BIOTECHNOLOGICAL APPROACHES ON THE FERMENTATIVE PRODUCTION OF BACTERIOCIN AS BIOPRESERVATIVE

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By

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DEDICATED TO MY BELOVED PARENTS
& WELL WISHERS

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DECLARATION

I hereby declare that the thesis entitled "**BIOTECHNOLOGICAL APPROACHES ON THE FERMENTATIVE PRODUCTION OF BACTERIOCIN AS BIOPRESERVATIVE"** submitted to the **University of Mysore** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** is the result of the research work carried out by me under the guidance and supervision of **Dr. ARUN CHADRASHEKAR**, Sr. Scientist; Central Food Technological Research Institute, Mysore- 570 020, India; during the period 1998-2004.

I further declare that the results presented in this thesis have not been submitted for the award of any other degree or with any similar title.

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CERTIFICATE

This is to certify that the thesis entitled "**BIOTECHNOLOGICAL APPROACHES ON THE FERMENTATIVE PRODUCTION OF BACTERIOCIN AS BIOPRESERVATIVE**" submitted by **Mr. Prakash M. Halami** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE** is the result of research work carried out by him under my guidance and supervision in the Department of Food Microbiology, CFTRI; during the period of 1998-2004.

ARUN CHANDRASHEKAR
(**Research Guide)**

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ABBREVIATIONS USED

Ab	Antibody
APS	Ammonium persulfate
ATP	Adenosine triphosphate
AU	Arbitrary Unit
BCP	Bromo cresol purple
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BHI	Brain-heart infusion broth
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CF	Culture filtrate
CFU	Colony forming unit
CIAP	Calf intestinal alkaline phosphatase
Da	Dalton
DIG	Digoxigenin
DMF	N, N'-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DTT	1, 4-Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
h	Hour (time)
IPTG	Isopropyl-β-D-thio galactopyranoside
IB	inclusion body
kb	kilobase
kDa	kilodalton
LAB	Lactic acid bacteria
LB	Luria Bertani media
MBP	Maltose binding protein
MCA	Mono clonal antibody

MDa	Mega dalton
MES	2-[N-morpholino]ethanesulfonic acid
MIC	Minimum inhibitory concentration
mM	millimolar
MOPS	3-[N-morpholino]-2-hydroxypropanesulfonic acid
MRS	De Man, Rogosa and Sharpe
MRVP	Methyl red voges proskauer reaction
MW	Molecular weight
MTCC	Microbial Type Culture Collection
NBT	Nitro blue tetrazolium
NCDO	National Collection of Dairy Organisms
NCFB	National Collection of Food Bacteria
NCIM	National Collection of Industrial Microorganisms
NRRL	Northern Regional Research Laboratory
OD	Optical density
ONPG	O-Nitrophenyl-□-D-galactopyranose
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
Rec-pediocin	Recombinant pediocin
RNase	Ribonuclease
rRNA	Ribosomal RNA
RP-HPLC	Reverse phase high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PEG	Polyethylene glycol

PMSF	Phenyl methyl sulfonyl fluoride
SSC	Saline sodium citrate
SSCP	Single stranded conformational polymorphism
TAE	Tris-acetate-EDTA buffer
Tricine	N-tris (hydroxymethyl) methylglycine
TCA	Tri chloro acetic acid
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-tetramethyl-1,2-diaminoethane
TFA	Trifluro acetic acid
Tris	Tris (hydroxymethyl) amino methane
TGE	Tryptone glucose yeast extract
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate
YE	Yeast extract
WP	Whey permeate
WT	Wild type

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INTRODUCTION AND REVIEW OF LITERATURE

1.0 INTRODUCTION

Lactic acid bacteria (LAB) have been used by man from as early as the start of humankind. They have been used for production of food and its preservation ever since man noticed that he could turn milk into curd. These bacteria are one of the best studied organisms as they have a number of applications in the food industry and are beneficial to human health. Of late, LAB have attracted world-wide attention as a model system for studying multidrug resistance, bacteriocin production, quorum sensing, osmoregulation, proteolysis, autolysis, bacteriophages, comparative genomics, live vaccine delivery, development of food-grade genetically modified LAB etc (Geisen and Holzapfel 1996; Hansen 2002; Konings *et al.* 2000; Renault 2002; de Vos 1999; Weimer and Mills 2002).

1.1 Classification of LAB

An earlier definition of LAB as a group was based on their ability to ferment and coagulate milk. The true lactic acid-producing bacteria were classified in the beginning of the last century by Orla-Jensen as a natural group of Gram-positive, non-motile, non-spore forming, rods, cocci or coccobacillus shaped organisms that ferment carbohydrates and higher alcohols to yield lactic acid as an end product. LAB were taxonomically classified by Orla-Jensen into seven different genera namely, a) Betabacterium: a group of rod shaped heterofermentors viz *Lactobacillus* and *Weisella*; b) *Thermobacterium*: a group of homofermentative rod-shaped lactobacilli; c) *Streptobacterium*: also a group of homofermentative rods viz *Lactobacillus*, *Carnobacterium;* d) *Streptococcus:* a group of homofermentative cocci viz *Streptococcus*, *Enterococcus*, *Lactococcus* and *Vagococcus;* e) *Betacoccus* are heterofermentative cocci viz *Leuconostoc*, *Oenococcus* and *Weissella;* f) The genus *Microbacterium* included homofermentative rods viz *Brochothrix* and g) the genus *Tetracoccus*, homofermentative cocci viz *Pediococcus* and *Tetragenococcus* (Axelsson 1993; Stiles and Holzapfel 1997).

The classical approach to taxonomy of the LAB was based on morphological and physiological features. Other methods of identification include composition of cell wall such as cellular fatty acid analysis, isoprenoid quinines etc. Recently, molecular techniques have become an important tool for identification of LAB. These methods include mol % G+C content of DNA, electrophoretic properties of total soluble proteins, Southern hybridization and ribotyping (Cocconcelli *et al.* 1991; Jager and Harlander 1992; Mora *et al.* 2000; Sharpe *et al.* 1966).

LAB or lactics generally refer to certain species of the genera *Lactobacillus*, *Lactococcus* (*Streptococcus*), *Leuconostoc* and *Pediococcus*. Chemotaxonomic and phylogenetic studies have resulted in changes in their nomenclature. Phylogenetically, the LAB belong to the clostridial branch of Gram-positive bacteria with less than 55 mol % G+C content in their DNA, except *Bifidobacterium* which has more than mol 55% G+C content (Figure 1.1a). The genera *Lactobacillus*, *Leuconostoc* and *Pediococcus* are traditionally treated separately because of their different morphology. However, phylogenetically they are intermixed. Based on 16S rRNA studies the genus *Lactobacillus* and related genera of LAB are classified into three groups. The first group comprises homofermentative lactobacilli. The second group so-called *Lact. casei-Pediococcus* group. This is the largest among the three groups and contains more than 30 *Lactobacillus* species and five of the six pediococcal species. The third group consists of the members of the genus *Leuconostoc* and some obligately heterofermentative lactobacilli (Schleifer *et al.* 1995). The LAB which are of importance in food systems belong to the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Stiles and Holzapfel 1997).

1.2 Natural habitat

LAB are widely distributed in nature and are usually found in fermented, decaying organic matter. They form a fastidious group of microorganisms that require supplementation of various vitamins, amino acids and fermentable carbohydrates for their growth. The most recognized habitat for lactococci is dairy products. However, subspecies of *Lactococcus lactis* has been commonly isolated from plant material. The genus *Enterococcus* inhabits human and other animal intestines including that of poultry. These bacteria are also found in clinical samples, grass, silage, soil and plant material. *Carnobacterium* is reported to have been isolated from vacuum packaged, chill-stored meat. Various species of this genus have been reported from meat, poultry, cheese, Salmonid fish, Antarctic lake etc. The genus *Vagococcus* has been reported from chicken faeces, river water and from diseased Salmonid fish. The genus *Lactobacillus* is associated with diverse habitat of human origin such as the oral cavity, intestinal tract and vagina. The other potential habitats include plant and plant material, soil, water, sewage, manure, fermented food, cereal products and silages. It is also found in spoilt beer, fruit and grain mashes, fish, processed sugar, milk and milk products and in fermented beverages. The genus *Pediococcus* has been commonly associated with breweries, wines and cider, beer, silages, saurkraut, vegetable material, fermented sausages, milk and dairy products, soy-sauce, pickled brine and meat products (Stiles and Holzapfel 1997).

1.3 Lactobacilli

Lactobacilli are Gram-positive, regular non-sporing rods and aerobic or aerotolerant bacteria. They are normal inhabitants of the oral cavity and the digestive tracts in humans. Based on fermentation characteristics, lactobacilli are classified as

1) Obligate homofermentative
2) Facultative heterofermentative and
3) Obligate heterofermentative

The genus *Lactobacillus* is heterogeneous with 33 to 35 mol % G+C content in the DNA. Phylogenetic analysis has affirmed the presence of 54 species of lactobacilli, 18 of which might be considered as probiotics (live microbial food ingredients that are beneficial to health). The lactobacilli are strictly fermentative and have complex nutritional requirements. Lactobacilli are aciduric or acidophilic, producing considerable amounts of acids in foods containing a fermentable carbohydrate. A low pH, thus induced, helps suppress the growth or kill other contaminating bacteria.

Table 1.1: Lactobacilli characterized from different sources

LAB	source	Purpose	Reference/s
1. *Lactobacillus acidophilus*	Chicken crop	Amylase activity	Champ *et al.* 1983
2. *Lact. casei*	Ready-to-use vegetable	Prevention of coliforms and *E. coli*	Vescovo *et al.* 1995
3. Lactobacilli	Chicken intestine	Acid and bile tolerance studies	Jin *et al*. 1998
4. *Lact. plantarum* *Lact. fermetum* *Lact. farciminis*	Chilli Bo	Enumeration and identification of LAB in Asian foods	Leisner *et al.* 1999
5. *Lact. plantarum*	Green Olive fermentation	Bacteriocin production	Jimenz-diaz *et al.* 1993
6. *Lact. acidophilus*	Fermented foods and feeds	Bacteriocin production	ten Brink *et al.* 1994
7. *Lact.* species	Fermented dry sausages	Antibiotic resistance	Gevers *et al.* 2003
8. *Lact. plantarum*	Fermented milk products	Probiotics	Cebeci and Guerakan 2003
9. *Lact. delbruecki* *Lact. bulgaricus*	Curd	Bacteriocin production	Varadaraj *et al.* 1993

These bacteria can grow upto a pH of 7.2. Lactobacilli are used as a starter culture for several varieties of cheese, fermented plant foods, and fermented meat production etc. Table 1.1 provides a detailed account of lactobacilli isolated from different sources and their applications.

1.4 Enterococci

At the end of nineteenth century, Thiercelin has recognized the genus *Enterococcus* as 'enterocoque' due to their intestinal origin. The taxonomy of enterococci has been always vague. It is known that there are no fixed phenotypic characteristics to separate the enterococci from other genera of Gram-positive, catalase negative cocci. Infact, phenotypic identification is generally a reverse identification (Stiles and Holzapfel 1997). Initially, enterococci were described based on growth at temperature of 10 and 45^{0}C, in 6.5% NaCl, at a pH of 9.6 and ability to survive heating at 60^{0}C for 30min and reaction with lancefied group D antisera. Enterococci produce L (+) lactic acid, are homofermentative and can derive energy from the degradation of amino acids. Most of the species of enterococci often survive at high temperature and hence can dominate the microbial population of heat treated food (Mundt 1986). Enterococci are routinely used as a starter culture in some foods and also are available as probiotics for prevention and treatment of intestinal disorders of human and animal. The strain of *Ent. faecium* is associated with the fermentation of a number of southern European cheese and is often applied in their processing. The genus *Enterococcus* is widely distributed in environment and often associated with plant, clinical samples, intestines of animals, human and poultry (De Vuyst *et al*. 2003).

The genus *Enterococcus* was initially included under the genus *Streptococcus*. Enterococci cells are spherical or ovoid, 0.6-2.0 X 0.6-2.5 mm, occur in pairs or in short chain in liquid media. They do not form endospores, are Gram-positive, non-motile, sometimes shows motility due to scanty flagella, lack capsules,

facultative anaerobes, chemo-organotrophs with fermentative metabolism. Enterococci can ferment wide range of sugars into lactic acid. The final pH of the fermentation broth often found is between 4.2 to 4.6.

Among the enterococci, *Ent. faecium* is unique because it is commonly used in production of fermented foods, and has been used as a probiotic bacterium. Many isolates of *Ent. faecium* have been shown to produce bacteriocins that are able to kill or inhibit growth of pathogens such as listeria, clostridia, bacillus and staphylococci (Aymerich *et al.* 1996; Cintas *et al.* 1997; Franz *et al.* 2003; Leroy *et al.* 2003).

The genus *Enterococcus* includes more than twenty species, with *Ent. faecium* and *Ent. faecalis* being the two species mostly found, especially in food and related habitats. A variety of DNA based methods for specific identification of enterococci are being developed. An *Enterococcus* species assay based on the hybridization of rRNA gene is commercially available for culture confirmation (Ke *et al.* 1999). The variety of conserved genes such as rRNA genes can be exploited to differentiate species of enterococci (Figure 1.1b).

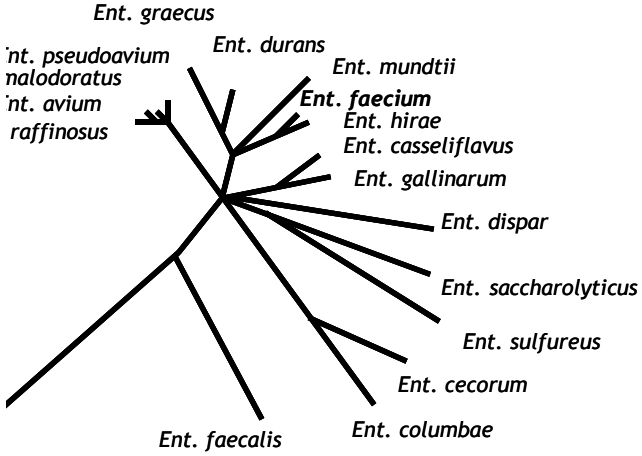


Figure 1.1: 16S rRNA-based distance matrix tree reflecting phylogenetic relationship of a) LAB and b) enterococci (Schleifer *et al*. 1995)

1.5 Pediococci

Pediococci are spherical cells, non-elongated, tetrads that divides in two plains, Gram-positive, non-motile, non-spore forming, facultative anaerobes (with tolerance to oxygen). Colonies are 1.0 to 2.5 mm in diameter, smooth, round, grayish white in colour. All species of *Pediococcus* grow at 30^0C, but optimum growth temperature ranges from 25 to 40^0C. Pediococci need a rich medium containing complex growth factors and amino acids (-aa). The growth of pediococci is dependent on the presence of a fermentable carbohydrate and glucose is fermented by Embedon-Meyerhof pathway to DL or L-(+) lactate (Sharpe *et al.* 1966). Based on its physiological characteristics and nutritional requirements, *Pediococcus* has been recognized as a separate genus. Pediococci are generally found as saprophytes in fermenting vegetable material, in summer sausage, cheese, rumen of cows, silages, beer and saurkraut. At least eight different species of pediococci have been identified till date which includes *Ped. pentosaceus*, *Ped. acidilactici*, *Ped. parvulus*, *Ped. inopinatus*, *Ped. damnosus*, *Ped. dextrinicus*, *Ped. halophilus* and *Ped. urinaeequii* (Garvie, 1986; Stiles and Holzapfel 1997).

Species of *Pediococcus* can be differentiated from each other by DNA-DNA homology and characteristic growth at different physiological conditions and acid production from different sugars. Growth at selective temperature is one of the important criteria for differentiating the species of pediococci. For example *Ped. acidilactici* grows at 45^0C while other species do not grow except *Ped. pentosaceus* whose temperature of growth varies with strain. Likewise, *Ped. dextrinicus* can utilize starch while other species cannot. Utilization of maltose by all species of pediococci except *Ped. acidilactici* is another feature that is used for its easy identification. Growth at pH 7.0 is a characteristic feature of all pediococcal strain except for *Ped. damnosus* which grows at pH 4.5. Nevertheless, utilization of a specific sugar is dependant on involvement of

genes encoding hydrolyzing enzyme. This being a transmissible character, can, but not necessarily be considered as a marker for identification.

1.6 Biochemical and molecular methods for strain differentiation

Owing to the occurrence of LAB in diverse ecological niche, it is difficult to limit a particular organism to specific environmental constraints that will enable its identification. Conventional methods are time consuming, tedious and lead to ambiguous results. Various biochemical and molecular methods have been developed for rapid identification and strain differentiation. Many of these methods are correlated to the production of specific metabolites. These methods offer the possibility of being a convenient and reliable basis for clustering similar strains and facilitate their tentative identification using available reference strains. These methods are highly useful while dealing with large number of environmental samples that have to be assessed simultaneously.

Biochemical methods include soluble protein profiles produced by one-dimensional SDS-PAGE using whole cells of bacteria and fatty acid analysis. Determination of the stereo isomers of lactic acid produced by LAB for strain identification, such as with *Sporolactobacillus* and *Bacillus coagulans* and comparison with a reference strain has also been employed (Manome *et al.* 1998). Gilarova *et al.* (1994) have used cellular fatty acid analysis for the identification of LAB and have investigated factors affecting the fatty acid composition. Plasmid profiling was carried out in order to demonstrate the presence of native plasmid DNA in indigenous *Lact. plantarum* strains isolated from green olive fermentation process (Ruiz-Barba *et al.* 1991).

The response of bacterial strains to a set of antibiotics is another method for strain differentiation. Limited studies on LAB indicate that antibiogram can facilitate differentiation of related strains. This procedure is important for the LAB which are clinically involved in infections and spread of antibiotic

resistance, especially that for vancomycin. The gene for resistance *van*A has been shown to occur in many *Ent. faecium* strains that are important in traditional cheese ripening and probiotics preparations (De Vuyst *et al.* 2003). Information on the profile of resistance to antibiotics can help track the spread of the character to other LAB along the food chain as seen in the case of the movement of tetracycline resistance among lactobacilli (Gevers *et al.* 2003). The RAPD (random amplified polymorphic DNA) fingerprint of house keeping genes was useful tool to investigate genetic variability among pediocin producing and non-producing strains of *Ped. acidilactici* (Mora *et al.* 2000). Multiplex PCR analysis is a rapid method which can readily and unambiguously identify species of pediococci based on amplification of 16S rRNA and D-lactate dehydrogenase encoding genes (Mora *et al.* 1997). DNA fingerprinting and ribotyping procedures were specifically followed in the characterization of *Ped. acidilactici* (Jager and Harlander 1992).

Rapid molecular biology techniques such as dot-blot, Southern hybridization, colony hybridization have been employed for the detection of pediocin and enterocin As-48 producers (Rodriguez *et al.* 1997; 1998). DNA probes are being used for the study of silage colonization by lactobacilli and pediococci as these bacteria represents 95% of the total bacterial population in silage. DNA probes have been found to be effective in monitoring these bacteria and evaluating the colonization properties of inoculants (Cocconcelli *et al.* 1991). Multiplex PCR analysis approach was effective in differentiation of *Lactobacillus* spp. by studying *rec*A gene sequences (Torriani *et al.* 2001). Bhunia and Johnson (1992) have developed monoclonal antibody based colony immuno-blot method which is specific for isolation of *Ped. acidilactici* from fermented meat. It was shown that a 116 kDa protein reacting with the antibody was specific for the screening of pediocin producing pediococci.

1.7 Starter and protective cultures

The spontaneous fermentation of food has been utilized by mankind for more than 5000 years in alcoholic beverages, dairy products like cheese, yogurt and kefir and meat products like salami and fermentation of olives. A starter culture can be defined as a microbial preparation of a large number of cells of atleast one microorganism added to a raw material to produce a fermented food by accelerating and steering its fermentation process (Leroy and De Vuyst 2004).

Table 1.2: LAB Starter Cultures for food Fermentation

LAB	Foods	Reference/s
Lactobacillus spp. *Lact. casei, Lact. bulgaricus, Lact. helveticus, Lact. brevis* ssp. *linens Lact. sanfrancisco, Lact. sake, Lact.* curvatus, Lact. plantarum	Butter, cheese, bread, vegetables, sausages & yogurt etc.	Hansen 2002
Lactococcus spp. *L. lactis.* ssp lactis, L. lactis ssp. *cremoris.*	*Butter, Cheese*	*Leroy and De Vuyst 2004*
Leuconostoc spp		
Leuc. mesenteroides *ssp. mesenteroides, Leuc. mesesnteroides* ssp. *cremoris, Leuc. oenos*	*Butter, cheese, wine* fermented vegetables	*Leroy and* De Vuyst 2004
Pediococcus spp. *Ped. acidilactici, Ped. pentosaceus*	Sausage, olive	Ray 1995
Streptococcus thermophillus	Yogurt	Sarkar and Misra 2002

LAB play a prime role in these processes and have a long and safe history of application in the production of fermented foods and beverages. The most

commonly used LAB starter cultures and the fermented foods derived from them are listed in Table 1.2.

Geisen and Holzapfel (1996) reported that microorganisms that are used as starter cultures would have to meet several requirements such as:

a) It should be wholesome ie. non-pathogenic and incapable of producing toxins
b) It should produce the desirable effect
c) It should neither affect the nature of the product nor its sensory character, and

Table 1.3: Antimicrobial products produced by LAB*

Products	Producer LAB	Target microorganism
Lactic acid	All LAB	All microorganisms
Acetic acid	Heterofermentative LAB	All microorganisms, pH dependent
Hydrogen peroxide	All LAB	All microorganisms
Alcohols	Heterofermentative LAB	All microorganisms
Carbon dioxide	Heterofermentative LAB	Most microorganisms
Other low molecular weight compounds		
Diacetyl	*Lactococcus* spp	Yeasts, Gram+ve and Gram-ve bacteria
Reuterin	*Lactobacillus reuteri*	Broad spectrum, Gram+ve Gram-ve bacteria, fungi
Benzoic acid	*Lactobacillus*	Certain Gram-ve bacteria
Mevalonic acid	*plantarum*	*Fusarium avenaceum*

Lactone, Methyldantoin		
Antimicrobial peptides		
Bacteriocins	Most LAB	Mostly Gram+ve bacteria

(*Ref: Helander *et al.* 1997; Leroy and De Vuyst 2004; Tyopponen *et al.* 2003)

Starter cultures should suppress the growth of specific undesirable microorganisms. A list of various antimicrobial products reported from different LAB is collated in Table 1.3.

LAB that produce natural antimicrobial compounds in food are referred to as protective cultures. Protective cultures can also act as starter cultures in food fermentation processes and there has been increasing demand for combining both these properties to form a starter and protective culture inoculum (Leroy and De Vuyst, 2004).

1.8 Traditional Food fermentation by LAB

Consuming traditional fermented foods (TFF) has been a hereditary practice of all Indians, the tropical climate that engulfs most of the country aiding easy fermentation. Although many of these TFFs have not been developed by using a defined culture or a specific starter or protective culture, a portion of the fermented material has always been used as an inoculum. In different regions of India, diversified TFFs are being consumed in which various LAB in combination with other microorganisms are involved in the process of fermentation.

TFFs consumed in South India such as *Idli* and *Dosa* are naturally fermented by LAB and yeasts using the substrates like rice and blackgram. Similarly *Ambali* is prepared by using millet and rice where LAB are the sole natural fermentors. *Kanji,* a strong flavoured alcoholic beverage is prepared using carrot/beet root where natural fermentation takes place by LAB and yeasts.

In north India many TFFs, which are consumed as snacks, are prepared using blackgram as a substrate, where in LAB and yeasts are involved in natural fermentation. These TFFs are *Ballae*, *Vadai*, *Papad*, *Wari* etc. Preparation of *Bhatura* and *Jalebi* involves natural fermentation of wheat flour by LAB and LAB/yeasts respectively. *Shrikhand*, *Masti dahi* are prepared by natural fermentation of milk using LAB. *Gundruk*, *Sinky*, *Mesu* and *Jaanr* is used as a pickles and beverage, fermented by LAB using various vegetables and bamboo shoots. In these fermentation processes species of *Lactobacillus, Leuconostoc, Lactococcus, Enterocccus* and *Pediococcus* are involved (Tamang 1998).

1.9 Antimicrobial properties of LAB

LAB starter cultures cause rapid acidification of the raw material by the production of organic acids such as lactic and acetic acids. The property of preservation of LAB based starter and protective culture in food and beverages may be attributed the combined action of a range of antimicrobial metabolites produced during the fermentation process. The various organic acids such as lactic, acetic and propionic acids produced as end products provide an acidic environment unfavourable for growth of many pathogenic and spoilage microorganisms. Acid generally interferes with cell membrane potential, inhibits active transport, reduces the intracellular pH and inhibits variety of metabolic activity. Growth of both Gram-positive and Gram-negative bacteria as well as yeasts and moulds are inhibited by acid. Ethanol produced by heterofermentative pathways, H_2O_2 produced during aerobic growth and diacetyl groups generated from excess pyruvate can effect membrane lipids and cellular proteins leading to cell death (Helander *et al*.1997).

Reuterin, one of the well known secondary metabolites produced by LAB is a broad spectrum, low molecular weight antimicrobial product that kills both Gram-positive and Gram-negative bacteria, fungi and protozoa. Chemically reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic diameric forms of □-hydroxypropionaldehyde. Recently, one of the first

antibiotics- reuterocyclin, produced by LAB was discovered. Reuterocyclin produced by *Lact. reuteri* is a negatively charged, highly hydrophobic antagonist, and a novel tetrameric acid. It acts against many Gram-positive bacteria such as *Lactobacillus* spp., *B. subtilis*, *B. cereus*, *Ent. faecalis*, *Staph. aureus* and *Listeria innocua* and has a synergistic effect against *Escherichia coli* and *Salmonella* (Ross and Hill 2002).

1.10 Bacteriocins

LAB are known to be a good source of antimicrobial peptides which have tremendous applications in microbial food systems. Colicin produced by *E. coli* was one of the first bacteriocins to be characterized. Colicins can kill target microorganism by various mechanisms such as inhibition of cell wall synthesis, permeabilization of the target cell membrane and inhibition of nuclease activity. Among the Gram-positive bacteria, LAB bacteriocins are significant due to their food-grade properties and direct involvement in food applications. Bacteriocins of LAB kill closely related bacteria and are safe, thus having a potential application as a natural food preservative (Abee *et al.* 1995; Cleveland *et al.* 2001; Jack *et al.* 1995).

Bacteriocins have applications in hurdle technology that utilizes synergies of combined treatments to effectively preserve food. These factors include N_2, CO_2, low temperature, hydrostatic pressure, sodium diacetate, sucrose fatty esters, modified atmosphere packaging (MAP) and Tween 80. Bacteriocins, the ribosomally synthesized antimicrobial peptides are sometimes misrepresented as clinical antibiotics. The main difference between a bacteriocin and clinical antibiotic are that bacteriocins are gene encoded, ribosomally synthesized peptides with narrow spectrum of activity while antibiotics are heat labile, active across a broad-spectrum and are a product of a multienzyme complex. Bacteriocins forms pores in target cells or even affect cell wall biosynthesis (Cleveland *et al.* 2001). For a bacteriocin to be exploited in food preservation, it should possess the following properties:

It should be a protein molecule that is inactivated by proteases of the gastrointestinal tract.
It should be heat stable to ensure its activity after cooking of food
It should possess a broad-spectrum of activity and
Should be active in a wide range of pH.

1.11 Classification of bacteriocins

Bacteriocins are commonly divided into three or four groups. Nisin, the antimicrobial peptide produced by N-Streptococci (*Lactococcus lactis*) was one of the first bacteriocins discovered as early as in 1928. A similar bacteriocin, subtilin was discovered in 1948 which is an analogue of nisin (Hurst, 1981). Both belong to class I group of bacteriocins and are linear, pentacyclic peptide antibiotics termed as lantibiotics, due to their lanthionine containing amino acids. Based on the structural differences, class I bacteriocins are further divided into class Ia and Ib. The class II contains small, heat-stable, non-modified peptides further divided into classes IIa and IIb bacteriocins. Class IIa includes pediocin-like anti-listerial peptides with a conserved N-terminal sequence of Tyr-Gly-Asn-Gly-Val and two S-S bridges in the N-terminal half of the peptide. However, class IIb bacteriocins consist of two different peptides which are needed for their activity. Class IIc bacteriocins were originally proposed to contain the bacteriocins that are secreted by the general *sec*-system. Later, studies showed that class IIa bacteriocins could also use this secretary pathway and therefore this class is no more valid in classification (Ennahar *et al.* 2000; Jack *et al.* 1995). The class III bacteriocins consist of large and heat labile molecules. The classification of bacteriocins is illustrated in Table 1.4, as proposed by Klaenhammer (1993).

Class IIa bacteriocins can be considered as the major subgroup of bacteriocins from LAB because of their wide diversity, broad spectrum of activity and potential applications in food systems. Class IIa bacteriocins have attracted particular

attention as listericidal compounds and are now believed to be the next in line, if more bacteriocins are to be approved by the FDA in future. Class IIa bacteriocins that are studied so far vary from 37 -aa (in case of leucocin A and mesentericin Y105) to 48 -aa (carnobacteriocin B2) and share considerable sequence similarity (Hastings *et al.* 1991). Class IIa bacteriocins were thought to possess the characteristic YGNGVXaaC motif at their N-terminal half which perhaps acts as a receptor sequence to the membrane of their target host (Fig 1.2). However as newer members of groups of class IIa bacteriocins have been added the N-terminal conserved motif is thought to be YGNGVXaaCXaa (K/N)XaaXaaCXaav (N/D) (W/K/R) Xaa (G/A/S) (A/N). The C-terminal portion of class IIa bacteriocins are low in sequence similarity. These bacteriocins share 34 to 80% sequence identity with exceptions of leucocin A and mesentericin Y105 which vary only in the amino acids at positions 22 and 26 respectively (Maftah *et al.* 1993).

Table 1.4: Classification of bacteriocins produced by LAB

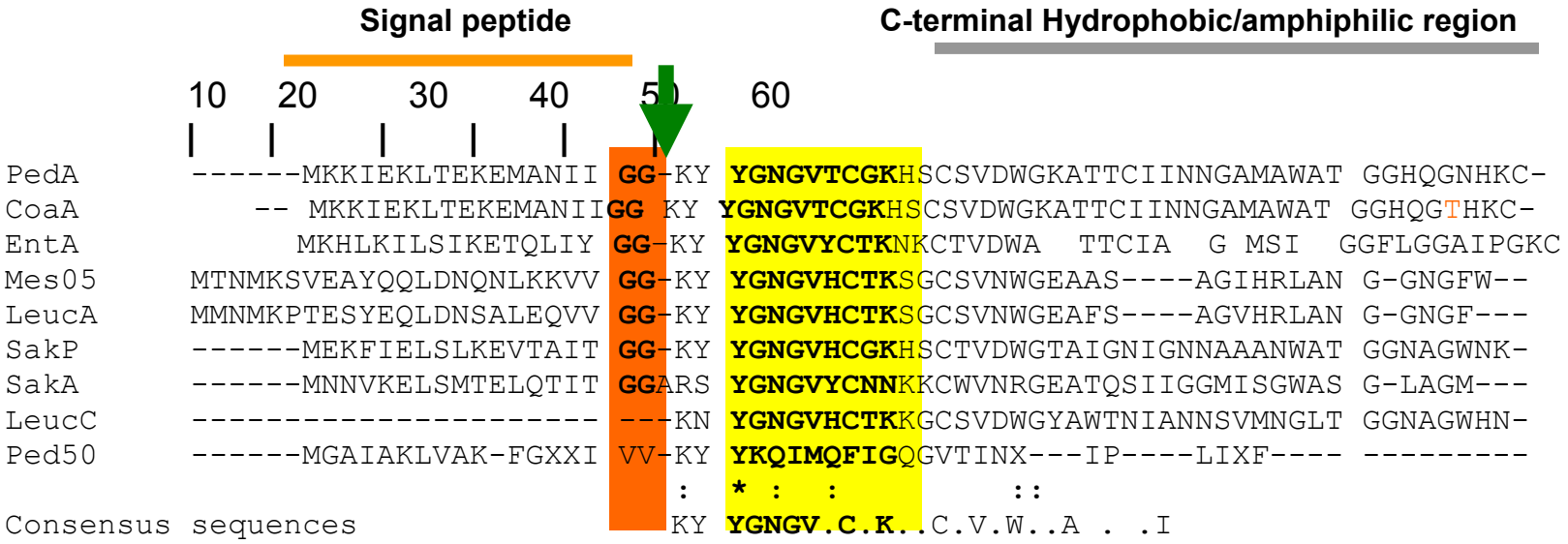
Group		Description	Example
I^A	Ia	Lantibiotics, small (<5 kDa) peptides containing lanthionine and β-methyllanthionine	Nisin
	Ib	Small globular peptides with no net charge or net negative charge	Mersacidin
II^B	IIa	Small heat stable peptides, synthesized as a precursor and active against *listeria* get processed after two glycine residues and have conserved sequences at the N-terminal	Pediocin PA-1/AcH Sakacin A and P Leucocin A, Carnobacteriocins
	IIb	**Two component systems: two different** peptides required to form an active poration complex	**Lactococcins G/F** Lacticin F Plantaricin EF, JK

III[C]	Large molecules sensitive to heat	Helveticins J and V-1829, lactacins A and B etc.

([A]: Hechard and Sahl 2002; Kuipers *et al.* 1993. [B]: Ennahar *et al.* 1999, 2000. [C]: Joerger and Klaenhammer 1986)

Class IIa bacteriocins have a net positive charge, with pI varying from 8.3 to 10.0. These classes of bacteriocins posses a high content of non-polar -aa residues and smaller -aa such as glycine which provide conformational freedom. Class IIa bacteriocins posses a highly conserved N-terminal hydrophilic domain and a moderately conserved hydrophobic and/or amphiphilic C-terminal domain which is associated with variation in the bacteriocin activity on the membrane. Class IIa bacteriocins exist primarily in unstructured confirmations, most often as random coils in aqueous solutions, which in non-aqueous solutions, adopt partial helical structures with varying amounts of hydrophobicity. This class of bacteriocins are cystibiotics i.e they have atleast two cysteine residues with disulfide bridges. The C-terminal half of Class IIa bacteriocins are predicted to adopt an amphiphilic □-helix. This helical portion is believed to be a transmembrane segment during pore-formation in a sensitive cell membrane (Aymerich *et al.* 1996; Bennik *et al.* 1998; Chen *et al.* 1997; Ennahar 2000; Fimland *et al.* 2000; Montville and Bruno 1994).

Figure 1.2: Prepeptide sequences of class IIa bacteriocins*



(*PedA: Marugg *et al.* 1992, Motlagh *et al.* 1994; CoaA: Le Marrec *et al*. 2000; EntA: Aymerich *et al*. 1996; Mes05: Fleury *et al.* 1996; LeucA: Hastings *et al.* 1991; SakP: Tichaczek *et al.* 1992; SakA: Holck *et al.* 1992; LeucC: Fimland *et al.* 2002 and Ped50: Cintas *et al.* 1995. Double glycine leader is highlighted with red colour and the pediocin motif box consisting of YGNGVXCXXK is highlighted with yellow colour. The signal peptide cleavage site is indicated by the green colour arrow. Pedioocin50 produced by *Ped. acidilactici* is known to be a different antimicrobial peptide with modified -aa and its alignment shows very low similarity with class IIa bacteriocins)

1.12 Gene organization of class IIa bacteriocins

The bacteriocin operon consists of divergently transcribing regions for bacteriocin production, extracellular translocation, immunity of the producers and, in several cases, regulation of bacteriocin synthesis. The presence of the regulatory genes has been convincingly demonstrated for a few of the class IIa bacteriocins eg. sakacin (Axelsson *et al.* 1998).

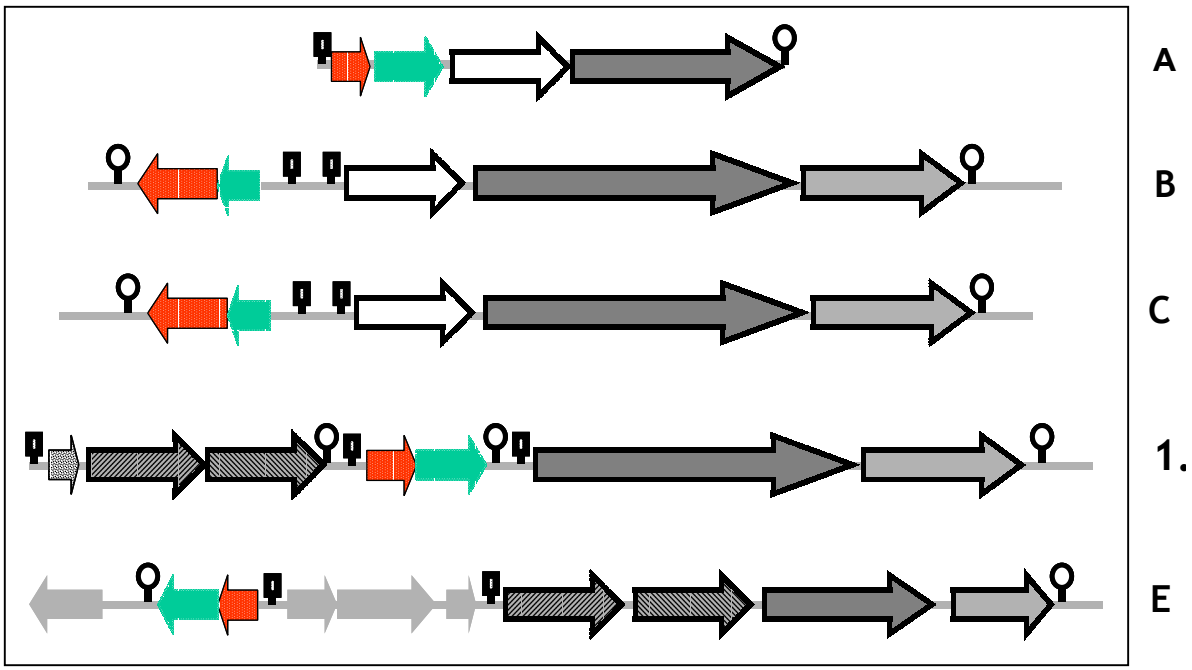


Figure 1.3: Operon organization of class IIa bacteriocin. A: the plasmid-borne pediocin operon of 3.5 kb, as described by Marugg *et al.* (1992); Motlagh *et al.* (1994). B: Mesentericin Y105 (Fleury *et al.* 1996, Maftah *et al.* (1993). C: Leucocin A (Hastings *et al.* 1991). D: Sakacin P (Tichaczek *et al.* 1992) and E: Sakacin A (Holck *et al.* 1992).

Bacteriocin pre-peptide, Immunity protein, Maturation protein, transport protein, Response regulator, Histidine kinase and unidentified ORF found in certain bacteriocin operons promoter and terminator elements etc.

The bacteriocin structural gene encodes a pre-form of the bacteriocin called 'pre-peptide' containing a leader sequence with two glycine residues at its C-terminal as a recognition signal for a *sec*-independent ABC-transporter. The ABC transporter's homologue and its accessory protein help in externalization of the bacteriocin. The bacteriocin structural gene is found preceding an immunity gene that is co-transcribed as a part of the small operon. Induction factors (IF), histidine kinase (HK) and response regulators are found to be present in the vicinity of the structural gene and work as transcriptional regulators (Tichaczek *et al.* 1992). Figure 1.3 shows the comparative overview of genetic organization of pediocin with other similar operons of class IIa bacteriocins.

1.13 Pediocin

Pediocin represents a class IIa heat stable, membrane acting antimicrobial peptide produced by various strains of *Pediococcus* (Daeschel and Klaenhammer 1985; Gonzalez and Kunka 1987; Ray *et al.* 1989). In the recent past, this bacteriocin has reached limelight due to its high antilisterial activity and stability at a neutral pH. Comprehensive information highlighting the different pediocins, source of their isolation and the strain that produce them is indicated in Table 1.5. As shown in the above table, pediocin is produced by various strains of pediococci notably *Ped. acidilactici*, *Ped. pentosaceus* and *Ped. damnosus*. Intergeneric pediocin AcH production, similar in activity to that produced by *Ped. acidilactici*, has been reported in *Lact. plantarum* and *Bacillus coagulans* (Ennahar *et al*. 1996; Le Marrec *et al*. 2000). The different pediocins refereed to in Table 1.5 share common characteristic such as retention of activity when subjected to heat at 100^0C for 60 min and 121^0C for 10 min indicating their heat stable nature. They can withstand wide pH range of 2-10 except pediocin N5p which is inactivated at pH 10. Pediocin PD-1 has been found to be resistant to many proteolytic enzymes such as pepsin, papain, trypsin, □-chymotrypsin and proteinase-K, though majority of the pediocins are inactivated by proteinase-K (Green *et al.* 1997).

Table 1.5: Pediocin-type bacteriocin producing strains of LAB from different food systems

Bacterial strain/s	pediocin-type	Source
1. *Pediococcus parvulus*	pediocin PA1	Minimally processed vegetables
2. *Pediococcus* sp.	pediocin K1	Fermented flat fish
3. *Ped. acidilactici* M	pediocin AcM	Fermented sausage
4. *Ped*. *pentosaceus*	pediocin N5p	Wine
5. *Ped*. *acidilactici*	pediocin L50	Spanish dry-fermented sausage
6. *Ped. damnosus* NCFB 1832	pediocin PD-1	NCFB*
7. *Ped. acidilactici* H	pediocin AcH	Fermented sausage
8. *Ped. acidilactici* PAC 1.0	pediocin PA-1	Fermenting vegetables
9. *Ped. acidilactici* JD-23	pediocin JD	NA
10. *Ped. acidilactici*	pediocin SJ-1	Naturally fermented meat products
11. *Ped. acidilactici* UL5	pediocin 5	Cheese
12. *Ped. pentosaceus* FBB-6	pediocin A	Cucumber fermentation
13. *Ped. acidilactici*	pediocin K7	Cucumber fermentation
14. *Lact. plantarum*	pediocin AcH1	Munster cheese
15. *Bacillus coagulans*	coagulin	Cattle faeces

1. Bennik *et al.* 1997a; 2. Kim *et al.* 2000; 3. Elegado *et al*. 1997; 4. Manca de Nadra *et al.* 1998; 5. Cintas *et al.* 1995; 6. Green *et al.* 1997; 7. Bhunia *et al.* 1987, 1988; 8. Gonzalez and Kunka 1987; 9. Richter *et al.* 1989; 10. Schved *et al.* 1993; 11. Huang *et al.*1996; 12. Daeschel and Klaenhammer 1985; 13. Ramesh 2000; 14. Ennahar *et al.* 1996; 15. Le Marrec *et al.* 2000. NA; Not available.

Pediocin N4p inhibited LAB which where isolated from wine and market yoghurt. *Ped. pentosaceus* E5p appeared to be most affected by pediocin N5p. Likewise, PD-1 exhibited a different antimicrobial spectrum compared to that of the other pediocins such as SJ-1, AcH, PA-1 and N5p (Green *et al.* 1997). The bacteriocidal mode of action of pediocin was seen against many strains of *List. monocytogenes (*Ennahar *et al.* 1996; Green *et al.* 1997; Manca de Nanda *et al.* 1998).

Table 1.6: Spectrum of antimicrobial activity of different pediocins

Pediocin	kDa	Antimicrobial activity against
1. pediocin PD-1.	3.5	*Clostridium sporogenes*, *Lact. buchneri*, *Lact. fermentum*, *Oenococcus oeni*, *Propionibacterium acidipropioni*
2. pediocin N5p	ND	*Lact. hilgardii* 5w, *Leuc. oenos* X2L, L10, *Ped. pentosacues* E5p, Xp
3. pediocin K1	4.2	*Lact. curvatus* KCA 170-12, *Ped. pentosaceus* FBB61-2*, Carn. piscicoli LV17, UAL26, List. monocytogenes* ATCC1911, *Ent. faecalis* ATCC 19433/11576
4. pediocin L50	5.2	*Lact. acidophilus* ATCC 4356, *Lact. bulgaricus* ATCC11842, *Lact. fermentum* ATCC 9338 *Clostridium sporogenes* TNO C222/10 *Propionibacterium acidipropionici* NCDO 563 *Staph. aureus* FRI 137
5. pediocin AcH/PA1 UL5, 5/SJ1/K7	4.6	*List. monocytogenes* 1/2a, 1/2b, 1/2c, 4d *List. seeligeri* 26 and many other listerial spp.
6. pediocin AcM	4.6	*Lact. diacetyllactis*, *Lact. plantarum*, *Lact. sake*, *Lact.fermentum*, *Ped. pentosaceus*, *Ped. acidilactici Staph. aureus*, *Ent. faecium*
7. leucocin C	4.6	*Lact. sake* NCDO 2714

(1, Green *et al.* 1997; 2, Manca de Nadra *et al.* 1998; 3, Kim *et al.* 2000; 4, Cintas *et al.* 1995; 5, Ramesh 2000; 6, Elegado *et al.* 1997 and 7, Fimland *et al.* 2002) ND, Not determined.

Fimland *et al.* (2002) have reported pediocin like antimicrobial peptide produced by *Leuc. mesenteroides* called leucocin C which has a high level of sequence homology with several pediocins. Pediocin AcM inhibited a large number of test bacteria including many species of the genera of *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Enterococcus*. It has been seen that the antimicrobial spectra of AcM is wider than that of pediocin PA-1. Pediocin K1 whose N-terminal sequence has been established and pediocin K7 whose nucleotide sequence data is available belong to the pediocin AcH/PA-1 group, though pediocin K1 has been estimated to be a 4.2 kDa protein on SDS-PAGE. Kim *et al.* (2000) demonstrated that pediocin K1 was found to be unique in its amino acid sequence, composition and heat stability as well as activity at a wider pH range. Pediocin L50 can be produced at low temperatures and hence finds a commercial advantage for use in the preservation of refrigerated foods (Cintas *et al.* 1995). The antimicrobial spectra of pediocins activities are indicated in Table 1.6.

1.14 Plasmids

Plasmids often found in LAB encode for many of the dispensable functions. Many plasmids are associated with carbohydrate fermentation, antibiotic and bacteriophage resistance. Strains may be discriminated by the size and number and perhaps sequence of the plasmids they contain. The number of native plasmids in LAB vary from one to sixteen (Ruiz-Barba *et al.* 1991). The first evidence of plasmid DNA in pediococci was reported by Gonzalez and Kunka (1983) in strains of *Ped. pentosaceus* and *Ped. acidilactici.* However, no information about their metabolic functions was reported. Many of the plasmids in pediococcal strains coding for bacteriocin production range in size from 4.5 to 39.5 MDa.

The number of naturally occurring plasmids in native strain can be deciphered by isolating the plasmid DNA and subsequent analysis in agarose gel

electrophoresis. Various methods have evolved for *in vitro* analysis of plasmids. O'Sullivan and Klaenhammer (1993) have described the rapid mini-prep protocol for the isolation of high quantities of plasmid DNA from lactococci and lactobacilli. However, this method failed to extract high molecular weight plasmid DNA which are often found in LAB. A simple and rapid method of isolation of large quantities of plasmid DNA larger than 30 Mda was developed by Anderson and Mckay (1983). This is a composite method where in cell lysis is followed by specific enrichment of large plasmid DNA and is useful on a preparative scale to isolate sufficient quantities of plasmid DNA required for restriction analysis, cloning, transformation and many other *in vitro* studies. Plasmid linked characters can be identified by various methods. Kim *et al.* (1992) demonstrated the conjugal transfer of *Ped. acidilactici* pPR72 plasmid into two other strains of *Ped. acidilactici* by filter mating experiments. The plasmid pPR72 is 53.7 kb in size and is associated with sucrose fermentation. Gonzalez and Kunka (1983) demonstrated intergeneric and intrageneric transfer of broad host range plasmid pIP501 in *Pediococcus* spp. This plasmid was associated with bacteriocin activity and carbohydrate fermentation. Plasmid curing is one of the more efficient methods of determining the plasmid linked phenotypes. Plasmid curing tentatively provides the physical evidence of loss of metabolic trait(s) with the concomitant loss of a particular plasmid.

Various plasmid curing agents have been employed for determining the bacteriocin producing phenotypes in the genus *Pediococcus*. Plasmid curing is normally carried out using chemical agent like ethidium bromide, novobiocin, acryflavin, sodium dodecyl-sulphate, ascorbic acid and acrydine orange (Ruiz-Barba *et al.* 1991, Hoover *et al.* 1988). Daeschel and Klaenhammer (1985) developed a novel method of curing the native plasmids by growing the bacteriocin producing pediococci in a chemostat. In this method, plasmid curing was achieved by elevating the growth temperature to 42^0C, limiting the availability of glucose, and diluting

Table 1.7: Pediocin encoding native plasmids

Bacterial strain	Plasmid	size (MDa)	Curing method/s	Traits
Ped. pentosaceus[a]	pMD136	13.6	Chemostat cultivation	Bac^+, Imm^+
Ped. acidilactici[b]	bacteriocin production	5.5	Acryflavine Acrydine orange	Bac^+
Ped. cerevisiae[c] FBB 63 (*acidilactici*)	-do-	10.5	Novobiocin	Bac^+
Ped. acidilactici[d] 23	pSRQ11	6.2 Suc^+	High temperature	Bac^+
Ped. acidilactici[e] H	pSMB7.4	5.7	Acryflavine, Ethidium bromide, high temp etc.	Bac^+ Bac^R
Ped. acidilactici[f] SJ-I	bacteriocin plasmid	2.9	Acryflavine and heat treatment	Bac^+ Bac^S
Ped. acidilactici K7[g]	bacteriocin plasmid	6.1	Ascorbic acid	Bac^+ Bac^R
Pediococcus sp. KCA 1303-10[h]	bacteriocin plasmid	5.9	Acrydine orange	Bac^+
Ped. acidilactici UL5[i]	pMJ5	8.4	Acryflavine	Bac^+
Lact. plantarum WHE 92[j]		7.1	NA	Bac^+
B. coagulans[k]	pI4	9.0	NA	Bac^+

([a], Daeschel and Klaenhammer, 1985; [b], Hoover *et al.* 1988; [c], Graham and Mckay 1985; [d], Gonzalez and Kunka 1985; [e], Ray *et al.* 1989; [f], Schved *et al.* 1993; [g], Ramesh 2000; [h], Kim *et al.* 2000; [i], Huang *et al.* 1996; [j], Ennahar *et al*. 1996; [k], Le Marrec *et al.* 2000); Bac^{+}, bacteriocin production, Suc^{+} sucrose fermentation; Bac^{R} and Bac^{S} are bacteriocin resistance and sensitive respectively. NA, Not available.

the cell concentration at a rate of 0.2 h^{-1}. The subsequent transfer in glucose limiting conditions at high rate exerts environmental pressure on the cells which favours plasmid loss. By employing this technique, a 13.6 MDa plasmid associated with bacteriocin production was identified. Gupta and Batish (1992) have shown protoplast induced curing of bacteriocin plasmid in *L lactis* ssp. *lactis* 484. It has been shown that many antibiotic resistance genes are plasmid-borne. The acquired resistance to vancomycin among *Ent. faecium* and *Ent. faecalis* is well known (Quintilioni and Courvalin 1994).

Danielsen (2002) reported the molecular characterization of the tetracycline resistant plasmid pMD5057 from *Lact. plantarum* strain. Nucleotide sequence analysis revealed that tetracycline region of *Lactobacillus* was of the IS-element type and has a high sequence homology to clostridial and staphylococcal tetracycline resistance. Various antibiotic resistance plasmids have been reported in literature from LAB since plasmid encoded antibiotic resistance genes may play an important role in horizontal gene transfer in these bacteria.

