

**EFFECT OF NISIN ON THE BEHAVIOUR AND
ULTRASTRUCTURE OF SELECTED LACTIC
AND NON-LACTIC MICRO-ORGANISMS**

**THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY**

**BY:
NARESH KUMAR**

**DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)**

KARNAL -132001(HARYANA), INDIA

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DEDICATED
TO
MY INTERNAL CONSCIOUSNESS
AND ITS CREATOR

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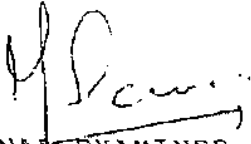
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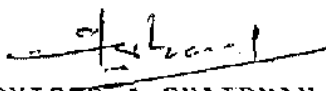
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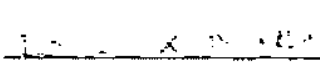
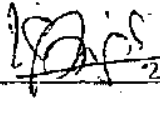
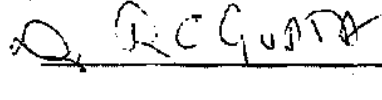
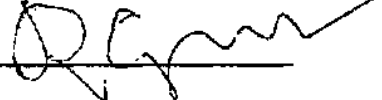
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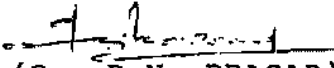
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This is to certify that the thesis entitled EFFECT OF NISIN ON THE BEHAVIOUR AND ULTRASTRUCTURE OF SELECTED LACTIC AND NON-LACTIC MICRO-ORGANISMS submitted by Mr. NARESH KUMAR in partial fulfilment of the requirement for the award of DEGREE OF DOCTOR OF PHILOSOPHY IN DAIRY MICROBIOLOGY of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.


(Dr. D.N. PRASAD)
MAJOR ADVISOR & CHAIRMAN
(GUIDE)

Dated: 30/12/93

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ABSTRACT

Nisin is a bacteriocin produced by some strains of *Lactococcus lactis* subsp. *lactis*. It inhibits most of the Gram positive bacteria, however, Gram negative, yeast and molds are not affected by nisin. Nisin has wide spread applications in canned foods, processed cheese, milk and milk products. Twenty six strains of Gram positive bacteria comprising of both lactic and non-lactics showed a wide variation in nisin sensitivity in broth and skim milk at genera, species and strains levels. *Lactococcus lactis* subsp. *cremoris* (C₃), *L. plantarum* (R) and *B. subtilis* (9144) were found to be most resistant strains with MIC of 175, 100 and 150 RU nisin/ml in broth respectively. On the other hand, *L. lactis* subsp. *diacetylactis* (ERC₁), *L. plantarum* (89) and *B. stearothermophilus* (37) were inhibited at very low levels of 5, 20 and 0.5 RU nisin/ml respectively. Activity of nisin was reduced significantly at higher pH, temperature, inoculum and incubation periods.

Mode of action of nisin appears to be non-specific as clearly revealed by light and electron microscopy. The various changes includes, clumping or aggregation, pore and bleb formation, acute perforation, rupturing of cell and subsequent release of protoplasmic contents and complete dis-integration can be correlated with bacteriostatic and bacteriocidal action of nisin.

Encouraging results of nisin addition in commercial pasteurised toned milk and Lassi were obtained. The increase in shelf-life of pasteurised milk containing 500 RU nisin/ml by 2½ days at 20°C can be of great significance especially in tropical countries like ours, where facilities for cold storage, transportation and distribution are inadequate. Nisin was found effective in controlling the excess of acid production in Lassi during storage and considerably improve the product acceptability.

The nisin resistant strains evaluated in the present study can find immense practical applications in dairy industry. The precise information on nisin sensitivity of lactic cultures will undoubtedly be very useful in enhancing the shelf-life and controlling the desirable qualities of fermented and non-fermented milk and milk products.

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

Nisin is a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*. It has wide spectrum of antibacterial activity and inhibits most of the Gram positive bacteria. The Gram-negative bacteria, yeast and molds are, however, not affected by nisin. The inhibitory effect of nisin was noticed about sixty years ago, when inhibitory streptococci were considered to be a problem in cheese making because of slow acid development.

The different groups among Gram positive bacteria i.e. *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Micrococcus*, *Staphylococcus*, *Listeria* and majority of spore forming species of *Bacillus* and *Clostridium* differ widely in their response towards nisin and depicts considerable variation in nisin tolerance at genera, species and strains level. The inhibitory action of nisin is greatly influenced by physical, chemical and microbiological environment in different kinds of food. The factors like pH, temperature, chemical composition, age, size of inocula and incubation periods have significant effect on efficacy of nisin as substantiated by comprehensive information in the present study.

A number of studies have been carried out on mode of action of nisin against susceptible vegetative cells and spores. The research on vegetative cells indicate that the initial point of action of nisin is the cytoplasmic membrane and subsequently undergoes disruption either, resulting in leakage of essential cellular material such as

adenosine triphosphate (ATP) or in more severe cases lysis of cells. Attempts were made in the present study to confirm the above biochemical observations through Scanning Electron Microscopy in terms of surface damage among different groups of Gram positive bacteria.

Nisin has received considerable attention regarding its application in food preservation. It is non-toxic as revealed by toxicological study and is being digested by pancreatic enzymes. Joint Food and Agricultural Organisation/World Health Organisation (FAO/WHO) has recommended the maximum acceptable daily intake (ADI) as 33,000 IU/kg of body weight. However, the available evidence indicate that a level of 33,000,000 Units nisin/kg of body weight has no adverse effect.

It has been successfully used in the preservation of number of thermally processed foods without increasing the risk of bacteriological spoilage. In fact, nisin was first used to control the blowing, oftenly caused by spores of anaerobic clostridia in processed cheese and cheese spreads. Presently, in certain parts of world, nisin has been used in preservation of canned foods, flavoured milk drinks, sterilised milk,, canned evaporated milk and other dairy products. The shelf-life of pasteurised whole milk having very low keeping quality can also be increased by application of nisin as evident from the present findings.

Nisin has great significance in industrial fermentation processes like wine, beer, ethyl alcohol, where lactic acid bacteria belonging to genus *Lactobacillus*, *Pediococcus* and *Leuconostoc* are potential contaminants and affects the yield and quality of the products. Nisin may prove successful to prevent lactic contaminants without affecting the growth of yeasts and molds.

In developing countries like, India, where facilities for cold storage, transportation and distribution are not adequate, nisin may be an invaluable preservative for precisely controlling the growth of lactic acid bacteria in fermented dairy products like Yoghurt, Dahi and Lassi etc. and therefore, the acid production can be controlled to an acceptable level.

The present project was successfully carried out with the following objectives:-

1. Comparative Nisin sensitivity of selected lactic and non-lactic cultures in broth and skim milk system at genera, species and strains level.
2. Factors affecting the inhibitory action of Nisin like pH, temperature, size of inoculum and incubation period.
3. Scanning Electron Microscopy (SEM) of sensitive and resistant strains among lactic and non-lactic cultures to study the nisin induced ultrastructural changes.
4. Preservative action of nisin on shelf-life of Pasteurised milk and Lassi at different temperatures and storage periods.

CHAPTER 2

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

2.1 BACTERIOCINS OF LACTIC ACID BACTERIA

Bacteriocins are proteins in nature and their action is bacteriocidal towards bacteria taxonomically close to the producer (Tagg et al., 1976). These were classified into 2 groups on the basis of their antibacterial spectrum of activity. One included the bacteriocins active against taxonomically close to the producer and other bacteriocins with a relatively broad spectrum (nisin and pediocins) of activity against Gram positive bacteria (Klaenhammer, 1988). Bacteriocins do not respond to well defined criteria, rather their biochemical properties, molecular weight, spectrum of activity, mode of action and genetic support are very heterogenous (Reeves, 1972 and Tagg et al., 1976). The study of bacteriocins in lactic acid bacteria has been actively developed over the past few years (Klaenhammer, 1988 and Schillinger, 1990) and these investigations have shown that bacteriocin production is an extensively prevalent phenotype in this group of bacteria.

The best known and studied bacteriocin produced by lactic acid bacteria is nisin. The initial interest in this substance arose from its bacteriocidal activity against spores of bacilli and clostridia, responsible for most of the spoilage in canned foods (Hurst, 1978; Somers and Taylor, 1987). Nisin is an inhibitory substance produced

by certain strains of *Lactococcus lactis* subsp. *lactis* and like other bacteriocins, possesses antimicrobial activity against a limited range of micro-organisms. It does not inhibit Gram negative bacteria, yeasts and fungi. Nisin does inhibit a wide range of Gram positive bacteria i.e. species of *Lactococcus*, *Streptococcus*, *Micrococcus*, *Lactobacillus*, *Staphylococcus* and majority of spore forming species of *Clostridium* and *Bacillus*, the spore being more sensitive than the vegetative cells. Recent studies have shown that strains of the food pathogen, *Listeria monocytogenes* are also sensitive to nisin (Harris et al., 1991).

2.2 NISIN SENSITIVITY OF LACTIC AND NON-LACTIC CULTURES IN BROTH

2.2.1 Lactic Cultures

Inhibition of lactic acid bacteria by a metabolite produced by one of the group members is detrimental type of antibiosis i.e. preventing the growth of desirable lactic organisms. This type of antibiosis generally cause huge losses to dairy industry associated with fermentation. However, in some fermented products, use of nisin is beneficial as it controls the acid production to acceptable levels.

2.2.1.1 Non-Nisin producers

Studies carried out by brewing Research Foundation in U.K. demonstrated that lactobacilli and pediococci, the principal spoilage bacteria of spirit like fruit brandies, were sensitive to nisin at 100 IU/ml in broth, under the laboratory and brewing situation (Ogden and Tubb, 1985). In a medium inoculated with approx. 10^5 cells/ml of a sensitive strain of *Lactobacillus* (BSO 375) and nisin added at level

recommended for commercial use (100 IU/ml) killed all the cells in 6 h. In control samples, this inoculum grew to a concentration of 10^{10} cells/ml in about 50h (Ogden, 1986). Controlling the contaminating bacteria by nisin addition to the fermenters is also an alternative method to acid washing which is known to effect yeast viability (Ogden, 1986) .

Nisin sensitivity of 30 strains of lactic acid bacteria isolated from cured and fermented meat products was carried out by Collins-Thompson et al. (1985). The maximum concentration of nisin permitting growth for 20 strains of lactic acid bacteria including *Lactococcus lactis*, *Lactobacillus plantarum* and *L. viridescens* was 50 IU/ml in MRS broth at 30°C and 24 h of incubation. A wide variation in nisin sensitivity among *L. brevis* strains was observed with MIC ranged from 10-200 IU nisin/ml.

Growth of *Streptococcus thermophilus* (ATCC 10987) was completely inhibited in MRS broth containing 200 IU nisin/ml (Lee and Kim, 1985). Other test organisms i.e. *L. bulgaricus*, *L. casei*, *L. plantarum*, *L. helveticus* and *L. acidophilus* were also inhibited at the same nisin concentration. Orberg and Sandine (1985) evaluated 26 strains of *S. cremoris* and 12 strains of *S. lactis* using Bauer-Kirby disk susceptibility test. *Streptococcus lactis* (ATCC 7962) was found most resistant with MIC atleast 40-fold higher (64 µg/ml) than other strains. This strain was potent nisin producer.

The inhibition of 31 strains of 11 species of lactic acid bacteria in M-17 broth was observed by Bossi et al. (1988) at very low nisin concentration i.e. 5 IU/ml, yet with significant difference in nisin sensitivity among the strains. Streptococci were found more nisin

tolerant than lactobacilli. A stimulating effect at nisin dose of 0.5 RU/ml was observed on some strains of *L. fermentum*. Nisin resistant strains among *L. helveticus* and *L. lactis* sp. were also discovered. A comprehensive information on nisin sensitivity of lactic and non-lactic micro-organisms has been reviewed by Gupta and Prasad (1989a). Eighteen strains of lactic acid bacteria were evaluated by Garg (1992) for nisin resistance in M-17 lactose broth at 32°C and 2% inoculum. *Lactococcus lactis* subsp. *diacetylactis* (DRC₁ and DRC₂) strains were completely inhibited at 100 RU nisin/ml, whereas in *Lactococcus* and *Lactobacillus* sp. slight growth was detected at same nisin concentration. Recently, Kumar and Prasad (1992a,b) reported the nisin sensitivity of 16 strains of *Lactococcus*, *Streptococcus* and *Lactobacillus* sp. in Elliker's and MRS broth with MIC ranged from 5-175 and 20-100 RU nisin/ml respectively at 1% inoculum and 24 h of incubation period.

2.2.1.2 Nisin-producers

Effect of secondary metabolites added to cultures of the organisms producing them was investigated by Hurst and Kruse (1972). Nisin was added to a growing culture of nisin-producing strain i.e. *Streptococcus lactis* (354/07) and observed that one quarter (12 µg/ml) of the amount of nisin formed by the cultures of *S. lactis* inhibited any lysed lag and log phase cells respectively. The same amount of nisin added before inoculation or at a time when nisin synthesis had started (in late log phase), caused only transient delay in growth.

In a similar study, Egorov et al. (1976) further substantiated the above findings and reported that addition of 100, 250, 500 and 1000

RU nisin/ml had no effect on the growth of *S. lactis* (MGU) strain. However, 1500 or 2000 units of nisin/ml inhibited growth during the first 24 h but thereafter, biomass increased at the control rate, or even exceeded it. Addition of 16000 units of nisin/ml increased the lag phase to 2 days but later growth was reduced by only 30%. Nisin synthesis by the culture was increasingly reduced with the increase in nisin addition.

The maximum production of nisin by nisin producing strain of *S. lactis* (MGU) occurred after 9 h at 28°C in a glucose yeast medium (Egorov et al., 1982). Further incubation resulted in inactivation of nisin, slowly if acidity was allowed to develop naturally (pH 4.5) and rapidly if pH was artificially maintained at 6.7. It is concluded from preliminary findings that the inactivation was of enzymatic nature.

2.2.1.3 Nisinase producers

Nisin is not inactivated by all the proteolytic digestive enzymes. Trypsin, elastase, carboxypeptidase⁻A, pepsin and erepsin were without effect (Jarvis and Mahoney, 1969). Both pancreatin and α -chymotrypsin inactivated nisin. Kooy (1952) reported the appearance of a nisin destroying enzyme Nisinase from *Lactobacillus plantarum*. Fifty percent of streptococci isolated from raw milk were able to destroy nisin (Galesloot, 1956). *Streptococcus lactis* was more active in this respect than *S. cremoris*. Alifax and Chevalier (1962) partially purified nisinase from the most active strain of *S. thermophilus* (TJ) which inactivated nisin but not other antibiotic including subtilin. Collins-Thompson et al. (1985) observed weak nisinase activity in most nisin tolerant strain of *Lactobacillus brevis*.

2.2.2 Non-lactic Cultures

Adequately heat processed canned foods which are regarded as commercially sterile may contain spore bearing rods (aero-anaerobic) and the spoilage like sweet curdling, bitterness or flat sour during storage commonly appears to be due to viable spores of *Bacillus subtilis*, *B. cereus*, *B. coagulans*, *B. megaterium* and *B. calidolactis*. Certain species of this group i.e. *B. cereus* may also cause food poisoning and intoxication, when survived the high heat treatments. The nisin was found to be effective against Bacilli and Clostridia (Mattick and Hirsch, 1947; Campbell and O'Brien, 1955) and therefore, these spore formers can be controlled by addition of nisin.

2.2.2.1 Bacilli

The inhibition of *B. coagulans* strains at 5.0 µg nisin/ml in basamin and tomato juice was observed by Campbell and Sniff (1959). The inhibitory action was significantly higher in basamin broth where 61 and 91% of the total 31 strains were inhibited at nisin dose of 0.1 and 1.0 µg/ml, respectively, whereas in tomato juice 12 and 61% inhibition was observed under similar conditions.

Denny et al. (1961) studied the effect of nisin on *B. stearothermophilus* (1518) strain. The inhibition in tryptone yeast extract broth was observed at 25 ppm of nisin. In a similar study, Jarvis (1967) observed very low sensitivity of *B. stearothermophilus* (NCIB 8157 and 8224) strains with MIC of 3 and 6 RU nisin/ml respectively.

The effect of nisin on growth pattern of *B. cereus* (NCIB 3329), *B. polymyxa* and *B. subtilis* (NCIB 8057) was demonstrated by Jarvis (1967). The addition of nisin to cultures in lag phase resulted in a

prolongation of that phase, while, in logarithmic phase caused rapid lysis of the organisms and introduced a prolonged secondary lag phase. The degree of lysis and the duration of the secondary lag phase were dependent upon the concentration of nisin added. When nisin was added at the end of logarithmic phase, little changes occurred either in the concentration of the organisms or in the rate of the growth.

The nisin sensitivity of *B. cereus* and *B. subtilis* was also studied by Jarvis (1967). The inhibition of *B. subtilis* (NCIB 8739 and 8057) strains was observed at 75 and more than 100 RU nisin/ml respectively, whereas *B. cereus* (NCIB 3329), was inhibited at more than 100 RU nisin/ml. Nisin was found most effective in inhibiting exponential growth phase of a steady state growing culture of *B. cereus* at 20°C and in presence of 20-100 mg nisin/ml (Shehata and Hassan, 1981). The complete inhibition of growth of *B. subtilis*, *B. megaterium* and *B. coagulans* strains was demonstrated by Lee and Kim (1985) at nisin dose of 200 IU/ml in broth.

Some *Bacillus* sp. were reported to produce nisinase which inactivated both nisin and subtilin (Jarvis, 1967). This enzyme reduced the C-terminal dehydroalanyl-lysine of nisin and was active on nisin A, B, C and E but not on D (Jarvis, 1970; Jarvis and Farr, 1971).

Effect of nisin sodium chloride interactions on the outgrowth of *B. licheniformis* spores was studied by Bell and De-Lacy, K.M. (1985) and observed that salt appears to antagonise the sporocidal action of nisin by interfering with nisin adsorption onto the spore. The complete inhibition of spore outgrowth was observed at 100 IU/ml of nisin whereas, detectable outgrowth was observed when 3% salt was incorporated along with 100 IU nisin/ml in growth medium.

Recently, in 1992b, Kumar and Prasad observed that among 8 strains of bacilli, *B. stearothermophilus* (37) and *B. subtilis* (9144) were the most sensitive and resistant strains with MIC of 0.5 and 150 IU nisin/ml in broth respectively at 1% inoculum and 24 h of incubation.

2.2.2.2 Clostridia

Considerable spoilage may occur when canned foods infected with *Clostridium* sp. or related strains are exposed to hot climates and stored there. Damage and loss as a result of hard swells due to gaseous fermentation by *Cl. thermosaccharolyticum* and *Cl. pasteurianum* present a world wide problem and cause serious damages. Spoilage resulting in profuse gas production and sometimes putrefactive digestion of the product can lead to various economic losses to dairy and other food industries. Nisin was found to be effective for controlling clostridial spoilage in these products. Ramseier, (1960) demonstrated the bactericidal effect of nisin on spores and vegetative cells of *Cl. butyricum*. The lytic effect and adsorption was maximum at pH 6.5 to 6.8. The sensitivities of proteolytic and non-proteolytic *Cl. botulinum* strains to nisin and other bacteriocins were investigated in tryptose peptone yeast extract glucose broth by Montville et al. (1992) and statistically different nisin sensitivities among 18 strains of *Cl. botulinum* were observed. However, these differences were not biotype associated. *Clostridium botulinum* (169 and 56A) strains were inhibited at nisin dose of 10 and 2500 IU/ml after 30 days of incubation. However, complete inhibition was observed at 10,000 IU/ml.

2.2.2.3 Listeria

Listeria monocytogenes has become a major concern of the food industry in many parts of the world. Recent well described outbreaks of

listeriosis have implicated a variety of foods particularly dairy products such as milk (Fleming et al., 1985) and cheese (Anon. 1985a and Ho et al., 1986) as Vectors of the bacterium. Benkerroum and Sandine (1988) evaluated 9 strains of *Listeria* sp. (*L. monocytogenes*, *L. ivanovii* and *L. saligary*) for nisin sensitivity and their MIC ranged from 1.85 to 3.36×10^3 and 740 to 1.18×10^5 IU/ml in MRS and Trypticase Soy Broth (TSB) respectively. Nisin activity was found considerably low in TSB in comparison to MRS broth.

A significant variation in nisin tolerance among *Listeria* strains was observed by Asperger et al (1989). The MIC and MBC ranged from 400 to 800 and 600 to 1000 IU/ml of nisin respectively. Contrary to this report, no significant difference in MIC and MBC of nisin among 21 *Listeria* strains was observed by Url, (1990). However, a linear relationship was observed between nisin concentration and number of *Listeria* killed/inhibited. Two different inocula of *Listeria* strains (1.0×10^3 and 5.0×10^7) were inhibited at nisin dose of 200 and 1000 IU/ml respectively.

Nisin resistance of *Listeria* strain was found much higher than lactic acid bacteria. Harris et al. (1991) demonstrated that nisin sensitivity of *L. monocytogenes* (ATCC 19135, Scott A and UAL 500) strains was found increased by addition of 2% sodium chloride or by reduction of the medium pH from 6.5 to 5.5 in presence of 10 µg nisin/ml.

Monticello and O'Connor (1990) demonstrated that *L. monocytogenes* counts decreased from about 10^6 to 10^3 /ml in 2-4 h in presence of 100 IU nisin/ml, but further lysis was not evident even after 24 h. After several days of incubation at 37°C, cell numbers begins to rise, eventually reaching levels equivalent to those in control cultures without nisin. The possible reason for increase in counts may be that with a large inoculum,

nisin is essentially used-up and cells which survive the initial event can then grow in a nisin reduced environment.

2.3 NISIN SENSITIVITY OF LACTIC AND NON-LACTIC CULTURES IN FLUID MILK

The activity of nisin is strongly influenced by chemical composition of a particular food to which it is added. It appears likely that binding or adsorption of the poly-peptide structure of nisin occurs with certain food components which makes it inactive or unavailable to inhibit micro-organisms.

Several studies have shown that nisin activity is diminished in foods that contains fat (Jones, 1974). Nisin has been observed consistently to be more active in low fat foods than in high fat foods. How fat or lipids interfere with nisin activity may relate to its mode of action (Daeschel et al., 1990). The reduction of nisin activity in presence of meat proteins was observed by Scott and Taylor (1981). Similarly, it may be possible that milk proteins can also bind or interact with nisin and may effect its antimicrobial potential.

2.3.1 Lactic Cultures

2.3.1.1 Non-nisin producers

The nisin sensitivity of 22 strains of lactic acid bacteria belonging to different species i.e., *Streptococcus lactis*, *S. cremoris* and *S. diacetylactis* was determined in skim milk at 30°C and 6 h of incubation. The MIC at 30% inhibition ranged from 0.1-20 RU nisin/ml (Keogh, 1956). The inhibition of *S. diacetylactis* (DRC₁ and DRC₂) strains was observed at 1.0 and 2.5 RU nisin/ml, whereas, *S. cremoris* (C₁ and C₃) strains were

inhibited at 0.5 and 1.0 RU nisin/ml respectively. *Streptococcus lactis* (C₂, C₆ and C₁₀) strains also depicted low nisin tolerance with MIC of 2.5 RU/ml.

The inhibitory effect of nisin producing strains of *S. lactis* as well as commercial nisin preparation (80 RU nisin/ml) was evaluated by Galeslout (1957) in milk. Some strains of *S. lactis* and *S. cremoris* were found capable of destroying the nisin rapidly, however, other strains were nisin sensitive. *Lactobacillus plantarum* strains were slightly inhibited, even those which destroyed nisin. In a similar study, Teply, (1962) demonstrated the effect of 10, 50 and 100 IU nisin/ml on acid production of yoghurt, acidophilus, cream and some pure *Lactobacillus* cultures and complete inhibition was observed at 100 IU/ml. Thermophilic streptococci were found significantly more resistant among the strains studied.

Effect of 2 nisin producing strains of *S. lactis* against dairy starters was further studied by Shahani, (1962) in milk medium containing 5% nisin broth. Nisin broth was found to have no effect on 3 strains of *S. lactis*, 2 strains each of *S. thermophilus* and *L. bulgaricus* and a mixed lactic culture. However, slight delay in cell multiplication and acid production of 2 strains of *S. thermophilus* was observed inhibitory but upon further incubation, cell count and acid production were not appreciably effected. It appears that initial nisin concentration used in the experiment was not sufficient to inhibit completely the growth and acid production of selected lactic cultures.

Nisin sensitivity of 26 strains of streptococci and lactobacilli at very low level of nisin i.e. 10 RU/ml in skim milk was investigated by Kalra and Dudani (1975). *Streptococcus lactis*-HL, *S. cremoris* (C₁₃),

L. bulgaricus (1373), *L. plantarum* (L-111) and *L. acidophilus* (L-1) did not appear to be inhibited whereas, *S. lactis* (C₁₀) and *S. diacetylactis* strains were inhibited to the extent of 50%, *Lactobacillus plantarum* (L-84) and *L. bulgaricus* (H and W) showed an inhibition of more than 30%.

The inhibition of *Streptococcus lactis* and *S. thermophilus* strains in presence of 50-1000 RU nisin/ml in skim milk was observed by Naquib et al. (1985). However, precise nisin doses for individual cultures were not determined. Similarly, in 1989, Mukundan also evaluated some lactic cultures i.e. *S. lactis* (SIR), *S. cremoris* (SC₁), CH₉, LF-40, *L. bulgaricus* (LB-4) and *L. casei* in presence of 100-500 RU nisin/ml in skim milk under different inocula and incubation periods. LF-40 was found to have higher resistance among strains studied.

The nisin sensitivity of 8 strains of lactococci and streptococci in skim milk was observed by Kumar and Prasad (1992a) at 1% inoculum and optimum growth conditions. The MIC ranged from 25-225 RU nisin/ml and 2-5 fold variation in nisin sensitivity of *Lactococcus* and *Streptococcus* sp. was observed in broth and skim milk study.

2.3.1.2 Nisin resistance in lactic cultures

2.3.1.2.1 Lactococci:

The sensitivity of nisin resistant streptococci to antibiotic used as drugs was demonstrated by Lipinska and Marta (1964). Two strains of *S. lactis* (3a and 2a₃) and one strain each of *S. cremoris* and *Leuconostoc citrovorum* were adapted to tolerate about 320 IU nisin/ml. These strains with the exception of *S. cremoris* were found significantly more sensitive to all antibiotics tested in comparison to parent sensitive strains.

In 1974, Lipinska further evaluated nisin resistant strains of *S. lactis* which do not destroy nisin by producing nisinase enzyme. Lactic

and propionic acid fermentation was determined in media containing 300 Units of nisin/ml where nisin sensitive strains of *S. lactis* (2a₃) were completely inhibited. Nisin resistant strains of *S. lactis* (2a₃) depicted higher lactic acid fermentation (acetoin, diacetyl and Co₂) and proteolytic activity. However, some common features i.e. higher metabolic activity in growth phases and greater sensitivity to temperature and salt concentration were also observed among nisin resistant and sensitive strains.

2.3.1.2.2 Lactobacilli:

The resistance of pure cultures of *L. casei* was increased by gradual adaptation to increasing concentration of nisin (Hylmar, 1970; and Hylmar et al, 1970). In a culture medium consisting initially of full cream milk enriched with 5% of molasses base, 200-300 Units of nisin/ml had ultimately shown no inhibitory effect. Adequate fermentation and proteolytic activity of this strain was retained in presence of 200 Units nisin/ml. However, the original *Lactobacillus* culture was shown to be inhibited completely by 50 Units nisin/ml in milk.

2.3.2 Non-lactic Cultures

2.3.2.1 Clostridia

A comparative inhibition of *Clostridium* sp. in milk or casein peptone medium containing lactate and small quantity of lactose or glucose (simulating conditions in cheese) was evaluated by Lind, (1958). A wide variation in nisin sensitivity was observed among strains studied. However, the inhibitory action of nisin was found less pronounced in casein peptone medium in comparison to milk. In the same year, Frohlich further reported that skim milk inoculated with 1 and 5% culture of

Clostridia and 50-100 RU nisin/ml was found to have no effect on butyric acid fermentation. The inhibition of *Cl. sporogenes* and *Cl. butyricum* spores at 100-120 RU nisin/ml in skim milk was also observed by Efimova et al. (1973).

2.3.2.2 Bacilli

The nisin sensitivity of 150 strains belonging to species, *B. cereus*, *B. subtilis*, *B. mesentericus*, *B. mycoides* and *B. megaterium* species in hydrolysed skim milk was evaluated by Efimova et al (1973) and observed that 100-120 RU nisin/ml decreased the heat resistance of spores. *Bacillus megaterium*, *B. cereus*, *B. mesentericus* and *B. B. circulans* spores were found comparatively more sensitive towards nisin. Wajid and Kalra (1974) observed complete inhibition of *B. subtilis* (9144) and *B. stearothermophilus* (I-63) strains in sterilised milk containing 100 RU nisin/ml. The reduction in D-values of spores in presence of nisin was further enhanced with the increase of temperature of heat treatment. A comparative nisin tolerance of *B. cereus* in nutrient broth and skim milk at 20 and 15°C respectively with inhibition at 20-40 mg nisin/ml was reported by Shehata (1981). The incorporation of 100 fg nisin/ml in skim milk decreased the heat resistance of *B. subtilis* spores. Germination of spores was also delayed (Lee and Kim, 1985).

2.3.2.3 Staphylococci

Apart from studies conducted on the fermentative lactic organisms, information is available on the inhibitory action of nisin against pathogenic organisms-like *Staphylococcus aureus* causing food poisoning. Jarchovska et al.(1974) found that a conc. of 500 RU.

nisin/ml was required to control the growth of this organism in sweetened condensed milk. Whereas, Jones (1974) reported the interference of butter fat on the inhibitory action of nisin against *S. aureus* when present in whole milk and cheese. The inhibition of *S. aureus* was also observed in skim milk by Naqib et al. (1985) at nisin concentration ranging from 50-1000 IU/ml. However, nisin tolerance was found comparatively very low in broth as reported by Lee and Kim (1985), where inhibition of *S. aureus* was observed at 200 IU/ml. In a similar study, Owens and Watts (1987) demonstrated that the activity of antimicrobials against *S. aureus* in milk was low when compared with the activity in Mueller-Hinton broth. However, the growth rate of the organism in both media was same. They observed that the lower effect appears to be due to action of milk on the antimicrobials and not on the growth rate of the organism.

A slightly higher nisin tolerance of *S. aureus* in milk in comparison to solid medium was also reported by Broadbent et al. (1989).

2.3.2.4 Listeria

After observing the widespread outbreaks of human listeriosis due to consumption of milk and milk products and the presence of *Listeria* in the environment of food factories, several efforts have been made to assure *Listeria*-free production (Anon, 1988a; Coleman, 1986 and WHO, 1988). Recently, the addition of bacteriocins of lactic acid bacteria, especially, nisin was found to be effective for inhibition or controlling the proliferation of *L. monocytogenes* in milk and milk products.

Some bacteriocins of lactic acid bacteria were found active against *L. monocytogenes*. Hoover et al. (1988) demonstrated that inhibitory activity of *Pediococcus acidolactici* (Po_2) towards

L. monocytogenes (ATCC 19111, 19113 and 19115). The decrease in viable counts and growth of *L. monocytogenes* in several refrigerated products was observed by Pucci et al. (1988) by addition of a crude preparation of bacteriocin PA-1 produced by *P. acidolactici* (PAC-1). The inhibitory effect of nisin against 9 strains of *L. monocytogenes* in sterilised and non-sterilised cottage cheese was determined by Bankerroum and Sandine (1988) with MIC of 37×10^2 and 2.55×10^3 IU/g respectively.

The inhibitory effect of fat on nisin activity was well demonstrated by Daeschel et al. (1990) and Jung et al. (1992). The initial activity of 10 Units nisin/ml in sterile milk containing 0 (skim milk), 1.15 and 11.5% fat was decreased by 30, 50 and 95% respectively (Daeschel et al., 1990). However, the initial viable counts of *L. monocytogenes* (7.6×10^3 c.f.u. log growth phase) in milk containing 0, 1.15 and 2.3% fat were decreased to 200, 2300 and 22,000/ml respectively at nisin dose of 50 Units/ml and 24 h of incubation.

Jung et al. (1992) subsequently reported that initial nisin activity of 50 IU/ml in milk containing 0 (skim milk) and 12.9% fat was decreased by 33 and more than 88% respectively. The initial viable counts (\log_{10} 7-7.5 c.f.u. log growth phase) of *L. monocytogenes* (Scott: A) were found decreased to \log_{10} 2.90 c.f.u. and \log_{10} 30 c.f.u./ml in skim milk at 10 and 50 Units of nisin/ml respectively after 2 h of inoculation.

2.4 FACTORS AFFECTING NISIN ACTIVITY

The various factors like composition of the medium, age and size of inoculum, incubation temperature and pH of the solution containing

nisin prior to its addition to the broth, affects the antimicrobial potential of nisin in foods (Ramseier, 1960; Mohamed et al., 1984; Benkerroum and Sandine, 1988). Among these factors, pH appears to be the most important, because nisin is acidic in nature and its stability and solubility are pH dependent (Tramer, 1964 and Hurst, 1978). At neutral or alkaline pH, nisin is both relatively insoluble and unstable (Hurst, 1983). Therefore, the best solvent for nisin is 0.02N HCl in comparison to buffer at pH 10, where 80% of the nisin get inactivated within 2 hours.

2.4.1 Effect of pH

2.4.1.1 Effect on nisin stability

Nisin exhibits ^{the} greatest stability under acidic conditions (Lipinska, 1977). In dilute HCl solution at pH 2.5 or below, solutions can be boiled without loss of activity and even autoclaving does not cause serious loss of activity (Hall, 1966). In a similar study, Tramer (1964) further reported that nisin was stable at autoclaving (115.6°C) at pH 2.0 but 40% of the activity was lost at pH 5.0. Holding a solution of nisin at 100°C for 30 min at pH range 2.0-6.0 produces no adverse effect. However, in most practical applications, it is preferable not to exceed pH 6.5 (Hawley, 1957). The destruction of nisin in presence of proteins is less dramatic (Tramer, 1964). The activity of nisin is rapidly lost under alkaline conditions (Tramer, 1964; Hurst, 1978). At pH 11.0, the activity may be completely destroyed at 63°C in just 30 min. Hall (1966) stated that nisin has a higher antibiotic activity when dissolved in 50M HCl than when assayed from solutions in distilled water. It may be because of the fact that the nisin molecules tend to polymerise with the increase of pH. However, the relationship between the biological activity and the polymeric form of nisin has not been established yet.

2.4.1.2 Effect on nisin solubility

The solubility of nisin also depends on the pH of the solution. At pH 2.5, its solubility is 12% and decreased to 4% at pH 5.0 (Hall, 1966). It is practically insoluble at neutral and alkaline pH values. Recently, in (1990), Liu and Hansen further reported that solubility of nisin dropped sharply and continuously from 57 mg/ml, at pH 2.0 to about 1.5 mg/ml at pH 6; it dropped again to 0.25 mg/ml, at pH 8.5, where upon it levelled off. It is interesting that the pH at which solubility leveled off (about pH 8.0) coincides with the pH at which nisin began to undergo pH induced modifications. The solubility properties and electrophoretic behaviour suggested that the iso-electric point of nisin might be in the alkaline range.

The mechanism of inactivation of nisin under alkaline conditions is unknown but could be a consequence of denaturation, chemical modification or a combination of both. The instability and decreased solubility of nisin at high pH may be because of Dehydroalanine (Dha) and Dehydrobutyrine (Dhb) which are susceptible to modifications by nucleophiles (hydroxyl groups or nucleophilic R groups) that are present at high pH (Liu and Hansen, 1990).

2.4.1.3 Effect on inhibitory action of nisin

2.4.1.3.1 Bacilli:

The increased effectiveness of nisin at low pH was reported by Campbell and Sniff (1959) in tomato juice and basamin broth adjusted to different pH. It was observed that 200 IU nisin/ml was enough to inhibit *B. coagulans* at pH 5.3, however, 560 IU nisin/ml failed to inhibit this bacterium at pH 7.2. In a similar study, Oscroft et al. (1990)

demonstrated that a decrease in 0.3 units to pH 5.7 enabled 500 IU/ml nisin to inhibit the growth of *Bacillus* spores (10^6 /ml) inoculum at 30°C whereas, at pH 6.0 no nisin concentration was effective. The challenge of low concentrations of nisin (125-250 IU/ml) with large inoculum (10^6 spores/ml) were ineffective at pH 5.4 or above, however, inhibition of growth was observed when pH was adjusted with HCl.

2.4.1.3.2 Clostridia:

The effectiveness of nisin in preventing the spores outgrowth of six strains of *Cl. botulinum* types A, B and E in trypticase-peptone-yeast extract-glucose (TYPG) broth was profoundly affected by pH (Scott and Taylor, 1981). Nisin was considerably more effective at pH 6.0 than at pH 7.0 or 8.0. The enhanced effectiveness of nisin under acidic pH conditions may be because of the increased stability of nisin in acidic solutions or some acid damaging effects on the spores of *Cl. botulinum*. In a similar study, Scmers and Taylor (1981) demonstrated that at pH 6.0, nisin level of 5000 IU/ml was insufficient to prevent spores outgrowth of *Cl. botulinum* (69A, 113B and 213B) in cooked meat medium. The decreased effectiveness of nisin in cooked meat medium was attributed to the binding of nisin to meat particles and this binding is apparently not affected by lowering of pH to 6.0.

A comparative study on the effect of nisin on *Cl. sporogenes* spores in liver veal (LV) agar and pork slurries adjusted to different pH range 6.0-7.2 and 5.7-6.6 was demonstrated by Rayman et al. (1981). The effectiveness of nisin in preventing outgrowth of spores decreased with the increase of pH in almost a linear fashion.

Prasad (1993). The MIC range of nisin in Elliker's broth were 2-60, 5-200 and 15-325 RU/ml at pH 5.5, 6.5 and 7.5 respectively at optimum growth temperature and 24 h of incubation. The inhibitory action of nisin was maximum at pH 5.5 and subsequently dropped sharply and continuously with the increase of pH upto 7.5.

2.4.2 Effect of Incubation Temperature

It has been a common knowledge that individual micro-organism has its own optimum growth temperature and the metabolic activity is hampered considerably at sub-optimum temperatures. The most of the studies in the past are, however, restricted to or near optimum growth temperature and an assumption is usually made that the physiological activities of micro-organisms at sub-optimum temperatures are qualitatively the same as manifested at optimum temperature.

The higher synthesis of unsaturated fatty acid (UFA) in cell membrane at sub-optimum temperature was observed by Rose (1968) and may cause different sensitivities among micro-organisms when treated with bacteriocidal substances (Dajani et al., 1970). The killing effect of Staphylococcin 1580 against *Streptococcus aureus* was adversely affected when the temperature of incubation was decreased from 37 to 20°C (Jetten and Vogels, 1974).

2.4.2.1 Effect of inhibitory action of nisin

The nisin sensitivity of 31 strains of *Bacillus coagulans* under different incubation temperatures i.e. 35, 45 and 55°C was evaluated by Campbell and Sniff (1959). However, the inhibition was observed at 14 µg/ml after 7 days of incubation irrespective of different temperature regimes. A substantial increase in inhibitory action of nisin against

In a comprehensive study, Kumar and Prasad (1993) reported higher nisin tolerance of 8 strains of *Lactococcus* and *Streptococcus* sp. at optimum growth temperature than sub-optimum temperatures. The MIC range of nisin for *Lactococcus* sp. were 2-65, 5-200 and 2-100 RU/ml at 25, 30 (optimum) and 37°C whereas, for *Streptococcus* sp. the respective figures were 10-15, 75-150 and 20-50 RU nisin/ml at 30, 37 (optimum) and 45°C.

2.4.3 Effect of Inoculum

A high initial inoculum necessitates the addition of higher level of nisin and therefore, the demand of nisin is related to the types and numbers of micro-organisms present in foods (Gupta et al., 1971 and Oscroft et al., 1990). Under appropriate conditions of heating, pH and spore loads, the amount of nisin required for inhibition of spore outgrowth can be lowered down considerably (Scott and Taylor, 1981).

The spore load was found as one of the key determining factor in controlling the amount of nisin necessary to prevent outgrowth in foods (Fowler, 1979). The effectiveness of nisin in preventing spore outgrowth decreased with the increase of spore load (Rayman et al., 1981; Scott and Taylor, 1981 and Oscroft et al., 1990). This inverse relationship between inhibition by nisin and spore load substantiated the observation of Gibbs and Hurst, (1964) that nisin is ineffective in preventing spoilage when the raw material of inferior quality are used.

Some lactic cultures, like *Streptococcus lactis* (SIR) and *Lactobacillus bulgaricus* (LB-4) were found to offer comparatively higher resistance with the increase of inoculum from 1 to 2% levels at nisin concentrations ranging from 100-500 RU/ml in skim milk after 24 h of incubation (Mukundan, 1989). The adverse effect of higher inoculum on

efficacy of nisin was also observed against *Leuconostoc oenos* in Model grapejuice system containing 100 RU nisin/ml (Splittstoesser and Stoyla, 1989).

A linear relationship was observed between nisin concentration and numbers of *Listeria* killed or inhibited (Url, 1990). It was observed that the requirement of nisin for complete inhibition of *Listeria* increased considerably from 200 to 1000 IU/ml with the increase of inoculum from 1.0×10^3 to 5.0×10^7 c.f.u./ml.

Among 8 strains of lactococci and streptococci, 1-2 folds increase in nisin tolerance with the increase of inoculum from 1 to 2% was observed by Kumar and Prasad (1993). The nisin tolerance of *Lactococcus* sp. (nisin and non-nisin producers) at 1 and 2% inoculum was 5-2000 and 10-2400 RU/ml respectively, whereas, for *Streptococcus* sp., the respective figures were 75-125 and 100-150 RU/ml.

2.5 MODE OF ACTION OF BACTERIOCINS

The most published work on bacteriocins of lactic acid bacteria revealed that the adsorption of bacteriocins on Gram positive bacteria whether, they are sensitive or resistant, is non-specific (Upreti and Hinsdill, 1975; Davey, 1981; Zajdel et al., 1985 and Bhunia et al., 1991). In case of Gram negative bacteria, however, there is no adsorption as shown in case of nisin by Anderson et al. (1988) and Pediocins Ac H by Bhunia et al. (1991).

The propensity of bacteriocins from lactic acid bacteria to adsorb non-specifically on cell envelopes could arise from the hydrophobic nature of bacteriocins which would hide the presence of specific

receptors. Muriana and Klaenhammer (1991) reported that lactacin F was bacteriocidal towards protoplasts, while intact cells of the corresponding strains were immune to the bacteriocins which suggest that cell wall is involved in immunity.

Bhunia et al. (1991) reported that the Lipotechoic acids (LTA) are present only in the cell wall of Gram positive bacteria and the involvement of these acids in the adsorption of pediocin ACH would thus be consistent with the fact that it adsorbs only in Gram positive bacteria.

2.5.1 Mode of Action of Nisin against Gram positive Bacteria

Nisin belongs to a class of amphiphilic peptide called LANTIBIOTICS with molecular masses between 2000 to 4000 Da (Schnell et al., 1988). Pep-5 subtilin, gallidermin and epidermin also belong to lantibiotic group and their common feature is the occurrence of α, β , unsaturated amino acids and rare thio ether amino acids lanthionine and 3-methylanthionine which forms several intramolecular thioether bridges.

Nisin and Pep-5 produced by *Lactococcus lactis* subsp. *lactis* strains and *Staphylococcus epidermidis* (5) are low molecular (3500) peptides with strong cationic properties and high content of hydrophobic amino acids. Both have been shown to be bacteriocidal for gram positive bacteria (Sahl and Brandis, 1982) and have been studied extensively.

A comprehensive information on mode of action of lantibiotics at molecular level is available and the following targets have been taken into considerations:

2.5.1.1 Effect of cytoplasmic membrane

The primary mechanism of the antibiotic action of the lantibiotics is probably their interaction with the cytoplasmic membrane of bacteria

(Sahl, 1985 and Kordel et al., 1988). The structural and functional properties of the cytoplasmic membrane is indispensable to understand the mode of action of nisin. This membrane is composed of a bilayer of phospho and glycolipids in which different proteins are embedded. The lipids vary in the chemical composition of the polar groups protruding into the water phase and in the nature of fatty acid groups forming the hydrophobic lipid bilayer.

Nisin can directly affect the permeability properties of the lipids bilayer and makes hole in this membrane through which ions including proton can diffuse. As a result, the proton motive force will be dissipated and the driving force for uptake of many solutes will collapse. Subsequently, gradient of solutes which have been built up by proton motive force driven transport system cannot be maintained and efflux of those solutes via their specific transport system will occur. Nisin can also make hole in the membrane which allow the diffusion of solutes.

The membrane disruption is now believed to be the result of incorporation of nisin into the membrane and subsequent ion channel or pore formation (Henning et al., 1986; Sahl et al., 1987; Kordel et al., 1989 and Gao et al., 1991). The membrane potential is destroyed in sensitive Gram positive cells as a result of the efflux of K^+ , amino acids and ATP through the membrane pores (Ruhr and Sahl, 1985; Kordel and Sahl, 1986). The mode of action of Pep-5, the staphylococcin -like peptide produced by *Staphylococcus epidermidis* 5 appears to be same as observed in nisin (Sahl and Brandis, 1981, 1983).

2.5.1.2 Effect on peptidoglycan synthesis

The synthesis of murein (Peptidoglycan) is the target of a large number of antibiotics. It is of vital importance for the vast majority of prokaryotes and is restricted to these organisms.

Using a *In vitro* system, Linnett and Strominger (1973) estimated that 40 µg/ml nisin caused a 50% inhibition of peptidoglycan synthesis catalysed by particulate enzyme proteins derived from either *Bacillus stearothermophilus* or *Escherichia coli*. In a similar study, Reisinger et al. (1980) reported that inhibition was due to the formation of a complex between nisin and the lipid intermediate of the protein murein biosynthetic pathway. The concentration of nisin necessary to bring about this inhibition was approximately 1000 folds higher than the MIC of *B. stearothermophilus* making it unlikely that inhibition of peptidoglycan synthesis was the primary site of nisin action. It is possible that a nisin-murein complex is involved in initial nisin-cell interaction and/or in transport to the membrane (Reisinger et al., 1980).

In another study, Henning et al. (1986) demonstrated that antimicrobial effect of nisin is caused by the interaction with phospholipid components of the cytoplasmic membrane followed by an interference with the membrane function.

2.5.1.3 Effect on autolytic enzymes

Nisin and Pep-5 are cationic peptide antibiotics which in addition to their membrane disruptive action induce autolysis in staphylococci. The enhanced hydrolysis of cell wall material is caused by stimulation of the activity of the autolytic enzymes of the cells (Bierbaum and Sahl, 1985).

Two autolysin of the Pep-5 indicator strains, *S. simulans* 22 were recently purified and identified as N-acetylglycosaminidase and N-acetylmuramoyl-L-alanine amidase (Bierbaum and Sahl, 1987 and 1988). Experiments with purified amidase, isolated cell walls and cationic peptides revealed that at low ionic strength, the amidase with an isoelectric point > 9.5 binds to the teichoic, teichuronic and lipoteichoic acids of the cell envelopes and is thereby inhibited.

2.5.1.4 Inactivation of sulfhydryl groups

The importance of sulfhydryl groups in membrane associated functions of active transport and oxidative phosphorylation has been recognised for many years (Hood and Harris, 1980). Antibiotic such as nisin may act as sulfhydryl agent (Gross and Morrell, 1967). The unusual dehydroalanine groups of nisin are potential Michael acceptors which might react with membrane sulfhydryl groups and may be useful for the identification or design of new antibiotic agents.

Morris et al. (1984) located and characterised the sulfhydryl groups in the membrane and further studied their reactivity with several sulfhydryl agents and outgrowth inhibitors. Nisin at very low concentrations inactivates the sulfhydryl sites towards sulfhydryl agents and therefore, suggesting that these membrane sulfhydryl groups are the natural targets of nisin bacteriostatic action.

