3. MATERIALS AND METHODS

3.1. CULTURE MEDIA USED

Anaerobic agar (Claus and Berkeley 1986)

Trypticase	20.0 g
Glucose	10.0 g
NaCl	5.0 g
Agar	15.0 g
Sodium thioglycolate	2.0 g
Sodium formaldehyde sulfoxylate	1.0 g
Distilled water	1000 ml
pH 7.2	

APT agar (HiMedia M226)

Arginine hydrolysis test medium (Thornley 1960)

Peptone	1.0 g
Arginine	10.0 g
NaCl	5.0 g
$K_2HPO_4.3H_2O$	0.3 g
Phenol red	0.01 g
Agar	4.0 g
Distilled water	1000 ml
pH 7.2-7.4	

Basal medium for acid and gas production from carbohydrates (Gordon *et al*. 1973)

Diammonium hydrogen phosphate	1.0 g
KCl	0.2 g

$MgSO_4.7H_2O$	0.2 g
Yeast extract	0.2 g
Bromocresol purple	0.4 g
Distilled water	1000 ml
pH 7.0	

Columbia blood agar base and **defibrinated sheep blood** (Oxoid CM331 and SR51)

Davis and Mingioli's broth (Davis and Mingioli 1950)

K_2HPO_4	7.0 g
KH_2PO_4	3.0 g
Sodium citrate.$3H_2O$	0.5 g
$MgSO_4.7H_2O$	0.1 g
$(NH_4)_2SO_4$	1.0 g
Glucose (Sterilized separately)	10.0 g
Distilled water	1000 ml
pH 7.0	

Esculin hydrolysis test medium

Esculin	1.0 g
Ferric chloride	0.5 g
Peptone	5.0 g
Yeast extract	1.0 g
Agar	20.0 g
Distilled water	1000 ml

Gelatin agar (Sneath and Collins 1974)

Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
Gelatin	10.0 g
Distilled water	1000 ml
pH 7.2	

Glucose-yeast extract-peptone (GYP)-$CaCO_3$ agar

(Okada *et al.* 1986)

Glucose	10.0 g
Yeast extract	5.0 g
Peptone	5.0 g
Tween 80	0.5 g
Salt solution	5.0 ml
$CaCO_3$ (Sterilized separately in an oven at 180°C for 6 h)	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH 6.8	

<u>Salt solution</u>

$MgSO_4.7H_2O$	4.0 g
$MnSO_4.4H_2O$	0.2 g
$FeSO_4.7H_2O$	0.2 g
NaCl	0.2 g
Distilled water	100 ml

Glucose-yeast extract-peptone water (Kreger-van Rij 1984)

Glucose	20.0 g
Peptone	10.0 g
Yeast extract	5.0 g
Distilled water	1000 ml
pH unadjusted	

Agar (2% w/v) was added to prepare glucose-yeast extract-peptone agar.

Gorodkowa agar (Kreger-van Rij 1984)

Glucose	1.0 g
Peptone	10.0 g
NaCl	5.0 g
Agar	20.0 g
Tap water	1000 ml
pH unadjusted	

Malt extract (Lodder and Kreger-van Rij 1952)

Malt extract powder	150.0 g
Demineralized water	1000 ml
pH 5.4	

Malt extract agar (Kreger-van Rij 1984)

Malt extract powder	100.0 g
Agar	20.0 g
Demineralized water	1000 ml
pH 5.4	

Milk agar (Gordon *et al*. 1973)

Skim milk powder	5.0 g in 50 ml distilled water
Agar	1.0 g in 50 ml distilled water

Autoclaved separately at 121°C for 20 min, cooled to 45°C, mixed together and poured into Petri dishes. The plates were allowed to stand at 37°C for 24 h to dry the surface of the agar.

MRS broth (de Man *et al*. 1960)

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
K_2HPO_4	2.0 g
Diammonium citrate	2.0 g
Glucose	20.0 g
Tween 80	1.0 g
Na acetate	5.0 g
$MgSO_4.7H_2O$	0.58 g
$MnSO_4.4H_2O$	0.28 g
Distilled water	1000 ml
pH 6.2-6.4	

Agar (2% w/v) was added to prepare MRS agar.

Nitrate broth (Gordon *et al.* 1973)

Peptone	5.0 g
Beef extract	3.0 g
KNO_3	1.0 g
Distilled water	1000 ml
pH 7.0	

Nutrient broth (Gordon *et al.* 1973)

Beef extract	3.0 g
Peptone	5.0 g
Distilled water	1000 ml
pH 6.8	

Agar (2% w/v) was added to prepare nutrient agar.

