REVIEW OF LITERATURE
2.1. Introduction

Man is a creature of his environment. He shares its hazards with many other living organisms and for most of the time achieves a measure of peaceful coexistence with them. Man is also a great manipulator of his environment and this manipulation may be simple or complicated. Micro-organisms are much smaller than men, but they are great opportunists. Give them the opportunity and they will flourish on man’s food (Dale, 2013).

The problem of diseases caused by food-borne pathogens remains largely unknown. Notably data representing trends in food-borne infectious Gastro-intestinal (GI) disease is limited to a few developed countries (Newell et al., 2010). Even though, bacterial food-borne agents have been the most well investigated and monitored causes of GI infectious disease, our understanding of the microbial agents of GI illness remains limited. Comprehensive diagnostic studies of intestinal infections (Tompkins et al., 1999) indicate that between 50 and 60% of all causative agents are unidentified. In addition, gastrointestinal illnesses caused by toxin producing bacteria, such as B. cereus, are almost certainly underestimated due to lack of diagnostic tools.

2.2. General pathogenic bacteria

Food pathogens mainly comprise of bacteria belonging to the genera Clostridium botulinum, Escherichia coli, Salmonella spp., Listeria monocytogenes, Yersinia enterocolitica, Staphylococcus aureus, Shigella spp., Bacillus cereus and Campylobacter jejuni. About 98% of microbes found in food commodities are non-pathogenic (SanathKumar et al., 2002). Species of Bacillus and related genera have long been a challenge to food producers because of their resistant endospores (Andersson et al., 1995; Ryu and Beuchat, 2005). These organisms have gone through huge taxonomic changes in the last three to four decades, with numbers of genera and species now amounting to 56 and over 545, respectively (Logan and Halket, 2011).
B. cereus is a large, Gram-positive, motile, aerobic-to facultative, spore-forming rod. The bacterial spores do not swell the sporangium and sporulate readily only in the presence of oxygen (Blackburn and McClure, 2005). The study of B. cereus in relation to food has gained importance in the light of its ability to form heat resistant endospores and its capacity to grow and produce toxins in a wide variety of foods. The Bacillus cereus sensu lato group contains varied Gram-positive spore-forming bacteria that are prevalent in the environment. This group comprises seven closely related species: B. mycoides, B. pseudomyxoides, B. weihenstephanensis, B. anthracis, B. thuringiensis, B. cytotoxicus and B. cereus sensu stricto (Guinebretière et al., 2008). Comparisons of genome sequence similarity between species have emphasized the close relationships between these bacteria, making their identification to species level difficult (Guinebretière et al., 2008). Converse to B. subtilis, a true soil saprophyte B. cereus species, have metabolic potential which is not suited for the utilization of plant material, or to metabolize a range of complex carbohydrate polymers. Certainly, the functions of the genes involved in complex carbohydrate metabolism seems to be restricted to the utilization of glycogen, starch, chitin and chitosan, all of which are important constituents of insect tissues (Ivanova et al., 2003). Furthermore, B. cereus contains numerous transporter genes of protease, peptide and amino-acid. Collectively, these observations indicate that B. cereus is better adapted to a protein based nutrition of probably animal tissues (Ivanova et al., 2003).

2.3. Historical view

The genus Bacillus was of significant importance in the early history of microbiology. Ferdinand in 1876 was able to discredit the theory of spontaneous generation after observing Bacillus subtilis and its spores and Robert Koch's (1876) study of the B. Anthracis marked the genesis of clinical bacteriology. According to Jackson et al., (1995), B. cereus is one of the most ubiquitous bacteria on earth and Todd et al., (1996), observed that the natural environment of B. cereus mainly consists of decaying organic material, fresh water, soil, marine water, vegetables and the intestinal tract of invertebrates and Rasko, (2005) and Bottone, (2010), declared that due to this ubiquitous
presence, soil and food is contaminated with *B. cereus* and the colonization of the human intestine is also possible.

Even though, there were reports in the European literature at the beginning of the 20th century about food-borne illness caused by *B. cereus* or *B. cereus*-like organisms, there was no explicit proof that *B. cereus* could cause food-poisoning. Hauge, (1955) was the first to establish *B. cereus* as a food-poisoning organism causing a diarrhoeal type of illness on the consumption of vanilla sauce. His findings were confirmed by other European workers in the early 1950s. In the United States and Canada, *B. cereus* food-poisoning was first documented in 1968 (Szabo *et al.*, 1984). Until 1970, outbreaks caused by *B. cereus* were only distinguished by watery diarrhea occurring 8-16 h after ingestion of the contaminated food, but in 1971, a new form of *B. cereus* food-poisoning characterized by nausea and vomiting was identified in UK following outbreaks associated with the consumption of rice from Chinese restaurants and take-away outlets. As many as 192 such incidences involving more than 1000 cases were reported in the UK between a period of 1971 and 1984 (Kramer and Gilbert, 1989). *B. cereus* was recorded to be the third most common cause of the food-poisoning outbreaks in Hungary (117 outbreaks) between 1960 and 1968, followed by Finland (82 outbreaks), Netherlands (11 outbreaks) and Canada (9 outbreaks) (Gilbert 1979; Shinagawa, 1990). Besides these, there are many reports of food-borne outbreaks of *B. cereus* from a large variety of foods in many countries including the USA (Bean and Griffin, 1999), United Kingdom, Scandinavia, Japan (Johnson, 1984) and Norway (Kotiranta, 2000). Although Norway is almost free of *Salmonella* and *Campylobacter* food poisoning, *B. cereus* is most commonly reported in food-poisoning syndrome (Blackburn and McClure, 2005). While summarizing the data generated on food-borne illnesses due to the consumption of Chinese-Indonesian food and meat products during 1991 to 1994 at the regional Food Inspection Services in the Netherlands, Simone *et al.*, (1997) reported 2,621 incidences, involving 7,567 ill people. Of the incidents of known etiological agent, 19% were attributed to *B. cereus*, which was the highest. Between 1991-94, *B. cereus* was the most common pathogen found in 3% of the samples taken from the hot meals served on aircraft (Hatakka, 1998).
Daniels et al., (2002) reported B. cereus to be the causative agent in 7% of school food borne disease outbreaks in North America over the period of 1998\textendash{}2000. Pirhonen et al., (2005) investigated food-poisoning outbreaks occurred after eating a dish of pasta and minced meat involving both emesis and diarrhea in two adult persons. Emetic toxin producing strains of B. cereus formed the majority (68%) of strains identified in tested food. Haemolytic diarrhoeal toxin was produced by 26% of the strains studied and 6% of the strains produced neither emetic nor haemolytic diarrhoeal toxin. According to the European Food Safety Authority (2007) report on food-borne outbreaks, B. cereus was stated to be the causative agent in 77 outbreaks and caused 17.1% of the cases due to bacterial toxins.

