REVIEW OF LITERATURE
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1. Production of antibacterial substances by microorganisms

An organism may elaborate specific toxic substances which interfere with the metabolism of other organisms so much so that the latter may either be killed or prevented from multiplying. These specific toxic substances are generally termed as 'antibiotics' and phenomenon of bacteriostatic or bactericidal action caused by such antibiotics is described as 'Antibiosis'.

The phenomenon of antibiosis however is by no means a new discovery since as early as 1877, Pasteur observed that certain air-borne saprophytic organisms caused inhibition in the growth of Bacillus anthracis. Later, Fleming (1929) working with agar plate culture of Staphylococcus aureus observed that contaminating mycel belonging to the genus Penicillium inhibited the growth of S. aureus culture in the plate. This eventually led to the discovery of the first successful antibiotic 'Penicillin'. Subsequently, several other antibiotics like streptomycin, aureomyein, subtilin, polymycin, etc. were discovered. Various species of microorganisms including fungi, actinomycetes and bacteria are now known to produce antibiotics and an extensive literature on the production and use of such antibiotics has been built up.

A number of lactic acid bacteria, some of which are normally present in dairy products as contaminants or used as starter cultures in the preparation of fermented milk products, are also known to produce antibacterial substances. These antibacterial substances have received considerable attention since
they may be involved in controlling the growth of other microorganisms during processing, or fermentation processes, in intestinal therapy and in preventing or retarding microbial spoilage of dairy products. These substances have been found to be mostly bacteriostatic in action.

Certain strains of *Streptococcus lactis*, an organism occurring naturally in raw milk have been known to produce an active antibacterial substance 'Nisin' inhibitory to *S. arboris*, *S. agalactiae*, organisms belonging to streptococcus groups A, B, E, F, G, H, K, M, and N and certain other gram-positive organisms including species of Bacillus, Clostridium and Lactobacillus (Mattick and Hirsch, 1949).

Certain strains of *Streptococcus arboris* have been shown to produce an antibiotic 'Diplococcin' effective against *S. lactis* (Oxford, 1944). Baribo and Foster (1961) have reported that some strains of *S. arboris* inhibited growth of strains of *Lactobacillus casei*.

Wager (1948) isolated four strains of facultative thermophilic streptococci which produced a substance inhibitory to *Sarcina lutea*. Similarly, Baribo and Foster (1949, 1961) have shown that eight strains of *S. lactis*, one strain of *S. arboris* and three cheese starters inhibited eight strains of *L. casei*. An inhibitory substance from silage streptococci which was designated as 'Streptocyme' was isolated by Hirsch and Wheater (1951).

Later, Hirsch and Grinstead (1951) examined twenty three strains of lactic streptococci and in addition to Nisin and Diplococcin new antibiotics were identified.
Some strains of lactobacilli are also known to elaborate antibacterial substances. Grosswicks et al. (1947) reported an antibacterial substance produced by a strain of Lactobacillus which was effective equally against gram-positive and gram-negative bacteria \textit{in vitro}. It was observed by Greaowieko et al. (1948) that yogurt had six times as much antibacterial activity as human milk and this was attributed to the substances elaborated by the lactobacilli in yogurt. Similarly, Wheater et al. (1981) isolated a large number of strains of lactobacilli from Gruyère cheese and reported forty antibiotic-producing strains. This antibiotic was named 'Lactobacillin', although later the authors (1982) withdrew this suggestion as the substance was found to be similar in properties to hydrogen peroxide. Kodama (1983) isolated a new antibiotic substance named 'lactolin' from a strain of \textit{L. plantarum}, whereas Polonskaya (1982) showed that a filtrate of \textit{L. acidophilus} inhibited growth of \textit{Escherichia coli}. Three strains each of \textit{L. helveticus} and \textit{L. acidophilus} which inhibited growth of seventeen strains of propionic acid bacteria were reported by Winkler (1983).

2. Production of inhibitory substances by \textit{Streptococcus lactis}.

Rogers and Whittier (1928) demonstrated that \textit{Streptococcus lactis} produced an inhibitory substance which diffused through a collodion membrane and which was inactivated by trypsin. Whitehead and Riddet (1933) observed that butter milk stored overnight sometimes inhibited growth of the starter culture used for cheese making and from this isolated a strain of \textit{S. lactis} which on inoculation into normal milk reproduced this phenomenon. Whitehead
(1933-a) investigated the nature of the inhibitory substance and showed that it had the characteristics of a protein and was possibly a polypeptide. Further observations were made by Whitehead (1933-b), Cox and Whitehead (1936) and Hunter and Whitehead (1942, 1944). Shattock and Mattick (1943), identified the strains producing the inhibitory substance as *S. lactis*. This inhibitory substance was found to be identical with that described later by Mattick and Hirsch (1944, 1947) and named as 'Nisin', being derived from group H streptococci.

3. Incidence of inhibitory strains of *S. lactis* in dairy products and other sources.

Chevalier, Fournaud, Lefebvre and Mocquot (1957) examined a number of individual producer's raw milk samples and reported that 41% contained one or more strains of inhibitory streptococci and 17 percent contained streptococci which were able to produce, in autoclaved milk, about 30 Reading Units (Ru) of Nisin per ml. Of raw milk cheese samples, 53% contained one or more strains of inhibitory streptococci and 22% of these streptococci could produce at least 50 Ru/ml of Nisin when cultivated in autoclaved milk. On an average, the number of inhibitory streptococci was 7% of the total bacterial population.

