Prevalence and characterization of Bacillus cereus group from various marketed dairy products in India

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ORIGINAL PAPER

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Abstract *Bacillus cereus* group, associated with foodborne outbreaks and dairy defects such as sweet curdling and bitterness of milk, is an indicator of poor hygiene, and high numbers are unacceptable. In the present study, the prevalence of *B. cereus* group was investigated in a total of 230 samples belonging to eight different types of dairy products marketed in India. The prevalence of *B. cereus* group in cheese, ice cream, milk powder, and milk was high (33%–55%), whereas it was low in butter and paneer samples (20% and 4%, respectively). None of the curd and khoa samples were found contaminated. The level of contamination in the various dairy products was up to 10^8 cfu.g^{-1} or mL^{-1}. An antibiogram of 144 isolates of *B. cereus* group was obtained using 14 different antibiotics commonly used against foodborne diseases. All the 144 isolates were multidrug (at least five antibiotics) resistant. Ninety-three percent of them exhibited β-hemolysis. Of the 144 isolates, 97%, 96%, and 63% were capable of producing protease, lipase, and amylase, respectively, indicating spoilage potentiality of the isolates. Seventy-one percent of the isolates formed biofilm at 4 °C. The principal component analysis allowed classifying different correlated variables into two types of risks (spoilage and food poisoning). Hierarchical cluster analysis classified isolates into four main groups on the basis of the studied characters. The present study will help to better assess the health and spoilage risk associated with *B. cereus* group in dairy environment and to incorporate adequate preventive measures.

Keywords *Bacillus cereus* group · Dairy product · Antibiogram · Extracellular enzyme · Hemolysis · Biofilm

1 Introduction

India is the largest milk-producing nation with estimated production of 132.4 million tons in 2012–2013 (NDDB 2013). The growing share of milk and milk products in food in developing countries has accelerated the demand for dairy products. With the

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increasing demand of dairy products, the need of extended refrigerated storage of raw milk before processing and the application of higher pasteurization temperatures and prolonged shelf-life requirements have enhanced the importance of thermoduric microorganisms (Meer et al. 1991).

The *Bacillus cereus* group sensu lato (s.l.) is now attracting the greatest interest among researchers working on bacilli as the members are not only responsible for spoilage of dairy products but also have been associated with foodborne outbreaks. The *B. cereus* group consists of seven closely related species: *B. cereus* sensu stricto (s.s.), *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, and *Bacillus cytotoxicus.* The group is composed of seven phylogenetic groups (Guinebretière et al. 2010, 2013). The strains of *B. cereus* s.s., *B. anthracis*, and *B. thuringiensis* are spreading over groups II, III, IV, and V. *B. pseudomycoides* belongs to group I; *B. weihenstephanensis* and *B. mycoides* belong to group VI; group VII consists of *B. cytotoxicus*.

There are two distinct syndromes caused by separate toxins produced by *B. cereus* group: emetic and diarrheal. The emetic type characterized by the occurrence of nausea and vomiting within 6 h after ingestion is caused by small cyclic heat-stable peptide, cereulide (Rajkovic et al. 2008), and the diarrheal type characterized by the occurrence of abdominal pain and watery diarrhea within 8 to 16 h after ingestion is caused by hemolysin BL (Beecher et al. 1995). Hemolysin is a three-component enterotoxin produced by *B. cereus* group which consists of two lytic components (L1 and L2) and a binding component B. It has hemolytic, dermonecrotic, and vascular permeability activities. Thus, it is considered as one of the potential virulence factors in *B. cereus*-mediated diarrhea (Beecher et al. 1995).

