Purification, Biochemical and Functional Characterization of Bacillomycin-type Lipopeptides of *Bacillus subtilis* RLID 12.1 and Optimization for the Selective Production of the Most Potent Antifungal variant

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RAMYA R

ID. No: 2011PHXF0005G

Under the Supervision of

UTPAL ROY, Ph.D.

BITS Pilani

Pilani | Dubai | Goa | Hyderabad

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CERTIFICATE

This is to certify that the thesis entitled **"Purification, Biochemical and Functional Characterization of Bacillomycin-type Lipopeptides of *Bacillus subtilis* RLID 12.1 and Optimization for the Selective Production of the Most Potent Antifungal variant"** submitted by Ramya R, ID No. 2011PHXF0005G for award of Ph.D. degree of the Institute embodies original work done by her under my supervision.

Utpal Roy

Signature of the Supervisor:

Dr. UTPAL ROY

(Associate Professor, Department of Biological Sciences)

Date: 17/04/2018

Dedicated to my parents

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ABSTRACT

An ever-escalating antifungal resistance has accentuated the compelling need to recover novel antifungal molecules that are insensitive to resistance processes to combat the multidrug resistant pathogens. In the pursuit of new antifungal compounds, garden soils from Dehradun and Goa were screened for the potential antifungal producer. As a result of primary and secondary screening, a wild-type soil isolate RLID 12.1 showed strong fungicidal action against different strains of *Candida albicans,* non-*albicans* and *Cryptococcus neoformans*. Based on the morphological and biochemical characterization, 16S rDNA sequencing and FAME analysis, the producer strain was identified as *Bacillus subtilis.* The accession number JX089317 was assigned for the wild-type soil isolate *B. subtilis* RLID 12.1 by NCBI.

Growth kinetics study of *B. subtilis* RLID 12.1 in modified tryptic soy broth (mTSB) media clearly indicated that the production of antifungal compounds began at the mid-exponential phase and was reached maximum at the late exponential phase and declined during the early stationary phase. The concentrated cell free supernatant of *B. subtilis* RLID 12.1 exhibited broad-spectrum antifungal activity against the total of 16 pathogenic yeast strains including *C. albicans, C. glabrata, C. krusei*, *C. neoformans, Aspergillus niger, A. flavus, Fusarium oxysporum*, *Curvularia* sp. *Bipolaris* sp. and *Mucor* sp. when tested by cut well agar assay. The antifungal compound was found to be stable over a wide range of pH (4.0-10.0), metals salts and relatively stable at higher temperature (70ºC). Studies on the effect of proteolytic (proteinase K and trypsin) and non-proteolytic (lipase and α-amylase) enzymes indicated the lipopeptide nature of the antifungal compound(s).

Five coproduced antifungal compounds (AF_1, AF_2, AF_3, AF_4 and AF_5) were purified using Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). All the five compounds were confirmed as lipopeptides by thin Layer chromatography and mass spectrometry analyses that included gas chromatography mass spectrometry (GC-MS). Out of five extracted lipopeptides, three lipopeptides AF_3, AF_4 and AF_5 were found to be effective in terms of antifungal potential against *Candida* and *Cryptococcus* spp. The lipopeptides AF_3, AF_4 and AF_5 were identified with the same peptide sequence Asn-Pro-Tyr-Asn-Gln-Thr-Ser showing variations in the fatty acid branching type and chain length (*anteiso*-C_{17}, *iso*-C_{17} and *iso*-C_{18} respectively). The amino acids in the

peptide moiety were found similar to bacillomycin class but the position of amino acid residues was found different from bacillomycin type of lipopeptides reported previously. PCR amplification studies using specific antimicrobial peptide primers also confirmed the presence of the bacillomycin operon in the genome of *B. subtilis* RLID 12.1.

Comparing the minimum inhibitory concentration (MIC) of AF_3, AF_4 and AF_5 against 81 *Candida* (64 no.) and *Cryptococcus* (17 no.) isolates, the antifungal efficiency of the lipopeptides were marked in the order AF_4>AF_5>AF_3. MIC and MFC determination studies revealed that the lipopeptide variant AF_4 was found to be the most promising antifungal lipopeptide where 93.8% of the tested strains exhibited geometric mean (MIC_g) ≤4 µg/mL at which the observed hemolysis was <5% and the IC_{50} values tested against four mammalian cell lines was 13.31µg/mL. In the time-kill kinetic study, AF_4 treatment reduced 99% viability ($2\log_{10}$ reduction) of *C. albicans* ATCC 24433 between 8-12 h of incubation. Studies on the biofilm formation inhibition potential of AF_4 showed that $SMIC_{50}$ was about 2-4 times higher than the MIC value obtained.

Interaction studies were evaluated among the lipopeptides AF_3, AF_4 and AF_5 against three *Candida* species along with the cytotoxicity studies. Five combinations exhibited good additive interaction effects: AF_3, AF_4 (4, 4 µg/mL), AF_3, AF_5 (4, 4 µg/mL), AF_3, AF_5 (2, 4 µg/mL) AF_4, AF_5 (4, 4 µg/mL) and AF_4, AF_5 (2, 4 µg/mL) in planktonic cell inhibition and AF_3, AF_4 (4, 4 µg/mL), AF_3, AF_5 (4, 4 µg/mL) and AF_3, AF_5 (2, 4 µg/mL) in the inhibition of biofilm formation. These interaction studies indicated that the two combinations, (AF_3, AF_4) and (AF_4, AF_5) might be used against the yeast strains which are less sensitive to AF_4 as they exhibit negligible hemolysis (<5%) and approximately 70% cell viability when tested against four mammalian cell lines. Taken together, the lead novel molecule AF_4 and the combinations (AF_3, AF_4) and (AF_3, AF_5) showing anti-*Candida* and anti-*Cryptococcal* potential along with anti-biofilm forming property with less cytotoxicity might promise a suitable substitute to confront the multidrug-resistance issues.

The yield of the antifungal (AF) lipopeptide variants were observed in the order AF_2>AF_1>AF_4>AF_3>AF_5 in mTSB. Investigation on the production conditions were performed aiming two outcomes, first was to optimize the media formulations and the production conditions to enhance the production of all the three variants AF_3, AF_4 and AF_5 and the second goal was to improve the selective production of the most

promisinglead molecule AF_4. Four media formulations, M_1 and M_2 (using Box-Behnken statistical methodology), M_3 and M_4 (by modifying M_2 and M_1 respectively) were designed during the optimization process. Significant finding derived from the optimization study was that the proportion of AF variants of *B. subtilis* RLID 12.1 can be controlled by temperature and metal ions under static and shaking growth conditions.

Targeting the selective augmented production of AF_4 variant, a new fermentation media (M_3) comprising malt extract (1.01%), dextrose (0.55%), peptone (1.79%), $MnSO_4$ (2mM) and NaCl (0.5%) was formulated. Maximum production of 954.8±10.8 mg/L was achieved with 44% selectivity at 30°C compared to unoptimized conditions (186.4±6.1 mg/L). Supplementing calcium alginate beads in the formulated media during the onset of lipopeptide production induced the selective production of AF_4 variant to 1170.9 ±10.2 mg/L. The selective improvement in the yield of the most efficacious AF_4 variant to about 72% was observed due to the partial immobilization (non-covalent adsorption) of other lipopeptide variants AF_1 and AF_2 into the beads. Non-covalent adsorption of lipopeptides varied with bead size, sodium alginate and calcium chloride concentrations. Use of Ca-alginate beads in the upstream production process of the lead AF_4 variant may be a considerable novel approach to address the challenge which might be faced during the scale-up and downstream processing steps.

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LIST OF ABBREVIATIONS

AMP - Antimicrobial peptide

AF - Antifungal

AFP - Antifungal peptide

APD - Antimicrobial Peptide Database

ATCC - American Type Culture Collection

BCA - Bicinchoninic Acid

BSA - Bovine Serum Albumin

CDC - Centre for Disease Control and Prevention

CFS- Cell Free Supernatant

CFU - Colony Forming Units

CID - Collision Induced Dissociation

CLSI - Clinical and Laboratory Standards Institute

CM - Cryptococcal meningitis

CNS-Central nervous system

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic Acid

ELISA - Enzyme-Linked Immunosorbent Assay

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

FAMEs - Fatty acid methyl esters

FBS - Fetal Bovine Serum

5-FC - 5-Flucytosine

IEC - Ion-exchange chromatography

MIC_g - Geometric Mean

HaCaT - Human keratinocytes

HIV - Human Immunodeficiency Virus

HC - Hemolytic concentration

HGT - Homologous Gene Transfer

IC - Inhibitory Concentration

GC/MS - Gas Chromatography/Mass spectrometry

LR - Log10 Reduction

m/z - mass-to-charge

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

MDR - Multi Drug Resistant

MFC - Minimum Fungicidal Concentration

MIC - Minimum Inhibitory Concentration

MTCC - Microbial Type Culture Collection

mTSB - modified Tryptone Soya Broth

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

NAC - Non-*albicans Candida*

NCBI - National Centre for Biotechnology Information

NCCPF - National Culture Collection of Pathogenic Fungi

NCIM - National Collection of Industrial Microorganisms

NRPs - Non-Ribosomal peptides

NRPS - Non-Ribosomal Peptide Synthases

OD - Optical Density

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PKs - Polyketides

PHYLIP - PHYlogeny Inference Package

SDA - Sabouraud dextrose agar

SE - Size exclusion chromatography

$SMIC_{50}$ - sessile cells MIC

TCA - Trichloroacetic acid

TFA - Trifluoroacetic Acid

TLC - Thin Layer Chromatography

TTC - Triphenyl Tetrazolium Chloride

XTT - [2,3-bis (2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]

WHO - World Health Organisation

Chapter 1

Introduction and Review of Literature

1.1. Introduction

1.1.1. History of antimicrobials

Discovery and usage of antimicrobials has been practiced since the ancient times to treat, control and manage infectious diseases. Although historical evidences such as tetracycline distribution in the human skeletal systems (bones) of ancient Sudanese Nubia about 350-550 CE, the Egyptian and Greek culture of applying old moldy bread or concoction made of honey, lint, and lard, and Jordanian culture of applying red soils on the infected area (or wounds) explain the practice of utilizing antimicrobials for some thousands of years, the systematic screening on antimicrobial compounds started only during the 'Antibiotic era" (Aminov, 2010).

Discovery of the first drug, Salvarsan, by Paul Ehrlich in 1909 against syphilis was considered as the start of the "Antibiotic era" (Ehrlich and Hata, 1910) and his drug screening strategies shaped the future approaches used for antimicrobial research. The second drug, prontosil, a sulfa drug was synthesized in 1935 by Josef Klarer, Fritz Mietzsch and Gerhard Domagk (Domagk, 1935). A major breakthrough in the history of medicine was the discovery of penicillin from *Penicillium* strain by Alexander Fleming in 1929. After 12 years of his discovery, penicillin was accepted for clinical use following several clinical trials (Fleming, 1929). These findings set an architype for the future antimicrobial research and resulted in the identification of variety of antimicrobial drugs from several/various natural sources. The period from 1940s to late 1960s was referred as the "golden era" (Walsh, 2003) where maximum number of antimicrobials were identified.

"*The time may come when penicillin can be bought by anyone in the shops. There is the danger that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."*

- Alexander Fleming

at his 1945 Nobel Prize lecture

As rightly predicted by Alexander Fleming, advancement in the modern medicine and increased use of antibiotics led to the development of antimicrobial resistance.

1.1.2. Fungal infections

Humans develop fungal infections *via* direct contact with yeasts or by inhaling the fungal spores. In general, human body can resist the primary fungal infections by powerful immune surveillance system along with the body temperature. Severe infections occur mostly in the immunocompromised humans where fungus get access to carbon and nitrogen sources for its survival (Papon et al., 2013). Among the five million fungal species reported worldwide, around 300 species are found to be causative agents of fungal infections. About 95% of the fungal infections are caused by *C. albicans*, *C. neoformans* and *A. fumigatus* (Khan et al., 2010). *Candida* spp. has unfortunately emerged to be a serious public threat and ranks fourth in causing invasive fungal infections particularly nosocomial blood stream infections (8-10%) which raised the mortality rates to about 15-35% (Pfaller MA and Diekema, 2007; Lai et al., 2012; Sardi et al., 2013). Even the vulnerable *Candida* strains become virulent in most of the critical care unit due to the increased antifungal usage (Rajgopal, 2017). Increased outbreaks of *Candida* infection, resistance development against the existing antifungal therapy, and therapeutic management are the great challenges faced by the clinicians in the last few decades. A combination therapy of the existing antifungals also has failed to combat the resistances developed by yeasts. Therefore, alternate antifungal medicines/molecules are the need of the hour.

"Human subtlety will never devise an invention more beautiful, more simple, or more direct than does Nature—because in her inventions, nothing is lacking—and nothing is superfluous..."

-Leonardo da Vinci

Nature has a reservoir of chemicals or compounds produced by both prokaryotes, and eukaryotes. These natural substances are strategically produced in response to the biological targets present in the diversified environment as a first line of defence without affecting the host. Such naturally available compounds may be utilized to specifically target the yeast pathogens. As a rule, higher the target specificity lesser is the toxicity. These naturally occurring antimicrobial molecules may be the ideal solution to the increasing incidences of antimicrobial resistance.

1.1.3. Alternate antifungal - Antimicrobial peptides

Antimicrobial peptides (AMPs) are the naturally occurring defence effector molecules produced by bacteria, fungi, insects, amphibians, plants, mammals, and humans. Currently more than 1000 AMPs are documented in the Antimicrobial Peptide Database (APD) (http://aps.anmc.edu/AP/main.php). Extensive research on AMPs as an alternate therapeutic option has resulted in the discovery of several novel antibacterial and antifungal compounds which are currently available in the market for clinical use (De Lucca and Walsh, 1999).

Antifungal peptides (AFPs) elicit their activity either through membrane disruptive action (barrel stave, toroidal and carpet) or through non-membrane disruptive action (intracellular targets) (Matejuk et al., 2010). Antifungal peptides following membrane lysis mechanism include defensins, cecropins, magainins, histatins and RsAFP-2. In the barrel stave mechanism, individual peptides aggregate and form a barrel like structure inside the membrane and damage it by forming pores. Toroidal mechanism entails the binding of AFP to the membrane lipid resulting in the pore formation leading to an inward curvature of the membrane with the positive head groups facing towards the center of the pore. The carpet mechanism on the other hand, involves the electrostatic binding of the peptide monomers to the membrane surface in a carpet form thereby disrupting the membrane without formation of any pores or channels. Non-membrane disruptive mechanisms target either DNA, RNA or protein synthesis, or essential cellular components for eg: echinocandins and nikkomycins (Shai, 2002; Yeaman and Yount, 2003).

1.1.4. AMPs produced by *Bacillus* sp.

Bacillus sp. is an aerobic, rod shaped omnipresent Gram-positive bacteria which produce various peptide based antifungals and antibiotics of different spectrum (Stein, 2005; Slepecky and Hemphill, 2006). Among bacteria, *Bacillus* sp. is known to adapt to various nutritional and environmental conditions and survive predominantly over other organisms present in the ecological habitats (Nicholson et al., 2002). This remarkable feature is mainly due to their ability to produce antimicrobial substances and sporulation property. The whole genome sequencing of *Bacillus* sp. revealed the presence of putative biosynthetic gene cluster which is an unexpected source of antimicrobial compounds. Although, 4% of its genome was annotated for the synthesis of polyketides (PKs), non-

ribosomal peptides (NRPs), bacteriocins, and other unusual antibiotics (Fickers, 2012; Zhao and Kuipers, 2016), very few antimicrobials from *Bacillus* sp. have been reported to date (Sumi et al. 2014; Zhao and Kuipers, 2016). Therefore, *Bacillus* sp. is a potential candidate to screen for potent and novel antifungals to combat the high prevalence of fungal infection.

1.2. Pathogenicity of *Candida* spp.

In a healthy human body, *Candida* spp. particularly *C. albicans* (70%), *C. glabrata* and *C. tropicalis* (together7%) are found to exist in mouth, gastrointestinal tract, anus, groin, and vaginal canal (Odds, 1988). *Candida* spp. acts like a saprophytic constituent of human microbial flora in the yeast form (blastospores) along with bacteria without causing any harm or disease (Odds et al., 2006). Infections caused by *Candida* may be categorized as endogenous or exogenous (Chakrabarti and Shivprakash, 2005). Endogenous infections occur when there is a change in the pH, oxygen, glucose concentration, and carbon dioxide in the human tissues and also when the microbial floral balance is disturbed by increased use of broad spectrum antibiotics. At this stage, the yeast cells of *Candida* are transformed to hyphae form and attains the potential to harm the host by producing adhesins and hydrolytic enzymes (Tampakakis et al., 2009; Khan et al., 2010; Kim et al., 2011). Exogenous fungal infections arise due to a prolonged stay in the hospital, central venous catheter, abdominal surgery, skin burns, steroids, dialysis, impaired immune system by HIV, diabetes or may be from health care workers. *Candida* spp. causes skin and nail infections (cutaneous as well as chronic mucocutaneous infections), urinary infections (vaginal/genital candidiasis or yeast infection), mouth and throat infections (oropharyngeal/esophageal candidiasis), and gastrointestinal infections (intestinal candidiasis). *Candida* infection of the blood is termed as candidemia while the systematic infection which affects the brain, heart, eyes, bones, and other organs of body via the blood stream is called invasive candidiasis or candidemia (Murzyn et al., 2010). Eventually, disturbances in the host's tissue environment and the immune surveillance system lead to the transition of commensal organism to an opportunistic pathogen.

Approximately seventeen species of *Candida* have been so far recognized as a etiological agents responsible for candidiasis and candidemia. Among them, *C. albicans* is the third leading agent for hospital acquired blood stream and urinary infections associated with central venous catheters, and bladder catheters usage (Horn et al., 2009;

Sardi et al., 2013). Various factors contributing to the pathogenicity of *C. albicans* include adhesins and invasins present on yeast cell surface that enable the pathogens to adhere on to the host cells; besides this, secretion of hydrolytic enzymes like proteases, phospholipases and lipases, biofilm formation ability, rapid adaptation to environmental conditions, yeast to hyphae transition, phenotypic switching, contact sensing and thigmotropism (directed hyphal growth), metabolic flexibility, and significant stress response machineries (Mayer et al., 2013) potentially arm these yeasts to cause pathogenicity.

Although *C. albicans* is the predominant causative agent of candidiasis, in the last two decades the isolation rates of *C. albicans* has decreased from 70 to 50% whereas that of non-albicans *Candida* (NAC) has increased from 40 to 60% which may be responsible for the high mortality rates observed (Deorukhkar et al., 2014). NAC species involved in candidiasis are *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. auris.* Shift in the epidemiology of *Candida* infections started in 1990s mainly due to the extensive use of azole type antifungals and further enhancement was attributed to the prolonged hospitalizations and illnesses arising due to high immunosuppression (Bassetti et al., 2006; Chakrabarti et al., 2009). Among the NAC species, *C. tropicalis*, and *C. parapsilosis* are emerging as a global pathogen exhibiting high resistance towards fluconazole, and Amphotericin B, and the second leading causative of candidemia (Silva et al., 2012). *C. tropicalis* infections are usually reported in adult patients with neutropenia (particularly acute leukaemia) and malignant cancers. These infections exhibit variability in both pathogenicity and antifungal susceptibility, rendering them difficult to cure. The incidence of *C. parapsilosis* infections is also reported to be higher in neonates as compared to adults (Trofa et al., 2008). *C. glabrata*, a non-pathogenic organism of human flora has emerged as a pathogen in recent years causing systemic infections mostly in adults than the neonates (Hajjeh et al., 2004). The antifungal resistance patterns as observed in *C. tropicalis* and *C. glabrata* are mainly due to their biofilm forming ability (Silva et al., 2012; Silva et al., 2015). Intrinsically fluconazole resistant *C. krusei* are reported mostly in the patients with hematological malignancies and bone marrow transplantation. The second case of *C. auris* infection was reported in India after the report on the first clinical case in Japan,2009 (Satoh et al., 2009). *C. auris* was found phenotypically similar to *C. heamulonii*. Among all the *Candida* spp. reported till date, *C. auris* is one of the difficult challenges for the clinicians worldwide as it

manifests drug resistance against all the three classes of antifungals (Sarma and Upadhyay, 2017).

1.3. Infections caused by *Cryptococcus* sp.

Two species of *Cryptococcus* are mainly responsible for cryptococcosis/cryptococcal meningitis (CM): *C. neoformans*, and *C. gattii*(May et al., 2016). Based on the whole genome sequencing, *C. neoformans* is classified as *C. neoformans* var. *grubii* (serotype A); *C. deneoformans* (serotype D); and *C. gatti* comprising serotype B and C is divided in to five: *C. gattii, C. bacillisporus, C. deuterogattii, C. tetragattii* and *C. decagattii* (Hagen et al., 2015). *Cryptococcus* spp. is an encapsulated basidiomycetous yeast found mostly in birds, for example pigeon and environment (soil). On exposure to *Cryptococcus* sp., human immune system generates antibodies and clears it from the body. However, in immunosuppressive individuals, this opportunistic pathogen enters the central nervous system (CNS) and damages the meninges and brain tissues (CM) (Heitman et al., 2010). In general, cryptococcosis infection is observed in immunocompromised patients with HIV infection, organ transplantation, or prolonged corticosteroids usage. However, recent studies have shown that immunocompetent individuals may also be prone to infections by *Cryptococcus* sp. like *C. tetragattii* and *C. bacillisporus* (Raberahona et al., 2016).

1.4. Current scenario of *Candida* and *Cryptococcus* infections

1.4.1. Foreign countries other than India

Both the developed and developing countries are facing the challenge to combat *Candida* infections whose incidence has increased five times in the past decade irrespective of the advancements in diagnostic process and various disease management strategies (Kaur and Chakrabarti, 2017). However, the occurrence and the type of species involved in spreading the disease varies with the infection site and geography. Studies on the incidence of ICU infections shows that globally 17% of infections are caused by *Candida* spp. that is about 1 to 10 per 1000 ICU patients are affected (Yapar, 2014; Kullberg and Arendrup, 2015). Candidemia is found to be about 4-15 times higher in developing countries as compared to developed countries. Isolation rates of *C. albicans* in developed nations was found to be 30-50% in USA, Spain, Italy, and Australia and 50-70% in Canada, Europe, Sweden, Norway, Switzerland, Germany and Belgium, where as

in developing countries 25-50% was found in China, Brazil, Latin America, Argentina, Columbia, Thailand, Turkey, and South Africa, and 50-70% in Asia pacific, Africa, and Middle East (Yapar, 2014; Tan et al., 2015; Kaur and Chakrabarti, 2017). Among the NAC species, a higher isolation rates for *C. glabrata*, and *C. krusei* were found in developed countries as compared to developing countries. Particularly in USA, patients undergoing fluconazole therapy and aged patients were found to be susceptible to *C. glabrata* infections (Pfaller et al., 2007; Kaur and Chakrabarti, 2017). Lower isolation rates (about 0-10%) of *C. glabrata* was reported in most of the developing countries with the exception of South Africa (20%), China (~13%), and Asia Pacific (~14%). A higher isolation rates was reported for *C. tropicalis*, and *C. parapsilosis* in developing countries such as, about 10-30% and <7% in South Africa (Yapar, 2014; Kaur and Chakrabarti, 2017). Overall, the literature survey on *Candida* infections shows that the developing countries suffer higher mortality rates (>50%) than the developed countries (<50%) (Kaur and Chakrabarti, 2017). Another threat growing across the globe is the emergence of multidrug resistant (MDR) superbug *C. auris* which has a higher incidence in Asian countries (Chowdhary et al., 2017; Rudramurthy et al., 2017). MDR *C. auris* isolates are found spreading in both developing and developed countries like Colombia, Germany, Canada, Kuwait, India, Israel, Japan, Kenya, Kuwait, Norway, Pakistan, Spain, South Africa, South Korea, the United Kingdom, and Venezuela, as well as the United States (Emara et al., 2014; Schelenz et al., 2016; Lockhart et al., 2016; Calvo et al., 2016; Vallabhaneni et al., 2017; Schwartz and Hammond, 2017).

Before the introduction of antiretroviral therapy (ART), mortality in AIDS-patients due to cryptococcosis was reported in about 371,500-1,544,000 cases each year (Park et al., 2009). Yearly on an average 222,000 cases of *Cryptococcus* infection were reported in HIV infected patients after ART expansion. Among them, 162,500 cases were found in sub-Saharan Africa (Rajasingham et al., 2017) where meningitis has been found to be a leading cause for the death in HIV-infected patients than tuberculosis. A higher incidence of *Cryptococcus* infections was reported in low and middle-income countries where limited health care resources (ART) are available for treatment of HIV/AIDS patients (Jarvis et al., 2009). Second highest incidence rates (43,200 cases) of secondary infection due to *Cryptococcus* spp. (cryptococcosis) in ART treated HIV +ve/AIDS patients was reported in developing countries like those in Asia-Pacific. Comparatively fewer cases were reported in developed countries like Europe (4,400 cases), and North/South America (9,700 cases) (Rajasingham et al., 2017). *C. gattii* isolates were

more prevalent in Europe, Asia, and Australia whereas *C. deuterogattii* and *C. bacillisporus* isolates exhibited higher prevalence in North and South America; while *C. tetragattii* was more prevalent in Southern part of Africa (Espinel-Ingroff and Kidd, 2015; Bielska E and May, 2016; Mukhopadhyay et al., 2017).

1.4.2. Prevalence of *Candida* infections in India

Outbreak of multidrug resistant *C. tropicalis*, *C. krusei*, *C. glabrata* and *C. auris* is a major clinical crisis faced in India (Deorukhkar et al., 2014; Chakrabarti et al., 2015; Duggal et al., 2015; Chowdhary et al., 2017). As compared to other developing countries, high prevalence of *C. tropicalis* (41.6%) was reported in India mainly in private or corporate ICUs. Among them 1.9% exhibited multi drug resistance, while 11.8% isolates were resistant to azole (Deorukhkar et al., 2014; Chakrabarti et al., 2015). Candidemia studies carried out in about 27 ICUs of India demonstrated that *C. auris* isolates were found in 19 ICUs comprising 5.2% of the total *Candida* sp. isolated. Among them, 58.1% were resistant to fluconazole, 13.5% to amphotericin B, and 9.5% to caspofungin. The prevalence of *C. auris* was higher in public sector hospitals in Northern India as compared to those in Southern India (Rudramurthy et al., 2017). The phenotypical and genotypical characteristics of about 26 Indian *C. auris* isolates (North and South India) were found to be different from that of Korean, and Japanese isolates (Chowdhary et al., 2014). In Indian scenario, amongst infections caused by *Cryptococcus*, *C. gattii* isolates were reported to be more prevalent as compared to *C. neoformans* which accounted for about 2-2.8% in Southern India and 8-8.8% in Northern India (Chowdhary et al., 2011; Cogliati et al., 2012). *C. tetragattii* was also found to be the predominant species, of which 20% showed fluconazole resistance (Mukhopadhyay et al., 2017).

1.5. Currently available antifungal drugs

Several topical drugs (like Nystatin, Griseofulvin, Clotrimazole, Tolnafate, Triacetin and many more) are available for superficial mycoses. Therapeutic antifungal drugs for systematic fungal infections may be divided into three main classes viz., (i) polyenes;(ii) azoles; and (iii) echinocandins. The antifungal development timeline is illustrated in Fig. 1.1.

Systematic antifungal therapy commenced with the discovery and approval of amphotericin B deoxycholate, a polyene in the 1950s followed by 5-Flucytosine in the

1960s. However, toxicity issues in the case of amphotericin B, and drug resistance development in the case of 5-FC were the major drawbacks. Later in the 1970s, azole derivatives were approved as a first line choice for treating systematic and invasive infections.

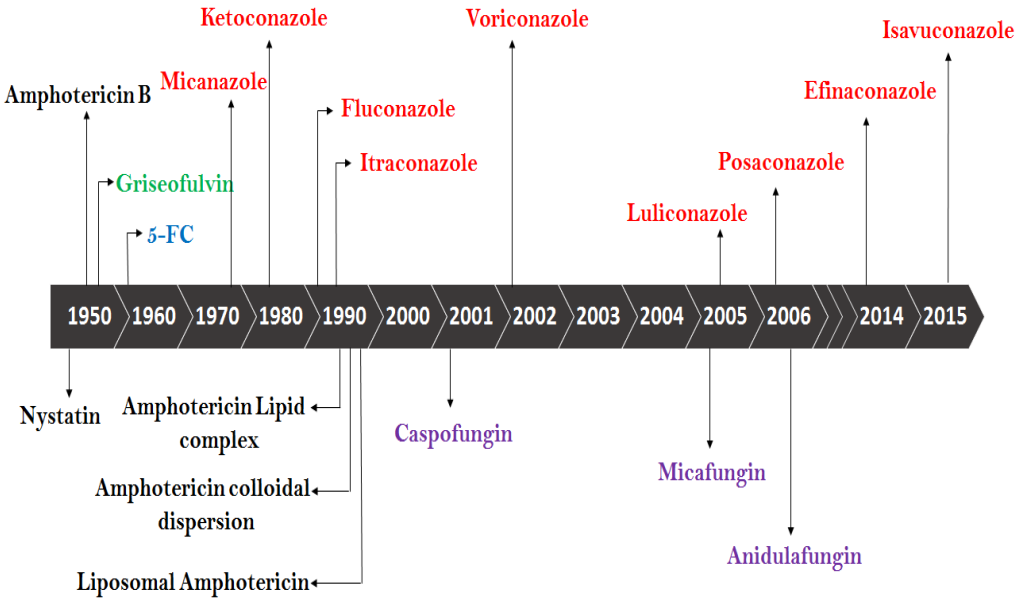


Fig. 1.1: Timeline of antifungal drug development from 1950-2017 (Perfect 2017)

Azole derivatives were mainly classified as Imidazoles and Triazoles. Ketoconazole and Miconazole are Imidazole derivatives. Triazole derivatives were further divided into second generation azoles (fluconazole and itraconazole), and third generation azoles (voriconazole, posaconazole, and isavuconazole). New formulations of azole derivatives showed an advanced antifungal action and pharmacokinetics with intravenous as well as oral applications. In the early 2000s (Fig. 1.1), echinocandin family of antifungals (caspofungin, micafungin and anidulafungin) were introduced, which exhibited lower toxicity as compared to azoles. Echinocandins are considered as the first line of drugs for treatment of nosocomial candidemia, and invasive candidiasis (Pappas et al., 2016). The molecular targets of polyenes, azoles, 5-FC, and echinocandins in the yeasts cells are elaborated in Table 1.1. After the introduction of different classes of antifungals and their prolonged use, yeast strains have developed resistance in the due course of time for their survival. The resistance mechanisms followed by yeasts against the existing antifungal drugs are mentioned in Table1.1 along with the limitations of these antifungal chemotherapies.

Table 1.1: Therapeutic antifungal drugs currently used in the hospitals

S.No	Antifungal drugs	Mode of action	Resistance	Limitations
1.	Polyenes	**Bind the ergosterol** moiety of the fungal membrane, create pores and leaks the cytoplasmic constituents of cells	Occurs very rarely- Replacement of ergosterol with another sterol and alterations in membrane fluidity	Nephrotoxicity
2.	Flucytosine (5-FC) (fluorinated cytosine analog)	Inhibits **nucleic acid synthesis** by converting the 5-FC to fluorodeoxyuridylic acid which is an inhibitor of thymidylate synthesis.	Mutation of cytosine permease which is responsible for the uptake of 5-FC for metabolization	Bone marrow toxicity with renal impairment and rapid development of drug resistance
3.	Azole derivatives	Inhibits **Cytochrome P-450 14*α*-demethylase** which converts lanosterol to ergosterol.	• Efflux pump activation • Overexpression or alteration of the target - ERG11 gene encoding 14-α-demethylase • Modifications in the sterol synthesis	Side effects (toxicity) and drug resistance due to the increased use of azoles
4.	Echinocandin	Inhibits the synthesis of a cell wall component, **β-(1,3)-D-glucan**	Mutation of the gene FKS1 encoding glucan synthase and reduce the echinocandin interaction with FKS1	• Not applicable to treat *Cryptococcus* infection • Increased drug resistance

1.6. Antimicrobial peptides

Development of novel antibiotics is evidently the need of the hour to replace the traditional antibiotics and its synthetic derivatives, plus combinations to combat the infectious pathogens and its resistance towards the currently used antifungal therapies (Hassan et al. 2012). AMPs are ubiquitous and promising natural antibiotics produced by various bacteria, fungi, plants, and animals as a part of their defence action (Pálffy et al. 2009; Sumi et al. 2014), of which bacterial AMPs, particularly play a major role (Cotter et al. 2013; Cochrane and Vederas, 2016). As the AMPs cause membrane disruption by pore formation, the chances of the target microbes to develop resistance towards them is inherently very less (Sang and Blecha 2008). Till date, thousands of AMPs have been reported and several have passed through clinical trials. (Jenssen et al. 2006; Sang and Blecha 2008; Sumi et al. 2014; Brogden and Brogden 2011).

Research on antimicrobial metabolites of endospore forming Gram-positive *Bacillus* sp. has been on-going from several decades and an average of 4-5% of its entire genome was found to be dedicated for antibiotic synthesis. Previous studies described the role of structurally differentiated secondary metabolites for the survival of *Bacillus* sp. in the naturally competitive habitat and its specific contribution in the morphology and physiology (Hassan et al. 2012; Paik et al. 1997; Li et al. 2012; Sabaté and Audisio 2013). Among the various classes of antibiotics produced by *Bacillus* sp., the peptide class of antibiotic was most abundant which exhibit a variety of structures comprising of unusual amino acids such as D-amino acids which confers stability even in the presence of proteolytic enzymes such as peptidases and proteinases (Kumar and Johri, 2012). *Bacillus* sp. produce antimicrobial peptides by one of the following pathways: (i) ribosomal peptide synthesis (linear type) with post-translational modification; and (ii) non-ribosomal peptide synthesis (both linear and cyclic type) by large non-ribosomal peptide synthetases (NRPSs) (Stein 2005; Abriouel et al. 2011; Finking and Marahiel, 2004).

1.6.1. Ribosomally synthesized peptides

Low molecular weight ribosomally synthesized AMPs, reported in both prokaryotic, and eukaryotic organisms, comprise of 12-50 amino acid residues. The gene-coded typical cationic AMPs exhibiting narrow antimicrobial spectrum can be subjected to mutagenesis or

cloning in heterologous expression systems to improve their antimicrobial spectrum as well as production (Caterina and Stahl, 2009). Bacteriocins are bacterial AMPs most commonly produced by *Bacillus* spp., and *Lactobacillus* spp. with either amphiphilic or hydrophobic property - that provides competitive advantage by inhibiting closely related organisms, and enabling occupation of the ecological niche (Motta and Brandelli, 2008). Bacteriocin follows the regular ribosomal mechanism of peptide and protein biosynthesis. The amino acid-tRNA synthetase loads the selected cognate amino acid in to the corresponding t-RNA while performing proof reading for insertion of correct amino acid, simultaneously. The elongation factor EF-Tu selects the suitable aa-tRNA and elongates the polypeptide according to the mRNA sequence. GTP hydrolysis of EF-Tu in the ribosomal subunit occurs after confirming the complementary base pairing (proof reading) between mRNA and aa-tRNA. Once the aa-tRNA accommodates the acceptor site (A-site) of ribosome, translocation and translation occurs leading to a peptide synthesis (McIntosh et al., 2009; Singh and Ghosh, 2012). Depending on the structure, size, stability, sensitivity, and function, the *Bacillus* bacteriocins are categorized in to three classes, viz.: Class I, Class II and Class III bacteriocins (Zhao and Kuipers, 2016).

- Class I: Post translationally modified bacteriocins-lantibiotics and lantipeptides, labyrinthopeptins and sactibioticseg: nisin, subtilin, clausin, subtilomycin, geobacillin, lactaticidin and mersidin
- Class II: unmodified bacteriocins-pediocin like peptideseg: pediocin PA1, sakacin A and P, curvacin A, mesentericin, carnobacteriocin, entericin A, and piscicolin, 2-peptide bacteriocins, circular bacteriocins, and linear non pediocin like 1-peptide bacteriocinseg: lactococcin A, G, and MN, enterocins 1071 and L50, lactacins F, mutacin IV, brochocin-C, and plantaracin C, EF, JK and S
- Class III: Bacteriocin like peptides or large antimicrobial proteins eg: helvetin and enterolysin

Ribosome synthesized bacterial AMPs show excellent antibacterial activity but do not possess antifungal potential (Sumi et al. 2014).

1.6.2. Non-ribosomally synthesized peptides

Non-ribosomal peptides are the unusual peptides synthesized in bacteria, fungi, and streptomycetes involving large complex multifunctional enzyme, non-ribosomal peptide synthetase (NRPS) which acts as a catalyst as wells as a template for peptide synthesis (Robert and Marahiel, 2004; Sieber and Marahiel 2005; Felnagle et al., 2008). A part of a polypeptide chain present in NRPSs is said to be a module which plays a role in building up of amino acid blocks to peptide form. NRPS modules are primarily subdivided as initiation, elongation, and termination modules, where the elongation module comprises of three core domains: adenylation domain (A), thiolation (T) or peptidyl carrier protein (PCP) domain and condensation domain (C), arranged in the order of C-A-T whereas the initiation module contains A-T domain without a C-domain. A-domain (containing 550 amino acids) recognizes the cognate amino acid and activates the substrate forming aminoacyl-adenylate intermediate utilizing ATP. This activated amino acid further binds covalently to the 4' phosphopantethiene carrier of PCP (containing 80-100 amino acids) catalyzed by phosphopantetheinyl transferase. The 4' phosphopantethiene cofactor helps the bound amino acyl and peptidyl substrate to move flexibly between the catalytic centers. The condensation domain transfers the amino acid to the PCPs of adjacent module and catalyzes the peptide bond formation between the amino acids (Hancock, 1997; Roongsawang et al., 2010; Jacques, 2011). Finally, thioesterase (TE) domain (containing 450 amino acids) of the termination module (C-terminal of NRPS) cyclize the peptide (by lactones or lactams linkage) or oligomerize the peptide units and release it from NRPS (Kopp and Marahiel, 2007).

Additionally, tailoring domains are found in the integral parts of NRPS which are involved in the modification of the primary peptide structure by methylation, oxidation, glycosylation, epimerization, oxidation, and fatty acid addition. Presence of unusual amino acids like D-amino acids in the non-ribosomal peptides is attributed to the presence of epimerization domain (E) in the NRPS module which converts the configuration of PCP bound L- amino acid to D- amino acid. Attachment process of fatty acid to peptide is catalyzed by a starter condensation domain (Felnagle et al., 2008; Roongsawang et al., 2010).

1.7. Lipopeptides

Lipopeptides are the structurally diverse biosurfactants produced by several bacteria, fungi, yeasts, and actinomycetes (Cameotra, 2015; Kumar and Johri, 2012). Lipopeptides are amphiphilic compounds comprising of hydrophilic amino acid moiety linked to hydrophobic fatty acid chain leading to a cyclic or linear structure. Research on lipopeptides in microbes was initiated during 1950s and currently more than 100 lipopeptides have been isolated and characterized which differ either in the peptide sequence or level of fatty acid chain (saturated or unsaturated; *n*-, *iso*-, *antesio*- branching) (Vater et al., 2002). Differences in the chemical structure are responsible for lipopeptides with distinct functions. Microbial lipopeptides are expressed in various species as a part of defence mechanism (antifungal, antibacterial, antiviral or anticancer property), for biofilm formation, and motility (Kumar and Johri, 2012). Among the bacteria, *Bacillus* sp. and *Pseudomonas* sp. are the most widely reported producers of lipopeptides (Cochrane and Vederas, 2016). Apart from antimicrobial and anti-biofilm applications, lipopeptides also find application as immunomodulators, enhancer for oil recovery, and template plus stabilizing agent in nanoparticle synthesis (Mulligan et al., 2014; Inès and Dhouha, 2015).

1.7.1. Lipopeptides from *Bacillus* sp.

Bacillus sp. are well-known producers of low molecular weight lipopeptide compounds which exhibit both antifungal as well as antibacterial properties (Bizani et al. 2005; Xie et al., 2009). Antimicrobial compounds may be produced either through ribosomal synthesis eg. bacteriocins, subtilin, subtilosin A, and sublancin or via non-ribosomal synthetic route eg. bacilysin, bacillaene, rhizoctins and lipopeptides (Moshafi et al. 2011). Non-ribosomal peptide synthetase (NRPS) plays a key role in diversified production of antimicrobial compounds by determining the nature of the fatty acid chain, D or L type amino acids in peptide sequence and structure (linear form or cyclized form) (Schallmey et al. 2004; Baltz, 2014).

Bacillus lipopeptides are majorly classified into four families: iturin, surfactin, fengycin and locillomycin. Surfactin and fengycin contain β-hydroxy linked fatty acid whereas iturin is comprised of β-amino linked fatty acid (Raaijmaker et al., 2010; Pathak and

Keharia, 2013). The diverse production of lipopeptides varies with the strains and culture conditions. Apart from antimicrobial action, lipopeptides contribute to some of the phenotypic features of the host (producer organism) as a part of survival and competence in the ecosystem (Xu et al., 2013; Luo et al., 2015). Surfactins and iturins (mycosubtilin and bacillomycin) play a major role in the biofilm formation, swarming motility, and hemolysis. Disruption of iturin, surfactin, and locillomycin gene cluster shows high impact on the colony morphology whereas fengycins and locillomycins has less influence on phenotypic functions (Luo et al., 2015). Synergistic action of surfactin and iturin, iturin and fengycin, surfactant and fengycin has been reported in *Bacillus* sp. against plant pathogens (Maget-Dana et al., 1992; Maget-Dana and Peypoux, 1994; Romero et al., 2007).

1.7.1.1. Surfactin family

Surfactin lipopeptide was first isolated from *B. subtilis* in 1968 (Arima et al., 1968, Kakinuma et al. 1968). Among the *Bacillus* lipopeptides, surfactin family possess excellent biosurfactant properties exhibiting strong antibacterial, antiviral, anticancer, and anticoagulant property with lower fungitoxicity (Jacques, 2011). Indeed, antifungal activity was observed owing to the synergistic action of surfactin and iturin A against plant pathogens (Maget-Dana et al., 1992). Surfactins produced by other *Bacillus* sp. are lichenysin (*B. licheniformis*), pumilacidin (*B. pumilus*), bamylocin (*B. amyloliquefacians*) etc. (Horowitz et al. 1990; Morikawa et al. 1992; Lee et al. 2007). Surfactin type lipopeptides follow detergent mechanism at high concentrations to kill the microbes whereas at moderate concentrations they cause membrane damage by creating ion-conducting pores (Grau et al., 1999; Heerklotz and Seelig, 2007).

1.7.1.1.1. Structure

Surfactin comprises of a cyclic structure with a conserved chiral peptide sequence of seven α amino acids (LLDLLDL; a heptapeptide) linked to a hydroxy fatty acid moiety with carbon chain (C_{12}-C_{16}) by a β-hydroxy linkage (ester bond). The molecular mass ions of surfactin family are in the range of m/z 994–1,065. General structure of surfactin family lipopeptide is shown in Fig. 1.2. Lipopeptides belonging to surfactin family are lichenycin (Jacques, 2011), pumilacidin (Morikawa et al. 1992), bamylocin A (Lee et al. 2007), Esperin

and Surfactin-O-methyl ester (Liu et al., 2009) which vary in structure and functional features (Fig.1.2). Broad antimicrobial spectrum of surfactin and its isoforms are due to its β-sheet structure with horse-saddle conformation in aqueous phase (Bonmatin et al., 1994).

