GENETIC AND BIOCHEMICAL CHARACTERIZATION OF BACTERIOCIN PRODUCED BY A SELECTED PEDIOCOCCUS SP.

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY

BY
NAGESWARA RAO KORASAPATI
B.Sc. (DAIRY TECHNOLOGY)
M. Sc. (DAIRY MICROBIOLOGY)

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NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL – 132001 (HARYANA), INDIA

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Dedicated
to my
Beloved Parents

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Thesis submitted to the National Dairy Research Institute (Deemed University), Karnal in partial fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY

Approved by

(External Examiner)

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CERTIFICATE

This is to certify that the thesis entitled **"GENETIC AND BIOCHEMICAL CHARACTERIZATION OF BACTERIOCIN PRODUCED BY A SELECTED *PEDIOCOCCUS* SP."** submitted by **MR. NAGESWARA RAO KORASAPATI** in partial fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in **DAIRY MICROBIOLOGY** of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India is bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any degree or diploma.

(R.K. Malik) 5.9.98
Major Advisor and Chairman
(Guide)

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05/09/98

(K. Nageswara Rao)

सारांश

पी.एच.डी. नागेश्वर राव के. डेरी सूक्ष्म जीवाणु

एक चयनित पीडियोकोकस जाति के द्वारा उत्पादित बैक्टीरियोसिन की आनुवांशिक एवं जैवरासायनिक विशेषताओं का लक्षणवर्णन

प्रस्तुत अनुसंधान बैक्टीरियोसिनोजेनिक पीडियोकोकल संवर्धनों के वियोजन, छंटाई एवं अभिनिर्धारण; बैक्टीरियोसिन के उत्पादन, आंशिक शोधन एवं लक्षणवर्णन तथा चयनित पीडियोकोकल वियोजन में बैक्टीरियोसिन के साथ सम्बंधित आनुवांशिक तत्वों के निर्धारण से सम्बंधित हैं.

पच्चीस विभिन्न दुग्ध एवं दुग्ध उत्पादों में से प्राप्त 45 पीडियोकोकल वियोजनों में से बैक्टीरियोसिन सक्रियता प्रदर्शित करने वाले 5 स्ट्रेन पीडियोकोकस पैन्टोसेशियस के विभिन्न स्ट्रेनों के रूप में निर्धारित किए गए. आगे अध्ययन के लिए चयन किये गए पी. पैन्टोसेशियस 34 का बैक्टीरियोसिन एम.आर.एस. ब्रॉथ (प्रारम्भिक पी.एच. 6.5) में 37° सैंटीग्रेड तापमान पर 16 से 18 घंटे तक उष्मायन के पश्चात उत्पादित किया गया. बैक्टीरियोसिन लॉग प्रावस्था में प्रमुख मैटाबोलॉइट के रूप में निस्सारित हुआ एवं लॉग प्रावस्था के अन्त तक इसका उच्चतम अनुमाप 78,000 ए.यू. प्रति मिलिलिटर तक पहुंच गया. इसे अमोनियम सल्फेट अवक्षेपण (30-60 प्रतिशत संतृप्ति) द्वारा 3.1 गुणा शोधन एवं 34.3 प्रतिशत प्रतिपूर्ति से अवक्षेपित किया गया. जैल फिलट्रेशन तथा आयन-विनिमय क्रोमैटोग्राफी द्वारा बैक्टीरियोसिन शोधन के प्रयास सफल नही हुए.

बैक्टीरियोसिन ने कई ग्रॉम पाज़िटिव जीवाणु जिनमें लिस्टीरिया मोनोसाइटोजीनस, बैसिलस सीरियस, स्टैफाइलोकोकस ऑरियस सम्मिलत है का निरोध कर एक व्यापक स्पैक्ट्रम प्रतिजीवाणु सक्रियता प्रदर्शित की. एस डी एस पेज प्रौद्योगिकी द्वारा बैक्टीरियोसिन का आणिवक भार 3500-6100 डाल्टन के बीच पाया गया. बैक्टीरियोसिन प्रोटियोलिटिक किण्वकों के लिए संवेदनशील था परन्तु नितान्त तापस्थाई (100° सैंटीग्रेड/30 मिनट) है तथा एक विस्तृत पी.एच. रेंज (1-11) में सक्रिय है. इसने एक नॉन बैक्टीरियोलाइटिक जीवाणु नाशी कार्यप्रणाली प्रदर्शित की. बैक्टीरियोसिन की सक्रियता एक प्रतिशत एस डी एस से उपचार द्वारा तीन गुणा बढ़ी परन्तु लवणीय विलयन (सोडियम क्लोराइड 0.5 एम या अधिक) में अस्थिर थी.

बैक्टीरियोसिन उत्पन्न करने वाले पी.पैन्टोसेशियस 34 में 11, 4.6, 3.6, 3.2 तथा 2.1 मैगाडॉल्टन भारों के 5 प्लाजमिड थे. मूल संवर्धक को 45° सैंटीग्रेड पर नोवोबॉयोसिन (40,45 एवं 50 माइक्रोग्राम प्रति मिलिलिटर) के साथ उपचारित करने के परिणामस्वरूप 15 बैक रहित परिवर्तों का वियोजन हुआ. मूल एवं उपचारित परिवर्तों के प्लाजमिड प्रोफाइल की तुलना एवं संयोजन द्वारा बैकसहित फीनोटाइप को प्लाजमिड-रहित बैक रहित परिवर्तों में स्थानान्तरित करने के प्रयास बैक्टीरियोसिन उत्पादन में प्लाजमिड डी. एन. ए .की भूमिका होने के कोई प्रमाण प्रदान करने में असफल रहे.

स्किम मिल्क (सप्रेटा दूध) में बैक्टीरियोसिन मिलाने पर 15 दिन के लिए 5° सैंटीग्रेड पर संग्रहण के दौरान एल. मोनोसाइटोजीनस का विकास महत्वपूर्ण रूप से नियन्त्रित हुआ.

ABSTRACT

Ph.D. Nageswara Rao, K. Dairy Microbiology

Genetic and Biochemical Characterization of Bacteriocin Produced by a Selected *Pediococcus* sp.

The present investigation deals with the isolation, screening and identification of bacteriocinogenic pediococcal cultures; production, partial purification and characterisation of bacteriocin; and determination of genetic elements associated with bacteriocin production in the selected pediococcal isolate.

Of the 45 pediococcal isolates obtained from 25 different milk and milk products, 5 strains exhibiting bacteriocin activity were identified as different strains of Pediococcus pentosaceus. *The bacteriocin of* P. pentosaceus *34 selected for further studies was optimally produced in MRS broth (initial pH 6.5) after 16-18 hr incubation at 37°C. The bacteriocin was secreted as a primary metabolite during the log phase reaching the highest titre (78,000 AU/ml) at the end of the log phase. It was precipitated by ammonium sulphate precipitation (30-60% saturation) with 3.1 fold purification and 34.3% recovery. Efforts to purify the bacteriocin further by gel filtration and ion-exchange chromatographies were not successful.*

The bacteriocin exhibited a broad spectrum antibacterial activity inhibiting numerous Gram +ve bacteria including Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus etc. *The molecular weight of bacteriocin was found to be between 3,500-6,100 Da as determined by SDS-PAGE technique. The bacteriocin was sensitive to proteolytic enzymes but extremely heat stable (100°C/30 min) and active over a wide pH range (1-11). It demonstrated a bactericidal, non-bacteriolytic mode of action. The activity of bacteriocin increased by 3-folds upon treatment with 1% SDS but was unstable in salt solutions (NaCl 0.5 M or more).*

The bacteriocin producing P. pentosaceus *34 harboured 5 plasmids with molecular weights of 11, 4.6, 3.6, 3.2 and 2.1 MDa. The parent culture treated with novobiocin (40, 45 and 50µg/ml) at 45°C resulted in the isolation of 15 Bac⁻ variants. The comparison of plasmid profiles of parent and cured variants as well as attempts to transfer the Bac⁺ phenotype to a plasmid free Bac⁻ variant by conjugation failed to provide any evidence for the involvement of plasmid DNA in bacteriocin production.*

The bacteriocin added to skim milk effectively controlled the growth of L. monocytogenes *during storage at 5°C for 15 days.*

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Chapter 1

Introduction

1. INTRODUCTION

Preservation of perishable foods in a sound and safe condition has ever remained a challenging subject for the mankind. Foods containing highly nutritious ingredients such as carbohydrates, proteins, fats, minerals etc. serve as excellent media for the proliferation of microorganisms some of which may cause the spoilage of foods or may prove a safety hazard to the consumers. Over the years, mankind has developed several processes, either by default or design, to enhance the safety and prolong the shelf life of perishable foods which otherwise are susceptible to the spoilage by the undesirable microflora. These food preservation methods can be broadly grouped as physical (heating, drying, freezing etc.), chemical (smoking, salting, synthetic preservatives etc.) and microbiological (fermentation) methods.

Although thermal processing is considered as one of the most effective means to control the undesirable bacteria, their very presence in foods suggests the post-processing contamination. The changing consumer requirements for fresh, highly nutritious foods further require a less severe processing treatment leaving many bacteria unaffected which may eventually grow and spoil the foods during subsequent refrigeration storage. As no single method is effective in providing protection against several types of microorganisms encountered in the spoilage of foods, "hurdle technology" involving the combined use of different physical, chemical and microbial factors is being widely practised for the preservation of foods.

Chemical preservatives such as lactate, sulfites, nitrites, sorbates, parabens etc. have been used as additional barriers in the preservation of foods by using hurdle technology. On the other hand, the consumers of present day are constantly looking for foods without any chemical preservatives, thus forcing the food scientists to search for naturally occurring antimicrobial compounds for preservation of foods. These naturally occurring antimicrobial compounds are termed as "biopreservatives" and include all those antimicrobials that are of plant, animal or microbial origin. Amongst all the biopreservatives, those from lactic acid bacteria (LAB) employed in the manufacture of fermented foods are being studied extensively for their application in the biopreservation of foods.

The diverse group of lactic acid bacteria involved in various food fermentations belong to the genera: *Lactococcus, Lactobacillus, Leuconostoc, Pediococcus, Streptococcus* and the newly recognised *Carnobacterium*. These bacteria alter the sensory characteristics of raw materials often resulting in foods with increased nutritive and economic values. In the third world countries, many of the fermented foods are produced under relatively unhygienic conditions and yet retain a good record with respect to microbiological safety. This has been attributed to the synthesis by LAB of a variety of inhibitory compounds which prevent the development of undesirable bacteria. These inhibitory compounds include organic acids (lactic, acetic, propionic etc.), carbon dioxide, hydrogen peroxide, diacetyl, bacteriocins or bactericidal proteins etc. The antimicrobial metabolites of LAB are unique with respect to their role in the biopreservation of foods.

Bacteriocins are small, single or complex proteins or proteinaceous substances that exhibit bactericidal activity against a limited range of organisms, usually closely related to the producer. Bacteriocin production has been detected among all the members of LAB. The bacteriocins of LAB form a heterogeneous group of antibacterial proteins with regard to their inhibitory spectrum, physical and biochemical properties, mode of action and genetic elements associated with their production. The bacteriocins with a wide inhibitory spectrum are interesting because of their potential to control the growth of spoilage and pathogenic flora. In the recent past, there has been a great deal of research on the bacteriocins of LAB for natural preservation of foods.

Nisin, the bacteriocin produced by certain strains of *Lc. lactis* subsp. *lactis*, is the only bacteriocin that has been permitted for use in certain types of foods in as many as 45 countries round the globe including India. The food microbiologists all over the world are engaged in search for a bacteriocin from LAB to overcome some of the inherent problems encountered with the use of nisin such as ineffectiveness in foods at near neutral pH and limited antibacterial spectrum. The fast accumulating literature suggests that bacteriocins produced by pediococci can be exploited as natural food preservatives, overcoming the problems associated with the use of nisin.

The pediococci comprise a group of bacteria that are of economic importance in the brewing and food industries. Several species and strains of pediococci have been used as starter cultures in the fermentation of vegetables, meats, sausage products, fermented milks and associated with the development of flavour in Cheddar and

other related cheese varieties. The pediococcal fermentations not only help in improving the sensory quality of foods but also enhance the safety and extend the shelf life of foods by inhibiting the growth of pathogenic and spoilage microorganisms. The inhibitory effect of pediococcal cultures is largely due to bacteriocins that are termed as pediocins to signify their origin. The sparse information available on pediocins suggests that they could also be used as natural food preservatives owing to their broad spectrum activity and high sensitivity to gastric proteolytic enzymes such as trypsin, chymotrypsin and pepsin. It is very likely that more future research on pediocins may aid their successful application in the biopreservation of foods.

The pediocins for food application should have (i) a broad spectrum of activity (ii) bactericidal rather bacteriostatic action (iii) good activity and stability in the environment encountered in foods (iv) a high consumer safety margin i.e. no toxicological effects. Therefore, thorough characterisation of pediocins with respect to their antibacterial activity spectrum, mode of action, physical and biochemical properties. etc. is necessary to facilitate the approval by regulatory agencies for their use in foods. The effective commercial application requires the production of pediocins in large amounts. Till date very few reports are available which deal with the cultural and environmental factors that influence the ability of the pediococcal strains to produce pediocins. Another important area that needs further investigation is the genetic elements associated with pediocin production which would further facilitate the improved function and wider application of this group of bacteria. Better understanding of pediocins would also lead to targeted

biocontrol of spoilage microflora and foodborne pathogens.

Keeping in view the ongoing global search for an ideal bacteriocin biopreservative and the importance of pediococci and pediocins in preserving the foods, the present investigation has been undertaken with the following objectives:

1. Isolation and identification of bacteriocinogenic pediococci
2. Optimisation of the conditions for maximum bacteriocin production by a selected *Pediococcus* sp.
3. Partial purification and characterisation of the bacteriocin
4. Delineation of the genetic control of bacteriocin production and genetic construction of starters.

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 BACTERIOCINS OF LACTIC ACID BACTERIA

Eversince the publication of the first review on the bacteriocins of Gram positive bacteria by Tagg *et al* (1976), there has been a renewed interest in the field of bacteriocins of Gram positive bacteria. The research on bacteriocins produced by a heterogeneous group of Gram positive bacteria comprising genera, *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus* and *Carnobacterium*, collectively known as lactic acid bacteria (LAB) has witnessed a tremendous growth in the past one and half decades as is evidenced by the publication of several review articles (Klaenhammer, 1988, 1993; Piard and Desmazeaud, 1992; Nettles and Barefoot, 1993; Malik *et al.*, 1994b; Jack *et al.*, 1995; Stiles, 1996) and books (Ray and Daeschel, 1992; Hoover and Steenson, 1993; Devuyst and Vandamme, 1994) dealing with various aspects of bacteriocins produced by lactic acid bacteria.

The potential for applications of bacteriocins and bacteriocins producing LAB in food preservation has lead to the isolation and characterisation of several bacteriocins. The bacteriocin producing LAB have been isolated from various sources such as vegetables, meat and meat products, milk and milk products and other sources such as dental plaque. In some cases an identical bacteriocin may be produced by different subspecies of the same species as observed for

lactococcin A (Neve *et al.*, 1984; Stoddard *et al.*, 1992), different strains of the same species as observed for lactococcin A (Neve *et al.*, 1984; Holo *et al.*, 1991). There are also incidents where a single strain produces more than one bacteriocin as recorded for *Lc. lactis* subsp. *cremoris* 9B4 (van Belkum *et al.*, 1991a, 1992) and *Lb. plantarum* LPC010 (Jimenez-Diaz *et al.*, 1993).

2.1.1 LACTOBACILLI

The bacteriocinogenicity has been described for several of the obligate homofermenters (*Lactobacillus acidophilus, and Lb. helveticus*), facultative heterofermenters (*Lb. plantarum* and *Lb. sake*) and for the heterofermentative *Lb. brevis*.

2.1.1.1 Lactobacillus helveticus

The bacteriocins described from the species include helveticin J by the strain 481 (Joerger and Klaenhammer, 1986) and helveticin V-1829 by the strain 1829 (Vaughan *et al.*, 1992). Recently, Thompson *et al* (1996) identified a bacteriocin in the culture supernatant of *Lb. helveticus* CNRZ450.

2.1.1.2 Lactobacillus acidophilus

Early investigations into the antimicrobial activities of *Lb. acidophilus* suffered due to insufficient characterization of the antagonistic agents, to determine whether or not bacteriocins are responsible for the observed inhibition (Klaenhammer, 1988). Barefoot and Klaenhammer (1983) provided a more definitive characterization of bacteriocins from the species with the description of lactacin B, a bacteriocin

produced by *Lb. acidophilus* N2. ten Brink *et al* (1994) reported the production of acidocin B, an atypical bacteriocin by *Lb. acidophilus* strain M46 isolated from human dental plaque and *Lb. acidophilus* TK9201 was found to produce a bacteriocin termed as acidocin A (Kanatani *et al.*, 1995).

2.1.1.3 Lactobacillus plantarum

Daeschel *et al* (1990) reported the production of plantaricin A by *Lb. plantarum* C-11 isolated from cucumber fermentations. It is interesting to note that *Lb. plantarum* LPC010 isolated from a green olive fermentation elaborated into the growth medium two bacteriocins designated as plantaricins S and T (Jimenez-Diaz *et al.*, 1993). A bacteriocin, plantaricin KW30, producing strain of *Lb. plantarum* has recently been isolated from fermented maize (Kelly *et al.*, 1996).

Bacteriocinogenic *Lb. plantarum* strains from dairy and meat products have also been reported. A *Lb. plantarum* strain LTF154 isolated from a fermented sausage produced a bacteriocin designated as plantacin 154 (Kanatani and Oshimura, 1994). Rekhif *et al* (1994) isolated a bacteriocin producing *Lb. plantarum* strain LC74 from goat raw milk and named the bacteriocin as plantaricin LC74. Recently Ennahar *et al* (1996) reported the production of a bacteriocin identical to pediocin AcH by a strain of *Lb. plantarum* WHE92 isolated from a soft cheese.

2.1.1.4 Lactobacillus sake

Bacteriocins produced by the strains of *Lb. sake* isolated from meat and fermented sausages include:

sakacin A by *Lb. sake* 706 (Schillinger and Lucke, 1989), lactocin S by *Lb. sake* L45 (Mortvedt and Nes, 1990)..

2.1.1.5 Lactobacillus brevis

Benoit (1994) identified an antibacterial protein produced by *Lb. brevis* SB27 isolated from dry cured sausage and designated it as brevicin 27. Production of brevicin 286 has been recently reported in *Lb. brevis* VB286 that was originally isolated from vacuum packaged meat (Coventry *et al.*, 1996).

2.1.1.6 Other Lactobacilli

Lactacin F producing *Lb. acidophilus* 11088 (Muriana and Klaenhammer, 1987) has recently been renamed as *Lb. johnsonii* as cited by Klaenhammer (1993).

2.1.2 LACTOCOCCI

Several workers (Oxford, 1944; Mattick and Hirsch, 1947; Neve *et al.*, 1984; Holo *et al.*, 1991 and Kojic *et al.*, 1991) have reported bacteriocinogenicity among the different strains of the three most economically important lactococcal species: *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*.

2.1.2.1 Lactococcus lactis subsp. lactis

Nisin, the most extensively characterised bacteriocin from lactic acid bacteria, is produced by several strains of *Lc. lactis* subsp. *lactis*. The word 'nisin' was coined to designate the group 'N' inhibitory substance in 1947 by Mattick and Hirsch. A bacteriocin,

lacticin 481, produced by *Lc. lactis* subsp. *lactis* CNRZ481 was reported by Piard *et al* (1990). The production of several lactococcins has been described in several other *Lc. lactis* subsp. *lactis* which include: Lactococcin by *Lc. lactis* subsp. *lactis* ADRI 85LJ30 (Dufour *et al.*, 1991); lactococcin G by *Lc. lactis* subsp. *lactis* LMG2081 (Nissen-Meyer *et al.*, 1992); lactococcin 972 by *Lc. lactis* subsp. *lactis* (Martinez *et al.*, 1996); lactococcin 484 by *Lc. lactis* subsp. *lactis* 484 (Gupta and Batish, 1992).

2.1.2.2 Lactococcus lactis subsp. cremoris

The first description of a proteinaceous inhibitor in lactococci was from *Lc. lactis* subsp. *cremoris*. The antimicrobial agent described by Whitehead in 1933 was later on partly purified and shown to be proteinaceous in nature. It was termed as 'Diplococcin' to signify the diplococcal arrangement of the producer cells (Oxford, 1944). A number of lactococcins have been described for *Lc. lactis* subsp. *cremoris*. These include: lactococcin A from strain LMG2130 (Holo *et al.*, 1991) and strain 9B4 (Neve *et al.*, 1984). Later it was found that *Lc. lactis* subsp. *cremoris* strain 9B4 produced two more bacteriocins termed as lactococcin M (van Belkum *et al.*, 1991a) and lactococcin B (van Belkum *et al.*, 1992). Huot *et al* (1996) described the production of a bacteriocin designated as Bacteriocin J46 by *Lc. lactis* subsp. *cremoris* J46.

2.1.2.3 Lactococcus lactis subsp. lactis biovar. diacetylactis

The bacteriocin described in *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* WM4 (Scherwitz *et al.*, 1983)

has been found to be identical to the lactococcin A produced by *Lc. lactis* subsp. *cremoris* strains 9B4 and LMG2030 (Stoddard *et al.*, 1992). Kojic *et al* (1991) reported the production of bacteriocin S50 by *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* S50. A strain of *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* UL720 isolated from raw milk has been found to produce a bacteriocin termed as diacetin B (Ali *et al.*, 1995). Morgan *et al* (1995) isolated *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* DPC398 from an Irish cheese factory and observed the effect of all the three lactococcins viz. A, B, and M in the strain DPC398.

2.1.3 LEUCONOSTOCS

The first evidence for bacteriocin production in *Leuconostoc* spp. was provided by Harding and Shaw in 1990. They reported the production of a heat stable protein by a strain of *Leu. gelidum* that was active against other lactic acid bacteria and three strains of *Listeria monocytogenes*. In recent years, a number of bacteriocin producing strains of *Leuconostoc* spp. have been isolated from various sources such as milk and meat products.

Hastings and Stiles (1991) reported the production of a bacteriocin designated leucocin A-UAL187 by *Leu. gelidum* UAL187 isolated from meat packed under elevated (30%) carbondioxide. *Leuconostoc paramesenteroides* OX isolated by Lewus *et al* (1991) from retail lamb was found to produce a bacteriocin named as leuconocin S (Lewus *et al.*, 1992). Bacteriocins, carnosin 44A, carnocin LA54A and leucocin B-Talla, produced by *Leu. carnosum* LA44a from vacuum packaged Vienna-type-sausage (van Laack *et al.*, 1992), *Leu. carnosum* LA54A from meat (Keppler *et*

al., 1994) and *Leu. carnosum* Talla isolated from vacuum packaged processed meat (Felix *et al.*, 1994), respectively, have been described in the strains of *Leu. carnosum*. Yang and Ray (1994a) observed the predominance of *Leu. carnosum* and *Leu. mesenteroides* in the spoiled low heat processed vacuum packaged meat products. The notable feature of many of these *Leuconostoc* isolates is their ability to produce bacteriocins.

Bacteriocinogenic strains of *Leuconostoc* spp. have also been isolated from milk and milk products. Strains of *Leu. mesenteroides* subsp. *mesenteroides*, Y105 from goat milk and FR52 from raw milk were found to produce bacteriocins, mesentericin Y105 (Hechard *et al.*, 1992)and mesenterocin 52 (Mathieu *et al.*, 1993), respectively. Dextranicin J24 was a bacteriocin produced by an isolate of *Leu. mesenteroides* subsp. *dextranicum* J24 from French soft cheese (Sudirman *et al.*, 1994). Malik *et al* (1994a) reported the detection and activity of a novel bacteriocin, leucocidin R1, produced by *Leu. paramesenteroides* NM14 isolated from an aged cream sample.

2.1.4 PEDIOCOCCI

2.1.4.1 Pediococcus pentosaceus

The bacteriocin produced by *P. pentosaceus* FBB61 from cucumber fermentations was designated as pediocin A (Daeschel and Klaenhammer, 1985). Hoover *et al* (1988) observed bacteriocinogenic activity in *P. pentosaceus* MCC3 isolated from pepperoni, a fermented sausage. Bacteriocin production in *P. pentosaceus* strain N5p from wine has been reported and the bacteriocin was named as

pediocin N5p (Strasser-de-Saad and Manca-de-Nadra, 1993).

2.1.4.2 Pediococcus acidilactici

The most extensively characterized bacteriocins, pediocin AcH and pediocin PA-1, after nisin have been produced by strains of *P. acidilactici*. Gonzalez and Kunka (1987) reported pediocin PA-1 production by *P. acidilactici* PAC1.0. Pediocin AcH producing *P. acidilactici* H was isolated by Bhunia *et al* (1987) from fermented sausage. Hoover *et al* (1988) observed the production of unnamed bacteriocins by three strains of *P. acidilactici* P02, B56 and PC. Coventry *et al* (1395) have designated the bacteriocin produced by *P. acidilactici* P02 as pediocin P02. Schved *et al* (1993) reported the isolation of *P. acidilactici* SJ1 from a naturally fermented meat product and designated its bacteriocin as pediocin SJ1 while pediocin L50 producing *P. acidilactici* L50 was obtained from Spanish dry fermented sausage (Cintas *et al.*, 1995).

2.1.4.3 Other Pediococci

Recently Kimura *et al* (1997) reported the isolation of bacteriocin producing *Pediococcus* spp. ISK-1 from Nukadoko, a bed of fermented rice bran.

2.1.4.4 Pediococci of Dairy Origin

Reports concerning the bacteriocinogenicity of pediococcal isolates from dairy origin are very scarce. Andric and Sutic (1980) reported that none of the 11 strains of *Pediococcus* spp. isolated from ewe's milk Kashkaval cheese showed antagonistic activity against 18 strains of lactobacilli and 8 of lactic streptococci.

Litopoulou-Tzanetaki *et al* (1989) also observed the inability of *Pediococcus* spp. obtained from American Cheddar cheese to elaborate the bacteriocin active against the indicator organism, *P. pentosaceus* FBB59.

Daba *et al* (1991) isolated a bacteriocin producing *Leu. mesenteroides* UL5 from Cheddar cheese and designated it as mesenterocin 5. This organism was later re-identified as *P. acidilactici* UL5 and bacteriocin produced was renamed as pediocin 5 (Huang *et al.*, 1994). Till date, the bacteriocin, pediocin 5 has been the only bacteriocin discovered in pedioccal isolates of dairy origin.

2.2 INHIBITORY SPECTRUM OF BACTERIOCINS OF LACTIC ACID BACTERIA

In the original definition of Jacob *et al* (1953), bacteriocins were characterised by predominate intraspecies killing activity. While this is true for most of the bacteriocins of LAB especially those produced by a large number of lactococci and lactobacilli, others have been found to exhibit a broad range of inhibitory activity extending across numerous Gram positive bacteria. Thus Klaenhammer (1988) defined two types of bacteriocins of lactic acid bacteria; one type exhibiting a classical bacteriocin antibacterial spectrum affecting only closely related bacteria and the second type effective against a wide range of Gram positive bacteria. Inhibition of Gram negative bacteria in their native state has not been reported for any of the purified and thoroughly characterised bacteriocin. Similarly, inhibition of yeast and molds has not been observed.

2.2.1 LACTOBACILLI

2.2.1.1 Lactobacillus helveticus

Bacteriocins, helveticin J and helveticin V-1829 have been reported to exhibit antagonistic activities directed against closely related *Lactobacillus* spp. (Joerger and Klaenhammer, 1986; Vaughan *et al.*, 1992). The bacteriocin from *Lb. helveticus* CNRZ450 was active against a narrow range of strains from closely related species of homofermentative lactobacilli (Thompson *et al.*, 1996).

2.2.1.2 Lactobacillus acidophilus

Lactacin B produced by *Lb. acidophilus* N2 was found to be inhibitory only to lactobacilli including *Lb. helveticus, Lb. delbrueckii* subsp. *lactis* and *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. leichmanii* (Barefoot and Klaenhammer, 1984).

In recent years, bacteriocins with a relatively broad spectrum of antibacterial activity have been described in *Lb. acidophilus*. ten Brink *et al* (1994) reported that the inhibitory spectrum of acidocin B included *Cl. perfringens* along with closely related lactobacilli. Kanatani *et al* (1995) reported that acidocin A from *Lb. acidophilus* TK9201 was active against closely related lactic acid bacteria and food-borne pathogens including *Listeria monocytogenes*.

2.2.1.3 Lactobacillus plantarum

Daeschel *et al* (1990) reported that plantaricin A was effective against some species of the four genera of

lactic acid bacteria: *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus*, and *Enterococcus faecalis*.

Plantaricin S and plantaricin T produced by *Lb. plantarum* LPC010 were observed to have a similar spectrum of inhibitory activity except that only plantaricin S was active against *Lb. curvatus*. The antibacterial activity of both the bacteriocins was directed against *Lb. plantarum, Propionibacterium* spp., *Cl. butyricum* and *Enterococcus faecalis*. It was also reported that plataricin T exhibited a lower level of activity than plataricin S against all the indicator strains tested (Jimenez-Diaz *et al.*, 1993). The antibacterial spectrum of plantacin 154 included taxonomically related lactic acid bacteria and undesirable bacteria for sausage fermentation such as *E. faecalis* and *Propionibacterium* spp. (Kanatani and Oshimura, 1994).

Rekhif *et al* (1994) reported the inhibition of several strains of mesophilic lactobacilli and *B. stearothermophilus* but not of staphylococci, listeriae and enterococci and lactococci by plantaricin LC74 from *Lb. plantarum* LC74. However, plantaricin KW30 inhibited other lactobacilli only (Kelly *et al.*, 1996).

2.2.1.4 Lactobacillus sake

Sakacin A displayed antagonism against lactobacilli, leuconostocs, carnobacteria, enterococci and *L. monocytogenes* (Schillinger and Lucke, 1989). In contrast to sakacin A, lactocin S exhibited a very narrow spectrum of activity inhibiting growth of selected species of the genera *Lactobacillus, Pediococcus* and *Leuconostoc* (Mortvedt and Nes, 1990).

2.2.1.5 Lactobacillus brevis

Benoit *et al* (1994) observed the inhibition by Brevicin 27 of numerous strains of heterofermentative lactobacilli, some strains of pediococci and *Bacillus*. A relatively narrow specrum of activity with a notable activity against *Listeria* spp. has been recorded for Brevicin 286. (Coventry *et al.*, 1996).

2.2.1.6 Other Lactobacilli

Lactacin F produced by *Lb. johnsonii* 11088 exhibited an inhibitory spectrum restricted to other lactobacilli as well as *E. faecalis* (Muriana and Klaenhammer, 1991a).

2.2.2 LACTOCOCCI

2.2.2.1 Lactococcus lactis subsp. lactis

Nisin, the most important and widely used bacteriocin, is normally ineffective against Gram negative bacteria, yeast and moulds, but is effective against a wide range of Gram positive bacteria including other lactic acid bacteria, *Staph. aureus* and *L.monocytogenes*. Gram positive spore formers i.e. *Bacillus* spp. and *Clostridium* spp. are particularly sensitive to nisin with spores being more sensitive than vegetative cells (Ray, 1992a; Delves-Broughton *et al.*, 1996).

Lacticin 481 from *Lc. lactis* subsp. *lactis* CNRZ481 was found to be effective against *Lactococcus* spp., some *Lactobacillus* spp., *Leuconostoc* spp. and *Clostridium* spp. (Piard *et al.* 1990). Lactococcin produced by *Lc. lactis* subsp. *lactis* ADRIA 85L030 has been found to inhibit

vegetative cells of *Cl. tyrobutyricum*, strains of *Streptococcus thermophilus* and *Lb. helveticus* but is rather inactive against other Gram positive and Gram negative genera (Thuault *et al.*, 1991). Lactococcin 484 from *Lc. lactis* subsp. *lactis* 484 has been reported to be effective against members of the *Lactococcus* group, *B. cereus*, *Staph. aureus* and *Salmonella typhi* (Gupta and Batish, 1992). Lactococcin 972 has been reported to be effective against lactococci only (Martinez *et al.*, 1996).

2.2.2.2 Lactococcus lactis subsp. cremoris

The inhibitory spectrum of diplococcin from *Lc. lactis* subsp. *cremoris* was restricted to lactococci only (Davey and Pearce, 1980). *Lactococcus lactis* subsp. *cremoris* strain 9B4 secreting Lactococcins A, B and M prevented the growth not only of other lactococci but also of some clostridia (Geis *et al.*, 1983). Holo *et al* (1991) purified lactococcin A and found that it inhibited the growth of only lactococci. Out of over 120 strains of different lactococci tested only one was insensitive to Lactococcin A as was the case with all other Gram positive bacteria included in the experiment. Bacteriocin *J46* had a wide spectrum of antibacterial activity including anticlostridial activity (Gonzalaz *et al.*, 1996).

2.2.2.3 Lactococcus lactis subsp. lactis biovar. diacetylactis

Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* WM4 producing lactococcin A was active against other lactococci (Scherwitz *et al.*, 1983). Bacteriocin S50 has a narrow antibacterial spectrum being

active only against *Lactococcal* species (Kojic *et al.*, 1991). The antibacterial activity of *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* DPC398 secreting at least three bacteriocins, lactococcins A, B and M was also restricted to lactococci only (Morgan *et al.*, 1995).

Ali *et al* (1995) reported that diacetin B had a broad spectrum of antibacterial activity inhibiting the growth of various pathogens including *L. monocytogenes*, *Staph. aureus* and clostridial strains.

2.2.3 LEUCONOSTOCS

Leucocin A-UAL187 produced by *Leu. gelidum* UAL-187 was active against leuconostocs, lactobacilli, pediococci, carnobacteria and a strain of *E. faecalis* and *L. monocytogenes* (Hastings and Stiles, 1991). Whereas leuconocin S had a broad spectrum of activity. It was inhibitory to *L. monocytogenes*, *Staph. aureus*, *Yersinia enterocolitica*, *Aeromonas hydrophila* and some strains of *Cl. botulinum* (Lewus *et al.*, 1991; Okereke and Montiville, 1993).

Inhibition of growth of various lactic acid bacteria, *L. monocytogenes* and *E. faecalis* by carnosin 44A (van Laack *et al.*, 1992) and carnocin LA54A (Keppler *et al.*, 1994) has been reported. A spectrum similar to that of carnocin LA54A and carnosin without *E. faecalis* was also observed for leucocin B-Ta11a produced by *Leu. carnosum* Ta11a (Felix *et al.*, 1994). Yang and Ray (1994) reported the inhibition of several lactic acid bacteria from spoiled meat products and *L. monocytogenes* by bacteriocins produced by *Leu. carnosum* and *Leu. mesenteroides* isolated from the same niche.

Mesenterícin Y105 from *Leu. mesenteroides* subsp. *mesenteroides* was observed to be specific for *Listeria* spp. as all of the 14 strains inhibited neither the Gram negative nor other Gram positive bacteria including closely related lactic acid bacteria species were inhibited under the test conditions (Hechard *et al.*, 1992). The antibacterial spectrum of mesenterocin 52 included other strains of *Leuconostoc* spp., several strains of *Enterococcus* and *Listeria* spp. None of the strains of *Lactococcus, Lactobacillus* and *Pediococcus* spp. tested were however, sensitive (Mathieu *et al.*, 1993). Dextranicin J24 exhibited a classical bacteriocin spectrum inhibiting only the strains of *Leuconostoc* spp. (Sudirman *et al.*, 1994). The broad spectrum of activity of leucocidin R1 from *Leu. paramesenteroides* NM14 comprised several food spoilage and food-borne pathogenic Gram positive as well as several Gram negative bacteria. However, it was not effective against many of the closely related lactic acid bacteria of commercial importance (Malik *et al.*, 1994a).

2.2.4 PEDIOCOCCI

2.2.4.1 Pediococcus pentosaceus

Pediocin A was active against a broad spectrum of Gram positive bacteria including pediococci, lactococci, lactobacilli, leuconostocs, micrococci, enterococci, staphylococci, bacilli, clostridia and listeriae (Fleming *et al.*, 1975; Daeschel and Klaenhammer, 1985; Spelhaug and Harlander, 1989). Strasser-de-Saad and Manca-de-Nadra (1993) reported that the antibacterial protein from *P. pentosaceus* N5P was effective against 19 strains of the three genera of lactic acid bacteria.

2.2.4.2 Pediococcus acidilactici

Pediocin PA-1 inhibited other pediococci, lactobacilli, leuconostocs, *L. monocytogenes* but strangely not staphylococci (Gonzalez and Kunka, 1987; Pucci *et al.*, 1988; Henderson *et al.*, 1992). The bacteriocin pediocin AcH, produced by *P. acidilactici* H exhibited a much more wider antibacterial spectrum. The inhibitory spectrum included lactobacilli, leuconostocs, *Staph. aureus, Cl. perfringens* and *L. monocytogenes*. It was not active against the Gram negative bacteria (Bhunia *et al.*, 1988; Ray, 1992b). Pediocins from *P. acidilactici* strains PC, PO2 and B5627 effectively inhibited other pediococci, leuconostocs, *E. faecalis* and *L. monocytogenes* (Hoover *et al.*, 1988).

The antibacterial spectrum of pediocin 5 comprised of *L. monocytogenes, Str. faecalis, Brevibacterium linens* and *P. pentosaceus*. Interestingly, it was not effective against several lactic acid bacteria tested (Daba *et al.*, 1991). The antibacterial protein from *P. acidilactici* PC was found to be active against members of the genera *Listeria, Clostridium, Leuconostoc* and *Pediococcus* but not against *Lactococcus, Streptococcus* and *Lactobacillus* strains tested (Jager and Harlander, 1992). Pediocin SJ-1 from *P. acidilactici* SJ-1 inhibited the growth of selected strains of *Lactobacillus* spp., *Cl. perfringens* and *L. monocytogenes* (Schved *et al.*, 1993). Recently Coventry *et al* (1995) reported the inhibitory spectrum of pediocin PO2 from *P. acidilactici* PO2. The spectrum comprised other pediococci, lactobacilli, leuconostocs, enterococci, a strain each of *B. coagulans* and *Staph. aureus*. The bacteriocin was notably more active against *Listeria* spp. including *L. monocytogenes*. The inhibitory spectrum of pediocin L50 included other lactic acid

bacteria, *Clostridium* spp., *Listeria* spp., *staphylococcus* spp., *E.faecalis* and *Propionibacterium* spp. (Cintas *et al.*, 1995).

2.2.4.3 Other Pediococci

Recently Kimura *et al* (1997) observed the inhibition of *P. acidilactici, Lc. lactis* subsp. *lactis, Lb. casei* subsp. *casei, Micrococcus luteus, B. subtilis* by a bacteriocin from *Pediococcus* spp.

2.3 CHARACTERISTICS OF BACTERIOCINS

Bacteriocins of LAB have been characterised with respect to their (i) sensitivity to various proteolytic and non-proteolytic enzymes (ii) stability to various heat treatments (iii) pH stability (iv) mode of action and (v) molecular weight etc. In most of these characterisation studies, either crude or partially purified bacteriocin preparations have been used.

The fact that bacteriocins are proteins renders them sensitive to at least one proteolytic enzyme. Apart from protein moiety, some bacteriocins have been found to contain an active lipid or carbohydrate moiety which is also required for antibacterial activity as revealed by loss of bacteriocin activity upon treatment with lipases or amylases (Lewus *et al.*, 1992; van Laack *et al.*, 1992; Jimenez-Diaz *et al.*, 1993; Schved *et al.*, 1993; Keppler *et al.*, 1994).

The term bacteriocin has been restricted to those antibacterial proteins that exhibit a bactericidal mode of action (Tagg *et al.*, 1976). Although a vast majority of bacteriocins of LAB exert a bactericidal mode of

action, but a few have been found to be bacteriostatic rather than bactericidal to the sensitive cells (Lewus *et al.*, 1992; Thompson *et al.*, 1996).

Most of the bacteriocins of LAB characterised to date are small (< 10 KDa)heat stable peptides. However, the occurrence of large (> 30 KDa) heat labile proteins has also been reported (Joerger and Klaenhammer, 1986; Vaughan *et al.*, 1992). Bacteriocins are extremely heat stable at low pH (Hurst, 1981; Hastings *et al.*, 1991; Felix *et al.*, 1994) becoming more sensitive to heat upon purification (Davey, 1981; Hastings *et al.*, 1991). Bacteriocins of LAB, in general, are active over a wide pH range with optimum being on acidic side.

2.3.1 BACTERIOCINS OF LACTOBACILLI

2.3.1.1 Helveticins

Apart from their sensitivity to various proteolytic enzymes, helveticin J and helveticin V-1829 were shown to be heat sensitive, the latter being more heat labile with total loss in activity at 60°C after 15 min (Joerger and Klaenhammer, 1986; Vaughan *et al.*, 1992). Although both of these bacteriocins were bactericidal, the un-named bacteriocin from strain CNRZ450 was found to be bacteriostatic in its mode of action (Thompson *et al.*, 1996). Helveticin J and CNRZ450 bacteriocin but not helveticin V-1829 formed aggregates.

2.3.1.2 Lactacin B

Lactacin B activity was eliminated by treatment with protease of *Streptomyces griesus*. It was a heat stable protein being unaffected by heating at 100°C for 60 min.

It was bactericidal but not bacteriolytic to the sensitive cells (Barefoot and Klaenhammer, 1983). The purified lactacin B was inactivated by proteinase-K (Barefoot and Klaenhammer, 1984). The molecular weight of the purified compound was determined to be 8100 Da as mentioned by Nettles and Barefoot (1993).

2.3.1.3 Acidocins A and B

While acidocin A retained its full activity after boiling for 20 min and hence described as a heat stable protein (Kanatani *et al.*, 1995), acidocin B was found to be moderately heat stable as boiling led to a partial loss of activity (ten Brink *et al.*, 1994). The molecular weight of acidocin A was 6500 Da, whereas in SDS-PAGE experiments of butanol extracts of acidocin B, a protein band corresponding to a molecular weight of 2400 Da possessed anticlostridial activity. Acidocin B was active within the pH range of 2-10.

2.3.1.4 Plantaricin A

Plantaricin A produced by *Lb. plantarum* C11 was shown to be susceptible to protease and bactericidal in mode of action. The bacteriocin was heat stable with no loss in activity at 100°C for 30 min and active from pH 4.0 to 6.5. The molecular weight was reported to be greater than 6000 Da (Daeschel *et al.*, 1990).

2.3.1.5 Plantaricins S and T

Jimenez-Diaz *et al* (1993) reported the characterisation of plantaricins S and T secreted by *Lb. plantarum* LPC010. Plantaricin S was inactivated not only by proteolytic enzymes but also by lipolytic and

glycolytic enzymes suggesting that it was a glycolipo protein. However, platnaricin T was distinguished from plantaricin S by its resistance to lipolytic and amylolytic enzymes. Plantaricin S was further characterised to be bactericidal, non-bacteriolytic, heat stable (60 min at 100°C) peptide active in the pH range of 3-7. The bacteriocin S migrated as a single band of approximately 2500 Da on SDS-PAGE gel while T migrated at a slightly lower position than S.

2.3.1.6 Plantaricins LC74 and KW30

Plantaricin LC74 from *Lb. plantarum* LC74 was susceptible to high temperature (95°C) and bacteriostatic against sensitive cells (Rekhif *et al.*, 1994). On the other hand, plantaricin KW30 was reported to be heat stable and bactericidal (Kelly *et al.*, 1996).

2.3.1.7 Sakacin A

Sakacin A, produced by *Lb. sake* 706, was inactivated by trypsin, pepsin, chymotrypsin or proteinase K (Schillinger and Lucke, 1989; Lewus *et al.*, 1991). It was heat stable. It remained active after 20 min at 100°C. The bacteriocin demonstrated a bactericidal mode of action (Schillinger and Lucke, 1989). The molecular weight of the purified preparation was estimated to be 4308 Da (Holck *et al.*, 1992).

2.3.1.8 Lactocin S

Trypsin and protease XIV sensitive antibacterial compound produced by *Lb. sake* strain 45 was moderately heat stable. The antagonistic activity was reduced by 50% upon heat treatment for 1 hr at 100°C (Mortvedt and

Nes, 1991). The molecular mass of the purified lactocin S was determined to be 13700 (Mortvedt *et al.*, 1991).

2.3.1.9 Brevicin 286

The culture supernatant of *Lb. brevis* VB286, brevicin 286 producer, lost its antibacterial activity upon treatment with β-chymotrypsin, pepsin and proteases I, XIV and XXIII, but was unaffected by lysozyme or lipase. The bacteriocin was found to be stable to heating at 100°C particularly under acidic conditions. Brevicin 286 retained 100% activity for at least 10 min, and suffered only minimal loss of activity after 30 min when heated at 100°C at pH $\leq$ 5.0. The loss in activity increased with both increase in pH and time of heating. Brevicin retained 50% of its activity after autoclaving (121°C, 15 min) at pH 5.0 (Coventry *et al.*, 1996).

2.3.1 10 Lactacin F

Lactacin F activity was completely eliminated with ficin, trypsin, proteinase K and subtilin (Muriana and Klaenhammer, 1987; 1991a). Lactacin F was heat stable, retaining activity after 20 min at 99°C or after autoclaving at 121°C for 15 min (Muriana and Klaenhammer, 1987). The purified compound had a molecular weight of 2500 Da as estimated by SDS-PAGE, which did not correlate with amino acid composition analysis indicating a total of 56 amino acids with a molecular weight of approximately 6300 Da (Muriana and Kleanhammer, 1991a). The discrepancy in estimated size was resolved by cloning and characterization of LaF gene (laf) which confirmed the size of 6300 Da (Muriana and Klaenhammer, 1991b).

2.3.2 BACTERIOCINS OF LACTOCOCCI

2.3.2.1 Nisin

Nisin is inactivated by α-chymotrypsin and ficin. In general pepsin, trypsin and erapsin do not inactivate nisin. The immunity of nisin to amylases and lipases suggests that no lipo- or glyco- portions are associated with the antibacterial property (Hurst, 1981; Ray, 1992a). The bacteriocin is more stable at acidic pH. As the pH increases above 7.0 the molecule undergo irreversible changes with loss of activity (Hurst, 1981; Ray, 1992a). Nisin is more stable to heat at low pH. It withstands prolonged heating at 115°C to 121°C at pH 2.0. In the pH range 5 to 7, nisin becomes progressively less stable to heating and significant losses in activity are to be expected when heated at elevated temperatures (Ray, 1992a; Delves-Broughton, 1996).

Nisin has a molecular weight of 3510 Da. It forms dimers and tetramers of 3500 monomer molecules and may show a molecular weight of 7000 and 14000 Da (Ray, 1992a).

2.3.2.2 Diplococcin

The purified diplococcin was found to be rather unstable, whereas partially purified preparation was stable at 100°C at pH 5.0 for at least 1 hr (Davey, 1981). Both preparations were sensitive to trypsin, pronase and α-chymotrypsin. Diplococcin had a molecular weight of 5300 Da. It had a bactericidal and non bacteriolytic effect against the sensitive cells (Davey, 1981; Davey and Richardson, 1981).

2.3.2.3 Lacticin 481

The cell free supernatant of *Lc. lactis* subsp. *lactis* CNRZ481, the organism producing lacticin 481, lost its antibacterial activity completely with ficin, proteinase K and partially with α-chymotrypsin and pronase (Piard *et al.*, 1990). It was not inactivated by trypsin, α-amylase and rennet. Lacticin 481 was heat stable compound showing no detectable loss of activity at 100°C for 1 hr at pH 4.5 or 7.0. It lost 50% of its inhibitory activity after 20 min at 115°C and at pH 2.0. The estimated molecular weight of the purified lacticin 481 was 1700 Da by SDS-PAGE, 1300 Da by gel filtration and 2400-2700 Da by amino acid composition analysis (Piard *et al.*, 1992).

2.3.2.4 Bacteriocin S50

Bacteriocin S50, the bacteriocin of *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* S50 was sensitive to various proteolytic enzymes, but not to lysozyme, α-amylase, DNase-I and RNase A. The bacteriocin retained its activity in the pH range 2 to 11. It was inactivated irreversibly after 30 min at pH 12.0. Bacteriocin S50 was heat stable, possessing its antibacterial activity after heating for upto 60 min at 100°C. The bacteriocin was bactericidal to the sensitive cells (Kojic *et al.*, 1991).

2.3.2.5 Lactococcin G

Nissen-Meyer *et al* (1992) observed that the activity of lactococcin G depends on the complementary action of two peptides termed α and β. The molecular weights as

determined by amino acid sequencing and mass spectrometry were 4346 and 4376 for α-peptide and 4110 and 4109 for β-peptide, respectively. The isoelectric points of α- and β-peptides were calculated to be 10.9 and 10.4, respectively (Nissen-Meyer *et al.*, 1992).

2.3.2.6 Diacetin B

This bacteriocin was inactivated by selected proteases, retained antibacterial activity after heat treatments of 100°C for 60 min and 121°C for 20 min and in the pH range of 2-11 (Ali *et al.*, 1995). The pure peptide had a molecular weight of 4292.32 or 4490.28 Da.

2.3.2.7 Lactococcin 972

Lactococcin 972 was reported to be heat sensitive, active in the pH range of 4.0 to 9.0, less susceptible to proteinases. The purified lactococcin 972 had a molecular weight of 7500 Da (Martinez *et al.*, 1996).

2.3.2.8 Bacteriocin J46

Bacteriocin J46 was sensitive to proteolytic enzymes, thermostable, more stable near neutral pH values than nisin and bactericidal to the sensitive cells (Gonzalez *et al.*, 1996).

2.3.2.9 Lactococcins A, B and M

Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* WM4, a producer of lactococcins, was reported to loose its inhibitory activity upon treatment with pronase, trypsin or α-chymotrypsin but retained the

activity upon heating at 80°C for 10 to 60 min (Scherwitz *et al.*, 1983). On the other hand, Geis *et al* (1983) observed the sensitivity of antibacterial activity demonstrated by *Lc. lactis* subsp. *cremoris* 9B4, a strain producing all the three lactococcins to trypsin but not to heat (100°C for 30 min) at pH 4.5, 7.0 and 9.4. A bactericidal, non-bacteriolytic activity was observed for purified lactococcin A (van Belkum *et al.*, 1991b) and lactococcin B (Venema *et al.*, 1993). However, the bactericidal and bacteriolytic activity exhibited by *Lc. lactis* subsp. *lactis* DPC398 on sensitive cultures has been attributed to the concerted action of all the three lactococcins A, B and M (Morgan *et al.*, 1995). A decrease in stability with increasing purity of lactococcin A was observed. The purified lactococcin A had a molecular weight of 5783 ± 4 as revealed by mass spectrometry and 5778 as calculated from the sequencing data (Holo *et al.*, 1993).

2.3.3 BACTRIOCINS OF LEUCONOSTOCS

2.3.3.1 Leucocin A-UAL187

Leucocin A-UAL187 produced by *Leu. gelidum* UAL187 completely lost its bacteriocinogenic activity upon treatment with protease I, IV, VIII, X, XIII or XIV, trypsin, α-or β-chymotrypsin, pepsin or papain (Hastings *et al.*, 1991). However, lysozyme, lipase and phospholipase did not affect the activity. Crude leucocin from ammonium sulfate precipitate was found to be stable at pH 2.0 and 3.0 even after samples were boiled for 20 min. Loss of activity was however, detected at pH 5.0 and above being most severe at a pH above 8.0. The purified preparation was less stable. There was rapid loss of activity of the pure sample at

all pH levels and temperatures tested. The average molecular weight of leucocin A-UAL187 was found to be 3930.3 ± 0.4 (Hastings *et al.*, 1991).

2.3.3.2 Leuconocin S

The sensitivity of leuconocin S to various proteolytic enzymes and α-amylase indicated that it was a glycoprotein which required both glyco protion and the protein portion of the molecule for acitivity. Leuconocin S was stable against heating at 60°C for 30 min. There was a loss of 50% of the activity after 60 min at 60°C. The bacteriocin was bacteriostatic to the indicator *Lb. sake* ATCC15521 and dissipated the proton motive force (Lewus *et al.*, 1992).

2.3.3.3 Carnosin 44A

This bacteriocin from *Leu. carnosum* LA44A exhibited sensitivity to trypsin and chymotrypsin but not to pepsin and other non-proteolytic enzymes. The culture supernatant lost its activity upon treatment with amylase from Fluka but not with amylase from Sigma making unsure whether the observed sensitivity to amylase from Fluka represented actual sensitivity or possible contamination of amylase by proteases. The bacteriocin retained its activity after 20 min at 100°C but completely inactivated after 15 min at 121°C. It was stable at pH values ranging from 2 to 9. The partially purified carnosin 44A had a molecular mass between 2510 and 6000 Da (van Laack *et al.*, 1992).

2.3.3.4 Carnocin LA54A

The bacteriocin completely lost its activity upon treatment with α-chymotrypsin, pronase-E and proteinase

and only partially with trypsin. The bacteriocin was also found to be sensitive to α-amylase indicating that carbohydrate moiety of the molecule is also important for activity. Carnocin LA54A was active and stable over a wide pH range with an optimum between pH 3 and 5. The activity decreased to 50% between pH 6-7 and to 10% at pH 9. No activity was however, detected at higher pH values. It was stable after heat treatment at 100°C for 15 min. The purified bacteriocin showed a molecular weight of about 4000 Da (Keppler *et al.*, 1994).

2.3.3.5 Leucocin B-Ta11a

The bacteriocin was susceptible to pronase, α-and β-chymotrypsin, papain, proteinase-K and trypsin but not to lysozyme. Crude bacteriocin extracts were stable at 100°C for upto 30 min in the pH range 2-9. However, the bacteriocin was stable to heating at 121°C for 15 min in the pH range 2-4 only. The bacteriocin exhibited a bacteriolytic mode of action against the indicator *Leu. mesenteroides* TA10C (Felix *et al.*, 1994).

2.3.3.6 Mesentericin Y105

Mesentericin Y105 was sensitive to pronase, proteinase-K, trypsin, chymotrypsin and pepsin. The bacteriocin remained active after 120 min at 60°C. The bacteriocinogenic peptide was more stable to heat at a low pH of 4.5 than at 6.8. The apparent relative molecular mass, as indicated by activity detection after SDS-PAGE was 2.5-3.0 KDa (Hechard *et al.*, 1992).

2.3.3.7 Mesenterocin 52

The bacteriocin was inactivated by trypsin, α-chymotrypsin, pronase-E, proteinase-K and pepsin

(Mathieu *et al.*, 1993). Sudirman *et al* (1994) observed complete inactivation of mesenterocin 52 by phospholipase A2 and partial inactivation by phospholipase D. The bacteriocin was relatively heat stable. It retained 80%, 50% and 20% of its original activity after heating at 100°C for 15 min and 60 min and 121°C for 15 min, respectively. Mesenterocin 52 was more stable to heat at acidic pH than at neutral pH (Mathieu *et al.*, 1993).

2.3.3.8 Dextranicin J24

It was inactivated completely by proteases, partially by lipase. Alpha-amylase displayed no action on dextranicin J24. It retained 7% of its original activity after autoclaving for 20 min at 121°C (sudirman *et al.*, 1994).

2.3.3.9 Leucocidin R1

Leucocidin R1, a novel bacteriocin identified by Malik *et al* (1994a), was completely inactivated by protease I and protease XXII and partially by trypsin and chymotrypsin. It was heat stable. It retained its activity after 30 min at 60°C and 5 min at 70° or 90°C.

