3
Materials and Methods

3.1. Materials

3.1.1. Culture media

Bacillus cereus selective agar
Bacillus cereus agar base (M833; HiMedia Laboratories Pvt Limited, India)
Polymyxin B selective supplement (HiMedia FD003)
Egg yolk emulsion (HiMedia FD045)

Baird-Parker agar
Baid-Parker agar base (HiMedia M043)
Potassium tellurite 3.5% (w/v) (HiMedia FD047)
Egg yolk emulsion (HiMedia FD045)

Brilliant green bile broth 2% (HiMedia M121)

Coagulase mannitol broth base (HiMedia M277)

DNase test agar with toluidine blue (HiMedia M1041)

HiCrome nickels and Leesment medium (HiMedia M1712)

Lactobacillus MRS agar (HiMedia M641)

Lactobacillus MRS broth (HiMedia M369)

MacConkey agar (HiMedia M008)

MRS - 0.2 agar (Schillinger and Lücke, 1989)
MRS - 0.2 broth supplemented with 1.5% (w/v) agar

MRS - 0.2 broth

Peptone	10.0 g
Meat extract	10.0 g
Yeast extract	5.0 g
K_2HPO_4	2.0 g
Diammonium citrate	2.0 g
Glucose	2.0 g
Tween 80	1.0 g
Sodium acetate	5.0 g
$MgSO_4.7H_2O$	100.0 mg
$MnSO_4.4H_2O$	50.0 mg
Distilled water	1000 ml
pH 6.2-6.4	

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

Nitrate broth (Claus and Berkeley, 1986)

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

Nutrient agar (HiMedia M561)

Nutrient broth (HiMedia M002)

Perfringens agar
Perfringens agar base (HiMedia M579)
Perfringens supplement I (HiMedia FD011)
Perfringens supplement II (HiMedia FD012)

Plate count agar (HiMedia M091A)

Purple agar
Purple agar base (HiMedia M098)
Glucose (5-10 g/l)

Tryptone soya agar (HiMedia M290)

Tryptone water (HiMedia M4631)

Violet red bile glucose agar w/o lactose (HiMedia M581)

Voges-Proskauer broth (Claus and Berkeley, 1986)

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

All the media mentioned above were sterilized by autoclaving at 1.1 kg/cm^2 pressure for 15 min, unless mentioned otherwise.

3.1.2. Reagents

Ehrlich-Böhme reagent

p-Dimethylaminobenzaldehyde	1.0 g
95% (v/v) ethanol	95.0 ml
Conc. HCl	20.0 ml

Nitrate reagent (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

a-Napthylamine	0.5 g
5N Acetic acid	100 ml

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

Peptone physiological saline

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

All the chemicals used were of highest purity grade.

3.1.3. Reference strains

The reference strains used in this study were *Bacillus cereus* s.l. ATCC 9139, *Bacillus subtilis* MTCC 1747 (DK-W1), *Escherichia coli* MTCC 119, *Staphylococcus aureus* MTCC 1430 and *Lactobacillus plantarum* MTCC 1407. These cultures were obtained from the Microbial Culture Collection of the Department of Botany, University of North Bengal.

3.2. Experimental

3.2.1. Survey

A moderate survey in various parts of India such as Bihar, Delhi, Maharashtra, Punjab, Sikkim, Tamil Nadu, Uttar Pradesh and West Bengal was conducted. The aim of this survey was to collect information about the various traditional legume-based foods, their consumption, popularity and methods of preparation.

3.2.2. Sampling

Samples for the study included market samples (Table 5), raw ingredients required for the preparation of some selected legume-based foods, freshly prepared intermediate products and the final products (Table 6).

Table 5. Sampling of legume-based food products

Date of collection	Sample No.	Kind of product	Place of collection	State	Open/Pkd (L/B)*
07.07.08	S1	Sattoo	Bidhan Market, Siliguri	West Bengal	Open
14.07.08	S2	Sattoo	Hakimpara, Siliguri	West Bengal	Pkd (L)
14.07.08	S3	Sattoo	Rathkhola, Siliguri	West Bengal	Pkd (L)
10.08.08	S4	Sattoo	P.B. Road, Kurseong	West Bengal	Open
11.08.08	S5	Sattoo	Shivmandir, Kadamtala	West Bengal	Pkd (L)
11.08.08	S6	Sattoo	Nimtala, Rangia	West Bengal	Pkd (L)
13.08.08	S7	Sattoo	Bangachatra Road, Cooch Behar	West Bengal	Pkd (B)
13.08.08	S8	Sattoo	Dhupguri Market, Dhupguri	West Bengal	Pkd (L)
13.08.08	S9	Besan	Dhupguri Market, Dhupguri	West Bengal	Open
13.08.08	S10	Besan	Bangachatra Rd, Cooch Behar	West Bengal	Pkd (B)
25.08.08	S11	Besan	Hakimpara, Siliguri	West Bengal	Open
25.08.08	S12	Besan	Shivmandir, Kadamtala	West Bengal	Open
25.08.08	S13	Papad	Khalpara, Siliguri	West Bengal	Pkd (L)
25.08.08	S14	Papad	Khalpara, Siliguri	West Bengal	Pkd (B)
25.08.08	S15	Sattoo	Raiganj	West Bengal	Pkd (L)
25.08.08	S16	Besan	Raiganj	West Bengal	Pkd (B)
26.08.08	S17	Besan	Kishanganj	West Bengal	Open
26.08.08	S18	Sattoo	Kishanganj	West Bengal	Pkd (B)
26.08.08	S19	Papad	Kishanganj	West Bengal	Pkd (B)
08.09.08	S20	Besan	Balurghat	West Bengal	Open
08.09.08	S21	Besan	Balurghat	West Bengal	Open
08.09.08	S22	Sattoo	Balurghat	West Bengal	Pkd (L)
12.09.08	S23	Sattoo	Shyamnagar, Kolkata	West Bengal	Pkd (B)
12.09.08	S24	Sattoo	Sealdah, Kolkata	West Bengal	Open
12.09.08	S25	Besan	Sealdah, Kolkata	West Bengal	Open
12.09.08	S26	Besan	Shyamnagar, Kolkata	West Bengal	Pkd (B)
12.09.08	S27	Besan	Gangtok	Sikkim	Open
12.09.08	S28	Sattoo	Gangtok	Sikkim	Pkd (L)
12.09.08	S29	Papad	Gangtok	Sikkim	Pkd (B)
20.09.08	S30	Sattoo	Chembur, Mumbai	Maharashtra	Pkd (L)
20.09.08	S31	Besan	Chembur, Mumbai	Maharashtra	Pkd (L)
03.11.08	S32	Besan	Amritsar	Punjab	Open
03.11.08	S33	Papad	Amritsar	Punjab	Open
03.11.08	S34	Besan	Pahar ganj bazaar, Delhi	Delhi	Open
03.11.08	S35	Papad	Pahar ganj bazaar	Delhi	Open
22.11.08	S36	Wadi	Bangachatra Rd, Cooch Behar	West Bengal	Open
22.11.08	S37	Sattoo	Allahabad	Uttar Pradesh	Pkd (B)
22.11.08	S38	Besan	Allahabad	Uttar Pradesh	Open
01.12.08	S39	Wadi	Matigara	West Bengal	Open
01.12.08	S40	Wadi	Dim Bazaar, Jalpaiguri	West Bengal	Pkd (L)

