3. MATERIALS AND METHODS

3.1. CULTURE MEDIA

Anaerobic agar (HiMedia M228)

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	1 L
pH	7.2-7.4

Ascospore agar (HiMedia M804)

Basal medium (Gordon *et al.*, 1973)

Diammonium hydrogen phosphate	1.00 g
KCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
Yeast extract	0.2 g
Bromocresol purple	0.4 g
Distilled water	1 L
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
$(NH4)_2 SO_4$	1.0 g
Glucose (sterilized separately)	10.0 g
Distilled water	1 L
pH	7.0

Fermentation basal medium (Wickerham, 1951)

Yeast extract powder	4.5 g
Peptone	7.5 g
Demineralised water	1 L
Bromothymol blue	
(Till sufficiently dense green colour appears)	

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	1 L
pH	7.2

Malt extract agar (HiMedia M137)

MRS agar (HiMedia M 641)

MRS broth (HiMedia M 369)

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
Sodium acetate	5.0 g
$MgSO_4.7H_2O$	0.58 g
$MnSO_4.4H_2O$	0.28 g
Distilled water	1 L
pH	6.2-6.4

Milk agar (Gordon *et al.*, 1973)

Skim milk powder (HiMedia RM1254)	5 g in 50 ml distilled water
Agar	1 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 dry the surface of the agar.

Nitrate broth (Gordon *et al.*, 1973)

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	1.0 g
Distilled water	1L
pH	7.0

Plate Count agar (HiMedia M091A)

Potato Dextrose agar (HiMedia M096)

Starch agar (Gordon *et al.*, 1973)

(a) Starch (HiMedia RM089)	10% (w/v)
(b) Tryptone	5.0 g
Yeast extract	15.0 g
Potassium dihydrogen phosphate	3.0 g
Agar	20.0 g

[(a) and (b) were mixed and autoclaved after making the volume up to 1L]

Yeast extract-malt extract (YM) agar (HiMedia M424)

Yeast Morphology agar (HiMedia M138)

Yeast Nitrogen Base (HiMedia M139)

3.2. REAGENTS

Burke's iodine solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Crystal violet stain (HiMedia S012)

Ehrlich-Böhme reagent (Iswaran, 1980)

p-Dimethylaminobenzaldehyde	1.0 g
95 % ethanol	95 ml
Concentrated HCl	20 ml

Malachite green (1 % w/v) solution (HiMedia S020)

Nitrate reduction test reagent

Solution A

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

Solution B

∝-Napthylamine	0.5 g
5 N acetic acid	100 ml

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

Reagents for alcohol determination

N/5 $K_2Cr_2O_7$

$K_2Cr_2O_7$	9.807 g
Distilled water	1 L

8% KI

KI	8.0 g
Distilled water	92 ml

(Store in a brown bottle)

N/10 $Na_2S_2O_3$

$Na_2S_2O_3$	25.0 g
Distilled water	1 L

Reagents for reducing sugar and total sugar estimation

Reagent A

Anhydrous sodium carbonate	25.0 g
Sodium potassium tartarate	25.0 g
Sodium hydrogen carbonate	20.0 g
Anhydrous sodium sulphate	200.0 g

These are dissolved in 800 ml distilled water and diluted to 1 L.

Reagent B

$CuSO_4.5H_2O$	30.0 g
Distilled water with 4drops of conc.H_2SO_4	200 ml

Reagent C

(a) Ammonium molybdate	25.0 g
Distilled water with 21ml of conc. H_2SO_4	450 ml
(b) Disodium hydrogen arsenate heptahydrate	3.0 g
Distilled water	25 ml

Solution (b) was added to solution (a) slowly with stirring, then diluted to 500 ml, kept at 37° C to 40° C overnight and stored in a brown bottle.

Reagent D (Somogy copper solution)

25 ml of Reagent A was mixed with 1 ml of Reagent B. Freshly prepared Reagent D was used.

Reagents for phosphorus

Molybdovanadate reagent

Forty g of NH_4 molybdate.$4H_4O$ was dissolved in 400 ml of hot distilled water and allowed to cool. Two g of NH_4 metavanadate was dissolved in 250 ml of hot distilled water and cooled; 200 ml 70 % $HClO_4$ was added. Molybdate solution was gradually added to vanadate solution with stirring and diluted to 2 L.

Phosphate standard solution

A pure 1.9174 g of dried (105° C for 2 h) KH_2PO_4 was dissolved in 1 L distilled water. Freshly prepared solution was used.