Yang and Ray (1994a) characterised bacteriocins produced by several strains of *Leu. mesenteroides* and *Leu. carnosum*. Bacteriocins from all strains exhibited similar properties. They were sensitive to various proteolytic enzymes but not to lipase and ribonuclease A. They retained activity after 30 min at 65°C and 10 min at 100°C but lost it at pH 10.0. Bacteriocins produced by two *Leu. carnosum* strains and one *Leu. mesenteroides* strain were partially purified. The analysis of the partially purified bacteriocins by SDS-PAGE for

inhibitory activity showed a molecular weight of less than 2500 Da for all the three bacteriocins (yang and Ray, 1994a).

2.3.4 BACTERIOCINS OF PEDIOCOCCI

2.3.4.1 Pediocin PA-1

One of the extensively studied bacteriocins of *Pediococcus spp.*, pediocin PA-1 was completely inactivated by protease, papain, pepsin and α-chymotrypsin. The activity was not adversely affected by other enzymes such as lipase, phospholipase C, lysozyme, RNase or DNase and by heating at 80°C for 60 min or at 100°C for 3 min or 10 min. However, exposure to 121°C for 15 min did partially inactivate (68% loss) the bacteriocin. The activity was most stable at pH 4 to 7, partially lost at pH 2, 3, 9 and 10 and mostly at pH 11.0 (Gonzalez and Kunka, 1987). The purified bacteriocin had a molecular weight and isoelectric pH of 4629 Da and 10.0 (Henderson *et al.*, 1992) and 4600 Da and 8.6 (Lozano *et al.*, 1992), respectively.

2.3.4.2 Pediocin AcH

Pediocin AcH from *P. acidilactici* H was sensitive to trypsin, papain, chymotrypsin, ficin, proteinase-K, protease IV, XIV and XXIV but not to lysozyme, lipase, RNase A and DNase (Bhunia *et al.*, 1988). Pediocin AcH activity was not lost after 15 min at 93°C but lost about 30-40% of original activity after heating at 121°C for 15 min. The bacteriocin was found to be stable at pH levels between 2.5 and 9.0. It lost activity at pH 10.0 and above after 24 hr at 25°C. It showed bactericidal, non bacteriolytic, mode of action on sensitive indicator, *Lb.*

plantarum WSO-39. The molecular weight of pediocin AcH as determined by SDS-PAGE was 2700 Da. However, the molecular weight was 4628 Da as determined by the amino acid composition and sequence and the estimated isoelectric pH was 9.6 (Bhunia *et al.*, 1988; Motlagh *et al.*, 1992; Ray, 1992b).

2.3.4.3 Pediocin 5

Pediocin 5 lost its activity following pronase treatment. It was relatively heat stable being affected very little after heating for 30 min at 100°C. Bacteriocin activity in SDS-PAGE gels was detected at an area corresponding to 4500 Da. The molecular weight of the pediocin 5 as determined by mass spectrometry was 4624 Da (Daba *et al.*, 1991, 1994).

2.3.4.4 Pediocin PO2

Besides loss of bactericidal activity by several proteases lysozyme also reduced the activity by 50%. It was stable within the pH range 2.5-12.0. Like other pedicoccal bacteriocins, this bacteriocin was also heat stable as it retained 100% activity after 60 min at 80°C and 50% activity after 15 min at 121°C. The molecular weight of pediocin PO2 was approximately 3200 Da (Coventry *et al.*, 1995).

2.3.4.5 Pediocin L50

Cintas *et al* (1995) reported that pediocin L50 was sensitive to several proteolytic enzymes such as trypsin, papain, pepsin, protease II, VI and XIV. However, treatment with lipolytic or amylolytic agents had no effect on bacteriocinogenic activity. The bacteriocin

was stable at pH levels between 2 and 11 for 24 hr at 25°C. Moderately heat stable showing detectable activity after 40 min of exposure to 100°C. Pediocin L50 was bactericidal to *P. acidilactici* 347 and *L. monocytogenes*. The molecular weight of pediocin L50 as determined by SDS-PAGE was about 3600 and 5250 as estimated by electrospray mass spectrometry.

2.3.4.6 Pediocin SJ-1

In addition to proteolytic enzymes such as trypsin, chymotrypsin or protease, pediocin SJ-1 was also found to be sensitive to α-amylase suggesting that a glycomoiety is also involved in the inhibitory activity of the molecule. Pediocin SJ-1 was stable over a wide pH range (3-9), apparently being more stable at lower pH values. Bacteriocin was more stable to heat at low pH than at neutral pH. It retained 100% activity at pH 3.6 after heating at 100°C for 30 min while 87.5% of the activity was lost when the same treatment was given at pH 7.0. Pediocin SJ-1 had a molecular weight of approximately 4000 Da (Schved *et al.*, 1993).

2.3.4.7 Pediocin A

Sensitivity to pronase, stability to heating at 100°C for 60 min, and a bactericidal mode of action were some of the characteristics observed by Reuchert (1979) in case of pediocin A. Further attempts to isolate and purify pediocin have been uniformly unsuccessful probably because routine laboratory manipulations are destabilizing or denaturing it in some manner (Daeschel, 1992).

2.3.4.8 ISK-1 Bacteriocin

The ISK-1 bacteriocin produced by *Pediococcus* sp. was inactivated by acid protease, α-chymotrypsin, pepsin, ficin and papain. Whereas it was not affected by lysozyme, lipase, ribonuclease-A and α-amylase. The bacteriocin was stable at acidic pH and more than 60% of the antimicrobial activity was still retained even after autoclaving at 121°C for 20 min in the pH range of 3-8 (Kimura, 1997).

2.4 PRODUCTION OF BACTERIOCINS

One of the most important steps in the study of bacteriocins is their production. The composition of culture medium and cultural conditions such as temperature, pH and time of incubation have profound effect on the production of bacteriocins. In general, conditions that provide high cell density favour high bacteriocin concentration.

The culture media generally employed for the growth of lactic acid bacteria such as MRS, APT, TGE, M17G, ELB etc. have also been found to support good bacteriocin production. Although bacteriocin production occurs over a wide temperature range, it is greater at the optimum temperature for the growth of the producer.

The production of bacteriocins by lactic acid bacteria is strongly influenced by the pH of the culture medium. The regulation of pH at a certain value during the course of fermentation has been found to have favourable (Hurst, 1981; Piard *et al.*, 1990) and detrimental (Biswas *et al.*, 1991; Coventry *et al.*, 1996)

effects on the final yield of bacteriocins of lactic acid bacteria.

The maximum production of bacteriocins occurs at different phases in the cell growth cycle. Most of the bacteriocins of lactic acid bacteria are secreted during the logarithmic growth phase with a slight decline in the activity of some of them during the stationary phase of the producer culture. However, some bacteriocins for e.g., nisin (Hurst, 1981), pediocin SJ-1 (schved *et al.*, 1993) are secreted as secondary metabolites. The termination of the incubation at appropriate time is essential to prevent the loss of bacteriocin activity.

2.4.1 GROWTH MEDIUM

Commonly used media for the production of bacteriocins by lactic acid bacteria include MRS (ten Brink *et al.*, 1994; Coventry *et al.*, 1996), TGE (Biswas *et al.*, 1991; Yang and Ray, 1994b), APT (Lewus *et al.*, 1992), GM17 (Parente and Hill, 1992), ELB (Geis *et al.*, 1983; Piard *et al.*, 1990) etc. with or without modifications. Although a large number of bacteriocins have been found to be identified and several media have been used for the production of bacteriocins, very few studies are available on the comparison of bacteriocin production in different media.

Geis *et al* (1983) compared various media including ELB, GM17, BHI, a synthetic medium and milk for their ability to support bacteriocin production by various lactococcal strains. All the strains produced antibiotic activities in milk. Highest bacteriocin activities were found in unbuffered ELB followed by BHI, buffered M17 and synthetic medium (Geis *et al.*, 1983).

Lactococcus lactis subsp. *lactis* CNRZ481 produced maximum bacteriocin (12800 AU/ml) in ELB buffered with sodium β-glycerophosphate. The observed titre was double than the value recorded when the culture was grown in M17 or unbuffered ELB (Piard *et al.*, 1990).

Parente and Hill (1992) formulated three media (Tryptone-Yeast Extract-Tween) TYT10, TYT11 and TYT30 and compared with seven different media [ELB, M17, M17 dialysate, tryptose phosphate (TP), tryptone yeast extract broth (TYB), yeast glucose lemco (YGL) broth and MRS] for the growth of and bacteriocin production by *Lc. lactis* subsp. *lactis* DPC3286 and *Lc. lactis* subsp. *cremoris* LMG2130. Good growth and bacteriocin production were obtained for both in the TYT, M17 and MRS media. Bacteriocin production was very poor in YGL. It was also observed that *Lc. lactis* subsp. *cremoris* LMG2130 could not grow or produce bacteriocins in M17 dialysate and TP media (Parente and Hill, 1992). Although the cell mass was greater in MRS broth, 15% less pediocin AcH production by *P. acidilactici* H was observed in MRS broth than it did in TGE (Biswas *et al.*, 1991). It was reported that *P. acidilactici* LB42-923 produced higher pediocin AcH titres in TGE broth than in buffered TGE broth (Yang and Ray, 1994b).

In contrast to pediocin AcH, higher levels of nisin, sakacin A and leucocin Lm1 were observed in TGE buffer broth than in TGE (Yang and Ray, 1994b). Earlier Hechard *et al* (1992) observed consistently higher levels of (x16) mesentericin Y105 in MRS broth than in a semi-defined medium.

2.4.2 pH

2.4.2.1 Lactobacilli Bacteriocins

Barefoot and Klaenhammer (1984) reported maximum lactacin B production when *Lb. acidophilus* N2 was grown in MRS broth regulated at pH 6.0. In contrast, lactacin F was produced maximally in MRS broth held at a constant pH of 7.0 rather than 7.5, 6.0 or 5.0 (Muriana and Klaenhammer, 1987). Production of helveticin J and helveticin V-1829 was observed to be greatest in anaerobic MRS cultures maintained at a pH 5.5 than at other pH values tested in the range of 5.0 to 7.0 (Joerger and Klaenhammer, 1986; Vaughan *et al.*, 1992). Vaughan *et al* (1992) also reported a two fold increase in helveticin V-1829 when MRS broth was held at a pH 5.0 than in pH unregulated cultures.

ten Brink *et al* (1994) observed that growth of *Lb. acidophilus* M46 in five fold concentrated MRS broth held at a constant pH 5.5 resulted in eight fold increase in acidocin B activity than that obtained after growth in normal MRS broth without pH control. Regulation of MRS broth at pH 5.0 resulted in maximum yield of acidocin A produced by *Lb. acidophilus* TK9201 (Kanatani *et al.*, 1995).

Maximum production of plantaricin S was obtained in a fermentor system in unregulated pH in MRS broth containing 4% NaCl. It was also reported that regulation of pH at 4.0-7.0 during fermentation had a detrimental effect on the production of plantaricin S by *Lb. plantarum* LPC010 (Jimenez-Diaz *et al.*, 1993).

Coventry *et al* (1996) studied the effect of pH on the production of brevicin 286 by *Lb. brevis* VB286. No

substantial cell growth or brevicin 286 activity was detected in MRS broth with an initial pH 4.5. In spite of substantial cell growth, brevicin 286 production was minimal at pH 5.0. Optimum production of brevicin 286 was observed in MRS broth at an initial pH of 6.0-6.5. It was also observed that regulation of pH at either 6.0 or 6.5 had no advantage over stirred culture without pH control with respect to brevicin 286 (Coventry *et al.*,1996).

2.4.2.2 Lactococcal Bacteriocins

Nisin production was greatest when the pH of the medium was maintained at about 6.0 alongwith a large cell mass (Hurst, 1981). Piard *et al* (1990) observed maximum lacticin 481 production when the producer strain *Lc. lactis* subsp. *lactis* CNRZ481 was grown in buffered ELB held at a constant pH of 5.5. Regulation of pH at 6.0 or 6.5 or growing the producer in pH non-regulated medium resulted in decreased bacteriocin yields (Piard *et al.*, 1990). Bacteriocin production by *Lc. lactis* subsp. *lactis* ADRI 85L030 was reported to be independent of the initial pH of the medium in the range 5.0 to 7.0 (Thuault *et al.*, 1991).

2.4.2.3 Leuconostocs Bacteriocins

Leuconostoc gelidum UAL187 produced leucocin A-UAL187 maximally in APT broth at pH 6.0 and 6.5. At a lower initial pH, growth of the producer organism was slower and concentration maxima was lower (Hastings and Stiles, 1991). Lewus *et al* (1992) studied the effect of initial pH of APT broth on the growth of and bacteriocin production by *Leu. paramesenteroides* OX. Leuconocin S

was produced in detectable amounts at pH 6.0 and appeared to be optimal (400 AU/ml) at pH 6.5 and 7.0. They observed slight depression in growth and leuconocin S production at pH 7.5. Recently Baker *et al* (1996) reported that the production of leuconocin S was maximum (2000 AU/ml) in fermentors maintained at pH 7.0 than at 6.0, 6.5 and 7.5.

van Laack *et al* (1992) observed a 50% decrease in the production of carnosin 44A when the initial pH of MRS broth was lowered from 6.0 to 5.1. Although *Leu.carnosum* Ta11a produced leucocin B-Ta11a in MRS broth with an initial pH in the range 4.5-7.5, the bacteriocin concentration was found to be optimal at pH 6.0-6.5 (Felix *et al.*, 1994).

2.4.4.4 Pediococcal Bacteriocins

Pediococcus acidilactici H produced maximum pediocin AcH when grown in TGE broth with an initial pH of 6.5. Pediocin AcH was produced in negligible amounts when the pH of TGE broth was maintained at a PH 5.0 or above. It was concluded that a terminal pH below 4.0 alongwith a large cell mass was essential for the production of pediocin AcH (Biswas *et al.*, 1991). High titres of pediocin N5P was observed when *P. pentosaceus* N5P was grown in TGE broth with an initial pH of 6.5 (Strasser-de-saad and Manca-de-Nadra, 1993). It was also reported that pediocin N5P could not be detected in TGE broth at an initial pH below 5.0. Liao *et al* (1993) reported optimum production of pediocin PO2 in whey

permeate medium with an initial pH of 6.5 without pH regulation during incubation.

2.4.3 TEMPERATURE

2.4.3.1 Lactococcal Bacteriocins

Nisin production was maximum when the culture was incubated between 25 and 30°C as opposed to 37°C. Incubation of nisin producer at 37°C resulted in 386 AU/ml of nisin as compared to 542 AU/ml at 26°C (Hurst, 1981). Thuault *et al* (1991) reported that the bacteriocin production by *Lc. lactis* subsp. *lactis* ADRI 85L030 was not significantly dependent on the incubation temperature in the range of 30 to 42°C.

2.4.3.2 Leuconostocs Bacteriocins

Leuconostoc carnosum Talla produced bacteriocin, leucocin B Talla over a wide range of temperature i.e. 0°C to 30°C, but the optimal production was observed at 25°C (Felix *et al.*, 1994). van Laack *et al* (1992) reported that *Leu. carnosum* LA44A could grow and produce bacteriocins in the temperature 4-10°C. Although bacteriocin production by various *Leuconostoc* spp. was observed both at 4°C and 25°C, the bacteriocin titres, in general, were 2-3 times higher at 25°C than at 4°C (Yang and Ray, 1994a). Leucocin A-UAL187 production by *Leu. gelidum* UAL187 was observed over a wide range of incubation temperatures (1-25°C) with more time taken at low temperatures (Hastings and Stiles, 1991).

2.4.3.3 Pediococcal Bacteriocins

Pediococcus acidilactici H produced same amounts of pediocin AcH after 16 hr of growth in TGE broth both at

30° and 37°C. The cell mass and bacteriocin production were slightly reduced at 40°C (Biswas *et al.*, 1991). Schved *et al* (1993) observed the production of pediocin SJ-1 at 20°, 30°, 40° and 45°C with optimal production in the range of 35-40°C. It was reported that the amount of pediocin L50 formed at 16°C was comparable to that formed at 32°C, while considerably less amount was produced at 8°C. The organism failed to produce detectable amounts of bacteriocin at 45°C (Cintas *et al.*, 1995).

2.4.4 GROWTH PHASE

2.4.4.1 Lactobacilli Bacteriocins

Joerger and Klaenhammer (1986) observed accumulation of helveticin J between late log phase and stationary phase of growth of *Lb. helveticus* 481. Helveticin V-1829 was produced from the middle log phase into the stationary phase of growth of *Lb. helveticus* V-1829 (Vaughan *et al.*, 1992).

Barefoot and Klaenhammer (1984) observed the production of lactacin B during the logarithmic phase of growth of *Lb. acidophilus* N2. *Lactobacillus acidophilus* M46 produced acidocin B continuously during the logarithmic growth phase. The level of inhibition reached maximum at the beginning of the stationary phase and maintained constant for at least 24 hr (ten Brink *et al.*, 1994).

Lactobacillus plantarum C-11 was found to accumulate maximum amount of plantaricin A during the mid log phase of growth with a decrease in activity thereafter (Daeschel *et al.*, 1990). Maximum production of plantaricin S was obtained in log phase cultures of *Lb.*

plantarum LPC010. It was also observed that *Lb. plantarum* LPC010 secreted another bacteriocin designated plantaricin T in the late-stationary phase (Jimenez-Diaz *et al.*, 1993). Rekhif *et al* (1994) reported plantaricin LC74 production in the exponential phase of growth of *Lb. plantarum*. However, the bacteriocin, plantaricin KW30, was maximally produced at the beginning of stationary phase cultures of *Lb. plantarum* KW30 (Kelly *et al.*, 1996).

It was reported that the concentration of brevicin 286 was highest at the late exponential growth phase (Coventry *et al.*, 1996).

2.4.4.2 Lactococcal Bacteriocins

Davey and Pearce (1980) observed diplococcin production by *Lc. lactis* subsp. *cremoris* 346 throughout the exponential growth phase. Nisin is synthesized as a secondary metabolite at a high rate when the cells have reached mid-exponential phase, and continues to be synthesized during a greater part of the stationary phase when the cells are grown at a constant pH of 6.8 at 30°C for 20-24 hr (Ray, 1992a).

Bacteriocin S50 by *Lc. lactis* subsp. *lactis* biovar. diacetylactis S50 was produced continuously during the growth, but the highest production was observed after 8 hr of incubation (Kojic *et al.*, 1991). Lacticin 481 production occurred in late-log phase of growth of *Lc. lactis* subsp. *lactis* 481 (Piard *et al.*, 1990).

2.4.4.3 Leuconostocs Bacteriocins

Mathieu *et al* (1993) reported that the biosynthesis of mesenterocin 52 and its secretion into the medium

started early in the growth phase, continued over the whole of that phase before reaching a maximum at the end. A decrease upto one to two orders of magnitude in the activity of carnocin LA54A was recorded during the stationary phase (Keppler *et al.*, 1994). Yang and Ray (1994a) observed the termination of bacteriocins production by various *Leuconostoc* spp. in the stationary phase of their growth. Leucocin B-Talla production occurred during the exponential phase of growth of the producer *Leu. carnosum* Talla (Felix *et al.*, 1994). Production of leucocin A-UAL187 occurred early in the growth cycle of the producer organism, rather than as a secondary metabolite of growth (Hastings and Stiles, 1991).

2.4.4.4 Pediococcal Bacteriocins

Biswas *et al* (1991) reported that about 60% of the pediocin AcH was produced by 8 hr and the rest 40% was produced during the next 8 hr (stationary phase). The authors suggested that pediocin AcH appeared to be a secondary metabolite. Later studies have shown that post translational processing of prepediocin to active pediocin AcH occurred efficiently at a pH below 5.0 (Johnson *et al.*, 1992). After 24 hr of growth, the pediocin AcH was slightly reduced at all the temperatures studied (Biswas *et al.*, 1991). Production of pediocin during the logarithmic and early stationary phases of growth suggested that pediocin SJ-1 was a secondary metabolite and after reaching maximum levels, in contrast to many bacteriocins, the antibacterial activity of pediocin SJ-1 remained stable in broth cultures over a period of upto 48 hr (Schved *et al.*, 1993). *Pediococcus acidilactici* L50 produced highest bacteriocin from the onset of stationary phase and it remained stable at 8°C and 16°C while at 32°C, a decrease in antibacterial

activity was seen throughout the stationary phase (Cintas *et al.*, 1995). Daba *et al* (1991) observed the secretion of pediocin 5 from *P. acidilactici* UL5 during the late exponential phase of growth and the activity dropped sharply (>90% in 24 hr) during the early stationary phase. However, experiments with pH controlled at 5.0 did not show this large decrease in activity during the stationary phase.

2.5 PURIFICATION OF BACTERIOCINS

An extensive characterisation with respect to physical and chemical properties of bacteriocins is necessary before considering them for application in foods. The availability of bacteriocins in a pure form is essential for characterisation studies.

Purification of bacteriocins is a difficult task for several reasons. Firstly, protein concentration in the supernatant is very high while bacteriocin concentration is low, meaning a very low specific activity. Secondly, bacteriocins form a heterogeneous group of substances, and the specific purification protocol has to be developed by trial and error for each bacteriocin. An additional problem encountered with the purification of bacteriocins of lactic acid bacteria is the use of media containing tween 80, a surfactant which has been shown to interfere with the precipitation procedures (Muriana and Klaenhammer, 1991a; van Laack *et al.*, 1992).

During the recent years, the above mentioned problems have been overcome and several bacteriocins of lactic acid bacteria have been purified to homogeneity by growing the producers in semi-defined media by minimising the level of contaminating proteins and peptides (Joerger and Klaenhammer, 1986; Hastings *et al.*, 1991; Hechard *et*

al., 1992). Also MRS broth has been generally modified by omission of tween 80 (van Laack *et al.*, 1992; Mortvedt *et al.*, 1991).

2.5.1 LACTOBACILLI BACTERIOCINS

Barefoot and Klaenhammer (1984) purified lactacin B by ion-exchange chromatography, ultrafiltration and gel filtration chromatography. Later, as mentioned by Nettles and Barefoot (1993) a simpler purification protocol was devised for lactacin B. The protocol involved lyophilisation of culture supernatants followed by ultrafiltration and preparative electrofocussing. Muriana and Klaenhammer (1991a) achieved a 474-fold increase in specific activity of lactacin F by ammonium sulfate precipitation, gel filtration and HPLC.

Lactocin S produced by *Lb. sake* L45 was purified to a 4000-fold increase in specific activity with a recovery of just 3.0% by ammonium sulfate precipitation, and sequential anion and cation exchange, hydrophobic interaction, gel filtration, phenyl superose and reverse-phase chromatographies (Mortvedt *et al.*, 1991). Holck *et al* (1992) purified sakacin A to a 9000-fold increase in specific activity and a very good recovery of about 80% was achieved by ammonium sulfate precipitation, ion exchange, hydrophobic interaction and reverse-phase chromatography.

Plantaricin S from *Lb. plantarum* LPC010 was purified to homogeneity by ammonium sulfate precipitation, binding to SP-sepharose fast flow, phenyl sepharose CL-4B and C2/C-18 reverse-phase chromatographies. The purification protocol resulted in a final yield of 91.6% and

352,617-fold increase in specific activity (Jimenez-Diaz *et al.*, 1995).

A purification protocol comprising ammonium sulfate precipitation and sequential cation exchange and reverse-phase chromatographies has been used for the purification of acidocin A with a recovery of about 10% (Kanatani *et al.*, 1995). The protocol resulted in a more than 3000-fold increase in the specific activity of acidocin A.

2.5.2 LACTOCOCCAL BACTERIOCINS

Diplococcin was purified from the supernatant of *Lc. lactis* subsp. *cremoris* 346. The procedure employed included ammonium sulfate precipitation (60% saturation) and cation exchange chromatography on carboxy methyl cellulose (CMC) resulting approximately 1000-fold purification (Davey and Richardson, 1981). Dufour *et al* (1991) purified lactococcin from culture supernatant of *Lc. lactis* subsp. *lactis*, as a single band by dialysis, cation exchange and gel filtration chromatographies. The procedure employed resulted in a 14.5-fold purification with about 3000-fold increase in specific activity.

Ammonium sulfate precipitation of culture supernatant obtained from *Lc. lactis* subsp. *lactis* CNRZ481 resulted in a 455-fold increase in the total lacticin 481 activity. Subsequent purification by gel filtration chromatography and C18 reverse-phase high performance liquid chromatography (HPLC) lead to a 107,506-fold increase in the specific activity of lacticin 481 (Piard *et al.*, 1992). Holo *et al* (1991) purified lactococcin A with about 2300-fold purification and a yield of 16% by a sequential protocol including ammonium sulfate precipitation, cation exchange

chromatography and reverse-phase HPLC. Lactococcin G was similarly purified to homogeneity by a four step protocol which included ammonium sulfate precipitation, binding to a cation exchanger and octyl-sepharose CL-4B and reversed-phase chromatography leading to a recovery of about 20% of the original activity and a 7000-fold increase in specific activity (Nissen-Meyer *et al.*,1992). The bacteriocin diacetin B produced by *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* UL720 was purified by a pH dependent adsorption-desorption procedure followed by a reverse-phase HPLC with a yield of just 1.25% of the original activity (Ali *et al.*, 1995).

2.5.3 LEUCONOSTOCS BACTERIOCINS

Leucocin A-UAL187 from *Leu. gelidum* UAL-187 was purified by ammonium sulfate precipitation followed by a sequential hydrophobic interaction, gel filtration and reverse-phase HPLC with a yield of 58% of the original activity and a purification fold of 4500 (Hastings *et al.*, 1991). Hechard *et al* (1992) employed a three step protocol for the purification of mesentericin Y105. The protocol included affinity chromatography on a blue agarose column, ultrafiltration through a 5-KDa cut off membrane and finally reverse-phase HPLC on a C4 column. The purification procedure resulted in a very low yield of 0.7% with a purification fold of about 420. The purification procedure consisting of ammonium sulfate precipitation, and a sequential gel filtration, cation exchange and hydrophobic interaction chromatography resulted in a satisfactory increase of specific activity (1,135-fold) but a very low recovery of 8% of mesenterocin 52 produced by *Leu. mesenteroides* FR52 (Sudirman *et al.*, 1994). Keppler *et al* (1994) reported the purification to homogeneity of carnocin LA54A by single step hydrophobic interaction chromatography using

amberlite XAD-2. Revol-Junelles and Lefebvre (1996) reported the purification of dextrancin J24 to homogeneity by desorbing the bacteriocin from the producer cells at pH 2.0 followed by a reverse-phase HPLC.

2.5.4 PEDIOCOCCAL BACTERIOCINS

Pediocin AcH from the culture supernatant of *P. acidilactici* H was purified by ammonium sulfate precipitation (70% saturation), fast protein liquid chromatography (FPLC), gel filtration and anion exchange chromatography leading to a 98.8-fold purification with a single protein band on SDS-PAGE gel (Bhunia *et al.*, 1988). Yang *et al* (1992) reported the purification of pediocin AcH to homogeneity as revealed by a single sharp band on SDS-PAGE gel by a pH dependent adsorption/desorption procedure. Pediocin AcH was adsorbed to the producer cells at a pH of 6.0-6.5, centrifuged, the bacteriocin adsorbed onto the cells was extracted at a low pH of 1.5-2.0. The purification protocol resulted in the recovery of almost all the bacteriocin produced. Henderson *et al* (1992) reported a 470-fold purification of pediocin PA-1 by gel filtration, ion-exchange chromatography, dialysis and HPLC, whereas Lozano *et al* (1992) achieved a 80,000-fold increase in specific activity of pediocin PA-1 by employing ammonium sulfate precipitation, chromatography with a cation exchanger and octyl sepharose and reverse-phase HPLC.

Daba *et al* (1994) employed the pH dependent adsorption/desorption procedure developed by Yang *et al* (1992) for the recovery of pediocin 5 produced by *P. acidilactici* UL5. The procedure resulted in a partial recovery of the cell associated bacteriocin fraction and

even longer desorption times exceeding 24 hr could not result in the recovery of more than 10% of the original activity. Further purification to homogeneity was however, achieved by reverse-phase HPLC (Daba *et al.*, 1994).

Schved *et al* (1993) reported a 262-fold purification with a recovery of 50% of pediocin SJ-1 by the direct application of cell free supernatant containing crude bacteriocin to a cation exchange chromatography column. The homogeneity of pediocin SJ-1 thus purified was confirmed by SDS-PAGE. Cintas *et al* (1995) purified pediocin L50 to homogeneity by ammonium sulfate precipitation, and sequential cation exchange, hydrophobic interaction and reverse-phase chromatographies resulting in the recovery of more than 80% of the starting material with a 114, 112-fold increase in specific activity.

2.6 GENETIC DETERMINANTS OF BACTERIOCIN PRODUCTION AND IMMUNITY

An understanding of the genetic control for bacteriocin production and host immunity might be beneficial for their effective use. This is also necessary for cloning and sequencing of genes involved, and application of genetic methods for the construction and improvement of bacteriocin producing strains of LAB.

The original criteria laid down for bacteriocins specify the plasmid borne genetic determinants of bacteriocin production and host cell immunity (Tagg *et al.*, 1976). Although most of the bacteriocins of lactic acid bacteria analysed to date adhere to this criterion, a very few, especially those produced by lactobacilli, have been found to have chromosomal borne genetic

determinants (Barefoot and Klaenhammer, 1983; Joerger and Klaeanhammer, 1986; Thompson *et al.*, 1996). The bacteriocin immunity genes are generally borne on the plasmids that encode bacteriocin production. However, bacteriocin plasmids that do not carry immunity genes have also been found in lactic acid bacteria (Gonzalez and Kunka, 1987; Schved *et al.*, 1993; Kanatani and Oshimura, 1994).

There are reports dealing with a single plasmid (p9B4-6) encoding the production of several bacteriocins (lactococcins A, B and M) and corresponding immunity proteins (van Belkum *et al.*, 1991a; 1992), separate plasmids ranging in size from 55 to 131 Kb carried in different strains and subspecies encoding the same bacteriocin, lactococcin A (Holo *et al.*, 1991; van Belkum *et al.*, 1991a; Stoddard *et al.*, 1992).

2.6.1 LACTOBACILLI BACTERIOCINS

Joerger and Klaenhammer (1986) reported the ineffectiveness of ethidium bromide, acriflavine and novobiocin in yielding variants lacking the production of helveticin J. *Lactobacillus helveticus* 481 was shown to harbour a single 8 MDa plasmid pMJ1008. Isolates cured of pMJ1008 were phenotypically identical to plasmid bearing cells in fermentation patterns, helveticin J production and immunity spectra providing the evidence for a chromosomal location of helveticin J activity and host immunity to helveticin J (Joerger and Klaenhammer, 1986). Similarly, genetic determinants for bacteriocin production in *Lb. helveticus* 1829 (Vaughan *et al.*, 1992) and *Lb. helveticus* CNRZ450 (Thompson *et al.*, 1996) were found to be located on chromosomal DNA.

Bacteriocin production and host immunity in the strains of *Lb. sake* have been reported to be encoded on extrachromosomal genetic elements. Schillinger and Lucke (1989) obtained a Bac$^-$ mutant which also became sensitive to the bacteriocin sakacin A after treatment of *Lb. sake* 706 with acriflavine and reported that a 27.7 Kb plasmid was associated with sakacin A production and immunity of host to sakacin A. However, later it was found that the structural gene for sakacin A was localised on a 60 Kb plasmid present in *Lb. sake* 706 (Holck *et al.*, 1992). Mortvedt and Nes (1990) observed the spontaneous appearance with high frequency of non-bacteriocin producing variants of lactocin S producing *Lb. sake* L45 grown in liquid broth. Genetic experiments suggested that a highly unstable 50 Kb plasmid, pCIM1, was involved in the production of bacteriocin and in immunity to the bacteriocin (Mortvedt and Nes, 1990).

Daeschel *et al* (1990) reported that attempts with plasmid curing agents such as ethidium bromide, acridine orange and novobiocin were not successful in the isolation of Bac$^-$ variants of *Lb. plantarum* C-11. However, they were successful in obtaining Bac$^-$ variants from a chemostat culture of *Lb. plantarum* C-11. The plasmid profile of both Bac$^+$ and Bac$^-$ variants remained identical revealing that the plasmid DNA was not involved in the bacteriocin production. The Bac$^-$ variants were not sensitive to the bacteriocin. *Lactobacillus plantarum* LPC010 grown in MRS broth containing novobiocin (0.125 to 0.5 µg/ml) resulted in the isolation of bacteriocin deficient variants that were still immune to both plantaricins S and T and it was also reported that the determinants for bacteriocin production did not appear to be plasmid encoded (Jimenez-Diaz *et al.*, 1993). Kanatani and Oshimura (1994) reported the isolation of non-bacteriocin producing mutants of *Lb. plantarum* CTF154

after treatment with acriflavine. The genetic determinants for plantacin 154 production in this strain were found to be encoded on a 9.5 MDa plasmid. However, the genes responsible for host immunity in *Lb. plantarum* CTF154 existed in chromosomal DNA as the plasmid free Bac$^-$ variants still retained their immunity to plantacin 154 (Kanatani and Oshimura, 1994).

Kelly *et al* (1996) obtained derivatives of *Lb. plantarum* KW30 that no longer produced bacteriocin, plantaricin KW30 but still retained immunity to it. The identical plasmid profiles of the parent and cured derivatives suggested a chromosomal location for the genes for bacteriocin production in *Lb. plantarum* KW30.

Lactobacillus acidophilus N2 was found to be plasmid free suggesting chromosomal determinants for lactacin B production and host immunity to the bacteriocin (Barefoot and Klaenhammer, 1983). Kanatani *et al* (1995) reported that genetic determinants for acidocin A production in *Lb. acidophilus* TK9201 and the immunity of the host to acidocin A were located on a 45-Kb plasmid, pLA9201. Vossen *et al* (1994) observed the loss of a 14-Kb plasmid (pCV461) in *Lb. acidophilus* M46 derivatives which were cured of acidocin B production. The Bac$^-$ derivatives, however, retained immunity to acidocin B.

Conjugation experiments showed that a 58-62 Kb plasmid was responsible for lactacin F production and host immunity in *Lb. johnsonii* VP11088 (Muriana and Klaenhammer, 1987). Klaenhammer (1993) reported that the plasmid encoding Laf$^-$ Imm$^+$ appeared to represent a transient state for an episome that is conjugally transfered in *Lb. johnsonii* VPI11088. Treatment of *Lb. brevis* SB27 with novobiocin resulted in the generation of

a non-bacteriocin producing mutant which was also sensitive to the bacteriocin brevicin 27 and a plasmid of about 3 MDa was reported to carry genes for both brevicin 27 activity and host immunity to brevicin 27 (Benoit *et al.*, 1994).

2.6.2 LACTOCOCCAL BACTERIOCINS

The genetic basis for nisin production has eluded researchers for many years. Initially, several workers reported that the production of nisin was associated with extrachromosomal DNA (Gonzalez and Kunka, 1985; Steele and McKay, 1986; Tsai and Sandine, 1987; Kaletta and Entian, 1989) as well as chromosomal DNA (Buchman *et al.*, 1988; Dodd *et al.*, 1990; Steen *et al.*, 1990). However, in recent years it has been conclusively proved that the structural gene for nisin production was located on a 70 Kb conjugative transposon (Horn *et al.*, 1991; Rauch and devos, 1992).

Neve *et al* (1984) reported that bacteriocin production in *Lc. lactis* subsp. *cremoris* 9B4 was plasmid encoded as evidenced by conjugal transfer and by analysis of cured bacteriocin negative mutants of donor strain and transconjugants. All the transconjugants acquired a 60 Kb plasmid from the donor strain 9B4 while the Bac^- variants obtained by incubation at an elevated temperature of parent and transconjugants were found to be deprived of 60 Kb plasmid (Neve *et al.*, 1984). Later it was found that the bacteriocin plasmid p9B4-6 of *Lc. lactis* subsp. *cremoris* 9B4 carried the genes for production of and immunity to lactococcins A and M (van Belkum *et al.*, 1991a) and lactococcin B (van Belkum *et al.*, 1992). By the use of a synthetic DNA probe, lactococcin A production in *Lc. lactis* subsp. *cremoris*

LMG2130 was found to be located on 55 Kb plasmid (Holo *et al.*, 1991). The cured variant obtained after treatment with novobiocin at 38°C was found to be deprived of the 55 Kb plasmid. Genes for diplococcin production and immunity in *Lc. lactis* subsp. *cremoris* 346 were found to be located on a 54 MDa conjugative plasmid (Davey, 1984).

Acriflavine, acridine orange and ethidium bromide were found to be ineffective in yielding Bac$^-$ mutants of *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* WM4. However, treatment with novobiocin resulted in one colony with Bac$^-$ phenotype (Scherwitz *et al.*, 1983). The analysis of plasmid content of parent and cured variants and conjugation experiments provided evidence for an 88 MDa plasmid linked bacteriocin production in the strain WM4.

Morgan *et al* (1995) reported the isolation of Bac$^-$ mutants of *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* DPC938 after growing it in the presence of acridine orange (20 µg/ml). Bac$^-$ mutants had lost immunity to the bacteriocins. The bacteriocin production and immunity were found to be associated with a 72 Kb plasmid pSM72.

Dufour *et al* (1991) observed the appearance of Bac$^-$ mutants that had also lost immunity after prolonged incubation for 96-480 hr of lactococcin producing *Lc. lactis* subsp. *lactis* ADRIA 85L030 and reported that a 70 Kb plasmid pOS5 encoded gene for lactococcin production and immunity. It was reported that lactococcin production and immunity were associated with a 2 MDa plasmid in *Lc. lactis* subsp. *lactis* 484 (Gupta and Batish, 1992). Novobiocin treatment of *Lc. lactis* subsp. *lactis* CNRZ481 resulted in the appearance of variants cured of bacteriocin production and immunity and both the

phenotypes were found to be associated with a 69 Kb plasmid (Piard *et al.*, 1993).

2.6.3 LEUCONOSTOCS BACTERIOCINS

The growth of *Leu. gelidum* UAL-187 in APT broth containing novobiocin (5 µg/ml) and SDS (0.002%) resulted in the generation of cured variants that have lost both bacteriocin production and immunity to the bacteriocin and a plasmid of 7.6 MDa was found to carry the genes responsible for leucocin A-UAL187 production and host immunity (Hastings and Stiles, 1991). The bacteriocin production in *Leu. carnosum* Talla was linked to an 8.9 MDa plasmid (Felix *et al.*, 1994). Sudirman *et al* (1994) obtained Bac$^-$ mutants of mesenterocin 52 producing *Leu. mesenteroides* subsp. *mesenteroides* FR52 after treatment with novobiocin (15-25 µg/ml). Some of the Bac$^-$ mutants became sensitive to the bacteriocin while others retained their immunity to mesenterocin 52. Analysis of the plasmid profiles revealed the loss of several plasmids in both types of Bac- mutants thus failing to assign a specific plasmid(s) to be responsible for bacteriocin production and host immunity.

2.6.4 PEDIOCOCCAL BACTERIOCINS

Graham and Mckay (1985) reported that acriflavine and acridine orange were ineffective in yielding Bac$^-$ variants of *P. cerevesiae* FBB63. However, treatment with novobiocin (75 µg/ml) did result in the isolation of variants that have lost bacteriocin production. The authors also provided physical evidence linking a 10.5 MDa plasmid in *P. cerevesiae* to bacteriocin production. Isolation of Bac$^-$ mutants of *P. pentosaceus* FBB61 was reported after propagation of the cultures at 42°C in a

glucose limited chemostat culture operating at a rate of $0.2h^{-1}$ (Daeschel and Klaenhammer, 1985). Bac^- variants of *P. pentosaceus* FBB61 have also lost immunity to the bacteriocin. A 13.6 MDa plasmid was found to carry the genetic determinants for bacteriocin production and immunity to the bacteriocin in *P. pentosaceus* FBB61. Hoover *et al* (1988) employed acriflavine and acridine orange in the curing experiments and reported the association of 5.5 MDa plasmid with bacteriocin production in *P. pentosaceus* MC03.

Growth at elevated temperature (42°C to 45°C) of the bacteriocin producing strains of *P. acidilactici* was employed by Gonzalez and Kunka (1987)and Jager and Harlander (1992), while Hoover *et al* (1988) generated Bac^- mutants after treatment with acriflavine and acridine orange. Gonzalez and Kunka (1987) reported the association of a 6.2 MDa plasmid with bacteriocin production but not host immunity in *P. acidilactici* PAC1.0. Bateriocin production in *P. acidilactici* strains P02, B5627 (Hoover *et al.*, 1988) and PC (Hoover *et al.*, 1988; Jager and Harlander, 1992) was reported to be encoded on a 5.5 Mda plasmid.

Ray *et al* (1989a) observed the spontaneous appearance of Bac^- variants of *P. acidilactici* H and reported that the occurrence of Bac^- variants could be increased after treatment of *P. acidilactici* H with chemical curing agents such as acriflavine, ethidium bromide and novobiocin or growth at an elevated temperature. In contrast to pediocin PA-1, the genes for pediocin AcH production and immunity were located on a 7.4 MDa plasmid pSMB74 (Ray *et al.*, 1989a). The conjugal transfer of pSMB74 to a Bac^- Bac^s recipient strain of *P. acidilactici* produced Bac^+ Bac^r strain that harbored

pSMB74 providing genetic evidence for plasmid encoded bacteriocin activity and immunity in *P. acidilactici* H (Ray *et al.*, 1989b).

Daba *et al* (1991) reported that high temperature (50°C) incubation failed to yield bacteriocin- negative mutants of *P. acidilactici* UL5. However, treatment with acriflavine (15 µg/ml) resulted in the isolation of a Bac$^-$ mutant which was not inhibited by pediocin 5, the bacteriocin produced by parent strain. Growth of *P. acidilactici* SJ-1 at an elevated temperature or in the presence of acriflavine yielded variants deficient in bacteriocin production. The genes for pediocin SJ-1 production but not host immunity in *P. acidilactici* SJ-1 were located on a 4.6 MDa plasmid (Schved *et al.*, 1993).

Chapter 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 ISOLATION OF PEDIOCOCCAL CULTURES

3.1.1 COLLECTION OF SAMPLES

A total number of 25 samples of different milk and milk products comprising 15 cheddar cheese, 3 *dahi*, 2 cream and 5 buffalo milk obtained from the Institute's Cattle Yard, Experimental Dairy and a local cheese manufacturing plant were collected in sterile 125 ml capacity sample bottles after taking appropriate precautions.

3.1.2 CULTURE MEDIUM

Rogosa (SL) Agar (Rogosa *et al.*, 1951) was employed for the isolation of pediococcal cultures from various milk and milk products.

3.1.2.1 Composition

Tryptone	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Potassium dihydrogen phosphate	6.0 g
Sodium acetate	25.0 g
Ammonium citrate	2.0 g
Magnesium sulfate. 7 H_2O	0.1 g
Manganese sulfate. 2 H_2O	0.12 g
Ferrous sulfate. 7 H_2O	0.034 g
Glacial acetic acid	1.32 ml
Tween 80	1.0 ml

Agar	20.0 g
Distilled water	1000 ml

pH 5.4 ± 0.2

3.1.2.2 Preparation of Medium

Dissolved the ingredients in cold distilled water. Heated to boiling to dissolve the ingredients completely. Added 1.32 ml of glacial acetic acid; mixed thoroughly and distributed into culture flasks. Heated to 90-100°C for 2-3 minutes. The medium should not be autoclaved.

3.1.3 PROCEDURE

Aseptically weighed 11 g of *Dahi*, cream and buffalo milk samples were transferred to 99 ml of sterile normal saline (0.85% sodium chloride) (warmed to 45°C for cream) and mixed well.

Cheese samples were prepared by transferring 11 g of aseptically weighed samples to 99 ml sterile 2% sodium citrate solution at 45 to 50°C and homogenised for 3 minutes using a Sorvall Omni-mixer.

In all the cases serial dilutions were subsequently prepared in sterile normal saline. Appropriate dilutions of the samples were plated out on Rogosa (SL) acetate agar supplemented with 100 mg / lit. of cycloheximide and the plates were incubated at 30°C for 3-5 days. Typical colonies were picked up randomly and transferred to MRS broth (de Man *et al.*, 1960) for microscopic examination and gas production subsequently.

3.2 BACTERIAL CULTURES

Bacterial cultures employed in this study, their source, growth media and maintenance are as given in Table 3.1.

3.3 COMPOSITION OF DIFFERENT CULTURE MEDIA

3.3.1 MRS MEDIUM (de Man *et al.*, 1960)*

Peptone	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Dipotassium hydrogen phosphate	2.0 g
Sodium acetate	5.0 g
Ammonium citrate	2.0 g
Magnesium sulfate	0.1 g
Manganese sulfate	0.05 g
Tween 80	1.0 g
Distilled water	1000 ml

pH 6.5 ± 0.2

3.3.2 ELLIKER MEDIUM (Elliker *et al.*, 1956)*

Tryptone	20.0 g
Yeast Extract	5.0 g
Gelatin	2.5 g
Sodium chloride	4.0 g
Dextrose	5.0 g
Lactose	5.0 g
Sucrose	5.0 g
Sodium acetate	1.5 g
Ascorbic acid	0.5 g
Distilled water	1000 ml

pH 6.8 ± 0.2

* Dehydrated media procured from various commercial sources such as Hi-Media, India; Difco, USA; BBL, USA; Biokar, France.

Table 3.1 Bacterial Cultures: Source, Growth and Maintenance

Culture	*Source*	*Growth Medium (Broth, Soft Agar & Agar)*	*Maintenance (at 5°C)*
Lactic culture			
Lb. plantarum NCDO955	Dr. Bibek Ray, University of Wyoming, USA	TGE	TGE Slants
Leu. mesenteroides LY			
P. acidilactici LB42			
P. acidilactici NCIM2292	National Chemical Laboratories, Pune	MRS/TGE	Yeast dextrose milk
P. pentosaceus NCIM2296			
P. cerevesiae NCIM2171			
Lc. lactis subsp. *lactis* NCDC91, 94, 96	National Collection of Dairy Cultures (NCDC), National Dairy Research Institute (NDRI), Karnal	Elliker	Chalk litmus milk
Lc. lactis subsp. *cremoris* NCDC82, 83, 86			
Lc. lactis subsp. *lactis* biovar. *diacetylactis* NCDC60, 61, 64			

Lb. acidophilus NCDC13, 14, 15			Chalk litmus milk
Lb. plantarum NCDC20, 21, 22			
Lb. casei subsp. *casei* NCDC19	NCDC, NDRI, Karnal	MRS	
Lb. delbrueckii subsp. *bulgaricus* NCDC26			
Leu. mesenteroides subsp. *dextranicum* NCDC30, 31, 34, 143	NCDC, NDRI, Karnal	MRS	Yeast Dextrose milk
Non Lactic Cultures:			
Gram Positive:			
L. monocytogenes Scott A, V7	Dr. Bibek Ray, USA		
MTCC657, 1143	Microbial Type Culture Collection, IMTECH, Chandigarh	BHI	BHI slants
L. ivanovii	Dr. Bibek Ray, USA		
L. innocua 111	Dr. Richards, INRA, France		
Enterococcus faecalis MB1	Dr. Bibek Ray, USA	BHI	BHI slants

E. faecalis NCDC116, 122, 119, 124	NCDC, NDRI, Karnal	M17 (Lactose)	Chalk litmus milk
B. cereus NCDC66 *B. subtilis* NCDC70, 71 *B. polymyxa* NCDC68	NCDC, NDRI, Karnal	Nutrient Medium	Nutrient agar slants
Staph. aureus NCDC109, 110			
Gram Negative:			
Escherichia coli NCDC134			Nutrient agar slants
Proteus vulgaris NCDC73 *Pseudomonas aeruginosa* NCDC105 *Salmonella typhi* NCDC113 *Serratia marcescens* NCDC108	NCDC, NDRI, Karnal	Nutrient medium	
E. coli V517 (plasmid DNA marker)	Dr. Bibek Ray, USA	LB	LB agar slants
Pediococcal cultures	Experimental Isolates	MRS/TGE	Yeast dextrose milk

3.3.3 M-17 MEDIUM (Terzaghi and Sandine, 1975)*

Tryptone	5.0 g
Peptone	5.0 g
Yeast Extract	2.5 g
Beef Extract	5.0 g
Lactose	5.0 g
Sodium-β-glycerophosphate	19.0 g
Ascorbic acid	0.5 g
Magnesium sulfate	0.25 g
Distilled water	1000 ml

pH 7.0 ± 0.1

3.3.4 APT MEDIUM (Evans and Niven, 1951)*

Tryptone	12.5 g
Yeast Extract	7.5 g
Sodium chloride	5.0 g
Dextrose	10.0 g
Sodium citrate	5.0 g
Dipotassium hydrogen phosphate	5.0 g
Sodium acetate	5.0 g
Sodium carbonate	1.25 g
Magnesium sulfate. 7 H_2O	0.8 g
Manganese chloride. 4 H_2O	0.14 g
Ferrous sulfate. 7 H_2O	0.04 g
Thiamine hydrochloride	0.001 g
Tween 80	0.2 g
Distilled water	1000 ml

pH 6.7 ± 0.2

3.3.5 TGE MEDIUM (Biswas *et al.*, 1991)

Tryptone	10.0 g
Glucose	10.0 g
Yeast Extract	10.0 g
Magnesium sulfate	0.05 g
Manganese sulfate	0.05 g

Tween 80	2.0 ml
Distilled water	1000 ml

pH 6.5 ± 0.2

3.3.6 TGE BUFFER MEDIUM (Yang and Ray, 1994a)

The TGE medium (3.3.5) was supplemented with dipotassium hydrogen phosphate (0.2%), sodium acetate (0.5%) and sodium citrate (0.5%). The pH of this medium was adjusted to 6.8.

3.3.7 NUTRIENT MEDIUM*

Peptone	5.0 g
Beef Extract	1.5 g
Yeast Extract	1.5 g
Sodium chloride	5.0 g
Distilled water	1000 ml

pH 7.4 ± 0.2

3.3.8 BHI MEDIUM*

Beef Heart, infusion from	250.0 g
Calf Brain, infusion from	200.0 g
Dextrose	2.0 g
Dipotassium hydrogen phosphate	2.5 g
Sodium chloride	5.0 g
Peptone	10.0 g
Distilled water	1000 ml

pH 7.4 ± 0.2

3.3.9 LB (LURIA-BERTANI) MEDIUM (Sambrook *et al.*, 1989)

Tryptone	10.0 g
Yeast Extract	5.0 g
Sodium chloride	10.0 g
Distilled water	1000 ml

pH 7.0

3.3.10 CHALK LITMUS MILK

Skim milk powder	110.0 g
Blue Litmus	5.0 g
Distilled water	1000 ml

A pinch of calcium carbonate was added to each litmus milk test tube.

3.3.11 YEAST DEXTROSE MILK

Skim milk powder	110.0 g
Yeast Extract	3.0 g
Dextrose	10.0 g
Distilled water	1000 ml

3.3.12 LOW GLUCOSE MEDIUM (Felton *et al.*, 1953)

Tryptone	10.0 g
Yeast Extract	5.0 g
Glucose	0.5 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.0 g
Distilled water	1000 ml

pH 6.8

3.3.13 CITRATE UTILISATION MEDIUM (Kempler and McKay, 1980)

3.3.13.1 Basal Medium (Modified)

Peptone	4.0 g
Beef Extract	3.0 g
Yeast Extract	5.0 g
Dextrose	5.0 g
Casein hydrolysate	2.5 g

Sodium acetate	5.0 g
Ascorbic acid	0.5 g

pH 5.8-6.0

3.3.13.2 Potassium ferricyanide- 10% (w/v) in water

3.3.13.3 Ferric citrate and sodium citrate- one gram each in 40 ml water

The basal medium was autoclaved, tempered at 45°C and then supplemented with 10 ml each of solutions 3.3.13.2 and 3.3.13.3 previously steamed (100°C for 30 min.

3.4 SCREENING OF PEDIOCOCCAL CULTURES FOR ANTIBACTERIAL ACTIVITY

Pediococcal cultures isolated in this study were screened for their antibacterial activity against *Lb. plantarum* NCDO955, *Leu. mesenteroides* LY, *P. acidilactici* IB42, *L. monocytogenes* MTCC657 and 1143, *B. cereus* NCDC66, *Staph. aureus* NCDC110 by using agar-spot assay (Fleming *et al.*, 1975), agar-well assay (Barefoot and Klaenhammer, 1983) and agar-disc assay (Ray *et al.*, 1989a).

3.4.1 AGAR-SPOT ASSAY

Pediococcal cultures for spot-inoculation of agar surfaces were grown in MRS broth for 18 hr at 30°C while indicator strains for adding to the soft agar overlay were grown in appropriate broth for 16-18 hr at their optimum growth temperatures. All the cultures were used undiluted in the assay.

The surface of the solidified and dried (one day at 37°C) MRS agar (1·5% agar) plates was spot inoculated with 5 µl of pediococcal cultures. A maximum of three cultures was spotted per dish. The inoculated agar dishes were incubated at 30°C for 18 hr and then overlaid with 5 ml of soft agar (0.8% agar) which had been seeded with 100 µl of indicator culture. A clear zone of 2 mm or greater extending laterally from the border of the *Pediococcus* colonies after incubation for one day at optimum temperature of indicator strains was recorded as positive inhibition.

3.4.2 AGAR WELL AND DISC ASSAYS

Pediococcal cultures were grown in MRS broth for 18 hr at 30°C. Cells were removed by centrifugation at 12,000 rpm for 20 min at 5°C. The culture supernatants thus obtained were sterilised by passing through a 0.22 µm membrane (Gelman Sciences Inc., Michigan, USA) and used in the assays. Alternately, cells were killed by boiling for 3-5 min and heat-killed culture broths were employed.

A fresh culture of indicator bacteria (10 µl) grown for 16-18 hr at their optimum growth temperature was mixed with 5 ml of soft agar, melted and cooled to 45°C, and poured into a petridish containing 20-25 ml of agar medium. The soft agar was allowed to solidify thus generating a potential mat of the indicator bacteria. The plates were refrigerated at 5°C for one hour before several wells were punched out of the agar with the broad end of a sterile Pasteur pipette. The agar buttons were removed and wells were filled with 100 µl of the prepared culture supernatants. The plates were once again refrigerated at 5°C for 3-4 hr to facilitate the diffusion of antibacterial compounds and then were

incubated at appropriate temperature for 18 hr. A clear zone of 1 mm or greater extending laterally from the edge of wells was considered positive inhibition.

In the case of disc assay instead of punching wells, sterile paper discs were placed on refrigerated indicator lawns and 20 µl of culture supernatants were spotted on the discs.

3.5 ENZYME SENSITIVITY OF ANTIBACTERIAL PRINCIPLE

3.5.1 CULTURE BROTHS

The pediococcal cultures showing antibacterial activity were grown in MRS broth at 37°C for 16-18 hr. The culture broths were adjusted to pH 7.0 and boiled for 5 minutes.

3.5.2 ENZYMES

Enzymes used were Trypsin, Chymotrypsin, Ficin, Papain, Proteinase-K, Pronase-E and Catalase (2,00,000 EU/ml).

All the enzymes, were dissolved in sterile 50 mM phosphate buffer (pH 7.0) at a concentration of 10 mg/ml.