Date of collection	Sample No.	Kind of product	Place of collection	State	Open/Pkd (L/B)*
02. 05.09	S41	Wadi	Bidhan Market, Siliguri	West Bengal	Pkd (L)
02. 05.09	S42	Wadi	Balurghat	West Bengal	Open
02. 05.09	S43	Wadi	Haat Bazaar, Kurseong	West Bengal	Open
02. 05.09	S44	Papad	Bhuvaneswar	Orissa	Pkd (B)
02. 05.09	S45	Papad	Guwahati	Assam	Pkd (B)
02. 05.09	S46	Papad	Ahmedabad	Gujarat	Open
11. 05.09	S47	Papad	Bengaluru	Karnataka	Pkd (B)
11. 05.09	S48	Wadi	Guwahati	Assam	Pkd (L)
11. 05.09	S49	Wadi	Imphal	Manipur	Pkd (L)
11. 05.09	S50	Wadi	Imphal	Manipur	Pkd (L)
11. 05.09	S51	Wadi	Hyderpur, Malda	West Bengal	Open
20. 05.09	S52	Dosa	Court More, Siliguri	West Bengal	Open
20. 05.09	S53	Dosa	Hakimpara, Siliguri	West Bengal	Open
20. 05.09	S54	Dosa	Seth Srilal Market, Siliguri	West Bengal	Open
20. 05.09	S55	Idli	Court More, Siliguri	West Bengal	Open
20. 05.09	S56	Idli	Hakimpara, Siliguri	West Bengal	Open
20. 05.09	S57	Idli	Seth Srilal Market, Siliguri	West Bengal	Open
30. 05.09	S58	Dosa	Shivmandir, Kadamtala	West Bengal	Open
30. 05.09	S59	Dosa	Shivmandir, Kadamtala	West Bengal	Open
30. 05.09	S60	Dosa	Airport More, Bagdogra	West Bengal	Open
30. 05.09	S61	Idli	Shivmandir, Kadamtala	West Bengal	Open
30. 05.09	S62	Idli	Shivmandir, Kadamtala	West Bengal	Open
30. 05.09	S63	Idli	Airport More, Bagdogra	West Bengal	Open
10.06.09	S64	Dhokla	Hakimpara, Siliguri	West Bengal	Open
10.06.09	S65	Dhokla	Airview More, Siliguri	West Bengal	Open
10.06.09	S66	Dhokla	Bidhan Market, Siliguri	West Bengal	Open
20.06.09	S67	Dhokla	Shivmandir, Kadamtala	West Bengal	Open
20.06.09	S68	Dhokla	Sevoke More, Siliguri	West Bengal	Open
20.06.09	S69	Dhokla	Hakimpara, Siliguri	West Bengal	Open
12.03.12	S70	Kinema	Haat Bazaar, Kurseong	West Bengal	Open
12.03.12	S71	Kinema	Bardhaman Road, Kurseong	West Bengal	Open
12.03.12	S72	Kinema	Dumaram Busty, Kurseong	West Bengal	Open

* Pkd, packed; L, locally packed; B, branded

A total of 72 samples of eight different kinds of legume-based traditional foods were collected from different market sources of India. Among the samples analyzed, there were 15 samples of besan, 6 samples each of dhokla, dosa and idli, 3 samples of kinema, 10 samples of papad, 16 samples of sattoo and 10 samples of wadi (Table 5).

Besides the market samples, three kinds of legume-based foods namely, sattoo, idli and wadi (Table 6) were collected during the processing stages from different sites of production. About 250 g of each sample was collected aseptically in sterile Nasco sampling bags (HiMedia PW389) or bottles from different markets and sites of production and transported immediately to the laboratory in an ice-box for analyses.

Table 6. Collection of samples at different stages of processing

Date of collection	Kind of food	Sample No.	Processing stage	Place of collection
16.11.08	Sattoo	St-1a	Raw Bengal gram	Bidhan Market, Siliguri
16.11.08	Sattoo	St-1b	Roasted Bengal gram	Bidhan Market, Siliguri
16.11.08	Sattoo	St-1c	Bengal gram dal	Bidhan Market, Siliguri
16.11.08	Sattoo	St-1d	Sattoo	Bidhan Market, Siliguri
22.12.08	Sattoo	St-2a	Bengal gram	Bidhan Market, Siliguri
22.12.08	Sattoo	St-2b	Roasted Bengal gram	Bidhan Market, Siliguri
22.12.08	Sattoo	St-2c	Bengal gram dal	Bidhan Market, Siliguri
22.12.08	Sattoo	St-2d	Sattoo	Bidhan Market, Siliguri
05.01.09	Sattoo	St-3a	Bengal gram	Bidhan Market, Siliguri
05.01.09	Sattoo	St-3b	Roasted Bengal gram	Bidhan Market, Siliguri
05.01.09	Sattoo	St-3c	Bengal gram dal	Bidhan Market, Siliguri
05.01.09	Sattoo	St-3d	Sattoo	Bidhan Market, Siliguri
02.02.09	Wadi	Wa-1a	Black gram dal	Matigara
02.02.09	Wadi	Wa-1b	Soaked black gram dal	Matigara
02.02.09	Wadi	Wa-1c	Batter	Matigara
02.02.09	Wadi	Wa-1d	Wadi	Matigara
09.02.09	Wadi	Wa-2a	Black gram dal	Matigara
09.02.09	Wadi	Wa-2b	Soaked black gram dal	Matigara
09.02.09	Wadi	Wa-2c	Batter	Matigara
09.02.09	Wadi	Wa-2d	Wadi	Matigara
18.02.09	Wadi	Wa-3a	Black gram dal	Matigara
18.02.09	Wadi	Wa-3b	Soaked black gram dal	Matigara
18.02.09	Wadi	Wa-3c	Batter	Matigara
18.02.09	Wadi	Wa-3d	Wadi	Matigara
02.04.09	Idli	Id-1a	Black gram dal	Airport More, Bagdogra
02.04.09	Idli	Id-1b	Soaked black gram dal	Airport More, Bagdogra
02.04.09	Idli	Id-1c	Rice	Airport More, Bagdogra
02.04.09	Idli	Id-1d	Soaked rice	Airport More, Bagdogra
02.04.09	Idli	Id-1e	Mixed batter	Airport More, Bagdogra
03.04.09	Idli	Id-1f	Fermented batter	Airport More, Bagdogra
03.04.09	Idli	Id-1g	Idli	Airport More, Bagdogra
13.04.09	Idli	Id-2a	Black gram dal	Court More, Siliguri
13.04.09	Idli	Id-2b	Soaked black gram dal	Court More, Siliguri,
13.04.09	Idli	Id-2c	Rice	Court More, Siliguri
13.04.09	Idli	Id-2d	Soaked rice	Court More, Siliguri
13.04.09	Idli	Id-2e	Mixed batter	Court More, Siliguri
14.04.09	Idli	Id-2f	Fermented batter	Court More, Siliguri
14.04.09	Idli	Id-2g	Idli	Court More, Siliguri
20.04.09	Idli	Id-3a	Black gram dal	Seth Srilal Market, Siliguri
20.04.09	Idli	Id-3b	Soaked black gram dal	Seth Srilal Market, Siliguri,
20.04.09	Idli	Id-3c	Rice	Seth Srilal Market, Siliguri
20.04.09	Idli	Id-3d	Soaked rice	Seth Srilal Market, Siliguri
20.04.09	Idli	Id-3e	Mixed batter	Seth Srilal Market, Siliguri
21.04.09	Idli	Id-3f	Fermented batter	Seth Srilal Market, Siliguri
21.04.09	Idli	Id-3g	Idli	Seth Srilal Market, Siliguri

3.2.3. Physicochemical analyses

3.2.3.1. Moisture content

Approximately 10 g of sample was accurately weighed 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, 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).

3.2.3.2. pH

Approximately 10 g of sample was mixed with 20-30 ml of CO_2-free distilled water and the pH was noted using a CyberScan pH 510 meter (Eutech Instruments Pvt. Ltd., Singapore).

