3
Materials and Methods

3.1. Materials

3.1.1. Culture media

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 form carbohydrates (Gordon *et al.*, 1973)

$(NH_4)_2HPO_4$	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	

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	

Salt solution

$MgSO_4.7H_2O$	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^{-1}) 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

***Lactobacillus* MRS agar**
(HiMedia M641)

***Lactobacillus* MRS broth**
(HiMedia M369)

Malt extract
(HiMedia RM004)

Malt extract agar (Kreger-van Rij, 1984)

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

pH 5.4

Nitrate broth
(HiMedia M439)

Nutrient agar
(HiMedia M561)

Nutrient broth
(HiMedia M002)

Plate count agar
(HiMedia M019)

Potato dextrose agar
(HiMedia M096)

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 (1.6% w v^{-1} in ethanol)	1.0 ml
Distilled water	1000 ml
pH 6.8	

Tributyrin agar
(HiMedia M157, FD081)

Tryptone-glucose-yeast extract broth
(HiMedia M592)

Urea agar
(HiMedia M112, FD048)

Yeast carbon base
(HiMedia M141)

Yeast extract-malt extract agar
(HiMedia M424)

Yeast nitrogen base
(HiMedia M139)

All media mentioned above were sterilized by autoclaving for 15 min, unless mentioned otherwise.

3.1.2. Reagents

Acrylamide solution

Acrylamide (SRL 014022)	300.0 g
N,N,-methylene bisacrylamide (SRL 134985)	8.0 g
Deionized water	1000 ml

Burke's iodine solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Coomassie brilliant blue solution

Coomassie brilliant blue R250 (SRL 024018)	0.4 g
Methanol (SRL 132977)	90 ml
Glacial acetic acid (Merck 60006325001046)	20 ml
Deionized water	90 ml

Crystal violet stain (Bartholomew, 1962)

Crystal violet	2.0 g
95% (v v^{-1}) ethanol	20.0 ml
Ammonium oxalate	80.0 ml
(1.0% w v^{-1} aqueous solution)	

Destaining solution

Methanol	40 ml
Glacial acetic acid	10 ml
Deionized water	50 ml

Ehrlich-Böhme reagent (Iswaran, 1980)

p-Dimethylaminobenzaldehyde	1.0 g
95% (v v^{-1}) ethanol	95.0 ml
Conc. HCl	20.0 ml

Folin-Ciocalteu reagent

(SRL 62015)

Peptone physiological saline (Nout *et al.*, 1998)

Peptone	1.0 g
NaCl	8.5 g
Distilled water	1000 ml
pH 7.0	

Phenolphthalein

Phenolphthalein	0.1 g
Ethanol (95% v v^{-1})	100 ml

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

Solution A:	Sulphanilic acid	0.8 g
	5 N Acetic acid	100 ml
	(Glacial acetic acid: water, 1:2.5)	
Solution B:	α-Napthylamine	0.5 g
	5 N Acetic acid	100 ml

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

Resolving gel buffer

Tris (hydroxymethyl) aminomethane (Tris-HCl; SRL 204991)	3.0 M
pH 8.9	

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

2.5% (w v^{-1}) safranin in 95% (v v^{-1}) ethanol	10.0 ml
Distilled water	100 ml

Sample buffer 2X

Sodium lauryl/dodecyl sulphate (SDS; SRL 1948101, 10% w v^{-1})	4.0 ml
Stacking gel buffer	2.5 ml
Glycerol (SRL 072483)	1.5 ml
Bromophenol blue (SRL 0240168)	10.0 mg
β-mercaptoethanol (SRL 1327198)	2.0 ml

Stacking gel buffer

Tris-HCl	0.5 M
pH 6.8	

Tank buffer

Tris-HCl	0.025 M
Glycine (Merck India 4201)	0.129 M
SDS	10% (w v^{-1})
pH 8.3	

3.2. Experimental

3.2.1. Survey

A moderate survey was conducted in different places of India including the States of Assam, Bihar, Karnataka, Meghalaya, Orissa, Sikkim, Tamil Nadu and West Bengal on the detailed information about the different types, traditional methods of preparation, modes of consumption, ethnic value (if any) and distribution of various legume-based indigenous fermented foods used by the local people. A format of survey sheet (Table 2) was prepared exclusively for this survey.

3.2.2. Sampling

Approx. 250 g of fermented batter, dough and freshly prepared products were collected aseptically in sterile Nasco sample bags (HiMedia PW389) or screw-capped glass bottles from different food outlets. The raw ingredients, used for the preparation of some selected legume-based fermented foods, were procured from local markets. Those were kept in an ice-box, transported immediately to the laboratory and analysed as early as possible.