3.5.3 PROCEDURE

To 100 µl of culture broths in a microfuge tube, 100 µl of enzyme preparation was added and mixed immediately. The microfuge tubes were allowed to float in a water bath maintained at 37°C for 1-3 hr. The microfuge tubes were then transferred to a boiling water bath for 5 minutes.

The controls consisted of culture broths added with phosphate buffer and enzyme solutions treated in the similar manner.

3.5.4 SPOT-ON-LAWN ASSAY

The loss of the activity of the culture supernatants was tested by spot-on-lawn assay. This assay is essentially the same as agar disc assay (3.4.2) except that 5-10 µl of the culture supernatants were directly applied onto the indicator lawn in stead of filter paper discs and examined for presence or absence of zones of inhibition.

3.6 MEASUREMENT OF ACTIVITY UNITS (AU)

Five microlitres of serial dilutions 10, 20, 30, 40 ...) of the culture broths (3.5.1) were used to determine the highest dilution that produced a 1 mm zone of growth inhibition using the spot-on-lawn assay (3.5.4). The activity units of the culture broths was calculated using the following formula and expressed as activity units per ml:

Activity Units per ml (AU/ml) = 200 X highest dilution that gave a clear zone

3.7 IDENTIFICATION OF PEDIOCOCCAL CULTURES

Apart from morphology, an array of physiological and biochemical tests were performed to identify the pediococcal strains isolated in this study.

3:7.1 PREPARATION OF CULTURES

Pediococcal cultures were grown in TGE buffer broth at 30°C for 18 or 24 hr with an inoculum rate of 1.0%, centrifuged at 12000 rpm for 15 min at 4°C; cells were washed twice with sterile 5 ml phosphate buffer (0.05 M, pH 7.0) and resuspended in the same buffer to the original volume and used as an inoculum for the various tests employed to identify the isolated cultures. An inoculum rate of 1.0% was used unless and otherwise stated.

3.7.2 MORPHOLOGY

The shape, arrangement and size of individual organisms were determined in Gram stained smears prepared from 24 hr old cultures fixed by heat.

3.7.3 PHYSIOLOGICAL AND BIOCHEMICAL TESTS

3.7.3.1 Growth at Different Temperatures

TGE buffer broth tubes were inoculated with the pediococcal cultures and incubated at 15°C for 3 days, 40° and 45°C for 24 hr, and 50°C for one week. Growth was estimated from the turbidity of the culture broths.

3.7.3.2 Growth at Different pH Values

TGE buffer broth tubes adjusted to different pH values, 4.2, 8.5 and 9.6 with either 3 N HCl or 3 N NaOH, were inoculated with pediococcal cultures and incubated at 30°C for 24-72 hr and the growth was assessed by observing the turbidity of the culture broths.

3.7.3.3 Growth in NaCl

TGE buffer broth tubes containing 4%, 6.5%, 15% and 18% NaCl were inoculated with the test cultures, incubated at 30°C for 24-72 hr and growth was visually examined by the turbidity in the culture broths.

3.7.3.4 Gas Production

Gas production by the pediococcal isolates was examined by Hot-Loop test described by Sperber and Swan (1976).

The cultures in the study were grown in HFT broth tubes for 24 hr at 30°C. A heated (red hot) inoculating loop was plunged immediately into the culture tube. Evolution of a stream of bubbles or copius amounts of gas was taken as positive reaction for gas production by test culture. Cultures giving a negative reaction at the end of 24 hr incubation were retested after additional 24 hrs.

3.7.3.5 Reaction in Litmus Milk

Litmus milk tubes without added calcium carbonate were inoculated with pediococcal cultures, incubated at 30°C, examined for reduction and curdling every day for 7 days.

3.7.3.6 Arginine Hydrolysis

TGE buffer broth, without citrate and glucose, supplemented with 0.3% arginine monohydrochloride was inoculated with pediococcal cultures incubated at 30°C for 48 hr. The production of ammonia from arginine was detected by adding Nessler's reagent (Hi Media

Laboratories, Bombay) to culture broth in a ratio of 1:1. The development of an orange or a brick-red colour constituted the positive reaction.

3.7.3.7 Catalase and Pseudocatalase Activity

Catalase and pseudocatalase activities of pediococcal cultures were tested by the method of Felton *et al* (1953).

3.7.3.7.1 Catalase Activity: Catalase activity was tested by streaking pediococcal cultures on APT agar and incubating the plates at 30°C for 24 hr. After incubation, the plates were flooded with 5 ml of 3% Hydrogen peroxide (10 vol.) and observed upto 30 min for the presence of visible gas bubbles which was considered a positive reaction.

3.7.3.7.2 Pseudocatalase Activity: The procedure is same as above except that a medium with low glucose (0.05%) was employed for streaking pediococcal cultures.

3.7.3.8 Carbohydrate Fermentation

The ability of pediococcal cultures to ferment and produce acid from various sugars was tested in a basal agar medium (TGE buffer agar without glucose) containing 0.004% bromocresol purple as an indicator. Carbohydrates tested were: glucose, galactose, fructose, mannose, ribose, xylose, arabinose, maltose, lactose, sucrose, rhamnose, salicin, mannitol, dulcitol and sorbitol.

The dried basal medium agar plates were overlaid with 5 ml of the same soft agar seeded with 100 µl of pediococcal cultures. Carbohydrate differentiation discs

(Hi Media Laboratories, Bombay) were placed on the culture lawns and the plates were incubated at 30°C and examined upto 5 days. The presence of yellowish zones around the discs indicated the fermentation of the carbohydrate with acid production.

Alternately, the basal agar medium was supplemented with membrane filtered carbohydrates at 1% level. The dried agar surface was spotted with 2 µl of pediococcal cultures and the observations were recorded as above.

3.7.3.9 Citrate Utilisation

Citrate utilisation by the pediococcal cultures was studied on a modified medium of Kempler and McKay (1980).

The basal agar medium supplemented with potassium ferricyanide and ferric and sodium citrates was poured and the plates were dried at 37°C for 24 hr. A loopful of the pediococcal culture was streaked on to the dried surface and plates were incubated at 30°C for 48 hr. The appearance of prussian blue colonies was taken as positive for citrate utilisation.

3.8 OPTIMISATION OF CONDITIONS FOR BACTERIOCIN PRODUCTION

The inoculum for production studies was prepared as given under section 3.7.1 and used at 1% level.

3.8.1 MEDIUM

TGE, MRS, APT and Elliker broth flasks inoculated with the culture were incubated at 37°C.

3.8.2 INITIAL pH

MRS broth adjusted to various initial pH values (4.0, 5.0, 6.0, 6.5, 7.0 and 8.0) was inoculated and incubated at 37°C.

3.8.3 TEMPERATURE

MRS broth inoculated with the producer strain was incubated at 25, 30, 37 and 45°C.

In all the above experiments, samples were drawn at 0, 8, 16 and 24 hr intervals and pH, O.D. (600 nm) and bacteriocin activity units were determined.

3.8.4 INCUBATION TIME

MRS broth with an initial pH 6.5 was inoculated at 1% level and incubated at 37°C. Samples drawn at regular intervals upto 72 hr were assayed for pH, O.D (600 nm), viable cell counts and bacteriocin activity units.

3.9 PROTEIN ESTIMATION

Protein content of samples was estimated by the method of Lowry *et al* (1951) using the bovine serum albumin (BSA) as the standard (Fig. 3.1).

3.10 DETERMINATION OF SPECIFIC ACTIVITY

Specific activity of the bacteriocin preparation was defined as activity units of the bacteriocin per milligram of protein.

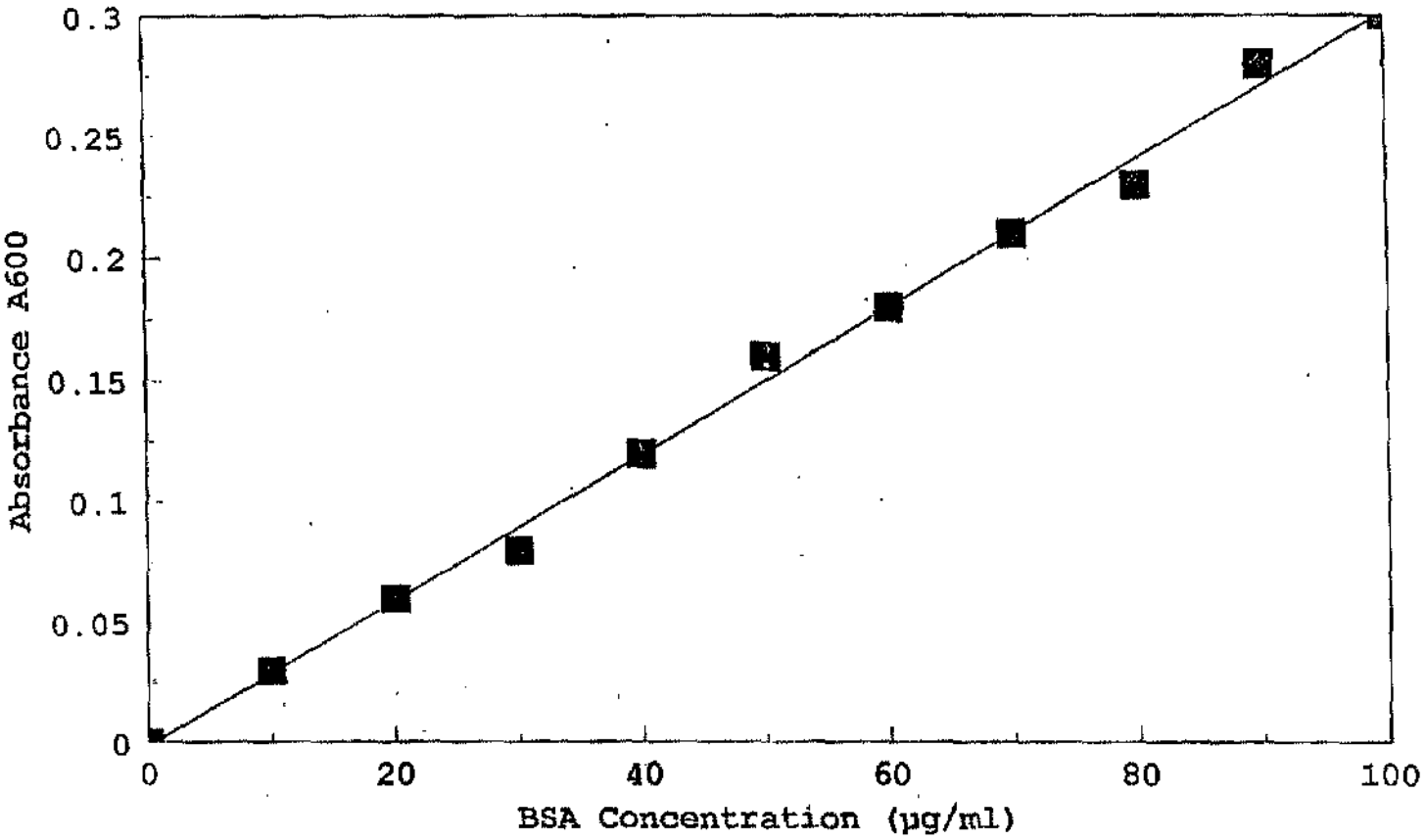


Fig. 3.1 Standard curve for protein estimation

3.11 PURIFICATION OF THE BACTERIOCIN

3.11.1 PURIFICATION OF THE BACTERIOCIN BY PH DEPENDENT ADSORPTION-DESORPTION METHOD

The purification of the bacteriocin was attempted by using the pH dependent adsorption-desorption method developed by Yang *et al* (1992). The schematic chart of the protocol is given in Fig. 3.2.

3.11.2 PRECIPITATION WITH ORGANIC SOLVENTS AND AMMONIUM SULFATE

MRS broth having an initial pH 6.5 was inoculated at 1% level with the overnight grown culture of *P. pentosaceus* 34 and incubated at 37°C for 18 hr. The pH of the grown culture was adjusted to 2.0 and heated at 75°C for 30 min after which it was stored at 5°C for 24 hr. The cells were then removed by centrifugation at 10,000 rpm for 20 min. The supernatant was filter sterilised by passing through a 0.2μ, 45 mm diameter membrane filter and used for purification studies. The bacteriocin activity (3.6) and protein content (3.9) of the supernatant were determined to calculate the specific activity (3.10).

3.11.2.1 Organic Solvents

To 50 ml of the culture supernatant, 50 ml of the chilled organic solvent (acetone, ethanol, isopropanol or methanol) was added at 0°C with constant stirring to obtain the supernatant to solvent ratio of 1:1. The mixture was stirred on magnetic stirrer for another one hour and left in the refrigerator overnight. The precipitate formed was separated by centrifugation and the supernatant obtained was further treated with

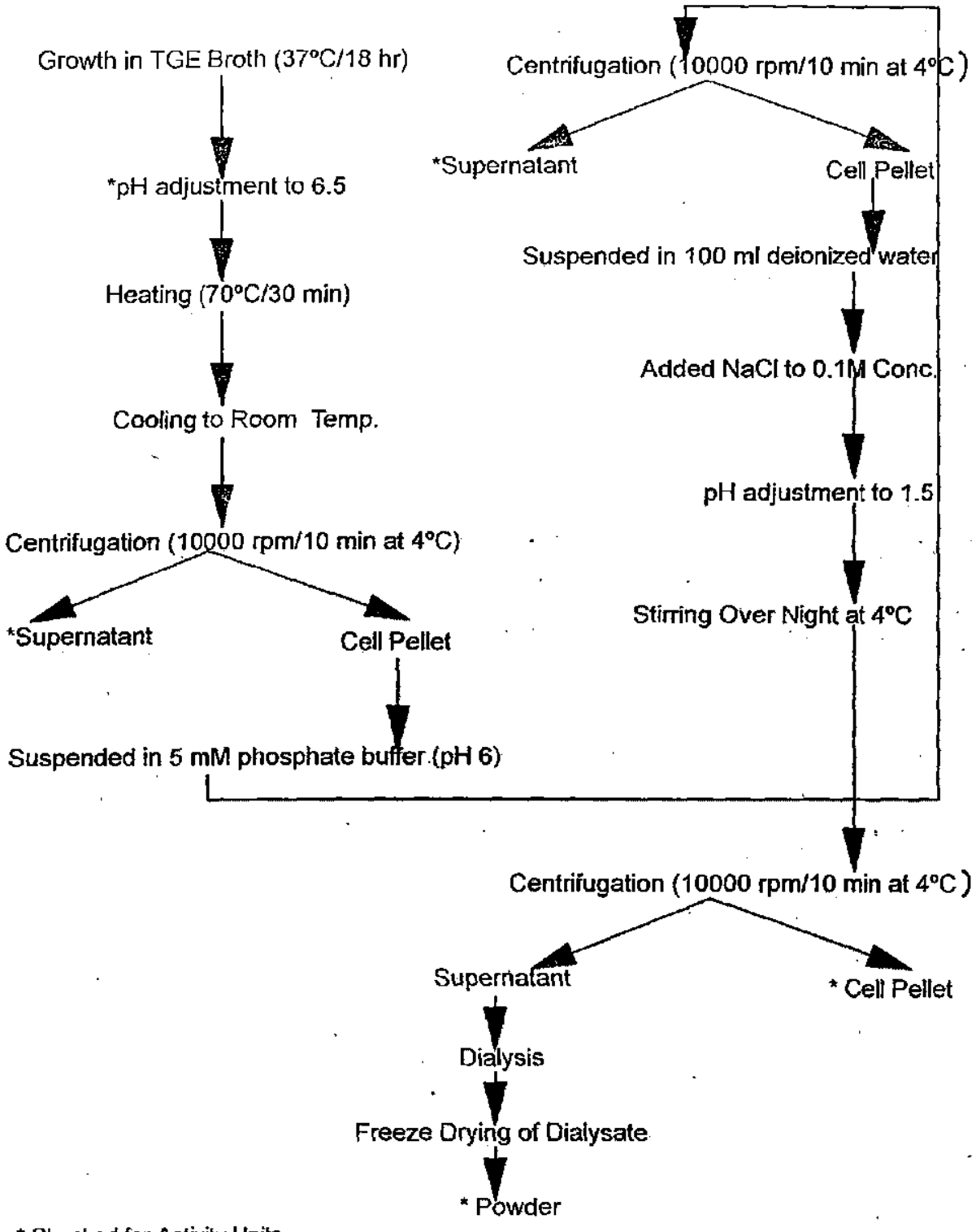


Fig. 3.2 Protocol for the Recovery of *P. pentosaceus* 34 Bacteriocin by pH Dependent Adsorption-Desorption Technique

incremental addition of the organic solvent to obtain the supernatant to solvent ratios of 1:2 and 1:3. The precipitate obtained every time was dissolved in distilled water and tested for protein content and bacteriocin activity.

3.11.2.2 Ammonium Sulfate

To 100 ml of supernatant, ammonium sulfate was added slowly with constant stirring to achieve a 40% saturation and stirring continued for another one hour in a cold room at 5-7°C. The mixture was then kept overnight in the cold room. It was then centrifuged at 16,000 g for 20 min and the precipitate was dissolved in distilled water. The supernatant was subsequently adjusted to 60, 80 and 100% saturation levels by further addition of solid ammonium sulfate. The pellet in each case was dissolved in distilled water.

The bacteriocin solution obtained after each ammonium sulfate fractionation step was dialysed using a 1000 MWCO (molecular weight cut off) cellulose acetate membrane for about 18 hr against distilled water with 3-4 intermittent changes of water.

The specific activity and recovery of the bacteriocin were calculated from the protein content and activity units of the samples.

In a further study, the supernatant was initially adjusted to 30% saturation. The supernatant obtained was further raised to 60% saturation by adding the required amount of ammonium sulfate. The precipitate obtained in both the cases was dissolved in distilled water, dialysed as described above, and the dialysates were assayed for protein content and bacteriocin activity.

3.11.2.2.1 Selection of Buffer:

The bacteriocin preparation from ammonium sulfate precipitation was mixed with 0.2 M acetate buffer (pH 4.0, 4.6, 5.2) or 0.2 M phosphate buffer (pH 5.8 and 6.4) to attain a final ionic strength of 0.1 M. The samples were kept at 5°C and 37°C and assayed for activity units after 24, 48 and 72 hr.

3.11.3 GEL FILTRATION CHROMATOGRAPHY

Sephadex G-25 (medium, particle size 50-150μ) and Sephadex G-50 (fine, particle size 20μ) obtained from Pharmacia Fine Chemicals, Uppasala, Sweden were soaked in 0.1 M acetate buffer (pH 5.2) overnight at room temperature and the fines were decanted off. The gel suspension after deaerating for 10-15 min was packed into a 55 cm X 2.5 cm glass column at the operating pressure head of 30 cm. The void volume of the packed column was determined by using blue dextran B-2000. The column was flushed with 3-4 volumes of the eluting buffer, 0.1 M acetate buffer containing 0.1% SDS and 0.004% sodium azide. The bacteriocin solution was carefully layered over the surface of the gel with the help of a glass pipette and the protein was eluted from the column at a flow rate of 30 ml/hr. Five millilitres fractions, were collected after draining 80% of the void volume and analysed for bacteriocin activity. The protein content of the fractions was monitored by determining O.D. at 280 nm.

3.11.4 ION-EXCHANGE CHROMATOGRAPHY

Further purification of the ammonium sulfate precipitated (30-60% saturation) bacteriocin was attempted by ion-exchange chromatography.

3.11.4.1 Selection of Ion-Exchanger and Starting pH of the Buffer

3.11.4.1.1 Ion-Exchanger:

CM-Sephadex C-25 and DEAE-Sephadex A-25 were used as cation and anion exchangers, respectively.

3.11.4.1.2 Buffers:

Acetate buffers (pH 4, 4.5, 5, 5.5) and phosphate buffers (pH 6, 6.5 and 7) were used for the cation-exchanger while Tris-HCl buffer (pH 7.2, 7.6 and 8) was used for the anion-exchanger.

3.11.4.1.3 Procedure:

Equilibrated 0.1 g ion-exchanger gel in each test tube to a different pH by washing 10 times with 10 ml of 0.5 M buffer followed by washing of the gel 5 X with 10 ml of buffer of the same pH but lower ionic strength (50 mM for cation exchanger and 25 mM for anion exchanger). One ml of the bacteriocin solution (20,000 IU/ml) in the low ionic strength buffer of the same pH was added to each of the tube. Stirred the gel for 5-10 min and allowed it to settle. The supernatant was assayed for the bacteriocin activity (3.6) to determine the amount of bacteriocin bound to the ion-exchanger.

3.11.4.2 Selection of Starting and Eluting Ionic Strength of the buffer

Test tube method was used to determine the starting and eluting ionic strengths to be employed in the subsequent column experiments.

3.11.4.2.1 Procedure:

Equilibrated 0.1 g DEAE-Sephadex A-25 gel in each tube by washing 10 X with 10 ml of 0.5 M Tris-HCl buffer (pH 7.2) followed by equilibration of the gel to different ionic strengths by washing 5 X with 10 ml of the same buffer containing different concentrations of NaCl (0.05 to 0.4 M, 0.05 M intervals). Added 1 ml of the bacteriocin (20,000 IU/ml) sample to the gel and stirred the mixture for 5-10 min. Then allowed the gel to settle and assayed the supernatant for the unbound bacteriocin activity as in 3.6.

3.12 CHARACTERISATION OF THE BACTERIOCIN PRODUCED BY *P. PENTOSACEUS* 34

The crude as well as partially purified bacteriocin preparations were used in the characterisation studies.

3.12.1 CRUDE BACTERIOCIN

P. pentosaceus 34 was grown in MRS broth at 37°C for 16-18 hr. The culture broth was boiled for 3-5 min in a boiling water bath to kill the cells unless or otherwise stated. The bacteriocin concentration in the culture supernatant was estimated to be 78,000 AU/ml.

3.12.2 PARTIALLY PURIFIED BACTERIOCIN

The bacteriocin precipitated with ammonium sulfate (30-60% saturation) was dissolved in deionised water and dialysed against the same overnight, and filter sterilised by passing through 0.22 µm membrane filter. The bacteriocin concentration was 2,00,000 AU/ml.

3.12.3 INHIBITORY SPECTRUM

The antibacterial activity against various lactic and non-lactic cultures of partially purified bacteriocin was determined by agar-well assay (3.4.2).

3.12.4 *DETERMINATION OF MOLECULAR WEIGHT BY SDS-PAGE*

The molecular weight of the *P. pediococcus* 34 bacteriocin was determined by the discontinuous procedure of Schagger and von Jagon (1987) with some modifications as given in Technical Bulletin No. MWM-100 of Sigma Chemical Co., USA. The bacteriocin purified by pH dependent adsorption and desorption method was made use in the determination of molecular weight of the bacteriocin.

3.12.4.1 Reagents

3.12.4.1.1 Acrylamide Solution:

Acrylamide	48 g
N, N' Methylene-bis-acrylamide	1.5 g

Dissolved in water to a final volume of 100 ml and the solution was stored for one month in an amber coloured bottle at 4°C. Warmed gently for dissolution after refrigeration.

3.12.4.1.2 Buffer:

Trizma Base	36.34 g
SDS	0.30 g

Dissolved by gentle warming in 60 ml water. Adjusted pH to 8.45 with concentrated HCl. Made up the final volume to 100 ml with water.

3.12.4.1.3 SDS Stock Solution 20%:

SDS	10 g

Dissolved completely in 50 ml water by gentle warming. Stored at room temperature.

3.12.4.1.4 Tris-HCl, 1M, pH 6.8:

Trizma Base	12.1 g

Dissolved in 80 ml water. Adjusted pH to 6.8 with concentrated HCl. Made up the final volume to 100 ml with distilled water.

3.12.4.1.5 Sample Buffer:

20% SDS Solution (3.12.4.1.3)	4.0 ml
Glycerol	2.4 ml
2-Mercaptoethanol	0.4 ml
Brilliant Blue G	2.0 ml
Tris-HCl, 1 M, pH 6.8 (3.12.4.1.4)	1.0 ml

Made up the final volume to 20 ml with water. Brilliant blue G served as marker dye.

3.12.4.1.6 Ammonium Persulfate Solution:

Prepared fresh each time by dissolving 100 mg ammonium persulfate in 1 ml of distilled water.

3.12.4.1.7 Anode Buffer:

Trizma Base	121.1 g

Dissolved in 1 lit. water. Adjusted pH to 8.9 with concentrated HCl. Made up the final volume to 5 lit. with distilled water.

3.12.4.1.8 Cathode Buffer:

Trizma Base	12.11 g
Tricine	11.92 g
SDS	1.0 g

Dissolved in 1 lit. water. The pH of the solution was not adjusted but was approximately 8.2.

3.12.4.1.9 Fixative Solution:

Methanol	50 ml
Glacial acetic acid	10 ml

Final volume made upto 100 ml with distilled water.

3.12.4.1.10 Staining Solution:

Brilliant BlueG	50 mg

Dissolved in 200 ml 10% glacial acetic acid. Stirred for 30 min and filtered through a filter paper. Stain was used twice.

3.12.4.1.11 Destaining Solution:

Glacial acetic acid	100 ml

Combined with 900 ml water. The solution was discarded after each use.

3.12.4.2 Sample Preparation

The bacteriocin sample was prepared at a concentration of 1 mg/ml in sample buffer (3.12.4.1.5).

3.12.4.3 Preparation of Molecular Weight Markers Solution

Dissolved the contents in 1 ml of sample buffer (3.12.4.1.5).

3.12.4.4 Treatment of Samples Prior to Electrophoresis

Incubated the sample and molecular weight markers solution for 30 min. at 40°C and then allowed to cool to room temperature.

3.12.4.5 Preparation of Electrophoresis Gels

	Spacer gel solution (10% T, 3% C)*	Separation gel solution (16.5% T, 3% C)
Acrylamide solution (ml) (3.12.4.1.1)	1.525	2.50
Gel buffer (ml) (3.12.4.1.2)	2.50	2.50
Glycerol (ml)	-	0.80
Water (ml)	3.475	1.70

$$*PercentT = \frac{[Acrylamide(g)+Bis\text{-}Acrylamide(g)]X100}{100ml}$$

$$PercentC = \frac{Bis\text{-}Acrylamide(g)X100}{Acrylamide(g)+Bis\text{-}Acrylamide(g)}$$

3.12.4.5.1 Procedure:

- Glass plates were thoroughly cleaned with an acetone-swabbed tissue paper, separated by 0.75 mm teflon spacers and fixed onto the gel casting assembly.

- Inserted an 8 well comb and placed a mark on the glass plate 1 cm below the teeth of the comb. Removed the comb.

- Pipetted the solutions as indicated in 3.12.4.5 and deaerated them.

- Final preparation of the separating gel solution was done by adding 0.1 ml ammonium persulfate (3.12.1.1.6) and 0.01 ml of N,N,N',N'-Tetramethylethylenediamine (TEMED) and the contents were mixed thoroughly by gentle stirring.

- Completed the preparation of spacer gel solution by adding 0.1 ml ammonium persulfate and 0.01 ml TEMED followed by thorough mixing by gentle swirling.

- Quickly poured the separating gel to the mark followed by careful overlaying with spacer gel solution. This operation was completed within 15 min.

- Inserted the well-forming comb into the spacer gel.

- After the gel had set, allowed to equilibrate by leaving it overnight at 4°C.

3.12.4.6 <u>Electrophoresis</u>

- Transferred the gel to the electrophoretic apparatus (Hoeffer, USA) and removed the comb.

- Rinsed well with distilled followed by cathode buffer (3.12.4.1.8)

- Filled the upper chamber with the cathode buffer (3.12.4.1.8) and lower chamber with anode buffer (3.12.4.1.7).

- Loaded 7 µl and 10 µl of the molecular weight markers (3.12.4.4) in Lane A and C, respectively.

- Loaded 10 µl of the treated bacteriocin sample (3.12.4.4) in Lane B and F and 10 µl of the 1:1 diluted sample in Lane D and H.

- The electrophoresis was carried out at a constant current of 30 mA until (approx. 4 hr) the marker dye was within 0.5 cm of the anodic end of the gel.

- Disconnected the power supply and removed the gel carefully. The gel was cut into two halves. One half was stained after fixing in the fixative and the other half was used to detect the bacteriocin activity.

3.12.4.7 <u>Fixing, Staining and Destaining of the Gel</u>

- The first half of the gel containing protein markers was transferred to staining solution (3.12.4.1.10) for 1.5 hr after fixing it in the fixative solution (3.12.4.1.9) for 30 min.

- Transferred the gel to destaining solution (3.12.4.1.11) in the glass tray for 2 hr. After every 30 min, the destaining solution in the tray was replaced with the fresh one.

- The destained gel was observed for the protein bands and photographed using a Kodak film 200 ASA.

3.12.4.8 Detection of the Bacteriocin Activity

- Washed the second half of the gel containing only bacteriocin sample in the two lanes with deionised water for 4 hr. The wash water was replaced every 30 min with the fresh one for effective cleaning of the gel.

- Transferred the washed gel to a dried TGE agar plate.

- Overlayed the gel with 10 ml of TGE soft agar (0.8% agar) containing 50 µl of freshly grown indicator strain *P. acidilactici* LB42.

- Incubated the plate overnight at 30°C and examined the gel for the zone of inhibition.

3.12.4.9 Estimation of Molecular Weight

- The stained gel was superimposed on the gel used to detect the bacteriocin activity.

- Approximate molecular weight of the activity band was estimated by comparing the centre of the zone of the inhibition with the molecular weight markers.

3.12.5 ENZYME SENSITIVITY

3.12.5.1 Enzymes

Proteolytic enzymes used were: Trypsin, Chymotrypsin, Pepsin, Ficin, Papain, Proteinase-K, Pronase-E, Protease I, IV, XIII, XVI and XXIII. The non-proteolytic enzymes included: α-amylase, β-amylase, Lysozyme, RNase-A Lipase and Catalase.

Catalase (2,00,000 EU/ml) was procured from BDH Chemicals Ltd., England while all other enzymes were obtained from Sigma Chemicals Co., USA.

All the enzymes, except pepsin, were dissolved in sterile 50 mM phosphate buffer (pH 7.0) at a concentration of 10 mg/ml, whereas pepsin was dissolved in 0.02 N HCl.

3.12.5.2 Procedure

One hundred microlitres of neutralised crude bacteriocin containing 7800 AU and 100 µl of 1:1 diluted purified bacteriocin containing 10000 AU were used and the rest of the procedure remained same as given in 3.5.3 except that before transferring to the water bath, the pH of the pepsin treated samples was brought to 7 with o.1 N NaOH.

The residual activity of the enzyme treated sample was estimated as given in 3.6.

3.12.5.3 Sensitivity of the Bacteriocin to Protease Inhibitors Treated α-amylase

3.12.5.3.1 Protease Inhibitors:

Two different protease inhibitors viz. phenylmethylsulfonylfluoride (PMSF) and iodoacetamide were used for inhibiting the contaminating protease (if any) in the amylase preparations. One hundred millimole solutions of each inhibitor were prepared fresh by dissolving 17.42 mg of PMSF and 18.5 mg of iodoacetamide in 1 ml of distilled water.

3.12.5.3.2 Procedure:

To 100 µl of α-amylase (10 mg/ml) in phosphate buffer (pH 6) added 15 µl of protease inhibitor(s) (3.12.5.3.1) individually or in combination and after mixing well incubated the contents for 1 hr at 30°C.

The α-amylase treated with protease inhibitors was used to inactivate the purified bacteriocin (3.12.2) as given in 3.5.3.

Controls consisted of protease inhibitors, bacteriocin treated with protease inhibitors, singly or in combination, bacteriocin treated with α-amylase and α-amylase.

3.12.6 HEAT STABILITY

One ml culture broth and one ml of 1:10 diluted partially purified bacteriocin were subjected to different heat treatments viz. 63°C/30 min, 75°C/30 min, 85°C/10 and 15 min, 90°C/10 and 15 min, 100°C (boiling)/5, 10, 15, 30 and 60 min and autoclaving at 121°C/15 min, cooled on ice immediately and assayed for the residual activities.

3.12.7 pH STABILITY

3.12.7.1 Crude Bacteriocin

The heat killed culture broths were adjusted to different pH values ranging from 1-13 with either sterile 3 N NaOH or 3 N HCl and the activity was assayed after 2, 8, 24, 168 and 360 hr of storage at 5°C.

3.12.7.2 Purified Bacteriocin

To 100 µl aliquots of the purified bacteriocin 900 µl of 50 mM buffers of different pH values in the range of 1-10 were added to attain a final bacteriocin concentration of 20,000 AU/ml and the bacteriocin activity was assayed after 24, 168 and 360 hr of storage at 5°C.

The buffers used were: HCl-KCl buffer for pH 1 and 2, glycine HCl buffer for pH 3, acetate buffer for pH 4 and 5, phosphate buffer for pH 6 and 7, Tris-HCl buffer for pH 8 and 9 and glycine-NaOH buffer for pH 10.

3.12.8 EFFECT OF pH ON THE HEAT STABILITY

The crude bacteriocin adjusted to different pH values in the range of 1-13 as in 3.12.7.1 was subjected to two different heat treatments viz. 75°C/30 min and 100°C/10 min, while the purified bacteriocin adjusted to different pH values in the range of 1-10 as in 3.12.7.2 was heated for 10 min at 100°C, cooled on ice immediately and the residual activity of both the preparations was determined after 8 hr of storage at room temperature.

3.12.9 EFFECT OF SURFACTANTS

The ammonium sulfate precipitated, dialysed bacteriocin preparation in distilled water, was mixed with a 2% solutions of Tween 80, Tween 20, Triton X-100 and SDS to attain a final concentration of 1.0% of the surfactants. The mixtures were stored at 5°C. The activity units of the treated samples were determined after 24 hr of storage.

The effect of 0.1% and 0.5% SDS was also studied in the similar manner.

3.12.10 EFFECT OF SODIUM CHLORIDE

One hundred microlitre portions of purified bacteriocin were added to 900 µl of 25 mM Tris-HCl buffer (pH 7.2) aliquots containing 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, and 3 M concentrations of NaCl. The residual activity of the added bacteriocin was assayed after incubation for 24 hr at 5°C.

3.12.11 MODE OF ACTION

The action of the bacteriocin on the resting cells *Lb. plantarum* NCDO955 was determined by the following method.

3.12.11.1 Preparation of the Cells

Lb. plantarum NCDO955 was grown in TGE broth for 16-18 hr at 30°C, centrifuged at 10,000 rpm for 10 min at 5°C, and the cell pellet washed twice with 50 mM phosphate buffer (pH 6) to remove any media ingredients.

3.12.11.2 Procedure

The cell pellet obtained from 1 ml TGE broth as described above was dissolved in 1 ml of 50 mM phosphate buffer (pH 6) containing four different concentrations of bacteriocin viz. 200, 2000, 20000 and 200000 AU, mixed well and the viable cell counts were determined after 30 min, 1, 2 and 4 hr by spreading 0.1 ml of the appropriate dilutions of each sample made in 0.85% sterile saline.

The control sample without bacteriocin and the sample with 2,00,000 units of bacteriocin were also monitored for changes in the optical density values at two different wavelengths viz. 600 and 260 nm after 0, 1, 2 and 4 hr.

3.12.11.3 Bacteriocin Sensitivity of the Survivors

The surviving cells were grown in TGE broth for 24 hr and assessed for bacteriocin sensitivity by spot-on-lawn assay (3.5.4).

3.13 EXTRACTION OF PLASMID DNA

3.13.1 PEDIOCOCCAL CULTURES

Plasmid extraction from the pediococcal isolates was carried out by the method of Anderson and McKay (1983) with slight modification.

3.13.1 1 Reagents

3.13.1.1.1 6.7% Sucrose-50 mM Tris-1 mM EDTA (pH 8.0)*:

Sucrose	6.70 g
Tris	0.606 g
EDTA	0.037 g
Distilled water	to 100 ml

3.13.1.1.2 25 mM Tris - pH 8.0*:

Tris	0.303 g
Distilled water	to 100 ml

3.13.1.1.3 0.25 M EDTA - 50 mM Tris (pH 8.0)*:

EDTA	9.306 g
Tris	0.606 g
Distilled water	to 100 ml

3.13.1.1.4 20% SDS - 50 mM Tris - 20 mM EDTA (pH 8.0)*:

SDS	20.00 g
50 mM Tris	0.606 g
20 mM EDTA	0.744 g
Distilled water	to 100 ml

3.13.1.1.5 3.0 N NaOH:

NaOH	1.20 g
Distilled water	to 10 ml

It was prepared fresh just before use.

3.13.1.1.6 2.0 M Tris - HCl (pH 7.0)*:

Tris	24.23 g (OR)
Tris-HCl	31.52 g
Water	to 100 ml

3.13.1.1.7 5.0 M Sodium Chloride (NaCl)*:

NaCl	29.22 g
Distilled water	to 100 ml

3.13.1.1.8 Phenol saturated with 3% NaCl:

To 300 ml of melted phenol, added an equal volume of 3% NaCl, stirred the mixture on a magnetic stirrer for 30 min. Allowed it to separate in a separating funnel for

30 min, collected the bottom. The whole procedure was repeated twice and the saturated phenol was stored in dark in an amber colour bottle at 4°C when not in use.

3.13.1.1.9 Chloroform: isoamyl alcohol (24:1):

Isoamyl alcohol	4.0 ml
Chloroform	96.0 ml

3.13.1.1.10 10 mM Tris - 1 mM EDTA (pH 7.5)*:

Tris	0.121 g
EDTA	0.037 g
Distilled water	to 100 ml

* Autoclaved at 121°C/15 min.

3.13.1.2 Growth and Harvesting of Cells

- Pediococcal cultures were grown overnight in TGE broth at 37°C.
- Inoculated overnight grown cultures in 10 ml of TGE broth containing 20 mM DL-threonine at the rate of 1.0%.
- Propagated the cultures for 4-5 hr at 37°C
- Harvested the cells by centrifugation at 10,000 rpm for 10 min at 4°C.

3.13.1.3 Lysis Protocol

Step	Details of Protocol
- Resuspended and harvested cells in 6.7% sucrose-50 mM Tris-1 mM EDTA, pH 8.0	379 µl
- Transferred the contents to 1.5 ml Eppendorf tube and warmed at 37°C	20 min

- Added lysozyme (15 mg/ml in 25 mM Tris, pH 8.0)	96.5 µl
- Incubated at 37°C	15 min
- Added 0.25 M EDTA-50 mM Tris, pH 8.0	48.2 µl
- Added sodium dodecyl sulfate (20% W/V in 50 mM Tris-20 mM EDTA, pH 8.0)	27.6 µl
- Mixed immediately	
- Incubated at 37°C to complete lysis	10 min
- Vortexed at highest setting	30 sec
- Added fresh 3.0 N NaOH	27.6 µl
- Mixed the contents gently by intermittent inversion or swirling	10 min
- Added 2.0 M Tris-hydro chloride, pH 7.0	49.6 µl
- Continued gently mixing	3 min
- Added 5.0 M NaCl	71.7 µl
- Added phenol saturated with 3% NaCl, mixed thoroughly	700 µl
- Centrifuged Removed upper phase to a fresh tube	12,000 rpm/5 min
- Added chloroform - iso amyl alcohol (24:1)	700 µl
- Centrifuged Upper phase transferred to a new tube	12,000 rpm/1 min

- Precipitated the DNA with isopropanol	1 Vol.
- Incubated at 0°C	Overnight
- Centrifuged	12,000 rpm/15 min
- Removed excess propanol and pellet resuspended in 10 mM Tris-1 mM EDTA, pH 7.5	20 µl

All the reagents were mixed immediately after addition by vortexing momentarily at low speed, with the exception of 3 N NaOH and the 2.0 M Tris-HCl, pH 7.0. These reagents were mixed by inversion. Centrifugations were performed at room temperature in an Eppendorf centrifuge.

3.13.2 *ESCHERICHIA COLI* V517

Plasmids of known molecular weight from *E. coli* V517 were isolated by Large Scale alkali lysis method described by Sambrook *et al* (1989).

3.13.2.1 Reagents

3.13.2.1.1 STE (0.1 M NaCl-10 mM Tris-1 mM EDTA, pH 8.0):

NaCl	0.584 g
Tris	0.121 g
EDTA	0.037 g
Distilled water	to 100 ml

3.13.2.1.2 Solution I (50 mM glucose-25 mM Tris-10 mM EDTA, pH 8.0):

Glucose	0.901 g
Tris	0.303 g

EDTA	0.372 g
Distilled water	to 100 ml

3.13.2.1.3 Lysozyme buffer (10 mM Tris, pH 8.0):

Tris	0.121 g
Distilled water	to 100 ml

3.13.2.1.4 Solution II (1% SDS in 0.2 N NaOH):

SDS	1.0 g
NaOH	to 100 ml

NaOH was freshly diluted from a 10 N stock.

3.13.2.1.5 Solution III (5 M Potassium acetate-acetic acid-water):

5M	Potassium acetate	60 ml
	Glacial acetic acid	11.5 ml
	Distilled water	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

5 M potassium acetate was prepared by dissolving 49.070 g in water and making the volume to 100 ml.

3.13.2.1.6 TE Buffer (10 mM Tris-1 mM EDTA, pH 8.0):

Tris	0.121 g
EDTA	0.037 g
Distilled water	to 100 ml

3.13.2.2 Growth and Harvesting of the Culture

- Inoculated 500 ml of LB broth with 5 ml of *E. coli* V517 culture grown overnight at 37°C in LB broth.
- Incubated the culture for 18 hr at 37°C with vigorous shaking (300 cycles/min) on a rotary shaker.
- Harvested the cells by centrifugation at 4,000 rpm for 15 min at 4°C.
- Resuspended the bacterial pellet in 100 ml of ice-cold STE.
- Collected bacterial cells by centrifugation at 4,000 rpm for 15 min at 4°C.

3.13.2.3 Lysis of the Cells

The harvested cells were lysed by alkali to extract plasmid DNA.

- Resuspended the washed bacterial pellet in 18 ml of Solution I.
- Added 2 ml of freshly prepared solution of lysozyme (10 mg/ml in lysozyme buffer).
- Added 40 ml of freshly prepared Solution II.
- Closed the bottle, mixed the contents thoroughly by gently inverting the bottle several times and stored the bottle at room temperature for 5-10 min.
- Added 20 ml of ice-cold Solution III.
- Closed the bottle, mixed the contents by shaking the bottle several times, and stored the bottle on ice for 10 min.
- A flocculant white precipitate of chromosomal DNA, high molecular weight RNA, and potassium/SDS/protein/ membrane was observed.
- Centrifuged the bacterial lysate at 4,000 rpm for 15 min at 4°C. Allowed the rotor to stop without braking.

- Filtered the supernatant through four layers of cheese cloth into a 250 ml centrifuge bottle.
- Added 0.6 volume of isopropanol, mixed well and stored the bottle for 10 min at room temperature.
- Recovered the nucleic acids by centrifugation at 5,000 rpm for 15 min at room temperature.
- Decanted off the supernatant carefully, inverted the open bottle to allow the last drops of supernatant to drain away. Rinsed the pellet and the walls of the bottle with 70% ethanol at room temperature. Drained off ethanol and placed the inverted, open bottle on a pad of paper towels for a few minutes at room temperature to allow the final traces of ethanol to evaporate.
- Dissolved the pellet of nucleic acid in 3 ml of TE buffer.

3.14 AGAROSE-GEL ELECTROPHORESIS OF PLASMID DNA

3.14.1 REAGENTS

3.14.1.1 Tris-Acetate-EDTA (TAE) Buffer

3.14.1.1.1 Stock Solution (50 X):

Tris	24.20 g
0.5 M EDTA (pH 8.0)	10.00 ml
Glacial acetic acid	5.71 ml
Distilled water	to 100 ml

pH of the above solution was not adjusted. The solution was sterilised by autoclaving at 121°C/15 min.

3.14.1.1.2 Working Solution (1 X):

Working solution was prepared by diluting 1 ml of stock solution to 50 ml with distilled water.

3.14.1.2 Gel Loading Solution pH 8.0

Bromophenol blue	0.05 g
Sucrose	40.00 g
EDTA	3.72 g
SDS	0.50 g
Distilled water	to 100 ml

3.14.1.3 Staining Solution

3.14.1.3.1 Stock Solution (10 mg/ml):

Ethidium bromide (EtBr) was prepared as a stock solution of 10 mg/ml in water, stored at room temperature in screw cap tubes wrapped in aluminium foil.

3.14.1.3.2 Working Solution (0.5 µg/ml):

Prepared by adding 50 µl of stock solution to 1 lit of distilled water.

3.14.1.4 Agarose Solution (0.7%)

Appropriate quantities of agarose to obtain a concentration of 0.7% was weighed into 1X TAE buffer in an Erlenmeyer flask. The mini gel (7 cm x 10 cm) required about 25-30 ml of agarose solution while it was 80-90 ml for maxi gel (15 cm x 10 cm). Agarose was melted by heating the flask in a microwave oven (BPL Sanyo, India) stopping the oven every 30 seconds and swirling the flask gently. The melted agarose was allowed to cool to 50°C before pouring into the gel tray.

3.14.2 PROCEDURE

Agarose solution cooled to almost 50°C was poured in the gel tray on a levelled surface with comb (8, 12, 15

or 20 teeth) positioned on the walls of the tray so that it was 1-2 mm above the base of the gel tray. Agarose was allowed to harden for 45-60 min at room temperature after which the comb was carefully removed taking care to prevent the whole gel being lifted out of the tray. The gel tray was then transferred to the centre of the electrophoresis buffer tank (Hoefer, USA for mini gel and Biorad, USA for maxi gel) and the tank was filled with 1X TAE buffer until there was a 1-2 mm layer of buffer over the surface of the gel. To each of 20 µl of plasmid DNA sample added was 5 µl of gel loading solution, gently mixed using a micro pipette and then was spinned momentarily. The sample was then loaded into the wells with marker plasmid DNA from *E. coli* V517 being loaded each time in the first well. The electrophoretic run was carried out at 45 V (3.75 v/cm) for 3 hr or 45 V (1.5 v/cm) for 7 hr for mini and maxi gels, respectively by which time the tracking dye, bromophenol blue, had reached the other end. The gels, after the run, were stained in ethidium bromide solution (0.5 µg/ml) for 30 min and briefly destained for 10 min in distilled water. The gels were then viewed under UV-light on a UV-transilluminator (Foto/UV-21, Fotodyne Inc., USA) for the visualisation of DNA bands and photographed using a polaroid camera (MP-ST Photographic System, Fotodyne Inc., USA) loaded with a polaroid type 667 pack film.

3.15 DETERMINATION OF MOLECULAR WEIGHT OF PLASMID DNA

The molecular weight of the plasmids extracted from the test cultures was determined by the method described by Rochelle *et al* (1985).

The distance moved by various plasmid bands on the agarose gels from the centre of the wells was recorded in mm. A standard curve was drawn by plotting the log

values of the molecular weights of marker plasmids from *E. coli* V517 on X-axis and the corresponding log values of distances moved on Y-axis. The molecular weight of the plasmids isolated from the pediococcal cultures was calculated by extrapolation of the log value of the distance moved by the respective plasmid band.

3.16 CURING OF BACTERIOCIN PRODUCTION (Bac$^+$) PHENOTYPE

3.16.1 GROWTH AT AN ELEVATED TEMPERATURE

TGE broth inoculated with the overnight grown parent culture was incubated at 45°C for 3 days.

3.16.2 GROWTH IN THE PRESENCE OF CHEMICAL AGENTS

TGE broth tubes containing different concentrations of various curing agents viz., acridine orange (50-250 µg/ml), acriflavine (10-50 µg/ml), ethidium bromide (10-50 µg/ml) and novobiocin (10-100 µg/ml) were inoculated with the parent culture and incubated at 37°C for 24 hr. The growth in the tubes was visually examined and three consecutive transfers of the parent culture were made in TGE broth containing 30 µg/ml acriflavine, 200 µg/ml acridine orange, 40 µg/ml ethidium bromide and 70µg/ml novobiocin with incubation at 37°C for 24 hr each time.

3.16.3 COMBINATION OF ELEVATED TEMPERATURE OF INCUBATION AND INTERCALATING AGENTS/ANTIBIOTICS

TGE broth tubes containing acriflavine (5-15 µg/ml), ethidium bromide (10-20 µg/ml) and novobiocin (30-50 µg/ml) were inoculated with the parent culture and incubated at 45°C/3 days.

3.16.4 SCREENING FOR BAC⁻ VARIANTS

3.16.4.1 Simultaneous Antagonism

Loopfuls of the culture from each treatment described above were streaked on TGE agar and incubated at 37°C for 24 hr. The colonies developed were stabbed with the help of a sterile tooth pick into the indicator lawn prepared by pouring 5 ml of TGE soft agar on a dried TGE agar surface seeded with the overnight grown indicator culture. The plates were incubated at 37°C overnight and examined for the colonies without zones of inhibition surrounding them and were designated as Bac⁻ variants.

3.16.5 CURING EFFICIENCY

Curing efficiency was calculated by using the formula:

$$\text{Percent Curing Efficiency} = \frac{\text{Number of cured variants}}{\text{Total number of colonies}} \times 100$$

3.16.6 CHARACTERISATION OF BAC⁻ VARIANTS

3.16.6.1 Plasmid Profile

Plasmid DNA was isolated from the Bac⁻ variants by the method of Anderson and McKay (1983) as described in 3.13.1 and the plasmid profile of both the parent and the cured variants was compared to ascertain the genetic elements involved in the bacteriocin production.

3.16.6.2 Sensitivity or Resistance to Bacteriocin

The sensitivity or resistance of Bac- variants to the bacteriocin produced by the parent culture was determined by the agar-well assay described in 3.4.2.

3.16.6.3 Antibiotic Sensitivity

The method employed was essentially the same as that of agar-disc assay described in 3.4.2 except that commercially available discs of different antibiotics (Hi Media, Bombay) were placed on the lawns of Bac^+ strain and Bac^- variants and antibiotic sensitivity was defined as the presence of at least a 2 mm zone of growth inhibition around a disc.

3.16.6.4 Carbohydrate Fermentation

The ability of the Bac^+ strain and Bac^- mutants to ferment and produce acid from various carbohydrates was examined by the method given in 3.7.3.8.

3.17 CONJUGATION STUDIES

3.17.1 DONOR

The bacteriocin producing culture, *P. pentosaceus* 34 (Bac^+ Bac^r Em^s) was used as the donor strain.

3.17.2 RECIPIENT

Plasmid free derivative (Bac^- Bac^- Em^-) of *P. pentosaceus* 34 used as the recipient was prepared as under:

The mutants resistant to erythromycin (20 µg/ml) of plasmid free Bac^- Bac^r Em^s derivative of *P. pentosaceus* 34 were isolated by sequential selection on MRS agar fortified with increasing concentrations of erythromycin.

It was made sure that the donor strain would not grow on the selective agar during conjugation experiments.

3.17.3 PREPARATION OF DONOR AND RECIPIENT STRAINS

The donor strain was grown in MRS broth while the recipient in MRS broth containing erythromycin (20 µg/ml) and the cultures were inoculated at 1% level and incubated at 37°C for 3.5-4.0 hr to grow to an optical density of 0.6 at 600 nm. The donor and the recipient cells were mixed in 1:1 and 1:10 ratios in 1.5 ml microfuge tubes and immediately centrifuged at 10,000 rpm for 5 min. The pellet was washed twice with 1 ml of saline (0.85% NaCl) and resuspended in 100 µl of the same for mating experiments.

3.17.4 SOLID SURFACE MATING

The donor and the recipient cell mixture (100 µl) prepared as above was spread on non-selective agar and incubated at 37°C for 24 hr.

3.17.5 FILTER PAPER MATING

The donor and the recipient mixture was placed on sterile 0.2 µm 25 mm diameter sterile membrane filter in TGE agar plate and the broth was allowed to diffuse through the agar for 1 hr after which the membrane was transferred to a fresh agar plate with upside down and incubated at 37°C for 24 hr.

3.17.6 SCREENING FOR BAC$^+$ TRANSCONJUGANTS

The growth from the above mating experiments was removed into saline; appropriate dilutions made in saline were plated on selective TGE agar containing erythromycin (20 µg/ml). The plates were incubated at 37°C for 48 hr and the developed colonies were screened for bacteriocin production by simultaneous antagonism as described in 3.16.4.1 and the plates were examined for colonies exhibiting zones of inhibition around them.

3.18 EFFECT OF BACTERIOCIN ON THE GROWTH OF *L. MONOCYTOGENES* IN SKIM MILK

The effectiveness of the bacteriocin in controlling the *L. monocytogenes* MTCC657 in sterile skim milk was assessed.

Listeria monocytogenes MTCC657 grown in BHI broth at 37°C for 16-18 hr was centrifuged (10,000 rpm/10 min) in refrigerated centrifuge, washed the pellet thrice with 5 mM sterile phosphate buffer (pH 6) and suspended in the same buffer to the original broth volume. The cells thus prepared were used to inoculate sterile skim milk (121°C/15 min) at the rate of 1%. The bacteriocin solution was added to the skim milk containing *L. monocytogenes* to obtain a final concentration of 200 and 2000 AU/ml. The viable cells of control and experimental samples were estimated by plating on BHI agar after 0, 1, 4, 7, 10 and 15 days of storage at 5°C. The control tube (without bacteriocin) contained only added *L. monocytogenes* MTCC657.

Chapter 4

Results and Dicussion

4. RESULTS AND DISCUSSION

The results obtained in the present investigation on the isolation of pediococci from milk and milk products, screening of pediococcal isolates for bacteriocin activity, optimisation of cultural and environmental parameters for bacteriocin production, partial purification and characterisation of the bacteriocin, genetic determinants encoding bacteriocin production and host cell immunity in the selected pediococcal isolate (*P. pentosaceus* 34) and effectiveness of the bacteriocin in controlling the growth of *L. monocytogenes* in milk are presented in Tables 4.1 to 4.30, Figs. 4.1 to 4.15 and Plates 1 to 18.

4.1 ISOLATION OF PEDIOCOCCI FROM MILK AND MILK PRODUCTS

A total number of 1030 well isolated colonies (Table 4.1) on Rogosa (SL) acetate agar plates obtained by plating 25 different milk and milk products comprising 5 buffalo raw milk, 2 raw cream, 3 dahi and 15 Cheddar cheese samples were transferred to MRS broth, incubated at 37°C and subsequently examined microscopically for shape and arrangement of cells after Gram's staining and gas production. As there is no selective medium available in the literature for the isolation of pediococci, the selective medium for lactobacilli developed by Rogosa *et al* (1951) which also permits the growth of pediococci and leuconostocs was used for the isolation of pediococci from different milk products. Since lactococci and other streptococci do not grow on Rogosa (SL) acetate agar, the gas forming Gram positive cocci and lenticular shaped

Table 4.1 : Distribution of lactic acid bacteria from different dairy products

Product	No. of samples	No. of different lactic acid bacteria			
		Lactobacilli	Leuconostocs	Pediococci	Total
Raw Milk	5	165	30	5	200
Cream	2	60	20	-	80
Dahi	3	135	15	-	150
Cheddar cheese	15	525	35	40	600
Total	25	885	100	45	1030

cells occurring in pairs and chains were counted as leuconostocs and the non-gas forming Gram positive rods were considered as lactobacilli. The Gram positive cocci that appeared in pairs and tetrads (Plate 1) without any gas production were taken to be pediococci. The distribution of lactic acid bacteria comprising lactobacilli, leuconostocs and pediococci isolated from milk and milk products in general and Cheddar cheese at different stages of ripening in particular is shown in Tables 4.1 and 4.2 and Figures 4.1 and 4.2, respectively.

