MATERIALS AND METHODS

CULTURE MEDIA

Anaerobic Agar (HiMedia M288)

Arginine Hydrolysis Medium (Thornley, 1960)

Peptone	10.0 g
Yeast extract	5.0 g
D (+) glucose	0.5 g
$K_2HPO_4.3H_2O$	2.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Sodium acetate	5.0 g
Tri-sodium citrate	20.0 g
Tween 80	1.0 ml
Arginine	0.3 %
Phenol red	0.01 g
Distilled water	1000 ml
pH	5.0

Ascospore Agar (HiMedia M804)

***Bacillus cereus* Agar Base** (HiMedia M833)

Baird Parker Agar Base (HiMedia M043)

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

Diammonium hydrogen phosphate	1.0 g
Potassium chloride	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

Biogenic Amine Sub-culturing Medium
(Bover-Cid and Holzapfel, 1999)

	g/l
MRS Broth (HiMedia M369)	52.2
D-Tyrosine (HiMedia RM 1520)	1.0
L-Histidine monohydrochloride (Merck)	1.0
L-Lysine monohydrochloride (Merck)	1.0
L-Ornithine monohydrochloride (Merck)	1.0
Pyridoxal-5-Phosphate (HiMedia RM 1554)	0.001
pH	6.00

Biogenic Amine Screening Medium
(Joosten and Northold, 1989; modified by Bover-Cid and Holzapfel, 1999)

	g/l
Tryptone	5.0
Yeast extract	5.0
Meat extract	5.0
Sodium chloride	2.5
Glucose	0.5
Tween 80	1.0
K_2HPO_4	2.0
Ammonium citrate	2.0
Calcium carbonate	0.1
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
$FeSO_4.7H_2O$	0.04
Thiamine	0.001
Pyridoxal-5-phosphate	0.005
Bromocresol purple	0.05
Agar	22.0
Amino acid	5.0

Amino acids are D-Tyrosine (pH 5.3) (HiMedia RM 1520); L-Histidine monohydrochloride (pH 5.0) (Merck); L-Lysine monohydrochloride (pH 5.15) (Merck); L-Ornithine monohydrochloride (pH 5.0) (Merck); No amino acid (pH 5.15).

Egg Yolk Emulsion (HiMedia FD045)

Egg Yolk Tellurite Emulsion (HiMedia FD046)

Fermentation Basal Medium (Wickerham, 1951)

Yeast extract	4.5 g
Peptone	7.5 g
Distilled water	1000 ml
Bromothymol blue (Till sufficiently dense green colour appears)	

Lactate Configuration Medium (Holzapfel, unpublished)

Peptone from casein	10.0 g
Yeast extract	4.0 g
Glucose	20.0 g
Di-potassium hydrogen phosphate	2.0 g
Tween 80	1.0 g
Di-ammonium hydrogen phosphate	2.0 g
Magnesium sulphate	0.2 g
Manganese sulphate	0.04 g
Distilled water	1000 ml

Malt Extract Agar (HiMedia M137)

Malt Extract Agar (Kreger-van Rij, 1984)

Malt extract	100.0 g
Agar	20.0 g
Distilled water	1000 ml
pH	5.4

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

Skim milk powder (HiMedia RM1254)	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 Agar (HiMedia M641)

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	1000 ml
pH	6.2-6.4

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

MRS Broth (HiMedia M369)

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

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

Nutrient Agar (HiMedia MM012)

Nutrient Broth (HiMedia M002)

Plate Count Agar (HiMedia M091)

Polymyxin B Selective Supplement (HiMedia FD003)

Potato Dextrose Agar (HiMedia M096)

Phytone broth (Nagai *et al.,* 1994)

Phytone peptone	15.0 g
Mono-Sodium glutamate	15.0 g
Sucrose	15.0 g
Potassium dihydrogen phosphate	2.5 g
Disodium hydrogen phosphate	1.7 g
Sodium chloride	0.05 g
Magnesium chloride	0.05 g
Biotin	0.1 µg/ml
Distilled water	1000 ml
pH 7.0	

