Enumeration and characterisation of *Bacillus cereus* strains in the dairy environment of the district of Darjeeling, India

Thesis submitted to the University of North Bengal for the Award of Doctor of Philosophy in Botany

BY

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Declaration

I declare that the thesis entitled, 'Enumeration and characterisation of *Bacillus cereus* strains in the dairy environment of the district of Darjeeling, India' has been prepared by me under the guidance of P.K. Sarkar, Professor of Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ENLIGHTENMENT TO PERFECTION

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Certified that the work presented in the thesis entitled, 'Enumeration and characterisation of *Bacillus cereus* strains in the dairy environment of the district of Darjeeling, India' has been carried out by Ms Sarita Kumari, MSc under my supervision at Microbiology Laboratory of the Department of Botany in the University of North Bengal. The results incorporated in the thesis have not been submitted for any other degree elsewhere.

Further certified that Ms Kumari has followed the rules and regulations laid down by the University of North Bengal in carrying out this work.

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Abstract

Bacillus cereus is one of the most important spoilage microorganisms in dairy environment and its growth may result in various dairy defects. Moreover, it is a great safety concern for dairy industry as it is associated with incidences of food poisoning by producing enterotoxin. Because of its outstanding ability to adhere to stainless steel surfaces of dairy plant and form biofilm, *B. cereus* can lead to serious hygiene problems and economic loss due to spoilage of dairy products and equipment impairment. Biofilms are more resistant to antimicrobials and cleaning regimes compared to planktonic cells, and this makes their elimination from dairy industry a big challenge. As explicit data on evidence for occurrence *of B. cereus* in dairy environment in India is lacking, the present work was undertaken with a view to investigate the prevalence of *B. cereus* in dairy environment of Darjeeling district in India, characterise isolated strains for health risk and spoilage risk assessment, optimise the existing cleaning-in-place (CIP) regimes for a better removal *B. cereus* biofilm from dairy processing environment and design and optimise alternative CIP regime in order to replace conventional CIP.

The prevalence of *B. cereus* was investigated in a total of 230 samples belonging to eight different types of marketed milk and dairy products. The prevalence of *B. cereus* in cheese, ice cream, milk powder and milk was high (33-55%), while it was low in butter and paneer (20% and 4%, respectively). None of the samples of curd and khoa were found contaminated. The level of contamination in various milk and dairy products ranged from 1-8 log cfu g^{-1} or ml^{-1}. The occurrence of a high level of *B. cereus* contamination in ice cream and cheese is of public health concern, and products can be considered unsafe for consumption.

Out of 144 isolates, 107 (74%) were able to grow at $\leq$7 ºC. Presence of such a large number of psychrotropic strains in dairy environment is of major concern mainly because of their potential for growth, spoilage and toxin production in chilled products. Twenty-one isolates (14.5%) were moderate thermotolerant. An antibiogram of 144 isolates of *B. cereus* was obtained using 14 different antibiotics commonly used against foodborne diseases. All the isolates were multi-drug (at least five antibiotics) resistant. Emergence of multi-drug resistance among high number foodborne bacterial pathogens can be a major health concern. Ninety-three percent of the isolates exhibited β-haemolysis. Of the isolates, 140 (97%) were positive for the production of diarrhoeal enterotoxin (NheA). All the isolates from milk powder, ice cream, paneer and butter produced diarrhoeal enterotoxin. However, 98% of the isolates from milk and 89% of cheese were found positive for diarrhoeal enterotoxin. The prevalence of high producers of Nhe among dairy isolates indicates high risk and public health concern. Of the 144 isolates, 97%, 96% and 63% were capable of producing protease, lipase and amylase, respectively, indicating spoilage potentiality of the isolates. The protease and lipase were thermostable. Thermostable enzymes can withstand milk heat treatments and remain active in dairy products and can be responsible for spoilage and reduction in shelf-life.

Seventy-one percent of the isolates formed biofilm at 4 ºC. Of the all 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. Formation of biofilm by such a high number of dairy isolates is of concern, as cells can detach from surfaces to milk and subsequently contaminate the processing lines. Moreover spores formed within biofilm are more resistant to cleaning, and can be responsible for (re)contamination and spoilage of dairy products as well as transmission of disease.

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. All the four clusters were heterogeneous, indicating the studied factors were associated with all kinds of dairy products studied.

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. *Bacillus cereus* was also recovered from 40% of the samples collected from stainless steel surfaces of pasteurised milk chilling tanks. The presence of *B. cereus* strains on chilling tank surface indicates that the current cleaning system or frequency and time interval between cleaning may not be satisfactory.

An *in vitro* model study was carried out to check biofilm formation by a selected strain of *B. cereus* isolated from a chilling tank where pasteurised milk was stored. The strain was able to form biofilm even at 4 ºC. From the study of different simulated conditions, it was concluded that *B. cereus* cell count could reach up to 6 log cfu cm^{-2} on the surface of a stainless steel tank, if inadequately cleaned tanker is left to stand empty at room temperature. Presence of *B. cereus* in such a high number is of significance as it can be an important source for food spoilage and can lead to foodborne outbreaks.

Response surface methodology (RSM) was used to optimise parameters influencing biofilm cell removal. The RSM results were used to design an optimised alkali 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. Effectiveness of reference 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) and optimised CIP was assessed against 24 h-old biofilm. While the reference CIP caused 3.29 log reduction of *B. cereus* biofilm cells cm^{-2}, the optimised alkali CIP achieved 4.77 log reduction cm^{-2}. Thus, the optimised alkali CIP regime was significantly (P <0.05) more effective in biofilm cell removal as compared to the reference CIP.

Another *in vitro* model study was carried out for response surface optimisation of *B. cereus* biofilm removal using protease. Exposure time, pH, enzyme concentration and pH-enzyme concentration interaction showed a significant positive effect on removal. The optimised condition (1.0 U ml^{-1} protease in pH 8.5 buffer at 60 °C for 20 min) for removal of biofilm in microtiter plate assay was tried with stainless steel coupons, the condition which mimics actual scenario in dairy industry. A CIP regime was designed by replacing caustic step with the optimised protease treatment followed by nitric acid treatment. Effectiveness of the optimised protease

CIP was compared with reference (currently practiced alkali) CIP and optimised alkali CIP. While the optimised protease CIP caused a complete removal of biofilm cells, the reference CIP caused a reduction of 4.08 log *B. cereus* biofilm cells cm^{-2} and the optimised alkali CIP was able to cause a reduction of 4.92 log cells cm^{-2}. Furthermore, the quantum of biofilm matrix removed in the optimised protease CIP was significantly higher than that of either reference CIP or optimised alkali CIP. So, an optimised protease CIP has a better acceptability than a caustic CIP.

A quantitative model was generated using RSM and Monte Carlo simulation to evaluate public health risks associated with the consumption of pasteurised milk contaminated with *B. cereus* stored in household refrigerators. Thirty percent of the samples stored in refrigerators contained *B. cereus* at a level of 3-5 log cfu ml^{-1}. The distribution of *B. cereus* ranged from 1.83 to 4.16 log cfu ml^{-1} (mean, 3 log cfu ml^{-1}). The storage temperature ranged from 3.54 to 12.84 ºC (mean, 8.2 ºC). The probability of temperature being ≤7 ºC was only 25%. The storage time ranged from 0.76 day to 3.55 days (mean, 2.16 days). After optimisation of the results considering the load of *B. cereus* M312 to be 3 log cfu ml^{-1}, the results showed that the cell count will reach the threshold level of >4 log cfu ml^{-1} after 47.5 h, 45.5 h, 41.6 h, 35.3 h, 25.3 h and 24 h at 7 ºC, 8 ºC, 9 ºC, 10 ºC, 11 ºC and 12-13 ºC, respectively. Thus, in the present scenario, storage of pasteurised milk under household refrigeration for more than 24 h increases *B. cereus*-associated health risk.

The present study will help to better assess the health and spoilage risk associated with *B. cereus* in dairy environment and to incorporate adequate preventive measures.

Preface

Dairy sector in India plays an important role in socio-economic development and provides livelihood to millions of homes in villages. Consumption of milk and dairy products is deeply rooted in Indian tradition as an important part of daily diet, essential items during rituals and festivals. Thus, microbiological safety, proper storage, and shelf-life of milk and dairy products are important issues to be considered. This research was therefore aimed at assessing health and spoilage risk associated with presence of *Bacillus cereus* strains in milk and dairy products, optimising the existing CIP regimes for a better removal *B. cereus* biofilm from dairy processing environment and designing optimised alternative CIP regime in order to replace conventional CIP.

My journey as a researcher was amazing learning experience and this would not have been possible without the people who have helped me to be what I am today. So, I take this opportunity to thank them all.

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Abbreviations

AHC	Agglomerative hierarchical clustering
ANOVA	Analysis of variance
CIP	Cleaning-in-place
CCP	Critical control point
cfu	Colony forming unit
CytK	Cytotoxin K
D-value	Decimal reduction time
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substances
HACCP	Hazard analysis and critical control point
Hbl	Haemolysin BL
HIMUL	Himalayan Milk Producers' Union Limited
ISO	International Organization for Standardization
Nhe	Nonhaemolytic enterotoxin
OD	Optical density
P-value	Calculated probability
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RSM	Response surface methodology
SE	Standard error
s.l.	*sensu lato*
s.s.	*sensu stricto*
UHT	Ultra high temperature

1 Introduction

India is the largest milk-producing nation with estimated production of 146.3 million tonnes in 2014–2015; its share in global milk production is 18.5% (NDDB, 2015). Dairy sector in India plays an important role in socio-economic development and provides livelihood to millions of homes in villages. Consumption of milk and dairy products (Fig. 1) is deeply rooted in Indian tradition as an important part of daily diet, essential items during rituals and festivals. Changing life style, rising income and urbanisation have affected consumption patterns and increased the demand for more value-added dairy products. Various cooperatives and private sector dairies are producing more dairy products like ghee (clarified butter), butter, yoghurt, khoa, milk

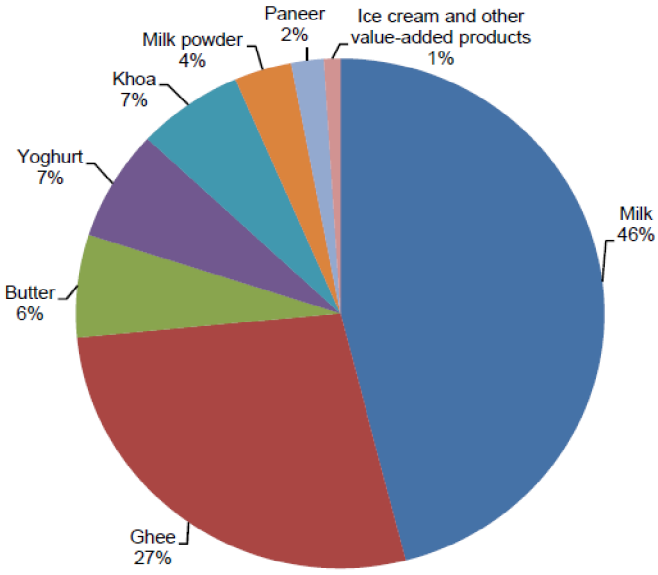

Fig. 1. Annual consumption of different dairy products in India (Source: NDDB, 2012)

powder, paneer (cottage cheese), ice cream, cheese and ethnic sweets to meet this demand. With an increasing demand of dairy products, the need of extended refrigerated storage of raw milk before processing and the application of higher pasteurisation temperatures for prolonged shelf-life requirements have enhanced the importance of thermoduric microorganisms.

Milk is an important source of essential nutrients containing lactose, proteins, fats, calcium, magnesium, selenium, riboflavin, vitamin B_{12} and pantothenic acid. Milk of good quality is a complete food with a slightly sweet taste, very little odour and a smooth, rich feel in the mouth that leaves only a clean, pleasing sensation. Liquid milk has white, glossy appearance (Phillips *et al.,* 1995). Bovine milk is composed of approximately 870 g water, 37-39 g fat, 32-35 g protein, 48-49 g carbohydrate (principally lactose) and 7 g ash per litre (Fernandes, 2009). High water content, abundant nutrients and near neutral pH (6.4–6.8) of milk make it an ideal medium for the growth of microorganisms.

Raw milk can be contaminated by endogenous path due to direct transfer from the blood to milk (systemic infection) or due to mastitis in udder. Exogenous contamination of raw milk can take place during or after milking by dirty udder, feeds, faeces, milking equipment and storage tanks. Diseased animals can shed *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis, Escherichia coli*, *Mycobacterium bovis*, *Brucella abortus*, *Coxiella burnetii* and *Listeria monocytogenes* into milk. Psychrotrophs associated with raw milk include members of the genera *Pseudomonas, Micrococcus, Aerococcus*, *Lactococcus* and of the family Enterobacteriaceae. *Pseudomonas* is of significance as it produces heat-stable enzymes, particularly proteases and lipases, during growth under refrigerated storage, which adversely affect quality of milk (Muir, 1996). Silage is an important source of *Bacillus* and *Clostridium* spores in raw milk (te Giffel *et al*., 2002; Vissers *et al*., 2006). Spores can survive pasteurisation and can be responsible for foodborne illness and spoilage of milk and dairy products.

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). *Bacillus cereus* is an important safety and shelf-life concern in dairy industry. It is associated with foodborne outbreaks by producing enterotoxins (Anderson Borge *et al.,* 2001) and is also responsible for decrease in the organoleptic quality of milk and dairy products by causing spoilage, like sweet curdling and bitterness of milk (Chen *et al.*, 2003). There are two distinct syndromes caused by separate toxins produced by *B. cereus*: emetic and diarrhoeal. The emetic type, characterised 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 diarrhoeal type, characterised by the occurrence of abdominal pain and watery diarrhoea within 8 to 16 h after ingestion, is caused by haemolysin BL (Beecher *et al.,* 1995). Haemolysin is a three-component enterotoxin produced by *B. cereus* which consists of two lytic components (L1 and L2) and a binding component B. It has haemolytic, dermonecrotic and vascular permeability activities. Thus, it is considered as one of the potential virulence factors in *B. cereus*-mediated diarrheoa (Beecher *et al.,* 1995). Although *B. cereus* is mainly associated with gastrointestinal disorders, it is an opportunistic human pathogen associated with a multitude of other infections such as severe eye infections, periodontitis, necrotising fasciitis, endocarditis, nosocomial acquired bacteraemia, osteomyelitis, sepsis, liver abscess, pneumonia and meningitis, particularly in postsurgical patients, immunosuppressed individuals, intravenous drug abusers and neonates. In the idiophase, it produces several compounds (degradation enzymes, cytotoxic factors and cell-surface proteins) that might contribute to virulence. However, there is still little recognition and appreciation of the role of *B. cereus* in these serious, and frequently fatal, clinical infections in humans (Ramarao and Sanchis, 2013).

In dairy environment, *B. cereus* can negatively affect product quality. It produces various extracellular enzymes which can be responsible for a decrease in the organoleptic quality of milk and dairy products. Production of protease, lipase and amylase by contaminating bacteria in dairy environment can be responsible for a decrease in the organoleptic quality of the products. The presence of protease can lead to bitter flavour, 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 flavour, such as rancid, butyric, buttery, unclean and soapy (Furtado, 2005). Starch has become an increasingly popular additive to

dairy products, such as ice cream and yoghurt because of its stabilising 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 products, is a matter of concern as they can survive processing temperatures and be responsible for spoilage even if vegetative cells are eliminated during processing.

Bacillus cereus can be introduced into the dairy environment from various sources during production, handling and processing, mainly from improperly cleaned and sanitised equipments (te Giffel *et al*., 1995). The hydrophobic properties of endospores and their resistance towards heat, desiccation and disinfectants allow them to attach to processing equipment and survive cleaning procedures (Andersson *et al.,* 1995; Simmonds *et al*., 2003; Ryu and Beuchat, 2005). Adherence to stainless steel surfaces of dairy plant can result in biofilm formation (Shaheen *et al.,* 2010) which can be an important reservoir for recurrent contamination of dairy products. Biofilms 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). As spore-forming bacteria are ubiquitous in nature, contamination has been shown to occur along the whole processing line. Pasteuriser, filling machine, packaging boards and blanks can also be a source of contamination (Svensson *et al.,* 2000; Eneroth *et al.,* 2001). However, an effective control of these bacteria in dairy products and processing environment is still a difficult task. In dairy, like any other food industry, an effective cleaning and sanitation program is a part of the process to eliminate microorganisms. Generally the sanitation agents are developed on the basis of studies utilising planktonic cells which are quite different from the biofilm cells due to their altered physiological status. Therefore, inactivation and removal of bacterial cells capable of forming biofilms deserve more attention (Peng *et al*., 2001). *Bacillus cereus* accounts for 12.4% of microbiota growing in biofilms in a commercial dairy plant (Sharma and Anand, 2002). Thus, an evaluation of cleaning regime in dairy plants for biofilm cell removal is very important. Optimisation of various factors affecting biofilm cell removal is of much importance to design an effective cleaning-in-place (CIP) regime. CIP is a process of cleaning the interior surface of tanks, pasteurisers, pipelines, process equipment and associated things without dismantling them (Thomas and Sathian, 2014). The traditional approach for optimising a multivariable system which consisted of one-factor-at-a-time is not only time-consuming but also inapplicable where factor interactions affect final response. Thus, the most efficient way to enhance the value of research and cut-down time in process development is through statistical experimental designs. Response surface methodology (RSM) is a useful statistical tool to evaluate the effect of different factors and their interactions on response variables, and can be effectively used to find out levels of factors required for optimum response. Thus optimisation of cleaning regimes using RSM can help to design more effective CIPs.

The safety and quality of milk and dairy products are based primarily on risk assessment, and the introduction of preventive measures at all stages of the dairy chain from farm to table where the producers, processors, sellers, consumers and governments are all required to play a vital role in ensuring safety and quality. *Bacillus cereus* in retail and consumer phase is of interest as it is less controllable, and storage temperatures may be insufficient to prevent its growth. With a better understanding of long-term survival of pathogens, risk assessment recommendations will have more valid scientific backing, and consumers will better understand the risk and danger of improper handling and storage.

Explicit data on evidence for the occurrence of *B. cereus* in dairy environment in developing countries, like India, are lacking and there are more chances of storage temperature abuse in retail outlets/households. The present work was undertaken with a view to (a) investigate prevalence of *B. cereus* in dairy environment of Darjeeling district in India, (b) characterise the isolated strains for health risk and spoilage risk assessment, (c) investigate the occurrence of *B. cereus* in dairy processing plants and households, (d) optimise the existing CIP regimes for a better removal *B. cereus* biofilm from dairy processing environment, (e) design and optimise alternative CIP regime(s) in order to replace conventional CIP, and (f) carry out quantitative risk assessment of exposure to *B. cereus* associated with the household refrigerated storage of pasteurised milk.

The above objectives were accomplished by adopting the following strategies:

(1) isolating and enumerating *B. cereus* in marketed dairy products and industrial dairy processing environment;
(2) evaluating susceptibility of isolated strains to antibiotics;
(3) determining potentiality of the dairy strains to produce various extracellular enzymes;

(4) determining ability of the dairy strains to produce enterotoxin;
(5) studying biofilm formation by the isolated strains;
(6) designing *in vitro* model for biofilm formation by *B. cereus* in dairy chilling tanks;
(7) removing biofilm by using response surface optimisation of the various factors involved in alkali-based CIP;
(8) removing biofilm by using response surface optimisation of the various factors involved in enzyme-based CIP; and
(9) undertaking risk assessment study for the presence of *B. cereus* in pasteurised milk stored in domestic refrigerators.

2
Review of literature

2.1. *Bacillus cereus* group

2.1.1. Taxonomy

The *Bacillus cereus* group, also known as *B. cereus sensu lato* (*s.l.*), consists of seven closely related species, namely *B. cereus sensu stricto* (*s.s.*), an opportunistic human pathogen associated with food poisoning syndromes (emetic and diarrhoeal) and spoilage of dairy products (Drobniewski, 1993; Helgason *et al*., 2000; Rajkovic *et al*., 2008); *Bacillus thuringiensis*, an entomopathogen used as a biopesticide (Aronson and Shai, 2001); *Bacillus anthracis,* the etiologic agent of anthrax (Mock and Fouet, 2001); *Bacillus mycoides* and *Bacillus pseudomycoides*, characterised by rhizoidal growth on agar plates (Nakamura, 1998); *Bacillus weihenstephanensis*, a psychrotolerant (Lechner *et al*., 1998); and *Bacillus cytotoxicus*, a moderate thermo-tolerant associated to food poisoning (Guinebretière *et al*., 2013) (Fig. 2). Ecological population of the *B. cereus* group is so closely related that they cannot be distinguished by 16S rRNA gene sequencing. Based on molecular data from fluorescent amplified fragment length polymorphism patterns, ribosomal gene sequences, partial *panC* (pantothenate synthetase) gene sequence, 'psychrotolerant' DNA sequence signatures and growth temperature, the *B. cereus* group has been divided into seven phylogenetic groups (Table 1). While all the mesophilic rhizoidal colony-forming strains of *B. mycoides* and *B. pseudomycoides* are clustered in group I, all the psychrotolerant strains of *B. mycoides*, *B. cereus s.s.* and *B. thuringinesis* along with *B. weihenstephanensis* are placed in group VI. *Bacillus cereus s.s.* and *B. thuringinesis* are highly polyphyletic, being spread over group II, III, IV, V and VI. The

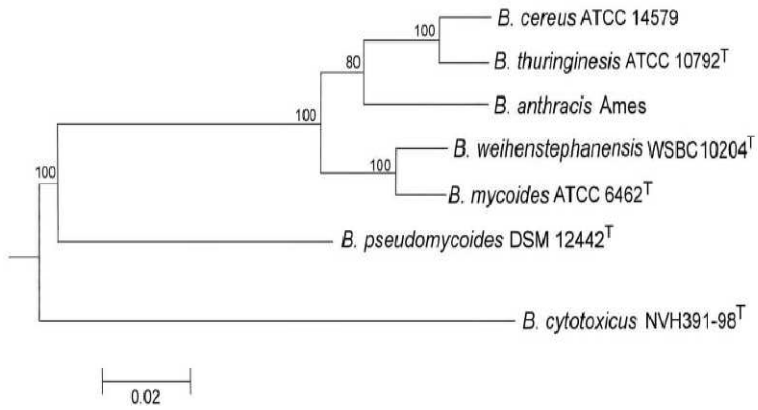

Fig. 2. The phylogenetic position of *Bacillus cereus* group species, based on concatenated sequences from genes included in MLST scheme of Tourasse *et al.* (2006). Bootstrap values above 75% are given at each branch point. Bar, 0.02 substitutions per site (based on Guinebretière *et al.*, 2013).

Table 1. Genetic diversity of the *Bacillus cereus* group[a]

Group	Growth temperature (°C)	Presence of psychrotolerant sequence signature[b]		% of strains associated with food poisoning	Species
		rrs	*cspA*		
I	10-43	C	-	0	*B. mycoides* *B. pseudomycoides*
II	7-40	B	-	21	*B. cereus s.s.* *B. thuringiensis*
III	15-45	C	-	33	*B. cereus s.s.* *B. thuringiensis* *B. anthracis*
IV	10-45	D	-	22	*B. cereus s.s.* *B. thuringiensis*
V	8-40	B	-	12	*B. cereus s.s.* *B. thuringiensis*
VI	5-37	A	A	0	*B. mycoides* *B. cereus s.s.* *B. thuringiensis* *B. weihenstephanensis*
VII	20-50	-	-	50	*B. cytotoxicus*

[a] Based on Guinebretière *et al.* (2008, 2013).

[b] A, 100% positive carrying the signature in abundance; B, a high number of strains carrying the signature in abundance; C, a low number of strains with the signature in low amount; D, a low number of strains with a significant amount of the signature; -, absence of the signature.

strains of *B. anthracis* are clustered in group III. The moderate thermotolerant *B. cytotoxicus* belongs to group VII (Guinebretière *et al.,* 2008, 2013). The '*B. cereus* group' is an informal, however, widely used term describing a genetically highly homogeneous subdivision of the genus *Bacillus* and caters a dilemma from taxonomic viewpoint (Stenfors Arnesen *et al.,* 2008). Genome sequencing data (gene content as well as synteny) have shown that *B. anthracis*, *B. cereus* and *B. thuringiensis* are closely related (Rasko *et al.*, 2004); their 16S rRNA gene sequences share >99% similarity (Ash *et al.*, 1991). The distinguishing features among the species are encoded by highly mobile genes located on plasmids (van der Auwera *et al.*, 2007). It has been proposed that these three species should form one species (Helgason *et al.*, 2000), but no consensus on this matter has been reached. This equivocal taxonomic state of the *B. cereus* group poses difficulties encountered with species definition within bacterial systematic, in particular in the genomic era (Stenfors Arnsen *et al.*, 2008). Although the *B. cereus* group could genetically be considered one species, a strong argument for retaining the current nomenclature is the principle that "medical organisms with defined clinical symptoms may continue to bear names that may not necessarily agree with their genomic relatedness so as to avoid unnecessary confusion among microbiologists and nonmicrobiologists" (Stackebrandt *et al.*, 2002).

2.1.2. Functional morphology of cells

Bacillus cereus group members are large (1.0-1.2 μm x 3.0-5.0 μm), rod-shaped, motile (excepting *B. anthracis*), endospore-forming, aerobe-to-facultative, gram positive bacteria which grow on common agar media to large colonies (3-8 mm dia) with a flat, greyish and 'ground-glass' appearance, often with irregular borders. In selective culture media, such as mannitol-egg yolk-polymyxin (MYP) agar, polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA) and Bacara medium, *B. cereus* colonies are pink, peacock blue and orange-pink, respectively, surrounded by a halo or precipitation zone (Tallent *et al.,* 2012). *Bacillus mycoides* and *B. pseudomycoides* exhibit rhizoidal growth on solid media. On blood agar, except *B. anthracis* strains, the colonies are surrounded by zones of β-haemolysis (Kramer and Gilbert, 1989). *Bacillus thuringiensis* produces intracellular parasporal toxin crystals during sporulation (Vachon *et al*., 2012). Spores are ellipsoidal, centrally or paracentrally placed and with net negative charge. The hydrophobic nature of *B. cereus* spores and presence of appendages (1-30 in number of 0.45-3.8 μm x 13.6 nm) on the surface result in firm adhesion to food processing surfaces, like stainless steel (Tauveron *et al.,* 2006; Ankolekar and Lebbé, 2010). Adhered cells and spores can act as initiation stage for the formation of biofilm which can be a source of recurrent contamination and reduced shelf-life of the product (Ryu and Beuchat, 2005). Generally, heat treatments like pasteurisation fail to effectively kill the heat-resistant endospores, limiting the possibilities of extending the shelf-life of pasteurised products.

Heat resistance of *B. cereus* spores is dependent on strain and sporulation medium, and the D_{100} ranges from 0.075 to 2.26 min when suspended in buffer. Faille *et al.* (2001) found that the spores of *B. cereus* CUETM 98/4, isolated from a milk product, were found to be highly resistant to heat (D_{100} 3.32 min in whole milk). Four of the 23 dairy silo spore isolates showed extremely high D_{75} value (>40 min in hot alkali, pH >13) (Shaheen *et al*., 2010). Heat-resistant spores can survive pasteurisation, be present in the milk, and will be able to germinate and grow during storage, thus limiting the keeping quality of pasteurised milk. Germination is the process whereby spores change from a dormant state to a metabolically active state. Heat, chemicals and a decrease in pH to 2-3 can activate spores (Ghosh and Setlow, 2009). In dairy industry, heat is the most likely mechanism of spore activation, because of the extensive use of heat as a preservation technology. Following activation, germination of *Bacillus* spores can be triggered by an interaction of specific nutrients, like L-alanine and inosine, with germinant receptors located in the inner membrane. The *B. cereus* group expresses a core group of five germinant receptors, GerR, GerL, GerK, GerS and GerI which recognise specific germinant as a signal of conditions suitable for growth (van der Voort *et al*., 2010). Subsequent activation of a transporter mediates transport of Ca-dipicolinate out of the spore with concomitant influx of water. Adhered spores show identical germination properties when compared to spores in suspension. Thus, an additional germination step using L-alanine/inosine-like germinant mixtures in cleaning-in-place (CIP) regime can be an effective measure to reduce the number of adhered spores (Hornstra *et al*., 2007).

2.1.3. Ecology

Members of the *B. cereus* group are found in various habitats in the environment. The natural reservoir for *B. cereus* consists of decaying organic matter, fresh and marine waters, vegetables, fomites and the intestinal flora of different animals, from which soil and food products may become contaminated, leading to a transient colonisation of the human intestine. Because of adhesive nature of its endospores, *B. cereus* is frequently present in food production environments. This character enables the bacterium to spread to all kinds of foods, such as milk and dairy products, vegetables, rice and rice dishes, meat and meat products, soups and spices (te Giffel *et al.,*1996; Organji *et al.,* 2015). *Bacillus thuringiensis* is ubiquitous in soil and also found on the phylloplane and in insects. Less is known on the ecology of other members of the *B. cereus* group (*B. mycoides*, *B. weihenstephanensis* and *B. pseudomycoides*), but they have been isolated from a wide variety of environmental niches, such as soils, sludge, arthropods, earthworm and rhizospheres (Jensen *et al.,* 2003). *Bacillus anthracis* shares the same ecological niche as *B. cereus* and *B. thuringiensis*, and can grow and persist outside the host, in rhizosphere of plants (Saile and Koehler, 2006).

2.1.4. Toxigenicity

Bacillus cereus-mediated food poisoning is highly under-reported due to its generally mild, short duration and self-limiting symptoms which do not motivate the patient to seek medical attention. Most of the *B. cereus* strains isolated from different food products and raw materials are multidrug-resistant (Banerjee and Sarkar, 2004; Roy *et al.*, 2007). Examples of outbreaks and incidences are in several publications (Table 2).There are two distinct symptoms of emesis and diarrhoea, caused by very different types of toxins produced by *B. cereus* (Table 3). The rapid on set of the emetic disease caused by *B. cereus*, generally within 5 h after consumption of the meal, indicates that this is due a toxin preformed in the food.