3.2.3. Analysis of proximate composition

3.2.3.1. Moisture content

Approx. 10 g sample was accurately weighed using a Sartorius (Göttingen, Germany) model CP224S balance into a cooled and weighed Petri dish, previously heated to 105± 1°C. The sample was uncovered and allowed to dry for 48-72 h at 105±1°C in a hot air oven. The dish was covered while still in oven, transferred to a desiccator and weighed soon after reaching room temperature. The process of drying,

Table 2. Format of survey on consumption of legume-based traditional fermented foods

State: District: Tehsil /Town: Village: Approx. population:

Name of informant: Status of informant: Age: Date of survey:

Sl No.	Name of product	Nature of product	Mode of consumption	Taste	Reason for consuming	Daily/ weekly/ monthly/ occasional	Shelf life	Household preparation /market purchase	If suitable for ailing/pre- or post-natal women/children	Market price	Source of the preparation procedure	Ethnic value, if any

cooling and weighing were repeated until two successive weighings reached a constant value. Moisture content was calculated by subtracting the final weight from the initial weight (AOAC, 1990; Roy *et al.*, 2007).

3.2.3.2. 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 at 600°C. The sample was held at that temperature for 2 h. The crucible was transferred directly to a desiccator, 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 <1 mg. The lowest mass was recorded (IS: 5162, 1980).

3.2.3.3. pH

Approx. 10 g sample was mixed with 20-30 ml of CO_2-free distilled water in a warring blender (Bajaj, India) for 1 min. The temperature of the slurry prepared was equilibrated to 25°C, and the pH was noted (AOAC, 1990) using a pH meter (model 335, Systronics, Naroda, India) calibrated with the standard buffer solutions (HiMedia BC004, BC007) (Roy *et al.*, 2007).

3.2.3.4. Titratable acidity

A well-mixed sample (10 g) was blended with 90 ml CO_2-free distilled water for 1 min. The mixture was filtered, and 25 ml of the filtrate was titrated with 0.1 N aqueous solution of NaOH to an end point of phenolphthalein (AOAC, 1990).

$$\% \text{ titratable acidity (as lactic acid)} = \frac{100 \times \text{ml of NaOH} \times \text{N of NaOH} \times 0.09}{\text{Weight of sample (g)}}$$

3.2.3.5. Free fatty acidity

An accurately weighed (*ca* 5 g) sample was dissolved in 25 ml of 95% (v v^{-1}) ethanol neutralized previously by 0.1 N aqueous solution of NaOH, using phenolphthalein. After mixing, the solution was heated to boiling on water-bath. The mixture was titrated with 0.1 N aqueous solution of NaOH until a faint pink colour persisted (Sarkar and Tamang, 1995; Shieh *et al.*, 1982).

$$\% \text{ fatty acid content} = \frac{100 \text{ x ml of NaOH x N of NaOH x } 0.192}{\text{Weight of sample (g)}}$$

3.2.3.6. Total nitrogen

The method (microKjeldahl method) as described in AOAC (1990) was followed. Approx. 1.0 g accurately weighed sample, taken in a digestion flask, was added with 0.7 g of HgO, 15 g powdered K_2SO_4 and 25 ml conc. H_2SO_4. The flask was heated gently until frothing ceased, boiled briskly until the solution became cleared and then continued the boiling for about 1 h. The solution was transferred quantitatively to a round bottom flask, and mixed with 100 ml distilled water and 25 ml 40 g Na_2S l^{-1} to precipitate mercury. A pinch of zinc granules to prevent bumping and a layer of 40% (w v^{-1}) aqueous

solution of 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^{-1} 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 reagent was considered for correction.

$$\%\text{nitrogen} = \frac{[(\text{ml of } H_2SO_4 \times \text{N of } H_2SO_4) - (\text{ml of NaOH} \times \text{N of NaOH})] \times 1.4007}{\text{Weight of sample (g)}}$$

3.2.3.7. Protein and nonprotein nitrogen

A sample (0.5 g) was mixed with 30 ml of 100 g cold trichloroacetic acid (SRL 204842) l^{-1} 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 (nonprotein nitrogen) and the precipitate (protein nitrogen) (Nirenberg and Matthaei, 1961) were measured by the microKjeldahl method as described in section 3.2.3.6.

3.2.3.8. 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 microKjeldahl method as described in section 3.2.3.6.

