4 Results

4.1. Prevalence and characterisation of *Bacillus cereus* isolates from marketed dairy products

4.1.1. Prevalence

Isolates with characteristic turquoise to peacock blue colonies surrounded by a zone of precipitate of the same colour on *Bacillus cereus* selective agar were regarded as presumptive *B. cereus s.l.* (Fig. 9A). Gram positive, motile, endospore-forming rods, which were positive for glucose fermentation, nitrate reduction and Voges-Proskauer reaction (Table 14), were regarded as confirmed *B. cereus s.l.* and selected for further study. In rest of the thesis, *B. cereus s.l.* is referred as *B. cereus*.

Out of 230 samples of milk and dairy products, *B. cereus* was detected in 73 (32%), from which a total of 144 isolates were obtained. The prevalence of *B. cereus* in cheese, ice cream, milk powder and milk was high (33–55%), while it was low in butter (20%) and paneer (4%). None of the curd and khoa samples were found contaminated. The level of population of *B. cereus* was high (maximum 6-8 log cfu ml^{-1} or g^{-1}) in ice cream and cheese, moderate (maximum 3-4 log cfu ml^{-1} or g^{-1}) in milk, milk powder and butter, and low (maximum 2.6 log cfu g^{-1}) in paneer (Table 15).

4.1.2. Characterisation

4.1.2.1. Growth temperature requirement

Out of 144 isolates, 107 (74%) were able to grow at $\leq$7 ºC and 21 (15%) at 20-50 ºC (Table 16).

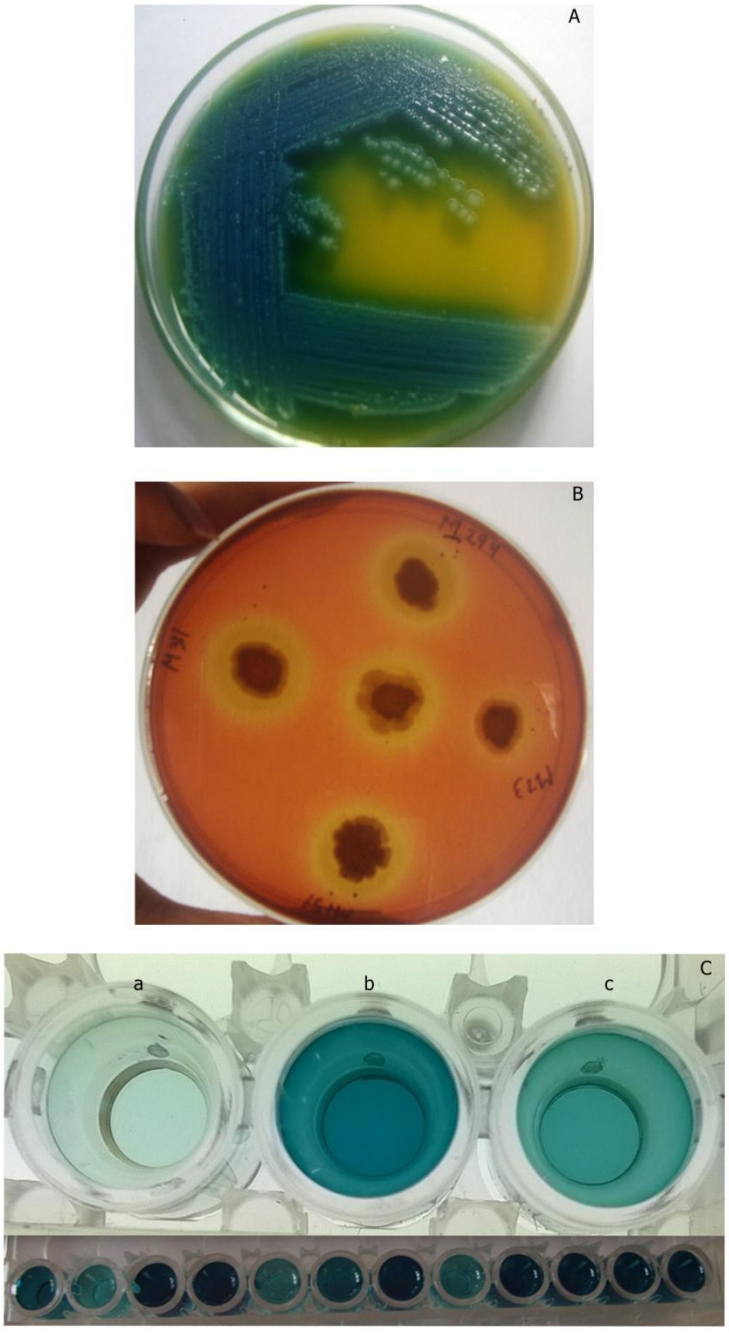

Fig. 9. *Bacillus cereus* isolation and characterisation. A, *Bacillus cereus* colonies on *Bacillus cereus* selective agar plate; B, haemolysis by some isolates on sheep blood agar plate; C, diarrhoeal enterotoxin production by some isolates detected by Tecra antibody test.

Table 14. Confirmation of the presumptive *Bacillus cereus* isolates, grown on *Bacillus cereus* selective agar plate[a]

Isolate code	Biochemical parameter[b]			% positive
	Nitrate reduction	Glucose fermentation	VP reaction	
M11, M12	+, +	+, +	+, +	100
M21, M22, M23, M24, M25	+, +, +, +, +	+, +, +, -, +	+, +, +, +, +	80
M61, M62, M63, M64, M65	+, +, +, +, +	+, +, +, +, +	+, +, -, +, +	80
M71, M72, M73, M74, M75	+, -, +, +, -	+, +, +, +, +	+, +, +, +, +	60
M81, M82, M83, M84, M85	+, -, -, -, +	+, -,+,+,+	+, -, +, -, +	40
M121	+	+	+	100
M131	+	+	+	100
M151, M152, M153	+, +, +	+, -, +	+, -, +	67
M161, M162, M163, M164	+, +, +, +	+, -, +, +	-, -, -, -	0
M171	+	+	+	100
M191, M192	-, -	-, +	-, -	0
M211, M212, M213, M214, M215	+, +, +, +, -	+, +, +, +, +	-, +, +, +, +	60
M221	+	+	+	100
M231, M232, M233	+, -, +	+, +, +	+, -, +	33
M241, M242	+, +	+, +	-, +	50
M251	-	+	+	0
M261, M262, M263, M264, M265	-, -, +, -, -	+, +, +, +, +	+, +, +, +, +	20
M28	+	+	+	100
M291, M292, M293, M294, M295	+, +, +, +, +	+, +, -, +, +	+, +, +, +, +	80
M29B1, M29B2, M29B3, M29B4, M29B5	+, +, +, -, +	+, +, +, +, +	+, +, +, -, +	80
M301, M302, M303, M304, M305	+, -, +, -, +	+, +, +, +, +	+, -, -, +, -	20
M311, M312, M313, M314, M315	+, +, -, +, +	+, +, +, +, +	+, +, +, -, +	60
M321, M322, M323, M324, M325	+, +, +, +, +	+, +, +, +, +	+, -, +, +, +	80
M361, M362, M363, M364, M365	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
M381, M382, M383, M384, M385	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
M411, M412, M413, M414, M415	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
M441, M442, M443, M444, M445	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
M451, M452, M453, M454, M455	+, +, +, -, -	+, +, +, +, +	+, +, +, +, +	60
M481, M482, M483, M484, M485	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
M491, M492, M493, M494, M495	+, +, +, +, +	+, +, +, +, +	+, +, +, -, +	80
M511, M512, M513, M514, M515	+, +, +, -, +	+, +, +, +, +	+, -, +, +, +	60
M521, M522, M523, M524, M525	+, +, -, -, -	+, +, +, +, +	+, -, +, +, +	20
M541, M542, M543, M544, M545	+, +, +, +, -	+, +, -, +, +	+, +, +, -, -	40
MP21, MP22, MP23, MP24, MP25	-, +, +, +, +	+, +, +, +, +	+, -, -, +, +	40
MP41, MP42, MP43, MP44, MP45	+, -, -, -, -	+, +, +, +, +	+, +, -, +, +	20
MP61, MP62, MP63, MP64, MP65	+, +, +, +, -	+, +, +, +, +	+, +, -, -, +	40
MP71, MP72, MP73, MP74, MP75	-, +, +, +, -	+, +, +, +, +	+, -, +, -, +	20
MP91, MP92, MP93, MP94, MP95	+, -, +, +, +	+, +, +, +,+	+, +, -, -, +	40
MP111, MP112, MP113, MP114, MP115	-, +, +, +, +	+, +, +, +,+	-, -, +, +, +	60
MP121, MP122, MP123, MP124, MP125	+, +, -, +, +	+, +, +, +, +	+, +, +, +, -	60
MP131, MP132, MP133, MP134, MP135	+, -, -, -, -	+, +, +, +, +	+, +, +, +, +	20
MP141, MP142, MP143, MP144, MP145	+, +, -, +, -	+, +, +, +, +	+, +, +, -, +	40
MP201, MP202, MP203, MP204, MP205	+, +, +, +, -	+, +, +, +, +	+, +, +, -, +	60
MP211, MP212, MP213, MP214, MP215	+, +, +, -, -	+, +, +, +, +	+, -, -, +, +	20
MP241, MP242, MP243, MP244, MP245	+, -, -, +, -	+, +, +, +, +	+, +, +, -, -	20
MP251, MP252, MP253, MP254, MP255	+, +, +, +, +	+, +, +, +, +	-, +, + ,+, -	60
MP261, MP262, MP263, MP264, MP264	+, -, -, +, +	+, +, +, +, +	+, +, +, -, -	20
MP271, MP272, MP273, MP274, MP275	+, +, -, -, -	+, +, +, +, +	+, -, +, +, +	20
MP281, MP282, MP283, MP284, MP285	+, +, -, -, +	+, +, -, +, +	+, +, +, -, -	40
MP311, MP312, MP313, MP314, MP315	-, -, -, -, +	+, +, +, +, +	-, +, +, +, +	20
MP341, MP342, MP343, MP344, MP345	+, +, -, -, -	+, +, +, +, +	+, +, +, +, -	40
Ic31, Ic32, Ic33, Ic34, Ic35	-, +, +, +, +	+, +, +, +, +	+, +, -, -, -	20
Ic61, Ic62, Ic63, Ic64, Ic65	+, +, -, -, -	+, +, +, +, +	+, +, +, +, +	40
Ic81, Ic82, Ic83, Ic84, Ic85	+, +, -, -, -	+, +, +, +, +	+, -, +, +, +	20
Ic121, Ic122, Ic123, Ic124, Ic125	+, -, -, -, -	+, +, +, +, +	+, +, +, +, +	20
Ic131, Ic132, Ic133, Ic134, Ic135	+, -, +, -, +	+, +, +, +, +	+, -, -, +, -	20
Ic141, Ic142, Ic143, Ic144, Ic145	+, +, +, -, +	+, -, +, +, +	+, -, -, +, -	20

