PARTIAL PURIFICATION, CHARACTERIZATION AND ANTIBIOSIS OF BACTERIOCINS PRODUCED BY SOME LACTIC ACID BACTERIAL ISOLATES

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN SCIENCE (BOTANY)
OF THE
UNIVERSITY OF NORTH BENGAL

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Certified that the work presented in the thesis titled, '*Partial purification, characterization and antibiosis of bacteriocins produced by some lactic acid bacterial isolates*' has been carried out by Ms Sharmistha Banerjee, M.Sc. under my supervision at the Department of Botany, University of North Bengal. The results incorporated in the thesis have not been submitted for any other degree elsewhere.

Further certified that Ms Banerjee has followed the rules and regulations laid down by the University of North Bengal in carrying out this work.

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Acknowledgments

I take this opportunity to offer my heartfelt gratitude to my supervisor Dr P.K. Sarkar, Reader and Head of the Department of Botany, University of North Bengal for constant guidance and encouragement throughout this work and also for providing me with necessary laboratory facilities.

I also extend my sincere gratitude to Prof. Samir Bhattacharya, Prof. Shelly Bhattacharya, Prof. S.C. Pal and Dr S.R. Biswas of Visva-Bharati, Santiniketan for providing guidance and laboratory facilities in bacteriocin purification.

I gratefully acknowledge the University Grants Commission, New Delhi for financially supporting the work.

I extend my thanks to all the faculty members of the Department of Botany, University North Bengal for extending their generous help, whenever needed.

I thankfully acknowledge the help I obtained from my labmates Dr B. Saha, Dr J.P. Tamang, Dr S.A. Hossian, Dr P.K. Pal, Dr J.K. Pal and Ms A. Das Gupta in carrying out various experiments and statistical analysis.

I extend my thanks to all my friends at Siliguri for their love and support. Specially, I wish to thank Ms Arpita Ray and Mr Subroto Saha for their love and care.

I am thankful to Mr Subhasis Kundu for typing the thesis.

Last, but not the least, I thank my parents for their constant inspiration, love and support. Their patience with me through this work is worth-mentioning

Siliguri
May 4, 1996

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Introduction

Bacteriocins from lactic acid bacteria have attracted much attention in recent years because of their potential as natural food preservatives and as phenotypic markers for the construction of food grade cloning vectors (Klaenhammer 1988; Nettles and Barefoot 1993). The study of bacteriocins began with the discovery by Gratia in 1925 of a highly specific antibiotic (principle V), produced by one strain of *Escherichia coli* and active against another strain of the same species. Gratia's publications described many features of what later turned out to be a general class of antibiotic-like proteins produced by Enterobacteriaceae, for which the generic name 'colicine' was proposed by Gratia and Fredericq (1946). With the discovery that the production of apparently similar agents is not limited to coliform organisms, Jacob *et al.* (1953) proposed the more general term 'bacteriocine' for highly specific antibacterial proteins, produced by certain strains of bacteria and active mainly against some other strains of the same species. Although this definition still holds good, both 'colicine' and 'bacteriocine' are now spelt without the final 'e'.

However, most of the bacteriocins produced by Gram positive bacteria do not fit the classical colicin mould. Rather, they tend to be more broadly active against strains of Gram positive species, with little evidence of their action being mediated by specific receptor molecules or their release from producer cells being enhanced by the action of lysins or bacteriocin release proteins. The absence of an outer membrane in Gram positive bacteria excludes any possibility of a modulating receptor molecule in the manner which applies to the interaction of colicins with sensitive bacteria. Rather, the potentially lethal interaction of bacteriocins of Gram positive bacteria with sensitive cells appears to be dependent upon a more general compatibility between surface charges and hydrophobic domains of the interacting molecules. Another difference is that the level of immunity of the producing strain to its own inhibitory product is generally less strong for bacteriocins of Gram positive bacteria than it is for the colicins (Tagg 1992).

Bacteriocinogeny has been ascribed to several species. However, in many instances the presence of bacteriocins has not been convincingly established, since it has been based solely on a finding of antagonism between two bacterial strains. It is thus important that claims of bacteriocinogeny be supported by determination of protein nature of the antagonist as well as a demonstration that the producing organism is insensitive (immune) to its action. Immunity is not always absolute. In some cases bacteriocinogenic cells have been reported to be sensitive to high concentration of homologous bacteriocins (Ivanovics and Alföldi 1954; Fredericq 1957; Levisohn *et al.* 1968).

The antibacterial activity of lactic acid bacteria is associated with the major end products of their metabolism, such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins (Klaenhammer 1982). Lactic acid bacteria are well recognized for their production of bacteriocins (Klaenhammer 1988; Lindgren and Dobrogosz 1990; Schillinger 1990). Bacteriocins have been found in all genera of lactic acid bacteria,

and most of the bacteriocin-producing strains have been isolated from foods (Schillinger *et al.* 1993).

The ability to produce bacteriocins has technological and scientific importance. Research on bacteriocins from lactic acid bacteria has expanded exponentially during the last decade, as these have several interesting application possibilities. Lactic acid bacteria are used for biological processing of many raw materials to produce acceptable foods which are with improved flavour and consumed for their prophylactic and therapeutic properties (Fernandes and Shahani 1989). Bacteriocins or bacteriocin producers occupy important position in fermented foods. Firstly, bacteriocin-producing starter cultures may result in a more reliable fermentation process preventing growth of spoilage bacteria (Mortvedt and Nes 1990). In mixed starter cultures, bacteriocin producers may dominate and affect the microbiological balance required to obtain products with defined characteristics. Bacteriocin production may offer certain advantages. Bacteriocins from food grade lactic acid bacteria are bactericidal to many Gram positive bacteria associated with food spoilage (Bhunia *et al.* 1987). Several unique properties, such as activity over a wide range of pH and high or low temperature treatment make them suitable as biological preservatives to extend the shelf life of refrigerated semi-preserved and canned foods. Their use in foods has an added advantage, because they are degraded by the proteolytic enzyme of the gastrointestinal tract and are nontoxic and nonantigenic to animals (Biswas *et al.* 1991). The most important compound of this type is nisin, produced by *Lactococcus* ssp. *lactis,* which has commercial importance in food industry. In many foods, higher levels of acids are undesirable. For such products, the inhibition of the undesired organisms must be achieved at least partially by means other than acidification. (Schillinger and Lücke 1989). The second reason for undertaking extensive research on bacteriocins is that the genetic determinants for bacteriocin production and immunity could be used in the construction of food grade vectors for strain improvement using r-DNA technology. (Mortvedt and Nes 1990; ten Brink *et al.* 1994). Thirdly, bacteriocin production or susceptibility could be used in taxonomic studies. (ten Brink *et al.* 1994).

Compared to other antimicrobial agents, studies on distribution of bacteriocin-producing microorganisms in nature, factors affecting bacteriocin production and their characterization are relatively rare. Therefore, the main objectives of the present investigation had been the folowing:

1) Isolation of lactic acid bacteria from natural habitats;
2) Detection and assay of bacteriocin activity;
3) Optimization of bacteriocin production;
4) Purification of bacteriocins;
5) Characterization of bacteriocins; and
6) Characterization of selected bacteriocin-producing isolates with a view to identify their taxonomic status.

2

Review of Literature

2.1. Bacteriocins from lactic acid bacteria

Majority of bacteriocins produced by lactic acid bacteria have been characterized by the initial definition of a proteinaceous inhibitor, crude estimation of molecular weight (via retention in dialysis membranes or ultrafiltration) and determination of susceptible strains. Lactic acid bacteria produce three general classes of antimicrobial proteins : (1) lantibiotics, (2) small hydrophobic heat-stable peptides (<13 kDa) and (3) large heat-labile proteins (>30 kDa) (Klaenhammer *et al.* 1992).

2.1.1. Lantibiotics

The term 'lantibiotics' has been coined for those bacteriocins in which the amino acid lanthionine is a main component (Hurst 1981). Bacteriocins such as nisin (Gross and Morell 1971), subtilin (Gross and Kiltz 1973), epidermin (Allgaier *et al.* 1985), gallidermin (Kellner *et al.* 1988) and PEP5 (Kellner *et al.* 1989) have been shown to contain amino acid lanthionine. Reports of lantibiotics within Lactobacillaceae are rare. However, Mortvedt *et al.* (1991a) reported that the *Lactobacillus sake* bacteriocin, lactocin S, contains lanthionine residue. The presence of lanthionine has also been detected in a bacteriocin of 4.63 kDa (33-35 amino acid residues) produced by *Carnobacterium* sp. isolated from fish. Incorporation of a lanthionine residue introduces a monosulphur bridge which results in unique peptide ring structures among the various lantibiotics. Foremost among the characterized bacteriocins is the lantibiotic nisin, produced by *Lactococcus lactis* subsp. *lactis*. This small 34 amino acid-containing peptide has two sulphur-containing amino acids, lanthionine and β-methyllanthionine. If shows antimicrobial activity against a range of Gram positive bacteria, particularly sporeformers (Delves-Broughton 1990). The nisin molecule is acidic in nature and exhibits greatest stability under acid conditions; it is more soluble in acidic pH (Hurst 1981). Due mainly to its relatively narrow antibacterial spectrum, low solubility in body liquids, susceptibility to digestive proteases and instability at physiological pH (7.0-7.5), it is found to be unsuitable for therapeutic effect for veterinary and clinical uses (Hurst 1983).

2.1.2. Small hydrophobic heat-stable peptides

The first of this class characterized and purified is a lipocarbohydrate-protein macromolecular complex produced by *Lactobacillus fermenti* (de Klerk and Smit 1967). This bacteriocin is relatively heat-stable (96°C for 30 min) and contains a high proportion of glycine (11.1%) and alanine (13.4%) residues. Lactocin 27 produced by *Lactobacillus helveticus* is a small (12.4 kDa) heat-stable glycoprotein, similar to the *Lb. fermenti*

bacteriocin. It was initially isolated as a large molecular weight complex (>200 kDa) while the active peptide was defined only at 12.4 kDa and contained unusually high concentrations of glycine (15.1%) and alanine (18.1%) residues (Upreti and Hinsdill 1973, 1975). Lactacins B and F produced by *Lactobacillus acidophilus* N2 and 11088, respectively are highly heat-stable (121°C for 15 min) (Barefoot and Klaenhammer 1983; Muriana and Klaenhammer 1991a). Lactacin B showed many similarities to lactocin 27 (Barefoot and Klaenhammer 1984). Both were initially isolated as large molecular weight complexes. The initial molecular weight estimates were 100 kDa and >200 kDa for lactacin B and lactocin 27, respectively. Lactacin B was later purified as a 6.3 kDa peptide. The antimicrobial peptides within this group are small, ranging in size between 3.7 and 6.3 kDa. Native lactacin F was sized at approximately 180 kDa by gel filtration. However, the lactacin F, purified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was identified as only a 2.5 kDa peptide. Compositional analysis indicates that lactacin F contains 56 amino acid residues; the peptide is composed primarily of hydrophobic and polar neutral residues (87.3%) which include glycine (21.6%), alanine (15.8%) and valine (8.8%) (Muriana and Klaenhammer 1991a, 1991b). Lactocin S, produced by *Lactobacillus sake* L45, is yet another bacteriocin in this group. Analysis of the amino acid composition and N-terminal sequencing of lactocin S reveals that 50% of the approximated 33 amino acids are hydrophobic and nonpolar residues of alanine, valine and glycine (Mortvedt *et al.* 1991b). The lower glycine content and hydrophobic propensity of lactocin S compared to lactacin F correlates with their relative heat stabilities (lactacin F7S). A number of additional heat-stable hydrophobic proteins have been purified subsequently. A number of carnobacteriocins produced by *Carnobacterium piscicola* (Ahn and Stiles 1990), brevicin 37 (Rammelsberg and Radler 1990) and gassericin A (Toba *et al.* 1991a) are small (~5 kDa) heat-stable (121°C for 20 min) peptides. Some of the recently reported *Lactobacillus* bacteriocins that can be enumerated within this category are sakacin B, a hydrophobic peptide (6.3 kDa) produced by *Lb. sake* (Samelis *et al.* 1994), acidocin B, a heat-stable, 2.4 kDa hydrophobic peptide produced by *Lb. acidophilus* M46 (ten Brink *et al.* 1994) and brevicin 27, a heat-stable hydrophobic peptide with an apparent molecular weight between 10 and 30 kDa for the crude inhibitory molecule produced by *Lactobacillus brevis* SB 27 (Benoit *et al.* 1994).

Majority of the lactococcal and pediococcal bacteriocins belong to this group. Lactococcins are heat-stable (100°C for 60 min), small hydrophobic peptide bacteriocins. Lactococcin A is produced by *Lactococcus lactis* subsp. *cremoris* LMG2130 (Holo *et al.* 1991) and bacteriocin S50 is produced by *Lactococcus lactis* subsp. *diacetylactis* S50 (Kojic *et al.* 1991). *Lactococcus lactis* CNRZ 481 produces lacticin 481, a 5.5 kDa heat-stable peptide (Piard *et al.* 1990). Parrot *et al.* (1989) reported four bacteriocins (mutacins) produced by different strains of *Streptococcus mutans*. The mutacins produced by the strains C67-1, Ny266 and T8 possess similar properties.

They are thermoresistant (100°C for 30 min) and low molecular weight (<3.5 kDa) peptides. The mutacin produced by the strain Ny 257-S is of lower thermoresistance (80°C for 30 min) but of a slightly higher molecular weight (8-14 kDa).

The pediococcal bacteriocins include pediocin PA-1, a 16.5 kDa peptide produced by *Pediococcus acidilactici* PAC 1.0 (Gonzales and Kunka 1987), pediocin AcH, a 2.7 kDa-peptide produced by *Pc. acidilactici* (Bhunia *et. al.* 1988) and pediocin N5p produced by *Pc. pentosaceus* N5p (Strasser de Saad and Manca de Nadra 1993). Pediocin N5p is somewhat lipophilic in nature and is inactivated by chloroform and ethanol.

2.1.3. Large heat-labile proteins

Recent reports of large heat-labile bacteriocins suggest that there are numerous members of this class. Acidophilucin A, lacticins A and B, and caseicin 80 appear to be large proteins, since it takes 10-15 min to be inactivated at 60°C (Rammelsberg and Radler 1990; Toba *et al.* 1991b, 1991c). To date, only helveticin J (37 kDa) produced by *Lactobacillus helveticus* has been purified and characterized at the genetic level. It is a heat-sensitive protein (inactivated at 100°C within 30 min) which retains activity after treatment with various dissociating agents (Joerger and Klaenhammer 1986). Another recently reported bacteriocin, helveticin V-1829, from *Lb. helveticus* 1829 is heat-labile. A partially purified sample approximates a molecular weight of more than 10 kDa (Vaughan *et al.* 1992). The biochemical properties and mechanism of action of many larger bacteriocins remain to be investigated. Considering their size and heat lability, the bactericidal activities of these proteins are likely to be affected by changes in conformation and secondary structure.

Table I lists bacteriocins reported from different lactic acid bacteria.

2.2. Optimization of bacteriocin production

Optimization of bacteriocin production by regulation of environmental growth parameters is important for effective commercial application.

2.2.1. Medium composition

This is one of the important environmental factors. Complex growth media commonly used for the growth of bacteriocin-producing lactic acid bacteria are MRS, M17, Elliker lactic, *Lactobacillus* selection (LBS), tryptone-yeast extract-Tween (TYT), APT and

Table 1. Bacteriocins from lactic acid bacteria

Producer	Bacteriocin (MW, kDa)	Activity spectrum	Reference
Lactobacillus acidophilus	Acidolin		Hamada *et al.* (1971)
	Acidophilin		Shahani *et al.* (1977)
	Lactacin B (6.3)	*Lactobacillus delbrueckii, Lactobacillus helveticus, Lactobacillus leichmannii*	Barefoot and Klaenhammer (1983, 1984)
	Lactacin F (6.3)	*Lactobacillus fermentum, Lactobacillus delbrueckii, Lactobacillus helveticus, Enterococcus faecalis, Acromonas hydrophila, Staphylococcus aureus*	Muriana and Klaenhammer (1987,1991a, 1991b); Lewus *et al. (1991)*
	Acidophilucin A	*Lactobacillus delbrueckii, Lactobacillus helveticus*	Toba *et al.* (1991b)
	Acidocin B (2.4)	*Lactobacillus, Clostridium sporogenes*	ten Brink *et al.* (1994)
Lactobacillus bavaricus	Bavaricin MN		Kaiser and Montville (1993)
Lactobacillus brevis	Lactobacillin		Wheater *et al.* (1951)
	Lactobrevin		Kavasnikov and Sodenko (1967)
	Brevicin 37	*Pediococcus damnosus, Lactobacillus brevis , Leuconostoc oenos*	Rammelsberg and Radler (1990)
	Brevicin 27 (10-30)	Heterofermentative	Benoit *et al.* (1994)

Contd....

Producer	Bacteriocin (MW, kDa)	Activity spectrum	Reference
		lactobacilli	
Lactobacillus casei	Caseicin 80 (42)	*Lactobacillus casei*	Rammelsberg and Radler (1990); Rammelsberg *et al.* (1990); Müller and Radler (1993)
Lactobacillus carnis	Bacteriocins (4.9)	*Lactobacillus, Carnobacterium, Pediococcus , Enterococcus*	Ahn and Stiles (1990); Schillinger and Holzapfel (1990)
Lactobacillus curvatus	Curvacin a	*Lactobacillus, Enterococcus faecalis, Listeria monocytogenes*	Vogel *et al. (1993)*
Lactobacillus delbrueckii subsp. *bulgaricus*	Bulgarican	*Staphylococcus aureus, Pseudomonas fragi*	Reddy *et al.* (1984)
Lactobacillus delbrueckii	Lacticin A, B	*Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* subsp. *delbrueckii*	Toba *et al.* (1991c)
Lactobacillus fermenti	Baccteriocin	*Lactobacillus fermenti*	de Klerk and Smit (1967)
Lactobacillus helveticus	Lactocin 27 (12.4)	*Lactobacillus acidophilus, Lactobacillus helveticus*	Upreti and Hinsdill (1975)

Contd....

Producer	Bacteriocin (MW, kDa)	Activity spectrum	Reference
	Helveticin J (37)	*Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii*, subsp. *bulgaricus*	Joerger and Klaenhammer (1986)
	Helveticin V-1829	*Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*	Vaughan *et al.* (1992)
Lactobacillus plantarum	Plantaricin SIK-83	*Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*	Andersson *et al.* (1988)
	Plantaricin A (>8)	*Lactobacillus plantarum*, *Leuconostoc sp.* *Pediococcus sp.*, *Lactococcus lactis*, *Enterococcus faecalis*	Daeschel *et al.* (1990)
	Plantacin B	*Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Pediococcus damnosus*	West and Warner (1988)
	Plantaricin C19 (3.5)	*Listeria* spp.	Atrih *et al.* (1993)
	Plantaricin-149 (2.2)	*Lactobacillus delbrueckii*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *Pediococcus cerevisiae*, *Enterococcus hirae*, *Lactococcus lactis*	Kato *et al.* (1994)
	Plantaricin LC74 (<5)		Rekhif *et al.* (1994)

Contd....

Producer	Bacteriocin (MW, kDa)	Activity spectrum	Reference
Lactobacillus sake	Lactolin		Kodama (1952)
	Sakacin A	*Carnobacterium piscicola, Enterococcus sp., Lactobacillus sake, Lactobacillus curvatus, Leuconostoc paramesentederoides, Listeria monocytogenes, Acromonas hydrophila, Staphylococcus aureus*	Schillinger and Lücke (1989); Lewus *et al.* (1991)
	Sakacin B (6.3)	*Lactobacillus sake, Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus minor, Lactobacillus farciminis, Lactobacillus halotolerans, Lactobacillus viridescens, Leuconostoc mesenteroides, Leuconostoc paramesenteroides, Lactococcus sp., Listeria monocytogenes, Listeria innocua*	Samelis *et al.* (1994)
	Lactocin S (3.7)	*Lactobacillus sp., Leuconostoc sp., Pediococcus sp.*	McCormick and Savage (1983); Mortvedt and Nes(1990); Mortvedt *et al.* (1990, 1991a, 1991b)
Lacobacillus salivarius	Salivaricin B	*Listeria monocytogenes, Bacillus cereus, Brocothrix thermosphacta Enterococcus faecalis, Lactobacillus sp.*	ten Brink *et al.* (1994)

Contd....

Producer	Bacteriocin (MW, kDa)	Activity spectrum	Reference
Lactococcus lactis subp. *lactis*	Nisin	*Staphylococcus, Listeria monocytogenes, Streptococcus, Micrococcus, Lactobacillus, Bacillus, Clostridium*	Hirsch (1950); Hirsch *et al.* (1951); Hurst (1981)
Lactococcus lactis subsp. *cremoris*	Lactococcin A	*Lactococcus lactis ssp. cremoris, Lactococcus lactis* subsp. *lactis*	Holo *et al.* (1991)
Lactococcus lactis subsp. *diacetylactis*	Bacteriocin S-50	*Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *diacetylactis*	Kojic *et al.* (1991)
Lactococcus lactis	Lacticin 481	*Lactococcus sp., Leuconostoc sp., Lactobacillus sp., Clostridium tyrobutyricum*	Piard *et al.* (1992)
Lactococcus mutans	Mutacins (<3.5)	*Neissaria subflava, Flavobacterium capsulatum*	Parrot *et al.* (1989)
Lactococcus cremoris	Diplococcin		Davey (1981)
Leuconostoc carnosum	Carnocin 54 (~4)	*Leuconostoc mesenteroides, Carnobacterium divergens, Enterococcus faecalis, Listeria monocytogenes Listeria innocua*	Keppler *et al.* (1994); Schillinger *et al.* (1995)

Contd....