2.4. Epidemiology

B. cereus food poisoning has been shown to occur year-round and no specific or particular geographic distribution has been known. Between 1973\textendash{}1985, in Finland B. cereus caused 17.8% of the total bacterial food poisonings, 11.5% in the Netherlands, 0.8% in Scotland, 0.7% in England and Wales, 2.2% in Canada, 0.7% in Japan, and 15.0% (between 1960\textendash{}1968) in Hungary (Kotiranta et al., 2000). In Norway, B. cereus was the most widespread microbe isolated from foodborne illnesses in 1990 (Kotiranta et al., 2000). From 1998 to 2000, in France, B. cereus represented 4 to 5% of food borne poisoning outbreaks of known origin (Haeghbaert et al., 2001, 2002a and 2002b). During 2008, 103 confirmed outbreak cases have been reported in the US (Venkitanarayanan et al., 2008). In Northern America, 1 to 2% of outbreaks of identified origin were found to be represented by B. cereus (Granum and Baird-Parker, 2000).

The genus Bacillus is ubiquitous in nature. It does not have complex and stringent nutrient requirements. It is frequently found in soils with low nutrients as well as on rice and straw (Kotiranta et al., 2000). Between 10^3 and 10^5 spores of B. cereus were observed per gram Soil (Guinebretiere and Nguyenthe, 2003). Bacillus species has also been isolated from extreme environments like hot lakes with temperature more than 60°C, deep sea
(Jannasch and Taylor, 1984; Gaill, 1993), refrigerated foods and high pressure environments (Csonka, 1989). Growth optimum has been found between pH 4.5 to 9.3; water activity being higher than 0.92 for growth and a wide temperature range for growth of 4°C to 50°C (Kramer and Gilbert, 1989). On the other hand, strains able to multiply below 7°C, and above 45°C, are not the most common. Emetic B. cereus is in all probability unable to grow and produce their toxin cereulide below 10°C, or in the absence of oxygen (EFSA, 2007).

B. cereus can easily spread to many types of foods, especially of plant origin, from its natural environment because of the resistance of its endospores to various stresses and their long term survival capacity. Its spores and vegetative form are frequent inhabitants of many food types, especially cereals and its derivatives (Blakey and Priest, 1980) rice, vegetables (Portnoy et al., 1976); spices, herbs and additives (Baxter and Holzapfel, 1982). These forms as also present in milk and dairy products (Ahmed et al., 1983); raw meat, eggs, and processed foods (Goepfert et al., 1972) as well as in ready to eat food stuffs (Kramer and Gilbert, 1989). It forms resistant spores and spreads easily, consequently, there is a danger in its spread through processed, pasteurized, sterilized, and heat-treated food products (Kotiranta et al., 2000). The primary mode of transmission has been shown to be via ingestion of B. cereus contaminated food. Emetic type of food poisoning has been largely related with the consumption of rice and pasta, whereas the diarrheal type is transmitted mostly by milk products, vegetables and meat (Murray et al., 2007; Logan et al., 2006).

A somewhat dissimilar occurrence between countries is observed for the emetic and diarrhoeal diseases, which could partially be a manifestation of the association of the two types of diseases with diverse food transmitters: in Japan and the UK, the emetic disease has been found to dominate (Gilbert and Kramer, 1986; Shinagawa et al., 1995), while the diarrhoeal illness is more prevalent in northern Europe and North America, (Kotiranta et al., 2000). Part of the difference in disease pattern may be probably due to different
eating habits, but it is difficult to establish whether the distribution is actually different and not a result of reporting differences (Kotiranta et al., 2000).

2.5. *B. cereus* and food-borne illness

Mainly two types of disease syndromes have been reported due to *B. cereus* infection. Diarrheal syndrome is due to the production of heat labile enterotoxins during growth of vegetative cells in the small intestine with an infective dose of $10^4-10^9$ cells per gram of food (Logan et al., 2006). However, this syndrome is mild and primarily manifested by abdominal cramps and diarrhea, having an incubation period of 8 to 16 h and lasting for 6 to 12 h (Murray et al., 2007; Logan et al., 2006). Generally diarrhea may be mild or profuse and watery. This type is referred to as the "long incubation" or diarrheal form of the disease and it is similar to food poisoning caused by *Clostridium perfringens* (Drobniewski, 1993). Emetic syndrome which is more severe and acute than diarrheal syndrome also referred to as "short-incubation" or emetic form of the disease. Emetic syndrome is distinguished by nausea and vomiting and abdominal cramps. The toxin responsible for this syndrome is a small cyclic heat-stable peptide which causes vomiting after 1 to 6 h of ingestion (average 2 to 5 h) (Mortimer and McCann, 1974). The toxin is preformed and indigested in food. In emetic type of illness, the dose is about $10^5-10^8$ cells per gram in order to produce sufficient toxin (Logan et al., 2006). It is similar to *Staphylococcus aureus* food poisoning in its symptoms and incubation period. The number of organisms necessary to cause this syndrome appeared to be higher than that of diarrheal syndrome (Gilbert, 1979). In either cases of syndrome, the illness usually was found to last for less than 24 hours. In a few patients symptoms lasted longer (Murray et al., 2007; Logan et al., 2006). Both syndromes occur as a result of the fact that *B. cereus* spores can survive normal cooking procedures. After cooking, under improper storage conditions, the spores would germinate and the vegetative cells would then multiply (Logan, 2011).
2.6. *B. cereus* toxins