B. cereus group members produce various extracellular enzymes. Production of protease, lipase, and amylase by contaminating bacteria in dairy environment can be responsible for a decrease in the organoleptic quality of milk and milk products. The presence of protease can lead to bitter flavor, clotting, and gelation of milk (Chen et al. 2003; Datta and Deeth 2003). On the other hand, lipases have been responsible for dairy defects such as bitty cream and also contribute to unpleasant flavor such as rancid, butyric, buttery, unclean, and soapy in milk and milk products (Furtado 2005). Starch has become an increasingly popular additive to dairy products such as ice cream and yoghurt because of its stabilizing properties, low cost, and availability. Thus, the presence of amylase can lead to potential spoilage of these products. The presence of heat-stable enzymes, especially protease and lipase in processed milk and milk products, can be a matter of concern as they can survive processing temperatures and be responsible for spoilage even if vegetative cells are eliminated during processing. Moreover *B. cereus* s.l. spores are hydrophobic in nature and can adhere to surfaces in dairy processing lines and form biofilm, resulting in the recurrent contamination of milk and poor-quality dairy products (Kumari and Sarkar 2014; Shaheen et al. 2010). This can lead to hygiene problems and economic losses due to spoilage and equipment impairment such as reduced flow through blocked tubes and reduced heat transfer through plate heat exchangers (Flint et al. 1997).

The incidence of foodborne illnesses has increased globally, and it becomes more important in developing countries where food products are exposed to contaminated environments in food processing industries and temperature abuse during transportation and storage at retail outlets (WHO 2007). As explicit data on the occurrence of

B. cereus group in dairy environment in India were lacking, the present investigation aimed to determine the prevalence of *B. cereus* group in Indian dairy markets. A series of relevant experiments to health risk and spoilage risk assessment, such as production of antibiogram, hemolysin, extracellular hydrolases, and biofilm, was carried out.

2 Materials and methods

2.1 Isolation and enumeration of *B. cereus* group

Members of the *B. cereus* group were isolated from samples (n=230) of various dairy products, such as pasteurized and sterilized milk, milk powder, ice cream, paneer, butter, cheese, curd, and khoa, collected from retail outlets in India.

Samples (10 g or mL) were homogenized with 90 mL sterile peptone physiological saline (1 g neutral peptone.L^{-1}, 8.5 g NaCl.L^{-1}, pH 7.2) using a Stomacher lab-blender 400 (Seward Medical, London, UK) at "normal" speed for 1 min. Appropriately diluted suspension (0.1 mL) was spread-plated on *B. cereus* selective agar (BCSA) base containing peptic digest of animal tissue, mannitol, NaCl, $MgSO_4$, Na_2HPO_4, KH_2PO_4, sodium pyruvate, and bromothymol blue (M833, HiMedia Laboratories Pvt. Limited, Mumbai, India), supplemented with sterile egg yolk (HiMedia FD045) and polymyxin B sulfate (100 U.mL^{-1}; HiMedia FD003), and incubated at 35 °C for 24–48 h. Characteristic turquoise to peacock blue colonies surrounded by a zone of precipitate of the same color were regarded as presumptive *B. cereus* s.l. The presumptive isolates were confirmed on the basis of motility, endospore formation, glucose fermentation, acetylmethylcarbinol production, and nitrate reduction (Claus and Berkeley 1986). The 144 isolates belonging to *B. cereus* group from various dairy products were maintained on nutrient agar (HiMedia M561; per liter: 5 g peptic digest of animal tissue, 3 g beef extract, and 15 g agar, pH 7) slants at 4 °C.

2.2 Antibiotic susceptibility test

Disc agar diffusion method (HiMedia 1998) was used to develop antibiogram of the *B. cereus* group isolates against 14 commonly used antibiotics (per disc: ampicillin (10 μg), carbenicillin (10 μg), cephalothin (30 μg), penicillin G (10 U), vancomycin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), polymyxin B (300 U), nalidixic acid (30 μg), metronidazole (5 μg), rifampicin (15 μg)) for treating gastroenteritis. Colonies, grown on tryptone soya agar (HiMedia M290) at 37 °C for 24 h, were transferred to about 5 mL tryptone soya broth (HiMedia M011) and incubated for 6–8 h. After incubation, inoculum was applied evenly onto Mueller-Hinton agar (HiMedia M173) plate (4 mm thick) with a sterile cotton swab (HiMedia PW005). After drying for 15 min, different antibiotic susceptibility test discs (HiMedia) were applied aseptically and the plates were incubated at 37 °C for 14–19 h. All the experiments were carried out in triplicate for 144 isolates belonging to the *B. cereus* group, and the results were expressed as diameter of inhibition zone.