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

Starch (HiMedia RM089)	1.0 g in cold distilled water
Tryptone	5.0 g
Yeast extract	15.0 g
Potassium dihydrogen phosphate	3.0 g
Agar	20.0 g
Distilled water	1000 ml

Tributyrin Agar (Stolp and Gadkari, 1981)

Peptone	5.0 g
Yeast extract	3.0 g
Tributyrin (HiMedia FD081)	10.0 g
Agar	12.0 g
Distilled water	1000 ml
pH	7.4-7.6

Tryptone Soya Agar (HiMedia 290)

Violet Red Bile Glucose Agar w/o Lactose (HiMedia M581)

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

Peptone	7.0 g
Glucose	5.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml
pH	6.5

Yeast Malt Agar (HiMedia M424)

Yeast Malt Broth (HiMedia M425)

Yeast Extract-Malt Extract 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	5-6

Yeast Morphology Agar (HiMedia M138)

Yeast Nitrogen Base (HiMedia M139)

REAGENTS

Acidic Ninhydrin

1-Butanol/water saturated	465 ml
Acetic acid	35 ml
Ninhydrin	2.5 ml

Burke's Iodine Solution (Bartholomew, 1962)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Gram's Crystal Violet (HiMedia S012)

Malachite Green (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.

Phenolphthalein (HiMedia I009)

Reagents for α-amylase assay

100 mM Tris - HCl buffer, pH 7.0

1.5% soluble starch was dissolved in 100 mM 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%

Reagents for Protease activity assay

100 mM phosphate buffer pH 6.8

0:1 M/L $Na_2HPO_4.12H_2O$
0.1 M/L KH_2PO_4
1% Azocasein was dissolved in 0.1M phosphate buffer (Sigma Chemicals, St. Louis, USA), pH 6.8

10% Trichloroacetic acid

1N NaOH

Safranin (HiMedia S027)

Voges-Proskauer test reagent

Potassium hydroxide (40%)
Ethanolic α-napthol (5%)

REFERENCE STRAINS

Reference Strains	Origin	Purpose in this experiment
Listeria monocytogenes 20600	DSM	Indicator strain (Antimicrobial activity)
Enterococcus faecium 20477	DSM	Indicator strain (Antimicrobial activity)
Streptococcus mutans 6178	DSM	Indicator strain (Antimicrobial activity)
Bacillus cereus 2010	CCM	Indicator strain (Antimicrobial activity)
Lactobacillus plantarum 20174	DSM	Standard (+) strain for meso-diaminopimelic acid determination

Originally these reference strains were obtained from DSM (Deutsche Sammlung von Mikroorganismen, Göttingen, Germany) and CCM (Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia). *Listeria monocytogenes* DSM 20600 and *Bacillus cereus* CCM 2010 were propagated in standard nutrient agar (HiMedia M002), *Enterococcus faecium* DSM 20477, *Streptococcus mutans* DSM 6178 and *Lactobacillus plantarum* DSM 20174 were cultivated in MRS broth (HiMedia M369). The cultures were maintained as frozen stocks at –20° C in 15% glycerol.

EXPERIMENTAL

Survey

A survey was conducted in different places of the Eastern Nepal, the Darjeeling hills, Sikkim, Assam, Meghalaya and Manipur to get detailed information on the types of fish products, traditional methods of preparation and mode of consumption of various fermented, smoked, dried/salted fish products used by the ethnic people. A questionnaire was prepared to collect information on the various types of fish products.

Collection of samples

Variety of fermented, smoked, dried/salted fish products samples used by the ethnic people were collected from different regions and markets of the Eastern Nepal in Maglung, Therathum and Aitabare; Gidhang village in Kalimpong of the Darjeeling hills, local "Lal Bazar" of Gangtok in Sikkim; local market of Shillong in Meghalaya, Ima market of Imphal in Manipur and local "Palten Bazar" of Guwahati. Samples were collected aseptically in pre-sterile poly-bags as well as sterile bottles, sealed, lebelled and stored at –20° C for analyses.

