CHAPTER 6

IN VITRO AND IN SITU CONJUGAL TRANSFER OF PEDIOCIN AMONG LAB
6.0. ABSTRACT

In the study, the transfer of the bacteriocin plasmid from donors to a recipient was investigated under natural in vitro and in situ conditions. The ability of the plasmid encoded pediocin transfer from *Pediococcus acidilactici* NCIM 5424, *Enterococcus faecium* NCIM 5423 and *Lactobacillus plantarum* Acr2 to rifapicin resistant, *Ent. faecalis* JH2-2 (recipient) was determined by conjugation. The in vitro filter mating method and in situ soymilk model system were used for conjugation experiment. Under in vitro conditions the donor (*Ped. acidilactici* NCIM 5424) and under in situ conditions the donors (*Ent. faecium* NCIM 5423 and *Ped. acidilactici* NCIM 5424) were able to transfer the bacteriocin. The in situ soymilk model that would have provided a suitable environment resulted in high transfer frequency rates when compared to in vitro conditions. About 33% high transfer frequency was observed for the mating pair *Ped. acidilactici* NCIM 5424 and *Ent. faecalis* JH2-2 in soymilk model. However, no transconjugants were observed for the mating pair *Lact. plantarum* Acr2: *Ent. faecalis* JH2-2 under both the methods used. The results of conjugation experiment demonstrated in this study explained the possibility of HGT of pediocin plasmid to *Ent. faecalis* JH2-2. Several molecular techniques were used to characterize the transconjugants. The production of pediocin by the transconjugants at different temperature and pH was found to vary by about 10 fold times. Our results indicated the natural transfer of pediocin PA-1 to other LAB present in their close vicinity by conjugation. The physiological conditions like pH and temperature were found to effect the production of pediocin in transconjugants. The dissemination of pediocin PA-1 under in situ conditions is noteworthy, and such intergeneric pediocin producers can be useful in the fermentation of dairy products and construction of novel cultures.
6.1. INTRODUCTION

LAB are considered as an important group of microbiota, which are widely used in food fermentations, biopreservation and also found to improve the health properties of fermented food products. Thus, they contribute towards benefiting the mankind, which includes alleviation of lactose intolerance, immune enhancement, reduction of colon-cancer, anti-cholesterol, anti-mutagenic effects, etc. (Papagianni and Anastasiadou 2009; Van Reenen and Dicks 2010). The genetic events like HGT including gene loss/gain, gene duplication, mutations, recombinations, etc., are found to be responsible for the genome structure within different species of LAB (Mayo et al. 2008; Horvath et al. 2009). The comparative genome analysis revealed that the LAB are the masters in adaptation and have the ability to adapt to diverse environmental conditions as described previously (Van Reenen and Dicks 2010). Because of HGT, large inter-intraspecies diversity was observed in the LAB genomes (Siezen et al. 2010).

The HGT is considered as an important phenomenon in the field of evolution, ecology, biotechnology and medicine (Keeling and Palmer, 2008). The process of HGT is mainly by three methods; conjugation, transformation and transduction, which are known to be facilitated by the association of plasmids, phages, mobile genetic elements (MGEs) like transposons, insertion elements, integrons, recombinases, etc. (Družina et al. 2009). Such MGEs serve as effective vectors in the transfer or spread of genes among different group of bacteria.

*Pediococcus acidilactici* is a primary constituent in the fermented dairy product (cheese), meat and vegetable products (Papagianni and Anastasiadou 2009). The *Ped. acidilactici* was found to produce a plasmid encoded broad spectrum antimicrobial peptide known as pediocin PA-1 (Miller et al. 2005). The pediocin PA-1 is widely used as a biopreservative and found it to inhibit several food-borne pathogenic organisms (Papagianni and Anastasiadou 2009; Kumar et al. 2011). Recently, Diez et
al. (2012) reported the use of pediocin PA-1 as a potential biopreservative in wine-making and found to be active against wine spoilage bacteria. However, the fermentation of milk-like products by the pediocin producing *Ped. acidilactici* was found to be difficult due to its lack of lactose fermenting ability (Somkuti and Steinberg, 2003; Somkuti and Steinberg, 2010).

