoskin & Ramamoorthy, 2008).

As the environmental factors and life style contributes to occurrence of so many diseases and cancer/tumours are also increasing in the population and a need of effective anticancer/tumour agents are also growing. A synthetic TH2-3 peptide and epinecidin-1 acted as potential antitumor agents after external application against human fibro sarcoma cells. TH2-3 and epinecidin-1 mediated tumor cell lysis, inhibition of cell proliferation and reduction in migration of tumor cells, which was demonstrated under in vitro conditions in human fibro sarcoma cells (Chen *et al*., 2009). AMPs with antitumor functions should act on tumor cells, but should not be detrimental to normal cells. This can be a promising approach in cancer research. In addition, an antitumor function against human leukemia U937 cells and an anti-necrotic function of epinecidin-1 were reported under *in vitro* conditions (Lin *et al.*, 2009).

2.11.3 Antioxidant peptides

Numerous publications point out that the most important problem associated with living organisms is posed by uncontrolled production of reactive oxygen species (ROS), which are very active byproducts having one or more unpaired electrons. ROS are generated during the normal cellular metabolism or as an outcome of exposition to some stress factors, such as changes in temperature, and the presence of metal ions or redox-cycling xenobiotics (Wu & Hansen, 2008; Fan *et al.*, 2012). As a protection system for maintaining a balance between the

production and inactivation of ROS, living organisms have evolved specific defense mechanisms, consisting of enzymatic and nonenzymatic antioxidants (Jamieson, 1995). An increase in the level of free radicals without any control can cause damage to numerous cellular components including DNA, proteins, rmembrane lipids, leading to many serious human diseases and disorders, such as atherosclerosis, coronary heart disease, cancer, impaired immune function or aging processes (Halliwell *et al*., 1997; Wu & Hansen, 2008; Thetsrimuang *et al*., 2011). This outcome suggests that, antioxidants having capacity to scavenge free radicals may protect organisms from oxidative stress-caused damage. A kind of active protein from curry leaves, with a molecular mass of about 35 kDa, had many kinds of antioxidant activities, such as inhibition of lipid oxidation, scavenging of hydroxyl and DPPH free radicals, reduction of ferri ion and cytopigment C (Mylarappa *et al*., 2008). Antioxidant peptides have also been identified and purified from sweet potato protein hydrolysates prepared from alcalase and obtained via trypsin hydrolysates of Tilapia frame proteins (Agyei & Danquah, 2012).

2.11.4 Antiviral peptides

Antiviral effects of AMPs have described in many studies. Some studies suggest a preferable mechanism of action of AMP as they integrate into the viral envelope or to the host cell membrane (blocking viral receptors). Some antiviral AMPs can prevent viral particles from entering host cells by occupying specific receptors on mammalian cells. AMPs can integrate into viral envelopes and cause membrane instability, making the viruses unable to infect host cells. AMPs can also reduce the binding of viruses to host cells. For example, defensins bind to the viral glycoproteins making herpes simplex viruses (HSV) unable to bind to the surface of host cells (Ganz, 2003). Some antiviral AMPs can cross the cell membrane and localize in the cytoplasm and organelles, causing changes in the

gene expression profile of the host cells, which can help the host defense system fight against viruses or block viral gene expression. For example, NP-1, an AMP from rabbit neutrophils, prevents Vero and CaSki cell lines from infection by herpes simplex viruses type 2 (HSV-2), by preventing the migration of a major viral protein, VP16, into the nucleus.

2.11.5 Antifungal peptides

Effective antifungal antibiotics are a few in numbers so a need for efficient drugs is in the market. Like antibacterial AMPs, antifungal peptides can kill fungi by targeting either the cell wall or intracellular components; however, bacterial membrane and fungi cell wall have different contents. For example, chitin is one of the major components of fungal cell walls and some of antifungal peptides are capable of binding to chitin. Such binding capability helps AMPs to target fungal cells effectively. Cell wall targeting-antifungal AMPs kill the target cells by disrupting the integrity of fungal membranes, by increasing permeability of the plasma membrane, or by forming pores directly. The peptide, piscidin-2, secreted by striped bass disrupts fungal membranes and acts as a fungicide (Sung *et al*., 2008).

2.11.6 Peptides as food preservatives

AMPs have captured much attention for use as food preservatives and they have recently been the subject of extensive reviews (Ibrahim *et al*., 2002). This concern may be due to several reasons: AMPs, being proteins, can be easily digested in the stomach so that it will not lead to residue build-up and some of them (e.g., lactoferrin and other bioactive peptides) may even contribute to probiotic effects, including prevention and treatment of diseases. AMPs tend to produce less undesirable effects on the sensory characteristics of food, compared with other antimicrobial systems such as phenolics or oils (Gould, 1996). AMPs

show rapid killing action, highly selective toxicity and low potential for development of resistance (Hancock & Scott, 2000). AMPs could be an alternative method for extending the shelf life of food. However, they would be best utilized for surface treatments or edible coatings and films because, when added as food ingredients, the antimicrobial activity is highly vulnerable to inactivation by other components or conditions within the food systems. If they are to be added to the bulk of the food, appropriate strategies must be designed to ensure their effectiveness. In addition, AMPs can serve as a source for non-contaminated coatings of food packages.

2.11.7 Other applications

Other than the antibacterial, antiviral and antifungal activities AMPs have also exhibited antiparasitic effects. The first antiparasitic peptide reported is magainin, which is able to kill *Paramecium caudatum.* Even though some parasitic microorganisms are multicellular, the mode of action of antiparasitic peptides is the same as other AMPs. They kill cells by directly interacting with cell membrane (Ullal & Noga, 2010).

In addition to direct destruction of pathogens, peptides enter a host cell or bind to membrane receptors of host cells. The recent reviews suggest that, deficiency of defensins results in Crohn's disease and Kostmann's syndrome. Modulation of pathogen responsive genes and protein expressions may reduce the severity of or enable the complete protection against pathogen infections. In addition, treatment of pathogens with AMPs induces adaptive immunity and reduces the severity of pathogen re-infection (Kindrachuk & Napper, 2010).

Adjuvants are used to boost the less-immunogenic potential of subunit vaccines or protein antigens (Kovacs *et al.*, 2009). Certain AMPs act as inducers of proinflammatory cytokines like TNF, COX-2, and IFN. Antigen-specific immunity which is achieved by promoting differentiation of particular cell lines, such as dendritic cells (DCs), as well as modulating cytokine expressions alters

the switch between Th1 and humoral Th2 polarization of adaptive immune responses. This property is exploited by adjuvants, and focused research with AMPs should contribute to the search toward safe alternative vaccine adjuvants (Kovacs *et al.*, 2009).

The other application of AMPs is the direct antimicrobial functions of AMPs. AMPs can be used to inactivate pathogens which then can be used as vaccines against specific pathogens. The widely used formalin-based inactivated vaccines are associated with problems of allergic reactions. Hence finding risk-free alternative compounds to produce inactivated vaccines can overcome the undesirable side effects of formalin. (Rajanbabu & Chen, 2011)

Apart from antimicrobial activities few peptides have been explored for their contraceptive potential. Spermicidal activity of magainins in both in vitro and in vivo conditions has been reported. Reports of the contraceptive efficacy of nisin both *in vitro* and *in vivo* are also in the literature. Nisin showed a time and dose dependent effect on sperm motility. A concentration of 300–400 μg/mL was found to be sufficient to inhibit human sperm motility with in 20 sec *in vitro* (Aranha *et al.*, 2004*). In vivo* studies in rabbits indicated that vaginal administration of 1mg of nisin stopped sperm motility completely and none of the treated animals became pregnant.

AMPs, nonlytic cell-penetrating property were used as drug delivery vector to treat and manage several diseases. Certain large hydrophilic drugs cannot easily penetrate through the cell membrane barriers. In such cases, AMPs with efficient membrane translocating property, which could enter the cells without causing damage to the membranes, were used as drug delivery vectors. The main feature of AMPs to serve as delivery vector is that they should be able to penetrate the cell membrane at very low concentrations without any specific receptors. They are capable of efficiently delivering electrostatically or covalently bound biologically active cargoes such as drugs into the cell interior. Antibacterial

peptides such as LL-37, TP10, and pVEC were associated with bacterial membrane damage shown to act as cell penetrating peptides (CPPs) without exhibiting toxicity to eukaryotic host cells (Jarver & Langel, 2006).

Most HDPs (host defense peptides) are involved in modulation of immune response as host defense and also act as modulators of signal transduction pathways by influencing the activity of intracellular signaling targets such as protein kinases. Defensins were known to involve in host cell receptor interaction, chemo attractant of immune cells, recruitment of neutrophils, mobilization of immunocompetent T-cells as well as enhancer of cell adhesion, and activation of classical complement pathways (Steinstraesser *et al*., 2011).

AMPs have important applications in plant biotechnology. Antimicrobial activity and their molecular studies may give insight into the active genes and this can used to produce plants which are resistant to specific bacteria and fungus. The AMPs such as D4E1, esculestin, MSI-999, human lactoferrin , shiva-1, and SB-37 have been reported to be expressed in plant systems such as tobacco, banana, and potato for the production of pharmaceutical peptides and to develop transgenic plants that confer resistance to several plant diseases (Tripathi *et al*., 2004).

In the light of the review, current research was focused on the production, purification and characterization of novel antimicrobial peptides with a potential therapeutic value from fungal source. The knowledge and understanding of the new peptides will be of great relevance in the development of new antibiotics, host defence and / or immunomodulatory agents in humans, and thereby augment endogenous antimicrobial activity.

3. MATERIALS AND METHODS

3.1 SCREENING FOR ANTIBACTERIAL PEPTIDE PRODUCING FUNGI FROM TERRESTRIAL AND MARINE SEDIMENT SAMPLES

3.1.1 SAMPLE COLLECTION

Humus rich soil samples from terrestrial environment were collected in sterile polythene bags from central region of Kerala - Ernakulam, and Alappuzha districts and marine sediment samples from onboard the research vessel FORV Sagar Sampada (Cruise No.271). Fungal isolates available in the Immuno Technology Laboratory, Department of Biotechnology, Cochin University of Science and Technology, India were also used for screening.

3.1.2 ISOLATION OF FUNGI

Sediment sample (10 g) collected from sampling sites were suspended in 100 mL sterile distilled water and incubated at 28^{o} C in a rotary shaker (Scigenics, India) at 125 rpm for one hour to get homogeneous soil suspension. Sediment suspensions after incubation were serially diluted in distilled water. Pour plate technique was done by transferring 0. 1 mL of the suspension from each tube on to Mycological agar plate (HiMedia, Mumbai, India) composition given in Appendix 1. The plates were incubated at 28^{o} C for 3-5 days. Fungal colonies obtained on the plates were picked and spotted on to Mycological agar plates to get a monospecific culture. The isolated colonies were streaked on to nutrient agar plates to check for bacterial contamination. Spores from pure colonies were streaked on to Mycological agar slants and were kept at 28 °C for 5 days for sporulation. The sporulated cultures were stored at 4 °C and sub cultured every 45 days.

3.1.3 PRIMARY SCREENING OF FUNGI FOR ANTIBACTERIAL ACTIVITY

Crude culture supernatant was prepared for antibacterial activity assay in primary screening. Direct plate assay method was used in the screening process. Mueller Hinton (MH) agar (HiMedia, Mumbai, India; Media composition given in Appendix 1) was used for the preparation of plates for susceptibility test.

3.1.3.1 Preparation of inoculum

Fungal spores inoculated on Mycological Agar (HiMedia, Mumbai, India) slants were incubated at 28°C (Media composition given in Appendix 1). The fully sporulated slants obtained after five days of incubation were stored at 4°C for short term preservation. For preparing the spore inoculum, sterile distilled water containing 0.01 % (w/v) tween- 80 was added to the five day old sporulated slants and the spores were dislodged into it by gentle pipetting under aseptic conditions. The suspension was appropriately diluted with sterile distilled water containing 0.01 % tween-80 to obtain the required spore count determined by microscopic counting using a cell-counting haemocytometer. One millilitre of this spore suspension containing 2.4 x 10^6 spores was used as inoculum.

3.1.3.2 Preparation of culture supernatant

Czapek-Dox (CD) minimal medium (composition as given in Appendix 1) was used as the production medum. The fungi were inoculated in 50 mL of sterile media taken in 250 mL Erlenmeyer flasks and incubated at 28 °C in a shaking incubator (Orbitek, Scigenics, Chennai, India) for 5 days at 150 rpm. Culture supernatant was obtained by filtering through four layers of cheese cloth and further centrifugation at 13000 rpm at 4°C (Sigma, Germany) for 15 min. This crude preparation was used to evaluate antibacterial activity using MH agar plate.

3.1.3.3 Disc diffusion assay

Disc diffusion assay was used to screen the isolates for peptide production (Bauer *et al*., 1966). For this, test organisms were spreaded on Mueller-Hinton (HiMedia) agar plate and then sterile discs were placed on the plate and 20 µL of culture supernatant was transferred on the disc. The plate was incubated at 37°C for 24 h and observed for the antimicrobial activity indicated by clear regions due to growth inhibition of the test organism around the disc.

3.1.3.4 Test organisms used in antimicrobial activity testing

Gram positive and gram negative bacteria were used as test organisms as listed in Table 3.1. The cultures were procured from National Centre for Industrial Microorganisms (NCIM**,** Pune, India) and from the culture collection of the Microbial Genetics Laboratory (MGL), Department of Biotechnology, CUSAT.

Table 3.1 Test organisms used to screen fungi for antibacterial peptide.

Test organisms	Isolate no.	Source
Pseudomonas aeruginosa	2863	NCIM
***Salmonella* Typhimurium**	2501	”
Escherichia coli	2343	”
***Salmonella* Abony**	2257	”
Klebsiella pneumoniae	2957	”
Proteus vulgaris	2027	”
Staphylococcus aureus	2127	”
Bacillus cereus	2155	”
Bacillus circulans	2107	”
Bacillus coagulans	2030	”
Bacillus macerans	2131	”
Bacillus pumilus	2189	”
Vibrio parahaemolyticus	CHAVA4(4)	MGL
Vibrio cholerae	EKM16	”
Vibrio alginolyticus	CHV2(2)	”
Vibrio vulnificus	AF7	”
Vibrio proteolyticus	AF4	”
Vibrio harveyi	P6	”
Vibrio furnisii	MUS13	”

3.1.4 SECONDARY SCREENING FOR PEPTIDE PRODUCERS

Following primary screening, the culture supernatants with antibacterial activity were subjected to acetone precipitation and ammonium sulphate precipitation to isolate proteinaceous antibacterial compounds. Isolates that produce antibacterial compounds, which could be precipitated by either acetone or ammonium sulphate precipitation were selected for further study.

3.1.4.1 Acetone precipitation

Crude extracts commonly contain substances that interfere with downstream processing. Several strategies exist for eliminating these substances from samples. One strategy for removing undesirable substances is to add a compound that causes precipitation of peptides. After centrifugation, the supernatant containing the interfering substance can be removed and the pellet is re-dissolved in buffer. The procedure is as detailed below.

The culture supernatant (prepared as described in section 3.1.3.2) was taken in acetone- compatible tube and added four times the sample volume of cold (-20°C) acetone (SRL) to the tube. Vortexed thoroughly and incubated for 60 min at -20°C, followed by centrifugation for 10 min at 16000 rpm. Decanted and properly disposed the supernatant. Then the air dried pellet was resuspended in 0.01 M phosphate buffer (pH 7.5; Appendix 2) and its antibacterial activity was checked.

3.1.4.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation of the culture supernatant (prepared as described in section 3.1.3.2) was carried out by the method of Englard and Siefter (1990). Ammonium sulphate (SRL) required to precipitate the fungal peptide was optimized by adding varying concentrations to the crude extract. To precipitate the peptide, sufficient ammonium sulphate salt to get 30% saturation was added with gentle stirring to the crude extract by keeping in ice. After complete

dissolution of ammonium sulphate salt, the solution was kept for precipitation at 4°C for overnight. After centrifugation (Thermo scientific, USA) at 13,000 rpm for 15 min at 4°C, the precipitated pellet was collected and ammonium sulphate salt required for next levels of 30- 60% and 60- 90% saturation was added to the supernatant and the procedure mentioned above was repeated.

3.1.4.2.1 Dialysis

The precipitate obtained as a result of ammonium sulphate precipitation was resuspended in minimum quantity of 0.01 M phosphate buffer (pH 7.5) and dialyzed against same buffer in order to remove the ammonium sulphate from the precipitate. The solution was transferred into the benzoylated dialysis tube with cut off value of 2 kDa (Sigma-Aldrich, USA) and dialyzed against 0.01 M solution of phosphate buffer (pH 7.5) for 24 h at 4°C with frequent changes of buffer. The partially purified fungal peptide obtained as a result was assayed for antibacterial activity and specific activity as described under sections 3.1.4.2.2 and 3.1.4.2.4 .

3.1.4.2.2 Antibacterial activity titre determination by critical dilution assay

The estimation of the antibacterial activity titre of the fungal peptide was performed by the critical dilution assay (Enan *et al*., 1996). Twofold serial dilutions were made from dialyzed fraction after ammonium sulphate precipitation of the fungal culture supernatant. From each dilution, 5µL was spotted on the surface of MH agar plates already swabbed with suspension of log-phase cells of test organism. Plates were incubated at 37^{o}C for 18h and zone of growth inhibition was observed.

One activity unit (AU) is defined as 5 µL of the highest dilution of peptide yielding a definite zone of inhibition on the lawn of the test organism. The highest dilution was multiplied by 200 to obtain the activity units per mL (AU/mL).

Highest dilution = 2^n ('n' is the number of times the sample was twofold diluted to give zone of inhibition)

Activity Units/mL = 2^n x 200

3.1.4.2.3 Protein estimation

Protein content was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard and the concentration was expressed in mg/mL.

Protein standards were prepared with 5 to 25 µg of bovine serum albumin (BSA) in 100 µL deionized water. 1 mL Bradford reagent was added, vortexed and the absorbance was measured at 595 nm in a UV-Visible spectrophotometer (Shimadzu, Japan). Standard graph was plotted and the slope was calculated. To determine the protein concentration of test sample, 10 µL sample was taken and made up to 100 µL with distilled water. Then 1mL of Bradford reagent was added and the absorbance was measured as above. The protein concentration of the test was calculated from the slope.

Bradford reagent

Hundred milligrams of Coomassie Brilliant Blue G-250 (Sigma Aldrich, USA) was dissolved in 50 mL 95% ethanol, added 100 mL of 85% (w/v) phosphoric acid and diluted to 1L. When the dye was completely dissolved, this was filtered through Whatman No.1 filter paper.

3.1.4.2.4 Specific Activity

Specific activity of the sample was calculated by dividing the inhibitory activity units (AU/mL) with the protein content (mg/mL) and was expressed as AU /mg protein.

$$\text{Specific activity} = \frac{\text{Activity Units (AU/mL)}}{\text{Protein (mg/mL)}}$$

As the values of specific activity obtained are very large, this was expressed as $\log_{10}$ (log specific activity) in this study.

3.1.4.2.5 Evaluation of antibacterial activity of the peptide using broth assay

The antibacterial activity of peptide against test organisms in liquid broth was studied by employing broth assay method described by Wang & Kuramitsu (2005). Where, 50 µL culture (OD_{600}~1) of the test organisms was added to 5 mL nutrient broth. The tubes taken as test (included 100 µL partially purified peptide) and control (without peptide) were incubated at 37^{o}C. Absorbance of the culture at 600nm was measured using UV-Vis spectrophotometer (Shimadzu, Japan) after 6 h and 24 h.

3.2IDENTIFICATION OF THE POTENT ISOLATE AFTER SECONDARY SCREENING

The fungal strain that produced antibacterial peptide that could be precipitated by ammonium sulphate salt was identified by morphological features and the identification was confirmed by molecular analysis.

3.2.1 Morphological identification of fungal strain

Vegetative and reproductive features of fungal strain were studied by microscopical observations. For identification of fungal taxa, morphological features were compared with fungal descriptions stated in standard books of mycology (Ainsworth, 1996; Alexopoulos *et al*., 1996).

3.2.2 Molecular identification of potential strain

Molecular identification was carried out by sequencing the amplified regions of 18S rRNA gene followed by BLAST analysis. ITS1 and ITS4 were the primer pair used to amplify internal transcribed spacers (ITS1 and ITS2) and 5.8S rDNA of

the genomic DNA (Nazar, 2003). The fungal DNA isolation, the primer sequences and PCR conditions were as follows:

3.2.2.1 Template preparation for PCR

Fungal strain was cultivated in Mycological broth (MB) by inoculating 50 mL sterile broth taken in 250 mL Erlenmeyer flasks with ~10^6 spores and incubated at room temperature (28 ºC ± 2 ºC) for 72 h (Rodrigues et al., 2007). One gram wet weight of the fungal mycelia frozen in liquid nitrogen was ground to fine powder and suspended in 1.5 mL of lysis buffer (250 mM NaCl, 25 mM EDTA, 0.5 % w/v SDS and 200 mM Tris–HCl, pH 8.5). The suspension was incubated at 68 ºC for 15 min with occasional gentle mixing. After centrifugation at 13,000 rpm for 15 min (4 ºC), the supernatant was transferred to a new tube and polysaccharides and proteins were precipitated by adding 750 µL of cold 4.0 M sodium acetate at pH 5.2. This solution was gently mixed by inversion, kept at -20 ºC for 20 minutes and then centrifuged at 13000 rpm for 15 min (4ºC). The supernatant was transferred in to a new tube and chromosomal DNA was precipitated by adding 1 volume of chilled 2-Propanol. This was gently mixed by inversion for a few minutes and incubated at -20 ºC for 10 minutes. DNA was recovered by centrifugation at 12000 rpm for 10 min. The precipitated DNA was washed twice with 70 % ethanol, allowed to air dry and was resuspended in 10 mM Tris-EDTA buffer (pH 8.0) and stored at -20 ºC.

3.2.2.2 Primer sequence

Primer	Sequence (5'-->3')
ITS1	**TCC GTA GGT GAA CCT TGC GG**
ITS4	**TCC TCC GCT TAT TGA TAT GC**

3.2.2.3 PCR Mix composition

10X PCR buffer	2 µL
2 mM dNTP mix	2 µL
Forward primer (10 µM)	2 µL
Reverse primer (10 µM)	2 µL
Taq DNA polymerase	1 U
Magnesium Chloride (25 mM)	1.8 µL
Template DNA (40 ng/µL)	5µL
Nuclease free water to a final volume of	25 µL

3.2.2.4 PCR conditions

Initial denaturation	95°C	for 3 min.
Denaturation	95°C	for 30 sec.
Annealing	62°C	for 30 sec.
Primer extension	72°C	for 90 sec.
Repeated 30 times		
Final extension	72°C	for 10 min.

PCR was performed in a thermal cycler (Bio-Rad).

3.2.2.5 Agarose gel electrophoresis

Standard procedure of Sambrook & Russell, 2001 was adopted for electrophoresis using an agarose gel (0.8%) for analysis of PCR products. Wells were loaded with 5 µL of the PCR products and electrophoresis was done at 80 V until the migrating dye (Bromophenol blue) had traversed two-thirds distance of the gel. The gel was stained in freshly prepared ethidium bromide solution (10 µg/mL) for

10 min and the bands were viewed using a UV- Transilluminator. Hundred base pair DNA ladder (Fermentas, India) was used as the marker.

3.2.2.6 DNA sequencing

Nucleotide sequences were determined by the ABI 3730*xl* cycle sequencer by using the big dye Terminator kit (Applied Biosystems) at SciGenom Labs Pvt Ltd., Cochin, India. The sequence obtained was analyzed online using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequence was determined by homology search with the available nucleotide database (Altschul *et al.,* 1990). The nucleotide sequence was converted into FASTA format and multiple sequence alignment for the assembled nucleotide sequences was done using the Clustal W program (Thompson *et al.,* 1994) in BioEdit software (Hall, 1999). Aligned sequences were imported into MEGA 5 software (Molecular Evolutionary Genetics Analysis version 5.0; Tamura *et al.,*

2011) software for further analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou & Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and non synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps.

3.2.2.7 Haemolytic activity of the selected strain BTMF9

Haemolytic activity is an indication of the potential for pathogenicity. Haemolytic screening was performed by the method of (Greenhill & Blaney, 2010). The screening was done on blood agar medium containing 5% human blood in agar base (Appendix 1) with the fungus strain identified to produce active peptide. 72 h culture grown in mycological agar medium was spot inoculated onto the blood agar plates and incubated at 28^{o}C. The blood agar plates were examined at regular intervals of 24 h, 48 h and 72 h. The formation of clearing zone/ green

coloration of the medium around the colonies indicated the hemolytic nature and such isolates were considered as pathogenic.

3.3 ANTIBACTERIAL PEPTIDE PRODUCTION BY SELECTED FUNGAL STRAIN: STUDY ON EFFECT OF BIOPROCESS VARIABLES BY 'ONE-FACTOR AT-A-TIME METHOD'

Various bioprocess parameters affecting maximum peptide production under submerged fermentation were studied by one-factor at-a-time method using CD medium. The parameters studied included temperature, inorganic nitrogen source, organic nitrogen sources, carbon source, initial pH, sodium chloride, incubation period, inoculum concentration, agitation, metal ions and additional inorganic nitrogen source. The effect of each parameter for maximal peptide production was evaluated and incorporated the same variable at its optimized level in the subsequent experiment to optimize next parameter.

Inoculum preparation and extraction of fungal peptide were performed as detailed under sections 3.1.3.1 and 3.1.3.2 respectively. In each case, samples were analyzed for antibacterial activity and protein concentration as detailed under sections 3.1.4.2.2 and 3.1.4.2.3 respectively, unless otherwise mentioned. All the experiments were carried out in triplicates. The results are an average of triplicate experiments and standard deviation was determined using Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA). The graphs were plotted with the help of Sigma plot for Windows Version 11.0 (Systat Software Inc., Germany).