Microbial analysis

Ten g of sample was suspended in 90 ml of 0.85 % (w/v) sterile physiological saline and homogenized in a stomacher lab-blender 400 (Seward, UK) for 1 min. For aerobic spore-counts, 1 ml dilution was mixed with 9 ml sterile physiological saline, and heated for 2 min in continuously boiling water (Tamang and Nikkuni, 1996). Decimal dilution series were prepared in sterile diluent and diluted suspension of

sample was mixed with the molten media and poured into plates. Lactic acid bacteria (LAB) were selectively isolated on MRS agar (HiMedia M641) plates supplemented with 1 % $CaCO_3$ and incubated under anaerobic condition in an Anaerobic Gas-Pack system (HiMedia LE002) at 30° C for 3 days. Spore-forming bacteria were isolated on nutrient agar (HiMedia MM012) and incubated at 37° C for 1 day. Total viable counts were determined using plate count agar (HiMedia M091A) and incubated aerobically 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. Colonies are either selected randomly or all sampled if the plate contained less than 10 colonies, according to Leisner *et al.* (1997).

Purity of the isolates was checked by streaking again on fresh agar plates of the isolation media, followed by microscopic examinations. Colonies appeared were counted as colony forming units (cfu) per g of sample. The isolated strains were picked up on slants of their respective media and kept at 4° C. Cultures were sub-cultured after every two months. Identified representative strains of the different groups of the isolates were deposited and preserved in the respective broth media with 15 % glycerol in cryotubes at –20° C.

Characterization of bacterial isolates

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 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 safranin for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

Cell morphology

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

Motility

A drop of a 24 h-old culture in MRS broth for LAB; nutrient broth for *Bacillus* and *Micrococcus*, was used to prepare a hanging drop in a cavity slide following the method of Harrigan (1998). The prepared culture was observed in a phase contrast microscope (Olympus CH3-BH-PC, Japan) for motility test of the strains

Production of catalase

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

Hydrolysis of arginine

Tubes of 5 ml arginine hydrolysis medium (Thornley, 1960) were inoculated with 24 h-old culture. The tubes were incubated at 30° C for 3 days and observed for the formation of ammonia from arginine (Schillinger and Lücke, 1987).

Gas ($C0_2$) production from glucose

For lactic acid bacteria, tubes of 10 ml MRS broth without citrate and containing inverted Durham tubes was inoculated with 24 h-old cultures and incubated at 30° C (Schillinger and Lücke, 1987). Accumulation of gas in the inverts indicated positive result.

Acid and gas production from glucose

For spore forming bacteria, tubes of 10 ml basal medium containing 0.5% w/v sugars and inverted Durham tubes were inoculated with the isolates and incubated at 37° C for 3 days (Norris *et al.*, 1981).

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 5 ml in each. They were autoclaved, cooled to room temperature and inoculated with 24 h-old MRS broth culture. The tubes were incubated at 30° C for 24 h and observed for growth (Dykes *et al.*,

1994). For spore forming bacteria the pH of nutrient broth was adjusted to 6.8, inoculated with 24 h-old cultures and incubated at 37° C for 3 days.

Growth at different temperatures

MRS broth and nutrient broth were inoculated with 24 h-old cultures and incubated at 10° C and 15° C for 7 days, 37° C and 45° C for 3 days, respectively and observed for growth (Dykes *et al.*, 1994).

Salt Tolerance

Salt tolerance was tested by inoculating a loop-full of culture in MRS broth supplemented with 6.5%, 10.0% and 18.0% NaCl, respectively, and incubated for 3 days at 30° C in a slanting position to improve aeration (Schillinger and Lücke, 1987). For spore forming bacteria, nutrient broth was supplemented with 7.0% w/v NaCl, inoculated with 24 h-old culture and incubated at 37° C for 7 days in a slanting position to improve aeration. Cultures were observed for growth after incubation.

Acid from carbohydrates

The method was based on Schillinger and Lücke (1987). Tubes of 5 ml MRS broth without beef extract and glucose containing 0.5% w/v of different carbohydrates and 0.004% phenol red indicator were inoculated and incubated at 30° C for 2-5 days. Colour change from red to yellow indicated acid production.

Voges-Proskauer reaction

Tubes of 10 ml Voges-Proskauer broth were inoculated with the isolates and incubated at 37° C for 7 days. 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 positive reaction. Initial and final pH of the broth was measured using pH meter (Gordon *et al.*, 1973).