In the genus *Pediococcus,* plasmids encoding bacteriocin production, carbohydrate fermentation and their method of curing is listed in Table 1.7. The well-characterized plasmids of LAB are used for construction of shuttle vectors, food-grade cloning and expression vectors. The food-grade cloning and expression vectors are devoid of any antibiotic resistance markers and are accepted in food systems (Platteeuw *et al.* 1996; Shimadzu-Kadota *et al.* 1991). Novel system for NICE (nisin inducible cassette expression) regulation was

developed based on various selective regions of Nisin biosynthesis. This is one of the important food-grade process in LAB for controlled over-production of heterologous proteins (de Vos 1999).

Plasmid encoded carbohydrate metabolizing genes can be incorporated in construction of food-grade vectors. Genes for fermentation of sugars such as raffinose and sucrose are known to be plasmid encoded traits. Such traits can be directly exploited for the purpose of construction of food grade vectors.

1.15 β-galactosidase

β-D-galactoside galactohydrolase, trivially lactase or β-galactosidase (β -Gal) (EC 3.2.1.23), the enzyme of LAB hydrolyzes lactose into its monosaccharide components glucose and galactose. This intracellular enzyme is required by bacteria for lactose utilization. The enzyme β–Gal also performs transgalactosylation reaction yielding galacto-oligosaccharides which has enormous potential for the synthesis of novel oligosaccharides having food, pharmaceutical and medical applications. The occurrence and importance of this enzyme in many LAB have been reported (Bhowmik and Marth, 1990).

The consumption of food containing LAB which provide β -Gal can help reduce the level of lactose present in many fermented dairy products and have many advantages as lactose has a relatively low level of sweetness and solubility in water. Lactose intolerance is a major problem among Asians which can be efficiently managed by consuming food-grade microorganisms which are a promising source for the production of β–Gal. In addition, the β –Gal encoding gene can be used as a selection marker for the construction of food-grade vectors. Plasmid encoded β–*gal* gene has been reported in certain species of LAB (Fernandez *et al.* 1999; Griffin *et al.* 1996). Table 1.8 summarizes the distribution of β-Gal in various lactics and their *E. coli* counterpart.

Table 1.8: β-galactosidase of LAB

LAB	No of amino acids	protein (kDa)	Method of Gene Isolation
E. coli[1]	1, 023	116	Gene complementation
Lact. bulgaricus[2]	1, 006	114	Complementation in *E. coli*
Bifidobacterium longum MB 219[3]	1, 023	116	Complementation in *E. coli*
L. lactis[4]	996		PCR cloning
Leuc. lactis[5]	600	75	Deletion and complementation
	321	36	analysis
Lact. sake[6]	625	72	DNA probe
32			
Lact. plantarum[7]	ND		Heterologous expression
Bif. Infantis[8]	ND		Gene complementation
Carn. piscicola BA[9]	668	76.8	Gene complementation

([1], Jacob and Monod 1961; [2], Schmidt *et al.* 1989, [3], Rossi *et al.* 2000; [4], Griffin *et al.* 1996; [5], David *et al.* 1992; [6], Obst *et al.* 1995; [7], Mayo *et al.* 1994; [8], Hung and Lee 1998 and [9], Coombs and Brenchley 1999; ND, not determined)

From Table 1.8, it is evident that β –Gal encoding gene of LAB belongs to two categories: the high molecular weight enzyme from *Lact. bulgaricus* and *L. lactis* and the low molecular weight one from *Leuc. lactis* and *Lact. sa*ke. However, the high molecular weight β–Gal shows atleast seven regions of high similarity common to its *E. coli* counterpart (Schmidt *et al.* 1989). The low mol wt β **-**Gal found in *Leuconostoc* group show overlapping ORFs (David *et al.* 1992). The β-Gal of *Bif. infantis* is secreted as an isozyme showing three different subunits. Multiple sequence alignment followed by homology search and degenerate primer synthesis for PCR proved to be an useful tool for a amplification of a 500 bp fragment of *L. lactis* β–*gal* gene (Griffin *et al.* 1996).

In many cases, the plasmid encoded β-*gal* gene was evident by plasmid curing (Fernandez *et al.* 1999; Griffin *et al.* 1996). However, duplication of the β-*gal*

gene in some *Lact. plantarum* strains has also been observed. Phylogenetic analysis of the β-*gal* gene shows that the bacterial β -galactosidase enzyme is divided into four distinct groups; the Lactococcal enzyme being most related to the enzyme from Gram-negative bacteria. However the β-Gal of *Lact. bulgaricus* and *Leuc. lactis* falls into a separate group. Like wise, β-*gal* from *B. stearothermophillus* does not appear to be related to the β-*gal* from any other bacterial species. The -aa sequence alignment of high molecular weight β –Gal of LAB with *E. coli* counterpart showed only 30-35% similarity. It is interesting to note that there are atleast seven regions of high similarity common in β-Gal of *L. lactis*, *E. coli* and *K. pneumonie*. The putative active site residues Glu-461 and Tyr 503 found in the in *E. coli lac*Z β-Gal are conserved among all forms of the enzyme. The conservation of active site -aa and large regions of similarity suggest that all four of these evolved from a common ancestral gene (Schmidt *et al.* 1989).

Recent reports indicate the development of processes for the production of commercial β-Gal enzyme from microbial sources. Vasiljevic and Jelen (2001) demonstrated an economically viable process for the production of β-Gal for lactose hydrolysis in dairy products using *Lact. delbrueckii* ssp *bulgaricus*. A suitable medium was formulated for the industrial production of β-Gal from thermophilic bacterium using whey permeate (WP) as the basal medium. In this process a thermostable enzyme was chosen, as high temperature conditions in a reactor enhances the rate of lactose hydrolysis as well as prevented the growth of undesirable microorganisms.

1.16 Production of bacteriocin in food-grade media

As stated previously, LAB are fastidious in nature and require many growth factors such as minerals and vitamins in addition to carbohydrates and a nitrogen source in their growth medium. Many of the LAB strains require 4 to 14 –aa depending on the strain. Bacteriocin production has been carried out mostly in MRS (de Man, Rogosa and Sharpe) medium where glucose (2%) is

supposed to be a good carbon source (de Man *et al*. 1960). MRS is a complex bacteriological broth which is generally used as a maintenance as well as bacteriocin production media for majority of the LAB. Use of MRS as a production medium has two major disadvantages: higher cost and interference of large number of exogeneous proteins/peptides present in culture filtrate during purification of bacteriocins. Nisin has been produced in whey permeate medium as lactococci mainly utilize lactose present in whey permeate as a carbon source.

Daba *et al.* (1993) have studied the influence of growth conditions on the production and activity of pediocin AcH/PA1 (mesenterocin 5) by a strain of *Ped. acidilac*tici (initially identified as *Leuc. mesenteroides*). Various media have been tested for their ability to support both the growth of producer microorganism and production of bacteriocin. The different media tested include MRS broth obtained from two different sources, Elliker broth, TSY broth, BHI broth, M_{17} broth, whey and whey permeate. All the above media were compared in their ability to support bacteriocin production. Growth was weak in whey, whey with 2% yeast extract (YE), whey permeate and whey permeate with 2% YE. Maximum growth and bacteriocin production was found when the cells were grown in MRS. Specific production rate was highest when whey permeate with 2% YE supplemented with Tween 80 was used while being slightly lower when cells were grown in Elliker broth with Tween 80. It was found that Tween 80 was a major factor in increasing bacteriocin production and even specific production rate. It was further found that large quantities of bacteriocin production was possible in whey and WP supplemented with YE in the presence of 0.1% surfactant. In another separate study, pediocin PO2 was produced in WP using a strain of *Ped. acidilactici* PO2, where, WP with 2% YE was found to favor high cell density and maximum antimicrobial activity of pediocin PO2. The initial pH which was adjusted to 6.5 dropped to a range of 5.2 –5.6 solely because of fermentation. Increase in YE concentration from 2 to 4% had positive effect in both biomass as well as bacteriocin production.

Production of bacteriocin in WP was possible only after supplementing a rich source of carbon and nitrogen in the form of YE (Liao *et al.* 1993).

Biswas *et al.* (1991) have demonstrated an inexpensive food-grade medium for the production of pediocin AcH produced by *Ped. acidilactici*. This medium consists of Trypticase, glucose and YE, (TGE) along with Tween 80 and the salts of manganese and magnesium. Pediocin AcH production was 15% less in MRS compared to TGE broth however, cell biomass was more in MRS broth. 1X TGE supports not only pediocin production but favours more cell biomass and acid production too. However, 2X concentration of TGE showed only slight increase in pediocin and biomass. Among the different sugars tested, glucose and sucrose showed highest level of pediocin production and cell biomass. Though pediocin producing pediococci are tolerant to high temperatures, reduction in cell biomass and pediocin production was seen at 40^0C when compared to 30 to 37^0C. In this study, low pH of 3.6 was found to be necessary for active pediocin synthesis.

Yang and Ray (1994) further studied the influence of the producer strain, the composition of broth and the final pH of broth cultures on the production of nisin, pediocin AcH, leuconocin LcmI and sakacin A. Production of all bacteriocins except pediocin was lower in TGE broth than in TGE buffer broth. This may be due to need of higher pH for the production of the other three bacteriocins. Na-acetate when incorporated in TGE broth showed an increase in the production of all three bacteriocins except pediocin AcH, though stimulatory effect of Na-acetate on cell growth of many LAB have been reported (de Man *et al.* 1960). It was found that maximum pediocin was produced when the terminal pH was 3.7. However, highest level of nisin, leuconocin LcmI and Sakacin A were produced at pH 5.8, 5.0 and 4.5 respectively.

Wan *et al.* (1995) demonstrated the continuous production of brevicin, Nisin and pediocin in a Calcium alginate-immobilized reactor. The concentration of all the

three bacteriocins tested was as high as those obtained from conventional free-cell batch fermentation. Ca-alginate is a food grade additive and its use in the encapsulation of viable cells is simple and economical. In cell immobilization technology, the cost of cell removal from the fermentation broth can be minimized during purification or concentration of bacteriocin. The cell immobilization in combination with continuous fermentation process offers a new approach for the commercial production of bacteriocin. Strasser de Saad *et al.* (1995) examined the suitability of grape juice as a production medium for pediocin N5p since effective application in the microbiological control of vinification is necessary to understand the production and stability of pediocin N5p in grape juice medium (GJM). GJM, the food grade medium was formulated by diluting commercial grape juice (1:8) and supplementing with 1% yeast extract. This medium was found to produce about 30% more pediocin N5p compared to tomato juice medium (TJM).

Goulhen *et al.* (1999) demonstrated mixed production of Nisin Z/pediocin in a supplemented whey permeate medium. As stated above WP was found to be one of the cheapest food-grade medium for the production of bacteriocin. An increased bacteriocidal activity against undesirable micro-organisms has been demonstrated using a mixture of pediocin and nisin as compared to their use singly.

1.17 Biopreservation using bacteriocins

Biopreservation refers to use of metabolites derived from living systems for the purpose of preservation, in contrary to use of chemical preservatives (Abee *et al.* 1995; Schillinger *et al.* 1996). Food safety is of international concern and due to infection by various food-borne pathogens and food-borne illnesses, use of natural food preservative such as bacteriocin derived from LAB has received a great attention. Bacteriocin, produced by various LAB found in numerous fermented and non-fermented foods, can be explored for the purpose of biopreservation. Nisin is currently the only bacteriocin widely used in food

preservation (Delves-Broughton *et al.* 1996). Recent out-breaks of *List. monocytogenes* as one of the prominent emerging food-borne pathogens have posed a serious concern in food industry (Kozak *et al.* 1996). Thus pediocin, an anti-listerial bacteriocin, finds wide application in the control of this pathogen. Either whole cells of pediocin producer, pediocin preparation or heterologously produced pediocin can be used for this purpose.

Buyong *et al.* (1998) has expressed plasmid-encoding pediocin into *Lact. lactis* to aid in the preservation of cheddar cheese and assured the anti-listerial quality of fermentation process. Coderre and Somkuti (1999) have expressed pediocin PA-1 into *Streptococcus thermophillus* which is an important dairy starter culture. Schoeman *et al.* (1999) have expressed pediocin PA-1 in yeast *Saccharomyces cerevisiae* for the purpose of preservation of wine, bread and other food. Table 1.9 provides the detailed information about the various uses of pediocin and its method of preparation.

From the Table 1.9 it is evident that pediocin has numerous applications for preservation of various food system such as meat, dairy and vegetable. A few US patents (nos. 4, 929, 445 and 5, 445, 835) have been granted for the biopreservation of various foods using pediocin (Vandenbergh *et al.* 1990; Vedamuthu 1995).

Table 1.9: Applications of pediocin

Pediocin	Target microorganism	Method of bacteriocin preparation	Food system
Pediocin PA1[A]	*Listeria monocytogenes*	MRS broth grown CF fortified with 10% Milk powder	Dressed cottage cheese half and half Cream, sauce etc
Pediocin P02[B]	*List. monocytogenes*	MRS broth grown CF Amm sulphate ppt	Cooked meat products
Pediocin PO2[C]	*List.* monocytogenes	Whey permeate supple-mented with yeast extract	liquid whole egg whole milk etc
Bacteriocin of *Ped. acidilactici* M [D]	*List. monocytogenes*	MRS broth grown CF ethanol precipitate and freeze dried	Kimchi fermentation
Pediocin K7[E]	*List. monocytogenes*	TGE broth	Shrikhand preservation

([A]: Pucci *et al.* 1988; [B]: [C]: Liao *et al.* 1993; [D]: Choi and Beuchat 1994 and [E]: Ramesh, 2000)

1.18 Heterologous production of bacteriocin

A heterologous system refers to the system other than the native organism in which the metabolite is produced. A well-characterized host-vector system is a prime requirement for the heterologous production/expression of foreign genes. Numerous prokaryotic Gram-positive and Gram-negative hosts have been developed for the purpose of expression. In general, heterologous expression systems are usually employed for three main purposes (Makrides 1996):

1. To assist in the l elucidation of the function of recombinant protein and peptides

2. To facilitate the transcriptional/translational control of recombinant gene expression and
3. To achieve a level of production higher than those of the native sources

The efficacy of bacteriocin is limited in food systems due to varied reasons which can be overcome by heterologous expression. Heterologous production of LAB bacteriocin may be used to construct multibacteriocinogenic strains or to confer antimicrobial properties to strains of technological interest such as those used as starter cultures (Axelsson *et al*. 1998). In Gram-negative bacteria, *Escherichia coli* has long since been considered the primary prokaryotic host for cloning and expressing heterologous genes due to its extensive genetic characterization. Besides, many of its biological processes are well understood and there are a wide range of genetic tools readily available for its manipulation. Due to this reason, *E. coli* has invariably been selected as a host for cloning a variety of genes involved in the biosynthesis of LAB bacteriocins. However, a single organism is unlikely to be suitable for every application and not every gene can be expressed efficiently in *E. coli*. In this Gram-negative microorganism, the organization of cell membrane differs from that of the Gram-positive LAB. Hence an *E. coli* host may lack the secretion mechanisms required for the efficient release of recombinant proteins into the culture medium.

Many of the class I bacteriocins of LAB have been heterologously expressed using native biosynthetic genes. Lactococcin S, a lantibiotic produced by *Lact. sake* L45 was expressed in *Lact. sakei* 790 and *Lact. plantarum* NC8 by subcloning a 16 kb DNA fragment containing eleven ORFs. This heterologous system was used to analyze a variety of mutations introduced into the gene cluster and it was easy to characterize and classify the essential genes involved in bacteriocin production. Lacticin 3147 was heterologously expressed in *Ent. faecalis* to compare the anti-microbial activities of lacticin 3147 and cytolysin.

Although lower levels of antimicrobial activity was observed, it was possible to conclude that lacticin machinery can function in other genera. The gene dosage dependant expression of acidocin B was activated in *Lact. plantarum* by using high and low copy no. of plasmid (Rodriguez *et al.* 2002b). O'Keeffe *et al.* (1999) cloned a 10.5kb amplicon comprising all structural genes and regulatory regions involved in enterocin A (inducible class II bacteriocin produced by *Ent. faecium*) biosynthesis into the *E. coli-L. lactis* shuttle vector pCI1372. In broth, enterocin A production was only observed after addition of exogenous induction factor. The production of enterocin A in *L. lactis* was only possible by constitutive expression of the four gene cassette *ent*AITD under the control of the lactococcal promoter P32.

For the expression of helveticin J, a 3.8 kb fragment that contains the helveticin J structural gene was cloned in a pTRK135 vector and transformed into *Lact. johnsonni* VP111088, which resulted in helveticin production. Expression of this class III heat labile bacteriocin was the first reported case of heterologous production of a LAB bacteriocin (Joerger and Klaenhammer 1986). Many of the bacteriocins have been heterologously expressed by exchanging leader peptides and/or ABC secretion and processing systems. Secretion and processing of most LAB bacteriocins requires a dedicated transport and processing system comprised of an ABC (ATP-binding cassette) export protein and an accessory protein. Exploitation of the significant -aa homologies in both the leader peptides and dedicated transporters of most class II bacteriocins, some lantibiotics and also colicin V revealed an attractive possibility for heterologous production of bacteriocins. Kuipers *et al.* (1993) demonstrated the function of leader peptide of Nisin Z by fusing it to subtilin. Results indicated that there was a recognition of the hybrid precursor by the nisin Z dedicated secretion system of lactococci. The precursor was exported but not processed, resulting in decreased antimicrobial activity with respect to that of the wild type nisin. The N-terminal leader sequences of the lacticin F peptide was substituted for carnobacteriocin A leader sequence and was co-expressed with

carnobacteriocin A in *Carn. piscicola* LA17, which resulted in the production of both the peptides of lacticin F while not affecting the expression of carnobacteriocin. However, lactocin F subunit was produced at low levels. This study indicates that for the production of both the peptides of lactocin F, secretion machinery of carnobacteriocin producing host can be used (Rodriguez *et al*. 2002b). van Belkum *et al.* (1997) carried out a detailed investigation of the role of the leader sequence of heterologous class II bacteriocin and their recognition by the dedicated translation machinery by different host organisms. Many of the bacteriocin are heterologously produced by adding signal peptides recognized by general secretory pathways.

A few examples of bacteriocins heterologously produced by LAB using general secretory pathway includes production of brochocin –C in *Carn. piscicoli* LV17C, carnobacteriocin B2 in *Carn. divergens* LV13, enterocin B in *Ent. faecalis* ATCC 19433 and mesenteriocin Y105 in *E. coli* DH5□. In the above studies, in-frame fusion between sequences encoding the general export pathway's signal-peptide was constructed with the mature part of a different LAB bacteriocins. Heterologous systems as described above, developed either using ABC transporter or *sec*-dependant secretion systems, can help overcome problems such as poor adaptability, low production or genetic instability in a native strain. Further, heterologous systems can be used for the production of hybrid bacteriocins and engineered molecules with enhanced properties (Rodriguez *et al.* 2002b).

Kuipers *et al.* (1998) have developed a food-grade overexpression system of the genes encoding the secretion and processing machinery based on nisin inducible PnisA system. Sanders *et al.* (1998) demonstrated a salt inducible Pgad system. The use of these promoters can be one of the interesting approaches for achieving a higher level of heterologous expression of bacteriocin genes in a cost effective way.

1.19 Heterologous expression of pediocin

Like other bacteriocins, pediocin has been studied in heterologous expression system either by using native biosynthetic genes, exchanging leader peptides/ABC secretion system or by adding signal peptides recognized by general secretary pathways.

Marugg *et al.* (1992) have cloned the pediocin PA-1 operon under the control of its own promoter and expressed it in *E. coli* and the sensitive strain of *Ped. pentosaceus* PPE1.2. They observed the functional production, processing and secretion of pediocin PA1. This study helped in the functional analysis of the pediocin operon. Sub-cloning of the *ped* operon without its promoter also resulted in pediocin PA-1 production and secretion in these heterologous hosts, although bacteriocin production was found to be very unstable (Bukhtiyarova *et al.* 1994).

Chikindas *et al.* (1995) reported, a four fold increase in pediocin PA1 for the production and secretion of pediocin PA-1 in *Ped. pentosaceus* PPE1.2 under the control of P32 promoter. However, when *L. lactis* IL1403 was used as a host, yield of pediocin was less than 1%. It was observed that the native promoter did not function efficiently in *L. lactis* background.

Coderre and Somkuti (1999) studied the expression of pediocin PA-1 in different hosts resulting in the production of variable amounts of bacteriocin. Further they have demonstrated that gradual loss of pediocin production phenotype was linked to plasmid instability. Martinez *et al.* (2000) reported the co-production of pediocin PA-1 and enterocin A in *L. lactis* IL1403 along with its immunity counterpart. However, the level of expressed bacteriocins was only 5 and 4% of WT in enterocin and pediocin respectively.

Various examples of heterologous expression of pediocin in different hosts is depicted in Table 1.10. Horn *et al.* (1998) reported the heterologous production

of pediocin PA-1 in *L. lactis* IL 1403. Mature pediocin PA-1 encoding gene was cloned in-frame with a leader sequence and expressed under the control of lactococcin A promoter.

Table 1.10: Heterologous expression of pediocin

Native host	Heterologous host	Vector	Reference
Pediocin PA-1 heterologously expressed using ABC dedicated transport system			
Ped. acidilactici PAC1.0	*E. coli* V850	pSRQ11.1	Marugg*et al.* (1992)
Ped. acidilactici PAC1.0	*E. coli* V850	pSRQ11.2	Marugg*et al.* (1992)
Ped. acidilactici LB42-923	*E. coli* JM109	pMBR1.0	Bukhtiyarova *et al.* (1994)
Ped. acidilactici PAC1.0 PPE1.2	*Ped. pentosaceus*	pMG117	Chikindas *et al.* (1995)
Ped. acidilactici PAC1.0	*L. lactis* IL1403	pMC117	Chikindas *et al.* (1995)
Ped. acidilactici PAC1.0	*Lact. sakei* Lb790	pPED1 (pSAK20)	Axelsson *et al.* (1998)
Ped. acidilactici 347	*Lact. lactis* IL 1403	pF12126	Horn *et al.* (1998)
Ped. acidilactici F	*E. coli* DH5α	pPC318	Coderre and Somkuti (1999)
Ped. acidilactici 347	*L. lactis* IL1403	pJM03	Martinez *et al.* (2000)
MBP-pediocin PA1 heterologously produced using general secretary pathway			
Ped. acidilactici LB2 923	*E. coli* E609L	pPR6821	Miller *et al.* (1998)

The lactococcal host was able to secrete pediocin PA-1 but the production level was ~ 25% less than that of the native pediocin producer.

Horn *et al.* (1999) co-produced nisin A and pediocin PA-1 in a nisin producing strain of *L. lactis* F15876. This was one of the first examples for the construction of LAB strains with the ability to co-produce two or more well characterized, wide spectrum bacteriocins belonging to different classes. Axelsson *et al.* (1998) developed an efficient system for heterologous production of the class II bacteriocins such as sakacin P, pediocin PA-1 and pisciolin 61. The system depends on trans activation of sakacin A promoter and also the secretion and processing of prebacteriocins. In case of pediocin PA-1 expression, when the *ped*A and *ped*B genes were expressed in *Lact. sake* under the sakacin A promoter, signal peptide and upstream regulatory elements, the expression was atleast two fold higher than the native bacteriocin producer. Thus, heterologously expressed system offered the advantage of purification from the culture medium of *Lact. sake* Lb790 since this system appeared to be more efficient than that of the original producing strain.

Heterologous expression of pediocin was achieved by adding the signal peptide of Omp (outer membrane protein) of *E. coli* and using its general secretary pathway. Pediocin has been expressed in the periplasmic-space of *E. coli*. Again, using *E. coli* as a leaky host, it was possible to obtain chimeric pediocin in the culture medium (Miller *et al.* 1998). These studies were useful for screening the mutants with altered antimicrobial activity, to understand that *ped*D and *ped*C are not essential for heterologous expression of pediocin in *E. coli* and to prove that pre-pediocin is biologically active.

1.20 Strategies of protein expression in *E. coli* and *in vitro* refolding

Large quantities of proteins intended for biophysical and structural studies are often difficult to obtain from native sources (Makrides, 1996). This is so for the Bacteriocins. Firstly, bacteriocins are low molecular weight peptides and secondly, they are produced at very low levels as secondary metabolites. As discussed in the previous section, many of class II bacteriocin genes are

heterologously expressed in other LAB strains in order to produce bacteriocin in food-grade microorganisms (Axelsson *et al*. 1998; Rodriguez *et al.* 2002a). However, the overall yield has been found to be low. In such cases, production of recombinant protein in *E. coli* is an easy solution for production of these low molecular weight polypeptides and their variants generated by genetic engineering. *E. coli* is the most widely and successfully used host for the production of large amounts of proteins for many biochemical investigations. Construction of fusion proteins through molecular cloning can facilitate their localization, purification, detection with antibodies and many other *in vitro* studies. Fusion proteins are extremely useful especially for expression and single step purification of low molecular weight proteins and peptides (Rogl *et al.* 1998).

The destination, location and state of protein produced by heterologous expression in *E. coli* varies due to the reasons such as the nature of the host strain used, nature of the promoter, growth conditions, origin and nature of the target protein. Proteins expressed in *E. coli* can be targeted to different compartments of the cell: cytoplasm, periplasm, inner membrane, cell surface and/or extracellular medium. Targeting the protein in different cell compartments has certain advantages and disadvantages. Insoluble aggregates in the cytoplasm called inclusion bodies (IBs) are formed when the protein expressed by the target gene reaches high levels of expression (De Bernardez Clark 2001; Patra *et al.* 2000).

Although IBs have often been considered as undesirable, dead end- product of protein expression, their formation can be an advantage as their isolation from the cell homogenate is convenient and their purification is also effective. The recombinant proteins deposited in IBs amount to be about 50% or more of the total cell protein and purity of recombinant protein may reach upto 90%.

Under appropriate conditions, these IBs often exclusively contains the over-expressed protein. Although the proteins in IBs can be inactive and insoluble, they can be an excellent starting juncture for many low molecular weight peptides of LAB origin; provided procedure can be developed to reconstitute them *in vitro* (Lilie *et al.* 1998). Production of recombinant/fusion protein as IBs offers several advantages such as:

1) IBs can be accumulated in the cytoplasm to a greater extent (>25%) than production in a soluble form
2) IBs are initially isolated in a highly purified, solid and concentrated state by simple centrifugation which can lower down the cost of down-stream processing
3) Since IBs do not have biological activity, the toxic proteins can be expressed in an inactive form and
4) As IBs are amorphous aggregates resistant to proteolysis by proteases of *E. coli,* high yields of recombinant protein can be achieved.

The general strategy employed to recover active protein from IBs involves three steps (De Bernardez Clark 2001)

a. Isolation of IBs and washing
b. Solubilization of the aggregated proteins and
c. Refolding of the solubilized protein

Although the efficiency of the first two steps can be relatively high, renaturation yields may be limited by the accumulation of inactive misfolded

and aggregated species. Refolding of the solubilized protein involves removal of the denaturant. The efficiency of refolding depends on the competition between correct folding and aggregation and hence refolding is usually carried out at a low protein concentration of around 10-100 mg ml^{-1} to help eliminate

aggregation. Further, it is necessary to optimize the refolding conditions for each individual protein. These conditions are buffer composition, temperature and additives for disulfide-bond formation. Protein refolding buffer should be supplemented with a redox system. The addition of a mixture of reduced and oxidized forms (1-3 mM reduced thiol and a 5:1 to 1:1 ratio of reduced to oxidized thiol) of low molecular weight thiol reagent provides a better redox condition for reshuffling and formation of disulfide bonds. The most commonly used redox shuffling reagents are reduced/oxidized glutathione, cysteine/cysteamine and β-mercaptoethanol (Lilie *et al*. 1998; Patra *et al*. 2000; Winter *et al*. 2002). There are different methods for refolding of proteins (De Bernardez Clark 1998). These includes:

Dialysis: This method is used for removal of the solubilizing agent used in solubilization of IBs. During dialysis, concentration of solubilizing agent decreases slowly which allows the protein to refold optimally.

Dilution: The concentration of solubilizing agent is decreased by dilution, allowing the protein to refold.

Pulse renaturation: In order to keep the concentration of the unfolded protein low and thus limiting the aggregation, this method is being used.

Chromatography: Different chromatography such as size exclusion, ion exchange and affinity chromatography are being employed to remove solubilizing agents. Refolding by chromatography gives high yields of active protein even at a protein concentration in the mg ml^{-1} range.

1.21 Bacteriocin immunity proteins

Bacteriocin producing LAB protect themselves from the lethal action of its own antimicrobial peptides with its immunity system. Most of bacteriocin biosynthesis operon contain immunity gene. The involvement of an immunity system was first described for colicins in the middle of last century by Frederich. Immunity mechanisms aer highly specific to a given bacterial strain and its associated bacteriocin (Dayem *et al.* 1996; Fimland *et al*. 2002a). Literature

indicates that very few immunity protein of LAB have been studied. Immunity protein directed against lactococin A and carnobacteriocin B2 have been studied in detail. Several immunity proteins of lantibiotics have been characterized and many of them consist of a two component system

a) Membrane associated lipoprotein and
b) ABC transporter

In case of lantibiotic Pep5, only PepI, a membrane integral protein has been associated with immunity function (Pag *et al*. 1999). Dayem *et al.* (1996) showed the localization of the immunity protein of mesentericin Y105 in a cytoplasmic compartment. The detection and localization of immunity protein was carried out by immunofluorescence and electron microscopy using antibodies directed against the C-terminal end of the predicted immunity protein.

McAuliffe *et al.* (2000) identified and overexpressed the LtnI, the immunity protein for two component lantibiotic lacticin 3147. It was found that LtnI encodes a 116 amino acid protein with a predicted membrane location which bears no homology to other bacteriocin immunity proteins. Disruption of the *ltn*I showed complete loss of immunity in the producer cells. By cloning of *ltn*I behind a nisin-inducible promoter, it was seen that level of immunity was dependent on concentration of nisin suggesting the potential use of LtnI as a food-grade selectable marker.

Stein *et al.* (2003) demonstrated the function of *L. lactis* nisin immunity genes *nis*I and *nis*FEG after coordinated expression in a heterologous host, *B. subtilis*. Different combinations of nisin immunity genes were integrated into the chromosome of the nisin sensitive host of *B. subtilis* under the control of an

inducible promoter. When all four genes were coordinately expressed, the highest level of acquired nisin tolerance was achieved. However, expression of only lipoprotein NisI or ABC transporter homologue system NisFEG was able to protect *Bacillus* host to certain extent from the exogeneously added nisin. It was found that protection by NisIFEG was specific to nisin and did not show

tolerance to subtilin. The function of NisFEG is known to transport nisin from the membrane to the culture filtrate. However, NisI which acts as an intercepting protein and can protect the producer cells from lethal action of nisin to a certain extent.

Venema *et al.* (1995) demonstrated the functional analysis of pediocin operon of *Ped. acidilactici* PAC1.0. By deletion analysis and over-expression of *ped*B using strong lactocoocal promoter P32 in *Ped. pentosaceus* they have demonstrated that the gene encodes the pediocin PA1 immunity protein.

Fimland *et al*. (2002) has investigated the functionality of immunity protein in pediocin like bacteriocins. It has been shown that bacteriocin sensitivity has direct correlation with presence of immunity gene along with the production of its own bacteriocin, presence of extra-immunity genes and the properties of membranes and receptors.

1.22 Importance and scope of the present investigation

The review of literature presented above suggests that LAB have prime importance in our daily food habits, both in enriching the nutrient content and in the preservation of food. Lactobacilli, pediococci and enterococci represent the major genera in LAB and have been isolated from various sources. Reports on isolation intestinal LAB are scanty and especially with reference to their characterization and bacteriocinogenic properties. A few reports indicate that LAB have been studied for antibiotic sensitivity in order to understand the spread of antibiotic resistance among food-grade microorganisms (Katla *et al.* 2001; Tynkkynen *et al.* 1998). However, intestinal microflora and fermenting mushroom were found to be unique source for isolation and characterization of LAB in scientific studies and food industrial applications.

Mostly food-borne pathogenic microorganisms are used for the assay of bacteriocins. For example, nisin bioassay is carried out with *Micrococcus luteus* and for pediocin bioassay, *List. monocytogenes* is the choice. Pathogenic bacteria are routinely used due to their clinical importance for study of antibiotic sensitivity. Bacteriocins produced by LAB are known to act against closely related microorganisms. Therefore it is important to develop a native strain of LAB with a bacteriocin and antibiotic assay properties.

Naturally occurring plasmids are known to encode for many metabolic traits and for production of bacteriocin and resistance to it. Plasmid associated traits are deciphered in many LAB especially pediococci. Pediocin encoding plasmids reported in literature are of various sizes ranging from 5 to 30 kb (Graham and Mckay 1985; Huang *et al.* 1996). However, high molecular weight pediocin-encoding plasmids have not been reported yet in literature. Use of gene specific primers for PCR and PCR based probes for dot-blot hybridization using plasmid DNA as a substrate are rapids methods of identifying plasmid linked traits (Bennik *et al.* 1997a; Bhunia *et al.* 1994). Alternatively, these methods are used

for deciphering the function in plasmid cured strains. Utility of different carbon sources for the fermentative production of pediocin has not been tested. Production of considerable amounts of pediocin in sugars like raffinose, mellibiose and lactose is important aspect from an economical point of view, as these sugars are abundantly present in plant based material and in dairy whey. Studies related to curing of high molecular weight plasmid using curing agents such as novobiocin in combination with high temperature have not been reported previously. Pediococcal strains isolated from vegetable, meat sources have been found to be potent pediocin producers. Most *Ped. acidilactici* clearly differ from their *Ped*. *parvulus* and *Ped*. *pentosaceus* counterparts. Intestinal isolate of *Enterococcus* sp., which can tolerate bile salts and can utilize sugars such as maltose, lactose has not been reported previously for production of pediocin. Molecular tools such as rRNA and *tuf* gene sequencing or ribotyping have been found to be reliable methods for the identification of species (Mora *et al.* 2000; Schleifer *et al*. 1995).

There are many reports of pediocin PA-1 production by *Ped. acidilactici*, while, reports of interspecific, intergeneric synthesis of pediocin PA-1 is rare. Production of pediocin-type bacteriocin by *B. coagulans* have been reported (Le Marrec *et al*. 2000). These studies indicate that there has been a natural spread of pediocin producers among the group of LAB and lactic acid producing bacteria. However, to date very few pediocin-type producers have been isolated worldwide.

It is known that pediocin producing LAB grow poorly in milk and milk based products. This is mostly due to their inability to use lactose as a carbon source. Lactose fermenting LAB will find enormous applications for fermentation of dairy products. Evaluating the genetic and biochemical basis for lactose utilization is an important aspect in this area. Use of whey permeate as a food-grade media has an advantage over other complex and expensive media (Biswas *et al.* 1991). Pediocin produced in either MRS or TGE has been applied

against *Listeri*a in many meat products, dairy and vegetable products. Many of such processes and products are covered under patents. Exploration of native strain for the development of such processes may therefore be important.

Pediocin has been heterologously expressed in different LAB, *E. coli* and yeast to produce biologically active proteins. However, the overall yield obtained was marginal. Pediocin production was found to be almost twice higher than in the native strain when *ped*A and *ped*B gene were expressed in *Lact. sake* using the sakacin promoter (Axelsson *et al.* 1998). However, in certain cases bacteriocin producing strains produce more than one bacteriocin which lead to over estimation of activity or in purification to homogeneity. Production of pediocin in heterologous host, which has natural resistance to pediocin, offers an advantage that immunity gene need not be co-expressed. Strong *E. coli* promoters such as P_{T7} have not been employed for high level expression of chimeric pediocin in IBs. The refolding of solubilized protein obtained from IBs, using β-mercaptoethanol as a catalyst, can be a low cost redox system for disulfide-bond proteins to regain their biological activities (Winter *et al.* 2002; Zang *et al.* 1998).

Nucleotide sequence alignment of *ped*B gene from all pediocin producers reported so far showed that *ped*B gene falls in to two groups based on exchange of nucleotide at position 201 (Mora *et al.* 2000b). This provides the basis that pediocin producing strains are widely distributed in nature, which can be confirmed only by nucleotide sequences. Hyper-expression of *ped*B has not been studied previously in *E. coli*. Expression of the protein as a fusion protein has its advantages over its native counterpart since it can be localized and purified easily. Limited information on the functional aspects of *ped*B in Gram-negative organism such as *E. coli* has been reported. The role of the PedB a membrane associated protein, its membrane spanning regions and hydrophobicity remains unexplored.

The present review of literature indicated that pediocin type of bacteriocin has a enormous potential in preservation of food. Limited studies from the same laboratory have been carried out in Indian context on the isolation and characterization of class IIa bacteriocin (Ramesh 2000). Pediocin producers reported so far do not found to have probiotic properties and hence exploitation of such cultures for pediocin production gain prime importance in bacteriocin research. Further, limited studies on production of pediocin in a food-grade media, which can be an important factor for large scale production of pediocin, have bee carried out. In order to study the *in vitro* interaction between pediocin and its immunity protein, it is important to produce both proteins in heterologous system. With this background of the literature presented as above, the objectives of the work presented in this study therefore are:

1. Isolation and identification of bacteriocin producing and indicator strains of lactic acid bacteria
2. Intergeneric pediocin PA-1 production by a native strain of *Enterococcus faecium* PH-1
3. Production of pediocin PA-1 for biopreservation and,
4. Molecular cloning and expression of *ped*A and *ped*B genes in *E. coli*

CHAPTER II

ISOLATION AND IDENTIFICATION OF BACTERIOCIN PRODUCING AND INDICATOR STRAINS OF LACTIC ACID BACTERIA

2.0 ABSTRACT

Lactic acid producing bacteria (LAB) isolated from fowl, fish intestine and mushroom have been identified and characterized with reference to the production of bacteriocins and their genetic markers. Among the isolates, two strains each of *Lactobacillus casei* ssp. *casei* C40 and M50, *Pediococcus pentosaceus* C6 and M10, and one isolate each of *Pediococcus acidilactici* F58, *Pediococcus* sp. F7 and *Enterococcus faecium* C20 (renumbered as PH-1) were found to be potent producers of bacteriocin active against standard cultures viz. *Lact. casei* NRRL B1922, *Leuc. mesenteroides* NRRL B640, *Ped. acidilactici* NRRL B1153 and related strains of LAB isolated from the same environment as were the bacteriocin producer belonging to the genera of *Lactobacillus* and *Pediococcus*. The potent cultures appeared to posses either one or two plasmids of 4 –8 kb and a megaplasmid. Curing of the 5.5 and 8 kb plasmids in the strain of *Lact. casei* ssp. *casei* C40 resulted in the loss of bacteriocin production and in altered pattern of sugar utilization. Antibiograms of bacteriocinogenic cultures of LAB and bacteriocin indicators were carried out to determine their genetic markers. Bacteriocinogenic cultures were resistant to most antibiotics, particularly those belonging to the □-lactam and cephalosporin groups. Besides, most of these cultures were sensitive to 30 mg of chloramphenicol and 15 mg of erythromycin.

A strain of *Enterococcus faecium* PH-1 (C20) that could hydrolyse many sugars including raffinose, maltose, melibiose and lactose, grow in bile salt and had a broad antimicrobial spectra was isolated. The strain was further characterized in detail for its unique features of γ-hemolysis, growth in bile esculin agar, growth in sodium azide, negative reaction to the MRVP test and sensitivity to vancomycin. The strain PH-1 was differentiated from pediococci due to its diplo-coccoid nature, tolerance to exposure at 60^0C for 30min, growth in the pH range of 4.5 to 9.6 and tolerance of up to 10% salt in the medium.

A native strain of *Lactobacillus farciminis* MD, isolated as an indicator strain from fermenting mushroom, exhibited a high degree of sensitivity to most of the bacteriocins produced by strains of lactobacilli, leuconostoc and pediococci. The sensitivity of *Lact. farciminis* MD to many antibiotics was established using different antibiotics including ampicillin, cefazoline, chloramphenicol and nitrofurantoin at a concentration of 30 mg each, producing an inhibition zone 30 mm diameter in all cases. The sensitivity of *Lact. farciminis* MD towards bacteriocins and antibiotics was made use of when the bacteria was used as an indicator strain.

2.1 INTRODUCTION

Lactic acid bacteria (LAB), a group of food-grade microorganisms, have tremendous potential applications in food industry and human health including preservation of foods. These bacteria are not only involved in food fermentation but also impart certain changes in structure and texture of food and add various vitamins and thus increases the nutritive value of food. LAB have often been isolated from varieties of natural sources for several scientific and commercial purposes (Abee *et al.* 1995; Axelsson 1993; Berg van den Dick *et al.* 1993). LAB are industrially employed chiefly for the production of fermented foods, organic acids, exopolysaccharides, antimicrobial peptides and probiotics formulations (Konings *et al.* 2000; Leroy and De Vuyst 2004).

Nutritional requirements of LAB are often fastidious and therefore it is essential to supplement fermentable carbohydrates with various vitamins and amino acids. However, they are common inhabitants in many plant materials as saprophytes and are also found in fermenting meat (Stiles and Holzapfel 1997). LAB exert a strong antagonistic activity against many microorganisms including food spoilage organisms and pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* etc. Production of the primary metabolite viz lactic and acetic acid results in decrease in pH is one of the main preservation factor in food fermentation. Besides, many LAB are known to produce antimicrobial peptides commonly knows as bacteriocins (Helander *et al.* 1997; Jack *et al.* 1995; Klaenhammer 1993).

Recently there has been an increasing consumer demand for natural food additives since many of the commonly used chemical preservatives are carcinogenic and mutagenic. Nisin (***N***-***S***treptococci ***in***hibition) produced by *Lactococcus lactis,* is one such bacteriocin that has been exploited by over 45 countries for preservation of canned foods and dairy products and has been accepted by WHO as a food biopreservative (Delves-Broughton *et al.* 1996; Hurst 1981). Nisin exhibits a broad spectrum of antimicrobial activity and display inhibition against many spore-formers and Gram-positive bacteria. However, many of other LAB derived bacteriocins are known to be active against closely-related organisms, which suggests that isolation of new bacteriocin producers is possible by using closely related LAB as an indicator.

Previously, it has been reported that many specific bacteriocin producers can be isolated from different environments. This indicates that either genetic determinants for the biosynthesis of some bacteriocins or the producers are wide-spread. For ex. Lactocin S producing lactobacilli and pediocin PA-1 producing pediococci grow in a different environmental niche and/or there is an apparent horizontal gene transfer among the genera (Ennahar *et al.* 1996; Rodriguez *et al.* 1995).

Conventional methods for identification of LAB involves isolation on selective media such as MRS (de Man *et al.* 1960) and examination of pure culture using several criteria such as morphology, biochemistry, substrate assimilation and product formation as given in the Bergey's manual (Sneath *et al.*1986). Since conventional methods are time consuming and are some times ambiguous, newer methods have come to the fore. DNA based methods such as PCR, colony hybridization, dot-blot etc are the choice due to their simplicity and reliability (Rodriguez *et al.* 1995; 1997; 1998). Analysis of the total number of resident plasmids by plasmid profiling and subsequent curing to demonstrate the plasmid linked characters is a common technique employed for detection of

bacteriocin encoding plasmids (Ruiz-Barba *et al.*1991). Antibiotic resistance markers are also important genetic tools that can be used for strain differentiation. Antibiotic resistance has a special reference with regard to vancomycin resistance as seen in the case of enterococci and lactobacilli (Cebeci and Guerakan 2003; Sozzi and Smiley 1980; De Vuyst *et al.* 2003).

Numerous strains of lactobacilli, enterococci and pediococci are known to produce class II bacteriocins and are involved in fermentation of various vegetables and meat products (Ennahar *et al.* 1999; 2000; Nes *et al*. 2002). Antilisterial bacteriocin such as pediocin have enormous potential application since pediocin producing strains have a wider adaptability due to their diverse origin (Bennik *et al.* 1997a; Rodriguez *et al*. 2002b).

Bacteriocin production among strains of LAB has been established primarily by carrying out assays against closely related LAB and a few important spoilage and pathogenic bacteria. The degree of inhibitory activity is dependent on the nature of the indicator organism (Bennik *et al.* 1997b; Rogers and Montville 1991). Similarly antibiotic sensitivity assays are performed against organisms such as *B. Stearothermophillus*, *B. subtilis*, *E. coli* (Quesada *et al.* 1996),

Klebsiella pneumoniae and *Mycobacterium smegmatis*. The potentiality of using a similar type of indicator organism for both bacteriocin and antibiotic assays, particularly among the group of LAB, will enhance the degree of sensitivity and facilitate antibiotic assays. This is in contrast to attempts made to increase sensitivity of the assays themselves as by enhancing diffusion through agar (Hurst 1981).

The present literature suggests that, work on isolation of bacteriocinogenic LAB from intestinal sources has been limited (Barnes 1979; Jin *et al*. 1998; Juven *et al.* 1991; Mauguin and Novel 1994). Further, there are no reports on the isolation of LAB associated with mushroom fermentation.

This chapter deals with the isolation and characterization of LAB populations from fowl and fish intestine and from mushroom fermentation to delineate bacteriocin producing isolates and strains sensitive to bacteriocin (bacteriocin indicators). These strains have also been characterized with regard to plasmids they bear and the antibiotics to which they are sensitive to. A native isolate of *Lactobacillus farciminis* MD was characterized by its high sensitivity to both, bacteriocins and to antibiotics. The use of this organism as an Indicator of bacteriocin activity is described.

2.2 MATERIALS AND METHODS

2.2.1. Materials

2.2.1.1 Fine chemicals

Agarose, bromo cresol purpule (BCP), sodium azide and antibiotics such as Cycloheximide, Cycloserine, Erythromycin, Novobiocin and sugars, sugars-discs, sterile discs, antibiotics discs and potassium tellurite were purchased from HiMedia (Mumbai; India). Nisin was from ICN Biomedicals Inc. (CA, USA), Ampicillin, Trypsin and Lysozyme were procured from Sigma Chemicals (St. Louis, MO, USA). Soluble starch and Hydrogen peroxide were obtained from Sd. Fine Chemicals

(Mumbai, India). Glutarldehyde was from Kodak (USA). Ethidium bromide and all other reagents for Molecular Biology work were from Sigma (USA). Antibiotic sensitivity ring was obtained from Dynamicro, Thane (India).

2.2.1.2 Bacterial strains

Standard bacterial strains used in this study were obtained from various culture collections and from different laboratories as enlisted here. Bacterial strains such as *Lactobacillus amylovorous* NRRL B4552, *Lact. casei* NRRL B1922, *Leuc. mesenteroides* NRRL B640, *Ped. acidilactici* NRRL B1153, B1325, B5627 and *Ped. pentosaceus* NRRL B11425 were obtained through the courtesy of Dr. L. K. Nakamura, Northern Regional Research Laboratory, Peoria, IL. USA. *Pediococcus parvulus* ATO77 was received as a gift from Dr. M. H. J. Bennik, ATO-DLO Centre, The Netherlands. A strain of *Lact. buchneri* NCIM 2357, *Lact. plantarum* NCIM 2083 and *Lact. viridescens* NCIM 2165 were received from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. A strain of *Lact. maltaromicus* MTCC 108 was received from Microbial Type Culture Collection and GenBank, IMTECH; Chandigarh. *Lactococcus lactis* ssp. *lactis* (nisin producer) was received from NDRI; Karnal, Haryana. *Pediococcus acidilactici* K7, K3 (Ramesh 2000) and other native strains mentioned in Table 2.1 and 2.11 were laboratory isolates.

The pathogenic bacterial strain of *Bacillus cereus* F4433 was a gift from Dr. J. M. Kramer, Central Public Health Laboratory, United Kingdom. *Escherichia coli* D21 was from Dr. M. A. Linggood, Unilever Research UK and *Staphylococcus aureus* FRI 722 was received from Dr. E. Notermans, National Institute of Public Health, The Netherlands.

2.2.1.3 Experimental samples

Intestine sample from freshly dissected fowl, *Gallus domestica* broiler of age 10 weeks, rohu fish, *Labeo rohita* and mushroom *Agaricus* spp. were procured from the local market and processed immediately for the isolation of LAB.

2.2.1.4 Microbiological media

All microbiological media were purchased from HiMedia and were sterilized by autoclaving at 121^0C (15psi) for 15-20min. For the isolation and cultivation of LAB, either the de Man, Rogosa and Sharpe (MRS) or the M_{17} media was used. These media consists of-

i) MRS: 10gm each of proteose peptone and beef extract; 5 gm each of yeast extract and sodium acetate; 20 gm dextrose; 1 gm poly sorbate-80; 2 gm each of ammonium citrate and dipotassium phosphate; 0.1 gm magnesium sulphate and 0.05 gm of manganese sulphate; dissolved in distilled water and made up to a final volume of 1000 ml. The pH of the media was adjusted to 6.5 (±0.1) with NaOH.

ii) M_{17} broth: 2.5 gm each of casein enzymic hydrolysate, papaic digest of animal tissue and yeast extract; 5.0 gm each of papaic digest of soyabean meal, beef extract and lactose. 0.5 gm of ascorbic acid. 0.25 gm of magnesium sulphate and 19 gm of disodium-b-glycerophosphate, the final pH 7.5 ± 0.2.

Pathogenic bacterial strains were cultivated in Nutrient broth (NB) or Brain heart infusion (BHI) broth

iii) Nutrient broth: Peptone, 5.0 gm; yeast and beef extract 1.5 gm each and sodium chloride, 5.0 gm $litre^{-1}$. The pH of the medium was adjusted to 7.4±0.2;

MRS, M_{17}, NB agar media were prepared by addition of 1.5% bacteriological agar and was sterilized by autoclaving.

iv) BHI broth: Brain infusion from calf, 200 gm; heart infusion from calf, 250 gm; protease peptone 10 gm; sodium chloride 5 gm; disodium phosphate 2.5 gm and dextrose 2 gm litre^{-1}. The pH of the medium was adjusted to 7.4.

For performing specific identification tests, various selective media were used and these media components were also obtained from HiMedia and include:

v) Arginine hydrolysis broth: Per liter it consists of tryptone 1 gm; yeast extract 0.1 gm; 0.3 gm each of beef extract, dipotassium hydrogen phospahate and L-arginine hydrochloride along with bromothymol blue (indicator dye) 0.04 gm, pH 6.6± 0.2.

vi) Simon citrate agar: Per liter it consists of magnesium sulphate 0.2 gm; ammonium dihydrogen phosphate 1.0 gm, dipotassium phosphate 1.0 gm; sodium citrate 2.0 gm; sodium chloride 5.0 gm; agar 15.0 gm and bromothymol blue 0.08 gm, pH 6.8± 0.2.

vii) Bile salt agar: consists of MRS agar 6.2 gm incorporated with 0.3% of sodium salt of OXgall.