3.2.3.3. a_w

Approximately 3 g of sample was taken in an a_w measuring container and readings recorded using a_w measuring system (Novasina model ms1-a_w, Axair Ltd., Pfäffikon, Switzerland) and sample sensor (Novasina model enBSK, Axair Ltd.).

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 sodium hydroxide (Merck 61757305001046) solution to an end point of phenolphthalein (0.1% w/v phenolphthalein in 95% v/v ethanol) (AOAC, 1990).

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

3.2.3.5. Free amino nitrogen

A 5 g sample was homogenized (RQ-122, Remi Laboratory Instruments, Mumbai, India) in distilled water to make up the final volume up to 20 ml and pH of that suspension was measured. Using 0.1 M NaOH the pH of this suspension placed over a magnetic stirrer was gradually increased to 8.5 after which 10 ml of 35% v/v formaldehyde (Merck 61780805001730) was added and the suspension stirred for 2 min. Stirring resulted in the release of protons which were titrated against 0.1 M NaOH. When the final pH of the suspension reached 8.5, it marked the end-point of the titration. Free amino nitrogen was calculated on the basis that 1 mol of NaOH is equivalent to 1 mol of amino group (Nout *et al*., 1998).

3.2.3.6. Proteolytic activity

In this assay, 3 g of sample were homogenized for 15 s with 5 ml of 0.05 M potassium phosphate buffer and the homogenate centrifuged at 13,000xg for 2 min. The supernatant

(0.12 ml) was a component of the assay mixture which contained 0.25 ml of azocasein (Sigma A2765; 2.5 g/l of 0.05 M potassium phosphate buffer, pH 7.0). The mixture in an Eppendorf tube was incubated at 37°C for 1 h after which the reaction was stopped by adding 0.75 ml cold 3 M trichloroacetic acid (Merck 82234205001730). The assay mixture was allowed to stand for another 1 h at 4°C during which the undigested proteins sedimented at the bottom and removed by centrifuging at 13,000xg for 10 min. A 0.5 ml of the resulting supernatant was mixed with 2 ml of purified water and absorbance measured at 400 nm using a spectrophotometer (type 166; Systronics, Ahmedabad, India) to detect the presence of any free dye. One unit of proteolytic activity was defined as the amount which produced an absorbance increase of 0.01 unit under the assay conditions (Sarkar *et al*., 1993).

3.2.4. Bacteriological analyses

3.2.4.1. Isolation of bacteria

About 10 g of each sample was aseptically weighed and homogenized with 90 ml of sterile PPS using a Stomacher lab-blender 400 (Seward Medical, London, UK) at 'normal' speed for 1 min. Serial decimal dilutions were made with the same diluent, and plating was done using appropriate dilutions. For pour plating, 1 ml of the dilutions was mixed with molten media and poured into plates. For surface seeding, 0.1 ml of the dilutions was spread on the surface of the dried media in plates. After incubation at appropriate temperatures, the colonies appearing on the plates were counted as cfu per gram fresh weight sample. The representative colonies of each type were picked and diluted by streaking out on plates of appropriate media. The purified colonies were observed microscopically and grown on slants of NA and stored at 4°C.

3.2.4.1.1. TAMB

The viable counts of TAMB were carried out in pour-plates of PCA which were incubated at 35°C for 18-24 h.

3.2.4.1.2. Mesophilic bacterial spores

For enumeration of aerobic mesophilic bacterial spores, 100 ml sample/l suspension was heated at 80°C for 30 min, suitably diluted and spread on PCA plates followed by incubation at 30°C for 72 h. For the anaerobic sporeformers the suspension was pour-plated with perfringens agar followed by incubation at 37°C for 48 h in an anaerobic jar with AnaeroHiGas Pack (HiMedia LE002A) (Kneifel and Berger, 1994).

3.2.4.1.3. *Bacillus cereus* group

Bacillus cereus group (s.l.) was enumerated on spread-plates of BCSA which was incubated at 35°C for 24-48 h (HiMedia, 1998). The presumptive colonies were purified on BCSA and finally on NA (Nout *et al*., 1998).

3.2.4.1.4. Enterobacteriaceae

Estimation of Enterobacteriaceae was carried out by pour-plating on TSA and incubating the plates for 1-2 h at room temperature followed by an overlay of VRBGA

without lactose and incubated at 35ºC for 18-24 h (HiMedia, 1998). The representative colonies were purified on TSA and finally on NA (Nout *et al*., 1998).

3.2.4.1.5. *Staphylococcus aureus*

Staphylococcus aureus was isolated on spread-plates of BPA that were incubated at 35ºC for 24-48 h (HiMedia, 1998). Representative colonies were purified on BPA and finally on NA (Nout *et al*., 1998).

3.2.4.1.6. LAB

LAB were enumerated in pour plates of Lactobacillus MRS agar, incubated at 37ºC for 48-72 h in an anaerobic jar with AnaeroHiGas pack. These bacteria were enumerated on spread plates of HiCrome Nickels and Leesment medium, incubated at 25-30ºC for 48-72 h.

3.2.4.2. Maintenance of pure cultures

All the isolates were maintained on NA slants except the LAB isolates that were maintained on MRS agar slants. The slants were stored at 4ºC.

3.2.4.3. Confirmatory tests

3.2.4.3.1. Gram staining

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 make smear. It was air-dried and heat-fixed, flooded with crystal violet stain (Merck 9218) for 1 min and washed for 5 s with water. The smear was flooded with Gram's iodine solution (HiMedia R044), allowed to react for 1 min, and washed again for 5 s with water. Then, 95% (v/v) ethanol (Merck 1.00983.0511) 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 (Merck 9127) for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

3.2.4.3.2. Motility

A hanging drop in a cavity slide was prepared by 24 h-old cultures in NB. The drop was observed using a phase-contrast microscope (BH2-PC-PA-1, Olympus, Tokyo, Japan).

3.2.4.3.3. Endospore

Six day-old cultures on NA at 30°C were observed under a phase-contrast microscope for endospore formation.

3.2.4.3.4. Glucose fermentation

Tubes containing 10 ml of purple agar base medium with 5-10 g sterile glucose/l medium were stabbed with the 24 h-old isolates and incubated at 30°C for 7 days. A change in colour from purple to yellow indicated the production of acids and cracking of the medium indicated gas production.

3.2.4.3.5. Nitrate reduction

24 h-old cultures were inoculated into 10 ml nitrate broth and incubated at 30°C. After 3, 7 and 14 days, 1 ml of the culture was mixed with 3 drops of nitrate reagent and observed for the development of a red or yellow colour. The latter indicated the presence of nitrite as a result of nitrate reduction. A small amount of zinc dust was added to the tubes that was negative even after 14 days of incubation and observed for the development of red colour, indicating the absence of reduction (Norris *et al*., 1981).

3.2.4.3.6. VP reaction

Cultures (24 h-old) were grown in 10 ml VP broth (Gordon *et al*., 1973) and incubated at 30°C for 3, 5 and 7 days. After incubation, 3 ml of 40% KOH (Merck 61781005001046) and 0.5-1.0 mg creatine monohydrate (HiMedia RM161) were added and shaken thoroughly for the production of pink colour, indicating the positive reaction.

3.2.4.3.7. Oxidase test

A well-isolated colony was evenly spread on an oxidase disc (HiMedia DD018) and observed for the formation of any deep purple or blue colouration within a few minutes at 25-30°C (HiMedia, 1998).

3.2.4.3.8. Lactose fermentation in presence of bile

Confirmed Enterobacteriaceae isolates were inoculated into 5 ml BGBB in culture tubes containing inverted Durham tubes and incubated at 37°C for 24-48 h. Production of acid and gas confirmed the presence of coliforms. To confirm the presence of faecal coliforms, BGBB tubes with inverted Durham tubes were inoculated with confirmed coliforms and incubated at 44°C for 24 h and examined for growth and gas production (Nout *et al*., 1998).

