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, Mumbai, India)
Polymyxin B selective supplement (HiMedia FD003)
Egg yolk emulsion (HiMedia FD045)

Blood agar (HiMedia M834)

Brain heart infusion broth (HiMedia M210)

J-broth (Claus and Berkeley, 1986)

Peptone	5.0 g
Yeast extract	15.0 g
K_2HPO_4	3.0 g
Glucose	2.0 g
Distilled water	1000 ml
pH 7.2	

Lipase production medium (Lee *et al*., 1999)

Tryptone	6.0 g
Yeast extract	2.0 g

Olive oil	15.0 ml
$CaCl_2.2H_2O$	0.2 g
$MgSO_4.7H_2O$	0.1 g
$FeCl_3.6H_2O$ (10 g l^{-1})	0.4 ml
Distilled water	1000 ml
pH 6.0	

Milk agar (HiMedia M163)

Mueller-Hinton agar (HiMedia M173)

Nitrate broth (HiMedia M439)

Nutrient agar (HiMedia M561)

Nutrient broth (HiMedia M002)

Protease production medium (Patel *et al*., 2005)

Peptone	5.0 g
Yeast extract	5.0 g
Beef extract	1.5 g
NaCl	5.0 g
Glucose	10.0 g
Distilled water	1000 ml
pH 7.0	

Purple agar base medium (HiMedia M098)

Skim milk (HiMedia M530)

Skim milk agar (HiMedia M163)

Starch agar (HiMedia M107)

Trybutyrin agar (HiMedia M157 and FD081)

Tryptone soya agar (HiMedia M290)

Tryptone soya broth (HiMedia M011)

Voges-Proskauer broth (HiMedia M070)

All the media mentioned above were sterilised by autoclaving at 1.1 kg cm^{-2} for 15 min, unless mentioned otherwise. Skim milk was sterilised by autoclaving at 1.1 kg cm^{-2} for 5 min.

3.1.2. Reagents

Gram's crystal violet solution (9218; Merck Specialities Pvt. Ltd, Mumbai, India)

Gram's iodine solution (HiMedia S057)

Lipase assay reagent

Solution A	
p-Nitrophenyl palmitate (Sigma-Aldrich N2752)	30 mg
iso-Propanol (Merck 1.94524.0521)	10 ml
Solution	
Gum acacia (Merck 61835005001730)	0.1 g
Triton X-100 (HiMedia MB031)	0.4 ml
Tris–HCl (HiMedia M631), 50 mM, pH 8.0	90 ml

Nitrate reagent (Norris *et al*., 1981)

Solution A	
Sulphanilic acid	0.8 g
Acetic acid (5 ml l^{-1})	100 ml
(Glacial acetic acid:water, 1:2.5 v/v)	
Solution B	
α-Napthylamine	0.5 g
Acetic acid (mol l^{-1})	100 ml

Neutralisation buffer (HiMedia M1334)

Peptone physiological saline

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

Voges-Proskauer reagent

Solution A	
α-Naphthol	5 g
Absolute alcohol	100 ml
Solution B	
Potassium hydroxide	40 g
Distilled water	100 ml

3.1.3. Antibiotic susceptibility test discs

Ampicillin (HiMedia SD002)
Carbenicillin (HiMedia SD004)
Cephalothin (HiMedia SD050)
Chloramphenicol (HiMedia SD006)
Erythromycin (HiMedia SD013)
Kanamycin (HiMedia SD017)
Metronidazole (HiMedia SD020)
Nalidixic acid (HiMedia SD021)
Penicillin G (HiMedia SD028)
Polymyxin B (HiMedia SD002)
Rifampicin (HiMedia SD030)
Streptomycin (HiMedia SD031)
Tetracycline (HiMedia SD037)
Vancomycin (HiMedia SD045)

All the chemicals used were of the highest purity grade.

3.2. Experimental

3.2.1. Isolation and enumeration of *Bacillus cereus* from milk and dairy products

3.2.1.1. Sampling

A total of 230 samples of pasteurised and sterilised milk, milk powder, ice cream, paneer, butter, cheese, curd and khoa were collected from retail outlets in Darjeeling district (Fig. 8 and Table 7). Samples were also collected aseptically from raw milk silo tanks and pasteurised milk chilling tanks of HIMUL (Himalayan Milk Producers' Union Limited) Dairy at Matigara, near to Siliguri, using sterile screw-capped glass bottles (30 ml). Sterile swabs were used to collect samples from stainless steel surfaces of the pasteurised milk storage chilling tanks.

The samples from different milk batches were collected for three months at regular interval. Immediately after collection, the samples were transported to the laboratory in insulated container containing ice gel packs for analyses.

3.2.1.2. Isolation

A 10 g or ml-sample was homogenised with 90 ml sterile peptone-physiological saline using a Stomacher lab-blender 400 (Seward Medical, London) at 'normal' speed for 1 min. A swab sample was dipped in sterile peptone-physiological saline. Appropriately diluted suspension (0.1 ml) was spread-plated on *Bacillus cereus* selective agar and incubated at 35 °C for 24-48 h. Characteristic turquoise to peacock blue colonies, surrounded by a zone of precipitate of the same colour, were regarded as presumptive *B. cereus* group (*s.l.*).