Table 2. Foodborne outbreaks/incidence caused by *Bacillus cereus*

Year	Place	Outbreaks /incidence	Reference
2007	India	1	Hussain *et al.* (2007)
2007	Australia	2	NSW Food Authority (2013)
2006-2008	India	54	Banerjee *et al.* (2011)
2005	Norway	50	EFSA (2005)
2003	Belgium	4	Dierick *et al.* (2005)
2001	Italy	94	Ghelardi *et al.* (2002)
2000-2011	USA	202	CSPI (2014)
2000-2002	New Zealand	10	Boxall and Ortega (2003)
1999	USA	38	Mead *et al.* (1999)
1996-2000	UK	342	Adak *et al.* (2002)
1995	India	1	Singh *et al.* (1995)
1992-1994	Netherlands	40	Simone *et al.* (1997)

Table 3. Characteristics of food poisoning caused and toxins produced by *Bacillus cereus*

Characteristics	Emetic syndrome	Diarrhoeal Syndrome
Primary cause	Preformed toxin in food	Ingestion of cells and spores and toxin production in small intestine
Foods mostly involved	Starch-rich foods: rice, pasta, potato, pastry and noodles	Proteinaceous foods: milk and dairy products, meat products, pudding, soups, sauces and vegetables
Symptoms	Nausea, vomiting, malaise and in some cases fatal liver failure	Watery diarrhoea and abdominal pain
Incubation time	≤5 h	8-16 h
Period of illness	6-24 h	12-24 h
Infective dose	0.02-1.83 µg kg^{-1} body weight (5-8 log cells g^{-1} implicated food)	5-8 log cfu g^{-1}
Toxin associated	Cereulide	Haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK)
Heat sensitivity of toxin	Extremely stable (even 121 °C for 90 min)	Labile
Genes	Cereulide synthetase (Ces) gene cluster (*cesHPTABCD* genes) *cesA* (10 kb) and *cesB* (8 kb): structural genes *cesP*: 4′-phosphopantetheinyl transferase *cesT*: a thioesterase *cesC* and *cesD*: ABC transporter	Hbl complex (*hblCDA* operon) *hblC*: L_2 (46 kDa) *hblD*: L_1 (38 kDa) *hblA*: B (37 kDa) Nhe complex (*nheABC* operon) *nheA*: NheA (41 kDa) *nheB*: NheB (40 kDa) *nheC*: NheC (36 kDa) cytK: CytK (34 kDa)
Genetic regulation	By Spo0A and AbrB	By PlcR
Mode of action	Acts as K-ionophore to inhibit fatty acid oxidation in mitochondria Binds to serotonin 5-hydroxytryptamine 3 (5-HT3) receptors, stimulates vagus afferent resulting in vomiting Brain oedema and fulminate liver failure due to inhibition of mitochondrial fatty-acid oxidation Inhibit the natural killer cells (T cells) of the human immune system	Hbl shows haemolytic, cytotoxic, dermonecrotic and vascular permeability activity Nhe is pore-forming toxin, structurally similar to Hbl CytK is dermonecrotic, cytotoxic and haemolytic

Based on Granum and Lund (1997), Fagerlund *et al.* (2004), Ehling-Schulz *et al.* (2006) and Stenfors Arnesen *et al.* (2008)

Cereulide, the emetic toxin, is a cyclic 1.2 kDa dodecadepsipeptide [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$ resembling valinomycin (Agata *et al.*, 1995). t is produced by a nonribosomal peptide synthetase, encoded by the 24 kb

cereulide synthetase (*ces*) gene cluster which is located on pCER270, a 270 kb pXO1-like megaplasmid (Ehling-Schulz *et al*., 2004, 2005, 2006). Emetic toxin is not inactivated during food processing or gastrointestinal passage due to its high resistance against heat treatments, pH extremes and proteolytic degradation (Rajkovic *et al*., 2008). The mechanism by which cereulide causes emesis in humans has not been definitely determined; however, animal model study has shown that 5-hydroxytryptamine 3 (a receptor), released from the stomach into the duodenum, binds with cereulide to stimulate vagus afferent causing vomiting (Agata *et al*., 1995). Gene cluster for cereulide production (*ces*) is mainly present in *B. cereus* isolates and some rare strains of *B. weihenstephanensis* (Thorsen *et al.,* 2006). Diarrhoeal syndrome is characterised by abdominal pain with watery diarrhoea, developed 8-16 h after ingestion of the contaminated food. The syndrome has been linked to two enterotoxin complexes, haemolysin BL (Hbl) and nonhaemolytic enterotoxin (Nhe), and a single protein, cytotoxin K (CytK) (Beecher and Macmillan, 1991; Beecher *et al*., 1995; Lund and Granum, 1996; Lund *et al*., 2000; Granum *et al*., 2014).

Hbl complex consists of three proteins, L_2, L_1 and B, encoded by the *hbl* operon. The optimal ratio of components L_2, L_1 and B for maximal toxicity is 1:1:1. The Hbl components independently bind to the cell membrane and after association and pore formation, osmotic cell lysis occurs (Beecher and Wong, 1997). Prevalence of *hbl* gene is rare in emetic strains and *B. anthracis.*

Nhe complex is the most important toxin in food poisoning. It is composed of NheA (cytolytic protein), NheB and NheC (binding proteins), encoded by the *nhe* operon (Lindbäck *et al*., 2010). All the three components are required for biological activity; maximal toxic effects are exerted by NheA, NheB and NheC in the ratio 10:10:1. The cytotoxic activity of Nhe on epithelial cells has been shown to be due to colloid osmotic lysis following pore formation in the plasma membrane. *Nhe* operon is ubiquitously found among B*. cereus* group members.

CytK, a β-barrel pore-forming protein, is cytotoxic due to its pore-forming ability in the cell membranes. This heptamer toxin is secreted in a soluble form, which is eventually converted into a transmembrane pore by the assembly of an oligomeric β-barrel, with the hydrophobic residues facing the lipids and the hydrophilic residues facing the lumen of the channel. Toxin occurs in two forms that have 89% amino acid sequence homology, CytK-1 and CytK-2 (Fagerlund *et al*., 2004). Gene *cytK-1* is specific to *B. cytotoxicus* (Guinebretière *et al*., 2010)

The enterotoxic potential of different phylogenetic groups of *B. cereus* has implications for health risk. Food poisoning risk is highest for group III (containing *B. cereus* III, *B. thuringensis* III and *B. anthracis*-like strains). The risk remains high for groups VII, IV and II, and decreases with group V. *Bacillus weihenstephanensis* and *B. mycoides* strains belonging to group VI are safest, representing a low level of risk (Guinebretière *et al*., 2010).

2.2. Role of the group in dairy production chain

2.2.1. The dairy production chain

A dairy chain involves milk production, transport, processing, packaging and storage; with each activity the product increases in value (Fig. 3). The dairy chain starts at raw milk production and ends when consumers utilise products that are created in the value chain. Indian dairy sector has a three-tier structure which consists of village cooperative society formed by milk producers, a district cooperative milk producers union owned by dairy cooperative societies and the state federation formed by cooperative milk producer's in a state. The union buys all the societies milk, then processes and markets fluid milk and products. The state federation is responsible for marketing the milk and products of member unions (NDDB, 2015) (Fig. 4).

2.2.2. Sources of contamination

Presence of *B. cereus* in the dairy environment is inevitable as it is ubiquitously present in soil, on grass, dairy cattle feed and dung, etc. Soil, feed (through excretion of spores in faeces) and bedding material are the major source of contamination of raw milk with *B. cereus* (Fig. 5). The concentration of *B. cereus* spores in raw milk

Fig. 3. Industrial dairy processing line

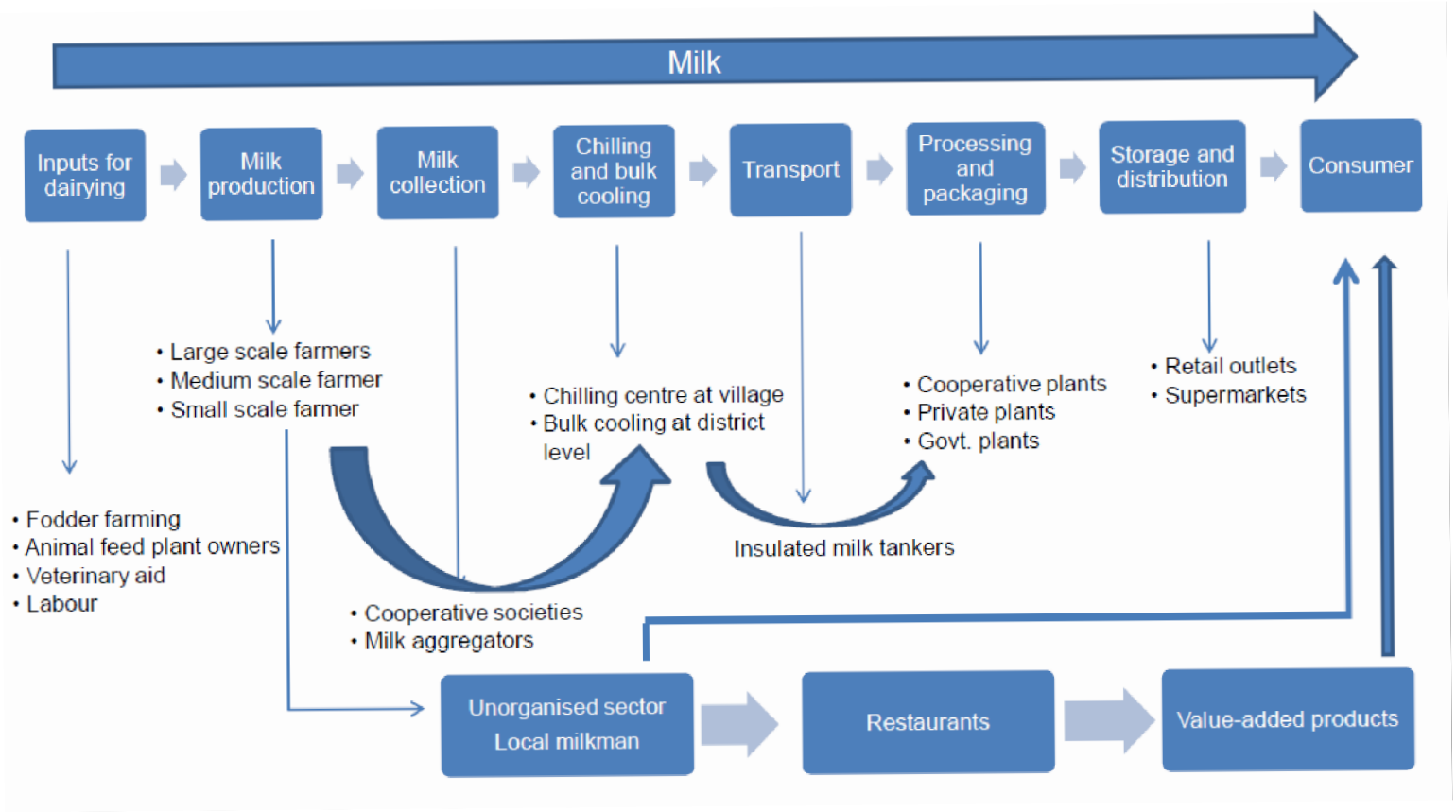

Fig. 4. Dairy supply chain in Indian scenario (Source: www.slideshare.net/sudarshanpatel5/dairy-presentation-pgdma; accessed on 13 May 2016)

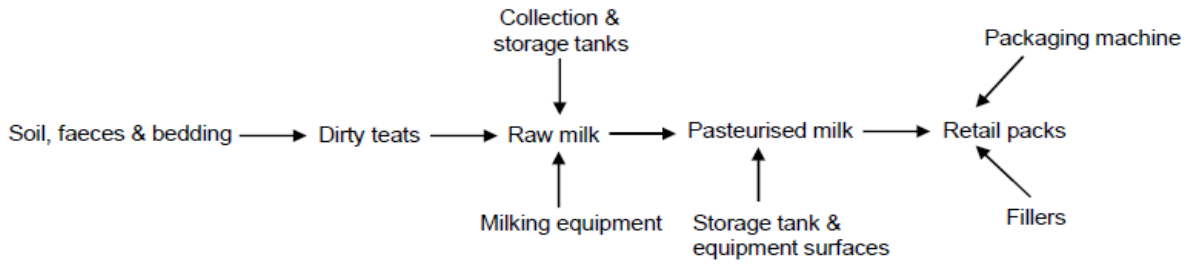

Fig. 5. Contamination routes of *Bacillus cereus* in dairy production chain (based on Heyndrickx, 2011)

is higher in summer than in winter (Bartoszewicz *et al.*, 2008). Their main contamination route to milk during the grazing season (summer) is via cow's udder contaminated by soil and faeces (Christiansson *et al.,* 1999; Vissers *et al.*, 2007). In the housing period of cows, feed is the only source of spores; udder becomes contaminated mainly through the bedding material that is contaminated with faeces. Milking equipment can also be a source of contamination for raw milk (Christiansson *et al.*, 1997). *Bacillus cereus* occurs in low numbers (2-3 log cfu l^{-1}) in farm-collected milk (Svensson *et al.*, 2004, 2006; Bartoszewicz *et al.*, 2008). However, higher levels of *B. cereus* contamination (up to 5 log cfu ml^{-1}) in pasteurised milk (Table 4) have been reported (te Giffel *et al.*, 1996; Larsen and Jørgensen, 1997). Studies by global typing methods, such as fatty acid profiling, biochemical

Table 4. Incidence of *Bacillus cereus* in dairy products

Type	Country	Positive samples (%)	Population (cfu ml^{-1} or g^{-1})	Reference
Raw (untreated) milk	Egypt	30	9×10^2	Hassan *et al.* (2010)
	India	66	10^5	Bedi *et al.* (2005)
	Sweden	-	0.198	Svensson *et al.* (2004)
Pasteurised /sterilised milk	Brazil	-	0.44-70	Salustiano *et al.* (2009)
	Thailand	100	10^2- 10^3	Chitov *et al.* (2008)
	Poland	-	0.04-0.17	Bartoszewicz *et al.* (2008)
	China	71	11.7 MPN	Zhou *et al.* (2008)
	Netherlands	35	10-10^4	te Giffel *et al.* (1996)
	Denmark	47	10^3-10^5	Larsen and Jørgensen (1997)
	Spain	25	10^3-10^5	van Netten *et al.* (1990)
Dried milk product	Chile	46	3-10^4	Reyes *et al.* (2007)
Ice cream	India	40	10-10^3	Warke *et al.* (2000)
Cheese	Turkey	12	-	Molva *et al.* (2009)

typing, RAPD-PCR and rep-PCR fingerprinting of the *B. cereus* isolates, have shown that the distribution of genotypes in the dairy and its products differed from that in raw milk (te Giffel *et al.,* 1997; Svensson *et al.,* 2004; Bartoszewicz *et al.,* 2008). Thus, the farms are not the sole source of *B. cereus* in milk. In addition, it is likely that 'in-house' microbiota present in silo tanks, pasteurisers and filling machines could contribute to post-pasteurisation contamination of milk (Svensson *et al.,* 2004). Contamination of milk with *B. cereus* by post-pasteurisation surface exposures can be demonstrated by using automated ribotyping. In a study, seven ribotypes were identified, demonstrating the genetic variability of the *B. cereus* group, isolated from pasteurised milk and different surfaces. Surfaces responsible for major contamination of pasteurised milk were pasteurised milk storage tank, packaging machine, levelling tank and the package-forming tube surfaces. Most of the isolates belonged to the same ribogroup (RIBO1222-73-S4), and they were found on four surfaces and also in the milk, indicating the role of the equipment surfaces as reservoirs for milk (re)contamination (Salustiano *et al.,* 2009).

2.2.3. Toxigenicity and spoilage

In dairy industry, sporeformers are important contaminants because they can significantly affect product safety and quality. The occurrence of emetic and diarrhoeal toxin-producing strains of *B. cereus* in dairy production

chain has been extensively reported. Majority of the strains isolated from dairy products are cytotoxic and PCR positive for *nhe* and *hbl* (Anderson Borge *et al*., 2001). Growth temperature affects toxin production in *B. cereus* group species in a strain-dependent manner. In an investigation of food poisoning potential of *B. cereus* strains from Norwegian dairies, while some of the 39 strains were moderately or highly cytotoxic when grown at 25 °C or 32 °C, none of those were highly toxic at human body temperature, i.e. 37 °C. Hence, those strains should be considered to pose a minor risk with regard to diarrhoeal food poisoning (Stenfors Arnesen *et al.,* 2007). The occurrence of emetic toxin-producing strains in milk and other sources at the farms is rare (1.9%) (Svensson *et al.,* 2006). Toxin profile of *B. cereus* group from the silo tanks is dominated by strains with the toxin profile 'C' (nhe^+, hbl^+, $cytK^-$ and ces^-) and 'F' (nhe^+, hbl^-, $cytK^-$ and ces^-) (Svensson *et al.,* 2004; Ehling-Schulz *et al.,* 2006).

Members of the *B. cereus* group and *Bacillus subtilis* group are the most important spoilage microorganisms in dairy environment (Lücking *et al*., 2013). They produce various extracellular enzymes such as protease, lipase and amylase significantly which contribute to the reduction of shelf-life of processed milk and dairy products by degrading milk components and additives (Table 5). The presence of protease can lead to bitter

Table 5. Effects of enzymes produced by *Bacillus cereus* on the organoleptic quality of milk and dairy products[a]

Enzyme	Principle of action	Product	Quality defect
Protease	Hydrolysis of casein, resulting in the formation of gel (coagulation)	Pasteurised/ sterilised milk	Sweet curdling, off-flavour, bitter, foreign, unclean
		Cream/butter	Off-flavours
		Cheese	Lower yield, shorter coagulation time due to higher concentration of free amino acids which stimulate the growth of starter culture
		Milk powder	Bitterness
Lipase	Hydrolysis of milk triacylglycerols, resulting in high concentration of free fatty acids	Pasteurised/ sterilised milk, cream, butter	Rancidity, off-flavour, bitterness, soapy
		Cheese	Rancidity, off-flavour, longer coagulation time as free fatty acids inhibit the growth of starter culture
		Milk powder	Rancidity
Lecithinase and phospholipases	Disruption of membrane structure of fat globules, resulting in degradation of milk fat	Milk	Bitty cream

[a] Based on Chen *et al.* (2003) and Samaržija *et al.* (2012)

flavour, clotting and gelation of milk (Chen *et al*., 2003; Datta and Deeth, 2003). The proteolytic changes caused by *Bacillus* spp. significantly increase the concentration of free tyrosine (Nabrdalik *et al.,* 2010), which can increase in milk up to 2.13 mg ml^{-1} in comparison with their initial level of *ca* 0.65 mg ml^{-1} (Janštová *et al*., 2006). On the other hand, lipases have been responsible for dairy defects such as bitty cream and also contribute to unpleasant flavour such as rancid, butyric, buttery, unclean and soapy in milk and dairy products (Furtado, 2005). Lipolysis is known to contribute both desirable and undesirable flavours to dairy products, initially through hydrolysis of milk triacylglycerols. Short-chain fatty acids, such as butyric acid, caproic acid and caprylic acid, give sharp and tangy flavour. Medium-chain fatty acids, such as capric acid and lauric acid, tend to impart a soapy taste, while long-chain fatty acids, such as myristic acid, palmitic acid and stearic acid contribute little to flavour. Unsaturated fatty acids, released during lipolysis, are susceptible to oxidation and the concomitant formation of aldehydes and ketones which give rise to off-flavour. Other unpleasant flavours, such as rancid, butyric, bitter, unclean, soapy and astringency in milk and dairy products have also been attributed to lipolysis. Production of protease by *B. cereus* induces proteolysis in sterilised milk (Murugan and Villi, 2009). Proteolytic and lipolytic changes, caused by the occurrence of *Bacillus* spp. in sterilised milk, can cause reduction of total protein from 34.6 g l^{-1} to 29.5-32.9 g l^{-1} and casein ratio reduction by 7.3% (Janštová *et al*., 2004). *Bacillus cereus* isolates from different dairy products are able to produce protease and lipase (Lücking *et al*.,

2013). These enzymes are sufficiently thermostable and remain active after pasteurisation and may cause defects in the final dairy products (Chen *et al*., 2004).

2.2.4. Biofilm

In milk storage and dairy processing operations, besides being present in the raw material, bacteria are associated with surfaces (Mittelman, 1998). The attachment of bacteria with subsequent development of biofilms in milk processing environments is a potential source of contamination of finished products that may shorten the shelf-life or facilitate transmission of diseases (Lindsay *et al*., 2006; Brooks and Flint, 2008). Thus, biofilm formation in dairy processing environment can lead to serious hygiene problems and economic loss due to spoilage of dairy products and equipment impairment (Bremer *et al.*, 2006). Formation of bacterial biofilms within food processing plants is a concern to processors, as bacteria within biofilms are more difficult to be eliminated than planktonic cells, and can act as a source of recurrent contamination to plant, product and personnel. Biofilm renders its inhabitants resistant to antimicrobial agents and cleaning (Srey *et al*., 2013). Other undesirable conditions associated with biofilms include reduced flow through blocked tubes, reduced plan run times, corrosion of stainless steel and reduced heat transfer through plate heat exchangers (Parkar *et al*., 2004).

Biofilm is a sessile microbial community adhered to a solid surface surrounded by a matrix which includes extracellular polymeric substances (EPS). Dairy biofilms are predominated by bacterial EPS and milk residues, mostly proteins and calcium phosphate (Flint *et al*., 1997; Mittelman, 1998). The biofilm formation is a stepwise and dynamic process consisting of initial attachment, irreversible attachment, and formation of microclonies, maturation and dispersion (Fig. 6).

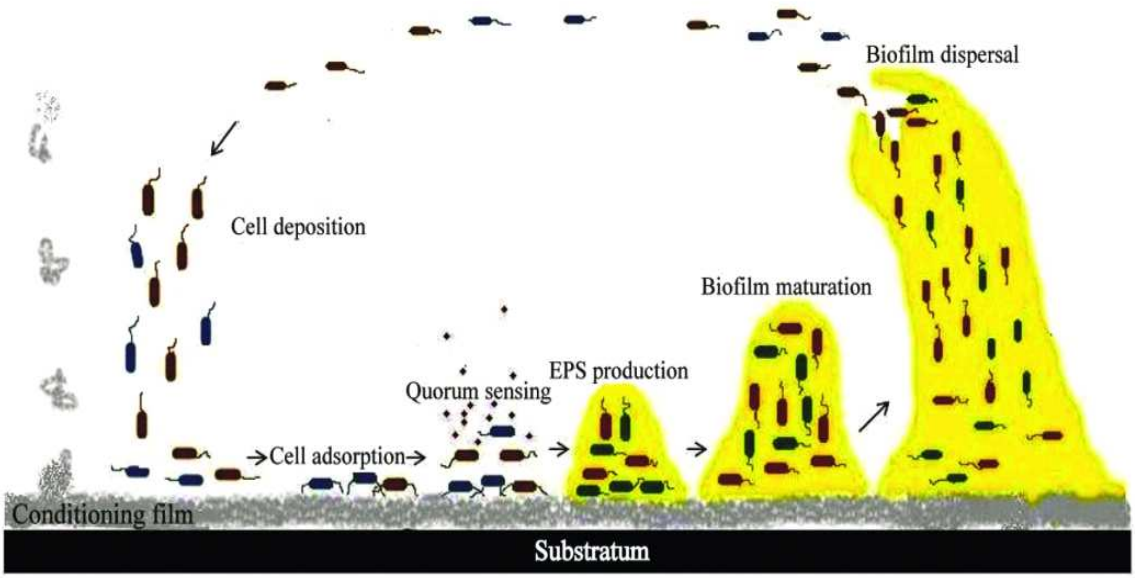

Fig. 6. Processes governing biofilm formation (based on Breyers and Ratner, 2004)

2.2.4.1. Attachment of planktonic cells to a solid surface

Attachment of microorganisms to surfaces and subsequent biofilm development are complex processes, and affected by the surface properties of attachment site and cell. *Bacillus cereus* forms a considerably higher amount of biofilm on stainless steel compared to polystyrene in terms of total biomass and number of cells attached (Hayrapetyan *et al.,* 2015). The observation triggered them to study the impact of iron, which is the main component in stainless steel, on biofilm formation. Although iron does not promote initial attachment of cells to the surface, its role in surface-associated behaviour, such as cell–cell interaction, is more likely than cell–

substratum interaction or production of matrix components in biofilm. Attachment occurs more readily on surfaces that are rougher, more hydrophobic and coated by surface conditioning films (Simões *et al.,* 2008). In dairy operations, the conditioning film mainly consists of organic milk components. The cell surface properties, particularly cell surface hydrophobicity and presence of extracellular filamentous appendages may influence the rate and extent of microbial attachment (Donlan, 2002; Klavenes *et al*., 2002). Among the various physiological stages of *B. cereus*, spores have the greatest biofilm formation potential (Pagedar and Singh, 2012). CalY and TasA, coded by gene *calY* and operon *sipW-tasA*, respectively, cooperate to assemble robust and stable fibres with amyloid properties and are important for *B. cereus* biofilm assembly (Caro-Astorga *et al.,* 2015). Adhesion of *B. cereus* to stainless steel surface increases with the increase in temperature, pH and time (Peña *et al.,* 2014).

In the attachment stage of biofilm formation, single bacterial cells are transported to surfaces and reversible bonds are formed between the cell wall and the substratum. Forces influencing the reversible adhesion process are the van der Walls forces, electrostatic forces and hydrophobic interactions. During this stage, bacteria still can be removed merely by rinsing. Next stage is a shift from a weak interaction of the bacteria with the surface to irreversible attachment to permanent bonding with the presence of EPS (Sauer *et al*., 2002).

2.2.4.2. Formation of microcolony

The irreversibly attached bacterial cells grow and divide leading to the formation of microcolonies which are discrete matrix-enclosed communities of bacterial cells that may include cells of one or many species. During this period, attached cells produce EPS which helps in the anchorage of the cells to the surface and to stabilise the colony from the fluctuations of the environment. EPS protects biofilm microorganisms against adverse conditions. The EPS matrix delays or prevents antimicrobials from reaching target microorganisms within the biofilm by diffusion limitation and/or chemical interaction with the extracellular proteins and polysaccharides. Moreover, within the EPS matrix the molecules required for cell–cell communication and community behaviour may accumulate at concentrations, high enough for the recruitment of planktonic cells from the surrounding environment (Sutherland, 2001).

2.2.4.3. Biofilm maturation

In the maturation step, biofilm develops into an organised structure which can be flat or mushroom-shaped (Chmielewski and Frank, 2003). The cell growth in the periphery is rapid and in the interiors is slow. Portion of mushroom may break off and repopulate other sites. The microorganisms within the biofilm grow in the matrix-enclosed microcolonies interspersed within highly permeable water channels (Davey and O'Toole, 2000). Most metabolically active bacteria remain at the top layers of the biofilm matrix near water channels which allow the dispersion and exchange of dissolved organics, metals cations and metabolites. Nutrients become trapped and concentrated in the biofilm matrix and move throughout the matrix by diffusion, which results in a stratified habitat that selects for different microbial species.

2.2.4.4. Biofilm dispersal

As the biofilm ages, attached cells revert into their planktonic form (Sauer *et al*., 2002). Increased fluid shear, endogenous enzymatic degradation, or release of EPS or surface-binding proteins is the possible cause of biofilm detachment (Stoodley *et al*., 2002). Starvation is considered as a reason of detachment and search for a nutrient-rich environment (O'Toole *et al*., 2000). Biofilm sloughing may also occur when there is an imbalance or fluctuation of nutrients. Low-carbon availability can cause increased EPS production which leads to detachment (Kim and Frank, 1995).

2.2.4.5. Biofilm hazard and control strategies

The ability of *B. cereus* spores to adhere and act as an initiation stage for biofilm formation on food processing plants is well known (Faille *et al*., 2001; Peng *et al*., 2001). The strong adhesion properties of *B. cereus* spores

have been attributed to the hydrophobic character of the exosporium, which varies from species to species and to the presence of appendages on the surface of the spores (Tauveron *et al*., 2006). BclA is the major glycoprotein of the *B. cereus* exosporium and plays an important role in spore interaction with materials, probably by providing a larger contact surface with stainless steel (Lequette *et al.,* 2011).Thicker biofilms of *B. cereus* develop at the air-liquid interface, compared to submerged systems. This suggests that *B. cereus* biofilms may develop particularly in industrial storage and piping systems that are partly filled during operation or where residual liquid has remained after a production cycle (Wijman *et al*., 2007). While for most of *Bacillus* strains negative effects of whole milk on biofilm formation have been observed (Flint *et al*., 1997; Wong, 1998), the study of Shaheen *et al.* (2010) illustrated that *B. cereus* was capable of forming biofilms in whole milk but not in water-diluted milk. *Bacillus cereus* biofilm formation is enhanced under low nutrient conditions and dependent on biosurfactant production, which can be directly or indirectly repressed by PlcR a pleiotropic regulator which controls the expression of a variety of genes, many of which encode potential virulence factors, including enterotoxins, haemolysins, phospholipases C and proteases (Hsueh *et al*., 2006).

Process biofilms are common in dairy industry where a single species predominates due to reduction of competition as a result of heat treatment of milk; 12.4% of microbiota growing in biofilms in a commercial dairy plant is *B. cereus* (Sharma and Anand, 2002). Biofilm formation even at lower temperatures can be a matter of concern. Presence of *B. cereus* biofilm in dairy processing line and raw milk chilling tankers can be a source of contamination. From various *in vitro* model studies it is evident that *B. cereus* biofilms are present even on internal surfaces of the tankers; cell count in the biofilm developed on the surface of stainless steel tanks can reach up to 8 log cfu cm^{-2}, if inadequately cleaned tanker is left to stand empty at room temperature (Teh *et al*., 2012). Thus, the presence of biofilm on internal surfaces of chilling tanks can lead to recurrent contamination and spoilage of fresh lot of milk collected into tank.

In dairy, like any other food industry, an effective cleaning and sanitation program is part of the process to eliminate microorganisms. CIP procedures are usually employed in milk processing lines (Table 6).

Table 6. Different clean-in-place (CIP) regimes in dairy environment for *Bacillus cereus* biofilm cell removal

CIP regime	Biofilm-forming strain	Reduction in biofilm cells (log cfu cm^{-2})	Reference
Phosphate buffer (pH 10) + subtilisin P2 at 45 °C for 30 min	BC98/4	0.92	Lequette *et al.* (2010)
Realco B (pH 10, containing anionic surfactant, dispersing and chelating agents, + subtilisin P2 at 45 °C for 30 min	BC98/4	0.56	Lequette *et al.* (2010)
10 g NaOH l^{-1} at 65 °C for 10 min – 10 ml HNO_3 l^{-1}at 65 °C for 10 min	Consortium	2	Bremer *et al.* (2006)
5 min water rinse – 5 g NaOH l^{-1} at 60 °C for 15 min - water rinse for 5 min	CUETM98/4	0.98	Faille *et al.* (2014)

In dairy industry, CIP systems generally involve the sequential use of caustic (sodium hydroxide) and acid (nitric acid) wash steps, selected for their ability to remove organic (proteins and fat) and inorganic (calcium and other minerals) fouling layers. The most common and aggressive caustic cleaner is sodium hydroxide (NaOH), which is typically used in 10-50 g l^{-1} concentrations for plate-type and tubular heat exchangers, and other heavily soiled surfaces and 10-20 g l^{-1} for general use (Flint *et al*., 1997). The primary role of the caustic (alkali) wash step is the removal of proteins and carbohydrates (Chisti, 1999). To enhance cleaning effectiveness, caustic detergents and caustic additives have been developed, which contain surfactants, emulsifying agents, chelating compounds and complexing agents (Bremer *et al*., 2006).Traditionally, chlorine (sodium hypochlorite)-based sanitisers have been used, however, a wide variety of sanitisers including quaternary ammonium compounds, anionic acids, iodophores and chlorine-based compounds are currently in use or being evaluated or use in CIP systems (Parkar *et al*., 2004; Bremer *et al*., 2006). The combined effects of the biocide, benzyldimethyldodecyl ammonium chloride (BDMDAC) with a series of increasing Reynolds number of agitation promotes *B. cereus* biofilm removal from stainless steel surface (Lemos *et al*., 2015).

