CHAPTER V:

MOLECULAR CLONING AND EXPRESSION OF *ped*A and *ped*B GENES IN *Escherichia coli*

5.0 ABSTRACT

A gene encoding the pre-pro-pediocin (*ped*A) was amplified in combination with *ped*B using PCR technique from *Ent. faecium* PH-1 into *E. coli* cloning vector pTZ57R/T. The DNA fragment of 304 bp in size was subcloned from pTZpedAB into the *E. coli* expression vector pRSET A. Nucleotide sequencing of pRpedA/A confirmed the restoration of the reading frame. The protein expression host, *E. coli* Bl21 (DE3) was transformed with the recombinant plasmid pRpedA/A and the T_7 promoter was induced with 1 mM IPTG. The presence of a 12.8 kDa fusion protein, localized in inclusion bodies (IB) at high concentration, was confirmed by SDS-PAGE. Identity of the recombinant fusion protein, *6XHis-Xpress-Ped*A, after purification with Ni-NTA beads, was confirmed by western blotting using anti-His antibody. Refolding of the recombinant pediocin (rec-pediocin) solubilized in 8M urea was carried out in a refolding buffer consisting of 5 mM of β-mercaptoethanol and 1M glycine. The results indicated that the refolded rec-pediocin eluted earlier on preparative RP-HPLC than did the unfolded protein and moreover, exhibited antimicrobial activity against *Listeria monocytogenes* Scott-A. This activity was approximately 25% less (per ng of protein, exhibiting definite zone of inhibition) than that of the native pediocin.

The *ped*B gene coding for pediocin immunity protein was amplified by PCR by using the primers PED.IMM.F and PED.IMM.R bearing restriction sites *Nco*I and *Bam*HI, respectively. The PCR amplicon 336 bp in size obtained, was double digested with *NcoI/Bam*HI and cloned in-frame in the pQE60-HA tag vector, that had been previously digested with the same enzymes. The pQE60 vector had been initially modified by addition of HA-tag at the C-terminal end of the cloning site, wherein the tag allows immunological characterization of the expressed protein. The recombinant plasmid pQEpedIMM was double digested with *Eco*RI/*Pvu*II and the released 1.2kb insert, sub-cloned into the pUC19 double digested with *Eco*RI/*Sma*I. The nucleotide sequences of recombinant

plasmid pUCpedIMM thus obtained confirmed restoration of the reading frame in the *ped*B. The pQEpedIMM recombinant was transformed into the *E. coli* M15 (pREP4) host and the gene was induced with 1 mM IPTG. Western blot using anti-HA antibody indicated the presence of a 15 kDa protein as expected and its multimeric forms of the recombinant protein in the total cell lysate, albeit at low levels.

It has been shown that the C-terminal of PedB is involved in biological activity and hence it was decided to fuse the tag at the N-terminal of pedB, to obtain 6Xhis-PedB fusion protein. The PCR primers, pedB.F and pedB.R as forward and reverse primers respectively bearing *Pst*I and *Bam*HI restriction sites were used for amplification of the gene. The amplicon and the pQE30 vector was cut with the same enzymes prior to ligation. The recombinant plasmid pQE30pedB was constructed and transformed into *E. coli* M15 (pREP4) host and gene expression was carried out by the addition of 1 mM IPTG. Expression of fusion protein was analysed by SDS-PAGE. Ni-NTA purification of 6X His tag PedB was also carried out. Dot-blot data indicated a high level expression of recombinant PedB in induced cells as compared to that in uninduced cells.

Hydrophobicity profile of PedB protein indicated that there are atleast four domains of amino acid sequence number 25-35, 40-60, 65-75 and 90-100 consisting of 10-20 amino acid each, representing membrane spanning regions, which keep the protein in the cellular compartment and provides protection by inhibiting pore formation in the membrane by its cognate pediocin.

5.1 INTRODUCTION

Antimicrobial peptides, bacteriocins produced by LAB have been the subject of considerable research and industrial interest due to their potential as food biopreservatives (Jack *et al.* 1995; Nes *et al.* 2002). These ribosomally synthesized antibiotics can be produced by heterologous expression. The development of such systems offer a number of advantages such as higher production levels over its native counterpart. Further, heterologous production by food-grade LAB facilitates biopreservative applications in food systems (Axelsson *et al.* 1998; Rodriguez *et al.* 2002a). Moreover these proteins can be obtained in large quantities for their structural and biophysical studies by the recombinant route. *E. coli* is the most widely and successfully used host for the production of large amounts of foreign protein for biochemical/biophysical investigations. Recombinant proteins can be expressed in their native, active states and purified by conventional means or by using purification tags. High level of recombinant proteins expressed in *E. coli* tend to accumulate in inclusion bodies (IB) due to the lack of required accessories for its folding into its native form.

Formation of IBs takes place mostly with cysteine containing proteins wherein the reducing environment of the bacterial cytosol inhibits formation of disulfide bonds (Makrides 1996; Patra *et al.* 2000, Xie *et al.* 1998). However, formation of IBs facilitates easy and simple recovery of highly expressed proteins since they can be solubilized by using high concentration of chaotropic agents such as urea, guanidine. hydrocloride (Gdn-HCl), thiocyanate, salts, SDS, N-cetyltrimethylammonium chloride and sarkosyl (sodium N-lauroyl sarcosine), in combination with reducing agents like β-mercaptoethanol, dithiothreitol and cysteine. The solubilized proteins have been refolded to their native state by using various refolding strategies. These include the use of immobilized minichaperones, size-exclusion

chromatography, redox- refolding buffer such as cysteine/cystine, glutathione redox buffer etc (Lilie *et al.* 1998; Patra *et al.* 2000).

Antilisterial bacteriocin, pediocin PA-1 displays broad-spectrum bacteriocidal activity against many Gram-positive and certain stressed Gram-negative bacteria associated with food-spoilage and human pathogenesis. Bacteriocins also have potential application in controlling topical infections caused by bacterial pathogens, and hence there is a need to produce these molecules in a large scale for their potential applications (Miller *et al.* 1998). The bacteriocin pediocin PA-1 operon encompasses four genes viz *ped*A, *ped*B, *ped*C and *ped*D. These genes are transcribed as two overlapping transcripts with a single promoter upstream of *ped*A (Marugg *et al.* 1992; Venema *et al.* 1995). Pediocin PA-1 is translated as a 62 -aa precursor. This precursor peptide is cleaved *in vivo* behind the double glycine residues of the leader peptide, resulting in formation of the mature pediocin PA-1 molecule of 44 - aa (Marugg *et al.*1992). The *ped*D gene product is known to be essential for the removal of 18 -aa leader peptide from the inactive pre-pediocin PA-1 precursor and generation of the active mature form of the peptide during membrane translocation (Venema *et al.* 1995).

It is known that *ped*A and *ped*D are essential for pediocin PA-1 production. The *ped*A encodes the precursor of pediocin PA-1 and the *ped*D gene product belongs to the group of ATP-dependant translocators (Bukhtiyarova *et al.* 1994). However the functions of these gene products could not be established in *Pediococcus* because of plasmid instability. Previously, it has been shown that pre-pediocin PA-1 can be secreted and processed in *E. coli* in presence of *ped*C and *ped*D protein upon co-expression in the host (Venema *et al.* 1995). Secretion and processing of pediocin requires a dedicated transport and processing system comprised of an ABC export protein and an accessory protein. It is important that the use of these molecules be explored for the heterologous expression of pediocin. Pediocin has been heterologously

expressed in several strains of *E. coli, Ped. pentosaceus, L. lactis. Lact. sake* etc., using the *ped*C and *ped*D genes. The antimicrobial activity of heterologously expressed pediocin was approximately 25% less than wild type (WT) to 2.5 fold higher depending on the expression system (Horn *et al.* 1998).

Further, pediocin has been heterologously expressed using general secretory pathways such as MBP-pediocin PA-1 in *E. coli* where in MBP-pediocin fusion protein has been exploited for screening of deletion mutants with varying antimicrobial activity and to demonstrate that the fusion of the maltose binding protein to the signal peptide of prepediocin does not interfere with the antimicrobial activity of the fusion protein (Ray *et al.* 1999). For heterologous expression of pediocin in LAB, the major drawback is that the *ped*A has to be expressed in combination with *ped*B due to natural sensitivity of many of LAB strains to pediocin (Axelsson *et al.* 1998).

Pediocin immunity protein (PedB) is a 112 -aa protein with a deduced MW of 13 kDa, an isoelectric point of 8.10 with N and C-terminal domain possessing hydrophilic pattern and central region that shows high degree of hydrophobicity. Preliminary sequence analysis indicated that this protein does not seem to be a membrane anchored lipoprotein and probably could be a membrane associated protein as seen in case of the PepI immunity protein (Pag *et al.*1999). *Ped*B has been heterologously expressed using a strong lactococcal promoter P32 in *Ped. pentosaceus* conferring resistance to the pediocin susceptible host. Point mutations inducing frame-disruptions of the *ped*B gene confirmed that PedB has a function in immunity (Venema *et al.* 1995).

The immunity proteins of the pediocin family range from 81 to 115 -aa residues in size and display 5-85% sequence similarity. Functional analysis of immunity protein of mesentericin Y105 and carnobacteriocin B2 indicated that these proteins were located intracellularly and their intracellular pool is

divided into a small membrane associated fraction and a large cytoplasmic fraction. The C-terminal part of immunity proteins are involved in specific recognition of cognate bacteriocins (Dayem *et al.* 1996; Fimland *et al.* 2002a; Johnsen *et al.* 2004).

The present literature suggests that mechanism of action/localization of PedB has not been demonstrated yet in detail. Further, there are no biochemical evidences showing its interaction with its target. Such studies would need production of large quantities of the protein that can be obtained through heterologous expression. Computational analysis provides an attractive tool for the understanding of the structure and function of biomolecules. The vast data available in data banks facilitates the analysis of structure and helps predict the functions of a given protein by comparison with those of a known protein.

In previous chapters, molecular and biochemical characterization of intergeneric pediocin PA-1 was carried out. Further, production of pediocin PA-1 in WP medium by *Ent. faecium* PH-1 was demonstrated and its possible use for biopreservation was studied. This chapter deals with the high level expression of chimeric pediocin in *E. coli*, followed by a simple single step purification and glycine/β-mercaptoethanol mediated *in vitro* refolding of.the recombinant protein. Heterologous expression of *ped*B in *E. coli* was also carried out in order to obtain the pediocin immunity protein free from pediocin. Further, computational analysis of the deduced -aa sequences of PedB was performed to predict possible transmembrane domains and its hydrophobicity profile.

5.2 MATERIALS AND METHODS

5.2.1 Materials

5.2.1.1 Fine chemicals

Ampicillin, Coomassie R250, PMSF, Triton X100 and TFA were purchased from Sigma Co. (USA), IPTG, X-Gal, Gelatin were purchased from Himedia, Mumbai. Ni^{2+} beads were purchased from Qiagen (Hilden, Germany). β-mercaptoethanol, DTT, Imidazole, TCA, Trizma-base and glycine were from SRL. BCIP, NBT reagents were purchased from Bangalore Genei.

5.2.1.2 DNA modifying enzymes and antibodies

Restriction enzymes such as *Acc*II, *Eco*RI, *Pvu*II, *Sma*I, *Nco*I, BamHI, *Pst*I (each 10U μl^{-1}) were procured from Promega (USA). T_4 DNA Ligase, (10U μl^{-1}), CIAP, 1 U μl^{-1}) from Promega (USA) and MBI Fermentas (Lithuania), respectively. Anti-HA epitope antibody was obtained from Boehringer Mannheim (Germany). Anti-His antibody was obtained from Research and Diagnostic Inc. (USA). The secondary anti-rabbit IgG was obtained from Bangalore Genei.

