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DETECTION OF *BACILLUS CEREUS* FROM IMPORTED RICE IN MALAYSIA

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Detection of *Bacillus cereus* from Imported Rice in Malaysia

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(Resource Biotechnology)

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Declaration

I hereby declare that this report entitled 'Detection of *Bacillus cereus* from Imported rice in Malaysia' submitted to the Faculty of Resource Science and Technology is presented of my original work except for the citations and references and never been before concurrently submitted for any other degree of qualification or other institutions. This work was submitted to partially fulfill the requirement for the degree of Bachelor of Science with Honors in Resource Biotechnology at Universiti Malaysia Sarawak.

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List of Abbreviations

%	Percentage
$^{\circ}C$	Degree Celsius
µl	Micro liters
AGE	Agarose Gel Electrophoresis
bp	Base pair
Cyt K	Cytotoxin K
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
EtBr	Ethidium Bromide
g	Gravity
*gyr*B	*Gyrase*B
Hbl	Haemolysin B, L1, L2
$MgCl_2$	Magnesium (II) Chloride
ml	Milliliter
MPN	Most Probable Number
Nhe	Nonhaemolysin
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
RTE	Ready-to-eat
TBE	Tris-Borate-EDTA
TSB	Tryptic Soy Broth
UV	Ultraviolet
V	Voltage

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ABSTRACT

Bacillus cereus is frequently associated with foodborne illness outbreak. The common food vehicles for transmission of *B. cereus* are rice, rice products and starchy foods. Hence, it is crucial to investigate the biosafety of *B. cereus* in rice. The bacteria were enumerated by performing Most Probable Number (MPN) approach followed by Polymerase Chain Reaction (PCR) Assay targeting *gyrase*B (*gyr*B) gene at 475 bp. A total of 20 imported raw rice samples from various countries were evaluated. The finding indicated that the occurrence of *B. cereus* was >1100 MPN/g for all the imported raw rice samples. In addition, 100% of the imported raw rice samples were detected with presence of *B. cereus*. The result highlights the potential risk of *B. cereus* to cause food poisoning in rice. Hence, extra precautions in preparing, handling and storage of the rice are necessary to prevent microbial contamination.

Keyword: *B. cereus,* foodborne illness outbreak, raw imported rice, MPN, PCR

ABSTRAK

Bacillus cereus biasanya dikaitkan dengan kes keracunan makanan. Antara makanan yang berisiko tinggi mengandungi B. cereus adalah makanan seperti nasi,hidangan nasi serta makanan yang mengandungi kanji. Oleh itu, adalah penting untuk menyelia biokeselamatan kandungan B. cereus dalam beras. Bakteria dikultur dengan MPN teknik dan seterusnya PCR teknik dengan sasaran gyrB gen pada 475 bp. Hasil kajian menunjukkan keberadaan B. cereus adalah >1100MPN/g untuk semua sampel beras import. Selain itu, 100% sampel didapati mengandungi B. cereus. Kesimpulannya, hasil kajian menunjukkan B. cereus berpotensi menyebabkan keracunan melalui nasi. Oleh itu, nasi yang telah dimasak hendaklah diurus dengan baik terutamanya dari segi penyediaan serta penyimpanan demi untuk mengelakkan kontaminasi mikcrob.

Kata Kunci: B. cereus, keracunan makanan, beras import, MPN, PCR

1.0 Introduction

Bacillus cereus is a rod shape, gram positive, motile and spore forming bacterium. It is ubiquitous and worldwide distributed in soil where transmission can be occurred readily to plants and subsequently to food such as meats, cereals, spices, dairy products, rice and rice products (Opinion of the Scientific Panel, 2005; Batt and Tortorello, 2014). In addition, *B. cereus* is able to grow in the temperature around 10-48 $^{\circ}$C and the optimum temperature range from 28-35 $^{\circ}$C. According to Ankolekar *et al.* (2009) *bacillus* is the dominant genus making up 90% of the paddy soil bacteria whereby *B. cereus* remains closely associated with the rice plant throughout its development. Hence, uncooked rice is most likely to be contaminated with *B. cereus*. Due to its widespread distribution as well as the ability of spores to survive under harsh condition, *B. cereus* is widely associated with foodborne illness.

