MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

ISOLATION AND IDENTIFICATION OF *Bacillus cereus* s.l. FROM VEGETABLES AND CEREALS FROM LOCAL MARKETS IN NEGERI SEMBILAN USING 16S rDNA AMPLICON SEQUENCING

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History
Received: 13 March 2023
Accepted: 20 November 2023

Keywords:

Bacillus cereus; Contaminated vegetables; Contaminated cereal; Food poisoning; 16S rDNA sequencing

Abstract

Bacillus cereus is a spore-forming food-borne pathogen that can cause food poisoning. The bacteria can produce toxins that lead to emetic and diarrheal in toxication. This study was designed to investigate the occurrence of *B. cereus* in different types of cereals and vegetables purchased from local markets in Negeri Sembilan using selective medium agar, biochemical approaches and bacterial species confirmation using 16S rDNA amplicon sequencing. Phylogenetic tree was then created using MEGA X software to identify relationship between different bacterial species. From this study, the prevalence of *B. cereus* in cereal and vegetables were recorded at 38.5% and 9.1%, respectively. These suggest that the presence of *B. cereus*-containing cereals and vegetables in diets may represent the risk in the case of inadequate heat treatment.

INTRODUCTION

Bacillus cereus is a ubiquitous bacterium that can easily be found naturally in the environment especially in soil, plant surfaces, contaminated foods or water [1]. The *Bacillus cereus* group, also called *Bacillus cereus sensu lato* (*s.l.*) is a subdivision of the genus *Bacillus* and currently consists of *Bacillus cereus sensu stricto (s.s.)*, *Bacillus anthracis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis, Bacillus. weihenstephanensis, Bacillus cytotoxicus* and *Bacillus toyonensis*. Among the species of this group, *B. anthracis* and *B. cereus* are well-known pathogenic to human and animals [2]. The bacterial spores from the soil can easily contaminate soil-based food including grains, cereal, vegetable, spices and some dairy products [3]. The spores will help them to survive in the unfavourable condition and can even survive in normal cooking temperature [3]. It is difficult to eliminate *B. cereus* spores in raw materials and food ingredients as they are common in the environment that must be considered in food production and food processing. Although some precautions have been implemented through food technological processing such as pasteurisation, sterilisation, gamma radiation and heating, *B. cereus* spores are partially resistant to these processes making it a great health risk for consumers [2].

B. cereus is thought to be responsible for 1.4% to 12% of all foodborne outbreaks worldwide [4]. In fact, in Malaysia, there were foodborne outbreaks related to the consumption of contaminated rice [5-6] and milk [7]. Previously, Sandra and her colleagues (2012) reported that there were 100% prevalence of *B. cereus* in chicken rice, 76.2% in white rice, 70.4% in *nasi lemak* (rice cooked in coconut milk), and 50% in *nasi biryani* [9]. The presence of *B. cereus* in cereal can be detected in four out of 30 samples [10]. The chances of cereals to be contaminated by *B. cereus* are high because cereals are grain-based food products such as from corn, grain, rice, wheat, and oats [11]. Nonetheless, in Malaysia, no data has been published regarding the prevalence of *B. cereus* in vegetables. The prevalence of *B. cereus* in different

vegetables recorded from 29.0 to 70.0% was reported in China [12], from 20% to 48% in Korea [13], 57% in Mexico City [14], and 52% in the southeast of Spain [15]. As the popularity of eating raw vegetables or locally known as '*ulam-ulaman*' grows here in Malaysia, the risk of *B. cereus* contamination in vegetables should be evaluated and determined specifically.

There were few studies conducted to investigate the prevalence of *B. cereus* in local food products. This study was intended to provide the current data on the occurrence of *B. cereus* contamination in vegetables and cereal products purchased from local markets in Negeri Sembilan, Malaysia. In fact, no study has been conducted as yet to check the *B. cereus* contamination in vegetables in Malaysia. Besides, this study serves as a relevant primary source of information for further investigation such as the presence of toxins related to *Bacillus* gastroenteritis which will lead to a more proper and efficient risk assessment and therefore prevention of *B. cereus*-related foodborne illness.

