Methodology
3. METHODOLOGY

The present work was carried out in the Department of Microbiology, C.C.S. University, Meerut during the period February 2004 to March 2007.

3.1 Collection of samples: - A total number of 137 samples, of food products and different milk and milk products comprising buffalo milk-31, cow milk-16, goat milk-16, curd-19, cheese-22, Pasteurized milk-10 (Parag, Kailash, Gopal ji, Amul, Madhusudan, Saras, Param), cream-5, sweets-8 (mava-burfi, mava-ladoo, ghevar), apple jam-5 and mango jam-5, were collected from various cattle yards, dairies and sweet shops in Meerut region.

25 ml of sample was collected in sterile 100 ml sample bottle after taking appropriate precautions. The sample was processed immediately for isolation of the lactic acid bacteria (LAB).

3.2 Isolation of Lactic acid bacteria: - LAB were isolated by different methods using MRS medium (de Man et al., 1960) (Appendix I).

3.2.1 Preparation of medium: - The ingredients were dissolved in cold distilled water, for mixing the constituents homogenously; the medium bottle was placed over magnetic stirrer for 15 minutes. The medium was sterilized by autoclaving at 15 pa (121°C) for 15 minutes.

The sterilized medium was allowed to cool down at 45°C and approximately 25 ml of the medium was poured in sterile glass Petri plates and allowed to solidify. For the preparation of broth 40 ml of medium (without agar) is dispensed in 250 ml conical flask and the opening is closed with cotton plug and covered with aluminium foil and then, sterilized by autoclaving at 15 pa (121°C) for 15 minutes. The slants of MRS and nutrient agar medium (Appendix I) were prepared by pouring approximately 10 ml of properly mixed medium (agar was previously dissolved in water bath) in screw capped culture tubes and these tubes were autoclaved at 15 pa (121°C) for 15 minutes.
Thereafter, the tubes were kept in slanting position until they got solidified. The medium plates, broth and slants were stored at 4°C until used.

3.2.2 Streak plate method: - The sample was streaked by triple streaking method with sterilized loop on MRS plates. The plates were then incubated at 37°C for 24 h. After 24 h isolated colonies were picked up randomly and subcultured on MRS plates for the isolation of pure culture.

3.2.3 Dilution plate method: - 10 g of a sample from each curd, cream, milk and jam were aseptically transferred to 90ml of sterile physiological saline (0.9% NaCl) (warmed to 45°C for cream) and mixed well.

    Cheese samples were prepared by transferring 10 g of aseptically weighed sample to 100 ml sterile 2% sodium citrate solution at 45 to 50°C and homogenized for 3 minutes.

    In all the cases, serial dilutions were subsequently prepared in sterile physiological saline. 1 ml sample by appropriate dilutions were plated out onto MRS medium. The plates were incubated at 37°C for 24 h. After 24 h, typical colonies were picked up randomly and transferred to MRS broth (de Man et al., 1960) (Appendix I) for microscopic examination and gas production subsequently.

3.2.4 The effect of incubation on bacteriocin production: - 100 ml of milk sample was taken in a beaker and with 2 g of curd sample incubated at 37°C and the sampling was done after every 1 h and centrifuged at 15,000 rpm for 30 minutes at 4°C in the high-speed refrigerated centrifuge (Sigma 2K15). The supernatant was then filtered through sterile membrane filter of 0.22 μm pore size (Millipore). Cell free filtrate, thus obtained was stored in vials, at -20°C Supernatant was used for screening for bacteriocin production. The pellet was then streaked with sterilized loop on MRS plates for isolation of LAB.
3.3 Procurement of strains: Bacteriocin sensitive strains *Lactococcus lactis* subsp. *lactis* MTCC3038, *Lactococcus lactis* subsp. *lactis* MTCC3041 and bacteriocin producer strain *Lactococcus lactis* subsp. *lactis* MTCC440, were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.4 Screening of LAB for antibacterial activity: The Lactic acid bacteria isolated in this study were screened for their antagonistic activity against bacteriocin sensitive strain MTCC3038, by using Waksman and Lechevalier method (1962), Yang et al., (1992) method and agar-well assay (Varadaraj et al., 1993) as follows:

3.4.1 Waksman and Lechevalier Method (1962): A MRS medium plate was taken and isolated Lactic acid bacteria was streaked once on the plate with the help of sterilized loop. Then bacteriocin sensitive strain *Lactococcus lactis* subsp. *lactis* MTCC3038 was streaked once on the plate at an angle of 90° C to the first streak crossing the first streak at one end as shown in Figure 14A. The plates were then incubated at 37°C for 24 h and examined for inhibition of the culture by bacteriocin positive isolates.