Reagents for α-amylase assay

100 mM Tris - HCl buffer, pH 7.0

Dissolve 1.5% soluble starch in 100mM Tris (hydroxymethyl) aminomethane – HCl buffer, pH was adjusted to 7.0.

Stop solution

0.5 N Acetic acid – 0.5 N HCl (5:1)

Iodine solution

I_2 = 0.01%
KI = 0.1%

Reagent for glucoamylase assay

100 mM Acetate buffer, pH 5.0

0.1 M solution of acetic acid
0.1 M solution of sodium acetate
pH was adjusted to 5.0

3.3. EXPERIMENTAL

3.3.1. Collection of samples

Marcha samples were collected directly from their place of preparation in different traditional marcha-making villages located in the Darjeeling hills and Sikkim. Fermented beverages samples were collected from different places and markets of the Darjeeling hills and Sikkim aseptically in pre-sterile bottles, which were kept in an icebox carrier and transported, immediately to the laboratory for analyses.

3.3.2. Survey

Survey was conducted in randomly selected 100 houses in three sub-divisions of the Darjeeling hills namely Darjeeling, Kalimpong and Kurseong and 100 houses in four districts of Sikkim - North, West, South and East, representing the major ethnic communities of the Nepalis, Bhutias and Lepchas. Information was collected on indigenous knowledge of traditional beverage fermentation technology, ingredient uses, equipment uses, mode of consumption and socio-cultural importance of the product, using an questionnaire lasting for 1 h. Amount of fermented beverages consumed in every meal by each person was weighed directly by a portable weighing balance (Ishida, Germany) and daily per capita consumption was estimated as g/capita/day. Feeding frequency of fermented beverage by each family was also recorded in percentage.

Table B. Questionnaire on consumption of fermented beverages in the Darjeeling hills and Sikkim

- Name of the Informant:
- Ethnic Group:
- Name of (i) Village/Revenue Block:; (ii) Sub-division; (iii)District:
- Approximate number of population of the Village
- Nearest Market
- Kindly provide information on fermented beverages:

Fermented beverages	Local name	Raw material	Consume (daily/times per week/occasionally)	Whether prepare at home or market purchase/both
Kodo ko jaanr				
Bhaati jaanr				
Makai ko jaanr				
Gahoon ko jaanr				
Other beverages				
Raksi				

- Method of preparation
- Mode of consumption
- Equipment uses
- Socio-economy of the product, if any
- Do you perform any ritual or worship any particular god(s) or goddess(es) with fermented products you consume/drink?

3.3.3. Microbial analysis

3.3.3.1. Isolation of microorganisms

Ten g of sample was blended with 90 ml of 0.85 % (w/v) sterile physiological saline contained on a rotary shaker (120 rpm) for 30 min. Decimal dilution series were prepared in sterile diluent. One ml of appropriately diluted suspension of sample was mixed with the molten media and poured into plates. Total viable counts were determined using plate count agar (HiMedia M091A) and incubated at 30° C for 2 days. Moulds and yeasts were isolated on potato dextrose agar (HiMedia M096) and yeast extract-malt extract agar (HiMedia M424), respectively supplemented with 10 IU/ml benzylpenicillin and 12 µg/ml streptomycin sulphate and incubated aerobically at 28°C for 3 days. The lactic acid bacteria (LAB) were selectively isolated on MRS agar (HiMedia M641) plates supplemented with 1 % $CaCO_3$ and incubated anaerobically in an Anaerobic Gas-Pack system (HiMedia LE002) at 30° C for 3 days. Colonies were either selected randomly or all sampled if the plate contained less than 10 colonies. The purity of the isolates was checked by streaking again on fresh agar plates of the isolation medium, followed by microscope examination. Colonies appeared were counted as colony forming units (cfu) per g sample. The isolated strains were picked up on slants of their respective media and kept at 4° C. Cultures were sub-cultured in every two months. Identified strains were deposited and preserved in cryotubes at -20° C at the Food Microbiology Laboratory of the Department of Botany, Sikkim Government College, Gangtok, Sikkim, India.

3.3.3.2. Characterisation of moulds

The general morphology of moulds was examined after growing on potato dextrose agar (HiMedia M096) for 2 days at 28° C. Presence or absence of rhizoid and stolon were observed. Size of sporangium and sporangiospore were measured with a standardised ocular micrometer.