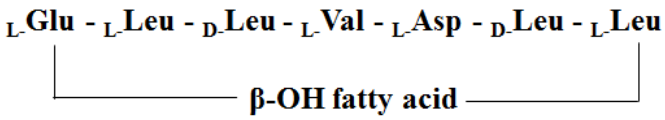


Fig. 1.2: Primary structure of surfactin

1.7.1.1.2. Genetic organization

Surfactin synthesis is controlled by three large open reading frame (ORF) of surfactin synthetase: SrfA-A, SrfA-B and SrfA-C (Galli et al., 1994). Surfactin synthetase contains a linear array of seven modules (one amino acid residue-one module), each SrfA-A and SrfA-BORF has three modules while SrfA-C has the last module. Studies on the specificity of adenylation domain revealed that A-domain of modules 1, 3, 5, and 6 specifically recognize L-Glu, L-Leu, L-Asp, and L-Leu, respectively whereas A-domain of modules 2, 4, and 7 have flexibility to accept any aliphatic amino acid substrate. E-domain of the modules 3, and 6 transform the configuration of L-leu and incorporate D-Leu. β-hydroxy fatty acid chain is attached to the activated amino acid by N-acyl domain present in the N terminal of the starter C-domain (first module) (Kraas et al., 2010). TE-domain cause cyclization by catalyzing the lactone linkage between COO^- group of the last amino acid and OH-group of the fatty acid chain and release the synthesized cyclic surfactin from the enzyme template (Peypoux et al., 1999). Thioesterase/acyltransferase enzyme SrfA-D stimulates surfactin synthetase and initiates the biosynthesis of surfactins (Steller et al., 2004). Non-specificity in the activation process of adenylation domain of several NRPSs results in the development of new surfactin structure with modified properties. For example, in lichenysin, Gln is found at position 1 instead of Glu, and Leu at position 7 instead of Ile, due to a small change in the primary sequence of modules 1 and 7. (Fig. 1.2). Lichenycin show higher bio-surfactant property as compared to surfactin (Jacques, 2011).

1.7.1.2. Iturin family

Iturin lipopeptide exhibits unique and strong antifungal activity against yeasts and filamentous fungus with restricted antibacterial nature (Maget-Dana et al., 1985). Diverse groups of iturin, Iturin A, C, D and E, bacillomycin D, F and L, bacillopeptin, mojavensin A, B, C and mycosubtilin were reported in *Bacillus* sp. (Pathak and kehari, 2013). Iturin interacts with the cytoplasmic membrane of the targets and enhance the membrane permeability by releasing more K^+ ions by forming ion-conducting pores and destroy the organisms (Maget-Dana et al., 1985 and 1989).

1.7.1.2.1. Structure

The noncationic cyclic iturin lipopeptide family comprises of a unique conserved chiral peptide sequence of seven α amino acids (LDDLLDL) linked to fatty acid by β-amino linkage. Cyclization (lactone ring) of iturin lipopeptides occur by amide linkage formed between α-COOH of the 7^{th} amino acid of the peptide moiety and β-NH_2 of fatty acid moiety. Molecular mass of iturin ranges from 1028-1100 Da. Antifungal efficiency of iturin derivatives varies based on the fatty acid chain length (C_{13}-C_{17}) and heterogeneity in peptide sequence which occurs either by a change in amino acid sequence or it positions (Koumoutsi et al., 2004). General structure of iturin is given below (Fig. 1.3). Peptide moiety of iturin type lipopeptides contain D-Tyr at the position 2 which plays a major role in antifungal activity (Maget-Dana et al., 1987).

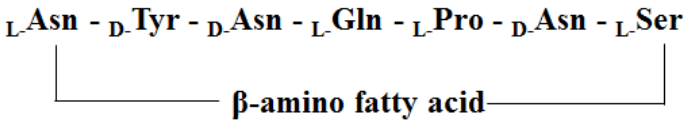


Fig. 1.3: Primary structure of iturin

Depending on the variation in the peptide sequence, iturin is further subdivided in to iturin A, C, D, and E. Iturin A exhibits the strongest antifungal action comparatively (Tsuge et al., 2001). Five homologues of Iturin A vary based on fatty acid chain length (β-amino fatty acids). Mass of the homologues [$M+H^+$] is1029 (A_1), 1043 (A_2), 1057 (A_3, A_4, and A_5), 1071 (A_6 and A_7), and 1085 (A_8), where A_3-A_5 and A_6-A_7 are identified as isomers with the

difference in the branching type of fatty acid chain (Isogai et al., 1982; Gong et al., 2006). New cyclic iturin A derivative was reported in *B. subtilis* SSE4 with unique unsaturated β-amino 13-methyltetradec-8-enoic acid (*iso*-C_{15}) (Thasana et al., 2010). Iturin C differs from Iturin A by the presence of aspartyl residue (Asp) in the amino acid position 1 rather than asparaginyl residue (Asn) which in turn blocks the antifungal action of iturin C (Table 1.2). Iturin D differs from Iturin A by the substitution Asp or Glu in place of Asn or Gln (Table1.2). Iturin E is the modified form of Iturin D sequence where carboxyl group is substituted with carboxy methyl ester (Table 1.2). Mycosubtilin differs from iturin A by shift of Ser and Asn in the position 6 and 7 (Table 1.2). However, a small variation in the position of amino acids exhibited an increase in the antifungal efficiency of mycosubtilin as compared to iturin A (Hansen et al., 2007).

Mojavensin, a relatively new antifungal lipopeptide, has Asn in place of Ser at position 7 of the iturin A peptide (Table 1.2). Mojavensin A isolated from marine derived *B. mojavensis* showed antifungal activity against phytopathogens but the antifungal effect was lower as compared to iturin A (Ma and Hu, 2014) but exhibited cytotoxicity against human leukemia cell lines. Bacillomycin belongs to the iturin family due to the presence of β-amino acid. Bacillomycinis further subdivided into bacillomycin D, bacillomycin F, and bacillomycin L (Table 1.2), of which bacillomycin D exhibits significant antifungal potential as compared to the other classes of bacillomycins. Bacillomycin D has shown a wide-spectrum antifungal activity against several fungi, such as *A. flavus*, *A. niger*, *C. albicans* and *F. oxysporum* (Moyne et al., 2004). This shows that variation in the amino acid sequence and composition determined the potency of antifungal lipopeptides. Apart from peptide sequence, fatty acid chain length and branching type also help in determining the antifungal action of lipopeptides. The thumb rule is that- longer the fatty acid chain length higher the antifungal activity (Maget-Dana et al., 1989). Based on the branching type, antifungal efficacy of the lipopeptides differ as follows: *n*- >*iso*- >*anteiso*- type (Bland, 1996).

In spite of strong antifungal potency, iturin type lipopeptides were not suitable for clinical trials due to their high hemolytic activity and cytotoxicity. Iturin A, bacillomycin D and mycosubtilin displayed 25, 28, and 100% hemolysis at their MIC value (~10 µg/mL) (Cochrane and Vederas, 2016).

1.7.1.2.2. Genetic organization

Both NRPS-PKS enzyme complexes are involved in biosynthesis of iturin as opposed to biosynthesis of other lipopeptides which uses only one of the enzyme complexes. NRPS gene clusters of iturin family lipopeptides contain four large ORFs: iturin A (itu D, itu A, itu B and itu C), mycosubtilin (fen F, myc A, myc B and myc C), and bacillomycin (bmy D, bmy A, bmy B and bmy C). The first ORF itu D, fen F, and bmy D codes for a putative malonyl-CoA transacylase gene (related to PKS) which plays a role in the last steps of fatty acid synthesis and β-amination before it is transferred to module 1 (Fernando et al., 2006). The ituA, myc A, and bmy A contains modules for β-ketoacylsynthetase, amino transferase, and A-domain coding for the first residue Asn. Itu B and myc B encodes four modules containing A-domain specific for Tyr, Asn, Gln, and Pro substrates, and bmy B for Tyr, Asn, Pro, and Gln, of which modules 2 (Tyr) and 3 (Asn) are flanked with the epimerization domain (for D-configuration). The last two modules of all the iturin family lipopeptides (itu C, myc C, and bmy C) display specificity and high degree of variability in activating the amino acid substrates. Modules 6 and 7 of Itu C gene codes for (Asn, Ser), Myc C for (Ser, Asn), and bmy C for (Ser, Thr). The E-domain and TE domain are common for all the three last gene clusters. E-domain flanking the module 6, incorporates D-configuration to the respective amino acids. TE-domain catalyzes the amide bond formation between COO^- of the last amino acid and β-amino group of fatty acid, and releases the cyclic peptide from the template. (Tsuge et al., 2001; Duitman et al., 1999; Hofemeister et al., 2004; Koumoutsi et al., 2004).

1.7.1.3. Fengycin family

Fengycin class of lipopeptides are the cyclic decapeptides possessing a lactone linkage between the phenol side chain of tyrosine and C-terminus amino acid, and an amide linkage with β-hydroxy fatty acid (Vanittanakom et al., 1986). Fengycin was reported to exhibit strong antifungal activity against filamentous fungi such as *Botrytis cinerea*, *Colletotrichum gleosporioides*, and *Podosphaera fusca* (Sun et al., 2006; Romero et al.,

Table 1.2: Iturin lipopeptides of *Bacillus* species

		Source	Sequence	M+H$^+$	M+Na$^+$	β-amino acid-isoforms
Iturin A	A_1	*B. subtilis*	$N_1Y_2N_3Q_4P_5N_6S_7$	1,029	1,051	*iso*-C_{13}
	A_2			1,043	1,065	*n*-C_{14}
	A_3, A_4, A_5			1,057	1,079	*anteiso*-C_{15}, *iso*-C_{15}, *n*-C_{15}
	A_6, A_7			1,071	1,093	*iso*-C_{16}, *n*-C_{16}
	A_8			1,085	1,107	*anteiso*-C_{17}
	Subtulene	*B. subtilis*	$N_1Y_2N_3Q_4P_5N_6S_7$	1,057	1,079	unsaturated *iso*-C_{15}
Iturin C		*B. subtilis*	$D_1Y_2N_3Q_4P_5N_6S_7$	1,044	1,066	*n*-C_{14}
				1,058	1,080	*anteiso*-C_{15}, *iso*-C_{15}
Iturin D		*B. subtilis*	$D_1Y_2D_3E_4P_5D_6S_7$	1,044	1,066	*n*-C_{14}
				1,058	1,080	*anteiso*-C_{15}, *iso*-C_{15}
				1,072	1,094	*iso*-C_{16}, *n*-C_{16}
Iturin E		*B. subtilis*	$D_1Y_2D_3E_4P_5N_6S_7$	1,058	1,080	*n*-C_{14}
				1,072	1,094	*anteiso*-C_{15}, *iso*-C_{15}
				1,086	1,108	*iso*-C_{16}, *n*-C_{16}
Mycosubtilin		*B. subtilis*	$N_1Y_2N_3Q_4P_5S_6N_7$	1,071	1,093	*iso*-C_{16}
				1,085	1,107	*anteiso*-C_{17}
Mojavensin	Mojavensin A	*B. mojavensis*	$N_1Y_2N_3Q_4P_5N_6N_7$	1,069	1,091	*iso*-C_{14}
	Mojavensin B			1,097	1,119	*iso*-C_{16}
	Mojavensin C			1,111	1,133	*anteiso*-C_{17}
Bacillomycin	Bacillomycin D	*B. subtilis*	$N_1Y_2N_3P_4Q_5S_6T_7$	1,031	1,053	*n*-C_{14}
		B. amyloliquefaciens		1,045	1,067	*anteiso*-C_{15}, *iso*-C_{15}
				1,059	1,081	*iso*-C_{16}, *n*-C_{16}
	Bacillomycin F	*B. subtilis*	$N_1Y_2N_3P_4Q_5N_6T_7$	1,085	1,107	*iso*-C_{16}
		B. amyloliquefaciens		1,099	1,121	*anteiso*-C_{17}, *iso*-C_{17}
	Bacillomycin L	*B. subtilis*	$D_1Y_2N_3S_4Q_5S_6T_7$	1,021	1,043	*n*-C_{14}
		B. amyloliquefaciens		1,035	1,057	*anteiso*-C_{15}, *iso*-C_{15}
				1,049	1,071	*iso*-C_{16}, *n*-C_{16}
	BacillomycinLc	*B. subtilis*	$N_1Y_2N_3S_4Q_5S_6T_7$	1,020	1,042	*n*-C_{14}
		B. amyloliquefaciens		1,034	1,056	*anteiso*-C_{15}, *iso*-C_{15}
				1,048	1,070	*iso*-C_{16}, *n*-C_{16}

2007; Pyoung et al., 2010) but are not effective against yeast and bacteria. Quite interestingly fengycin exhibited lower hemolytic activity - at least 40 and 70 times lesser than surfactin and iturin, respectively, which induce 100% hemolysis (Vanittanakom et al., 1986; Malfanova et al., 2012). The antifungal mechanism of fengycin is poorly understood. Like other lipopeptides, fengycin has been shown to disrupt the cell membrane in a dose dependent manner (Deleu et al., 2008).

1.7.1.3.1. Structure

Structure of fengycin shows a cyclic peptide ring comprising of eight amino acids linked via lactone linkage between Tyr^3 and Ile^{10}, while the lipid moiety is attached to the peptide sequence by amide bond formed between β-hydroxy fatty acid and Glu^1. The molecular mass of fengycin type lipopeptides falls within the range of 1400-1521 Da. Primary structure of fengycin type lipopeptide is illustrated in Fig. 1.4.

1.7.1.3.2. Genetic organization

Fengycin synthetase gene comprises of five ORFs assembled in the linear order of Fen C, Fen D, Fen E, Fen A, and Fen B. Like surfactin gene clusters, fengycin also contains N-acyl domains, E domains and type I TE domain. First three ORFs code for three proteins containing two modules with adenylation domains specific for two amino acid substrates. Fen C codes for glutamate (Glu) and ornithine (Orn) (amino acid position 1 and 2) which forms a side chain, while Fen D encodes tyrosine (Tyr) and allo-threonine (*allo*-Thr) (amino acid position 3 and 4). One module of Fen D codes for Glu while the other module contains motif for activation of both alanine (Ala) and valine (Val) substrate leading to the different sequence (fengycin A and B, respectively; amino acid position 5 and 6). TE-domain of the Fen B gene catalyzes the lactone linkage between COO^- group of Ile and OH^- group of Tyr and terminates the peptide from the template. Similar to surfactin biosysnthesis, β-hydroxy fatty acid is transferred to the first amino acid module (Glu) by acyl transferase (Steller et al, 1999).

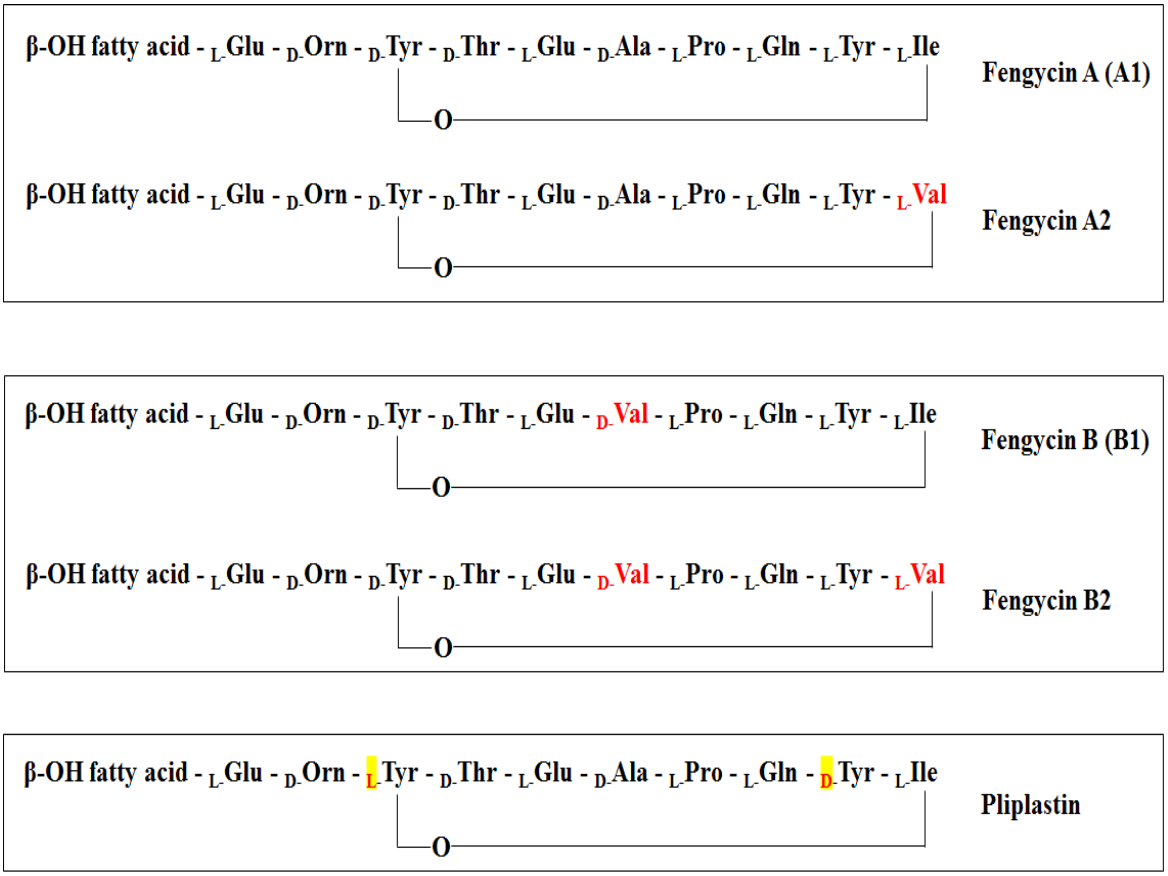


Fig. 1.4: Primary structure of pliplastin and fengycin A1, A2, B1, and B2

Fengycin family lipopeptides are mainly classified as fengycin or pliplastin based on the tyrosine chirality at the position 3 and 9 of the peptide sequence. Conserved chiral sequence of fengycin and pliplastin are LDDDLDLLLL and LDLDLDLLDL, respectively. Various other lipopeptides, derivatives and isoforms of fengycin/pliplastin have been reported which vary in fatty acid chain length (C_{14}-C_{19}), branching type (*n*-, *iso*- and *anteiso*-), double bonds in lipid tailor amino acid sequence of the peptide. Fengycin and piplastin with variability in the amino acid sequence of Ala, Val, Ile or Leu at position 6 and Val and Ile in position 10 has been reported (Bie et al., 2009; de Faria et al., 201; Pathak et al., 2012). Fengycin/pliplastinare classified in to four classes: (i) fengycin A/pliplastin A; and (ii) fengycin B/pliplastin B, based on the difference in the amino acid at 6thposition; (iii) fengycin A2; and (iv) fengycin B2, based on the variation at the 10th amino acid position of the peptide ring. Ala present at the 6th amino acid position in fengycin A/ pliplastin A is replaced with Val in fengycin B/ pliplastin B, while the isoleucine at the 10th position in fengycin A/pliplastin A and fengycin B/pliplastin B is replaced by Val, forming fengycin A2/pliplastin A2 and fengycin B2/pliplastin B2 (Pathak et al., 2012).

1.7.1.4. Locillomycin family

Locillomycin is a new family of cyclic lipopeptides produced by *B. subtilis* 916 with unique structure which follow novel biosynthetic non-linear biosynthetic mechanism (Luo et al., 2015). Locillomycin exhibit strong antifungal, antibacterial, and antiviral properties.

1.7.1.4.1. Structure

Locillomycin is categorized as a new family of lipopeptide as it possesses a unique nonapeptide sequence with chirality (LDLLLLLLL) and exhibits macrocyclization. Locillomycin comprises of nine amino acids with peptide ring (lactone linkage) formed between side chain of Thr and N- termini of Val. Molecular mass values [$M+H^+$] of 1,145.6, 1159.6, and 1,173.6 corresponds to C_{13}, C_{14}, and C_{15} isoforms of locillomycin, respectively (Luo et al., 2014). The general structure of locillomycin is shown in Fig. 1.5.

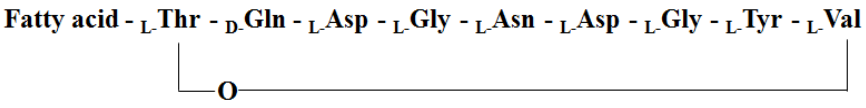


Fig. 1.5: Primary structure of Locillomycin

1.7.1.4.2. Genetic organization

Locillomycin gene cluster contains four ORFs in the following order: Loc D, Loc A, Loc B, and Loc C with hexamodular structure. loc D gene encodes modules of PKs for fatty acid synthesis, acyl-coenzyme synthetase (ACS) domain, PCP-domain, and β-ketoacylsynthetase domain for initiating the biosynthesis of locillomycin. Loc A contains one module specific for Thr, Loc B gene consists of three modules specific for Gln and Asn, Asp (2) and Gly (2) which do not follow co-linearity rule. Domain (A) specific for Gln residue in the module 2 is flanked with E-domain for incorporating D-configuration. TE domain in Loc C is involved in the cyclization process between Val and Thr and terminating the synthesis process thereby releasing the peptide (Luo et al., 2015).

1.7.1.5. Other lipopeptides

The other noncationic cyclic lipopeptides reported are kurstakins from *B. thurinjenisis* subsp. *kurstaki* HD-1, and marihysin A from*B. marinus* B-9987. Kurstakins is a heptapeptide with the amino acid sequence, Thr-Gly-Als-Ser-His-Gln-Gln partially cyclized with the ester linkage between side chain (OH group) of Ser residue and C-terminal Gln residue. Fatty acid

is attached to the Thr residue present at the 1st position *via* an amide linkage. Four lipopeptides with same amino acid and different fatty acid length and branching have been reported (Hathout et al., 2000). Marihysin is another cyclic heptapeptide with the sequence, Pro-Gln-Asn-Ser-Asn-Tyr-Asn-β-amino tetradecanoic acid cyclized by a lactone linkage between fatty acid β-amino group and Pro (Liu et al., 2010). Although kurstakins and marihysin have been tested against limited fungi, they have exhibited good antifungal activity, however they were found to be less effective than iturin and fengycin (Cochrane and Vederas, 2016).

Gageostatins produced by marine *B. subtilis* is a novel linear heptalipopeptide with the sequence Leu-Leu-Asp-Val-Leu-Leu-Glu-β-hydroxy fatty acid. Three gageostatins A, B, and C have been identified with a similar amino acid sequence but with different fatty acid (β-hydroxy-11-methyltridecanoic acid, β-hydroxy-9,11-dimethyltridecanoic acid, and conjugated double bond *(E)*-7,9-dimethylundec-2-enoic acid). Gageostatins showed significant antifungal activity against *R. solani*, *B. cinerea*,and *C. acutatum* with the MIC ranging from 4-32 µg/mL. Combination of gageostatins A and B exhibited synergistic effect in improving the antifungal action, while reducing the cytotoxicity (Tareq et al., 2014).

1.7.2. Antifungal lipopeptides produced by other microbes

1.7.2.1. *Paenibacillus* sp.

Fusaricidin produced by *Paenibacillus polymyxa* is a unique antifungal heptapeptide with the sequence cyclo (guanidylated β-hydroxy fatty acid-Thr-Val-Val-Thr-Asn-Ala). Four fusaricidins A, B, C, and D have been characterized and all four had shown strong activity against plant pathogenic fungi such as *F. oxysporum*, *A. niger*, and *A. oryzae.* Particularly fusaricidin B, which exhibited good antifungal activity against *C. albicans* and *Saccharomyces cerevisiae* (Kajimura and Kaneda,1996; Kajimura and Kaneda,1997; Raza et al., 2009; Lee et al., 2013).

1.7.2.2. *Pseudomonas* sp.

A total of three *Pseudomonas* related antifungal lipopeptides are reported till date. Pseudomycin A-C produced by *P. syringae* showed selective phytotoxicity and significant activity against *C. albicans* (Harrison et al., 1991). Three cyclic lipodepsinoapeptide, syringomycin E, syringotoxin B and syringostatin A produced by *P. syringae* py. s*yringae* are water soluble and displayed unique mode of action. Syringomycin E and syringotoxin A are

more effective as compared to syringotoxin B (Sorensen et al., 1996). A cyclic antifungal lipodepsipeptide, viscosinamide produced by *P. fluorescens* DR54 is active against *Pythium ultimum* and *R. solani* (Nielsen et al., 1999).

1.7.2.3. *Streptomyces* sp.

Nikkomycin X and Z are peptidyl nucleosides produced by *Streptomyces tendae* which target the chitin biosynthesis in fungi. They exhibited very strong antifungal activity against *Coccidiodes immitis* and *Blastomyces dermatitidis* without any cytotoxicity against human cells (Hector et al., 1990). A peptide antibiotic, valinomycin, produced by *Streptomyces* sp. strain M10 shows activity against phytopathogen, *B. cinerea,* and not against any animal fungal pathogens (Park et al., 2008).

1.8. Glycosylated antifungal peptides

Cepacidines obtained from *Burkholderi acepacia* exhibit antifungal activity against *Candida* sp, *A. niger*, *C. neoformans,* and *F. oxysporum* (Lee et al., 1999; Lim et al., 1994). Occidiofungin produced by *B. contaminans* is a unique cyclic glycopeptide containing eight amino acids, Asn-NAA-Trp-BHT-Lys-Gly-Asn-Ser where BHT is β-hydroxy tyrosine and NAA is novel amino acid which acts as a scaffold for xylose and short acyl chain attachment to the peptide sequence. Two occidiofungins have been reported, Occidiofungin A (1199.5 Da) and Occidiofungin B (1215.5 Da) showing strong antifungal activity against both plant and animal fungal pathogens like *R. solani*, invasive pulmonary aspergillosis causing *A. fumigatus* and *A. niger,* and dermatophytosis causing *Microsporum gypseum* and *Trichophyton mentagrophytes* (Lu et al., 2009).

Hassallidin is a novel glycosylated cyclic lipopeptide produced by epilithic cyanobacterium *Hassallia* sp. Based on the variation in fatty acid chain length, sugars, amino acid composition and their degree of acetylation, hassallidin is classified in to four, viz., A, B, C, and D, of which hassallidin D is very effective with a IC_{50} ranging from 0.29-1.0 µM against *C. albicans* (Vestola et al., 2014).

1.9. Antifungal drug development

In the process of antifungal drug development, few compounds have been screened and identified as novel antifungals against various/several biological targets. The potent antifungal drugs and their molecular targets are shown in Fig. 1.6.

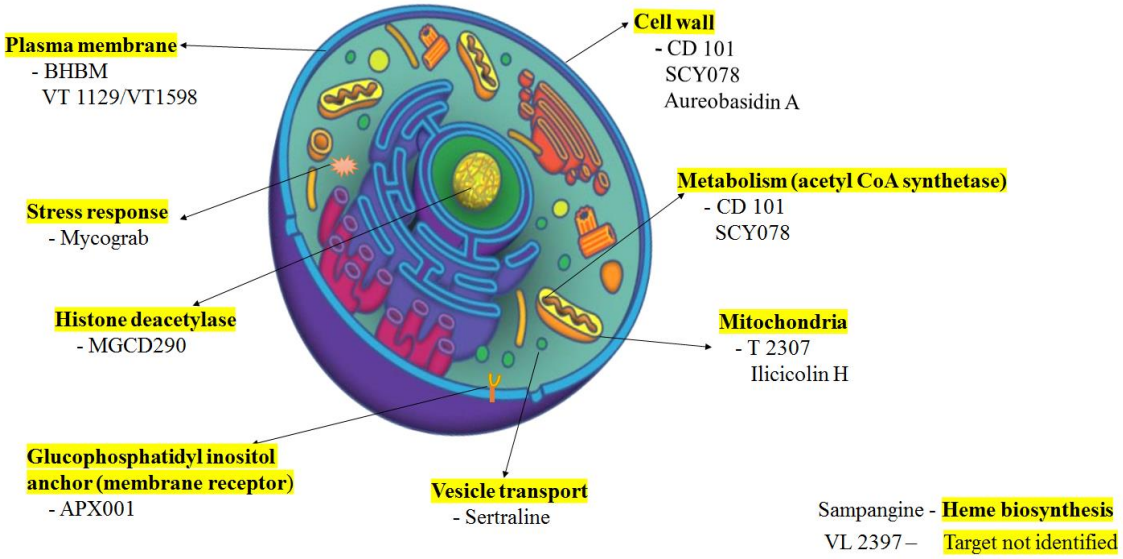


Fig. 1.6: A fungal cell showing the molecular targets (highlighted) of the newly identified drugs which is undergoing different phases of clinical trials.(https://cdn.thinglink.me/api/image/713393433326125057/230/230/thumbnail, Scorzoni et al., 2017; Chang et al., 2017; Perfect et al., 2017).

A total of 15 drugs are currently being investigated and are at various stages of clinical trials with the aim of developing novel drug against *Candida* and *Cryptococcus* species (Table 1.3). Apart from VT 1161/VT1129 (azole derivative - tetrazole), SCY-078 (echinocandin type) and T-2307 (allylamine), all the other drugs exhibit a different mechanism of action which include targeting mitochondria and its metabolism, heme biosynthesis, histone deacetylase, membrane receptor, vesicle transport and stress response (Fig. 1.6 and Table 1.3). All the drugs listed in Table 1.3 are either in phase 1 and 2 or in preclinical levels of the clinical trials (Roemer and Krysan, 2014; Pianalto and Alspaugh, 2016; Scorzoni et al., 2017; Chang et al., 2017; Perfect et al., 2017).

Table 1.3: Different phases of clinical trials followed for the new antifungal drugs in specific to *Candida* sp. and *Cryptococcus* sp. infections

S.No	Novel compounds	Organisms	Phase trials	Company
1.	CD101/Biafungin	Candidemia causing isolates	Phase 2	Cidara Therapeutics
2.	SCY-078	Invasive candidiasis	Phase 2	Scynexis
3.	T-2307	*Candida* spp. *Cryptococcus* sp.	Phase 1	Toyama Chemicals, Tokyo, Japan
4.	Ilicicolin H	*Candida* spp.	Preclinical	-
5.	BHBM	*C. neoformans, C. gattii C. glabrata*	Preclinical	-
6.	VT-1129	*C. neoformans, C. gattii*	Phase 1	Viamet Pharmaceuticals
7.	VT-1161	Fluconazole-resistant *Candida* isolates	Phase 2b	
8.	AR-12	*C. neoformans C. albicans* biofilms	Completed phase I for oncology indications	Arno Therapeutics, Flemington, USA
9.	Sampangine	*C. neoformans, C. albicans C. glabrata, C. krusei*	Preclinical	-
10.	Sertraline	*Cryptococcus* sp.	Phase 3	
11.	VL-2397	*C. glabrata C. kefyr C. neoformans*	Phase 1	Vical, San Diego, CA, USA
12.	APX001	*Candida* species	Phase I; phase II planned	Amplyx Pharmaceuticals
13.	Aureobasidin A	Broad spectrum	Preclinical	AureoGen Biosciences
14.	Efungumab (Mycograb)	*Candida* species	Phase 2	NeuTec Pharma/Novartis
15.	MGCD290	Broad spectrum	Phase 2	Mirati Therapeutics

1.10. Gaps in the existing antifungal research

1) For more than 40 decades, the medical field is confronting the rise in antifungal resistance, worldwide which apparently increased the morbidity and mortality by more than thousands every year. This alarming situation compelled clinicians to

address this medical emergency either by modifying the existing antifungals (derivatives) or using them in combinations. Despite the significant antifungal action, microbes gain resistance even to the derivatives or the combinational therapy in due course of time (Coates et al. 2002). In addition to drug-resistance, drug combinations also led to adverse side effects driving the researches to hunt for new antifungal compounds as an alternate with selective cytotoxicity (Chopra et al., 2002).

2) Over the last few decades, researchers have launched a massive hunt for novel antibacterial and antifungal compounds to combat the issue of drug-resistance. Unfortunately, antifungal pipeline has shown a lag in the antifungal drug development in comparison to antibiotics. A new antifungal drug Isavuconazole was recently (2015) approved by FDA for treating fungal infections. However, Isavuconazole is an azole derivative (modified azole) and not a new antifungal drug with a different mechanism of action. The discovery of novel class of antifungals has been halted since the 2000s with the discovery and approval of echinocandin type antifungals (Roemer and Krysan, 2014).
3) The usage of existing antifungal compounds is limited due to their cytotoxic property and increased prevalence of resistance due to the unconditional usage of antifungals.
4) To date, only one microbially synthesized antifungal lipopeptide, echinocandin has been approved for therapeutic use; however, echinocandin has proven to be less effective against *Cryptococcus* sp. and *C. glabrata*. Irrespective of its excellent fungicidal potency, the clinical use of the lipopeptides of *Bacillus* sp. is hindered mainly due to its high haemolytic property.
5) Antifungal peptides get degraded or inactivated due to action of the proteolytic enzymes present in the blood stream.
6) Scale-up of microbial products showing high-end antimicrobial applications have been a challenge preventing the translation of production from lab-to-industrial scale. This is due to the lack of knowledge regarding specific media, production conditions, and intensification process which selectively induce an enhanced production of the target antimicrobial compound.

Acknowledging these limitations, the objectives were framed to screen natural bioactive compounds from the potent bacterial isolates (recovered from various soil sources) that could produce potential antifungal compound(s) and render a mighty contribution towards the antifungal research for combating antifungal resistance.

1.11. Objectives of the Research Work:

The following objectives were undertaken:

1) To screen and identify the promising wild-type *Bacillus* isolate producing broad-spectrum antimicrobial activity against different multidrug-resistant fungal pathogens.

2) To purify and characterize biochemically the potent antifungal compound(s).

3) To examine the antifungal potency of the purified antifungal compound(s).

4) To optimize the production conditions of the antifungal compound(s).

Chapter 2

Screening and Identification of potent antifungal producing isolates

2.1. INTRODUCTION

In 2016, World Health Organization (WHO) and Centres for Disease Control and Prevention (CDC) alerted the hospitals regarding the emergence of the deadly and drug resistant yeast infection which will lead to a serious global health threat. An ever-escalating antibiotic resistance accentuates the compelling need to recover novel molecules that are insensitive to the resistance mechanisms in order to combat the MDR pathogens. Most of the antimicrobial productions by organisms are due to the defensive action for maintaining their ecological niche in an established microbial community structure (Boman, 1995). Exploration of different ecological niches and ferreting out of the novel antifungals from the diversified microbial group will be an alternate and considerable approach to tackle this global antifungal resistance issue. A global report published by the World Health Organization (WHO) (WHO, 2014) revealed that only three classes of antifungal drugs, azoles, polyenes and echinocandins are currently in use to treat invasive candidiasis. The alarming rise of antifungal drug-resistance might be potentially addressed by alternative antifungal agents.

In this chapter, antagonistic wild type bacterial isolate showing significant antifungal activitywas screened. Potent antifungal producer was selected based on the antifungal potency against a total of eleven *Candida* sp. and *Cryptococcus* sp. The producer organism was identified by biochemical characterization and 16S rDNA gene sequencing.

2.2. EXPERIMENTAL METHODS

2.2.1. Microbial Cultures, Media and Growth Conditions:

Antifungal compound producing isolates were grown in tryptic soy broth (TSB) media and the indicator yeasts including *Candida* and *Cryptococcus* species used for screening were grown in MGYP media (0.3% malt extract, 1% dextrose, 0.3% yeast extract and 0.5% peptone, pH 6.5-6.8) at 37°C. All the cultures were maintained as glycerol stocks (20%) at −80°C. Indicator organisms used in this study was procured from Microbial Type Culture Collection (MTCC), Chandigarh and National Collection of Industrial Microorganisms (NCIM), Pune.

2.2.2. Isolation and screening of antifungal compound producing organism from soil

Preliminary screening for antifungal producers were carried out using *C. albicans* SC5314 as an indicator organism in crowded plate technique. MGYP plates with 1% *C. albicans* SC5314 was prepared by pour plate method. Soil samples (1g) collected from the garden soil of Goa and East Dehradun were dissolved in sterile saline solution (0.9%) and about 100 µL was spread on the dried MGYP plates (pre-inoculated with *C. albicans*) and incubated at 37˚C for 72 h.

2.2.3. Selection of potent antifungal producer

Colonies showing zone of clearance in MGYP plate were picked up, purified and further tested for antifungal potency. The purified colonies were grown and maintained in TSB media. Secondary screening was performed by spot agar assay using eleven yeasts which include *C. albicans* MTCC 3958, *C. albicans* SC5314, *C. albicans* MTCC 227, *C. albicans* MTCC 854, *C. albicans* DI (clinical isolate), *C. albicans* WT (clinical isolate), *C. glabrata* NCIM 3019, *C. glabrata* MTCC 3814. *C. krusei* NCIM 3129, *C. neoformans* NCIM 3541 and *C. neoformans* NCIM 3378 (Table2.1). Spot agar assay was carried out by inoculating 5 µL of freshly grown test strains (screened isolates) on the MGYP plates which was inoculatedwith 1% of the indicator organisms (yeasts) and allowed to grow for 72 h at 37˚C. The isolate was selected based on the zone of inhibition and reproducibility of antifungal activity against the tested yeasts.

2.2.4. Identification of the antifungal producer organism

2.2.4.1. Biochemical studies

The selected antifungal producer RLID 12.1 were identified by various biochemical tests like Methyl Red and Voges-Proskauer tests, production of indole and catalase, growth at different temperaturesand different concentrations of NaCl according to Bergey's Manual of Determinative Bacteriology (Bergey, 1994) (Table 2.2). Carbohydrate fermentation tests were done using KB009 Kit from Hi Media Pvt. Ltd., India.

2.2.4.2. 16S rRNA gene analysis

Total genomic DNA of the selected soil isolates were extracted from 24 h grown RLID 12.1 culture. PCR with 50 µL reaction volume was performed using about 100 ng

of genomic DNA, 10X reaction buffer, 10 mM (each) deoxynucleoside triphosphates, 1.5 mM $MgCl_2$ and 1.0 U of Taq polymerase (Bangalore GeneI, India). The universal eubacterial primers 27F (5´-AGA GTT TGA TCC TGG CTC AG-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACT T-3´) (Lane 1991) were used at the rate of 10 picomoles to amplify small-subunit rRNA (16S rRNA) gene sequences of the wild-type soil isolates. PCR was carried out under the following cycling conditions: initial denaturation at 95 ºC for 5 min, followed by 30 cycles of 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min, with a final extension at 72 ºC for 1 min. The expected PCR product of around 1.5 Kb was checked by gel electrophoresis using 5 µL of the PCR product on 1% agarose gel in 1X TBE buffer. The sequencing of the target gene (amplicon) was done using BigDye Chemistry, and performed as per the manufacturer's protocols (Applied Biosystems 3730xl DNA Analyzer) in Royal Life Sciences Pvt. Ltd., Secunderabad, India.

2.2.4.3. Computational analysis and phylogenetic tree construction

Sequence data analysis was done using Chromas Pro and sequencing analysis software like BLAST to compare the sequences in the GenBank nucleotide sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). The 16S rRNA sequence of RLID 12.1 strain were aligned using the CLUSTALX 2.1 program (Larkin et al., 2007) against the corresponding DNA sequences of representatives of the genus *Bacillus* retrieved from GenBank. Phylogenetic tree was inferred using PHYLIP (PHYLogeny Inference Package) and the neighbour-joining method with a bootstrap value of 1000 replicates (Felsenstein, 1989) for inferring evolutionary trees (phylogenies). All 16S rRNA gene sequences of the isolate described in this study have been deposited in the GenBank nucleotide sequence database.

2.2.4.4. FAME analysis

The analysis of the cellular fatty acid methyl esters (FAMEs) was performed at Royal Life Sciences Pvt. Ltd., Secunderabad, India using MIDI Sherlock software. The FAME extracts were prepared by a procedure that consisted of cell harvesting, saponification, methylation, and extraction from the aqueous phase and finally analysis was done by gas chromatography (Pendergrass, 1998). FAME profiles achieved were analysed by comparing with Sherlock MIS RTSBA6 identification library. Further cluster analysis was carried using dendrogram tool with the FAME profile data for confirmation of species.

2.2.5. Time course of growth and production of antifungal compound

Freshly grown antifungal producing RLID 12.1 was inoculated in 100 mL of modified Tryptic soya broth (mTSB stands for TSB with 0.5% yeast extract) in 250 mL Erlenmeyer flask and incubated at 37±0.5 ºC for 72 h at 105 rpm. At 12 h interval, 2 mL of sample was withdrawn, centrifuged at 10000 rpm for 15 min at 4ºC and the supernatant assayed for antifungal activity against *C. albicans* SC5314 in Sabouraud Dextrose agar. Simultaneously, pH and the growth of *B. subtilis* RLID 12.1 at OD_{600} were noted at the respective time intervals.

2.2.6. Antifungal activity of RLID 12.1 by cut well agar assay

After 60 h of incubation of RLID 12.1, the cell free supernatant was collected by centrifugation and was membrane filtered through 0.45 µm disposable polysulphonate membrane filter (Axiva). Cell free supernatant was concentrated about 5 times by ultrafiltration usingMillipore membrane (Molecular weight cut-off 3 kDa) in a stirred cell device (Millipore) and antifungal activitywas evaluated by testing against 16 organisms includingyeasts such as *C. albicans*, non-*albicans* and *Cryptococcus* sp. and filamentous fungi (clinical isolates) such as *Bipolaris* sp., *Curvularia* sp., *Mucor* sp., *A. niger*, *A. flavus* and *F. oxysporum* by cut well agar method (Table 2.4). Freshly grown fungal cultures (100 µL) were swabbed over dried Sabouraud dextrose agar plates and 100 µLof concentrated supernatant was added in the well (8 mm diameter).

2.2.7. Preliminary characterization of the antifungal compound

2.2.7.1. pH and thermal stability of the antifungal compound

To determine the thermal stability, aliquots of concentrated supernatants were incubated at different temperatures: 37˚C for 9 h, 50˚C for 5 h, 60˚C for 2 h and 70 ˚C for 1 h. pH stability was analysed using 0.1M buffers of different pH: Citrate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0 and 7.0), Tris buffer (pH 8.0 and 9.0) and glycine NaOH buffer (pH 10.0) and were incubated at 37˚C for 2 h. After incubation, antifungal activity was tested against *C. albicans* SC5314.

2.2.7.2. Effect of surfactants and other chemicals on the antifungal compound

Effect of surfactants like sodium dodecyl sulfate (SDS), Tween 20, Tween 80 and Triton X-100 at a final concentration of 1% (v/v), β-mercaptoethanol (β-ME) at 10%

concentration and EDTA at 2, 5 and 50 mM concentration were analysed by incubating at 37 ˚C for 5 h (Kayalvizhi and Gunasekaran, 2010). Chemicals at the rate of 1% and 10% in mTSB broth and untreated concentrated supernatant were used as negative and positive controls respectively. Effect of trichloroacetic acid (TCA, 10 mg/mL) on antifungal compound was tested at 37 ˚C for 5 h. After treatment with TCA, the samples were centrifuged at 10000 rpm for 5 min and the supernatant was adjusted to pH 8.0 and checked for antifungal activity (Kayalvizhi and Gunasekaran, 2010).

2.2.7.3. Effect of metal salts on the antifungal compound

Effect of metal salts ($AgNO_3$, $MgSO_4$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $CdCl_2$, $FeSO_4$ and $CaCl_2$) at a final concentration of 1mg/mL on the concentrated CFS was analysed by incubating at 37°C for 1 h. The untreated concentrated supernatant and solutions of metals salts were used as positive and negative controls respectively (Larsen et al., 1993).

2.2.7.4. Effect of enzymes on the antifungal compound

To check the sensitivity of enzyme, the cell free supernatant concentrated by ultrafiltration was treated with filter-sterilized trypsin (0.5 M Tris HCl buffer, pH 8.0), lipase (100 mM phosphate buffer, pH 7.4) and α-amylase (20 mM phosphate buffer, pH 7.0) at a final concentration of 1 mg/mL at 37 ºC and proteinase K (50 mM Tris HCl buffer, pH 7.5) at a final concentration of 1 mg/mL at 55 ºC for 2 h. The concentrates without enzyme treatment and buffers were used as positive and negative controls respectively. The residual activity was determined by agar well diffusion method against *C. albicans* SC5314 (Shekh et al., 2011).