An appraisal of the data recorded in Table 4.1 indicates that the lactobacilli constituted the majority of the lactic acid bacteria examined followed by leuconostocs and pediococci. The respective numbers of lactobacilli, leuconostocs and pediococci isolates were 885, 100 and 45 and similarly the percent distribution for the three genera was 85.9, 9.7 and 4.4.

The percent distribution of lactobacilli, leuconostocs and pediococci in raw milk, cream, dahi and Cheddar cheese is presented in the form of a pie diagram (Fig. 4.1). The percent distribution of lactobacilli was 82.5 (raw milk), 75 (cream), 90 (dahi) and 87.5 (Cheddar cheese). The corresponding values for leuconostocs in the respective products were 15, 25, 10 and 5.8. Although pediococci could not be isolated from cream and dahi under the isolation conditions employed in the present study, they constituted about 2.5% and 6.7% of the total isolates obtained from raw milk and cheddar cheese (Fig. 4.1). It can also be observed from the table (4.1) that out of the total number of 45 pediococcal isolates, 40 isolates (89%) were from 15 Cheddar cheese samples and the remaining 5 isolates (11%) were from 5 raw milk samples.

Plate 1. Photomicrograph showing the shape and arrangement of cells of a pediococcal isolate (X 2000)

(Arrow indicates a typical tetrad pattern)

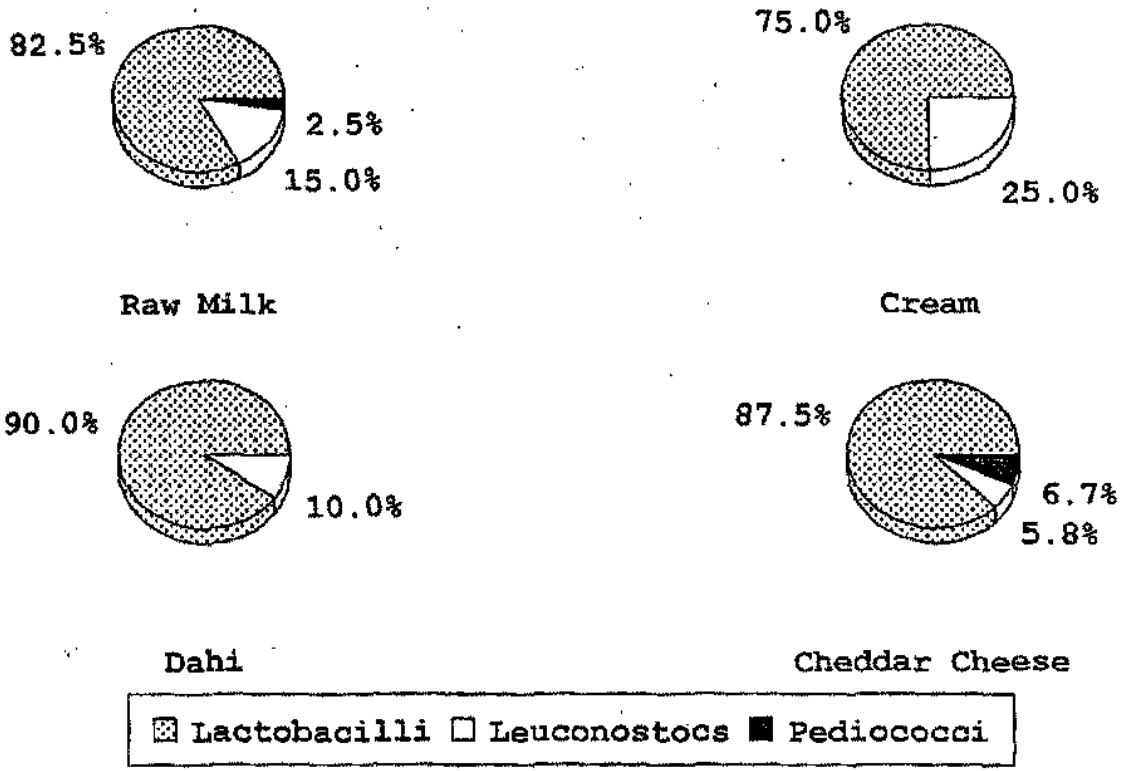


Fig. 4.1 Distribution of lactic acid bacteria from different dairy products

The distribution of lactic acid bacteria in Cheddar cheese at different stages of ripening is given in Table 4.2 and Fig. 4.2. The perusal of the data presented in Table 4.2 indicates that out of a total number of 600 isolates obtained from 15 Cheddar cheese samples, majority of the isolates (525) were found to be lactobacilli. The number of isolates belonging to the genus *Leuconostoc* were 35 and to the *Pediococcus* were 40. The percent distribution of lactobacilli, leuconostocs and pediococci was 87.5, 5.8 and 6.7, respectively. It is also evident from the Fig. 4.2 that lactobacilli remained the dominant non-starter lactic acid bacteria of Cheddar cheese at all stages of ripening period as they constituted 100%, 98.3%, 95%, 80%, 79.2% and 70% of the total isolates examined at 1, 2, 3, 6, 9 and 12 mo intervals of the ripening period, respectively. The leuconostocs were not found in 1 mo old cheese samples. However, they accounted for 1.7%, 5%, 5%, 10% and 22.5% of the total isolates obtained from cheeses of 2, 3, 6, 9 and 12 m of age. On the other hand, pediococci were absent in cheeses of 1, 2 and 3 months of ripening. They appeared late in the cheese ripening period since they could be isolated from 6, 9 and 12 months old ripened cheese samples. The percent distribution of pediococci was 15, 10.8 and 7.5 in cheese samples after 6, 9 and 12 months of ripening, respectively.

Lactic acid bacteria belonging to the genera *Lactobacillus, Leuconostoc* and *Pediococcus* were isolated from different milk and milk products. The pediococci could not be isolated from dahi and cream with the media and other cultural conditions employed in the isolation procedure.

The fact that milk is free from lactobacilli when it leaves the udder but gets easily contaminated with

Table 4.2 : Distribution of lactic acid bacteria isolated from Cheddar cheese at different stages of ripening

Age of cheddar cheese (months)	No. of samples	No. of different lactic acid bacteria			
		Lactobacilli	Leuconostocs	Pediococci	Total
1	2	80	–	–	80
2	3	118	2	–	120
3	2	76	4	–	80
6	4	128	8	24	160
9	3	95	12	13	120
12	1	28	9	3	40
Total	15	525	35	40	600

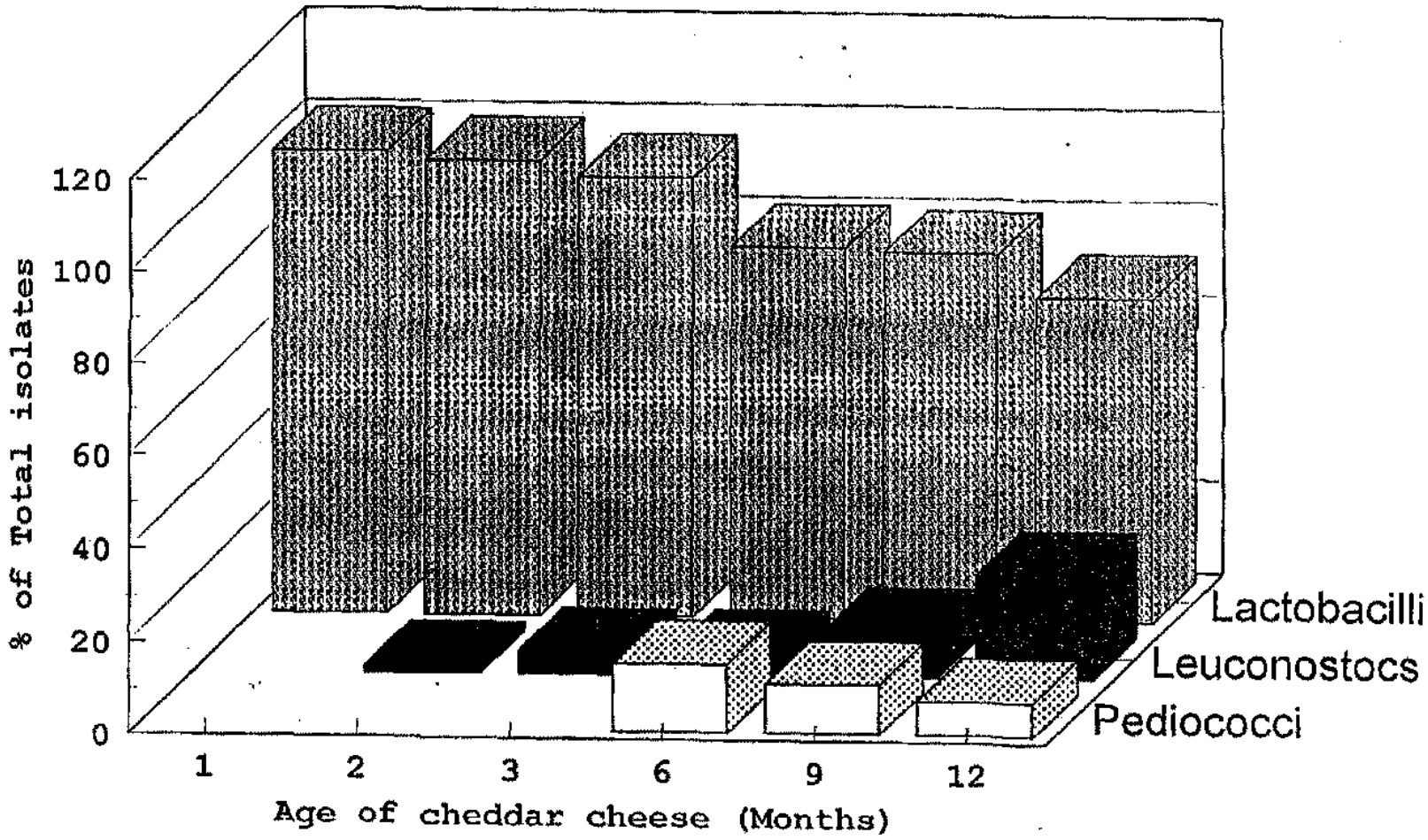


Fig. 4.2 Distribution of lactic acid bacteria from cheddar cheese at different stages of ripening

lactobacilli with feeds, silage, dust, dairy utensils etc. (Kandler and Weiss, 1986) may also hold true for leuconostocs and pediococci which also occur along with lactobacilli in plant environments (Garvie, 1986a,b). The pediococci are generally present in silage (Langston and Bouma, 1960), the rumen and saliva of cattle (Gunther and White, 1961). The presence of pediococci in raw milk reflects the contamination of milk from any or all of the above sources. Relatively lesser occurrence of leuconostocs (15%) and pediococci (2.5%) in comparison to lactobacilli (82.5%) in raw milk could be due to the fact that milk is a poor growth medium for leuconostocs (Garvie, 1986a) and pediococci (Garvie, 1986b since organisms of both these genera can not utilise lactose and also other required growth factors are unavailable for their growth. The presence of pediococci in low numbers (13 isolates) has also been reported in raw goat milk (Tzanetakis and Litopoulou-Tzanetaki, 1989).

Attempts to isolate pediococci from dahi and cream were not successful and the microflora of both the products consisted primarily of lactobacilli and leuconostocs. In addition to the lactic streptococci, the starter cultures of dahi consist of aroma producing leuconostocs with or without *Lactobacillus* sp. (IS: 7035, 1973). The general method of dahi manufacture involves the heating of milk at 80-90°C for 15-30 min which destroys the pediococci, *P. pentosaceus* and *P. acidilactici*, commonly found in milk and milk products (Garvie, 1986b). The isolation of lactobacilli and leuconostocs from cream would be attributed to the contamination of cream with lactobacilli from cream contact surfaces and also with leuconostocs which are components of butter cultures. The very low numbers of dahi (3) and cream (2) samples used for the isolation of

pediococci could also be one of the reasons for failure to isolate pediococci from these sources.

Ripening of cheese is a very dynamic system and the microflora of Cheddar cheese keeps on changing regularly during this process. The lactic streptococci added as starters in the manufacture of cheese die out rapidly during the early stages of ripening whereas the non-starter lactic acid bacteria (NSLAB) comprising [illegible] lactobacilli, leuconostocs and pediococci predominate the microflora of cheese at later stages of ripening. The lactobacilli however, dominated the NSLAB of Cheddar cheese at all the stages of ripening (Fig. 4.2) in this study which is also in agreement with the earlier findings (Darce, 1958b; Prentice and Brown, 1983; Litopoulou-Tzanetaki *et al.*, 1989; Jordan and Cogan, 1993; McSweeney *et al.*, 1993). In the present study leuconostocs could be isolated from cheeses even after one year. Similar observations were earlier made by Prentice and Brown (1983) and Litopoulou-Tzanetaki *et al* (1989).

Fifteen Cheddar cheese samples at 6 different stages of ripening were examined and 40 (6.7%) pediococcal strains among 600 non-starter lactic acid bacteria were isolated. The pediococci were found in the 6 mo to 1 yr ripened cheese samples. Litopoulou-Tzanetaki *et al* (1989) also observed late appearance (6-12 mo) with a low incidence (1%) of pediococci in American Cheddar cheese. The slight difference observed in the occurrence of pediococci reflects the degree of contamination of raw milk employed in the cheese manufacture. On the other hand, Darce (1958b) had reported a very high incidence (25-33%) and early appearance (1-12 mo) of pediococci in New Zealand Cheddar cheese samples. The observed anomaly could be attributed to the ripening of Cheddar cheese at

room temperature. Elliott and Mulligan (1968) also observed low levels (1%) of pediococci in the young (4 weeks old) Canadian Cheddar cheese.

The isolation of pediococci from several other cheeses has also been described including Grana (Bottazzi, 1959), Parmesan (Bottazzi, 1959; 1960), 59 isolates from UK Cheddar (Fryer and Sharpe, 1966), 20 isolates from Canadian Cheddar (Elliott and Mulligan, 1968), 15 from mild to sharp (refer to age) American Cheddar cheese (Keenan *et al.*, 1968), 21 from Manchego (Nunez, 1976), Kashkaval (Andric and Sutic, 1980) and 24 isolates from Feta and 46 from Kaseri (Tzanetakis and Litopoulou-Tzanetaki, 1989).

4.2 SCREENING OF PEDIOCOCCAL ISOLATES FOR THEIR ANTIBACTERIAL ACTIVITY

Forty five pediococcal isolates obtained from buffalo raw milk and Cheddar cheese samples were screened for their antibacterial activity against various lactic and non-lactic cultures by three different assays viz. agar-spot assay, agar-well assay and agar-disc assay. The observations made in these studies are presented in Tables 4.3, 4.4 and 4.5 and Plates 2, and 3.

Out of the total number of 45 pediococcal isolates, only five isolates viz. isolate numbers 12, 16, 21, 26 and 34 showed antibacterial activity against all the four lactic cultures made use in the agar-spot assay (Table 4.3). The inhibition by all the five isolates was very strong against *Lb. plantarum* NCDO955 and *P. acidilactici* LB42 as diameter of the zone of inhibition ranged from 31-40 mm. The pediococcal isolate No. 34 exhibited a strong inhibitory activity (dia. of zone of inhibition was 21-30 mm) against *Lb. plantarum* NCDC21 and *Leu.*

Table 4.3 : Screening of pediococcal isolates for antibacterial activity by agar-spot assay*

Isolate No.	Indicator Bacteria							
	Lb. plantarum		*Leu. mesenteroides*	*P. acidilactici*	*L. monocytogenes*		*B. cereus*	*S. aureus*
	NCDO 955	NCDC 21	LY	LB 42	MTCC 657	MTCC 1143	NCDC 66	NCDC 110
1-11	-	-	-	-	+	+	+	+
12	+ + +	+	+	+ + +	+ + +	+ + +	+ +	+ +
13-15	-	-	-	-	+	+	+	+
16	+ + +	+	+	+ + +	+ + +	+ + +	+ +	+ +
17-20	-	-	-	-	+	+	+	+
21	+ + +	+	+	+ + +	+ + +	+ + +	+ +	+ +
22-25	-	-	-	-	+	+	+	+
26	+ + +	+	+	+ + +	+ + +	+ + +	+ +	+ +
27-33	-	-	-	-	+	+	+	+
34	+ + +	+ +	+ +	+ + +	+ + +	+ + +	+ +	+ +
35-45	-	-	-	-	+	+	+	+

* Diameter of inhibitory zone (mm) : 11-20 +
21-30 + +
31-40 + + +
Absence of zone -

mesenteroides LY while the same indicators were moderately inhibited (zone of the inhibition 11-20 mm) by the isolates 12, 16, 21 and 26. None of the remaining 40 pediococcal isolates showed inhibitory activity against any of the lactic indicator strains in the agar-spot assay. All the 45 pediococcal isolates displayed moderate to very strong antibacterial activity against all the non-lactic cultures in the agar-spot assay. However, among them the inhibition by the five isolates i.e. isolate numbers 12, 16, 21, 26 and 34, that had inhibited all the lactic cultures, was relatively much stronger against *L. monocytogenes* MTCC657 and *L. monocytogenes* MTCC1143 followed by *B. cereus* NCDC66 and *Staph. aureus* NCDC110. While the pediococcal isolates that had failed to inhibit any of the lactic indicator organisms exhibited a moderate antibacterial activity against all the non-lactic cultures in the agar-spot assay.

In the agar-well and agar-disc assays, the centrifuged and filter sterilised cell free supernatants of pediococcal isolates grown in MRS broth (pH 6.5) at 37°C for 24 hr were used in checking the antibacterial activity against all the eight indicator strains. The pH of the culture supernatants was in the range of 3.6-4.0 and the results are given in Tables 4.4 and 4.5, respectively.

The pediococcal isolate numbers 12, 16, 21, 26 and 34 displayed a very strong inhibitory activity (dia. of inhibitory zone 20-25 mm) against *Lb. plantarum* NCDO955 and *P. acidilactici* LB42 in the agar-well assay (Table 4.4). The inhibition of *Lb. plantarum* NCDC21 and *Leu. mesenteroides* LY by the isolate 34 was rather less strong (dia. of the inhibitory zone 15-19 mm), while these two indicator strains were moderately (dia. of the zone of

Table 4.4 : Screening of pediococcal isolates for antibacterial activity by agar-well assay*

Isolate No.	Indicator Strains							
	Lb. plantarum		*Leu. mesenteroides*	*P. acidilactici*	*L. monocytogenes*		*B. cereus*	*S. aureus*
	NCDO 955	NCDC 21	LY	LB 42	MTCC 657	MTCC 1143	NCDC 66	NCDC 110
1-11	-	-	-	-	-	-	+	+
12	+ + +	+	+	+ + +	+ + +	+ + +	+	+
13-15	-	-	-	-	-	-	+	+
16	+ + +	+	+	+ + +	+ + +	+ + +	+	+
17-20	-	-	-	-	-	-	+	+
21	+ + +	+	+	+ + +	+ + +	+ + +	+	+
22-25	-	-	-	-	-	-	+	+
26	+ + +	+	+	+ + +	+ + +	+ + +	+	+
27-33	-	-	-	-	-	-	+	+
34	+ + +	+ +	+ +	+ + +	+ + +	+ + +	+	+
35-45	-	-	-	-	-	-	+	+

* Diameter of inhibitory zone (mm) : 10-14 +
15-19 + +
20-25 + + +
Absence of zone (< 9 mm) -

inhibition 10-14 mm) inhibited by isolate numbers 12, 16 21 and 26. The remaining 40 isolates, as in agar-spot assay, failed to inhibit any of the four strains of lactic indicator organisms.

The five isolates showing the inhibition of the four lactic strains displayed a very strong inhibitory activity against *L. monocytogenes* MTCC657 and *L. monocytogenes* MTCC1143 when tested by agar-well assay (Table 4.4). However, they were found to be moderately inhibitory to *B. cereus* NCDC66 and *Staph. aureus* NCDC110. The remaining 40 isolates of pediococci that had shown a moderate zone of inhibition against the two strains of *L. monocytogenes* in agar-spot assay, however, did not show inhibition of both the strains in agar-well assay and were observed to be rather moderately inhibitory to *B. cereus* NCDC66 and *Staph. aureus* NCDC110.

The results pertaining to the screening of pediococcal isolates for antibacterial activity by agar-disc assay are presented in Table 4.5. The pattern of inhibition of indicator strains by the culture supernatants of the pediococcal isolates observed by agar-disc assay (Table4.5) was almost similar to the one observed by the agar-well assay (Table 4.4).

The inhibition of lactic acid non-lactic indicator strains by the pediococcal isolates in the three assay systems has also been shown in Plates 2 and 3, respectively.

Agar-spot assay is one of the most sensitive and widely used bioassays for the detection of microbial antagonism. The antagonistic activity observed against various indicator strains in this assay is the cumulative effect of all the antimicrobial metabolites secreted by

Table 4.5 : Screening of pediococcal isolates for antibacterial activity by agar-disc assay*

Isolate No.	Indicator Strains							
	Lb. plantarum		*Leu. mesenteroides*	*P. acidilactici*	*L. monocytogenes*		*B. cereus*	*S. aureus*
	NCDO 955	NCDC 21	LY	LB 42	MTCC 657	MTCC 1143	NCDC 66	NCDC 110
1-11	-	-	-	-	-	-	+	+
12	+ + +	+	+	+ + +	+ + +	+ + +	+	+
13-15	-	-	-	-	-	-	+	+
16	+ + +	+	+	+ + +	+ + +	+ + +	+	+
17-20	-	-	-	-	-	-	+	+
21	+ + +	+	+	+ + +	+ + +	+ + +	+	+
22-25	-	-	-	-	-	-	+	+
26	+ + +	+	+	+ + +	+ + +	+ + +	+	+
27-33	-	-	-	-	-	-	+	+
34	+ + +	+ +	+ +	+ + +	+ + +	+ + +	+	+
35-45	-	-	-	-	-	-	+	+

* Diameter of inhibitory zone (mm) : 10-14 +
15-19 + +
20-24 + + +
Absence of zone (< 9 mm) -

Plate 2. Antibacterial activity of pediococcal isolates against lactic cultures

A : *Lactobacillus plantarum* NCDO955
B : *Pediococcus acidilactici* LB42

a) Agar-spot assay
b) Agar-well assay
c) Agar-disc assay

1) Pediococcal isolate 33
2) Pediococcal isolate 35
3) Pediococcal isolate 34

A

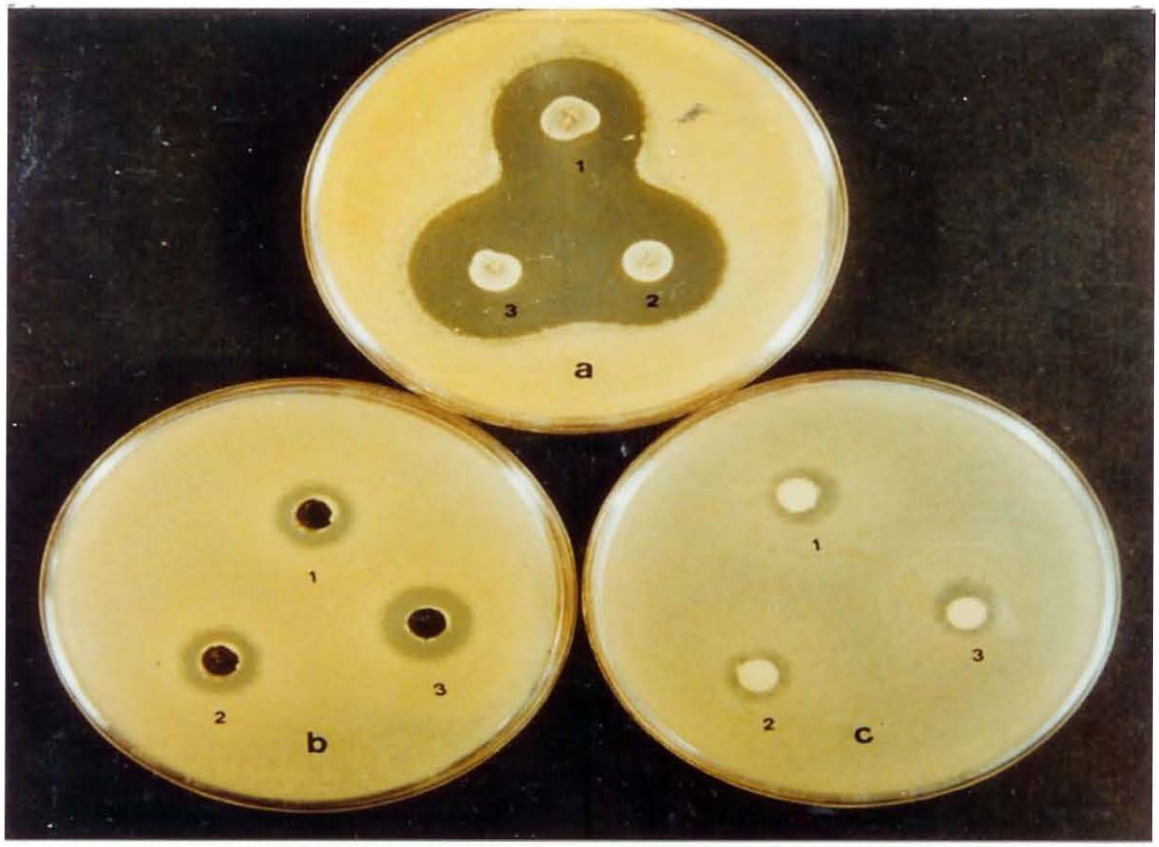
B

Plate 3. Antibacterial activity of pediococcal isolates against pathogenic bacteria

A : *Bacillus cereus* NCDC66
B : *Listeria monocytogenes* MTCC657

a) Agar-spot assay
b) Agar-well assay
c) Agar-disc assay

1) Pediococcal isolate 33
2) Pediococcal isolate 35
3) Pediococcal isolate 34



A

B

the test culture into the medium that get concentrated and diffused around the spot of the test culture (producer strain) much before the indicator is added. Out of 45 isolates, the failure of 40 pediococcal isolates to inhibit lactic indicator strains in the agar-spot assay reveals that the concentration of antibacterial substances such as organic acids, bacteriocin etc, secreted by them was not sufficient to antagonise the lactic cultures. The pH of all the pediococcal culture supernatants used in the agar-well and agar-disc assays was in the range of 3.6-4. In spite of this low pH, the same 40 isolates once again failed to inhibit lactic cultures in both the assays, further substantiating the fact that indicator strains were insensitive to the concentration of organic acids produced by the test cultures.

A large number of bacteriocins of lactic acid bacteria have a narrow spectrum of antibacterial activity and are usually active against closely related bacteria. Thus, selection of lactic acid bacteria which are relatively insensitive to the concentration of organic acids encountered in the screening procedures as indicator strains enhances the probability of the detection of bacteriocin-like activity. Lactic acid bacteria have been widely used as indicators of antagonistic activity while screening isolates belonging to the genera *Lactobacillus* (Garriga *et al.*, 1993; Jimenez-Diaz *et al.*, 1993; Vignolo *et al.*, 1993; ten Brink *et al.*, 1994), *Leuconostoc* (Mathieu *et al.*, 1993; Yang and Ray, 1994a), *Pediococcus* (Strasser-de-Saad and Manca-de-Nadra, 1993; Cintas *et al.*, 1995) and *Lactococcus* (Kozak *et al.*, 1978; Geis *et al.*, 1983; Morgan *et al.*, 1995 and Rodriguez *et al.*, 1995).

Though all the 45 pediococcal isolates exhibited antagonistic activities against both the strains of *L. monocytogenes* in the agar-spot assay but examination of the culture supernatants by agar-well and agar-disc assays showed inhibition of listerial strains by five isolates only (Plate 3 B). The more or less same pH (3.6-4) of the culture supernatants shows the insignificant role of organic acids in the inhibition of listerial indicator strains by the cultural supernatants of five pediococcal isolates. The failure of the 40 isolates to exert antagonistic activity against *Listeria* cultures in the well- and disc-assays could be due to the insufficient quantities of antibacterial substances present in the culture supernatants used for detecting inhibitory activity or that the antagonistic compounds are secreted only in the agar medium but not in the liquid medium. Schillinger and Holzapfel (1990) observed the inhibition of *L. monocytogenes* DSM20600 in the well diffusion assay only by a tenfold concentrated supernatant of *Carnobacterium piscicola* LV61. Although 65 strains of lactic streptococci exhibited antagonistic effect on the agar but among them only 16 strains showed inhibitory activity in the liquid medium (Geis *et al.*, 1983). Similarly, Barefoot and Klaenhammer (1983) reported the detection of inhibitors of some strains of *Lb. acidophilus* only on agar media but not in the liquid media. West and Warner (1988) also could not detect the antagonistic activity of *Lb. plantarum* NCDO1193 in a liquid culture, although this strain produced inhibition zone against the other lactic acid bacteria on the agar.

The discrepancy in the observations between agar-spot assay and agar-well diffusion assay has been widely reported. Although 24 strains of carnobacteria showed antagonistic activity in the agar-spot assay, only 18 were found inhibitory to the indicator strains in

agar-well assay (Schillinger and Holzapfel, 1990). Similarly, Thuault *et al* (1991) reported that *Cl. tyrobutyricum* was inhibited by 33 strains of lactococci in the agar-overlay method but when the culture supernatants were tested only 4 of them showed inhibition zones in the well diffusion assay. Of the 55 strains of lactobacilli exhibiting antibacterial activity in the spot assay, only 6 produced inhibition zones in the well assay (Garriga *et al.*, 1993) while Cintas *et al* (1995) reported the inhibition by only 12 isolates of the indicator strains in the well assay even if as many as 55 strains showed antagonistic activities in the spot-assay.

Although the 45 pediococcal isolates exhibited a relatively less inhibitory activity against *B. cereus* NCDC66 and *Staph. aureus* NCDC110, only five isolates that produced inhibition zones against all the eight indicator strains in all the three different assays were considered for further characterisation of the antibacterial substances.

4.3 IDENTIFICATION OF PEDIOCOCCAL ISOLATES

The detailed taxonomic studies involving various morphological, physiological and biochemical characteristics to identify the pediococcal isolates to species level are documented in Table 4.6 and the proposed identity of the isolates is given in Table 4.7.

It may be observed from Table 4.6 that all the isolates were Gram positive cocci with cells being arranged in pairs and tetrads. All showed growth at 45°C. Two isolates viz. isolates 43 and 45 failed to grow at 12°C but exhibited growth at 50°C. All the isolates were able to grow at pH 4.2 and 8.5 but not at pH 9.6, and in 6.5% NaCl but not in 10% or above.

Table 4.6 : Morphological, physiological and biochemical characteristics of some selected pediococcal isolates*

Test	Isolate No.																	
	8	9	12	13	16	19	21	26	28	33	34	35	36	37	38	40	43	45
Morphological																		
Shape	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Arrangement of cells	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T	P&T
Gram's reaction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Physiological																		
Growth at 12°C	+	+	+	+	+	+	+	+	+	ND	+	ND	+	+	+	+	-	-
45°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Growth at pH 4.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl 6.5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Biochemical																		
Gas production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	?	+	+	+	+	+	+	+	+	+	+	+	+	?	?	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudocatalase	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+

Reaction in litmus milk (7 days)																			
Reduction	ND	+	+	-	-	ND	-	-	ND	+	-*	-	ND	ND	ND	ND	ND	ND	
Curdling	ND	+	-	-	-	ND	-	-	ND	-	-*	-	ND	ND	ND	ND	ND	ND	
Sugar Fermentation																			
Arabinose	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Lactose	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Maltose	-	+	+	-	+	+	+	+	-	-	+	-	+	+	-	-	-	-	
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

C : Cocci; P&T : Pairs and tetrads;

+ : Positive reaction; - : Negative reaction; ND : Not determined; ' : After 30 days

Isolate 34 was citrate positive

Isolates 33, 34 and 35 were salicin positive and negative for sorbitol, dulcitol and mannitol.

Biochemical characterisation of the pediococcal isolates showed that all the isolates were non-gas producers, arginine positive (Plate 4), catalase negative but possessed variable pseudo-catalase activity (Table 4.6). None of the isolates except No. 9 curdled litmus milk and only three isolates viz. numbers 9, 12 and 33 reduced litmus milk after 7 days of incubation. Isolate No. 34 neither reduced nor curdled litmus milk on incubation for 30 days.

The carbohydrate fermentation pattern revealed the utilisation of ribose, xylose, dextrose, galactose, mannose, fructose and cellobiose by all the isolates. None of the isolates produced acid from sucrose and rhamnose, while a variable reaction was observed with arabinose, lactose and maltose. Isolate numbers 9, 12, 16, 19, 21, 26, 34, 36 and 37 were maltose positive while others were maltose negative, whereas isolate numbers 33, 34 and 35 were positive for salicin but negative for sorbitol, mannitol and dulcitol. Utilisation of these three sugars by the remaining pediococcal cultures was not assessed. Fermentation of various carbohydrates by pediococcal isolate No. 34 is depicted in Plate 5.

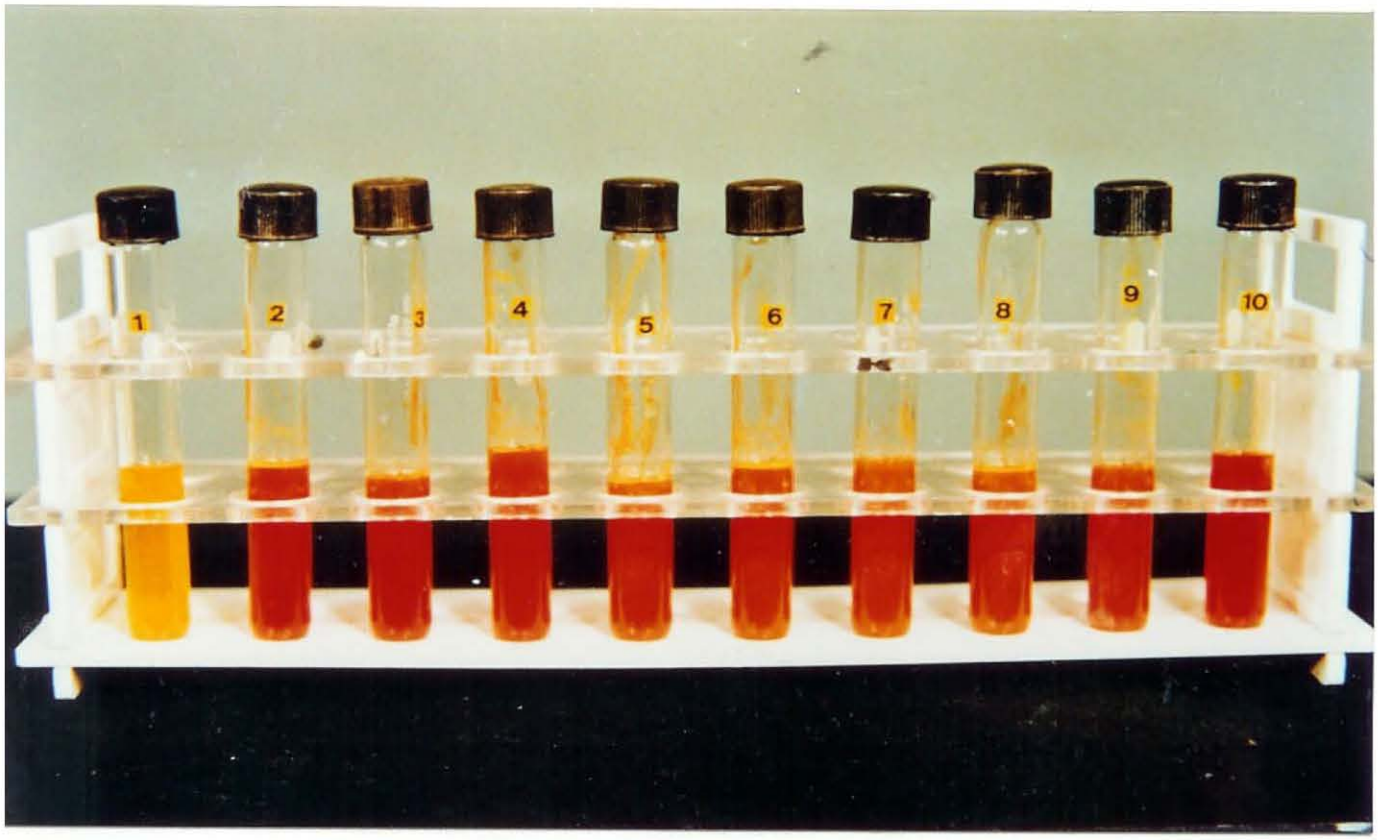
Out of the total number of 18 pediococcal isolates subjected to detailed taxonomic studies, 2 were identified as the strains of *P. acidilactici*, 9 were identified as *P. pentosaceus* and 7 could not be assigned to any of the above or any other species of the genus *Pediococcus* (Table 4.7). The five isolates, i.e. numbers 12, 16, 21, 26 and 34 which had shown inhibition of all the indicator strains in all the three assays belonged to *P. pentosaceus*.

Plate 4. Arginine hydrolysis reaction by pediococcal isolates

Tube No.1 : Control showing -Ve reaction
Tube No.2-10 : Pediococcal isolates showing +ve reaction

Plate 5. Carbohydrate fermentation by *Pediococcus pentosaceus* 34

1. Sorbitol	-	4. Maltose	+
2. Dulcitol	-	5. Lactose	-
3. Mannitol	-	6. Sucrose	-



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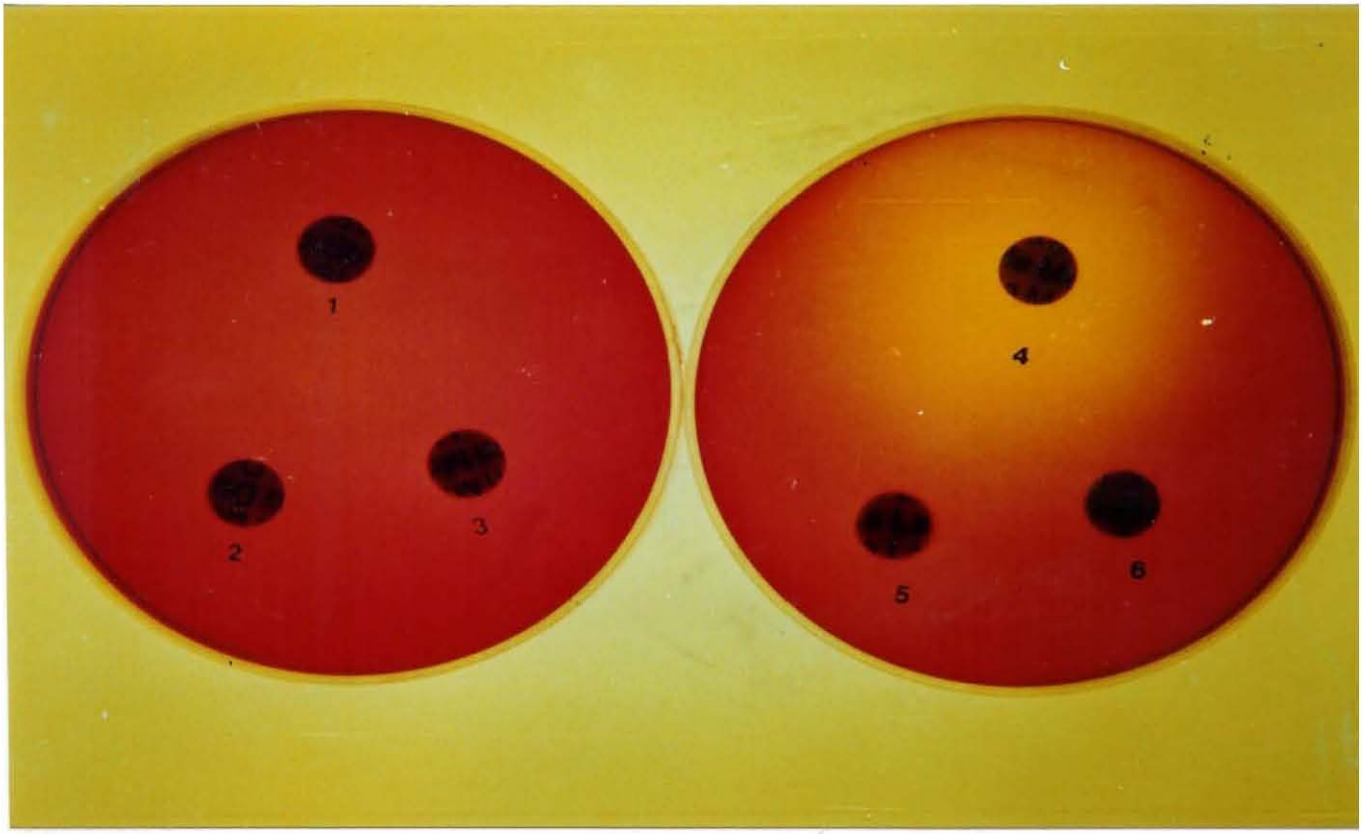

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Table 4.7 : Identity of pediococcal isolates

Isolate No.	Identity	No. of isolates	% selected isolates
8	Unidentified		
13	"		
28	"		
33	"		
35	"		
38	"		
40	"	7	38.88
9	*P. pentosaceus*		
12	"		
16	"		
19	"		
21	"		
26	"		
34	"		
36	"		
37	"	9	50.00
43	*P. acidilactici*		
45		2	11.11
Total		**18**	**100**

Eight species of the *Pediococcus* genus have been recognised in the latest edition of Bergey's Manual of Systematic Bacteriology (Garvie, 1986b) and Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). They include *P. acidilactici, P. damnosus, P. dextrinicus, P. halophilus, P. inopinatus, P. parvulus, P. pentosaceus* and *P. urinaeequi*. The strains previously included as *P. cerevisiae* have been distributed among *P. damnosus, P. pentosaceus* and *P. acidilactici* (Ray, 1995).

Pediococci are the only lactic acid bacteria that divide alternately in two planes at right angles and occur in pairs and tetrads with single cells being rare and no chains formed. The ability to use pentoses such as arabinose, xylose and ribose is limited to *P. pentosaceus, P. acidilactici, P. urinaeequi* and some strains of *P. halophilus* (Garvie, 1986b). None of the isolates in the study could be identified as *P. halophilus* as they failed to grow at 18% NaCl. *P. pentosaceus* and *P. acidilactici* are the only pediococci that can grow in Rogosa (SL) acetate agar at 45°C and hydrolyse arginine. Thus all the isolates can be assigned to either of the two species. Growth at 50°C and ability to ferment maltose differentiates between the two species. The strains of *P. pentosaceus* can not grow at 50°C but ferment maltose while *P. acidilactici* which can grow at 50°C fail to ferment maltose.

Therefore, on the basis of the taxonomic schemes of Garvie (1986b) and Holt *et al* (1994) the two isolates (No. 43 and 45) which could grow at 50°C but failed to ferment maltose were classified as *P. acidilactici*. Half of the total 18 isolates obtained in the present study were identified as *P. pentosaceus* (Table 4.7) as they could utilise maltose with no growth being observed at

50°C. The remaining 7 isolates could not be assigned to either of the species as they could neither exhibit growth at 50°C nor ferment maltose. It is interesting to note that all the pediococcal isolates i.e. isolate numbers 12, 16, 21, 26 and 34 (that inhibited all the indicators in all the assays) were found to be different strains of *P. pentosaceus*.

P. pentosaceus and *P. acidilactici* may occur in milk and milk products (Garvie, 1986b). The results pertaining to the identification of pediococcal strains isolated from Indian Cheddar cheese as *P. pentosaceus* and *P. acidilactici* are in agreement with several other earlier reports. The characteristic pediococcal strain isolated from New Zealand Cheddar cheese was identified as *P. cerevisiae* (Darce, 1958a) while Thomas *et al* (1985) isolated *P. pentosaceus* strains from the same source. All the 59 pediococcal strains from UK (Fryer and Sharpe, 1966), 20 from Canadian (Elliott and Mulligan, 1968) and 15 from American (Keenan *et al.*, 1968) Cheddar cheese samples were identified as *P. cerevisiae*. However, Litopoulou-Tzanetaki *et al* (1989) characterised the 4 pediococcal strains from American Cheddar cheese and identified them to be *P. pentosaceus*.

The pediococcal strains from other cheese varieties were also identified by several other workers. The pediococcal strains from Manchego cheese were identified as *P. pentosaceus* (Nunez, 1976) while Andric and Sutic (1980) reported the identification of both *P. pentosaceus* and *P. acidilactici* from Kashkaval cheese. Seventy five out of 83 pediococcal strains from raw goat milk, Feta and Kaseri cheeses were identified as *P. pentosaceus* (Tzanetakis and Litopoulou- Tzanataki, 1989).

4.4 CHEMICAL NATURE OF THE ANTIBACTERIAL SUBSTANCE PRODUCED BY PEDIOCOCCAL CULTURES

Culture broths were adjusted to pH 7, treated with catalase and different proteolytic enzymes viz. trypsin, chymotrypsin, ficin, papain, pronase-E and proteinase-K and subsequently assayed for the residual antibacterial activity against *P. acidilactici* LB42. It may be observed from Table 4.8 and Plate 6 that all the five strains of *P. pentosaceus* retained their activity upon treatment with catalase indicating that the inhibition of the indicator strain was not due to hydrogen peroxide. However, the inhibitory activity of all the supernatants was lost upon treatment with all the six proteolytic enzymes pointing to the involvement of active protein moiety in the antagonism and indicating that the antagonistic activity is due to the presence of bacteriocin in the culture broths.

This is the second report, and of course first in India, till date concerning the bacteriocinogenicity among the pediococcal isolates of dairy origin. Earlier, Daba *et al* (1991) isolated a bacteriocin producing *Leu. mesenteroides* UL5 from Cheddar cheese and the producing strain was later reidentified as *P. acidilactici* UL5 (Huang *et al.*, 1994). However, other efforts to isolate bacteriocinogenic pediococci from Kashkaval (Andric and Sutic, 1980) and Cheddar (Litopoulou-Tzanetaki *et al.*, 1989) cheese were not successful probably because of the very few number (11 and 4, respectively) of strains examined or due to several other factors such as the method employed, the number and inherent susceptibility of the indicator strains, and the criteria adopted to define the antagonism.

Table 4.8 : Enzyme sensitivity on the antibacterial substances produced by selected pediococcal isolates

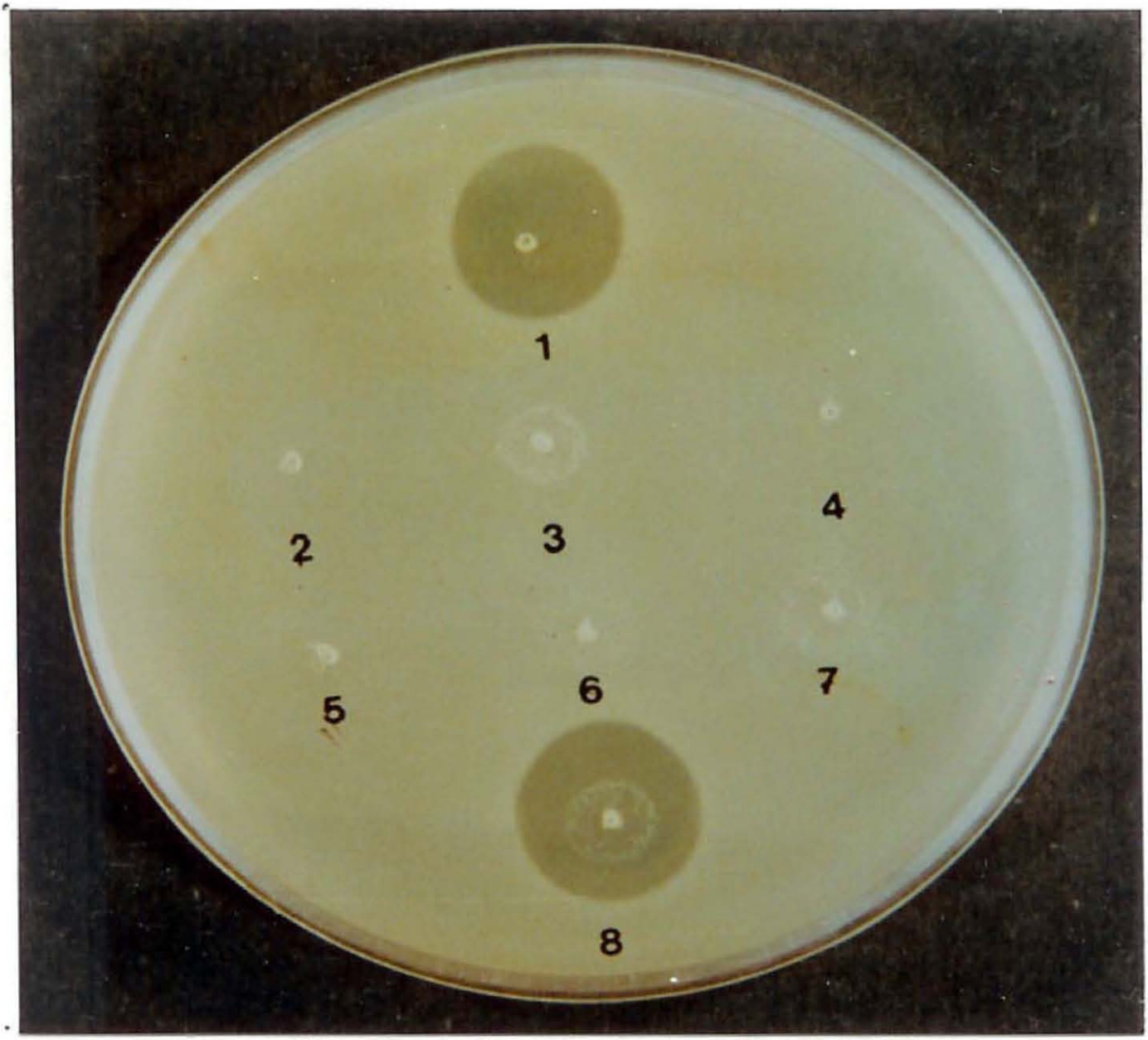
Enzyme	Strains of *Pediococcus pentosaceus*				
	12	16	21	26	34
Catalase	R	R	R	R	R
Chymotrypsin	S	S	S	S	S
Ficin	S	S	S	S	S
Papain	S	S	S	S	S
Pronase-E	S	S	S	S	S
Proteinase-K	S	S	S	S	S
Trypsin	S	S	S	S	S

Pediococcus pentosaceus LB42 was used as the indicator organism, using spot-on-the-lawn assay system.

R : Resistant; S : sensitive

Plate 6. Enzyme sensitivity of the antibacterial substance produced by *Pediococcus pentosaceus* 34

1. Control
2. Trypsin
3. Chymotrypsin
4. Pronase-E
5. Proteinase-K
6. Ficin
7. Papain
8. Catalase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7
8

Although reports concerning the isolation of pediococci from dairy sources are rather scarce, the bacteriocinogenic pediococci have been isolated from various other sources such as vegetables (Daeschel and Klaenhammer, 1985), meats (Bhunia *et al.*, 1987; Hoover *et al.*, 1988; Schved *et al.*, 1993 and Cintas *et al.*, 1995), wine (Strasser-de-Saad and Manca-de-Nadra, 1993) etc.

Bacteriocinogenic lactic acid bacteria isolated from dairy products include lactococci (Piard *et al.*, 1990; Thuault *et al.*, 1991; Gupta and Batish, 1992; Ali *et al.*, 1995; Morgan *et al.*, 1995 and Martinez *et al.*, 1996), lactobacilli (Muriana and Klaenhammer, 1987; Vaughan *et al.*, 1992; Kanatani and Oshimura, 1994 and Rekhif *et al.*, 1994) leuconostocs (Hechard *et al.*, 1992; Mathieu *et al.*, 1993; Malik *et al.*, 1994a and Sudirman *et al.*, 1994).

4.5 SELECTION OF THE PRODUCER STRAIN AND INDICATOR ORGANISM

Selection of the producer as well as indicator strains was done by estimating the bacteriocin titres of the culture broths of the producers against all the four lactic indicator organisms employed in the screening process and expressing the bacteriocin titre as Arbitrary or Activity Units (AU) per millilitre of the culture broth. The results are presented in Table 4.9.

The bacteriocin activity of *P. pentosaceus* strains 12, 16, 21, 26 and 34 was 2000, 4000, 3000, 4000 and 6000, respectively against both *Lb. plantarum* NCDC21 and *Leu. mesenteroides* LY. All the pediococcal cultures displayed higher antibacterial activity against *P. acidilactici* LB42 ranging from 22000 to 78000 AU/ml followed by *Lb. plantarum* NCDO955 with an activity range

Table 4.9 : Sensitivity of lactic indicator organisms to the bacteriocins produced by different isolated strains of *Pediococcus pentosaceus*

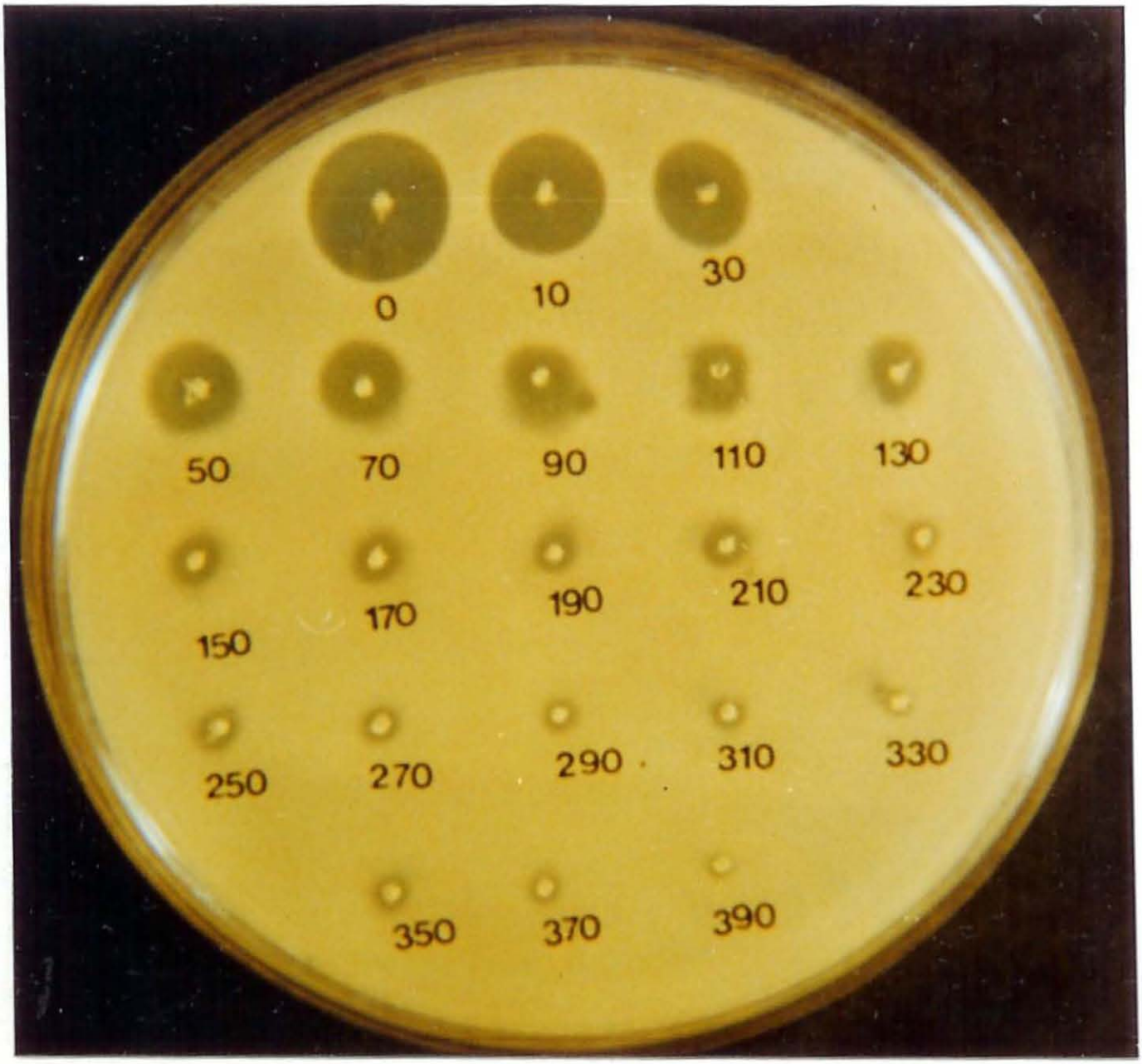
Strains of *P. pentosaceus*	Activity (AU)/ml ($X10^3$)			
	Lb. plantarum		*Leu. mesenteroides*	*P. acidilactici*
	NCDC21	NCDO955	LY	L3-42
12	2	14	2	22
16	4	26	4	42
21	3	28	3	38
26	4	30	4	46
34	6	54	6	78

of 14000 to 54000 AU/ml. Of all the bacteriocin producing pediococcal cultures, *P. pentosaceus* 34 resulted in the maximum activity of 78000 AU/ml against *P. acidilactici* LB42 (Plate 7) whereas the bacteriocin activity of the same producer strain against *Lb. plantarum* NCDO955 was estimated to be 54000 AU/ml (Table 4.9).