Fig. 8. Milk and dairy products, as marketed

Table 7. Sampling of marketed milk and dairy products

Date of collection	Sample No.	Kind of product	Place of collection	Open/Pkd (L/B)[a]
06.11.10	S1	Pasteurised milk	Matigara	Pkd (B)
09.11.10	S2	Pasteurised milk	Matigara	Pkd (B)
13.11.10	S3	Pasteurised milk	Khaprail	Pkd (B)
14.11.10	S4	Pasteurised milk	Matigara	Pkd (B)
15.11.10	S5	Pasteurised milk	Shivmandir	Pkd (B)
15.11.10	S6	Pasteurised milk	Shivmandir	Pkd (B)
17.11.10	S7	Pasteurised milk	Shivmandir	Pkd (B)
28.12.10	S8	Pasteurised milk	Matigara	Pkd (B)
31.12.10	S9	Pasteurised milk	Khaprail	Pkd (B)
31.12.10	S10	Pasteurised milk	Shivmandir	Pkd (B)
05.01.11	S11	Pasteurised milk	Hakimpara, Siliguri	Pkd (B)
06.01.11	S12	Pasteurised milk	Shivmandir	Pkd (B)
10.01.11	S13	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
19.01.11	S14	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
27.01.11	S15	Pasteurised milk	Khaprail	Pkd (B)
11.02.11	S16	Pasteurised milk	Matigara	Pkd (B)
12.02.11	S17	Pasteurised milk	Matigara	Pkd (B)
23.02.11	S18	Pasteurised milk	Khaprail	Pkd (B)
25.02.11	S19	Pasteurised milk	Shivmandir	Pkd (B)
02.03.11	S20	Pasteurised milk	Shivmandir	Pkd (B)
03.03.11	S21	Pasteurised milk	Matigara	Pkd (B)
04.03.11	S22	Pasteurised milk	Khaprail	Pkd (B)
05.03.11	S23	Pasteurised milk	Khaprail	Pkd (B)
06.03.11	S24	Pasteurised milk	Matigara	Pkd (B)
09.03.11	S25	Pasteurised milk	Sevoke More, Siliguri	Pkd (B)
13.03.11	S26	Pasteurised milk	Sevoke More, Siliguri	Pkd (B)
14.03.11	S27	Pasteurised milk	Shivmandir	Pkd (B)
15.03.11	S28	Pasteurised milk	Shivmandir	Pkd (B)
21.03.11	S29	Pasteurised milk	Matigara	Pkd (B)
21.03.11	S30	Pasteurised milk	Matigara	Pkd (B)
28.03.11	S31	Pasteurised milk	Matigara	Pkd (B)
28.03.11	S32	Pasteurised milk	Matigara	Pkd (B)
29.03.11	S33	Pasteurised milk	Matigara	Pkd (B)
29.03.11	S34	Pasteurised milk	Matigara	Pkd (B)
30.03.11	S35	Pasteurised milk	Matigara	Pkd (B)
04.04.11	S36	Milk powder	Bidhan Market, Siliguri	Pkd (B)
04.04.11	S37	Milk powder	Bidhan Market, Siliguri	Pkd (B)
04.04.11	S38	Milk powder	Bidhan Market, Siliguri	Pkd (B)
05.04.11	S39	Milk powder	Matigara	Pkd (B)
08.04.11	S40	Milk powder	Matigara	Pkd (B)
09.04.11	S41	Milk powder	Airport More, Bagdogra	Pkd (B)
13.04.11	S42	Milk powder	Kurseong	Pkd (B)
13.04.11	S43	Milk powder	Kurseong	Pkd (B)
13.04.11	S44	Milk powder	Kurseong	Pkd (B)
04.05.11	S45	Milk powder	Court More, Siliguri	Pkd (B)
05.05.11	S46	Milk powder	Hakimpara, Siliguri	Pkd (B)
11.05.11	S47	Milk powder	Matigara	Pkd (B)
12.05.11	S48	Milk powder	Matigara	Pkd (B)
12.05.11	S49	Milk powder	Shivmandir	Pkd (B)
15.05.11	S50	Milk powder	Mirik	Pkd (B)
15.05.11	S51	Milk powder	Mirik	Pkd (B)
20.05.11	S52	Milk powder	Airport More, Bagdogra	Pkd (B)
20.05.11	S53	Milk powder	Airport More, Bagdogra	Pkd (B)
22.05.11	S54	Milk powder	Kalimgpong	Pkd (B)
22.05.11	S55	Milk powder	Kalimgpong	Pkd (B)
22.05.11	S56	Milk powder	Kalimgpong	Pkd (B)
28.05.11	S57	Milk powder	Airview More, Siliguri	Pkd (B)
30.05.11	S58	Milk powder	Shivmandir	Pkd (B)