The consumption of fermented foods leads to the development of a major pathway for the dissemination of antibiotic resistant bacteria to animals and human populations (Toomey et al. 2010). Since, the bacteriocins produced by the Gram-positive and Gram-negative bacteria have shown potential activity against bacteria that are resistant to antibiotics, the use of bacteriocin producers may reduce the of antibiotic resistant bacteria present in a food system (Nes et al. 2002; Cotter et al. 2005). Thus in this context, the pediocin PA-1 like bacteriocin which are found to have broad application in the field of food industry, can also be used in inhibiting the antibiotic resistant bacteria in fermented food products.

Pediocin PA-1 is produced by a number of LAB strains and was initially reported in *Ped. acidilactici* strain PAC 1.0 and *Ped. acidilactici* H. Subsequently, it was reported in *Lactobacillus plantarum* WHE 92, *Ped. parvulus* ATO34 and ATO77; Plantaricin 423 from *Lact. plantarum* 423 and as coagulin in *Bacillus coagulans* I₄, *Ped. pentosaceus* S34; *Ped. pentosaceus* ST44AM of 6.5 KDa bacteriocin isolated from marula; *Lact. plantarum* ST202Ch and ST216Ch isolated from pork products; *Lact. casei*, *Lact. paracasei* and *Lact. rhamnosus* isolated from retail cheddar cheese (refer Chapter 2, section 2.2.3). Le Marrec et al. (2000) reported the about the possible dissemination of pediocin PA-1 by a phenomenon of horizontal gene transfer to several LAB. However, the experimental evidences were lacking. Hence, the use of intergeneric/interspecific pediocin PA-1 like bacteriocin producers in dairy products, help in extending the shelf-life and improves the food safety of the end-product.
A wide distribution of bacteriocin encoded genes among LAB is basically due to the presence of plasmids or conjugative transposons or MGEs. Coakley et al. (1997) reported the conjugal transfer of lantibiotic “lacticin 3147” on a 60 Kb conjugative plasmid into *Lactococcus lactis* DPC3147, a commercial starter culture. Similarly, nisin, enterocin, ruminococcin, bacteriocin 43 were found to be transferred to recipient strains of *Enterococcus faecalis* by *in vitro* filter mating conjugal experiment (Dougherty et al. 1998; Balla et al. 2000; Gomez et al. 2002, Todokoro et al. 2006). It was also observed that the antibiotic resistance genes and carbohydrate utilization genes are found to be linked to the MGEs, plasmids etc., and proved to be disseminated by conjugation process under *in vitro, in vivo* and *in situ* methods (Macheilsen et al. 2011, Gazzola et al. 2012).

The conjugal transfer of pediocin PA-1 encoded plasmid pSMB74 from *P. acidilactici* H to a gentamicin-neomycin resistant *P. acidilactici* was performed (Ray et al. 1989b). Subsequently, Kim et al. (1992), reported the transfer of different plasmids within the pediococcal strains by conjugation and electroporation methods, however the transfer of the pediocin encoded plasmid was not successful. As pediocin PA-1 was often found in other genera of LAB, the present study focussed on the conjugal transfer of the pediocin PA-1 like bacteriocin from the native producer, i.e., *Ped. acidilactici* NCIM 5424, *Ent. faecium* NCIM 5423 and *Lact. plantarum* Acr2 to a recipient *Ent. faecalis* JH2-2 was performed. The *in vitro* filter mating method and *in situ* soymilk model system were used to prove the conjugation process. The production of the bacteriocin in the obtained transconjugants was also discussed.
6. 2. MATERIALS AND METHODS

6. 2. 1. Materials

6. 2. 1. 1. Materials used for the microbiological and molecular assays

- Soy bean seeds (*Glycine max*) from local market as described previously in Chapter 5.
- 0.45 µm filter membranes were purchased from Whatman, Schleicher and Schuell, 25mm, Germany.
- Rifampicin and fusidic acid were obtained from HiMedia, Mumbai.
- DIG high prime DNA labeling and detection kit II was used for the Southern hybridization and purchased from Roche Inc., Germany.

6. 2. 1. 2. Bacterial strains used for conjugation experiment

Previously, three LAB strains (*Pediococcus acidilactici* NCIM 5424, *Enterococcus faecium* NCIM 5423 and *Lactobacillus plantarum* Acr2) isolated and characterized as plasmid encoded pediocin producers in chapter 2 were used as donors. The recipient *Ent. faecalis* JH2-2 was obtained from C.A.M.P Franz, Federal Research Centre for Nutrition and Food, Institute of Hygiene and Toxicology, Karlsruhe, Germany. All the donors were grown in MRS broth and the recipient in BHI broth at 37 ºC under static condition. The *Listeria monocytogenes* ScottA was used as an indicator for bacteriocin activity and were grown in BHI broth at 37 ºC under shaking conditions. The culture maintenance and growth conditions were followed accordingly as described previously (section 3. 2. 1. 3).