2.5.2 Mode of Action of Nisin against Gram negative Bacteria

The Gram negative bacteria in general, are insensitive to bacteriocins of lactic acid bacteria as they do not have specific receptors (Bhunia et al., 1991), however, their inner membrane can be destabilised by these bacteriocins (Gao et al., 1991). The sensitivity

of some Gram negative bacteria to both nisin and pediocin ACH has been reported earlier by Mattick and Hirsch, (1947); Bhunia et al. (1988); Blackburn et al. (1989) and Stevens et al. (1991), however, the exact mechanisms of sensitivity have not been determined.

The destruction of outer membrane can be achieved by osmotic shock, formation of cytoplasmic membrane vesicles or by procedures which affect the lipo-polysaccharide components of the outer membrane like treatment with EDTA. The mutants of *Salmonella typhimurium* which have reduced membrane lipo-polysaccharide are sensitive to nisin (Stevens et al., 1991).

Kalchayanand et al. (1992) hypothesise that adsorption of a bacteriocin on a sensitive cells impairs the barrier functions of the wall allowing the bacteriocins molecules to enter the cell and come in contact with the membrane. If this hypothesis is correct, then sub-lethally injured Gram negative and Gram positive bacteria with impaired cell wall barriers will be sensitive to bacteriocins (Ray, 1989).

The validity of hypothesis was tested by injuring Gram negative and bacteriocins resistant Gram positive bacteria by freezing, heating and acid treatment and measuring their subsequent loss in viability caused by nisin and pediocin ACH (Kalchayanand et al., 1992). It was observed that sub-lethal injury impaired the wall function probably by some conformational alterations and as a result the bacteriocins molecules enter through the impaired wall.

2.6 ELECTRON MICROSCOPY (EM) OF BACTERIOCINS TREATED LACTIC AND NON-LACTIC CULTURES

2.6.1. Bacteriocins of Lactic Cultures

The most published work revealed that the mode of action of bacteriocins have been studied at molecular levels by biochemical studies and very little efforts were made to corroborate these informations through EM (Scanning and Transmission) which is also an indispensable tool to know the surface and internal details of bacteriocins treated cultures. However, few reports on cationic lantibiotics i.e. nisin and Pep-5 and some other bacteriocins are available.

2.6.1.1 Nisin

A bacterial culture of *Clostridium butyricum* when treated with nisin undergoes bacteriolysis and appears more intense during logarithmic phase of growth as reported by Ramseier (1960). The vegetative cells following nisin treatment undergo some changes in surface tension of cytoplasmic membrane, which results in disturbance in cell permeability leading to bacteriolysis. The lysed bacterial cells were also found to release some substances absorbing at 260 nm. The lysis of nisin producing strain of *Streptococcus lactis* was also observed by Hurst and Kruse (1972), when it was treated with nisin during logarithmic phase of growth.

Lactobacillus cells (BSO 28 343, 375) following treatment with nisin at concentration of 10,000 units/ml undergo clumping as observed under light microscopy (Ogden and Waites, 1986). There was no evidence of cell lysis even after one hour following treatment. The extent of cell aggregation appears to depend on both the cell and nisin concentrations. The majority of cells clumped after 15-20 min. of treatment and no further aggregation was seen.

2.6.1.2 Pep-5

The effect of cationic peptide, Pep-5 on staphylococci was studied by Brandis and Sahl (1984) and reported that destruction primarily occurs in septum area as revealed by EM.

2.6.1.3 Pediocin -AcH

The ultrastructure changes in *Lactobacillus* and *Leuconostoc* sp. were studied by Bhunia et al. (1991). *Leuconostoc mesenteroides* following treatment with Pediocin AcH at concentration of 10,240 AU/ml undergoes lysis and revealed the presence of ghost cells. On the other hand, *L. plantarum* (NCDO 955) did not lyse, however, the cytoplasm became denser and darker.

The presence of ghost cells in *Listeria monocytogenes* strains treated with 1500 AU/ml of Pediocin AcH was demonstrated by Motlagh et al. (1991) under phase contrast microscopy.

2.6.1.4 Lactococcin A

Lactococcin A is a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*. The electron microscopy of lactococcin A treated *L. lactis* subsp. *lactis* cells revealed no lysis or other morphological alteration (Van-Belkum et al., 1991). However, the efflux of accumulated substrates, suggested that lactococcin A permeabilised the cytoplasmic membrane by pore formation. In contrast to nisin, lactococci A is, however, active in absence of proton motive force and its activity is mediated by a receptor present in the membrane of sensitive cells (Van-Belkum et al., 1991).

2.6.2 Bacteriocins of Non-lactic Cultures

2.6.2.1 Staphylococcin G-55

Staphylococcin is a bacteriocidal substance isolated from phage type 71 *Staphylococcus aureus* and has been studied for its mechanism of action

on staphylococcal cells (Clawson and Dajani, 1970). The morphological changes observed included the condensation of nuclear material, partial loss of ribosomes, modification of mesosomes and eventual dissolution of cell contents. The cell wall was, however, not affected. Almost similar kinds of changes were also detected in sensitive bacteria treated with streptococcin AFF-22 (Tagg et al., 1973).

2.6.2.2 Bacitracin

It is a cyclic peptide antibiotic, first isolated from a strain of *Bacillus licheniformis* in 1945. Siewart and Stronminger (1967) discovered that bacteriocin inhibits a crucial step in cell wall biosynthesis i.e. the enzymatic dephosphorylation of the G 55 isoprenyl pyrophosphate.

Bacitracin is generally considered to have activity against Gram positive bacteria, however, its effects on Gram negative bacteria has received very little attention. *Escherichia coli* strains, following the treatment with zinc bacitracin in logarithmic phase were examined electron microscopically using a negative staining technique which revealed the presence of structural lesions in defined area of cell wall (Walton, 1976). The lesions described were, however, not present on all the cells examined.

2.6.2.3 Polymyxin

Polymyxin is a polycations decapeptide antibiotic and generally destroy the barrier properties of cytoplasmic membrane by its affinity to bind with phospholipids (Teuber and Bader, 1976; Storm et al., 1977). It induced extensive visible alternations in the outer membrane of *Salmonella* sp. as observed under electron microscope (Schindler and Tuber, 1975; Lounatama and Sarvas, 1976).

2.6.2.4 Megacin

Megacin is a bacteriocidal substance with narrow antibacterial activity against producing organisms i.e. *Bacillus megaterium*. Ivanovics et al. (1959) reported that megacin either act like an enzyme which breaks the osmotic barrier of sensitive cells or activate the autolytic enzymes of cells which leads to an autolysis of cytoplasm.

The megacin treated *B. megaterium* were examined at intervals under phase contrast microscope. It was observed that the homogeneity of the cytoplasm gradually decreased and appeared as distinct granules in the centre. The microscopic and chemical investigations of cultures containing megacin suggested that there was a marked change in the osmotic barrier of the organisms. In a similar study, Holland, (1962) reported that *B. megaterium* strain 207K, when treated with megacin at 37°C and examined under phase-contrast microscope showed a gradual loss of intracellular materials. Bacteriocidal amounts of megacin transform protoplasts of sensitive organisms to empty spherical ghosts and also appear to disrupt the permeability barrier of whole organisms.

2.6.2.5 Bacteriocin 28

Bacteriocin 28 induced spheroplast formation in sensitive strains of *Clostridium perfringens* with some loss of cytoplasmic contents as revealed by phase contrast and electron microscopy (Mahony et al., 1971) The rigid cell wall of the bacterium was missing in these spherical forms and small pleomorphic structures with membrane could also have been seen to bud off from the larger spheroplast.

In another study, Ellison et al. (1971) reported extensive structural changes like aggregation of nucleoid deoxyribonucleic acid,

vesicular configuration of mesosomes and dissolution of cell contents with remainants of cytoplasmic membrane and internal structures were observed in *Cl. botulinum* type E strains following treatment with Botocin S51

2.6.2.6 Colicin M

The mode of action of colicin M against *E. coli* K-12 32T 19F/T₁ was studied by Braun et al. (1974). It induced the formation of spheroplast in presence of 15% sucrose. The electron microscopic examination revealed bulging of cell envelope either equatorially or at sites all over the cell.

2.7 APPLICATION OF NISIN IN FOOD PRESERVATION

During the past several years, considerable attention has been given to nisin for its use as a food preservative. Initially, it was used in processed cheese and cheese products to combat bacteriological spoilage caused by clostridia. Now a days, nisin has been permitted in varieties of foods in dairy and canning industries. This expansion of nisin usage has occurred during a period in which ultra high temperature (UHT) treatments have come into vogue. However, it has not been proved satisfactory or efficient for many products such as acid canned foods.

Nisin is being used in processed food under three main categories: (a) To control an existing spoilage problem, (b) To allow a reduction in heat processing conditions without creating extensive spoilage problems, and (c) in the formulation of new products, especially where quality demands a minimum of heat treatment and risk of bacterial spoilage would otherwise , be a limiting factor. The product treated with nisin could

attain commercial sterility with less heat treatment and improved nutritional value, flavour, texture and appearance.

2.7.1 Processed Cheese and Cheese Spreads

Nisin was proved an effective preservative in pasteurised processed cheese and cheese spreads. The raw ingredients used in the manufacture of processed cheese products are typically raw cheese, butter, skim milk, whey powder, various flavour additives, emulsifying salts and water. The clostridial spores often unavoidably present in some of these raw ingredients can survive the heat treatment of 85-105°C during melt process. The composition of processed cheese products in terms of increased pH moisture content and ultimate anaerobiosis favour the outgrowth of spores which may result in subsequent spoilage due to the production of gas and off odours and liquefaction of cheese.

The anaerobic spore formers particularly associated with spoilage of processed cheese are *Clostridium butyricum*, *Cl. tyrobutyricum* and *Cl. sporogenes* (Meyer, 1973, and Thomas, 1977). The research carried out in U.K (Anon, 1991) indicated that 250 mg Nisaplin/kg in processed cheese and cheese spreads which were artificially inoculated with spores of aforementioned *Clostridium* sp. at level of 200/g, prevented the spoilage at 37°C, however, the partial control was observed at 100 mg/kg of Nisaplin. Somers and Taylor (1987) demonstrated that Nisaplin is effective in pasteurised processed cheese spreads in delaying or preventing the growth of *Cl. botulinum* strains A and B and its subsequent formation of toxins.

2.7.2 Canned foods

Many bacterial species are found in foods depending upon the type of foods and its method of production. There is wide variation in the ability of these organisms to survive and grow in different temperatures ranges.

The processing of low pH foods packed in hermetically sealed container is designed to kill the heat resistant pathogenic bacterium, i.e. *Cl. botulinum*. However, the bacterial spores can survive the heat treatment and may cause food spoilage during storage, especially at elevated temperature. The spores of thermophilic bacteria like *Bacillus stearothermophilus* and *Cl. thermosaccharolyticum* are also significant cause of spoilage during hot climates or slow cooling in manufacture.

Nisaplin was found a powerful inhibitor of heat resistant and thermophilic bacterial spores in processed foods. It has widespread application in canned foods like canned mushrooms, canned soups, canned tomato products and canned tomato products (Anon, 1988b) In most of the canned foods, Nisaplin treatment of 50-250 mg/kg was found quite effective.

2.7.3 Milk and Milk products

Dairy products comprise a variety of foods based on milk or its by products as a major ingredients. Some of these foods are pasteurised and have a limited shelf-life under refrigerated conditions, other receive more drastic heat treatments which often extended shelf-life in both hot and temperate climates. The different conditions of climate, milk supply, transport and processing throughout the world bring diverse bacteriological problems to the dairy industry. Nisaplin has a significant role in solving many storage problems.

2.7.3.1 Pasteurised whole milk

The climatic conditions and the demands of transporting milk over a long distances produce short-term spoilage problems in many countries. Extending the shelf-life by 2 days with a preservative like Nisaplin is

an obvious advantage. Vanini and Moro (1967) showed that low concentration of nisin i.e. 2, 4 and 8 μg in pasteurised milk at 32°C significantly reduced the bacterial counts from $3.28 \times 10^4/\text{ml}$ to 1.19×10^4 , 8.0×10^3 and 7.8×10^3 respectively. Clotting time was same in each case. Lactobacilli and streptococci were prevalent in the samples containing nisin. In a similar study, Anon (1988c) demonstrated that Nisaplin at concentration of 30-50 mg/litre doubled the shelf-life of pasteurised milk stored at 35 and 50°C . The pasteurised milk which stored alternately at 10°C for 16 h and then 8 h at ambient temperature, the Nisaplin concentration of 10 mg/litre trebled the shelf life in comparison to 2 days of control samples.

A nisin concentration of 100-1000 RU/ml in commercially pasteurised milk with or without *Bacillus subtilis* spores (1000/ml) was found to have no effect on keeping quality (K.Q.) at 32°C . However, the K.Q. increased from 6-7 to 10 days at 6°C (Mahmoud et al., 1976). The Gram negative bacteria like *Escherichia coli* Type I *Aerobacter aerogenes* Type I and *Pseudomonas fluorescens* commonly found as contaminants in commercially pasteurised milk were not affected by nisin concentration of 100-1000 RU/ml. The K.Q. of laboratory pasteurised milk at 32°C was extended from 1 day (control) to 8, 15 and 26 days at 100, 500 and 1000 RU nisin/ml respectively (Mahmoud et al., 1976).

The initial heat shock of 85°C with no hold before pasteurisation significantly affects the preservative action of nisin in pasteurised milk (Magdoub et al., 1984). The K.Q. of standardised/recombined pasteurised milk containing 3% fat, 0.1% alanine and 100 RU nisin/ml at 7 and 20°C was found increased from 3 to $5\frac{1}{2}$ and $1\frac{1}{2}$ to $2\frac{1}{2}$ days respectively.

2.7.3.2 Evaporated milk

The evaporated milk, properly manufactured may not be necessarily sterilised, however, it may contain only few spores of the heat resistant bacilli and clostridia. Spoilage due to non-sporing bacteria has been reported, however, the defects were either due to faulty heat treatment or defective cans.

According to published reports, spoilage appears to be due to aerobic spore formers belonging to *B. subtilis*, *B. cereus*, *B. megaterium*, *B. coagulans* and *B. calidolactis* species. Some of these organisms cause sweet curdling or bitterness and flat sours. Nisaplin concentration of 80-100 mg/litre in evaporated milk inhibited the typical spores and reduced the process time by 10 min (Radaeva et al., 1976). The National Institute for Research in Dairying in U.K. also found Nisaplin useful in reducing the process time without increasing the spoilage rate (Gregory et al., 1964)

2.7.3.3 Sterilised milk

In commercial term sterilised milks are free of bacterial spoilage only in the context of elevated temperature and storage times. The sterilised milk can contain heat resistant thermophilic bacterial spores, which can spoil the milk stored at elevated temperature.

The addition of 100 RU nisin/ml in sterilised milk was found effective in preventing the outgrowth of bacterial spores and increased the shelf-life from 3-7 days to 60 days (Wajid and Kalra, 1976). In a similar study conducted in France, it was demonstrated that addition of Nisaplin 20 mg/litre in sterilised milk completely suppressed the growth of thermophilic *Bacillus* spores which survive the heat treatment of 115°C/15 min (Anon, 1988c) The sterilisation of milk in presence of nisin

was found to be more effective in preventing spoilage of milk drinks as reported by Shehata et al. (1977). The addition of 10 RU nisin/ml in whole buffalo milk or chocolate flavoured milk products, resulted in commercially sterilised milk products, which did not exhibit any spoilage during storage at 37 and 55°C for 21 days.

2.7.3.4 Flavoured milk

Chocolate milks are more susceptible to storage losses than plain milk because of the additional bacterial load in the cocoa powder, sugar and dried milk solid used as ingredients. Heinemann et al. (1964) showed that Nisaplin at 80 mg/litre allowed chocolate milk which had been heat treated to destroy *Clostridium botulinum* to be stored for six months at 35°C or for three weeks at 55°C and 10% loss after six months at 35°C. The addition of nisin reduced the F-value (centre of the can held at 250°C/3min as opposed to 11 min) significantly and the product had a better flavour with improved nutritional quality.

The shelf-life of chocolate dairy desserts increased from 7 to 21 days with 50 mg Nisaplin/kg and further extended to 35 days at 7°C when 150 mg/kg was added. At higher storage temperature (12 and 25°C), 150 mg/kg of Nisaplin doubled the shelf-life (Anon, 1985b).

2.7.3.5 Cream products

The stability and characteristic properties of cream products depends crucially on the heat and other treatment used. Nisin has been determined in specific products and their particular conditions of production, transport and storage. Phillips et al. (1983) in their study on pasteurised cream in presence of 25-100 RU nisin/ml obtained a significant decrease in mesophilic, psychrotrophic and thermophilic counts

and shelf-life was increased from 9-19 days when stored at 12°C.

2.7.3.6 Reconstituted and recombined milk products

Countries without an indigenous dairy industry often import milk powders and butter oil for local manufacture of dairy products. These products experience the same bacteriological hazards and sensitivity to excessive heat treatments as fresh milk products. Many of these countries, however, have the additional problem of ambient temperatures which means that any thermophilic bacteria surviving processing can reproduce rapidly and hamper the shelf-life.

The addition of nisin to dried milk might permit diminished heating without loss in nutritive value.

2.7.4 Fermented Milk products

2.7.4.1 Yoghurt

The palatability of fermented milk products is very low due to high acid development during storage. In tropical countries like India, where facilities for cold storage, transportation and distribution are not sufficient and therefore, to avoid the cold storage of the product, nisin was used in stirred Yoghurt stored at room temperature and shelf-life of the product increased from < 7 days to 10 days without much change in flavour, body, texture and consistency (Gupta and Prasad, 1989b).

CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 NISIN SENSITIVITY OF LACTIC AND NON-LACTIC CULTURES

3.1.1 Collection of Cultures

Twenty six strains belonging to genus *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Bacillus* were collected from National Collection of Dairy Cultures (NCDC) at N.D.R.I., Karnal, as well as Microbial Type Culture Collection (MTCC, at Chandigarh.

3.1.1.1 Cultures collected from NCDC

Lactococcus lactis subsp. *lactis* (C₁₀, ML₈), *L. lactis* subsp. *lactis* 496 (nisin producer); *L. lactis* subsp. *diacetylactis* DRC₁, DRC₂; *L. lactis* subsp. *cremoris* C₁, C₃; *Streptococcus salivarius* subsp. *thermophilus* H, I; *Lactobacillus delbrueckii* subsp. *bulgaricus* 1373, RTS, W; *L. acidophilus* 1899, R; *L. plantarum* R, 89; *Bacillus cereus* 10876; *B. subtilis* 9144, 6633 and *B. stearothermophilus* (953).

3.1.1.2 Cultures collected from MTCC

Lactococcus lactis subsp. *lactis* 440 (nisin producer), *L. acidophilus* 447, *B. cereus* 430 and *B. stearothermophilus* 37, 38.

3.1.2 Maintenance of Cultures

3.1.2.1 Lactic cultures

Lactococcus, *Streptococcus* and *Lactobacillus* sp. were maintained and sub-cultured in fresh sterile Litmus milk at fortnightly intervals and stored in refrigerator at 4 ± 1°C after 16-18 h growth at their optimum temperatures.

Composition of litmus milk

Reconstituted skim milk	11% w/v
Blue litmus	4% aqueous
Solution	1.5%

A pinch of calcium carbonate (0.5-1.0 g) was dispensed in each tube. Sterilisation was carried out at 1.1 kg/cm² for 15 min.

3.1.2.2 Non-lactic cultures

Bacillus cereus, *B. subtilis* and *B. stearothermophilus* strains were maintained and subcultured on nutrient agar slant at one month intervals and stored at 4 ± 1°C after 18-24 h growth at 35, 37 and 55°C respectively.

NUTRIENT AGAR

Composition	g/L
Yeast extract	2
Beef extract	1
Peptone	5
Sodium chloride	5
Agar	15
pH	7.4 ± 0.2

Sterilisation by autoclaving at 1.1 kg/cm² for 15 min.

3.1.3 Preparation of Cell Suspension

The lactic and non-lactic cultures were grown in their respective medium (given below) at optimum growth temperatures for 18-20 h. The cells were harvested at 3000 rpm for 15 min in IEC CU-5000 centrifuge to get the cell pellet. The pellet was washed with 0.9 percent saline and again centrifuged at higher speed i.e. 4000 rpm for 15 min to

collect the cells following each washing. These were then suspended in sterile saline (0.9% NaCl), until 50% transmission was visible at 750 nm using Spectrophotometer (SP-30). This suspension was used for studying the effect of nisin.

3.1.3.1 Medium used for lactic cultures

a) Lactococci and streptococci

Elliker's broth (Elliker et al., 1956)

Composition	g/L
Tryptone	20
Yeast extract	5
Glucose	10
Sodium chloride	4
Sodium acetate	1.5
Ascorbic acid	0.5
pH	7.0 ± 0.1

Sterilised by autoclaving at 1.1 kg/cm² for 15 min.

b) Lactobacilli

De Man Rogosa Sharpe (MRS) medium (DeMan et al., 1960)

Composition	g/L
Proteose-peptone	10
Beef extract	10
Yeast extract	5
Di-potassium hydrogen phosphate	2
Ammonium citrate	2
Tween-80	1
Sodium acetate	5
Magnesium sulphate	0.1
Manganese sulphate	0.05
pH	6.4 ± 0.1

Sterilised by autoclaving at 1.1 kg/cm^2 for 15 min.

3.1.3.2 Medium used for non-lactic cultures

a. *Bacillus cereus* and *B. subtilis*

NUTRIENT BROTH

Composition	g/L
Beef extract	1
Yeast extract	2
Peptone	5
Sodium chloride	5
pH	7.4 ± 0.2

Sterilised by autoclaving at 1.1 kg/cm^2 for 15 min.

b. *Bacillus stearothermophilus*

Peptone Yeast Extract Medium

Composition	g/L
Peptone	10
Yeast extract	5
Sodium chloride	5
pH	7.2 ± 0.1

Sterilised by autoclaving at 1.1 kg/cm^2 for 15 min.

3.1.4 Nisin

Nisaplin brand with an activity of 1.0×10^6 Reading Units (RU) or International Units (IU)/g was obtained from M/s Aplin and Barrett Ltd., Trowbridge, Wiltshire, U.K. (Batch No.2 BP). The International Unit is defined as 0.001 mg of this preparation. The activity of 1g pure nisin is 40 times that of 1 g of nisaplin. The activity of nisin can be expressed as IU/g, mg/kg, ppm or % nisin. The relationship between these

terms are as follows:

<u>PURE NISIN</u>			<u>NISAPLIN</u>		
mg/kg	or	ppm	mg/kg	or	IU/g
2.5	=	2.5	100	=	100
5.0	=	5.0	200	=	200
12.5	=	12.5	500	=	500

3.1.4.1 Preparation of standard stock solution of Nisaplin

The stock solution of nisaplin with concentration of 1000 RU/ml was prepared by suspending 0.1 g Nisaplin in 80 ml of 0.02 N HCl and final volume (100 ml) was made after 2 h storage at room temperature and the pH was adjusted to 3.0 with 0.1N NaOH. The solution was filter sterilised (0.25 µm pore), stored at 5°C and used within one week.

3.1.5 Determination of Minimal Inhibitory Concentration(MIC) of Nisin

3.1.5.1 Definition of MIC

The MIC is defined as the lowest concentration of antibiotic in µg/ml that prevent the in-vitro growth of bacteria. In the present study, the MIC was interpreted as the concentration of the antibiotic contained in the first tube in the series that completely inhibited the growth of micro-organisms.

3.1.5.2 MIC in broth

The nisin sensitivity of lactic and non-lactic cultures was determined using BROTH DILUTION SUSCEPTIBILITY TECHNIQUE. The test tubes containing 8 ml of broth were inoculated with filter sterilised Nisaplin with final concentrations ranging from 0.5-2000 RU/ml. Thereafter, the tubes were inoculated with cell suspension of lactic and non-lactic

cultures at 1% inoculum. The inoculated tubes without Nisaplin and uninoculated tubes containing Nisaplin were included as controls. The tubes were incubated aerobically at optimum temperature and pH for 24 h.

3.1.5.3 Criteria used for MIC

The criteria used for determining the MIC of lactic cultures i.e. Lactococcus, Streptococcus and Lactobacillus sp. were optical density (O.D.), standard plate counts (SPC) and pH whereas, for non-lactic cultures i.e. Bacillus cereus, B. subtilis and B. stearothermophilus, was SPC/ml.

3.1.5.3.1 O.D.:

The O.D. of Lactococcus, Streptococcus and Lactobacillus sp. was determined at wavelength 650 nm using SP 30 UV Spectrophotometer (PYE UNICHAM).

3.1.5.3.2 SPC:

The SPC of lactic cultures i.e. lactococci and streptococci in Ellikers agar and lactobacilli in MRS agar were determined after incubating the plates at optimum growth temperatures for 48 h of incubation. Likewise, the SPC of non-lactic cultures i.e. Bacillus cereus and B. subtilis in Nutrient agar and B. stearothermophilus in Peptone Yeast Extract agar were determined at their optimum growth temperatures and 48 h of incubation.

3.1.5.3.3 pH:

The pH was determined using EC Digital pH Meter (pH 5651).

3.1.5.4 MIC in skim milk

The nisin sensitivity of lactic and non-lactic cultures was also determined in skim milk system for comparative information using the same

technique as explained earlier. The criteria used for determining the MIC for lactics were titrable acidity (% lactic acid) and pH whereas, for non-lactics was SPC/ml.

3.1.5.4.1 Titrable acidity:(IS:1479, Part II, 1961):

Ten millilitre of thoroughly mixed sample of milk was transferred to a 25 ml beaker. Thereafter, few drops of phenolphthalein indicator solution were added and titrated against standard sodium hydroxide solution to a pink colour. The titrable acidity was calculated from the titre value as percent lactic acid.

3.2 FACTORS AFFECTING THE INHIBITORY ACTION OF NISIN

The efficacy of nisin under 3 different pH and temperatures (given below) against lactic and non-lactic cultures was evaluated in broth inoculated at 1 and 2% levels. The MIC were determined on the basis of O.D. for lactics and SPC for non-lactics. The effect of incubation periods i.e. 12, 24 and 48 h on inhibitory action of nisin was also studied in skim milk on the basis of titrable acidity (% lactic acid) and pH for lactics and SPC for non-lactics.

Cultures	Different pH		
	Minimum	Optimum	Maximum
Lactococci and Streptococci	5.5	6.5	7.5
Lactobacilli	5.0	6.0	7.0
Bacilli	6.5	7.0	7.5

Cultures	Different Temperatures (°C)		
	Minimum	Optimum	Maximum
Lactococci	25	30	37
Streptococci	30	37	45
Lactobacilli			
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	37	42	48
<i>L. acidophilus</i> and <i>L. plantarum</i>	30	37	45
Bacilli			
<i>B. cereus</i>	25	35	45
<i>B. subtilis</i>	30	37	45
<i>B. stearothermophilus</i>	50	55	60

3.3 SCANNING ELECTRON MICROSCOPY OF LACTIC AND NON-LACTIC CULTURES

3.3.1 Selection of Cultures

The most sensitive and resistant strains among 3 groups of lactococci, lactobacilli and bacilli (given below) were selected for SEM study.

3.3.1.1 Lactococci

L. lactis subsp. *lactis* (C₁₀, ML₈) (non-nisin producer),

L. lactis subsp. *lactis* 440 (Nisin producer)

L. lactis subsp. *diacetylactis* DRC₁, DRC₂.

3.3.1.2 Lactobacilli

L. delbrueckii subsp. *bulgaricus* RTS, W.

L. acidophilus R

L. plantarum 89

3.3.1.3 Bacilli

B. cereus 10876

B. subtilis 6633

B. stearothermophilus 38

3.3.2 Preparation of Reagents

3.3.2.1 Cacodylate buffer

To prepare 0.1M Cacodylate buffer, 1.6 g of sodium cacodylate (Fluka Batch No.135301-120) was dissolved in 100 ml glass distilled water. The pH was adjusted to 7.2 using EC-Digital pH meter.

3.3.2.2 Buffered glutaraldehyde solution (Sabitini et al., 1963)

Following composition was adopted to prepare 2.5% buffered glutaraldehyde.

2.5% Glutaraldehyde EM grade (Poly Science, Batch No. 99C-5029)	2.5 ml
0.1 M Cacodylate buffer	12.5 ml
Distilled water	10.0 ml
Total volume	<u>25.0 ml</u>

3.3.2.3 Graded series of ethanol: water mixtures

For dehydration of cells, graded series of ethanol:water mixtures of 50, 70, 90, 95 and absolute alcohol were prepared. Propylene oxide was used subsequently for final dehydration.

3.3.3 Preparation of Cell Suspension

The cell suspension of selected culture was prepared as explained earlier.

3.3.3.1 Nisin treatment of cell suspension

The cell suspension of lactic and non-lactic cultures was treated with filter-sterilised Nisaplin at their MIC as well as 1-10,000 times

MIC doses. Thereafter, the treated cells were kept at optimum growth temperature for 4-6 h, centrifuged 2-3 times at 4500 rpm for 15 min each and washed with distilled water to obtain clean cells.

3.3.4 Scanning Electron Microscopy

3.3.4.1 Fixation

Nisin treated cells were transferred to micro beakers containing 2.5% buffered glutaraldehyde fixative and kept for 3 h in cold.

3.3.4.2 Drying of samples

The samples for SEM observations, both freeze-drying and conventional air drying techniques were standardised in our laboratory.

3.3.4.2.1 Freeze-drying:

A smear of fixed cells were prepared on cover glass and freeze-dried using Toshniwal Lyophiliser. The processed samples were kept in desiccator till examination.

3.3.4.2.2 Conventional air drying:

A smear of the fixed cells similarly prepared was gradually dehydrated through ethanol:water series as under:-

<u>Graded ethanol: water series</u>	<u>Duration</u>
50%	10 min.
70%	10 min
90%	10 min.
95%	10 min.
100 (Absolute) -I	30 min.
100 (Absolute) -II	14 min.(room temp.)

After dehydration in absolute alcohol, these were subsequently dehydrated in propylene oxide for 15 min and allowed to dry. The processed samples were kept in desiccator till use.

3.3.4.3 Coating of specimens

Dried samples were mounted on aluminium stubs with silver paint and sputter coated with gold at approximately 200 Å thickness in Hitachi IB-3 Ion coater. The ion current was maintained at 6 mA at fine vacuum of 0.05-0.07 torr for 4 min.

3.3.4.4 SEM observations

The samples on aluminium stubs were placed in specimen holder and inserted into chamber under vacuum. Hitachi S-405A Scanning Electron Microscope was operated at 15 KV using secondary electron mode.

3.3.5 Electron Micrography

3.3.5.1 Processing of photographic films

The observations were recorded on ORWO 35 mm perforated film (50 ASA) with the help of attached camera assembly. The film was developed in film developer (Kodak D-19) to 8-10 min at 20°C and fixed in acid-hardening fixer (Agfa-301) for 10-15 min. The film was thoroughly washed in running water and dried in air.

3.3.5.2 Printing of electron micrographs

Enlarged prints were made on Agfa-brovira normal/hard photographic paper. Standard (Agfa-100) and high contrast (Agfa-108) developers were used for developing the prints. Developing was followed by fixation in acid fixing bath (Agfa-300) and washing thoroughly under running water. The prints were finally dried on glazing machine. A number of electron micrographs of each treatment were studied for interpretation.

3.4 SHELF-LIFE OF MILK AND MILK PRODUCTS CONTAINING NISAPIN

3.4.1 Collection of Toned milk

Fresh toned milk containing 3.0% fat, 8.6% SNF and pasteurised at high temperature short time (HTST) i.e. 71°C for 15 sec, was procured from Experimental Dairy Plant at N.D.R.I., Karnal.

3.4.2 Collection of Lassi

Fresh Lassi packets were also procured from Experimental Dairy Plant of the Institute. The steps involved in Lassi preparation as per flow diagram.

3.4.3 Treatment with Nisaplin

The pasteurised milk and Lassi were incorporated with the filter sterilised Nisaplin with the help of pre-sterilised syringe at concentration ranging from 100-500 RU/ml. The pasteurised milk stored at 3 different temperatures i.e. 37, 30 and 20°C was analysed upto 3 days at 8, 12 and 16 h intervals respectively. Lassi was stored at 20 and 30°C and evaluated upto 2 days at 8 and 12 h intervals respectively.

3.4.4 Shelf-life

The shelf-life was assessed on the basis of chemical and microbiological tests as under:

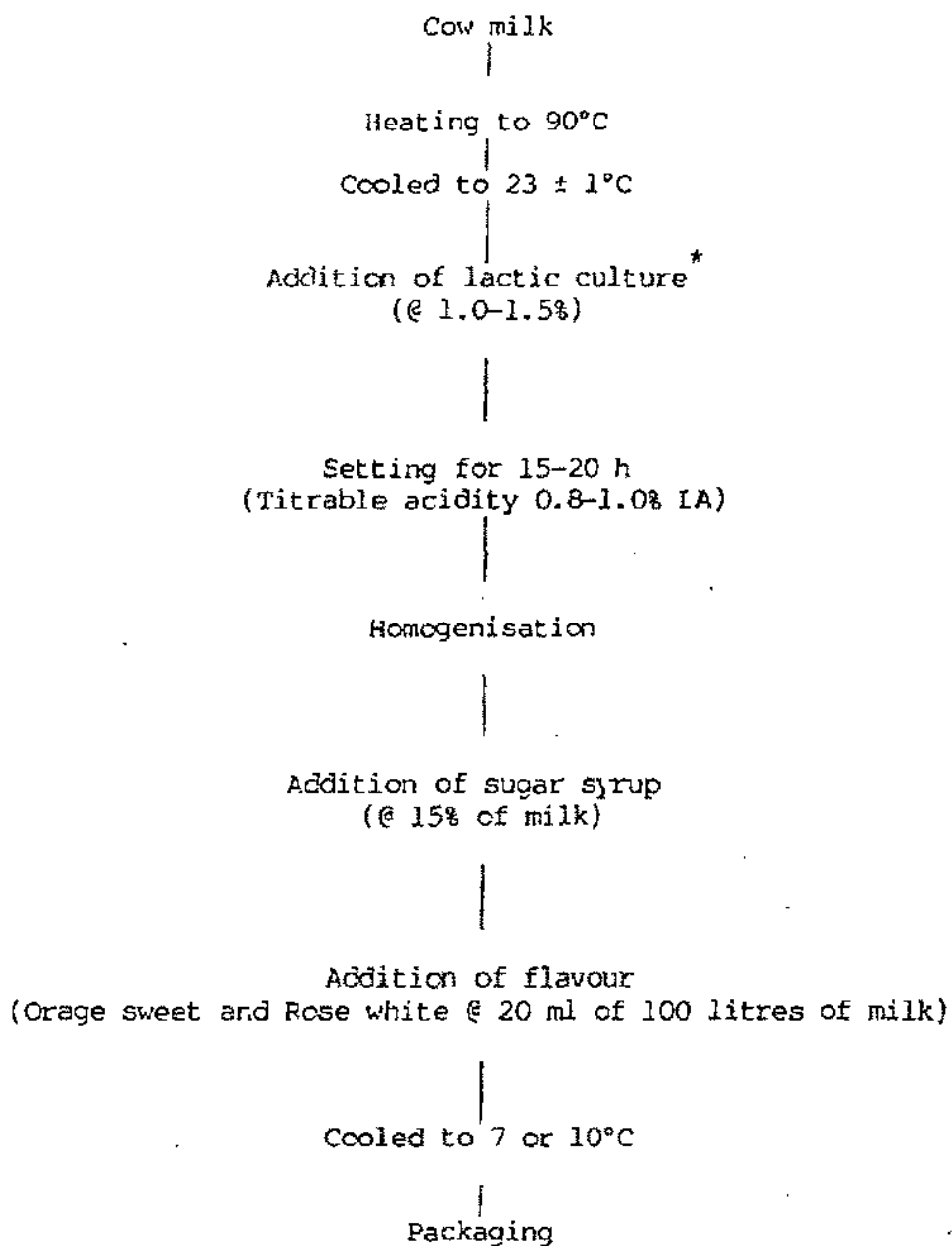
Pasteurised milk:

- i) Sensory evaluation
- ii) Titrable acidity (% lactic acid)
- iii) Standard plate count (SPC)

Lassi:

- i) Sensory evaluation
- ii) Titrable acidity (% lactic acid)

Flow Diagram of Lassi Preparation



* BD-4 containing

Lactococcus lactis subsp. *lactis*, *L. lactis* subsp. *cremoris*,
L. lactis subsp. *diacetylactis* and *Leuconostoc* sp.

iii) Yeast and mold counts

iv) Lactic counts

3.4.4.1 Sensory evaluation

Sensory evaluation was done as per the following score card.

3.4.4.1.1 Score card for pasteurised milk or Lassi:

Name of the Judge

Batch No.

Date:

Incubation temperature

Storage periods

							Sample No.
1	2	3	4	5	6	7	

Scores

Comments if any

Scores for judging

Scores to be allotted:

Grade

40-38

Excellent

37-35

Good

34-32

Poor

Possible defects

Changes from the natural colour, loss of sweetness and freshness, sediment formation, whey/fat separation, rancidity, acidity, any other off flavour, change in consistency/viscosity or any other remarks.

3.4.4.2 Chemical analysis

3.4.4.2.1 pH:

The pH of the sample was measured using EC Digital pH meter (pH 5651).

3.4.4.2.2 Titration acidity:

The titration acidity (% lactic acid) was determined according to I.S.I. (I.S. 1479, part II, 1961).

3.4.4.3 Microbiological analysis

3.4.4.3.1 Standard plate counts (SPC):

The SPC of control and treated toned pasteurised milk samples was determined according to standard procedures (APHA, 1972) using Tryptone Dextrose Agar.

Tryptone dextrose agar

Composition	g/L
Tryptone	5
Beef extract	3
Dextrose	1
Agar	15
pH	7.0 ± 0.1

Sterilised at 1.1 kg/cm² for 15 min.

3.4.4.3.2 Yeast and mold counts:

Yeast and mold counts in Lassi were determined according to APHA (1978) using Potato Dextrose Agar.

Potato Dextrose Agar

Composition

Infusion of 200 g potatoes in 800 ml distilled water.

Dextrose	20.0 g
Agar	20.0 g

The volume was made upto 1000 ml using distilled water and sterilised at 1.1 kg/cm^2 for 20 min, cooled to 45°C and tartaric acid (10%) was added at the rate of 1 ml per 100 ml of the medium to adjust its pH to about 3.5 at the time of pouring of medium in the plates.

3.4.4.3.3 Lactic counts:

The counts of lactic acid bacteria in *Lassi* were taken using Lactic indicator agar (McKay et al., 1970).

Lactose Indicator Agar

Composition	g/L
Tryptone	20
Yeast extract	5
Gelatin	2.5
Lactose	10
Sodium chloride	4
Sodium acetate	1.5
Ascorbic acid	0.5
Bromo-cresol purple	0.04
Agar	15
pH	7.0 ± 0.1

Sterilised at 1.1 kg/cm^2 for 15 min.

CHAPTER 4

RESULTS AND DISCUSSION

**I. NISIN SENSITIVITY OF LACTIC AND
NON-LACTIC CULTURES IN BROTH
AND SKIM MILK**

4.0 RESULTS AND DISCUSSION

4.1 NISIN SENSITIVITY OF LACTIC AND NON-LACTIC CULTURES IN BROTH

Twenty six strains of Gram positive bacteria belonging to groups lactococci, streptococci, lactobacilli and bacilli were evaluated for minimal inhibitory concentration of nisin (MIC) on the basis of optical density, pH and standard plate count (SPC) for lactic and SPC for non-lactic cultures.

A wide variation in nisin sensitivity was observed among different genera, species and strains. The MIC of 26 strains ranged from 0.5-2000 RU nisin/ml at 24 h incubation and optimum growth conditions as presented in Table 1. Out of these, *Lactococcus lactis* subsp. *cremoris* (C₃) was found most resistant strain whereas, *Bacillus stearothermophilus* (37), the most sensitive among strains studied. However, nisin producing strains of *L. lactis* subsp. *lactis* behaved conspicuously and depicted unusually high nisin tolerance.

4.1.1 Lactic Cultures

4.1.1.1 Lactococci and streptococci

The MIC of 10 strains of *Lactococcus* and *Streptococcus* sp. ranged from 5-175 RU nisin/ml in Elliker's broth at 1% inoculum and optimum growth conditions. Among lactococci, *Lactococcus lactis* subsp. *diacetylactis* (DRC₁) was found most sensitive with inhibition at very low level of 5 RU nisin/ml, whereas, *L. lactis* subsp. *diacetylactis* (DRC₂) depicted higher tolerance with MIC of 25 RU/ml (Table 1).

Table 1. Minimal inhibitory concentration (MIC) of nisin against lactic and non-lactic cultures in BROTH at optimum pH, temperature and 1% inoculum

Sl. No.	Cultures	MIC ^a (RU/ml) at 24 h	Cultures	MIC ^a (RU/ml) at 24 h	Cultures	MIC ^a (RU/ml) at 24 h
	LACTOCOCCUS and STREPTOCOCCUS sp.		LACTOBACILLUS sp.		BACILLUS sp.	
1.	<i>L. lactis</i> subsp. <i>lactis</i> (C ₁₀)	35	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (1373)	75	<i>B. subtilis</i> (9144)	150
2.	<i>L. lactis</i> subsp. <i>lactis</i> (NL _g)	75	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (RTS)	45	<i>B. subtilis</i> (441)	135
3.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)	5	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (W)	35	<i>B. subtilis</i> (6633)	125
4.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)	25	<i>L. acidophilus</i> (447)	50	<i>B. cereus</i> (10876)	75
5.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)	35	<i>L. acidophilus</i> (1899)	35	<i>B. cereus</i> (430)	45
6.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₂)	175	<i>L. acidophilus</i> (R)	25	<i>B. stearothersophilus</i> (953)	45
7.	<i>L. lactis</i> subsp. <i>lactis</i> (496) (nisin producer)	1600**	<i>L. plantarum</i> (R)	100	<i>B. stearothersophilus</i> (38)	1.0
8.	<i>L. lactis</i> subsp. <i>lactis</i> (440) (nisin producer)	2000**	<i>B. plantarum</i> (89)	20	<i>B. stearothersophilus</i> (37)	0.5
9.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (R)	75				
10.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (I)	125				

^a Arithmetic means of three determinations

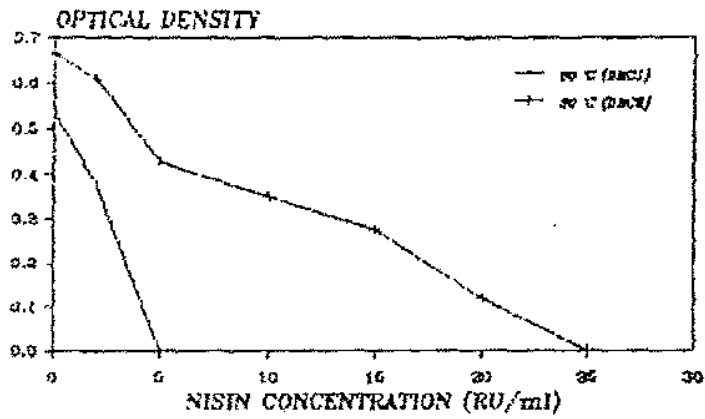
** MIC at 18 h of incubation

A 5 fold variation in nisin sensitivity was observed at strain level (Fig. 1a). Complete inhibition of *L. lactis* subsp. *diacetylactis* (DRC₁ and DRC₂) strains was observed by Garg in 1992 at 100 RU nisin/ml in M-17 Lactose broth using 2% inoculum. The higher MIC may be because of higher inoculum and compositional difference of medium used in the study.

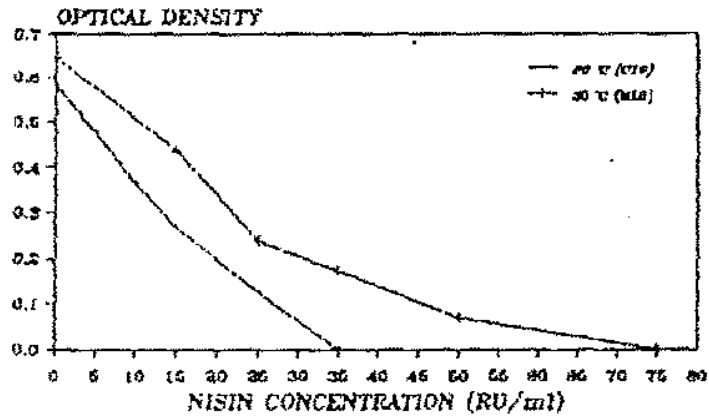
Nisin and non-nisin producing strains of *L. lactis* subsp. *lactis* differ widely in behaviour towards nisin. The MIC of non-nisin producing strains of *L. lactis* subsp. *lactis* (C₁₀ and ML₈) were 35 and 75 RU nisin/ml respectively in Elliker's broth at 24 h incubation and more than 2 fold variation in nisin sensitivity among strains was observed (Fig.1b). Collins-Thompson et al. (1985) evaluated 30 strains of lactic acid bacteria (LAB) and observed that growth of 20 strains of LAB including *L. lactis* was inhibited at 50 IU nisin/ml in MRS broth at 30°C and 24 h incubation. Recently, in 1992, Garg evaluated 18 strains of LAB in M-17 Lactose broth at 32°C, 2% inoculum and observed limited growth of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains in presence of 100 RU nisin/ml at 24 h incubation.

Two nisin producing strains of *L. lactis* subsp. *lactis* (496 and 440) were investigated in the present study and their MIC were 1600 and 2000 RU/ml in Elliker's broth respectively at 18 h incubation period. The growth of these cultures was initially inhibited at 18 h but as the incubation period increased further, the growth could be inhibited only at very high dose of 13,200 and 14,000 RU nisin/ml respectively at 24 h incubation. The addition of 200 and 400 RU nisin/ml was found to have stimulating effects on growth of *L. lactis* subsp. *lactis* (496 and 440) strains respectively (Fig. 1c).

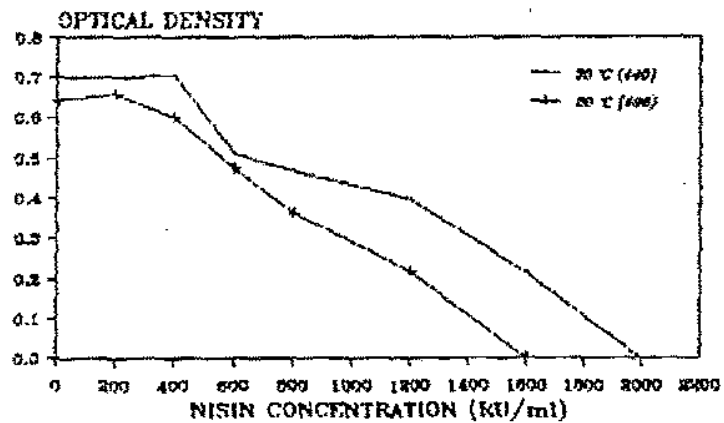
Fig 1(a-c) : EFFECT OF NISIN ON THE BEHAVIOUR OF L. LACTIS STRAINS IN BROTH AT OPTIMUM pH, TEMPERATURE & 1% INOCULUM



(a) *L. LACTIS* SUBSP. *DIACETYLACTIS*



(b) *L. LACTIS* SUBSP. *LACTIS*



(c) *L. LACTIS* SUBSP. *LACTIS*
(NISIN PRODUCER)

The increase in resistance of nisin producing strains after 18 h appears to be due to production of nisin degrading enzyme i.e. nisinase. Moreover, the inhibitory action is largely dependent upon the stage when nisin is added, either before inoculation or at a time when nisin synthesis has started (Hurst and Kruse, 1972). In our experiment, nisin was added before inoculation and hence caused only transient delay in growth.