Date of collection	Sample No.	Kind of product	Place of collection	Open/Pkd (L/B)[a]
03.06.11	S59	Milk powder	Shivmandir	Pkd (B)
05.06.11	S60	Milk powder	Airport More, Bagdogra	Pkd (B)
05.06.11	S61	Milk powder	Shivmandir	Pkd (B)
05.06.11	S62	Milk powder	Shivmandir	Pkd (B)
10.06.11	S63	Milk powder	Hakimpara, Siliguri	Pkd (B)
10.06.11	S64	Milk powder	Hakimpara, Siliguri	Pkd (B)
10.06.11	S65	Milk powder	Bidhan Market, Siliguri	Pkd (B)
10.06.11	S66	Milk powder	Bidhan Market, Siliguri	Pkd (B)
17.06.11	S67	Milk powder	Sevoke More, Siliguri	Pkd (B)
20.06.11	S68	Milk powder	Sevoke More, Siliguri	Pkd (B)
22.06.11	S69	Milk powder	Khaprail	Pkd (B)
22.06.11	S70	Milk powder	Khaprail	Pkd (B)
01.07.11	S71	Ice cream	Matigara	Pkd (B)
01.07.11	S72	Ice cream	Matigara	Pkd (B)
05.07.11	S73	Ice cream	Shivmandir	Pkd (B)
05.07.11	S74	Ice cream	Shivmandir	Pkd (B)
08.07.11	S75	Ice cream	Shivmandir	Pkd (L)
08.07.11	S76	Ice cream	Shivmandir	Pkd (L)
10.07.11	S77	Ice cream	Matigara	Open
10.07.11	S77	Ice cream	Matigara	Open
14.07.11	S78	Ice cream	Bihar More, Bagdogra	Pkd (B)
14.07.11	S79	Ice cream	Bihar More, Bagdogra	Pkd (L)
14.07.11	S80	Ice cream	Bihar More, Bagdogra	Open
18.07.11	S81	Ice cream	Khaprail	Pkd (L)
18.07.11	S82	Ice cream	Khaprail	Pkd (L)
21.07.11	S83	Ice cream	Matigara	Pkd (L)
21.07.11	S84	Ice cream	Matigara	Pkd (L)
25.07.11	S85	Ice cream	Seth Srilal Market, Siliguri	Pkd (B)
25.07.11	S86	Ice cream	Seth Srilal Market, Siliguri	Pkd (B)
25.07.11	S87	Ice cream	Seth Srilal Market, Siliguri	Pkd (L)
30.07.11	S88	Ice cream	Shivmandir	open
30.07.11	S89	Ice cream	Shivmandir	open
03.08.11	S90	Ice cream	Matigara	open
04.08.11	S91	Ice cream	Matigara	open
04.08.11	S92	Ice cream	Matigara	open
08.08.11	S93	Ice cream	Bengdubi, Bagdogra	Pkd (B)
08.08.11	S94	Ice cream	Bengdubi, Bagdogra	Pkd (B)
10.08.11	S95	Ice cream	Shivmandir	Pkd (B)
20.08.11	S96	Paneer	Matigara	Pkd (L)
20.08.11	S97	Paneer	Matigara	Pkd (B)
20.08.11	S98	Paneer	Matigara	Open
22.08.11	S99	Paneer	Shivmandir	Open
22.08.11	S100	Paneer	Shivmandir	Open
25.08.11	S101	Paneer	Bidhan Market, Siliguri	Open
25.08.11	S102	Paneer	Bidhan Market, Siliguri	Pkd (B)
29.08.11	S103	Paneer	Mirik	Open
29.08.11	S104	Paneer	Mirik	Open
02.09.11	S105	Paneer	Khaprail	Pkd (B)
0.2.09.11	S106	Paneer	Khaprail	Open
15.09.11	S107	Paneer	Kalimgpong	Open
15.09.11	S108	Paneer	Kalimgpong	Open
15.09.11	S109	Paneer	Kalimgpong	Open
15.09.11	S110	Paneer	Kalimgpong	Open
17.09.11	S111	Paneer	Matigara	Pkd (B)
17.09.11	S112	Paneer	Bengdubi, Bagdogra	Pkd (B)
25.09.11	S113	Paneer	Kurseong	Open
25.09.11	S114	Paneer	Kurseong	Open
25.09.11	S115	Paneer	Kurseong	Open
29.09.11	S116	Paneer	Court More, Siliguri	Pkd (B)

Date of collection	Sample No.	Kind of product	Place of collection	Open/Pkd (L/B)[a]
02.10.11	S117	Paneer	Naxalbari	Open
02.10.11	S118	Paneer	Naxalbari	Open
04.10.11	S119	Paneer	Sevoke More, Siliguri	Pkd (B)
04.10.11	S120	Paneer	Airview More, Siliguri	Pkd (B)
15.10.11	S121	Khoa	Matigara	Open
15.10.11	S122	Khoa	Matigara	Open
19.10.11	S123	Khoa	Shivmandir	Open
19.10.11	S124	Khoa	Shivmandir	Open
21.10.11	S125	Khoa	Sukna	Open
21.10.11	S126	Khoa	Sukna	Open
23.10.11	S127	Khoa	Junction, Siliguri	Open
23.10.11	S128	Khoa	Airview More, Siliguri	Open
23.10.11	S129	Khoa	Airview More, Siliguri	Open
28.10.11	S130	Khoa	Naxalbari	Open
28.10.11	S131	Khoa	Naxalbari	Open
3.11.11	S132	Khoa	Mirik	Open
3.11.11	S133	Khoa	Mirik	Open
3.11.11	S134	Khoa	Mirik	Open
8.11.11	S135	Khoa	Bidhan Market, Siliguri	Open
8.11.11	S136	Khoa	Bidhan Market, Siliguri	Open
14.11.11	S137	Khoa	Kurseong	Open
14.11.11	S138	Khoa	Kurseong	Open
14.11.11	S139	Khoa	Kalimgpong	Open
14.11.11	S140	Khoa	Kalimgpong	Open
19.11.11	S141	Curd	Matigara	Pkd (B)
20.11.11	S142	Curd	Matigara	Open
23.11.11	S143	Curd	Shivmandir	Open
25.11.11	S144	Curd	Shivmandir	Open
29.11.11	S145	Curd	Khaprail	Open
29.11.11	S146	Curd	Khaprail	Open
2.12.11	S147	Curd	Matigara	Pkd (B)
4.12.11	S148	Curd	Naxalbari	Pkd (L)
4.12.11	S149	Curd	Naxalbari	Open
6.12.11	S150	Curd	Bidhan Market, Siliguri	Open
6.12.11	S151	Curd	Bidhan Market, Siliguri	Open
7.12.11	S152	Curd	Matigara	Pkd (B)
10.12.11	S153	Curd	Junction, Siliguri	Open
10.12.11	S154	Curd	Junction, Siliguri	Open
10.12.11	S155	Curd	Airview More, Siliguri	Pkd (L)
10.12.11	S156	Curd	Airview More, Siliguri	Pkd (L)
14.12.11	S157	Curd	Matigara	Pkd (B)
15.12.11	S158	Curd	Bihar More, Bagdogra	Pkd (L)
15.12.11	S159	Curd	Bihar More, Bagdogra	Pkd (L)
15.12.11	S160	Curd	Bihar More, Bagdogra	Open
19.12.11	S161	Cheese	Matigara	Pkd (B)
19.12.11	S162	Cheese	Matigara	Pkd (B)
19.12.11	S163	Cheese	Matigara	Pkd (B)
19.12.11	S164	Cheese	Matigara	Pkd (B)
20.12.11	S165	Cheese	Shivmandir	Pkd (B)
24.12.11	S166	Cheese	Ghum	Pkd (L)
24.12.11	S167	Cheese	Ghum	Open
24.12.11	S168	Cheese	Darjeeling Town	Pkd (L)
24.12.11	S169	Cheese	Darjeeling Town	Open
29.12.11	S170	Cheese	Khaprail	Pkd (B)
29.12.11	S171	Cheese	Khaprail	Pkd (B)
02.01.12	S172	Cheese	Bidhan Market, Siliguri	Pkd (B)
02.01.12	S173	Cheese	Bidhan Market, Siliguri	Pkd (B)
03.01.12	S174	Cheese	Kurseong	Pkd (L)
03.01.12	S175	Cheese	Kurseong	Pkd (L)