Hirsch and Wheater (1951) isolated 853 pure cultures from raw and pasteurised milk, human, bovine and pig faeces as well as human nose and throat swabs. These workers also tested a large number of samples for occurrence of antibiotic-producing streptococci but came across only a very few Nisin producing strains of *S. lactis*.
4. Production of Nisin by streptococci in relation to their growth and metabolism

a) Biosynthesis of Nisin

Hurst (1966 b) during the course of his work on biosynthesis of Nisin observed that by the end of the lag phase of growth when active DNA and RNA synthesis was already proceeding, Nisin carried over with the inoculum could not be detected although the cocci contained other basic peptides. Rapid Nisin synthesis started after an increase of about 50% in the dry weight of the cocci had taken place. Initially high molecular weight Nisin was made which decreased on subsequent incubation, followed by the production of lower molecular weight Nisin which could be recovered 24 hr after the end of incubation period. Electrophoretic studies revealed that only the faster bands (lower molecular weight) were inhibitory.

The biosynthesis of Nisin was inhibited by antibiotics such as chloramphenicol, puromycin and terramycin which also inhibited protein synthesis. The messenger RNA also appeared to be involved which suggests that Nisin synthesis occurs by a mechanism similar to that of protein synthesis (Hurst, 1966 a).

Later, White and Hurst (1966) studied distribution of Nisin in the cell walls, membranes, ribosomes and the cell sap of the producer organism both in the un-controlled as well as controlled growth media where pH was maintained at 6.8. The major difference with regard to Nisin content was only in respect of the concentration of Nisin in the cell sap in the two cases. Analysis of the cell wall indicated that it was composed of mucopeptide and polysaccharide.
Burst and Lazarus (1968) demonstrated that high cellular concentration of Ca\(^{++}\) occurred during the lag phase which corresponded with apparent low Nisin content. During stationary phase Ca\(^{++}\) content was found to be low and the cellular Nisin content was high. This would appear to establish a complex inverse relationship between antibiotic activity and calcium content.

b) Relationship between growth and Nisin inactivation

Burst (1968) observed that when stationary phase culture of *S. lactis* was inoculated into fresh medium maintained at pH 6.8, the maximum dry weight of cells was obtained after 11 hr. It was seen that 50% of Nisin had disappeared at 0 hr and continued to decline for 120 min. (lag period) when a minimum value was obtained and fresh Nisin synthesis started again. This was obviously due to the fact that the cells had to inactivate the Nisin they contained before initiation of fresh growth.

It was further observed that the autoclaved cells could destroy as much Nisin as the fresh cells and destruction occurred equally well at 2°C and 30°C. It showed that destruction of Nisin was non-enzymatic. Gross and Merrel (1967) observed that mercaptacetamide gave a biologically inactive addition product with Nisin (carboxy amido methyl thiol Nisin) which was later confirmed by Burst (1968). Occasional occurrence of Ca\(^{++}\) in the Nisin peaks from chromatographic columns also suggested non-enzymatic inactivation of Nisin.

c) Role of Nisin and Nisinoids in the metabolism of *S. lactis*

Burst (1967) observed that during the period of lag, Nisin carried over with the inoculum disappears before
exponential growth starts. It was further seen that lag phase was directly related to the Nisin content of the cells. From the results of these studies it was concluded that basic proteins like Nisin are made by *S. lantana* for the purpose of cell regulation, particularly in the processes associated with the initiation and cessation of growth. The antibiotic activity of these basic proteins was thus thought to be incidental. The non-antibiotic producing strains of *S. lantana* synthesized similar 'Nisinoid' proteins which could have the same function in the cell regulation as Nisin. These small molecular weight basic proteins (Nisinoids) resembled Nisin in Sephadex chromatography and electrophoretic behaviour.

Hurst (1968) has reported that when chloramphenicol was added in small amounts, Nisin produced in relation to dry weight of the cells decreased and the resulting culture had a shorter lag phase. These results also support the view that in the case of Nisin producing strains, the antibiotic has a cell regulatory function.

5. Isolation of Nisin

a) From the culture fluid

Mattick and Hirsch (1944) concentrated Nisin from the broth culture of the Nisin producing streptococci and this was found to inhibit *Streptococcus alactiae* at a dilution of 1:640,000. Later the same authors (1947) described the production of the concentrates of Nisin having a specific activity of the two '1947 units' per μg with a yield of 20%. Since
adequate precautions were not observed, it was likely that the concentration of Nisin was much less and probably the concentrates contained only about 4% of actual Nisin produced. Barridge (1949) purified crude concentrates of Nisin from the cultures by fractional precipitation from an acid solution and final crystallization from 80% ethanol. The overall yield in this case was assumed to be around 10%.

Preparation of the culture in a dehydrated form facilitates its storage in an active state and its addition to cheese, has been subject matter of a British patent (1952). Later a method was described by the same patentees (1955) for manufacture of a dry preparation containing Nisin in an edible absorbent material.

Cheeseman and Barridge (1957) using high Nisin yielding medium of Hirsch and by adopting an improved method of concentration, succeeded in developing a product which gave about 80% of the brew activity was reported to be nearly free from inactive material. This crude preparation was further purified by counter-current distribution method. The preparation thus obtained yielded 65% of the biological activity as Nisin A(Barridge et al 1952). The rest of the activity was accounted for by other peptides with only a small amount of Nisin B being detected.

