International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.11 pp 132-141, 2016

Random Amplified Polymorphic-Dinucleic Acids (RAPD-PCR)and16S rDNAPhylogenetic analysis of Bacillus cereusIsolated fromfried rice samples

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Abstract : *Bacillus cereus* can cause two types of food poisoning as emetic or diarrheal syndrome. Fried rice is recognized as one of potential risk food especially in tropical countries. This study was conducted to detect and characterize *B. cereus* isolated from fried rice. A total of 70 fried rice samples were purchased from restaurants at Bangi (20), Kajang (20) and UKM's (30) cafeteria. The isolation of *B. cereus* was done onto Mannitol egg yolk polymyxin medium and the presence of *B. cereus* ranged from 1.2×10^4 to 1.6×10^6 cfu/g of fried rice samples. Of more than 110 colonies of presumptive *B. cereus* tested onto chromogenic *B. cereus* agar, 35 colonies were identified as *B. cereus* using biochemical test and partial sequence of 16S rDNA sequences analysis. 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 shown the amplicons of *B. cereus* isolates using 16S rDNA universal primers which produced 711 bp in size. Genetic fingerprinting of 35 *B. cereus* isolates was examined by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) using primer (RAPD1). The results of the RAPD-PCR were analyzed using GelCompare software. RAPD-PCR with primer RAPD1 discriminated the *B. cereus* isolates into 5 clusters and 4 single isolates at 70% similarity level.
Keywords : *Bacillius cereus, isolated, fried rice, RAPD-PCR.*

Introduction

Bacillus cereus is regarded as a gram positive bacterium that causes severe food poisoning. This bacterium is found to be rod shaped and forms spores. The spores can stay alive in hot and dry conditions, and remain dormant for many years[1]. The bacterium normally exists in soil, however, it can be found in foods, such as dairy products, rice, cereals and cereals derivatives, dried foods, spices, eggs, vegetables and meats[2]. Food poisoning concerning the outbreaks from the past comprises boiled and fried rice, vegetables, cooked meats, soups, and raw vegetable sprouts[3].According to Food and Drug Administration of the United Stated, food poisonings cause by *B. cereus* is presented into two different clinical syndromes as diarrheal and emetic syndrome. The emetic type results in vomiting after 0.5–6 h of ingestion[4] and diarrhoeal type leads to abdominal pain and diarrhoea after 8 to 16 h of consumption. The diarrheal syndrome has found to be associated with a broad category of food consisting meats, milk, vegetables and fish. On the other hand, the emetic syndrome has been usually correlated with rice products, starchy foods such as potato, pasta, noodles, spaghetti, pastry and cheese products[5].The diarrheal poisoning is caused by heat-labile enterotoxins produced during vegetative growth of *B. cereus* in small intestine[6].There are three different enterotoxins in *B. cereus* involved in food poisoning outbreaks are known. Two protein complexes, hemolysin BL (HBL) and nonhemolytic enterotoxin

(NHE)[7] and the single protein cytotoxic CytK[8]. HBL is a three-component hemolysin that consist of two lytic components and a binding protein B. The two lytic component are encoded by *hblC* and *hblD*. Whereas, protein B is encoded by *hblA*. Similar with NHE, it also has three component but non hemolytic, encoded by *nheA*, *nheB* and *nheC*. The emetic syndrome is caused by a single heat stable peptide toxin called cereulide encoded by cesgene[9]. Bacterial typing will be examined in this study using random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR).Typing of the bacteria is necessary for epidemiological control since it allows cases to be linked, outbreaks to be traced with precision, changing pattern of disease to be monitored, appearance of new strains and to identify the presence of virulent isolates. To date several typing methods base on genotypic approaches have been introduced which involving the application of nucleic analysis of chromosomal DNA or extra-chromosomal element such as plasmid or insertion sequence (IS). Most analysis is using chromosomal DNA such as random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)[10], Enterobacteria Repetitive Intergenic consensus (ERIC-PCR),Pulse Field Gel Electrophoresis (PFGE),[11]ribotyping restriction fragment length polymorphisms (PCR-RFLP), IS200 typing and plasmid profiling.