3.3.3.3. Characterisation of yeasts

3.3.3.3.1. *Cell morphology*

Sterile yeast morphology agar (Hi Media M138) slants were inoculated with an actively growing (24 h-old) yeast culture and incubated at 28° C for 3 days and observed for cell morphology and mode of vegetative reproduction (Kreger-van Rij, 1984). Dimension of cells were measured with a standardized ocular micrometer.

3.3.3.3.2. *Pseudo- andTrue-mycelium*

For observation of pseudo-mycelium and true-mycelium of yeast isolates, slide culture method described by Kreger-van Rij (1984) was followed. A petri-dish, containing U-shaped glass rod supporting two glass slides, was autoclaved at 121° C for 20 min. The molten potato dextrose agar (HiMedia M096) was poured onto the slides. The solidified agar on the slides was inoculated very lightly with yeast isolates in two lines along each slide. Four sterile coverslips were 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. 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 coverslips were examined microscopically for the formation of pseudo-mycelium or true- mycelium.

3.3.3.3.3. *Characteristics of asci and ascospore*

Sterile ascospore agar (HiMedia M804) slants were streaked with a 24 h-old yeast isolates, incubated at 28° C for 3 days and examined at weekly intervals up to 4 weeks for observation of asci and ascospores. A heat fixed smear was flooded with 5 % w/v aqueous malachite green (HiMedia S020) for 30 to 60 sec, heated to steaming 3 to 4 times over the flame of a spirit lamp and counterstained with safranin (HiMedia S027) for 30 sec and observed under the microscope (Kreger-van Rij, 1984).

3.3.3.3.4. *Reduction of nitrate*

Cultures were grown in 5 ml nitrate broth incubated at 28° 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, indicating the presence of nitrate. 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).

3.3.3.3.5. *Growth at 37° C*

Slants of malt-extract agar (HiMedia M137) were inoculated with cells of young yeast isolates and incubated at 37° C for 4 days and then observed the growth (Yarrow, 1998).

3.3.3.3.6. *Sugar fermentation*

The method was based on Kreger-van Rij (1984). Cells were grown at 28° C on yeast extract-malt extract agar (HiMedia M424) slants for 3 days. Tubes of 10 ml of fermentation basal medium (Wickerham, 1951) supplemented with 2 % w/v sterile sugars containing Durham tubes, were inoculated with the above yeast culture and incubated at 28° C and were shaken regularly to observe gas accumulation in the inverts.

3.3.3.3.7. *Sugar assimilation*

The method was based on Kreger-van Rij (1984). Yeast isolates were grown at 28° C on yeast extract-malt extract agar (HiMedia M424) slants for 3 days. Tubes containing 5 ml mixture of yeast nitrogen base (HiMedia M139) and carbon source were inoculated with cultures and incubated at 28° C for 3 to 7 days. Control test tube was made by adding 0.5 ml of yeast nitrogen base (HiMedia M139) in 4.5 ml of sterilized distilled water (devoid of any carbon source). Assimilation of carbon sources was observed by comparing with the control.

3.3.3.4. Characterization of bacteria

3.3.3.4.1. *Gram staining*

The method of Bartholomew (1962) was followed. A 48 h-old drop of cell suspension of bacterial culture was taken on grease-free slide and a smear was made. It was then heated-fixed, flooded by crystal violet stain for 1 min, and washed for 5 sec with water. The smear was flooded with Burke's

iodine solution, allowed to react for 1 min, and washed again for 5 sec with water. Holding the slide against a white surface, 95 % ethanol was poured drop-wise 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.

3.3.3.4.2. *Cell morphology*

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

3.3.3.4.3. *Motility*

A drop of a 24 h-old culture in MRS (HiMedia M369) broth was used to prepare a hanging drop in a cavity slide. The drop of culture was observed in a phase contrast microscope (Olympus CH3-BH-PC, Japan) for motility test following the method of Harrigan (1998).

3.3.3.4.4. *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).

3.3.3.4.5. *Gas ($C0_2$) production from glucose*

It was observed in MRS broth without citrate and containing inverted vials (Schillinger and Lücke, 1987). Accumulation of gas in the inverts indicates positive result.

3.3.3.4.6. *Anaerobic growth*

Anaerobic agar (HiMedia M228) was put into culture tubes in sufficient amount so that to give 7.5 cm depth of the medium and sterilized by autoclaving at 121° C for 20 min. Tubes were inoculated with a small (outside diameter 1.5 mm) loopful of 24 h-old MRS broth culture by stabbing up to the bottom of the column. They were incubated at 30° C for 3 and 7 days and observed for growth along the length of the stab (anaerobic) (Claus and Berkeley, 1986).