3.3.1 Effect of temperature on peptide production

Optimal incubation temperature for maximum fungal peptide production was studied by incubating the inoculated CD media at different temperatures - 30°C, 35°C, 40°C , 45°C, 50°C and 55°C at 150rpm. Antibacterial activity and specific activity was determined after 5 days of incubation.

3.3.2 Effect of inorganic nitrogen sources on peptide production

Effect of inorganic nitrogen source on fungal protein production was studied by the addition of ammonium sulphate, ammonium nitrate, potassium ferricyanide, ammonium vanadate, sodium azide, ammonium carbonate and ammonium molybdate individually at 0.3% (w/v), replacing the inorganic nitrogen source (sodium nitrate) of fungal protein production medium. The sample was analyzed after incubation at 30^{o}C at an agitation of 150 rpm for 5 days. A control was also kept with sodium nitrate as nitrogen source.

3.3.3 Effect of organic nitrogen sources on peptide production

Effect of complex organic nitrogen source on fungal peptide production was studied using tryptone, beef extract, peptone, yeast extract, malt extract, soybean casein hydrolysate, and casein added individually at 0.3% (w/v) of production medium described earlier, having sodium nitrate as inorganic nitrogen source. The culture broth was incubated at 30^{o}C with agitation of 150rpm for 5 days.

3.3.4 Effect of carbon source on peptide production

Effect of various carbon sources on fungal peptide synthesis was studied by the addition of glucose, lactose, mannitol, starch, maltose, glycerol, galactose, cellobiose, inositol and pectin used individually at 3% (w/v), replacing the carbon source (sucrose) of the fungal protein production medium consisting of sodium nitrate as inorganic nitrogen source. The culture was incubated at 30^{o}C and at an agitation of 150rpm for 5 days. A control was also kept with sucrose as carbon source.

3.3.5 Effect of initial pH on peptide production

Different pH may have an effect on the growth and production of the desired compound. The initial pH of CD medium that support maximum peptide

production was analysed by adjusting the pH of the medium with 1N NaOH and 1N HCl. Different pH levels studied were from a range of 3-9.

3.3.6 Effect of sodium chloride on peptide production

The effect of sodium chloride for maximum fungal peptide production was evaluated using different sodium chloride concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% & 5%). The media contained sucrose as carbon source, and sodium nitrate as inorganic nitrogen source. The culture was incubated at 30^{o}C for 5 days at 150rpm.

3.3.7 Optimization of incubation period for peptide production

Optimum incubation time required for maximum peptide production was determined by incubating the inoculated media for a total period of 7 days and analyzing the samples at a regular interval of 24 h for antibacterial activity.

3.3.8 Effect of agitation on peptide production

In submerged fermentation aeration is an important factor that would affect the growth and metabolite production by the fungus. Production medium containing sucrose as carbon source, sodium nitrate as inorganic nitrogen source and 0.5% NaCl concentration was provided agitation of 75rpm, 100rpm, 125rpm, 150rpm, 175rpm, and 200rpm within shaker incubator for 3 days at 30^{0} C.

3.3.9 Effect of metal ions on peptide production

The effect of various metal ions on the peptide production was evaluated. The metal ions in 2mM concentration were supplied to the production medium in the form of salts, which included sodium chloride, calcium chloride, magnesium sulphate, ferric chloride, manganese chloride, nickel chloride, barium chloride, cadmium sulphate, zinc sulphate, copper sulphate, cobalt chloride and aluminum

sulphate which supplied the metal ions, Na^{+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, , Ba^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Al^{3+} respectively.

3.3.10 Effect of additional inorganic nitrogen source on peptide production

Effect of additional inorganic nitrogen source on fungal peptide production was studied by the addition of ammonium sulphate, ammonium bi carbonate, ammonium phosphate, ammonium ferrous sulphate and ammonium chloride individually at 0.1% (w/v) along with sodium nitrate in fungal peptide production medium. The sample was analyzed after incubation at 30^{o}C at an agitation of 150 rpm for 3 days.

3.3.11 Effect of inoculum concentration on peptide production

Optimal inoculum concentration that support maximum fungal peptide production was evaluated using different concentration of initial inoculum (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% & 1.0%) to the media containing sucrose as carbon source, sodium nitrate as inorganic nitrogen source, and 0.5% NaCl. Culture was incubated for 3 days in 30^{0}C at 150 rpm.

3.3.12 Time course study under optimized conditions

Time course experiment was conducted with the Czapek- Dox medium and optimized medium after one- factor- at - a - time method to ascertain the rate of peptide production. The optimized medium with selected conditions included 30^{o}C, sodium nitrate (0.3%) as inorganic nitrogen source, sucrose (3%) as carbon source, initial pH 7, sodium chloride (0.5%), inoculum concentration (0.4%), Ca^{2+} as metal ion (2mM), ammonium phosphate (1%) as additional inorganic nitrogen source incubated at 150 rpm for 168 h. Samples were taken for antibacterial analysis at a regular interval of 24 h for antibacterial activity.

3.4 PURIFICATION OF THE PEPTIDE

The antibacterial peptide isolated from the selected fungal strain was designated as MFAP9. Purification of fungal peptide was done by standard protein purification methods, which included ammonium sulphate precipitation followed by dialysis and ion exchange chromatography. All purification steps were carried out at 4°C unless otherwise mentioned.

3.4.1 AMMONIUM SULPHATE PRECIPITATION

Ammonium sulphate precipitation was carried out (Englard and Seifter, 1990) as described earlier under section 3.1.4.2.

3.4.2 ION EXCHANGE CHROMATOGRAPHY

The active fungal peptide obtained after the dialysis of ammonium sulphate precipitated fraction was further purified by ion exchange chromatography, using DEAE sepharose (Sigma-Aldrich) as the anion exchanger. Peptides and proteins, due to surface charge, bind to ion-exchangers. These reversibly adsorbed proteins were eluted out using a salt gradient.

3.4.2.1 Purification using DEAE sepharose column

DEAE sepharose was packed in Bio-Rad econo column (25 X 1.5 cm) ensuring the absence of any air bubble and the column was equilibrated with five column volumes of 0.01 M phosphate buffer pH 7.5. Dialyzed sample, prepared as mentioned in section 3.2.2 was lyophilized (Yamato, Japan) and resuspended in 1mL 0.01 M phosphate buffer with a protein content of 4 mg, was applied to the pre equilibrated DEAE sepharose column. After the complete entry of sample into the column, the column was connected to the reservoir containing 0.01 M phosphate buffer, pH 7.5 with a flow rate of 1 mL / min to wash out unbound peptides till the OD_{280} reached near zero. The bound peptides were eluted by a stepwise gradient of 0.1 M, 0.3 M and 0.5 M NaCl in the same buffer. Absorbance

of each fraction was measured at 280nm. Peak fractions were pooled and dialyzed against deionized water. The dialyzed fraction was lyophilized and assayed for antibacterial activity as described in section 3.1.4.2.2. The fraction with antibacterial activity was analyzed for its protein content and specific activity determined as explained under sections 3.1.4.2.3 and 3.1.4.2.4 respectively.

3.4.3 REVERSE-PHASE HPLC

The purified active fraction after ion exchange chromatography (20 μL, 0.1 mg/mL) was rechromatographed by reversed phase HPLC (Schimadzu LC 2010) using Phenomenex C18 HPLC column (22.5 mm ID X 250 mm length). Chromatography was done at a flow rate of 1 mL/min with 100% solvent A (0.1% trifluoroacetic acid (TFA) in water) for 10 min and a linear gradient (0–100%) of solvent B (80% acetonitrile) over 45 min. Proteins were detected by monitoring the absorbance at 214 nm.

3.4.4 CALCULATION OF FOLD OF PURIFICATION

Fold of purification in each step was calculated by dividing the specific activity (section 3.1.4.2.4) of the respective fraction with that of the crude extract as follows

$$\text{Fold of purification} = \frac{\text{Specific activity of the purified fraction}}{\text{Specific activity of the crude extract}}$$

3.5 CHARACTERIZATION OF THE ACTIVE PEPTIDE

Purified peptide was further characterized for their biophysical and physicochemical properties like molecular weight, isoelectric point, stability at different temperature and pH, action of different proteases, effect of detergents, metal ions, reducing agents, oxidizing agents and amino acid modifiers as described in the following sections.

3.5.1 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The active fraction of the peptide after ammonium sulphate precipitation and ion exchange chromatography was subjected to electrophoretic analysis by Glycine SDS-PAGE in a vertical slab electrophoresis (Mini-PROTEAN Tetra cell, BioRad). Electrophoresis was carried out in a 18% polyacrylamide gel according to the method described by (Laemmli, 1970).

3.5.1.1 Glycine SDS-PAGE

The purified peptide fraction was subjected to SDS-PAGE to analyze and visualize protein and peptide pattern. Reagents for glycine SDS-PAGE gels are given in Table 3.2 and the preparation of reagents given in Appendix 3

Gel preparation

The gel plates were cleaned and assembled before gel casting.

Resolving gel - The resolving gel was prepared combining all reagents given in Table 3.2 except APS and TEMED in a beaker. Mixed gently, APS and TEMED added and again mixed well. Immediately poured the mixture into the assembled glass plates and pipetted out a layer of water over the gel and allowed to polymerize at least for 45 min.

Stacking gel - The components of stacking gel (Table 3.2) except APS and TEMED were added into a beaker, mixed gently and finally APS and TEMED were added. After casting the mixture above the resolving gel, the comb was immediately inserted between the glass plates and allowed to polymerize for atleast 30 min.

Table 3.2 Reagents for the preparation for Glycine SDS-PAGE gel

CONSTITUENTS	RESOLVING GEL (18%) (mL)	STACKINGGEL (5%) (mL)
Acrylamide-bisacrylamide	6.0	0.666
Buffer	2.5	1
Water	1.32	2.27
SDS	0.1	0.04
APS	0.075	0.02
TEMED	0.005	0.004
Total	10.0	4.0

Gel was placed in the electrophoresis apparatus and reservoir was filled with electrode buffer for SDS-PAGE (Appendix 3). Protein samples were loaded to the wells in the gel. The current at 80 V was applied till the sample entered the resolving gel portion. When the dye front entered the resolving gel, the current was increased to 120 V. The electric run was stopped when the dye front reached 1 cm above the lower end of the glass plate. The apparatus disassembled and removed the gel from glass plate. For Coomassie brilliant blue staining, the gel was immersed for at least 1 h in the staining solution. The gel was destained till the protein bands became clear.

Sample preparation

5X sample buffer (Appendix 3) was mixed with the test protein sample and 20 μL sample was applied in the well of the gel.

3.5.1.2 Silver staining

The silver staining method developed by Blum *et al.* (1987) with slight modifications was used to visualize the protein profile after SDS- PAGE. The preparation of the solutions is provided in Appendix 3.

Procedure

The SDS-PAGE gel was incubated in the fixing solution for 30 min. Then the gel was washed in wash solution for 15 min, followed by five washes (5 min interval) with deionized water. The sensitizer solution was applied to gel for exactly 60 s and washed twice at 60 s intervals with deionized water. The gel was immersed in chilled silver nitrate solution for 25 min and washed two times for 60 s with deionized water. The gel was transferred to developer solution and kept until protein bands were developed. Reaction was arrested by adding stop solution.

3.5.1.3 Protein Markers for SDS-PAGE

Medium range molecular weight protein marker of GeNei (Bangaluru) and Broad range molecular weight protein marker mix of New England BioLabs (UK) were used for detecting the approximate size. Details of marker are given in Appendix 3.

3.5.2 GLYCINE SDS-PAGE AND DETECTION OF ANTIBACTERIAL ACTIVITY ON GEL TO DETERMINE APPROXIMATE MASS OF THE PEPTIDE

The partially purified peptide was subjected to SDS-PAGE using 18% polyacrylamide gel as described in section 3.5.1.1. The protein was run in two lanes along with protein molecular weight marker (GeNei, Bangaluru, India). After the electrophoretic run, the gel was removed and cut into two parts. One half containing the sample and molecular weight marker was coomassie stained. The other half with sample was washed thrice with 0.1% tween 80 (30 min each), followed by washing with deionized water in order to remove SDS. The gel was then placed on MH agar base plate, overlaid with soft MH agar (0.8 % agar) seeded with 50 µL (OD_{600} ~ 1) of test organisms (Yamamoto *et al*., 2003) and visualised zone of clearance at the corresponding band position due to the antibacterial activity after overnight incubation at 37^{o} C.

3.5.3 ELECTRO ELUTION OF ANTIBACTERIAL PEPTIDE

The partially purified peptide was subjected to SDS-PAGE using 18% polyacrylamide gel and the gel was removed from the apparatus and cut into two. One portion containing the sample and molecular weight marker was silver stained. The other half with sample was washed thrice with 0.1% Tween 80 (30 min each), followed by washing with deionised water in order to remove SDS. The gel portion corresponding to the active band was excised into small pieces. The gel pieces were transferred in to a 2 kDa benzoylated dialysis tubing (Sigma Aldrich, USA) and 0.01 M phosphate buffer (2mL) added and placed in an electrophoretic tank containing same buffer. For this a voltage of 30 V was applied overnight at 4^{o} C, followed by a reversal of the electrodes with voltage of 30 V applied for 30 min (Lei *et al.*, 2007). The solution containing the peptide was dialysed against deionized water to remove the salts. Then the sample was lyophilized and used for molecular mass determination by MALDI-TOF MS.

3.5.4 INTACT MASS BY MALDI-TOF MS

The purified peptide was subjected to intact mass analysis by MALDI-TOF mass spectrometry. The intact molecular mass of the purified peptide was determined by MALDI TOF/TOF (Bruker Daltronics, Germany) at Indian Institute of Science (IISc), Bengaluru, India.

3.5.5 PEPTIDE MASS FINGERPRINTING

Peptide was reduced, alkylated with iodoacetamide and trypsin digested. Then resultant peptides were extracted according to standard techniques and were analyzed by MALDI-TOF-TOF mass spectrometer using a 4800 Proteomics Analyzer (Applied Biosystems). Spectra were analyzed to identify peptide of interest using Mascot sequence matching software (Matrix Science) with Swiss-Prot and NCBInr database.

3.5.6 ISOELECTRIC FOCUSING

Isoelectric point (pI) of the purified peptide was determined by isoelectric focusing, performed using isoelectric focusing unit (Bio-Rad, USA). Immobilized pH gradient (IPG) strip of pH 3-10 (Bio-Rad, USA) was used for the purpose. The detailed procedure is as given below.

3.5.6.1 Rehydration of sample with IPG strip

The lyophilized sample (0.1 mg/ mL) was dissolved in 125 µL rehydration buffer (Appendix 3) and loaded on to the equilibration tray. Two IPG strips of pH 3-10 were taken for both IEF followed by 2D gel electrophoresis and strips were gently placed gel side down in the equilibration tray. Air bubbles removed if any and the strips were overlaid with 2-3 mL of mineral oil to prevent evaporation during rehydration process. Covered the equilibration tray and left the tray overnight to rehydrate IPG strips.

3.5.6.2 Isoelectric focusing

Paper wicks were placed at both ends of the clean, dry IEF focusing tray covering the wire electrodes and were made wet with nano pure water. IPG strips were taken out from the rehydration tray and drained the mineral oil by holding the strip vertically for 8 s and positioned the IPG strips in the focusing tray. Placed in the PROTEAN IEF cell, IPG strips overlaid with mineral oil and closed the cover. Program was set in the IEF cell as given below in Table 3.3 and run the electrophoresis.

Table 3.3 Steps involved in isoelectric focusing

	Voltage	Time	Volt-Hours	Ramp
Step 1	250	20 min	---	Linear
Step 2	4000	2 h	---	Linear
Step 3	4000	---	10,000 V-h	Rapid

The cell temperature was maintained at 20^{o}C with maximum current of 50 µA/strip and no dehydration in all steps.

3.5.6.3 Staining and 2-D electrophoresis after IEF

IPG strips were removed from focusing tray after electrophoresis. Drained mineral oil and pressed the strip against a wet blotting paper. One of the strips were transferred to Coomassie stain tray and kept for 1 h and destained the gel for 10 min. The other IPG strip was transferred to the equilibration tray and 2.5mL equilibration buffer I was added and kept at orbital shaker for 10 min. The tray was decanted to discard the equilibration buffer I (Appendix 3) completely from the strip. Equilibration buffer II (2.5mL) (Appendix 3) was added then and shaking was applied for 10 min. IPG strip was taken and rinsed in a 1X Tris-glycine buffer and placed on the precast 16% SDS-PAGE gel (A portion of the gel was left at the top, according to the dimension of IPG strip during the casting itself of SDS-PAGE gel). IPG strip was overlaid with molten agarose and allowed to set. The electrophoresis was carried out at 200 V. The gel was silver stained after electrophoresis.

3.5.7 MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC).

The microtitre-plate based resazurin assay method with slight modifications was used to determine MIC and MBC values of the peptide against test organism (Sarker *et al.*, 2007). For this 30 µL deionized water was added to

each well. Then 30 µL purified peptide (0.04mg/mL) was added in the first column and double diluted vertically. 100 µL nutrient broth was added in each well. 10 µL test organisms (OD at 600 nm ~1) were also added to each well. The microtitre plate was incubated at 37^{o}C for 18- 24 h. Each well of the negative control contained 10µL of microbial suspension and 100µL double strength nutrient broth. Ampicillin (0.1mg/mL) was taken as the positive control .After incubation, 1 µL resazurin (1% stock) was added to each well and observed for colour change by incubating at 37^{o}C for 30 min. Blue coloured resazurin turns pink/colourless in wells which contain living cells. The highest dilution where colour not changed from blue to pink was noted and considered as the MIC value.

The MBC value was determined by inoculating 10 µL sample from wells in microtitre plate that showed no apparent growth when MIC assay was done on to MHA plates. The plates were incubated at 37ºC for 24h and were examined for colony growth or lack of growth for each peptide dilution. The lowest concentration of peptide that killed all or >99.9% of the total bacteria was taken as MBC value (Powthong *et al*., 2012).

3.5.8 EFFECT OF VARIOUS PHYSICO-CHEMICAL PARAMETERS ON PEPTIDE STABILITY

The effect of various physicochemical factors like temperature, pH, proteases, detergents, metal ions, reducing agents, oxidizing agents and amino acid modifiers on activity of peptide was studied. The effects of different parameters were expressed in terms of relative activity and residual activity.

3.5.8.1 Relative activity

Relative activity is the percent antibacterial activity of the sample with respect to the activity of the sample for which maximum activity was obtained.

$$\text{Relative activity} = \frac{\text{Activity of the sample (AU / mL)}}{\text{Activity of the sample with maximal activity (AU /mL)}} \times 100$$

3.5.8.2 Residual activity

Residual activity is the percent antibacterial activity of the sample with respect to the activity of the control sample.

$$\text{Residual activity} = \times 100 \frac{\text{Activity of the sample (AU / mL)}}{\text{Activity of the control (AU / mL)}}$$

3.5.8.3 Effect of temperature on the stability of the peptide

Thermal stability of the peptide was studied by incubating at different temperatures ranging from 4°C-100^{o}C for 1 h. After incubation, the antibacterial activity of each sample was assessed by conducting the critical dilution assay as described under section 3.1.4.2.2 and relative activity was determined.

3.5.8.4 Effect of pH on the stability of peptide

To study the effect of different pH on the stability of the peptide, the purified peptide was incubated with equal amount of buffers with pH range starting from 1 to13, with an interval of 1 pH and kept for 18 h at 4°C. The buffer systems (Vincent and John, 2009) used included hydrochloric acid- potassium chloride buffer (pH 1 and 2), citrate buffer (pH 3, 4, 5 and 6), phosphate buffer (pH 7), Tris (hydroxy methyl amino methane) buffer (pH 8 and 9), carbonate-bicarbonate buffer (pH 10 and 11), sodium hydroxide-potassium chloride buffer (pH 12 and 13). Composition of each buffer is charted in appendix 2. The samples after incubation were tested for the relative activity.

3.5.8.5 Action of proteases on the peptide

Confirmation of proteinaceous nature of active compound was done by treatment with proteolytic enzymes. Sensitivity to different proteases was assessed by incubating the dialyzed peptide fraction with different concentrations of proteases. Varying concentration of proteinase K, pepsin and trypsin (from Bovine pancreas, SRL, India) ranging from 20, 40, 60, 80 and 100 µg was used for incubation with partially purified fraction for 1 h at 37°C. The antibacterial

activity was tested as described under section 3.1.4.2.2 and residual activity was calculated.

3.5.8.6 Effect of various detergents on stability of peptide

Effect of various non-ionic and ionic detergents such as triton X-100, tween 80, tween 20, SDS and CTAB (0.1% each w/v) on peptide activity was determined by incubating the purified peptide in each detergent for 1h. This was followed by dialysis against 0.01 M phosphate buffer having pH 7.5. The samples were then lyophilized and tested for antibacterial activity as described under section 3.1.4.2.2 and residual activity was estimated.

3.5.8.7 Effect of various metal ions on the activity of peptide

Effect of various metal ions was evaluated by incubation along with 1 mM concentrations of various metals ions for 1 h followed by testing the antibacterial activity as described under section 3.1.4.2.2. The metal ions were supplied in the form of salts, which included sodium chloride, calcium chloride, magnesium sulphate, ferric chloride, manganese chloride, nickel chloride, barium chloride, cadmium sulphate, zinc sulphate, copper sulphate, cobalt chloride and aluminum sulphate which supplied the metal ions, Na^{+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, , Ba^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, $Co^{2+,}$ Cr^{2+} and Al^{3+} respectively.

3.5.8.8 Effect of reducing agents on the stability of fungal peptide

The effect of reducing agents on the activity was studied by incubating the peptide with β-mercaptoethanol at a concentration of 20, 40, 60, 80 & 100 mM and dithiothreitol at a concentration of 2, 4, 6, 8 & 10 mM. After 1 h incubation, the antibacterial activity was assayed as described under section 3.1.4.2.2 and residual activity was calculated.

3.5.8.9 Effect of oxidizing agents on activity of fungal peptide

Impact of oxidizing agents on the activity of peptide was studied by incubating the purified peptide with dimethyl sulfoxide (1-5%, v/v) and sodium hypochlorite at a concentration of 20, 40, 60, 80 and 100 mM for 1h followed by testing the antibacterial activity as described under section 3.1.4.2.2.

3.5.8.10 Effect of chemical amino acid modifiers on activity of fungal peptide

Effect of amino acid modifiers on the activity of peptide was studied by incubating the purified peptide with diethylpyrocarbonate (0.1Tris HCl, pH- 7) at 30°C for 30 min, PMSF (0.05M Tris HCl, pH- 7.8) at 25°C for 120 min, iodoacetamide (0.1 M Tris HCl, pH- 8) at 35 °C for 60 min at a concentration of 5, 10, 15, 20 and 25 mM and N- Bromosuccinamide (0.1Tris HCl, pH- 7) at concentrations of 1, 2, 3, 4 and 5mM at 30 °C for 30 min followed by testing the antibacterial activity as described under section 3.1.4.2.2.

3.5.9 HEMOLYTIC ACTIVITY ASSAY

Human red blood cells were centrifuged and washed three times with phosphate-buffered saline (Appendix 3). Method of Park *et al.* (2008) with slight modifications was used. The hemolytic activity was evaluated by measuring the release of hemoglobin from fresh human erythrocytes. For this assay, 100 µL deionized water was taken in microfuge tubes and 100 µL peptide sample was added to the first tube and two fold serial dilutions was carried out. Then, aliquots (100 µL) of an 8% suspension of red blood cells were transferred to each tube and incubated for 30 min at 37 °C. After the incubation period, the reaction mixtures were centrifuged at 1,000 x g for 10 min to remove intact erythrocytes and then supernatant containing released hemoglobin was measured at 414nm. No hemolysis (0%) and full hemolysis (100%) were determined in the presence of PBS and 0.1% Triton X-100, respectively. Hemolytic activity was expressed as a percentage hemolysis, which was calculated using the following equation:

$$\% \text{ hemolysis} = \frac{(A_{414nm} \text{ with peptide solution} - A_{414nm} \text{ in PBS})}{(A_{414nm} \text{ with 0.1\% Triton-X 100} - A_{414nm} \text{ in PBS})} X100$$

3.6 APPLICATION STUDIES

3.6.1 PEPTIDE FOR THE CONTROL OF BACTERIAL BIOFILMS

The organisms used for biofilm inhibition assay were studied for their capacity to form biofilms and observed as strong biofilm producers (Laxmi & Sarita, 2014). Biofilm formation by bacterial isolates was quantified by microtitre plate assay of Rode *et al.* (2007).

The experiment was carried out in triplicates to determine the ability of the fungal peptide to inhibit biofilm formation. The wells of the sterile 96 well polystyrene microtitre plates were filled with 220 µL of tryptone soya broth (TSB). 20µL test bacterial cultures (OD_{600} ~1) was added into each well separately in both positive control wells and the experimental wells, then incubated at 37^{o}C for 24 h for biofilm formation. Wells with only TSB was maintained as negative control. After 24 h of incubation, 30µL of purified fungal peptide (0.1mg/mL) was added in experimental wells. Positive control was added with 30 µL of TSB. The microtitre plates were further incubated aerobically for 24 h at 37^{o}C. For antibiofilm assay, the contents of the plates were poured off and the wells were washed 3 times with 300 µL of phosphate buffer (0.01 M, pH 7.5). Remaining bacteria were fixed with 250µL of methanol per well. After 15 min, the plates were emptied, air dried and stained with 250 µL of 1% crystal violet for 5 minutes. The excess stain was rinsed off by placing the microtitre plates under running tap water. After the plates were air dried, the dye bound to the adherent cells was extracted with 250 µL of 33% (v/v) glacial acetic acid per well and the absorbance of each well was measured at 570nm using a UV-Vis spectrophotometer.

3.6.1.1 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to visualize the effect of peptide MFAP9 on biofilm formed by the most sensitive *Bacillus pumilus* BT3 on cover slip by the technique of Lembke *et al*., 2006. Cover slip was kept in culture broth for biofilm formation for 24 hours at 37^{O}C. After incubation, peptide sample was added and incubated under same conditions. The fixation of culture was done with 2.5% gluteraldehyde for 1h at room temperature. The cover slips were rinsed three times using 0.1M sodium phosphate buffer (pH 7.3). They were then dehydrated in a graded series of alcohol (25, 50, 75 and 90%) for 5 min each. Further dehydration was done with 100% ethanol two times each for 10 min followed by SEM analysis (JEOL Model JSM - 6390LV).

