PREVALENCE, CHARACTERIZATION AND BIOFILM FORMATION OF TOXIGENIC *Bacillus cereus* ISOLATED FROM READY-TO-EAT COOKED RICE IN PENANG

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PREVALENCE, CHARACTERIZATION AND BIOFILM FORMATION OF TOXIGENIC *Bacillus cereus* ISOLATED FROM READY-TO-EAT COOKED RICE IN PENANG

by

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LIST OF SYMBOLS &ABBREVIATIONS

°C	Degree Celsius
%	Percentage
α	Alpha
ß	Beta
γ	Gamma
x*g*	Centrifugal force
µg	microgram
µl	microlitre
µM	micromolar
AFLP	Amplified Fragment Length Polymorphism
AGs	Aminoglycosides
Ala	Alanine
AMP	Antimicrobial Peptides
ANOVA	Analysis of Variance
APC	Aerobic Plate Count
ARI	Antibiotic Resistant Infections
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual
BceT	*Bacillus cereus* enterotoxin T
BCET-RPLA	*Bacillus cereus* Enterotoxin-Reverse Passive Latex Agglutination
bp	Base pairs
$CaCl_2.6H_20$	Calcium chloride hexahydrate
CDC	Centre for Disease Control and Prevention
Ces	Cereulide synthethase
CFU/g	Colony forming unit per gram
CFS	Centre for Food Safety
CLSI	Clinical and Laboratory Standard Institute
CytK	Cytotoxin K
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicyclic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EFSA	European Food Safety Agency
EntFM	Enterotoxin FM
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
$FeCl_3$	Iron (III) chloride
GDP	Gross Domestic Product
g/L	Gram per litre
GR	Germinant receptors
HACCP	Hazard Analysis and Critical Control Point
Hbl	Haemolysin BL

HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
ISO	International Organization for Standardization
k	Kilo
KCl	Potassium chloride
KFDA	Korean Food and Drug Administration
LB	Luria-Bertani
Leu	Leucine
Log	Logarithm
MDR	Multiple Drug Resistance
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MHA	Mueller-Hinton Agar
mg	milligram
ml	millilitre
MLST	Multilocus Sequence Typing
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
Nhe	Non-haemolytic enterotoxin
nm	Nanometer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEMBA	Polymyxin B Egg Yolk Mannitol *Bacillus cereus* Agar
PFGE	Pulse Field Gel Electrophoresis
rRNA	Ribosomal Ribonucleic acid
r_s	Spearman correlation coefficient
rpm	Rotations per minute
RTE	Ready to eat
TAE	Tris-Acetate-EDTA
TECRA BDE-VIA	*Bacillus cereus* Diarrhoeal Enterotoxin Visual Immuno Assay
TSB	Tryptic Soy Broth
UHT	Ultra High Temperature
US	United States
UV	Ultraviolet
V	Volts
Val	Valine
VP	Voges-Proskauer
v/v	Volume per volume
w/v	Weight per volume

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KELAZIMAN, PENCIRIAN DAN PEMBENTUKAN BIOFILM *Bacillus cereus* TOKSIGENIK YANG DIASINGKAN DARIPADA NASI YANG SEDIA DIMAKAN DI PULAU PINANG

ABSTRAK

Bacillus cereus merupakan patogen makanan penting yang menyebabkan intoksikasi and toksiko-infeksi kepada manusia. *B. cereus* boleh bertahan di dalam makanan dan alam sekitar kerana keupayaannya menghasilkan spora dan tumbuh dalam biofilm, yang membolehkan ia hadir di dalam makanan yang diproses seperti nasi yang dimasak. Pengendalian makanan yang tidak betul terutama dalam keadaan suhu yang salah mencipta persekitaran yang baik untuk pertumbuhan *B. cereus* yang kemudiannya menghasilkan toksin emetik dan enterotoksin. *B. cereus* juga memainkan peranan yang penting dalam penyebaran gen yang rintang antibiotik dan kemunculan strain yang rintang terhadap antibiotik. Selain itu, *B. cereus* boleh menyebabkan kerosakan makanan melalui aktiviti enzimnya. Oleh sebab itu, kajian ini dijalankan untuk menentukan kelaziman *B. cereus* dalam 100 sampel nasi yang dimasak, dan yang dikumpul secara rawak dari kedai-kedai makanan di Pulau Pinang. Selain itu, kajian ciri-ciri strain *B. cereus* yang berbeza yang berkaitan dengan keracunan makanan, kerosakan makanan, rintangan antibiotik dan potensi pencemaran berulang turut dijalankan. Kaedah kiraan plat aerobik menggunakan media selektif (PEMBA) digunakan untuk pengasingan dan penghitungan *B. cereus* dan identiti isolat disahkan menggunakan ujian morfologi, ujian biokimia, 16S rRNA gen PCR dan jujukan DNA dengan menggunakan kaedah Sanger. Analisis PCR juga digunakan untuk mengesan 9 gen yang mengkod toksin. Aktiviti amilolitik, proteolitik dan

lipolitik ditentukan secara kualitatif dengan menggunakan media ujian seperti agar kanji, susu skim dan agar tributyrin, masing-masing dan isolat yang positif digunakan dalam ujian enzim secara kuantitatif. Antibiogram isolat *B. cereus* terhadap 13 antibiotik dan keupayaan pembentukan biofilm ditentukan melalui kaedah Penyebaran Disc Kirby-Bauer dan ujian biofilm yang terendam,. Sejumlah 34 sampel positif *B. cereus* telah dikenalpasti dengan kandungan bakteria antara 3.51-5.95 log CFU / g. Peratusan yang tinggi (82.4%) daripada isolat mempunyai sekurang-kurangnya 1 gen toksin, dengan kadar pengesanan yang lebih tinggi terhadap gen diarrheal (2.8-76.5%), berbanding dengan gen toksin emetik (14.7%). Sejumlah 4, 33 dan 6 isolat menunjukkan aktiviti amilolitik, proteolitik dan lipolitik masing-masing, dan ujian enzim pada isolat yang positif enzim mendedahkan aktiviti amilolitik yang lebih tinggi (11.1-47.3 U/ml) daripada aktiviti lipolitik (0.03-1.42 U/ml) dan aktiviti proteolitik (0.089- 0.21 U/ml). Aktiviti enzim yang diperhatikan menunjukkan potensi isolat untuk menyebabkan kerosakan makanan. Peratusan isolat bakteria yang memperlihatkan kerintangan terhadap ampisilin, penisilin dan trimethoprim berada dalam lingkungan 79.4-94.1%. Peratusan yang tinggi diperhatikan untuk sensitiviti terhadap antibiotik seperti gentamicin (88.2%), kanamisin (82.4%), ciprofloxacin (94.1%), vancomycin (82.4%) dan tetracyline (73.5%). Rintangan pelbagai drug (MDR) diperhatikan dalam 12 isolat. Rintangan antibiotik terutamanya rintangan pelbagai drug dalam kalangan *B. cereus* dalam kajian ini amat membimbangkan kerana ciri-ciri rintangan itu dapat dipindahkan kepada patogen lain yang penting secara klinikal melalui sistem makanan. Ujian biofilm menunjukkan bahawa kira-kira 55.9% daripada isolat dalam kajian ini mampu membentuk biofilm, iaitu satu fenotip yang menggalakkan pencemaran berulang dalam rangkaian pemprosesan makanan. Kesimpulannya, sampel nasi boleh menjadi vektor bagi *B. cereus* dan ciri-ciri yang