6. 2. 2. Methods

6. 2. 2. 1. Inoculum preparation

Prior to mating experiments, strains were plated from -20 ºC stored glycerol stocks for single colonies. After 12-16 h of incubation at 37 ºC, single colony was picked and inoculated in 5 ml of broth. Donors were grown in MRS broth, whereas recipients were grown in BHI broth containing 50 µg/ml of rifampicin and 25 µg/ml.
of fusidic acid and incubated at 37 °C for 16 h. From the grown cultures, a 2% of inoculum was used in fresh broth without antibiotics. A cell suspension was prepared at an OD value of 1.0 and 1.2 by centrifugation at 8000 rpm at 4 °C for 10 min. The cell pellet was washed with physiological saline solution (PPS- 0.85% NaCl and 0.1% neutralized bacteriological peptone) twice and resuspended in 1 ml of PPS.

6.2.2.2. Extraction of bacteriocin

The extraction of bacteriocin was performed by chloroform as described previously (section 3.2.2.7). The protein concentration of the extracted bacteriocin was determined according to Bradford (1976). The bacteriocin of 10 µg/ml (100 AU/µl) was used as a selective marker for transconjugants.

6.2.2.3. In vitro filter mating conjugation experiment

The donors were examined for their ability to transfer the pediocin encoded plasmid using in vitro filter mating conjugation technique as described earlier (Gevers et al. 2003). The 1:1 and 10:1 ratio of donor and recipient were filtered through the 0.45 µm membrane filter. The filtered cells were washed by passing 10 ml of PPS to attach the cells tightly to the membrane. The filter was aseptically transferred on the MRS agar with cells side upward and incubated at 37 °C for 12-16 h. The filters were placed in 2 ml of PPS and the filters were mixed by vortex. Serial dilutions were done and plated with appropriate dilutions on media selective for donors, recipients and transconjugants in their selective media. The plates were incubated for 2-3 days and observed for the growth transconjugants on 50 µg/ml of rifampicin, 25 µg/ml of fusidic acid and 10 µg/ml of pediocin. The colonies were selected randomly and checked for production of pediocin by overlaying with the indicator organism (L. monocytogenes ScottA).
6. 2. 2. 4. **In situ soymilk model system for conjugal transfer of pediocin**

The extraction of soymilk was performed as described previously (section 5. 2. 2. 1) and was used for the experiment. One ml of freshly grown donors in MRS broth and recipient in BHI broth were taken, and the cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. The collected cell pellet was washed twice with the same initial volume of sterile distilled water. In the present study, 1% (v/v) of this cell suspension having 10^8 cfu/ml was used as inoculum. The viable counts of the donors and recipients present in the fermented soymilk were calculated as described earlier (section 5. 2. 2. 3). For in situ conjugal mating experiment, the cell suspension of donors and recipients with 1.0 and 1.2 OD values were inoculated into the sterilized soymilk. The samples were withdrawn at 4, 6, 8 and 12 h, appropriate dilutions were made and plated on the selective media independently for donors, recipients and transconjugants as described above.

6. 2. 2. 5. **Total DNA and plasmid DNA preparation**

The total DNA and plasmid extraction procedures were followed as described earlier (section 3. 2. 2. 3. and 4. 2. 2. 1).

6. 2. 2. 6. **PCR detection of pedB gene**

The amplification of the immunity gene (pedB) with total DNA as template was performed for donor, recipient and transconjugant as described previously (section 3. 2. 2. 4).

6. 2. 2. 7. **RAPD fingerprinting for transconjugants**

Random Amplified Polymorphic DNA (RAPD) PCR was performed by using total DNA as a template with either M13 (5’-GAGGGTGGCGGTTCT-3’) or Col 1 (5’-AGCAGCGTGG-3’) primer (Cocconcelli et al. 1995; Schillinger et al. 2003). The PCR conditions were the same as described earlier (section 3. 2. 2. 10).
6. 2. 2. 8. Southern transfer and hybridization

The Southern transfer and hybridization was performed by using DIG-high prime DNA labelling kit and the procedure was followed as described previously (section 4. 2. 2. 4).