A method for the commercial production of Nisin using a milk culture of the Nisin producing organism was subject matter of a further British Patent (1960). Agnet and CaCl₂ were added to precipitate para-casein and Nisin was collected by salting out, dissolving in methanol and precipitating with
acetone. This was followed by another patent in 1963. The milk before inoculation was treated with papain, pepsin or trypsin to partly digest the milk protein and heated to 100°C to destroy the enzyme. The precipitated protein was separated out, the liquid was then sterilized at 115°C and inoculated.

b) From the cells

Falconer (1952) has described a method for extraction of Nisin from the cells of the producer organism, wherein Nisin remained with the bacterial cells the pH being maintained around 6.0. After collecting the cells Nisin was extracted from them with acidified aqueous acetone or other solvents and further purified by methods similar to those described by Berridge (1949). Considerable increase in the yield was reported. It is probable that the increase in yield obtained was due to higher activity produced in the brew by techniques described by Hirsch (1951).

6. Chemical nature of Nisin

Berridge (1949) crystallized Nisin in a purified form by fractional precipitation with salt and on hydrolysis, it was found to contain alanine, valine, leucine, isoleucine and possibly cystine and aspartic acid. This worker further observed that the Nisin isolated contained at least two substances, the less soluble of which amounted to about two-thirds of the whole. Hirsch (1951) from his studies with different batches of Nisin concluded that he was dealing with several antibiotics. Berridge et al (1953) on the basis of their studies on several preparations of Nisin observed that some of the preparations
contained two active components. Similar observations were also made by Bavin, Beach, Paleconer and Freidmann (1952). The antibacterial properties of the different components were described by Gowans, Smith and Florey (1952). Berridge et al. (1953) also made counter-current distribution studies of the Nisin preparation and reported that it was a complex mixture containing 49% A, 32% B, 12% C, 5% D and possibly 3% of another component termed E. The specific activity of A was reported to be similar to that of B and about 5 times that of C and D.

The paper chromatographic studies of these different components revealed that the chromatograms of A, B and C were strikingly similar and slightly different from that of D.

Leucine and or isoleucine, alanine, glycine, proline, aspartic acid, histidine and lysine were present in all the four components. Valine and methionine were present in A, B and C, but were probably absent from D. In addition to methionine (A, B and C) and histidine (A, B, C and D), all the four Nisins contained two other amino acids containing sulphur lanthanine and cystathionine which are not normally present in protein hydrolysates. Various commercial preparations of Nisin were found to be similar in amino acid composition, but some contained glutamic acid and serine, which are absent from Nisin A, B and C.

Berridge (1953) also studied the amino acid composition of the hydrolysates of several preparations of concentrated Nisin and compared these with that of another antibiotic, subtilin. Whereas subtilin contained amino acids tryptophan, glutamic
acid and phenylalanine which are absent from Nisin, the latter contained sulphur-containing amino acids histidine and methionine which were not present in subtilin. Nisin resembles several other antibiotics peptide in nature obtained from bacteria, the gramicidins, tyrocidines (Craig, Gregory and Barry, 1949), licheniformins (Callow and Work, 1982) and bacitracins (Newton and Abraham, 1980, 1982).

Cheeseman and Berridge (1957) suggested that the molecular weight of Nisin was 7,000. This was challenged by Bodansky and Perlman (1964) who suggested that the molecular weight was more likely to be one third of the figure reported by Cheeseman and Berridge. The recent work on the migration of peptides and small proteins in polyacrylamide gels (Ingram, Tombs and Hurst, 1967) has however, confirmed the molecular weight to be 7,000.

Hurst (1968) reported that since Nisin was found in the cell wall of the producer organism (White and Hurst, 1968), it was probable that it occurred there as a Ca-Nisinate complex. In order to confirm this suggestion, this worker used calcium precipitating acidic solvents and reported that none of the four acids used inactivated pure Nisin. It was also observed that acetic acid was a poor solvent and could extract only 7% of available Nisin, subsequent extraction with hot HCl recovering most of the Nisin, when 0.1 N H$_2$SO$_4$ was used, 90% of Nisin being lost irreversibly. Similarly, extraction with oxalic acid prevented subsequent extraction of the Nisin with hot HCl. This seemed to indicate that at least some of the Nisin was bound to the other polymers of the cell wall in the form of Ca-Nisinate. Hot HCl
also hydrolysed a bond after treatment with cold HCl or acetic acid for releasing the antibiotic. In case of \( \text{H}_2\text{SO}_4 \) and oxalic acid, \( \text{CaSO}_4 \) and \( \text{CaC}_2\text{O}_4 \) were formed which being insoluble formed complex salts of Nisin which are antibiologically inactive and stable to hydrolysis by hot HCl.

7. Detection of inhibitory streptococci in milk and milk products

A simple technique for detection and isolation of inhibitory streptococci has been described by Chevallier et al. (1985). This consists of making a suitable dilution of the sample to be tested and inoculating at the same time into an agar medium a suspension of sensitive lactobacillus which serves as a test-organism. The plates are first incubated at 22°C which enables only the streptococci to develop and form colonies. This also results in diffusion of inhibitory substance into the agar medium. Subsequent incubation at 30-37°C permits growth of the test organism. The medium shows uniform opacity but certain colonies may be surrounded by zones of inhibition due to production of inhibitory substances by test streptococcus strains.

8. Quantitative estimation of Nisin

The quantitative estimation of Nisin produced by \textit{Streptococcus lactis} in culture media or the concentration of the inhibitory substance present in food materials has been estimated by several methods. However all of these are based on the measurement of the inhibitory effect of Nisin on the growth of a particular sensitive test organism either in a liquid or a solid medium.
Several procedures involving the observation of the growth of the sensitive test organism in litmus milk or broth containing the test material in serial dilutions, have been described. By observing the highest dilution in which the organism failed to show growth and interpolating with the standard curve prepared by using graded amounts of Nisin, the amount of inhibitory substance was calculated. Using a somewhat similar method, Mattick and Hirsch (1944) followed the growth of Streptococcus salivarius in nutrient broth tubes containing different dilutions of the test material. The amount of inhibitory substance needed to inhibit the growth of *S. salivarius* in 1 ml of broth, after 16 to 20 hr incubation at 37°C was considered to represent one unit of Nisin. Hoyle and Nichols (1946) described a method in which cultures of inhibitory strains were heated and added to growth medium to give serial dilutions (1/10, 1/20 to 1/1200). The test organism, *S. cremoris* - IP5, grown in yeast-dextrose broth was diluted in quarter strength Ringer's Solution and inoculated into the medium. The highest dilution in which no growth of test organism was apparent after incubation for 16-24 hr at 30°C was recorded.