*B. cereus* produces four types of toxins, one emetic toxin (ETE) and three different enterotoxins. Three pore forming toxins, responsible for the diarrhoeal type of food poisoning are Hemolysin BL (HBL), Non-haemolytic enterotoxin (Nhe), and Cytotoxin K (CytK). HBL and Nhe each consist of three different protein components, named L\(_2\), L\(_1\), and B, and NheA, NheB and NheC, respectively, while CytK is a single-component toxin (Stenfors Arnesen *et al.*, 2008; Fagerlund *et al.*, 2010). The emetic type is an illness caused by highly heat-, proteolysis-, acid- and alkali-resistant toxin, that is pre-formed when consumed, leading to swift onset of the syndrome. The emetic toxin (ETE) is dodecadepsipeptide, cereulide (Shinagawa *et al.*, 1995; Agata *et al.*, 1995) and having a ring-shaped structure of three repeats of four amino acids having a molecular weight of 1.2 kDa. The mechanism and site of action of emetic toxin have not been deciphered, although the small molecule appears to form ion channels and holes in membranes. The long-incubation form of illness is mediated by the diarrhoeagenic enterotoxin Nhe and/or hemolytic enterotoxin HBL, which cause intestinal fluid secretion, possibly by a number of mechanisms, including pore formation and activation of adenylate cyclase enzymes (Jalalpour, 2012). L\(_1\), L\(_2\) and B, the three protein components, constitute the haemolytic toxin, where B is the binding; L\(_1\) and L\(_2\) are lytic components. It is a proteinaceous toxin that also has dermonecrotic and vascular permeability activities. It causes fluid accumulation in ligated rabbit ileal loops. Genes encoding *hbl* were found in about 50-66% of strains tested (Granum 2002; Ngamwongsatit *et al.*, 2008; Ankolekar *et al.*, 2009), and it was previously believed to be the primary virulence factor in *B. cereus* diarrhoea. However, outbreaks associated with strains lacking this toxin have been reported (Granum *et al.*, 1996). It is supposed to cause osmotic lysis by forming pore in the transmembrane, after independent binding of its three components B, L\(_1\) and L\(_2\) (Stenfors Arnesen *et al.*, 2008).

Non-haemolytic enterotoxin (Nhe) is another three component proteinaceous, pore forming toxin that is structurally similar to HBL; including a cytolytic protein NheA and two binding components NheB and NheC. Almost all *B.
cereus strains have genes encoding Nhe (Ngamwongsatit et al., 2008; Stenfors Arnesen et al., 2008; Ankolekar et al., 2009). It was noticed that following a Norwegian outbreak caused by a HBL-negative strain, has been believed to be the most dominant diarrhoeal toxin Nhe (Stenfors Arnesen et al., 2008). Production of both HBL and Nhe is presumed to be limited to members of the B. cereus group (From et al., 2005). A single-component, β-barrel pore-forming Cytotoxin K (CytK) belongs to the similar family of toxins as Clostridium perfringens β-toxin. It is dermonecrotic, cytotoxic and haemolytic, and almost 90% B. cereus strains might carry the gene for it (Ngamwongsatit et al., 2008). The toxin is able to form weakly anion selective pores and exhibit an open channel probability close to one. CytK is a potent cytotoxin against human intestinal Caco-2 epithelia. CytK, like other L-barrel pore-forming toxins, immediately forms oligomers that are resistant to sodium dodecyl sulphate (SDS), but not to boiling (Stenfors Arnesen et al., 2008, Fagerlund et al., 2010). This toxin occurs in two forms CytK-1 and CytK-2 which have 89% amino acid sequence homology, (Fagerlund et al., 2004).

Another toxin, first isolated from the B. cereus FM1 strain, named enterotoxin FM (entFM), at high doses was suspected to cause fluid accumulation in rabbit and mouse ligated intestinal loop tests (Boonchai et al., 2008; Tran et al., 2010). However, very few studies have been performed on this protein, and its specific role during B. cereus virulence has not been reported. The entFM gene has been found to be located on the chromosome and appears to be common to B. thuringiensis and B. cereus strains. Prevalence studies revealed entFM is detected in most outbreak-associated strains (Ngamwongsatit et al., 2008).

### 2.7. Regulation of toxin production

Regulation of B. cereus toxins production and implications of toxins on food safety were reviewed by Ceuppens et al., (2011); It is observed that the toxin expression complexity is still not clearly understood and the influences of food components, temperature and other environmental factors need detailed investigation. The capacity of the contaminating strain to produce toxin(s)
influences the infective or intoxicating dose in both syndromes. Only strain producing both diarrhoeal and emetic toxins may cause illnesses with both diarrhoeal and emetic symptoms or the presence of separate diarrhoeal strains and emetic strains in the food may be responsible (Pirhonen et al., 2005).

2.8. *B. cereus* in food and food products

Among various food-borne pathogens known to cause food-borne illnesses, *B. cereus* has been generally found in most of the cases to be responsible for food-borne outbreaks (Velusamy et al., 2010). *B. cereus* being ubiquitous in the environment has made it difficult to link clinical cases to its environmental sources. Because it easily contaminates various food samples and as its elimination is not guaranteed by pasteurization and sanitation procedure, it causes spoilage and food-poisoning by its proteolytic, lipolytic and saccharolytic activities (Kalogridou-vassiliodou, 1992). The factors that make *B. cereus* a potential threat to processing of food is its capacity to grow, survive and form thermoduric endospore, at refrigeration temperatures with toxin production (McKillip, 2000). Milk and rice are probably the two most commonly contaminated food items.