6. 2. 2. 9. Pediocin production by the transconjugants at different temperature and pH

The production of pediocin by the donor and transconjugants was analyzed at different temperature (16, 37 and 50 ºC) and pH (4, 8, 10) as described previously (section 3. 2. 2. 13). The anti-listerial activity was expressed in arbitrary unit per ml (AU/ml) and is defined as the highest dilution of test sample exhibiting the zone of inhibition against indicator *L. monocytogenes* ScottA.

6. 3. RESULTS AND DISCUSSION

6. 3. 1. In vitro and in situ conjugal filter transfer of bacteriocin encoded plasmids

In the present study, the ability of the pediocin PA-1 like bacteriocin encoded plasmid transfer to a non-bacteriocin recipient was investigated. The *in vitro* conjugal transfer of the pediocin encoded plasmid was performed between the selected three donors (*Ped. acidilactici* NCIM 5424, *Ent. faecium* NCIM 5423 and *Lact. plantarum* Acr2) and the recipient (*Ent. faecalis* JH2-2). The 1:1 ratio of donor: recipient with 1.0 OD and 1.2 OD value (600 nm wavelength) yielded around $10^{-7}$ cfu/ml and $10^{-9}$ cfu/ml for 1:1 and 10:1 donor: recipient respectively. The transconjugants for the mating pair *Ped. acidilactici* NCIM 5424: *Ent. faecalis* JH2-2 with a transfer frequency of around $10^{-6}$ and $10^{-4}$ transconjugants/recipient were obtained for 1:1 and 10:1 donor: recipient ratios. Whereas, no transconjugants were observed for mating pairs *Ent. faecium* NCIM 5423 and *Lact. plantarum* Acr2 with *Ent. faecalis* JH2-2 with either of ratios 1:1 and 10:1.

The transfer of the bacteriocin encoded plasmids under *in situ* conditions was investigated. The survival of donor and recipients in soymilk was examined by inoculating 1% of cell suspension for a period of 24 h as described previously (Chapter
5). The *Ent. faecium* NCIM 5423 and *Lact. plantarum* Acr2 were found to survive with an average viability of \(10^8\) cfu/ml, and curdling of soymilk was observed within 6 h of incubation whereas, *Ped. acidilactici* NCIM 5424 was unable to survive. The *Ped. acidilactici* NCIM 5424 was able to grow in soymilk with 1% glucose. Hence, 1% glucose was used for the *in situ* conjugal transfer of the pediocin like bacteriocin encoded plasmid from *Ped. acidilactici* NCIM 5424 to *Ent. faecalis* JH2-2. The three LAB mating pairs were inoculated into soymilk model and examined their ability to transfer the pediocin encoded plasmids in this environment. Low transfer frequency was observed for 10:1 (donor: recipient) ratio for the mating pairs tested. The transconjugants were observed after 6 h of incubation of donor and recipient and were found to maintain their viability in the soymilk. Table 6.3.1.1 represents the mating pairs with different donor: recipient ratios and transfer frequencies under both *in vitro* and *in situ* conditions. The obtained transconjugants were found to grow in 50 µg/ml of rifampicin and 10 µg/ml of crude pediocin and are represented as *Ent. faecalis* JH2-2(5424) ped\(^+\) rif\(^+\) and *Ent. faecalis* JH2-2(5423) ped\(^+\) rif\(^+\). The donors used in the present study were unable to grow in 50 µg/ml of rifampicin.

