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## 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% (NDDDB, 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

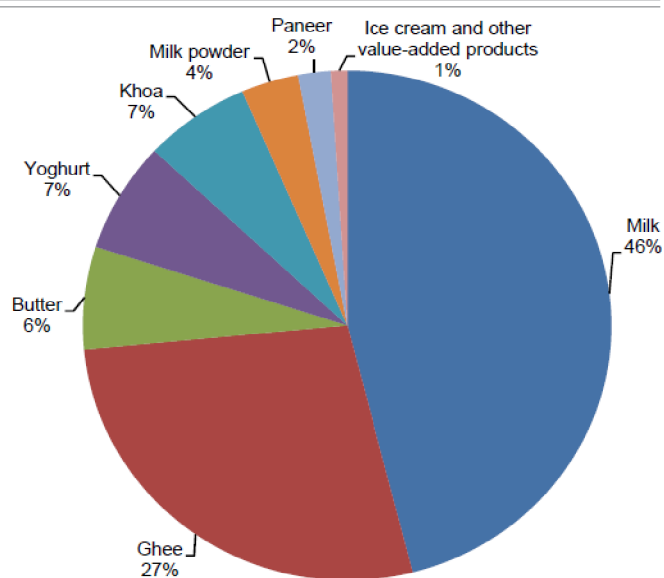


Fig. 1. Annual consumption of different dairy products in India (Source: NDDDB, 2012)

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 thermotolerant microorganisms.

Milk is an important source of essential nutrients containing lactose, proteins, fats, calcium, magnesium, selenium, riboflavin, vitamin B<sub>12</sub> 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 diarrhoea (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.