3.2.3.9. Total protein

Total protein contents in the sample were determined by multiplying total nitrogen, estimated using microKjeldahl method by 5.95 for rice (STFCJ, 2000), 5.7 for the soybean products and 6.25 for the others (AOAC, 1990).

3.2.3.10. Crude fat

Crude fat content of the sample was determined by ether extraction method using Soxhlet apparatus (AOAC, 1990). One round bottom flask was oven dried and kept in the desiccator. Accurately weighed (*ca* 2.0 g) dried sample was transferred to the dry and fat-free absorbent cotton which was placed into the fat extraction tube of the Soxhlet apparatus. Crude fat was extracted by using petroleum ether (Merck 61784925001046); b.p., 40-60°C) on a heating mantle at 60°C for 4-5 h. A pinch of carborandum powder was added to prevent bumping. At the end of extraction, the flask was kept on a steam-bath at low heat to evaporate the ether. The flask was then dried for 1 h at 100°C in a hot air oven, cooled in a desiccator and weighed. Crude fat was calculated in percentage.

$$\%\,\text{crude fat} = \frac{\text{Weight of ether-soluble material} \times 100}{\text{Weight of sample(g)}}$$

3.2.3.11. Carbohydrate

The carbohydrate content was calculated by difference (Standal, 1963):

$$\%\text{carbohydrate} = 100 - (\%\text{protein} + \%\text{fat} + \%\text{ash})$$

3.2.3.12. Energy value

The Energy value of the sample was estimated 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 (100 g)$^{-1}$ (4184 kcal = 1 Joule) dry matter (Gopalan *et al.*, 1995)

3.2.4. Microbial analysis

3.2.4.1. Isolation and maintenance

A representative 10 g of sample was suitably homogenized with 90 ml of sterile peptone physiological saline using a Stomacher lab-blender (Seward Medical, London, UK) at 'normal' speed for 1 min. Serial decimal dilutions were made with the same diluent. Appropriate dilutions were used in duplicate for the isolation of microbiota by pouring or surface seeding. Total aerobic mesophilic bacteria were enumerated by pour-plating using plate count agar and incubated at 35°C for 18-24 h. In order to estimate aerobic mesophilic bacterial spores, 10% (v v^{-1}) sample suspension was heated at 80°C for 30 min, suitably diluted and spread on plate count agar plates followed by incubation at 30°C for 72 h (Banerjee and Sarkar, 2003). Yeasts and moulds were isolated on yeast-extract malt-extract agar and potato dextrose agar, respectively, supplemented with benzylpenicillin (10 IU ml^{-1}) and streptomycin sulphate (12 µg ml^{-1}), and incubated at 28 °C for 48 h. Lactic acid bacteria were enumerated in pour plates of MRS *Lactobacillus* agar, incubated at 37°C for 48-72 h in an anaerobic jar with AnaeroHiGas pack (HiMedia LE002A). Usually, the plates containing 50-300 colonies were selected for enumeration. The colonies appeared were counted as colony forming units (cfu) g^{-1} fresh weight sample. Five colonies of each morphotype appearing from each sample were picked up randomly and the purity of the isolates was checked by streaking them on fresh agar plates of the isolation media followed by microscopic examination. The purified colonies were grown on suitable slants or in broth. Homogeneity of the isolates under each morphotype was checked using a few selected parameters. One of the five isolates of each morphotype from each of the positive samples was tagged randomly as a representative strain and stored at 4°C by subculturing after every two months (15-20 days for lactic acid bacteria). All the representative strains of different groups of isolates were deposited in the Culture Collection of the Microbiology Laboratory of the Department of Botany, University of North Bengal.

3.2.4.2. Taxonomical studies on bacterial isolates

3.2.4.2.1. Gram staining

The method of Bartholomew (1962) was followed. A suspension of 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 spread to smear. It was heat-fixed, flooded with 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% (v v^{-1}) 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 slide was air-dried and observed under an oil-immersion objective.

3.2.4.2.2. General morphology

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

3.2.4.2.3. 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 (model BH2-PC-PA-1, Olympus, Tokyo, Japan).

3.2.4.2.4. Production of catalase

A 24 h-old slant culture was flooded with 0.5 ml of 100 ml H_2O_2 l^{-1} solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.*, 1981).

3.2.4.2.5. Growth in sodium chloride

Tubes containing 5 ml of tryptone-glucose-yeast extract broth (HiMedia M592) supplemented with 40, 65 and 180 g of NaCl l^{-1} medium were inoculated each with a loopful of culture and incubated at 30°C for 7 days (Norris *et al.*, 1981).

