Studies on production of Nisin and Nisin-like substances by lactic streptococci and their use in preservation of milk products
MATERIALS AND METHODS

1. Isolation of lactic streptococci producing inhibitory substances.

   a) Samples of dairy products

   The following samples of milk and milk products were obtained from the local market or from the farm of the Institute and examined for the presence of streptococci producing inhibitory substances:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo milk (market)</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Cow milk (farm)</td>
<td></td>
<td>120</td>
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<tr>
<td>Goat milk (farm)</td>
<td></td>
<td>20</td>
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<tr>
<td>Dahi (market)</td>
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<td>50</td>
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   All the above samples were collected in sterile glass bottles and held at 2-5°C in refrigerator till required for analysis.

   b) Cultures

   The following cultures maintained at the Type Cultures Collection of the Microbiology Division, National Dairy Research Institute, Kamal were used in the study:

   *Streptococcus lactis* 496 (Standard Nisin producing strain)

   Lactobacillus bulgaricus- Wisconsin (Test organism used for assay of Nisin)

   Micromonas clavia- NCIB 8106 (Standard Nisin sensitive strain used for assay of Nisin)

   *Obtained from National Institute for Research in Dairying, Shinfield, Reading, England.*
c) Media

The composition of various media used in the study was as follows:

1) **Litmus milk**

Fresh cow's skimmed milk containing 1 to 2% litmus and sterilised by steaming.

2) **Tryptone-glucose-vegetable (TGV) broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (Oxoid)</td>
<td>1.0%</td>
</tr>
<tr>
<td>Meat extract (ACAS)</td>
<td>1.0%</td>
</tr>
<tr>
<td>Yeast extract (Oxoid)</td>
<td>0.5%</td>
</tr>
<tr>
<td>Glucose (BDH)</td>
<td>2.0%</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>4.0%</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

Distilled water up to 100 ml
pH 7.0 ± 0.1
Sterilised at 15 lbs psi for 20 min.

3) **T.G.V. agar**

T.G.V. broth containing 1.5% agar.
Sterilised at 15 lbs psi for 20 min.

4) **Misin agar medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Bact.peptone (Oxoid)</td>
<td>1.0%</td>
</tr>
<tr>
<td>Bact. Beef extract (ACAS)</td>
<td>0.3%</td>
</tr>
<tr>
<td>Yeast extract (Oxoid)</td>
<td>0.15%</td>
</tr>
<tr>
<td>NaCl (BDH)</td>
<td>0.3%</td>
</tr>
<tr>
<td>Unrefined sugar</td>
<td>0.1%</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5%</td>
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</tbody>
</table>

Distilled water up to 100 ml
pH 7.5 ± 0.1
Sterilised at 15 lbs psi for 20 min.
v) **Yeast-dextrose agar**

Peptone (Oxoid)  
Yeast extract (Oxoid)  
Sod. acetate hydrated (BDH)  
Glucose (BDH)  
Agar  
Distilled water up to 100 ml  

pH 7.0 ± 0.1  

Sterilised at 15 lbs psi for 20 min.

**Streptococcus lactis-496** was maintained in plain litmus milk by weekly transfer. **Mesorococcus flavus** was maintained on Assay medium slants and transferred every 3 weeks. Between transfers the cultures were kept in a refrigerator at 2.5°C after 16-18 hr growth at 30.0°C ± 0.5°C. **Lactobacillus bulgaricus** was grown at 37.0°C ± 0.5°C on TGV agar and transferred at fortnightly intervals.

2. **Characterisation and identification of the isolates.**

   a) **Morphology**

   The isolates were examined for shape, size and arrangement of cells in freshly curdled milk cultures by microscopic examination of the smear, defatted with xylene, fixed in alcohol and stained with borax methylene blue (Davis, 1940).

   b) **Cultural characteristics**

   i) **Catalase test**

   A drop of 30% hydrogen peroxide was placed on each colony on the yeast-dextrose agar plate and observations made
regarding formation of gas bubbles due to decomposition of 
$H_2O_2$ in the presence of enzyme catalase. The isolates which 
gave a negative reaction were considered to be catalase nega-
tive which is a characteristic property of lactic acid 
bacteria.

ii) Growth at different temperatures

Young cultures of the organisms were inoculated into 
plain litmus milk and incubated at 40° and 45°C. The rate of 
acid production as indicated by colour of litmus milk and the 
time of curdling of milk were noted.

iii) Alkali tolerance

This was determined by inoculating a young culture of 
the organism into yeast-dextrose broth, adjusting to pH 9.2 
and 9.6 and observing growth by visual turbidity.

iv) Salt tolerance

Salt tolerance of the organism was studied by inocula-
ting the culture into yeast-dextrose broth containing NaCl 
(2.4 and 6.5%) and observing growth by visual turbidity.

Biochemical characteristics

i) Action on litmus milk

The cultures soon after curdling were inoculated into 
plain litmus milk and incubated at 30°C. Observations were 
made regarding acid production, reduction of litmus and coagu-
lation of milk.

ii) Fermentation of maltose

The cultures were inoculated into sugar fermentation 
media containing 1% maltose, with BC as indicator and were 
incubated at 30°C. Production of acid (indicated by deep
Yellow colour of BCP) and gas (in the Durham's tube) was observed. Controls (uninoculated sugar fermentation media tube as well as tubes containing the basal medium without any sugar and inoculated with cultures) were run simultaneously.