2.3. RESULTS

2.3.1. Isolation and primary screening of antibiotic producing genus *Bacillus* from soil

63 single colonies of genus *Bacillus* were isolated as pure cultures based on the standard phenotypic (morphological) characterization. During the primary screening process, two organisms, INK and RLID 12.1 exhibited good antifungal activity (Fig. 2.1). Selection of the potent antifungal producer among the screened organism was carried out by spot agar against 11 selected yeasts (indicator organism) listed in Table 2.1. Among the two organisms, the soil isolate RLID 12.1 from East Dehradun showed clear zones of

inhibition against all the 11 selected yeasts. INK showed significant activity against *C. albicans* however no antifungal activity was observed against *C. glabrata* and *C. neoformans* tested (Table 2.1).

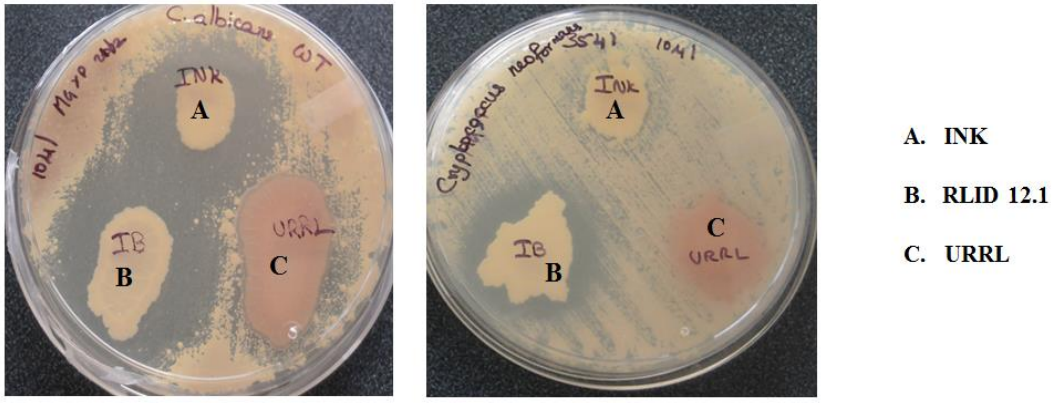


Fig. 2.1: Antifungal potential of the two screened organisms **A. INK**, **B. RLID 12.1** and the non-producer organism URRL against *C. albicans* WT (clinical isolate) and *C. neoformans* MTCC 3541

Table 2.1: Antimicrobial activity of the selected RLID 12.1 and INK strains by Spot agar method

S.No	Indicator organism	RLID 12.1	INK
1.	*Candida albicans* DI (clinical isolate)	+++	++
2.	*C. albicans* WT (clinical isolate)	+++	+++
3.	*C. albicans* MTCC 3958	+++	++
4.	*C. albicans* SC5314	+++	++
5.	*C. albicans* MTCC 227	+	+
6.	*C. albicans* MTCC 854	+++	+
7.	*C. glabrata* NCIM 3019	+	-
8.	*C. glabrata* MTCC 3814	++	-
9.	*C. krusei* NCIM 3129	++	+
10.	*C. neoformans* NCIM 3541	++	-
11.	*C. neoformans* NCIM 3378	+	-

+++ indicates very strong activity, ++ indicates strong activity, + indicates presence of activity and – indicates no activity

2.3.2. Identification of RLID 12.1

RLID 12.1 was selected for further identification as it showed strong and reproducible antifungal activity. From the microscopic examination, it was found that RLID 12.1 is a rod shaped, endospore containing gram-positive bacteria belonging to the genus *Bacillus* (Table 2.2). RLID 12.1 showed positive for acetoin production (Voges-Proskauer test), amylase and catalase production but negative for indole and mixed acid production (Methyl Red test). Carbohydrate fermentation and growth at various salt concentrations (NaCl) are summarized in Table 2.2.

Table 2.2: Biochemical characterization of *Bacillus* strain RLID 12.1

S. No	**Tests**	***Bacillus* sp. RLID 12.1**
Morphology		
1.	Colony morphology	Wrinkled
2.	Colour	White
3.	Gram staining	positive
4.	Shape of the cells	Rod shaped
5.	Spores	+
6.	Pigment production on glutamate glycerol agar media	No pigment
NaCl		
7.	2%	+
8.	5%	+
9.	7%	+
10.	10%	+
Biochemical Tests		
11.	Catalase	+
12.	Indole	-
13.	Starch	+
14.	Production of mixed acid (MR test)	-
15.	Acetoin production (VP test)	+
16.	Lactose	-
17.	Tween 80	-
18.	Sorbitol	+
19.	Mannitol	+
20.	Adonitol	-
21.	Arabitol	-
22.	Erythritol	-
23.	Methyl-D-glucoside	-
24.	Rhamnose	-

25.	Cellobiose	+
26.	Melezitose	-
27.	Methyl-D-mannoside	-
28.	Xylitol	-
29.	Ortho-Nitro phenyl-ß-galactoside (ONPG)	-
30.	Esculin hydrolysis	+
31.	D-Arabinose	-
32.	Citrate utilization	-
33.	Malonate utilization	-
34.	Sorbose	-
35.	Tween 20	+
36.	Xylose	+
37.	Ribose	+
38.	Maltose	-
39.	Fructose	+
40.	Dextrose	+
41.	Galactose	+
42.	Raffinose	+
43.	Trehalose	-
44.	Melibiose	-
45.	Sucrose	+
44.	L-Arabinose	-
45.	Mannose	+
46	Inulin	+
47	Sodium gluconate	-
48	Glycerol	+
49	Salicin	-
50	Dulcitol	-
51	Inositol	-

Species level identification was carried out from the partial sequence analysis of 16S rDNA gene of RLID 12.1. Based on the BLAST analysis of the partial 16S rDNA sequence, the wild-type isolate RLID 12.1 was identified as *B. subtilis*. Multiple sequence alignment was performed using CLUSTAL W and the alignment is shown in the Fig. 2.2. The phylogenetic tree was constructed using PHYLIP (Fig. 2.3). Further confirmation was done by FAME analysis. Comparing the FAME profiles (Table 2.3) of the Sherlock MIS RTSBA6 identification library, FAME profile of RLID 12.1 was found similar to *B. subtilis*. The accession number JX089317 was assigned for RLID 12.1 by NCBI.

```
   1 gcacaagcggtggagcatgtggtttaatttgaagcaacgcgaagaaccttaccaggtctt
  61 gacatcctctgacaatcctagagataggacgtccccttcgggggcagagtgacaggtggt
 121 gcatggttgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaac
 181 ccttgatcttagttgccagcattcagttgggcactctaaggtgactgccggtgacaaacc
 241 ggaggaaggtggggatgacgtcaaatcatcatgccccttatgacctgggctacacacgtg
 301 ctacaatggacagaacaaagggcagcgaaaccgcgaggttaagccaatcccacaaatctg
 361 ttctcagttcggatcgcagtctgcaactcgactgcgtgaagctggaatcgctagtaatcg
 421 cggatcagcatgccgcggtgaatacgttcccgggccttgtacacaccgcccgtcacacca
 481 cgagagtttgtaacacccgaagtcggtgaggtaacctttatggagccagccgccgcaaag
 541 gtgggaacaaggaatg
```

Sequence 1: Partial 16S rDNA sequence of *B. subtilis* RLID 12.1

```
URID 12.1      GGCCCGCACAAGCGCTCGAGCAGGCGGTTTAACTCAAAGCTAACGCGAAAAACCTTACCA
DSM            GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA-ACGCGAAGAACCTTACCA
407D3          GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA-ACGCGAAGAACCTTACCA
RLID12-1       -----GCACAAGCGGTGGAGCATGTGGTTTAATTTGAAGCA-ACGCGAAGAACCTTACCA
                    ********* * ***** * ******* *  ****  ******* **********

URID 12.1      GCTCTTGACAACCGCGGACAATCACTAGAAACAGGACAACCCCTTCGAGGGCAGAAGAGA
DSM            GGTCTTGACATCCTCTGACAATCCTAGAGA-TAGGACGTCCCCTTCGGGGGCAGAGTGAC
407D3          GGTCTTGACATCCTCTGACAATCCTAGAGA-TAGGACGTCCCCTTCGGGGGCAGAGTGAC
RLID12-1       GGTCTTGACATCCTCTGACAATCCTAGAGA-TAGGACGTCCCCTTCGGGGGCAGAGTGAC
               * ******** ** * *******      *  *****  ******** *******

URID 12.1      AAAGTGGAATTCCACGTGTAGCCGTCAAATCGCGTAGTGAGATGTGGAGGTAACACCAGC
DSM            AG-----GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
407D3          AG-----GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
RLID12-1       AG-----GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
               *                ** * *****  *** ** ********* * * ***  ** **

URID 12.1      AACGAACGCAACCCTCTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTAACTG
DSM            AACGAGCG-CAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG
407D3          AACGAGCG-CAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG
RLID12-1       AACGAGCG-CAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG
               ***** **  * **  ****************************************** ****

URID 12.1      ACGCTGACAAACCGGAGAAAGCGTGGGGAGCAAACAAAATCAGCATACCCCTGATAACCC
DSM            CCGGTGACAAACCGGAGGAAGGTGGGGATGACGT-CAAATCATCATGCCCCTTATGACCT
407D3          CCGGTGACAAACCGGAGGAAGGTGGGGATGACGT-CAAATCATCATGCCCCTTATGACCT
RLID12-1       CCGGTGACAAACCGGAGGAAGGTGGGGATGACGT-CAAATCATCATGCCCCTTATGACCT
                ** ************** ***   ***  *      ****** *** ***** ** ***

URID 12.1      ACGCCACAAACGATGAGTGCTACAATGGACAGAACAAAGGGCAGCCAAACCGCAAGGCTA
DSM            GGGCTAC-----ACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTA
407D3          GGGCTAC-----ACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTA
RLID12-1       GGGCTAC-----ACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTA
                 ** **     *   ******************************** ******* *** **

URID 12.1      CAGCCAATCCCACAAATATGCACTCAGCCCGGGGAGCACAGTCTGCAACTCGACTGCGTG
DSM            A-GCCAATCCCACAAATCTG--TTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTG
407D3          A-GCCAATCCCACAAATCTG--TTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTG
RLID12-1       A-GCCAATCCCACAAATCTG--TTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTG
                 *************** **   **       *** * ***********************

URID 12.1      AAACTCAAATCGCAAGTAATCGCGGACCAGCACAACGCGGTGAATACGTTCCCGGGCCTT
DSM            AAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT
407D3          AAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT
RLID12-1       AAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT
               ** **  ****** ************ *****   **************************

URID 12.1      GTACACACCGCCTAATCAAAAAACAACAACCTGGAAGGAAAAA----------------
DSM            GTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTT
407D3          GTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTT
RLID12-1       GTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTT
               ************      *  *  *        *      **

URID 12.1      ------------------------------------------------------------
DSM            TTAGGAGCCAGCCGCCGAAGGTGGGACAGATGA--TTGGGGTGAAGTCGTAACAAGGTAG
407D3          TATGGAGCCAGCCGCCGCAAGGTGGGACAGCATGATTGGGGTGAAAGTCCGTTAAA----
RLID12-1       TATGGAGCCAGCCGCCGCAAAGGTGGGAACAAGGAATG----------------------
```

Fig. 2.2: Multiple sequence alignment showing the similarity of RLID 12.1 with *B. subtilis* (URID 12.1, DSM, 407D3).

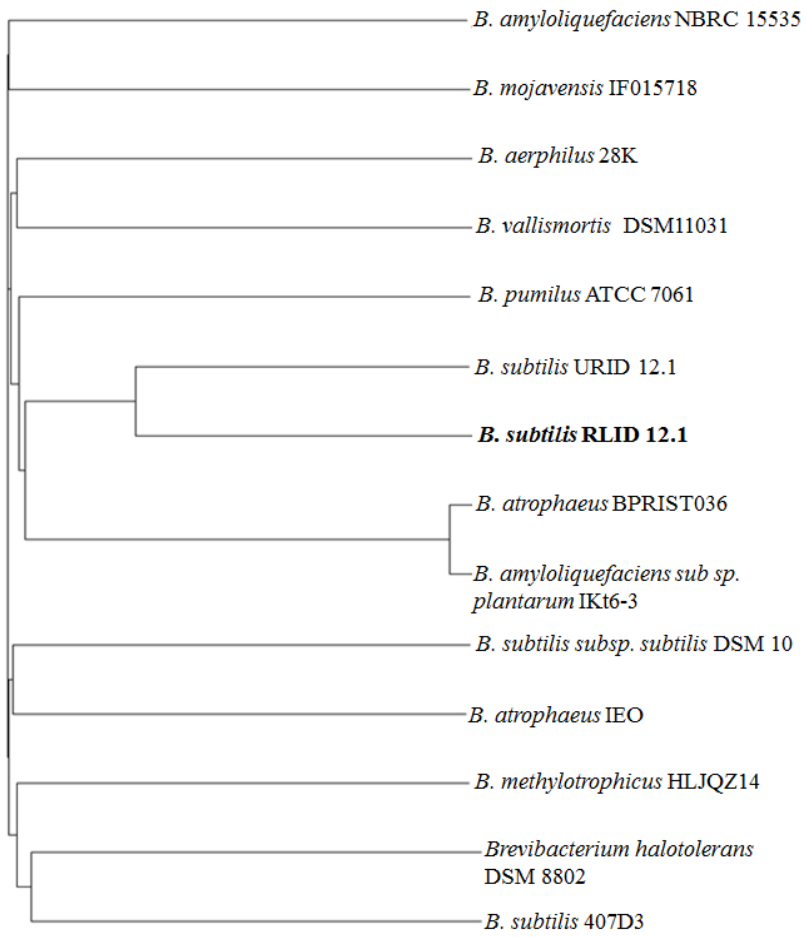


Fig. 2.3: Distance tree of the test strain *Bacillus* sp. RLID 12.1 constructed using PHYLIP (Ramya et al. 2013).

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Commen
0.2249	1085	0.026	----	3.1120		----	< min rt	
0.3680	876	0.019	----	4.1655		----	< min rt	
0.4334	1021	0.022	----	4.6471		----	< min rt	
0.4907	927	0.020	----	5.0692		----	< min rt	
0.5470	1015	0.017	----	5.4835		----	< min rt	
0.6049	784	0.018	----	5.9100		----	< min rt	
0.6523	946	0.020	----	6.2585		----	< min rt	
0.7042	337961	0.005	----	6.6413		----	< min rt	
0.7127	1.136E+9	0.018	----	6.7039	SOLVENT PEAK	----	< min rt	
0.9995	522	0.013	----	8.8132		----	< min rt	
1.1229	1066	0.015	----	9.7223		----		
1.1610	690	0.015	1.188	10.003	10:0	0.38	ECL deviates 0.003	Referenc
1.2108	947	0.017	----	10.288		----		
1.2530	871	0.017	----	10.529		----		
1.2787	661	0.017	----	10.676		----		
1.3036	842	0.018	----	10.819		----		
1.3928	610	0.014	----	11.269		----		
1.5497	3074	0.012	1.052	11.998	12:0	1.48	ECL deviates -0.001	Referenc
1.5975	843	0.020	1.041	12.190	11:0 2OH	0.40	ECL deviates -0.001	
1.7045	984	0.009	1.020	12.618	13:0 iso	0.46	ECL deviates -0.005	Referenc
1.7286	399	0.009	1.016	12.714	13:0 anteiso	0.19	ECL deviates 0.000	Referenc
1.7815	550	0.012	----	12.925		----		
1.8577	851	0.009	0.994	13.206	12:0 2OH	0.39	ECL deviates 0.003	
1.9362	1170	0.009	0.983	13.487	12:0 3OH	0.53	ECL deviates 0.005	
1.9751	3274	0.009	0.978	13.627	14:0 iso	1.47	ECL deviates -0.001	Referenc
2.0793	2747	0.009	0.966	14.000	14:0	1.22	ECL deviates 0.001	Referenc
2.2674	36556	0.009	0.948	14.631	15:0 iso	15.88	ECL deviates -0.001	Referenc
2.2952	80481	0.008	0.945	14.724	15:0 anteiso	34.88	ECL deviates -0.001	Referenc
2.3772	908	0.011	0.939	14.999	15:0	----	ECL deviates -0.001	
2.5053	1487	0.011	0.931	15.413	16:1 w7c alcohol	0.63	ECL deviates -0.001	
2.5386	1629	0.011	0.930	15.521	Sum In Feature 2	0.69	ECL deviates 0.006	14:0 3OH
2.5734	7373	0.009	0.928	15.633	16:0 iso	3.14	ECL deviates 0.000	Referenc
2.6192	3585	0.009	0.926	15.781	16:1 w11c	1.52	ECL deviates -0.001	
2.6369	3822	0.010	0.925	15.838	Sum In Feature 3	1.62	ECL deviates -0.001	16:1 w7c
2.6873	25096	0.009	0.923	16.001	16:0	10.62	ECL deviates 0.001	Referenc
2.7397	825	0.013	0.921	16.169	15:0 iso 3OH	0.35	ECL deviates 0.007	
2.7696	316	0.009	0.921	16.265	15:0 2OH	0.13	ECL deviates 0.010	
2.8167	1776	0.010	0.919	16.416	17:1 iso w10c	0.75	ECL deviates 0.002	
2.8462	1254	0.009	0.919	16.510	Sum In Feature 4	0.53	ECL deviates -0.002	17:1 ante
2.8859	13642	0.009	0.918	16.637	17:0 iso	5.74	ECL deviates 0.001	Referenc
2.9163	21055	0.009	0.917	16.735	17:0 anteiso	8.85	ECL deviates 0.002	Referenc
3.0864	772	0.015	----	17.280		----		
3.1718	695	0.012	0.917	17.554	16:0 3OH	0.29	ECL deviates 0.006	
3.2469	10013	0.010	0.917	17.795	18:1 w9c	4.21	ECL deviates 0.001	
3.2622	827	0.008	0.918	17.844	Sum In Feature 8	0.35	ECL deviates -0.003	18:1 w7c
3.3116	1361	0.010	0.918	18.002	18:0	0.57	ECL deviates 0.003	Referenc
3.3710	1097	0.019	0.920	18.197	17:0 iso 3OH	0.46	ECL deviates 0.004	
3.4019	1226	0.012	0.921	18.298	17:0 2OH	0.52	ECL deviates 0.011	
3.4327	842	0.018	0.921	18.399	18:0 10-methyl, TBSA	0.36	ECL deviates 0.005	
3.5470	364	0.008	0.925	18.774	Sum In Feature 6	0.15	ECL deviates -0.001	19:1 w11
3.6597	824	0.015	0.930	19.145	18:1 2OH	0.35	ECL deviates 0.000	
3.6878	741	0.013	----	19.239		----		
3.7101	1255	0.014	----	19.313		----		
3.7531	846	0.014	0.935	19.456	20:4 w6,9,12,15c	0.36	ECL deviates -0.009	
3.7645	493	0.010	----	19.494		----		
3.8170	915	0.012	----	19.669		----		
3.8659	798	0.013	----	19.832		----		
3.9154	1239	0.013	0.945	19.997	20:0	0.54	ECL deviates -0.003	Referenc
3.9589	1403	0.014	----	20.142		----	> max rt	
4.0110	1047	0.013	----	20.315		----	> max rt	
4.0575	1020	0.017	----	20.469		----	> max rt	
4.1033	1379	0.015	----	20.622		----	> max rt	
4.1440	1041	0.015	----	20.758		----	> max rt	
4.1926	798	0.013	----	20.919		----	> max rt	
4.2304	1025	0.014	----	21.045		----	> max rt	
----	1629	---	----	---	Summed Feature 2	0.69	12:0 aldehyde ?	unknown
----	----	---	----	---		----	16:1 iso I/14:0 3OH	14:0 3OH
----	3822	---	----	---	Summed Feature 3	1.62	16:1 w7c/16:1 w6c	16:1 w6c
----	1254	---	----	---	Summed Feature 4	0.53	17:1 iso I/anteiso B	17:1 ante
----	364	---	----	---	Summed Feature 6	0.15	19:1 w11c/19:1 w9c	19:1 w9c

Table 2.3: Analysis of Fatty acid methyl esters extracted from *B. subtilis* RLID 12.1.

2.3.3. Time course of growth and production of antifungal compound

The production of antifungal compound was determined based on the zone of inhibition (diameter in millimeter) against *C. albicans* SC5314. It was observed that the formation of the zone of inhibition by the concentrated culture filtrate actually started at 36th h, attained a maximum diameter after 60th h of growth of the producer strain in mTSB and the zone diameter started reducing from 64 h (Fig. 2.4). This observation shows that the production of antifungal compound(s) in the broth began at the mid log phase, reached maximum in the late log phase and declined during the initiation of the stationary phase. pH value increased gradually from the initial pH of 7.4 to 9.5 till the death phase but maintained pH ~8.5 during the antifungal production period (Fig. 2.4).

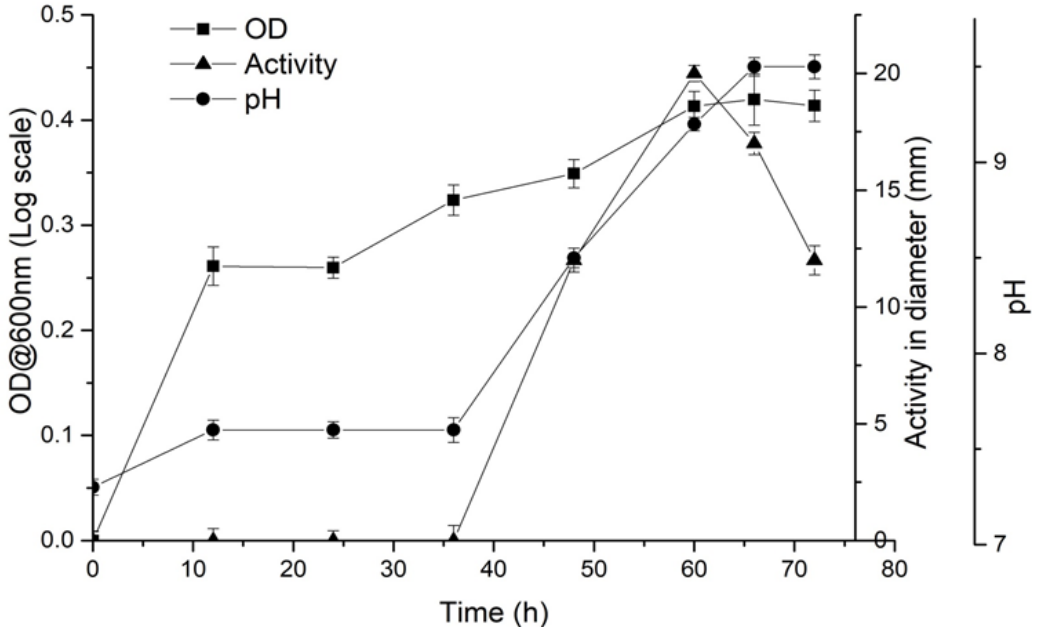
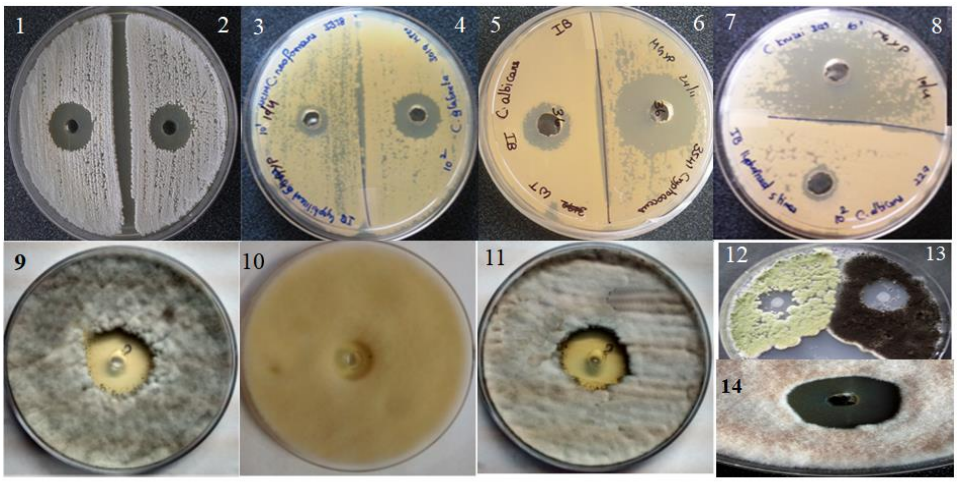


Fig. 2.4: Time course of growth of *B. subtilis* RLID 12.1, pH recorded during the production of antifungal compound and the antifungal activity was checked using *C. albicans* SC5314 as an indicator organism (Ramya et al., 2018).

2.3.4. Antifungal activity of *B. subtilis* RLID 12.1 by cut well agar assay

The compound(s) produced by *B. subtilis* RLID 12.1 displayed a broad antifungal spectrum against the yeasts and filamentous fungi tested (Fig. 2.5; Table 2.4). Antifungal activity of the ultrafiltered cell free supernatant of *B. subtilis* RLID 12.1 was determined by cut well agar method and expressed as the inhibition zone diameter in millimetres (mm) (Shekh et al., 2011; Ramya et al. 2013).



1) *C. albicans* ATCC 24433
2) *C. albicans* SC5314
3) *Cryptococcus neoformans* NCIM 3378
4) *C. glabrata* NCIM 3019
5) *C. albicans* WT (clinical isolate)
6) *Cryptococcus neoformans* NCIM 3541
7) *C. krusei* NCIM 3129
8) *C. albicans* MTCC 227
9) *Curvularia* sp.
10) *Mucor* sp
11) *Bipolaris* sp.
12) *Aspergillus flavus*
13) Aspergillus niger
14) Fusarium oxysporum

Fig.2.5: Antifungal potency of *B. subtilis* RLID 12.1 against yeasts and filamentous fungi (Ramya et al. 2013)

Table 2.4: Antifungal spectrum of ultrafiltered cell free supernatant of *B. subtilis* RLID 12.1 by cut well agar method (Ramya et al. 2013)

S.No	**Indicator organism**	**Inhibition zone diameter (mm)**
1	*Candida albicans* ATCC 24433	18
2	*C. albicans* SC5314	18
3	*C. neoformans* NCIM 3378	15
4	*C. glabrata* NCIM 3019	20
5	*C. glabrata* MTCC 3814	17
6	*C. albicans* WT (clinical isolate)	17
7	*C. albicans* DI (clinical isolate)	14
8	*C. neoformans* NCIM 3541	24
9	*C. krusei* NCIM 3129	23
10	*C. albicans* MTCC 227	14
11	*Curvularia* sp.	21
12	*Mucor* sp.	12
13	*Bipolaris* sp.	21
14	*A. flavus*	12
15	*A. niger*	18
16	*F. oxysporum*	21

2.3.5. Preliminary characterization

Preliminary characterization of the antifungal compound is summarized in Table 2.5.

2.3.5.1. Effect of pH andtemperature on the antifungal compound

The antifungal activity was recorded over a wide range of pH (2.0-10.0) (Fig.2.6a). 100% activity was found between pH 6.0 and 10.0 whereas reduced activity (61% and 83%) was observed at the pH 2.0 and 4.0 respectively. Antifungal activity of about 90% was retained at 37 ºC for 9 h, 70% at 50ºC and 60 ºC when incubated for 5 h and 2 h respectively and 46% of antifungal activity at 70ºC when incubated for 1 h (Fig. 2.6b and Table 2.5).

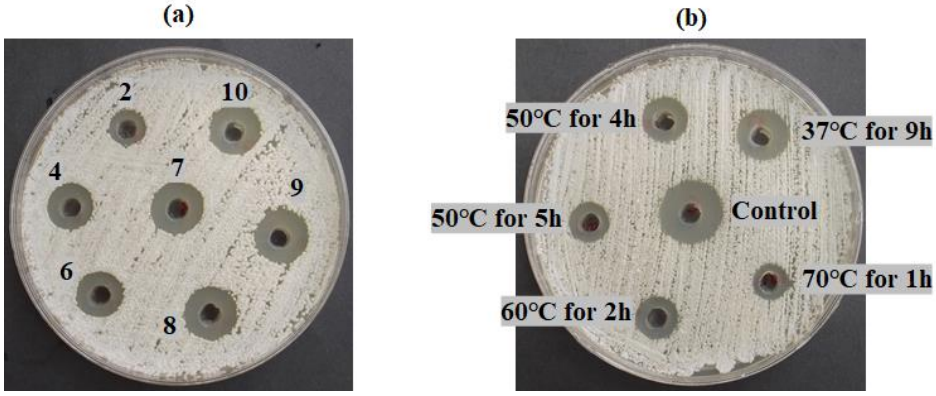


Fig. 2.6: Effect of pH and temperature on the antifungal activity of the concentrated CFS.

2.3.5.2. Effect of surfactants, chemicals and metal salts on the antifungal compound

When the ultrafiltered concentrate (retentate) was treated with β-ME, TCA and surfactants like SDS, Triton-X-100, tween 20 and tween 80, a complete loss of antifungal activity was observed (Table 2.5). Biological activity of the antifungal compound remained the same when exposed to 2 mM EDTA whereas at 5 and 50 mM EDTA concentration, increased activity was observed equal to EDTA control indicating the loss of biological activity (Fig. 2.7). Antifungal activity was found to be stable (100 %) after treating the concentrate with seven different metal salts (Fig. 2.8) except $CdCl_2$ where 30% biological activity was recorded (Table 2.5).

Table 2.5: Preliminary characterization of the concentrated antifungal compound (ultrafiltered retentate of CFS)

Treatment	**Concentration**	**Residual activity (%)**
Control		100
pH		
2.0		61
4.0		83
6.0		100
7.0		100
8.0		100
9.0		100
10.0		100
Temperature		
9 h at 37˚C		90
5 h at 50˚C		70
2 h at 60˚C		70
1 h at 70˚C		46
Metal salts		
$CdCl_2$	1 mg/mL	30
$FeSO_4$		100
$AgNO_3$		100
$MgSO_4$		100
$MnCl_2$		100
$ZnSO_4$		100
$CuSO_4$		100
$CaCl_2$		100
Other reagents		
SDS	1% (v/v)	0
β-ME	10% (v/v)	0
TCA	10 mg/mL	0
EDTA	2 mM	100
	5 mM	0
	50 mM	0
Tween 20	1% (v/v)	0
Tween 80	1% (v/v)	0
Triton X-100	1% (v/v)	0
	Enzymes	
Proteinase K	1 mg/mL	83
Trypsin		72
Lipase		0
α-Amylase		100

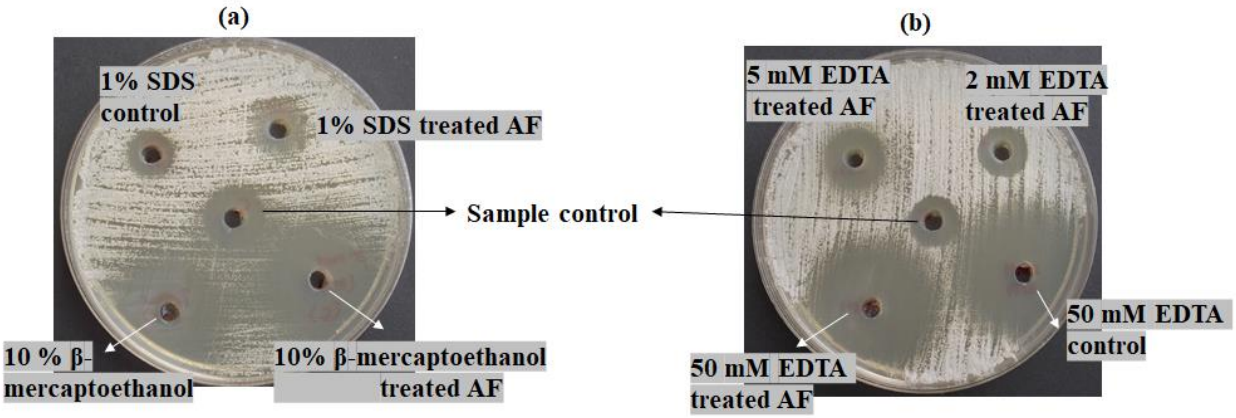
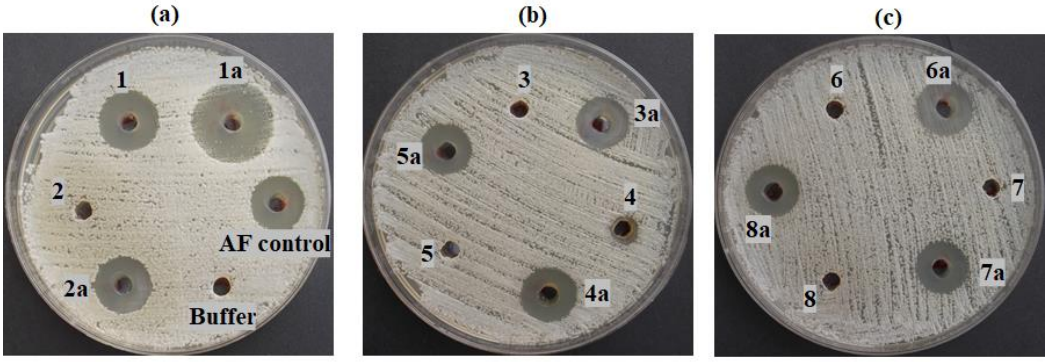


Fig. 2.7: Effect of SDS, β-mercaptoethanol and EDTA on the antifungal activity of the concentrated CFS



1 = $CdCl_2$ control	1a = $CdCl_2$ treated AF
2 = $FeSO_4$ control	2a = $FeSO_4$ treated AF
3 = $MgSO_4$ control	3a = $MgSO_4$ treated AF
4 = $AgNO_3$ control	4a = $AgNO_3$ treated AF
5 = $MnCl_2$ control	5a = $MnCl_2$ treated AF
6 = $CaCl_2$ control	6a = $CaCl_2$ treated AF
7 = $ZnSO_4$ control	7a = $ZnSO_4$ treated AF
8 = $CuSO_4$ control	8a = $CuSO_4$ treated AF

Fig. 2.8: Effect of metal salts on the antifungal activity of the concentrated CFS.

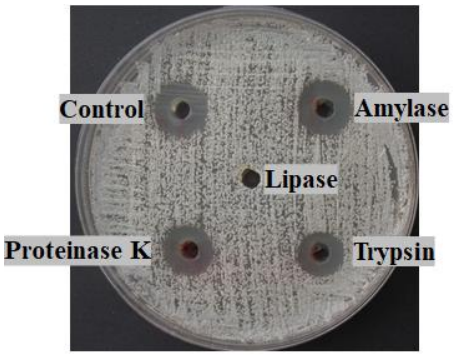


Fig. 2.9: Effect of enzymes on the antifungal activity of the concentrated CFS.

2.3.5.3. Effect of proteolytic and non-proteolytic enzymes

The concentrated antifungal compound was tested for sensitivity to proteolytic and non-proteolytic enzymes (Table 2.5). No antifungal activity was obtained after lipase treatment; however, the antifungal activity was retained up to 83% and 72% when treated with proteinase K and trypsin (Table 2.5 and Fig. 2.9). No loss of antifungal activity was observed upon exposure to α- amylase (Fig. 2.9).

2.4. Discussion

An increasing incidence of antifungal resistance is the urgent requirement to develop novel molecules with different mode of action to target the multidrug-resistant pathogens. In this context, soils from two places were screened and the organism showing significant antifungal activity was selected. Based on the morphological, biochemical studies (Table 2.2), BLAST analysis of partial sequence of the 16S rDNA gene of RLID 12.1 and FAME analysis, its taxonomic identity was identified as *B. subtilis*. Differential sugar fermentation and salt tolerance (2-10%) studies added more to the fact that *B. subtilis* RLID 12.1 can adopt to different habitat condition by regulating its metabolism required for its survival.

The genus *Bacillus*, one of the most abundant soil bacteria, is capable of producing several dozens of antibiotics with various chemical properties, among which peptide derivatives are elaborately studied (Stein T, 2005; Mannanov and Sattarova, 2010). In a natural environment, these soil bacteria generate peptide antibiotics using different mechanisms to compete with other organisms for an ecological niche or a substrate or as an induced systemic resistance (Abriouel et al., 2011). When two species of peptide-antibiotic producing bacilli coexist, they inactivate or shield the peptide antibiotics of the other as a survival strategy (Vlok, 2005). *B. subtilis* is regarded as a main representative for the production of vast array of structurally unrelated antimicrobial compounds. Studies on the whole genome sequencing of *B. subtilis* revealed that 4-5 % of its genome were devoted to ribosomal and non-ribosomal antibiotic synthesis (Fickers, 2012; Zhao and Kuipers, 2016). Non-ribosomal synthesized peptide antimicrobials include lipopeptides, glycopeptides and glycolipopeptides whereas the ribosomal peptides include bacteriocins and unusual antibiotics (Abriouel et al., 2010; Stein T, 2005; Mannanov and Sattarova, 2010). The production of lipopeptides allows certain *B. subtilis* strain to modify their outer surface which permits them to regroup together in a biofilm in

order to proliferate and spread in the territory. Biofilm formation also contributes to the defence and resistance mechanism of *B. subtilis* species towards other peptide or antibiotic producing organisms (Morikawa, 2006). Although, *B. subtilis* is commonly investigated, the production of antimicrobial compounds varies with the strain based on its competition in the ecological niche, which thereby tempt us to speculate novel compounds with broad-spectrum potential.

Antimicrobial compound produced by *B. subtilis* RLID 12.1 was found to exhibit antifungal activity against cryptococcosis caused by *C. neoformans*, candidiasis or candidosis caused by *C. albicans* and non-*albicans*, for example, *C. krusei* and *C. glabrata* (Fig. 2.5). Strong inhibitory action against the filamentous fungi *Aspergillus* sp., *Bipolaris* sp., *Mucor* sp. and *Fusarium* sp. indicate the broad-spectrum potential of the antifungal compound produced by *B. subtilis* RLID 12.1 (Fig. 2.5). Broad inhibitory spectral property had been found to be reported previously in many *B. subtilis* LFB 112 and *B. amyloliquefaciens* MBL27 (Xie et al., 2009; Vijayalakshmi et al., 2011). In this connection the next chapters dwell upon the purification processes, identification and characterization of the antifungal compound(s) produced by the producer strain *B. subtilis* RLID 12.1 used throughout the study.

Antifungal compound produced by RLID 12.1 was found to be stable over wide pH range, metal salts and relatively stable at higher temperatures. The ultrafiltered concentrate was found to be partially sensitive to proteinase K and trypsin which retained about 83 and 72% activity (partial loss of 17 and 28%) but the 100% loss of activity observed with TCA treatment confirmed the proteinaceous nature of the antifungal compound produced by RLID 12.1. A complete loss of activity recorded after the lipase treatment indicated the presence of the lipid moiety and its involvement in the fungicidal action of the antifungal compound of RLID 12.1. Resistance to α-amylase indicated the absence of glucosidic moiety in the antifungal compound. Study on the effect of proteolytic and non-proteolytic enzyme on the antifungal compound provides a hint about the lipopeptide nature of the antifungal compound. Failure of proteolytic digestion might be due to the presence of unusual amino acids with D-conformation in the peptide structure (Boman, 1991) due to the presence of proline residues or cyclic N and/or C terminally blocked peptides (Galati et al., 2003) which are in consonance with data reported from many *Bacillus* spp. (Aunpad and Bangchang, 2007; Kayalvizhi and Gunasekaran, 2010).

Chapter 3

Purification and Identification of antifungal variants

3.1. INTRODUCTION

Antimicrobial compounds particularly AMPs are well documented for the members of *Bacillus* species. Particularly *B. subtilis* are considered as a microbial factory for the production of structural and functional variant biomolecules which potentially inhibit bacteria, fungi, viruses and insects (Sumi et al., 2015). Antifungal peptides reported in *B. subtilis* belong to lipopeptide, glycopeptide and glycolipopeptides type. These peptides are produced non-ribosomally by large modular enzymes like NRPS and PKs (Stein, 2005). The remarkable regulatory biosynthetic system of multi-complex enzymesis responsible for the heterogeneity among the non-ribosomal peptides with regard to amino acid sequence and its chirality, fatty acid chain length and branching type, sugars and their degree of deacetylation and linearity/cyclization of the peptide (Ongena and Jacques, 2007). Among them, iturin (cyclopeptide) and fengycin (macrolactone) family lipopeptides are well known for its antifungal property (Romero et al., 2007). Iturin was found active mostly against yeasts and filamentous fungi and very less against the bacteria. Fengycin displayed excellent antifungal activity against plant filamentous fungal pathogens. Iturin family lipopeptides includes iturin (A,C,D,E), mycosubtilin, bacillomycin (D,F,L,Lc), mojavensin (A,B,C), bacillopeptin. Main variants of fengycin family are fengycin A/B and pliplastin A/B (Deleu et al., 2003). The fungicidal activity of iturins and fengycins are mainly due to the membrane permeabilization and pore formation (Maget-Dana et al., 1985; Deleu et al., 2003).

In general, antimicrobial peptides of *Bacillus* sp. are extracted from cell free supernatant (CFS) using the organic solvents or ammonium sulphate precipitation (Youssef et al., 2005; Yakimov et al, 1995). Nature of the extracted antifungal compounds are analysed by Thin Layer Chromatography (TLC) using various spraying and staining reagents. Bioautography is a technique similar to zymogram process which is used to recognise the active fungicidal spot in TLC plate. Further the purification was carried out using adsorption (silica) column chromatography or ion-exchange chromatography (IEC) and size exclusion chromatography (SE).

In case of *Bacillus* genus, coproduction of different families of antifungal lipopeptides was reported for e.g. surfactin and iturin, iturin and fengycin or surfactin and fengycin which entail a synergistic action against fungal pathogens (Koumoutsi et al., 2004; Romero et al., 2007; Tanaka et al., 2015). Also, various homologues and isoforms

of lipopeptides with different fungicidal action were coproduced by *Bacillus* sp. (Bie et al., 2009; Pathak and Keharia, 2012 and 2014). However, the above-mentioned chromatography techniques may not be always found suitable for separating the individual homologues/isoforms from the lipopeptide mixtures. In this context, the High-Performance Liquid Chromatography (HPLC) was found to be the most efficient method for purifying those antimicrobials. In particular, reversed-phase HPLC (RP-HPLC) was used extensively to achieve high purity of the antifungal homologues and isoforms (Sivapathasekaran et al., 2009) without losing their biological activity. Especially in case of biomolecules like lipopeptides, good resolution among the homologues or isomers was observed as the separation is based on the polarity.

Mass spectrometry techniques facilitate the rapid identification of the structure of low molecular weight antifungal compounds of *Bacillus* sp. Mass spectrometry like Matrix Assisted Laser Desorption/Ionization-Time of Flight mass spectrometry (MALDI-TOF-MS), Electrospray Ionization mass spectrometry (ESI-MS/MS), Electrospray Ionization Fourier Transform Ion Cyclotron Resonance mass spectrometry (ESI-FT-ICR-MS) were used generally for molecular characterization of *Bacillus* antifungal peptides. For structural characterization of lipopeptide or glycopeptide type compounds, Gas Chromatography/Mass spectrometry (GC/MS) has to be performed additionally to identify the fatty acid or sugar moiety respectively attached to the peptide sequence.

In this study, antifungal compounds of *B. subtilis* RLID 12.1 were purified using acid precipitation, solvent extraction, adsorption chromatography and RP-HPLC. Identification was achieved by mass spectrometry analysis using MALDI-TOF-MS, ESI-FT-ICR-MS and GC/MS techniques.

3.2. EXPERIMENTAL METHODS

In order to perform the molecular characterization, purification of antifungal compound(s) to near homogeneity was essential. Two different purification strategies were attempted initially as mentioned in the Fig. 3.1. After each step of purification, bioassays were performed.

