7 SUMMARY OF THESIS

Microbial diversity is huge and exploitation of available microbes is very important for biotechnological applications. Microbes existing in complex environments produce antimicrobial substances to protect them from the growing competition. Since, these antimicrobial substances are effective against specific microbes they can be used to treat the drug-resistance pathogenic and opportunistic pathogenic bacteria. Thus, in the present study we made an attempt to isolate antimicrobial producing strains from samples collected from different complex ecosystems and performed the molecular characterization of antimicrobial peptides.

Among the various isolates, three strains designated as GI-9, IE-3 and SKA24 that revealed high identity with *Brevibacillus laterosporus*, *Pediococcus pentosaceus* and *Streptomyces griseoflavus* respectively, were selected for further processing. The basis for selection was novel taxonomic status, antimicrobial spectrum and potential of industrial applications. Among these, strain GI-9 and SKA24 appear to be novel isolates as they differed in biochemical and molecular characteristics with their close phylogenetic relatives. Strain IE-3 was recognized as GRAS organism.

The genome of strain GI-9 and IE-3 had been sequenced and deposited at EMBL under accession numbers CAGD01000001 to CAGD01000061 and CAHU01000001 to CAHU01000091 respectively.

The antimicrobial substance produced by strain GI-9 was identified as a peptide, which are usually called as bacteriocins. The antimicrobial peptide was purified using combinations of chromatography and named as laterosporulin. Molecular weight analysis revealed it as a low molecular weight peptide (5.6 kDa), a characteristic observed for bacteriocins. Other bacteriocins properties displayed by the peptide were resistant to proteases treatment, thermo-stable, broad spectrum antimicrobial activity with wide pH range. Interestingly, it also killed non-multiplying cells of *S. aureus* and *P. aeruginosa*.

The partial N-terminal sequencing of peptide (19 amino acid) obtained was instrumental in identifying the putative biosynthetic gene of laterosporulin from the genome sequence of strain GI-9. Further analysis of flanking regions of putative biosynthetic gene provided information about the presence of components like ABC transporter, regulator and maturation genes which are essentially required for biosynthesis of a bacteriocin. Bioinformatic analysis of gene cluster showed conserved region of immunity protein associated with ABC transporter. Another gene annotated as alkyl hydroperoxide reductase exhibited similarity with disulfide oxidoreductases which is known to form the disulfide bonds.

Further crystal structure of laterosporulin at 2.0 Å resolution reveals an all-β conformation of this peptide with four beta strands forming a twisted anti-parallel β-sheet. All the six cysteine residues are intramolecular disulfide bonded, with two disulfides constraining the N-terminus of the peptide and the third disulfide crosslinks the extreme C-terminus resulting in the formation of a cyclic structure. Significance of disulfides in maintaining the in-solution assembly of the peptide was confirmed by the circular dichroism and fluorescence spectrometry analyses. Although it was found that disulfide play crucial role in maintaining the structural integrity, it did not show effect on antimicrobial property of the peptide. Despite poor sequence conservation, laterosporulin has disulfide connectivity (I-V, II-IV, III-VI) like β-defensins and a striking architectural similarity with α-defensins. Laterosporulin thus presents a missing link between bacteriocins and mammalian defensins and is a potential antimicrobial lead against especially non-multiplying bacteria. On the basis of biophysical and molecular characteristics the peptide was categorised into class IId bacteriocins.

Killing kinetics study showed it as an efficient antimicrobial with cidal killing mechanism for both Gram-positive and Gram-negative bacteria. Mode of action study with the help of fluorescence and electron microscopy suggest that growth inhibition occurs due to increased membrane permeability. Permeabilization of membrane was also found in protein leakage assay. On the basis of structural compositions, bioinformatic analysis (docking) and microscopic observation (fluorescence and electron microscopy) we predicted a possible mode of action for laterosporulin, where it was assumed that laterosporulin (monomer or trimer) cross the cell wall barrier through passive diffusion (or membrane transporters in case of Gram-negative bacteria). After that it interacts with negatively charge pyrophosphate group of lipid II or phospholipid heads, the subsequent cell lysis may be initiated with the peptide interacting with cell membrane resulting in leakage similar to the 'carpet model' of AMP action.

Laterosporulin was also explored for the killing to non-multiplying cells (dormant) of bacteria. The prepared dormant cells of *S. aureus* and *P. aeruginosa* showed increased tolerance to the antibiotics like ampicillin and rifampicin. Laterosporulin killed very efficiently the prepared non-multiplying cells of both test strain at their MIC values calculated for vegetative cells. The cytotoxicity of laterosporulin was evaluated against RBCs of rabbit and found non haemolytic.

A low molecular weight (1.7 kDa) antimicrobial peptide was purified from the supernatant of *P. pentosaceus* strain IE-3 using cation exchange chromatography and reversed-phase high-performance liquid chromatography techniques. The *de novo* sequence analysis performed using MALDI-TOF-MS showed 16 amino acid sequence as APVPFSCTRGCLTHLV for the peptide, which revealed no similarity with any reported bacteriocins. The low molecular weight peptide displayed thermo-stability and resistance to different proteases activity. Interestingly peptide treated

with reducing agent like dithiothreitol (DTT) exhibited increased activity against both Gram-positive and Gram-negative test strains in comparison to native peptide. However, peptide was found to be sensitive to oxidation as significant reduction in antimicrobial activity was observed after incubation with H_2O_2. Additionally, LMW antimicrobial peptide did not show haemolytic activity suggesting its non-cytotoxic nature. The properties like increased activity under reduced conditions and non-hemolytic nature suggests its applicability in food processing industry.

Antimicrobial peptide purified from strain SKA24 was active against bacterial and fungal test strains including fungal spores. MALDI-TOF MS revealed the molecular mass of peptide as 4.3 kDa. The peptide was active at wide pH range and up to 80 °C temperature for 30 minute. Though it was found to be haemolytic in nature, the effective killing of saprophytic fungi (*A. flavus*, *A. niger*) along with their spores reveal its potential biotechnological applications.

Overall, from this study we conclude that three novel antimicrobial peptides have been characterised. The peptide produced by strain GI-9 revealed similarities with defensin molecules produced by mammalians that are in clinical trials for therapeutic applications. Since, it is easy to express prokaryotic genes in recombinant systems, laterosporulin of this study can be taken forward for recombinant DNA technology to produce antimicrobial peptides with improved and selective inhibition spectrum. Moreover, results obtained on laterosporulin characterization in this study also suggest that it can be used for topical applications. The peptide produced by strains IE-3 and SKA24 find potential applications in food processing and other biotechnological applications.