3.2.4.2.6. Acid and gas from carbohydrates

Tubes containing 10 ml sugar basal broth supplemented with 20 g sugar l^{-1} medium with inverted Durham tubes were inoculated and incubated at 30°C for 10 days. Any changes in the colour of basal broth and accumulation of gas in the inverts indicated positive result (Okada *et al.*, 1986; Pederson and Albury, 1950)

3.2.4.2.7. Production of indole

Cells were grown at 30°C in 10 ml Davis and Mingioli's broth (Davis and Mingioli, 1950), prepared by replacing $(NH_4)_2SO_4$ with 1.0% (w v^{-1}) L-tryptophan and supplementing with yeast extract (0.02%, w v^{-1}). 1-2 ml of Ehrlich-Böhme reagent was gently 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).

3.2.4.2.8. Reduction of nitrate

Cultures (24 h-old) 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 the nitrate reduction test and observed for the development of a red or yellow colour indicating the presence of nitrite. A small amount of zinc dust was added to the tube that was negative even after 14 days of incubation and observed for the development of red colour indicating the presence of nitrate i.e., absence of reduction (Norris *et al.*, 1981).

3.2.4.2.9. Hydrolysis of gelatin

The streaked MRS agar plates containing 10 g gelatin l^{-1} medium were incubated at 30°C for 3 and 5 days. Plates were flooded with 10 ml 1 N H_2SO_4 saturated with $(NH_4)_2SO_4$. Hydrolysis was indicated by clear zone, under and around the growth, in contrast to the opaque precipitation of unchanged gelatin (Sneath and Collins, 1974).

3.2.4.2.10. 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).

3.2.4.2.11. Hydrolysis of fat

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

3.2.4.2.12. 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).

3.2.4.2.13. Growth at different pH

The pH of MRS broth was adjusted to different levels using 1 N HCl or 100 g NaOH l^{-1} solution. The medium was distributed into tubes containing 10 ml in each. Those were autoclaved, cooled to room temperature and inoculated with 0.2 ml of 48 h-old MRS broth cultures. The tubes were incubated at 30°C for 24 h and observed for any growth (Hesseltine and Ray, 1988).

3.2.4.2.14. Growth at different temperatures

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.

3.2.4.3 Taxonomical studies on yeast isolates

3.2.4.3.1. General morphology

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

Malt extract and glucose-yeast extract-peptone water (30 ml each) containing in separate Erlenmeyer flasks (100 ml) were inoculated with 24 h-old yeast cultures. They were incubated at 28°C

for 3 days and observed for cellular morphology and mode of vegetative reproduction (Kreger-van Rij, 1984). The dimension of cells was measured with a standardized ocular micrometer.

3.2.4.3.2. Formation of pseudomycelium and true mycelium

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

3.2.4.3.3. Characteristics of asci and ascospores

A yeast extract-malt extract agar or yeast malt agar (presporulation medium) slant was streaked with a 24 h-old culture of the yeast isolate 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 5.0 g malachite green l^{-1} solution, heated to 3-4 times steaming over the spirit lamp, washed for 30 s and counterstained with 5.0 g safranin l^{-1} solution for 30 s (Wickerham, 1951).

3.2.4.3.4. Reduction of nitrate

The method was the same as described in section 3.2.4.2.9, excepting that the temperature of incubation was 28°C.

3.2.4.3.5. Hydrolysis of urea

Christensen's urea agar plates were streaked with 24 h-old yeast cultures, incubated at 28°C for 3 days and observed for any change in colour of medium (Christensen, 1946).

3.2.4.3.6. Hydrolysis of fat

The method was the same as described in section 3.2.4.2.12, excepting that the plates were incubated at 28°C.

3.2.4.3.7. Growth at 37°C

Yeast extract-malt extract agar slants were inoculated with 24 h-old yeasts and incubated at 37°C for 48 h (Kreger-van Rij, 1984).

3.2.4.3.8. Fermentation of carbohydrates

The method was based on Kreger-van Rij (1984). Cells were grown at 30°C for 18 h on malt extract agar plates or yeast extract-malt extract agar plates overlaid with cellophane. The growth was harvested in 2.5 ml of 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 2.0% (w v^{-1}) filter-sterilized sugars containing Durham tube 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.

