3

Materials and Methods

3.1. Culture media used

3.1.1. All purpose Tween (APT) agar

(M226) - HiMedia, Bombay, India

3.1.2. All purpose Tween (APT) broth

(HiMedia M227)

3.1.3. Gelatin broth

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

3.1.4. Glucose-yeast extract agar

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

3.1.5. Milk agar

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

3.1.6. MRS agar

Lactobacillus MRS agar (HiMedia M641)

3.1.7. MRS basal medium

Peptone	10 g
Yeast extract	5 g

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

3.1.8. MRS broth

Lactobacillus MRS broth (HiMedia M369)

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

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

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

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

3.1.11. MRS soft agar

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

3.1.12. Nitrate broth

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

3.1.13. Nutrient agar

(HiMedia M001)

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

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

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

3.1.15. Semi-defined (SD)-MRS broth

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

pH	6.0

3.1.16. Starch agar

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

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

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

3.1.18. Tributyrin agar

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

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

3.1.19. Tryptophan broth

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

3.2. Reagents used

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

Iodine	1 g
KI	2 g
Distilled water	100 ml

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

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

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

p-Dimethylaminobenzaldehyde	10 g
HCl concentrated	100 ml

3.2.4. Physiological saline

0.85% (w/v) NaCl in distilled water

3.2.5. Protein precipitating reagent

1 N sulphuric acid saturated with ammonium sulphate

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

Solution A:

Sulphanilic acid	800 mg
5 N acetic acid	100 ml

(glacial acetic acid : water, 1 : 2.5)

Solution B:

α-Napthylamine	500 mg
5 N Acetic acid	100 ml

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

3.2.7. Reagents for protein estimation

Reagent A:

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

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

Reagent B:

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

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

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

3.3. Buffers used

3.3.1. 0.2 M Ammonium bicarbonate buffer (pH 8.0)

Ammonium bicarbonate	15.8 g
Distilled water	1000 ml

3.3.2. 50 mM sodium phosphate buffer (pH 7.2)

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

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

3.3.3. 20 mM sodium phosphate buffer (pH 8.0)

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

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

3.3.4. 20 mM Tris buffer (pH 7.5)

Tris	2.4 g
Distilled water	1000 ml

3.4. Organisms used

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

3.5. Experimental

3.5.1. Collection of sample

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

3.5.2. Isolation of lactic acid bacteria

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

3.5.3. Characterization of isolates

3.5.3.1. Cell morphology

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

3.5.3.2. Gram staining

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

3.5.3.3. Production of catalase

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

3.5.3.4. Production of gas from glucose

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

3.5.3.5. Growth in 6.5% NaCl

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

3.5.3.6. Hydrolysis of casein

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

3.5.3.7. Production of indole

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

3.5.3.8. Reduction of nitrate

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

3.5.3.9. Hydrolysis of starch

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

3.5.3.10. Hydrolysis of fat

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

3.5.3.11. Liquefaction of gelatin

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

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

3.5.3.12. Utilization of sugars

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

3.5.4. Effect of anaerobiosis on lactic acid bacterial growth

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

3.5.5. Detection of antibacterial activity

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

3.5.6. Preparation of cell-free culture supernatants

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

3.5.7. Well diffusion assay

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

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

3.5.8. Determining antagonism due to phage

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

3.5.9. Determining antagonism due to hydrogen peroxide

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

3.5.10. Determining antagonism due to bacteriocin

3.5.10.1. Treatment with pepsin

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

3.5.10.2. Treatment with trypsin

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

3.5.11. Determining antagonism due to acids

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

3.5.12. Elimination of Bac⁻ variants

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

3.5.13. Optimization of bacteriocins production

3.5.13.1. Type of medium

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

3.5.13.2. pH of medium

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

3.5.13.3. Period of incubation

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

3.5.13.4. Temperature of incubation

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

3.5.14. Characterization of bacteriocins

3.5.14.1. Thermostability

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

3.5.14.2. pH stability

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

3.5.14.3. Storage stability

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

3.5.14.4. Stability against organic solvents

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

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

3.5.15. Purification of bacteriocin

3.5.15.1. Concentration

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

3.5.15.2. Dialysis

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

3.5.15.3. Gel filtration

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

3.5.15.4. Ion exchange chromatography

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

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

3.5.15.5. Desalting column chromatography

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

3.5.15.6. Protein estimation

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

3.5.15.7. Activity assay

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

3.5.16. Mode of action

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

3.5.17. Activity spectrum

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

3.5.18. Statistical analysis

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