Reduction of nitrate

Cultures were grown in 5 ml nitrate broth incubated at 37° 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 of 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).

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).

Anaerobic growth

Anaerobic agar (HiMedia M228) 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 m) loop-full 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).

Lactic acid configuration

The configuration of lactic acid produced was determined enzymatically using D-lactate and L-lactate dehydrogenase kits (Boehringer-Mannheim GmbH, Cat. No. 1112821, Germany) based on the method of Boehringer-Mannheim (1989). Lactic acid bacteria strains were grown in lactate configuration medium (Holzapfel, unpublished) at 37^0 C overnight. One ml culture was centrifuged in a microcentrifuge (Heraeus, Germany) at 10,000 rpm for 5 min. The 20 µl of the supernatant was mixed with 980 µl of redistilled water to obtain 1:50 sample dilution. The 1 ml of Solution (1), 0.2 ml of Solution (2), 0.02 ml of Suspension (3), 0.1 ml sample solution and 0.9 ml of redistilled water was pipetted into a cuvette, followed by gentle swirling to mix the contents of the cuvette after closing it with parafilm. Similarly, a blank was prepared by adding all the reagents except the sample solution being replaced with 1.0 ml of redistilled water. After 5 minutes the absorbance of the solutions (A_1) was measured in UV-VIS Spectrophotometer (Analytik Jena, Germany) at 340 nm. The absorbance differences (A_2-A_1) for both, blank and sample was determined and the difference of the absorbance difference of the blank from that of the sample ($\Delta A_{D\text{-lactic acid}}$) was calculated. The reaction was started by adding 0.02 ml of Solution (4) to the sample as well as to the blank. The cuvettes were swirled gently to mix the contents by closing it with parafilm. After 30 minutes the absorbance (A_2) of the sample and

the blank were measured immediately one after another at 340 nm. The 0.02 ml of Solution (5) was added to both the sample and the blank followed by mixing. These were allowed to stand for 30 minutes. The absorbance (A_3) was measured immediately one after another for the sample as well as for the blank at 340 nm. The absorbance differences (A_3-A_2) for both, blank and sample was determined and the difference of the absorbance difference of the blank from that of the sample ($\Delta A_{L\text{-lactic acid}}$) was calculated. The lactic acid isomer concentration was calculated as:

$$c = \frac{V \times MW \times \Delta A}{\varepsilon \times d \times v \times 1000} \text{ (g/l)}$$

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight of lactic acid = 90.1 (g/mol)

d = light path = 1 cm

ε = extinction coefficient of NADH at 340 nm = 6.3 (l/mmol × cm)

The result was multiplied by the dilution factor.

meso-Diaminopimelic acid (meso-DAP)

The presence of meso-diaminopimelic acid in the cell walls of lactic acid bacteria was determined using thin-chromatography on cellulose plate (Schillinger and Lücke, 1987). Cells were grown in 5 ml MRS broth for 48 hours and were harvested by centrifuging at 13,000 rpm for 5 min, and washed with 3.0 ml of distilled water. The sediment was resuspended in 1.0 ml 6 N HCl and transferred to screw-capped tubes. The cells were hydrolysed overnight at 100° C in a water-bath. The contents of the tubes were blow-dried while immersed in boiling

water. The sediment was resuspended in 1.0 ml of distilled water and blow dried again and oven dried for 1 h. Finally, the sediment was suspended in 0.1 ml of distilled water and each sample (5 µl) was spotted on thin-layer chromatography plates on cellulose plates (Merck, Germany). Descending one-dimensional chromatography was done by keeping the plates in a TLC chamber in a solvent solution containing methanol: pyridine: 10 N HCl: water (32:4:1:7). The solvent solution was prepared 1 hour before use. After keeping for 4-5 h the plates were dried with a hair drier and the chromatograms were developed by spraying acidic ninhydrin and when almost dried, placed for 5 minutes in 100° C oven. Spots representing meso-diaminopimelic acid appeared dark green to grey and turned yellow within 24 hour. *Lactobacillus plantarum* DSM 20174 was used as standard (meso-DAP positive).