Different studies simulating CIP regimes in dairy industry showed varied results. A CIP regime against dairy biofilms (water rinse -10 g NaOH l^{-1} at 70 °C for 10 min - water rinse – 8 ml HNO_3 l^{-1} at 70 °C for 10 min - water rinse) followed by exposure to either chlorine or combinations of nisin, lauricidin and the lactoperoxidase system for defined exposure periods was inefficient in the total biofilm control (maximum log reduction of 2) (Dufour *et al*., 2004). The additional antimicrobial treatment resulted in a maximum log reduction of 2.8, verified 2 h after chlorine exposure. The standard CIP regime (water rinse - 10 g NaOH l^{-1} at 65 °C for 10 min - 10 ml HNO_3 l^{-1} for 10 min - water rinse), commonly followed in dairy industry, is inefficient to remove bacteria attached to surfaces, as only removal of 2 log reduction in bacterial numbers can be achieved (Bremer *et al*., 2006). The germination response of *B. cereus* adhered spores can be an additional strategy to improve commonly used CIP regime (water rinse - 10 g NaOH l^{-1} at 65 °C for 10 min - 10 ml HNO_3 l^{-1} for 10 min - water rinse) in dairy industry (Hornstra *et al*., 2007). Implementation of a germination-inducing step in CIP helps to reduce the number of spores attached to processing equipment surfaces and additional 3 to 4 log unit removal of cells can be achieved. Thus, optimisation of existing CIP regimes is important to achieve maximum biofilm removal. Use of higher concentration of NaOH (20 g l^{-1}) and HNO_3 (18 ml l^{-1}) at a high temperature (75 °C) can enhance biofilm removal (Parker *et al*., 2004). CIP regimes, commonly used in dairy industry, showed a varied effectiveness in eliminating biofilms. This may be due to the resistance of biofilms to the chemical and physical treatments applied during cleaning and sanitising procedures in the food industry (Chmielewski and Frank, 2003). Therefore, the use of enzyme treatments to break down EPS in biofilms is a possible alternative when standard cleaning agents do not give satisfactory results in removing biofilms. Cleaning regime should break-up or dissolve the EPS matrix associated with biofilm, so that disinfectants can gain access to bacterial cells (Simões *et al*., 2006, 2010). Proteases and polysaccharidases can be used as potential agents for biofilm removal (Meyer, 2003). Proteases in commercially available detergents are already used to clean, for example, ultrafiltration units, contact lenses, medical apparatus and laundry. The combination of proteolytic enzymes with surfactants increase the wettability of biofilms formed by a thermophilic *Bacillus* sp. and, therefore, enhanced cleaning efficiency can be achieved (Parkar *et al*., 2004; Lequette *et al*., 2010). An use of biomimetic superhydrophobic surfaces formed via the self-assembly of paraffin and fluorinated wax crystals inhibited biofilm formation by *B. cereus*. 3D crystalline wax surfaces form a heterogeneous surface that combines wax and air pockets, reducing the contact area between a bacterial cell and the surface and thereby interrupting bacterial adhesion, thus preventing the initial step of biofilm formation (Pechook *et al*., 2015).

Hazard analysis and critical control point (HACCP) in dairy processing lines is required to minimise the initial load of *B. cereus* in finished products (Fig. 7). Examination of raw milk from sensorial, physicochemical and microbiological points of view at the reception, pasteurisation, temperature of pasteurised milk storage tank, maintenance of aseptic conditions during packaging and storage temperature of the packaged products are the critical control points (CCPs) in dairy industry (Tamime, 2009). For each step, critical control limit should be set and monitored. The maximum levels of *B. cereus* spores in raw milk and finished dairy products should be 10^3 l^{-1} and 100 g^{-1}, respectively (EU, 2005).

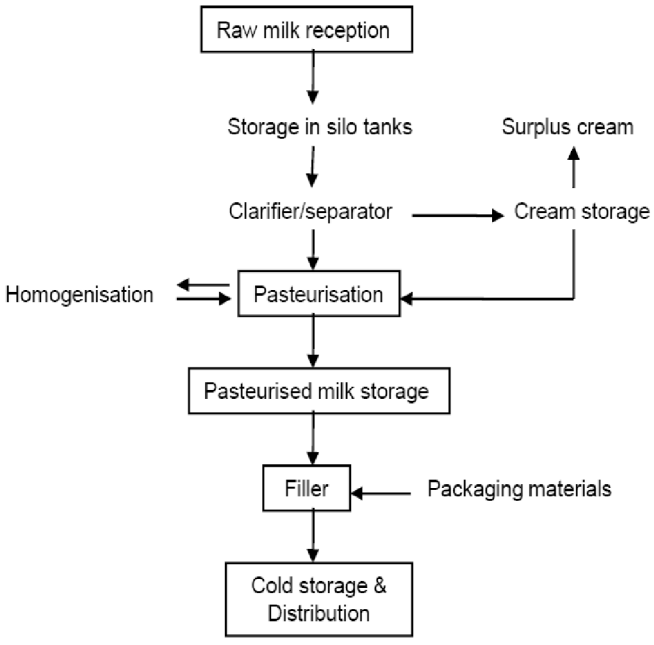

Fig. 7. Flow diagram and critical control points (CCPs) for pasteurised milk. The text within a box indicates CCP (based on ANZDAC, 2011)

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.

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).

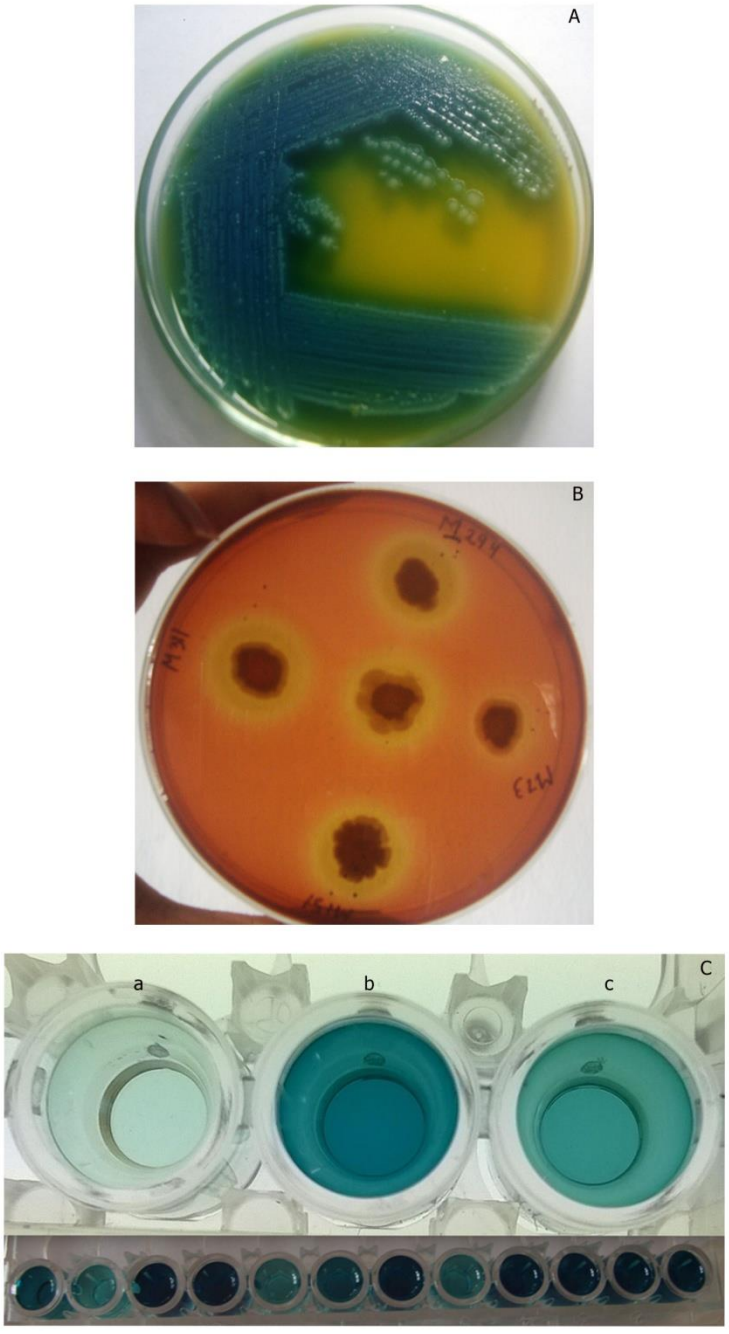

Fig. 9. *Bacillus cereus* isolation and characterisation. A, *Bacillus cereus* colonies on *Bacillus cereus* selective agar plate; B, haemolysis by some isolates on sheep blood agar plate; C, diarrhoeal enterotoxin production by some isolates detected by Tecra antibody test.

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.

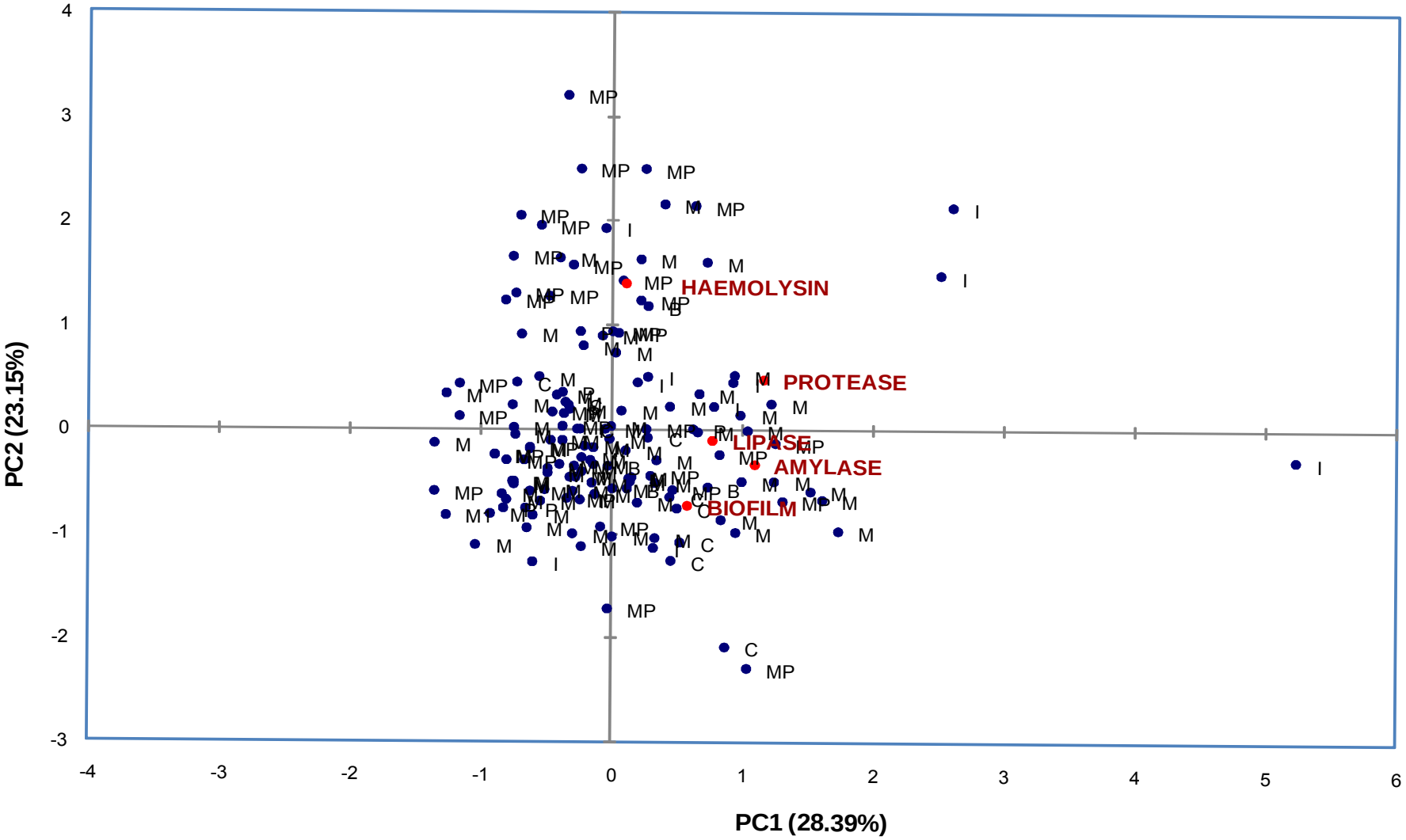

Fig. 10. Score biplot for principal component analysis showing observations (M, milk; MP, milk powder; C, cheese; I, ice cream; P, paneer; B, butter isolates) and variables (production of protease, amylase, lipase, haemolysin and biofilm) together for 144 isolates of *Bacillus cereus*

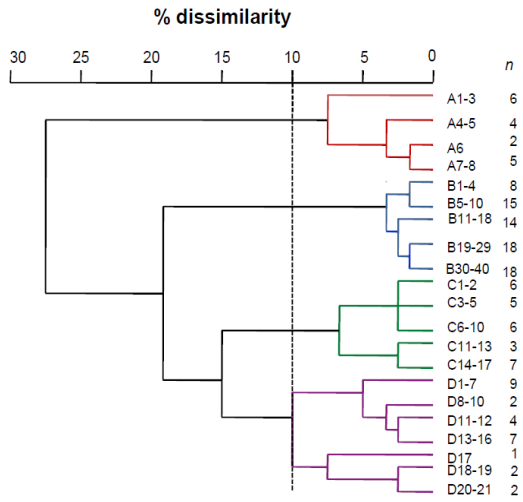

Fig. 11. Simplified dendrogram based on wards clustering of dissimilarity coefficient generated by agglomerative hierarchical clustering. Based on studied characters (production of protease, amylase, lipase, haemolysin and biofilm), the 144 isolates of *Bacillus cereus* were grouped into four major clusters,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

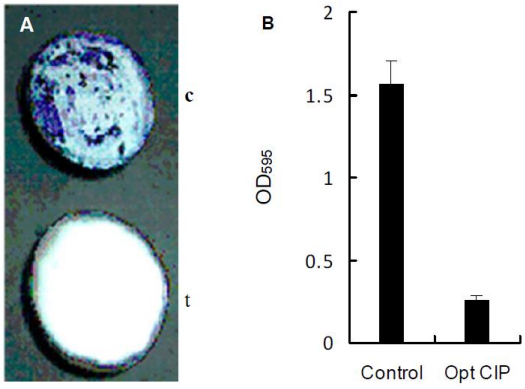

Fig. 13. Crystal violet-stained biofilms present on stainless steel coupons (A) and OD_{595} of stained biofilms from coupons before (c) and after (t) treatment with optimised cleaning regime (B). Error bars represent mean ± *SE*, obtained from triplicate sets of experiment.

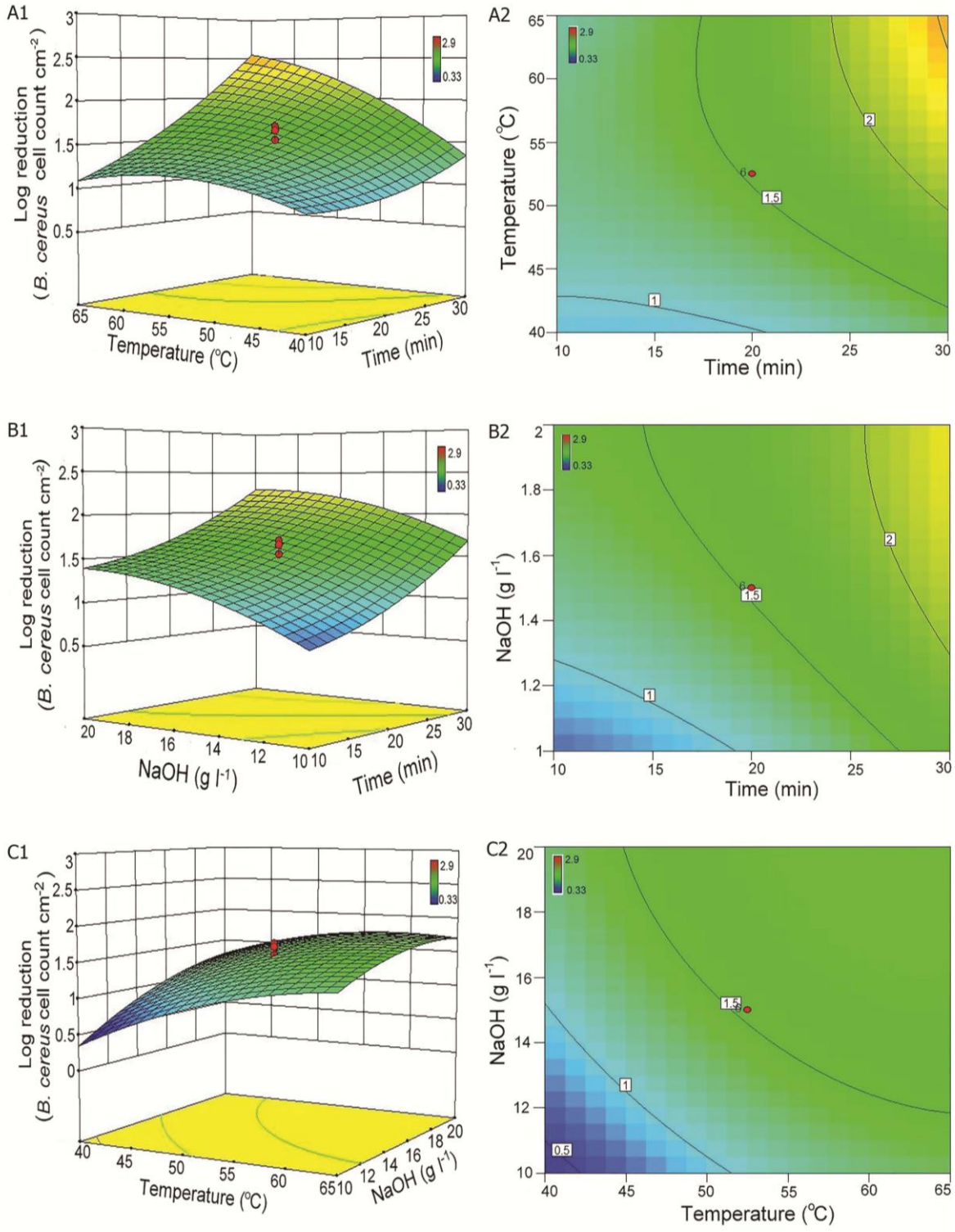

Fig. 12. 3-D (column 1) and contour response surface (column 2) plots on removal of *Bacillus cereus* biofilm. A1 and A2 show effect of time and temperature, when exposed to 15 g NaOH l^{-1}. B1 and B2 show effect of time and NaOH at 52.5 °C. C1 and C2 show effect of temperature and NaOH, when exposure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

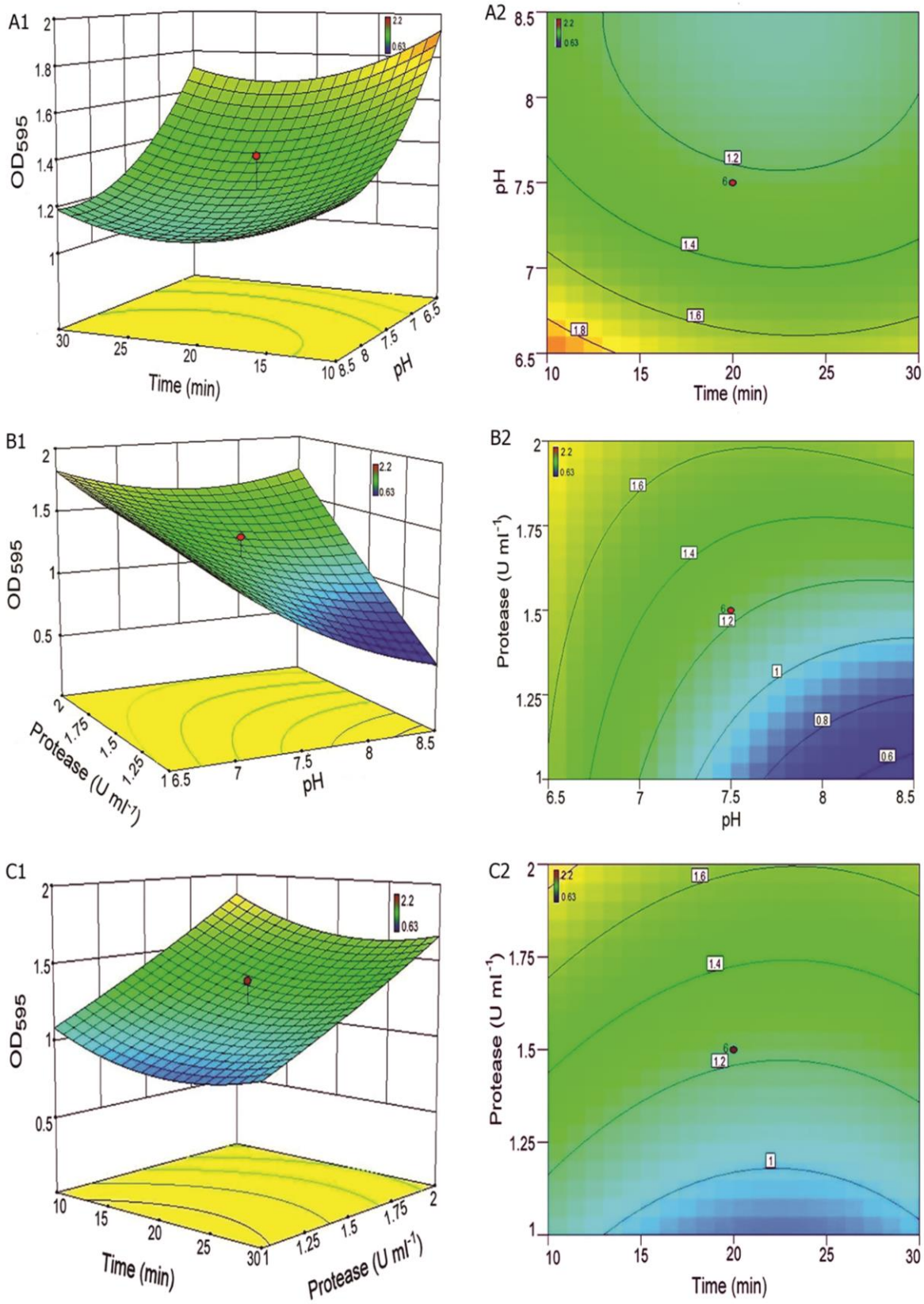

Fig. 14. 3-D (column 1) and contour response surface (column 2) plots on removal of *Bacillus cereus* M28 biofilm from the wells of microtiter plates. A1 and A2 show the effect of exposure time and pH, when protease concentration was 1.5 U ml^{-1}. B1 and B2 show the effect of pH and protease concentration,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

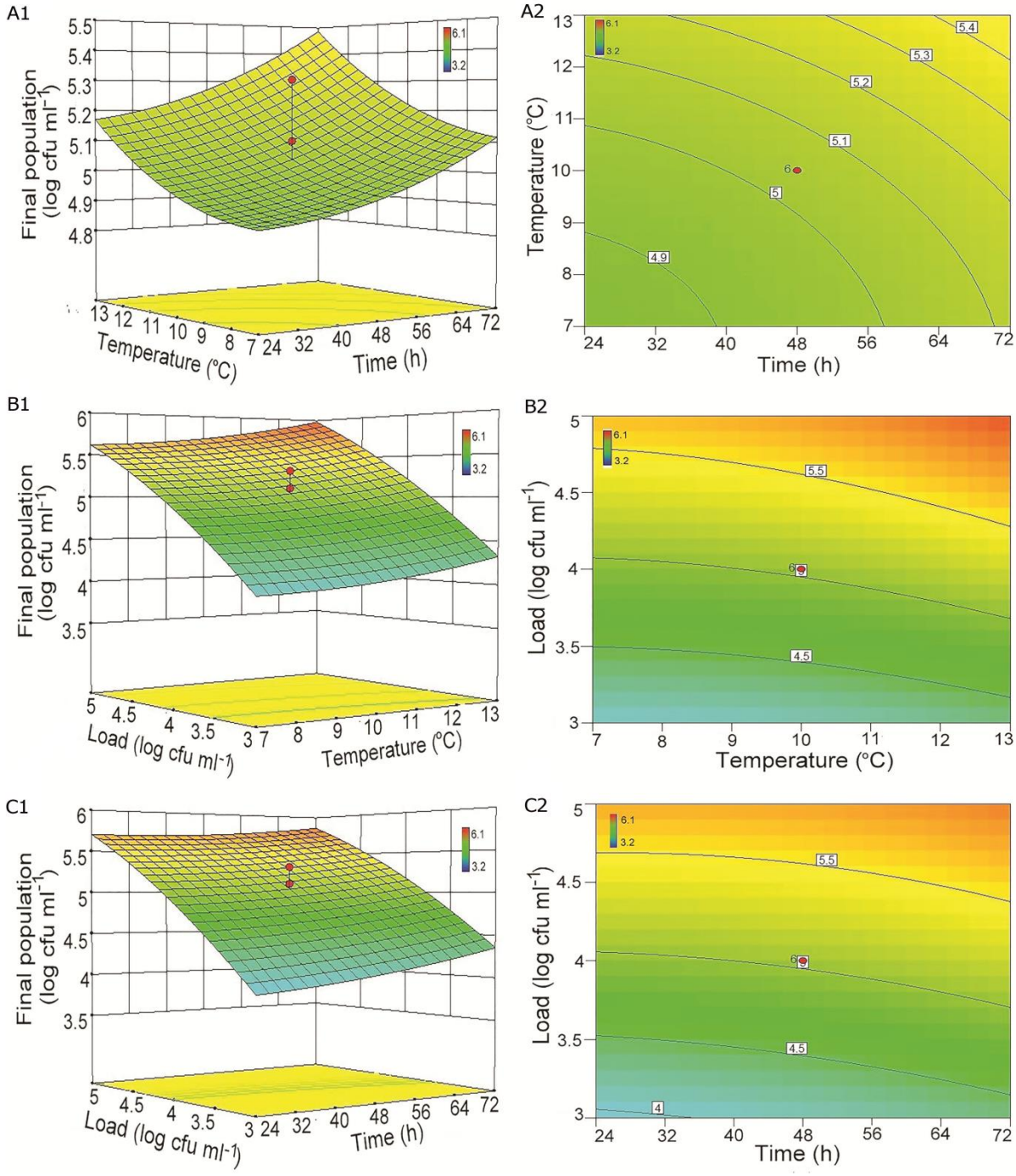

Fig. 15. 3-D (column 1) and contour response surface (column 2) plots on final population of *Bacillus cereus*. A1 and A2 show the effect of storage time and temperature when the load was 4 log cfu ml^{-1}. B1 and B2 show the effect of storage temperature and load after 48 h of storage. C1 and C2 show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

5
Discussion

5.1. Prevalence and characterisation of *Bacillus cereus* in dairy environment

Bacillus cereus is ubiquitous in environment, however underestimated as a foodborne pathogen. It is important to understand the behaviour of *B. cereus* in the dairy environment, since it can grow at a low temperature and play a role in limiting the products' shelf-life and causing potential human health hazards. This study was undertaken to establish its presence in marketed milk and dairy products and during processing, and also to assess potential hazard posed to consumers in the district of Darjeeling. Further, investigation was aimed at optimising existing cleaning-in-places (CIPs) and designing alternative CIP regimes.

Bacillus cereus occurred in six out of eight different dairy products marketed in the district. In case of pasteurised/sterilised milk, 55% of the samples contained *B. cereus* at a level up to 4 log cfu ml^{-1}. In a similar study in Denmark, 47% of the pasteurised milk samples were found to be contaminated by *B. cereus* at a level of 3–5 log cfu ml^{-1} (Larsen and Jørgensen, 1997). In another study in the Netherlands, 35% of the pasteurised milk samples contained *B. cereus* at a level of 1-4 log cfu ml^{-1} (te Giffel *et al.,* 1996). In Poland, Bartoszewicz *et al.* (2008) reported pasteurised milk to contain relatively low counts (1-2 log cfu l^{-1}). The difference in percentage of contaminated samples and level of contamination in different studies may be attributed to the degree of post-pasteurisation contamination and/or storage temperature abuse and seasonal variation in sampling. In case of milk powder, 52% of the samples contained *B. cereus* at a level of 2–3 log cfu g^{-1}. As milk powder

contains an elevated level of carbohydrates and minerals, which can promote *B. cereus* cell proliferation and enterotoxin production when they are reconstituted and held at ambient temperature for extended periods, even low levels of *B. cereus* in milk powder can act as potential vehicles for foodborne diseases (Reyes *et al.*, 2007). In Chile, Reyes *et al.* (2007) found 10% of milk powder samples contaminated with *B. cereus* at a level up to 3 log cfu g^{-1}. The high incidence of *B. cereus* in milk powder samples in the present study is likely due to monospecies biofilm formation on the milk evaporators which can be a source of recurrent contamination of the final product, as stated by Burgess *et al.* (2010). The results were in agreement with a previous report where 60% of the milk powder samples were found contaminated with *B. cereus* (te Giffel *et al.,* 1996). In case of ice cream, 40% of the samples contained *B. cereus* at a level as high as 8 log cfu ml^{-1}. In a similar study made with the samples from retail outlets in Mumbai, India, the organism was prevalent in 40% of the unpackaged samples and 27% of the packaged samples, at a level of 1–3 log cfu ml^{-1} (Warke *et al.,* 2000). The high prevalence and population level of *B. cereus* may be attributed to post-production handling of the products and temperature abuse, which is likely to occur during frozen storage or transportation and unhygienic conditions prevailing during distribution or sale in the ice cream parlours. Thirty-three percent of the cheese samples analysed contained *B. cereus* at a level of 2–6 log cfu g^{-1}, while Molva *et al.* (2009) reported only 12% of Turkish cheese samples to be contaminated with *B. cereus*. This difference might be attributed to type of cheese samples analysed and post-production contamination. In case of butter, 20% of the samples were found to be contaminated with *B. cereus* at a level of 3-4 log cfu g^{-1}. This may be due to the use of contaminated milk or due to biofilm formation by *B. cereus* on centrifugal separators and recycle loops in butter manufacturing plants and subsequent contamination of finished products. In khoa and curd samples analysed, *B. cereus* was not detected. Khoa is a partially desiccated milk which is prepared by condensing milk through regular heating (90–95 ºC) 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 cause for its absence. A high level of *B. cereus* in ice cream and cheese is a matter of great public health concern as its level reported in food poisoning is 2–8 log cfu g^{-1} or ml^{-1} (Beattie and Williams, 2000). This is because a food exceeding 4–5 log cells g^{-1} or ml^{-1} is considered unsafe for consumption (Notermans *et al.,* 1997).

