SYNOPSIS

Molecular genetic studies of pediocin-like bacteriocin in *Pediococcus, Lactobacillus* and *Enterococcus* sp.

Bacteriocins are ribosomally synthesized antimicrobial peptides usually inhibitory to the species that are closely related to the producing bacteria. Pediocin PA-1/AcH (pediocin PA-1) is a class IIa bacteriocin produced by a lactic acid bacteria (LAB), *Pediococcus acidilactici*. The pediocin PA-1 bacteriocin was found to have a strong anti-listerial activity and a very conserved N-terminal consensus motif – YGNGV-. Several species of *Pediococcus* genera are known to produce this bacteriocin. The pediocin PA-1 molecule targets the cell membrane of other bacteria with their amphiphilic helical structure and gets inserted into the membrane, leading to the formation of pores and cell lysis. The genetic determinants for the biosynthesis of the pediocin PA-1 is encoded in an operon, comprising of four genes *pedA*, *pedB*, *pedC* and *pedD*. The pediocin PA-1 was initially isolated from *Pediococcus acidilactici* H and PAC 1.0 strains. Subsequently, several reports on the production of intergeneric and interspecific pediocin PA-1 in different species of LAB, like *Ped. parvulus*, *Ped. pentosaceus*, *Lactobacillus plantarum*, *Lact. rhamnosus*, *Lact. casei* and *Lact. Paracasei*, and non-LAB *Bacillus coagulans* was observed. This indicates that there is a natural distribution of pediocin PA-1 among and between species of LAB. However, the role of mobile genetic elements (MGEs) and the mechanism of transfer of the bacteriocin in such intergeneric and interspecific LAB has not been described.

The production of the same bacteriocin by different bacteria exists in nature because of their plasmid encoded phenotype; hence characterization of these plasmids is necessary. As a consequence, there is a need to portray the MGEs involved in the transfer of such bacteriocin among LAB at their genetic level by using molecular biology techniques. Plasmids with bacteriocin encoding genes and MGEs facilitates in
the expansion of novel cultures and in developing cloning vectors, as well as starter cultures with bio-preservative and bio-protective ability in the fermentation of dairy and meat products. The pediocin bacteriocin is a good bio-preservative and can be used to improve the shelf life of any food system like vegetable, meat, fish, cheese, etc.

Considering the natural spread of pediocin PA-1 like bacteriocin among LAB, limited studies are carried out on intergeneric and interspecific pediocin PA-1 like bacteriocin producers. In addition, the characterization of MGEs in the flanking regions of the pediocin operon has not been accomplished to reveal the phenomenon of horizontal gene transfer (HGT). The application of *Ped. acidilactici* producing pediocin PA-1 like bacteriocin is very limited in the fermentation of milk. Hence, there is a need to use the pediocin like bacteriocin producing cultures in the fermentation of milk. Studies on the natural transfer of bacteriocin encoded plasmid among different LAB under *in vitro* and *in situ* conjugal transfer methods to understand the mechanism of HGT has not been investigated. Considering the fact that pediocin PA-1 like bacteriocin distribution among different LAB is an important aspect for development of novel bacteriocinogenic cultures, this study was carried out to obtain better insight with the following objectives.

**OBJECTIVES**

1. Molecular characterization of pediocin-like bacteriocin production in native bacterial cultures.
2. To provide molecular evidences for interspecific and intergeneric pediocin PA-1 production.
3. To study the distribution of pediocin PA-1 production in soya milk fermentation.
Chapter – 1 : General introduction and objectives of the study

A concise general introduction about LAB and different types of bacteriocins produced by different genera of LAB is described in this chapter. The mobile genetic elements associated with HGT of bacteriocin encoded genes in LAB are also illustrated. The distribution of pediocin PA-1 among different genera and species of LAB are enlisted, and the reason behind the study has been furthermore described. The applications of bacteriocins in different food systems are described for apprehensive use of pediocin producers in any food system. The objectives of the study are also enlisted.