Food poisoning associated with *B. cereus* generally manifested by diarrheal or emetic symptom. However, certain patients could possibly experience both syndromes at the same time due to presence of both toxins. Emetic syndrome has shorter incubation period, about 1-5 hours whereas diarrheal symptom might take about 12 hours or longer (Batt and Tortorello, 2014). The toxin that causes emesis is known as cereulide whereas toxins that lead to diarrheal symptom comprise of cytotoxins haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K (Cyt K) (Opinion of the Scientific Panel, 2005; Batt and Tortorello, 2014).

Rice is a very important staple food in many countries including Malaysia. Despite that Malaysia cultivate different varieties of rice; the total amount of production is unable to reach self-sufficient level (Hanafi *et al.*, 2009). Therefore, imported rice still play a critical role in determining the country's food security as more than quarter of the rice requirement is met by

imported rice (BERNAS, 2015). Majority of the rice is imported from countries such as Vietnam, Thailand, Cambodia, India, Pakistan, China, Brunei and Japan.

In Malaysia, there were a few studies on biosafety of *B. cereus* in food such as noodle, spices and legumes by Rusul and Yaccob (1995), ready-to-eat (RTE) cereals (Lee *et al.*, 2009; Lesley *et al.*, 2013), local raw rice and RTE cooked rice by Sandra *et al.* (2012). However, there is still a gap remain on the knowledge of prevalence of *B. cereus* in imported rice in Malaysia. This study addresses the problem of investigating the biosafety of *B. cereus* in the imported rice. The outcome of this study is to provide a baseline data for risk assessment study of *B. cereus* from imported rice in Malaysia. The objectives of this study are as the following:

(i) To enumerate the *B. cereus* from various types of imported raw rice samples by using MPN method.

(ii) To detect the presence of *B. cereus* from the imported raw rice samples by using PCR targeting at the 475 bp of *gyr*B gene.

2.0 Literature Review

2.1 The genus *Bacillus*

According to Todar (2012), the genus *Bacillus* is introduced in the year of 1872 with the identification of *Bacillus* (*B. subtilis*) species. *Bacillus* genus constitutes a group of bacteria which exhibit properties such as gram-positive, rod-shaped, form endospore either aerobically or facultatively, and motile (Todar, 2012). As more genetic information has been discovered, *B. subtilis* is further classified into subgroups which comprise of *B. cereus, B. thuringiensis, B. anthracis*, *B. weihenstephanensis*, *B. mycoides* and *B. pseudomycoides*. This group of members are phenotypic and genotypic indistinguishable and their 16S rRNA sequences are very similar up to 99% (Yamada *et al.*, 1999; Opinion of the Scientific Panel, 2005). Therefore, it is vital to isolate and identify these members with precision as different members have different usage and properties. Currently, only *B. cereus* is implicated as food borne disease agent. Meanwhile, *B. thuringiensis* is commonly used as a bio-pesticide, *B. anthracis* which produce anthrax is used to make biological weapon whereas *B. mycoides* is identified with the ability to promote plant growth associated with conifer roots (Opinion of the Scientific Panel, 2005; Park *et al.*, 2007).

2.2 *Bacillus cereus*

B. cereus is ubiquitous in nature especially in soil and plants. The bacterium is able to form heat-resistance spores. It is gram-positive, motile, rod shape and grows well in both aerobic and anaerobic condition. Apart from that, it is also able to grow at optimal temperature about 28-35 $^{\circ}$C as well as minimum temperature about 4-5 $^{\circ}$C. The organism adapts well in a wide range of pH from 4.9-9.3 and salt concentration up to 7.5% (Batt and Tortorello, 2014).

2.3 Virulence factors of *Bacillus cereus*

B. cereus is associated with 2 types of food poisoning - emetic which is caused by pre-formed toxin produced by the bacteria that grows in the food and the second type, diarrheal which is caused by enterotoxins produced during vegetative growth in the small intestine. Patients normally suffered from emesis within 1-5 hours after consumption of food whereas diarrheal symptoms might occur after 12 hours or even longer period (Batt and Tortorello, 2014). In some cases, patients also experience both diarrheal and emetic symptoms which might due to production of both types of toxins. Diseases associated with emetic syndrome are acute attack of nausea and vomiting which resemble *Staphylococcus aureus* intoxification whereas the diarrheal syndrome is characterized by abdominal pain and diarrheal which resembles the symptom of *Clostridium perfringens* food poisoning (Ankolekar *et al.*, 2009).