Bacterial cultures for bacteriocin production and characterisation studies have been selected on the basis of their broader antibacterial spectrum of activity (Piard *et al.*, 1990; Jimenez-Diaz *et al.*, 1993; Schved *et al.*, 1993; Cintas *et al.*, 1995 and Rodriguez *et al.*, 1995). Since no differences in the inhibitory activity among the five producing strains against the lactic and non-lactic indicators employed in the present screening studies were observed, the bacteriocin activity present in the respective culture broths was quantified using the four lactic indicator strains. *P. pentosaceus* 34, however, exhibited highest activity against all the lactic cultures and was, therefore, retained for further studies. *P. acidilactici* LB42 was found to be the most sensitive organism to the bacteriocin produced by *P. pentosaceus* 34 and hence used as the indicator strain for the bacteriocin assays. In some earlier studies, *Lactococcus* spp. have been used as indicators for the bacteriocins of different lactococcal strains (Piard *et al.*, 1990; Kojic *et al.*, 1991 and Rodriguez *et al.*, 1995), *Leuconostoc* spp. for bacteriocins of leuconostocs (van Laack *et al.*, 1992 and Keppler *et al.*, 1994) and *Lactobacillus* spp. for the bacteriocins of lactobacilli (Daeshel *et al.*, 1990; Joerger and Klaenhammer, 1986). *Lactobacillus* strains have also been used as indicator organisms in the characterisation of bacteriocins of

Plate 7. Quantitative determination of bacteriocin activity by spot-on-lawn assay

Producer strain : *P. pentosaceus* 34
Indicator strain : *P. acidilactici* LB42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10
30
50
70
90
110
130
150
170
190
210
230
250
270
290
310
330
350
370
390

pediococci such as pediocin AcH (Bhunia *et al.*, 1988) and pediocin SJ-1 (Schved *et al.*, 1993).

P. pentosaceus 34 grown in MRS broth exhibited a maximum activity of 54000 AU/ml against *Lb. plantarum* NCDO955 using the spot-on-lawn assay (Table 4.9). On the other hand, pediocin SJ-1 produced by *P. acidilactici* SJ-1 exhibited 25600 AU/ml (Schved *et al.*, 1993) and pediocin AcH produced by *P. acidilactici* H exhibited 36000 AU/ml (Biswas *et al.*, 1991)) against the same indicator strain also using the spot-on-lawn assay. Since both of these cultures were grown in TGE broth, the differences in the bacteriocin activity could be attributed to the media employed in the production. However, it was reported that the pediocin AcH production was maximum in TGE broth than in MRS broth (Biswas *et al.*, 1991). Different strains of *P. acidilactici* though produced identical pediocin AcHs, they have exhibited varying bacteriocin titres (Yang and Ray, 1994b) against *Lb. plantarum* NCDO955 (range 22000 - 30000 AU/ml). Different titres of the bacteriocin activity (14000 - 54000 AU/ml) by different strains of *P. pentosaceus* against the same indicator were also observed in the present investigation.

4.6 OPTIMISATION OF PARAMETERS FOR BACTERIOCIN PRODUCTION

The bacteriocin production by the selected pediococcal culture, *Pediococcus pentosaceus* 34, was optimised with respect to the culture medium, initial pH of the culture medium, temperature of growth of the producer culture, and the time of incubation. In each experiment, changes in pH, growth in terms of absorbance at 600 nm and bacteriocin activity expressed in terms of AU/ml were recorded at regular intervals. The results

obtained during the optimisation of bacteriocin production parameters are given in Tables 4.10-4.12 and Figures 4.3 to 4.7.

4.6.1 CULTURE MEDIUM

It may be seen from the Table 4.10 that there was a gradual decrease in the initial pH of all the growth media (APT, Elliker, MRS and TGE broths) with the decline being very sharp in the first 8 hr of incubation and there after it was only marginal. The pH of APT, Elliker, MRS and TGE broths dropped to 4.18, 4.94, 3.87 and 3.60 from the initial pH values of 6.6, 6.3, 6.5 and 6.5, respectively at the end of the 24 hr incubation period. Conversely, the bacterial growth exhibited a gradual increase as the incubation period proceeded. The absorbance values (O.D.) measured at 600 nm after 24 hr incubation at 37°C were 3.14, 1.10, 5.77 and 2.76 in APT, Elliker, MRS and TGE broths, respectively (Table 4.10). However, a sharp increase in O.D. values was observed after first 8 hr of incubation and there after the increase was rather gradual.

The bacteriocin production was observed to be maximum in MRS broth followed by TGE, APT and Elliker broths (Table 4.10). The activity in MRS, TGE, APT and Elliker broths after 24 hr of incubation was 78,000, 30,000, 18,000 and 14,000 AU/ml, respectively. The bacteriocin titres obtained in TGE, APT and Elliker broths were about 38.5%, 18% and 23% of the highest titres reached in MRS broth. It was also observed that about 65.00% of the total bacteriocin was secreted into MRS broth after first 8 hr of incubation at 37°C and the remaining amount during the next 8 hr of incubation. The bacteriocin titres obtained in APT, Elliker and TGE broths at the end of 8 hr incubation were calculated to

Table 4.10 : Influence of culture medium on pH, growth and bacteriocin production by *Pediococcus pentosaceus* 34

Parameter	Time (Hr)	Growth Medium			
		APT	Elliker	MRS	TGE
pH	0	6.60	6.30	6.50	6.50
	8	4.70	4.94	4.30	3.90
	16	4.16	4.94	3.99	3.66
	24	4.18	4.94	3.87	3.60
Absorbance (600 nm)	0	0.05	0.04	0.04	0.04
	8	2.00	0.96	5.41	2.10
	16	3.03	1.16	5.72	2.70
	24	3.14	1.10	5.77	2.76
Activity Units (A.U.) per ml ($x 10^3$)	0	0.00	0.00	0.00	0.00
	8	4.00	6.00	50.00	14.00
	16	14.00	14.00	70.00	26.00
	24	18.00	14.00	78.00	30.00

be about 22, 43 and 47%, respectively of the highest titres reached in the respective media and they increased to 78%, 100% and 87% after 16 hr of growth. It is evident from the Fig. 4.3 that bacteriocin activity remained highest in MRS broth at all the three time intervals employed in the experiment.

MRS broth was found to be the most suitable medium for optimal production of the bacteriocin by *P. pentosaceus* 34 followed by TGE, APT and Elliker broths. The variation in the bacteriocin titres observed could be due to the different cell densities attained in each of the broths at the end of the incubation period. MRS broth medium in the present study supported good growth of the test culture and hence the maximum bacteriocin production. Although the growth was slightly more in APT broth (A_{600} = 3.14) as compared to TGE broth (A_{600} = 2.76), the bacteriocin production was almost double in TGE than in APT medium. The difference in bacteriocin titres may be attributed to the lesser availability of some ingredients such as Tween 80 to this bacteriocinogenic culture. It was observed that addition of Tween 80 to a final concentration of 0.1% in the APT broth resulted in the increased production of pediocin AcH by *P. acidilactici* F (Richards, INRA, France, Personal Communication). Among the four broth media, Elliker broth was the least supportive in bacteriocin production by the test culture which is probably due to the poor growth of the producer strain in the medium as two of three sugars viz. lactose and sucrose in the medium are not utilised by *P. pentosaceus* 34 (Table 4.6 and Plate 5). It may also be likely due to lack of certain essential ingredients such as Tween 80, $MnSO_4$, $MgSO_4$ etc in the menstrum. In case of TGE broth, the dramatic decline in pH could be due to lack of any buffering component in the medium.

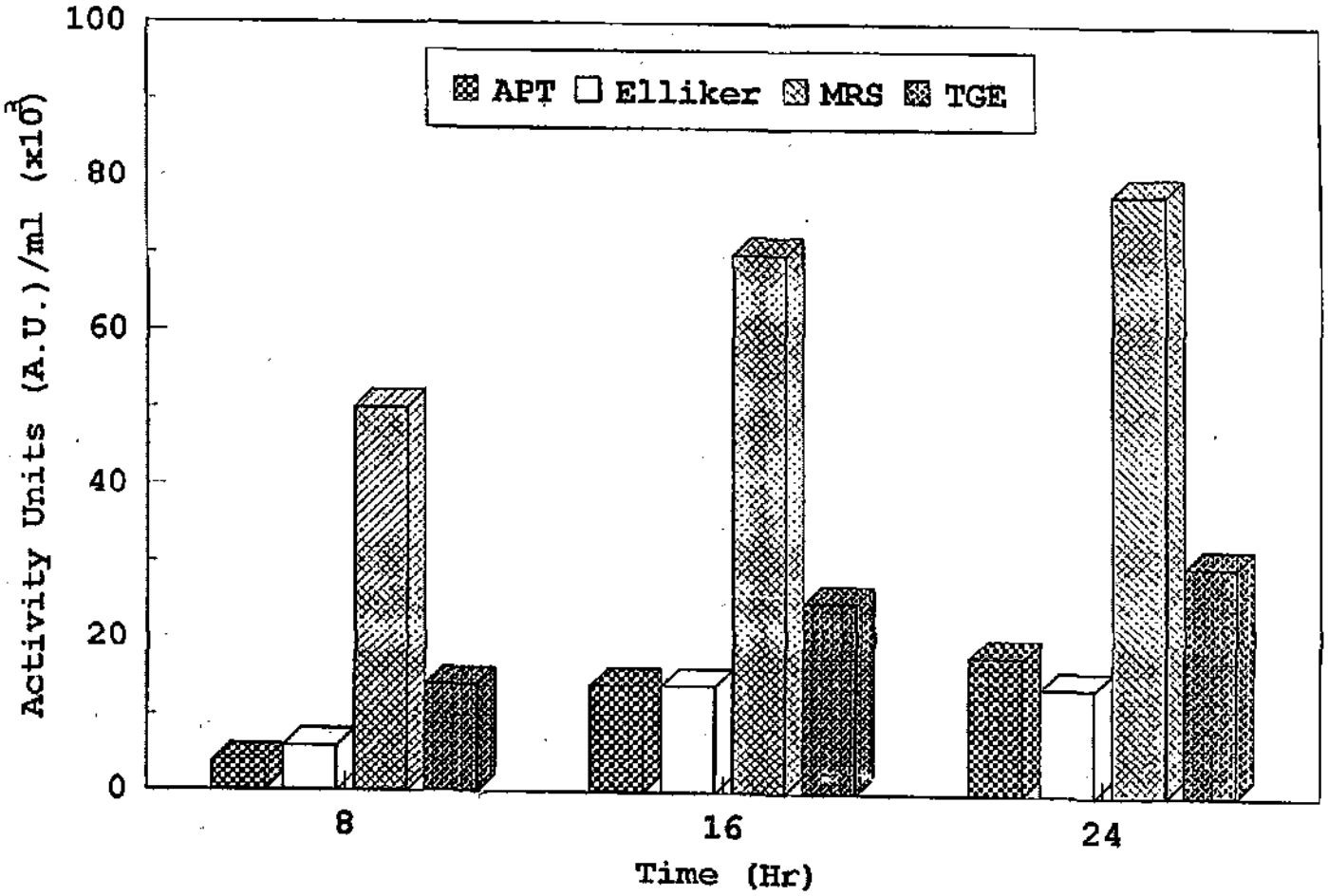


Fig. 4.3 Influence of culture medium on bacteriocin production by *Pediococcus pentosaceus* 34

MRS medium has also been reported to support maximum production of bacteriocins like mesentericin Y105 (Hechard *et al.*, 1992), piscicocin VI and divercinV41 (Pilet *et al.*, 1995) and pediocin L50 (Cintas *et al.*, 1995). Biswas *et al* (1991), however, reported a 15% less pediocin AcH production in MRS broth than in TGE broth in spite of higher cell mass of the producer strain in MRS broth. It was also observed that the incorporation of acetate, citrate and phosphate together into TGE broth though increased the cell mass, it reduced bacteriocin production. Yang and Ray (1994b) also observed maximum pediocin AcH titres in plain TGE broth than in buffered TGE broth. Geis *et al* (1983) reported that bacteriocin production by several strains of lactococci was maximum in Elliker broth while Piard *et al* (1990) observed maximum lacticin 481 production in Elliker broth buffered with sodium β-glycerophosphate .

4.6.2 INITIAL PH OF THE GROWTH MEDIUM

The bacteriocin producing *Pediococcus pentosaceus* 34 was grown in MRS broth adjusted to different initial pH values viz. 4, 5, 6, 6.5, 7 and 8 and changes observed with respect to pH, absorbance and bacteriocin activity during the course of incubation at 37°C are given in Table 4.11.

The growth of the bacteriocin producer in MRS broth resulted in lowering of the initial pH from 4 to 3.38 after 24 hr cell growth. The absorbance expressed in terms of O.D. reached upto 2.04 and the bacteriocin activity reached to a maximum extent of 50,000 AU/ml during the same time. When the initial pH of the MRS broth media ranged from 5 to 8, the corresponding pH

Table 4.11 : Effect of initial pH of MRS broth on pH, growth and bacteriocin production by *Pediococcus pentosaceus* 34

Parameter	Time (Hr)	Initial pH					
		4.0	5.0	6.0	6.5	7.0	8.0
pH	0	4.01	5.07	5.92	6.53	6.96	8.01
	8	3.95	4.13	4.18	4.30	4.33	4.80
	16	3.66	3.62	3.80	3.84	3.85	3.95
	24	3.38	3.54	3.70	3.70	3.79	3.81
Absorbance (600 nm)	0	0.04	0.04	0.08	0.06	0.10	0.08
	8	0.20	3.00	4.00	5.23	5.40	5.80
	16	1.21	3.73	5.18	5.75	6.03	6.13
	24	2.04	3.84	6.13	5.83	6.04	6.25
Activity Units (A.U.) per ml ($\times 10^2$)	0	0.00	0.00	0.00	0.00	0.00	0.00
	8	2.00	50.00	50.00	50.00	50.00	50.00
	16	34.00	70.00	70.00	78.00	70.00	70.00
	24	50.00	78.00	78.00	78.00	78.00	78.00

decreased was in the range of 3.5 to 3.8 after 24 hr at 37°C while the absorbance values recorded were in the range of 3.8 to 6.25 (Table 4.11). It was also observed that there was no difference in the bacteriocin titres reached after 8 hr in MRS broth with an initial pH ranging from 5 to 8 and the highest titres of 78,000 AU/ml were attained after 24 hr at all these initial pH values in the range of 5 to 8 except at 6.5 in which case the highest activity was attained after 16 hr of growth and remained at the same level even after 24 hr of incubation. The Fig. 4.4 also shows that the bacteriocin titres at the end of 8 and 24 hr incubation remained same at all the initial pH values in the range of 5 to 8. The highest activity of 78,000 AU/ml was attained after 16 hr at pH 6.5 while the same value was reached after 24 hr at other initial pH values of MRS medium.

The bacteriocin production by *P. pentosaceus* 34 was not significantly dependent on the initial pH of the MRS medium between 5 and 8. However, there was a slight decrease in bacteriocin titres when the test culture was grown in MRS broth with an initial pH 4. The decreased titres of bacteriocin could be due to the reduced cell growth (A_{600} = 2.04) at rather lower initial pH of the medium. Therefore, normal pH (6.5) of MRS medium was employed as an initial pH for the production of the bacteriocin by the test strain.

Thuault *et al* (1991) have also reported that the bacteriocin production by *Lc. lactis* subsp. *lactis* ADRI 85L030 is independent of initial pH of the medium in the range of 5 to 7. Coventry *et al* (1996) observed no detectable levels of brevicin 286 when the producer strain was grown in MRS broth at an initial pH 4.5, but the production was optimum at a pH 6 - 6.5. Lower cell densities and consequently lower bacteriocin titres were

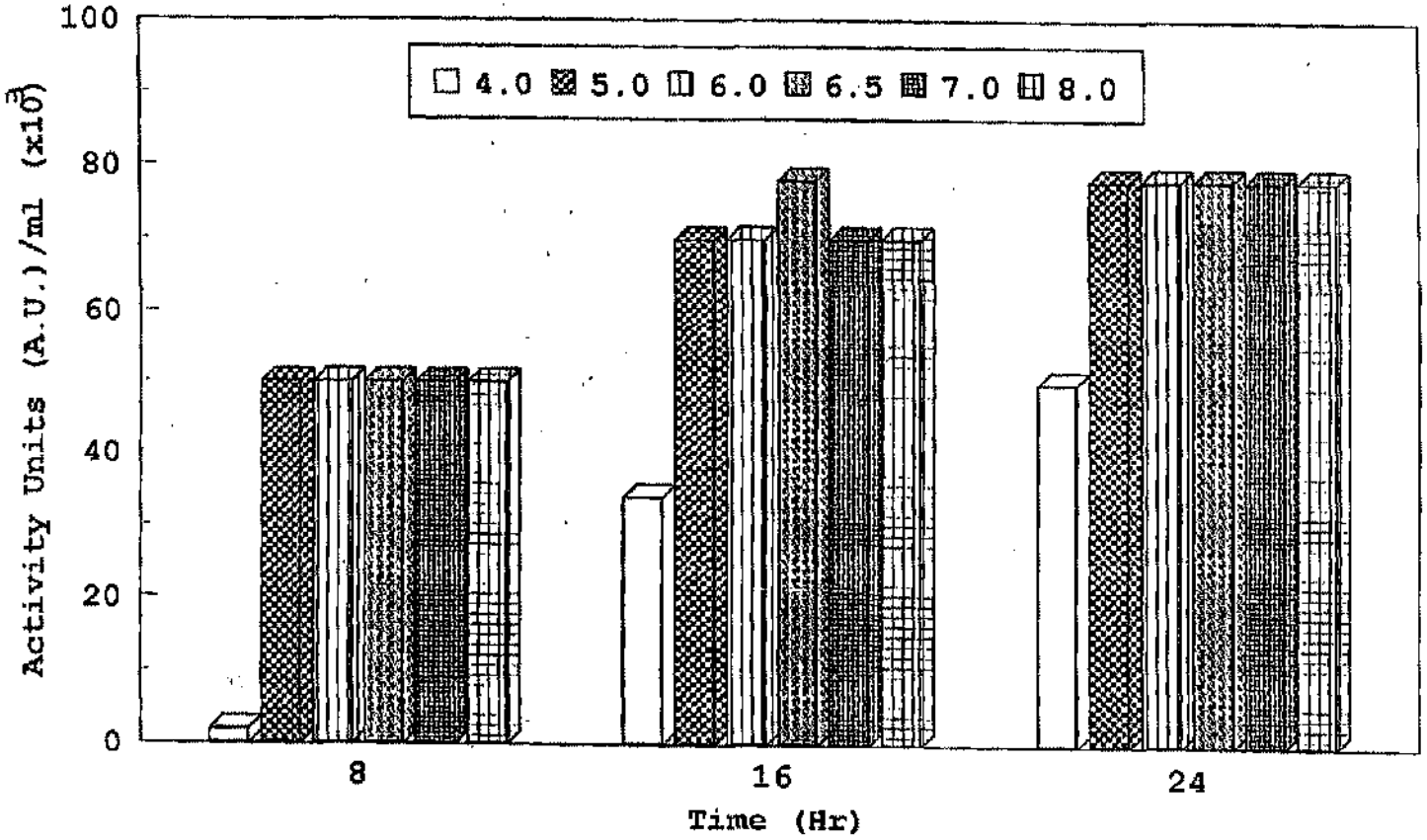


Fig. 4.4 Effect of initial pH of MRS broth on bacteriocin production by Pediococcus pentosaceus 34

observed when *Leu. gelidum* was grown in APT broth at an initial pH lower than 6 and 6.5 (Hastings and Stiles, 1991). Similar reports concerning the effect of initial pH of the growth medium on the bacteriocin production include a decreased production of leuconocin S in APT with an initial pH 7.5 (Lewus *et al.*, 1992) and a 50% decrease in carnosin 44A production by *Leu. carnosum* LA44A when the initial pH of the APT medium was lowered from 6 to 5. The decreased bacteriocin production in most of the cases has been attributed to the reduced cell mass.

It has been observed by several workers (Hurst, 1981; Barefoot and Klaenhammer, 1984; Muriana and Klaenhammer, 1987; Piard *et al.*, 1990; [illegible]; ten Brink *et al.*, 1994 and Kanatani *et al.*, 1995) that maintenance of final pH of the growth medium at a particular level results in increased levels of bacteriocin titres.

4.6.3 INCUBATION TEMPERATURE

It may be seen from the Table 4.12 that *Pediococcus pentosaceus* 34 incubated at 25, 30 and 37°C in MRS broths did not show any variation in the pH, growth and bacteriocin activity after 24 hr of incubation. The pH values recorded at three different incubation temperatures after 24 hr were 3.8, 3.78 and 3.75, respectively while the O.D. values were 5.78, 5.8 and 5.85 at the three respective temperatures. The highest bacteriocin activity attained after 24 hr was 78,000 AU/ml at all the three temperatures. Although there were no differences in bacteriocin production by the selected strain after 24 hr at all the three incubation temperatures, the drop in pH, increase in absorbance and the bacteriocin activity differed significantly after 8

Table 4.12 : Effect of incubation temperature on pH, growth and bacteriocin production by *Pediococcus pentosaceus* 34 in MRS broth

Parameter	Time (Hr)	Temperature (°C)			
		25	30	37	45
pH	0	6.50	6.50	6.50	6.50
	8	5.70	4.82	4.22	5.32
	16	3.98	3.89	3.83	4.43
	24	3.80	3.78	3.75	4.43
Absorbance (600 nm)	0	0.03	0.03	0.03	0.03
	8	0.80	2.67	5.35	0.89
	16	5.18	5.70	5.68	1.37
	24	5.78	5.80	5.85	1.20
Activity Units (A.U.) per ml ($\times 10^3$)	0	0.00	0.00	0.00	0.00
	8	0.20	14.00	50.00	0.20
	16	70.00	70.00	78.00	0.20
	24	78.00	78.00	78.00	0.20

hr of growth. The pH dropped to 5.7, 4.82 and 4.2 at 25, 30 and 37°C, respectively after 8 hr while the absorbance (O.D.) values recorded at the corresponding time were 0.8, 2.67 and 5.35 at the respective temperatures. The bacteriocin activity after 8 hr of incubation at 25, 30 and 37°C was found to be 0.25, 18 and 64 percent of the total bacteriocin activity observed at the respective temperatures after 24 hr incubation. It can also be seen that the highest bacteriocin titre of 78,000 AU/ml was attained after 24 hr of growth at 25°C as well as at 30°C whereas it was attained after 16 hr at 37°C and remained at the highest level without any further decline even at the end of 24 hr of incubation. The incubation of *Pediococcus pentosaceus* 34 at 45°C resulted in a bacteriocin activity of 200 AU/ml after 8 hr and remained at the same level with neither increase nor decrease in the activity upto 24 hr incubation. After 16 hr growth at 45°C in the MRS broth, the pH value dropped to 4.43 and O.D. increased to 1.37. Though pH value remained constant at 4.43 but the absorbance value decreased to 1.2 after 24 hr.

The bacteriocin production as affected by temperature of incubation is given Fig. 4.5. It is evident that though there was no difference in the bacteriocin activity after 24 hr at all the three temperatures of incubation, the activity was maximum at 37°C after 8 hr as well as after 16 hr of incubation.

There were no significant differences in the growth, drop in pH and bacteriocin production when the test culture was grown in MRS broth at either 25°, 30°c or 37°C. However, relatively faster growth (A_{600} = 5.35) at 37°C resulted in the synthesis and secretion of a large proportion (64%) of the bacteriocin into the growth medium in the first 8 hr of the incubation period. The

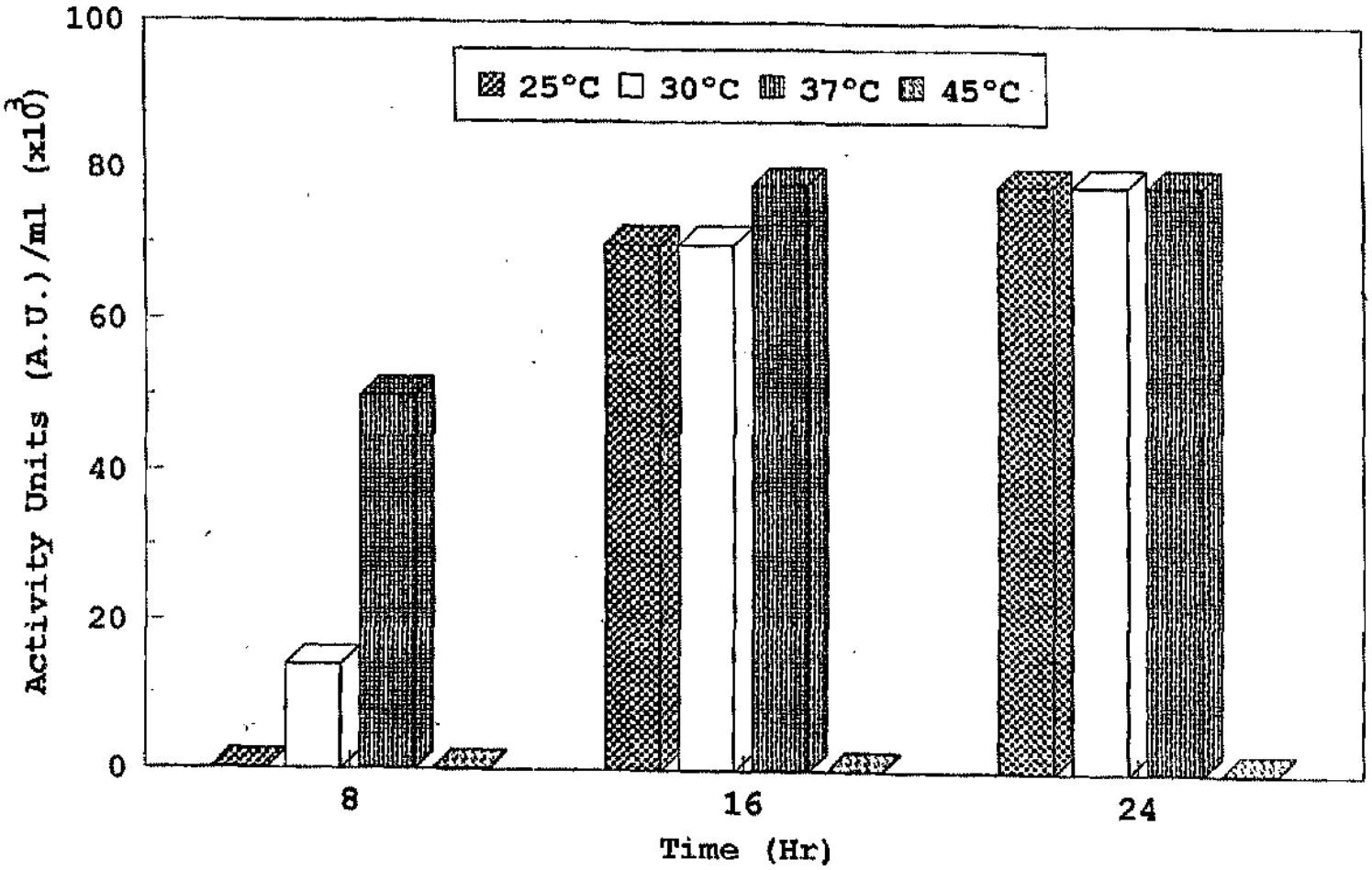


Fig. 4.5 Effect of incubation temperature on bacteriocin production by Pediococcus pentosaceus 34

bacteriocin production was adversely affected when the test culture was grown at 45°C and this could be attributed to very little growth, rather lysis of the producer strain at the end of incubation.

The bacteriocin production by *Lc. lactis* subsp. *lactis* ADRI 85L030 was not significantly dependent on the incubation temperature in the range of 30 to 42°C (Thuault *et al.*, 1991). Biswas *et al* (1991) though reported an identical pediocin AcH titres at 30 and 37°C but there was a slight decrease in producer's cell mass and bacteriocin production at 40°C. Similarly, Schved *et al* (1993) reported the production of pediocin SJ-1 by *P. acidilactici* SJ-1 over the entire temperature range of 30 to 45°C. On the other hand, no detectable pediocin L50 was observed when the producing strain *P. acidilactici* L50 was grown at 45°C. Similar reports concerning the effect of temperature on the bacteriocin production include, the production of carnosin 44A at a temperature of 4-10°C (van Laack *et al.*, 1992), leucocin BTa11a over a wide temperature range of 0-30°C (Felix *et al.*, 1994), and a number of bacteriocins by several *Leuconostoc* spp. (yang and Ray, 1994a). The general observation was that although the bacteriocin production was detected over a wide temperature range, the production was maximum at the optimum temperature of growth of the producer strain and a relatively longer incubation times were needed to achieve the highest bacteriocin titres at low temperatures.

4.6.4 INCUBATION TIME

Pediococcus pentosaceus 34 was grown in MRS broth with an initial pH 6.5 and incubated for upto 72 hr at 37°C. The samples were drawn at regular intervals and

analysed for pH, absorbance and bacteriocin activity. The results are shown in Figs. 4.6 and 4.7.

There was a sharp decline in the pH value in the first 8 hr during which period the pH dropped to 4.2 from an initial value of 6.5. The decrease in the pH thereafter was quite marginal, reaching upto 3.37 at the end of 16 hr incubation and remained more or less constant at the same value upto the 72 hr incubation period.

It may be observed from the Fig. 4.6 that there was no increase in the absorbance value in the first 2 hr. The increase was, however, maximum between 4 and 8 hr of incubation during which the absorbance value increased from 0.65 to 5.4. The increase, thereafter, was marginal reaching a value of 5.76 after 16 hr incubation. The highest value of 6.14 was recorded after 36 hr with a slight decline in absorbance after 48 and 72 hr of incubation.

The changes in the viable cell counts expressed as log cfu/ml are presented in Fig. 4.7. No change in the log cfu/ml during the first 2 hr of incubation was observed. The culture entered the log phase after 4 hr and remained in that phase upto 16 hr as evidenced by a gradual increase in log cfu/ml from 8.8 at 4 hr to 15.1 after 16 hr of growth. There was, however, a brief stationary phase between 16th and 24th hr as a decrease in log cfu/ml to 14.8 was recorded when the culture was plated out on MRS agar at the end of 24 hr. The cells remained in the death phase thereafter with a sharp decline in log cfu/ml after 36 hr of incubation at 37°C.

It is evident from both the Figs. 4.6 and 4.7 that bacteriocin was detected in the growth medium after 4 hr

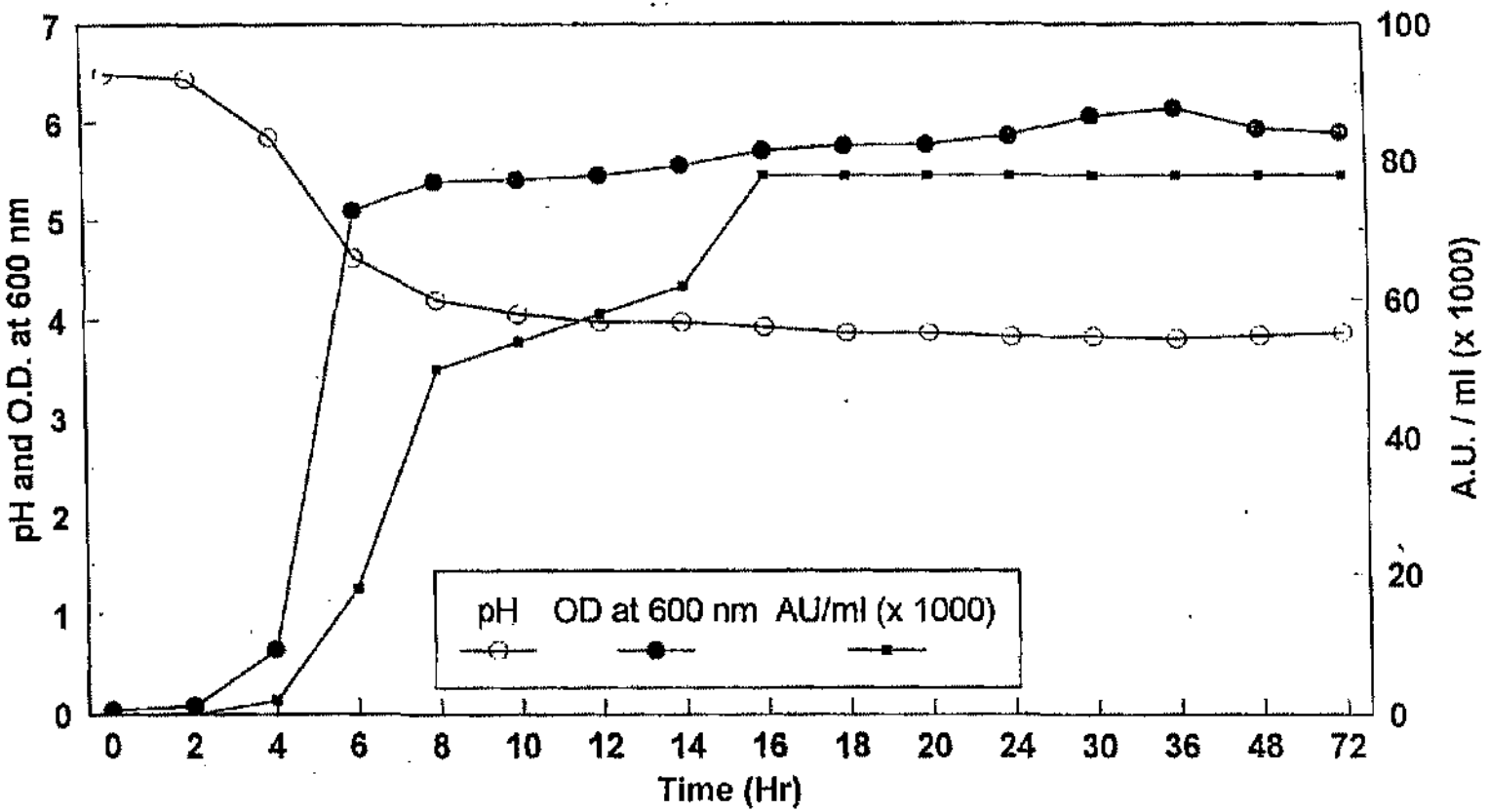


Fig. 4.6 Effect of incubation period on pH, growth and bacteriocin production by *Pediococcus pentosaceus* 34

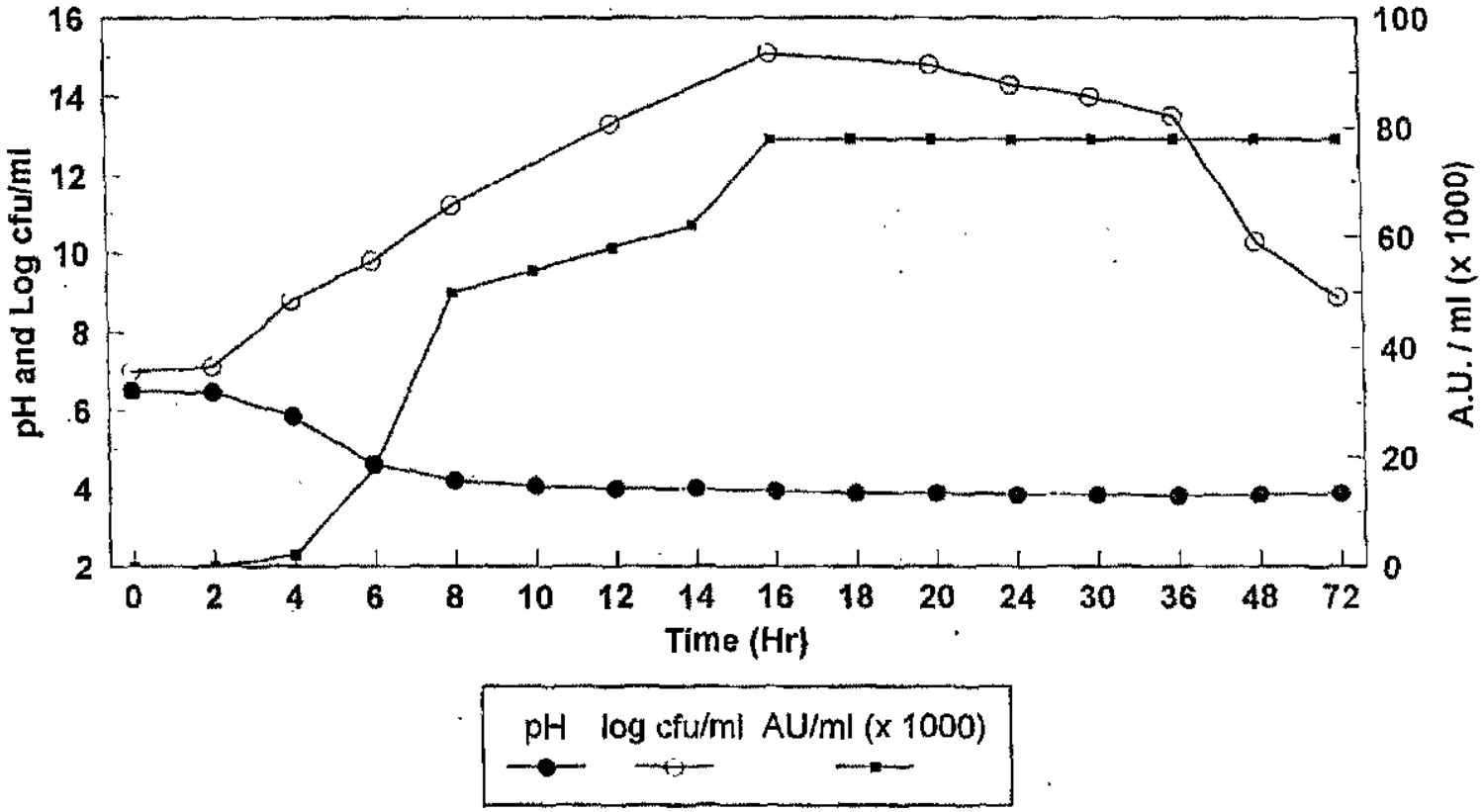


Fig. 4.7 Effect of incubation period on pH, growth and bacteriocin production by *Pediococcus pentosaceus* 34

of incubation coinciding with the onset of log phase of growth of the culture. The bacteriocin production continued throughout the log phase of growth reaching the highest value of 78,000 AU/ml after 16 hr. The bacteriocin titres remained at the highest level with no further decline in the activity during stationary and death phases of growth of the culture upto 72 hr incubation.

The bacteriocin production by *P. pentosaceus* 34 was observed to be growth associated as it was secreted into the growth medium continuously throughout the log phase reaching the highest titres at the end of this phase, thus, indicating it to be a primary metabolite. It was also observed that there was no detectable loss in the bacteriocin activity in the stationary as well as death phases. The maximum production (64%) of the bacteriocin occurred in the first 8 hr of growth while the rest (36%) being produced in the next 8 hr of the incubation.

Pediocin AcH was also secreted as a primary metabolite (Yang and ray, 1994b) with the maximum production being observed in the first 8 hr of incubation but a slight decrease in the bacteriocin activity was also observed after 24 hr growth. The other pediocins such as pediocin 5 (Daba *et al.*, 1991), and pediocin L50 (Cintas *et al.*, 1995) were also produced in the log phase of growth of test cultures, but the bacteriocin titres of both the bacteriocins decreased during the extended periods of incubation. However, the activity of pediocin SJ-1, which was secreted as a secondary metabolite, remained constant for upto 48 hr (Schved *et al.*, 1993).

Earlier, Davey and Pearce (1980) also observed production of diplococcin in the exponential growth phase. However, classical bacteriocin, nisin, has been

described as a secondary metabolite (Hurst, 1981; Ray, 1992a). Although bacteriocin S50 was secreted throughout the log phase, the highest production occurred in the first 8 hr of incubation (Kojic *et al.*, 1991). The production of several bacteriocins of leuconostocs such as leucocin A-UAL187 (Hastings and Stiles, 1991), mesenterocin 52 (Mathieu *et al.*, 1993), carnosin LA54A (Keppler *et al.*, 1994), leucocin B-Talla (Felix *et al.*, 1994) have also been reported to be secreted in the log phase of the growth of the respective producing strains. *Lb. plantarum* LPC010 produced plataricin S in the log phase while plantaricin T was produced by the same strain in the stationary phase (Jimenez-Diaz *et al.*, 1993).

Although not conclusively proved, the decline in the bacteriocin activity reported in the above studies during the extended periods of incubation has been believed to be due to the secretion of inhibitors such as proteolytic enzymes that degrade active bacteriocin molecules.

4.7 PURIFICATION OF THE BACTERIOCIN

The purification of bacteriocin from the culture broth was attempted by different methods viz. pH dependent adsorption-desorption procedure, precipitation with organic solvents and fractionation with ammonium sulfate followed by gel filtration and ion-exchange chromatography for further purification of the bacteriocin.

4.7.1 pH DEPENDENT ADSORPTION-DESORPTION PROCEDURE

It may be observed from the Table 4.13 that harvesting of cells after adjusting the pH of the culture broth to 6.5 resulted in the adsorption of 92.3% of the total bacteriocin on the producer cells while the rest

Table 4.13 : Recovery of bacteriocin at different stages of extraction and purification by pH dependent adsorption-desorption technique

S.No.	Stage	Activity Units (AU)	% AU (Based on AU in culture broth as 100%)
1.	pH adjusted culture broth (500 ml)	1,30,00,000	100
2.	Lost in supernatant	10,00,000	7.69
3.	Lost in phosphate buffer	-	-
4.	Lost with cells after extraction	1,00,00,000	76.92
5.	Dried preparation	16,94,000	13

(7.69%) being lost in the supernatant. The acidic extraction involving stirring of the bacteriocin adsorbed on to producer cells overnight in 0.1 M NaCl solution (pH 1.5) and subsequent freeze-drying of the extracted bacteriocin resulted in an overall recovery of about 13% of the total bacteriocin activity in broth. A 77% loss of the bacteriocin activity alongwith the cells was observed under desorption conditions.

The recovery of the bacteriocin by pH dependent adsorption-desorption technique was not satisfactory as only 13% of the activity could be recovered in the process. Most of the bacteriocin adsorbed onto the cell surface at pH 6.5 could not be desorbed even after 18 hr under acidic conditions (pH 1.5). Yang *et al* (1992) on the other hand have reported more than 90% recovery of the bacteriocin for pediocin AcH, nisin and leuconocin LCm1, and 44% for sakacin A. A similar approach, used with cultures of *Streptococcus pyogenes*, permitted high recoveries of bacteriocin SA-FF22 (Jack and Tagg, 1992). However, using the same method a relatively lower yields of pediocin 5 (10%) (Daba *et al.*, 1994) and diacetin B (Ali *et al.*, 1995) were reported. The 10% recovery of the bacteriocin in case of pediocin 5 was observed after extended period (24 hr) of desorption time as 2 hr desorption period yielded just 3% of the total bacteriocin.

It has been proposed by Jack and Tagg (1992) that cell-associated bacteriocins are probably displaced by the protons under acidic conditions indicating that the cell walls of Gram-positive bacteria may act as a cation exchangers. In the present study, the ionic strength of the acid used may not be sufficient to replace the bacteriocin molecules adsorbed onto the cells surface.

The adsorption of some bacteriocins has been shown to be cell surface receptor mediated. Bhunia *et al* (1991) reported that lipoteichoic acids present in the cell walls of Gram-positive bacteria act as receptors for pediocin AcH and also observed that cell wall preparations after treatment with ethanol : chloroform and hot 20% TCA, completely lost the ability to adsorb pediocin AcH. Therefore, the other possible reason for non-desorption of bacteriocin in this study could be that the receptors, if involved, retained their property to adsorb the bacteriocin molecules under the conditions employed for the desorption.

It may also be possible that the major forces responsible for binding of the bacteriocin molecules to the producer cell wall may be hydrophobic, rather than ionic / electrostatic interactions, making it difficult for desorption of the bacteriocin from cell surface of the test culture.

Further studies are thus needed for the effective extraction of the bacteriocin adsorbed onto the cell surfaces and to employ this cost effective method for the recovery of the bacteriocin of *P. pentosaceus* 34 culture.

4.7.2 SOLVENT FRACTIONATION OF THE BACTERIOCIN

The bacteriocin from the culture supernatant was recovered by fractionation with various organic solvents viz. acetone, ethanol, isopropanol and methanol (Table 4.14). The bacteriocin precipitated between 0.0 to 1.0 volume of ethanol and methanol exhibited a recovery of 5.7 and 4.57%, and 5.5 and 8.21-fold increase in specific activity, respectively. The fractionation of the bacteriocin between 1.0-2.0 and 2.0-3.0 volumes of ethanol and methanol and at all the three volumes i.e.

Table 4.14 : **Fractionation of the bacteriocin of *Pediococcus pentosaceus* 34 with different organic solvents**

Solvent	Solvent volume per volume of culture supernatant	Total bacteriocin (AU)	Total protein (mg)	Specific activity (A.U./mg protein)	Purification (fold)	Recovery (%)
		35,00,000	485	7,216.5	1	100
Isopropanol	0.0-1.0	40,000	2.0	20,000	2.77	1.14
	1.0-2.0	4,000	11.2	357	0.04	0.11
	2.0-3.0	8,000	20.1	398	0.05	0.23
Methanol	0.0-1.0	1,60,000	2.7	59,259.3	8.21	4.57
	1.0-2.0	40,000	3.4	11,764.7	1.63	1.14
	2.0-3.0	40,000	3.4	11,764.7	1.63	1.14
Ethanol	0.0-1.0	2,00,000	5.0	40,000	5.50	5.70
	1.0-2.0	4,000	2.35	1,702:12	0.24	0.11
	2.0-3.0	4,000	4.65	860.21	0.12	0.11
Acetone	0.0-1.0	24,000	3.7	6,486.5	0.90	0.69
	1.0-2.0	12,800	20.0	640	0.09	0.37
	2.0-3.0	4,000	5.6	714.29	0.10	0.11

0.0-1.0, 1.0-2.0 and 2.0-3.0 of isopropanol and acetone resulted in a very low recovery and a decrease in the specific activity of bacteriocin.

The fractionation of the bacteriocin produced by *P. pentosaceus* 34 using different organic solvents viz. acetone, methanol, ethanol, isopropanol not only afforded very low yields but also resulted in a decrease in the specific activity of bacteriocin preparations, indicating the adverse effect of the organic solvents on the bacteriocin activity of the culture supernatants.

Although recovery of enzymes by fractionation with organic solvents is a common practice, virtually no such reports are available on the solvent fractionation of bacteriocins of lactic acid bacteria.

4.7.3 AMMONIUM SULFATE PRECIPITATION

The results presented in the Table 4.15 show that fractionation of the bacteriocin between 0-40% and 40-60% ammonium sulfate saturation exhibited a recovery of 5.71 and 20% of the total bacteriocin activity with a 1.58 and 3.53-fold increase in specific activity, respectively. Ammonium sulfate saturation at higher levels i.e. 60-80% and 80-100% resulted in very low bacteriocin recovery as well as a decrease in the specific activity. It was however, observed that the bacteriocin precipitated between 30-60% ammonium sulfate saturation exhibited 34.3% recovery of the bacteriocin concomitant with 3.1-fold purification.

Ammonium sulfate precipitation is the most common and widely used method to concentrate the antibacterial proteins from the culture supernatants of bacteriocin

Table 4.15 : Ammonium sulfate fractionation of the bacteriocin of *Pediococcus pentosaceus* 34

Ammonium sulfate saturation (%)	Total Bacteriocin (AU)	Total Protein (mg)	Specific Activity (A.U./mg protein)	Purification (fold)	Recovery (%)
-	70,00,000	970	7,216.5	1	100
0-40	4,00,000	35	11,428.6	1.58	5.71
40-60	14,00,000	55	25454.54	3.53	20.00
60-80	1,00,000	43.5	229.9	0.32	1.43
80-100	1,600	29.6	54.05	0.01	0.02
0-30	2,20,000	13.75	16,000	2.20	3.14
30-60	24,00,000	108	22,222.2	3.10	34.30

producing lactic cultures and the procedure results in varying degrees of recovery and purity of different bacteriocins.

Lozano *et al* (1992) obtained a 4-fold purified pediocin PA-1 with a 40% recovery using a 0-60% ammonium sulfate saturation while a 3-fold increase in the activity of pediocin PO2 upon precipitation with ammonium sulfate (0-60% saturation) was reported by Coventry *et al* (1995). A slightly higher levels of purity (10-fold) was observed when a 70% ammonium sulfate saturation was used to precipitate pediocin AcH (Bhunia *et al.*, 1988). Similarly, Cintas *et al* (1995) observed a 9-fold purification of pediocin L50 upon ammonium sulfate precipitation.

The other bacteriocins of LAB precipitated with ammonium sulfate include: lactococcin G, which was purified to a 35-fold with a recovery of 57% (Nissen Meyer *et al.*, 1992), carnosin 44A with 4-fold increase in the activity using 0-60% saturation (vaan Laack *et al.*, 1992) while Piard *et al* (1992) obtained a 455-fold purified lacticin 481 preparation upon reprecipitation with ammonium sulfate (0-80%) of the pellet recovered from the culture supernatant using 0-60% saturation.

The variations observed in the degree of purity among various bacteriocins of LAB upon concentration with ammonium sulfate is probably due to the differences in concentration of contaminating proteins which lead to decrease in the specific activity of the bacteriocin preparations.

4.7.4 GEL FILTRATION AND ION-EXCHANGE CHROMATOGRAPHY

4.7.4.1 Effect of Buffers on the Activity of the Bacteriocin

The bacteriocin dissolved in various buffers such as acetate, phosphate and Tris-HCl having different pH values of 4.0, 4.6 and 5.2; 5.8, 6.4 and 7.0; and 7.2, 7.6 and 8.0, respectively retained 100% activity both at refrigeration (5°C) as well as at room (37°C) temperatures even after 3 days of storage (Table 4.16).

The bacteriocin of *P. pentosaceus* remained stable at both the room as well as at refrigeration temperatures in all the buffers in the pH range 4-8 thus allowing the use of any such buffer in the further purification studies. The buffers that have been used in the purification of bacteriocins by some workers include acetate buffer for mesentericin Y105 (Hechard *et al.*, 1992), pediocin SJ-1 (Schved *et al.*, 1993), phosphate buffer for lacticin 481 (Piard *et al.*, 1992), pediocin PA-1 (Lozano *et al.*, 1992), pediocin L50 (Cintas *et al.*, 1995) and Tris-HCl buffer was used in the purification of pediocin AcH (Bhunia *et al.*, 1988).

4.7.4.2 Gel Filtration through Sephadex G-25 and G-50

The bacteriocin dissolved in 0.1 M phosphate buffer (pH 6.0) containing 0.1% SDS and 0.04% sodium azide was applied on to a column of Sephadex G-25 as well as Sephadex G-50 which were equilibrated with the same buffer containing both SDS and sodium azide. The bacteriocin was eluted from both the columns in the first

Table 4.16 : Effect of different buffers on the bacteriocin activity of *Pediococcus pentosaceus* 34

Buffer	pH	Activity Units (AU/ml)					
		5°C			37°C		
		24 Hr	48 Hr	72 Hr	24 Hr	48 Hr	72 Hr
Acetate	4.0	20,000	20,000	20,000	20,000	20,000	20,000
	4.6	20,000	20,000	20,000	20,000	20,000	20,000
	5.2	20,000	20,000	20,000	20,000	20,000	20,000
Phosphate	5.8	20,000	20,000	20,000	20,000	20,000	20,000
	6.4	20,000	20,000	20,000	20,000	20,000	20,000
	7.0	20,000	20,000	20,000	20,000	20,000	20,000
Tris	7.2	20,000	20,000	20,000	20,000	20,000	20,000
	7.6	20,000	20,000	20,000	20,000	20,000	20,000
	8.0	20,000	20,000	20,000	20,000	20,000	20,000

few fractions immediately after the void volume. Almost whole of the bacteriocin activity was obtained in these fractions without any increase in the specific activity .

Sephadex G-25 and Sephadex G-50 gel resins have molecular weight fractionation ranges of about 1500-5000 and 1500-30000 Daltons, respectively. The bacteriocin applied onto the gel columns did not get fractionated as was revealed by the estimation of protein content and bacteriocin activity of the pooled fractions obtained during the gel filtration experiment. It was also observed that both the ammonium sulfate precipitated material and concentrated pooled gel filtration fractions showed an identical protein profile on the SDS-PAGE gel.

The formation of aggregates having a molecular weight in excess of 30000 with other bacteriocin molecules or other proteins present in the sample could be one of the reasons for the non-fractionation of the bacteriocin sample by the gel resins. The formation of large aggregates of bacteriocins has also been observed for other bacteriocins such as helveticin J (Joerger and Klaenhammer, 1986) and lactacin F (Muriana and Klaenhammer, 1991). SDS used at 0.1% level in the eluent was not sufficient to dissociate the bacteriocin aggregates. However, helveticin J was purified by gel filtration through sephadex G-200 using 0.1% SDS as the dissociating agent for the bacteriocin aggregates. Several other bacteriocins of LAB that have been purified by gel filtration include lactocin S (Mortvedt *et al.*, 1991), lactacin F (Muriana and Klaenhammer, 1991),

lacticin 481 (Piard *et al.*, 1992) and pediocin PA-1 (Lozano *et al.*, 1992).

4.7.4.3 Selection of pH of the Buffer and Type of Ion-exchanger

The bacteriocin suspended in various buffers of different pH values was added to ion-exchange resins in the test tubes equilibrated with the corresponding buffer having the respective pH value.

It may be seen from the Table 4.17 that the bacteriocin failed to adsorb to the cation-exchanger (CM-Sephadex C-25) at all the pH values in the pH range of 4-7. Since the total bacteriocin (20,000 AU) added to the ion-exchanger remained in the supernatant, it did not get adsorbed to the resin. However, the bacteriocin adsorbed on to the anion exchanger (DEAE-Sephadex A-25) equilibrated with Tris-HCl buffer having pH values of 7.2, 7.6 and 8. Thus a starting pH of 7.2 and anion-exchanger (DEAE-Sephadex A-25) were selected for further purification of the bacteriocin.

