NBU Journal of Plant Sciences

Vol. 10, No. 1, March 2016 p. 49-54
ISSN No. 0974-6927

Research Article

In vitro production of diarrhoeal enterotoxin by *Bacillus cereus* isolates from milk and dairy products

Sarita Kumari and Prabir K. Sarkar*
Microbiology Laboratory, Department of Botany, University of North Bengal, Siliguri-734 013

Abstract

Bacillus cereus is a great safety concern for dairy industry as it is associated with incidences of food poisoning by producing enterotoxins. In the present study, growth temperature profile and enterotoxin production potential of 144 strains of *Bacillus cereus* isolated from milk and dairy products were investigated. Out of them, 107 (74.3%) were able to grow at ≤7 °C. Presence of such a large number of psychrotolerant/psychrotrophic strains in dairy environment is of major concern mainly because of their potential for growth, spoilage and toxin production in chilled products. Out of 144 isolates, 134 (93%) exhibited *β*-haemolysis. While 98% of the isolates from milk and 89% from cheese were positive for diarrhoeal enterotoxin, all the isolates from milk powder, ice cream, paneer and butter were positive. The prevalence of potent producers of enterotoxin among dairy isolates poses a high health risk.

Key words: *Bacillus cereus*, Growth temperature requirement, Enterotoxin, Haemolysin, Dairy product.

Introduction

Endospore-forming bacteria are important contaminants in dairy industry and significantly affect the quality of milk and dairy products. Particularly, members of *Bacillus cereus sensu lato* (*s.l.*) are significant, as they are associated with foodborne outbreaks by producing enterotoxins (Anderson Borge *et al.*, 2001) as well as 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).

Bacillus cereus may enter 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 and Beuchat, 2005). Adherence to stainless steel surfaces of dairy plants can result in biofilm formation which can be an important reservoir for recurrent contamination of milk and dairy products (Kumari and Sarkar, 2014a, 2016; Shaheen *et al.*, 2010). With an increasing demand of milk and dairy products, the need of extended refrigerated storage of raw milk before processing and the application of higher pasteurization temperatures for prolonged shelf-life requirements leads to serious concerns, like tolerance, adaptation or resistance of spores or vegetative cells to conditions of low temperature or low pH that were previously presumed to stop growth, or to treatments such as ultrahigh heat treatment (UHT) that were expected to inactivate all living materials (Heyndrickx, 2011). Thus, it has become important to characterize spore-formers in the dairy sector.

The incidence of foodborne illnesses has increased globally, and it becomes more important in developing countries where food products are frequently exposed to contaminated environments in food processing industries and temperature abuse during transportation and storage at retail outlets (WHO, 2007). Hence, *B. cereus* is an important safety and shelf-life concern in dairy industry. It is associated with two types of food poisoning: the diarrhoeal type by enterotoxin production in the small intestine and the emetic type by toxin which is formed in food. The rapid onset of the emetic disease, generally within 5 h after consumption of a

*Corresponding author
E-mail: pksarkar.nbu@gmail.com

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). 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) (Lund *et al.*, 2000; Stenfors Arnesen *et al.*, 2008). 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, necrotizing fasciitis, endocarditis, nosocomial acquired bacteraemia, osteomyelitis, sepsis, liver abscess, pneumonia and meningitis, particularly in postsurgical patients, immunosuppressed individuals, intravenous drug abusers and neonates (Ramarao and Sanchis, 2013).

Since pathogenic potential of the different *B. cereus* isolates is highly variable, from nontoxic to highly toxic strains, and toxin expression is influenced by food components, temperature and other environmental factors, there is need to study toxigenic potential of strains within the relevant food products. Therefore, aim of this research was to study the growth temperature limits and enterotoxin production potential of strains, previously isolated from milk and dairy products.

Materials and methods

Microorganisms

A total of 144 strains of *B. cereus*, isolated from milk and dairy products (Kumari and Sarkar, 2014b), were evaluated for growth temperature limits and production of enterotoxins.

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 (Guinebretière *et al.*, 2008).