Egorov et al. (1976) demonstrated that addition of 100, 250, 500 and 1000 RU nisin/ml had virtually no effects on growth of *Str. lactis* (MGU). However, 1500 or 2000 units of nisin/ml inhibited the growth during the first 24 h, but thereafter, biomass increased at the controlled rate or even surpassed it. Addition of 16,000 units of nisin/ml increased the lag phase of growth to 2 days but later growth was reduced by only 30%. Orberg and Sandine (1985) also reported 40 fold higher MIC(64 µg/ml) of nisin producing strain of *S. lactis* (ATCC 7962) than other strains.

Lactococcus lactis subsp. *cremoris* (C₃) depicted highest tolerance of nisin among strains studied showing inhibition at 175 RU/ml whereas, *L. lactis* subsp. *cremoris* (C₁) was inhibited at comparatively very low concentration of 35 RU/ml in Elliker's broth (Fig. 2a). The possible explanation for higher nisin tolerance appears to be nisinase production by *L. lactis* subsp. *cremoris* (C₃) strain during growth of the organism. Rapid destruction of nisin by *S. lactis* and *S. cremoris* strains was observed by Galescot (1957) in presence of 80 RU/ml in milk. Lipinska and Marta (1964) further demonstrated that *Str. cremoris* strain was adapted to tolerate 320 IU nisin/ml.

Streptococcus salivarius subsp. *thermophilus* strains depicted comparatively higher nisin tolerance than other *Lactococcus* strains

Fig 2a : EFFECT OF NISIN ON THE BEHAVIOUR OF *L. LACTIS SUBSP CREMORIS* IN BROTH AT OPT. pH, TEMP. & 1 % INOCULUM

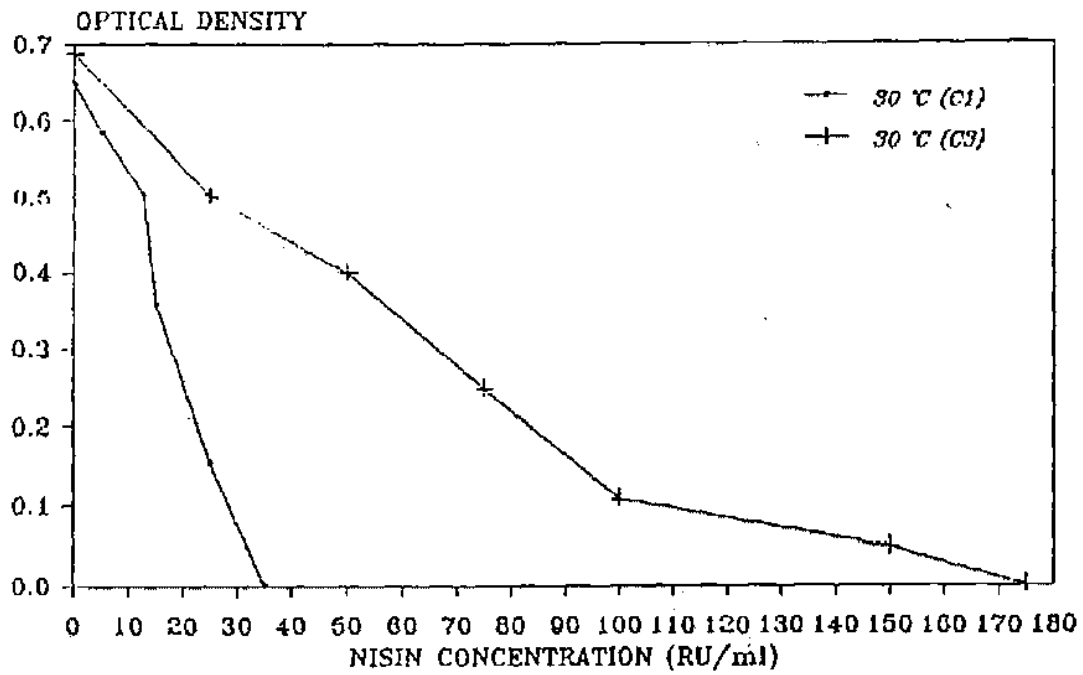


Fig 2b : EFFECT OF NISIN ON THE BEHAVIOUR OF *STR. SALIVARIUS SUBSP THERMOPHILUS* IN BROTH AT OPT.pH, TEMP. & 1 % INOCULUM

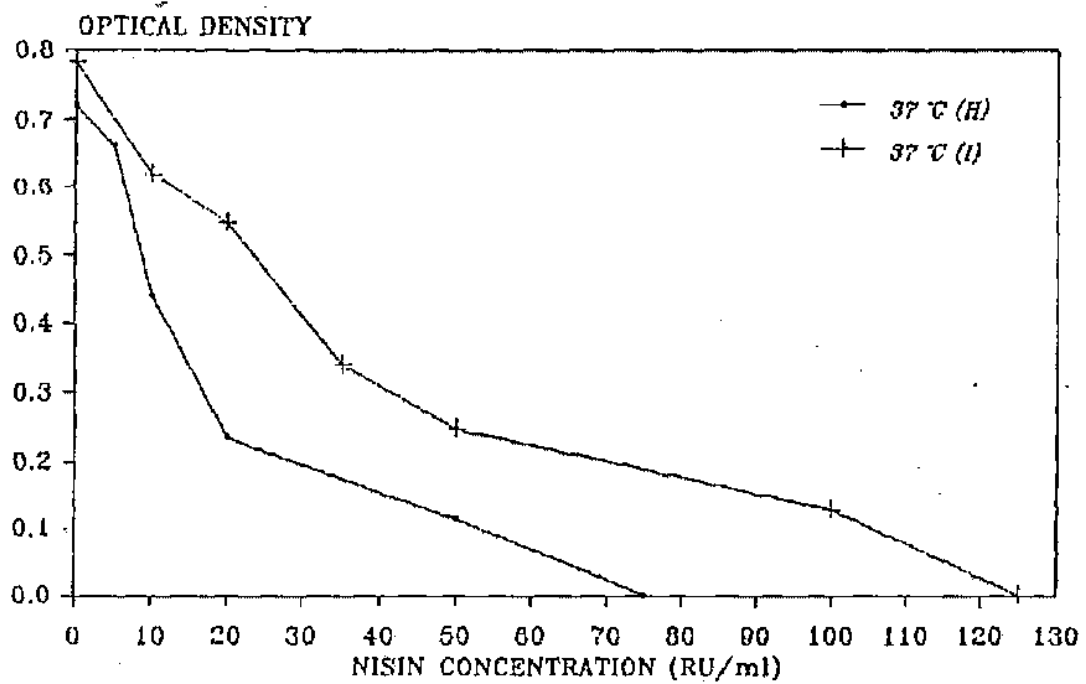
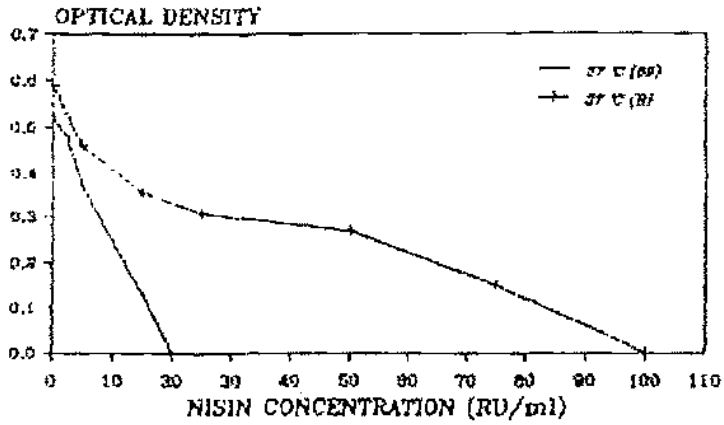
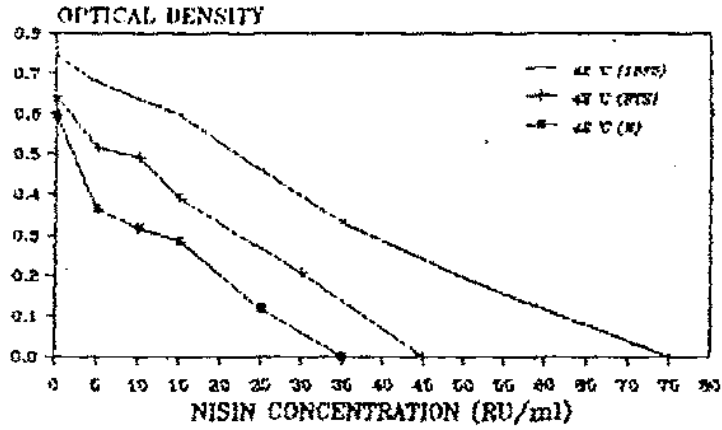


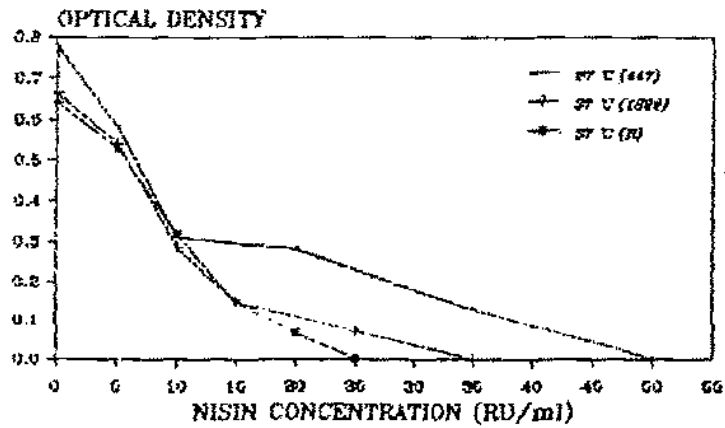
Fig 3(a-c) : EFFECT OF NISIN ON THE BEHAVIOUR OF LACTOBACILLUS STRAINS IN BROTH AT OPT. pH. TEMP. & 1 % INOCULUM



(a) *L. PLANTARUM*



(b) *L. DELBRUECKII* SUBSP. *BULGARICUS*



(c) *L. ACIDOPHILUS*

to be relatively low and appears to be more nisin sensitive than *L. delbrueckii* subsp. *bulgaricus* strains. The MIC of *L. acidophilus* (R, 1899 and 447) strains were 25, 35 and 50 RU nisin/ml respectively in MRS broth (Fig. 3c) at 37°C and 1% inoculum. In a similar study, Collins-Thompson et al. (1985) evaluated 30 strains of lactic acid bacteria and reported that 20 strains of LAB comprising mainly of *Lactobacillus* strains were inhibited at 50 RU nisin/ml in MRS broth at 30°C and 24 h incubation. However, very high nisin tolerance in *L. bulgaricus* and *L. acidophilus* was observed at 200 IU/ml in MRS broth by Lee and Kim (1985). Such variation may exist on account of strain characteristics.

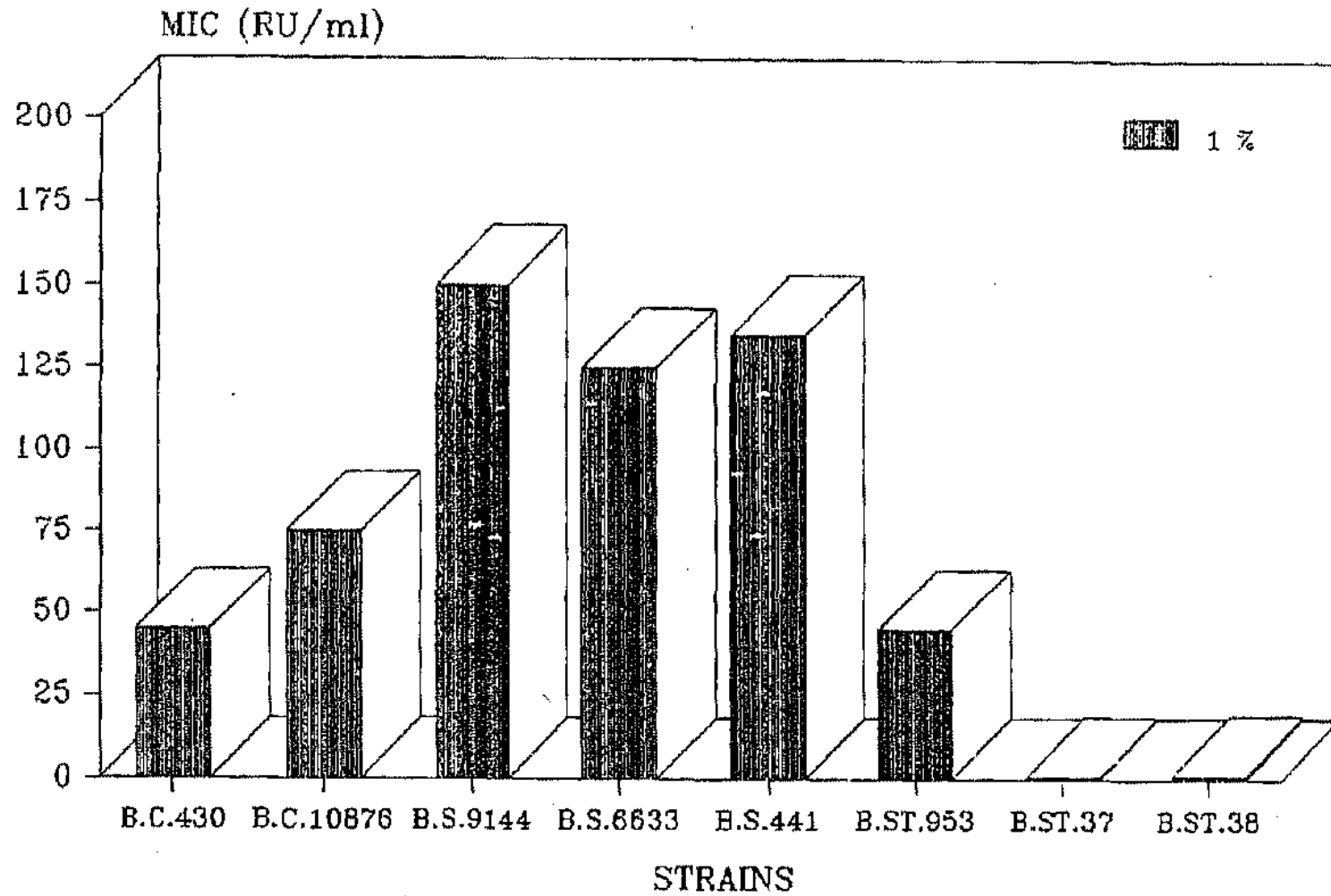
In general, lactococci and streptococci appears to be more tolerant to nisin than lactobacilli. Bossi et al. (1988) evaluated 31 strains of lactic acid bacteria and demonstrated that streptococci were more resistant to nisin than lactobacilli.

4.1.2 Non-lactic Cultures

4.1.2.1 Bacilli

The MIC of 8 strains of bacilli belonging to *B. subtilis*, *B. cereus* and *B. stearothermophilus* species ranged from 0.5-150 RU nisin/ml in broth at their optimum growth conditions (Table 1). *Bacillus subtilis* (9144) was found to be the most resistant among strains studied and complete inhibition was observed at 150 RU/ml in nutrient broth. However, other strains of *B. subtilis* (441 and 6633) were inhibited at slightly lower nisin concentration of 135 and 125 RU/ml respectively (Fig.4) at 37°C and 24 h incubation. Generally, *Bacillus cereus* strains were found comparatively more nisin sensitive than *B. subtilis* strains. The MIC of *B. cereus* (430 and 10876) strains were 45 and 75 RU/ml in

Fig 4 : EFFECT OF NISIN ON THE BEHAVIOUR OF *BACILLUS* STRAINS IN BROTH AT OPTIMUM pH, TEMPERATURE AND 1 % INOCULUM



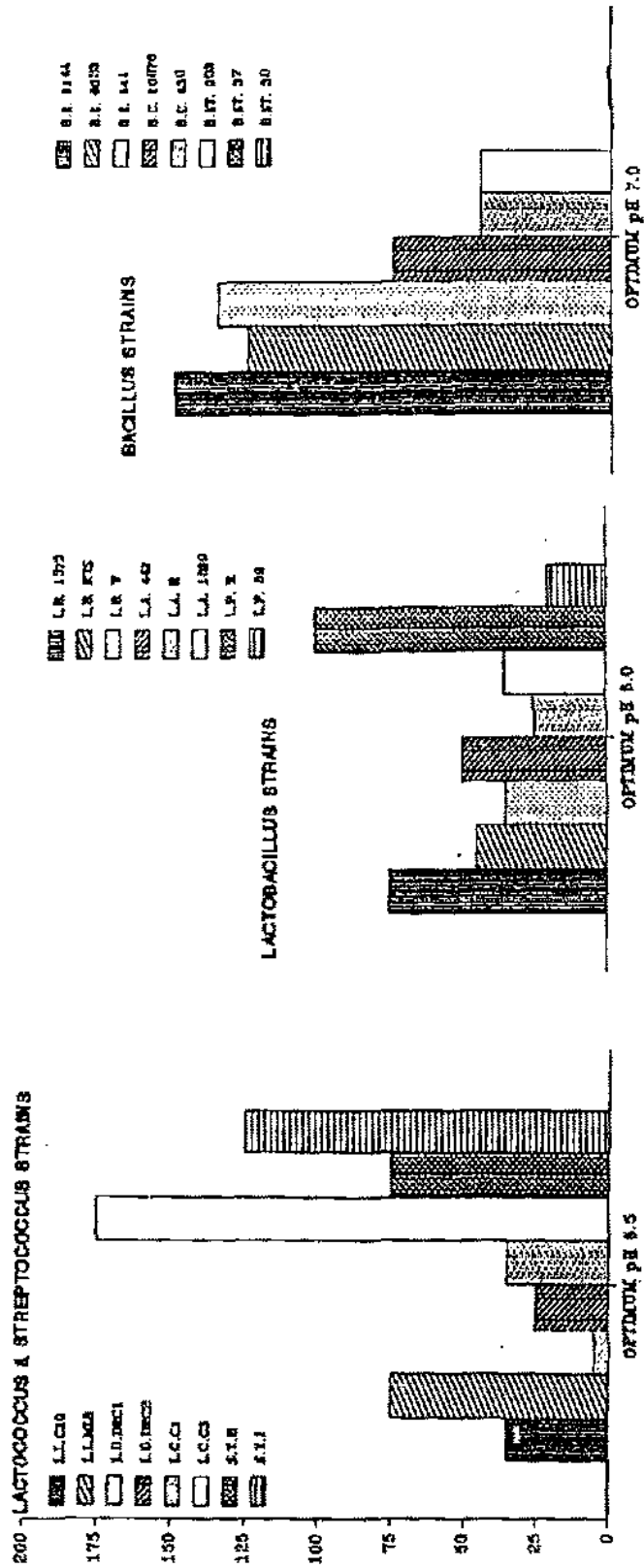
nutrient broth respectively at 35°C and 24 h incubation.

In 1967, Jarvis examined 14 strains of 9 species of *Bacillus* for nisin resistance and production of nisin-inactivating enzymes. Jarvis observed that *B. subtilis* (NCIB 8739 and 8057) strains were inhibited at nisin concentration of 75 and more than 100 RU/ml respectively. Whereas, *B. cereus* (NCIB 3329) was inhibited at above 100 RU nisin/ml. Shehata and Hassan (1981) further demonstrated the effect of nisin (20-100 mg/ml) against *B. cereus* in a steady state condition at 20°C and observed that nisin was most effective in inhibiting exponential growth phase.

Bacillus stearothermophilus (37 and 38) strains were found most sensitive not only in *Bacillus* groups, but among all different groups studied. Their inhibition was observed at extremely low level of 0.5 and 1.0 RU nisin/ml in peptone-yeast-extract broth respectively at 55°C and 24 h incubation. Interestingly, under similar conditions, one strain of *B. stearothermophilus* (953) showed comparatively very high nisin tolerance and was inhibited at 45 RU/ml in peptone-yeast-extract broth. The probable reason for its higher nisin tolerance could be the heat resistant source of this particular strain. The inhibition of *B. stearothermophilus* (1518) strain at 25 ppm of nisin in tryptone-yeast-extract broth was also observed by Denny et al (1961). Further, *Bacillus stearothermophilus* (NCIB 8157 and 8224) were also found to behave similar to *B. stearothermophilus* (37 and 38) strains showing complete inhibition at only 3 and 6 RU nisin/ml (Jarvis, 1967).

A comparative nisin sensitivity of different groups of lactic and non-lactic cultures is depicted in Fig. 5. It appears that lactococci and streptococci are invariably more tolerant to nisin than lactobacilli,

Fig. 5 : COMPARATIVE NISIN SENSITIVITY OF LACTIC AND NON-LACTIC CULTURES IN BROTH AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM



whereas, bacilli are more resistant among the different groups studied. However, it is difficult to conclude because of varying conditions of pH used for different groups of cultures. Therefore, the higher nisin resistance in lactococci, streptococci and bacilli may be because of decrease in the inhibitory action of nisin with the increase of of pH.

The valuable information on nisin sensitivity of lactococci, streptococci and lactobacilli along with 3 nisin resistant strains of *L. lactis* subsp. *cremoris* (C₃), *Str. salivarius* subsp. *thermophilus* (I) and *L. plantarum* (R) can be of great significance in preparation and preservation of fermented milk and milk products. This can also be beneficial in brewing industry where lactobacilli and pediococci are principal spoilage organisms.

In heat processed canned foods, the spoilage like sweet curdling, bitterness or flat sour, commonly appear due to *B. subtilis*, *B. cereus* (food poisoning), *B. coagulans* and *B. megaterium* etc. Certain species of this group also cause food poisoning and intoxication when survived the high heat treatments. The present findings on nisin sensitivity of bacilli with defined temperature, pH, inoculum and incubation period will be beneficial in controlling spoilage in milk and milk products as well as canned foods.

4.2 NISIN SENSITIVITY OF LACTIC AND NON-LACTIC CULTURES IN SKIM MILK

The inhibitory action of nisin is strongly influenced by chemical composition of the particular food to which it is added. Earlier, it has been well demonstrated that nisin activity is diminished in foods that

contains fat as reported by Jones (1974). Effect of milk components other than fat e.g. milk proteins on nisin sensitivity has also been observed (Scott and Taylor, 1981).

In present study, 26 strains belonging to different groups of lactococci, streptococci, lactobacilli and bacilli were evaluated to study the effect of skim milk on their nisin sensitivities. A considerable variation in nisin sensitivity was observed among most of the strains in broth and skim milk.

4.2.1 Lactic Cultures

4.2.1.1 Lactococci and streptococci

Skim milk was found to have considerable effect on the inhibitory action of nisin and 2-5 fold variation in nisin sensitivity of *Lactococcus* and *Streptococcus* sp. was observed in skim milk in comparison to broth study. The MIC of *Lactococcus* and *Streptococcus* sp. in skim milk ranged from 25-225 RU/ml (Table 2). Whereas, in broth it was 5-175 RU nisin/ml.

A five fold variation in nisin sensitivity was observed in *L. lactis* subsp. *diacetylactis* (DRC₂) with MIC of 25 RU/ml in skim milk. On the other hand, *Lactococcus lactis* subsp. *lactis* (C₁₀ and ML₈) and *L. lactis* subsp. *cremoris* (C₁) strains depicted 2-3 fold variation in nisin sensitivity with inhibition at 75, 175 and 100 RU nisin/ml respectively (Fig. 6a). Keogh (1956) evaluated 22 strains of lactic acid bacteria and demonstrated 30% inhibition of *S. diacetylactis* (DRC₁ and DRC₂) at 1 and 2.5 RU/ml, whereas, *S. lactis* (C₂, C₆ and C₁₀) strains were inhibited at 2.5 RU nisin/ml in skim milk respectively at 6 h of incubation. In a similar study, Kalra and Dudani (1975) also observed 50% inhibition of *S. diacetylactis* and *S. lactis* (C₁₀) strains. However,

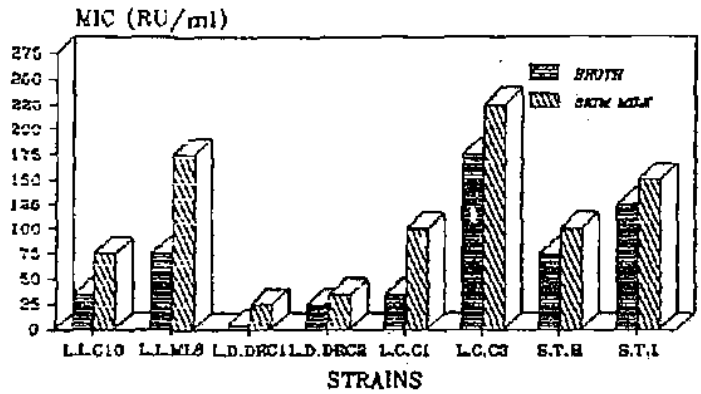
Table 2. Minimal inhibitory concentration (MIC) of nisin against lactic and non-lactic cultures in SKIM MILK at optimum pH, temperature and 1% inoculum

Sl. No.	Cultures	MIC ^a (RU/ml) at 24 h	Cultures	MIC ^a (RU/ml) at 24 h	Cultures	MIC ^a (RU/ml) at 24 h
	LACTOCOCCUS and STREPTOCOCCUS sp.		LACTOBACILLUS sp.		BACILLUS sp.	
1.	<i>L. lactis</i> subsp. <i>lactis</i> (C ₁₀)	75	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (1373)	100	<i>B. subtilis</i> (9144)	175
2.	<i>L. lactis</i> subsp. <i>lactis</i> (HC ₈)	175	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (RFS)	75	<i>B. subtilis</i> (441)	150
3.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)	25	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (N)	50	<i>B. subtilis</i> (6633)	150
4.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)	35	<i>L. acidophilus</i> (447)	75	<i>B. cereus</i> (10876)	100
5.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)	100	<i>L. acidophilus</i> (1899)	50	<i>B. cereus</i> (430)	75
6.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₃)	225	<i>L. acidophilus</i> (81)	50	<i>B. stearothermophilus</i> (953)	50
7.	<i>L. lactis</i> subsp. <i>lactis</i> (496) (nisin producer)	2000**	<i>L. plantarum</i> (8)	150	<i>B. stearothermophilus</i> (38)	2.5
8.	<i>L. lactis</i> subsp. <i>lactis</i> (448) (nisin producer)	2400**	<i>L. plantarum</i> (89)	50	<i>B. stearothermophilus</i> (37)	1.0
9.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (8)	100				
10.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (1)	150				

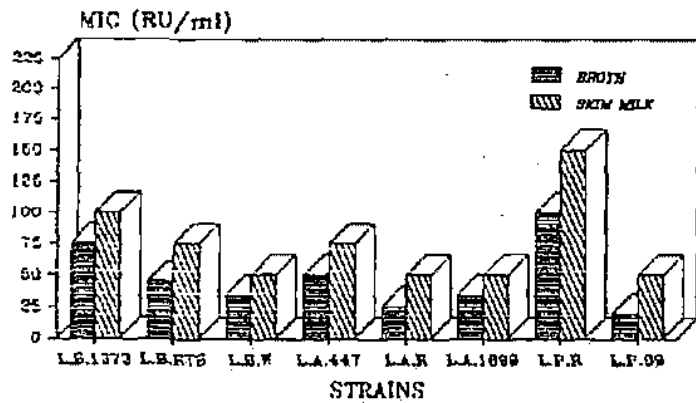
^a Arithmetic means of three determinations

** MIC at 18 h of incubation

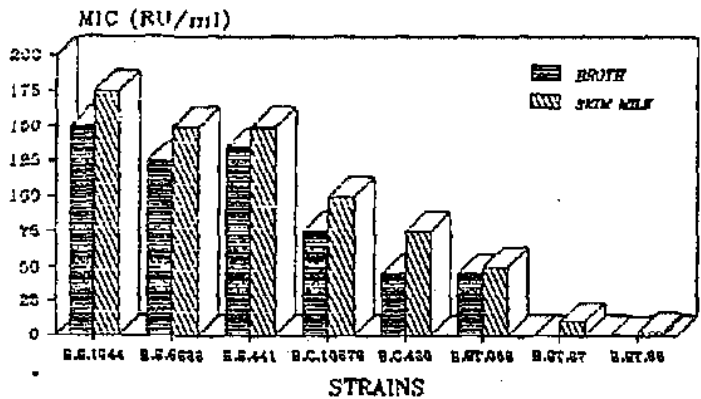
Fig 5(a-c) : COMPARATIVE NISIN SENSITIVITY OF LACTIC & NON-LACTIC CULTURES IN BROTH & SKIM MILK AT OPT.TEMP.& 1% INOC.



(a) LACTOCOCCUS & STREPTOCOCCUS STRAINS
OPTIMUM pH IN BROTH (6.5)



(b) LACTOBACILLUS STRAINS
OPTIMUM pH IN BROTH (6.0)



(c) BACILLUS STRAINS
OPTIMUM pH IN BROTH (7.0)

S. lactis-III and *S. cremoris* (C₁₃) were not inhibited at all. The inhibitory action of nisin in skim milk was found comparatively low against *L. lactis* subsp. *diacetylactis* (DRC₂), *Str. salivarius* subsp. *thermophilus* (H and I) and *L. lactis* subsp. *cremoris* (C₃) strains with MIC of 35, 100, 150 and 225 RU nisin/ml respectively. The respective MIC of aforesaid cultures in broth were 25, 75, 100 and 175 RU nisin/ml. Among *Lactococcus* and *Streptococcus* sp., it was observed that effect of nisin in skim milk was relatively more on some strains which showed that in addition to fat some other factors like milk proteins also influence the inhibitory action of nisin. The differential binding of milk proteins to *Lactococcus* and *Streptococcus* sp. or nisin itself may be responsible for different skim milk effect. A reduction in nisin activity in presence of meat proteins was observed by Scott and Taylor (1981). The possibility of binding of milk proteins with nisin was also reported by Jung et al (1992).

Nisin producing strains, *L. lactis* subsp. *lactis* (496 and 440) in skim milk behaved in a similar fashion as observed in broth. However, the inhibition was observed at relatively higher nisin concentration of 2000 and 2400 RU/ml respectively at 18 h incubation. Further increase in incubation from 18 to 24 h resulted in higher tolerance of *L. lactis* subsp. *lactis* (496 and 440) strains and growth could be inhibited only at 13,500 and 14,200 RU nisin/ml respectively. Similar trends were also recorded in broth study.

4.2.1.2 Lactobacilli

Among 8 strains of lactobacilli, 1-2 fold variation in nisin sensitivity was observed in broth and skim milk which was considerably low when compared with lactococci and streptococci. The MIC of *Lactobacillus* sp. ranged from 50-150 RU nisin/ml in skim milk at 18

inoculum, whereas, in broth it was 20-100 RU nisin/ml (Table 2). Among lactobacilli, equal or 2 fold variation in nisin sensitivity was observed in *L. plantarum* (89) and *L. acidophilus* (R) with nisin tolerance of 50 RU/ml each at 37°C and 24 h incubation. Other strains of *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. plantarum* depicted relatively small variation in nisin sensitivity in broth and skim milk (Fig. 6b).

The respective MIC for *L. delbrueckii* subsp. *bulgaricus* (W, RTS and 1373) strains were 50, 75 and 100 RU nisin/ml, whereas, for *L. acidophilus* (1899 and 447) strains were 50 and 75 RU nisin/ml in skim milk. *Lactobacillus plantarum* (R) depicted maximum nisin tolerance among lactobacilli and could be inhibited at 150 RU/ml in skim milk. It appears that lactobacilli are more sensitive than lactococci and streptococci as well supported by broth study. Earlier, in 1962 Teply also demonstrated that thermophilic streptococci were significantly more resistant than lactobacilli. Some information on nisin tolerance of *Lactobacillus* sp. in milk was given by Galesloot (1957) who reported that *L. plantarum* strains in presence of 80 RU nisin/ml were slightly inhibited even those which destroyed the nisin.

The complete inhibition of acid production by yoghurt, *acidophilus*, cream and some pure *Lactobacillus* culture was observed at 100 IU nisin/ml by Teply (1962). *Lactobacillus bulgaricus* (1373), *L. plantarum* (L-111) and *L. acidophilus* (L-1) were not inhibited in presence of 10 RU nisin/ml in skim milk as observed by Kalra and Dudani, (1975). However, there is no comparative information on nisin sensitivity of *Lactobacillus* sp. in broth and skim milk.

Streptococcus sp., followed by *Lactobacillus* and *Bacillus* sp. The possible explanation for higher decrease in nisin activity against lactococci and streptococci groups may be related to specific optimum pH (6.5) employed in both broth and skim milk systems. Whereas, in lactobacilli and bacilli, the optimum pH were 6.0 and 7.0, respectively as used in broth study. However, in skim milk system, the pH in both cases were kept 6.5 which was not optimum for *Lactobacillus* and *Bacillus* sp. and hence differential drop in nisin activity against different groups was observed. A wide variation in nisin sensitivity in skim milk among lactic and non-lactic cultures at genera, species and strains level suggest the possible involvement of milk proteins in effecting the nisin activity. The valuable data obtained on comparative nisin sensitivity in broth and skimmilk would be of immense use for controlling the growth of desirable and non-desirable organisms.

**II. FACTORS AFFECTING THE INHIBITORY
ACTION OF NISIN**

The activity of nisin in different kinds of food is considerably influenced by physical, chemical and microbiological environments. The fluid milk containing fat and proteins appears to have an adverse effect on nisin activity as observed earlier in broth and skim milk study. The number and type of micro-organisms, pH and incubation temperature were also found to have considerable effect on nisin action. However, there is scarcity of information on these factors and needs to be explored further. Among these factors, pH is considered as the most crucial because nisin itself is acidic in nature and its stability and solubility is affected under acidic, neutral and alkaline pH conditions.

4.3 FACTORS AFFECTING THE ACTIVITY OF NISIN IN BROTH

4.3.1 Effect of pH

The inhibitory action of nisin against lactic and non-lactic cultures at optimum growth temperatures was evaluated in broth under 3 different pH (minimum, optimum and maximum). The criteria for determining the MIC were optical density for lactic and SPC for non-lactic. The MIC range of nisin for *Lactococcus* and *Streptococcus*, *Lactobacillus*, and *Bacillus* sp. were 2-325, 5-150 and 15-225 RU/ml within pH range of 5.5-7.5, 5.0-7.0 and 6.5-7.5 respectively (Tables, 3-5). In general, the inhibitory action of nisin decreased with the rise of pH.

4.3.1.1 Lactococci and streptococci

The nisin tolerance of *Lactococcus* and *Streptococcus* sp. at respective optimum growth temperature, 30 and 37°C was studied in Elliker's broth adjusted to different pH 5.5, 6.5 (optimum) and 7.5 using HCl or NaOH. Nisin exhibits maximum inhibitory action under most acidic condition.

Subsequent increase of pH upto 7.5 appears to have adverse effect on nisin action. The MIC range of nisin for *Lactococcus* and *Streptococcus* sp. were 2-60, 5-200 and 12.5-325 RU/ml at pH 5.5, 6.5 and 7.5 respectively (Table 3).

The increase of pH from 5.5 to 6.5 decreased the vulnerability of *Lactococcus* and *Streptococcus* sp. to inhibitory action of nisin by approx. 2-17 folds. However, the increase in nisin tolerance among few strains of lactococci namely *L. lactis* subsp. *cremoris* (C₁) and *L. lactis* subsp. *lactis* (C₁₀ and ML₈) was comparatively high. Their MIC at pH 5.5 were 2, 2.5 and 10 RU/ml whereas, at pH 6.5, the respective values were 35, 35 and 75 RU nisin/ml (Figs. 7a and 8a). Other strains of lactococci and streptococci like, *L. lactis* subsp. *diacetylactis* (DRC₁), *Str. salivarius* subsp. *thermophilus* (H and I) and *L. lactis* subsp. *cremoris* (C₃) were comparatively less affected with the increase of pH from 5.5 to 6.5. Their MIC were 2, 15, 25 and 50 RU/ml at pH 5.5 and 5, 75, 125 and 175 RU/ml at pH 6.5 respectively (Figs. 7b and 8a,b).

The nisin tolerance of lactococci and streptococci declined further by 1-2 folds, when the pH of Elliker's broth was raised by one unit between 6.5 to 7.5. The inhibition of *L. lactis* subsp. *lactis* (C₁₀ and ML₈) at pH 7.5 was observed at 12.5, 35, 75 and 150 RU nisin/ml respectively (Fig. 7a,b). However, other strains of *L. lactis* subsp. *cremoris* (C₁ and C₃) and *Str. salivarius* subsp. *thermophilus* (H and I) were inhibited at 50, 300, 125 and 200 RU nisin/ml at pH 7.5 respectively (Fig. 8a,b). Nisin producing strains also behaved similarly and 1-3 folds increase in nisin tolerance was observed within pH range of 5.5-7.5. The MIC range of nisin for *L. lactis* subsp. *lactis* (496 and 440) were 600-800, 1600-2000 and 2000-2400 RU/ml at pH 5.5, 6.5 and 7.5 respectively (Fig. 9).

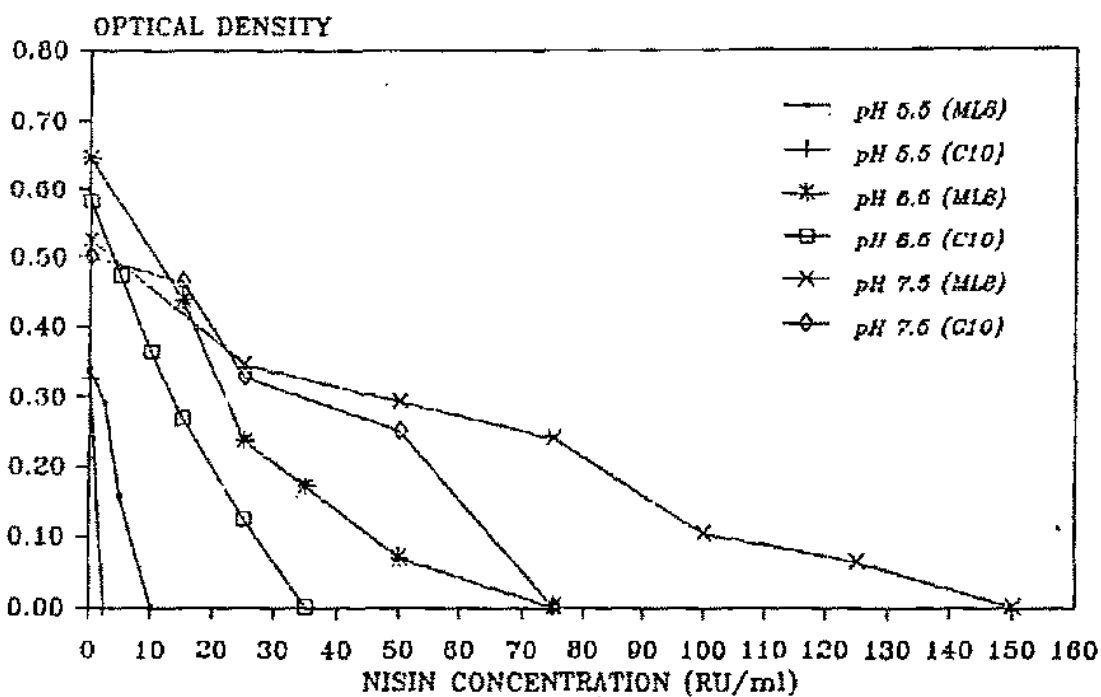
Table 3. Effect of pH on inhibitory action of nisin against *Lactococcus* and *Streptococcus* strains at optimum temperature and different inoculum levels

Sl. No.	Cultures	Inoculum levels (%)	MIC* (RU/ml) in broth at 24 h		
			5.5	Different pH 6.5	7.5
1.	<i>L. lactis</i> subsp. <i>lactis</i> (C ₁₀)	1	2.5	35	75
		2	3.5	50	100
2.	<i>L. lactis</i> subsp. <i>lactis</i> (ML ₈)	1	10	75	150
		2	15	100	175
3.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)	1	2.0	5.0	12.5
		2	2.5	10	15
4.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)	1	3.0	25	35
		2	5.0	27.5	50
5.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)	1	2.0	35	50
		2	2.5	45	80
6.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₃)	1	50	175	300
		2	60	200	325
7.	<i>L. lactis</i> subsp. <i>lactis</i> (440)** (nisin producer)	1	800	2000	2400
		2	1000	2400	2800
8.	<i>L. lactis</i> subsp. <i>lactis</i> (496)** (nisin producer)	1	600	1600	2000
		2	800	2000	2400
9.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (H)	1	15	75	125
		2	20	100	150
10.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (I)	1	25	125	200
		2	40	150	225

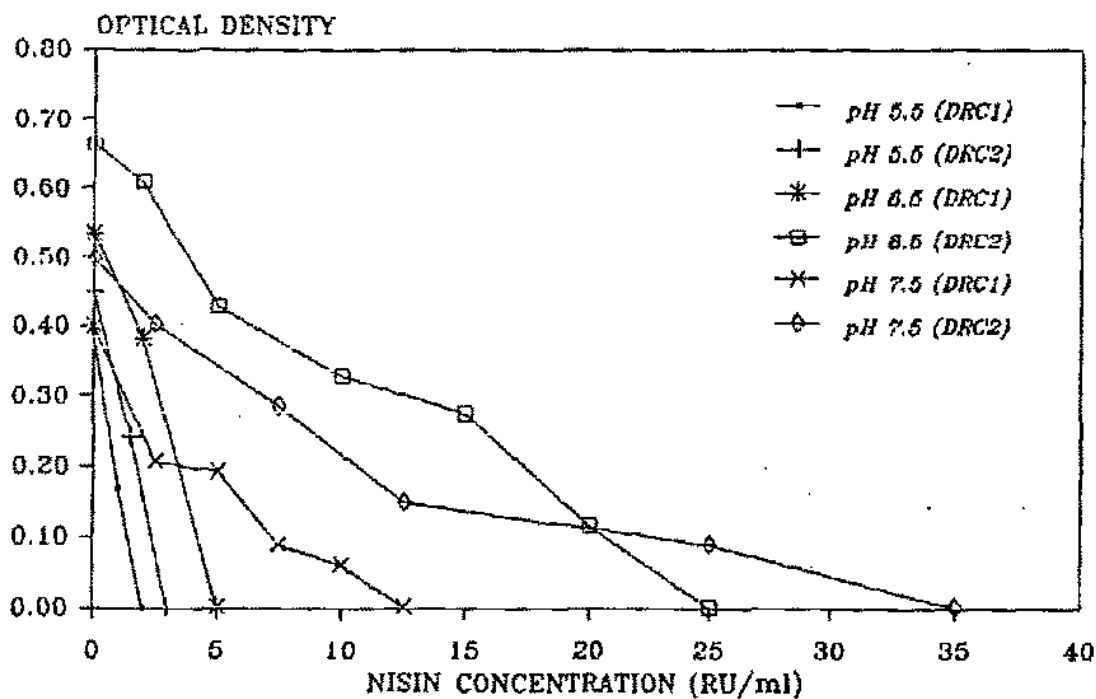
* Arithmetic means of three determinations

** MIC at 18 h for nisin producer

Fig 7 (a & b) : EFFECT OF pH ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM TEMPERATURE AND 1 % INOCULUM

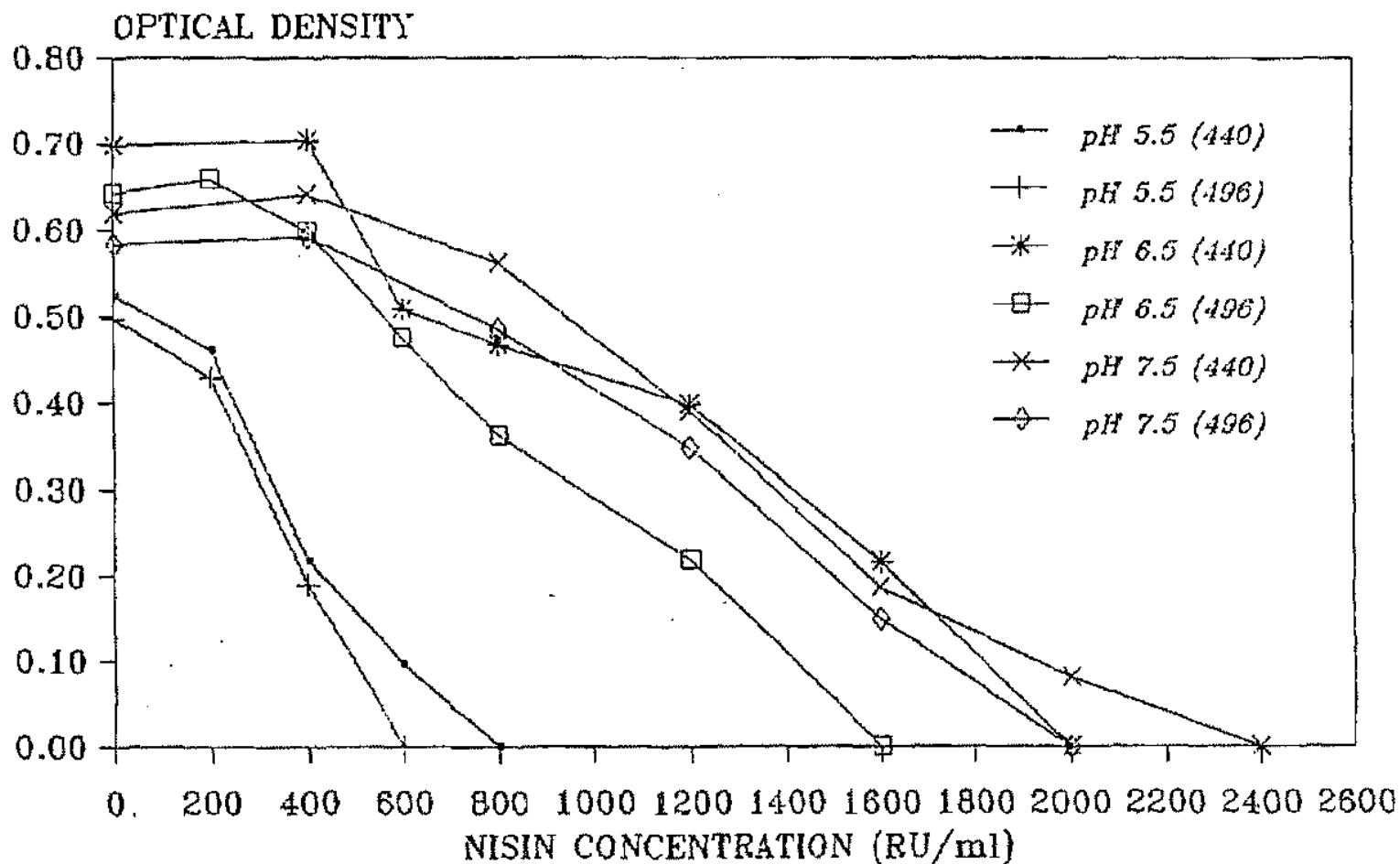


(a) *L. LACTIS SUBSP LACTIS*



(b) *L. LACTIS SUBSP DIACETYLACTIS*

Fig 9: *EFFECT OF pH ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM TEMPERATURE AND 1 % INOCULUM*



(a) *L. LACTIS SUBSP LACTIS*
(NISIN PRODUCER)

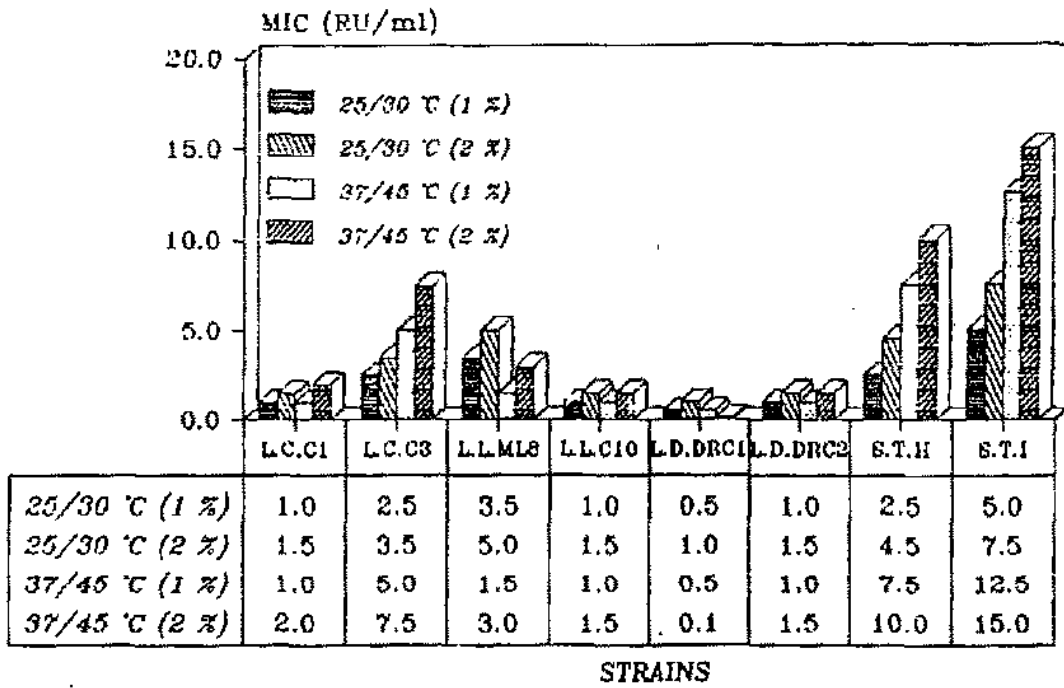
The antimicrobial potential of nisin against *Lactococcus* and *Streptococcus* sp. under different pH was adversely affected at sub-optimum growth temperatures (Fig. 10). The MIC range of nisin for *Lactococcus* sp. at pH 5.5 and 7.5 were 0.5-5 and 7.5-135 RU/ml at 25°C. On the other hand, the respective values at 37°C were 0.5-7.5 and 2.5-175 RU/ml at pH 5.5 and 7.5. Similarly, the MIC range for *Streptococcus* sp. were 2.5-7.5 and 25-32.5 RU/ml at 30°C whereas, the respective values at 45°C were 7.5-15 and 45-75 RU nisin/ml at pH 5.5 and 7.5. The lower values of MIC at sub-optimum temperatures in comparison to optimum temperature perhaps due to their adverse effects on growth pattern of the *Lactococcus* and *Streptococcus* sp. (for details refer to 4.3.2).