3.4.2 Yang, Johnson and Ray's Method (1992): In this method, the bacteriocin producing strain was inoculated in nutritional broth containing 2% glucose. It was then incubated at 37°C for 18 h. The culture broth was then serially diluted and 1ml was inoculated into sterile plates on which sterile MRS medium was poured. The plates were then incubated at 37°C for 24 h to obtain 50-100 isolated colonies per plate. The plate with desirable colonies were selected and 5 ml of melted soft agar MRS medium was inoculated with 5 μl of an overnight culture of bacteriocin sensitive strain was overlaid on precultured plate. The plate was incubated at 37°C for 24 h. After incubation the plates were examined for presence of circular and clear zones of growth inhibition around bacteriocin producer's colony as shown in Figure 14B.
3.4.3 Preparation of cell free filtrate (CFF):- Fresh 40 ml of MRS broth in 250 ml flask was prepared and inoculated with a loop full of 24 h old bacteriocin producing culture from MRS agar plates. The flask was incubated at 37°C at 150 rpm on incubator shaker (Carlon, Orbital Shaking incubator cum B.O.D. incubator) for 24 h. From this 24 h old culture, 1 ml was inoculated into another fresh 40 ml sterilized MRS broth flask and incubated at 37°C on incubator shaker for 24 h. About 20 ml of broth from each flask was then centrifuged at 15000 rpm for 30 minutes at 4°C in the high-speed refrigerated centrifuge (Sigma 2K 15). The supernatant was then filtered through membrane filter of 0.22 μm pore size (Millipore), cell free filtrate, thus obtained was used in the assay.

3.4.4 Agar-well assay: - Inhibitory activity titers against the indicator bacteria were determined by agar diffusion well assay (Varadaraj et al., 1993) with slightly modification. Fresh culture of bacteriocin sensitive strain (Lactococcus lactis subsp. lactis MTCC3038) was inoculated in 40 ml of sterilized MRS broth and incubated at 37°C for 24 h. 100 μl of broth culture was spread on fresh MRS medium plates. Wells were cut with sterile cork borer (4 mm in diameter).

50 μl of CFF which was serially diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 (two fold dilution) was placed in each well with the help of micropipette using sterilized tips. The plates were then incubated at 37°C for 24 h, without inversion. After incubation the plates were observed for clear circular zones of inhibition around the wells. The diameter of zone of inhibition was measured in mm with the help of standard scale (Hi Antibiotic Zone Scale-c PW297, Hi Media). The highest dilution that gave a well-defined zone of inhibition of growth was used to calculate AU ml⁻¹ (highest dilution that showed a distinct zone of inhibition X 20 (1000 μl/50 μl).
AU ml⁻¹ = highest dilution that showed clear well-defined zone of inhibition x 1000 μl / Volume (μl) used in the well (Figure 15).

Arbitrary unit or Activity unit = $2^x \times \frac{1000}{50 \, \mu l}$

(x= highest dilution that gave minimum visible clear zone of inhibition, 50 μl volume used in wells)

3.4.5 Antibacterial activity of isolated bacteriocin: - Antibacterial activity of bacteriocin was tested against some pathogenic and nonpathogenic bacteria like Lactococcus lactis subsp. lactis MTCC3038, Bacillus polymyxa, Bacillus subtilis MTCC441, Salmonella typhi MTCC734, Shigella sonnei MTCC2957, Escherichia coli MTCC119, Staphylococcus aureus MTCC96, Clostridium perfringens MTCC450, Enterobacter faecalis MTCC439, Enterobacter aerogenes MTCC111, Streptococcus pneumoniae MTCC1935, Pseudomonas aeruginosa MTCC2581 and Listeria monocytogenes MTCC657, Micrococcus MTCC106, Klebsiella pneumoniae MTCC109, Proteus vulgaris MTCC744. These cultures were collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India and were maintained in 30% sterile skimmed milk in small vials of 2ml, at -20°C in the Department of Microbiology, C.C.S. University Meerut (Figure 16).

3.5 Identification and characterization of the bacteriocin producing strains: - The finally selected strains were further characterized using different tests, by taking Lactococcus lactis subsp. Lactis MTCC440 as a standard. The strains were compared with standard description of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

3.5.1 Colony morphology: - The size, shape, colour and appearance of the colony were visualized by hand lence on MRS medium (Appendix I).
3.5.2 Microscopic characterization: - The isolates were further studied for cellular characterization by Gram’s staining, motility by wet mount technique and endospore staining.