2.8.1. Meat and meat products

*B. cereus* was found to be present in several spices, additives and other samples from the environment with an increase in contamination of meat with *B. cereus* with each additional stage in the processing of the raw meat (Volkova et al., 1971). Increase in *B. cereus* load in final products of vegetables, cheese and spices were of 14, 10.3 and 10% respectively, and load was 500/g in Hamburgers and minced meat (Cantoni et al., 1994). This bacteria was found to survive food processing where *B. cereus* was recovered from 28% of the meat products samples including heat-treated products (Schlegelova et al., 2003). *B. cereus* also occurs in frozen food and food products. Mira and co-workers, (2006) showed 100% prevalence rate of *B. cereus* in five types of ready-to-eat chicken products and frozen half cooked chicken products. Highest incidence of *B. cereus* from ready-to-eat chicken
product was found and followed by frozen half cooked chicken products samples. 60 samples of five chicken meat products were analyzed by Smith et al., (2004) for the presence of \textit{B. cereus} and 27 found were found harbouring the organism. In retail samples of meat and meat products, Guven et al., (2006); Kursun et al., (2011) found 22.4% and 36% incidence of \textit{B. cereus} respectively.

2.8.2. Milk and milk products

Most of the \textit{B. cereus} contamination has been found in the raw milk where the organism is partly present as spores and able to survive pasteurization. In the dairy industry, \textit{B. cereus} group, especially psychrothrophic strains, have been distinguished to limit the keeping quality of pasteurized milk (Svensson et al., 2004; Hanson et al., 2005; Barbano and Santos, 2006; Aires et al., 2009). Contamination of pasteurized milk has been mainly traced to raw milk and/or equipment surfaces (Lin et al., 1998; Huck et al., 2007; Banyko and Vyletelova, 2009). The role of processing equipment (Svensson et al., 2004; Schlegelova et al., 2010) as a reservoir for \textit{B. cereus} milk recontamination is well recognized especially in pasteurized milk (Eneroth et al., 2001; Sharma and Anand, 2002; Salustiano et al., 2009). \textit{B. cereus} at high incidences were recovered in milk, milk products and cakes (Smykal et al., 1976) and Soegaard et al., (1977) observed \textit{B. cereus} in whole milk, low-fat milk, skimmed milk and cream samples obtained from 5 dairies. \textit{B. cereus} was detected mastitis milk, commercial milk products, farm milk samples, pasteurized milk samples, pasteurized cream samples and dried milk samples (Raevuori and Koiranen, 1978). El-Naway et al., (1982) found market milk; scalded cream and Domiati cheese contained \textit{B. cereus}. Various kinds of baby food products produced at a factory in Hradec Kralove, Czechoslovakia contained \textit{B. cereus} (Jarchovska, 1987). Nagarajan et al., 1990, isolated psychrotrohpic \textit{B. cereus} from milk was found to survive pasteurization, mainly as endospores. Homleid, (1993) observed samples of skim, low-fat and whole milk, whipping cream and 20% fat cream from Norwegian dairies contained \textit{B. cereus} in about 8-10% of each type samples, except 20%-fat cream. Carp et al., (2000) reported its presence of \textit{B. cereus} in milk powder
and observed that *B. cereus* was identified in 76.1% (51 of 67) of the samples. Floristean *et al.*, (2004) found 17.59% of milk and dairy products samples and 37.5% in powdered milk samples to be positive for *B. cereus*. Reyes *et al.*, (2007) noticed prevalence of *B. cereus*, in a total of 175 samples out of 381 samples of dried milk products (milk with rice, milk substitute, milk powder, milk-cereal-rice, pudding milk, flan, and mousse) used by the Chilean School Feeding Program. Chitov *et al.*, (2008) investigated dairy and cereal products in Thailand and found that the number of *B. cereus* cells was ranging from 50 to $1.7 \times 10^3$ cfu/g.

### 2.8.3. Other types of foods

*B. cereus* has been isolated from a wide variety of foods such as desert mixes (Warburton *et al.*, 1987), infant foods (Becker *et al.*, 1994), spices (Choo *et al.*, 2007), ready to serve foods (Harmon and Kautter, 1991), seafood (Wijnands *et al.*, 2006; Rahmati and Labbe, 2008), pulses, cereals and cereal derivates (Te Giffel *et al.*, 1997), fresh vegetables (Valero *et al.*, 2002) and rice (Sarrias *et al.*, 2002), dried foods and wet wheat noodles (Rusul and Yaacob, 1995), bakery products, yeast, flour, cocoa, chocolate, pasta products, Chinese meals, meat products, herbs and species (Te Giffel *et al.*, 1996), traditional Indian food samples (Desai and Varadaraj, 2009), infant foods in Iran (Rahimi *et al.*, 2013).