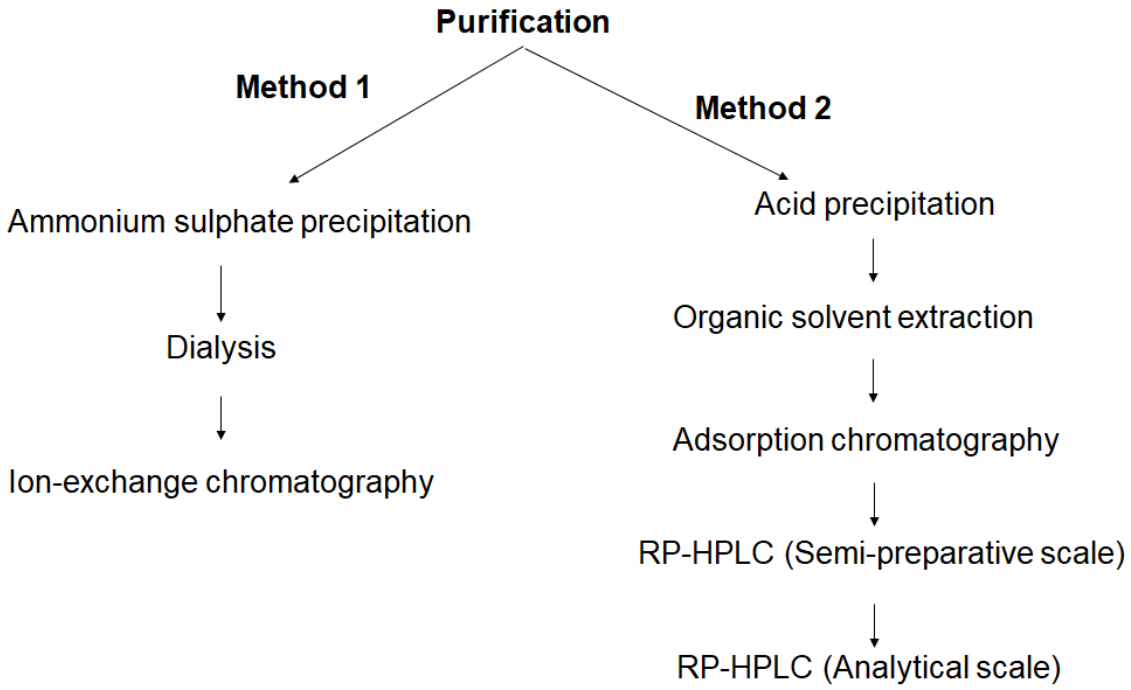


Fig. 3.1: Flow chart illustrating the purification steps

3.2.1. Method 1

3.2.1.1. Ammonium sulphate fractionation

After 60 h of growth, CFS of *B. subtilis* RLID 12.1 was collected after centrifugation at 12000 rpm for 15 min at 4 °C. CFS was subjected to ammonium sulphate fractionation (30, 50 and 80% saturation) at 4 ˚C. Then pellet was resuspended in 20 mM Tris buffer (pH 8.0) and was dialysed using 2 kDa molecular weight cut-off membrane (Spectrapor) against the same buffer for 24 h with four changes at 4 ˚C. The dialyzate was stored at -20˚C for further analysis.

3.2.1.2. Purification of antifungal compound by ion-exchange chromatography

For the ion-exchange chromatography, the dialysed sample (1mg/mL) was applied to diethylaminoethyl (DEAE) - Sepharose Fast flow (GE Healthcare) column (110 mm x 17 mm) pre-equilibrated with 20 mM phosphate buffer (pH 8.0) and protein were eluted with same buffer followed by a gradient from 0 - 0.5 M at a flow rate of 1mL/min. Protein in the effluent was monitored by determining the absorbance at 280 nm. A total of 90 fractions (2 mL) were collected, and 100 µL aliquot of each fraction was tested for

antifungal activity by cut well agar assay. Phosphate buffer was used as a negative control.

Ion exchange purified fractions were subjected to molecular mass determination by SDS-PAGE 15% gel in accordance with the procedure of Laemmli and Favre (1973). Electrophoresis was carried out at 60 V and the protein bands were visualized by silver staining method (Ramya et al., 2013). Low molecular weight marker (3.5–43 kDa - GeneI) was used.

3.2.2. Method 2

Antifungal compound produced by *B. subtilis* RLID 12.1 was purified to near homogeneity by four step processes: acid precipitation, solvent extraction, silica adsorption chromatography and RP-HPLC.

3.2.2.1. Selection of organic solvents

To screen the solvent essential for the maximum recovery of antifungal compound of *B. subtilis* RLID 12.1, different solvents like methanol, chloroform, n-butanol and t-butanol were selected. *B. subtilis* RLID 12.1 was grown in mTSB media at 37±0.5 °C. for 60 h at 105 rpm. After 60 h, culture was centrifuged at 10000 rpm at 4 °C for 15 min and the CFS was collected. Organic solvent was added to the CFS in the equal volume (1:1) and kept in stirring condition for 3 h. After 3 h, the mixtures were centrifuged at 10,000 rpm for 10 min and the solvents were evaporated completely at 55±0.5 °C. The residue was dissolved in 10 mM sodium phosphate buffer pH 7.0 and tested for activity against *C. albicans* SC5314 by spot agar assay/spot-on-lawn-assay.

3.2.2.2. Thin Layer Chromatography (TLC) analysis

Initially, the pre-coated silica gel 60 plate on the aluminium support (7 × 4 cm; layer thickness, 0.20 mm, Merck) was activated by heating at 110 °C. The solvent system n-butanol: methanol: water at the ratio 5:1:1 (v/v/v) was used as a mobile phase. The TLC chamber was saturated with 5mL of solvent system for 20-30 min. The solvent extracted sample (3 µL) was spotted on to the activated TLC plate and allowed for drying. Then the plate was placed in the saturated chamber for 30-45 minutes until the solvent reaches the developing line. The developed plates were completely dried of solvents and one part of the TLC plate was subjected to bioautography and the other part for analysing the nature

of the compound. The retention factor (R_f) value of the active spot was determined using the formula stated below

$$R_f = \frac{Distance\ travelled\ by\ the\ compound}{Distance\ travelled\ by\ the\ solvent}$$

3.2.2.3. Bioautography

In this study the bioautography was carried out to identify the active spot in the developed TLC plate using the redox indicator Triphenyl tetrazolium chloride (TTC) dye. Stock solution (2%) of TTC was prepared using the sterile distilled water, filtered through 0.22 μm membrane and stored at 4°C. The indicator organism *C. albicans* SC 5314 (1%) and TTC (final concentration, 0.2 %) were added to the SDA media prepared with 0.8% agar. After TLC run, the developed plate was dried completely in the sterile condition and placed in the Petri plate base. The inoculated media with the colourless TTC dye was poured on the TLC plate, mixed gently and incubated at 37±0.5 °C for 24-48 h. The active compound diffuses to the agar layer and forms an inhibition zone in the respective position. Bioautography was also performed against *A. niger* and incubated at 30±0.5 °C for 48-72 h for antifungal spot detection (Tabbene et al., 2009; Afsharmanesh et al., 2014).

3.2.2.4. Identification of the compound

The other part of the developed TLC plate after complete drying of solvents was exposed to staining and spraying reagents to determine the nature of the compound. The TLC plate was sprayed with a) distilled water and heated at 100 ºC for 5 min to detect the hydrophilic nature of the bioactive compound; b) exposed to iodine vapour to detect the presence of lipid moiety; and c) treated with Serva blue W stain for the detection of peptide moiety. Serva blue W staining was carried out by immersing the developed plate first in 20% trichloroacetic acid (TCA) until the white spot appears and then in 0.3% Serva Blue W solution for 10-15 min until the dark blue spots were visualized clearly (Tabbene et al., 2009).

3.2.2.5. Acid precipitation

After 60 h of growth, CFS of *B. subtilis* RLID 12.1 was collected after centrifugation at 12000 rpm for 15 min at 4 °C. The pH of the collected CFS was

adjusted to pH 2.0 by adding 12N HCl slowly in a dropwise manner at 4 °C under stirring condition and left for 8-10 h in the cold room (4°C) under mild stirring condition (Yakimov et al., 1995) (for 1.0 L CFS, 12 mL of HCl was added to adjust the pH to 2.0). After 10 h, centrifugation was carried out at 10000 rpm for 20 min at 4 °C and the pellet obtained was dissolved in 50 mM sodium phosphate buffer of pH 8.0 to neutralize the acid precipitated pellet.

3.2.2.6. Solvent Extraction

After the complete dissolution of the pellet in buffer, the antifungal sample was extracted with the equal volume of n-butanol solvent in the constant stirring condition for 3 h at room temperature and then centrifuged at 10000 rpm for 5 min at 30 °C. As n-butanol is immiscible with water, two layers were separated. The upper butanol layer containing the antifungal sample was separated from lower aqueous phase and the butanol was evaporated completely at 55±0.5 °C (Baindara et al., 2013).

3.2.2.7. Silica Column Chromatography

Silica adsorption column chromatography was performed using silica gel of 230-400 mesh size as a stationary phase and chloroform with methanol in the mobile phase at different ratios. Silica powder of about 20 g was equilibrated with the chloroform for 30 min and packed in the glass column uniformly using chloroform. Antifungal sample was loaded on to the column using dry sampling method. Chloroform equilibrated silica of about 0.2 g was added to the evaporated antifungal residue obtained after butanol extraction, mixed uniformly with the silica powder and dried at 55 °C. Dried antifungal silica powder was loaded on to the packed silica bed surface (stationary phase) and different ratios of chloroform and methanol were added for eluting the antifungal compound(s). Following were the chloroform (C) and methanol (M) ratios used for elution (Table 3.1).

Table 3.1: Different ratios of the mobile phase used in the adsorption chromatography

C: M	Amount with respect to column volume
100: 0	1 volume
80:20	1.5 volumes
60:40	2 volumes
55:45	1 volume

Eluted fractions were collected in 2 mL sterile micro-centrifuge tubes and the solvents were evaporated completely at 55°C. The evaporated residue in the tubes were dissolved using 200 µL of 10 mM sodium phosphate buffer of pH 7.0 and tested for antifungal activity against *C. albicans* SC5314. Samples were stored at 4°C after each step of purification.

3.2.2.8. Purification of antifungal compound using semi-preparative scale RP-HPLC

The pooled active fractions obtained after adsorption chromatography was dried completely using speed vacuum evaporator and dissolved in 50% methanol (about 10 mL) and fractionated by reversed phase-HPLC (RP-HPLC) at a semi-preparative scale using Eclipse XDB-C18 column (9.4 mm × 250 mm, particle size 5 µm). The sample injection volume was 200 µL and the solvent system used was (A) water with 0.1% trifluoroacetic acid (TFA) (HPLC-grade) and (B) acetonitrile (HPLC-grade) containing 0.1% TFA. The gradients of solvent B used for purification were shown in the Table 3.2.

Table 3.2: Solvent system used to separate antifungal compounds at the semi-preparative scale RP-HPLC.

Time (min)	Water (A%)	Acetonitrile (B%)	Flowrate (mL/min)
0-10	0	45	1
10-20	45	54	0.5
20-48	54	60	0.5

All the fractions eluted from the HPLC column were monitored at 214 nm in a diode array detector and all the peaks were collected. Fractions of multiple runs were pooled, concentrated by speed vacuum and tested for antifungal activity against *C.*

albicans SC5314. The active fractions were subjected to TLC analysis and bioautography assay as stated previously.

3.2.3. Determination of lipopeptide concentration

The concentration of the purified compound was determined by microplate assay using Bicinchoninic acid (BCA) method using Pierce™ BCA protein assay kit. Different dilutions of the bovine serum albumin (BSA) ranging from 0-1000 µg/mL were prepared for plotting a standard curve (Mukherjee and Das, 2005; Smyth et al., 2010; Baindara et al, 2016; Wei and Latour, 2010; Faber et al., 2003). BCA working reagent was prepared by mixing 50 volumes of reagent A (BCA) and 1 volume of reagent B ($CuSO_4$). Sample and working reagent were added in the 96 well microtitre plate in the ratio of 1:8 (25µL:200 µL) respectively. The plate was incubated for 30 min at 37±0.5 °C. After 30 min, the plate was cooled to room temperature and the OD was recorded at 562 nm using Multiskan Thermoscientific microplate reader. Standard curve was prepared using the absorbances (y-axis) obtained for different BSA concentrations (x-axis). This standard curve was used to determine the concentration of the antifungal fractions eluted from HPLC.

3.2.4. Re-chromatography of partially purified antifungal fractions

MIC and toxicity studies of all the antifungal fractions are discussed in chapter 4. Based on that, the identification and characterization studies were further focussed on the potent and less toxic AF_3, AF_4 and AF_5 lipopeptides. Re-chromatography of AF_3, AF_4 and AF_5 fractions were carried out in the analytical scale HPLC to improve the purity for molecular identification studies. The partially purified antifungal fractions, AF_3, AF_4 and AF_5 were re-chromatographed using the analytical column (4.6 mm × 250 mm, particle size 5 µm and the injection volume, 100 µL) with a gradient of 0-50% for 22 min at 0.7 mL/min for AF_3 and AF_4 fraction and 0-55% for 25 min at 0.7 mL/min for AF_5 fraction.

3.2.5. Identification of the antifungal compounds

3.2.5.1. Mass spectrometry analysis by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI TOF/TOF)

Mass spectrometry analysis was performed by a Ultraflex MALDI-TOF/TOF mass spectrometer (Brukerdaltonics) with nitrogen laser operating at 337 nm. For mass

spectrometric (MS) analysis, the purified active fractions (1 µL aliquot) were mixed with an equal volume of 0.1% solution of α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile-water-TFA [50:50:0.1 (v/v/v)] used as the matrix. Mass spectra were accumulated over 100 individual laser shots and obtained in the reflector mode at an initial accelerating voltage of 20 kV. A molecular mass gate of 350 Da improved the measurement by filtering out most matrix ions (Pathak and Keharia, 2014).

3.2.5.2. Mass spectrometry analysis by Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR-MS)

Analysis was performed on an ESI-FT-ICR mass spectrometer (SolariX, Bruker) in a positive ion mode at a concentration of 9.3 µM in acetonitrile-water-formic acid [50:50:0.1 (v/v/v)]. Analysis of spectra and elemental compositions were generated using the data analysis (X-mass acquisition) software vs 4.4 (Bruker).

3.2.5.3. Fatty acid analysis by Gas Chromatography/Mass Spectrometry (GC/MS)

Lipopeptides (1 mg) were incubated with 0.5 mL of 6 M HCl at 110°C for 16 h in sealed tubes for acid hydrolysis. Then the fatty acids were extracted with ether, treated with 0.95 mL methanol and 0.05 mL of 98% H_2SO_4 at 65°C for 6 h. Finally, the fatty acid methyl esters were obtained by n-hexane extraction and analyzed on GC-MS-QP 2010 quadruple mass spectrometer (SHIMADZU) with DB-5 column (0.25x30x0.25). The carrier gas used was helium with a flow rate of 1.0 mL/min. The column temperature was maintained at 100°C for 4 min and thereafter gradually increased (5°C/min) to 280°C and held for 4 min (Peng et al., 2008). The spectrum was analysed using NIST05 library.

3.2.6. Extraction of genomic DNA

Genomic DNA from strain RLID 12.1 was prepared as described by Neumann et al., (1992). *B. subtilis* was grown in 10 mL of Trypticase Soya Broth, pH 7.2 at 37^{o}C$\pm$0.5 for 24 h. The cells were harvested by centrifugation at 10000 rpm for 10 min and cell pellet was suspended in 1mL of TE (Tris buffer 10mM: 0.1mM EDTA) buffer pH 8.0 followed by 800µl of SET buffer, pH 7.5. Freshly prepared lysozyme was added to the suspended pellet at a concentration of 1mg/mL and incubated at 37$\pm$0.5 ^{o}C for 1h. After incubation, 1/10th volume of 10% SDS and 1 mg/mL proteinase K were added and incubated at 55^{o}C with occasional inversion for 2 h. Sodium chloride (5M, 1/3rd volume) and chloroform (1 volume) were added and incubated at room temperature for 30 min

with frequent but gentle inversion and centrifuged at 7000 rpm for 15 min. The upper aqueous phase was separated followed by PCI (phenol, chloroform and isoamylalcohol, 25:24:1) extraction. Ribonculease A (10 µg/mL) treatment was carried out at 37 ^{o}C for 40 min followed by PCI (25:24:1) extraction. The upper aqueous layer containing the genomic DNA was precipitated by adding 2.5 volumes of absolute alcohol and kept for overnight at -20^{o}C. Then DNA was pellet down by centrifugation at 7000 rpm for 15 min at 4^{o}C, rinsed with 70% ethanol and the pellet was dried. After complete drying, the DNA pellet was suspended in 0.1 mM TE (10:0.1) and stored at -20 ℃ for PCR studies.

3.2.7. Molecular detection of non-ribosomal peptide genes

PCR amplifications were carried out using the specific sets of primers to analyze the genes related to the antagonistic property of *B. subtilis* RLID 12.1. Six primers corresponding to the antifungal peptides iturin C (ItuC), fengycin D (FenD) and bacillomycin (bmyB) and antibacterial peptides bacilysin (BacA) and surfactin (SUR3 and srfA) were selected (Table 3.3).

Table 3.3: Specific PCR primers used for detecting non-ribosomal peptide genes of *B. subtilis* RLID 12.1

S.No	Primer used	Primer sequence (5' to 3')	T_A (ºC)	Product size(bp)	References
1.	BmyB F BmyB R	GAATCCCGTTGTTCTCCAAA GCGGGTATTGAATGCTTGTT	49.0	370	Athukorala et al., 2009 Mora et al., 2011
2.	FenD F FenD R	GGCCCGTTCTCTAAATCCAT GTCATGCTGACGAGAGCAAA	52.0	269	
3.	ItuC F ItuC R	GGCTGCTGCAGATGCTTTAT TCGCAGATAATCGCAGTGAG	50.4	423	
4.	BacA F BacA R	CAGCTCATGGGAATGCTTTT CTCGGTCCTGAAGGGACAAG	50.4	498	
5.	srfA F srfA R	TCGGGACAGGAAGACATCAT CCACTCAAACGGATAATCCTGA	49.9	201	
6.	SUR3 F SUR3 R	ACAGTATGGAGGCATGGTC TTCCGCCACTTTTTCAGTTT	48.7	441	

The PCR mix in a 50 μL reaction volume was as follows: 100 ng of genomic DNA as template, 2.5 U of Taq DNA polymerase (EMerck), 5 μL reaction buffer, MgCl2

(25 mM), deoxynucleoside triphosphate solution (10 mM each), and of each primer (100 pico moles). The PCR conditions included an initial denaturing step for 2 min at 94 ºC followed by 30 cycles of denaturing at 94 ºC for 1 min, annealing at the specific temperature (Table 3.3) for 45 s, elongation at 72 ºC for 45 s followed by a final extension of 7 min at 72 ºC. The PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel. Amplification was accomplished in a thermal cycler (MJ Research, Biorad). The gel was run at 80 V for approximately 90 min and 100 bp DNA ladder (Merck) was used as the molecular weight marker.

3.3. RESULTS

3.3.1. 1st method of purification: Ammonium sulphate fractionation and Ion-Exchange Chromatography

Application of salting out methods using ammonium sulphate precipitation was performed to concentrate the antifungal compound due to the proteinaceous nature. Recovery of antifungal compound was found more in the dialyzate of 30% ammonium sulphate saturated fraction compared to 50 and 80% saturation. Use of anionic exchangers particularly DEAE ion-exchange chromatography were reported for antimicrobial peptides' purification (Gordillo and Maldonado, 2012). The dialyzate of 30% ammonium sulphate saturated fraction applied onto a DEAE-Sepharose column showed the elution of two fractions (b) and (c) (Fig. 3.2) with the increased gradient of sodium chloride (0 to 0.5 M NaCl). The unbound/washed out fraction (a) was also collected and checked for antifungal activity (Fig. 3.2). All the three eluted fractions failed to show antifungal activity whereas the fraction (c) exhibited antibacterial activity against *Streptococcus pyogenes* MTCC 442.

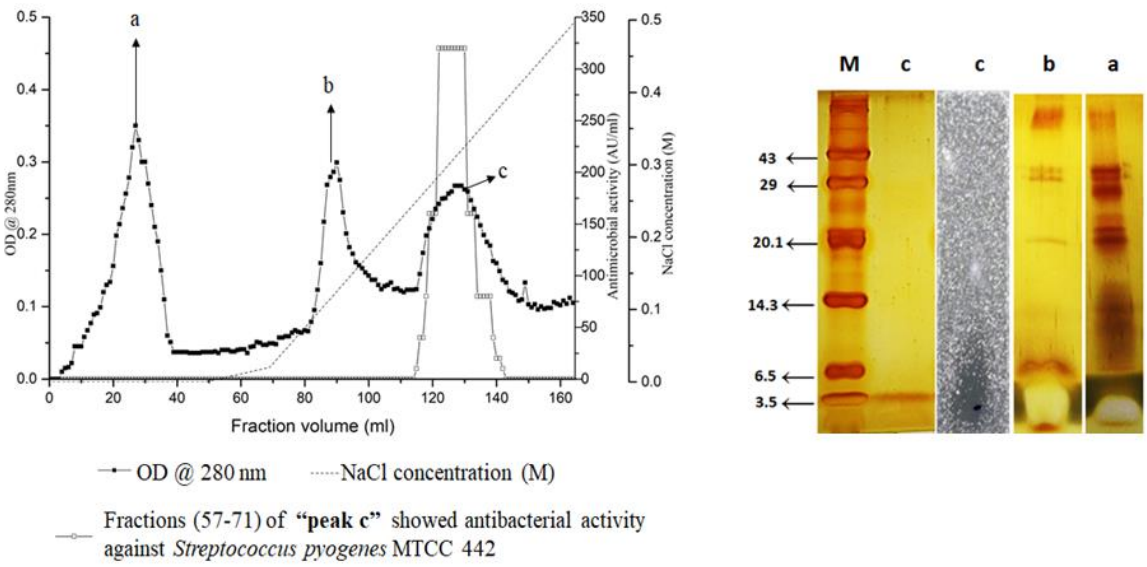


Fig. 3.2: Ion-exchange chromatography elution profile and SDS-PAGE profile of unbound fractions (a) and eluted fractions (b) and (c). Zymogram figure of the fraction c displays the antibacterial activity against *S. pyogenes* MTCC 442.

It is evident from the above observation that the first method of purification steps which included ammonium sulphate fractionation, dialysis and ion-exchange chromatography failed to separate and recover the antifungal compounds. Therefore, a 4-step method involving acid precipitation, solvent extraction, adsorption chromatography and RP-HPLC were carried out to achieve purification to near homogeneity**.**

3.3.2. 2nd method of purification

Maximum recovery of antifungal compound was obtained when n-butanol was used for extraction followed by *tert*-butanol. Recovery was less in methanol and no antifungal compound was extracted when chloroform was used.

3.3.2.1. TLC analysis of solvent extracted solvents

When water was sprayed over the developed plate and heated at 100 ºC, white patches were observed at the R_f values0.45, 0.51, 0.63, 0.72 and 0.86 and soon it disappeared. Then the plate was kept in the closed chamber for iodine vapour exposure. Yellow spots were observed in the same R_f values as observed for water spray except 0.45 R_f value (Fig. 3.3). Once the yellow spot disappeared from the plate, it was transferred to TCA solution. White patches were observed at the spots wherever the peptides were present. When the plate was stained with Serva Blue W dye, dark blue spots appeared in the light background at the R_f values 0.51, 0.45 and 0.39 (Fig. 3.3).

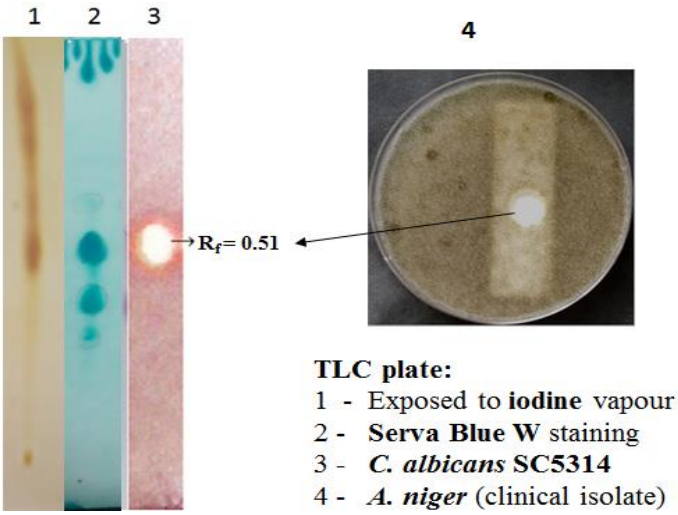


Fig. 3.3: TLC analysis of solvent extracted sample

For bioautography assay, TTC dye was used to check the viability of cells. The colourless dye is reduced to red TPF (1,3,5-triphenylformazan) by various dehydrogenases enzymes involved in the cellular metabolism of the viable cells. TTC dye also helps to differentiate the zone of inhibition spot from the white background of TLC plate (Fig. 3.3). A clear zone of inhibition was observed when the TLC plate was exposed to *C. albicans* SC5314 at the R_f value 0.51 in the red background. Similarly, on exposure to *A. niger* (clinical isolate), distinct zone of clearance was observed at the same R_f value of 0.51 (Fig. 3.3). Yellow and blue spots observed corresponding to the R_f value 0.51 indicate the presence of lipid and peptide moieties of the antifungal compound respectively.

3.3.2.2. Purification of antifungal compound

After acid precipitation, antifungal compound was extracted using n-butanol and was partially purified in silica-based adsorption column chromatography using chloroform and methanol. Antifungal fractions were eluted at the ratio of 60:40 of chloroform and methanol respectively. The eluted fraction showing antifungal activity is shown below in the Fig. 3.4.

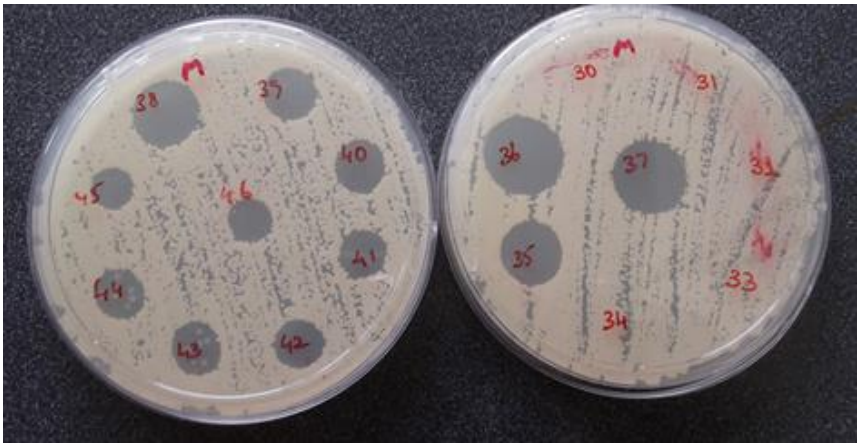


Fig. 3.4: Antifungal activity of the fractions eluted from silica-based adsorptioncolumn chromatography against *C. albicans* SC5314.

The active fractions were pooled and evaporated in the speed vacuum. The dried sample was dissolved in 50% methanol or acetonitrile and further purified by semi-preparative scale RP-HPLC. All the peaks in the chromatogram were collected and tested for fungicidal action against *C. albicans* SC5314, out of which five fractions (AF_1, AF_2, AF_3, AF_4 and AF_5) were found to be bioactive (Fig. 3.5). The retention times of AF_1, AF_2, AF_3, AF_4 and AF_5 were noted as 31.3, 34.2, 39.1, 40.8 and 44.8 min.

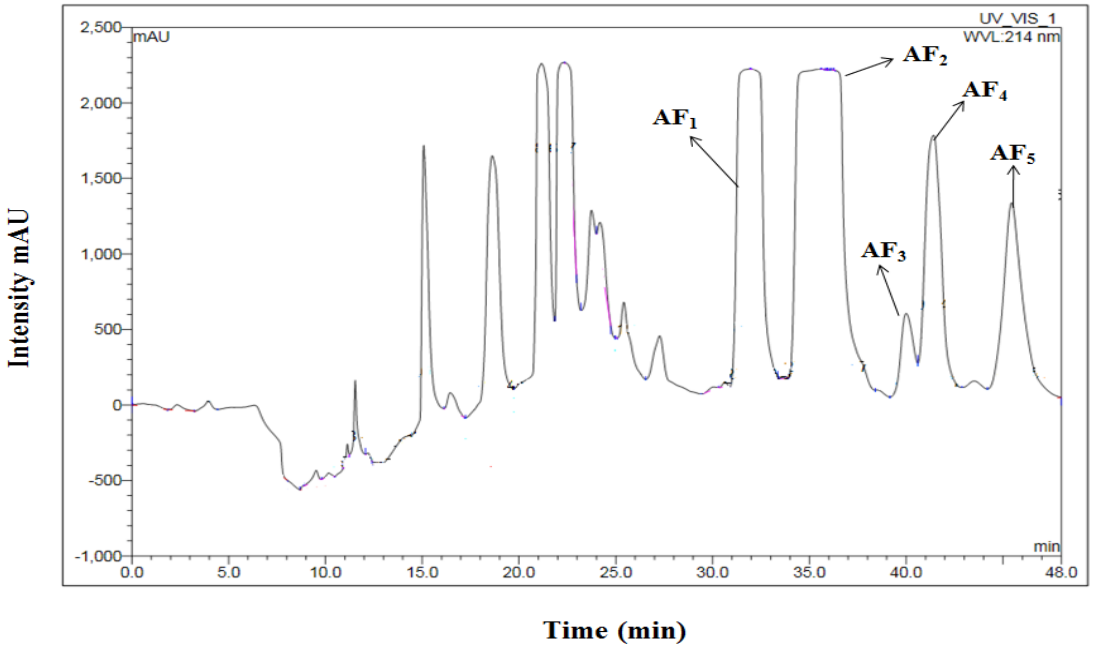


Fig. 3.5: Reversed-phase HPLC chromatogram of the antifungal compounds purified at the semi-preparative scale

3.3.2.3. TLC analysis of HPLC purified fractions

Thin layer chromatography (TLC) of all the five different HPLC fractions were carried out and R_f values of bioactive fractions were confirmed by bioautography assay. The fraction AF_1 showed two spots at the R_f of 0.51 and 0.43, AF_2 at 0.51 and 0.39, AF_3 at 0.53, AF_4 at 0.53 and AF_5 at 0.55. The fractions AF_1, AF_2, AF_3, AF_4 and AF_5 showed white patches on water spraying, yellow spots when exposed to iodine vapour and the blue spots after Serva blue W staining suggesting their lipopeptide nature (Fig.3.6).

3.3.3. Mass analysis of RP-HPLC purified fractions

Further mass spectrometry analysis was performed for all the five fractions. The major peaks of AF_1, AF_2, AF_3, AF_4 and AF_5 were obtained at m/z $[M+H]^+$ 1043.59, 1057.69, 1,071.57, 1,071.68 and 1085.61 respectively in MALDI-TOF-MS (Fig.3.7). There was an exact 14 Da difference among the peaks of AF_1, AF_2, AF_3/AF_4 and AF_5 corresponding to the mass of CH_2, indicating the homologous nature of the compounds. Identical molecular mass (m/z 1,071) of AF_3 and AF_4 describes the isomeric nature of the two lipopeptides. This mass analysis also confirms the lipopeptide nature of the antifungal compounds.

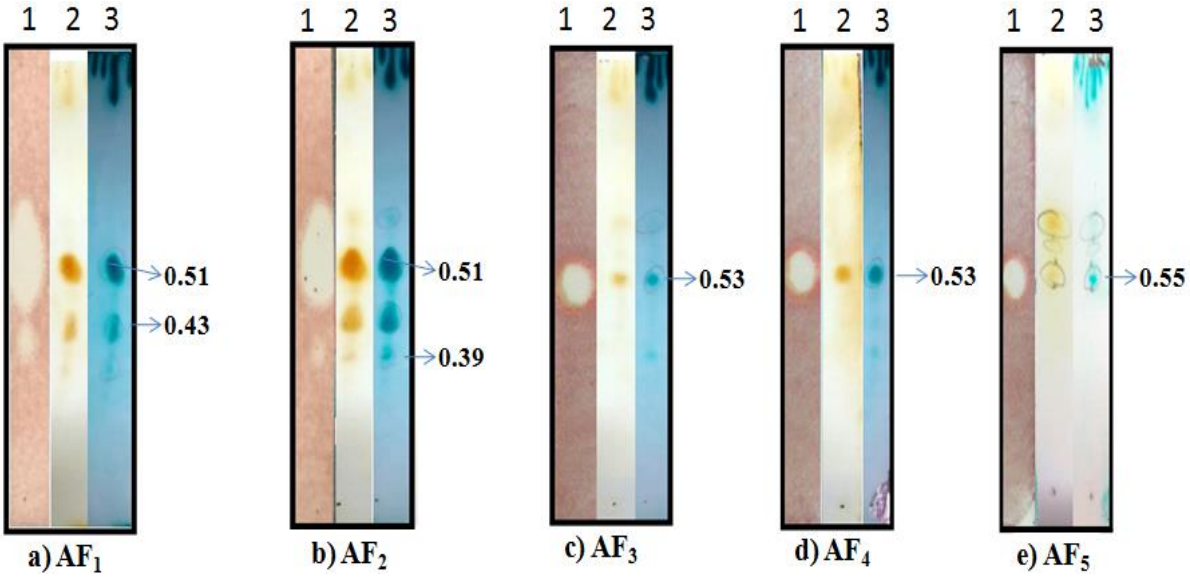


Fig. 3.6: TLC profiles of five different bioactive fractions purified by RP-HPLC showing Lane 1) activity against *C. albicans* SC5314. 2) presence of lipid moiety when exposed to iodine vapour and 3) peptide moiety in Serva blue W staining.

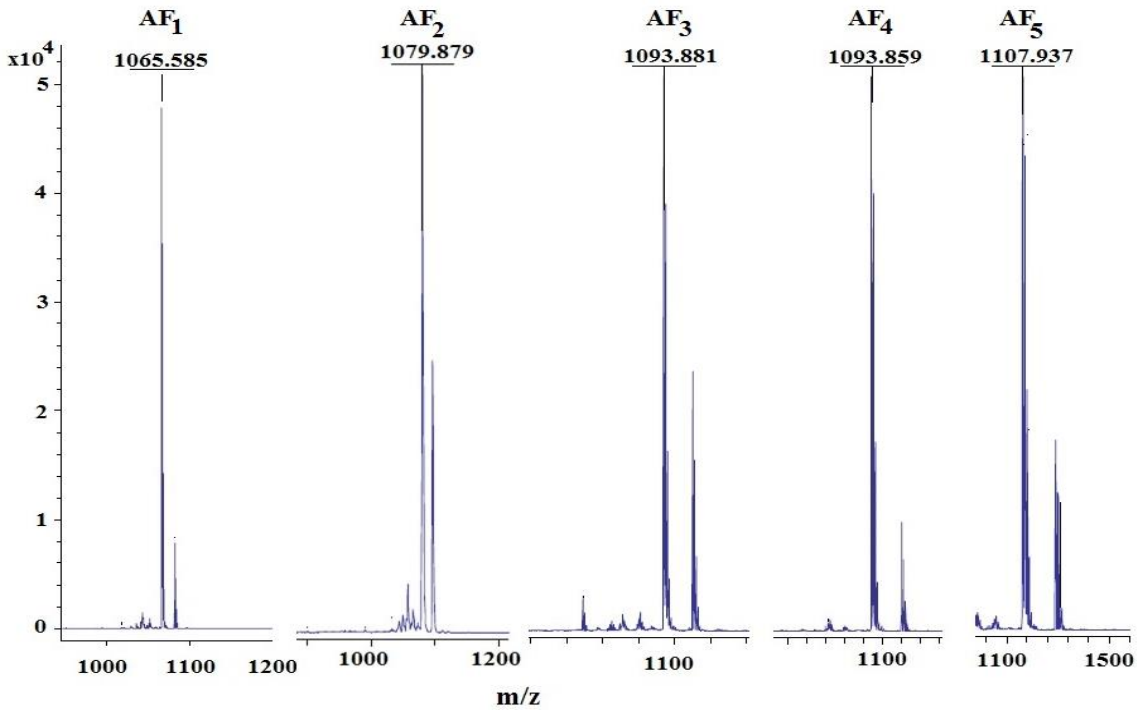


Fig. 3.7: MALDI-TOF MS profile showing m/z ratios of HPLC purified active antifungal fractions

3.3.4. Protein estimation

A standard curve was plotted with the BSA standards of known concentration (µg/mL) by BCA method (Fig. 3.8). Quantification of antifungal HPLC fractions was carried out using the formula y = 0.0014x+0.0095.

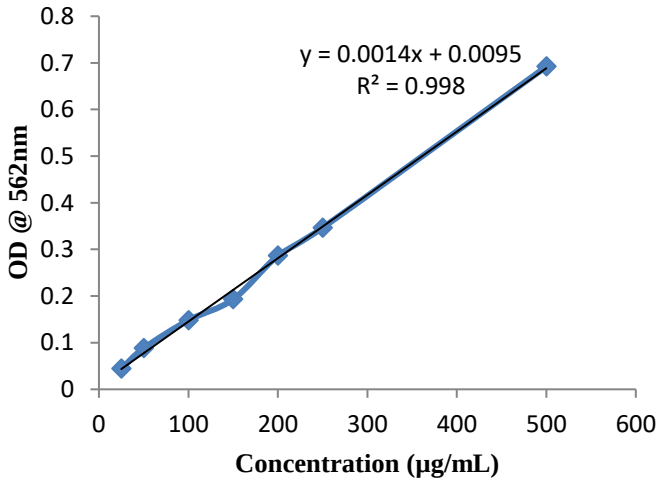


Fig.3.8: BSA standard curve to determine the peptide concentration by BCA method.

3.3.5. Re-chromatography of antifungal fractions

For molecular characterization studies, completely purified antifungal fractions were required. So, the re-chromatography of the selective lipopeptides, AF_3, AF_4 and AF_5 was performed in the analytical scale HPLC (Fig. 3.9). Two different gradients were used: 0-50% for AF_3 and $AF_4$and 0-55% for AF_5 at 0.7 mL/min flowrate for 22 and 25 min respectively. In the first method, AF_3 was eluted at the retention time 18.3 min and AF_4 at 19.2 min. In the second method, AF_5 got eluted at 20.8 min. As the fractions AF_1 and AF_2 exhibited less antifungal potency, no further characterization work was carried out on these two lipopeptide homologues.

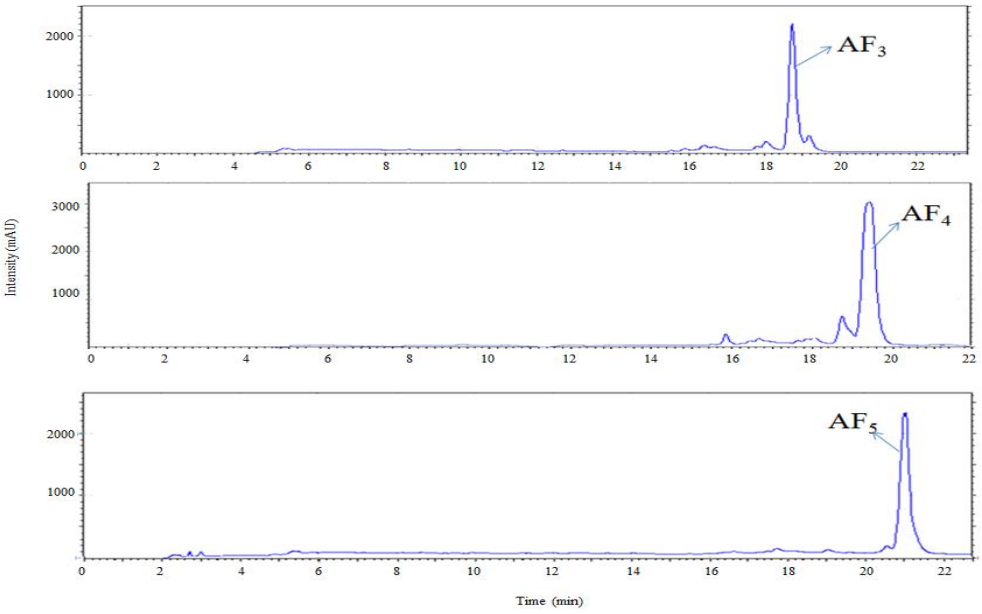


Fig.3.9: Purification (re-chromatography) of individual compounds at an analytical scale RP-HPLC for molecular characterization.

3.3.6. Identification of antifungal fractions

3.3.6.1. MALDI-TOF MS/MS analysis

AF_3 and AF_4 shared similar m/z value with identical product ions (*b* ions and *y* ions) in MALDI-TOF-MS/MS. Series of *b*-type ions at m/z 243.15 (b_2), 406.21 (b_3), 503.261 (b_4), 617.41(b_5) and 1053.5 (b_9) and *y* type ions at m/z 1,071.08 (y_9), 828.8 (y_7), 665.8 (y_6), 568.8 (y_5) and 454.8 (y_4) respectively were obtained in the MS/MS spectra (Fig. 3.11a and 3.11b). As they share similar *b*-type and *y* type ions, the difference in the antifungal nature and the mass of these two isomers were presumably due to the fatty acid

chain. The peptide sequences of the lipopeptide isomers, AF_3 and AF_4 were predicted as Q-N-Y-P-N-X_1-X_2-Xaa_3 and Q-N-Y-P-N-X_1-X_2-Xaa_4 respectively, where Xaa_3 and Xaa_4 correspond to the fatty acid moiety. When analysing the MS/MS spectra of AF_5, series of *b*-type ions at m/z 244.16 (b_2+H_2O), 407.23(b_3+H_2O), 502.25 (b_4-H_2O), 617.28 (b_5-H_2O) and 1067.47.30 (b_9) and *y* type ions at m/z 1,085.48 (y_9), 957.38 (y_8), 843.33 (y_7-NH_3), 680.28 (y_6), 583.22 (y_5) and 469.19 (y_4) were observed (Fig. 3.11c). Assigning *b*-type and *y*-type ions, the amino acid sequence Q-N-Y-P-N-X_1-X_2-Xaa_5 was predicted for AF_5 where Xaa_5 corresponds to the fatty acid. Difference of 14 Da between AF_3/AF_4 and AF_5 denotes the presence of an additional CH_2 group in AF_5. Taken together, the MS/MS analyses indicate that all three lipopeptides contain the identical peptide sequence of Q-N-Y-P-N-X_1-X_2-Xaa (Fig. 3.11) differing in fatty acid chain. Further to confirm the fatty acid variant, GC/MS analysis was performed.

3.3.6.2. GC/MS analysis

Fatty acid identification in GC/MS was achieved by comparing a mass spectrum of AF_3, AF_4 and AF_5 with the reference mass spectra in the NIST 05 library. Mass spectra of AF_3 and AF_4 showing the ion at m/z 298 (M^+) corresponded to 3-amino-15-methyl hexadecanoic acid methyl ester and 3-amino-14-methyl hexadecanoic acidmethyl ester respectively whereas m/z 312 (M^+) in the mass spectrum of AF_5 indicated the presence of 3-amino-16-methyl heptadecanoic acid methyl ester. In conclusion, GC/MS analysis revealed that Xaa_3, Xaa_4 and Xaa_5 represent β-amino fatty acids of *anteiso*-C_{17}, *iso*-C_{17} and *iso*-C_{18} respectively for AF_3, AF_4 and AF_5.