ditunjukkan oleh isolat dalam kajian ini mencerminkan peranan mereka dalam kerosakan makanan serta potensi patogenisiti, kerana kerintangan dan kehadiran pelbagai gen toksin dalam organisma ini.

PREVALENCE, CHARACTERIZATION AND BIOFILM FORMATION OF TOXIGENIC *Bacillus cereus* ISOLATED FROM READY-TO-EAT COOKED RICE IN PENANG

ABSTRACT

Bacillus cereus is an important foodborne pathogen causing intoxication and toxico-infection in human. *B. cereus* can persist in food and environment due to its ability to produce spore and grow in biofilm, resulting in its presence even in processed food such as cooked rice. Mishandling of foods especially in time-temperature abused condition creates favourable conditions for the growth of *B. cereus* which subsequently produce emetic toxin and enterotoxins. *B. cereus* also plays significant roles in dissemination of resistant genes and emergence of antibiotic resistant strains. Moreover, *B. cereus* can cause food spoilage through its enzymatic activities. Hence, this study was undertaken to determine the prevalence of *B. cereus* in 100 cooked rice samples randomly collected from food outlets in Penang Island. Moreover, the studies on the different *B. cereus* strains' characteristics that are relevant in food poisoning, food spoilage, antibiotic resistance and potentiality to promote recurrent contamination were also carried out. Aerobic plate count method using selective media (PEMBA) was employed for isolation and enumeration of *B. cereus* and the identities of the isolates were confirmed through morphological, biochemical tests, 16S rRNA gene PCR and DNA sequencing by using Sanger method. PCR analysis was also used to detect 9 toxin-encoding genes. Amylolytic, proteolytic, and lipolytic activities were determined qualitatively by using test media such as starch, skim milk and tributyrin agars, respectively and the positive isolates were subjected to enzyme assays for quantitative study. The antibiogram of the *B. cereus* isolates against 13 antibiotics and biofilm-forming ability were determined by Kirby-Bauer Disc Diffusion

method and submerged-biofilm assay respectively. A total of 34 *B. cereus* positive samples were identified with the bacterial loads ranging from 3.51-5.95 log CFU/g. High percentage (82.4%) of the isolates carried at least 1 toxin gene, with higher detection rates of diarrhoeal genes (2.8-76.5 %), than emetic toxin genes (14.7%). A total number of 4, 33 and 6 isolates exhibited amylolytic, proteolytic and lipolytic activities respectively, and the enzyme assays on corresponding enzymes-positive isolates revealed higher amylolytic activity (11.1-47.3 U/ml) than both lipolytic (0.03-1.42 U/ml) and proteolytic (0.089- 0.21 U/ml) activities. The enzyme activities observed indicate food spoilage potential of the isolates. The percentage of bacterial isolates displaying resistance against ampicillin, penicillin and trimethoprim were in the range of 79.4-94.1%. High percentage was observed for sensitivity to antibiotics such as gentamicin (88.2%), kanamycin (82.4%), ciprofloxacin (94.1%), vancomycin (82.4%) and tetracyline (73.5%). Multiple drug resistance (MDR) was observed in 12 isolates. The antibiotic resistance especially multiple drug resistance among *B. cereus* in this study is of utmost concern as such resistant property can be transferred to other clinically significant pathogens via food systems. The biofilm assay revealed that about 55.9% of the isolates in this study were capable of biofilm formation, a phenotype that promotes recurrent contamination in food processing lines. In conclusion, rice samples may become a vehicle for *B. cereus* and the characteristics displayed by the isolates in this study reflect on their role in food spoilage as well as potential pathogenicity, due to the organism's resistance and multiple toxin genes.

CHAPTER 1

INTRODUCTION

1.1 General Backgrounds

Bacillus cereus has been recognized as the causative agent of food spoilage caused by extracellular enzymatic actions (Roy et al., 2007; Toh et al., 2004) and foodborne illnesses, classified as emetic and diarrhoeal syndromes (Carter et al., 2018; Ghelardi et al., 2002;). Both syndromes are mild and self-limiting (24-48 hours) (Arnesen et al., 2008; Carter et al., 2018) but in certain instances, deaths among the affected patients have been reported (Sandra et al., 2012). *B. cereus* has gained much significance in relation to food safety issue due to its ability to exist in both vegetative and spore forms (Desai and Varadaraj, 2010; Tewari and Abdullah, 2015).

The spores of *B. cereus* are resistant to stresses like heat, desiccation, antimicrobials, and radiation (Setlow, 2014a; Soni et al., 2016). Moreover, biofilm-forming ability of *B. cereus* further renders higher survival capacity through genotypic and phenotypic modifications (Auger et al., 2009; Majed et al., 2016) by facilitating horizontal gene transfer (Cloete, 2009). Biofilm-mediated resistance to antimicrobials, differing from that of planktonic cells is one such example of the advantages of biofilm-inhabiting cells (Hall and Mah, 2017). The unique ability of *B. cereus* to exist in different cellular states, growth modes as well as regulation of metabolism does not only result in *B. cereus* resistance and survival but also play key roles in activation of its virulence factors (Duport et al., 2004; Duport et al., 2016; Zigha et al., 2006).

As a result, efficient elimination of *B. cereus* from food processing lines and food commodities becomes unlikely and consequently, presents increased risks of foodborne illnesses and spoilage arising from omnipresence of *B. cereus* with

toxigenic and spoilage properties (Ryu and Beuchat, 2005). Rice is one of the foodstuffs frequently contaminated with *B. cereus* (Tewari and Abdullah, 2015), often implicated as the vehicle in 95% of *B. cereus* gastroenteritis particularly that of emetic nature (Ankolekkar and Labbe, 2009; Horwood, 2005).