Organisms used for biofilm inhibition study

- *Bacillus altitudinis* BT1
- *Bacillus pumilus* BT3
- *Brevibacterium casei* BTDF1
- *Staphylococcus warneri* BTDF2
- *Bacillus niacini* BTDP3
- *Micrococcus luteus* BTFF1
- *Geobacillus stearothermophilus* BTFF2

3.6.2 *IN VITRO* ANTIPROLIFERATIVE STUDIES OF PEPTIDE ON CULTURED CELL LINES

3.6.2.1 Cell culture maintenance

L929 fibroblast (Normal cell line) and A549 (lung carcinoma cell line) were the cell lines used for this study and were purchased from National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's modified Eagles media

(HiMedia) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) was used for cell line maintenance and grown to confluency at 37°C in 5 % CO_2 (NBS, Eppendorf, Germany) in a humidified atmosphere in a CO_2 incubator.

Procedure

The cells were trypsinized (500 µL of 0.025% Trypsin in PBS/ 0.5 mM EDTA solution (HiMedia)) for 2 min and passaged to T flasks in complete aseptic conditions. Peptide sample was added to grown cells at a final concentration of 6.25 µg/ml, 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL from a stock of 0.1 mg/mL and incubated for 24 h. The percentage difference in cell viability was determined by standard MTT assay after 24 h of incubation as given below. The morphological characteristics of cells were imaged using inverted phase contrast microscope (Olympus CKX41, Japan) with Optika Pro5 CCD camera.

3.6.2.2 MTT Assay (Arung *et al*., 2006)

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4,5dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with DMSO and the released, solubilized formazan reagent was read spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is inturn a measure of the viability of the cells.

The cells were washed with 1X PBS and then added 30 µL of MTT solution (MTT -5 mg/mL dissolved in PBS) to the culture. It was then incubated at 37^0C for 3 h. MTT was removed by washing with 1X PBS and 200 µL of DMSO was added to the culture. Incubation was done at room temperature for 30 min until the cells got lysed and colour was obtained. The solution was transferred to centrifuge

tubes and centrifuged at top speed for 2 min to precipitate cell debris. Absorbance was read at 540 nm using DMSO as blank in a microtitre plate reader (ELISASCAN, ERBA).The percent viability was calculated by the following equation:-

% viability = (OD of Test/ OD of Control) X 100

3.6.2.3 Determination of apoptosis and necrosis by acridine orange (AO) and ethidium bromide (EB) double staining method

DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells (Attari *et al*., (2009)). AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. A549 cells were washed by cold PBS and then stained with a mixture of AO (100 µg/mL) and EB (100 µg/mL) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).

3.6.3 ANTIOXIDANT ACTIVITY

DPPH radical scavenging by purified peptide was estimated using the method of Liyana-Pathirana & Shahidi, (2005). .A solution of 0.135 mM DPPH in methanol was prepared and 1.0 mL of this solution was mixed with 100 µL of peptide at different concentrations (0.8, 1.6, 2.4, 3.2 and 4 µg). The standard antioxidant ascorbic acid in the same concentrations as that of peptide was used as positive controls. The reaction mixture was vortexed thoroughly and left in the

dark at room temperature for 30 min. The absorbances of the samples were measured at 517 nm. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{Free radical scavenging activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

where $A_{control}$ is the absorbance of the free radical solution (DPPH) + methanol, and A_{sample} is the absorbance of the free radical solution with peptide/standard antioxidant.

3.6.4 DETERMINATION OF INVITRO ANTIINFLAMATORY EFFECT OF FUNGAL PEPTIDE ON CULTURED THP1 CELL LINES

The anti inflammatory effect of peptide was determined by assessing the inhibition of cycloxygenase enzyme (COX-2) when THP1(Human monocytic cell lines) THP1 treated with LPS (Raheema *et al*., 2014). cell line was cultured in RPMI 1640 [HiMedia] media, supplemented with 10% heat inactivated FBS, antibiotics (Penicillin and Streptomycin) and 1.5% sodium bicarbonate. The media was filtered using 0.2µm pore sized cellulose acetate filter (Sartorius) in completely aseptic conditions. The cells were then grown till 60% confluency followed by activation with 1µl LPS (1µg/mL). LPS stimulated THP 1 cells were exposed with different concentrations of peptide samples (6.25µg/ml, 12.5µg/mL 25µg/mL, 50µg/mL and 100µg/mL) from a stock of 1mg/ml dissolved in 1% DMSO and incubated for 24 hours.

The crude enzyme isolation was done by spinning at 6000 rpm for 10 minutes. Supernatant was discarded and 200µl of cell lysis buffer (1MTris HCl, 0.25M EDTA, 2M NaCl, 0.5% Triton) was added .The incubation was done for 30 minutes at 4°C and enzyme assay was done in pellet suspended in a small amount of supernatant.

3.6.4.1 Assay of cycloxygenase (COX-2) activity

The assay mixture contained 100 mM Tris- HCl buffer (pH. 8), 5mM glutathione, 5µM hemoglobin and crude enzyme supernatant. The reaction was started by the addition of 200µM arachidonic acid and incubated at 37ºc for 20 min. Reaction was terminated by adding 0.2mL of 10% TCA in 1N HCl, mixed and 0.2mL of 1% thiobarbituric acid was added. Reaction mixture kept in a boiling water bath for 20 min, cooled and centrifuged at 1000 rpm for 3 min. The absorbance was measured at 632 nm for COX activity.

4.1SCREENING FOR ANTIBACTERIAL PEPTIDE PRODUCING FUNGI FROM TERRESTRIAL AND MARINE SEDIMENT SAMPLES

4.1.1 ISOLATION OF FUNGI

A total of one hundred and twenty terrestrial and ten marine isolates were obtained from different samples. These isolates were screened first by disc diffusion assay using culture supernatant to pick out fungi capable of producing antagonistic molecules for inhibiting the growth of test organisms.

4.1.2 PRIMARY SCREENING OF FUNGI FOR ANTIBACTERIAL ACTIVITY

The primary screening resulted in the segregation of fifteen isolates with antibacterial activity against at least four test organisms (Table 4.1).

Table 4.1 Primary screening by disc diffusion method for antibacterial activity

Sl. No	Source	*B.cereus*	*B.coagulans*	*S.aureus*	*S.typhimurium*	*V.cholerae*	*V.parahaemolyticus*	*V.alginolyticus*	*K.pneumoniae*	Total Positive
1	BTAM114	-	-	+	-	-	+	+	+	**4**
2	BTAM50	+	+	+	+	-	+	+	+	**7**
3	BTAM97	-	+	+	-	-	+	+	+	**5**
4	BTSA103	-	+	+	-	-	-	+	+	**4**
5	BTA1	+	-	+	-	+	-	+	+	**5**
6	BTAM80	+	-	+	-	+	-	+	+	**5**
7	BTAM94	+	-	+	-	+	-	+	+	**5**
8	BTUK2	+	-	+	-	+	+	+	+	**6**
9	BTAM84	+	-	+	-	+	+	+	-	**5**
10	BTAM224	+	-	+	-	+	+	+	-	**5**
11	BTAM223	+	-	+	-	+	+	+	-	**5**
12	BTAM123	+	-	+	-	+	+	+	-	**5**
13	BTAM122	+	-	+	-	+	+	+	-	**5**
14	BTMF3	+	+	+	+	-	-	-	+	**5**
15	BTMF9	+	+	+	+	-	-	-	+	**5**

+ = Activity
- = No activity

BTAM- isolate from terrestrial sediment
BTA1 - "
BTUK - "
BTSA - "
BTMF - isolate from marine sediment

4.1.3 SECONDARY SCREENING FOR PEPTIDE PRODUCERS

Secondary screening was performed by testing the antibacterial activity of the acetone precipitated and that of the ammonium sulphate precipitated protein fraction. The culture supernatants of the fifteen isolates were used for both acetone precipitation and ammonium sulphate precipitation and antibacterial activity was checked by disc diffusion assay against all test bacteria. Out of the fifteen isolates screened, ammonium sulphate precipitate (90% saturation) of only single isolate BTMF9 from marine sediment showed antimicrobial activity against *Bacillus cereus, Bacillus circulans, Bacillus coagulans, Bacillus pumilus and Staphylococcus aureus.* No activity was observed against the gram- negative test bacteria.

The quantitative estimation of antibacterial activity of the ammonium sulphate fraction of BTMF9 isolate was determined by the critical dilution assay against the sensitive test bacteria. From the results obtained, the test organism *Bacillus circulans* (NCIM 2107) showed more consistent sensitivity towards the peptide than others and was selected as the antibacterial activity indicator organism for further studies (Table 4.2; Fig. 4.1).

Table 4.2 Secondary screening for peptide production by Critical dilution method (Antibacterial activity of ammonium sulphate precipitated protein fraction)

Test Organism	Antibacterial activity (Activity units/milli litre) (AU/mL)
B.circulans	**3200**
B.coagulans	**1600**
S.aureus	**3200**
B.pumilus	**1600**
B.cereus	**800**

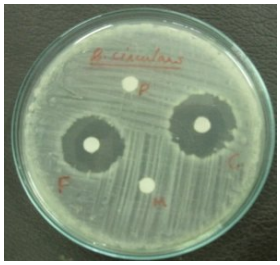


Fig: 4.1 Antimicrobial activity of 30-90% ammonium sulphate fraction against *Bacillus circulans* by disc diffusion assay: F- 30-90% fraction, C- Ampicillin, P- Buffer control and M- Medium control.

4.1.4 Growth inhibition assay in broth culture

From the experiment it was observed that the absorbance measured at 600nm of all the test organisms viz. *B. coagulans*, *B. circulans*, *B. cereus, B.pumilus* and *S. aureus* did not increase significantly when the partially purified sample was added into the growth media at the time of inoculation (Fig. 4.2). This indicates that the growth of these test organisms was inhibited. In the control experiment, there was significant growth and raise in absorbance after six and twenty four hours of incubation. From the experiment it was concluded that the antibacterial peptide is active against all test organisms in broth culture.

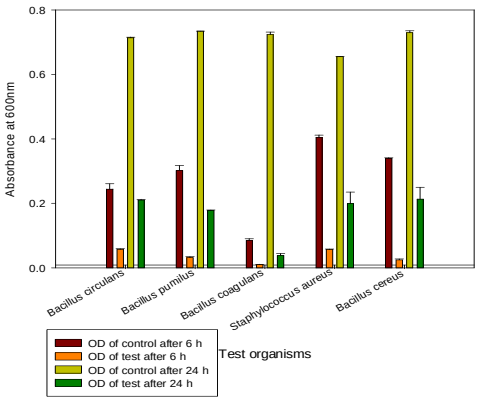
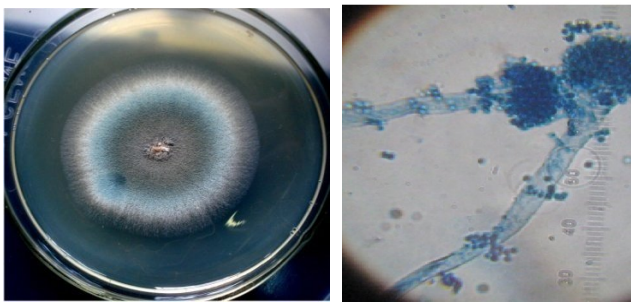


Fig: 4.2 Change in absorbance at 600 nm when the test organisms were inoculated in media supplemented with partially purified sample.

4.2 IDENTIFICATION OF THE SELECTED FUNGAL STRAIN

The selected marine fungal isolate BTMF9 developed as greyish green colony on mycological agar plate. The mycelial growth was limited and sporulation occurred on the 3rd day of spore inoculation. The fungal mycelia were septate with typical columnar, erect, uniseriate conidial heads. Conidiophores were short, smooth-walled with hemispherical terminal vesicles. A single row of phialides were seen on the upper two thirds of the vesicle. The conidia were elliptical and were borne as chains on the phialides. Based on these macroscopic and microscopic characteristics as in Fig. 4.3a&b, it was confirmed that BTMF9 belongs to the genus *Aspergillus*.



a **b**

Fig. 4.3 a) Plate showing fungal colony .b) Microscopic view of conidiophore and conidia

Partial nucleotide sequences encoding 18S rRNA gene and internal transcribed spacer 1; 5.8S r RNA gene and internal transcribed spacer 2 complete sequence; and partial sequence of 28S r RNA gene, were amplified and resolved at a size of ~750 bp on 1.2% agarose gel (Fig. 4.4a).

The amplicon was subjected to sequencing and identity was established by homology search and analysis using BLAST software. The obtained partial sequence given below in Fig. 4.4b showed 100% similarity with already available sequences of *Aspergillus fumigatus* strains and designated as *Aspergillus fumigatus* BTMF9 (NCBI GenBank accession No.HQ285882).

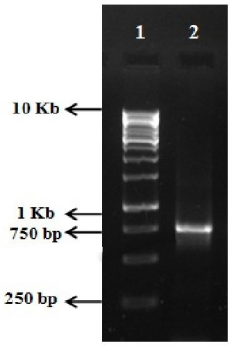


Fig. 4.4 a

5'-
CGTAGGTGACCTGCGGAAGGATCATACCGAGTGAGGGCCCTCGGGTCCAACCTCCCACCCGT
GTCTATCGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCTTGC
GCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCT
GAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTG
AACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCT
CAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAG
CGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGC
CGGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCC
GCTGAACTTAAGCATATCATG-3'

Fig. 4.4 b

Fig. 4.4 a) PCR amplicon of 18S rDNA segment. Lanes 1. 100 bp ladder 2. Amplicon. 4.4b) DNA sequence of amplicon.

Phylogenetic tree was constructed based on the 18S rRNA gene sequences to analyse the relatedness of isolated fungus *Aspergillus fumigatus* BTMF9 with other reported *Aspergillus* sps (Fig. 4.5). All the strains grouped together into a major clade, except BTMF9, which formed a separate clade, indicated its unique identity. From the phylogenetic tree, it can be observed that all strains grouped

together to form a single clade as they are related and distinct from the out group. The clonal origin of strains was evidenced from the phylogram even though they exhibited intraspecies variations.

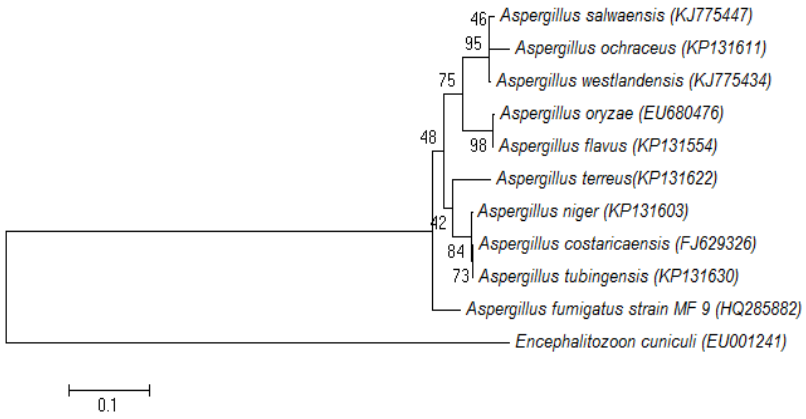


Fig. 4.5 Neighbour-joining phylogenetic tree based on 18S rRNA gene sequences, showing the phylogenetic relationship between strain BTMF9 and other members of the genus *Aspergillus*.

The taxonomic hierarchy of *Aspergillus fumigatus* BTMF9 is presented in the Table 4.3.

Table 4.3Taxonomic hierarchy of *Aspergillus fumigatus* BTMF9

Kingdom	-	Mycota
Division	-	Eumycota
Subdivision	-	Ascomycotina
Class	-	Ascomycetes
Subclass	-	Plectomycetes
Order	-	Eurotiales
Family	-	Eurotiaceae
Genus	-	*Aspergillus* Micheli
Species	-	*Aspergillus fumigatus* Fresenius

4.2.1 Hemolytic activity

The selected potent strain *Aspergillus fumigatus* BTMF9 did not show any hemolytic activity on blood agar plates (Fig. 4.6). As positive haemolysis indicates pathogenicity and isolated strain is non- haemolytic.

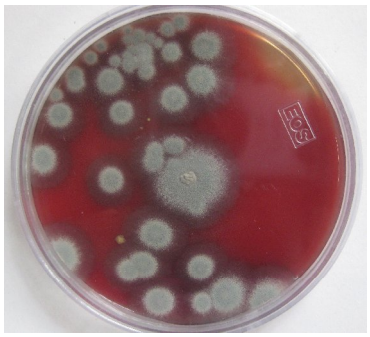


Fig. 4.6 Haemolytic activity assay: Fungal colony on blood agar plate

4.3 ANTIBACTERIAL PEPTIDE PRODUCTION BY *ASPERGILLUS FUMIGATUS* BTMF9: STUDY ON EFFECT OF BIOPROCESS VARIABLES BY 'ONE-FACTOR AT-A-TIME METHOD'

4.3.1 Effect of temperature on peptide production

Optimum temperature is an important factor in the growth of organism and the production of desired compound. Maximum antibacterial activity (800AU/mL) and specific activity of $\log_{10}$ 4.3 ± 0.1 AU/mg protein was observed at a temperature of 30°C. A higher incubation temperature (>45^{0}C) had an adverse effect on growth and antibacterial peptide production (Fig. 4.7)

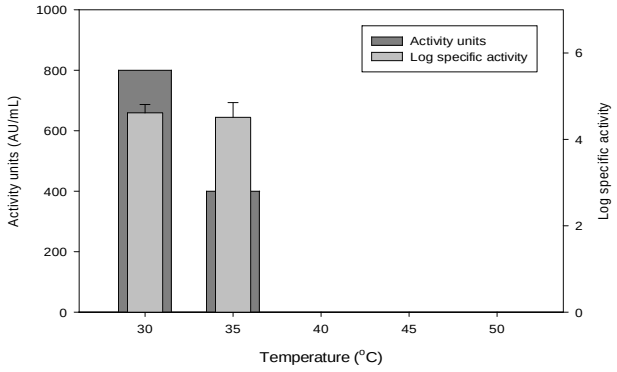


Fig: 4.7 Effect of temperature on peptide production by BTMF9

4.3.2 Effect of inorganic nitrogen sources on peptide production

Nitrogen sources widely influence the growth and production of specific metabolites. Various inorganic nitrogen sources were checked for production optimization. In case of inorganic nitrogen sources, it can be inferred that sodium nitrate is a very good nitrogen source which gave both higher inhibitory activity (800AU/mL) and specific activity ($\log_{10}$ 3 .2 ± 0.002) than the other nitrogen sources (Fig. 4.8). However a very low activity was noted with ammonium molybdate.

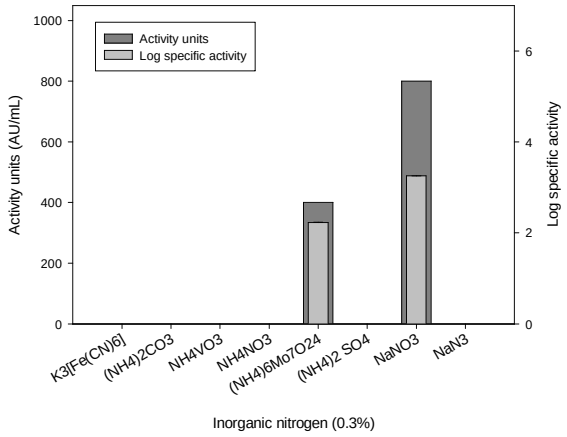


Fig: 4.8 Effect of inorganic nitrogen sources on peptide production by BTMF9

4.3.3 Effect of organic nitrogen sources on peptide production

Effect of complex organic nitrogen source on fungal peptide production was studied using tryptone, beef extract, peptone, yeast extract, malt extract, soybean casein hydrolysate and casein. Organic nitrogen sources were found to be least significant for production optimization (Fig.4.9) as activity was obtained only for soy casein hydrolysate (400AU/mL) and malt extract (400AU/mL). Due to this reason organic nitrogen source was not incorporated in medium for further studies.

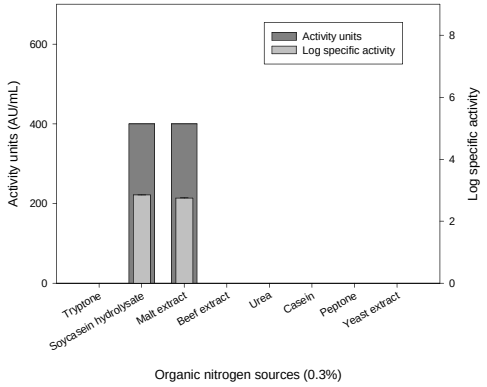


Fig: 4.9 Effect of organic nitrogen sources on peptide production by BTMF9

4.3.4 Effect of carbon source on peptide production

The influence of different carbon sources on production of antibacterial peptide is shown in Fig. 4.10. In case of extract obtained from culture grown separately in broth containing sucrose and glucose as carbon sources, inhibitory activity (800AU/mL) observed was same but the log specific activity was maximum ($\log_{10}$ 4.5 ± 0.00 AU/mg protein) in presence of sucrose than glucose. Therefore sucrose can be considered as the best carbon source. There was no peptide production when all other carbon sources were incorporated in the medium.

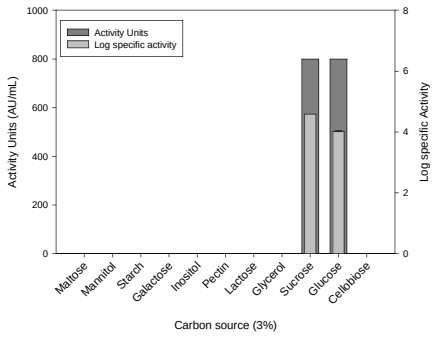


Fig: 4.10 Effect of carbon sources on peptide production by*Aspergillus fumigatus* BTMF9

4.3.5 Effect of initial pH on peptide production

Since inhibitory activity and specific activity was highest (1600AU/mL and $\log_{10}$ 4.4 ± 0.05 AU/mg protein) with pH 7, it was taken as the optimum pH for the Peptide production. At pH 4 and pH 5, less inhibitory activity was observed (400AU/mL) and at pH 6 the activity further increased to 800AU/mL. Production decreased and no production occurred when the pH was lowered and raised from seven respectively (Fig.4.11).

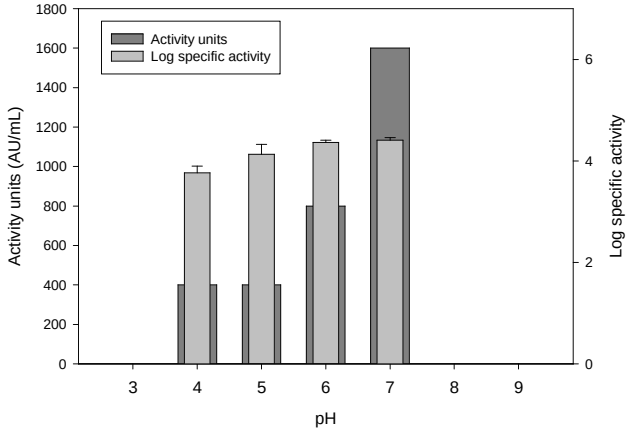


Fig: 4.11 Effect of initial pH on peptide production by BTMF9

4.3.6 Effect of sodium chloride on peptide production.

Maximum antibacterial activity (1600 AU/mL) with a specific activity of $\log_{10}$ 4.15 ± 0.06 AU/mg protein was obtained when sodium chloride was supplied at a concentration of 0.5 % (Fig.4.12). Specific activity was little higher ($\log_{10}$ 4.3 ± 0.1 AU/mg protein) when 1% NaCl was provided but inhibitory activity was only 800AU/mL. So 0.5% concentration of sodium chloride was considered optimum for peptide production. As the concentration of sodium chloride increased, the production of peptide decreased.

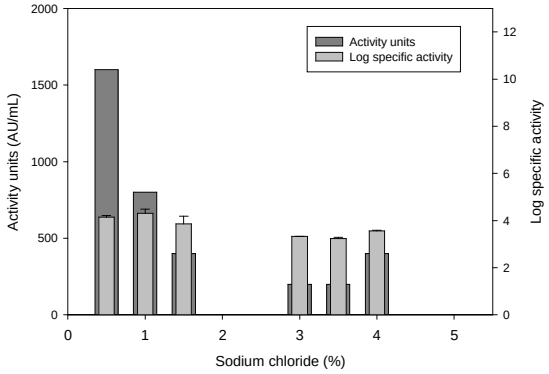


Fig: 4.12 Optimization of sodium chloride concentration for peptide production by BTMF9

4.3.7 Optimization of incubation period for peptide production

The incubation period at which maximum peptide production occurred was studied by incubating the cultures for 180 h and sampling at 24 h interval (Fig. 4.13). The peptide production started when the culture was incubated for 72 h and antibacterial activity (1600AU/mL) remained stable throughout the incubation time. However since specific activity was highest ($\log_{10}$ 4.13 ± 0.006 AU/mg protein) at 72 h, it was taken as the optimum incubation period for the peptide production.

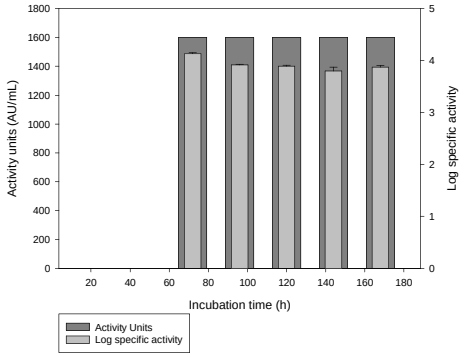


Fig: 4.13 Effect of incubation time on peptide production by BTMF9

4.3.8 Effect of agitation on peptide production

Agitation plays a major role by providing proper aeration for peptide production during submerged fermentation. Maximum peptide production of 1600AU/mL with specific activity, $\log_{10}$ 4.71 ± 0.05 AU/mg was obtained when the culture was incubated with an agitation of 150 rpm (Fig. 4.14). The activity was very low (400AU/mL) when the culture was kept static.