### iii) Ammonia production from arginine

(Niven et al, 1942) The cultures were inoculated into arginine broth and incubated for 7 days. The supernatant was taken after centrifugation and tested for ammonia production using Messeiller's reagent. The composition of Arginine broth was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>1.0%</td>
</tr>
<tr>
<td>Maltose</td>
<td>1.0%</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.3%</td>
</tr>
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**d) Screening of Nisin-producing streptococci**

The procedure adopted by previous workers (Chevalier, Fourmaud and Moquet, 1966; Galesloot, 1967) was followed in the preliminary trials. One ml of a 16 hr old TGV broth culture of *Lactobacillus bulgaricus* W and one ml of 10^-7 and 10^-8 dilutions of the sample containing antibiotic producing cultures were inoculated into petridishes and 15 ml of melted TGV agar was poured. The plates were incubated at 30°C for 2 days. This permits the colonies of antibiotic culture, if any, to develop and permit Nisin to diffuse into the agar. After 2 days, the plates were incubated at 37°C for 16 hr. The agar became turbid due to the growth of lactobacilli, throughout except around the Nisin-producing colonies which were surrounded by a clear zone of inhibition. This procedure permitted isolation of Nisin-producing streptococci.
The above procedure was however, found to be quite laborious and time consuming method. Accordingly, a modified procedure using *H. flavus* as test organism was adopted for the isolation of streptococci producing Misin-like substances. This consisted in plating out the serial dilution of the sample along with the test organism (*H. flavus*) on Assay medium and incubating the plate at 22°C for 48 hr. In the modified procedure the plates were required to be incubated at one temperature (22°C) and the results were obtained in 24-48 hr. Another advantage of this method was that the colonies with clear zone of inhibition against the yellow background could be easily seen. Fifty nine isolates of streptococci showing inhibition of *H. flavus* were isolated from different samples. The distribution of the isolates according to their source of isolation is given in Table 1.

3. Screening of streptococci for Misin-producing ability

All the 59 streptococci isolated from dairy products on the basis of their ability to produce inhibitory substances were examined for their relative efficiency in the production of Misin-like substances in broth as well as milk cultures. Since the inhibitory material secreted by strains of *S. lactis* has been generally denoted by the term 'MISIN' by earlier workers. The same terminology will be used in the present study also for indicating the inhibitory substances produced in cultures of streptococci.

a) Broth cultures of streptococci

The Misin producing lactic streptococci were grown in
yeast-dextrose broth for 16 hr at 30°C. After the incubation was over, the cultures were kept in boiling water for 5 min., cooled in ice water and then centrifuged at 3,000 rpm for 10 min. The supernatant in each case was tested for Nisin production using standard cup assay procedure and L. flavus as test organism.

b) **Milk cultures of streptococci**

The Nisin-producing lactic cultures were grown in plain skim milk for 16 hr at 30°C. After the incubation, the cultures were held in boiling water for 5 min. and finally cooled in ice-water. These were then centrifuged at 3,000 rpm for about 10 min. The clear supernatant obtained at the top was tested for Nisin production by the standard cup assay procedure.

c) **Inoculum of assay organism (L. flavus-NCIB 8166)**

Growth on a 24 hr old agar slope culture was washed in 10 ml of normal saline and centrifuged at 3,000 rpm for 10 min. The supernatant was poured off and the cells were resuspended in normal saline, centrifuged and again resuspended in 10 ml of normal saline. A uniform cell suspension was obtained by breaking the cell sediment with the help of an inoculating needle and subsequently shaken thoroughly. It was ensured that the cell suspension did not contain any clumps of cells by making microscopic examination. To standardise the cell suspension, 0.5 ml of the cell suspension was transferred into another tube and diluted suitably to 50% transmittance at 430 m/μ in Hilger absorptiometer. The number of viable cells in 1 ml of suspension giving 50% transmittance was then determined by plating and the number of cells in the original suspension calculated.
This cell suspension was then diluted in such a way that when added at the rate of 2% of the volume of the medium, it would give initial concentration of about 50 million cells per ml.

d) **Zip assay procedure**

The procedure recommended in British Standard Methods (B.S. 4020:1966) was generally followed. The assay medium was melted and cooled to 50 ± 2°C to which was added 2% of a 50% solution of polyoxyethylene sorbitan monolaurate (Tween 20) previously held at 50 ± 2°C for 20 to 30 min. and thoroughly mixed with the medium. Into the above medium, 2% of the inoculum of the assay organism (M. flava) prepared as described above was added and mixed well. The inoculated medium was poured to a depth of 3.4 mm into petri dishes and allowed to solidify. The plates were then transferred into a refrigerator (2°C) for 1 hr to facilitate the boring of holes. Four wells were made on the solidified medium in each plate using a sterile hollow borer (3 mm in diameter).

With a standardised pasteur pipette 0.01 ml of the broth or milk culture of the isolate (prepared as above) was transferred into one of the wells and the plates were then incubated at 30 ± 1°C and left overnight undistributed. On the following day the diameters of the zones of inhibition formed round the wells were measured accurately by means of callipers.

e) **Preparation of standard curve**

Graded amounts of Nisin* (0.01 ml each of solutions containing concentrations 1.25, 2.5, 5.0 and 10 BU/ml

* 'Nisaplin' obtained from M/s Nisaplin and Barret Ltd., England.
respectively) were added into the wells on the agar medium seeded with the assay medium. After incubation of the plates over night the diameters of zones of inhibition were measured. A standard curve was prepared by plotting log Nisin concentration against diameter of zone of inhibition which yielded a straight line graph.

f) Estimation of antibiotic activity

The antibiotic activity of the different cultures in terms of Nisin was calculated by interpolating the diameters of zones of inhibition formed by these against the standard curve.

4. Dye reduction methods for assay of Nisin

With a view to develop a simple, rapid procedure for assaying Nisin-like substances produced by streptococcus cultures and also for detecting the residual amount of Nisin in dairy products preserved with this antibiotic, dye-reduction tests using triphenyl tetrazolium chloride were compared with the standard cup assay method.

a) Test Organisms

1) Various isolates of Enterococci were screened for sensitivity to Nisin by tetrazolium reduction in order to find out a suitable strain which could grow fast enough and reduce tetrazolium rapidly in a broth culture and also be sensitive to Nisin.

Different cultures were grown in Neo-peptone broth at 37°C. The experimental tube contained 1.0% of Nisin whey* containing 1,000 RU/ml of Nisin while the control tube did not

*Nisin whey was prepared from 24 hours old milk culture of Nisin producing culture of S. lactis-4. It was kept in boiling water for 5 min, cooled immediately in ice-water and centrifuged. The clear whey thus obtained was neutralised and standardised to contain 1,000 RU/ml of Nisin/ml by adding sterilised distilled water.
contain any Nisin. After 2 hr of incubation TTC solution (1\%) was added to the cultures at 1% level. The tubes were incubated further and observed upto 8 hr for reduction of tetrasodium to red colour.

ii) The following cultures, which were found to be sensitive to the action of Nisin at low concentration and capable of reducing tetrasodium to the red compound, formazan rapidly, were used as test organisms:

- Lactobacillus hilgardii- Wisconsin
- Streptococcus aureus- IP.S.
- Streptococcus faecalis- NCDO 681
- Streptococcus faecalis- DB 2112

b) Test media
1) Skim milk
2) Yeast-dextrose broth
3) Tryptone-neopeptone-dextrose (TND) broth

- Tryptone (Oxoid) 1.7%
- Neopeptone (Difco) 0.3%
- Dextrose (BDH) 0.25%
- NaCl (BDH) 0.5%
- K_{2}HPO_{4} (BDH) 0.25%

Distilled water upto 100 ml.

pH 7.3 ± 0.1

Sterilised at 15 lbs psi for 20 min.