The contamination of rice with *B. cereus* usually commences during its growth in paddy field as this organism is commonly present in soil, water and air (Arnesen et al., 2008; Vilain et al., 2006). The high resistance and adaptability of *B. cereus* enable easy and rapid transmission of this organism along food processing lines, leading to subsequent and recurrent contaminations during harvesting, milling and other agricultural operations (Sawei and Sani, 2016; Haque and Russell, 2005). Thermal processing such as cooking are also ineffective to completely kill the resistant *B. cereus* spores, resulting in its occurrence in ready-to-eat cooked rice (Ryu and Beuchat, 2005; Sandra et al., 2012).

This is further worsened by mishandling of foods such as time and temperature abuse during storage (> 4°C and < 60°C with optimal temperature in the range of 30-37°C), poor hygiene during food preparation and in serving environments (CFS, 2014; Tewari and Abdullah, 2015). These provide favourable conditions for outgrowth, proliferation and synthesis of plethora of toxins and degradative enzymes by the surviving *B. cereus* cells and thereby, establishing *B. cereus* as a notorious foodborne pathogen (Arnesen et al., 2008; Majed et al., 2016).

1.2 Problem statement

In recent times, the trend of "eating out" culture among Malaysians due to factors such as affluence and urbanization, is on the rise. This makes the food handlers the front-line stakeholders in ensuring food safety. However, poor health education and awareness among the food handlers and consumers in developing nation, as well as close association of spore-forming *Bacillus cereus* with staple food such as rice give rise to concern regarding microbiological quality of cooked rice dishes sold in the eateries across Malaysia. Unfortunately, there is paucity on *B. cereus* contamination levels in food products in Malaysia with only a number of studies carried out in certain regions in Selangor (Sandra et al., 2012) Sabah (Sawei and Sani, 2016), Sarawak (Bilung et al., 2016), and Kelantan (Aklilu et al., 2016).

The data on *Bacillus cereus* contamination levels in ready-to-eat cooked rice samples from eateries and food outlets in Penang as well as its associated risk factors are hitherto unavailable. The present *B. cereus* strains may possess various toxigenic properties and spoilage potential due to possession of toxin-encoding genes and activity of extracellular enzymes (Amor et al., 2019; Techer et al., 2014). Apart from its role in foodborne illnesses and food spoilage leading to health issues and economical losses, this highly adaptive organism thrives well under the antibiotic pressure arising from frequent, extensive use of antibiotics in agricultural setting and in turn, mediate the dissemination of resistance genes to other clinically relevant pathogens leading to antibiotic-resistant infections (Amor et al., 2019; Fiedler et al., 2019). The dissemination of resistance genes and subsequent emergence of resistance phenomenon are enhanced by the mobile genetic elements possessed by *B. cereus* (Zhu et al., 2016)

Furthermore, *B. cereus* cells which strongly form biofilm on various surfaces in food systems are difficult to eradicate by cleaning and disinfection procedures carried out. As a result, the food processing environment also become reservoir for *B. cereus* and causes recurrent contamination. Biofilms also often serve as route for transmission of genetic materials, crucial for microbial survival and virulence, including novel patho-types.

1.3 Research Objectives

The main objective of the study is to investigate and provide information on *Bacillus cereus* contamination levels in RTE cooked rice in Penang and the characteristics of the foodborne organism. The specific research objectives are as follows:

a) To determine the prevalence of *Bacillus cereus* in the ready-to-eat cooked rice.

b) To determine *Bacillus cereus* toxin production potential through PCR-based detection of toxin-encoding genes

c) To determine *Bacillus cereus* enzyme activity through phenotypic characterization

d) To determine the antibiotic resistance profiles of *Bacillus cereus*

e) To determine the varying ability of *Bacillus cereus* isolates to form surface-associated (submerged) biofilm

CHAPTER 2

LITERATURE REVIEW

2.1 *Bacillus cereus sensu lato*

Bacillus cereus sensu lato (B. cereus s.l.) refers to a group of endospores forming organisms which are homogenous within the *Bacillus* genus. This *Bacillus cereus* group consists of eight closely related species which are *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus toyonensis* and *Bacillus cytotoxicus* (Forghani et al., 2016; Jeßerger et al., 2015). Many of them are significant as they possess medical and economic importance (Jensen et al., 2003).

B. cereus is a microscopically large (normally 3.0 -5.0 μm in length with diameter in the range of 1.0-1.2 μm), rod-shaped, Gram-positive bacterium which is facultatively anaerobic (Ghelardi et al., 2002; Jawad and Mutalib, 2016; Sandra et al., 2012). *Bacillus cereus* species can grow in the temperature range of 4-55 °C at the pH of 4.3 – 9.3 with minimum water activity (a_w) of 0.93 (Jääskeläinen, 2008). *B. cereus* cells are usually motile, haemolytic, resistant to penicillin and bacteriophage gamma and do not produce parasporal crystals (Altayar and Sutherland, 2006). In terms of colonial morphologies, *B. cereus* have irregular colonies with fimbriated edges and appear greyish, flat and with ground-glass texture on common solid media such as nutrient agar (Altayar and Sutherland, 2006; Kumari and Sarkar, 2016).

B. thuringiensis which is another closely related species to *B. cereus*, is also an important foodborne pathogen synthesizing several virulence factors including diarrhoeal toxins similar to *B. cereus* (Frederiksen et al., 2006). This organism differs from *B. cereus* by production of proteinaceous parasporal crystal (Frederiksen et al.,

2006). Due to the insecticidal properties of the proteinaceous crystals, this organism is commercialized and widely used as biopesticide and these crystalline proteins have also been expressed in crops for innate resistance against pest (Sanahuja et al., 2011).

B. anthracis is a non-motile, non-haemolytic, penicillin-sensitive, anthrax-causing organism, appearing with more spiking and tailing along the inoculation lines (Logan and Rodriguez-Diaz, 2006; Spencer, 2003). This organism causes anthrax by synthesizing anthrax toxin, the genetic determinants of which are harboured on two large plasmids, known as pXO1 and pXO2 which are typical virulence markers of this organism (Arnesen et al., 2008). The individual components of anthrax toxin known as protective antigen (PA), edema factor (EF) and lethal factor (LF), are encoded by pXO1 for formation of functional toxin (Jensen et al., 2003). pXO2-borne genes are responsible for poly-γ-D-glutamic acid capsule formation which is essential for *B. anthracis* to evade phagocytosis by host immune system cells (Friebe et al., 2016; Jensen et al., 2003).

Other non-motile members of *B. cereus s.l.* are *B. mycoides* and *B. pseudomycoides* which are easily distinguished by its characteristic growth pattern on agar resembling that of fungal-like (rhizoidal) pattern (Ehling-Schulz et al., 2019; Franco et al., 2002). In spite of similar phenotypic characteristics shared by these two organisms, genetic relatedness studies and fatty acid composition analysis clearly revealed that these two belong to different species (Jensen et al., 2003).