Date of collection	Sample No.	Kind of product	Place of collection	Open/Pkd (L/B)*
03.01.12	S176	Cheese	Kurseong	Pkd (L)
06.01.12	S177	Cheese	Sevoke More, Siliguri	Pkd (B)
06.01.12	S178	Cheese	Sevoke More, Siliguri	Pkd (B)
11.01.12	S179	Cheese	Mirik	Pkd (L)
11.01.12	S180	Cheese	Mirik	Pkd (L)
11.01.12	S181	Cheese	Mirik	Pkd (L)
15.01.12	S182	Cheese	Bengdubi, Bagdogra	Pkd (B)
15.01.12	S183	Cheese	Bengdubi, Bagdogra	Pkd (B)
02.02.12	S184	Cheese	Kurseong	Pkd (B)
02.02.12	S185	Cheese	Kurseong	Pkd (L)
02.02.12	S186	Butter	Kurseong	Pkd (L)
02.02.12	S187	Butter	Kurseong	Pkd (B)
8.02.12	S187	Butter	Matigara	Pkd (B)
8.02.12	S187	Butter	Matigara	Pkd (B)
9.02.12	S188	Butter	Shivmandir	Pkd (B)
9.02.12	S189	Butter	Shivmandir	Pkd (B)
15.02.12	S190	Butter	Mirik	Pkd (B)
15.02.12	S191	Butter	Mirik	Pkd (B)
15.02.12	S192	Butter	Mirik	Pkd (B)
18.02.12	S193	Butter	Khaprail	Pkd (B)
18.02.12	S194	Butter	Khaprail	Pkd (B)
21.02.12	S195	Butter	Bidhan Market, Siliguri	Pkd (B)
21.02.12	S196	Butter	Bidhan Market, Siliguri	Pkd (B)
21.02.12	S197	Butter	Bidhan Market, Siliguri	Pkd (B)
24.02.12	S198	Butter	Bengdubi, Bagdogra	Pkd (B)
24.02.12	S199	Butter	Bengdubi, Bagdogra	Pkd (B)
25.02.12	S200	Butter	Matigara	Pkd (B)
02.03.12	S201	Butter	Kalimgpong	Pkd (B)
02.03.12	S202	Butter	Kalimgpong	Pkd (B)
02.03.12	S203	Butter	Kalimgpong	Pkd (B)
05.03.12	S204	Butter	Siliguri Junction	Pkd (B)
05.03.12	S205	Butter	Siliguri Junction	Pkd (B)
07.03.12	S206	Butter	Hakimpara, Siliguri	Pkd (B)
07.03.12	S207	Butter	Hakimpara, Siliguri	Pkd (B)
10.03.12	S208	Butter	Sevoke More, Siliguri	Pkd (B)
10.03.12	S209	Butter	Sevoke More, Siliguri	Pkd (B)
15.03.12	S210	Butter	Pradhan Nagar	Pkd (B)
05.01.14	S211	Sterilised milk	Matigara	Pkd (B)
08.01.14	S212	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
10.01.14	S213	Sterilised milk	Matigara	Pkd (B)
10.01.14	S214	Sterilised milk	Matigara	Pkd (B)
13.01.14	S215	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
15.01.14	S216	Sterilised milk	Matigara	Pkd (B)
14.01.14	S217	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
16.01.14	S218	Pasteurised milk	Hill Cart road, Siliguri	Pkd (B)
19.01.14	S219	Sterilised milk	Shivmandir	Pkd (B)
19.01.14	S220	Sterilised milk	Shivmandir	Pkd (B)
21.01.14	S221	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
22.01.14	S222	Pasteurised milk	Hill Cart road, Siliguri	Pkd (B)
24.01.14	S223	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
27.01.14	S224	UHT milk	Matigara	Pkd (B)
30.01.14	S225	Pasteurised milk	Khalpara, Siliguri	Pkd (B)
02.02.14	S226	UHT milk	Matigara	Pkd (B)
05.02.14	S227	Pasteurised milk	Hill Cart road, Siliguri	Pkd (B)
08.02.14	S228	UHT milk	Shivmandir	Pkd (B)
08.02.14	S229	UHT milk	Shivmandir	Pkd (B)
10.02.14	S230	UHT milk	Matigara	Pkd (B)

* Pkd, packed; L, locally packed (not branded); B, branded

3.2.1.3. Maintenance of pure cultures

The isolates were maintained on nutrient agar slants at 4 °C with subculturing after every six months.

3.2.1.4. Confirmation of the presumptive isolates

The presumptive isolates were confirmed on the basis of gram reaction, motility, endospore formation, glucose fermentation, nitrate reduction and acetylmethylcarbinol production (Claus and Berkeley, 1986).

3.2.1.4.1. Gram staining

A 24 h-old bacterial culture was used to prepare a suspension in distilled water. A drop of the suspension was used to prepare smear on a grease-free slide. The smear was air-dried, heat-fixed, flooded with gram's crystal violet solution for 1 min and washed for 5 s with water. The smear was flooded with gram's iodine solution, allowed to react for 1 min and washed for 5 s with water. Then, 950 ml l^{-1} ethanol (Merck, Germany 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 counter-stained with safranine (Merck, India 9127) for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

3.2.1.4.2. Motility

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

3.2.1.4.3. Formation of endospore

A 6 d-old culture on nutrient agar at 30 °C was observed under a phase-contrast microscope for endospore formation.

3.2.1.4.4. Fermentation of glucose

A 24 h-old broth culture was stabbed in a tube containing 10 ml of purple agar base medium supplemented with 5-10 g sterile glucose l^{-1} 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 the production of gas.

