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Identification of *Bacillus cereus* isolates from cooked rice by biochemical test and 16s rDNA sequences

Nisreen Jawad and Sahilah Abd. Mutalib

School of Chemical Sciences and Food Technology, Faculty of Science and Technology Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

Abstract: *Bacillus cereus* isolates are considered to be toxigenic and can lead to food poisoning. Cooked rice is a potentially risky food, particularly while grown in tropical countries. The present study aims to explore the prevalence of *B. cereus* in cooked rice from several restaurant and stalls in the area (Bangi, Kajang and UKM) in Selangor Malaysia. The isolation was conducted using the standard procedure for detection of *B. cereus.* Of seventy (n= 70) cooked rice samples examined, 42.8% were positive for *B. cereus* indicated bright pink colonies when it grown onto mannitol egg yolk polmyxin medium. The thirty five (35) colonies were selected and identified as *B.cereus* by biochemical test and 16s rDNA sequences. The results in the presence study, showed the cooked rice samples were potentially can cause food poisoning to the public consumers.
Keywords: *Identification, Bacillus cereus, cooked rice, biochemical test, 16s rDNA sequences.*

Introduction

The genus *Bacillus cereus* is described as having rod shape, gram positive and aerobic spore which forms the microorganisms which are widely found in the environment in the soil, water and food[1].believe that the *B. cereus* is an essential pathogen which is responsible for food poisoning and food spoilage. In addition, it produces two different kinds of toxin. The most widespread one is the 'emetic' or vomiting toxin, a peptide also referred to as cereulide. As it suggests, the emetic toxin involves quick onset (0.5-6 hours) nausea and vomiting, which sometimes leads to abdominal cramps and diarrhea. These symptoms do not usually continue more than 24 hours and the related complications are rare. *B. cereus* which results in emetic poison which is usually seen in starchy food, such as boiled or fried rice, potatoes, pasta and noodles.

B. cereus is also can be observed in foods such as pasteurized milk, hazard products or cooked chill foods, as well as in refrigerated or dehydrated foods such as milk powder, powder used for dairy desserts or dehydrated soups or spices and pasture. These spores are said to be able to maintain germination, growth and toxin which in turn are produced by *B. cereus* during storage time or after reformation in products, which are recognized in foods such as dairy products, potato puree, meals containing rice or pasta or salads, pasteurized milk and milk powder. In general, the diarrhea syndrome is considered as a toxic infection, which is constructed from the ingestion of vegetative cells or spores of *B. cereus*. Its existence is dependent on the type of the food, and handling of a food product as well, whether it is *B. cereus* vegetative cells or spores or the presence of both in food. On the other hand as[2]. And[3] point out, this suggests the possibility of producing diarrheal toxin in the small intestine, in relation to the survival, germination, growth and intestinal adhesion of *B. cereus* spores and vegetative cell.

Rarely, this syndrome occurs as intoxication, which encompasses the passage of biologically active toxin through the stomach. The other circumstances are related to people with low stomach acidity. The alternative dose needed for diarrheal syndrome is rated to be 10^5-10^8 cells.[8]report a new record of presented the model of *B. cereus* attitude and production of diarrhea enterotoxins in the host gastrointestinal tract. As the European Food Safety Agency (2005) suggests 1-33% of food-born poisonings are resulted from the *B. cereus* which in turn lead to food-borne in the United States from 1973-1987, manifesting roughly 3% of the total spread. According to[4], in 1997, it caused about 27,360 cases of food-borne diseases. Moreover, the emetic type performed in food is a heat-stable emetic toxin which are similar to those of *Staphylococcus aurous* intoxication, and is distinguished by a short incubation period.

In Malaysia there was no report on the outbreak by *B. cereus*. However, the incidence of *B. cereus* in cooked rice was described in year 2012 by[5].In this study, we continue to assess the prevalence of *B. cereus* in cooked rice by isolating the *B. cereus* strains and identified using biochemical test and 16s rDNA sequences.

Materials and Methods

Samples collection and preparing

For the purpose of sample collection and bacteria identification, a total of (n=70) cooked rice samples were bought randomly from different restaurants in Selangor, Malaysia from August 2013 until June 2014. All samples were immediately transported to the laboratory and were analyzed within 24 hours.