API Tests

The ability to ferment various carbon sources by lactic acid bacteria was determined using API 50 CHL system (bioMérieux, France) according to manufacturer's instructions and also based on the method described by Tamang and Holzapfel (1999). Cultures were grown on MRS agar at 30° C for 48 hour. The growth was harvested in 2 ml sterile normal NaCl solution 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 suspensions. The inoculated strips were kept slightly tilted and incubated at 30° C for 48 h. The results were read by referring to the manufacturer's interpretation table

at 24 hour and 48 hour, respectively. All spontaneous reactions were recorded.

Characterisation of yeast isolates

Cell morphology

Sterile yeast morphology agar (HiMedia 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 (Yarrow, 1998). Dimensions of cells were measured with a standardized ocular micrometer.

Pseudo- and True-mycelium

For observation of pseudo-mycelium and true-mycelium of yeast isolates, the 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. 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.

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 (Yarrow, 1998).

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 (Yarrow, 1998).

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 observed for growth (Yarrow, 1998).

Sugar fermentation

The method was based on Kreger-van Rij (1984) and Yarrow (1998). 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 inverted 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.

Sugar assimilation

The method was based on Kreger-van Rij (1984) and Yarrow (1998). 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.

Pathogenic contaminants

Samples were tested for enumeration of pathogenic contaminants such as *Bacillus cereus* using selective *Bacillus cereus* agar base (HiMedia M833), *Staphylococcus aureus* using Baird Parker agar base (HiMedia M043) and enterobacteriaceae using Violet Red Bile Glucose agar w/o lactose (HiMedia M581) (Nout *et al.*, 1998). Ten g of sample was blended with 90 ml of peptone-physiological saline (0.1% neutral peptone, 0.85% NaCl) in a stomacher lab-blender 400 (Seward, UK) for 1 min. Serial decimal dilution series was prepared in the same diluent in duplicates.

***Bacillus cereus*:** Selective enumeration was carried out on spread plates of *Bacillus cereus* agar base (HiMedia M833) with appropriate additions of Polymyxin B Selective Supplement (HiMedia FD003) and Egg yolk emulsion (HiMedia FD045). The inoculated plates were incubated at 30° C for 24 h to 48 h. Characteristic turquoise to peacock blue colonies surrounded by zone of precipitate of the same colour were regarded as presumptive *Bacillus cereus*. A representative number (usually five per plate counted) were isolated and purified on *Bacillus cereus* agar base, followed by nutrient agar. Confirmation was on the basis of endospore formation, fermentation of glucose, xylose and arabinose and ability to grow at 50° C.

***Staphylococcus aureus*:** Selective enumeration was carried out on spread plates of Baird Parker agar base (HiMedia M043) with appropriate additions of Egg yolk tellurite emulsion (HiMedia FD046) and incubated at 30° C for 24 h to 48 h. The black colonies appeared which were regarded as presumptive *Staphylococcus aureus*.

Enterobacteriaceae: Sample dilutions in Tryptone soya broth (HiMedia M011) were allowed to resuscitate on thin Tryptone soya agar (HiMedia 290) plates for 1-2 h at 27° C, followed by a thick overlay of selective Violet Red Bile Glucose agar (without lactose) medium and incubated at 30° C for 20 h. Colonies appeared were regarded as presumptive enterobacteriaceae.

Identification

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. Endospore-forming rod-shaped bacteria were identified according to the keys based on Claus and Berkeley (1986), Slepecky and Hemphill (1992) (Table B). Yeast strains were identified according to the criteria laid down by Kreger-van Rij (1984), and Kurtzman and Fell (1998).