Isolate code	Biochemical parameter[b]			% positive
	Nitrate reduction	Glucose fermentation	VP reaction	
Ic171, Ic172, Ic173, Ic174, Ic175	+, +, +, -, +	+, -, +, -, +	+, -, -, -, -	20
Ic181, Ic182, Ic183, Ic184, Ic185	-, +, +, -, +	+, -, +, -, +	+, -, -, -, +	20
Ic191, Ic192, Ic193, Ic194, Ic195	+, -, +, -, +	+, +, +, +, +	+, -, -, +, -	20
Ic201, Ic202, Ic203, Ic204, Ic205	+, -, +, -, +	-, +, +, +, +	+, -, -, +, +	20
P41, P42, P43, P44, P45	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
C11, C12, C13, C14, C15	+, -, +, -, +	+, +, +, +, -	+, -, -, +, +	20
C31, C32, C33, C34, C35	+, +, +, +, +	+, +, +, +, +	+, -, -, -, -	20
C41, C42, C43, C44, C45	+, -, -, +, +	+, +, +, +, +	+, -, +, -, -	20
C51, C52, C53, C54, C55	+, +, -, +, -	+, +, +, +, +	+, +, +, -, +	40
C91,C92,C93,C94,C95	+, +, -, +, -	+, +, +, +, +	+, -, +, -, +	20
C101, C102, C103, C104, C105	-, +, -, +, -	+, +, +, +, +	+, -, +, -, -	0
C121, C122, C123, C124, C125	+, -, +, -, +	+, +, +, +, +	+, -, -, +, -	20
C141, C142, C143, C144, C145	+, -, +, -, +	-, +, +, +, +	+, -, -, +, +	20
C171, C172, C173, C174, C175	+, +, +, -, +	+, +, +, +, +	+, -, -, -, -	20
B11, B12, B13, B14, B15	-, -, +, +, +	+, +, +, +, -	+, +, -, -, -	0
B21, B22, B23, B24, B25	+, +, +, +, +	+, +, +, +, +	-, -, -, -, +	20
B51, B52, B53, B54, B55	-, -, +, +, -	+, +, +, +, +	+, -, +, -, +	20
B111, B112, B113, B114, B115	-, +, +, -, -	+, +, +, +, +	+, +, +, -, +	20
B131, B132, B133, B134, B135	-, +, +, +, +	+, +, +, +, +	+, -, -, -, +	20
R41, R42, R43, R44, R45	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
R51, R52, R53, R54, R55	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
R111, R112, R113, R114, R115	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
R121, R122, R123, R124, R125	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
R141, R142, R143, R144, R145	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
R151, R152, R153, R154, R155	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
R171, R172, R173, R174, R175	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp51, Pbp52, Pbp53, Pbp54, Pbp55	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp71, Pbp72, Pbp73, Pbp74, Pbp75	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp111, Pbp112, Pbp113, Pbp114, Pbp115	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp141, Pbp142, Pbp143, Pbp144, Pbp145	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp171, Pbp172, Pbp173, Pbp174, Pbp175	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp181, Pbp182, Pbp183, Pbp184, Pbp185	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp191, Pbp192, Pbp193, Pbp194, Pbp195	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
Pbp201, Pbp202, Pbp203, Pbp204, Pbp205	+, +, +, +, +	+, +, +, +, +	+, +, +, +, +	100
PT11, PT12, PT13	+, +, +	+, +, +	+, +, +	100
PT4	+	+	+	100
PT81, PT82, PT83, PT84	+, +, +, +	+, +, +, +	+, +, -, +	75
PT91, PT92	+, +	+, +	+, +	100

[a] The isolates in a row were the maximum number of colonies obtained from the same plate. All the isolates were motile, endospore-forming and gram positive rods.
[b] +, positive reaction; -, negative reaction. M, milk; MP, milk powder; Ic, Ice cream; P, paneer; C, cheese; B, butter; R, raw milk; Pbp, pasteurised milk before packaging; PT, pasteurised milk chilling tanks.

Table 15. Prevalence and population of *Bacillus cereus* in market samples (n = 230) of various dairy products

Product	No. of samples	Positive samples (%)	Population (log cfu)
Milk (pasteurised/sterilised)	55	55	1-4 ml^{-1}
Milk powder	35	52	2-3 g^{-1}
Ice cream	25	40	2-8 ml^{-1}
Paneer	25	4	1.3-2.6 g^{-1}
Khoa	20	0	<dl[a]
Curd	20	0	<dl
Cheese	25	33	2-6 g^{-1}
Butter	25	20	3-4 g^{-1}

[a] dl, detection limit (1 log cfu g^{-1})

4.1.2.2. Susceptibility to antibiotics

The results for susceptibility of the 144 isolates to 14 different antibiotics, including *b*-lactams (4), benzene derivative (1),aminoglycosides (2), macrolide (1), peptide (1), glycopeptide (1), naphthyridone (1),nitro-imidazole (1), rifampicin and tetracycline are shown in Table 17. All the isolates were multi-drug resistant; each of

Table 16. Range of growth temperatures of *Bacillus cereus* isolates from dairy products

Source	No. of isolates	Temperature range (% of positive isolates)			
		4-40 °C	7-40 °C	10-45 °C	20-50 °C
Milk	83	71	12	5	12
Milk powder	32	31		34.6	34.4
Ice cream	11	90	10		
Paneer	5	17	83		
Butter	4	100			
Cheese	9	89		11	

Table 17. Antibiogram of 144 isolates of *Bacillus cereus* from dairy products

Mechanism of action	Antibiotic disc^{-1}	Percent score[a]		
		Sensitive	Intermediate	Resistant
Inhibition of cell wall synthesis	Ampicillin (A; 10 µg)	1		99
	Carbenicillin (Cb; 10 µg)	1	3	96
	Cephalothin (Ch; 30 µg)	7	8	85
	Penicillin G (P; 10 U)		2	98
	Vancomycin (Va; 10 µg)	50	11	39
Inhibition of protein synthesis	Chloramphenicol (C; 30 µg)	88	3	9
	Erythromycin (E; 15 µg)	50	42	8
	Kanamycin (K; 30 µg)	69	13	18
	Streptomycin (S; 10 µg)	89	2	9
	Tetracycline (T; 30 µg)	75	12	13
Damage to cell membrane	Polymyxin B (Pb; 300 U)	67	17	16
Inhibition of nucleic acid synthesis	Nalidixic acid (Na; 30 µg)	37	41	22
	Metronidazole (Mt; 5 µg)			100
	Rifampicin (R; 15 µg)	15	12	73

[a] The inhibition zone size (diameter in mm) interpretation was based on HiMedia instruction sheet (the following values are upper and lower cut-off lines for resistant and sensitive, respectively): A, 28 and 29; Cb, 19 and 23; Ch, 14 and 18; P, 19 and 28; Va, 14 and 17; C, 12 and 18; E, 13 and 23; K, 13 and 18; S, 11 and 15; T, 14 and 19; Pb, 8 and 12; Na, 13 and 19; Mt, 8 and 13; R, 16 and 20.

those was resistant to at least five different antibiotics used. Most of the isolates were resistant to β-lactams (ampicillin, carbenicillin, cephanothin and pencillin G), but susceptible to protein synthesis inhibitors. Only 16% of the isolates, initially enriched on *Bacillus cereus* selective agar (containing 100 U polymyxin B l^{-1}), were resistant to a higher concentration of polymyxin B (300 U disc^{-1}).