2.3 Hemolysis

Broth cultures (24 h old) of 144 isolates were spotted on blood agar (HiMedia M834) plates containing 5% of defibrinated sheep blood and incubated for 16–18 h at 30 °C (Prüß et al. 1999). All the experiments were carried out in triplicate, and the results were expressed as ratio of clear zone diameter to diameter of the spot.

2.4 Production of extracellular enzymes

Production of protease, lipase, and amylase by the 144 isolates was determined using skim milk agar (HiMedia M163), trybutyrin agar base (HiMedia M157) supplemented with 1.0% $v.v^{-1}$ of trybutyrin (HiMedia FD081), and starch agar (HiMedia M107), respectively. Plates were spotted with 24 h-old cultures using a 2-mm diameter loop and incubated for 18–20 h at 37 °C. The diameter of clear zone was measured directly in case of skim milk agar and trybutyrin agar plates. The starch agar plates were flooded with Lugol's iodine solution to obtain zone of clearance. All the experiments were carried out in triplicate, and the results were expressed as ratio of clear zone diameter to diameter of the spot.

2.4.1 Assay of protease and determination of thermostability

The experiments were done with the six isolates selected on the basis of the largest zone of clearance on skim milk agar. Inoculation by the selected isolates was made into a medium which contained (per liter): 5 g peptone, 5 g yeast extract, 1.5 g beef extract, 5 g NaCl, and 10 g glucose, pH 7.0 (Patel et al. 2005). After incubation for 48 h at 37 °C under shaking condition (100 rpm), the cultures were centrifuged (7,800×g for 10 min) at 4 °C to obtain a crude enzyme extract.

Relative proteolytic activity was measured according to Thys et al. (2004) with some modifications. The crude enzyme extract (120 μL) was mixed with 250 μL of azocasein (A2765, Sigma-Aldrich Corporation, St. Louis, MO, USA; 2.5 $g.L^{-1}$) 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 M trichloroacetic acid. After 1 h standing at 4 °C, the mixture was centrifuged at 13,000×*g* for 10 min. The supernatant (50 μL) was mixed with 2 mL of water and analyzed 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 conditions.

Thermostability of protease was determined by treating the crude enzyme extract for 10 min at 37, 40, 50, 60, 70, 80, and 90 °C, followed by estimating residual relative proteolytic activity as described above. All the experiments were carried out in triplicate.

2.4.2 Assay of lipase and determination of thermostability

The experiments were done with the six isolates selected on the basis of the largest zone of clearance on trybutyrin agar. Inoculation was made into a medium which contained (per liter): 6 g tryptone, 2 g yeast extract, 15 mL olive oil, 0.2 g $CaCl_2.2H_2O$, 0.1 g

$MgSO_4.7H_2O$, 0.4 mL 1% $FeCL_3.6H_2O$, pH 6.0. After incubation for 48 h at 37 °C under shaking condition (150 rpm), the cultures were centrifuged (2,800×*g* for 30 min) at 4 °C to obtain a crude enzyme extract (Lee et al. 1999).

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 solution A (30 mg of *p*-nitrophenyl palmitate (PNPP, Sigma-Aldrich N2752) in 10 mL of isopropanol (1.94524.0521; Merck Specialities Pvt. Ltd., Mumbai, India)) with solution B (0.1 g gum acacia (Merck 61835005001730) and 0.4 mL of Triton X-100 (HiMedia MB031) in 90 mL of 50 mM Tris–HCl buffer, pH 8.0 (HiMedia M631)), and 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 conditions.