Production of haemolysin

A 24 h-old nutrient broth (HiMedia M002) culture was spotted on blood agar plate containing 50 ml defibrinated sheep blood l^{-1} blood agar base (HiMedia M834) 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.

Production of Nhe 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 (HiMedia M210), supplemented with 10 g glucose l^{-1}, was inoculated with a 24 h-old culture, incubated at 37 °C for 24 h and centrifuged at 7830 *g* for 10 min. The supernatant was used for enterotoxin detection as per manufacturer's instructions. Amounts of produced enterotoxin were evaluated with index values derived from the Tecra reading scale; indices from 1 to 5 corresponded to the colouration intensity. According to the manufacturer's instructions, strains with an index of <3 were considered negative.

Results

Growth temperature requirement

Out of 144 isolates, 74% were able to grow at ≤7 °C and 15% at 20-50 °C (Table 1). Majority of milk (83%) and cheese (89%) isolates and all the isolates from ice cream, paneer and butter were able to grow at ≤7 °C.

Production of haemolysin

Out of 144 isolates, 93% exhibited *β*-haemolysis on sheep blood agar and showed a discontinuous haemolytic pattern (Fig. 1), characteristic for Hbl (Table 2). While majority of the isolates from milk were weak haemolytic, the same from rest of the products were moderate haemolytic.

Production of Nhe enterotoxin

Production of diarrhoeal enterotoxin component, NheA, was measured using Tecra

Bacillus diarrhoeal enterotoxin visual immunoassay kit (Fig. 2). Out of 144 isolates, 97% were positive for the production of diarrhoeal enterotoxin (Table 3). While majority of the isolates from milk, ice cream, paneer, cheese and butter were strong producers (colouration index, >4-5) of NheA enterotoxin, only 32% of the same from milk powder were strong producers.

Fig. 1. Haemolysis on sheep blood agar plate by some *Bacillus cereus* isolates.

Discussion

For the growth of *B. cereus* isolates, different ranges of temperature were observed, indicating their wide diversity and ecotype. Majority (74%) of the strains were able to grow at refrigeration temperature (≤7 °C), the criterion for considering those as psychrotrophic (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 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 pasteurized 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 isolates from milk powder were moderate thermotolerant (20-50 °C). This may be attributed to adaptation or selection of thermotolerant strains during drying and heating processes generally used to make milk powder.

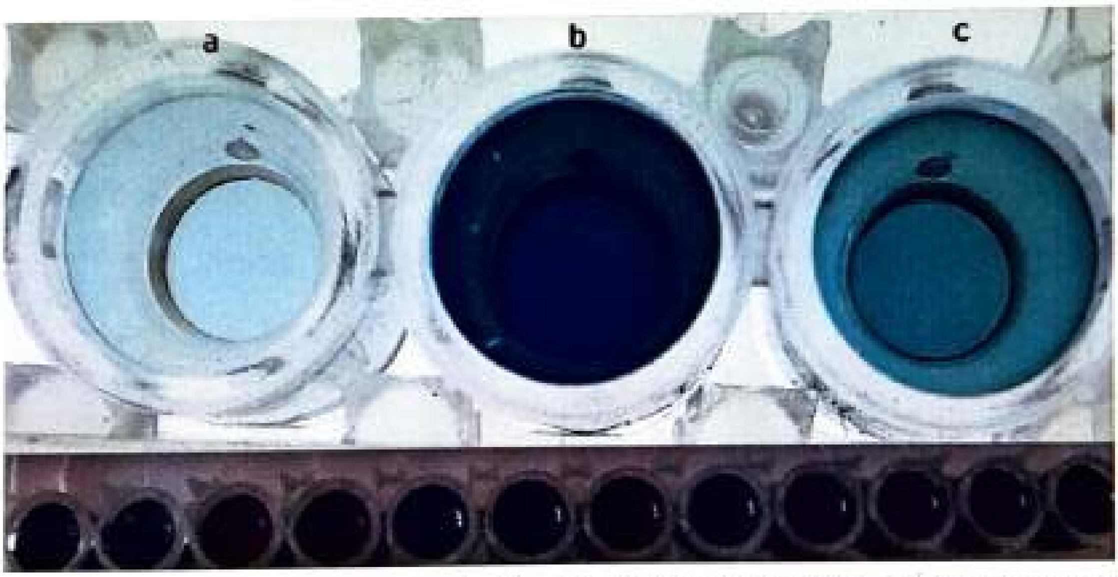

Fig. 2. Diarrhoeal enterotoxin production by some *Bacillus cereus* isolates, detected by Tecra antibody test: a, negative control; b, test; c, positive control.