Potato dextrose agar (PDA) (APHA 1967)

Potatoes, peeled and sliced	200.0 g
D-glucose	20.0 g
Agar	20.0 g
Distilled water	1000 ml

Potatoes (200 g) were peeled, sliced and boiled for one h in 1000 ml distilled water. The solution was filtered. The filtrate was added with glucose and agar, and boiled again to dissolve the agar. The volume was made up to 1000 ml.

Sugar basal broth (for lactic acid bacteria) (Garvie 1984)

Peptone	10.0 g
Yeast extract	2.5 g
Tween 80	0.1 g
Bromocresol purple	1.0 ml
(1.6% w/v in ethanol)	
Distilled water	1000 ml
pH 6.8	

Tributyrin agar (Stolp and Gadkari 1981)

Peptone	5.0 g
Yeast extract	3.0 g
Tributyrin	10.0 g
Agar	12.0 g
Distilled water	1000 ml
pH 7.4-7.6	

Tryptone-glucose-yeast extract (TGYE) broth
(Mukherjee *et al*. 1965)

Tryptone	5.0 g
Glucose	15.0 g
Yeast extract	2.5 g
Tween 80	1.0 ml
Salt A	5.0 ml
Salt B	5.0 ml
Distilled water	1000 ml

Salt A

K_2HPO_4	100.0 g
KH_2PO_4	100.0 g
Distilled water	1000 ml

Salt B

$MgSO_4.7H_2O$	40.0 g
NaCl	2.0 g
$FeSO_4.4H_2O$	2.0 g
$MnSO_4.7H_2O$	2.0 g
Distilled water	1000 ml

Urea medium (Christensen 1946)

Part A

Peptone	1.0 g
Glucose	1.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Phenol red	0.012 g
Agar	20.0 g
Distilled water	1000 ml

pH 6.8

Part B

Urea	40.0 g
Distilled water	1000 ml

(Sterlized by filtration)

After sterilization of Part A in test tubes (5 ml/ tube), 0.25 ml of sterile Part B was added and made slants.

Voges-Proskauer (VP) broth (Gordon *et al.* 1973)

Peptone	7.0 g
Glucose	5.0 g
NaCl	5.0 g
Distilled water	1000 ml
pH 6.5	

Yeast extract-malt extract (YM) agar (Wickerham 1951)

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	20.0 g
Distilled water	1000 ml
pH 5.0-6.0	

Yeast nitrogen base (Difco 0392-15-9)

All the media mentioned above were sterilized by autoclaving at 121°C for 15 min, unless mentioned otherwise.

3.2. REAGENTS USED

Burke's iodine solution (Bartholomew 1962)

Iodine	1.0 g
KI	2.0 g
Distilled water	100 ml

Crystal violet stain (Bartholomew 1962)

Crystal violet	2.0 g
95% Ethanol	20 ml
Ammonium oxalate (1% w/v aqueous solution)	80 ml

Ehrlich-Böhme reagent (Iswaran 1980)

p-Dimethylaminobenzaldehyde	1.0 g
95% Ethanol	95 ml
HCl concentrated	20 ml

Physiological saline (Karki *et al.* 1983d)

NaCl	8.5 g
Distilled water	1000 ml

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

Solution A

Sulphanilic acid	0.8 g
5 N Acetic acid (Glacial acetic acid : water, 1 : 2.5)	100 ml

Solution B

α-Naphthylamine	0.5 g
5 N Acetic acid	100 ml

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

Safranin stain (Norris *et al.* 1981)

2.5% w/v Safranin in 95% ethanol	10 ml
Distilled water	100 ml

All the chemicals used were of the highest purity grade.

3.3. EXPERIMENTAL

3.3.1. Survey on traditional fermented foods

A survey was conducted in different places of Darjeeling hills and Sikkim to get detailed information on the types, traditional methods of preparation, modes of consumption, ethnic value and distribution of various fermented foods used by the local people. A format (Table 3) was specially prepared for the survey.

3.3.2. Collection of sample

Samples of kinema, sinki, mesu and murcha were collected aseptically in separate sterile glass bottles from different periodic markets, locally called 'hats', of Darjeeling, Kalimpong, Gangtok and Rongli .

Table 3. Format of survey on consumption of fermented foods in Darjeeling hills and Sikkim

District :

Tehsil/town :

Village :

Approximate population :

Name of informant :

Date of survey :

Sl No.	Fermented products	Consumption (Yes/No)	Daily/Weekly/ Monthly/ Occasional	Household preparation/ Market purchase	Raw materials: Home cultivation/ Cash purchase

The bottles of kinema and mesu were kept in an ice-box and transported immediately to the laboratory for analyses. The raw ingredients used for the preparations of kinema, sinki and mesu were also collected for analyses.