5.2.1.3 Bacterial strains and cultivation medium

The bacterial strains of *E. coli* DH5α, Bl21 (DE3): (F^-*ompT gal*[*dcm*](*lon*) *hsdS*$_B$ ($r_B^- m_B^-$, an *E. coli* B strain) a λ prophage carrying the T_7 RNA polymerase gene) obtained from In Vitrogen (The Netherlands) and M15: pREP4, *lacI*^{q} (Qiagen, Germany) were used. These strains were grown in LB broth medium as given in section (3.2.1.2). LAB strain of *Ent. faecium* PH-1 was grown in MRS broth and *List. monocytogenes* Scott-A was grown in BHI broth as mentioned previously (3.2.1.2).

5.2.1.4 Plasmid vectors

The *E. coli* plasmid vectors were used in this study are enlisted in Table 5.1.

Table 5.1: Vectors used for cloning and expression of pediocin genes.

Vector	Size (kb) and selection marker	Purpose	Reference/Source
pUC19	2.6, Ampicillin	General Cloning vector	New England Biolab
pTZ57R/T	2.6, Ampicillin	PCR product cloning vector	MBI
pRSET-A	2.9, Ampicilin	Expression vector	In Vitrogen
pQE60	3.4, Ampicillin	Expression vector	Qiagen
pQE30	3.4, Ampicillin	Expression vector	Qiagen
pREP4	3.7, Kanamycin	RNA polymerase expression vector	Qiagen

5.2.1.5 Oligonucleotide primers

For the amplification of the *ped*AB gene, the following primers were used

PedA.F 5' TTT GCC ATG GAA AAA ATT GAA AAA TTA AC3' and
PED.IMM-R 5' CCC TTT ATC AGG ATC CTT GGC TAG GCC 3'

For the amplification of *ped*B gene the following two sets of primers were used. The restrictions site introduced are shown as bold italics.

Set I: PED.IMM-F 5' GGG TGA TT***C CAT G***GG TAA GAC TAA GTC GG 3"
PED.IMM-R 5' CCC TTT ATC A***GG ATC C***TT GGC TAG GCC 3' and

Set II: PedB.F 5' G GTG ATT T***GG ATC C***TA AGA CTA AGT CG 3'
PedB.R 5' CCC CTT ***CTG CAG*** TAC TAT TGG C3'

The *ped*A gene amplifying the forward primer (PedA.F) was synthesized by Sigma-Aldrich (USA) and the *ped*B gene amplifying primers were synthesized at SRD, Frankfurt University, Germany. T7 reverse primer: 5'TAA TAC GAC TCA CTA TAG GG 3' (NEB 1248). This primer was provided by the DNA sequencing facility of DBT, New Delhi.

5.2.2 Methodology

5.2.2.1 Molecular Biology techniques

5.2.2.1.1 DNA isolation

Plasmid DNA of pediocin producing strain of *Ent. faecium* PH-1 was isolated by the method described previously (Section 2.2.2.8.1). Plasmid DNA from recombinant *E. coli* was isolated by the method described by Birnboim and Doly (1979).

5.2.2.1.2 PCR

Plasmid DNA of PH-1 was used as a template for in vitro amplification. PCR was carried out using the standard protocol described previously (section 3.2.2.6.5) for amplification of *ped*AB and *ped*B genes. The PCR components and the conditions were same as described in section **3.2.2.6.5.2** and Table 3.2.

5.2.2.1.3 Cloning and sub-cloning

The *ped*A and pedB genes were amplified together by PCR using primers PedA.F and PED.IMM-R as forward and reverse primers and initially cloned into the pTZ57R/T vector and subsequently sub-cloned into the *KpnI/ Hin*dIII site of pRSET-A. The subcloned fragment consists of gene coding for precursor pediocin and the N-terminal portion of *ped*B gene.

The *ped*B gene amplified using primers PED.IMM-F and PED.IMM-R (set I) was double digested with the enzyme *Nco*I and *Bam* HI and gel purified using a

Qiagen column. It was then ligated to double digested (*NcoI*/*Bam*HI), dephosphorylated vector pQE60.

The *ped*B gene amplified using the primers PedB-F and PedB-R (set II) was double digested with the enzymes *Bam* HI and *PstI* and gel purified using the Qiagen column. It was then ligated to double digested (*Bam*HI/*PstI*) dephosphorylated vector pQE30. The recombinant plasmid pQEpedIMM was double digested with the enzyme *Eco*RI and *Pvu*II and the insert release (~600bp) was gel purified and was ligated with the pUC19 vector which had already been double digested with *Eco*RI/*SmaI* and dephosphorylated. The presence of recombinant plasmid pUCpedimmQ thus constructed was confirmed by *AccII* digestion followed by gel analysis. pUCpedimmQ was subjected to bi-directional DNA sequencing.

5.2.2.1.4 DNA manipulation

pUC derived recombinants were transformed in *E. coli* DH5α and were selected on LB agar, X-Gal, Ampicillin (100 μg ml^{-1}) by blue/white screening as given in 3.2.2.6.3. *E. coli* DH5α was used for the plasmid amplification and either Bl21 or M15 host was used for gene expression studies. The modified protocol described in previous section (3.2.2.6.2) was used for the development of competency of *E. coli* strains.

5.2.2.1.5 Gene expression studies

Gene expression was carried out using the inducer IPTG. The cells were grown till absorbance of 0.5 OD at 600nm. Subsequently, 1 mM IPTG was added and the cell were additionally grown for 4 to 6h. The expressed protein was analysed by SDS-PAGE and/or by western blotting described in subsequent section.

5.2.2.1.6 Nucleotide sequencing and data analysis

For oligonucleotide designing, fusion protein translation, nucleotide sequence analysis etc. Clone Manager (Ver.5) programme was used. Nucleotide sequencing of *ped*A gene was carried out at the DBT sponsored facility at University of Delhi, New Delhi and the *ped*B gene sequencing was carried out at the University of Frankfurt/M, Germany.

5.2.2.1.7 Computation analysis

Hydrophobicity plot was determined by the method of Kyte and Doolittle (1982). The Tmpred was calculated using the programme available on the web: www.ch.embnet.org

5.2.2.2 Biochemical and immunological techniques

5.2.2.2.1 RP-HPLC

Preparative RP-HPLC was carried out as described previously for purification of the pediocin (3.2.2.5.3). Refolded and unfolded rec-pediocins were also analysed by semi-preparative RP-HPLC.

5.2.2.2.2 SDS-PAGE

The procedure described by Laemmli (1970) was followed with certain modifications (Sambrook and Russell 2001). The stocks of acrylamide mixture (30:0.6%), 10% each of APS and SDS were prepared as described previously (Section 4.2.2.5.2). Separating gel buffer of 1.5M Tris HCl (pH 8.8) was prepared. 1M Tris HCl (pH 6.8) buffer was used for the stacking gel buffer. All the stock solutions except SDS were stored at 4^0C. The components described in Table 5.2 were used for the preparation of gel.

The Tris glycine buffer was used for electrophoresis as a running buffer. It consists of 25mM Tris HCl pH8.3; 200mM Glycine and 0.1% SDS. Electrophoresis conditions, staining, destaining was carried out as described previously (Section 3.2.2.5.4).

Table 5.2: Components of Tris Glycine SDS-PAGE gel

Components (ml)	Separating gel (10ml) 15%	Stacking gel (5ml) 5%
H_2O	2.3	3.4
30% Acrylamide mix	5.0	0.83
1.5M Tris (pH8.8)	2.5	-
1M Tris (pH 6.8)	-	0.63
SDS 10%	0.1	0.05
APS 10%	0.1	0.05
TEMED	0.004	0.005

5.2.2.2.3 Protein dot-blotting

5.2.2.2.3.1 Sample spotting

The total cell lysate was prepared in lysis buffer containing 50 mM Tris (pH 7.5), 2 mM EDTA, 2 mM PMSF and 0.1% Triton X100) from the test strain by sonication, as described in 4.2.2.5.2. It was spotted on nitrocellulose (NC) membrane in different quantities, allowed to dry and was then used in reaction with antibody. The membrane was rinsed with a 1X Tris buffered saline-Tween 20 (TBST), containing Tris HCl 20mM, NaCl 0.9%, Tween 20, 0.1% and pH adjusted to 7.4 with HCl. The membrane was blocked by incubating with 2% gelatin (prepared in 1X TBST) for 1h. The membrane was again washed twice with TBST buffer for 15min each.

5.2.2.2.3.2 Antibody reaction

- The primary antibody (Ab) diluted 5000 times in TBST was incubated with the membrane at room temperature (RT) with gentle shaking for 1-2h.
- The Ab solution was decanted and the membrane washed twice with TBST buffer for 15 min to remove unbound Ab.

- Subsequently, the membrane was incubated with the secondary Ab (prepared in TBST 1:5, 000 dilution) with gentle shaking at RT for 1-2h.
- The Ab was decanted and the membrane was washed gently for 30min with TBST, with atleast three changes of the wash buffer.

5.2.2.2.3.3 Colour development

The membrane was equilibrated with alkaline phosphatase buffer for the colour reaction. The buffer consists of:

NaCl	100mM
$MgCl_2$	5mM
Tris HCl	100mM. The pH was adjusted to 9.5

The color was developed by adding 1 ml of 1X BCIP/NBT substrate and the reaction was incubated in the dark. Once the desired band appeared, reaction was stopped by adding 200 µl of 0.5 M EDTA pH 8.0, in 50 ml of 0.9% NaCl or by simply washing the membrane with sterile distilled water.

5.2.2.2.3.4 Electrophoretic transfer to proteins

The protocol described by Towbin *et al.* (1979) was followed, with certain modifications, for the electrophoretic transfer of protein from the acrylamide gel to the NC membrane. The LKB 2117 Multiphor II electrophoresis apparatus (Pharmecia) was used for this purpose. A filter paper pad of Whatman No. 3 was prepared by cutting it to the exact dimensions of the gel to be transferred. The NC membrane to be used for transfer was also cut to the same size as the gel. The Whatman No. 3 filter paper and the membrane were presoaked in Transfer buffer (TRB), containing:

Glycine	3.0gm
Tris HCl	5.8gm
SDS	0.37gm
Methanol	200 ml (was added just before use)

The final volume of the TRB was made to 1 litre using double distilled water.

Eight strips of Whatman paper (presoaked in TRB) were placed on graphite plate (cathode). The air-bubbles trapped inside were removed by rolling a glass rod over it. The NC membrane, which was charged by soaking in TRB, was also placed on the filter paper pad kept on the cathode plate. Above this, the protein gel detached from stacking gel was placed. End eight presoaked Whatman sheets were stacked on the gel. Above that, the anode graphite plate was mounted carefully and the assembly was connected to the electrophoretic power supply.

Current (0.8 mA/cm$^{2)}$) was passed through the circuit for 90 min using a Nova blot LKB apparatus. After the transfer, the stacks of the papers and the gel were removed carefully the right side of the membrane was marked for identification of the ends and it was subjected for immunological characterization. The procedure for immunological detection was the same as described in the section **5.2.2.2.3.2** and **5.2.2.2.3.3.**

5.2.2.3 Ni-NTA purification

The Ni^{2+} beads (size 45-165 μm) were washed twice with sterile water by keeping the tube in ice and were equilibrated with the lysis buffer. These beads were mixed with the cell lysate and/or supernatant and incubated at 4^0C for 1h with intermittent shaking.