B. weihenstephanensis is a psychrotolerant member of this group with the ability to grow at temperatures below 7°C but not at 43°C (Stenfors et al., 2001). This psychrotolerant member of *B. cereus s.l* can also be distinguished from other members by genotypic detection of its specific signature 16S rRNA sequences and cold shock protein A (*cspA*) gene (Soufiane and Cote, 2013). As a result of its ability to grow at

low temperatures, this bacterium is often associated with the contamination of dairy products and refrigerated foods (Soufiane and Cote, 2013).

B. cytotoxicus is a thermotolerant member of this group with the growth temperatures in the range of 20-50 ℃ and frequently associated with foods such as mashed potato and vegetable puree (Bagcioglu et al., 2019). Apart from the growth temperatures, phenotypic characteristics discriminating *B. cytotoxicus* from other members of *B. cereus s.l.* include its inability to utilize trehalose and growth impairment in the absence of tryptophan (Guinebretiere et al., 2013).

B. toyonensis is known for its probiotic properties and hence commonly used in animal feed (Ehling-Schulz et al., 2019). The bacterium has been authorized by government bodies such as Japanese Ministry of Agriculture and Forestry and European Commission for commercial use in animal husbandry and aquaculture in several countries (Jimenez et al., 2013). *B. toyonensis* is the only member of *B. cereus s.l* which possesses the abilities to metabolize methyl-α-D-glucopyranoside and D-turanose as well as tolerant to high salinity level of up to 5% (Jimenez et al., 2013).

2.2 *Bacillus cereus*-related food poisoning

2.2.1 Overview

In 1950s, Hauge who had investigated four food poisoning outbreaks followed by consumption of vanilla sauce in Norway, was successful in establishing *Bacillus cereus* as a recognized food poisoning pathogen (Drobniewski, 1993; Kramer and Gilbert, 1989). However, the incidence of *Bacillus cereus* food poisonings are very much underestimated and under-reported due to *Staphylococcus aureus* and *Clostridium perfringens* food poisoning symptoms which resemble the emetic and diarrhoeal syndromes respectively (Forghani et al., 2016; Organji et al., 2015). The emetic syndrome (intoxication) manifests with symptoms such as vomiting and nausea upon ingestion of pre-formed toxin (Messelhäusser et al., 2014) whereas diarrhoea and abdominal cramps are common in diarrhoeal syndromes (toxicoinfections) following toxins' production in host's intestine by the ingested cells (Carroll et al., 2019).

Hence, the classification of *B. cereus* as either emetic or diarrhoeal depends on the type of toxins produced by the organisms (Carroll et al., 2019). The prevalence of the two types of illnesses caused by *B. cereus* may vary from country to country (Granum, 2007). The difference in distribution between countries is probably a reflection of the vehicles used by the organism (Marrollo, 2016) and varying reporting procedures between countries (Horwood, 2005). The diarrhoeal syndrome is more dominant in the United States and Northern Europe whereas emetic form of foodborne illness is more prevalent in countries such as Japan and United Kingdom (Ankolekkar and Labbe., 2009; Jung et al., 2017). It was in United Kingdom where the emetic form of *B. cereus* gastroenteritis was initially identified and reported (Kramer and Gilbert, 1989).

Due to the ubiquity of *B. cereus*, this bacterium is often isolated from natural habitats such as soil and growing plants (Fangio et al., 2010; Sandra et al., 2012) and also from a variety of foods (Grande et al., 2006) such as fresh and minimally processed vegetables, honey, anchovies, raw and pasteurized milk, cereals and cereals by-products (Fangio et al., 2010) , chicken dishes, rice-based products such as fried rice, pasta and noodles (Altayar and Sutherland, 2006; Grande et al., 2006), dairy products, egg, meat (Wong et al., 1988), bread, cakes, seafood, legumes, grains (Sandra et al., 2012) spices and infant formula (Carter et al., 2018).

However, only proteinaceous foods such as vegetables and meats are often incriminated in diarrhoeal syndrome whereas farinaceous foods particularly cooked rice are associated with emetic syndrome (Arnesen et al., 2008; Schoeni and Wong, 2005). This is due to the ability of each food matrix to support specific toxin production. Proteinaceous foods have been shown to enhance *B. cereus* enterotoxins' production under anaerobic conditions (Ceuppens et al., 2011; Jeßerger et al., 2017) whereas starchy foods support cereulide production in the presence of oxygen (Agatha et al., 1999; Agatha et al., 2002).

In both syndromes, the reported infective dose is approximately 10^5-10^8 although doses as low as 10^3 CFU/g in foods had been found sufficient to cause illness in certain foodborne outbreaks (Arnesen et al., 2008). The variation in infective dose occurs mainly due to *B. cereus* strain-specific differences in terms of toxin production level which could vary up to 100-fold between individual strains (Apertroaire-Constantine et al., 2008).

2.2.2 Epidemiology of *Bacillus cereus*

Bacillus cereus-induced food poisoning occurs worldwide and throughout the year (Tewari et al., 2015). Table 2.1 shows some of the food poisoning episodes and brief details of those incidents which occurred in various countries. The European Food Safety Agency (EFSA) stated that 1-33% of foodborne outbreaks are caused by *B. cereus* in the year 2005 (Sandra et al., 2012). In 2011, the statistics of *B. cereus* gastrointestinal and non-gastrointestinal infections in Europe reported by EFSA increased to as high as 122.2 % (Messelhäusser et al., 2014). A total of 413 foodborne outbreaks affecting 6657 individuals from European Union (EU) was reported in 2016 alone (EFSA, 2016). In specific European countries, *B. cereus* caused 117 food poisoning outbreaks between 1960 and 1968 and was ranked third most cause of foodborne diseases in Hungary (Tewari and Abdullah, 2015). During 1973-1985, 17.8% of total bacterial food poisoning cases in Finland, 11.5% in Netherlands, 0.8% in Scotland, 0.7 % in. England and Wales were caused by *B. cereus* (Kotiranta et al., 2000). In 2014, 12 members of EU reported 287 *B. cereus*- related foodborne outbreaks which resulted in 89 hospitalizations with no casualties (FSAI, 2016).