Hailbard (1956) described a simple and a rapid test for determination of Nisin using a thermophilic, aerobic test organism, *Thermobacillus harzianus*, which grows at 60°C and produces lactic acid by fermentation of sucrose. The concentration of Nisin was calculated from the change in pH of the test medium.
Toply et al. (1981) described a dilution method for estimation of Nisin in foods using *Lactobacillus var. paracasei* as test organism. This method has been claimed to reliably detect Nisin up to a concentration of 0.8 unit/ml and takes only 5.5 to 7 hr.

The dilution methods are reasonably satisfactory in regard to speed, but errors due to time of culture propagation, kind of indicators and interpretation of colour changes and low sensitivity are serious limitations of these procedures.

b) Dye reduction method

More rapid methods of detecting residual amounts of penicillin and other antibiotics, based on the dye reducing activities of sensitive organisms, have been recommended by several workers (Reid and Brewer 1946; Schipper and Peterson, 1951; Neal and Calbert, 1955; Parks and Donn, 1969; Proud, 1960; British Standards Institution, 1968). The application of this principle for the estimation of Nisin has also been suggested.

Hirsch (1958) and Freidman and Epstein (1951) suggested reduction of methylene blue and resazurin respectively using *Streptococcus cremoris* L.P.S. strain for assay of Nisin. Both these methods were claimed to permit rapid assay of Nisin. With resazurin reduction test it was possible to detect Nisin levels as low as 0.5 units/ml. Conditions of propagation and size of the inoculum were also standardised to make these tests sensitive and reproducible. Tramer and Pouder (1964) successfully used method of Wright and Tramer (1961) of testing penicillin in milk for estimation of Nisin based on reduction of
triphenyl tetraselium to a red coloured compound, formazan chloride. Using *S. thermophillus* 'BC' as test organism it was possible to test quantitatively solutions containing 5 to 30 units/ml Nisin. The tubes contained 5 ml of antibiotic free sterilised milk inoculated with the culture and Nisin suspension made to 2 ml with 0.02 N HCl for each level. The tubes were incubated for 16 hr at 44 °C and 1 ml of TTC was then added and the colours measured after further one hr.

c) Graded response method

Berridge and Barrett (1962) developed a method in which a nutrient broth culture of *S. armanis*, in its lag growth phase, was distributed in test tubes into which serial dilutions of Nisin were incorporated. Growth was terminated in 20-60 min. by the addition of a bactericidal agent, merthiolate and the turbidity measured in a photoelectric colorimeter. Optical density was plotted against dilution and the Nisin content was calculated from the standard curve. A modification of the above method has been described by Chevalier and Noquist(1959) according to which *S. armanis* inoculated into pasteurised milk was used as test organism and the differences in milk clotting with rennet enzyme due to changes in acid production, were taken to indicate concentration of Nisin.

Shahni (1962) determined Nisin production of bacterial cultures by centrifuging out the cells from 14 to 16 hr old broth cultures. The clear broth was heated for 2-3 min. at 80-90 °C to destroy the remaining viable cells. Different
concentrations of Nisin broth under test were then introduced into separate fresh broth tubes and inoculated with 1% broth suspension of *Lactobacillus lactis* used as test organism. Turbidity measurements were taken in a colorimeter after 14 to 16 hr incubation at 32°C.

Hurst (1966) followed the method of Berridge and Barrett (1962) for estimation of Nisin except that an actively growing culture of *S. cremoris* I.P.5 was used as test organism in suitable growth medium.

d) Plate assay

Inhibitory zones on agar provided another means of measuring the potency of Nisin. The method in principle, is similar to the disc assay test method commonly used for assay of antibiotics. As diffusion of Nisin in agar is relatively slow, low temperature storage of the agar plates containing the antibiotic for several hr is necessary, before incubation at a more favourable temperature for growth.

A procedure to detect Nisin in cheese using diffusion in agar plate has been described (Moquot and Lefebvre, 1986). In order to facilitate diffusion of Nisin, Tween 80 was incorporated in the assay medium. Sixteen to Eighteen hr old skim milk culture of *S. cremoris* I.P.5, was mixed with melted agar and poured into petri plates. The plates were kept in a refrigerator for hardening the agar and then holes were made aseptically with a 13 mm borer. Cheese was mixed and macerated in a waring blender, with 10 ml. of 0.02 NHCl, the mixture kept in boiling water for 5 min. with occasional stirring, chilled
in ice, centrifuged and 0.01 ml of the supernatant transferred into the wells. The plates were then kept for 8 to 12 hr at 40 F for diffusion and incubated further at 21 C for 18 hr. Zones of inhibition were measured with slide callipers and Nisin content estimated from the standard curve prepared by using Nisin from 0.2 g to 0.001 g/1000 ml and plotting log concentration of Nisin against diameter of inhibition zone in mm. This procedure of Moquot and Lefebvre (1966) was not found very satisfactory because in many foods, where Nisin was used as a preservative or for reducing the heat treatment and where residual amounts of Nisin were required to be estimated, it needs to be separated from protein materials. In addition, interfering substances were encountered which influenced zone sizes and made the interpretations difficult. Tramer and Fowler (1964) described a modified procedure for the quantitative estimation of Nisin in foods. This method required no pre-diffusion, as it relied on incorporation into the medium of 2% (1+1 dilution in distilled water) of Tween 20 of the volume of the assay medium and using Micrococcus flavus NCIB 8166 as test organism. Nisin was freed from proteins by acidifying the medium to pH 2 and boiling. Compensation in food extracts for the effect of interfering substances on the plate assay was made by an alkali treatment, and using these treated extracts as a basis for controls. British Standards Institution (BS 4020; 1966) have adopted the above procedure for estimation of Nisin in cheese, with minor modifications with regard to preparation of acid cheese extract and suitable controls to compensate influence of interfering substances which affect the zones of inhibition.
e) Reverse-phase disc assay technique