3.3.3.4.7. *Hydrolysis (decomposition) of casein*

Milk agar plates were streaked with 24 h-old cultures at the centre and examined after incubation at 30° C for 7 days for any clearing of casein around and underneath the growth (Gordon *et al.*, 1973).

3.3.3.4.8. *Hydrolysis of gelatin*

MRS agar (HiMedia M641) plates containing 1 % w/v gelatin were streaked with bacterial culture at the centre and incubated at 30° C for 3 and 5 days. Plates were then flooded with 10 ml of 1 N sulphuric acid saturated with ammonium sulphate. Hydrolysis was indicated by a clear zone, under

and around the growth, in contrast to opaque precipitate of unchanged gelatin (Sneath and Collins, 1974).

3.3.3.4.9. *Hydrolysis of arginine*

Culture tubes containing arginine hydrolysis test medium were inoculated by stabbing and immediately after this, a layer (~ 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).

3.3.3.4.10. *Hydrolysis of starch*

Starch agar plates were streaked with 24 h-old cultures and incubated at 30° C for 3 days. Flooded the plates with iodine solution for 15-30 min and observed and measured the clear zone underneath (after the growth was scrapped off).

3.3.3.4.11. *Production of indole*

Cells were grown at 30° C 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).

3.3.3.4.12. *Reduction of nitrate*

The method followed was the same as described in 3.3.3.3.4.

3.3.3.4.13. *Salt tolerance*

Salt tolerance was tested by inoculating a loopful of culture in MRS broth (HiMedia M369) supplemented with 4 %, 6.5 %, 10 % and 18 % sodium chloride, respectively, and incubated for 3 days at 30° C in a slanting position to improve aeration (Schillinger and Lücke, 1987).

3.3.3.4.14. *Growth at different pH*

The pH of MRS broth (HiMedia M369) was adjusted to different levels using 1 N HCl or 10 % w/v NaOH. The medium was then distributed into tubes containing 10 ml in each tube. They were autoclaved, cooled and inoculated with 0.2 ml of 48 h-old cultures. The tubes were incubated at 30° C for 24 h-old and observed for growth (Hesseltine and Ray, 1988). The different pH used was 4.2, 7.5 and 8.5.

3.3.3.4.15. *Growth at different temperatures*

MRS broth (HiMedia M369) was inoculated with cultures and incubated at 15° C and 45° C for 3 days, respectively and the growth was observed (Schillinger and Lücke, 1987).

3.3.3.4.16. *Sugar Fermentation*

Sugar fermentation test of bacterial cultures for 49 sugars were carried out by using API 50 CHL system (bioMérieux, France). Cultures were grown at

30° C for 48 h on MRS agar (HiMedia M641). The growth was harvested in 2 ml sterile normal saline which was used to prepare suspensions, corresponding to 10^7 cells/ml. The incubation box was prepared by distributing about 10 ml of sterile water into the honeycombed base of the 50 CHL trays. The strips were unpacked, placed them in the trays and the tubes were filled with the bacterial suspension. The inoculated strips were kept slightly tilted and incubated at 30° C for 48 h. The strips were read by referring to the manufacturer's interpretation table. All spontaneous reactions were recorded.

3.3.3.5. Identification

Moulds were identified according to Schipper (1976, 1984) and Hesseltine (1991). Identified representative strains of moulds were sent to International Institute of Mycology, Surrey, U.K. for confirmation. Yeast strains were identified according to the criteria laid down by Kreger-van Rij (1984) and Kurtzman and Fell (1998). For identification of bacterial species, taxonomic keys laid down in Bergey's Manual of Systematic Bacteriology, volume 2 (Sneath *et al.*, 1986) and keys described by Wood and Holzapfel (1995) were followed.

3.3.3.6. Preliminary screening of amylolytic activity

All isolates were streaked on surface-dried plates of starch agar and incubated at 28° C for mould and yeasts and at 30° C for lactic acid bacteria. After 3 days plates were flooded with iodine solution and observed for clear zone in and around the colony. If positive, diameter of

the clearing zones was measured by a scale, which was used as an assessment of amylolytic activity.