The failure of the bacteriocin to adsorb onto the cation-exchager (CM-Sephadex C-25) at pH values ranging from 4-7 indicates that the bacteriocin under these conditions carries a net negative charge and such a bacteriocin must be rich in acidic amino acid residues like asparatic acid and glutamic acid and also it may contain very less number of basic amino acids like arginine and lysine. The present observation is, however, at variance with several other bacteriocins including diplococcin (Davey and Richardson, 1981), lactococcin (Dufour *et al.*, 1991), lactococcin A (Holo *et*

Table 4.17 : Selection of pH of the buffer and type of ion exchanger for the purification of bacteriocin of *Pediococcus pentosaceus* 34

Type of ion-exchager	Buffer	pH	Bacteriocin adsorbed (AU)	Bacteriocin in supernatant (AU)
Cation Exchanger (CM Sephadex C-25)	Acetate	4.0	0	20,000
		4.5	0	20,000
		5.0	0	20,000
		5.5	0	20,000
	Phosphate	6.0	0	20,000
		6.5	0	20,000
		7.0	0	20,000
Anion Exchanger (DEAE Sephadex A-25)	Tris	7.2	16,000	4,000
		7.6	16,000	4,000
		8.0	16,000	4,000

al., 1991), lactococcin G (Nissen-Meyer *et al.*, 1992) produced by lactococcal strains, mesenterocin 52 (Sudirman *et al.*, 1994) of *Leuconostoc* sp., lactocin S (Mortvedt *et al.*, 1991) and acidocin A (Kanatani *et al.*, 1995) of lactobacilli and pediocin PA-1 (Henderson *et al.*, 1992; Lozano *et al.*, 1992), pediocin SJ-1 (Schved *et al.*, 1993) and pediocin L50 (Cintas *et al.*, 1995) produced by different strains of *Pediococcus* sp.

The bacteriocin of *P. pentosaceus* 34 did bind to the anion-exchanger (DEAE-Sephadex A-25) at pH values ranging from 7.2-8 indicating again that it carries a net negative charge at the above range of pH values. Bhunia *et al* (1988) reported the purification of pediocin AcH by binding it to the anion-exchanger.

Bacteriocins of LAB in general are cationic hydrophobic peptides and have been purified to a great extent by using a cation-exchange chromatography. In the present study, the bacteriocin did not bind to the cation-exchanger but was bound to the anion-exchanger. The abnormal behaviour of the bacteriocin of *P. pentosaceus* 34 may also be due to its formation of aggregates with other media components that carry a net negative charge. Henderson *et al* (1992) observed that the aggregation apparent in crude preparations of pediocins was more likely of heterogeneous nature involving other media components rather than self aggregation.

Some bacteriocins such as a highly hydrophobic gassericin A did not adsorb either to DEAE- or CM-exchangers (Kawai *et al.*, 1994).

4.7.4.4 Selection of the Starting Ionic Strength to Adsorb and Elute the Bacteriocin

The bacteriocin in Tris-HCl buffer (pH 7.2) was added to the DEAE-Sephadex A-25 anion exchange resin in test tubes which was equilibrated with increasing ionic strengths of NaCl and the bacteriocin activity in the supernatant was assayed. It may be seen from the Table 4.18 that the bacteriocin adsorbed equally well to anion-exchange resin at all the concentrations of NaCl (0.1-0.4 M) employed in the study.

The bacteriocin of *P. pentosaceus* 34 did bind to the DEAE-Sephadex A-25 resin equilibrated with various concentrations (0.05-0.4 M) of sodium chloride. A moderate ionic strength of 0.5 M is generally employed to elute the proteins adsorbed on to Sephadex ion-exchange resin. Thus, the binding of bacteriocin at a moderately higher concentration of NaCl (0.4 M) indicates a relatively large negative charge density on the molecule. Salt solutions of higher ionic strength (> 0.5 M), however, could not be used as the bacteriocin was found to be unstable in salt solutions of higher concentration. Bhunia *et al* (1988) eluted the pediocin AcH bound to the anion-exchanger column using a linear salt gradient of 0-1 M.

It may be suggested from the binding profiles of the *P. pentosaceus* 34 bacteriocin onto the ion-exchanger and bacterial cell wall that a significant portion of the bacteriocin is hydrophobic in nature and at the same time it contains relatively more number of acidic amino acids.

Table 4.18 : Selection of the starting and eluting ionic strengths for the purification of *Pediococcus pentosaceus* 34 bacteriocin by DEAE-Sephadex ion exchanger

S.No.	Ionic strength of NaCl (M)	Bacteriocin activity in the supernatant (AU)
1	0.00	4,000
2	0.05	4,000
3	0.10	4,000
4	0.15	4,000
5	0.20	4,000
6	0.25	4,000
7	0.30	4,000
8	0.35	4,000
9	0.40	4,000

4.8 CHARACTERISATION OF BACTERIOCIN

The partially purified bacteriocin was characterised with respect to its inhibitory spectrum, molecular weight, enzyme sensitivity, heat and pH stability, mode of action and the effect of surfactants and salt on the bacteriocin activity (Tables 4.19 to 4.23).

4.8.1 INHIBITORY SPECTRUM

The inhibitory spectrum of partially purified bacteriocin was determined by agar-well assay (3.4.2) against several bacterial species and a few yeasts. It may be seen from the Table 4.19 that out of the nine lactococcal cultures tested, only two viz. *Lc. lactis* subsp. *cremoris* NCDC83 and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* NCDC64 were found to be sensitive to the action of the bacteriocin. The bacteriocin was, however, found to be ineffective against all the strains of *Lb. acidophilus, Lb. casei* subsp. *casei, Lb. sporogenes, Lb. delbrueckii* subsp. *bulgaricus* and several other *Lactobacillus* spp. isolated from meat products. The bacteriocin inhibited *Lb. plantarum* NCDC21 whereas two other strains of the same species were resistant to it. A high percentage (63.6%) of the leuconostocs were found to be sensitive to the bacteriocin action. Out of the 22 strains of *Leuconostoc* spp. tested for their sensitivity to the bacteriocin as many as 14 were inhibited by the bacteriocin. The bacteriocin exerted antibacterial activity against a majority of leuconostocs isolated from spoiled meat products. The bacteriocin inhibited only one strain of pediococci i.e. *Pediococcus* sp. M3 while the other four strains used were insensitive to the bacteriocin.

Table 4.19 : Inhibitory spectrum of *Pediococcus pentosaceus* 34 bacteriocin

Bacteria	Inhibition
LACTIC ORGANISMS:	
Lc. lactis subsp. *cremoris*	
NCDC 82	-
83	+
86	-
Lc. lactis subsp. *lactis*	
NCDC 91	-
94	-
96	-
Lc. lactis subsp. lactis biovar. *diacetylactis*	
NCDC 60	-
61	-
64	+
Lb. acidophilus	
NCDC 13	-
14	-
15	-
Lb. casei subsp. *casei*	
NCDC 17	-
19	-
Lb. plantarum	
NCDC 20	-
21	+
22	-
Lb. sporogenes	
NCDC 20	-
Lb. delbrueckii subsp. *bulgaricus*	
NCDC 9	-
Lactobacillus sp.	
Lb 1	-
Lb 2	-
4 L	-
5 L	-
21 L	-
25 L	-
Leu. mesenteroides subsp. *dextranicum*	
NCDC 30	+

34	–
143	+
Leu. mesenteroides subsp. *mesenteroides*	
NCDC 31	–
Leuconostoc sp.	
30 L	+
3 L	+
4 L	+
5 L	+
M 8	+
29 L-1	+
29 L-2 to 29 L-7	+
TOM 1	–
TOM 5	–
LM 1	–
LM 2	+
TA 5	+
CTA 5	+
AL	–
B 3	+
1 H 2	–
T 7 L	–
Pediococcus sp.	
M 3	+
P. pentosaceus	
NCDC 35	–
P. acidilactici	
NCIM 2272	–
P. pentosaceus	
NCIM 2296	–
P. cerevesiae	
NCIM 2171	–
NON-LACTIC CULTURES	
Gram Positive Bacteria	
Enterococcus faecalis	
MB 1	+
NCDC 116	+
NCDC 119	+
NCDC 122	+
E. faecium	
NCDC 124	+

Bacillus cereus	
NCDC 66	+
Bacillus polymyxa	
NCDC 68	-
Bacillus subtilis	
NCDC 71,70	-
staphylococcus aureus	
NCDC 109	+
NCDC 110	+
Micrococcus flavus	
NCDC 131	+
Listeria monocytogenes	
MTCC 657	+
MTCC 1143	+
Scott A	+
V 7	+
L . ivanovii	+
L . innocua	
111	+
Gram Negative Bacteria:	
Escherichia coli	
NCDC 134	-
Ps. aeruginosa	
NCDC 105	-
Proteus vulgaris	
NCDC 73	-
Serratia marcescenes	
NCDC 108	-
Salmonella typhi	
NCDC 113	-
Enterobacter aerogenes	
NCDC 106	-
Yeasts:	
Saccharomyces cerevesiae	
NCDC 45	-
Saccharomyces cerevesiae	
NCDC 47	-

The bacteriocin inhibited all the four strains of *Enterococcus faecalis* and one strain of *E. faecium*. Among the *Bacillus* spp., *B. cereus* NCDC66 was inhibited while *B. polymyxa* NCDC68 and *B. subtilis* NCDC71 and 70 were insensitive. The partially purified bacteriocin was effective against both the strains of *Staph. aureus* and also *Micrococcus luteus* NCDC131. All the *Listeria* Spp. (four strains of *L. monocytogenes*, one strain each of *L. ivanovii* and *L. innocua*) included in the spectrum of bacteriocin activity were found to be sensitive to the bacteriocin (Table 4.19).

All the Gram negative bacteria and yeasts tested were insensitive to the action of the bacteriocin.

Bacteriocins of LAB on the basis of their inhibitory spectrum have been divided into two major classes: one includes bacteriocins active against bacteria that are taxonomically related to the producer while the second class is composed of those bacteriocins that exhibit a relatively broad range of activity extending across numerous Gram positive bacteria. The bacteriocin produced by *P. pentosaceus* 34 exhibited a wide spectrum of activity inhibiting not only closely related lactic acid bacteria but also other Gram positive bacteria including *B. cereus*, *Staph. aureus* etc. The remarkable feature of the bacteriocin is its extreme antagonistic activity against different strains of *L. monocytogenes* but at the same time it was relatively ineffective against most of the strains of LAB which are used as starter cultures in the manufacture of fermented milk products. Among the LAB, leuconostocs were found to be the most sensitive with lactococci, lactobacilli and pediococci being relatively insensitive to the bacteriocin. In most cases the bacteriocin action was

found to be strain specific, rather than species specific. It failed to inhibit Gram negative bacteria and yeasts.

Although bacteriocins of pediococci described till date possess a broader spectrum of antibacterial activity, there are variations in their inhibitory pattern against several indicator bacteria. In contrast to *P. pentosaceus* 34 bacteriocin, one of the two most thoroughly characterised bacteriocins, pediocin PA-1 was ineffective against the strains of *Staph. aureus* (Henderson *et al.*, 1992). Similarly, pediocin SJ-1 was also reported to be not active against *Staph. aureus* (Schved *et al.*, 1993). Though pediocin AcH produced by *P. acidilactici* H also has a broader spectrum of antibacterial activity (Bhunia *et al.*, 1988), but its action against strains of *B. cereus* has not been reported. However, pediocin L50 was active against a strain of *B. cereus* (Cintas *et al.*, 1995). The wider spectrum of antibacterial activity of *P. pentosaceus* 34 inhibiting numerous Gram positive foodborne pathogenic organisms makes it a potent candidate for the biopreservation of foods.

Similarly, other bacteriocins of LAB exhibiting a wide spectrum of activity include nisin (Hurst, 1981; Ray, 1992a), lacticin 481 (Piard *et al.*, 1990) and bacteriocin J46 (Gonzalez *et al.*, 1996) from lactococci, plantaricin S (Jimenez-Diaz *et al.*, 1993) and acidocin B (ten Brink *et al.*, 1994) from lactobacilli. Bacteriocins of *Leuconostoc* spp. described till date besides being effective against closely related species (mostly strains of leuconostocs) have also been found to exhibit antilisterial activity. Such bacteriocins include mesenterocin 52 (Mathieu *et al.*, 1993), carnocin LA54A

(Keppler *et al.*, 1994) and dextranicin J24 (Sudirman *et al.*, 1994). The antibacterial activity of mesentericin Y105 was restricted only to listerial strains (Hechard *et al.*, 1992).

4.8.2 MOLECULAR WEIGHT

The partially purified bacteriocin preparation obtained by pH dependent adsorption-desorption procedure was run on SDS-PAGE. One part of the gel with molecular weight markers was stained with Brilliant Blue G and one part of the gel was used to determine bacteriocin activity.

It may be observed from the Plate 8 A that the gel stained with the dye had shown three protein bands with molecular weights between 6.1 and 14 KDa in the bacteriocin sample (Lane B and D). The gel overlaid with the indicator strain gave clear zones of inhibition (Plate 8 B). Super imposing of stained gel over the gel used to detect the bacteriocin activity revealed that the three protein bands detected in the stained gel did not possess the antibacterial activity (Plate 8 C). The protein band corresponding to the inhibition zone could have molecular weight between 3.5 to 6.1 KDa (Plate 8 C). The active protein band, however, could not be detected by Brilliant Blue G staining of the gel.

The failure to visualise the active protein band in the SDS-PAGE gel in this study may be due to very low concentration of the bacteriocin protein or the poor binding of the dye Brilliant Blue G. This phenomenon was also observed while estimating the molecular weights of mesentericin Y105 (Hechard *et al.*, 1992) and carnosin LA44A (van Laack *et al.*, 1992) by SDS-PAGE.

Plate 8. Determination of molecular weight of partially purified *P. pentosaceus* 34 bacteriocin by SDS-PAGE

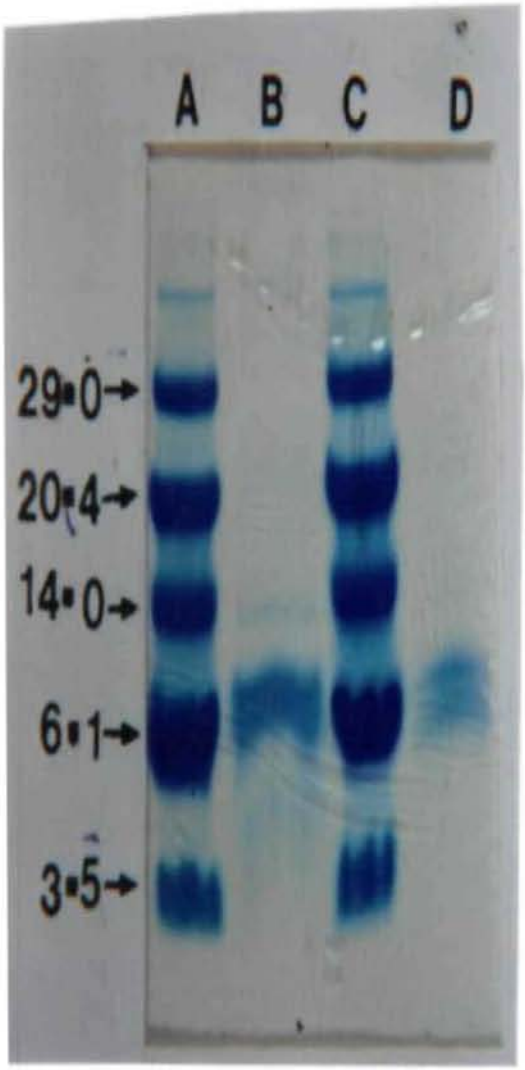
A) Gel stained with Brilliant Blue G

B) Gel depicting the bacteriocin activity

C) Gel stained with Brilliant Blue G superimposed on gel depicting the bacteriocin activity

Lane A & C : Molecular weight markers (MDa)

Lane B & D : Bacteriocin preparation



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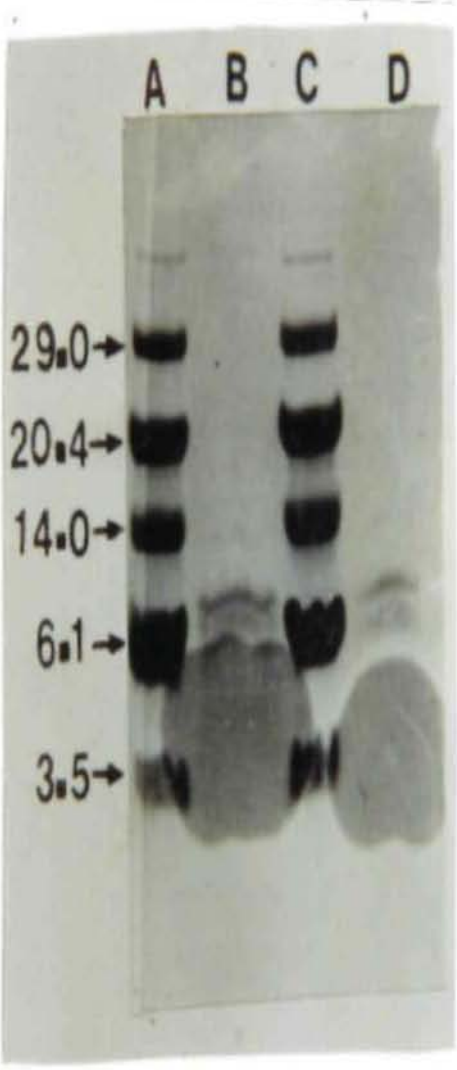


C

The bacteriocin preparation purified by pH dependent adsorption-desorption method had shown three major protein bands. Similarly, sakacin A and leuconocin LCM1 purified by the same method showed several protein bands in the SDS-PAGE gels (Yang *et al.*, 1992). These bands are probably surface or cell wall proteins. Since the active protein band could not be stained, the direct detection of bacteriocin activity in the unstained SDS-PAGE gels and the subsequent comparison with the stained gel allowed the estimation of the apparent molecular weight of the bacteriocin of *P. pentosaceus* 34 to be in the range of 3500-6100 Da. Using a similar approach, the molecular weights of mesentericin Y105 and carnosin LA44A were estimated to be 2.5-3 KDa (Hechard *et al.*, 1992) and 2.5-6 KDa (van Laack *et al.*, 1992), respectively. The molecular masses of several other bacteriocins viz. pediocin 5 (Daba *et al.*, 1991), acidocin B (ten Brink *et al.*, 1994) have also been estimated by this method.

Bacteriocins of LAB in general have been characterised as low molecular weight substances. Molecular weights reported for various pediocins are in the range 3-6 KDa. Pediocin AcH, pediocin PA-1 as well as pediocin 5 have a molecular weight of about 4.6 KDa (Motlagh *et al.*, 1992; Henderson *et al.*, 1992; Lozano *et al.*, 1992; Daba *et al.*, 1994).

Several other low molecular weight (< 10,000 Da) bacteriocins of LAB include nisin (Hurst, 1981), diplococcin (Davey and Richardson, 1981), lacticin 481 (Piard *et al.*, 1992), lactococcin G (Nissen-Meyer *et al.*, 1992), diacetin B (Ali *et al.*, 1995) from lactococci, leucocin A-UAL187 (Hastings and Stiles, 1991), mesentericin Y105 (Hecahrd *et al.*, 1992), carnocin LA54A

(Keppler *et al.*, 1994) from leuconostocs and lactacin B (Barefoot and Klaenhammer, 1984) and acidocin B (ten Brink *et al.*, 1994), plantaricins S and T (Jimenez-Diaz *et al.*, 1993). Very few bacteriocins of lactobacilli have been reported to have high molecular weights in excess of 30,000 Da (Joerger and Klaenhammer, 1986; Vaughan *et al.*, 1992).

4.8.3 ENZYME SENSITIVITY

The sensitivity i.e. loss of activity of the crude and partially purified bacteriocins of *P. pentosaceus* 34 to various proteolytic and non-proteolytic enzymes is shown in Plates 9 A and 9 B and the residual activity of the bacteriocin after treatment with enzymes is given in the Fig. 4.8. It may be seen from the Plate 9 A that the bacteriocin was sensitive to all the 12 proteolytic enzymes viz. trypsin, chymotrypsin, pepsin, proteinase-K, pronase-E, ficin, papain and proteases I, IV, XIII, XVI and XXIII (spots 3-14). The non-proteolytic enzymes such as catalase, ribonuclease A, lipase (spots 2, 16 and 17, respectively in Plate 9 A), β-amylase and lysozyme (spots 4 and 5, respectively in Plate 9 B) were ineffective against the bacteriocin, both in crude and partially purified forms. However, the bacteriocin was inactivated by two different α-amylase preparations obtained from Sigma Chemical Co., USA (spot 15 in Plate 9 A and spots 2 and 3 in Plate 9 B).

It may be seen from the Fig. 4.8 that there was a complete loss in activity upon treatment of both crude and partially purified bacteriocins with all the proteolytic enzymes (bars 3-14) and α-amylase (bar 15). The bacteriocin preparations treated with other non-proteolytic enzymes such as catalase, β-amylase,

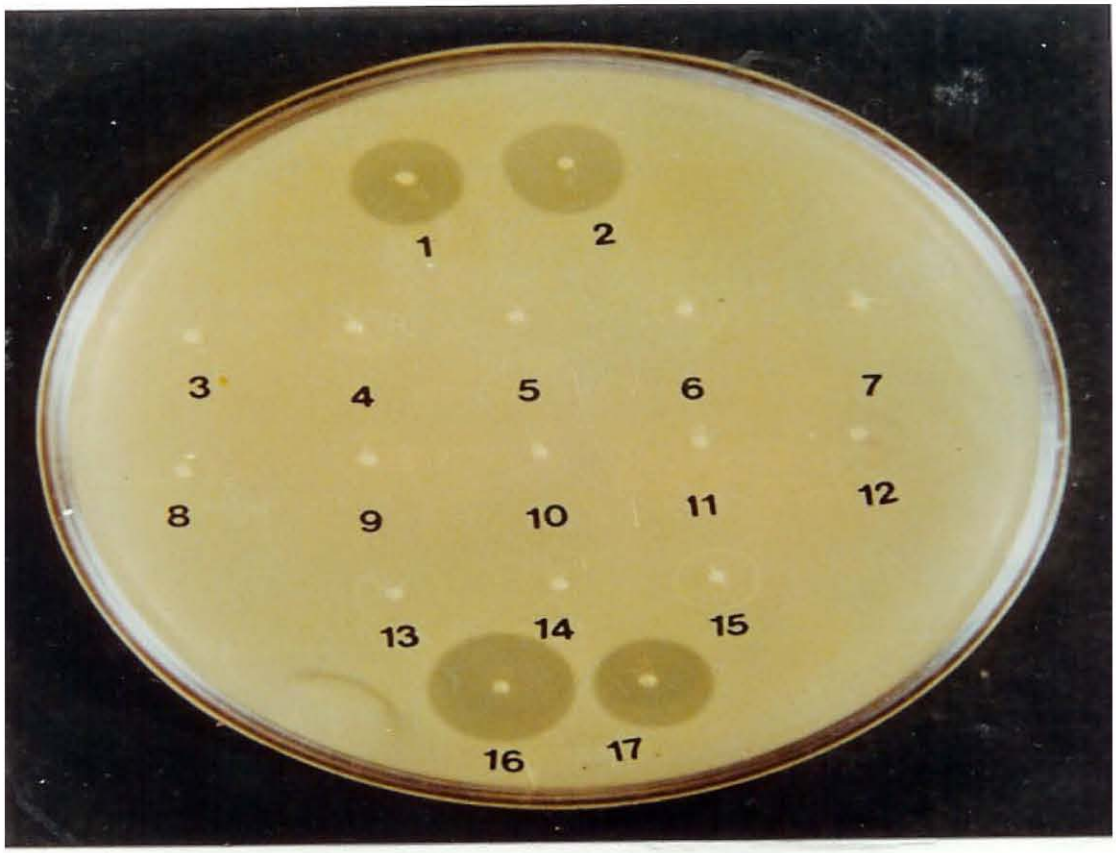
Plate 9. Sensitivity of *Pediococcus pentosaceus* 34 bacteriocin to different proteolytic and non-proteolytic enzymes

A

1. Control
2. Catalase
3. Trypsin
4. Chymotrypsin
5. Pepsin
6. Proteinase-K
7. Pronase-E
8. Ficin
9. Papain
10. Protease I
11. Protease IV
12. Protease XIII
13. Protease XVI
14. Protease XXIII
15. α-amylase
16. Ribonuclease A
17. Lipase

B

1. Control
2. α-amylase (Preparation i)
3. α-amylase (Preparation ii)
4. β-amylase
5. Lysozyme



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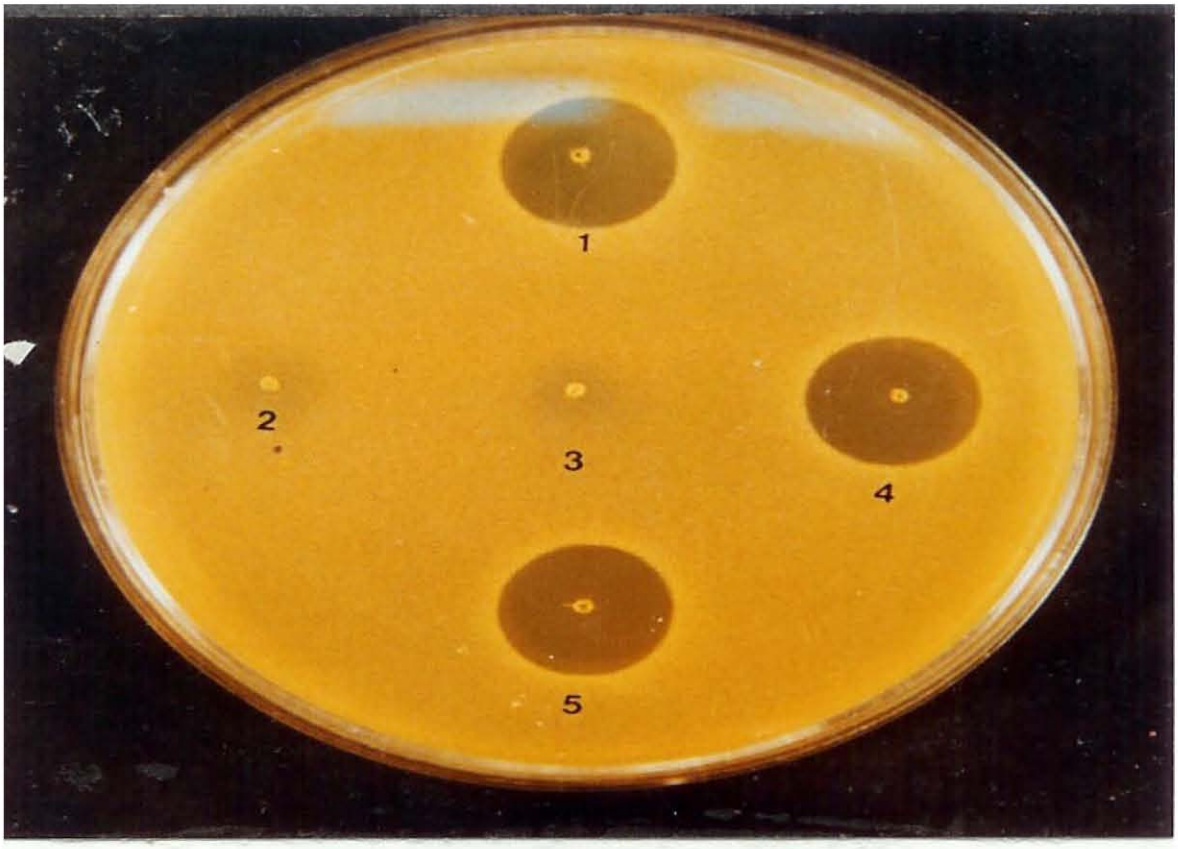

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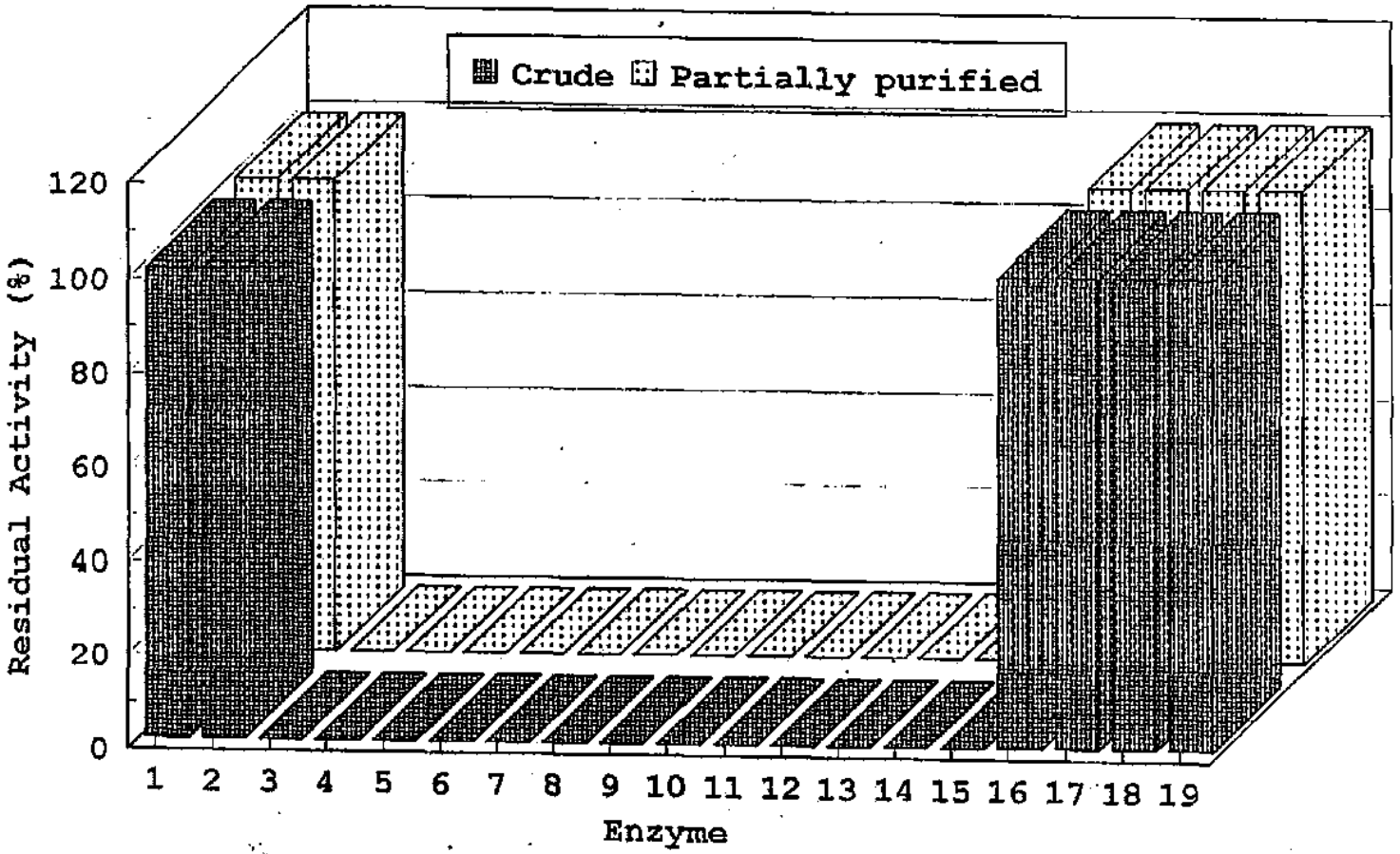

Fig. 4.8 Effect of enzymes on the activity of Pediococcus pentosaceus 34 bacteriocin

lipase, lysozyme and ribonuclease A (bars 2 and 16-19, respectively) retained 100% of their initial activity.

As bacteriocins are proteinaceous in nature, they are inactivated by at least one protease. The bacteriocin of *P. pentosaceus* - 34 was found to be sensitive to several proteolytic enzymes. It was not susceptible to the action of lipase thus indicating that no lipid moiety is associated with the antibacterial property and insensitivity to lysozyme reveals that there is no role for peptidoglycon moieties in the inhibitory activity. However, the bacteriocin was inactivated by two different α-amylase preparations thereby indicating it to be a glycoprotein. The insensitivity of the bacteriocin to β-amylase shows that maltose residues at the non-reducing end of glycoprotein, if any, is not associated with the antagonistic property.

Alpha-amylase sensitivity of the bacteriocins of LAB is an uncommon phenomenon. Leuconocin S produced by *Leu. paramesenteroides* OX was reported to be susceptible to the action of α-amylase (Lewus *et al.*, 1992). Bacteriocins, carnosin LA44A (van Laack *et al.*, 1992) and carnocin LA54A (Keppler *et al.*, 1994) both produced by different strains of *Leu. carnosum* also lost their activity upon treatment with α-amylase. Similarly, pediocin SJ-1 produced by *P. acidilactici* SJ-1 was also characterised as glycoprotein as it lost its activity after treatment with α-amylase (Schved *et al.*, 1993). Plantaricin S but not plantaricin T, produced by *Lb. plantarum* LPC010 was reported to be sensitive to α-amylase and lipase suggesting that it was glycolipoprotein (Jimenez-Diaz *et al.*, 1993).

The sensitivity of bacteriocins of LAB to various proteolytic enzymes has been reported widely. All pediocins, with the exception of pediocin SJ-1, have, however, been reported to be resistant to non-proteolytic enzymes (Gonzalez and Kunka, 1987; Bhunia *et al.*, 1988; Daba *et al.*, 1991; Schved *et al.*, 1993; Coventry *et al.*, 1995).

4.8.4 EFFECT OF PROTEASE INHIBITORS ON THE INACTIVATION OF BACTERIOCIN BY α-AMYLASE

The results pertaining to the effect of protease inhibitors on the inactivation of bacteriocin by α-amylase is given in Table 4.20 and Plate 10. Although the bacteriocin was totally inactivated by α-amylase (spot 5 in Plate 10), it was insensitive to the action of α-amylase when the later was pretreated with either PMSF or a combination of PMSF and iodoacetamide as is evident by the presence of zones of inhibition (spot 3 and 4, respectively) and also by the retention of 100% of the original bacteriocin activity (Table 4.20). However, pretreatment with iodoacetamide alone did not have any adverse effect on the inactivation of the bacteriocin by α-amylase.

Iodoacetamide inhibits the cysteine proteinases, whereas PMSF is generally used as inhibitor of serine proteases. Treatment of both the α-amylase preparations with both inhibitors prior to their use in the inactivation assay did not result in the loss of bacteriocin activity indicating that the enzyme preparation was contaminated with either cysteine proteinase or serine proteinase or both. However, α-amylases treated with PMSF, but not iodoacetamide, were

Plate 10. Effect of protease inhibitors on the inactivation of *P. pentosaceus* 34 bacteriocin by α-amylase

1. Bacteriocin

2. Bacteriocin + α-amylase treated with Iodo-acetamide

3. Bacteriocin + α-amylase treated with PMSF

4. Bacteriocin + α-amylase treated with both PMSF and Iodoacetamide

5. Bacteriocin + α-amylase

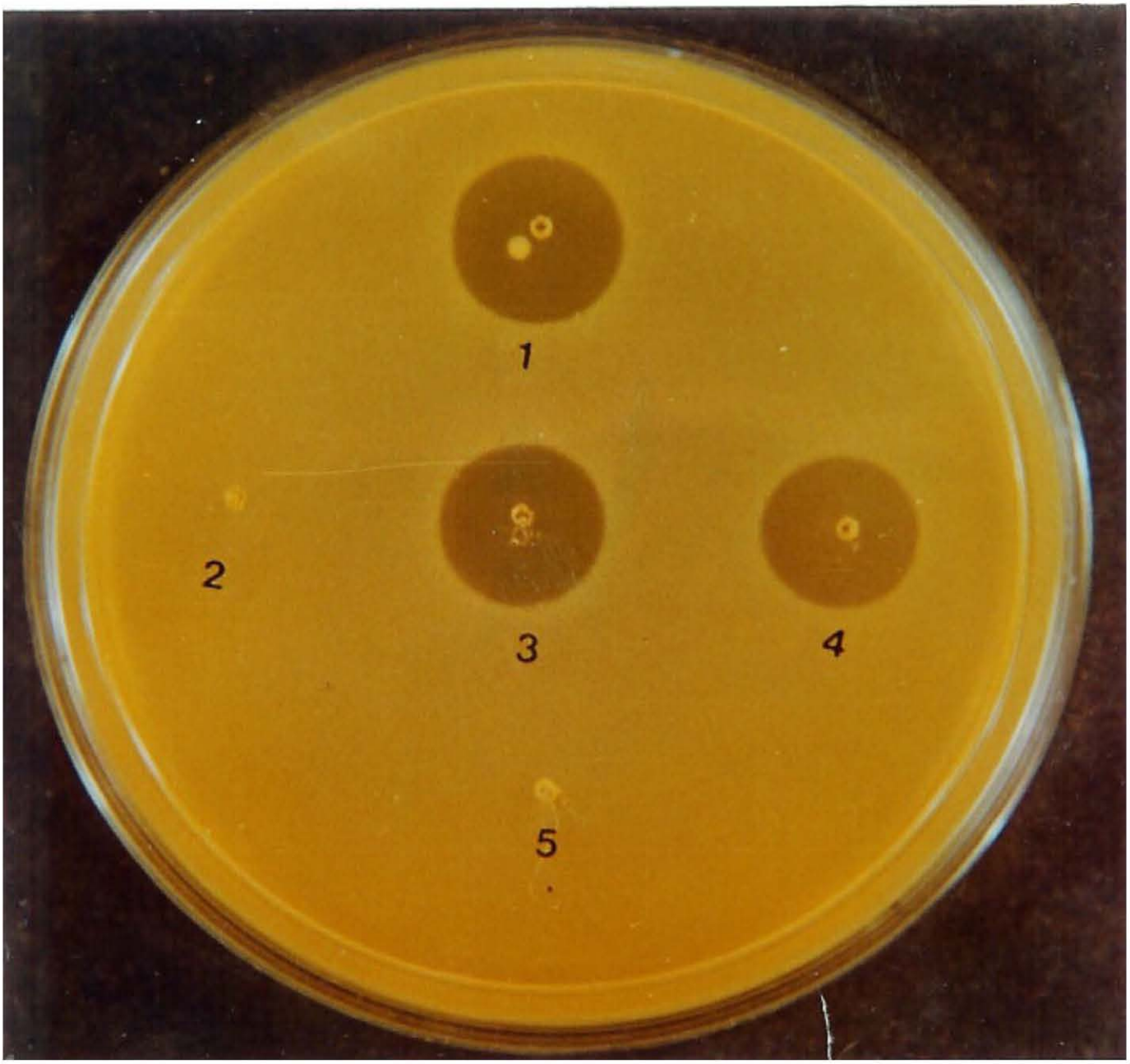
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Table 4.20 : Effect of protease inhibitors on the activity of α-amylase in the inactivation of *Pediococcus pentosaceus* 34 bacteriocin

S.No	Sample	Activity Units (AU/ml)
1	Bacteriocin	20000
2	Bacteriocin + α-amylase	0
3	Bacteriocin + α-amylase + PMSF	20000
4	Bacteriocin + α-amylase + Iodoacetamide	0
5	Bacteriocin + α-amylase + PMSF + Iodoacetamide	20,000

ineffective in destroying the bacteriocin activity, thus providing evidence that contaminating serine proteinases but not cysteine proteinases were actually responsible for the loss of activity of the bacteriocin treated with α-amylases.

Of the two α-amylase preparations used in the inactivation studies of carnosin LA44A, van Laack *et al* (1992) reported that the enzyme preparation obtained from Fluka, but not from Sigma, inactivated the bacteriocin and were unsure whether the observed sensitivity to α-amylase represented the actual sensitivity or possible contamination of α-amylase with certain proteases. Schved *et al* (1993) used the protease inhibitors such as iodoacetamide and PMSF to destroy the contaminating proteases and found that α-amylase was still active against pediocin SJ-1 thus confirming the glycoprotein nature of the bacteriocin produced by *P. acidilactici* SJ-1.

4.8.5 HEAT STABILITY

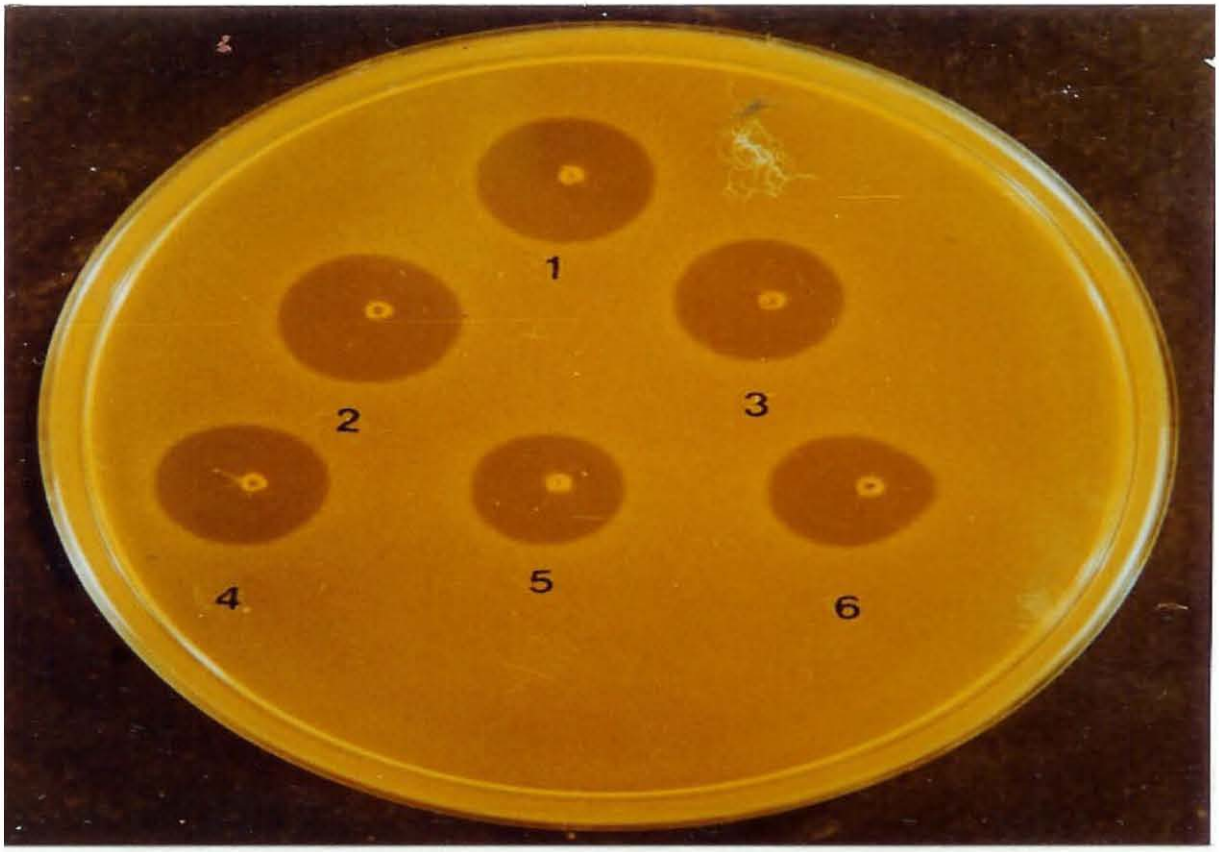
P. pentosaceus 34 bacteriocin preparation in both crude and partially purified forms was found to be stable to different heat treatments viz. 63°C for 30 min, 75°C for 30 min, 85°C for 10 and 15 min, 90°C for 10 and 15 min, 100°C for 5, 10, 15, 30 and 60 min and 121°C for 15 min. The stability of both the preparations to the last two treatments i.e. heating at 100°C for 5, 10, 15, 30 and 60 min and 121°C for 15 min is presented in Plates 11 A and 11 B.

It is also shown in the Fig. 4.9 that the bacteriocin in both the forms completely retained their respective activities at 100°C for 30 min, but lost

Plate 11. Heat stability of *Pediococcus pentosaceus* 34 bacteriocin

A : Crude bacteriocin
B : Partially purified bacteriocin

1. 100°C/5 min
2. 100°C/10 min
3. 100°C/15 min
4. 100°C/30 min
5. 100°C/60 min
6. 121°C/15 min



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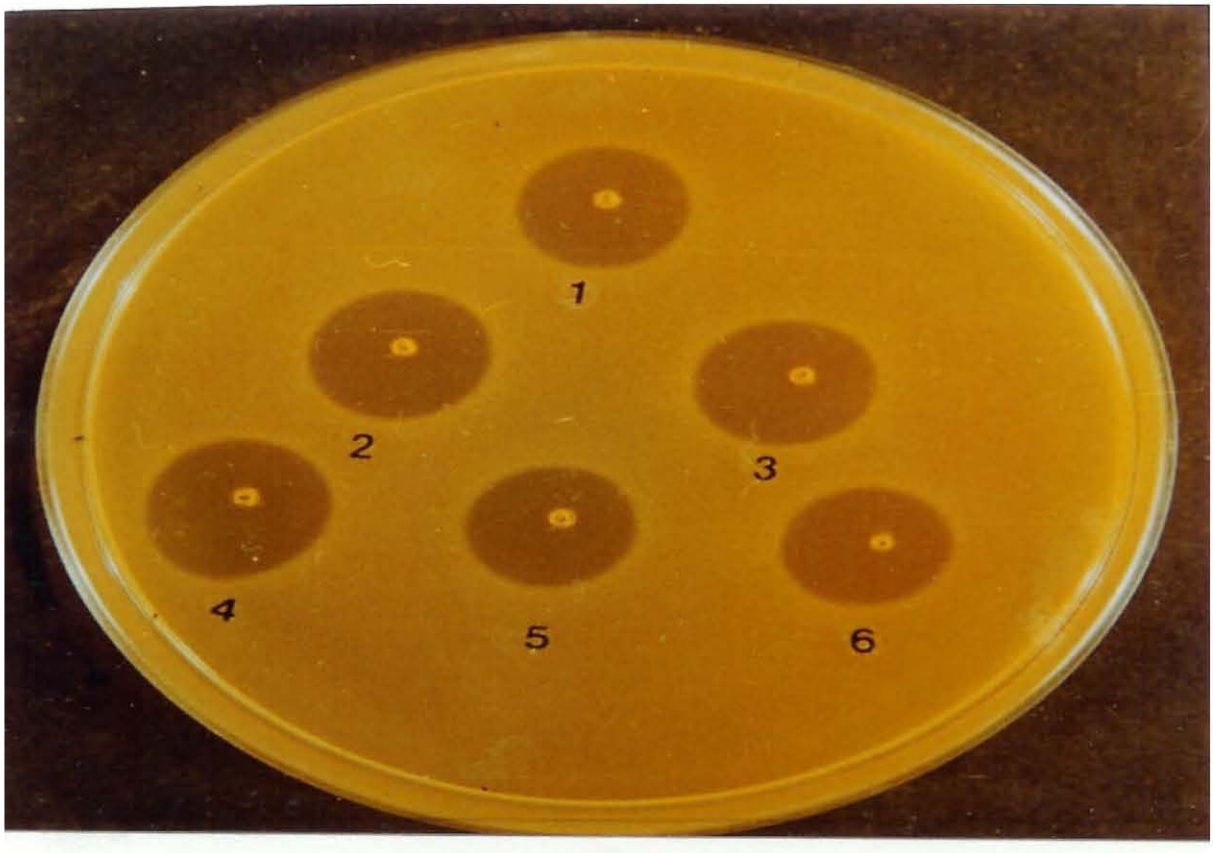

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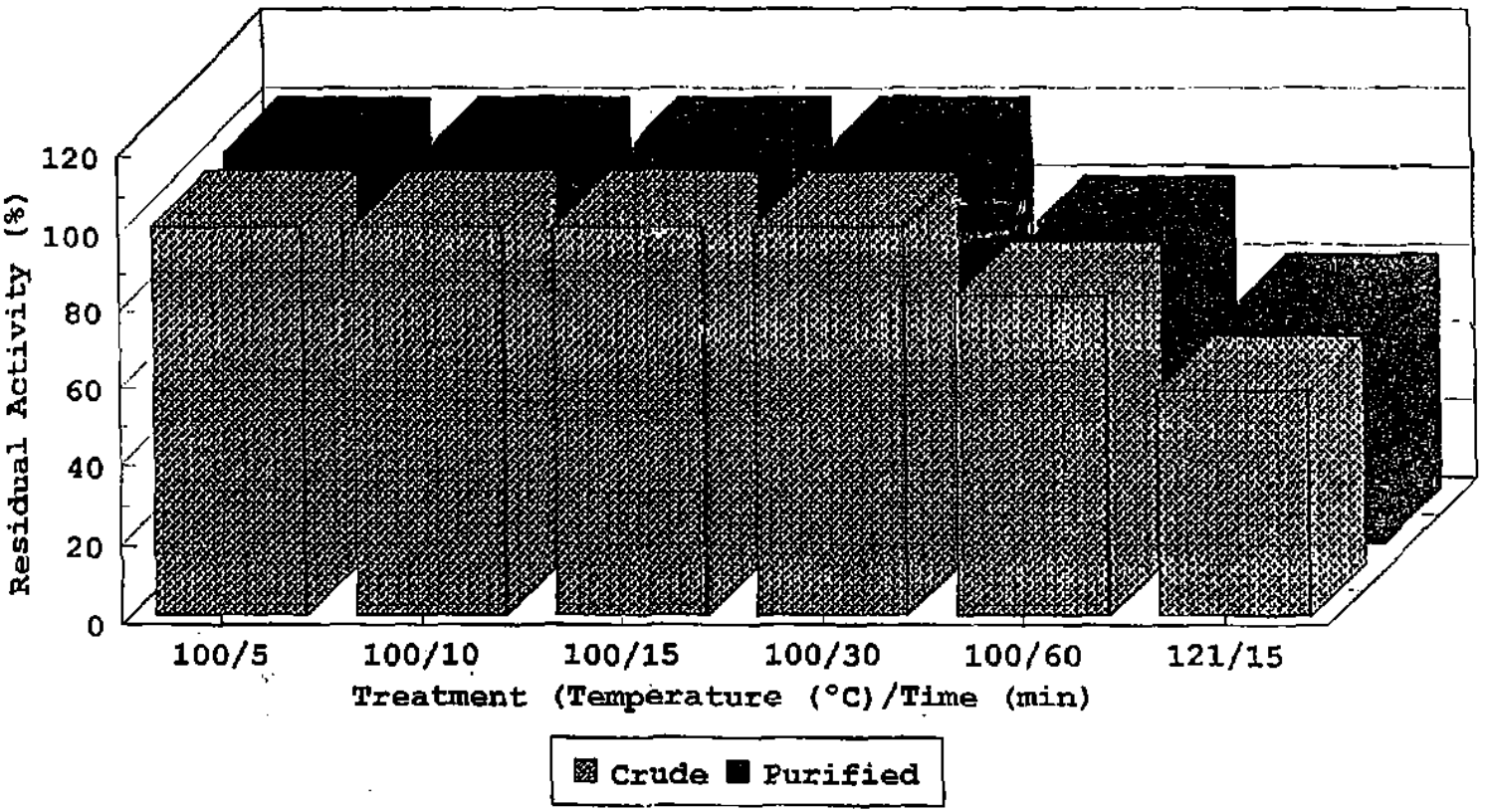


Fig. 4.9 Effect of heat on the antimicrobial activity of Pediococcus pentosaceus 34 bacteriocin

around 20% of the activity after 60 min. Autoclaving (121°C for 15 min) of the bacteriocin preparations resulted in a loss of about 40% of the initial activity.

The retention of the bacteriocin activity after different heat treatments amply shows that the bacteriocin of *P. pentosaceus* 34 is extremely heat stable. Bacteriocins of LAB in general are heat stable antibacterial substances. The extreme heat stability is believed to be because of their simple structure. All the pediocins described till date have been found to be low molecular weight heat stable peptides (Bhunia *et al.*, 1988; Daba *et al.*, 1991; Schved *et al.*, 1993). The activity of pediocin 5 (Daba *et al.*, 1991), pediocin SJ-1 (Schved *et al.*, 1993) and pediocin L50 (Cintas *et al.*, 1995) was also not affected after heat treatment for 30 min at 100°C. It was also reported that pediocin SJ-1 retained 75% of the initial activity after heating for 15 min at 121°C at pH 3.6. However, bacteriocins of lactobacilli such as helveticin J (Joerger and Klaenhammer, 1986) and helveticin V-1829 (Vaughan *et al.*, 1992) were reported to be heat labile. The heat sensitivity of these bacteriocins is apparent from their size and the apparent complexity of their protein structure in contrast to other bacteriocins of LAB.

4.8.6 pH STABILITY

The results relating to the pH stability of crude bacteriocin are shown in Plate 12. The bacteriocin remained stable at pH 12 for 2 hr (Plate 12 A), 11 for 24 hr (Plate 12 B), 10 for 7 days (Plate 12 C) and at pH 9 for 15 days (Plate 12 D).