The method of Kosikowski and Ledford (1960) for detection of antibiotics in milk has been modified by Tramer and Fowler (1964) for semi-quantitative estimation of Nisin in food extracts. This modification consisted of incorporating heat-shocked spores of *Lactobacillus* into a non-nutrient saline agar. Specially prepared nutrient discs were dipped into test solution, placed on agar and incubated. Inhibitory substance, if present, would diffuse into agar and produce clear zones of inhibition. Solutions containing 0.5 to 10 units/ml could be semi-quantitatively assayed by this method by comparison with suitable controls.

9. Influence of various factors on production of Nisin

a) Effect of temperature

Since Nisin is a normal metabolic product of Nisin producing strain of *Lactis*, it was reasonably assumed that optimum temperature for Nisin production corresponded to its optimum growth temperature at 30 °C. Hirsch (1961) reported 28-30 °C as the optimum temperature for Nisin production. At 37 °C there was about 30% less yield as compared to that at 28.5 °C, though growth was more rapid. Caissar and Pulay (1966) also observed that Nisin production was at its optimum between 20-30 °C. Shkundova et al. (1965) reported 24-27 °C to be the optimum temperature both for growth and Nisin production; at 30-31 °C the decrease was by 20% and at 20-37 °C this declined by about 50%.
b) Effect of growth medium

Effect of composition of medium, on the growth of streptococci in general and their requirements for various vitamins and growth factors has been studied extensively. However, the specific nutritional requirements of Nisin-producing strains of *S. lactis* in relation to production of this antibiotic have received very little attention.

Formation of antibiotics which are polypeptide in nature is closely associated with the nitrogen metabolism of the producing organism. The majority of lactic acid bacteria can normally grow on media containing organic forms of nitrogen, although various species are known to have different requirements for nitrogen sources. In this respect lactic streptococci are considered to be least demanding (Niven, 1944; Bogdanov, 1965).

Several media in which peptone and yeast autolysate are used as nitrogen source have also been suggested for Nisin production (Mattick and Hirsch, 1947; Hirsch, 1961). Hirsch (1961) further observed that maximum yield of Nisin could be obtained by addition of sufficient glucose (2%) and calcium pantothenate (0.6 mg/ml) with pH being kept constant at 6.6, which was the optimum pH for Nisin production. Similarly, Caissar and Puly (1966) also observed favourable effect of incorporation of glucose in the medium on Nisin production. Aqueous glucose yeast medium containing Ca-pantothenate and sulphate at pH 5.9-6.0 has been reported to give high yield of Nisin (Brit. Patent 682,483). On the other hand, Shkundova et al. (1965) concluded from their studies that addition of Ca-pantothenate
or glucose had no effect on Nisin production nor concentrated skim milk medium improved Nisin production.

Egorov and Baranova (1967) reported that ammonium salts of inorganic acids like $\text{NH}_4\text{Cl}$ had no effect either on growth of \textit{S. lactis} or Nisin production since the organism could not utilise inorganic ammonium salts as nitrogen sources. These workers further observed that addition of ammonium salts of organic acids increased both bacterial population as well as Nisin production.

Hirsch (1951) observed that acetate had no specific effect on Nisin production as suggested earlier by Quirard, Snell and Williams (1946). This worker further reported that both acetate and citrate at 1.5% level were most effective in maintaining the medium near the optimum pH for Nisin production and this increase in Nisin production was attributed to the buffering action of these salts in the growth medium. This was supported further by the observation that combination of acetate, citrate and phosphate resulted in highest yield of Nisin since this combination was more effective in maintaining the medium near optimum pH for Nisin synthesis. On addition of inorganic salts to the growth medium it was observed that increased concentrations of $\text{NaCl}$ or $\text{NaNO}_3$ diminished rather than improved growth as well as yield of Nisin. There was, however, enormous increase in Nisin production on substitution of $\text{NaCl}$ or $\text{NaNO}_3$ by $\text{Na}_2\text{SO}_4$. This was suspected to be due to alternative specific diversion of some enzyme system into Nisin production, since Nisin is known to contain 5.7% sulphur (Falconer, 1949).
Egorov and Baranova (1968) also studied the effect of sodium salts of organic acids and observed that these salts with the exception of sodium lactate stimulated both growth and Nisin production by S. lactis. Malonic acid (0.3%) gave highest yield in cell population while highest yield of Nisin was obtained with 0.3% succinic, 0.5% malic or 0.3% acetic acid. Maximum increase in Nisin production and bacterial population occurred with 0.3-0.6% sodium acetate and with any further increase in this concentration both Nisin production as well as the cell mass decreased. The unfavourable effect of lactate on the growth of Nisin producing S. lactis has also been reported by Hirsch (1931) who observed that addition of even 1% lactate before growth began diminished the rate of growth and at 4-6% growth was virtually stopped whereas if added after the growth had begun even 10% did not completely stop growth.