Chapter – 2 : Review of literature

This chapter mainly emphasizes about the distribution of LAB, general features of LAB, bacteriocins produced by them, etc. The classification of bacteriocins based upon their mode of action is also described. A detailed description on the pediocin PA-1, biosynthesis and mode of action of pediocin PA-1 molecule has been explained. The dissemination of this bacteriocin among LAB as well as genetically modified pediocin to enhance the production for application to a food system is described. Several phenotypic methods like morphological, physiological and biochemical properties of some of the genera of LAB have been illustrated. The molecular methods like PCR based identification, DNA-DNA hybridization, Random Amplified Polymorphic DNA (RAPD), Restriction fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Multiple Locus Sequence Typing (MLST), etc., used in the identification and characterization of LAB producing bacteriocins are portrayed. The different methods of HGT and various MGEs like transposons, insertion elements, mobilization regions, plasmids, etc., are described in this chapter. These MGEs involved in the transfer of genes encoding bacteriocin, antibiotic resistance, carbohydrate utilization etc., among LAB are also depicted. The application of bacteriocin producing LAB in soymilk fermentation, which would enhance the nutritional value of the end product, has been illustrated.
Chapter – 3: Isolation and characterization of pediocin PA-1 like bacteriocin producers

This chapter accentuates with the isolation of bacteriocinogenic LAB from different sources. The work carried out on the characterization of LAB producing pediocin PA-1 like bacteriocin was performed by using several morphological, physiological, biochemical and molecular techniques. The results presented here helped in the rapid detection of putative pediocin PA-1 like bacteriocin producers by PCR and dot blot hybridization. The factors that effect the bacteriocin production such as temperature, pH and salt concentration were studied and the findings are reported in this chapter.

An attempt was made to isolate the potential pediocin PA-1 like bacteriocin producers from different sources, which include raw milk, curd, carrot, cucumber, beans, chicken intestine, idli batter and dosa batter. The initial screening was based on the LAB inhibiting the growth of most important food borne and spoilage pathogen Listeria monocytogenes. The isolates showing a strong anti-listerial and anti-microbial activity were selected for further studies. The PCR (polymerase chain reaction) of pedB and pedAB gene and dot-blot hybridization revealed that, out of 55 anti-listerial bacteriocin producing isolates, eight isolates gave positive amplification for both the gene specific PCR and showed an intense signal to the immunity gene probe. The partial characterization of the bacteriocin from the selected isolates revealed that the peptide exhibited strong anti-listerial property and was found to be heat-stable, active in acidic to alkaline pH, proteinaceous in nature and had a molecular weight around 4.5 kDa. Hence, indicating the properties of class IIa pediocin-family in the selected isolates.

These eight isolates are further characterized to identify the LAB at their species level by conventional morphological, physiological and biochemical properties and molecular tools like RAPD, RFLP and 16S rDNA gene sequencing. The selected isolates belonging to different genera and species of LAB are identified as Ped. acidilactici (2), Ped. pentosaceous (2), Lact. plantarum, Enterococcus faecium
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(3) and *Streptococcus equinus*. The bacteriocin producing LAB isolates reported in this chapter are deposited at the National Collection of Industrial Microorganisms (NCIM) at the National Chemical Laboratory, Pune, India. The cultures *Strep. equinus* Ac1, *Ped. acidilactici* Cb1, *Ped. pentosaceus* Cb4, *Ent. faecium* V3, *Ent. faecium* BL1, *Ent. faecium* Acr4, *Ped. acidilactici* K7 and *Ped. pentosaceus* R38 were given the accession numbers from NCIM 5418 to 5425, respectively. The production of bacteriocin at different temperatures, pH and NaCl concentrations suggested that some of the isolates can be exploited as a starter culture or as a bio-preservation agent in acidic as well as alkaline foods. The *Ped. acidilactici* NCIM 5424, *Ent. faecium* NCIM 5423, *Lact. plantarum* Acr2 and *Ped. pentosaceus* NCIM 5420 were selected for further studies.