MATERIALS AND METHODS

Materials

Selective and differential agar Mannitol Yolk Polymyxin (MYP), peptone water and Tryptone Soy Broth were purchased from Oxoid™ (United Kingdom). PrimeWay Genomic DNA extraction kit and exTEN 2X PCR Master Mix were purchased from 1st Base, Apical Scientific (Malaysia). Ready-made sheep blood agar plates were purchased from Thermo Scientific™. Others include Agarose powder (Thermo Scientific™), 1kb DNA ladder (SMOBIO, Technology inc), and SYBR™ Safe DNA Gel stain (Invitrogen).

Sample Collection

A total of twelve samples of vegetables and thirteen samples of cereals were randomly purchased from different local supermarkets in Negeri Sembilan, Malaysia. The vegetable samples include tomato, coriander, leaf celery, cucumber, green spinach, Vietnamese coriander ('*kesum*' leaves), cabbage, water spinach, Chinese kale, mustard green and red spinach. These vegetable samples were grown locally by local farmers and not imported from other countries. Samples were quickly stored in a refrigerator (4°C) until further analysis. For cereal products, there were three samples of black glutinous rice purchased from different markets, two samples of barley that bought separately from two different markets, corn, glutinous rice, two samples of wheat grains, granola, oat, and two branded cereal products (corn-made cereal and honey-made cereal). The expiry dates were checked before buying them. Cereal samples were manufactured locally as indicated in the food labelling.

Isolation and Identification of *Bacillus cereus* by plating, Gram-staining and Catalase Test

MYP agar is a selective agar used to isolate vegetative cells of *B. cereus*. Prior to analysis, 90 ml of sterile peptone saline solution was added with 10 g of samples, homogenised using a Stomacher at 230 rpm for two minutes. Serial dilutions were prepared until 10^{-5} and 0.1ml of each diluted sample was plated onto Mannitol Yolk Polymyxin (MYP) (Oxoid™, United Kingdom) agar medium. Plates were incubated for overnight at 37°C. The ingredients of MYP agar contain egg yolk and mannitol supplementation, with additional Polymyxin B that inhibits Gram-negative bacteria. Presumptive *B. cereus* colonies are characterised as bright-pink and uniform as they do not ferment mannitol, surrounded by a zone of precipitation indicating lecithinase production [16]. From each sample, a typical colony presumed to belong to the *B. cereus* was then re-streaked on blood agar (Thermo Scientific™) and incubated for overnight at 37°C. *B. cereus* exhibited ß-hemolytic property where the colonies produce 2-4 mm zone of complete hemolysis surrounding growth [9].

Furthermore, those presumptive colonies for *B. cereus* were selected for Gram staining followed by catalase test. *B. cereus* appears as a Gram positive with rod-shaped with the formation of bubbles when added to the 3% hydrogen peroxide [18].

Confirmation of Bacterial Species using 16S rDNA Gene Amplification by PCR

B. cereus-like isolates were grown in 10 ml of tryptic soy broth (Oxoid™, United Kingdom) overnight at 37°C incubation. Genomic DNA was then extracted using the commercial kit PrimeWay Genomic DNA Extraction Kit (1st Base, Apical Scientific, Malaysia) according manufacturer's instruction given in the manual kit. The extracted DNA was then subjected to Polymerase Chain Reaction (PCR) targeting bacterial 16S rDNA using exTEN 2X PCR Master Mix (1st Base, Apical Scientific, Malaysia). The sequence for the forward primer (primer 27F) used was 5' AGA GTT TGA TCC TGG CTC AG 3' [18], while the reverse primer (primer 13B) has the following sequence 5' AGG CCC GGG AAC GTA TTC AC 3' [19]. Final primers concentration used were at 600nM. The PCR reaction begins with the initial denaturation at 95℃ for 2 minutes, followed by 35 cycles of three-step reaction (95℃ for 30 seconds, 54℃ for 30 seconds, and 72℃ for 90 seconds) and a final extension of 10 minutes at 72℃. The PCR amplicons were then subjected to gel electrophoresis (2.5% TAE agarose gels (Thermo Scientific™)), and DNA was visualised by adding 1X SYBR™ Safe DNA Gel stain (Invitrogen). The electrophoresis was carried out for 45 minutes at 80V. Next, 5µl of 1kb DNA ladder (SMOBIO, Technology inc) was