For the growth of *B. cereus* isolates, a large temperature range was observed, indicating a wide diversity and ecotype. The majority (74%) of the strains were able to grow at $\leq$7 ºC. To be considered as psychrotrophic, an organism should be able to grow at 7 ºC or less (te Giffel *et al*., 1995; Francis *et al.,* 1998). Presence of such a large number of psychrotropic strains in dairy environment is of major concern mainly because of their potential for growth, spoilage and toxin production in chilled products, such as milk and dairy products (Anderson Borge *et al.,* 2001). Other studies also showed pasteurised milk and refrigerated food to frequently harbour psychrotrophic strains of *B. cereus* (te Giffel *et al*., 1997; Svensson *et al*., 2004). Thirty-four percent of the *Bacillus cereus* isolates from milk powder were able to grow up to 50 ºC. This may be attributed to adaptation or selection of thermotolerant strains during drying and heating process generally used to make milk powder.

All the *B. cereus* isolates were multi-drug resistant. Each of those was resistant to at least five different antibiotics used. Most of the isolates were resistant to β-lactams (ampicillin, carbenicillin, cephalothin 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 $disc^{-1}$). However, all the 48 isolates of *B. cereus* from legume-based fermented food products were resistant against this higher concentration of polymyxin B (Roy *et al.,* 2007). As expected, all the isolates were resistant to metronidazole. An investigation on antibiotic-resistance profiles of *B. cereus* isolates from different food products in Morocco showed that the isolates were resistant to ampicillin, tetracycline and penicillin, but susceptible to chloramphenicol and erythromycin (Merzougui *et al*., 2014). Thus, emergence of multi-drug resistance among foodborne bacterial pathogens can be a major health concern.

Enzymes, such as protease, lipase and amylase significantly contribute to the reduction of shelf-life of processed milk and dairy 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 produced all the three enzymes. This indicates potentiality of

majority of the isolates for spoilage of dairy products, which in turn can be responsible for the reduction in shelf-life of the products. The high spoilage potential of *B. cereus* isolates is also emphasised by a previous work, analysing spore-formers isolated from dairy processing environments for spoilage-associated enzyme activities, where all the tested strains showed a high proteolytic activity (Lücking *et al*., 2013). In another study on legume-based fermented food products, 50% of the 48 isolates of *B. cereus* were capable of producing one of these enzymes and 23% of producing 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 hydrolyse starch has been indicative of emetic subtype. This indicates a possible high prevalence of emetic subtype in dairy products analysed in the present study.

The protease and lipase produced by the representative isolates of *B. cereus* were thermostable. Thermal stability of these enzymes from *B. cereus* has been reported previously (Chen *et al.,* 2004; Akanbi *et al.,* 2010). The presence of thermostable protease and lipase increases spoilage potentiality of the isolates as these enzymes retain their activity even after heat treatments, such as pasteurisation and spray drying. Thermostable enzymes can withstand milk heat treatments, remain active in dairy products, and can provoke changes in texture up to structural defects and typical off-flavours. Well-known are the 'bitty cream' and 'sweet curdling' defects, caused by the lecithinase and proteolytic activity of *B. cereus*. Lipolysis causes bitter taste of dairy products, making them unacceptable to consumers and lead to a significant economic loss and/or reputational damage of food companies.

Haemolysin is a three-component enterotoxin produced by *B. cereus*, which is one of the potential virulence factors in *B. cereus*-mediated diarrhoea (Beecher *et al.,* 1995). Ninety-three percent of the *B. cereus* isolates exhibited β-haemolysis which was a discontinuous pattern in blood agar. This is as a result of a mutually inhibitory effect of B and L1 components and the slow reaction between the B component and the erythrocyte membrane (Stenfors Arnesen *et al.,* 2008). This is in consistence with the report of β-haemolytic activity exhibited by 92% of the *B. cereus* isolates from food ingredients and products in Brazil (Chaves *et al.,* 2011).

Majority (97%) of the isolates were positive in the Tecra antibody test for the production of diarrhoeal enterotoxin. Results were in consistence with the earlier reports, where 96% of the isolates from various food products and 74% of the isolates from dairy production chain were Nhe positive (Moravek *et al.*, 2006; Svensson *et al.,* 2007). Semi-quantitative production index indicated 94% of the isolates were high producers (index 4-5) of NheA. The prevalence of high producers of Nhe among dairy isolates is of significance as Moravek *et al.* (2006) found cytotoxicity on Vero cells to be dominated by Nhe, indicating a high diarrhoeic potential of the toxin.

Majority (72%) of the isolates were able to form biofilm even at 4 ºC. Biofilm formation by *B. cereus* isolates from dairy origin has been previously reported by Shaheen *et al.* (2010). In another study, 98% of the 56 isolates of *B. cereus* from foods and clinical specimens were able to form biofilm (Wijman *et al.,* 2007). As bacteria within biofilms are more resistant to antimicrobial agents and cleaning, it is more difficult to eliminate biofilm cells than planktonic ones (Faille *et al.,* 2001; Hornstra *et al.,* 2007). Thus, biofilm formed by the cells in dairy processing lines can be responsible for recurrent contamination and spoilage of dairy products or facilitate transmission of diseases.

The principal component (PC) analysis allowed classifying correlated variables (production of exo-enzymes, biofilm and haemolysin) into two types of adversities (spoilage and food poisoning). It is evident that majority of the isolates from cheese, butter, ice cream and a few from milk and milk powder were dominant in the 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 haemolysin. Many milk isolates were predominant in the PC1 and PC2 negative sides, and thus characterised by low production of enzymes and biofilm. Agglomerative hierarchical clustering (AHC) resulted in four heterogeneous clusters.

In the study for prevalence of *B. cereus* along the dairy processing line, 35% of raw milk samples collected from silos were found contaminated with *B. cereus*. The prevalence of positive samples increased up to 40% during the processing of pasteurised milk. In a similar study, 35% of the raw milk samples and 70% of the pasteurised milk samples were found contaminated with *B. cereus* when samples were collected from dairy

processing plant (te Giffel *et al*., 1996). The one-day in-depth sampling study indicated that contamination of milk by *B. cereus* may occur in dairy plants. *Bacillus cereus* was also isolated from surfaces of the pasteurised milk storage chilling tanks. Thus, it seems likely that the source of contamination for pasteurised milk was present in the production line, possibly in the pasteurised milk storage tank. The presence, adhesion and biofilm formation of *B. cereus* strains on stainless steel surface of dairy tanks may represent a mechanism for survival and dispersal of spores with rinse water from one location to another (Shaheen *et al.*, 2010).

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

Presence of *B. cereus* biofilm in dairy processing line, chilling tanks in particular, can be a source of post-pasteurisation contamination. An *in vitro* model was designed to study biofilm formation by *B. cereus* PT4, isolated from a chilling tank where pasteurised milk was stored. The selected strain was able to form biofilm even at 4 °C. The study on different simulated conditions indicated that the *B. cereus* cell count in the biofilm developed on the surface of stainless steel chilling tanks could reach up to 6 log cfu cm^{-2}, if inadequately cleaned tanker was left to stand empty at room temperature. In a study simulating the surface of a raw milk tanker, bacterial cells found in the biofilm reached up to 8 log cfu cm^{-2} (Teh *et al.,* 2012). This is a matter of concern, as the presence of biofilm on internal surfaces of chilling tanks can lead to sporulation within biofilm. As environmental conditions in biofilm affect sporulation and heat resistance, these spores pose quality issues and safety risk either by directly contaminating food through contact or germinate on the surfaces of equipment to form new biofilms (Faille *et al.*, 2014; Hayrapetyan *et al.*, 2016). Enzymes produced in the biofilms on the internal surfaces of chilling tanks can be responsible for spoilage of milk (Teh *et al.,* 2012, 2014).

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

The spores of *B. cereus* possess a pronounced ability to adhere to stainless steel surface, a common food processing material (Peng *et al*., 2002). *Bacillus cereus* biofilm can be a recurrent source of food spoilage and outbreaks of food poisoning. Thus, biofilms have been a major food safety concern for dairy industry. The technique used for biofilm cell removal in dairy industry mainly consists of CIP regimes which commonly consist of alkali and acid washes (Chisti, 1999). Effectiveness of CIP regimes against *B. cereus* biofilm is not reported extensively. Peng *et al.* (2002) reported requirement of a long-hot CIP for an effective removal of *B. cereus* biofilm. Efficacy of CIP regimes greatly depends on exposure time, temperature and concentration of cleaning agent (Bremer *et al*., 2002; Parkar *et al*., 2004). Thus, for designing an effective CIP, optimisation of these parameters is essential. The caustic step in CIP regimes is believed to be predominantly responsible for biofilm removal (Chisti, 1999). So, use of response surface methodology (RSM) to optimise parameters influencing biofilm cell removal during caustic step is of significance. All the variables used in the present study significantly contributed to biofilm removal, and an interaction between time and temperature was the main influencing factor. A linear increase in biofilm removal was observed when exposure time and temperature were increased. Maximum removal was predicted when the biofilm was exposed to 15 g NaOH l^{-1} for 30 min at 65 °C, where 2.55 log reduction cm^{-2} in biofilm cell count was achieved. Thus, RSM was successfully deployed to obtain conditions which influenced efficacy of the caustic step. The RSM results were used to design an optimised CIP regime which consisted of 15 g NaOH l^{-1} for 30 min at 65 °C - water rinse – 10 ml HNO_3 l^{-1} for 10 min at 65 °C- water rinse. Effectiveness of reference CIP (10 g NaOH l^{-1} for 10 min at 65 °C- water rinse – 10 ml HNO_3 l^{-1} for 10 min at 65 °C - water rinse) was compared with that of optimised CIP against 24 h-old biofilm. It was found that the reference CIP achieved 3.29 log reduction cm^{-2} in the number of *B. cereus* cells recovered, as compared to control. This result was in consistence with the findings of Bremer *et al.* (2006) who reported 2 log reduction cm^{-2} in biofilm cells after reference CIP regime. Difference in the reduction of cells recovered may be attributed to the fact that Bremer *et al.* (2006) used biofilm formed by consortium. The optimised CIP achieved 4.77 log reduction of biofilm cells cm^{-2}. From crystal violet staining of coupons it was evident that not only biofilm cells were inactivated by optimised CIP, but biofilm matrix also got removed from coupons. Thus, the optimised CIP in the present study effectively removed biofilm. The concentration of NaOH used was within

the permissible limit and usually used in dairy industry for CIP; 10-50 g NaOH l^{-1} is used for plate-type heat exchangers and 10-20 g l^{-1} for general cleaning (Flint *et al.,* 1997). An effective CIP regime should achieve maximum removal of biofilm cells within the shortest possible time and at a low temperature. The optimised CIP regime achieved a significant increase in log reduction of biofilm cells (4.77 cm^{-2}). Parker *et al.* (2004) reported the use of 20 g NaOH l^{-1}, 18 ml HNO_3 and a temperature of 75 °C for the removal of *Bacillus flavothermus* biofilm. The optimised CIP achieved a significant reduction in biofilm cells at lower concentrations of NaOH (15 g l^{-1}) and HNO_3 (10 ml l^{-1}), and at a lower temperature (65 °C). As evaluation of biofilm status and development of an effective CIP regime is part of HACCP plan development and ISO:9000 specifications for food processing industry to make them more meaningful (Sharma and Anand, 2002), the optimised CIP regime established can be an effective tool for *B. cereus* biofilm cell removal.

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

Alkali and acid treatments, practiced in dairy industry to achieve standard CIPs, are not always sufficient for removing biofilms (Antoniou and Frank, 2005). So, an effective alternative can be a use of enzymes to breakdown extracellular polymeric substances (EPS) network and remove biofilms (de Carvalho, 2007). Since proteases were more efficient in removing cells of *B. cereus* biofilms than polysaccharidases (Lequette *et al.,* 2010), RSM was deployed to study the influence of individual factors and their interaction on *B. cereus* biofilm removal using protease only. Biofilm removal increased with the increase in pH, indicating pH had a major role in biofilm removal. This was substantiated by *ANOVA* which showed that pH significantly (P <0.05) affected biofilm removal. EPS is insoluble at an acidic pH and responsible for increase in compactness of biofilm, making it more resistant to cleaning (Dogsa *et al.,* 2005). Alkaline pH reduces biofilm cohesiveness and facilitates removal of biofilm by increasing solubility of EPS and inducing swelling of EPS network (Lequette *et al.*, 2010).

Since biofilm formation and removal are greatly influenced by the physicochemical properties of the attachment surface (Donlan, 2002), the results of the microtiter plate assay were compared with those of the biofilms developed on stainless steel coupons in skim milk which mimics the actual environment in dairy industry. The type of assay used to study biofilm formation is of great importance, since *B. cereus* shows a preference to form biofilms at an air-liquid interface (Wijman *et al*., 2007). Using submerged assays might lead to an underestimation of the possible number of biofilm cells in a system. Factors contributing to the formation of biofilms at the air-liquid interface may involve oxygen availability at the surface, causing aerotaxis of *B. cereus* towards oxygen (Laszlo *et al.*, 1984). Thus, biofilm formation was carried out by placing coupons at an air-liquid interface. The biofilms developed on coupons were more resistant than those in microtiter plates. This may be attributed to the fact that stainless steel provides more favourable conditions for *B. cereus* biofilm formation and maturation compared to polystyrene (Hayrapetyan *et al*., 2015).

In dairy industry, an effective CIP is essential as it greatly affects the final product quality (Bremer *et al.,* 2006). Since conventional CIP using chemical agents do not provide satisfactory hygienic results, enzymic control of biofilms would present a prospective alternative (Lequette *et al.,* 2010). The RSM results were used to design an 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). The optimised protease CIP was able to completely remove *B. cereus* biofilm cells from coupons, while non-optimised protease treatment (pH 10-45 °C - 30 min) caused a reduction of 0.92 log *B. cereus* BC98/4 cells cm^{-2} in biofilm (Lequette *et al.*, 2010). The optimised protease CIP not only removed biofilm cells completely, but also removed biofilm matrix significantly (P <0.05), as compared to both reference and optimised alkali CIPs. NaOH could not remove biofilm matrix so effectively. Cleaning regime should break-up or dissolve the EPS matrix associated with biofilm, so that disinfectants can gain access to bacterial cells (Simões *et al.* 2006). Compared to other CIPs, the optimised protease CIP had an added advantage of a significantly reduced (near neutral) pH level. Thus, a significant benefit could be achieved by replacing caustic-based cleaning solutions with enzymes. This is because, in contrast to concentrated NaOH and other caustic detergents, enzymes are non-corrosive and their use leads to reduced rinsing volumes and easier disposal without neutralisation (Boyce *et al.,* 2010). To apply these results, further industrial-scale studies and economic feasibility are warranted.

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

Incidence and level of *B. cereus* contamination in milk have been reported by various researchers and those have been found to be associated with different foodborne outbreaks (Boxall and Ortega, 2003; EFSA, 2005). Thus, the presence of *B. cereus* in pasteurised milk is regarded as a potential microbial hazard. Milk and dairy products are purchased from retail outlets and subsequently stored under different conditions prior to consumption. So, a risk assessment study at consumer level is of paramount importance.

Thirty percent of the pasteurised milk samples in 2–4 h-old stored packages from 50 household refrigerators in the present study were found to be contaminated with *B. cereus*, and the level of contamination was 3–5 log cfu ml^{-1}. The presence of such a high prevalence of *B. cereus* in pasteurised milk is a matter of concern as milk is usually stored in households for more than two days. The critical limit for *B. cereus* is 4 log cfu ml^{-1} (Notermans *et al.,* 1998), since the infective dose of *B. cereus* to cause foodborne illnesses is 5–8 log cfu g^{-1} or ml^{-1} (Notermans *et al.,* 1997; Granum and Baird-Parker, 2000). However, in the context of a dose–response model, this level cannot be considered as threshold for illness. It is used as an alternative since the development of dose–response models for toxigenic spore-forming microorganisms is complex.

The results of 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}. In a study in the Netherlands, 40% of the pasteurised milk samples stored in the household refrigerators were found contaminated with *B. cereus* (te Giffel *et al.,* 1997). Storage temperature in the refrigerators ranged from 3.54 °C to 12.84 °C with the mean of 8.2 °C, having probability of only 5% refrigerators operating at 5 °C. In an investigation in Greece, 25% of the 136 domestic refrigerators and 13.6% of the 228 supermarket refrigerators were found to be operating at temperatures higher than 10 °C (Sergelidis *et al.,* 1997). In a survey in Sweden, on the top shelf, the mean temperature was found to exceed 8 °C in more than 37% of the cases and on the middle shelf, it exceeded 8 °C in 11% of cases. Almost 33% of the bottom shelves tested had a temperature higher than 8 °C (Marklinder *et al.,* 2015). Results indicate that there are chances that milk and dairy products are being stored at higher temperatures; this may be due to over stacking of refrigerators, preventing proper circulation of chilled air.

Only 1% of the stored milks was found to be used within the day of purchase, while 45% within 2 days and to the maximum within 3 days of purchase. In a similar study in Slovakia, the storage time in domestic refrigerators was reported to vary from 1 day to 11 days, with the mean of 3.11 days (Acai *et al.,* 2014).

Quantitative exposure assessment provides numerical estimates of exposure and requires development of mathematical models in which relationship between factors affecting exposure can be studied (FAO/WHO, 1995). 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 cell population in milk. The model predicts *B. cereus* population will reach the threshold level (>4 log cfu ml^{-1}) after 47.5 h, 45.5 h, 41.6 h, 35.3 h, 25.3 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}. Thus if milk is stored for more than 24 h in refrigerators, the chance of consumers being exposed to *B. cereus* more than the threshold level is likely to occur which can lead to foodborne illness. Results showed that for safer pasteurised milk consumption, a lower initial load from industry part and a better temperature control and sanitation of domestic refrigerators can be effective measures to control *B. cereus*-associated hazard.

From the distribution study it is evident that there are chances that 50% of the consumers may be exposed to a high level (4.5 log cfu ml^{-1}) of *B. cereus* cells if milk is stored for more than two days. Actual scenario can be even worse as the risk associated with per capita intake of 322 g (NDDB, 2015). A similar study in the Netherlands reported 11% of portions of the milk consumed contained >4 log *B. cereus* cells ml^{-1} (Notermans *et al.,* 1997). Another study in Slovakia reported 14% of pasteurised milk to contain *B. cereus* at a level of >4 log cfu ml^{-1} (Acai *et al.,* 2014). Thus, *B. cereus* was identified as a microbiological risk in pasteurised milk presently stored in domestic refrigerators. The temperature control of refrigerators is important to prevent the growth of bacteria. To maintain a low refrigerator temperature, one should leave enough space in the refrigerator to allow the cool air to circulate. In addition, consumers should maintain proper hygienic

conditions to prevent cross contamination in the refrigerator. This study can be a valuable tool for risk management with a comprehensive picture of the key factors in the system of interest. In addition to an initial low spore count, cooling after pasteurisation and limited exposure to storage time-temperature, as set by such predictive and probabilistic modellings, will help to control the growth of this pathogen below the critical limit.

Thus, to limit the presence of *B. cereus* in dairy processing environment, there is need to gain a better insight into the whole contamination flow of endospore-formers originating from soil as well as in the conditions permitting their proliferation. Good manufacturing practices (GMPs) in farms during the production and storage of milk should be implemented and strategies aiming at reducing the population of spore-forming bacteria in raw milk should be reinforced. Better implementation of HACCP in dairy processing lines should be given importance so that the initial load in finished products could be minimised. CCPs, such as storage temperature and time, should be properly defined. More research on better understanding of the structure of *B. cereus* biofilms in the context of milk processing environment is needed to develop better CIP regimes for eliminating biofilm from dairy processing lines. Furthermore, optimisation of the existing cleaning processes and development of novel and effective strategies are of great importance to the dairy industry, as these may lead to quality improvements of products and processes. Future research could also focus on coating strategies to reduce microbial attachment on dairy equipment and on food grade quorum inhibitors as an intervention strategy which can offer new opportunities for the dairy industry in the coming years.

6

Bibliography

Acai, P., Valík, L. and Liptáková, D. (2014) Quantitative risk assessment of *Bacillus cereus* in pasteurised milk produced in the Slovak Republic. *Czech Journal of Food Sciences* 32, 122–131

Adak, G.K., Long, S.M. and O'Brien, S.J. (2002) Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut* 51, 832–841

Agata, N., Ohta, M. and Mori, M. (1996) Production of an emetic toxin, cereulide, is associated with a specific class of *Bacillus cereus*. *Current Microbiology* 33, 67–69

Agata, N., Ohta, M., Mori, M. and Isobe, M. (1995) A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiology Letters* 129, 17–19

Akanbi, T.O., Kamaruzaman, A.L., Abu Bakar, F., Sheikh Abdul Hamid, N., Radu, S., Abdul Manap, M.Y. and Saari, N. (2010) Highly thermostable extracellular lipase-producing *Bacillus* strain isolated from a Malaysian hotspring and identified using 16S rRNA gene sequencing. *International Food Research Journal* 17, 45–53

Anderson Borge, G.I., Skeie, M., Sørhaug, T., Langsrud, T. and Granum, P.E. (2001) Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. *International Journal of Food Microbiology* 69, 237–246

Andersson, A., Rönner, U. and Granum, P.E. (1995) What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *International Journal of Food Microbiology* 28, 145–155

Ankolekar, C. and Lebbé, R.G. (2010) Physical characteristics of spores of food-associated isolates of the *Bacillus cereus* group. *Applied and Environmental Microbiology* 76, 982-984

Antoniou, K. and Frank, J.F. (2005) Removal of *Pseudomonas putida* biofilm and associated extracellular polymeric substances from stainless steel by alkali cleaning. *Journal of Food Protection* 68, 277–281

ANZDAC (2011) *Pathogen Management Guidelines*. Australia-New Zealand Dairy Authorities' Committee, 33 p.

Aronson, A.I. and Shai, Y. (2001) Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiology Letters* 195, 1–8

Ash, C., Farrow, J.A., Dorsch, M., Stackebrandt, E. and Collins, M.D. (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *International Journal of Systematic Bacteriology* 41, 343–346

Banerjee, M., Nair, G.B. and Ramamurthy, T. (2011) Phenotypic & genetic characterization of *Bacillus cereus* isolated from the acute diarrhoeal patients. *Indian Journal of Medical Research* 133, 88–95

Banerjee, M. and Sarkar, P.K. (2004) Antibiotic resistance and susceptibility to some food preservative measures of spoilage and pathogenic micro-organisms from spices. *Food Microbiology* 21, 335–342

Bartoszewicz, M., Hansen, B.M. and Swiecicka, I. (2008) The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. *Food Microbiology* 25, 588–596

Beattie, S.H. and Williams, A.G. (2000) Detection of toxins. In: Robinson, R.K., Batt, C.A. and Patel, P.D. (Eds), *Encyclopedia of Food Microbiology*, pp. 141–158. Academic Press, San Diego, CA, USA

Bedi, S.K., Sharma, C.S., Gill, J.P.S., Aulakh, R.S. and Sharma, J.K. (2005) Incidence of enterotoxigenic *Bacillus cereus* in milk and milk products. *Journal of Food Science and Technology* 42, 272–275

Beecher, D.J. and Macmillan, J.D. (1991) Characterization of the components of hemolysin BL from *Bacillus cereus. Infection and Immunity* 59, 1778–1784

Beecher, D.J., Schoeni, J.L. and Wong, A.C. (1995) Enterotoxic activity of hemolysin BL from *Bacillus cereus*. *Infection and Immunity* 63, 4423–4428

Beecher, D.J. and Wong, A.C.L. (1997) Tripartite hemolysin BL from *Bacillus cereus*: hemolytic analysis of component interactions and a model for its characteristic paradoxical zone phenomenon. *Journal of Biological Chemistry* 272, 233–239

Bhatnagar, P., Khan, A.A., Jain, M., Kaushik, S. and Jain, S.K. (2007) Microbiological study of khoa sold in Chambal region (Madhya Pradesh): a case study. *Indian Journal of Microbiology* 47, 263–266

Boxall, N. and Ortega, J. (2003) Annual summary of outbreaks in New Zealand in 2002. *ESR Clint Report FW0151*. Institute of Environmental Science and Research Ltd, Porirua, New Zealand

Boyce, A., Piterina, A.V. and Walsh, G. (2010) Assessment of the potential suitability of selected commercially available enzymes for cleaning-in-place (CIP) in the dairy industry. *Biofouling* 26, 837–850

Bremer, P.J., Fillery, S. and McQuillan, A.J. (2006) Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology* 106, 254–262

Bremer, P.J., Monk, I., Butler, R., (2002) Inactivation of *Listeria monocytogenes/Flavobacterium* spp. biofilms using chlorine: impact of substrate, pH, time and concentration. *Letters in Applied Microbiology* 35, 321– 325

Breyers, J.D. and Ratner, B.D. (2004) Bioinspired implant materials befuddle bacteria. *ASM News* 70, 232–237

Brooks, J.D. and Flint, S.H. (2008) Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science and Technology* 43, 2163–2176

Burgess, S.A., Lindsay, D. and Flint, S.H. (2010) Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology* 144, 215–225

Caro-Astorga, J., Pérez-García, A., de Vicente, A. and Romero, D. (2015) A genomic region involved in the formation of adhesion fibers in *Bacillus cereus* biofilms. *Frontiers in Microbiology* 5, 745

Chaves, J.Q., Pires, E.S. and Vivoni, A.M. (2011) Genetic diversity, antimicrobial resistance and toxigenic profiles of *Bacillus cereus* isolated from food in Brazil over three decades. *International Journal of Food Microbiology* 147, 12–16

Chen, L., Coolbear, T. and Daniel, R.M. (2004) Characteristics of proteinases and lipases produced by seven *Bacillus* sp. isolated from milk powder production lines. *International Dairy Journal* 14, 495–504

Chen, L., Daniel, R.M. and Coolbear, T. (2003) Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal* 13, 255–275

Chisti, Y. (1999) Modern systems of plant cleaning. In: Robinson, R., Batt, C. and Patel, P. (Eds.), *Encyclopedia of Food Microbiology*, pp. 1086-1815. Academic Press, London, UK

Chitov, T., Dispan, R. and Kasinrerk, W. (2008) Incidence and diarrhegenic potential of *Bacillus cereus* in pasteurized milk and cereal products in Thailand. *Journal of Food Safety* 28, 467–481

Chmielewski, R.A.N. and Frank, J.F. (2003) Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety* 2, 22–32

Christiansson, A., Bertilsson, J. and Svensson, B. (1999) *Bacillus cereus* spores in raw milk: factors affecting the contamination of milk during the grazing period. *Journal of Diary Science* 82, 305–314

Christiansson, A., Ekelund, K. and Ogura, H. (1997) Membrane filtration method for enumeration and isolation of spores of *Bacillus cereus* from milk. *International Dairy Journal* 7, 743–748

Claus, D. and Berkeley, R.C.W. (1986) Genus *Bacillus* Cohn (1872), 174. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (Eds), *Bergey's Manual of Systematic Bacteriology*, vol. II, pp. 1105-1139. Williams and Wilkins, Baltimore, MD, USA

CSPI (2014) *A review of foodborne illness in America from 2002-2011*. Center for Science in the Public Interest, Washington, DC, USA

Datta, N. and Deeth, H.C. (2003) Diagnosing the cause of proteolysis in UHT milk. *LWT – Food Science and Technology* 36, 173–182

Davey, M.E. and O'Toole, G.A. (2000) Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* 64, 847–867

de Carvalho, C.C.C.R. (2007) Biofilms: recent developments on an old battle. *Recent Patents Biotechnology* 1, 49–57

Dierick, K., van Coillie, E., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M. and Mahillon, J. (2005) Fatal family outbreak of *Bacillus cereus*-associated food poisoning. *Journal of Clinical Microbiology* 43, 4277–4279

Dogsa, I., Kriechbaum, M., Stopar, D. and Laggner, P. (2005) Structure of bacterial extracellular polymeric substances at different pH values as determined by SAXS. *Biophysical Journal* 89, 2711–2720

Donlan, R.M. (2002) Biofilms: microbial life on surfaces. *Emerging Infectious Diseases* 8, 881–890

Drobniewski, F.A. (1993) *Bacillus cereus* and related species. *Clinical Microbiology Reviews* 6, 324–338

Dufour, M., Simmonds, R.S. and Bremer, P.J. (2004) Development of a laboratory scale clean-in-place system to test the effectiveness of "natural" antimicrobials against dairy biofilms. *Journal of Food Protection* 67, 1438–1443

EFSA (2005) Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp in foodstuffs. *EFSA Journal* 175, 1–48

Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M. and Scherer, S. (2006) Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. *BMC Microbiology* 6, 20

Ehling-Schulz, M., Fricker, M. and Scherer, S. (2004) *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. *Molecular Nutrition & Food Research* 48, 479–487

Ehling-Schulz, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Märtlbauer, E., Nguyen-The, C. Salkinoja-Salonen, M. and Scherer, S. (2005) Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Applied and Environmental Microbiology* 71, 105–113

Eneroth, Å., Svensson, B., Molin, G. and Christiansson, A. (2001) Contamination of pasteurized milk by *Bacillus cereus* in the filling machine. *Journal of Dairy Research* 68, 189–196

EU (2005) Commission Regulation (EC) No.2076/2005 of 5 December 2005 laying down transitional arrangements for the implementation of Regulations (EC) No. 853/2004, (EC) No. 854/2004 and (EC) No.853/2004 and (EC) No.854/2004. *Official Journal of the European Commission* L338, 83-88

Fagerlund, A., Ween, O., Lund, T., Hardy, S.P. and Granum, P.E. (2004) Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. *Microbiology* 150, 2689–2697

Faille, C., Bénézech, T., Midelet-Bourdin, G., Lequette, Y., Clarisse, M., Ronse, G., Ronse, A. and Slomianny, C. (2014) Sproulation of *Bacillus* spp. within biofilms: a potential source of contamination in food processing environments. *Food Microbiology* 40, 64-74

Faille, C., Fontaine, F. and Bénézech, T. (2001) Potential occurrence of adhering living *Bacillus* spores in milk product processing lines. *Journal of Applied Microbiology* 90, 892–900

FAO/WHO (1995) Application of risk analysis to food standards issues. Report of the Joint FAO/WHO Expert Consultation, pp. 13–17. World Health Organization, Geneva, Switzerland

Fernandes, R., Ed. (2009) *Microbiology Handbook - Dairy Products.* Leatherhead Publishing, Leatherhead, UK & Royal Society of Chemistry, Cambridge, UK

Flint, S.H., Bremer, P.J. and Brooks, J.D. (1997) Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling* 11, 81–97