Producer	Bacteriocin (MW, kDa)	Activity spectrum	Reference
Pediococcus acidilactici	Pediocin PA-1		Gonzales and Kunka (1987)
	Pediocin AcH (2.7)	*Lactobacillus, Leuconostoc, Staphylococcus aureus, Clostridium perfrinngens, Listeria monocytogenes, Pseudomonas putida*	Bhunia *et al.* (1988)
	Pediocin L50		Cintas *et al.* (1995)
Pediococcus pentosaceus	Pediocin N5p	*Pediococcus pentosaceus, Lactobacillus hilgardii, Leuconostoc oenos*	Strasser de Saad and Manca de Nadra (1993)

tomato juice broths. However media components, especially peptides, which are present in relatively high concentration interfere with subsequent bacteriocin purification (Barefoot and Klaenhammer 1984). Dialysates of complex media containing only low molecular weight fractions were found to be effective for the production of pediocin AcH (Bhunia *et al.* 1988), but not for lactococcal bacteriocins (Geis *et al.* 1983). Semidefined media, containing casitone (a pancreatic digest of casein), yeast extract, glucose and several growth factors are used for the production of lactacin B (Barefoot and Klaenhammer 1984), helveticin J (Joerger and Klaenhammer 1986) and helveticin V-1829 (Vaughan *et al.* 1992). Studies on the comparison of different media or the effect of media ingredients for bacteriocin production are scarce. Geis *et al.* (1983) compared bacteriocin production by 16 strains of lactococci in Elliker lactic broth (ELB), M17, brain heart infusion, a synthetic medium and litmus milk. ELB followed by M17 supported the highest bacteriocin production. Biswas *et al.* (1991) studied the effect of the ingredients of a complex medium, trypticase-glucose-yeast extract (TGE) broth on the production of pediocin AcH. Glucose was found to be the best carbon source. Tween 80 and Mn^{2+} allowed optimal biomass and bacteriocin production, which were stabilized by the presence of Mg^{2+}. However, Tween 80 sometimes interferes with subsequent ammonium sulphate precipitation of bacteriocins (Mortvedt *et al.* 1991b). Parente and Hill (1992) studied the effects of tryptone, yeast extract and Tween 80 on the production of enterocin 1146 and lactocin D in TYT medium using factorial experiments and emperical modelling. On the basis of predictions of the models developed, three TYT media (TYT10, TYT11 and TYT30) were designed to maximize bacteriocin production, while minimizing the amount of peptides in the medium. Bacteriocin production in TYT media is comparable with that in M17 and MRS broths which have higher peptide content.

2.2.2. pH of medium

A number of studies have shown that control of the medium pH is a critical factor in bacteriocin production (Goebel *et al.* 1955; Joerger and Klaenhammer 1986). APT broth adjusted at an initial pH of 6.0 - 6.5 allowed optimal production by *Carnobacterium piscicola* (Ahn and Stiles 1990) and *Leuconostoc gelidium* (Hastings and Stiles 1991). Production of pediocin AcH was maximum in TGE broth adjusted at an initial pH of 6.0-6.5. External pHcontrol can provide further improvement of bacteriocin production. The optimal pH varies among strains: lactacin B production was optimal at pH 6.0 (Barefoot and Klaenhammer 1984), helveticin J at pH 5.5 (Joerger and Klaenhammer 1986), lacticin B at pH 5.5 (Piard *et al.* 1990), helveticin V-1829 at pH 5.5 (Vaughan *et al.* 1992) and bavaricin MN at pH 6.0 (Kaiser and Montville 1993). Production of lactacin F is also pH dependent; maximum levels of lactacin F are obtained in MRS broth

maintained at pH 7.0, whereas negligible activity is found in fermentors held at pH 7.5 or 6.5 (Muriana and Klaenhammer 1987).

2.2.3. Duration and temperature of incubation

The period and temperature of incubation needed to achieve maximum yield are usually different for different strains. Studies on the production of bacteriocins with respect to growth of the producer strains are thus common. Most of the bacteriocins of lactic acid bacteria are produced during late exponential phase, indicating that a high amount of biomass was essential for bacteriocin synthesis (Piard and Desmazeaud 1991). About 60% of the pediocin AcH was produced within 8 h, and the final 40% was produced during the next 8 h (stationary phase) at 37°C. Thus, pediocin AcH appears to be a secondary metabolite (Biswas *et al.* 1991) produced by *Pc. acidilactici* H. In *Lb. sake* LB706 producing sakacin A, no activity was detected during the first 8 h of incubation. However, significant activity was detected after 23 h at 25°C when the cells were in the mid or late logarithmic growth phase. A loss of activity of Lb 706 supernatant was observed after 47 h at 25°C (Schillinger and Lücke 1989). The highest activity of *Lactobacillus casei*, producing caseicin 80, is observed when the cells reach the stationary phase usually after 3 d at 30°C (Rammelsberg and Radler 1990). Helveticins J and V-1829 produced by *Lb. helveticus* (Joerger and Klaenhammer 1986; Vaughan *et al.* 1992), sakacin B produced by *Lb. sake* 251 (Samelis *et al.* 1994) and acidocin B produced by *Lb. acidophilus* M46 (ten Brink *et. al* 1994) are produced during late logarithmic and begining of stationary phases. However, excretion during early growth phase has also been reported (Ahn and Stiles 1990; Hastings and Stiles 1991). Production of bacteriocin by *Pc. pentosaceus* N5p occurs early in the growth cycle of the organism, rather than as a secondary metabolite of growth (Strasser de Saad and Manca de Nadra 1993). Thus, the timing of bacteriocin harvest must also be determined emperically for each different organism, method and set of conditions.

2.3. Purification of bacteriocins

Since bacteriocins are proteins or peptides, the methods applied to bacteriocin purification are those which are generally used in protein purification.

2.3.1. Ultrafiltration

Ultrafiltration achieves concentration of large molecules, but not of small ones. This

method is not practical for the concentration of large volumes. Ultrafiltration was carried out with crude lactacin F by sequentially filtering through membranes of decreasing pore sizes (Muriana and Klaenhammer 1991a). To concentrate the antimicrobial activity of *Lb. casei*, culture supernatants were lyophilized and later dissolved in a small volume of water or buffer, followed by ultrafiltration (Rammelsberg and Radler 1990). Cell-free culture supernatant of *Lb. plantarum* SIK83, producing plantaricin SIK83, was concentrated 50 times by ultrafiltration using a 10 kDa molecular weight cut off membrane (Andersson *et al.* 1988). Besides concentration and partial purification, sequential ultrafiltration through membranes of decreasing molecular weight cut off helps to approximate the size of the bacteriocin, as in helveticin J (Joerger and Klaenhammer 1986).

2.3.2. Ammonium sulphate precipitation

This method is frequently used as a preliminary concentration step and also achieves some degree of purification. The reason for the choice of ammonium sulphate is that it is highly soluble, and the solubility does not vary much with temperature. The amount of ammonium sulphate needed to precipitate different bacteriocins varies. Ammonium sulphate precipitation was used as the preliminary purification step by Bhunia *et al.* (1988) for pediocin AcH, Mortvedt *et al.* (1991b) for lactocin S, Muriana and Klaenhammer (1991a) for lactacin F, Vaughan *et al.* (1992) for helveticin V-1829 and Samelis *et al.* (1994) for sakacin B. The pellet that is received after precipitation was dialysed exhaustively against large volumes of buffer or deionized water to remove ammonium sulphate.

2.3.3. Gel chromatography

Molecular exclusion chromatography or gel chromatography is another method that is frequently employed for the purification of bacteriocins. The active fraction may be passed successively through columns with different packing materials. Joerger and Klaenhammer (1986) applied helveticin J concentrate to columns containing Sephadex G-200-120 (a cross-linked dextran). The active fraction from this column was pooled, concentrated and then applied to a Sephadex G-200-50 column for further purification.

2.3.4. Ion exchange chromatography

Ion exchange chromatography of both types i.e. cation and anion is frequently used

for bacteriocin purification. The most widely used materials in this process are DEAE cellulose, Q sepharose, CM cellulose and Mon QHR5/5. Ion exchange chromatography was used as one of the steps for the purification of lactacin B (Barefoot and Klaenhammer 1984), pediocin AcH (Bhunia *et al.* 1988), lactocin S (Mortvedt *et al.* 1991b) and sakacin B (Samelis *et al.* 1994).

2.3.5. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is often used as the terminal purification step. SDS is an anionic detergent that disrupts all noncovalent interactions in native protein, and SDS-PAGE is performed mainly for determining the molecular weight of the purified bacteriocin. Direct detection of the peptide subunit responsible for bacteriocin activity is possible (Bhunia *et al.* 1987).

Judicious combination of the methods described above are used for purification of various bacteriocins. Properties of the bacteriocin in question are often exploited for their purification. Acidocin B was purified (>90%) by butanol extraction in a single step from concentrated cell suspensions in a chemically defined medium (ten Brink *et al.* 1994).

2.3.6. Activity assay

Activity of the active fraction is assayed after each purification step, mostly by critical dilution method described by Mayr-Harting *et al.* (1972), and then amount of protein measured. Progress of purification at each step is indicated by drawing up a table which gives the information of the following types: volume (ml), concentration (units/ml), total activity (arbitrary units), protein (mg/ml), specific activity (units/mg), yield (%) and degree of purification (%).

2.4. Mode of action

Although the pioneering work of Fredericq (1946) pointed out a narrow range-killing specificity among the colicins (i.e. they are active on strains of the same or related species), bacteriocins produced by Gram positive organisms are often active on a wide variety of Gram positive bacteria (Hamon and Peron 1963; Hamon 1964).

In most cases, mode of action studies have been carried out with a single susceptible strain. Studies on the mode of action of many lactic acid bacterial bacteriocins implicate bactericidal or bacteriostatic action.

Quite often the killing kinetics or even the designation of a bactericidal versus bacteriostatic effect are dependent upon aspects of the assay system such as the concentration and purity of the inhibitor, the type of buffer or broth, the sensitivity of the indicator species and the density of cell suspension used (Lewus *et al.* 1991). The physiological state of an indicator culture has been shown to have a strong influence on susceptibility to the lethal action of a bacteriocin, with actively multiplying cells being the most sensitive (Tagg *et al.* 1976). Helveticin V-1829 showed more pronounced lethal action on susceptible cells in logarithmic phase than stationary phase of growth (Vaughan *et al.* 1992). Lacticin showed an action similar to helveticin V-1829 (Piard *et al.* 1990).

The pH of a bacteriocin preparation plays an important role on its action on susceptible cells. The killing kinetics of carnocin 54 were affected by the pH. When susceptible cells of *Listeria innocua* WS2257 were treated with carnocin 54, a higher inactivation rate was observed at pH 4.9 than at pH 6.5. The higher killing rate at acidic pH may either be the result of the higher activity of carnocin 54 or may be due to higher susceptibility of the indicator strain at lower pH (Schillinger *et al.* 1995). Similarly, Harris *et al.* (1991) observed an increase in the effectiveness of nisin when the pH of the medium was decreased from 6.5 to 5.5.

The killing of sensitive bacteria is thought to be a two-step process; the initial adsorption of bacteriocin by cell receptors being followed by transfer of a 'lethal' message to specific biochemical targets (Nomura 1974). A number of biochemical targets have been identified with principal effects in energy production, macromolecular synthesis (or membrane transport) and permeability (Reeves 1972).

The common mechanism of action which has been determined for other bacteriocins of lactic acid bacteria is disruption of the electrochemical gradients across the cytoplasmic membrane by pore formation (Klaenhammer 1993). Exposure of sensitive cells to pediocin SJ-1 caused rapid leakage of potassium ions and amino acids, leading to rapid depolarization of the cytoplasmic membrane, finally leading to cell death (Schved *et al.* 1994).

Plantaricin SIK 83 has been shown to bind specifically to sensitive cells, but not to non-sensitive lactic acid bacteria and Gram negative bacteria. Sensitive cells, after exposure to bacteriocin, could be rescued by treatment with proteolytic enzymes. Morphological strains are observed within 2 h after the cells are exposed to bacteriocins. A lethal mode of action appears to be due to damage to the cell membrane, resulting in cell lysis (Andersson *et al.* 1988).

The bactericidal effect of salivaricin B and acidocin B on sensitive lactobacilli in the absence of cell lysis suggests that their mode of action is not impairment of cell wall biosynthesis (ten Brink *et al.* 1994). Also, sakacin B and pediocin N5p exhibit a bactericidal mode of action, without causing cell lysis (Strasser de Saad and Manca de Nadra 1993; Samelis *et al.* 1994). Pediocin AcH is bactericidal to sensitive bacterial cells, and this effect is produced within a few minutes. Cell death is not associated

with lysis or leakage of the cell membrane. The effect of pediocin AcH could be related to inhibition of the synthesis of ATP and/or cellular macromolecules such as proteins and nucleic acid (Bhunia *et al.* 1988).

2.5. Activity spectrum

Antibacterial spectrum is one of the criteria in the characterization of a bacteriocin and determining its novelty. According to the definition of Tagg *et al.* (1976), bacteriocins are proteinaceous compounds with a bactericidal mode of action against a limited range of organisms closely related to the producer. Several different bacteriocins produced by the same species are differentiated on the basis of their antibacterial spectrum. In spite of several similarities, plantaricin 149 produced by *Lb. plantarum* is considered different from plantaricins A, B, S and T based on differences in antibacterial activity, towards *Enterococcus, Lactococcus* and *Micrococcus* strains (Kato *et al.* 1994). Acidocin B, produced by *Lb. acidophilus* M46, is considered unique and atypical when compared to other *Lb. acidophilus* bacteriocins such as lactacin B, lactacin F, acidophilucin A and acidocin 8912, since it combines inhibition of *Clostridium sporogenes* with a narrow activity spectrum within the genus *Lactobacillus* (ten Brink *et al.* 1994). The antimicrobial substance from *Pc. pentosaceus* N4p has specific activity against other strains of *Pediococcus*, whereas the inhibitor produced by *Pc. pentosaceus* N5p has an antagonistic effect on large number of other lactic acid bacteria (Strasser de Saad and Manca de Nadra 1993).

In general, bacteriocins found in lactobacilli display a narrow range of inhibitory activity, towards closely related species within Lactobacillaceae (Klaenhammer 1988). However, *Lactobacillus* bacteriocins showing a broad activity spectrum has been reported. *Lactobacillus salivarius* M7 produces a broad spectrum bacteriocin, salivaricin B, which inhibits the growth of *Listeria monocytogenes, Bacillus cereus, Brocothrix thermosphacta* and *Enterococcus faecalis* (ten Brink *et al.* 1994). Sakacin A, (Schillinger and Lücke 1989) produced by *Lb. sake* possess a relatively broad spectrum activity that includes activity against certain pathogens. *Listeria monocytogenes* is most commonly inhibited, probably because of its taxonomic relation to Lactobacillaceae (Wilkinson and Jones 1977). Pediocins, produced by pediococci, have a wide spectrum of bactericidal activity against Gram positive bacteria (Gonzales and Kunka 1987; Hoover *et al.* 1988; Bhunia *et al.* 1988; Ray *et al.* 1989).

3

Materials and Methods

3.1. Culture media used

3.1.1. All purpose Tween (APT) agar

(M226) - HiMedia, Bombay, India

3.1.2. All purpose Tween (APT) broth

(HiMedia M227)

3.1.3. Gelatin broth

Lactobacillus MRS broth (HiMedia M369) supplemented with 12% (w/v) gelatin

3.1.4. Glucose-yeast extract agar

Glucose	10 g
Yeast extract	10 g
Agar	15 g
Distilled water	1000 ml
pH	6.8

3.1.5. Milk agar

MRS agar supplemented with 5% (w/v) skimmed milk

3.1.6. MRS agar

Lactobacillus MRS agar (HiMedia M641)

3.1.7. MRS basal medium

Peptone	10 g
Yeast extract	5 g

K_2HPO_4	2 g
Diammonium citrate	2 g
Tween 80	1 g
Sodium acetate	1 g
$MgSO_4 . 7H_2O$	100 mg
$MnSO_4 . 4H_2O$	50 mg
Bromothymol blue	500 mg
Distilled water	1000 ml
pH	6.2 - 6.4

3.1.8. MRS broth

Lactobacillus MRS broth (HiMedia M369)

3.1.9. **MRS-0.2 agar** (Schillinger and Lücke 1989)

MRS-0.2 broth supplemented with 1.5% (w/v) agar

3.1.10. **MRS-0.2 broth** (Schillinger and Lücke 1989)

Peptone	10 g
Meat extract	10 g
Yeast extract	5 g
K_2HPO_4	2 g
Diammonium citrate	2 g
Glucose	2 g
Tween 80	1 g
Sodium acetate	5 g
$MgSO_4 . 7H_2O$	100 mg
$MnSO_4 . 4H_2O$	50 mg
Distilled water	1000 ml
pH	6.2 - 6.4

3.1.11. MRS soft agar

MRS broth supplemented with 1% (w/v) agar

3.1.12. Nitrate broth

Meat extract	10 g
Yeast extract	10 g
K_2HPO_4	2 g
Sodium citrate	2 g
Sodium acetate	5 g
Glucose	2 g
Tween 80	1 g
$MgSO_4.7H_2O$	100 mg
$MnSO_4.4H_2O$	50 mg
Distilled water	1000 ml
pH	6.5

3.1.13. Nutrient agar

(HiMedia M001)

3.1.14. Nutrient broth (Gordon *et al.* 1973)

Beef extract	3 g
Peptone	5 g
Distilled water	1000 ml
pH	6.8

Agar (15.0 g/l) was added to prepare nutrient agar.

3.1.15. Semi-defined (SD)-MRS broth

Casein acid hydrolysate	10 g
Sodium acetate	5 g
Yeast extract	5 g
Glucose	10 g
K_2HPO_4	2 g
Tween 80	1 g
$MgSO_4.7H_2O$	80 mg
$FeSO_4.7H_2O$	4 mg
$MnCl_2.4H_2O$	8 mg
Distilled water	1000 ml

pH	6.0

3.1.16. Starch agar

Peptone	10 g
Beef extract	10 g
Yeast extract	10 g
K_2HPO_4	2 g
Ammonium citrate	2 g
Tween 80	1 g
Sodium acetate	5 g
$MgSO_4.7H_2O$	100 mg
$MnSO_4.4H_2O$	50 mg
Starch	20 g
Agar	15 g
Distilled water	1000 ml
pH	6.5

3.1.17. Tomato juice medium (after Rammelsberg and Radler 1990)

Peptone	20 g
Yeast extract	5 g
Glucose	5 g
Tween 80	50 mg
Tomato juice (centrifuged)	250 ml
Distilled water	1000 ml
pH	5.5 adjusted with KOH

3.1.18. Tributyrin agar

Peptone	10 g
Beef extract	10 g
Yeast extract	10 g
K_2HPO_4	2 g
Ammonium citrate	2 g
Glucose	20 g
Sodium acetate	5 g

$MgSO_4.7H_2O$	1 g
$MnSO_4.4H_2O$	50 mg
Tributyrin	10 g
Agar	15 g
Distilled water	1000 ml
pH	6.5

3.1.19. Tryptophan broth

Beef extract	10 g
Yeast extract	10 g
K_2HPO_4	2 g
Ammonium citrate	2 g
Glucose	20 g
Tween 80	1 g
Sodium acetate	5 g
$MgSO_4.7H_2O$	100 mg
$MnSO_4.4H_2O$	50 mg
Tryptophan	1 g
Distilled water	1000 ml
pH	6.5

3.2. Reagents used

3.2.1. **Burke's iodine solution** (Bartholomew 1962)

Iodine	1 g
KI	2 g
Distilled water	100 ml

3.2.2. **Crystal violet stain** (Bartholomew 1962)

Crystal violet	2 g
95% ethanol	20 ml
Ammonium oxalate (1% w/v aqueous solution)	80 ml

3.2.3. **Ehrlich Böhme reagent** (Plummer 1978)

p-Dimethylaminobenzaldehyde	10 g
HCl concentrated	100 ml

3.2.4. Physiological saline

0.85% (w/v) NaCl in distilled water

3.2.5. Protein precipitating reagent

1 N sulphuric acid saturated with ammonium sulphate

3.2.6. Reagents for nitrate reduction test (Norris *et al.* 1981)

Solution A:

Sulphanilic acid	800 mg
5 N acetic acid	100 ml

(glacial acetic acid : water, 1 : 2.5)

Solution B:

α-Napthylamine	500 mg
5 N Acetic acid	100 ml

The solution A and B were mixed in equal quantities just before use.

3.2.7. Reagents for protein estimation

Reagent A:

i) 2% (w/v) Na_2CO_3 in 0.4% (w/v) NaOH
ii) 2% (w/v) NaK tartrate in distilled water
iii) 1% (w/v) $CuSO_4$ in distilled water

Reagent A was prepared just before use by adding the solutions i, ii and iii in the ratio 100:1:1.

Reagent B:

Commercially available Folin-ciocalteu reagent was diluted with double volume of distilled water.

3.2.8. Safranin stain (Norris *et al.* 1981)

2.5% (w/v) safranin in 95% (v/v) ethanol	10 ml
Distilled water	100 ml

3.3. Buffers used

3.3.1. 0.2 M Ammonium bicarbonate buffer (pH 8.0)

Ammonium bicarbonate	15.8 g
Distilled water	1000 ml

3.3.2. 50 mM sodium phosphate buffer (pH 7.2)

Solution A : NaH_2PO_4	6.9 g
Distilled water	1000 ml
Solution B : $Na_2HPO_4.7H_2O$	13.3 g
Distilled water	1000 ml

28.0 ml of Solution A and 72.0 ml of Solution B were mixed.