3.3.4. Physico-chemical analysis

3.3.4.1. *pH*

Ten g of sample was blended with 20 ml of carbon-dioxide free-distilled water in a homogeniser for 1 min and the pH of the slurry was determined directly using pH-meter (Type 361, Systronics) calibrated with standard buffer solutions (Merck).

3.3.4.2. *Temperature*

The temperature (° C) change of the fermenting substrates during beverage fermentation was recorded directly by a thermometer.

3.3.4.3. *Moisture*

Moisture content of sample was calculated by drying 2.5–3.0 g of well-mixed sample at 135 ± 1° C for 2 h to constant weight (AOAC, 1990).

3.3.4.4. *Titratable acidity*

Titratable acidity of sample was calculated by titrating the filtrates of a well blended 10 g sample in 90 ml carbon-dioxide free distilled water with 0.1 N sodium hydroxide to end point of phenolphthalein (0.1 % w/v in 95 % ethanol) (AOAC, 1990).

3.3.4.5. *Ash*

A sample (~ 2 g) was accurately weighed into a previously dried and weighed porcelain crucible and placed in a muffle furnace preheated to 550° C for 3 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 weighing was ≤ 1 mg.

3.3.4.6. *Alcohol*

Alcohol content of sample was determined by dichromate oxidation method (AOAC, 1990). The 10 ml of extract was pipetted in a 500 ml round-bottomed flask where 1 g of $CaCO_3$ and 100 ml of distilled water was added and distilled. The distillate was collected for 15 min and diluted to 100 ml with distilled water (after coming to room temperature). Diluted distillate was pipetted out into a conical flask with stopper to which 10 ml of N/5 $K_2Cr_2O_7$ and 10 ml of concentrated H_2SO_4 were added and allowed to stand for 1 h. After this, stopper was removed and 100 ml of distilled water was added, followed by addition of 8 % KI and immediately titrated with N/10 $Na_2S_2O_3$ using freshly prepared 1 % starch (HiMedia RM089) solution as the indicator. Alcohol content was calculated in percentage.

Alcohol (%) =

$(V_1-V_2) \times f_2 \times 0.00115 \times 100/V_3$ 100/S (multiply by 250/E of diluted extract used).

V_1 = titration volume of N/10 $Na_2S_2O_3$ against 10 ml of N/5 $K_2Cr_2O_7$ (blank test without sample)

V_2 = titration volume of N/10 $Na_2S_2O_3$ against the distillate

f_2 = factor of N/10 $Na_2S_2O_3$

100 = total volume of the distillate

V_3 = pipetting volume of the distillate for the reaction

100 = %

S = sample size

250 = total volume of the diluted extract

E = ml of extract taken for alcohol distillation.

3.3.4.7. *Reducing sugar*

Reducing sugar content of sample was determined by modified colorimetric method of Somogyi (1945) using glucose as standard solution. To 1 ml of extract in a 20 ml capped glass tube, 1 ml of Reagent D was added and heated in a vigorously boiling water-bath for 20 min. Allowed to cool for 5 min in running tap water, and 1 ml of Reagent C was added and shaken the test tube until no bubbles were evolved. After standing for 20 min diluted to 25 ml with distilled water and absorbance was measured at 520 nm in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). Reducing sugar was calculated in percentage.

$$\text{Glucose (\%)} = (As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 250/V_2 \times 100/10$$

As = absorbance of sample

Ab = absorbance of blank

Ag = absorbance of glucose

[G] = concentration of glucose solution (µg/ml)

10^{-3} = mg to g

V_1 = total dilution volume for reaction (ml)

1= 1 ml for reaction

V_2 = pipetting volume of extract for dilution (ml)

250 = total volume of extract (ml)

100 = %

10 = sample size for preparation of extract

3.3.4.8. *Total Sugar*

Total sugar was determined by determining reducing sugar in hydrolysed sample with HCl (AOAC, 1990). In a 300 ml conical flask fitted with condenser, 2 g of sample was blended in 20 ml of distilled water to which 160 ml of distilled water and 20 ml of HCl (25 %) were added. It was heated in vigorously boiling water bath for 3 h, cooled in a running tap water, neutralized with 10 % NaOH using pH meter (Type 361, Systronics) and diluted to 500 ml with distilled water. It was filtered and the filtrate was taken for determining reducing sugar as described above. Total sugar was calculated in percentage.