Thermostability of lipase was determined by treating the crude enzyme extract for 10 min at 37, 40, 50, 60, 70, 80, and 90 °C, followed by estimating residual relative lipolytic activity as described above. All the experiments were carried out in triplicate.

2.5 Assay of biofilm formation

Biofilm-forming ability of the isolates was carried out following the method described by Harvey et al. (2007). Biofilm was allowed to develop by inoculating overnight culture of an isolate grown on nutrient agar into microtiter wells (initial total cell count, 10^5.well^{-1}) containing 150 μL of reconstituted skim milk (HiMedia M530). The plates were incubated at 4 °C for 24 h. The medium from the wells was drained out. The wells were washed three times with distilled water to remove non-biofilm cells, allowed to dry for 30 min at 30 °C, added with 1% w.v^{-1} of crystal violet, and held at 20 °C. After 45 min, excess crystal violet was removed 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 95% v.v^{-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 (microplate reader iMark, Bio-Rad, Tokyo, Japan). To correct background staining, the mean OD value obtained for control (without biofilm) was subtracted from the OD value obtained from each condition. Biofilm formation assay was carried out in triplicate for all the 144 isolates belonging to the *B. cereus* group.

2.6 Statistical analysis

Experimental data were analyzed 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, hemolysin, and biofilm, by the isolates were subjected to PCA. In the present study, PCA was undertaken to understand relationship among five different variables which can act as evaluation indices for food spoilage and poisoning potentialities. The suitability of our data for structure detection was subjected to Kiser-Meyer-Olkin measure of sampling adequacy and Bartlett's test of sphericity. Small value 0.001 ($P<0.05$) of significance level in Bartlett's test of sphericity indicated that factor analysis could be useful with our data. Varimax rotation method was used to

produce orthogonal transformations which make component matrix easier to interpret than unrotated matrix.

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

3 Results and discussion

3.1 Prevalence of *B. cereus* group

B. cereus group was found to occur in six out of eight different dairy products marketed in India (Table 1). Out of 230 samples, 73 (32%) contained *B. cereus* s.l. cells. Their incidence in cheese, ice cream, milk powder, and pasteurized/sterilized milk was fairly high (33%–55%). In case of pasteurized/sterilized milk, 55% of the samples contained *B. cereus* s.l. cells. In a study in Denmark, 47% of the pasteurized milk samples were found to be contaminated by *B. cereus* group at a level of 10^3–10^5 cfu.mL^{-1} (Larsen and Jørgensen 1997). In another study in the Netherlands, 35% of the pasteurized milk samples contained *B. cereus* group at a population level of 10–10^4 cfu.mL^{-1} (Te Giffel et al. 1996b). The difference in percentage of contaminated samples may be attributed to the degree of post-pasteurization contamination and/or storage temperature abuse. In case of milk powder, 52% of the samples contained *B. cereus* group at a population level of 10^2–10^3 cfu.g^{-1}. The results were in agreement with a previous report where

Table 1 Prevalence and population level of *B. cereus* group in market samples (n=230) of various dairy products in India

Product	No. of samples	Positive samples (%)	*B. cereus* s.l. population level (cfu)
Milk (pasteurized/sterilized)	55	55	10–10^4 mL^{-1}
Milk powder	35	52	10^2–10^3 g^{-1}
Ice cream	25	40	10^2–10^8 mL^{-1}
Paneer	25	4	20–40 g^{-1}
Khoa	20	0	<dl[a]
Curd	20	0	<dl
Cheese	25	33	10^2–10^6 g^{-1}
Butter	25	20	10^3–10^4 g^{-1}

[a] dl, detection limit (10 cfu.g^{-1})