3.3.3. Biochemical analysis

3.3.3.1. Moisture

A well-mixed sample (*ca* 2 g) was accurately weighed into a cooled and weighed Petri dish (provided with cover), previously heated to 130±1°C. The sample was uncovered and allowed to dry for 1 h at 130±1°C in a hot air oven. The dish was covered while still in oven, transferred to a dessicator, and weighed soon after reaching room temperature. The process of drying, cooling and weighing was repeated until the two successive weights reached a constant value. Moisture content was calculated by subtracting the final weight from the initial weight (AOAC 1990).

3.3.3.2. Ash

A well-mixed sample (*ca* 2 g) was accurately weighed into a previously dried and weighed porcelain crucible and placed in a muffle furnace preheated to 600°C. The sample was held at that temperature for 2 h. The crucible was transferred directly to a dessicator, allowed to cool to room temperature and weighed immediately (AOAC 1990). The process of heating for 30 min, cooling and weighing was repeated until the difference between two successive weighings was $\leq$1 mg. The lowest mass was recorded (IS: 5162 1980).

3.3.3.3. pH

A sample (100 g) was mixed with 20-50 ml carbon dioxide-free distilled water in a waring blender (Bajaj, India) for 1 min. The temperature of the paste prepared was equilibrated to 25°C and the pH was noted (AOAC 1990) using a pH meter (Systronics type 335).

3.3.3.4. Titratable acidity

A well-mixed sample (10 g) was blended with 90 ml carbon dioxide-free distilled water for 1 min. The mixture was filtered, and 25 ml of the filtrate was titrated with 0.1 N NaOH to an end point of phenolphthalein (0.1% w/v in 95% ethanol) (AOAC 1990).

$$\underset{\text{(as lactic acid)}}{\text{\% Titratable acid content}} = \frac{100 \text{ x ml of NaOH x N of NaOH x } 0.09}{\text{Weight of sample (g)}}$$

3.3.3.5. Total nitrogen

The method as described in AOAC (1990) was followed. Approximately 1 g accurately weighed sample, taken in a digestion flask, was added with 0.7 g HgO, 15 g powdered K_2SO_4 and 25 ml concentrated H_2SO_4. The flask was heated gently until frothing ceased, boiled briskly until the solution became clear, and then continued the boiling for about 1 h. The solution was transferred quantitatively to a round bottom flask, and mixed with approximately 100 ml distilled water and 25 ml 4% w/v aqueous Na_2S to precipitate mercury. A pinch of zinc granules to prevent bumping and a layer of 40% w/v aqueous NaOH were added carefully. The flask was immediately connected to a distillation apparatus and the tip of the condenser was immersed in standard (0.1-0.5 N) H_2SO_4 containing

about 5 drops of methyl red indicator (0.5% w/v methyl red in ethanol). The flask was rotated to mix the contents thoroughly and heated until all the ammonia had distilled. The receiver was removed and the tip of the condenser was washed with distilled water. The remaining acid in the receiver was titrated with standard (0.1-0.5 N) NaOH solution. The blank determination on reagents was considered for correction.

% N = [(ml of standard acid x N of standard acid) - (ml of standard NaOH x N of standard NaOH)] x 1.4007 / Weight of sample (g)

3.3.3.6. Protein and non-protein nitrogen

A sample (0.5 g) was mixed with 30 ml 10% w/v cold trichloroacetic acid and allowed to stand at 5°C for 20 h. The solution was centrifuged at 8500 g for 10 min. Nitrogen contents in the supernatant fraction (non-protein nitrogen) and the precipitate (protein nitrogen) (Nirenberg and Matthaei 1961) were measured by micro-Kjeldahl method as described in Section 3.3.3.5.

3.3.3.7. Soluble nitrogen

A well-mixed sample (*ca* 2 g) was shaken with 100 ml distilled water for 45 min on a rotary shaker (120 rpm) and centrifuged at 8000 g for 10 min. The supernatant was filtered through Whatman No. 2 filter paper (Shieh *et al.* 1982) and the nitrogen in the known volume of the filtrate was determined using the micro-Kjeldahl procedure as described in Section 3.3.3.5.

3.3.3.8. Protein

Protein contents in the sample were determined by multiplying total nitrogen value with 5.7 for the soya bean product and 6.25 for the others (AOAC 1990).