Table 2.1: Worldwide scenarios of *B. cereus*- related food poisoning

Continents	Country	Year(s)	Other details	References
Europe	Austria	2013	Three outbreaks from May to July. Symptoms including diarrhoea and vomiting within 24 hours due to consumption of mashed potatoes, pancake strip soup and fruit salad.	Schmidt et al. (2015)
	Belgium	2005 - 2011	Fatal outcomes involving 5 members of a family (consumed salad pasta stored in refrigerator for 3 days) and a young adult (consumed pasta with tomato sauce left at room temperature for 5 days) in a separate incident	Dierick et al. (2005) Naranjo et al. (2011)
	England	2012	Vomiting symptoms within an hour of ingestion of contaminated lunch meals in 5 nurseries in South East England due to consumption of *B. cereus*-contaminated shepherd's pie and haricot beans (soaked at 22°C)	Nicholls et al. (2016)
	France	2007-2014	74 outbreaks strictly due to *B. cereus* whereas 66 other outbreaks involving simultaneous detection of *Clostridium perfringens.* Most outbreaks related to consumption of starchy foods and vegetables	Glasset et al. (2016)
Americas	Argentina	2014	Development of emesis and watery diarrhoea, leading to severe dehydration in a 39 year old woman. Cooked chicken was confirmed as food poisoning vehicle of this case,	Lopez et al. (2015)
	Brazil	2000-2015	*B. cereus* was identified as causative agent in about 330 poisoning cases linked to consumption of cereals products and sauces.	Lentz et al. (2018)
	USA	2016	An outbreak involving 160 *B. cereus* food poisoning cases in New York was reported due to consumption of refried beans served in a Mexican restaurant	Food Safety News (2016)
Asia & Oceania	Australia	2017	Patrons of a restaurant in Cranberra developed diarrhoea (9 cases) and vomiting (6 cases) with *B. cereus* detected at unsatisfactory level (1.9 x 10^4 CFU/g) in beef.	Thirkell et al (2019)

Continents	Country	Year(s)	Other details	References
Asia & Oceania	China	2013	A major outbreak involving 139 individuals who suffered nausea, vomiting and diarrhoea after consuming fermented black beans (*douchi*)	Zhou et al. (2014)
	India	2017	A *B. cereus* outbreak in working men's hostel due to consumption of cooked chicken.	Grewal and Khera (2020)
	Indonesia	2019	An outbreak involving 188 affected villagers in Bantul, Yogyakarta was due to consumption of contaminated chicken satay.	Son et al. (2019)
	Malaysia	1984 & 2012	Only 2 outbreaks reported in 28 years due to consumption of school canteen foods	Rampal et al. (1984) Jeffree and Mihat (2016)

In the United States, though the *B. cereus* food poisoning cases from the year 1972-1982 was low (1.3%), there was a major outbreak in 1989 involving 100 people (Kotiranta et al., 2000). From the year 1998-2008, a total of 235 (19%) foodborne outbreaks due to *B. cereus* were documented in US (Bennett et al., 2013). *B. cereus* was ranked only fourth most notorious pathogen, being implicated in 3.1% of foodborne diseases in whole of Brazil (Lentz et al., 2018). However, in Southern region of Brazil named Porto Alegre, *B. cereus* was identified as the main etiological agent in as many as 32.2% foodborne cases studied from 2003 to 2013 with most of the incriminated foods were of cereal types and sauces (Lentz et al., 2018).

B. cereus was identified as one of the main foodborne pathogens causing food poisoning in Australia with a total of 114 cases recorded over a period of 2001-2013 (May et al., 2016). Recent studies revealed that *B. cereus*-mediated food poisonings are very common in Australia with about 3350 cases occur annually (Thirkell et al., 2019). A total of 145 out of 1082 foodborne diseases which occurred in China from

1994-2005 were associated with *B. cereus* (Yu et al., 2019). This organism was identified as third most prevalent pathogen in the food commodities in China in a period of 2006- 2016 (Paudyal et al., 2018).

These statistics however may not be a depiction of actual scenario. This is because *B. cereus* related food poisoning may be underestimated and under-reported due to lack of effective surveillance especially in developing countries, self-limiting nature of the illnesses and resemblance of *B. cereus* food poisoning symptoms to that of *Staphylococcus aureus* and *Clostridium perfringens* (Organji et al, 2015; Sandra et al , 2012). Moreover, detection of *B. cereus* in any foodborne outbreaks may not be of priority as this pathogen is not zoonotic and thus receive less attention than other prominent foodborne pathogens (Ceuppens et al., 2013).

The lack of data on statistics of *B. cereus* related foodborne outbreaks and prevalence in food commodities in Malaysia is one of the examples of *B. cereus* underestimation. The first food poisoning case due to *B. cereus* in Malaysia was reported by Rampal (1984) which affected 114 Malay students of a religious secondary school, Klang. In 2012, another episode of *B. cereus* food poisoning case was documented in a primary school in Sabah (Jeffree and Mihat, 2016).

As for incidence, there are limited reports on detection of *B. cereus* in Malaysian food commodities such as legumes, spices, noodles (Rusul and Yaacob, 1995) cereals, honey, chocolate, milk (Lee et al., 2009), and cooked chicken meat (Aklilu et al., 2016). The report on *B. cereus* prevalence in both raw and cooked rice in Malaysia was firstly documented in 2012 (Sandra et al., 2012) and followed by a number of other studies (Bilung et al., 2016, Sawei and Sani, 2016).

2.3 *Bacillus cereus* spores and its germination

2.3.1 Endospore structures and properties

All *Bacillus* species are able to form endospores, an attribute which is a survival strategy in harsh conditions as the endospore is a metabolically dormant, tough, non-reproductive structure with extremely high resistance towards heat, radiation, desiccation and chemicals (Setlow, 2013; Shaheen., 2008; Wei et al., 2010). Hence, endospore formation is of great research interests as endospore formation plays roles in adherence of spores to substrates and hosts as well as ensures survival of *B. cereus* which is a causative agent of food spoilage, foodborne diseases and other diseases in human and animals (Setlow, 2013; Shaheen, 2008; Stalheim and Granum, 2001).

So, bacterial spore formation is regarded as a threat to food safety even though the dormant spores do not directly contribute to foodborne illnesses but rather pave way for persistence and colonization of disease-causing *B. cereus* in wide range of environments and food products (Setlow, 2014a; Majed et al., 2016). An endospore typically consists of inner protoplasts in which DNA, ribosomes, inert enzymes and other cellular components are located (De Vries, 2006; Priest, 1993 ; Soni et al., 2016). The bacterial DNA is associated with highly conserved small acid-soluble spore proteins (SASPs). These proteins which are present in all endospores have molecular size of 12-15 kDa and exist in two forms, which are α or β type (Setlow, 2014b).