included in the electrophoresis to indicate the size of the bands. Amplification of 16S rDNA from bacterial DNA results in a band product of around 1300 bp and samples were sent for sequencing (Apical Scientific Sdn Bhd, Malaysia).

BLAST Analysis and Phylogenetic Tree

The sequence results received from the provider were first trimmed and checked for quality. To confirm the bacterial species, the sequences of 16S rDNA were imported to NCBI BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database to search for the similarities in bacterial species. The 16S rDNA sequences of the related bacteria in *B. cereus s.l.* as listed in a Table 1 were searched and downloaded from the GenBank. All the sequences were aligned using BioEdit software and maximum likelihood (ML)-based phylogenetic analyses were performed with MEGA 4 using default parameters (bootstrap=100). *B. weihenstephanensis* was not present in the phylogenetic tree as the 16S rDNA sequence is not available upon this study. Meanwhile, *B. anthracis* was not included as the bacteria do not exhibit beta-hemolytic property on the blood agar [20].

Table 1. List of *B. cereus* s.l. group species and strains where the 16S rDNA gene sequences were retrieved from the database and used in the phylogenetic tree

Bacillus cereus	***Bacillus thuringiensis***	***Bacillus mycoides***	***Bacillus cytotoxicus***	***Bacillus toyonensis***	***Bacillus pseudomycoides***
NBRC 15305	NBRC 101235	ATCC 6462	NVH 391-98	P18	NBRC 101232
CCM 2010	ATCC 10792	DSM 11821			DSM 12442
ATCC 14579	IAM 12077	NBRC 101238			
JCM 2152	BCT-7112	273			
IAM 12605					

RESULTS AND DISCUSSION

Table 2 found that out of 11 samples of fresh vegetables, three showed presumptive *B. cereus* based on the characteristics of pink colonies (mannitol negative), with a precipitation zone surrounding them, suggesting lecithinase production on Mannitol-yolk Polymyxin Agar (MYP). Meanwhile, there were nine out of 12 samples of cereal products that were found as presumptive *B. cereus*. Although MYP plates are selective and differential for the isolation of *B. cereus*, it has been reported that other species in *B. cereus s.l.* group namely *B. thuringiensis* and *B. weihenstephanesis* as well as some bacteria from other genera such as *Staphylococcus* spp. would give similar morphology of presumptive *B. cereus* on MYP agar [16]. Other saprophytic bacteria or ubiquitous bacteria that are present in the environment could mask the growth of the *B. cereus sensu stricto* and compete to grow on the MYP plate agar. The selectivity of this plating medium alone is therefore could be insufficient in food with high background microflora such as vegetables and cereal products.

These presumptive colonies were then proceeded with Gram staining procedure, catalase test and streaking on the blood agar. For the vegetable samples, only one out of three samples was indicated as *B. cereus*-like which is isolate SB from tomato. Meanwhile isolate SD and SK showed cocci shaped under the microscope and exhibited alpha hemolysis on the blood agar suggesting non-*B. cereus.* For the cereal product samples, out of nine, five isolates (FF, FG, FH, FI and FK) were shown as Gram positive with rod-shaped bacteria, catalase positive and exhibited beta-hemolytic properties. Isolates FC and FD were Gram-positive bacteria with cocci shaped, meanwhile isolate FB was observed as Gram-negative under the microscope. Although the presence of polymyxin B inhibits the Gram-negative bacteria, however the Gram staining found there was a lacking in the selectivity for the Gram-positive bacteria. It has been suggested that additional of trimethoprim in the agar could improve the selectivity by inhibiting competing flora [21]. Besides, these three isolates FC, FD and FB were only partial-haemolysis on blood agar proposing these isolated bacteria were non-*B. cereus*. This indicated that selectivity by MYP alone is not fully sufficient and therefore additional molecular approaches such as using 16S rDNA amplicon sequencing is essential to confirm the bacterial species.