3.2.4.3.9. Assimilation of carbon compounds

The method was based on Wickerham and Burton (1945) and Wickerham (1951). The tests were done in rimless tubes each containing 5 ml of filter-sterilized yeast nitrogen base added with 2.0% (w v^{-1}) of test substrate, i.e. carbon compound. The cells were grown at 28°C for 24-48 h on yeast malt agar plates. Growth was harvested in about 3 ml of filter-sterilized yeast nitrogen base, taking care to avoid carrying over any of the nutrient medium. A white card with black lines of approx. 0.75 mm in width drawn on it was held behind the tube. The suspension was diluted aseptically until the lines become visible through the tubes as dark bands. Each of the tubes containing the test media was inoculated with 0.1 ml of the above-mentioned suspension and incubated at 28°C, usually up to 21 days. The degree of growth was assessed visually by placing the tubes, after they have been shaken well to thoroughly disperse the cells, against a white card with black lines.

3.2.4.3.10. Assimilation of nitrogen compounds

A 0.1-ml aliquot of yeast cell suspension was used to inoculate the tube containing 5 ml filter-sterilized yeast carbon base supplemented with required amount of the nitrogen sources to be tested. The tubes were then incubated at 28°C for 3-7 days and the results were read in the same way as descried in section 3.2.4.3.9.

3.2.4.3.11. Growth in vitamin-free medium

Tubes of 5 ml vitamin-free medium were inoculated with the cells from young and actively growing cultures and incubated at 28°C for 2-3 days with regular shaking.

3.2.4.3.12. Formation of extracellular amyloid compounds (starch formation)

Ammonium sulphate-glucose agar plates were inoculated with actively growing (24 h-old) yeast cultures and incubated at 28°C for 1-2 weeks. The plates were flooded with Lugol's iodine solution and inspected for formation of any blue to green colouration.

3.2.4.4. Taxonomical studies on mould isolates

3.2.4.4.1. General morphology

The moulds were grown on potato dextrose agar plates at 28°C for 48 h, and the general morphology of the mycelium and reproductive structures were studied.

3.2.4.4.2. Hydrolysis of starch

Starch agar (20 g starch and 20 agar l^{-1}) plates were streaked with the mould isolates and incubated at 28°C for 2-4 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.

3.2.4.5. Identification of isolates

All the representative strains of the bacterial isolates 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 Kurtzman and Fell (2000) and Barnett *et al.* (2000). Moulds were identified according to the criteria laid down by Samson and van Reenen-Hoekstra (1988) and Hesseltine (1983)

3.2.5. Optimization of process parameter

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 score cards (Tables 3 and 4), specially prepared for the purpose, were based on the score card prepared by Patil and Gupta (1986).

3.2.6. Microbial and biochemical changes accompanying fermentation

The succession of microbial and biochemical changes during fermentation of some selected fermented foods was studied following their traditional methods of preparation in the laboratory. While dhokla and wadi were studied under semicontrolled conditions, idli and papad preparations were optimized before this study.

For the preparation of dhokla, Bengalgram dal and white polished rice (4:1 proportion) were cleaned, washed and allowed to soak separately in excess of tap water at room temperature (~25°C) for 10 h. Excess water was decanted. While soaked dal was ground to a smooth paste, rice was coarsely ground using a waring blender (Bajaj, India). The slurries were mixed thoroughly along with common salt (8 g kg^{-1}). The thick batter, in 100 ml aliquots, was dispensed into presterilized 250 ml beakers, the mouths of which were then wrapped with aluminium foil. The batches of batter were incubated at 32°C for 15 h. Studies on microbial and biochemical changes were carried out at every 3 h-interval within a range of 0-15 h. The fermented batter was dispensed in greased cups (7 cm in diameter having holding capacity of 40 ml) and steamed for 15 min to prepare dhokla.

Employing the traditional techniques, wadi was prepared in laboratory under semicontrolled conditions. Blackgram dal was washed and allowed to soak in excess of tap water at 25°C for 10 h. The soaked dal was ground to a thick, smooth and mucilaginous paste. The thick batter was incubated in a sterile glass beaker (500 ml), the mouth of which was then wrapped with aluminium foil, at 32°C for 10 h. The fermented batter was manually beaten continuously for 30 min, and hand-moulded to small cones (3-5 cm in diameter) and deposited on a greased bamboo mat and sun-dried (29-33°C) for 8 h followed by 16 h shade-drying at room temperature (28-30°C) daily for three successive days. Studies on microbial and biochemical changes of batter was carried out after 10 h of fermentation while drying wadis were analysed at every 12 h-interval within a range of 0-60 h.