When compared to *in vitro* and *in situ* methods, the soymilk model showed high transfer frequency, suggesting a suitable condition for conjugation. No transconjugants were observed for the mating pairs *Lact. plantarum* Acr2 and *Ent. faecalis* JH2-2 under both *in vitro* and *in situ* conditions. The transconjugants obtained by both the *in vitro* and *in situ* methods were able to inhibit the growth of *L. monocytogenes* ScottA, indicating the production of the bacteriocin (Fig. 6.3.1.1). The pediocin activity was observed to be more for donors (around 25600 AU/ml) when compared to transconjugants (around 6400 AU/ml). Similar observation was reported for the transconjugants obtained by the transfer of 60 Kb plasmid encoding lacticin 3147 from *Lactococcus lactis* DPC4268 to *Lac. lactis* MG1363. Also, small and large zone of inhibitions against the parental strains were obtained for the transconjugants used, indicating variation in the production of bacteriocin lacticin 3147 (Trotter et al. 2004). Hence, the transconjugants producing a bacteriocin was found to vary at their production level when compared to the parental donors.
This is the first report on the *in situ* soymilk model for the conjugal transfer of pediocin PA-1 like bacteriocin encoded plasmid. The failure of conjugation under *in vitro* and *in situ* methods for the mating pair *Lact. plantarum* Acr2 and *Ent. faecalis* JH2-2, suggests the influence of several environmental factors, as reported by McMahon et al. (2007). Similarly, Lampkowska et al. (2008) suggested that the *in vitro* conjugation was influenced by various factors like culture age, ratios of donor and recipient cultures in solid and liquid mating models for antibiotic resistance dissemination among LAB. Vogel et al. (1992) observed similar conjugal transfer rates under both *in vitro* and *in situ* sausage fermentation from the starter *Lact. curvatus* LTH1432.

The pediocin PA-1 bacteriocin was found to be widely distributed among LAB. The effectiveness of conjugation and electroporation of sucrose hydrolysis encoded plasmid (pPR72, 53.7 Kb) was observed between pediocin producing *Ped. acidilactici* M to other plasmid free pediococcal recipient strains. However, the transfer of pediocin encoded plasmid (pSMB74) was not successful (Kim et al. 1992). Later on, the *in vitro* conjugal transfer of a 50 Kb plasmid encoding the enterocin 1071A and 1071B from *Ent. faecalis* BFE 1071 to *Ent. faecalis* OGX1 was observed by Balla et al. (2000). Hence, the use of both *in vitro* and *in situ* methods helped in the transfer of bacteriocin encoded plasmids from the donors (*Ped. acidilactici* NCIM 5424 and *Ent. faecalis* NCIM 5423) to a recipient strain.

### Table 6.3.1.1 Conjugal transfer frequencies of pediocin PA-1 encoded plasmid by different methods

<table>
<thead>
<tr>
<th>Mating pairs</th>
<th>Transfer frequency</th>
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<tr>
<td></td>
<td><em>In vitro</em> filter mating method</td>
</tr>
<tr>
<td>Donor: recipient</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>10:1</td>
</tr>
<tr>
<td>NCIM 5424: JH2-2</td>
<td>8.4 x 10^{-7}</td>
</tr>
<tr>
<td>NCIM 5423: JH2-2</td>
<td>3.03 x 10^{-5}</td>
</tr>
<tr>
<td>Acr2: JH2-2</td>
<td>-</td>
</tr>
</tbody>
</table>

Transfer frequency = Number of transconjugants/ Number of recipients
The conjugal transfer in both *in vitro* and *in situ* methods of pediocin PA-1 was found to be due to conjugation process facilitated by conjugative plasmids or MGEs involved in the HGT. The MGEs as described in chapter 4, play a major role in the transfer of this bacteriocin to other LAB strains. The presence of theta-type replicating *rep* genes (putative *oriT* region) and tyrosine recombinase (integrase) found in the plasmid of pSMB74 of *Ped. acidilactici* H suggests the possible involvement of such MGEs in the conjugal transfer of pediocin. The ISLpl1 and mob regions detected in the flanking region of the *Ent. faecium* NCIM 5423 as described previously, must have facilitated an efficient transfer of the pediocin encoded plasmid to the *Ent. faecalis* JH2-2 under both *in vitro* and *in situ* conditions (Chapter 4). In contrast, Danielsen, (2002) observed no conjugal transfer of tetracycline resistance plasmid pMD5057 (around 10 Kb of size), also encoding putative *oriT* and integrases/recombinases from *Lact. plantarum* 5057 to *Ent. faecalis* JH2-2. However, a co-mobilization of chloramphenicol resistance plasmid from several strains of *Lact. plantarum* with the use of pAMβ1-like plasmid from *Enterococcus* sp. was observed from *Lact. plantarum* 5057 to *Ent. faecalis* JH2-2 (Danielsen, 2002; Ahn et al. 1992). The
presence of a mobilization protein (Mob) in \textit{B. coagulans} I$_4$ and \textit{Lact. plantarum} 423 suggests the integration of pediocin operon in the plasmids of these bacteria (Le Marrec et al. 2000; Van Reenen et al. 2006). Fig 6. 3. 1. 2. represents the MGEs involved in the transfer of pediocin among different genera of LAB.