4.3.1.1.1 Effect on growth pattern:

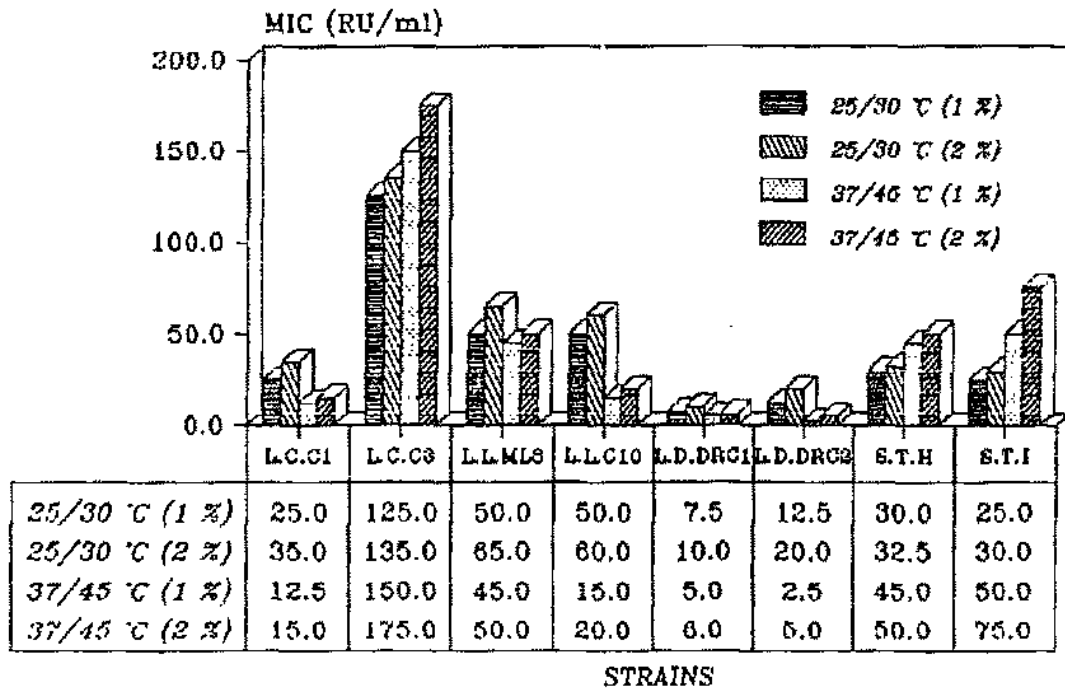
A considerable variation in growth behaviour of *Lactococcus* and *Streptococcus* sp. was recorded in control samples under different pH conditions (Fig.11). At pH 5.5, the growth of cultures reduced significantly in comparison to optimum pH 6.5. However, the reduction in growth of *L. lactis* subsp. *lactis* (C₁₀ and ML₈) at pH 5.5 was comparatively higher than other strains of lactococci and streptococci. It may be the possible explanation for their high nisin tolerance within pH range of 5.5-6.5. The drastic reduction in the growth of *Lactococcus* and *Streptococcus* sp. in broth at pH 5.5 demonstrate the possibility of some acid damaging effects on micro-organisms and may be partially responsible for such enhanced antimicrobial action of nisin under acidic conditions.

At alkaline pH 7.5, the growth of lactococci and streptococci was also affected to some extent, however, it was not as drastic as observed at pH 5.5. It shows that in addition to the effects of pH on growth

Fig 1C(a & b):INHIBITORY ACTION OF NISIN AGAINST *LACTOCOCCUS* AND *STREPTOCOCCUS* STRAINS IN BROTH AT MIN. AND MAX. pH

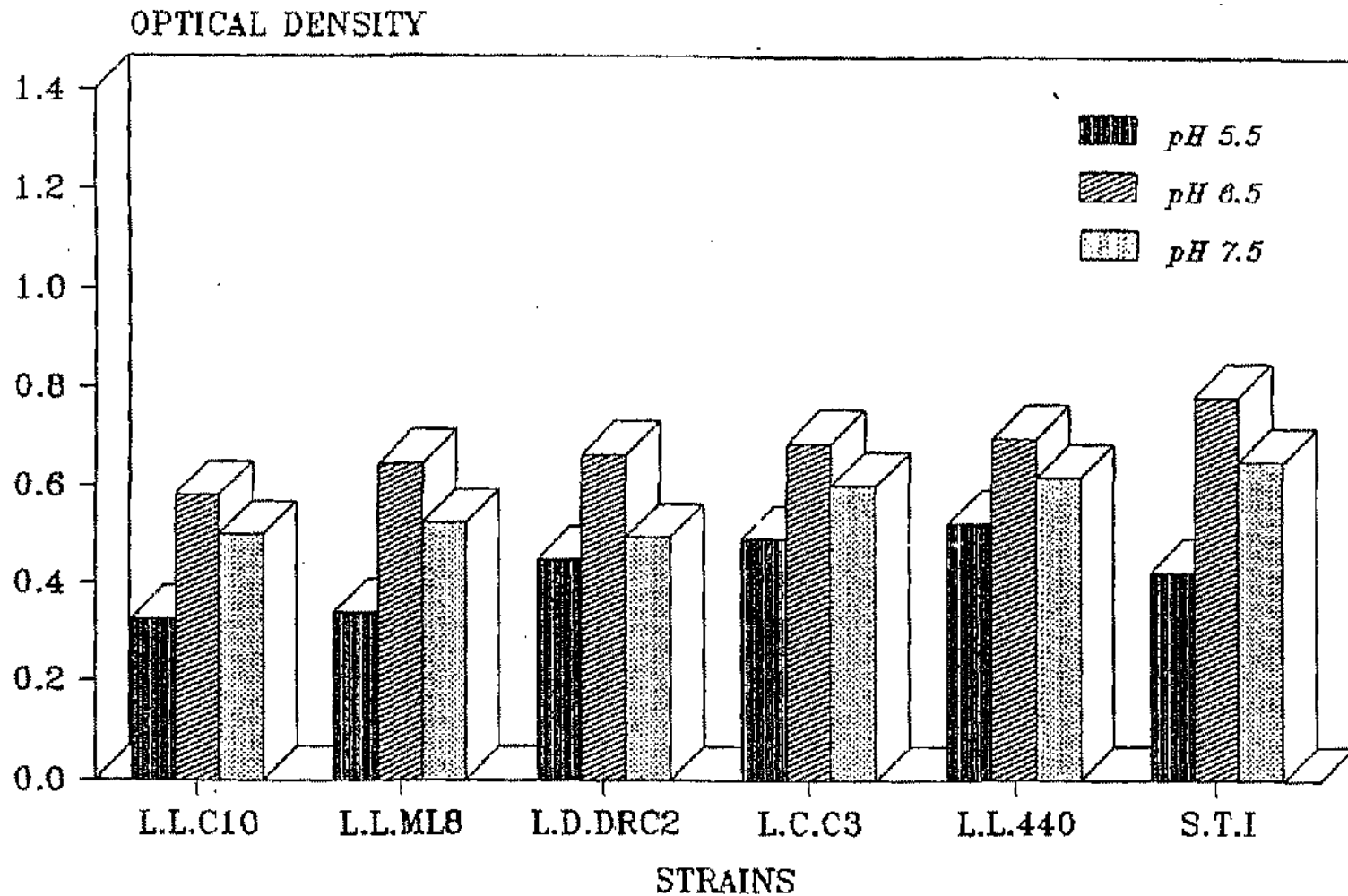


(a) MINIMAL pH (5.5)



(b) MAXIMAL pH (7.5)

Fig 11: EFFECT OF pH ON GROWTH PATTERN OF LACTOCOCCUS AND STREPTOCOCCUS STRAINS IN BROTH AT OPTIMUM TEMP. AND 1 % INOCULUM



behaviour of the organisms, some other factors like solubility and stability of nisin may be influenced by acidic, neutral and alkaline pH of the medium.

As reported earlier, nisin exhibits maximum stability under acidic conditions and its activity is rapidly lost under alkaline pH (Tramer, 1964; Lipinska, 1977 and Hurst, 1978). At pH 2.5, the solubility of nisin is 12% and decreased to 4% at pH 5.0. Nisin is being practically insoluble at neutral or alkaline pH as reported by Hall (1966).

Although specific information on effect of pH on inhibitory action of nisin against lactic cultures is not available, except a few reports on food borne pathogen, *Listeria monocytogenes*. A 16 fold increase in nisin tolerance of *L. monocytogenes* (4379) was observed by Mohamed et al. (1984) within pH range of 5.5-7.4. In a similar study, Benkerroum and Sandine, (1988) further demonstrated the pronounced inhibitory action of nisin at pH 3.5, 4.0 and 4.5 in comparison to pH 5.0, 6.5 and 7.0.

4.3.1.1.2 Mechanism of pH effect on inhibitory action of nisin:

The action of pH may effect either the growth pattern of the organisms or the solubility and stability of nisin. The enhanced effectiveness of nisin under acidic conditions may as well be due to some acid damaging effects on the growth of lactococci and streptococci or higher stability and solubility of nisin. However, it is difficult to conclude which of the two factors is significant. Earlier in 1981, Scott and Taylor; Mohamed et al., 1984 and Benkerroum and Sandine, 1988 also attributed to the similar effects of low pH for enhanced effectiveness of nisin.

The neutral or alkaline pH appears to have considerable effects on stability and solubility of nisin, however, the actual mechanism is yet to be known. In one report, Liu and Hansen (1990) demonstrated that inactivation of nisin at high pH may be because of Dehydroalanine (Dha) and Dehydrobutyrine (Dhb) which are susceptible to modification by nucleophiles (hydroxyl groups or nucleophilic R groups) that are present at high pH. The polymerisation of nisin molecules may also occur with the increase of pH as reported by Hall (1966). However, the relationship between the biological activity and the polymeric form of nisin has not been established yet.

4.3.1.2 Lactobacilli

The inhibitory action of nisin against 8 strains of *Lactobacillus* belonging to species, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. plantarum* was carried out in MRS broth at optimum temperature and under different pH 5.0, 6.0 (optimum) and 7.0. The MIC of nisin for *Lactobacillus* sp. ranged from 5-45, 20-125 and 35-150 RU/ml at pH 5.0, 6.0 and 7.0 respectively (Table 4). The nisin sensitivity of *Lactobacillus* sp. dropped by approx. 2-10 folds within pH range of 5.0-7.0.

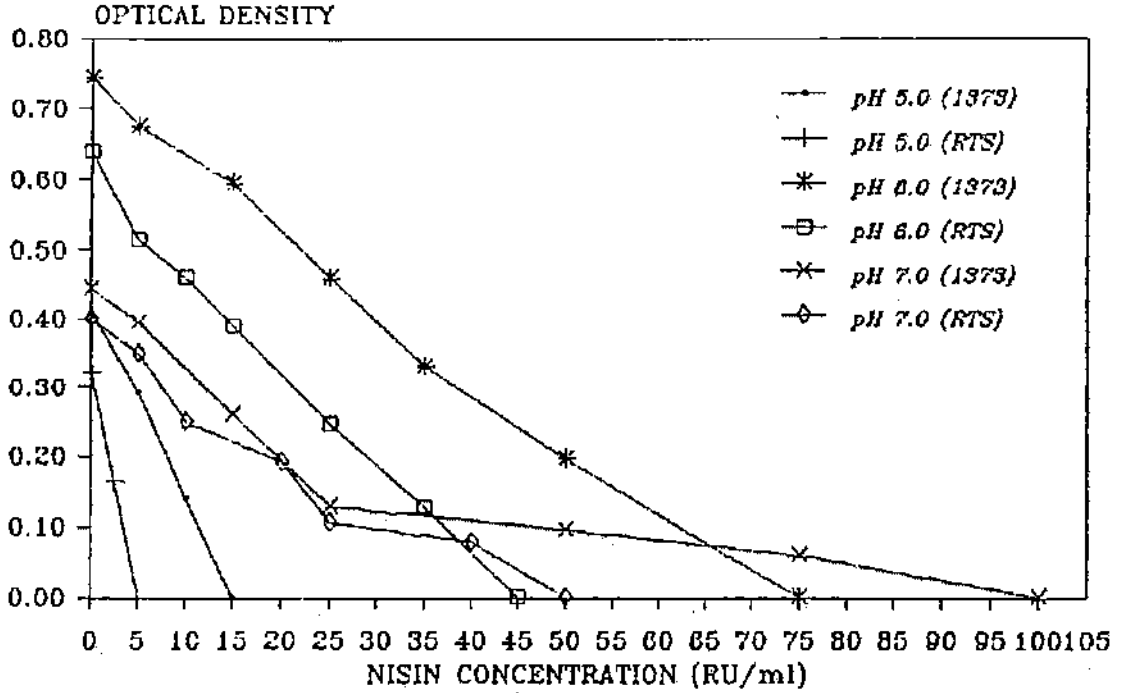
The increase of pH by one unit from 5.0 to 6.0 increased the nisin tolerance of *Lactobacillus* strains. However, the increase was comparatively higher in case of *L. delbrueckii* subsp. *bulgaricus* strains with 5-9 folds followed by 2-4 folds in *L. plantarum* and 1-3 folds in *L. acidophilus* strains. At pH 5.0 and 6.0, the MIC of nisin for *L. delbrueckii* subsp. *bulgaricus* (RTS, 1373) strains were 5, 15 and 45, 75 RU/ml. Whereas, for *L. acidophilus* (R, 447) strains the respective values were 10, 30 and 25, 50 RU/ml (Fig. 12a,b). *Lactobacillus*

Table 4. Effect of pH on inhibitory action of nisin against *Lactobacillus* strains at optimum temperature and different inoculum levels

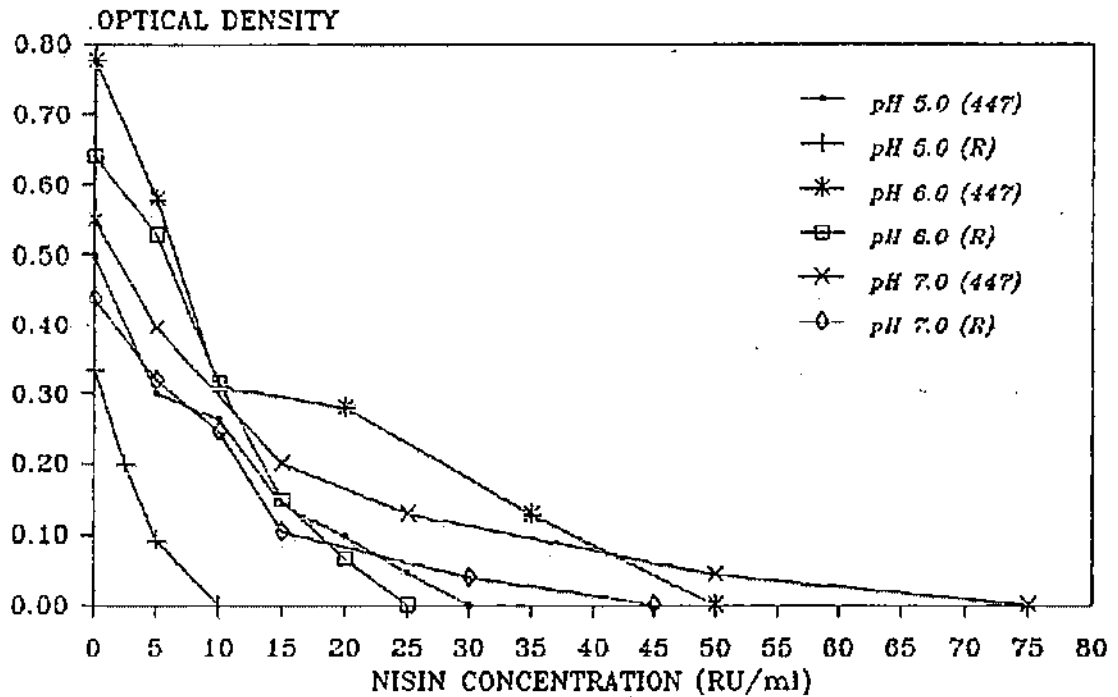
Sl. No.	Cultures	Inoculum levels (%)	MIC* (RU/ml) in broth at 24 h		
			5.0	Different pH 6.0	7.0
1.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (1373)	1	15	75	100
		2	20	100	125
2.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (RTS)	1	5.0	45	50
		2	10	60	75
3.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (W)	1	7.5	35	50
		2	10	50	75
4.	<i>L. acidophilus</i> (447)	1	30	50	75
		2	45	75	100
5.	<i>L. acidophilus</i> (R)	1	10	25	45
		2	15	45	60
6.	<i>L. acidophilus</i> (1899)	1	15	35	50
		2	20	50	75
7.	<i>L. plantarum</i> (R)	1	30	100	125
		2	45	125	150
8.	<i>L. plantarum</i> (89)	1	5.0	20	35
		2	10	25	50

* Arithmetic means of three determinations

Fig 12(a & b) : EFFECT OF pH ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM TEMPERATURE AND 1 % INOCULUM



(a) *L. DELBRUECKII SUBSP BULGARICUS*



(b) *L. ACIDOPHILUS*

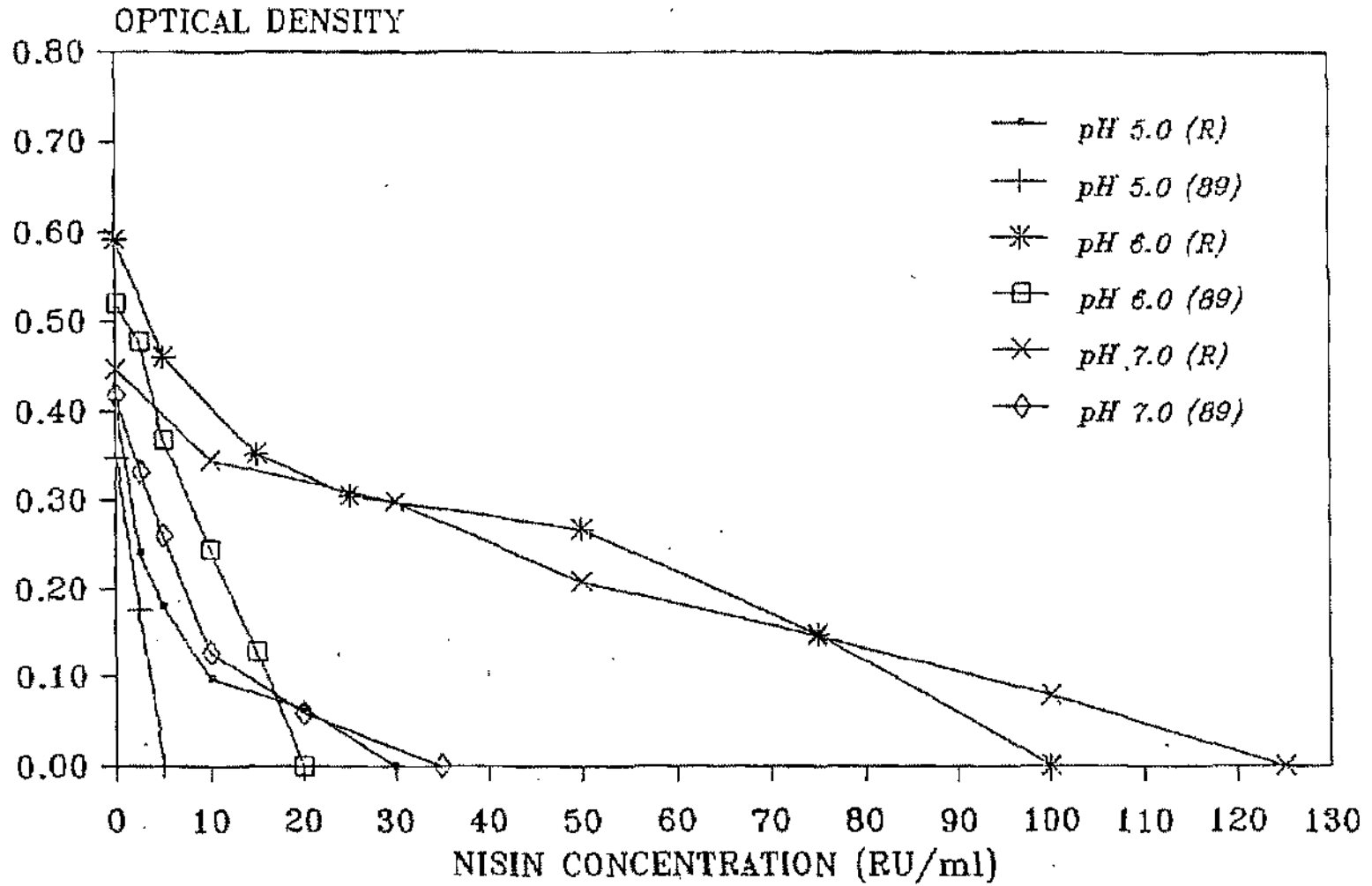
plantarum (89, R) differ widely in nisin tolerance at strain level and their MIC were 5, 30 and 20, 100 RU nisin/ml at pH 5.0 and 6.0 respectively (Fig. 13). At neutral pH, the nisin tolerance of *Lactobacillus* strains further increased by 1-2 folds and the MIC range of nisin for *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum* and *L. acidophilus* strains were 50-100, 35-125 and 45-75 RU/ml respectively (Figs. 12a,b and 13).

The effect of pH on nisin tolerance of *Lactobacillus* strains at sub-optimum temperatures was also studied. It was observed that sub-optimum temperatures affect the growth behaviour of the *Lactobacillus* strains adversely and therefore, relatively low MIC values were observed at pH 5.0, or 7.0 as compared to MIC at optimum temperature (Fig. 14a,b). The MIC range of nisin for *L. delbrueckii* subsp. *bulgaricus* strains at pH 5.0 and temperatures 37 and 48°C were 2.5-12.5 and 2.5-10 RU/ml whereas, the respective values at pH 7.0 were 25-75 and 10-30 RU nisin/ml. Similarly, the MIC range of *L. acidophilus* and *L. plantarum* strains at 30 and 45°C were 2.5-25, 2.5-10 RU/ml and 2.5-10, 2-15 RU/ml at pH 5.0. The respective values at pH 7.0 were 10-75, 20-50 RU/ml and 10-25, 10-100 RU/ml (Fig. 14a,b).

4.3.1-2.1 Effect on growth pattern

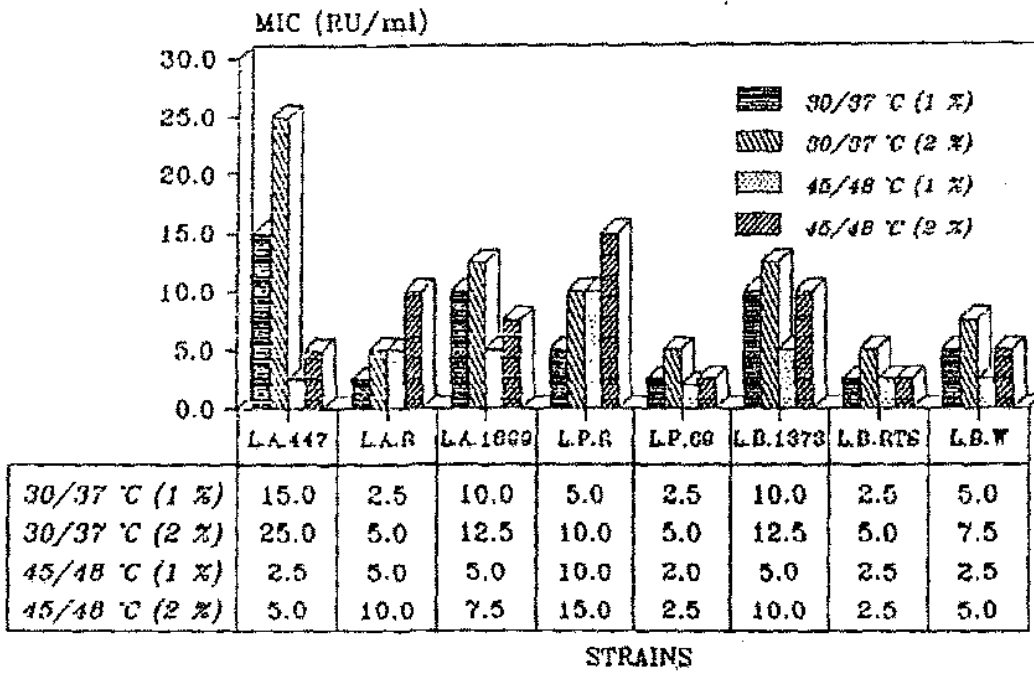
The metabolic activities of *Lactobacillus* sp. in MRS broth were maximum at pH 6.0. However, *L. delbrueckii* subsp. *bulgaricus* (1373), *L. acidophilus* (447) and *L. plantarum* (R) strains showed comparatively higher growth among 3 groups of *Lactobacillus* sp. The superior growth of these strains at optimum pH may be possible due to their higher nisin tolerance among the respective group (Fig. 15a,b).

Fig13: *EFFECT OF pH ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM TEMPERATURE AND 1 % INOCULUM*

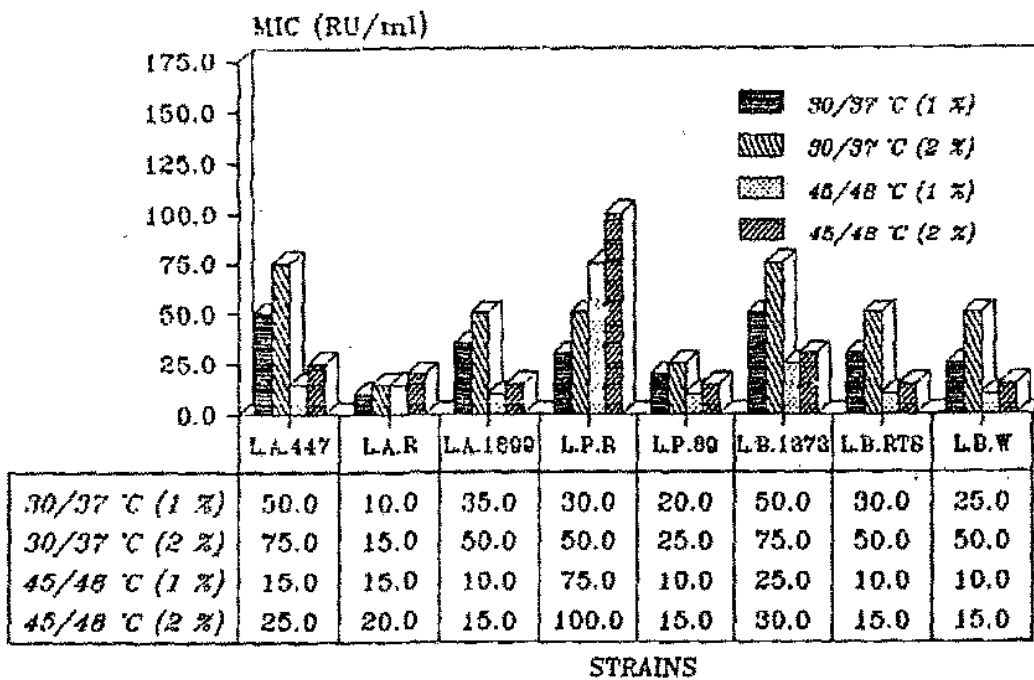


L. PLANTARUM

Fig 14 (a & b) : INHIBITORY ACTION OF NISIN AGAINST *LACTOBACILLUS* STRAINS IN BROTH AT MINIMAL AND MAXIMAL pH

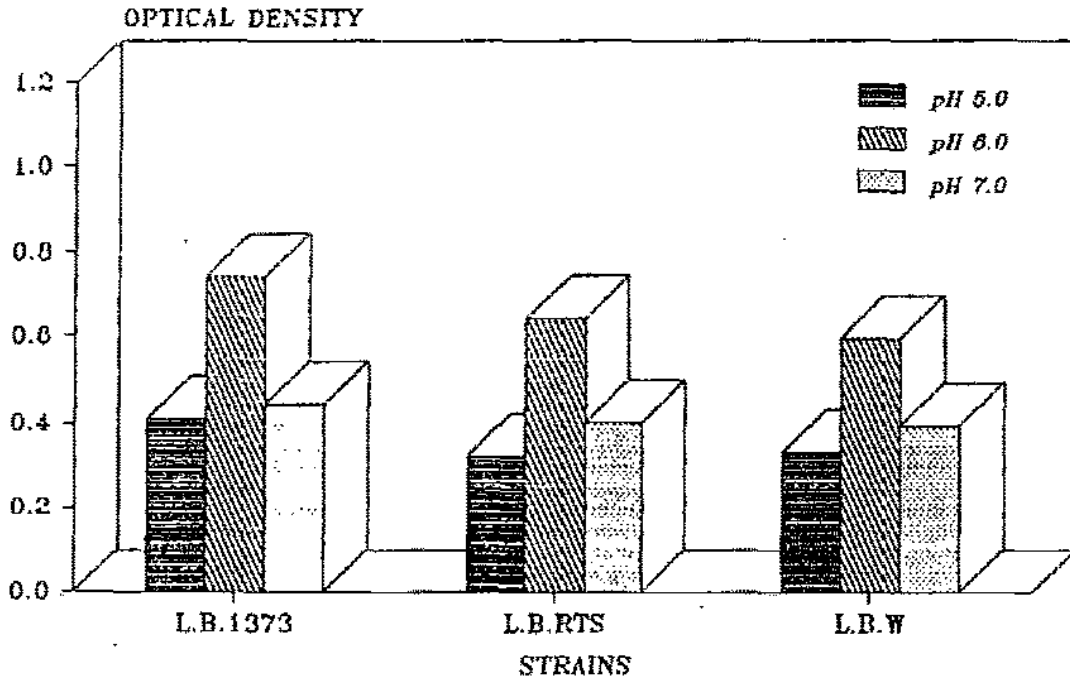


(a) MINIMAL pH (5.0)

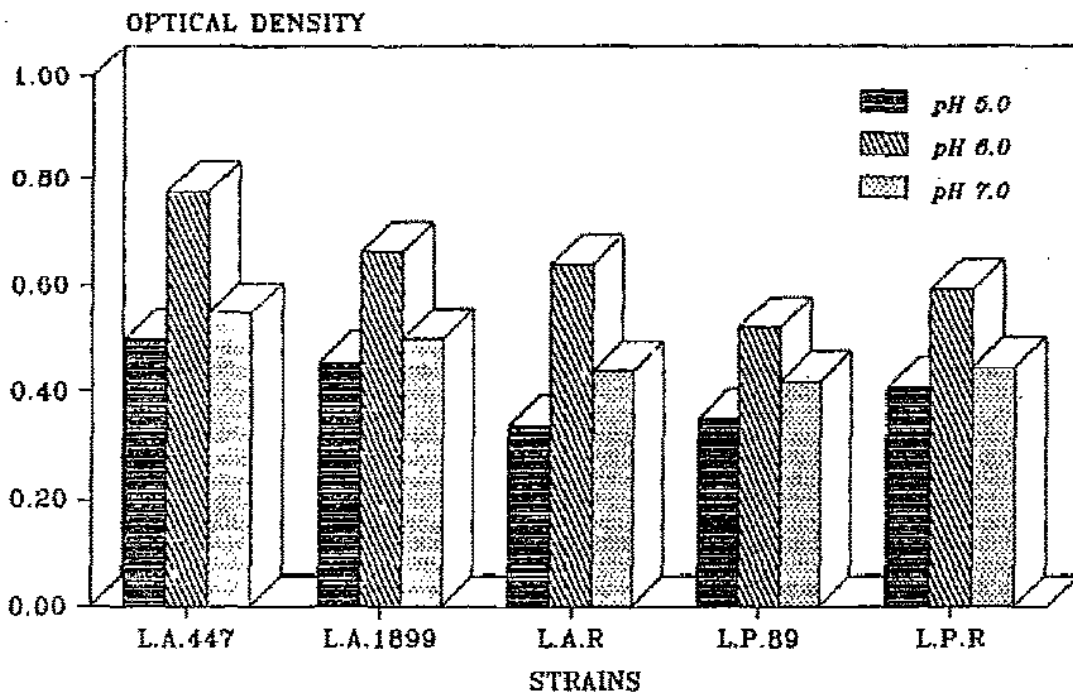


(b) MAXIMAL pH (7.0)

Fig15 (a & b) : EFFECT OF pH ON GROWTH PATTERN OF LACTOBACILLUS STRAINS IN BROTH AT OPT. TEMPERATURE & 1 % INOCULUM



(a) *L. DELBRUECKII SUBSP BULGARICUS*



(b) *L. ACIDOPHILUS & L. PLANTARUM*

The increase or decrease over optimum pH 6.0 by one unit impeded the growth of the organisms considerably. However, the effect was more or less comparable at both pH. The inhibitory mechanism of nisin against *Lactobacillus* sp. appears to be similar as observed in lactococci and streptococci.

4.3.1.3 Bacilli

Six strains of bacilli belonging to species, *B. cereus*, *B. subtilis* and *B. stearothermophilus* were evaluated for nisin tolerance in broth at neutral pH i.e. optimum and sub-optimum pH (6.5 or 7.5). The nutrient broth tubes inoculated with 1 and 2% cultures of *B. cereus* and *B. subtilis* were incubated at their optimum growth temperatures 35 and 37°C. However, *B. stearothermophilus* strains were incubated at thermophilic growth temperature 55°C for 24 h after inoculation in peptone-yeast-extract broth.

Nisin exhibited higher inhibitory action at pH 6.5 and progressively decreased with the rise of pH upto 7.5. The increase of pH by 0.5 units from 6.5 to 7.0 and 7.5 increased the nisin requirement from 15-75 to 45-175 and 60-225 RU/ml respectively for complete inhibition of *Bacillus* sp. (Table 5). The increase in nisin tolerance among bacilli within pH range of 6.5-7.5 was higher in *B. subtilis* strains (3-8 folds) followed by *B. cereus* (4-5 folds) and *B. stearothermophilus* (3 folds).

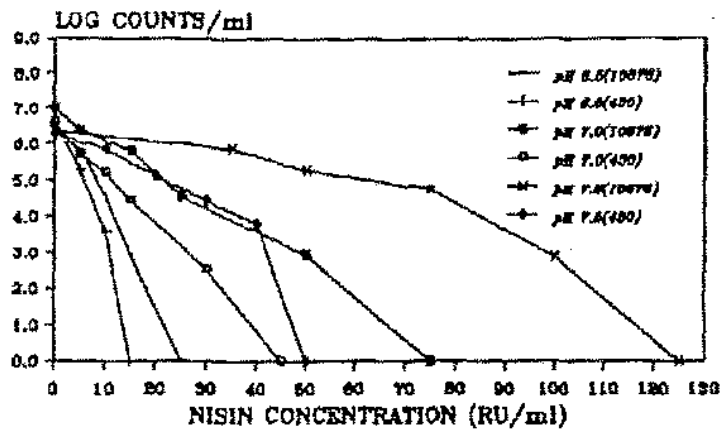
Bacillus cereus (430, 10876) strains were found comparatively more sensitive than *B. subtilis* and their MIC were 15-25, 45-75 and 60-125 RU/ml at pH 6.5, 7.0 and 7.5 respectively (Fig. 16a). Under similar conditions, *B. subtilis* (441, 9144) strains were inhibited with MIC range of 35-50, 135-150 and 175 RU/ml each at pH 6.5, 7.0 and 7.5 respectively

Table 5. Effect of pH on inhibitory action of nisin against *Bacillus* strains at optimum temperature and different inoculum levels

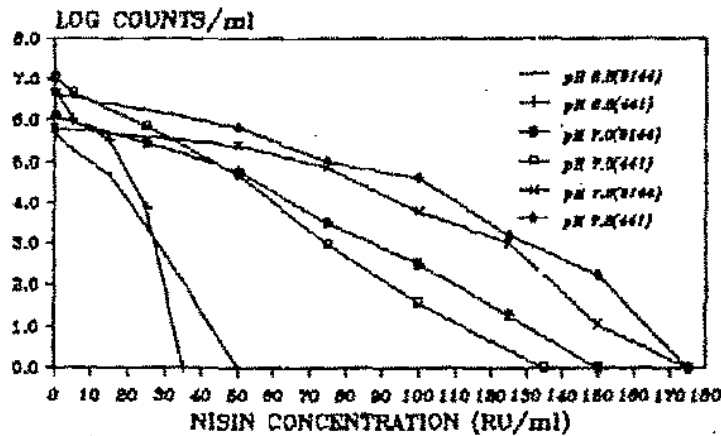
Sl. No.	Cultures	Inoculum levels (%)	MIC* (RU/ml) in broth at 24 h		
			6.5	Different pH 7.0	7.5
1.	<i>B. subtilis</i> (9144)	1	50	150	175
		2	75	175	200
2.	<i>B. subtilis</i> (441)	1	35	135	175
		2	50	150	200
3.	<i>B. subtilis</i> (6633)	1	25	125	200
		2	50	150	225
4.	<i>B. cereus</i> (10876)	1	25	75	125
		2	50	100	150
5.	<i>B. cereus</i> (430)	1	15	45	60
		2	20	60	75
6.	<i>B. stearothermophilus</i> (953)	1	20	45	60
		2	25	60	75

* Arithmetic means of three determinations

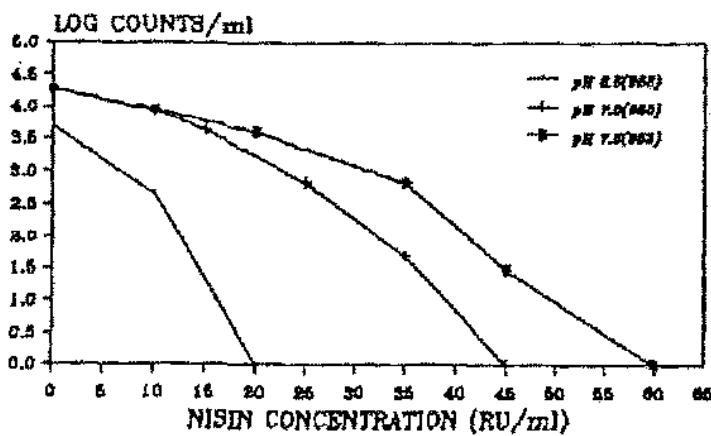
FIG 16(a-c) : EFFECT OF pH ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM TEMPERATURE AND 1 % INOCULUM



(a) *B. CEREUS*



(b) *B. SUBTILIS*



(c) *B. STEAROTHERMOPHILUS*

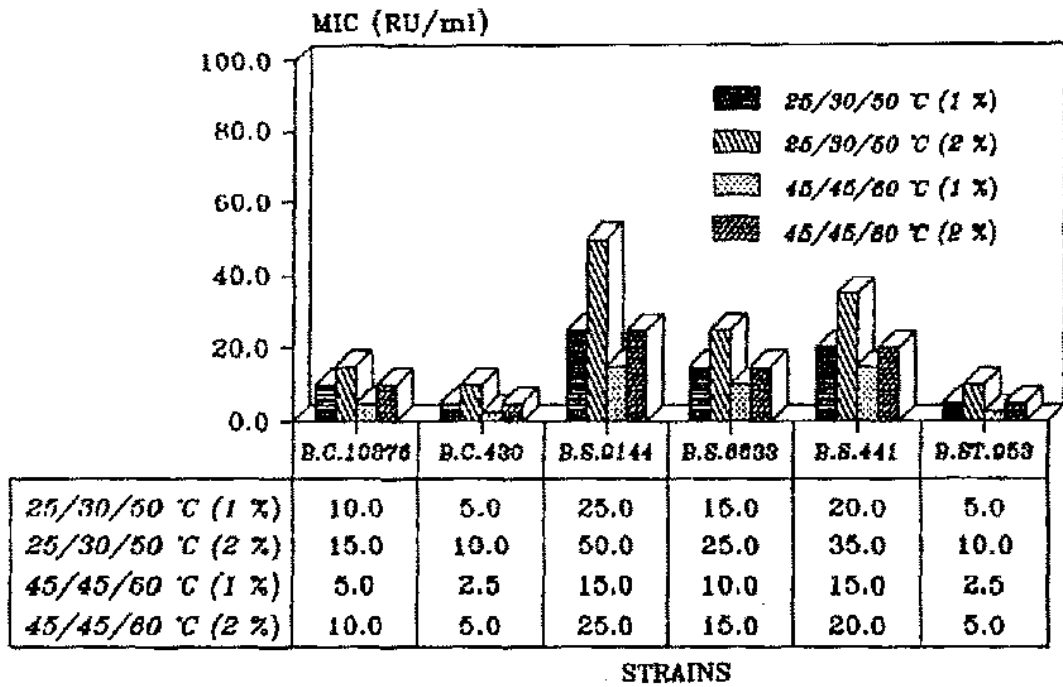
(Fig. 16b). As reported earlier, *B. stearothermophilus* strains were the most sensitive among *Bacillus* sp. However, their response was also similar under different pH conditions. The nisin tolerance of *B. stearothermophilus* (953) increased from 20 to 40 and 60 RU/ml with the increase of pH from 6.5 to 7.0 and 7.5 (Fig. 16c).

The information on the effect of pH on vegetative cells is meagre with few reports on spores of bacilli and clostridia. Complete inhibition of 31 strains of *B. coagulans* at 200 and 560 IU/ml in basamin broth at pH 5.3 and 7.2 was observed by Campbell and Sniff (1959). A decrease of 0.3 units to pH 5.7 enabled 500 IU nisin/ml to inhibit the growth of *Bacillus* spores (10^6 /ml) inoculum at 30°C, however, at pH 6.0 no nisin concentration was effective (Oscroft et al., 1990).

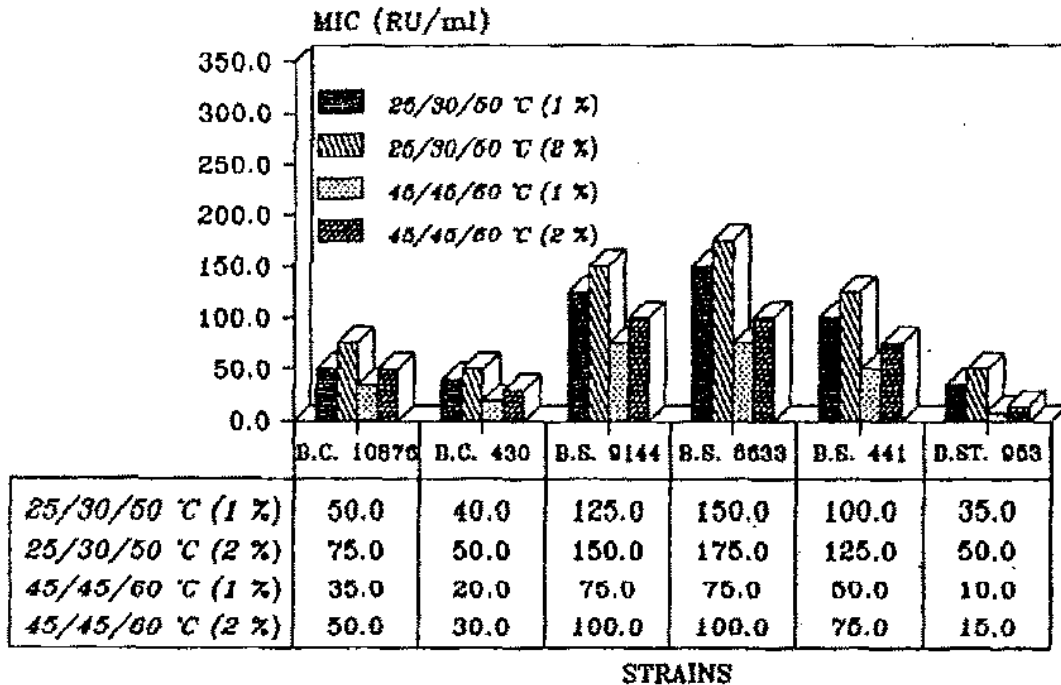
The effectiveness of nisin further improved when the optimum temperatures was either decreased or increased to sub-optimum levels under similar pH conditions. The increase in pH by one unit from 6.5 to 7.5 at 25°C increased the nisin tolerance of *B. cereus* from 5-15 to 40-75 RU/ml. Whereas, at 45°C it increased from 2.5-10 to 20-50 RU/ml respectively (Fig. 17a,b). Similarly, for *B. subtilis* strains at 30 and 45°C, the MIC increased from 15-50 to 100-175 and 10-25 to 50-100 RU/ml respectively with the increase of pH from 6.5 to 7.5. The inhibitory action of nisin at 45°C was considerably higher than 25 and 30°C within 6.5-7.5 pH range.

The nisin requirement for complete inhibition of *B. stearothermophilus* (953) at 50°C increased from 5-10 to 35-50 RU/ml with the increase of pH from 6.5 to 7.5. However, at 60°C the effect was low and MIC varied from 2.5-5 to 10-15 RU/ml within pH 6.5-7.5.

Fig17(a & b) : INHIBITORY ACTION OF NISIN AGAINST *BACILLUS* STRAINS IN BROTH AT MINIMAL AND MAXIMAL pH



(a) MINIMAL pH (6.5)



(b) MAXIMAL pH (7.5)

The increase or decrease of optimum pH 7.0 by 0.5 units adversely affects the growth behaviour of *B. cereus*, *B. subtilis* and *B. stearothermophilus* strains. The factors responsible for enhanced antimicrobial action of nisin under acidic conditions appears to be similar as observed by Scott and Taylor (1981) who demonstrated that some factors like acid damaging effects on the spores of *Cl. botulinum* and higher stability of nisin were responsible for enhanced effectiveness of nisin under acidic conditions.