3.5.2.1 Gram’s staining:- One drop of physiological saline (0.9% NaCl) was placed in the center of plane glass slide. A small mass of the bacterial colony was picked up with the help of sterile loop. A thin smear was made in approximately 1 cm area and allowed to air dry and heat fixed. It was stained with Gram’s staining solutions (Crystal violet for 1 minute, Gram’s iodine for 1 minute, Decolorize for 10 second with acetone: alcohol (25 ml: 75 ml) and counterstained with Saffranin for 1 minute). (Appendix II) Figure 12.

3.5.2.2 Motility Test: - One drop of the broth culture of isolated strain was placed on cover glass in the center and cavity slide was placed carefully over it so as to keep facing the drop towards the cavity. The slide was inverted quickly and was examined immediately under the 45X objective of Sterioscopic binocular (Carl Zeiss) microscope to observe motility.

3.5.2.3 Endospore staining: - The air-dried and heat fixed isolated culture on plain microscopic slide was flooded with malachite green, (Appendix II). The slide was heated to steaming for 5 minute and more stain was added to the smear from time to time. The slide was washed under slowly running tap water, and counterstained with saffranin for 30 sec. Smear was washed with distilled water and examined under 100X objective of Stereoscpic binocular microscope (Carl Zeiss) for the presence of endospores.

3.5.3 Biochemical characterization: - The isolated cultures were further identified by following biochemical tests.
3.5.3.1 Catalase test: - Small biomass of 24 h old bacterial culture was picked up from the center of a colony with a sterile loop. It was placed in the center of a microscopic plain slide. One drop of 3% hydrogen peroxide was placed and observed the culture for the production of bubbles, which are produced due to break down of H₂O₂ in presence of the enzyme catalase. Liberation of gas bubbles is taken as positive reaction for the production of the enzyme.

\[
\text{Catalase} \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

3.5.3.2 Cytochrome oxidase test: - One drop of freshly prepared oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) (Appendix II) was put on a small piece of filter paper strip (Whatman No. 1). Colony was picked up with the help of sterile loop and rubbed gently over the reagent placed on the filter paper. Change of colour of the reagent from grey to blue indicates the presence of oxidase in the test organism. The cytochromes are iron-containing hemoproteins that act as the last link in the chain of aerobic respiration by transferring electrons to oxygen. The test is performed with the dye, as a substitute for oxygen. In the reduced state the dye is colourless, in the presence of cytochrome oxidase and oxygen it is oxidized, forming blue colour.

3.5.3.3 Gelatin liquefaction test: - Approximately 5 ml of gelatin agar medium (Appendix I) was prepared and poured into each test tubes and autoclaved at 15 pa (121°C) for 15 minutes. After sterilization, medium was allowed to solidify at room temperature. Stab inoculation with the help of sterile needle, was made from culture into tubes and incubated at 37°C for 4 to 7 days. After incubation, the tubes were placed into a refrigerator at 4°C for 15 minute and were examined for the liquefaction of the gelatin for positive cultures. Hydrolysis (liquefaction) of gelatin is bough about by microorganisms capable of producing a proteolytic exoenzyme known as gelatinase, which acts to hydrolyze the gelatin to amino acids.
3.5.3.4 Nitrate reduction: - Approximately 5 ml of potassium nitrate medium (Appendix I) was prepared and poured into each test tube and autoclaved at 15 Pa (121°C) for 15 minute and incubated for 96 h. 0.1 ml of test reagent (Appendix II) was added to the test culture. Red colour developing within few minutes indicates the presence of nitrate, which shows the ability of the organism to reduce nitrate. This is a test for the presence of the enzyme nitrate reductase, which causes the reduction of nitrate, in the presence of a suitable electron donor, to nitrite.

\[
\text{NO}_3^- + 2\text{H}^+ + 2e^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O} \quad \text{Nitrate ion} \quad \text{Nitrite ion}
\]

\[
\text{NO}_2^- + 7\text{H}^+ + 7e^- \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2 \quad \text{Nitrous oxide} \quad \text{Nitrogen gas}
\]

3.5.3.5 Arginine hydrolysis test: - Arginine broth (Appendix I) was inoculated with bacteriocin producing strain incubated at 37°C for 48 h. The production of ammonia from arginine was detected by adding Nessler’s reagent (Hi Media Laboratories, Bombay) to culture broth in a ratio of 1:1. The development of an orange or a brick-red colour suggests the hydrolysis of arginine by the test organism. (Figure 17A).