3.3.3. 20 mM sodium phosphate buffer (pH 8.0)

Solution A : NaH_2PO_4	2.7 g
Distilled water	1000 ml
Solution B : $Na_2HPO_4.7H_2O$	5.2 g
Distilled water	1000 ml

19.0 ml of Solution A and 81.0 ml of Solution B were mixed.

3.3.4. 20 mM Tris buffer (pH 7.5)

Tris	2.4 g
Distilled water	1000 ml

3.4. Organisms used

The indicator organisms and the reference strain used in this study were obtained from the culture collection of the Microbiology laboratory, Department of Botany, University of North Bengal.

3.5. Experimental

3.5.1. Collection of sample

Samples of curd, cheese, chhana (acid-and-heat coagulated traditional milk product), spoiled milk, spoiled vegetables, silage and putrid fish and meat were collected in sterile glass bottles from different shops and houses nearby. They were transported immediately (within 3 h) to the laboratory and processed for analysis. For silage enrichment, the method of Whittenbury (1955) was followed. Grasses and plant materials were collected, cut into pieces, placed into sterile glass tubes and compressed. The tubes were sealed in a way that permitted gas under pressure to escape but prevented the entry of oxygen. These were incubated at 30°C to produce silage.

3.5.2. Isolation of lactic acid bacteria

Samples (10 g) were homogenized at 'normal' speed for 30 s to 2 min with 90 ml of physiological saline using a Stomacher lab-blender Model 400 (Seward Medical, London, England). One millilitre of appropriately diluted sample was mixed with 15 ml of molten MRS agar, poured to plates and incubated in a candle jar for 48 h at 32°C. The isolates were maintained in MRS broth at 5°C with fortnightly transfers.

3.5.3. Characterization of isolates

3.5.3.1. Cell morphology

Morphology of the isolates was studied using a phase contrast microscope (Olympus BH2-PC-PA-1). An air-dried (not heat-fixed) smear of a 24 h-old culture of the isolates, grown on MRS agar, was stained for 30 s with safranin, washed in water, air-dried and observed under oil-immersion objective. Cell dimension was measured using a standardized ocular micrometer.

3.5.3.2. Gram staining

The method of Bartholomew (1962) was followed for this purpose. A suspension of a 24 h-old culture on MRS agar was prepared in distilled water. A drop of that suspension was smeared on a grease-free slide. The smear was air-dried, heat-fixed, flooded with crystal violet stain for 1 min, and washed for 5 s with water. The smear was then flooded with Burke's iodine solution, allowed to react for 1 min, and washed again for 5 s with water. Holding the slide against a white surface, 95% (v/v) ethanol was poured dropwise from the top edge of the slide until no more colour came out from the lower edge of the slide. After washing with water, the smear was stained with safranin for 1 min and washed again with water. The smear was air-dried and observed under oil-immersion objective.

3.5.3.3. Production of catalase

A loopful of a 24 h-old culture on MRS agar slant was placed onto a clean slide, flooded with 10% (v/v) hydrogen peroxide solution, covered with a cover glass and observed under microscope for the production of effervescence for up to 5 min, indicating the presence of catalase (Feresu and Muzondo 1990).

3.5.3.4. Production of gas from glucose

MRS broth (10 ml) in culture tubes with inverted Durham tubes were inoculated, incubated at 32°C for 24 h and observed for any accumulation of gas in the inverts.

3.5.3.5. Growth in 6.5% NaCl

MRS broth supplemented with 6.5% (w/v) NaCl was inoculated with a 24 h-old culture and incubated at 32°C. The growth was checked after 7 d.

3.5.3.6. Hydrolysis of casein

Dried milk agar plates were streaked with 24 h-old cultures and examined after incubating for 3 d at 30°C for any clearing of casein around and underneath the growth (Gordon *et al.* 1973).

3.5.3.7. Production of indole

Cells were grown at 32°C in 10 ml tryptophan broth. Ehrlich Böhme reagent (1-2 ml) was layered on 2 d-old broth cultures and checked for the formation of any red ring at the culture-reagent interface, indicating the presence of indole (Iswaran 1980).

3.5.3.8. Reduction of nitrate

Cultures were grown at 32°C in 5 ml nitrate broth. After 2 d, 1 ml of the culture was mixed with 3 drops of the reagent for nitrate reduction test and observed for the development of any red or yellow colour, indicating the presence of nitrate (Norris *et al.* 1981).

3.5.3.9. Hydrolysis of starch

Starch agar plates were streaked with the isolates and incubated at 30°C for 1-3 d. The growths were flooded with Burke's iodine solution and observed for the formation of any clear zone around and underneath the growth, indicating the presence of amylase.

3.5.3.10. Hydrolysis of fat

Surface-dried plates of tributyrin agar were streaked with 24 h-old culture, incubated at 32°C for 24 h and checked for the formation of any clear zone around the growth, indicating lipolytic activity.

3.5.3.11. Liquefaction of gelatin

Gelatin broth in tubes were stabbed with 24 h-old cultures, incubated for 2 d, kept in

an ice-bath for 15 min and observed for the physical state of the medium.

3.5.3.12. Utilization of sugars

Filter-sterilized solutions of the test sugars were added (final concentrations being 1% w/v) to MRS basal medium. The inoculated broths were incubated at 32°C, and results were recorded upto 7 d (Kandler and Weiss 1986).

3.5.4. Effect of anaerobiosis on lactic acid bacterial growth

Two isolates were randomly selected for inoculating MRS agar plates with the same amount of inocula. One set was kept inside a candle jar and the other remained outside. Following incubation at 32°C for 48 h, the colonies developed per plate were counted and compared.

3.5.5. Detection of antibacterial activity

For the detection of antibacterial activity, a modification based on the 'spot on the lawn' method of Fleming *et al.* (1985) and Schillinger and Lücke (1989) was made. Plates of MRS-0.2 agar were dried overnight, spotted with producing cultures (5 spots per plate) and incubated in a candle jar for 18-24 h at 32°C. MRS soft agar was tempered to 45°C and seeded with 10^5-10^6 sensitive organisms per ml. The spotted plates were overlaid with 8 ml of the seeded MRS soft agar, incubated for 18-24 h at 32°C and observed for inhibition zones. The score was positive if the width of the clear zone around the colonies of producer strain was > 0.5 mm.

3.5.6. Preparation of cell-free culture supernatants

Cell-free supernatant from 18 h-old MRS broth culture was collected by centrifugation at 10,200 X g for 10 min. The supernatant was adjusted to pH 6.5, concentrated 10 times using a rotary evaporator at 50°C and sterilized by passing through a Borosil (India) Porosity grade 5 sintered glass filter (Lewus *et al.* 1991).

3.5.7. Well diffusion assay

MRS agar plates were overlaid with 8 ml of MRS soft agar seeded with 10^5-10^6 sensitive

organisms per millilitre. Wells (6 mm dia) were cut into these agar plates, and 0.1 ml of the cell-free culture supernatant of the test bacterium was placed into each well. The plates were incubated at 32°C for 24 h and observed for the formation of any clear zone around the wells (Schillinger and Lücke 1989).

3.5.8. Determining antagonism due to phage

Wells (7 mm dia) were cut in MRS agar plates and sealed at the bottom by pouring 1-2 drops of molten agar. Cell-free culture supernatants (0.1 ml) were placed in the sealed wells. After diffusion of the extract into the agar (6 h at 25°C), the entire agar disc of the plate was inverted and overlaid with 8 ml of MRS soft agar seeded with 18 h-old 10^5-10^6 indicator cells per millilitre. The plates were incubated at 32°C for 24 h and observed for the formation of any clear zone around the wells (Tagg and McGiven 1971).

3.5.9. Determining antagonism due to hydrogen peroxide

The culture supernatant was added with 5 g/ml of catalase (Himedia RM446), incubated at 37°C for 1 h, concentrated *in vacuo* and sterilized by passing through a bacterial filter. The filtrate was assayed by well diffusion method as described in section 3.5.7.

3.5.10. Determining antagonism due to bacteriocin

3.5.10.1. Treatment with pepsin

A 10 fold-concentrated culture supernatant was treated with pepsin (HiMedia RM084; 60 U/ml) at pH 1.5, incubated for 1 h at 37°C, after which the pH was adjusted to 6.5 and the activity assayed by well diffusion method (Rammelsberg and Radler 1990).

3.5.10.2. Treatment with trypsin

The 10 fold-concentrated culture supernatant was treated with trypsin (Merck Art 8367.02 Anson E/g; 40 U/ml) at pH 7.0, and incubated for 1 h at 37°C. The activity was assayed by well diffusion assay (Rammelsberg and Radler 1990).

3.5.11. Determining antagonism due to acids

Well diffusion assay was carried out with culture supernatants of pH unadjusted.

3.5.12. Elimination of Bac⁻ variants

For the elimination of non-bacteriocin (Bac⁻) variants, the culture was grown in MRS broth to the stationary phase of growth. Then the culture was diluted and plated on MRS agar to obtain 20-30 colonies per plate. After 1-2 days of incubation, replica plating was done. The master plate was overlaid with 8 ml of MRS soft agar seeded with 10^5-10^6 cells of 18 h-old sensitive organisms per millilitre, and incubated at 32°C for 18-24 h. Colonies with inhibition zones around them were isolated from the replica plate (Mortvedt and Nes 1990).

3.5.13. Optimization of bacteriocins production

3.5.13.1. Type of medium

The producer strains were grown for 24 h at 32°C in five different broths, namely MRS broth, MRS-0.2 broth, APT broth, tomato juice medium and SD-MRS broth. Antibacterial activity was assayed by the well diffusion method.

3.5.13.2. pH of medium

SD-MRS broth was adjusted to different pH values ranging from 5.0 through 8.0. Activity was assayed following the well diffusion method with the culture supernatant after adjusting it to pH 6.5 and concentrating 10 times in a rotary evaporator at 55°C.

3.5.13.3. Period of incubation

SD-MRS broth was inoculated with 5% (v/v) of a 24 h-old inoculum of a producer strain, and incubated at 32°C for different periods. Activity was assayed by well diffusion method.

3.5.13.4. Temperature of incubation

SD-MRS broth was inoculated with 5% (v/v) of a 24 h-old inoculum of a producer strain, and incubated for 2 d at different temperatures. Activity was assayed by well diffusion method.

3.5.14. Characterization of bacteriocins

3.5.14.1. Thermostability

Cell-free culture supernatant from SD-MRS broth was collected by centrifugation at 10,200 X g for 10 min. The supernatant was adjusted to pH 6.5. After 10-fold concentration in a rotary evaporator at 50°C, the crude bacteriocin was subjected to different temperatures for different time periods.

3.5.14.2. pH stability

Samples of crude bacteriocin extract were dissolved in sodium phosphate buffer (50 mM) at different pH, so that the final concentration of bacteriocin in each pH solution was 40 AU/ml. The solutions were kept for 24 h at 4°C, readjusted to pH 6.5, and activities determined by critical dilution assay (Samelis *et al.* 1994).

3.5.14.3. Storage stability

Producer strains were grown in SD-MRS broth for 2 d. One part of the culture was stored at 37°C. From the other part cells were removed by centrifugation followed by filtration through a bacterial filter. The filtrate was stored at different temperatures. At different time intervals, samples were taken out to determine activity by well diffusion assay (ten Brink *et al.* 1994).

3.5.14.4. Stability against organic solvents

Various organic solvents were added to culture supernatant of producer strains in 1 : 1 ratio. After thorough shaking the mixture was centrifuged at 5000 X g for 10 min. The organic phase and the aqueous phase were collected separately, and the solvents removed by evaporation at 45°C in a rotary evaporator. The residues were suspended

in distilled water to make volumes equal to 10% (v/v) of the initial volume of the culture supernatant. The activity was determined by well diffusion assay (ten Brink *et al.* 1994).

3.5.15. Purification of bacteriocin

3.5.15.1. Concentration

To concentrate antimicrobial activity, cell-free culture supernatants were adjusted to pH 6.5, freeze-dried at -20°C and dissolved in a small volume of buffer (50 mM phosphate buffer, pH 7.2).

3.5.15.2. Dialysis

One millilitre of 10-fold concentrated culture supernatant was dialyzed using 3.5 and 12 kDa molecular cut-off tubings (Sigma Chemical Company, St Louis, MO) against a 200-fold more volume of water at 4°C. Both retentate and dialysate were assayed for activity by well diffusion method. The dialysate was concentrated before assay.

3.5.15.3. Gel filtration

A glass column (25.4 cm height, 2.02 cm dia, 81.4 ml volume) was packed with Sephadex G-25 medium (Pharmacia, Uppsala, Sweden). The column was equilibrated with 50 mM (pH 7.2) sodium phosphate buffer. At the top, it was loaded with 1 ml of crude bacteriocin. The eluent used was 50 mM (pH 7.2) sodium phosphate buffer containing 0.6% (w/v) NaCl. Fractions of 1.0 ml were collected and read at 280 nm using a Beckman (Beckman Instruments, Inc., CA) model DU640 spectrophotometer Fractions at each peak were pooled, freeze-dried at -20°C followed by dissolving in 0.5 ml of 50 mM (pH 7.2) sodium phosphate buffer. The activity was determined by well diffusion assay.

3.5.15.4. Ion exchange chromatography

The active fractions derived after gel filtration were subjected to ion exchange chromatography. A fraction (2 ml) was applied to a column (15 cm height, 1.5 cm dia, 30 ml volume) packed with DEAE cellulose (Sigma Chemical Company, St Louis, MO)

and equilibrated with 20 mM (pH 7.5) Tris buffer. The column was washed with the same buffer until the absorbance at 280 nm returned to zero. Fractions of 1 ml were collected. Activity was eluted by applying a linear salt gradient (0 to 0.5 M NaCl). Fractions showing maximum absorbance were pooled and freeze-dried.

3.5.15.5. Desalting column chromatography

The freeze-dried sample was dissolved in minimum volume of distilled water, and 1.25 ml of it was applied on a prepacked Exocellulose GF-5 (Pierce Chemical Company, Rockford, IL) desalting column (107 mm x 12 mm dia; gel volume, 5 ml; gel particle size, 40-100 μm) equilibrated with 0.2 M (pH 8.0) ammonium bicarbonate buffer. Activity was eluted with the same buffer, and protein in the elution was determined by monitoring absorbance of the pooled fractions at 280 nm using the Beckman spectrophotometer (Pierce Manual 1988). Activity for each fraction was assayed by well diffusion method.

3.5.15.6. Protein estimation

The amount of protein after each purification step was estimated by the method based on that described by Lowry *et al.* (1951). Required volume of distilled water was added to different volumes (0-500 μl) of stock solution to make the total volume of 0.25 ml. Then, 0.9 ml of reagent A was added in each of the tubes incubated for 20 min, added with 0.1 ml of reagent B and incubated for 15 min. After this, 5 ml water was added in each of the tubes. The intensity of the characteristic blue colour that developed was measured in the Beckman spectrophotometer at 620 nm.

3.5.15.7. Activity assay

Bacteriocin activity of the culture supernatant was assayed using the critical dilution method described by Barefoot and Klaenhammer (1983). MRS agar plates were overlaid with MRS soft agar, seeded with 18 h-old 10^5-10^6 indicator cells/ml. A 20 μl volume of a serial two-fold dilution of cell-free culture supernatant was spotted onto these lawns. After incubation for 24 h at 32°C, the assay plates were examined for inhibition of the bacterial lawn. One arbitary activity unit (AU) was defined as the reciprocal of the highest dilution yielding a definite zone of inhibition on the indicator lawn.

3.5.16. Mode of action

Cell-free culture supernatants of 48 h-old cultures of producer strains grown in SD-MRS broth were adjusted to pH 6.5, filter-sterilized and added to four times-volume of SD-MRS broth. In control experiments, no such culture supernatant was added. Indicator organisms of different ages were added to yield a starting cell density of approximately 4 x 10^6 cfu/ml. During incubation at 32°C, sampling was done at regular intervals to determine the optical density at 660 nm and the cfu/ml was counted (ten Brink *et al.* 1994).

3.5.17. Activity spectrum

The antagonistic effect of the neutralized culture supernatant of the three selected *Lactobacillus casei* strains on various Gram positive and Gram negative bacteria as well as yeasts was tested by well diffusion assay.

3.5.18. Statistical analysis

The data obtained were analysed statistically by determining standard error (SE) and analysis of variance (ANOVA) (Snedecor and Cochran 1989).

4

Results

4.1. Isolation of lactic acid bacteria

The non-motile, non-sporeforming catalase negative and Gram positive cells, denoted as lactic acid bacteria were screened out of the total bacterial isolates on MRS agar. They were further characterized on the basis of cell morphology, gas from glucose and growth in 6.5% (w/v) sodium chloride. Following the taxonomic keys of Sneath *et al.* (1986), lactic acid bacterial isolates were identified up to their generic level. A total of 171 lactic acid bacteria were isolated form 74 samples of different kinds (Table 2; Fig. 1). Lactobacilli constituted 62% of the total lactic acid bacterial isolates and predominated in whey, curd, chana and putrid fish, followed by lactococcal isolates (31%) which occured predominantly in rotten vegetables, putrid meat, silage and cheese. Besides, isolates of leuconostocs were isolated from chhana and rotten vegetables, and pediococci from silage, which together constituted only 7% of the total lactic acid bacterial isolates.

During enrichment of lactic acid bacteria, the plates were incubated in candle jar. But, since the number of pure culture colonies developed on plates incubated microaerophilically (in candle jar) did not differ significantly from those developed on plates incubated aerobically (data not shown), in all subsequent experiments the cultures were grown aerobically.

4.2. Screening and nature of antimicrobial substance

In agar spot test (Fig. 2a), 14% of the total lactic acid bacterial isolates showed antibacterial activity (Table 3). Comparisons of the results in well diffusion assay (Fig.2b) with unadjusted and adjusted pH of the culture supernatants showed that the antibacterial activity of most of the strains was due to organic acid production (Table 4). Out of 24, seven strains were found positive in well diffusion assay when the pH of their cell-free culture supernatants were neutralized. They scored positive even when they were treated with catalase, confirming the nature of antibacterial compounds produced by these seven isolates to be other than acid and hydrogen peroxide. These seven strains tested positive in the reverse-side technique (Tagg and McGiven 1971), establishing the fact that the antibacterial activity was not even due to lytic phages. When the culture supernatants were treated with proteolytic enzymes and the activity assayed by well diffusion method, their antibacterial property was lost (Tables 4 and 5; Fig. 2c). The results of pepsin treatment resembled those of a trypsin one (Fig. 3), confirming the protein or peptide nature of the antibacterial compounds.

4.3. Selection of Bac$^+$ cells from a mixed population

Table 2. Lactic acid bacterial isolates from different sources

Source	No. of isolates			
	Lactobacillus	*Lactococcus*	*Leuconostoc*	*Pediococcus*
Curd (13)	29	3	0	0
Chhana (16)	17	6	4	0
Whey (11)	35	0	0	0
Cheese (7)	5	7	0	0
Silage (5)	1	10	0	6
Rotten vegetables (16)	8	14	2	0
Putrid fish (2)	10	2	0	0
Putrid meat (4)	1	11	0	0
Total (74)	106	53	6	6

Figures in parentheses indicate number of samples analysed.

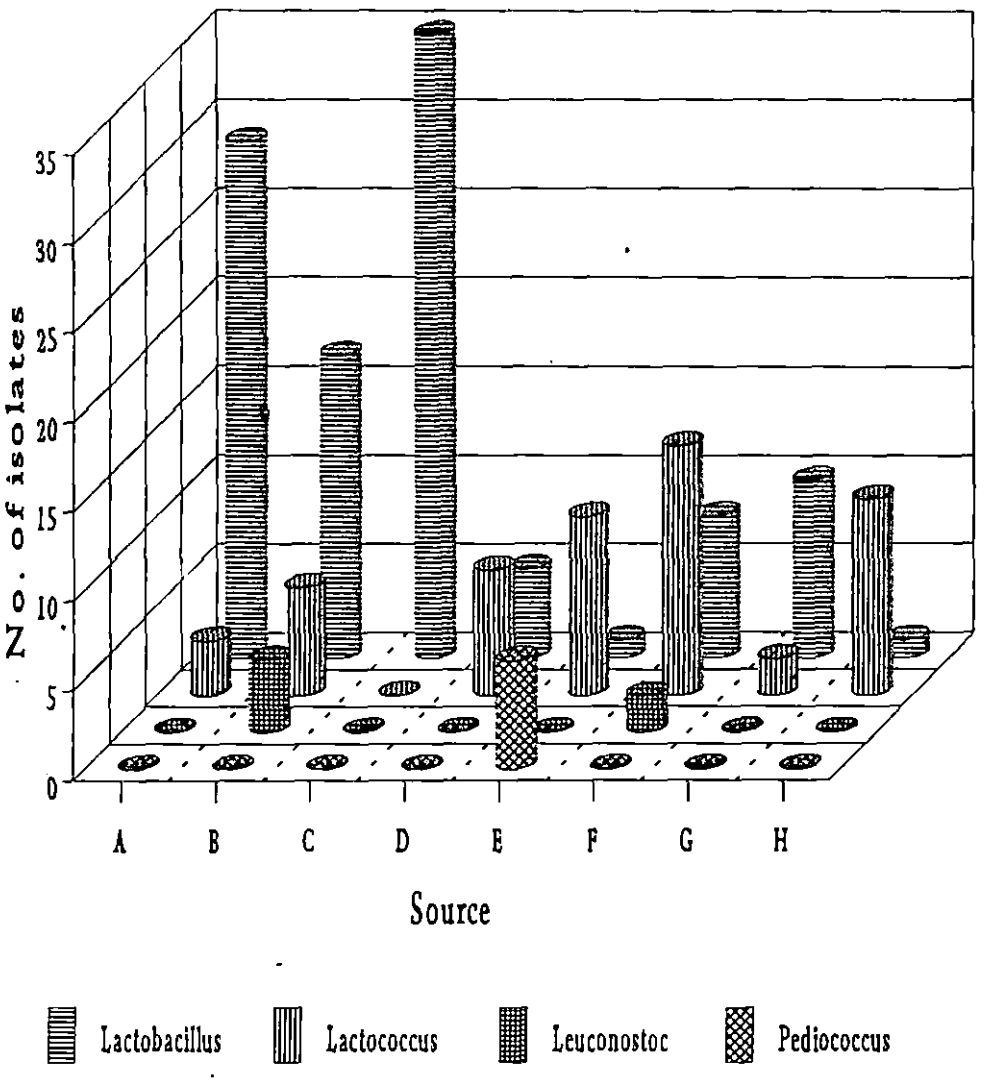

Fig. 1. Lactic acid bacterial isolates from different sources. A, curd; B, chhana, C, whey; D, chhese; E, silage; F, rotten vegetables; G, putrid fish; H, putrid meat.