3.2.4.3.9. Production of indole

Tryptone water (5 ml) was inoculated with 24 h-old cultures and incubated at 30°C. Ehrlich-Böhme reagent (1-2 ml) was gently poured over the 3, 5 and 7 days-old broth culture and observed for the formation of red ring at culture-reagent interface (Ishrawan, 1980).

3.2.4.3.10. Coagulase and mannitol fermentation

Coagulase mannitol broth base (5 ml), containing pretested EDTA; HiMedia RM678)-treated rabbit plasma (150 ml plasma/l broth base) was inoculated with 2 drops of 24 h-old culture in NB and incubated at 35°C. Coagulase activity in which the broth turned opaque due to coagulated plasma was observed after 2-5 h incubation. Mannitol fermentation in which a change in colour from red-orange to yellow occured was examined after 18-48 h at 35°C (HiMedia, 1998).

3.2.4.3.11. Thermostable DNase

DNase test agar with toluidine blue was spotted with a 10 min-boiled culture supernatant,

incubated at 35°C for 18-24 h and examined for the formation of a bright pink zone around the colony (HiMedia, 1998).

3.2.4.3.12. Production of catalase

A 24 h-old slant culture was flooded with 0.5 ml of 10% (v/v) hydrogen peroxide (Merck 61765305001046) solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al*., 1981).

3.2.5. Response surface optimization of different food process parameters

Experiments were performed according to the experimental design generated by the software MINITAB 15.1.1.0. (Minitab Inc., State College, PA) and the results analyzed to obtain the optimum processing conditions.

3.2.5.1. Optimization of soaking conditions of blackgram

3.2.5.1.1. Soaking

Blackgram dal, purchased from a local market in Siliguri, was soaked in distilled water under different conditions as per the experimental design. The pH was adjusted to the desired values using 0.1 N acetic acid (Merck 61780705001046) and 0.1 N sodium hydroxide. The temperatures were controlled by placing the soaked samples in incubators set at the desired levels.

3.2.5.1.2. Preparation of sample

The soaked beans were ground to a fine paste, frozen overnight at -20°C and lyophilized (Eyela freeze dryer, model FDU-506, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The dried product was ground to fine powder which was then defatted with distilled petroleum ether (Merck 61782225001730) in Soxhlet extractor. The extract was evaporated at below 45°C in a rotary vacuum evaporator and quantified gravimetrically to use for fat correction during calculation of the RFO content of defatted samples on dry weight basis.

Defatting was followed by deproteination and RFO extraction by a procedure based on that of Knudsen (1986). Approximately 1 g of the defatted sample was mixed with 10 ml water and brought just to boiling. The mixture was shaken in a 60°C water bath for 5 min, made up to 10 ml with distilled water, and centrifuged at 1,100xg for 10 min. The supernatant (3.5 ml) was mixed thoroughly with 6.5 ml acetonitrile (HPLC grade SDFCL 25251 L25) and left overnight at 4°C. After filtering through a G3 sintered glass filter, an aliquot of the filtrate was placed in a 5-ml glass vial for HPLC analysis.

3.2.5.1.3. Chromatographic analysis

The estimation of RFOs was done following the method described by Sarkar *et al*. (1997). The chromatographic system (Waters Associates, Milford, MA, USA) consisted of Waters isocratic 515 pump, a Rheodyne manual injector equipped with a 5-µl sample loop, a Waters column heating attachment, a Waters carbohydrate column (3.9 mm i.d. x 30 cm), a Waters column heating arrangement, a Waters guard-pak column, and a refractive index detector model 2414 (Waters). The mobile phase used for elution was acetonitrile-water (HPLC grade Merck 61765010001730) (65:35, v/v) and the flow rate was constant

at 1.0 ml/min. The column and detector were maintained at 31°C. The run time for the chromatogram was maintained for 15 min.

Identification and quantification of the sugars present in the samples were done by comparing each peak retention time and area with those of the standards. The quantity of each sugar was corrected based on the recovery ratio of the internal standard. The standard sugars used were D(+)-sucrose (Fluka, Germany, 84100), D(+)-raffinose (Fluka, Japan 83400), stachyose (Sigma, UK, S4001) and verbascose (Fluka, Ireland, 56217). D(+)-xylose (Sigma, China, 95729) was used as an internal standard since it does not interfere with the RFOs. The chromatographic data were collected and plotted using Waters Empower 2 software.

3.2.5.1.4. Experimental design

RSM was used for optimizing the soaking conditions of raw blackgram dal and a CCRD was used to study the effect of four independent variables (bean-water ratio, and soaking temperature, time and pH) on the total RFO content of the soaked beans. The experimental data were fitted to a quadratic model using the least square regression (Kong *et al*., 2010).

Table 7. Variables and their levels for the experimental design

Independent variable	Symbol coded	Level[a]				
		+2	0	-2	+1	-1
Bean:water ratio	X_1	1:10	1:6.5	1:3	1:8.25	1:4.75
Temperature (°C)	X_2	35	25	15	30	20
Time (h)	X_3	24	12	0	18	6
pH	X_4	8	6	4	7	5

[a] Corner (±1), central (0) and axial (±2).

The design comprised of 31 experimental runs with 16 factorial points, 8 axial points at a distance of 2 from the design centre and 7 replicates at the centre point. Each experimental run was conducted in triplicate. The variables optimized were bean-water ratio (1:3-1:10, w/v), temperature (15-35°C), time (0-24 h), and pH (4.0-8.0) each at five levels, viz. -2, -1, 0, +1, and +2. The corner (±1), central (0), and axial (±2) levels of each variable were designated as +1 (1:8.25, 30°C, 18 h, pH 7.0), -1 (1:4.75, 20°C, 6 h, pH 5.0), 0 (1:6.5, 25°C, 12 h, pH 6.0), +2 (1:10, 35°C, 24 h, pH 8.0), and -2 (1:3,15°C, 0 h, pH 4.0), respectively. Table 7 shows the levels of variables and codes used in optimizing the soaking conditions of blackgram. The coded values and the factor levels used in the analysis are given in Table 8. Raffinose, stachyose, verbascose and ajugose profiles of the soaked beans under different soaking conditions were studied and quantified, and RSM plots were constructed based on the total RFO content present in the soaked beans.

3.2.5.2. Optimization of mechanized drying of wadi

3.2.5.2.1. Preparation of wadi

For the optimization process, the traditional method of preparing wadi was slightly modified. The recent trend in its preparation eliminates the natural fermentation for 18-