3.3.3.9. Fat (crude)

The method as described in IS: 1167 (1965) was followed. Approximately 5 g accurately weighed sample was carefully placed in about 20 ml concentrated HCl containing in a beaker and boiled for 10 min. After cooling to room temperature, the content was quantitatively transferred to a Rose-Gottlieb fat extraction tube using 25 ml diethyl ether. The tube was stoppered with a bark cork and shaken vigorously for 1 min. The beaker was washed with 25 ml petroleum ether and the wash liquid was poured into the tube. After vigorous shaking, the tube was allowed to stand until the upper liquid was clear. The ether solution was decanted into a preweighed Erlenmeyer flask. The tube was washed with a mixture of equal parts of both the solvents, and the washings were added to the flask. Extraction of the liquid remaining in the tube was repeated twice, using 25 ml petroleum ether. The solvent was evaporated completely on a steam bath. The fat was dried in an oven at 100°C to constant weight.

3.3.3.10. Free fatty acidity

An accurately weighed (*ca* 5 g) sample was dissolved in 25 ml 95% ethyl alcohol neutralized previously by 0.1 N NaOH, using phenolphthalein (0.01% w/v in 95% ethanol). After mixing, the

solution was heated to boiling on water bath. The mixture was titrated with 0.1 N NaOH until a faint pink colour persisted (Shieh *et al.* 1982).

$$\text{\% Fatty acid content (as linoleic acid)} = \frac{100 \times \text{ml of NaOH} \times \text{N of NaOH} \times 0.192}{\text{Weight of sample (g)}}$$

3.3.3.11. Carbohydrate

The carbohydrate content was calculated by difference (Standal 1963).

100 - (% protein + % fat + % ash)

3.3.3.12. Energy value

The energy value of a sample was determined by multiplying its percent protein, fat and carbohydrate contents by the factors 4, 9 and 4, respectively, and adding all the multiplication values to get kcal (4184 kcal = 1 Joule) per 100 g.

3.3.4. Microbial analysis

3.3.4.1. Isolation and maintenance

A sample (10 g) was mixed with 90 ml sterile physiological saline containing in an Erlenmeyer flask by placing it on a rotary shaker (120 rpm) for 1 h. Decimal dilution series was prepared in physiological saline. One ml of appropriately diluted suspension of sample was mixed well with molten (45°C) medium and poured into plates. Nutrient agar plates were incubated at 37°C for 18- 24 h; MRS, APT and GYP-$CaCO_3$ agar plates were incubated at 30°C in a spontaneously extinguished candle jar for 48-72 h; and YM agar and

PDA (supplemented with 10 IU/ml benzylpenicillin and 12 μg/ml streptomycin sulphate; Batra and Millner 1974) plates were incubated at 28°C for 48 h. Usually, the incubated plates containing 50-300 colonies were selected for enumeration. The colonies appeared were counted as colony forming units (cfu) per g sample. The isolated colonies of bacteria, yeasts and moulds were checked for purity and picked up on slants of their respective media on which they developed during isolation. The slant cultures were maintained at 4°C by subculturing after every two months. All the representative strains of the different groups of the isolates were deposited in the Culture Collection of the Microbiology Laboratory of the Department of Botany, University of North Bengal.

3.3.4.2. Taxonomic studies on bacterial isolates

3.3.4.2.1. Gram staining

The method of Bartholomew (1962) was followed. A suspension of a 24 h-old bacterial culture on slant was prepared in distilled water. A drop of that suspension was taken on a grease-free slide and a smear was made. It was then heat-fixed, flooded by crystal violet stain for 1 min, and washed for 5 s with water. The smear was 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% 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 safranine for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

3.3.4.2.2. General morphology

An air-dried (not heat-fixed) smear of a 24 h-old bacterial culture was stained for 30 s with safranine, washed in water, air-dried (Norris *et al.* 1981) and observed under oil-immersion objective. Cell dimension was measured with a standardized ocular micrometer.

3.3.4.2.3. Motility

A drop of a 24 h-old culture in nutrient or MRS broth was used to prepare a hanging drop in a cavity slide. The drop was observed using a phase contrast microscope (Olympus BH2-PC-PA-1).

3.3.4.2.4. Production of catalase

A 24 h-old slant culture was flooded with 0.5 ml of 10% hydrogen peroxide solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.* 1981).

3.3.4.2.5. Growth in sodium chloride

Nutrient broth (3 ml/tube) containing 5 and 7% w/v sodium chloride were inoculated with a loopful of culture, and incubated for 3 days at 37°C in a slanting position to improve aeration (Norris *et al*. 1981). For enterococci and lactic acid bacteria, tubes of TGYE broth (5 ml/tube) containing 4, 6.5 and 18% w/v sodium chloride were inoculated with a loopful of culture and incubated at 30°C for 7 days.