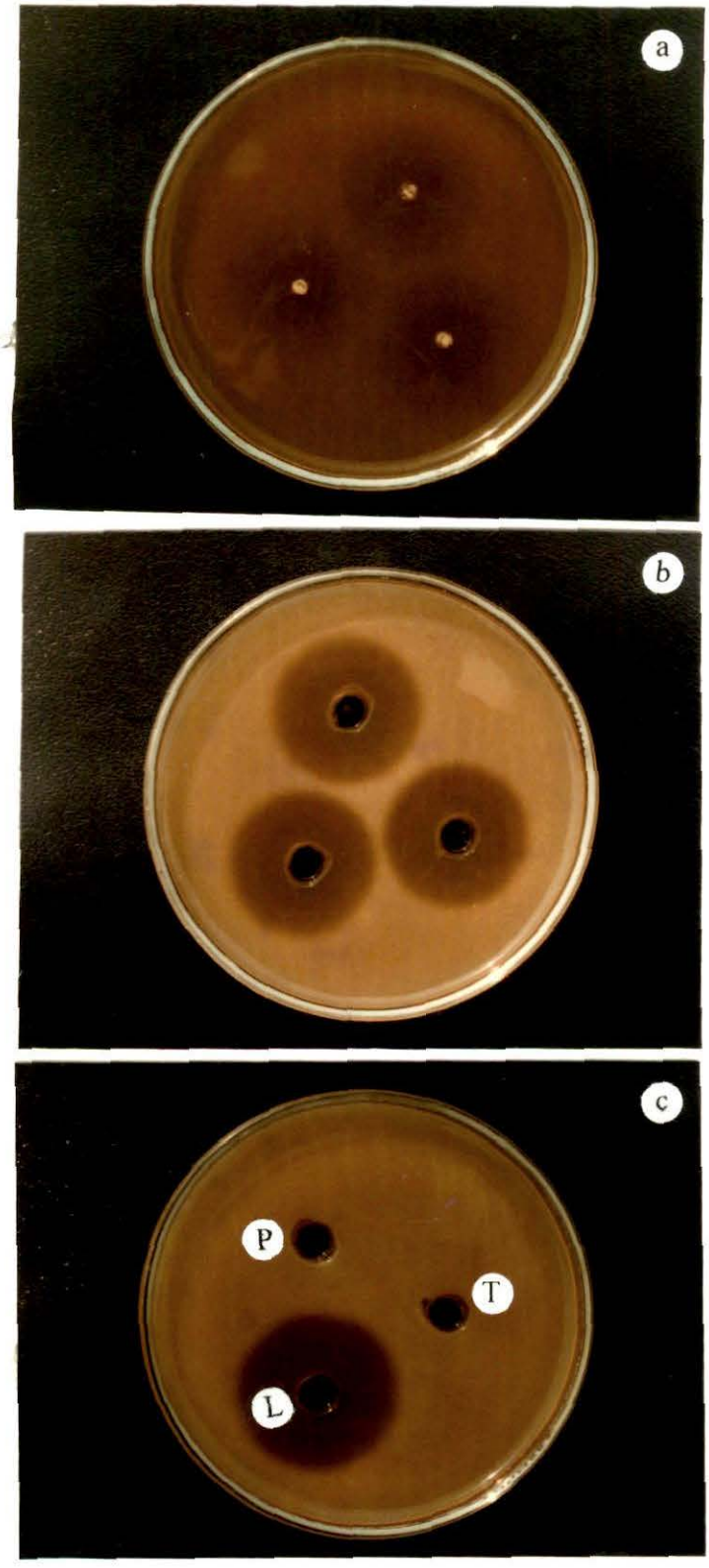

Fig. 2. Testing antimicrobial activities: (a) agar spot test showing growth inhibition by A, B and C; (b) well diffusion assay showing growth inhibition zones caused by neutralized, 10 fold-concentrated and filter-sterilized culture filtrates of A, B, and C; (c) response of neutralized, 10 fold-concentrated and filter-sterilized culture filtrates of C to catalase (L), pepsin (P) and trypsin (T) treatments. Producers: *Lactobacillus casei* W25B (A), W26B (B) and W28 (C). Indicator organism: *Lactobacillus plantarum* GM-R1.

Table 3. Antibacterial activity of lactic acid bacterial isolates determined by agar spot test

Genera	No. of isolates (A)	Positive isolates (B)	Percent frequency (B/A X 100)
Lactobacillus	106	17	16
Lactococcus	53	4	8
Leuconostoc	6	2	33
Pediococcus	6	1	17

Table 4. Nature of antibacterial principles in concentrated culture supernatants

Isolates	Treatment of culture supernatants				
	Unadjusted (pH<3.0)	Neutralized	Neutralized and treated with catalase	Neutralized and treated with protease	Neutralized and subjected to reverse-side technique
Lactobacillus	17	7	7	0	7
Lactococcus	4	0	0	0	0
Leuconostoc	2	0	0	0	0
Pediococcus	1	0	0	0	0

Data indicate the number of strains positive in well diffusion assay.

Table 5. Effect of proteolytic treatment on antibacterial principles of the culture supernatants

Producer (*Lactobacillus*)	Indicator (*Lb. plantarum*)	Width of inhibition zone (mm)*		
		Control	Trypsin treated	Pepsin treated
C34	GMR1	2.62 (0.06)	0.00	0.00
W25B	GMR1	3.80*(0.04)	3.10**	0.00
W25C	GMR1	2.30*(0.04)	2.80**	0.00
W26B	GMR1	3.10 (0.03)	0.00	0.00
W28	GMR1	3.50 (0.13)	0.00	0.00
W30A	GMR1	6.10 (0.28)	0.00	0.00
W30B	MTCC1325	3.60 (0.10)	0.00	0.00

* Data represent the means (with standard error) of triplicate sets in well diffusion assay.
** Values in rows are not significantly different ($P<0.05$).

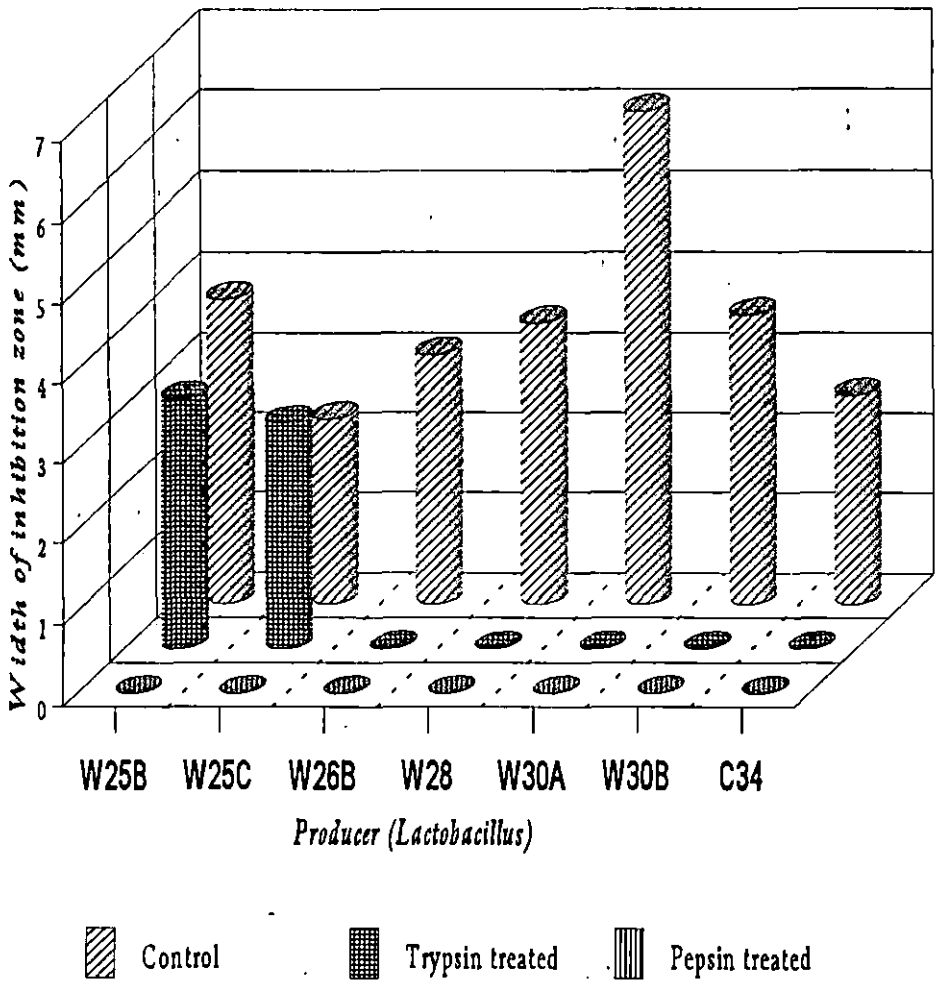

Fig. 3. Effect of proteolytic treatment on antibacterial principles of cell-free culture supernatants, evidenced in well diffusion assay

In the course of study with bacteriocins, the inhibitory activity was found to be gradually diminished and finally disappeared in case of all the producers. This finding led us to often search for the spontaneous loss of bacteriocin-producing bacteria within the populations of positive isolates. In order to regain the original bacteriocin titre, the colonies of Bac$^-$ variants were eliminated by replica plating technique. The picked up positive colonies from the replica plates resumed the original titres. However, activity could be revived in only three of the seven positive isolates.

4.4. Identification of selected Bac$^+$ strains

Characteristics of the three selected *Lactobacillus* isolates were analysed following the keys of Kandler and Weiss (1986). All the three isolates exhibited similar characteristic features. They were rod with square ends, with 2-4 cells in chains (Fig. 4). Their size was 0.7 μm X 2.2-2.4 μm. They were Gram positive and able to grow at 15°C. The isolates were negative for production of catalase and gas from glucose, growth in 6.5% NaCl and at 45°C, liquefaction of gelatin, production of indole, reduction of nitrate and hydrolysis of starch, lipid and casein. They were positive for the utilization of sugars including ribose, glucose, mannose, galactose, sucrose, lactose, maltose, cellobiose, mannitol and sorbitol, but negative for the utilization of arabinose, xylose, fructose, rhamnose, melibiose, raffinose, salicin and starch. These characters were in full agreement with those of co-culture of a reference strain, *Lactobacillus casei* subsp. *casei* ATCC 393. Based on these criteria, the three selected isolates were identified as *Lactobacillus casei*.

4.5. Optimization of bacteriocin production

Bacteriocin titres in all these experiments were determined by well diffusion assay, against *Lactobacillus plantarum* GMR1 as the indicator strain.

4.5.1. Type of medium

Five different media, used frequently for the growth of lactobacilli, were tried to search for the most suitable one for bacteriocin production (Table 6). Among these, SD-MRS broth as well as MRS broth were found to be the best media for this purpose (Fig. 5). Since, the mean bacteriocin titres in SD-MRS broth were higher than those in MRS broth, in all subsequent experiments SD-MRS broth was selected for all

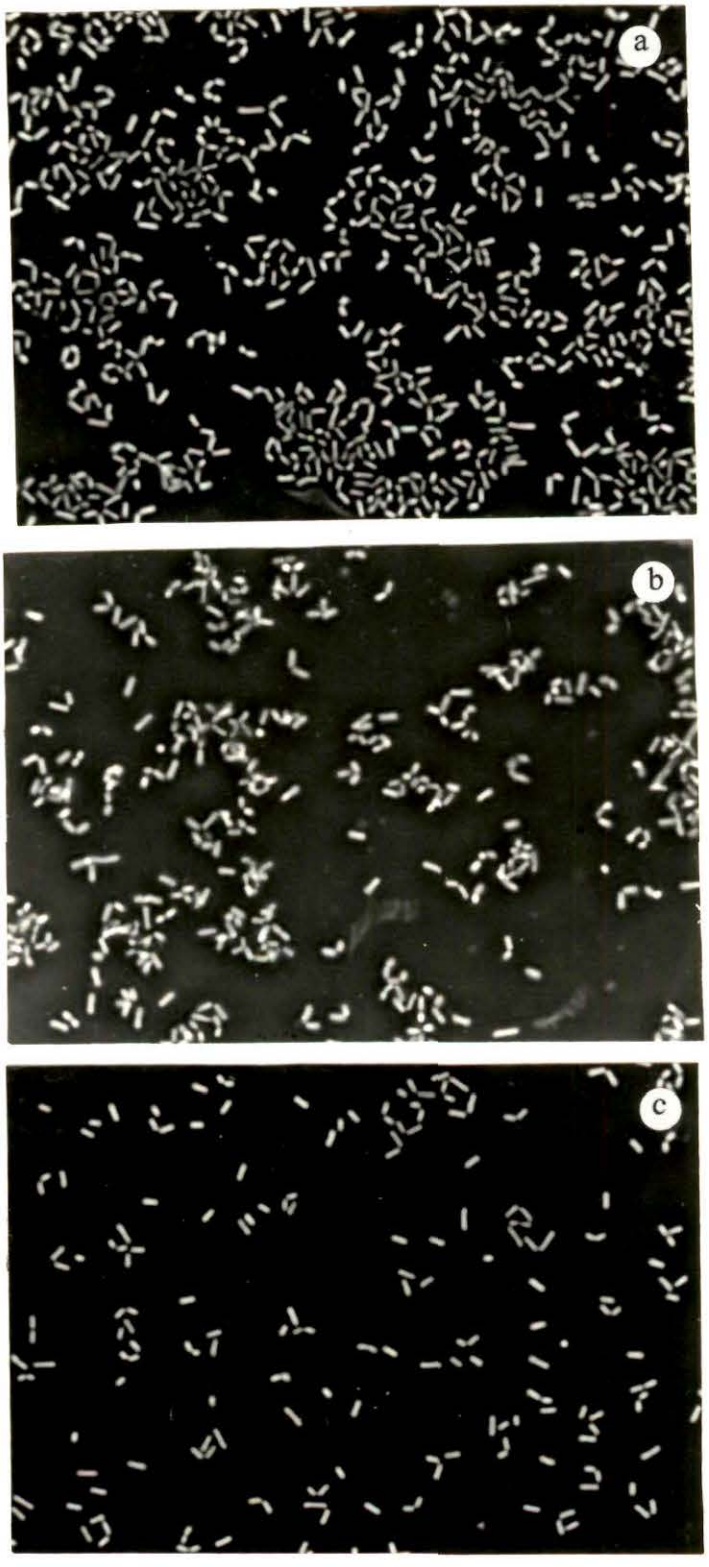

Fig. 4. Phase-contrast micrographs (x 1300) of cells of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

Table 6. Bacteriocin titres in different broth cultures

Producer (*Lactobacillus casei*)	Width of inhibition zone (mm)*				
	MRS broth	MRS-0.2 broth	APT broth	Tomato juice medium	SD-MRS broth
W25B	2.00[a] (0.11)	2.00[a](0.11)	0.35[b] (0.07)	1.00[a] (0.11)	3.90[a] (0.04)
W26B	1.71[a] (0.14)	2.00[a](0.16)	2.00[a] (0.06)	0.00[b]	3.10[a] (0.04)
W28	2.58[a] (0.19)	1.04[b](0.04)	0.00[c]	0.00[c]	3.90[a] (0.04)

*Data represent the means (with standard error) of triplicate sets in well diffusion asay. Values bearing different superscripts in each row differ significantly ($P<0.05$).

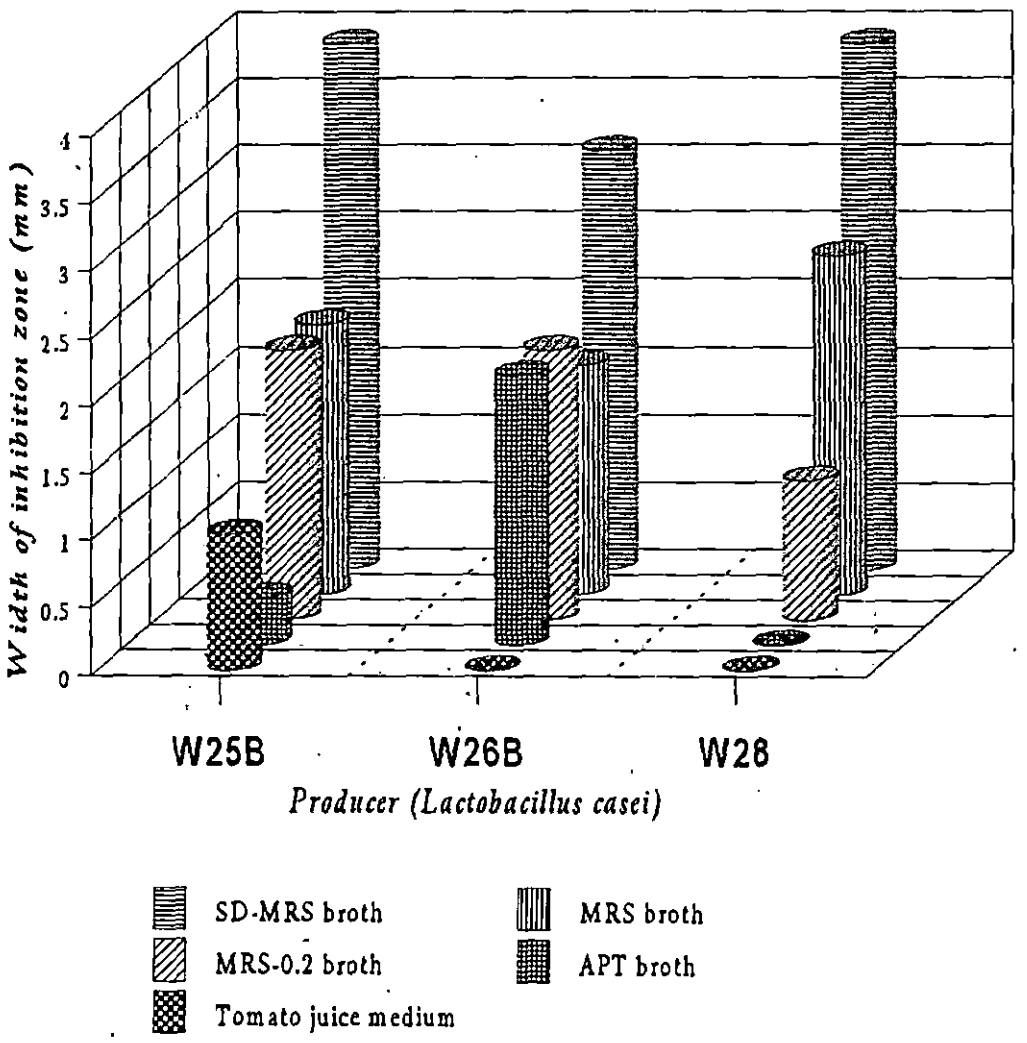

Fig. 5. Bacteriocin titres in different broth cultures, determined by well diffusion assay.

subsequent experiments.

4.5.2. pH of medium

Table 7 and Fig. 6 show the growth and production of inhibitory substance in SD-MRS broth adjusted initially to different pH values. *Lactobacillus casei* W25B showed maximum growth at pH 6, but maximum bacteriocin titre at pH 6.0-6.4. In *Lb. casei* W26B maximum growth was at pH 6.0-6.4, but highest bacteriocin titre was at pH 5.5-6.2. In *Lb. casei* W28 maximum growth as well as bacteriocin production occurred at pH 5.0 - 6.4. When the initial pH of the medium was raised to 8.0 no activity was detected. However, absence of activity was not due to lack of producer growth, since confluent growth occured at all the initial pH levels (5.0-8.0).

4.5.3. Temperature of incubation

Table 8 and Fig. 7 show the viable count and the bacteriocin production ~~after~~ at different temperatures. The maximum growth as well as bacteriocin production in W25B, W26B and W28 were at 30-32°C.

4.5.4. Period of incubation

Tables 9 and 10 and Figs 8 and 9 show the effect of incubation period on growth and bacteriocin production. In *Lb casei* W25B, viable count increased rapidly to reach its maximum in 1-2 d and the maximum bacteriocin titre was achieved between 1-3 d of incubation. Further incubation had an adverse effect on growth as well as bacteriocin titre. *Lactobacillus casei* W26B showed maximum growth after 1 d of incubation, while the maximum bacteriocin titre was obtained between 1 to 4 d of incubation. While the growth of *Lb. casei* W28 was maximum in 1-2 d of incubation, the maximum bacteriocin titre was obtained between 1 and 4 d of incubation.