3.3.6.3. ESI-FT-ICR MS/MS analysis of AF_3 and AF_4 isomers

ESI-FT-ICR MS/MS was performed for the purified AF_3 and AF_4 to determine the entire sequence. In the collision induced dissociation (CID) profile of the ion at m/z 1071.58 (Fig. 3.10), connected losses of ions were spotted by the fragments m/z 1054.5 (loss of H_2O), 943.5 (Q), 829.5 (Q-N), 666.4 (Q-N-Y), 569.5 (Q-N-Y-P) and 455.3 (Q-N-Y-P-N). Also, there were two connected losses at m/z 842.48 from the precursor ions which corresponds to Q-T. Similar to the MALDI results, the fragmentation patterns were identical for AF_3 and AF_4. Altogether, the sequences of AF_3 and AF_4 were assigned as T-Q-N-Y-P-N-X_2-Xaa_3/Xaa_4.

The remaining sequence was deduced based on the GC/MS results. Removal of methyl ester (CH_2= 14 Da) from the fatty acids of AF_3 and AF_4 led to the m/z value 284 (298-14) for each isomer. Lactone linkage formed between Ser-fatty acid and fatty acid-Asn led to the loss of 17 Da. Therefore, the molecular masses of AF_3 and AF_4 become m/z value 267 (284-17) for each isomer. Inclusion of the Xaa_3/Xaa_4 of m/z 267 to the predicted sequence leads to the difference of 87 (that corresponds to the m/z of Ser) which proves that the X_2 is Ser in both AF_3 and AF_4. Combining the ESI-FT-ICR MS/MS spectra and GC-MS data, the sequence was predicted as T-S-βAA-N-P-Y-N-Q. Similarly, AF_5 sequence was also deduced using GC/MS results. The difference in the molecular masses between AF_3/AF_4 and AF_5 by 14 Da (because of the CH_2 group) explains the presence of fatty acid chain length of C_{17} and C_{18}respectively. MS/MS spectrum combining the results of GC/MS, MALDI-TOF-MS/MS and ESI-FT-ICR MS/MS are exemplified in Fig. 3.11.

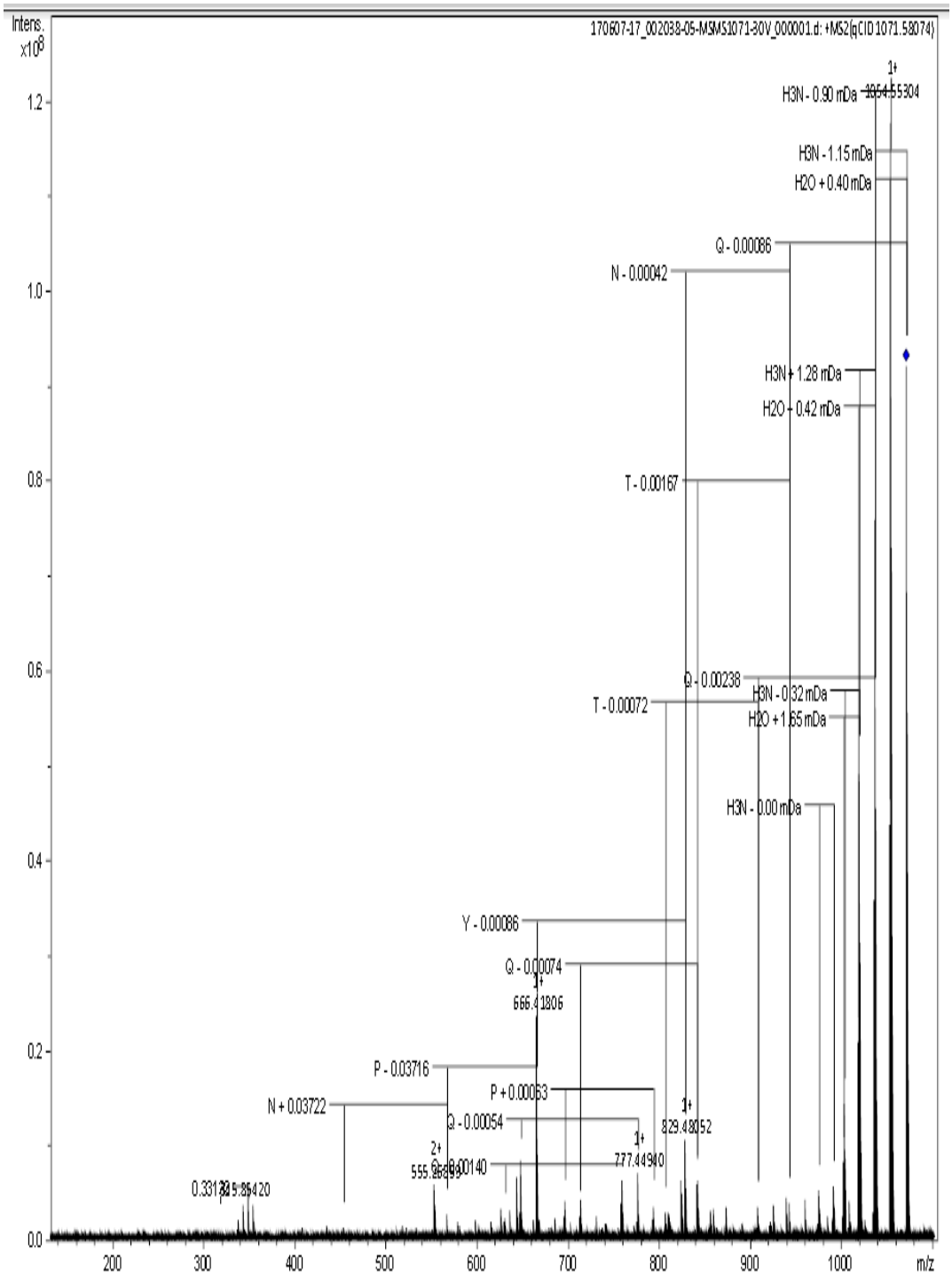
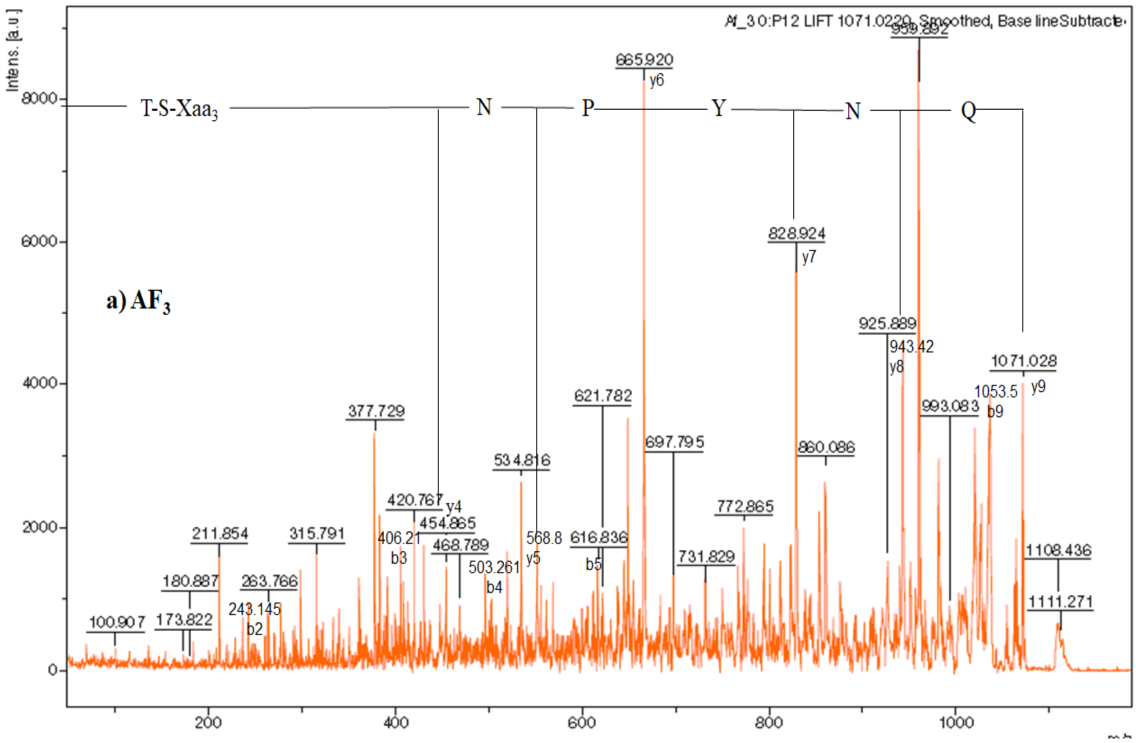


Fig. 3.10: ESI-FT-ICR MS/MS spectra analysis of AF_3 and AF_4 (both showed similar type of CID spectra).


Intens. [a.u.]
AF_30:P12 LIFT 1071.0220, Smoothed, Base lineSubtracte
a) AF3
T-S-Xaa3
N
P
Y
N
Q
665.920
y6
959.892
828.924
y7
925.889
943.42
y8
1071.028
y9
1053.5
b9
993.083
621.782
697.795
860.086
534.816
377.729
420.767
y4
454.865
406.21
b3
468.789
503.261
b4
568.8
y5
616.836
b5
772.865
731.829
211.854
315.791
180.887
263.766
243.145
b2
173.822
100.907
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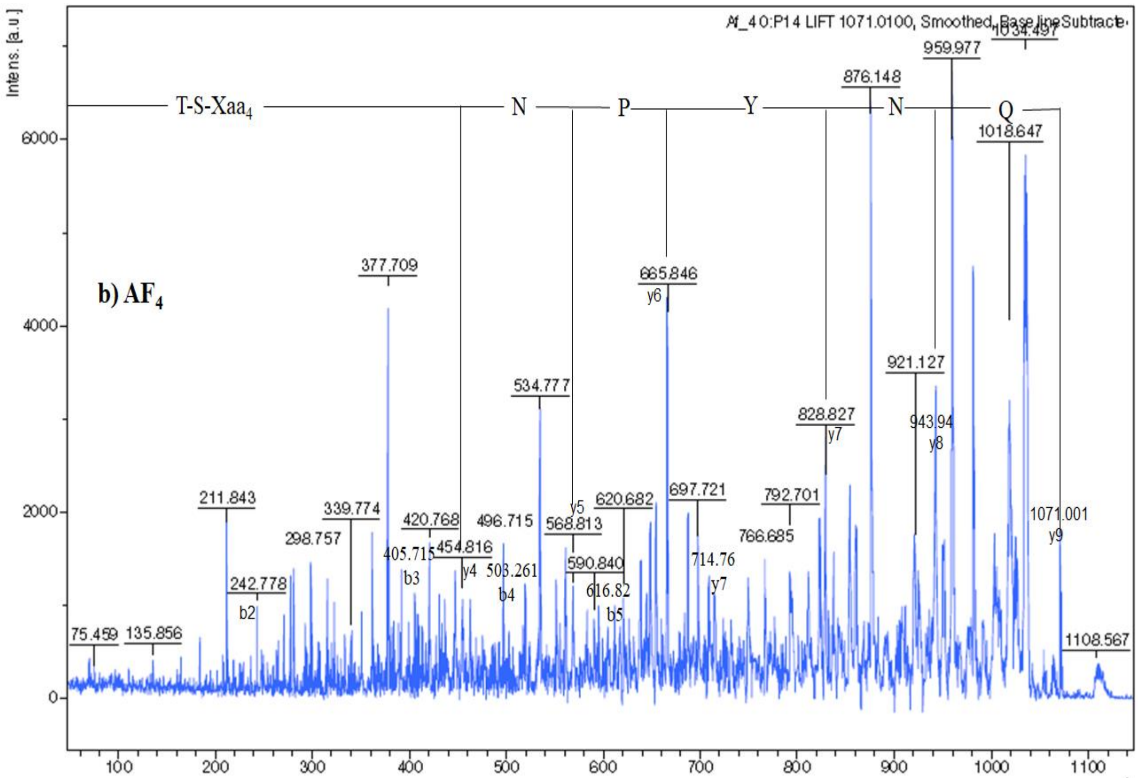
1111.271
8000
6000
4000
2000
0
200
400
600
800
1000


Intens. [a.u.]
AF_40:P14 LIFT 1071.0100, Smoothed, Base lineSubtracte
b) AF4
T-S-Xaa4
N
P
Y
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Q
1034.497
959.977
876.148
1018.647
377.709
665.846
y6
534.777
921.127
828.827
y7
943.94
y8
211.843
339.774
298.757
420.768
496.715
y5
568.813
620.682
697.721
792.701
766.685
1071.001
y9
405.715
b3
454.816
y4
503.261
b4
590.840
616.82
b5
714.76
y7
242.778
b2
75.459
135.856
1108.567
6000
4000
2000
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900
1000
1100

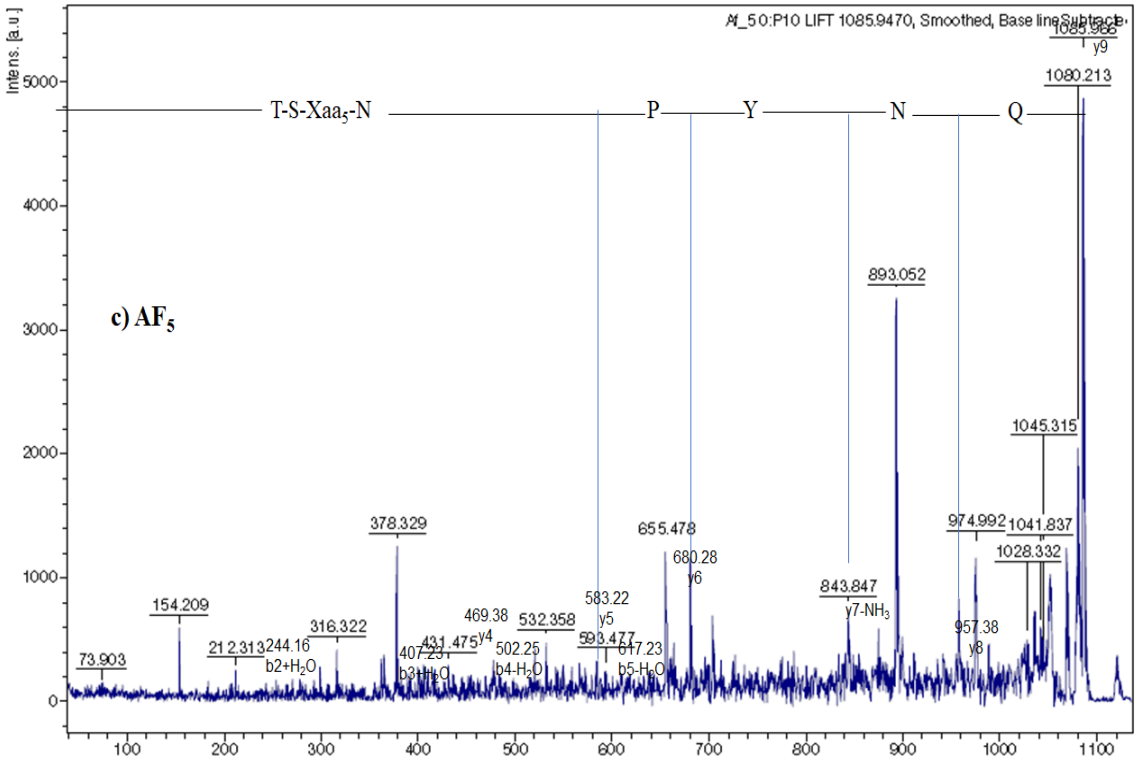

Fig.3.11: MS/MS spectra showing the predicted sequences of a) AF_3, b) AF_4 and c) AF_5. The m/z differences between the product ions were calculated to deduce the compositions combining the results of MALDI-TOF MS/MS, ESI-FT-ICR MS/MS and GC/MS analyses. Xaa_3, Xaa_4 and Xaa_5 represent β-amino fatty acids with *anteiso*-C_{17}, *iso*-C_{17} carbons and *iso*-C_{18} respectively.

3.3.7. Molecular detection of NRPS antibiotic genes

A 100bp ladder was used to detect the size of PCR products and sizes of all the AMPs synthesizing genes were found between 200-500 bp. Genome of the producer strain was found positive for all the tested iturin, fengycin, bacillomycin, bacilysin and surfactin non-ribosomal peptide biosynthetic genes (Fig.3.12). Single clear PCR product of ~380 bp was observed when BmyB primers were used and ~260 and 420 bp bands were observed when FenD and ItuC primers were used, indicating the most likely presence of iturin, bacillomycin and fengycin operons in the chromosome. PCR products amplified using SUR and srfA primers showed 440 bp and 200 bp size bands respectively proving the presence of surfactin operons. RLID 12.1 also showed positive for bacilysin biosynthetic gene with ~500 bp band when specific BacA gene primer was used.

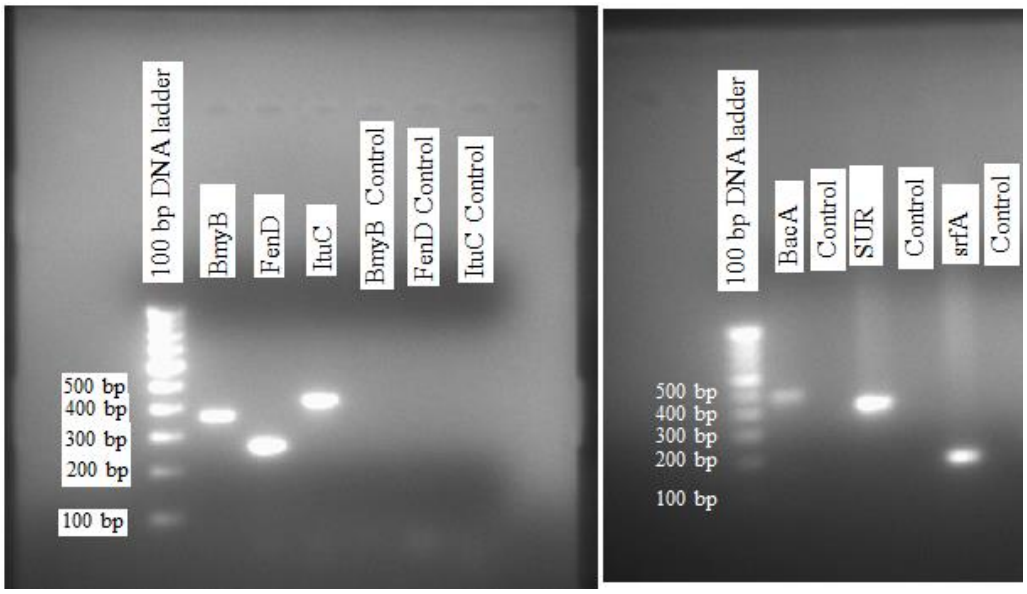

Fig. 3.12: PCR amplification of genes corresponding to antifungal peptides, bacillomycin (bmyB), fengycin (FenD) and iturin (ItuC) and antibacterial peptides, bacilysin (BacA) and surfactin (SUR and srfA) (Ramya et al., 2013)

3.4. DISCUSSION

By applying ammonium sulphate fractionation, dialysis followed by ion-exchange chromatography, the separation and purification of antifungal compounds were difficult to achieve. Therefore, a four-step method entailing acid precipitation, solvent extraction, adsorption chromatography and RP-HPLC were carried out to achieve purification to near homogeneity.

RP-HPLC has been preferred for the separation and purification of lipopeptide homologues (Dhanrajan et al. 2016). Based on the TLC and mass spectral analysis, the RP-HPLC purified antifungal compounds produced by *B. subtilis* RLID 12.1 were confirmed as lipopeptides. Antifungal lipopeptide mixtures(AF) were separated in to five variants AF_1, AF_2, AF_3, AF_4 and AF_5 where AF_3/AF_4 were identified as isomers showing similar m/z value while the rest of the lipopeptides m/z value differed by 14 Da pointing toward the homologous nature of lipopeptides. Lipopeptide AF variants were eluted in the retention time (RT) between 30-48 min with the linear increase of the acetonitrile in RP-HPLC. The equation obtained from linear regression analysis was $y = 3.36x + 27.96$ with the R^2 value 0.985 where y is the retention time and x is the fatty acid carbon number (Fig. 3.5). Linear shift in the retention time of the lipopeptides variants showed

that the difference in RT was mainly due to the addition of CH_2 group in the fatty acid and not by the amino acid sequence.

Non-ribosomally processed amphipathic antimicrobial lipopeptides with condensed beta-amino fatty acids are prevalent (Stein 2005) and these lipopeptides are coproduced in the form of isomers or homologues which differ in amino-acid substitutions or by the length and branching of fatty acid side chains (Vater et al. 2002). Sequences of AF_3, AF_4 and AF_5 were predicted combining the results of the MALDI-TOF-MS, ESI-FT-ICR-MS and GC/MS. Due to high haemolytic nature (Fig.4.1), molecular characterization of AF_1 and AF_2 were not carried out except the molecular mass. Spectral analyses of the antifungal lipopeptides revealed that the three lipopeptides AF_3, AF_4 and AF_5 share the same amino acid sequence with the long fatty acid variant where AF_3 and AF_4 differ by fatty acid branching type and AF_3/AF_4 from AF_5 by fatty acid chain length. The m/z $[M+H]^+$ of AF_1, AF_2, AF_3, AF_4 and AF_5 (Fig.3.7)were found exactly similar to iturin A m/z $[M+H]^+$ 1043.59 (A_2), 1057.69 (A_3, A_4, A_5), 1,071.57 (A_6), 1,071.68 (A_7) and 1085.61 (A_8) (Isogai et al., 1982). But the mass spectrometry analysesof AF lipopeptides revealed different amino acid sequence from iturin A with long fatty acid chain length and the amino acid variation is exemplified below in the Fig. 3.13. Presence of threonine and the fatty acid chain length were the major differences found between iturin A and AF lipopeptides. Indeed, the deduced amino acid sequence of AF lipopeptides was found similar to bacillomycin class of iturin lipopeptides.

a)	*Asn-Tyr-Asn-Gln-Pro-Asn-Ser*-Fatty acid	Iturin A
b)	*Asn-Tyr-Asn-Pro-Gln-Asn-Thr*-Fatty acid	Bacillomycin F
c)	*Asp-Tyr-Asn-Ser-Gln-Ser-Thr*-Fatty acid	Bacillomycin L
d)	*Asn-Tyr-Asn-Ser-Gln-Ser-Thr*-Fatty acid	BacillomycinLc
e)	***Asn-Tyr-Asn-Pro-Gln-Ser-Thr*-Fatty acid**	**Bacillomycin D**
f)	***Asn-Pro-Tyr-Asn-Gln-Thr-Ser*-Fatty acid**	**Predicted AF sequence**

Fig. 3.13: Variation in the amino acid sequences of iturin A, bacillomycin class lipopeptides and AF lipopeptides of *B. subtilis* RLID 12.1.

Four different classes of bacillomycins are bacillomycin D, bacillomycin F, bacillomycin L and bacillomycin Lc (Fig.3.13). These bacillomycins differ by amino acid sequences which presumably changed their antifungal potential (Besson and Michel, 1988; Moyne et al., 2001; Lee et al., 2008; Tabbene et al., 2011). The sequences of

bacillomycin class and the predicted sequence of antifungal lipopeptide of *B. subtilis* RLID 12.1 are shown (Fig. 3.13).

The amino acid sequences of AF_3, AF_4 and AF_5 lipopeptides were found similar to bacillomycin D homologues with several alterations in the amino acid positions (Fig 3.13). Bacillomycin D homologues were reported as cyclic lipopeptides with the sequence N-$N_1Y_2N_3P_4Q_5S_6T_7$-Xaa-C where Xaa is the β-amino fatty acid varying from C_{14} to C_{17} with *n*-, *iso*-, or *anteiso*- terminal structures (Peypoux et al., 1987; Tanaka et al., 2014). Our analyses reveal the predicted sequence of AF_3, AF_4 and AF_5 as N-$N_1P_2Y_3N_4Q_5T_6S_7$-Xaa-C. When compared to the bacillomycin D sequence (Peypoux et al., 1987), two changes were observed in AF_3, AF_4 and AF_5 amino acid sequences: shift of Ser and Thr in the position 6 and 7 and change in the alignment of Y-N-P to P-Y-N in the position 2, 3 and 4. AF differ from bacillomycin F and L/Lc due to the absence of serine and proline residues in the peptide sequence (Peypoux et al., 1984; Peypoux et al., 1985; Besson and Michel, 1988; Lee et al., 2008). Another difference with bacillomycin L is the replacement of asparagine in the position 1 by aspartic acid (Peypoux et al., 1984). The long fatty acid chain of C_{17} and C_{18} were identified in AF_3/ AF_4 and AF_5 lipopeptides respectively compared to other bacillomycin lipopeptides (Tanaka et al., 2014).

Positional variation in the amino acid sequence of bacillomycin D and AF was found similar to iturin A and mycosubtilin. Although iturin A and mycosubtlin share the same amino acid sequence, shift in the position of serine and aspargine improved the antifungal potential of mycosubtilin compared to iturin A (Cochrane SA and Vederas, 2016). Similarly, antifungal activity and haemolytic nature of AF varied from bacillomycin D perhaps due to the difference in the position of amino acid in the peptide sequence which is discussed in the Chapter 4.

Molecular detection studies of genomic DNA using the gene specific primers revealed the presence of five antimicrobial peptide (AMP) operons for iturin, bacillomycin, fengycin, surfactin and bacilysin. Although RLID 12.1 strain showed positive for all the three antifungal peptide genes (ItuC, BmyB and FenD) (Fig. 3.12), purification and identification studies of the active antifungal fractions proved that the genes specific for bacillomycin only has been expressed in the given production condition whereas the genes specific for iturin and fengycin have not been expressed. Combined

role of all non-ribosomal peptide biosynthetic genes might assist *Bacillus* sp. to fit in the competitive natural environments and enable them to maintain the microbial community structure. In addition to the antimicrobial function, surfactin and bacillomycin particularly bacillomycin D exhibit a vital role in biofilm formation (Luo et al., 2015). Finally, the peptide sequence identification results and the PCR-based detection of genes help conclude that the antifungal compounds produced by *B. subtilis* RLID 12.1 belong to the non-ribosomal peptides synthesized by NRPS/PK pathway (Stein 2005; Abriouel et al. 2011, Finking and Marahiel, 2004).

Chapter 4

Functional Characterization of antifungal variants

4.1. INTRODUCTION

Over the last three decades, the incidence of invasive fungal infections is on the rise across the globe. Among the fungi causing invasive fungal infections (IFIs), yeasts including *Candida* spp. and *Cryptococcus* sp. are the common agents (Chakrabarti et al., 2014; Espinel-Ingroff et al., 2012). The management of IFIs is difficult due to the change in epidemiology, less optimum diagnostic capability and the emergence of antifungal resistance (Calandra et al., 2016). In India in a recent study over 27 intensive care units (ICUs) 1400 candidemia cases were reported. Two major issues had emerged from the study; multidrug-resistant *C. auris* has spread across India and 11.8% *Candida* isolates are resistant to azoles. Only 55% isolates were clearly susceptible, rest others are in susceptible dose-dependent range (Chakrabarti et al., 2014). Other than azoles, the rise of echinocandin-resistant *C. glabrata* in many countries has become a matter of worry while managing invasive candidiasis (Pham et al., 2014).

The majority of the existing antifungal agents are fungistatic, toxic and create problems during management due to drug-interaction. Lipopeptides of *Bacillus* sp. such as bacillomycin, iturin A, mojavensin, mycosubtilin, bacillopeptin and fengycin homologues have been reported to have antifungal property (Cochrane and Vederas, 2016). These lipopeptides target the cytoplasmic membrane by forming ion conducting pores in the lipid membrane (Maget-Dana and Peypoux, 1994). However, their strong hemolytic activity at their corresponding MIC values and limited antifungal activity have restricted their clinical applications (Maget-Dana and Peypoux, 1994; Kajimura et al., 1995; Moyne et al., 2001).

In this chapter, the purified antifungal lipopeptides of *B. subtilis* RLID 12.1 were studied for their therapeutic efficiency based on MIC and cytotoxicity studies. Additionally, interactive effects of AF_3, AF_4 and AF_5 homologues/isomers were exemplified with respect to MIC and cytotoxicity.

4.2. EXPERIMENTAL METHODS

4.2.1. Microbial Cultures and Media

The yeasts cultures including *Candida* and *Cryptococcus* species were grown in Sabouraud Dextrose agar (SDA) media at 37 °C. All the cultures were maintained as glycerol stocks (20%) at −80 °C. Indicator organisms used in this study were procured

from National Culture Collection of Pathogenic Fungi (NCCPF)-Post Graduate Institute of Education and Medical Research (PGIMER), Chandigarh, Microbial Type Culture Collection (MTCC), Chandigarh and National Collection of Industrial Microorganisms (NCIM), Pune, India.

4.2.2. Media preparation

RPMI 1640 with L-glutamine and phenol red indicator and without bicarbonate was selected for susceptibility studies. RPMI 1640 media was dissolved in MOPS buffer ((3-(N-morpholino) prophane sulfonic acid) of 0.165 mol/L concentration and pH of the media was adjusted to pH 7.0 using sodium hydroxide. Media becomes reddish pink once it reaches pH 7.0. Then it was filtered in the sterile condition using 0.22 µm membrane filter and stored at 4 °C in the sterile bottles. *Cryptococcus* sp., requires high glucose content (of 2%) for its growth. In general, RPMI 1640 contains 0.2% of dextrose so additionally 1.8 % of dextrose was added, filtered and stored separately.

4.2.3. Minimum inhibitory concentration of antifungal lipopeptides against yeasts

4.2.3.1. Inoculum preparation

Inoculum was prepared as per the recommendations of CLSI (M27-A3). The test organisms were grown in SDA media at 37 °C for 24-48 h. 24 h old inoculum was used in case of *Candida* sp. and 48 h old culture for *Cryptococcus* sp. One or two colonies of test strains were suspended in 1 mL of sterile saline solution (0.9%) and mixed gently. The cell density in the suspension was adjusted to an absorbance of 0.08 - 0.1 at OD_{530} for *Candida* sp. and 0.257 for *Cryptococcus* sp. in the sterile plastic cuvettes. This absorbance adjustment method in the spectrophotometer is equivalent to the turbidity of 0.5 McFarland standard. This yields a yeast stock suspension of $1x10^6$ to $5x10^6$ cells/mL. Stock suspension was first diluted in 1:20 ratio and then in 1:50 ratio using the RPMI 1640 media to obtain a working suspension of 1×10^3 to 5×10^3 cells/mL which is two times the test inoculum.

4.2.3.2. Preparation of antifungal lipopeptide fractions

Concentrations of the RP-HPLC purified antifungal lipopeptides, AF_1, AF_2, AF_3, AF_4 and AF_5 were estimated by BCA assay using the BSA standard curve (Fig.3.8). Final concentration ranging from 0.5-32 µg/mL was selected in case of AF_3, AF_4 and AF_5

whereas the two concentration ranges, 2.5-40 µg/mL and 0.5-32 µg/mL were chosen for AF_1 and AF_2. First, 100 µL of RPMI 1640 media was dispensed in to wells. Then 100 µL of lipopeptide fractions at 4x concentration were added to the 1st well and two-fold dilutions were carried out with the media forming 2x concentration of lipopeptide in each well. For example, 4x concentration (128 µg/mL) of AF_3 was diluted two-fold in the media to achieve the 2x concentration ranging from 64 to 1 µg/mL. Similarly, 4x concentration (160 µg/mL) of AF_1 was diluted to the 2x concentration ranging from 80 to 5 µg/mL.

4.2.3.3. Preparation of standard antifungal agents

As the assay units of the antimicrobial agents vary from the actual weight of the powder, standardization of antimicrobial solution is preferred in all the laboratories. Formula given below was used to determine the powder amount required for the preparation of antimicrobial solutions with the potency details given by the manufacturers.

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

Antifungal stock solutions of amphotericin B (1600 µg/mL) and fluconazole (6400 µg/mL) were prepared 100 times more than the working concentration in dimethyl sulphoxide (DMSO), aliquoted and stored at -20 or -60 °C. Aliquots removed from the storage conditions were used immediately after thawing. Stock solutions of amphotericin B (1600 µg/mL) and fluconazole (6400 µg/mL) were diluted two-fold in RPMI 1640 media to get a 2x concentration ranging from 32-0.06 µg/mL and 128-0.250 µg/mL respectively (100x to 2x) and dispensed in to microplate wells (CLSI guidelines, M27-A3).

4.2.3.4. Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution protocol as per the CLSI recommendations (M27-A3) against 81 yeasts strains including *Candida* spp. (no. 64) and *Cryptococcus* spp. (no. 17) (Table 4.1). Amphotericin B and fluconazole as antifungals were used as positive controls for assessing the antifungal susceptibility of all the strains tested. 100 µL of diluted cell suspension of 1 x 10^3 to 5 x 10^3 cells/mL were added to the 100 µL of 2x concentration of

antifungal compound, leading to a final concentration (1x) of suspension of 0.5 x 10^3 to 2.5 x 10^3 cells/mL. Antifungal concentrations reached a final concentration (1x) of 32 to 0.5, 40 to 2.5, 16 to 0.03 and 64 to 0.125µg/mL for AF_3 to AF_5, AF_1 and AF_2, amphotericin B and fluconazole respectively. The plates were incubated at 37 °C for 24-48 h for *Candida* spp. and 48-72 h for *Cryptococcus* spp. MIC_{50} and MIC_{90} values were calculated as the concentrations of fluconazole and amphotericin B that could inhibit 50 and 90% of the isolates, respectively. Geometric mean MICs (MIC_g**)** were used to facilitate the comparisons of the antifungal activities of HPLC purified active compounds. The results were processed statistically using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (Graph Pad Prism).

4.2.3.5.Determination of minimum fungicidal concentration (MFC)

MFC is the lowest concentration of the antifungal compound which kills 99% of yeasts or fungus. After 48 h and 72 h of lipopeptides treatment, 10 µL from the test wells of *Candida* spp. and *Cryptococcus* sp. were withdrawn, streaked on the SDA plates and incubated at 37±0.5 °C for 24-48 h for *Candida* spp. and 48-72 h for *Cryptococcus* sp. The concentration showing no growth or colony formation in the plate was considered as MFC of the respective antifungal compound.

4.2.4. Hemolytic assay

Hemolytic activity of the purified compounds on human erythrocytes was determined as reported previously (Jiang et al., 2014). Two mL of blood was collected in heparinized vial and centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet was washed withphosphate buffered saline (PBS) (5 times the blood volume) for at least 3 times. After the 3rd wash, 10% suspension was prepared and stored at 4 °C. Suspension (10%) was diluted to 5% by the addition of 2X concentrated antifungal compounds. Eventually, 5% v/v of a suspension of fresh human erythrocytes was incubated with each antifungal compound at a final concentration ranging from 0.5 to 32 µg/mL for 1 h at 37 ºC with gentle mixing. Then the tubes were centrifuged at 3000 rpm for 10 min at room temperature and the absorbance of the supernatants were measured at 450 nm in the microplate reader (Multiskan, Thermoscientific). 1% Triton X-100 and PBS were used as positive and negative controls respectively. Percentage of hemolysis was calculated using the formula,

$$\frac{A450\ of\ lipopeptide\ treatment - A450\ of PBS\ treatment}{A450\ of\ TritonX\ treatment - A450\ of PBS\ treatment} * 100$$

4.2.5. Time killing kinetics assay

Inoculum of $1x10^6$ to $5x10^6$ cells/mL suspension was prepared equal to 0.5 McFarland standard (turbidity) and diluted 10-fold to achieve the cell suspension of $1x10^5$ to $5x10^5$ cells/mL. Killing kinetics of the AF_3, AF_4 and AF_5 on *C. albicans* ATCC 24433 were determined by treating the lipopeptides at 2x MIC concentration with 10^5 cells/mL in RPMI medium at 37±0.5 °C (final total volume was 300 µL). Cells without the lipopeptide addition were used as a control. Aliquots of 10 µL was withdrawn at 4h interval period, serially diluted and spread on the Sabouraud dextrose agar (SDA) plates. Plates were incubated at 37 °C and the number of colony forming unit (CFU/mL) was determined after 48h (Lum et al., 2015). Results were obtained from three independent experiments.

The colony counts showing the microbial population obtained before and after lipopeptide treatment and from the control (without lipopeptide) were converted in to log_{10} scale. The log reduction (LR) was calculated using the formula

$$LR = Mean\ log_{10}\ (initial\ microbial\ population) - Mean\ log_{10}\ (surviving\ microbial\ population)$$

Percentage reduction (%) was calculated using the formula

$$Percent\ reduction\ (\%) = 100 * (1 - 10^{-LR})$$

4.2.6. Inhibition of biofilm formation

4.2.6.1. XTT preparation

The tetrazolium dye XTT [2,3-bis (2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] was prepared at 1 mg/mL concentration in sterile phosphate buffered saline (PBS) and menadione solution of 0.4 mM concentration was prepared in acetone. Both solutions are filter sterilized using 0.22 µm membrane filters, aliquoted and stored at −80 °C (Pierce et al., 2010). Prior to each XTT reduction assay, XTT and menadione were mixed in the ratio of 20:1. For example, 200 µL of XTT-menadione mixture was prepared from 158 µL of PBS, 40 µL of XTT and 2 µL of menadione solution (Lum et al., 2015).

4.2.6.2. Selection of biofilm forming yeasts

100 µL of 10^6 yeast suspension was dispensed into each well of a microtitre plate and the plates were incubated at 37±0.5 °C for 8-10 h. After cell adhesion, media with the unadhered cells were removed and each well was washed twice with 150-200µL of PBS. Then, fresh RPMI 1640 media was added to each well and incubated at 37°C for 48 h. Following the biofilm phase (after 48 h), medium was aspirated and each well was washed twice gently with 200 µL PBS to remove non-adherent cells.200 µL of XTT-menadione mixture was added to the plate and was incubated in the dark for 2 h. 100 µL of the solution was transferred to new wells and the absorbance was measured at 490 nm wavelength using microtiter plate reader (Rautela et al., 2014).

4.2.6.3. Effect of lipopeptide on the biofilm formation

Seven species of *Candida* were selected for biofilm inhibition studies. AF_3, AF_4 and AF_5 of 1xMIC, 2xMIC and 4xMIC concentration were prepared in RPMI 1640 medium and 100 µL of sample was transferred to pre-adhered cells (Rautela et al., 2014; Tabbene et al., 2016). The plates were incubated at 37±0.5 °C for 48 h following which the medium was aspirated and washed twice with PBS. Biofilm without any lipopeptide treatment was taken as a positive control. After treatment with lipopeptides, each well was washed with PBS as mentioned above. Each experiment was repeated three times in duplicate. XTT reduction assay was used to quantify the biofilm formation. Percentage of inhibition/suppression of biofilm formation was determined using the formula

$$Inhibition\ \%\ of\ biofilm\ formation = \frac{A490\ of\ Control}{A490\ of\ lipopeptide\ treatment} * 100$$

The antifungal concentration which inhibits 50% of the biofilm formation was marked as $SMIC_{50}$ (sessile MIC).

4.2.7. *In vitro* cytotoxicity assay

Cultured cell lines namely Human Embryonic Kidney (HEK 293), Human immortalized keratinocyte cell line (HaCaT), Human cervical cancer cell line (HeLa) and Human type II alveolar epithelial (A549 cell line) were cultured in a complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum) in a humidified atmosphere of 5% CO_2 at 37±0.5 °C. Cells were seeded in 96-well

microplates at 0.1mL/well at a density of 1.5 x 10^4 cells/well. After 24 h of incubation, media was removed without disturbing the cells and the fresh media containing serially diluted peptide (final concentrations in the range of 0.5-32 µg/mL) were added and incubated for another 24 h at 37±0.5 ºC. Cell viability was determined by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Hi-Media). After 24 h of lipopeptide treatment, MTT (final concentration, 0.5 mg/mL) solution was added to each well and incubated for 3-4h in the dark condition. Oxidoreductase enzymes present in the viable cells reducethe MTT to insoluble formazan crystals. Formazan crystal formation was proportional to the number of viable or metabolically active cells. 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The plates were read on a microplate reader at the wavelength of 550 nm to check the cell viability (Thasana et al., 2010). Cells without lipopeptide treatment were used as the control.

$$Viability\ \% = \frac{A550\ of\ lipopeptide\ treated\ cells}{A550\ of\ Control} * 100$$

4.2.8. Interaction effect of three lipopeptides

4.2.8.1. MIC determination for lipopeptide interaction studies

To study the interaction effects among the three lipopeptide homologues/isomers, three *Candida* sp., *C. albicans* ATCC 24433, *C. parapsilosis* NCCPF 450033 and *C. krusei* NCCPF 440022 were selected based on their sensitivity (MIC and $SMIC_{50}$ values) to AF_3, AF_4 and AF_5. For interaction study, final inoculum size was raised to 1x10^5 to 5x10^5 CFU/mL instead of 0.5x$10^3$to 2.5x10^3 CFU/mL. Inoculum was prepared using 0.5 McFarland standard and serially diluted 10-fold to achieve the cell suspension of 1x10^5 to 5x10^5. MICs studies of AF_3, AF_4 and AF_5 were determined as per the CLSI recommendations (M27-A3) using RPMI 1640 medium with the final concentration ranging from 0.5-32 µg/mL.

4.2.8.2. Interaction study using checkerboard method

Purified peptide-peptide interactions were assessed for AF_3, AF_4 and AF_5 at a final concentration of 0.5-32 µg/mL (AF_3), 0.5-16 µg/mL (AF_4) and 0.5-32 µg/mL (AF_5) for planktonic cells in sterile 96-well polystyrene microplates using a checkerboard method. First antifungal lipopeptide of 50 µL (two-fold diluted) was dispensed into to the well along the ordinate while 50 µL of the second antifungal

lipopeptide (two-fold diluted) was added in the abscissa (Moody, 1992). 100 µL of suspension comprising $1x10^5$ to $5x10^5$ cells/mL were added to each well. Untreated cells were considered as a control. Also, the individual MIC concentrations were checked for each tested culture. Inhibition (50% and 80%) was quantified using XTT. The fractional inhibitory concentration (FIC), defined as summation of MIC of the drug used in combination divided by the MIC of drug tested alone. The effects of the peptide-peptide combinations were expressed in term of FIC index (FICI). The FICI ≤ 0.5 was interpreted as synergistic interaction; while FICI > 0.5–4 was an indication of additive interaction; and FICI> 4 was interpreted as antagonism.

$$FICI = \frac{MIC\ of\ drug\ A\ in\ combination}{MIC\ of\ drug\ A\ alone} + \frac{MIC\ of\ drug\ B\ in\ combination}{MIC\ of\ drug\ B\ alone}$$

4.2.8.3. Interactive effect of lipopeptides in preventing the biofilm formation

Interactive effect of the three lipopeptides were tested for the inhibition of biofilm formation of three *Candida* strains, *C. albicans* ATCC 24433, *C. parapsilosis* NCCPF 450033 and *C. krusei* NCCPF 440022. An inoculum equal to 0.5 McFarland standard containing $1x10^6$ to $5x10^6$ cells/mL was prepared and 100 µL were transferred to the wells. Cells were allowed to adhere on the surface of the wells of microtitre plates for 8-10 h at 37±0.5 °C. Media containing unadhered cells were aspirated and washed twice with PBS (150-200µL). The lipopeptides, AF_3, AF_4 and AF_5 ranging from 0.5-32 µg/mL (final concentration) were added along the ordinate and abscissa by checkerboard method. The plates were incubated at 37 °C for 48 h. After 48 h, spent media was removed and the wells were washed twice with 200 µL of PBS. XTT reduction assay was carried out to quantify the biofilm formation.

4.2.8.4. Hemolytic activity of the screened lipopeptide combinations

Combinations showing potent antifungal action were tested for its haemolytic activity and *in vitro* cytotoxicity assays. Combinations containing different concentrations of (AF_3, AF_4), (AF_3, AF_5) and (AF_4, AF_5) were prepared in the wells and 5% human erythrocytes (final concentration) were added to each well. Then the plates were incubated at 37 °C for 1 h. After incubation, plate was centrifuged and the supernatant was transferred to the new wells and the absorbance at 450 nm was recorded. Percentage of hemolysis was calculated using the formula mentioned above.