Francis, K.P., Mayr, R., von Stetten, F., Stewart, G.S.A.B. and Scherer, S. (1998) Discrimination of psychrotrophic and mesophilic strains of the *Bacillus cereus* group by PCR targeting of major cold shock protein genes. *Applied and Environmental Microbiology* 64, 3525-3529

Furtado, M.M. (2005) *Main problems of cheeses: causes and prevention*. Fonte Press, São Paulo, Brazil

Ghelardi, E., Celandroni, F., Salvetti, S., Barsotti, C., Baggiani, A. and Senesi, S. (2002) Identification and charaterization of toxigenic *Bacillus cereus* isolates responsible for two food-poisoning outbreaks. *FEMS Microbiology Letters* 208, 129–134

Ghosh, S. and Setlow, P. (2009) Isolation and characterization of superdormant spores of *Bacillus* species. *Journal of Bacteriology* 191, 1787–1797

Granum, P.E. and Baird-Parker, T.C. (2000) *Bacillus* species. In: Lund, B.M., Baird-Parker, T.C. and Gould, G.W. (Eds), *The Microbiological Safety and Quality of Food,* pp. 1029–1056. Aspen Publishers, New York, NY, USA

Granum, P.E. and Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters 157*, 223–228

Granum, P.E., Stenfors Arnesen, L. and From, C. (2014) Bacteria: *Bacillus cereus* and other pathogenic *Bacillus* species. In: Motarjemi, Y. (Ed.), *Encyclopedia of Food safety*, vol. 1. *History, Science and Methods*, pp. 356-363. Elsevier, Amsterdam, The Netherlands

Guinebretière, M.H., Auger, S., Galleron, N., Contzen, M., de Sarrau, B., de Buyser, M.L., Lamberet, G., Fagerlund, A., Granum, P.E., Lereclus, D., de Vos, P., Nguyen-The, C. and Sorokin, A. (2013) *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. *International Journal of Systematic and Evolutionary Microbiology* 63, 31–40

Guinebretière, M.H., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M. and De Vos, P. (2008) Ecological diversification in the *Bacillus cereus* group. *Environmental Microbiology* 10, 851–865

Guinebretière, M.H., Velge, P., Couvert, O., Carlin, F., Debuyser, M. and Nguyen-The, C. (2010) The food poisoning power of *Bacillus cereus* group strains varies 1 according to phylogenetic affiliation (groups I-VII), not to species affiliation. *Journal of Clinical Microbiology* 48, 3388–3391

Gupta, N., Rathi, P. and Gupta, R. (2002) Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Analytical Biochemistry* 31, 98–99

Harvey, J., Keenan, K.P. and Gilmour, A. (2007) Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiology* 24, 380–392

Hassan, G.M., Al-Ashmawy, M.A.M., Meshref, A.M.S. and Afify, S.I. (2010). Studies on enterotoxigenic *Bacillus cereus* in raw milk and some dairy products. *Journal of Food Safety* 30, 569–583

Hayrapetyan, H., Abee, T. and Nierop Groot, M.N. (2016) Sporulation dynamics and spore heat resistance in wet and dry biofilms of *Bacillus cereus*. *Food Control* 60, 493-499

Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T. and Nierop Groot, M.N. (2015) Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. *International Journal of Food Microbiology* 200, 72-79

Helgason, E., Caugant, D.A., Olsen, I. and Kolstø A.B. (2000). Genetic structure of population of *Bacillus cereus* and *Bacillus thuringiensis* isolates associated with periodontitis and other human infections. *Journal of Clinical Microbiology* 38, 1615–1622

Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I. and Kolstø, A.B. (2000) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Applied and Environmental Microbiology* 66, 2627-2630

Heyndrickx, M. (2011) The importance of endospore-forming bacteria originating from soil for contamination of industrial food processing. *Applied and Environmental Soil Science* 2011, Article ID 561975, 11 pages

HiMedia (1998) *The HiMedia Manual for Microbiology Laboratory Practices*. HiMedia Laboratories Pvt Limited, Mumbai, India

Hornstra, L.M., De Leeuw, P.L.A., Moezelaar, R., Wolbert, E.J., De Vries, Y.P., De Vos, W.M. and Abee, T. (2007) Germination of *Bacillus cereus* spores adhered to stainless steel. *International Journal of Food Microbiology* 116, 367–371

Hsueh, Y.H. Somers, E.B., Lereclus, D. and Wong, A.C.L. (2006) Biofilm formation by *Bacillus cereus* is influenced by PlcR, a pleiotropic regulator. *Applied and Environmental Microbiology* 72, 5089–5092

Hussain, S.A., Munshi, Z.H. and Hussain, I. (2007). Food poisoning due to *Bacillus cereus*: a case report. *Journal of Veterinary Public Health* 5, 57–59

Janštová, B., Draèková, M. and Vorlová, L. (2006) Effect of *Bacillus cereus* enzymes on milk quality following ultra high temperature processing. *Acta Veterinaria Brno* 75, 601–609

Janštová, B., Lukášová, J., Draèková, M. and Vorlová, L. (2004) Influence of *Bacillus* spp. enzymes on ultra high temperature-treated milk proteins. *Acta Veterinaria Brno* 73, 393–400

Jensen, G.B., Hansen, B.M., Ellenberg, J. and Mahillon, J. (2003) The hidden lifestyles of *Bacillus cereus* and relatives. *Environmental Microbiology* 5, 631–640

Kim, K.Y. and Frank, J.F. (1995) Effect of nutrients on biofilm formation by *Listeria monocytogenes* on stainless steel. *Journal of Food Protection* 58, 24–28

Klavenes, A., Stalheim, T., Sjøvold, O., Josefsen, K. and Granum, P.E. (2002) Attachment of *Bacillus cereus* spores with and without appendages to stainless steel surfaces. *Food and Bioproducts Processing* 80, 312-318

Kramer, J.M. and Gilbert, R.J. (1989) *Bacillus cereus* and other *Bacillus* species. Ch. 2. In: Doyle, M.P. (Ed.), *Foodborne Bacterial Pathogens* , pp. 21–70. Marcel Dekker, New York, NY, USA

Larsen, H.D. and Jørgensen, K. (1997) The occurrence of *Bacillus cereus* in Danish pasteurized milk. *International Journal of Food Microbiology* 34, 179–186

Laszlo, D.J., Niwano, M., Goral, W.W. and Taylor, B.L. (1984) *Bacillus cereus* electron transport and proton motive force during aerotaxis. *Journal of Bacteriology* 159, 820–824

Lechner, S., Mayr, R., Francis, K.P., Prüss, B.M., Kaplan, T., Wießner-Gunkel, E., Stewart, G.*S*.A.B. and Scherer, S. (1998). *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. *International Journal of Systematic Bacteriology* 48, 1373–1382

Lee, D., Koh, Y., Kim, K., Kim, B., Choi, H., Kim, D., Suhartono, M.T. and Pyun, Y. (1999) Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiology Letters* 179, 393–400

Lemos, M., Gomes, I., Mergulhão, F., Melo, L. and Simões, M. (2015) The effects of surface type on the removal of *Bacillus cereus* and *Pseudomonas fluorescens* single and dual species biofilms. *Food and Bioproducts Processing* 93, 234–241

Lequette, Y., Boels, G., Clarisse, M. and Faille, C. (2010) Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. *Biofouling* 26, 421–431

Lequette, Y., Garénaux, E., Tauveron, G., Dumez, S., Perchat, S., Slomianny, C., Lereclus, D., Guérardel, Y. and Faille, C. (2011) Role played by exosporium glycoproteins in the surface properties of *Bacillus cereus* spores and in their adhesion to stainless steel. *Applied and Environmental Microbiology* 77, 4905–4911

Lindbäck, T., Hardy, S.P., Dietrich, R., Sødring, M. Didier, A., Morvavek, M., Fagerlund, A., Bock, S. Nielsen, C. Casteel, M., Granum, P.E. and Märtlbauer E. (2010) Cytotoxicity of the *Bacillus cereus* Nhe enterotoxin requires specific binding order of its three exoprotein components. *Infection and Immunity* 78, 3813-3821

Lindsay, D., Brözel, V.S. and von Holy, A. (2006) Biofilm-spore response in *Bacillus cereus* and *Bacillus subtilis* during nutrient limitation. *Journal of Food Protection* 69, 1168–1172

Lücking, G., Stoeckel, M., Atamer, Z., Hinrichs, J. and Ehling-Schulz, M. (2013) Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. *International Journal of Food Microbiol*ogy 166*,* 270–279

Lund, T., de Buyser, M.L. and Granum, P.E. (2000) A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Microbiology* 38, 254–261

Lund, T. and Granum, P.E. (1996) Characterisation of a nonhaemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiology Letters* 141, 151–156

Marklinder, I. and Erikkson , M.K. (2015) "Best-before date – food storage temperatures recorded by Swedish students". *British Food Journal* 117, 1764–1776

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999) Food-related illness and death in the United States. *Emerging Infectious Diseases* 5, 607-625

Merzougui, S., Lkhider, M., Grosset, N., Gautier, M. and Cohen, N. (2014) Prevalence, PFGE typing, and antibiotic resistance of *Bacillus cereus* group isolated from food in Morocco. *Foodborne Pathogens and Disease* 11, 145-149

Meyer, B. (2003) Approaches to prevention, removal and killing of biofilms. *International Biodeterioration & Biodegradation* 51, 249–253

Mittelman, M.W. (1998) Structure and functional characteristics of bacterial biofilms in fluid processing operations. *Journal of Dairy Science* 8, 2760–2764

Mock, M. and Fouet, A. (2001) Anthrax. *Annual Review of Microbiology* 55, 647–671

Molva, C., Sudagidan, M. and Okuklu, B. (2009) Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey. *Food Control* 20, 829–834

Moravek, M., Dietrich, R., Buerk, C., Broussolle, V., Guinebretière, M.H., Granum, P.E., Nguyen-The, C. and Märtlbauer, E. (2006) Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. *FEMS Microbiology Letters* 257, 293-298

Muir, D.D. (1996) The shelf-life of dairy products: 1. Factors influencing raw milk and fresh products. *International Journal of Dairy Technology* 49, 24–32

Murugan, B. and Villi, R.A. (2009) Lipolitic activity of *Bacillus* species isolated from milk and dairy products. *Indian Veterinary Journal* 86, 80–81

Nabrdalik, M., Grata, K. and Latala, A. (2010). Proteolytic activity of *Bacillus cereus* strains. *Proceedings of ECO-pole* 4, 273-276

Nakamura, L.K. (1998) *Bacillus pseudomycoides* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 48, 1031-1035

NDDB (2012) Presentation at the International Dairy Federation Summit, November 6, 2012 in Cape Town, South Africa. National Dairy Development Board, Anand, Gujarat, India

NDDB (2015) Annual report 2014–2015. National Dairy Development Board, Anand, Gujarat, India

Norris, J.R., Berkeley, R.C.W., Logan, N.A. and O'donnel, A.G. (1981) The genus *Bacillus* and *Sporolactobacillus*. In: Starr, M.P., Stolp, H., Trüper, H.G., Ballows, A. and Schlegel, H.G. (Eds), *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*, pp. 1711-1742. Springer-Verlag, New York, NY, USA

Notermans, S., Dufrenne, J., Teunis, P., Beumer, R., te Giffel, M. and Weem, P.P. (1997) A risk assessment study of *Bacillus cereus* present in pasteurised milk. *Food Microbiology* 14, 143–151

Notermans, S., Nauta, M.J., Jansen, J., Jouve, J.L. and Mead, G.C. (1998) A risk assessment approach to evaluating food safety based on product surveillance. *Food Control* 9, 217–223

NSW Food Authority (2013) Details - Register of offences (prosecutions). http://www.foodauthority.nsw.gov.au/news/offences/prosecutions/offences-details-tablesrestaurant/. Accessed 25 January 2013

Organji, R.S., Abulreesh, H.H., Elbanna, K., Osman, G.E.H. and Khider, M. (2015) Occurrence and characterization of toxigenic *Bacillus cereus* in food and infant feces. *Asian Pacific Journal of Tropical Biomedicine* 5, 515–520

O'Toole, G., Kaplan, H.B. and Kolter, R. (2000) Biofilm formation as microbial development. *Annual Review of Microbiology* 54, 49–79

Pagedar, A. and Singh, J. (2012) Influence of physiological cell stages on biofilm formation by *Bacillus cereus* of dairy origin. *International Dairy Journal* 23, 30-35

Parkar, S.G., Flint, S.H. and Brooks, J.D. (2004) Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *Journal of Applied Microbiology 96*, 110–116

Patel, R., Dodia, M. and Singh, S.P. (2005) Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: production and optimization. *Process Biochemistry 40*, 3569–3575

Pechook, S., Sudakov, K., Polishchuk, I., Ostrov, I., Zakin, V., Pokroy, B. and Shemesh, M. (2015) Bioinspired passive anti-biofouling surfaces preventing biofilm formation. *Journal of Materials Chemistry B* 3, 1371–1378

Peña, W.E.L., de Andrade, N.J., Soares, N.F.F., Alvarenga, V.O., Rodrigues Jr, S., Granato, D., Zuniga, A.D.G. and de Souza Sant'Ana, A. (2014) Modelling *Bacillus cereus* adhesion on stainless steel surface as affected by temperature, pH and time. *International Dairy Journal* 34, 153-158

Peng, J.S., Tsai, W.C. and Chou, C.C. (2001) Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. *International Journal of Food Microbiology 65*, 105–111

Peng, J.S., Tsai, W.C. and Chou, C.C. (2002) Inactivation and removal of Bacillus cereus by sanitizer and detergent. *International Journal of Food Microbiology 77*, 11–18

Phillips, L.G., Mcgiff, M.L., Barbano, D.M. and Lawless, H.T. (1995) The influence of fat on the sensory properties, viscosity, and color of low fat milk. *Journal of Dairy Science* 78, 1258-1266

Prüß, B.M., Dietrich, R., Nibler, B., Martlbauer, E. and Scherer, S. (1999) The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. *Applied and Environmental Microbiology* 65, 5436–5442

Rajkovic, A., Uyttendaele, M., Vermeulen, A., Andjelkovic, M., Fitz-James, I., In't Veld, P., Denon, Q., Vérhe, R. and Debevere, J. (2008) Heat resistance of *Bacillus cereus* emetic toxin, cereulide. *Letters in Applied Microbiology* 46, 536–541

Ramarao, N. and Sanchis, V. (2013) The pore-forming haemolysins of *Bacillus cereus*: a review. *Toxins* 5, 1119-1139

Rasko, D.A., Ravel, J., Økstad, O.A., Helgason, E., Cer, R.Z., Jiang, L., Shores, K.A., Fouts, D.E., Tourasse, N.J. Angiuoli, S.V., Kolonay, J., Nelson, W.C., Kolstø, A.B., Fraser, C.M. and Read, T.D. (2004) The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucleic Acids Research* 32, 977–988

Reyes, J.E., Bastías, J.M., Gutiérrez, M.R. and de la O. Rodríguez, M. (2007) Prevalence of *Bacillus cereus* in dried milk products used by Chilean school feeding program. *Food Microbiology* 24, 1-6

Roy, A., Moktan, B. and Sarkar, P.K. (2007) Characteristics of *Bacillus cereus* isolates from legume-based Indian fermented foods. *Food Control* 18, 1555–1564

Ryu, J.H. and Beuchat, L.R. (2005) Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. *Journal of Food Protection* 68, 2614–2622

Saile, E. and Koehler, T.M. (2006) *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. *Applied and Environmental Microbiology* 72, 3168–3174

Salustiano, V.C., Andrade, N.J., Soares, N.F.F., Lima, J.C., Bernardes, P.C., Luiz, L.M.P. and Fernandes, P.E. (2009) Contamination of milk with *Bacillus cereus* by post-pasteurization surface exposure as evaluated by automated ribotyping. *Food Control* 20, 439–442

Samar•ija, D., Zamberlin, S. and Pogacic, T. (2012) Psychrotrophic bacteria and milk and dairy products quality. *Mljekarstvo* 62, 77-95

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W. and Davies, D.G. (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology 184*, 1140–1154

Schaffner, D.W., McEntire, J., Duffy, S., Montville, R. and Smith, S. (2003) Monte Carlo simulation of the shelf life of pasteurized milk as affected by temperature and initial concentration of spoilage organisms. *Food Protection Trends* 23, 1014–1021

Sergelidis, D., Abrahim, A., Sarimvei, A., Panoulis, C., Karaioannoglou, P. and Genigeorgis, C. (1997) Temperature distribution and prevalence of *Listeria* spp. in domestic, retail and industrial refrigerators in Greece. *International Journal of Food Microbiology* 34, 171-177

Shaheen, R., Svensson, B., Andersson, M.A., Christiansson, A. and Salkinoja-Salonen, M. (2010) Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. *Food Microbiology* 27, 347–355

Sharma, M. and Anand, S.K. (2002) Biofilms evaluation as an essential component of HACCP for food/dairy processing industry - a case. *Food Control* 13, 469–477

Simmonds, P., Mossel, B.L., Intaraphan, T. and Deeth, H.C. (2003) Heat resistance of *Bacillus* spores when adhered to stainless steel and its relationship to spore hydrophobicity. *Journal of Food Protection* 66, 2070–2075

Simões, M., Simões, L.C., Cleto, S., Pereira, M.O. and Vieira, M.J. (2008) The effects of a biocide and a surfactant on the detachment of *Pseudomonas fluorescens* from glass surfaces. *International Journal of Food Microbiology* 121, 335–341

Simões, M., Simões, L.C., Machado, I., Pereira, M.O. and Vieira, M.J. (2006) Control of flow-generated biofilms with surfactants: evidence of resistance and recovery. *Food and Bioproducts Processing* 84, 338–345

Simões, M., Simões, L.C. and Vieira, M.J. (2010) A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology* 43, 573–583

Simone, E., Goosen, M., Notermans, S.H.W. and Borgdorff, M.W. (1997) Investigations of foodborne diseases by Food Inspection Services in the Netherlands, 1991 to 1994. *Journal of Food Protection* 60, 442–446

Singh, B.R., Kulshreshtha, S.B. and Kapoor, K.N. (1995) A bakery product associated *B. cereus* food poisoning outbreak. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* 16, 151–152

Srey, S., Jahid, I.K. and Ha, S.D. (2013) Biofilm formation in food industries: a food safety concern. *Food Control* 31, 572–585

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kämpfer, P., Maiden, M.C.J., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C. and Whitman, W.B. (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* 52, 1043–1047

Stenfors Arnesen, L.P., Fagerlund, A. and Granum, P.E. (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews* 32, 579–606

Stenfors Arnesen, L.P., O'Sullivan, K. and Granum, P.E. (2007) Food poisoning potential of *Bacillus cereus* strains from Norwegian dairies. *International Journal of Food Microbiology* 116, 292–296

Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. (2002) Biofilms as complex differentiated communities. *Annual Review of Microbiology* 56, 187–209

Sutherland, I.W. (2001) The biofilm matrix – an immobilized but dynamic microbial environment. *Trends in Microbiology* 9, 222–227

Svensson, B., Ekelund, K., Ogura, H. and Christiansson, A. (2004) Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different diary plants. *International Dairy Journal* 14, 17–27

Svensson, B., Eneroth, Å., Brendehaug, J., Molin, G. and Christiansson, A. (2000) Involvement of a pasteurizer in the contamination of milk by *Bacillus cereus* in a commercial dairy plant. *Journal of Dairy Research* 67, 455–460

Svensson, B., Monthán, A., Guinebretière, M.H., Nguyen-The, C., Christiansson, A. (2007) Toxin production potential and the detection of toxin genes among strains of the *Bacillus cereus* group isolated along the dairy production chain. *International Dairy Journal* 17, 1201-1208

Svensson, B., Monthán, A., Shaheen, R., Andersson, M.A., Salkinoja Salonen, M. and Christiansson, A. (2006) Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. *International Dairy Journal* 16, 740–749

Tallent, S.M., Kotewicz, K.M., Strain, E.A. and Bennett, R.W. (2012) Efficient isolation and identification of *Bacillus cereus* group. *Journal of AOAC International* 95, 446–451

Tamime, A.Y., Ed. (2009) *Milk Processing and Quality Management*. Blackwell Publishing, Oxford, UK

Tauveron, G., Slomianny, C., Henry, C. and Faille, C. (2006) Variability among *Bacillus cereus* strains in spore surface properties and influence on their ability to contaminate food surface equipment. *International Journal of Food Microbiology* 110, 254–262

te Giffel, M.C., Beumer, R.R., Granum, P.E. and Rombouts, F.M. (1997) Isolation and characterisation of *Bacillus cereus* from pasteurised milk in household refrigerators in the Netherlands. *International Journal of Food Microbiology* 34, 307–318

te Giffel, M.C., Beumer, R.R., Leijendekkers, S. and Rombouts, F.M. (1996). Incidence of *Bacillus cereus* and *Bacillus subtilis* in foods in the Netherlands. *Food Microbiology* 13, 53-58

te Giffel, M.C., Beumer, R.R., Slaghuis, B.A. and Rombouts, F.M. (1995) Occurrence and characterization of (psychrotropic) *Bacillus cereus* on farms in the Netherlands. *Netherlands Milk and Dairy Journal*, 49, 125–138

te Giffel, M.C., Wagendorp, A., Herrewegh, A. and Driehuis F. (2002) Bacterial spores in silage and raw milk. *Antonie van Leeuwenhoek* 81, 625-630

Teh, K.H., Flint, S., Palmer, J., Andrewes, P., Bremer, P. and Lindsay, D. (2012) Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. *International Journal of Food Microbiology* 157, 28-34

Teh, K.H., Flint, S., Palmer, J., Andrewes, P., Bremer, P. and Lindsay, D. (2014) Biofilm: an unrecognised source of spoilage enzymes in dairy products? *International Dairy Journal 34*, 32-40

Thomas, A. and Sathian, C.T. (2014) Cleaning-in-place (CIP) system in dairy plant - review. *IOSR Journal of Environmental Science, Toxicology and Food Technology* 8*,* 41-44

Thorsen, L., Hansen, B.M., Nielsen, K.F., Hendriksen, N.B., Phipps, R.K. and Budde, B.B. (2006) Characterization of emetic *Bacillus weihenstephanensis*, a new cereulide-producing bacterium. *Applied and Environmental Microbiology* 72, 5118–5121

Thys, R.C., Lucas, F.S., Riffel, A., Heeb, P. and Brandelli, A. (2004) Characterization of a protease of a feather-degrading *Microbacterium* species. *Letters in Applied Microbiology* 39, 181–186

Tourasse, N.J., Helgason, E., Økstad, O.A., Hegna, I.K. and Kolstø, A.B. (2006) The *Bacillus cereus* group: novel aspects of population structure and genome dynamics. *Journal of Applied Microbiology* 101, 579–593

Vachon, V., Laprade, R. and Schwartz, J.L. (2012) Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. *Journal of Invertebrate Pathology* 111, 1-12

Valero, M., Hernandez-Herrero, L.A., Fernandez, P.S. and Salmeron, M.C. (2002) Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. *Food Microbiology* 19, 491–499

van der Auwera, G.A., Timmery, S., Hoton, F. and Mahillon, J. (2007) Plasmid exchanges among members of the *Bacillus cereus* group in foodstuffs. *International Journal of Food Microbiology* 113, 164–172

van der Voort, M., García, D., Moezelaar, R. and Abee, T. (2010) Germinant receptor diversity and germination responses of four strains of the *Bacillus cereus* group. *International Journal of Food Microbiology* 139, 108–115

van de Weyer, A., Devleeschhouwer, M.J. and Dony, J. (1993) Bactericidal activity of disinfectants on *Listeria*. *Journal of Applied Bacteriology* 74, 480-483

van Netten, P., van de Moosdjik, A., van Hoensel, P., Mossel, D.A.A. and Perales, I. (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology* 69, 73–79

Vissers, M.M.M., Driehuis, F., te Giffel, M.C., De Jong, P. and Lankveld, J.M.G. (2006) Improving farm management by modeling the contamination of farm tank milk with butyric acid bacteria*. Journal of Dairy Science* 89*,* 850-858

Vissers, M.M.M., te Giffel, M.C., Driehuis, F., De Jong, P. and Lankveld, J.M.G. (2007) Minimizing the level of *Bacillus cereus* spores in farm tank milk. *Journal of Dairy Science* 90, 3286-3293

Warke, R., Kamat, A., Kamat, M. and Thomas, P. (2000) Incidence of pathogenic psychrotrophs in ice creams sold in some retail outlets in Mumbai, India. *Food Control* 11, 77–83

WHO (2007) Food safety and food-borne illness. Fact sheet no. 237. World Health Organization, Geneva, Switzerland

Wijman, J.G.E., de Leeuw, P.P.L.A., Moezelaar, R., Zwietering, M.H. and Abee, T. (2007) Air-liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. *Applied and Environmental Microbiology* 73, 1481–1488

Wong, A.C.L. (1998) Biofilms in food processing environments. *Journal of Dairy Science* 81, 2765–2770

Zhou, G., Liu, H., He, J., Yuan, Y. and Yuan, Z. (2008) The occurrence of *Bacillus cereus, B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. *International Journal of Food Microbiology* 121, 195–200

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- Kumari, S. and Sarkar, P.K. (2016) *Bacillus cereus* hazard and control in industrial dairy processing environment. *Food Control* 69, 20-29

- Sharma, A., Kumari, S., Wongputtisin, P., Nout, M.J.R. and Sarkar, P.K. (2015) Optimization of soybean processing into kinema, a *Bacillus*-fermented alkaline food, with respect to a minimum level of antinutrients. *Journal of Applied Microbiology* 119, 162-176

- Kumari, S. and Sarkar, P.K. (2014) *In vitro* model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology. *Food Control* 36, 153–158

- Kumari, S. and Sarkar, P.K. (2014) Prevalence and characterization of *Bacillus cereus* group from various marketed dairy products in India. *Dairy Science and Technology* 94, 483–487

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In vitro model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology

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ABSTRACT

An *in vitro* model study was carried out to check biofilm formation by a selected strain of *Bacillus cereus* isolated from a chilling tank where pasteurized milk was stored. The strain was able to form biofilm even at 4 °C. From the study of different simulated conditions it was found that *B. cereus* cell count reached up to 10^6 cfu/cm^2 of the surface of stainless steel tank if inadequately cleaned tanker is left to stand empty at room temperature. Prevalence of biofilm in dairy environment is a matter of concern as it can be an important reservoir for food spoilage and can lead to foodborne outbreaks. In the present study response surface methodology (RSM) was used to optimize parameters influencing biofilm cell removal. The RSM results were used to design an optimized clean-in-place (CIP) regime which consisted of 1.5% NaOH at 65 °C for 30 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse. Effectiveness of reference CIP (1% NaOH at 65 °C for 10 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse) and optimized CIP was assessed against 24 h-old biofilm. While the reference CIP caused 3.29 log reduction/cm^2 in *B. cereus* biofilm cells, the optimized CIP achieved 4.77 log reduction/cm^2 in biofilm cell count. Thus, the optimized CIP regime designed in the present study was found to be significantly ($p < 0.05$) more effective in biofilm cell removal as compared to the reference CIP.

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1. Introduction

Formation of bacterial biofilms within food processing plants is of concern to processors, as bacteria within biofilms are more difficult to eliminate than planktonic cells, and can act as a source of recurrent contamination to plant, product and personnel. Biofilm renders its inhabitants resistant to antimicrobial agents and cleaning (Costerton, Stewart, & Greenberg, 1999; Srey, Jahid, & Ha, 2013). Other undesirable conditions associated with biofilms include reduced flow through blocked tubes, reduced plan run times, corrosion of stainless steel (SS) and reduced heat transfer through plate heat exchangers (Parkar, Flint, & Brooks, 2004). Deciphering the role of bacterial biofilms in the post-processing area of plants is a novel aspect that can contribute to the HACCP plan development (Sharma & Anand, 2002). Endospore-forming bacteria cause problems in dairies, where they form biofilms in pasteurizers and post-pasteurization line, resulting in the contamination of milk and poor quality dairy products. Since *Bacillus* spp. are resilient, ubiquitous sporeformers, and many are psychro-tolerant, they proliferate at low temperatures when nutrients are available (Swiecicka & Mahillon, 2005). *Bacillus cereus* is a great safety concern for dairy industry as it is associated with outbreaks of food poisoning by producing enterotoxin (Borge, Skeie, Sorhaug, Langsrud, & Granum, 2001; Granum & Lund, 1997; Te Giffel, Beumer, Granum, & Rombouts, 1997). It also causes spoilage, like sweet curdling and bitterness of milk (Huang, Lai, Shin, Liau, & Peng, 1999). It can be introduced into dairy environment from various sources during production, handling and processing mainly from improperly cleaned and sanitized equipments (Te Giffel, Beumer, Slaghuis, & Rombouts, 1995). Once in the dairy environment, cells of *B. cereus* can adhere to SS surfaces of dairy plant and form biofilm (Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010; Wijman, de Leeuw, Moezelaar, Zwietering, & Abee, 2007) which can be an important reservoir for recurrent contamination of dairy products; but it has received relatively little attention. Hence, this study was conducted to design an *in vitro* model to study biofilm formation by a selected strain of *B. cereus* isolated from a chilling tank where pasteurized milk was stored.

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Table 1
Results of *in vitro* model study.

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

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

In dairy, like any other food industry, an effective cleaning and sanitation program is part of the process to eliminate microorganisms. Generally the sanitation agents are developed on the basis of studies utilizing planktonic cells which are quite different from the biofilm cells due to their altered physiological status. Therefore, inactivation and removal of bacterial cells capable of forming biofilms deserves much more attention (Peng Tsai, & Chou, 2002). *B. cereus* accounts for 12.4% of microbiota growing in biofilms in a commercial dairy plant (Sharma & Anand, 2002). Thus, an evaluation of cleaning regimes in dairy plants for biofilm cell removal is very important. Hence, the second objective of our study was to optimize the clean-in-place (CIP) regimes.

The reference CIP regimes followed in dairy plants exhibited varied results in eliminating biofilm (Bremer, Fillery, & McQuillan, 2006; Dufour, Simmonds, & Bremer, 2004; Faille, Fontaine, & Benezech, 2001). Hence, optimization of various factors affecting biofilm cell removal is of much importance to design an effective CIP regime. The traditional approach for optimizing a multivariable system which consisted of 'one factor at a time' is not only time consuming but also inapplicable where factor interactions affect final response. Thus, the most efficient way to enhance the value of research and cut down time in process development is through statistical experimental designs. Response surface methodology (RSM) is a useful statistical tool to evaluate the effect of different factors and their interactions on response variables, and can be effectively used to find out levels of factors required for optimum response. Therefore, in the present study RSM was used to analyse the effects of time, temperature and NaOH concentration on the removal of biofilm.

