Chapter – 7

Summary and Conclusion
Summary and Conclusion

The increasing consumer awareness and desire for natural products and processes have given emphasis to the search for natural alternatives to traditional techniques, thus preventing the use of chemical additives in processed foods to reduce contamination. In this context the thesis entitled “Antibacterial peptides of Bacillus species active against food-borne pathogens” presents the details of the study carried out to isolate Bacillus spp. from food with potent antibacterial property which can find application in food industry.

Initially, the Bacillus cultures with antibacterial activity were selectively screened from various food sources to obtain food grade cultures. Screening of Bacillus isolates from different fermented and non-fermented foods revealed that food is a novel source for isolation of bacteriocinogenic Bacillus species. The biochemical tests and molecular approaches, such as RAPD were useful tools for claiming the diversity of the Bacillus spp. in food systems. The morphophysiological and biochemical parameters, as well as genetic analysis were used for the identification of selected isolates successfully. The seven selected isolates identified includes B. subtilis Ec1, B. thuringiensis Ik15, B. licheniformis Me1, B. cereus Ik11, B. flexus Hk1, B. megaterium Pk12 and B. amyloliquefaciens Bk1. The agar streak assay method allowed the determination of the antibacterial potential of the isolates. These Bacillus isolates exhibited inhibitory activity against a broad spectrum of food-borne pathogenic and spoilage organisms, which is of crucial importance for application of these isolates and their antibacterial substance in food and therapeutic purpose.

Partial characterization of the ABPs of the seven selected isolates led us to the conclusion that they are extracellular, proteinaceous in nature with an apparent MW falling in the range of 3 to 6 kDa, and are stable at a wide range of temperature and pH. Among these seven isolates, the ABP of the culture B. licheniformis Me1 was found to be more potential than other isolates, in terms of technological properties and inhibitory spectrum against pathogens. Thus, further studies including production, purification, characterization and mode of action (MOA) of this culture’s ABP was performed. The purification scheme (ammonium sulphate (65%) precipitation - extraction with n-butanol-RP-HPLC using C-18 column) employed confirmed that the
ABP was purified and the specific activity was increased to 68817 AU/mg as compared to culture supernatant with an yield of 0.4%. The SDS-PAGE analysis of the purified ABP of *B. licheniformis* Me1 revealed an apparent MW ranging between 3 to 3.5 kDa. The production studies led to the conclusion that a higher yield of ABP (51200 AU/ml) from *B. licheniformis* Me1 was achieved when grown in an economical formulated media under optimized growth conditions. Several approaches (assays using cell reporter strains, FTIR and release of UV absorbing materials) adapted to study the MOA revealed that the target of inhibition of the ABP was on cell wall. Cell reporter based analysis also revealed that the ABP is a Lipid II interacting cationic lantibiotic and it is not bacitracin. The effect of ABP on the growth kinetics of tested pathogens indicated the bacteriolytic nature of the ABP. These observations indicate the potential use of the ABP of *B. licheniformis* Me1 for application as food biopreservatives, which was later confirmed by evaluating the biopreservative efficacy using two different methods; direct application and active packaging.

The ABP from *B. licheniformis* Me1 proved to be an efficient antimicrobial agent against food-borne pathogens in milk and milk-based food products. The particular properties of this ABP (pH and temperature tolerance, proteolytic inactivation, wide range of inhibitory activity, stability during storage) including control of pathogens in food systems and sensorily acceptability in milk, makes a convincing evidence for the safe use of this bacteriocin-like substance as biopreservatives in food products. The application of the ABP for making active films for food packaging and the potential application of such antimicrobial packages for biopreservation of food products also demonstrated promising results. Both the type of activated films (LDPE and cellulose) showed potential reduction in the population of the indicator organism, *L. monocytogenes* Scott A in packed food (cheese and panner), which signifies the use of this ABP in packaging material to control spoilage and pathogenic organisms in food. These observations indicates that the ABP from *B. licheniformis* Me1 can be used as an alternative to commonly used chemical preservatives (e.g., nitrate, NaCl) and biological antimicrobial agents, such as nisin and pediocin for food preservation. Furthermore, the application of this ABP in food system either directly or through antimicrobial packaging can be an efficient way of extending shelf-life and for providing food safety through the inhibition of spoilage and pathogenic bacteria, without altering the nutritional quality of raw materials and food products.
Chapter 7

Summary and conclusion

The toxicological safety assessment including acute and subchronic toxicity studies in rat, micronucleus test in mice, and skin and eye irritation studies in rabbits showed that the culture B. licheniformis Me1 was safe for application in food industry, and can be labelled as non-toxic, non-irritant and non-mutagenic. Furthermore, the comparison of in vitro probiotic properties of the selected Bacillus species led to the conclusion that all the culture exhibited promising probiotic properties. However, among the tested cultures, B. licheniformis Me1 exhibited remarkable in vitro probiotic properties and thus can be considered a positive trait for supplementation in food products as probiotic.

Overall, the ABP from B. licheniformis Me1 has bright prospects to be used as a food biopreservative as it displays desirable characteristics, such as strong inhibitory activity against a broad range of food-borne pathogenic/spoilage microorganisms, bactericidal MOA, stability in antibacterial activity at wide range of temperature and pH, degradation in the presence of proteolytic enzymes. Furthermore, the culture is safe for food industry application.

Future perspectives

- The isolation of potent bacteriocinogenic Bacillus cultures from different foods resulted in adding novel cultures to the culture collection centre. These isolates can be further characterized in terms of their application in food industry as biopreservatives or probiotics, and in clinical settings as alternatives to antibiotics.

- Keeping in mind, the practical interest of the potent ABP production by these isolated Bacillus strains, physical as well as genetic investigations can be carried in order to characterize and identify the ABPs for its application in food system as a novel antibacterial agent. The function of the enzymes responsible for modification reactions in the biosynthesis of ABPs produced and mechanism of the producer’s immunity can also be performed.

- DNA micro array analysis can be performed to analyse the MOA in-depth and transcriptional profiling of stress response will help develop novel reporter assays for the rapid identification of specific antibacterial substance.
Although both the packaging material proved to be an efficient carrier of the ABP, future studies with respect to physiochemical properties of the film material after incorporation of ABP are required. Furthermore, the development of active films with the ABP of \textit{B. licheniformis} Me1 on industrial scale by direct incorporation into polymer matrix and studies for determining its efficacy in control of pathogens in several processed food products are required.

Detailed toxicological studies would need to be done to obtain GRAS status of \textit{B. licheniformis} Me1 and the ABP prior to its use as a probiotic or biopreservative. Furthermore, \textit{in vivo} efficacy studies in animal models and human are required before classifying the use of this culture in food as probiotic.