This information on effect of pH on nisin action can be of great significance in low and high acid foods where the spoilage like sweet curdling, bitterness or flat sour are commonly caused by aerobic and anaerobic spore formers.

4.3.2 Effect of Incubation Temperature

The nisin tolerance of lactic and non-lactic cultures was evaluated in broth at optimal and sub-optimal growth temperatures. An 18-20 h old culture pre-grown at optimum temperature, centrifuged and washed with saline was inoculated in broth containing different concentrations of nisin, at 1 and 2 % levels. The MIC were determined at 24 h of incubation on the basis of optical density for lactics and SPC/ml for non-lactics. In general, the efficacy of nisin against lactic and non-lactic cultures increased with the decrease or increase of optimum growth temperature.

4.3.2.1 Lactococci and streptococci

Any increase or decrease of optimum growth temperature decreased the nisin tolerance of lactococci and streptococci by approx. 2-12 folds. The complete inhibition of *Lactococcus* sp. was observed at MIC range of 2-65, 5-200 and 2-100 RU/ml at 25, 30 (optimum) and 37°C whereas,

Streptococcus sp. were inhibited at MIC values of 10-15, 75-150 and 20-50 RU/ml at 30, 37 (optimum) and 45°C as presented in Table 6.

At sub-optimum temperatures of 25 or 37°C, the decrease in nisin tolerance among *L. lactis* subsp. *lactis* (nisin and non-nisin producing strains), *L. lactis* subsp. *diacetylactis* was comparatively more at 37°C. *Lactococcus lactis* subsp. *lactis* (ML₉) was inhibited at 40, 100 and 45 RU/ml at 25, 30 and 37°C (Fig. 18a) whereas, *L. lactis* subsp. *lactis* (C₁₀) was inhibited at 30, 50 and 15 RU/ml respectively under similar conditions. Likewise, the MIC values for *L. lactis* subsp. *diacetylactis* (DRC₂) were 7.5, 27.5 and 2.5 RU/ml at 25, 30 and 37°C respectively (Fig. 18b).

Nisin producing strains of *L. lactis* subsp. *lactis* albeit, responded similarly, however, the inhibition was observed only at very high concentrations of nisin as explained earlier. The MIC values for *L. lactis* subsp. *lactis* (440) were 1200, 2400 and 800 RU/ml at 25, 30 and 37°C respectively (Fig. 18c).

The response of few strains among *Lactococcus* and *Streptococcus* sp. like *L. lactis* subsp. *cremoris* (C₃) and *Str. salivarius* subsp. *thermophilus* (H, I) was relatively different at sub-optimum temperatures and exhibited lower nisin tolerance at 25 or 30°C instead of 37 or 45°C. The nisin tolerance of *L. lactis* subsp. *cremoris* (C₃) at optimum temperature 30°C was 200 RU/ml whereas, at sub-optimum temperatures of 25 and 37°C was 65 and 100 RU/ml (Fig. 19a). Similarly, for *Str. salivarius* subsp. *thermophilus* (I) the inhibition at 37°C was observed at 150 RU/ml whereas, at 30 and 45°C, the inhibition occurred at comparatively low nisin concentration of 15 and 50 RU/ml (Fig. 19b). Teply (1962) observed an enhanced effect of nisin action against nisin

Table 6. Effect of temperature on inhibitory action of nisin against *Lactococcus* and *Streptococcus* strains at optimum pH and different inoculum levels

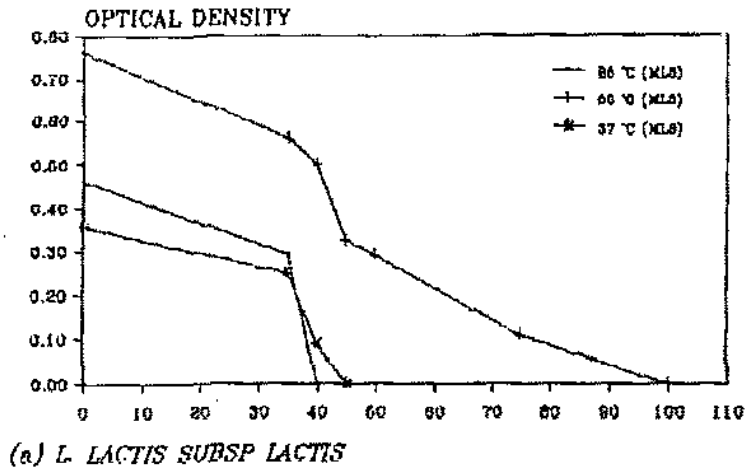
Sl. No.	Cultures	Inoculum levels (%)	MIC* (RU/ml) in broth at 24 h		
			25/30°C**	Different temperature 30/37°C**	37/45°C**
1.	<i>L. lactis</i> subsp. <i>lactis</i> (C ₁₀)	1	25	35	10
		2	30	50	15
2.	<i>L. lactis</i> subsp. <i>lactis</i> (ML ₈)	1	35	75	25
		2	40	100	45
3.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)	1	2.0	5.0	2.0
		2	5.0	10	2.5
4.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)	1	5.0	25	2.0
		2	7.5	27.5	2.5
5.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)	1	12.5	35	5.0
		2	20	45	6.0
6.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₃)	1	50	175	75
		2	65	200	100
7.	<i>L. lactis</i> subsp. <i>lactis</i> (440)*** (nisin producer)	1	800	2000	600
		2	1200	2400	800
8.	<i>L. lactis</i> subsp. <i>lactis</i> (496)*** (nisin producer)	1	600	1600	400
		2	800	2000	600
9.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (H)	1	10	75	20
		2	12.5	100	22.5
10.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (I)	1	10	125	35
		2	15	150	50

* Arithmetic means of three determinations

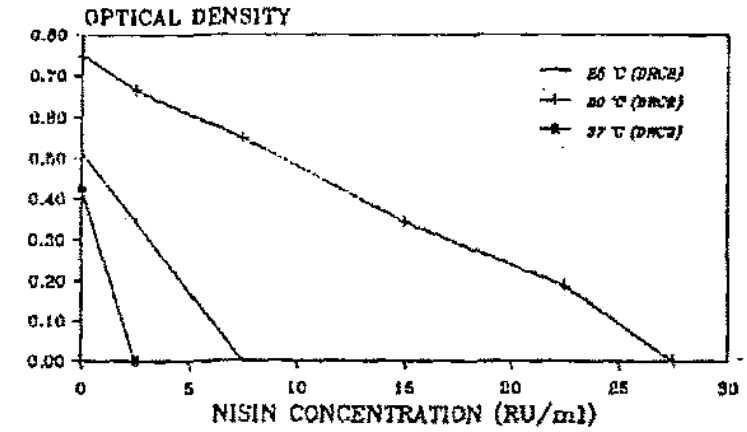
** *Lactococcus*/*Streptococcus*

*** MIC at 18 h for nisin producer

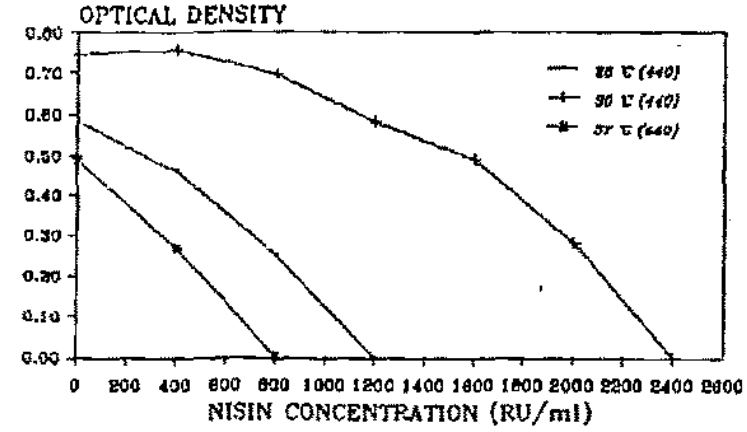
Fig 18 (a-c) : EFFECT OF TEMPERATURE ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM pH AND 2 % INOCULUM



(a) *L. LACTIS SUBSP LACTIS*

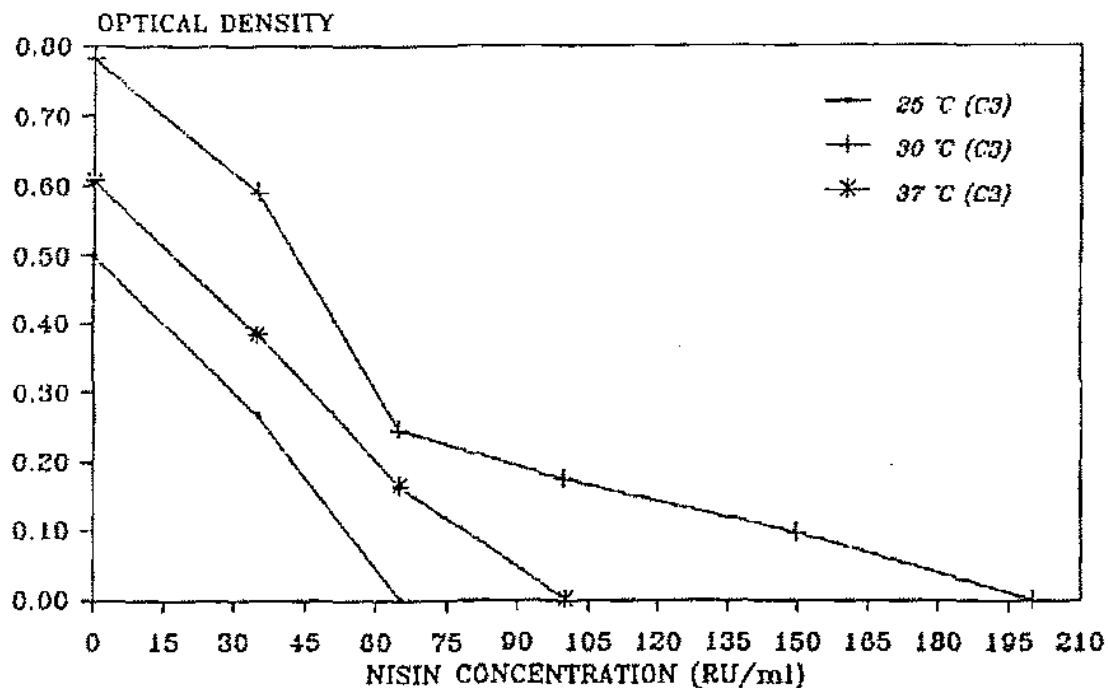


(b) *L. LACTIS SUBSP DIACETYLACTIS*

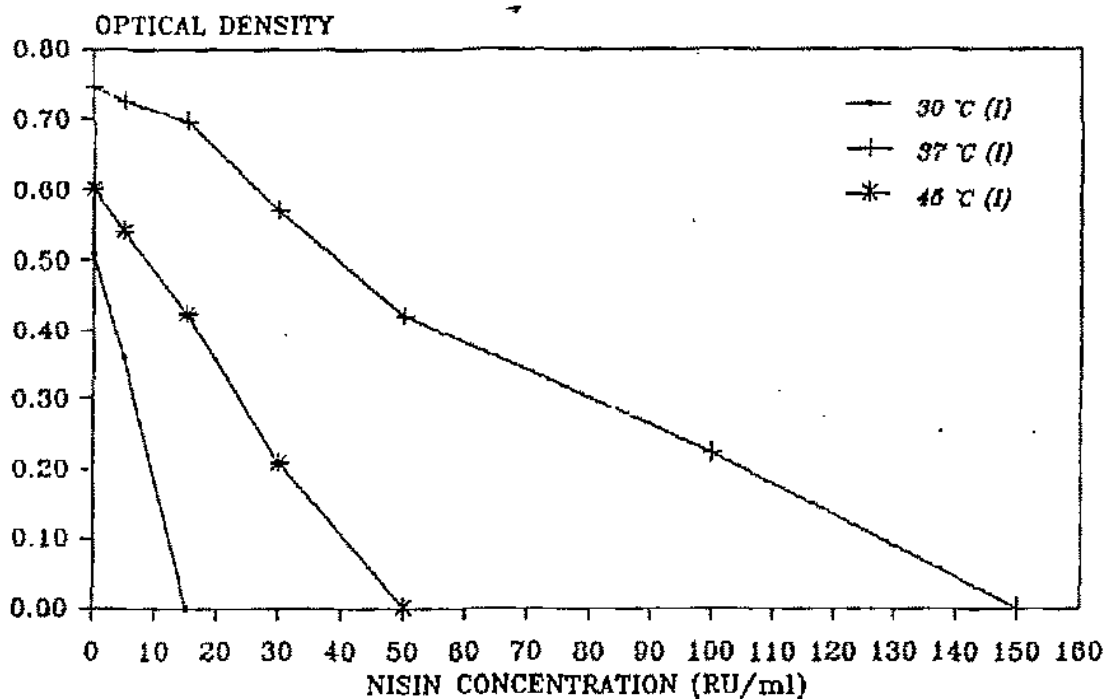


(c) *L. LACTIS SUBSP LACTIS*
(NISIN PRODUCER)

Fig19(a & b) : EFFECT OF TEMPERATURE ON
INHIBITORY ACTION OF NISIN IN BROTH AT
OPTIMUM pH AND 2 % INOCULUM



(a) *L. LACTIS SUBSP CREMORIS*



(b) *STR. SALIVARIUS SUBSP THERMOPHILUS*

producing strain of *Streptococcus lactis*, when the temperature of incubation was increased from 30 to 55°C. In a similar study, Mohamed et al. (1984) further reported 8 folds increase in inhibitory action of nisin against *Listeria monocytogenes* (4379) with the increase of temperature from 22 to 37°C and the respective MIC values were 256 and 32 IU/ml.

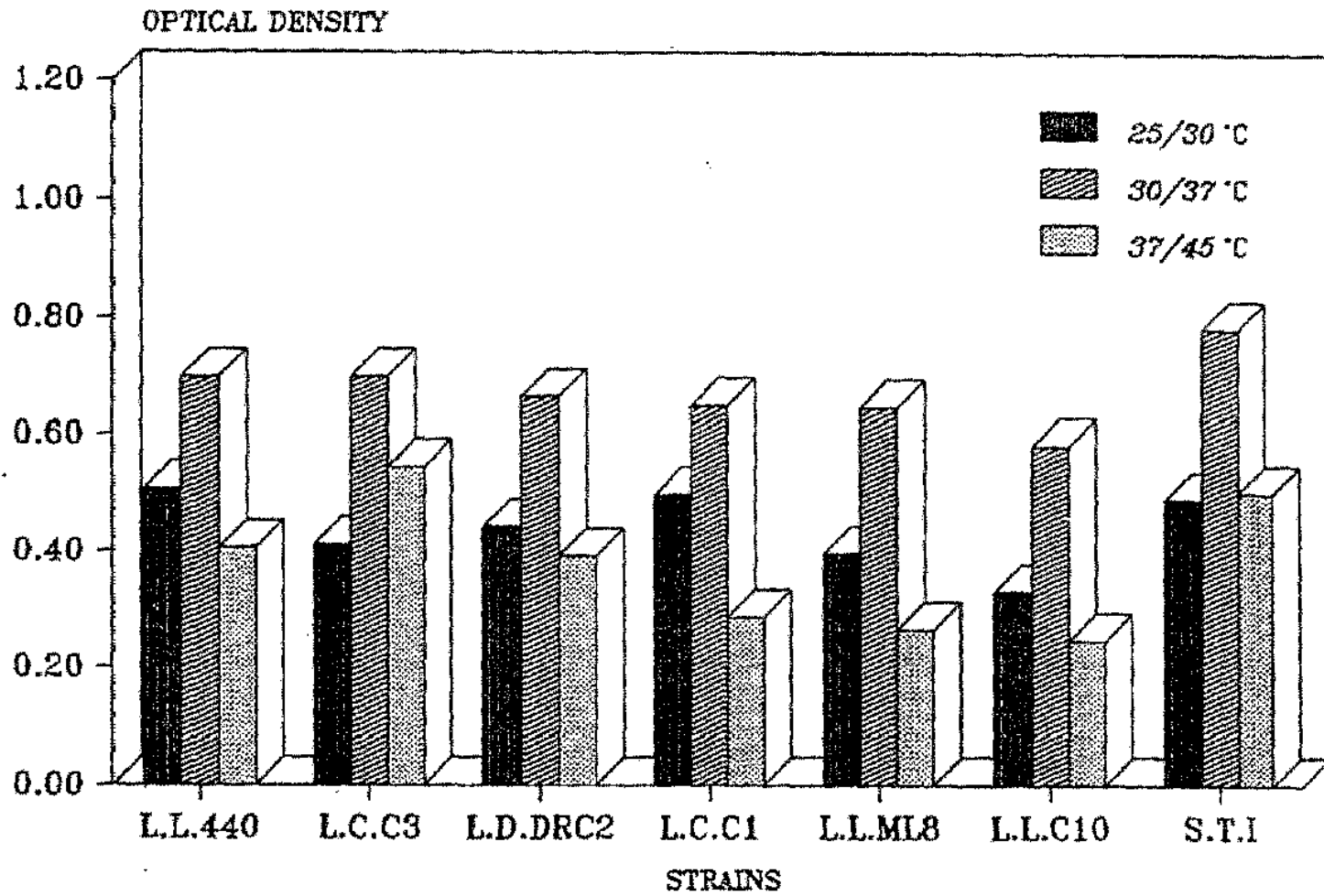
4.3.2.1.1 Effect on growth pattern:

The growth of *Lactococcus* and *Streptococcus* sp. in control samples was remarkably high at optimum growth temperatures; 30 and 37°C. However, a pronounced adverse effect on growth pattern was observed with either, the decrease or increase of optimum temperature (Fig. 20). Among lactococci, the adverse effect of sub-optimum temperatures 25 or 37°C, with the exception of *L. lactis* subsp. *cremoris* (C₃) was prominent at 37°C. However, in streptococci, the inhibition was relatively more at 30°C than 45°C.

The higher synthesis of unsaturated fatty acid (UFA) at sub-optimum temperature was observed by Rose (1968) and this may cause different sensitivities among micro-organisms when treated with bacteriocidal substances (Dajani et al., 1970). However, the mechanism is not understood. The correlation between the nisin tolerance and the growth pattern of lactococci and streptococci i.e. higher is the growth, more will be the nisin tolerance and vice-versa sounds valid to some extent, however, may not be sufficient per se for complete and explicit delineation of the fact.

The higher nisin tolerance of *Lactococcus* and *Streptococcus* sp. at optimum growth temperature albeit, difficult to explain. It may be possible that after initial bacteriostatic action of nisin on lactococcal

Fig20 : EFFECT OF TEMPERATURE ON GROWTH PATTERN OF LACTOCOCCUS & STREPTOCOCCUS STRAINS IN BROTH AT OPT.pH & 1% INOCULUM



and streptococcal cells which occurs maximally in log phase along with few cells in lag or stationary phase, and enter lag phase again (Jarvis, 1967). Henceforth, the incubation of these lag phase cells under different temperatures, higher cell counts will be expected at optimum growth temperature and hence, the requirement of nisin for complete inhibition of large inoculum at optimum temperature within 24 h will be more in comparison to sub-optimum temperatures.

4.3.2.2 Lactobacilli

The behaviour of 8 strains of lactobacilli belonging to species *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. plantarum* was evaluated in MRS broth inoculated at 1 and 2% levels under different concentrations and temperatures. The optimum and sub-optimum temperatures used for *L. delbrueckii* subsp. *bulgaricus* were 42 and 37 or 48°C whereas, for *L. acidophilus* and *L. plantarum* were 37 and 30 or 45°C respectively. The response of *Lactobacillus* sp. showing maximum nisin tolerance at optimum temperature was comparable as observed earlier in lactococci and streptococci.

The MIC range of nisin for *L. delbrueckii* subsp. *bulgaricus* strains at optimum 42°C and sub-optimum temperatures 37 and 48°C were 35-100, 15-60 and 5-20 RU/ml respectively. Whereas, for *L. acidophilus* strains the respective figures were 25-75, 10-50 and 10-30 RU/ml at 37, 30 and 45°C (Table 7). *Lactobacillus plantarum* strains were the most resistant and sensitive among 8 strains of lactobacilli and their inhibition was observed at 20-100, 10-50 and 5-75 RU nisin/ml at 37 (optimum), 30 and 45°C respectively.

Any increase or decrease of optimum growth temperature in general, enhanced the inhibitory action of nisin against *Lactobacillus* sp. The

Table 7. Effect of temperature on inhibitory action of nisin against *Lactobacillus* strains at optimum pH and different inoculum levels

Sl. No.	Cultures	Inoculum levels (%)	MIC* (RU/ml) in broth at 24 h		
			30/37°C**	Different temperatures 37/42°C**	45/48°C**
1.	<i>L. acidophilus</i> (447)	1	35	50	10
		2	50	75	20
2.	<i>L. acidophilus</i> (R)	1	10	25	15
		2	15	45	30
3.	<i>L. acidophilus</i> (1899)	1	25	35	10
		2	40	50	20
4.	<i>L. plantarum</i> (R)	1	25	100	50
		2	50	125	75
5.	<i>L. plantarum</i> (89)	1	10	20	5
		2	15	25	10
6.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (1373)	1	50	75	15
		2	60	100	20
7.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (RTS)	1	30	45	10
		2	40	60	20
8.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (W)	1	15	35	5
		2	30	50	10

* Arithmetic means of three determinations

** *L. delbrueckii* subsp. *bulgaricus* strains : 37/42/48°C

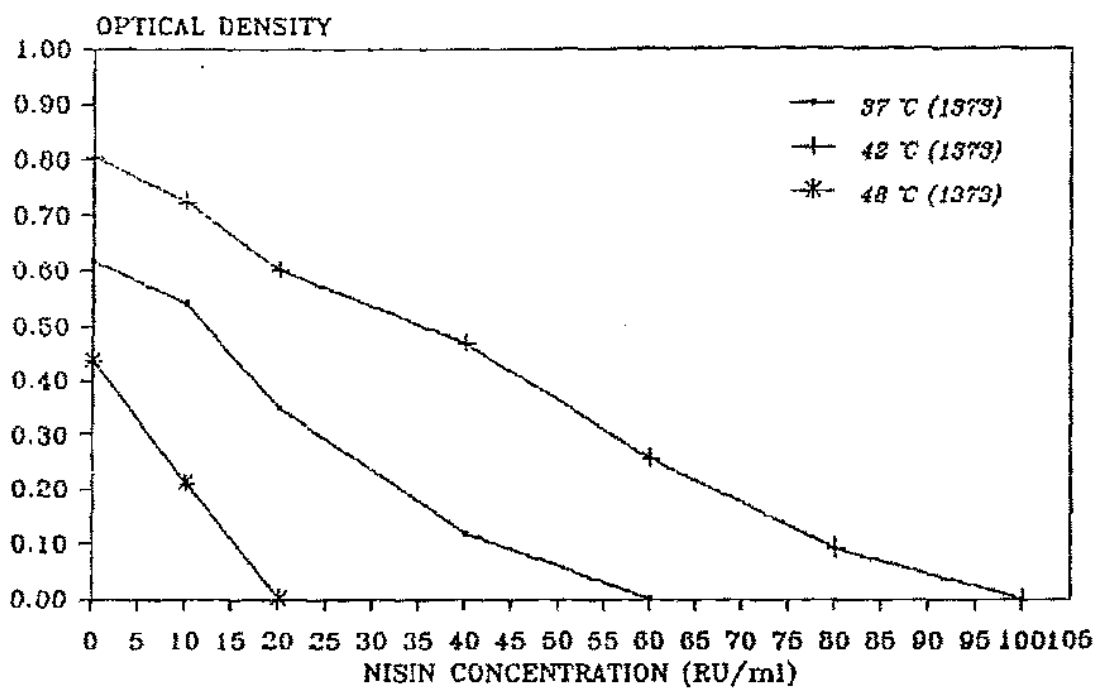
decrease in nisin tolerance of *L. delbrueckii* subsp. *bulgaricus* strains at sub-optimum growth temperature 37 or 48°C, was relatively higher at 48°C. The inhibition of *L. delbrueckii* subsp. *bulgaricus* (RTS, 1373) strains was observed at 60, 100; 40, 60 and 20 RU/ml each at 42, 37 and 48°C respectively (Fig. 21a,b). Similar response was obtained among *L. acidophilus* and *L. plantarum* strains at sub-optimum temperatures with the exception of *L. acidophilus* (R) and *L. plantarum* (R) where the inhibitory action of nisin was relatively higher at 30°C than 45°C. The requirement of nisin for complete inhibition of *L. acidophilus* (1899, 447) strains were 50, 75; 40, 50 and 20 RU/ml each at 37, 30 and 45°C respectively (Fig. 22a,b). Whereas, *L. plantarum* (89, R) strains were inhibited at 25, 125; 15, 50 and 10, 75 RU/ml at 37, 30 and 45°C respectively (Fig. 23a, b).

Like lactococci and streptococci, the importance of effect of temperature on inhibitory action of nisin against *Lactobacillus* sp. was overlooked in the past. However, in an exclusive report, Lipinska (1977) demonstrated that the nisin tolerance of *L. acidophilus* strain which was initially resistant to 200 IU nisin/ml at optimum growth temperature, dropped by 10 folds with the increase of temperature to 55°C.

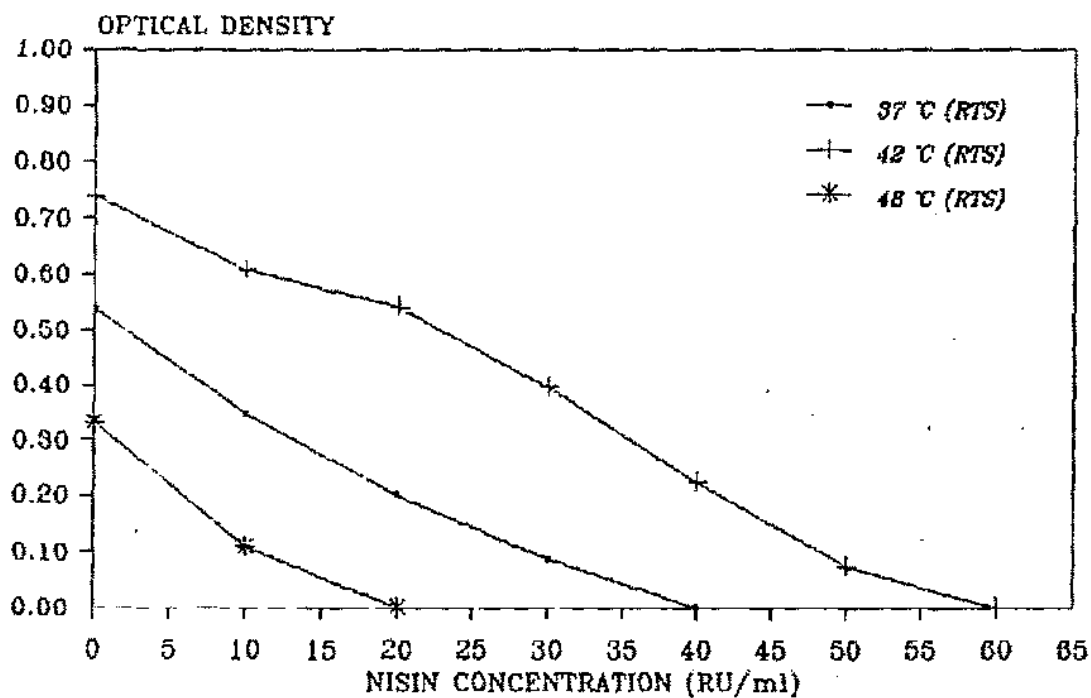
4.3.2.2.1 Effect of growth pattern:

In overall, the growth of *Lactobacillus* sp., especially *L. delbrueckii* subsp. *bulgaricus* (1373), *L. acidophilus* (R) and *L. plantarum* (R) was stupendous at optimum growth temperature (Fig. 24a,b). The increase or decrease of optimum growth temperature adversely affected the growth pattern of *Lactobacillus* strains. The effect was more pronounced with the increase of optimum temperature with the exception of *L. acidophilus* (R) and *L. plantarum* (R).

Fig 21(a & b) : EFFECT OF TEMPERATURE ON
INHIBITORY ACTION OF NISIN IN BROTH AT
OPTIMUM pH AND 2 % INOCULUM

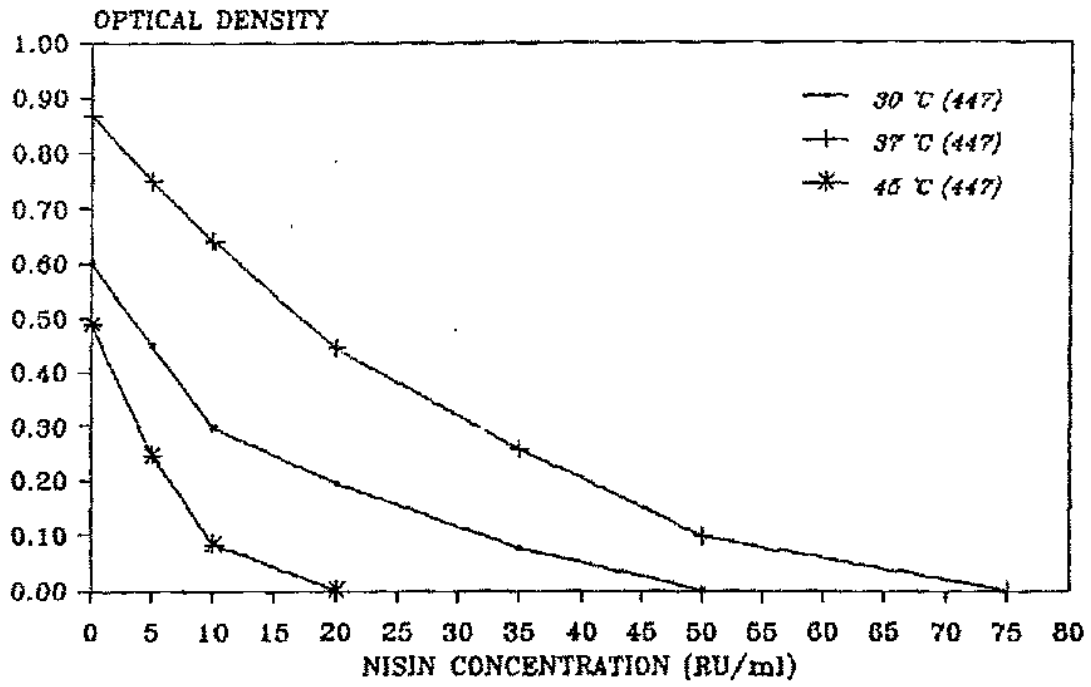


(a) *L. DELBRUECKII* SUBSP *BULGARICUS*

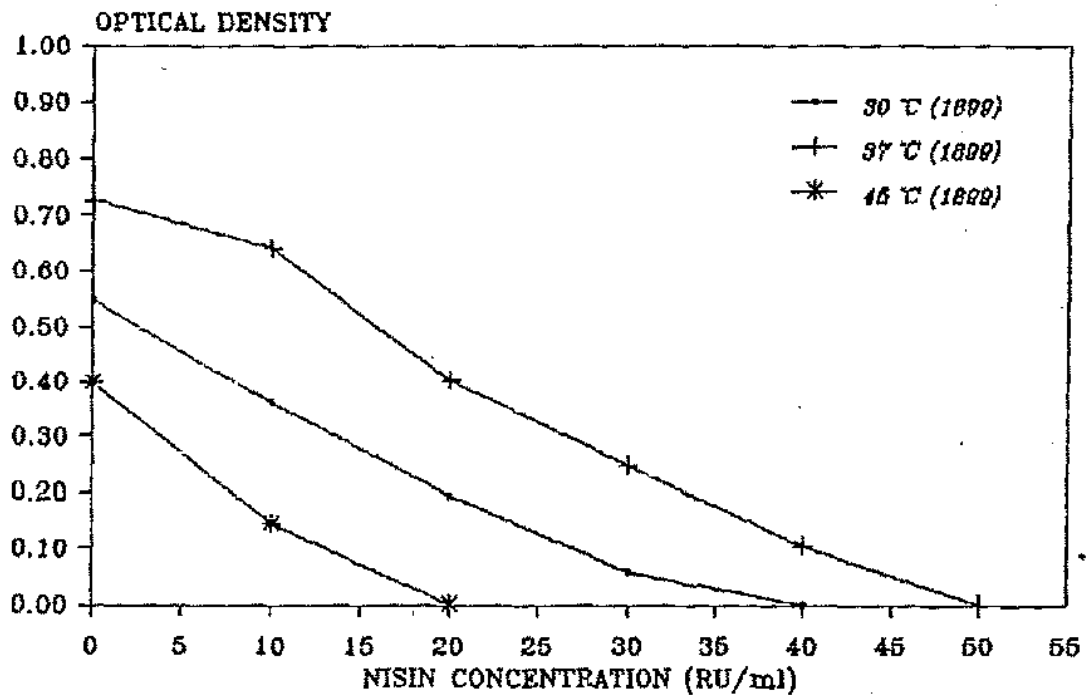


(b) *L. DELBRUECKII* SUBSP *BULGARICUS*

Fig 22(a & b) : EFFECT OF TEMPERATURE ON
INHIBITORY ACTION OF NISIN IN BROTH AT
OPTIMUM pH AND 2 % INOCULUM

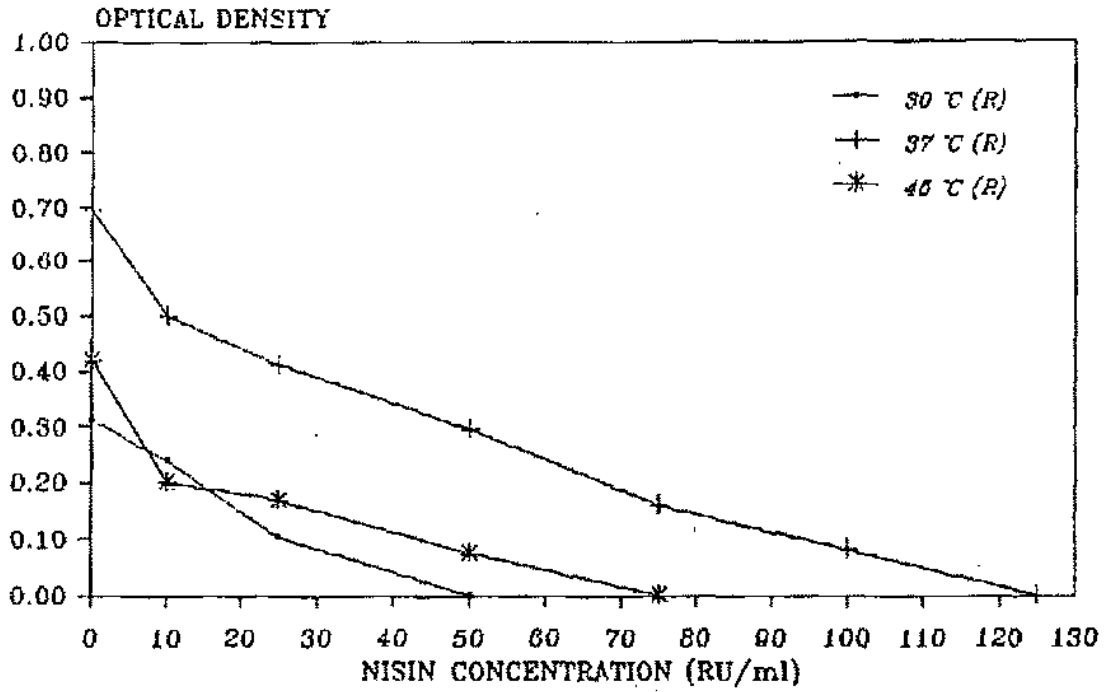


(a) *L. ACIDOPHILUS*

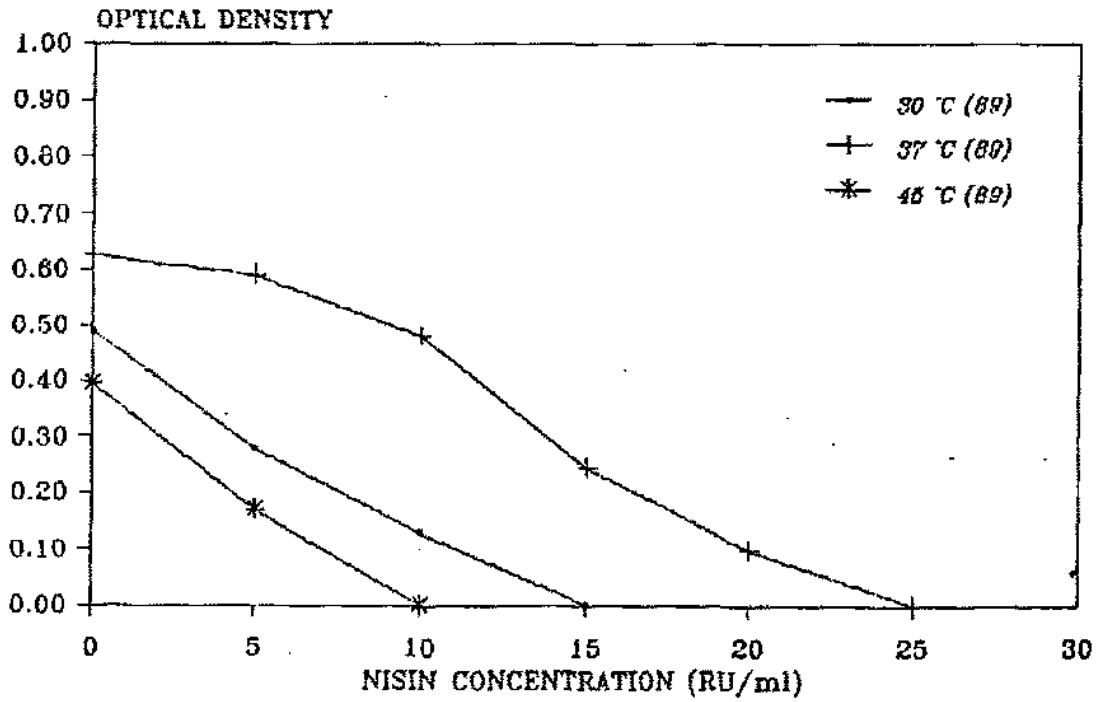


(b) *L. ACIDOPHILUS*

Fig23 (a & b) : EFFECT OF TEMPERATURE ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM pH AND 2 % INOCULUM

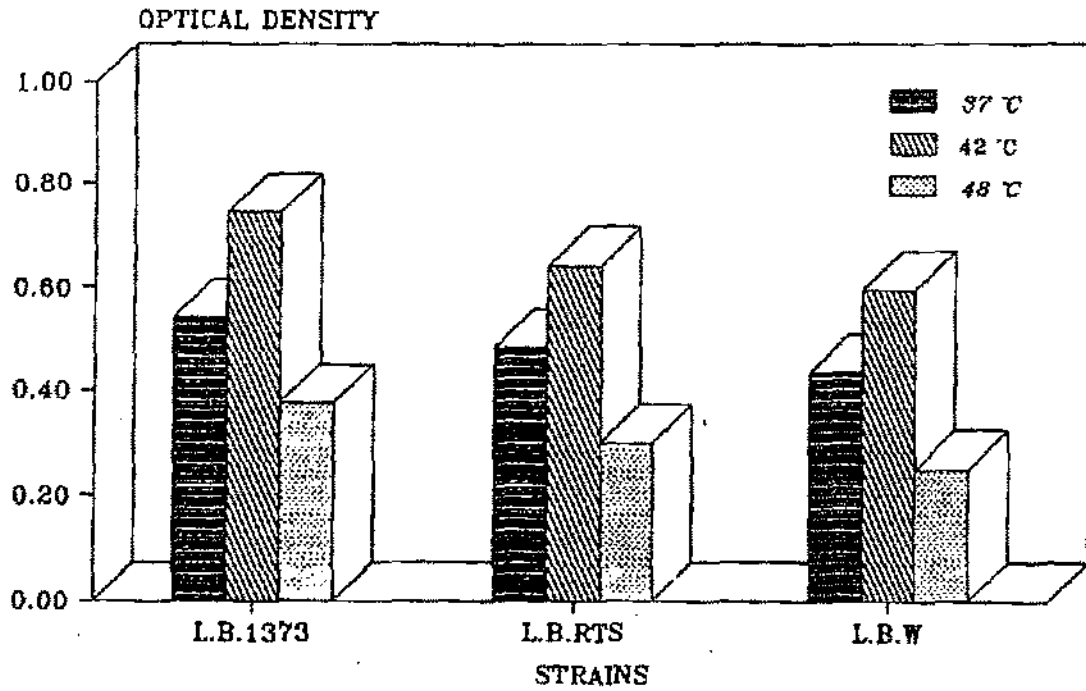


(a) *L. PLANTARUM*

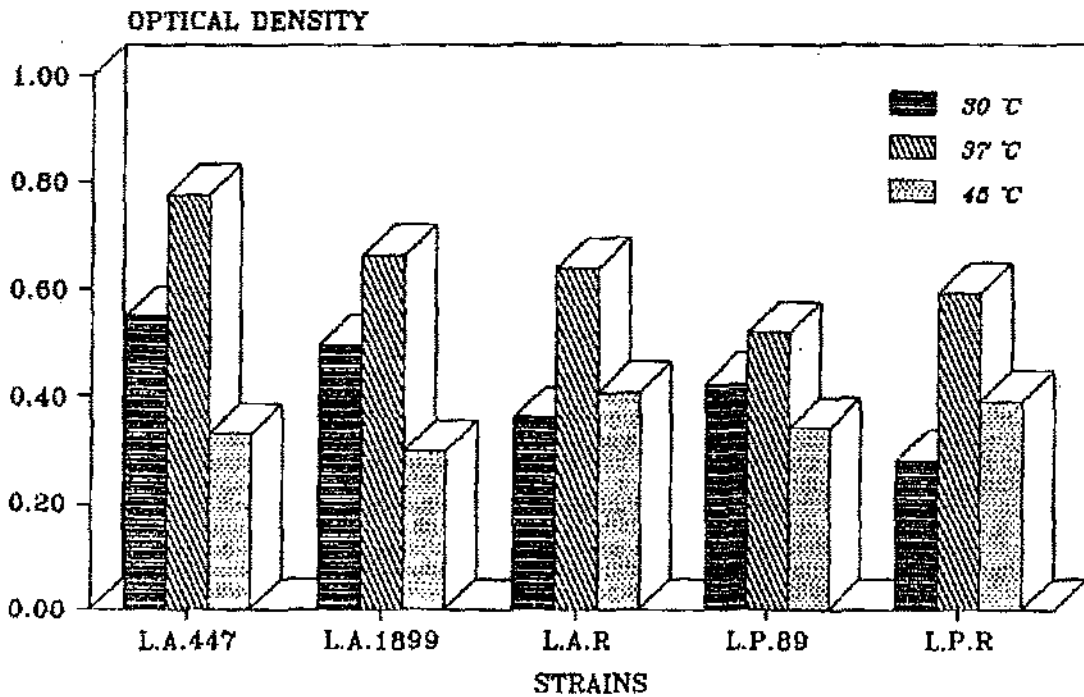


(b) *L. PLANTARIUM*

FIG 24(a & b) : EFFECT OF TEMPERATURE ON GROWTH PATTERN OF LACTOBACILLUS STRAINS IN BROTH AT OPTIMUM pH AND 1 % INOCULUM



(a) *L. DELBRUECKII SUBSP BULGARICUS*



(b) *L. ACIDOPHILUS AND L. PLANTARUM*

The variation in growth behaviour and nisin tolerance observed in *Lactobacillus* sp. followed the same pattern as observed in lactococci and streptococci and therefore, the possible reasons for differential nisin sensitivity at optimum and sub-optimum temperatures could be explained on similar lines as discussed earlier.

4.3.2.3 Bacilli

Six strains of bacilli belonging to species, *B. cereus*, *B. subtilis* in nutrient broth and *B. stearothermophilus* in peptone-yeast-extract broth were evaluated for their nisin tolerance at 1 and 2% inoculum and different temperatures. The optimum temperature used for *B. cereus*, *B. subtilis* and *B. stearothermophilus* strains were 35, 37 and 55°C, while sub-optimum temperatures were 25, 45; 30, 45 and 50, 60°C respectively.