4.1.2.3. Production of extracellular enzymes

The results for the production of extracellular enzymes are presented in Table 18. Among the 144 isolates, 97%, 96% and 63% produced protease, lipase and amylase, respectively, and 60% produced all the three enzymes.The maximum clear zone-producing isolates from each product on skim milk and tributyrin agar plates were selected for the assay of protease and lipase, respectively, and evaluation of thermostability (Table 19).

Table 18. Production of extracellular enzymes and enterotoxins by the strains of *Bacillus cereus*, isolated from different dairy products

Source	No. of isolates	% of positive isolates				
		Protease	Lipase	Amylase	Haemolysin	Enterotoxin[a]
Milk	83	92	100	82	90	98
Milk powder	32	100	97	50	84	100
Ice cream	11	100	100	75	90	100
Paneer	5	100	100	100	67	100
Butter	4	100	100	0	100	100
Cheese	9	100	50	23	100	89

[a] *Bacillus* diarrhoeal enterotoxin, detected by 3M™ Tecra™ *Bacillus* diarrhoeal enterotoxin VIA kit.

Table 19. Relative activities and thermostability of the crude enzymes from selected isolates of *Bacillus cereus* from dairy products

Isolate No.	Source	Ratio[a]	Temperature (°C)[b]						
			37	40	50	60	70	80	90
Proteolytic activity									
M312	Milk	2.2	1.5a ± 0.1	1.5a ± 0.1	1.4a ± 0	1.4a ± 0	1.2b ± 0.3	1.1b ± 0	1.1b ± 0.1
MP113	Milk powder	2.6	1.9a ± 0.1	1.9a ± 0.1	1.8a ± 0	1.8a ± 0	1.5b ± 0.1	1.6b ± 0.1	1.6b ± 0.1
IC63	Ice cream	3.2	2.4a ± 0.2	2.4a ± 0.2	2.2a ± 0.1	2.2a ± 0.1	1.8b ± 0.1	1.7b ± 0.1	1.6b ± 0
P23	Paneer	2.2	1.5a ± 0.1	1.4a ± 0.1	1.4a ± 0	1.3b ± 0.1	1.3b ± 0.1	1.2b ± 0	1.1b ± 0.1
B3	Butter	2.0	2.0a ± 0.2	1.9a ± 0.1	1.8a ± 0	1.7b ± 0	1.7b ± 0	1.7b ± 0	1.6b ± 0
C3	Cheese	1.6	2.2a ± 0.1	2.2a ± 0.1	2.2a ± 0	2.2a ± 0.1	2.0a ± 0.1	2.0a ± 0.1	2.0a ± 0.1
Lipolytic activity									
M144	Milk	2.7	33.0a ± 0.7	33.0a ± 0	32.6a ± 0.3	23.3b ± 1.0	21.0b ± 0	20.6c ± 0.3	15.0d ± 0.1
MP251	Milk powder	2.4	35.0a ± 0.6	33.0a ± 1.0	33.0a ± 0	33.3a ± 1.0	23.3b ± 1.0	15.3c ± 0.9	8.3d ± 0.9
IC65	Ice cream	3.1	46.0a ± 1.0	42.0a ± 1.0	42.0a ± 1.0	34.0b ± 0.6	35.0b ± 1.0	31.0b ± 1.0	10.0c ± 0.1
P22	Paneer	1.7	11.3a ± 0.8	11.7a ± 0.3	12.0a ± 0	8.6b ± 0.3	7.3b ± 0.3	8.3b ± 0.3	2.7c ± 0.3
B5	Butter	1.5	12.0a ± 1.0	10.7a ± 0.6	10.3a ± 0.1	7.7b ± 0.3	6.7b ± 0.9	5.3c ± 0.3	1.6d ± 0.3
C51	Cheese	1.5	11.3a ± 0.3	11.7a ± 0.3	12.0a ± 0	11.0a ± 0.3	8.6b ± 0	8.3b ± 0.3	2.3c ± 0.3

[a] Diameter of zone of clearance to that of colony spot on skim milk agar (proteolytic activity) and tributyrin agar (lipolytic activity), incubated at 37 °C.
[b] Values, showing mean ± *SE*, were obtained from triplicate sets. Means, sharing a common alphabet in each row, are not significantly (P <0.05) different.

At least 75% of the initial proteolytic activity of the isolates, except the one from cheese, was retained even at 90 °C. However, in the cheese isolate, there was no change in the activity. In case of isolates from cheese and paneer, 73% and in isolates from milk and ice cream, more than 60% of the initial lipolytic activities were retained even at 80 °C. However, in the isolates from milk powder and butter, more than 40% of the activity was retained.

4.1.2.4. Production of enterotoxins

Out of 144 isolates, 134 (93%) exhibited β-haemolysis on sheep blood agar and showed a discontinuous haemolytic pattern (Fig. 9B), characteristic for heamolysin BL (Table 18).

Production of diarrhoeal enterotoxin component, NheA, was measured (Fig. 9C). Out of 144 isolates, 140 (97%) were positive for the production of diarrhoeal enterotoxin. While 98% of the isolates from milk and 89% of cheese were found positive for diarrhoeal enterotoxin, all the isolates from milk powder, ice cream, paneer and butter produced diarrhoeal enterotoxin (Table 18).

4.1.2.5. Formation of biofilm

The results of biofilm formation assay by the isolates are given in Table 20. Of the 144 isolates, 78 (54%) were found to be weak biofilm formers, 13 (9%) were assessed as moderate and 12 (8%) as strong biofilm formers. Majority (71–90%) of the isolates from milk, cheese and ice cream were biofilm formers, while all the isolates from butter were positive.

Table 20. Clustering of 144 isolates of *Bacillus cereus* from dairy products on the basis of biofilm-forming ability at 4 °C

Group[a]	% of isolates					
	Milk	Milk powder	Ice cream	Paneer	Cheese	Butter
Non-biofilm former	29	69	10	83	11	
Weak biofilm former	54	15	80	17	22	25
Moderate biofilm former	9	8			11	
Strong biofilm former	8	8	10		56	75

[a] Isolates were designated as non-biofilm (<0.2), weak (0.2-0.6), moderate (>0.6-1.2) and strong (>1.2) biofilm formers, according to OD_{595} readings.

4.1.2.6. Relationship among characteristics

Principal component analysis allowed transformation of a large number of putative correlated variables into a smaller number of variables, called principal components (PCs) (Fig. 10). The first two PCs explained 51% of the variance of the whole data. The PC1 was strongly correlated with protease (CC = 77.5%), amylase (CC = 68.5%), lipase (CC = 48.9%) and biofilm formation (CC = 35.3%). On the other hand, the PC2 was strongly correlated to haemolysis (CC = 88.8%).

The results obtained from agglomerative hierarchical clustering (AHC) are shown in Fig. 11 and Table 21. All the 144 isolates were grouped into four major clusters. Cluster A contained 17 isolates; 29% from milk, 24% from milk powder, 35% from cheese and 12% from butter. The predominant cluster B contained 73 isolates. Although this cluster contained isolates from different products, 77% of them were from milk. Cluster C contained 27 isolates, of which 52% were from milk powder, 22% from milk, 15% from ice cream, 7% from paneer and 4% from butter. In cluster D, majority (56%) of the isolates were from milk.

4.2. Prevalence of *Bacillus cereus* in dairy processing environment

Bacillus cereus was present in 35% of raw milk samples collected from silo tanks where the level of contamination was up to 7 log cfu ml^{-1}. On the other hand, 40% of the pasteurised milk samples, collected before packaging, were positive. Few instances of increase in population level during processing was observed and the level of contamination was as high as 9 log cfu ml^{-1}. It was also recovered from 40% of the samples collected from stainless steel surfaces of pasteurised milk chilling tanks.