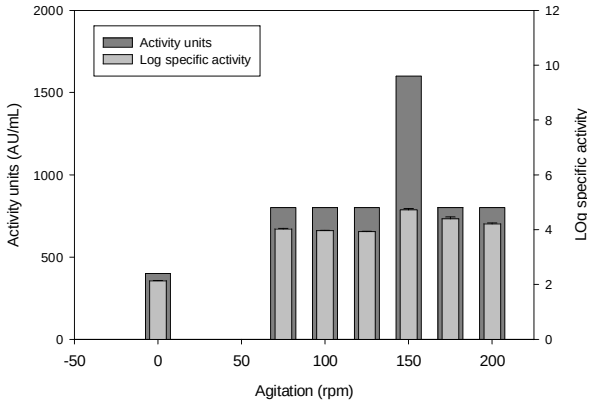


Fig: 4.14 Effect of agitation speed on peptide production by BTMF9

4.3.9 Effect of metal ions on peptide production

The effect of various metal ions on the peptide production was evaluated and presented in Fig. 4.15 . The metal salts in 2mM concentration were added to the production medium to supply following metal ions, Ca^{2+}, Cr^{2+} , Mn^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Al^{3+} for utilization of fungus for peptide production. Among all metal ions, activity was observed when Ca^{2+}, Ba^{2+} , Cr^{2+} , Mn^{2+} ,Cu^{2+} and Al^{3+} were added in the media. However a drastic increase in activity was obtained in presence of Ca^{2+} with activity of 3200AU/mL and specific activity of $\log_{10}$ 4.83 ± 0.02 AU/mg.

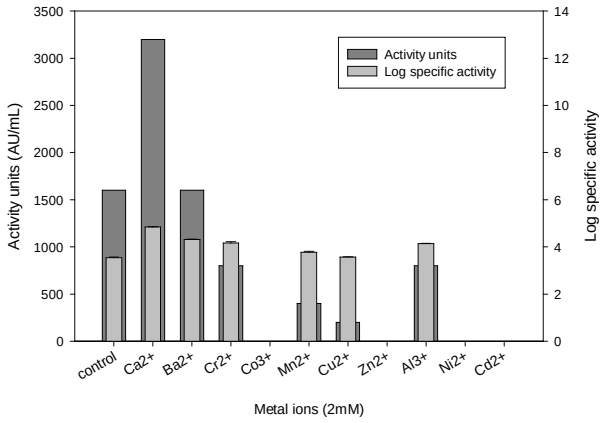


Fig: 4.15 Effect of metal ions on peptide production by BTMF9

4.3.10 Effect of additional inorganic nitrogen source on peptide production

The result presented in the Fig. 4.16 indicate that among various additional inorganic nitrogen sources tested, ammonium phosphate exhibited maximum activity, 3200AU/mL and specific activity of $\log_{10} 4.69 \pm 0.16$ AU/mg.

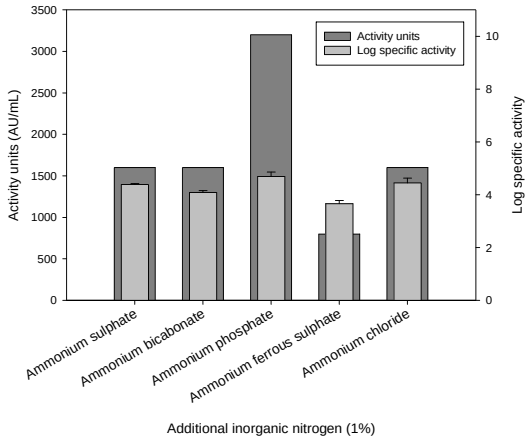


Fig: 4.16 Effect of inorganic nitrogen source on peptide production by BTMF9

4.3.11 Effect of inoculum concentration on peptide production

The inoculum concentration that supports fungal peptide production was found to be ranged from 0.3% to 1% (1600 AU/mL). However the optimal inoculum concentration for maximum peptide production was 0.4% at which antibacterial activity, 3200 AU/mL and specific activity (log_{10} 4.53 ± 0.12 AU/mg.) was maximum and all other concentrations exhibited a stationary manner with lesser activity (Fig. 4.17).

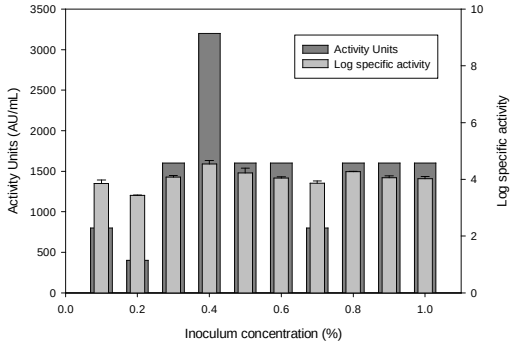


Fig: 4.17 Effect of inoculums concentration on peptide production by BTMF9

4.3.12 Time course experiment

The results obtained for the time course experiment for peptide production after medium optimization is shown in Fig. 4.18 and it is evident that a significant increase in activity was occurred in optimized medium compared to Czapek –Dox medium. The peptide production was started at 72h for both media, however the activity obtained in optimized medium (6400AU/mL) was far higher than in unoptimized medium (1600AU/mL). In optimized medium, the activity decreased after 120h whereas in other case activity was steady throughout the incubation time. Maximum specific activity was recorded on 72h for both media even though specific activity was higher in optimized medium (log_{10} 4.63 ± 0.00 AU/mg).

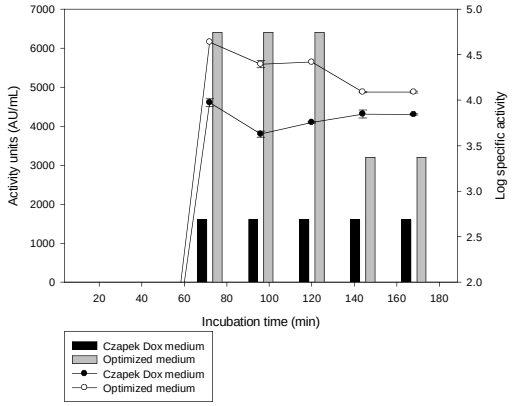


Fig: 4.18 Time course experiment for peptide production by BTMF9

4.4 PURIFICATION OF THE ANTIBACTERIAL PEPTIDE

The purified antibacterial peptide from BTMF9 was designated as MFAP9. The specific activity increased gradually after each purification process. The details of each purification steps are depicted in Table 4.5.

The proteins and peptides in the crude culture supernatant was concentrated and fractionated using ammonium sulphate precipitation. The precipitate obtained after 30- 60% and 60-90% saturation of ammonium sulphate showed antibacterial activity. There was a significant increase in activity in the 30-90% precipitated fraction and this fraction was collected in further experiment.

Table 4.4 Fold of purification of peptide MFAP9 at each step of purification

Purification step	Protein conc. mg/mL	Activity AU/mL	Specific activity AU/mg	Fold of purification
Crude	0.1	1600	16000	1*
Ammonium sulphate	0.29	6400	22068.96	1.38
Ion exchange	0.13	12800	45176.47	6.15

*Values taken arbitrarily

4.4.1Ion exchange chromatography

The dialysate obtained after ammonium sulphate saturation (30-90% fraction) was subjected to ion exchange chromatography using DEAE sepharose. Two millilitre (1.5 mg) of ammonium sulphate (30- 90%) fraction was loaded to DEAE sepharose column (25 X 1.5 cm). Peak fractions were pooled, dialysed and lyophilized. The active peptide MFAP9 was eluted out when 0.3 M NaCl containing phosphate buffer was used for elution (Fig.4.19).

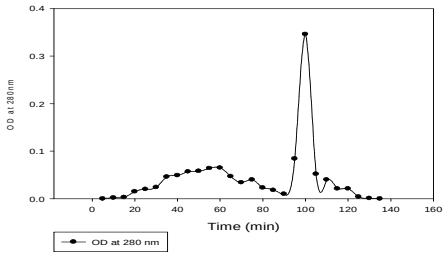
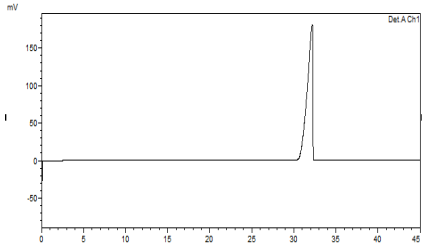


Fig: 4.19 Ion exchange elution profile of fungal peptide (0.3M NaCl eluted fraction)

4.4.2 HPLC profile of the purified peptide

The active fraction obtained after ion exchange chromatography was rechromatographed in a reversed phase HPLC system (Schimadzu LC 2010, Japan) using Phenomenex C18 column. A single homogenous peak with retention time 32.5 min was observed in the chromatogram (Fig. 4.20).



Retention time (min)

Fig: 4.20 Reverse phase HPLC profile of peptide MFAP9

4.5 CHARACTERIZATION OF THE PEPTIDE MFAP9

4.5.1 TRIS- GLYCINE SDS- PAGE

Glycine SDS- PAGE was performed to check purity of the sample after each stage of purification. The protein profile of ammonium sulphate fraction (30-90%) showed multiple bands (Fig. 4.21B). The position of peptide band inhibiting the growth of test organisms was determined using the method of agar overlaying on the gel with test organisms (zymogram). As the zone of clearing due to growth inhibition was detected at the dye front region corresponding to the marker band size 3 kDa in Fig. 4.21A, it was confirmed that the peptide MFAP9 produced by the strain *Aspergillus fumigatus* BTMF9 is a low molecular weight peptide.

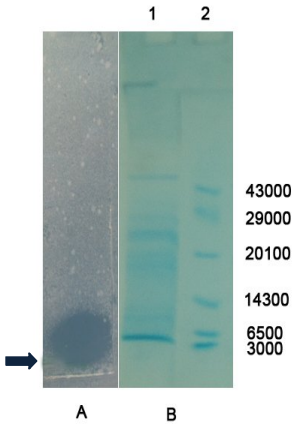
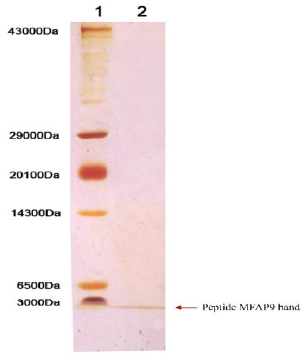


Fig: 4.21 Tris-Glycine SDS PAGE of (30-90%) fraction of the peptide MFAP9 and the agar overlay assay on gel . (B) Glycine SDS-PAGE of Ammonium sulphate fraction and (A) detection of protein band with antibacterial activity.

After purification of the peptide by ion exchange chromatography, single band was obtained at the dye front region is depicted in the Fig. 4.22. This infers purification of the peptide to homogeneity which was in same line correspond to 3 kDa band (lowermost band) of GeNei protein marker.



▼

Fig: 4.22 Glycine SDS-PAGE of the peptide after ion exchange chromatography.

Lane 1 – GeNeI protein marker; Lane 2- Purified MFAP9

4.5.2 INTACT MASS DETERMINATION BY MALDI-TOF MASS SPECTROMETRY

The intact mass of the peptide MFAP9 was determined by MALDI-TOF MS. From the mass spectrum it was evident that the mass of the peptide MFAP9 as 3.055 kDa (Fig. 4.23). The mass obtained in MALDI was in agreement with the value corresponded to the active band in SDS – PAGE gel (3kDa).

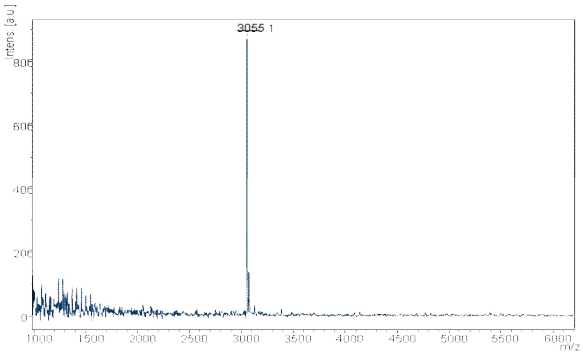


Fig: 4.23 Mass spectrum of peptide MFAP9 obtained by MALDI-TOF MS

4.5.3 Peptide mass fingerprinting

4.5.3 PEPTIDE MASS FINGERPRINTING

Peptide mass fingerprint of MFAP9 generated by MALDI-TOF-TOF analyzed with the MASCOT search tool in Swiss-Prot database (Fig. 4.24). The analysis of mass values obtained for different fragments revealed that the peptide showed some degree of similarity to FMRFamide-like neuropeptide from *Arthurdendyus triangulates* (FARP ARTTR), Cytotoxin NN-32 from *Naja naja* (3SAW NAJNA), Ranatuerin-1C from *Rana clamitans* (RN1C RANCL) , Ponericin-G4 from *Pachycondyla goeldii* (PCG4 PACGO) and Antimicrobial peptide THP3 (Fragment) of *Meleagris gallopavo* (AMP3 MELGA), suggesting that MFAP9 is a previously unknown antibacterial peptide from fungal species.

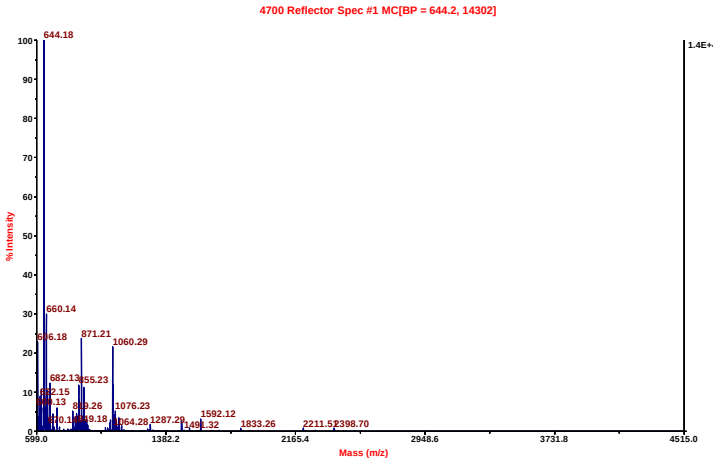


Fig. 4.24 Peptide mass finger print of the peptide

4.5.4 ISOELECTRIC FOCUSING (IEF) & 2D GEL ELECTROPHORESIS

Isoelectric focusing was carried out in pre-casted IPG strips. The peptide MFAP9 was visualized as blue coloured band on the IPG strip after staining with

Coomassie brilliant blue. The 7cm IPG strip was equally divided into seven points to represent each pH value in the range 3-10. The isoelectric point (pI) was calculated by observing the position of the band on the strip. The pI of MFAP9 was determined as 6 (Fig. 4.25a). Another strip which was not stained subjected to 2D gel electrophoresis to verify the presence of multiple proteins with same pI. The result depicted in Fig. 4.25b showed a single spot after silver staining.

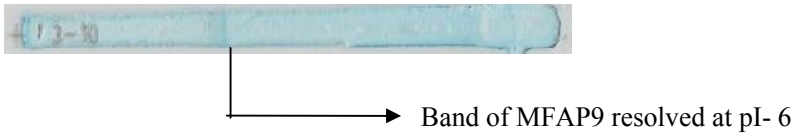


Fig. 4.25a

Fig. 4.25b

Fig: 4.25 IPG strips stained after IEF; (a) Coomassie stained IPG strip loaded with MFAP9. (b) 2-D gel profile of fungal peptide MFAP9.

4.5.5 HAEMOLYTIC ACTIVITY ASSAY OF MFAP9 AGAINST hRBC

The cytotoxicity studies of various concentrations of the peptide with human red blood cells (hRBCs) showed that the peptide exhibits no damage to mammalian RBC. The peptide was nontoxic to human RBCs even at higher

concentration (100µg/mL) than the concentration required for causing killing effects in test bacteria (Fig. 4.26) as at which the heamolysis was 0.6% only. The results clearly indicated the reliability of using MFAP9 as a therapeutic agent.

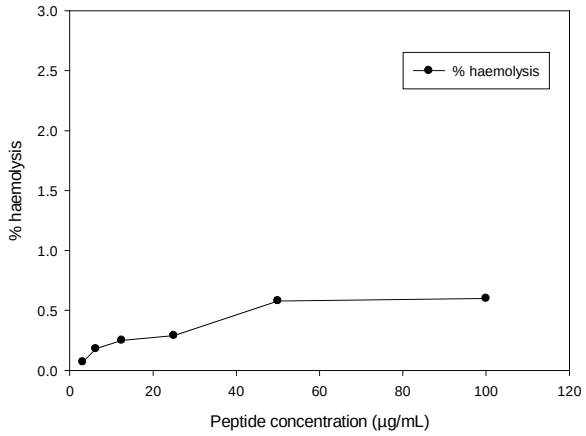


Fig: 4.26 Haemolytic assay showing % hemolysis of human RBC by MFAP9

4.5.6 EFFECT OF PHYSICO-CHEMICAL PARAMETERS ON THE STABILITY OF MFAP9

4.5.6.1 Action of proteases on the peptide

Effect of three proteases viz. trypsin, pepsin and proteinase K on the stability of the peptide MFAP9 is depicted in Fig. 4.27. When the peptide was treated with trypsin at concentrations ranging from 20-100 µg, the activity decreased even though complete loss of activity was not observed up to maximum concentration. When treated with pepsin in same concentrations, the activity of the peptide started to decrease at lowest concentration and complete lost was not observed. The proteinase K acted on the peptide in a different pattern. Only 25% activity of peptide was retained even at 40 µg concentration of proteinase K and activity was completely lost at 100 µg concentration.

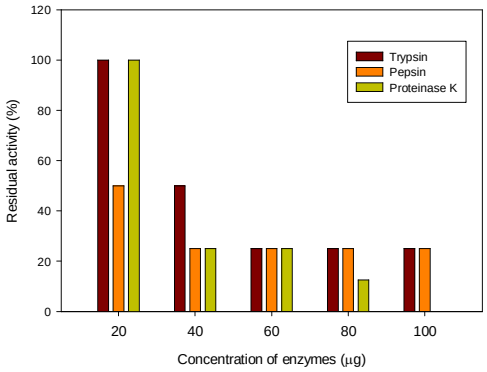


Fig: 4.27 Action of proteases like trypsin, pepsin and proteinase K on MFAP9

4.5.6.2 Effect of temperature on the stability of peptide

The effect of temperature on peptide stability was studied and depicted in Fig. 4.28. It was noticed that the peptide MFAP9 was stable over a wide range of temperature from 4°C to 90°C with optimum activity at 50°C. The peptide MFAP9 was found to be thermo stable due to the reason that 25% antibacterial activity was retained even after 1h incubation at 100°C.

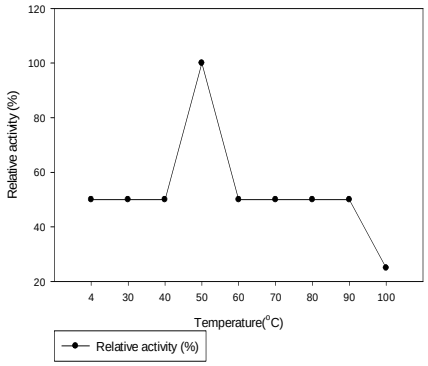


Fig: 4.28 Effect of temperature on the stability of MFAP9

4.5.6.3 Effect of pH on the stability of peptide

Studies for evaluating the effect of pH on stability of peptide MFAP9 was done using different buffer systems, showed that the peptide was stable over a wide range of pH. The peptide was found to be very stable even at very low pH, but unstable in alkaline pH range and complete loss of activity at pH 13. From the results presented in Fig. 4.29, it was inferred that there was a maximal activity was recorded at pH 4.

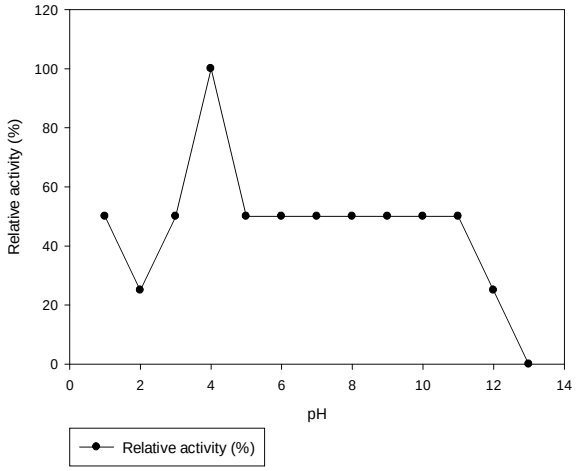


Fig: 4.29 Effect of pH on the stability of MFAP9

4.5.6.4 Effect of detergents on the stability of peptide

Non-ionic detergents are normally considered as mild detergents and that they do not interact extensively with the protein surface, whereas ionic detergents generally bind non-specifically to the protein surface. From the results presented in Fig. 4.30, it could be noted that the non-ionic detergents like tween 20, tween 80 and triton X 100 had no effect on the activity of peptide .Where as anionic detergent SDS and cationic detergent CTAB had very significant effect on peptide as complete inactivation was observed.

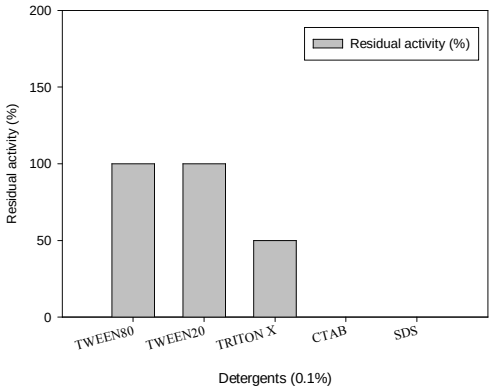


Fig: 4.30 Effect of detergents on the stability of MFAP9

4.5.6.5 Effect of metal ions on the activity of peptide

Twelve different metal ions were used for the evaluation of change of activity. The activity of peptide was enhanced (200%) in presence of Ni^{2+}, Cd^{2+} and Mg^{2+} and activity was reduced when incubated with Cu^{2+}, Al^{3+} and Na^{+}. Further it was observed that the ions of Ba^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Cr^{2+} and Ca^{2+} did not affect the activity of the peptide (Fig. 4.31).

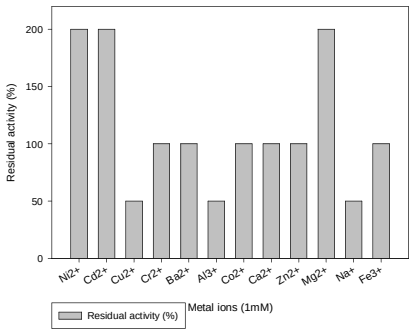


Fig: 4.31 Effect of metal ions on the stability of MFAP9

4.5.6.6 Effect of reducing agents on the stability of peptide

The effect of reducing agents such as dithiothreitol and β-mercaptoethanol on peptide stability was studied and the results were documented in Fig. 4.32. The DTT at a concentration range of 2-10 mM was used and β- mercaptoethanol was used in the range of 20-100 mM concentration. Actvity of MFAP9 remained stable till exposure upto 6 mM DTT and above that concentration the activity reduced quickly (Fig 4.32a). Whereas in the case of β-mercaptoethanol, activity started to decrease at 40mM concentration and complete inactivation occurred at a concentration of 80 mM (Fig 4.32b).

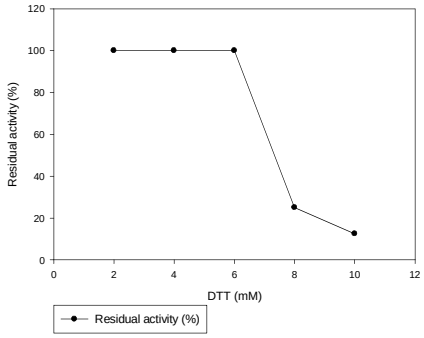


Fig. 4.32a

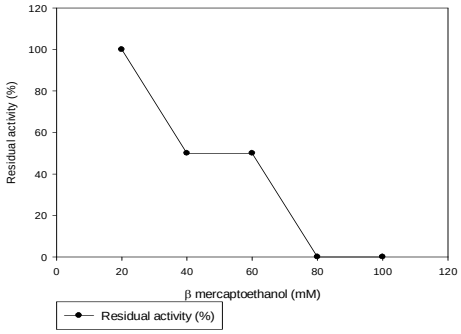


Fig. 4.32b

Fig: 4.32 Effect of reducing agents on the stability of MFAP9 (a) Effect of DTT (b) Effect of β -mercaptoethanol

4.5.6.7 Effect of oxidising agents on the stability of peptide

The oxidising agents used for stability study were dimethyl sulfoxide (DMSO) and sodium hypochlorite. The peptide was treated with 1-5% DMSO (v/v) and obtained results indicated that the activity was not affected by this oxidising agent. Whereas in the case of other strong oxidising agent sodium hypochlorite, initial decline of 50% activity was observed at concentrations 20mM and 40mM (Fig. 4.33). Then the activity further decreased to 25% when the concentration increased from 40mM to 60mM.

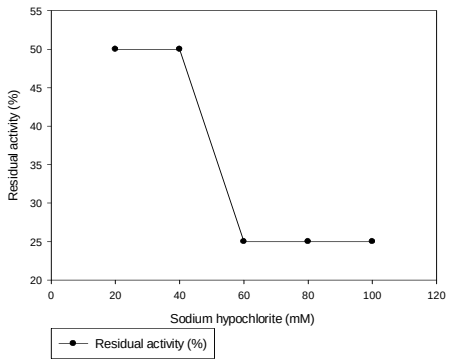


Fig: 4.33 Effect of sodium hypochlorite on the stability of BTMF9

4.5.6.8 Effect of aminoacid modifiers

Amino acids were individually modified using specific chemical modifiers and their effect on activity of peptide was determined and the results are presented in Fig. 4.34. Amino acid modifiers used for the study were PMSF, DEPC, *N*-Bromosuccinamide and Iodoacetamide. Among the four chemical modifiers used, PMSF had no effect on activity of peptide. When the peptide was modified with DEPC, the activity was drastically declined initially to 25% at 5 mM concentration itself and was found stable up to 25 mM concentration (Fig. 4.34a). Similar results were obtained in the case of reduction in activity of peptide when modified with iodoacetamide at all concentrations. Modification of tryptophan residue by N-Bromosuccinamide showed significant effect on activity

of peptide in a concentration dependent mode. Initially 50% reduction in the activity of peptide was observed at 1mM concentration of *N*-Bromosuccinamide and further increase in concentration resulted in decreased activity of peptide as only 12.5% activity was retained at 4- 5 mM concentration.