Table 8. CCRD arrangement for soaking of blackgram

Run order	Bean:water (w/v)[a]	Temp. (°C)[a]	Time (h)[a]	pH[a]
1	1:6.5 (0)	25 (0)	12 (0)	6 (0)
2	1:6.5 (0)	25 (0)	0 (-α)	6 (0)
3	1:4.75 (-1)	20 (-1)	6 (-1)	5 (-1)
4	1:8.25 (+1)	20 (-1)	18 (+1)	7 (+1)
5	1:6.5 (0)	25 (0)	12 (0)	4 (-α)
6	1:4.75 (-1)	20 (-1)	18 (+1)	5 (-1)
7	1:6.5 (0)	15 (-α)	12 (0)	6 (0)
8	1:8.25 (+1)	30 (+1)	18 (+1)	5 (-1)
9	1:4.75 (-1)	30 (+1)	6 (-1)	7 (+1)
10	1:4.75 (-1)	30 (+1)	18 (+1)	7 (+1)
11	1:8.25 (+1)	20 (-1)	6 (-1)	5 (-1)
12	1:8.25 (+1)	30 (+1)	6 (-1)	5 (-1)
13	1:6.5 (0)	25 (0)	12 (0)	6 (0)
14	1:4.75 (-1)	30 (+1)	6 (-1)	5 (-1)
15	1:8.25 (+1)	30 (+1)	6 (-1)	7 (+1)
16	1:6.5 (0)	25 (0)	12 (0)	6 (0)
17	1:6.5 (0)	25 (0)	12 (0)	6 (0)
18	1:4.75 (-1)	20 (-1)	18 (+1)	7 (+1)
19	1:3 (-α)	25 (0)	12 (0)	6 (0)
20	1:8.25 (+1)	30 (+1)	18 (+1)	7 (+1)
21	1:4.75 (-1)	30 (+1)	18 (+1)	5 (-1)
22	1:6.5 (0)	25 (0)	12 (0)	6 (0)
23	1:8.25 (+1)	20 (-1)	6 (-1)	7 (+1)
24	1:4.75 (-1)	20 (-1)	6 (-1)	7 (+1)
25	1:6.5 (0)	25 (0)	12 (0)	6 (0)
26	1:6.5 (0)	25 (0)	24 (+α)	6 (0)
27	1:6.5 (0)	25 (0)	12 (0)	6 (0)
28	1:6.5 (0)	25 (0)	12 (0)	8 (+α)
29	1:10 (+α)	25 (0)	12 (0)	6 (0)
30	1:8.25 (+1)	20 (-1)	18 (+1)	5 (-1)
31	1:6.5 (0)	35 (+α)	12 (0)	6 (0)

[a] Corner (±1), central (0) and axial (±2) levels of each variable are designated as +1, -1, 0, +α, -α.

24 h as fermentation accompanies drying. Blackgram dal was washed and soaked in tap water for 10 h and ground to a smooth thick batter. This batter was hand-beaten for about 30 min for proper aeration, hand-moulded into cones of varied sizes as per the experimental design and placed over nylon meshes. While placing the batter, a cardboard with circles of various diameters drawn on it as per the experimental design was placed below the mesh. To obtain a cone of the desired size, known amount of batter was carefully placed on the mesh within the boundary of the circle drawn on the board below. The cardboard was removed once the cones were placed on the mesh and ready to be air-dried inside a dehumidifying chamber (Bry-Air model FFB-170, Bry-Air (Asia) Pvt. Ltd., Gurgaon, India), set at different RH levels for varying time periods. The RH inside the dehumidifying chamber was verified using a hygrometer, and the desired levels of RH inside the chamber were controlled using a humidistat.

3.2.5.2.2. Experimental design

A 5-level, three-factor CCRD was used for investigating the effect of three independent variables, viz. RH, time and size in diameter on the response variables, viz. OAA and a_w of wadi. The design comprised of 20 experimental runs having 8 factorial points, 6 axial points at a distance of 1.68 from the design centre and 6 replicates at the centre point. All the experimental runs were conducted in triplicate. The variables that were optimized were RH (20–65%), time (24–120 h) and diameter(15–35 mm) each at five levels, viz. -1.6818, -1, 0, +1 and +1.6818. The corner (±1.6818), central (0) and axial (±1) levels of each variable were designated as -1 (29.12% RH, 43.46 h, 19.05 mm), +1 (55.88% RH, 100.54 h, 30.95 mm), 0 (42.50% RH, 72 h, 25 mm), +1.6818 (65% RH, 120 h, 35 mm) and -1.6818 (20% RH, 24 h, 15 mm), respectively. Table 9 shows the levels of variables and codes used in the determination of optimum drying conditions for wadi. The coded values and the factor levels used in the analysis are given in Table 10.

Table 9. Variables and their levels for the experimental design

Independent variable	Symbol coded	Level[a]				
		+1	0	-1	+1.6818	-1.6818
Relative humidity (%)	X_1	55.88	42.5	29.12	65	20
Time (h)	X_2	100.54	72	43.46	120	24
Dia (mm)	X_3	30.95	25	19.05	35	15

[a] Corner (±1.6818), central (0) and axial (±1).

Table 10. CCRD for mechanized drying of wadi

Experimental order	RH[a] (%)	Time[a] (h)	Diameter[a] (mm)
1	42.50 (0)	72.00 (0)	25.00 (0)
2	42.50 (0)	24.00 (-α)	25.00 (0)
3	42.50 (0)	72.00 (0)	25.00 (0)
4	55.88 (+1)	100.54 (+1)	19.05 (-1)
5	42.50 (0)	72.00 (0)	25.00 (0)
6	29.12 (-1)	43.46 (-1)	19.05 (-1)
7	42.50 (0)	72.00 (0)	25.00 (0)
8	29.12 (-1)	100.54 (+1)	19.05 (-1)
9	55.88 (+1)	43.46 (-1)	30.95 (+1)
10	29.12 (-1)	100.54 (+1)	30.95 (+1)
11	42.50 (0)	72.00 (0)	25.00 (0)
12	42.50 (0)	72.00 (0)	25.00 (0)
13	65.00 (+α)	72.00 (0)	25.00 (0)
14	42.50 (0)	72.00 (0)	35.00 (+α)
15	20.00 (-α)	72.00 (0)	25.00 (0)
16	55.88 (+1)	43.46 (-1)	19.05 (-1)
17	55.88 (+1)	100.54 (+1)	30.95 (+1)
18	42.50 (0)	120.00 (+α)	25.00 (0)
19	29.12 (-1)	43.46 (-1)	30.95 (+1)
20	42.50 (0)	72.00 (0)	15.00 (-α)

[a] Corner (±1.6818), central (0) and axial (±1) levels of each variable are designated as +1, -1, 0, +α and –α.

3.2.5.2.3. Sensory analysis

Fifteen trained judges evaluated the sensory quality of wadi consisting of flavour (10), body and texture (10), and colour and appearance (5), using a 25-point score card (Table 11). The judges scored the samples in separate booths without discussing in a group.

Table 11. Sensory score card for wadi

Name: Date: Time:

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

Attributes	Defects	Slight	Distinct	Pronounced	Sample No.
Flavour [10]	Highly acidic/sour	6	4	1	
	Bitter	5	2	1	
	Oxidized	6	4	1	
	Mouldy/yeasty	5	2	0	
	Rancid	5	3	1	
	Beany	9	7	1	
	Bland	1	0	0	
Body & Texture [10]	Hard	7	3	1	
	Soggy	6	4	2	
	Granular	7	6	4	
	Very brittle	1	0	0	
	Non-porous	6	4	1	
Colour and appearance [5]	Yellow to brown	3	2	0	
	Mouldy	2	1	0	
	Unclean	1	0	0	
Total score [25]:					

Signature of the judge

Grading of wadi:

Total score	Grade
20-25	Excellent
14-19	Good
8-13	Fair
3-7	Poor
<2	Bad

Requirements of high grade wadi:

Flavour: Fermented, slightly acidic, slightly beany

Body & texture: Porous, smooth to slightly granular surface, slightly brittle, crunchy

Colour & appearance: Off-white, clean, cones/umbonate

3.2.5.2.4. Comparative study of sun-dried and machine-dried wadi

Samples of wadi prepared by sun-drying the cones on a greased bamboo mat at 25-30°C for 72 h, and that dried inside the dehumidifying chamber at the optimized drying conditions were analyzed for their physicochemical attributes and bacteriological quality after drying was complete.

3.2.6. Survival and growth of foodborne bacterial pathogens in idli and wadi

The fate of some bacterial pathogens such as *E. coli*, *B. cereus* s.l. and *S. aureus* and were studied during the mechanized drying of wadi and its storage at ambient room temperature and during the production of idli.