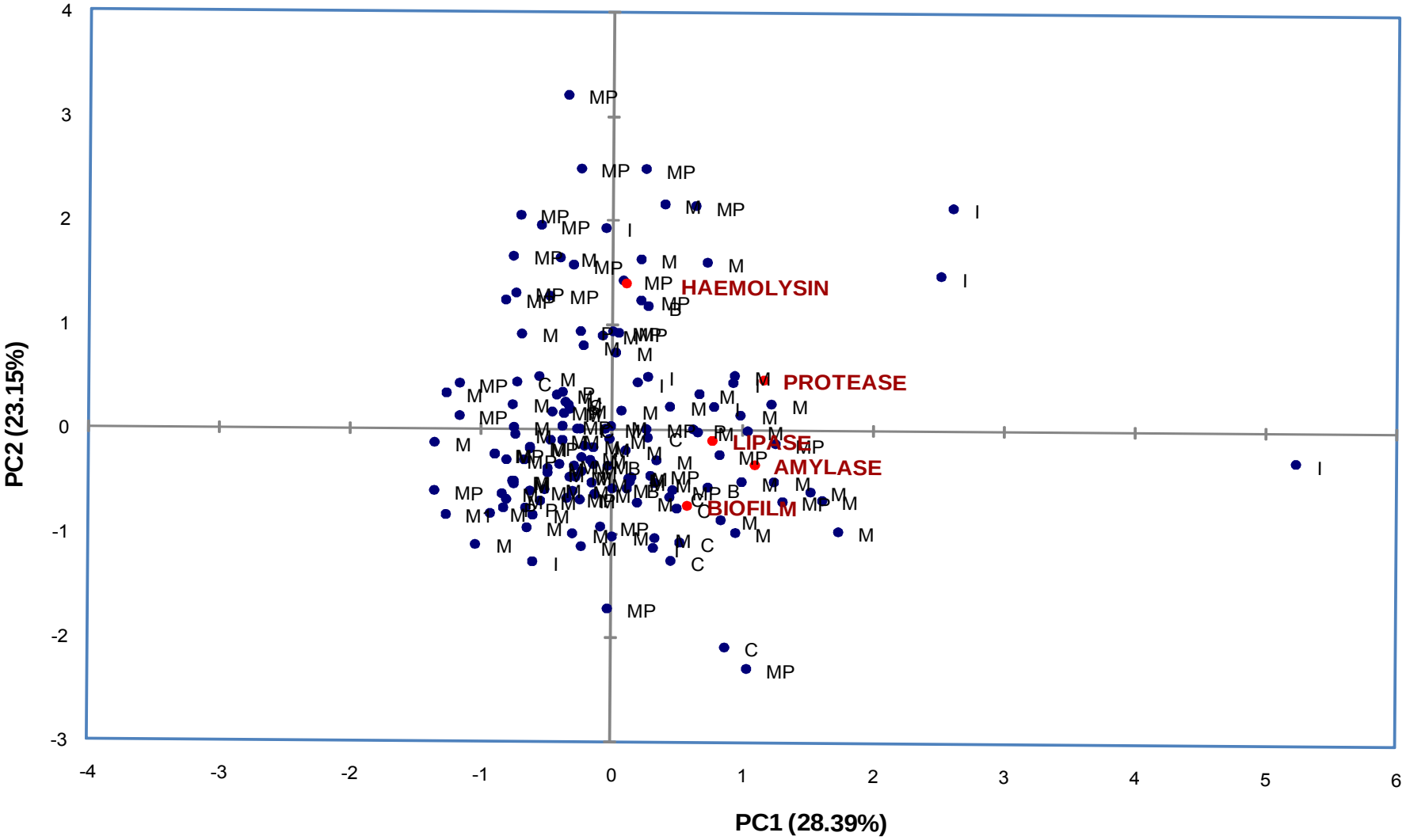

Fig. 10. Score biplot for principal component analysis showing observations (M, milk; MP, milk powder; C, cheese; I, ice cream; P, paneer; B, butter isolates) and variables (production of protease, amylase, lipase, haemolysin and biofilm) together for 144 isolates of *Bacillus cereus*

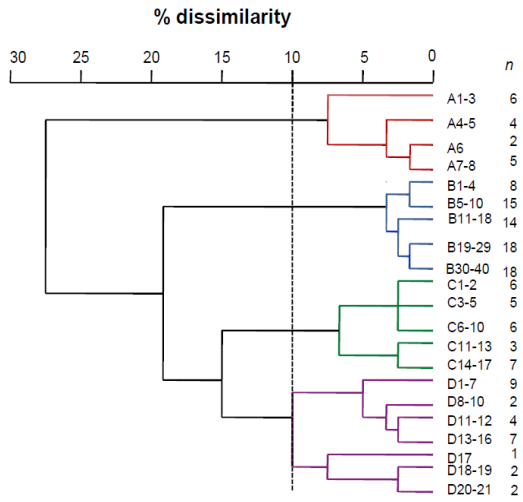

Fig. 11. Simplified dendrogram based on wards clustering of dissimilarity coefficient generated by agglomerative hierarchical clustering. Based on studied characters (production of protease, amylase, lipase, haemolysin and biofilm), the 144 isolates of *Bacillus cereus* were grouped into four major clusters, designated A through D. *n*, number of isolates in (sub)clusters

Table 21. Distribution of 144 isolates of *Bacillus cereus* from different dairy products among the clusters generated

Cluster	% of isolates					
	Milk	Milk powder	Ice cream	Paneer	Cheese	Butter
A	29	24			35	12
B	77	11	4	4	4	
C	22	52	15	7		4
D	55	18	15	4	4	4

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

Results of *in vitro* model study are shown in Table 22. Among the various isolates from pasteurised milk chilling tanks, *B. cereus* PT4 was selected for *in vitro* model study, as it exhibited maximum proteolytic activity and was multi-drug resistant. It was able to adhere and form biofilm on stainless steel coupons in an *in vitro* model, with 3 log cfu cm^{-2} recovered in scenario 1 and 2, and 6 log cfu cm^{-2} in scenario 3.

Table 22. Results of *in vitro* model study

In vitro model	Biofilm cell[a] (log cfu cm^{-2})
Scenario 1 Storage of milk in chilling tank (Stainless steel coupons in skim milk inoculated with 10^4 total cells of *B. cereus* ml^{-1}, incubated at 4 °C for 24 h)	$3.37^{b} \pm 0.12$
Scenario 2 Inadequately cleaned tanker with subsequent milk collection (Stainless steel coupons from scenario 1 transferred to fresh skim milk and further incubated at 4 °C for 24 h)	$3.11^{b} \pm 0.11$
Scenario 3 Inadequately cleaned tanker left to stand empty (Stainless steel coupons from scenario 1 transferred to a centrifuge tube and further incubated at 27 °C for 24 h)	$6.16^{a} \pm 0.07$

[a] Values, showing mean ± *SE*, were obtained from triplicate sets. Means sharing a common superscript are not significantly ($P < 0.05$) different.

4.4. Optimisation of *Bacillus cereus* biofilm removal by alkali-based cleaning-in-place

4.4.1. Influence of NaOH treatment on biofilm removal

The results of experiments conducted according to response surface methodology (RSM) design for biofilm cell removal, are shown in Table 23. The results for analysis of variance (*ANOVA*) are given in Tables 24 and 25.

Table 23. Design of RSM, and its actual and predicted values for *Bacillus cereus* biofilm cell removal

Run	*A*: Time (min)	*B*: Temperature (°C)	*C*: NaOH (g l^{-1})	Log reduction[a] in *B. cereus* cell count cm^{-2}	
				Experimental[b]	Predicted
1	20.00	52.50	15.0	1.49 ± 0.05	1.53
2	20.00	52.50	23.4	1.76 ± 0.10	1.64
3	30.00	40.00	20.0	1.62 ± 0.03	1.62
4	30.00	40.00	10.0	0.66 ± 0.05	0.75
5	30.00	65.00	20.0	2.31 ± 0.05	2.55
6	10.00	65.00	10.0	0.69 ± 0.12	0.71
7	36.82	52.50	15.0	2.90 ± 0.20	2.73
8	20.00	52.50	15.0	1.54 ± 0.03	1.53
9	10.00	40.00	10.0	0.44 ± 0.07	0.23
10	20.00	52.50	6.6	0.44 ± 0.02	0.52
11	20.00	52.50	15.0	1.32 ± 0.10	1.53
12	10.00	40.00	20.0	1.20 ± 0	1.25
13	20.00	73.52	15.0	1.65 ± 0.05	1.56
14	3.18	52.50	15.0	0.99 ± 0.03	1.12
15	20.00	52.50	15.0	1.47 ± 0.20	1.53
16	20.00	31.48	15.0	0.33 ± 0.03	0.38
17	20.00	52.50	15.0	1.70 ± 0.10	1.53
18	30.00	65.00	10.0	2.26 ± 0.06	2.24
19	10.00	65.00	20.0	1.21 ± 0.03	1.15
20	20.00	52.50	15.0	1.65 ± 0	1.53

[a] Initial count, 5.3-5.5 log cfu cm^{-2}.
[b] Values, showing mean ± *SE*, of experiments were carried out in triplicate.