4.6. Characterization of bacteriocins

4.6.1. Thermostability

The bacteriocins were found totally stable at 98°C for 2 h, the maximum temperature-time treatment applied. Treatment at 121°C for 15 min also did not result in any loss

Table 7. The effect of initial pH of SD-MRS broth on growth and bacteriocin production of the three isolated strains of *Lactobacillus casei**

pH	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
5.0	335.0^{d} (0.4)	3.12^{c} (0.06)	322.0^{c} (0.5)	3.96^{ab} (0.04)	343.3^{a} (1.8)	5.25^{a} (0.16)
5.5	337.0^{d} (0.4)	3.46^{b} (0.04)	335.0^{b} (0.7)	4.17^{a} (0.05)	356.0^{a} (8.3)	5.33^{a} (0.08)
6.0	395.5^{a} (2.9)	4.17^{a} (0.05)	377.0^{a} (0.5)	4.42^{a} (0.05)	392.0^{a} (2.1)	5.96^{a} (0.12)
6.2	388.0^{b} (5.0)	3.92^{a} (0.05)	375.5^{a} (0.6)	4.37^{a} (0.06)	391.0^{a} (2.4)	6.08^{a} (0.14)
6.4	350.0^{c} (1.0)	3.83^{a} (0.12)	374.0^{a} (0.9)	4.12^{ab} (0.06)	389.0^{a} (1.8)	5.96^{a} (0.12)
7.0	224.2^{e} (0.9)	3.17^{c} (0.05)	205.0^{d} (1.7)	3.17^{b} (0.12)	224.0^{b} (0.6)	3.00^{b} (0.09)
8.0	3.7^{f} (0.1)	0.00^{d}	2.8^{e} (0.1)	0.00^{c}	3.7^{c} (0.1)	0.00^{c}

* Incubated at 32°C for 24 h. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

†Determined by well diffusion assay.

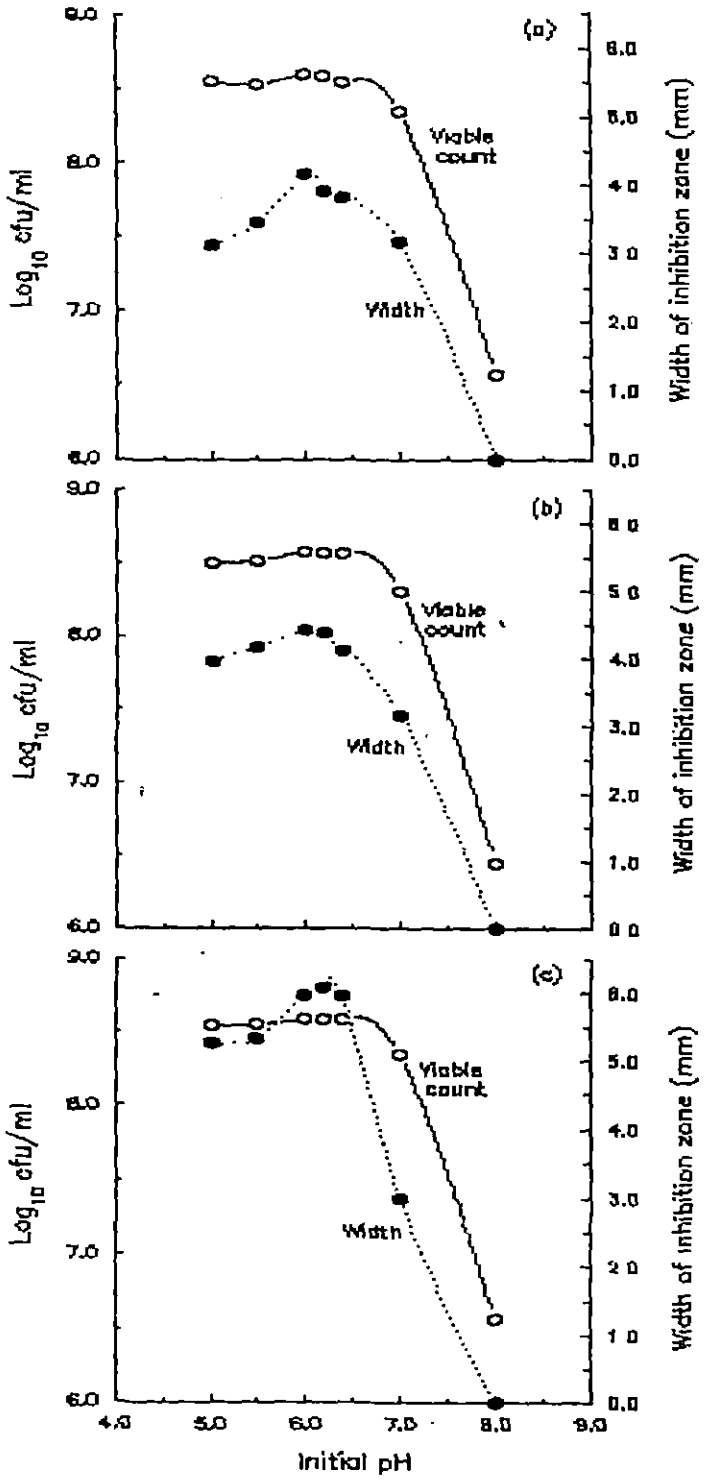

Fig. 6. The effect of initial pH of SD-MRS broth on viable count and bacteriocin titre (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B and W28 (c).

Table 8. The effect of temperature of incubation on growth and bacteriocin production of the three isolated strains of *Lactobacillus casei**

Temperature (°C)	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
20.0	36.0^{d} (1.4)	0.00^{e}	44.0^{e} (0.6)	0.00^{d}	36.0^{d} (1.1)	0.00^{d}
25.0	211.8^{c} (3.2)	1.33^{d} (0.05)	118.0^{d} (0.4)	1.12^{c} (0.06)	212.0^{c} (3.2)	2.16^{c} (0.05)
27.5	302.0^{b} (4.9)	2.33^{c} (0.05)	254.0^{c} (0.5)	3.17^{b} (0.05)	302.0^{b} (4.5)	4.16^{b} (0.05)
30.0	332.0^{ab} (8.8)	3.87^{ab} (0.06)	402.0^{a} (2.5)	4.29^{a} (0.10)	345.3^{a} (8.3)	5.62^{a} (0.08)
32.0	388.0^{a} (3.6)	4.54^{a} (0.14)	409.0^{a} (0.9)	4.33^{a} (0.05)	388.0^{a} (3.6)	5.87^{a} (0.12)
35.0	211.0^{c} (1.8)	3.67^{b} (0.05)	308.0^{b} (1.0)	3.83^{ab} (0.08)	211.0^{c} (2.1)	4.37^{b} (0.06)

* Grown in SD-MRS broth for 24 h. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

†Determined by well diffusion assay.

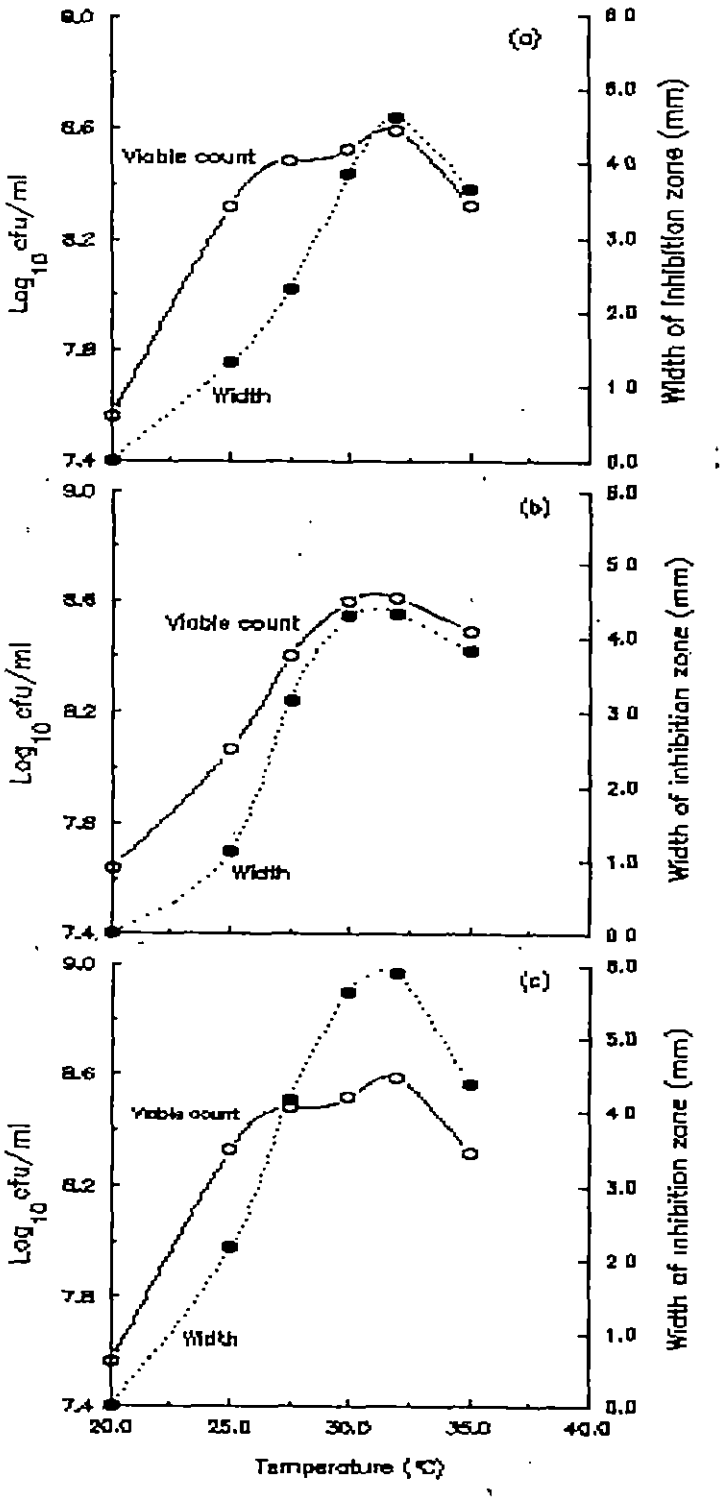

Fig. 7. The effect of temperature of incubation on growth and bacteriocin production (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

Table 9. The influence of incubation period on growth and bacteriocin production of the three isolated strains of *Lactobacillus casei**

Incubation period (d)	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
0	46.0^{f} (1.4)	0.00^{d}	36.0^{f} (1.5)	0.00^{d}	38.0^{e} (1.6)	0.00^{d}
1	489.0^{a} (2.2)	3.96^{a} (0.04)	399.0^{a} (1.2)	4.04^{a} (0.04)	406.0^{a} (2.4)	4.08^{a} (0.08)
2	462.0^{ab} (2.7)	3.92^{a} (0.05)	379.0^{b} (1.9)	3.92^{a} (0.08)	366.7^{ab} (7.5)	4.29^{a} (0.08)
3	426.0^{b} (3.4)	3.25^{ab} (0.11)	315.3^{c} (1.1)	3.83^{a} (0.05)	318.0^{b} (1.4)	4.08^{a} (0.08)
4	383.0^{c} (5.6)	2.83^{bc} (0.05)	303.0^{c} (0.4)	3.37^{ab} (0.08)	218.0^{c} (3.2)	4.04^{a} (0.04)
5	225.0^{d} (2.5)	2.62^{bc} (0.08)	249.0^{d} (1.2)	2.33^{b} (0.16)	120.0^{d} (3.7)	3.08^{b} (0.08)
6	109.0^{e} (2.1)	1.96^{c} (0.04)	114.0^{e} (0.9)	1.62^{c} (0.08)	105.0^{d} (0.4)	2.62^{c} (0.06)

* Grown in SD-MRS broth at 32°C. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

†Determined by well diffusion assay.

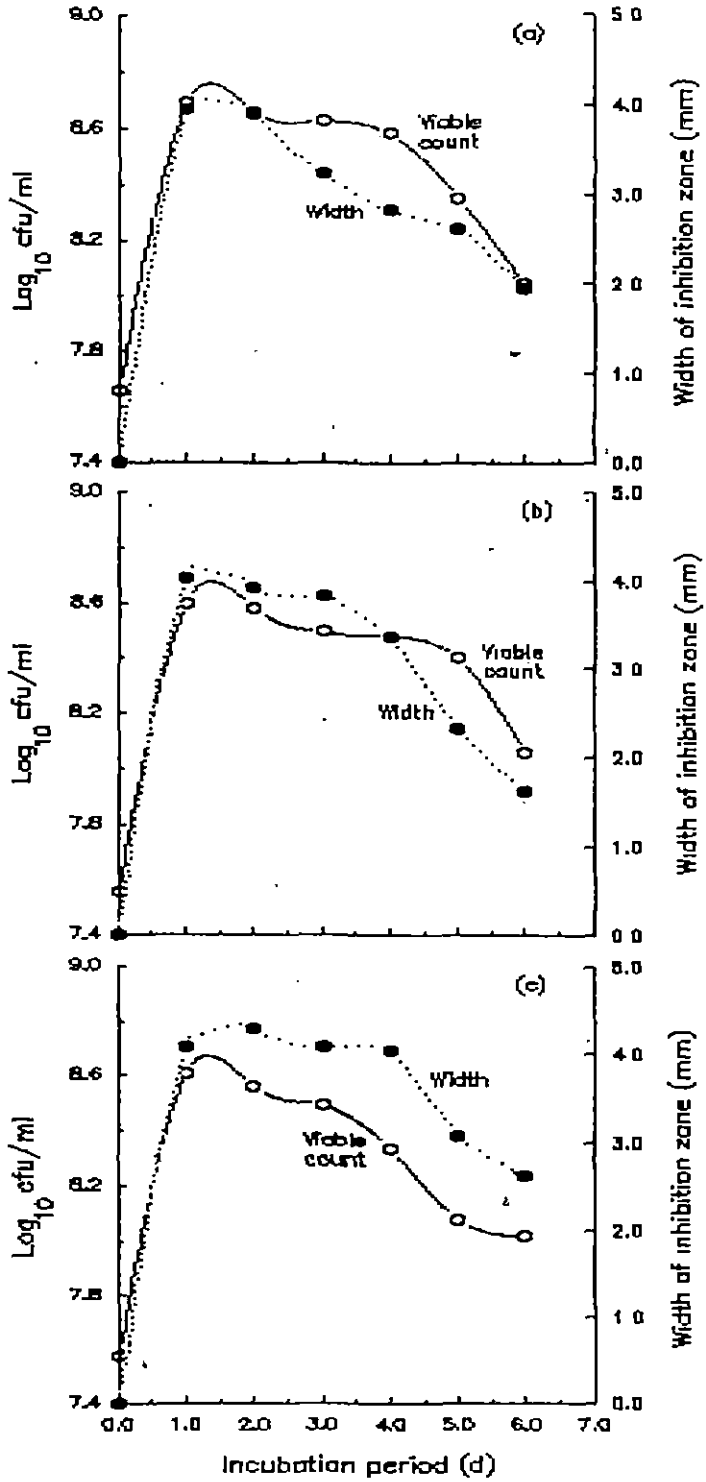

Fig. 8. The effect of period of incubation on growth and bacteriocin production (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

Table 10. Growth and bacteriocin production of the three isolated strains of *Lactobacillus casei* during 36-h incubation period*

Incubation period (h)	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
0	35.3^{d} (1.9)	0.00^{d}	40.0^{d} (3.3)	0.00^{d}	42.2^{e} (1.7)	0.00^{d}
6	116.0^{c} (2.6)	0.00^{d}	112.2^{c} (3.3)	0.00^{d}	126.0^{d} (5.9)	0.00^{d}
12	295.0^{b} (5.4)	1.78^{c} (0.16)	281.0^{b} (5.8)	1.30^{c} (0.03)	301.0^{c} (3.6)	1.83^{c} (0.08)
18	377.8^{a} (3.2)	2.40^{b} (0.04)	371.0^{a} (6.0)	2.30^{b} (0.04)	396.0^{b} (5.7)	2.60^{b} (0.12)
24	397.8^{a} (4.7)	4.62^{a} (0.13)	386.0^{a} (2.8)	4.40^{a} (0.06)	431.3^{a} (8.8)	5.90^{a} (0.09)
30	395.0^{a} (4.0)	4.60^{a} (0.13)	382.0^{a} (2.9)	4.40^{a} (0.10)	427.8^{a} (2.0)	5.90^{a} (0.09)
36	380.0^{a} (2.5)	4.93^{a} (0.07)	376.0^{a} (4.3)	4.60^{a} (0.11)	418.0^{a} (2.5)	6.00^{a} (0.11)

* Grown in SD-MRS broth at 32°C. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
†Determined by well diffusion assay.

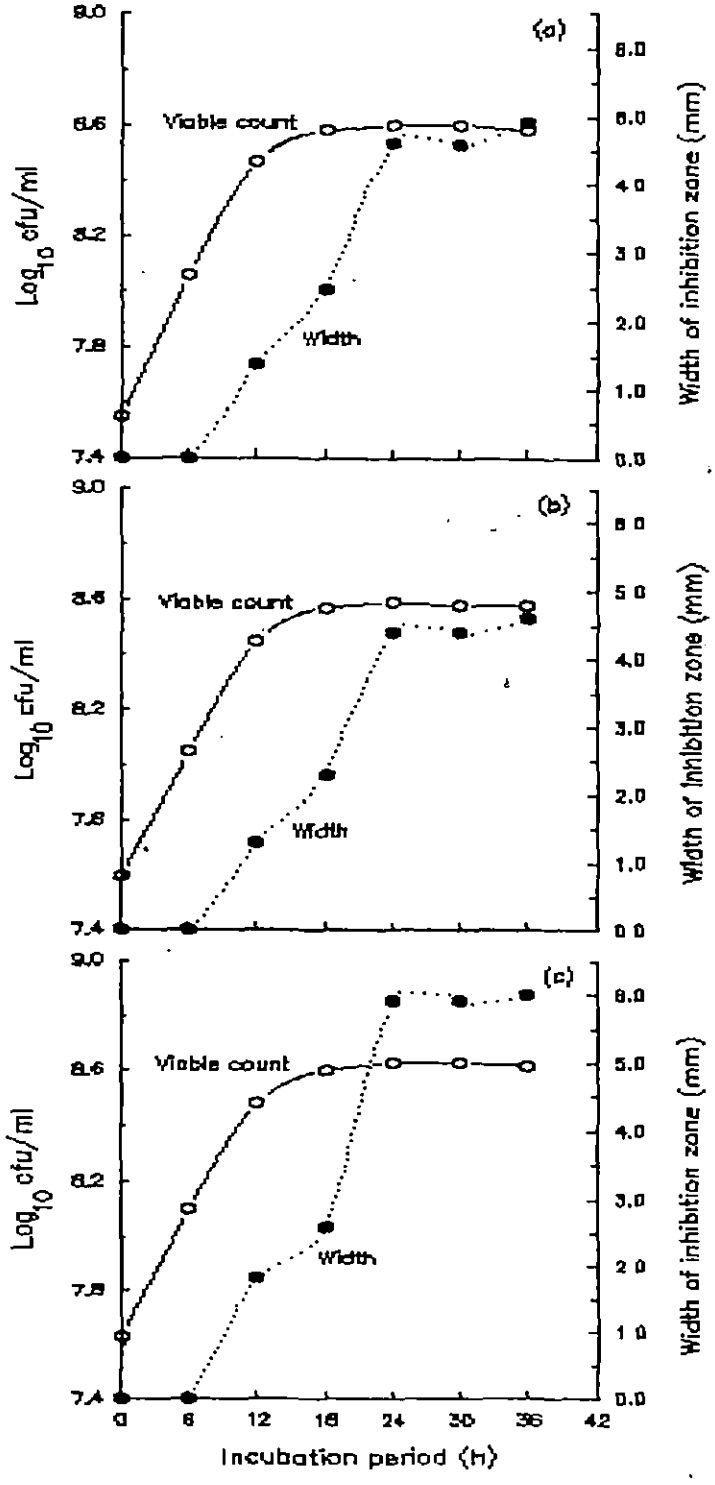

Fig. 9. The effect of period of incubation on growth and bacteriocin production (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

of activity (Table 11).

4.6.2. pH stability

While the bacteriocin activity present in the crude extract of *Lb. casei* W25B and W28 was fully stable at pH 2-8, the same in *Lb. casei* W26B was at pH 2-9 (Table 12; Fig. 10)

4.6.3. Storage stability

The effect of storage on bacteriocin activity is presented in Tables 13A-C and Fig. 11. On storing the culture broth at 37°C, a rapid loss of activity was recorded with time. Strains W25B, W26B and W28 lost complete activity at a storage period of 30, 20 and 60 d, respectively. On storing the cell-free culture supernatant at 37°C again a loss of activity with time was observed, but the complete loss of activity was after a longer period of time, when compared to culture broth stored at the same temperature. There was no significant loss of activity when the cell-free culture supernatant was stored at -85 to 4°C over a period up to 60 d.

4.6.4. Stability against organic solvents

Various organic solvents were tested for their effect on bacteriocin stability (Table 14). Treatment with benzene, propane-2-ol, iso-amyl alcohol, diethyl ether, acetone, formaldehyde, n-hexane and chloroform did not result in any loss of bacteriocin activity in all the three producer strains. However, when treated with n-butanol, the activity was reduced and a part of the activity was recovered from the organic phase.