Viii) MRS starch agar: MRS starch agar was prepared by addition of 1% starch solution to MRS agar medium. Starch and MRS agar were sterilized individually and mixed just before pouring the plates.

ix) Carbohydrate fermentation test medium: the basal medium of MRS broth with 2X concentration (devoid of glucose and beef extract) was reconstituted by incorporating BCP (0.04 gm litre^{-1}) and was supplemented with 1% each sugar solution, individually (described in section 2.2.2.4).

x) Skim milk and milk medium: skim milk (purchased from local market) medium was prepared by dissolving 10 gm of skim milk powder in 100 ml distilled water and steamed in a pressure cooker for 30min. For the preparation of milk medium, fresh market milk was used and also sterilized by steaming.

xi) MRS Gelatin agar medium: MRS agar 6.8 gm was prepared separately and mixed with 12 gm of gelatin per 100 ml, which was weighed and melted in boiling water bath.

xii) Potassium tellurite reduction test medium:, 0.04% of potassium tellurite (1% filter sterilized stock) was added to 10 ml of autoclaved MRS broth.

xiii) Homofermentative test: A Durham tube was placed inverted into 10ml of MRS broth in a tube and media was sterilized by autoclaving.

xiv) Blood agar medium: Sterilized nutrient broth agar was mixed with 7% of human blood and poured into petriplates.

xv) MRVP medium: Methyl red Voges-Proskauer (MRVP) medium consisting of 5 gm each of glucose, Proteose peptone and K_2HPO_4 was prepared and sterilized by autoclaving.

xvi) Bile esculin agar: Beef extract 3.0 gm, Peptone 5.0 gm, Oxgall 40gm, Esculin 1.0 gm, ferric citrate 0.5 gm and agar 15 gm Final pH 6.6± 0.2. The medium was sterilized by autoclaving and plates were prepared.

xvii) Sodium azide medium: Filter sterilized sodium azide was added to sterilized NB @ 0.04% .

2.2.2 Methodology

2.2.2.1 Maintenance of the cultures

All the LAB were cultivated and maintained in MRS broth, except *L. lactis* ssp. *lactis* which was cultivated in M_{17} broth, with repeated subculturing every 15 days. The cultures were maintained at 4^0C in MRS soft agar (0.8% agar). For

long term storage, cultures were frozen at –20^{0}C in 10% glycerol along with 10% skim milk medium.

The pathogenic bacterial strains were grown in BHI broth and sub-cultured in 15d intervals. The cultures were maintained at 4^{0}C in BHI soft agar and for long term storage, they were stored in 50% each BHI and glycerol at –20^{0}C .

2.2.2.2 Preparation of antibiotic solutions

Ampicillin, 100 mg ml^{-1}; cycloheximide, 10 mg ml^{-1}; cycloserine 10 mg ml^{-1}; novobiocin 10 mg ml^{-1} were dissolved in sterile distilled water. Erythromycin 10 mg ml^{-1} was dissolved in absolute ethanol. All the stocks of antibiotics were sterilized by using 0.22 m filter (Millipore, India) and stored at 4^{0}C.

2.2.2.3 Preparation of nisin stock

Nisin standard was prepared by dissolving 0.1g of nisin in 10 ml of 0.02 mol l^{-1} HCl, 0.75% NaCl and the pH was adjusted to 5.3. The mixture was centrifuged at 10, 000rpm for 10 min at room temperature and the supernatant sterilized through a 0.22 m membrane filter and stored at -20^{O}C in sterile screw-capped tubes. This stock of nisin corresponded to 10,000 International Unit per ml (IU ml^{-1}).

2.2.2.4 Preparation of sugar solutions

A 10% stock of different sugars (referred in table 2.2, 2.3 and 2.8) was prepared by dissolving 10gm of sugar in 100ml of sterile water, filter sterilized and stored at 4^{0}C.

2.2.2.5 Isolation of bacteriocinogenic and bacteriocin sensitive LAB

The vertically dissected Intestinal (caecal content of the fowl and alimentary canal of the fish) samples and mushroom samples were added to MRS broth and incubated for enrichment of resident LAB, for around 2h. The enriched broth was serially diluted in saline (0.8%) and pour-plated on MRS agar supplemented with 200 mg ml^{-1} of cyclohexamide. The plates were incubated at 37^{0}C for 16h

until colonies appeared. The bacterial colonies that were very small in size were presumed to indicate inhibition by adjacent larger (prominent) colonies which were tested for their ability to produce bacteriocins against the local isolates (purified small colonies) and standard lactic cultures as shown in Table 2.1. Thus the smaller colonies were tested for their susceptibility to bacteriocins as "indicators".

2.2.2.6 Identification of the native isolates

The bacterial strains which were selected as potential bacteriocin producers and indicator of bacteriocin production were subjected to morphological, cultural and biochemical characterization and identified according to Bergey's manual of Determinative Bacteriology and the methods given by Sneath *et al.* (1986), Stiles and Holzapfel (1997) and Mundt (1986). The methods include the Gram's staining, test for catalase, luxuriant growth on MRS agar, CO_2 production from glucose in Durham tubes, growth in different sugars and salt concentrations, growth at different temperatures, in bile salt, hydrolysis of arginine and utilization of citrate, hemolysis in blood agar, methyl red and VP reaction, growth in sodium azide, etc. The procedures described for various tests are as follows:

2.2.2.6.1 Grams staining and motility

A loopful of freshly grown test culture was smeared onto a clean glass slides, heat fixed and stained with crystal violet (HiMedia) (crystal violet 2.0 g, ethyl alcohol 20 ml, Ammonium oxalate 0.8 gm in 80ml of distilled water) for 1min. Excess of stain was washed with water and Lugol's iodine (Mordant), (iodine 1 gm, potassium iodide 2 gm in 300 ml distilled water) solution was added and allowed to react for 1min. The slide was washed with water and the smear was rinsed first with ethanol to remove excess of crystal violet and then with water. The slide was counter stained with safranin for 30sec followed by washing with water. It was then air dried and examined with an oil immersion

objective of a microscope. Colour and morphology of the cells were recorded. Motility was recorded on a cavity slide using the hanging drop technique.

2.2.2.6.2 Catalase reaction

Test cultures were grown in MRS broth for 15h and a loopful of it was taken on a glass slide. A drop of 3% H_2O_2 (prepared from 30% stock by diluting in distilled water) was added and allowed to react with the cells. Emission of effervescence was recorded as catalase positive and absence as catalase negative. *Bacillus* culture was used as a positive control and standard LAB B640 was used as a negative control.

2.2.2.6.3 Ammonia production from Arginine hydrolysis

Arginine hydrolase broth in tubes were prepared and inoculated with 100ml of 15h broth culture of the test strains, mixed well and kept for incubation in static condition at 37^0C for 24h. Production of ammonia was monitored by the change in the colour of the medium reflected by the indicator dye.

2.2.2.6.4 Growth at different temperatures

MRS broth (5 ml in test tubes) was inoculated with 100 ml of 15h old test culture. The inoculated tubes were grown at different temperature viz 10, 37 and 50^0C and change in the turbidity was observed visually after 24-48h.

2.2.2.6.5 Citrate utilization tests

Simmon's citrate agar tubes were inoculated with test cultures and incubated at 37^0C for 24-48h. The tubes were observed for colour change in the medium due to citrate utilization.

2.2.2.6.6 Starch hydrolysis test

A loopful of freshly grown test culture was streaked on a MRS starch agar plate and incubated at 37^0C for 24h. After growth, the plates were flooded with

drops of iodine and observed for clear zone around the colony. *B. subtilis* culture was used as the positive control.

2.2.2.6.7 Heat tolerance test

The test culture was grown in MRS broth for 15h and the cells were pelleted by centrifugation at 10, 000 rpm for 10min. The cell pellet was suspended in 5 ml of sterile peptone water (peptone 1.0 gm, NaCl 5.0 gm litre^{-1} pH 7.0). Aliquots of 1 ml were taken in sterile glass tubes and subjected to heat in a thermostatically controlled water bath (Newtronic, India) at 60 and 70^{0}C for 15min and 60^{0}C for 30min. The contents were transferred into 5 ml of MRS broth and observed for growth (change in turbidity) after incubation at 37^{0}C for 24-48h. The results were documented as: growth (+) or and no growth (-).

2.2.2.6.8 Growth in different pH

MRS broth with different pH viz 4.4, 5.0 and 9.6 was prepared and distributed into test tubes and sterilized. These tubes were inoculated with 100ml of 15h grown test cultures and incubated under static condition at 37^{0}C for 24-48h. Growth as indicated by change in turbidity of medium was observed.

2.2.2.6.9 Growth at different salt concentration

MRS broth containing sodium chloride of different concentrations viz. 2, 4, 6.5 and 10% was prepared and inoculated with 100 ml of test cultures and incubated at 37^{0}C for 24-48h. The presence of turbidity was taken as a measure of growth.

2.2.2.6.10 Carbohydrate fermentation test

The test cultures were inoculated into the carbohydrate fermentation medium and incubated at 37^{0}C for 24-48h. The change in colour of the indicator dye from purple-violet to yellow due to acid production was recorded and classified as indication of growth (+) and of no growth (-).

2.2.2.6.11 Growth in skim milk

The test culture was inoculated into skim milk medium and incubated at 37^0C for 24-48h. Curdling of milk was considered a positive reaction (+) as growth and no curdling as a negative (-) reaction.

2.2.2.6.12 Potassium tellurite reduction

Reduction of the potassium tellurite resulting in blackening of the medium was recorded visually.

2.2.2.6.13 Bile salt tolerance

Bile salt medium was inoculated with the test culture and incubated at 37^0C for 24 and growth was recorded.

2.2.2.6.14 CO_2 production from glucose

The medium was inoculated with the test culture and after 24h of growth, accumulation of CO_2 at the tip of Durham tube was recorded. Heterofermentative cultures produce CO_2 while homofermentative cultures do not.

2.2.2.6.15 Hemolysis

The test culture was pour-plated on NB blood agar plates and incubated at 37^0C for 24h and the presence and type of clearance zone around the colony was observed (clear zone, □□ fuzzy zone, □ and no zone, γ hemolysis).

2.2.2.6.16 MRVP test

The test cultures were grown in MRVP medium and subjected for the MRVP test. Change in color of the medium from pink to red indicates the presence of acetyl-methylcarbinol. The presence of acid was tested by adding few drops of an alcoholic solution of methyl rest to the culture. Production of a distinct red

colour was taken as positive for acid while yellowing of the medium indicated absence of acid.

2.2.2.6.17 Bile esculin agar

The test culture was pour-plated in bile esculin agar and incubated at 37^0C for 24h. Growth in bile salt was observed in ferric reduction by change in colour around the colony to black.

2.2.2.6.18: Acid production in milk

The milk medium was inoculated with the test culture and the acid production was recorded by drop in pH using a pH meter.

2.2.2.6.19: Growth in 0.04% sodium azide

The medium was inoculated with the test culture and turbidity was recorded.

2.2.2.7 Bacteriocin assay

2.2.2.7.1 Agar well diffusion assay

The method described by Geis *et al.* (1983), for testing the presence of bacteriocins in the culture filtrate (CF) of potential bacteriocinogenic strains, was followed with certain modifications

- All the test cultures were grown in MRS broth at 37^0C for 15-20h. The cells were separated by centrifugation at 10, 000 rpm at 4^0C for 10min.
- The CF was neutralized by the addition of 6 N NaOH to pH 6.5 and was heated in a boiling water bath for 10min followed by filter sterilization with a 0.22 m filter and stored at -20^0C till further use.
- The assay plates were prepared with a bottom layer of MRS agar for LAB and NA for pathogenic bacteria in which requisite number of holes of 6mm diameter were punched with a suction borer.
- The test CF (40 ml) was loaded in each of the numbered holes and the plates were placed at 4^0C for 2-3h.

- The CF diffused plates were dried at 37^0C for 20-30min and overlaid with either MRS or BHI soft agar seeded with freshly grown (10^6 cells ml^{-1}) indicator strain (10 ml) as listed in Table 2.1
- The overlaid plates were incubated at 37^0C for 15-20h and examined for zone of inhibition around the wells.
- An inhibitory zone of 8 mm greater in diameter was considered positive.

2.2.2.7.2 Proteolytic inactivation of CF

The CF of test strain was subjected to proteolytic inactivation. Trypsin (10 ml :0.4% stock prepared in distilled water) was mixed with 250 ml of CF. The reaction was incubated for 1h at 37^0C and assayed (40 ml) for antimicrobial activity as described in section 2.2.2.7.1. The results were compared with the antimicrobial activity of the CF treated with trypsin.

2.2.2.7.3 Sensitivity of bacteriocin indicators

The Sensitivity of *Ped. pentosaceus* C6 and *Lact. farciminis* MD to the CF and trypsin treated CF from different LAB (listed in Table 2.9) was determined by the method described in **2.2.2.7.1** and **2.2.2.7.2**.

2.2.2.7.4 Agar disc assay

Sensitivity of indicator strain C6 and strain MD to commercial nisin and other antibiotics to *Lact. farciminis* MD was determined by using sterile 6 mm filter discs in an agar disc assay (Bhunia *et al*. 1987).

2.2.2.8 DNA isolation

2.2.2.8.1 Plasmid DNA isolation

Native plasmids from LAB were isolated according to the method described by Anderson and Mckay (1983) with certain modifications. All the reagents were prepared in deionised distilled water and sterilized by autoclaving. Following are the steps involved for plasmid DNA isolation:

- The test strains were cultivated in 5 ml of MRS broth at 37^0C for 10h.
- The cells were harvested by centrifugation at 10, 000 g for 10min in 1.5 ml eppendorf tubes. The cell pellet was washed with 200 ml of 10% sucrose solution, suspended in 400 ml of 6.7% sucrose, 50mM Tris, 1mM EDTA (pH 8.0) and then warmed at 37^0C for 10min.
- Cell wall was degraded by adding lysozyme (1 mg ml^{-1}). The reaction was carried out at 37^0C for 20-30min.
- Activity of the lysozyme was terminated by adding 50 ml of 0.25 M EDTA and 50 mM Tris pH 8.0.
- Cell lysis was achieved with the addition of SDS 20% (w/v) prepared in 50 mM Tris, 20 mM EDTA followed by further incubation at 37^0C for 20 min.
- The mixture was vortexed at the highest setting for 30sec.
- 3 N NaOH (30 ml) of was added and gently mixed by intermittent inversion for 10min.

The pH of the mixture was neutralized by adding 50 ml of 2 M Tris HCL (pH 7.0) and mixed gently for 3-5min.

The mixture was equilibrated with 75 ml of 5 M NaCl and kept in 4^0C for ½h and was extracted with phenol (700 ml) saturated with 3% NaCl, emulsified by vortexing and the phenol phase was separated by centrifugation at 10, 000 rpm for 15min.

The upper phase was removed separately and was further extracted with chloroform: isoamyl alcohol (24:1) by vortexing followed by centrifugation at 10, 000 rpm for 15min.

The plasmid DNA was precipitated by 1 volume of isopropanol followed by incubation at –20^0C for about 1h. The DNA was pelleted by centrifugation at 10, 000 rpm for 15min at 4^0C followed by washing with 70% ethanol and traces of ethanol evaporated by air-drying.

The DNA was dissolved in 20 ml of TE (10 mM Tris, 1 mM EDTA; pH 8.0).

The DNA samples were mixed with 1X conc. of loading dye (6X loading dye consists of 0.25% each of bromophenol blue and xylene cyanol and 30% glycerol) and were heated at 70^0C in water bath for ½h prior to loading into the agarose gel. Separations were carried out on agarose 1% gels as described by Sambrook and Russell (2001) using 1X TAE buffer (50X TAE stock: 24.2 g of Tris base, 5.71 ml glacial acetic acid and 10 ml of 0.5M EDTA per 100ml). □ DNA *Eco* RI /*Hin*dIII double digest (Bangalore Genei, India) was used as a standard molecular size marker. Electrophoresis was carried out at 100 Volts (3.5 V/cm) for 3-4h. Gels were stained with 0.5 mg ml^{-1} of ethidium bromide and observed under a UV transilluminator (Photodyne USA). The results were documented using a Gel documentation system (Hero Lab, Germany).

2.2.2.8.2 Plasmid curing

Novobiocin was used for plasmid curing of the strain *Lact. casei* ssp. *casei* C40. The procedure of Ruiz-Barba *et al.* (1991) was followed for plasmid curing with certain modifications. The test culture was grown in MRS broth containing different concentrations of novobiocin (0.12 to 8 mg ml^{-1}). After 24h of growth, the cells from an appropriate concentration of novobiocin (wherein ~90% lethality was visually observed) was taken, pour-plated in MRS agar and incubated at 37^0C for 24-48h. The plates consisting well separated colonies were overlaid with the indicator strain of C6 and zone of inhibition observed. The colonies with no zones of inhibition were considered putatively as plasmid cured colonies. Such cured colonies were separated aseptically, purified and were tested for their ability to produce bacteriocin against strain C6. The colonies was also analyzed for concomitant plasmid loss by agarose gel electrophoresis, sugar utilization profile and sensitivity to antibiotic using the methods described by Ray *et al.* (1989) and Ruiz-Barba *et al.* (1991).

2.2.2.8.3 Genomic DNA isolation

LAB genomic DNA was isolated according to the method of Lewington *et al.* (1987) with certain modifications.

The stationary phase culture of test strains grown in MRS broth was harvested by centrifugation in a 1.5 ml eppendorf tube.

The cell pellet was resuspended in 200 ml of 0.25 M sucrose, 50 mM Tris HCl (pH8.0) and vortexed briefly.

The 50 ml of 5 mg ml^{-1} lysozyme (prepared in TE buffer) was added and the tubes were incubated at 37^0C for 10-20 min.

The cells were lysed by adding 100 ml of 20% SDS (w/v) prepared in TE buffer and rocked gently until complete cell lysis.

Cold sodium chloride (50 ml- 5 M) was added and mixed thoroughly and the tubes were placed on ice for at least 1h.

Cell debris were removed by centrifugation at 10, 000 for 10min at 4^0C.

The supernatant was transferred into a fresh tube and extracted with chloroform :isoamyl alcohol (24:1).

DNA was precipitated on addition of absolute ethanol (0.4 ml) and $^1/_{10}$ volume of 3 M sodium acetate and incubation at -20^0C for 1h.

DNA was recovered by centrifugation at 10, 000 rpm 4^0C for 15min followed by removal of excess of salt by washing with 70% ethanol.

The DNA pellet was air-dried and dissolved in 20 ml of TE buffer.

The quality and quantity of the chromosomal DNA was analyzed by agarose gel electrophoresis.

2.2.2.9 Antibiotic sensitivity test

Antibiotic sensitivity rings containing twenty different antibiotics (shown in Table 2.4; 2.5; 2.7 and Figure 2.4) was used for studying the response of native isolates to the antibiotics.

Molten MRS agar medium was poured in 180 mm diameter glass petriplates.

The media was allowed to set and was then overlaid with 10 ml of MRS soft agar seeded with early log-phase (10^6 cells ml^{-1}) test culture (10 ml).

The Antibiotic sensitivity ring was placed in the center of the petriplate facing upward.

These plates were incubated at 4^0C for 2h for the antibiotics to diffuse into the medium and then at 37^0C for 12h. Similar procedures was followed for the antibiotic disc assay.

The diameter of zone of inhibition around each antibiotic disc was measured and the results recorded according to the rating chart and the manufacturer's instructions. The strains were classified as resistant (R), sensitive (S) and moderately resistant (M).

2.2.2.10 Relative sensitivity and MIC

The relative sensitivity (R.S.) of *Lact. farciminis* MD against bacteriocins produced by *Enterococcus faecium* C20, *Lact. casei* ssp. *casei* C40, *Ped. parvulus* ATO 77 and *L. lactis*. ssp. *lactis* was determined. These bacteriocin producing strains were grown in MRS broth, except for *L. lactis* ssp. *lactis* which was grown in M_{17} broth, at 30^0C for 24h. The CF was separated by centrifugation and pH was adjusted to 5.5 and assayed for bacteriocin activity as described in section 2.2.2.7.4. Bacteriocin activity was expressed as Arbitrary Unit (AU) ml^{-1}, which was defined as the highest dilution of the CF that produced a zone of inhibition of 8 mm. The relative sensitivity of strain MD was determined based on the bacteriocin that causing the greatest zone of inhibition.

The MIC of erythromycin, ampicillin, cycloserine and nisin for each strain was determined by exposing the strain to various concentrations of the filter sterilized antibiotic. The MIC for each antibiotic was defined as the lowest concentration (mg ml^{-1}) of antibiotic that can suppress growth of the indicator strain *Lact. farciminis* MD completely. The international unit (IU) activity of nisin was converted to mg ml^{-1}.

2.2.2.11 Scanning Electron Microscopy (SEM)

The protocol described by McDougall *et al.* (1994) was followed with certain modifications (Ramesh 2000).

2.2.2.11.1 Sample preparation

1. The samples to be scanned were collected in eppendorf tubes as pellets.
2. The pellets were washed and centrifuged twice with 1X PBS Buffer (KCl, 0.2 gm; NaCl, 8.0 gm; KH_2PO_4, 0.2 gm and Na_2HPO_4, 1.15 gm $litre^{-1}$ pH 7.4) at 1000 rpm for 10 minutes.
3. The cells were fixed in 2% glutraldehyde
4. The cells were then dried in an ethanol gradient varying from 10-100%.
5. Finally, the cells were washed with methanol for dehydration.

2.2.2.11.2 Loading onto the SEM

1. A carbon-coated membrane was placed on an aluminum disk and the sample was smeared on the lining.
2. An inert metal gold was coated on the sample using a sputter coater (Poluron Sputter coat system, Model 5001, England). The coated sample was taken for viewing in the SEM (Leo Electron Microscopy Ltd., U. K.).

2.3 RESULTS AND DISCUSSION

2.3.1 Antimicrobial properties of native strains

In a random screening process, a total of 650 lactic acid bacterial isolates were tested for their ability to produce bacteriocin. Three isolates from fowl intestine, as indicated by the letter 'C'; two from fish intestinal source as indicated by the letter 'F' and three isolate from fermenting mushroom indicated by the letter 'M' were bacteriocinogenic when tested against the indicator strains as shown in Table 2.1. In general all the isolates were more antagonistic to the local isolate than to the standard lactic cultures. The CF from strain C20 showed the zone of growth inhibition against *Lact casei* NRRL V1922, *Leuc. mesenteroides* NRRL B640, *Ped. acidilactici* NRRL B1153. Further, strain C20 inhibited the growth of many local isolates when tested in an agar well diffusion assay. Strain *Lact. maltaromicus* MTCC 108 was sensitive to only C6 where as *Lact. casei* NRRL B1922 was sensitive to all the fowl intestinal isolates. Strain C6 was found to act as a good indicator for the bacteriocins of C20, C40 and M10 (data not shown). The mushroom isolate M31, M50 and M10 failed to inhibit the growth of the standard cultures. None of the isolates, except strain C20, were able to inhibit the growth of pathogenic bacteria such as *Bacillus cereus*, *E. coli* and *Staph. aureus*. The zone of inhibition produced by the CF from various isolates against *Lact. casei* NRRL B1922 is shown in Figure 2.1. The CF, that had been heated in a boiling water bath for 15min to remove H_2O_2 and neutralized with NaOH to eliminate acid, exhibited strong inhibitory activity, No inhibition of the growth of the indicator was seen when the CF of C20 and C40 was treated with the proteolytic enzyme, trypsin (Figure 2.1, wells 3 & 5). These results indicate that the compound inhibiting microbial growth in the CF was proteinaceus and not other organic or low molecular weight compounds which is commonly produced by many LAB (Helander *et al*. 1997). The testes used here are in accordance with those for bacteriocin secreted in the CF (Geis *et al.* 1983; Jack *et al.* 1995; Varadaraj *et al*. 1993; Ramesh 2000). Strains C40 and

C20 were taken for further studies as they appeared to produce a potent bacteriocin.

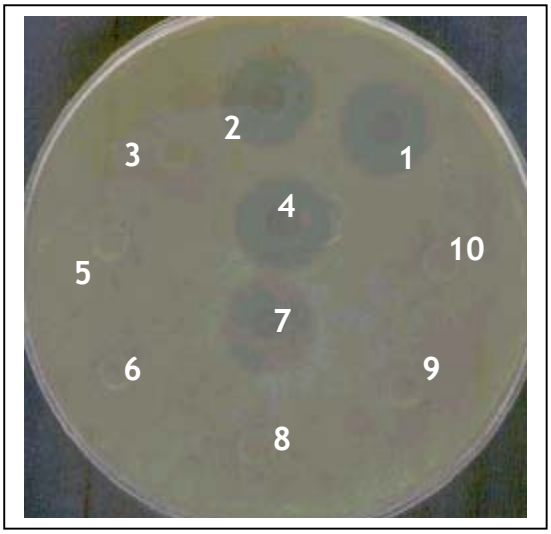


Figure 2.1: Agar-well diffusion assay of native bacteriocinogenic cultures against the indicator *Lact. casei* B1922. The CF and trypsin treated CF of various native bacteriocinogenic strains of LAB were tested against the indicator B1922. Well 1, CF of C6; 2, C20; 3, trypsin treated CF of C20; 4, C40; 5, trypsin treated C40; 6, F7; 7, F58; 8, M10; 9, M31 and 10, CF of M50.

Usually bacteriocins of Gram-positive bacteria do not inhibit the growth of Gram-negative bacteria such as *E. coli* (Bennik *et al.* 1997a; Schillinger *et al.* 1996). Geis *et al.* (1983) showed that the bacteriocins produced by the strains of *Streptococcus* do not inhibit the growth of *Staph. aureus* and *B. subtilis*. In the present study, most of the strains exhibited antimicrobial property against closely related bacteria.

Bacteriocin producers isolated from the intestine could kill readily indicators isolated from intestinal microflora and similar results were obtained for isolates from fish and from mushroom. These results indicate that the isolates obtained in this investigation were primarily acting against other strains isolated from the same source indicating the environmental effect on antagonism. Strain MD seems to be easily killed by all the producers tested

(Table 2.1). It was presumed that this strain could be a potential candidate for use as an indicator for isolating bacteriocinogenic LAB (see below).

TABLE 2.1: Antimicrobial activity of selected native lactic isolates against different lactics and pathogenic bacteria

I. Standard Cultures

Indicator strain/s	**Strain No.**	**Native bacteriocinogenic cultures**							
		C6	**C20**	**C40**	**F7**	**F58**	**M10**	**M31**	**M50**
Lactobacillus amylovorous	NRRL B4552	-	-	-	-	-	-	-	-
Lact. buchneri	NCIM 2357	-	-	-	-	-	-	-	-
Lact. casei	NRRL B1922	+	+	+	-	+	-	-	-
Lact. maltaromicus	MTCC 108	+	-	-	-	-	-	-	-
Lact. plantarum	NCIM 2083	-	-	-	-	-	-	-	-
Lact. viridescens	NCIM 2165	-	-	-	-	-	-	-	-
Leuc. mesenteroides	NRRL B640	-	+	-	-	-	-	-	-
Pediococcus acidilactici	NRRL B1153	-	+	-	-	-	-	-	-
Ped. acidilactici	NRRL B1325	-	-	-	-	-	-	-	-
Ped. pentosaceus	NRRL B11465	-	-	-	-	-	-	-	-
II. Pathogenic bacteria									
Bacillus cereus	F 4433	-	-	-	-	-	-	-	-
E. coli	D 21	-	-	-	-	-	-	-	-
Staph. aureus	FR 1722	-	+	-	-	-	-	-	-

III. Local isolates

Indicator strains	Strain No*	C6	C20	C40	F7	F58	M10	M31	M50
Lact. sp	CC	+	+	+	+	-	-	-	-
Lact. sp	MC	-	-	-	-	-	+	+	+
Lact. sp	MD	+	+	+	+	+	+	+	+
Ped.sp	CA	-	+	+	-	-	+	-	-
Ped. sp	F1	+	-	-	-	+	-	-	-
Ped. sp	F4	-	+	-	+	+	-	-	-
Ped. sp	MC	-	-	-	-	-	+	+	+

*alphabets were used to designate the fowl and mushroom indicator strains
(+ Zone of inhibition due to CF activity; - no inhibition)

2.3.2 Physiological and biochemical characteristics of native isolates

2.3.2.1 Characteristic features of bacteriocin producers

The selected isolates grown in MRS agar plate were typical LAB. A tentative identification scheme for the identification of bacteriocins producers at generic and species level is presented in Table 2.2. Indicators are identified at a generic level except strain MD which was identified at the species level (see below). The bacteriocin producers were all able to utilize sugars such as maltose, sucrose, lactose, cellibiose, fructose, glucose, mannose, galactose and trehalose. Inositol, arabinose, xylose, glycerol, cellulose, starch and inulin were not utilized by any of the isolates. No strain could liquefy gelatin or reduce potassium tellurite.

Other characteristic features are shown in Table 2.2. Based on the Bergey's manual of determinative Bacteriology and the battery of tests conducted as given by Sneath *et al.* (1986) and Stiles and Holzapfel (1997), eight strains were

identified to the species level as listed in Table 2.8. The identified local isolates used as a indicator are enlisted in Table 2.1. *Ped. pentosaceus*

M10, a mushroom isolate was able to utilise citrate. Citrate utilization by pediococci has been earlier reported by Litopoulou-Tzanetaki *et al.* (1989). None of the other strains could utilize citrate.

All intestinal isolates were found to grow in bile salt. It is known that LAB isolated from intestinal sources has a property to grow at low pH and in bile salt (Jin *et al.* 1998; O'Sullivan *et al.* 1992). Such intestinal strains could thus be exploited for their probiotics properties (Juven *et al.* 1991; Leroy *et al*. 2003). The principal habitat of lactobacilli includes intestinal tract, fermented food materials and being associated with food spoilage (Barnes 1979; Jin *et al.* 1998; Juven *et al.* 1991; Stiles and Holzapfel 1997). In the present study two lactobacilli were identified these are *Lact. casei* ssp. *casei* strain C40 and M50 which were primarily isolated from intestinal tract and mushroom fermentation, respectively. The other identified isolates reported in this study are mainly pediococci and *Enterococcus* sp. Similar observations for the presence of *Lactobacillus* and *Pediococcus* spp. in intestinal tract of fowl has been made by Juven *et al.* (1991); Jin *et al*. (1998). Isolates of the genus *Lactococcus*, *Leuconostoc* and *Lactobacillus* have been reported from sea food by Mauguin and Novel (1994. *Carnobacterium* spp was isolated by Mauguin and Novel (1994), Stoffels *et al.* (1992).

Among the bacteriocinogenic cultures, C20 was found to be a potent bacteriocin producer and was able to grow at high temperature, salt concentration and pH (Table 2.1 and 2.2), and hence has been taken up for further studies.

Table 2.2: Biochemical and physiological characteristics of bacteriocin producing and indicator LAB

Character-istics	C6	C20	C40	F7	F58	M10	M31	M50	MD
Sugar Utilization									
Mannitol	+	+	+	-	+	+	+	+	+
Mellibiose	+	+	-	+	+	-	+	+	-
Raffinose	+	+	-	-	+	-	+	-	-
Rhamnose	+	-	+-	+-	-	-	-	-	+
Sorbitol	+	+	+	-	-	-	+	+	-
Morpho-logy	Tetrad	Diplo-cocci	Rod	Tetrad	Tetrad	Tetrad	Tetrad	Rod	Rods
Growth in NaCl (%w/v)									
10	+	+-	-	-	-	-	+-	-	+-
18	-	-	-	-	-	-	-	-	-
Growth in pH									
4.5	+	+	+	+	+	+	+	+	-
9.2	+	+	+	+	+	+	+	+	+
9.6	+	+	+	+	+	+	+	+	+
Growth at ^{0}C									
10	+	+	+	+	+	-	+	+	+
50	-	+	+	+	+	-	+	+	-
Growth in skim milk	+	+	+	-	+	+	+	+	-
Arginine hydrolysis	+	+	-	-	+	+	+	+	+
Citrate utilization	-	-	-	-	-	+	-	-	ND
Bile salt growth	+	+	+	+	+	-	-	-	ND

(+ Growth of the test strain; – no growth; +- weak growth and ND not determined)

2.3.2.2 Characteristic features of *Ent. faecium* C20

In order to identify and characterize the native strain of C20, selected microbiological, physiological and biochemical tests that differentiate enterococci were carried out and the results are presented in Table 2.3. The *Ped. acidilacitici* NRRL B5627 was used as a control for comparison.

Table 2.3: Specific tests of *Enterococcus faecium* C20

Test/s	C20	NRRL B5627
Microscopy and motility	Cocci and diplococci chains, non-motile	Diplococci and tetrad, non-motile
Hemolysis	- (no clear zone, thus □ hemolysis)	- (No growth of the organism was seen)
Heat tolerance test (60^0C for 30 min)	+	-
Methyl red test	+	-
VP test	-	-
Fermentation of ribose	+	+
Growth in 0.04% sodium azide	+	-
Acid production in milk 8h at 37^0C (pH)	6.12	6.34
Acid production in milk 18h at 37^0C (pH)	5.8	6.3
Acid production in milk 7 days at 37^0C (pH)	4.8	6.0
Growth in bile esculin agar	+	±

(+ growth observed by turbidity, - no growth and ±weak growth)

Strain C20 retained crystal violet and hence is a Gram-positive bacteria. There was no movement of cells as determined by the hanging drop method. The cells were not flagellated and were clusters of cocci. The cells numbering 4-5 cells in short chains, two cells (diplococci) was a very common feature noticed. Chains of cells numbering 3 to 5 arranged one above the other in a straight or a slightly curved line was also found. A specific heat tolerance test was performed at 60^0C for 30min. This test physiologically discriminates between Enterococcus spp and *Ped. acidilactici*. It was observed that the strain of C20 survived the heat treatment and grew luxuriantly in MRS agar medium. However the strain of *Ped. acidilactici* NRRL B5627 did not show any growth after treatment at that temperature.

The strain C20 grew on NB blood agar plate but with no clearance zone around the colonies indicating γ-hemolysin production (De Vyust *et al.* 2003). However the strain of *Ped. acidilactici* NRRL B5627 did grow on the NB blood agar. Based on above tests given in Table 2.2 and 2.3 and in with the tests given in Bergeys Manual of Systematic Bacteriology, the native isolate of C20 was identified as *Enterococcus faecium* C20.

The strains of *Ent. faecium* are often inhabitants of intestine. They survive at high pH, temperature and salt concentration. They are catalase negative, diplococci, negative for hemolysis, potassium telurite reduction and gelatin liquification. They do not hydrolyse the starch. The typical enterococci can be easily distinguished from other Gram-positive, catalase negative, homofermentative cocci such as streptococci and lactococci by their characteristic growth at extreme temperature, pH and salt concentration and in presence of bile (Franz *et al.* 2003). Various strains of enterococi are known to produce class II bacteriocins, which has potential commercial application in food preservation and fermentation (Ennahar *et al.* 2000; Franz *et al.* 2003; Leroy *et al*. 2003; Nes *et al.* 2002).

Scanning electron microscopy was used to check purity of the isolate and to identify the morphological features of the cell. Cells from strain *Ent. faecium* C20 were found to be cocci, in bunches of two commonly known as diplococci, round and smooth (Figure 2.2). This study further revealed that the culture was pure.

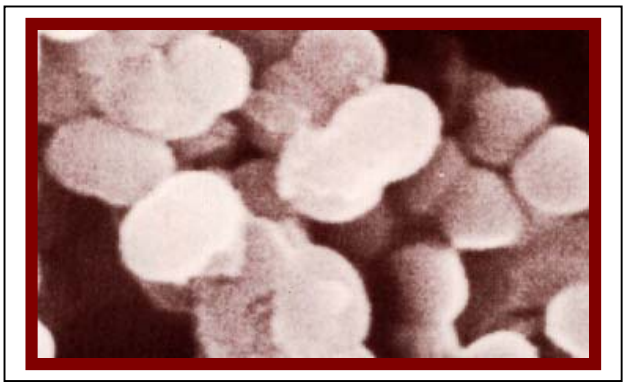


Figure 2.2: Scanning electron microscopy of *Enterococcus faecium* C20

2.3.2.3 Characteristic features of native strain *Lactobacillus farciminis* MD

Prior to the exploitation of the native isolate MD which was found to be highly sensitive to the antibiotics and bacteriocins, a detailed identification was carried out. Carbohydrate fermentation tests revealed that the strain was able to ferment cellibiose, esculin, glucose, maltose, mannitol, mannose, rhamnose, salicin, sucrose and trehalose. The strain grew weakly in lactose and was unable to ferment arabinose, dulcitol, galactose, glycerol, inulin, mellibiose, dulcitol, pyranose, raffinose, sorbitol and xylose. Strain MD grew in MRS at neutral to alkaline pH range of 6.5 to 9.6. However the cells did not grow at the acidic pH of 4.5. Strain MD could tolerate salt concentrations from 2 to 6%, grew weakly in 10% salt and was unable to grow when the salt concentration of the media was raised to 18%. The cells were unable to hydrolyze gelatin and starch and to grow in a skim milk medium. Hydrolysis of arginine was observed. The optimum temperature for growth was between 10 to 40^0C. No growth occurred at 50^0C. Scanning electron microscopy of the native strain MD is presented in Figure 2.3. The cells were found to be rods, and the culture was pure. Based on the

above physiological and biochemical tests and the tests given in Bergey's manual of Systematic Bacteriology for *Lactobacillus* (Sharpe *et al.* 1966; Sneath *et al*. 1986) the native isolate was identified as *Lactobacillus farciminis* MD.

Figure 2.3: Scanning electron microscopy of *Lactobacillus farciminis* MD.

2.3.3 Antibiogram

The responses of various isolates, bacteriocin producers as well as indicators to a wide range of antibiotics are presented in Table 2.4 and 2.5. Antibiogram was carried out with the idea of differentiating the strains and to identify plasmid-borne markers for genetic studies. It was found that most strains were resistant to most of antibiotics, particularly those belonging to the □-lactam and cephalosporin group of antibiotics. This was particularly true of those isolated from fowl intestine. Many strains were susceptible to erythromycin and all were susceptible to chloramphenicol at a concentration of 15 and 30 mg respectively. Most bacterial isolates were found to be resistant to antibiotics such as ceptadizime, penicillin, amikasin, nalidixic acid, chlotriaxazole and cephaloridine. Moreover their degree of resistance based on the diameter of zone of inhibition varied (data not shown). This kind of resistance could develop in the intestinal microflora following feeding of poultry with antibiotics. Similar kind of observations has been earlier reported by Curragh and Collins (1992).

Among the three different sources studied in the present work, isolates from mushroom were most sensitive to antibiotics. Among them, strain *Lact*. *farciminis* MD was found to be particularly sensitive to most antibiotics (see below). Data on the sensitivity of the different strains to antibiotics indicated great differences between the isolates. It is suggested that antibiotic markers can be used as a tool for further genetic studies.

Table 2.4: Antibiogram of native bacteriocin producing lactic isolates determined by antibiotic sensitivity megadisc.

Antibiotics	**Letter code**	**C6**	**C20**	**C40**	**F7**	**F58**	**M10**	**M31**	**M50**
Class of antibiotics				Mode of action					
CEPHALOSPORINS				Cell wall synthesis					
Cefazoline	CZ	R	R	R	R	R	S	R	S
Cephaloridine	CE	R	R	R	R	R	R	R	S
Cefuroxime	CR	R	R	R	M	M	S	M	S
Ceptadizime	CPZ	R	R	R	R	R	R	R	M
Ceftriaxone	CTX	R	R	R	R	R	M	M	R
BETA-LACTAMS				Cell wall synthesis					
Cloxicillin	CX	R	R	R	R	R	S	M	S
Cefotaxime	CF	R	R	R	R	R	M	R	S
Ampicillin	A	S	S	R	S	S	S	M	S
Penicillin	P	R	R	R	R	R	R	R	R
MACROLIDE				Protein synthesis, ribosome					
Erythromycin	E	M	M	M	S	S	S	M	R
AMINOGLYCOSIDE				Protein synthesis					
Amikasin	AN	R	R	R	R	R	R	R	R

Gentamycin	G	R	R	M	R	R	R	R	S
ALKYLBENZENE		Protein synthesis, 50S ribosome							
Chloramphenicol	CH	S	S	S	S	S	S	S	S
TETRACYCLINE		Protein synthesis, 30S ribosome							
Tetracycline	T	R	S	S	M	M	S	S	R

Antibiotics	**Letter code**	**C6**	**C20**	**C40**	**F7**	**F58**	**M10**	**M31**	**M50**
QUINOLONE		Nucleic acid synthesis, DNA gyrase							
Nalidixic acid	NA	R	R	R	R	R	R	R	R
IMIDAZOLE		Cell wall biosynthesis							
Chlotrimaxazole	CTX	R	R	R	R	R	R	R	R
Netilmycin	NET	R	R	R	R	R	M	R	S
NITROFURANTOIN		Nucleic acid synthesis							
Nitrofurantoin	NF	R	M	M	S	M	R	R	S
FLUOROQUINOLONES		Nucleic acid synthesis, DNA gyrase							
Ciprofloxacin	CIP	M	R	R	R	R	R	M	S
Norfloxacin	NR	M	R	M	R	R	R	M	S

(R, Resistant; S, Sensitive and M, moderate reaction)

It is known that enterococci are intrinsically resistant to a number of antibiotics which are mediated by chromosomally encoded genes. Such intrinsic antibiotic resistance includes resistance to cephalosporins, □-lactams, sulfonamide and low levels of clindamycin and aminoglycosides. The acquired resistance usually

mediated by the genes residing on plasmids or on transposons exhibits resistance to the antibiotics such as chloramphenicol, erythromycin, tetracycline and high levels of □-lactam, fluoroquinolones and glycopeptides such as vancomycin. Resistance of the enterococci to vancomycin is of special concern since this antibiotic has been considered as a last resort for the treatment of multiple resistant in enterococal infections (Franz *et al*. 2003).

Table 2.5: Antibiogram of native indicator lactic isolates determined by antibiotic sensitivity megadisc.

Antibiotics	**Letter code**	**CA**	**CC**	**MA**	**MC**	**MD**	**F1**	**F4**
Class of antibiotics CEPHALOSPORINS				Mode of action Cell wall synthesis				
Cefazoline	CZ	R	R	R	R	S	R	R
Cephaloridine	CE	R	R	R	R	R	R	R
Cefuroxime	CR	R	R	R	M	S	R	R
Ceptadizime	CPZ	R	R	R	R	R	R	R
Ceftriaxone	CTX	R	R	R	R	R	R	M
BETA-LACTAMS				Cell wall synthesis				
Cloxicillin	CX	R	R	R	R	S	R	R
Cefotaxime	CF	R	R	R	R	S	R	R
Ampicillin	A	R	R	R	R	S	R	R
Penicillin	P	R	R	R	R	M	R	R

MACROLIDE				Protein synthesis, ribosome				
Erythromycin	E	M	S	S	S	S	R	S
AMINOGLYCOSIDE				Protein synthesis				
Amikasin	AN	R	R	R	R	S	R	R
Gentamycin	G	R	R	R	R	S	R	R
ALKYLBENZENE				Protein synthesis, 50S ribosome				
Chloramphenicol	CH	S	S	S	S	S	S	S
TETRACYCLINE				Protein synthesis, 30S ribosome				
Tetracycline	T	R	R	R	R	S	M	R
QUINOLONE				Nucleic acid synthesis, DNA gyrase				
Nalidixic acid	NA	R	R	R	R	R	R	R

Antibiotics	**Letter code**	**CA**	**CC**	**MA**	**MC**	**MD**	**F1**	**F4**
IMIDAZOLE				Cell wall biosynthesis				
Chlotrimaxazole	CTX	R	R	R	R	S	R	R
Netilmycin	NET	R	R	R	R	S	R	R
NITROFURANTOIN				Nucleic acid synthesis				
Nitrofurantoin	NF	M	R	R	R	S	R	R
FLUOROQUINOLONES				Nucleic acid synthesis, DNA gyrase				
Ciprofloxacin	CIP	M	R	R	R	S	R	R
Norfloxacin	NR	R	R	R	R	S	R	R

Several lactobacilli strains with potential to be used as probiotics are known to be resistant to various antibiotics such as vancomycin and tetracycline (Cebeci and Guerakan, 2003). Antibiotic resistance can possibly be transferred between and within species especially by conjugation (Quintilioni and Courvalin 1994).

In clinical samples, resistance to antibiotics in many pathogenic bacteria are often spread by conjugation between antibiotic resistant and sensitive strains.

Danielsen (2002) has reported the presence of a plasmid pMD5057 from *Lact. plantarum* 5057 conferring tetracycline resistance to the host bacteria. A strain of *Leuconostoc* is known to be intrinsically resistant to vancomycin (Tynkkynen *et al.* 1998), while many lactobacilli and lactococcci are found to be susceptible to vancomycin (Orberg and Sandine 1985). Vancomycin resistant enterococci isolated from different sources have been well documented in literature (De Vuyst *et al.* 2003). Such antibiotic resistance is always undesirable in strains used as starter cultures, co-culture or as probiotics.

2.3.4.Comparative antibiotic sensitivity of *Enterococcus faecium* C20 with *Pediococcus acidilactici* NRRL B5627

The native isolate of C20 was further characterized with the selected set of antibiotics. The data is presented in Table 2.6. The pediocin PA-1 producing strain of *Ped. acidilactici* NRRL B5627 has been used for comparison.

Table 2.6: Comparative antibiotic sensitivity of potent bacteriocin producers

Antibiotic/s	Concentration (mg)	*Ent. faecium* C20		Ped. acidilactici NRRL B5627	
		Inhibition zone (mm)	R/S	Inhibition zone (mm)	R/S
Cephalothin	30	20	S	30	S
Clindamycin	2	20	S	30	S
Lincomycin	2	20	S	36	S
Oxacillin	1	12	M	20	S
Ofloxacin	5	18	S	20	S
Vancomycin	30	17	S	0	R

From the above table it is clear that the *Ent. faecium* C20 is sensitive to the antibiotic vancomycin. Resistance to vancomycin is usually govern by the presence of *van* gene. Genetic basis of vancomycin sensitivity can be confirmed by the PCR by using *van* gene specific primers (De Vuyst *et al*. 2003; Franz *et al.* 2003).

2.3.5 Antibiotic sensitivity of *Lact. farciminis* MD

Although a preliminary study of the sensitivity of the strain *Lactobacillus farciminis* MD has been presented in Table 2.5, the results of a more detailed investigation is presented in Table 2.7 *Lact. farciminis* MD was highly sensitive

to all antibiotics used in the investigation, except for 30 mg ceptazidime and 1mg cloxacillin. The maximum zone of growth inhibition was obtained for chloramphenicol (30 mg) followed by ampicillin (10 units), Cefoperozone (75 mg), cefozoline (30 mg), novobiocin (30 mg), norfloxacine (10

mg) and some other antibiotics as shown in Table 2.7. Ciprofloxacin (5 mg) and pipercillin (100 mg) exhibited zones of inhibition of 25mm in diameter and 30mm in diameter. The antibiotics such as penicillin (10 Units), neomycin (30 mg) reacted moderately against *Lact. farciminis* MD showing zone of inhibition of 25mm diameter. Resistance of starter cultures to antibiotics has been reported in many LABs by Reinbold and Reddy (1974), Sozzi and Smiley (1980). Orberg and Sandine (1985) observed trimethoprim, sulfathiazole resistance in N-streptococci. Further, variability in resistance to gentamycin, kanamycin, lincomycin, nafcillin, neomycin, nisin, rifamycin and streptomycin has also been reported.

Vescovo *et al.* (1982) have shown that *Lact. reuteri* had a chromosomal-borne resistance to antibiotics like cloxacillin, streptomycin, neomycin, gentamycin, kanamycin and a strain of *Lact. acidophilus* was similarly resistant to penicillin, ampicillin, cloxacillin, cephaloridine, streptomycin, neomycin, kanamycin, gentamycin, erythromycin, chloramphenicol and polymixin-B. Several reports indicated that LAB are resistant to major classes of antibiotics like β-lactam, cephalosporins, aminoglycosides, quinoline, imidazole, nitrofurantoin and fluroquinolines. This is in contrast to the results obtained in case of strain *Lact. farciminis* MD which was susceptible to many antibiotics. These results suggest that strain MD could be exploited as an indicator organism to develop new antibiotic assays. The susceptibility of LAB to different types of antibiotics including bacteriocin was mainly due to membrane composition (Montville and Bruno 1994; Bennik *et al*. 1997b). The differences in the degree of inhibition observed in this study with various antibiotics could possibly be

attributed to differences at the molecular and biochemical level such as in the synthesis of cell wall, protein and nucleic acids (Neu 1992).

Table 2.7: Antibiogram pattern of *Lact. farciminis* MD as tested using antibiotic sensitivity ring and discs

Antibiotics	Concentration in disc (mg)	Inhibition zone (mm)	Resistant / Sensitive/ Moderate
1. Sensitivity ring			
Amikasin	30	17	S
Ampicillin	10 Units	32	S
Cefoperozone	75	30	S
Cefotaxime	30	28	S
Ceforoxime	30	28	S
Cefozoline	30	30	S
Cetadroxil	30	18	S
Ceftriaxone	30	28	S
Ceptazidime	30	-	R
Chloramphenicol	30	35	S
Ciprofloxacin	5	25	S
Erythromycin	15	22	S
Gentamicin	10	15	S
Lomefloxacin	10	22	S

Netilmycin	30	25	S
Nitrofurantoin	30	30	S
Norfloxacin	10	30	S
Penicillin	10 Units	22	M
Piperacillin	100	30	S
Roxythromycin	15	30	S
2. Discs			
Cloxacillin	1	-	R
Kanamycin	30	18	S
Neomycin	30	14	M
Novobiocin	30	30	S
Polymixin B	300	12	S
Streptomycin	10	18	S
Tetracycline	30	26	S

R= Resistant, S=Sensitive and M= Moderate

A representative antibiogram for the strain *Lact. farciminis* MD is presented in Figure 2.4. It can be clearly seen that there is a definite zone of growth inhibition around the antibiotic discs. Such kind of zones inhibition of test strain was not seen while testing many of the other bacteriocin producers and indicator strains.

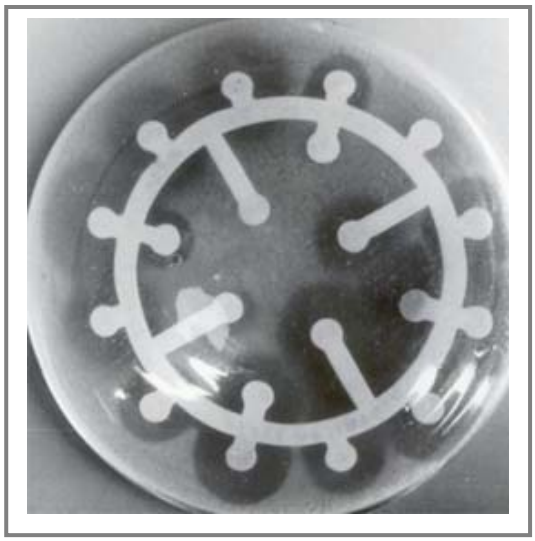


Figure 2.4: Antibiogram of *Lactobacillus farciminis* MD using antibiotic sensitivity ring

2.3.6 Plasmid profiles of native strains

The plasmid profiles of the bacteriocin producers are summarized in Table 2.8. It is interesting to note that most strains harbour mega-plasmids. Strain F7 was devoid of plasmid (lane 5) while strain M50 harbors five naturally occuring plasmids. The presence of mega-plasmid was revealed by the method of Anderson and Mckay (1983). Very small molecular weight plasmids were observed in strains such as *Lact. casei* ssp. *casei* M50, *Ped. pentosaceus* C6 (Fig. 2.5; lane 3 &9) and in several indicator strains too (data not shown).