3.2.1.4.5. Reduction of nitrate

A 24 h-old culture was 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 freshly mixed solutions A and B in equal proportions. Formation of red or yellow colour indicated the presence of nitrite as a result of nitrate reduction. A small amount of zinc dust (Merk, Germany 61762805001046) was added to the tube that was negative even after 14 days of incubation. Development of red colour indicated the absence of reduction (Norris *et al*., 1981).

3.2.1.4.6. Voges-Proskauer reaction

A 24 h-old isolate was grown in 10 ml Voges-Proskauer broth and incubated at 30 °C for 48 h. A few drops of α-naphthol, followed by 400 g KOH (Merck, Germany 61781005001046) l^{-1} and 0.5-1.0 mg creatine monohydrate (HiMedia RM161) were added to it and shaken thoroughly for the production of pink colour, indicating the positive reaction.

3.2.2. Characterisation of the *Bacillus cereus* isolates

3.2.2.1. Growth temperature requirement

Growth temperature was determined by inoculating J-broth supplemented with 1 g l^{-1} agar with a 24 h-old culture (Claus and Berkeley, 1986). The tubes were incubated at 4-55 °C, and examined after every seven days up to 21 days for the low temperatures (4-20 °C) and after 5 days for the higher temperatures.

3.2.2.2. Susceptibility to antibiotics

Disc agar diffusion method (HiMedia, 1998) was used to develop antibiogram of the *B. cereus* isolates against 14 antibiotics (per disc: 10 µg ampicillin, 10 µg carbenicillin, 30 µg cephalothin, 10 U penicillin G, 10 µg vancomycin, 30 µg chloramphenicol, 15 µg erythromycin, 30 µg kanamycin, 10 µg streptomycin, 30 µg tetracycline, 300 U polymyxin B, 30 µg nalidixic acid, 5 µg metronidazole and 15 µg rifampicin), commonly used for treating gastroenteritis. Colonies, grown on tryptone soya agar at 37 °C for 24 h, were transferred to about 5 ml tryptone soya broth and incubated for 6–8 h. After incubation, inoculum was applied evenly onto Mueller-Hinton agar plate (4 mm thick) using a sterile cotton swab (HiMedia PW005). After drying for 15 min, different antibiotic susceptibility test discs were applied aseptically and the plates were incubated at 37 °C for 14–19 h. All the experiments were carried out in triplicate and the results were expressed as diameter of inhibition zone.

3.2.2.3. Production of extracellular enzymes

Production of protease, lipase and amylase by the isolates was determined using skim milk agar, trybutyrin agar and starch agar, respectively. Plates were spotted with 24 h-old cultures using a 2 mm-diameter loop and incubated for 18-20 h at 37 °C. Diameter of clear zone was measured directly in case of skim milk agar and trybutyrin agar plates. The starch agar plates were flooded with gram's iodine solution to obtain zone of clearance, if any. The results were expressed as ratio of clear zone diameter to diameter of the spot.

3.2.2.3.1. Thermostability of protease

The experiment was done with one respective isolate, selected from each product type on the basis of largest zone of clearance on skim milk agar. Inoculation was made into the protease production medium. After incubation for 48 h at 37 °C under shaking condition (100 rpm), the culture was centrifuged (7800 *g* for 10 min) at 4 °C to obtain a crude enzyme extract (Patel *et al.*, 2005).

Relative proteolytic activity was measured according to Thys *et al*. (2004) with modification. The crude enzyme extract (120 µl) was mixed with 250 µl of azocasein (2.5 g l^{-1}; A2765, Sigma-Aldrich Corporation, St. Louis, MO, USA) in 0.05 M potassium phosphate buffer (pH 7.0) and incubated at 37 °C for 1 h. The reaction was terminated by adding 750 µl cold 3 mol l^{-1} trichloroacetic acid (HiMedia GRM6274). After standing for 1 h at 4 °C, the mixture was centrifuged at 13,000 *g* for 10 min. The supernatant (50 µl) was mixed with 2 ml of purified water and analysed for free dye by measuring the absorbance at 400 nm (UV-Vis spectrophotometer 118; Systronics, Ahmedabad, India). One unit of proteolytic activity was defined as the amount which caused an absorbance increase of 0.01 unit under the assay condition.

Thermostability of protease was determined by treating the crude enzyme extract for 10 min at different temperatures (40-90 °C), followed by estimating residual relative proteolytic activity as described above.

3.2.2.3.2. Thermostability of lipase

The experiment was done with one respective isolate selected from each product type on the basis of largest zone of clearance on trybutyrin agar. Inoculation was made into the lipase production medium. After incubation for 48 h at 37 °C under shaking condition (150 rpm), the culture was centrifuged (2800 *g* for 30 min) at 4 °C to obtain a crude enzyme extract.

Lipase activity was measured according to Gupta *et al.* (2002). The crude enzyme extract (1 ml) was mixed with 9 ml of substrate solution, prepared by freshly mixing lipase assay reagent solutions A and B, incubated at 37 °C for 15 min, and the absorbance was measured at 410 nm. One unit of lipolytic activity was defined as the amount which caused an absorbance increase of 0.01 unit under the assay condition.

Thermostability of lipase was determined by treating the crude enzyme extract for 10 min at different temperatures (40-90 °C), followed by estimating residual relative lipolytic activity as described above. All the experiments were carried out in triplicate.

3.2.2.4. Production of haemolysin

A 24 h-old nutrient broth culture was spotted on blood agar plate containing 50 ml l^{-1} defibrinated sheep blood and incubated for 16-18 h at 30 °C (Prüß *et al*., 1999). The results were expressed as ratio of clear zone diameter to diameter of the spot.

3.2.2.5. Production of enterotoxin

Enterotoxin production by the isolates was checked by using 3M Tecra *Bacillus* diarrhoeal enterotoxin visual immunoassay kit (3M Australia Pty Limited, Frenchs Forest, NSW, Australia). Brain heart infusion broth, supplemented with 10 g glucose l^{-1}, was inoculated with a 24 h-old culture, incubated at 37 ºC for 24 h and centrifuged at 7830 *g* for 10 min. The supernatant was used for enterotoxin detection as per manufacture's instruction. Amounts of produced enterotoxin were evaluated with index values derived from the Tecra reading scale; indices from 1 to 5 corresponded to the coloration intensity. According to the manufacturer's instructions, strains with an index of <3 were considered negative.