3.3.4.2.6. Acid and gas from carbohydrates

Tubes of 10 ml basal medium (for acid and gas production from carbohydrates) containing 0.5% w/v of different carbohydrates and

inverted Durham tubes were inoculated with the isolates and incubated at 37°C for 3 days (Gordon *et al.* 1973). For enterococci and lactic acid bacteria, the method was based on Pederson and Albury (1950) and Okada *et al.* (1986). Tubes of 10 ml sugar basal broth containing 2% w/v sugar and inverted Durham tubes were inoculated and incubated at 30°C for 10 days. Accumulation of gas in the inverts indicated positive result.

3.3.4.2.7. Production of indole

Cells were grown at 37°C (30° for enterococci and lactic acid bacteria) in 10 ml Davis and Mingioli's broth, prepared by replacing ammonium sulphate with L-tryptophan (0.1% w/v) and supplementing with yeast extract (0.02% w/v). Ehrlich-Böhme reagent (1-2 ml) was layered on 3,5 and 7 days-old broth culture. Formation of a red ring at the culture-reagent interface was considered as an indication of positive result (Iswaran 1980).

3.3.4.2.8. Voges-Proskauer reaction

Tubes of 10 ml Voges-Proskauer broth were inoculated with the isolates and incubated at 37°C for 3 days (30°C for 7 days for enterococci and lactic acid bacteria). To the culture, 0.6 ml 5% w/v ethanolic α-naphthol and 0.2 ml 40% w/v aqueous potassium hydroxide were added and kept for 1 h at room temperature for the production of a pink colour, indicating the positive reaction. Initial and final pH of the broth were measured using the pH meter.

3.3.4.2.9. Hydrolysis of gelatin

Gelatin agar plates were streaked with the isolates and incubated

at 37°C for 24 h. For enterococci and lactic acid bacteria, the streaked MRS agar plates containing 1% w/v gelatin were incubated at 30°C for 3 and 5 days. Plates were then flooded with 10 ml 1 N sulphuric acid saturated with ammonium sulphate. Hydrolysis was indicated by a clear zone, under and around the growth, in contrast to the opaque precipitate of unchanged gelatin (Sneath and Collins 1974).

3.3.4.2.10. Hydrolysis of arginine

Tubes of 5 ml arginine hydrolysis test medium were inoculated by stabbing. Immediately after inoculation, a layer (*ca* 1 cm) of sterile mineral oil was added over the stab. The tubes were incubated at 30°C for 3 days and observed for the change in colour from yellow to red, indicating the formation of ammonia from arginine (Lelliott *et al.* 1966).

3.3.4.2.11. Reduction of nitrate

Cultures were grown in 5 ml nitrate broth incubated at 30°C. After 3,7 and 14 days, 1 ml of the culture was mixed with 3 drops of the reagent for nitrate reduction test and observed for the development of a red or yellow colour, indicationg the presence of nitrite. A small amount of zinc dust was added to the tube that was negative even after 14 days and observed for the development of red colour, indicating the presence of nitrate i.e., absence of reduction (Norris *et al.* 1981).

An alternative method was also followed. A strip of filter paper moistened with 10% w/v aqueous potassium iodide and then with a few drops of 1 N hydrochloric acid was touched with a drop

of the culture. It was observed for the production of purple colour, indicating the presence of nitrite (Claus and Berkeley 1986).

3.3.4.2.12. Hydrolysis of fat

Surface-dried plates of tributyrin agar were streaked with the isolates and incubated at 37°C for 24 h (30°C and 4 days for enterococci and lactic acid bacteria). Lipolytic activity was indicated by the formation of clear zones around the streaks (Stolp and Gadkari 1981).

3.3.4.2.13. Anaerobic growth

Anaerobic agar medium was distributed into culture tubes in amount sufficient to give 7.5 cm depth of the medium and sterilized by autoclaving at 121°C for 20 min.

The tubes were inoculated with a small (outside diameter 1.5 mm) loopful of 24 h-old nutrient broth culture by stabbing up to the bottom of the column. They were incubated at 37°C for 3 and 7 days, and observed for growth along the length of the stab (anaerobic) and on the surface of the agar (aerobic) (Claus and Berkeley 1986).

3.3.4.2.14. Decomposition of casein

Milk agar plates were streaked with 24 h-old cultures and examined after incubating at 37°C for 3 days (30°C and 7 days for enterococci and lactic acid bacteria) for any clearing of casein around and underneath the growth (Gordon *et al.* 1973).

3.3.4.2.15. Hydrolysis of esculin

Slants of esculin hydrolysis test medium were inoculated and incubated at 30°C for 7 days. Esculin hydrolysis was indicated by blackening of the medium (Facklam and Wilkinson 1981).