3.5.3.6 Carbohydrate fermentation test: - Approximately 5 ml of carbohydrate fermentation broth (Appendix II) was poured into each fermentation tubes (culture tube with inverted Durham tube) and autoclaved at 15 Pa for 15 minutes. After sterilization, tubes were inoculated with test isolates and incubated at 37°C for 24 to 48 h. The tubes were examined for acid and gas production. Yellow colour indicated acid production while the bubble formation in Durham tube showed gas production. (Figure 17B).
3.5.4 Growth under different conditions:

3.5.4.1 Preparation of inoculum: - 40 ml MRS broth was added in 250 ml conical flask and inoculated with isolated colony of bacteriocin producing strain and incubated at 37°C for 24 h. After 24 h O.D. was taken at 600 nm by Spectrophotometer (Systronics UV-VIS double-beam Spectrophotometer model 2201). Dilution was made to an absorbance of 0.5 at 600 nm with sterile MRS broth pH 6.5.

3.5.4.2 Effect of temperature on growth: - 250 ml conical flasks each with 40 ml MRS broth were inoculated with 100 μl inoculum (O.D. 0.5 at 600 nm) and incubated at 10°C, 25°C, 30°C and 45°C. These tubes were checked for turbidity (O.D. at 600 nm) after 24 and 48 h of incubation period.

3.5.4.3 Effect of Sodium chloride on growth: - MRS broth flasks containing 4% and 6.5% NaCl were inoculated with 100 μl inoculum (O.D. 0.5 at 600 nm) and incubated at 37°C for 24-72 h and growth was examined spectrophotometrically (O.D. at 600 nm) in the culture broth.

3.5.4.4 Effect of pH on growth: - 40 ml MRS broth in 250 ml conical flasks were adjusted to pH values, 4.2, 8.5 and 9.6 with either 3N HCl or 3N NaOH, were inoculated with 100 μl inoculum (O.D. 0.5 at 600 nm) and incubated at 37°C for 24-72 h and growth was assessed spectrophotometrically (O.D. at 600 nm) in the culture broth.

3.6 Maintenance of cultures: - Pure cultures were maintained on MRS slants in universal screw capped culture tubes at 4°C for shorter duration of 1 to 2 months. For longer duration, the organism was stored frozen at -20°C in 30% sterile skimmed milk in small vials of 2 ml.
3.7 Optimization of conditions for bacteriocin production: - The culture conditions for maximum production of bacteriocin by bacteriocin producing strain using Agar-well assay (Varadaraj et al., 1993).

3.7.1 Effect of different culture media on bacteriocin production: -
The bacteriocin producing strain was inoculated in different culture media and the bacteriocin production was assayed by agar well diffusion method as described in paragraph 3.4.4. Samples were drawn at 0, 8, 16, 24, 32, 40 h intervals and pH, absorbance (O.D. at 600 nm) and bacteriocin activity units (AU/ml) were determined. The following culture medium (Appendix I) were used.
1. MRS (de Man Rogosa Sharpe Medium).
2. MRS with 1.5% Tween.
3. MRS I (In MRS media, peptone was replaced by Soya nuggets extract).
4. MRS II (In MRS media, yeast extract was replaced by Soya nuggets extract)
5. T.G.Y.A (Tryptone Glucose Yeast Agar Medium)
6. T.G.E.A (Tryptone Glucose Extract Agar Medium)
7. Elliker's broth.
8. NAM (Nutrient Agar Medium)

3.7.2 Effect of initial pH: - 40 ml MRS, MRS I and MRS II broth in 250 ml conical flasks adjusted to various initial pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0) was inoculated 1 ml of bacteriocin producing strain (absorbance of 0.5 at 600 nm) and incubated at 37°C. Sample were drawn at 0, 8, 16, 24, 32, 40h intervals and pH, absorbance (O.D. at 600nm) and bacteriocin activity units (AU/ml) were determined.

3.7.3 Effect of incubation temperature: - 1 ml of bacteriocin producing strain (O.D. 0.5 at 600 nm) was inoculated in 40 ml sterilized MRS, MRS I and MRS II broth (pH 6.5) in 250 ml conical flask and incubated at 25°C, 30°C, 37°C, 40°C, 45°C and 50°C. Sample were drawn at 0, 8, 16, 24, 32, 40 h
intervals and assayed for pH, absorbance (O.D. at 600 nm) and bacteriocin activity units (AU/ml).

3.7.4 Effect of incubation period:- 40 ml sterilized MRS, MRS I and MRS II broth with initial pH 6.5 in 250 ml conical flask were inoculated 1 ml bacteriocin producing strain (O.D. 0.5 at 600 nm) and incubated at 37°C. Each flask drawn at 2 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 28 h, 32 h and 36, 40 h were assayed for pH, O.D. at 600 nm and bacteriocin activity units by using agar-well diffusion method.