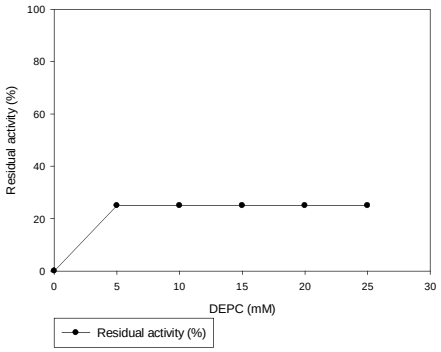


Fig. 4.34a

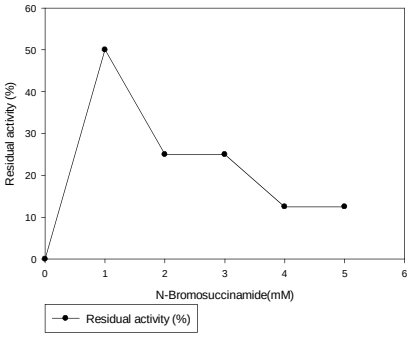


Fig. 4.34b

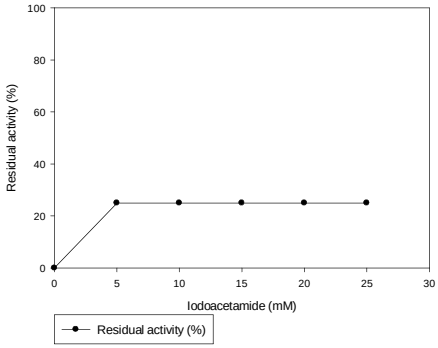


Fig. 4.34c

Fig: 4.34 Effect of chemical modification on peptide activity by a) DEPC. b) *N*-Bromosuccinamide. c)Iodoacetamide

Table 4.5 Summary of the effect of enzymes and physico-chemical parameters on the stability and activity of peptide

Physico chemical parameters	MFAP9
Temperature	Maximum activity observed at 50^{o}C and decreased at 100^{o}C
pH	Very stable at basic range of pH; complete loss of activity at pH 2 and pH- 13
Detergents (0.1%)	Reduction in activity when treated with Triton X and loss of activity with SDS & CTAB
Metal ions (1 mM)	Ni^{2+}, Cd^{2+} and Mg^{2+} promotes the action. Cu^{2+}, Al^{3+} and Na^{+} reduce the activity
DTT (2-10 mM)	Activity reduces when treated with 8 and 10mM conc.
β-mercaptoethanol (20-100mM)	Complete loss of activity when treated with 80 and 100mM conc.
Sodium hypochlorite (20-100mM)	Activity decreased at higher concentrations
DMSO (5-25mM)	No effect
DEPC (5-25mM)	Activity decreased at all concentrations
PMSF (5-25mM)	No effect
Iodoacetamide (5-25mM)	Activity decreased at all concentrations
N-bromosuccinamide (1-5mM)	Activity decreased at higher concentrations

4.5.7 MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC).

Minimum concentration of peptide required to inhibit the growth of test organism *Bacillus circulans* was determined using modified microtitre plate assay incorporating resazurin dye. The microtitre well with highest dilution of the peptide that inhibited the growth of test organism remained blue and the wells having next dilution onwards turned pink as no inhibition of the growth of test organism occurred. The protein concentration in the well was calculated by dividing the total content of purified peptide added in the first well with that of the dilution factor of the well (Fig. 4.35) and expressed in μg/mL. This value is considered as the MIC of the peptide. Here, the total protein content of MFAP9

taken was 0.7 µg and the dilution factor was found to be 3 and the MIC was determined as 0.525 µg/mL. For MBC determination, direct plating of sample on Mueller Hinton agar plates from wells that showed no apparent growth in MIC assay in microtitre plate was done and the MBC value was 4.2 µg/mL. MIC and MBC values for ampicillin which was kept as standard were 0.937 µg/mL and 3.75 µg/mL respectively.

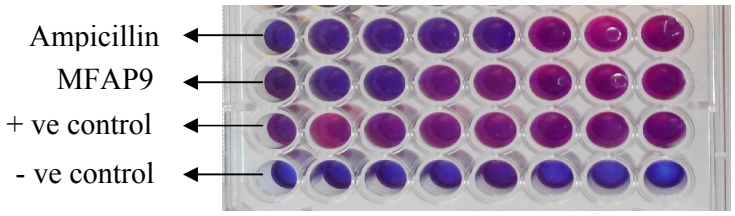


Fig: 4.35 Microtitre plate showing colour change of resazurin from blue to pink in the presence of living cells

4.6 APPLICATION STUDIES

4.6.1 EFFECT OF PEPTIDE FOR THE CONTROL OF BIOFILM FORMATION

In vitro biofilm formation by the seven test organisms under study was experimentally checked and were categorised as strong biofilm producers. The effect of peptide MFAP9 tested at 3 µg concentration on viability of established biofilms of test organisms are shown in Fig. 4.36. For antibiofilm activity assay, test organisms were cultured in 96 wells of microtitre plate allowing the formation of biofilm by the production of an extracellular matrix of various polysaccharides and macromolecules. Then purified peptide MFAP9 was applied to evaluate inhibitory effect on the biofilm growth. This study revealed that MFAP9 had significant inhibitory effect on biofilm formation of test organisms because biofilm inhibition percentage was more than 85% against all tested bacteria. MFAP9 exhibited maximum biofilm inhibitory activity against *Bacillus pumilus*

BT3 (99%) and was used for SEM analysis to substantiate biofilm inhibitory activity of peptide.

A clear image of the superficial structure and morphology of multilayered bacterial biofilm without the treatment of peptide can be seen in Fig. 4.37a. However, incubation of *Bacillus pumilus* BT3 with peptide fraction resulted in significant loss of biofilm organization (Fig. 4.37b). The aggregated cells got completely disintegrated and bacterial cells lost their original shape showing a distorted, irregular structure including the formation of grooves and pore-like lesions on the surface. Thus it was experimentally proved that the peptide perform very well in the control of biofilm formation.

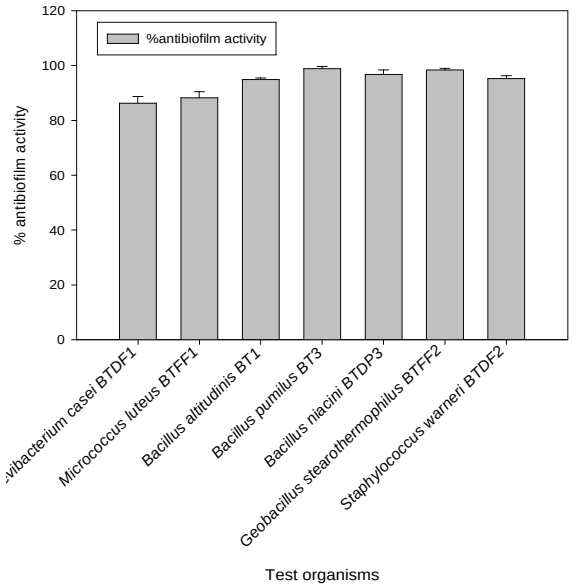
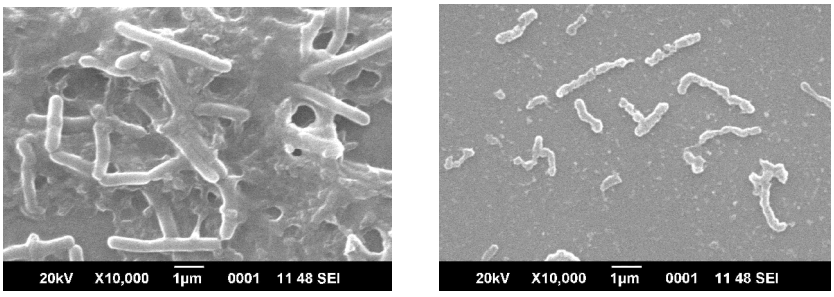


Fig: 4.36 Control of biofilm formation of strong biofilm producers by the peptide MFAP9



4.37a 4.37b

Fig: 4.37 SEM images of anti biofilm activity of MFAP9 on biofilm of *Bacillus pumilus BT3* (a) Control (b) treated with MFAP9

4.6.2 *IN VITRO* ANTIPROLIFERATIVE ACTIVITY STUDY OF MFAP9

The normal L929 fibroblast cell line and lung cancer cell line A549 was used under current study to evaluate the cytotoxic properties of peptide MFAP9 by employing standard microculture tetrazolium (MTT) assay method. The percentage viability of cells of normal L929 fibroblast cell line after peptide treatment was depicted in Fig. 4.38. The IC_{50} value of MFAP9 was determined as 113.78 µg/mL. The microscopic observation of L929 cell lines with and without peptide treatment was shown in Fig. 39 and from that it was evident that MFAP9 having less cytotoxic activity but at high concentration only.

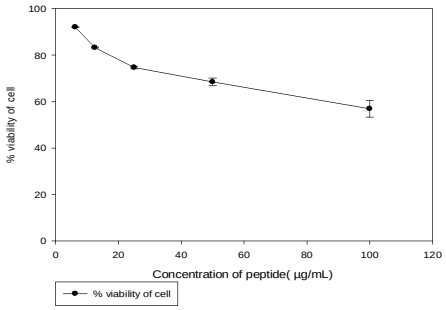
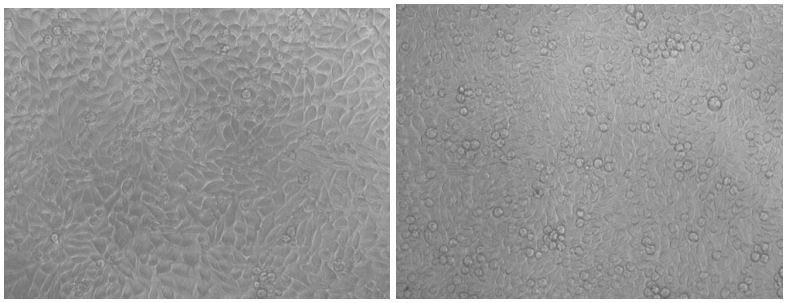


Fig: 4.38 *In vitro* cytoyoxicity study of peptide MFAP9 on normal L929 cell lines.



4.39a 4.39b

Fig: 4.39 Cytoyoxicity study of peptide MFAP9 on normal L929 cell lines. (a) Control cells (b) MFAP9 treated cell lines

A549 adenocarcinomic human alveolar basal epithelial cells were used to study antiproliferative activity of MFAP9 and Fig. 4.40 shows the percentage viability of cells after peptide treatment. The results revealed that the peptide exhibited significantly higher cytotoxicity on cancer cells and the IC_{50} value was determined as 29μg/mL. The cell viability decreased considerably in a concentration-dependent manner.

The morphological image of A549 cells after peptide MFAP9 treatment showed distorted cells with globular shape. Also cells got fragmented into membrane-bound particles producing irregular shapes that are smaller in size than the untreated cells (Fig. 4.41). It was also found that the quantities of the cells decreased when treated with MFAP9**.**

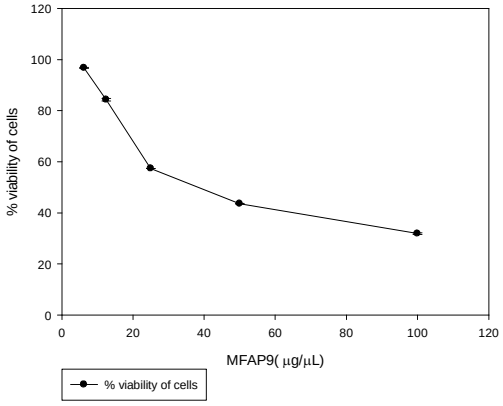
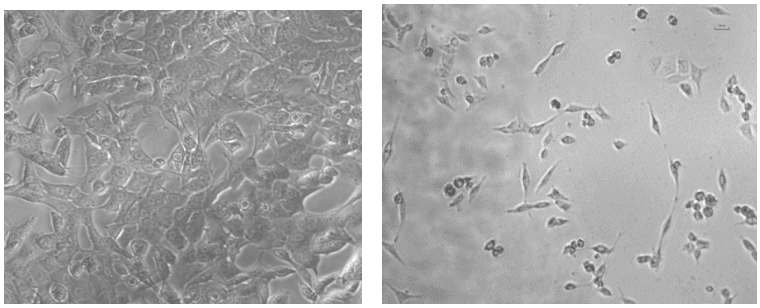


Fig: 4.40 *In vitro* antiproliferative effect of peptide MFAP9 on A549 cancer cell lines.

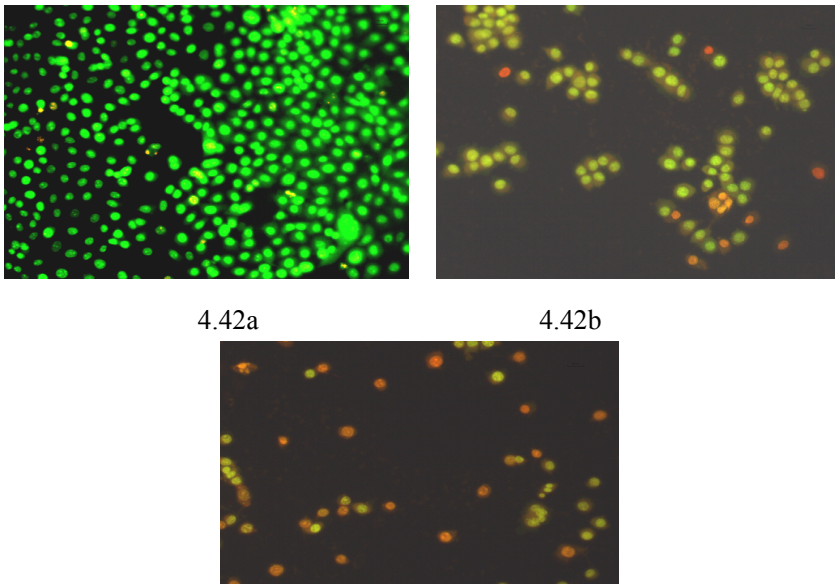


4.41a 4.41b

Fig: 4.41 Cytoyoxicity of peptide MFAP9 on A549 cancer cell lines.
(a) Control cells (b) MFAP9 treated cell lines

The type of cell death induced by MFAP9 in A549 cells was further assessed by investigating the morphological changes after double staining with acrydine orange (AO) and ethidium bromide (EB). As in (Fig. 4.42) AO penetrates into living cells, emitting green fluorescence due to intercalation into DNA while EB emits red fluorescence in the cells with an altered cell membrane.

Fluorescence microscopic analysis revealed that features of apoptosis such as chromatin condensation and alterations in the size and the shape of cells after treatment with MFAP9 at a concentration 50 µg/mL. The peptide treatment also resulted in the depletion of cell count. From the results it was evident that necrosis occurred at high concentration as increased percentage of necrotic red fluorescent cells were observed after treatment with 100 µg/mL concentration of MFAP9.



4.42a 4.42b

4.42c

Fig: 4.42 Morphological changes in A549 cells after treatment with MFAP9 and then staining with acridine orange (AO)/ethidium bromide (EtBr). (a) Control cells (b) 50µg/mL MFAP9 treated cell line (c) 100µg/mL MFAP9 treated cell line

These data indicated that the peptide MFAP9 caused damage to normal cell line only at higher concentration (IC_{50} - 113.78 µg/mL) while cytotoxic to cancer cell line at lower concentration (IC_{50} - 29 µg/mL) . The apoptotic effect on cancer cell line suggests the possible application in cancer therapy.

4.6.3 ANTIOXIDANT ACTIVITY STUDY OF MFAP9 USING DPPH ASSAY

The DPPH radical scavenging activity of the antibacterial peptide MFAP9 compared with that of standard antioxidant ascorbic acid. The results revealed that the peptide had a little higher scavenging activity compared to standard antioxidant at same concentration level. From the Fig. 4.43, it was apparent that activity of both standard and peptide was based on a concentration dependent manner.

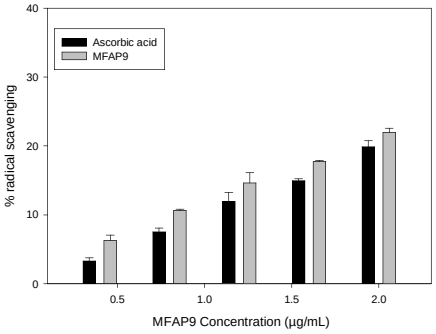


Fig: 4.43 DPPH assay using standard ascorbic acid and peptide MFAP9.

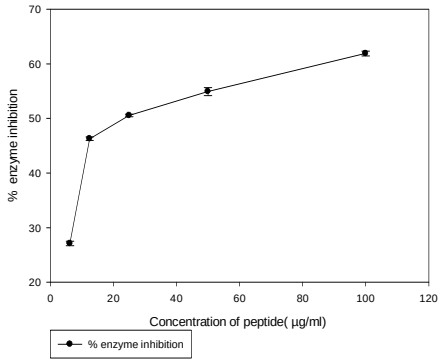


Fig: 4.44 Anti-inflammatory assay derived by spectroscopic analysis: Inhibition of COX-2 by MFAP9.

4.6.4 DETERMINATION OF *IN VITRO* ANTI-INFLAMMATORY EFFECT OF MFAP9 ON CULTURED THP1 CELL LINES

LPS stimulated THP1 cells were exposed with different concentrations of peptide sample. The spectroscopic analysis showed loss of activity of inflammatory enzyme COX-2 (Fig. 4.44) when culture supernatant incubated with peptide MFAP9 in the presence of the substrate, arachidonic acid revealed the anti-inflammatory property of MFAP9 by competing with the substrate. The IC_{50} value was determined to be 24.8 µg/mL.

DISCUSSION

Antimicrobial peptides are a universal feature of the defence systems of virtually all forms of life. They form part of the nonspecific innate immune system, which is the primary defence system for the majority of living organisms (Jenssen *et al*., 2006). In many cases, the primary role is to kill the invading pathogenic organisms, however, it is recognized as modulators of the innate immune response in higher organisms. The present study focuses on peptide antibiotics that are widespread in nature where they play an important role in the innate immune systems protecting living organisms from microbial infection. The advantage of peptide antibiotics is the capability to neutralize bacterial toxicity by disrupting the outer cell membrane and thereby causing bacterial cell death.

5.1 SCREENING OF FUNGI FOR ANTIBACTERIAL PEPTIDE AND IDENTIFICATION OF THE SELECTED STRAIN

Fungi proved to be capable of biosynthesizing metabolites bearing obvious structural diversity, which could be further enlarged by structure modification. Over the years, numerous fungal defensins have been identified, mostly within the phyla of *Ascomycota* and *Zygomycota*. These findings point out that antimicrobial peptides (AMPs) are key components of the innate immune system of fungi active against competing microbes. Only a limited number of peptides with antibacterial activity have so far been obtained from filamentous fungi even though this restraint family represents a valuable source of antimicrobial peptides. The first isolated antifungal peptide, called AFP, was produced by *Aspergillus giganteus* (Moreno *et al*., 2005). Secondly, antifungal peptide PAF, was isolated from *Penicillium chrysogenum* and later viz. antifungal peptides like AnAFP isolated from *Aspergillus niger* , NAF peptide identical to PAF isolated from *Penicillium nalviogense,* AcAFP, an antifungal peptide from *Aspegillus clavatus* VR1 strain were identified (Odell, 2002; Osusky *et al*., 2004; Ki *et al*., 2013). All these reports dealt with the antifungal activity and no

antibacterial activity was described for these strains. Plectasin from the saprophytic fungus *Pseudoplectania nigrella* (Mygind *et al*., 2005), micasin from the dermatophytic fungus *Microsporum canis* (Zhu *et al*., 2012)), AcAMP from *Aspergillus clavatus* ES1 **(**Hajji *et al*., 2010), eurocin from *Eurotium amstelodami* (Oeemig *et al*., 2012) and copsin from *Coprinopsis cinerea* (Essig *et al*., 2014) are some of the peptides showing antibacterial activity hitherto studied.

Current study was focused on the production, purification and characterization of an antibacterial peptide from a fungus isolated from the marine environment. Marine environment represents an enormous pool of microbial biodiversity as 70% of the earth's surface is covered by the ocean representing 80% of life on earth; oceans have been explored to a much lesser extent and therefore open the possibility for discovery of new natural products (Kennedy *et al*., 2008). Among marine microorganisms, principally fungi have gained an important role as a source of biologically active secondary metabolites (Gamal-Eldeen *et al*., 2009). Das *et al*.(2009) reported the isolation and identification of 90 different deep sea fungal isolates, where *Aspergillus* was found to be the dominant genus (33%) followed by *Penicillium* (13%) *Lulworthia* (8%), others (40%) and non-sporulating fungi (6%).

Primary screening with a total of one hundred and twenty isolates was done in the present study for their antibacterial potency, among them fifteen isolates inhibited the growth of gram-positive/ gram-negative bacteria. Fungal isolates were grown in Czapek- Dox media at 28 ℃ in a shaking incubator (Orbitek, Scigenics, Chennai, India) for 5 days at 150 rpm. Fungi as a source for AMPs has not been much exploited. Competition among microbes for space and nutrient is the dominant selection force that flourishes marine microorganisms with the ability to produce natural products possessing industrial and medical values. The preliminary screening of extracts from a total of fourteen fungal strains for antimicrobial activity showed that eleven strains possessed antibacterial activity (Fagade & Oyelade, 2009). Similarly, Barakat & Gohar ,(2012) screened

26 fungal isolates collected from different locations of Alexandria coast, Egypt to obtain new biologically active metabolites against some virulent fish pathogens (*Edwardsiella tarda, Aeromonas hydrophila, Vibrio ordalli and Vibrio angularium*) and the strain *Aspergillus terreus* var. africanus was identified as the most potent isolate. In another study, the preliminary screening of 43 endophytic and epiphytic fungi isolated from the intertidal zone of the Fujiazhuang coastline (121° 36′ 13.82″ E, 38° 48′ 36.66″ N) of Dalian revealed that 88.4% of the total isolates possessed inhibitory activities against one or more test organisms, 79.1% inhibited gram-positive bacteria (*Staphylococcus epidermidis* and/or *Bacillus subtilis*), 62.8% inhibited gram-negative bacteria (*Pseudomonas fluorescens*, *P. aeruginosa*, and/or *Escherichia coli*), and 48.9% inhibited both (Zhang *et al* ., 2009).

The secondary screening found that ammonium sulphate precipitated fraction obtained from only a single marine isolate BTMF9 displayed proteinaceous compound with antibacterial activity among other fungal isolates shortlisted after primary screening. The peptide in the ammonium sulphate precipitate inhibited the growth of only gram-positive test organisms, majority of which belonged to genus *Bacillus* viz. *Bacillus circulans*, *B.cereus*, *B.coagulans*, *B.pumilus* and *Staphylococcus aureus* was *a*lso inhibited. *Bacillus circulans* was selected for further activity assays as activity indicator organism because it showed much more consistent zone of inhibition. Similarly, ammonium sulphate precipitated fraction from *Streptomyces fulvissimus* had no activity against gram negative bacteria (Malik *et al.*, 2008) and also a fungal defensin eurocin was found to be active even at low concentrations against gram-positive bacteria rather inactive against gram-negative bacteria used for antibacterial activity assay (Oeemig *et al.*, 2012). The exAP-AO17 protein from *Aspergillus oryzae* strongly inhibited pathogenic microbial strains, including pathogenic fungi, *Fusarium moniliform* var. *subglutinans* and *Colletotrichum coccodes*, showed antibacterial activity against bacteria, including *E. coli* O157 and *Staphylococcus aureus* (Park

et al., 2008). From the growth inhibition assay, the strong inhibitory activity of peptide in liquid broth was clear against the test organisms *S. aureus*, *B. circulans*, *B. coagulans* , *B. cereus* and *B. pumilus*. Most of the bacteriocins from *Bacillus* sp. showed a bactericidal effect (Naclerio *et al*., 1993; Hyronimus *et al*., 1998; Cherif *et al*., 2003; Gray *et al.*, 2006 ; Sharma *et al*., 2006; Aunpad & Na-Bangchang, 2007) except in some cases like bacteriocin from *Lactococcus lactis* exhibited bacteriostatic effect (Ivanova *et al.*, 2000).

The isolated fungus in this study was determined as *Aspergillus fumigatus* BTMF9 by observing colonial and microscopic morphology as well as by molecular analysis of fungal rDNA at the sequence level. The rDNA internal transcribed spacer (ITS) region was amplified with the conserved fungal primers ITS1 and ITS4 and the partial nucleotide sequence obtained after sequencing was of 500 bp. The identity of the sequence was determined by BLAST software and the resultant sequence showed 100% identity with already available sequences of *Aspergillus fumigatus* in the GenBank (Altschul *et al.*, 1990). For molecular identification, nowadays the most commonly sequenced DNA region in fungi is ITS region as it has been possibly most useful for molecular systematics at the species level, and even within species level identification **(**Bhimba *et al*., 2012). Among the regions of the ribosomal cistron, the ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. The eukaryotic rRNA cistron consists of the 18S, 5.8S, and 28S rRNA genes transcribed as a single unit by RNA polymerase I and the post transcriptional processes split the cistron, removing two internal transcribed spacers. These two spacers, including the 5.8S gene, are typically referred to as the ITS region (Schoch *et al.*, 2012). Phylogenetic tree was constructed with already reported strains by using neighbour joining method to study their relatedness or variability. It was apparent from the phylogram that the peptide producing strain BTMF9 in the present study grouped together as clade with all other selected strains. But,

within the clade the selected potent strain BTMF9 remained as a separate unique isolate relative to other strains.