Table 24. *ANOVA* results for response surface quadratic model

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value Prob > *F*	Comment
Model	8.24	9	0.92	31.93	<0.0001	Significant
A- Time (min)	3.11	1	3.11	108.61	<0.0001	
B- Temp. (°C)	1.67	1	1.67	58.09	<0.0001	
C- NaOH (g l^{-1})	1.49	1	1.49	51.93	<0.0001	
AB	0.52	1	0.52	17.96	0.0017	
AC	9.112E-033	1	9.112E-033	0.32	0.5854	
BC	0.17	1	0.17	5.76	0.0373	
A^2	0.28	1	0.28	9.84	0.0106	
B^2	0.56	1	0.56	19.65	0.0013	
C^2	0.36	1	0.36	12.68	0.0052	
Residual	0.29	10	0.029			
Lack of fit	0.19	5	0.039	2.09	0.2182	Not significant**
Pure error	0.093	5	0.019			
Core total	8.53	19				

* Values of Prob > *F* less than 0.0500 indicate model terms are significant.
** Non-significant (lack of fit is good).

Table 25. *ANOVA* results for the equations of the Design Expert for studied responses[a].

Response	R^2	Adj R^2	Pred R^2	Adeq precision	*SD*	*CV*%	*PRESS*
Log reduction in *B. cereus* cell count cm^{-2}	0.9664	0.9361	0.8011	20.848	0.17	12.26	1.70

[a] *SD*, Standard deviation; *CV*, Coefficient of variation; *PRESS*, Predicted residual error sum of squares

The *ANOVA* of the quadratic regression model for biofilm cell removal were significant (P <0.05) with F-values of 31.93 and P-values of 0.0001. The predicted R^2 of 0.8011 was in reasonable agreement with the adjusted R^2 of 0.9361, and there was no significance in the lack of fit (P = 0.2182). This indicated that the model can be used to predict responses. The regression equation coefficient was calculated and data were fitted to a second order polynomial equation:

$$\text{Log reduction in biofilm cells cm}^{-2} = 1.53 + 0.48A + 0.35B + 0.33C + 0.25AB - 0.034AC - 0.14BC + 0.14A^2 - 0.20B^2 - 0.16C^2$$

where A was time (min), B was temperature (ºC) and C was NaOH concentration (g l^{-1}).

In order to determine the optimal levels of each variable for maximum biofilm cell removal, three-dimensional response surface and contour plots were generated by using Design Expert. Figure 12 shows the effect of two factors, while the other factor held at zero level. The results indicated that the interaction between time and temperature was an important parameter for biofilm cell removal, and maximum removal was predicted when the biofilm was exposed to 15 g NaOH l^{-1} at 65 °C for 30 min where 2.55 log reduction cm^{-2} in biofilm cell count was achieved.

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

The RSM results were used to design an optimised alkali cleaning-in-place (CIP) regime which consisted of 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. Results for the effectiveness of reference and optimised CIP regimes are given in Table 26. 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) achieved only 3.29 log
reduction in the number of *B. cereus* cells recovered from the stainless steel coupons when compared to control coupons (without treatment).

Table 26. Effect of different cleaning regimes on *Bacillus cereus* biofilm cell removal

Cleaning regime	Number of cells recovered from biofilm on stainless steel coupons (log cfu cm^{-2})[a]		Log reduction in cell count
	Without treatment	With treatment	
Reference CIP[b]	5.33 ± 0.33	2.03 ± 0.03	3.29 ± 0.34
Optimised CIP[c]	5.10 ± 0.10	0.33 ± 0.03	4.77 ± 0.22

[a] Values, showing mean ± *SE*, were obtained from triplicate sets.
[b] biofilm containing stainless steel coupons were treated with 10 g NaOH l^{-1} at 65 °C for 10 min, followed by rinsing with water, treating with 10 ml HNO_3 l^{-1} at 65 °C for 10 min, and again rinsing with water.
[c] biofilm containing stainless steel coupons were treated with 15 g NaOH l^{-1} at 65 °C for 30 min, followed by rinsing with water, treating with 10 ml HNO_3 l^{-1} at 65 °C for 10 min, and again rinsing with water.

On the other hand, the optimised CIP designed led to 4.77 log reduction. From crystal violet staining of the coupons it was evident that biofilm cells were not only inactivated by optimised CIP, but biofilm matrix also got removed from the coupons (Fig. 13A). This was substantiated by significantly different (P = 0.03) OD-value of elute from biofilm-containing coupons which underwent optimised CIP when compared to that of control coupons (Fig. 13B). Thus, optimised CIP was found to be significantly (P <0.05) more effective in biofilm removal as compared to reference CIP.

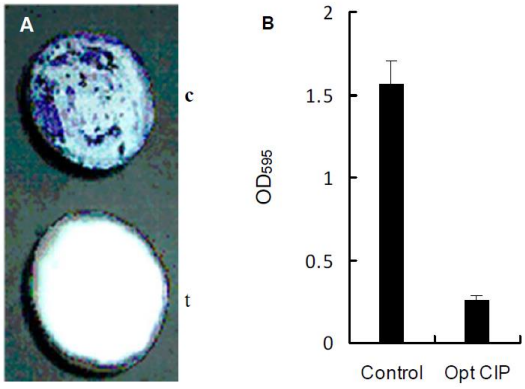

Fig. 13. Crystal violet-stained biofilms present on stainless steel coupons (A) and OD_{595} of stained biofilms from coupons before (c) and after (t) treatment with optimised cleaning regime (B). Error bars represent mean ± *SE*, obtained from triplicate sets of experiment.

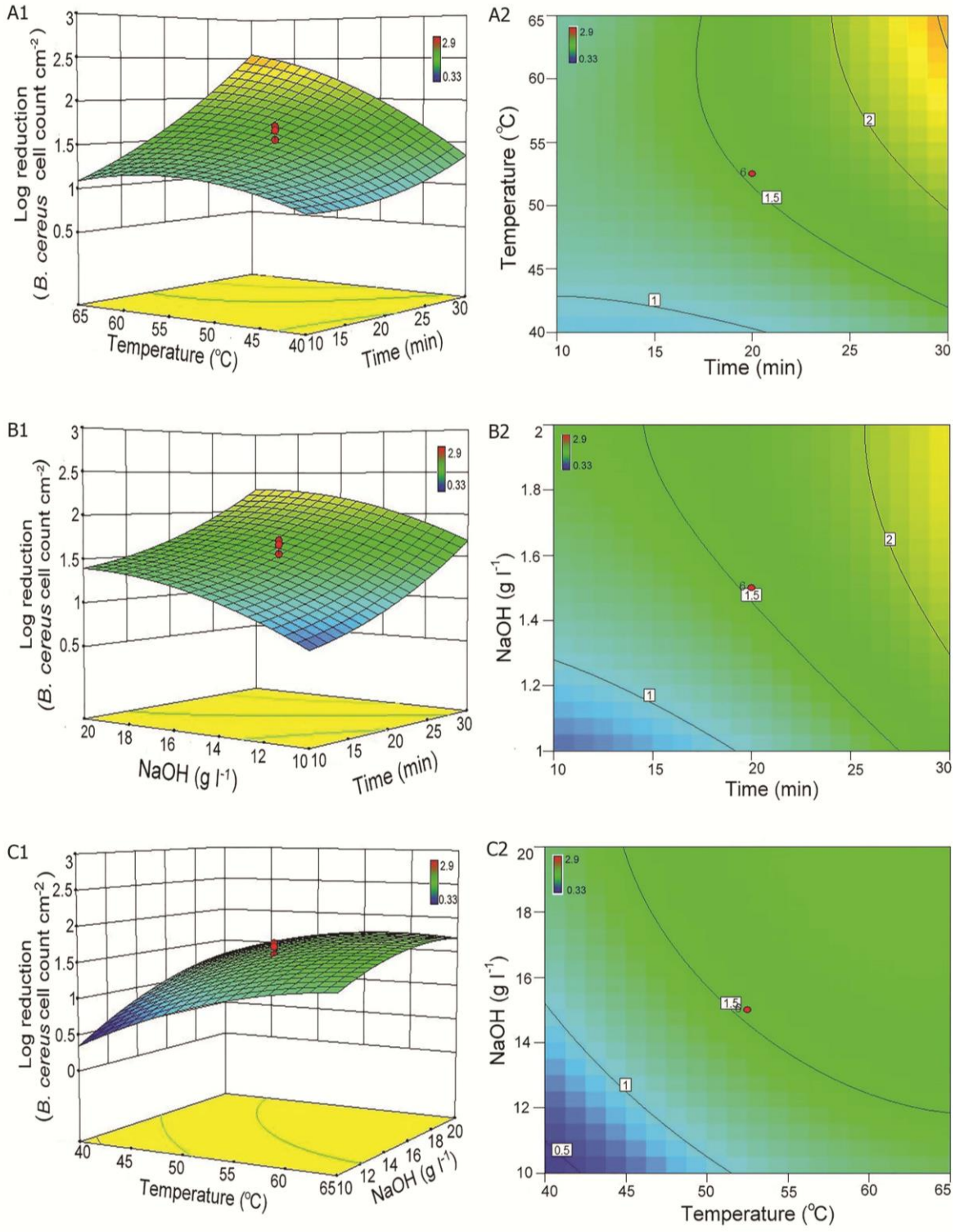

Fig. 12. 3-D (column 1) and contour response surface (column 2) plots on removal of *Bacillus cereus* biofilm. A1 and A2 show effect of time and temperature, when exposed to 15 g NaOH l^{-1}. B1 and B2 show effect of time and NaOH at 52.5 °C. C1 and C2 show effect of temperature and NaOH, when exposure time was 20 min.