3.8 Optimization of medium: - In this part of study, Lactococcus lactis subsp. lactis CCSUB202 (isolated bacteriocin producing strain) culture was grown in soya nutri nuggets extract medium with different concentration of various nutrients (carbon, salts and Tween--80). Samples were taken after 24 h and incubated at 37°C and assayed for pH and bacteriocin activity units. In this study, a particular ingredient, which showed maximum bacteriocin production, was considered as an optimum ingredient and incorporated in the medium, while designing next experiment for other nutrients to achieve maximum activity. Using this approach, the different nutrients or nutritional factors were studied for the optimization of the conditions for bacteriocin production in soya nutri nuggets extract medium.

3.8.1 Preparation of soya nutri nuggets extract:- 1 gm Soya nutri nuggets boiled for 10-20 minutes in 10 ml distilled water, after that filter with whatman no.1. Filtrate was used as extract of Soya nutri nuggets.

3.8.2 Bacteriocin production on soya nutri nuggets extract:- 1 ml producer strain Lactococcus lactis subsp. lactis CCSUB202 culture was inoculated into 40 ml sterilized Soya nutri nuggets extract medium in 250 ml conical flask and incubated at 37°C for 24 h.
3.8.3 Effect of supplementation of various nutrients in soya nutri nuggets extract on bacteriocin production: - 1 ml producer strain *Lactococcus lactis* subsp. *lactis* CCSUB202 culture was inoculated into 40 ml sterilized soy nutri nuggets extract medium with various nutrients in 250 ml conical flask and incubated at 37°C for 24 h, samples were taken and assayed for pH, absorbance (O.D. at 600 nm and bacteriocin activity units.

3.8.3.1 Effect of soya nutri nuggets extract: - Soya nutri nuggets extract was added at 25, 50, 75, and 100% level individually.

3.8.3.2 Effect of glucose:- Carbon sources i.e., glucose was added at 0, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10% level individually.

3.8.3.3 Effect of salts:-- Different salts (MgSO₄, KH₂PO₄ and Sodium acetate) were added at 0.01, 0.02, 0.03, 0.04 and 0.05% level individually.

3.8.3.4 Effect of Tween-80:- Tween-80 was added at 0.025, 0.05, 0.075 and 0.1% level individually.

3.8.4 Effect of supplementation of combination of nutrients in soya nutri nuggets extract on bacteriocin production:-- Soya nutri nuggets extract medium (*Appendix I*) was supplemented with various nutrients such soya nutri nuggets extract (1000 ml), glucose (40 g), sodium acetate (3.0 g), KH₂PO₄ (2.0g), MgSO₄ (0.2 g), Tween-80 (1 ml). The medium was inoculated and incubated at 37°C for 24 h to study their effect on bacteriocin production in the developed medium.

3.8.5 Optimization of physical factors (pH, Temperature and Incubation period) for bacteriocin production on soya nutri nuggets extract medium: -

3.8.5.1 Effect of initial pH: - 40 ml soya nutri nuggets extract medium in 250 ml conical flasks adjusted to various initial pH values (5.5, 6.0, 6.5, 7.0, 7.5,
8.0, 8.5, 9.0) was then sterilized by autoclaving at 15 Pa (121°C) for 15 minutes and inoculated 1 ml of bacteriocin producing strain (O.D. 0.5 at 600 nm) then incubated at 37°C. Sample were drown at 0, 8, 16, 24, 32, 40 h intervals and pH, absorbance (O.D. at 600 nm) and bacteriocin activity units (AU/ml) were determined.

3.8.5.2 Effect of incubation temperature:- 1 ml of bacteriocin producing strain (O.D. 0.5 at 600 nm) was inoculated in 40 ml sterilized soya nutri nuggets extract medium (pH 6.5) in 250 ml conical flask and incubated at 25°C, 30°C, 37°C, 40°C, 45°C and 50°C. Sample were drown at 0, 8, 16, 24, 32, 40 h intervals and assayed for pH, absorbance (O.D. at 600 nm) and bacteriocin activity units (AU/ml).

3.8.5.3 Effect of incubation time:- 40 ml sterilized soya nutri nuggets extract medium with initial pH 6.5 in 250ml conical flask were inoculated 1 ml bacteriocin producing strain (O.D. 0.5 at 600 nm) and incubated at 37°C. Each flask drown at 2 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 28 h, 32 h and 36, 40 h were assayed for pH, O.D. at 600 nm and bacteriocin activity units by using agar-well diffusion method.

3.9 Mass production of bacteriocin in soya nutri nuggets extract medium: - Bacteriocin was produced in soya nutri nuggets extract medium by batch fermentation in a 2-litre volume batch fermenter (LAB FORS AG CH-4103 Bottmingen/Switzerland) with Lactococcus lactis subsp. lactis CCSUB202.