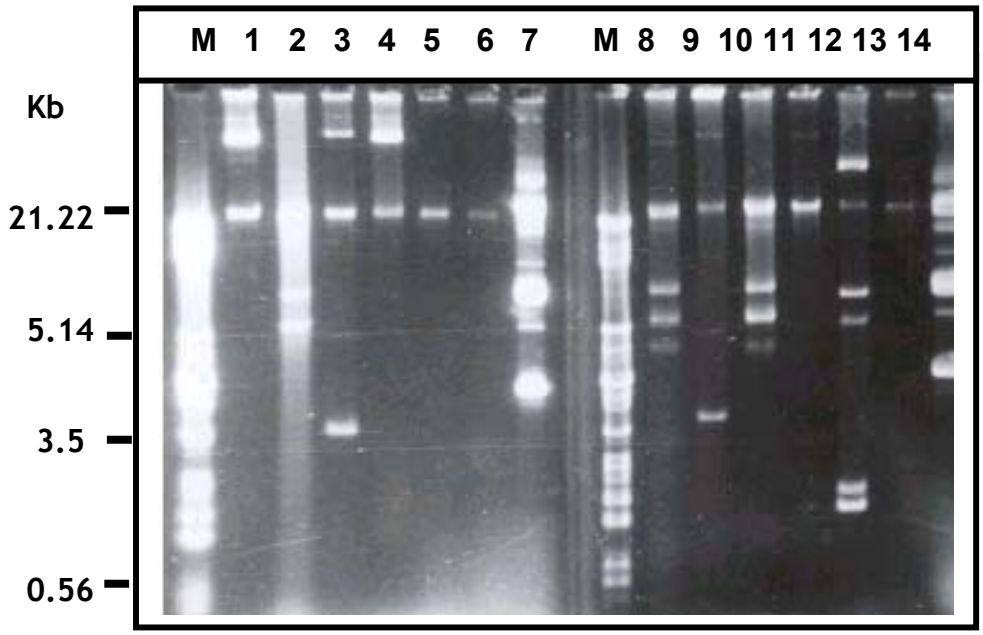


Figure 2.5: Agarose gel analysis of plasmid DNA isolated from bacteriocin producing native strains of LAB. lane 1, C20; 2, C40, 3 and 9, C6; lane 4, F58; 5, F7; 6, C67; lane 7 & 14, pUC18 (control); lane 8, C75; 10, C75; 11, M31; 12, M50; 13, M10; M, λ DNA *Eco*RI/*Hin*dIII digest.

Table 2.8: Plasmid profiles of bacteriocin producing strains as determined by 1% agarose gel electrophoresis

SN	Strain No.	Strain identity	Total no. of plasmids	Plasmids in Kb (approximately)
1	C6	**Pediococcus pentosaceus**	Two	*Megaplasmid, 4.0
2	C20	**Enterococcus faecium**	One	Megaplasmid

3	C40	*Lactobacillus casei* ssp *casei*	Two	8, 5.5
4	F7	*Pediococcus* sp.	None	-
5	F58	**Pediococcus acidilactici**	One	Megaplasmid
6	M10	**Pediococcus pentosaceus**	One	Megaplasmid
7	M31	**Pediococcus acidilactici**	One	Megaplasmid
8	M50	*Lactobacillus casei* ssp. *casei*	Five	Megaplasmid, 9.4, 5.1, 3.5 and 2.0

(*size not determined)

2.3.7 Characteristic features of *Lact. casei* ssp. c*asei* DC40

The culture *Lact. casei* ssp. *casei* C40 was chosen for plasmid curing experiment since this culture contains low MW plasmids, characteristic sugar fermentation markers and exhibits detectable antimicrobial activity against *Ped. pentosaceus* C6. The curing results indicated that atleast 10-20% of novobiocin treated C40 colonies were unable to exhibit zones of inhibition against the indicator C6. Such representative colonies were analyzed for the presence of native plasmids, utilization of sugars and sensitivity to antibiotics. The cured strain of C40 (denoted as DC40) was devoid of plasmids of molecular weight 8 and 5.5 kb (Figure 2.6), indicating elimination of these plasmids due to the action of the curing agent. Novobiocin inhibits DNA gyrase and thus hinders plasmid DNA replication (Kornberg 1980). Novobiocin has been used as a plasmid curing agent for removal of various native plasmids in the species of pediococci and lactobacilli (Graham and Mckay 1985; Ruiz-Barba *et al*. 1991).

Curing studies with *Lact. casei* ssp *casei* C40 revealed that bacteriocin production and sugar utilization are plasmid-borne traits. Strain DC40 was unable to utilize many sugars such as arabinose, glycerol, mannitol, mellibiose, raffinose and sorbitol indicating non-fermentation of these carbohydrates has direct linkage with plasmid loss (Table 2.9) The cured strain grew slowly in

lactose and sucrose suggesting that the genes encoding for these enzyme probably duplicated and reside in the chromosome as well as in the plasmid. Fernandez *et al.* (1999) demonstrated that the genes coding for □-galactosidase in some strains of *Lact. plantarum* was both chromosomally and plasmid encoded. The presence of such duplicated genes can be confirmed by Southern hybridization using gene specific probe.

A strains of *Lact. casei* ssp *casei* C40 and DC40 strain were similarin their pattern of sensitivity to antibiotic (Table 2.10). These results implies that antibiotic resistance was mostly a chromosomal encoded character as suggested by an earlier report (Sozzi and Smiley 1980). The antibiogram data suggest however strain DC40 exhibited increased sensitivity to cefotaxime, cefuroxime, ceftriaxone, erythromycin. The loss of native plasmid appeared to be directly correlated with reduced resistance to these antibiotics.

Plasmid associated bacteriocin production and sugar utilization has been widely reported in *Ped. acidilactici* (Gonzalez and Kunka 1987) and in *Lact. plantarum* (Ray *et al.* 1989; Ruiz-Barba *et al.* 1991). However, the strain *Ped*. sp F7 (Figure 2.5) while not bearing any plasmid produced antimicrobial compounds (Table 2.1). Although it is well established that bacteriocins production is a plasmid-borne character (Ray *et al.* 1989), chromosomal encoded bacteriocin production has been found in *Ped. acidilactici* and *Ped. pentosaceus* as reported by Jager and Harlander (1992).

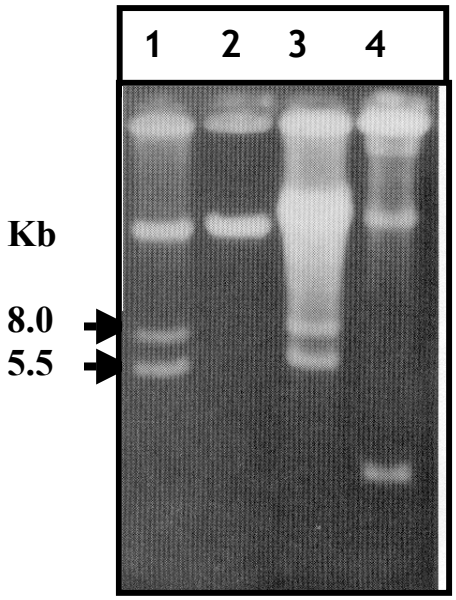


Figure 2.6: Agarose gel analysis of Bact^{+} and Bact^{-} phenotypes of strain C40 by plasmid DNA isolation. Lane 1. C40 Bact^{+}; 2. C40 Bact^{+-}; 3. genomic DNA of C40 and lane 4. plasmid DNA preparation from C6 indicator strains used for selection of C40 Bact^{-}.

The eight strains studied in detail for their plasmid profiles show remarkable differences among each other in respect to their total number of plasmid molecules and their sizes. Plasmid profiling thus helped in strain differentiation and characterization. Similar studies on plasmid profiling and strain differentiation have been carried out in lactic streptococci by Davies *et al.* (1981).

Plasmid curing and analysis by plasmid DNA isolation provides the physical evidence for plasmid loss. Plasmids are known to encode variety of metabolic traits such as antibiotic resistance, carbohydrate fermentation, bacteriocin production. Such phenotypes can be easily determined by plasmid curing. Various species of lactobacilli are known to produce class II bacteriocin (ten Brink *et al*. 1994; Jimenez-diaz *et al*. 1993; Jack *et al*. 1995). The present study

provides physical and genetic evidence for the plasmid linked bacteriocin production and sugar fermentation in the strain *Lact. casei* ssp. *casei* C40.

Table 2.9: Carbohydrate fermentation pattern of C40 and DC40 strains

Sugar/s	WT C40	DC40
Arabinose	**+**	**-**
Cellibiose	+	+
Esculin	+	+
Galactose	+	+
Glucose	+	+
Glycerol	**+**	**-**
Inulin	-	-
Lactose	**+**	±
Maltose	+	+
Mannitol	+	-
Mannose	+	+
Mellibiose	+	-
Pyranose	-	-
Raffinose	+	-
Rhamnose	+-	±
Sorbitol	+	-
Sucrose	+	±
Trehalose	+	+
Xylose	-	-

(+ Growth; - No growth and ± weak growth)

Well characterized low molecular weight plasmids are often useful for the construction of food-grade vectors and/or bacteriocin encoding plasmids may be

modified by inserting a suitable food-grade marker (Motlagh *et al.* 1994; Platteeuw *et al*. 1996).

Table 2.10: Comparison of antibiotic sensitivity between C40 and ∆C40 strain

Antibiotics (code)	**C40** Inhibition zone (mm)	R/S/M	**DC40** Inhibition zone (mm)	R/S/M
A	11	R	-	R
An	12	R	-	R
Ce	-	R	-	R
CF	-	R	13	R
Ch	25	S	23	S
Cip	14	R	-	R
CPZ	10	R	-	R
CR	**-**	**R**	**15**	**M**
CT	-	R	-	R
CTX	**-**	**R**	**15**	**M**
Cx	-	R	-	R
Cz	15	R	12	R
E	**17**	**M**	**23**	**S**
G	13	M	12	R
NA	12	R	-	R
Net	**12**	**R**	**13**	**M**
NF	15	M	12	R
NR	13	M	-	R
P	12	R	12	R

	25	S	12	R
T				

R, resistant; S, sensitive and M, moderate

2.3.8 Bacteriocin sensitivity of *Lact. farciminis* MD

The sensitivity pattern of strain *Lact. farciminis* MD to various bacteriocins is presented in Table 2.11. A strain of *Ped. pentosaceus* C6 was used as a reference strain. The CF of 23 isolates belonging to genera *Lactobacillus, Leuconostoc* and *Pediococcus* were tested against *Pediococcus pentosaceus* C6 and *Lactobacillus farciminis* MD. Among all the strains tested, the CF of

2 strains of *Lactobacillus* species and 1 strain of *Pediococcus* were found to be inactive against *Lact. farciminis* MD (Table 2.11). The CF derived from other 20 organisms exhibited profound effect on the growth of this culture. It is also evident from the results that the CF of *Ent. faecium* C20 and *Ped. acidilactici* K7 inhibited the growth of *Lact. farciminis* MD strongly, followed by moderate inhibition by the CF of the native isolate of *Lact. casei* ssp *casei* C40. The trypsin treated CF of these bacteria did not inhibit the growth of either *Ped. pentosaceus* C6 or *Lact. farciminis* MD indicating that the antimicrobial activity was from a protein (Geis *et al.* 1983; Klaenhammer 1993; Ramesh 2000).

Several strains of *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* have been used as indica tor organisms for bacteriocin assay (Bennik *et al.* 1997b). In this study, the use of *Lact. farciminis* MD, a sensitive organism for bacteriocin assay has been reported. The sensitivity of *Lact. farciminis* MD and *Ped. pentosaceus* C6 to different concentration of commercial nisin (conc. 10 to 1000 IU ml^{-1}) was tested. The inhibition zone formed against *Lact. farciminis* MD and against *Ped. pentosaceus* C-6 by a commercial preparation of nisin (1000 IU ml^{-1}) was 10 mm and 7 mm diameter, respectively. These results indicate that strain MD is more sensitive than strain C6 to nisin.

However, LAB strains with higher sensitivity to similar concentrations of nisin has been reported in the literature. The efficacy of nisin depends upon its storage and solubility, a fact also observed by Orberg and Sandine (1985). Similar pattern of growth inhibition by nisin was reported by Rogers and Montville (1991) for *Micrococcus luteus* 10240 and for *Lact. sake* ATCC 15521.

Table 2.11: Sensitivity pattern of *Ped. pentosaceus* C6 and *Lact. farciminis* MD against the culture filtrate (Control) and trypsin treated culture filtrate (Exptl.) of different LAB

Bacteriocin producing strains	Source/ Strain No.	Inhibition zone against indicator bacteria			
		Ped. pentosaceus C6		*Lact. farciminis* MD	
		Control	Exptl.	Control	Expt
Lact. amylovorous	NRRL B4552	-	-	+	-
Lact. buchneri	NCIM 2357	+	-	+	-
Lact. casei	NRRL B1922	-	-	+	-
Lact. maltaromicus	MTCC 108	+	-	+	-
Lact. plantarum	NCIM 2083	-	-	+	-
Lact. viridescens	NCIM 2165	-	-	+	-
Leuc. mesenteroides	NRRL B640	+	-	+	-
Ped. acidilactici	NRRL B1325	-	-	+	-
Ped. pentosaceus	NRRL 11465	+	-	+	-
Lact. casei ssp *casei* C40	Native isolate	+	-	+	-

Lact. sp. F-58	-do-	-	-	+	-
Lact. sp A	-do-	-	-	+	-
Lact. sp C	-do-	-	-	-	-
Lact. sp J	-do-	-	-	+	-
Lact. delbrueckii FMD	-do-	-	-	-	-
Ent. faecium C20	-do-	+	-	+++	-
**Ped. pentosaceus* C6	-do-	-	-	+	-
Pediococcus sp F7	-do-	-	-	+	-
Ped. sp. M10	-do-	+	-	+	-

Bacteriocin producing strains	Source/ Strain No.	Inhibition zone against indicator bacteria			
		Ped. pentosaceus C6		*Lact. farciminis* MD	
		Control	Exptl.	Control	Expt
Ped. sp. I	-do-	+	-	++	-
Ped. sp. UNK	-do-	-	-	-	-
Ped. acidilactici K3	Ramesh 2000	+	-	++	-
Ped. acidilactici K7	-do-	++	-	+++	-

Degree of inhibition:
+ moderate inhibition zone, 6 to 9 mm; ++ strong inhibition zone, 10 to 14 mm; +++ very strong inhibition zone, 15 to 18 mm; - no inhibition zone; *strain was tested for its potential for bacteriocin production against *Lact. farciminis* MD

2.3.9 Relative sensitivity

Since *Lact. farciminis* MD was very sensitive to nisin, the effect of pediocin PA-1 of *Ped. parvulus* ATO 77 (Bennik *et al.* 1997a), the bacteriocins from strain *Ent. faecium* C20 and from *Lact. casei* ssp *casei* C40 and nisin from *L. lactis* ssp *lactis* on the growth of *Lact. farciminis* MD was investigated (Figure 2.7).

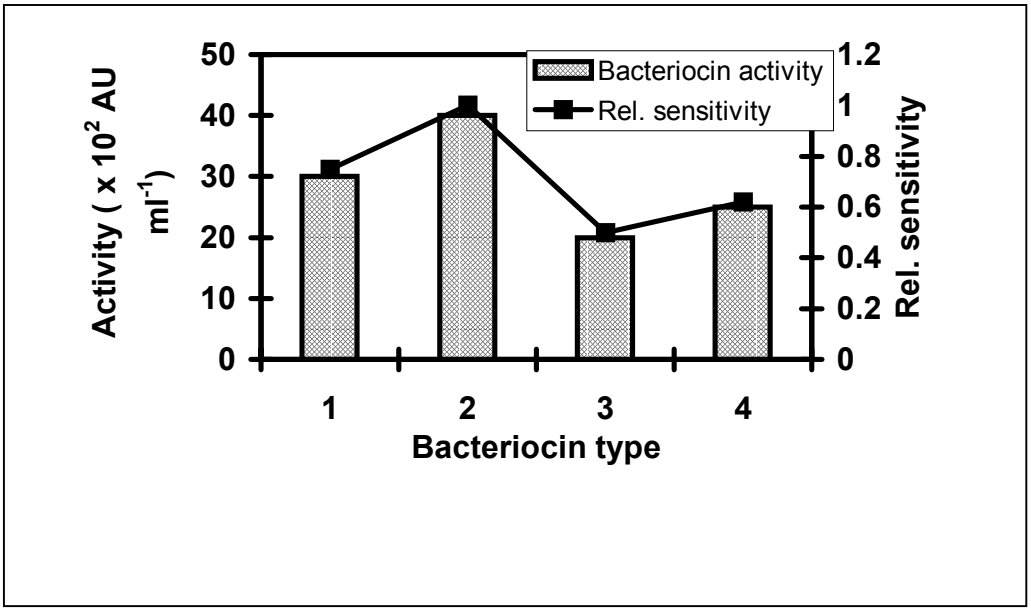


Figure 2.7: Relative sensitivity of *Lact. farciminis* MD against different bacteriocins 1, Pediocin PA-I; 2, Bacteriocin C20; 3, nisin produced by *L. lactis* ssp *lactis* and 4, Bacteriocin from *Lact. casei* ssp. *casei* C-40

The greatest inhibition was observed in that order, with bacteriocin produced by C20 followed by pediocin PA-1, bacteriocin of *Lact. casei* C40 and finally the culture filtrate of *L. lactis* ssp. *lactis*. This result indicated that *Lact. farciminis* MD was more sensitive to class IIa heat stable bacteriocins than to nisin a class 1 bacteriocin. Bennik *et al.* (1997b) have shown that many of the strains of *Lact. sake* exhibited higher degree of sensitivity to pediocin PA-1 than to nisin.

2.3.10 MIC of antibiotics against *Lact. farciminis* MD

The MIC of three different commercially available antibiotics and for nisin was determined on *Lact. farciminis* MD. Erythromycin at a concentration of 0.4 mg and 0.7 mg ml^{-1} inhibited the growth of this bacteria to an extent of 50% and 100%, respectively. Ampicillin at a concentration of 1.32 mg ml^{-1} and 6.0 mg ml^{-1} inhibited the growth by 50 and 100%, respectively. The MIC for cycloserine was found to be 35 mg ml^{-1} and that for nisin to be 85 mg ml^{-1} exhibiting 100% inhibition. Quesada *et al.* (1996) have shown that minimum active concentration (MAC) of penicilin-G towards *E. coli* pen-3 was 200 mg ml^{-1} and 0.4 mg ml^{-1} for parental and hypersensitive strains respectively. Antibiotics such as chloramphenicol, gentamycin, neomycin, erythromycin, tetracycline, nalidixic acid and novobiocin at a concentration of 5mg ml^{-1} had no effect on hypersensitive strain *E. coli* pen-3. It is important to note that native isolate of *Lact. farciminis* MD was sensitive to all the above antibiotics at a concentration of 10-30 mg ml^{-1}. These results suggest that the native strain of *Lact. farciminis* MD is very sensitive to antibiotics and to bacteriocins and thus can be explored as a potential candidate as an indicator bacterium.

2.4 CONCLUSION

A isolate from fowl intestine identified was *Lactobacillus casei* ssp. *casei* C40 based on conventional microbiological techniques. Plasmid curing in this strain indicated the plasmid-borne nature of the bacteriocin that it produced.

A mushroom isolates identified while using conventional tools was *Lactobacillus farciminis* MD, found to be highly sensitive to most of antibiotics and the bacteriocins (produced by LAB) that were tested. This bacterium thus can be adopted as an indicator organism for the assay of bacteriocin, antibiotics and also screening of novel bacteriocinogenic strains from different sources.

Among the different bacteriocin producing cultures isolated, the fowl intestinal isolate C20 was identified as *Enterococcus faecium* based on its morphology, tolerance to high temperature, pH and salt concentration, 0.04% sodium azide and to bile salts. The strain was negative to the VP test. This strain of *Ent. faecium* C20 harbors a high molecular weight plasmid and the CF exhibited strong antimicrobial activity. The culture was found to be resistant to many antibiotics that were tested while remains sensitive to vancomycin. Since, *Ent. faecium* C20 exhibited a wide-spectrum of antimicrobial activity against the indicators used, this strain was taken up for detailed investigation and is referred henceforth as *Enterococcus faecium* PH-1.

CHAPTER III

INTERGENERIC PEDIOCIN PA-1 PRODUCTION BY A NATIVE STRAIN OF *Enterococcus faecium* PH-1

3.0 ABSTRACT

The 16S rRNA gene from native strain of PH-1 was amplified by PCR technique and the 1.2 kb PCR product obtained was cloned in to a pTZ57R/T vector and the partial nucleotide sequences determined (GenBank Acc. No. AY723748). The sequence was highly homologous on BLAST and on phylogenetic analysis to the sequence of the 16S rRNA gene from *Ent. faecium* and those from other enterococcal species. These results were further substantiated with sequences of the *tuf* gene (GenBank Acc. No. AY753890), encoding the putative elongation factor-Tu isolated from a ~1.2kb clone, derived from the isolate PH-1. Sequence analysis of this gene also indicated that strain PH-1 was indeed *Enterococcus faecium*.

Plasmid curing experiments employing novobiocin in combination with high temperatures indicated that the genetic determinants for bacteriocin production, immunity function and several sugar utilization resided in the megaplasmid of strain *Ent. faecium* PH-1. Bacteriocin production with different sugars was tested and the culture filtrate (CF) activity varied from 2,500 to 3,500 AU ml^{-1} when the bacteria was grown with a 1% percent carbon source in the medium.

The strain of *Ent. faecium* PH-1 was grown in MRS broth for 24h and the CF was subjected to antimicrobial assay against a series of listerial strains and to various treatment such as incubation with organic solvents, proteolytic inactivation, incubation with reducing agents and heating to a high temperature. These results suggested that the antimicrobial activity in CF was due to a

proteinaceous, thermo-tolerant, disulfide bonded, anti-listerial bacteriocin. The bacteriocin adsorbed to the cells of both the indicator and the bacteriocin producing host. This property was employed to concentrate the bacteriocins. A 4.6kDa protein band that exhibited antimicrobial activity was observed when the concentrated bacteriocin was analysed for antimicrobial activity on tricine SDS-PAGE. The bacteriocin was further purified by preparative RP-HPLC. A single peak eluting at 25min on a 28% acetonitrile gradient exhibited strong antimicrobial activity against *Listeria monocytogenes* V7. The specific activity of the bacteriocin increased 1830 folds during the purification process.

PCR was carried out using two sets of *ped*A gene specific primers in order to provide molecular evidences for bacteriocin production by strain PH-1. The size of the amplicon (260 and 186bp) corresponded to that of the PCR amplicon was obtained for *Ped. parvulus* ATO 77, a pediocin PA-1 producing strain. A PCR generated DIG labeled probe exclusively reacted with the plasmid isolated from the PH-1 and did not react with DNA isolated from the cured strain (∆PH-1), indicating plasmid linked pediocin PA-1 type bacteriocin production by *Enterococcus faecium* PH-1. The plasmid-linkage of pediocin PA-1 type bacteriocin production was further evident on Southern hybridization. The PCR amplified *ped*AB gene of strain PH-1 was partially sequenced and the nucleotide sequences of 450 bp indicated cent-percent homology to the gene from *Ped. acidilactici* for pediocin PA-1/AcH, suggesting an intergeneric production of pediocin PA-1 by the native strain of *Enterococcus faecium* PH-1.

3.1 INTRODUCTION

Enterococci are an important group of generally recognized as safe (GRAS) LAB (De Vuyst *et al.* 2003). The species of *Ent. faecium* and *Ent. faecalis* are more frequently encountered in the gastro-intestinal tract of vertebrates. Enterococci persist in the extraenteral environment and are ubiquitous in food processing establishments and hence become an important part of the food microflora. Enterococci display desirable metabolic activities such as lipolytic

and esterolytic activities, citrate utilization and bacteriocin production. Species of enterococci play an important role in the ripening and the development of aroma and flavour of traditional cheese, development of probiotic cultures in human and animal health promotion (Leroy *et al*. 2003).

As ecological adaptation is important for the application of bacteriocinogenic strains as protective cultures in biopreservation (Bennik *et al*. 1999), there is a need to isolate potent bacteriocin producing LAB and to elucidate the involvement of plasmids and genetic transfer systems for economically important functions. Plasmid encoding sugar utilizing markers for sucrose and lactose can be used in basic studies for constructing food-grade vectors (Platteeuw *et al.* 1996) and for designing media using cheaper carbon sources such as milk whey or molasses for industrial scale production of bacteriocin (Daba *et al*. 1993; Liao *et al.* 1993).

Bacteriocins are gene encoded, ribosomally synthesized, antimicrobial peptides exhibiting antimicrobial activity against several pathogenic, food-spoilage and closely related bacteria. Bacteriocins produced by LAB have an eminent economic importance in food preservation (Abee *et al*. 1995; Nes *et al*. 2002). Bacteriocins from LAB have been divided into three distinct classes using biochemical and genetic methods (Klaenhammer 1993). However, bacteriocins of class I and II are the most studied because of their abundance and prominence for industrial application. Unlike Class I bacteriocins, class II bacteriocins lack modified residues, and hence are better candidates for heterologous expression in other LAB. The pediocin like bacteriocins constitute the largest subgroup with in the class II bacteriocins. These are small heat stable, membrane active peptides bearing a YGNGVXC consensus motif. This class of bacteriocins is widely spread among LAB and include pediocins AcH/PA-1, mesentericin Y105, enterocin A, carnobacteriocin BM1 and B2, piscicocin VIa and divercin V41 etc (Aymerich *et al.* 1996; Ennahar *et al*. 1999; Rodriguez *et al*. 2002b)

Antilisterial bacteriocin, pediocin PA-1 produced by the strains of *Ped. acidilactici* has been isolated from meat and green olive fermentation. Pediocin acts against many Gram-positive pathogenic and food spoilage bacteria (Bhunia *et al*. 1988; Daeschel and Klaenhammer 1985; Gonzalez and Kunka 1987). Plasmids are linked to pediocin production, immunity, antibiotic resistance and sugar utilization have been reported in several strains of pediococci and the molecular weight of these plasmids range from 2.0 to 50 MDa (Gonzalez and Kunka 1987; Kim *et al.* 1992; Ray *et al.* 1989).

Conventionally, bacteriocin producers are isolated and characterized by microbiological assays that are time consuming, tedious and sometimes ambiguous. Rapid molecular biology techniques such as colony hybridization, PCR and DNA probes have been recently employed to overcome these inconveniences (Bennik *et al*. 1997; Martinez *et al.* 1998; Ramesh 2000; Rodriguez *et al.* 1997).

Many strains of pediococci are known to produce bacteriocins known as pediocin. Different pediocins have been isolated and characterized from *Ped. acidilactici* strains, these includes pediocin AcH, PA-1, JD, SJ-1 and K7. However, at the molecular level all these bacteriocins are identical (Mora *et al*. 2000a). This indicates that there is a need to characterize native pediocin producers at molecular level and to evolve a common nomenclature for bacteriocins identical in sequence (Ray 1996). Pediocin A and N5p have been reported to be produced by strains of *Pediococcus pentosaceus* (Daeschel and Klaenhammer 1985; Manca de Nadra *et al*. 1998) The antimicrobial spectra of all these bacteriocins are very similar since the genes coding for their production share many conserved regions (Daeschel and Klaenhammer 1985; Manca de Nadra *et al*. 1998).

A pediocin like antimicrobial peptide leucocin C produced by *Leuconostoc mesenteroides* has been isolated (Ennahar *et al.*1996; Fimland *et al.* 2002b). Recently, intergeneric pediocin PA-1 production by *Lactobacillus plantarum* WHE 92 isolated from cheese has been reported (Ennahar *et al.* 1996). Likewise, production of coagulin, a pediocin family of bacteriocins is produced by the lactic acid producing bacterium *Bacillus coagulans* I_4 was reported (Le Marrec *et al.* 2000). Coagulin and pediocin differed only in a single amino acid at their C-terminal. The genes coding for coagulin production are present on a 10 kb stretch containing four genes syntenic with genes involved in the production of pediocin PA-1 (Marugg *et al*. 1992). It has been shown that only *ped*B and *ped*D are different in nucleotide sequence. By using SSCP and restriction digestion of the pediocin operon one can distinguish different pediocin PA-1 producers (Mora *et al.* 2000b). Interspecific pediocin PA-1 production was also observed in the case of *Pediococcus parvulus* (Bennik *et al.* 1997a). Since pediocin production is a plasmid encoded phenotype, spread of such character among LAB is possible (Kim *et al.* 1992). This indicates that there is a need to characterize the bacteriocin producing strains of LAB using molecular biology techniques such as comparison of rRNA and protein coding gene sequences (*gro*EL, *tuf*). Such a study would help to determine phylogeny and help assess natural spread of the gene for pediocin production among LAB (Mora *et al*. 1997; 2000a). Analysis of rRNA gene sequence is a standard method (ribotyping) of confirming and documenting strain identity, since alteration of the rRNA gene sequence does not induce phenotypic changes. This technique is more sensitive and reproducible than other conventional methods. rRNA gene sequences are often useful in determining genetic relatedness among strains and investigating their phylogenetic relationship and evolution (Jager and Harlander 1992; Schleifer *et al.* 1995).

Recently, use of the *tuf* gene sequences in the molecular identification of enterococci has received world wide attention. The *tuf* gene is highly conserved and ubiquitous among eubacteria rendering it a better candidate

for taxonomic classification in LAB (Ke *et al.* 1999; Ventura *et al*. 2003). The *tuf* gene encodes for the elongation factor-Tu (EF-Tu), which is a GTP binding protein and loads the amino-acyl tRNA molecule on to the ribosome during translation of protein. The *tuf* gene is present in either one or two copies per bacterial genome. In several enterococci it has been demonstrated that two divergent copies of genes encoding EF-Tu are present (Ke *et al.* 1999; 2000).

Analysis of the *tuf* gene sequences in LAB species can complement the use of 16S rRNA markers for taxonomic characterization. Combination of these approaches can further help distinguish closely related species. Experimental evidences proved that there being two copies of *tuf* genes in many enterococci, these are *tuf*A and *tuf*B. Sequence data revealed that both the genes are highly conserved at the amino acid level. The *tuf*A gene is known to be found in most of enterococci while *tuf*B is present only in few species (Ke *et al*. 1999).

In the previous chapter, the potent bacteriocinogenic strain of *Ent. faecium* C20 (PH-1) was identified and characterized by using conventional microbiological tools. This chapter deals with the studies on molecular identification of native *Ent. faecium* PH-1, analysis of plasmid linked traits and use of biochemical and molecular tools for studying pediocin PA-1 production.

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 Fine chemicals

Agarose, Ampicillin, Nisin, novobiocin, glutarldehyde and other fine chemical/reagents required in molecular biology experiments are the same as enlisted in the previous chapter (section **2.2.1.1**). Acyrlamide, bis-acrylamide, glycine, tricine and various proteolytic enzymes such as trypsin, pronase and proteinase-K, and TEMED, DTT and APS were purchased from SRL (Mumbai). β-mercaptoethanol was purchased from Merck (Mumbai). X-Gal, IPTG, rubidium chloride was purchased from HiMedia. Different organic solvents such as

acetone, chloroform, methanol, ethanol, isopropanol, acetonitrile, etc. were obtained from Qualigens fine chemicals (Mumbai). MOPS buffer, TFA and calcium chloride were obtained from Sigma. Restriction enzyme *Eco*RI was purchased from Promega, and CIAP was from MBI Fermentas (Lithuania).

3.2.1.2 Bacterial strains and cultivation media

The LAB bacteriocin indicator strains used in this study were *Lactobacillus farciminis* MD, *Pediococcus pentosaceus* C6, *Leuconostoc mesenteroides* NRRL B640, *Ped. acidilactici* NRRL B1153, and *Ent. faecalis* ATCC344. The bacteriocin producing strains were *Ent. faecium* PH-1 (referred as C20 in chapter 2), *Ped. acidilactici* CFR K7 (Ramesh 2000) and *Ped. parvulus* ATO 77 (Bennik *et al.* 1997a). All LAB were cultivated in MRS medium as described in section **2.2.2.1**. The food-borne pathogenic indicator strains like *Listeria monocytogenes* FMD (laboratory isolate), *List. monocytogenes* V7 and Scott-A were received from Dr. A. K. Bhunia, Purdue University, USA and other listerial strains shown in Table 3.7 were from the culture collection of the department of Food Microbiology, CFTRI. These pathogenic strains were grown in BHI medium as described previously (**2.2.2.1**). Prior to use in experiments, the cultures were propagated twice in appropriate media. *E. coli* DH5□. F$^-$ D*lac*U169 (∅80d *lac* Z DM15) *rec* AI end AI *hsd* R17 *sup* E44 *thi*$^{-1}$ *gyr* A96 *rel* AI □$^-$) used in transformation experiment was received from New England Biolabs (Inc). It was cultivated in Luria-Bertani (LB) medium. LB broth consists of 1% each of peptone and sodium chloride and 0.5% yeast extract, (pH 7.2) was prepared and sterilized by autoclaving.

3.2.1.3 Oligonucleotide primers

The chemically synthesized oligonucleotide primers were obtained from Sigma Aldrich (USA). The primers used in this study are mentioned in Table 3.1.

Table 3.1: Oligonucleotide primers used for amplification of *ped*A, *ped*AB and rRNA genes and sequencing

Primer Name	Primer sequence (5'-3')	Position (bp)	Expected size of amplicon (bp)	Source/ Reference
2A	TAAGGATAATTTAAGAAGAAGGAG	1043-1066	260	Bennik *et al.* 1997a
2B	TAAAATCACCCCTTTAATGA	1279-1299		
A5	GATGAAAAAAATTGAAAAAATAACT	1074-1096	186	Bennik *et al.* 1997a
A3	CATTTATGATTACCTTGATGTCCA	1236-1260		
KAS-RNA. F	CGA CGT CGG CTC AGG ATG AAC GCT GGC GGC	15-43	1,200	This study
KAS-RNA. R	GCT CTA GAG CGA TTA CTA GCG ATT CCG ACT TCG	1324-1353		
M13F*	CGCCAGGGTTTTCCCAGTCACGAC	24 MER	-	NEB 1224 & 1233
M13R*	AGCGGATAACAATTTCACACAGGA	24 MER		
PED. IMM-R	CCC TTT ATC AGG ATC CTT GGC TAG GCC	1616-1643	581 (with 2A primer)	This study

*Sequencing primers from the DNA sequencing facility of UDSC, New Delhi, India

3.2.1.4 Bacteriocin production media

For the production of bacteriocin, the test strains were grown in MRS broth. The influence of carbon source on bacteriocin production was studied by growing *Ent. faecium* PH-1 in MRS broth with different sugars. The MRS as production medium was constituted without glucose and was substituted with different carbon sources at 1% level as shown in Table 3.5.

3.2.2 Methodology

3.2.2.1 Bacteriocin assay

Bacteriocin activity in the culture supernatant of the producer strains and the cured strain was tested by spot-on-lawn method described by Venema *et al.* (1993) with certain modifications. The strains were grown in MRS broth and/or bacteriocin production media, for 18h at 37^{o}C. Cells were separated by

centrifugation at 10,000 g for 15 min at 4^{o}C. The CF was filtered through 0.22 m filter and used for bacteriocin assay. An aliquot of the CFS (5 ml) was spotted on MRS soft agar seeded with ~10^6 cells of the appropriate indicator strain. Plates were incubated at 37^{0}C and examined for the presence of clearing zones of at least 8 mm in diameter. Bacteriocin activity was expressed in terms of arbitrary units per ml (AU ml^{-1}), which was defined as the highest dilution of the culture filtrate which gave an inhibition zone of 8 mm.

3.2.2.2 Plasmid curing and analysis

The protocol described in a previous chapter for curing of plasmid from the strain *Lactococus casei ssp casei* C40 has also been used for the removal of plamsid from *Ent. faecium* PH-1 (section **2.2.2.8.2**) was used with some modifications. MRS broth (5 ml) containing 1 mg ml^{-1} novobiocin was inoculated with an overnight grown culture of strain PH-1 and incubated at 45^{0}C for 24h. The culture was serially diluted in saline and pour plated on MRS agar plates and incubated at 37^{0}C till colonies appeared. The plates bearing separated colonies were selected and overlaid with 10^6 cells of freshly grown *Lact. farciminis* MD. Plates were incubated at 37^{0}C for 12-15h and checked for zone of growth inhibition. Colonies not inhibiting growth of the indicator (absence of inhibition zone) were considered as those which had been cured. These colonies were purified on MRS agar plates and propagated in MRS broth. Plasmid DNA from both the parental and cured strain was isolated by the method described by Anderson and McKay (1983) and the presence of plasmid was checked on agarose gel electrophoresis.

3.2.2.3 Carbohydrate fermentation test

Carbohydrate fermentation test of strain PH-1 and its cured derivative was carried out as described in previous chapter (section **2.2.2.6.10**) and the protocol described by Ray *et al.* (1989). Various sugars shown in Table 3.3. were used in the experiment.

3.2.2.4 Immunity function

For testing immunity of strain PH-1 and its cured derivative (DPH-1) to bacteriocin, CF and ammonium sulphate precipitate of the CF from strain PH-1 were used. Ammonium sulphate of 65% saturation was used to precipitate the bacteriocin from 100 ml of CF. The precipitate was recovered by centrifugation at 10, 000 rpm at 4^0C for 30min. The resultant pellet was washed with water and finally dissolved in 20 ml of sterile water. The concentrated bacteriocin was about five fold more active than the CF against the indicator strain *Lact. farciminis* MD. Effect of nisin (0.1 gm of nisin in 10 ml of 0.002 N HCl, 0.75% NaCl, prepared as described in section **2.2.2.3**) was tested against the WT PH-1 and DPH-1 strains.

3.2.2.5 Biochemical techniques

3.2.2.5.1 Bacteriocin adsorption studies

Adsorption of bacteriocin to cell membrane was carried out according to the method of Elegado *et al*. (1997). Briefly, cells of *Lact. farciminis* MD were grown in MRS broth at 37^{o}C for 18h. The cells were harvested by centrifugation, washed twice in sterile phosphate buffer 5 mM (pH 6.0) and resuspended in I0 ml CF of strain PH-1 pre-adjusted to pH 6.0. The cells were stirred at room temperature for 1h. The mixture was centrifuged and residual bacteriocin activity in the culture supernatant was determined. For testing the adsorption of bacteriocin to producer strain, overnight grown cells of strain PH-1 were used. The culture broth was heated to 70^{o}C for 30 min, cooled to room temperature and pH was adjusted to 6.0. Cells were stirred at room temperature for 1h. Cells were separated by centrifugation and residual bacteriocin activity in the culture supernatant was assayed. The amount of bacteriocin adsorbed was calculated as from the difference in activity of the CF to the residual activity after adsorption.

3.2.2.5.2 Bacteriocin purification

Native bacteriocin was prepared by growing the *Ent. faecium* PH-1 in 1000 ml reconstituted MRS broth supplemented with 2% lactose. The Cell adsorption method as described in section **3.2.2.5.1** was followed to concentrate the bacteriocin. The cell-bacteriocin complex was suspended in 20 ml of 100 mM NaCl, the pH adjusted to 2.0 with phosphoric acid and the mixture stirred at 4^0C for 2 to 10h to desorp the bacteriocin. The cell suspension was centrifuged at 10,000 rpm for 30min at 4^0C. The supernatant was filtered through a 0.22 m membrane filter, extensively dialyzed through a 1000 MWCO dialysis bag (Spectra Por7, Fisher Scientific, USA) against distilled water for 24h at 4^0C. The solution was freeze dried and dissolved in sterile distilled water.

3.2.2.5.3 RP-HPLC

The protocol described by Elegado *et al.* (1997) was used for subsequent purification by RP-HPLC. The bacteriocin was purified using a semi-preparative reverse phase column C18 (25 cm X 4.6 m̩) in a preparative chromatography Class VP system (LC8A, SPD-MOAVP, Schimadzu, Japan). The conditions for the gradient elution of solvent B (99.9% acetonitrile with 0.1% TFA) against solvent A (water with 0.1% TFA) at a flow rate of 1.5 ml min^{-1} were as follows: 0 to10 min, 99.7% solvent A and 0.3% solvent B; 10 to 20 min, 70% A and 30% B; 20 to 30 min, 40% solvent B and 60% A; 30 to 40 min, 40% solvent B and 60% solvent A; 40 to 50 min, 50% each solvent B and A; 50 to 55 min, 100% solvent B; 55 to 58 min, 100% solvent A. All protein peaks were collected manually, vacuum evaporated with a speed Vac concentrator (Biotron ECOSPIN 3180C) and subjected to bioassay (**2.2.2.7.4**) to identify the bacteriocin peak. Finally the active fraction was freeze dried and stored in a vacuum dessicator prior to analysis.

3.2.2.5.4 Tricine SDS-PAGE

The initial protocol described for denaturing acrylamide gel by Laemmli (1970) with modification for tricine gel as described by Schägger and von Jagow (1987) was followed.

The resolving gel was casted after mixing the following components:

Acrylamide	4.8 ml (*Acrylamide monomer solution (40% T/ 6% C)*
Gel buffer	3.5 ml (4X stock: 3M Tris HCl, pH 8.45 and 0.4% SDS)
TEMED	30 ml
APS (40%)	25 ml
Ultrafiltered water	3.7 ml

The mixture was poured between the glass plates. A thin layer of t-butanol was added over the gel to prevent contact with air. The gel was allowed to polymerize at room temperature for 15 min.

The butanol layer was removed and the stacking gel was cast with a mixture of the following components:

Acrylamide	0.5 ml
Gel buffer	1.2 ml
TEMED	15 ml
APS (40%)	8 ml
Ultrafiltered water	2.3 ml

The mixture was poured between the glass plates on the separating gel. The comb was inserted carefully without entrapping air bubbles. The gel was allowed to polymerize at room temperature for 15min. The sample was prepared by mixing loading dye (5X dye consisting of Tris-HCl 60 mM pH 6.8; glycerol 25%; SDS 2%; □-mercaptoethanol 14.4 mM; bromophenol blue 0.1%) with the sample (1:4 ratio) and heating in a boiling water bath for 15min. Ultralow molecular weight markers (Sigma, MO,USA) consisting of 26,600;17,000; 14,200; 6,500; 3,496 and 1,060 Da proteins were used for comparison.

Vertical gel electrophoresis was carried out using Hoefer Mini electrophoresis system (Hoefer, Germany). The two buffer tanks, anodal and cathodal were filled with 1X anode buffer (10X consists of 2 mM Tris-HCl, pH 8.9, 1% SDS) and 1X cathode buffer (10X consists of 0.2 M Tris, 1.6 M tricine, 1% SDS) respectively, and electrophoresis was carried out at 50 V for 6h till the dye-front migrated

through 90% of the gel. One half of the gel was cut out and used for staining while the other half was processed for bacteriocin K7 gel assay.

The gel was washed in ultrafiltered water for 5-10min and fixed in freshly prepared 5% glutaraldehyde solution for 1h. The gel was again washed with ultrafiltered water for 5min with three changes and was stained for 1h with Coomasie Brilliant Blue G250 (0.025% Coomasie Brilliant Blue G250 solution prepared in 10% acetic acid). The gel was destained in 10% acetic acid solution with several changes of destaining solution and photographed.

3.2.2.5.5 Gel assay of partially purified bacteriocin

The method described by Bhunia *et al.* (1987) for direct detection of antimicrobial compounds on SDS-PAGE was followed with certain modifications. One half of the gel that was not stained was fixed for 2h in isopropanol, acetic acid and water (20:10:70). The gel was washed in deionized water for 6h with frequent changes. The gel was placed in a sterile petriplate and overlaid with 5 ml of MRS soft agar containing 10^6 cells of freshly grown cells of *Lact. farciminis* MD. The plate was incubated at 37°C for 15h and examined for zone of growth inhibition.

3.2.2.5.6 Effect of temperature, pH, proteolytic enzymes and solvents on CF of *Ent. faecium* PH-1

Proteolytic enzymes and various solvents as described in Table 3.6 were used for this experiment. CF of PH-1 (90 ml) was mixed with 10 ml of proteolytic enzyme or organic solvent, incubated at room temperature for half an hour prior to checking for residual activity by the spot-on-lawn assay as described in section 3.2.2.1 against *Lact. farciminis* MD. The solvents used were methanol, ethanol, isopropanol, acetonitrile, cloroform, DMSO and acetone

along with reducing agents such as □-mercaptoethanol and 1 M DTT. The bacteriocin was heated at 100^0C for 30min and at 121^0C for 15min for

temperature tolerance tests. The antimicrobial activity of the bacteriocin was tested over a range of pH from 2 to 9.

3.2.2.6 Molecular biology techniques

3.2.2.6.1 DNA isolation

Genomic DNA from test strains of LAB were isolated by the method of Lewington *et al.* (1987) as described in section **2.2.2.8.3**. The plasmid DNA from all the test strains of LAB was isolated by the method of Anderson and Mckay (1983) as described in previously (section **2.2.2.8.1**).

For rapid plasmid DNA isolation from *E. coli,* the protocol described by Birnboim and Doly (1979) was followed with certain modifications:

- The *E. coli* DH5□ harbouring recombinant plamid was grown overnight (12-15h) in 2 ml of LB broth with appropriate antibiotic.
- The cells were harvested by centrifugation for 3min at 13, 000 rpm and the cell pellet resuspended in 0.2 ml of TED buffer (25mM Tris, 10 mM EDTA and 5 mM glucose).
- After 10min, 0.2 ml of lysis mix (0.2 N NaOH and 1% SDS) was added and were kept on ice for 10-15min.
- A portion of 0.3 ml chilled potassium acetate (3 M, pH 4.8) was added and samples were kept on ice for 15min.
- The mixture was centrifuged for 10min and the aquous phase was transfered to a fresh tube and DNA precipitated with 0.6 volume of isopropanol.
- The DNA was recovered by centrifugation at 13, 000 rpm for 15min and the pellet was washed with 70% chilled ethanol.
- The DNA pellet was dissolved in 20 ml of sterile water or in 1X TE buffer and analyze by agarose gel electrophoresis.

3.2.2.6.2 Preparation of *E. coli* competent cells

The protocol described by Hanahan (1983) was followed with certain modifications.

- ⇨ A single colony of *E coli* was grown overnight in 5 ml of LB broth.
- ⇨ It was subcultured in 1: 200 ml LB (1 litre flask) and grown till an OD of 0.4 to 0.6 at 600nm was attained and the inoculum was chilled on ice for 10 – 15min.
- ⇨ The cells were harvested by centrifugation at 4000 rpm at 4^0C for 12—15min.
- ⇨ The cell pellet was resuspended in 20 ml of **RF1 buffer** (100 mM Rb Cl_2; 50 mM $MnCl_2$; 30 mM potasium acetate; 10 mM $CaCl_2$; 15% glycerine, pH5.8, filter sterilised and stored at 4^0C) and kept on ice for 1-2h with intermitant mixing.
- ⇨ Later, it was centrifuged at 4000 rpm at 4^0C for 12—15min. The cell pellet was resuspended in **RF2 buffer** (10 mM MOPS; 10 mM $RbCl_2$; 75 mM $CaCl_2$ and 15% glycerine pH 6.8, filter sterilised and stored at 4^0C) and kept on ice for 15min.
- ⇨ The cell suspension was aliquoted in 200 ml volumes, frozen in liquid nitrogen (if possible) and stored at -20^0C.

3.2.2.6.3 DNA ligation and transformation of *E. coli*

A Vector/inert ratio of 1:2 to 1:3 was used for ligation. The reaction mixture was mixed in an eppendorf tube by addition of vector, insert (PCR product), 10X ligation buffer, BSA, PEG, 10 U of T_4 DNA ligase (MBI) and deionized water. The reaction mixture was incubated at 16^0C for blunt ended ligation and at 22^0C for 4h or at 4^0C overnight for cohesive end ligation.

For transformation of *E. coli* standard protocol was followed (Hanahan 1983; Sambrook and Russell 2001). One hundred ml of competent cells and 10 ml of plasmid DNA or ligation mixture were mixed together and kept on ice for 30min with intermitant shaking. The cells were subjected to heat shock at 42^0C for 45sec and cooled again on ice. Prewarmed 1 ml LB was added to the

transformation mix and incubated at 37^0C for 60min with shaking. The transformation mixture was plated in LB agar plates containing 50 mg ml^{-1} X-Gal (prepared in dimethylformamide) and 1mM IPTG (prepared in distilled water and filter sterilized) for use in selection of white, transformed colonies.

3.2.2.6.4 Shot-gun cloning

Total genomic DNA isolated from the strain *Ent. faecium* PH-1 was digested with the restriction enzyme *Eco* RI. Fragments ranging from 0.5 to 5 kb were gel eluted, purified using the QIA Quick gel extraction kit (Qiagen, Germany) and ligated to the pUC19 vector pre-digested with *Eco* RI and dephosphorylated with CIAP. Inserts from selected colonies were taken for sequencing.

3.2.2.6.5 Polymerase chain reaction

Standard protocol was followed (Sambrook and Russell, 2001) with certain modifications for carrying out the polymerase chain reaction (PCR). For *in vitro* amplification, *Taq* DNA polymerase (Bangalore Genei) was used.

Table 3.2: Standard PCR components

Components	Vol (ml)	Final concentration
Template	4.0	~1-2 ng
Primers (F/R)	2.0	0.2 mM
dNTPs mix (10 mM each)	1.0	0.2 mM each
10X reaction buffer with 15 mM $MgCl_2$	5.0	1X
Taq DNA polymerase	0.5	0.03 U/ml
Sterile deionized water	X ml	
Total volume of reaction	25 ml	

Each PCR component was taken in a thin-walled 0.2 ml reaction tube with the help of fresh sterilized tips, mixed by brief spinning in a microcentrifuge tube.

The reaction was carried out in a Thermocycler Gene Amp PCR system 9700 (Perkin –Elmer, USA) with following parameters given for each target gene:

3.2.2.6.5.1 PCR of rRNA gene

Total genomic DNA was used as a template for amplification of rRNA gene from *Ent. faecium* PH-1. The primers used were KAS-RNA.F and KAS-RNA.R as forward and reverse primers (Table 3.1).

Following cycle parameters were used for PCR

	Temp (^{0}C)		Time (min)	
Initial denaturation 95		3		
Denaturation	94		0.4	35 cycles
Primer annealing	48		0.4	
Extension	72		2.0	
Final extension	72		10.0	

3.2.2.6.5.2 PCR of *ped*A and *ped*AB gene

Total genomic DNA was used as a template along with the components described in Table. 3.2 for PCR amplification of *ped*A gene from strain *Ent. faecium* PH-1.

The two sets of primers used were 2A and 2B as forward and reverse primer and A5 and A3 as forward and reverse primer (as shown in Table 3.1) *Ped. parvulus* ATO 77 was used as a reference strain.

	Temp (^{0}C)		Time (mins)	
Initial denaturation 95		3		
Denaturation	94		0.4	35 cycles
Primer annealing	50		0.4	
Extension	72		0.3	
Final extension	72		10.0	

The primers used were 2A and PED.IMM-R for PCR amplification of *ped*AB genes of *Ent. faecium* PH-1. The conditions were same as described for

amplification of *ped*A, except that the extension time was a min per cycle. The PCR product was purified and subsequently cloned in pTZ57R/T vector prior to sequencing.

3.2.2.6.5.3 PCR for probe preparation

Amplification of *ped*A gene of strain *Ped. parvulus* ATO 77 was carried out for generation of a DNA probe. The PCR primers used were 2A and 2B. The PCR conditions were as described in 3.2.2.6.5.2. After amplification, the PCR

product was analyzed by agarose gel electrophoresis and purified using Wizard PCR preps DNA purification system (Promega, Madison, WI, USA).