Sterilised skim milk was used for observing the reduction of tetrasodium by cultures of L.hilgardii and S. aureus since they failed to show appreciable reducing activity in broth media. In the case of cultures of S.faecalis-
NCDO 801 and S. faecalis. DB 2112 yeast-dextrose broth and tryptone-neopeptone-dextrose broth respectively were used for estimating reduction of tetrazolium. The latter culture (DB 2112 strain) did not produce appreciable growth and reduction of tetrazolium in any other medium.

\[ \text{(c) Determination of minimum inhibitory concentration of Nisin} \]

Sixteen-hour old milk or broth cultures were used for inoculation into milk or broth media respectively. The amount of inoculum was adjusted so as to give initial concentration of about 5 million cells per ml in the test medium. Graded amounts of Nisin were added to 10 ml of the test media containing the culture. The test tubes were incubated in a water bath maintained at 37°C except in case of S. cremoris, which was incubated at 30°C. At the end of 2 hr, 1 ml of sterile 0.1% solution of triphenyl tetrazolium chloride (BDH) was added into each of the tubes and these were reincubated in the water bath. Development of red colour due to the formation of formazan was observed in the tubes at various intervals.

In the preliminary experiments reduction of tetrazolium as indicated by the development of red colour due to formazan in different tubes was found to be inversely proportional to the concentration of Nisin in the medium. Minimum concentrations at which the reducing activity was completely inhibited, as indicated by the absence of colour development, was also determined.

In order to assess the reliability and reproducibility of the method for assay of Nisin in milk cultures, recovery
experiments were conducted by addition of known amounts of Nisin into milk and separating the whey by acidification of milk and centrifugation, incorporating the whey into the test media and determining the minimum amount of whey at which the reduction was inhibited. The amount of Nisin in a sample of whey was calculated on the basis of minimum concentration of Nisin required for bringing about inhibition in the corresponding control tubes. Values for Nisin concentration obtained by this method were compared with those given by the conventional agar cup assay method.

d) Application of the method for assay of Nisin

For these trials, reduction of tetrazolium by L. bulgaricus in milk and by S. faecalis-DB 2112 in tryptone-neopeptone-dextrose broth was followed.

Graded amounts of whey obtained from milk cultures of Nisin producing streptococci were added to the test media containing the above two cultures and minimum concentrations, at which there was no reduction at the end of incubation for 2 hr, was noted. From this the Nisin content of the material was calculated as indicated earlier under (c). These values were also compared with the results of the agar cup assay method.

e) Colorimetric estimation

The procedure adopted was similar to one described earlier, the test organism used being Streptococcus faecalis-DB 2112. Graded amounts of Nisin were added to give a final concentration of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 Ryu/ml in the
medium. The red colour of formazan was extracted with 5 ml of N-Butanol and read in Hilger absorbptiometer at 430 n/m. A straight line response was obtained by plotting per cent transmittance against Nisin concentration.

5. Relationship between rate of growth and Nisin production

A 16 hr old broth culture centrifuged and washed twice was used as inoculum. The cell suspension was so adjusted that 1 ml of it when added to 100 ml of broth media would give a final concentration of approximately 5 million cells/ml. The inoculated broth was then distributed into tubes, incubated at 30°C and examined at different intervals (0, 2, 4, 6, 8, 10, 12, 24 and 48 hr) for bacterial count, Nisin production and pH.

The bacterial count was determined by Standard Plate Count method (APHA, 1967). Nisin was assayed by standard cup assay procedure as described earlier. pH was determined using Beckman pH meter.

In the case of cultures or samples containing unknown amounts of Nisin, 1 ml of the sample (in 2 or more dilutions) was added to the broth containing the test organism and the amount of formazan formed at the end of 4 hr was estimated colorimetrically. The corresponding values of Nisin concentration were obtained by interpolation against standard curve.

6. Effect of various factors on production of Nisin

The effect of various factors on production of Nisin by selected strains of *Streptococcus lactis* (4, 6 and 10) in milk and broth cultures was studied using the cultures of *M. flavus* as test organism. Nisin was assayed by the standard cup assay procedure described earlier.
a) **Amount of inoculum**

Effect of inoculum on Nisin production by the isolates *S. lactis*-4 and *S. lactis*-6 in milk cultures and broth cultures was studied. The amount of inoculum used both for milk and broth cultures was 1, 2, 4, 6, 8 and 10 percent of the volume of the test media. The cultures were incubated at 30°C for 16 hr. Nisin was assayed by the cup assay procedure as described earlier.

b) **Effect of age of culture**

Effect of age of culture at the time of inoculation on Nisin production by *S. lactis*-4 was studied in milk and broth cultures. The cultures grown for different periods i.e., 24, 48, 72 and 96 hr were used in the study, Nisin content in each case being determined at the end of 24 hr incubation at 30°C.

c) **Effect of temperature of incubation**

Different incubation temperatures (22, 30, 37 and 40°C) were used to study the effect on Nisin production in broth cultures using *S. lactis*-6. The rate of inoculum used was 1% of the volume of the test media. The incubation was stopped after 24 hr and Nisin content of the culture held at different temperatures determined.

d) **Period of incubation**

Effect of incubation period on production of Nisin in milk and broth were made at 30°C. Shorter incubation periods employed were 0, 2, 4, 6, 8 and 24 hr while for longer incubation readings were taken at the end of 24, 48, 72 and 96 hr. The isolates *S. lactis*-4 and *S. lactis*-6 were used for observing effect of incubation time on Nisin production.
e) **Effect of medium**

The Nisin producing organisms *S. lactis*-4, *S. lactis*-6 and *S. lactis*-496 were grown in various milk and broth media. The Nisin content in different growth media was assayed at the end of 24 hr. The composition of the various media (sterilized at 15 lbs psi for 20 min.) was as follows:

1) **Milk media**

   a) **Skim milk**

   b) **Yeast-dextrose milk**

   - Yeast extract (Oxoid) 0.5%
   - Dextrose (BDH) 1.0%

   c) **Fortified milk**

   - Skim milk was fortified with 0.75% milk solids not fat.

   d) **Enriched milk**

   - Milk powder 0.75%
   - Glucose (BDH) 0.5%
   - Yeast extract (Oxoid) 0.5%
   - Tryptone (Oxoid) 0.5%
   - Peptone (Oxoid) 0.5%

2) **Broth media**

   a) **Yeast-dextrose broth**

   - Peptone (Oxoid) 0.5%
   - Yeast extract (Oxoid) 0.5%
   - Sod. acetate hydrated (BDH) 1.6%
   - Glucose (BDH) 1.0%

   - Distilled water up to 100 ml.

   - pH 7.0 ± 0.1
Medium 22 (Hirsch, 1951)

Meat extract (ACAS) 1.0%
Peptone (Oxold) 1.0%
Glucose (BDH) 2.5%
Sod.acetate hydrated (BDH) 1.5%
Sod.citrate (E.Merck) 1.5%
Disod.hydrogen phosphate (BDH) 0.5%
Ca-pantothenate 1μg/ml

Distilled water up to 100 ml.

pH 7.0 ± 0.1

f) Addition of various organic and inorganic compounds

i) Sodium salts of organic acids

Sodium salts of various organic acids were added to yeast-dextrose broth. Thirty percent solution of each (Sod. acetate, Sod.citrate, Sod.tartarate, Sod.oxalate and Sod. lactate) was prepared and one ml of each added to 100 ml of yeast-dextrose broth, pH adjusted to 7.0 ± 0.1 and sterilized at 15 lbs psi for 20 min. Thus the final concentration of each of these sodium salts was 0.3%. The effect of these salts on Nisin production by S.lactis-6, corresponding bacterial count and pH was also observed, after incubation for 24 hr at 30°C. Nisin was estimated by cup assay method and the bacterial count by the standard plate count by plating out serial dilutions of the sample on yeast-dextrose agar.

The change in pH of the growth media was recorded by using Beckman pH meter.

Similar observations were made using different concentrations of sodium acetate i.e. 0.0, 0.3, 0.6, 0.9, 1.2 and 1.5% (W/v)
ii) Acetate and phosphates

To compare the effect of organic and inorganic salts on Nisin production, sodium acetate, sodium dihydrogen phosphate and disodium hydrogen phosphate were added at a concentration of 0.3%. The Nisin content and pH were determined as before.

iii) Purine and pyrimidine bases

Adenine, guanine, xanthine, uracil and guanosine were added to skim milk at two different levels of 10 and 100 µg/ml. Nisin production and percent titratable acidity were determined after 24 hr. Skim milk without any addition of purine or pyrimidine base served as control both for Nisin and titratable acidity determinations.

Stock solutions of purine and pyrimidine bases were prepared in hot distilled water. Adenine and guanine required weak alkaline solution (KOH) for complete solubility. Each test solution and controls were autoclaved for 15 min. at 121°C.

iv) Monovalent salts

To see the effect of monovalent salts on Nisin production, sodium chloride, potassium chloride and ammonium chloride were added to broth cultures of S. lactis-6 at a level of 0.0, 0.5, 1.0 and 2.0%. The Nisin content and pH of the cultures with different levels of these salts were determined and results compared.

v) Divalent salts

Similarly, studies were conducted with the divalent salts of calcium chloride and magnesium chloride using different levels as described before.
vi) Acetate and citrate in milk

Effect of addition of sodium acetate along with sodium citrate on Nisin production by *S. lactis*-4 in milk was determined. The salts were used at all level of 0.6% (w/v). Nisin content and pH at the end of incubation (24 hours at 30°C) were recorded.

vii) Calcium carbonate in milk

Effect of calcium carbonate on Nisin production was studied by using 3 levels of CaCO₃ viz. 1.0, 2.0 and 3.0% in the milk cultures of *S. lactis*-4. Effect of CaCO₃ on bacterial count and change in pH of the cultures were also studied at 24, 48, 72 and 96 hr. Skim milk without addition of CaCO₃ served as controls.

g) Effect of aeration

The broth cultures were aerated to see its effect on Nisin production. The cultures were first aerated slowly in a thermostatically controlled water bath maintained at 30°C. In a second set of trials, to see the effect of vigorous aeration, the culture flasks were put on a shaker. The Nisin content in either case was determined after an incubation period of 16 hr at 30°C and the results compared with non-aerated samples which served as control.

h) Effect of carbon dioxide

Carbon dioxide was passed into milk cultures of *S. lactis*-6 in screw cap media bottles. The gas was passed at the rate of 2 litres/min. for two min. after which the cultures were incubated for 24, 48, 72 and 96 hr at 30°C. The Nisin content was determined at different intervals and results compared with control cultures.
1) Effect of repeated sub-culturing on Nisin production

1) Daily sub-culturing

For studying the effect of repeated sub-culturing on Nisin production, Nisin-producing cultures, S. lactis-6 and S. lactis-10 were employed. The cultures were maintained by daily transfers in plain skim milk. Each day the culture was inoculated (at the rate of 15%) into sterile skim milk tubes and incubated at 30°C. At the end of 16 hr the growth of the cultures was terminated by immersion of the tubes in boiling water for 5 min. and Nisin assayed by standard cup Assay procedure as described earlier.

In order to check the activity of the culture every time, one of the inoculated milk tubes was examined for titratable acidity at the end of 8 hr incubation. The acidity was determined by titrating against N/9 alkali using phenolphthalein as indicator and the values expressed as percent lactic acid.

ii) Weekly sub-culturing

For studying the effect of weekly sub-culturing on Nisin production, the cultures were carried in plain skim milk and sub-cultured once a week up to 5 weeks. Before testing the week-old cultures, these were first sub-cultured twice in plain skim milk and then inoculated into milk and 16 hr old culture examined for Nisin production as above.

iii) Monthly sub-culturing

The Nisin producing cultures were carried in chalk milk and transferred at monthly intervals. Before testing for Nisin

*(Freeze-dried cultures after 3 sub-cultures in plain skim milk were used).*
production the month-old culture was passed twice in plain
skim milk and then examined as described earlier.