Isolation and morphological characterization

Samples were analyzed using the standard procedure for detection of *B.cereus*[6] with modifications described by[6]. A total of 25 g of each sample was placed in a stomacher bag added with 225 ml of Tryptic Soy Broth (TSB; Bacto™,) and homogenized in a stomacher (Interscience, France) for 60 s followed by incubation at 30°C for 12 h. The determination of *B. cereus* count has been done according to ISO 7932l2004 by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (Oxide CM0929).The dilutions of the stomached fluid were prepared with Moreover, Tryptic Soy Broth (TSB; Bacto™).then 0.1 ml portions of each dilutions of the fluid were transferred into three tubes and incubated at 30°C for 18 to 24 h. A loopful of culture from each tube was streaked onto Mannitol Egg Yolk Polymyxin Agar Base (MYP; Difco) added with sterile Polymyxin B Selective Supplement (Difco) and sterile Egg-Yolk Tellurite Emulsion 20% (V/V) (Merck) which is a specific media for the isolation of the *B. cereus* and therefore identification is confirmed by microscopic. The biochemical tests were conducted as described by[7].

Isolation of genomic DNA

The bacterial DNA was extracted from 1 ml of the overnight culture grown of 30^0C on orbital shaker (200 rpm) and purified using a DNA Extraction Kit (Promega). The concentration and purity of the extracted DNA were determined by absorbance at 260 nm and 280 nm using Maestro Nano Spectrophotometer (Maestro Gen, USA). The extracted DNA was stored at -20°C until used.

PCR Amplification

PCR amplification was performed in a 25 µl reaction volume, containing a mixture of DreamTaq™ PCR MasterMix (Fermentas), forward and reverse oligonucleotide primer (1st Base, Malaysia), nuclease free water (NFW) and the extracted DNA as described by the manufacturer's instruction. All mixture was prepared in a 0.2 ml sterile PCR tubes. Negative control was prepared by substituting the extracted DNA with nuclease free water (NFW) whereas positive control using *Bacillus cereus* ATCC 11778 DNA Further PCR reaction was carried out using Eppendorf Gradient Thermocycler (Eppendorf, Germany) with a temperature program consisting of the initial heat activation at 94°C for 3 minutes, with 25 cycles were programmed as follows: 94°C for 1 minutes, 50°C for 1 minutes, 72°C for 2 minutes, and a final extension 72°C for 10 minutes[8]. The amplified PCR product (amplicon) was stored at -20°C for electrophoresis purpose. The extracted DNA from bacteria were separated by electrophoresis technique on 1.5 % (w/v) agarose gel in 1X TAE buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.0) (1st Base, Malaysia) at 100 V for 40 minutes. The gel was pre-stained with

Maestrosafe^{TM} Nucleic Acid (V-Bio Science, Malaysia) while GeneRuler^{TM} 1 kb DNA ladder (Fermentas) was used as DNA size marker. Finally, all gels were viewed and captured by UV trans-illuminator Gel Documentation System (Syngene, UK).

16s r DNA gene Sequencing analysis

Genomic DNA was extracted using DNA extraction kit (Promaga). The 16s r DNA gene from the genomic DNA was amplified by using the following primers; 5-AGAGTTTGATCCTGGCTCAG-3 and 5-AAGGAGGTGATCCAGCCGCA-3 corresponding to the forward and reverse of 16s rDNA, respectively[9] .The amplicons were sequenced and blasted on online Genbank of NCBI (National Center for Biotechnology Information. (http//www.ncbi.gov).

Results and Discussion

Isolation of *Bacillus cereus*

A total of cooked rice (n=70) samples were purchased from three different locations of Kajang (20), Bangi (20), and UKM (30), respectively. A total of 43.3% samples of cooked rice were positive for *B. cereus* from Kajang, Bangi 33.3%, and 16.7% from UKM. The presumptive *B. cereus* isolated from cooked rice is shown in Table 1.

Table 1. Prevalence of *Bacillus cereus* in cooked rice samples.