3.2.2.6.6 DNA dot-blot hybridization

3.2.2.6.6.1 Non-radioactive labeling of the probe

Labelling of 260 bp PCR amplicon was carried out using DIG dUTP DNA labeling and detection kit purchased from Boehringer Mannheim (Mannheim Germany). The labeling kit mainly consists of Hexanucleotide mix, dNTPs mix (with DIG dUTP) and Klenow enzyme.

The purified PCR product (15 ml) was taken and denatured by heating for 15 min in boiling water bath followed by snap cooling in an ice-bath. The labeling components consisting of denatured target DNA, 2.0 ml hexanucleotide mix, 2.0 ml of dNTP mixture, 1.0 ml of Klenow enzyme were mixed together briefly spun and incubated at 37^0C for 12h in order to incorporate DIG dUTP during synthesis of complementary strand *in vitro*. The reaction was terminated by adding 2.0 ml of 0.2 M EDTA. The free nucleotides and labeled DNA were separated by overnight precipitation with $1/10^{th}$ volume of LiCl and 2.5 volumes of absolute ethanol at -20^0C. The DNA was recovered by centrifugation at 10, 000 rpm for 15min at 4^0C, followed by washing with 100 ml of 70% ethanol.

The dried pellet of the labeled DNA was dissolved in 50 ml of TE buffer and stored at –20^{0}C till further use.

3.2.2.6.6.2 DNA spotting on the membrane

The crude plasmid preparation from strain *Ped. acidilactici* CFR K7, *Ent. faecium* PH-1 and its cured derivative (DPH-1) were used for dot-blot experiments. Heat denatured test DNA sample of 10 ml were spotted on Hybond Nylon membrane (Amersham International, UK) according to the method described in Sambrook and Russell (2001) prior to hybridization with *ped* A probe.

3.2.2.6.6.3 Prehybridization of the membrane

The membrane was transferred to a heat-resistant polyethylene bag. The hybridization buffer 5X SSC, *(10X SSC consists of 1.5M NaCl and 0.15M Sodium citrate pH 7.2)*, 0.1% (w/v) N-Laurylsarcosine and 0.02% (w/v) SDS was pre-warmed to 68^{0}C and the desired quantity of the same was added to the polyethylene bag containing the membrane. The bag was sealed and incubated overnight at 68^{0}C in a hybridization oven (GATC-HYBE, Germany).

3.2.2.6.6.4 Hybridization with probe

The pediocin gene (*ped*A) probe (5 ml) was mixed with 50 ml hybridization solution and heat denatured in a boiling water bath for 10min, followed by snap-cooling in an ice-bath. The denatured probe was added on to the membrane and the bag was resealed. The reaction of hybridization was continued at 68^{0}C in hybridization oven for another 6h.

3.2.2.6.6.5 Post-hybridization

The hybridization buffer was discarded and the membrane washed twice with 50 ml of high stringency wash buffer (2X SSC, 0.1% SDS) at room temperature with gentle shaking. Followed by washing twice with 50 ml of low stringency wash buffer (0.1% each SSC and SDS) for 15min at 68^{0}C with mild agitation.

3.2.2.6.6.6 Color development

After post-hybridization washes, the membrane was rinsed briefly at room temperature in maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5) and incubated in 50 ml of 1X blocking solution (10X stock: blocking reagent 10% (w/v) was dissolved in maleic acid buffer) for 30min. Subsequently, the membrane was incubated in 10 ml of antibody solution (1: 5000 Anti-DIG-AP conjugate prepared in 1X blocking solution) at room temperature for 45min under gentle shaking. The membrane was washed twice for 15min in 50 ml of maleic acid buffer and equilibrated with 20 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 5min. The detection buffer was discarded and the membrane incubated in the dark in 10 ml of freshly prepared color solution (45 ml NBT and 35 ml X-phosphate per 10 ml of detection buffer). After the desired band appeared, the reaction was terminated by washing the membrane for 5min with excess of deionized water. The results were documented using a Hero Lab gel documentation unit.

3.2.2.6.7 Southern hybridization

3.2.2.6.7.1 Capillary transfer of plasmid DNA

Plasmid DNA isolated from test strains was transferred from agarose gels to nylon membrane using the capillary method. For this procedure and for subsequent hybridization with probe, the protocol described by Southern (1975) was followed with certain modifications as described by Sambrook and Russell (2001).

3.2.2.6.7.2 Procedure for transfer

After electrophoresis (0.8% agarose gel), the gel was stained with ethidium bromide and viewed under transilluminator to ensure separation. The gel was rinsed with deionized water and DNA in the gel was subjected to depurination with 0.2 N HCl treatment with mild agitation till the loading dye changed its color. After depurination, the gel was again rinsed with deionized water and subjected

to denaturation treatment with 1.5 M NaCl and 0.5 M NaOH. The reaction was carried out at room temperature for about 20 min with mild agitation. The process of denaturation was continued for another 20min by replacing with fresh denaturation solution. The gel was again rinsed with deionized water and incubated with 100 ml of neutralization solution (1.5 M NaCl, 0.5 M Tris HCl pH 7.0). The neutralization was carried out for another 20min by replacing with fresh solution.

Meanwhile, the two troughs in the transfer tank were filled with 75 ml of 10 X SSC. A Whatmann (3 MM thickness) filter paper having dimension of 14.8 X 22 cm was placed on the platform of the transfer tank and the ends of the filter paper were dipped into the buffer. The filter paper was allowed to get wet completely with SSC buffer and any air bubble on it was carefully removed by rolling a glass rod over it. The nylon membrane and Whatmann sheets (3MM) were cut to the exact size of the gel and were equilibrated with 10X SSC solution for 5min. Two of the wet Whatmann sheets were placed in the middle of the platform, over which the gel was placed in an inverted fashion. The right top side of the gel was cut to serve as an identification mark. Parafilm strips were aligned all around the gel so that efficient capillary transfer of buffer takes place through out the gel. The nylon membrane (which was also marked at right side top) was placed over the gel. The other four Whatmann filter papers pre-soaked in 10X SSC were placed on the membrane and the air-bubbles if any on this stacking of transfer were removed by rolling the glass rod over it. Stacks of dry hand made filter paper were placed over the Whatmann filter paper and a weight of ~500 gm was kept over the entire stack. The capillary transfer of buffer from troughs of the transfer tank to the dried filter paper through the gel was allowed for around 24h with intermittent changes of dry paper towel and refilling of transfer buffer. After sufficient transfer, weight, paper towels and the Whatmann papers were removed and the position of the wells was marked on the membrane. The nylon membrane was carefully separated from the gel and submerged in the solution of 6X SSC. The membrane was incubated in above

solution for 5min and air-dried. The efficiency of transfer of DNA from the gel was checked by restaining in ethidium bromide. The membrane was subjected for cross-linking of the DNA by using an UV transilluminator (Photodyne, USA) for 2min. The membrane was then stored under vacuum in room temperature till it was used for hybridization.

3.2.2.7 Protein estimation

The proteins were estimated by the method of Bradford (1976). The preparation of the reagents and protocol followed is described as follows.

1. The dye reagent was prepared by vigorous homogenization or agitation of 100 mg of Commassie Brilliant Blue G-250 in 50 ml of 95% ethanol. This solution was mixed with 100 ml of 85% phosphoric acid and diluted with water to 1 Litre and filtered. This reagent is stable at room temperature for atleast 2 weeks.
2. Standard assay was performed by addition of 5 ml of dye reagent into 0.1 ml protein samples containing 20 to 140 mg of protein, and/or
3. Microassay was performed by addition of 0.2 ml of dye reagent to 0.8 ml sample containing 1-20 mg of protein.
4. The reaction was mixed and incubated for 5-30min.
5. The absorbance was measured at 595nm using quartz cuvette in a spectrophotometer, against a blank prepared from 0.1 ml (0.8 ml for microassay) of sample buffer and 5 ml (0.2 ml for the microassay) of reagent. BSA was used as a standard for protein estimation.

3.2.2.8 Nucleotide sequence analysis

3.2.2.8.1 DNA sequencing

Unidirectional DNA sequencing was carried out using M13F and M13R primers by dideoxy chain termination method of Sanger *et al.* (1977). The reaction was carried out in a automatic DNA sequencer (ABI prism, Applied Biosystem, Perkin Elmer, USA) with fluorescent dideoxy chain terminators at the DBT supported facility; UDSC, New Delhi. The sequences were analyzed using Clone Manager

Version 5. The BLAST search was carried out by the method described by Altschul *et al*. (1997). Multalin was carried using the method of Corpet (1988).

3.2.2.8.2 Nucleotide sequence deposition

rRNA gene sequences reported in this chapter have been deposited in the GenBank under Accession No. AY723748, and the partial *tuf* gene sequences are under Accession No. AY753890.

3.2.2.8.3 Bacterial strain deposition

The native pediocin PA-1 producing strain of *Ent. faecium* PH-1 has been deposited at Microbial Type Culture Collection and GenBank at the facility of IMTECH Chandigarh (India) under the Accession No. *Pediococcus acidilactici* PH-1 MTCC 5153.

3.3 RESULTS AND DISCUSSION

3.3.1 Molecular cloning of rRNA gene

Nucleotide sequences of rRNA gene of selected LAB strains viz *Ped. acidilactici* DSMZ20284 (acc. no:M58833), *Ped. acidilactici* (Mora *et al*. 2000a) acc. No: AJ249539, *Lactococcus lactis* (AY675242) and *E. coli* (NC_000913) were aligned by using multiple sequence alignment programme (Morgenstern 1999) and highly conserved region were selected for designing the forward and reverse primers as shown in Figure 3.1.

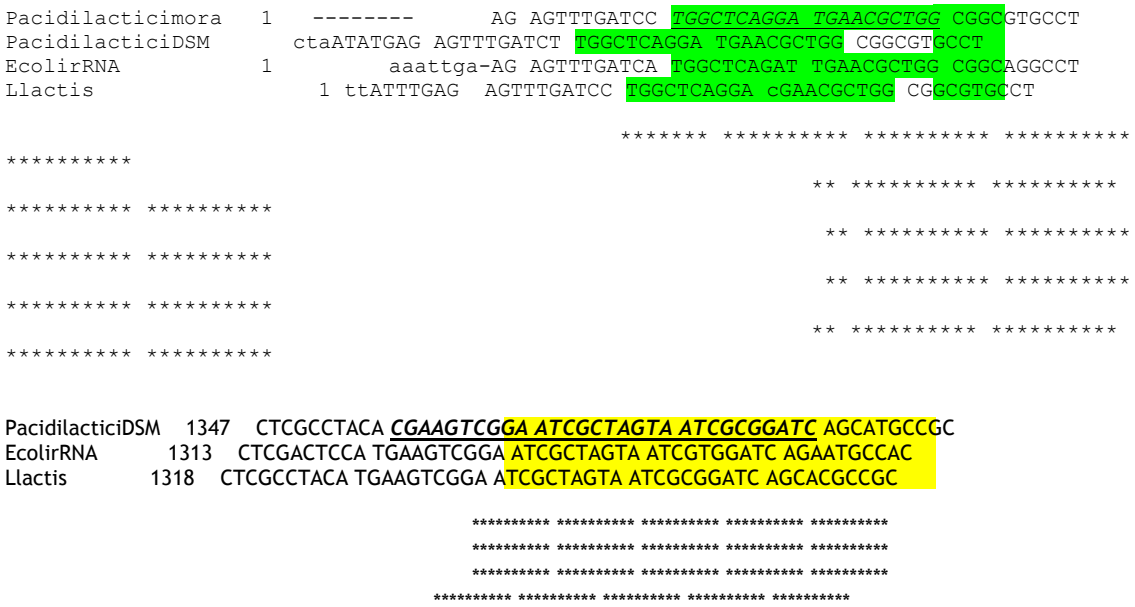


Figure 3.1: Multiple sequence alignment of 16S rRNA genes of *E. coli*, *L. lactis* and *Ped. acidilactici*. (coloured region indicates the region selected for primer designing based on conserved sequences)

The nucleotide sequences of these primers are given in Table 3.1. Average length of the primers are 30 bases since restriction sites were introduced into these primers. The rRNA of *Ped. acidilactici* sequences were considered since the native strain PH-1 was initially mis-identified as *Ped. acidilactici* and the the sequences of *E. coli* rRNA were selected for comparison since *E. coli* represents an important bacterium in molecular biology. The rRNA sequence from *L. lactis* was inducted as a representative of the LAB. Total genomic DNA of PH-1 was used For PCR amplification. The primer annealing temperature of

48^0C was found to be suitable for clear amplification of 1.2 kb PCR product as expected (Figure 3.2). There was no amplification at higher annealing temperatures while non-specific amplification occurred at lower annealing temperature of 45-47^0C.

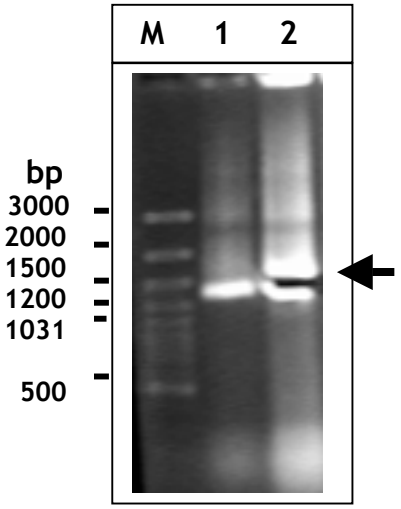


Figure 3.2: Agarose (1%) gel analysis of PCR amplicon of rRNA gene of *Ent. aecium* PH-1. Lane 1, PCR product of 1,200 bp product obtained and lane 2 is a corresponding eluted band for cloning. M is a Generuler^{TM} 1000 bp ladder (MBI). Arrow indicates amplicon of the expected size.

Gel analysis of the PCR product is shown in Figure 3.2. The desired fragment was excised from the gel and purified using an QIAQuick gel extraction kit and ligated to pTZ57R/T vector using 5U of T_4 DNA ligase. The recombinants were selected based on blue/white colonies and restriction analysis. One of the clones after confirmation of the insert was sent for DNA sequencing. The sequence data obtained using M13 forward (~450bp) and reverse primer (~450bp) was subjected to BLAST. The BLAST search data indicated that the analysed sequence of DNA was high in similarity with that of rRNA genes from *Ent. faecium* followed by *Ent. hirae.*

The restriction map of the rRNA gene from PH-1 is presented in Figure 3.3 and compared with that from *Ent. faecium*. It is clear that both the organisms have identical restriction sites, since their sequences share 99.5% sequence homology.

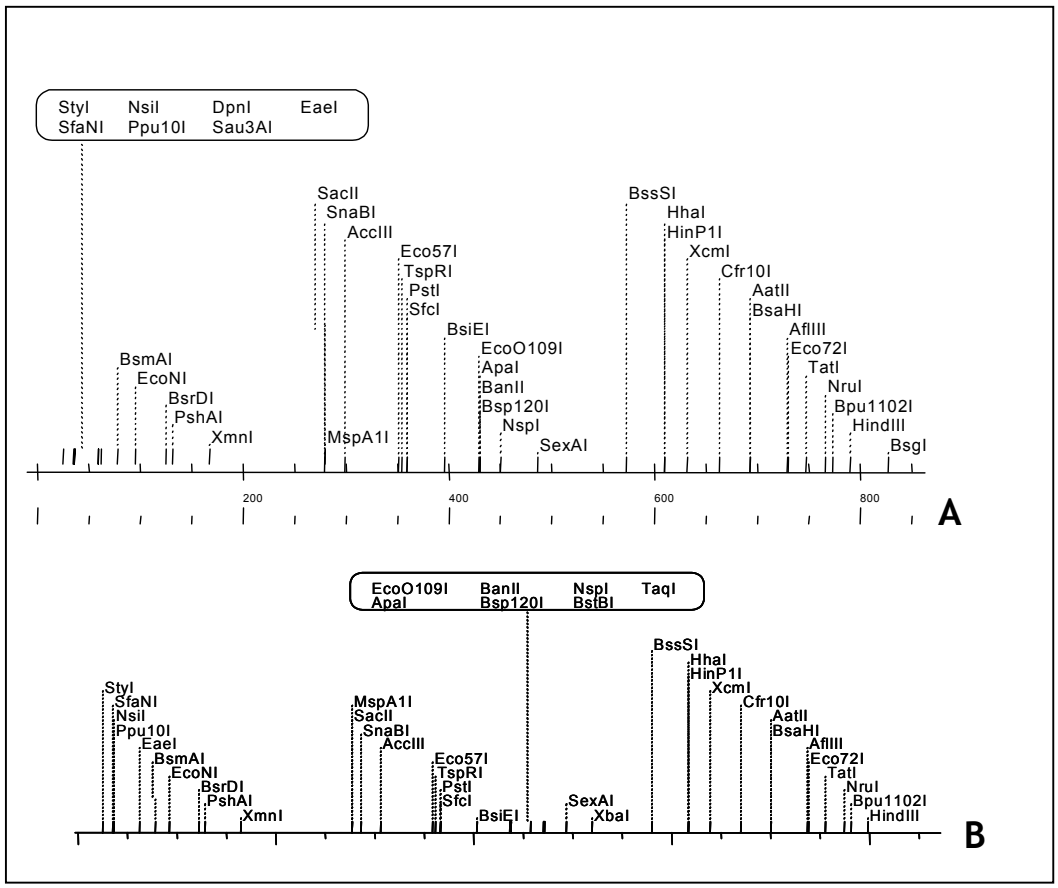


Figure 3.3: Comparison of restriction map of (A) *Ent. faecium* PH-1 with that of (B) *Ent. faecium* (GenBank Acc. No. AJ276355).

3.3.2 Homology sequence of rRNA gene of PH-1

The sequence of the rRNA gene from PH-1 was homologous to an extent of 99% with that of many *Ent. faecium* strains. These include acc. nos. AJ276355;

AY172570; AF070223, Y18294; AY675247 etc where in the E value also was 0.0 indicating greatest similarity with sequence of rRNA from PH-1. rRNA sequences from strains of *Ent. hirae*, Y17302 and AJ276356 and *Ent. azikeevi* acc. No. AJ309563 were also similar (99%) to that of PH-1. Multiple sequence alignment using CLUSTAL W (1.82) was carried out using the rRNA gene sequences of strain PH-1 with those it was highly homologous. Sequences from the rRNA gene of *Ent. faecium* acc. no. AJ276355. *Ent. faecalis* acc. No. AJ271856. *Ent. mundt* acc. No. AB066266, *Ped. acidilactici* acc no. : AJ249893. and *E. coli* acc. No. AJ605115 were used for alignment (Figure 3.4).

```
PH-1                          ---------------CGAGCTCGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGC
45
Enfaem                                  ----------------------
TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 39
Entfacalis                       ---------
AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 51
Entmundt                            -------------
TTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGC 47
Pacidltc              NCTAATATGAGAGTTTGATCTTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGC 60
Ecoli                   ------------------------------------------------------------

PH-1            AAGTCGAACG--CTTCTTTT--TCCACCGGAGCTTGCTC-CACCGGA------------A 88
Enfaem          AAGTCGAACG--CTTCTTTT--TCCACCGGAGCTTGCTC-CACCGGA------------A 82
Entfacalis      AAGTCGTACG--CTTCTTTT--TCCACCGGAGCTTGCTC-CACCGGA------------A 94
Entmundt        AAGTCGAACG--CTTCTTTT--CCCACCGGAGCTTGCTC-CACCGGG------------A 90
Pacidltc        AAGTCGAACGAACTTCCGTTAATTGATCAGGACGTGCTTGCACTGAATGAGATTTTAAYA 120
Ecoli           -------------------------ACAGGAAGCAGCTT--GCTGTTT-----------C 22
                                          *   *     ***    * *

PH-1            AAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA 148
Enfaem          AAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA 142
Entfacalis      AAAGAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA 154
Entmundt        AGAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA 150
Pacidltc        YGAAGTNAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGCAGGGGATAA 180
Ecoli           GCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAAC-TGCCTGATGGAGGGGGATAA 81
                       ******* ************    **** *** ****     *  ********

PH-1            CACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAA 208
Enfaem          CACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAA 202
Entfacalis      CACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAA 214
Entmundt        CACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTCGTTTTGAAA 210
Pacidltc        CACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAA 240
Ecoli           CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGA---------GGGG 132
                *   ******* *  ********** *****     **  *    *

PH-1            GGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAA 268
Enfaem          GGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAA 262
Entfacalis      GGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAA 274
Entmundt        GGCGCTTTACGGTGCCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAA 270
Pacidltc        GATGGCT-CTGCTATCACTTCTGGATGGACCCNCGGCGCATTAGCTAGTTGGTGAGGTAA 299
Ecoli           GACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAA 192
                *           *    *    *****  ***     * ********** **** *****

PH-1            CGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACT 328
Enfaem          CGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACT 322
Entfacalis      CGGCTCACCAAGGCTACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACT 334
Entmundt        CGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACT 330
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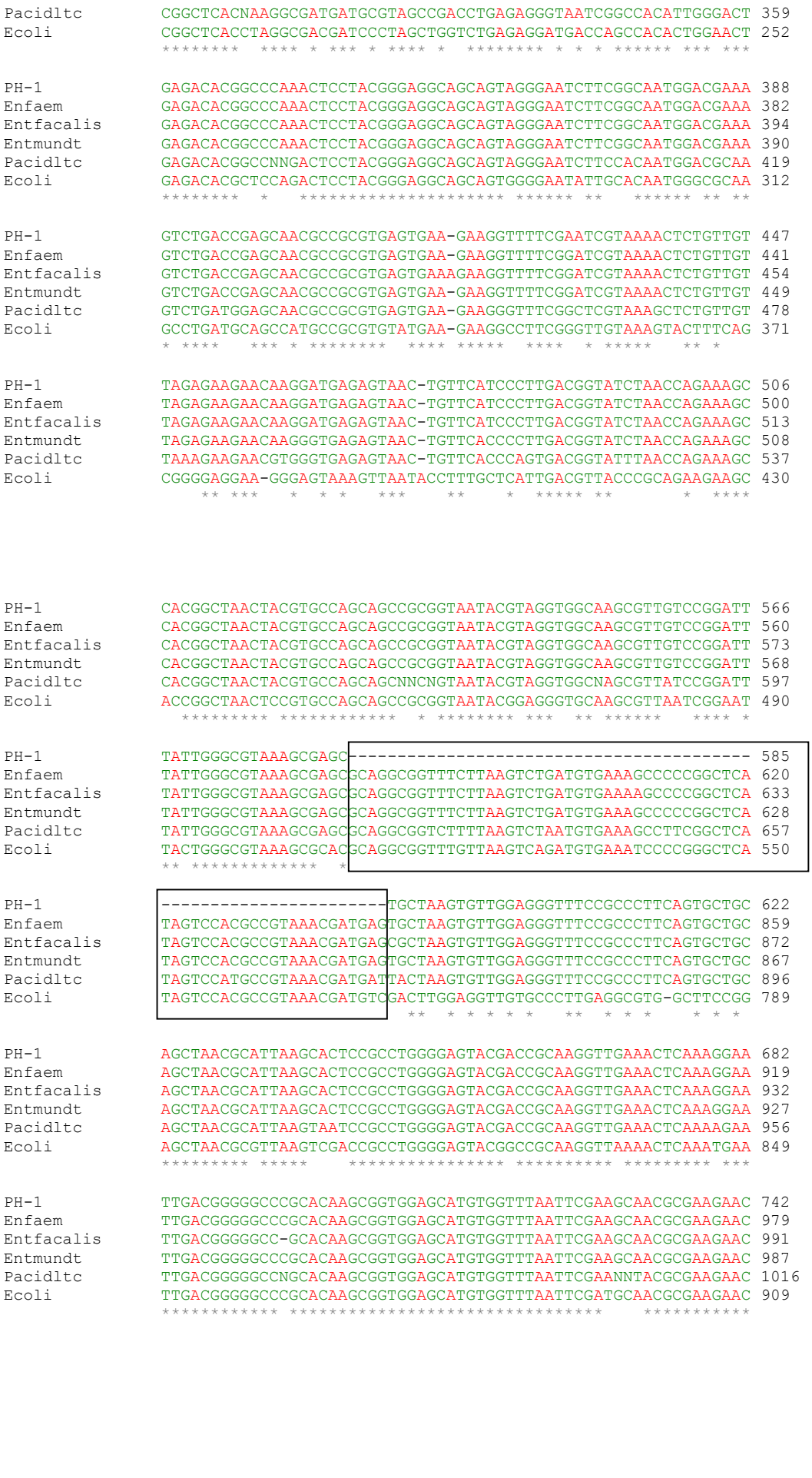
Pacidltc CGGCTCACNAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACT 359
Ecoli CGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACT 252
PH-1 GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAA 388
Enfaem GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAA 382
Entfacalis GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAA 394
Entmundt GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAA 390
Pacidltc GAGACACGGCCNNGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAA 419
Ecoli GAGACACGCTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA 312
PH-1 GTCTGACCGAGCAACGCCGCGTGAGTGAA-GAAGGTTTTCGAATCGTAAAACTCTGTTGT 447
Enfaem GTCTGACCGAGCAACGCCGCGTGAGTGAA-GAAGGTTTTCGGATCGTAAAACTCTGTTGT 441
Entfacalis GTCTGACCGAGCAACGCCGCGTGAGTGAAAGAAGGTTTTCGGATCGTAAAACTCTGTTGT 454
Entmundt GTCTGACCGAGCAACGCCGCGTGAGTGAA-GAAGGTTTTCGGATCGTAAAACTCTGTTGT 449
Pacidltc GTCTGATGGAGCAACGCCGCGTGAGTGAA-GAAGGGTTTCGGCTCGTAAAGCTCTGTTGT 478
Ecoli GCCTGATGCAGCCATGCCGCGTGTATGAA-GAAGGCCTTCGGGTTGTAAAGTACTTTCAG 371
PH-1 TAGAGAAGAACAAGGATGAGAGTAAC-TGTTCATCCCTTGACGGTATCTAACCAGAAAGC 506
Enfaem TAGAGAAGAACAAGGATGAGAGTAAC-TGTTCATCCCTTGACGGTATCTAACCAGAAAGC 500
Entfacalis TAGAGAAGAACAAGGATGAGAGTAAC-TGTTCATCCCTTGACGGTATCTAACCAGAAAGC 513
Entmundt TAGAGAAGAACAAGGGTGAGAGTAAC-TGTTCACCCCTTGACGGTATCTAACCAGAAAGC 508
Pacidltc TAAAGAAGAACGTGGGTGAGAGTAAC-TGTTCACCCAGTGACGGTATTTAACCAGAAAGC 537
Ecoli CGGGGAGGAA-GGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGC 430
PH-1 CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATT 566
Enfaem CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATT 560
Entfacalis CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATT 573
Entmundt CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATT 568
Pacidltc CACGGCTAACTACGTGCCAGCAGCNNCNGTAATACGTAGGTGGCNAGCGTTATCCGGATT 597
Ecoli ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT 490
PH-1 TATTGGGCGTAAAGCGAGC---------- 585
Enfaem TATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCA 620
Entfacalis TATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAAGCCCCGGCTCA 633
Entmundt TATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCA 628
Pacidltc TATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCA 657
Ecoli TACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCA 550
PH-1 ----------TGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGC 622
Enfaem TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGC 859
Entfacalis TAGTCCACGCCGTAAACGATGAGCGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGC 872
Entmundt TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGC 867
Pacidltc TAGTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGC 896
Ecoli TAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTG-GCTTCCGG 789
PH-1 AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA 682
Enfaem AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA 919
Entfacalis AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA 932
Entmundt AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA 927
Pacidltc AGCTAACGCATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAA 956
Ecoli AGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA 849
PH-1 TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 742
Enfaem TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 979
Entfacalis TTGACGGGGGCC-GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 991
Entmundt TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 987
Pacidltc TTGACGGGGGCCNGCACAAGCGGTGGAGCATGTGGTTTAATTCGAANNTACGCGAAGAAC 1016
Ecoli TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC 909

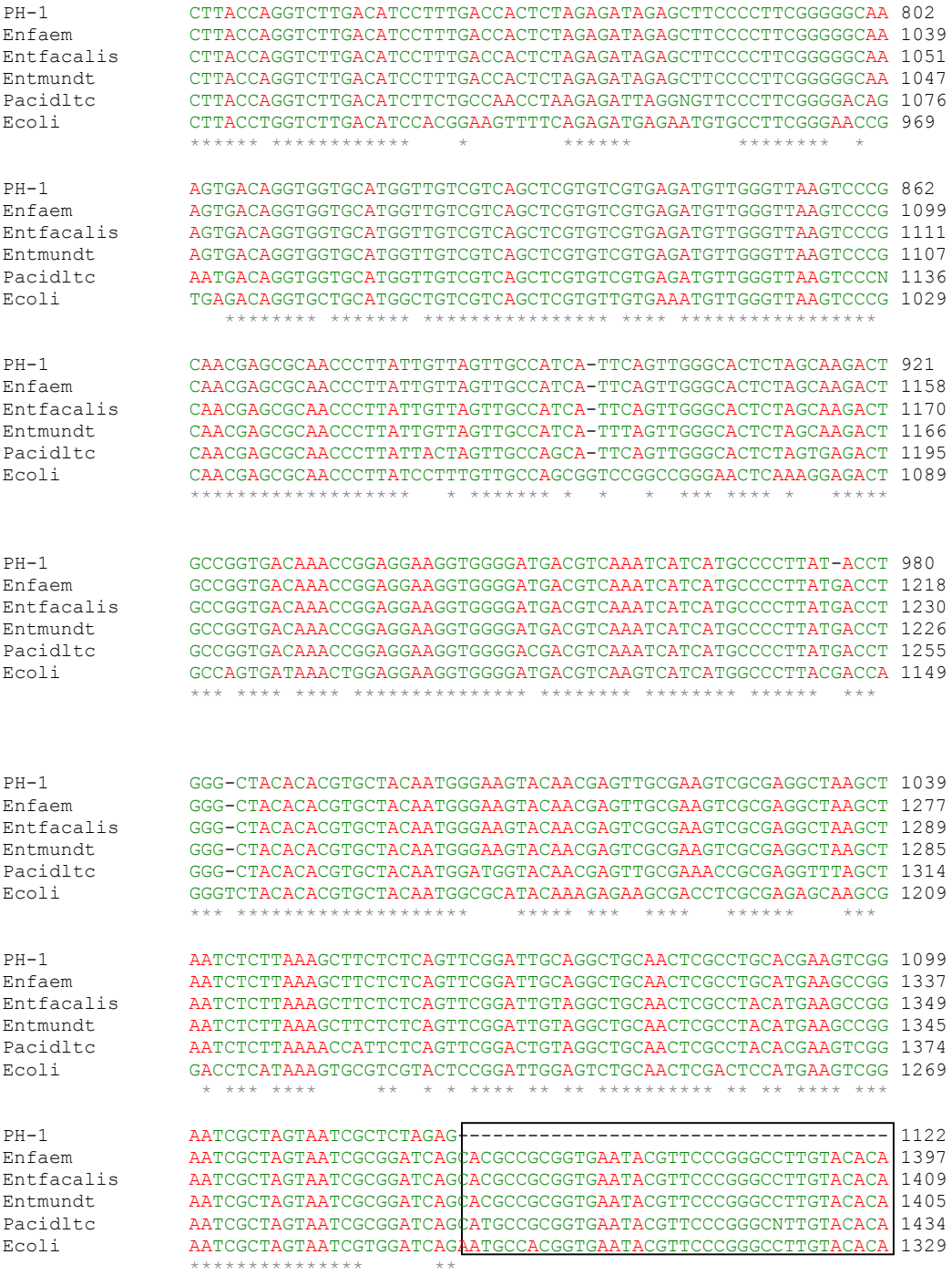

Figure. 3.4: Multiple sequence alignment of rRNA gene from PH-1 and enterococci, *Ped. acidilactici* and *E. coli*. ☐ region of rRNA not yet sequenced in PH-1. * indicates conserved region

A phylogenetic map was constructed using the rRNA gene sequences from the six strains listed above (Figure 3.5). Among the rRNA gene sequences

analyzed, *E. coli* is separated from the rest with *Ped. acidilactici* as its closest relative. PH-1 and *Ent. faecium* occupy one end of the tree while *Ent. mundti* occupies the middle position between *Ent. faecium* and *Ent. faecalis*. These results are in agreement with the data presented by Schleifer *et al*. (1995) on the phylogenetic tree of enterococci.

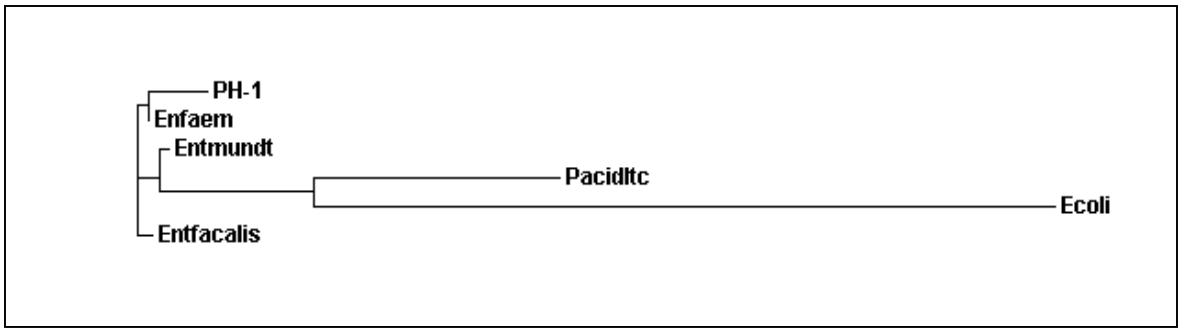

Figure 3.5: Phylogenetic analysis of rRNA gene sequences. PH-1: *Ent. faecium* PH-1; Enfaem: *Ent. faecium*; Entmundt: *Ent. mundtii*; Entfacalis: *Ent. faecalis;* Paciditc: *Ped. acidilactici* and Ecoli: *E. coli.*

3.3.3 Novel signature sequences of rRNA gene of PH-1

Three other strains of *Pediococcus* were used as a reference cultures for rRNA gene analysis. The sequences of their rRNA genes was aligned with the partial sequence from PH-1 as described by Morgenstern (1999). The results are presented in Figure 3.6.

```
                                                                                  253   257
PacidiDSMZ       238   TCTGCTATCA CTTC-TGGAT GGACCCGCGG CGCATTAGCT AGTTGGTGAG
DSMZ             247   TCTGCTATCA CTTC-TGGAT GGACCCNCGG CGCATTAGCT AGTTGGTGAG
PAC1.0           238   TCTGCTATCA CTTC-TGGAT GGACCCGCGG CGCATTAGCT AGTTGGTGAG
PH-1             234   ttcgggtgtc gctgaTGGAT GGACCCGCGG TGCATTAGCT AGTTGGTGAG

                       ********** **** ***** ********** ********** **********
                                       ***** ********** ********** **********
                                       ***** ****** *** ********** **********
                            271   273                                      307
PacidiDSMZ       287   GTAACGGCTC ACCAAGGCGA TGATGCGTAG CCGACCTGAG AGGGTAATCG
DSMZ             296   GTAACGGCTC ACNAAGGCGA TGATGCGTAG CCGACCTGAG AGGGTAATCG
PAC1.0           287   GTAACGGCTC ACCAAGGCGA TGATGCGTAG CCGACCTGAG AGGGTAATCG
PH-1             284   GTAACGGCTC ACCAAGGCCA CGATGCATAG CCGACCTGAG AGGGTgATCG
                       ********** ********** ********** ********** **********
                       ********** ********** ********** ********** **********
                       ********** ********** ********** ********** ***** ****
                       ********** ** ******* ********** ********** ***** ****
                              314      324  327
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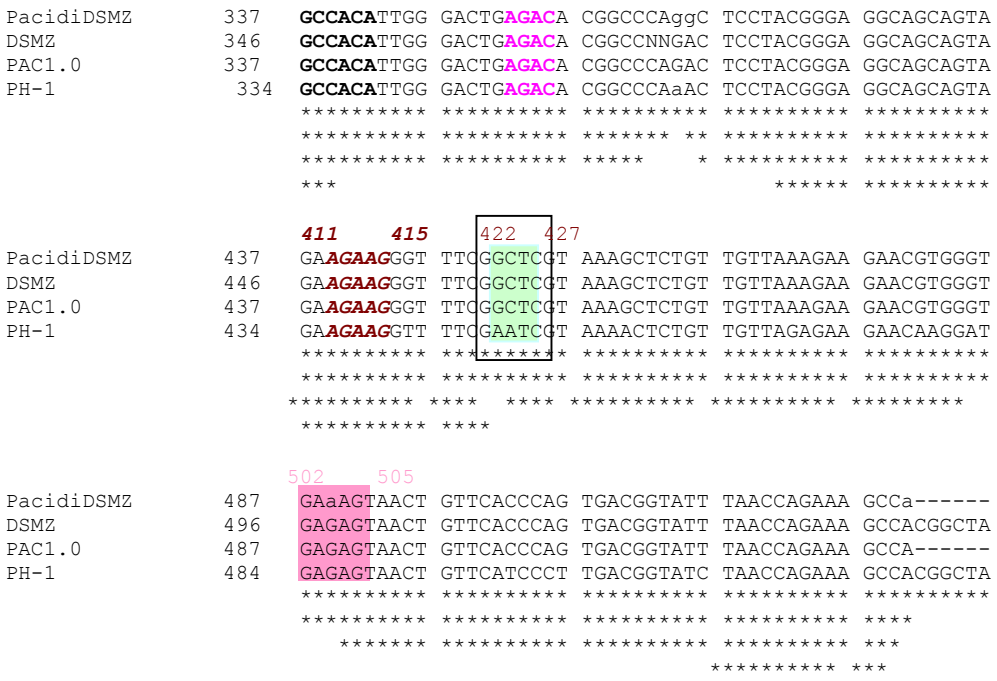

Figure 3.6: Multiple sequence alignment of rRNA gene of *Ent. faecium* PH-1 and other pediocin producing pediococci. Signature sequences in shaded with *E. coli* numbering given as described by Mora *et al.* (2000a).□Region showing difference nucleotide sequences.

Nucleotide sequence analysis of important house-keeping genes serves as a modern method of bacterial classification and determining phylogenetic relationships of many native isolates. The bacterial small ribosomal RNA (16S rRNA) gene transcribing 1, 500 NTPs component forms an important molecule for these studies. This gene is found in multiple copies and has highly conserved consensus sequences in the genome of organisms. Hence it is suitable for analysis as compared to other genes routinely used for multilocus typing approach in strain identifications (Mora *et al.* 2000a).

3.3.4 Molecular cloning of *tuf* gene

A shot gun cloning approach was followed in the cloning of DNA from total genomic DNA of strain PH-1 into the vector pUC19. The clones were randomly sequenced using M13 primers. The partial nucleotide sequences (452bp) of a 1.2 kb clone is shown in Figure 3.7 with its translated ORF.

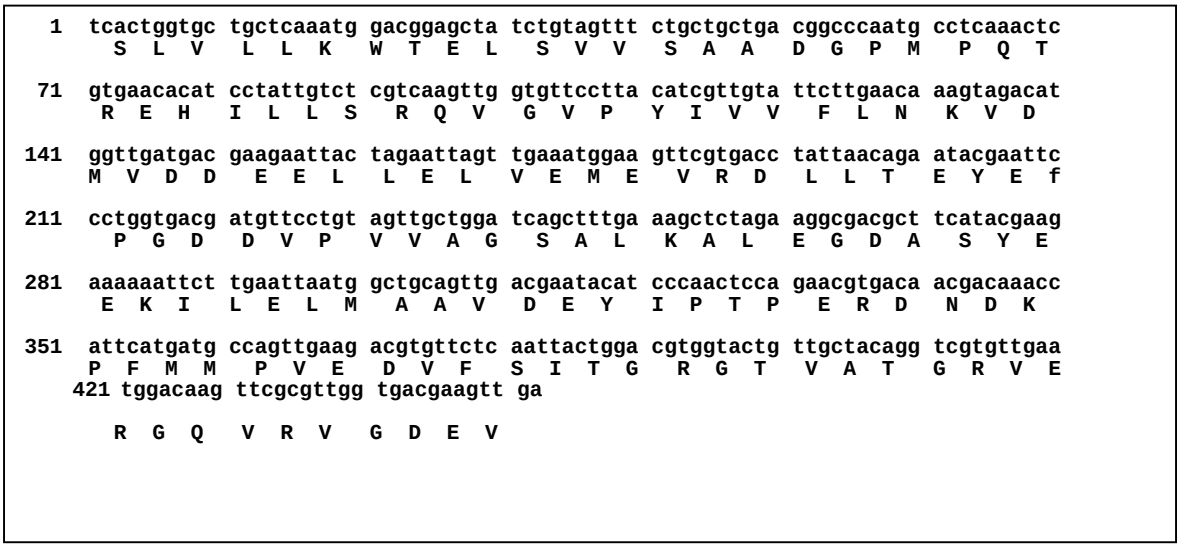

Figure 3.7: Partial nucleotide sequences of the *tuf* gene of PH-1. The putative ORF is also shown with single amino acid code.

BLAST search results indicated that *tuf* gene sequences of the strain PH-1 exhibited 99% sequence homology with the *tuf* gene sequence from *Ent. faecium* (acc.no. AF124222); 95% with that from *Ent. durans* (acc no. AF 274722); 94% with that from *Ent. hirae* (acc. No. AF274726) and *Ent. mundtii* (acc no. 274730) and 89% *with* that from *Ent. faecalis* V583 (AE016947). However the deduced – aa sequences of all the enterococcal strains listed above was homologous to an extent of 83% sequence homology with that of the PH-1 strain. The multiple sequence alignment of the *tuf* gene sequences from PH-1 strain using the CLUSTAL W (1.82) program is shown in Figure 3.8.

```
Eduran          TAGTAGTTTCTGCTGCTGATGGCCCTATGCCTCAAACTCGTGAACATATCCTATTATCTC 71
Enhirae         TAGTAGTTTCTGCTGCTGATGGTCCTATGCCTCAAACTCGTGAACATATCCTAYTATCTC 71
Enmndt          TAGTTGTTTCTGCTGCTGACGGCCCTATGCCTCAAACTCGTGAACACATCCTATTATCTC 71
Entfm           TGGTAGTTTCTGCTGCTGACGGCCCAATGCCTCAAACTCGTGAACACATCCTATTGTCTC 71
Entfaclis       TAGTAGTTTCTGCTGCTGATGGTCCTATGCCTCAAACACGTGAACATATCTTATTATCAC 71
Ecoli           TGGTAGTTGCTGCGACTGACGGCCCGATGCCGCAGACTCGTGAGCACATCCTGCTGGGTC 240
PH-1            TGGTAGTTTCTGCTGCTGACGGCCCAATGCCTCAAACTCGTGAACACATCCTATTGTCTC 231
                * ** *** ****  **** ** ** ***** ** ** ***** ** *** *  *    *

Eduran          GTCAAGTTGGTGTTCCTTACATCGTYGTATTCTTGAACAAAGTAGATATGGTCGATGACG 131
Enhirae         GTCAAGTTGGTGTTCCATACATCGTTGTATTCTTGAACAAAGTAGATATGGTTGACGACG 131
Enmndt          GTCAAGTTGGTGTACCATACATCGTTGTATTCTTGAACAAAGTAGATATGGTTGATGACG 131
Entfm           GTCAAGTTGGTGTTCCTTACATCGTTGTATTCTTGAACAAAGTAGACATGGTTGATGACG 131
Entfaclis       GTAACGTTGGTGTACCATACATCGTTGTATTCTTAAACAAAATGGATATGGTTGATGACG 131
Ecoli           GTCAGGTAGGCGTTCCGTACATCATCGTGTTCCTGAACAAATGCGACATGGTTGATGACG 300
PH-1            GTCAAGTTGGTGTTCCTTACATCGTTGTATTCTTGAACAAAGTAGACATGGTTGATGACG 291
                ** * ** ** ** ** ****** * ** *** * ******   ** ***** ** ****
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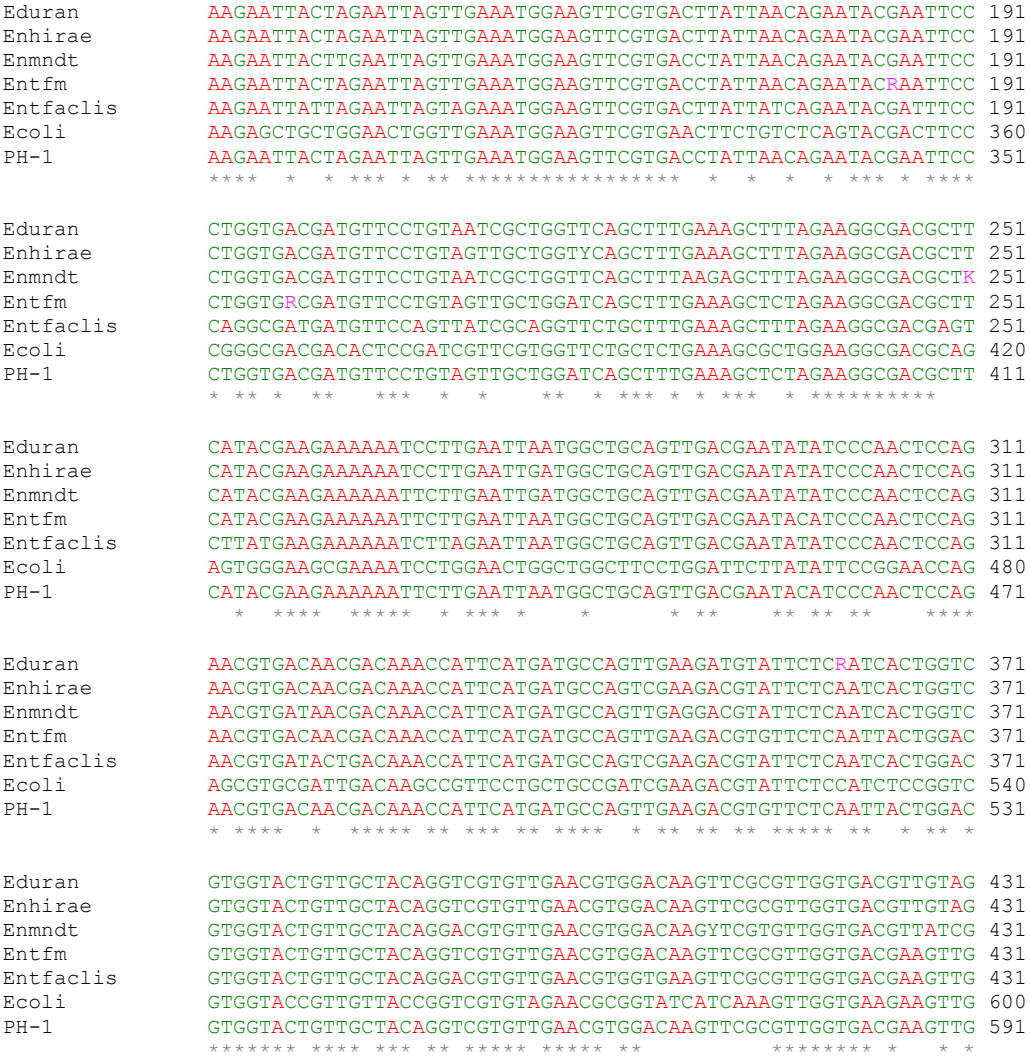
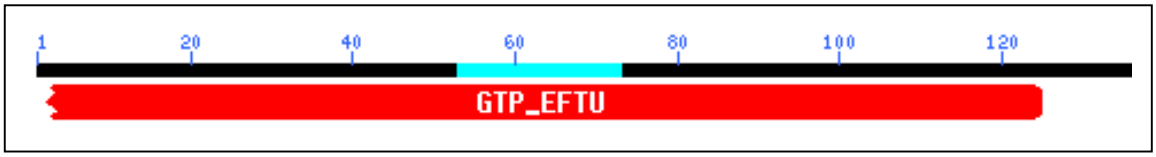


Figure. 3.8: Multiple sequence alignment of *tuf* genes from Eduran: *Ent. durans*; Enhirae: *Ent. hirae*; Enmndt: *Ent. mundtii*; Entfm, *Ent. faecium*; Entfaclis: *Ent. faecalis*; Ecoli: *Escherichia coli* (acc no. J01690) and PH-1, *Ent. faecium* PH-1, this study). * Conserved consensus sequences.

The putative conserved domain detected by BLAST search is shown in Fig. 3.9a indicating that the -aa sequence of PH-1 strain has high similarity with that of the GTP binding protein of EF-Tu.



1.