7. Inhibitory effect of Nisin on various dairy microorganisms

a) Lactic acid bacteria

For studying the inhibitory effect of Nisin on various lactic acid bacteria, 6 cultures of lactic streptococci and 20 cultures of lactobacilli were taken. The cultures were grown in plain skim milk distributed in 9 ml quantities. One ml amounts of 16 hr old milk cultures diluted 10 times were inoculated into 9 ml of sterile skim milk. One set of tubes was kept as control. To the other set, 1% of sterile Nisin-whey was incorporated into each tube. The inoculated milk tubes were held in a water bath maintained at 30°C. After 8 hr incubation, the tubes were removed from the water bath, cooled immediately in ice-water and titrated against N/9 NaOH using phenolphthalein as indicator. Growth inhibition of the cultures by Nisin was expressed as percent titratable acidity compared to that of the corresponding control.

b) Other dairy micro-organisms

For observing inhibitory effect of Nisin on various other dairy micro-organisms, two cultures each of Escherichia- aerobacter, Staphylococci, Bacilli, Clostridia, Aspergillus and Saccharomyces sp and one culture of Penicillium Syncephalastrum and Torulopsis were studied.

* Nisin-whey was prepared from 24 hr old milk culture of Nisin producing culture of S. lactis-4. It was kept in boiling water for 5 min., cooled immediately in ice-water and centrifuged. The clear whey thus obtained was neutralised and standardised to contain 1000 R/u of Nisin/ml by adding sterile water.
The cultures of anaerobic spore-formers were grown in Differential Reinforced Clostridial Medium while the other bacterial cultures were grown in nutrient broth. The yeast and the mold cultures were grown in Csapek’s solution. The experimental tubes contained 1% of skim-whey. The tubes were incubated at 37°C for 24 hr. Growth was indicated by development of black colour in the medium in the case of clostridia and turbidity in other cases. The composition of the media used was as follows:

1) **Nutrient broth** (A.P.H.A, 1967)

2) **Differential Reinforced Clostridial Medium (DRCM)** (Gibbs and Frame, 1965)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Peptone (Oxoid)</td>
<td>1%</td>
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<tr>
<td>Lactemco (Oxoid)</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium acetate hydrated (BDH)</td>
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<tr>
<td>Yeast extract (Oxoid)</td>
<td>0.15%</td>
</tr>
<tr>
<td>Soluble starch</td>
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<tr>
<td>Cystein hydrochloride</td>
<td>0.05%</td>
</tr>
<tr>
<td>Distilled water up to 100 ml</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.1 to 7.2</td>
</tr>
</tbody>
</table>

Sterilized at 15 lbs psi for 20 min.

Equal volume of sodium sulphite (4%) and ferric citrate (7%) were mixed and added at the rate of 2%, just before the medium was to be used.

3) **Csapek's solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrate</td>
<td>0.3%</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.05%</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.05%</td>
</tr>
</tbody>
</table>
Ferrous sulphate 0.001%
Potassium phosphate 0.1%
Sucrose 3%
Distilled water up to 100 ml.
Sterilized at 15 lbs psi for 20 min.

8. Effect of incorporating Misin producing streptococci on starter activity and quality of fermented milks

a) Effect of Misin producing culture on starter organisms

For studying the effect of Misin producing cultures on production of acid by single strain starter cultures, the following cultures were used.

**Misin-producing cultures**
- *Streptococcus lactis*-4
- *Streptococcus lactis*-6
- *Streptococcus lactis*-496 (Standard Misin producing culture)

**Single strain starter cultures**
- *Streptococcus lactis* - ML2
- *Streptococcus lactis* - ML3
- *Streptococcus lactis* - C2
- *Streptococcus lactis* - C10
- *Streptococcus lactis* - Hansen
- *Streptococcus diacetylmethane* - DRC 1
- *Streptococcus cremoris* - CL
- *Streptococcus cremoris* - ML1
- *Streptococcus cremoris* - KH

Individual starter cultures as well as cultures of both starter organisms and Misin-producing strains together were grown in milk and the difference in acid production by the two
sets of cultures was examined. The cultures were grown in plain skim milk for 16 hr at 30°C and then used for inoculating at 1% level. One ml of it was diluted with 9 ml of saline and 1 ml of culture thus diluted was inoculated into 9 ml of plain skim milk in duplicate. In the case of mixed culture, the inoculum consisted of 1 ml of the two cultures mixed in equal proportions.

The tubes were incubated in a water bath maintained at 30°C for 8 hr, and acidity determined by titration against N/9 alkali using phenolphthalein as indicator and the values expressed as lactic acid.

b) Effect of Nisin producing culture on common dahi starter cultures

The Nisin producing culture of *S. lactis* was employed in combination with some of the single strain starter cultures commonly used for preparation of dahi. The following dahi cultures were used.

- **Streptococcus lactis** - C 2
- **Streptococcus lactis** - ML 3
- **Streptococcus cremoris** - C 1
- Mixed strains lactic culture - LF

Whole milk from National Dairy Research Institute farm, steamed for 30 min. in sample bottles, was used for preparation of dahi. Sixteen hr old culture of the organism was inoculated into milk at 1% level. The two cultures were mixed in equal proportion and used at the rate of 1%. The samples were incubated at 30°C and titratable acidity determined after 24, 48, 72 and 96 hr, by titration against N/9 alkali using phenolphthalein as indicator.
c) Effect of Nisin-producing culture in preparation of dahi using market dahi cultures

Nisin producing culture *S. lactis*-4 was incorporated along with 8 market dahi cultures and dahi was prepared in 2 parallel sets. One set was incubated for 24 hr and the other for 48 hr at 30 °C. The control samples were inoculated with dahi cultures alone, while in the experimental dahi samples, dahi culture and Nisin-producing culture were used in equal proportion at the rate of 1%. The acidity of different dahi samples was determined as usual by titration against standard alkali.