2. Materials and methods

2.1. Microorganism

Strains of *B. cereus* were isolated from a chilling tank, where pasteurized milk was stored at 4 °C, in the HIMUL (Himalayan Milk Producers' Union Limited) Dairy at Matigara, near to Siliguri. Samples were collected from SS surfaces of the chilling tank by using sterile swabs and spread on the surface of plates of B. cereus selective agar (HiMedia M833, FD003 and FD045). The inoculated plates were incubated at 35 °C for 24–48 h. The presumptive identification was confirmed on the basis of endospore formation, glucose fermentation, acetylmethylcarbinol production, nitrate reduction and motility, following standard methods (Claus & Berkeley, 1986). The isolates were maintained on nutrient agar (HiMedia M561) slants with subculturing after every six months.

Among the isolates, *B. cereus* PT4 was found to exhibit maximum proteolytic activity and resistance against multiple antibiotics (data not shown). Hence, this strain was selected for the present study.

2.2. Biofilm formation assay

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

B. cereus PT4 (initial total count: 10^4/ml) was inoculated into reconstituted skim milk (HiMedia M530) containing sterilized SS coupons (grade 304 with 2B finish) and incubated at 4 °C for 24 h. Three sets of experiment were designed, each in triplicates (Table 1). Biofilm cells were recovered from the SS 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. Those were then transferred to peptone saline, vortexed with glass beads for 2 min, decimally diluted and plated on milk agar (HiMedia M163). 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 SS coupons from the second set of scenario 1 were transferred to sterilized centrifuge tube containing fresh sterile reconstituted skim milk and further incubated at 4 °C for 24 h; this simulated scenario 2. The SS 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 SS coupons of scenario 2 and 3 were recovered as in case of scenario 1.

2.3. Influence of NaOH treatment on biofilm cell removal

The effectiveness of NaOH was determined according to Sharma and Anand (2002). Biofilms were developed on SS coupons by inoculating overnight culture of *B. cereus* PT4 on nutrient agar (initial total count: 10^4/ml) into reconstituted skim milk containing sterilized SS coupons placed at air–liquid interface and incubated at 30 °C for 24 h. SS coupons with 24 h-old biofilm were washed with sterile distilled water thrice to remove non-biofilm cells and exposed to varying concentrations of NaOH, time and temperature. The SS coupons were then rinsed thrice with neutralization buffer (Himedia M1334), 10 s for each time. Survivors after treatment were determined by vortexing the SS 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 SS coupons was determined by the following equation:

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

where N is the count of untreated control cells and n is the count of cells recovered after treatment (Van de Weyer, Devleeschhouwer, & Dony, 1993).

2.4. Response surface optimization for biofilm cell removal

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. A total of 20 experiments

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Table 2
Levels of variables in the experimental design.

Independent variables	Coded levels[a]				
	−1.682	−1	0	1	1.682
Time (min)	3.18	10	20	30	36.82
Temperature (°C)	31.48	40	52.50	65	73.52
NaOH (%, w/v)	0.66	1	1.50	2	2.34

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

were designed by using the statistical software package Design Expert version 8.0 (Stat-Ease Inc., Minneapolis, USA). The experimental designs are shown in Tables 2 and 3.

2.5. Effectiveness of reference and optimized CIP

To determine the efficacy of the reference CIP (1% NaOH at 65 °C for 10 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse) and optimized CIP (1.5% NaOH at 65 °C for 30 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse) 24 h-old biofilm was developed on SS coupons and these coupons underwent treatment with reference and optimized CIP regimes. Following each cleaning regime biofilm cells were recovered according to Teh et al. (2012) and spread on milk agar plates which were then incubated at 30 °C for 24 h.

Crystal violet staining of SS coupons which underwent optimized cleaning regime and SS coupons containing 24 h-old biofilm without treatment was performed to check whether biofilm matrix material really removed or are only cells in the biofilm inactivated (Harvey, Keenan, & Gilmour, 2007: Wijman et al., 2007). The SS coupons were added with 3 ml of 1% crystal violet and incubated at 20 °C for 45 min. After staining, excess crystal violet was removed and coupons were washed three times with sterile distilled water and air-dried at 30 °C for 30 min. Each coupon was added with 3 ml of 95% ethanol and left for 30 min to elute the stain, if any. Intensity of the stain was monitored by measuring the optical density 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.

Table 3
Design of RSM, and its actual and predicted values.

Run	A: Time	B: Temperature	C: NaOH	Log reduction[a] in *B. cereus* cell count/cm^2	
				Experimental[b]	Predicted
1	20.00	52.50	1.50	1.49	1.53
2	20.00	52.50	2.34	1.76	1.64
3	30.00	40.00	2.00	1.62	1.62
4	30.00	40.00	1.00	0.66	0.75
5	30.00	65.00	2.00	2.31	2.55
6	10.00	65.00	1.00	0.69	0.71
7	36.82	52.50	1.50	2.90	2.73
8	20.00	52.50	1.50	1.54	1.53
9	10.00	40.00	1.00	0.44	0.23
10	20.00	52.50	0.66	0.44	0.52
11	20.00	52.50	1.50	1.32	1.53
12	10.00	40.00	2.00	1.20	1.25
13	20.00	73.52	1.50	1.65	1.56
14	3.18	52.50	1.50	0.99	1.12
15	20.00	52.50	1.50	1.47	1.53
16	20.00	31.48	1.50	0.33	0.38
17	20.00	52.50	1.50	1.70	1.53
18	30.00	65.00	1.00	2.26	2.24
19	10.00	65.00	2.00	1.21	1.15
20	20.00	52.50	1.50	1.65	1.53

[a] Initial count, 5.3–5.5 log cfu/cm^2.
[b] Mean values of experiments carried out in triplicates.

Table 4
ANOVA results for response surface quadratic model.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value Prob > *F*[a]	Comment
Model	8.24	9	0.92	31.93	<0.0001	Significant
A – Time	3.11	1	3.11	108.61	<0.0001	
B – Temperature	1.67	1	1.67	58.09	<0.0001	
C – NaOH	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[b]
Pure error	0.093	5	0.019			
Core total	8.53	19				

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

2.6. Statistical analysis

Analysis of variance (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 optimized CIPs.

3. Results

3.1. Biofilm formation assay

Results of *in vitro* model study are shown in Table 1. *B. cereus* strain was able to adhere and form biofilm on SS coupons in the *in vitro* model, with the number of bacterial cells recovered (10^3 cfu/cm^2) in scenario 1 and 2, and 10^6 cfu/cm^2 in scenario 3.

3.2. Response surface optimization of biofilm cell removal

Results for analysis of variance are given in Tables 4 and 5. 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 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:

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

where A is time, B is temperature and C is NaOH%.

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 software. Fig. 1 represents the effect of two factors while the other factor held at zero level. The results indicated that interaction between time

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Table 5
ANOVA results for the equations of the Design Expert for studied responses.[a]

Response	*R*-squared	Adj *R*-squared	Pred *R*-squared	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, Predicated residual error sum of squares.

and temperature is an important parameter for biofilm cell removal, and maximum removal was predicated when the biofilm was exposed to 1.5% NaOH for 30 min at 65 °C where 2.55 log reduction/cm^2 in biofilm cell count was achieved.

3.3. Effectiveness of reference and optimized CIP

Results for effectiveness of reference and optimized cleaning regimes are given in Table 6. The reference CIP regime (1% NaOH for 10 min at 65 °C – water rinse – 1% HNO_3 for 10 min at 65 °C –

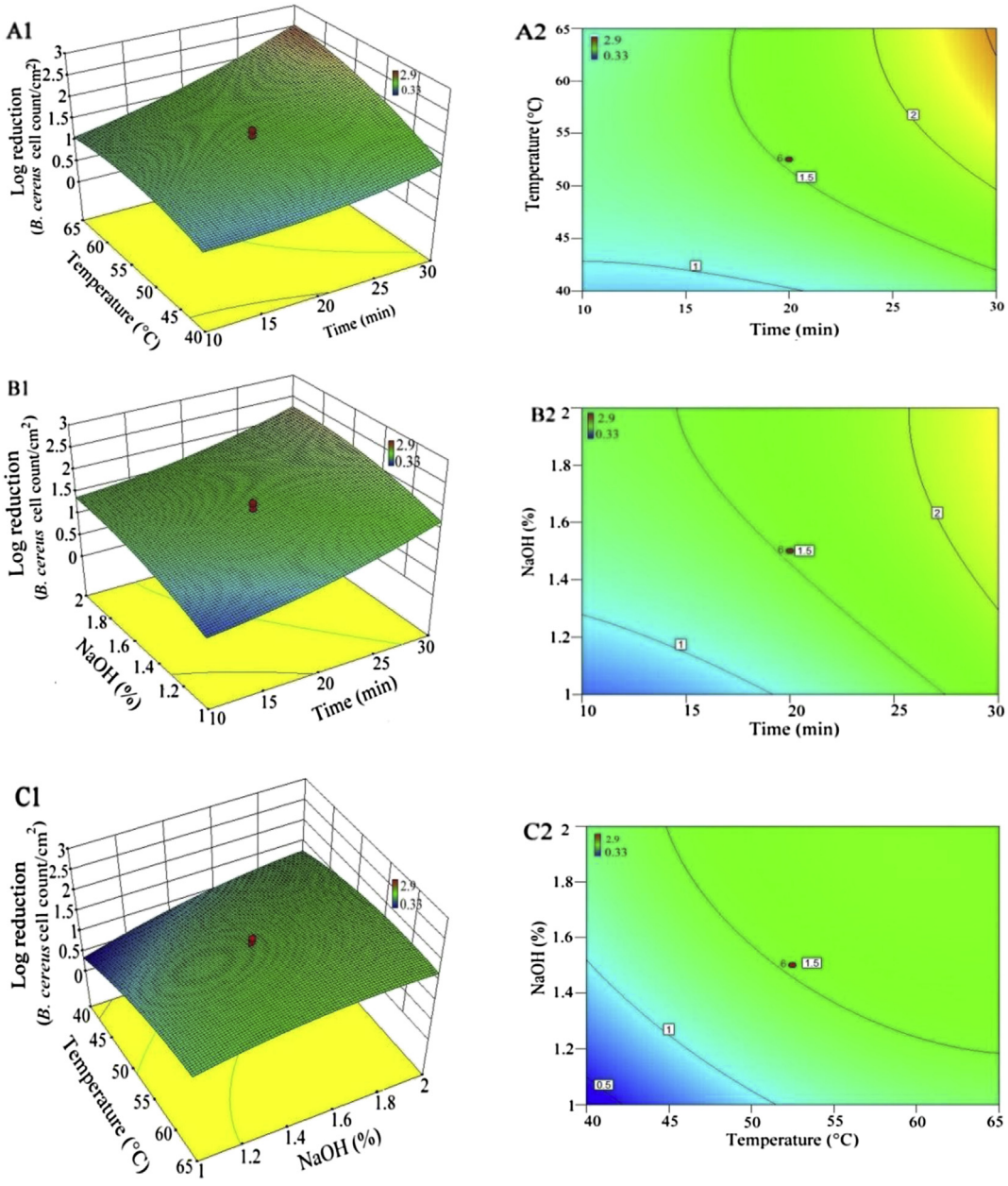

Fig. 1. A1 and A2 show 3D and contour response surface plots, respectively, for biofilm removal showing effect of time and temperature when 1.5% NaOH was used. B1 and B2 show 3D and contour response surface plots, respectively, for biofilm removal showing effect of time and NaOH at 52.5 °C. C1 and C2 show 3D and contour response surface plots, respectively, for biofilm removal showing effect of temperature and NaOH when the exposure time was 20 min.

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Table 6
Effect of different cleaning regimes on biofilm cell removal.

Cleaning regimes	Count of *B. cereus* cells recovered from biofilm on SS coupons (log cfu/cm^2)[a]		Log reduction in *B. cereus* cell count
	Without treatment	With treatment	
Reference CIP[b]	5.33 ± 0.33	2.03 ± 0.03	3.29 ± 0.34
Optimized 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 SS coupons were treated with 1% NaOH at 65 °C for 10 min, followed by rinsing with water, treating with 1% HNO_3 at 65 °C for 10 min, and again rinsing with water.
[c] Biofilm containing SS coupons were treated with 1.5% NaOH at 65 °C for 30 min, followed by rinsing with water, treating with 1% HNO_3 at 65 °C for 10 min, and again rinsing with water.

water rinse) achieved only 3.29 log reduction in the number of *B. cereus* cells recovered from the SS coupons when compared to control coupons (without treatment). On the other hand, optimized CIP designed in the present study led to 4.77 log reduction in the number of *B. cereus* cells. From crystal violet staining of SS coupons it was evident that biofilm cells were not only inactivated by optimized CIP but biofilm matrix also got removed from SS coupons (Fig. 2a). This was substantiated by the OD obtained in case of biofilm-containing SS coupons which underwent optimized CIP was significantly different ($p = 0.03$) from that of control SS coupons (Fig. 2b). Thus, optimized CIP was found to be significantly ($p < 0.05$) more effective in biofilm cell removal as compared to reference CIPs.

4. Discussion

Presence of *B. cereus* biofilm in dairy processing lines and chilling tanks can be a source of post-pasteurization contamination. In the present study we designed an *in vitro* model to study biofilm formation by *B. cereus* strain isolated from a chilling tank where pasteurized milk was stored. From the results it was evident that the selected strain was able to form biofilm even at 4 °C. From the study of different simulated conditions it was found that the *B. cereus* cell count in the biofilm developed on the surface of SS chilling tanks can reach up to 10^6 cfu/cm^2 if inadequately cleaned tanker is left to stand empty at room temperature. This is a matter of concern as the presence of such a high number of cells on internal surfaces of chilling tanks can lead to recurrent contamination and spoilage of fresh lot of milk collected into tank.

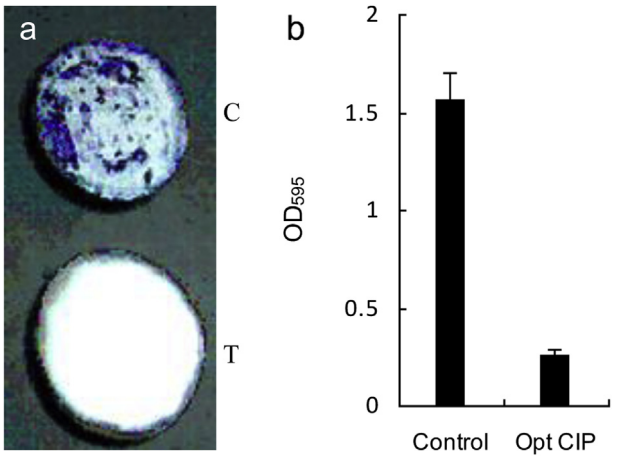

Fig. 2. Crystal violet-stained biofilms present on SS coupons (a) and OD_{595} of stained biofilms from SS coupons before (C) and after (T) treatment with optimized cleaning regime (b). Error bars represent mean ± SE, obtained from triplicate sets of experiment.

B. cereus biofilm is of great significance to dairy industry as it can be a recurrent source of food spoilage and outbreaks of food poisoning. The spores of *B. cereus* possess a pronounced ability to adhere to the surface of SS, a common food processing material (Peng et al., 2002). Thus, biofilms have been a major food safety concern for dairy industry. The technique used for biofilm cell removal in dairy industry mainly consists of CIP regimes which commonly consist of alkali and acid wash (Chisti, 1999). Effectiveness of CIP regimes against *B. cereus* biofilm is not reported extensively. Peng et al. (2002) reported requirement of long-hot CIP for effective removal of *B. cereus* biofilm. Efficacy of CIP regimes greatly depends on exposure time, temperature and concentration of cleaning agent (Bremer, Monk, & Butler, 2002; Parkar et al., 2004). Thus for designing an effective CIP, optimization of parameters, like time, temperature and concentration of agent is essential. Caustic step in CIP regimes is believed to be predominantly responsible for biofilm removal (Chisti, 1999). So, use of RSM to optimize parameters influencing biofilm cell removal during caustic step in the present study can be very useful. From the results it was evident that all the variables used in the study significantly contributed to biofilm removal, and interaction between time and temperature was the main influencing factor. A linear increase in biofilm removal was observed when exposure time and temperature were increased. Maximum removal was predicated when the biofilm was exposed to 1.5% NaOH for 30 min at 65 °C where 2.55 log reduction/cm^2 in biofilm cell count was achieved. Thus, in the present study, RSM was successfully deployed to obtain conditions which influenced efficacy of caustic step. The RSM results were used to design an optimized CIP regime which consisted of 1.5% NaOH for 30 min at 65 °C – water rinse – 1% HNO_3 for 10 min at 65 °C – water rinse. Effectiveness of reference CIP (1% NaOH for 10 min at 65 °C – water rinse – 1% HNO_3 for 10 min at 65 °C – water rinse) was compared with that of optimized CIP against 24 h-old biofilm. It was found that the reference CIP achieved a mean 3.29 log reduction in the number of *B. cereus* cells recovered as compared to control. This result was in consistence with the findings of Bremer et al. (2006) who reported mean 2 log reduction in biofilm cells after reference CIP regime. Difference in the reduction of cells recovered may be attributed to the fact that Bremer et al. (2006) used biofilm formed by consortium. Optimized CIP achieved 4.77 log reduction of biofilm cells/cm^2. From crystal violet staining of SS coupons it was evident that biofilm cells were not only inactivated by optimized CIP but biofilm matrix also got removed from SS coupons. Thus, the optimized CIP in the present study effectively removed biofilm. Concentration of NaOH used in this study was within the permissible limits and usually used in dairy industry for CIP, 1–5% concentrations of NaOH is used for plate-type heat exchangers and 1–2% for general cleaning (Flint, Bremer, & Brooks, 1997).

An effective CIP regime should achieve maximum removal of biofilm cells in the shortest possible time and at lower temperature. Optimized CIP regime in the present study achieved a significant increase in log reduction of biofilm cells (4.77 cells/cm^2) at low concentration of NaOH and HNO_3 (1.5% and 1%, respectively), and at a lower temperature (65 °C). Parker et al. (2004) reported the use of higher concentration of NaOH and HNO_3 (2% and 1.8%, respectively), and a higher temperature (75 °C) for the removal of *Bacillus flavothermus* biofilm. As evaluation of biofilm status and development of an effective CIP regime are part of the HACCP plan development and ISO:9000 specifications for food processing industry to make them more meaningful (Sharma & Anand, 2002), the optimized CIP regime established in the present study can be an effective tool for *B. cereus* biofilm cell removal.

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5. Conclusions

In the present study, we were able to successfully deploy RSM to optimize conditions for biofilm cell removal. The maximum biofilm cell removal was achieved using 1.5% NaOH at 65 °C for 30 min. The results of RSM were used to design optimized CIP which consisted of 1.5% NaOH at 65 °C for 30 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse. Optimized CIP was found to be significantly ($p < 0.05$) more effective in biofilm cell removal as compared to the reference CIP usually used in dairy industry.

References

Borge, G. I. A., Skeie, M., Sorhaug, T., Langsrud, T., & Granum, P. E. (2001). Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. *International Journal of Food Microbiology, 69*, 237–246.

Bremer, P. J., Fillery, S., & McQuillan, A. J. (2006). Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology, 106*, 254–262.

Bremer, P. J., Monk, I., & Butler, R. (2002). Inactivation of *Listeria monocytogenes/Flavobacterium spp.* biofilm using chlorine: impact of substrate, pH, time and concentration. *Letters in Applied Microbiology, 35*, 321–325.

Chisti, Y. (1999). Modern systems of plant cleaning. In R. Robinson, C. Batt, & P. Patel (Eds.), *Encyclopedia of food microbiology* (pp. 1086–1815). London: Academic Press.

Claus, D., & Berkeley, C. W. (1986). The genus *Bacillus*. In P. H. A. Sneath (Ed.), *Bergey's manual of systematic bacteriology* (Vol. 2; pp. 1105–1139). Baltimore, MD: Williams and Wilkins.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science, 284*, 1318–1322.

Dufour, M., Simmonds, R. S., & Bremer, P. J. (2004). Development of a laboratory scale clean-in-place system to test the effectiveness of "natural" antimicrobials against dairy biofilms. *Journal of Food Protection, 67*, 1438–1443.

Faille, C., Fontaine, F., & Benezech, T. (2001). Potential occurrence of adhering living bacillus spores in milk product processing lines. *Journal of Applied Microbiolology, 90*, 892–900.

Flint, S. H., Bremer, P. J., & Brooks, J. D. (1997). Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling, 11*, 81–97.

Granum, P. E., & Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters, 157*, 223–228.

Harvey, J., Keenan, K. P., & Gilmour, A. (2007). Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiology, 24*, 380–392.

Huang, C. C., Lai, S. H., Shin, S. L., Liau, S. M., & Peng, J. S. (1999). A study on the spoilage of aseptic packaged long life milk caused by *Bacillus cereus*. *Journal of the Chinese Society of Animal Science, 28*, 249–260.

Parkar, S. G., Flint, S. H., & Brooks, J. D. (2004). Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *Journal of Applied Microbiology, 96*, 110–116.

Peng, J.-S., Tsai, W.-C., & Chou, C.-C. (2002). Inactivation and removal of *Bacillus cereus* by sanitizer and detergent. *International Journal of Food Microbiology, 77*, 11–18.

Shaheen, R., Svensson, B., Andersson, M. A., Christiansson, A., & Salkinoja-Salonen, M. (2010). Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. *Food Microbiology, 27*, 347–355.

Sharma, M., & Anand, S. K. (2002). Biofilms evaluation as an essential component of HACCP for food/dairy processing industry – a case. *Food Control, 13*, 469–477.

Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: a food safety concern. *Food Control, 31*, 572–585.

Swiecicka, I., & Mahillon, J. (2005). The clonal structure of *Bacillus thuringiensis* isolates from north-east Poland does not correlate with their *cry* gene diversity. *Environmental Microbiology, 7*, 34–39.

Te Giffel, M. C., Beumer, R. R., Granum, P. E., & Rombouts, F. M. (1997). Isolation and characterization of *Bacillus cereus* from pasteurized milk in household refrigerations in the Netherlands. *International Journal of Food Microbiology, 34*, 307–318.

Te Giffel, M. C., Beumer, R. R., Slaghuis, B. A., & Rombouts, F. M. (1995). Occurrence and characterization of (psychrotropic) *Bacillus cereus* on farms in the Netherlands. *Netherlands Milk Dairy Journal, 49*, 125–138.

Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., & Lindsay, D. (2012). Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. *International Journal of Food Microbiology, 157*, 28–34.

Van de Weyer, A., Devleeschhouwer, M. J., & Dony, J. (1993). Bactericidal activity of disinfectants on Listeria. *Journal of Applied Bacteriology, 74*, 480–483.

Wijman, J. G., de Leeuw, P. P., Moezelaar, R., Zwietering, M. H., & Abee, T. (2007). Air–liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. *Applied and Environmental Microbiology, 73*, 1481–1488.

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ORIGINAL PAPER

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

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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

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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.

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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

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$MgSO_4.7H_2O$, 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

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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})

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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

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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

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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.

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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

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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.

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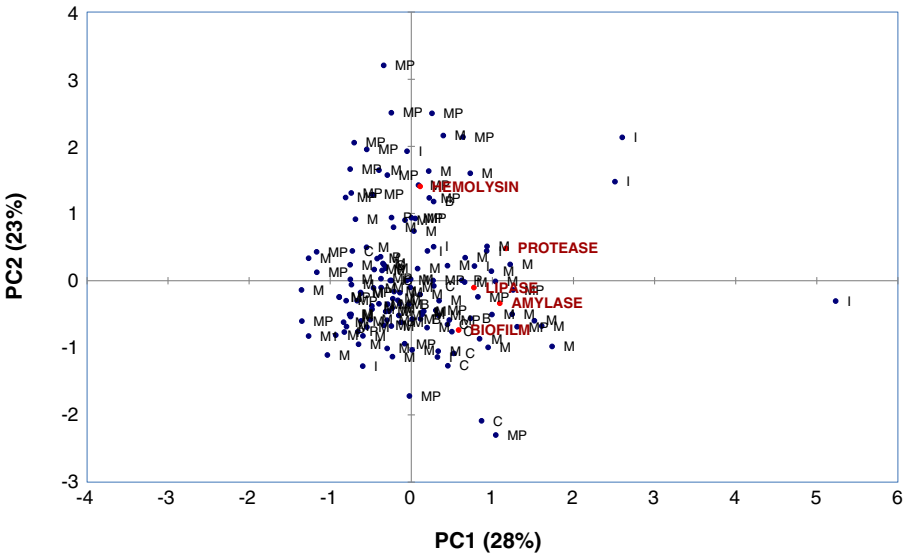

Fig. 1 Score biplot for principal component analysis showing observations (*M* milk, *MP* milk powder, *C* cheese, *I* ice cream, *P* paneer, *B* butter isolates) and variables (production of protease, amylase, lipase, hemolysin, and biofilm) together for 144 isolates belonging to *B. cereus* group

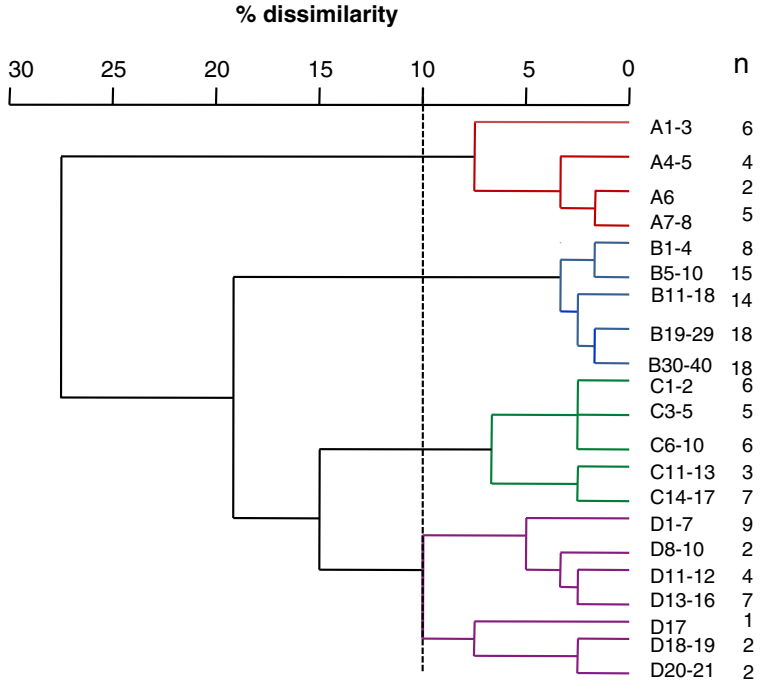

Fig. 2 Simplified dendrogram based on wards clustering of dissimilarity coefficient generated by agglomerative hierarchical clustering. Based on studied characters (production of protease, amylase, lipase, hemolysin, and biofilm), the 144 isolates belonging to *B. cereus* group were grouped into four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

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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.

References

Agata N, Ohta M, Mori M (1996) Production of an emetic toxin, Cereulide, is associated with a specific class of *Bacillus cereus*. Curr Microbiol 33:67–69

Akanbi TO, Kamaruzaman AL, Abu Bakar F, Sheikh Abdul Hamid N, Radu S, Abdul Manap MY, Saari N (2010) Highly thermostable extracellular lipase-producing *Bacillus* strain isolated from a Malaysian hotspring and identified using 16S rRNA gene sequencing. Int Food Res J 17:45–53

Banerjee M, Sarkar PK (2004) Antibiotic resistance and susceptibility to some food preservative measures of spoilage and pathogenic micro-organisms from spices. Food Microbiol 21:335–342

Beattie SH, Williams AG (2000) Detection of toxins. In: Robinson RK, Batt CA, Patel PD (eds) Encyclopedia of food microbiology. Academic Press, San Diego, pp 141–158

Beecher DJ, Schoeni JL, Wong ACL (1995) Enterotoxin activity of hemolysin BL from *Bacillus cereus*. Infect Immun 63:632–639

Bhatnagar P, Khan AA, Jain M, Kaushik S, Jain SK (2007) Microbiological study of khoa sold in Chambal region (Madhya Pradesh): a case study. Ind J Microbiol 47:263–266

Chaves JQ, Pires ES, Vivoni AM (2011) Genetic diversity, antimicrobial resistance and toxigenic profiles of *Bacillus cereus* isolated from food in Brazil over three decades. Int J Food Microbiol 147:12–16

Author's personal copy

Chen L, Daniel RM, Coolbear T (2003) Detection and impact of protease and lipase activities in milk and milk powder. Int Dairy J 13:255–275

Chen L, Coolbearb T, Daniela RM (2004) Characteristics of proteinases and lipases produced by seven *Bacillus* sp. isolated from milk powder production lines. Int Dairy J 14:495–504

Claus D, Berkeley RCW (1986) Genus *Bacillus* Cohn 1872, 174. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1105–1139

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Datta N, Deeth HC (2003) Diagnosing the cause of proteolysis in UHT milk. Leb Wiss U Technol 36:173–182

Flint SH, Bremer PJ, Brooks JD (1997) Biofilms in dairy manufacturing plant description, current concerns and methods of control. Biofouling 11:81–97

Furtado MM (2005) Main problems of cheese: causes and prevention. Fonte Press, São Paulo

Guinebretière MH, Velge P, Couvert O, Carlin F, Debuyser ML, Nguyen-The C (2010) Ability of *Bacillus cereus* group strains to cause food poisoning varies according to phylogenetic affiliation (groups I to VII) rather than species affiliation. J Clin Microbiol 48:3388–3391

Guinebretière MH, Auger S, Galleron N, Contzen M, De Sarrau B, De Buyser ML, Lamberet G, Fagerlund A, Granum PE, Lereclus D, De Vos P, Nguyen-The C, Sorokin A (2013) *Bacillus cytotoxicus* sp. nov. is a new thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. Int J Syst Evol Microbiol 63:31–40

Gupta N, Rathi P, Gupta R (2002) Simplified para-nitrophenyl palmitate assay for lipases and esterases. Anal Biochem 31:98–99

Harvey J, Keenan KP, Gilmour A (2007) Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiol 24:380–392

HiMedia (1998) The HiMedia manual for microbiology laboratory practices. HiMedia Laboratories Pvt. Limited, Mumbai

Kiessling CR, Cutting JH, Loftis M, Kiessling WM, Dutta AR, Sofos JN (2002) Antimicrobial resistance of food-related *Salmonella* isolates, 1999–2000. J Food Prot 65:603–608

Kumari S, Sarkar PK (2014) *In vitro* model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology. Food Control 36:153–158

Larsen HD, Jørgensen K (1997) The occurrence of *Bacillus cereus* in Danish pasteurized milk. Int J Food Microbiol 34:179–186

Lee O-W, Koh Y-S, Kim K-J, Kim B-C, Choi H-J, Kim D-S, Suhartono MT, Pyun Y-R (1999) Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol Lett 179: 393–400

Meer RR, Baker J, Bodyfelt FW, Griffiths MW (1991) Psychrotrophic *Bacillus* spp. in fluid milk products: a review. J Food Prot 54:969–979

Molva C, Sudagidan M, Okuklu B (2009) Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey. Food Control 20: 829–834

NDDB (2013) Annual report 2012–2013. National Dairy Development Board, Anand, Gujarat, India

Notermans S, Dufrenne J, Teunis P, Beumer R, Te Giffel M, Weem PP (1997) A risk assessment study of *Bacillus cereus* present in pasteurised milk. Food Microbiol 14:143–151

Patel R, Dodia M, Singh SP (2005) Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: production and optimization. Process Biochem 40:3569–3575

Prüß BM, Dietrich R, Nibler B, Martlbauer E, Scherer S (1999) The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. Appl Environ Microbiol 65:5436–5442

Rajkovic A, Uyttendaele M, Vermeulen A, Andjelkovic M, Fitz-James I, In't Veld P, Denon Q, Vérhe R, Debevere J (2008) Heat resistance of *Bacillus cereus* emetic toxin, cereulide. Lett Appl Microbiol 46: 536–541

Roy A, Moktan B, Sarkar PK (2007) Characteristics of *Bacillus cereus* isolates from legume-based Indian fermented foods. Food Control 18:1555–1564

Shaheen R, Svensson B, Andersson MA, Christiansson A, Salkinoja-Salonen M (2010) Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. Food Microbiol 27:347–355

Te Giffel MC, Beumer RR, Bonestroo MH, Rombouts FM (1996a) Incidence and characterization of *Bacillus cereus* in two dairy processing plants. Neth Milk Dairy J 50:479–492

Te Giffel MC, Beumer RR, Granum PE, Leijendekkers S, Rombouts FM (1996b) Incidence of *Bacillus cereus* and *Bacillus subtilis* in food in the Netherlands. Food Microbiol 13:53–58

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Thys RCS, Lucas FS, Riffel A, Heeb P, Brandelli A (2004) Characterization of a protease of a feather-degrading *Microbacterium* species. Lett Appl Microbiol 39:181–186

Valero M, Hernandez-Herrero L, Fernandez P, Salmeron M (2002) Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. Food Microbiol 19:491–499

Warke R, Kamat A, Kamat M, Thomas P (2000) Incidence of pathogenic psychrotrophs in ice creams sold in some retail outlets in Mumbai, India. Food Control 11:77–83

WHO (2007) Food safety and food-borne illness. Fact sheet no. 237. World Health Organization, Geneva

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Review

Bacillus cereus hazard and control in industrial dairy processing environment

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ABSTRACT

Bacillus cereus is one of the most important spoilage microorganisms in dairy environment and its growth may result in various dairy defects. Moreover, it is a great safety concern for dairy industry as it is associated with incidences of food poisoning by producing enterotoxin. Because of its outstanding ability to adhere to stainless steel surfaces of dairy plant and form biofilm, *B. cereus* can lead to serious hygiene problems and economic loss due to spoilage of dairy products and equipment impairment. Biofilms are more resistant to antimicrobials and cleaning regimes compared to planktonic cells, and this makes their elimination from dairy industry a big challenge. *B. cereus* biofilms may develop particularly in storage and piping systems that are partly filled during operation or where residual liquid has remained after a production cycle. These biofilms in pasteurizer and storage tanks can be a source of post-pasteurization recurrent contamination. Cleaning-in-place (CIP) regimes, commonly used in dairy industry, showed a varied effectiveness in eliminating *B. cereus* biofilms. Optimization of alkali-based CIP significantly increases *B. cereus* biofilm cell removal, as compared to the reference CIP usually used in dairy industry. Thus, optimization of the existing cleaning processes and development of novel and effective strategies as well are of utmost importance to the dairy industry, as these may lead to quality improvement of products and processes. This review discusses the characteristics, and spoilage and toxigenic potential of *B. cereus* in dairy industry, with an emphasis on biofilm development and emerging control strategies.