15%–75% of the milk powder samples were contaminated with *B. cereus* group with a population level of 5–10^3 cfu.g^{-1} (Te Giffel et al. 1996a). The high incidence of *B. cereus* s.l. in milk powder samples is likely due to monospecies biofilm formation on the milk evaporators which can be a source of recurrent contamination of the final product (Shaheen et al. 2010). In case of ice cream, 40% of the samples contained *B. cereus* s.l. at a population level of 10^2–10^8 cfu.mL^{-1}. In a similar study made with the samples from retail outlets in Mumbai, India, the organism was found to be prevalent in 40% of the unpackaged samples and 27% of the packaged samples, with a population level of 10–10^3 cfu.mL^{-1} (Warke et al. 2000). The high prevalence and population level of *B. cereus* group may be attributed to post-production handling of the products and/or temperature abuse which is likely to occur during frozen storage or transportation. Thirty-three percent of cheese samples analyzed contained *B. cereus* group with a population level of 10^2–10^6 cfu.g^{-1}, while Molva et al. (2009) reported only 12% of Turkish cheese samples to be contaminated with *B. cereus* group. This difference might be attributed to type of cheese samples analyzed and post-production contamination. In case of butter, 20% of the samples were found to be contaminated with *B. cereus* group with a population level of 10^3–10^4 cfu.g^{-1}. This may be due to biofilm formation by *B. cereus* group on centrifugal separators and recycle loops in butter manufacturing plants and subsequent contamination of finished products.

In khoa and curd samples analyzed, *B. cereus* group was not detected. Khoa is a partially desiccated milk which is prepared by condensing milk through regular heating (90–95ºC) till total solid reaches 65%–70% (Bhatnagar et al. 2007). Heating and dehydration during the preparation of khoa and low pH (3.5–4.5) in curd and various organic acids, peroxides, and antibacterial agents produced by lactic acid bacteria during fermentation might be the likely causes for its absence.

A high prevalence of *B. cereus* group in ice cream and cheese is a matter of great public health concern as the level of *B. cereus* group reported in food poisoning is 10^2–10^8 cfu.g^{-1} or mL^{-1} (Beattie and Williams 2000), and generally, any food exceeding 10^4–10^5 cells.g^{-1} or mL^{-1} is considered unsafe for consumption (Notermans et al. 1997).

3.2 Susceptibility to antibiotics

The results for susceptibility of the 144 isolates of the *B. cereus* group to 14 different antibiotics, including β-lactam (4), benzene derivative (1), aminoglycoside (2), macrolide (1), peptide (1), glycopeptide (1), naphthyridone (1), nitro-imidazole (1) rifampicin, and tetracycline are shown in Table 2. All the isolates were multidrug resistant; each of these 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 BCSA (containing 100 U polymyxin B per liter) were resistant to higher concentration of polymyxin B (300 U per disc). An earlier study reported susceptibility of only 8% of the 84 isolates of *B. cereus* group from spices to this higher concentration of polymyxin B (Banerjee and Sarkar 2004). However, all the 48 *B. cereus* group isolates from legume-based fermented food products were resistant against this higher concentration of polymyxin B (Roy et al. 2007). As expected, all the

Table 2 Antibiogram of 144 isolates of *B. cereus* group from Indian marketed dairy products

Mechanism of action	Antibiotic (quantity per disc)	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 cutoff 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

isolates were resistant to metronidazole. Emergence of multidrug resistance among foodborne bacterial pathogens can be a major health concern (Kiessling et al. 2002).

3.3 Hemolysis

Ninety-three percent isolates of the *B. cereus* group exhibited β-hemolysis (Table 3). This is in consistence with the report of β-hemolytic activity exhibited by 92% isolates of the *B. cereus* group from food ingredients and products in Brazil (Chaves et al. 2011).