3.9.1 Preparation of fermentation medium: - Soya nutri nuggets extract medium (SNNEM) (Appendix-I) was supplemented with 40 g glucose, 3.0 g sodium acetate, 2.0 g K2HPO4, 0.2 g MgSO4, 1 ml Tween-80 to made up the volume 1 litre. For mixing the constituents homogenously, the medium bottle was placed over magnetic stirrer for 15 minutes. The medium was sterilized in
Figure 10A: Mass production of Bacteriocin in batch fermenter.

Figure 10B: Dialysis process at 4°C.
the fermenter vessel by autoclaving at 121°C for 30 minutes as recommended by the manufacture of the fermenter.

3.9.2. Preparation of inoculum: - This consisted of two steps; first 10 ml of SNNEM was inoculated with 0.1 ml of a freshly prepared Lactococcus lactis subsp. lactis CCSUB202 culture and incubated at 37°C for 24 h with shaking at 150 rpm. This pre-culture was used to inoculate 90 ml SNNEM. After incubated at 37°C for 24 h, this second pre-culture was used to inoculate the fermenter vessel. 100 ml of the inoculum with 0.5 O.D. was inoculated in total 1000 ml of the culture medium aseptically.

3.9.3 Preparation of acid, base & pH buffers: -
**Acid:** 200 ml I.0 N HCl was prepared in acid reservoir (3.65 ml HCl in 100 ml D.W.)

**Base:** 200 ml of I.0 N NaOH was prepared in base reservoir (4 gm NaOH in 100 ml D.W.)

**pH buffers:** pH buffers of pH 4.0, 7.0 & 9.2 were prepared by dissolving corresponding pH tablets in 100 ml of double distilled water separately.

3.9.4 Sterilization of fermenter vessel by autoclaving: - The fermenter vessel with medium was then autoclaved at 121°C for 30 minutes. The vessel was allowed to cool completely after autoclaving before inoculated the inoculum.

3.9.5 Inoculation of the bacteriocin producer strain: - The inoculum (as paragraph 3.9.2) was aseptically transferred to a sterile, disposable syringe of a suitable size. The inoculum port fitting was removed and the syringe quickly inserted through the membrane and the inoculum pushed into the vessel. The syringe needle was quickly withdrawn and port fitting replaced.

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3.9.6 Fermentation experiment: - Fermentation experiment was carried out in a 2-litre volume laboratory batch fermenter (LAB FORS AG CH-4103 Bottmingen/Switzerland) with a working volume of 1 litre (Figure 10). Preparation of the fermenter and on-line control of the fermentation process. The temperature was set to 35°C, pH to 7.5, pO₂ to 100% and agitator speed to 150 revolutions per minute.

The samples were aseptically withdrawn at regular intervals (2 h, 4 h, 8 h, 10 h, 12 h, 16 h, 20 h, 24 h, 32 h, 40 h). The culture was finally harvested after 24h of fermentation from fermentation vessel to determine the absorbance (O.D. at 600nm) and bacteriocin activity (AU/ml). After the harvesting, the culture was examined microscopically and biochemically and checked for the inoculated strains of Lactococcus lactis subsp. lactis CCSUB202.

3.10 Plate assay of bacteriocin: - To study the effect of bacteriocin produced by Lactococcus lactis subsp. lactis CCSUB202 on sensitive cells. 2 ml of CFF (pH 6.5) was added to 10 ml of growing cells of sensitive strain (Lactococcus lactis subsp. lactis MTCC3038) in MRS broth medium in early exponential phase. The absorbance was determined at 600 nm after appropriate intervals of 4 h and the culture after 24 h period of incubation was plated onto agar plates and examined after incubation of 24 h.

3.11 Protein estimation: - Protein concentration was determined by the method of Lowry, et. al., (1951) using bovine serum albumin (BSA) as standard. 1 ml of Lowry C (Appendix II) was added to 200 µl CFF (cell free filtrate) and keeps it for 10 minute at room temperature. Add 100 µl folin reagent (preparation in Appendix II) and keep it for 30 minute at room temperature. The protein content of the sample was monitored at 750 nm. Figure 11 (Appendix III).
Figure 11: Standard curve for protein estimation (Lowry et al., 1951).
3.12 Determination of specific activity: Specific activity of bacteriocin is defined as activity units of the bacteriocin per milligram of the protein and was calculated using the formula:

\[
\frac{\text{Total activity units in fractions}}{\text{Specific activity}} = \frac{\text{Total amount of protein in fraction}}{}
\]

3.13 Determination of fold purification: -

Fold purification = \[
\frac{\text{Specific activity of fraction}}{\text{Specific activity of original sample (culture supernatant)}}
\]

3.14 Determination of activity recovery: -

Activity recovery or Yield = \[
\frac{\text{Total activity units in fraction}}{\text{Total activity units in culture supernatant}}
\]

3.15 Partial purification of bacteriocin: - The purification of the bacteriocin produced by Lactococcus lactis subsp lactis CCSUB202 was done by different methods viz: precipitation with organic solvents and fractionation with ammonium sulphate followed by dialysis and gel filtration (HPLC, AKTA prime Amersham Bioscience, Sweden). Molecular weight determined by SDS-PAGE. (Appendix III).