Total sugar (%) = $(As-Ab)/(Ag-Ab) \times [G] \times 10^{-3} \times V_1/1 \times 500/V_2 \times 100/S$

As= absorbance of sample

Ab = absorbance of blank

Ag = absorbance of glucose

[G]= concentration of glucose solution (µg/ml)

10^{-3} = mg to g

V_1 = total dilution volume for reaction (ml)

1= 1 ml for reaction

V_2 = pipetting volume of extract for dilution (ml)

500 = total volume of extract (ml)

100 = %

S = sample size for preparation of extract

3.3.4.9. *Nitrogen*

The method described in AOAC (1990) was followed. Approximately 1 g of sample was taken in a digestion flask, 0.7 g catalyst ($CuSO_4$: K_2SO_4, 1:9) and 25 ml of concentrated H_2SO_4 were added to it. 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-bottomed flask, and mixed with approximately 100 ml of 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 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 N H_2SO_4 containing about 5 drops of methyl red indicator (HiMedia I007). 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 N NaOH solution. The blank determination on reagents was considered for correction. Nitrogen was calculated in percentage.

N (%) =

(ml of standard acid × N of standard acid) – (ml of standard NaOH × N of standard NaOH) ×1.4007/weight of sample (g).

3.3.4.10. *Protein*

Protein content was determined by multiplying total nitrogen, estimated by micro-Kjeldahl method, by 6.25 (AOAC, 1990).

3.3.4.11. *Fat*

Fat content was determined by ether extraction using glass soxhlet (AOAC, 1990). Flat-bottomed flask was oven dried and kept in the desiccator for cooling. The weight (W_1) of the round-bottomed flask was taken. A cellulose thimble (dry and fat free) was taken and in which ~ 2 g of sample was placed and put in the soxhlet. Fat was extracted by using petroleum ether having boiling range 40-60° C, on heating mantle at 60° C for 5 h. The flat bottomed flask was dried for 1 h at 100° C to evaporate ether and moisture, cooled in desiccator and weighed (W_2). Fat was calculated in percentage.

Fat (%) = $W_2 - W_1$/Sample weight x 100

3.3.4.12. *Crude fibre*

Crude fibre content was determined using fibertec extraction (Tecator, model 1010 M6, Sweden) following the method of AOAC (1990). One g of the pre-dried sample was weighed and weight of the sample was noted (W_1). The 150-ml of hot 0.128 N H_2SO_4 was added into the column. Tap

water was opened for cooling of the condensers. Drops (2-4) of octanol was added to prevent foaming and heated to boiling for 30 min. Cold water tap was opened for the water suction pump and filtration was started. Sediment was removed from the filter surface by applying reversed pressure, washed three times with hot deionized water in each column. The 150 ml of 0.223 M KOH solution was added to sample and a few drops of octanol was added and boiled for 30 min. As in the previous case here also the heater was put off and filtered and if necessary sediments were removed by applying reversed pressure, washed 3 times with hot deionized water in each column. It was made sure that no residue remained on the inside wall of the column. The crucibles were removed from the Hot Extractor Unit to the Cold Extractor Unit by using the crucible holder. The valves of Cold Extractor Unit were closed and filled each crucible containing the sample with 25 ml of acetone, filtered it out by placing the valve in vacuum position. It was repeated for three times for each sample, removed the crucibles and transferred them to a crucible stand, left them at room temperature, until nearly all acetone was is gone in order to avoid the burning of the fibres during the drying process. The crucibles were dried at 100° C overnight, cooled to room temperature in a desiccator and weighed them afterwards (W_2). The sample was ashed in the crucible at 500° C for at least 3 h and final weight was taken (W_3). Crude fibre was calculated in percentage.

Crude fibre (%) = $W_2 - W_3/W_1 \times 100$

W_1 = sample weight

W_2 = crucible + residue weight

W_3 = crucible +ash weight

3.3.4.13. *Carbohydrate*

Carbohydrate content was calculated by difference (Standal, 1963):

100 – (% protein + % fat + % ash)

3.3.4.14. *Minerals*

The method was based on AOAC (1990). The ash after heating the sample (2 g) at 550° C for 3 h was dissolved in 5 ml of 20 % HCl. The solution was evaporated to dryness on a hot plate at a temperature of 100-110° C and in an oven at 110° C for 1 h. The minerals in the dried residue were dissolved in about 10 ml of 100 % HCl and the solution was heated on a hot plate at a temperature of 100-110° C for 3-4 times. The solution was made up to 100 ml with 1 % HCl. Calcium, magnesium, manganese, copper, iron, zinc, sodium and potassium were estimated in an atomic absorption spectrophotometer (Model 2380, Perkin-Elmer).