3.3.4.2.16. Growth at different pH

The pH of MRS broth was adjusted to different levels using 1 N HCl or 10% w/v NaOH. The medium was distributed into tubes containing 10 ml in each. They were autoclaved, cooled to room temperature and inoculated with 0.2 ml of 48 h-old MRS broth culture. The tubes were incubated at 30°C for 24 h and observed for growth (Hesseltine and Ray 1988).

3.3.4.2.17. Growth in 0.1% methylene blue milk

Tubes containing 10 ml 5% w/v milk and 0.1% w/v aqueous methylene blue were sterilized, inoculated and tightly capped. Following incubation at 30°C for 3 days, the tubes were observed for decolouration, indicating the growth of inoculum and the consequent reduction of methylene blue.

3.3.4.2.18. Growth at different temperatures

Nutrient agar slants were prepared to determine ability to grow at 5°C intervals within a range of 10-60°C. The slants were immersed in water bath at appropriate temperatures until equilibrated, and then inoculated. Growth of the culture was observed after 3 days at 55°C or higher, after 5 days at 30-50°C, after 14 days at 20 and 25°C, and after 21 days at 10 and 15°C (Claus and Berkeley

1986). For lactic acid bacteria, tryptone-glucose-yeast extract broth was inoculated and incubated at 15, 30, 37, 45 and 50°C for a maximum period of 21 days. The growth was checked visually.

3.3.4.2.19. Haemolysis

The method was based on Hardie (1986). Sterile and molten Columbia blood agar base was cooled to 50°C, mixed with defibrinated sheep blood and poured into Petri dishes. The plates were streaked with the isolates and incubated under semi-anaerobic condition (candle jar) for 48 h at 37°C.

3.3.4.2.20. Requirement of growth factors

The test was performed by growing the culture through several transfers of it in Davis and Mingioli's broth at 37°C, and monitoring visually.

3.3.4.2.21. API tests

For API tests, the isolates were grown on sterile cellophane (Grade 325P British Cellophane, Avonmouth) overlaid plates to avoid nutrient contamination. The *Bacillus* strains were grown at 37°C for 16 h on nutrient agar and the *Enterococcus* strains were grown on MRS agar. The growth was harvested in 2 ml sterile normal saline which was used to prepare two further suspensions, corresponding to 10^8 cells/ml: (i) for API 50CH strips, in 10 ml of respective API 50CHB (*Bacillus*) or API 50CHS (*Streptococcus*) medium, and (ii) for API 20E strips, in 5 ml sterile distilled water. The incubation box was prepared by distributing about 10

and 5 ml of sterile water into the honey combed base of the 50CH and 20E trays, respectively to create humid chambers. The strips were unpacked and placed them in the trays. In API 50CH tests, only the tubes (not the cupules) were filled with the bacterial suspension. For the *Bacillus* strains the cupules were left blank, whereas for the *Enterococcus* strains they were filled with sterile mineral oil. The inoculated 50CH strips were incubated at 30°C for 48 h and read at 24 and 48 h. During incubation, the strips were kept tilted approximately 5° away from the cupules in order to trap any gas evolved. A test scoring positive at either reading time was considered positive. For the API 20E tests, filling of tubes and cupules were made following the manufacturer's instruction. The box was incubated at 37°C. After 24 h, the strips were read by referring to the manufacturer's interpretation table. All spontaneous reactions were recorded. Tests for VP, TDA, IND, nitrate and N_2 were done by adding the prescribed chemicals or reagents.

3.3.4.3. Taxonomic studies on yeast isolates

3.3.4.3.1. General morphology

Malt extract and glucose-yeast extract-peptone water (30 ml each) containing in separate Erlenmeyer flasks (100 ml) were inoculated with an actively growing (24 h-old) yeast culture. They were incubated at 28°C for 3 days and observed for cellular morphology and mode of vegetative reproduction (Kreger-van Rij 1984). Dimension of cells was measured with a standardized ocular micrometer.

Malt extract agar and glucose-yeast extract-peptone agar were streaked with actively growing (24 h-old) yeast isolates, incubated at 28°C for 3 days and observed for the colony characteristics.

3.3.4.3.2. Formation of pseudomycelium and true mycelium

For slide culture preparation, a Petri dish, containing a U-shaped glass rod supporting two glass slides, was autoclaved at 121°C for 15 min. Molten (45°C) potato dextrose agar was poured onto the slides. The solidified agar on the slides was inoculated very lightly with a yeast in two lines along each slide. A sterile coverslip was placed over part of the lines. Some sterile water was poured into the Petri dish to prevent the agar from drying out. The culture was then incubated at 28°C for 4 days. For observation, the slides were taken out of the Petri dish and the agar was wiped off the back of the slide. The edges of the streak under and around the coverslip were examined microscopically for the formation of pseudomycelium or true mycelium (Kreger-van Rij 1984).