4.7. Purification of bacteriocin

4.7.1. Dialysis

Bacteriocin activity in cell-free culture supernatants was not retained after exhaustive dialysis against 50 mM (pH 7.2) phosphate buffer using membrane tubings with molecular exclusion limits of 12 kDa. However, when dialysis was done using membrane tubings with molecular exclusion limit of 3.5 kDa, activity was detected in the retentate. This

Table 11. Temperature stability of the bacteriocins in crude extracts of the three isolated strains of *Lactobacillus casei*

Treatment of crude extract		Width of inhibition zone (mm)*		
Temperature (°C)	Time (min)	W25B	W26B	W28
Control		4.04 (0.04)	4.29 (0.04)	4.79 (0.04)
70	60	3.75 (0.13)	4.17 (0.05)	4.58 (0.14)
80	60	3.75 (0.11)	4.17 (0.05)	4.54 (0.15)
90	60	3.62 (0.06)	4.17 (0.05)	4.58 (0.08)
98	120	3.79 (0.04)	4.17 (0.05)	4.42 (0.05)
121	15	3.42 (0.05)	4.12 (0.08)	4.62 (0.08)

* Grown in SD-MRS broth at 32° C for 24 h. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values in each column are not significantly different ($P<0.05$).

Table 12. pH stability of the bacteriocins in crude extracts of the three isolated strains of *Lactobacillus casei*

Adjusted pH of the crude extract*	Width of inhibition zone (mm)†		
	W25B	W26B	W28
2	6.17^{ab} (0.08)	6.29^{a} (0.08)	8.92^{a} (0.05)
3	6.17^{ab} (0.08)	6.33^{a} (0.08)	8.96^{a} (0.04)
4	6.17^{ab} (0.08)	6.33^{a} (0.08)	9.04^{a} (0.04)
5	6.21^{ab} (0.10)	6.50^{a} (0.16)	8.88^{a} (0.08)
6	6.17^{ab} (0.08)	6.87^{a} (0.12)	8.87^{a} (0.06)
7	6.25^{a} (0.09)	6.87^{a} (0.12)	8.83^{a} (0.05)
8	6.12^{ab} (0.06)	6.29^{a} (0.14)	8.83^{a} (0.10)
9	4.79^{b} (0.04)	5.12^{a} (0.06)	7.62^{b} (0.12)
10	3.17^{c} (0.08)	2.12^{b} (0.08)	6.12^{c} (0.06)
11	1.96^{c} (0.04)	1.21^{b} (0.14)	3.62^{d} (0.06)

* Incubated at the pH for 24 h at 4°C.

† Grown in SD-MRS broth at 32°C for 24 h. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

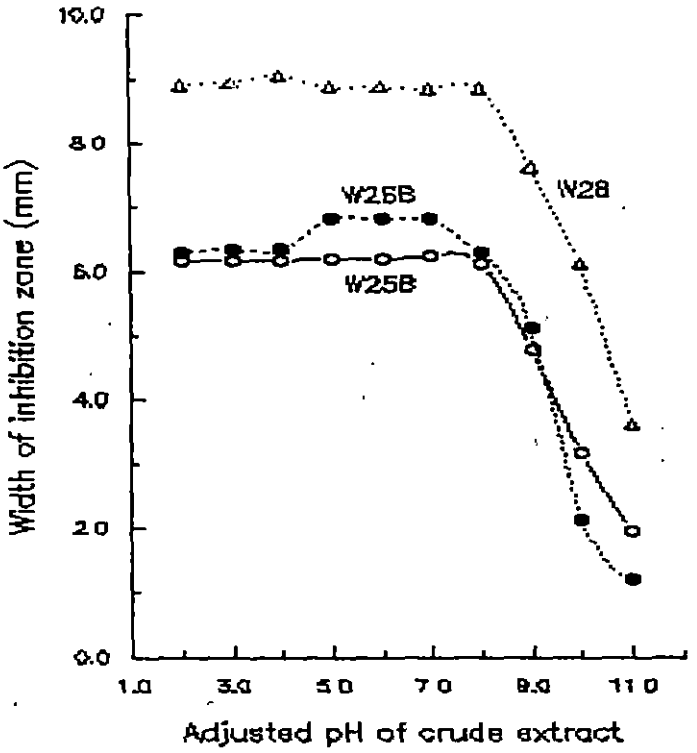

Fig. 10. The effect of pH on bacteriocin activity of the three isolated strains of *Lactobacillus casei*, as determined by well diffusion assay.

Table 13A. The influence of time-temperature treatments on the stability of bacteriocin of *Lactobacillus casei* W25B

Days	Width of inhibition zone (mm)*				
	Culture broth	Cell-free culture supernatant			
	37°C	37°C	4°C	-20°C	-85°C
1	6.42[a] (0.05)	6.46[a] (0.42)	6.46[a] (0.04)	6.45[a] (0.04)	6.42[a] (0.05)
5	5.12[b] (0.06)	5.92[b] (0.05)	6.46[a] (0.04)	6.54[a] (0.04)	6.96[a] (0.04)
10	3.62[c] (0.06)	4.87[c] (0.06)	6.33[a] (0.05)	6.46[a] (0.04)	6.92[a] (0.05)
20	1.54[d] (0.10)	3.72[d] (0.06)	6.33[a] (0.05)	6.62[a] (0.06)	6.46[a] (0.04)
30	0.00[e]	0.00[e]	6.33[a] (0.05)	6.67[a] (0.05)	6.67[a] (0.05)
40	0.00[e]	0.00[e]	6.17[a] (0.05)	6.46[a] (0.04)	6.46[a] (0.04)
50	0.00[e]	0.00[e]	6.17[a] (0.05)	6.46[a] (0.04)	6.42[a] (0.05)
60	0.00[e]	0.00[e]	6.42[a] (0.05)	6.67[a] (0.05)	6.67[a] (0.05)

* Grown in SD-MRS broth for 24 h at 32°C. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 13B. The influence of time-temperature treatments on the stability of bacteriocin of *Lactobacillus casei* W26B

Days	Width of inhibition zone (mm)*				
	Culture broth	Crude extract			
	37°C	37°C	4°C	-20°C	-85°C
1	6.50[a] (0.16)	6.67[a] (0.05)	6.71[a] (0.04)	6.67[a] (0.05)	6.67[a] (0.05)
5	4.83[b] (0.05)	5.37[b] (0.06)	6.71[a] (0.04)	6.62[a] (0.06)	6.58[a] (0.08)
10	2.17[c] (0.05)	4.12[c] (0.06)	6.62[a] (0.06)	6.62[a] (0.06)	6.62[a] (0.12)
20	0.00[d]	2.87[d] (0.06)	6.67[a] (0.05)	6.71[a] (0.04)	6.62[a] (0.17)
30	0.00[d]	1.54[e] (0.08)	6.67[a] (0.05)	6.62[a] (0.06)	6.67[a] (0.05)
40	0.00[d]	0.00[f]	6.67[a] (0.05)	6.62[a] (0.06)	6.67[a] (0.08)
50	0.00[d]	0.00[f]	6.62[a] (0.06)	6.62[a] (0.06)	6.46[a] (0.08)
60	0.00[d]	0.00[f]	6.62[a] (0.06)	6.54[a] (0.12)	6.46[a] (0.12)

* Grown in SD-MRS broth for 24 h at 32°C. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 13C. The influence of time-temperature treatments on the stability of bacteriocin of *Lactobacillus casei* W28

Days	Width of inhibition zone (mm)*				
	Culture broth	Crude extract			
	37°C	37°C	4°C	-20°C	-85°C
1	8.92[a] (0.08)	8.96[a] (0.04)	8.87[a] (0.06)	8.96[a] (0.04)	8.96[a] (0.04)
5	7.37[b] (0.06)	8.92[a] (0.05)	8.83[a] (0.05)	8.96[a] (0.04)	8.96[a] (0.04)
10	5.12[c] (0.06)	7.62[b] (0.12)	8.79[a] (0.08)	8.92[a] (0.08)	8.96[a] (0.04)
20	4.83[c] (0.05)	7.04[b] (0.04)	8.79[a] (0.08)	8.92[a] (0.08)	8.92[a] (0.05)
30	4.12[cd] (0.06)	6.92[b] (0.08)	8.46[a] (0.04)	8.92[a] (0.08)	8.92[a] (0.08)
40	3.67[d] (0.12)	6.67[b] (0.08)	8.54[a] (0.04)	8.87[a] (0.06)	8.92[a] (0.08)
50	1.83[e] (0.05)	5.00[c] (0.06)	8.54[a] (0.04)	8.87[a] (0.06)	8.92[a] (0.12)
60	0.00[f]	4.08[c] (0.05)	8.62[a] (0.06)	8.83[a] (0.05)	8.92[a] (0.12)

* Grown in SD-MRS broth for 24 h at 32°C. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

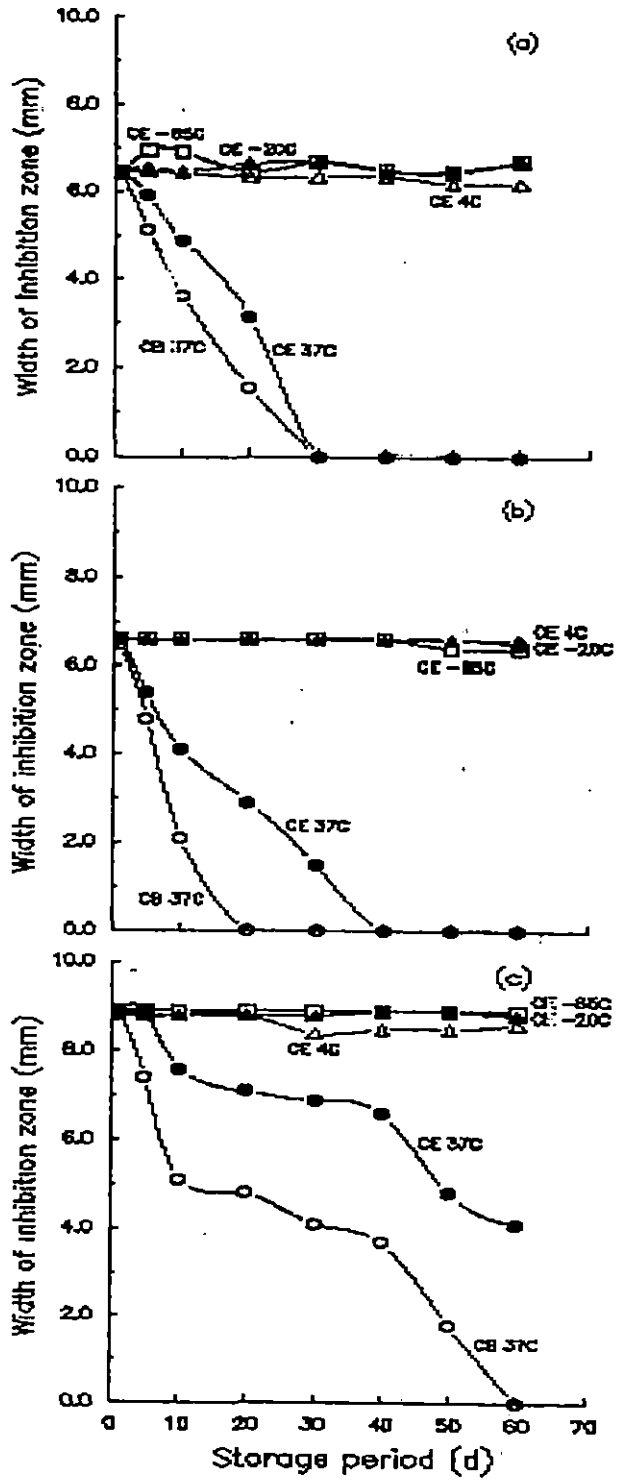

Fig. 11. The effect of time-temperature treatment on the stability of bacteriocins of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c), as determined by well diffusion assay. CB, culture broth; CE, crude extract (cell-free culture supernatant).

Table 14. Solubility of bacteriocins in different organic solvents

Organic solvent	Width of inhibition zone (mm)*					
	W25B		W26B		W28	
	Aqueous phase	Organic phase	Aqueous phase	Organic phase	Aqueous phase	Organic phase
Control	5.10 [a] (0.04)	0.00	4.10 [a] (0.04)	0.00	6.07 [a] (0.23)	0.00
Benzene	4.62 [a] (0.11)	0.00	4.00 [a] (0.09)	0.00	5.62 [a] (0.17)	0.00
Propane-2-ol	4.25 [a] (0.09)	0.00	3.83 [a] (0.05)	0.00	6.04 [a] (0.19)	0.00
iso-Amyl alcohol	4.75 [a] (0.09)	0.00	3.62 [a] (0.06)	0.00	5.92 [a] (0.26)	0.00
n-Butanol	2.08 [b] (0.05)	2.38 [a] (0.45)	1.04 [b] (0.04)	2.29 [b] (0.08)	2.12 [b] (0.06)	3.67 [a] (0.10)
Diethyl ether	4.71 [a] (0.08)	0.00	3.75 [a] (0.09)	0.00	5.92 [a] (0.23)	0.00
Acetone	5.00 [a] (0.09)	0.00	3.79 [a] (0.12)	0.00	6.29 [a] (0.22)	0.00
Formaldehyde	4.71 [a] (0.08)	0.00	3.75 [a] (0.09)	0.00	5.92 [a] (0.19)	0.00
n-Hexane	4.75 [a] (0.10)	0.00	3.83 [a] (0.10)	0.00	6.17 [a] (0.23)	0.00
Chloroform	5.03 [a] (0.26)	0.00	3.70 [a] (0.16)	0.00	6.00 [a] (0.18)	0.00

* Grown in SD-MRS broth at 32°C for 24 h. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

activity was lost on proteolytic treatments. To check the possibility of adsorption of the bacteriocin to dialysis membrane, the membranes were cut after dialysis (0.5 cm X 2.0 cm) and placed on MRS agar plates exposing the inner side up and overlaid with seeded MRS soft agar. No zone of inhibition around the membrane was observed after incubation.

4.7.2. Gel filtration

Gel filtration through Sephadex G-25 was the first step involved in purification of W-28 bacteriocin (Fig. 12). Activity was eluted with 50 mM (pH 7.2) sodium phosphate buffer containing 0.6% sodium chloride in the last 12 ml. Following purification, the total activity recovered was 24%, and the specific activity increased from 2.28 AU/mg before gel filtration to 148.15 AU/mg. The sample was 65-fold purified after filtration (Table 15).

4.7.3. Anion-exchange and desalting column chromatographies

The purified sample after gel filtration was subjected to anion-exchange chromatography (Fig. 13). The bacteriocin was adsorbed strongly at pH 8 to DEAE cellulose (Sigma, USA as the column material, and was released only in the flow through fractions by applying 0.5 M sodium chloride in the elution buffer. Active fractions were run through Exocellulose GF-5 desalting columns to remove sodium chloride (Fig. 14), and freeze-dried. Under these conditions, an extensive removal of other contaminants was achieved. Activity was eluted in the last 4 ml fractions, which were combined and found to contain only 0.06 mg/ml protein (11% of the sample protein was recovered from the active fraction). However, loss of activity was also observed (4% recovery). The specific activity increased to 666.67 AU/mg and 292-fold purification was achieved (Table 15). When the above purification step was repeated under the same conditions, but at pH 7.2, adsorption did not occur. Exposure of active samples to high sodium chloride concentrations showed the W28 bacteriocin to be stable.

4.8. Mode of action

Mode of action studies were done for all the three selected producer strains which showed similar action (Tables 16A-C, 17A-C, 18A-C; Figs. 15-17). After addition of cell-free culture supernatant, a strong decrease in the number of viable cells of the indicator organism was observed within 1.5 h, and 100% killing was observed after 4.5 h. The killing effect was dependent on the stage of the indicator cells; it was most

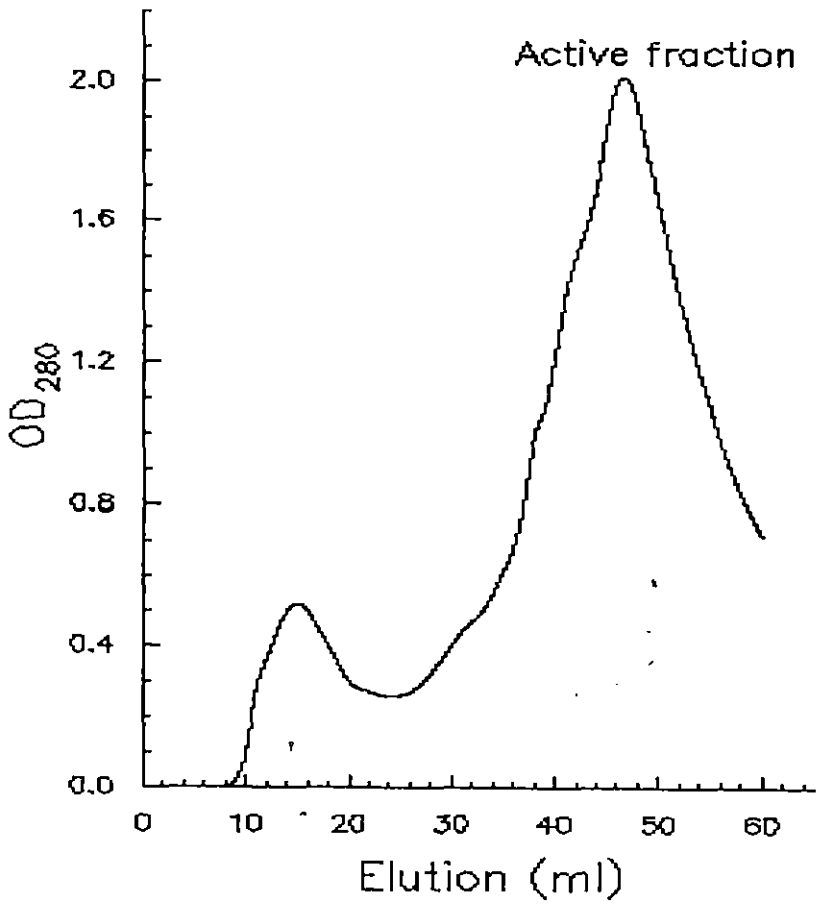

Fig. 12. Elution of *Lactobacillus casei* W28 bacteriocin from Sephadex G-25. A 2 ml-volume of culture supernatant was applied to Sephadex G-25 column equilibrated with 50 mM (pH 7.2) phosphate buffer. Activity was eluted with 0.6% (w/v) NaCl in the buffer. Fractions (1 ml) were collected and assayed for bacteriocin activity.

Table 15. Purification of *Lactobacillus casei* W28 bacteriocin

Sample*	Volume (ml) (A)	Activity (AU/ml) (B)	Total activity (C=A x B)	Protein concentration (mg/ml) (D)	Specific activity (AU/mg) (E=B/D)	Activity recovered (%) (C_2/C_1x100)	Fold purification (E_2/E_1)
1	100	40	4000	17.53	2.28	100.0	1.0
2	12	80	960	0.54	148.15	24.0	65.0
3	4	40	160	0.06	666.67	4.0	292.4

*1, 10-fold concentrated culture supernatant (pH 6.5); 2, G-25-purified sample; 3, DEAE cellulose and GF-5 Exocellulose-purified sample.

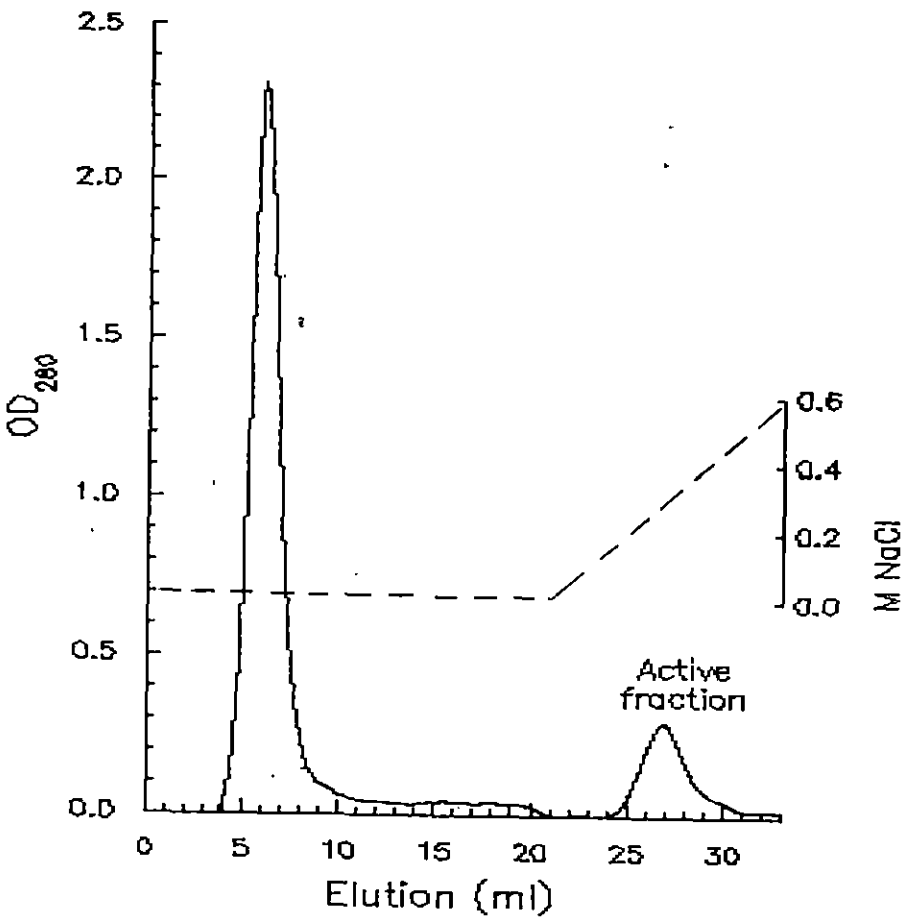

Fig. 13. Elution of *Lactobacillus casei* W28 bacteriocin from DEAE cellulose. Cell-free culture supernatants (12 ml), purified through G-25 Sephadex, were applied to DEAE cellulose column equilibrated with 20 mM (pH 8.0) phosphate buffer. The column was washed until absorbance at 280 nm returned to zero. Bacteriocin was eluted with a linear gradient of 0-0.6 M NaCl in 20 mM Tris. Fractions (1 ml) were collected and assayed for bacteriocin activity.