5.2.2.4 Solubilization of IBs and purification of rec-pediocin

Cells were harvested by centrifugation and the cell pellet was **washed** with 0.9% NaCl and resuspended in 50 mM Tris, 2 mM EDTA, 2 mM PMSF and 0.1% Triton X100 (pH 7.5). The resuspended cells were broken down by sonication for 1 min and freeze thawed twice at -20^0C. The crude IBs were precipitated by centrifugation and the impurities solubilized in 2M Urea along with 0.1% Triton X-100 and centrifuged at 10, 000 rpm. The resultant pellet was washed twice with sterile distilled water. The pellet was resuspended in 5mM EDTA, incubated for 10-20min and was centrifuged. It was washed with

sterile water and partially purified IBs were solubilized in different molar strengths of Urea in 25 mM Tris at a pH range of 6 to 9; for studying solubilization patterns and effect of urea on solubility in different molar strengths ranging from 0 to 10 M at fixed pH. The absorbance was recorded at 280nm by using UV Spectrophotometer.

5.2.2.5 *In Vitro* refolding

The urea denatured, completely-solubilized IBs were refolded as described by Eisenmesser *et al.* (2000). Essentially, the urea dissolved IBs were suspended slowly in the refolding buffer consisting of 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 M glycine, 5 mM β-mercaptoethanol and 5 mM immidazole and stirred for 18-20h at room temperature. Any precipitate that was formed, was removed by filtration through a 0.45 μ filter and the filtrate was mixed with the refolding buffer charged Ni^{2+} beads. Nickel beads-bound-proteins were removed by centrifugation and beads were washed with refolding buffer consisting of 20 mM immidazole and pure 6X His tagged proteins were eluted with 0.5 M immidazole in refolding buffer.

5.2.2.6 Protein estimation and pediocin assay

The proteins were estimated by the method of Bradford (1976) using microassay described in section 3.2.2.5.4.

Pediocin was assayed for its anti-listerial activity against *List. monocytogenes* Scott-A by the method described previously (2.2.2.7.4).

5.3 RESULTS AND DISCUSSION

5.3.1 Molecular cloning of pediocin structural gene

The *ped*A and pedB genes were amplified as described in section **3.3.12**, by using the primers PedA.F and PED.IMM-R. The diagrammatic representation of cloning of pedAB gene from pediocin operon is presented in Figure 5.1. To facilitate further sub-cloning of *ped*A gene, the PCR product was purified and cloned into the T-tail (pTZ57R/T) vector. The putative recombinants (pTZpedAB) were selected by blue/white screening and white colonies were isolated and subjected to restriction digestion with *Hind*III (Fig. 5.2A). The results indicated that about 50% of white colonies released insert. It was expected that there was one *Hind*III site each in *ped*B gene and in the vector.

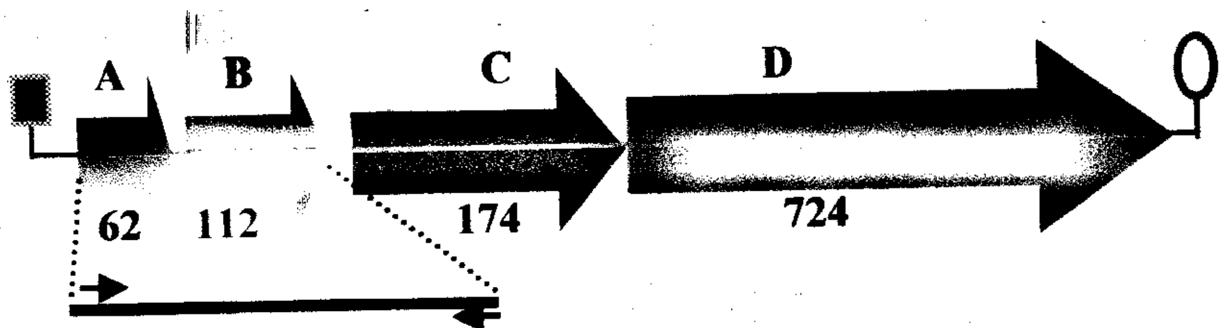

Figure 5.1: Gene organization for pediocin production. **A** to **D** are the genes coding for structural, immunity, maturation and transport proteins, respectively. Size of each protein is shown in number of -aa in each case. Promoter (rectangle box) and terminator (lollipop symbol) is also shown. Arrow indicates the position of forward and reverse primers used for PCR amplification of the *ped*AB gene.

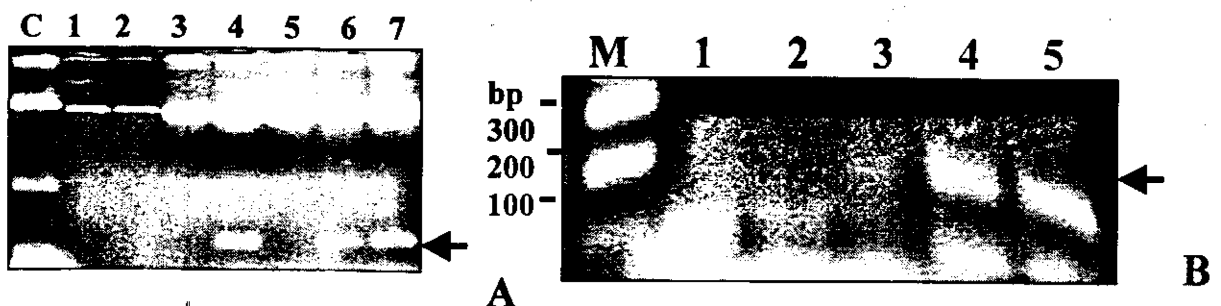

Figure 5.2: Molecular cloning of *ped*A gene. a) Agarose gel (1.5%) electrophoresis of the pTZpedAB recombinants by *Hind*III digestion and b) confirmation of gene (insert release) after sub-cloning into pRSET A vector. Arrow indicates the desired size of band obtained. M is a 100 bp ladder.

The *KpnI/Hind*III fragment from pTZpedAB was released, purified and ligated to double digested (*KpnI/Hind*III), CIAP treated vector pRSET A. Thus this fragment consists of gene coding for pre-pediocin and the N-terminal portion of *ped*B gene. The molecular map of pRpedA/A (pRSET A vector harbouring *ped*A gene) was constructed using the nucleotide sequences from the pediocin operon (Marugg *et al.* 1992) and is shown in Figure 5.3. The features of the same are shown in Table 5.3.

5.3.2 Construction of pRpedA/A recombinant

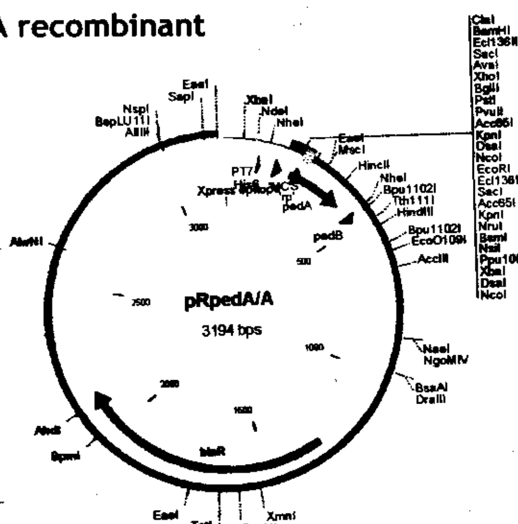

Figure 5.3: Molecular map of pRpedA/A

Table 5.3: Molecular features of pRpedA/A

Type	Start	End	Name	Description
Region	88	96	P_{T7}	IPTG inducible PT7 promoter
Gene	112	119	6X*his*	Tag for affinity purification
Gene	169	193	Xpress	Epitope for western analysis
Gene	227	416	*ped*A	Strutural gene for pre-pediocin
Region	237	271	MCS	Multiple cloning site
Marker	239	271	rp	rep (pMBI)
Gene	1290	2150	blaR	Ampicillin resistance

The *ped*A gene is under the control of pT7 promoter with 6X His-X-press tag at the N-terminal of the pre-pediocin. The release of insert 186 bp in size only from The pRpedA/A recombinant plasmid after restriction digestion with *Kpn*I/*Hind*III verified the clone (Figure 5.2B).

Pediocin is a small (4.2kDa) heat stable anti-listerial bacteriocin. It is encoded by a *ped*A gene as a precursor. Due to its small molecular weight and the size of the corresponding gene, cloning by PCR is often difficult. Therefore the *ped*A gene was cloned in combination with the N-terminal of *ped*B in the present study.

5.3.3 Nucleotide sequence analysis of pediocin fusion protein

The nucleotide sequences are presented in Figure 5.4. The deduced -aa sequences are also shown. As it may be seen the ORF of 6Xhis-Xpress-prepedA is continuous. After initiation codon of prepediocin encoding gene glutamic acid (E) was incorporated due to change in one nucleotide in primer PedA.F. The three different molecules of pediocin that can be expected are: 1. prepediocin, 6.4; 2. matured pediocin, 4.6 and 3. chimeric pediocin, 12.8kDa

In the present study, the fusion tag was cloned at the N-terminal of *ped*A because the C-terminal of the peptide is known to display antimicrobial activity. Previous evidences shown in literature suggest that the N-terminal of pediocin is involved in recognition of the receptors present in the indicator strain. Prepediocin tagged to maltose binding protein displayed activity (Miller et al 1998;Ray *et al.* 1999). Figure 5.5 shows the -aa sequences of the cloned and expressed pediocin fusion protein. Small molecular weight peptides that are generally produced at a very low level as a secondary metabolites are difficult to purify.