### 2.9. *B. cereus* food poisoning in different countries

#### 2.9.1. India

In India, majority of outbreaks of food borne disease go unreported, unrecognized or un-investigated. The reported bacterial food borne disease outbreaks in India during 1980-2009 pointed to 24 outbreaks involving 1,130 persons. One third of total pediatric admissions in hospitals in India were due to diarrheal diseases and in indoor pediatric patients, 17% of all deaths were diarrhea related (Park, 2011). However, the exact identification of the causative agent pointing to *B. cereus* has been very difficult. In 1978, Kulshreshtha (1978) reported the first outbreak of *B. cereus* food-poisoning in
children due to the consumption of milk powder in India, where *B. cereus* was isolated from stools, vomitus of the victims and from implicated food. Lakhani, (1979) reported a case of food poisoning due to *B. cereus* in a village near Poona where around 500 people of different age groups developed nausea and vomiting after consuming contaminated rice having viable count ranging from 2.0 to 7.0x10^7 cfu/g at a religious function. Chopra *et al.*, (1980) found contamination of *B. cereus* in all of the 10 milk, 8 of 10 burfi and 7 of 10 milk cakes obtained from Ludhiana city market. An episode of gastrointestinal illness was recounted by Hussain *et al.*, (2007), due to consumption of *B. cereus* contaminated food in a fast food restaurant in India. The contaminated food included hot cholapuri, made of flour and Bengal gram. 35 of 50 students, who had attended a party suffered from food poisoning (Ram Shoba *et al.*, 1987). Symptoms appeared 2 h after ingestion. The foods contaminated were gulab jamun and samosa. Singh *et al.*, (1995) reported that six person of a family were involved in food poisoning who had consumed bakery bun contaminated with *B. cereus*. Bachhil and Jaiswal, (1988), isolated *B. cereus* from fresh buffalo meat and cooked and semi-cooked buffalo meat samples while, Konuma *et al.*, (1988) found *B. cereus* in meat products and raw meat. Fish, chicken and meat products (Kamat *et al.*, 1989), fried rice and chow mein have also been contaminated with *B. cereus* (Yadav, 2004). Anamika *et al.*, (2004) found that samples of khoa, paneer and mushroom, obtained from local and standard shops in Ranchi, Jharkhand, India, were contaminated with *B. cereus*. Bedi *et al.*, (2004) found an incidence of *B. cereus* in chicken, mutton, butter chicken, chicken soup and mutton soup. Bedi *et al.*, (2005) reported an incidence of *B. cereus* in raw milk, burfi and skimmed milk powder samples and the level of *B. Cereus* contamination was more than 10 cfu/g. Raw and cooked mutton (Willayat *et al.*, 2007), Fish, shrimp and clam, milk samples procured from local markets of Cochin (Das, 2011), meat samples (mutton tikka and chutney samples) collected from Kashmir valley (Hafiz *et al.*, 2012), raw milk (Altarf *et al.*, 2012), poultry based street food (Sudershan *et al.*, 2012), various meat and meat products (raw and processed) (Tewari *et al.*, 2013) reported incidence of *B. cereus*. 
2.9.2 Outbreaks in other countries

*Bacillus cereus* a major cause of foodborne disease worldwide (Clavel et al., 2007; Granum, 2007), many go under-reported in official lists. In the European Union, *Bacillus* species (including non-cereus) were responsible for 1.4% of foodborne outbreaks in 2005 (Anonymous, 2006). Nearly 45 outbreaks of gastroenteritis attributed to *Bacillus* spp during 1992–2006, in England and Wales (www.hpa.org.uk/infections/topics_az/bacillus/fp/fpdata.htm). Schmidt, (2001) have reported 12% of foodborne disease outbreaks between 1993 and 1998 in the Netherlands, where *B. cereus* has been accounted as a causative agent. The number of cases of *B. cereus* foodborne disease are reportedly increasing in industrialized countries (Kotiranta et al., 2000).

The *B. cereus* illnesses vary from country to country. In Japan the emetic type was observed to be ten types more frequent than diarrhoeal type (Kramer and Gilbert, 1989). Conversely, in Europe and North America, diarrhoeal type has been frequently and widely reported (Kramer and Gilbert, 1989). In most of the countries the illness has not been reported and only a few countries in Europe have reported the onset of infection due to *B. cereus*. Of the total cases of food poisoning, 33% in Norway between 1988-93, 47% in Iceland between 1985–92, 22% in Finland during 1992, 8.5% in the Netherlands during 1991, 5% in Denmark between 1990–92 (Schmidt 1995), 0.7% in England and Wales, 0.8% in Japan, 1.3% in USA and 2.2% in Canada (Kramer and Gilbert 1989).

2.10. Detection of *B. cereus*

2.10.1. Traditional microbiological methods for detection of pathogens in food

Monitoring and identification of food-borne pathogenic bacteria are complicated due to many intrinsic and extrinsic factors (Mead et al., 1999). Rigorous adherence to sanitary practices in a food-processing environment necessitates rapid assay results (Marriott 1999).
2.10.2. Standard/Aerobic Plate Counts and relevant variations

Detection of viable bacteria is conventionally carried out by culturing/measuring growth of individual microorganisms. Many commonly used bacteriological media and culture specific media are used to monitor for spoilage and (or) pathogenic bacteria in food (Harrigan, 1998). The use of routine non-selective media known as the aerobic plate count (APC) or standard plate count (SPC) is in practice since a long time. Increased sensitivity of SPC/APC has been achieved using a selective agar overlay approach designed to recover a larger proportion of bacteria from food matrices (Harrigan, 1998). Detecting these bacteria is of vital significance in the food industry, since the selective agar overlay technique aids in the revival of bacterial cells from food, which are viable. Slight variations on the recovery technique have been employed, including a membrane or solid-support-based transfer (Blackburn and McCarthy, 2000). Another approach to enhance the number of bacteria in a food matrix is pre-enrichment of the suspected food sample in a non-selective or selective broth culture enabling to obtain viable target bacteria in detectable levels (Zhao and Doyle, 2001). Depending on the food product being analyzed, the enrichment step(s) may require an additional 8–24 h before enumeration. In many cases, analyst may find it necessary to implement a bacterial concentration or immobilization step immediately prior to plating to sequester cells within an otherwise polluted and heterogenous food matrix. Several general methods have been described for selective removal of cells from liquid food systems (Sharpe, 1997). Immunomagnetic separation (IMS) is one such technique employing bacterial specific antibodies linked to magnetic beads. The food matrix is then exposed to a magnetic field that essentially pulls the bacteria out of suspension for plating or other techniques (Tomoyasu, 1998). An effective alternative to IMS is the use of metal hydroxide based bacterial concentration, where cells may either bind to titanous, hafnium or zirconium hydroxide suspensions and may be separated using low-speed centrifugation, resuspended, and directly plated (Cullison and Jaykus, 2002; Jaykus, 2003). The selective media used for Bacillus cereus detection is Polymixin pyruvate egg yolk mannitol agar (PMBA) and BHI agar supplemented with 0.1% glucose. Fluorogenic and
chromogenic media for *Bacillus cereus*, is based on fact that *B. cereus* may produce a phosphatidylinositol-specific phospholipase c, the key enzyme reacting with 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate, giving distinctive turquoise colonies (Restainoa et al., 2000). Dry plate culturing (e.g., 3M Petrifilm or similar) is yet another widely used means of assessing microbiological quality of a wide range of foods for coliforms, aerobic mesophilic bacteria, psychrotrophs, and staphylococci (Silbernagel and Lindberg, 2001; Ellis and Meldrum, 2002). Although the plate count method is dynamic, sensitive, specific the time it takes to obtain data prevents the SPC/APC technique from being considered among the “rapid methods”