4.5. Optimisation of *Bacillus cereus* biofilm removal by enzyme-based cleaning-in-place

4.5.1. Biofilm removal from microtiter plate using protease

The results of experiments conducted according to Design of RSM for *B. cereus* biofilm removal from microtiter plate by protease are given in Table 27. The *ANOVA* of the quadratic regression model for biofilm removal were significant

Table 27. Design of RSM for *Bacillus cereus* biofilm removal from microtiter plate by protease

Run	*A*: Time (min)	*B*: pH	*C*: Enzyme (mU ml^{-1})	OD_{595} of biofilm elute after treatment	
				Experimental[a]	Predicted
1	20.00	7.5	1500	1.20 ± 0.03	1.23
2	20.00	7.5	1500	1.20 ± 0.05	1.23
3	30.00	6.5	1000	1.76 ± 0.03	1.64
4	10.00	6.5	2000	2.09 ± 0.07	2.08
5	20.00	7.5	1500	1.20 ± 0.09	1.23
6	20.00	7.5	1500	1.20 ± 0.04	1.30
7	10.00	8.5	2000	1.83 ± 0.03	1.94
8	10.00	8.5	1000	1.94 ± 0.03	1.80
9	20.00	9.1	1500	1.89 ± 0.08	1.75
10	30.00	6.5	2000	1.84 ± 0.05	1.85
11	20.00	7.5	659	0.68 ± 0.03	0.69
12	30.00	8.5	1000	0.63 ± 0.07	0.62
13	20.00	7.5	2340	1.91 ± 0.09	1.91
14	3.18	7.5	1500	1.89 ± 0.06	1.76
15	20.00	7.5	1500	1.38 ± 0.03	1.23
16	30.00	8.5	2000	1.94 ± 0.05	1.80
17	20.00	5.8	1500	2.19 ± 0.03	2.17
18	20.00	7.5	1500	1.20 ± 0.06	1.23
19	10.00	6.5	1000	1.68 ± 0.08	1.80
20	36.82	7.5	1500	1.36 ± 0.05	1.50

[a] Values, showing mean ± *SE*, were obtained from triplicate sets. OD_{595} of *B. cereus* biofilm elute in control well without treatment was 2.52 ± 0.03.

(P <0.05) with F-values of 35.92 and P-values of 0.0001 (Table 28). Exposure time, pH, enzyme concentration and pH-enzyme concentration interaction had a significant (P <0.05) positive effect on biofilm removal. The predicted R^2 (0.7983) was in reasonable agreement with the adjusted R^2 (0.9430) and there was no significance in the lack of fit (P = 0.0868), indicating applicability of the model to predict responses.

Table 28. *ANOVA* results of quadratic model for *Bacillus cereus* biofilm removal from microtiter plate by protease

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value Prob > *F*	Comment
Model	4.143	9	0.460	35.92	<0.0001	Significant
A – Time (min)	0.079	1	0.079	6.14	0.0327	
B - pH	1.165	1	1.165	90.93	<0.0001	
C - Enzyme (mU ml^{-1})	1.819	1	1.819	141.94	<0.0001	
AB	0.006	1	0.005	0.451	0.5171	
AC	0.003	1	0.003	0.234	0.6387	
BC	0.478	1	0.477	37.29	0.0001	
A^2	0.288	1	0.288	22.49	0.0008	
B^2	0.364	1	0.364	28.40	0.0003	
C^2	0.009	1	0.009	0.688	0.4261	
Residual	0.128	10	0.013			
Lack of fit	0.101	5	0.020	3.75	0.0868	Not significant**
Pure error	0.027	5	0.005	35.92		
Core total	4.271	19				
R^2						0.9699

* Values of Prob > *F* less than 0.05 indicate model terms are significant.
** Lack of fit is good.

The regression equation coefficient was fitted to a second order polynomial equation:

$$OD_{595} \text{ of } B.\ cereus \text{ biofilm elute} = 17.98 - 0.078A - 3.46B - 0.003C + 0.002AB - 3.87E - 06AC + 0.0005BC + 0.0014A^2 + 0.1598B^2 + 9.90E - 08C^2$$

where A was time (min), B was pH and C was protease concentration (mU ml^{-1}).

Three-dimensional response surface and contour plots were generated, representing the effect of two factors, while the other factor held at zero level (Fig. 14). When the biofilm was exposed to 1.5 U ml^{-1} protease for 20 min at pH 8.5 (Fig. 14A), the OD was 1.096. Biofilm removal increased with the increase in pH from 6.5 (OD, 1.91) to 8.5 (OD, 1.28). Maximum biofilm removal was predicted when the biofilm was exposed to 1.0 U ml^{-1} protease at pH 8.5 for 20 min, where the OD decreased to 0.52 (Fig. 14B). When the effect of exposure time and protease concentration was studied at pH 7.5, the biofilm exposed to 1.0 U ml^{-1} protease for 20 min caused a partial removal (OD, 0.89) (Fig. 14C).

Thus, the response surface plots as well as numerical optimisation predicted a maximum biofilm removal when the biofilm was exposed to 1.0 U ml^{-1} protease at pH 8.5 for 20 min. Experimentally obtained values were close to the predicted ones.

4.5.2. Biofilm removal from stainless steel coupons using optimised protease treatment

When the biofilm-coated stainless steel coupons were exposed to 1.0 U ml^{-1} protease in pH 8.5 at 60 ºC for 20 min, the OD-value of the elute was 0.396 as compared to control (OD, 1.2). With similar treatment of the biofilm in microtiter plate, the OD decreased to 0.3 as compared to control (OD, 2.52).

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

The RSM results were used to design an optimised protease CIP by replacing caustic step with the optimised protease treatment followed by nitric acid treatment. The optimised protease CIP (1.0 U ml^{-1} protease in 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) was compared with the reference (currently practiced alkali) CIP (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). Results for the comparative effectiveness of reference, optimised alkali and optimised protease CIPs are shown in Table 29. Efficacy of the optimised protease CIP was compared with that of the optimised alkali CIP (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) and that of the reference CIP. While the optimised alkali CIP caused a reduction of ≥4.92 log cfu cm^{-2} and the reference CIP achieved 4.08 log reduction, the optimised protease CIP was able to completely remove *B. cereus* biofilm cells from the coupons. In contrast to both reference and optimised alkali CIPs, the optimised protease CIP caused a complete removal of biofilm cells as well as removal (P <0.05) of biofilm matrix (Table 29).

Table 29. Effect of different cleaning-in-place (CIP) regimes on *Bacillus cereus* biofilm cell and matrix removal from stainless steel (SS) coupons[a]

Treatment	Count of *B. cereus* cells recovered from biofilm on SS coupons (log cfu cm^{-2})		Reduction in *B. cereus* count (log cfu cm^{-2})	Absorbance of crystal violet eluted from stained matrix (OD_{595} coupon^{-1})		Reduction in absorbance (OD_{595} coupon^{-1})
	Without treatment	With treatment		Without treatment	With treatment	
Reference CIP[b]	6.54 ± 0.03	2.46 ± 0.08	4.08c ± 0.12	1.15 ± 0.05	0.90 ± 0.03	0.25b ± 0.03
Optimised alkali CIP[c]	6.42 ± 0.06	<dl[e]	≥4.92b ± 0.06	1.20 ± 0.05	0.79 ± 0.07	0.41b ± 0.07
Optimised protease CIP[d]	6.54 ± 0.03	0[f]	6.54a ± 0.03	1.20 ± 0.05	0.49 ± 0	0.71a ± 0

[a] Values, showing mean ± *SE*, were obtained from triplicate sets. Means, within columns, sharing a common alphabet are not significantly (P <0.05) different.

[b] Biofilm containing SS coupons were treated with 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.

[c] Biofilm containing SS coupons were treated with 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.