Traditional process parameters for the preparation of idli were optimized with the ingredients ratios, fermented temperature and period. Blackgram dal and parboiled rice were cleaned, washed

Table 3. Format of sensory score card for idli

Name: Date: Time:

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

Attribute	Defect	Intensity			Sample No.		
		Slight	Distinct	Pronounced	A	B	C
Taste, 35	Bland	23	20	17			
Normal range: 28-34	Rancid	19	17	16			
Flavour (smell), 30	Starchy	20	18	15			
Normal range: 22-28	Beany	18	16	13			
Body and texture, 30	Hard	20	17	15			
Normal range: 25-28	Sticky	18	15	14			
Colour, 5	Yellowish	3	2	1			
Normal range: 4-5							
Total score, 100							

(Signature of the judge)

Grading of idli:

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

Requirement of high-grade idli:
Taste: sour
Flavour: acidic
Body and texture: spongy (honeycomb inside)
Colour: white

and allowed to soak separately in excess of tap water at room temperature (~25°C) for 10 h. Excess water was decanted. While soaked dal was ground to a smooth paste, rice was coarsely ground using a waring blender. The slurries were mixed thoroughly along with common salt (8 g kg^{-1}). The thick batter, in 100 ml aliquots, was dispensed into presterilized 250 ml beakers, the mouths of which were then wrapped with aluminium foil. The batches of batter were incubated at 30°C for 18 h. Studies on microbial and biochemical changes were carried out at every 3 h-interval within a range of 0-18 h. The fermented batter was dispensed in the idli pan (7 cm in diameter having holding capacity of 40 ml) and steamed for 15 min to prepare idli.

Traditional process parameters for the preparation of papad were optimized for the ratio of ingredients, fermentation period and drying period of papad sheets under laboratory conditions.Properly mixed ingredients were hand-kneaded and pounded with a cylindrical stone pestle to attain homogenous and stiff lump of dough and left at 30°C for 3 h. The fermented dough was drawn into a rope and manually made into small balls (4-5 g each) between the palms, and rolled over 'bellani' (wooden roller) into circular flat sheets (9-10 cm in diameter and 0.7-1.0 mm in thickness). Circular papad discs were dried under controlled conditions (70±5% relative humidity and 30±1°C) to final moisture content of 14-16%. Dried papad was fried in refined groundnut oil. Studies on microbial and biochemical changes of papad dough were carried out after 3 h of fermentation, and the drying papad sheets were analysed at every 2 h-interval within a range of 0-8 h.

Table 4. Format of sensory score card for papad

Name: Date: Time:

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

Attribute	Defect	Intensity			Sample No.		
		Slight	Distinct	Pronounced	A	B	C
Hand feel, 25	Hard	17	14	<11			
Normal range: 20-23	Non cohesive	18	13	<10			
Rolling, 25	Tough with cracked edges	15	12	<10			
Normal range: 18-22	Sticky	16	13	<9			
Colour, 15	Dull	9	7	<5			
Normal range: 10-14	Yellowish brown	8	7	<5			
Texture, 20	Hard	12	10	<9			
Normal range: 14-18	Sticky	13	11	<11			
Taste, 15	Bland and floury	8	6	<5			
Normal range: 10-13	Salty	7	5	<5			
Total score, 100							

(Signature of the judge)

Grading of papad:

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

Requirement of high-grade papad:
- Hand feel (dough): soft
- Rolling (dough): smooth and easy
- Texture (papad): crisp and brittle
- Appearance/colour (papad): bright and light yellowish

3.2.7. SDS-PAGE

SDS-PAGE was carried out following the method described by Laemmli (1970) in a vertical gel electrophoresis system (cat. no. 05-03, Genei, Bangalore, India). A 10% (w v^{-1}) solution of running gelwas prepared by taking appropriate volume of acrylamide solution, resolving gel buffer, 10% (w v^{-1}) SDS solution and deionized water. Excess N,N,N′,N′-tetramethyl ethylenediamine (TEMED; SRL 202788) and a pinch of ammonium persulphate (APS; SRL 0148134) were added to set the gel suitably. When the resolving gel set, 4% (w v^{-1}) of stacking gel prepared by taking appropriate volume of solution A, stacking gel buffer, aqueous solution of 10% SDS, deionized water, TEMED and APS, was poured over the resolving gel.