The SASPs makes up about 5-12 % of the total spore protein which is ample for the saturation and protection of spore DNA by enhancing spore resistance (Priest, 1993, Setlow and Setlow, 1995). The binding of SASPs to DNA causes conformational changes of DNA such as right-handed helices (A- or B- forms) and left-handed helices (Z- form). The difference between A- form and B- form lies in the number of base

pairs which is higher in the former form (Priest., 1993). These conformational changes provide protection against UV radiation (De Vries., 2006; Setlow, 2014a,b).

Dipicolinic acid (DPA) chelated with divalent cations, mainly Ca^{2+} and other ions such as Mn^{2+} and Mg^{2+}, is present in the protoplast in high levels (Priest, 1993; Setlow, 2013.) This molecule makes up about 5-15% of the *Bacillus* spores' dry weight (De Vries, 2006; Setlow, 2006). Low water content in spore core due to the presence of DPA (pyridine-2,6-dicarboxylic acid) during sporulation and chelation of DPA with divalent cations affect wet heat resistance and UV photochemistry of spore DNA (Nicholson et al., 2000).

Inner membrane separating germ cell from central spore core contains many crucial germination proteins in or adjacent to inner membrane either as peripheral or integral membrane proteins (Setlow, 2013). Properties of inner membrane such as higher viscosity of inner membrane of spore than that of germinated cell, low permeability even to water and immobility of lipid molecules indicate that this membrane probably plays role in inhibiting access of bactericidal agents to spore core (Setlow, 2014b). Although the lipid molecules of inner membrane are highly immobile, the mobility of lipid molecules is restored when the process of spore germination completes (Cowan et al., 2004; Setlow, 2014a). The function of outer membrane, which lies beneath spore coat is not precisely known (Setlow, 2014b).

Germ cell wall which lies under the cortex, will become the outgrowing spore's cell wall. This germ cell wall is composed of peptidoglycan that is structurally similar to peptidoglycan of vegetative cells (Setlow, 2006). Cortex which is below the spore coat, is mainly composed of a thick layer of peptidoglycan comprising of a backbone of repeating disaccharide and cross-linking oligopeptides (Setlow, 2006; Setlow, 2013). The cortex is pivotal for the dormant state and low water content of the spore

core and protects the core from chemical damages caused by organic solvent (Henriques and Moran, 2000; Soni et al., 2016).

Unlike the peptidoglycan of germ cell wall and vegetative cells, cortic-specific modifications such as conversion of muramic acid residues in the polysaccharide to muramic acid-δ-lactam (MAL) occur in cortical peptidoglycan (Setlow, 2013). MALs aid cortic-lytic enzymes (CLEs) in peptidoglycan recognition and cleavage. These enzymes degrade the cortex to permit spore core expansion and further outgrowth (Setlow, 2006; Setlow, 2013; Setlow, 2014).

The spore coat consists of multiple layers of several proteins which are involved in the assembly of spore coat and exosporium (Setlow, 2014). As major components of spore, these proteins comprise 40-80% of total spore protein (Priest, 1993). Although spore coat has no significance in developing spore resistance towards heat, radiation and some chemicals, it is essential in spore resistance to some other agents like exogenous lytic enzyme and predation by protozoa (Nicholson et al., 2000; Setlow, 2006).

The exosporium is a large, loose-fitting, irregularly shaped, balloon-like structure with crystalline basal layer. The basal layer of exosporium is surrounded by hair-like projections in which collagen-like glycoprotein BclA is the main component that serves as a immunodormant spore antigen (Priest, 1993; Setlow, 2006; Stewart, 2015; Todd et al., 2003). This structure forms the outermost layer of spores of *B. cereus* and its close relatives although many species such as *B. subtilis* either do not possess exosporium in its spores or if they do, the size of the exosporium is too small (Setlow, 2006). The anchorage of exosporium to spore coat occurs through a number of protein-protein interactions (Stewart, 2015).

The major component of *B. cereus* spore's exosporium is enzyme alanine racemase and it also contains purine nucleoside hydrolase. These enzymes can modify germinants and thus play role in modulating germination (Setlow, 2013; Yan et al., 2007). The composition of exosporium also include other proteins like ExsFA and ExsFB which are required for maintaining stability of exosporium structure (Faille et al., 2007) The hydrophobic nature of this outermost layer suggests that exosporium may have significance in the interaction of spore with target organisms or hosts and spore pathogenicity (Todd et al., 2003).

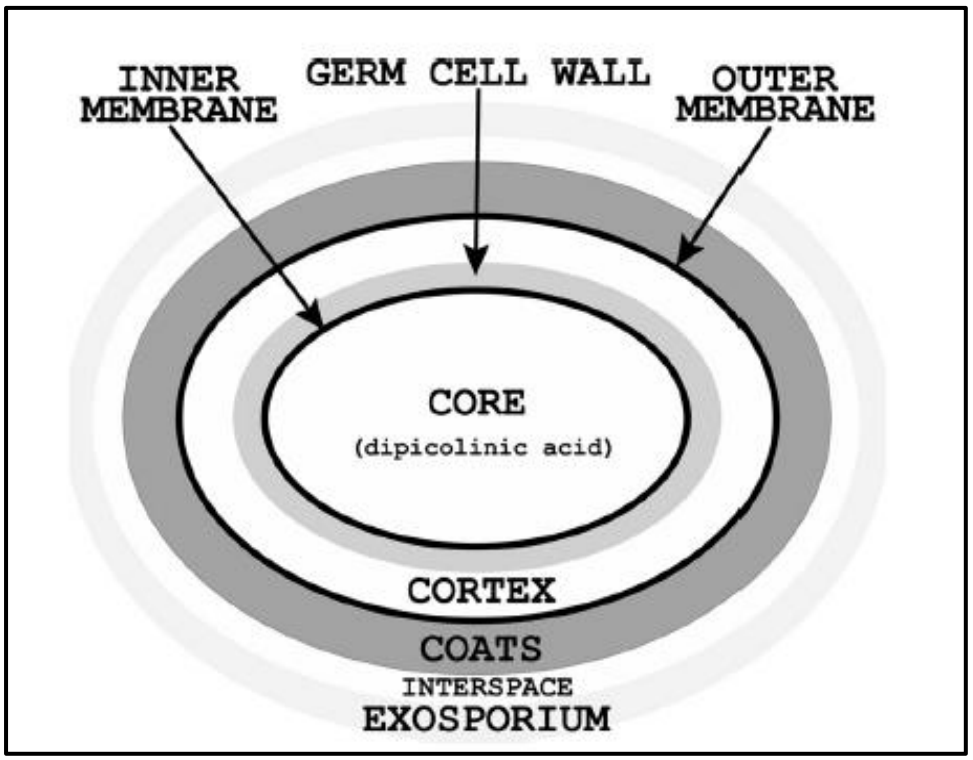

Figure 2.1: Spore structure. The various labelled spore layers are not drawn to scale, and the sizes of the various layers in particular the exosporium vary significantly between spores of different species (Setlow, 2014a)

2.3.2 Germination of spores

The conversion of dormant spores into vegetative cell is achieved through a process known as germination, during favourable conditions (Hornstra et al., 2007; Ramirez-Peralta et al,. 2013). Spore germination is an important event with respect to food safety as the metabolically active vegetative cells contribute to food spoilage and poisoning (Setlow, 2014a). Signaling molecules (germinants) which trigger germination can be divided into nutrient and non-nutrient germinants. Nutrients such as amino acids, sugars and purine nucleosides are known as nutrient germinants whereas non-nutrient germinants include heat, lysozyme, high pressure, mechanical abrasion specific bile salts and cationic surfactants such as dodecylamine (Setlow, 2003; Setlow, 2014; Vepachedu et al., 2007).