Chapter – 4: Detection of mobile genetic elements in the intergeneric / interspecific pediocin PA-1 like producers

In the present chapter, the bacteriocin encoding genes in the pediocin operon of selected isolates are characterized by PCR using several sets of primers. It was observed that these isolates had a conserved pediocin operon and gave an expected amplicon size as that of the positive control (*Ped. acidilactici* NCIM 5424). The flanking regions were also characterized to detect MGEs present in the intergeneric and interspecific pediocin PA-1 like bacteriocin producers. The results obtained by Inverse PCR showed an amplicon size ranging from 12 Kb to 6 Kb in the selected isolates. In *Ent. feacium* NCIM 5423 and *Lact. plantarum* Acr2 a variation in the amplicon size in the downstream region of the operon was identified, suggesting a deletion in this region. The plasmid profiling and Southern hybridization in the wild type (bacteriocin producers) and mutants (plasmid cured) strains revealed that the selected isolates produced pediocin like bacteriocin on plasmids of varying sizes from 12 to 3.5 Kb. In addition, the MGEs like ISLpl1 and mobilization genes (mob) have been detected by Southern hybridization on the plasmids encoding pediocin PA-1 like bacteriocin.
The presence of replicative (rep) genes in the upstream and tyrosine recombinase (integrase) in the downstream region of the pediocin operon suggests either the excision or insertion of the operon in different genera of LAB. The phylogeny of ISLpl1 in Ent. faecium NCIM 5423 suggests the transfer of this bacteriocin from Lactobacillus strains producing pediocin PA-1 like bacteriocin present in their ecological niche. The MGEs like rep, tyrosine recombinase, ISLpl1 and mob genes are associated with the transfer of genes linked with the bacteriocin production, antibiotic resistance, sugar utilization. Hence, suggesting a mechanism for the natural distribution of this bacteriocin among different LAB. The production of bacteriocin from Ent. faecium NCIM 5423 makes it an ideal organism because of its wider adaptability to the environment. Such plasmids with intergeneric transferring ability play a major role for the development of potential starter cultures with bio-preservative and carbohydrate utilization properties. The isolates Ped. acidilactici NCIM 5424, Ent. faecium NCIM 5423 and Lact. plantarum Acr2 were selected to observe the production of pediocin PA-1 like bacteriocin in soymilk.

Chapter – 5: In situ production of pediocin PA-1 like bacteriocin in soymilk

The production of pediocin PA-1 like bacteriocin by intergeneric and interspecific LAB is of required because of their ‘Generally Regarded As Safe’ (GRAS) status, antagonistic effect on the food borne pathogens. This chapter focused on the application of intergeneric pediocin PA-1 like bacteriocin producer in the fermentation of soymilk. The Ped. acidilactici NCIM 5424, Ent. faecium NCIM 5423 and Lact. plantarum Acr2 were used in the present study to observe the fermentation as well as production of the bacteriocin in soymilk. The survival of the native pediocin producer Ped. acidilactici in milk was not accomplished much, because of its requirement for high levels of carbohydrates and lack of lactose fermenting ability. The obtained end product from Ent. faecium NCIM 5423 was subjected to the bacteriocin production, safety during the storage period, and also observed for the sensory properties of the obtained end product.
Results of this study indicated that the isolate *Ped. acidilactici* NCIM 5424 was unable to survive and ferment the soymilk even after incubation for 24 h at 37 °C. Whereas the isolates *Ent. faecium* NCIM 5423 and *Lact. plantarum* Acr2 was able to survive with $10^8$ cfu/ml for a period of 15 days at 4 °C. The fermentation of the soymilk was obtained by these two isolates within 6 h of incubation at 37 °C. The co-cultivation of bacteriocin producers with *L. monocytogenes* ScottA, revealed a decline in the *Listeria* cell count within 6 h in the presence of *Ent. faecium* NCIM 5423 and 8 h in the case of *Lact. plantarum* Acr2. The quality parameters of the fermented soymilk were considered to be better for *Ent. faecium* NCIM 5423 when compared to *Lact. plantarum* Acr2 and commercially fermented soymilk based upon their solidity, pediocin activity, pH, titratable acidity, total carbohydrates, reducing sugars and antioxidant property. Hence, the sensory evaluation of *Ent. faecium* NCIM 5423 was performed for a period of 15 days at 4 °C. An overall score of 8.4 was obtained for *Ent. faecium* NCIM 5423 and 7.6 for commercially fermented soymilk. The yogurt fermented by the *Ent. faecium* NCIM 5423 had a fat content of 24.2% and a moisture content of 8.71%. The direct use of intergeneric pediocin PA-1 like bacteriocin producers helps in the production of bacteriocin in soymilk, thus extending the shelf life of the soy end-product. This culture has a potential to be used as a biopreservative agent in any food system because of its wider adaptability and broad spectrum of activity.