d) Effect of Nisin producing culture on the quality of yoghurt

In order to study the effect of Nisin on keeping quality of yoghurt, Nisin-producing isolate, *S. lactis*-4 was incorporated along with yoghurt culture in the preparation of yoghurt. Yoghurt culture consisting of *S. thermophilus* and *L. bulgaricus* was used in equal proportion at 3% level. In the other set in addition to yoghurt culture, Nisin-producing culture was also incorporated at 1% level. The samples were incubated in a water bath maintained at 45 °C for 3½ hr when the milk just curdled. The samples were then removed from the water bath and cooled to 5 °C. These were then transferred to incubators maintained at 22 °C and 30 °C in order to see the effect of Nisin on keeping quality of yoghurt at different temperatures.
9. Effect of incorporation of Nisin on shelf life of processed cheese

a) Use of Nisaplin (Applin and Barret Ltd., England)

i) Incorporation of Nisin

Lots of cheddar cheese at various stages of ripening were obtained and ground together after scraping off the paraffin layer. The approximate weight of the ground cheese was 16 kg sufficient to fill 48 tins of 300 g capacity. The ground cheese was divided into 2 parts. One part was used as such to serve as control. To the other part Nisaplin (Nisin activity 10 units/g) was added to give a concentration of 100 mg per kg of cheese. Nisaplin was incorporated along with the melting salts containing 2 parts of sodium citrate and 1 part of disodium orthophosphate (30 g per kg of cheese). The above mixture was added slowly into the kettle containing cheese, melted at 170°F. After the cheese was processed, the hot melt was transferred to sterilized tins to fill completely and the tins were sealed. The other lot was melted and mixed with the salts without Nisin and then filled in the tins.

The experimental and control samples of processed cheese were stored at 10°C and 30°C and examined at various intervals for bulging of tins or other defects in cheese and also for microbiological quality.

ii) Microbiological analysis of the samples

The processed cheese tin was thoroughly cleaned and then the lid of the tin was sterilized by applying alcohol and then flaming it. The top layer of the cheese was separated and 11.0 g of the material was weighed and transferred into a
sterile glass mortar and emulsified with 99 ml of 2% sodium citrate solution already held at 45°C. This gave 1:10 dilution of the product which was used for preparing further serial dilutions.

**Total plate count**

Total plate count was determined by plating suitable dilutions of the cheese sample using tryptone-dextrose agar. The plates were incubated at 37°C for 48 hr and colony counts made according to standard procedures (A.P.H.A. 1967).

**Yeast and mold counts**

Yeast and mold counts were determined by plating out cheese suspension using acidified potato-dextrose agar. The counts were made after 3 days of incubation at 22°C.

**Coliform count**

For presumptive test 1 ml of 1:10 suspension of cheese sample was inoculated into tubes of McConkey's broth, in duplicate. For enumeration, the first two dilutions were plated out using violet red bile agar. Surface growth was prevented by using 3-4 ml of the medium as an overlay, completely covering surface of solidified agar. The tubes and the plates were incubated at 37°C for 24 hr.

**Anaerobic count**

The cheese suspension was heated in a water bath at 80°C for 10 min. to destroy non-spore forming organisms. For enumeration, Most Probable Number (MPN) method was adopted. 10 ml, 1.0 ml and 0.1 ml of the sample were inoculated into 10 ml of the Different Reinforced Clostridial Medium (DRCM). For 10 ml of the sample, double strength DRCM broth was used. The tubes
in triplicate were incubated at 37°C for 24 hr and longer for observing delayed and doubtful reactions which were further confirmed by sub-culturing in fresh DRGM tubes. The number of clostridia was determined by using the MPN table (Jacobs and Gerstein, 1960).

b) Use of Nisin produced by S. lactis-4 (NDRI)

Nisin was isolated from milk cultures of S. lactis-4 and obtained in a dry form according to procedure described in Section 12. This preparation showed an activity of 250,000 IU/g. The method of incorporating Nisin into processed cheese and the study of the shelf life was the same as followed in the case of Misaplin preserved cheese.

10. Use of Nisin for improving shelf life of Khoa

a) Preservation of Khoa and incorporation of Nisin

Buffalo milk, standardized to 4.5% fat and 8.5% solids-not-fat, was pasteurised and used for Khoa making in a jacketed pan according to procedure described by De and Ray (1962). Khoa was divided into two parts. One part was directly distributed in 300 g quantities into sterilized tins so as to fill them up to the brim leaving very little air space. The filling was done when the Khoa was hot (70-75°C). The tins were then sealed and used as controls.

To the other part of Khoa, calculated amount of Nisin (to give a final concentration of about 100 IU/g) dissolved in sterile water was added and thoroughly mixed. The Khoa was then packed in tins as in the case of the control sample.

The Khoa samples prepared as above were found to contain 27% fat and 28% moisture meeting the requirements of Indian Standard (IS:2785-1964)
Both the experimental and control samples of khas were incubated at 10\(^{\circ}\), 22\(^{\circ}\) and 30\(^{\circ}\) C and examined at intervals for changes in texture and flavour and visible signs of spoilage. Similarly, standard plate count on tryptone-dextrose agar, yeast and mold count on potato dextrose agar (pH 3.5) and presumptive coliform test in McConkey's broth were determined according to standard procedures (A.P.H.A., 1967).

For the estimation of anaerobic count the procedure recommended by Gibbs and Frame (1965), was followed. Khas samples were ground with 2% sodium citrate solution, tempered to 45\(^{\circ}\) C, and then the suspension diluted in normal saline, the diluted (1:10) sample was heated in a water bath at 80\(^{\circ}\) C for 10 min. to destroy non-spore-forming organisms. The sample was then cooled to room temperature and 1 ml, 0.1 and 0.01 ml amount of the sample were transferred to tubes of DRGM in triplicate. The tubes were incubated at 37\(^{\circ}\) C for 24 hr or longer and examined for development of black colour. Doubtful reactions were confirmed by further sub-culturing in fresh DRGM tubes. On the basis of the number of positive tubes, the anaerobic count was calculated by the Most Probable Number method.

b) Microbiological Analysis of Khas

i) Preparation of sample

The tin was washed with soap and water and dried. The alcohol was then applied on the lid of the tin with a cotton swab and burnt. The top layer of khas was removed with a sterile spatula and then 11.0 g of khas was weighed and transferred into a sterile glass mortar. The uniform suspension was obtained by grinding the sample using 99 ml of sterile solution of 2% sodium
citrate tempered to 45°C. This gave 1:10 dilution which was used for preparing further serial dilutions. Usual aseptic precautions were observed during the entire procedure.