![Fig. 6. 3. 1. 2. Schematic representation of transfer of pediocin PA-1 bacteriocin by conjugation in different genera of LAB.](image)

6. 3. 2. Molecular characterization of transconjugants

The obtained transconjugants were subjected to PCR by the pediocin specific gene \((\text{pedB})\) as described earlier (Chapter 3, section 3. 2. 2. 4). A product of expected size (362 bp) was observed for the donor \textit{Ped. acidilactici} NCIM 5424, \textit{Ent. faecium} NCIM 5423, \textit{Lact. plantarum} Acr2 and transconjugants (\textit{Ent. faecalis} JH2-2 5424 ped$^+$ rif$^+$ and \textit{Ent. faecalis} JH2-2 5423 ped$^+$ rif$^+$), whereas the recipient showed no PCR amplicon (Fig. 6. 3. 2. 1 A). For further confirmation, the plasmid DNA was extracted and subjected to Southern hybridization. Two small molecular sized
plasmids of 9 and 3 Kb were observed in donor, and were also found to be present in the transconjugant. The presence of these two extra plasmid bands in the transconjugants obtained by Ped. acidilactici NCIM 5424 corresponds to transfer of the pediocin plasmid. The pedB probe reacted to the 9 and 3 Kb plasmids in both donor and transconjugants, confirming the conjugation event from Ped. acidilactici NCIM 5424 to Ent. faecalis JH2-2 (Fig. 6. 3. 2. 1. B, C). It was also observed that the 9 and 3 Kb plasmids of Ent. faecium NCIM 5423 reacted to the pedB probe by Southern hybridization, as described earlier (Chapter 4). Similar observation was reported for the enterocin P, where the 19, 26 and 35-38 Kb sized plasmid encoding the bacteriocin, were detected by Southern hybridization in Ent. faecium UJA15, Ent. faecalis strains UJA52 and UJA56 (Abriouel et al. 2006). This confirms that the pediocin encoded plasmid gets transferred from one bacteria to other present in a same ecological niche under suitable conditions.
Fig. 6.3.2.1. Molecular detection of transconjugant producing bacteriocin by pedB gene PCR (A), plasmid profiling (B) and Southern detection (C) of the immunity gene in the donor, recipient and transconjugants.

(A) – Lane 1: 10 Kb molecular marker, lane 2: *Ped. acidilactici* NCIM 5424 (donor), lane 3: *Ent. faecalis* JH2-2 5424 ped" rif" (transconjugant), lane 4: *Ent. faecalis* JH2-2 (recipient), lane 5: *Ent. faecium* NCIM 5423 (donor), lane 6: *Ent. faecalis* JH2-2 5423 ped" rif" (transconjugant).

(B) & (C) – Lane1: λ *HindIII* molecular marker, lane2: *Ped. acidilactici* NCIM 5424, lane 3: *Ent. faecalis* JH2-2, lane 4: *Ent. faecalis* JH2-2 5424 ped" rif", lane 5: *Ent. faecalis* JH2-2 5423 ped" rif", lane 6-10 Kb molecular marker.
The obtained transconjugants were subjected to RAPD PCR with M13 and col1 primers to differentiate among the pediocin producers. The RAPD profiling of the transconjugant *Ent. faecalis* JH2-2 5423 ped<sup>+</sup> rif<sup>+</sup> and *Ent. faecalis* JH2-2 5424 ped<sup>+</sup> rif<sup>+</sup> showed similar banding pattern to the parental *Ent. faecalis* JH2-2 with both M13 and Col1 primers. The banding pattern of the donor *Ped. acidilactici* NCIM 5424 and *Ent. faecalis* NCIM 5423 was found to be different from *Ent. faecalis* JH 2-2 (Fig. 6.3.2.2). The analysis by RAPD PCR suggests that the transconjugants showing similar pattern to the parent recipient strain indeed produces pediocin PA-1 with anti-listerial activity.

![Fig. 6.3.2.2. Analysis of transconjugants by RAPD-PCR with M13 primer (A) and Col1 primer (B)](image)

(A) & (B)- Lane 1- 10 Kb molecular marker, lane 2- *Ent. faecium* NCIM 5423, lane 3- *Ent. faecalis* JH 2-2 (recipient), lane 4- *Ent. faecalis* JH2-2 5424 ped<sup>+</sup> rif<sup>+</sup>, lane 5- *Ent. faecalis* JH2-2 5423 ped<sup>+</sup> rif<sup>+</sup>, lane 6- *Ped. acidilactici* NCIM 5424.