**Chapter – 6 : In vitro and in situ conjugal transfer of pediocin like bacteriocin among LAB**

To prove the transferability of the plasmid encoding pediocin PA-1 like bacteriocin under *in vitro* and *in situ* conditions, conjugation experiments were carried out. For *in vitro* experiment the filter mating method was followed and for *in situ* soymilk model system is used. The pediocin producing donors (*Ped. acidilactici* NCIM 5424, *Ent. faecium* NCIM 5423 and *Lact. plantarum* Acr2) and a recipient *Ent.
faecalis JH2-2 are used for conjugation experiments. Earlier, the transfer of pediocin plasmid and antibiotic resistance encoded plasmids among closely related pediococcal strains was performed by conjugation and electroporation. However, the transfer of pediocin PA-1 like bacteriocin encoded plasmid to different genera of LAB was not performed till date.

It was observed that the bacteriocin was able to get transferred from the cultures *Ped. acidilactici* NCIM 5424 to *Ent. faecalis* JH2-2 under both *in vitro* and *in situ* conditions. However, *Ent. faecium* NCIM 5423 was able to transfer the bacteriocin only under *in situ* conditions. No transconjugants were observed between *Lact. plantarum* Acr2 and *Ent. faecalis* JH2-2. The conjugation experiment demonstrated the HGT of bacteriocin encoded plasmid to *Ent. faecalis* JH2-2 and observed more transfer frequency under *in situ* conditions when compared to *in vitro* method, suggesting a suitable environment for the conjugal transfer of the bacteriocin encoded plasmid. The production of the bacteriocin by the transconjugants was found to vary at different temperature and pH, suggesting the drastic effect of physiochemical environmental factors on the production of bacteriocin as well as growth of the bacteria. Several molecular techniques like, *pedB* gene PCR and Southern hybridization of the immunity gene were used to characterize the transconjugants producing the bacteriocin. The results obtained for RAPD PCR of the donors, recipients and transconjugants, suggested that the transconjugants producing bacteriocin showed the similar banding pattern to the recipient *Ent. faecalis* JH2-2.

From the result presented here, both *in vitro* and *in situ* conjugal mating techniques were found to be useful in the construction of transconjugants with bacteriocin encoded plasmids which help in the development of starter and probiotic cultures. The presence of the pediocin PA-1 like bacteriocin in diverse genera of LAB gives an idea of transfer of this bacteriocin by conjugative mode of HGT by MGEs in their ecological
niche. The distribution of pediocin like bacteriocin among different genera of LAB helps in the development of cloning vectors, starter cultures with bio-preservative and bio-protective culture ability in the fermentation of dairy and meat products.

**Chapter – 7: Summary and conclusion**

- The LAB cultures producing pediocin PA-1 like bacteriocin were isolated in the laboratory from different sources.
- The molecular techniques used in the present work helped in the rapid detection of pediocin PA-1 like bacteriocin producing bacteria.
- This is the first report on the production of pediocin PA-1 like bacteriocin by *Streptococcus equinus* NCIM 5418 from milk, *Enterococcus faecium* NCIM 5423 and *Lactobacillus plantarum* Acr2 from vegetable origin.
- The detection of the MGEs like ISLpl1 and mob genes on the plasmids encoding the bacteriocin by Southern hybridization helped in the rapid identification of such elements.
- The production of pediocin by the intergeneric pediocin producers in soymilk fermentation by *Ent. faecium* NCIM 5423 helped in improving the flavour, texture and taste of the finished product with longer keeping quality.
- An acceptable sensory score for the fermented soymilk by *Ent. faecium* NCIM 5423 reveals that this organism has a prospective use in the fermentation of dairy products where the application of the native pediocin producer *Ped. acidilactici* is limited.
- The *in vitro* and *in situ* conjugal methods provided an evidence of HGT phenomenon of this bacteriocin in different genera of LAB.
- This is the first report on the *in situ* conjugal transfer of pediocin PA-1 like bacteriocin in soymilk model and provided a better transfer efficiency and scope for explaining the natural distribution or dissemination of this bacteriocin among different genera of LAB.
Bibliography

The latest review articles and book chapters, publication from the peer-reviewed scientific journals, are utilized for gaining the national and international perspective on the bacteriocin producing LAB, their diversity and properties. The articles cited in all the chapters are compiled together in the bibliography section.

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