3.3.4.3.3. Characteristics of asci and ascospores

A yeast extract-malt extract agar (presporulation medium) slant was streaked with a 24 h-old culture of yeast and incubated at 28° C for 2 days. This was used as an inoculum for gorodkowa agar and potato dextrose agar (sporulation medium) slants. They were incubated at 28°C for 3 days and examined at weekly intervals up to 4 weeks for the observation of asci and ascospores. A heat-fixed smear was covered with 5% w/v aqueous malachite green for 30 s, heated to steaming 3-4 times over the flame of a spirit lamp and counterstained with safranin for 30 s.

3.3.4.3.4. Reduction of nitrate

The method followed was the same as described in Section 3.3.4.2.11, excepting that the temperature of incubation was 28°C.

3.3.4.3.5. Hydrolysis of urea

Christenses's urea agar plates were streaked with 24 h-old yeast, incubated at 28°C for 3 days and observed for any change in colour of the medium.

3.3.4.3.6. Hydrolysis of fat

The method followed was the same as described in Section 3.3.4.2.12, excepting that the plates were incubated at 28°C.

3.3.4.3.7. Growth at 37°C

Malt extract agar slants were inoculated with 24 h-old yeasts, and incubated at 37°C for 2 days (Kreger-van Rij 1964).

3.3.4.3.8. API tests

For API tests, the isolates were grown at 30°C for 24 h on yeast extract-malt extract agar overlaid with cellophane. The growth was harvested in 20 ml yeast nitrogen base. After thorough shaking, the suspension was incubated at 30°C for 4 h and used for inoculating the API 50CH strips. Following incubation at 30°C up to 10 days, the strips were observed for any change in colour in the tubes.

3.3.4.3.9. Fermentation of sugars

The method was based on Kreger-van Rij (1984). Cells were grown at 30°C for 18 h on malt extract agar plates overlaid with cellophane. The growth was harvested in 2.5 ml filter-sterilized yeast nitrogen base. After thorough mixing, the cell suspension was incubated at 30°C for 4 h. Tubes of 10 ml yeast nitrogen base supplemented with 2% w/v filter-sterilized sugars containing Durham tubes were inoculated with the above-mentioned inoculum preparation. The tubes were incubated at 25°C and shaken regularly to observe for any accumulation of gas in the inverts.

3.3.4.4. Taxonomic studies on mould isolates

3.3.4.4.1. General morphology

The moulds were grown on potato dextrose agar plate at 28°C for 2 days. The size of the sporangiospores was measured with a standardized ocular micrometer.

3.3.4.4.2. Hydrolysis of starch

Starch agar (2% w/v starch and 2% w/v agar) plates were streaked with the mould isolates and incubated at 28°C for 3 days. The plates were flooded with Burke's iodine solution and observed for any formation of a clear zone around and underneath the growth, indicating the presence of amylase.

3.3.4.5. Identification of isolates

Bacteria were identified following the taxonomic keys of Starr *et al.* (1981) and Sneath *et al.* (1986). Yeasts were identified

according to the criteria laid down by Barnett *et al.* (1983) and Kreger-van Rij (1984). Moulds were identified according to Zycha ans Siepmann (1969) and Hesseltine and Ellis (1973).

3.3.5. Optimization of traditional process parameters

Various process parameters involved in the production of fermented foods were considered for optimization. The sensory attributes of the laboratory-made samples were evaluated immediately after sampling by a panel of seven trained judges. The formats of the score cards (Tables 4-6), specially prepared for the purpose, were based on the score card prepared by Patil and Gupta (1986).

3.3.6. Microbial and biochemical changes accompanying fermentation

The selected fermented foods were prepared in the laboratory under optimized conditions and studied for succession of the microorganisms and selected biochemical parameters.

3.3.7. Testing each of the isolates or combinations thereof for producing kinema

Bacillus , *Enterococcus* and yeast inocula were prepared by introducing approximately 5 ml sterile distilled water onto 24 h-old (37°C) nutrient agar, 48 h-old (37°C) MRS agar and 48 h-old (28°C) yeast extract-malt extract agar slant cultures, respectively, isolated previously from the fermenting materials. The tubes were agitated for 30 s with a cyclomixer (Remi, India). The number of cells in the suspension was determined using a Neubauer's counting chamber and a phase contrast microscope. The suspensions were used

Table 4. Format of sensory score card for kinema

Name .... Date .... Time ....