Those presumptive *B. cereus*es (isolates SB, FF, FG, FH, FI and FK) were then subjected to DNA isolation and 16S ribosomal gene (16S rDNA) sequencing to confirm the bacterial species between phenotypically identical bacteria. The 16S rDNA gene region is highly conserved in all bacteria as this encodes protein that is essential in ribosomal assembly, yet this gene contains variable regions that can be used as fingerprints for specific species and phylogenetic classification of bacteria [22]. As shown in Figure 1, all samples were successfully amplified producing bands at approximately 1300 base pairs.

Table 2. Description on colony morphology of isolated bacteria from vegetables and cereal products on the selective and differential MYP agar

Isolate code	Sample description	Morphology on MYP plates	Mannitol utilisation	Lecithinase production	CFU/ml	Presumptive *Bacillus cereus*
VEGETABLE PRODUCTS						
SA	Vietnamese Coriander	Yellow, small colonies	Yes	No	TMTC	No
SB	Tomato	Pink, large colonies	No	Yes	TMTC	Yes
SC	Cabbage	Yellow, big colonies	Yes	Yes	TMTC	No
SD	Coriander	Pink, small colonies	No	Yes	TMTC	Yes
SE	Leaf celery	Yellow, big colonies	Yes	Yes	TMTC	No
SF	Green spinach	Yellow, small colonies	Yes	No	TMTC	No
SG	Water spinach	Yellow, big colonies	Yes	Yes	TMTC	No
SH	Chinese Kale	Yellow, small colonies	Yes	No	TMTC	No
SI	Mustard green	Yellow, small colonies	Yes	No	TMTC	No
SJ	Cucumber	Yellow, big colonies	Yes	Yes	TMTC	No
SK	Red spinach	Pink, small colonies	No	Yes	TMTC	Yes
CEREAL PRODUCTS						
FA	Black glutinous rice	Pink, large colonies	No	Yes	TMTC	Yes
FB	Barley packed	Pink, small colonies	No	Yes	2×10^7	Yes
FC	Dried corn	Pink, small colonies	No	Yes	TMTC	Yes
FD	Glutinous rice	Pink, small colonies	No	Yes	TMTC	Yes
FE	Wheat seed	Yellow, large colonies	Yes	Yes	TMTC	No
FF	Corn-made cereal	Pink, small colonies	No	Yes	TMTC	Yes
FG	Granola	Pink, large colonies	No	Yes	6×10^2	Yes
FH	Oat	Pink, large colonies	No	Yes	TMC	Yes
FI	Honey-made cereal	Pink, large colonies	No	Yes	TMC	Yes
FJ	Wheat seed	No growth	N/A	N/A	N/A	N/A
FK	Unpacked barley market A	Pink, large colonies	No	Yes	TMC	Yes
FL	Unpacked barley market B	No growth	N/A	N/A	N/A	N/A

TMTC: Too much too count colonies

Table 3. Observation of isolated colonies on Gram staining, catalase test and hemolytic properties on blood agar