```
                  130       140       150       160       170       180
                    |         |         |         |         |         |
Ecoli_tuf     VAATDGPMPQTREHILLGRQVGVPYIIVFLNKCDMVDDEELLELVEMEVRELLSQYDFPG
PH_1xx1       VSAADGPMPQTREHILLSRQVGVPYIVVFLNKVDMVDDEELLELVEMEVRDLLTEYEFPG
Consensus     VaAaDGPMPQTREHILLgRQVGVPYI!VFLNKcDMVDDEELLELVEMEVR#LLs#Y#FPG
Prim.cons.    V2A2DGPMPQTREHILL2RQVGVPYI2VFLNK2DMVDDEELLELVEMEVR2LL22Y2FPG

                  190       200       210       220       230       240
                    |         |         |         |         |         |
Ecoli_tuf     DDTPIVRGSALKALEGDAEWEAKILELAGFLDSYIPEPERAIDKPFLLPIEDVFSISGRG
PH_1xx1       DDVPVVAGSALKALEGDASYEEKILELMAAVDEYIPTPERDNDKPFMMPVEDVFSITGRG
Consensus     DDtP!VaGSALKALEGDAewEaKILELaaalDeYIPePERanDKPF$$P!EDVFSIsGRG
Prim.cons.    DD2P2V2GSALKALEGDA22E2KILEL2222D2YIP2PER22DKPF22P2EDVFSI2GRG

                  250       260       270       280       290       300
                    |         |         |         |         |         |
Ecoli_tuf     TVVTGRVERGIIKVGEEVEIVGIKETQKSTCTGVEMFRKLLDEGRAGENVGVLLRGIKRE
PH_1xx1       TVATGRVERGQVRVGDEV----------------------------------------
Consensus     TVaTGRVERGq!rVG#EV
Prim.cons.    TV2TGRVERG222VG2EVEIVGIKETQKSTCTGVEMFRKLLDEGRAGENVGVLLRGIKRE
```

B

Figure 3.9: Reading frame of EF-Tu strain PH-1: amino acid BLAST search (A) and homology with *E. coli* (B) using Multalin (Corpet 1988).

The *tuf*A gene of *E. coli* codes for 409 -aa protein (EF-Tu) which is a GTP binding protein involved in the translation of RNA to protein. The EF-TU protein from *E. coli* has been well characterized. The region spanning 25-219 –aa at the protein is involved in GTP binding activity, 240-309 –aa comprise the EF-Tu domain 2 and the 313-408 -aa constitute the EF-Tu C-terminal domain. The cloned gene product of PH-1 appear to consist of a GTP binding domain and the EF-Tu domain-2. Multalin constructed by using the method described by Corpet (1988). The partially aligned EF-Tu sequence of PH-1 and *E. coli* EF-Tu are shown in Fig. 3.9B. Phylogenetic map was constructed using the nucleotide sequences of *tuf* genes and the results are shown in Figure 3.10.

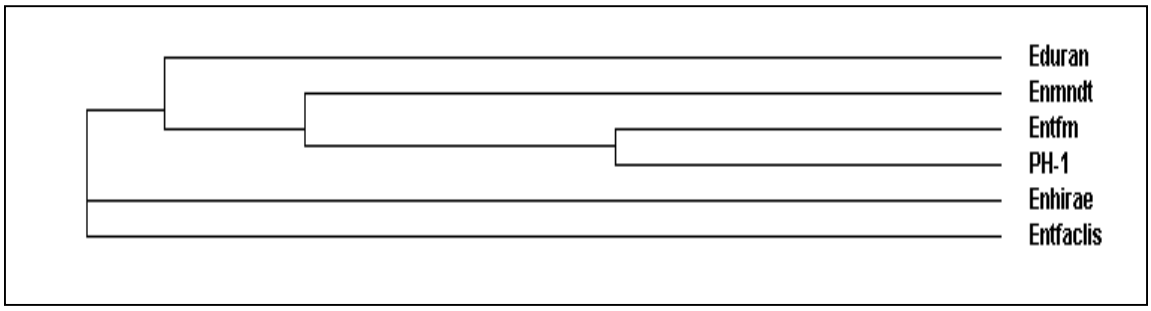


Figure 3.10: Phylogenetic analysis of *tuf* gene sequences of strain PH-1 and *Enterococcus* spp.

Nucleotide sequences of *tuf* gene from strain PH-1 and *Enterococcus* spp. of *mundtii*, *hirae*, *durans*, *faecalis* and *faecium* were used for phylogenetic analysis. The phylogenetic analysis of *tuf* gene sequences are in agreement with the results obtained for phylogenetic analysis of rRNA genes and provided a better insight for the molecular identification of *Ent. faecium* PH-1.

Various workers have used sequences or rRNA, *ldh*, *tuf*, *rpo*C, *mle*, *sod*, *hsp*60 genes , for identification and characterization of bacterial species (Mora *et al.* 1997, 2000a; Ke *et al*. 1999). In the present investigation, 16S rRNA gene and *tuf* gene sequences were used for detailed phylogenetic characterization of *Ent. faecium* PH-1, which enabled the correct identification of the strain.

Use of polyphasic taxonomy has been recognized by the International Committee on Systematic Bacteriology as one of the modern tools for bacterial strain identification. Protein coding genes such as *tuf* gene has been found to be an effective candidate for such purpose. For the determination of phylogenetic markers, *tuf* gene sequences can be often combined with the arsenal of rRNA sequence data base, and the sequences of small protein encoding genes such as *gro*EL and *rec*A (Ke *et al*. 1999; Torriani *et al.* 2001; Ventura *et al*. 2003).

3.3.5 Plasmid linked phenotypes in *Ent. faecium* PH-1

For plasmid curing, the strain PH-1 was grown in presence of novobiocin at

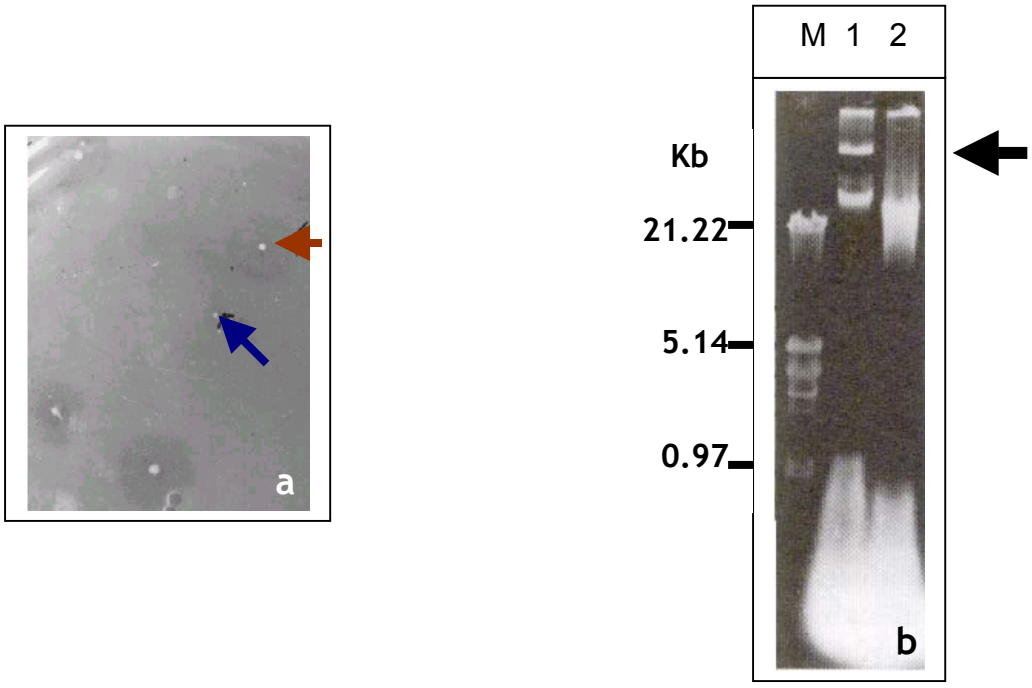


Figure 3.11: Plasmid curing of *Ent. faecium* PH-1

a) MRS agar plate assay showing Bact$^+$ and Bact$^-$ colonies of PH-1.
b) Plasmid profiling of Bact$^+$ and Bact$^-$ colonies. Lane 1, bact$^+$ and lane 2 bact$^-$ colonies. M is a λ DNA *Eco* RI/ *Hind* III double digest marker. Arrow indicates loss of the high molecular weight plasmid.

at 45^{0}C, was pour-plated, and overlaid with the indicator strain of *Lact. farciminis* MD. It was found that around 20-30% colonies showed no zone of inhibition against the indicator (Figure 3.11a). Such colonies grew slower than those exhibiting zones of inhibition.

Plasmid linked functions are generally ascertained by curing experiments using curing agents such as ethidium bromide, acridine orange, mitomycin, novobiocin, acriflavine and high temperature (Ray *et al*. 1989; Kim *et al*. 2000; Huang *et al*.

1996). In this study, the strategy of employing novobiocin at a concentration of 1 mg ml^{-1} and growing the strain at an elevated temperature (45^{o}C) cured strain PH-1 of the megaplasmid. Figure 3.11b reveals physical loss of the megaplasmid from strain PH^{-}, as indicated by an arrow (lane 2). Gupta and Batish (1992) had indicated that conventional curing agents are not effective for the elimination of highly stable plasmids. Although only novobiocin was found to be effective for plasmid curing in *Lact. casei* subsp. *casei* C40 (**2.3.7**). Novobiocin alone could not cure strain PH-1. Novobiocin is a DNA gyrase inhibitor and is widely used as a curing agent (Kornberg 1980). Ruiz-Barba *et al.* (1991) showed novobiocin induced loss of plasmid DNA from strains of *Lact. plantarum* isolated from green olive fermentation. Gonzalez and Kunka (1987) had grown *Ped. acidilactici* at elevated temperature to accomplish plasmid curing.

The pattern of sugar utlization by *Ent. faecium* PH-1 and its cured derivative (DPH-1) to grow in presence of different sugars was studied (Table 3.3). The results were compared with the key given in Bergey's Manual for *Enterococcus faecium* (Mundt 1986). A pediocin producing strain of *Ped. acidilactici* K7 (Ramesh 2000) that had been isolated in this Laboratory in previous studies was used for comparison.

Table 3.3: The ability of of *Ent. faecium**, native isolate of *Ent. faecium* PH-1, its plasmid cured derivative and *Ped. acidilactici* K7 to utilize different sugars.

Sugar/s	*Bergey's Manual	PH-1	ΔPH-1	K7
Lactose	+	+	-	-
Maltose	NA	+	-	-
Mannitol	+	+	-	-
Melibiose	+	+	-	-
Raffinose	-	+	-	NA
Sorbitol	-	+	-	NA
Sucrose	+-	+	-	+
Trehalose	NA	+	+	+

(+) growth, (-) no growth, (NA) and not available (*Mundt 1986)

It was observed that the media color changed to yellow within 20h of growth indicating that the strain PH-1 readily utilized all the sugars used for the test of growth and acid production. The ability of strain PH-1 to ferment sucrose and raffinose offers considerable potential for its use as starter culture in vegetable based products, where these sugars are in abundance (Gonzalez and Kunka 1986). Strain PH-1 was capable of fermenting lactose, which is an important feature in bacteriocin producing strains. The ability to use Lactose is an entercooccal character required for its growth in its natural habitat. Strain PH-1 might have acquired wide range of sugar utilizing capability especially for raffinose, maltose, sucrose and sorbitol through natural genetic transfer. Intrageneric and intergeneric transfer of certain plasmid linked characters are well documented (Gonzalez and Kunka 1983, Ennahar *et al.* 1996; Le Marrec *et al.* 2000). It may also be seen from Table 3.3 that most of the sugar utilizing genes from PH-1 are plasmid borne, since the DPH-1 was unable to ferment any of the sugars tested except for trehalose.

Table 3.4: Immunity of parental and cured strain of *Ent. faecium* PH-1 and DPH-1 to bacteriocin preparations.

	Bacteriocin activity against strain	
Bacteriocin	PH-1 (AU ml^{-1})	ΔPH-1 (AU ml^{-1})
CF PH-1	-	500
Ammonium sulphate Precipitate of CF	-	2, 000
Nisin	5, 000	6, 000

- no inhibition

After identifying that the bacteriocin production and sugar fermentation are plasmid encoded phenotypes, the strain was tested for its immunity function. The results are shown in Table 3.4. WT PH-1 was used for comparison. From Table 3.4 it can be seen that bacteriocin activity against the DPH-1 was 500 AU ml^{-1} indicating tentative loss of immunity to bacteriocin in the cured strain. This inhibition is more prominent when an ammonium sulphate

precipitate of the CF of PH-1 was used as a bacteriocin concentrate. However both the parental and the cured strain were inhibited by nisin. The latter showed marginally higher degree of inhibition. These results indicate that the immunity function is encoded by the megaplasmid in strain PH-1, which also encodes for bacteriocin production. Ray *et al*. (1989) have provided evidence for residence of bacteriocin activity and immunity function on the same plasmid in *Ped. acidilactici*.

The bacteriocin immunity system has been well characterized (Fimland *et al*. 2002a; Johnsen *et al.* 2004). It has been seen that immunity system has a very high degree of specificity. Immunity system of very closely related bacteriocins do not cross react with each other. However, exceptions have been seen only in case of natural variants such as nisin A and Z, gallidermin etc; where in

differences can be found in structural gene of bacteriocin and rest of the gene cluster is homologous (Stein *et al.* 2003).

3.3.6 Influence of carbon source on bacteriocin production

Table 3.5 reveals the yield of bacteriocin produced by PH-1 strain following 20 h of fermentation in MRS broth incorporated with different sugars at 1% level. The sugars were chosen based on earlier experiments on carbohydrate fermentation pattern for strain identification (section **2.3.2.1**). The time of fermentation was chosen based on the results obtained from carbohydrate fermentation experiments where 20h was found to be sufficient for BCP to change its color indicating metabolism of the sugar to acid.

The highest activity of bacteriocin (3500 AU ml^{-1}) was obtained with cells grown in sucrose whereas cells grown in raffinose, mannose and fructose gave the lowest yields. Earlier report of Biswas *et al.* (1991) revealed that pediocin AcH production was greatest when glucose was used at 1% level. In the present study, the use of sucrose resulted in marginally higher yields as compared to glucose.

Table 3.5: Bacteriocin production by *Ent. faecium* PH-1 in presence of different sugars

Sugar/s	*Bacteriocin activity (AU ml^{-1})
Celibiose	2, 500
Fructose	2, 000
Galactose	3, 000
Glucose	3, 000
Lactose	3, 000
Maltose	3, 000
Mannitol	3, 000
Mannose	2, 000
Melibiose	3, 000
Raffinose	2, 000
Sucrose	3, 500
Trehalose	3, 000

*Antimicrobial activity was checked against *Lact. farciminis* MD

This might be accounted for high sucrose hydrolase activity in strain PH-1. Earlier work of Gonzalez and Kunka (1987) reports high sucrose hydrolase activity in a strain of *Ped. pentosaceus* when grown in presence of sucrose. The fact that lactose and glucose supported production of bacteriocin equally suggests the possibility of formulating an alternative cheaper media like whey permeate. This would improve the economy of the commercial production of bacteriocin and allow the exploitation of pediocin for the preservation of food.

3.3.7 Biochemical characteristic features of bacteriocin

The effect of various organic solvents on bacteriocin activity was tested. Solvents such as methanol, ethanol, isopropanol, acetonitrile, cloroform, DMSO and acetone did not reduce the zones of inhibition. The other compound such as β-mercaptoethanol, 1 M DTT has completely destroyed the antimicrobial activity (Table 3.6) of the bacteriocin. Proteolytic enzymes such as trypsin, pronase, proteinase-K and pepsin inactivated the antimicrobial compound. However, RNase, lysozyme and urea did not affect the antimicrobial activity of the bacteriocin type. Incubation of the bacteriocin at 121^0C for 15min and at different pH ranges from 4 to 8 did not lower the bacteriocin activity. However, at pH 2 to 3 and 9, 50% activity was seen.

Table 3.6: Effect of different organic solvents and enzymes on activity of the bacteriocin produced by *Ent. faecium* PH-1

Organic solvent	AU ml^{-1}
Methanol	3, 000
Ethanol	3, 000
Isopropanol	3, 000
Acetonitrile	3, 000
Chloroform	3, 000
DMSO	3, 000
Acetone	3, 000
□-mercaptoethanol	0
Urea	3, 000
Lysozyme	3, 000
RNase	3, 000
1M DTT	0
pH 4-8	3, 000
temperature (121^0C, 15 min and 100^0C 30min)	3, 000
Control (untreated)	3, 000

Bacteriocin from strain PH-1 readily adsorbed to both the indicator and the producer strain (Figure 3.12). Adsorption to indicator bacterial strain *Lact. farciminis* MD was 100% at pH 6.0 whereas only 75% of the added bacteriocin was adsorbed by PH-1 cells. These results indicate higher affinity of bacteriocin for the indicator strain MD. A similar preference of bacteriocin (pediocin) for the indicator strain has been reported by Bhunia *et al.* (1991). pH dependant adsorption of bacteriocin to cell surface has been observed earlier and has been exploited for the purification of some bacteriocins (Daba *et al.* 1994; Elegado *et al.* 1997; Yang *et al*. 1992).

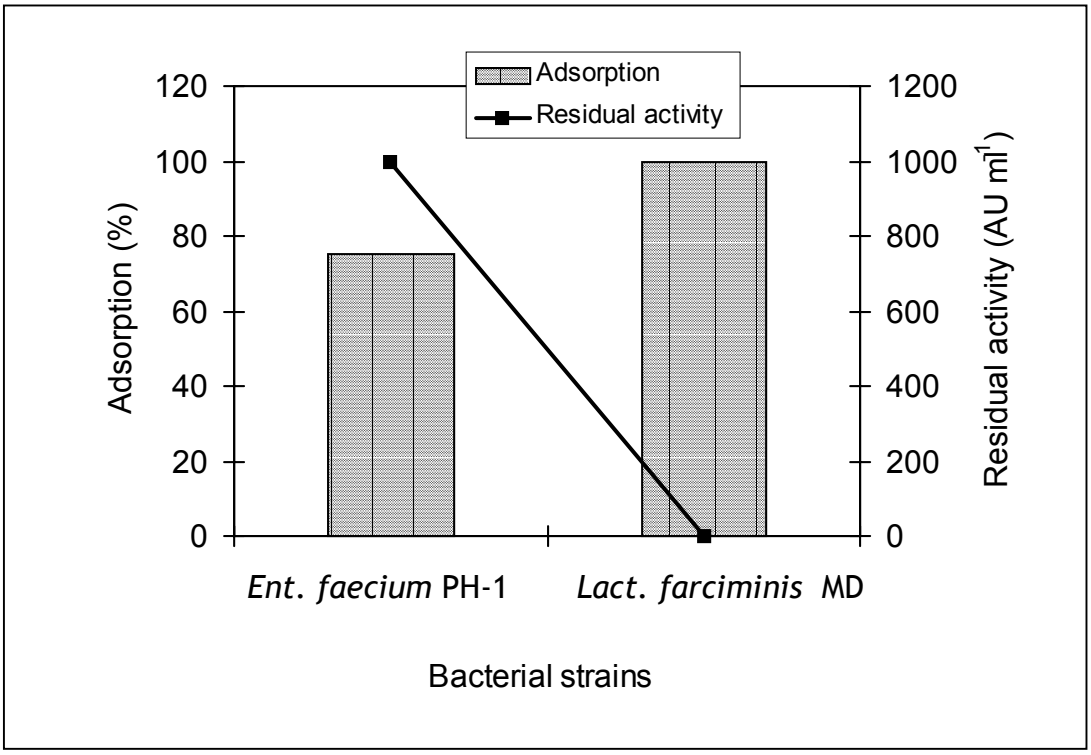


Figure 3.12: Adsorption (▩) and residual activity (■) of pediocin to host and sensitive strains of LAB.

CF activity was completely eliminated on treatment with proteolytic enzymes clearly indicating its proteinaceous nature of antimicrobial compound as generally seen in case of bacteriocin identification. Retention of bacteriocin activity was observed even after autoclaving, implying that the molecule was thermostable. The activity of bacteriocin was lost on treatment with β-mercaptoethanol and DTT indicating involvement of disulfide bonds in the antimicrobial activity of the molecule.

3.3.8 Bacteriocin produced by PH-1 strain is a potent anti-listerial bacteriocin

it is evident from Table 3.7 that the antimicrobial activity of bacteriocin produced by strain PH-1 was more potent than that of strain CFR K7 against most

indicators, except against *Leuc. mesenteroides* NRRL B640 and some of the species of listeria. The differences in the bacteriocin activity of the two strains tested could be possibly accounted for by differences in their specificity towards the indicators that were used. Most of the indicator strains used were meat isolates.

Table 3.7: Comparison of bacteriocin activity in the culture filtrate (CF) of strain PH-1 and CFR K7 against different indicator strains

Indicator	Bacteriocin activity in CF (AU ml^{-1})	
	PH-1	*Ped. acidilactici* CFR K7
Lact. farciminis MD	4, 000	2, 000
Leuc. mesenteroides B640	3, 000	6, 400
List. monocytogenes V7	3, 500	3, 200
List. monocytogenes Scott-A	25, 600	12, 000
List. monocytogenes FMD	12, 800	6, 400
List. sielegeri	3, 200	3, 200
List. greyi	3, 200	6, 400
List. ivanovi	0	6, 400
List. innocua	0	0
List. murrayi	3, 200	6, 400
Pediococcus pentosaceus C6	3, 000	2, 000
Ped. acidilactici B1153	3, 200	3, 200
Ent. faecalis ATCC 344	3, 200	3, 200
Staph. aureus FR1722	1, 200	1, 600

Strain PH-1 being a meat isolate was more potently able to kill other meat isolate than did the strain CFR K7, which was derived from cucumber (Ramesh 2000). A similar phenomenon was reported by Bennik *et al*. (1997b) wherein isolates from the same environmental niche exhibited greater antagonism to one another. In above study, it can be seen that strain PH-1 exhibited strong anti-listerial activity against Scott-A and V7 as compare to strain K7.

3.3.9 Purification of bacteriocin

Concentration of bacteriocin by cell-adsorption was carried out to recover bacteriocin from CF. This method was initially developed by Daba *et al*. (1994) for the recovery and purification of pediocin UL5. The MRS broth grown inoculum of PH-1 was adjusted to pH 6.0 and the cell-adsorbed bacteriocin released at pH 2.0. The results are shown in Figure 3.13. The pH 2.0 extract fraction was directly applied to a C18 semi-preparative column and the HPLC peaks at different time intervals were collected. One of the peaks corresponding to 25min was found to be highly active against the indicator bacteria indicating purification of bacteriocin (Figure 3.14). This active fraction was flash evaporated and the dried powder was dissolved in sterile deionised water.

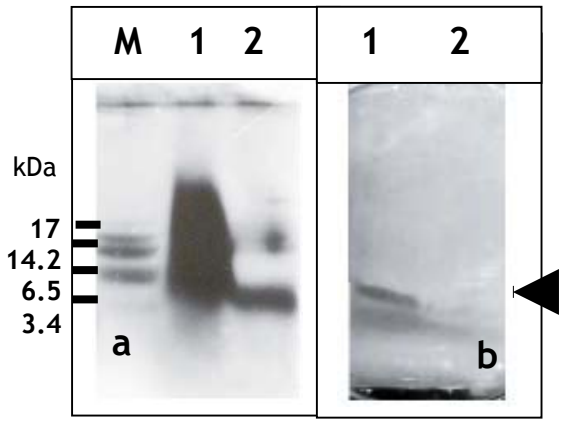


Figure 3.13: Analysis of bacteriocin on denaturing acrylamide gel. A)Tricine SDS-PAGE (18%) and b) Direct detection of antimicrobial peptide on gel. Arrow indicates the position of antimicrobial peptide. a) 1, Ammonium sulphate precipitated CF; 2, pH2.0 extract of CF of PH-1. b) lane 1, pH 2.0 extract and 2, trypsin treated pH 2.0 extract. M is a ultra low MW marker. Arrow indicate the position of the protein band exhibiting antimicrobial activity.

Since bacteriocins are the secreted peptides, many purification procedures begin with concentration of the antimicrobial compound before performing any chromatographic purification. Various workers have followed different procedures for purification. These include ammonium sulphate precipitation followed by chromatography, gel filtration in combination with chromatography, cell-adsorption in combination with HPLC and ethanol precipitation followed by

ultra-filtration, immuno-affinity etc. However, in many of the protocols, the final yield was found to be low and involve high cost and non-reproducibility of results (Rodriguez *et al.* 2002b). The purification protocol described in the present study is cheaper, reproducible and the yields are considerable higher.

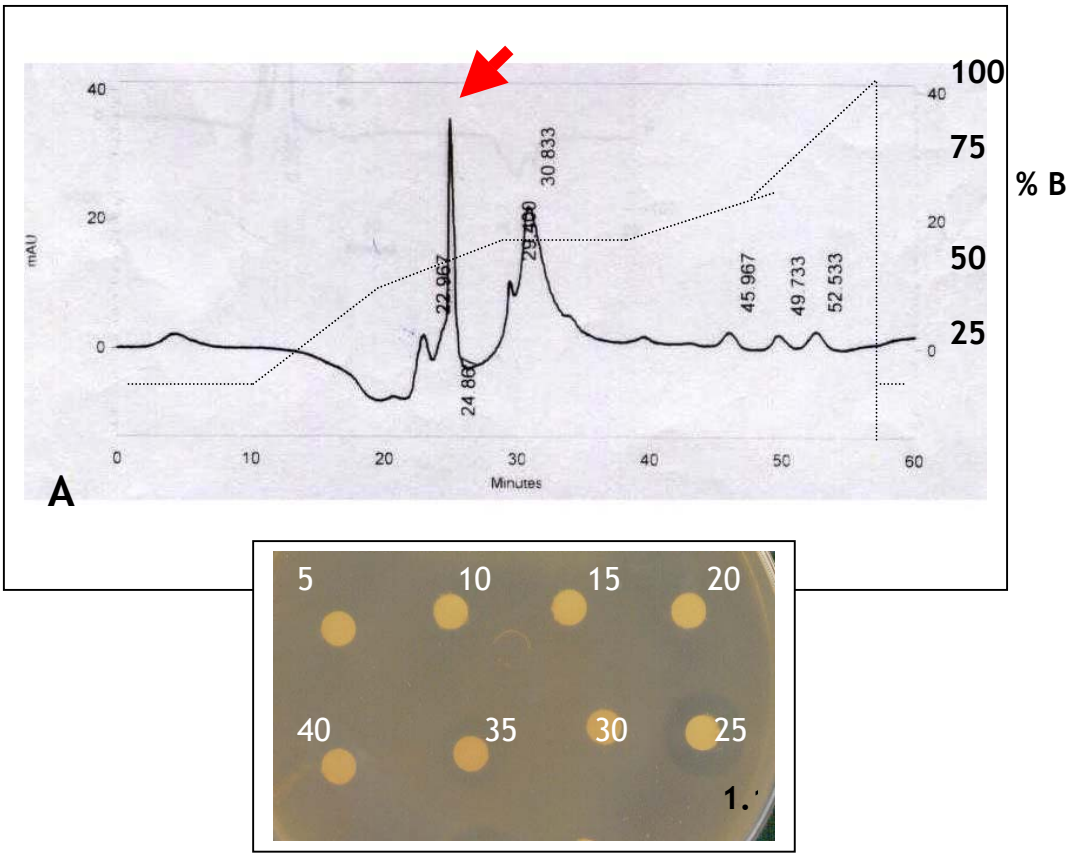


Figure 3.14: Purification of bacteriocin produced by *Ent. faecium* PH-1 to homogeneity. A) Chromatograph of bacteriocin C20 obtained by semi-preparative RP-HPLC C18 column and
B) Bioassay of HPLC fractions against *List. monocytogenes* V7.

Tricine SDS-PAGE indicated that the antimicrobial peptide is ~4.6 kDa in size and had a elution profile similar to that reported for pediocin AcM. These biochemical results indicated that the bacteriocin from strain PH-1 is similar to the pediocins AcH/PA-1. Results of step wise purification of bacteriocin produced by *Ent. faecium* PH-1 is shown in Table 3.8.

Table 3.8 : Purification of bacteriocin from *Ent. faecium* PH-1

Step	volume (ml)	protein mg ml^{-1}	activity AU ml^{-1}	total activity (AU)	Specific activity	Yield (%)	purification fold
CF	1000	3.2	3,500	3,50,0000	1093	100	1
pH 2.0 extract	20	0.5	21,0000	420,0000	42000	120	399
RP-HPLC	2	0.2	40,0000	80,0000	200,0000	22	1830

A three step procedure was used to purify the bacteriocin produced by the stain PH-1. The property of adsorption and desorption of the bacteriocin to the host cell as it shown in section **3.3.7** and the HPLC was used in purification. The desorbed material exhibited a specific activity 399 times while after preparative HPLC, specific activity was increased to 1830. Thus the pH dependant adsorption followed by acidic pH desorption of bacteriocin helps in selective purification of the bacteriocin. Purification of bacteriocin to homogeneity by cell adsorption was found to be extremely useful in purifying the bacteriocin from LAB, since the bacteriocin production media contains large number of media components which can contaminate the preparation during purification (Elegado *et al.* 1997).

3.3.10 Molecular basis of bacteriocin production by PH-1 strain

It is clear from Figure 3.15 that using pediocin PA-1 (*ped*A) gene specific primers A5 and A3 as forward and reverse, a PCR product of size 186 was obtained with strain PH-1 (lane 2). By using primers 2A and 2B an amplicon 260 bp in size was obtained (lane 4). The sizes of these PCR products corresponded with the sizes obtained from PCR of DNA from *Ped. parvulus* ATO 77, a pediocin PA-1 producing strain (lanes 1 and 3) indicating the presence of pediocin PA-1 type bacteriocin encoding genes in PH-1. PCR based detection of pediocin gene

has been reported earlier (Bennik *et al.* 1997a; Ramesh 2000; Rodriguez *et al.* 1997). The DNA based methods facilitate rapid enumeration of target microorganism and circumvent routine microbial assays and biochemical analysis.

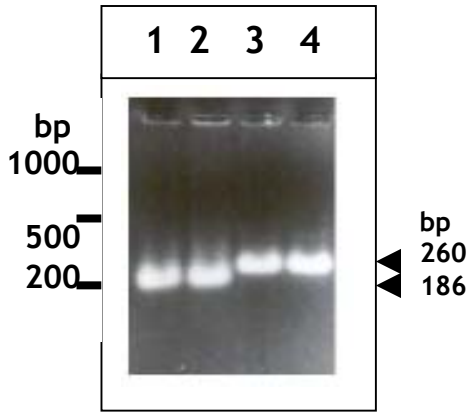


Figure 3.15: Agarose gel (1.2%) electrophoresis of PCR amplicons obtained while *ped* A gene specific primers. Lanes 1 and 3: Amplification of *ped A* gene of *Ped*. *parvulus* ATO 77 (positive control) with A5, A3 and 2A, 2B primers respectively. Lanes 2and 4: Amplification of *ped A* gene of PH-1 with A5, A3 and 2A, 2B primers respectively. The position of 100 bp ladder is on left side of the gel.

In the present study, the results of PCR were further validated by using a DNA probe. Figure 3.16 depicts that the plasmid DNA of strain PH-1 (lane 2) reacting with the DIG-labelled *ped*A gene probe, indicating the presence of pediocin gene in the megaplasmid of strain PH-1. The cured sample (DPH-1) failed to react in the blot (lane 3) clearly indicating the loss of megaplasmid bearing pediocin gene. Plasmid encoding pediocin production is well established in pediocin producers. The cured organism is unable to produce bacteriocin and hence provides a phenotypic means for plasmid loss (Kim *et al*. 2000).

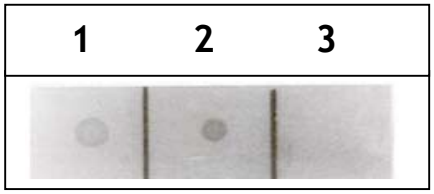


Figure 3.16: Dot-blot hybridization of plasmid DNA with *ped*A gene probe. Lane 1, control ATO 77 DNA, 2, PH-1 and 3, ΔPH-1.

3.3.11 Southern detection of bacteriocin encoding plasmid

In order to ascertain the plasmid encoded character for pediocin production, Southern hybridization experiment was carried out. The *ped*A gene probe derived from pediocin PA-1 producing strain of *Ped. parvulus* ATO77 reacted strongly with a plasmid DNA isolated from PH-1 strain. The probe however did not react with the chromosomal DNA from PH-1 indicating that pediocin PA-1 encoding gene is a plasmid-borne character in *Ent. faecium* PH-1. It may be seen from Figure 3.17 that the reaction with DNA from *Ent. faecium* PH-1 was positive but smeared. The smear may have be due to shearing of the high molecular wt plasmid during isolation and electrophoresis or due to the inefficient transfer of supercoiled megaplasmid to the membrane. There ws no reaction with control DNA from another bacterium C6 The results presented in Figure 3.17 confirm that bacteriocin production by *Ent. faecium* PH-1 is associated with high molecular weight plasmid. Previously, Southern hybridization technique has been employed to prove the presence of bacteriocins encoding plasmids in *Ped. acidilactici* (Bhunia *et al.* 1994; Ramesh 2000). Southern detection is a valuable tool especially when bacteriocin producer harbour multiple plasmids. Further, this technique overcomes the drawback of conventional plasmid curing methods for determining plasmid linked phenotypes, where native plasmid is not lost.

It is well-known that pediocin production is a plasmid –encoded trait and the plasmid size varies in different pediococcal strains. It has been shown that plasmids ranging from 8.8 to 11 kb in size are known to be involved in pediocin production (Gonzalez and Kunka 1987, Kim *et al.* 1992).

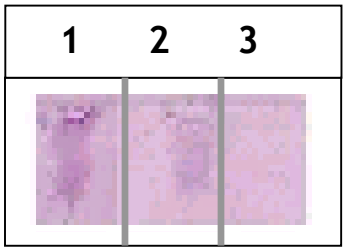


Figure 3.17: Southern hybridization of Bacteriocin encoding plasmids of PH-1 by *ped*A gene probe. Lane 1 & 2, 2 and 1 mg (approx) plasmid DNA of strain PH-1 was separated on 1% gel. Lane 3, C6 plasmid DNA

Ennahar *et al.* (1996) showed 11kb size plasmid associated with intergeneric pediocin AcH production in a *Lact. Plantarum* WHE 92 strain. It is postulated that intergeneric plasmid transfer followed by modification in size could be responsible for the same bacteriocin being coded by plasmids of varying sizes (Ennahar et al. 1996; Le Marrec *et al*. 2000).

3.3.12 Intergeneric pediocin PA-1 production by *Ent. faecium* PH-1

The *ped*AB gene of *Ent. faecium* PH-1 was amplified using the primer 2A and PED.IMM-R as forward and reverse primers, respectively. As expected, a 581 bp amplicon was obtained (Figure 3.18). These results are in agreement with the sequences from the pediocin PA-1/AcH operon described previously (Marugg *et al*. 1992; Motlagh *et al*. 1994). The PCR product 581 bp in size comprising *ped*AB gene was cloned into pTZ57R/T vector and was sequenced partially using a M13 forward primer. The nucleotide sequences of *ped*A gene and partial *ped*B gene are presented in Figure 3.19.

Nucleotide sequence analysis of the 540bp fragment of *ped*AB gene indicated cent percent homology with pediocin AcH/PA-1 and with *ped*B to the extent of the sequence. It was seen that the nucleotide at position 201 of *ped*B gene sequence, PH-1 is similar to that of *ped*B of *Ped. acidilactici* PA-1/AcH and not to that of *Ped. parvulus*/ *Lact. plantarum* (Figure 3.20). This result clearly indicates that the native isolate of *Ent. faecium* PH-1 contains the genes necessary for synthesis of intergeneric pediocin PA-1.

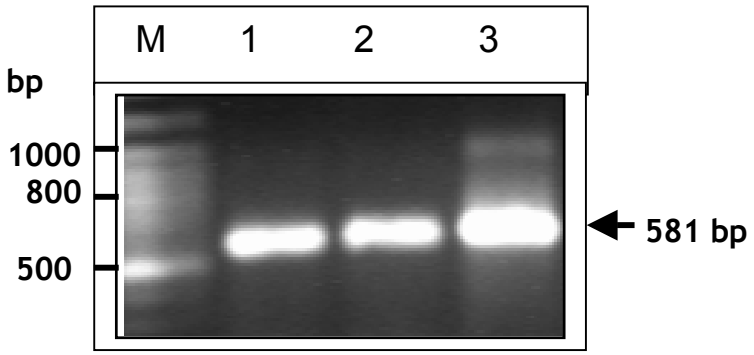


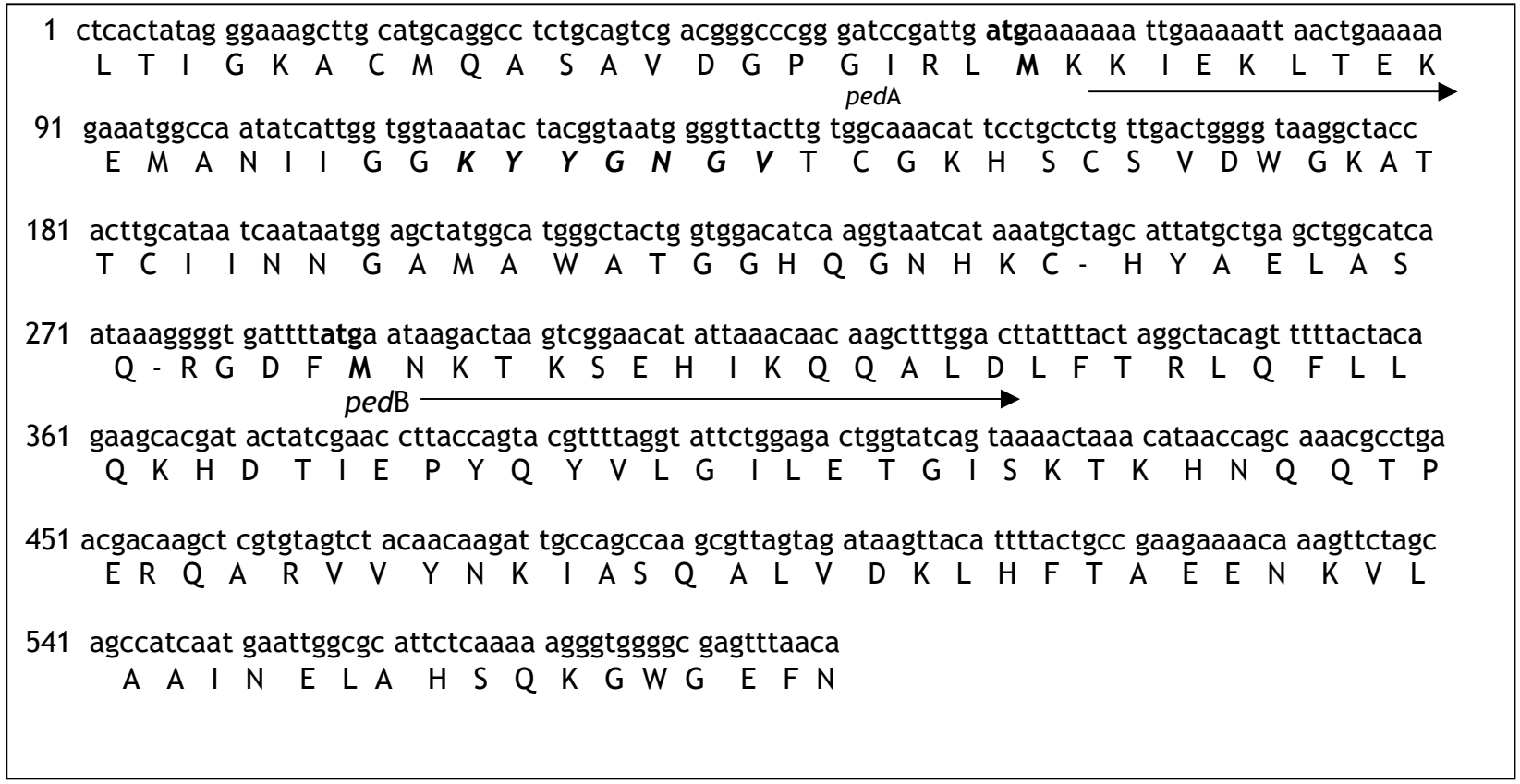
Figure 3.18: PCR analysis of *ped*AB gene of *Ent. faecium* PH-1 strain. Lane 1 to 3 DNA from of PH-1 strain used as template. M, 100 bp ladder

Reports of intergeneric pediocin production has been reported previously only in two cases. Pediocin PA-1 type bacteriocin production by *Lact. plantarum* was reported by Ennahar *et al.* (1996), where in a single nucleotide difference in *ped*B gene as compared to that of *ped*B of *Ped. acidilactici* PA-1/AcH was reported. The intergeneric production of coagulin (pediocin type bacteriocin) by lactic acid producing bacterium *B. coagulans* was reported by Le Marrec *et al*. (2000). However, the structural gene of *coa*A which exhibits high degree of homology with *ped*A gene differed in only one –aa at the C-terminal end of the protein from that of pediocin PA-1/AcH.

The results presented in this chapter with combination of biochemical and molecular techniques showed that the native strain of *Ent. faecium* PH-1 indeed produced pediocin PA-1 bacteriocin.

It is class IIa heat stable, low molecular weight, wide pH range antilisterial bacteriocin (Rodriguez et al. 2002b).

Figure 3.19: Partial nucleotide sequences of *ped*AB genes of *Ent. faecium* PH-1.



The first ORF *ped*A codes for prepeptide of pediocin structural gene; the second ORF *ped*B shown was partial sequenced

→ indicates starts of ORF of *ped*A and *ped*B genes.

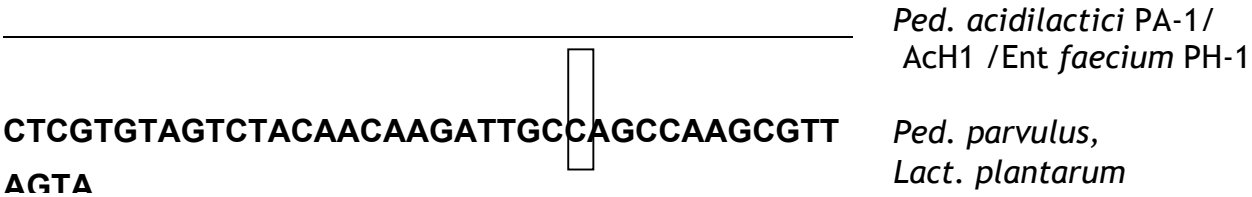


Figure 3.20: Comparative difference in sequence of *ped*B among pediocin producers

3.4 CONCLUSION

A native bacteriocinogenic strain of *Ent. faecium* PH-1 was characterized while using the tools of molecular biology. The 16S rRNA gene sequences of ~1Kb in combination with 450bp *tuf* gene sequences provided detailed evidence that the native strain is indeed *Ent. faecium*. Native plasmid curing was carried out to demonstrate megaplasmid associated bacteriocin production, immunity and carbohydrate fermentation in the strain of *Ent. faecium* PH-1. Bacteriocin from strain PH-1 exhibited strong anti-listerial acitivity: such antagonism to listeria is a characteristic feature of class IIa bacteriocins.

PCR, DNA dot-blot and Southern hybridization techniques were employed to determine genetic basis of pediocin PA-1 production. The pediocin PA-1 was purified by a cell-adsorption desorption technique followed by RP-HPLC. A single peak eluting at 25min in 30% acetonitrile exhibited strong antilisterial activity. The single active band was observed on gel assay corresponding to a MW of 4.6 kDa. The deduced amino acid sequence of 450 bp PCR product of *ped*AB gene showed cent percent homology with pediocin AcH/PA-1. The native strain of *Ent. faecium* PH-1 offers great potential, from a biotechnological point of view, due to its tolerance to high temperature, pH, salt concentration, bile salt and its ability to produce pediocin PA-1.

CHAPTER 4

PRODUCTION OF PEDIOCIN PA-1 FOR BIOPRESERVATION

4.0 ABSTRACT

The gene (β-*gal*) coding for β-galactosidase from strain *Ent. faecium* PH-1 was cloned by a shot-gun cloning approach and functional complementation was done in *E. coli* DH5αby using vector δpUC19, bearing disrupted *lac*Z gene. The nucleotide sequences of one of the putative recombinant (H3B) was determined using M13 primers. Multiple sequence alignment with *lac*Z genes available in GenBank of other LAB indicated that β-*gal* of PH-1 had least similarity with the aligned sequences. However, maximum similarity with *Carn. maltaroniaticum*, was observed followed by *lac*Z of *Bif. breve*.

The recombinant (H3B) was qualitatively analyzed for the production of the enzyme β-Gal using the ONPG as substrate. The results indicated that faster degradation of substrate (as seen by appearance of yellow colour) by the recombinant occurred indicating the presence of putative □-*gal* coding enzyme. The results for lactose hydrolysis were further substantiated by molecular and biochemical evidences. The □-*gal* gene probe derived from *Lact. plantarum* reacted with the total plasmid DNA of strain PH-1 and did not react with DPH-1, indicating the presumptive plasmid linked □-*gal* gene. The native □-Gal enzyme activity was studied by using total cell lysate prepared from lactose grown culture. The native gel assay indicated that a single florescence band of faster migration (as compare to *E. coli* □-Gal) was observed as suggesting low molecular weight single subunit enzyme.

The ability of *Ent. faecium* PH-1, to produce quantifiable amounts of pediocin PA-1 in whey permeate was studied. Production of pediocin PA-1 was found to be 1 to 1.5 folds greater in lactose based medium than in a medium containing glucose. Optimized whey permeate supplemented with 2% yeast extract supported a cell growth of absorbance of 3.5, $OD_{600.}$ Pediocin PA-1 activity of 150 $X10^3$ AU ml^{-1}, equivalent to that obtained by growth in commercial MRS broth was observed.

Limited biopreservation studies were carried out using pediocin PA-1 preparation in a listerial spiked vegetable salad. It was found that upon addition of pediocin. The viable count of Scott-A decreased from $7.6\log_{10}$ to $3.4\log_{10}$ in a span of nine hour of storage at room temperature indicating effectiveness of pediocin PA-1 in increasing shelf-life of the veg-salad.

4.1 INTRODUCTION

Bacteriocins of LAB are of scientific and commercial important because of their abilities to preserve food. These ribosomally synthesized peptides are bacteriocidal to many Gram-positive and pathogenic bacteria. They posses the novel property of heat tolerance, activity at acidic pH and can be inactivated by proteolytic enzymes present in gastrointestinal tract. Bacteriocins derived from LAB that have a GRAS (generally regarded as safe) status seem to be non-toxic and non-antigenic to animals; thus, they can be used to enhance the safety and shelf life of many foods (Biswas *et al*. 1991; Liao *et al*. 1993; Marugg *et al*. 1992).

Besides pediococci, *Lact. plantarum*, *B. coagulans* and *Ent. faecium* (present study) are known to produce pediocin PA-1 type bacteriocin. Pediocin inhibits the growth of several bacteria including *Listeria monocytogenes* which causes listeriosis and is of great concern as a potential contaminating food-borne pathogen. Recently this psychotropic pathogen has become a major challenge to food industry and regulatory agencies, as listeriosis caused by this organism is estimated to cost much more per incident than any other common but less serious food-borne disease, because of high fatality rate (Biswas *et al.* 1991).

Food grade LAB such as *Pediococcus* sp. and recombinant *Streptococcus thermophillus* could be used for the production of pediocin with low cost food ingredients such as whey permeate (WP) or tryptone, glucose yeast extract (TGE) and such a product can be directly used as a natural biopreservative to control undesirable microorganisms in food (Biswas *et al.* 1991; Liao *et al.*

1993; Somkuti and Steinberg 2003). Furthermore, consumption of foods containing lactose utilizing LAB has been credited with many health benefits in humans, including enhanced digestion of lactose in lactose–intolerant individuals (Bhowmik *et al.* 1987). Characterization of sugar utilizing markers in a bacteriocin producing strain can facilitate the formulation of an economically viable medium for large scale production of bacteriocins and the construction of food-grade vector system in lactic transformation (Platteeuw *et al.* 1996).

Lactose utilization by LAB has been an important attribute for selection of strains as probiotics and the use of lactose as a carbon source in the formulation of economical viable media. Lactose is hydrolysed by either the enzyme □-galactosidase (□-Gal) or Phospho □-galactosidase (□-PGal) in many LAB, however, □-Gal is known to be the predominant enzyme in these bacteria (David *et al.*1992; Obst *et al.*1995). The gene encoding for □-Gal has been isolated from various LAB which includes *Bif. infantis* (Hung and Lee 1998); *Lact. sake* (Obst *et al.* 1995); *Lact. plantarum* (Fernandez *et al.*1999; Mayo *et al.*1994) using either PCR or gene complementation (Griffin *et al.*1996; Hung and Lee 1998).

For the production of □-galactosidase, thermophilic bacteria have become an object of interest. Since LAB have a GRAS status one should often exploit these bacteria for the commercial preparation of □-Gal enzyme. The thermophilic bacteria such as *Stre. thermophilus* and its □-Gal have been characterized and its activity and stability at temperatures above 50^0C was studied (Vasiljevic and Jelen 2001). These conditions can enhance the rate of lactose hydrolysis as well as prevent the growth of undesirable microorganisms. This indicates that the use of thermophilic bacteria, which can grow in a medium consisting of cheaper carbon source such as WP, provides an alternative source for the commercial production of □-Gal.

For effective commercial application of bacteriocins, optimization of their production by modification of both genetic regulation and environmental growth parameters are important (Biswas *et al*. 1991). Such factors have been elucidated for the production of nisin. Such studies have rarely been made with pediocin. A few reports indicate the use of WP (Liao *et al.* 1993) and YE or WP supplemented with glucose as a carbon source in the production medium (Goulhen *et al.* 1999). Production of pediocin solely on WP or using lactose as a carbon source have not been examined so far. Two US patents (Nos. 4, 929, 445 and 5, 445, 835) describing the use of pediocin for inhibiting *List. monocytogenes* in food or other materials and/or production of yogurt containing bacteriocin PA-1 have been filed. However theses reports relied entirely on the use of MRS medium, MRS base with a simple sugar like glucose or galactose and/or WP containing glucose. Most of the pediocin producing strains used so far, are unable to hydrolyze milk sugar viz. lactose and hence, glucose has to be added to the medium (US patent nos. 4, 929, 445 and 5, 445, 835). However, *Lact. plantarum* WHE92 an intergeneric pediocin producing strain has an inherent capacity to use lactose (Ennahar *et al*. 1996).

Over the past ten years, *List. monocytogenes* has been recognized as an important food-borne pathogen. It is an ubiquitous pathogen often found in raw and ready to eat meat, poultry, sea food, vegetables, raw milk, dairy products and other foods etc. (Kozak *et al.* 1996).

Pediocin has followed nisin in being used as a food preservative. Either the whole cell or the bacteriocin preparation has been used as a source of antimicrobial compound. Pediocin has been widely used in the preservation of variety of meats (Liao *et al.* 1993), fermented dairy products such as shrikhand (Ramesh 2000), minimally processed vegetables (Bennik *et al.* 1999) and kimchi

fermentation (Choi and Beuchat 1994). Use of pediocin AcH producer *Lact. plantarum* WHE92 as spray on the munster cheese surface at the beginning of

the ripening period to prevent outgrowth of the *List. monocytogenes* has been demonstrated (Ennahar *et al.* 1998). Likewise, for the preservation of wine and baked products, pediocin has been heterologously expressed in yeast *Saccharomyces cerevisiae* (Schoeman *et al*. 1999). The use of pediocin in a mixed food system with both vegetables and dairy products have been not yet demonstrated. it is vital to study the effectiveness of biopreservative since many vegetables and dairy products also support the growth and survival of *Listeria*.

In the previous chapter, detailed characterization of intergeneric pediocin PA-1 production was presented. It was found that *Ent. faecium* PH-1 had an ability to ferment a wide range of sugars including lactose and mellibiose. WP, a dairy waste which consists of lactose (as a principal carbon source) and the minerals and trace elements was employed as a production medium for the enhanced pediocin PA-1 production. The pediocin PA-1 has been used for the increasing shelf life of vegetable salad where listeria is a common pathogen. The genetic and molecular basis of lactose utilization by *Ent. faecium* PH-1 was also studied.

4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 Chemicals

Agarose, Ampicillin, acrylamide, IPTG and X-GAL were as described in section **3.2.1.1**. Low melting agarose, 4-O-methylumbelliferyl-□-D-galactoside, citric acid and glycine were purchased from SRL. The sugars such as glucose, lactose, surfactant Tween 80, and ONPG discs were from HiMedia. Restriction enzymes *Eco* RI, *Pst*I, *Hin*dIII, *Hinc*II, *Sma*I were from Promega (USA).T_4 DNA ligase and CIAP were same as indicated in section **3.2.2.1.**

The DIG-dUTP random primed DNA labelling and detection kit was from Boehringer Mannheim. All the fine chemicals used in the experiments were of analytical grade. Skim milk powder was procured from local market.

4.2.1.2 Bacterial strains, cultivation media and plasmid vectors

The LAB strains *Ent. faecium* PH-1, its plasmid cured derivative (DPH-1), *Ped. acidilactici* CFR K7 and *Lact. plantarum* C3.8 were used in this study. *List. monocytogenes* Scot A was used as a pediocin PA-1 indicator bacteria. *E. coli* DH5□ was used for cloning and transformation purpose. All the bacterial strains were cultivated and maintained in their respective media as described previously (**3.2.1.2**). The plasmid vectors pUC18 and 19 were used in this study for gene cloning and expression purpose. A 30 kb plasmid DNA of *Lact. plantarum* C3.8 coding for □-galactosidase was procured from Dr. Baltasar Mayo, Instituto de Productos Lates de Asturias, Villaviciosa, Spain.

4.2.1.3 Pediocin PA-1 production media

For pediocin PA-1 production following media were used:

4.2.1.3.1 MRS broth: As referred in section **2.2.1.4**

4.2.1.3.2 TGE broth: It consists of 1% each of Tryptone, YE and glucose, 0.005% each of $MgSO_4$ $7H_2O$ and $MnSO_4$ and 0.2% Tween 80. The pH of the medium was adjusted to 6.8 and was sterilized by autoclaving.

4.2.1.3.3 TYE broth: Similar components as in TGE broth (**4.2.1.3.2**) except that glucose was replaced with the same quantity of lactose.

4.2.1.3.4 WP medium: Whey permeate (WP) 10% stock was prepared by coagulating skim milk powder and filter sterilized. This stock corresponds to 2% lactose. The working volume for the experiments was adjusted to 10.0 ml and the medium pH was adjusted to 6.5. Tween 80 (0.1%) was added to WP that had been supplemented with 2% YE. WP medium containing varying concentrations of YE along with 0.1% Tween 80 (Table 4.2) was also prepared.

4.2.2 Methodology

4.2.2.1 Preparation of pediocin-rich crude preparation

A strain of *Ent. faecium* PH-1 was grown in WP broth supplemented with YE at incubated at 37^0C for 18h. The cells were separated by centrifugation at 10, 000

g for 10min at 4^0C. The pH of the CF was adjusted to 6.0, it was filtered through 0.22 mm filter and was stored at -20^0C till further use.

4.2.2.2 Effect of pediocin PA-1 on growing cells of *List. monocytogenes* Scott-A

The freshly grown *List. monocytogenes* was sub-cultured into 5 ml of BHI broth at 1% level and was incubated at 37^0C in 200rpm in the incubator shaker and after 2h the crude pediocin PA-1 preparation was added at a concentration of 10^2 AU ml^{-1} when the OD_{600} was 0.4. The samples were drawn periodically at different time intervals and the OD at 600nm was measured. The culture without pediocin was used as control.

4.2.2.3 Enumeration of *Listeria monocytogenes* Scott-A

Listeria Oxford medium base (HiMedia) was used for the enumeration of *List. monocytogenes* Scott-A. This medium consists of peptone special 23.0 gm, lithium chloride 15.0 gm, sodium chloride 5.0 gm, corn starch and esculin 1.0 gm each, ferric ammonium citrate 0.5 gm and agar 10.0 gm $liter^{-1}$. The pH of the medium was adjusted to 7.0 ± 0.2 and sterilized by autoclaving.

4.2.2.4 Molecular Biology techniques

4.2.2.4.1 DNA isolation

Total genomic DNA and plasmid DNA of LAB strain was isolated by the method described in section **2.2.2.8**. *E. coli* plasmids were isolated by the method of Birnboim and Doly (1979) as described in previous section **3.2.2.6.1**.

4.2.2.4.2 DNA manipulation

For restriction digestion, ligation and transformation of *E. coli*, standard protocols was followed as described by Sambrook and Russell (2001) and discussed previously in the section **3.2.2.6.3.** The restriction digested DNA was eluted from the agarose gel and purified using Qiagen column. The pUC19 vector was digested overnight with *Hinc*II and *Sma*I restriction enzymes, it was gel purified

and was blunt end ligated. The total genomic DNA of *Ent. faecium* PH-1 strain was separately digested with *Eco*RI, *Pst*I and *Hin*dIII separated on 0.8% agarose gel and the DNA fragments of sizes 0.5 to 1.5kb were eluted from the gel and purified. These DNA fragments were used as a source for cloning of □-*gal* gene.

4.2.2.4.3 Labeling of probe

Recombinant pUC18 plasmid bearing the □-*gal* gene of *Lact. plantarum* C3.8 was used as a source of the □-*gal* DNA probe. The plasmid was digested with *Eco* RI to rescue an internal 1.5 kb fragment of the □ -*gal* gene of strain C3.8. This fragment corresponded to probe A for □-*gal* gene as described by Fernandez *et al*. (1999). It was labeled using the DIG-dUTP random primed DNA labeling and detection kit (Boehringer) as described in section (3.2.2.6.6.1) and used as a probe for □-gal.

4.2.2.4.4 DNA dot-blot hybridization

The procedure described in section **3.2.2.6.6** was followed for DNA dot-blot hybridization. The plasmid DNA from strains PH-1, its cured derivative (DPH-1) described in section (**3.3.5**) was used for dot-blot experiment. A cesium chloride purified plasmid DNA of *Lact. plantarum* C3.8 was used as a positive control. Heat-denatured test DNA (conc.~2-5 mg) was spotted on a nylon membrane (Amersham) and hybridized with the dig-labelled □-*gal* gene probe. Hybridization and stringency washes were carried out at 45^0C according to the instructions of the manufacturer (Boehringer).

4.2.2.4.5 Nucleotide sequencing and data analysis

Nucleotide sequencing was carried out by SRD, University of Frankfurt/M, Germany with M13 primers. Multiple sequence alignment was carried out using the programme clustalW (1.82). The □-*gal* gene sequences used for comparison were taken from the GenBank.

4.2.2.5 Biochemical techniques

4.2.2.5.1 Enzyme assay

The *E. coli* DH5□ expressing □-Gal of *Ent. faecium* PH-1 and the strain harbouring control vector was grown in LB broth till the optical density reached 0.5 at 600nm. The cells were induced with 1mM IPTG and additionally grown for 4h. The cells were harvested by centrifugation, washed with potassium phosphate buffer (pH 6.8) and lysed with 0.5% Triton X100 added to 500ml of cell suspension. A disc containing ONPG was added and the reaction incubated at 37^0C water bath. Change in colour was noted visually.

4.2.2.5.2 Native gel electrophoresis

The protocol described by Laemmli (1970) for acrylamide gel electrophoresis was followed. The SDS was omitted from the separating, stacking gel, running buffer and sample buffer. The components of native acrylamide gel are shown in Table. 4.1. The stocks of acrylamide mixture (30:0.6%), 10% of APS was and Tris buffer 1.5M (pH8.8), 1M (pH 6.8) was prepared and stored at 4^0C.

The Tris glycine buffer was used for electrophoresis as a running buffer. It consists of, 25mM Tris HCl pH 8.3 and 200mM glycine. Staining, destaining were carried out as described previously (Section **3.2.2.5.3**).

Activity of the native □-galactosidase was tested in the cell lysates of test strains since the enzyme is intracellular (Bhowmik and Marth 1990). Strain PH-1, CFR K7 and *E. coli* DH5□ harboring the pUC18 plasmid were used.

Table 4.1: Components of native PAGE

Components (ml)	Separating gel (10ml) **12%**	Stacking gel (5ml) **5%**
H_2O	3.3	3.4
30% Acrylamide mix	4.0	0.83
1.5M Tris (pH8.8)	2.5	-
1M Tris (pH 6.8)	-	0.63
APS 10%	0.1	0.05
TEMED	0.004	0.005

Recombinant *E. coli* was used as a positive control in the experiment to test □-Gal activity. Strains PH-1 and CFR K7 were grown overnight at 37^0C in 50 ml MRS broth having 1% lactose as carbon source. The cells were subsequently used to inoculate 50 ml of the same medium at 1% level. The cultures were grown for 12h at 37^0C under static condition. Cells were harvested by centrifugation at 10, 000 g for 10min at 4^0C, washed twice with 0.05 mM potassium phosphate buffer (pH 6.8) and then resuspended in 1 ml of the same buffer. The cell suspensions were placed in an ice-water bath and sonicated by using an ultrasonicator (dr. Hielscher, GmbH, Germany) at 20 kHz for 15min, consisting of 1min cycle each followed by cooling with an interval of 1min. The disrupted cells were centrifuged at 10, 000 g for 15min at 4^0C and 0.05 ml of the supernatant was loaded on to a non-denaturing polyacrylamide gel (Table 4.1).

In case of *E. coli* DH5α, the strain was grown in 50 ml of LB broth supplemented with 100 mg ml^{-1} ampicillin for 3h. The culture was then induced by adding 0.5 mM IPTG and grown for additional 6h. Cells were harvested, sonicated and the lysates were loaded on to a non-denaturing polyacrylamide gel as described above. Electrophoresis was conducted at 150 V for 3h. The enzyme β-Gal activity was subsequently detected by incubating the gel for 30 min in 4-O-methylumbelliferyl-β-D-galactoside solution prepared in McIlvaine citrate phosphate buffer (pH 6.5) as described by Hung and Lee (1998). Fluorescent

bands were visualized under UV light on a transilluminator (Photodyne, USA) and photographed.

4.2.2.6 Pediocin PA-1 production

MRS, TGE and WP were used as basal media for testing the production of pediocin PA-1. The production media were formulated as follows: a) MRS broth along with 2% glucose, MRS along with 1 with 2% lactose replacing glucose as a carbon source, b) TGE broth and its double strength (2X TGE). TYE and 2X TYE contain of 1 & 2% lactose as a carbon source; respectively, and c) WP supplemented with 2% YE and 0.1% Tween 80. WP medium was optimized while varying concentrations of WP and YE along with 0.1% Tween 80 (Table 4.2). *Ent. faecium* PH-1 was grown in the MRS medium overnight at 37^0C and then used at 1% level (v/v) to inoculate 100 ml of each production media. Cells were grown for 24h (unless and otherwise mentioned) at 37^0C. Samples were withdrawn at regular intervals of 5h from the fermenting broth, to measure cell growth and production of pediocin PA-1 which was expressed in terms of its anti-listerial activity as described in subsequent section. Cell growth was monitored by measuring optical density at 600nm (OD_{600}) by Spectrophotometer (Shimadzu, Japan) and acidity (pH) was measured by pH meter (Control Dynamics, India).

4.2.2.7 Pediocin assay

Antimicrobial activity of pediocin PA-1 in the CF was tested by the modified spot-on-lawn method as described in section (**3.2.2.1**). Pediocin PA-1 activity was expressed in terms of arbitrary units per ml (AU ml^{-1}) which was defined as the highest dilution of the CF that gave an inhibition zone of 2 mm against *List. monocytogenes* Scott- A.

4.2.2.8 Preparation of vegetable salad

Vegetables such as cucumber, onion, tomato were cut into fine pieces and were mixed with the market curd (dahi) in the ratio of 50:20:10:20 proportion and used in shelf-life studies.

4.2.2.9 Biopreservation studies

Vegetable salad was inoculated with the overnight grown culture of *Listeria monocytogenes* Scot A (4.5 X10^7 cfu gm^{-1}) and kept at room temperature. The control was without Scot A. The pediocin PA-1 preparation of 10^4 AU gm^{-1} was added into the experimental sample. Samples were withdrawn periodically and serially diluted in saline and were spread plated on listeria oxford agar plates. These plates were incubated at 37^0C for 12-15h and the listerial colonies were counted and expressed as CFU gm^{-1}.

4.3 RESULTS AND DISCUSSION

4.3.1 Construction of *lacZ*⁻ pUC19 vector

Scheme for the construction of disrupted reading frame of *lac*Z gene in pUC19 vector is shown in Figure 4.1. Thus the vector designed was denoted as □pUC19 having disrupted reading frame of *lac*Z gene. During □-complementation, the *lac*Z of *E coli* DH5□translates a 1, 023 -aa product of 116 kDa protein which trans-complements with the 107 -aa product of 12 kDa from the pUC vector. For this study the reading frame of □-Gal coding *lac*Z in pUC19 was disrupted after the vector has been digested with *Hin*cII and *Sma*I and blunt-end ligated to yield □pUC19. The vector □pUC19 had a deletion of 17bp at the N-terminal end (Figure 4.1). □pUC19 upon transformation into *E. coli* DH5□yielded only white colonies with X-Gal-IPTG since the fragment of the □-*gal* gene in the plasmid was unable to complement functionally the host fragment (Coombs and Brenchley 1999).

A

*Sac*I *Sma*I *Xba*I *Sbf*I

*Eco*RI *Kpn*I *Bam*HI *Sal*I *Pst*I *Sph*I *Hin*dIII

agtgAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtaatcatggtcat

400 410 420 430 440 450 460

…..S N S S P V R P D E L T S R C A H L S P T I M M

B

agtgAATTCGAGCTCGGTACCC**D**GACCTGCAGGCATGCAAGCTTGGcgtaatcatggtcat (17 ntp deleted by *Hin*cII/*Sma* I digestion followed by blunt end ligation)

Daa C A H L S P T I M T **M**

Figure 4.1: Construction of □pUC19 vector. A) ORF of *lac*Z gene and B) ORF disrupted *lac*Z gene. D, 17bp deleted region; Daa disrupted reading frame of *LacZ*.

4.3.2 Molecular cloning of □-Gal encoding gene

The □pUC19 vector that had digested with three separate enzymes viz *Pst*I, *Hin*dIII and *Eco*RI was used for cloning. This vector after dephosphorylation and purification was ligated with the DNA fragments 0.5 to 1.5 kb in size obtained by digestion of DNA from PH-1 strain with *Pst*I, *Hin*dIII and *Eco*RI (figure 4.2). Several thousands of transformants were obtained on transformation of the ligated vector in *E. coli* DH5□. Among 1000 to 1200 transformants, 5 to 10 colonies were blue in colour. Such blue colonies were separated and purified on the same plate and used for further studies. The blue colonies obtained from the recombinant clones were considered as □-Gal proficient colonies complementing the recombinant □-Gal of the strain PH-1.

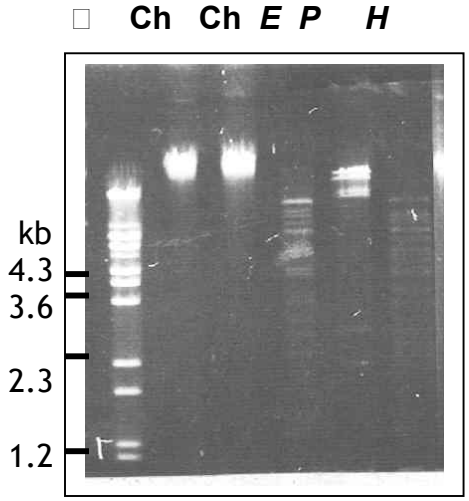


Figure 4.2: Agarose (0.8%) gel analysis of chromosomal DNA isolated from PH-1 strain and restriction digested. □, □DNA *Eco*911 marker; Ch, chromosomal DNA; lane *E*, *P* and *H* are the chromosomal DNA digested with *Eco*RI, *Pst*I and *Hin*dIII.

Plasmids were isolated from these colonies digested with the appropriate enzyme for release on insert and separated on agarose gels. The results of recombinant analysis are shown in Figure 4.3. The presence of blue colored colonies indicated that either the cloned gene complemented functionally the □-Gal of *E. coli* or that the □-Gal of *Ent. faecium* PH-1 was being expressed in the recombinant.

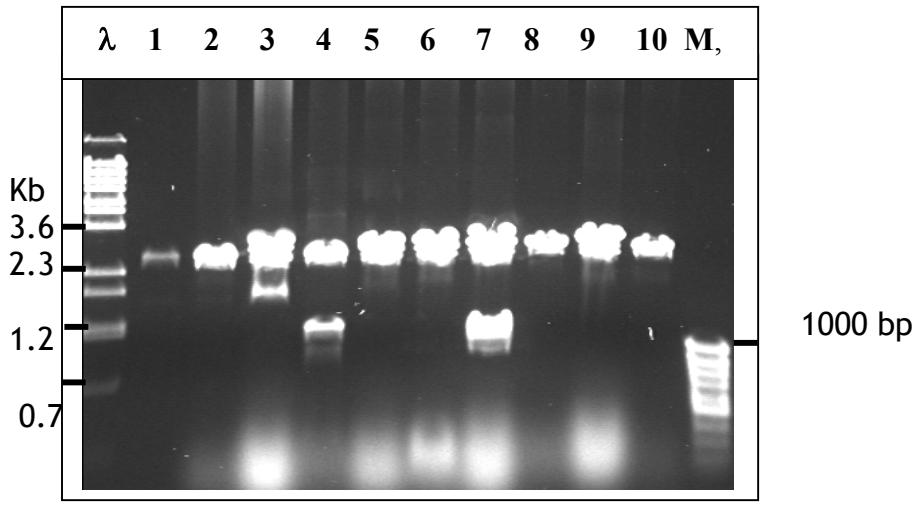


Figure 4.3: Gel analysis of □-Gal proficient recombinant of *E. coli.* The recombinants (lane 1 to 10) had been digested with the enzyme that had been used for cloning. □is a *Eco*911 marker *Bst*II digest and M is a 100 bp ladder. Recombinants 3,4 and 7 show release of insert upon restriction digestion.

Several genes coding for □-galactosidase were isolated from LAB using the complementation technique in *E. coli* (Schmidt *et al.* 1989; Rossi *et al*. 2000; Coombs and Brenchley 1999). This technique offer the advantage of cloning functional gene from the total genomic DNA upon selection in a suitable substrate that facilitate phenotypic difference. Thus, gene complementation technique is more common among other methods such as PCR cloning or Southern detection.

4.3.3 Biochemical and molecular analysis of recombinant

The □-*gal* recombinants obtained in *E. coli* were first tested for their potentiality to convert ONPG to the yellow NPG. In the present study the observations were made visually. Among the three recombinants (lane 3, 4 and 7) selected, one of the clone H3B (showed in Figure 4.3, lane 4) exhibited dense yellow color within 1-2min indicating its strong □-Gal activity. Such qualitative rapid color change was not seen in other clones as well as the pUC18 transformed cells. With only vector □pUC19 transformed *E. coli* cells, no hydrolysis of the substrate was observed.

The enzyme □-galactosidase hydrolyses the chromogenic substrate ONPG into ortho- nitrophenyl (ONP) and such end product can be measured spectrophotometrically at 420nm and thus helping in measuring the enzyme activity (Bhowmik *et al.* 1987; Bhowmik and Marth 1990; Griffin *et al.* 1996).

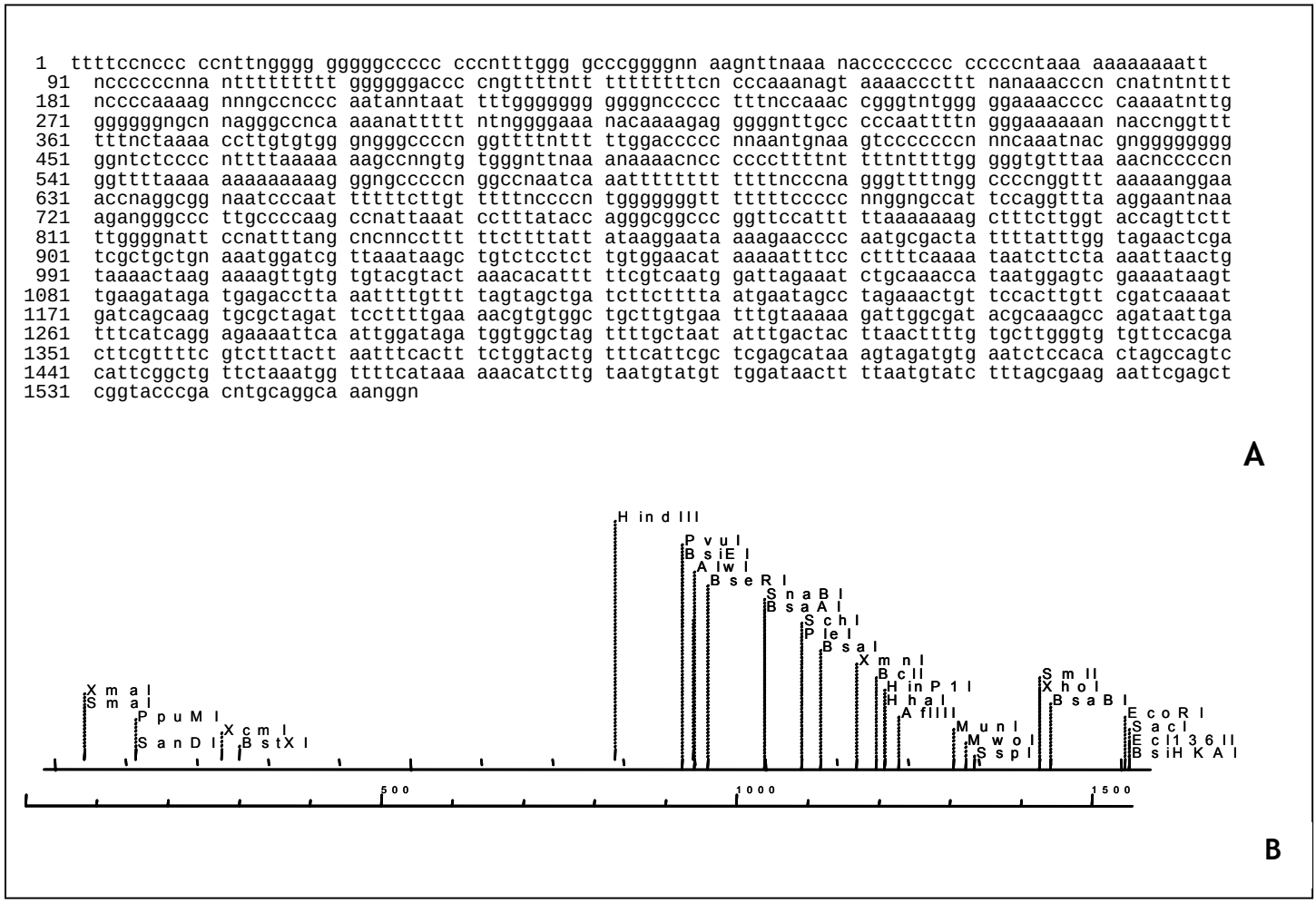


Figure 4.4: Analysis of nucleotide sequences of □ -*gal* of *Ent. faecium* PH-1. (The nucleotide sequences presented are the reverse complement of the M13R generated sequences). A, nucleotide sequences; B, restriction map generated using the programme Clone Manager Ver. 5.

The ~1.2kb insert from one of the recombinants (□-Gal expressing) was subjected for bi-directional sequencing using the M13 primers. The partial nucleotide sequences and restriction map is presented in Figure 4.4.

Multiple sequence alignment of □-*gal* of PH-1 was carried out against the □-*gal* of LAB those encoding low as well as high molecular weight □-Gal proteins. Only the regions of high similarity are shown in Figure 4.5.