As reported earlier, *B. subtilis* strains were the most nisin resistant among *Bacillus* sp. followed by *B. cereus* and *B. stearothermophilus*. The nisin tolerance of *Bacillus* sp. was maximum at optimum growth temperature, however, the adverse effects observed at sub-optimum temperatures were similar to lactic cultures. The MIC of nisin for *B. subtilis* strains were 125-175, 50-100 and 25-60 RU/ml at 37, 30 and 45°C respectively whereas, for *B. cereus* strains the respective figures were 45-100, 15-50 and 10-25 RU/ml at 35, 25 and 45°C (Table 8). The efficacy of nisin against *Bacillus* sp. in general, increased with either increase or decrease of optimum growth temperature. The decrease of optimum temperature 37°C of *B. subtilis* (441, 9144) strains to 30°C decreased the nisin tolerance from 150 and 175 RU/ml to 75 and 100 RU/ml. However, the effect was comparatively more when the temperature was increased to 45°C and the inhibition was observed at 50 RU/ml each (Fig. 25a,b). Similarly, the inhibition of *B. cereus* (10876), strain was observed at 50, 100 and 25 RU/ml at 25, 35 and 45°C respectively (Fig. 26a). *Bacillus*

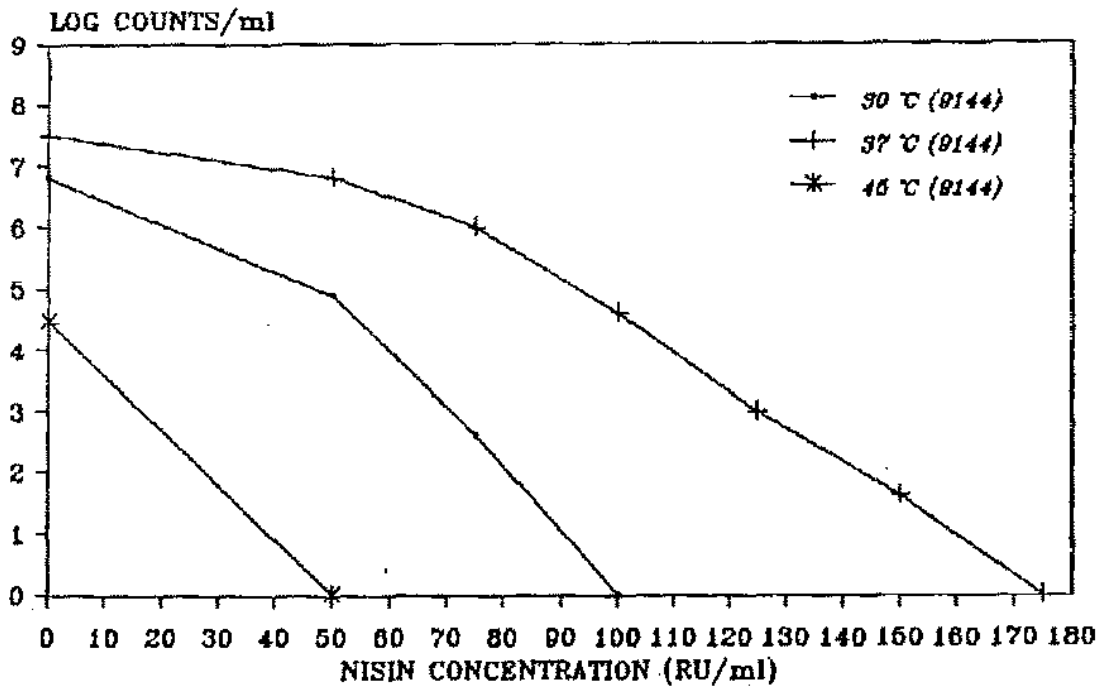
Table 8. Effect of temperature on inhibitory action of nisin against *Bacillus* strains at optimum pH and different inoculum levels

Sl. No.	Cultures		Inoculum levels (%)	MIC* (RU/ml) in broth at 24 h		
				25/30/50°C**	Different temperatures 35/37/55°C**	45/45/60°C**
1.	<i>B. cereus</i>	(10876)	1	25	75	15
			2	50	100	25
2.	<i>B. cereus</i>	(430)	1	15	45	10
			2	25	60	15
3.	<i>B. subtilis</i>	(9144)	1	75	150	35
			2	100	175	50
4.	<i>B. subtilis</i>	(441)	1	50	135	25
			2	75	150	50
5.	<i>B. subtilis</i>	(6633)	1	60	125	40
			2	80	150	60
6.	<i>B. stearothermophilus</i>	(953)	1	25	45	10
			2	35	60	15

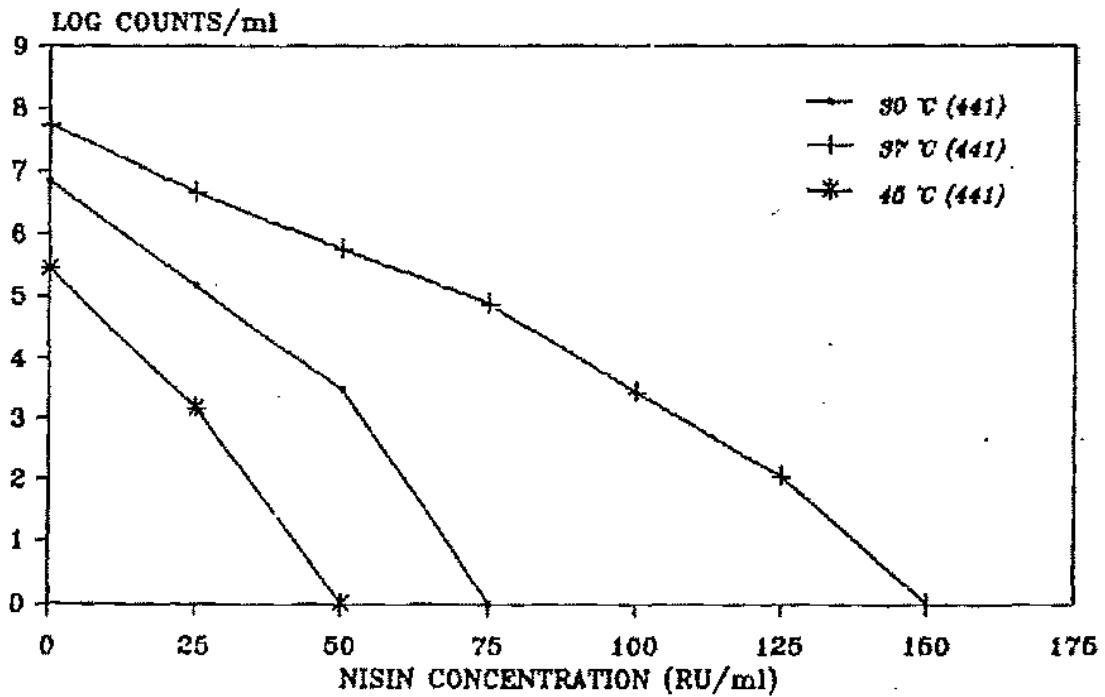
* Arithmetic means of three determinations

** *B. cereus*/*B. subtilis*/*B. stearothermophilus*

Fig.25(a & b) : EFFECT OF TEMPERATURE ON INHIBITORY ACTION OF NISIN IN BROTH AT OPTIMUM pH AND 2 % INOCULUM



(a) *B. SUBTILIS*



(b) *B. SUBTILIS*

stearothermophilus (953) strain also followed a similar trend and was inhibited at nisin concentration of 35, 60 and 15 RU/ml at 50, 55 and 60°C respectively (Fig. 26b).

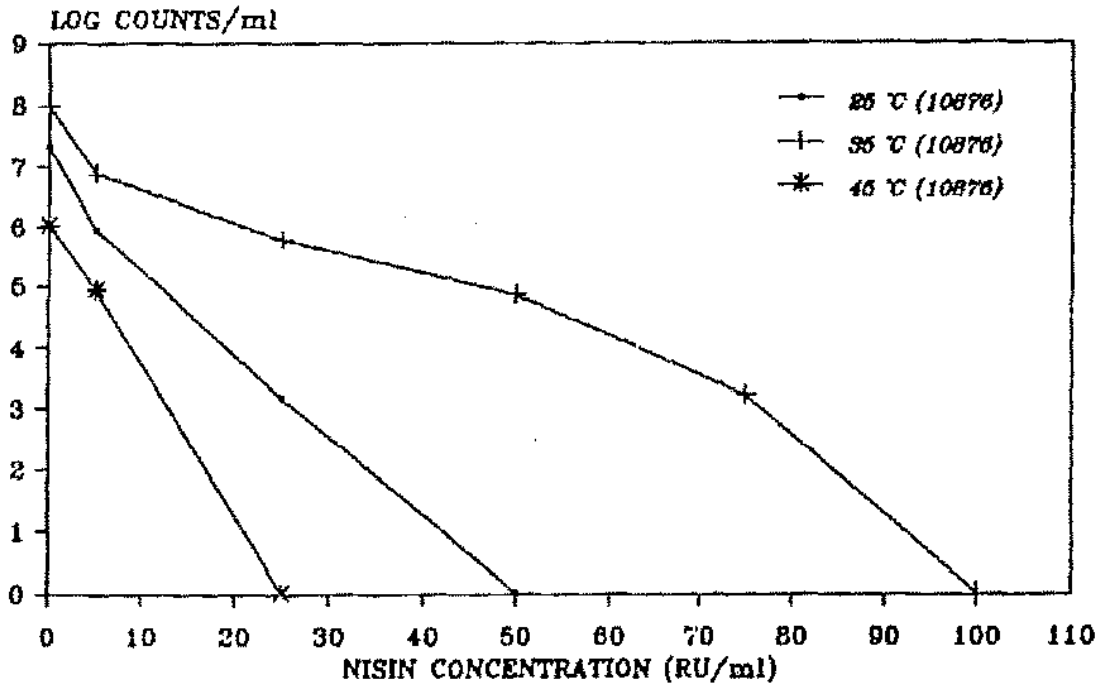
Campbell and Sniff (1959) studied the effect of different temperatures 35, 45 and 55°C on 31 strains of *B. coagulans* and reported that the inhibition was observed at 14 µg/ml after 7 days of incubation, irrespective of different temperatures. In another study, Oscroft et al. (1990) reported that the efficacy of nisin was adversely affected at or near optimum growth temperature. The germination and outgrowth of *Bacillus* spores at 12°C was severely restricted in comparison to 20 or 30°C.

The salient findings on nisin tolerance of lactic and non-lactic culture at their optimum and sub-optimum growth temperatures can be of great value for controlling the growth of these micro-organisms in fermented and non-fermented milk and milk products.

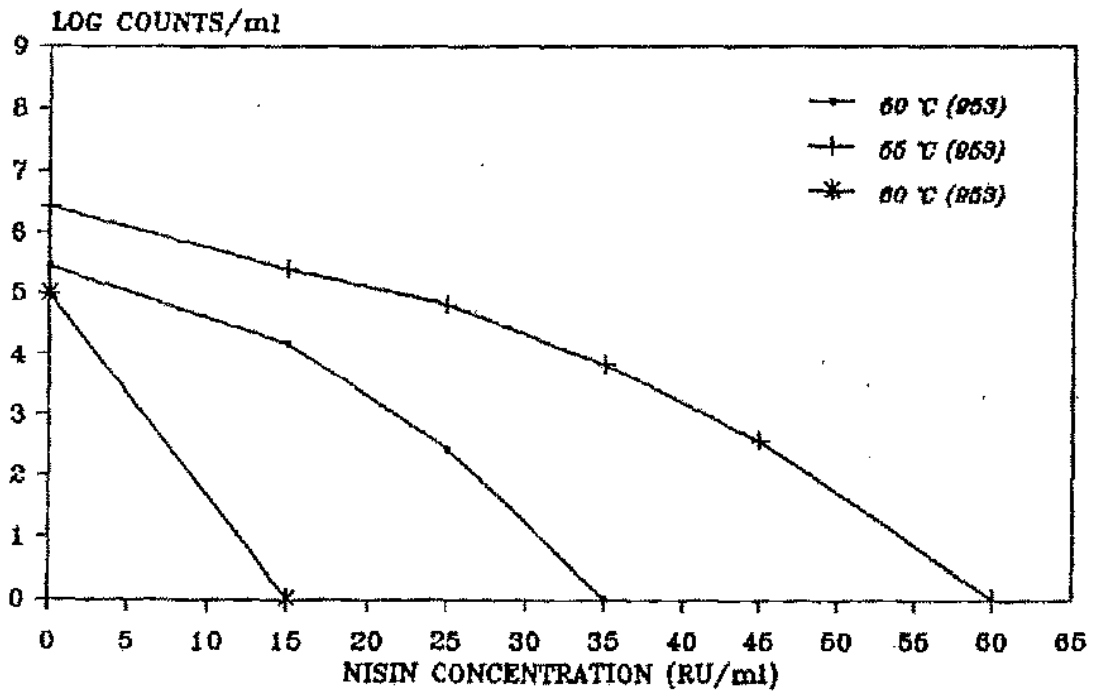
4.3.3 Effect of Inoculum

The behaviour of lactic and non-lactic cultures in broth containing different concentrations of nisin was evaluated under optimum growth conditions and two different inocula i.e. 1 and 2% levels. The efficacy of nisin was adversely affected with the increase of inoculum and 1-2 folds increase in nisin tolerance was observed among the strains studied. Similar variation in nisin tolerance was also observed when broth adjusted to different pH and inoculated with cultures at 1 and 2% levels was incubated at sub-optimum temperatures as reported earlier (Tables 3-8). The nisin tolerance of 26 strains of lactic and non-lactic cultures was found to increase from 0.5-2000 to 2.0-2400 RU/ml with the increase of 1 to 2% inoculum (Table 9).

FIG 26(a & b) : EFFECT OF TEMPERATURE ON
INHIBITORY ACTION OF NISIN IN BROTH AT
OPTIMUM pH AND 2 % INOCULUM



(a) *B. CEREUS*



(b) *B. STEAROTHERMOPHILUS*

Table 9. Effect of inoculum on inhibitory action of nisin against lactic and non-lactic cultures in BROTH at optimum pH, Temperature and 24 h of incubation

Sl. No.	Cultures	MIC* (RU/ml)		Cultures	MIC* (RU/ml)		Cultures	MIC* (RU/ml)	
		@ 1h	@ 2h		@ 1h	@ 2h		@ 1h	@ 2h
	LACTOCOCCUS and STREPTOCOCCUS sp.			LACTOBACILLUS sp.			BACILLUS sp.		
1.	<i>L. lactis</i> subsp. <i>lactis</i> (C _{1D})	35	50	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (1373)	75	100	<i>B. subtilis</i> (9144)	150	175
2.	<i>L. lactis</i> subsp. <i>lactis</i> (NL ₈)	75	100	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (RTS)	45	60	<i>B. subtilis</i> (441)	135	150
3.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)	5	10	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (W)	35	50	<i>B. subtilis</i> (6633)	125	150
4.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)	25	275	<i>L. acidophilus</i> (447)	50	75	<i>B. cereus</i> (10876)	75	110
5.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)	35	45	<i>L. acidophilus</i> (1899)	35	50	<i>B. cereus</i> (430)	45	60
6.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₂)	175	200	<i>L. acidophilus</i> (R)	25	45	<i>B. stearothermophilus</i> (953)	45	60
7.	<i>L. lactis</i> subsp. <i>lactis</i> (nisin producer) (496)	1600**	2000**	<i>L. plantarum</i> (R)	100	125	<i>B. stearothermophilus</i> (38)	1.0	2.5
8.	<i>L. lactis</i> subsp. <i>lactis</i> (nisin producer) (440)	2000**	2400**	<i>L. plantarum</i> (89)	20	25	<i>B. stearothermophilus</i> (37)	0.5	2.0
9.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (R)	75	100						
10.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (I)	125	150						

* Arithmetic means of three determinations

** MIC at 18 h of incubation

4.3.3.1 Lactococci and streptococci

The nisin tolerance of 8 strains of lactococci belonging to species, *L. lactis* subsp. *lactis* (Nisin and non-nisin producers), *L. lactis* subsp. *diacetylactis* and *L. lactis* subsp. *cremoris*, increased from 5-2000 to 10-2400 RU/ml with the increase of inoculum from 1 to 2% levels. The adverse effect of higher inoculum on nisin efficacy was comparatively more against least resistant strain of lactococci i.e. *L. lactis* subsp. *diacetylactis* (DRC₁) and the inhibition at 1 and 2% inoculum was observed at 5 and 10 RU/ml respectively. Among other lactococci strains, the effect of higher inoculum was comparable to non-nisin producing strains of *L. lactis* subsp. *lactis* (C₁₀, ML₈) and inhibition was observed at 35, 75 and 50, 100 RU/ml at 1 and 2% respectively (Table 9). Likewise, the MIC values for *L. lactis* subsp. *cremoris* (C₁, C₃) at 1 and 2% inoculum were 35, 175 and 45, 200 RU/ml respectively.

The nisin producing strains of *L. lactis* subsp. *lactis* (496, 440) which were capable of tolerating very high doses of nisin, also depicted the same behaviour at higher inoculum. Their inhibition was observed at 1600, 2000 and 2000, 2400 RU/ml at 1 and 2% inoculum respectively.

The adverse effect on nisin efficacy at higher inoculum was also observed among streptococci and nisin tolerance of *Str. salivarius* subsp. *thermophilus* (H and I) increased from 75 and 125 to 100 and 150 RU/ml with the increase of 1 and 2% inoculum respectively.

4.3.3.2 Lactobacilli

Increasing inoculum from 1 to 2% involved an additional nisin requirement from 25-80% among 8 strains of *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *L. plantarum*. The response of *L. delbrueckii*

subsp. *bulgaricus* strains towards nisin at higher inoculum was comparable and their MIC values increased from 35-75 to 50-100 RU/ml with the increase of 1 to 2% inoculum (Table 9). The adverse effect of inoculum on nisin among *Lactobacillus* sp. was maximum in *L. acidophilus* (R). The MIC values for *L. acidophilus* strains at 1 and 2% levels ranged from 25-50 and 45-75 RU/ml respectively. The effect of increase of inoculum on nisin tolerance among most sensitive and resistant strains of *L. plantarum* (89, R) was found similar. Their inhibition at 1 and 2% inoculum was observed at 20, 100 and 25, 125 RU/ml respectively depicting 25% increase in their nisin tolerance.

In similar studies, lactic cultures, like *Streptococcus lactis* (SIR) and *Lactobacillus bulgaricus* (LB-4) in skim milk were found to offer comparatively higher resistance (Mukundan, 1989). He reported this based on increase of inoculum from 1 to 2% at nisin concentrations ranging from 100-500 RU/ml after 24 h of incubation. The adverse effect of higher inoculum on efficacy of nisin against *Leuconostoc oenos* was also observed by Splittstoesser and Stoyla (1989) in Model grape juice system containing 100 IU nisin/ml. Url, (1990) reported that under two different inocula 1.0×10^3 and 5.0×10^7 c.f.u./ml, the inhibition of *Listeria* sp. was observed at 200 and 1000 RU nisin/ml respectively, depicting 5 folds variation in nisin tolerance.

4.3.3.3 Bacilli

The effect of inoculum on vegetative cells of 8 bacilli strains belonging to species, *B. subtilis*, *B. cereus* and *B. stearothermophilus* was studied in broth at 24 h of incubation and their nisin tolerance was found increased from 0.5-150 to 2.0-175 RU/ml with the increase from 1 to 2% inoculum (Table 9). The response of higher nisin tolerance as

offered by bacilli at higher inoculum was maximum in *B. stearothermophilus* followed by *B. cereus* and *B. subtilis* strains. The nisin tolerance of *B. subtilis* strains at two different inocula ranged from 125-150 and 150-175 RU/ml respectively whereas, for *B. cereus* strains, the respective figures were 45-75 and 60-100 RU/ml. Among *B. stearothermophilus* strains, 1-4 folds increase in nisin tolerance was observed with the increase of inoculum and their MIC values ranged from 0.5-45 to 2.0-60 RU/ml at 1 and 2% inoculum respectively.

The information on inoculum effect against vegetative cells of aerobic spore formers, however, is scanty with the exception of few reports on spores of bacilli and clostridia. The spore load as observed by Fowler (1979) was one of the key determining factors in controlling the amount of nisin necessary to prevent outgrowth in foods. The effectiveness of nisin in preventing spore outgrowth was found to decrease with the increase of spore load as reported by Rayman et al. (1981), Scott and Taylor (1981) and Oscroft et al. (1990).

4.3.4 Effect of Incubation Period in Skim Milk

The nisin sensitivity of lactic and non-lactic cultures at 1% inoculum was evaluated in skim milk incubated at optimum growth temperature for different incubation periods i.e. 12, 24 and 48 h. The MIC were determined on the basis of titrable acidity for lactic and SPC for non-lactic as enclosed in Appendix (Tables xi-xx). A continuous increase in nisin tolerance of 26 strains of *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Bacillus* sp. was observed with the increase of incubation from 12 to 24 and 48 h.

4.3.4.1 Lactococci and streptococci

Among 8 strains of lactococci and streptococci, 1-4 folds decrease in nisin activity was observed with the increase of incubation from 12

12 to 48 h. However, the effect was pronounced against *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *diacetylactis* strains (Table 10). The MIC values of nisin for *L. lactis* subsp. *cremoris* (C₁, C₃) were 50, 75; 100, 225 and 150, 300 RU/ml at 12, 24 and 48 h respectively. Whereas, for *L. lactis* subsp. *diacetylactis* (DRC₁, DRC₂) strains, the respective figures were 10, 15; 25, 35 and 35, 50 RU/ml. The non-nisin producing strains of *L. lactis* subsp. *lactis* (C₁₀, ML₈) also behaved similarly and their nisin tolerance 50, 150 at 12 h increased to 75, 175 and 100, 200 RU/ml with the increase of incubation to 24 and 48 h respectively.

On the other hand, nisin producing strains of *L. lactis* subsp. *lactis* (496, 440) were inhibited at 1200, 1600 and 2000 RU/ml at 12 and 18 h respectively. Subsequent increase in incubation resulted in tremendous growth of these cultures and could be inhibited only at 13,500, 14,200 RU/ml at 24 h of incubation respectively (Appendix Table, xiv).

Among streptococci, 2 folds increase in nisin tolerance of *Str. salivarius* subsp. *thermophilus* (H, I) strains was observed with the increase of incubation periods and their MIC values were 75,100; 100,150 and 150, 200 RU/ml at 12, 24 and 48 h respectively (Table 10).

4.3.4.2 Lactobacilli

The loss of nisin activity among 8 strains of lactobacilli was 2-4 folds with the increase of incubation from 12 to 48 h. However, the adverse effect on nisin efficacy at higher incubation was comparatively more against *L. delbrueckii* subsp. *bulgaricus* (RTS), *L. acidophilus* (1899) and *L. plantarum* (R) strains (Table 11). The MIC range of nisin for *L. delbrueckii* subsp. *bulgaricus* strains were 25-50, 50-100 and 75-

Table 10. Effect of incubation period on inhibitory action of nisin against *Lactococcus* and *Streptococcus* strains in SKIM MILK at optimum pH, temperature and 1% inoculum

Sl. No.	Cultures	MIC* (RU/ml)		
		Different incubation periods (h)		
		12	24	48
1.	<i>L. lactis</i> subsp. <i>lactis</i> (C ₁₀)	50	75	100
2.	<i>L. lactis</i> subsp. <i>lactis</i> (ML ₈)	150	175	200
3.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)	10	25	35
4.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)	15	35	50
5.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)	50	100	150
6.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₃)	75	225	300
7.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (H)	75	100	150
8.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (I)	100	150	200

* Arithmetic means of three determinations

Table 11. Effect of incubation period on inhibitory action of nisin against *Lactobacillus* strains in SKIM MILK at optimum pH, temperature and 1% inoculum

Sl. No.	Cultures	MIC* (RU/ml)		
		Different incubation periods (h)		
		12	24	48
1.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (W)	25	50	75
2.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (1373)	50	100	150
3.	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> (RTS)	25	75	100
4.	<i>L. acidophilus</i> (447)	50	75	125
5.	<i>L. acidophilus</i> (1899)	25	50	100
6.	<i>L. acidophilus</i> (R)	25	50	75
7.	<i>L. plantarum</i> (R)	50	150	200
8.	<i>L. plantarum</i> (89)	25	50	75

* Arithmetic means of three determinations

150 RU/ml at 12, 24 and 48 h respectively whereas, for *L. acidophilus* strains, the respective figures were 25-50; 50-75 and 75-125 RU nisin/ml.

The response of other *Lactobacillus* sp. like *L. plantarum* (89, R) was also similar and their nisin tolerance at 12, 24 and 48 h was 25, 50; 150 and 75, 200 RU/ml respectively.

The present findings were supported by Shahani (1962), who studied the effect of 10 units of nisin against *Streptococcus lactis*, *L. bulgaricus* and *Str. thermophilus* strains in skim milk and reported that cell counts of the organisms decreased continuously for the first 6 h of incubation but thereafter, the surviving organisms multiplied normally and proximated the counts of the control samples at the end of 24 to 36 h.

In a similar study, Mukundan (1989) studied the effect of different incubation periods of 6, 9, 12, 24 and 48 h on nisin tolerance of *S. lactis* (SIR), *S. cremoris* (SC₁, CH₉, LF-40), *L. bulgaricus* (LB-4) and *L. casei* in skim milk containing 100-500 RU nisin/ml. The nisin tolerance of lactic cultures increased continuously with the increase of incubation periods.

4.3.4.3 Bacilli

The response of 8 strains of *Bacillus* belonging to *B. subtilis*, *B. cereus* and *E. stearothermophilus* species in skim milk at different incubation periods was comparable to lactic cultures. Increase upto 3 folds in their nisin tolerance against *B. subtilis* (9144) and *B. stearothermophilus* (953) was observed with the increase of incubation from 12 to 48 h (Table 12). The nisin tolerance of *B. subtilis* strains from 75-100 at 12 h, increased to 150-175 and 175-225 RU/ml at 24 and 48 h respectively. Similarly, for *E. cereus* (430, 10876) strains, the

Table 12. Effect of incubation period on inhibitory action of nisin against *Bacillus* strains in SKIM MILK at optimum pH, temperature and 1% inoculum

Sl. No.	Cultures		MIC* (RU/ml)		
			Different incubation periods (h)		
			12	24	48
1.	<i>B. subtilis</i>	(6633)	100	150	200
2.	<i>B. subtilis</i>	(9144)	75	175	225
3.	<i>B. subtilis</i>	(441)	100	150	175
4.	<i>B. cereus</i>	(10876)	75	100	150
5.	<i>B. cereus</i>	(430)	50	75	100
6.	<i>B. stearothermophilus</i>	(953)	25	50	75
7.	<i>B. stearothermophilus</i>	(37)	1.0	1.0	2.5
8.	<i>B. stearothermophilus</i>	(38)	2.5	2.5	5.0

* Arithmetic means of three determinations

nisin tolerance 50, 75 at 12 h, increased to 75, 100 and 100, 150 RU/ml at 24 and 48 h respectively. *Bacillus stearothermophilus* strains also required more concentration of nisin with the increase of incubation periods and their MIC values varied from 1.0-25, 1.0-50 and 2.5-75 RU/ml at 12, 24 and 48 h respectively.

In the present study, the growth of 26 strains of lactic and non-lactic cultures was found increase with the increase of incubation periods as evident from titrable acidity and standard plate counts enclosed in Appendix Table xi-xx.

The loss of nisin activity at higher incubation periods was difficult to explain, whether it was a chemical inactivation or because of additional growth of the micro-organisms. However, based on the present findings, it is likely that after initial bacteriostatic action of nisin against growing micro-organisms, the residual nisin in milk appears to be insufficient for complete inhibition. As a result, higher nisin concentration would be required as the incubation period is extended.

The comparative higher loss of nisin activity observed among *Lactobacillus plantarum* (R) and *B. subtilis* (9144) perhaps be due to inactivating enzymes produced by these strains as reported earlier.

**III. SCANNING ELECTRON MICROSCOPY (SEM)
OF NISIN TREATED LACTIC AND NON-
LACTIC CULTURES**

Most published work on bacteriocins of lactic acid bacteria especially nisin related to mode of action is confined to biochemical investigations. However, very little efforts were made to study at the Electron Microscope level which is an indispensable tool for fine structural morphology.

In the present study, attempts were made to ascertain the mode of nisin action against most sensitive and resistant strains of *Lactococcus*, *Lactobacillus* and *Bacillus* sp. through SEM. Nisin was found to execute its bacteriocidal action only at very high doses ranging from 1-10,000 times of minimal inhibitory concentration and brought about extensive surface damages in and around bacterial cells as clearly revealed by SEM. At MIC doses, however, only nominal changes like, clumping or aggregation of cell was observed under both light as well as scanning microscope .

4.4. SCANNING ELECTRON MICROSCOPY OF LACTIC CULTURES

4.4.1 Lactococci

4.4.1.1 *L. lactis* subsp. *diacetylactis* (DRC₁)

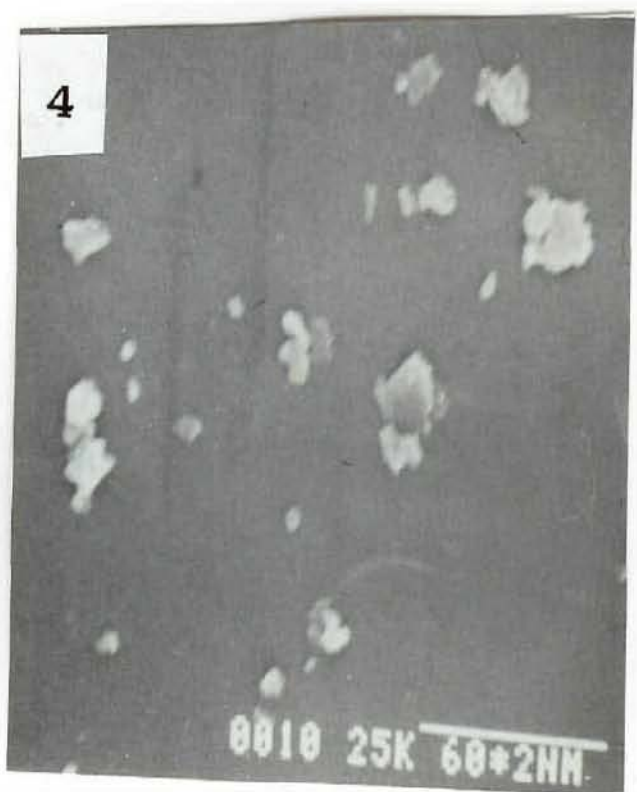
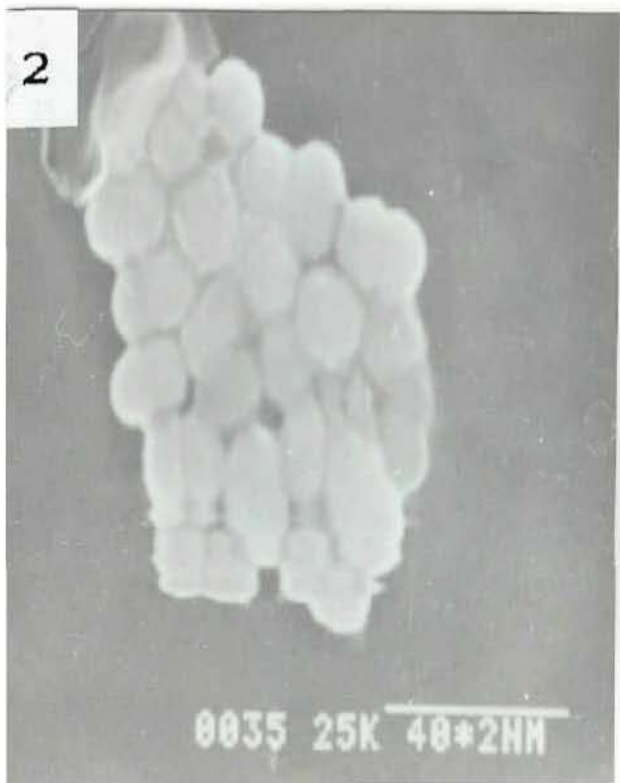
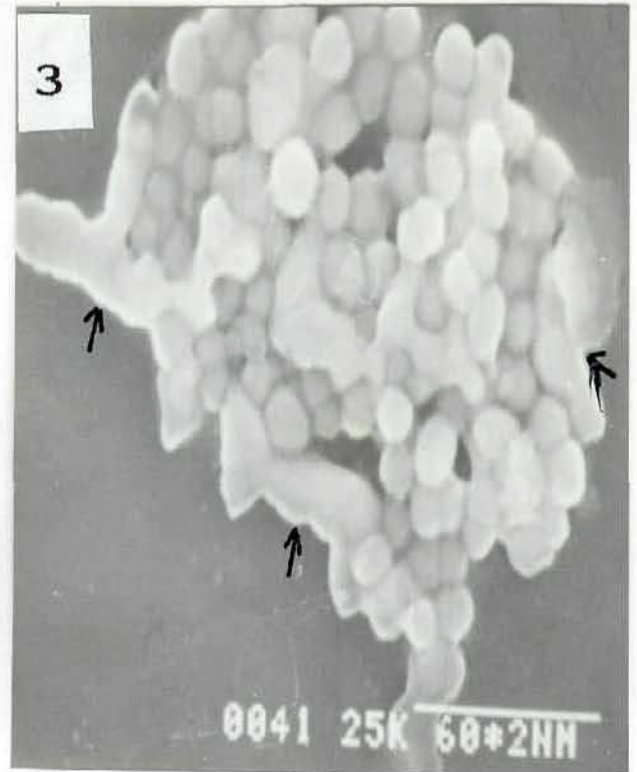
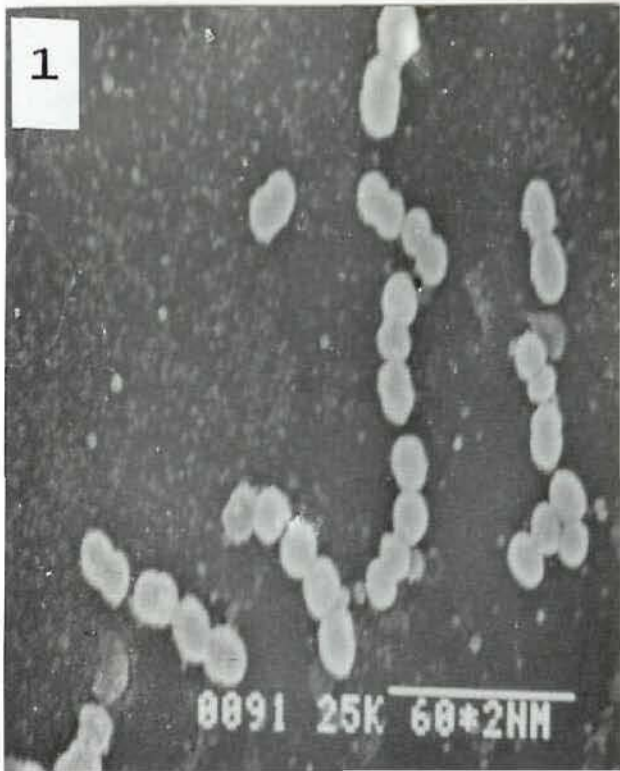
The most sensitive strain among lactococci was *L. lactis* subsp. *diacetylactis* (DRC₁). The untreated cells were maximally in pairs or small chains (Plate I, Fig.1). When exposed to nisin at MIC dose of 5 RU/ml, cells tend to aggregate at few points resulting in development of big clumps. This response of the cells further, intensified with the increase of nisin concentration to 500 times of MIC. However, fusion of proximal cells could be seen quite frequently within a lump (Figs. 2 and 3). Whereas, complete disintegration of cells was observed at one

Plate I Scanning Electron Micrographs of normal and
(Figs. 1-4) NISIN treated cells of *Lactococcus lactis*
 subsp. *diacetylactis* (DRC₁)

- Fig.1 Control (Untreated) sample showing intact cocci
 in pair and small chains*.
- Fig.2 Nisin treated at MIC (5 RU) cells depicting
 clumping or aggregation, forming a compact mass.
- Fig.3 MIC (x 500) treated showing aggregation and
 fusion of cells (solid arrows) at several places.
- Fig.4 MIC (x 1000) treated showing complete
 disruption of cells into scattered remnants.
-

* 25 kV operating voltage

60* 2NM = 60×10^2 NM corresponding to the length of the
bar above it (detailed chart is printed in Table XXI).



thousands times of MIC concentration as evident in Fig. 4. Cellular fragments following disintegration could also be frequently seen.

4.4.1.2 L. lactis subsp. diacetylactis (DRC₂)

Untreated cells of *L. lactis* subsp. *diacetylactis* (DRC₂) were maximally found in long chains consisting of around 15-20 cells (Plate II, Fig.1). This strain forms still longer chains as examined under light microscope.

At MIC of nisin with 25 RU/ml, normally aggregation or clumping of cells was observed (Fig. 2). However, the effect was not as intense as observed earlier in *L. lactis* subsp. *diacetylactis* (DRC₁). The increase in nisin concentration to one thousand times MIC brought about stretching of cells resulting in lengthening of chain length as could be seen in Fig. 3. In addition, complete dissolution of cytoplasmic contents was also observed (Fig. 4). The bacteriocidal action of nisin was intensified considerably at two thousand times MIC depicting star and V-shaped structures, lump formation, protoplast-like structures and appearance of fragments of cellular components (Figs. 5-7). Complete disintegration of cells and its subsequent fusion could be seen at very high nisin concentration of five thousand times MIC (Fig. 8).

4.4.1.3 L. lactis subsp. lactis (C₁₀)

The untreated cells of non-nisin producing strain of *L. lactis* subsp. *lactis* (C₁₀) occurs maximally in relatively small chains (Plate III, Fig.1). Cells treated with MIC 35 RU nisin/ml appears to aggregate involving few cells at places in microscopic field as observed in Fig. 2. However, it was not as prominent as observed earlier in *L. lactis* subsp. *diacetylactis* strains.

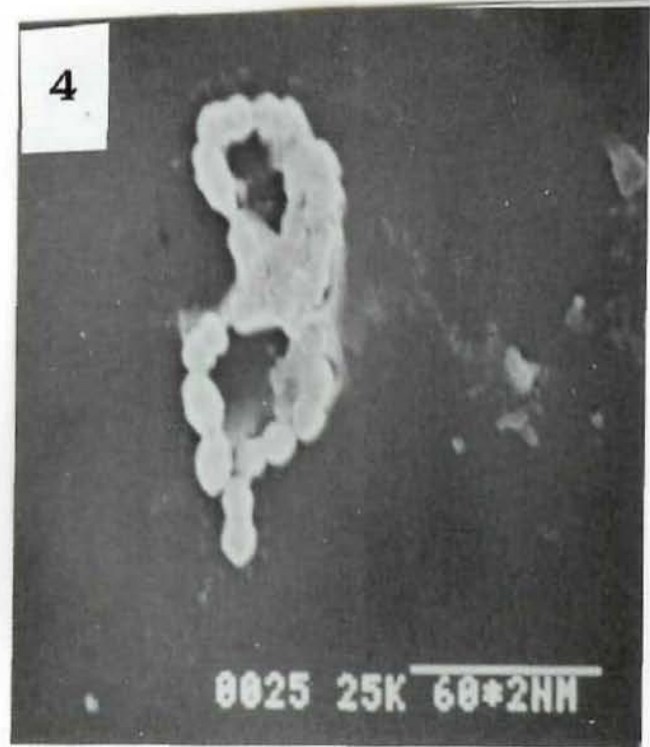
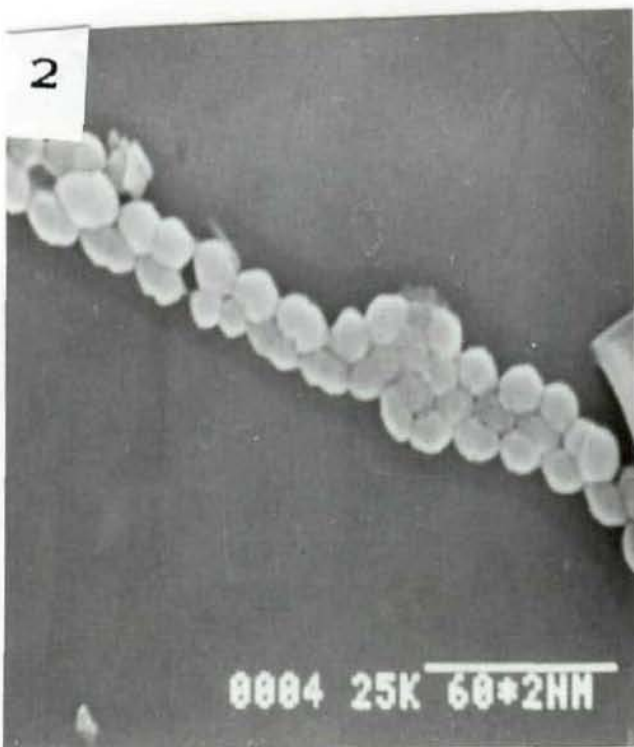
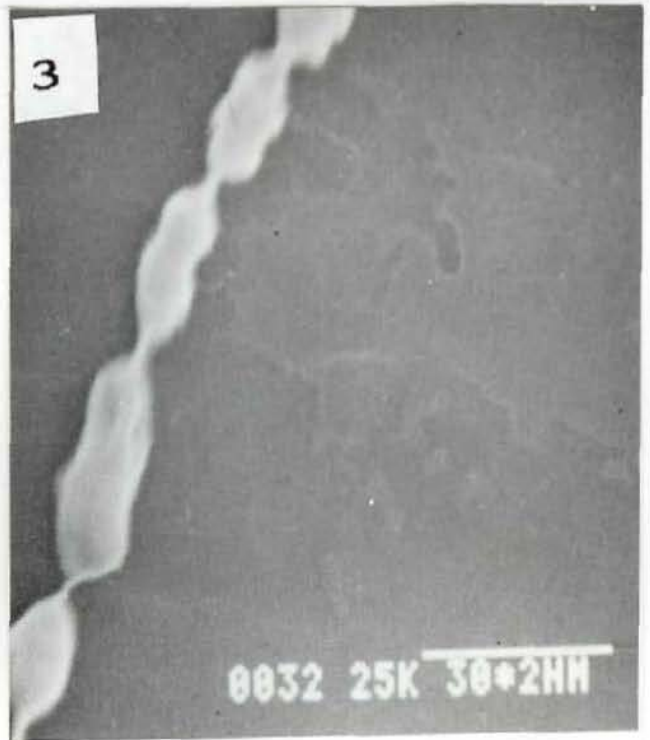
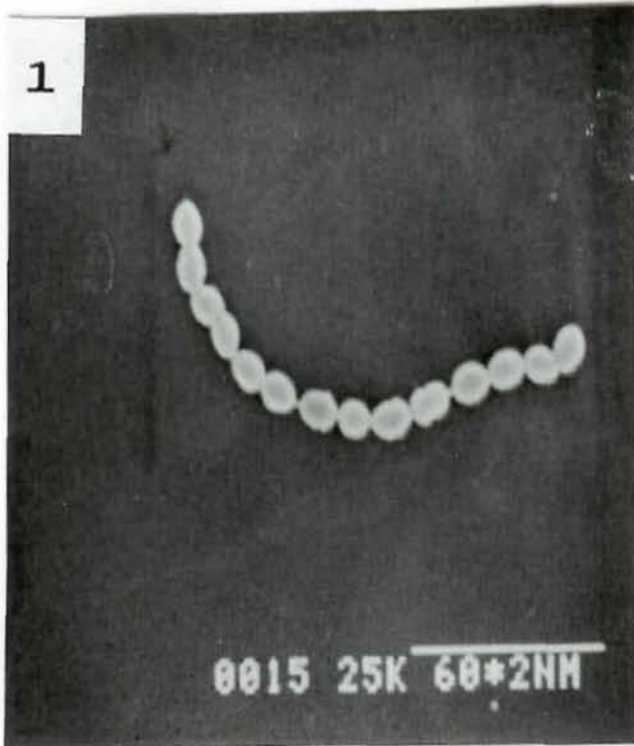
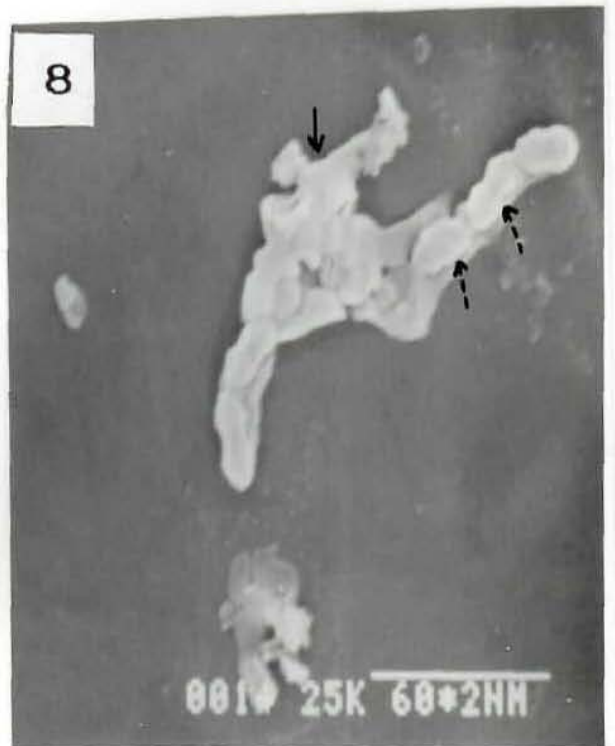
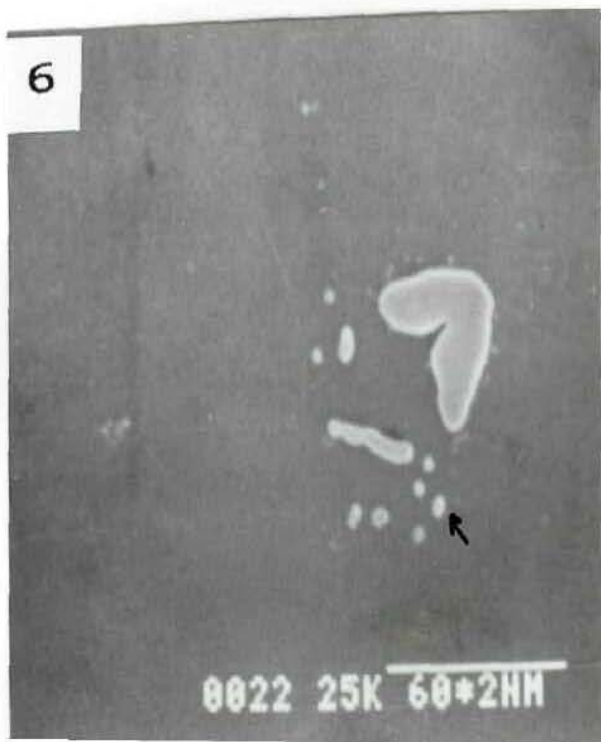
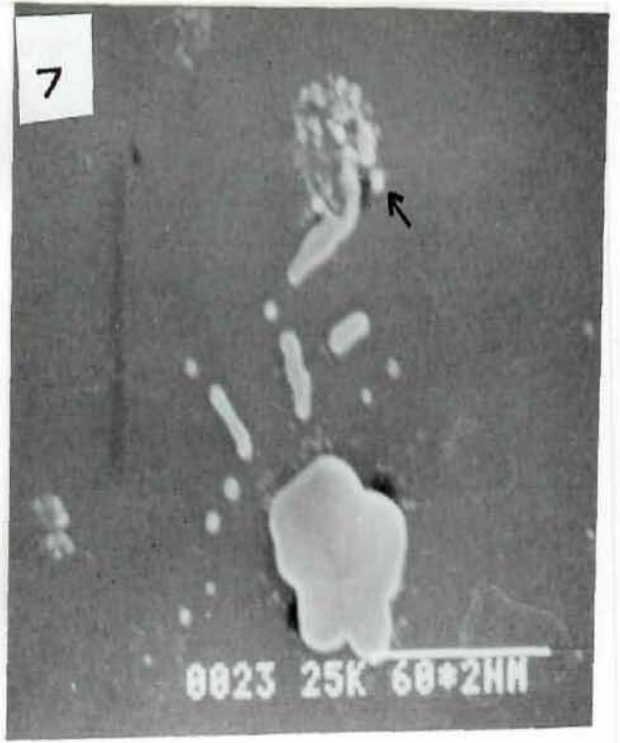
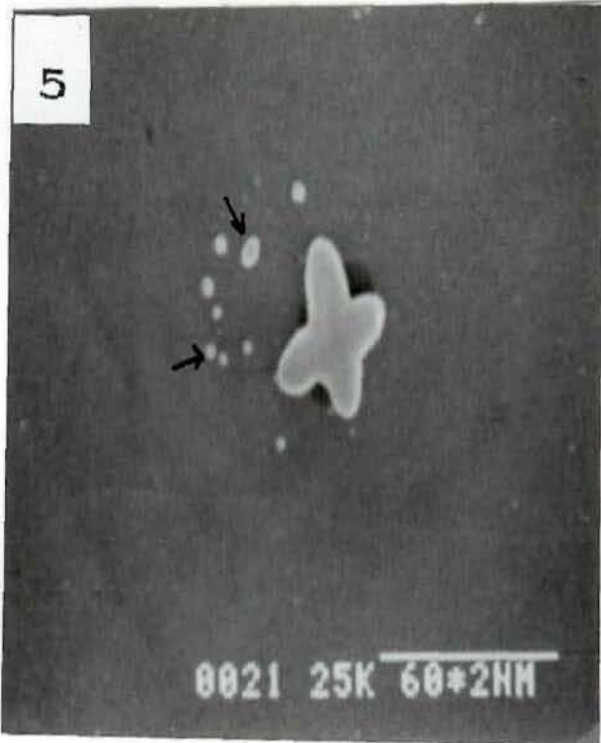


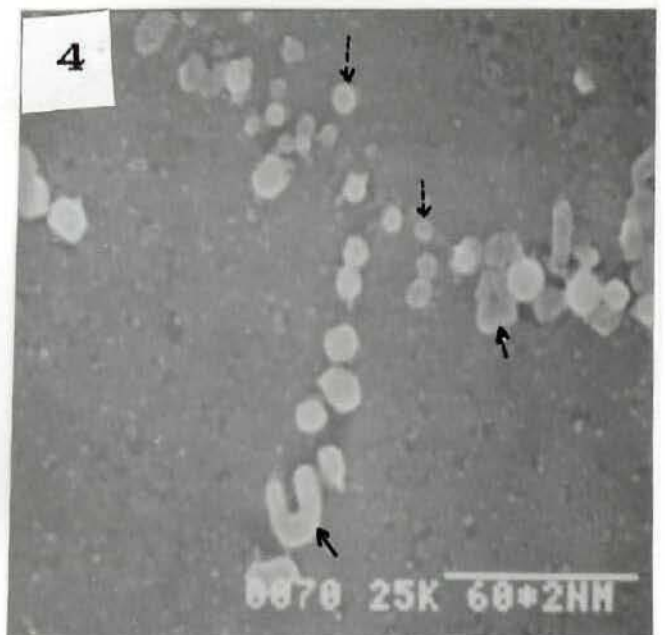
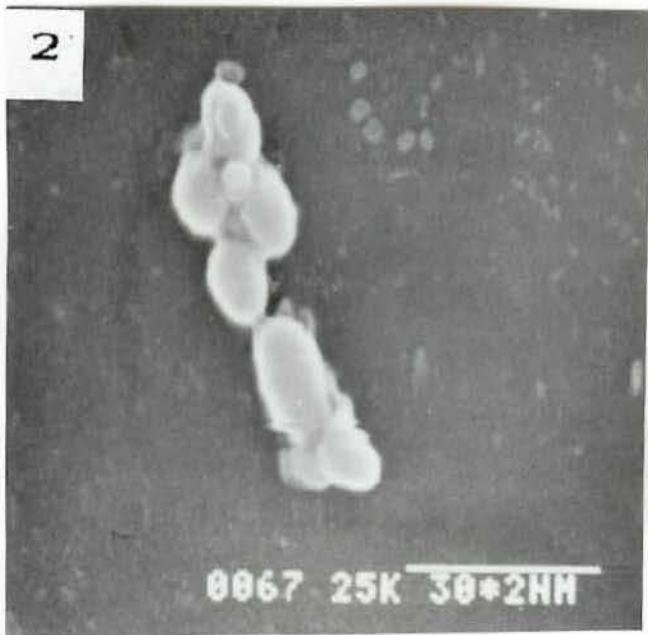
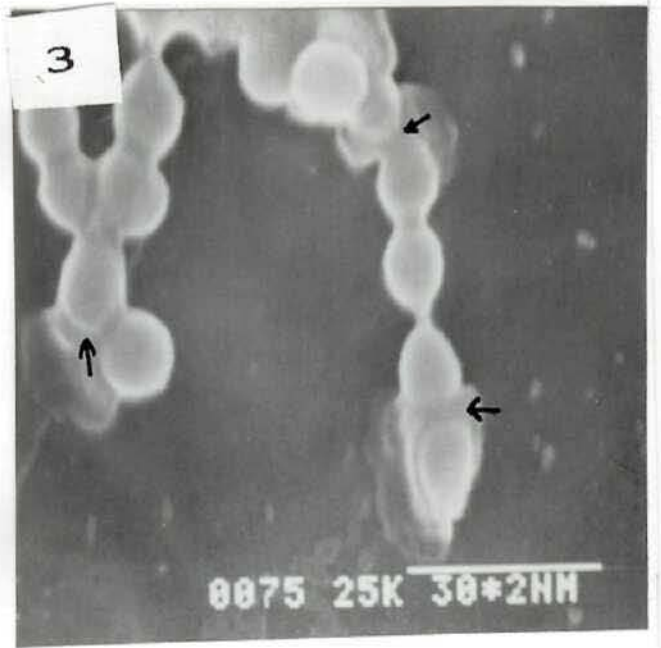
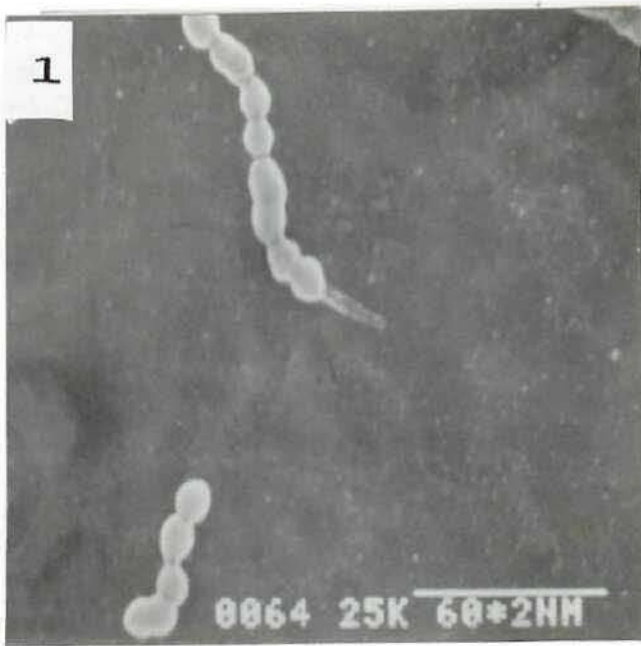
Plate II (Contd.)