3.2.2.6. Biofilm formation assay

Biofilm formation and quantification were carried out following the method modified after Harvey *et al.* (2007). An overnight-grown culture of the isolates on nutrient agar was resuspended in sterile distilled water. Biofilm was allowed to develop by inoculating 150 µl reconstituted skim milk per well with the resuspended culture of 10^5 total cells. Following incubation of the plates at 30 °C for 24 h, the wells were washed thrice with distilled water, allowed to dry at 30 ºC for 30 min, added with gram's crystal violet solution and held at 20 ºC. After 45 min, excess stain was pipetted out, and the wells were washed thrice with distilled water and air-dried at 30 °C for 30 min. Each well was added with 100 µl of 950 ml l^{-1} ethanol and left for 30 min to elute the stain. Intensity of the stain was measured by taking optical density (OD) readings at 595 nm using a microplate reader (iMark; Bio-Rad, Tokyo, Japan). OD was assumed to be proportional to the amount of biofilm. To correct background staining, the mean OD-value obtained for well without biofilm (wells containing reconstituted milk, not inoculated but treated similar to wells containing biofilm) was subtracted from the OD-value obtained in each condition. All the experiments were carried out in triplicate sets.

3.2.3. *In vitro* model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks

Among the nine strains of *B. cereus* isolated from chilling tanks, PT4 was found to exhibit maximum proteolytic activity and resistance against multiple antibiotics. Hence, this strain was selected for the model study.

An *in vitro* model to simulate the conditions in chilling tanks was set up: scenario 1: biofilm formation in chilling tanks where pasteurised milk is stored at 4 °C; scenario 2: inadequately cleaned chilling tanks with subsequent pasteurised milk collection and storage at 4 °C; and scenario 3: inadequately cleaned chilling tanks left to stand at room temperature for subsequent milk collection.

PT4 (initial total cell count: 10^4 ml^{-1}) was inoculated into reconstituted skim milk containing sterilised stainless steel coupons (2 cm dia, grade 304 with 2B finish) and incubated at 4 °C for 24 h. Three sets of experiment, each in triplicate, were designed. Biofilm cells were recovered from the coupons following the method based on Teh *et al*. (2012). The coupons were rinsed in sterile distilled water for three consecutive times to remove non-biofilm cells, transferred to peptone physiological saline and vortexed with glass beads for 2 min. The saline was decimally diluted and plated on milk agar. The plates were incubated at 30 °C for 24 h, and the colonies were counted and expressed as log cfu cm^{-2}. This represented scenario 1. The coupons from the second set of scenario 1 were transferred to sterilised centrifuge tube containing fresh sterile reconstituted skim milk and further incubated at 4 °C for 24 h; this simulated scenario 2. The coupons from the third set of scenario 1 were transferred to sterile empty centrifuge tubes and further incubated at 27 °C for 24 h. Biofilm cells from the coupons of scenario 2 and 3 were recovered as in case of scenario 1.

3.2.4. Response surface optimisation for *Bacillus cereus* biofilm removal

3.2.4.1. Alkali-based cleaning-in-place

3.2.4.1.1. Influence of NaOH treatment on biofilm removal

The effectiveness of NaOH was determined according to Sharma and Anand (2002). Biofilms were allowed to develop on coupons by inoculating overnight culture of PT4 on nutrient agar (initial total count: 10^4 ml^{-1}) into reconstituted skim milk containing sterilised coupons placed at air-liquid interface and incubated at 30 °C for 24 h. The coupons with 24 h-old biofilm were washed thrice with sterile distilled water to remove non-biofilm cells and exposed to varying concentrations of NaOH, time and temperature.

The coupons were then rinsed thrice with neutralisation buffer, 10 s for each time. The survivors after treatment were estimated by vortexing the coupons with glass beads for 2 min in peptone saline, further diluting and plating on milk agar. The plates were incubated at 30 °C for 24 h, and the colonies were counted and expressed as log cfu cm^{-2}. Log reduction of biofilm cells recovered from the coupons was determined by the following equation:

$$\text{Log reduction} = \text{Log } N - \text{Log } n$$

where N was the count of untreated control cells and n was the count of cells recovered after treatment (van de Weyer *et al.*, 1993).

Response surface methodology (RSM) was used for investigating the influence of three independent variables (time, temperature and NaOH concentration) on biofilm cell removal. The low, middle and high levels of each variable were designated as -1, 0 and +1, respectively, and alpha 1.681 is the axial distance from the centre point (Table 8). A total of 20 experiments were designed by using Design Expert version 8.0 (Stat-Ease Inc., Minneapolis, MN, USA). The experimental design is shown in Table 9

Table 8. Levels of variables in experimental design for *Bacillus cereus* biofilm cell removal from stainless steel coupon using alkali

Independent variable	Coded level[a]				
	-1.682	-1	0	1	1.682
Time (min)	3.18	10	20	30	36.82
Temp. (°C)	31.48	40	52.50	65	73.52
NaOH (g l^{-1})	6.6	1	15	20	23

[a] Low, middle and high levels, of each variable were designated as -1, 0 and +1, respectively.

3.2.4.1.2. Effectiveness of reference and optimised cleaning-in-places

To determine the efficacy of the reference cleaning-in-place (CIP; 10 g NaOH l^{-1} at 65 °C for 10 min - water rinse – 10 ml HNO_3 l^{-1} at 65 °C for 10 min – water rinse) and optimised CIP (15 g NaOH l^{-1} at 65 °C for 30 min - water rinse – 10 ml HNO_3 l^{-1} at 65 °C for 10 min - water rinse), 24 h-old biofilms were allowed to develop on the coupons, which then underwent treatment with reference and optimised CIP regimes. Following each cleaning regime, biofilm cells were recovered according to Teh *et al.* (2012), spread on milk agar plates and incubated at 30 °C for 24 h. Crystal violet staining of the coupons, which underwent optimised cleaning regime and also which contained 24 h-old biofilm but without treatment, was performed to check whether biofilm matrix material was really removed or were only cells in the biofilm inactivated (Harvey *et al.*, 2007; Wijman *et al.*, 2007).