```
lactdel          AGGACTTCTGGCGCATGT-CTGGTTTG--TTCAGATCAGTGACTCTTCAGGCCAAGCCGC 673
Streptthерm      AAGACTTCTGGAGACTTT-ACGGTATT--TTTAGAGATGTTTACTTGTATGCTATTCCAA 808
Blongum          AGGACTTCTGGCGTCTGC-ACGGCCTG--TTCCGCTCCGTCGAACTCGCCGCCCGCCCGC 682
lactsake         AAGATTTCTTCCGCTTCT-TCGGGATT--TTCCGGGATGTTAAGTTATTAGCTAAACCCC 869
leuconost        AAGATATGTTCCGTTTCT-CTGGTATT--TTCCGTGACGTCAATATACTGGCGGAGCCTG 955
Llacis           AAGATATGTGGCGAATGT-CAGGTATT--TTTCGTTCAGTGAACTTACAATGGCTTCCAG 649
Ecoli            AGGATATGTGGTGGTCAG-CGGGGATC--TTCCGCGATGTTTATCTGGTCGGAAAACACC 622
PH-1             AAGTTCTTTTGGGTATTC-CATTTATG--CTCCTCCTTTTTCTTTTATTATAAGAATAAA 582
Carnmalt         ATAGTCAATGGGACGAGA-TATTCTTG--CCTAAAGAAATGCCAACTTTTAAAAATCCAG 645
Bifbrev          ATGGTCTCCGCCGGCGGTGCCGGCATCGATTACGACAAGTGGGGTTACGACGTGGACTTC 1071
                 *                          *           *

lactdel          AGCGAAGCAACATCA-ATGCTGTCCGCTGCTCTCACTACC-CGAACCAGTCCCTCTTTTA 1181
Streptthерm      AACAACATAACATCA-ATGCTGTTCGTACTTCACACTATC-CTAACCAAACACGTTGGTA 1316
Blongum          AGCGCCACAACATCA-ACTCCATCCGCACCTCGCACTACC-CGAACCAGGAACGCTGGTA 1193
lactsake         AGCGCAACCATATCA-ATGCCGTCCGAACATCGCATTATC-CAGATCGGTTATCCTTTTA 1371
leuconost        TAGCTAACAATATCA-ATGCCGATCGGACCTGCCATTATC-CTGATCAATTACCTTGGTA 1463
Llacis           AAGAACATAATTTTA-ATGCGGTCCGCTGCTCTCATTATC-CTAATGATTCACGCTGGTA 1136
Ecoli            AGCAGCACAATATCA-ACTCCGTGCGTACCGCTCACTACC-CGAACGATCCGCGTTTTTA 1151
PH-1             NCCCCAAAAACCCNN-ANAAAATTGGGNTTCCTTCCCGGG-GGNAAAAATCCNATTTGGG 1080
Carnmalt         ATAGTCGGATTTTTA-AAGAAGTC-GTTCAATTAGGAACT-GAAATGAGCCAGTTAGACG 1161
Bifbrev          GCTCGCGTGGTTCCGCGCGCTGGCGGACAACGGTCTGACCGCAGACGTGGTGCCGGTTCG 1604
                                         *                 *