Phosphorus was determined by colorimetric method (AOAC, 1990). A series of standard solutions (0.0, 0.1, 0.2, 0.3 and 0.4 mg P_2O_5/ml) were prepared. Five ml aliquots was pipetted into 100 ml volumetric flasks, and 50 ml distilled water and 4 ml 70 % $HClO_4$ were added. To each flask 20 ml molybdovanadate reagent was added, diluted to volume with distilled water, thoroughly shaken and allowed to stand for 15 min. Determination of blank as 'A' and standards in set of matched cells against distilled water as reference was done. Standards for 'A' were corrected and was plotted against concentration in mg P_2O_5/ml solution. The ash after heating the

sample (5 g) at 550° C for 3 h was dissolved in 4 ml of 70% $HClO_4$ and 20 ml of distilled water. The solution was transferred to 100 ml volumetric flask and 20 ml molybdovanadate reagent was added, diluted to 100 ml, mixed thoroughly and absorbance at 400 nm was measured in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). Blank and standard were carried through entire determination. 'A' of blank was subtracted from that of sample.

3.3.4.15. *Energy*

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 per 100 g (Gopalan *et al.*, 1995).

3.3.5. Enzymatic activities

3.3.5.1. *Enzymatic profiles*

The enzymatic profiles of selected strains of moulds, yeasts and bacteria isolates were assayed following the method of Arora *et al.* (1990) in API zym (bioMérieux, France) galleries by testing for the activity of the following 19 enzymes: phosphatase alkaline, esterase (C4), esterase lipase (C8), lipase (C14), leucine, valine and cystine arylamidase, trypsin, chymotrypsin, phosphatase acid, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase. Cultures were grown on MRS agar (HiMedia M641) and growth was

harvested in 2 ml sterile normal saline which was used to prepare suspension of 10^7 cells/ml. The strip was unpacked and 2 drops of cell suspensions was inoculated in each cupule of the strip containing ready-made enzyme substrates and incubated at 30° C for 6 h. After incubation, 1 drop of ready-made zym A and zym B reagents was added and observed for colour development based on the manufacturer's colour chart.

3.3.5.2. *α-amylase activity assay*

The blue value method of Fuwa (1954) as modified by Kawaguchi *et al.* (1992) was followed for α-amylase activity. Cultures were grown on broth medium (1.0 % soluble starch, 1.0 % yeast extract, 1.0 % peptone, and 0.3 % NaCl, pH 7.2) in shaking incubator (180 rpm) at 28° C for 48 h. The cultures were immediately centrifuged in refrigerated automatic centrifuge (C-24, Remi) at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The enzyme solution and 1.5 % soluble starch dissolved in 100 mM Tris-HCl buffer, pH 7.0 were pre-incubated separately at 37° C for 5 min in water-bath shaker (RSB-12, Remi). Then, the reaction mixture was started by adding 1.0 ml of 1.5 % soluble starch (HiMedia RM089) to 0.5 ml enzyme solution and incubated at 37° C for 10 min. The reaction was stopped by the addition of 2.5 ml of stop solution. The 100 ml of the reaction mixture was added to potassium iodide solution, left at room temperature for 20 min and absorbance at 660 nm of the resulting solution was measured in UV-VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). One unit of α-amylase activity (liquefying

activity) was defined as the amount of α-amylase which produced 10 % fall in the intensity of blue colour at the above condition.

3.3.5.3. *Glucoamylase activity assay*

Glucoamylase activity was determined according to modified method of Ueda and Saha (1983). Cultures were grown on broth medium (1.0 % soluble starch, 1.0 % yeast extract, 1.0 % peptone, and 0.3 % NaCl, pH 7.0) in shaking incubator (180 rpm) at 28° C for 48 h and were immediately centrifuged in refrigerated automatic centrifuge (C-24, Remi) at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The reaction mixture containing 2 ml of 1 % soluble starch (HiMedia RM 089) in 2 ml of 100 mM acetate buffer (pH 5.0) and 0.5 ml of enzyme solution was pre-incubated separately at 40° C for 5 min in a water-bath shaker (RSB-12, Remi). The 2 ml of 1 % soluble starch dissolved in 100 mM acetate buffer was added to enzyme solution and incubated at 40° C for 10 min. After a 10 min reaction, 1 ml of the reaction mixture was taken and glucose was determined by calorimetric method (Somogyi, 1945). One unit of glucoamylase activity (saccharifying activity) was defined as the amount of enzyme, which liberated 1 mg glucose in 1 min under the above condition. Unit of activity was expressed as mg glucose released per ml per 10 min.