***B. cereus* strains**	**Presumptive of *B. cereus***	**CFU / ml grow On MYP ager**	**Location**	**Number of samples**
BC 1	ve+	cfu 10^6×1.4	Kajang	1
-	ve-	0	UKM	2
-	ve-	0	Bangi	3
BC 2	ve+	cfu $10^6$6 ×1.	Hentian Kajang	4
BC 3	ve+	cfu 10^6 ×.11	Pasar Bangi	5
-	ve-	0	UKM	6
-	ve-	0	Kajang	7
BC 4	ve+	cfu $10^6$2×.1	Bangi	8
BC 5	ve+	1 cfu 10^6 1 ×	UKM	9
-	ve-	0	Kajang	10
BC 6	ve+	cfu $10^6$3 ×.1	Bangi	11
BC 7	ve+	cfu 10^6 ×5 .1	Hentian Kajang	12
-	ve-	0	Pasar Bangi	13
-	ve-	0	UKM	14
-	ve-	0	Hentian Kajang	15
-	ve-	0	Kajang	16
-	ve-	0	Bangi	17
-	ve-	0	Hentian Kajang	18
-	ve-	0	Pasar Bangi	19
BC 8	ve+	cfu 10^6 1 ×	UKM	20
BC 9	ve+	cfu 10^6 ×1.4	Kajang	21
BC 10	ve+	cfu 10^6×.3 1	Bangi	22
-	ve-	0	Hentian Kajang	23
-	ve-	0	Pasar Bangi	24
BC 11	ve+	cfu^5 104 ×.1	UKM	25
BC 12	ve+	cfu 10^6 ×1.4	Kajang	26
-	ve-	0	Bangi	27
-	ve-	0	Hentian Kajang	28
-	ve-	0	Pasar Bangi	29
BC 13	ve+	cfu $10^5$3 ×.1	Kajang	30

-	ve-	0	Bangi	31
BC 14	ve+	cfu $10^5$5 ×.1	Hentian Kajang	32
-	ve-	0	Pasar Bangi	33
BC 15	ve+	cfu $10^6$1 ×	UKM	34
BC 16	ve+	cfu 10^6 ×1.4	Kajang	35
-	ve-	0	Bangi	36
BC 17	ve+	cfu 10^6 ×.71	Hentian Kajang	37
-	ve-	0	Pasar Bangi	38
-	ve-	0	UKM	39
BC 18	ve+	cfu 10^4 ×.51	Pasar Bangi	40
BC 19	ve+	cfu 10^4 ×.81	Kajang	41
-	ve-	0	Bangi	42
BC20	Ve+	cfu 10^6 ×.61	Hentian Kajang	43
-	Ve-	0	UKM	44
-	ve-	0	Pasar Bangi	45
BC 21	ve+	Cfu $10^4$2×1.	Hentian Kajang	46
BC 22	ve+	cfu $10^6$6×1.	Kajang	47
BC 23	ve+	Cfu $10^4$6×	Bangi	48
-	ve-	0	UKM	49
BC 24	ve+	cfu $10^4$3×.1	Pasar Bangi	50
-	ve-	0	UKM	51
BC 25	ve+	cfu $10^5$7×1.	Kajang	52
BC 26	ve+	cfu 104×.1	Bangi	53
BC 27	ve+	cfu $10^6$1×1.	Hentian Kajang	54
-	ve-	0	Pasar Bangi	55
BC 28	ve+	cfu $10^6$1×1.	Kajang	56
-	ve-	0	Bangi	57
-	ve-	0	Hentian Kajang	58
BC 29	ve+	4cfu^4 10×.91	Pasar Bangi	59
BC 30	ve+	4cfu^4 105×.1	UKM	60
-	ve-	0	Kajang	61
BC 31	ve+	cfu 10^4×.61	Bangi	62
BC 32	ve+	cfu $10^6$3×1.	Hentian Kajang	63
BC 33	ve+	cfu 10^4×.91	Pasar Bangi	64
-	ve-	0	UKM	65
BC 34	ve+	cfu $10^6$2×1.	Kajang	66
-	ve-	0	Bangi	67
BC 35	ve+	cfu $10^6$6×1.	Hentian Kajang	68
-	ve-	0	Pasar Bangi	69
-	ve-	0	UKM	70

Biochemical test and 16s r DNA sequences

All *B. cereus* isolates (n =35) showed similar biochemical characteristics :100% of isolate were able to produce acidic fermentation from glucose, fructose and lactose. But none were from mannitol ,mannose, arabinose and xylose[10]. Those also isolates showed positive results on citrate, reduce nitrate into nitrite, motile, starch hydrolysis, catalase, Indol test. While, the cells morphology observed under microscope was shown as rod shape.

All *B. cereus* isolates specifically known as BC1 to BC35, were further identified by 16S rDNA with similarity of 80%-100% when it blast in the NCBI data base (Table 2), (Figure 1) shown the amplicons of *B. cereus* isolates using 16s rDNA universal primers which produced 711 bp in size. The 35 *B. cereus* isolates

amplicons were sequences and blast with available data in the GenBank database (Table2). It was revealed that all 35 isolates were *Bacillus cereus* 80-100%.

When amplicons were sequenced and blasted on online genbank of N*CBI (National Center for Biotechnology Information. (http//www.ncbi.gov),* all were identified as B. cereus. The isolation *B. cereus* in cooked rice is expected. This result is in agreement with[11,12] and[9] whose reported the isolation of *B. cereus* in cooked rice.

