CHAPTER-9

SUMMARY

Probiotics are generally regarded as safe microorganism and are now widely used in aquaculture, poultry and other livestock farming as well as in human beings for control of disease, enhancement of growth and stimulating immune response. Probiotics are replacing antibiotics in aquaculture and other livestock farming as they are becoming ineffective due to the development of antibiotics resistance among pathogens. The present study was undertaken to optimize the culture parameters, media, bacteriocin, viable cell and EPS production by the two probiotic strains *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13. Also long term preservation of the probiotics with cryoprotectants was studied. Both probiotics were isolated from the gut of shrimp *Penaeus indicus* and fish *Mugil cephalus*. The *S. phocae* PI80 and *E. faecium* MC13 secreted antimicrobial compound like bacteriocins which showed broad spectrum inhibition against diverse group of Gram positive and Gram negative bacterial strains.

This bacteriocin and biomass production by probiotic strains mainly depends on the culture parameters such as temperature, pH, salinity and media components. Hence, the environmental conditions such as temperature, pH and salinity were optimized for enhancement of bacteriocin and biomass production by probiotic strains. Temperature 35°C, pH 6.5 and salinity 1-2% were found to be optimum for increasing yield of bacteriocin and viable cells. The commercially available Lactobacillus MRS growth medium was used for media optimization. The MRS media components were replaced by some other carbon and nitrogen sources. The concentrations of medium components were optimized by statistical based experimental designs.
such as two level factorial design and central composite design. Optimum MRS medium components revealed three fold higher bacteriocin activity than normal MRS medium.

The MRS medium is cost expensive medium for large scale bacteriocin and biomass production by probiotic bacteria. Therefore, two low cost culture media were formulated by response surface methodology using central composite design. Optimum media components showed higher bacteriocin activity which was two fold higher than normal MRS medium as well as three fold less cost than commercially available MRS medium.

However, mass culture of probiotic bacteria is required for the control of pathogenic Vibrio spp., and Listeria spp., in various shrimp hatcheries and sea food industries. Hence, various carbon and nitrogen sources were tested along with low cost medium 1. At every 6 h intervals, carbon and nitrogen sources were added with medium and also the pH of the medium was adjusted to neutral pH for a period of 48 h. The carbons sources such as lactose (20 g L⁻¹) and sucrose (20 g L⁻¹) were found to increase the maximum biomass (Optical density, wet weight, dry weight and total viable cells) production by S. phocae PI80 and E. faecium MC13. Moreover, the nitrogen source yeast extract (30 and 20 g L⁻¹) was found to be optimum for mass culture of S. phocae PI80 and E. faecium MC13. The combination of lactose (20 g L⁻¹) and yeast extract (30 g L⁻¹) were found to improve the biomass production by S. phocae PI80, which is five times higher than the biomass obtained from normal medium 1. Similarly the mass culture of E. faecium MC13 was increased (five times) by the fed batch fermentation through the addition of sucrose (20 g L⁻¹) and yeast extract (20 g L⁻¹).

The bacteriocin gene present in the probiotic bacteria were confirmed by amplification and sequencing of bacteriocin genes. Gene specific primers were used for amplification of
bacteriocin gene from probiotic bacterium. Bacteriocin genes of *S. phocae* PI80 and *E. faecium* MC13 showed high level similarity to the genes of bacteriocin thermophilin and enterocin.

Probiotic strains *S. phocae* PI80 and *E. faecium* MC13 also secrete exopolysaccharides (EPS). Exopolysaccharides from probiotic bacteria are having potential applications in various industries. Hence, culture parameters were optimized for enhancement of EPS production. Temperature 35°C, pH 6.5 and salinity 1-2% were found to be optimum for increasing yield of exopolysaccharide from *S. phocae* PI80 and *E. faecium* MC13. The carbons sources such as lactose (20 g L\(^{-1}\)) and sucrose (30 g L\(^{-1}\)) were found to increase the maximum exopolysaccharide production. Moreover, EPS from *S. phocae* PI80 and *E. faecium* MC13 were purified through the gel filtration chromatography using protein purification system. Sugar molecules present in the exopolysaccharides were analyzed by thin layer chromatography (TLC), which revealed arabinose, fructose and galactose for EPS of *S. phocae* PI80. In contrast, EPS of *E. faecium* MC13 revealed two sugars spots such as glucose and galactose in TLC plate. The molecular mass of EPS from *S. phocae* PI80 and *E. faecium* MC13 were found to be 2.8 × 10\(^5\) Da and 2.0 × 10\(^5\) Da. EPS of *S. phocae* PI80 and *E. faecium* MC13 was found to be high viscous in nature and also expressed better antioxidant and antibiofilm activity. Both exopolysaccharide also showed good bioflocculating and emulsifying activities than the commercial polysaccharides such as xanthan gum, guar gum and gelatin. In addition, thermal properties of EPSs were analyzed using differential scanning calorimetry (DSC). Melting point of EPS from *S. phocae* PI80 and *E. faecium* MC13 were found to be different from the EPS of other lactic acid bacteria.

Due to wide industrial applications at various conditions, the maintenance of high cell viability of probiotic bacteria becomes important. Hence, the cells of *S. phocae* PI80 and *E. faecium* MC13 were freeze dried with different cryopreservatives. The freeze dried cells were
stored at different temperatures. At every one month intervals, total viable cells were counted for the period of six months. More than 70% relative cell viability of *S. phocae* PI80 and *E. faecium* MC13 were observed when freeze dried with trehalose at storage temperatures -20°C and 4°C. Also, the cells of *S. phocae* PI80 and *E. faecium* MC13 were freeze dried with different combinations of cryoprotectants. The combinations such as trehalose + skim milk and trehalose + sucrose were found to retain maximum relative cell viability of *S. phocae* PI80 and *E. faecium* MC13 at storage temperature -20°C.

Recently, the probiotic bacteria are preserved using microencapsulation techniques. Also, most of the probiotic bacteria were dead when suddenly introduced in to the gastric and intestinal system. Hence, the cells of *S. phocae* PI80 and *E. faecium* MC13 were entrapped in to the alginate-chitosan capsules. Half of the microcapsules were dried at 37°C and subsequently stored at different temperatures. At every one month intervals, total viable cells and bacteriocin activity of preserved cells were observed for the period of six months. Microcapsules successfully preserved probiotic bacterial cells during the long term storage and also effectively protected it against simulated gastric and intestinal fluid at 37°C. Moreover, stability of microcapsule was analyzed in *in vivo* conditions using male wistar rats. Here, capsules were broken and directly released probiotic cells to the intestinal tract of rats within 6 h of oral administration period. This was an important feature of alginate bead microencapsulation as it both protected probiotic cells from gastro-intestinal juice and released the probiotic strains at appropriate place i.e. intestine so to adhere there and carry out their probiotic role.