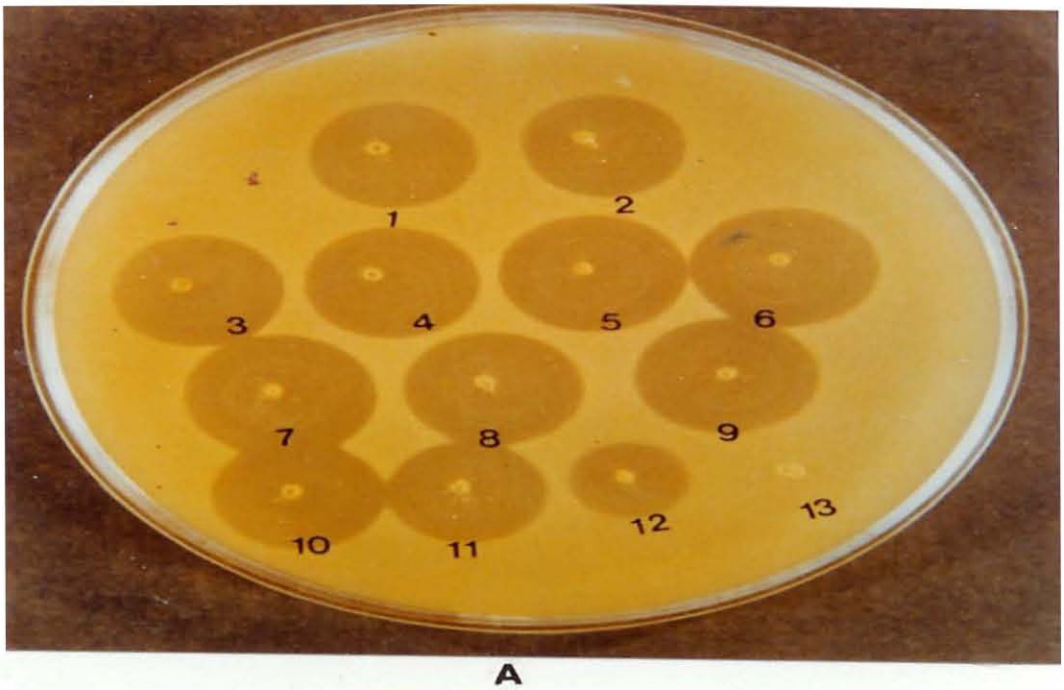
The estimation of the residual activities of the pH adjusted culture broths (Fig. 4.10) revealed that the

Plate 12. pH stability of the bacteriocin of *Pediococcus pentosaceus* 34

A. Crude after 2 hr
B. Crude after 24 hr
C. Crude after 168 hr

(Numerals indicate pH values)

Continued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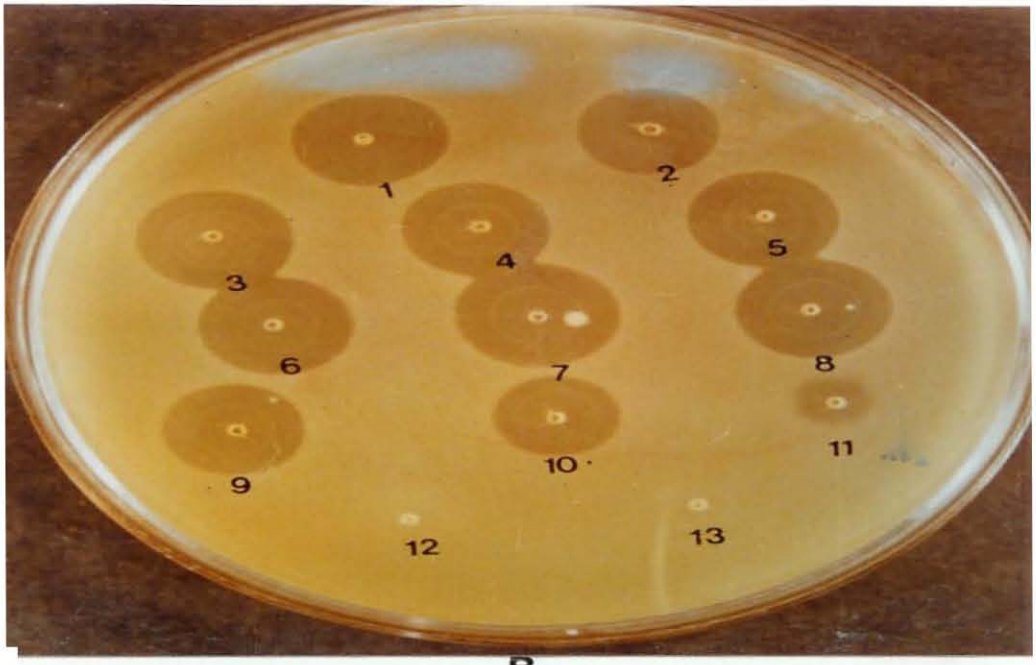
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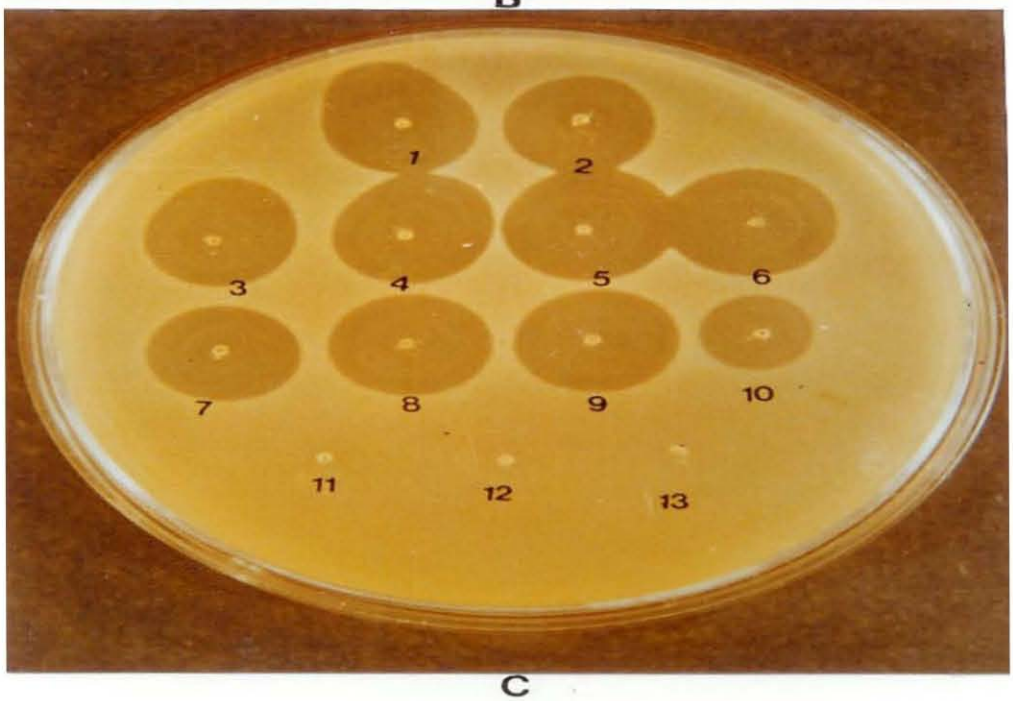
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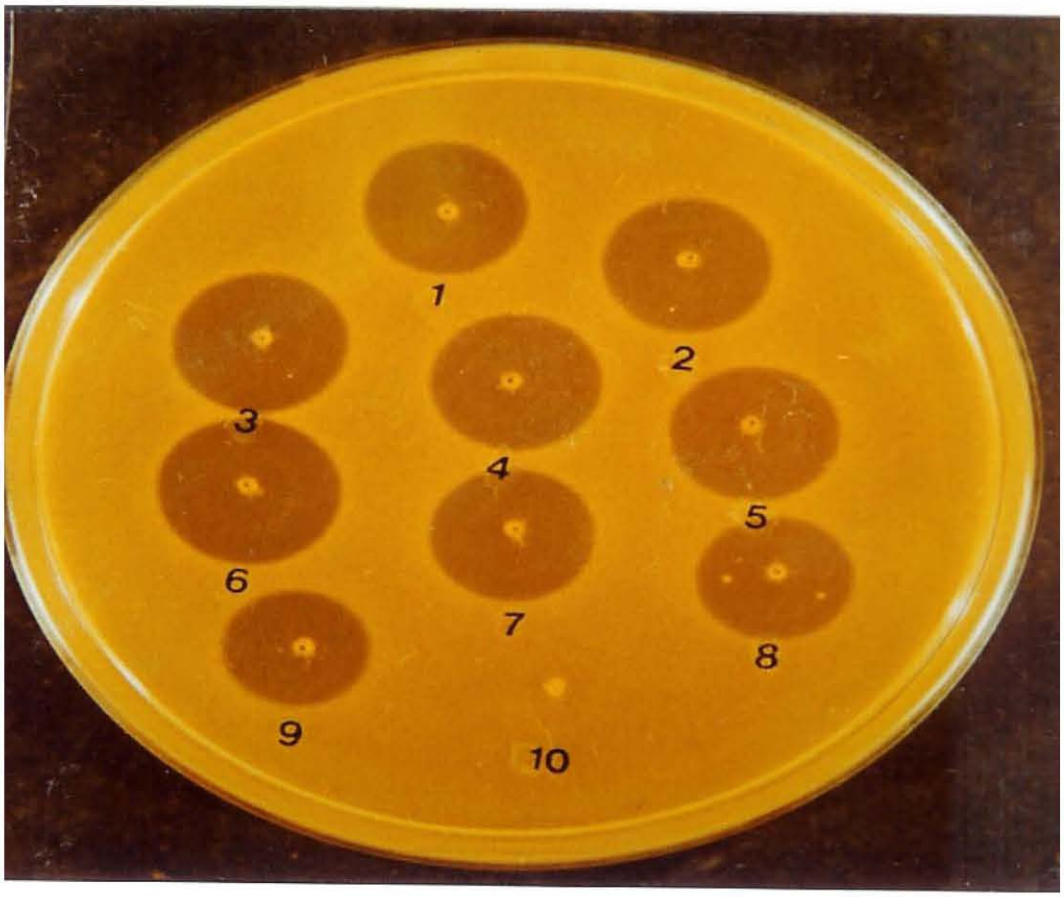
Plate 12. pH stability of the bacteriocin of *Pediococcus pentosaceus* 34 (contd.)

D : Crude after 360 hr

(Numerals indicate pH values)

E : Purified after 360 hr

(Numerals indicate pH values)



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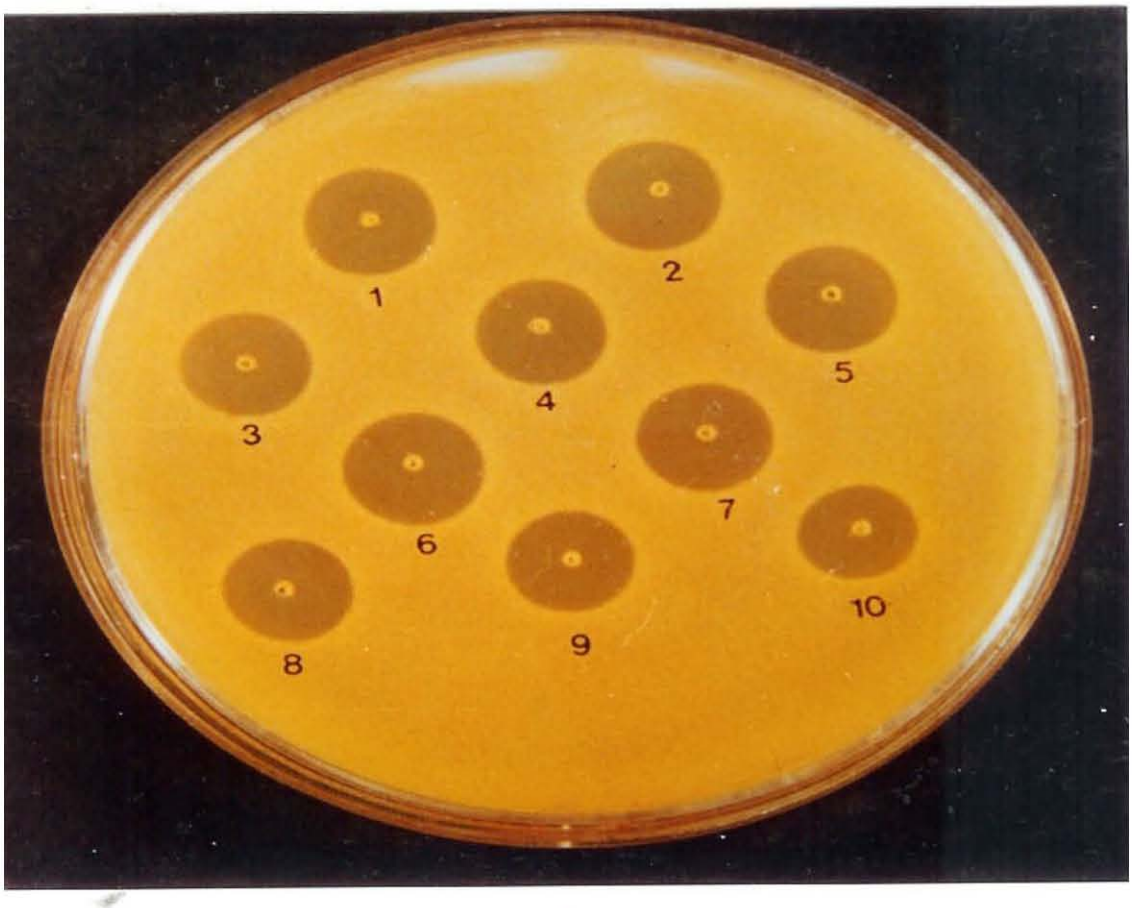


E

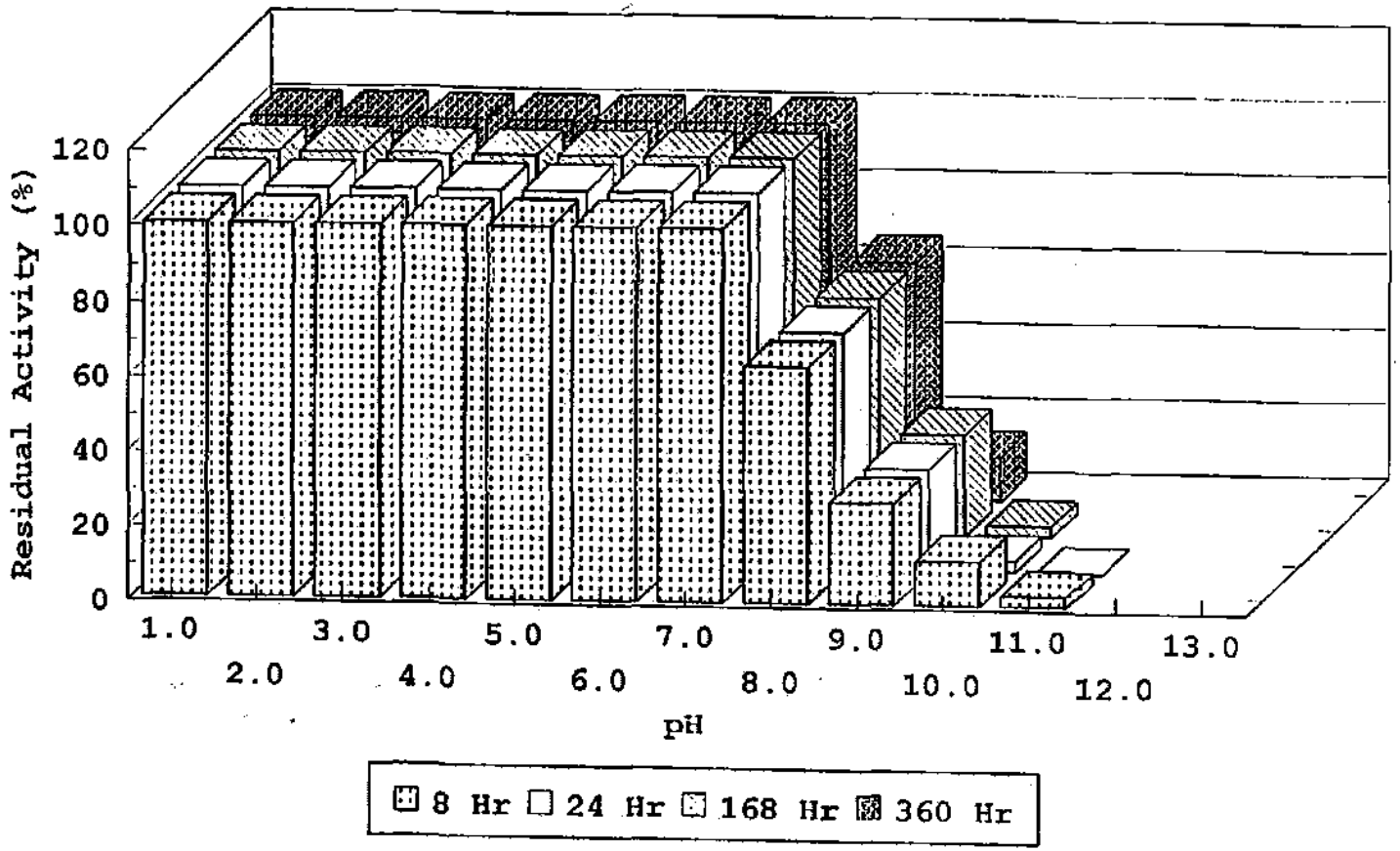


Fig. 4.10 Effect of pH on the activity of crude bacteriocin produced by *Pediococcus pentosaceus* 34

bacteriocin in crude form was extremely stable to pH in the range 1-7 retaining 100% activity throughout the 15 days storage period. It lost about 36.4% of the initial activity after 8 hr at pH 8 with no further loss of activity after 15 days of storage. At pH 9, the residual bacteriocin activity was found to be only 4.12% after 15 days of storage. There was no detectable bacteriocin activity after 15 days of storage at pH 10, 7 days at pH 11 and after 8 hr at pH 12. The estimated residual activities of the bacteriocin at pH 10 were 12.1, 3 and 3% after 8 hr, 1 and 7 days of storage, respectively, while the residual activity at pH 11 decreased from 3 to 0.15% during the storage of 24 hr.

The estimation of residual activities of the partially purified bacteriocin of *P. pentosaceus* 34 adjusted to different pH values revealed that the bacteriocin retained total activity in the pH range 1-8 even after 15 days of storage at 5°C (Plate 12 E, Fig. 4.11). There was about 60% loss of the bacteriocin activity at pH 9 and 10 after 24 hr, and 70% after 15 days.

When the pH of the crude bacteriocin adjusted to 12 or above was lowered to the initial value (pH 4) and the pH readjusted samples were assayed for their inhibitory activity against the indicator strain, no inhibitory activity was noticed.

The bacteriocin preparations of *P. pentosaceus* 34 thus remained stable and active over a wide range of pH. The bacteriocins of some of the lactococcal strains such as lactococcin produced by *Lc. lactis* subsp. *lactis* (Thuault *et al.*, 1991) and bacteriocin S50 produced by *Lc. lactis* subsp. *lactis*. biovar. *diacetylactis* (Kojic *et al.*, 1991) also did not loose activity after exposure for

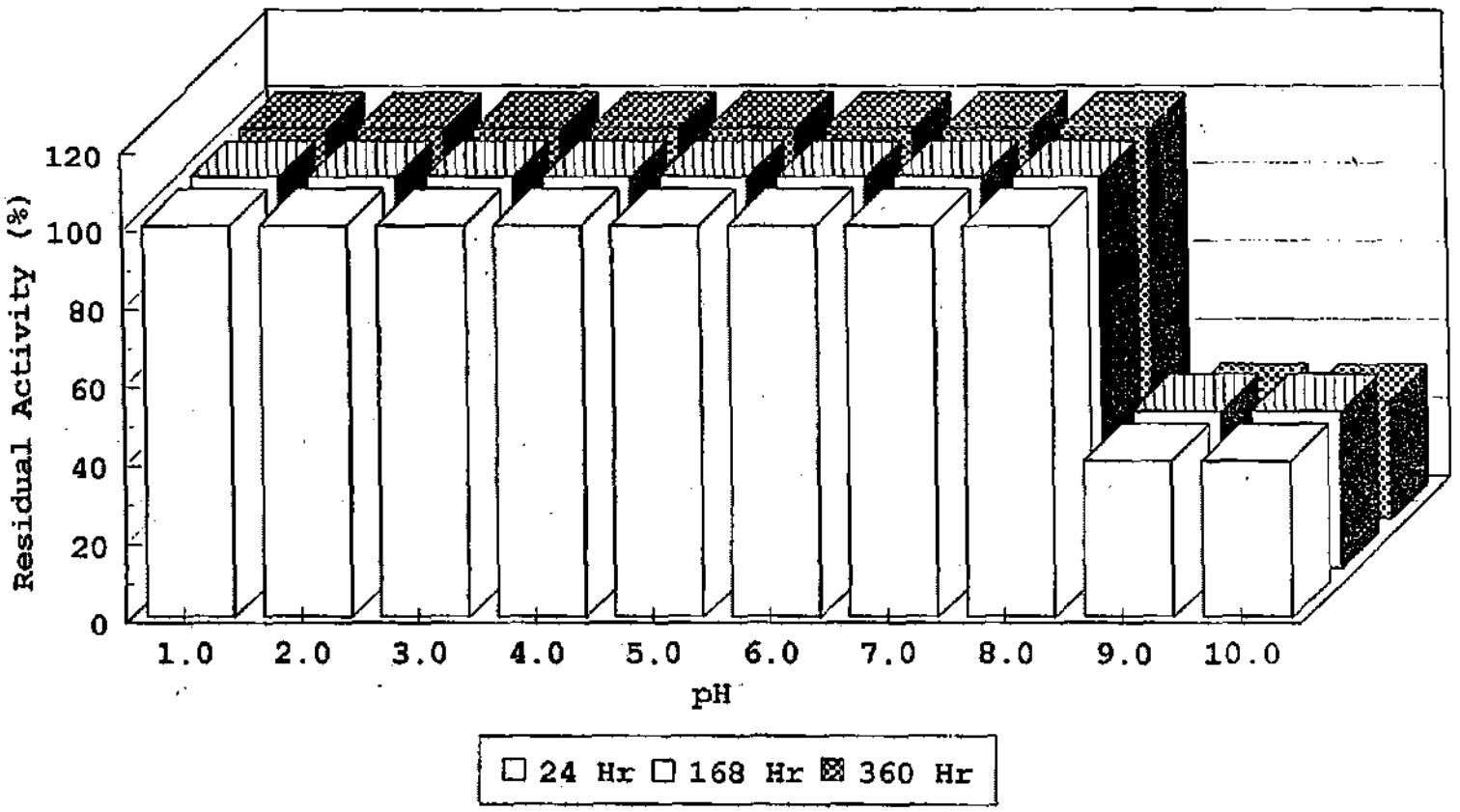


Fig. 4.11 Effect of pH on the activity of the *Pediococcus pentosaceus* 34 bacteriocin

24 hr between pH values 2 and 11. In contrast to pediocin AcH which lost its activity after 24 hr at 25°C at pH 10 and above, the bacteriocin of *P. pentosaceus* 34 remained active for more than 10 days at 5°C at pH 10. Bacteriocin S50 was also reported to lose the activity at pH 12 within 30 min. The activity of lactostrepcins, however, was completely lost when pH was raised to 7 or above (Kozak *et al.*, 1978). Likewise, carnocin LA54A lost about 50% activity between pH 6-7 and 90% at pH 10 (Keppler *et al.*, 1994). Bacteriocin in the present study did not get destabilised during storage for 15 days at 5°C over a pH range of 1-7 (crude) and 1-8 (partially purified). The heat treated crude lacticin 481 also did not show any decrease in the activity both at pH 4 and 7.5 after 15 days of storage at 5°C. However, unheated lacticin 481 lost 50% and 87.5% of the initial activity after 2 and 7 days, respectively at 4°C.

The fact that *P. pentosaceus* 34 bacteriocin activity could not be revived when the pH of the samples adjusted to 12 and 13 were brought down to 4 indicated that the loss in its activity was irreversible.

4.8.7 EFFECT OF pH ON THE HEAT STABILITY OF THE BACTERIOCIN

The results pertaining to the impact of heat treatment on the crude bacteriocin adjusted to different pH values are delineated in Plate 13 and Fig. 4.12. The crude bacteriocin subjected to heat treatment at 75°C for 30 min and 100°C for 10 min retained its total activity in the pH range 1-7 but lost about 41.1% and 96.67% of the activity at pH 8 and 9, respectively, with no detectable activity at pH 10 and above. However, the crude bacteriocin which was not given any heat treatment retained total activity in the pH range 1-7 but the loss

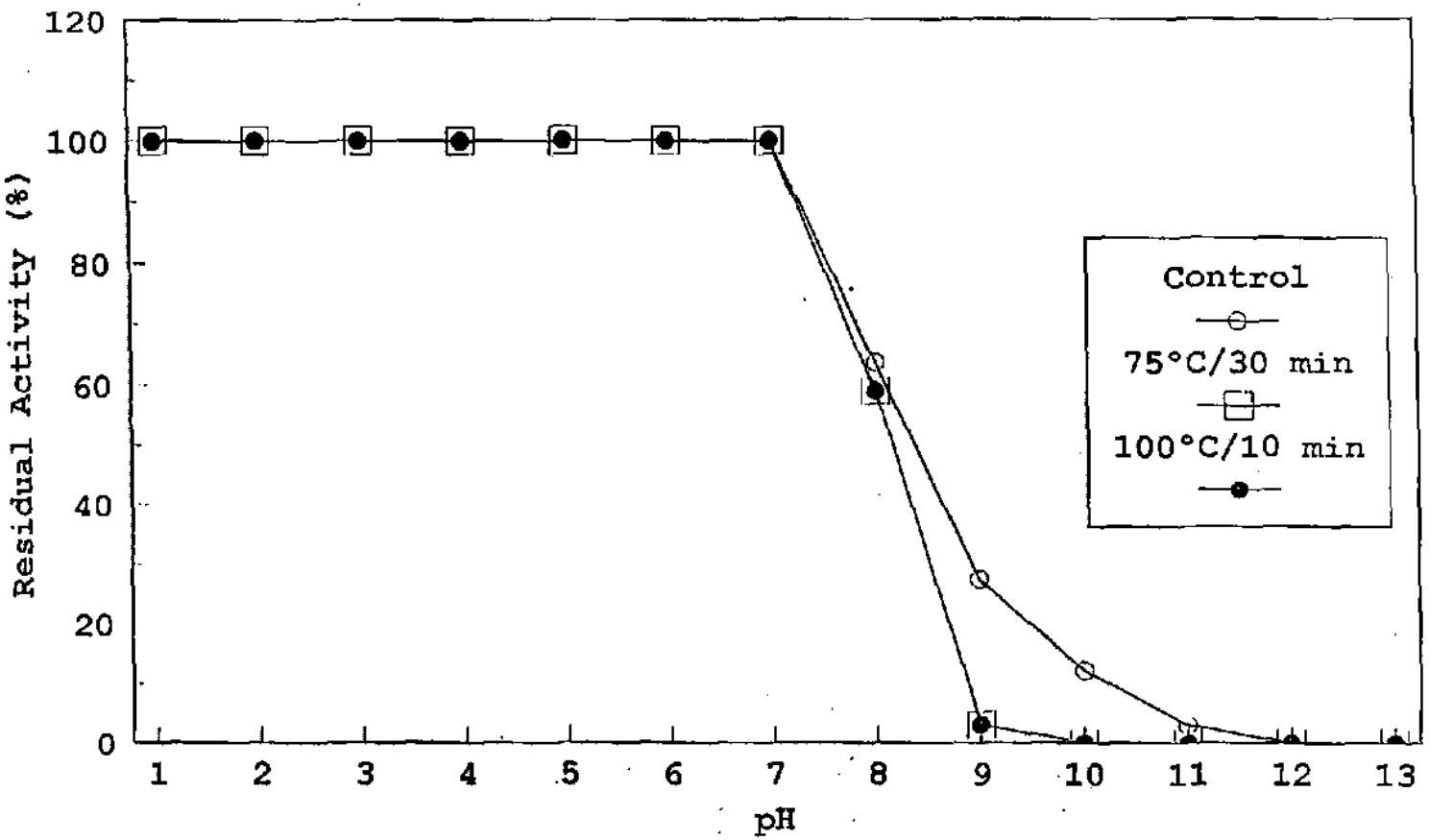


Fig. 4.12 Effect of pH on the heat stability of the crude *Pediococcus pentosaceus* 34 bacteriocin

of activity was found to be about 36.4%, 72.7%, 87.9% and 97% at pH 8, 9, 10 and 11, respectively. The bacteriocin adjusted to pH 11 produced a zone of inhibition in the spot-on-lawn assay (spot 11, Plate 13 A) while the bacteriocin given the two heat treatments exhibited a zone of inhibition only at pH 9 (spot 9, Plate 13 B).

The ammonium sulfate fractionated bactericcin remained active retaining total activity in the pH range 1-8 and residual activity at pH 9 and 10 was calculated to be about 40% (Fig. 4.13, Plate 14 A). The same preparation heated at 100°C for 10 min retained 100% activity upto pH 7 only, beyond which the residual activities were 80, 20 and 1% at pH 8, 9 and 10, respectively. Almost complete loss of activity of the heated bacteriocin at pH 10 may also be visualised by a very small zone of inhibition (spot 10, Plate 14 B).

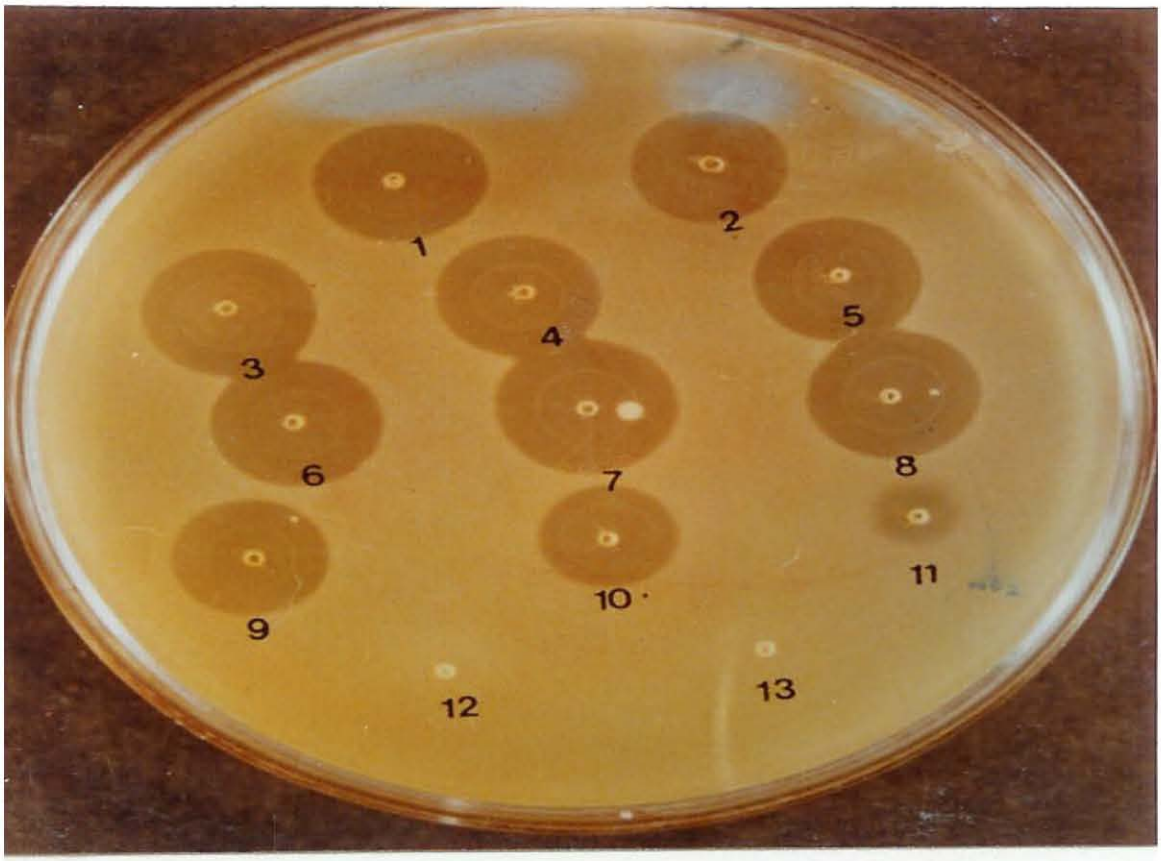
Pediococcus pentosaceus 34 bacteriocin was found to show heat stability over a wide range of pH. The bacteriocins of several LAB have, however, been reported to be heat stable under acidic conditions only. An 80% loss of activity of mesenterocin 52 has been reported at pH 7 after a heat treatment of 100°C/15 min (Mathieu *et al.*, 1993). The same heat treatment resulted in 50% loss of activity of pediocin PO2 at pH 2.5 (Coventry *et al.*, 1995). In a similar study, it was observed that though pediocin SJ-1 retained total activity at pH 3.6 after 30 min at 100°C, but lost about 90% of its activity at pH 7 (Schved *et al.*, 1993). Likewise, brevicin 286 lost substantial amount of antibacterial activity when heated at 100°C at pH $\geq$ 6 (Coventry *et al.*, 1996). The loss of inhibitory activity of heated *P. pentosaceus* 34 bacteriocin at pH 10 is in agreement with the loss of activity of pediocin AcH at the same pH when heated for 15 min at 93°C (Bhunia *et al.*, 1988).

Plate 13. Effect of pH on the heat stability of the *Pediococcus pentosaceus* 34 crude bacteriocin

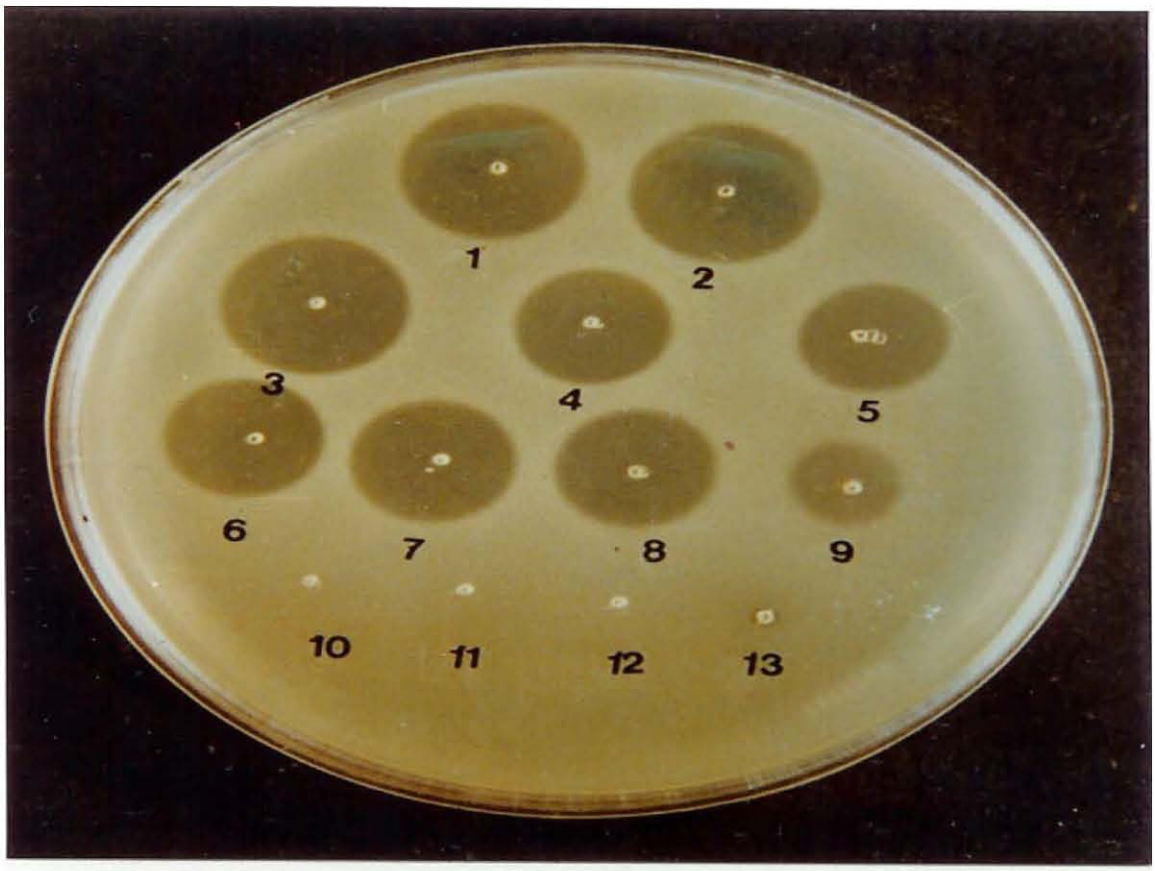
A : Control

B : Heat treated (75°C for 30 min/100°C for 10 min)

(Numerals indicate pH values)



A



B

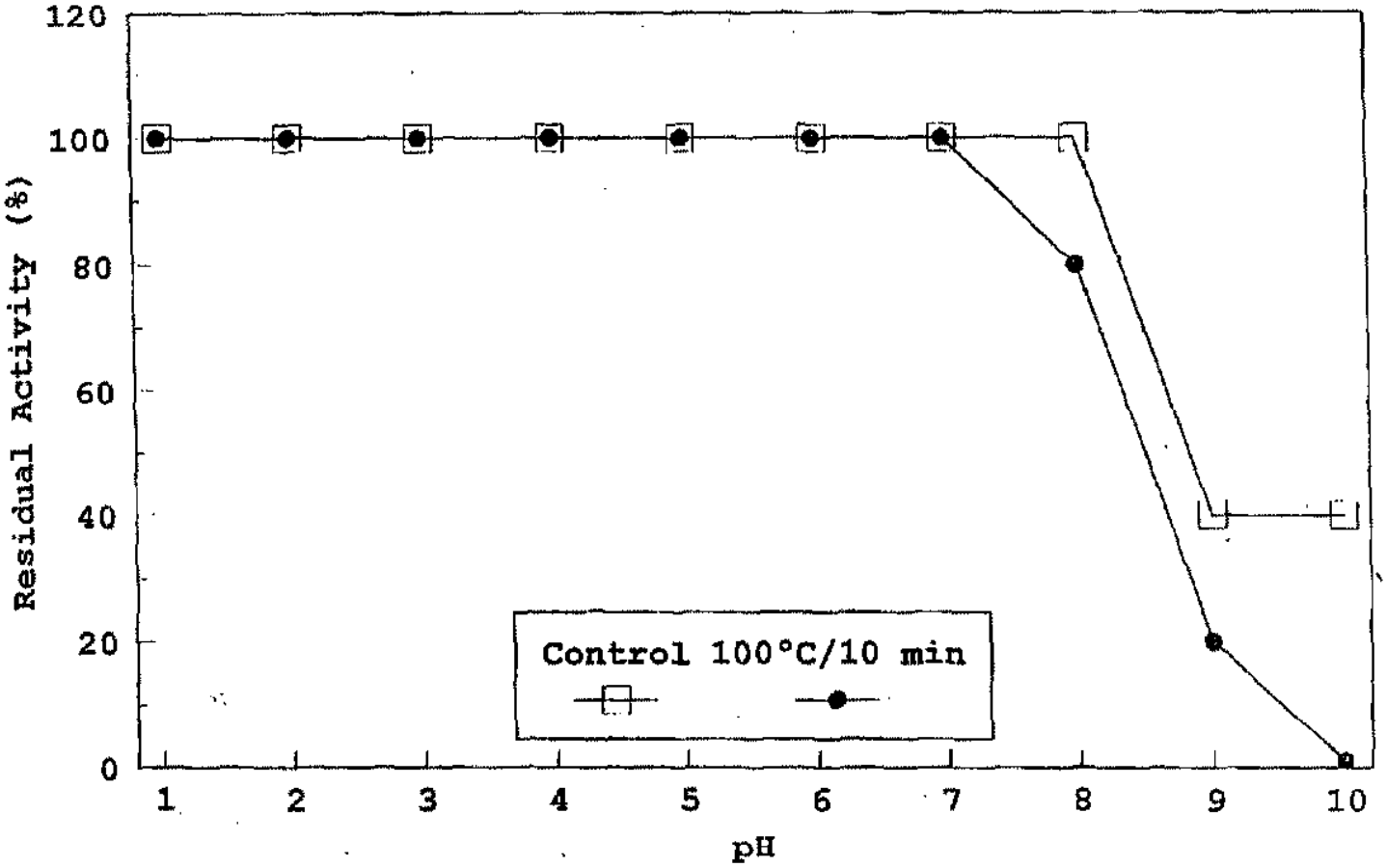


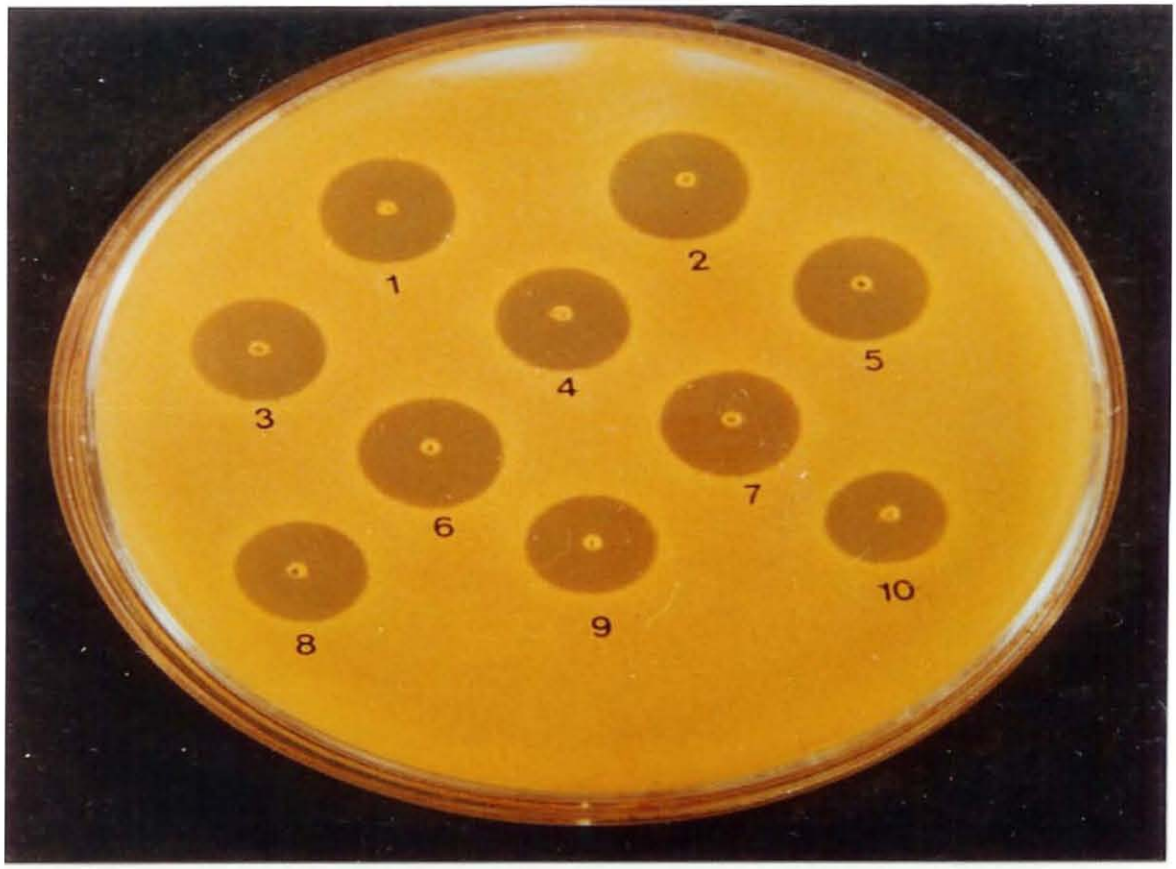
Fig. 4.13 Effect of pH on the heat stability of *Pediococcus pentosaceus* 34 bacteriocin

Plate 14. Effect of pH on the heat stability of the partially purified *P. pentosaceus* 34 bacteriocin

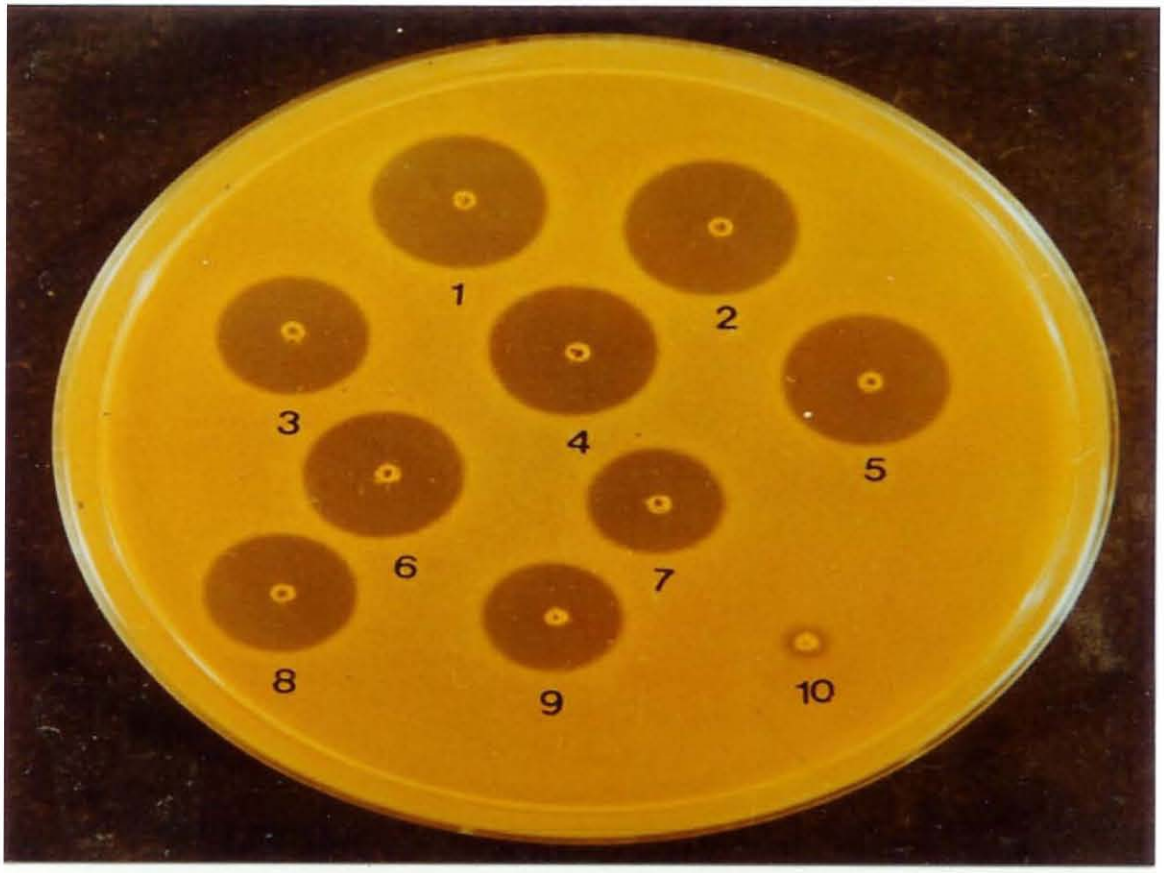
A : Control

B : Heat treated (100°C for 10 min)

(Numerals indicate pH values)



A



B

The ammonium sulfate precipitated bacteriocin of *P. pentosaceus* 34 was also found to be heat stable at pH values from 1 to 7 with loss of activity being detected at pH 8 and complete loss in activity at pH 10. In contrast to this observation, loss of activity of ammonium sulfate precipitate of leucocin A-UAL187 was detected at pH 5 and was very severe at 8 when boiled for 20 min (Hastings *et al.*, 1991).

The activity and stability of *P. pentosaceus* 34 bacteriocin over a wide range of pH and also its thermostability at or near neutral pH values may make this bacteriocin serve as an effective natural biopreservative.

4.8.8 MODE OF ACTION

The results regarding the mode of action of bacteriocin on the cells of *Lb. plantarum* NCDO955 is depicted in Fig. 4.14. It may be discerned that addition of higher concentrations of bacteriocin brought about complete destruction of *Lb. plantarum* NCDO955 in less than 30 min as no survivors could be detected when 0.1 ml of undiluted sample was spread on TGE plates. The bacteriocin added at lower concentrations viz. 200 and 2000 AU/ml resulted in about 3 and 3.2 log cycle reduction, respectively in the first half-an-hour of addition reaching to 4 and 4.3 log cycle reduction at the corresponding concentrations at the end of 4 hr incubation period at 30°C. The control sample without any bacteriocin showed a 0.7 log cycle reduction in the viable cell counts at the end of the 4 hr incubation period. The difference in the viable cell counts between control and experimental (bacteriocin added at a

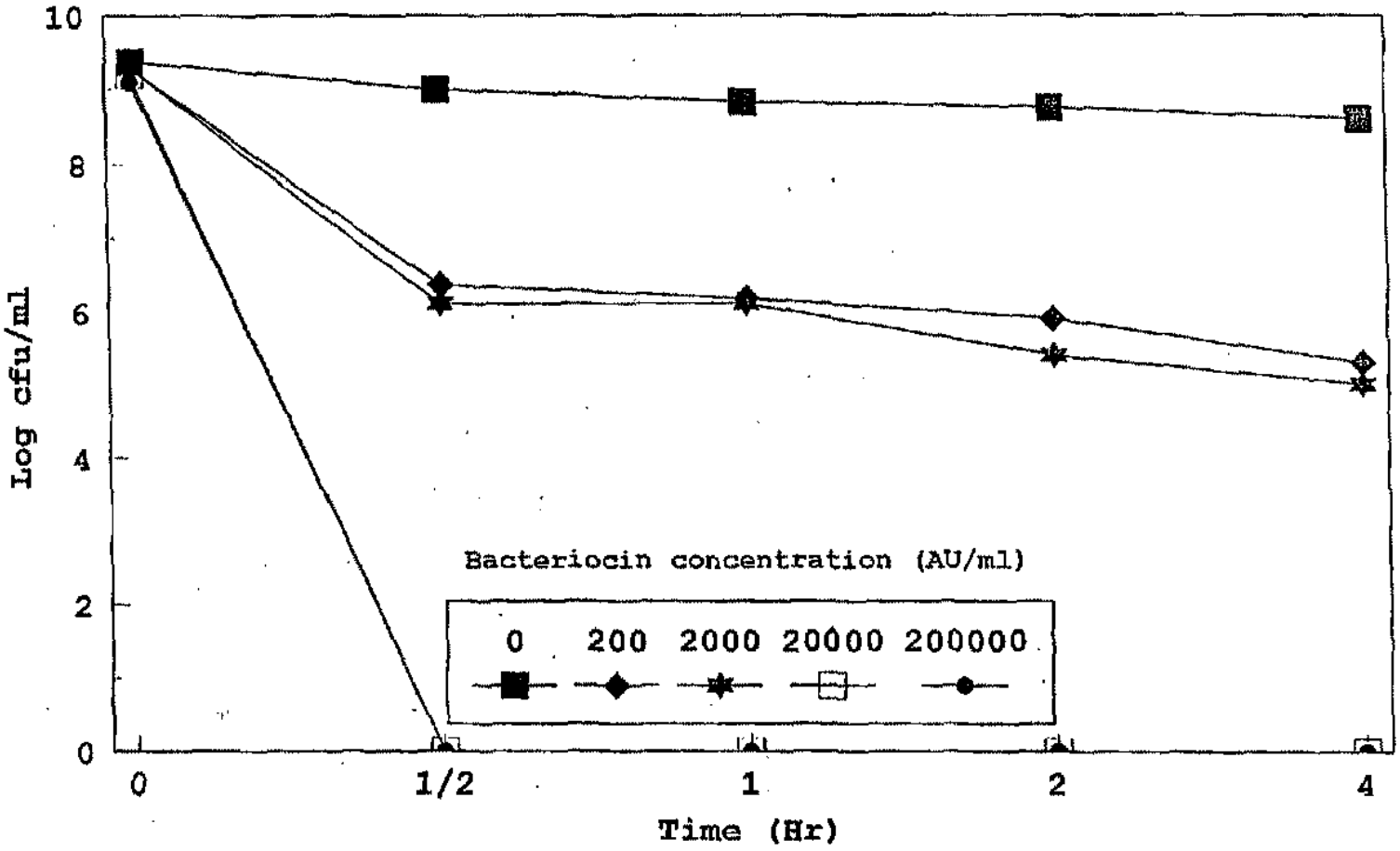


Fig. 4.14 Effect of the *Pediococcus pentosaceus* 34 bacteriocin on the resting cells of *Lb. plantarum* NCDO955 at 30°C

concentration of 200 and 2000 AU/ml) samples was observed to be 3.3 and 3 log cycle after 4 hr of incubation.

To find out any lysis or leakage of the susceptible cells due to the activity of the bacteriocin, changes in the absorbance values of the cell suspension (5 x 10^3 cells) were monitored at two different wavelengths viz. 260 and 600 nm during the 4 hr incubation period at 30°C. It may be seen from the Table 4.21 that optical density values of the bacteriocin in phosphate buffer (20,000 AU/ml) remained constant (0.05 and 1.71 at 600 and 260 nm, respectively) at both 0 and 4 hr of incubation. The cell suspension in phosphate buffer also did not show either decrease at 600 nm or increase at 260 nm in the optical density values. The O.D. at 0 hr was 0.57 and after 4 hr it was found to be 0.53. Similarly, the values of O.D at 260_{λ}^{nm} were 1.93 and 1.94 at 0 and 4 hr of incubation, respectively. The cell suspension in phosphate buffer containing bacteriocin showed an O.D. value at 600 nm of 0.64 in the beginning and 0.6 at the end of 4 hr incubation period. The O.D. values at 260 nm were 3.25 and 3.37 at start and end of the incubation period, respectively. Thus, there were no detectable changes in the O.D. 260 and O.D.600 values of all the three samples during the incubation period for 4 hr at 30°C.

The bactericidal action was observed within a few minutes (Fig. 4.14) after the addition of the bacteriocin as revealed by a 99% reduction in the viable cell counts in the first 30 min. At the end of 4 hr incubation period, the viable cell counts in the bacteriocin added samples were 0.02-0.04% of the control samples. It was also observed that cell death was not accompanied by the lysis of the cells (Table 4.21) since there were no changes in the absorbance values at 260 and 600 nm at the

Table 4.21 : Changes in the optical density values of *Lactobacillus plantarum* NCDO955 incubated with bacteriocin in phosphate buffer (pH 6) at 30 C

Sample	O.D. at 600 nm				O.D. at 260 nm			
	0 Hr	1 Hr	2 Hr	4 Hr	0 Hr	1 Hr	2 Hr	4 Hr
Phosphate buffer + bacteriocin	0.052	-	0.05	0.05	1.71	-	1.72	1.71
Phosphate buffer + cells	0.57	0.56	0.54	0.53	1.93	1.94	1.96	1.94
Phosphate buffer + bacteriocin + cells	0.64	0.63	0.62	0.60	3.25	3.25	3.37	3.37

end of 4 hr incubation period even in the presence of very high concentrations (2,00,000 AU/ml) of *P. pentosaceus* 34 bacteriocin. It may be concluded that *P. pentosaceus* 34 bacteriocin exhibited a bactericidal non-bacteriolytic mode of action on the sensitive *Lb. plantarum* NCDO955 cells. The indicator cells that survived the bacteriocin treatment were, however, found to be sensitive to the bacteriocin.

Except for a few exceptions, bacteriocins of LAB, in general, are bactericidal in their mode of action. In some strains, the bactericidal effect is associated with the lysis of cells. The bacteriocins of LAB exhibiting a bactericidal, non-bacteriolytic mode of action include pediocin AcH (Bhunia *et al.*, 1988), pediocin SJ-1 (Schved *et al.*, 1993), helveticin J (Joerger and Klaenhammer, 1986), helveticin V-1829 (Vaughan *et al.*, 1992), diplococcin (Davey and Richardson, 1981), bacteriocin S50 (Kojic *et al.*, 1991) etc. Some bacteriocins such as leuco cin B-Talla bring about the killing effect by lysing the cells and thereby exhibiting a bactericidal, bacteriolytic effect (Felix *et al.*, 1994).

Some bacteriocins that exhibit bacteriostatic mode of action include leuconocin S (Lewus *et al.*, 1992) and helveticin CNRZ450 (Thompson *et al.*, 1996).

4.8.9 EFFECT OF SURFACTANTS

The ammonium sulfate precipitated bacteriocin was treated with a variety of surfactants. It may be noted from the Table 4.22 that treatment of the bacteriocin with Tween 20, Tween 80 and Triton X-100 registered an increase of 20, 20 and 60% in the activity, respectively. The bacteriocin activity increased by 20, 200 and 300%

Table 4.22 : Effect of surfactants on the activity of *Pediococcus pentosaceus* 34

Surfactant	Concentration (%)	Activity Units (AU/ml)	
		Surfactant	Bacteriocin + Surfactant
None	-	-	1,00,000
Tween 20	1.0	0	1,20,000
Tween 80	1.0	0	1,20,000
Triton X-100	1.0	0	1,60,000
SDS	0.1	0	1,20,000
	0.5	200	2,00,000
	1.0	200	3,00,000

upon treatment with 0.1, 0.5 and 1% SDS, respectively. Among the different concentrations of surfactant as controls, only SDS at 0.5 and 1% level exhibited a negligible bacteriocin titre of 200 AU/ml on the indicator strain.