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TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCGGGGTTCTCATCAT
 S  R  N  N  F  V  -  L  -  E  G  D  I  H  M  R  G  S  H  H
CATCATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATCTGTAC
 H  H  H  H  G  M  A  S  M  T  G  G  Q  Q  M  G  R  D  L  Y
GACGATGACGATAAGGATCGATGGGGATCCGAGCTCGAGATCTGCAGCTGGTACCTCGCG
 D  D  D  D  K  D  R ▲ W  G  S  E  L  E  I  C  S  W  Y  L
A AATGCATCTAGATTTTTTGCCATGGAAAAAATTGAAAAATTAACTGAAAAAGAAATGGCC
 N  A  S  R  F  F  A  M  E  K  I  E  K  L  T  E  K  E  M  A
AATATCATTGGTGGTAAATACTACGGTAATGGGGTTACTTGTGGCAAACATTCCTGCTCT
 N  I  I  G  G  K  Y  Y  G  N  G  V  T  C  G  K  H  S  C  S
GTTGACTGGGGTAAGGCTACCACTTGCATAATCAATAATGGAGCTATGGCATGGGCTACT
 V  D  W  G  K  A  T  T  C  I  I  N  N  G  A  M  A  W  A  T
GGTGGACATCAAGGTAATCATAAATGCTAGCATTATGCTGAGCTGGCATCAATAAAGGGG
 G  G  H  Q  G  N  H  K  C  -  H  Y  A  E  L  A  S  I  K  G
TGATTTTATGAATAAGACTAAGTCGGAACATATTAAACAACAAGCTTGATCCGGCTGCTA
 -  F  Y  E  D  -  V  G  T  Y  -  T  T  S  L  I  R  L  L
```

Figure 5.4: Nucleotide sequences of 6X *his-Xpress-pedA* of *Ent. faecium* PH-1. The deduced most probable translated product is also shown. Nucleotide sequence result confirmed the correct restoration of reading frame of the fusion construct. The primer generated changed amino acid 'E' is shown after methionine of prepro pedA. The other modules highlighted are; Initiation codon methione of fusion protein; 6X *his* tag (bold letters), ▲ Enterokinase cleavage site, X-press epitope, ☐ Signal peptide of pediocin

MR GSHHHHHHGM ASMTGGQQMG RDLYDDDDKD RWGSELEICSYLANASRFFA ***MEKIEKLTEK*** **EMANIIGGK^{+1}Y YGNGVTCGKH SCSVDWGKAT TCIINNGAMA WATGGHQGNH KC^{+44}-**

Fig.5.5: Amino acid sequences of the cloned and expressed recombinant pre-pediocin. Authentic portion of the sequences are shown in bold. The added tags are in italics and the changed nucleotide primers generated -aa in bold italics.

The Expression of such epitopes as a fusion protein is preferred to circumvent these problems (Makrides 1996). Construction of pediocin fusion protein in the present study was taken-up in order to produce pediocin in large quantities and to achieve high levels of rapid purification.

4.3.4 Heterologous expression and analysis of recombinant pediocin

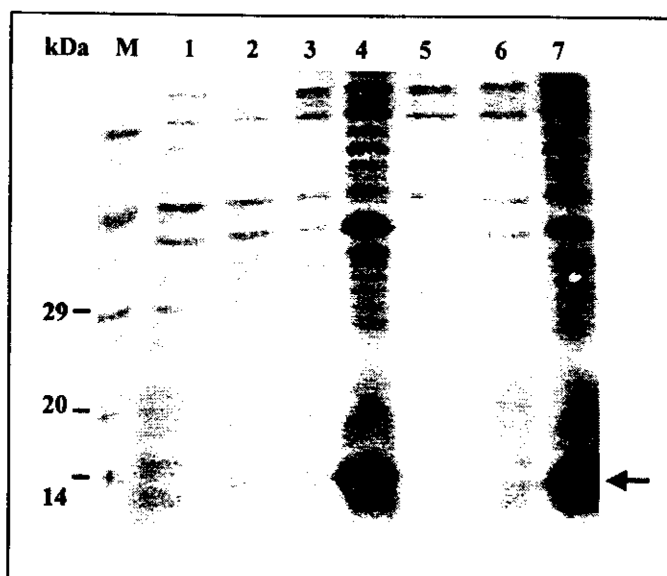

Figure 5.6: Analysis of total cell lysate of pediocin expressing *E. coli* SDS-PAGE (15%) analysis of ***His6-Xpress-PedA*** recombinant in *E. coli* Bl21. Lane 1, total cell lysate prepared from 2h grown preinduced r4 recombinant. Lane 2, lysate of r4 recombinant prepared directly in 1X loading buffer. 3, Sonicated lysate of r4. 4, The pellet of sample 3 dissolved in 6 M urea; 5, supernatant of sample 3; 6, lysate of r4 recombinant prepared same as lane2; and lane 7, sonicated sample as in lane 6, centrifuged and the pellet solubilised in 6 M urea. Lane 2-7 are induced with 1 mM IPTG and grown for 4h (lane 2) and 6h (lane 6). M is a medium range protein MW marker (Bangalore Genei), Arrow indicates recombinant protein of the expected size (12.8 kDa), being detected in protein solubilised by 8M urea from inclusion bodies.

After confirmation of the pediocin structural gene and restoration of the reading frame by sequencing, the recombinant plasmid was transformed into the *E. coli* expression host Bl21 (DE3) and gene expression induced by 1mM IPTG for 2, 4 and 6h. B21 host tranfomed with only vector was taken as control.

Protein from the total cell lysate was separated on a 15% denaturing gel (Figure 5.6). Hyperexpression of the protein of ~12.8kDa (expected size) was observed in recombinants induced with IPTG. However, un-induced recombinant and induced vector were unable to synthesize a band of this size. The precipitate obtained after centrifugation of recombinant bacterial lysates that had been induced was washed with 2M urea and dissolved in 8M urea. The proteins thus solubilised contained an intense 12.8 kDa protein band indicating that the protein had been targeted to IBs. In the recombinant *E. coli.* Induction with 1 mM IPTG for 4h allowed for high accumulation of recombinant protein. Induction for longer periods or the addition of higher concentrations of inducer reduced the cell growth drastically.

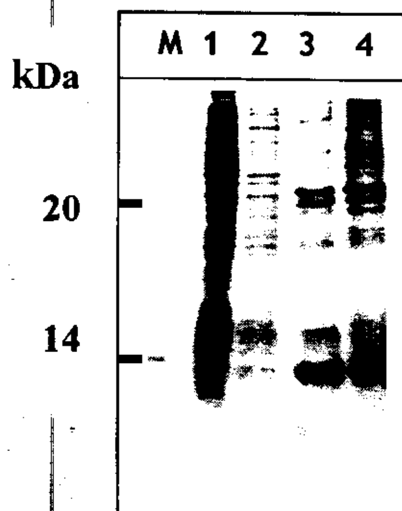

Figure 5.7: Isolation and purification of IBs of *E. coli* expressing rec-pediocin. Gel analysis of IBs purified from hyper expressing *E. coli*. Lane 1, induced cells (control); 2, induced (frozen at -20^{0}C), 3, purified IBs from (2) and 4, purified IBs from sample 1.

Recombinant E. *coli* Bl21 cells stored in 20% glycerol at -20^{0}C for more than a week grew very slowly on revival while the recombinant protein expression accounted for almost 50% of the total cellular protein (Figure 5.7). The reasons for this are not clear and bear investigation.

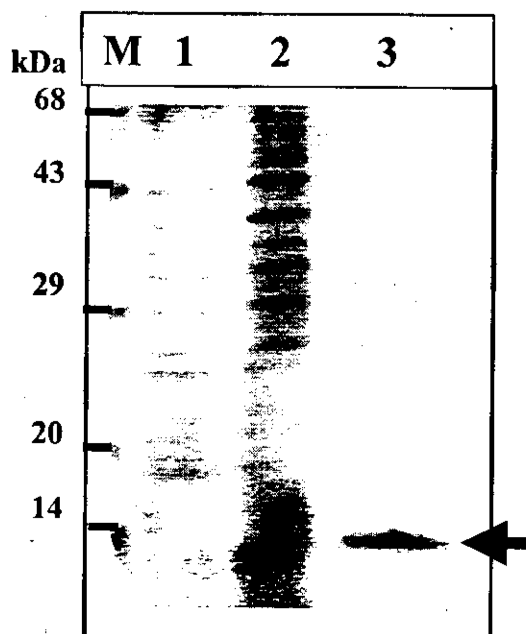

Figure 5.8: Single step purification of recombinant pediocin. SDS-PAGE (15% acrylamide) analysis of 6X histidine -Xpress-prepediocin produced in *E. coli.* Lane 1, total cell lysate uninduced ; 2, Lysate, induced recombinant and lane 3, Ni-NTA beads absorbed IBs protein eluted with 0.5 M imidazole. M, medium range protein marker

Heterologously expressed rec-pediocin was induced by 1 mM IPTG and accumulated in IBs of *E. coli.* The IBs were purified and the 6X His tagged protein was immobilized to the Ni^{2+} beads pre-equlibrated with lysis buffer. A 12.8 kDa protein was eluted with 0.5 M immidazole from the gel as evidenced on SDS-PAGE gel (Figure 5.8, lane 3). This would indicate the purification of the hexa-histidine tag fusion protein in a single step.

Immunological characterization of 6Xhis-Xpress-pediocin was carried out and the results are presented in Figure 5.9.

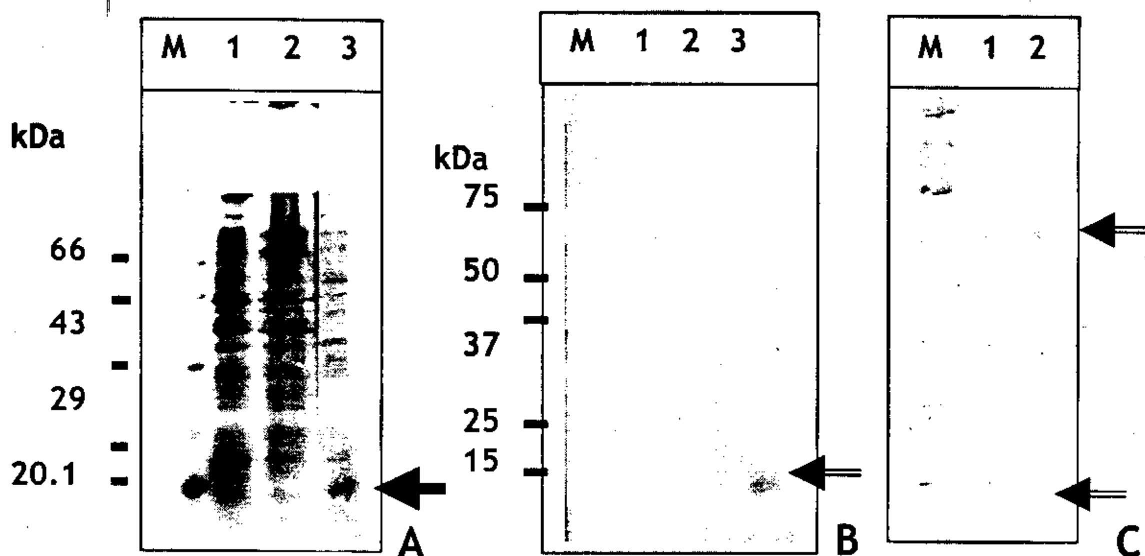

Figure 5.9: Immunlogical detection of rec-pediocin by anti-6XHis Ab.
A) SDS-PAGE analysis, **B)** & **C)** immunological detection by Ab.
← Indicates IBs of *E. coli.* ⟸ indicates putative monomeric and multimeric fusion protein detected in western blot, M in figure B and C is a prestained see blue molecular size marker (MBI). Lane 1, vector, 2 & 3 un-induced and IBs produced from induced culture.

The recombinant reacted with the His tag antibody indicating its chimeric nature (Figure 5.9). In certain cases, multimeric forms of the fusion protein were also observed (Figure 5.9C). 6X His tag proteins are know to occur in multimeric forms due to intermolecular hydrogen bonding. These multimeric forms of fusion protein can be resolved on SDS-PAGE by proper boiling of the sample in the presence of β-mercaptoethanol.

5.3.5 Solubilization of rec-pediocin Inclusion bodies

The solubility of the IBs from the recombinant *E.coli* was tested vaying the pH of extraction from pH 6 to 9 and urea concentration from 0 to 10M. Tris buffer at pH 7.5 along with 8 M urea concentration was found to solublise most protein as indicated by maximum absorbance at 280nm. Addition of DTT

increased solubility of IB over two folds due to reduction on disulfide bonds of prepediocin (Figure 5.10).

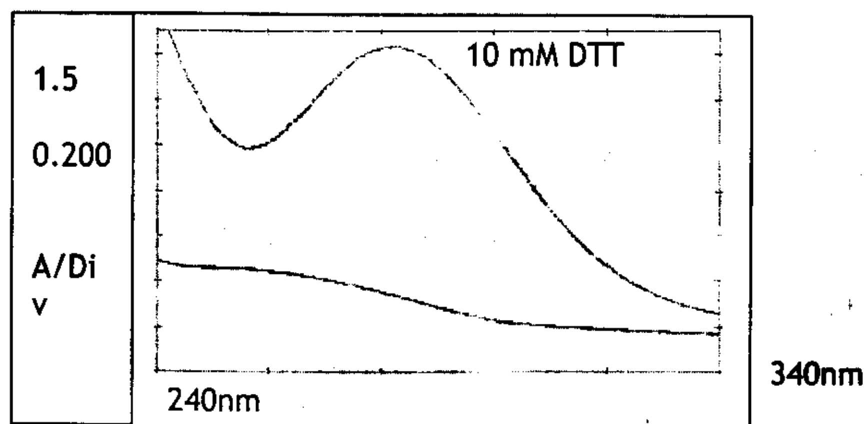

Figure 5.10: Effect of DTT on solubility of IBs of *E. coli.*

Xie *et al.* (1998) have demonstrated that using 10mM DTT increased the solubilization of human RBP (retinal binding protein) by 2.5 folds. A similar effect of DTT was seen in our study with rec-pediocin increasing solubility by atleast 2 fold.

5.3.6 *In Vitro* refolding of chimeric protein

The hexahistidine tagged pediocin was purified using Ni^{2+} beads, since Ni^{2+} beads have a high affinity to 6Xhis (5-10 mg ml^{-1}). The refolded rec-pediocin was eluted as a single major peak of 25min. However, the unfolded fraction showed late elution of major peak at 30min in a preparative RP-HPLC (Figure 5.11). The single peak of refolded recombinant pediocin was comparable with the active fraction of native pediocin which was shown a similar pattern of retention of 22min upon analysis. A 12.8 kDa protein was pediocin was eluted (3^{rd} fraction) by 0.5 M immidazole. The eluted fraction was capable of