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Contents

1. Introduction

Bacillus cereus comprises the largest group of endospore-forming bacteria and is now attracting a great interest among researchers as the members are not only associated with foodborne

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outbreaks but also responsible for spoilage of food products. The *B. cereus* group, also known as *B. cereus sensu lato* (*s.l.*), consists of seven closely related species, namely *B. cereus sensu stricto* (*s.s.*), *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus anthracis*, *Bacillus weihenstephanensis* and *Bacillus cytotoxicus*. Since a close genetic relationship exists among all the seven species of the *B. cereus* group (Fig. 1), it was suggested that the entire group should represent a single species (Guinebretière et al., 2013).

Although *B. cereus s.s.*, or *B. cereus* as it is usually called, is mainly associated with gastrointestinal disorders, it is an opportunistic human pathogen associated with a multitude of other infections, such as severe eye infections, periodontitis, necrotizing fasciitis, endocarditis, nosocomial acquired bacteraemia, osteomyelitis, sepsis, liver abscess, pneumonia and meningitis, particularly in postsurgical patients, immunosuppressed individuals, intravenous drug abusers and neonates. In the idiophase, it produces several compounds, like degradation enzymes, cytotoxic factors and cell-surface proteins, which might contribute to virulence. However, there is still little recognition and appreciation of the role of *B. cereus* in these serious, and frequently fatal, clinical infections in humans (Ramarao & Sanchis, 2013).

In dairy environment, *B. cereus* can negatively affect product quality. It produces various extracellular enzymes which can be responsible for a decrease in the organoleptic quality of milk and dairy products.

B. cereus can be introduced into the dairy environment from various sources during production, handling and processing, mainly from improperly cleaned and sanitized equipments. The hydrophobic properties of endospores and their resistance towards heat, desiccation and disinfectants allow them to attach to processing equipment and survive cleaning procedures (Ryu & Beuchat, 2005; Simmonds, Mossel, Intaraphan, & Deeth, 2003). Adherence to stainless steel surfaces of dairy plants can result in biofilm formation which can be an important reservoir for recurrent contamination of dairy products (Kumari & Sarkar, 2014a; Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010). An effective control of these bacteria in dairy products and processing environment is still a challenging task. As spore-forming bacteria are ubiquitous in nature, contamination has been shown to occur along the whole processing line; pasteurizer, filling machine, packaging boards and blanks can be a source of contamination (Eneroth, Svensson, Molin, & Christiansson, 2001).

The aim of this review was to give an overview of hazards caused by *B. cereus* in dairy environment and its control strategies.

2. Taxonomy

B. cereus group consists of seven closely related species, as mentioned earlier. Ecological populations of the *B. cereus* group are so closely related that they cannot be distinguished by 16S rRNA gene sequencing. Based on molecular data from fluorescent amplified fragment length polymorphism patterns, ribosomal gene sequences, partial *panC* (pantothenate synthetase) gene sequence, 'psychrotolerant' DNA sequence signatures and growth temperature, the *B. cereus* group has been divided into seven phylogenetic groups (Table 1). While all the mesophilic rhizoidal colony-forming strains of *B. mycoides* and *B. pseudomycoides* are clustered in group I, all the psychrotolerant strains of *B. mycoides*, *B. cereus s.s.* and *B. thuringinesis* along with *B. weihenstephanensis* are placed in group VI. *B. cereus s.s.* and *B. thuringinesis* are highly polyphyletic being spread over group II, III, IV, V and VI. The strains of *B. anthracis* are clustered in group III. The moderate thermotolerant *B. cytotoxicus* belongs to group VII (Guinebretière et al., 2008, 2013). Hence, the '*B. cereus* group' is an informal, however, widely used term describing a genetically highly homogeneous subdivision of the genus *Bacillus* and caters a dilemma from the taxonomic viewpoint (Stenfors Arnesen, Fagerlung, & Granum, 2008).

3. Functional morphology of cells

B. cereus is a large (1.0–1.2 μm by 3.0–5.0 μm), rod-shaped, motile, endospore-forming, aerobe-to-facultative, gram-positive bacterium which grows on common agar media, such as nutrient agar and plate count agar, to large colonies (3–8 mm dia) with a flat, greyish and 'ground-glass' appearance, often with irregular borders. On blood agar, the colonies are surrounded by zones of β-haemolysis. In selective culture media, such as mannitol-egg yolk-polymyxin (MYP) agar, polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA) and Bacara medium, *B. cereus* colonies are pink, peacock blue and orange-pink, respectively, surrounded by a halo or precipitation zone (Tallent, Kotewicz, Strain, & Bennett, 2012). Its spores are ellipsoidal, centrally or paracentrally placed and have net negative charge. The hydrophobic nature of *B. cereus* spores and presence of appendages (1–30 in number of 0.45–3.8 μm × 13.6 nm) on the surface result in firm adhesion to food processing surfaces, like stainless steel (Ankolekar & Lebbé, 2010; Tauveron, Slomianny, Henry, & Faille, 2006).

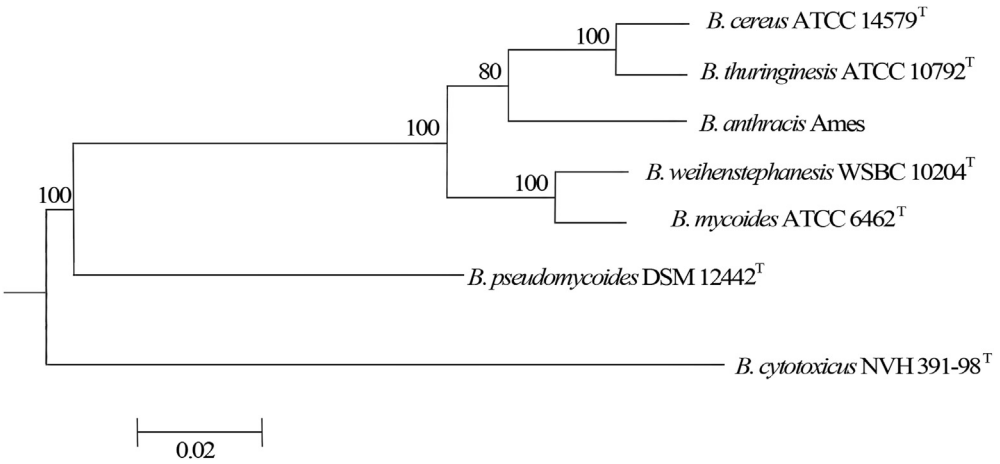

Fig. 1. The phylogenetic position of *Bacillus cereus* group species, based on concatenated sequences from genes included in MLST scheme of Tourasse, Helgason, Økstad, Hegna, and Kolstø (2006). Bootstrap values above 75% are given at each branch point. Bar, 0.02 substitutions per site (based on Guinebretière et al., 2013).

Table 1
Genetic diversity of the *Bacillus cereus* group[a].

Group	Growth temperature (°C)	Presence of psychrotolerant sequence signature[b]		% of strains associated with food poisoning	Species
		rrs	*cspA*		
I	10–43	C	–	0	*B. mycoides* *B. pseudomycoides*
II	7–40	B	–	21	*B. cereus s.s.* *B. thuringiensis*
III	15–45	C	–	33	*B. cereus s.s.* *B. thuringiensis* *B. anthracis*
IV	10–45	D	–	22	*B. cereus s.s.* *B. thuringiensis*
V	8–40	B	–	12	*B. cereus s.s.* *B. thuringiensis*
VI	5–37	A	A	0	*B. mycoides* *B. cereus s.s.* *B. thuringiensis* *B. weihenstephansis*
VII	20–50	–	–	50	*B. cytotoxicus*

[a] Based on Guinebretière et al. (2008, 2013).
[b] A, 100% positive carrying the signature in abundance; B, a high number of strains carrying the signature in abundance; C, a low number of strains with the signature in low amount; D, a low number of strains with a significant amount of the signature; –, absence of the signature.

Adhered vegetative cells and spores can act as an initiation stage for the formation of biofilm which can be a source of recurrent contamination and reduced shelf life of the product (Ryu & Beuchat, 2005). Generally, heat treatments, like pasteurization fail to effectively kill the heat-resistant endospores, limiting the possibilities of extending shelf life of pasteurized products.

Heat resistance of *B. cereus* spores is dependent on strain and sporulation medium, and D_{100} ranges from 0.075 to 2.26 min when suspended in buffer. Faille, Fontaine, and Bénézech (2001) found that the spores of *B. cereus* CUETM 98/4 isolated from a dairy product, were highly resistant to heat (D_{100} 3.32 min in whole milk). Four of the 23 dairy silo spore isolates showed an extremely high D_{75} value (> 40 min in hot alkali, pH > 13) (Shaheen et al., 2010). Heat-resistant spores can survive pasteurization, be present in the milk, and able to germinate and grow during storage, thus deteriorating the organoleptic quality and shelf life of pasteurized milk. Heat, chemicals and a decrease in pH to 2–3 can activate spores (Ghosh & Setlow, 2009). Because of the extensive use of heat as a preservation technology, heat is the most likely mechanism of spore activation in the dairy industry. Following activation, germination of *Bacillus* spores can be triggered by an interaction of specific nutrients, like L-alanine and purine ribonucleoside inosine, with germinant receptors located in the inner membrane. The *B. cereus* group expresses a core group of five germinant receptors, GerR, GerL, GerK, GerS and GerI which recognize a specific germinant as a signal of conditions suitable for growth (van der Voort, García, Moezelaar, & Abee, 2010). Subsequent activation of a transporter mediates transport of Ca-dipicolinate out of the spore with concomitant influx of water. Adhered spores show identical germination properties when compared to spores in suspension. Thus, an additional germination step using L-alanine/inosine-like germinant mixtures in cleaning regime can be an effective measure to reduce the number of adhered spores (Hornstra et al., 2007).

4. Ecology

The natural reservoir for *B. cereus* consists of decaying organic matter, fresh and marine waters, vegetables, fomites and the intestinal flora of different animals, from which soil and food products including milk and dairy products may become contaminated, leading to a transient colonization of the human intestine. Because of adhesive nature of its endospores, *B. cereus* is frequently present in food production environments. This character enables the bacterium to spread to different kinds of food, such as milk and dairy products, vegetables, rice and rice dishes, meat and meat products, soups and spices (te Giffel, Beumer, Bonestroo, & Rombouts, 1996; Organji, Abulreesh, Elbanna, Osman, & Khider, 2015).

5. Toxigenicity

There are two distinct syndromes of emesis and diarrhoea, caused by different types of toxins produced by *B. cereus* (Table 2). The rapid onset of the emetic disease, generally within 5 h after consumption of a meal, indicates that this is due to a toxin preformed in the food. This toxin is not inactivated during food processing or gastrointestinal passage due to its high resistance against heat treatments, pH extremes and proteolytic degradation (Rajkovic et al., 2008). Cereulide, the emetic toxin, is a cyclic 1.2-kDa dodecadepsipeptide [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$ resembling valinomycin and produced by a nonribosomal peptide synthetase, encoded by the 24-kb cereulide synthetase (*ces*) gene cluster which is located on a 208-kb pXO1-like megaplasmid (Ehling-Schulz et al., 2006). Animal model study has shown that 5-hydroxytryptamine 3 (a receptor), released from the stomach into the duodenum, binds with cereulide to stimulate vagus afferent causing vomiting (Agata, Ohta, Mori, & Isobe, 1995).

Diarrhoeal syndrome, which develops 8–16 h after ingestion of the contaminated food, has been linked to two enterotoxin complexes, haemolysin BL (Hbl) and nonhaemolytic enterotoxin (Nhe), and a single protein, cytotoxin K (CytK) (Beecher, Schoeni, & Wong, 1995; Lund, De Buyser, & Granum, 2000). Hbl complex consists of three proteins, L_2, L_1 and B, encoded by the *hbl* operon. The optimal ratio of components L_2, L_1 and B for maximal toxicity is 1:1:1. The components independently bind to the cell membrane and after association and pore formation, osmotic cell lysis occurs (Beecher & Wong, 1997). Nhe complex, the most important toxin in food poisoning, is composed of NheA (cytolytic protein), NheB and NheC (binding proteins), encoded by the *nhe* operon. All the three

Table 2
Characteristics of food poisoning caused and toxins produced by *Bacillus cereus*[a].

Characteristics	Emetic syndrome	Diarrhoeal syndrome
Primary cause	Preformed toxin in food	Ingestion of vegetative cells and spores and toxin production in small intestine
Foods mostly involved	Starch-rich foods: rice, pasta, potato, pastry and noodles	Proteinaceous foods: milk and dairy products, meat products, pudding, soups, sauces and vegetables
Symptoms	Nausea, vomiting, malaise and in some cases fatal liver failure	Watery diarrhoea and abdominal pain
Incubation time	≤5 h	8–16 h
Period of illness	6–24 h	12–24 h
Infective dose	0.02–1.83 μg/kg body weight (10^5–10^8 cells/g implicated food)	10^5–10^8 cfu/g
Toxin associated	Cereulide	Haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK)
Heat sensitivity of toxin	Extremely stable (even 121 °C for 90 min)	Labile
Genes	Cereulide synthetase (Ces) gene cluster (*cesHPTABCD* genes) *cesA* (10 kb) and *cesB* (8 kb): structural genes *cesP*: 4′-phosphopantetheinyl transferase *cesT*: a thioesterase *cesC* and *cesD*: ABC transporter	Hbl complex (*hblCDA* operon) *hblC*: L_2 (46 kDa) *hblD*: L_1(38 kDa) *hblA*: B (37 kDa) Nhe complex (*nheABC* operon) *nheA*: NheA (41 kDa) *nheB*: NheB (40 kDa) *nheC*: NheC (36 kDa) *cytK*: CytK (34 kDa)
Genetic regulation	By Spo0A and AbrB	By PlcR
Mode of action	Acts as K-ionophore to inhibit fatty acid oxidation in mitochondria Binds to serotonin 5-hydroxytryptamine 3 (5-HT3) receptors, stimulates vagus afferent resulting in vomiting Brain oedema and fulminate liver failure due to inhibition of mitochondrial fatty acid oxidation Inhibits natural killer cells (T cells) of human immune system	Hbl shows haemolytic, cytotoxic, dermonecrotic and vascular permeability activity Nhe is pore-forming toxin, structurally similar to Hbl CytK is dermonecrotic, cytotoxic and haemolytic

[a] Based on Ehling-Schulz et al. (2006), Fagerlund et al. (2004), Granum and Lund (1997), Stenfors Arnesen et al. (2008).

components are required for biological activity; maximal toxic effects are exerted when their ratio is 10:10:1. The cytotoxic activity of Nhe on epithelial cells has been shown to be due to colloid osmotic lysis following pore formation in the plasma membrane (Lindbäck et al., 2010). CytK is cytotoxic due to its pore-forming ability in the cell membranes. This heptamer protein toxin is secreted in a soluble form, which is eventually converted into a transmembrane pore by the assembly of an oligomeric β-barrel, with the hydrophobic residues facing the lipids and the hydrophilic residues facing the lumen of the channel. The toxin occurs in two forms that have 89% amino acid sequence homology, CytK-1 and CytK-2 (Fagerlund, Ween, Lund, Hardy, & Granum, 2004).

6. Contamination and risk in the dairy production chain

Soil, feed (through excretion of spores in faeces) and bedding material are the major source of *B. cereus* contamination of raw milk (Fig. 2). The *B. cereus* spore concentration in raw milk is higher in summer than in winter (Bartoszewicz, Hansen, & Swiecicka, 2008). Their main contamination route to milk during the grazing season (summer) is via cow's udder contaminated by soil and faeces (Christiansson, Bertilsson, & Svensson, 1999). In the housing period of cows, feed is the only source of spores; udder becomes contaminated mainly through the bedding material that is contaminated with faeces. Milking equipment can also be a source of contamination for raw milk (Christiansson, Ekelund, & Ogura, 1997). *B. cereus* occurs in low numbers (10^2–10^3/l) in farm-collected milk (Bartoszewicz et al., 2008; Svensson, Ekelund, Ogura, & Christiansson, 2004; Svensson et al., 2006). However, higher levels of *B. cereus* contamination (up to 10^4 cfu/ml) in pasteurized milk (Table 3) have been reported (te Giffel et al., 1996; Kumari & Sarkar, 2014b; Larsen & Jørgensen, 1997). Studies by global typing methods, such as fatty acid profiling, biochemical typing, RAPD-PCR and rep-PCR fingerprinting, have shown that the distribution of genotypes in the dairy and its products differed from that in raw milk (Bartoszewicz et al., 2008; te Giffel, Beumer, Granum, & Rombouts, 1997; Svensson et al., 2004). Thus, the farms are not the sole source of *B. cereus* in dairy milk. In addition, it is likely that 'in-house' microbial flora present in silo tanks, pasteurizers and filling machines could contribute to post-pasteurization contamination of milk (Svensson et al., 2004). Contamination of milk with *B. cereus* by post-pasteurization surface exposures can be demonstrated by using automated ribotyping. In a study, seven ribotypes were identified, demonstrating the genetic

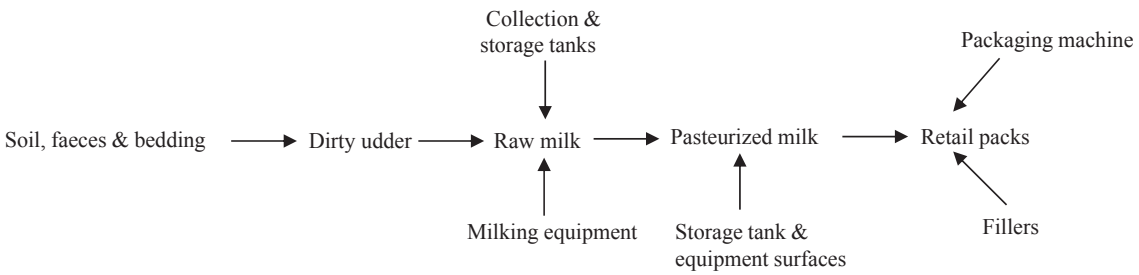

Fig. 2. Contamination routes of *Bacillus cereus* in dairy production chain (based on Heyndrickx, 2011).

Table 3
Incidence of *Bacillus cereus* in dairy products.

Type	Country	Positive samples (%)	*B. cereus* population (cfu/ml or g)	Reference
Pasteurized/sterilized milk	India	55	$10–10^4$	Kumari and Sarkar (2014b)
	Brazil	–	0.44–70	Salustiano et al. (2009)
	Thailand	100	$10^2–10^3$	Chitov, Dispan, and Kasinrerk (2008)
	Poland	–	0.04–0.17	Bartoszewicz et al. (2008)
	China	71	11.7 MPN	Zhou, Liu, He, Yuan, and Yuan (2008)
	Netherlands	35	$10–10^4$	te Giffel et al. (1996)
	Denmark	47	$10^3–10^5$	Larsen and Jørgensen (1997)
	Spain	25	$10^3–10^5$	van Netten, van de Moosdjik, van Hoensel, Mossel, and Perales (1990)
Dried milk product	Chile	46	$3–10^4$	Reyes, Bastías, Gutiérrez, de la, and Rodríguez (2007)
	India	52	$10^2–10^3$	Kumari and Sarkar (2014b)
Ice cream	India	40	$10^2–10^8$	Kumari and Sarkar (2014b)
	India	40	$10–10^3$	Warke, Kamat, Kamat, and Thomas (2000)
Cheese	Turkey	12	–	Molva, Sudagidan, and Okuklu (2009)
	India	33	$10^2–10^6$	Kumari and Sarkar (2014b)
Raw (untreated) milk	Egypt	30	9×10^2	Hassan, Al-Ashmawy, Meshref, and Afify (2010)
	India	66	10^5	Bedi, Sharma, Gill, Aulakh, and Sharma (2005)
	Sweden	–	0.198	Svensson et al. (2004)

variability of the *B. cereus* group, isolated from pasteurized milk and different surfaces. Surfaces responsible for major contamination of pasteurized milk were pasteurized milk storage tank, packaging machine, levelling tank and the package-forming tube surfaces. Most of the isolates belonged to the same ribogroup (RIBO1222-73-S4), and they were found on four surfaces and also in the milk, indicating the role of the equipment surfaces as reservoirs for milk recontamination (Salustiano et al., 2009).

Hazard analysis and critical control point (HACCP) in dairy processing lines is required to minimize the initial load of *B. cereus* in finished products (Fig. 3). Examination of raw milk, from sensorial, physicochemical and microbiological points of view at the reception, pasteurization, temperature of pasteurized milk storage tank, maintenance of aseptic conditions during packaging and storage temperature of the packaged products are the critical control points (CCPs) in dairy industry. For each step, critical control limit should be set and monitored. The maximum levels of *B. cereus* spores in raw milk and finished dairy products should be 10^3/l and 100/g, respectively (EU, 2005).

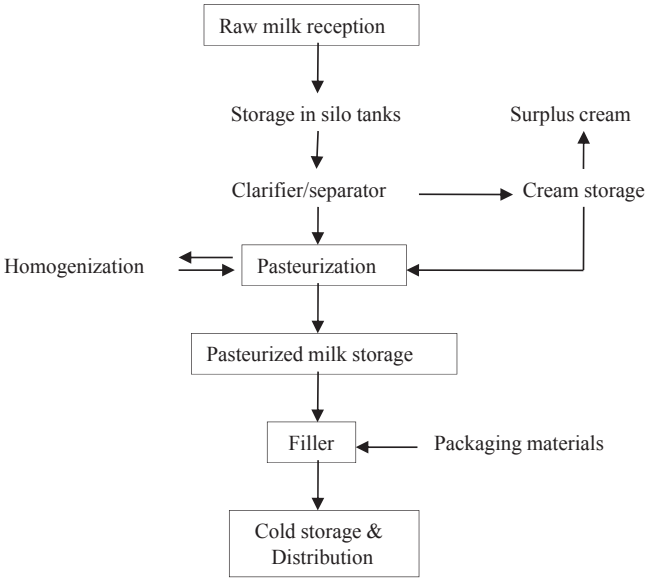

Fig. 3. Flow diagram and critical control points (CCPs) for pasteurized milk. The text within a box indicates CCP (based on ANZDAC, 2011).

7. Role in toxigenicity and spoilage of dairy products

In dairy industry, sporeformers are important contaminants because they can significantly affect product safety and quality. The occurrence of emetic and diarrhoeal toxin-producing strains of *B. cereus* in dairy production chain has been extensively reported. Majority of the strains, isolated from dairy products, are cytotoxic and PCR positive for *nhe* and *hbl* (Borge, Skeie, Sørhaug, Langsrud, & Granum, 2001). Growth temperature affects toxin production in *B. cereus* group species in a strain-dependent manner. In an investigation of food poisoning potential of *B. cereus* strains from Norwegian dairies, while some of the 39 strains were moderately or highly cytotoxic when grown at 25 °C or 32 °C, none of those were highly toxic at human body temperature, i.e. 37 °C. Hence, those strains should be considered to pose a minor risk with regard to diarrhoeal food poisoning (Stenfors Arnesen, O'Sullivan, & Granum, 2007). The occurrence of emetic toxin-producing strains in milk and other sources at the farms is rare (1.9%) (Svensson et al., 2006). Toxin profile of *B. cereus* group from the silo tanks is dominated by strains with the toxin profile 'C' (nhe^+, hbl^+, $cytK^-$ and ces^-) and 'F' (nhe^+, hbl^-, $cytK^-$ and ces^-) (Ehling-Schulz et al., 2006; Svensson et al., 2004).

Members of the *B. cereus* group and *Bacillus subtilis* group are the most important spoilage microorganisms in dairy environment (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013). They produce various extracellular enzymes which contribute to the reduction of shelf life of processed milk and dairy products by degrading milk components and additives (Table 4). The presence of protease can lead to bitter flavour, clotting and gelation of milk (Chen, Daniel, & Coolbear, 2003; Furtado, 2005). The proteolytic changes caused by *Bacillus* spp. significantly increase the concentration of free tyrosine, which can increase in milk up to 2.13 mg/ml in comparison with their initial value of approximately 0.65 mg/ml (Janštová, Dračková, & Vorlová, 2006). On the other hand, lipases have been responsible for dairy defects, such as bitty cream, and also contribute to unpleasant flavour, such as rancid, butyric, buttery, unclean and soapy in milk and dairy products (Furtado, 2005). Lipolysis is known to contribute both desirable and undesirable flavours to dairy products, initially through hydrolysis of milk triacylglycerols. Short-chain fatty acids, such as butyric acid, caproic acid and caprylic acid, give a sharp and tangy flavour. Medium-chain fatty acids, such as capric acid and lauric acid, tend to impart a soapy taste, while long-chain fatty acids, such as myristic acid, palmitic acid and stearic acid, contribute little to flavour.

Table 4
Effects of enzymes produced by *Bacillus cereus* on the organoleptic quality of milk and dairy products[a].

Enzyme	Principle of action	Product	Quality defect
Protease	Hydrolysis of casein, resulting in the formation of gel (coagulation)	Pasteurized/sterilized milk	Sweet curdling, off-flavour, bitter, foreign, unclean
		Cream/butter	Off-flavours
		Cheese	Lower yield, shorter coagulation time due to higher concentration of free amino acids which stimulate the growth of starter culture
		Milk powder	Bitterness
Lipase	Hydrolysis of milk triacylglycerols, resulting in high concentration of free fatty acids	Pasteurized/sterilized milk, cream, butter	Rancidity, off-flavour, bitterness, soapy
		Cheese	Rancidity, off-flavour, longer coagulation time as free fatty acids inhibit the growth of starter culture
		Milk powder	Rancidity
Lecithinase and phospholipases	Disruption of membrane structure of fat globules, resulting in degradation of milk fat	Milk	Bitty cream

[a] Based on Chen et al. (2003); Samaržija, Zamberlin, & Pogačić (2012).

Unsaturated fatty acids, released during lipolysis, are susceptible to oxidation and concomitant formation of aldehydes and ketones which give rise to off-flavour. Other unpleasant flavours, such as rancid, butyric, bitter, unclean, soapy and astringency in milk and dairy products have also been attributed to lipolysis. *B. cereus* isolates from different dairy products are able to produce protease and lipase which are sufficiently thermostable, remain active after pasteurization and may cause defects in the final products (Kumari & Sarkar, 2014b; Lücking et al., 2013).

8. Biofilm and its hazard in the dairy environment

In milk storage and dairy processing operations, besides being present in raw material, bacteria are associated with surfaces (Mittelman, 1998). The attachment of bacteria with subsequent development of biofilms in milk processing environments is a potential source of contamination of finished products that may shorten the shelf life or facilitate transmission of diseases (Brooks & Flint, 2008). Thus, biofilm formation in dairy processing environment can lead to serious hygiene problems and economic loss due to spoilage of dairy products and equipment impairment (Bremer, Fillery, & McQuillan, 2006). Formation of bacterial biofilms within food processing plants is a matter of great concern to processors, as bacteria within biofilms are more difficult to be eliminated than planktonic cells, and can act as a source of recurrent contamination to plant, product and personnel. Biofilm renders its inhabitants resistant to antimicrobial agents and cleaning (Srey, Jahid, & Ha, 2013). Other undesirable conditions associated with biofilms include reduced flow through blocked tubes, reduced plant run times, corrosion of stainless steel and reduced heat transfer through plate heat exchangers (Parkar, Flint, & Brooks, 2004).