Bacteriocins of LAB have a tendency to form large macro-molecular complexes after aggregating with other bacteriocin molecules or medium components. Non-ionic detergents such as Tween 20, Tween 80 and Triton X-100 at 1% level did not result in significant increase in bacteriocin activity indicating that these agents are not capable of dissociating aggregates of *P. pentosaceus* 34 bacteriocin. However, anionic detergent, SDS used at 0.5% and 1% level resulted in a 200 and 300% increase in bacteriocin activity, respectively. The increase in bacteriocin activity could be attributed to the dispersion of bacteriocin complex thereby releasing more units for the activity.

The formation of bacteriocin aggregates has also been reported in bacteriocins such as helveticin J (Joerger and Klaenhammer, 1986), pediocin AcH (Bhunia *et al.*, 1988) and lactacin F (Muriana and Klaenhammer, 1991). Whereas, SDS had a favourable effect on the dissociation and a consequent increase in the activity (40%) of lactacin F (Muriana and Klaenhammer, 1991), it had a detrimental effect on the activity of helveticin V-1829 (Vaughan *et al.*, 1992).

4.8.10 EFFECT OF SODIUM CHLORIDE

It may be seen from the Table 4.23 that the sodium chloride upto a concentration of 0.4 M did not have any deleterious effect on the bacteriocin activity. Treatment of the bacteriocin with 0.5 M and 1 M NaCl,

Table 4.23 : Effect of sodium chloride (NaCl) on the activity of *Pediococcus pentosaceus* 34 bacteriocin

S.No.	Concentration of NaCl (M) in 25 mM Tris-HCl Buffer; pH 7.2	Bacteriocin activity (AU/ml)
1	0.0	20,000
2	0.1	20,000
3	0.2	20,000
4	0.3	20,000
5	0.4	20,000
6	0.5	14,000
7	1.0	14,000
8	2.0	8,000
9	3.0	8,000

however, resulted in 30% loss in the activity which increased to 70% with an increase in the concentration of NaCl to 2 or 3 M. The salt solutions as control failed to exhibit any inhibitory activity on the indicator organisms. It seems that NaCl at relatively higher concentration exerts a toxic effect on the bacteriocin molecule and thus, leads to loss of the antagonistic activity.

4.9 PLASMID PROFILES OF SELECTED PEDIOCOCCAL CULTURES

It may be seen from Plate 15 and Table 4.24 that the number of plasmids in pediococcal cultures ranged from none in *P. pentosaceus* 37 (Lane I) to a maximum number of 8 in three cultures viz. *P. pentosaceus* 36 (Lane J), *Pediococcus* sp. 38 (Lane H) and *P. acidilactici* 45 (Lane E). The size of plasmids ranged from 1.6 to 49 MDa. The plasmid profile of *P. pentosaceus* 36 and *Pediococcus* sp. 38 were identical while six of the eight plasmids found in *P. acidilactici* 45 shared an identical size to those found in *P. pentosaceus* 36 and *Pediococcus* sp. 38.

Out of the three pediococcal cultures viz. *P. cerevisiae* NCIM2171, *P. acidilactici* NCIM2292 and *P. pentosaceus* NCIM2296, the latter two cultures i.e. NCIM2292 and NCIM2296 were found to contain 5 plasmids identical in molecular size, while the third culture NCIM2171 contained 6 plasmids, three of which differed in size from that of the plasmids present in the other two cultures.

The bacteriocin producing cultures of *P. pentosaceus* 12, 16, 21, 26 and 34 (Lane P, N, M, L and K, respectively) contained 5-7 plasmids ranging in size from 1.6 MDa to 49 MDa (Table 4.24, Plate 15). The largest

Plate 15. Agarose gel electrophoresis of plasmid DNA from selected pediococcal cultures

Lane A - *E. coli* V517/Marker DNA
D - *P. pentosaceus* NCIM 2296
C - *P. acidilactici* NCIM 2292
B - *P. cerevesiae* NCIM 2171
E - *P. acidilactici* 45
F - *P. acidilactici* 43
G - *Pediococcus* sp. 40
H - *Pediococcus* sp. 38
I - *P. pentosaceus* 37
J - *P. pentosaceus* 36
K - *P. pentosaceus* 34
L - *P. pentosaceus* 26
M - *P. pentosaceus* 21
N - *P. pentosaceus* 16
O - *Pediococcus* sp. 13
P - *P. pentosaceus* 12
Q - *P. pentosaceus* 8

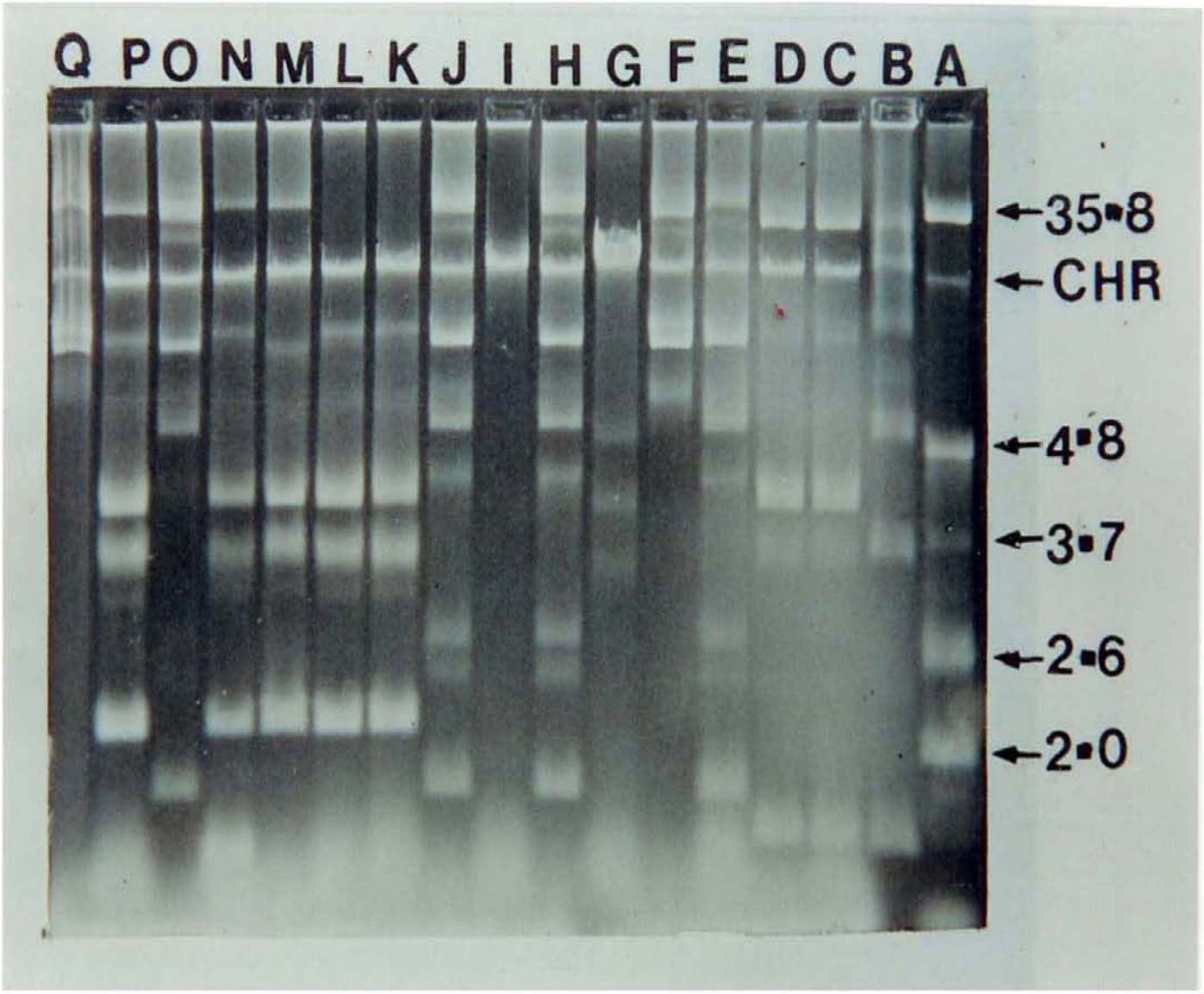

Q P O N M L K J I H G F E D C B A
35·8
CHR
4·8
3·7
2·6
2·0

Table 4.24 : Plasmid profile of selected pediococcal isolates

Isolate	No. of plasmids	Size of the plasmid (MDa)							
Pediococcus sp. 8	2	49	10						
P. pentosaceus 12*	6	49	10	4.6	3.6	3.2	2.1		
Pediococcus sp. 13	5	35.5	27.5	10	6	1.8			
P. pentosaceus 16*	7	49	11	4.6	3.6	3.2	2.1	1.6	
P. pentosaceus 21*	6	49	10	4.6	3.6	3.2	2.1		
P. pentosaceus 26*	5	11	4.6	3.6	3.2	2.1			
P. pentosaceus 34*	5	11	4.6	3.6	3.2	2.1			
P. pentosaceus 36	8	41	32.4	11	6.5	4.9	2.8	2.5	1.9
P. pentosaceus 37	0								
Pediococcus sp. 38	8	41	32.4	11	6.5	4.9	2.8	2.5	1.9
Pediococcus sp. 40	3	6	4.3	3.5					
P. acicilactici 43	4	40.7	32.3	11	7.2				
P. acicilactici 45	8	49	27.5	11	6.5	4.9	2.8	2.5	1.9
P. cerevisiae 2171	6	35.5	12.3	8.1	6	3.8	1.7		
P. acicilactici 2292	5	35.5	11	4.3	3.8	1.7			
P. pentosaceus 2296	5	35.5	11	4.3	3.8	1.7			

* Bacteriocin producers

plasmid of 49 MDa was found in *P. pentosaceus* 12, 16 and 21 while the smallest plasmid of 1.6 MDa was found in *P. pentosaceus* 16 only. It may also be observed that the size of the 5 plasmids present in all the five bacteriocin positive cultures was identical and the approximate molecular size of the five plasmids was calculated to be 2.1, 3.2, 3.6, 4.6 and 11 MDa.

The first evidence of plasmid DNA in pediococci was reported by Gonzalez and Kunka (1983) who observed the presence of one or two plasmids in the strains of *P. acidilactici* and *P. pentosaceus*. Later, Graham and McKay (1985) observed the presence of three to six resident plasmids ranging in size from 4.5-39.5 MDa in each of five parental pediococcal cultures examined. In the present investigation, the number and size of plasmids obtained is quite similar to those reported by Graham and McKay (1985) in different pediococcal cultures.

Five bacteriocin producing cultures of *P. pentosaceus* isolated in the study were found to contain five identical plasmids in them, indicating that they are different strains of the same species. Similar observations have been made by Hoover *et al* (1988) and Ray *et al* (1989a) in different species of *Pediococcus* and three different strains of *P. acidilactici*, respectively.

4.10 GENETIC DETERMINANTS OF BACTERIOCIN PRODUCTION

The results pertaining to the curing experiments are presented in Tables 4.25 and 4.26.

4.10.1 CURING BY CHEMICAL AGENTS

Pediococcus pentosaceus 34 was grown at 37°C in TGE broth containing different concentrations of various

intercalating agents such as acridine orange (50-200 µg/ml) acriflavine (10-30 µg/ml), ethidium bromide (10-40 µg/ml) and the antibiotic novobiocin (10-70 µg/ml). The effect of these curing agents in generating cured variants lacking bacteriocin production is shown in Table 4.25. It may be deduced from the data that none of the 960 colonies screened for bacteriocin production were Bac$^-$ thereby indicating the ineffectiveness of these curing agents in the generation of Bac$^-$ variants.

The reports concerning the effect of different curing agents in the generation of Bac$^-$ variants of Bac$^+$ pediococcal strains are conflicting. Whereas Graham and McKay (1985) reported the ineffectiveness of acriflavine and acridine orange in yielding Bac$^-$ variants of *P. cerevisiae* FBB63, Hoover *et al* (1988) were successful in isolating Bac$^-$ variants by growing the bacteriocinogenic *Pediococcus* spp. in the presence of the very same agents. Other workers (Ray *et al.*, 1989a; Daba *et al.*, 1991; Schved *et al.*, 1993) have also reported the effectiveness of acriflavine in obtaining bacteriocin negative variants. Graham and McKay (1985) and Ray (1989a) reported a favourable effect of novobiocin at various concentrations in curing pediococcal cultures of Bac$^+$ phenotype. Similarly, several other workers have reported the ineffectiveness of acriflavine, acridine orange and ethidium bromide to yield Bac$^-$ variants of various bacteriocin producing lactic cultures (Scherwitz *et al.*, 1983; Joerger and Klaenhammer, 1986 and Daeschel *et al.*, 1990).

Table 4.25 : Effect of various chemical agents on the curing of Bac^+ phenotype in *Pediococcus pentosaceus* 34

Chemical agent	Concentration (µg/ml)	No. of colonies screened for bacteriocin production	No. of Bac^- colonies
Acridine orange	50-200	224	0
Acriflavine	10-30	192	0
Ethidium bromide	10-40	224	0
Novobiocin	10-70	320	0
	Total	960	0

4.10.2 ELEVATED TEMPERATURE WITH OR WITHOUT CURING AGENTS

The results relating to the efficiency of elevated (45°C) temperature of incubation and combination of elevated temperature and chemical curing agents in curing the bacteriocinogenic test strain *P. pentosaceus* 34 of bacteriocin production are presented in Table 4.26. It was observed that the incubation of the parent strain at 45°C was not effective in generation of Bac⁻ variants. Incubation of the parent test strain at 45°C in the presence of different concentrations of intercalating agents such as acriflavine (5-15 µg/ml) and ethidium bromide (10-20 µg/ml) also failed to yield variants lacking ability to produce bacteriocin. None of the 672 colonies screened from the above treatments were Bac⁻ in nature. Although growth at 45°C in TGE broth containing 30 and 35 µg/ml novobiocin could not cure the parent of Bac⁺ phenotype, novobiocin at higher concentrations (40, 45 and 50 µg/ml) was effective in the generation of a few Bac⁻ variants. A total number of 15 cured variants in all were obtained which failed to inhibit the growth of the indicator strain *P. acidilactici* LB42 in the simultaneous antagonism method (Plate 16). The combined effect of sublethal temperature (45°C) and different concentrations of novobiocin viz. 40, 45 and 50 µg/ml yielded 1, 6 and 8 Bac⁻ variants, respectively and the corresponding curing efficiencies were calculated to be 1.67, 6 and 3.76%.

The inability of the cured variants to produce bacteriocin was further confirmed by growing them in MRS broth at 37°C for 18 hr and assessing the bacteriocin activity of the heat killed culture broths against *P. acidilactici* LB42 by spot-on-lawn assay (Plate 17).

Table 4.26 : Effect of elevated/sub-lethal temperature (45°C) with or without chemical agents on the curing of Bac$^+$ phenotype in *Pediococcus pentosaceus* 34

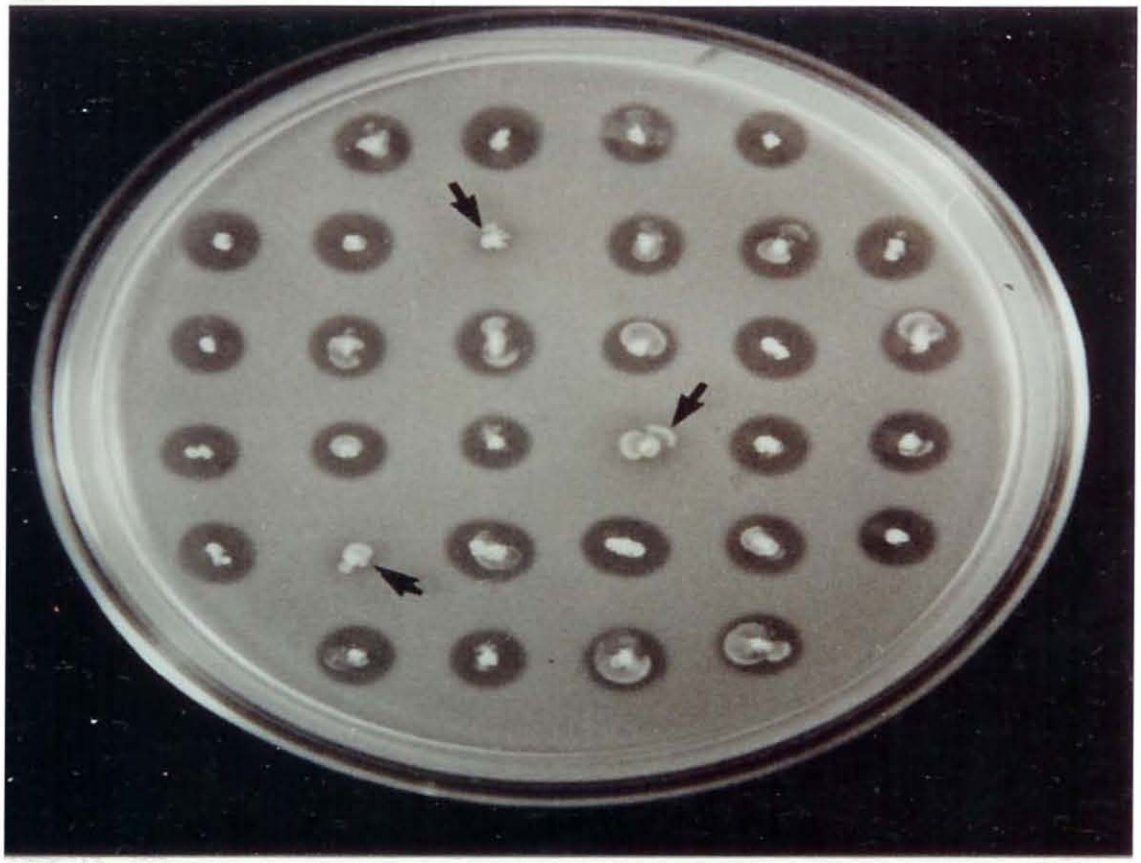
Chemical agent	Concentration (µg/ml)	No. of colonies screened for bacteriocin production	No. of Bac$^-$ colonies	Curing efficiency (%)
-	-	320	0	-
Acriflavine	5	32	0	-
	10	32	0	-
	15	96	0	-
Ethidium bromide	10	32	0	-
	15	32	0	-
	20	128	0	-
Novobiocin	30	41	0	-
	35	72	0	-
	40	60	1	1.67
	45	100	6	6
	50	198	8	3.76

Plate 16. Screening of colonies of Bac$^+$ *Pediococcus pentosaceus* 34 exposed to curing agents for bacteriocin activity by simultaneous antagonism method.

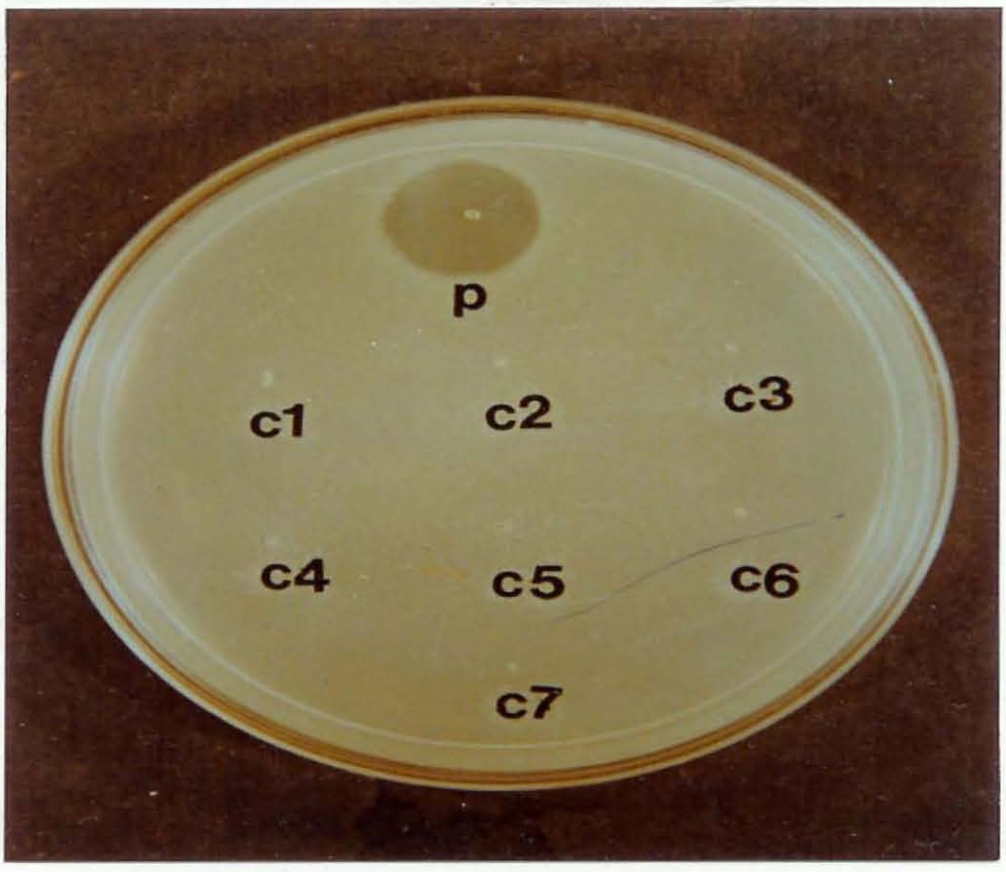
Arrow indicates variants that have been cured of bacteriocin production.

Plate 17. Antibacterial activity of parent (Bac$^+$) and cured (Bac$^-$) variants of *Pediococcus pentosaceus* 34.

P Parent
C1 to C7 - Cured (Bac$^-$) variants



16



17

Bacteriocin negative variants could not be isolated even after growing *P. pentosaceus* 34 at 45°C in the presence of acriflavine or ethidium bromide (Table 4.26) indicating the highly stable nature of Bac$^-$ gene. However, growing the culture at 45°C in the presence of novobiocin resulted in the generation of variants lacking bacteriocin production. While Gonzalez and Kunka (1987) and Schved *et al* (1993) were successful in isolating Bac$^-$ variants of different strains of *P. acidilactici* by growing the cultures only at elevated temperature, Daba *et al* (1991) failed to obtain Bac$^-$ variants of *P. acidilactici* UL5 even at 50°C. Graham and McKay (1985) reported that growing *P. pentosaceus* FBB63 in the presence of novobiocin at its optimum growth temperature resulted in the generation of Bac$^-$ variants.

4.11 CHARACTERISATION OF BAC$^-$ VARIANTS

The Bac$^-$ variants obtained in the curing experiments were characterised for their plasmid profile, resistance to parent bacteriocin, carbohydrate fermentation and antibiotic susceptibility pattern in relation to the parent culture *P. pentosaceus* 34 and the results are presented in Plate 18, Fig. 4.15 and Tables 4.27 and 4.28.

4.11.1 PLASMID PROFILES AND BACTERIOCIN RESISTANCE

The plasmid profile of the parent and all the 15 Bac$^-$ variants is given in Plate 18. The parent culture contained 5 plasmids and molecular sizes of the individual plasmids were: 2.1, 3.2, 3.6, 4.6 and 11 MDa (Plate 18 A). It may be observed that some Bac$^-$ variants were devoid of none (Lane C) to all the five (lane D and E) plasmids present in the parent (Lane B), whereas some

Plate 18. Agarose gel electrophoresis of plasmid DNA

a : Bac$^+$ *Pediococcus pentosaceus* 34

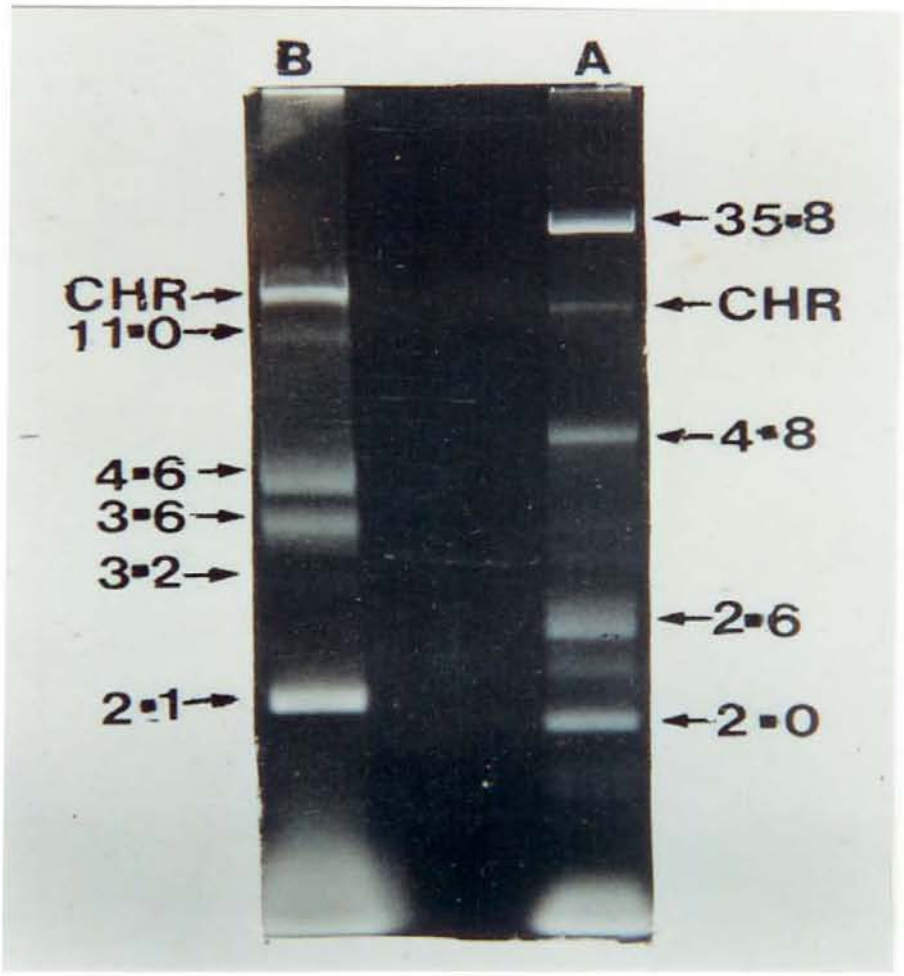
Lane A - *E. coli* V517 (Marker Plasmid DNA)
Lane B - Bac$^+$ *Pediococcus pentosaceus* 34

(Molecular weights in MDa)

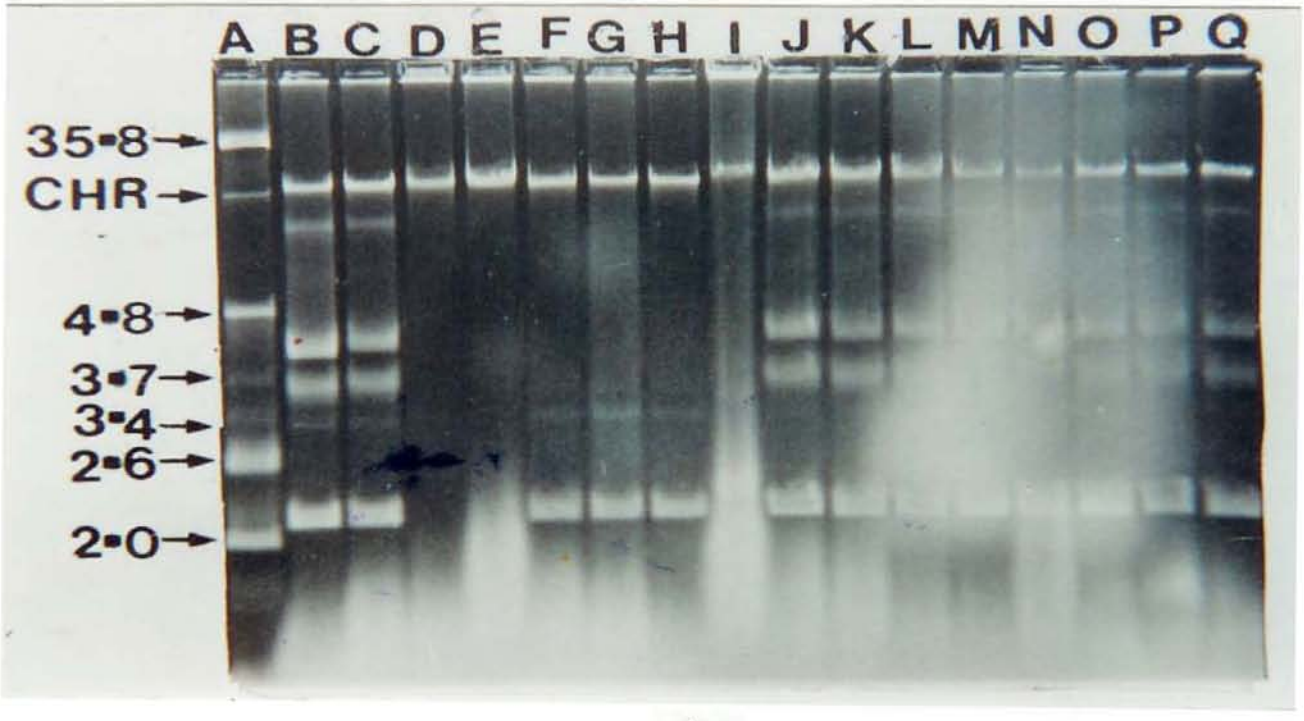
b : Bac$^+$ *P. pentosaceus* 34 and its cured variants

Lane A - *E.coli* V517 (Marker Plasmid DNA)
Lane B - Bac$^+$ *Pediococcus pentosaceus* 34
Lane C to Q - Bac$^-$ cured variants (C1 - C15)

(Molecular weights in MDa)


B
A
35•8
CHR
11•0
CHR
4•8
4•6
3•6
3•2
2•6
2•1
2•0

a


A B C D E F G H I J K L M N O P Q
35•8
CHR
4•8
3•7
3•4
2•6
2•0

b

lost one (Lane J-Q) while some other Bac⁻ variants lost 3 (Lane F-I) plasmids found in the parent culture (Plate 18b).

The grouping and designation of the cured variants is given Table 4.27. The cured variants were assigned to four groups on the basis of the plasmids they retained. The cured variant designated C1 contained all the five plasmids found in the parent and assigned to Group-I. Group-II consisted of the maximum number of cured variants, designated C8, C9, C10, C11, C12, C13, C14 and C15, all of which had lost a single plasmid of 3.2 MDa. The four variants designated as C4, C5, C6 and C7 in Group-III were devoid of 3 plasmids found in the parent strain. The molecular weights of the three missing plasmids were 11, 4.6 and 3.6 MDa. The cured variants designated as C2 and C3 lost all the five plasmids present in the parent strain and were assigned to Group-IV.

It may also be seen from the Table 4.27 that all the 15 cured variants were resistant to the bacteriocin produced by the parent strain *P. pentosaceus* 34.

The loss of all the plasmids in the two Bac⁻ variants indicates that any of the missing plasmid might be involved in the Bac⁺ phenotype. Whereas the loss of a single plasmid (3.2 MDa) in the eight variants (group III) narrowed down the possibility of involvement of that plasmid in Bac⁺ phenotype. However, the presence of 3.2 MDa plasmid in four variants of group III shows that this plasmid is devoid of genes necessary for bacteriocin production. Moreover, the lone Bac⁻ variant C1 of group I contained all the plasmids present in the parent culture. No physical evidence, therefore, could be obtained for the plasmid involvement in bacteriocin production by the test culture. All the 15 Bac⁻ variants retained their immunity to parent bacteriocin. The retention of immunity by Bac⁻ cured variants to the parent bacteriocin suggests that the mutation responsible for Bac⁻ phenotype affected the gene for bacteriocin

production or for the excretion of bacteriocin but did not affect the immunity. A similar profile of Bac- variants retaining all plasmids found in the parent culture, was observed in the curing studies of *Lb. plantarum* C11 (Daeschel *et al.*, 1990). However, the retention of immunity by the variants lacking all the plasmids clearly (Table 4.27, Group IV) indicates the chromosomal linkage of the bacteriocin immunity.

In contrast to these findings, a direct physical evidence for the involvement of plasmid DNA in bacteriocin production by different strains of pediocccci has been obtained (Gonzalez and Kunka, 1987; Ray *et al.*, 1989a; Schved *et al.*, 1993). The loss of Bac$^+$ phenotype sometimes associates with the loss of immunity. Thus, the bacteriocin plasmid pSMB74 of pediocin AcH also carried the genes for bacteriocin immunity in the *P. acidilactici H* (Ray *et al.*, 1984a). But the other two bacteriocin plasmids of pediocin PA-1 and pediocin SJ-1 did not contain the genes for immunity to the host bacteriocins (Gonzalez and Kunka, 1987; Schved *et al.*, 1993).

4.11.2 CARBOHYDRATE FERMENTATION AND ANTIBIOTIC SUSCEPTIBILITY

The association of any of the lost plasmid with the carbohydrate fermentation phenotype was investigated by assessing the ability of the parent and selected Bac$^-$ variants representing all the 4 major groups to ferment a number of carbohydrates and the data obtained in the experiment are presented in the Table 4.28.

It can be seen that out of the 17 sugars, 10 sugars viz. arabinose, ribose, xylose, dextrose, galactose, mannose, fructose, maltose, salicin and cellobiose were

Table 4.27 : Grouping of cured variants on the basis of their plasmid DNA content

Group	Bac⁻ cured variant	Sensitivity/ Resistance to parent bacteriocin	No. of plasmids	Size of plasmids* (MDa)
Parent	Bac⁺		5	11 4.6 3.6 3.2 2.1
I	C1	Resistant	5	11 4.6 3.6 3.2 2.1
II	C8, C9, C10, C11, C12, C13, C14, C15	Resistant	4	11 4.6 3.6 - 2.1
III	C4, C5, C6, C7	Resistant	2	- - - 3.2 2.1
IV	C2, C3	Resistant	None	- - - - -

Table 4.28 : Carbohydrate fermentation pattern of bacteriocin producing *Pediococcus pentosaceus* 34 and its selected Bac⁻ variants

Carbohydrate	Parent/cured variants							
	34	C1	C2	C3	C4	C5	C8	C9
Arabinose	+	+	±	+	+	+	+	+
Ribose	+	+	±	+	+	+	+	+
Xylose	+	+	±	+	+	+	+	+
Dextrose	+	+	-	+	±	+	±	+
Galactose	+	+	-	+	+	+	+	+
Mannose	+	+	±	+	±	+	+	+
Fructose	+	+	±	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-
Maltose	+	+	±	+	+	+	±	+
Sorbitol	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	ND	ND
Mannitol	-	-	-	-	-	-	-	-
Salicin	+	+	+	+	+	+	+	+
Cellobiose	+	+	±	+	+	±	±	+
Inositol	-	-	-	-	-	-	-	-

utilised by the parent strain as was observed by the appearance of yellowish zones around the sugar discs in the BCP-sugar-fermentation basal medium agar plates. It was also observed that all the selected Bac⁻ variants viz. C1, C2, C3, C4, C5, C8 and C9 were also able to utilise all the sugars which were fermented by the parent strain. The parent as well as the cured variants failed to ferment the remaining 7 sugars namely lactose, sucrose, rhamnose, sorbitol, dulcitol, mannitol and inositol.

The antibiotic susceptibility of the parent and selected Bac⁻ variants is represented in Table 4.29. A total number of 14 antibiotics were used. There was no difference in the antibiotic susceptibility pattern between the parent and Bac⁻ variants. All the cultures were resistant to polymyxin (50 U, 300 U), nalidixic acid (30 µg), vancomycin (30 µg) and kanamycin (5 µg) and all were sensitive to ampicillin (10 µg), bacitracin (10 µg), erythromycin (10 µg), streptomycin (10 µg), chloramphenicol (5 µg), clindamycin (2 µg), tetracycline (30 µg) and novobiocin (5 µg). The most effective antibiotics were clindamycin (2 µg) and chloramphenicol (10 µg) that gave a zone of inhibition of about 30 mm dia. followed by erythromycin (10 µg), bacitracin (10 µg) and tetracycline (30 µg) with a 20 mm dia. of zone of inhibition. The dia. of the zone of inhibition was about 15 mm for ampicillin (10 µg). The antibiotics novobiocin (5 µg) and kanamycin (30 µg) were found to be least antagonistic showing a 10 mm dia. of zone of inhibition.

Identical patterns of carbohydrate utilisation (Table 4.28) and antibiotic susceptibility (Table 4.29) of the parent and the representative cured variants of each group reveals that none of the plasmid controls either of the two properties indicating the cryptic

Table 4.29 : Antibiotic susceptibility of bacteriocin producing *Pediococcus pentosaceus* 34 and its selected Bac⁻ variants

Antibiotic (Concentration)	Diameter of zone of inhibition (mm)*							
	Parent/cured variants							
	34	C1	C2	C3	C4	C5	C8	C9
Ampicillin (10 µg)	15	16	16	15	15	15	15	15
Bacitracin (10 U)	20	20	20	21	19	20	20	20
Erythromycin (10 µg)	22	22	22	23	23	23	21	22
Polymyxin B (50 U)	-	-	-	-	-	-	-	-
(300 U)	-	-	-	-	-	-	-	-
Streptomycin (10 µg)	11	11	11	11	11	12	11	11
Nalidixic acid (30 µg)	-	-	-	-	-	-	-	-
Vancomycin (30 µg)	-	-	-	-	-	-	-	-
Chloramphenicol (10 µg)	30	28	27	29	30	31	28	30
Kanamycin (5 µg)	-	-	-	-	-	-	-	-
(30 µg)	10	10	10	11	10	10	11	11
Clindamycin (2 µg)	29	30	29	27	30	31	29	30
Tetracycline (30µg)	20	20	20	20	21	20	19	21
Novobiocin (5 µg)	11	11	11	11	11	11	11	11

* Diameter of the disc : 8 mm

nature of all plasmids present in the parent culture *P. pentosaceus* 34. However, plasmids have often been associated with the utilisation of various sugars such as sucrose (Gonzalez and Kunka, 1987; Kim *et al.*, 1992) and raffinose (Gonzalez and Kunka, 1986) in different strains of *P. acidilactici*.

4.12 CONJUGAL TRANSFER OF Bac^+ PHENOTYPE

The observations made during conjugal transfer of Bac^+ phenotype experiments are recorded in Table 4.30. Screening of a large number of recipients / suspected transconjugants developed on MRS agar plates for bacteriocin production failed to yield any transconjugant which could inhibit the indicator strain *P. acidilactici* LB42 in the simultaneous antagonism method.

Conjugal transfer of Bac^- phenotype using two different methods of conjugation was not successful in transferring the Bac^- variant to a Bac^+ transconjugant, thus providing evidence for the chromosomal location of genes in bacteriocin synthesis by *P. pentosaceus* 34. This also substantiates the earlier observations made while comparing the plasmid profile of the parent culture and its different groups of Bac^- cured variants.

Although bacteriocin production has been reported to be encoded by various plasmids in different species of pediococci (Daeschel and Klaenhammer, 1985; Gonzalez and Kunka, 1987; Ray *et al.*, 1989a; Schved *et al.*, 1993), the conjugal transfer of Bac^+ plasmid to a Bac^- pediococcal

Table 4.30 : Conjugal transfer of Bac$^+$ phenotype from *Pediococcus pentosaceus* 34 to its plasmid free Ery^s derivative

Method	Donor : Recipient*	No.of recipients/ transconjugants screened for bacteriocin production	No. of colonies showing bacteriocin production	Conjugal transfer frequency
Filter paper mating	1 : 1	128	0	-
	1 : 10	216	0	-
Solid surface mating	1 : 1	288	0	-
	1 : 10	384	0	-

* cfu/ml : Donor : 1.8×10^9
Recipient : 1.4×10^7

strain has been reported only with pSMB74 plasmid encoding pediocin AcH production (Ray *et al.*, 1992b).

4.13 EFFECT OF BACTERIOCIN ON THE GROWTH OF *LISTERIA MONOCYTOGENES* MTCC657 IN STERILE SKIM MILK

The growth of *L. monocytogenes* MTCC657 in sterile skim milk in the presence or absence of the *P. pentosaceus* 34 bacteriocin was measured during storage for 15 days at 5°C. It may be seen from the Fig. 4.15 that cell counts increased by approximately 2 log cycles after 15 days in the control sample but decreased substantially by 3 and 3.4 log cycles, respectively in the presence of 200 and 2000 AU/ml bacteriocin, during the first day of storage. The survivors were able to multiply and eventually increased by 1.6 log cycles at both the levels of bacteriocin employed in the experiment after 15 days of storage. A difference in cell counts of 3.3-3.7 log cycles between the control and experimental samples was observed after 15 days of storage at 5°C.

The bacteriocin of *P. pentosaceus* 34 used at two different concentrations displayed a strong bactericidal effect on *L. monocytogenes* MTCC657 in milk, and hence decreased the viable numbers by almost 3 log cycles. The bactericidal effect, was however, followed by a progressive growth of those *L. monocytogenes* cells that survived the bacteriocin treatment. These findings are in agreement with the earlier reports on nisin inhibiting *L. monocytogenes* Scott A in skim milk (Jung *et al.*, 1992) and pediocin 5 affecting three different strains of *L. monocytogenes* (Huang *et al.*, 1994).

The present investigation was primarily of basic nature, limited to production, purification,

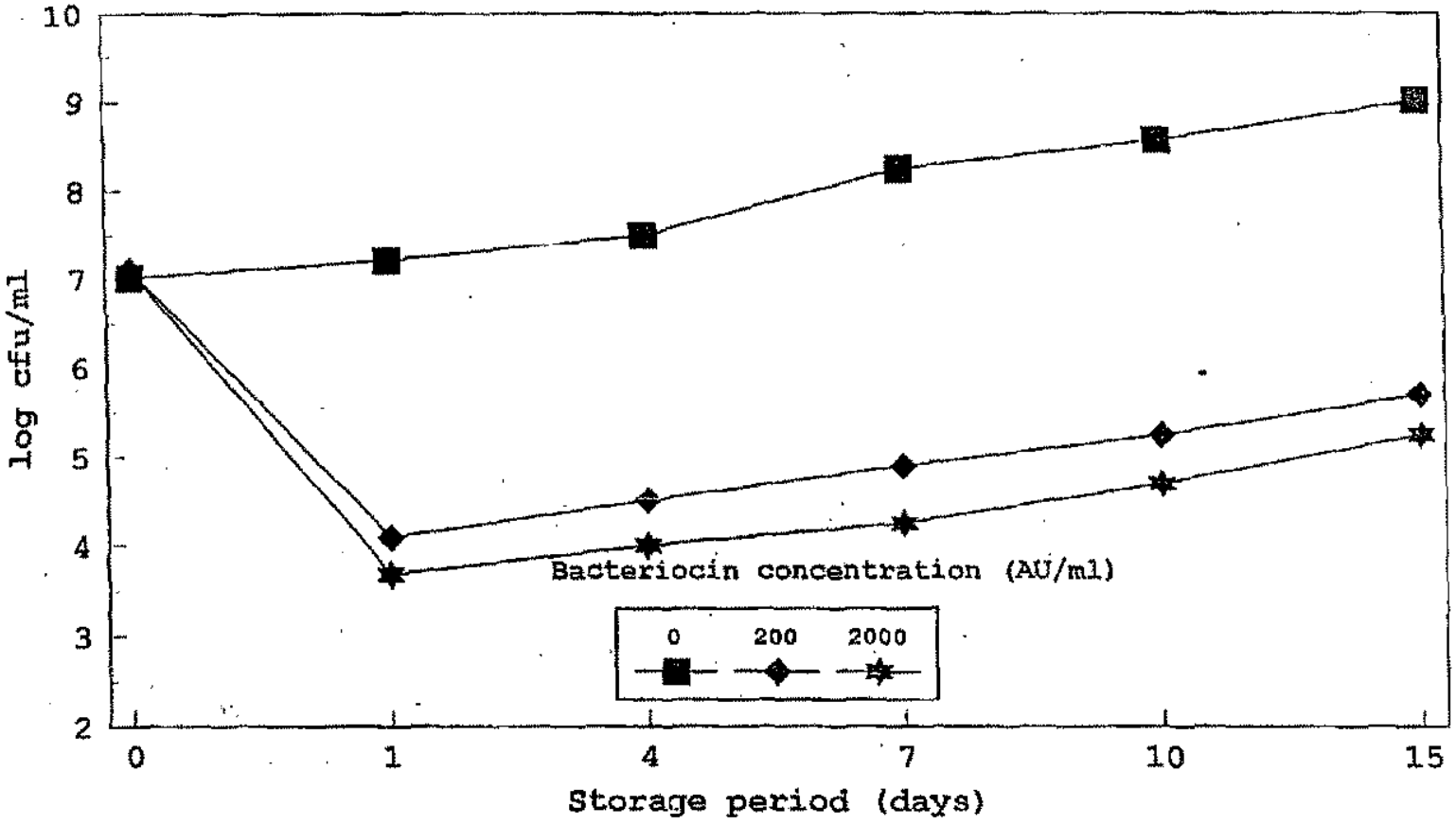


Fig. 4.15 Effect of *Pediococcus pentosaceus* 34 bacteriocin on the growth of *Listeria monocytogenes* MTCC657 in sterile skim milk

characterisation of bacteriocin and delineation of genetic elements associated with its production in a selected pediococcal isolate (*P. pentosaceus* 34) of Cheddar cheese origin. Such an investigation is deemed necessary to facilitate the approval for its use in foods to enhance their shelf life and safety by controlling the growth of spoilage and pathogenic bacteria. Bacteriocins for use in foods are required in large amounts. Thus, there is a need to develop a cost effective procedure such as pH dependent adsorption-desorption technique. However, the relatively low recoveries obtained in the present study necessitates further improvements in the method to desorb the adsorbed bacteriocin. Although foods are manufactured under hygienic conditions, their involvement in the outbreaks of foodborne diseases suggests that post processing contaminants are responsible for such outbreaks. It, therefore, becomes necessary to prevent the entry of post processing contaminants into processed foods or to find suitable means of inactivating such microflora. Besides, use of pediocinogenic pediococci or their pediocins in dairy and food industry has also been indicated. Though it was beyond the scope of present investigation, exploitation of pediocins in food and dairy industry would no doubt make an interesting and useful study.

Chapter 5

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

SUMMARY

The salient findings of the present investigation encompassing the isolation of pediococci from milk and milk products, screening of pediococcal isolates for bacteriocin activity, optimisation of cultural and environmental parameters for bacteriocin production, partial purification and characterisation of the bacteriocin, genetic determinants encoding bacteriocin production and host cell immunity in the selected pediococcal isolate and effectiveness of the bacteriocin in controlling the growth of pathogenic bacteria in milk are summarised in this section.

5.1 A total number of 1030 isolates of LAB comprising 885 (85.9%) lactobacilli, 100 (9.7%) leuconostocs and 45 (4.4%) pediococci were isolated from 25 samples of different milk and milk products.

5.2 Lactobacilli were the predominant microflora in all the milk product samples and also at all the stages of ripening of Cheddar cheese.

5.3 Pediococcal isolates were obtained only from raw milk (5) and Cheddar cheese (40), constituting about 2.5% and 6.7%, respectively of the total isolates from these products.

5.4 Pediococci appeared rather late in the ripening period (6-12 months) of Cheddar cheese.

5.5 Out of the 45 pediococcal isolates, only 5 cultures i.e. isolate numbers 12, 16, 21, 26 and 34, all from Cheddar cheese, exhibited antagonistic activity against all the indicator bacteria in the three different bioassays.

5.6 Morphological, physiological and biochemical characterisation of 18 pediococcal isolates revealed that 9 belonged to *Pediococcus pentosaceus*, 2 to *P. acidilactici*, whereas 7 could not be assigned to any of the two species.

5.7 The five pediococcal isolates exhibiting antibacterial activity against all the indicator organisms were identified as different strains of *P. pentosaceus*.

5.8 The culture supernatants of the five strains of *P. pentosaceus* retained their antibacterial activity upon neutralisation to pH 7.0 or treatment with catalase but their activity was lost on treatment with several proteases, indicating the role of bacteriocin in the inhibitory activity.

5.9 *Pediococcus pentosaceus* 34 exhibited maximum bacteriocin activity against the four lactic indicator organisms and , therefore, was selected for further studies.

5.10 *Pediococcus acidilactici* LB42 was the most sensitive indicator bacteria followed by *Lb. plantarum* NCDO955 and, hence, the former was routinely used as the indicator organism.

5.11 There was no difference in the bacteriocin titre at the end of 24 hr incubation period when the test

culture was grown at 25, 30 or 37°C. However, about 65% of the total bacteriocin was detected in the first 8 hr of growth at 37°C as against 0.25% and 18% at 25 and 30°C, respectively.

5.12 MRS broth was found to be the most suitable medium for bacteriocin production.

5.13 The initial pH of the MRS medium (5-8) did not have any influence on the bacteriocin production.

5.14 *Pediococcus pentosaceus* 34 when grown in MRS broth (pH 6.5) at 37°C produced about 65% of the total bacteriocin in the first 8 hr of growth whereas the rest was produced in the next 8 hr.

5.15 The bacteriocin by the test culture was secreted as a primary metabolite reaching maximum at the end of the logarithmic growth phase with no decline in the activity during the stationary and death phases.

5.16 The purification of bacteriocin by pH dependent adsorption-desorption technique resulted in the poor recovery (13%) of the total bacteriocin in the culture broth.

5.17 Fractionation of the bacteriocin from the culture supernatant with various organic solvents such as isopropanol, methanol, ethanol and acetone also resulted in a very low recovery and purification fold.

5.18 Ammonium sulfate fractionation (30-60% saturation) resulted in 34.3% recovery with 3.1-fold purification.

5.19 The bacteriocin bound itself to the anion exchanger but not to the cation exchanger.

5.20 The bacteriocin adsorbed to the DEAE-Sephadex (A-25) ion exchanger however, could not be eluted with 0.1-0.4 M NaCl.

5.21 The partially purified bacteriocin was found to exhibit a broad spectrum of antibacterial activity against numerous Gram positive bacteria viz. *B. cereus*, *Staph. aureus*, *Listeria* spp. including several strains of *Listeria monocytogenes*, *Enterococcus* spp. etc. It was relatively less active against several LAB. None of the Gram negative bacteria and yeasts were inhibited by the test bacteriocin.

5.22 The SDS-PAGE gel stained with brilliant blue G showed 2-3 protein bands in the partially purified preparation.

5.23 The SDS-PAGE gel overlaid with the indicator strain showed a discrete zone of inhibition.

5.24 Comparison of the stained SDS-PAGE gel (5.22) with that of the overlaid gel (5.23) demonstrated that the molecular weight of the bacteriocin is in the range of 3,500-6,100 Da.

5.25 The bacteriocin produced by *P. pentosaceus* 34 was sensitive to several proteolytic enzymes but resistant to non-proteolytic enzymes such as β-amylase, lipase, lysozyme and ribonuclease.

5.26 The observed sensitivity of the bacteriocin to α-amylase was shown to be due to the contamination

of the enzyme preparation with certain serine proteases.

5.27 The crude and partially purified bacteriocin preparations were extremely thermostable retaining 100% activity after 30 min at 100°C. However, there was a loss of 20% and 40% activity of both the preparations after 60 min at 100°C and 15 min at 121°C, respectively.

5.28 The crude bacteriocin remained active for 2, 24 and 168 hr at pH 12, 11 and 10, respectively. It was extremely stable in the pH range 1-8 while the residual activity dropped from 27.3% after one day to 12% after 15 days at pH 9.

5.29 The purified preparation was also extremely stable in the pH range 1-8. However, a marginal decrease in the activity was observed during its storage for 15 days at pH 9.

5.30 The inactivation of the bacteriocin observed at various pH values was irreversible.

5.31 Both the preparations were extremely heat stable over a wide pH range (1-8).

5.32 The bacteriocin exhibited a rapid bactericidal, non-bacteriolytic mode of action against the sensitive indicator bacteria.

5.33 The indicator cells surviving bacteriocin treatment were not resistant to the action of bacteriocin.

5.34 There was 300% increase in bacteriocin activity upon treatment with 1% SDS of the partially purified bacteriocin.

5.35 The bacteriocin was unstable in NaCl solutions of 0.5 M or more ionic strength.

5.36 Addition of bacteriocin at two different levels i.e. 200 and 2,000 AU/ml, in sterile milk inoculated with *Listeria monocytogenes* MTCC657 (10^7 cfu/ml) resulted in a substantial reduction of viable cell counts by about 3 and 3.4 log cycles, respectively, at 5°C on the first day of storage. The difference between the control and treated samples at the end of 15 days storage at 5°C was about 3.3-3.8 log cycles.

5.37 The pediococcal cultures were found to contain a maximum of eight plasmids with molecular weights ranging from 1.6 to 49 MDa.

5.38 The bacteriocin producing *P. pentosaceus* 34 harboured 5 plasmids with molecular weights of 11, 4.6, 3.6, 3.2 and 2.1 MDa.

5.39 Growth at 37°C of *P. pentosaceus* 34 in the presence of acridine orange (50-200 µg/ml), acriflavine (10-30 µg/ml), ethidium bromide (10-40 µg/ml) and novobiocin (10-70 µg/ml) did not yield any Bac^- variant.

5.40 The parent culture when incubated at 45°C with acriflavine (5-15 µg/ml) or ethidium bromide (10-20 µg/ml) also did not yield any Bac^- variant.

5.41 A total number of 15 Bac^- variants were obtained when the parent culture was grown at 45°C in TGE broth containing different concentrations of novobiocin (40, 45 and 50 µg/ml).

5.42 Comparison of plasmid profiles of parent and the Bac$^-$ variants revealed the loss of either all, some or none of the plasmids in the Bac$^-$ variants.

5.43 All the Bac$^-$ variants retained their immunity to the host bacteriocin and carbohydrate fermentation and the antibiotic susceptibility patterns of all the Bac$^-$ variants were identical with that of the parent culture.

5.44 Attempts made to transfer the Bac$^+$ phenotype from the parent to a plasmid free Bac$^-$ variant by conjugation technique were not successful thus indicating chromosomal mediated bacteriocin production and host cell immunity.

CONCLUSIONS

The present investigation afforded a potent bacteriocinogenic strain, *P. pentosaceus* 34 capable of producing high concentrations of pediocin. The pediocin produced by *P. pentosaceus* 34 is unique for several reasons. Firstly, it is extremely antagonistic to Gram-positive food spoilage bacteria as well as pathogenic organisms such as *L. monocytogenes*, yet relatively ineffective against several strains of lactic acid bacteria which are commonly used as dairy starters in the manufacture of fermented dairy foods. Secondly, it is extremely thermostable thereby indicating that it can be used in thermally processed foods. Thirdly, the bacteriocin is not only active and stable over a wide pH range but it is also extremely heat stable at neutral pH values also indicating that it can be useful in acidic and non-acidic foods. This characteristic is different from nisin which is unstable at or near neutral pH values. All these desirable features would no doubt make *P. pentosaceus* 34 bacteriocin a highly suitable candidate

to serve as an effective natural biopreservative in dairy and food industry.

The relative ineffectiveness of *P. pentosaceus* 34 bacteriocin towards lactic acid bacteria and poor growth of *P. pentosaceus* 34 in milk would further facilitate the use of bacteriocinogenic strain as starter adjuncts in the fermented dairy products. The chromosomal location of genetic determinants for bacteriocin production by the *P. pentosaceus* 34 strain further shows that this characteristic is relatively stable and is not likely to be lost upon repeated subculturing.

Chapter 6

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