inhibiting the growth *List. monocytogenes* ScotA indicating that the chimeric pediocin had refolded to its active confirmation in the refolding buffer.

In the present study, suspending protein isolated from IBs in refolding buffer in the presence of reducing agent β-mercaptoethanol and 1M glycine as a additive found to be undergo refolding to its biological active state. Further, affinity chromatography with Ni^{2+} beads helped refold the protein at a high level. It has been previously suggested that the choice of refolding buffer and immobilization on Ni-NTA beads itself facilitates in renaturation of proteins (Rogl *et al.* 1998). The thiol compound such as β-mercaptoethanol at a concentration of 5-15 mM have been found to act as a oxido-shuffling agents during renaturation and disulfide bond formation. The low MW additive like glycine may help reduce protein aggregation during the refolding process (De Bernardez Clark 1998; 2001). Eisenmesser *et al.* (2000) used either 5mM β-mercaptoethanol as a reducing agent along with 1M glycine or 1mM oxidized glutathione as a redox buffer in refolding of IL-13 expressed in IBs of *E. coli*. The former found to helped in increasing the yield of refolding fusion protein of IL-13 by a factor of two.

Recently, human proinsulin obtained from IBs of *E. coli* was renatured in presence of suitable redox conditions. The renatured and denatured proinsulin was characterized by RP-HPLC and comparison with native insulin was made (Winter *et al.* 2002). In the present study, a similar strategy was adopted to analyse unfolded and refolded pediocin by RP-HPLC followed by antimicrobial assay. Pediocin has been expressed in *E. coli* by fusing with MBP (Miller *et al.* 1998; Ray *et al.* 1999) to get an active protein. The protein was expressed without the need for the export or immunity functions of the *ped* operon. This protein was correctly disulfide bonded in the periplasmic space

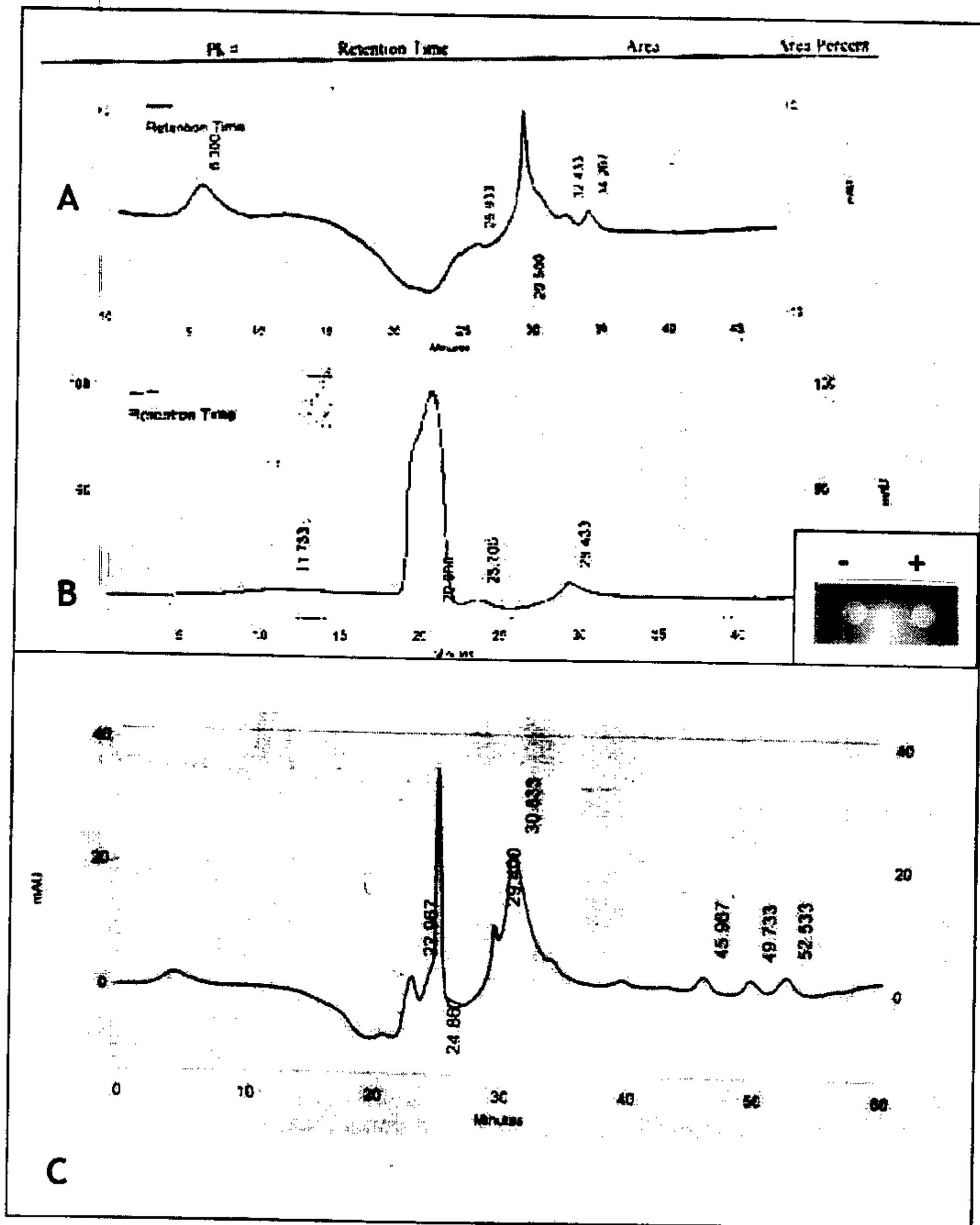

Figure 5.11: Semi-preparative RP-HPLC analysis of recombinant and native pediocin. A: unfolded, B: refolded recombinant pediocin, C: pediocin preparation from native source and D: pediocin assay by agar disc assay of recombinant pediocin (+) with trypsin and (-) without trypsin. Reduced zone of inhibition of trypsin treated pediocin indicates proteolytic inactivation of recombinant pediocin.

of *E. coli*. The property of MBP directed protein targeting to the periplasmic space of *E.coli* upon fusion to its C-terminal end has been exploited in this

study. The periplasmic space contains many of the proteins of the Dsb family which are involved in disulfide bond formation (Winter *et al.* 2002). However, in the present study, high level expression in IBs followed by refolding using cheaper redox agent has been employed. The MIC of native pediocin was 2 ng while for recombinant pediocin it was 50 ng against Scott-A. This value shows recombinant pediocin was atleast 25 times less active than its native counterpart. Comparison of the two protein needs to be repeated taking into account the yield and protein content.

4.3.7 UV adsorption studies of rec-pediocin

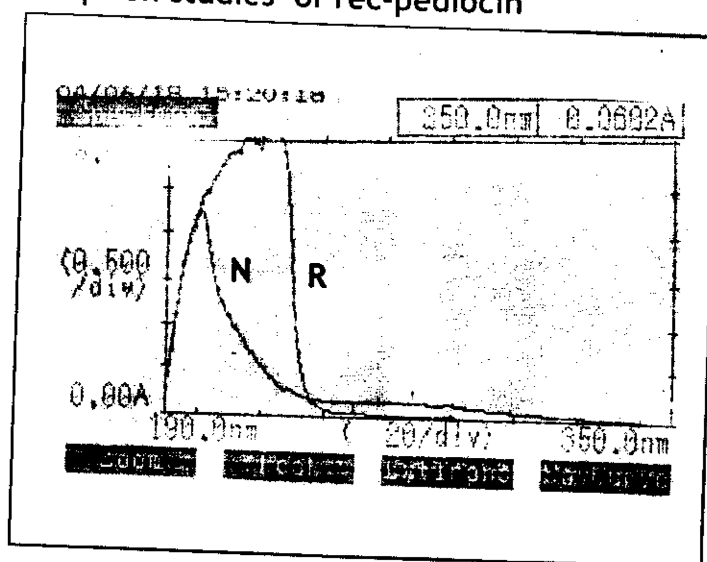

Figure 5.12: Physicochemical characterization of purified rec-pediocin using UV spectrum. (N) native and (R) rec-pediocin.

The UV absorption spectrum of the purified recombinant pediocin solubilized (scanned between 190 and 350nm) as compared to that of the with native pediocin (Figure 5.12). The absorbance maxima of the recombinant and native pediocin differ. The difference may be attributed to the N-terminal extension of prepeptide sequence and the tag. However, a similar shouldering was seen with both proteins at 190nm.

Previously, Patra *et al.* (2000) had used of spectroscopic analysis to study refolding of r-HGH (recombinant-human growth hormone) comparing pure r-

HGH and the native HGH. It was seen that the absorbance maxima of the purified r-HGH was at 276.8nm with a shoulder at 283nm.

5.3.8 Molecular cloning of pediocin immunity gene

In order to express *ped*B in *E. coli* as a fusion protein, two different constructs were made by cloning *ped*B into the vector pQE. The two vectors pQE30 and pQE60 add the tag at N and C-terminal of the target protein respectively, upon cloning the gene in its correct reading frame.

5.3.8.1 C-terminal tag of *ped*B

In order to obtain fusion tag at C-terminal the *ped*B gene was amplified using the set I primers (Ped.IMM.F and Ped.IMM.R). As expected, the amplification of 336 bp PCR product was obtained (Figure 5.13). The PCR product was double digested with *NcoI*/*Bam*HI restriction enzymes, gel purified and ligated to the vector pQE60, which had been previously double digested with same enzymes prior to dephosphorylation. This vector had been initially modified by introducing *ha*-tag so that a fusion protein bearing a tagged sequence recognized by a anti HA antibody (Figure 5.14).

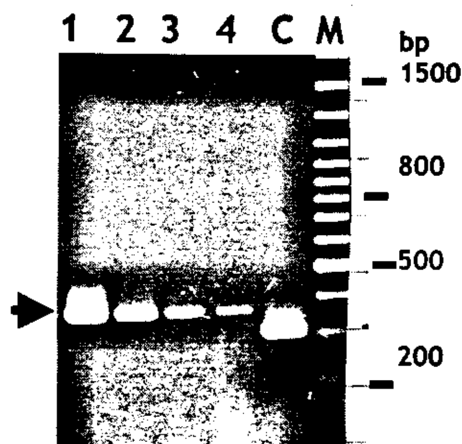

Figure 5.13: Amplification of *ped*B gene by PCR. Agarose (1.5%) gel electrophoresis of pediocin immunity gene amplified from the native PH-1 strain using templates (lane 1 to 4). Arrow indicates the position of the PCR product of expected size obtained. M, 1kb ladder (MBI). C, control PCR of 300bp

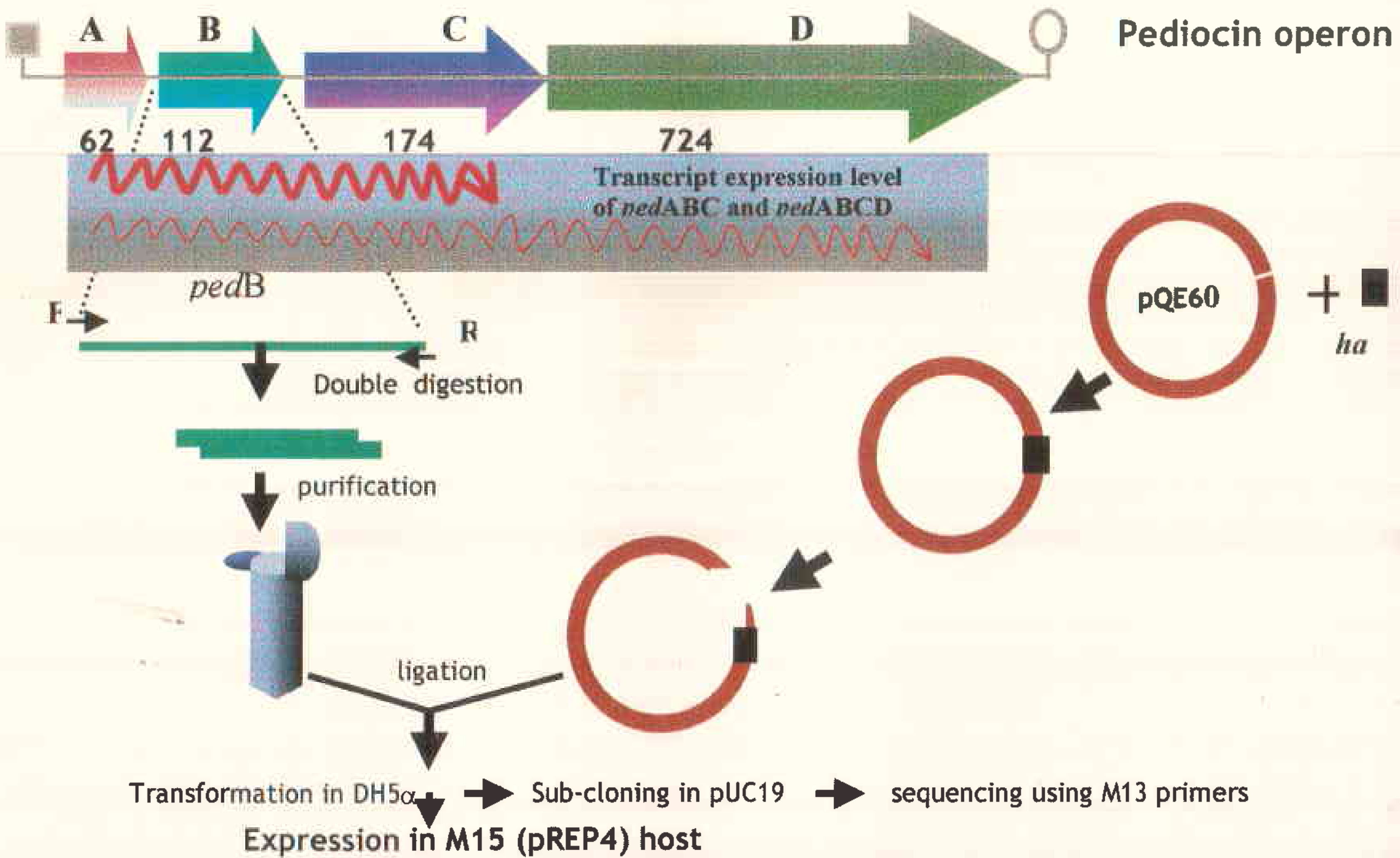

Figure 5.14: Strategy for the construction of a vector for expressing PedB along with a with C-terminal tag

The cloning strategy of pedB in pQE60-HA (ha-tag bearing pQE60 vector) is shown in Figure 5.14. Upon cloning of pedB into pQE60-HA, the recombinant thus obtained was designated as pQEpedIMM (Figure 5.15A). The results of the restriction analysis of pQEpedIMM and molecular map constructed is presented in Figure 5.15 A and B. The insert release of ~336 bp in size of (the size of *ped*B PCR product) can be seen in atleast 50% recombinant (Figure 5.15B).

Figure 5.15: Molecular map and restriction analysis of pQEpedIMM. A)The nucleotide sequences of *ped*B gene conferring immunity function against pediocin was taken from the GeneBank (Acc. No. M83924). B) Agarose (1.5%) gel electrophoresis of *Nco*I/*Bam*HI double digest of the putative recombinant (3-14). Arrow indicates the position of the insert release upon double digestion. M, PCR ladder and λ is a lambda *Eco*911 digest.

The *Eco*RI/*Pvu*II fragment from pQEpedIMM was sub-cloned into *Eco*RI/*Sma*I sites of pUC19 and taken for sequencing while using M13 forward and reverse primers. The nucleotide and deduced -aa sequences is presented in Figure 5.16.

Figure 5.16: Nucleotide sequences of *ped*B ha 6X *his*

1 ggnnnnnttt ttntgctgct gcggtcgact ctagaggatc cccgggtacc gagctcgaat tcattaaaga ggagaaatta accatgggta
? ? F ? A A A V D S R G S P G T E L E F I K E E K L T M G

91 aagactaagtc ggaacatatt aaacaacaag ctttggactt atttactagg ctacagtttt tactacggaa gcacgatact atcgaacctt
K T K S E H I K Q Q A L D L F T R L Q F L L R K H D T I E P

181 accagtacgt tttagatatt ctggagactg gtatcagtaa aactaaacat aaccagcaaa cgcctgaacg acaagctcgt gtagtctaca
Y Q Y V L D I L E T G I S K T K H N Q Q T P E R Q A R V V Y

271 acaagattgc *cagc*caagcg ttagtagata agttacattt tactgccgaa gaaaacaaag ttctagcagc catcaatgaa ttggcgcatt
N K I A S Q A L V D K L H F T A E E N K V L A A I N E L A H

361 ctcaaaaagg gtggggcgag tttaacatgc tagatactac caatacgtgg cctagccaag gatcctaccc atacgacgtc
S Q K G W G E F N M L D T T N T W P S Q G S Y P Y D V P D Y

451 ccagactacg ctagcagatc tcatcaccat caccatcact aagcttaatt agctgagctt ggactcctgt tgatagatcc agtaatgacc
A S R S H H H H H H - A - L A E L G L L L I D P V M

441 tcagaactcc
T S E L

Nucleotide sequences of the recombinant *ped*B gene was obtained after sub-cloning of the *Eco*RI/*Pvu*II fragment of pQEpedIMM recombinant into the pUC19 vector. The translated -aa sequences and the fusion protein at the C-terminal end is also shown. M is a methionine followed by glycine which was introduced in place of lysine, while adding restrictions sites to the forward primer.

5.3.8.2 Western analysis of PedB fusion protein