Materials and Methods

Sample collection

In the present study, a total of (n=70) cooked rice samples have been bought randomly from different restaurants in Selangor, Malaysia from August 2013 until June 2014. All samples have been immediately transported to the laboratory and have been analyzed within 24 hours.

Isolation and morphological characterization

Samples have been analyzed, using the standard procedure for the detection of *B. cereus* [10]with modifications which have been explained in details by[11]. A total of 25 g of each sample has been put in a stomacher bag and it has been added with 225 ml of Tryptic Soy Broth (TSB; Bacto™), and it has been homogenized in a stomacher (Interscience, France) for 60 s followed by incubation at 30°C for 12 h. *B. cereus* count has been determined on the basis of ISO 7932l2004 by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (Oxide CM0929). In addition, the dilutions of the stomached fluid have been prepared. Tryptic Soy Broth (TSB; Bacto™).0.1 ml portions of each dilutions of the fluid have then been transmitted into three tubes and incubated at 30°C for 18 to 24 h. Then aloopful of culture from each tube has been streaked onto Mannitol Egg Yolk Polymyxin Agar Base (MYP; Difco) and added with sterile Polymyxin B Selective Supplement (Difco) and sterile Egg-YolkTellurite Emulsion 20% (V/V) (Merck) which is a particular media for the isolation of the Rough and bright pink colonies with a zone of egg yolk precipitation,and use chromogenic medium (chromagar™*B. cereus* base)add supplement(France) have then been transmitted to nutrient agar slants, *B. cereus.* Identification is consequently confirmed by microscopic tests. The biochemical tests have been conducted as described by[12].

Isolation of genomic DNA

The bacterial DNA was extracted from 1 ml of the overnight culture grown of 30°C 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^{o}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 (1stBase, 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[15] The

amplified PCR product (amplicon) was stored at -20^{o}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 rDNA gene sequencing analysis

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

Partial 16S rDNA Sequences Analysis

The amplicons were sent to MyTACG Bioscience Enterprise laboratory and 16S rDNA sequenced data were compared with in the NCBI (National Center for Biotechnology Information) GenBank databases using BLAST (Basic local alignment search tool) software available at https://www.ncbi.nlm.nih.gov/. A phylogenetic tree that consisted of 16S rDNA***B. cereus*** isolates and other 16S rDNA from different microorganisms available from the NCBI database (Table 2) was constructed using ClustalX[17] 1.8. Evolutionary history was inferred using the neighbour-joining method in MEGA5. The 1000 bootstrap replicates of the original sequence data were run to assess the confidence value of individual branches[18,19,20,21].

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

Number of strains	Description	Accession No.	Identification (% identity)
BC 1	*Bacillus cereus*	JF509359	100%
BC 2	*Bacillus cereus*	HE610815	93%
BC 3	*Bacillus cereus*	DQ339679	90%
BC 4	*Bacillus cereus*	GQ214131	98%
BC 5	*Bacillus cereus*	KX369567	93%
BC 6	*Bacillus cereus*	KT380673	98%
BC 7	*Bacillus cereus*	KJ729602	87%
BC 8	*Bacillus cereus*	KU510055	99%
BC 9	*Bacillus cereus*	KU510057	99%
BC 10	*Bacillus cereus*	KT380824	99%
BC 11	*Bacillus cereus*	EU161996	95%
BC 12	*Bacillus cereus*	KT720351	89%
BC 13	*Bacillus cereus*	CP001177	99%
BC 14	*Bacillus cereus*	KT380824	99%
BC 15	*Bacillus cereus*	JX025736	92%
BC 16	*Bacillus cereus*	KC545042	99%
BC 17	*Bacillus cereus*	KM391942	99%
BC 18	*Bacillus cereus*	KT150198	95%
BC 19	*Bacillus cereus*--	KT380824	99%
BC 20	*Bacillus cereus*--	JQ824137	99%
BC 21	*Bacillus cereus*	KJ605415	95 %
BC 22	*Bacillus cereus*	KT719668	92%
BC 23	*Bacillus cereus*	KP813819	88%
BC 24	*Bacillus cereus*	CP009968	81%
BC 25	*Bacillus cereus*--	KU510055	97%
BC 26	*Bacillus cereus*	KC736480	97%
BC 27	*Bacillus cereus*	JQ773351	98%