c) Effect of neutralization of lactic acid during incubation

Rogers and Whittier (1923) and Bernheimer and Pappenheimer (1942) had shown earlier that considerable growth was obtained when NaOH was periodically added to growing cultures of streptococci. Similar observations were made by Hirsch (1951) who reported that periodic neutralization and presence of sufficient glucose increased amounts of growth and Nisin yield. White and Hurst (1968) also observed that yield of Nisin/unit dry weight in a glucose containing medium maintained at pH 6.8 was 3.7 times higher as compared to that when pH was allowed to fall to 4.2 during the course of growth. Hurst and Dring (1968) also observed that neutralization of the culture increased the dry weight of the organism 2-fold and also Nisin yield/unit dry weight of the organism.
d) Effect of aeration

Aeration has a significant effect on the growth and biochemical processes of microorganisms. Some reports indicate that conditions of aeration also affect the growth and lactic acid formation of *Streptococcus lactis*, a facultative anaerobe. Vigorous aeration has been reported to retard growth of *S. lactis* (Rahn and Richardson, 1942). Hirsch (1951) also reported that when air was vigorously bubbled, the growth was delayed and with it Nisin production also, but the end-product appeared normal.

Similarly Shkundova et al. (1965) reported decrease of up to 50% in biosynthesis of Nisin on aeration for 3 days at 30-31 C. These workers further observed that under anaerobic and semi-anaerobic conditions there was no change in Nisin production, whereas severe agitation considerably reduced level of Nisin production. Similar observations were made by Egorov and Baranova (1957) who reported that increase in intensity of aeration depressed Nisin production from 7-15% with corresponding decrease in cell population being more significant.

e) Effect of ultra-violet irradiation

Csizsar and Pulay (1956) reported that exposure of some Nisin-producing strains to ultra-violet irradiation resulted in an increase of 10-15 times in production of Nisin.

f) Inactivation of Nisin activity by microorganisms

A number of dairy microorganisms are known to produce Nisin-inactivating substances which can completely destroy or inhibit its action. This inactivation is believed to be brought about by elaboration of 'Nisinase' enzyme.
Kooy (1983) reported isolation of certain strains of *L. plantarum* which could inhibit the action of Nisin. Similarly Havley (1955) observed that strains of *L. plantarum* might destroy Nisin but this action could be prevented by strains of *L. casei*. It was reported by Galee-loot (1956) that about 50% of the lactic streptococci isolated from raw milk destroyed Nisin when grown in milk with Nisin-producing starters. Majority of these organisms were found to belong to *S. lactis-avermiria* group although one strain of *S. faealalis* and four Leuconostocs were also found to be active. The 'Nisinase' responsible for the inactivation of the antibiotic was found to be active at pH 6.9 but not at pH 4.3. Gaiee-loot (1957) further observed that some strains of *S. lactis, S. arvermiria, Leuconostoc sp.* and *L. plantarum* and one strain of *S. faealalis* were able to destroy Nisin rapidly.

Occurrence of *Staphylococcus aureus* strains responsible for production of Nisinase was reported by Carlson and Barner (1957). It was observed by Lind (1958) that some electridia might destroy Nisin until they were themselves killed by Nisin.

Remmier (1960) confirmed that *E. coli* and *L. aerogenes* were not responsible for inactivation of Nisin but attributed the decrease in activity of Nisin to the medium used. Alifax and Chevalier (1962) reported that Nisinase produced by *S. thermophilus* was most active out of all lactic acid bacteria that produced it and further observed that Nisinase had no action on other antibiotics including subtilins. These workers also isolated and purified this enzyme and studied its properties.
10. Effect of sub-culturing and maintenance on the stability of nisin-producing characteristic

Maintenance of cultures in liquid media is the most commonly used method in bacteriological laboratories (Hammer and Habel, 1957). In case of lactic acid bacteria, litmus milk is generally used for transfers of cultures, the cultures being generally sub-cultured after 1-2 weeks of storage in the refrigerator. This interval can be increased to 2-4 weeks by incorporating CaCO₃ to litmus milk. Repeated sub-culturing of nisin-producing strains is generally believed to have adverse effect on their capacity to produce nisin. Hirsch (1951) has described inconsistent behaviour of nisin producing cultures during maintenance and observed that sometimes the cultures showed a decline in nisin production after the 8th subculture. In certain cases it was found that the culture continued to produce maximum amount of nisin even after 29th sub-culture. Galesleot (1955) reported that after 65 transfers of a nisin-producing culture, non-nisin producing strains developed. The rate of development of non-nisin producing strains was 12% in case, 13.5 and 90% in the case of 197 transfers. Pettos (1964) also observed that nisin-producing cultures lost their nisin producing property on frequent sub-culturing. It was further observed that an active nisin-producing strain can be freed from variants by plating, but generally, new cultures thus obtained were weak acid producers.
Hoyle and Nichols (1943) found that antibiotic producing lactic streptococci generally dominated over the non-antibiotic producing lactic streptococci when grown together. Lightbody and Meanwell (1955) reported that the early domination of some strains in one or two daily subcultures among cultures of lactic streptococci was usually due to production of antibiotics by the antibiotic-producing strains. The above findings were supported by Collins (1961) who further observed that slower domination of particular strains could not be attributed to production of detectable amounts of antibiotics since even the non-antibiotic producing strains had the ability to dominate and also the strains that produced the same antibiotic. The latter type of dominance was thus attributed to differences in competitive growth which may be influenced by factors such as differences in lag phase, tolerance to fermentation end products and/or nutritional requirements. The competitive growth ability may further be influenced by differences in physiological state of the microorganisms at the time of testing and due to differences in the composition of milk used as growth medium. This worker supported the earlier findings that the strains of rapid acid producing lactic streptococci could not grow together for long periods in a fixed proportion (CsulaK and Hammond, 1954; Lightbody and Meanwell, 1955). It was also observed that mixtures in which the strains of bacteria would grow competitively for at least 2 weeks could be prepared. The mutual inhibitions of antibiotic
producing strains observed by Mattick and Hirsch (1947) and Hirsch and Grinsted (1951) and Collins (1961) apparently indicated that a given strain was resistant to the particular type of antibiotic produced by itself, and that this resistance did not constitute protection against antibiotics that were of different types.