3.4 Production of extracellular enzymes

The results for the production of extracellular enzymes are presented in Table 3. Enzymes such as protease, lipase, and amylase significantly contribute to the reduction of shelf-life of processed milk and milk products by degrading milk components and additives (Chen et al. 2003; Datta and Deeth 2003). In the present study, 97%, 96%, and 63% of the isolates produced protease, lipase, and amylase, respectively, and 60% of the isolates in the present study produced all the three enzymes. This proves potentiality of majority of the isolates for spoilage of dairy products, which can be responsible in turn for the reduction in shelf-life of the products. In an earlier study on 48 isolates of the *B. cereus* group from legume-based fermented food products, 50% of

Table 3 Production of extracellular enzymes and hemolysin by the isolates of *B. cereus* group from Indian marketed dairy products

Source	No. of isolates	Percent of positive isolates			
		Protease	Lipase	Amylase	Hemolysin
Milk	83	92	100	82	90
Milk powder	32	100	97	50	84
Ice cream	11	100	100	75	90
Paneer	5	100	100	100	67
Butter	4	100	100	0	100
Cheese	9	100	50	23	100

the isolates were capable of producing one of these enzymes and 23% produced all the three enzymes (Roy et al. 2007). Interestingly, in the present study, 37% of the isolates were amylase negative. According to previous reports (Agata et al. 1996; Valero et al. 2002), the inability to hydrolyze starch has been indicative of emetic subtype. This indicates high prevalence of emetic subtype in dairy products analyzed in the present study.

The maximum clearing zone-producing isolates from each product on skim milk agar plate were selected for protease assay and evaluation of thermostability (Table 4). 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. The results indicate thermostable nature of the protease. Chen et al. (2004) reported *Bacillus* strains to be thermostable, where at 70 °C at least 50% of the initial activity was retained. The presence of thermostable protease increases spoilage potentiality of the isolates as these enzymes retain their activity even after heat treatments such as pasteurization and spray drying.

The maximum clearing zone-producing isolates from each product on tributyrin agar were selected for lipase assay and evaluation of thermostability (Table 4). 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. The thermal stability of lipase from *B. cereus* group isolates has been previously reported by Akanbi et al. (2010). Thermostable lipase can withstand milk heat treatments and remain active in dairy products and can result into lipolysis that causes bitter taste of dairy products, making them unacceptable to consumers.

3.5 Biofilm formation

The results of biofilm formation assay by the isolates of the *B. cereus* group are given in Table 5. Of them, 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 144 isolates from milk, cheese, and ice cream were biofilm formers, while in butter, 100% of the isolates were positive. The biofilm-forming ability of the *B. cereus* group in dairy environment was reported earlier (Kumari and Sarkar 2014; Shaheen et al.

Table 4 Relative proteolytic and lipolytic activities and thermostability of the crude enzymes from selected isolates of *B. cereus* group from Indian marketed dairy products

Isolate no.	Source	Ratio[a]	Temperature (°C)[b]						
			37	40	50	60	70	80	90
Proteolytic activity									
M312	Milk	2.2	1.46a±0.06	1.46a±0.06	1.43a±0.03	1.40a±0	1.23b±0.33	1.13b±0.04	1.06b±0.06
MP113	Milk powder	2.6	1.90a±0.05	1.86a±0.05	1.83a±0.03	1.83a±0.03	1.53b±0.06	1.60b±0.11	1.60b±0.10
IC63	Ice cream	3.2	2.36a±0.20	2.43a±0.20	2.20a±0.15	2.20a±0.15	1.76b±0.08	1.70b±0.05	1.57b±0.03
P23	Paneer	2.2	1.50a±0.05	1.43a±0.05	1.40a±0	1.30b±0.05	1.30b±0.05	1.23b±0.03	1.06b±0.06
B3	Butter	2.0	2.00a±0.21	1.90a±0.05	1.83a±0.03	1.73b±0.03	1.73b±0.03	1.73b±0.03	1.60b±0.03
C3	Cheese	1.6	2.20a±0.10	2.16a±0.14	2.23a±0.14	2.16a±0.14	2.03a±0.08	2.03a±0.08	2.00a±0.11
Lipolytic activity									
M144	Milk	2.7	33.00a±0.66	33.00a±0	32.60a±0.33	23.30b±1.00	21.00b±0	20.60c±0.33	15.00d±0.06
MP251	Milk powder	2.4	35.00a±0.57	33.00a±1.00	33.00a±0	33.30a±1.00	23.30b±1.00	15.30c±0.88	8.33d±0.88
IC65	Ice cream	3.1	46.00a±1.00	42.00a±1.00	42.00a±1.00	34.00b±0.60	35.00b±1.00	31.00b±1.00	10.00c±0.06
P22	Paneer	1.7	11.33a±0.80	11.66a±0.33	12.00a±0	8.60b±0.33	7.30b±0.33	8.30b±0.33	2.66c±0.30
B5	Butter	1.5	12.00a±1.00	10.66a±0.60	10.33a±0.05	7.66b±0.33	6.66b±0.88	5.33c±0.33	1.60d±0.33
C51	Cheese	1.5	11.33a±0.33	11.66a±0.33	12.00a±0	11.00a±0.33	8.60b±0.03	8.30b±0.33	2.30c±0.33