Samples were prepared by taking four loopful of ingredient slurry or fermenting batter, and washing it thrice with 0.2 M phosphate buffer-saline, pH 7.0, by centrifugation at 3000g for 15 min each. The pellet was suspended in stacking gel buffer and boiled in a boiling water-bath for 10 min after adding equal volume of 2x sample buffer. A discontinuous buffer system was used. Samples were stacked at constant current of 15 mA and resolved at 25 mA until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was fixed in 10% glacial acetic acid for 30 min, stained with coomassie brilliant blue solution for 12 h and washed in a destaining solution until the protein bands became clearly visible in a colourless gel matrix.

3.2.8. Preparation of different food samples for the antioxidant activity assay

Dohkla, idli and kinema were selected for the evaluation of antioxidant activities during their different stages of preparation.

3.2.8.1. Dhokla

Employing the traditional process parameters, dhokla was prepared at laboratory under semicontrolled conditions. A portion of nonfermented and fermented dhokla batters, used for successional study of the dominant microbiota and their proximate composition, and the corresponding steam-cooked dhokla were assayed for their antioxidant activities.

3.2.8.2. Idli

Following the traditional techniques, the preparation process of idli was optimized at laboratory under semicontrolled conditions. A portion of nonfermented and fermented batters, used for successional study of the dominant microbiota and their proximate composition, and the corresponding steam-cooked idli were assayed for their antioxidant activities

3.2.8.3. Kinema

Although there are slight variations in the traditional method of kinema-making, the optimized process (Sarkar and Tamang, 1994) was followed in this study. *Bacillus subtilis* DK-W1 (MTCC 1747), used for the preparation of kinema, was maintained in the laboratory on nutrient agar slants. For inoculum preparation, the activated culture was streaked onto a nutrient agar slant and incubated at 37°C for 16 h. The cells were harvested in sterile distilled water, and after adjusting to a concentration of 10^7-10^8 total cells ml^{-1}, the suspension was used to inoculate cooked soybeans for fermentation.

Soybeans of 'local yellow' cultivar were purchased from Kalimpong market in the district of Darjeeling. Approximately 250 g of the beans were washed thoroughly under tap water and then with distilled water. Those were soaked in 1 l distilled water at 25°C for 16 h. after decanting the water, 100 g of the soaked beans were put into each of several 500 ml-capacity screw-capped bottles and 250 ml of distilled water was added. Those were autoclaved at 0.7 kg cm^{-2} for 10 min, cooled to about 50°C, and the water was decanted off. The beans from all the bottles were transferred into a sterile polyethylene bag and pestled from outside the bag in a mortar, so that about, two-thirds of the beans were dehulled and the cotyledons separated and crushed to give grits of mainly half-cotyledons. The beans were distributed into two portions; one was used for kinema preparation, while the other was dried directly for the extraction process.

A 2 ml-suspension of the organism was mixed with the cooked beans to make a load of 10^5-10^6 total cells g^{-1}. The inoculated beans were then distributed in approximately 100 g amounts to sterile glass bottles (500 ml) plugged with cotton wool and incubated at 37°C and 75± 5% relative humidity for 48 h to produce kinema (Sarkar *et al.*, 1993).

3.2.9. Preparation of methanolic extracts of different food samples

Samples of nonfermented and fermented substrates and fermented steam-cooked products were dried separately for 24 h at 60°C in a hot air oven and pulverized to powder using mortar and pestle. The

ground powder was extracted with 10 vol. of methanol for 5 h at room temperature with continuous stirring. The extraction process was repeated thrice, and the extracts were pooled together and filtered through Whatman no. 1 paper. The filtrates were concentrated at 40°C under vacuum and freeze-dried (model FDU-506, Eyela freeze dryer, Japan). The lyophilized extracts were stored in a desiccator at 4°C. Prior to use, the lyophilized extract was dissolved in methanol

3.2.10. Assay of antioxidant activities of crude methanolic extracts

3.2.10.1. Total phenol content

Total soluble phenolics in the extracts were assessed using the method described by Yen and Hsieh (1998). A 0.1-ml aliquot of lyophilized extract solution (10-50 mg ml^{-1} methanol) was added to 2 ml aqueous solution of 20 g sodium carbonate (HiMedia RM861) l^{-1}. After 2 min, 0.1 ml of 1 N Folin-Ciocalteu reagent (SRL 62015) was added to the mixture and the absorbance was read after 30 min at 750 nm. The concentration of the total phenolics was expressed as gallic acid equivalents, GAE (mg gallic acid g^{-1} extract), using the standard curve of gallic acid (HiMedia RM 233).