4.2.8.5. *In vitro* cytotoxicity studies of the screened lipopeptide combinations

Cytotoxicity assay was performed for the antifungal combinations obtained from checkerboard method against the human cell lines, HaCaT, HEK, A549 and HeLa. Cells at a density of 1.5 x 10^4 cells/well were seeded and allowed to grow for 24 h. Once the well reaches 70% confluent, media was aspirated and media with different combinations of lipopeptides were added. The plate was incubated for 24 h and MTT reduction assay was carried out to check the cell viability. Viability % was calculated using the formula mentioned above.

4.3. RESULTS

4.3.1. Determination of MIC and MFC

MIC and MFC values of the purified compounds were evaluated against 81 yeast strains, which included standard and clinical isolates of *Candida* spp. (no. 64) and *Cryptococcus* spp. (no. 17) (Table 4.1 and 4.2). Amphotericin B and fluconazole were also tested simultaneously. The MIC_{90} value of Amphotericin B ranged from 0.06 – 1.0 µg/mL (Table 4.1). For fluconazole, MIC_{50} value ranged from 0.25- 4 µg/mL; two clinical isolates of *C. albicans,* four isolates of *C. krusei* and three isolates of *C. parapsilosis* had shown higher MIC_{50} range of about 8-64 µg/mL (Table 4.1). MIC ranges of RP-HPLC purified fractions for the test compounds AF_1, AF_2, AF_3, AF_4 and AF_5 against *Candida* spp. were 8-20 µg/mL, 8-20 µg/mL, 4-16 µg/mL, 2-4 µg/mL (except one *C. parapsilosis* 16 µg/mL) and 2-16 (except one *C. parapsilosis* 32 µg/mL) respectively whereas for the same fractions MIC range recorded against *Cryptococcus* sp. were 8-16 µg/mL, 8-10 µg/mL, 2-8 µg/mL, 1-4 µg/mL (except one *C. neoformans var grubii* 8 µg/mL) and 2-4 µg/mL (except one strain of *C. neoformans var grubii and C. neoformans var gattii* 8 µg/mL) respectively (Table 4.1).

MIC and MFC values of all the compounds were found to be same for the majority of the organisms tested (Table 4.1 and 4.2). Comparisons of MICs of five different compounds were statistically significant ($P < 0.05$). No trailing endpoints were encountered for any of the five lipopeptides. MIC_g was calculated for the *Candida* and *Cryptococcus* spp. where the number of isolates are ≥10.

Table 4.1: MIC and MFC studies of HPLC purified antifungal fractions along with Amphotericin B and Fluconazole (ND-not determined)

				AF_1		AF_2		AF_3		AF_4		AF_5		Amphotericin B	Fluconazole
S.No	**Organisms**	**Source**	**Strain no.**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	100%	50%
1	*C. albicans*	NCCPF	400051	20	20	10	10	16	16	4	16	16	16	1	8
2		NCCPF	400034	20	20	10	10	8	8	4	4	4	4	0.25	1
3		NCCPF	400035	20	20	10	10	8	8	4	4	4	4	0.125	1
4		NCCPF	400036	20	20	10	10	8	8	4	4	4	4	0.25	1
5		NCCPF	400053	20	20	10	10	8	8	4	4	4	4	0.25	1
6		ATCC	90028	20	20	10	10	8	8	4	4	4	4	0.5	0.5
7		ATCC	24433	20	20	10	10	8	8	4	4	4	4	0.5	2
8		ATCC	MYA-2876	20	20	10	10	8	8	2	2	4	4	0.5	1
9		ATCC	10231	20	20	10	10	8	8	4	4	8	8	0.25	0.25
10		ATCC	96901	20	20	20	20	4	4	2	2	2	2	0.06	>64
11		MTCC	1637	10	10	10	10	4	4	2	2	2	2	0.125	0.25
12	*C. tropicalis*	NCCPF	B-28	20	20	20	20	8	8	4	4	4	4	0.25	0.5
13		NCCPF	11148	20	20	20	20	8	8	4	4	4	4	0.25	1
14		NCCPF	273324	20	20	20	20	8	8	2	2	8	8	0.5	2
15		NCCPF	420177	20	20	20	20	8	8	4	4	4	4	0.5	0.5
16		NCCPF	420179	20	20	20	20	8	8	4	4	8	8	0.25	0.5
17		NCCPF	10679	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
18		NCCPF	9652	ND	ND	ND	ND	8	8	4	4	4	4	ND	ND
19		NCCPF	702	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
20		NCCPF	8387	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
21		NCCPF	10315	ND	ND	ND	ND	4	4	4	4	4	4	ND	ND
22		ATCC	13803	20	20	20	20	4	4	2	2	2	2	0.25	0.5
23		ATCC	20336	20	20	20	20	8	8	2	2	4	4	0.5	2
24		ATCC	750	20	20	20	20	8	8	4	4	4	4	0.25	2
25	*C. krusei*	NCCPF	440022	20	20	20	20	16	16	4	4	8	8	0.5	16
26		NCCPF	440029	20	20	20	20	16	16	4	4	8	16	0.5	16

27		NCCPF	440030	20	20	20	20	8	8	4	4	4	4	0.5	16
28		ATCC	6258	20	20	20	20	8	8	4	4	4	8	0.5	8
29	*C. glabrata*	NCCPF	100026	10	10	10	10	8	16	4	4	4	4	0.5	2
30		NCCPF	100028	10	10	10	10	8	8	4	4	8	8	0.5	4
31		NCCPF	100029	10	10	10	10	8	8	2	2	8	8	0.06	4
32		NCCPF	IL 2029	ND	ND	ND	ND	4	4	4	4	4	4	ND	ND
33		NCCPF	ILK746B	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
34		NCCPF	IL 2355	ND	ND	ND	ND	4	4	4	4	8	8	ND	ND
35		NCCPF	2007	ND	ND	ND	ND	8	8	2	4	8	8	ND	ND
36		NCCPF	2580	ND	ND	ND	ND	8	8	4	4	4	4	ND	ND
37		ATCC	2001	10	10	10	10	8	8	4	4	4	4	1	4
38		ATCC	90030	10	10	10	10	8	8	2	2	8	8	0.5	4
39		MTCC	3814	10	10	10	10	8	8	4	4	4	4	0.5	2
40	*C. haemulonii*	NCCPF	470012	8	8	8	8	8	8	4	4	4	4	0.25	2
41		NCCPF	470018	8	8	8	8	8	8	4	4	4	4	0.5	2
42		NCCPF	470026	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
43		NCCPF	47001	ND	ND	ND	ND	8	8	4	4	16	32	ND	ND
44		NCCPF	470015	8	8	8	8	8	8	4	4	4	4	0.5	4
45		ATCC	22991	8	8	8	8	8	8	4	4	4	4	0.25	1
46	*C. parapsilosis*	NCCPF	450032	20	20	20	20	16	16	4	8	8	16	0.125	16
47		NCCPF	450033	10	10	10	10	16	16	8	8	8	16	0.125	16
48		NCCPF	450034	20	20	20	20	8	16	4	4	4	8	0.125	16
49		NCCPF	IL2256	ND	ND	ND	ND	16	16	16	16	32	32	ND	ND
50		NCCPF	IL2286	ND	ND	ND	ND	16	16	8	8	16	16	ND	ND
51		NCCPF	398500	ND	ND	ND	ND	16	16	8	8	8	8	ND	ND
52		ATCC	22019	10	10	10	10	8	8	4	4	8	8	0.25	1
53		ATCC	90018	ND	ND	ND	ND	8	8	8	8	16	16	ND	ND
54	*C. auris*	NCCPF	470067	10	10	10	10	8	16	2	2	4	4	1	8
55		NCCPF	470068	20	20	20	20	8	16	4	4	4	4	0.125	1
56		NCCPF	470069	20	20	10	10	8	8	2	2	4	4	1	2

57		NCCPF	ILK2386	ND	ND	ND		16	16	4	4	8	8	ND	ND
58		NCCPF	IL23318	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
59		NCCPF	470142	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
60		NCCPF	470000	ND	ND	ND	ND	8	16	4	4	8	8	ND	ND
61		NCCPF	1002	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
62		NCCPF	470994	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
63		NCCPF	470140	ND	ND	ND	ND	8	8	4	4	8	8	ND	ND
64	*C. viswanathii*	MTCC	1629	10	10	10	10	4	4	2	2	2	2	0.25	4
65	*C. neoformans var grubii*	NCCPF	250512	8	8	8	8	8	8	4	4	4	4	0.125	2
66		NCCPF	25.52	ND	ND	ND	ND	4	4	2	2	2	2	ND	ND
67		NCCPF	IL 2540	ND	ND	ND	ND	4	4	2	2	2	2	ND	ND
68		NCCPF	25.128	ND	ND	ND	ND	8	8	4	4	2	2	ND	ND
69		NCCPF	25.339	ND	ND	ND	ND	8	8	2	2	2	2	ND	ND
70		NCCPF	25.338	ND	ND	ND	ND	8	8	8	8	8	8	ND	ND
71		NCCPF	250509	20	20	10	10	8	8	2	2	2	2	0.25	4
72		NCCPF	250508	20	20	10	10	4	4	2	2	2	4	0.25	2
73		ATCC	34664	8	8	8	8	2	2	1	1	2	2	0.25	2
74		ATCC	32045	8	8	8	8	2	2	1	1	2	2	0.25	1
75		NCIM	3378	8	8	8	8	4	4	2	2	4	4	0.25	1
76	*C. neoformans var gattii*	NCCPF	250488	16	16	8	8	4	4	2	2	2	4	0.5	2
77		NCCPF	W-71	ND	ND	ND	ND	8	8	4	4	4	4	ND	ND
78		NCCPF	1.2496	ND	ND	ND	ND	8	8	4	8	4	8	ND	ND
79		NCCPF	517	ND	ND	ND	ND	8	8	2	4	2	4	ND	ND
80		NCCPF	250528	16	16	8	8	4	4	2	2	4	4	0.25	0.5
81		NCCPF	250190	10	10	10	10	8	8	4	4	8	8	0.25	1

Table 4.2: MIC ranges and geometric mean (* denotes MIC_g) of *Candida* spp. and *Cryptococcus* sp. (Ramya et al., 2018)

Yeasts	MIC range and Geometric Mean (MIC_g)					
	AF_3		AF_4		AF_5	
	MIC	MFC	MIC	MFC	MIC	MFC
***Candida* spp. (64)**						
**C. albicans* (11)	7.51	7.51	3.31	3.76	4.26	4.26
**C. tropicalis* (13)	7.19	7.19	3.41	3.41	4.95	4.95
C. krusei (4)	8-16	8-16	4	4	4-8	4-16
**C. glabrata* 11)	7.05	7.51	3.31	3.53	5.84	5.84
C. haemulonii (6)	8	8	4	4	4-16	4-32
C. parapsilosis (8)	8-16	8-16	4-16	4-16	4-32	8-32
**C. auris* (10)	8.57	10.55	3.48	3.48	6.498	6.498
C. viswanathii (1)	4	4	2	2	2	2
***Cryptococcus* spp. (17)**						
**C. neoformans var grubii* (11)	6.17	6.17	2.83	2.83	2.59	2.83
C. neoformans var gattii (6)	4-8	4-8	2-4	2-8	2-8	2-8

4.3.2. Hemolytic assay

To determine the effects of any potential cytotoxicity, the effects of various concentrations of all the five purified antifungal compounds on 5% human erythrocytes were studied (Fig. 4.1). Lipopeptide concentration exhibiting 10% (HC_{10}) and 50% (HC_{50}) hemolysis were analysed. AF_3 showed HC_{10} and HC_{50} at 8 and 16.4 µg/mL respectively, however, AF_4 displayed $HC_{<10}$ (~5.6%)at 8 µg/mL and HC_{50} at 17.7 µg/mL whereas AF_5 showed HC_{50} at 8 µg/mL (Fig. 4.1).On the contrary, AF_1 and AF_2 exhibited 100% hemolysis at the concentration less than their MIC values.

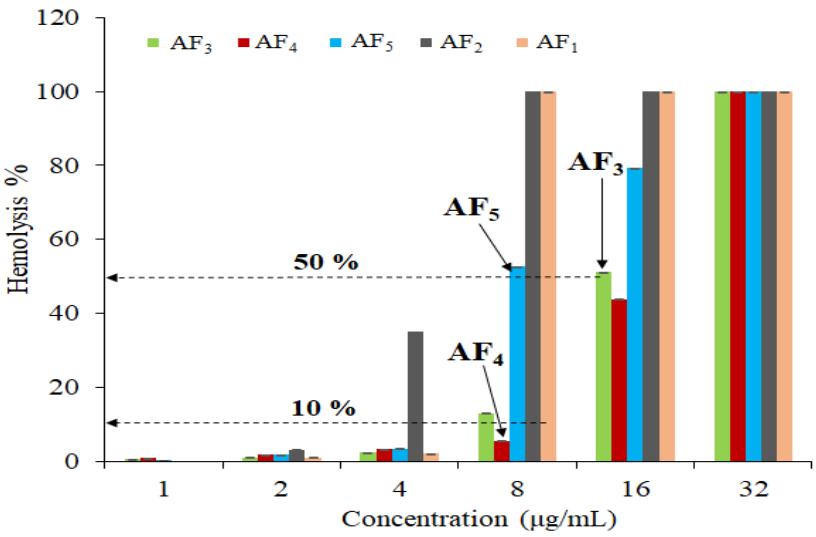

Fig. 4.1: Hemolytic activities of AF_1, AF_2, AF_3, AF_4 and AF_5 fractions against human erythrocytes. Each datapoint represents mean result ± standard deviation (error bars) of experiments performed in triplicate (Ramya et al., 2018).

4.3.3. Time killing assay

Killing kinetics studies were performed with 2xMIC concentration of AF_3, AF_4 and AF_5 on *C. albicans* ATCC 24433 in RPMI 1640 medium. AF_3 and AF_5 displayed $2\log_{10}$ decrease (99%) in CFU/mL at 8 h treatment whereas AF_4 decreased the number of viable cells to 99% ($2\log_{10}$) between 8 to 12 h of treatment (Fig. 4.2).

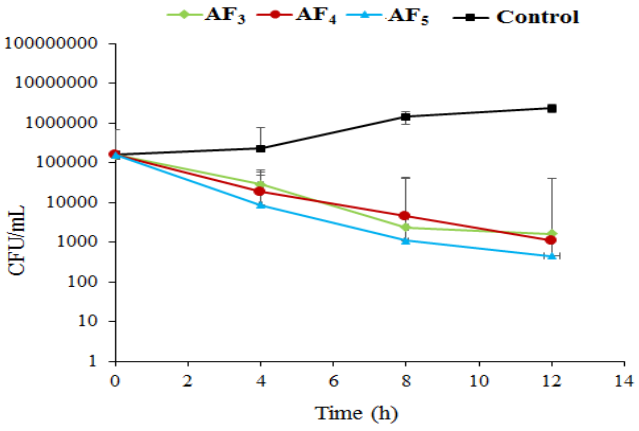

Fig. 4.2: Time killing kinetic studies of AF_3, AF_4 and AF_5 against *C. albicans* ATCC 24433. Each data point represents mean result ± standard deviation (error bars) of experiments performed in triplicate (Ramya et al., 2018).

4.3.4. Inhibition of biofilm formation

Using XTT reduction assay, seven *Candida* sp., *C. albicans* SC5314, *C. albicans* ATCC 24433, *C. auris* NCCPF 470067, *C. haemulonii* ATCC 22991, *C. krusei* NCCPF 440022, *C. glabrata* ATCC 2001 and *C. parapsilosis* NCCPF 450033 showing the absorbance ≥0.5 at 490 nm were selected for biofilm studies (Fig. 4.3).

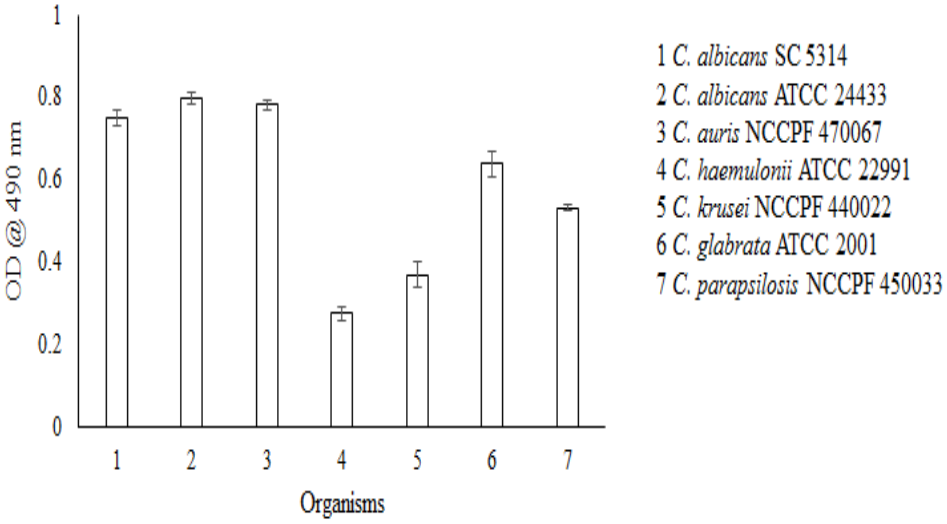

Fig. 4.3. Quantification of biofilm formation using XTT method (Ramya et al., 2018)

Biofilm formation was quantified using XTT dye reduction assay for the selected seven *Candida* species after 48 h (Fig. 4.3). AF_3, AF_4 and AF_5 were tested for its ability to inhibit biofilm formation (to about 50%) by the pre-adhered cells. It was found that AF_3 reduced the biofilm formation by the adhered cells at the concentrations equal to MIC for *C. albicans* SC5314 and *C. glabrata* ATCC 2001 and at 2xMIC for the other tested organisms (Table 4.3). In case of AF_4, the inhibition of the biofilm formation by the adhered cells was observed at 4x concentration of MIC for *C. krusei* NCCPF 440022 and *C. parapsilosis* NCCPF 450033 whereas for the remaining organisms, 2xMIC concentration showed 50% inhibition (Table 4.3). AF_5 showed 50% inhibition at the concentration of 1x and 4x for *C. parapsilosis* NCCPF 450033 and *C. auris* NCCPF 470067 respectively and 2xMIC for the remaining tested organism (Table 4.3). Effect of lipopeptide variant AF_4 on biofilm formation inhibition is shown in Fig. 4.4.

Table 4.3. Effect of three lipopeptides on the biofilm formation inhibition ($SMIC_{50}$) (Ramya et al., 2018)

Organisms	Strain no.	MIC (Planktonic cells)			$SMIC_{50}$ (Biofilm)		
		AF_3 (µg/mL)	AF_4 (µg/mL)	AF_5 (µg/mL)	AF_3 (µg/mL)	AF_4 (µg/mL)	AF_5 (µg/mL)
C. albicans	SC5314	8	2	4	8(1x)	4(2x)	8(2x)
C. albicans	ATCC 24433	8	4	4	16 (2x)	8 (2x)	8 (2x)
C. auris	NCCPF 470067	8	2	4	16(2x)	4 (2x)	16 (4x)
C. haemulonii	ATCC 22991	8	4	4	16 (2x)	8 (2x)	8 (2x)
C. krusei	NCCPF 440022	16	4	8	32 (2x)	16 (4x)	16 (2x)
C. glabrata	ATCC 2001	8	4	4	8 (1x)	8 (2x)	8 (2x)
C. parapsilosis	NCCPF 450033	16	4	8	32 (2x)	16 (4x)	8 (1x)

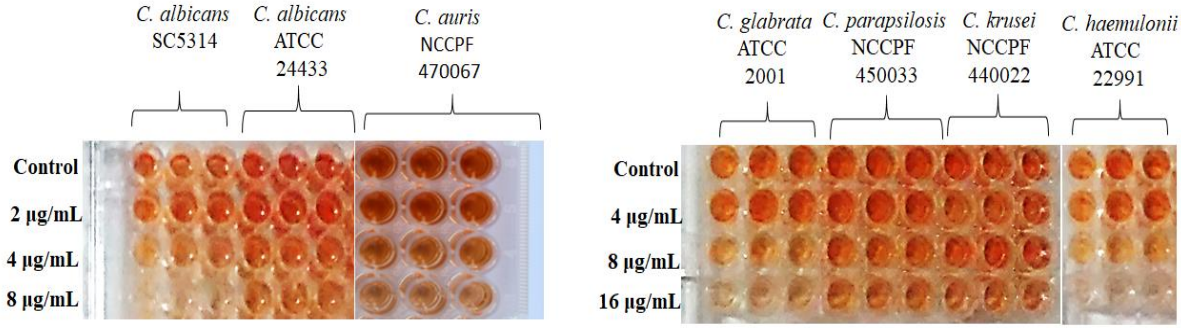

Fig. 4.4. Effect of lipopeptide variant AF_4 on biofilm formation inhibition tested using XTT method.

4.3.5. Cytotoxicity assay

The cytotoxic activity of all the antifungal compounds against HEK 293, HaCaT, HeLa and A549 cell lines were assessed using MTT assays (Fig. 4.5). IC_{50} values varied depending on the cell lines used (Table 4.4). All the three compounds showed dose dependent cytotoxicity effects against the tested cell lines. HaCaT and A549 cell lines showed 70% viability at 8 µg/mL concentration for all the three lipopeptides. (Fig. 4.5). AF_4

showed IC_{50} value of 13.31 µg/mL for the untransformed cell lines but 23.18 and 34 µg/mL for HeLa and A549 cell lines respectively whereas AF_3 exhibited IC_{50} value of 13-15.5 µg/mL for normal and HeLa cell lines. IC_{50} value of AF_5 were in the range of 9-13 µg/mL.

Table 4.4: IC_{50} values of AF_3, AF_4 and AF_5 against four mammalian cell lines

Cell lines	IC_{50} (µg/mL)		
	AF_3	**AF_4**	**AF_5**
HEK 293	14.53	13.12	9.5
HaCaT	15.69	13.33	11.45
A549	>32	34.4	>32
HeLa	13.04	23.18	12.57

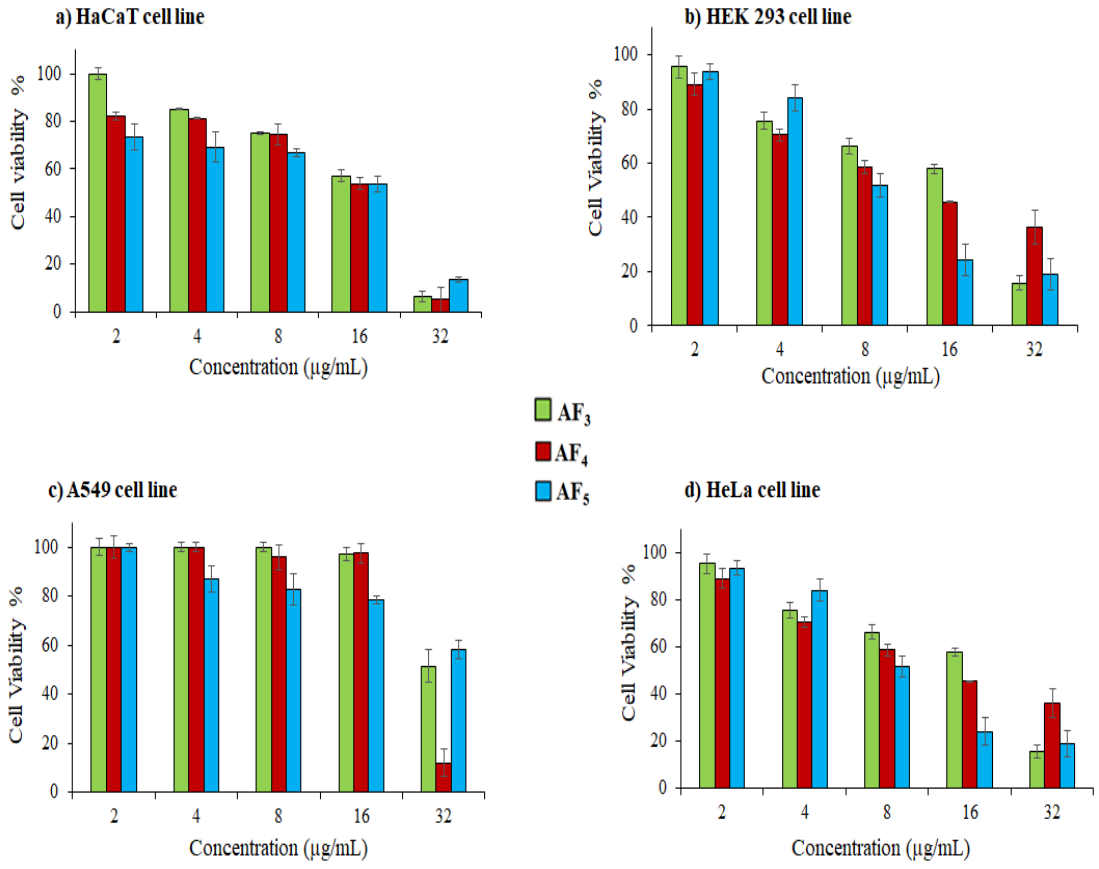

Fig. 4.5: Cytotoxicity profile of AF_3, AF_4 and AF_5 lipopeptides at various concentrations against the mammalian cell lines: a) HaCaT, b) HEK 293 c) A549 and d) HeLa. Each datapoint represents mean result ± standard deviation (error bars) from experiments done in triplicate (Ramya et al., 2018).

4.3.6. Interaction studies of three lipopeptides

4.3.6.1. Determination of MIC values of three lipopeptides against the increased number of yeasts cells

MIC_{80} values of AF_3, AF_4 and AF_5 lipopeptides tested against *C. albicans* ATCC 24433, *C. parapsilosis* NCCPF 450033 and *C. krusei* NCCPF 440022 at a final suspension of 10^5 cells/mL were almost double the $MIC_{>90}$ values obtained against 10^3 cells/mL suspension of the same cultures (Table 4.1 and 4.5). MIC_{50} values against three *Candida* sp. obtained with the cell suspension of 10^5 cells/mL were found similar to $MIC_{>90}$ values observed with 10^3 cells/mL suspension. Out of three tested cultures, two clinical isolates *C. parapsilosis* NCCPF 450033 and *C. krusei* NCCPF 440022 had shown high MIC values compared to *C. albicans* ATCC 24433.

Table 4.5: Comparison of MIC values of three lipopeptides with the cell suspension of 10^5 and 10^3 CFU/mL of *C. albicans* ATCC 24433 **(A)**, *C. parapsilosis* NCCPF 450033 (**B)** and *C. krusei* NCCPF 440022 (**C)**

Organism	**10^3 cells/mL**			**10^5 cells/mL**					
	AF_3 µg/mL	**AF_4 µg/mL**	**AF_5 µg/mL**	**AF_3 µg/mL**		**AF_4 µg/mL**		**AF_5 µg/mL**	
	$MIC_{>90}$	**$MIC_{>90}$**	**$MIC_{>90}$**	**MIC_{80}**	**MIC_{50}**	**MIC_{80}**	**MIC_{50}**	**MIC_{80}**	**MIC_{50}**
A	*8*	*4*	*4*	16	8	8	4	8	4
B	*16*	*8*	*8*	32	16	16	8	16	8
C	*16*	*4*	*8*	32	16	8	4	16	8

4.3.6.2. Interaction Study of three lipopeptides against planktonic cells and biofilm formation

Interactions among AF_3, AF_4 and AF_5 were studied using checkerboard analysis against three organisms which was selected based on their sensitivity/susceptibility to three homologues/isomers. Different combinations showing 80% (MIC_{80}) and 50 % (MIC_{50}) inhibition with different fractional inhibitory concentration index (FIC) values are listed in Table 4.6 for both planktonic cells and biofilm formation inhibition.

Table 4.6. Fractional inhibitory concentration (FIC) indices for the interaction effects of homologues AF_3, AF_4 and AF_5 by using *C. albicans* ATCC 24433, *C. parapsilosis* NCCPF 450033 and *C. krusei* NCCPF 440022 as the target organisms (Ramya et al., 2018)

		***C. albicans* ATCC 24433**				***C. parapsilosis* NCCPF 450033**				***C. krusei* NCCPF 440022**			
Combinations	**Concentration (µg/mL)**	**Planktonic cells**		**Biofilm formation**		**Planktonic cells**		**Biofilm formation**		**Planktonic cells**		**Biofilm formation**	
		Inhibition %	FIC	Inhibition %	FIC	Inhibition %	FIC	Inhibition %	FIC	Inhibition %	FIC	Inhibition %	FIC
AF_3, AF_4	**4,4**	80	0.75	50	0.75	50	0.75	50	0.375	80	0.625	50	0.375
	2,4	80	0.625	50	0.625	<50	-	<50	-	<50	-	<50	-
AF_3, AF_5	**4,4**	80	0.5	50	0.375	50	0.531	<50	-	50	0.75	50	0.375
	0.5,8	80	0.53	50	0.5	<50	-	<50	-	80	0.515	<50	-
	2,4	80	0.375	50	0.3125	<50	-	<50	-	<50	-	50	0.375
AF_4, AF_5	**4,4**	80	0.75	50	1	50	0.75	<50	-	80	0.75	<50	-
	4,2	80	0.625	50	0.75	<50	-	<50	-	<50	-	<50	-
	2,4	80	0.5	50	0.75	50	0.5	<50	-	80	0.5	<50	-

Eight combinations showing 80% (MIC_{80}) and 50 % (MIC_{50}) inhibition with different fractional inhibitory concentration index (FIC) values are listed in Table 4.6 for both planktonic cells and biofilm formation inhibition. All the eight combinations (Table 4.6) exhibited 80% inhibition in case of *C. albicans* ATCC 24433 with additive interaction. Among the eight, four combinations AF_3 and AF_4 (4,4 µg/mL), AF_4 and AF_5 (2,4 µg/mL) (4,4 µg/mL) and AF_3 and AF_5 (0.5,8 µg/mL) displayed 80% inhibition against *C. krusei* NCCPF 440022 wherein 50% inhibition was observed with AF_3 and AF_5 (4,4 µg/mL) combination (Table 4.6). The combination of AF_3 and AF_4 (4,4 µg/mL), AF_4 and AF_5 (4,4 µg/mL) (2,4 µg/mL), AF_3 and AF_5 (4,4 µg/mL) showed additive interaction with 50% inhibition of *C. parapsilosis* NCCPF 450033 (Table 4.6). The combinations of AF_3 and AF_5 inhibited the biofilm formation to about 50% with considerable additive effect against *C. albicans* ATCC 24433. The combination AF_3 and AF_4 (4,4 µg/mL) showed additive interaction in inhibiting both planktonic cells and biofilm formation in case of all the three selected strains (Table 4.6). On the other hand, AF_4 and AF_5 combination did not inhibit the biofilm formation effectively. AF_3 and AF_5 combination exhibited $SMIC_{50}$ at (4,4 µg/mL) and (2,4 µg/mL) against *C. krusei* NCCPF 440022.

4.3.6.3. Cytotoxicity of the selected potent lipopeptide combinations

Cytotoxic nature of the effective combinations obtained from checkerboard study was determined based on hemolytic activity with 5% human erythrocytes and *in vitro* cytotoxicity against human cell lines. All the effective combinations listed in Table 4.6 exhibited less hemolysis (<5%) except AF_3 and AF_5 combination of (0.5,8 µg/mL) where >10% hemolysis was observed. Although the combinations AF_3 and AF_4 (2,4 µg/mL) and AF_4 and AF_5 (4,2µg/mL) exhibited less hemolysis property, further cytotoxicity studies were not conducted as the overall antifungal efficiency was found less compared to the other combinations. Henceforth, five effective combinations (listed in Table 4.7) were selected and the cytotoxic property was evaluated. All these five combinations showed negligible hemolysis (<5%) against erythrocytes and ≤30% cytotoxicity against untransformed and the cancer cell lines tested in the present study (Table 4.7).

Table 4.7. Hemolytic and cytotoxic effects of the AF_3, AF_4 and AF_5 combinations in interaction study. Each data point with (±) represents standard deviation from three experiments (Ramya et al., 2018).

Combinations	Concentration (µg/mL)	Hemolysis %	Cell viability %			
			HaCaT	HEK 293	A549	HeLa
AF_3, AF_4	4,4	3.87±3.7	68.05±2.3	68.81±0.9	76.61±5.4	90.04±3.8
AF_3, AF_5	4,4	3.43±3.1	78.27±1.7	70.42±5.8	84.07±3.5	93.16±4.6
	2,4	4.11±5.9	75.56±2.5	72.58±1.8	63.17±5.2	70.16±6.4
AF_4, AF_5	4,4	3.12±3.4	73.08±3.9	60.90±2.6	60.93±2.8	69.01±0.5
	2,4	2.96±4.1	70.31±6.4	71.98±4.2	72.94±3.3	77.26±6.9

4.4. DISCUSSION

Due to the high MIC values (Table 4.1) obtained with AF_1 and AF_2, no studies on the inhibition of biofilm formation, time killing and additive interaction studies were conducted on these two lipopeptide homologues. The MIC and MFC values of AF_4 (Table 4.1) against *C. albicans, C. tropicalis, C. auris* and *C. neoformans* are very encouraging. The MIC and MFC values were found higher for AF_3 but comparatively less in case of AF_5. In the context of Indian subcontinent (Chowdhary et al., 2014), the rising infections caused by *C. auris* and associated drug-resistance have raised concerns amongst the clinicians (Chakrabarti et al., 2015); the MIC_g of AF_4 at 3.48 µg/mL against the clinical isolates of *C. auris* (Table 4.1) indicate that this compound might have the potential to combat the emerging drug-resistant *C. auris* (Chakrabarti et al., 2015). In a recent study from Hong Kong, an alarming rise in resistance has been noted with 40 % of *C. albicans*, 10% of *C. tropicalis*, 11.1% of *C. parapsilosis* and 100% of *C. glabrata* reported as fluconazole-resistant (Seneviratne et al., 2016). In the present study, fluconazole resistant *C. albicans* (1 no.), *C. parapsilosis* (1 no.) and *C. krusei* (4 no.) displayed less MIC values in case of AF_4 (Table 4.1). MIC range of 2-4 µg/mL was observed in case of *C. glabrata*, which is encouraging as limited treatment options are presently available for *C. glabrata* infection due to its azole and echinocandin resistance (Pham et al., 2014).

Emerging resistance of *C. neoformans* to fluconazole is a matter of great concern, as the drug is recommended for maintenance therapy for the treatment of cryptococcosis (Lewis, 2011). Reports on lipopeptide activity against *Cryptococcus* spp. are scanty as compared to *Candida* spp. Echinocandin lipopeptides were reported to show poor antifungal activity against *C. neoformans* (Lewis, 2011). The anti-*Cryptococcal* prowess of AF_4 and AF_5 with less MIC range is either superior or comparable to antifungal tested (Table 4.1) in the present study. Like the present work, a previous study reported promising antifungal activity of a novel membrane-active peptide (MP) and its D-enantiomer, (D-MP) with MIC in the range of 2-4 µg/mL against pathogenic clinical isolates of *C. neoformans* (Hong et al., 1999). YM-47522 compound produced by *Bacillus* sp. showed antifungal activity against *C. neoformans* at around 6.25 µg/mL (Sugawara et al., 1996). Overall, 95% of MIC and MFC endpoint values of AF_4 were the same, except for the MFC of two clinical isolates of fluconazole resistant *C. parapsilosis,* and *C. albicans* where it was marked as one or two-fold higher compared to the respective MIC value. MIC values of amphotericin B and fluconazole did rise with the increased incubation period whereas MIC of AF_3, AF_4 and AF_5 remained same even after 72 and 96 h of incubation in presence of *Candida* spp. and *Cryptococcus* sp. respectively which is an important aspect of these lipopeptides.

Iturin like lipopeptides disrupts the membrane integrity of mammalian cells at different concentrations (dose dependent) depending on the nature of the compound and its critical micelle concentrations (Aranda et al., 2005), leading to limited clinical usage. The hemolytic study was conducted using all the five compounds wherein AF_4 exhibited a negligible 0 to 5.6% hemolysis at the MIC range of 1-8 µg/mL (Fig.4.1) and the recorded cell survival of about 80% at 4 µg/mL (Fig. 4.5) unequivocally suggests this compound's antimicrobial efficacy. In the previous studies, the MIC values of iturin A and fengycin and lipopeptide 6-2 antiCA produced by *B. subtilis* and *B. amyloliquefaciens* against *C. albicans* were found to be 5-10, 15.62 and 7.0 µg/mL, respectively (Anupam et al., 2013; Song et al., 2013; Pathak and Keharia, 2014). However, percentage of hemolysis recorded was high for all these compounds at its respective MIC values. Iturin A, bacillomycin D, bacillomycin Lc, and mycosubtilin were reported to induce 25, 28, 75, and 100% hemolysis respectively at the MIC concentrations of 10 µg/mL indicating limited therapeutic applications of these compounds (Cochrane and Vederas, 2016). Similarly, three bacillomycin D like lipopeptides

a_1, a_2 and a_3 from *B. subtilis* B38 had been reported with different antimicrobial potency where a_3 was found to be very active but with 50% hemolysis at the reported MIC range (Tabbene et al., 2011).

IC_{50} varied depending on the nature of the cell lines tested. IC_{50} values of AF_3, AF_4 and AF_5 on A549 cell line was found to be high as compared to other cell lines tested in the present study. It is worth noting that the IC_{50} values of AF_4 is ~13.3 µg/mL (Table 4.4) and the MIC_g values of *Candida* spp. and *Cryptococcus* sp. are between 3.31-3.48 and 2.83 µg/mL respectively (Table 4.2) with hemolysis values not exceeding 4% at the MIC of 4 µg/mL (Fig. 4.1). Therefore, considering the low MIC values, low hemolytic and cytotoxicity values, AF_4 is found to be the most potent compound among the five-extracted antifungal lipopeptides of *B. subtilis* RLID 12.1.

Irrespective of its strong antifungal action, iturin and fengycin have not stepped in to clinical trials due to its strong haemolytic nature. From the natural isomers, it was found that antifungal activity is proportional to the side chain length (C_{18}> C_{17}>C_{16}> C_{15}> C_{14}) and branching type (*normal*>*iso*>*anteiso*) (Bland, 1996). Based on the MIC values, antifungal potentials of the HPLC-purified compounds AF_3, AF_4 and AF_5 were marked in the order of AF_4 > AF_5 > AF_3. The low MIC values produced by AF_4 compoundas compared to AF_3 might have resulted from the variations in the type of branching. Irrespective of the presence of long fatty acid chain, antifungal activity of AF_5 was found to be less compared to AF_4 which might be due to the presence of *iso*-C_{18} branched fatty acid. However, the antifungal activity of *iso*-C_{18} (AF_5) is greater than *anteiso*-C_{17} (AF_3). Antagonistic nature of AF_4 against *Candida* spp. and *Cryptococcus* spp. with reduced cytotoxicity was found to be effectual as compared to the other antifungal lipopeptides reported in *Bacillus* sp. (Cochrane and Vederas, 2016; Tabbene et al., 2011).

Although the MIC values of AF_4 for planktonic cells were found to be low, the MIC of biofilm formation inhibition ($SMIC_{50}$) was found to be high (4-16 µg/mL) about 4xMIC (Table 4.3). AF_4 showed 50% cytotoxicity for HEK 293, HaCaT and HeLa as well as hemolytic at its 4xMIC concentration (Fig. 4.5) making it less applicable for biofilm inhibition. Despite this, it is worth mentioning that AF_4 at its 1x and 2xMIC showed $SMIC_{50}$ against a *C. glabrata* strain (Table 4.3) which is positive as the biofilm of *C. glabrata* is

unmanageable by antimicrobial therapy due to the natural properties of growth (Rodrigues et al., 2017).

Synergistic action is a very well-known property of antifungal lipopeptides, which enables their broad antimicrobial spectrum applications (Kim et al., 2010). To overcome the cytotoxic effect of AF_4 at high $SMIC_{50}$ and low IC_{50} value of AF_5 as indicated in Table 4.3and 4.4, we attempted to study the interactive effects of three homologues of AF_3, AF_4 and AF_5 which has not been reported before. Combining the MIC and $SMIC_{50}$ values of all the three lipopeptides and all the tested yeasts, three *Candida* sp. were selected for interactions studies.*C. albicans* ATCC 24433 was found to be less sensitive as was determined from the previous MIC study whereas the other two clinical isolates *C. parapsilosis* NCCPF 450033 and *C. krusei* NCCPF 440022 were found to be less sensitive to AF_3, AF_4 and AF_5 in terms of both MIC and $SMIC_{50}$ (Table 4.1 and 4.3).

Interaction studies were performed with increased number of cells (10^5 cells/mL) to determine the effective combinations which might help to treat severe infections or resistant pathogens. Eight combinations were obtained with varying FIC values (Table 4.6) based on the sensitivity of three selected organisms to AF_3, AF_4 and AF_5 lipopeptides. Although some combinations exhibited FIC values $\leq$0.5, altogether interactions were found to have additive effect rather than synergistic since all the lipopeptides follow the same mechanism of action. Lipopeptide molecules penetrate the cytoplasmic membrane and form oligomer like structures which form ion conducting pores in the target cells (Maget-Dana and Peypoux, 1994). All five combinations, AF_3 and AF_4 (4, 4 µg/mL), AF_3 and AF_5 (4, 4 µg/mL), AF_3 and AF_5 (2, 4 µg/mL), AF_4 and AF_5 (4, 4 µg/mL and AF_4 and AF_5 (2, 4 µg/mL) showed effective additive actions in terms of planktonic cell inhibition whereas for biofilm formation inhibition, AF_3 and AF_4 (4, 4 µg/mL), AF_3 and AF_5 (4, 4 µg/mL) and AF_3 and AF_5 (2, 4 µg/mL) combinations were found suitable (Table 4.6). This observation is a pointer towards the fact that interactions among the lipopeptide homologues eventuate during their coproduction.

Working on the similar line, additive type interaction was reported with [Ile^7] surfactin and bacillomycin D in suppressing the gray mold disease on the cucumber leaves by *B. cinerea* (Tanaka et al., 2015). Maget-Dana *et al.* (1992) explained the mechanism of

the synergistic effect of surfactin on the biological properties of iturin A. Synergistic action of surfactin and fengycin as well as iturin and fengycin were also reported previously (Maget-Dana and Peypoux, 1994; Romero et al., 2007). Antifungal lipopeptides bacillomycin D and fengycin of *B. amyloliquefaciens* FZB42 showed a synergistic effect against *F. oxysporum* strain (Koumoutsi et al., 2004). Xianyang et al. reported a synergistic study between two surfactin homologues (C_{14} and C_{15}) with ketoconazole against *C. albicans* with MIC values of 12.5 and 6.25 µg/mL (Liu et al., 2012). Tabbene *et al.* (2015) in a separate study reported bacillomycin D showing synergistic effect with amphotericin B against *Candida* strains, with FIC indices ranging from 0.28 to 0.5. Interestingly, when these two drugs were used together at those dosages (displaying a synergism in the anti-*Candida* activity), cytotoxic effect was reduced (Olfa et al., 2015). Synergistic antifungal effect of gageostatins A and B were also reported in marine *B. subtilis* (Tareq et al., 2014).