Table B: Phenotypic key used for tentative identification of Gram-positive endospore forming rod-shaped bacteria[a]

1	Allantoin or urate required	Positive	*Bacillus fastidiosus*
		Negative	2
2	Catalase	Positive	3
		Negative	20
3	Voges-Proskauer	Positive	4
		Negative	11
4	Growth in anaerobic agar	Positive	5
		Negative	10
5	Growth at 50° C	Positive	6
		Negative	7
6	Growth in 7% NaCl	Positive	*Bacillus licheniformis*
		Negative	*Bacillus coagulans*
7	Acid and gas from glucose	Positive	*Paenibacillus polymyxa*
		Negative	8
8	Reduction of NO_3^- to NO_2^-	Positive	9
		Negative	*Paenibacillus alvei*
9	Parasporal body in sporangium	Positive	*Bacillus thuringiensis*
		Negative	37
10	Hydrolysis of starch	Positive	*Bacillus subtilis*
		Negative	*Bacillus pumilus*
11	Growth at 65° C	Positive	32
		Negative	12
12	Hydrolysis of starch	Positive	13
		Negative	17
13	Acid and gas from glucose	Positive	*Paenibacillus macerans*
		Negative	14
14	Width of rod ≥ 1.0 mm	Positive	34
		Negative	15

15	Growth at pH 6.8	Positive	16
		Negative	*Bacillus alcalophilus*
16	pH in VP broth < 6.0	Positive	28
		Negative	26
17	Growth in 10 % NaCl	Positive	*Bacillus pasteurii*
		Negative	18
18	Growth in anaerobic agar	Positive	*Brevibacillus laterosporus*
		Negative	19
19	Acid from glucose	Positive	30
		Negative	24
20	Growth at 65° C	Positive	33
		Negative	21
21	Growth in anaerobic agar	Positive	22
		Negative	*Bacillus azotoformans*
22	Decomposition of casein	Positive	35
		Negative	23
23	Parasporal body in sporangium	Positive	*Paenibacillus popilliae*
		Negative	35
24	Growth at 50° C	Positive	*Bacillus badius*
		Negative	25
25	Growth at 5° C	Positive	*Bacillus insolitus*
		Negative	*Bacillus sphaericus*
26	Acid from arabinose	Positive	*Bacillus lentus*
		Negative	27
27	Growth at 5° C	Positive	30
		Negative	31
28	Growth at 5° C	Positive	*Paenibacillus macquariensis*
		Negative	29
29	Growth in 10% NaCl	Positive	*Virgibacillus pantothenticus*
		Negative	*Bacillus circulans*
30	Hydrolysis of urea	Positive	*Bacillus globisporus*
		Negative	*Bacillus marinus*
31	pH in VP broth > 7	Positive	*Brevibacillus brevis*
		Negative	*Bacillus firmus*
32	Hydrolysis of starch	Positive	33
		Negative	*Bacillus schlegelii*
33	Growth at pH 6.8	Positive	*Bacillus stearothermophilus*
		Negative	*Alicyclobacillus acidocaldarius*
34	Growth in anaerobic agar	Positive	*Bacillus thuringiensis*
		Negative	*Bacillus megaterium*
35	Growth in 10% NaCl	Positive	*Bacillus pasteurii*
		Negative	36
36	Growth at 40° C	Positive	*Paenibacillus larvae*
		Negative	*Paenibacillus lentimorbus*
37	Colony rhizoidal	Positive	*Bacillus mycoides*
		Negative	38
38	Cells motile	Positive	*Bacillus cereus*
		Negative	*Bacillus anthracis*

[a] Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by a species name (based on Claus and Berkeley, 1986; Slepecky and Hemphill, 1992)

Enzymatic activity

Proteolytic activity

Surface-dried plates of milk agar (Gordon *et al.*, 1973) were streaked with 24 h-old cultures, incubated at 30° C for 4 days (lactic acid bacteria) and 37° C for 2 days (spore forming bacteria), and examined for any clearing of casein around and underneath the growth for assessment of proteolytic activity.

Amylolytic activity

Surface-dried plates of starch agar (Gordon *et al.*, 1973) were streaked with 24 h-old cultures, incubated at 30° C for 4 days (lactic acid bacteria) and 37° C for 2 days (spore forming bacteria). After incubation the plates were flooded with iodine solution for 15-30 min and examined the clear zone underneath (after the growth was scrapped off) for amylolytic activity.

Lipolytic activity

Surface-dried plates of tributyrin agar (Stolp and Gadkari, 1981) were streaked with 24 h-old culture and incubated at 30° C for 4 days (lactic acid bacteria) and 37° C for 2 days (spore forming bacteria). Lipolytic activity was detected by a clear zone surrounding the culture in the turbid tributyrin agar (Leuschner *et al.*, 1997).