2.10.3 Molecular-based detection formats

2.10.3.1. Immunoassays

Among the extensively used molecular methods for the detection of food pathogens is the enzyme-linked immunosorbent assay (ELISA), which potentially offers much greater specificity compared with SPC due to the antibody–antigen (target) interaction. ELISA has been successfully used to detect either whole-cell antigen targets or products (e.g., virulence determinants) of pathogens such as *Salmonella* spp., *E. coli* O157:H7, *Campylobacter* spp., *B. cereus*, and *L. monocytogenes*, in a more user-friendly ELISA is automatable and is convenient for large sample numbers with relative ease. In spite of these advantages, ELISA methods may still suffer in terms of desired sensitivity, with a typical detection limit of $10^4$ CFU/mL, depending on the food being analyzed (Cox et al., 1987; Hartman et al., 1992).

A total of five hybridoma cell lines that produced monoclonal antibodies against the components of the hemolysin BL (HBL) enterotoxin complex and sphingomyelinase produced by *Bacillus cereus* were characterized (Dietrich et al., 1999). Specificity of antibodies indicated that the monoclonal antibody 2A3 was specific for the B component, antibodies 1A12 and 8B12 were specific for the L2 component, and antibody 1C2 was specific for the L1 protein of the HBL enterotoxin complex (Dietrich et al., 1999). With other
proteins produced by different strains of \textit{B. cereus}, no cross-reactivity was observed for monoclonal antibodies 2A3, 1A12, and 8B12, whereas antibody 1C2 cross-reacted with an uncharacterized protein of one component of the nonhemolytic enterotoxin complex. Antibody 2A12 finally showed a distinct reactivity with \textit{B. cereus} sphingomyelinase (Dietrich \textit{et al.}, 1999).

\textbf{2.10.3.2. Recombinant antibodies as diagnostic tools}

Recombinant antibody fragments are gaining importance as better alternatives to monoclonal and polyclonal antibodies in diagnostics (Emanuel \textit{et al.}, 2000). Antibody fragments can be readily expressed in \textit{Escherichia coli} allowing low-cost production and purification, a significant advantage for many applications including proteomics (Hayhurst and Georgiou, 2001).

Specific and efficient detection of \textit{Bacillus anthracis} spores was developed and applied using a single-chain Fv (scFv) antibody. The antibody was isolated by using phage display library scFv antibody did not cross-react with representatives of some phylogenetically related \textit{Bacillus} spores (Mechaly \textit{et al.}, 2008).

Three distinct monoclonal phage antibodies against surface epitope protein components of \textit{S. Suis} were generated. Recombinant antibodies directed against surface components of a pathogenic strain of \textit{Streptococcus suis} serotype 2 was selected from a semi synthetic antibody phage display library (de Greeff \textit{et al.}, 2000).

Single domain antibodies (sdAbs) are recombinant antibodies which are derived from a novel class of immunoglobulins present in camelids have also been referred to as heavy-chain only antibodies (Hamers-Casterman \textit{et al.}, 1993; Ghahroudi \textit{et al.}, 1997), a product of numerous mutations that evade interaction with the light chain subunit (Vu \textit{et al.}, 1997). The proteins BclA, gerQ, SODA1, SOD15, BxpB and the protein p5303, the components of the \textit{B. anthracis} spore were used as targets for the detection of spores using a direct ELISA. The sdAbs were specific, demonstrating binding only to \textit{B. anthracis} spores and not to other \textit{Bacillus} species (Walper \textit{et al.}, 2013).
2.10.3.3. Nucleic acid based methods

Although there has been improvement in traditional methods, clinical laboratories have started to employ nucleic acid-based tests (NATs) to identify pathogens rapidly and consistently. A technological breakthrough in molecular biology came in 1983 with the development of polymerase chain reaction (PCR). Techniques based on PCR have been developed for different bacterial pathogens.

2.10.3.4. PCR, real-time PCR and RT-PCR

Amplification of target sequence by conventional PCR using two primers is detected and visualized by gel electrophoresis using DNA-binding fluorescent dyes.

A rapid real-time PCR detection assay for *B. anthracis* utilizing the LightCycler instrument (LightCycler Bacillus anthracis kit; Roche) was used. Specific PCR primers were designed for the protective antigens (plasmid pX01 and pX02) (Bell *et al.*, 2002). *HblA* gene specific PCR was employed for differentiation of enterotoxigenic and non-enterotoxigenic *B. cereus* isolates (Sanjoy Das *et al.*, 2009). For detecting anthrax spores, PCR assays developed showed that the heat treatment brought out the inactivation of *B. anthracis* spores and did not affect the efficiency of PCR assays (Fasanella *et al.*, 2003).

PCR assay for the detection and identification of *Bacillus cereus* in foods was developed (Schraft and Griffiths, 1995). Three primers for cereolysin AB and hemolytic activities of *B. cereus* were designed. The accuracy of the primers was tested with 39 isolates of *B. cereus* group, 17 other *Bacillus* spp., and with 21 non-*Bacillus* strains using PCR. With a combined PCR-hybridization assay, 1 CFU/mL of milk could be detected (Schraft and Griffiths, 1995). mPCR for the detection of enterotoxic *B. cereus* group strains were attempted by selecting *hbl A*, *nhe A* and *cyt K* genes. Validation of multiplex PCR assay was performed and simultaneous comparisons were made with the results of single-target. PCR assays and correlated to the conventional identification
tests. Contamination in food samples such as vegetable biriyani and milk were analysed. (Kalyan Kumar et al., 2010). B. cereus enterotoxin genes encoding the hbl and nhe complexes in strains of ground and roasted coffee samples showed enterotoxin production in the samples (Souza and Abrantes, 2011). A ready-to-use format for the isolation and the detection of B. cereus using PCR without enrichment are available with Norgen Biotech, Takara. A multiplex real-time PCR SYBR Green based assay was developed by Wehrle et al., (2010). Cereulide-producing Bacillus cereus was detected by real-time PCR assay in food within 2 h (Fricker et al., 2007).