Isolate code	Sample description	Gram-staining	Catalase test	Hemolytic properties on blood agar	Presumptive *Bacillus cereus*
		VEGETABLES			
SB	Tomato	Gram positive, cocci shaped	Bubbles produced	Beta hemolysis	Yes
SD	Coriander	Gram positive, cocci shaped	Bubbles produced	No hemolysis	No
SK	Red spinach	Gram positive, cocci shaped	Bubbles produced	No hemolysis	No
		CEREAL PRODUCTS			
FA	Black glutinous rice	Gram positive, cocci shaped	No bubbles	Alpha hemolysis	No
FB	Barley packed	Gram negative, cocci shaped	No bubbles	Alpha hemolysis	No
FC	Dried corn	Gram positive, cocci shaped	No bubbles	Alpha hemolysis	No
FD	Glutinous rice	Gram positive, cocci shaped	No bubbles	Alpha hemolysis	No
FF	Corm-made cereal	Gram positive, bacilli shaped	Bubbles produced	Beta hemolysis	Yes
FG	Granola	Gram positive, bacilli shaped	Bubbles produced	Beta hemolysis	Yes
FH	Oat	Gram positive, bacilli shaped	Bubbles produced	Beta hemolysis	Yes
FI	Honey-made cereal	Gram positive, bacilli shaped	Bubbles produced	Beta hemolysis	Yes
FK	Unpacked barley market A	Gram positive, bacilli shaped	Bubbles produced	Beta hemolysis	Yes

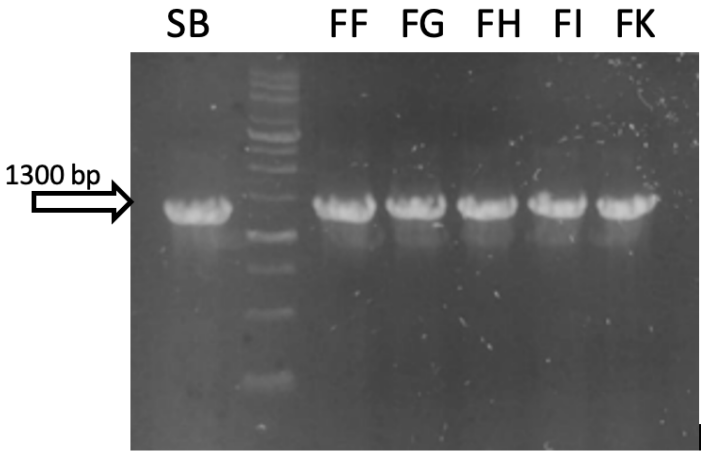

Figure 1. Gel electrophoresis of 16S rDNA amplification of the presumptive B. cereus in sample vegetable (SB) and cereal products (FF: corn-made cereal, FG: granola, FH: Oat, FI: honey-made cereal and FK: unpacked barley from market A).

Sequencing analysis obtained that all isolates SB (tomato), FF (corn-made cereal), FG (granola), FH (oat), FI (honey-made cereal) and FK (unpacked barley from market A) were belonged to *B. cereus s.l.* with 100% similarity. Based on the 16S rDNA sequencing and the phylogenetic tree (Figure 2) proved that isolates SB, FF, FG, FH, FI and FK were very closely related to *B. cereus sensu stricto*, yet it is neither obviously distinguishable from other *B. cereus* group species. As been mentioned by Tallent and colleagues previously, *B. cereus sensu stricto, B. thurienginesis* and *B. weihenstephanensis* would give similar colony morphology on MYP agar plates [16]. However, *B. thuringiensis* and *B. weihenstephanensi*s are less commonly found as compared to *B. cereus sensu stricto*. In fact, due to high genome similarity of *B. cereus sensu stricto* and *B. thuringiensis*, it has been suggested to treat them as one species [2,23].

Therefore, the 16S rDNA gene can only classify the isolated bacteria to the *B. cereus* group, but cannot assign it definitely to a certain species according to its low discrimination. Alternatively, more robust genome analysis approach such as whole genome sequencing or multilocus sequence typing (MLST) could produce higher degree of identification of bacterial isolates [23]. Though some reports suggested that amplification of *cry/cyt*- genes could be used to differentiate *B. thuringiensis* from *B. cereus sensu stricto* which eventually absent in *B. cereus sensu stricto* [24]. However, negative amplification of *cry/cyt*- genes does not necessarily indicate the absent of *B. thuringiensis* as some of the strains do not accommodate the gene. Besides, these gene regions have a huge gene diversity and it is really hard to detect all variants. In fact, based on ISO 7932:2004 criteria for identification and enumeration of *B. cereus* in feed and food do not differ between the species of *B. cereus* but instead classify all *B. cereus* as presumptive *B. cereus* [25]. Other recommendation that can be made is all isolates SB, FF, FG, FH, FI and FK can be assigned to different phylogenetic group which is from group I-IIV. This was due to because each phylogenetic group have different behaviour, characteristics and have different risk of food poisoning [26]. This will help to identify the risk of food poisoning when consuming cereal products.