The toxin which causes emesis is known as cereulide. It has very special properties which includes heat stable at 121 $^{\circ}$C for 90 minutes, pH stable range from 2-11, resistant to proteolysis and not antigenic (Ankolekar *et al.*, 2009). On the other hand, diarrheal syndrome is caused by a few enterotoxins: cytotoxins haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe), and cytotoxin K (Cyt K) (Opinion of the Scientific Panel, 2005; Batt and Tortorello, 2014). Unlike the emetic toxin, these enterotoxins are heat labile and susceptible to protease activity.

2.4 Imported Rice

Rice is an important staple food in most of the country as people needs it to obtain calories and protein. Each year, the total production of rice in Malaysia is approximately two million metric tons but the amount of production only manages to cater for 60-65% of the community

which is insufficient (Abu, 2012). Hence, imported rice play an important role to fully meet the rice requirement for the community in Malaysia and it is monopolized by National Paddy and Rice Authority (BERNAS). According to BERNAS (2015), the current policy for imported rice is depending on the level of production of local rice. Hence, Malaysia imported approximately 30-40% domestic rice demand annually so as to provide sufficient supply to the consumers (BERNAS, 2015). Apart from that, specialty rice such as basmati and fragrant rice which cannot be cultivated locally are also imported. In the year of 2012, Malaysia approximately imported 539 thousand metric tons of rice from Vietnam, 112 thousand metric tons from Pakistan, 56 thousand metric tons from Thailand, 19 thousand metric tons from Cambodia and 7 thousand metric tons from India (USDA Foreign Agricultural, 2013).

2.5 Most Probable Number (MPN)

The theory of MPN is based on the estimation on concentration of viable microorganisms in a sample with replication of liquid broth growth in ten-fold dilution (Sutton, 2010). Enrichment media is usually used where it allows the bacteria to grow upon incubation period. Then, the concentration of the bacteria in the sample is evaluated by observing the patterns of tubes which turns turbid after incubation period (Sutton, 2010). The occurrence of the bacteria can be obtained by referring to the MPN tables (refer to Appendix II) or by utilizing the formula:

$$MPN = \frac{\text{Number of positive tubes}}{\sqrt{\text{Number of ml of sample in negative tubes x Number of ml of sample in all tubes}}}$$

The density of microorganism in the original dilution is expressed as MPN per gram (MPN/g). This method is usually used to enumerate the microorganisms in low concentrations, which is less than 100 CFU/g (Pouillot *et al.*, 2013). Apart from that, several assumptions are taken into considerations which include (Sutton, 2010):

(i) The microorganisms are distributed randomly during dilution of sample and the inoculums will contain but not always viable organism.

(ii) The microorganism separated through dilution is not affected by each other (attract or repel).

(iii) Every tube whose inoculums has single viable microorganism will give visible growth result.

2.6 Polymerase Chain Reaction (PCR)

Rapid detection of *B. cereus* in food is important to facilitate the application of quality control measures to eliminate *B. cereus* from food and enhance diagnosis of food poisoning outbreak. The advent of gene probe and PCR technique has allowed the development of molecular techniques by which particular bacterial strains can rapidly be identified without the need for isolating pure culture (Patel, 1994). PCR is a rapid, efficient and reliable diagnostic tool to detect the presence of pathogen from food samples (Patel, 1994). The members of *B. subtilis* group are closely related species where 16S rRNA gene sequences are 98.1-99.8% similar (Wang *et al.*, 2007). Likewise, 23S rRNA gene sequences were also reported to be unable to differentiate *B. cereus* from *B. anthracis* (Yamada *et al.*, 1999).

Previous studies had proven that *gyrase* B (*gyr*B) is more suitable and efficient to use as molecular diagnostic marker to differentiate *B. cereus* from the members of *B. subtilis* group (Yamada *et al.*, 1999; Wang *et al.*, 2007). This is due to higher substitution rate of bases as well as higher genetic variation in *gyr*B gene (Wang *et al*., 2007). According to Yamada *et al.* (1999), *gyr*B gene provides higher resolution as compared to 16S rRNA. The *gyr*B gene is

responsible for encoding type II DNA topoisomerase, which is important for its DNA replication (Wang *et al*., 2007).