A comprehensive TaqMan probe for real-time PCR assay was developed by Reekmans et al., (2009). Quantitative real-time PCR (qRT-PCR) was used to detect and quantify the Bacillus cereus group species including B. cereus, B. anthracis, B. thuringiensis, B. weihenstephanensis, B. mycoides, and B. pseudomyoides. Based on three probes (MotB-FAM-1, MotB-FAM-2, and Bpm-FAM-1), differentiation between Bacillus cereus group members was possible (Oliwa-Stasiak et al., 2011). Ueda et al., (2013) used TaqMan probe and SYBR green I based real-time PCR assays, to target cereulide synthetase genes (ces genes) for rapid, reliable and sensitive identification of the emetic strains. Lim et al., (2011), developed Duplex real-time polymerase chain reaction (PCR) method for detection and identification of emetic and non-emetic Bacillus cereus strains in foods without enrichment using an improved triple-primer polymerase chain reaction (PCR) assay. This PCR assay has been shown to be a suitable method for the quick screening and identification of B. cereus strains producing emetic toxin in food (Kim et al., 2013).

Possession of genes for the nonhaemolytic enterotoxin (Nhe) and haemolysin BL (HBL) having the potential to cause diarrhoeal illness in humans was studied by RT-PCR. Endophytic B. cereus isolates that possessed genes for enterotoxin production were present in agronomic produce and other endophytic B. cereus isolates lacked all nhe and hbl genes. In addition, no impact on the enterotoxin genes was observed due to host, country of origin and tissue of origin (Melnick et al., 2012).
RNA-based diagnostic method for the specific, direct detection of viable bacterial spores has been developed (Martinez-Blanch et al., 2009). Total analysis time was 2–8 h depending on the concentration of spore in samples. The developed procedure was optimized using *Bacillus subtilis* spores but could be applicable to other organisms. In baby food, about 60 CFU/mL of *Bacillus cereus* was detected (Martinez-Blanch et al., 2009).

### 2.10.3.5. Nested PCR

Nested PCR is a PCR method that amplifies a selected region of DNA with an outer primer pair in the first reaction, followed by an amplification using an internal primer pair. Due to its enhanced sensitivity over a single amplification, it is useful for pathogen detection in clinical specimens. But this can be problematic due to contamination that is carried over from the first reaction to the second (Apfalter et al., 2002). Nested PCR technique has also been employed for the identification of the 16S and 23S rRNA genes from an array of bacteria and it provides many overlapping amplicons for accurate sequencing of these genes. Nested PCR procedures also have been used to confirm the results of PCR findings (Wang et al., 1997). Spores from several *Bacillus* spp., *B. subtilis*, *B. cereus*, and *B. megaterium*, were amplified by PCR and sensitivity of the PCR-based assay was increased using nested PCR (Keith et al., 2004).

### 2.10.3.6. Colony PCR

Digoxigenin (DIG)-labeled dUTP was used as a DNA probe for Colony blots, dot blots to detect *B. cereus* and *B. thuringiensis* (Wiwat and Boonchaisuk, 2009). Using PCR and hybridization, the specificity of the primers was tested for 39 isolates of the *B. cereus* group, 17 other *Bacillus* spp., and 21 non-*Bacillus* strains. Detection limit for *B. cereus* in artificially contaminated milk was 1 CFU/ml using this combined PCR-hybridization assay (Schraft and Griffiths, 1995).

Isolates of *Bacillus cereus* from traditional Indian foods were detected by colony hybridization using the PCR-generated phospholipase (PL-1) probe.
29 isolates picked up by the probe were confirmed as *B. cereus* by conventional cultural and biochemical characteristics (Radhika, *et al.*, 2001).

### 2.10.3.7. Isothermal and other amplification technologies

The loop-mediated isothermal amplification (LAMP) method was developed as an isothermal nucleic acid amplification technique by Notomi *et al.*, (2000). This technique is based on the principle of strand displacement DNA synthesis by the *Bst* DNA polymerase with distinct six primers that recognize eight independent sites. DNA amplification is performed under isothermal conditions (60\(^\circ\)C to 65\(^\circ\)C), thereby precluding the need for a thermal cycler. Moreover, the LAMP method generates an increase in turbidity in positive samples, allowing detection by visual judgement and by real-time monitoring based on the turbidity of the reaction mixture as well as agarose gel electrophoresis. The major advantages of LAMP method are its rapidity, simplicity and high sensitivity for detecting the target genes. LAMP assays have been used for rapid detection of several pathogenic viruses, bacteria and blood protozoa (Iwamoto *et al.*, 2003; Hong *et al.*, 2004; Poon *et al.*, 2006; Kurosaki *et al.*, 2009). LAMP primer sets were chosen based on 16S rDNA and pCER270 plasmid cesA sequence. Under optimal conditions the LAMP reaction was carried out with 2 mM of Mg\(^{2+}\) at 65\(^\circ\)C for 1 h. Specificity of LAMP primers were validated by analysing 19 *B. cereus* isolates with and without pCER270 plasmid and 42 non-*B. cereus* isolates. 1 cfu/mL of *B. cereus* without pCER270 plasmid and 11 cfu/mL of pathogenic *B. cereus* with pCER270 plasmid was observed to be the detection limits of the LAMP assay. Also, liquid milk samples were detected by the LAMP method (Liu *et al.*, 2011). Similarly *B. anthrasis* was detected by using LAMP method (Dugan *et al.*, 2012) without sample preparation.