3.2.6.1. Preparation of idli

For idli preparation, polished rice and blackgram dal were soaked for 5-10 h separately. They were ground separately, with occasional addition of water during the process. Rice was coarsely ground, while blackgram was ground to a fine paste. Both the batters were mixed (rice-bean ratio, 2:1 w/w) and 1% w/w salt added to it. The mixed batter was fermented at 30°C for 18 h. Finally, the fermented batter was placed in idli pans and steamed for 15 min.

3.2.6.2. Preparation of wadi

Wadi was prepared by shaping well-beaten blackgram batter into cones with a diameter of 25 mm and dried in a dehumidifying chamber at 42% RH for 72 h as per the specifications obtained from the optimization study.

3.2.6.3. Challenge study on idli and wadi

Freshly prepared idli and wadi batter were divided into 10 sets. One set was inoculated with *B. cereus* s.l. and another with both *B. cereus* s.l. and LAB. Similarly, the other sets were inoculated with *S. aureus*, *E. coli* and all the three pathogens together, with and without LAB. Two control sets were taken, one contained the natural microbiota and the other contained the natural microbiota along with inoculated LAB. The pathogens *E. coli* 40E4, *B. cereus* 36B1 and *S. aureus* 36S3 were obtained from the market samples of wadi while LAB Lb5 was isolated from one of the processing stages of idli. All the pathogenic cultures were maintained at 4°C on NA slants and LAB cultures on MRS agar slants. Before using, they were activated by streaking them on NA and MRS agar slants, respectively, followed by incubation at 35°C. A suspension of 18 h-old cultures in sterile distilled water having a concentration of 10^7-10^8 cells/ml was prepared. A measured volume of these suspensions were inoculated into the batter to obtain an inoculation level of approximately 10^4 cells/ml.

For idli, sampling was done at every 6 h of fermentation while for wadi, sampling was done at every 12 h interval of drying and at every 7 days interval during storage at 25°C.

3.2.6.4. Antibacterial activity of LAB

LAB isolates obtained from the different processing stages of wadi and idli were tested for their antibacterial activity against the pathogens used in the challenge study. The antibacterial activity was detected by the 'spot on the lawn' method of Fleming *et al*. (1985) and Schillinger and Lücke (1989). Plates of MRS-0.2 agar were dried overnight and spotted with the test cultures (5 spots per plate) and incubated in a candle jar for 18-24 h at 32°C. MRS soft agar, after sterilization, was cooled to 45°C and seeded with 10^5-10^6 sensitive pathogenic bacterial cells/ml. The spotted plates were overlaid with 10 ml of the seeded MRS soft agar, incubated for 18-24 h at 32°C and observed for inhibition zones. The results were considered positive if the width of the clear zone was >0.5 mm.

3.2.6.4.1. Preparation of cell-free culture supernatant

MRS broth culture (18 h old) was centrifuged at 10,200×g for 10 min and the cell-free supernatant was collected. The pH of the supernatant was adjusted to 6.5 and concentrated 10 times using a rotary evaporator at 50°C. Then, it was filter sterilized through a 0.45 µm cellulose acetate membrane (Septrane 71-45-02).

3.2.6.4.2. Well diffusion assay

MRS agar plates overlaid with 10 ml of MRS soft agar seeded with 10^5-10^6 cells of sensitive pathogens/ml was prepared. Wells of 6 mm diameter were cut into these plates, and 0.1 ml of the cell-free culture supernatant of the test bacterium was placed into each well. The plates were incubated at 32°C for 24 h and observed for the formation of any clear zone around the wells (Schillinger and Lücke (1989).

3.2.6.4.3. Determination of antagonism due to acids

In order to determine the antagonism due to acids, well diffusion assay was carried out without adjusting and after adjusting the pH of the cell-free culture supernatant.

3.2.6.4.4. Determination of antagonism due to hydrogen peroxide

To the culture supernatant, 5 µg catalase (Sigma 81H7146)/ml was added, incubated at 37°C for 1 h, concentrated in rotary evaporator and filter-sterilized through a cellulose acetate membrane (0.2 µm, Sartorius, Germany). The filtrate was then assayed by the well diffusion method described earlier.

3.2.6.4.5. Determination of antagonism due to phage

Antagonism due to lytic bacteriophages was determined by the reverse-side technique (Parrot *et al*., 1990). MRS agar plate was prepared and wells of 7 mm diameter were cut in it. The bottom of the well was sealed with 1-2 drops of molten agar. 0.1 ml of cell-free culture supernatant was placed in the sealed wells and incubated at 25°C for 6 h. After diffusion of the supernatant into the agar, the entire agar disc of the plate was inverted on the lid of the Petri dish with a sterile spatula and overlaid with 10 ml of MRS soft agar seeded with 18 h-old 10^5-10^6 sensitive cells/ml. The plates were incubated at 32°C for 24 h and observed for the formation of any clear zone around the wells.

3.2.6.4.6 Determination of antagonism due to bacteriocin

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

3.2.7. Modulation of traditional processing for enhancing safety and shelf-life of kinema

3.2.7.1. Aseptic preparation of kinema

Kinema was prepared in the laboratory with a slight modification in the traditional method optimized by Sarkar and Tamang (1994). For inoculum preparation, *B. subtilis* DK-W1 (MTCC 1747) was streaked onto NA slant and incubated at 37°C for 16 h. A suspension of the cells in sterile distilled water having a concentration of 10^7-10^8 total cells/ml was used to inoculate cooked soybeans for fermentation.

Soybeans with yellow seed coat were purchased from Kurseong in the district of Darjeeling. Approximately 250 g of the beans were washed thoroughly under tap water and then with distilled water. The beans were soaked in distilled water (bean-water ratio, 1:4 w/v) 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 glass bottles and distilled water (bean-water ratio, 1:2 w/v) was added. The beans were autoclaved at 121°C for 15 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 the beans were dehulled and the cotyledons separated and crushed to give grits of mainly half-cotyledons (Sarkar, 2000).

A suspension (2 ml) of the organism was mixed with the cooked beans to make a load of 10^5-10^6 total cells/g grits. The inoculated beans were distributed in approximately 50 g amounts to sterile glass bottles (250 ml), capped loosely and incubated at 37°C for 48 h to produce kinema (Sarkar *et al*., 1993). As a modulation to the traditional method of kinema preparation, salt (NaCl) was added in varying concentrations (1-5%, w/w) to the cooked beans prior to fermentation.

3.2.7.2. Effect of salt during fermentation and storage

Monoculture kinema samples were kept in 250 ml glass bottles (50 g in each), capped tightly and stored at 20°C up to 7 days.

3.2.7.2.1. Sensory analysis

Salt-free kinema and kinema containing 1-5% w/w salt produced by the monoculture fermentation of soybeans was subjected to a sensory analysis by a panel of 10 trained judges who were also frequent consumers of kinema. They evaluated the overall sensory quality consisting of flavour (50), texture (45) and colour (5), using a 100-point sensory score card (Table 12) (Sarkar and Tamang, 1994). All the analyses were conducted in triplicate.

Table 12. Sensory score card for kinema

Name: Date: Time:

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

Attributes	Defects	Slight	Distinct	Pronounced	Sample No.
Flavour (smell)	Flat	39	35	33	
[50]	Rotten	35	30	25	
Normal range:	Raw beany	34	28	26	
(38-47)					
Body & texture	Dry	37	34	32	
[45]	Watery	35	30	20	
Normal range:					
(38-44)					
Colour	Whitish	3	2	1	
[5]					
Normal range:					
(4-5)					
Total score [100]:					

Signature of the judge

Grading of kinema:

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

Requirements of high grade kinema:

Flavour: Nutty with ammoniacal odour

Body & texture: Highly sticky or mucilaginous and slightly pasty

Colour: Brown

3.2.7.2.2. Viable cell count

Beans (10 g wet wt) were homogenized using a Stomacher lab-blender for 1 min at 'normal' speed with 90 ml sterile PPS, and decimal dilutions were made with the same diluent. Suitable dilutions (0.1 ml) were spread plated on PCA and incubated at 37°C for 16-20 h to obtain the viable count.