Biofilm is a sessile microbial community adhered to a solid surface surrounded by a matrix which includes extracellular polymeric substances (EPS). Dairy biofilms are predominated by bacterial EPS and milk residues, mostly proteins and calcium phosphate (Flint, Bremer, & Brooks, 1997; Mittelman, 1998). The biofilm formation is a stepwise and dynamic process consisting of initial reversible attachment of cells, their irreversible attachment, formation of microclonies, maturation and dispersion (Fig. 4). Attachment of microorganisms to surfaces and subsequent biofilm development are complex processes, and affected by the surface properties of attachment site and cell. *B. cereus* forms a considerably higher amount of biofilm on stainless steel compared to polystyrene in terms of total biomass and number of cells attached (Hayrapetyan, Muller, Tempelaars, Abee, & Groot, 2015). The observation triggered them to study the impact of iron, which is the main component in stainless steel, on biofilm formation. Although iron does not promote initial attachment of cells to the surface, its role in surface-associated behaviour, such as cell–cell interaction, is more likely than cell–substratum interaction or production of matrix components in the biofilm. Attachment occurs more readily on surfaces that are rougher, more hydrophobic and coated by surface conditioning films (Simões, Simões, Cleto, Pereira, & Vieira, 2008). In dairy operations, the conditioning film mainly consists of organic milk components. The cell surface properties, particularly cell surface hydrophobicity and presence of extracellular filamentous appendages may influence the rate and extent of microbial attachment (Donlan, 2002). Among the various physiological stages of *B. cereus*, spores have the greatest biofilm formation potential (Pagedar & Singh, 2012). CalY and TasA, coded by gene *calY* and operon *sipW-tasA*, respectively, cooperate to assemble robust and stable fibres with amyloid properties and are important for *B. cereus* biofilm assembly (Caro-Astorga, Pérez-García, de Vicente, & Romero, 2015). Adhesion of *B. cereus* to stainless steel surface increases with the increase in temperature, pH and time (Peña et al., 2014).

The ability of *B. cereus* spores to adhere and act as an initiation stage for biofilm formation on food processing plants is well known. The strong adhesion properties of *B. cereus* spores have been attributed to the hydrophobic character of exosporium which varies from species to species and to the presence of appendages on the surface of spores. BclA is the major glycoprotein of the *B. cereus* exosporium and plays an important role in spore interaction with materials, probably by providing a larger contact surface with stainless steel (Faille et al., 2001; Lequette et al., 2011; Peng, Tsai, & Chou, 2001; Tauveron et al., 2006).

A thicker biofilm of *B. cereus* develops at an air-liquid interface, compared to a submerged system. This suggests that *B. cereus* biofilms may develop particularly in industrial storage and piping systems which are partly filled during operation or where residual liquid has remained after a production cycle (Wijman, de Leeuw, Moezelaar, Zwietering, & Abee, 2007). While for most of the *Bacillus* strains, negative effects of whole milk on biofilm formation have been observed (Flint et al., 1997), the study of Shaheen et al. (2010) showed that *B. cereus* was capable of forming biofilms in whole milk, but not in water-diluted milk. Whole milk contains natural surfactants, and also phospholipid is a surface active compound which might work as a surfactant needed for the biofilm of

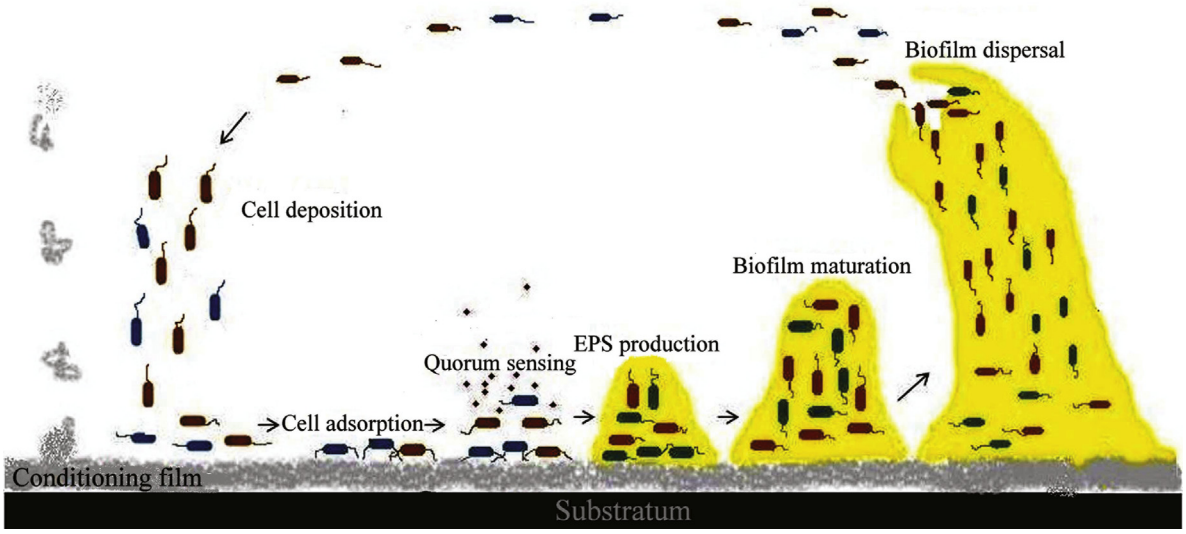

Fig. 4. Processes governing biofilm formation (based on Breyers & Ratner, 2004).

certain strains of *B. cereus*. *B. cereus* biofilm formation is enhanced under low nutrient conditions and dependent on biosurfactant production, which can be directly or indirectly repressed by PlcR, a pleiotropic regulator which controls the expression of a variety of genes, many of which encode potential virulence factors, including enterotoxins, haemolysins, phospholipase C and proteases (Hsueh, Somers, Lereclus, & Wong, 2006).

Process biofilms are common in dairy industry where a single species predominates due to reduction of competition as a result of heat treatment of milk; 12.4% of the microbiota growing in biofilms in a commercial dairy plant belonged to *B. cereus* (Sharma & Anand, 2002). Biofilm formation even at lower temperatures can be a matter of concern. Majority (71–90%) of the 144 isolates from milk, cheese and ice cream were able to form biofilm even at 4 °C, while in butter, 100% of the isolates were positive (Kumari & Sarkar, 2014b). The presence of *B. cereus* biofilm in dairy processing lines and chilling tanks can be a source of post-pasteurization contamination. From various *in vitro* model studies it is evident that *B. cereus* biofilms are present even on internal surfaces of chilling tanks, and *B. cereus* cell count in the biofilm developed on the surface of stainless steel chilling tanks can reach up to 10^6 cfu/cm^2, if inadequately cleaned tanker is left to stand empty at room temperature (Kumari & Sarkar, 2014a; Teh et al., 2012). Thus, the presence of biofilm on internal surfaces of chilling tanks can lead to recurrent contamination and consequent spoilage of fresh lot of milk collected into the tank.

9. Biofilm control strategies

Biofilms are more resistant to antimicrobials and cleaning regimes compared to planktonic cells, and this makes their elimination from dairy industry a big challenge (Srey et al., 2013). *B. cereus* has been found to be involved in biofilm formation in different dairy processing surfaces. Unfortunately, both spores and vegetative cells of bacteria embedded in biofilms are a matter of great concern because of their increased resistance to cleaning and disinfection procedures. In dairy, like any other food industry, an effective cleaning and sanitation program is part of the process to eliminate microorganisms. Cleaning-in-place (CIP) is a process of cleaning the interior surface of tanks, pasteurizers, pipelines, process equipment and associated things without dismantling them (Thomas & Sathian, 2014). CIP procedures are usually employed in milk processing lines (Table 5). In dairy industry, CIP systems generally involve the sequential use of caustic (sodium hydroxide) and acid (nitric acid) wash steps, selected for their ability to remove organic (protein and fat) and inorganic (calcium and other minerals) fouling layers. The most common and aggressive caustic cleaner is sodium hydroxide (NaOH), which is typically used in 1–5% concentrations for plate-type and tubular heat exchangers, and other heavily soiled surfaces and 1–2% for general use (Flint et al., 1997). The primary role of the caustic (alkali) wash step is the removal of proteins and carbohydrates. To enhance cleaning effectiveness, caustic detergents and additives have been developed, which contain surfactants, emulsifying agents, chelating compounds or complexing agents (Bremer et al., 2006). Traditionally, chlorine (sodium hypochlorite)-based sanitizers have been used, however, a wide variety of sanitizers including quaternary ammonium compounds, anionic acids, iodophores and chlorine-based compounds are currently in use or being evaluated for use in CIP systems. The combined effects of the biocide, benzyldimethyldodecyl ammonium chloride with a series of increasing Reynolds number of agitation promote *B. cereus* biofilm removal from stainless steel surfaces (Lemos, Gomes, Mergulhão, Melo, & Simões, 2015).

Different studies simulating CIP regimes in dairy industry showed varied results. A CIP regime against dairy biofilms (water rinse – 1% sodium hydroxide at 70 °C for 10 min – water rinse - 0.8% nitric acid at 70 °C for 10 min – water rinse) followed by exposure to either chlorine or combinations of nisin, lauricidin and lactoperoxidase system for defined exposure periods was inefficient in the total biofilm control (maximum log reduction of 2) (Dufour, Simmonds, & Bremer, 2004). The additional antimicrobial treatment resulted in maximum log reduction of 2.8, verified 2 h after chlorine exposure. The standard CIP regime (water rinse – 1% sodium hydroxide at 65 °C for 10 min – 1% nitric acid for 10 min – water rinse), commonly followed in dairy industry, is inefficient to remove bacterial cells attached to surfaces, as only removal of 2 log reduction in cell numbers can be achieved (Bremer et al., 2006). The germination response of *B. cereus* adhered spores could be an additional strategy to improve commonly used CIP regime (water

Table 5
Cleaning-in-place (CIP) regimes in dairy environment for *Bacillus cereus* biofilm cell removal.

Regime	Biofilm-forming strain	Reduction in biofilm cells (log cfu/cm^2)	Reference
Phosphate buffer (pH 10) + subtilisin P2 at 45 °C for 30 min	BC98/4	0.92	Lequette et al. (2010)
Realco B (pH 10, containing anionic surfactant, dispersing and chelating agents) + subtilisin P2 at 45 °C for 30 min	BC98/4	0.56	Lequette et al. (2010)
1% NaOH at 65 °C for 10 min – 1% HNO_3 at 65 °C for 10 min	Consortium	2	Bremer et al. (2006)
1% NaOH at 65 °C for 10 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse	PT4	3.29	Kumari and Sarkar (2014a)
1.5% NaOH at 65 °C for 30 min – water rinse –1% HNO_3 at 65 °C for 10 min – water rinse	PT4	4.77	Kumari and Sarkar (2014a)
Water rinse – 0.5% NaOH at 60 °C for 15 min – water rinse	CUETM98/4	0.98	Faille et al. (2014)

rinse – 1% sodium hydroxide at 65 °C for 10 min – 1% nitric acid for 10 min – water rinse) in dairy industry (Hornstra et al., 2007). Implementation of a germination-inducing step in CIP helps to reduce the number of spores attached to processing equipment surfaces and additional 3 to 4 log unit removal of cells can be achieved. Thus, optimization of the existing CIP regimes is very important to achieve maximum biofilm removal. Recently, response surface methodology (RSM) was used to optimize parameters influencing mono-species biofilm cell removal (Kumari & Sarkar, 2014a). Optimized CIP regime (1.5% NaOH at 65 °C for 30 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse) achieved 4.77 log reduction/cm^2 in biofilm cell count, as compared to reference CIP (1% NaOH at 65 °C for 10 min – water rinse – 1% HNO_3 at 65 °C for 10 min – water rinse) which caused only 3.29 log reduction/cm^2 in *B. cereus* biofilm cells. CIP regimes commonly used in dairy industry showed a varied effectiveness in eliminating biofilms, which may be due to the resistance of biofilms to the chemical and physical treatments applied during cleaning and sanitizing procedures in the food industry (Chmielewski & Frank, 2003). Therefore, the use of enzyme treatments to break down EPS in biofilms is a prospective alternative when standard cleaning agents do not give satisfactory results in removing biofilms. A cleaning regime should break-up or dissolve the EPS matrix associated with biofilm, so that disinfectants can gain access to bacterial cells (Simões, Simões, Machado, Pereira, & Vieira, 2006). Proteases and polysaccharidases can be used as potential agents for biofilm removal (Meyer, 2003). Proteases in commercially available detergents are already used to clean, for example, ultrafiltration units, contact lenses, medical apparatus and laundry. A combination of proteolytic enzymes with surfactants increases the wetability of biofilms formed by a thermophilic *Bacillus* sp., and therefore, an enhanced cleaning efficiency can be achieved (Lequette, Boels, Clarisse, & Faille, 2010; Parkar et al., 2004). Thus, a significant benefit could be achieved by replacing caustic-based cleaning solutions with enzymes. This is because in contrast to concentrated NaOH and other caustic detergents, enzymes are non-corrosive and their use leads to reduced rinsing volumes and easier disposal without neutralization. However, specific mode of action and lower prices of the chemicals currently used compared to the costs of enzymes are the major limitations in their current usage against different types of biofilms. Thus, further industrial-scale studies are necessary for optimization of the enzyme concentration of the cleaning solution, and to confirm economic feasibility (Boyce, Piterina, & Walsh, 2010; Simões, Simões, & Vieira, 2010).

A novel approach for the prevention of surface biofilm attachment using biomimetic superhydrophobic surfaces formed via the self-assembly of paraffin and fluorinated wax crystals showed an exceptional ability to inhibit biofilm formation by *B. cereus*. 3D crystalline wax surfaces form a heterogeneous surface that combines wax and air pockets, reducing the contact area between a bacterial cell and the surface and thereby interrupting bacterial adhesion, thus preventing the initial step of biofilm formation (Pechook et al., 2015).

10. Outlook

To limit the presence of *B. cereus* in dairy processing environment there is a need to gain a better insight into the whole contamination flow of endospore-formers originating from soil as well as in the conditions permitting their proliferation. Good manufacturing practices (GMPs) in farms during the production and storage of milk should be implemented and strategies aiming at reducing the population of spore-forming bacteria in raw milk should be reinforced. Better implementation of HACCP in dairy processing lines should be given importance so that the initial load in finished products could be minimized. CCPs, such as storage temperature and time, should be properly defined. More research on better understanding of the structure of *B. cereus* biofilms within the context of milk processing environment is needed to develop better CIP regimes for eliminating biofilm from dairy processing lines. Furthermore, optimization of the existing cleaning processes and development of novel and effective strategies are of great importance to the dairy industry as these may lead to quality improvements of products and processes. Future research could also focus on coating strategies to reduce microbial attachment on dairy equipment, use of enzymes and food grade quorum inhibitors as an intervention strategy can offer new opportunities for the dairy industry in the coming years.

References

Agata, N., Ohta, M., Mori, M., & Isobe, M. (1995). A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiology Letters, 129*(1), 17–19.

Ankolekar, C., & Lebbé, R. G. (2010). Physical characteristics of spores of food-associated isolates of the *Bacillus cereus* group. *Applied and Environmental Microbiology, 76*(3), 982–984.

ANZDAC. (2011). *Pathogen managemnt guidelines*. Australia-New Zealand Dairy Authorities' Committee, 33 pp.

Bartoszewicz, M., Hansen, B. M., & Swiecicka, I. (2008). The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. *Food Microbiology, 25*(4), 588–596.

Bedi, S. K., Sharma, C. S., Gill, J. P. S., Aulakh, R. S., & Sharma, J. K. (2005). Incidence of enterotoxigenic *Bacillus cereus* in milk and milk products. *Journal of Food Science and Technology, 42*(3), 272–275.

Beecher, D. J., Schoeni, J. L., & Wong, A. C. (1995). Enterotoxic activity of hemolysin BL from *Bacillus cereus*. *Infection and Immunity, 63*(11), 4423–4428.

Beecher, D. J., & Wong, A. C. L. (1997). Tripartite hemolysin BL from *Bacillus cereus*: hemolytic analysis of component interactions and a model for its characteristic paradoxical zone phenomenon. *Journal of Biological Chemistry, 272*(1), 233–239.

Borge, G. I. A., Skeie, M., Sørhaug, T., Langsrud, T., & Granum, P. E. (2001). Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. *International Journal of Food Microbiology, 69*(3), 237–246.

Boyce, A., Piterina, A. V., & Walsh, G. (2010). Assessment of the potential suitability of selected commercially available enzymes for cleaning-in-place (CIP) in the dairy industry. *Biofouling, 26*(7), 837–850.

Bremer, P. J., Fillery, S., & McQuillan, A. J. (2006). Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on

the removal of dairy biofilms. *International Journal of Food Microbiology, 106*(3), 254–262.
Breyers, J. D., & Ratner, B. D. (2004). Bioinspired implant materials befuddle bacteria. *ASM News, 70*(5), 232–237.
Brooks, J. D., & Flint, S. H. (2008). Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science and Technology, 43*(12), 2163–2176.
Caro-Astorga, J., Pérez-García, A., de Vicente, A., & Romero, D. (2015). A genomic region involved in the formation of adhesion fibers in *Bacillus cereus* biofilms. *Frontiers in Microbiology, 5*, 745.
Chen, L., Daniel, R. M., & Coolbear, T. (2003). Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal, 13*(4), 255–275.
Chitov, T., Dispan, R., & Kasinrerk, W. (2008). Incidence and diarrhegenic potential of *Bacillus cereus* in pasteurized milk and cereal products in Thailand. *Journal of Food Safety, 28*(4), 467–481.
Chmielewski, R. A. N., & Frank, J. F. (2003). Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety, 2*(1), 22–32.
Christiansson, A., Bertilsson, J., & Svensson, B. (1999). *Bacillus cereus* spores in raw milk: factors affecting the contamination of milk during the grazing period. *Journal of Diary Science, 82*(2), 305–314.
Christiansson, A., Ekelund, K., & Ogura, H. (1997). Membrane filtration method for enumeration and isolation of spores of *Bacillus cereus* from milk. *International Dairy Journal, 7*(11), 743–748.
Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging Infectious Diseases, 8*(9), 881–890.
Dufour, M., Simmonds, R. S., & Bremer, P. J. (2004). Development of a laboratory scale clean-in-place system to test the effectiveness of "natural" antimicrobials against dairy biofilms. *Journal of Food Protection, 67*(7), 1438–1443.
Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M., & Scherer, S. (2006). Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. *BMC Microbiology, 6*, 20.
Eneroth, Å., Svensson, B., Molin, G., & Christiansson, A. (2001). Contamination of pasteurized milk by *Bacillus cereus* in the filling machine. *Journal of Dairy Research, 68*(2), 189–196.
EU. (2005). Commission Regulation (EC) No.2076/2005 of 5 December 2005 laying down transitional arrangements for the implementation of regulations (EC) no. 853/2004, (EC) no. 854/2004 and (EC) no.853/2004 and (EC) no.854/2004. *Official Journal of the European Commission, L338*, 83–88.
Fagerlund, A., Ween, O., Lund, T., Hardy, S. P., & Granum, P. E. (2004). Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. *Microbiology, 150*, 2689–2697.
Faille, C., Bénézech, T., Midelet-Bourdin, G., Lequette, Y., Clarisse, M., Ronse, G., et al. (2014). Sporulation of *Bacillus* spp. within biofilms: a potential source of contamination in food processing environments. *Food Microbiology, 40*, 64–74.
Faille, C., Fontaine, F., & Bénézech, T. (2001). Potential occurrence of adhering living *Bacillus* spores in milk product processing lines. *Journal of Applied Microbiology, 90*(6), 892–900.
Flint, S. H., Bremer, P. J., & Brooks, J. D. (1997). Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling, 11*(1), 81–97.
Furtado, M. M. (2005). *Main problems of cheeses: Causes and prevention*. São Paulo, Brazil: Fonte Press.
Ghosh, S., & Setlow, P. (2009). Isolation and characterization of superdormant spores of *Bacillus* species. *Journal of Bacteriology, 191*(6), 1787–1797.
te Giffel, M. C., Beumer, R. R., Granum, P. E., & Rombouts, F. M. (1997). Isolation and characterisation of *Bacillus cereus* from pasteurised milk in household refrigerators in the Netherlands. *International Journal of Food Microbiology, 34*(3), 307–318.
te Giffel, M. C., Beumer, R. R., Leijendekkers, S., & Rombouts, F. M. (1996). Incidence of *Bacillus cereus* and *Bacillus subtilis* in foods in the Netherlands. *Food Microbiology, 13*(1), 53–58.
Granum, P. E., & Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters, 157*(2), 223–228.
Guinebretière, M. H., Auger, S., Galleron, N., Contzen, M., De Sarrau, B., De Buyser, M. L., et al. (2013). *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. *International Journal of Systematic and Evolutionary Microbiology, 63*(1), 31–40.
Guinebretière, M. H., Thompson, F. L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., et al. (2008). Ecological diversification in the *Bacillus cereus* group. *Environmental Microbiology, 10*(4), 851–865.
Hassan, G. M., Al-Ashmawy, M. A. M., Meshref, A. M. S., & Afify, S. I. (2010). Studies on enterotoxigenic *Bacillus cereus* in raw milk and some dairy products. *Journal of Food Safety, 30*(3), 569–583.
Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T., & Groot, M. N. (2015). Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. *International Journal of Food Microbiology, 200*, 72–79.
Hendrickx, M. (2011). The importance of endospore-forming bacteria originating from soil for contamination of industrial food processing. *Applied and Environmental Soil Science, 2011*. Article ID 561975, 11 pages.
Hornstra, L. M., de Leeuw, P. L. A., Moezelaar, R., Wolbert, E. J., de Vries, Y. P., de Vos, W. M., et al. (2007). Germination of *Bacillus cereus* spores adhered to stainless steel. *International Journal of Food Microbiology, 116*(3), 367–371.
Hsueh, Y. H., Somers, E. B., Lereclus, D., & Wong, A. C. L. (2006). Biofilm formation by *Bacillus cereus* is influenced by PlcR, a pleiotropic regulator. *Applied and Environmental Microbiology, 72*(7), 5089–5092.
Janštová, B., Dračková, M., & Vorlová, L. (2006). Effect of *Bacillus cereus* enzymes on milk quality following ultra high temperature processing. *Acta Veterinaria Brno, 75*(4), 601–609.
Kumari, S., & Sarkar, P. K. (2014a). *In vitro* model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology. *Food Control, 36*(1), 153–158.
Kumari, S., & Sarkar, P. K. (2014b). Prevalence and characterization of *Bacillus cereus* group from various marketed dairy products in India. *Dairy Science and Technology, 94*(5), 483–497.
Larsen, H. D., & Jørgensen, K. (1997). The occurrence of *Bacillus cereus* in Danish pasteurized milk. *International Journal of Food Microbiology, 34*(2), 179–186.
Lemos, M., Gomes, I., Mergulhão, F., Melo, L., & Simões, M. (2015). The effects of surface type on the removal of *Bacillus cereus* and *Pseudomonas fluorescens* single and dual species biofilms. *Food and Bioproducts Processing, 93*, 234–241.
Lequette, Y., Boels, G., Clarisse, M., & Faille, C. (2010). Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. *Biofouling, 26*(4), 421–431.
Lequette, Y., Garénaux, E., Tauveron, G., Dumez, S., Perchat, S., Slomianny, C., et al. (2011). Role played by exosporium glycoproteins in the surface properties of *Bacillus cereus* spores and in their adhesion to stainless steel. *Applied and Environmental Microbiology, 77*(14), 4905–4911.
Lindbäck, T., Hardy, S. P., Dietrich, R., Sødring, M., Didier, A., Moravek, M., et al. (2010). Cytotoxicity of the *Bacillus cereus* Nhe enterotoxin requires specific binding order of its three exoprotein components. *Infection and Immunity, 78*(9), 3813–3821.
Lücking, G., Stoeckel, M., Atamer, Z., Hinrichs, J., & Ehling-Schulz, M. (2013). Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. *International Journal of Food Microbiology, 166*(2), 270–279.
Lund, T., De Buyser, M. L., & Granum, P. E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Microbiology, 38*(2), 254–261.
Meyer, B. (2003). Approaches to prevention, removal and killing of biofilms. *International Biodeterioration & Biodegradation, 51*(4), 249–253.
Mittelman, M. W. (1998). Structure and functional characteristics of bacterial biofilms in fluid processing operations. *Journal of Dairy Science, 8*(10), 2760–2764.
Molva, C., Sudagidan, M., & Okuklu, B. (2009). Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey. *Food Control, 20*(9), 829–834.
van Netten, P., van de Moosdjik, A., van Hoensel, P., Mossel, D. A. A., & Perales, I. (1990). Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. *Journal of Applied Bacteriology, 69*(1), 73–79.
Organji, R. S., Abulreesh, H. H., Elbanna, K., Osman, G. E. H., & Khider, M. (2015). Occurrence and characterization of toxigenic *Bacillus cereus* in food and infant feces. *Asian Pacific Journal of Tropical Biomedicine, 5*(7), 515–520.
Pagedar, A., & Singh, J. (2012). Influence of physiological cell stages on biofilm formation by *Bacillus cereus* of dairy origin. *International Dairy Journal, 23*(1), 30–35.
Parkar, S. G., Flint, S. H., & Brooks, J. D. (2004). Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *Journal of Applied Microbiology, 96*(1), 110–116.
Pechook, S., Sudakov, K., Polishchuk, I., Ostrov, I., Zakin, V., Pokroy, B., et al. (2015). Bioinspired passive anti-biofouling surfaces preventing biofilm formation. *Journal of Materials Chemistry B, 3*(7), 1371–1378.
Peña, W. E. L., de Andrade, N. J., Soares, N. F. F., Alvarenga, V. O., Rodrigues, S., Jr., Granato, D., et al. (2014). Modelling *Bacillus cereus* adhesion on stainless steel surface as affected by temperature, pH and time. *International Dairy Journal, 34*(1), 153–158.
Peng, J. S., Tsai, W. C., & Chou, C. C. (2001). Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. *International Journal of Food Microbiology, 65*(1–2), 105–111.
Rajkovic, A., Uyttendaele, M., Vermeulen, A., Andjelkovic, M., Fitz-James, I., In't Veld, P., et al. (2008). Heat resistance of *Bacillus cereus* emetic toxin, cereulide. *Letters in Applied Microbiology, 46*(5), 536–541.
Ramarao, N., & Sanchis, V. (2013). The pore-forming haemolysins of *Bacillus cereus*: a review. *Toxins, 5*(6), 1119–1139.
Reyes, J. E., Bastías, J. M., Gutiérrez, M. R., & Rodríguez, M. O. (2007). Prevalence of *Bacillus cereus* in dried milk products used by Chilean school feeding program. *Food Microbiology, 24*(1), 1–6.
Ryu, J. H., & Beuchat, L. R. (2005). Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. *Journal of Food Protection, 68*(12), 2614–2622.
Salustiano, V. C., Andrade, N. J., Soares, N. F. F., Lima, J. C., Bernardes, P. C., Luiz, L. M. P., et al. (2009). Contamination of milk with *Bacillus cereus* by post-pasteurization surface exposure as evaluated by automated ribotyping. *Food Control, 20*(4), 439–442.
Samaržija, D., Zamberlin, S., & Pogačić, T. (2012). Psychrotrophic bacteria and milk and dairy products quality. *Mljekarstvo, 62*(2), 77–95.
Shaheen, R., Svensson, B., Andersson, M. A., Christiansson, A., & Salkinoja-Salonen, M. (2010). Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. *Food Microbiology, 27*(3), 347–355.

Sharma, M., & Anand, S. K. (2002). Biofilms evaluation as an essential component of HACCP for food/dairy processing industry - a case. *Food Control, 13*(6–7), 469–477.

Simmonds, P., Mossel, B. L., Intaraphan, T., & Deeth, H. C. (2003). Heat resistance of *Bacillus* spores when adhered to stainless steel and its relationship to spore hydrophobicity. *Journal of Food Protection, 66*(11), 2070–2075.

Simões, M., Simões, L. C., Cleto, S., Pereira, M. O., & Vieira, M. J. (2008). The effects of a biocide and a surfactant on the detachment of *Pseudomonas fluorescens* from glass surfaces. *International Journal of Food Microbiology, 121*(3), 335–341.

Simões, M., Simões, L. C., Machado, I., Pereira, M. O., & Vieira, M. J. (2006). Control of flow-generated biofilms with surfactants: evidence of resistance and recovery. *Food and Bioproducts Processing, 84*(4), 338–345.

Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology, 43*(4), 573–583.

Srey, S., Jahid, I. K., & Ha, S. D. (2013). Biofilm formation in food industries: a food safety concern. *Food Control, 31*(2), 572–585.

Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews, 32*(4), 579–606.

Stenfors Arnesen, L. P., O'Sullivan, K., & Granum, P. E. (2007). Food poisoning potential of *Bacillus cereus* strains from Norwegian dairies. *International Journal of Food Microbiology, 116*(2), 292–296.

Svensson, B., Ekelund, K., Ogura, H., & Christiansson, A. (2004). Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different diary plants. *International Diary Journal, 14*(1), 17–27.

Svensson, B., Monthán, A., Shaheen, R., Andersson, M. A., Salkinoja-Salonen, M., & Christiansson, A. (2006). Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. *International Dairy Journal, 16*(7), 740–749.

Tallent, S. M., Kotewicz, K. M., Strain, E. A., & Bennett, R. W. (2012). Efficient isolation and identification of *Bacillus cereus* group. *Journal of AOAC International, 95*(2), 446–451.

Tauveron, G., Slomianny, C., Henry, C., & Faille, C. (2006). Variability among *Bacillus cereus* strains in spore surface properties and influence on their ability to contaminate food surface equipment. *International Journal of Food Microbiology, 110*(3), 254–262.

Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., & Lindsay, D. (2012). Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. *International Journal of Food Microbiology, 157*(1), 28–34.

Thomas, A., & Sathian, C. T. (2014). Cleaning-in-place (CIP) system in dairy plant - review. *IOSR Journal of Environmental Science, Toxicology and Food Technology, 8*(6 v. III), 41–44.

Tourasse, N. J., Helgason, E., Økstad, O. A., Hegna, I. K., & Kolstø, A. B. (2006). The *Bacillus cereus* group: novel aspects of population structure and genome dynamics. *Journal of Applied Microbiology, 101*(3), 579–593.

van der Voort, M., García, D., Moezelaar, R., & Abee, T. (2010). Germinant receptor diversity and germination responses of four strains of the *Bacillus cereus* group. *International Journal of Food Microbiology, 139*(1–2), 108–115.

Warke, R., Kamat, A., Kamat, M., & Thomas, P. (2000). Incidence of pathogenic psychrotrophs in ice creams sold in some retail outlets in Mumbai, India. *Food Control, 11*(2), 77–83.

Wijman, J. G. E., de Leeuw, P. P. L. A., Moezelaar, R., Zwietering, M. H., & Abee, T. (2007). Air-liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. *Applied and Environmental Microbiology, 73*(5), 1481–1488.

Zhou, G., Liu, H., He, J., Yuan, Y., & Yuan, Z. (2008). The occurrence of *Bacillus cereus, B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. *International Journal of Food Microbiology, 121*(2), 195–200.