[d] Biofilm containing SS coupons were treated with 1.0 U protease ml^{-1} in 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.

[e] dl, detection limit, 1.5 log cfu cm^{-2}

[f] Zero indicates no viable cells detected in enrichment broth.

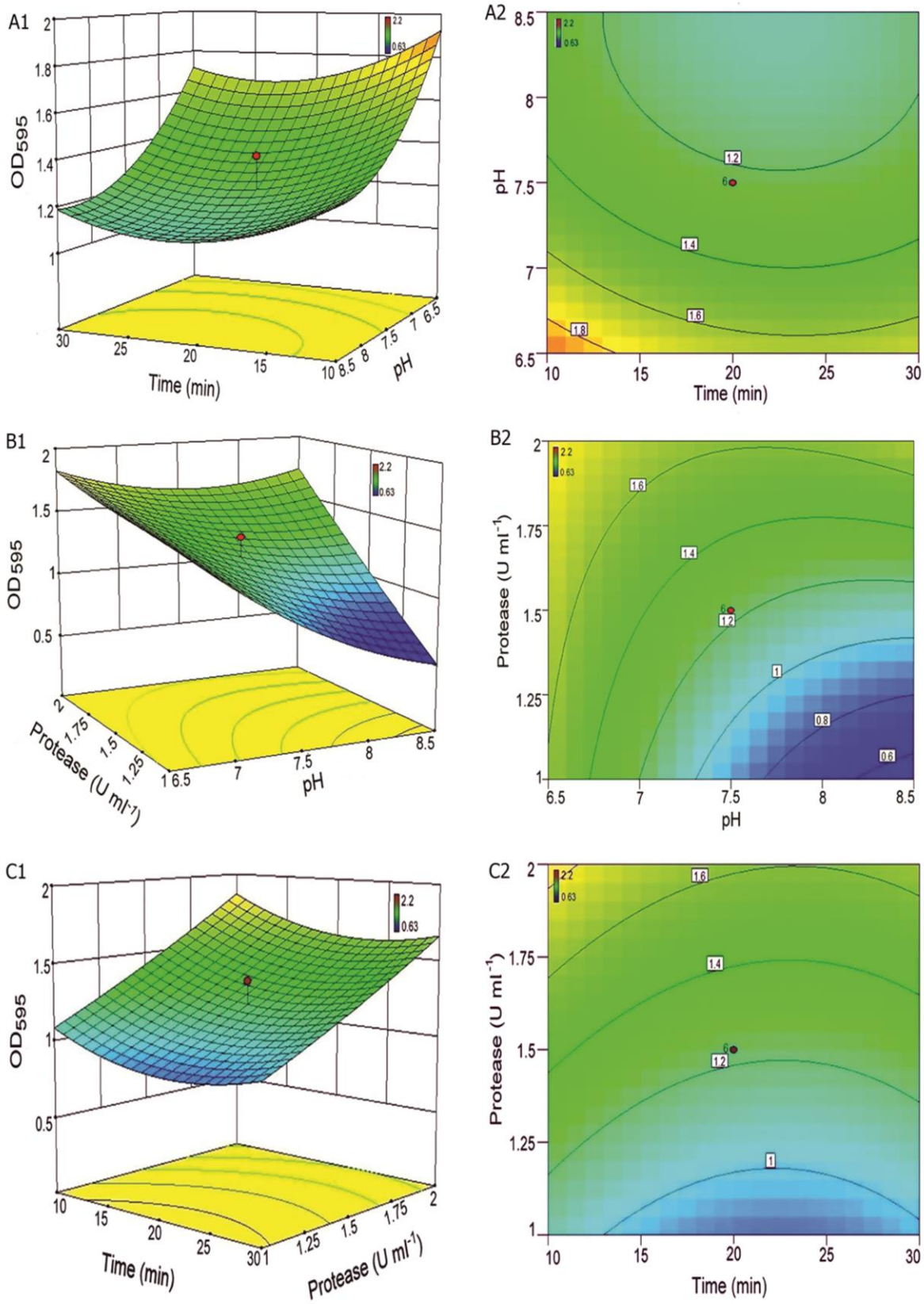

Fig. 14. 3-D (column 1) and contour response surface (column 2) plots on removal of *Bacillus cereus* M28 biofilm from the wells of microtiter plates. A1 and A2 show the effect of exposure time and pH, when protease concentration was 1.5 U ml^{-1}. B1 and B2 show the effect of pH and protease concentration, when exposure time was 20 min. C1 and C2 show the effect of exposure time and protease concentration at pH 7.5.

4.6. Quantitative risk assessment of human exposure to *Bacillus cereus*

4.6.1. Survey

A survey on the temperature of domestic refrigerators showed significant differences in temperatures between the different sections of the refrigerators; the warmest place was door shelf (10-12 °C), followed by the upper and the middle shelf (7-8 °C), while the lower shelves (6-7 °C) were the coldest positions. Milk stored in household refrigerator was usually consumed within three days.

4.6.2. Prevalence of *Bacillus cereus* in pasteurised milk stored in domestic refrigerators

Thirty percent of the pasteurised milk samples in 2-4 h-old stored packages from 50 household refrigerators were found to be contaminated with *B. cereus*, and the level of contamination was 3-5 log cfu ml^{-1}.

4.6.3. Monte Carlo simulation of the data on storage conditions

The distribution of *B. cereus* cells during storage of milk in household refrigerators is shown in Table 30. The population was 1.83-4.16 log cfu ml^{-1}, with the mean of 3, median of 2.9 and standard deviation of 0.5 log cfu ml^{-1}. The results of the Monte Carlo simulation showed that the 95th and 99th percentiles of the load of *B. cereus* in stored milk were 3.82 and 4.16 log cfu ml^{-1}, respectively, and only 1% of the stored milk had contamination of less than 2 log cfu ml^{-1}. The storage time for milk in household refrigerators ranged from 0.76 day to 3.55 days with the mean of 2.16, median of 2.1 and standard deviation of 0.6 day (Table 30). Only 1% of the stored milks were found to be used within the day of purchase, while 45% within 2 days and 90% within 3 days of purchase.

Table 30. Distribution of risk factors in pasteurised milk stored in domestic refrigerators against exposure to high levels of *Bacillus cereus*, determined using Monte Carlo simulation with 10,000 iterations

Distribution percentile	Storage temp. (°C)	Storage time (day)	Log cfu ml^{-1}
1	3.54	0.76	1.83
5	4.91	1.73	2.17
10	5.63	1.39	2.35
15	6.12	1.53	2.48
20	6.51	1.65	2.57
25	6.85	1.75	2.66
30	7.15	1.84	2.73
35	7.42	1.92	2.80
40	7.69	2.00	2.87
45	7.94	2.08	2.93
50	8.20	2.16	2.99
55	8.45	2.23	3.06
60	8.70	2.31	3.12
65	8.97	2.39	3.19
70	9.24	2.47	3.26
75	9.54	2.56	3.33
80	9.88	2.66	3.42
85	10.27	2.78	3.51
90	10.76	2.92	3.64
95	11.48	3.14	3.82
99	12.84	3.55	4.16

4.6.4. Exposure assessment

A predictive model was developed using RSM to study individual effects and interaction of three risk factors (storage time, storage temperature and load of *B. cereus* cells) on the final population of cells in milk (Table 31).

The *ANOVA* results of the quadratic regression model for *B. cereus* final population were significant ($P < 0.05$) with *F*-values of 57.42 and *P*-values of 0.0001 (Table 32). There was no significance in the lack of fit ($P = 0.4847$). The predicted R^2-value of 0.9132 was in reasonable agreement with the adjusted R^2-value of 0.9639. So, that the model can be used to predict responses. Quadratic model had R^2-value of 0.9810, which implies that the regression model explained 98.1% of the total variability in the final population. The regression equation coefficients were calculated and the data were fitted to a second order polynomial equation:

$$\textit{B. cereus} \text{ final population (log cfu ml}^{-1}) = 5.04 + 0.14A + 0.16B + 0.81C + 0.016AB - 0.0664AC + 0.008BC + 0.049A^2 + 0.067B^2 - 0.12C^2$$

where *A* was storage time (h), *B* was storage temperature (ºC) and *C* was *B. cereus* load (log cfu ml^{-1}).