(Figs. 5-8)

Figs. 5-7 MIC (x 2000) treated showing various degrees of cellular fusion involving few to several cells forming star, V and irregular lump. Fragments of cellular components are also seen (Solid arrows).

Fig. 8 MIC (x 5000) treated depicting compact mass of fused cells (Solid arrow) with partially damaged cells under fusion (Broken arrow).





The partial disintegration of few cells within lactococcal chain in specific mid region and its subsequent release of protoplasmic contents could be seen clearly at two thousands times MIC of nisin as apparent in Fig. 3. Thereafter, loss of chain character and appearance of protoplast-like structures along with fused cells were observed at six thousands times MIC of nisin.

4.4.1.4 L. lactis subsp. lactis (ML_g)

A considerable variation in nisin tolerance of *L. lactis* subsp. *lactis* (ML_g) was observed at strain level. The untreated cells were diplo or in small chains (Plate IV, Fig. 1).

Partial disintegration could be seen in one thousands times MIC of nisin. Release of protoplasmic contents was seen in the vicinity of the damaged cells (Fig. 2). In other microscopic fields, protoplast-like spherical structures were also observed (Fig. 3).

At six thousands times MIC of nisin, fusion of cells to complete disintegration was commonly observed (Fig. 4).

4.4.1.5 L. lactis subsp. lactis (440)

It is a nisin producing strain and the cells are highly resistant to nisin with MIC of 2000 RU/ml. The untreated cells occur largely in small chains, however, few cells in pairs could also be seen (Plate V, Fig.1).

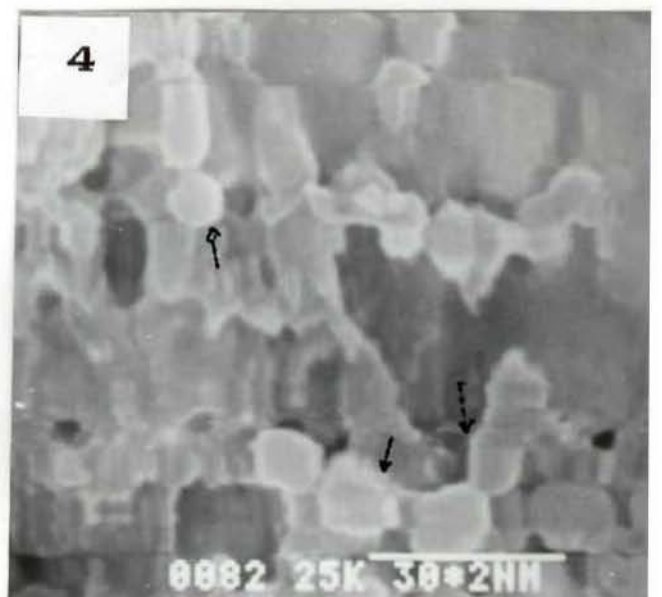
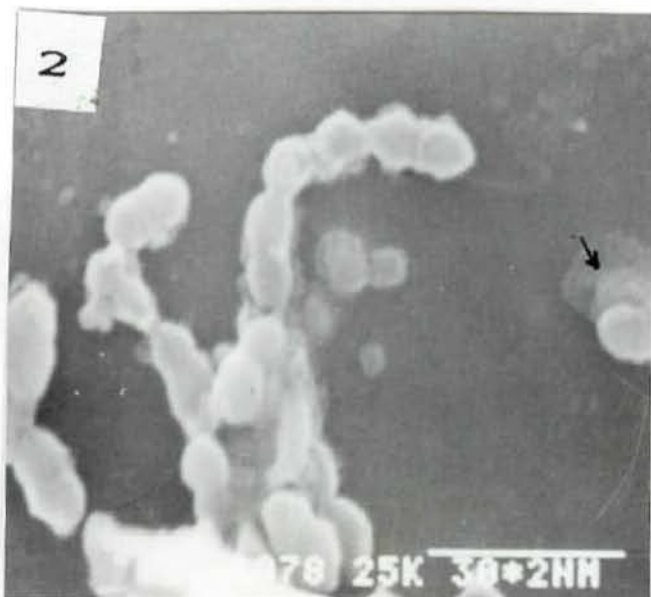
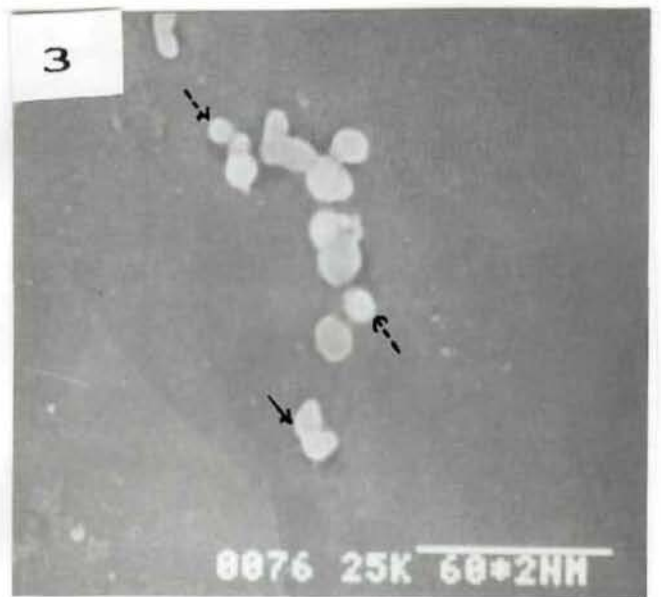
The partial deformation of cells within chain was observed at two thousands times of MIC (Fig. 2). Whereas, extensive bacteriocidal action of nisin with complete disintegration of cells was observed only at four thousands times MIC of nisin (Fig. 3).

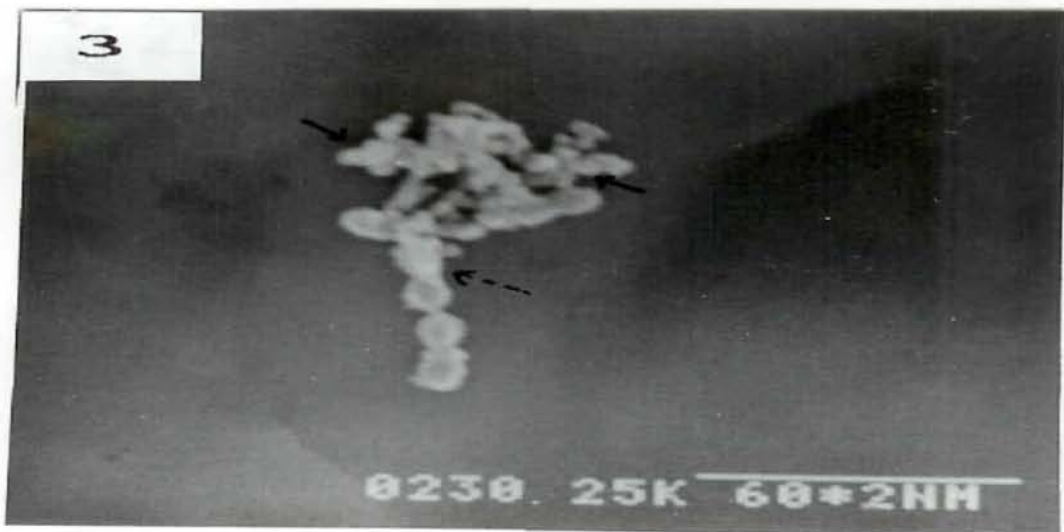
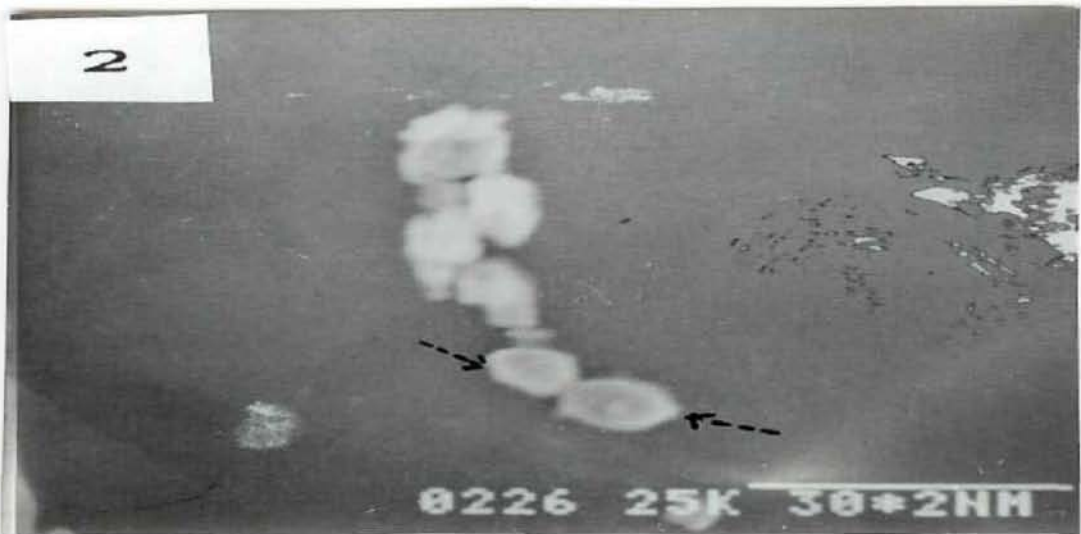
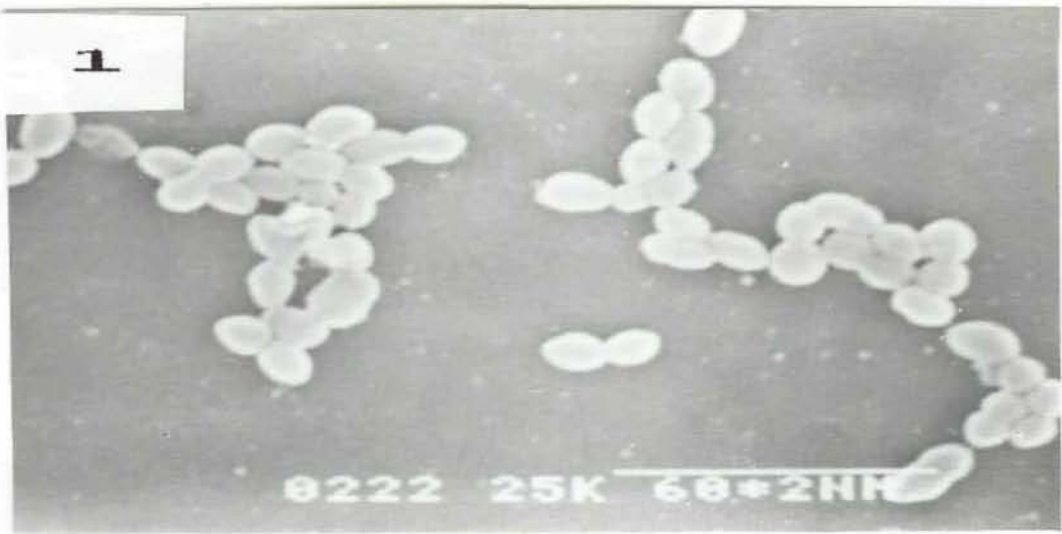
The basic information on Electron Microscopy of nisin treated lactic cultures is lacking. However, preliminary studies on the

Plate IV
(Figs. 1-4)

Scanning Electron Micrographs of normal and
NISIN treated cells of *Lactococcus lactis*
subsp. *lactis* (ML₈)

- Fig.1 Control sample showing intact and well preserved cocci in pairs and small chains.
- Fig.2 MIC (x 1000) treated showing onset of partial disintegration with leakage protoplasmic contents from the damaged cells (Solid arrow)
- Fig.3 MIC (x 1000) treated showing fused cells (Solid arrow) and formation of protoplast-like structures (Broken arrow)
- Fig.4 MIC (x 6000) treated showing complete disintegration along with few partially deformed cells (Broken arrows)





bacteriolysis of nisin producing strains of *Streptococcus lactis* with nisin during logarithmic phase was reported by Hurst and Kruse (1972). Recently, Van-Belkum et al (1991) studied the effect of lactococcin A against its producing strains i.e. *L. lactis* subsp. *lactis* through EM. However, they could not find any lysis or other morphological alterations in treated bacterial cells.

Other bacteriocins of Gram positive bacteria like staphylococcin C-55, a bacteriocidal substance produced by *Staphylococcus aureus* was earlier studied for its mode of action against streptococcal cells electron microscopically (Clawson and Dajani, 1970). The sub-cellular changes observed included the condensation of nuclear materials, partial loss of ribosomes, modification of mesosomes and eventual dissolution of cell contents. Similar kinds of changes were also observed in sensitive bacteria treated with streptococci AFF-22 (Tagg et al., 1973).

A cationic peptide Pep-5 with similar mode of action of nisin was also studied by Sahl and Brandis (1983) and Brandis and Sahl (1984) and reported that destruction primarily occurs in septum area in staphylococcal cells as revealed by SEM.

On the basis of present findings, it can be concluded that the lactococcal cells following treatment with nisin in increasing concentration undergo following surface changes in sequence as follows:

1. Clumping or aggregation of cells
2. Stretching of cells in chain
3. Formation of protoplast-like structures
4. Partial disintegration of cells with expulsion of protoplasmic contents
5. Complete disintegration of cells forming structure less matrix.

In general, the aggregation and stretching of lactococcal cells without any surface damage following treatment with MIC doses indicated the bacteriostatic action of nisin. On the other hand, the partial or complete disintegration of cells and appearance of protoplast-like structure under SEM revealed bacteriocidal action.

4.4.2 Lactobacilli

4.4.2.1 L. delbrueckii subsp. bulgaricus (RTS)

Untreated normal cells invariably appeared as long rods with round ends and occur largely in short chains with defined intercellular gaps (Plate VI, Fig. 1). When exposed to MIC of 45 RU nisin/ml, the cells loosely aggregated in clumps as first visible effect. Partial initiation of surface damage of cells is also discernible (Fig. 2).

Extensive surface damages in *L. delbrueckii subsp. bulgaricus* (RTS) were also observed at very high dose of nisin. A well defined pore through cell wall with subsequent release of cytoplasmic contents can be clearly seen. The cells, usually showed uneven undulation with the formation of typical blebs at the terminal region at three thousand MIC of nisin. The intercellular distances also become indistinguishable (Fig. 3). However, complete disintegration of few cells was observed, when treated with ten thousand times MIC (Fig. 4).

4.4.2.2 L. delbrueckii subsp. bulgaricus (W)

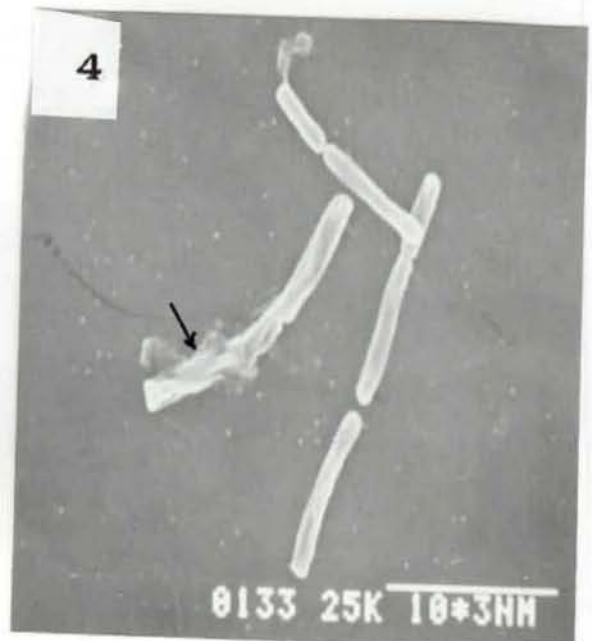
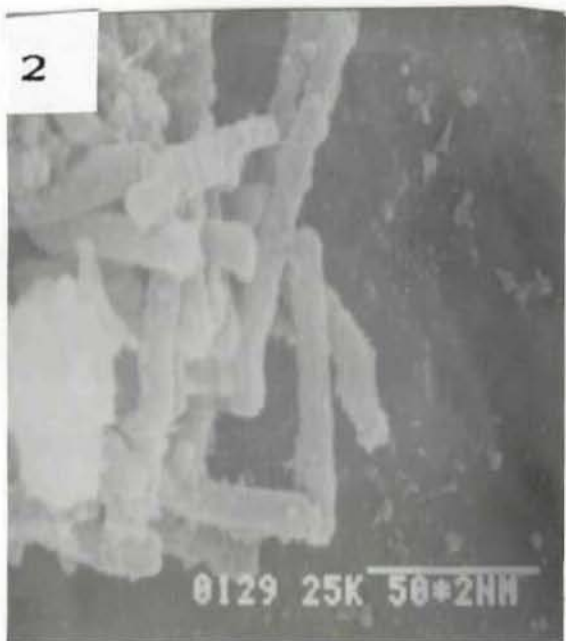
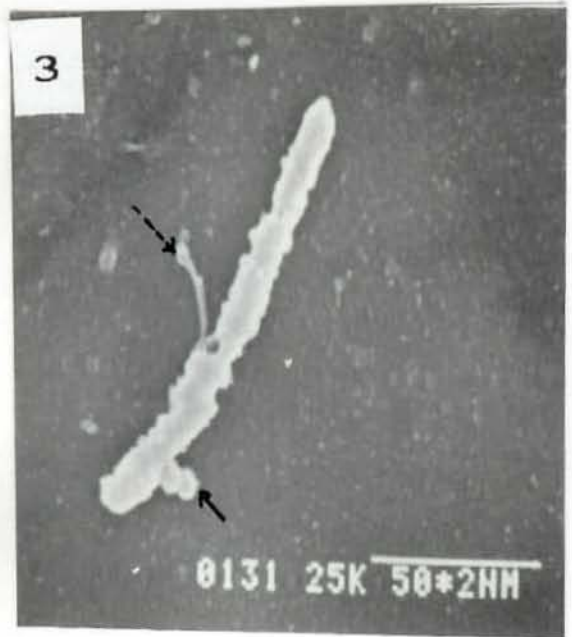
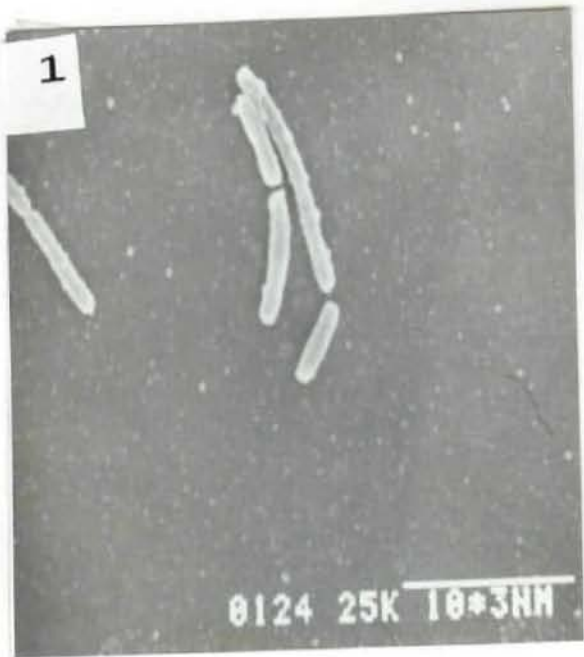
Untreated cells were relatively bigger rods than *L. delbrueckii subsp. bulgaricus* (RTS) and found maximally in single or pairs (Plate VII, Fig. 1).

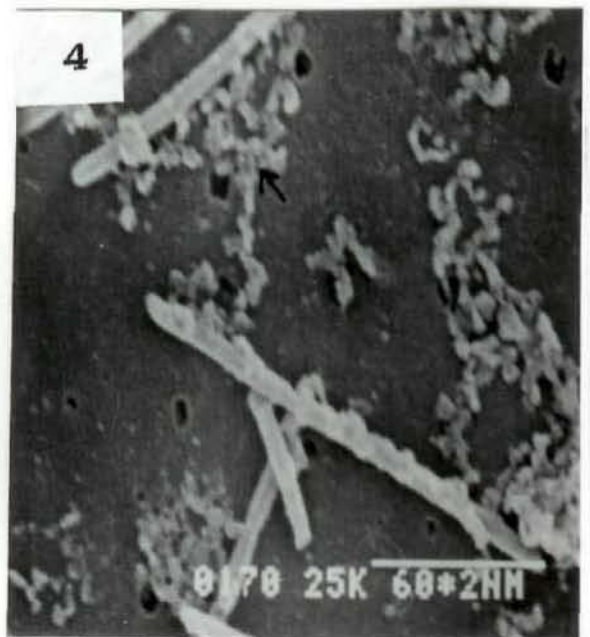
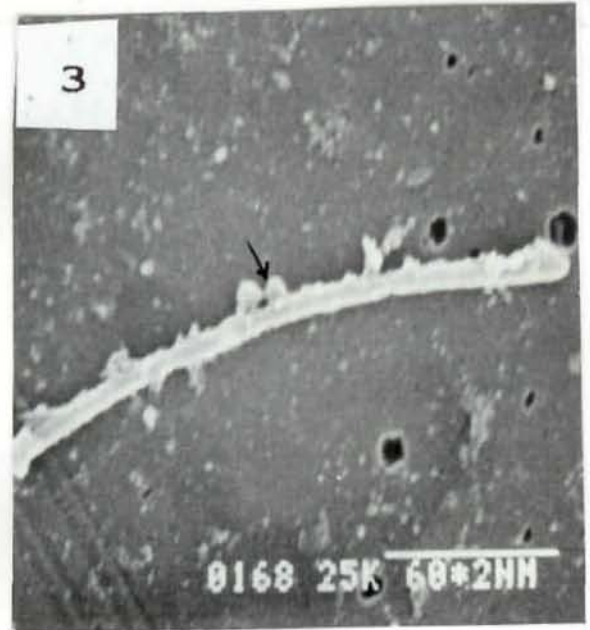
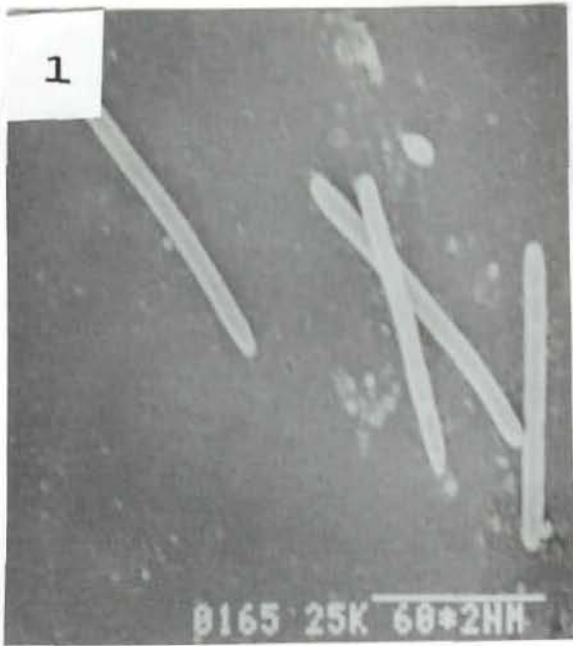
Bacterial cells treated with three thousand times MIC of nisin induced extensive surface damages with numerous blebs formation as

Plate VI
(Figs.1-4)

Scanning Electron Micrographs of normal and
NISIN treated cells of *Lactobacillus delbrueckii*
subsp. *bulgaricus* (RTS)

- Fig.1 Control sample showing intact and well preserved lactobacilli.
- Fig.2 Nisin treated at MIC (45 RU) showing loose aggregation.
- Fig.3 MIC (x 3000) treated depicting well defined pores. A number of blebs could be seen on the bacterial surface (Solid arrow). Loss of cytoplasmic fluid is seen through one of the pores (Broken arrow)
- Fig.4 MIC (x 10,000) treated showing sequence of cellular disintegration (Solid arrow)





depicted in Fig. 2. The bleb formation becomes more prominent (Fig. 3) with the increase of nisin concentration to ten thousand times of MIC. Whereas, cells in advanced stage of disintegration was observed in some microscopic fields. Fragments of disintegrated cells could also be seen forming the background (Fig. 4). The blebs formation appears to be a common feature particularly among *L. delbrueckii* subsp. *bulgaricus* strains.

4.4.2.3 *L. acidophilus* (R)

The control sample of *L. acidophilus* (R) under SEM depicted medium size rods in single and short chains (Plate VIII, Fig. 1). Partial disintegration of cells with pore and bleb formation were some of the prominent surface changes observed in *Lactobacillus* cells treated with three thousands times MIC (Fig. 2). In some microscopic fields, fusion of cells leading to filamentous structures could also be seen in Fig.3.

The vulnerability of bacterial cells increased further with much higher nisin concentration at ten thousands times MIC. The treated cells undergo complete loss of original rod character as a result of extensive deformation (Fig. 4).

4.4.2.4 *L. plantarum* (89)

The Scanning Electron Microscopy of *L. plantarum* (89) revealed usually thin cylindrical rods with rounded ends. These occur invariably in small chains (Plate IX, Fig. 1). Inter-cellular chains links appear to get dissolved following treatment with three thousand times MIC of nisin. Slight perforation and prominent blebs could also be seen (Fig.2). Further increase in nisin concentration to ten thousand times MIC brought about extensive rupturing of cells. As a result, the protoplasmic contents around the damaged cells could be seen (Fig. 3). Extensive

Plate VIII
(Figs. 1-4)

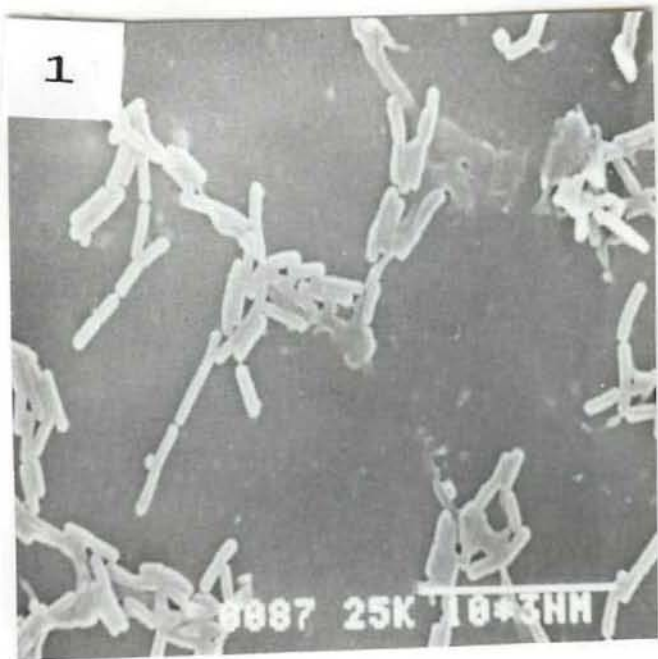
Scanning Electron Micrographs of normal and
NISIN treated cells of *Lactobacillus*
acidophilus (R)

- Fig.1 Control sample showing intact and well preserved cells of lactobacilli singly or in short chains.
- Fig.2 MIC (x 3000) treated depicting pore formation on the surface as indicated by Solid arrow.
- Fig.3 MIC (x 3000) treated showing filamentous appearance of cells. Initiation of blebs on the surface could also be seen (Solid arrow)
- Fig.4 MIC (x 10,000) treated showing fused cells covered with blebs.

Plate IX
(Figs. 1-4)

Scanning Electron Micrographs of normal and
NISIN treated cells of *Lactobacillus*
plantarum (89)

- Fig.1 Control sample showing intact and well preserved slender rods in chain and groups.
- Fig.2 MIC (x 3000) treated showing intensive undulation and bleb formation (Solid arrow)
- Fig.3 MIC (x 10,000) treated showing partially damaged cells with release of protoplasmic contents from one end.
- Fig.4 MIC (x 10,000) treated showing acute perforation of cell surface with a large bleb.



damage through acute perforation at this high dose of nisin was frequently observed (Fig. 4).

Few reports on light and electron microscopy of nisin treated *Lactobacillus* cells are available. In one study, Ogden and Waites (1986) demonstrated that *Lactobacillus* cells (Eso, 28, 348 and 375) following treatment with nisin at 10,000 Units/ml undergo clumping as revealed under light microscope. Lysis of treated cell was, however, not reported by them.

The aggregation of *L. delbrueckii* subsp. *bulgaricus* (RTS) at MIC was most commonly observed in the present study. Similar aggregation pattern in other *Lactobacillus* species was also confirmed by light microscopy.

The ultrastructure changes in *Lactobacillus* and *Leuconostoc* sp. were studied by Bhunia et al. (1991). *Leuconostoc mesenteroides* (Ly) following treatment with pediocin ACh at 10, 240 AU/ml resulted in lysis and revealed the presence of ghost cells. On the other hand, in *L. plantarum* (955) lysis of cell was not observed. However, they described condensation of cytoplasm to form dense and darker cells.

On the basis of present findings, it is convincing that *Lactobacillus* strains undergo some well defined nisin-induced surface changes like pore and bleb formation. These findings are consistent with the earlier reports of Henning et al. (1986); Sahl et al. (1987); Kordel et al. (1989) and Gao et al. (1991). They observed that nisin bring about -disruption in membrane function due to ion channel or pore formation. The subsequent release of low molecular compounds like K^+ , amino acids and ATP through these pores was also reported based on biochemical analysis.

4.5 SCANNING ELECTRON MICROSCOPY OF NON-LACTIC CULTURES

4.5.1 Bacilli

4.5.1.1 B. cereus (10876)

Untreated cells under SEM revealed small rods in short chains (Plate X, Fig. 1). At MIC dose of 75 RU/ml, visible changes could not be observed under light as well as SEM. However, rupturing of cell specifically in the mid region was noticed at three thousand times MIC. Protoplasmic fluid along with cell fragments could also be seen near the disrupted cells (Fig. 2).

The bacteriostatic action of nisin becomes more drastic at ten thousands times MIC. Complete disintegration involving only a few cells within chain could be seen. Fragments of disintegrated cells and prominent bleb formation in few cells were evident (Figs. 3 and 4).

4.5.1.2. B. subtilis (6633)

Bacillus subtilis (6633) was found to be resistant strain among bacilli. It occurs maximally in long chains (Plate XI, Fig. 1). The chain character of the organisms appears to be lost following treatment with three thousands times of MIC (Fig. 2). It is likely that the bacterial chain links are first target of nisin-induced changes at low concentrations. Partial disintegration of cells was observed at five thousands times of MIC. However, a peculiar clamp connection was noted. Whether such a structural modification is functional or a chance factor cannot be ascertained (Fig. 3). In addition, few cells were in relatively advanced stage of disintegration as seen in Fig. 4.

4.5.1.3. B. stearothermophilus (38)

These rods were relatively thick, small and curved (Plate XII, Fig. 1). *Bacillus stearothermophilus* (38) was inhibited at very low level

Plate X
(Figs. 1-4)

Scanning Electron Micrographs of normal and
NISIN treated cells of *Bacillus cereus* (10876)

- Fig.1 Control sample showing intact and well preserved small rods in pairs, chains and in groups.
- Fig.2 MIC (x 3000) treated depicting acute surface rupture of the intercalary cells in chain (Solid arrow).
- Fig.3 MIC (x 10,000) treated showing disintegration of cells in chain (Solid arrow).
- Fig.4 MIC (x 10,000) treated showing fragments of disintegrated cells (Solid arrow). Bleb formation (Broken arrow) can also be seen.

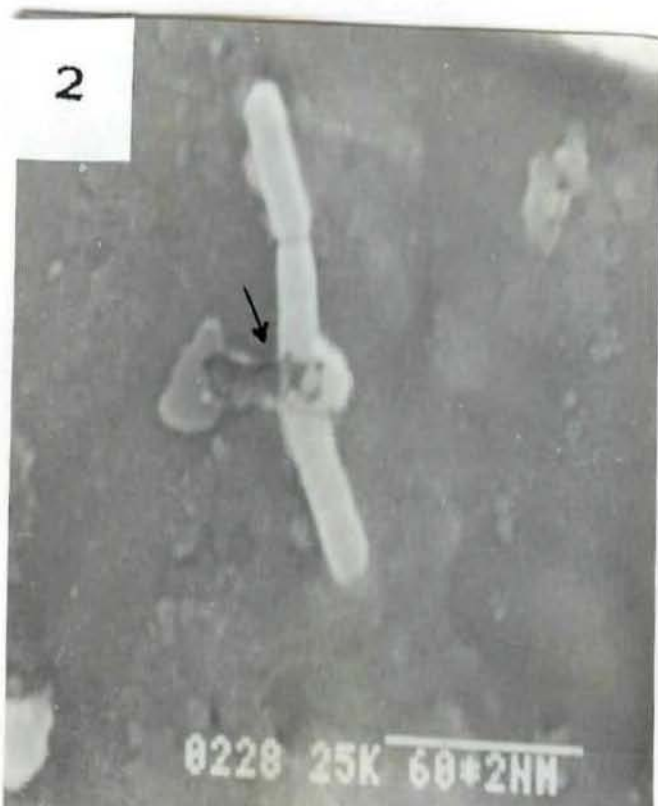
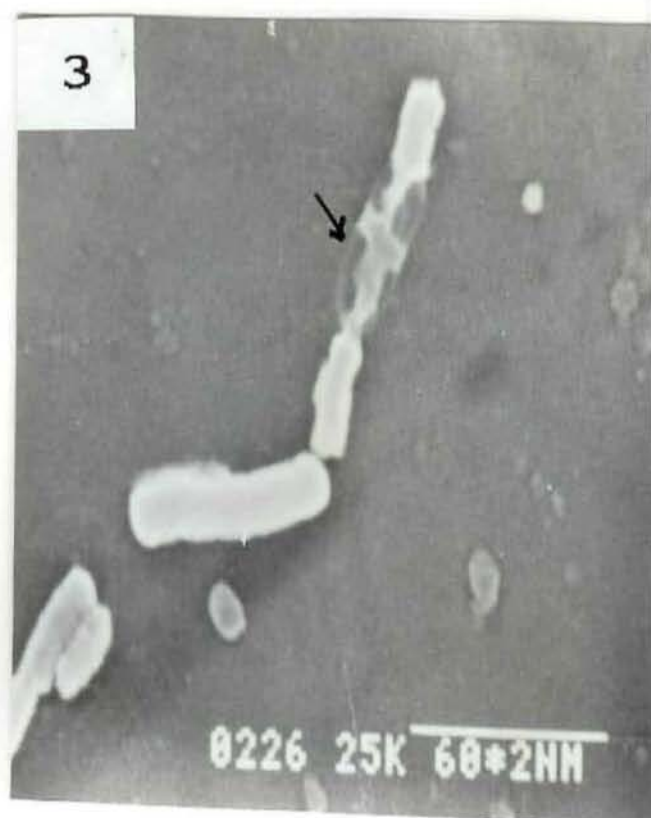
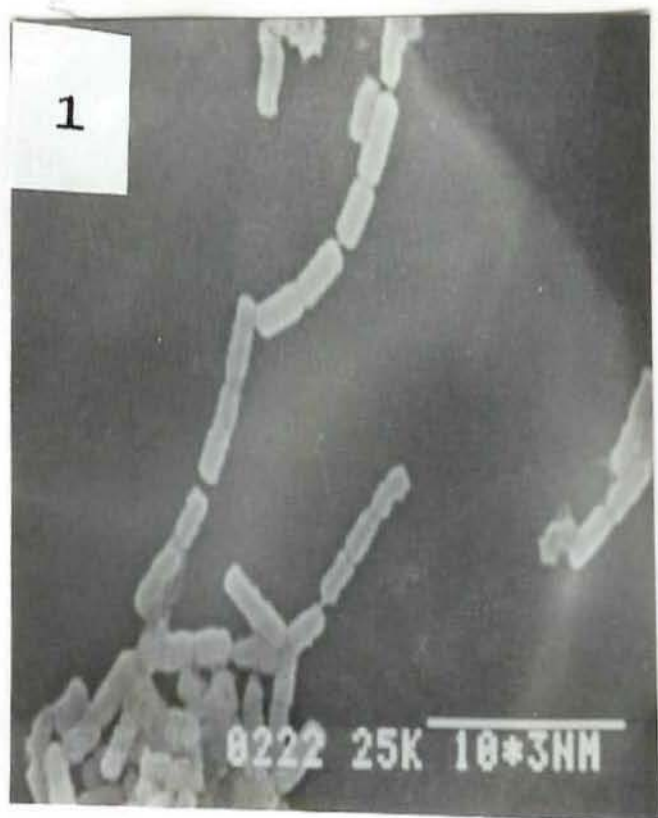


Plate XI
(Figs. 1-4)

Scanning Electron Micrographs of normal and
NISIN treated cells of *Bacillus subtilis* (6633)

- Fig.1 Control sample showing intact rods in chains.
- Fig.2 MIC (x 3000) treated showing partial dissolution of chain links (Solid arrow). Surface undulation in some cells are also visible.
- Fig.3 MIC (x 5000) treated showing conspicuous clamp connection (Solid arrow) amidst partially damaged cells.
- Fig.4 MIC (x 5000) treated showing relatively advanced stage of cell disintegration.

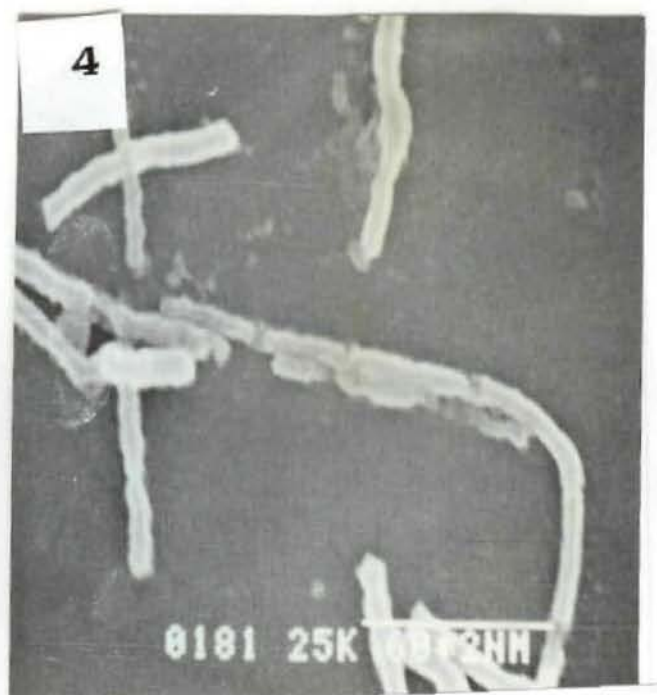
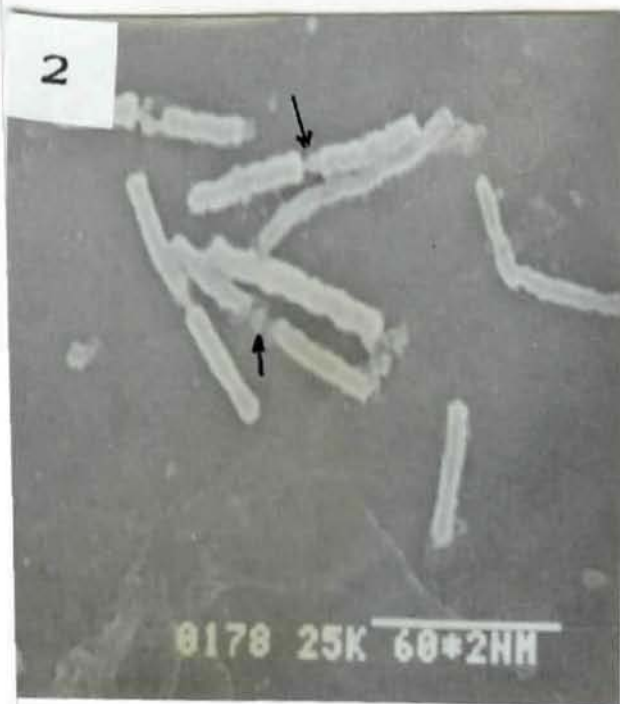
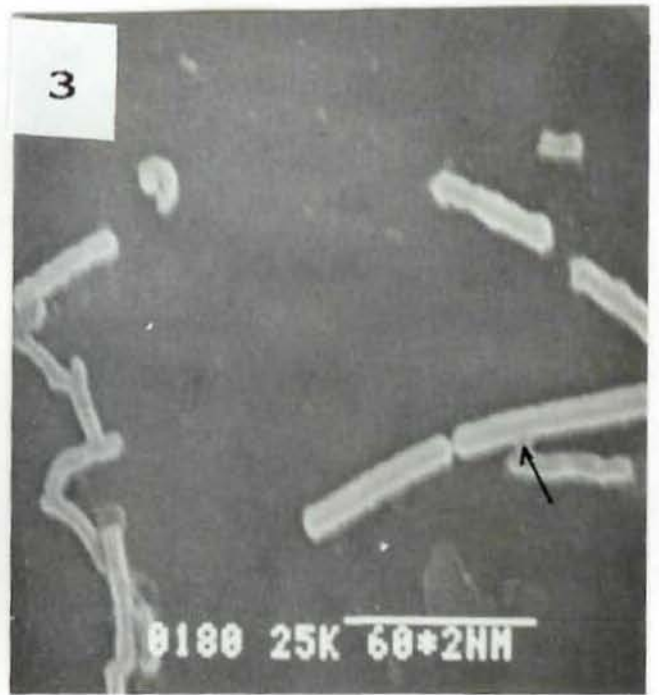
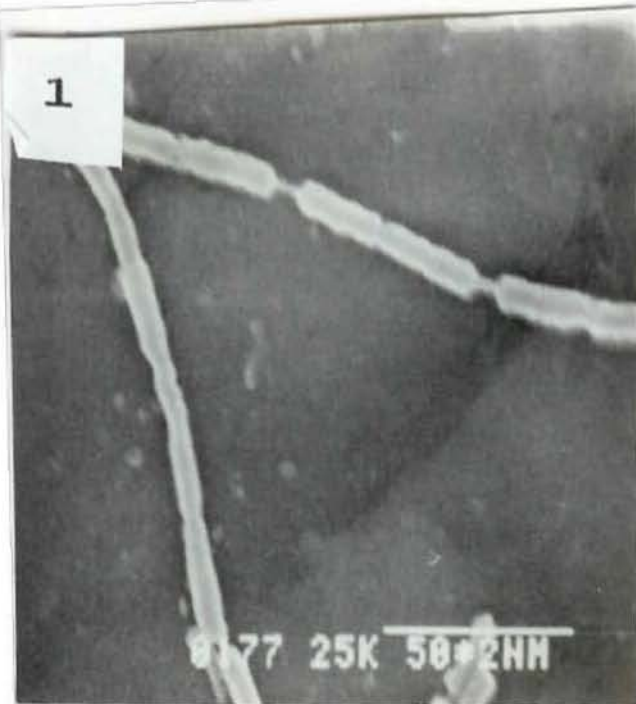
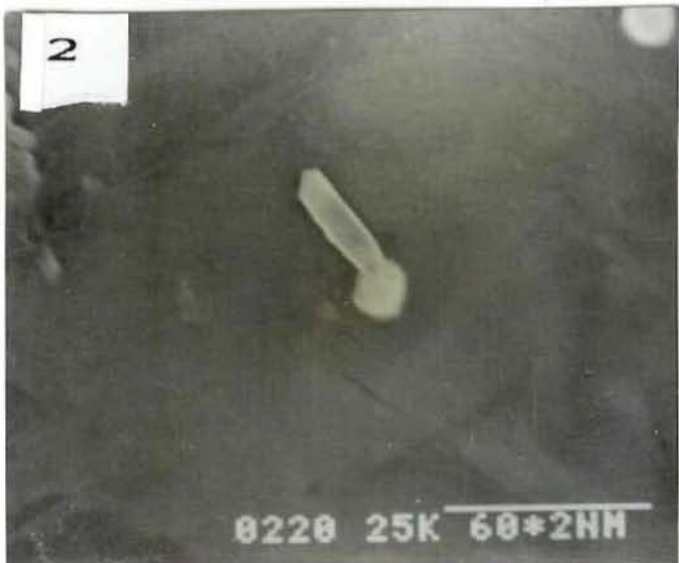
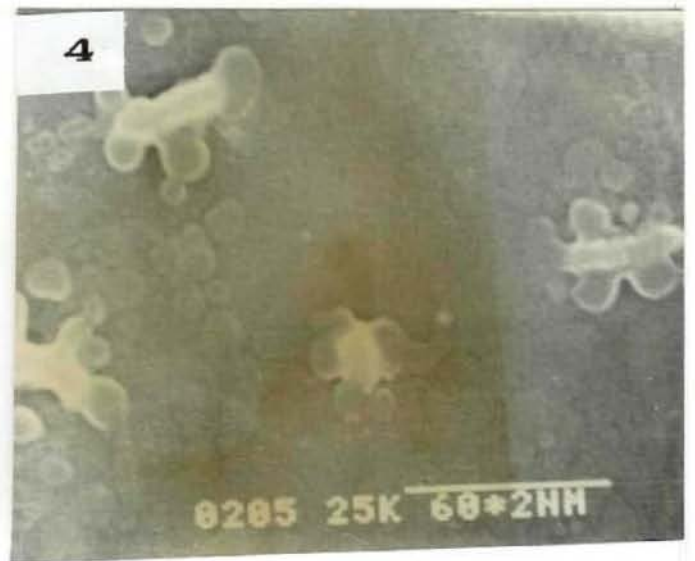
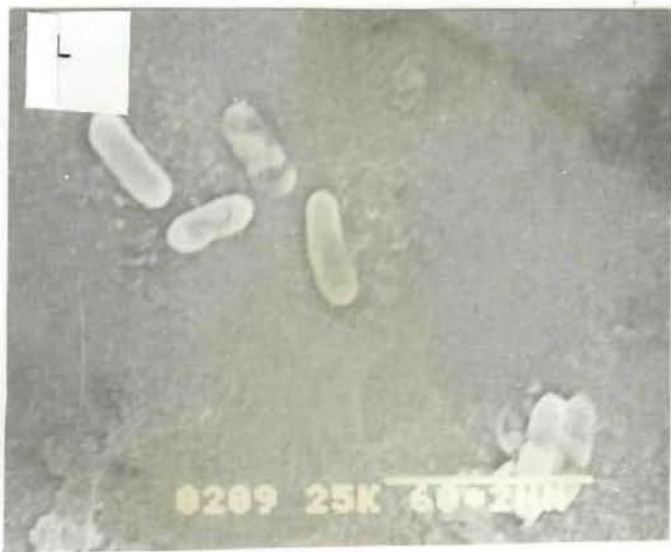


Plate XII Scanning Electron Micrographs of normal and NISIN
(Figs. 1-6) treated cells of *Bacillus stearothermophilus* (38)

- Fig.1 Control sample showing intact small rods.
- Fig.2 MIC (x 1000) treated showing rod with
 swollen tips at one end.
- Figs. 3 & 4 MIC (x 1000) treated showing partial to
 complete disintegration with balloon like
 protrusions.
- Fig.5 MIC (x 3000) treated showing dis-
 integration throughout the surface.
- Fig. 6 MIC (x 3000) treated showing complete
 disintegration of cells.



of 1.0 RU nisin/ml as reported earlier (Chapter I). Normally, the tip of cell was found more prone to nisin action. At one thousand times MIC, swelling of bacterial cells specifically in the tip region was observed (Fig. 2). It may perhaps arise due to weakening of the cell tips resulting in accumulation of protoplasmic contents. Appearance of Balloon-like eruptions could be explained on similar lines (Figs. 3 and 4).