The fungal genus *Aspergillus* has been reported to produce a considerable number of cytotoxic compounds as well as other bioactive compounds. The mold *Aspergillus giganteus*, isolated from the soil of a farm in Michigan, has been reported to produce a basic low-molecular weight protein (51 amino acids) showing antifungal properties, the so-called antifungal protein (AFP) (Nakaya *et al*., 1990). The filamentous fungus *Aspergillus fumigatus* produces a variety of enzymes and toxins. Antibacterial substances fumigacin and fumigatin (Waksman *et al*., 1942) and gliotoxin (Menzel *et al*., 1944) were the earlier reported bioactive compounds from this fungus. Though *Aspergillus fumigatus* also known to produce various bioactive metabolites like an extracellular polysaccharide named AFEPS, pyripyropenes (strong inhibitor of acyl-CoA cholesterol acyltransferase) from *Aspergillus fumigatus* FO-1289 (Sunazuka *et al*., 2009), fumagillin with therapeutic potential for topical application in cases of microsporidial keratoconjunctivitis in humans (Grossnikiaus *et al*., 1993), there was no reports regarding antibacterial peptide production have been noticed in literature.

As hemolysis on blood agar plate is an indication of the potential for pathogenesis, the selected strain BTMF9 was spotted on blood agar plate and was found to be non-hemolytic, indicating its non-pathogenic nature. The hemolysin produced by *A. fumigatus* (asp-hemolysin) promotes Aspergillosis or may promote opportunistic infections (Ebina *et al*., 1982; Young *et al*., 1970). Moreover, it was indicating that the various hemolysin can induce genotoxicity of dietary carcinogens *in vitro* considering that the degree of induction was strongly species dependent (Ebina *et al*., 1982; Ebina *et al*.,1994; Berne *et al*., 2005). Some other studies pointed out that hemolysin may not be a main virulence factor but a compound that increases the effects of other toxic factors involved in pathogenicity (Ebina *et al*., 1982; Fukuchi *et al.*, 1996; Maličev *et al*., 2007).

5.2 STUDIES ON EFFECT OF BIOPROCESS VARIABLES BY 'ONE-FACTOR AT-A-TIME' METHOD

The fungal producers may be capable of changing the nature of the end synthesized products depending on the environmental conditions, moreover an increase in amount of bioactive compounds may be found after optimization of cultural conditions. Though the metabolite biosynthesis in microbes is strongly controlled by regulatory mechanisms to avoid overproduction, these regulatory mechanisms often limit the discovery of novel metabolites by affecting the yield of bioactive metabolites. A well understanding of the effect of culture conditions on biosynthesis may direct to better utilization of microbial sources. The availability of reports are fewer regarding the studies on effect of nutritional and cultural conditions on mycelial growth and antimicrobial metabolite production by the antagonistic fungal strains (Vahidi *et al*., 2005; Gogoi *et al*., 2008; Ritchie *et al*., 2009; Jain & Pundir, 2011).

Initially the culturing of *Aspergillus fumigatus* BTMF9 was done in liquid broth of Czapek-Dox minimal media for five days in a shaker incubator (150rpm) at 28°C. After incubation, the filtrate was collected and antibacterial assay and protein estimation were done. The use of different parameters in the same culture medium led to variations in the antimicrobial activity of the *A. fumigatus* obtained extracts (Furtado *et al*., 2002). Optimization of culture conditions was the next step towards getting maximum peptide yield in the present study. The variables studied were temperature, inorganic nitrogen source, organic nitrogen source, carbon source, initial pH, sodium chloride, incubation period, agitation, metal ions, additional inorganic nitrogen source and inoculum concentration.

Obtained results in the current study indicated that the peptide production was observed to be comparatively more at an incubation temperature of 30°C. The loss of production and decrease in growth was observed as incubation temperature increased. Adinarayana *et al.* (2003) reported that maximum cephalosporin production by *Acremonium chrysogenum* was reached at 30°C, but in the case of

antibiotic production by a marine fungus *Penicillium citrinum* S36 through solid state fermentation, the production was maximum at 25℃. B**e**sides, decrease in bacteriocin production with increase in incubation temperature was reported by Iyapparaj *et al*. (2013).

Amongst various inorganic nitrogen sources used, sodium nitrate was found to be the most significant for influencing the antibacterial peptide production. From this result it can be inferred the fungus is highly specific for inorganic nitrogen. The other sources studied didn't allow the fungus to grow sufficiently for the production. Then the assessment of effect of organic nitrogen sources was done which results in the addition of organic nitrogen sources were less effective and showed more biomass production, but very less or no peptide production. But in presence of ammonium phosphate as an additional nitrogen source, maximum peptide production was observed. The results obtained in the case of organic nitrogen sources were not much significant and have to assume that the inorganic nitrogen sources mostly favour this fungus for metabolite production. Thakur *et al*. (2009) inferred that asparagine was found to be good nitrogen source and some sources didn't support the growth of *Streptomyces* sp. 201 for antimicrobial metabolite production. A study by Jain & Pundir, (2011) on effect of nitrogen source on antimicrobial metabolite production by *Aspergillus terreus* revealed that, sodium nitrate favoured maximum production.

Knight *et al*. (2003) recorded that the presence or absence, as well as the difference in concentrations of media components (peptones, yeast extract and vitamins) may lead to the biosynthesis of different compounds. An another study revealed that potassium nitrate was a good inorganic nitrogen source on the antimicrobial agent production by a fungal isolate SS2, whereas no obvious effect was detected when organic nitrogen sources were added. It was well known that in most microorganisms both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. Though, it was found that some carbon and nitrogen sources had an

inhibitory effect on the production of antimicrobial compounds, may be due to organic acid accumulation, oxygen depletion or sugar catabolic repression (Rajasekar *et al.*, 2012).

Addition of glucose and sucrose resulted in maximum production of peptide in the present study but specific activity was greater when sucrose was added as carbon source and this was fixed as carbon source for further optimization. Conversely, other sources such as starch, mannitol, lactose, galactose, inositol, maltose, cellobiose, pectin and glycerol did not support the production of peptide. Furthermore previous reports suggested that the addition of glucose resulted in peak growth of the fungus, but significantly in many fermentation processes higher concentration of glucose has a suppressive effect on production of bioactive metabolites (Gogoi *et al.*, 2008). When sucrose was used as sole carbon source at a concentration of 2.0 g/L was seemed to be optimum for maximum growth and bioactive metabolite production by *Aspergillus* strain TSF 146 (Bhattacharyya & Jha, 2011).

The peptide production in this study was found to occur at initial pH varying between 4 and 7, where pH 7 was observed to be optimum for the production while no production occurred above optimum pH value. It has been reported that optimum production of bioactive agents by *Streptomyces violates* (Hassan *et al.*, 2004) and *Pseudonocardia* sp. VUK-10 (Kiranmayi *et al.*, 2011) occurred at pH 7.0. Optimization studies for growth and bioactive metabolites production by *Fusarium* sp. DF2 revealed that initial pH 6 of the medium was found to be optimal and also seen that the neutral pH supported the growth (3.2 mg/ml) and bioactive metabolite production of the strain. In addition, there was no growth observed at pH <3 and pH >11 (Gogoi *et al.*, 2008).The production of antibacterial metabolite was maximum when *Aspergillus terreus* grown in potato dextrose broth at pH 6.0 (Jain & Pudir, 2011). Variations in pH during fermentation processes strongly influence many microbiological processes and

transport of various components across the cell membrane (Cuomo *et al*., 1995; Christophersen *et al*., 1998).

Maximum production of antibacterial peptide in the present study was obtained when sodium chloride was supplied at a concentration of 0.5 % to the medium. Specific activity was little higher when 1% NaCl was provided but inhibitory activity was very less. The concentration of 0.5% sodium chloride was considered optimum for peptide production. As the concentration of sodium chloride increased, the production of peptide decreased. Results in the present study displayed an agreement with the results recorded by Bhattacharyya & Jha, (2011), where the NaCl concentration of 0.5% was found to be optimal for maximum mycelial growth and active metabolite production by the strain TSF 146, whose values inversely related to salt concentration in the basal media. Optimization of NaCl concentration for the production of antimicrobial agents by *B. licheniformis* SN2 was done by El- Sersy *et al*. (2010) and found that the optimum NaCl concentration was 0.3%.

From the result, it was noted that the peptide production was started on the 3rd incubation day (72 h) and remained in a stationary mode till the last incubation time studied (168 h). As the specific activity was highest at 72 h, that particular incubation time was considered as optimum for peptide production in present study. Atalla *et al*. (2008) showed that the antibiotic compound production by a marine isolate *Varicosporina ramulosa* increased gradually until reached the maximum after 8 days and then decreased. Kuznetsova *et al*. (1998) recorded the maximum production of bioactive compounds from marine *Cladosporium sphaerospermum* after 8 days of incubation. The requirement of 9 day of incubation for the production of diketopiperazines was reported by Zheng *et al*. (2009). Earlier optimization studies illustrated that maximum antimicrobial agent production was achieved after 4 days of incubation of *Cladosporium* sp. (Miao & Qian, 2005), 5 days of incubation of *Penicillium corylophilum* (Silva *et al*., 2004) and 14 days of incubation of *Penicillium waksmanii* (Petit *et al*., 2004).

In shake flask cultures agitation is an important factor to get a growth and production of the compounds. It was observed that agitation at almost all rotation rate ranging from 50 to 200 rpm gave a stable yield but it was higher for 150 rpm. At static culture conditions the production was very low. Higher production under shake culture conditions compared to static condition indicating the supportive role of oxygen in peptide production. The generation will be increased if agitation is provided. For instance, the effect of agitation, temperature and initial pH on *Lentinula edodes* (Berk.) Pegler, the shiitake mushroom was studied and the result agreed with the general concept that aeration enhances fungal growth. Leatham & Griffin (1984) related that *L. edodes* growth rate in liquid media increased under agitation and the generation time was 1.5 days (Hiroko Hassegawa *et al*., 2005).

Effect of various metal ions on production of the peptide was studied and calcium was observed to produce a higher yield and this was incorporated into the optimized medium. In case of enzymes and its subunits metal ions is a limiting factor. But for peptides this may not be a critical factor. An eight-fold decrease in the activity of microplusin was observed when the medium was supplemented with Cu^{2+}, nonetheless, the antimicrobial activity of microplusin didn't affect when other metal ions such as Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} were added to the media. Overall analysis of the data indicated that the peptide exerts its activity by chelating copper ions. As a consequence the disruption of copper homeostasis by microplusin results in deleterious changes of the bacterial respiratory profile (Silva *et al*., 2004).

Inoculum concentration is one of the important factors in the growth of the fungal mycelia and production of the compound. The medium should contain sufficient nutrients for the growth of the fungus. If inoculum concentration is very much higher this may lead to competition and the culture may attain a stable phase and this may lead to death of the fungus, thus production efficiency may get reduced. The lower inoculum concentration may not provide sufficient growth in the time period of the study. There the concentration of inoculum is a limiting

factor. The optimum inoculum concentration of 0.4% was observed to be favourable for production of peptide MFAP9.

Time course experiment with optimized and unoptimized media was performed and it was noted that a several fold increase in production of peptide occurred in optimized medium. The maximal antibacterial acivity as well as specific activity was observed on the 3rd day of incubation in both media. The time course for antimicrobial agent production varies according to the strain and cultivation conditions. As evidenced that the maximum antimicrobial agent production was achieved after 4 days of incubation of *Cladosporium* sp (Miao & Qian, 2005), 5 days of incubation of *Penicillium corylophilum* (Radu & Kqueen, 2002) and for a fungal isolate SS2, production seems to be stable on the 5th and 6th days of the incubation with maximum production on the 5th day (Rajasekar *et al.*,2012). A typical time course study between statistically optimized and unoptimized medium was compared in case of exopolysaccharide (EPS) production by *Schizophyllum commune* AGMJ-1 for 18 days in shake flask conditions and found that under statistically optimized medium there was a 4 fold increase in EPS production was obtained (Joshi *et al*., 2013).

5.3 PURIFICATION AND CHARACTERIZATION OF PEPTIDE

Standard purification methods were used to separate out the fungal peptide from crude extract. The fungal mycelium was separated out by filtration and this was then subjected to ammonium sulphate precipitation for concentrating antibacterial peptide. Precipitate collected after dialysis was assayed for antibacterial activity and a significant increase was observed. It was further purified to homogeneity by ion exchange chromatography using DEAE sepharose column. A step by step increase in the specific activity was observed in each stage of purification. After ammonium sulphate precipitation, the partially purified fraction showed a 4 fold increase in antibacterial activity. The bound peptide from anion exchange column was eluted with 0.3M NaCl containing phosphate buffer

(pH 7) resulted in single peak. Then it was observed that peptide MFAP9 was six fold purified after ion exchange chromatography.

Purification includes combination of several strategies. Based on differences in molecular mass or charge, a further purification may then be obtained respectively by gel filtration or ion exchange chromatography. Same conditions were used for the elution of 50 kDa antifungal protein from *Sesbania virgata* (Praxades *et al*., 2011). From *Aspergillus aculeatus*, aspergillusol and AcAFP from *Aspergillus clavatus* were separated using C18 reverse phase HPLC (Ingavat *et al*., 2009; Skouri-Gargouri & Gargouri, 2008). An antimicrobial protein of 17 kDa was isolated from growth medium containing the filamentous fungus *Aspergillus oryzae* by extracting the supernatants from the culture media, ion exchange chromatography on CM-sepharose, and C18 reverse-phase high-performance liquid chromatography (Park *et al*., 2008). Purification of PcPAF of *P. citrinum* W1 was carried out by initially loading the ammonium sulphate precipitated fraction onto a DEAE Sepharose Fast Flow column followed by the reloading of partially purified fraction onto a CM Sepharose Fast Flow column (Wen & Chen, 2014). An earlier report by Chen *et al.* (2013) also revealed that same procedure was done for purifying Pc-Arctin from Arctic *P. chrysogenum*.

The analysis of purity of sample was further confirmed by employing analytical RP-HPLC, where a single peak with a retention time of 32.5 min. was obtained. Most known antimicrobial peptides have been purified mainly by using reversed phase chromatography. Some antimicrobial substances are very hydrophilic and will consequently not be retained on reversed phase column followed by the purification of an antioxidant protein by gel filtration chromatography. Huang *et al*. (2010) used RP-HPLC of high resolution for the identification of protein purity and observed that the protein G4b had only one elution peak in RP-HPLC, with the retention time of 16.4 min.

Characterization of the purified peptide was done using polyacrylamide gel electrophoresis. Approximate molecular mass was determined using a protein

marker along with the sample of our interest in a SDS-PAGE gel and either silver staining method or Coomassie brilliant blue staining was carried out to detect the active peptide band in the gel after electrophoresis. Approximate molecular mass of the peptide was observed to be 3 kDa and this was confirmed using zymography, where bacterial growth inhibitory zone was observed to be corresponded to the 3 kDa band of protein marker. Moreover, the intact molecular mass of MFAP9 was determined by MALDI-TOF MS as 3.055 kDa showed an unambiguous agreement with the relative mass indicated by mobility on SDS-PAGE gel. Similarly, a recently discovered peptide eurocin from fungus has a molecular mass of 4.3 kDa (Oeemig *et al*.,2012). Whereas the molecular mass of the extracellular, defensin-like antifungal proteins secreted by filamentous fungi ranges from 5.8 kDa to 6.6 kDa (Galgóczy *et al*., 2010). In *Aspergillus oryzae* a peptide with similar mass was characterized using SDS-PAGE and the effect of various culture conditions for production was also studied (Park *et al*., 2008). Peptide mass fingerprint of MFAP9 was analysed with the MASCOT search tool in Swiss-Prot database and one of the fragments of the peptide showed similarity to FMRFamide-like neuropeptide from *Arthurdendyus triangulates*, Cytotoxin NN-32 from *Naja naja* , Ranatuerin-1C from *Rana clamitans*, Ponericin-G4 from *Pachycondyla goeldii* and Antimicrobial peptide THP3 (Fragment) of *Meleagris gallopavo*. However, the peptide under study showed limited homology with existing peptides in the database and therefore it can be a novel peptide having distinct structure and sequence homology. In an earlier report, MASCOT search tool analysis of the mass spectra obtained after peptide mass fingerprinting of an inhibitor isolated from *Solanum tuberosum* did not show resemblance to any of the inhibitors of other plants (Obregón *et al*., 2012).

The isoelectric point (pI) of the peptide was carried out using isoelectric focusing and a single band was obtained at about a pI value of 6. However, the pI value of PgAFP produced by *Penicillium chrysogenum* was estimated as 9.22 according to the electrophoretic mobility of marker proteins (Rodríguez-Martín *et*

al., 2010). Besides, the theoretical pI values of most of the defensin-like antifungal proteins secreted by filamentous fungi were found to be in a range from 7.14 to 9.27 (Galgóczy *et al*., 2010).Thus the obtained pI of 6 in the present study highlights the unique character of MFAP9. To confirm the purity of peptide, two dimensional (2-D) gel electrophoresis was performed, displayed the resolved peptide as a single spot on SDS- PAGE gel.

Hemolytic activity is considered to be a factor of toxicity and thereby act as a limiting factor for the development of therapeutic compounds. Earlier reports suggested that the testing of those activities like hemolytic or hemagglutination remains a fundamental strategy for determining safety and selectivity of proteins for potential applications (Theis & Stahl 2004). Proteins which are non-cytotoxic to RBCs can be preliminarily regarded as safe (Sila *et al.,* 2014). So the present study was focused on the cytotoxicity of peptide against hRBCs and proved to be non haemolytic even at a higher concentration of 100 µg/mL. An extracellular antimicrobial protein from *A. oryzae* (exAP-AO17) possessed antimicrobial activity but lacked hemolytic activity (Park *et al*., 2008). Similarly, the cytotoxicity of Pc-Arctin was assessed by Chen *et al*. (2013), for that hemagglutination and hemolysis assays were done using RBCs from rabbits, rats, and guinea pigs and found that both hemagglutination and hemolytic activities were nil at the studied concentrations of Pc-Arctin (0.3 to 20 µg/mL).

The stability of peptide towards the action of proteases was studied using different proteases like proteinase K, pepsin and trypsin. It was observed that treatment with trypsin and pepsin resulted in the reduction of antibacterial activity rather than complete loss of activity. But activity of peptide disappeared at high concentration of proteinase K. Thus the obtained result showing sensitivity of peptide towards proteases confirmed its proteinaceous nature. In case of plectasin, its activity against *S. aureus* was not affected by pepsin and papain (Zhang *et al*., 2011). Cheikhyoussef *et al.* (2007) reported partial inactivation of a 3.0 kDa bacteriocin of *B. infantis* BCRC 14602 after treatment with proteolytic enzymes.

Purified antimicrobial peptide AcAMP from *Aspergillus clavatus* ES1 was treated with several enzymes and its antimicrobial activity was assayed against *Bacillus cereus*. The results indicated that the antimicrobial substance was proteinaceous due to the reason that the antimicrobial activity of AcAMP was totally lost when treated with the proteolytic enzymes alcalase, trypsin, chymotrypsin, pepsin, and proteinase K. In this study, a 6.0 kDa antimicrobial peptide from *Aspergillus clavatus* ES1, designated as AcAMP was isolated by a one-step heat treatment. AcAMP was sensitive to proteolytic enzymes **(**Hajji *et al*., 2010). Stability studies by Chen *et al.* 2013 showed that Pc- Arctin was most sensitive to proteinase K followed by trypsin, but insensitive to papain. The different sensitivity of Pc-Arctin to the proteases may be due to the specific digestion nature of these proteases. Alternatively, some antifungal proteins themselves possess protease inhibitory activities (Das *et al.*, 2011). Likewise the activity of an antifungal protein PcPAF from a *Penicillium citrinum* W1 was not affected after being treated with different proteases, except a weak inhibition shown by proteinase K (Wen & Chen, 2014). PAF retained its antifungal activity and the cytotoxicity after treatment with pepsin and resisted proteinase K and pronase digestions for 3–9 h. The prolonged exposure of PAF to pronase for 12 h and to proteinase K for 24 h significantly reduced the protein activity proves a specific gradual inactivation (Batta *et al*., 2009).

The purified peptide MFAP9 was found to be thermostable as it was stable over a wide range of temperature and complete loss of activity did not occur even at 100℃ for 1h. Also in another study, AcAMP peptide was highly heat-stable in the temperature ranging from 50℃ to 100℃ **(**Hajji *et al*., 2010). The antifungal activity of PAF was retained even after exposure to 80 ℃ for 60 min, and it was extensively reduced only after treatment at 95 and 100 ℃ for at least 60 min. The loss of protein activity was irreversible after cooling the sample to room temperature. Also in the case of fungal peptide, it was found to be heat stable as well as pH-stable (Zhang *et al*., 2011). Studies have discovered that cysteine

residues present in AFP from *A. giganteus* and PAF from *P. chrysogenum* were involved in the formation of disulfide bridges contributing to the heat stability of each antifungal protein (Marx *et al*. 2008; Meyer, 2008). The thermostable nature of Pc-Arctin from *Penicillium chrysogenum* therefore, supposed that the high temperature tolerance might be attributed to the formation of disulfide bridges (Chen *et al*., 2013).

pH stability analysis of antibacterial peptide MFAP9 in the current study depicts tolerance to a wide pH range and maximum activity at pH 4 and complete loss of activity at pH 13. Similarly, bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454 showed maximum pH stability in the range from 2.0 to 12.0 (Yildirim *et al*., 1999). A different study demonstrated that the activities of histidine-rich, amidated alpha-helical antimicrobial peptides were considerably greater at pH 5.5 than pH 7.4 (Lee *et al*., 1997). Likewise, Minahk *et al*. (2003) showed that the anti-listerial activity of enterocin CRL35 was higher at acidic condition when compared to neutral or basic. AcAMP peptide was observed to be relatively stable at pH values below 6.0 and above 10.0 **(**Hajji *et al*., 2010). The activity of antimicrobial substance produced by *Bacillus subtilis* RLID 12.1 was recorded over a wide range of pH (3.0−10.0), while 100% activity was found between pH6.0 and 8.0 (Ramachandran *et al*., 2014). Previous reports indicated that intramolecular disulfide bridges are presumably responsible for the functional stability of the proteinaceous protease inhibitor in the presence of physical and chemical denaturants such as temperature, pH and reducing agents (Oliveira *et al*., 2007)

Nonionic detergents are generally considered as mild detergents and that they do not interact extensively with the protein surface, whereas non-specific binding of ionic detergents, particularly SDS to the protein surface may lead to protein unfolding (Mogensen *et al*., 2005). Tween 20, tween 80 and triton X-100 were the non- ionic detergents used to evaluate the effect on peptide. The study revealed that Triton X influences the peptide by 50% reduction in activity when

compared to other mild detergents. In contrast, treatment with ionic detergents like SDS and CTAB resulted in complete inactivation of peptide. In case of Plantaricin C19 produced by *L. plantarum* C19 lost its activity after treatment with SDS or Triton X-100 (Atrih *et al.*, 2001). The results obtained after treatment with surfactants, like SDS, Triton-X-100, tween 20, and urea on ultrafiltered concentrate of *Bacillus subtilis* RLID 12.1 revealed that there was no inhibitory effect on its antimicrobial activity (Ramachandran *et al.*, 2014). Wen *et al.* (2014) demonstrated that the antifungal activity of PcPAF could be 35% inhibited by 0.5% SDS compared with the control while no change was happened on the activity of PcPAF when treated with 0.5% Triton X-100 and 1% Tween-20. It was noticed that the activity of plantaricin SR18 produced by *Lactobacillus plantarum* SR18 was increased in presence of tween 80 and SDS whereas Triton X-100 lowered the activity of the bacteriocin (*El*-Shouny *et al.*, 2013).

Antimicrobial peptides need to be stable in physiological conditions for therapeutic use against local or systemic infections due to the reason that the antagonism between the peptides and ionic strength in their environment may inhibit the development as novel antibiotics. In present study, the activity of peptide was enhanced by the addition of metal ions Ni^{2+}, Cd^{2+} and Mg^{2+}, whereas activity decreased in presence of Cu^{2+}, Al^{3+} and Na^{+}. Furthermore it was noted that the activity of peptide was not at all affected by the other ions such as Ba^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Ca^{2+}. AFP of *Aspergillus giganteus* was found to act by binding to the membrane or cell wall of sensitive fungi, and it was revealed that protein activity was severely reduced with 0.1 M KCl or NaCl (Theis *et al.*, 2003).Instead, only a limited decline of the potency by KCl and a minimal influence by NaCl were detected in case of PAF from *Penicillium chrysogenum* (Kaiserer *et al.*, 2003). The antifungal activity of Pc-Arctin produced by *Penicillium chrysogenum* from an Arctic sediment remained intact in the presence of different ions (0.5 M K^{+}, 0.5M Ca^{2+}, and 0.01 M Cu^{2+}, Co^{2+}, and Ni^{2+}) but was impaired when incubated with 0.5 M $Mg2^{+}$, 0.5 M Mn^{2+}, and 0.03 M Zn^{2+} (Chen

et al., 2013). One possible reason behind the influence of metal ions is that certain types of ions may interact with specific receptors that are targeted by antimicrobial proteins, and the saturation of these receptors could reduce the interaction between the receptors and the proteins (Kaiserer *et al*., 2003; Skouri-Gargouri & Gargouri., 2010; Theis *et al*., 2003).