12. **Inhibitory effect of Nisin on dairy microorganisms**

Hirsch (1947) observed that while Nisin was able to inhibit the growth of many strains of gram-positive organisms it had no inhibitory effect against gram-negative bacteria. Similarly Gowans et al. (1953) reported that Nisin had a strong inhibitory effect in *vitro* against several strains of *Streptococcus pyogenes* and *Staphylococcus aureus* and under certain conditions it was also effective against experimental infections with these organisms in mice. It was however, less effective against *Mycobacterium tuberculosis* whereas it was totally ineffective against infection with a bovine strain of *M. tuberculosis* in rabbits. Hawley (1957) reported that Nisin was inhibitory against several streptococci, lactobacilli, clostridia, staphylococci and bacilli. The effect of Nisin on bacteria involved in ripening of cheese was studied by Winkler et al. (1957) who reported *Lactobacillus casei* and *L. plantarum* to be only slightly sensitive to Nisin while *S. lactis* was found to be strongly inhibited. Caleslcool (1957) during the course of his studies on the effect of Nisin on bacteria concerned in cheese, observed that some strains of *S. lactis* and *S. cremoris* and all strains of *S. diacetilactis* studied were inhibited. *S. liquefaciens* was insensitive and *S. faecalis* and *S. durans* were either insensitive or only slightly inhibited. *L. casei* was not
affected but all strains of *L. plantarum* were slightly inhibited. The heterofermentative and lactate fermenting lactobacilli, the propionic acid bacteria and clostridia were all strongly inhibited. On the other hand, the *coll.* aerogenes bacteria were not inhibited at all. Ramsier (1960) studied the effect of Nisin on spores and vegetative cells of *M. butyricus*. It was shown not to have any effect on un-germinated spores though it was observed to have significant bactericidal effect on vegetative cells of all ages examined. It was reported by Pulvay and Katana (1960) that *M. butyricus* was inactivated in milk samples at a concentration of more than 2000 cells per ml was not completely inactivated by Nisin, but development of tuberculosis was retarded in guinea-pigs. Shahani (1962) observed that Nisin did not inhibit three strains of *S. lactis*, two strains of *L. bulgaricus* and two strains of *S. thermophilus* and on longer incubation the total cell count and acid production were either just about the same or slightly higher than in the controls. Out of 11 strains of *S. aureus* tested, only two cultures were sensitive to the antibiotic. Both these strains were found to be either weak or negative coagulase-producers. Studying the effect of Nisin on acid production by yoghurt, acidophilus and cream starter cultures as also pure cultures of lactobacilli, Toply et al (1961) observed that Nisin at a concentration of 10/ug/ml had no effect. At 50/ug/ml acid production was inhibited and at 100/ug/ml it was completely suppressed. Reeves et al (1964) also studied the effect of various concentrations of Nisin on germination and growth of spores of *B. licheniformis*. Nisin added
prior to germination with L-alanine had some destructive effect, but addition of Nisin in small concentrations after germination had variable effect while in higher concentrations it prevented growth of spores.

13. Use of Nisin for controlling clostridia in cheese

The inhibitory action of Nisin-producing strains of *Streptococcus lactis* is usually harmful against susceptible bacteria including some starter cultures in cheese making. This property of Nisin-producing organisms has been successfully used to combat the problem of late gas formation particularly in natural gruyère cheese.

Hirsch and associates in 1951 conducted experiments on natural gruyère cheese with milk experimentally contaminated with a suspension of clostridia isolated from a blown gruyère cheese. One lot of cheese was prepared from the milk which had additional inoculum of a Nisin-producing streptococcus. In the control lot, the cheese was found to be spoiled within 17-22 days, while the lot containing Nisin-producing culture remained normal. This indicated the beneficial use of Nisin-producing starter culture in preventing gas production in Swiss-type cheese without interfering with the normal propionic acid fermentation. Similarly encouraging results were obtained by different workers in both naturally as well as artificially infected cheese with clostridia. (McClintock *et al.*, 1952; Pette and Kocy, 1953; Berridge, 1953; Bylund *et al.*, 1954; Hawley, 1955). Hawley (1955) further observed that though Nisin-producing cultures could prevent blowing in gruyère
cheese, the inhibition of Lactobacilli disturbed the ripening process to some extent, although Edam cheese could be prepared successfully. It has also been shown that some strains of \textit{L. plantarum} occurring commonly in raw milk could destroy Nisin, but this neutralising effect could be prevented in the presence of some strains of \textit{L. casei} (Hawley, 1955; Galesloot, 1957).

Galesloot (1957) also observed that in cheese some strains of streptococci producing Nisin-inactivating substance were themselves inhibited by Nisin-producing starter cultures and they could not, therefore, decrease Nisin content of cheese.