Boguslawska et al. (2009) determined the transconjugants obtained by *in vitro* and *in vivo* methods by PCR assays, RAPD amplification and PFGE pattern, where the primer-specific PCR confirmed the transfer of the transposon Tn916 in the transconjugants from donors. Similarly, Toomey et al. (2010) characterized the
transconjugants by performing the gene specific PCR for antibiotic resistance genes, E-test results for resistance genes and rep-PCR fingerprinting to identify the strains. Further, the transconjugants obtained displayed DNA profiles similar to that of recipients by rep-PCR analysis. In the present study, the transconjugants obtained by both in vitro and in situ methods were characterized by anti-listerial assay, pediocin specific gene PCR, RAPD-PCR and Southern hybridization. The hybridization with the immunity gene suggests the transfer pediocin encoded plasmid from Ped. acidilactici NCIM 5424 to Ent. faecalis JH2-2.

6.3.3. Bacteriocin production by the transconjugants

The transconjugants Ent. faecalis JH2-2 5423 ped⁺ rif⁺ and Ent. faecalis JH2-2 5424 ped⁺ rif⁺ producing the pediocin PA-1 were subjected to study their production at different temperature (16, 37, 50 °C) and pH (4, 8, 10). It was observed that the recipient Ent. faecalis and both the transconjugants was unable to grow at 50 °C. The production of pediocin was observed at 16 and 37 °C temperature and at different pH tested by the transconjugants. The bacteriocin activity at different temperatures and pH were recorded in Arbitrary Units (AU) per ml against ScottA and represented in Fig. 6. 3. 3. 1. The production of bacteriocin at different pH (4, 7 and 10) for the donors and transconjugants is represented in Fig 6. 3. 3. 2.
Fig. 6.3.4.1. Effect on the production of pediocin PA-1 by the transconjugants at different pH and temperature. Results are the mean of two independent trials (±SD)
The production of pediocin is greatly influenced by the nutritional parameters, pH temperature, salt concentrations and aeration levels (Papagianni and Anastasiadou, 2009). The optimal temperature of 30-35 °C and pH 5-7 were reported for the pediocin AcH, SA-1, PA-1 etc. (Papagianni and Anastasiadou, 2009). The pediocin PA-1/AcH production was more when the pH of the culture medium was <5.0 for *Ped. acidilactici* H, whereas the more efficient production of pediocin was observed at pH 6.0 for *Lact. plantarum* WHE 92 (Ennahar et al. 1996). The transconjugants obtained in our study were able to produce low levels of pediocin PA-1 when compared to the parental donors under different temperatures and pH tested. An enhanced production of nisin by the transconjugant containing the protease-lactose plasmid pLP712 in *Lac. lactis* NCDO712 in milk was reported by Garcia Parra et al. (2010). The horizontal transmissions also vary from one bacterium to other and are found to be more under natural environmental stress like low pH, high salt, low temperature, etc., as reported for the antibiotic resistance gene transfer (Mc Mahon et al. 2007). Hence, the growth conditions of the bacteria and the production of the bacteriocin vary from one organism to other and are influenced by several environmental factors.
6. 4. CONCLUSION

Both *in vitro* and *in situ* conjugal mating techniques can be useful in the construction of transconjugants with bacteriocin encoded plasmids. The presence of pediocin PA-1 in intergeneric and interspecific LAB gives an idea of either transfer of entire plasmid by conjugative mode of HGT in their ecological niche or with other mobile genetic elements present in the pediocin encoded plasmid. The adaptability of the transconjugants to different environmental conditions was found to be different. In general, the soymilk model gave better transfer efficiency and suggested a suitable environment for the conjugal transfer of plasmid. Hence, such intergeneric pediocin producing cultures can be used in the production of bacteriocin in milk products, where the application of the natural pediocin producer, i.e. *Ped. acidilactici* was found to be very limited. The distribution of pediocin under natural conditions helps in the development of starter cultures, and/or as a biopreservative and bio-protective culture in the fermentation of dairy and meat products.

Such naturally modified LABs play a major role in the food industry which leads to the improvement of shelf-life because of their antagonistic nature, and their inherent ability of enhancing the flavour and texture of the fermented end-products, and also can be applied as an alternative of genome modification through genetic engineering.