3.2.7.2.3. Challenge study

Sterile bean grits were at first inoculated with 10^6 cells of the functional microorganism, *B. subtilis* DK-W1/g. The inoculated beans were then divided into two equal lots. Salt (4% NaCl, w/w) was added to one lot before the start of fermentation and mixed

thoroughly, while the other lot contained no salt. Salt was added at the beginning and not partway through fermentation so that the fermenting microbiota does not get disturbed and affect the quality of the final product. Each lot was divided into eight equal sets containing 50 g (wet wt) beans in 250 ml screw-capped glass bottles and incubated at 37°C for 48 h. Each set was intentionally inoculated with 10^4 cells of the foodborne bacterial pathogens like *B. cereus* s.l., *E. coli* and *S. aureus* separately and also in their various combinations/g grits. The bottles were loosely capped. After incubation at 37°C for 48 h, triplicate bottles from each set were examined for the recovery of the functional microorganism as well as the pathogens. In order to understand the reason for the complete removal of *S. aureus*, five separate experimental set-ups containing cooked beans were inoculated with only *S. aureus* at the same inoculum level as before and incubated at 37°C for 48 h. Among the five, one set-up contained no added salt while the other four contained salt in order of increasing concentration from 1 to 4 g/100 g.

Viable cell counts of *B. subtilis* were performed by the same procedure as described earlier. Colonies of the pathogens like *B. cereus* s.l. and *S. aureus* and *E .coli* were counted by spread plating 0.1 ml of appropriate dilutions of the beans in PPS onto plates of sterile BCSA, BPA and MacConkey agar plates, respectively. The inoculated BCSA and BPA plates were incubated at 30°C for 24-48 h, while the MacConkey plates were incubated at 30°C for 18-24 h. The log cfu of the microorganisms per gram of the beans were calculated after incubation.

The a_w of fresh kinema samples was reduced to 0.5 by air-drying it in a dehumidifying chamber, set at 30% RH (20 ± 2°C) for a period of 72 h. Survivability of *E. coli* cells was determined in these dried samples.

3.2.7.2.4. Microbial interference study

A microbial interference study between *E .coli* and *B. cereus* s.l. was undertaken in order to understand the effect of the former on the latter. For this, a cell-free culture supernatant was prepared by centrifuging (10,200×g for 10 min) an 18 h-old *E. coli* culture in NB. One-half of the supernatant was neutralized to pH 6.5 with 1 N NaOH while the other half was left non-neutralized. The supernatants were concentrated 10 times in a rotary evaporator at 50°C and filter-sterilized with a cellulose acetate membrane, 0.45 µm. The supernatants were then used for the well diffusion assay in which NA plates were overlaid with 10 ml of NB with 1% w/v agar seeded with 10^4 cells of *B. cereus*/ml. Wells of 6 mm diameter were cut into these plates and 0.1 ml of the cell-free supernatants of *E. coli* was placed into these wells. The plates were incubated at 30°C for 24 h and observed for the formation of any clear zone around the wells (Schillinger and Lücke, 1989).

3.2.8. Moisture sorption study of kinema and wadi

A moisture sorption study was performed on kinema and wadi during their storage at different temperatures and a_w.

3.2.8.1. Determination of EMC

EMC was determined by the static gravimetric method used by Wolf *et al*. (1985). The sorption apparatus used for this study was slight modified. Wide-mouthed glass bottles with air-tight lids were used as sorbostats. Saturated solutions of LiCl (Merck

10567901001046), $MgCl_2$ (Merck 60583305001730), K_2CO_3 (s.d. fine 20196), $Mg(NO_3)_2$ (Merck 61776905001046), $NaNO_2$ (Merck 61806805001046), NaCl (Merck 60640405001046), $(NH_4)_2SO_4$ (Merck 61783405001046), KCl (Merck 61779205001730), $BaCl_2$ (Merck 61777105001730) and K_2SO_4 (Merck 6177740 5001730), providing a_w 0.11-0.97, were prepared. Each salt solution, poured into a separate sorbostat, acted as the source of sorbate. The salts were dissolved in distilled water a week ahead, and occasionally mixed daily in order to bring stability.

Kinema was prepared aseptically in the laboratory, whereas blackgram wadi was purchased from the local market of Siliguri. Wadi was powdered and used for the experiment. Desorption isotherms were determined on fresh kinema, while adsorption isotherms were determined on kinema samples that were air-dried to a constant weight in a dehumidifying chamber, set at 30% RH, 20 ± 2°C for a period of 72 h. Approximately 2 g finely powdered, fresh or dried sample were weighed into tared weighing bottles (10 ml) in triplicate and placed over glass supports inside the sorbostats. Before putting the samples, the weighing bottles were allowed to come to equilibrium with the surrounding environment inside the sorbostat for 24 h. Approximately 5 mg potassium sorbate (HiMedia RM1311) was added to each sample to prevent microbial growth. The sorbostats were tightly closed and placed inside incubators set at different temperatures. The samples were weighed periodically until (20-30 days) the difference between the two successive weighings was $\leq$1 mg. The samples were exposed for less than 10 s while weighing them to avoid adsorption or desorption of moisture from the surrounding which can lead to erroneous results. The moisture content in the samples was determined gravimetrically (Helrich, 1990).

3.2.8.2. Moisture sorption models

The different models selected to fit the experimental moisture sorption data of kinema and wadi are shown in Table 13**.** The GAB and modified Mizrahi models have three parameters, while the rest have two parameters each. The most commonly used 2-parameter model is BET, but its applicability is limited only to the lower (0.1-0.5) a_w. On the other hand, the GAB model is the most successful 3-parameter model applicable even at a higher (0.1-0.9) a_w, and has been recommended by the European Project Group COST '90 on physical properties of foods (Wolf *et al*., 1985). All the equations were converted into linear form, and the moisture sorption data were analyzed and fitted to different equations either in the whole range of isotherm or part of it. The goodness of fit for each equation was evaluated in terms of coefficient of determination (r^2), obtained by plotting the experimental (M_{exp}) and calculated (M_{cal}) sorption moisture and by $\%rms$:

$$\%rms = \sqrt{\frac{1}{n}\sum_{1}^{n}\left(\frac{M_{\exp} - M_{cal}}{M_{\exp}}\right)^2} \times 100 \qquad (1)$$

where, n denotes the number of observations.

The appropriate constants of the models were determined by regression analysis using MS Excel software.

Table 13. Sorption isotherm models for fitting experimental data

Model	Equation	Constants	Reference
Smith	$M = b - a\mathrm{a_w}$	*a*, *b*	Chirife and Iglesias (1978)
Bradley	$\ln\left(\frac{1}{\mathrm{a_w}}\right) = ab^M$	*a*, *b*	Chirife and Iglesias (1978)
Iglesias and Chirife	$\ln\left(M + \sqrt{M^2 + M_{0.5}}\right) = a + b\mathrm{a_w}$	*a*, *b*	Chirife and Iglesias (1978)
BET	$M = \frac{M_b C_b}{(1-\mathrm{a_w})(1+(C_b-1)\mathrm{a_w})}$	M_b, C_b	Caurie *et al.* (1976)
Freundlich	$M = b(\mathrm{a_w})^{\frac{1}{a}}$	*a*, *b*	Ertugay *et al.* (2000)
Oswin	$M = a\left(\frac{\mathrm{a_w}}{1-\mathrm{a_w}}\right)^b$	*a*, *b*	Chirife and Iglesias (1978)
Caurie	$\frac{1}{M} = \frac{1}{C_c M_c}\left(\frac{1-\mathrm{a_w}}{\mathrm{a_w}}\right)^{\frac{2C_c}{M_c}}$	M_c, C_c	Caurie (1981)
Modified Mizrahi	$M = \frac{a + \mathrm{a_w}(c\mathrm{a_w} + b)}{\mathrm{a_w} - 1}$	*a*, *b*, *c*	Rao *et al.* (2006)
GAB	$\frac{M}{M_g} = \frac{C_g k\mathrm{a_w}}{(1-k\mathrm{a_w})(1-k\mathrm{a_w}+C_g k\mathrm{a_w})}$	M_g, C_g, *k*	Bizot (1983)