The total cell lysate of PedB expressing recombinant was separated on SDS-PAGE, subsequently transferred to a NC membrane and probed with anti-HA MCA. Western analysis results are presented in Figure 5.17. The anti-HA reacted specifically with a 15 kDa protein band corresponding to that expected for the PedB fusion protein and the multimeric forms of fusion protein. Proteins from the un-induced cells also reacted feebly with the antibody. Protein isolated from the control recombinant (SpaS-HA-6X His) also reacted with the antibody (lane C).

Tagging of protein helps in both purification and identification of the protein. The HA epitope is a nona-peptide sequence (YPYDVPDYA) derived from the influenza hemagglutinin protein. Epitope tagging offers a method for identification and localization of several proteins expressed at a very low level and whose localization is unknown (Makrides 1996).

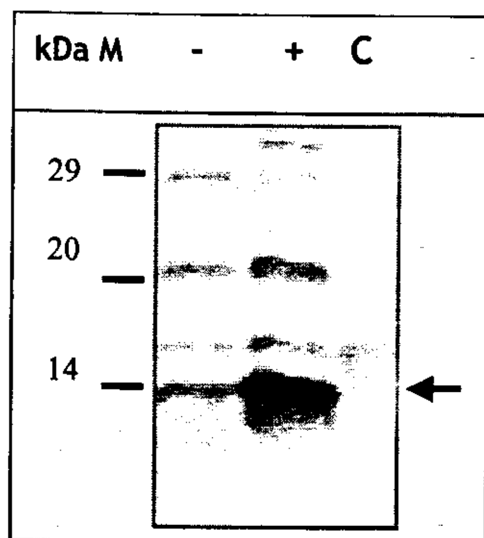

Figure 5.17: Western-blot analysis of PedB-HA-6XHis fusion protein in *E. coli M15*(pREP4). - uninduced, + induced with 1 mM IPTG. C is a positive control of SpaS-HA-6XHis detected by anti-HA antibody. Arrow indicates the expected size (~15 kDa) of fusion protein. M is a medium range protein MW marker in corresponding gel.

However, in the present study the yields of the PedB fusion protein was very low and further experiments with this construct were discontinued.

4.3.8.2 N-terminal tagging of *ped*B

The *ped*B gene was cloned into the expression vector pQE30 so as to obtain protein tagged with 6Xhis at its N-terminal prior to undertaking expression studies. The PCR product of *ped*B obtained as expected by using set II primers (PedB.F and PedB.R), was double digested with *Bam*HI/*Pst*I restriction enzyme, gel purified and ligated to the vector pQE30 which had also been double digested with the same enzymes and dephosphorylated. The results of the PCR amplification of *ped*B gene and the preparation of vector pQE30 for cloning of *ped*B are shown in Figure. 5.18.

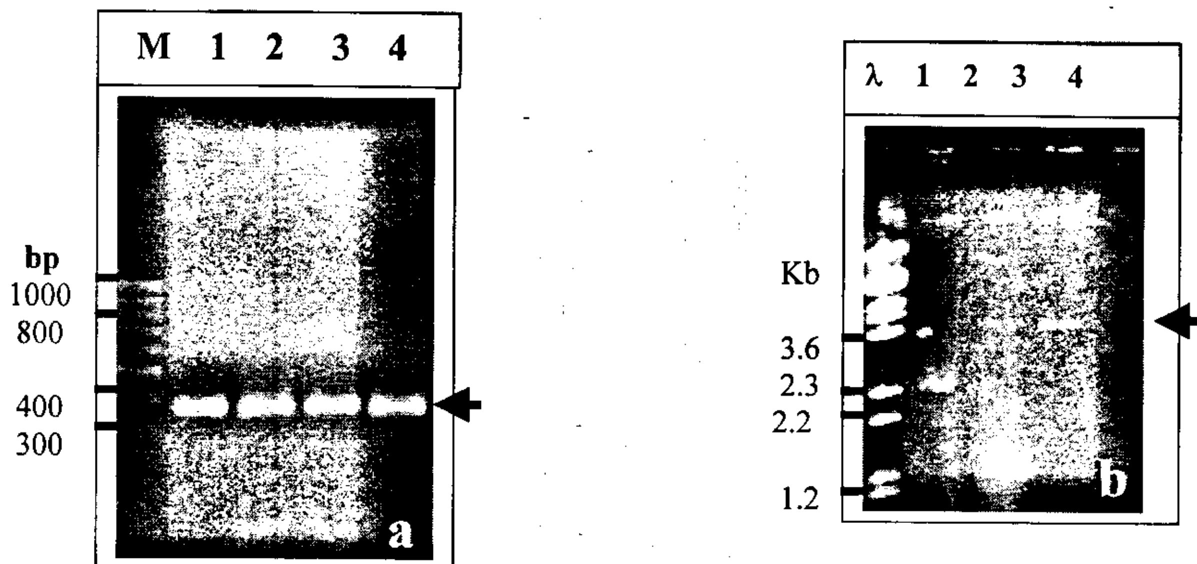

Figure 5.18: PCR cloning of *ped*B into pQE30
a: Agarose gel (1.5%) electrophoresis of PCR amplicon of *ped*B gene. Lane 1, PCR product undigested; 2, digested with *Bam*H1; 3, with *Pst*1 and lane 4, double digested with *Bam*HI/*Pst*1. M is a 100 bp ladder (MBI).
b: Agarose gel (0.8%) electrophoresis of pQE30 plasmid vector. Lane 1, undigested vector; 2, digested with *Bam*HI; 3, with *Pst* 1 and 4, double digested with both the enzymes. λ *Eco*911 digested marker DNA. Arrow indicates the position of the double digested vector which was eluted, treated with CIAP and used for cloning.

Insert release from recombinant plasmid pQEpedB30 thus obtained after digestion with *Hin*dIII and *Hin*dIII/*Pst*I are shown in Figure 5.21. A molecular map was constructed by using the programme Clone Manager Ver. 5 using the nucleotide sequences of pQE30 vector (Qiagen) and *ped*B gene sequences (Marugg *et al.* 1992). The expected sizes of the native and recombinant pediocin immunity proteins are 13 and 13.8kDa, respectively.

Figure 5.19 : Molecular map of recombinant plasmid pQEpedB30

MRGSHHHHHHDP KTKSEHIKQQALDLFTRLQFLLQKHDTIEPYQYVLDILETGI SKTKHNQQTPERQARVVYNKIASQALVDKLHFTAEENKVLAAINELAHSQKGWG EFNMLDTTNTWPSQ -

Figure 5.20: Deduced amino acid sequences of 6XHis-PedB fusion construct. The tag introduced at N-terminal of fusion protejn is shown in italics.

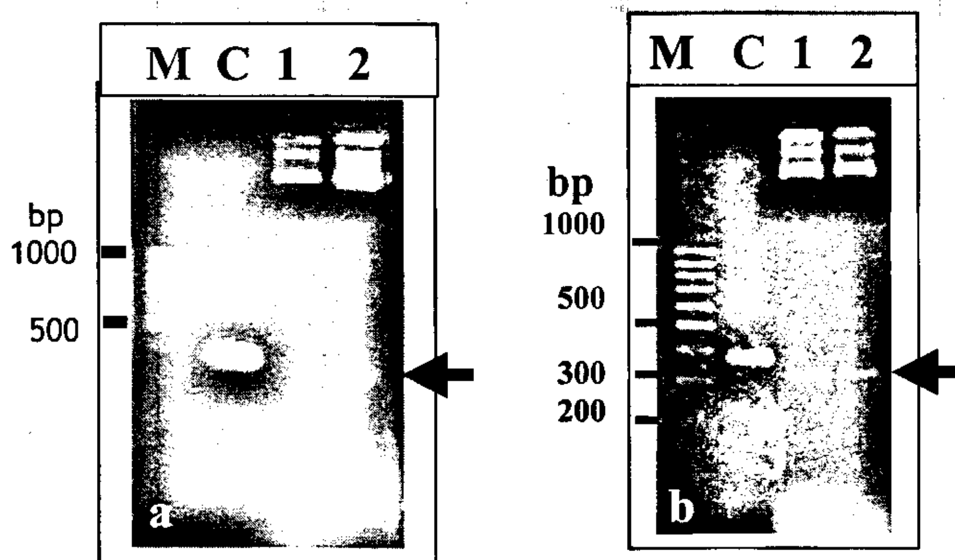

Figure 5.21: Restriction digestion of the pQEpedB30 recombinant. The putative recombinant, were digested with *Hind* III/*Pst* I (a) and *Hind* III (b). Lane 1 is the *ped*B PCR product. M, 100 bp ladder. Two putative recombinants were analyzed for insert release.

5.3.9 Heterologous expression of 6XHis-PedB in *E. coli* M15

The recombinant plasmid pQEpedB30 was transformed into Kan$^+$ M15 host for the expression of PedB fusion protein in *E. coli* and the gene induced with IPTG. The total cell lysate was first analysed by denaturing SDS-PAGE (Figure 5.22) and then by dot-blot hybridization of total cell lysate using anti-His Ab (Figure 5.23). Proteins from recombinant and induced cells reacted more densely with the Ab than proteins from control non recombinant cells.

Since, C-terminal tag of PedB could have interfered in the biological activity of the PedB, N-terminal tagging was carried out. The N-terminal tag was smaller in size and was expected not to interfere with activity of the protein. Recent report by Johnsen *et al.* (2004) on functional analysis of suggests that the biological activity of the immunity resides at the C-terminal end of the molecule.

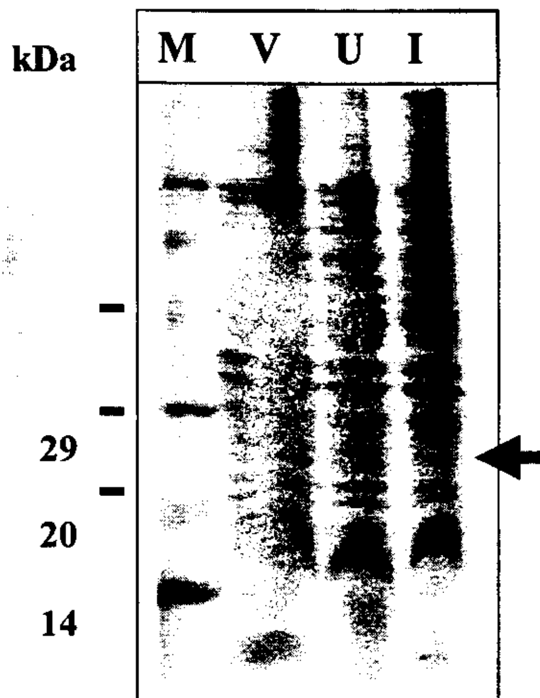

Figure 5.22: SDS-PAGE (15% gel) s of total lysate prepared from the PedB expressing *E. coli* M15 cells harboring pQEpedB30 plasmid. Lane M, Protein MW marker; V, vector lysate, U, un-induced and I, Induced with 1 mM IPTG recombinant.

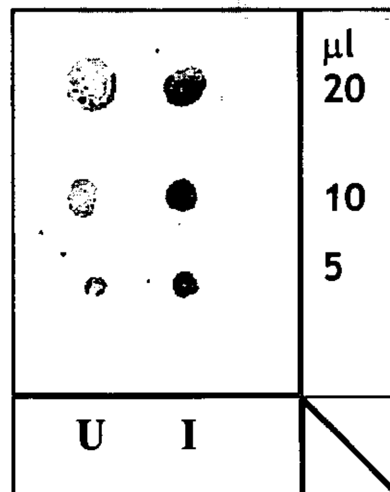

Figure 5.23: Dot-blot hybridization of the 6XHis-PedB expressing recombinants. Total cell lysate of the recombinant plasmid transformed *E. coli* M15 cells. U, un-induced and I, induced with IPTG. Three different concentrations of cell lysate were spotted (5, 10 and 20 µl) on NC membrane and probed with rabbit anti-His antibody. The color was developed using BCIP/NBT substrate in alkaline phosphatase buffer.

The PedB fusion protein was purified on Ni^{2+} beads. Lysate from cells that had been induced was centrifuged at a high speed. The supernatant and the pellet dissolved in lysis buffer was incubated with Ni^{2+} beads. The beads were separated and were analyzed in acrylamide gel for the presence of 6XHis tag protein (Figure 5.24).

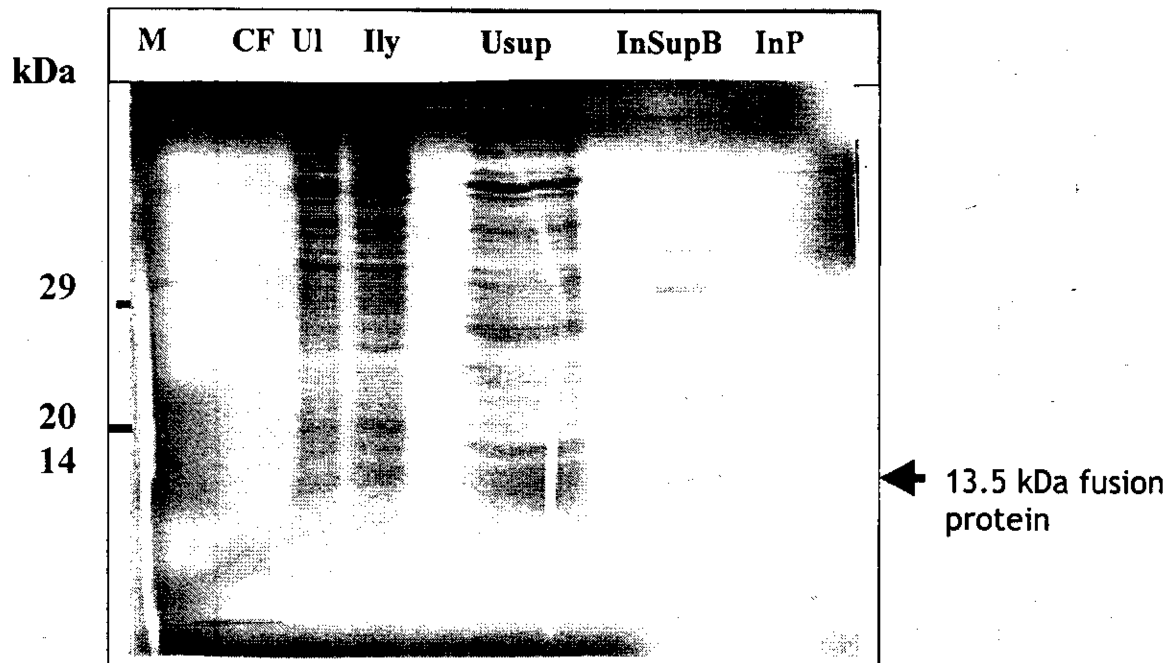

Figure 5.24: SDS-PAGE (15% acrylamide) gel analysis of Ni^{2+} NTA bead partially purified 6X His tagged Ped B. Lane CF, TCA precipitated culture filtrate; UI, uninduced and Ily, induced total cell lysate; Usup, Lysate from uninduced cells ; InsupB, Affinity purified supernatant from recombinant cells Inp, pellet from cells that had been induced. Arrow indicates the probable position of 13.5 kDa fusion protein, absorbed on Ni^{2+} NTA beads.

5.3.8.3 Tmpred prediction

The Tmpred program makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on statistical analysis of Tmbase a database of naturally occurring transmembrane proteins. The programme indicated that the PedB is not a transmembrane protein (Figure 5.25A). Spal

the immunity protein from *B. subtilis* which is transmembrane protein was taken for comparison. The computer Programme predicted the membrane spanning nature of this protein (Figure 5.25B).

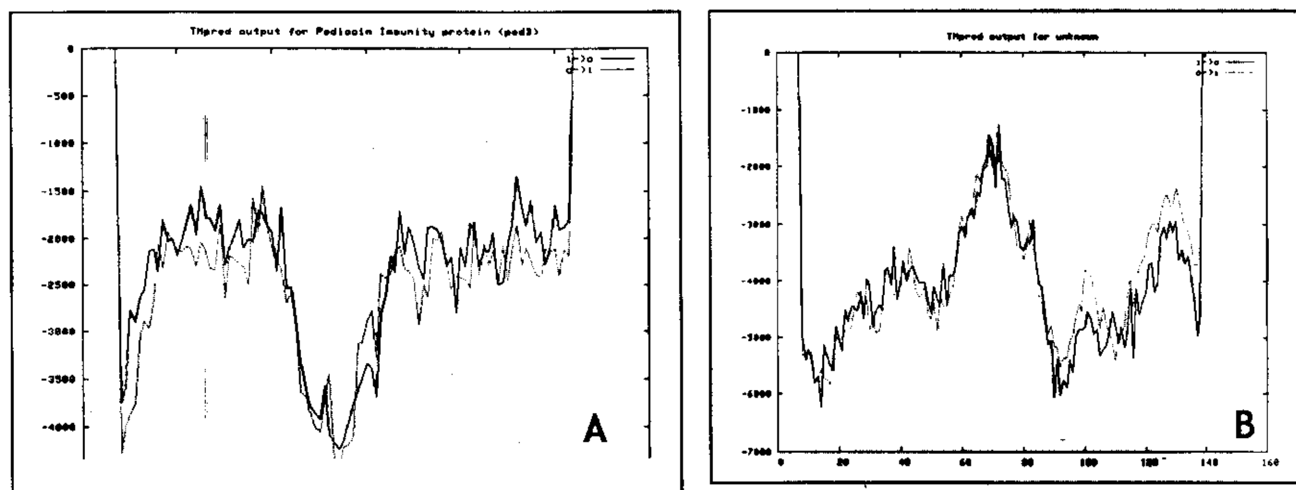

Figure 5.25: Tmpred analysis of PedB and SpaI proteins (acc no. U09819). A, PedB; B, SpaI.

5.3.8.4 Hydrophobicity profile

In this study, four bacteriocin immunity proteins were taken for computational analysis based on the similarity of their subunits and absence of signal peptides. The Molecular weight and other characteristic features of the different immunity proteins taken for this study is shown in Table 5.4.

Table 5.4: Bacteriocin immunity proteins

Immunity protein	cognate bacteriocin	No. of AA	MW (kDa)	% homology	Membrane span region (nos.)
PedB	Pediocin	112	13	100	4
*PedBr	pediocin	132	15	100	4
Spla	Sakacin	98	~100	34	5
LtnI	Lacticin3147	116	~120	35	3
PepI	Pep5	69	~72	50	2

*recombinant immunity protein of pediocin, with C-terminal tag.

The sequence alignment of these four proteins was carried out by using Multalin (Corpet 1988) and the results are presented in Figure 5.26. Results indicates conserved lysine (K) and leucine (L) residues and a leucine zipper motif. These motif was found more in the C-terminal end of the LtnI protein associated with the immunity of lacticin 3147. In this study, the leucine zipper motif was also found in N-terminal of PedB. Leucine zipper motifs are known to be associated with DNA-binding proteins such as transcription factors and are important for dimerization of such proteins (McAuliffe *et al.* 2000).