Protease Activity Assay

Protease activity was measured by a modification of the method of Maeda *et al.* (1993). Cultures were grown in phytone broth (Nagai *et al.*, 1994) on a rotary shaking incubator at 30° C at 180 rev/min for 72 h. Cultures were immediately centrifuged at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. Then, the enzyme solution and the substrate solution containing 1% Azocasein (Sigma Chemical Co., USA) was dissolved in 0.1 M phosphate buffer, (pH 6.8) were pre-incubated separately at 37° C for 5 min in a water-bath incubator (Remi, India). The enzyme reaction was started by adding 2 ml of 1% Azocasein to 1 ml of enzyme solution and incubated at 37° C for 20 min. The reaction was quenched by the addition of 2.5 ml of 10% (w/v) trichloroacetic acid. After centrifugation at 15,000 rpm for 10 min, 2 ml of supernatant was neutralized with equal amount of 1N NaOH and the absorbance was measured at 450 nm in UV-VIS Spectrophotometer (Analytik Jena, Germany). One unit of protease activity was defined as the quantity required to increase the absorbance by 0.1 under the above conditions.

α-Amylase Activity Assay

The blue value method of Fuwa (1954) as modified by Kawaguchi *et al.* (1992) was followed for determination of α-amylase activity. Cultures were grown on broth medium (1.0% soluble starch, 1.0% beef extract, 1.0% peptone, and 0.3% NaCl, pH 7.0) on a rotary shaking incubator at 30° C at 180 rev/min for 48 h. The cultures were immediately centrifuged 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 0.1M Tris-HCl buffer (pH 7.0) were

pre-incubated separately at 37° C for 5 min in water-bath incubator. Then, the reaction mixture was started by adding 1 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 (0.5 N acetic acid-0.5 N HCl 5:1). The 100 ml of the reaction mixture was added to 5 ml of 0.01% I_2 – 0.1% KI solution, left at room temperature for 20 min and the absorbance of the resulting solution was measured at 660 nm in UV-VIS Spectrophotometer (Analytik Jena, Germany). One unit of α-amylase activity (dextrinizing power) was defined as the amount of α-amylase which produced 10% reduction in the intensity of blue colour at the above conditions.

Enzymatic profiles by API-zym system

The enzymatic profile of selected strains of lactic acid bacteria were assayed following the method of Arora *et al.* (1990) uisng 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 broth and growth was harvested in 2 ml sterile distilled water which was used to prepare suspension of 10^7 cells/ml. The API zym 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.

Antimicrobial Activity

Agar Spot Test

The method was based on Schillinger and Lücke (1989) and Uhlman *et al.* (1992). Cultures were grown on the respective broth media for 24 h. Sterilized petriplates were plated with MRS agar (containing 0.2% glucose) and allowed to dry. These were spotted with a drop of the broth culture of the producer strain and incubated at 30° C for 24 h. The indicator strains *Listeria monocytogenes* DSM 20600 and *Bacillus cereus* CCM 2010 were propagated in standard nutrient agar (HiMedia M002), *Enterococcus faecium* DSM 20477 and *Streptococcus mutans* DSM 6178 were cultivated in MRS broth (HiMedia M369). The 0.1 ml of an overnight culture (~10^7 cells) of each indicator strain was inoculated into 7 ml of soft MRS agar (containing 0.7% agar) and poured over the plate on which the producer was grown, respectively. These were incubated at 30 °C for 24 h. After incubation the plates were checked for inhibition zones (clearing of the medium) around the producer colony. Inhibition was scored positive if the width of the clear zone around the colonies of the producer strain was 1 mm or larger.

Bacteriocin Activity

Bacteriocin activity was estimated using an agar spot assay as described by Schillinger *et al.* (1993). The antimicrobial-positive strains were grown in MRS broth at 30° C for 24 h and a cell-free extract was obtained by centrifuging the culture in a microcentrifuge (Heraeus,

Germany). The supernatant was heated at 100° C for 5 min in blockthermostat (Staurt Scientific, UK). The cell-free supernatant was adjusted to pH 6.5 by addition of 1 N NaOH. Agar plates overlaid with 7 ml soft MRS agar (containing 0.7% agar) were inoculated with 0.1 ml of an overnight culture of the indicator strains (as mentioned above), respectively. After incubation at 25° C for 24 h, 0.01 ml of the culture supernatant was spotted onto the agar surface. The plates were incubated at 30° C for 24 h and subsequently examined for zones of inhibition.