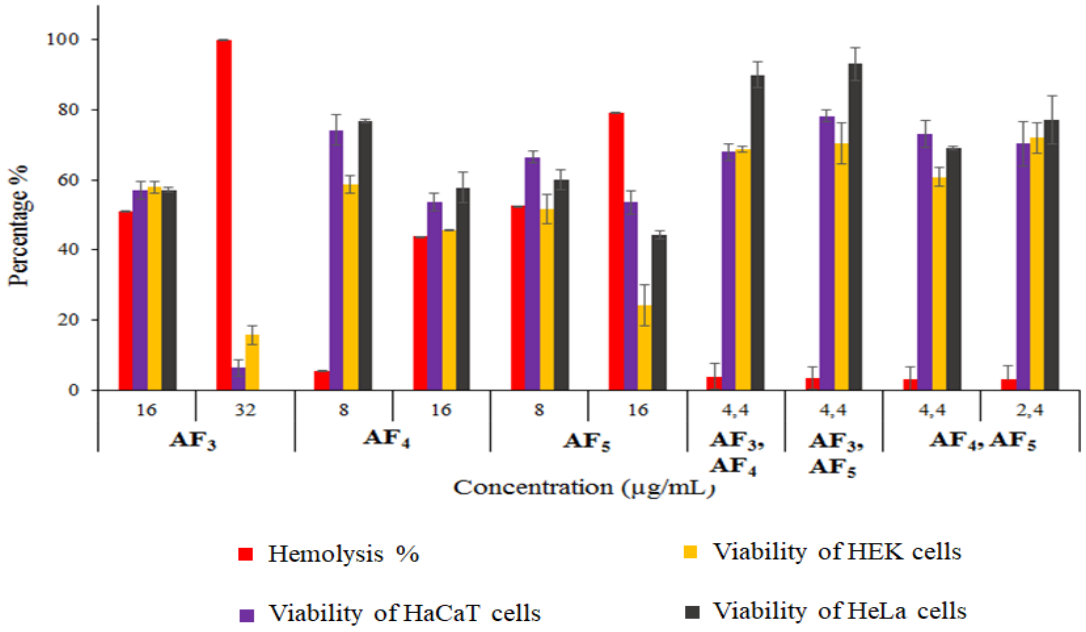

Fig. 4.6: Comparison of the cytotoxicity of the combinations with the individual MICs (determined against 10^5 *Candida* cells/mL) of AF_3, AF_4 and AF_5 lipopeptides.

Comparing the antifungal potency and cytotoxicity level of the combinations and individual MICs of three lipopeptides (Fig. 4.6), the additive interactions of (AF_3, AF_4) and (AF_3, AF_5) were found to be more effectual with strong anti-biofilm forming potential and fungicidal action with ≥ 70% cell survival and < 4% hemolysis.

Chapter 5

Optimization of the Production of Antifungal variants

5.1. INTRODUCTION

Bacilli in general are familiar for their inherent ability to produce a large number of antibiotics either by ribosomal or non-ribosomal metabolism. Bioactive lipopeptides from microbial origin have already curved their niches in industry for their biopharmaceutical potentials (Gudina et al., 2013). Coproduction of lipopeptides, for example, surfactin and iturin like compounds by microbes were considered as a remarkable trait for a potential biocontrol agent (Sandrin et al., 1990; Ahimou et al., 2000). Different lipopeptide homologues with a linear or branched fatty acid moiety or different peptide sequences are generally co-produced by *Bacillus* sp. in different ratios based on the environmental and nutrient conditions (Ohno et al., 1995). Antimicrobial efficacy of each homologues or isoforms differs based on its fatty acid tail length or amino acid sequence (Bland, 1996). Media formulation for such type of variants based on the overall antifungal activity (Pathak, 2011) may affect the production of therapeutically important lipopeptide variant. Specific media formulation for the potent lipopeptide homologue/isoform after evaluating its antagonistic efficacy and implementation of new process intensification strategies might improve the selective lipopeptide yield. This approach is likely to refine the production of selective potent metabolite, reduce the impurity load on downstream processing steps and support the lipopeptide marketing for antimicrobial applications (Rangarajan and Clarke, 2015).

In this chapter, a comparative study on the production conditions required for the biosynthesis of lipopeptide variants AF_3, AF_4 and AF_5 were targeted, and two steps of optimization process were carried out. First, the production conditions that influence the production of AF_3, AF_4 and AF_5 variants were formulated. Second, the production condition required for the selective improvement of the most potent AF_4 variant was analysed.

5.2. EXPERIMENTAL METHODS

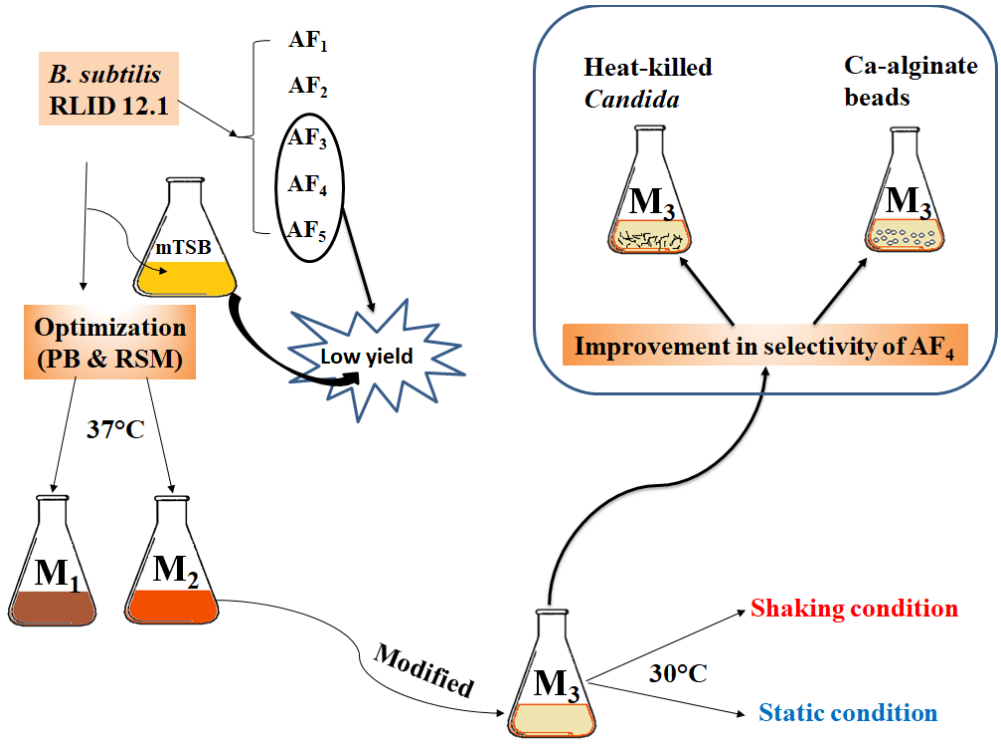

Fig. 5.1: Steps followed in the optimization process

5.2.1. Fractionation of antifungal compounds

As explained in the chapter 3, the antifungal substances were extracted using n-butanol (1:1), evaporated at 55±0.5 ºC and the dried sample obtained was dissolved in 1 mL of 10 mM phosphate buffer (pH 7.0). Quantitative analysis of the extracted sample (of about 100 µL) was performed using analytical RP-HPLC (4.6 mm × 250 mm, particle size 5 µm). The solvent system used was (A) water with 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% TFA. The gradients of solvent B used for purification were as follows: 0-50% for 0-25 min at the flow rate of 1ml/min, 50% from 25-30 min at 0.5 mL/min, 50-55% from 30-40 min at 0.5 mL/min, 55-60% from 40-45 min at 1mL/min and 60-100% from 45-65 min at 1mL/min and monitored at 214 nm (Deepika et al., 2014). Peaks relative to AF_3, AF_4 and AF_5 was confirmed by m/z using Electrospray Ionization Liquid Chromatography-Mass Spectrometry (ESI/LC/MS).

5.2.2. Quantification of the active antifungal fraction

AF_3, AF_4 and AF_5 fractions were further purified to homogeneity with the gradient of 0-50% for 20 min and 0-55% of acetonitrile for 25 min at 0.7 mL/min flow rate

respectively using the analytical HPLC column. Concentrations of these lipopeptides were determined by Bicinchoninic acid *(*BCA) method. Calibration curve was plotted using different concentrations of AF_3, AF_4 and AF_5 (x-axis) and its respective peak area (y-axis) obtained from HPLC system (Bie et al., 2005). The relation between the peak area (y) and the concentration of the antimicrobial substance (x) were expressed in equation (1) for all the three compounds,

$$\boldsymbol{y = (2 * 10^7)x + (2 * 10^8)} \qquad \textbf{..... (1)}$$

Using this standard equation, AF_3, AF_4 and AF_5 concentration were determined from the peak areas of AF_3, AF_4 and AF_5 obtained from HPLC chromatographic profiles of the various production conditions.

5.2.3. Media optimization

5.2.3.1. Preliminary screening of variables

Variation in the production of the antifungal variants was quantified using the equation obtained from the calibration curve except the initial media and inoculum volume screening. In the process of production enhancement of three antifungal variants, *B. subtilis* RLID 12.1 was grown in mTSB media at different volumes (50, 75, 100, 150 and 200 mL) in 250 mL Erlenmeyer flasks using varying inoculum volume (0.5, 1.0 and 2%). Inoculum volume and media volume influencing the production of antifungal variants were determined initially using cut well agar method by measuring the zone of inhibition. The fermentation medium used for optimization in shaker flask cultivation was 0.5% carbon source, 0.5% nitrogen source, 0.25% dipotassium hydrogen phosphate (K_2HPO_4) and 0.5% sodium chloride (NaCl) at 37°C. Inoculum (of 1% at OD_{600}=0.3) was transferred aseptically to a 250 mL Erlenmeyer flask containing 75 mL fermentation medium and incubated at 37±0.5 °C for 60 h on a rotary shaker (105 rpm). After 60 h, grown culture was centrifuged at 10,000 rpm for 15 min and the cell free supernatant was processed for n-butanol extraction for HPLC analysis. The cell pellet was dried completely, and the biomass yield was noted to correlate the growth and production.

Preliminary screening of variables was carried out in the fermentation media as follows:

a) Carbon sources (maltose, fructose, sucrose, starch, glycerol, dextrose, mannose, mannitol, xylose, lactose and malt extract) at 0.5% in fermentation media.

b) Nitrogen sources (yeast extract, casein, peptone, potassium nitrate, sodium nitrate, sodium nitrite, ammonium nitrate, ammonium chloride, ammonium dihydrogen phosphate and magnesium nitrate) at 0.5% in fermentation media.
c) Metal salts like ferrous sulphate (0.025, 0.3 and 2mM), manganese sulphate (0.025, 0.2 and 2mM) and magnesium sulphate (0.8 and 4mM) (Gudiña et al., 2015).

5.2.3.2. Screening of process variables using Plackett-Burman Design

Plackett-Burman (PB) statistical experimental design was applied to screen and evaluate the most essential factors which might influence the response. In the present work, eleven (n) variables were screened in 12 experimental runs (n+1) for AF_3, AF_4 and AF_5 variants (Table 5.1). Significant variables were selected based on the effects in Pareto chart and the insignificant variables were eliminated to obtain a less set of factors for BBD study. Pareto effect portrays the main effects (positive or negative) of the independent variables and their interaction with dependent variables where the bar represents the standardized effect of the factors based on the responses. The low and high levels of each factor are listed in (Table 5.1). The Minitab software (trial version 17) was used for generating experimental designs and analysing the experimental data. Factors with confidence levels higher than 85% were considered to have significant effect on AF_3, AF_4 and AF_5 production and selected for further optimization (Gudiña et al., 2015).

5.2.3.3. Statistical optimization using Box-Behnken Design (BBD)

Based on the preliminary studies four variables, peptone, malt extract, dextrose and ferrous sulphate ($FeSO_4$) were selected for Box-Behnken experimental design (BBD). BBD experiments were performed with three levels and the complete design consisted of 29 experiments with five replicates (used to estimate experimental error) of the centre point (Table 5.2). Statistical software package 'Design Expert 8' was used for analysing the experimental data. The experimental data were analysed by multiple regression analysis through the least square method. Quadratic polynomial equation was expressed in terms of both response surface and contour plots for determining optimum conditions and for predicting the relationships between the responses and the independent variables (Gu et al., 2005). Media formulations obtained from statistical analysis was termed as M_1 and M_2.

5.2.4. Standardization of media conditions to improve the selective production of AF_3, AF_4 and AF_5 variants

Increase in the lipopeptide yield with a small change in the process variables or novel production approach promotes the selective induction of lipopeptides (Rangarajan and Clarke, 2015). Selective induction of AF_4 compared to other variants calculated using the formula given below with the peak area of individual lipopeptide variants obtained in each step of optimization process.

$$\text{Selectivity } \% = \frac{\text{Peak area of } AF_4 \text{ variant}}{\text{Total peak area of four other variants}} * 100$$

An attempt was made to standardize the fermentation conditions by altering the media composition designed from the statistical analysis. The two media formulations selected from the statistical analysis based on the improvement of AF_3, AF_4 and AF_5 variants production were

M_1: dextrose (0.4%), peptone (2.28%), malt extract (0.9%) and $FeSO_4$ (0.55 mM)

M_2: dextrose (0.55%), peptone (1.79%), malt extract (1.01%) and $FeSO_4$ (0.74 mM)

5.2.4.1. Modification of production conditions and media formulations

Previous studies showed that manganese showed significant effect on the lipopeptide biosynthesis similar to the effect made by $FeSO_4$ (Eisenstadt et al., 1973; Mhatre et al., 2016). To achieve the improved selectivity, $FeSO_4$ in M_1 and M_2 media composition was replaced with $MnSO_4$ (0.5, 1 and 2.0 mM) and NaCl (0.5%) and the production was monitored under static and shaking conditions at different temperatures (30 and 37ºC). The new formulations obtained were termed as M_3 and M_4 respectively.

M_3: dextrose (0.55%), peptone (1.79%), malt extract (1.01%), $MnSO_4$ (2 mM), NaCl (0.5%)

M_4: dextrose (0.4%), peptone (2.28%), malt extract (0.9%), $MnSO_4$ (2 mM),NaCl (0.5%)

5.2.4.2. Heat-killed *C. albicans*

Heat inactivation was performed by autoclaving overnight grown *C. albicans* SC5314 at 121°C for 20 min (Leães et al., 2016). *B. subtilis* RLID 12.1 was grown for 24 h in M_3 media composition under shaking conditions at 30±0.5°C in 250 mL flasks. After

24 h, 1% of heat-killed C. *albicans* SC 5314 was added to the flask and *B. subtilis* RLID 12.1 were allowed to grow till 60 h. Production of AF variants was recorded after 60 h of incubation using RP-HPLC chromatographic profiles. Flasks without the addition of heat-killed *C. albicans* SC5314 were used as controls.

5.2.4.3. Calcium-alginate beads

Calcium (Ca) alginate beads were prepared by adding 20 mL of sterile aqueous solution of sodium alginate aseptically into 100 mL of $CaCl_2.2H_2O$ solution under constant stirring condition. Different concentrations of sodium alginate (1.3% and 2%), calcium chloride (1, 2 and 3%) (Serp et al., 2000) and bead sizes (1.0–1.5 mm and 2.7-3.0 mm diameter) were used to determine the optimum conditions required for the selective production of AF_4 variant in the CFS extract. The beads prepared using different conditions were stored at 4 ºC for 8 h and then washed twice in 0.9% sterile saline solution. Sterile Ca-alginate beads were added to the flask containing 24 h old *B. subtilis* RLID 12.1 and the culture was allowed to grow till 60 h in the presence of beads. Culture flasks without addition of beads were used as controls. After 60 h of incubation, beads were transferred to another 250 mL sterile flask and washed thrice with sterile distilled water. Simultaneously, CFS collected from the culture broth was processed and extracted with n-butanol as described previously. Production of AF lipopeptide variants was analysed in the CFS extract using RP-HPLC chromatographic profiles.

5.2.4.3.1.Bead extraction

In order to investigate the immobilization of antifungal variants, the transferred beads were treated with 50% methanol at 37 ºC for 2 h at 120 rpm. The methanol extracted sample was evaporated completely at 55±0.5 ºC and the residue was subjected to acid precipitation and n-butanol extraction. Production of AF lipopeptide variants was analysed in the bead extracts using RP-HPLC chromatographic profiles.

5.2.4.3.2.Effect of calcium ions on the production of antifungal variants

To determine the influence of calcium ions, two different concentrations (0.5 and 1mM) of $CaCl_2.2H_2O$ were added to the M_3 media without the addition of Ca-alginate beads. The CFS was processed as mentioned above and the production of antifungal variants was analysed using RP-HPLC chromatographic profile.

5.3. RESULTS

B. subtilis RLID 12.1 produced five lipopeptide variants with the yield rate in the order of AF_2>AF_1>AF_4>AF_3>AF_5 in mTSB (Fig.5.2). Using the m/z $[M+H]^+$values 1043.59, 1057.69, 1,071.57, 1,071.68 and 1085.61 obtained from ESI/LC/MS the peaks for AF_1, AF_2, AF_3, AF_4 and AF_5 respectively were confirmed and the respective retention times of all the variants were noted.

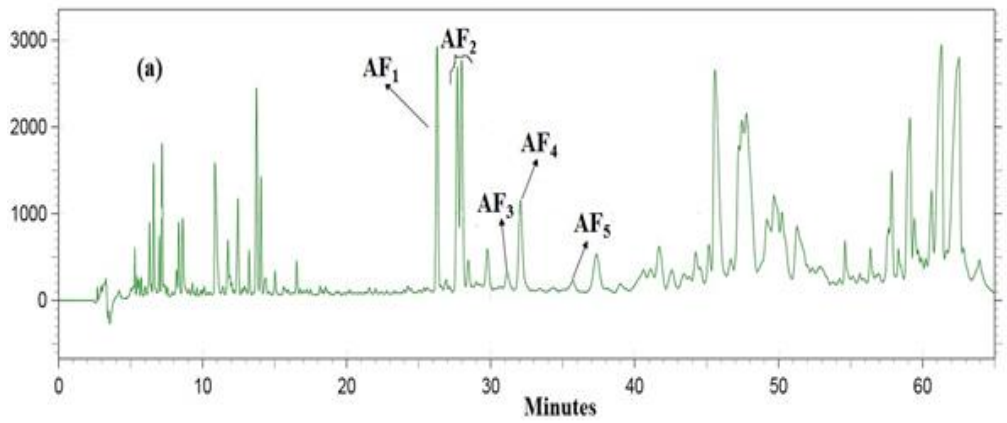

Fig. 5.2: Analytical HPLC chromatogram depicting the proportions of AF_1, AF_2, AF_3, AF_4 and AF_5 production in mTSB media.

5.3.1. Media standardization

5.3.1.1. Preliminary experiments

Media utilization in terms of growth and production of antimicrobial compounds varies with respect to strain. To understand the roles of ingredients for achieving the production of AF_3, AF_4 and AF_5 variants to a significant level, PB and BBD were carried out. Initially, inoculum volume (0.5, 1.0 and 2%) and media volume (50, 75, 100, 150 and 200 mL) were screened for enhancing the production. Antimicrobial activity was determined by cut well agar method measuring the zone of inhibition of butanol extracted sample. Comparatively, an improved antimicrobial activity was recorded when 1% culture (OD_{600}=0.3) was inoculated in 75 mL mTSB media grown at 37°C. In order to formulate the specific medium, peak area obtained from HPLC chromatogram was calculated to estimate the concentration of AF_3, AF_4 and AF_5 produced in each step of optimization. It was observed that dextrose was the best carbon source for all the three variants followed by malt extract and fructose (Fig. 5.3a, 5.3c and 5.3e). Biomass yield was found relatively high when lactose, sucrose, malt extract and maltose were used as

carbon sources whereas moderate biomass yield was recorded when starch, mannose, glycerol and xylose were used. Dextrose, fructose and mannitol showed less biomass yield comparatively (Fig. 5.3a, 5.3c and 5.3e).

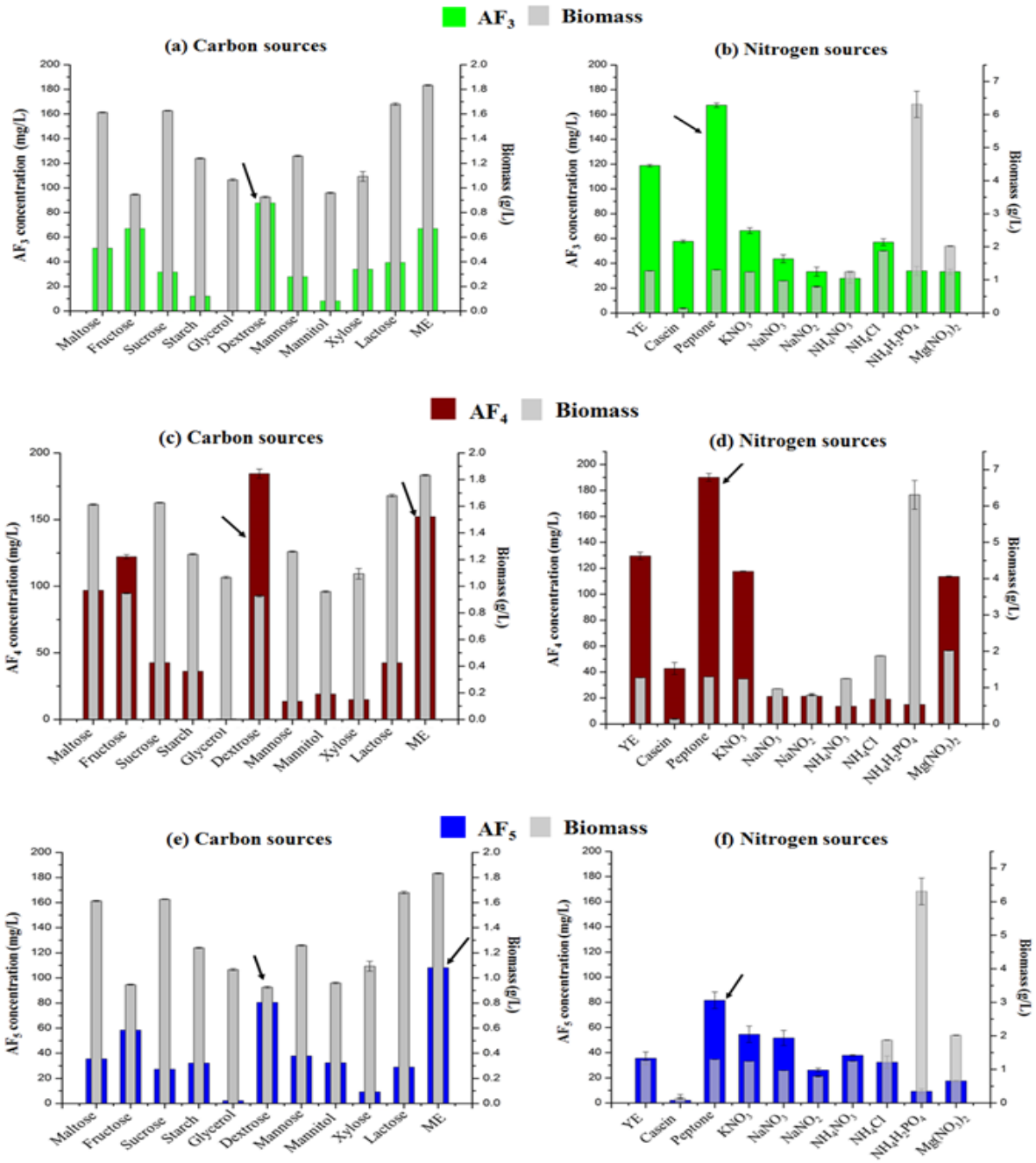

Fig. 5.3: Effects of carbon sources on the production of (a) AF_3, (c) AF_4 and (e) AF_5 and nitrogen sources on (b) AF_3, (d) AF_4 and (f) AF_5 variants production with respect to biomass yield (ME and YE denote malt extract and yeast extract respectively).

Among the nitrogen sources tested, peptone significantly influenced the production of all the three variants followed by yeast extract compared to the other nitrogen sources (Fig. 5.3b, 5.3d and 5.3f). Biomass yield was high only when

ammonium dihydrogen phosphate ($NH_3H_2PO_4$) was used compared to all the other nitrogen sources. From carbon and nitrogen source screening results, it was clear that the yield of AF_3, AF_4 and AF_5 were not correlated with the biomass yield. Out of three metal salts screened, ferrous ion induced the higher production of all the three variants followed by manganese and magnesium ions (Fig. 5.4).

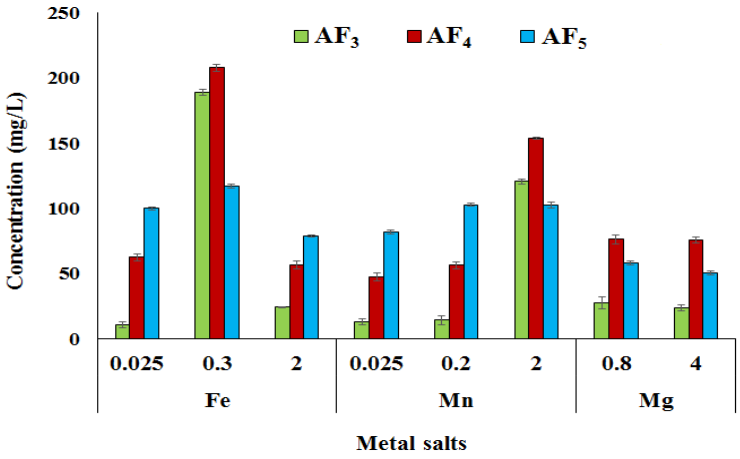

Fig. 5.4: Effects of metal salts on the production of AF_3, AF_4 and AF_5variants. Each data point represents mean result ± standard error of experiments performed in triplicate.

5.3.1.2. Evaluation of PB Design for important process variable

Based on the preliminary studies, dextrose, fructose, malt extract and peptone were chosen for PB design. In total, eleven process variables such as dextrose, fructose, peptone, malt extract, tryptone, NaCl, pH, $FeSO_4$, $MgSO_4$, $MnSO_4$ and K_2HPO_4 were used for screening. Production of AF_3, AF_4 and AF_5 variants in the PB experimental design is shown in Table 5.1. Pareto chart of AF_3, AF_4 and AF_5 (Fig. 5.5) with the standardised effect of the selected factors and the bar crossing the reference line shows the significance of the individual factors. The positive coefficients of the model components were found to be dextrose, peptone, malt extract and $FeSO_4$ and it explains the interactive effects on of AF_3, AF_4 and AF_5 production. The negative coefficients of fructose, tryptone, NaCl, pH, $MgSO_4$, $MnSO_4$ and K_2HPO_4 demonstrates the antagonistic effect on the production of both the fractions. Based on the standardised effect and positive coefficients, dextrose, peptone, malt extract and $FeSO_4$ were identified as significant factors and used further for optimization using Box–Behnken design.

Table 5.1: PB design and experimental response obtained for AF_3, AF_4 and AF_5 production

Run	Tryptone (%)	Dextrose (%)	Fructose (%)	Peptone (%)	Malt extract (%)	NaCl (%)	pH	$MnSO_4$ (mM)	$FeSO_4$ (mM)	$MgSO_4$ (mM)	K_2HPO_4 (%)	AF_3 concentration (µg/mL)	AF_4 concentration (µg/mL)	AF_5 concentration (µg/mL)
1	4	2	0.5	2	2	2	6.0	2	0.05	4	0.25	68.01±4.9	112.86±4.4	72.85±1.5
2	2	0.5	0.5	0.5	0.5	0.5	6.0	2	0.05	2	0.25	129.27±9.4	149.92±2.9	137.49±2.6
3	4	2	0.5	0.5	0.5	2	6.0	4	0.3	2	0.5	28.50±2.1	30.23±3.3	56.47±6.2
4	4	2	2	0.5	0.5	0.5	9.0	2	0.3	4	0.25	32.19±2.3	120.05±5.7	65.21±5.3
5	4	0.5	2	2	2	0.5	6.0	2	0.3	2	0.5	51.42±3.7	115.55±4.3	82.57±4.5
6	2	2	2	2	0.5	0.5	6.0	4	0.05	4	0.5	15.27±1.1	34.97±3.2	12.55±2.3
7	4	0.5	0.5	0.5	2	0.5	9.0	4	0.05	4	0.5	0	90.86±1.2	8.14±1.5
8	2	0.5	0.5	2	0.5	2	9.0	2	0.3	4	0.5	0	57.79±1.2	19.79±2.9
9	4	0.5	2	2	0.5	2	9.0	4	0.05	2	0.25	0	13.88±1.4	0
10	2	2	0.5	2	2	0.5	9.0	4	0.3	2	0.25	189.14±3.7	174.42±3.2	180.17±4.1
11	2	0.5	2	0.5	2	2	6.0	4	0.3	4	0.25	29.75±2.2	41.41±2.9	39.02±3.3
12	2	2	2	0.5	2	2	9.0	2	0.05	2	0.5	0	45.11±5.6	0

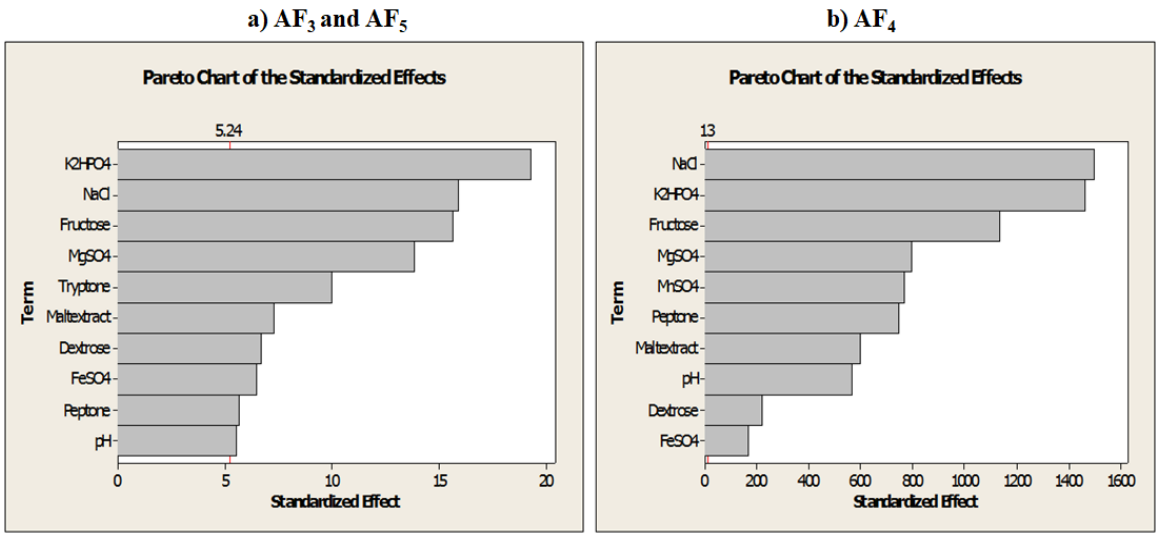

Fig. 5.5: Pareto chart showing the effect of process variable on a) AF_3 and AF_5 and b) AF_4 production based on the observations of PB design.

5.3.1.3. Box-Behnken design (BBD) - Mathematical models and statistical analysis

In this study, BBD was selected to study the interactions among four influencing media components such as peptone, malt extract, dextrose and $FeSO_4$ and to design the formulation media suitable for the enhanced production of AF_3, AF_4 and AF_5 variants. The experimental and predicted values are summarized in Table 5.2. The quadratic model was found suitable for AF_3, AF_4 and AF_5 production with maximum R^2, adjusted R^2 ($AdjR^2$) and predicted R^2 ($Pre\ R^2$) with low p-values greater than F value.

Through regression fitting, a second-order polynomial equation (eqn. 2, 3 and 4) were obtained to demonstrate the influence of four input variables and their interactions based on Box–Behnken experimental design model for AF_3, AF_4 and AF_5 production.

$$\frac{1}{Sqrt(Y_1)} = 0.05381 + 0.005621A + 0.008788B - 0.0021C + 0.000139D - 0.00965AC + 0.009188AD + 0.009431BD + 0.006646CD + 0.005412A^2 + 0.011384B^2 + 0.019839D^2 \quad \textbf{.... (2)}$$

$$Y_2 = 686.06 - 52.17A - 73.17B - 36.79C + 18.22D + 197.85AC - 97.73BC - 126.49BD - 160.49A^2 - 228.73B^2 - 90.89C^2 - 243.74D^2 \textbf{.... (3)}$$

$$Log_{10}(Y_3) = 2.383 - 0.076A - 0.121B - 0.008C + 0.0189D + 0.073AC - 0.089BC - 0.117BD - 0.103B^2 - 0.053C^2 - 0.277D^2 \textbf{.... (4)}$$

Table 5.2: BBD matrix with actual and coded values with observed and predicted AF_3, AF_4 and AF_5 production response.

Run	Dextrose	Peptone	Malt extract	$FeSO_4$	AF_3 concentration (mg/L)		AF_4concentration (mg/L)		AF_5concentration (mg/L)	
					Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	1.5	1.5	1.5	0.55	248.973	349.167	285.043	362.716	219.323	231.037
2	1.5	3	1.5	0.55	136.422	126.524	160.049	216.482	118.198	97.268
3	1	2.25	2	1	141.962	161.938	263.246	377.836	102.162	153.6
4	0.5	2.25	1.5	1	260.080	242.099	408.838	337.176	182.779	181.481
5	1	2.25	1.5	0.55	356.646	349.167	662.056	686.064	286.789	260.698
6	1.5	2.25	1.5	0.1	160.511	176.68	255.757	256.403	106.735	131.251
7	1	2.25	1	1	208.313	207.115	300.953	391.407	143.441	178.593
8	1.5	2.25	1.5	1	101.230	113.446	384.237	292.805	95.745	61.279
9	1	3	2	0.55	214.944	220.393	137.483	203.334	172.033	115.667
10	1	2.25	1.5	0.55	308.015	349.167	662.890	686.064	217.786	211.146
11	1	3	1.5	0.1	130.749	141.286	294.081	218.711	103.818	109.332
12	0.5	2.25	1.5	0.1	163.102	147.394	230.712	300.775	139.279	111.097
13	1	2.25	2	0.1	229.684	236.589	348.596	341.434	102.149	95.189
14	1	1.5	1	0.55	335.858	343.223	476.057	363.685	261.371	206.043
15	0.5	2.25	2	0.55	281.253	265.212	304.729	222.214	207.184	211.849
16	1	3	1.5	1	99.182	93.768	64.325	79.536	62.255	104.516
17	1	2.25	1.5	0.55	388.783	349.167	769.454	686.064	236.150	235.428
18	1	1.5	1.5	1	266.127	223.897	381.439	401.447	169.878	208.039
19	1	2.25	1.5	0.55	336.545	349.167	677.756	686.064	241.875	202.543
20	0.5	1.5	1.5	0.55	243.073	276.996	379.030	407.188	308.992	301.279
21	0.5	3	1.5	0.55	202.097	206.184	220.345	260.854	141.838	158.562
22	1	2.25	1.5	0.55	363.884	349.167	658.162	686.064	257.165	210.038
23	1	3	1	0.55	147.399	152.052	438.491	412.818	103.835	145.370
24	1.5	2.25	1	0.55	171.275	169.364	164.261	191.414	151.740	141.279
25	1	1.5	2	0.55	282.536	284.129	565.981	545.581	189.467	120.799
26	0.5	2.25	1	0.55	305.848	366.687	696.045	711.493	294.320	220.025
27	1	1.5	1.5	0.1	135.003	137.687	105.230	112.063	96.820	121.279
28	1	2.25	1	0.1	164.694	146.923	350.016	355.006	105.510	141.279
29	1.5	2.25	2	0.55	438.010	351.821	424.361	543.55	208.496	197.588

where Y_1, Y_2 and Y_3 are the responses of AF_3, AF_4 and AF_5 production and A, B, C and D denote dextrose, peptone, malt extract and $FeSO_4$ respectively. Inverse square root and Log data transformation were recommended for AF_3 and AF_5 from Box-Cox plot for the designed model whereas no data transformation was recommended for AF_4 variant.

Table 5.3: Analysis of variance (ANOVA) for response surface quadratic model for AF_3

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob> F
Model	*0.005726*	*11*	*0.000520*	*21.00529*	*< 0.0001*
A-Dextrose	0.000379	1	0.000379	15.29733	0.0011
B-Peptone	0.000927	1	0.000927	37.40048	< 0.0001
C-Malt extract	5.28E-05	1	5.28E-05	2.129779	0.1627
D-$FeSO_4$	2.31E-07	1	2.31E-07	0.009337	0.9242
AC	0.000373	1	0.000373	15.04567	0.0012
AD	0.000338	1	0.000338	13.62723	0.0018
BD	0.000356	1	0.000356	14.35624	0.0015
CD	0.000177	1	0.000177	7.128834	0.0162
A^2	0.000197	1	0.000197	7.951738	0.0118
B^2	0.000872	1	0.000872	35.18111	< 0.0001
D^2	0.002648	1	0.002648	106.8484	< 0.0001
Residual	0.000421	17	2.47E-05		
Lack of Fit	0.000399	13	3.07E-05	5.496463	0.0563
Pure Error	2.23E-05	4	5.58E-06		
Cor Total	0.006147	28			

R^2 = 0.9314 Adj R^2=0.8871 Pre R^2=0.7561

The results of Analysis of Variance (ANOVA) for Response Surface quadratic model of AF_3, AF_4 and AF_5 variants are summarized in the ANOVA Tables 5.3, 5.4 and 5.5. The F values 21.01, 15.35 and 39.4 (AF_3, AF_4 and AF_5 respectively) imply that the model was significant indicating merely 0.01% chance for noise. The regression equations indicate that the coefficient values of R^2 for AF_3, AF_4 and AF_5 are 0.9314, 0.9243 and 0.9563 respectively. In case of AF_3, AF_4 and AF_5, the Pred R^2 values (0.7560, 0.7237 and 0.8925) were in reasonable agreement with adj R^2 (0.9192, 0.8738 and 0.932). Adequate (Adeq) precision measures the signal to noise ratio which generally should be greater than 4 for the effective model. The estimated Adeq precision ratio of 17.908, 16.305 and 23.892 for AF_3, AF_4 and AF_5 production respectively indicated an adequate signal which proved that both the developed models were significant. We found that A, B, AC, AD, BD, CD, A^2, B^2 and D^2 were the significant model terms for AF_3 fraction (Table 5.3).

Table 5.4: Analysis of variance (ANOVA) for response surface quadratic model for AF_4

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob> F
Model	*1035972.46*	*11.00*	*94179.31*	*15.35*	*< 0.0001*
A-Dextrose	32655.68	1.00	32655.68	5.32	0.0339
B-Peptone	64240.94	1.00	64240.94	10.47	0.0049
C-Malt extract	16238.06	1.00	16238.06	2.65	0.1222
D-$FeSO_4$	3983.68	1.00	3983.68	0.65	0.4315
AC	156584.87	1.00	156584.87	25.52	< 0.0001
BC	38207.07	1.00	38207.07	6.23	0.0232
BD	64000.35	1.00	64000.35	10.43	0.0049
A^2	167080.59	1.00	167080.59	27.23	< 0.0001
B^2	339341.38	1.00	339341.38	55.31	< 0.0001
C^2	53587.20	1.00	53587.20	8.73	0.0089
D^2	385360.38	1.00	385360.38	62.81	< 0.0001
Residual	104304.99	17.00	6135.59		
Lack of Fit	95390.18	13.00	7337.71	3.29	0.1297
Pure Error	8914.81	4.00	2228.70		
Cor Total	1140277.45	28.00			
R^2 = 0.9243	**Adj R^2=0.8486**		**Pre R^2=0.7237**		

Table 5.5: Analysis of variance (ANOVA) for response surface quadratic model for AF_5

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob> F
Model	*0.898439257*	*10*	*0.089843926*	*39.4062467*	*< 0.0001*
A-Dextrose	0.068919671	1	0.068919671	30.22870559	< 0.0001
B-Peptone	0.176446577	1	0.176446577	77.39084542	< 0.0001
C-Malt extract	0.00077383	1	0.00077383	0.33940786	0.5674
D-$FeSO_4$	0.004269765	1	0.004269765	1.872752333	0.1880
AC	0.02109196	1	0.02109196	9.251098179	0.0070
BC	0.032218911	1	0.032218911	14.13146586	0.0014
BD	0.054352082	1	0.054352082	23.83924731	0.0001
B^2	0.071940245	1	0.071940245	31.55355272	< 0.0001
C^2	0.019022322	1	0.019022322	8.343338742	0.0098
D^2	0.516049754	1	0.516049754	226.343448	< 0.0001
Residual	0.041038942	18	0.002279941		
Lack of Fit	0.033072756	14	0.00236234	1.186183645	0.4793
Pure Error	0.007966185	4	0.001991546		
Cor Total	0.939478198				
R^2 = 0.9563		**Adj R^2 = 0.9320**			**Pre R^2 = 0.8925**

In this case of AF_4 variant, A, B, AC, BC, BD, A^2, B^2, C^2 and D^2 were found to be significant model terms (Table 5.4) whereas A, B, AC, BC, BD, B^2, C^2, D^2 were found to

be the significant model terms for AF_5 variant (Table 5.5). For achieving the significant model, both Adj R^2 and Pred R^2 should be high. Therefore, the model reduction was carried out for all the three fractions which have $p<0.05$ from the model which render the developed models more robust (Table 5.3, 5.4 and 5.5). The statistical results (Tables 5.3, 5.4 and 5.5) also signify that the designed models were reasonably good to predict the responses of the systems. The contour plots (Fig. 5.6, 5.7, 5.8) have been constructed to illustrate the individual and interactive effects of dextrose, peptone, malt extract and $FeSO_4$ on AF_3, AF_4 and AF_5 production. Each contour plot represents the effects of two variables while the other two were kept constant (at mid-point).

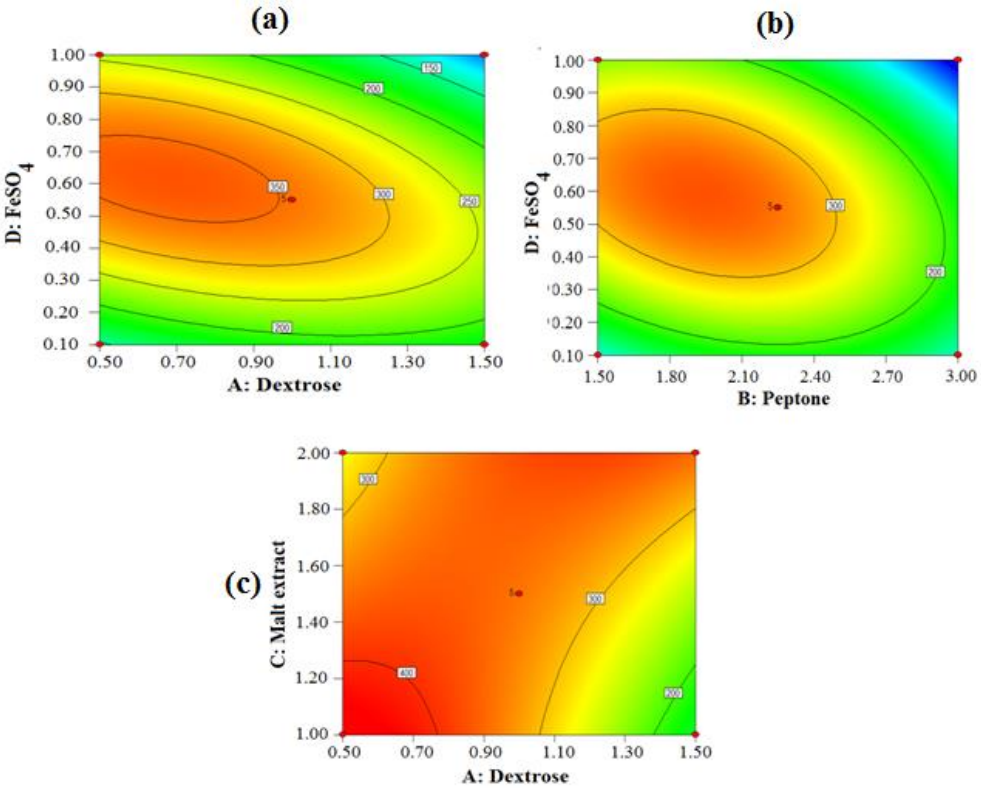

Fig. 5.6: Response surface plots showing the effects of (a) dextrose and $FeSO_4$, (b) peptone and $FeSO_4$ and (c) malt extract and dextrose on AF_3 production.