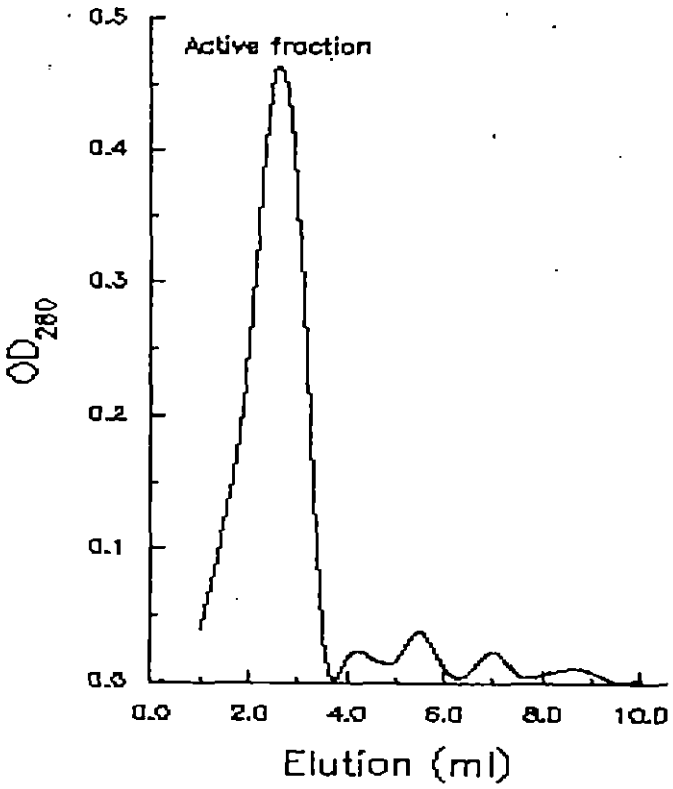

Fig. 14. Elution of *Lactobacillus casei* W28 bacteriocin from Exocellulose G-5 desalting column. Cell-free culture superantant (1.25 ml), purified through Sephadex G-25 and DEAE cellulose, was applied to Exocellulose G-5 equilibrated with 20 mM (pH 8.0) ammonium bicarbonate buffer. Activity was eluted with the same buffer until absorbance at 280 nm returned to zero.

Table 16A. The effect of crude extract of *Lactobacillus casei* W25B on growth of 6 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 ^{c} (0.001)	42.33 ^{e} (1.45)	0.051 ^{a} (0.001)	42.00 ^{a} (0.06)
1.5	0.066 ^{c} (0.001)	101.00 ^{d} (1.73)	0.053 ^{a} (0.001)	2.22 ^{b} (0.05)
3.0	0.086 ^{bc} (0.002)	166.33 ^{c} (2.96)	0.058 ^{a} (0.001)	0.18 ^{c} (0.02)
4.5	0.155 ^{b} (0.001)	217.33 ^{b} (1.45)	0.070 ^{a} (0.006)	0.02 ^{c} (0.00)
6.0	0.259 ^{a} (0.006)	275.33 ^{a} (2.91)	0.070 ^{a} (0.006)	0.02 ^{c} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 16B. The effect of crude extract of *Lactobacillus casei* W25B on growth of 12 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.050 ^{c} (0.001)	38.67 ^{d} (0.88)	0.050 ^{a} (0.003)	37.67 ^{a} (0.88)
1.5	0.064 ^{c} (0.006)	99.67 ^{c} (5.21)	0.057 ^{a} (0.002)	3.47 ^{b} (0.13)
3.0	0.095 ^{c} (0.001)	171.00 ^{b} (4.73)	0.064 ^{a} (0.000)	0.22 ^{b} (0.00)
4.5	0.166 ^{b} (0.002)	211.00 ^{b} (5.29)	0.082 ^{a} (0.001)	0.02 ^{b} (0.00)
6.0	0.259 ^{a} (0.006)	277.33 ^{a} (2.91)	0.101 ^{a} (0.001)	0.01 ^{b} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 16C. The effect of crude extract of *Lactobacillus casei* W25B on growth of 22 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 [d] (0.001)	43.0 [d] (0.6)	0.051 [cd] (0.001)	40.7 [c] (0.9)
1.5	0.051 [d] (0.001)	45.3 [d] (1.2)	0.050 [d] (0.000)	40.0 [c] (2.5)
3.0	ND [†]	117.3 [c] (3.5)	ND	55.7 [c] (3.8)
4.5	ND	154.0 [b] (0.6)	ND	115.3 [b] (2.3)
6.0	0.060 [c] (0.001)	183.0 [b] (6.2)	0.057 [c] (0.002)	163.3 [b] (0.9)
12.0	0.080 [b] (0.001)	329.0 [a] (4.4)	0.077 [b] (0.001)	310.3 [a] (0.2)
24.0	0.135 [a] (0.001)	ND	0.133 [a] (0.001)	ND

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
[†] ND, not determined.

Table 17A. The effect of crude extract of *Lactobacillus casei* W26B on growth of 6 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.052 ^{e} (0.001)	42.67 ^{e} (1.45)	0.051 ^{b} (0.001)	41.00 ^{a} (1.00)
1.5	0.067 ^{d} (0.001)	108.33 ^{d} (0.88)	0.053 ^{ab} (0.001)	2.20 ^{b} (0.01)
3.0	0.083 ^{c} (0.002)	166.33 ^{c} (2.40)	0.058 ^{ab} (0.002)	0.19 ^{c} (0.02)
4.5	0.153 ^{b} (0.002)	216.00 ^{b} (2.65)	0.063 ^{ab} (0.001)	0.02 ^{c} (0.00)
6.0	0.260 ^{a} (0.006)	279.33 ^{a} (5.46)	0.070 ^{a} (0.001)	0.01 ^{c} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 17B. The effect of crude extract of *Lactobacillus casei* W26B on growth of 12 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.045 ^{c} (0.002)	33.00 ^{e} (1.53)	0.044 ^{a} (0.002)	32.00 ^{a} (1.15)
1.5	0.064 ^{c} (0.003)	74.33 ^{d} (5.18)	0.049 ^{a} (0.001)	2.76 ^{b} (0.04)
3.0	0.095 ^{bc} (0.004)	119.33 ^{c} (2.85)	0.065 ^{a} (0.002)	0.18 ^{b} (0.00)
4.5	0.138 ^{b} (0.004)	194.00 ^{b} (3.61)	0.086 ^{a} (0.002)	0.01 ^{b} (0.00)
6.0	0.231 ^{a} (0.005)	242.67 ^{a} (6.44)	0.088 ^{a} (0.003)	0.01 ^{b} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 17C. The effect of crude extract of *Lactobacillus casei* W26B on growth of 22 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 [c] (0.001)	43.3 [d] (1.2)	0.051 [d] (0.001)	42.0 [c] (0.6)
1.5	0.051 [c] (0.001)	45.0 [d] (1.5)	0.051 [d] (0.001)	42.7 [c] (2.2)
3.0	ND †	118.7 [c] (2.2)	ND	54.0 [c] (3.6)
4.5	ND	155.0 [bc] (2.5)	ND	115.0 [b] (3.8)
6.0	0.060 [bc] (0.001)	176.0 [b] (2.3)	0.054 [c] (0.001)	154.0 [b] (6.0)
12.0	0.079 [b] (0.001)	355.0 [a] (1.7)	0.078 [b] (0.002)	295.7 [a] (2.6)
24.0	0.134 [a] (0.001)	ND	0.134 [a] (0.001)	ND

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
† ND, not determined.

Table 18A. The effect of crude extract of *Lactobacillus casei* W28 on growth of 6 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.052 ^{c} (0.001)	42.00 ^{e} (1.00)	0.052 ^{a} (0.001)	43.00 ^{a} (1.00)
1.5	0.063 ^{c} (0.001)	122.00 ^{d} (1.86)	0.053 ^{a} (0.002)	2.20 ^{b} (0.06)
3.0	0.082 ^{bc} (0.001)	170.00 ^{c} (1.00)	0.061 ^{a} (0.002)	0.19 ^{c} (0.01)
4.5	0.150 ^{b} (0.001)	218.00 ^{b} (1.53)	0.066 ^{a} (0.002)	0.02 ^{c} (0.00)
6.0	0.252 ^{a} (0.001)	287.00 ^{a} (2.52)	0.073 ^{a} (0.002)	0.01 ^{c} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 18B. The effect of crude extract of *Lactobacillus casei* W28 on growth of 12 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.043 [b] (0.001)	34.00 [c] (0.58)	0.044 [a] (0.002)	33.33 [a] (0.67)
1.5	0.065 [b] (0.003)	52.33 [c] (2.03)	0.048 [a] (0.003)	2.62 [b] (0.22)
3.0	0.022 [b] (0.003)	103.67 [b] (6.94)	0.053 [a] (0.003)	0.23 [b] (0.01)
4.5	0.129 [a] (0.001)	175.00 [a] (5.57)	0.086 [a] (0.001)	0.02 [b] (0.00)
6.0	0.131 [a] (0.001)	223.33 [a] (5.92)	0.095 [a] (0.004)	0.01 [b] (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 18C. The effect of crude extract of *Lactobacillus casei* W28 on growth of 22 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 ^{b} (0.001)	44.0 ^{de} (1.2)	0.050 ^{d} (0.001)	42.0 ^{c} (1.2)
1.5	0.050 ^{b} (0.001)	36.0 ^{e} (2.3)	0.051 ^{d} (0.001)	46.0 ^{c} (2.3)
3.0	ND †	49.7 ^{d} (2.9)	ND	53.3 ^{c} (1.7)
4.5	ND	120.7 ^{c} (4.6)	ND	123.0 ^{b} (5.0)
6.0	0.062 ^{b} (0.001)	161.0 ^{b} (1.5)	0.059 ^{c} (0.000)	161.0 ^{b} (1.5)
12.0	0.080 ^{b} (0.001)	303.0 ^{a} (2.3)	0.075 ^{b} (0.002)	302.0 ^{a} (2.5)
24.0	0.136 ^{a} (0.001)	ND	0.133 ^{a} (0.001)	ND

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
† ND, not determined.

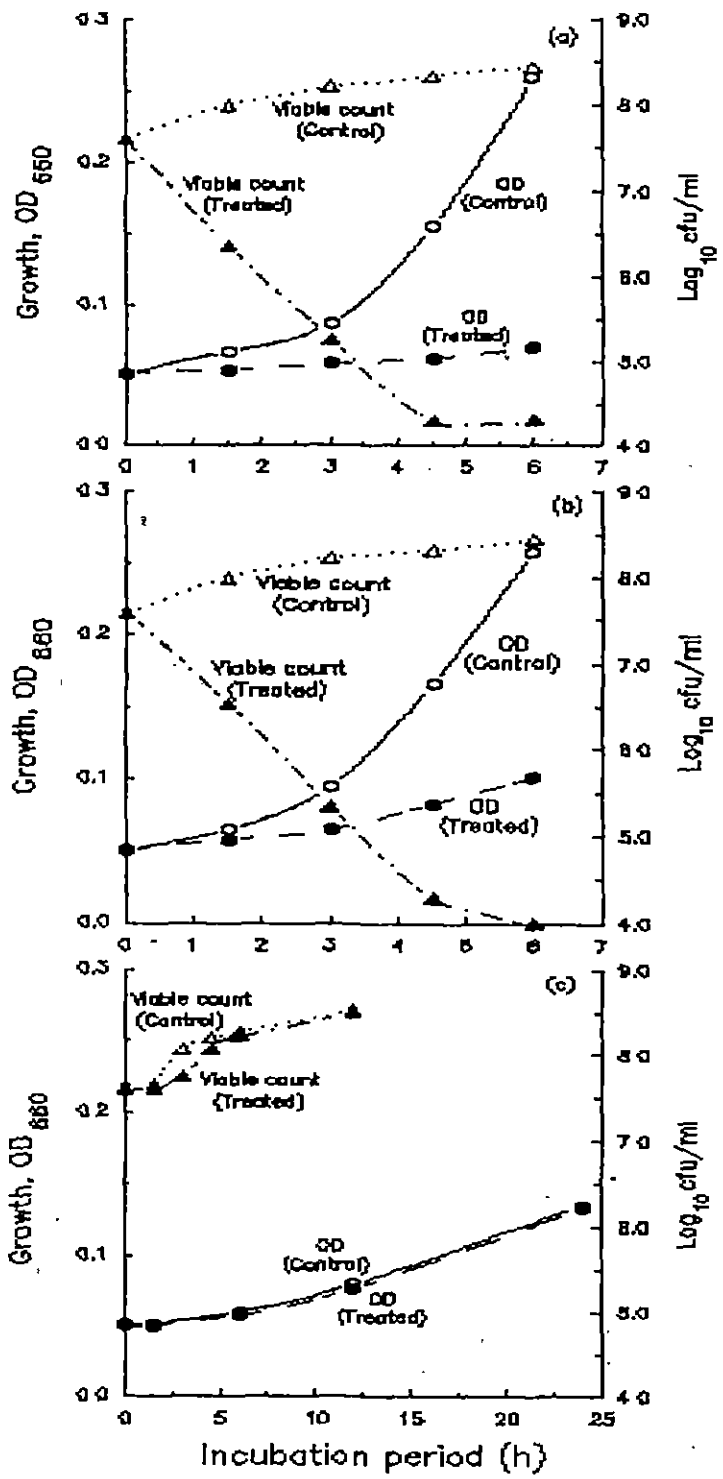

Fig. 15. The effect of crude extract of *Lactobacillus casei* W25B on growth of *Lactobacillus plantarum* GM-R1, aged 6 h (a), 12 h (b) and 22 h (c).

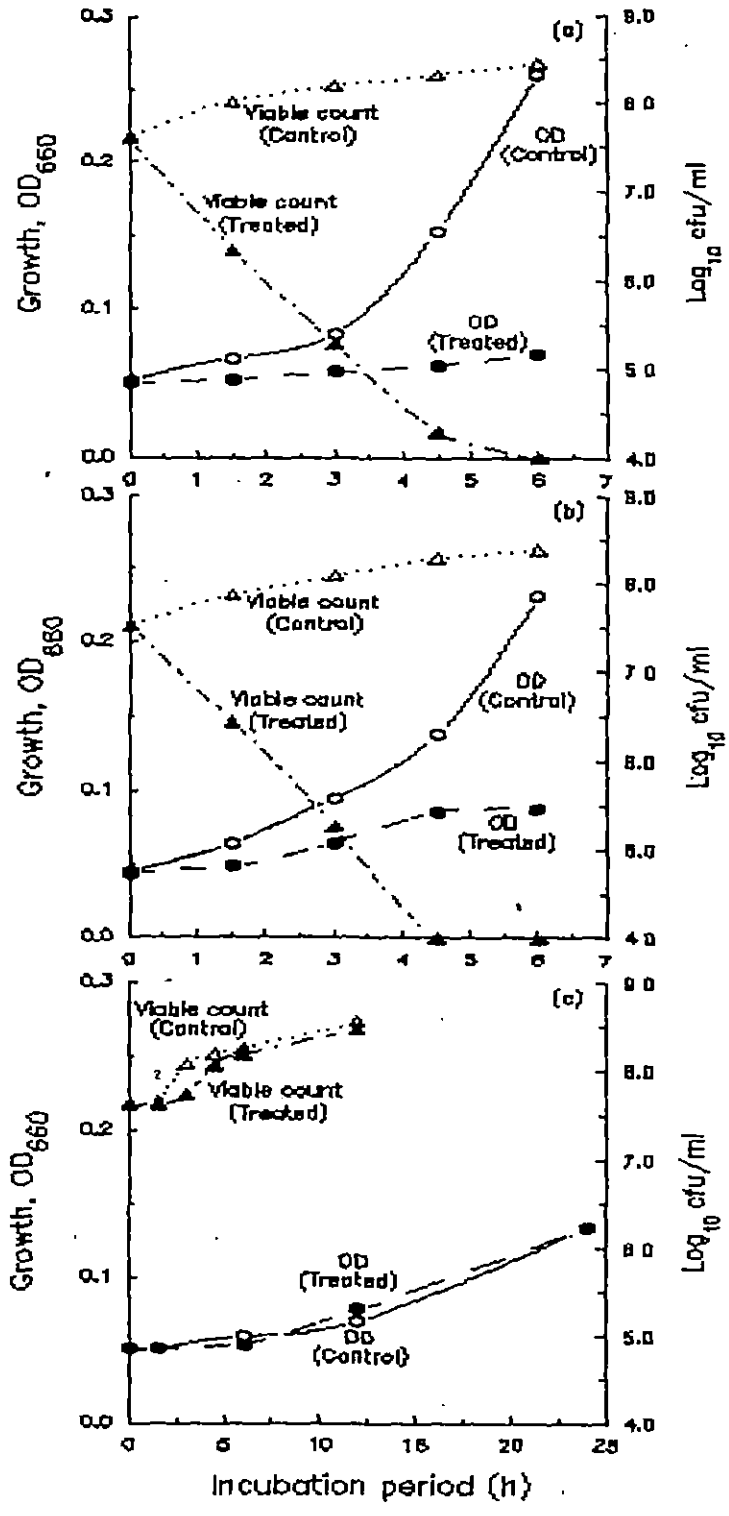

Fig. 16. The effect of crude extract of *Lactobacillus casei* W26B on growth of *Lactobacillus plantarum* GM-R1, aged 6 h (a), 12 h (b) and 22 h (c).

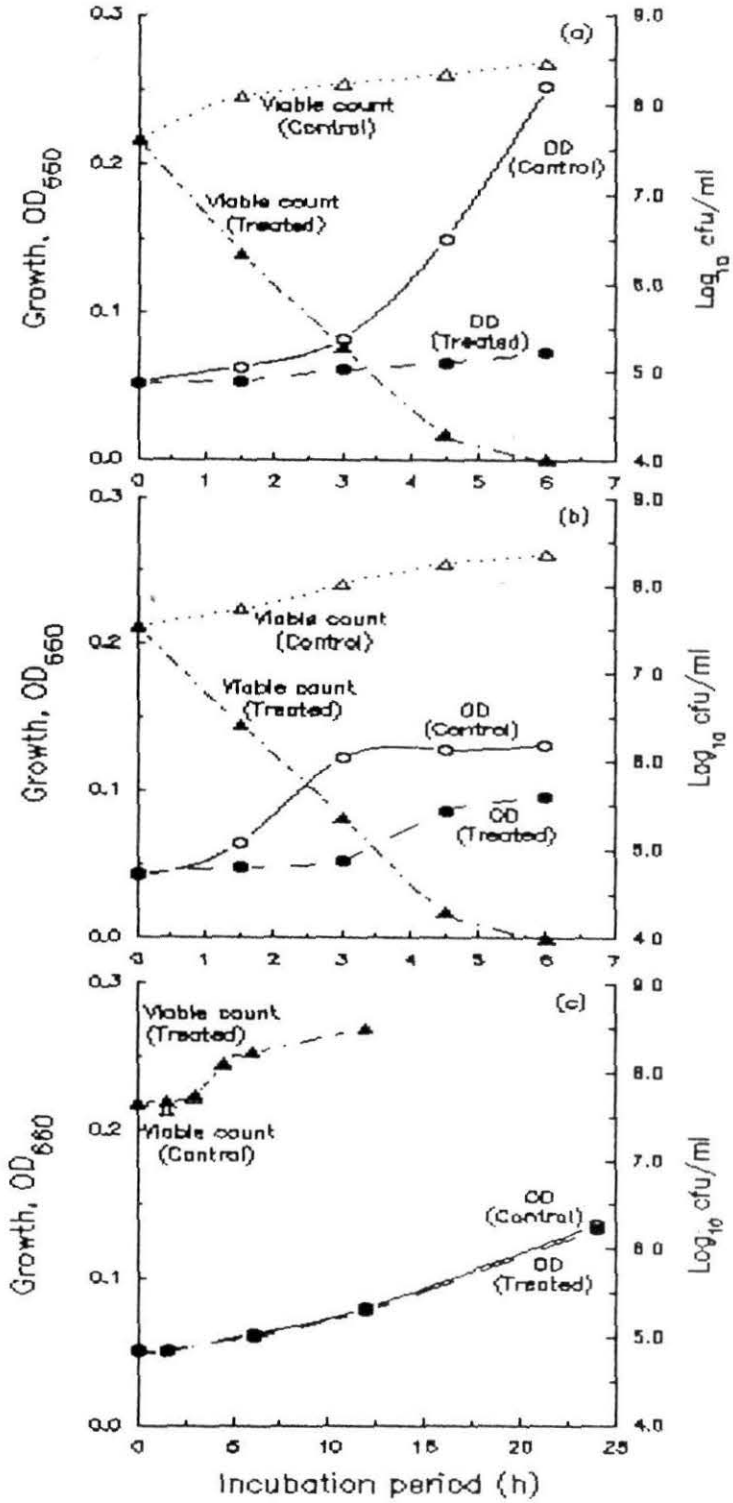

Fig. 17. The effect of crude extract of *Lactobacillus casei* W28 on growth of *Lactobacillus plantarum* GM-R1, aged 6 h (a), 12 h (b) and 22 h (c).

pronounced on logarithmic phased cells, and no action was observed on stationary phased cells.