Table 31. 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})	Log cfu ml^{-1}	
				Experimental[a]	Predicted
1	72.00	7.00	3.00	4.30 ± 0.03	4.24
2	24.00	13.00	5.00	6.00 ± 0.06	5.93
3	48.00	10.00	4.00	5.30 ± 0.07	5.04
4	88.36	10.00	4.00	5.40 ± 0.05	5.42
5	48.00	10.00	4.00	5.00 ± 0.05	5.04
6	48.00	4.95	4.00	4.80 ± 0.03	4.94
7	72.00	7.00	5.00	5.80 ± 0.06	5.73
8	24.00	7.00	3.00	4.00 ± 0.03	3.86
9	48.00	10.00	4.00	4.90 ± 0.10	5.04
10	72.00	13.00	3.00	4.63 ± 0.05	4.59
11	48.00	10.00	4.00	5.10 ± 0.06	5.04
12	48.00	10.00	4.00	4.99 ± 0.05	5.04
13	48.00	15.04	4.00	5.50 ± 0.03	5.51
14	48.00	10.00	4.00	4.99 ± 0.08	5.04
15	48.00	10.00	5.68	6.00 ± 0.06	6.05
16	24.00	7.00	5.00	5.70 ± 0.03	5.61
17	24.00	13.00	3.00	4.20 ± 0.04	4.14
18	72.00	13.00	5.00	6.10 ± 0.06	6.12
19	7.63	10.00	4.00	4.80 ± 0.05	4.94
20	48.00	10.00	2.31	3.20 ± 0.03	3.30

[a] Values, showing mean ± *SE*, were obtained from triplicate sets.

Table 32. *ANOVA* results for quadratic model of exposure assessment with *Bacillus cereus*

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value Prob > *F*[*]	Comment
Model	10.22	9	1.1300	57.420	<0.0001	Significant
A- Time (min)	0.27	1	0.2700	13.910	0.0039	
B- Temp. (°C)	0.38	1	0.3800	19.700	0.0013	
C- Cell load (log cfu ml^{-1})	9.15	1	9.1500	462.550	<0.0001	
AB	0.002	1	0.0020	0.106	0.7506	
AC	0.035	1	0.0350	1.770	0.2123	
BC	0.0006	1	0.0006	0.030	0.8638	
A^2	0.035	1	0.0350	1.810	0.2078	
B^2	0.065	1	0.0650	3.320	0.0981	
C^2	0.231	1	0.2310	11.720	0.0065	
Residual	0.197	10	0.0190			
Lack of fit	0.100	5	0.0200	1.030	0.4847	Not significant
Pure error	0.097	5	0.0190			
Core total	10.42	19				

[*] Values of Prob > *F* less than 0.0500 indicate model terms are significant.

In order to determine the effect of each variable in increasing *B. cereus* population, three-dimensional response surface and contour plots were generated. Figure 15 represents the effect of two factors, while the other factor is at zero level. The results indicate that the cell load and storage temperature were the main influencing factors in increasing the microbial population during storage in domestic refrigerators. On the other hand, the storage time did not have much effect on the final population. The cell load individually was the most significant factor in increasing the final population, which was also substantiated by the *ANOVA* results. Interaction of storage temperature and *B. cereus* load also had a positive effect on the final population. *Bacillus cereus* population reached the maximum level (5.97 log cfu ml^{-1}) when the reconstituted skim milk with *B. cereus* cells (5 log cfu ml^{-1}) was incubated at 13 ºC for 48 h (Fig. 15).

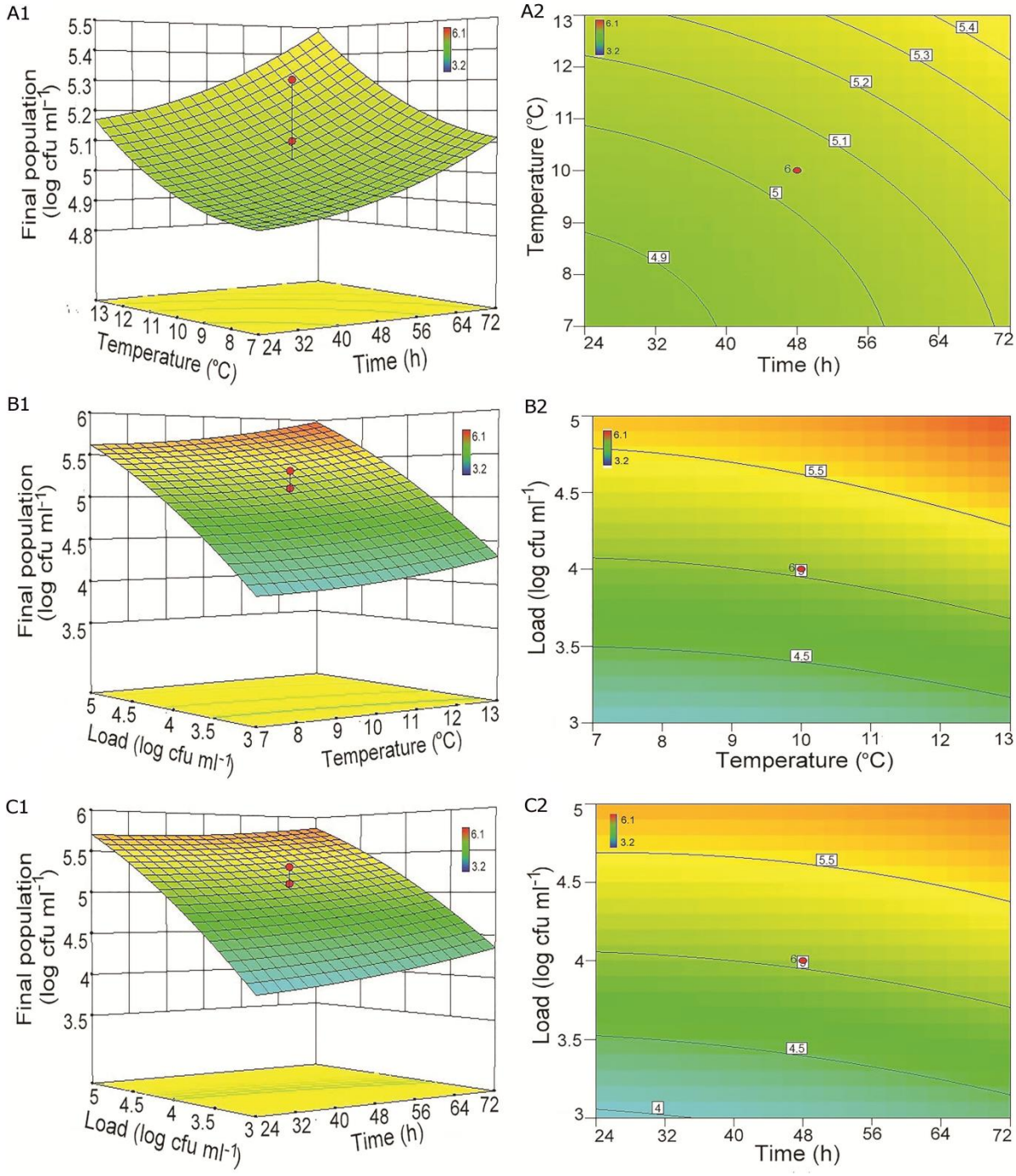

Fig. 15. 3-D (column 1) and contour response surface (column 2) plots on final population of *Bacillus cereus*. A1 and A2 show the effect of storage time and temperature when the load was 4 log cfu ml^{-1}. B1 and B2 show the effect of storage temperature and load after 48 h of storage. C1 and C2 show the effect of storage time and load when milk was stored at 10 °C.

The results of numerical optimisation are presented in Table 33. The model predicted *B. cereus* population will reach the threshold level (>4 log cfu ml^{-1}) after 47.51 h, 45.54 h, 41.59 h, 35.26 h, 25.31 h and 24 h at 7 ºC, 8 ºC, 9 ºC, 10 ºC, 11 ºC and 12–13 ºC, respectively, when the load of *B. cereus* cells in milk is 3 log ml^{-1}.

Table 33. Final population of *Bacillus cereus* at different time-temperature exposures

Temp. (°C)	Storage time (h)	Log cfu ml^{-1}[a]	
		Predicted	Experimental[b]
7	47.51	4.00	4.02 ± 0.02
8	45.54	4.00	4.03 ± 0.03
9	41.59	4.00	4.00 ± 0
10	35.26	4.00	4.02 ± 0.01
11	25.31	4.00	4.01 ± 0
12	24.00	4.00	4.00 ± 0
13	24.00	4.00	4.02 ± 0.01

[a] Load was 3 log cfu ml^{-1}
[b]Values, showing mean ± *SE*, were obtained from triplicate sets.

To verify the predicted results experimentally,laboratory experiments were carried out. The results obtained (Table 33) were close to the predicted values, confirming the efficiency of the present model.

4.6.5. Hazard characterisation and risk calculation

Mean storage temperature (8.2 °C), storage time (2.16 days) and *B. cereus* load (3 log cfu ml^{-1}), obtained from Monte Carlo simulation of actual data collected (Table 30), were used to predict the level of risk being exposed to *B. cereus* cells at the time of intake of milk. When the above parameters were used for numerical optimisation, the results indicate that the level of *B. cereus* cells will reach up to 4.5 log cfu ml^{-1} if milk is stored for 2.16 days at 8.2 ºC.