Table 9. Design of RSM for *Bacillus cereus* biofilm cell removal from stainless steel coupon using alkali

Run	*A*: Time (min)	*B*: Temp. (°C)	*C*: NaOH (g l^{-1})
1	20.00	52.50	15
2	20.00	52.50	23
3	30.00	40.00	20
4	30.00	40.00	10
5	30.00	65.00	20
6	10.00	65.00	10
7	36.82	52.50	15
8	20.00	52.50	15
9	10.00	40.00	10
10	20.00	52.50	6.6
11	20.00	52.50	15
12	10.00	40.00	20
13	20.00	73.52	15
14	3.18	52.50	15
15	20.00	52.50	15
16	20.00	31.48	15
17	20.00	52.50	15
18	30.00	65.00	10
19	10.00	65.00	20
20	20.00	52.50	15

The coupons were added with 3 ml of 10 g crystal violet (HiMedia GRM114) l^{-1} and incubated at 20 °C for 45 min. After staining, the excess stain was removed, and the coupons were washed thrice with sterile distilled water and air-dried at 30 °C for 30 min. Each coupon was added with 3 ml of 950 ml ethanol l^{-1} and left for 30 min to elute the stain, if any. Intensity of the stain was monitored by measuring OD at 595 nm. To correct background staining, the mean OD-value obtained for control (without biofilm) was subtracted from the mean OD-value obtained from each condition.

3.2.4.2. Enzyme-based cleaning-in-place

3.2.4.2.1. Optimisation of biofilm removal in microtiter plate using protease

Serine protease (subtilisin A; P4860; Novozymes) of ≥2.4 U g^{-1} was obtained from Sigma-Aldrich, St Louis, MO, USA. According to the manufacturer, the enzyme was active at pH 6.5–8.5 and enzyme activity was optimal at 60 ºC. The enzyme solution was diluted appropriately using 0.1 mol l^{-1} phosphate buffer (for pH 5.8–8.0) and 0.1 mol l^{-1} bicarbonate buffer (for pH 8.5 and pH 9.1). *Bacillus cereus* M28, isolated from one sample of pasteurised milk, was selected for the study as it exhibited a strong biofilm-forming ability in microtiter plates. RSM was used for investigating the influence of three variables, namely time, pH and protease concentration on biofilm removal. A total of 20 experiments with 8 factorial points, 6 axial points and 6 replicates at the central

Table 10. Levels of variables in experimental design for *Bacillus cereus* biofilm removal from microtiter plate using protease

Independent variable	Coded level[a]				
	-1.682	-1	0	1	1.682
Time (min)	3.18	10	20	30	36.82
pH	5.81	6.5	7.5	8.5	9.18
Enzyme (mU ml^{1})	659	1000	1500	2000	2340

[a] Low, middle and high levels of each variable were designated as -1, 0 and +1, respectively.

points based on low (-1), middle (0) and high (+1) levels of each variable with 1.681 axial distance from the centre point were designed by using Design Expert. The experimental designs are shown in Tables 10 and 11.

Table 11. Design of RSM for *Bacillus cereus* biofilm removal from microtiter plate using protease

Run	*A*: Time (min)	*B*: pH	*C*: Enzyme (mU ml^{-1})
1	20	7.5	1500
2	20	7.5	1500
3	30	6.5	1000
4	10	6.5	2000
5	20	7.5	1500
6	20	7.5	1500
7	10	8.5	2000
8	10	8.5	1000
9	20	9.1	1500
10	30	6.5	2000
11	20	7.5	659
12	30	8.5	1000
13	20	7.5	2340
14	3.18	7.5	1500
15	20	7.5	1500
16	30	8.5	2000
17	20	5.8	1500
18	20	7.5	1500
19	10	6.5	1000
20	36.82	7.5	1500

Randomised experiments were conducted. After numerical optimisation for maximum biofilm removal, validation of the optimised models was carried out.

Biofilm formation and quantification were carried out as discussed in section 3.2.2.6. Biofilm on microtiter plate was exposed to varying concentrations of protease (0.659-2.3 U ml^{-1}), pH (5.8-9.1) and time (3.2-36.8 min) at 60 ºC. After reaction, the enzyme solution was pipetted out and biofilm removal was quantified by crystal violet staining method as described in section 3.2.2.6. OD was assumed to be proportional to the amount of biofilm; lower the OD, higher is the biofilm removal. To correct background staining, the mean OD-value obtained for well without biofilm (wells containing reconstituted milk, not inoculated but treated similar to wells containing biofilm) was subtracted from the OD-value obtained in each condition. All the experiments according to experimental runs were carried out in triplicate sets in independently treated microtiter plates.

3.2.4.2.2. Removal of biofilm developed on stainless steel coupons using optimised protease treatment

An overnight-grown culture of *B. cereus* M28 on nutrient agar (initial total cell count: 10^5 ml^{-1}) was used to inoculate reconstituted skim milk containing sterilised coupons placed at air-liquid interface and incubated at 30 ºC. The coupons with 24 h-old biofilm were washed thrice with sterile distilled water to remove the non-biofilm cells, exposed to protease (1.0 U ml^{-1} buffer, pH 8.5) for 20 min at 60 ºC and rinsed thrice with sterile distilled water. Biofilm cells were recovered from the coupons following the method described in section 3.2.4.1.1. To cross check if the coupons contained any viable cells, the treated coupons were placed into nutrient broth for enrichment. The broth was incubated at 37 °C for 48 h, plated on *Bacillus cereus* selective agar and incubated further. When coupons in the enrichment broth showed growth, biofilm cells were considered to be present, i.e. below the limit of detection, and no growth indicated complete removal of biofilm cells.