```
                 10        20        30        40        50        60
        ....|....|....|....|....|....|....|....|....|....|....|....|
PedB   1   --------MNKTKSEHIKQQALLLFTRLQFLLQKHDTIEPYQYVLDILETGISKTKHNQQ
PedBR  1   --------MGKTKSEHIKQQALDLFTRLQFLLRKHDTIEPYQYVLDILETGISKTKHNQQ
SpIa   1   ----MKILKWYSGGKDRGERANDIIGQLLLDLNHDPKNEHLEAILINYQNEIKRKES--S
Pep    1   --------------------------MNIYLKVILTSLFFALIIFIVTYITTKQWG---
LtnI   1   MKNENINTFSESLFYSLKEWAEGSANNYNILLGLSFIFVMSSVVFFVTISTKIGKKDERT

                 70        80        90       100       110       120
        ....|....|....|....|....|....|....|....|....|....|....|....|
PedB   53  TPERQARVVYNKIASQALVDKLHFIAFENKVLAAINELAHSQKGWGEFNMLDTT[illegible]TWPSQ
PedBR  53  TPERQARVVYNKIASQALVDKLHFIAFENKVLAAINELAHSQKGWGEFNMLDTT[illegible]TWPSQ
SpIa   55  VFFILSRMN-ISIANTIRRDRLILIDFQEDKLKLLTALSNIRYCY---------------
Pep    31  [illegible]SLGFSSLS--FIGNFIYDYSTKLSDKKYEKRINSNKKDKL-------------------
LtnI   61  [illegible]KISLYSAYCVLITLIICDIIFPKGYLIQPFFMLKYGFSCLVGCIYCLLKYIKD[illegible]K----