Please rate these samples for quality attributes according to the following grade descriptions and scoring:

Attributes	Defects	Slight	Distinct	Pronounced	Sample No. A	B	C
Flavour (smell), 50	Flat	39	35	33			
Normal range: 38-47	Rotten	35	33	31			
	Raw beany	34	28	26			
Body and texture, 45	Dry	37	34	32			
Normal range: 38-44	Watery	35	30	20			
Colour, 5 Normal range: 4-5	Whitish	3	2	1			
TOTAL SCORE:							

Grading of kinema:

Total score	Grade
92-100	Excellent
82- 91	Good
72- 81	Fair
62- 71	Poor
< 61	Bad

....
(Signature of the judge)

Requirements of high grade kinema:

Flavour: Nutty with ammoniacal odour

Body and texture: Highly sticky or mucilaginous and slightly pasty

Colour: Brown

Table 5. Format of sensory score card for sinki

Name .... Date .... Time ....

Please rate these samples for quality attributes according to the following grade descriptions and scoring:

Attributes	Defects	Slight	Distinct	Pronounced	Sample No.		
					A	B	C
Taste, 60	Bitter	47	44	38			
Normal range: 50-57							
Flavour (smell), 35	Flat	27	26	22			
Normal range: 28-34	Raw radish	25	23	21			
	Rotten	20	18	15			
Colour, 5	Yellowish	3	2	1			
Normal range: 4-5							
TOTAL SCORE:							

Grading of sinki:

Total score	Grade
92-100	Excellent
82- 91	Good
72- 81	Fair
62- 71	Poor
< 61	Bad

....
(Signature of the judge)

Requirement of high grade sinki:

Taste : Soury

Flavour: Acidic (typical sinki flavour)

Colour : White, when fresh

Table 6. Format of sensory score card for mesu

Name .... Date .... Time ....

Please rate these samples for quality attributes according to the following grade descriptions and scoring:

Attributes	Defects	Slight	Distinct	Pronounced	Sample No. A	B	C
Taste, 60	Bitter	47	44	38			
Normal range: 50-57							
Flavour (smell), 35	Flat	27	26	22			
Normal range: 28-34	Rotten	25	23	21			
Colour, 5	Yellowish	3	2	1			
Normal range: 4-5							
TOTAL SCORE:							

Grading of mesu:

Total score	Grade
92-100	Excellent
82- 91	Good
72- 81	Fair
62- 71	Poor
< 61	Bad

....
(Signature of the judge)

Requirements of high grade mesu:

Taste : Soury

Flavour : Acidic (typical mesu flavour)

Colour : White

as inocula, so that the concentration of *Bacillus* cells and spores, *Enterococcus* cells and yeast cells became 10^5-10^6, 10^4-10^5 and 10^2-10^3, respectively per g of soaked and sterilized (121°C for 15 min) beans kept in a 300 ml glass jar with cotton plug. The rationale behind selecting the inoculum load was based on the observation on the initial microbial load in soya beans during kinema production.

The products were evaluated organoleptically using the method as described in Section 3.3.5.

3.3.8. Growth of the *Bacillus* isolate at different oxygen levels

Three sets of nutrient agar plates which had been dried at 37°C for 24 h after preparation were streaked and three sets of nutrient broth in test tubes were inoculated with *Bacillus subtilis* DK-W1. One of the three sets was incubated semi-anaerobically in a candle jar and the third anaerobically with CO_2, H_2 and N_2 in the proportion of 10 : 10 : 80, all at 37°C. Average sizes of the colonies developed under each oxygen tension condition were recorded. Counts of the organisms in the incubated nutrient broth in test tubes were determined after 24 h using pour plate method (Ogbadu and Okagbue 1988).

3.3.9. Pure culture fermentation for kinema production

The proven producing strain (DK-W1) of *Bacillus subtilis* was grown on nutrient agar slant for 24 h. A cell-spore suspension prepared at a concentration of 10^8-10^9/ml was used as an inoculum. Two hundred g of 8 h-soaked soya beans were sterilized at 121°C for 15 min and mixed with 0.5 ml of the inoculum suspension (Sakurai

1960). The inoculated beans were allowed to ferment at 45°C for determining the optimum period of fermentation. The samples were evaluated organoleptically following the method as described in Section 3.3.5.

3.3.10. Microbial and biochemical changes during fermentation of sterilized soya beans inoculated with *Bacillus subtilis* DK-W1

Two hundred g of 8 h-soaked soya beans were sterilized at 121°C for 15 min, and incubated with 24 h-old *Bacillus subtilis* DK-W1 culture at a load of 10^4-10^5 cells-spores/g fresh weight. The inoculated beans in a glass jar with cotton plug were allowed to incubate at 45°C till 18 h. Sampling was made at certain intervals for analysis.

3.3.11. Statistical analysis

The data obtained were analyzed statistically by determining analysis of variance using the least square design technique (Snedecor and Cochran 1967).