2.7 *Bacillus cereus* outbreak

B. cereus foodborne illness had been occurred without geographic distribution. It was recognized as food poisoning agent since 1955 and is frequently found in foods with improper handling, preparation and storage. An incident happened in Napa Country California at the year of 1989. At least 55 people were affected by *B. cereus* food poisoning with the combination of symptoms such as abdominal cramps, nausea, vomiting and diarrhea after having the buffet. Data showed that 82% of the victims had diarrhea symptom followed by abdominal cramps which contributed 80% (Slaten *et al.*, 1992). Meanwhile, fever was not prominent in this case.

In August 2003, five children from a family started to vomit after several hours of consumed the pasta salad. The 7 year old girl suffered from more severe illness. She experienced muscle cramps, pulmonary hemorrhage, and coma. The girl was dead after 13 hour consumed the meals (Dierick *et al.*, 2005). Further investigation showed that she had metabolic acidosis as well as liver failure. Apart from that, the PCR result also confirmed that the cereulide were presence in the pasta salad. According to Dierick *et al.* (2005), some *B. cereus* were psychotropic and able to have high production of cereulide at 12-15 $^{\circ}$C whereby the temperature of fridge that stored the pasta salad was 14 $^{\circ}$C, which fulfill the condition for the growth of *B. cereus*.

Another similar outbreak happened in Bari, Italy, January 2012 which involved 12 patients among the 13 customers. All the patients were reported that they had consumed

basmati rice as well as sweet and sour vegetables. In this outbreak, most of the victims (83%) suffered from emesis, 75% of them had nausea, 50% had abdominal pain and 42% had diarrhea (Martinelli *et al.*, 2013). From the investigation, *B. cereus* was isolated from basmati rice and faecal specimens. Poor food handling and storage were suspected to be the most probable reasons for this outbreak.

There was not much *B. cereus* outbreak reported in Malaysia. This was due to underreport and no further investigation of the outbreak (Sharifa *et al.*, 2013). The first reported case was in 1984 which affected 114 female Malay students in Klang, Malaysia. Most of the students experienced abdominal pain (85.1%) whereas only about 3% of the students experienced diarrhea (Rampal *et al.*, 1984). Further investigation shows that there was 2.3 x 10^6 of *B. cereus* per gram of fried noodles (Rampal *et al.*, 1984). Another recent case at Ipoh, reported that 36 students suffered from headaches, abdominal pain, vomiting and diarrhea after consumption of food in canteen (BERNAMA, 2013). The Indian food *idli*, which is made from rice flour, was suspected to be contaminated with *B. cereus* and caused food poisoning among the students (BERNAMA, 2013).

3.0 Materials and Methods

3.1 Materials

The materials used in this study were listed in Appendix I.

3.2 Methods

3.2.1 Sample Collection

A total of 20 raw imported samples were collected from the retail shops and hypermarkets in Malaysia. The samples consists of seven imported rice from Thailand, five from India, three from Vietnam, one from Pakistan, one from Cambodia, one from China, one from Brunei and one from Japan. The experiment was conducted with three replicates.

3.2.2 Sample Preparation

This method was conducted based on procedure described by Sandra *et al.* (2012). Twenty grams of raw rice sample was placed into a sterile stomacher bag. Then, 180 ml of Tryptic Soy Broth (TSB) was added into the stomacher bag. After that, the sample was incubated at 37 $^{\circ}$C for 20 hours.

3.2.3 Most Probable Number (MPN)

The enriched broth was proceeded with MPN three tubes serial dilution. Then, the tubes were incubated with 37 $^{\circ}$C for 18-24 hours. A loopful of culture from each tube was streaked onto *Bacillus cereus* selective Agar Base.

3.2.4 DNA Extraction

DNA extraction was performed according to the boiled cell method described by Lee *et al.* (2009). Two tubes were randomly chosen from each replicate. Hence, there were 6 tubes from each sample. 1 ml of culture was aliquot into 1.5 ml microcentrifugation tube. The tubes were centrifuged at 12,000 xg for 1 minute. Then, the pellet was resuspended with 500 µl of sterile distilled water. After that, the mixture was boiled for 20 minutes followed by immediate cooling at -20 °C for 10 minutes. Then, the mixture was centrifuged again at 12,000 xg for 5 minutes. The supernatant was collected and the DNA template was used for PCR assay.