3.15.1 Selection of buffer: - For the selection of buffer, precipitates obtained after ammonium sulphate fractionation step were dissolved in sterile distilled
water. 250 μl of this bacteriocin preparation was mixed with 750 μl of 0.2M sodium phosphate buffer (pH 7.0, 7.5), 0.2M potassium phosphate buffer (pH7.0), 0.2M citrate buffer (pH 5.0), 0.2M acetate buffer (pH 5.0), and 0.2M phosphocitrate buffer (pH 7.0) (Appendix II) to attain a final ionic strength of 0.05M of buffer. The samples were kept at 4°C, 37°C and assayed for bacteriocin activity units after 24, 48 and 72 h.

3.15.2 Solvent fractionation of the bacteriocin: - To 50 ml of the culture supernatant, 50 ml of solvent (isopropanol, ethanol, methanol or acetone) was added with constant stirring to obtain the supernatant to solvent ratio 1:1. The mixture was stirred on magnetic stirrer for another one hour and left in the refrigerator overnight. The precipitate formed was separated by centrifugation (at 15000 rpm for 30 minutes at 4°C) and the supernatant obtained was further treated with incremental addition of the organic solvent to obtain the supernatant to solvent ratios of 1:2 and 1:3. The precipitate obtained every time was dissolved in sodium phosphate buffer and tested for protein content and bacteriocin activity.

15.3 Ammonium Sulphate precipitation: - To the 100 ml of supernatant, for protein precipitation, ammonium sulphate was added slowly with constant stirring to achieve 40% saturation. The mixture was then kept overnight in the refrigerator at 4°C. It was then centrifuged at 15000 rpm for 30 minutes at 4°C and the precipitate was dissolved in sodium phosphate buffer 0.05M (pH 7.0). The supernatant was subsequently adjusted to 60%, 80% and 100% saturation levels by further addition of solid ammonium sulphate. The precipitates in each case were dissolved in sodium phosphate buffer.

In a further study, the culture supernatant was adjusted to 0-60% saturation with ammonium sulphate and precipitates obtained were dissolved in sodium phosphate buffer.

The bacteriocin solution obtained after each ammonium sulphate fractionation step was dialysed.
3.15.4 Dialysis:- The bacteriocin solution obtained after each ammonium sulphate fractionation step was dialysed using 10 kDa molecular weight cut off semi-permeable membrane such as cellophane (cellulose acetate) against 0.05M phosphate buffer (pH 7.0) with 4 to 5 changes over a period of 24 h at 4°C, to remove ammonium sulphate salt which might interfere in subsequent experiments.

In this, a dialysis tubing of suitable length was soaked and boiled for 10 minutes in a solution of 2% (w/v) sodium bicarbonate and 0.05% EDTA and cooled. The dialysis tubing was again boiled twice for 10 minutes in distilled water and then used for study.

An aliquot was analyzed for the protein content by Lowry et al. (1951) method, bacteriocin activity, specific activity and recovery of the bacteriocin as describe in paragraph 3.11, 3.12, 3.13 and 3.14. The protein fraction obtained from saturated ammonium sulphate precipitation showed highest bacteriocin activity and it was used for further purification.

3.15.5 Gel filtration chromatography:- The bacteriocin preparation obtained after ammonium sulphate precipitation followed by dialysis was further purified by gel filtration chromatography in HPLC using superdex 75.

14 ml Superdex 75 (fine, particle size 13μ) obtained from AKTA Prime Amersham Biosciences, Sweden were mixed with 0.05M sodium phosphate buffer (pH 7.0) upto 28-30 ml. The gel suspension after deaerating for 5-10 minutes was packed in HR 10/30-glass column at the operating pressure 0.5MPa. The column was flushed with 3 volume of the eluting buffer, 0.05M sodium buffer containing 0.004% sodium azide. The bacteriocin solution was carefully injected in the system with the help of a disposable syringe and the protein was eluted from the column at a flow rate 0.5 ml per minute. 1 ml fractions were collected after draining 80% of the void volume and analysed for bacteriocin activity. Determining O.D. at 280 nm monitored the protein content of the fractions.
3.16 Characterization of the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSUB202: - The crude as well as partially purified bacteriocin preparations were used in the characterization studies.