3.3.6. Microbial and physico-chemical changes during fermentation

Jaanr was prepared in the laboratory following traditional method. Dry seeds of finger millet {*Eleusine coracana* (L.) Gaertn.), local variety

'mudke kodo}, rice (*Oryza sativa* L.) and dry seeds of maize (*Zea mays* L.) were purchased from Gangtok market. Seeds were cleaned, washed, cooked and powdered marcha (2 %), collected from Aho village, was added when the temperature of cooked substrates was around 30° C. Inoculated seeds were put into pre-sterile bottles with loosely covered lids and fermented at 28° C for 2 days for saccharification. After 2 days, lids were tightly capped to make anaerobic condition and kept for 8 days at 28° C. Samplings were made at every one day interval till 10^{th} day for microbial, physico-chemical and enzymatic analysis.

3.3.7. Testing of isolates for producing kodo ko jaanr

3.3.7.1. *Preparation of inocula*

Kodo ko jaanr was prepared in the laboratory using selected strains of moulds, yeasts and lactic acid bacteria, previously isolated from marcha samples. The suspension of mould cultures was made by adding 5 ml of sterile distilled water to each 4-day-old slant culture on potato dextrose agar (HiMedia M096), and mycelia and sporangia were scraped off the agar with an sterile inoculating wire. Yeasts and lactic acid bacterial inocula were prepared by introducing 5 ml each of sterile distilled water onto 48 h-old slant culture on yeast extract-malt extract agar (HiMedia M424) and 72 h-old slant culture on MRS (HiMedia M641), respectively. Tubes were agitated for 30 sec in a cyclomixer (Remi). Number of cells in the suspension was determined using a Neubauer's counting chamber and a phase contrast microscope (Olympus CH3-BH-PC, Japan). Cell suspensions of selected strains were prepared (10^5 to 10^6 cells/ml) and 2 ml

of each mixture was inoculated to 100 g sterilised (121° C for 15 min) and cooled to ~ 40° C seeds of finger millet. Inoculated seeds were saccharified in loosely capped pre-sterile bottles at 28° C for 2 days, and after saccharification, lids of the bottles were tightly capped and fermented at 28° C for 6 days. The rationale behind selecting the inoculum size was based on the observation on conventional method of adding ~ 2 % of marcha in boiled seeds of finger millets during kodo ko jaanr preparation.

The products were evaluated physico-chemically and organoleptically using the method as described in this chapter.

3.3.7.2. *Sensory evaluation*

Sensory properties of product was evaluated in terms of aroma, taste, texture, colour and general acceptability as method described by Meilgaard *et al.* (1990). Kodo ko jaanr samples produced by selected strains of moulds, yeasts and lactic acid bacteria, previously isolated from marcha were organoleptically evaluated by a panel of 7 judges with score rate of 1, bad and 5, good considering market jaanr as control with scoring rate of 3, moderate (Table C).

Table C. Format for sensory evaluation of kodo ko jaanr produced by selected strains, isolated from marcha

Please use market kodo ko jaanr as a control with scoring rate of 3 (moderate)

Sample code:..... Name:.....

Attribute	Score					Comment
Aroma:						
Mild alcoholic	Weak				Strong	
	1	2	3	4	5	
Taste:						
Mild sweet	Weak				Strong	
	1	2	3	4	5	
Slightly acidic (sour)	Weak				Strong	
	1	2	3	4	5	
Texture:	Hard				Soft	
	1	2	3	4	5	
Colour:	Bad				Good	
	1	2	3	4	5	
General Acceptability:	Bad				Good	
	1	2	3	4	5	

3.3.8. Consumers' Preference Trial

Market samples of kodo ko jaanr as well as kodo ko jaanr prepared in the laboratory by using a mixture of selected isolates were served to 50 consumers representing different ethnic groups of people of the Sikkim Himalayas who were familiar with jaanr. The 9-point scale used in this study ranged from 'dislike extremely' (score, 1) to 'like extremely' (score, 9) (IS, 1971).

3.3.9. Statistical analysis

The data were analysed by determining standard deviation (SD), standard error of measurement (SEM) and analysis of variance (ANOVA) (Snedecor and Cochran, 1989).