In nutrient-dependent germination, the signals to initiate germination is sent by the germinants via receptor proteins known germinant receptors (GRs) which are found on the inner membrane of spores (Setlow, 2013). The activation of germination by non-nutrient germinant however can be achieved with and without germinant receptors. Heat activation usually requires GR-dependent pathway (Setlow, 2014a) while high pressures (300-500 MPa) and mechanical abrasion do not require GRs for initiate germination (Reineke et al., 2011; Vepachedu et al., 2007). The levels of GR not only vary between different spore populations but also between individual spores of a population due to stochastic effect on the level of germination proteins and GRs expression, eventually resulting in heterogeneity in germination rates (Setlow, 2013). Hence, spores with increased numbers of GRs will germinate much faster as compared to spores with reduced GRs (Kong et al., 2014).

Germination usually begins with the release of Ca^{2+}-DPA from spore core via numerous channels consisting of SpoVA proteins involved in the transport of DPA

(Setlow, 2014a; Vepachedu et al., 2007). The approximate time for the efflux of more than 90% DPA is 2 minutes even though individual spores may exhibit variation in DPA release time which may range from minutes to hours (Kong et al., 2011; Setlow, 2013) Stage I of germination becomes complete upon hydration of spore core by uptake of water (Kong et al., 2014). Stage II begins when cortex-lytic enzymes (CLEs) degrade cortical peptidoglycan. The spores of many *Bacillus* species have two types of CLEs which are Cw1J and SleB (Setlow, 2014a).

The presence of either one CLE alone is ample to permit spore germination to complete (Setlow., 2014a). Once the cortical peptidoglycan has been hydrolysed, the germ cell wall and spore core begin to expand and take up more water. The surface area of inner membrane increases 1.5- 2 fold in this process without synthesizing new membrane (Cowan et al., 2004). At this stage enzymes become active in the core, leading to a degradation of small, acid-soluble proteins in the spore core. Then, metabolism and synthesis of macromolecules are initiated in core (Setlow, 2003; Setlow, 2013). At the end of stage II of germination, germinated spore is converted to vegetative cell by outgrowth (Paidhungat and Setlow, 2002).

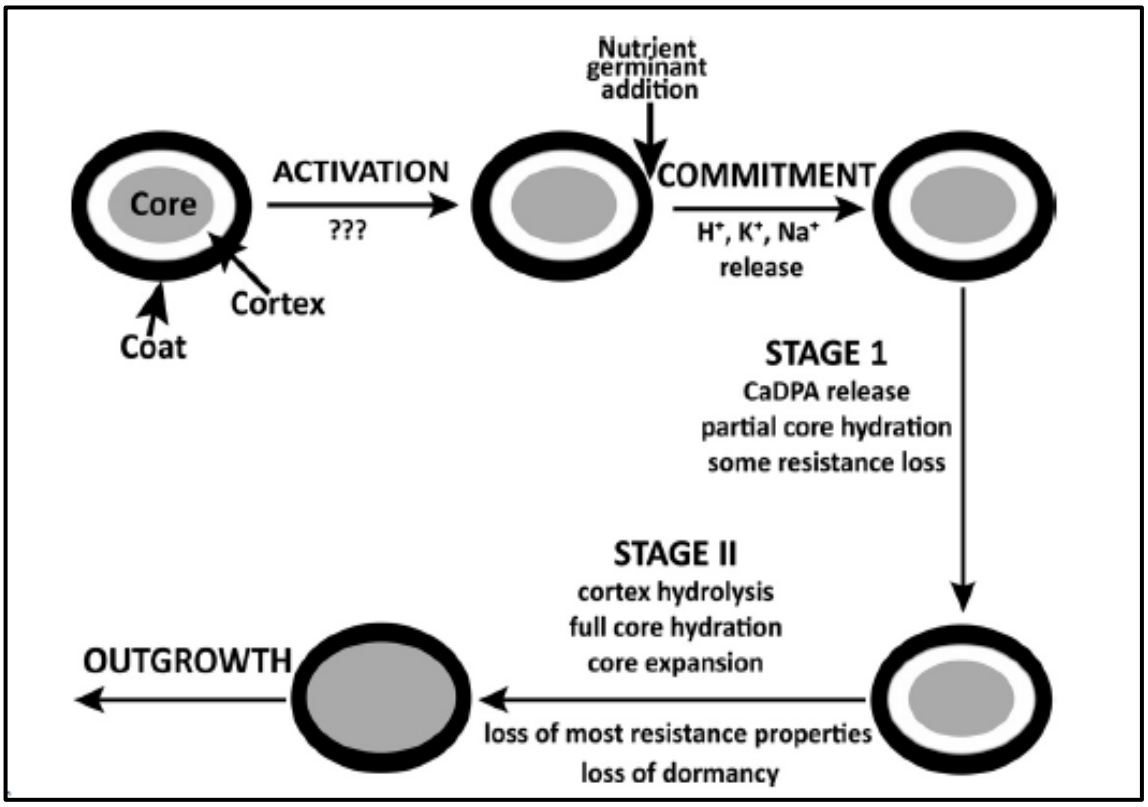

Figure 2.2: Events taking place during spore germination. The uncertainty regarding precise events in the activation step is denoted as question marks. (Setlow, 2014a)

2.4 Isolation and identification of *Bacillus cereus*

2.4.1 Culture-based methods

Standard method of quantitative evaluation for *Bacillus cereus* in foods, environment and clinical settings involve culture-based methods using selective media as recommended by several food authorities such as US Food and Drug Administration and International Organization for Standardization 7932 (ISO 7932) (Chon et al., 2012a; Ehling-Schulz and Messelhauser, 2013; Fricker et al., 2008). Two standard plating media recommended by these food authorities are mannitol-egg yolk-polymyxin B (MYP) agar and polymyxin B- egg yolk-mannitol-bromothymol blue (PEMBA) (Chon et al., 2014; Fricker et al., 2008).