Table 2: Isolation strains in this study and GenBank accession similarity for 16S rDNA sequences

Number of strains	Description	Identification (% identity)
BC 1	*Bacillus cereus*	100%
BC 2	*Bacillus cereus*	99%
BC 3	*Bacillus cereus*	99%
BC 4	*Bacillus cereus*	93%
BC 5	*Bacillus cereus*	82%
BC 6	*Bacillus cereus*	91%
BC 7	*Bacillus cereus*	87%
BC 8	*Bacillus cereus*	80%
BC 9	*Bacillus cereus*	97%
BC 10	*Bacillus cereus*	97%
BC 11	*Bacillus cereus*	89%
BC 12	*Bacillus cereus*	98%
BC 13	*Bacillus cereus*	98%
BC 14	*Bacillus cereus*	99%
BC 15	*Bacillus cereus*	94%
BC 16	*Bacillus cereus*	82%
BC 17	*Bacillus cereus*	80%
BC 18	*Bacillus cereus*	99%
BC 19	*Bacillus cereus*	81%
BC 20	*Bacillus cereus*	99%
BC 21	*Bacillus cereus*	99 %
BC 22	*Bacillus cereus*	83%
BC 23	*Bacillus cereus*	99%
BC 24	*Bacillus cereus*	94%
BC 25	*Bacillus cereus*	94%
BC 26	*Bacillus cereus*	93%
BC 27	*Bacillus cereus*	94%
BC 28	*Bacillus cereus*	98%
BC 29	*Bacillus cereus*	87%
BC 30	*Bacillus cereus*	98%
BC 31	*Bacillus cereus*	91%
BC 32	*Bacillus cereus*	99%
BC 33	*Bacillus cereus*	99%
BC 34	*Bacillus cereus*	99%
BC 35	*Bacillus cereus*	80%
Positive Control	*Bacillus cereus*	95%

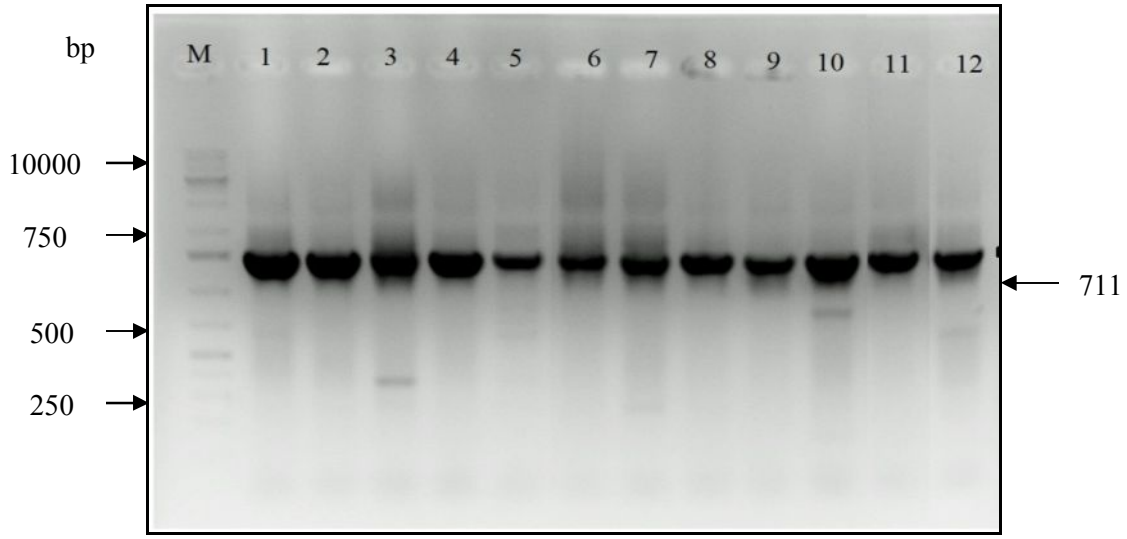

Figure 1: Amplicons of *Bacillus cereus* isolates using 16s rDNA universal primers on 1.5 % (w/v) agarose gel. Lane M: 1 kb DNA ladder; Lane1-11: *B. cereus* isolates (BC1-BC11); Lane 12: Positive control

Conclusion

In general, this present study offers an overview of distribution and presence of *B. cereus*. in cooked rice. Rice is a staple food and has a large number of consumers. Therefore, it is essential for the local public health authorities to monitor the cooked rice offer to the public.

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