Further intensive structural damages were noted at three thousands times MIC. Partial to complete disintegration of cells and loss of cellular outlines was observed. Complete rupturing of cells and splattered protoplasmic contents could be seen around damaged cells (Figs. 5 and 6). In one report, Ramseier (1960) demonstrated that *Clostridium butyricum* after treatment with nisin undergo bacteriolysis and appears more intense during logarithmic phase of growth. The lysed bacterial cells were also found to release some substances absorbing at 260 nm.

The presence of structural lesions in defined areas of cell wall of *Escherichia coli* following treatments with Bacitracin a bacteriocin produced by *B. licheniformis* were observed by Walton (1976) under EM using negative staining technique. In another study, Holland (1962) reported that *B. megaterium* (207N) strain when treated with megacin at 37°C showed a gradual loss of intra-cellular material examined under phase contrast microscope.

Based on overall informations from the present study, it can be concluded that the action of nisin particularly at MIC concentrations is bacteriostatic. Most bacterial strains studied at MIC undergo loose or compact aggregation preceding loss of chain. The precise surface changes at cellular level were, however, not observed.

The bacteriocidal action of nisin was quite apparent at high doses of nisin ranging from 1-10,000 times of MIC. The initial nisin-induced surface changes like pore and bleb formation, specific rupturing of cells and its subsequent release of protoplasmic contents, acute perforation of cells and dissolution of links, were frequently observed among the strains studied. The partial or complete disintegration of cells and appearance of protoplast-like structures were observed at very high concentrations of nisin. However, the action of nisin on lactic and non-lactic cells is non specific. Moreover, it is difficult to explain the nature of surface damages following nisin treatment.

**IV. PRESERVATIVE EFFECT OF NISIN
INCORPORATION IN MILK AND
MILK PRODUCTS**

4.6 SHELF-LIFE OF MILK AND MILK PRODUCTS

The dairy products comprise a variety of foods based on milk or its by-products as major ingredients. Some of these foods are pasteurised and have a limited shelf-life under refrigerated conditions. Different conditions of climate, milk supply, transport and processing throughout the world bring about diverse nature of bacteriological problems to the dairy industry. Addition of nisin appears to have significant role in solving many storage problems in pasteurised milk and milk products.

4.6.1 Shelf-life of Pasteurised and Nisin treated Pasteurised milk

The commercial pasteurised toned milk procured from Experimental Dairy Plant of the Institute, was incorporated with nisin at concentrations ranging from 100-500 RU/ml and shelf-life was assessed on the basis of sensory scores, titrable acidity (% lactic acid) and standard plate counts (SPC) at three temperatures, 37, 30 and 20°C at 8, 12 and 16 h intervals respectively (Tables 13-15). The preservative action of nisin was found significantly higher at 20°C followed by 30 and 37°C.

4.6.1.1 Shelf-life of pasteurised milk at 37°C

The initial quality of the pasteurised milk at 0 h (4.96 c.f.u/ml) was not as per the prescribed standard (4.47 c.f.u./ml) and the control sample was spoiled in less than 8 h (Table 13). The sensory evaluation of the untreated sample at 8 h of storage revealed some changes in consistency of the product, flakes formation (clot on boiling) and slight bitter taste. On subsequent storage for 16, 24 and 32h resulted in whey formation, bitterness or sweet curdling and off odours in the spoiled products.

Table 13. Preservative effect of nisin addition in 'Toned pasteurised milk' at 37°C and different storage periods

Nisin concentration (RU/ml)	Sensory scores*				Titrable acidity (pH) % lactic acid				SPC Log counts/ml			
	Storage period (h)				Storage period (h)				Storage period (h)			
	8	16	24	32	8	16	24	32	8	16	24	32
	37.5***				0.17*** (6.69)				4.96***			
0	34	32	32	32	0.23** (6.48)	0.28 (6.26)	0.35 (5.91)	0.41 (5.72)	5.91	6.48	7.59	8.14
100	36.6	33	32	32	0.19 (6.66)	0.22** (6.54)	0.26 (6.36)	0.32 (6.09)	5.38	5.88	6.58	7.02
200	37	33	32	32	0.17 (6.69)	0.19 (6.65)	0.21** (6.56)	0.25 (6.40)	4.83	5.44	5.98	6.74
300	37.3	36.5	32	32	0.17 (6.68)	0.18 (6.67)	0.20 (6.61)	0.23** (6.49)	4.07	4.76	5.30	6.00
400	37.5	37	34	33	0.17 (6.69)	0.18 (6.68)	0.18 (6.67)	0.21 (6.57)	3.92	4.17	4.88	5.32
500	37	37	36	34	0.18 (6.68)	0.18 (6.68)	0.18 (6.67)	0.19 (6.66)	2.80	3.44	3.91	4.55

* 40-38, excellent; 37-35, good; 34-32, poor

** Clot-on-Boiling (COB)

*** Control sample at 0 h

The shelf-life of pasteurised milk containing 100 or 200 RU nisin/ml was not significantly affected. However, the product remained unchanged upto 8 h and subsequent increase in storage periods for 16, 24 and 32 h emulate the similar changes, albeit, at a slow rate, as observed in untreated sample.

The preservative effect of nisin in pasteurised milk enhanced significantly at higher doses and increased the shelf-life from initial 8 to 16 h at 300 and 32 h at 500 RU nisin/ml. These products were highly acceptable in terms of sensory, chemical and microbiological quality upto 24 h. However, at 32 h of storage, some changes in consistency of the product was observed which appears to be due to the presence of high levels of proteases and lipases in initial raw milk used for pasteurisation.

4.6.1.2 Shelf-life of pasteurised milk at 30°C

The decrease in incubation temperature from 37 to 30°C, resulted in slightly improved shelf-life of un-treated sample (Table 14). Subsequent increase of storage periods to 24, 36 and 48 h brought about similar kinds of organoleptic, chemical and microbiological changes as observed at 37°C.

The addition of 100 RU nisin/ml was found to have very little effect on shelf life of pasteurised milk at 30°C, however, 200 and 300 RU/ml increased the acceptability of the product to 12 and 24 h respectively. The inhibitory effect of nisin at higher doses 400 or 500 RU/ml in pasteurised milk was rather encouraging and further improved the acceptability of the product upto 48 h. A small change in consistency of the product was, however, observed at 48 h of storage.

Table 14. Preservative effect of nisin addition in 'Toned pasteurised milk' at 30°C and different storage periods

Nisin concentration (RU/ml)	Sensory scores*				Titrable acidity (pH) % lactic acid				SPC Log counts/ml			
	Storage period (h)				Storage period (h)				Storage period (h)			
	12	24	36	48	12	24	36	48	12	24	36	48
	37.5***				0.17*** (6.68)				4.84***			
0	33.5	33	32.5	32	0.23** (6.50)	0.29 (6.22)	0.36 (5.89)	0.42 (5.75)	5.85	6.39	6.94	7.69
100	34	33.5	33.5	33	0.19 (6.66)	0.23** (6.50)	0.28 (6.26)	0.33 (6.02)	5.25	5.72	6.08	6.81
200	36.5	34	32	32.5	0.17 (6.70)	0.19 (6.65)	0.22** (6.53)	0.25 (6.40)	4.73	5.25	5.83	6.46
300	37	36.5	34	33.5	0.17 (6.69)	0.18 (6.68)	0.20 (6.61)	0.23** (6.50)	3.96	4.54	5.00	5.74
400	37.5	36	34.5	34	0.18 (6.68)	0.18 (6.68)	0.19 (6.66)	0.20 (6.61)	2.44	2.78	3.51	4.88
500	37	37	36.5	35	0.18 (6.68)	0.18 (6.68)	0.18 (6.67)	0.19 (6.67)	1.30	1.79	2.27	3.63

* 40-38, excellent; 37-35, good; 34-32, poor

** Clot-on-Boiling (COB)

*** Control sample at 0 h

4.6.1.3 Shelf-life of pasteurised milk at 20°C

The shelf-life of un-treated sample increased by 2 folds with the decrease of storage temperature from 37 to 20°C (Table 15). The bacteriological analysis of the un-treated spoiled sample at 32, 48 and 64 h storage indicated the pre-dominance of yeast and moulds at 20°C which may be partially responsible for spoilage of the product.

A considerable enhancement in shelf-life of nisin treated sample was observed with the decrease of incubation temperature from 37 to 20°C. Nisin concentration of 200 RU/ml in pasteurised milk doubled the shelf-life and the acceptability of the product in terms of sensory, chemical and microbiological quality upto 32 h storage (Table 15).

Further increase in nisin concentration to 500 RU/ml in pasteurised milk, increased the shelf-life upto 2½ days. However, the changes observed in the consistency of the product at higher temperatures 30 or 37°C were not detected at 20°C during sensory evaluation of products.

On the basis of above results, it can be concluded that the preservative action of nisin at higher temperature 37 or 30°C was effective only at very high doses of approximately 500 RU/ml and the shelf-life could be increased upto 1 and 1½ days respectively. Nisin concentration of 100-1000 RU/ml in commercial pasteurised milk was found to have no effect on the keeping quality at 32°C as reported by Mahmoud et al. (1976). However, in other reports, Anon (1988a) demonstrated that a nisin concentration of 35-50 mg/litre in pasteurised milk was effective at 35 and 50°C and shelf life doubled in comparison to control sample. This may be because of better initial quality of the raw milk, environmental factors and its subsequent processing conditions.

Table 15. Preservative effect of nisin addition in 'Toned pasteurised milk' at 20°C and different storage periods

Nisin concentration (RU/ml)	Sensory scores*				Titrable acidity (pH) % lactic acid				SPC Log counts/ml			
	Storage period (h)				Storage period (h)				Storage period (h)			
	16	32	48	64	16	32	48	64	16	32	48	64
	38.5***				0.17*** (6.68)				4.92***			
0	34	33	32.5	32	0.23** (6.50)	0.28 (6.26)	0.34 (5.94)	0.39 (5.78)	5.78	6.38	6.95	7.72
100	38.7	32.5	32.5	32	0.20 (6.62)	0.23** (6.50)	0.27 (6.31)	0.32 (6.09)	5.28	5.79	6.34	6.83
200	38	34.8	33.5	32.5	0.17 (6.70)	0.20 (6.62)	0.23** (6.50)	0.27 (6.31)	4.39	4.94	5.70	6.00
300	38.5	37.2	34	33	0.17 (6.69)	0.18 (6.69)	0.21 (6.58)	0.23** (6.50)	3.83	4.39	4.99	5.51
400	39.7	38	35	34	0.18 (6.69)	0.18 (6.69)	0.19 (6.67)	0.21 (6.59)	2.57	2.78	3.50	4.95
500	38.7	38.5	37	36	0.18 (6.68)	0.18 (6.68)	0.18 (6.68)	0.19 (6.67)	1.06	1.89	2.62	3.86

* 40-38, excellent; 37-35, good; 34-32, poor

** Clot-on-Boiling (COB)

*** Control sample at 0 h

The spoilage like, bitterness or sweet curdling, off odours and whey separation was observed in pasteurised milk at higher temperatures 37 or 30°C in the present study. This may appear due to higher counts of heat resistant and thermophilic bacteria in pasteurised milk or the Gram negative bacteria which are known to be unaffected by nisin, as a result of post-pasteurisation contamination or their presence in high numbers in initial raw milk. In a similar study, Mahmoud et al. (1976) demonstrated that Gram negative bacteria like *Escherichia coli*, *Aerobacter aerogenes* and *Pseudomonas fluorescens* were not inhibited in presence of 100-1000 RU nisin/ml in pasteurised milk and were mainly responsible for the spoilage.

In the present study, the preservative effect of nisin in pasteurised milk was encouraging at 20°C and the product was acceptable for more than 1 and 2½ days at 200 and 500 RU nisin/ml respectively.

In a similar study, Magdoub et al. (1984) demonstrated that the shelf-life of pasteurised milk containing 100 RU nisin/ml and 0.1% alanine extended to 2½ days at 20°C. However, prior to pasteurisation, additional flash heat shock at 85°C was also given which may have contributed towards enhanced preservative action at low level of nisin.

4.6.2. Fermented Milk Products

The fermented milk products have very low keeping quality because of high acid development during storage which makes the product unacceptable. In tropical countries like India, where facilities for cold storage, transportation and distribution are not sufficient and therefore, to avoid the cold storage of these products, altogether, nisin can play an important role in controlling the excess of acid production and thus the shelf-life can be increased considerably.

4.6.2.1 Shelf-life of Lassi and nisin treated Lassi

In the present study, an attempt was made to enhance the shelf-life of commercial product Lassi procured from Experimental Dairy Plant of the Institute. Nisin was incorporated at concentrations ranging from 100-500 RU/ml and the shelf-life was assessed on the basis of sensory scores, titrable acidity (% lactic acid), lactic and yeast and mold counts at two temperatures i.e. 30 and 20°C at 8 and 12 h intervals respectively. The preservative action of nisin was found superior at 20°C in comparison to 30°C.

4.6.2.1.1 Shelf-life of Lassi incubated at 30°C:

The control Lassi sample was acceptable upto 8 h and thereafter, with the increase of storage, the product becomes unacceptable due to tremendous growth of lactic acid bacteria (7.76 to 9.54 c.f.u. log counts/ml). As a result, the titrable acidity increased from 0.85 to 1.05% lactic acid. The yeast and mold counts were also found to increase from 2.47 to 3.79 c.f.u. log counts/ml (Table 16).

The major defects observed in spoiled samples during sensory evaluation were sour taste, whey separation and changes in consistency of the products.

The addition of 200 to 300 RU nisin/ml was found to have significant effect on shelf-life of Lassi and increase the acceptability of the product by 3 folds. The lactic counts and the titrable acidity of Lassi containing 200 and 300 RU nisin/ml were 7.07, 6.77 c.f.u./ml and 0.85, 0.82 respectively at 24 h storage.

The growth of yeast and molds in nisin treated samples was slightly higher in comparison to control samples. However, the presence of higher yeast and mold counts in Lassi samples containing 400 or 500 nisin/ml

Table 16. Preservative effect of nisin addition in 'lassi' at 30°C and different storage periods

Nisin concentration (RU/ml)	Sensory scores*				Titrable acidity (pH) % lactic acid				Yeast and molds (lactic) Log counts/ml			
	Storage period (h)				Storage period (h)				Storage period (h)			
	8	16	24	32	8	16	24	32	8	16	24	32
	38**				0.78** (4.55)				2.32** (6.69)			
0	38	34	33	32.5	0.85 (4.43)	0.89 (4.31)	0.96 (4.12)	1.05 (3.86)	2.47 (7.76)	2.69 (8.03)	3.00 (8.93)	3.79 (9.54)
100	38	33.5	33	32.5	0.82 (4.48)	0.85 (4.43)	0.91 (4.25)	0.96 (4.17)	2.51 (6.91)	2.69 (7.37)	3.08 (7.85)	3.83 (8.34)
200	38	36	35	33.5	0.80 (4.52)	0.83 (4.45)	0.85 (4.43)	0.90 (4.29)	2.54 (6.20)	2.71 (6.86)	3.25 (7.07)	3.90 (7.89)
300	37.5	36.5	35	34	0.78 (4.55)	0.79 (4.52)	0.82 (4.46)	0.85 (4.43)	2.54 (5.76)	2.71 (6.10)	3.39 (6.77)	3.99 (7.34)
400	38	37.5	37	34.5	0.78 (4.55)	0.79 (4.52)	0.81 (4.48)	0.83 (4.45)	2.55 (5.30)	2.74 (5.81)	3.51 (6.30)	4.11 (6.78)
500	38	37.5	37	35	0.79 (4.52)	0.79 (4.52)	0.80 (4.50)	0.82 (4.47)	2.55 (4.91)	2.74 (5.54)	3.54 (5.93)	4.17 (6.44)

* 40-38, excellent; 37-35, good; 34-32, poor

** Control sample at 0 h

Table 17. Preservative effect of nisin addition in 'lassi' at 20°C and different storage periods

Nisin concentration (RU/ml)	Sensory scores*				Titrable acidity (pH) % lactic acid				Yeast and molds (lactic) Log counts/ml			
	Storage period (h)				Storage period (h)				Storage period (h)			
	12	24	36	48	12	24	36	48	12	24	36	48
	39**				0.75** (4.61)				2.41** (6.61)			
0	38.5	34	33	33	0.80 (4.50)	0.83 (4.45)	0.89 (4.30)	0.95 (4.13)	2.76 (7.07)	3.27 (7.76)	3.72 (8.07)	4.07 (8.69)
100	38	37	33.3	32	0.79 (4.53)	0.81 (4.50)	0.85 (4.43)	0.89 (4.30)	2.77 (6.91)	3.30 (7.34)	3.72 (7.78)	4.09 (8.17)
200	37	37	33.5	31.5	0.75 (4.60)	0.78 (4.54)	0.82 (4.46)	0.85 (4.43)	2.79 (6.40)	3.39 (6.83)	3.73 (7.57)	4.17 (7.91)
300	37	37.5	36	33.5	0.75 (4.60)	0.76 (4.57)	0.79 (4.53)	0.83 (4.45)	2.81 (5.92)	3.44 (6.41)	3.76 (6.89)	4.25 (7.55)
400	37	37.6	38	35	0.76 (4.58)	0.76 (4.58)	0.78 (4.54)	0.80 (4.52)	2.83 (5.32)	3.47 (5.80)	3.77 (6.25)	4.34 (6.74)
500	37.5	37	37.2	35	0.76 (4.58)	0.77 (4.56)	0.78 (4.55)	0.80 (4.52)	2.83 (4.82)	3.48 (5.00)	3.77 (5.71)	4.36 (6.07)

* 40-38, excellent; 37-35, good; 34-32, poor

** Control sample at 0 h

RU nisin/ml could be kept upto 8-10 days without much change in acceptability.

However, the scope for nisin incorporation in commercial products like Lassi and pasteurised toned milk and such others appears to be very encouraging as evident from the increase in their shelf-life at ambient temperature. Moreover, the efficacy of nisin can be further improved if the hygienic conditions are properly maintained during the processing of raw milk and its subsequent conversion into products.

The information so generated can be of great significance in dairy industry especially in our context, where the facilities for refrigeration, transportation and distribution are extremely limited. The shelf-life of milk and milk products like flavoured, pasteurised and UHT milk is not satisfactory because of poor initial quality of raw milk and therefore, the addition of nisin in these products can improve the shelf-life considerably without much addition in the cost.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.0 SUMMARY AND CONCLUSIONS

The present research project was undertaken with definite objectives in mind to study the behaviour of different groups of Gram positive bacteria at genera, species and strains levels when treated with nisin. The comparative information in broth and skim milk presented a wide variation in nisin sensitivity among various strains studied. The inhibitory action of nisin was adversely affected at higher pH, temperature, inoculum and incubation periods. Bacteriostatic and bacteriocidal action of nisin at Minimal Inhibitory Concentrations (MIC) and higher doses respectively was confirmed by light and scanning electron microscopy. Finally, a considerable enhancement in shelf-life of commercial pasteurised toned milk and Lassi was achieved without much additional cost. The salient features of research findings are summarised as follows:

Twenty six strains of Gram positive bacteria belonging to different groups, lactococci, streptococci, lactobacilli and bacilli were evaluated for nisin sensitivity in broth at 1% inoculum under optimum growth conditions. *Lactococcus lactis* subsp. *cremoris* (C₃), *Str. salivarius* subsp. *thermophilus* (1), *L. plantarum* (R) and *B. subtilis* (9144) were found to be the most nisin resistant strains among their respective groups. Their MIC were 175, 125, 100 and 150 RU nisin/ml in broth at 20h incubation. *Lactococcus lactis* subsp. *diacetylactis* (DRC₁), *L. plantarum* (89) and *B. stearothermophilus* (37 and 38) were highly sensitive and inhibited at very low level having MIC of 5, 20, 0.5 and 1.0 RU nisin/ml respectively.

The behaviour of nisin producing strains of *L. lactis* subsp. *lactis* (496 and 440) was very different. These depicted a very high nisin tolerance of 1600 and 2000 RU nisin/ml at 18 h of incubation. However, their inhibition was observed only with 13,200 and 14,000 RU/ml respectively at 24 h. Higher nisin tolerance among nisin and non-nisin producing strains may be related to inactivating enzyme NISINASE during their growth. A number of factors were found to be associated with a wide response in nisin sensitivity.

Nisin sensitivity in skim milk was also carried out on similar lines for comparative information. A significant reduction in inhibitory action of nisin by approx. 2-5 folds was observed in comparison to broth study. The drastic decrease in nisin activity may be assigned to the binding of milk proteins either, with bacterial cultural or nisin itself. The involvement of fat in skim milk in inhibiting the nisin activity also cannot be ruled out.

Activity of nisin in low or high acid foods is greatly influenced during storage at different incubation temperatures. Further, nisin is acidic in nature and its stability and solubility is largely affected by different pH of solution. The increase of pH from 5.5 to 7.5 in Elliker's broth increased the nisin requirements among lactococci and streptococci by approx. 2-19 folds. Their MIC were 2-60, 5-200 and 12.5-325 RU nisin/ml at pH 5.5, 6.5 (optimum) and 7.5 respectively.

The response among lactobacilli was relatively low in MRS broth. Approximately, 2-10 folds increase in nisin tolerance was noted with the rise of pH from 5.0 to 6.0 (optimum) and 7.0. However, inhibition of bacilli was observed at MIC range between 15-75, 45-175 and 60-225 RU nisin/ml at pH 6.5, 7.0 (optimum) and 7.5 respectively.

The enhanced effectiveness of nisin under acidic conditions appears to be due to acid damaging effects on the growth or higher stability and solubility of nisin. As reported in literature earlier, nisin contains some unusual amino acids like Dehydroalanine (Dha) and Dehydrobutyrine (Dhb). Susceptibility of these amino acids to hydroxyl groups at alkaline pH may be responsible for its loss of activity.

In general, the requirement of nisin was found maximum at optimum growth temperature for all the strains. However, a considerable enhancement in activity of nisin was observed with either, the decrease or increase of optimal temperature.

Complete inhibition of lactococci was observed at MIC range between 2-65, 5-200 and 2-100 RU nisin/ml at 25, 30 (optimum) and 37°C respectively. Whereas, streptococci were inhibited at 10-15, 75-150 and 20-50 RU/ml at 30, 37 (optimum) and 45°C respectively. However, the behaviour of lactobacilli and bacilli under 3 different temperature regimes was comparable with lactococci and streptococci. Higher cell counts of the organism at optimum growth temperature, perhaps increase the nisin requirement considerably as compared to sub-optimal temperatures. So also, the requirement of nisin was found to increase with the increase of inocula. Approximately, 1-2 folds increase in inhibitory dose of nisin was observed with the rise of inoculum from 1 to 2% level.

A continuous loss of nisin activity among lactococci, streptococci, lactobacilli and bacilli was observed with the rise of incubation period from 12 to 24 and 48 h. Residual nisin in milk after initial bacteriostatic action appears to be insufficient for the complete inhibition of growing microflora at higher incubation periods.

Mode of action of nisin was studied through light and Scanning Electron Microscopy (SEM). Most sensitive and resistant strains, selected on the basis of preliminary findings were evaluated for nisin-induced surface changes. Information revealed under light and scanning electron microscope indicated that action of nisin appears to be bacteriostatic at MIC treatments. The tendency of cells to form a loose or compact aggregates resulting in development of clumps was most commonly observed. However, the surface damages were not detected in any strain at MIC of nisin.

Bacteriocidal action of nisin was quite apparent at very high doses of nisin ranging from 1-10,000 times of MIC. The initial nisin-induced surface damages include pore and bleb formation, rupturing of cells and subsequent release of protoplasmic contents, acute perforation and dissolution of chain links. Partial to complete disintegration of cell was usually observed at very high doses of nisin. Protoplast-like structures were most commonly observed particularly in lactococci and streptococci. In fact, the bacteriocidal action of nisin can be conveniently explained in terms of such structural damages at cellular levels. It is also apparent from the above that the cellular damages are random and no specific site for nisin action was identified.

The study also contributed knowledge to applied aspects through selective use of nisin. Incorporation of nisin in commercial Pasteurised toned milk exhibited much success. Shelf-life of pasteurised milk containing 500 RU nisin/ml was extended upto 2½ days at ambient temperature. The increase in shelf-life of raw and pasteurised milk by 2-3 days can be of great significance in tropical countries like ours, where the facilities for refrigeration, transportation and distribution are not satisfactory. During sensory evaluation of pasteurised milk at higher

temperatures, changes in consistency of nisin treated product was observed. Perhaps these may be associated with high level of proteases and lipases present in initial raw milk.

Nisin can play an important role in enhancing the shelf-life of fermented milk products. Addition of 400 to 500 RU nisin/ml in commercial product Lassi was effective at 20°C. The product could be kept for over 2 days without any deterioration in sensory, chemical and microbiological quality.

The valuable information generated in the present study can find enormous applications. During the preliminary screening of lactic cultures, *Lactococcus lactis* subsp. *cremoris* (C₃) was found to be the most resistant strain. However, there are common problem of starter failure in dairy industry due to inhibitory substances especially on account of non-judicious use of nisin which can otherwise be controlled precisely. Other nisin resistant strains screened in the present investigation, can be advantageously used as starters in various fermented dairy and non-dairy products. The relative growth of the organisms can be controlled suitably to improve the cheese ripening and fermented beverages.

Further, in various industrial fermentation processes like wine, beer, ethyl alcohol fermentation etc. lactic acid bacteria especially lactobacilli appears as contaminants and affects the yield drastically. Based on results of present study, growth of this potential contaminant can be fully checked in such fermentations.

Lactic and non-lactic organisms which constitute the main microflora of various milk and milk products are generally sensitive to nisin. The minimal inhibitory concentration (MIC) of nisin therefore, will be directly helpful in increasing the shelf-life and retaining the desirable quality

of raw, pasteurised milk, sterilised/UHT and processed milks. Similarly, the shelf-life of several other fermented milk products like Dahi, Kefir, Kumiss and Shrikhand can be considerably improved by regulating the acid production without affecting their organoleptic properties.

The use of SEM for study of bacterial cultures has been fully justified while explaining the nature of fine structural damages at cellular levels as a result of nisin action. This opens up a wide scope for the study of their mode of action by other bacteriocins.

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APPENDICES

TABLE I. EFFECT OF NISIN ON THE BEHAVIOUR OF *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>L. lactis</i> subsp. <i>lactis</i> (C ₁₀)				<i>L. lactis</i> subsp. <i>lactis</i> (MLg)			
	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml
1.	0	0.582	4.84	7.51	0	0.646	4.72	8.03
2.	5	0.476	5.30	6.08	15	0.438	5.43	6.58
3.	10	0.364	5.64	5.59	25	0.237	6.07	5.71
4.	15	0.269	5.93	3.45	35	0.171	6.20	4.00
5.	25	0.124	6.30	1.89	50	0.069	6.38	2.90
6.	35	0.000	6.48	0.00	75	0.000	6.48	0.00

TABLE II. EFFECT OF NISIN ON THE BEHAVIOUR OF *LACTOCOCCUS LACTIS* SUBSP. *DIACETYLLACTIS* STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)				<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)			
	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml
1.	0	0.664	4.64	7.66	0	0.535	5.09	6.55
2.	2.5	0.609	4.76	6.50	2.5	0.382	5.60	3.63
3.	5	0.428	5.48	5.46	5.0	0.000	6.49	0.00
4.	10	0.349	5.70	4.41				
5.	15	0.275	5.92	2.61				
6.	20	0.116	6.38	1.28				
7.	25	0.000	6.48	0.00				

TABLE III. EFFECT OF NISIN ON THE BEHAVIOUR OF LACTOCOCCUS LACTIS SUBSP. CREMORIS STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₃)				<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)			
	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml
1.	0	0.688	4.62	8.92	0	0.649	4.70	8.43
2.	25	0.502	5.20	6.73	5	0.584	4.92	7.39
3.	50	0.399	5.56	5.36	10	0.506	5.19	6.08
4.	75	0.246	5.99	4.49	15	0.358	5.68	4.98
5.	100	0.108	6.34	3.82	25	0.152	6.24	2.26
6.	125	0.086	6.38	2.46	35	0.000	6.49	0.00
7.	150	0.049	6.41	1.29				
8.	175	0.000	6.48	0.00				

TABLE IV. EFFECT OF NISIN ON THE BEHAVIOUR OF *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>L. lactis</i> subsp. <i>lactis</i> (440) (NISIN PRODUCER)				<i>L. lactis</i> subsp. <i>lactis</i> (496) (NISIN PRODUCER)			
	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml
1.	0	0.698	4.60	8.67	0	0.658	4.68	8.13
2.	400	0.704	4.58	8.71	200	0.698	4.61	8.35
3.	600	0.569	4.92	7.67	400	0.475	5.30	7.97
4.	800	0.468	5.35	5.68	600	0.362	5.64	6.58
5.	1200	0.398	5.56	3.90	800	0.219	6.10	4.69
6.	1600	0.216	6.12	2.04	1200	0.048	6.38	2.92
7.	2000	0.000	6.45	0.00	1600	0.000	6.46	0.00

TABLE V. EFFECT OF NISIN ON THE BEHAVIOUR OF *STREPTOCOCCUS SALIVARIUS* SUBSP. *THERMOPHILUS* STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (H)				<i>Str. salivarius</i> subsp. <i>thermophilus</i> (I)			
	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml
1.	0	0.720	4.58	7.83	0	0.782	4.45	8.38
2.	5	0.662	4.74	7.43	25	0.529	5.10	6.19
3.	10	0.440	5.43	5.90	50	0.248	5.98	4.50
4.	25	0.216	6.10	4.29	75	0.168	6.21	2.99
5.	50	0.116	6.31	3.61	100	0.129	6.32	1.84
6.	75	0.000	6.48	0.00	125	0.000	6.48	0.00

TABLE VIII. EFFECT OF NISIN ON THE BEHAVIOUR OF *LACTOBACILLUS PLANTARUM* STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>L. plantarum</i> (R)				<i>L. plantarum</i> (89)			
	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml	Nisin concentration (RU/ml)	Optical density (650 nm)	pH	Log counts/ml
1.	0	0.592	4.76	7.02	0	0.520	4.88	7.49
2.	5	0.460	5.08	6.18	2.5	0.478	5.05	6.62
3.	15	0.353	5.29	5.29	5	0.368	5.28	5.40
4.	25	0.305	5.40	4.82	10	0.244	5.56	4.60
5.	50	0.267	5.50	3.43	15	0.129	5.84	2.33
6.	75	0.148	5.80	1.98	20	0.000	5.99	0.00
7.	100	0.000	5.98	0.00				

TABLE IX. EFFECT OF NISIN ON THE BEHAVIOUR OF *BACILLUS SUBTILIS* STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>B. subtilis</i> (9144)		<i>B. subtilis</i> (441)		<i>B. subtilis</i> (6633)	
	Nisin concentration (RU/ml)	Log counts/ml	Nisin concentration (RU/ml)	Log counts/ml	Nisin concentration (RU/ml)	Log counts/ml
1.	0	6.07	0	7.04	0	6.68
2.	25	5.46	5	6.64	25	5.57
3.	50	4.77	25	5.83	50	4.90
4.	75	3.50	50	4.68	75	3.83
5.	100	2.49	75	2.98	100	2.20
6.	125	1.28	100	1.51	125	0.00
7.	150	0.00	135	0.00		

TABLE X. EFFECT OF NISIN ON THE BEHAVIOUR OF *BACILLUS CEREUS* AND *BACILLUS STEAROTHERMOPHILUS* STRAINS AT 1% INOCULUM AND 24 H OF INCUBATION IN BROTH AT OPTIMUM pH AND TEMPERATURE

Sl. No.	<i>B. cereus</i> (10876)		<i>B. cereus</i> (430)		<i>B. stearothermophilus</i> (953)	
	Nisin concentration (RU/ml)	Standard plate count/ml	Nisin concentration (RU/ml)	Standard plate count/ml	Nisin concentration (RU/ml)	Standard plate count/ml
1.	0	7.00	0	6.59	0	4.27
2.	5	6.38	5	5.71	10	3.94
3.	15	5.81	10	5.20	15	3.63
4.	25	4.54	15	4.44	25	2.84
5.	50	2.96	30	2.55	35	1.69
6.	75	0.00	45	0.00	45	0.00

TABLE XI. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *L. LACTIS* SUBSP. *LACTIS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>L. lactis</i> subsp. <i>lactis</i> (C ₁₀)				<i>L. lactis</i> subsp. <i>lactis</i> (MLg)			
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48
1.	0	0.46 (5.76)	0.72 (4.92)	0.81 (4.65)	0	0.45 (5.78)	0.75 (4.84)	0.89 (4.50)
2.	25	0.28 (6.25)	0.56 (5.49)	0.64 (5.24)	100	0.29 (6.21)	0.52 (5.62)	0.68 (5.12)
3.	50	0.19* (6.48)	0.36 (6.05)	0.55 (5.52)	125	0.25 (6.28)	0.40 (5.97)	0.59 (5.40)
4.	75		0.19* (6.48)	0.38 (6.00)	150	0.19* (6.48)	0.32 (6.12)	0.46 (5.75)
5.	100			0.19* (6.47)	175		0.19* (6.48)	0.32 (6.13)
6.					200			0.19* (6.47)

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XII. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *L. LACTIS* SUBSP. *DIACETYLLACTIS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₁)				<i>L. lactis</i> subsp. <i>diacetylactis</i> (DRC ₂)			
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48
1.	0	0.37 (6.00)	0.64 (5.14)	0.71 (4.92)	0	0.42 (5.81)	0.68 (5.08)	0.74 (4.78)
2.	5	0.30 (6.16)	0.50 (5.57)	0.62 (5.50)	10	0.26 (6.25)	0.43 (5.78)	0.57 (5.48)
3.	10	0.19* (6.49)	0.30 (6.15)	0.46 (5.70)	15	0.19* (6.49)	0.32 (6.09)	0.45 (5.72)
4.	15		0.23 (6.31)	0.30 (6.14)	20		0.26 (6.24)	0.36 (6.00)
5.	25		0.19* (6.48)	0.23 (6.30)	35		0.19* (6.48)	0.24 (6.25)
6.	35			0.19* (6.48)	50			0.19* (6.48)

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XIII. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *L. LACTIS* SUBSP. *CREMORIS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>L. lactis</i> subsp. <i>cremoris</i> (C ₁)				<i>L. lactis</i> subsp. <i>cremoris</i> (C ₃)			
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48
1.	0	0.57 (5.47)	0.82 (4.64)	0.96 (4.42)	0	0.58 (5.49)	0.85 (4.56)	0.98 (4.40)
2.	25	0.31 (6.14)	0.64 (5.26)	0.76 (4.80)	25	0.35 (6.09)	0.69 (5.09)	0.76 (4.80)
3.	50	0.19* (6.48)	0.45 (5.81)	0.56 (5.50)	75	0.19* (6.48)	0.50 (5.64)	0.62 (5.50)
4.	75		0.34 (6.10)	0.39 (6.02)	150		0.32 (6.10)	0.46 (5.78)
5.	100		0.19* (6.48)	0.30* (6.16)	225		0.19* (6.48)	0.30 (6.19)
6.	125			0.19* (6.47)	300			0.19* (6.47)

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XIV. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *L. LACTIS* SUBSP. *LACTIS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>L. lactis</i> subsp. <i>lactis</i> (496)				<i>L. lactis</i> subsp. <i>lactis</i> (440)			
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)		
		12	18	24		12	18	24
1.	0	0.55 (5.50)	0.73 (4.82)	0.81 (4.52)	0	0.56 (5.40)	0.76 (4.59)	0.92 (4.28)
2.	400	0.38 (5.92)	0.75 (4.72)	0.78 (4.66)	800	0.50 (5.51)	0.70 (4.83)	0.78 (4.56)
3.	800	0.26 (6.28)	0.61 (5.36)	0.72 (4.86)	1200	0.44 (5.76)	0.67 (5.04)	0.75 (4.62)
4.	1200	0.20* (6.46)	0.42 (5.92)	0.55 (5.43)	1600	0.20* (6.46)	0.54 (5.45)	0.72 (4.80)
5.	1600		0.30 (6.14)	0.50 (5.52)	2000		0.37 (6.00)	0.70 (4.79)
6.	2000		0.20* (6.46)	0.43 (5.79)	2400		0.20* (6.46)	0.58 (5.40)

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XV. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *STR. SALIVARIUS* SUBSP. *THERMOPHILUS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>Str. salivarius</i> subsp. <i>thermophilus</i> (H)				<i>Str. salivarius</i> subsp. <i>thermophilus</i> (I)			
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48
1.	0	0.42 (5.89)	0.58 (5.40)	0.86 (4.52)	0	0.46 (5.75)	0.62 (5.10)	0.91 (4.46)
2.	50	0.26 (6.27)	0.43 (5.87)	0.65 (5.20)	25	0.32 (6.09)	0.47 (5.74)	0.74 (4.84)
3.	75	0.18* (6.48)	0.28 (6.24)	0.46 (5.80)	50	0.24 (6.29)	0.32 (6.10)	0.50 (5.62)
4.	100		0.18* (6.48)	0.32 (6.10)	100	0.18* (6.48)	0.26 (6.28)	0.38 (5.99)
5.	125			0.24 (6.30)	150		0.18* (6.48)	0.30 (6.12)
6.	150			0.19* (6.47)	200			0.19* (6.47)

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XVI. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>L.delbrueckii</i> subsp. <i>bulgaricus</i> (1373)			<i>L.delbrueckii</i> subsp. <i>bulgaricus</i> (RTS)			<i>L.delbrueckii</i> subsp. <i>bulgaricus</i> (W)					
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48		12	24	48
1.	0	0.50 (5.65)	1.18 (4.08)	1.56 (3.86)	0	0.42 (5.90)	0.98 (4.40)	1.38 (4.00)	0	0.56 (5.49)	1.26 (3.98)	1.67 (3.70)
2.	25	0.37 (6.03)	0.84 (4.60)	0.92 (4.44)	5	0.27 (6.28)	0.61 (5.36)	0.96 (4.50)	5	0.40 (5.93)	0.92 (4.45)	1.21 (3.91)
3.	50	0.19* (6.48)	0.48 (5.72)	0.56 (5.50)	25	0.19* (6.29)	0.39 (5.98)	0.56 (5.49)	15	0.28 (6.23)	0.54 (5.56)	0.86 (4.52)
4.	75		0.27 (6.25)	0.42 (5.90)	50		0.30 (6.14)	0.36 (6.00)	25	0.19* (6.48)	0.34 (6.08)	0.44 (5.84)
5.	100		0.19* (6.48)	0.32 (6.12)	75		0.19* (6.48)	0.28 (6.22)	50		0.19* (6.48)	0.36 (6.01)
6.	125			0.19* (6.48)	75			0.19* (6.48)	75			0.19* (6.48)

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XVII. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *LACTOBACILLUS ACIDOPHILUS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>L. acidophilus</i> (447)			<i>L. acidophilus</i> (R)			<i>L. acidophilus</i> (1899)					
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48		12	24	48
1.	0	0.38 (6.00)	0.88 (4.50)	0.92 (4.44)	0	0.32 (6.16)	0.78 (4.72)	0.81 (4.68)	0	0.36 (6.04)	0.82 (4.64)	0.86 (4.52)
2.	25	0.27 (6.25)	0.58 (5.46)	0.66 (5.15)	5	0.26 (6.26)	0.50 (5.65)	0.56 (5.49)	5	0.32 (6.15)	0.69 (5.10)	0.72 (5.01)
3.	50	0.19* (6.48)	0.29 (6.21)	0.40 (5.92)	15	0.24 (6.29)	0.39 (5.94)	0.42 (5.90)	15	0.26 (6.29)	0.42 (5.91)	0.56 (5.50)
4.	75		0.19* (6.48)	0.34 (6.04)	25	0.19* (6.48)	0.26 (6.27)	0.30 (6.14)	25	0.19* (6.48)	0.24 (6.28)	0.35 (6.00)
5.	100			0.28 (6.23)	50		0.19* (6.48)	0.24 (6.30)	50		0.19* (6.48)	0.26 (6.28)
6.	125			0.19* (6.48)	75			0.19* (6.48)	100			0.19* (6.47)

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XVIII. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *LACTOBACILLUS PLANTARUM* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>L. plantarum</i> (R)				<i>L. plantarum</i> (189)			
	Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid			Nisin concentration (RU/ml)	Titrable acidity (TA) % lactic acid		
		Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48
1.	0	0.42 (5.90)	1.04 (4.36)	1.06 (4.32)	0	0.36 (6.07)	0.88 (4.69)	0.96 (4.42)
2.	25	0.26 (6.28)	0.72 (5.02)	0.83 (4.63)	5	0.30 (6.19)	0.60 (5.43)	0.69 (5.10)
3.	50	0.19* (6.48)	0.58 (5.45)	0.66 (5.15)	15	0.24 (6.32)	0.46 (5.75)	0.50 (5.65)
4.	75		0.39 (5.98)	0.47 (5.74)	25	0.19* (6.48)	0.28 (6.26)	0.36 (5.98)
5.	100		0.25 (6.29)	0.35 (6.02)	50		0.19* (6.48)	0.25 (6.27)
6.	150		0.19* (6.48)	0.27 (6.28)	75			0.19* (6.47)
7.	200			0.19* (6.47)				

* Minimal inhibitory concentration
 Figures in parentheses indicate pH values

TABLE XIX. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *BACILLUS SUBTILIS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>B. subtilis</i> (9144)			<i>B. subtilis</i> (6633)			<i>B. subtilis</i> (441)					
	Nisin concentration (RU/ml)	Log counts/ml			Nisin concentration (RU/ml)	Log counts/ml			Nisin concentration (RU/ml)	Log counts/ml		
		Incubation period (h)				Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48		12	24	48
1.	0	4.80	6.59	6.83	0	5.16	6.60	6.84	0	5.54	6.85	7.92
2.	25	2.74	5.08	5.46	50	3.94	5.03	5.88	75	3.77	5.04	5.94
3.	75	0.00*	4.81	4.90	75	2.57	3.93	4.69	100	0.00*	3.92	4.65
4.	125		2.65	3.17	100	0.00*	2.60	3.58	125		2.05	3.84
5.	175		0.00*	1.50	150		0.00*	1.69	150		0.00*	2.02
6.	225			0.00*	200			0.00*	175			0.00*

* Minimal inhibitory concentration

TABLE XX. EFFECT OF INCUBATION PERIOD ON INHIBITORY ACTION OF NISIN AGAINST *BACILLUS CEREUS* AND *B. STEAROTHERMOPHILUS* STRAINS IN SKIM MILK AT OPTIMUM pH, TEMPERATURE AND 1% INOCULUM

Sl. No.	<i>B. cereus</i> (10876)			<i>B. cereus</i> (430)			<i>B. stearothermophilus</i> (953)					
	Nisin concentration (RU/ml)	Log counts/ml			Nisin concentration (RU/ml)	Log counts/ml			Nisin concentration (RU/ml)	Log counts/ml		
		Incubation period (h)				Incubation period (h)				Incubation period (h)		
		12	24	48		12	24	48		12	24	48
1.	0	5.66	6.66	7.07	0	4.17	5.55	5.77	0	4.98	6.82	7.02
2.	50	2.44	5.43	5.76	25	2.94	3.25	3.42	5	2.23	4.70	5.18
3.	75	0.00*	3.06	3.67	50	0.00*	2.84	2.95	25	0.00*	2.95	3.86
4.	100		0.00*	2.81	75		0.00*	1.76	50		0.00*	2.06
5.	125			1.01	100			0.00	75			0.00*
6.	150			0.00*								

* Minimal inhibitory concentration

TABLE XXI. NANOMETER/MICRON MARKER SCALE FOR HITACHI S-405 A-SEM

Magnification	Marker position	Microns (μ)
X 100	30 x 4 NM	300
X 150	20 x 4 NM	200
X 200	15 x 4 NM	150
X 300	10 x 4 NM	100
X 400	75 x 3 NM	75
X 500	60 x 3 NM	60
X 1,000	30 x 3 NM	30
X 1,500	20 x 3 NM	20
X 2,000	15 x 3 NM	15
X 3,000	10 x 3 NM	10
X 4,000	75 x 2 NM	7.5
X 5,000	60 x 2 NM	6.0
X 6,000	50 x 2 NM	5.0
X 7,500	40 x 2 NM	4.0
X 10,000	30 x 2 NM	3.0
X 15,000	20 x 2 NM	2.0
X 20,000	15 x 2 NM	1.5
X 30,000	10 x 2 NM	1.0
X 40,000	75 x 1 NM	0.75
X 50,000	60 x 1 NM	0.60

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