3.2.10.2. Free radical-scavenging activity

The antioxidant activity of the extracts was measured in terms of hydrogen-donating or radical-scavenging ability using the DPPH· method (Sanchez-Moreno *et al.*, 1998). A 0.1 ml methanolic solution of the lyophilized extract (10-50 mg ml^{-1}) was added to 2.9 ml of 60 mM methanolic solution of DPPH· (HiMedia RM2798). The mixture was shaken immediately and allowed to stand at room temperature in dark. The decrease in absorbance at 517 nm was measured using a Systronics spectrophotometer, at 10 min-interval up to 60 min till a plateau was reached.

The scavenging percentage of DPPH·, as calculated according to Shyu and Hwang (2002), is as follows:

$$\%\text{scavenging} = \frac{A_0 - (A - A_b)}{A_0} \times 100$$

where A_0 was the A_{517} of DPPH· without sample (control), A was the A_{517} of sample and DPPH·, and A_b was the A_{517} of sample without DPPH· (blank).

3.2.10.3. Reducing power

The ability of the extracts to reduce Fe^{3+} was assessed according to the method of (Oyaizu, 1986). A 1.0-ml aliquot of lyophilized extract solution (10-50 mg ml^{-1} methanol) was mixed with 2.5 ml of 0.2 M phosphate buffer, pH 6.6 and 2.5 ml aqueous solution of 10 g potassium ferricyanide (HiMedia RM1034) l^{-1}. The mixture was incubated at 50°C for 20 min, added with 2.5 ml aqueous solution of 100 g trichloroacetic acid (SRL 204842) l^{-1} and centrifuged at 1200 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 1.0 g ferric chloride (SRL 64765) l^{-1}, and the absorbance was measured at 700 nm. The reducing power was expressed as ascorbic acid equivalents, ASE (mg ascorbic acid g^{-1} extract) using standard curve of ascorbic acid (SRL 0149100).

3.2.10.4. Metal-chelating activity

The Fe^{2+}-chealting ability by the extracts was carried out according to Carter (1971). The ability was monitored by measuring the formation of Fe^{2+}-ferrozine complex. A 200-ml aliquot of a methanolic

solution of the lyophilized extract (10-50 mg ml^{-1}) was added to 100 ml of 2.0 mM aqueous ferrous chloride (Merck 1.03861.0250) and 900 ml methanol. After incubation for 5 min, the reaction was initiated by adding 400 ml of 5.0 mM ferrozine (SRL 64956). The mixture was shaken and left at room temperature for 10 min to equilibrate. The absorbance of the resulting solution was recorded at 562 nm. A lower absorbance indicates a stronger Fe^{2+}-chelating ability which was calculated as follows:

$$\text{Chelating effect (\%)} = [(1 - A_s)/A_c] \times 100$$

where A_s was absorbance of the sample and A_c was that of the control.

3.2.10.5. Lipid peroxidation inhibitory activity

The total antioxidant activity of the lyophilized extracts was assessed by thiocyanate method of Duh *et al.* (1997). A 50-ml emulsion of linoleic acid was prepared by mixing and homogenizing 155 µl linoleic acid (HiMedia RM566) and 175 µg Tween 20 (HiMedia RM156) as emulsifier and 0.02 M potassium phosphate buffer, pH 7.0. The emulsion was mixed with different concentrations of the lyophilized extract solutions (10-50 mg ml^{-1} methanol) and incubated at 37°C. A 0.1-ml aliquot was withdrawn at every 24 h interval, and added sequentially with 5 ml of solution of 750 ml ethanol (Merck 1.00983.0511) l^{-1}, 0.1 ml of aqueous solution of 300 g ammonium thiocyanate (SRL 0149153) l^{-1} and 0.1 ml of 0.02 M ferrous chloride in 35 ml hydrochloric acid l^{-1} aqueous solution. The absorbance was measured at 500 nm. The lipid peroxidation inhibition activity (LPIA) in percent was calculated by the following equation:

$$\text{LPIA (\%)} = [(A_s/A_c)] \times 100$$

where A_s was the absorbance of the sample and Ac was that of the control.

3.2.11. Statistical analysis

The experimental results are expressed as means± SEM. Data were processed using Microsoft Excel 2002 and subjected to analysis of variance (ANOVA) after converting the microbial counts to a logarithmic (log_{10}) scale by using SPSS v. 12.0. For the assay and evaluation of antioxidant activities, the data were subjected to correlation and regression, t-test and ANOVA. Bivariate regression equations between total phenol content and different antioxidant parameters, and also coefficients of correlations among the different antioxidant parameters of the methanolic extracts at different concentrations were determined. P values of <0.05 were regarded as significant and of <0.01 as highly significant.