3.2.5 Polymerase Chain Reaction (PCR)

The pair of primers that was used in PCR assay was shown in Table 3.1. The primers used were targeting the *gyr*B gene at 475 bp for *B. cereus* (Wu *et al.,* 2006). The PCR amplification was operated in a total of 20 µl reaction mixture and the respective components was listed in Table 3.2. Apart from that, Table 3.3 showed the specific PCR condition applied in this study.

Table 3.1 The oligonucleotide sequences of the primers used in PCR assay.

Target gene	**Primer**	**Sequence**	**Product Size (bp)**	**Reference**
*gyr*B gene	BCJH-F	5’ TCATGAAGAGCCTGTGTACG 3’	475	Wu *et al.* (2006)
	BCJH-1R	5’ CGACGTGTCAATTCACGCGC 3’		

Table 3.2 The PCR mixture component and their respective amount needed.

Component	**Volume (µl/ reaction)**
5x PCR buffer	5.0
1.5 mM $MgCl_2$	1.2
0.2 mM Deoxynucleoside triphosphate mix	0.4
1.0 µM primer (Forward)	2.0
1.0 µM primer (Reverse)	2.0
0.2 U/µl Taq polymerase	0.8
DNA template	2.0
Double Distilled water (ddH_2O)	6.6
Total	**20**

Table 3.3 The specific PCR condition for amplification of *gyr*B gene of *B. cereus.*

Stage	**Temperature (°C)**	**Period**	**Cycle**
Initial denaturation	94	3 minutes	1
Denaturation	94	45 seconds	35
Annealing	63	1 minutes	35
Elongation	72	1 minute	35
Extension	72	7 minutes	1

3.2.6 Agarose Gel Electrophoresis (AGE)

Five microliters of PCR product was loaded into 1.0 % of agarose gel and run at 90 V for 1 hour 15 minutes. A 100 bp of DNA ladder was included in each gel. After that, the gel was stained with Ethidium Bromide (EtBr) together with 1x Tris Borate EDTA (TBE) buffer for 15 minutes. The gel was visualized under UV transilluminator (Maestrogen, USA).

4.0 Results

4.1 Enumeration of *Bacillus cereus*

All of the enriched tubes turns into turbid and therefore the occurrence of *B. cereus* for all the imported raw rice samples were >1100 MPN/g. The occurrence and enumeration of *B. cereus* in various imported raw rice samples are summarized in Table 4.1.

Table 4.1 The occurrence and enumeration of *B. cereus* in imported raw rice samples.

No	Types of Samples	Origin Country	MPN/g
1	Happy Rose Quality Thai Fragrant Rice	Thailand	>1100
2	Liansin Butterfly	Thailand	>1100
3	Liansin Mr. Thai	Thailand	>1100
4	Liansin Cap Amoi Super Siam	Thailand	>1100
5	Happy Bamboo Thai	Thailand	>1100
6	Liansin Beras Pulut Susu	Thailand	>1100
7	NutriRice Thai Fragrant Brown	Thailand	>1100
8	Jasmine Pusa Gold 1121	India	>1100
9	Liansin Bryani King	India	>1100
10	Pusa Creamy Sella Basmati Rice	India	>1100
11	Heera IDLI rice	India	>1100
12	Liansin Sonna Mahsuri Beras Herbal	India	>1100
13	Tulip Vietnam Premier Rice	Vietnam	>1100
14	Teana Sargon Super Imported Rice	Vietnam	>1100

15	Lap Padi Emas Premium Quality White Rice	Vietnam	>1100
16	Beras Taj Mahal Beras Faiza Herda Ponni	Pakistan	>1100
17	Sushi Rice	Japan	>1100
18	Brunei Rice	Brunei	>1100
19	Great Wall 5A Tuang Rice	China	>1100
20	Bird of Paradise Phkarkhei Organically Grown Cambodian rice	Cambodian	>1100

4.2 Detection of *Bacillus cereus* by Polymerase Chain Reaction (PCR)

PCR was performed to specifically detect the presence of *B. cereus* from the imported raw rice samples. The primers BCJH-F and BCJH-1F were used to target the *gyr*B gene in *B. cereus*. The amplicon size of the PCR product was 475 bp. Figure 4.1 and 4.2 illustrated the representative of agarose gel electrophoresis (AGE) for detection of *gyr*B gene of *B. cereus*. In addition, Table 4.2 summarizes the result of detection of *gyr*B gene of *B. cereus* according to respective samples. The AGE results revealed that 100% of the imported raw rice samples were detected with presence of *B. cereus*.