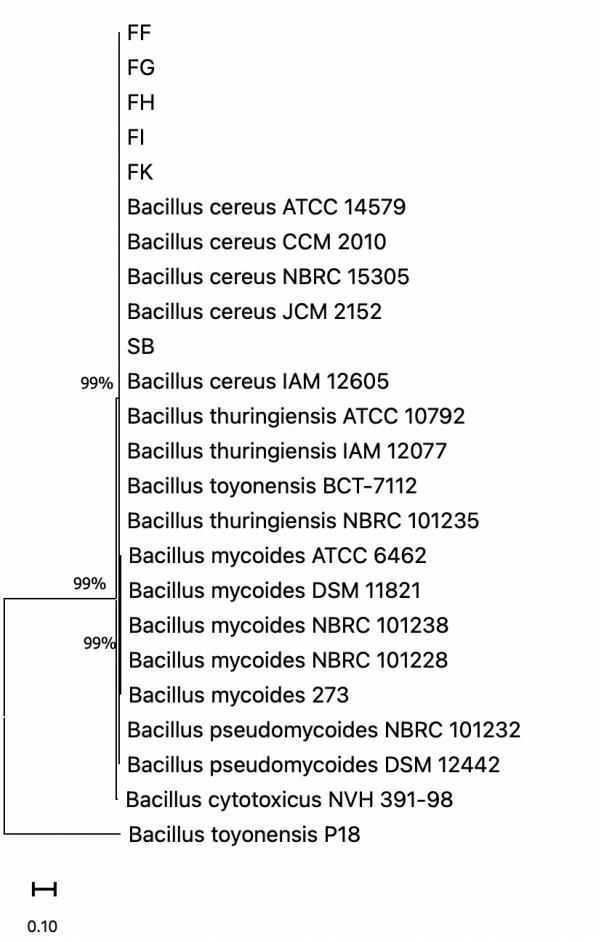

Figure 2. Phylogenetic tree showing the phylogenetic positions of the six presumptive B. cereus samples (SB, FF, FG, FH, FI and FK) and other type strains of species of the B. cereus group based on 16S rDNA sequences.

From this present study, the prevalence of *B. cereus s.l.* in vegetables and cereal products that bought from local market Negeri Sembilan reported to be 9.1% and 38.46% respectively. Five out of six isolates (SB, FF, FH, FI and FK) were considered as unsafe to be consumed as these samples contain more than 10^4 CFU/g or ml. It has been stated by Food Standards Australia New Zealand (2001), food that contains more than 10^4 CFU/g or ml were unsafe for human consumption as it may lead to food poisoning [27]. These findings imply that consuming *B. cereus*-containing vegetables on a daily basis may pose a risk in the event of insufficient heat treatment. For the occurrence of *B. cereus* in cereal products, the prevalence found in this study is higher than previously reported occurrence of *B. cereus* in ready-to-eat cereals purchased from Sarawak, Malaysia with a prevalence of 13.33% [10]. Majority of the positive

samples here were packed cereal products (isolate FF, FH and FI), which supposedly considered as highly hygienic and safe to be consumed since these products were manufactured under strict processing conditions [28]. Nevertheless, *B. cereus* can cross-contaminate the packed cereal during the processing line in the factories by chance. Since these spores are highly resistant even under the harsh processing environments, these bacterial spores can survive and germinate when conditions become more favourable [29,30]. The findings in this study highlight the occurrence of *B. cereus* in vegetables and cereal products from local market where precautious awareness are therefore highly recommended before consuming.

ACKNOWLEDGEMENTS

We are grateful to School of Biology, Universiti Teknologi MARA (UiTM), Kampus Cawangan Negeri Sembilan for the facilities in this research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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