lactdel          CAGCACTATGAAGGGGTGACCCACAACCGGAAGTTTGACGA---CGCCACCCAGATTGAA 1518
Streptthерm      GTTCACTATGAAGGTGTTGCATGGTGTCGTGAGTTTGATTA---CATTACAGACATCGAA 1650
Blongum          GTGCACTACGAGGGCGTGACCCACAACCGTGACTACGATGA---CGTCACCGACATCGAG 1530
lactsake         GTGCATTATGAAGGGGTCTTCCGGGCGCCCGAGTATAAGGCGACGATTTCGGATGTTGAA 1708
leuconost        GTCCACTACGAAGGCGTTTTCTACACACCAGAATTAAAAGATCGCATTTCTGATGTTGAA 1800
Llacis           GTTCATTATGAGGGGGGAGATGACGCAAGTAGAGGAGCAACAGATGCCACTGATATCATT 1449
Ecoli            GTGCATTACGAAGAAGATCGCGATGCTGAAGTGGTCGATAT---TATTTCCACCATGTAC 1470
PH-1             TNTTTTT----GGGNGTAACCCNCTCNAAAAGGGACTNTTT-----TTGCGGAGAAAAAN 1396
Carnmalt         ACTTTTTTTT--AGCGGTATTGTAGATGAATATGACCGGGTTTATCTTGGCGGTTATCCAG 1506
Bifbrev          ATCACCTCCGTGGCCGATACC---GCTCACGTGGTTGCTTCCTTCAAGGCTGATAAGTGG 1956
                       *     *  *                                  *
```

Figure 4.5: Multiple sequence alignment of a DNA fragment from PH-1 expressing □-Gal like activity with *lac*Z sequenced from other LAB. (lactdel: *Lact delbrueckii* acc no. M55068; *Stre. Thermophillus*: acc no. M63636; Blongum, *Bif. Longum*: AJ242596; lactsake: *Lact. sake* X82287; leuconost: *Leuc. lactis* M92281; Llactis: *L. lactis* X80037; E.coli: *Esch. coli* X52031; PH-1: Ent. faecium PH-1 (this study); Carnmalt: *Carn. maltaroniaticum* AF184246; Bifbrev: *Bif. breve* E05040) * indicates the conserved nucleotide found among aligned sequences.

Phylogenetic analysis of the β-*gal* like gene sequences from PH-1 indicated high degree of homology with *Carn. maltaroniaticum*, followed by *Bif. breve* (Figure 4.6). Better sequencing data could provide a detailed information on evolution of β-*gal* gene of *Ent. faecium*, which is of intestinal origin.

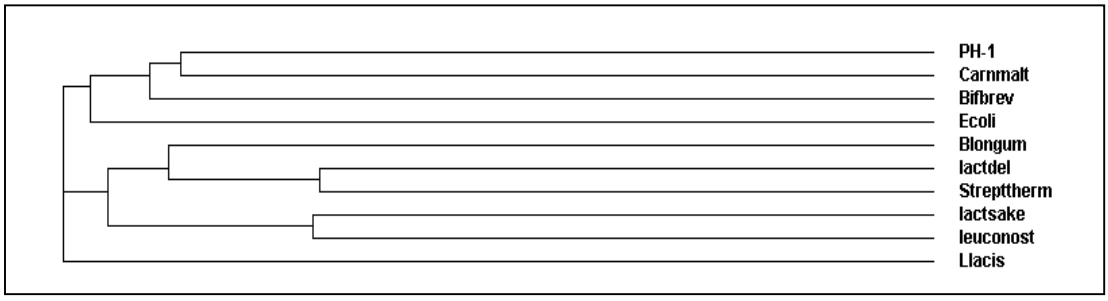


Figure 4.6: Phylogenetic analysis of β-*gal* like sequences from strain PH-1 with the *lac*Z gene sequences from other LAB. Abbreviation used are same as described in the Figure 4.5.

4.3.4 Molecular detection of native β-*gal* gene

The DNA dot-blot technique was employed to provide a molecular basis for the presence of β-*gal* gene sequences. The heat-denatured high molecular weight plasmid DNA and chromosomal DNA of strain PH-1 reacted with a DNA probe derived from *Lact. plantarum* β-*gal* gene. However, DPH-1 did not react with the probe and hence there was no signals of hybridization when approx. same quantity of DNA preparation was spotted (Figure 4.7). Hybridization signal with PH-1 DNA indicates a considerable homology between β-*gal* of C3.8 and PH-1, and found to be a plasmid encoded character.

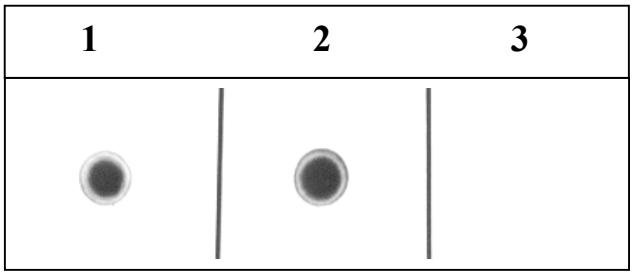


Figure 4.7: Dot-blot hybridization of DNA preparation using *Lact. plantarum* β-*gal* probe. 1, PH-1; 2, *Lact. plantarum* C3.8 and 3, DPH-1.

In the previous section **3.3.5**, it has been demonstrated that the pediocin production, immunity and carbohydrate fermentation such as lactose, maltose, raffinose and sucrose are plasmid encoded characters in *Ent. faecium* PH-1. The *Lact. plantarum* □-*gal* probe did not react with DNA from DPH-1 indicating the plasmid borne nature of the □-*gal* gene in strain PH-1. These results also suggested that there was no duplication of this gene into the chromosome as seen in dairy lactobacilli (Fernandez *et al*. 1999). The □-*gal* gene in *L. lactis* is plasmid encoded as reported by Griffin *et al*. (1996). *Bif. infantis* has two different □-*gal* genes encoding isozymes of □Gal (Hung and Lee,1998). However, in *Lact. sake,* two different genes, *lac*Z and *lac*M codes for a single active □-Gal (Obst *et al*. 1995). Rossi *et al*. (2000) showed that *lac*Z of *Bifidobacterium longum* shares high degree of homology with that of the well studied *E. coli lac*Z. The isozyme forms of □-Gal separable on native gels have been reported from *B. infantis* (Hung and Lee 1998). The amino acid sequence of □Gal from different organisms varies in sequence identity from 100 to 28% and in molecular size from 100 to 36 kDa (Obst *et al*. 1995; Rossi *et al.* 2000) indicating wide diversity among the different species. These observations indicated that □-*gal* is widely distributed in many LAB and the strain PH-1 would have acquired this genotype during the course of evolution in its natural habitat.

4.3.5 Characterization of the □-galactosidase enzyme

Activity staining with 4 MeUmG was performed to assess the presence of active enzyme in growing cells of PH-1 after separation of the total cell lysate on native acrylamide gels (Figure 4.8). The high molecular weight □-Gal of *E. coli* (MW 128 kDa) moved very slowly whereas the □-Gal of PH-1 was found to move faster. A similar assay performed with cell lysates from the control strain of CFR K7 was negative (lane 2). The inability of the strain CFR K7 to hydrolyze lactose has been documented (Ramesh 2000). Induction of the □-Gal enzyme from PH-1 in comparison with the enzyme from *E. coli* by lactose is shown in Figure 4.8b. Lactose inducible intracellular □-Gal was demonstrated by Bhowmik and Marth (1990) in few strain of pediococci. Moreover unlike as in *E.*

coli, IPTG a lactose analogue did not induce □-Gal activity in these bacteria. The purified □-Gal from *Ped. pentosaceus* on separation by SDS-PAGE was found to be 66 kDa protein. In the present study, only one fluorescent band was seen in native gels (Figure 4.8b, lane 3), indicating probably the presence of a single subunit enzyme.

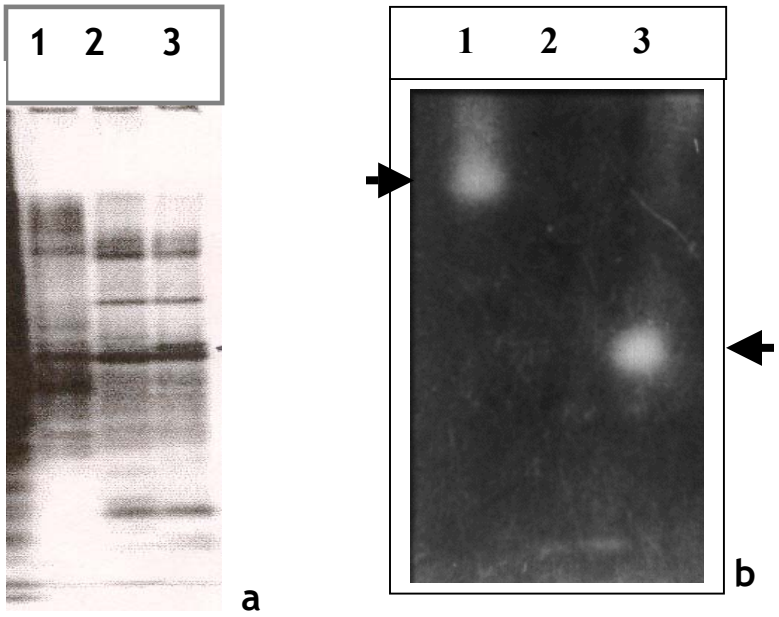


Figure 4.8: Activity staining on non-denaturing poly-acrylamide gel. Lane 1, the *E. coli* □-galactosidase; lane 2, Cell lysate from K7 and lane 3, cell lysate from PH-1. Arrow indicates the florescence □-Gal band **a)** native gel after staining with coomasie blue and **b)** activity staining of a gel run parallel with the stained gel.

4.3.6 Fermentative production of pediocin PA-1

After having identified the ability of strain PH-1 to ferment lactose, it was decided to explore the use of a lactose-based medium for theproduction of pediocin. Three basal media were used for production of the pediocin and the results are

graphically represented in Figure 4.9. It was found that the highest cell biomass was obtained when PH-1 was grown in MRS broth supplemented with 2% glucose. Pediocin production by PH-1 was highest when grown in MRS broth with 2% lactose (240 X 10^3): a yield comparable to that obtained when the cells were grown in commercially available MRS which contains 2% glucose as carbon source. These results show the preference for lactose by PH-1 strain for the production of pediocin. Low cost food-grade medium such as TGE, which is a non-buffered medium, was found suitable for pediocin AcH production (Biswas *et al*.1991). In another experiment glucose in TGE was substituted with lactose and a non-buffered medium TYE formulated. Pediocin production in this medium was considerably low. However doubling the strength of the same medium and raising lactose concentration to 2% enabled PH-1 to increase pediocin production 3 fold. This increase in pediocin production indicated the importance of nitrogen in the form of tryptone and YE.

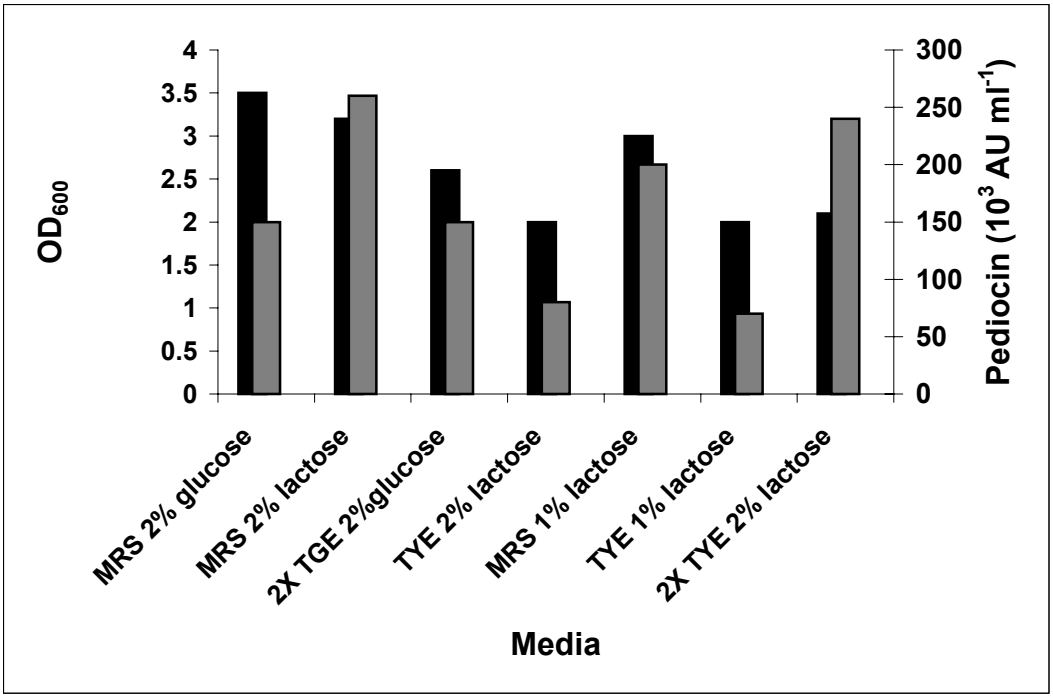


Figure 4.9: Growth (OD_{600}) and pediocin PA-1 production by *Ent. faecium* PH-1 in MRS, TGE and TYE broth supplemented with lactose.
(■ Growth and ■ Pediocin Production)

Based on the above results and the utilization of lactose as a carbon source, production of pediocin in WP was studied. Poor cell growth and a low titre of 1000 AU ml^{-1} pediocin PA-1 was obtained in WP alone having around 1.2% endogenous lactose (Table 4.2). These results are in contrary to the results obtained by *Liao et al*. (1993) for pediocin PO2 production in WP.

Table 4.2: Growth (OD_{600}) of *Ent. faecium* PH-1 and pediocin production (AU ml^{-1}) in WP supplemented with varying concentration of YE and 0.1% Tween 80.

Whey Permeate (ml)	YE (%)	Growth# (OD_{600})	Pediocin# (X 10^3AU ml^{-1})
6.0	-	0.3	1
6.0	0.5	2.2	40
6.0	1.0	2.9	140
6.0	2.0	3.5	150
6.0	3.0	3.42	150
4.0	2.0	3.1	140
8.0	2.0	3.3	150
10.0	2.0	3.1	150

#Values represents the results of mean of two independent experiments.

In a mixed production of pediocin and nisin, Goulhen *et al.* (1999) had used 0.5% glucose in WP medium, since *Ped. acidilactici* UL5 could not utilize lactose. In this experiment, to facilitate healthy cell growth, 6 ml WP was supplemented with different concentrations of YE ranging from 0.5 to 3.0%.

The highest yield of 150 $X10^3$ pediocin PA-1 with highest cell biomass was obtained in WP supplemented with 2% YE (Table 4.2). This yield was equal to that obtained in commercially available MRS broth. However, further increase in WP and YE concentration failed to enhance pediocin PA-1 production. Production of pediocin PA-1 in optimized WP supplemented with YE was studied at different time intervals and it was found that 50% of the pediocin was produced during first 10h of fermentation (pH 5.15) while the rest was recovered during the next 12h of fermentation when the pH dropped to 4.2 (Figure 4.10). However, Biswas *et al*. (1991) reported that 60% of pediocin AcH was produced during the first 8h while the remaining 40% was recovered during the next 8h of fermentation in TGE medium with the pH dropping from 6.5 to 3.6. It was suggested that a pH of 3.6 was necessary for active pediocin AcH synthesis.

The results presented here are in partial agreement with that of Ennahar *et al*. (1996) demonstrating that acidic pH is not needed for the production of pediocin AcH by *Lact. plantarum* WHE92. Moreover when pediocin PO2 was produced in a WP medium supplemented with 2% YE, 50% of the bacteriocin was produced during the first twelve hour of fermentation while the rest was secreted during the next 12h of fermentation. In comparison to above, this study suggests that WP with YE provides for healthy cell growth (2.5X more) leading to decrease in pH to 4.6 and production of highly active pediocin PA-1.

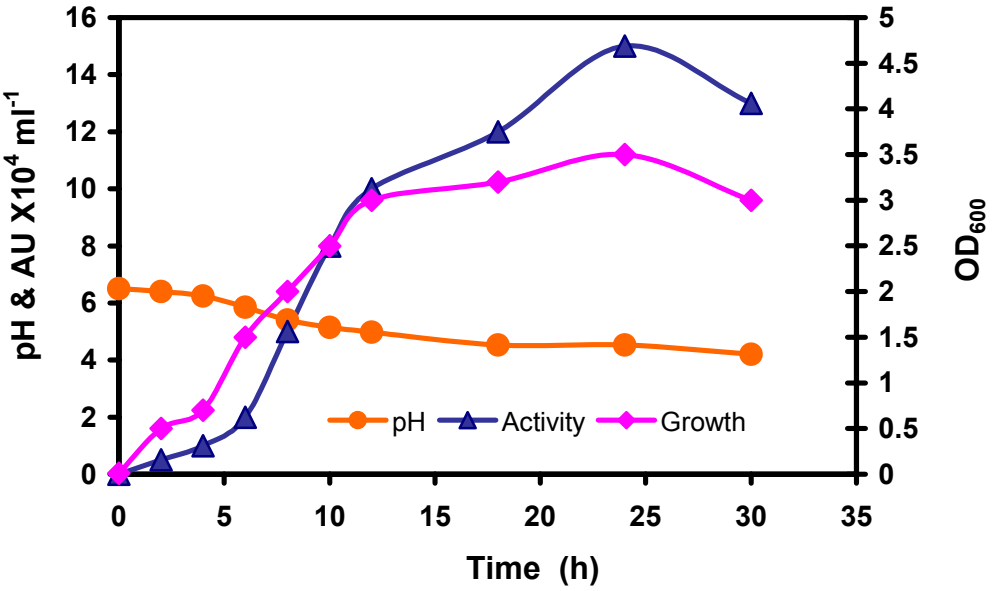


Figure 4.10: Changes in pH (acidity), cell density (OD_{600}) and pediocin PA-1 production (antimicrobial activity, AU ml^{-1}) during growth of *Ent. faecium* PH-1 in WP supplemented with 2% YE along with 0.1% Tween 80 at 37^0C for 30h.

Well characterized pediocin AcH/PA1 producing *Ped. acidilactici* strains cannot use lactose and hence WP could not be used for the production of these bacteriocins (Biswas *et al.*1991; US patent Nos. 4, 929, 445 and 5, 445, 835).

4.3.7 Growth inhibition of Scott-A by pediocin PA-1

The filter sterilized crude pediocin PA-1 was added into the growing cells of Scott-A and its inhibition was monitored by measuring the OD at 600nm. The inhibitory activity of pediocin against the *List. monocytogenes* Scott-A is shown in figure 4.11. The results suggest that the growth of exponential growing cells of Scott-A was restricted by the addition of pediocin. Pediocin PA-1 lysed cells of *List. monocytogenes* as evidenced by decrease in OD.

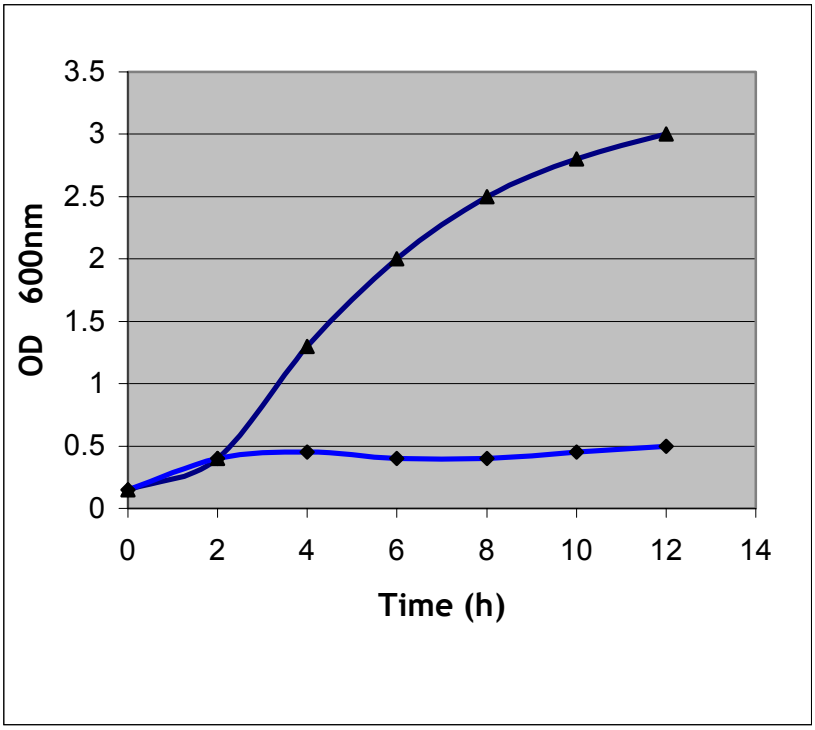


Figure 4.11: Effect of pediocin PA-1 on the growing cells of *List. monocytogenes* Scott-A. (with pediocin; without pediocin)

Pucci *et al*. (1998) had demonstrated the lytic activity of pediocin PA-1 against *List. monocytogenes* LM01. However, Ramesh (2000) has shown that pediocin K7 exhibits non-lytic inhibition to the growing cells of listeria. These results indicate that the antagonistic activity of pediocin may differ due to various factors such as strain specificity, initial dose of antimicrobial compound used and the medium for cultivation of the target bacterium.

4.3.8 Survival of *Listeria monocytogenes* Scott-A in vegetable salad

The effect of pediocin PA-1 on the survival of Scott-A in a vegetable salad kept at room temperature is presented in Figure 4.12 The results indicate that in the control experiment the population of Scott-A decreased from 7.6$\log_{10}$ to 3.4$\log_{10}$. However, when the pediocin PA-1 (1200 AU gm^{-1}) was added to the vegetable salad, viability of Scott-A was drastically reduced to 2$\log_{10}$. It was found that there was a 3 fold decrease in viability in 3h. These results indicate that pediocin

causes rapid cessation of Scott-A count due to the inhibitory action of anti-listerial bacteriocin. It would be desirable to continue these studies at different levels of pediocin and different storage temperatures. Such studies perhaps would helped in understanding the behavior of Scott-A in this food system.

Vegetable salad is routinely prepared by mixing culinary vegetables and market curd. In India generally yogurt is not prepared with a well defined starter culture and strict hygienic conditions are not often employed. The natural flora present in curd would also have contributed to the inhibition of Scot A, whose viability decrease even in the control salad. Vegetable salad especially consumed freshly. However, in certain cases, it is prepared well in advance in order to meet the requirements in bulk since preparation involves lot of labor.

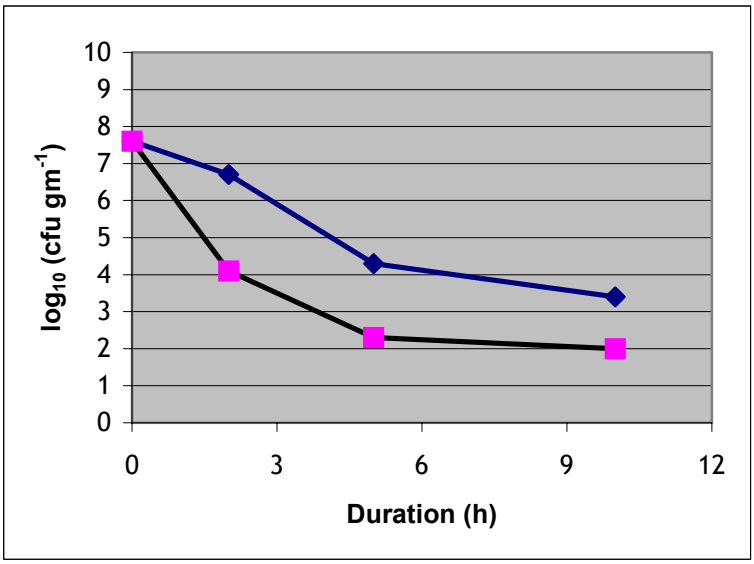


Figure 4.12: Survival of *List. monocytogenes* Scott-A in vegetable salad (■ with and ◆ without pediocin PA-1)**.**

Ramesh (2000) had previously shown the decrease in viability of *List. monocytogenes* in the fermented milk product (Srikhand) upon storage without pediocin K7. This indicate that the indigenous fermented milk products do not provide congenial environment for either further proliferation or the viability of

listeria. Although this pathogen has been known to be associated with raw milk samples and other dairy products such as ice-cream (Kozak *et al*. 1996). The antilisterial property of LAB found in yogurt (Dahi) has been described by Varadaraj *et al.* (1993). Many vegetables also contain antilisterial pediococci which can also contribute towards inhibition of listerial growth (Ramesh 2000).

Varieties of cut vegetables are highly prone to microbial spoilage due to its high moisture content and numerous cut surfaces that provides optimal conditions for growth of Gram-negative spoilage microorganisms such as *Pseudomonas* spp and the species of Enterobacteriaceae (Vescovo *et al.* 1995). Use of broad-spectrum bacteriocin such as pediocin PA-1 can exhibit strong antimicrobial activity against such undesirable microflora and could contribute in increasing the shelf-life of the vegetable salads, where the refrigeration is not available for storage.

4.4 CONCLUSION

In the present study attempts to clone and characterize □-*gal* gene from PH-1 have been made. The nucleotide sequence data suggest low homology of □-*gal* with those of other LAB used for comparison. However, dot-blot and native gel assay provided the molecular and biochemical evidences for the presence of a □-Gal encoding gene and that it was induced by lactose. Production of pediocin PA-1 was studied in WP and it was found that this food-grade low cost medium not only supported healthy cell growth but also higher amounts of pediocin production.

Shelf-life studies on vegetable salad were carried out in the presence of pediocin against the pathogen *List. monocytogenes* Scott-A. It was found that pediocin PA-1 is highly effective against this pathogen in a food-system suggesting the possible use of pediocin PA-1 in Indian foods, where cold-storage facilities are limited.

CHAPTER 5

MOLECULAR CLONING AND EXPRESSION OF *ped*A AND *ped*B GENES IN *E. 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 □-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 *Nco*I/*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 e*t 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 □-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/□-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). □-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 ml^{-1}) were procured from Promega (USA). T_4 DNA Ligase, (10U ml^{-1}), CIAP, 1 U ml^{-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* DH5□, Bl21 (DE3): (F^-*omp*T *gal*[*dcm*](*lon*) *hsd*S_B ($r_B^-m_B^-$, an *E. coli* B strain) a □ prophage carrying the T_7 RNA polymerase gene) obtained from In Vitrogen (The Netherlands) and M15: pREP4, *lac*I^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 GG***G 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 *Kpn*I/ *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 (*Nco*I/*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 *Pst*I and gel purified using the Qiagen column. It was then ligated to double digested (*Bam*HI/*Pst*I) 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/*Sma*I and dephosphorylated. The presence of recombinant plasmid pUCpedimmQ thus constructed was confirmed by *Acc*II 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* DH5□ and were selected on LB agar, X-Gal, Ampicillin (100 mg ml^{-1}) by blue/white screening as given in **3.2.2.6.3.** *E. coli* DH5□ 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	Separating gel (10ml)	Stacking gel (5ml)
(ml)	**15%**	**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 ml 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 mm) 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^{0}C. 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 □-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 m 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.

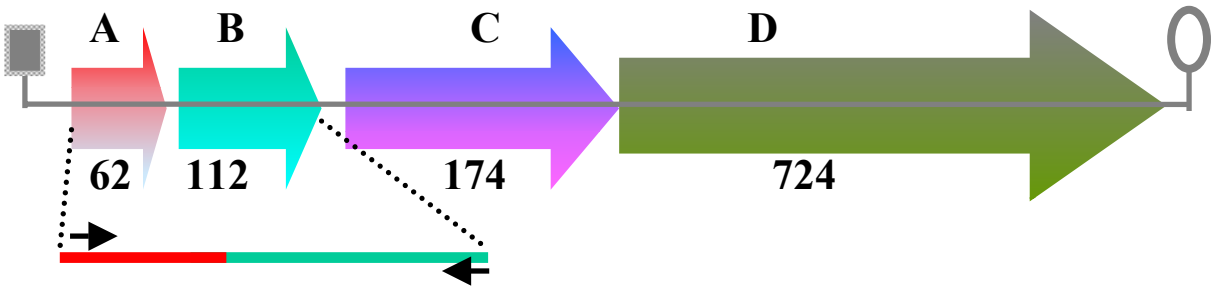


Figure 5.1: Gene organization for pediocin production. A to D are the genes coding for structural, immunity, maturation and transport proteins, respectively. Size of each protein is shown in number of –aa in each case. Promoter (rectangle box) and terminator (lollipop symbol) is also shown. Arrow indicates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

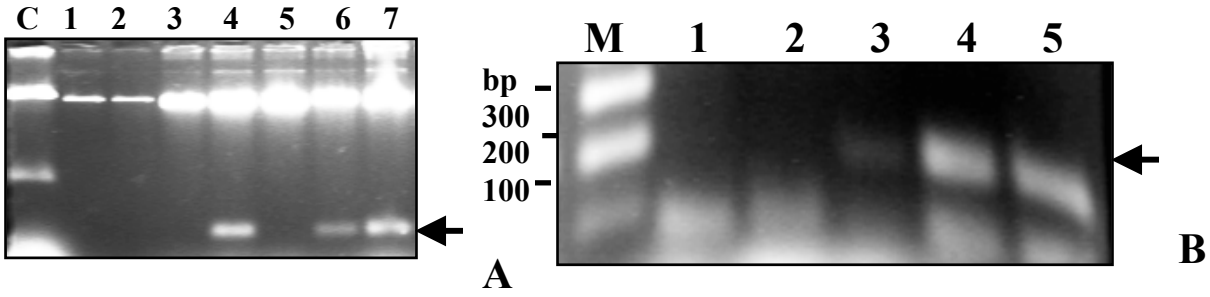


Figure 5.2: Molecular cloning of *ped*A gene. a) Agarose gel (1.5%) electrophoresis of the pTZpedAB recombinants by *Hind*III digestion and b) confirmation of gene (insert release) after sub-cloning into pRSET A vector. Arrow indicates the desired size of band obtained. M is a 100 bp ladder.

The *Kpn*I/*Hin*dIII fragment from pTZpedAB was released, purified and ligated to double digested (*Kpn*I/*Hin*dIII), 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

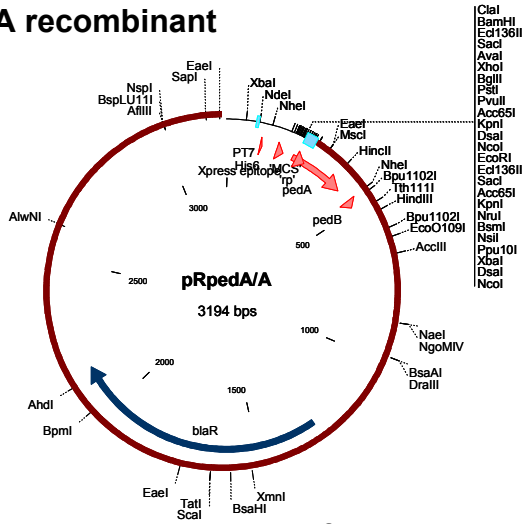


Figure 5.3: Molecular map of pRpedA/A

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/*Hin*dIII 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.

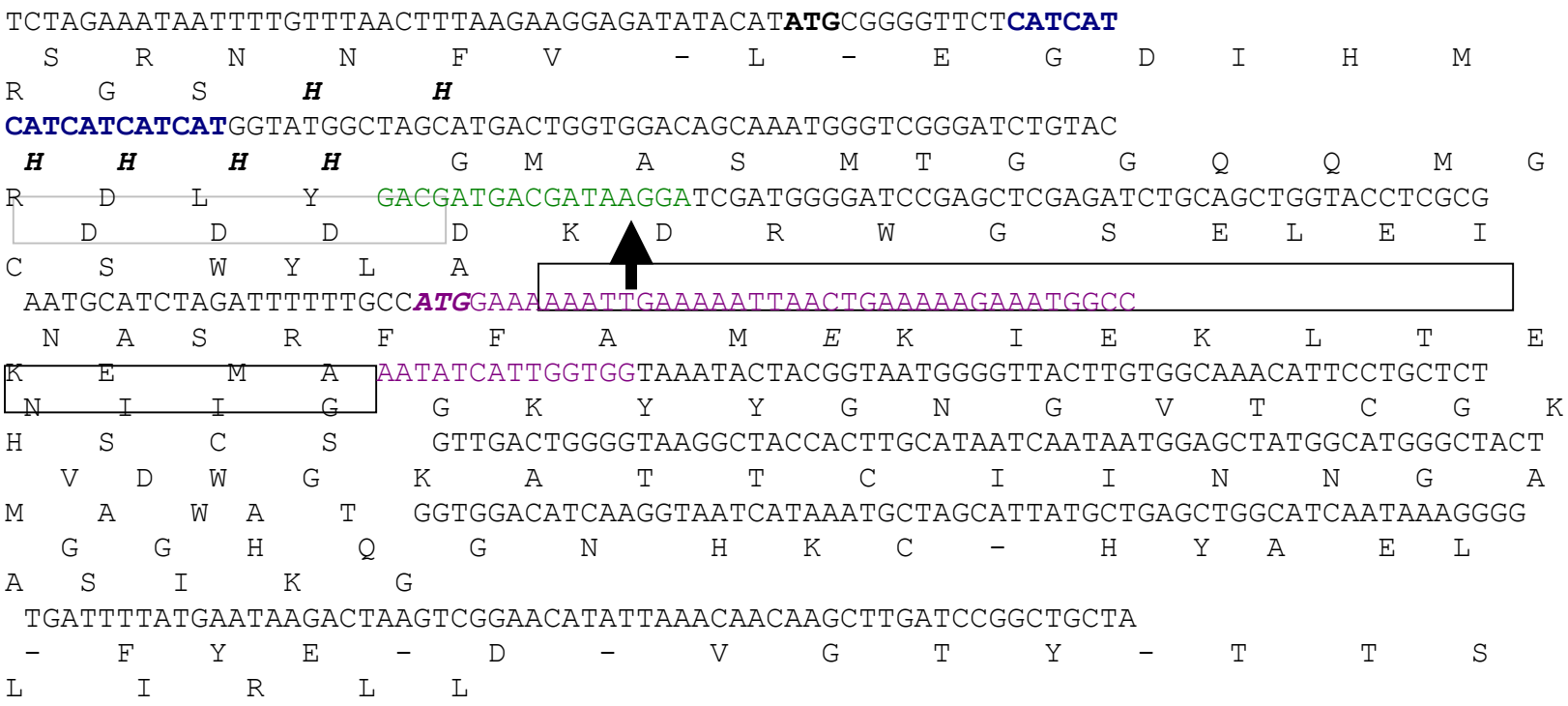


Figure 5.4: Nucleotide sequences of 6X *his*-X*press-ped*A of *Ent. faecium* PH-1. The deduced most probable translated product is also shown. Nucleotide sequence result confirmed the correct restoration of reading frame of the fusion construct. The primer generated changed amino acid 'E' is shown after 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 **M*E*KIEKLTEK 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

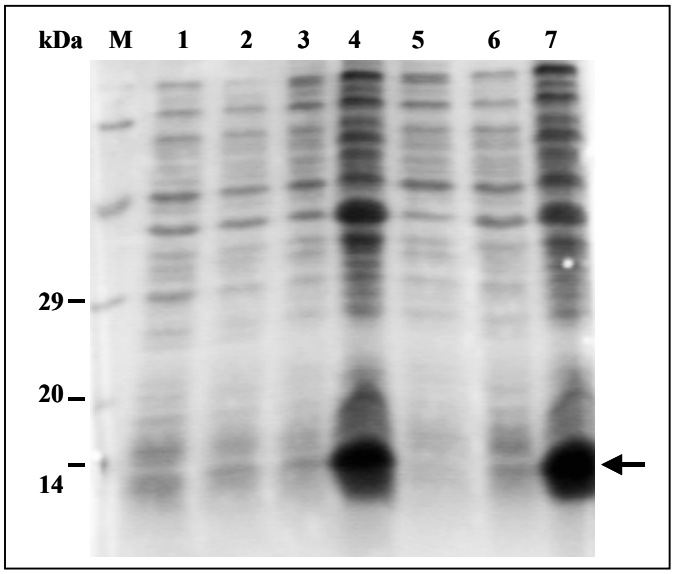


Figure 5.6: Analysis of total cell lysate of pediocin expressing *E. coli*
SDS-PAGE (15%) analysis of ***His6-Xpress-PedA*** recombinant in *E. coli* Bl21. Lane 1, total cell lysate prepared from 2h grown preinduced r4 recombinant. Lane 2, lysate of r4 recombinant prepared directly in 1X loading buffer. 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.

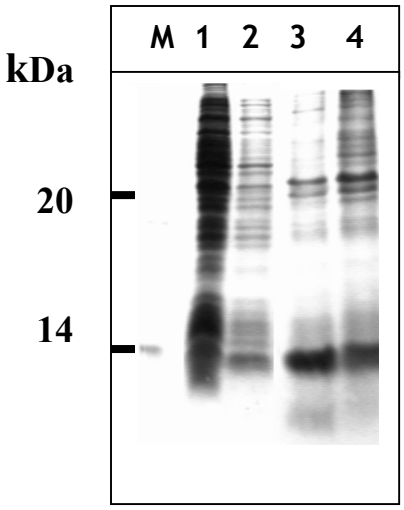


Figure 5.7: Isolation and purification of IBs of *E. coli* expressing rec-pediocin. Gel analysis of IBs purified from hyper expressing *E. coli*. Lane 1, induced cells (control); 2, induced (frozen at -20^0C), 3, purified IBs from (2) and 4, purified IBs from sample 1.

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.

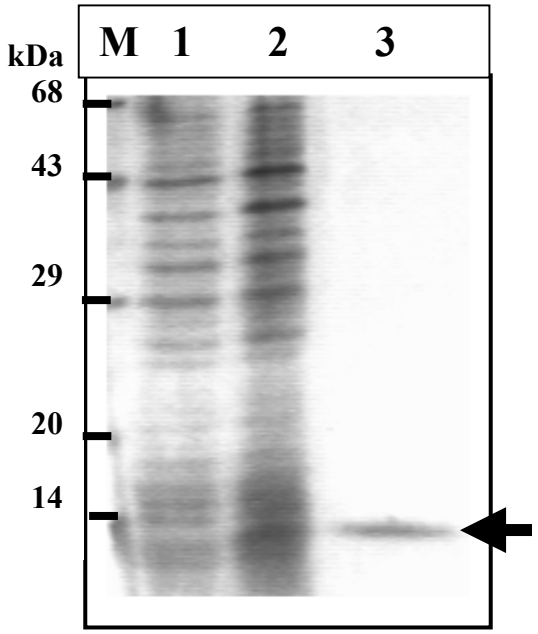


Figure 5.8: Single step purification of recombinant pediocin. SDS-PAGE (15% acrylamide) analysis of 6X histidine –Xpress-prepediocin produced in *E. coli.* Lane 1, total cell lysate uninduced ; 2, Lysate, induced recombinant and lane 3, Ni-NTA beads absorbed IBs protein eluted with 0.5 M imidazole. M,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

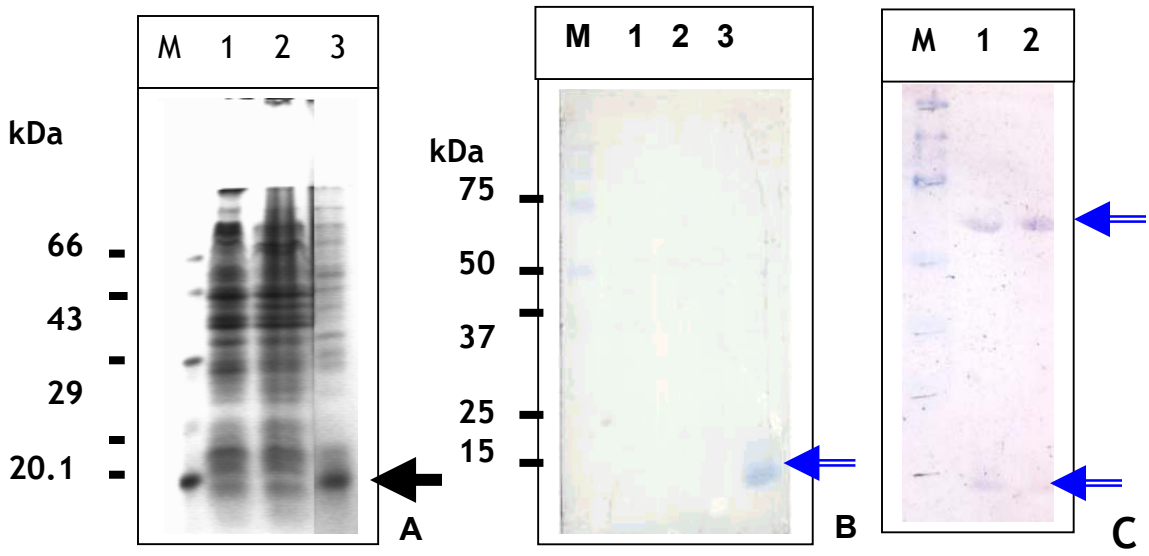


Figure 5.9: Immunlogical detection of rec-pediocin by anti-6XHis Ab.
A) SDS-PAGE analysis, **B)** & **C)** immunological detection by Ab.
← Indicates IBs of *E. coli*. ⇐ indicates putative monomeric and multimeric fusion protein detected in western blot, M in figure B and C is a prestained see blue molecular 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 □-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).

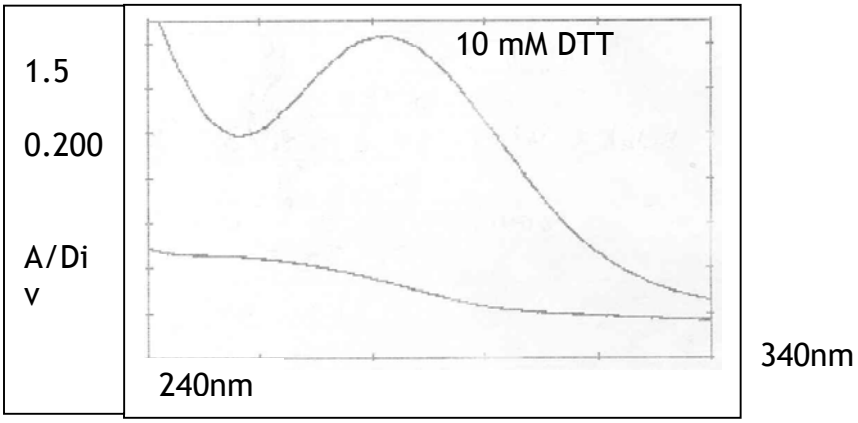


Figure 5.10: Effect of DTT on solubility of IBs of *E. coli*.

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* Scott-A 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 □-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 □-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 □-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

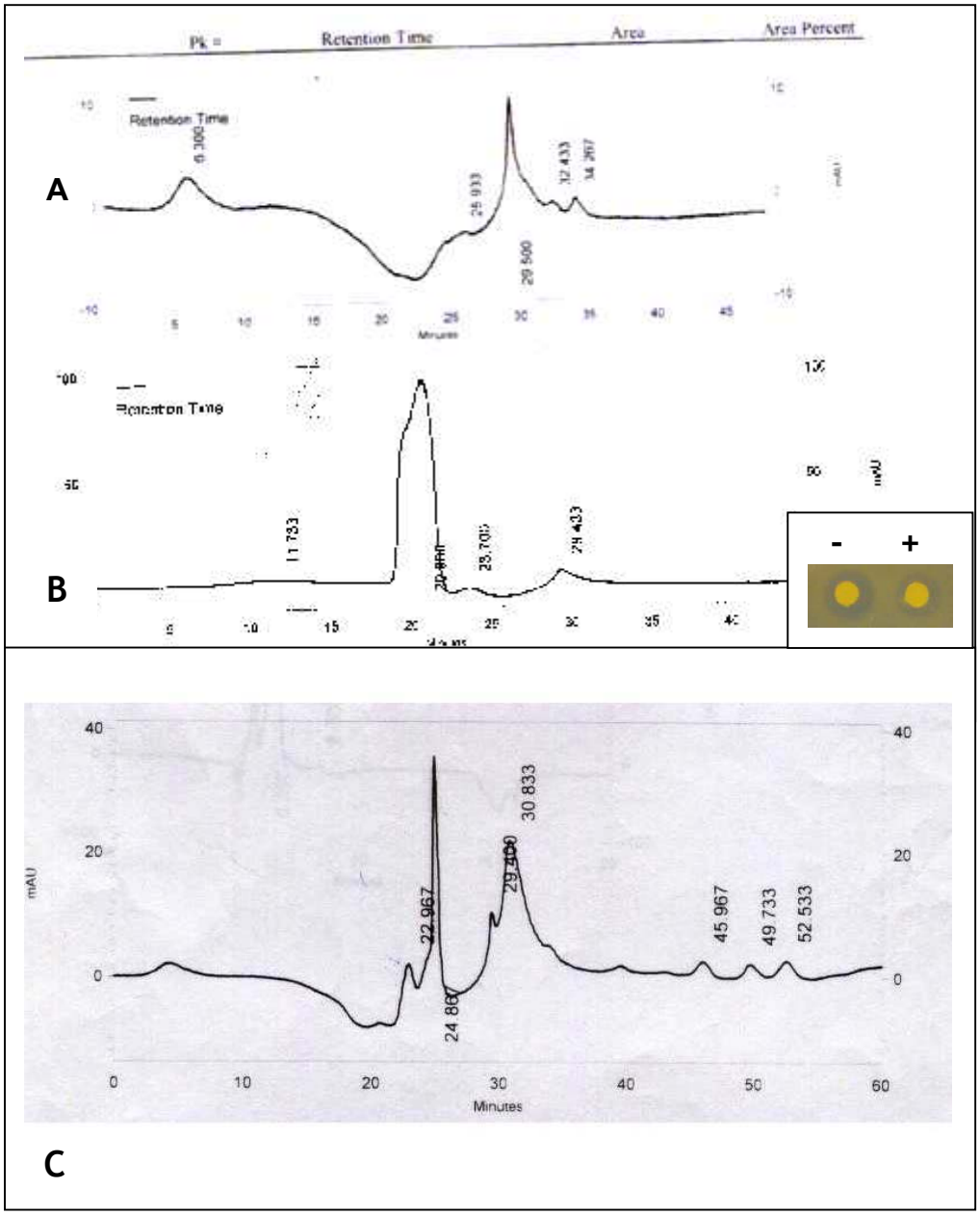


Figure 5.11: Semi-preparative RP-HPLC analysis of recombinant and native pediocin. A: unfolded, B: refolded recombinant pediocin, C: pediocin preparation from native source and D: pediocin assay by agar disc assay of recombinant pediocin (+) with trypsin and (-) without trypsin. Reduced zone of inhibition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

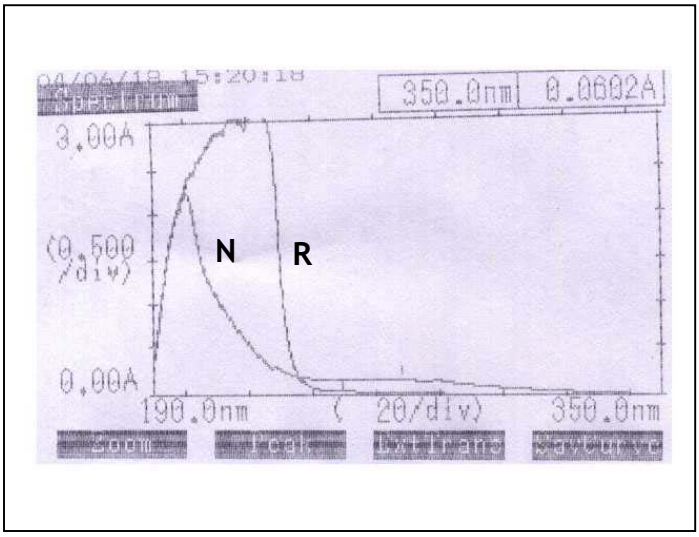


Figure 5.12: Physicochemical characterization of purified rec-pediocin using UV spectrum. (N) native and (R) rec-pediocin.

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 *Nco*I/*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).

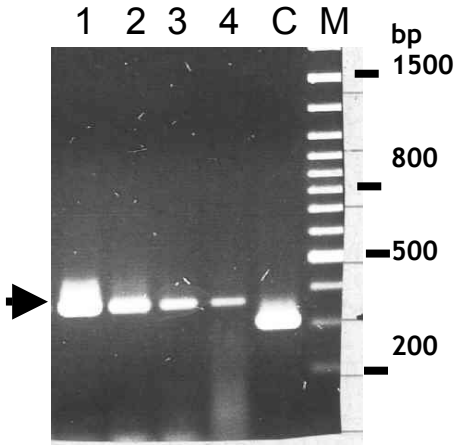


Figure 5.13: Amplification of *ped*B gene by PCR. Agarose (1.5%) gel electrophoresis of pediocin immunity gene amplified from the native PH-1 strain using templates (lane 1 to 4). Arrow indicates the position of the PCR product of expected size obtained. M, 1kb ladder (MBI). C, control PCR of 300bp

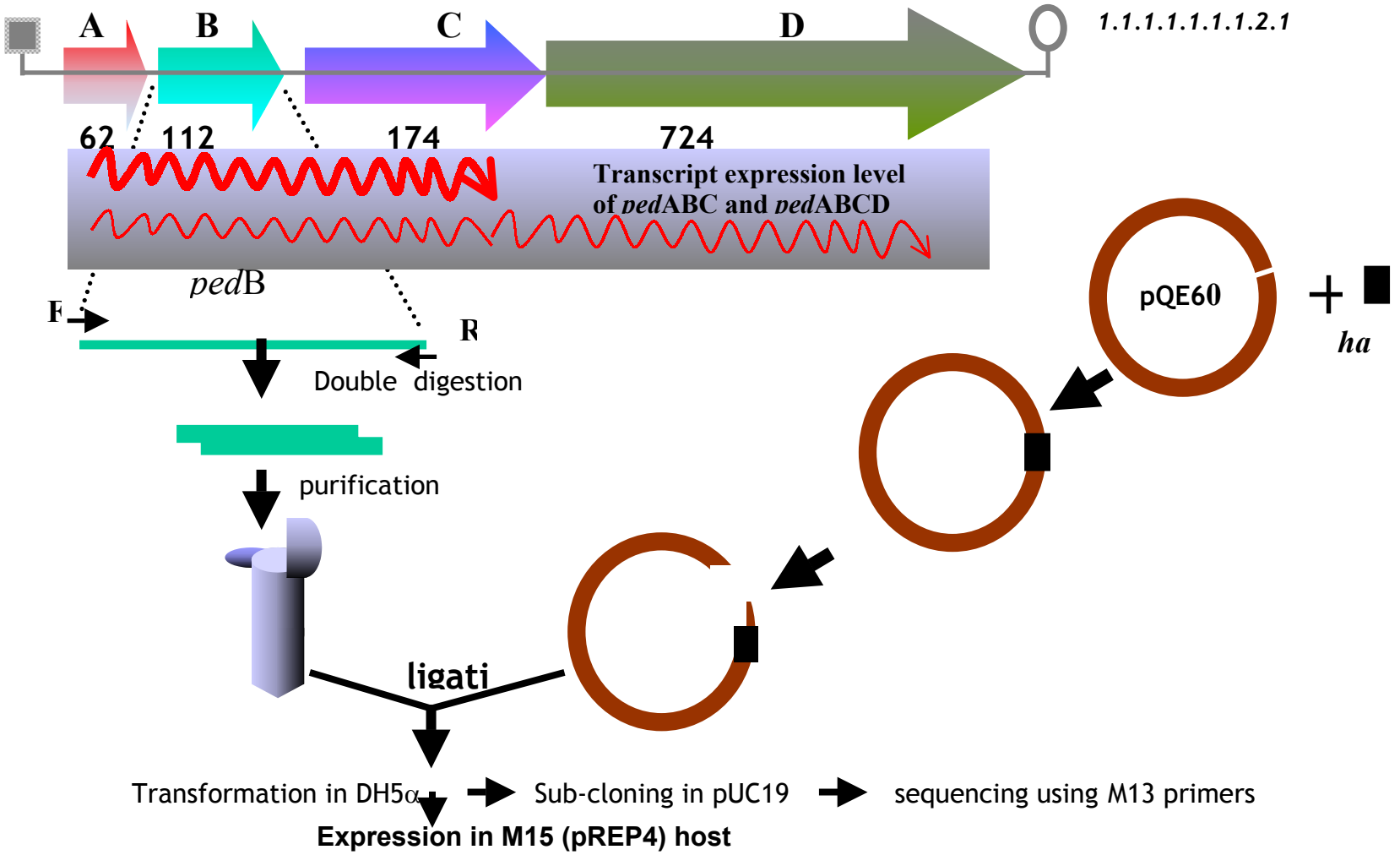


Figure 5.14: Strategy for the construction of a vector for expressing PedB along with a with C-terminal tag

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).

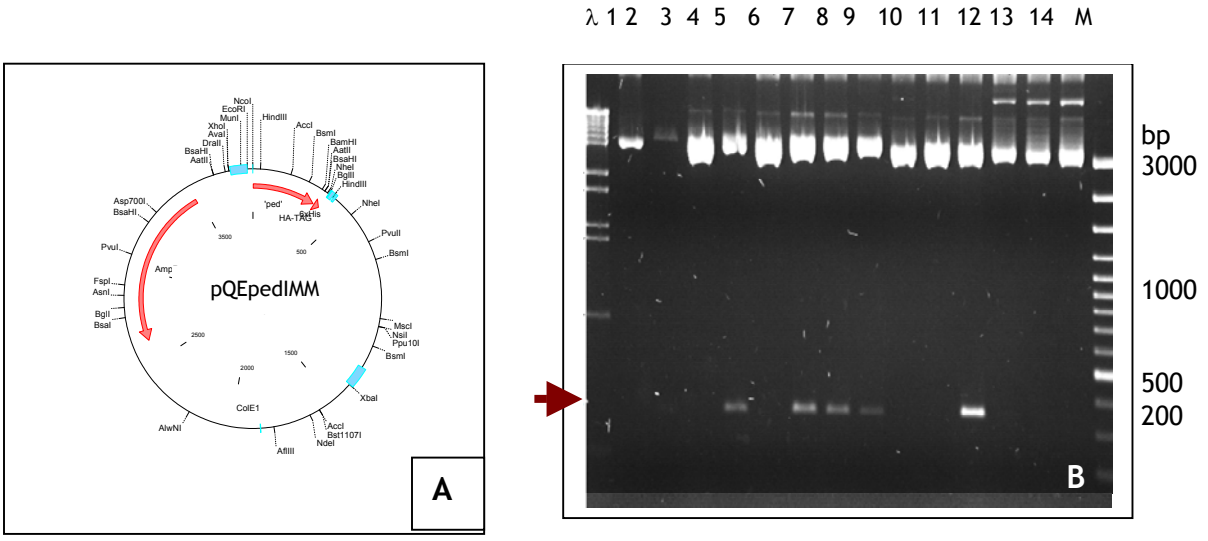


Figure 5.15: Molecular map and restriction analysis of pQEpedIMM. A)The nucleotide sequences of *ped*B gene conferring immunity function against pediocin was taken from the GeneBank (Acc. No. M83924). B) Agarose (1.5%) gel electrophoresis of *Nco*I/*Bam*HI double digest of the putative recombinant (3-14). 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 agactaagtc 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

181accagtacgt 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 c*agc*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).

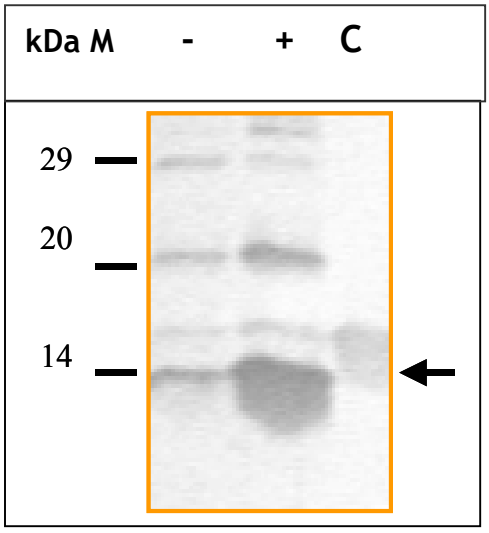


Figure 5.17: Western-blot analysis of PedB-HA-6XHis fusion protein in *E. coli M15*(pREP4). – uninduced, + induced with 1 mM IPTG. C is a positive control of SpaS-HA-6XHis detected by anti-HA antibody. Arrow indicates the expected size (~15 kDa) of fusion protein. M is a medium range protein MW marker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

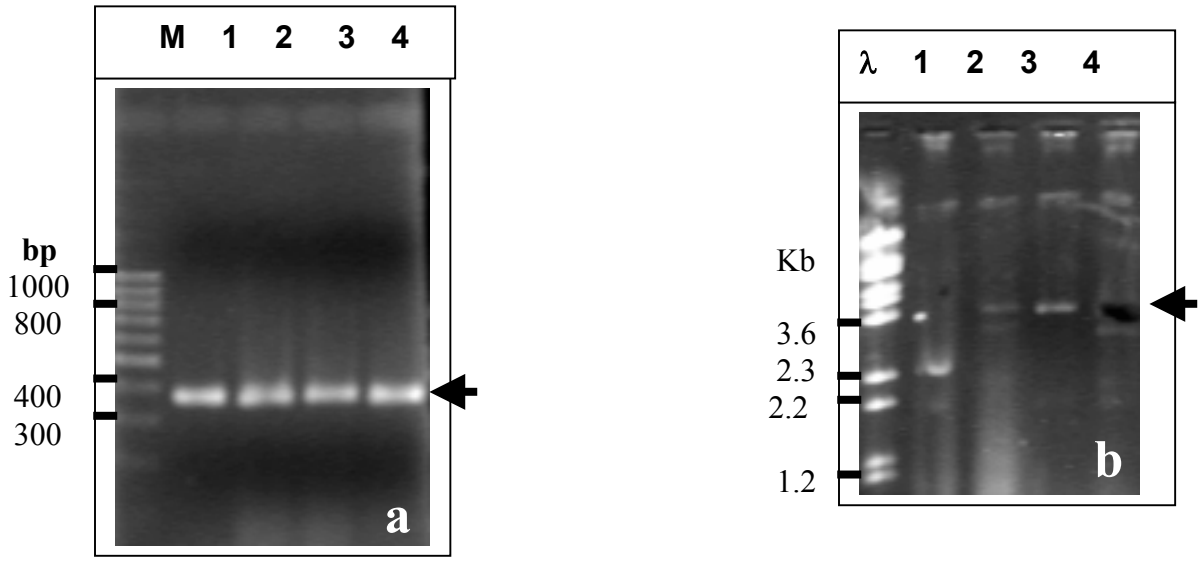


Figure 5.18: PCR cloning of *ped*B into pQE30
a: Agarose gel (1.5%) electrophoresis of PCR amplicon of *ped*B gene. Lane 1, PCR product undigested; 2, digested with *Bam*H1; 3, with *Pst*1 and lane 4, double digested with *Bam*HI/*Pst*1. M is a 100 bp ladder (MBI).
b: Agarose gel (0.8%) electrophoresis 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. □ *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.

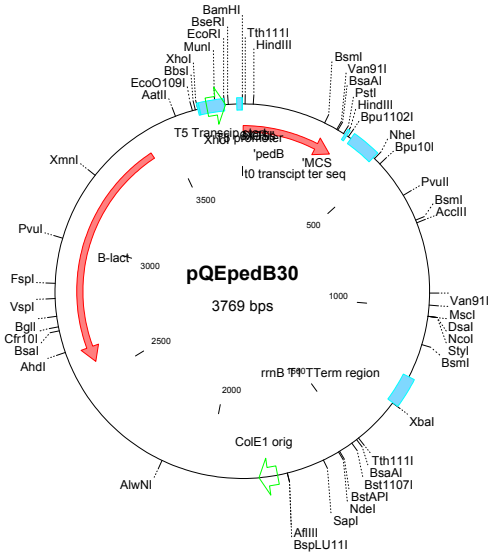


Figure 5.19 : Molecular map of recombinant plasmid pQEpedB30

MRGSHHHHHHDP KTKSEHIKQQALDLFTRLQFLLQKHDTIEPYQYVLDILETGI SKTKHNQQTPERQARVVYNKIASQALVDKLHFTAEENKVLAAINELAHSQKGWG EFNMLDTTNTWPSQ -

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

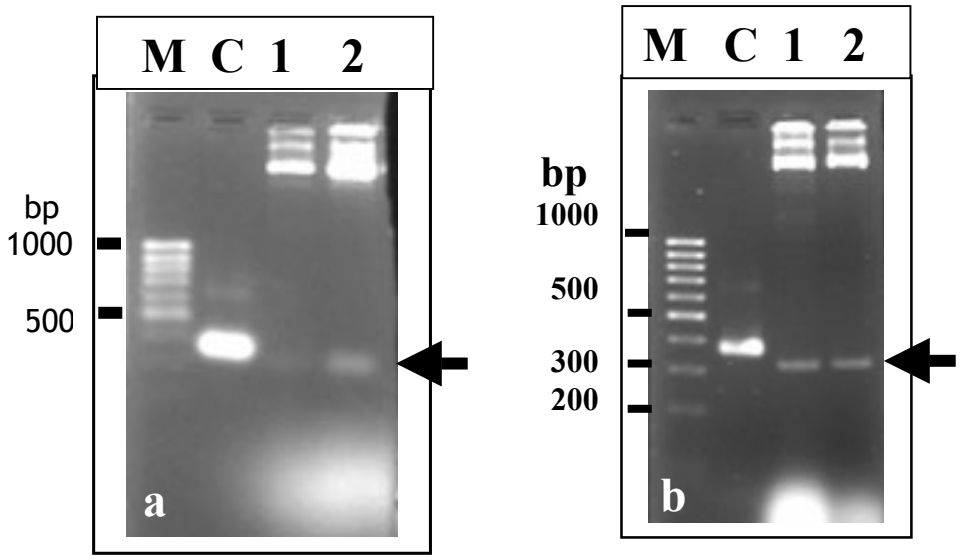


Figure 5.21: Restriction digestion of the pQEpedB30 recombinant. The putative recombinant, were digested with *Hin*d III/*Pst* I (a) and *Hin*d III (b). Lane 1 is the *ped*B PCR product. M, 100 bp ladder. Two putative recombinants were analyzed for insert release.

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.

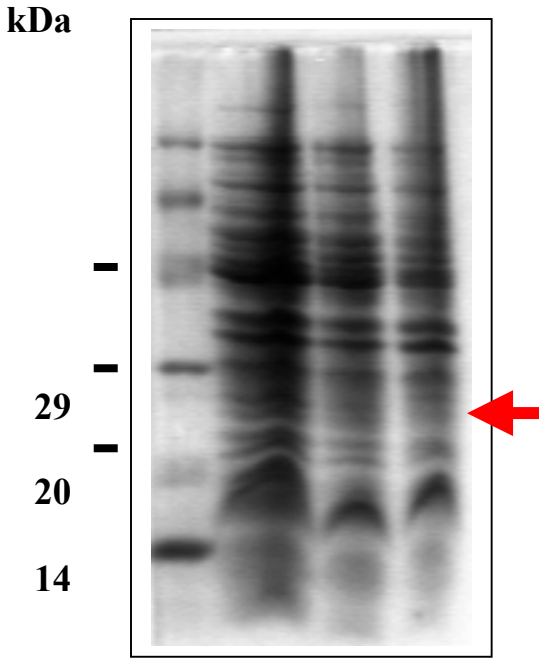


Figure 5.22: SDS-PAGE (15% gel) s of total lysate prepared from the PedB expressing *E. coli* M15 cells harboring pQEpedB30 plasmid. Lane M, Protein MW marker; V, vector lysate, U, un-induced and I, Induced with 1 mM IPTG recombinant.

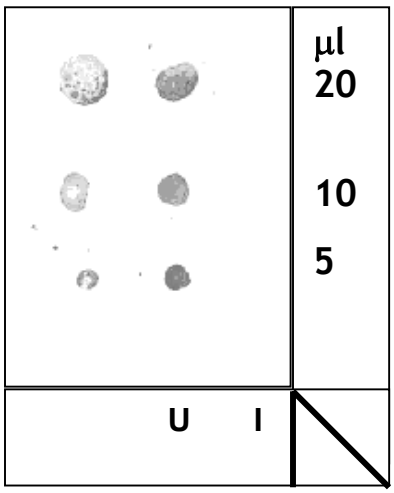


Figure 5.23: Dot-blot hybridization of the 6XHis-PedB expressing recombinants. Total cell lysate of the recombinant plasmid transformed *E. coli* M15 cells. U, un-induced and I, induced with IPTG. Three different concentrations of cell lysate were spotted (5, 10 and 20 ml) on NC membrane and probed with 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).

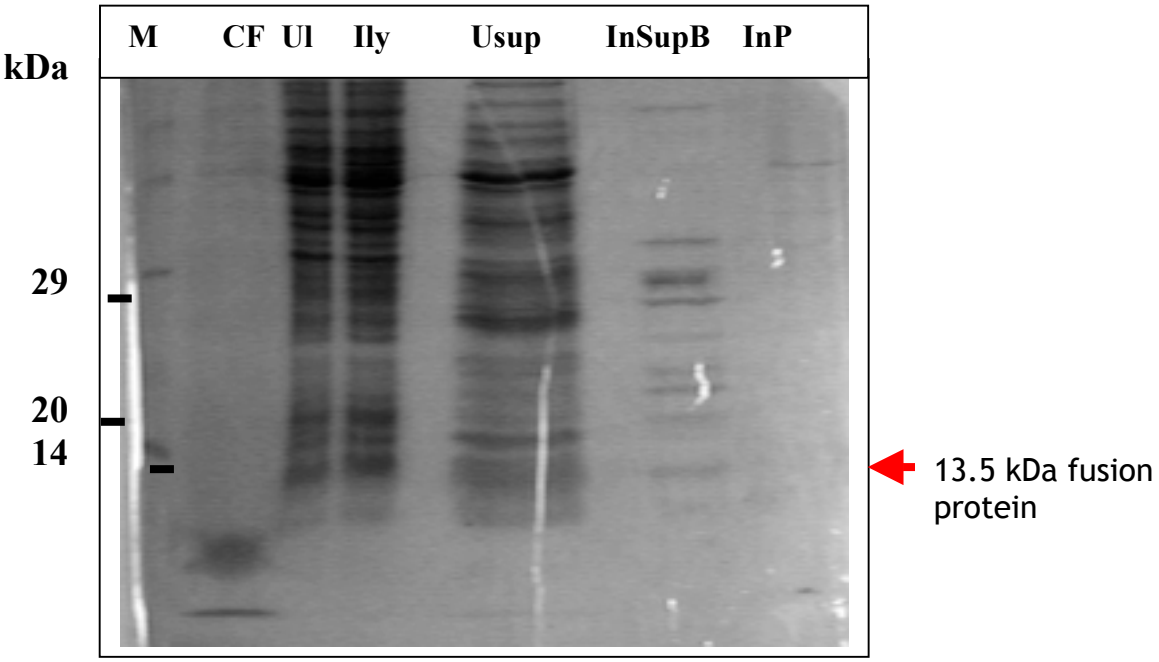


Figure 5.24: SDS-PAGE (15% acrylamide) gel analysis of Ni^{2+} NTA bead partially purified 6X His tagged Ped B. Lane CF, TCA precipitated culture filtrate; UI, uninduced and Ily, induced total cell lysate; Usup, Lysate from uninduced cells ; InsupB, Affinity purified supernatant from recombinant cells 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). SpaI 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).

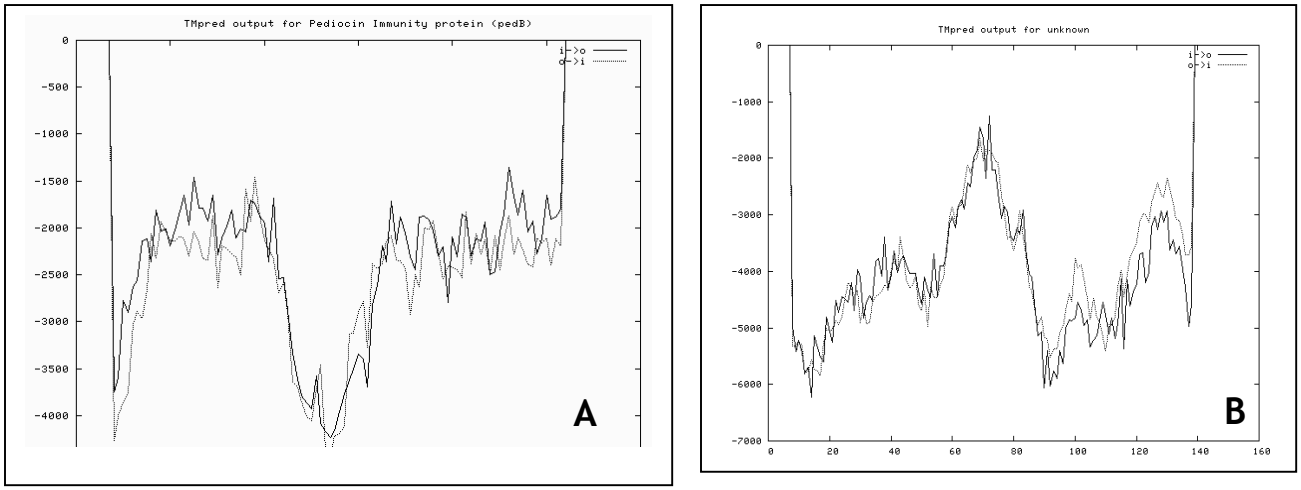


Figure 5.25: Tmpred analysis of PedB and SpaI proteins (acc no. U09819). A, PedB; B, SpaI.

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
SpIa	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   --------MNKTKSEHIKQQALDLFTRLQFLLQKHDTIEPYQYVLDILETGISKTKHNQQ
PedBR   1   --------MGKTKSEHIKQQALDLFTRLQFLLRKHDTIEPYQYVLDILETGISKTKHNQQ
Spla    1   ----MKILKWYSGGKDRGERANDIIGQLLLDLNHDPKNEHLEAILINYQNEIKRKES--S
Pep     1   ---------------------------MNIYLKVILTSLFFALIIFIVTYITTKQWG---
LtnI    1   MKNENINTFSESLFYSLKEWAEGSANNYNILLGLSFIFVMSSVVFFVTISTKIGKKDERT

                70        80        90       100       110       120
        ....|....|....|....|....|....|....|....|....|....|....|....|
PedB    53
TPERQARVVYNKIASQALVDKLHFTAEENKVLAAINELAHSQKGWGEFNMLDTTNTWPSQ
PedBR   53
TPERQARVVYNKIASQALVDKLHFTAEENKVLAAINELAHSQKGWGEFNMLDTTNTWPSQ
Spla    55  VPFILSRMN-ISIANTIRRDRLILTDFQEDKLKLLTALSNIRYGGY----------------
Pep     31  TSLGFSSLS--FIGNFIYDYSTKLSDKKYEKRINSNKKDKL--------------------
LtnI    61  TKISLYSAYCVLITLIICDIIFPKGYLIQPFFMLKYGFSCLVGGIYCLLKYIKDNK----

                130       140
        ....|....|....|....|
PedB    112 --------------------
PedBR   113 GSYPYDVPDYASRSHHHHHH
Spla    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 SpIa protein involved in sakacin immunity although total length of this protein is only 97 –aa (Figure 5.27e). LtnI and PepI 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).

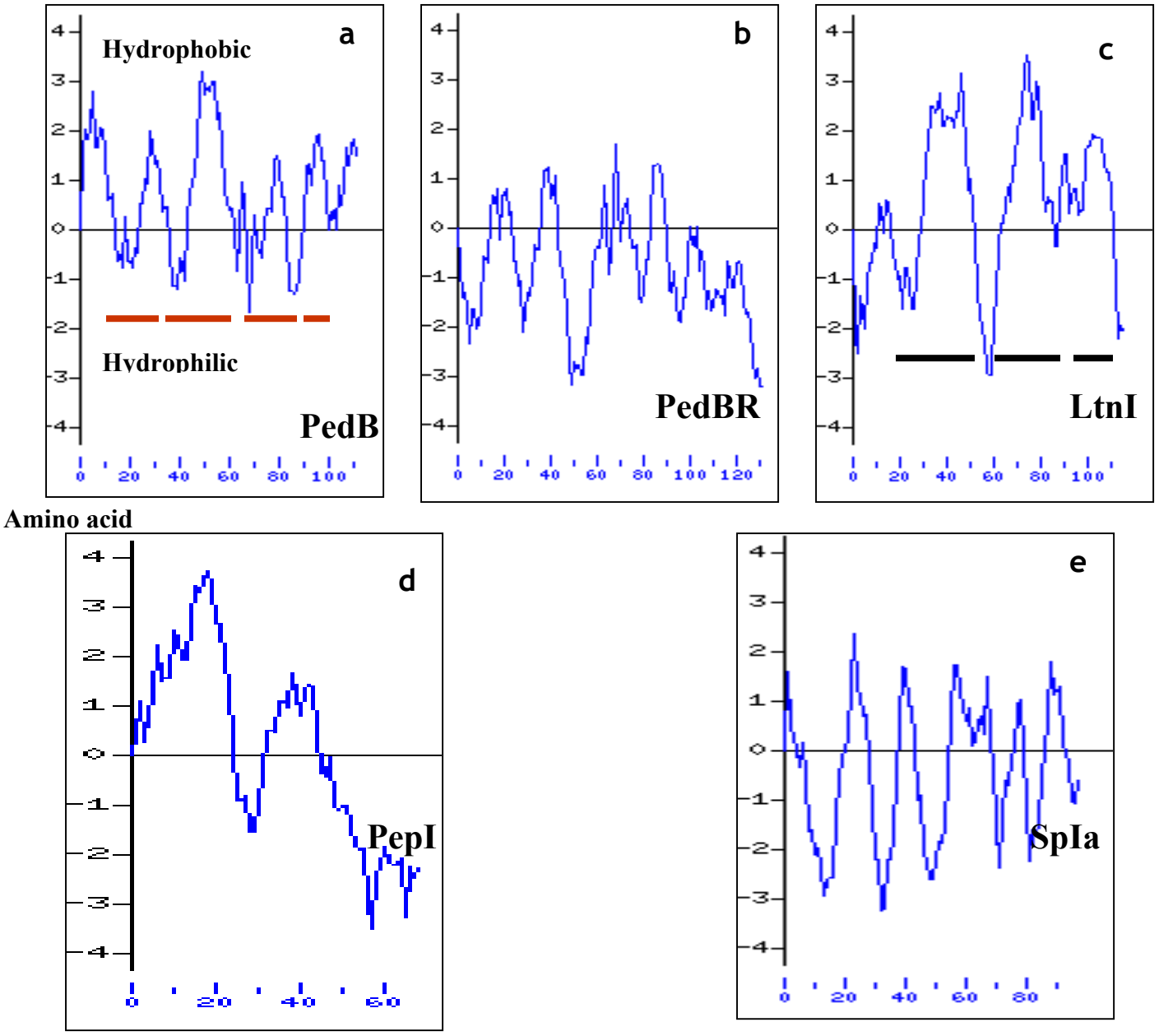


Figure 5.27: Hydrophobicity profiles of single subunit immunity proteins of Bacteriocins. Bars in the figure indicate membrane spanning region of hydrophobic segment. Red colour bars in a) are the proposed membrane spanning region identified in this study. B) is a hydrophobic profile of the recombinant 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.

FUTURE PERSPECTIVE

FUTURE PERSPECTIVE

The thesis presents a detailed survey of class IIa bacteriocins with greater emphasis on anti-listerial bacteriocins, and pediocin PA-1 type in particular. Among the many bacteriocinogenic strains tested, strains C40 and C20 (PH-1) were found to be potent against most of the indicator strains used. The strain C40 identified as *Lactobacillus casei* ssp. *casei* had plasmid linked bacteriocin production and carbohydrate fermentation features. Majority of the intestinal isolates were found to be resistant to several antibiotics as compared to the LAB isolated from mushroom fermentation.

The strain C20 identified as *Enterococcus faecium* PH-1, a colonic resident, posses a strong anti-listerial bacteriocin production, had properties of bile salt, temperature and pH tolerance and hence can be exploited as probiotic culture. The rRNA and *tuf* gene sequences reported for identification and characterization of *Ent. faecium* PH-1 was found to be a valuable tool for identification and characterization of new species of bacteria, whose species determination is often cumbersome and complicated.

The intergeneric pediocin PA-1 production by *Ent. faecium* PH-1 was reported. This is the first report for the production of pediocin PA-1 by a bacterium which can survive at extremes of pH, temperature and salt concentrations etc.

The megaplasmid encoding bacteriocin production reported in this study is unusual in nature, especially its molecular size. Nucleotide sequencing of the flanking region of the pediocin operon will establish the possible mechanism that took place in acquiring intergeneric pediocin production. Further characterization of sugar fermenting enzymes could help to understand the nature of these enzymes due to their unusual source.

Since strain PH-1 had an lactose hydrolyzing ability, the WP media was designed and the production of pediocin PA-1 was studied. This food-grade medium was found to be economical for the production of pediocin.

Further scale-up studies should be made by different fermentation trials. Further advanced methods of mathematical modeling should be evaluated for maximal production of pediocin PA-1. Isolation and molecular characterization of full length □-*gal* encoding genes find its application in basic studies. The native strain of PH-1 can even be employed for the commercial production of □-Gal due to its food-grade property and growth at high temperature. For studying the effectiveness of pediocin PA-1 in Indian food-systems, predictive models should be developed and its efficacy against various pathogenic bacteria should be evaluated.

Use of *Ent. faecium* PH-1 as a starter culture or co-culture cum bioprotective culture in vegetable and dairy product fermentation could be exploited since this culture has an ability to utilize melibiose, raffinose and lactose; the principal carbon sources in these food commodities.

The gene coding for the pediocin precursor and its immunity counterpart was cloned and expressed as a fusion protein in *E. coli*. The results showed that high level of pediocin was found in IBs of *E. coli* and its subsequent isolation, purification, solubilization and *in vitro* refolding yielded a biological active pediocin. Low levels of expression of immunity gene was observed upon cloning in pQE vector system having C- and N-terminal tagging. Further scale-up studies for the production of recombinant pediocin and optimization of *in vitro* refolding parameters are needed to obtain quantifiable yield. The recombinant pediocin and its immunity counterpart produced in *E. coli* could be a suitable material for further biophysical and biochemical investigations.

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