**Crude bacteriocin:** *Lactococcus lactis* subsp. *lactis* CCSUB202 was grown in SNREM broth at 37°C for 24 h. The culture broth was boiled for 3-5 minutes in a boiling water bath to kill the cells then centrifuged at 15000 rpm for 30 minutes at 4°C. The bacteriocin concentration in the culture supernatant was estimated to be 5280 AU/ml.

**Partially purified bacteriocin:** The bacteriocin preparation obtained after ammonium sulphate precipitation followed by dialysis against 0.05M sodium phosphate buffer (pH 7.0) overnight, the filter sterilized by passing through 0.22 μm membrane filter (Millipore).

The concentrated bacteriocin preparation obtained after gel-filtration on superdex 75 was used in the characterization study.

3.16.1 Inhibitory spectrum of partially purified bacteriocin: - The antibacterial activity against various lactic and non-lactic cultures of partially purified bacteriocin was determined by agar-well assay (3.4.4)


3.16.2.1 Sodium dodecyl sulphate-polyarylamide gel electrophoresis (SDS-PAGE) in a 12% discontinuous gel (Appendix II) was performed. 50 μl of bacteriocin was mixed in 1:1 ratio with sample buffer (Appendix II) and prepare the sample by heating them to 100°C for 3-4 minutes and cooled. Molecular weight standard (Sigma) and 30 μl of the sample were applied to
the gel. The electrophoresis buffer (Appendix II) was then carefully layered over the applied samples.

Electrophoresis was carried out at constant current 50 A (150) till the sample reached the lower end of the gel. After electrophoresis was over, removed the gel carefully and stained with coomassie brilliant blue R-250 solution (Appendix II) after destain it in the destaining solution (Appendix II) with 3-4 changes at room temperature. Finally, the destained gel was observed for the protein bands.

3.16.3 Heat stability of bacteriocin:- One ml centrifuged and filtered (0.22 μm Millipore filter) culture broth and one ml of 1:10 diluted partially purified bacteriocin were subjected to different heat treatments, viz, 65°C/30 minutes, 75°C/30 minutes, 85°C/10 and 15 minutes, 90°C/10 and 15 minutes, 100°C (boiling in water bath) for 5, 10, 15, 30, and 60 minutes and 121°C (autoclaving) for 15 minutes, cooled and assayed for bacteriocin activity units after 2h. Residual activity (%) was calculated using the formula:

\[
\text{Residual activity (\%) = } \frac{\text{AU/ml of the sample at different time intervals}}{\text{AU/ml of sample at zero hour}}
\]

3.16.4 pH stability of bacteriocin: -

Crude bacteriocin: - The centrifuged and filtered culture broth were adjusted to different pH values ranging from 2-13 with either sterile 1N NaOH or 1N HCl and the activity was assayed after 0, 2, 8, 24, 168 and 360 h of storage at 4°C

Purified bacteriocin: - To 100 μl aliquots of the purified bacteriocin in 900 μl of 0.05M buffers of different pH values in the range of 2-13 were added to attain a final bacteriocin concentration of 10560 AU/ml and the bacteriocin activity was assayed after 0, 2, 8, 24, 168 and 360 h of storage at 4°C.
The buffer used were: HCl-KCl buffer for pH 1 and 2, glycine HCl buffer for pH 3, acetate buffer for pH 4 and 5, sodium phosphate buffer for pH 6 and 7, Tris-HCl buffer for pH 8 and 9 and glycine-NaOH buffer for pH 10 and 11.

3.16.5 Effect of pH on the heat stability of the bacteriocin: - The crude bacteriocin adjusted to different pH values in the range of 2-13 as in paragraph 3.16.4 was subjected to two different heat treatment viz: 75°C/30 minutes and 100°C/10 minutes, while the purified bacteriocin adjusted to different pH values in the range of 2-13 as in paragraph 3.16.4 was heated for 10 minutes at 100°C, cooled and residual activity of both the preparation was determined after 8h of storage at room temperature.

3.16.6 Effect on surfactant on the bacteriocin: - The ammonium sulphate precipitated dialysed bacteriocin preparation in sodium phosphate buffer, was mixed with a 2% solution of Tween 80, Tween 20, Triton X-100 and SDS to attain a final concentration of 1.0% of the surfactants. The mixtures were stored at 4°C. The activity units of the treated samples were determined after 24 h of storage. The effect of 0.1% and 0.5% SDS was also studied in similar manner.

3.16.7 Effect of sodium chloride on the bacteriocin: - 1 ml portion of partially purified bacteriocin were added 9 ml of 0.05mM sodium phosphate buffer (pH 7.0) aliquots containing 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0 and 3.0M concentrations of NaCl. The residual activity of the added bacteriocin was assayed after incubation for 24 h at 4°C.