ii) Total plate count

Total plate count was determined by plating suitable dilutions of Khoa suspension using tryptone-dextrose agar. The plates were incubated at 37°C for 48 hr and colony counts were made according to standard procedures. (A.P.H.A., 1967).

iii) Yeast and mold counts

Yeast and mold counts were determined by plating suitable dilutions of the Khoa suspensions using acidified potato-dextrose agar. The counts were made after 3 days of incubation at 22°C.

iv) Coliform count

For presumptive test 1 ml of 1:10 dilution of Khoa sample was inoculated into tubes of McConkey's broth in duplicate. For enumeration, the first two dilutions were plated out using violet red bile agar. Surface growth was prevented by using 3.4 ml of medium as an overlay, completely covering surface of solidified agar. The tubes and the plates were incubated at 37°C for 24 hr.

v) Anaerobic count

The sample (1:10 suspension of Khoa) was heated in a water bath at 80°C for 10 min. to destroy non-sporo forming organisms. For enumeration, Most Probable Number (MPN) method was adopted. 1 ml, 0.1 ml and 0.01 ml of the sample were inoculated into 10 ml of the Differential Reinforced Clostridial Medium (DRCM). The tubes in triplicate were incubated at 37°C for 24 hr or longer for delayed and doubtful reactions which were further confirmed by sub-culturing in fresh DRCM tubes.
11. Isolation and characterization of Nisin

a) Extraction of Nisin from the cell wall of the producer organism

1.25 g of cell paste harvested from 24 hr old broth cultures of the Nisin-producing strains (*S. lactis* 4, 6 and 496) was introduced in 50 ml of 10 mM phosphate buffer at pH 6.7 and mixed thoroughly. The suspension was treated in a MSE ultrasonic disintegrator at 4°C for 30 min. Four equal portions were centrifuged at 15,000 g in Savarr Centrifuge for 30 min. The precipitate obtained in each tube was extracted separately with 5 ml of HCl, HNO₃, acetic acid and oxalic acid for 24 hr at 2°C. The supernatant in each case, obtained after centrifugation at 15,000 g for 30 min., was bio-assayed for Nisin by the standard Car Assay procedure. The supernatant after the cold extraction was resuspended in an equal volume of 0.05 NHCL placed in a boiling water bath for 5 min. centrifuged and the clear supernatant bio-assayed as before.

b) Percent distribution of Nisin in the medium and the cell wall

1.25 g of cell mass of the Nisin producing culture *S. lactis* 6 was collected after centrifugation of 1 litre of the broth culture of the organism and sonicated as described earlier. The broth culture after removal of the cells was used for assaying the Nisin produced in the medium.

Nisin was bio-assayed in the broth culture and in the supernatant obtained on centrifugation of the cell mass after sonication and extraction with cold and hot HCl. The percent distribution of Nisin excreted in the medium and that associated with the cell wall was calculated.
c) Amino acid composition of Nisin from different cultures

The conventional method of paper chromatography was used to elucidate the amino-acid make up of the Nisin isolated from the cultures \textit{S. lactis-4} and \textit{S. lactis-6} and their comparison with that of the material obtained from a culture of the standard strain \textit{S. lactis-496} and the standard Nisin preparation Wisaplin.

1) Hydrolysis of the test materials

A 10 ml pipette sealed from one end was used. The material was transferred into the pipette to which 6 NHCL was added and sealed from the other end over the flame. The pipette was then kept in the oven at 100°C for 24 hr for hydrolysis. After hydrolysis acid was removed by repeated drying and wetting process over boiling water bath in a porcelain dish.

2) Preparation of paper chromatograms

Whatman No.1 paper was used. 10/µg of the material was applied on strips of paper (30 x 15 cm) and chromatograms developed with butanol-acetic acid-water (4:1:1) and phenol-water (4:1) in closed jars using ascending technique. This method was used initially to separate out different amino-acids in the test materials and also to find out the Rf values of different amino-acids in the both solvents under the conditions of the experiment.

Finally two dimensional paper chromatography was used for better separation of the constituent amino-acids in the test samples. 100/µg of the material was applied to the paper for each chromatogram. The chromatogram was first developed
with a butanol-acetic acid-water mixture (4:1:1) (Woold, 1949). They were then dried in a current of air until most of the acetic acid had been removed. Phenol-water (4:1) was used as the second solvent (Dent, 1949). After the phenol had run, the papers were dried in a stream of air. The chromatograms were sprayed with Ninhydrin to detect the presence and position of the amino-acids (Woold, 1949).

4) Total lipids present in the cells of Misin-producing strains

i) Extraction of lipids

Total lipids were extracted by the method of Polach et al. (1957). Dried bacterial cells were ground with sand in a pestle. The ground material was transferred to an air tight glass stoppered iodometric flask (250 ml capacity) and to this a volume of chloroform-methanol (2:1 v/v), equal to 20 times the weight of bacteria was added. The contents of the flask were shaken on an electric shaker for two to three hr and filtered through sintered glass-funnel. The filtrate was distilled under vacuum till the lipids were completely free from the solvent.

To remove the water soluble impurities, the crude lipids so obtained were weighed and to it a volume equal to twenty times its weight of chloroform-methanol (2:1 v/v) and five times its weight of 0.9 percent sodium chloride solution were added. The contents were transferred to a separatory funnel, shaken well and allowed to stand till two distinct layers were formed. The lower chloroform layer containing the lipids was removed. The extraction
was repeated 4 times with 5 ml portions of chloroform. The extracts were combined and dried over anhydrous sodium sulphate. The solvent was distilled under vacuum and recovered lipids were weighed.

ii) Separation of different classes of polar and non-polar lipids by thin-layer chromatography

Preparation of thin-layer plates and application of the sample

Silica gel G plates with 250 µ layer thickness were prepared by the method of Lees and de Muria (1962). The plates were activated in a hot air oven at 100°C for 45 min before use. Solution of the lipid (1% approximately) was prepared in chloroform and 10 µl of the sample was applied, with the help of a Lambda pipette about 1.5 cm above the lower edge of the plate.

Thin-layer chromatography

The plates were developed by ascending chromatography up to 13-15 cm from the origin. The time required for development up to this height was about 55 and 35 min. for polar and non-polar lipids respectively. The developed chromatoplates were air dried for 15-20 min and visualised with iodine vapours except in case where the plates were sprayed with various spray reagents for identification of different components.