Biogenic Amine

The ability to produce biogenic amines was determined qualitatively on an improved screening medium as described by Bover-Cid and Holzapfel (1999) using a 'cocktail' of four precursor amino acids (histidine, lysine, ornithine and tyrosine). Freshly grown cultures were sub-cultured in 5 ml of biogenic amine Sub-culturing medium (Bover-Cid and Holzapfel, 1999) twice and incubated at 30° C for lactic acid bacteria and 37° C for *Bacillus* for 24 h to promote decarboxylase activity. The modified biogenic amine screening medium of Joosten and Northold (1989) (Bover-Cid and Holzapfel, 1999) was prepared, poured on to sterilized Petri-plates and allowed to dry. Bromocresol purple was used as pH indicator. These plates were streaked with the broth cultures in duplicates and incubated at 30° C for lactic acid bacteria and 37° C for *Bacillus* species for 4 days under aerobic and anaerobic conditions. Control plate lacked the amino acid. After incubation observation for positive reaction was made by the purple coloration of the colony and in case of tyramine production a clear halo due to tyrosine precipitate disappearance.

Hydrophobicity

The degree of hydrophobicity of the strains was determined by employing the methods described by Rosenberg (1984) and Ding and Lämmler (1992). These methods were based on adhesion of cells to hexadecane droplets. Cultures were grown in 5 ml of MRS broth (HeMedia M369). The 4 ml of this broth culture was centrifuged at 7,500 rpm for 5 min and the supernatant was discarded. The cell pellet was washed with 9 ml of Ringer solution (Merck), resuspended in a cylomixer and again centrifuged at 7,500 rpm for 5 min. The supernatant was again discarded; the cell pellet was washed with 9 ml of Ringer solution (Merck) and resuspended in a cylomixer. The 1 ml of this suspension was taken and the absorbance at 580 nm was measured in UV-VIS Spectrophotometer (Analytik Jena, Germany). The 1.5 ml of the suspension was mixed with 1.5 ml of n-Hexadecane (HiMedia RM 2238) in duplicates and mixed thoroughly in a cylomixer for 2 min. The two phases were allowed to separate for 30 min. The 1 ml of the lower phase was taken and the absorbance was measured at 580 nm in UV-VIS Spectrophotometer. The percentage hydrophobicity of strain adhering to hexadecane was calculated using the equation:

$$\text{Hydrophobicity (\%)} = \frac{OD_{580}\,(\text{initial}) - OD_{580}\,(\text{with hexadecane})}{OD_{580}\,(\text{initial})} \times 100$$

Adherence value greater than 75% were considered hydrophobic, less than 25% as hydrophilic and those between 25% and 75% as intermediate.

Proximate composition

pH

Ten g of sample was mixed with 20 ml carbon dioxide-free distilled water in a blender for 1 min and the pH of the slurry was determined directly (AOAC, 1990) using a μ pH meter (Systronics, Type.361) calibrated with standard buffer solutions (Merck).

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).

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).

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.

Protein

Total nitrogen of sample was determined following the method described in AOAC (1990). 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.

Total nitrogen (%) =

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

Protein content was determined by multiplying total nitrogen value with 6.38 (AOAC, 1990).

Protein (%) = Total Nitrogen (%) × 6.38

Fat

Fat content was determined by ether extraction using glass soxhlet (AOAC, 1990). Flat-bottomed flask was oven dried and kept in a 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 with boiling range 40-60° C, on a 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.

$$\text{Fat (\%)} = W_2 - W_1/\text{Sample weight} \times 100$$

Minerals

The method was based on AOAC (1990). The ash after heating the sample 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, iron, magnesium, manganese and zinc was estimated in an atomic absorption spectrophotometer (Model 3110, Perkin-Elmer).