Both DTT and β-mercaptoethanol as reducing agents had a prominent influence on the activity of peptide MFAP9. It was evidenced that DTT caused significant reduction of activity, whereas complete inactivation of peptide occurred using 80mM concentration of β-mercaptoethanol. The sensitivity of peptide towards β-mercaptoethanol confirmed the presence of intra disulfide bond and its vital role for the stability of peptide. It was noted that DTT reduced the activity of the bacteriocin produced by *B. subtilis* LFB112 (Xie *et al*., 2009).On the other hand, β-mercaptoethanol (0.2%) increased the activity of bacteriocin produced by *Lactobacillus plantarum* SR18 to 1.2-1.4 fold (El-Shouny *et* al., 2013). Moreover it was observed that the biological activity of concentrate from *Bacillus subtilis* RLID 12.1 remained the same when exposed to *β*-mercaptoethanol (Ramachandran *et al*., 2014). Effect of oxidizing agents was studied by incubating the peptide with DMSO that acts as a mild oxidant and another strong oxidant sodium hypochlorite. The results shown that DMSO had no influence on the activity of peptide, whereas high concentrations of sodium hypochlorite caused decline in the activity of MFAP9. An assessment of effect of oxidizing agents on inhibitory activity of protease inhibitor isolated from *Moringa oleifera* revealed that the activity declined in a concentration dependent manner for both oxidizing agents DMSO and H_2O_2 (Bijina *et al*., 2011).

Results obtained for the effect of chemical amino acid modifiers on the activity of MFAP9 indicated the presence of tryptophan, cysteine and histidine residues in the peptide and also their importance in the function of antibacterial activity. Modification of histidine residues with DEPC resulted in a sudden reduction in the activity of peptide and also observed insignificant role of higher

concentrations as the inhibition percentage remained same at all the concentrations. Similar effect was observed when modification of cysteine residues with iodoacetamide was done. On the contrary, the modification with PMSF had no effect on the activity of the peptide suggested that the amino acid serine may either not present or having no role in the activity of peptide. The activity of peptide gradually decreased along with increase in concentrations of N-Bromosuccinamide, indicated the presence and significance of tryptophan residue. Similarly, modification studies by Bhugaloo-Vial *et al*. (1999) reported the importance of tryptophan residues for the inhibitory activity of class IIa bacteriocins since those residues are known to play an indispensable role in the adsorption and orientation of amphiphilic peptides and proteins in membranes due to their capability to form both hydrogen and hydrophobic bonds with the polar and nonpolar groups of polar lipids.

Minimun inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of peptide to inhibit the sensitive indicator *Bacillus circulans* was determined by using modified microtitre plate assay, where MIC value was 0.525µg/mL and MBC value was 4.2µg/mL. Likewise, copsin exhibited MIC values in the low microgram range for gram-positive bacteria, such as *B. subtilis*, *Listeria* sp, and *Enterococcus* sp, including a vanA type vancomycin resistant *Enterococcus faecium* strain with a strong activity against *Listeria monocytogenes* with MIC values of 0.25-0.5 µg/mL (Essig *et al*., 2014). In case of eurocin, the more susceptible species were *Streptococcus pneumonia*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* with MIC values of 0.06–1 µg/mL. A larger variation was seen among other species; MIC for *Staphylococcus* sp. was found to be in the range (0.5–128 µg/mL) and for *Enterococcus* (0.25–128 µg/mL). Furthermore the MIC of eurocin against reference strains of important Gram-positive human pathogen bacteria were as follows: *S. aureus* (ATCC29213), 16 µg/mL; *Staphylococcus epidermidis* (ATCC12228), 16 µg/mL;

Enterococcus faecium (ATCC49624), 16 μg/mL; *E. faecalis* (ATCC29212), 2 μg/mL; *S. pneumonia* (ATCC49619), 0.25 μg/mL (Oeemig *et al*., 2012).

5.4 APPLICATION STUDIES

Antibiofilm activity of fungal peptide MFAP9 was evaluated by biofilm inhibition assay in a microtitre plate showed significant inhibitory activity. The average inhibition percentage of 94% against bacteria categorised as strong biofilm producers under study. In the microtitre plate the bacterial cells can adhere and form biofilm. Treatment with the fungal peptide was found to be very effective in the destruction or inhibition of the pre-grown biofilm. Extended growth of bacterial cells results in adherence to animal tissues and inorganic materials (Costerton *et al*., 1981). Sequentially, this allows the formation of a biofilm, which represents a multilayered community of sessile bacterial cells. Bacteria in biofilms are able to afford a survival advantage over planktonic or free-floating bacteria by enhancing nutrient trapping and colonization (Costerton, 1999). Currently, biofilms are a widespread problem in hospitals and healthcare facilities. There is increasing evidence that biofilms are often associated with dermal wounds and their presence causes prolonged infection exhibits delayed wound healing (Percival *et al*., 2012). In reality, it is estimated that biofilms are responsible for up to 65% of all infections in humans. Hence, specifically targeting bacterial cells within a biofilm may be an efficient strategy to combat serious biofilm-associated infections (de la Fuente-Núñez *et al*., 2013; Haney *et al*., 2015). Bacteria associated with biofilm structures are protected from environmental conditions, antimicrobial agents, and host immune responses, and further exhibit up to 1000-fold-increase in antibiotic resistance to a wide range of antimicrobial agents (Haussler & Fuqua, 2013) due to the antimicrobial tolerance, persister cells and the exopolysaccharide matrix which are provided by the sedentary lifestyle in biofilms (Bordi & de Bentzmann**,** 2011). The development of antibiofilm therapeutics has generally focused on interfering with quorum

sensing, inhibition of adhesion, enhancement of dispersion, or bacteriophage-based treatments. The selective targeting of antimicrobial peptides now becomes a new option for the species-specific control of biofilms (Chen & Wen, 2011). Another practical way to overcome biofilm associated resistance is through synergistic effects of antimicrobial agents. The results in the rapid inhibition of biofilm facilitate prevention or delay in the emergence of resistance. *Pseudomonas aeruginosa* is the significant pulmonary pathogen affecting patients with cystic fibrosis (Moreau-Marquis *et al*., 2008), and this organism forms a biofilm on medical devices and tissues. LL-37, a human cationic host defense peptide, showed a potent inhibitory activity in biofilm formation at a concentration of 0.5 µg/mL against *P. aeruginosa* biofilm and reduced pre-grown biofilms (Overhage *et al*., 2008).

The effect of the purified peptide for the control of biofilm and also on the morphology of bacterial cells was analyzed using Scanning Electron Microscopy (SEM). For this, the most sensitive strain *Bacillus pumilus* BT3 was selected. It was observed that in control without peptide treatment, the biofilm has been formed complete multilayered bacterial growth, whereas the peptide treatment inhibited the strain from forming biofilm by about 99 % and the aggregated cells got completely disintegrated and bacterial cells lost their original shape showing a distorted, irregular structure. Images of the untreated control cells revealed that cells were generally smooth-walled bodies, in shape entangled in a thick biofilm mass. However, the peptide treated biofilms, displayed the cells with morphological changes including formation of grooves and pore-like lesions with ruptured, damaged or a rough, irregular topograpic features. SEM provides high resolution images to visualize protuberances or blebs related to a local destabilization of the bacterial cell envelope by AMPs. This clear morphological disruption of the bacterial cell strongly suggests the therapeutic potential of the peptide.

A large number of studies have shown that some of the cationic antimicrobial peptides, which are toxic to bacteria but non-toxic to normal mammalian cells, exhibit a broad spectrum of cytotoxic activity against cancer cells. Such studies have considerably enhanced the significance of AMPs, both synthetic and from natural sources, which have been of importance for their potential as clinical antibiotics (Hoskin & Ramamoorthy, 2008). The *in vitro* antiproliferative activity of peptide was studied by using A549 lung carcinoma cell line and it was observed that the peptide was cytotoxic with an IC_{50} value of 29µg/mL indicating promising application in cancer therapy. In addition, the influence of peptide on A549 cell death was studied by acridine orange (AO)/Ethidium bromide (EB) double staining method and fluorescence microscopic analysis revealed the features of apoptosis such as chromatin condensation and alterations in the size and the shape of cells suggested that MFAP9 could induce cell death through apoptosis.

There were reports suggesting the process of excess lecithin synthesis by A549 cells resulted in pulmonary surfactant synthesis. Those surfactants may impede therapeutics during pulmonary disease treatment (Jensen & Meckling,, 1976). Because of this reason anticancer drugs could not function properly on A549 cell lines. In the present study it was expected that the intervention of peptide with the surfactants resulted in activity against cell lines. It was also observed that the peptide acts in a concentration dependent mode. Similar case where avrainvillamide has a dose-dependent cytotoxic effect against a variety of tumor cell lines including human colon HCT116 cells (IC_{50}, 2.0 µg/mL), melanoma MALME-3M cells (IC_{50}, 53nM), and two types of breast cancer cells, βT-549 (IC_{50}, 34 nM) and T-47D (IC_{50}, 72 nM) (Fenical *et al*., 2000). Aspergillamide A, which is a peptide isolated from the mycelium of a marine sediment-derived *Aspergillus* strain found to demonstrate modest cytotoxicity (IC_{50}, 16µg/mL) to human colon carcinoma HCT-116 cells (Lee *et al*., 2013). Calcaelin is a ribosome inactivating protein (RIP) from the puffball mushroom

Calvatia caelata with an N-terminal sequence resembling those of *American ginseng* and *Chinese ginseng* RIPs, expressed anti-mitogenic activity toward spleen cells and antiproliferative activity towards tumor cells (Ng *et al*., 2006). There are reports suggesting that glycoproteins kill the tumor cells directly by arresting the cell cycle and/or inducing the apoptosis of tumor cells (Zhao *et al*., 2010; Pohleven *et al*., 2009). The results of the present study demonstrated significant inhibition of tumor cell proliferation by using direct tumor cell lysis by target cell membrane disruption.

Antioxidant is defined as a substance which significantly delays or inhibits oxidation process. The antioxidant activity is measured by determining the inhibition rate of oxidation processes in the presence of an antioxidant (Antolovich *et al*., 2002). Epidemiology and investigational studies have concerned about oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems, the primary cause of cardiovascular diseases, cancer, aging etc (Halliwell *et al.*, 1997). Because of the complexity of oxidative reactions taking place in biological systems, numerous chemical methods with different mechanisms of action have been developed to measure antioxidant potential of peptides. Among them, the DPPH-based assay was the first one to be used extensively for screening antioxidant peptides (Chakrabarti *et* al., 2014). Scavenging of stable free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH), an organic stable radical in its crystalline form is widely used to determine the antiradical activity of a given compound or extract. The antioxidant activity of a given compound or extract is also often associated with its radical scavenging activity (Karimi *et al.*, 2010; Diouf *et al.*, 2009). Current study demonstrates the free radical scavenging activity of peptide MFAP9 in comparison with standard antioxidant ascorbic acid at different concentrations. The results showed that the peptide had a comparable activity with that of standard antibiotic and the activity followed a concentration dependent mode.

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) are among the most primarily used therapeutic invention for the treatment of pain and inflammation. This is particularly used for the treatment of arthritis. In the 1970s, it was verified that aspirin and other NSAIDs block the formation of prostaglandins (PGs) produced from arachidonic acid by the enzyme cyclooxygenase (COX), otherwise called as prostaglandin synthase. It was observed that consumption of NSAIDs can lead to high incidence of gastrointestinal irritation due to the development of life-threatening gastrointestinal ulcers and also causes abnormal renal physiology. Use of glucocorticoids and immunosuppressive as more advanced means of treatment of chronic inflammatory diseases possess an even greater array of side effects. The hypothesis that selective inhibitors of COX-2 would be antiinflammatory with diminished side effects was supported by a number of reports that have emerged on the preparation and biological activity of selective COX-2 inhibitors (Talley *et al*., 2000).

In this context, the relevance of anti-inflammatory potential of peptide gets high priority. The spectrophotometric analysis showed that the peptide treatment on LPS induced cell lines could inhibit the activity of COX-2 enzyme, indicative of its anti-inflammatory potential. Das *et al*. (2011) reported the isolation and characterization of a novel 14 kDa protein with antifungal and anti-inflammatory properties from *Aloe vera* leaf gel. The spectroscopic analysis displayed 84.6% loss of activity of inflammatory enzymes, LOX and 73% with COX-2 when incubated with Aloe protein in the presence of substrates, linoleic acid and arachidonic acid respectively. Azonazine (107) exhibited anti-inflammatory activity by suppressing NF-κB luciferase with an IC_{50} value of 8.37 μM and nitrite production (IC_{50}, 13.7 μM) in RAW264.7 murine macrophage cells (Wu *et al*., 2010).

6. SUMMARY AND CONCLUSION

Antibiotics have been used to treat infections for more than half a century since the discovery of penicillin G. Many different types of antibiotics have been discovered or synthesized to control pathogenic microorganisms, but the rate of development of antibiotic resistance by pathogenic bacteria is increasing at an alarming rate forcing scientists to find new ways to combat these microbes. Rapid development of more effective antimicrobial compounds is required to keep pace with demand. Generally, the discovery of new antimicrobials from natural sources involves bulk screening of crude extracts for antimicrobial activity, purification and isolation of the active compounds from the crude extract, and final compound identification. The methods of extraction, separation, or isolation can be different dependent on the nature of sample.

Fungi were isolated from terrestrial and marine sediment samples collected from various locations using pour plate technique. One hundred and twenty isolates were obtained and were screened for antibacterial activity. Following primary screening, fifteen isolates exhibiting antimicrobial activity were selected for secondary screening. The secondary screening was mainly focused on identifying fungal isolates producing proteinaceous inhibitory compounds and resulted in the selection of a single strain BTMF9.

By employing growth inhibition assay in liquid broth, the nature of activity of MFAP9 was determined to be bactericidal to all sensitive gram-positive test organisms studied.

The selected isolate BTMF9 inhibited the growth of gram-positive test organisms such as *Bacillus cereus*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus pumilus* and *Staphylococcus aureus*. Among all the test organisms,

Bacillus circulans displayed consistant sensitivity towards the peptide and was selected as antibacterial activity indicator organism for further studies.

Based on the colony characteristics and microscopic morphology as well as by molecular analysis of fungal rDNA using 18S ribotyping the identity of the isolate was determined as *Aspergillus fumigatus.* The nucleotide sequences were submitted in GenBank and accession number obtained. Proof for non-pathogenic nature of the selected strain was indicated by its non-haemolytic activity on blood agar plate.

The effect of bioprocess variables for peptide production by selected strain BTMF9 was studied. For that eleven different factors were taken into account. The optimized bioprocess conditions are given in Table 6.1.

Table 6.1 Summary of the optimized bioprocess variables for enhanced peptide production

Bioprocess Variables	Optimum values
Incubation period	72 h
Incubation temperature	30°C
Agitation	150 rpm
Inoculum concentration	0.4%
Initial pH of media	7
Metal ion	Ca^{2+}
NaCl Concentration	0.5%
Carbon source	Sucrose (3%)
Inorganic nitrogen source	Sodium nitrate (0.3%)
Organic Nitrogen source	No significant effect
Additional inorganic nitrogen source	Ammonium phosphate (0.1%)

Purification process of peptide included concentration and partial purification of the culture supernatant using ammonium sulphate followed by ion exchange chromatography using DEAE sepharose. Ammonium sulphate precipitated fraction (30-90%) showed inhibitory activity with a purification fold value 1.38 against the test organisms under study. Further purification of peptide by employing ion exchange chromatography resulted in an increase in the fold of purity up to 6.15 and the peptide was designated as MFAP9. Additionally, the presence of a single peak in the chromatogram after reverse phase high pressure liquid chromatography (RP-HPLC) confirmed the purity of peptide.

The specific band having antibacterial activity was determined using the method of overlaying the SDS-PAGE gel with test organisms (zymogram) and observed that the active peptide MFAP9 is a low molecular weight peptide as a clearing zone was obtained at the dye front region corresponded to 3 kDa molecular weight marker protein band. In addition, the intact mass of MFAP9 was determined as 3.055kDa from the mass spectrum of MALDI-TOF mass spectrometry. The isoelectric point was determined as 6 and the peptide was resolved as a single spot after 2D-gel electrophoresis further confirming the homogeneity of MFAP9. Peptide mass fingerprint of MFAP9 generated by MALDI-TOF-TOF analyzed with the MASCOT search tool in Swiss-Prot database peptide showed a low degree of similarity to FMRFamide-like neuropeptide from *Arthurdendyus triangulates*, Cytotoxin NN-32 from *Naja naja*, Ranatuerin-1C from *Rana clamitans*, Ponericin-G4 from *Pachycondyla goeldii* and Antimicrobial peptide THP3 (Fragment) of *Meleagris gallopavo*.

The cytotoxicity studies of MFAP9 at varying concentrations on human red blood cells (hRBCs) showed that the peptide exhibits no damage to mammalian RBC. The peptide was nontoxic to human RBCs as even at higher concentration (100µg/mL) the heamolysis was only 0.6%.

The action of proteases on the stability of peptide was studied and it was observed that the activity was reduced or lost when treated with proteases like proteinase K, pepsin and trypsin. The activity of MFAP9 was lost completely when treated with proteinase K whereas only reduction of activity was observed on treatment with trypsin and pepsin. This confirmed the proteinaceous nature of the active compound.

The study on the effect of temperature on the stability of MFAP9 revealed that it is thermostable without any loss of activity till 90^{o}C. Moreover, 25% antibacterial activity was retained even after 1h incubation at 100^{o}C.

The peptide MFAP9 was found to be very stable at very low pH, but unstable in alkaline pH range and complete loss of activity was observed at pH 13. The optimum antibacterial activity was displayed at pH 4.

The non-ionic detergents tween 20, tween 80 had no effect on MFAP9 whereas activity reduced in presence of triton X-100. The ionic detergents like SDS and CTAB completely deactivated the peptide.

When MFAP9 was exposed to metal ions like Ba^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Ca^{2+}, the activity of the peptide was not affected. The activity of peptide was enhanced in presence of Ni^{2+}, Cd^{2+} and Mg^{2+} and activity was reduced when incubated with Cu^{2+}, Al^{3+} and Na^{+}.

The effect of two reducing agents DTT and β-mercaptoethanol were analysed. The activity of MFAP9 reduced when it was exposed to β-mercaptoethanol at a concentration of 6mM and above. In the case of treatment

with β-mercaptoethanol, reduction in activity started at 40mM and complete loss of activity occurred at 80mM concentration.

Oxidizing agents used to study the stability of MFAP9 were DMSO and sodium hypochlorite. The weak agent DMSO had no effect on MFAP9 while sodium hypochlorite reduced the activity at higher concentration only.

Amino acid modifiers used in the study of effect on MFAP9 were PMSF, DEPC, N-Bromosuccinamide and Iodoacetamide. Among the four chemical modifiers used, PMSF had no effect on activity of peptide and in case of DEPC and iodoacetamide, the activity was drastically reduced initially to 25% at 5mM concentration but was found stable up to 25mM concentration. Modification of tryptophan residue by N-Bromosuccinamide showed significant effect on activity of peptide in a concentration dependent manner.

The MIC and MBC of MFAP9 was determined as 0.525µg/mL and 4.2 µg/mL respectively compared to standard antibiotic with MIC and MBC values of 0.937 µg/mL and 3.75 µg/mL respectively.

Application studies of peptide MFAP9 included control of biofilm formation of strong biofilm producers, study on *in vitro* antiproliferative activity against A549 lung carcinoma cell line, antioxidant activity study using DPPH radical scavenging assay and anti-inflammatory potential determination by assessing cycloxygense (COX2) activity of peptide treated LPS stimulated THP1 cell lines.

The addition of MFAP9 on preformed biofilm of test bacteria showed a significant inhibitory activity with an average inhibition percentage of 94%. Moreover, the visual analysis of antibiofilm activity against the most sensitive *Bacillus pumilus BT3* (99%) using scanning electron microscopy (SEM) revealed a severe destruction of biofilm with distorted cells due to grooves and pore like

lesions on bacterial surface. Thus the high potential of MFAP9 to destroy biofilm was experimentally proved in the present study.

The *in vitro* antiproliferative activity of MFAP9 was studied by using A549 lung carcinoma cell line. From the MTT assay results, it was observed that the peptide was cytotoxic with an IC_{50} value of 29µg/mL indicating its promising application in cancer therapy. Additionally, the effect of peptide on A549 cell death was studied by double staining method using acridine orange (AO)/ethidium bromide (EB) dyes and further fluorescence microscopic analysis revealed the features such as chromatin condensation and alterations in the size and the shape of cells suggested that MFAP9 could induce cell death through apoptosis and necrosis. The cytotoxicity study on normal cell line L929 showed that MFAP9 became toxic only at high concentration as evident with an IC_{50} value 113.78 µg/mL. The results of the present study demonstrated significant potential of peptide on inhibition of tumor cell proliferation.

The antioxidant activity of MFAP9 was determined by the free radical scavenging assay in comparison with standard antioxidant ascorbic acid at different concentrations showed that the peptide had a comparable activity with that of standard antibiotic and the activity increased with the increasing concentration. Furthermore, the peptide treatment on LPS induced cell lines could inhibit the activity of inflammatory enzyme COX-2, indicated its anti-inflammatory potential.

CONCLUSION

MFAP9, an antibacterial peptide isolated from the culture supernatant of fungus *Aspergillus fumigatus* BTMF9 isolated from marine sediment sample was purified and characterized in the present study. This low molecular weight peptide is highly efficient, active even at very low (in µg) concentrations, inhibiting an array of gram-positive pathogenic organisms including *Bacillus cereus*, *Bacillus*

circulans, *Bacillus coagulans*, *Bacillus pumilus* and *Staphylococcus aureus*, all of which are concerned either in gastroenteritis and/or food poisoning. The characterization of the peptide indicated that it is new with novel characteristics. In addition to antimicrobial activity, the peptide showed significant antibiofilm, antiproliferative, antioxidant and anti-inflammatory activities. The outcome of the experiments involving the application of this peptide, indicate the various fields like food, agriculture and pharmaceutical where this small molecule can be applied.

Filamentous fungi possess an important value as numerous fungal products are exploited for social benefit and moreover it is conceivable that many of bioactive compounds are yet to be discovered. As such, antimicrobial peptides from *Ascomycetes* bear great potential, whereas the science is still in its infancy compared with the information regarding other well studied antimicrobials for its activity and species specificity. The stability of peptide in a wide range of pH and temperature may be advantageous for scaled-up production and formulation into deliverable products. Hopefully, with the utilization of developments in proteomics, bioinformatics, and modification strategies, the fungal peptide in the current study could emerge as novel promising therapeutic drug in future for various clinical applications.

7. REFERENCES

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APPENDIX – 1

NUTRIENT MEDIUM

Ingredients		g/L
Peptone	-	5
Sodium chloride	-	5
Beef extract	-	1
Yeast extract	-	2

Suspended 13g of media (Himedia, Mumbai, India) in 1000mL distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55℃. When used as solid agar medium, 2.0% agar (w/v) was added to the medium. Final pH 7.4 ± 0.2.

MUELLER HINTON BROTH

Ingredients		g/L
Beef infusion	-	300
Casein acid hydrolysate	-	17.5
Starch	-	1.5

Suspended 21 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes. When used as solid agar medium, 2.0% agar (w/v) was added to the medium for agar plate preparation. Final pH (at 25^{o}C) -7.4 ± 0.2.

MYCOLOGICAL BROTH

Ingredients		g/L
Papaic digest of soyabean meal	-	10
Dextrose	-	40

Suspended 50g of media (Himedia, Mumbai, India) in 1000mL distilled water. Heat if necessary to dissolve the medium completely. Dispense and autoclaved at 15 lbs pressure (121℃) for 15 minutes. and cooled to 50-55℃. When used as solid agar medium, 2.0% agar (w/v) was added to the medium. Final pH 7.0 ± 0.2.

CZAPEK- DOX AGAR MEDIUM

Ingredients	**g / L**
Sucrose	30.000
Sodium nitrate	2.000
Dipotassium phosphate	1.000
Magnesium sulphate	0.500
Potassium chloride	0.500
Ferrous sulphate	0.010
Agar	15.000

Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121℃) for 15 minutes. Mix well and pour into sterile Petri plates. Final pH (at 25℃) 7.3±0.2

BLOOD AGAR MEDIUM

Ingredients		**g/L**
Enzymatic digest of Casein	-	15
Enzymatic digest of animal tissue	-	4
Yeast extract	-	2
Starch	-	1
Sodium Chloride	-	5
Agar	-	14

Suspended 42 g of the blood agar base in one liter of purified water. Heated the agar base with frequent agitation and boiled for one minute to completely dissolve the medium. Final pH: 7.0 ± 0.2 at 25℃. Autoclaved the medium at 121℃ for 15 minutes and prepared 10% blood agar by aseptically adding the appropriate volume of sterile defibrinated blood to melted sterile agar medium, cooled to 45 – 50℃ and poured into sterile petriplates.

SOYABEAN CASEIN DIGEST MEDIUM (TRYPTONE SOYA BROTH)

Ingredients		**g/L**
Pancreatic Digest of Casein	-	17
Papaic digest of soyabean meal	-	3
Sodium chloride	-	5
Dipotassium hydrogen phosphate	-	2.5
Dextrose (Glucose)	-	2.5

Suspended 30 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Final pH is 7.3 ± 0.2.

APPENDIX – 2

1X Phosphate Buffered Saline (PBS Buffer)

NaCl	-	8 g
KCl	-	0.2 g
Na_2HPO_4	-	1.44 g
KH_2PO_4	-	0.24 g

Dissolve the following in 800ml distilled H_2O. Adjust pH to 7.4 with HCl and adjust volume to 1L with additional distilled H_2O.Sterilize by autoclaving

0.01M Phosphate buffer (pH 7.5)

Solution A: 0.2 M NaH2PO4
Solution B: 0.2 M Na2HPO4

Mixed 16 mL of solution A with 84 mL of solution B and the volume was made up to 200mL with distilled water. This is made upto 1L to get 0.01 M buffer.

Sodium dodecyl sulphate (SDS) – 10%

SDS	-	10 g
Distilled water	-	100 mL

Gently swirl.

3M Sodium acetate (pH 5.2)

Sodium acetate.3H2O	-	408.3 g
Distilled water	-	800 mL

pH adjusted to 5.2 with glacial acetic acid. Adjusted the volume to 1 litre with distilled water. Sterilized by autoclaving.