For separation of various classes of non-polar lipids, following solvent system was used:

Pet.ether - ethyl ether-acetic acid (80:30:1)

(Williams et al, 1960)
For separation of various classes of polar lipids following solvent system was used:
Chloroform-Methanol - 7N ammonia (65:25:1

Two dimensional chromatographic technique was used to get the complete separation of various components of the polar lipid fraction. Glass plates (20 x 20 cm size) were developed first in CHCl₃-CH₂OH-H₂O (65:25:4 v/v). After drying these in air for 20 min, the plates were developed in second direction using CHCl₃-CH₂OH-7N NH₃ (65:25:4 v/v) system.

**Detection and identification of individual lipid components**

Lipid components were detected and tentatively identified on the chromatoplates with the help of iodine vapours (Sims and Larose, 1962) and cupric acetate-phosphoric acid reagent (Fewster et al, 1969).

**Spray sequence for the detection of mono and diglycerides and glyco-lipids**

Mono and diglycerides were identified by the method of Clark (1961), but instead of periodate-shiff's spray, periodate-benzidine spray of Gifonelli and Smith (1954) was used. Sequence of spray reagents were as follows:

(a) Alkaline hydroxylamine solution-mixture of aqueous solution of 2.5 M sodium hydroxide and 2.0 M hydroxylamine hydrochloride (1:1 v/v).
(b) 3 M hydrochloric acid.
(c) 0.5% v/v solution of sodium periodate.
(d) Benzidine reagent—mixture of 100 ml 0.1 M benzidine (in 50% alcohol), 20 ml of acetone and 10 ml of 0.2 N HCl.

Solvent free chromatoplate was sprayed first with (a) followed by (b) after 30 min, dipped in water for 5 sec. and allowed to dry. It was then sprayed with (c) followed by (d) after 5 min. White spots on a blue background showed the presence of diglycerides. In a similar way, monoglycerides and glycolipids were detected by spraying the chromatoplate with (c) and (d) only.

**Densitometry for quantitative analysis**

(Fevster et al., 1969)

The developed chromatoplates were sprayed with cupric acetate-phosphoric acid reagent and charred at 160°C for 30 min. The intensity of charred spots was measured densitometrically. The plate area representing integrated optical density values was measured by means of a planimeter.

**iii) Gas-liquid chromatography (GLC) for fatty acid analysis**

The lipids were methylated and the methyl esters thus prepared were identified by GLC.

For the preparation of methyl esters, method of Luddy et al. (1968) was used with slight modification. Lipid sample (1 to 20 mg) was taken in a 15 x 45 mm
centrifuge tube provided with an aluminium foil wrapped cap. To it 0.25 ml of 0.4 N sodium methyleate (prepared according to Luddy et al., 1963) in anhydrous methanol was added and securely covered with cap. The capped tube was then immersed to a depth of 12 mm for 30 sec. in water bath maintained at 65°C. It was constantly shaken during this period. When the mixture of lipid and the reagent became homogenous, the conversion to methyl esters was considered complete. After this stage the heating was continued without shaking for an additional period of 90 sec. The tube was then removed from the waterbath and cooled to the room temperature. It was opened and 0.6 g of a 1:1 mixture of silica gel and anhydrous CaCl₂ was quickly added and the contents were thoroughly stirred with a small rod. Three ml CS₂ was added and the cap was replaced. The tube was then shaken by hand for 1-2 min and then centrifuged. The clear CS₂ layer was withdrawn and evaporated, allowing the methyl esters to be isolated.

For the preparation of methyl esters of free fatty acids, 0.1 ml benzene was added to 1-30 mg of the sample in a centrifuge tube and its contents were slightly warmed to dissolve the acids. To it 0.3 ml of 4% H₂SO₄ in CH₃OH was added. The tube was tightly capped and was immersed in a water bath maintained at 65°C. The procedure from this point onwards was the same as that described above.

The esters thus prepared were analysed using Aerograph Hi P1 (Model 600 c) GLC apparatus-10 ft x 1/8"
stainless steel column, packed with 20% diethylene glycol succinate (DKGS) on 60-80 mesh chromosorb 'W' was used to fractionate the methyl esters. The conditions for the separation were:

Column temp. 192 ± 1°C, hydrogen flow 30 ml/min.

The peaks were tentatively identified by comparison of their retention time with those of standard fatty acid esters. Planimeter was used to calculate area under the peaks and the percent area converted into weight percent directly by area normalization method.

12. Production of antibiotic (Nisin-like substance) by *Streptococcus lactis-4*

Based on the results of various trials the following procedure was adopted for the production of Nisin.

Steam sterilized skim milk containing CaCl₂ (1g/l) and rennet (0.01g/l) was inoculated with a 24 hr old milk culture of *S. lactis-4* at the rate of 40 g/l. After mixing thoroughly, the inoculated milk was incubated at 30°C for 24 hr. The curd was then cut and warmed to 45°C for 10 min to expel the whey completely. The clear whey was decanted off and filtered through Whatman No. 1 filter paper under suction. The antibiotic in the whey was then precipitated out using sodium chloride (350 g/l). The salt was added gradually to the whey which was stirred continually. It was left overnight at 5°C and then filtered on Whatman No.1 filter paper under suction to recover the antibiotic in the form of precipitate. The precipitate was dried under vacuum and
ground into a fine powder in a pestle and mortar. It was stored in glass bottles at room temperature. For removing excess of the salt, the precipitate was dissolved in N/50 HCl and dialysed in a cellophane bag in running water. Undialysed material was concentrated and dried under vacuum and ground into a fine powder.

**Addition of CaCO₃**

In the subsequent trials CaCO₃ was added to skim milk to substitute CaCl₂. Rest of the procedure was same as described above for preparation of Nisin. The procedure has been illustrated in the form of a flow sheet diagram (Chart I).

**Testing the crude preparation for its potency**

In order to assay its potency, 100 mg of the powder was weighed, dissolved in 30 ml of N/50 HCl and boiled for 5 min. The volume was made up to 100 ml with N/50 HCl in a volumetric flask. Subsequent dilutions were made in N/50 HCl and Nisin assayed by the tetrasolium reduction as well as standard cup assay methods as described earlier.