                130       140
        ....|....|....|....|
PedB   112 --------------------
PedBR  113 GSYPYDVPDYASRSHHHHHH
SpIa   98  --------------------
Pep    69  --------------------
LtnI   116 --------------------
```

Figure 5.26: Multiple sequence alignment of immunity proteins. Conserved regions are highlighted in dark/faint colours.

Hydrophobicity profile of these immunity proteins was determined by the method of Kyte and Doolittle (1982). The hydrophobicity plot for LtnI revealed presence of three hydrophobic domains comprising of 30-49, 68-81 and 91-112 -aa suggesting a transmembrane location for this protein. The pedB

contains atleast four hydrophobic domains of aa 25-35, 40-60, 65-75 and 90-100 consisting of 10-20 -aa each, representing membrane spanning regions. The recombinant PedB protein, whose C-terminal was tagged with anti-HA 6X His, showed a similar hydrophobicity profile. The C-terminal end was hydrophilic. Since there was no difference in the profile of the recombinant and the native protein. A large number of membrane spanning domains are predicted in most of the immunity proteins. Five membrane spanning domains were predicted in Spla protein involved in sakacin immunity although total length of this protein is only 97 -aa (Figure 5.27e). Ltnl and Pepl contained lesser number of membrane spanning regions (3 and 2 respectively) but each domain consists of atleast 20 -aa (Pag *et al.* 1999). The proteins analysed in this study are most likely localized on the cytoplasmic face of the membrane, as suggested by the presence of several putative membrane spanning regions. Cytoplasmic membrane associated immunity protein conferring resistance to mesenterocin was shown by Dayem *et al.* (1996). The absence of typical signal sequences implies that all these proteins are neither secreted not are they lipoproteins. Therefore these may serve by hindering insertion of the bacteriocin molecule into the membrane or by interaction directly and inactivation of the bacteriocin (Fimland *et al.* 2002a; Johnsen *et al.* 2004; Pag *et al.*1999).

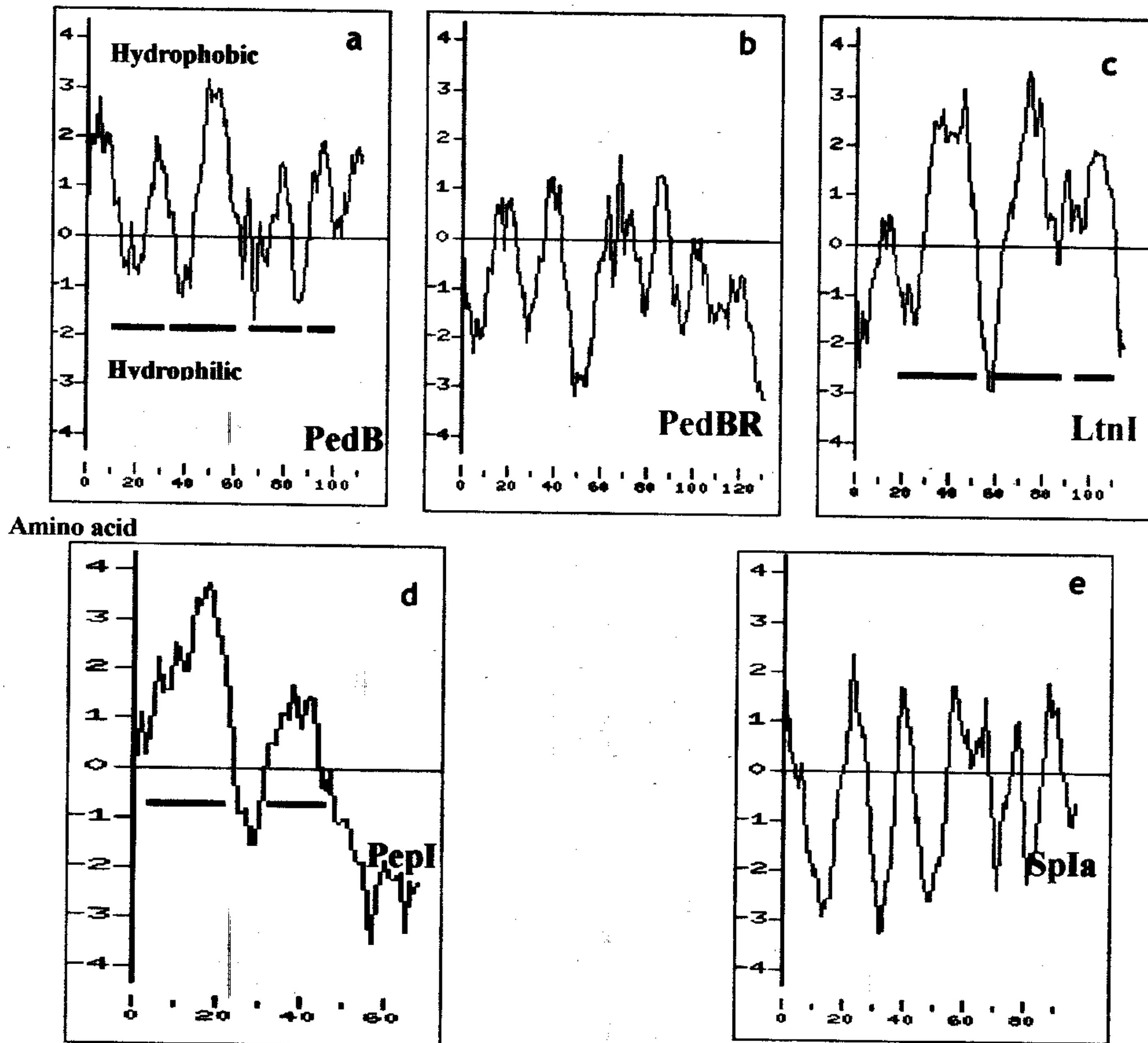

Figure 5.27: Hydrophobicity profiles of single subunit immunity proteins of Bacteriocins. Bars in the figure indicate membrane spanning region of hydrophobic segment. Red colour bars in a) are the proposed membrane spanning region identified in this study. B) is a hydrophobic profile of the recombinant PedB Protein where in the C-terminal tag exhibits a hydrophilic pattern.

The hypothesis on membrane spanning region presented as above, suggests that each hydrophobic region could react with the bacteriocin molecule and with the bacteriocin receptor and protect the cells from the formation of pores in the cytoplasmic membrane.

5.4 CONCLUSION

In this study the pre-pediocin encoding gene was cloned by PCR and was inserted into the *E. coli* expression vector, pRSET-A. Nucleotide sequencing was performed in order to verify the restoration of reading frame between the fusion tag and pediocin structural gene. The recombinant construct was transformed into protein expression host and gene expression was induced by the addition of IPTG. Hyperexpression of 12.8 kDa protein localized in IBs was observed. The chimeric pediocin upon refolding exhibited biological activity against the indicator Scott-A. This result provides the basis for large scale production of pediocin and for studying expression of mutants of pediocin in *E. coli*.

The pediocin immunity protein expressed in *E. coli* M15 showed a very low level of expression. Hydrophobicity profile of PedB suggested that there are atleast four membrane spanning regions. Each region consists of 10-20 -aa spanning across cytoplasmic membrane. Heterologously expressed *ped*A and *ped*B gene products can form the initial starting material for many biochemical and biophysical *in vitro* studies.