3.2.8.3. Properties of sorbed water

The monolayer moisture content (M_c) and density (C_c) were calculated from Caurie's model. The number of adsorbed monolayers (N) was calculated using the formula (Caurie, 1981):

$$N = \frac{M_c}{C_c} \qquad (2)$$

where, M_c (g/100 g) calculated from Caurie's model and C_c was the Caurie's constant. The bound water content was expressed as $M_c\, x\, N$ and assuming Caurie's constant (C_c) to be equivalent to the density of adsorbed water in the monolayer, the surface area of adsorption (A) was calculated using the formula (Sahu and Jha, 2008):

$$A = \frac{M_c}{C_c \, x \, d \, 10^8} \quad (3)$$

where, d is the diameter of a water molecule (3.673 x 10^{-10} m).

3.2.8.4. Thermodynamic functions

3.2.8.4.1. Free energy changes

Free energy changes occurring due to change in the moisture content of kinema were calculated by the relation:

$$\Delta G = -RT \ln a_w \quad (4)$$

where R is the universal gas constant (8.314 kJ/kmol K), T is the temperature in K and the a_w-values were obtained from the Oswin equation which showed the best fit to the experimental data for both adsorption and desorption. Free energy changes were calculated at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 g moisture/100 g solids at all the test temperatures.

3.2.8.4.2. Net isosteric heat of sorption and sorption entropy

The net isosteric sorption heat (q_{st}, MJ/mol) gives an insight into the heat and free energy changes occurring in food during a sorption process (Sopade and Ajisegiri, 1994). It is defined as the total isosteric sorption heat minus the heat of vapourization of pure water at the system temperature. It is the amount of heat needed to be liberated in the form of latent heat of vapourization of water in order to remove the absorbed water molecules at particular moisture content. Net isosteric sorption heat was calculated using the Clausius-Clapeyron equation:

$$\left[\frac{\partial(\ln a_w)}{\partial\left(1/T\right)}\right] = -\frac{\Delta q_{st}}{R} \quad (5)$$

$$\ln a_w = \left(\frac{\Delta q_{st}}{R}\right)\left(\frac{1}{T}\right) + c \quad (6)$$

where, c is a constant. Plotting ln a_w against $1/T$ at certain moisture content gave a straight line with slope $\Delta q_{st}/R$. On multiplying each slope with the R-value, q_{st} at given moisture content was obtained.

The differential entropy is directly proportional to the number of free soption sites available for moisture adsorption. It was calculated from the Gibbs-Helmholtz relationship (Lamharrar *et al.*, 2007):

$$\ln a_w = \left(\frac{q_{st}}{R}\right)\left(\frac{1}{T}\right) - \frac{\Delta S}{R} \quad (7)$$

where, ΔS is the change in differential entropy (J/mol K). By plotting ln a_w against $1/T$ at constant moisture content, the entropy change was calculated from the intercept value ($\Delta S/R$).

3.2.8.4.3. Fractionation of bound water based on binding energy

Water is bound to food in three different forms viz. primary, secondary and tertiary, and each fraction has a characteristic binding energy (Soekarto and Steinberg, 1981). Bound water can be fractionated quantitatively based on binding energy. The binding energy for primary bound water was obtained from the following equations (Brunauer *et al*., 1938):

$$C_b = K \exp\left(H_1 - H_2\right)/RT \qquad (8)$$

If binding energy H_B is defined as $H_1 - H_2$, where H_1 is the heat of absorption of water by a solid (MJ/mol) and H_2 is the heat of condensation of water vapour (MJ/mol) then Eq. (8) becomes,

$$C_b = K \exp\left(\Delta H_B / RT\right) \qquad (9)$$

Taking 'ln' on both sides of Eq. (9):

$$\ln C_b = \ln K + \left(\frac{\Delta H_B}{R}\right)\left(\frac{1}{T}\right) \qquad (10)$$

where, C_b was a binding energy constant. The above equation yielded an Arrhenius plot of ln C_b against $1/T$ in which a straight line with slope = $\Delta H_B/R$ was obtained. The slope was multiplied by R to obtain the binding energy for primary bound water. The binding energy for secondary and tertiary bound waters was calculated using the equation:

$$\ln a_w = \left(\frac{\Delta H_B}{R}\right)\left(\frac{1}{T}\right) + c \qquad (11)$$

3.2.8.4.4. Equilibrium spreading pressure

Spreading pressure (φ) is defined as the excess free energy at the surface and denotes an increase in the surface tension of the free sorption sites due to adsorption (Fasina *et al*., 1999). Spreading pressure can be calculated from the following relation:

$$\varphi = \frac{K_B T}{A_m} \int_0^{a_w} \frac{\theta}{a_w} d\left(a_w\right) \qquad (12)$$

where, φ was the spreading pressure, K_B was the Boltzmann constant (1.380 × 10^{-23} J/ A_m was the area of a water molecule (1.06 × 10^{-19} m^2) and ¯ (moisture ratio) was

M_{exp}/M_g. By combining the equation for GAB model and eq. 12 the above integral was expressed as:

$$\varphi = \frac{K_B T}{A_m} \ln\left(\frac{1 - K a_w + K C a_w}{1 - K a_w}\right) \quad (13)$$

where, the *K* and *C* were GAB constants.

3.2.9. Statistical analyses

Data were analyzed using Microsoft Excel 2007 and SPSS v. 12.0., expressed as means ± SEM and subjected to one-way ANOVA and paired *t*-test after converting the microbial counts to a logarithmic scale. For the moisture sorption study, regression analysis was performed which facilitates the estimation of the different parameters of the sorption models. All the experimental results obtained from the response optimization study were analyzed using MINITAB 15.1.1.0. (Minitab Inc., State College, PA) software and expressed as mean ± standard error. Quadratic models were used to fit the experimental data given in Tables 8 and 10 and the models for the responses described the effect of the independent variables in terms of linear, quadratic and cross product terms (Giovanni, 1983).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (14)$$

where Y was the predicted response for RFO content; X_1, X_2, X_3 and X_4 were the independent variables; β_0 was a constant at the centre point of the design; β_1, β_2, β_3 and β_4, respectively, were the regression coefficients for the linear effect terms; β_{11}, β_{22}, β_{33} and β_{44}, respectively, were for the quadratic effect terms and β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} were for the cross product effect terms.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (15)$$

where *Y* was the predicted response for OAA and a_w; X_1, X_2 and X_3 were the independent variables; β_0 was a constant at the centre point of the design; β_1, β_2 and β_3 were the regression coefficients for the linear effect terms; β_{11}, β_{22} and β_{33} were for the quadratic effect terms and β_{12}, β_{13} and β_{23} were for the cross product effect terms. The software generated these coefficients, and their significance was determined using the *p*-value generated through the *t*-test. The fitness of the overall models along with the term reduction was also expressed by the coefficient of determination (r^2), *t*-test and the standard errors of the estimate. Higher r^2-values indicated none of the predicted models had a significant lack of fit. The fitted quadratic model equation was expressed as three-dimensional response surface plots using the same software in order to visualize the relation between the independent variables at each level and the responses and to obtain the optimum conditions. One-way ANOVA was also performed for each response variable and the *p*-values indicated which terms were significant.