### 2.11. Biosensors

Applications of biosensors are almost 50 years old. After the International Union of Pure and Applied Chemistry defined a biosensor (Nagel *et al.*, 1992), and then further clarified its identity (Thevenot *et al.*, 1999), Leland C. Clark Jr. was recognized as the father of biosensors (Renneberg *et al.*, 2008). Karl
Cammann has been attributed with coining the term “biosensor” (Thevenot et al., 1999; Renneberg et al., 2008; Cammann, 1977). According to Thevenot et al., (1999), a biosensor is, an integrated device consisting of a biological component that is kept in spatial contact with an electrochemical transducer. The biosensor works when the biological component interacts specifically with its target, resulting in a signal change that is proportional to the test sample concentration which is measured by a transducer (Cock et al., 2009).

Since 2007 there have been articles related to food-borne pathogen detection using biosensors. Electrochemical (amperometric) (Poehlmann et al., 2009), optical (luminescence) (Cheng et al., 2009) and SPR (Linman et al., 2010), mass/acoustic (piezoelectric) (Chen et al., 2008) and mass (cantilever) (Xu and Mutharasan, 2009) transducers, have been developed for pathogens detection. Enrichment or culturing steps is being done by using magnetic beads. Antibodies that are specific for surface antigens on bacteria have been immobilised to these magnetic particles, which would then be mixed with the sample. The samples would then be passed through a magnetic field, which will attract these particles and thus concentrate and purify the bacteria from the matrix before biosensor analysis (Cheng et al., 2009; Zordan et al., 2009; Branen et al., 2007). Bruno and Yu, (1996) reported strain-dependent detection limits of $10^2$ to $10^5$ *Bacillus anthracis* spores/mg of soil suspension using immunomagnetic separation combined with an electro-chemiluminescence sensor.

A biosensor with antibodies for sensing and polyaniline nanowire as the electrical transducer for the detection of *B. cereus* was developed. The detection technique involved capillary flow of the liquid sample to move from one membrane to another. The principle involved antigen-antibody interaction and consequent electron charge flow generating a resistance signal that was recorded in pure cultures of *B. cereus* with a sensitivity of $10^1$ to $10^2$ CFU/ml (Pal et al., 2007).

A single-strand (ss) DNA biosensor implementing the ss-probe DNA was proposed by Velusamy et al., (2009). A direct-charge transfer conductometric
biosensor was developed for the detection of *Bacillus cereus* in various food samples (Pal *et al.*, 2008).

Cell-based biosensors (CBBs) are gaining importance for bio-security and rapid diagnostic applications in food microbiology. Cytotoxicity imparted by toxins of *Bacillus* sp. was checked using B-cell hybridoma, Ped-2E9 in 96 well formats (Banerje *et al.*, 2008). Potentiometric microbial biosensors with Urea as target have been developed for *Bacillus* sp. using NH$_4^+$ ion selective electrode. The limit of detection was 0.55–550 µM (Verma and Singh, 2003).

Phage-displayed peptide probes to detect biological threat agents (BTA) such as *B. anthracis* spores were developed. Several classes of pVIII- or pIII-displayed oligopeptides that recognized *B. anthracis* spores were identified (Brigati *et al.*, 2004; Turnbough, 2003; Uithoven *et al.*, 2000; Williams *et al.*, 2003). Regrettably, none of the peptides demonstrated 100% specificity for the target spores, which appears to be most likely due to a strong structural similarity of glycoproteins surrounding *Bacillus* spp. spores. A recent experiment using ruthenium red to stain *Bacillus* spp. spores demonstrated a glycoprotein layer surrounding both *B. subtilis* and *B. anthracis*, and suggested a higher ultrastructural similarity among various *Bacillus* species than had been recognized previously (Waller *et al.*, 2004).

The multivalent burrs are peptide/antibody probes and oligonucleotide tags conjugated to a protein base. Binding of multivalent burrs to an analyte (a cell or spore) resulted in oligonucleotide tags being brought together in proximity to generate a unique amplicon via ligation. Presence of this amplicon will then be detected via real-time PCR with high sensitivity. Pai *et al.*, (2005) successfully detected as few as 10 spores for *B. subtilis* and *B. cereus*, and 100 spores for *B. anthracis* using peptide/antibody probes and oligonucleotide tags conjugated to a protein base. Binding of multivalent burrs to an analyte (a cell or spore) results in oligonucleotide tags being brought together in close proximity to generate a unique amplicon via ligation. Presence of this amplicon will then be detected via real-time PCR with high sensitivity. Non-specific detection of cells has been carried out on both dip and peak type
Metal clad waveguide (MCWG) with a DL of 8–9 cells mm\(^{-2}\) (Skivesen et al., 2007). A DL of \(8 \times 10^4\) spores/mL for *Bacillus subtilis* var. *niger* bacterial spores were also demonstrated in a peak-type waveguide sensor (Zourob et al., 2005).

### 2.12. Conclusions and future perspectives

*Bacillus cereus* demonstrates a wide range of variation both phenotypically and genotypically. The highly heterogeneous genus *Bacillus* encompasses the largest species group of endospore forming bacteria. Because of their ubiquitous nature, *Bacillus* spores can enter food production chain at several stages resulting in significant economic losses and also posing a impending risk to consumers due the capacity of certain *Bacillus* strains to produce toxins. Previously, food microbiological diagnostics were focused on the determination of species using traditional culture-based methods, which are still widely practised. On the other hand, due to the extreme intra-species diversity found among the genus *Bacillus*, immuno- and DNA-based identification and typing methods are acquiring mounting significance in routine diagnostics. Several studies have shown that some of the characters are rather strain-dependent rather than species-specific. Therefore, the challenge for current and future *Bacillus* diagnostics is expected to be not only efficient but also accurate in identification at species level. Also the development of quicker methods to identify species with precise characteristics would help in tracing contamination sources, and last but not least differentiate potential hazardous strains from non-toxic strains.
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