It has also been reported that some strains of \textit{S. lactis}, \textit{S. cremoris}, \textit{S. faecalis} and leuconostoc produced 'Nisinase' in cheese (Galesloot, 1956, 1957). Another important observation made (Galesloot, 1955) was that Nisin-producing cultures were highly sensitive to phage attack for which reason their regular use as cheese starters would be difficult. Pulay (1956) reported unfavourable effects of Nisin-producing starter cultures on quality of cheese since they caused slow acid production and slow breakdown of sugars thus encouraging growth of \textit{E. coli} resulting in blowing of cheese.

Winkler and Frolich (1957) from their studies on Emmental cheese concluded that \textit{L. casei} and \textit{L. plantarum} were only slightly inhibited. Use of Nisin-producing starter in combination with the commercial starter and \textit{L. casei} was recommended (Stüber and Hinder, 1959) for successful utilisation of silage milk for manufacturing of Edam and Gouda cheese.
Nisin has also been extensively used in various countries for improving shelf life of processed cheese (Pette and Kooy, 1963; Berridge, 1963; Blylund, 1964; Hawley, 1955; Galea, 1957). However, no work has been reported on its use for improving keeping quality of indigenous dairy products in this country.

14. Application of Nisin in other canned products

Nisin has been shown to be effective against certain heat resistant bacteria which cause spoilage in canned products. It can aid in heat treatment and thus prevent overcooking of some canned foods and also increase the shelf life of the products making the process economically viable.

O'Brien and Coworkers (1959) reported that there was apparent reduction in D values of *B. coagulans* and *B. stearothermophilus* by 7 and 30% respectively using Nisin. Gillespie (1957) reported that addition of Nisin at a level of 100 ppm to beans and peas prevented any spoilage in the cans and helped to reduce the heat treatment. Similarly, Michener and Coworkers (1959) reported a 30% reduction in D value of *C. botulinum* using 20 ppm of Nisin. Campbell, Sniff and O'Brien (1959) also observed considerable reduction in D value of *B. coagulans*. In an experiment with tomato juice inoculated with the spores of *B. coagulans* and processed at 260 F for 30 min., these workers found 77% spoilage in cans without Nisin, while those containing 14 ppm of Nisin showed no spoilage even after processing for 7 min. Campbell and Sniff (1959) also observed encouraging results with 5 ppm Nisin using 31 strains of
*B. coagulans* inoculated in tomato juice at a pH of 5.3. It was further observed that Nisin at low pH had an increased effectiveness and that Nisin was neither sporidial nor sporistatic but inhibited the growth of germinated spores. Hawley (1961) observed that other organisms might not respond similarly. It was for instance reported that the spores of *B. coagulans* were insensitive to 25 ppm Nisin even though 2.5 ppm could reduce the thermal death time of their spores from 45 min. to 6 min. at 225°F. Campbell and Sniff (1959) reported that actual heat resistance of spores was not affected by Nisin or subtilin. Campbell and O'Brien (1955) had earlier observed an apparent heat resistance of spores of *M. butyricum*, *B. stearothermophilus* and *B. coagulans* in the presence of Nisin or subtilin at a pH range of 4.5-7.5, various recovery media, different suspending menstrua and spore levels. From these studies it was concluded that apparent reduction in thermal resistance was largely due to germination of the surviving spores with subsequent bacterial action of Nisin and subtilin.

Gillespy (1957) reported that use of 200 Ru/ml of Nisin prevented spoilage of canned beans in tomato sauce with *M. thermosaccharolyticus* and flat sour spoilage of green peas was prevented by 250 Ru/ml of Nisin in the brine.

The importance of contamination of raw material was emphasized by Burdley (1962) while studying the effect of Nisin in preventing flat sour spoilage of soup. It was observed by this worker that in control samples with and without additional load of spores of *B. stearothermophilus*, the rate of spoilage...
was 93 and 79% respectively whereas in the cans containing Nisin (125 Ru) there was no spoilage.

Heinemann et al. (1964) used Nisin to aid heat treatment of chocolate flavoured milk. It was observed that addition of 80 Ru/ml of Nisin at an F value of 3, was sufficient to prevent thermophilic spoilage and obtain commercially sterile chocolate flavoured milk without any sedimentation problems common at high heat treatment.

Use of Nisin in canned and bottled creams of various fat content has also been favoured in order to reduce the otherwise excessive heat treatment which results in cooked flavour in the product. This method can be profitably used in evaporated milk to reduce heat treatment thereby conserving vitamin B6, B12 and thiamine content. It also prevented the loss in true digestibility of the proteins of evaporated milk. (Gregory, Henry and Kon, 1964).

15. Public health aspects of using Nisin as a food preservative

No harmful effects on public health following consumption of foods containing Nisin have been reported and Nisin has been considered to be a safe preservative for use in foods (Fraser et al., 1962). It is not known to create any problems of sensitization since unlike antibiotics such as penicillin it has not found application in medical treatment. Whitehead (1933) reported that Nisin was hydrolysable by trypsin. Since it is destroyed by enzymes in the intestinal tract, it would be unable to influence intestinal flora and therefore has no
physiological significance (Heinemann and Williams, 1966). Further, consumption of a food-product containing Nisin would not be expected to alter the nature of bacterial flora in the oral cavity because residence time of Nisin in oral cavity is less than 10 min. this short exposure not being sufficient to promote a change in the bacterial flora (Claypool et al, 1966).

Legislation in respect of food additives in the United Kingdom, Australia, France, Italy, Israel, Mexico and Sweden at present permits the use of Nisin as a preservative in various food stuffs, including certain dairy products (Hall and Fowler, 1964). According to McClintock et al, 1962, the addition of Nisin to give 100 to 200 units of Nisin per gram of processed cheese provides good protection against gas-blowing by clostridia.