Ethidium Bromide (10mg/mL)

Ethidium bromide	-	10 mg
Distilled water	-	1mL

Dissolved well and stored by wraping in aluminium foil (to be kept in dark)

TE buffer

1M Tris-Cl (pH 8)	-	10mL
500mM EDTA (pH 8.0)	-	2mL

1M Tris-HCl (pH 8)

Tris base	-	60.57 g
Deionised water	-	500mL

Adjusted to desired pH using concentrated HCl

0.5M EDTA (pH 8)

EDTA	-	18.6 g
Deionised water	-	100mL

Adjust the pH to 8.0 with NaOH

50X TAE Buffer

Tris base	-	121 g
Glacial acetic acid	-	28.6mL
0.5M EDTA pH 8.0	-	50 mL

Deionised water added to make volume to 500mL.

1X TAE Buffer

50X TAE buffer	-	10mL
Deionised water	-	490mL

6x Gel-loading buffer

0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
40% (w/v) sucrose in H_2O
Stored at 4°C.

Hydrochloric acid- potassium chloride buffer (pH 2)

Solution A: 0.2 M KCl
Solution B: 0.2 M HCl
Mixed 50 ml of solution A with 10.6 ml of solution B and made up to 200ml with distilled water.

Citrate buffer (pH 3 - 6)

Solution A: 0.1 M Citric acid
Solution B: 0.1 M sodium citrate
Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to make up volume to 200 ml and then filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
3	46.5	3.5
4	33.0	17
5	20.5	29.5
6	9.5	41.5

Phosphate buffer (pH 7)
Solution A: 0.2 M NaH_2PO_4
Solution B: 0.2 M Na_2HPO_4
Mixed 39 ml of solution A with 61 ml of solution B and the volume was made up to 200ml with distilled water, followed by filter sterilization.

Tris (hydroxymethylamino methane buffer system (pH 8 and 9)
Solution A: 0.2 M Tris buffer
Solution B: 0.2 M HCl
Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
8	50	26.8
9	50	5

Carbonate – bicarbonate buffer (pH 10 and 11)
Solution A: 0.2 M Na_2CO_3
Solution B: 0.2M $NaHCO_3$
Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
10	27.5	22.5
10.7	45.0	5

Sodium hydroxide - Potassium chloride buffer (pH 12 and 13)
Solution A: 0.2 M KCl
Solution B: 0.2M NaOH
Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and then filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
12	50	12
13	50	132

APPENDIX 3

REAGENTS FOR GLYCINE SDS-PAGE

1. Stock acrylamide- bisacrylamide solution (30% T and 0.8% C)

Acrylamide (T)	-	30 g
Bis-acrylamide (C)	-	0.8 g
Distilled water (DW)	-	100 mL

Stored at 4℃ in amber coloured bottle

2. Stacking gel buffer stock

Tris buffer (0.5 M) - 6.05 g in 40 mL DW

Titrated to pH 6.8 with 1M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 (Whatman, England) filter paper and stored at 4℃.

3. Resolving gel buffer stock

Tris buffer (1.5 M) - 18.15 g

Titrated to pH 8.8 with 1M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 filter paper and stored at 4℃.

4. Running buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3 g
Glycine	-	14.4 g
SDS	-	1 g

Dissolved and made up to 1L with DW. Prepared in 10X concentration and stored at 4℃.

5. Sample buffer for Non- Reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	1.25 mL
Glycerol	-	2.5 mL
SDS (10%, W/v)	-	2 mL
Deionised water	-	3.55 mL
Bromophenol blue (0.5%, w/v)	-	0.2 mL

Samples were diluted with sample buffer prior loading into the gel

6. **SDS (10%)** - 1 g in 10 mL DW

7. **Ammonium persulfate** (10%, w/v) - 0.1 g of ammonium persulfate was dissolved in 1mL DW (prepared freshly).

REAGENTS OF 2D GEL ELECTROPHORESIS

Equlibration buffer I

Urea	-	6M
SDS	-	2%
Tris HCl (pH 8.8)	-	0.375M
Glycerol	-	20%
DTT	-	2%

Equlibration buffer II

Urea	-	6M
SDS	-	2%
Tris HCl (pH 8.8)	-	0.375M
Glycerol	-	20%
Iodoacetamide	-	0.5g

COOMASSIE STAINING SOLUTIONS

Protein staining solution

Coomassie brilliant blue (0.1%)	-	100 mg
Methanol (40%)	-	40 mL
Glacial acetic acid (10%)	-	10 mL
DW	-	50 mL

Destaining solution

Methanol (40%)	-	40 mL
Glacial acetic acid (10%)	-	10 mL
DW	-	50 mL

REAGENTS OF SILVER STAINING

1. Fixer

Methanol (50%, v/v)	-	50 mL
Acetic acid (5%, v/v)	-	5 mL
Milli Q water	-	45 mL

2. Wash

Methanol (50%, v/v)	-	50 mL
Milli Q water	-	50 mL

3. Sensitizing solution

Sodium thiosulfate (0.02%, w/v)	-	20 mg
Milli Q water	-	100 mL

4. Silver nitrate solution

Silver nitrate (0.2%, w/v)	-	200 mg
Milli Q water	-	100 mL

5. Developer

Sodium carbonate (6%, w/v)	-	3 g
Formaldehyde	-	12.5 µL
Milli Q water	-	100 mL

6. Stop solution

Sodium-EDTA	-	1.4 g
Milli Q water	-	100 mL

PROTEIN MARKERS

a. Medium range protein molecular weight marker (GeNei) was mixed by tapping and 10µL taken. Mixed with 10µL sample buffer and 30µL distilled water. Incubated in boiling water bath for one minute and loaded on to SDS-PAGE.

Protein	**MW (Da)**
Phosphorylase b	97,400
Bovine serum albumin	66,000
Ovalbumin	43,000
Carbonic anhydrase	29,100
Soyabean trypsin inhibitor	20,100
Lysozyme	14,300

REHYDRATION BUFFER FOR IEF

Urea	8 M
CHAPS	2%
DTT(50mM)	0.2%
Ampholyte	
Bromophenol (Trace)	

EQUILIBRATION BUFFER I

Urea	6 M
SDS	2%
Tris HCl (pH 8.8)	0.375 M
Glycerol	20%
DTT	2%

EQUILIBRATION BUFFER II

Urea	6 M
SDS	2%
Tris HCl (pH 8.8)	0.375 M
Glycerol	20%
Iodoacetamide	0.5g

LIST OF PUBLICATIONS

FULL PAPER IN PEER-REVIEWED JOURNALS

1. Rekha Mol K.R, Manzur Ali P.P, Sapna K, Abraham Mathew, Preethi G.U, Sarita G.Bhat and Elyas K.K. Production optimization of an antibacterial peptide MFAP9 from marine *Aspergillus fumigatus* BTMF9. *Current Research in Microbiology and Biotechnology* 11/2014; 2(6): 530-535
2. Manzur Ali P. P, Sapna. K, Rekha Mol K.R, Chandrasekaran M and Elyas K. K "Trypsin specific Inhibitor from edible mushroom *Pleurotus floridanus* active against proteases of microbial origin" *Applied Biochemistry and Biotechnology* 02/2014; 172(04)
3. Manzur Ali P. P, Sapna. K, Abraham Mathew, Rekha mol K. R and Elyas K. K Screening and activity characterization of protease inhibitor isolated from mushroom *Pleurotus floridanus*. *Advanced Biotechnology* 10/2012; 12(04): 27-30
4. Sapna. K, Manzur Ali P. P, Abraham Mathew, Rekha mol K. R, Sarita G. Bhat, Chandrasekaran. M and Elyas K. K. "Marine *Pseudomonas mendocina* BTMW 301 as a potential source for Extracellular Proteinaceous Protease Inhibitor"(2012)*Advanced Biotechnology* 06/2012; 11(12):16-19
5. Elyas K.K.; Abraham Mathew; Rajeev K Sukumaran; Manzur Ali P.P.; Sapna K.; Ramesh Kumar S.; Rekha Mol K.R.(2010) "Production optimization and properties of β-glucosidases from a marine fungus *Aspergillus* -SA 58". *New Biotechnology* 02/2010; 27(4):347-51

FULL PAPERS IN THE PROCEEDINGS OF INTERNATIONAL/ NATIONAL SYMPOSIA, CONFERENCES AND SEMINARS

Rekha Mol K. R, Manzur Ali P.P , Abraham Mathew, Smitha S, Sapna K, Sarita G Bhat and Elyas K.K (2011) "Screening of various biological sources for antibacterial peptides." Proceedings of National symposium on "Emerging Trends in Biotechnology" conducted by Department of Biotechnology, CUSAT, 1st and 2nd September, 2011. Sarita G bhat (edr) ISBN number : 978-93-80095-30-1

Smitha S., Rekhamol K.R and Sarita G Bhat, (2011) Partial characterization of antibacterial proteins from *Bacillus licheniformi*s. Proceedings of 2 day National symposium on "Emerging trends in Biotechnology" conducted by Department of Biotechnology, CUSAT, 1st & 2nd September, 2011. ISBN number : 978-93-80095-30-1

POSTERS / ABSTRACTS IN NATIONAL / INTERNATIONAL SYMPOSIA

1. **Smitha S.** and Sarita G Bhat, (2008) Exploitation of marine bacteria for novel bioactive compounds. Proceedings of BIOCAM 2008, Cochin, Kerala. International conference on biodiversity conservation and management, Kochi, from3-6th Feb, 2008.

2. Helvin Vincent, **S. Smitha** and Sarita G Bhat (2009) Isolation of a novel protease gene using metagenomic approach, Book of abstracts of MECOS 09,

Cochin, Kerala. International symposium on Marine Ecosystems challenges and Opportunities, Cochin from 9-12 Feb, 2009.

GENBANK SUBMISSIONS

1. Smitha, S., Jeena, A. and Sarita, B.G. (2010) **HM030818**- *Bacillus subtilis* subsp. *subtilis* strain BTFK101 16S ribosomal RNA gene, partial sequence.

2. Smitha, S., Raghul, S.S. and Sarita, B.G. (2010) **HM030819**- *Bacillus licheniformis* strain BTHT8 16S ribosomal RNA gene, partial sequence.

3. Smitha, S., Helvin, V., Linda, L. and Sarita, B.G (2010) **HM030820**- *Bacillus licheniformis* strain BTKM4 16S ribosomal RNA gene, partial sequence.

4. Smitha, S., Siju, V.M., Alphonsa, V.J. and Sarita, B.G (2010) **HM030821**- *Bacillus licheniformis* strain BTEK16 16S ribosomal RNA gene, partial sequence.

5. Smitha, S., Manzur, A.P.P. and Sarita, B.G. (2010) **HM030822**- *Bacillus subtilis* subsp. *subtilis* strain BTSB22 16S ribosomal RNA gene, partial sequence.

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Production optimization of an antibacterial peptide MFAP9 from marine *Aspergillus fumigatus* BTMF9

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ABSTRACT

Aspergillus fumigatus BTMF9 produces an antimicrobial peptide designated as MFAP9. This study deals with the optimization of culture conditions for production of peptide MFAP9 by varying one parameter at a time. The effects of various culture conditions such as temperature, pH, nitrogen sources, carbon sources and sodium chloride on antibacterial activity and specific activity were investigated. The candidate organism tested was Bacillus circulans (NCIM 2107). Maximum antibacterial activity and specific activity was obtained at 30 °C, pH 7, sodium nitrate as nitrogen source, sucrose as carbon source and 0.05% sodium chloride. After optimization, the inhibitory activity increased from 800AU/mL to 1600 AU/mL. The results showed obvious significance of production optimization of bioactive peptide by fungus relative to inhibitory activity and specific activity.

Keywords: *Aspergillus fumigatus;* Antibacterial peptide; Optimization.

INTRODUCTION

Since antibiotic resistance increasing at an alarming rate in virtually all clinically important pathogens, there is an urgent need for alternatives with new/novel antimicrobial mechanism. This in turn has triggered considerable interest in the isolation of new compounds from biological resources. Natural Antimicrobial peptides (AMPs) typically not only have broad-spectrum activity against multiple classes of bacteria and fungi at very low concentrations including drug-resistant strains, but are also nontoxic to host cells.

In recent years, marine microbial diversity was much sought after in the area of drug discovery [1]. Marine fungi have long been recognized as potential source of novel and biologically potent metabolites [2], and promising source of novel anticancer, antibacterial, antiplasmodial, anti-inflammatory and antiviral agents [3]. Genus *Aspergillus* are one of the major contributors to the antimicrobial metabolites of fungal origin [4].

Secondary metabolites are synthesized by several pathways and both genetically distinct strains and altered environmental conditions affect its production. Fermentation parameters such as time, temperature, pH and nutrients can be modified to increase the variety of the produced secondary metabolites [5]. So the aim of the present work was to optimize the production medium, Czapek-Dox (CD) minimal medium such that maximum product yield is obtained.

MATERIALS AND METHODS

Microorganisms

Aspergillus fumigatus BTMF9, the antibacterial peptide producer obtained from marine sediment (unpublished data), was used in this work. *Bacillus circulans* (NCIM 2107) was used as the indicator organism in the antibacterial activity assay.

Culture conditions and crude extract preparation
A. fumigatus conidia (2 x 10^6 conidia/ mL) were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of Czapek Dox minimal medium [6] incubated at 30°C in a shaking incubator (Orbitek, Scigenics, Chennai, India) at 150 rpm. After three days of incubation, the culture supernatant was obtained by filtering through four layers of cheese cloth and centrifugation at 13000 rpm at 4°C (Sigma, Germany) for 15 min. This supernatant with antibacterial activity was used as the crude preparation.

Antibacterial activity assay
Antibacterial activity was determined by both disc diffusion assay [7] and quantitative estimation of the antibacterial activity was by critical dilution assay [8]. For that the protein sample was double diluted. 5 µL of each dilution was spotted on the surface of Mueller-Hinton agar (HiMedia) plates, previously swabbed with log-phase cells of test organism. Plates were incubated at 37 °C for 18 h. One activity unit (AU) was defined as 5 µL of the highest dilution of filtrate yielding a definite zone of growth inhibition on the lawn of the test organism. The highest dilution was multiplied by 200 to obtain the activity units per mL (AU/mL). Protein concentration was determined using the method of Bradford [9] with BSA as standard. Specific activity of the sample was calculated by dividing the inhibitory activity units (AU/mL) with the protein content (mg/mL) and was expressed as AU /mg protein.

Effect of temperature on fungal peptide production
Effect of temperature on fungal peptide production was studied by growing the fungus in Czapek Dox medium at different temperature such as 30 °C, 35 °C, 40 °C , 45 °C and 50 °C.

Effect of inorganic nitrogen sources on fungal peptide production
Effect of inorganic nitrogen source on fungal peptide production was studied by the addition of ammonium sulphate, ammonium nitrate, potassium ferricyanide, ammonium vanadate, sodium azide, ammonium carbonate and ammonium molybdate individually at 0.3% (w/v), replacing the inorganic nitrogen source (sodium nitrate) of production medium. The culture was incubated at 30 °C and at an agitation of 150 rpm for 3 days.

Effect of organic nitrogen sources on fungal peptide production
Effect of complex organic nitrogen source on antibacterial peptide production was studied using tryptone, beef extract, peptone, yeast extract, malt extract, soybean casein hydrolysate, and casein added individually at 0.3% (w/v) of production medium described earlier, having sodium nitrate as inorganic nitrogen source. The culture broth was incubated at 30 °C and at an agitation of 150 rpm for 3 days.

Effect of carbon source on fungal peptide production
Various carbon sources added in to the production medium were glucose, lactose, mannitol, starch, maltose, glycerol, galactose, cellobiose, inositol and pectin used individually at 3% (w/v), replacing the carbon source (sucrose) of medium consisting of sodium nitrate as inorganic nitrogen source. The culture was incubated at 30 °C and at an agitation of 150 rpm for 3 days. A control was also kept with sucrose as carbon source.

Effect of pH on fungal peptide production
Different pH may have an effect on the growth and production of the desired compound. This was analysed by adjusting the pH of the medium with 1N NaOH and 1N HCl. Different pH studied were from a range of 3-9.

Effect of sodium chloride (NaCl) concentration on fungal peptide production
Addition of sodium chloride for maximum fungal peptide production was evaluated using different concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% & 5%). The media contained sucrose as carbon source, sodium nitrate as inorganic nitrogen source and pH was adjusted to 7. The culture was incubated at 30 °C for 3 days at 150 rpm.

Statistical analysis
The inhibitory activity and specific activity was performed in triplicates. The results are presented as Mean ± Standard deviation.

RESULTS AND DISCUSSION

Marine fungi are considered as an incredible source of biologically active natural products with chemically new structures [4,10]. Therefore developments of different approaches to mine for marine fungal species producing biologically active secondary metabolites are required. Facultative marine strains such as *Aspergillus, Penicillium, Verticillium* and *Phoma* are producing structural novelty products are often isolated [11, 12]. *Aspergillus fumigatus* is known as an opportunistic airborne pathogen affecting immune-compromised patients [13]. However, some strains of *A. fumigatus* produce broad range of biologically active metabolites [14,15]. Crude extract prepared by culturing *Aspergillus fumigatus* BTMF9 exhibited inhibitory action against Gram positive bacteria (data not shown) and among them, more sensitive *Bacillus circulans* was selected as test organism for present study (Fig. 1).

Optimum temperature is an important factor in the growth of organism and the production of desired compound. Maximum antibacterial activity (800 AU/mL) and specific activity of $\log_{10}$ 4.3 ± 0.1 AU/mg protein was observed at 30 °C. A higher incubation temperature (>45 °C) had an adverse effect on growth and antibacterial peptide production (Fig. 2).

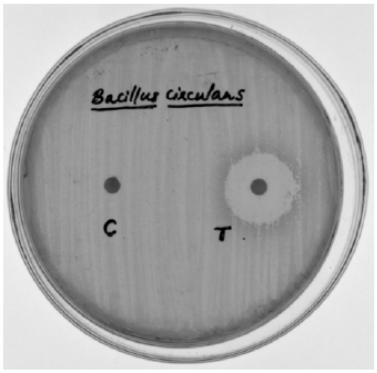

Figure 1. Inhibitory activity of crude extract from *Aspergillus fumigatus* BTMF9 against *Bacillus circulans* (C- Control, T- Crude extract)

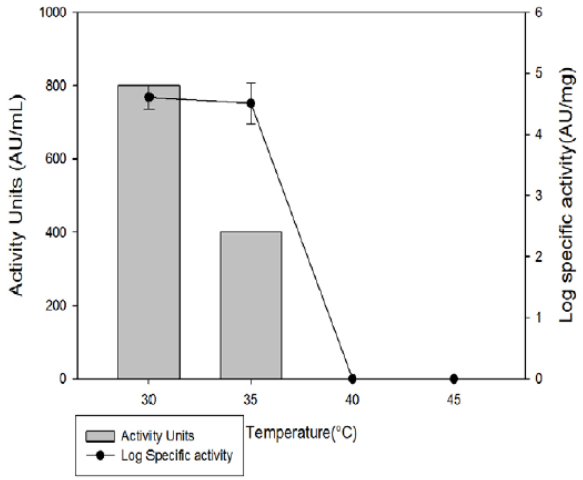

Figure 2. Effect of temperature on antibacterial peptide production by *Aspergillus fumigatus* BTMF9

Nitrogen sources widely influence the growth and production of the specific metabolites. Various inorganic and organic nitrogen sources were checked for production optimization. In case of inorganic nitrogen sources, it can be inferred that sodium nitrate was a very specific nitrogen source which gave both higher inhibitory activity (800AU/mL) and specific activity (log_{10} 3.2 ± 0.002) than the other nitrogen sources, which showed no activity at all (Fig. 3). However a very low activity was noted in the presence of ammonium molybdate. Organic nitrogen sources were least significant for production optimization (Fig.4) as activity was obtained only for soy casein hydrolysate (400AU/mL) and malt extract (400AU/mL).

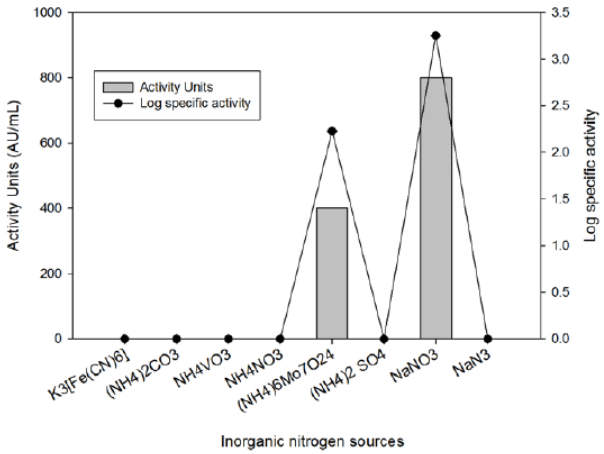

Figure 3. Effect of different inorganic nitrogen sources on antibacterial peptide production by *Aspergillus fumigatus* BTMF9.

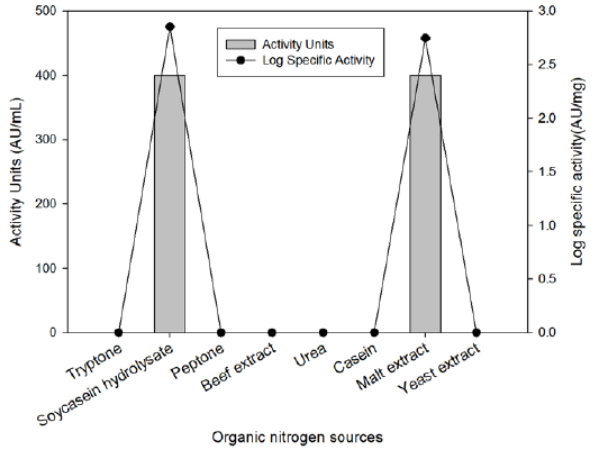

Figure 4. Effect of different organic nitrogen sources on antibacterial peptide production by *Aspergillus* fumigatus BTMF9.

The influence of different carbon sources on production of antibacterial peptide is shown in Fig. 5. In case of extract obtained from culture grown in broth containing both sucrose and glucose as carbon sources, inhibitory activity (800AU/mL) observed was same but the Log specific activity was maximum ($\log_{10}$ 4.5 ± 0.00 AU/mg protein) in presence of sucrose than glucose. Therefore sucrose can be considered as the best carbon source. There was no peptide production when other carbon sources were incorporated.

Since inhibitory activity and specific activity was highest (1600AU/mL and $\log_{10}$ 4.4 ± 0.05 AU/mg protein) with pH 7, it was taken as the optimum pH. At pH 4 and pH 5 there was reduced inhibitory activity (400AU/mL) and at pH 6 the activity increased to 800AU/mL. Production decreased, or there was no production when the pH was lowered and raised from seven respectively (Fig.6). However, there are reports of microorganisms with the ability to synthesize antimicrobial compounds at pH ranging from 5.5 to 8.5 [16].

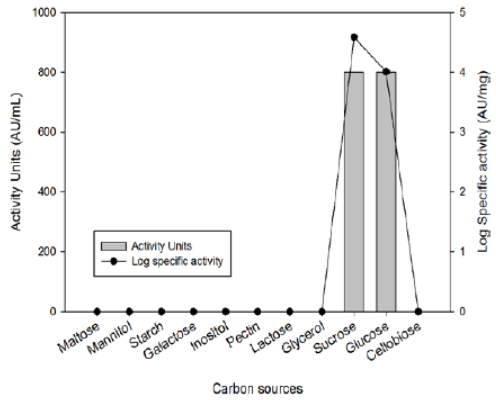

Figure 5. Effect of different carbon sources on antibacterial peptide production by *Aspergillus fumigatus* BTMF9.

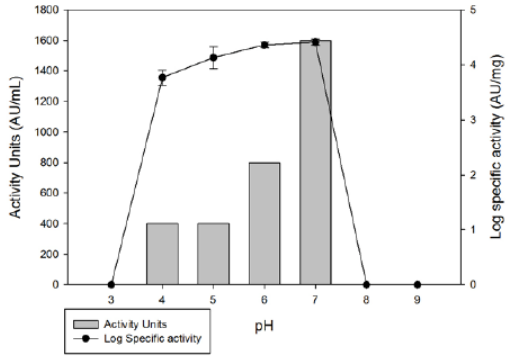

Figure 6. Effect of pH on antibacterial peptide production by *Aspergillus fumigatus* BTMF9.

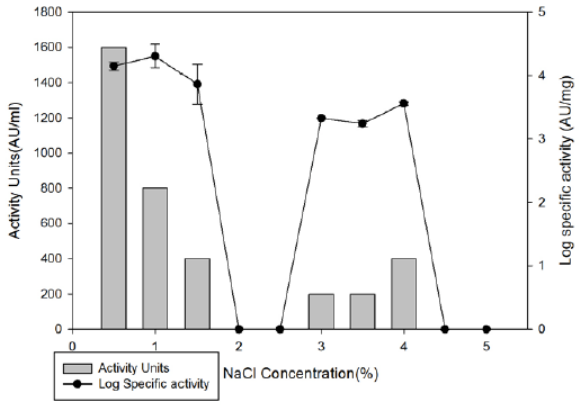

Figure 7. Effect of sodium chloride on antibacterial peptide production by *Aspergillus fumigatus* BTMF9.

Maximum antibacterial activity (1600 AU/mL) with a specific activity of $\log_{10}$ 4.15 ± 0.06 AU/mg protein was obtained when sodium chloride was supplied at 0.5 % (Fig.7) in the medium. Specific activity was a little higher ($\log_{10}$ 4.3 ± 0.1 AU/mg protein) when 1% NaCl was provided but inhibitory activity was only 800AU/mL. Therefore 0.5% concentration of sodium chloride was considered optimum for peptide production. As the concentration of sodium chloride increased, the production of peptide decreased.

CONCLUSION

Each organism requires very specific nutrients and environmental conditions for maximum production of a particular metabolite. The sequential medium optimization strategy utilized in the current work appeared to promote antibacterial peptide yield to a considerably higher level. These findings are of importance for the further study of peptide biosynthesis regulation and for improvement of production yields.

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