The detection or diagnostic features for *B. cereus* are i) characteristic appearance of colonies, ii) abilities to hydrolyze egg yolk, which is an indication of lechitinase and phospholipase C activity and iii) inabilities of the organism to ferment mannitol (Fricker et al., 2008; Netten and Kramer, 1992). *B. cereus* forms eosin-pink (Tallent et al., 2001), crenate to fimbirate colonies of 3-6 mm in diameter surrounded by precipitation zone on MYP agar (Netten and Kramer, 1992; Tallent et al., 2012). The reliance of this medium on egg yolk reaction as a diagnostic feature would cause weak or lecithinase-negative *B. cereus* strains, which already have been described, to be misidentified or overlooked (Chon et al., 2014; Netten and Kramer, 1992).

The selectivity of this medium is insufficient in food with high competing microflora (Chon et al., 2012a) as polymyxin B (antibiotic supplement) suppresses only Gram-negative bacteria by disrupting the structures and functions of their exterior and cytoplasmic membrane (Netten and Kramer, 1992). Moreover, drawbacks such as poor differentiation of organisms on MYP and weak egg yolk reactions led Holbrook and Anderson to develop new selective medium called PEMBA (Netten and

Kramer, 1992). Although the detection principles of PEMBA are same as MYP which are based on mannitol-negative and lecithinase-positive nature of *B. cereus*, the double diagnostic system of MYP is altered by replacing phenol red with bromothymol blue in PEMBA (Netten and Kramer, 1992).

The distinct morphology of colonies on PEMBA also allowed the detection of strains that do not precipitate egg yolk (Holbrook and Anderson, 1980; Kramer and Gilbert, 1989). The basal medium was reformulated with 0.1% w/v of peptone; sodium pyruvate to enhance egg yolk reaction, reduce the size of *B. cereus* colonies and induce spore formation; and phosphates to buffer the medium. PEMBA appears as a semi-opaque yellow-greenish medium (Netten and Kramer, 1992). In PEMBA, the colonies are crenate to slightly rhizoid (Kramer and Gilbert, 1989) with peacock blue colour and surrounded by a blue zone of precipitation which indicates egg yolk hydrolysis (Netten and Kramer, 1992). The diameter of the colonies was reported to be in the range of 2-5 mm (Kramer and Gilbert, 1989); 3-7 mm (Sawei and Sani, 2016).

Other isolation media for *B. cereus* include modified MYP (mMYP) agar and chromogenic media such as Brilliance *B. cereus* agar and *B. cereus* Rapid Agar (BACARA). The MYP medium is modified by supplementing the medium with trimethoprim. The combination of polymyxin B and trimethoprim exhibits broad-spectrum of antimicrobial activity against various bacteria (Chon et al., 2012a). Chromogenic plating media such as BACARA and Brilliance *B. cereus* agar were determined to be more efficient for isolation of *B. cereus* from complex food matrices with high loads of competing microflora which usually interferes with development of typical characteristics on conventional media (Chon et al., 2014; Kabir et al., 2017).

B. cereus colonies appear as turquoise green and orange-pink on Brilliance *B. cereus* agar and BACARA, respectively. due to the enzymatic cleavage of

chromogenic substrates added into the media (Chon et al., 2014; Tallent et al., 2012). API 50CHB and 20E kits in combination are used for confirmation and identification of *Bacillus* spp based on their utilization of various carbohydrates (Aruwa and Olatope, 2015). However, similar biochemical characteristics between closely related species (*B. cereus* and *B. thuringiensis*) as observed in some studies (Guinebretiere et al., 2013; Liu et al., 2017b) cause poor differentiation among the *Bacillus* species (Altayar and Sutherland, 2006). Aruwa and Olatope. (2015) also observed that while identification of several *Bacillus* spp to the strain level was possible by using API systems, four *B. thuringiensis* isolates were unable to be identified.

2.4.2 Molecular methods

Various molecular methods are employed to further confirm the identity and characterize the isolates obtained from selective media and they are also feasible for analyses of non-culturable bacteria (Ehling-Schulz and Messelhäusser, 2013). A molecular approach commonly used in research and clinical field is the sequencing of polymerase chain reaction-amplified 16S rRNA gene which serves as a rapid tool for bacterial identification (Hakovirta et al., 2016). Apart from its universal distribution and large size, this gene is highly conserved and only poorly affected by horizontal gene transfer, thus making it a suitable target gene in phylogenetic studies (Vetrovsky and Baldrian, 2013).

Bacterial 16S rRNA gene usually consists of nine hypervariable (V1-V9) regions, fringed by conserved regions (Bukin et al., 2019). Various 16S rRNA universal primers targeting specific region(s) of 16S rRNA gene have been developed for amplification (Chakravorthy et al., 2007). The selection of 16S rRNA region is crucial as amplification of different fragment(s) of the gene results in different profiles

of microbial community, thus making accurate determination of bacterial species difficult upon sequencing (Bukin et al., 2019). The amplification of entire or nearly full length of 16S rRNA gene by using 27F and 1492R universal primers is preferred for sequencing as more genetic information can be obtained for identification of microbial species (Fredriksson et al., 2013).

Multiple Locus Sequence Typing (MLST) is a method of molecular nature based on 7 housekeeping genes (Akamatsu et al., 2019). Five different MLST schemes involving alleles of different housekeeping genes were developed for *B. cereus* group for better understanding of the genetic diversity and species classification of the genetically similar members of *Bacillus* (*cereus, thuringiensis* and *anthracis*) (Helgason et al., 2004; Tourasse and Kolsto, 2008). Contrary to MLST, Amplified Fragment Length Polymorphism (AFLP) fingerprinting method does not require laborious sequencing and thus, suitable for high throughput analyses (Ehling-Schulz et al., 2013).

Based on AFLP analysis, members of *B. cereus* group were classified in to 7 major phylogenetic classes with members of each class exhibiting different physiological characteristics and virulence potential, thus also serving as a good tool for risk assessment (Ehling-Schulz and Messelhäusser 2013; Guinebretiere et al., 2010). Pulse Field Gel Electrophoresis (PFGE) is another genotyping method commonly used for characterization of foodborne pathogens in epidemiological studies (Ehling-Schulz and Messelhäusser, 2013) and regarded as ”gold standard” in DNA fingerprinting (Merzougui et al., 2013). In spite of its increased discriminatory capacity, the laboriousness of this method becomes its disadvantage (Merzougui et al., 2013).