3.2.4.2.3. Comparative efficiency of alkali- and optimised protease-based cleaning-in-places

The RSM results were used to design an optimised protease CIP regime (1.0 U ml^{-1} protease - pH 8.5 buffer at 60 ºC for 20 min - water rinse – 10 ml HNO_3 l^{-1} at 65 ºC for 10 min - water rinse) which was compared with the reference CIP regime (10 g NaOH l^{-1} at 65 ºC for 10 min - water rinse – 10 ml HNO_3 l^{-1} at 65 ºC for 10 min - water rinse) which is commonly practiced and optimised alkali CIP regime (15 g NaOH l^{-1} at 65 ºC for 30 min - water rinse – 10 ml HNO_3 l^{-1} at 65 ºC for 10 min - water rinse). To determine the efficiency of reference CIP, optimised alkali CIP and optimised protease CIP regimes, 24 h-old biofilms were allowed to develop on coupons. Those coupons then underwent treatment with alkali and optimised protease regimes. Following cleaning, biofilm cells were recovered as described in section 3.2.4.1.1 and spread on milk agar plates which were incubated at 30 ºC for 24 h. The coupons containing 24 h-old biofilms, which underwent treatment as well as those without treatment, were stained with crystal violet solution as described in section 3.2.4.1.2.

3.2.5. Quantitative risk assessment of human exposure to *Bacillus cereus* associated with household refrigerated storage of pasteurised milk

3.2.5.1. Survey

A survey was conducted on domestic refrigeration storage conditions of 50 randomly selected households in the district of Darjeeling. The temperatures of the top, middle and lower parts of the refrigerators were recorded. Questionnaires were used to collect information on the position in the refrigerators where pasteurised milks were stored and the storage time of those.

3.2.5.2. Exposure assessment

A quantitative risk assessment model was developed to evaluate public health risks associated with the consumption of pasteurised milk contaminated with *B. cereus*. RSM was used for investigating the influence of three risk factors (storage time, storage temperature and load of *B. cereus* cells) on the final population of *B. cereus* M312, one of the most potent producers of enterotoxin. A total of 20 experiments were designed by using Design Expert. Based on the survey of domestic refrigeration storage conditions, low (-1), middle (0) and high (+1) levels of each variable were selected with 1.681 axial distance from the centre point. The experimental designs are shown in Tables 12 and 13.

Table 12. Levels of variables in the experimental design for exposure assessment with *Bacillus cereus*

Independent variable	Level[a]				
	-1.682	-1	0	1	1.682
Time (h)	7.64	24	48	72	88.36
Temp. (ºC)	4.95	7	10	13	15.05
Load (log *N* ml^{-1})	2.32	3	4	5	5.68

[a] Low, middle and high levels of each variable were designated as -1, 0 and +1, respectively.

Table 13. RSM design and experimental values of exposure assessment with *Bacillus cereus*

Run	*A*: Time (h)	*B*: Temp. (°C)	*C*: Cell load (log cfu ml^{-1})
1	72.00	7.00	3.00
2	24.00	13.00	5.00
3	48.00	10.00	4.00
4	88.36	10.00	4.00
5	48.00	10.00	4.00
6	48.00	4.95	4.00
7	72.00	7.00	5.00
8	24.00	7.00	3.00
9	48.00	10.00	4.00
10	72.00	13.00	3.00
11	48.00	10.00	4.00
12	48.00	10.00	4.00
13	48.00	15.04	4.00
14	48.00	10.00	4.00
15	48.00	10.00	5.68
16	24.00	7.00	5.00
17	24.00	13.00	3.00
18	72.00	13.00	5.00
19	7.63	10.00	4.00
20	48.00	10.00	2.31

Numerical optimisation was carried out using Design Expert to determine optimum levels of the independent variables leading to a minimum response. A cell count of 4 log cfu ml^{-1} was specified as the highest acceptable upper limit (Notermans *et al.*, 1998).

3.2.6. Statistical analyses

Experimental data were analysed statistically using Microsoft Excel 2007 and SPSS v. 16.0. Principal component analysis (PCA) was conducted to examine relationship between the variables and original data set. Five different variables, namely production of protease, amylase, lipase, haemolysin and biofilm by the isolates were subjected to PCA. Varimax rotation method was used to produce orthogonal transformations which make component matrix easier to interpret than unrotated matrix.

Agglomerative hierarchical clustering was applied to data set to cluster different isolates of the *B. cereus* based on studied characters in PCA by XLSTAT v. 14. It is an iterative classification method. The process started by calculating the dissimilarity between the *N* objects. Then two objects, which when clustered together minimise a given agglomeration criterion, were clustered together thus creating a class comprising these two objects. Then, the dissimilarity between this class and the *N*-2 other objects was calculated using the agglomeration criterion. The two objects or classes of objects whose clustering together minimises the agglomeration criterion were then clustered together. This process continued until all the objects could be clustered. The results are presented in the form of a dendrogram to facilitate the visualisation of the sample relationships.

Data for biofilm formation were analysed using Excel and SPSS, expressed as mean ± *SE*, and subjected to one-way analysis of variance (*ANOVA*) and paired *t*-test. *ANOVA* was conducted on log transformed data to determine if biofilm formation in *in vitro* model had any significant differences ($P < 0.05$). *T*-test was performed to find out whether there was significant difference ($P < 0.05$) between reference and optimised CIPs.

All the experimental results obtained from the response optimisation study were analysed using Design Expert and expressed as mean ± *SE*. Quadratic models were used to fit the experimental data and the models for the responses described the effect of the independent variables in terms of linear, quadratic and cross product terms. 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 *SE* of the estimate. One-way *ANOVA* was also performed for each response variable and the *P*-values indicated which terms were significant.

For quantitative risk assessment of human exposure to *B. cereus* associated with household refrigerated storage of pasteurised milk, the data on storage time and temperature, and load of *B. cereus* cells were fitted and quantified by normal distribution (Schaffner *et al.,* 2003) with subsequent Monte Carlo simulation with 10,000 iterations for probability calculations using Excel with an add-in XLsim.