[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

Table 5 Clustering of 144 isolates of *B. cereus* group from Indian marketed dairy products on the basis of biofilm-forming ability at 4 °C

Group[a]	Percent 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

2010). As bacteria within biofilms are more resistant to antimicrobial agents and cleaning, it is more difficult to eliminate biofilm than planktonic cells (Costerton et al. 1999). Hence, their presence in dairy can be a matter of concern. Since a majority of the isolates in the present study were biofilm former, biofilm formed by them in dairy processing lines can be responsible for recurrent contamination and spoilage of dairy products or facilitate transmission of diseases.

3.6 Relationship among characteristics

In the present study, PCA allowed transformation of a large number of putative correlated variables into a smaller number of variables called principal components (PC) (Fig. 1). 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 hemolysis (CC=88.8%). The PC analysis allowed classifying these correlated variables into two types of risks (spoilage and food poisoning). From the PCA score biplot (Fig. 1), it is evident that majority of the isolates from cheese, butter, ice cream, and a few from milk and milk powder were dominant in positive side of PC1 and closer to variables such as biofilm, amylase, lipase, and protease production. On the other hand, majority of the isolates from milk powder were grouped in the positive side of PC2 which mainly consists of variable hemolysin. Many milk isolates were predominant in the PC1 and PC2 negative sides and thus characterized by low production of enzymes and biofilm.

The results obtained from AHC are shown in Fig. 2 and Table 6. All the 144 isolates belonging to *B. cereus* group from different dairy products were grouped into four major clusters. Cluster A contained 17 isolates from milk (29%), milk powder (24%), cheese (35%), and butter (12%). 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. From the AHC results, it is evident that each of the four clusters is heterogeneous.

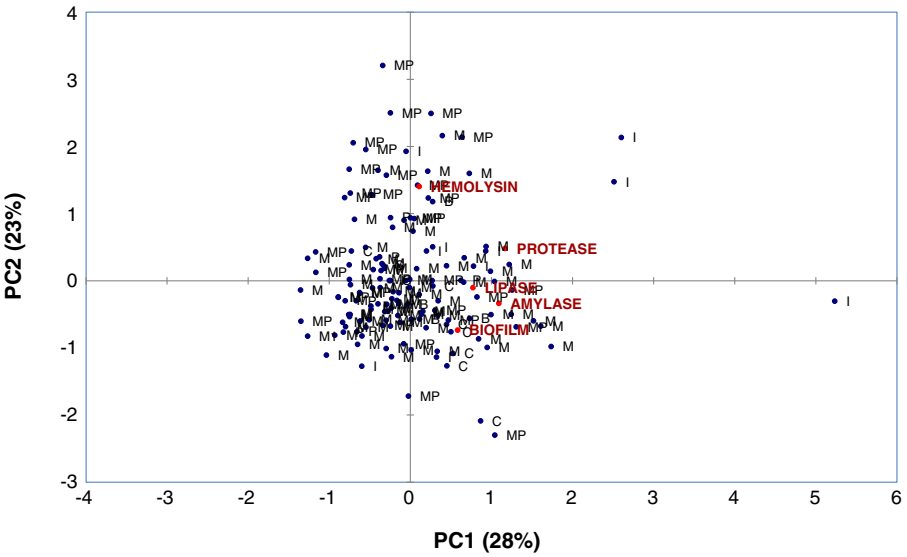

Fig. 1 Score biplot for principal component analysis showing observations (*M* milk, *MP* milk powder, *C* cheese, *I* ice cream, *P* paneer, *B* butter isolates) and variables (production of protease, amylase, lipase, hemolysin, and biofilm) together for 144 isolates belonging to *B. cereus* group