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.*

Table 1. Range of growth temperatures of *Bacillus cereus* isolates from milk and dairy products

Source	No. of isolates	% of positive isolates			
		4-40 ºC	7-40 ºC	10-45 ºC	20-50 ºC
Milk	83	71	12	5	12
Milk powder	32	31	0	34.6	34.4
Ice cream	11	90	10	0	0
Paneer	5	17	83	0	0
Butter	4	100	0	0	0
Cheese	9	89	0	11	0

Table 2. Clustering of 144 isolates of *Bacillus cereus* from milk and dairy products on the basis of haemolysin production ability

Group*	% of isolates					
	Milk	Milk powder	Ice cream	Paneer	Cheese	Butter
Non-haemolytic	10	16	10	33	0	0
Weak haemolytic	71	19	27	27	56	50
Moderate haemolytic	11	41	45	40	44	50
Strong haemolytic	8	24	18	0	0	0

*Isolates were designated as non-haemolytic (1), weak (>1-1.5), moderate (>1.5-2) and strong (>2) haemolysin producers, according to ratio of diameter of clear zone to that of colony spot, developed on blood agar after incubation at 30 °C.

Table 3. Clustering of 144 isolates of *Bacillus cereus* from milk and dairy products on the basis of diarrhoeal enterotoxin production ability

Group*	% of isolates					
	Milk	Milk powder	Ice cream	Paneer	Cheese	Butter
Non-producer	2	0	0	0	11	0
Weak producer	38	68	45	0	0	0
Strong producer	60	32	55	100	89	100

*Isolates were designated as non-producers (<3), weak producers (3-4) and strong producers (>4-5). Indices from 1 to 5 (within parentheses) correspond to colouration intensity according to the manufacturer's instructions.

cereus isolates from food ingredients and products in Brazil (Chaves *et al.,* 2011).

All the isolates from milk powder, ice cream, paneer and butter produced diarrhoeal enterotoxin. However, 98% of the isolates from milk and 89% from cheese were found positive for diarrhoeal enterotoxin. Results are 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 58% isolates were high producers (index >4-5) of NheA. Prevalence of high producers of Nhe among the 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. However, an in-depth investigation of the individual and synergistic effect of toxins in combination with other known virulent determinants and effect of various environmental factors needs to be further studied.

Conclusion

Majority of the *B. cereus* strains isolated from milk and dairy products were able to grow at refrigeration temperature and were potent producers of enterotoxin Hbl and Nhe. The prevalence of potent producers of enterotoxin among dairy isolates having ability to grow at refrigeration temperature indicates high risk and public health concern. Observations in the present study may be important when considering microbiological criteria for *B. cereus* in pasteurized milk and dairy products and possibly in other foods stored at low temperature.

Acknowledgments

This work was supported by grant, F.3–4/2013 (SAP-II), from the University Grants Commission, New Delhi, India.

References

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.

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.

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., 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.

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

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.

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.

Heyndrickx, M. (2011). The importance of endospore-forming bacteria originating from soil for contamination of industrial food processing. *Applied and Environmental Soil Science*, doi: http://dx.doi.org/10.1155/2011/561975

Kumari, S. and 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: 153-158.

Kumari, S. and Sarkar, P.K. (2014b). Prevalence and characterization of *Bacillus cereus* group from various marketed dairy products in India. *Dairy Science and Technology*, 94: 483-497.

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

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.

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.

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.

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.

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.

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.

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., Monthán, A., Guinebretière, M.H., Nguyen-The, C. and 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.

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., 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.

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