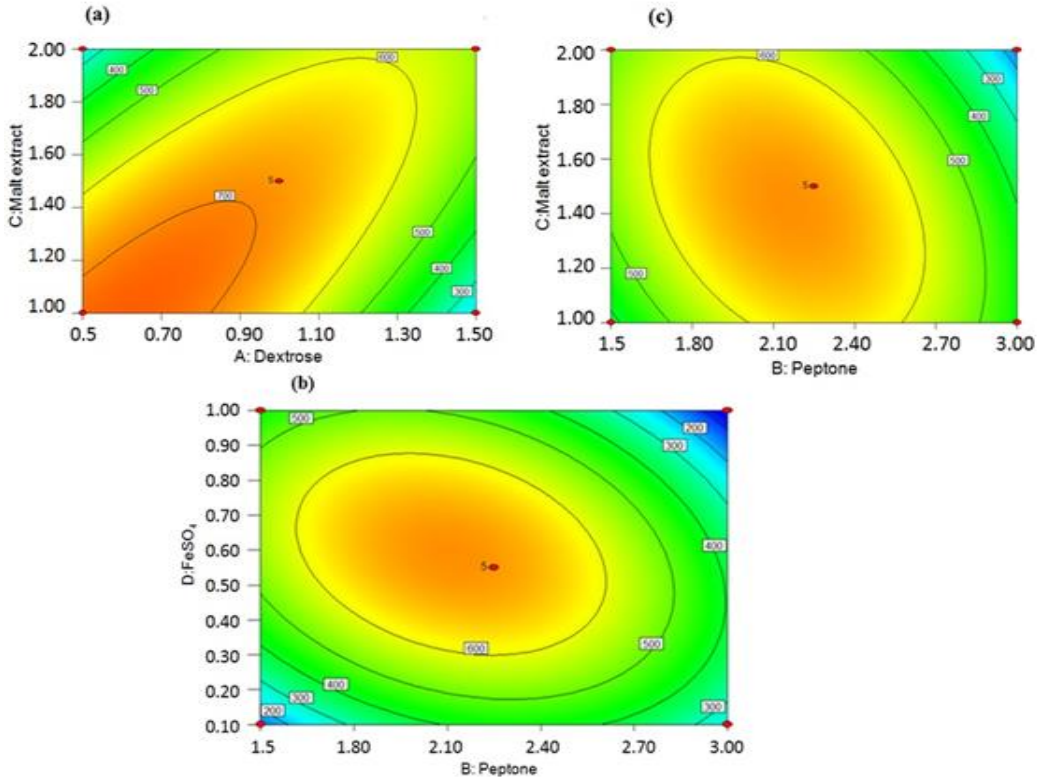

Fig. 5.7: Response surface plots showing the effects of (a) dextrose and malt extract, (b) peptone and $FeSO_4$ and (c) peptone and malt extract on AF_4 production.

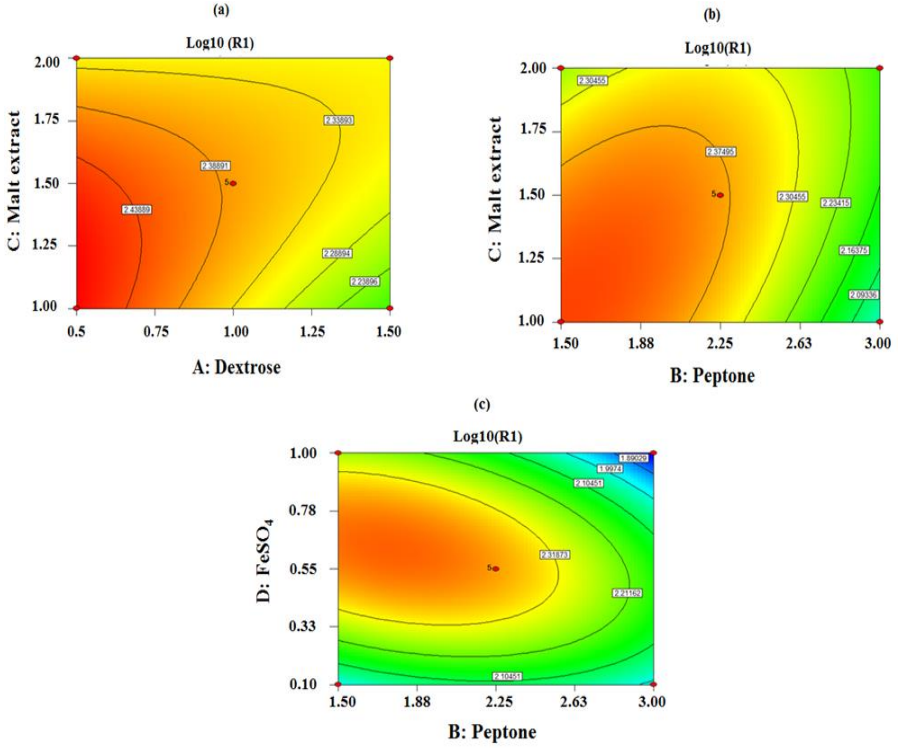

Fig. 5.8: Response surface plots showing the effects of (a) dextrose and malt extract, (b) peptone and malt extract and (c) peptone and $FeSO_4$ on AF_5 production.

5.3.1.4. Validation of the designed model

HPLC chromatograms corresponding to the production of three antifungal variants in mTSB, M_1 and M_2 media formulations are shown in Fig. 5.9a, 5.9b and 5.9c respectively. Two media formulations M_1 and M_2 were selected from Box-Behnken statistical analysis (Table 5.6). Production of AF_4 was found maximum in the M_1 media formulation that tantamounted to 702.96±9.5 mg/L and was found closer to the predicted value 732.64 mg/L (Table 5.6). M_2 media formulation enhanced the production of AF_4 variant to about 441.9±15 mg/L which is in close agreement to the predicted value 472.39 mg/L. Production of AF_4 increased to about 3.9 and 2.5-fold in M_1 and M_2 production media respectively compared to the mTSB media (186.4±6.1 mg/L) (Fig. 5.9a and 5.9b). The predicted and experimental values are in agreement (Table 5.6) to indicate that the developed model was adequate and reliable for predicting the production of AF_4 variant.

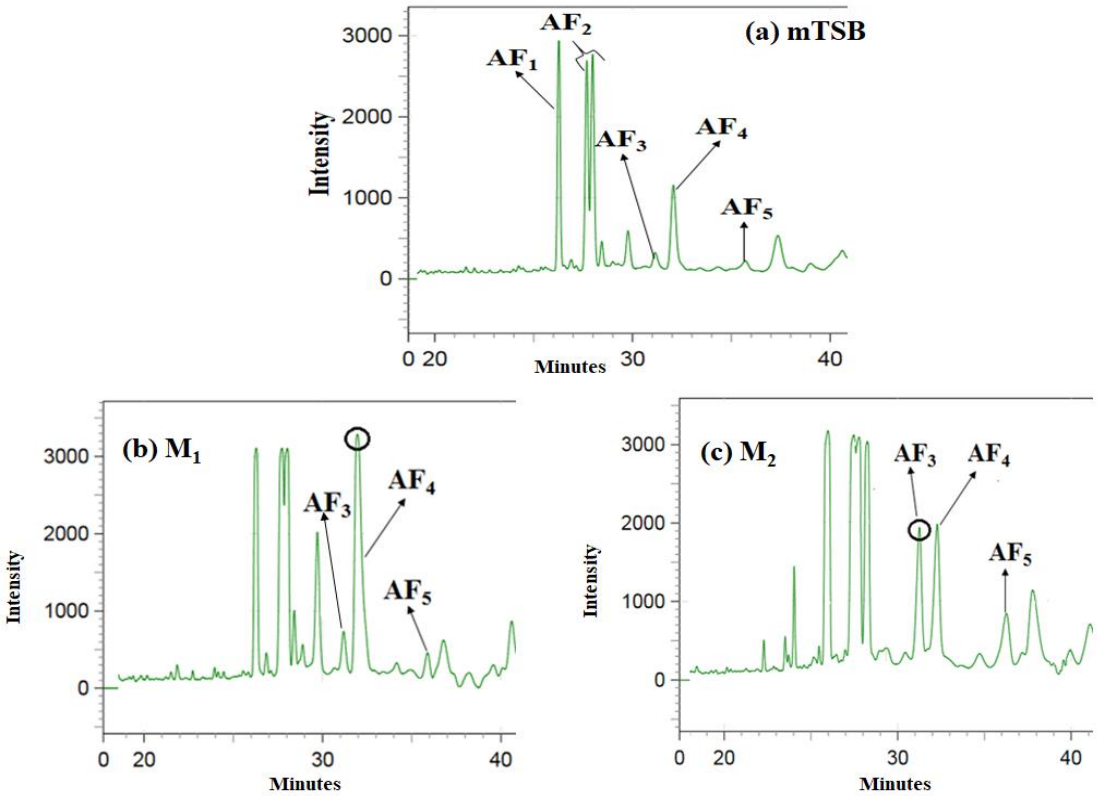

Fig. 5.9: RP-HPLC chromatograms showing the production of AF variants in mTSB, M_1 and M_2 media formulations

Likewise, *B. subtilis* RLID 12.1 produced 441.94 mg/L and 263.15 mg/L of AF_3 and AF_5 variants respectively in the M_2 media composition which were in close agreement with the predicted values of 497.63 mg/L and 328.01 mg/L respectively (Table

5.6). Production of AF_3 and AF_5 augmented to about 11.9 and 3.9-fold in M_2 production media respectively compared to the mTSB media (37.26 mg/L and 67.48 mg/L) for AF_3 and AF_5 respectively (Fig. 5.9a and 5.9c). No significant improvement in the production of AF_3 and AF_5 was observed in the M_1 formulation (Fig. 5.9b).

Table 5.6: Validation of AF_3, AF_4 and AF_5 production in the respective optimized media in comparison to mTSB (Each data point with (±) represents standard error from three experiments).

	Media	**Concentration (mg/L)**		**Fold increase**
		Experimental	**Predicted**	
AF_4	mTSB	186.40 ± 2.1		**3.8**
	M_1	702.96 ± 7.2	732.64	
AF_3	mTSB	37.26 ± 3.6		**11.9**
	M_2	441.94 ± 5.7	497.63	
AF_5	mTSB	67.48 ± 2.6		**3.9**
	M_2	263.15 ± 5.2	328.01	

5.3.2. Standardization of production media conditions for selective enhancement of AF variants

Standardization of production conditions were performed for two purposes, first was to improve the yield of AF_3 and AF_5 and second to increase the selective enhancement of AF_4 variant production.

5.3.2.1. Effect of aeration condition with respect to temperature

Production of AF_3, AF_4 and AF_5 varied under shaking and static condition at 30 and 37°C$\pm$0.5 in both M_1 and M_2 media formulations. AF_3 production was found to rise under static condition at both 30±0.5 and 37±0.5 °C (Fig.5.10a and 5.10b) being maximum at 30 ±0.5 °C about 515 ± 15.5 mg/L (Fig. 5.10b). Production of AF_5 was also found to be high at 30±0.5 °C under static condition about 312 ± 7.6 mg/L (Fig. 5.10b). Nonetheless, the production of AF_4 was found high (702.96 ± 7.2mg/L) in M_1

formulation under shaking condition at 37°C rather than at 30°C and static condition (Fig. 5.10a).

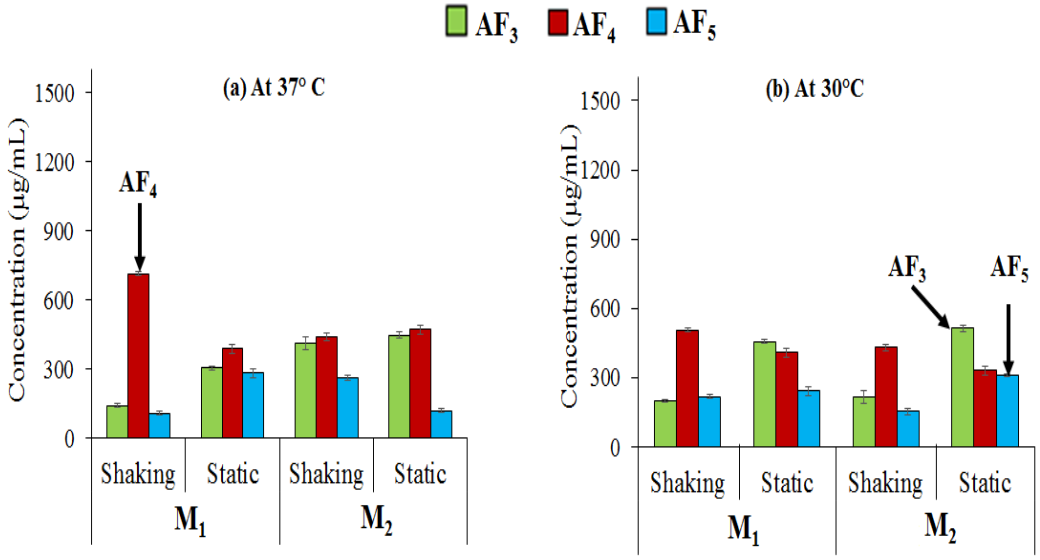

Fig. 5.10: Effects of shaking and static conditions in the production of AF_3, AF_4 and AF_5 fractions at a) 37°C and (b) 30°C. Each data point represents mean result ± standard error (error bars) of experiments performed in triplicates. (Arrow denotes the increase in production in the respective condition).

5.3.2.2. Effect of $MnSO_4$ and NaCl

$MnSO_4$ (2mM) and 0.5% NaCl displayed significant impacts on the production of three variants at 30°C (Fig. 5.11). New formulations derived modifying M_2 and M_1 were termed as M_3 and M_4 respectively. Under shaking condition (Fig. 5.11), the maximum production of 954.8±10.8 mg/L was achieved in M_3 media composition at 30°C. AF_4 variant production improved by 5.1-fold when its production was compared using mTSB (Table 5.7). The HPLC chromatogram showing the improvement in the production of AF_4 variant is marked in Fig. 5.12b. The production of AF_3 and AF_5 variants were found to increase by about 25.8 and 7.4-fold under the static condition in M_3 media formulation compared to mTSB (37.26 ± 3.6 and 67.48 ± 2.6 mg/L respectively) and the corresponding HPLC chromatogram is shown in the Fig. 5.12a. The maximum production of AF_3 and AF_5 variants were found to be 920.3±18.7 and 501.2 ± 12.3 mg/L respectively (Fig. 5.11). No significant improvement in the production of AF_4 variant was observed in the M_3 media composition under static condition (Fig.5.11).

Similar to M_1, M_2 and M_3 the proportions of three variants varied in responses to static and shaking conditions when M_4 media composition was used. However, M_3 media formulation was found superior to M_4 as it showed the elevated production of all the three variants substantially as compared to M_4 media composition (Fig. 5.11). The production of all the three variants was relatively very less when M_3 and M_4 formulations was used at 37°C.

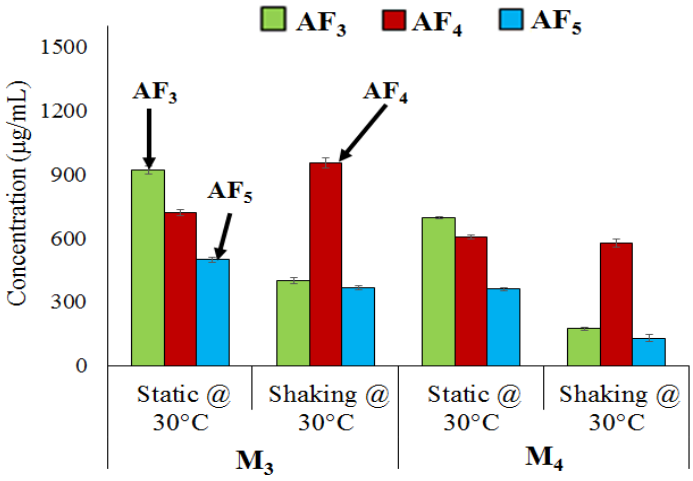

Fig. 5.11: Effects of 0.5% NaCl and 2mM $MnSO_4$ in the production of AF_3, AF_4 and AF_5 variants. Each data point represents mean result ± standard error (error bars) of experiments performed in triplicates (Arrow denotes the increase in production in the respective condition).

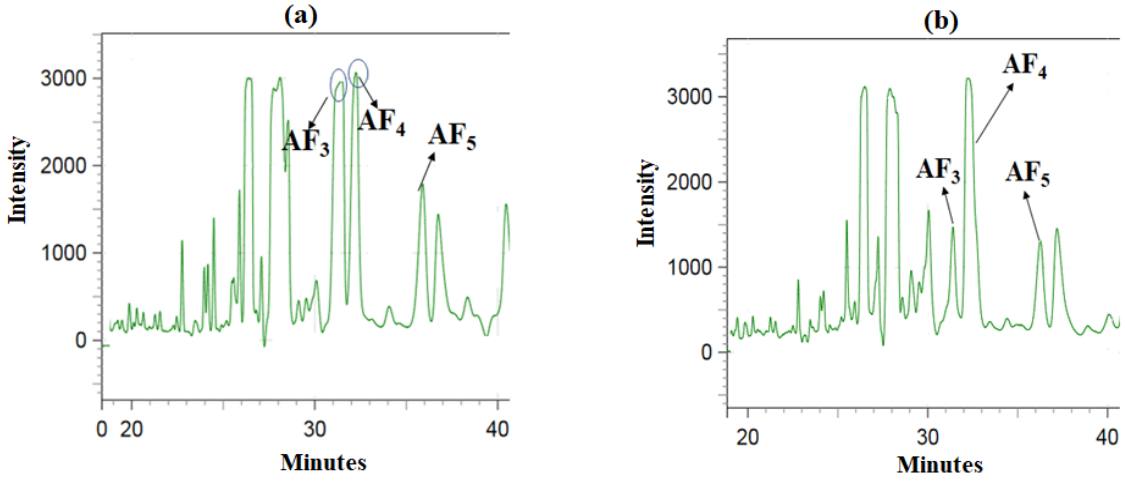

Fig: 5.12: HPLC chromatograms showing the variation in the production of AF_3, AF_4 and AF_5 variants in M_3 formulation under (a) static and (b) shaking conditions.

5.3.2.3. Exposure to heat-killed *C. albicans*

In the process of selective enhancement of AF_4 production, the *B. subtilis* RLID 12.1 culture was exposed to heat inactivated *C. albicans* in the optimized production media conditions (M_3 media at 30°C±0.5 under shaking condition). On exposure to heat-inactivated *C. albicans* (Fig. 5.13b), the production of AF_4 variant reduced to about 805.7±11.7 mg/L with 43% selectivity (Table 5.7).

5.3.2.4. Exposure to Calcium-alginate beads

Increase in the $CaCl_2.2H_2O$ concentration from 1 to 3% did not influence significant changes in case of AF_1, AF_4 and AF_5 production although 2% $CaCl_2.2H_2O$ was found to be optimum as the bead extract exhibited increased immobilization of AF_2 variant ranging from 33-44%. Increase in the sodium alginate concentration from 1.3 to 2% enhanced the immobilization of AF_1 (31 to 33%) and AF_2 (38-51%) into the beads. Change in the sodium alginate concentration from 1.3 to 2% improved the selective yield of AF_4 variant (of about 1170.9 ±10.2 mg/L) in the CFS extract from 66 to 72%. Production of AF_3 variant was found to get suppressed in the presence of Ca-alginate beads. HPLC chromatogram displaying the proportions of AF variants in the CFS extract and bead extract is shown in Fig. 5.13c and 5.13d.

Less amounts of AF_4 and AF_5 (7-10%) were observed in the extract of bead size (1.0-1.5 mm diameter) whereas bead size of 2.7-3.0 mm diameter increased the immobilization of AF_4 variant (31%) along with AF_1 and AF_2 variants. Immobilization of AF_5 was found to be less (18%) as compared to other AF variants.

5.3.2.4.1. Dose dependent study of $CaCl_2.2H_2O$

Addition of 0.5 and 1 mM $CaCl_2.2H_2O$ in M_3 media improved the production of all the five variants (Fig. 5.13e and 5.13f respectively). However maximum AF_4 variant production (of about 1137.5±8.4 mg/L) was achieved at 1mM concentration with 37% selectivity (Table 5.7).

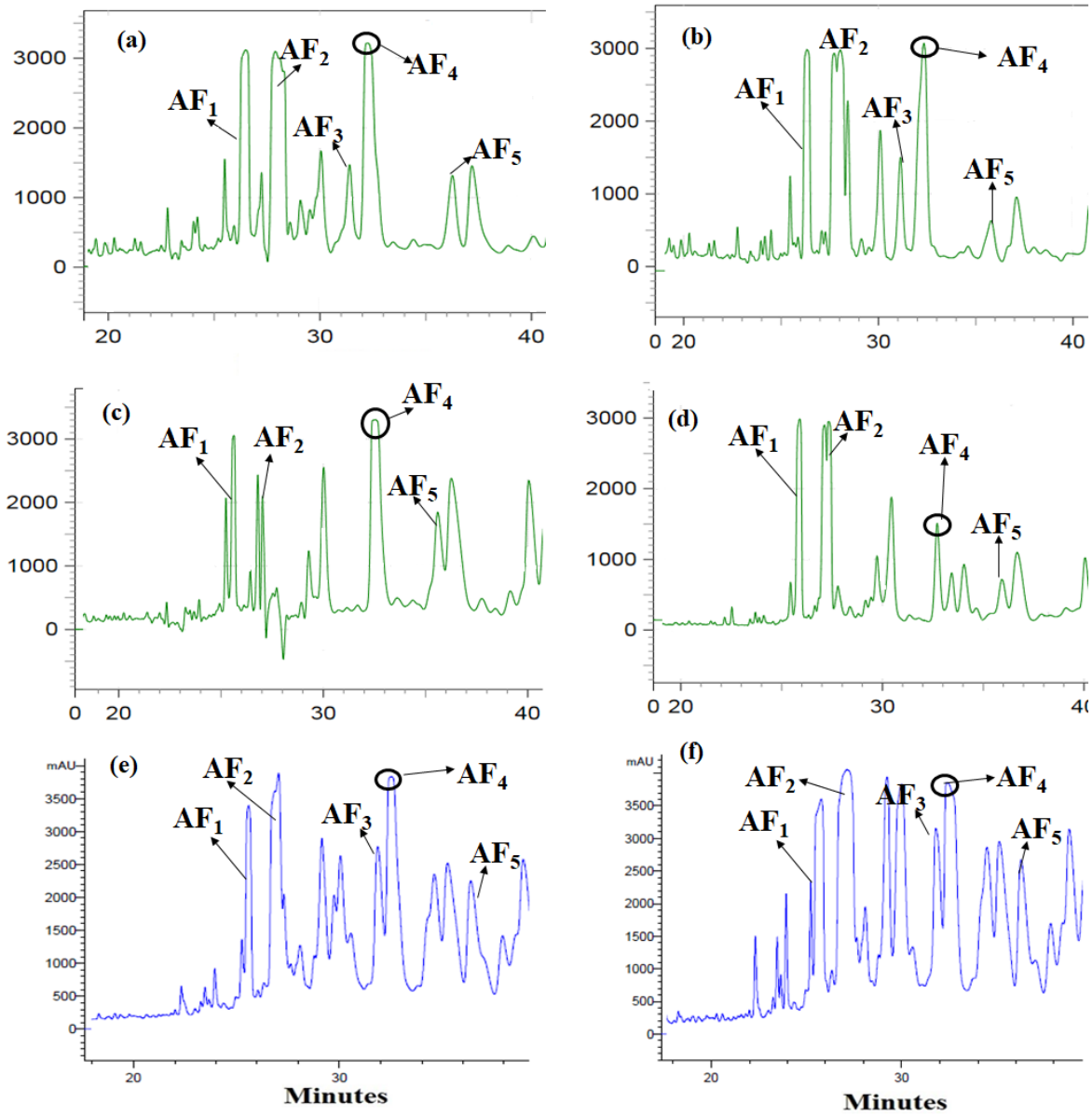

Fig.5.13. HPLC chromatograms depicting the proportions of AF variants (under shaking condition at 30 °C) in M_3 media (Control) (a), in the presence of heat-killed *C. albicans* (b), in the presence of Ca-alginate beads (2% sodium alginate and 2% $CaCl_2.2H_2O$): CFS extract (c) and bead extract (d) and calcium ions at 0.5mM (e) and 1mM (f) concentrations.

5.4. DISCUSSION

From BBD, the specific media composition suitable for AF_4 production was identified as M_1 formulation with 3.8-fold increase in production compared to mTSB and M_2 formulation (Table 5.6). M_2 media composition was found specific for AF_3 and AF_5 with the significant increase in the production (11.8 and 3.9-fold respectively) compared

to mTSB and M_1 (Table 5.6). Although the factors influencing the production of three lipopeptide variants were found similar (Fig. 5.3), BBD statistical method clearly shows that nutrient requirements of lipopeptide variants were different as their yield was determined by different proportion of the selected factors. Dextrose was considered as the main carbon source due to its size, rapid uptake, rapid metabolism and cellular energy conversion (Kumar and Johri, 2012). Dextrose was also found to be the best carbon source for antifungal lipopeptide production in *B. amyloliquefaciens* and *B. subtilis* K1 (Chollet-Imbert et al., 2009; Pathak, 2011). Peptone which is a mixture of several kinds of amino acids acts as a key nitrogen sources in the biosynthesis of antifungal lipopeptides (Davis et al., 1999). Role of peptone in antifungal lipopeptide production has been reported in *B. subtilis* TrigoCor 1448 and *B. subtilis* S499 (Pryor et al., 2007; Jacques et al., 1999). Of note, malt extract plays a role of both carbon and nitrogen sources as it contains enriched and easily fermentable carbohydrate source (>=70%, maltose and dextrose), protein (>=5.0%), vitamins and cofactors necessary for the growth of microorganisms (Heron, 1966; Wokes and Klatzkin, 1949). Use of malt extract in the improvement of the antifungal lipopeptide production by *Bacillus* spp. has not been reported before, rather maximum bacteriocin production by *Enterococcus faecium* BS13 was reported with 1.07% malt extract (Vandana, 2015). Shih et al., (2009) observed that 0.93% of maltodextrin (a product of malt extract) had increased the production of antifungal lipopeptide in *B. subtilis* S3. Like the earlier report (Mizumoto and Shoda, 2007), organic nitrogen sources have more positive effect on the production of AF_3, AF_4 and AF_5 compared to inorganic nitrogen sources. (Fig. 5.3b, 5.3d and 5.3f).

Although significant increase in the production of AF_3 and AF_5 was observed in M_2 media formulation, the yield was still found less. Similarly, in case of AF_4, the production was induced substantially in M_1 and M_2 media formulation compared to AF_3 and AF_5 variants, but the production of AF_1 and AF_2 were still found considerably high which limits the increase of selectivity % (Fig. 5.9b and 5.9c). To overcome these limitations, further investigation was carried out by modifying M_1 and M_2 media formulation by adding NaCl and Mn^{2+} ions and changing the environmental conditions like aeration with respect to temperature. Interestingly, it was observed that in M_3 media composition, the AF_4 production was higher in shaking condition at 30°C whereas the production of AF_3 and AF_5 were higher under static condition at 30°C (Fig. 5.12). In static condition, *Bacillus* spp. produce biofilms, where the cells remain in their

metabolically active state for a longer period and delay the sporulation which ultimately results in the maximum utilization of nutrients (particularly more carbon sources are utilized) increasing the production of iturin A (Mizumoto and Shoda, 2007; Mizumoto et al., 2007; Zohora et al., 2013). But in submerged condition, rapid nutrient utilization, oxygen, heat and mass transfer occur which results in sporulation (Mizumoto et al., 2007; Zohora et al., 2013). Perhaps, AF_3 might influence the biofilm formation whereas AF_4 production is commensurate with sporulation. In this regard, the contribution of bacillomycin D, bacillomycin L and surfactin in biofilm formation by *Bacillus* sp. was already reported (Xu et al., 2013; Luo et al., 2015).

In general, *B. subtilis* possess active transport systems for both manganese and iron ions which act as cofactors for many enzymes involved in the metabolite production (Eisenstadt et al., 1973; Miethke et al., 2006). In our study, we found that $FeSO_4$ at moderate concentration of about 0.55 mM and 0.74 mM ameliorated the production of AF_4 and AF_3/AF_5 respectively under shaking condition at 37°C wherein $MnSO_4$ at 2mM concentration along with 0.5% NaCl improved the production of AF variants with respect to aeration condition at 30°C (Fig. 5.10 and 5.11). At 37°C, $MnSO_4$ and 0.5% NaCl (M_3 formulation) showed insignificant effect on the production of AF variants as compared to $FeSO_4$. Contrarily, the combination of $FeSO_4$ and NaCl in M_1 media formulation plummeted the production of all the three compounds at 37°C. Sodium chloride has been reported to regulate the production of extracellular matrix necessary for biofilm formation in the bacterial cells (Kavamura and Melo, 2014). Combination of solutes (sugar or salt) and manganese ions were reported to promote the biofilm formation *via* histidine protein kinase pathway (Shemesh and Chai, 2013; Mhatre et al., 2016). Fickers and his colleagues (2008) found that the proportions of odd and even numbered mycosubtilin homologues vary depending on the temperature. According to Ohno et al. (1993) both temperature and aeration conditions affect the production of iturin lipopeptides. However, according to our findings described above, we hypothesize that the metabolic flux and the proportion of the coproduced antifungal variants can be controlled by the temperature and metal ions under static and shaking conditions. Influence of ferrous and manganese ions as supplements were also recorded in case of lipopeptides production in *B. amyloliquefaciens* B128, *B. amyloliquefaciens* Q-426 and *B. subtilis* ATCC 21332 (Lin et al., 2007; Zhao et al., 2013; Wei and Chu, 1998; Huang et al., 2015).

The major challenge in the industrial production of lipopeptides belonging to *Bacillus* spp. is the selectivity as they co-produce several lipopeptide homologues/isoforms with different therapeutic efficacy (Rangarajan and Clarke, 2015). Designing the media targeting the selective enhancement of specific lipopeptide variant helps the production scale up in the bioreactor and reduces the purification steps and cost. The results summarized in Table 5.7 indicate the improvement of AF_4 production selectivity at different stages of optimization process. Two media formulations M_1 and M_2 selected from Box-Behnken statistical analysis showed increase in selectivity of AF_4 variant production to about 33% and 23% compared to the mTSB media (17% selectivity). High production of cytotoxic and less effective AF_1 and AF_2 variants in mTSB, M_1 and M_2 media formulations limited the increase of the selectivity % of AF_4 variant. Importantly, the use of M_3 media formulation increased the selectivity of AF_4 variant to about 44% (Table 5.7). Two different supplementations were made for improving the selectivity of AF_4 variant, heat-killed *C. albicans* and Ca-alginate beads. Production of antifungal lipopeptides of *B. subtilis* RLID 12.1 begins from 36 h of incubation (Ramya et al., 2018), therefore the supplementation was carried out at the 24^{th} h of incubation of the bacterial culture (just before the lipopeptide production starts).

As the antimicrobials in the eco-habitat are produced or induced as a part of defence mechanism against the competing microorganisms to maintain the community structure, we used heat-inactivated *C. albicans* as an external stimulus for induction. Supplementation of heat-inactivated *C. albicans* in M_3 media formulation affected the selectivity (43%) with the nominal decrease in the production of AF_4 variant (Table 5.7), although a slight increase in AF_3 production was observed (Fig. 5.13b). Similar induction study was reported in case of *B. amyloliquefaciens* P11 where the heat-inactivated *A. parasitic*us was used and the expression of genes related to sfp (surfactin), lpa-14 (iturin A), ituD (iturin A), sboA (subtilosin A) and fenA (fengycin A) genes were studied using quantitative Real Time PCR methodology (Leães et al., 2016).

Process conditions involving Ca-alginate beads was found to be effective as they enabled the selective and augmented production of AF_4 variant. Analysing the HPLC chromatogram of CFS extract and the respective bead extract (Fig. 5.13c and 5.13d), revealed the partial immobilization of AF variants (particularly increased amount of AF_1 and AF_2) in to the beads by non-covalent adsorption (Palmieri et al., 1995) resulting in the increase of selective yield of AF_4 variant in the CFS extract to about 72% (Table 5.7). In addition to the selective production, the enhancement of AF_4 variant (of about 6.3-fold)

was also observed in the presence of Ca-alginate (Fig. 5.13c). By varying the bead preparation conditions, it was observed that the immobilization process of antifungal variants can be controlled by bead size, and sodium alginate and calcium chloride concentration. Improvement in the selectivity of AF_4 variant in the CFS extract was observed when bead size of 1.0-1.5 mm diameter, calcium chloride (2%) and sodium alginate (2%) were used. Reduced selectivity (37%) with improved AF_4 production (6.1 fold) observed in the presence of calcium ions (Table 5.7) also proves that the calcium alginate beads are responsible for the selective augmented production of AF_4 variant.

Table 5.7. Increment in the production of AF_4 variant attained using different optimization process showing the increase in fold and selectivity %.

S.No	**Growth conditions**	**AF_4 concentration (mg/L)**	**Increase in fold (compared to mTSB media)**	**Selectivity %**
1.	mTSB	186.4 ± 6.1	1	17
2.	M_3 formulation	954.8±10.8	5.1	44
3.	Addition of heat-killed *C. albicans* in M_3 media	805.7±11.7	4.3	43
4.	Addition of Ca-alginate beads in M_3 media	1170.9 ±10.2	6.3	72
5.	Addition of calcium ions ($CaCl_2.2H_2O$ - 1mM concentration)	1137.5±8.4	6.1	37

Summary and Conclusion

SUMMARY

In search of new antifungal compound producing isolates, soil sources from Goa and Dehradun were screened and a wild-type soil isolate RLID 12.1 showing antifungal activity against pathogenic *Candida* sp. and *Cryptococcus* sp. was isolated. By morphological and biochemical characterization, 16S rDNA sequencing and FAME analysis, the active strain was identified as *B. subtilis*. In *B. subtilis* RLID 12.1, the antifungal compound production was found maximum during the late logarithmic phase and declined at the early stationary phase. The antifungal compound of *B. subtilis* RLID 12.1 exhibited stability over wide pH range, temperature and metals salts and partially sensitive to proteolytic enzymes.

Five co-produced antifungal compounds (AF_1 to AF_5) were purified from wild-type *B. subtilis* RLID 12.1 using RP-HPLC and was identified as lipopeptides by TLC and MS analysis. Out of five, biochemical and molecular characterization of two variants AF_1 and AF_2 were not studied in detail as they exhibited strong hemolysis and less antifungal potency. Sequence analysis of the extracted antifungal lipopeptide homologues (AF_3, AF_4 and AF_5) of *B. subtilis* RLID 12.1 revealed a novel peptide sequence N-P-Y-N-Q-T-S-Xaa with fatty acid variant (Xaa) belonging to bacillomycin class. GC-MS analysis described that Xaa of AF_3, AF_4 and AF_5 corresponds to long carbon chain fatty acids, *anteiso*-C_{17}, *iso*-C_{17} and *iso*-C_{18} respectively where AF_3 and AF_4 differ by branching type and AF_5 by chain length. Presence of bacillomycin operon in the genome of *B. subtilis* RLID 12.1 was confirmed using the specific primer (marker).

MIC studies against 81 *Candida* species (64 no. including *albicans* and non-*albicans*) and *Cryptococcus* (17 no.) species marked the antifungal potency of the three lipopeptides in the order $AF_4 > AF_5 > AF_3$ with the MIC range 2-16, 1-8 and 2-16 µg/mL respectively. C_{17} isomers, AF_3 and AF_4 displayed quite difference in their antifungal and cytotoxicity potential. Hemolysis and cytotoxicity were observed in the order of $AF_4 < AF_3 \leq AF_5$ at its respective MIC range. In comparison, AF_4 was recognised as a promising antifungal lipopeptide which exhibited MIC_g of 3.31, 3.41, 3.48, 3.31 and 2.83 µg/mL showing 100% inhibition against *C. albicans*, *C. tropicalis*, *C. auris*, *C. glabrata* and *C. neoformans* respectively with low hemolysis value (<5%) and IC_{50} values (13.31µg/mL).

As an alternate to high $SMIC_{50}$ values of AF_4 and to handle the severe infections, studies on the interactive effect among the three variants AF_3, AF_4 and AF_5 were performed against three *Candida* sp. using checkerboard method. To date this is the first study which describes the additive effects of lipopeptide variants showing reduced cytotoxicity against mammalian cells and these combinations might serve as a potent anti-biofilm forming substitute. AF_4 and the additive combinations may be considered as an alternative to conventional antifungal agents although more trials are warranted. Based on these findings, the combinations determined can be further evaluated for its synergistic action with existing antifungal drugs as well, which can ameliorate the antifungal efficacy reducing the $SMIC_{50}$ as well as the toxicity which in turn may help to combat the rising antifungal resistance.

The major outcome of the optimization study is the design of the suitable fermentation condition for all the three variants as well as the formulation of selective production condition for the enhancement of novel and less cytotoxic AF_4 antifungal variant coproduced in *B. subtilis* RLID 12.1 with the introduction of new supplement. Increase in the yield of AF_4 variant with the improved selectivity of about 72% was achieved by using Ca-alginate beads in the formulated media comprising dextrose, peptone, malt extract, NaCl and $MnSO_4$ at 30°C under shaking condition. The selective enhancement of AF_4 variant is due to the partial immobilization of other antifungal variants AF_1 and AF_2 in to the Ca-alginate beads by non-covalent adsorption. Use of Ca-alginate beads in the bioreactor is likely to be a novel upstream production approach for maximising the yield and the productivity of AF_4 variant selectively which will help to minimize the challenges faced in downstream processing operations in case of coproduced lipopeptides. Increment in the production of AF_3 (25.8-fold) and AF_5 (7.4-fold) was attained when *B. subtilis* RLID 12.1 was grown in the same media formulation under static condition. These observations explained the combined role of temperature, metal ions and aeration conditions in controlling the proportion of lipopeptide variants of *B. subtilis* RLID 12.1. Eventually our results clearly suggest that the media optimization should be specific with respect to individual lipopeptide as it solves the limitation of selectivity, production scale up and purification cost.

Taken together, the present study reveals the finding of the most efficacious compound AF_4, finding of additive combinations with more than one antifungal peptide variants along with the negligible cytotoxicity and antibiofilm forming potential and

optimization of the media condition necessary to improve the selective production of antifungal variants. AF_4 and the additive combinations may be considered as an alternative to conventional antifungal agents although more trials including the pharmacokinetic-pharmacodynamic studies are warranted.

CONCLUSION

- Five antifungal compounds were co-produced by *B. subtilis* RLID 12.1, out of which three were identified as the novel lipopeptides with long fatty acid chain (C_{17} and C_{18}) belonging to bacillomycin class.
- Compared to the other reported antifungal lipopeptides of *Bacillus* genus, AF_4 lipopeptide variant produced by *B. subtilis* RLID 12.1 exhibited good antifungal potency with less cytotoxicity at its respective MIC range.
- MIC and MFC values of all the AF variants were found to be same for almost 95% of the tested isolates.
- C_{17} isomers (AF_3 and AF_4) which varies in the fatty acid branching type exhibited vast differences in their fungicidal action and cytotoxicity.
- Anti-*Cryptococcal* potency of AF_4 with low MIC and MFC values might render a potential candidate as the existing antifungals against the *Cryptococcus* sp. are facing limitations.
- The interactive studies performed among the three lipopeptides yielded two effective combinations (AF_3, AF_4) and (AF_3, AF_5) which exhibited an additive interactive fungicidal effect with negligible hemolysis and less cytotoxicity against four tested mammalian cell lines.
- To the best of our knowledge, this is the first report on the interactive effect among the lipopeptide isomers and homologues along with the hemolytic and cytotoxicity studies.
- Proportions of the AF variants of *B. subtilis* RLID 12.1 can be controlled by varying the temperature and metal salts under static and shaking conditions.
- Increment in the selective production of the most potent AF_4 variant (72%) was achieved using calcium alginate beads in the optimized media under shaking conditions.
- Immobilization of antifungal variants in to the Ca-alginate beads varies with bead size, sodium alginate concentration and calcium chloride concentration.

- Use of Ca-alginate beads in the bioreactor is likely to be a novel upstream production approach for selective enhancement of the lead antifungal variant

FUTURE SCOPE OF WORK

- Further structural characterization of AF variants by NMR studies
- Study related to the molecular mechanism of action of AF variants and its combinations.
- Production scaleup of the potent AF variants to the bioreactor level using the optimized media formulations for mass production.
- Study of the *in vivo* antifungal activity of the AF_4 variant and its combinations using mouse model and the analysis of their pharmacokinetic and pharmacodynamic properties.
- Study of anti-adhesive property of the evaluated AF combinations for pre-coating applications against *Candida* biofilm

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LIST OF PUBLICATIONS AND PRESENTATIONS

1. **Ramya R**, Manjari S, Namitha NN, Chakrabarti A, Thakur RL, Roy U. Evaluation of antifungal efficacy of three new cyclic lipopeptides of the class Bacillomycin from *Bacillus subtilis* RLID 12.1. Antimicrobial Agents and Chemotherapy. 2018, 62, e01457-17. doi:10.1128/AAC.01457-17. SCI Impact factor: 4.303, Scimago H index-218, SJR ranking-2.21

2. **Ramya R**, Ajay Ghosh Chalasani, Ram Lal and Utpal Roy "A broad-spectrum antimicrobial activity of *Bacillus subtilis* RLID 12.1", The Scientific World Journal, Volume 2014, Article ID 968487. http://dx.doi.org/10.1155/2014/968487. Scimago H index-56, SJR ranking-0.341.

3. **Ramya R**, U. Roy, 'Antimicrobial prowess of a *Bacillus* soil isolate' published in the Proceedings Book entitled: "Worldwide Research Efforts in the Fighting against Microbial Pathogens: From Basic Research to Technological Developments" pp.72-77, (Ed. A. Mendez-Vilas; Pub. Brown Walker Press; ISBN-13: 978-1-61233-636-7). July 2013. (Conference Proceeding).

Paper Presentations in the International Conferences held in India and abroad

1. **Ramya R**, Ram Lal Thakur and Utpal Roy 'Antimicrobial Prowess of a *Bacillus* soil isolate' a paper presentation in 2nd "International Conference on Antimicrobial Research (ICAR- 2012)" held at Lisbon University, Portugal, 21st -23rd November 2012.

2. **Ramya R** and Utpal Roy 'Antifungal substance produced by a soil isolate *Bacillus* sp. proved highly toxic against *Candida* spp. and *Aspergillus* sp.' a paper presentation in "Toxinological Society Conference on Natural Toxins (TSICON, 2013)" held at BITS Pilani Goa Campus, 19-21st November 2013.

3. Utpal Roy, Raeesh Sheikh, **Ramya** R, Ram Lal Thakur 'Antimycotic substances from wild-type *Enterococcus faecalis* APR 210 and *B. subtilis* RLID 12.1'in "World Congress of Microbes' (WCM-2) on Antifungal Drug Discovery" held at Guangzhou, China, 30th July to 1stAugust 2012.

Appendix II

Brief Biography of the Candidate

Name	Ms. Ramya R
Education	M. Tech. (Biotechnology), Kumaraguru College of Technology, Coimbatore, Tamil Nadu (2010)
	B. Tech. (Biotechnology), Bharathidasan University, Trichy, Tamil Nadu (2008)
Email	ramya.rbt@gmail.com

Work Experience:

Ms. Ramya R joined BITS Pilani K K Birla Goa campus as a research scholar in the Department of Biological Sciences. She has worked as a JRF in the DST sponsored project entitled "Screening of extremophile isolate for novel antimicrobial substances: Cloning and characterization of selected antimicrobial substances against multidrug resistant human pathogens" during 2011-2012. She was awarded with CSIR-SRF fellowship from 2013-2017. She has worked in the purification and characterization of an antifungal peptide from a wild type *Bacillus* soil isolate showing activity against yeasts. She has published two research articles as first author and one conference proceeding. She has presented her work in three conferences. She has worked as an Assistant Professor in Bannari Amman Institute of Technology, Sathyamangalam, Tamil Nadu from 2010 to 2011.

Appendix III

Brief Biography of the Supervisor

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