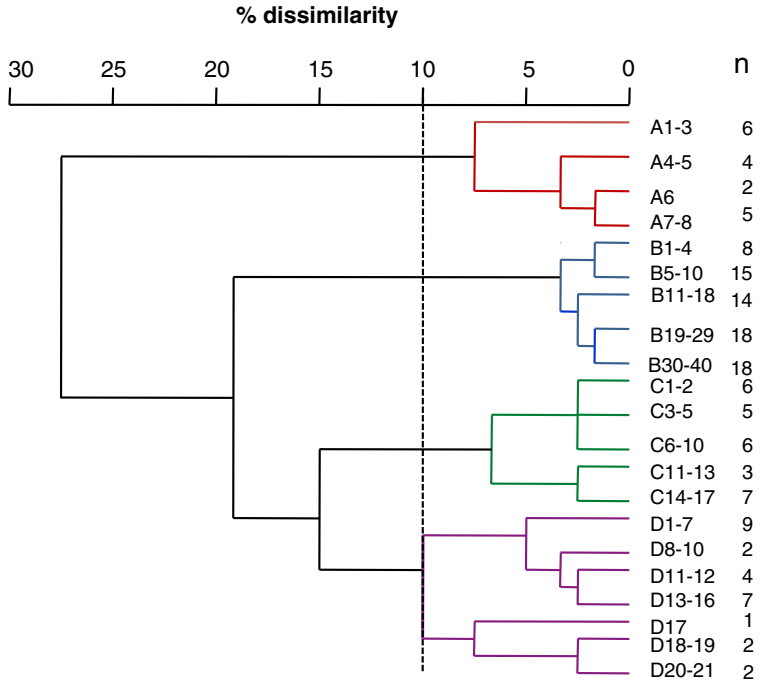

Fig. 2 Simplified dendrogram based on wards clustering of dissimilarity coefficient generated by agglomerative hierarchical clustering. Based on studied characters (production of protease, amylase, lipase, hemolysin, and biofilm), the 144 isolates belonging to *B. cereus* group were grouped into four major clusters, designated A through D. *n* number of isolates in (sub)clusters

Table 6 Distribution of 144 isolates of *B. cereus* group from different Indian marketed dairy products among the clusters generated through Fig. 2

Cluster	Percent 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 Conclusions

In the present study, prevalence of *B. cereus* group in cheese, ice cream, milk powder, and milk was high (33%–55%). On the other hand, the same in case of butter and paneer samples was found to be 20% and 4%, respectively. The level of contamination in various dairy products ranged from 10 to 10^8 cfu.g^{-1} or mL^{-1}. All the 144 isolates were multidrug (at least five antibiotics) resistant; 93% of the isolates exhibited β-hemolysis and 71% formed biofilm at 4 °C. Thus, from the present study, it is evident that there is a high incidence of *B. cereus* s.l. among most of the dairy products marketed in India. High level of contamination in dairy products can cause health concern and economic loss due to spoilage of dairy products by heat-stable enzymes. So, there is need for better implementation of hazard analysis and critical control points (HACCP) in dairy processing lines so that the initial load of *B. cereus* group in finished products could be minimized. Critical control points such as storage temperature and time should be properly defined. Most importantly, better clean-in-place (CIP) regimes to eliminate biofilm from dairy processing lines should be developed and implemented. Implementation of good manufacturing practices (GMP) in farms during the production, storage of milk also must be designed, constructed, and maintained in a manner that will prevent the introduction of members of *B. cereus* group to raw milk.

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