4.9. Inhibitory spectra

Inhibitory spectra of the crude bacteriocins of the three selected strains were examined against the test organisms listed in Table 19. The bacteriocins from the strains W25B and W28 were inhibitory to four, and W26B to five of the 10 lactic acid bacteria tested. Activity was restricted to Gram positive bacteria, including *Bacillus subtilis, Bacillus cereus, Enterococcus faecium, Staphylococcus aureus* and a few actinomycetes.

Table 19. Antimicrobial spectrum of bacteriocins from the three isolated strains of *Lactobacillus casei*

Indicator strains	Source	Sensitivity† to bacteriocin activity of		
		W25B	W26B	W28
Lactic acid bacteria:				
Lactobacillus acidophilus 447	MTCC	-	-	-
Lactobacillus casei 1423	MTCC	+	+	+
Lactobacillus casei ssp. *rhamnosus*1408	MTCC		-	- -
Lactobacillus fermentum LS-MS12	NBU	-	-	-
Lactobacillus lactis 1484	MTCC	-	-	-
Lactobacillus maltaromicus 108	MTCC	-	-	-
Lactobacillus plantarum GM-R1	NBU	+	+	+
Lactobacillus plantarum LM-R1	NBU	+	+	-
Lactobacillus plantarum LS-R1	NBU	-	+	+
Lactobacillus plantarum 1325	MTCC	+	+	+
Gram positive eubacteria:				
Bacillus cereus 07M1	NBU	(+)	(+)	+
Bacillus licheniformis 04M1	NBU	-	-	+
Bacillus mycoides 06M1	NBU	-	-	-
Bacillus subtilis DK-W1	NBU	+	+	+
Bacillus subtilis var. natto	NBU	+	+	+
Bacillus subtilis 026M1	NBU	(+)	+	+
Enterococcus faecium DK-C1	NBU	(+)	(+)	+
Staphylococcus aureus 96	MTCC	+	+	+
Actinomycetes:				
Amycolata autotrophica 031M6	NBU	-	-	-
Microbispora rosea 033M6	NBU	-	-	-
Nocardia asteroides 034M6	NBU	-	-	-
Rhodococcus rhodochrous 030M6	NBU	-	-	-

Contd....

Indicator strains	Source	Sensitivity† to bacteriocin activity of		
		W25B	W26B	W28
Streptomyces coelicolor 028M6	NBU	+	+	+
Saccharopolyspora hirsuta 032M6	NBU	-	-	-
Streptosporangium roseum 029M6	NBU	(+)	(+)	+
Gram negative bacteria:				
Escherichia coli 118	MTCC	-	-	-
Escherichia coli K-12	NBU	-	-	-
Myxococcus fulvus 49305	ATCC	-	-	-
Paracoccus denitrificans 035M1	NBU	-	-	-
Pseudomonas putida 102	MTCC	-	-	-
Salmonella sp. 05M1	NBU	-	-	-
Salmonella typhimurium 98	MTCC	-	-	-
Yeast :				
Candida parasilopsis 1744	MTCC	-	-	-
Geotrichum candidum 1735	MTCC	-	-	-
Saccharomyces cerevisiae 173	MTCC	-	-	-

*MTCC, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India; NBU, Microbiology Laboratory, Department of Botany, University of North Bengal, Siliguri, India; ATCC, American Type Culture Collection, Maryland, USA.

† Symbols for degree of inhibition in well diffusion assay: + large inhibition zone (width, ≥ 3.0 mm); (+), small inhibition zone (width, <3.0 mm); -, no inhibition zone.

5

Discussion

Among the different lactic acid bacteria, lactobacilli were isolated from all of the possible sources tried. This is because lactobacilli grow in a variety of habitats, wherever high levels of sugars, peptides, amino acids, vitamins and a low oxygen tension occur. Since they are aciduric and acidophilic, different species have adapted themselves to grow under widely different environmental conditions, and their production of high levels of lactic acid lowers the pH of the substrate and suppresses many other bacteria; these features contribute to the wide distribution of lactobacilli and their successful establishment in many markedly different habitats (Sharpe 1981; Kandler and Weiss 1986). Moreover, while in cold temperate climate mesophilic bacteria such as species of *Lactococcus* and *Leuconostoc* are predominant, in regions with a hot, subtropical or tropical climate, like that in India, thermophilic bacteria which include mostly *Lactobacillus* spp. prevail (Kurmann 1984; Thomas 1985; Tamine and Robinson 1988).

The lactic acid bacterial isolates were initially screened for antagonistic effect against the growth of 19 strains of lactic acid bacteria. Only 14% of the total lactic acid bacterial isolates showed antagonistic activity in agar spot test. The cause of this antagonism was thought to be either nutrient competition or induced antagonism by producing organic acid, hydrogen peroxide and/or antimicrobial protein or peptide compounds or the action of lytic bacteriophages. Even though no zone of inhibition was obtained with most of the isolates, it can not be considered that they were incapable of forming antibacterial compounds. Use of proper target organism, pH of the medium and temperature of incubation exerts the antagonistic effect of the potential producers. The concentrated culture supernatants of the positive 24 isolates also produced inhibition zones in well diffusion test against the respective indicator strains which responded positive to the agar spot test. Of them, only 7 strains, all belonged to *Lactobacillus* were positive in well diffusion test when their concentrated culture supernatants were neutralized. Comparison of the results with unadjusted and adjusted pH suggests that the antibacterial activity of most of the isolates was due to the production of organic acids. The acids decrease the pH and make the environment antagonistic to less acid tolerant bacteria. Organic acids inhibit microorganisms by entering the cell in the undissociated form and then dissociating within the cell (Levine and Fellers 1940). This causes acidification of the cytoplasm and collapse of the proton motive force, resulting in inhibition of nutrient transport. Thus, organic acids in the medium may contribute to growth inhibition because of increased energy consumption to maintain pH homeostasis (Herrero 1983).

Hydrogen peroxide produced by lactic acid bacteria has been indentified as contributing to their inhibitory activity (Gibbs 1987). *Staphylococcus aureus* is inhibited by peroxide produced by *Lactococcus lactis* and *Lactobacillus bulgaricus* (Dahiya and Speck 1968). For determining antibacterial activity due to hydrogen peroxide, culture supernatants were treated with catalase and assayed in well diffusion test. Cell-free culture supernatants (neutralized and concentrated) of seven isolates were positive in this experiment, confirming the nature of antibacterial compound to be other than acid

and hydrogen peroxide.

The inhibition zones of these seven strains were still present when the indicator strains were applied to the opposite side of agar discs, indicating that lytic bacteriophages were not responsible for these zones, as this reverse-side technique precludes contact between bacteriophages if present in culture supernatants or indicator strains (Tagg and McGiven 1971).

However, the inhibitory effect of the cell-free culture supernatants was eliminated by proteolytic enzymes. While pepsin could inhibit the antibacterial activity in all the seven cultures, trypsin inhibited five of them. Preliminary studies indicate that the antibacterial compound produced by these seven isolates is proteinaceous in nature, which is the key character of a bacteriocin (Tagg *et al.* 1976). Hence, about 4% of the total lactic acid bacterial isolates were producers of bacteriocin-like substances, active against the indicator strains used. This is in agreement with the findings of Geis *et al.* (1983) who, in an extensive survey, obtained about 5% of the total 280 lactococcal strains isolated. Under strictly controlled screening conditions detection of bacteriocin producers is normally rare. Sometimes, not more than 1% of the strains tested have been recorded as positive (Schillinger 1990).

With the passage of time, a gradual decrease in bacteriocin titre was encountered in all the seven producing strains. This observation led us to search for spontaneous loss of bacteriocin-producing character within the populations of positive isolates. In order to regain the original titre of bacteriocinogenic principles, the Bac$^-$ variants were eliminated by applying replica plating technique time to time. Because of this autocuring property, it is assumed that these bacteriocins are encoded by plasmids. Loss of bacteriocin plasmid in several strains frequently produce variants that results in loss of production (Deschel and Klaenhammer 1985; Ray *et al.* 1989; Hastings and Stiles 1991; van Belkum *et al.* 1992; Noerlis and Ray 1994).

Although factors affecting the production of the peptide antibiotic nisin have been studied, information regarding those affecting the production of other bacteriocins from lactic acid bacteria is scarce (Parente and Hill 1992). Limited studies are available on the effect of some media ingredients (Biswas *et al.* 1991) or on comparison of bacteriocin production in different media (Geis *et al.* 1983). Comparisons of bacteriocin production in different media, commonly used for the growth and production of bacteriocins, showed varied levels of bacteriocin titres. The media generally used for growth of lactic acid bacteria have a high peptone content which poses subsequent purification of bacteriocins difficult. Therefore, the objective of this study was to select a medium that would allow a maximum bacteriocin production with a minimum input of peptone. SD-MRS broth was found to be most suitable in all respects for further studies. Peptone is completely absent in this medium; instead casein acid hydrolysate (a pancreatic digest of casein) and yeast extract provide the organic nitrogen source. This may be particularly advantageous in media for bacteriocin production because most

of the organic nitrogen is in peptides or amino acids with molecular weight lower than 1 kDa (Bridson 1990) which makes them suitable to be dialyzed out during purification of bacteriocin. On the other hand, MRS, APT, tomato juice medium (TJM) and MRS-0.2 broths have a high content of peptone. The SD-MRS broth was used for the production of lactacin B (Barefoot and Klaenhammer 1984) and helveticin J (Joerger and Klaenhammer 1986). In comparison with the MRS broth and TJM the sugar content of the SD-MRS broth is low which ensures low levels of acid production. Another merit in using this medium is that the interference of colour in the diffusing broth to the zone of inhibition was markedly less in this medium, producing a zone with better contrast against the surrounding.

Effect of pH of the medium on bacteriocin production is well documented (Goebel *et al.* 1955; Joerger and Klaenhammer 1986). Production of bacteriocins in this study as well was dependent on initial medium pH, suggesting that manipulation of pH in broth might facilitate their production. In general, a high amount of bacteriocin titre was achieved when the initial pH of the medium was adjusted to 5.5-6.4, corresponding to a high cell mass. However at initial pH levels higher than this, both the growth of producer strains and the bacteriocin titres were adversely and irreversibly affected. This phenomenon is most likely due to the acidophilic nature of the microorganism, as lactobacilli tend to be inhibited by alkaline or neutral medium (Kandler and Weiss 1986). The high titre of bacteriocin at pH 5.0-6.4 may be partly attributed to its stability at lower pH values, because of its adsorption to the cells of the producing organisms, possibly followed by enzymatic digestion either in the cell or medium at this specific pH.

The producer strains showed optimal growth at 32°C, generation time being 3 h 55 min. In SD-MRS broth having pH 6.2, the period of incubation at 32°C was optimized. The excretion of bacteriocin in MRS broth was dependent on the phase of bacterial growth.

The bacteriocins were produced entirely during the late logarithmic phase. Production started after 6 h of incubation at 32°C in SD-MRS broth and reached the maximum level at 24 h. Further incubation did not increase the bacteriocin titre. The bacteriocins were produced continuously when nutrients were available for metabolic activity. Incubation for periods beyond 2 d yielded low bacteriocin titres, which may be attributed to proteolytic degradation, like most bacteriocins which show a more or less sharp decrease in activity at the end of growth (Barefoot and Klaenhammer 1984; Joerger and Klaenhammer 1986; Piard *et al.* 1990; Parente and Hill 1992; Daba *et al.* 1993; Parente and Ricciardi 1994; Parente *et al.* 1994). Production occured over the entire pH range, which lactobacilli normally encounter (4.0-6.5).

Since during purification the antibacterial compounds need to be exposed to various conditions, it was essential to determine the stability against pH, temperature and organic solvents of the crude extracts. The bacteriocins were found to be heat-stable;

there was no loss of activity even after autoclaving at 121°C for 15 min. In this respect, these bacteriocins resemble brevicin 37, nisin, lactacin F and lactocin 27. The bacteriocins of W25B, W26B and W28 were stable at pH 2.0-8.0 for 24 h at 4°C. Readjustment of samples at pH 9-11 back to pH 6.5 failed to restore activity.

The bacteriocins remained active after high temperature treatment and at a relatively low pH. This is similar to the properties reported for other bacteriocins, such as nisin (Bailey and Hurst 1971), lactocin 22 (Upreti and Hinsdill 1973, 1975), acidolin (Hamdan and Mikolazcik 1974), diplococcin (Davey and Richardson 1981), pediocin A (Daeschel and Klaenhammer 1985) and pediocin PA-1 (Gonzales and Kunka 1987).

Treatment with most organic solvents did not cause any loss of activity, probably because of absence of lipid moieties in the molecule. However, on treatment with n-butanol, a part of the activity was recovered in the organic phase. Complete recovery of the bacteriocin activity was not possible as in acidocin B (ten Brink *et al.*1994).

Since it was not possible to obtain sufficiently concentrated source of bacteriocin preparation, large initial volumes of the culture supernatants were concentrated. Lyophilization proved to be the best method of concentration. Freeze-dried culture supernatants, dissolved in small volume of distilled water, were used as the source of bacteriocin purification. Initial attempts to purify the active fraction by dialysis using tubings with molecular exclusion limits of 12 kDa failed, because the membrane was incapable of retaing the active fraction. On concentrating the dialysate, activity was found. This activity was lost when treated with proteolytic enzymes trypsin and pepsin, confirming the size of the bacteriocins as less than 12 kDa. It was mainly due to this reason that ammonium sulphate precipitation was not attempted as the active fraction was lost while removing the ammonium sulphate by dialysis. Dialysis using a tubing with molecular exclusion limits of 3.5 kDa retained the active fraction, suggesting the molecular weight of the active fraction to be between 3.5 and 12 kDa. Since *Lb. casei* W28 produced high bacteriocin titres in comparison to the other producer strains, purification was attempted only with this strain. Great difficulties were encountered in purifying this bacteriocin further. One of the reasons was the production of bacteriocin in low amounts ($\leq$1.7 mg/l) that occured during each purification step employed. This is not unique, for otner authors have similar problems with bacteriocins from Gram positive organisms (Sahl and Brandis 1981; Barefoot and Klaenhammer 1984; Rammelsberg and Radler 1990). The antimicrobial preparation was 292-fold purified after passage through gel filtration column followed by elution from an anion-exchange and desalting columns. In spite of low (4%) activity recovery following these methods, there was a marked increase in the specific activity (666.67 AU/mg). Though cation-exchange chromatography has been used extensively for bacteriocin preparation (Holo *et al.* 1991; Mortvedt *et al.* 1991b; Stoffels *et al.* 1992), successful application on an anion-exchanger has also been reported (Bhunia *et al.* 1988). Substantial losses during purification by ion-exchange chromatography have been reported; 97.5% and 97% of

the activity were lost during ion-exchange purification of sakacin B and lactacin B, respectively (Barefoot and Klaenhammer 1984, Samelis *et al.* 1994). With the semipurified preparation obtained, some characteristics of the antibacterial activity, previously established with the crude extract were confirmed. Since Exocellulose GF-5 has an exclusion limit of 5 kDa, the molecular weight of *Lb. casei* W28 bacteriocin is greater than 5 kDa.

Limited information is available on the mechanism of action of bacteriocins of the genus *Lactobacillus*. Information regarding the kinetics and mechanism of lethal action of bacteriocins is primarily based on studies of colicins and bacteriocins produced by lactococci. A widely accepted hypothesis is that the mode of action occurs in two steps. In the first step, the bacteriocin is adsorbed to specific receptors on the cell surface and, after a time the second develops which results in cell death. Bacteriocins differ in their lethal action and can initiate reactions which inhibit energy production, synthesis of protein or nucleic acids or alter membrane permeability and transport. Cell death with lysis or leakage of cell membrane is associated with the drop in optical density of the cell suspension and decrease in viable count (Piard *et al.* 1990). Exposure of *Lb. plantarum* GMR1 to active cell-free culture supernatant of the producer strains resulted in a 91-92% decrease after 1.5 h and 100% decrease after 4.5 h in the number of viable cells. This suggests that these bacteriocins are bactericidal, since the affected indicator cells could not revive after withdrawl of bacteriocins. The bactericidal mode of action and the proteinaceous nature of the substance are typical characteristics of a bacteriocin (Tagg *et al.* 1976). No change in the optical density was observed in the control and treated sets. Optical density in the treated set remained constant throughout. Therefore, it may be concluded that cell death occured due to reasons other than lysis or leakage of cellular materials through the cell membrane. The results were in agreement with the findings of Barefoot and Klaenhammer (1984) for salivaricin B, Joerger and Klaenhammer (1986) for helveticin J, Bhunia *et al.* (1988) for pediocin AcH, Piard *et al.* (1990) for lactacin 481, Samelis *et al.* (1994) for sakacin B and ten Brink *et al.* (1994) for acidocin B.

The physiological state of the indicator cells has been shown to have a strong influence on susceptibility of the lethal action of a bacteriocin. Decrease in the number of viable cells to 99% within 1.5 h was achieved only when the target cells were in the logarithmic phase of growth. No action of the bacteriocin preparation was found when the target cells were in stationary phase. Similar results were obtained by Vaughan *et al.* (1992) on the mode of action studies of helveticin V-1829. According to Tagg *et al.* (1976), actively multiplying cells are most sensitive to bacteriocin action.

The three bacteriocins from *Lb. casei* inhibited only strains of *Lb. casei* and *Lb. plantarum* among all the lactic acid bacteria tested. In most cases, the activity of the three bacteriocins was strain specific rather than species specific. The range of their inhibitory activity was broader than that reported for antimicrobial compounds produced

by many other lactic acid bacteria. Though bacteriocins found in lactobacilli display inhibitory activity within Lactobacillaceae (Klaenhammer 1988), *Lactobacillus* bacteriocins showing a broad activity spectrum have been reported for salivaricin B and sakacin A (Schillinger and Lücke 1989, ten Brink *et al.* 1994). Inhibition was scored positive against a few Gram positive bacteria which included strains of *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus* and *Enterococcus faecium. Bacillus licheniformis* was inhibited by one bacteriocin . All the three bacteriocins showed activity against the two actinomycetes, *Streptomyces coelicolor* and *Streptosporangium roseum.* None of the Gram negative bacteria and yeasts was susceptible to any of the three bacteriocins, sharing the property with other lactic acid bacterial bacteriocins. Comparisons of the antimicrobial spectra of the culture supernatants suggest that the inhibitory compounds are not identical. Bacteriocins produced from six strains of *Lb. sake* have been distinguished on the antibacterial spectra of the culture supernatant (Schillinger and Lücke 1989).

There is report of just one bacteriocin (caseicin 80) from *Lb. casei.* (Rammelsberg *et al.* 1990; Mueller and Radler 1993). A closer comparison of the physical properties of *Lb. casei* W28 bacteriocin and caseicin 80 show several differences. Whereas caseicin 80 is a large compound (42 kDa) thermolabile (20 min at 100°C) molecule, *Lb. casei* W28 bacteriocin is a low molecular weight (5-12 kDa) compound and heat-stable (15 min at 121°C). Studies on production of bacteriocin show that *Lb. casei* W28 bacteriocin is produced in the late logarithmic phase of growth, whereas caseicin 80 is produced usually after 3 d at 30°C, when the cells reach stationary phase. Another dissimilarity is the time taken for killing target cells; caseicin 80 requires days to demonstrate its activity on slow-growing indicator, *Lb. casei* B109 (Rammelsberg and Radler 1990), whereas *Lb. casei* W28 bacteriocin shows an irreversible bactericidal effect, exhibiting 99% killing within 1.5 h.

Because of these unique characteristics, the bacteriocin *Lb. casei* W28 is designated as caseicin-W which is different from the bacteriocins hitherto known.

In view of the interesting inhibition spectrum of caseicin-W and its technological properties (good pH, temperature-and storage stability and its inactivation by digestive proteases such as pepsin and trypsin), this novel bacteriocin has an application potential as a safe biopreservative.

6

Summary

A total of 171 isolates of lactic acid bacteria belonging to the genera, *Lactobacillus* (106), *Lactococcus* (53), *Leuconostoc* (6) and *Pediococcus* (6) were obtained from different sources including curd, chhana (acid-and-heat coagulated milk product), cheese, whey, rotten vegetables, putrid meat, putrid fish and silage. Only 24 of them were inhibitory against 19 strains of lactic acid bacteria as evaluated by agar spot test and well diffusion assay. Under conditions eliminating the effect of organic acids, hydrogen peroxide and bacteriophages, seven isolates of *Lactobacillus* showed antibacterial activity due to the exocellular production of bacteriocins, because this activity was lost when the culture supernatants were treated with proteolytic enzymes. An attempt was made to characterize crude bacteriocins and optimize their production with three producer strains of *Lactobacillus casei*. All the three bacteriocins were remarkably heat-stable and active over a wide range of pH. Treatment with many organic solvents did not cause any loss of activity. Comparisons between different growth media showed that semi-defined (SD)-MRS broth was most suitable for bacteriocin production. Maximum bacteriocin production was detected when the culture had passed the logarithmic phase of growth and when the culture was grown in SD-MRS broth with an initial pH of 6.5 at 32°C. No bacteriocin was produced at pH 8.0. Antibacterial activity was most pronounced on indicator cells at their logarithmic phase of growth, with 99% killing within 1.5 h. There was no activity on indicator cells which reached stationary phase of growth. The bacteriocins showed a bactericidal mode of action on indicator cells without causing cell lysis. The range of inhibitory activity was moderate, including a few food-borne pathogens such as *Bacillus cereus, Staphylococcus aureus* and *Enterococcus faecium*. Partial purification was achieved for *Lb. casei* W28 bacteriocin using gel filtration and anion-exchange chromatography. Molecular weight of the partially purified *Lb. casei* W28 bacteriocin, designated caseicin-W, was between 5 and 12 kDa.

7

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