BC 28	*Bacillus cereus*	KT922033	98%
BC 29	*Bacillus cereus*	FJ932655	89%
BC 30	*Bacillus cereus*	KF494193	95%
BC 31	*Bacillus cereus*	EU430093	92%
BC 32	*Bacillus cereus*	GU056810	99%
BC 33	*Bacillus cereus*	EF472263	80%
BC 34	*Bacillus cereus*	KU512628	88%
BC 35	*Bacillus cereus*	KT982245	99%
Positive Control	*Bacillus cereus*	DQ466089	95%
*Bacillus cereus*Fck		KX350005	94%
Bacillus cereus BS1		KR063194	98%
Bacillus cereus NC7		AP007209	94%
Bacillus cereus CC2H2L N		KP940382	97%
Bacillus cereus D26		KC441765	99%
*Bacillus cereus*BC-1		KF835390	99%
*Bacillus cereus*ML208		KC692193	99
*Bacillus cereus*ATCC 21281		FJ501984	99%
*Bacillus cereus*MI_RH03		KT380673	99%
Bacillus cereus KES7		KP202304	99%
*Bacillus cereus*BA9		KU510057	97%
Bacillus cereus JY2		HQ833023	99%
Bacillus cereus B2A22		KX023349	99%
Streptomyces sp.		KF892538	99%
Enterobacter cloacae		KF697363	94%
Desulfovibrio vulgaris		KC462187	97%
Clostridium acetobutylicum		JQ086380	99%
Microbacteriumoxydans		LN890040	99%
Carduusnutans		KT176586	99%
Syzygium sp.		KJ709117	100%

Randomly Amplified Polymorphic DNA (RAPD) analysis using RAPD1 Primer

The discriminatory ability and stability of RAPD-PCR fingerprinting were conducted with primers RAPD1[22], as shown in (Table 3) which showed the greatest stability and discriminatory ability among the *B. cereus* isolates and was therefore used in this study. The RAPD-PCR fingerprinting assay was performed in a 25 µl volume containing GoTaq green master mix (MyTACG Bioscience Enterprise, MY) 12.5 µl and 1.0 µl of 100 mM primer (RAPD1),7.5 µl water nuclease-free and 5 µl of (50-100 ng) DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water[10]. The amplicon was purified using a PCR purification kit (Promega, MY). Amplification was performed in an Eppendorf Mastercycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 4 min followed by 38 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 50°C and polymerization at 72°C for 2 min. Final elongation was at 72°C for 5 min. Negative controls (NFW) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination.

Table 3: RAPD primers and their sequences used in this study

Amplification Target gene	**Primer codes**	**Primer sequences (5'-3')**	**References**
Random	RAPD-1	CCGAGTCCA	22

Gel Electrophoresis

The extracted DNA from the 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 min. following PCR amplification. The gel was pre-stained with Maestrosafe^{TM} Nucleic Acid (V-Bio Science, Malaysia) while GeneRuler^{TM} 1 kb DNA ladder and 100 bp (Fermentas, MY) were used as DNA size marker. Finally, all gels were viewed and captured by UV trans-illuminator Gel Documentation System (Syngene, UK).

Results and Discussion

Biochemical testand 16S rDNA partial DNA sequence

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[13]. 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 (Table 2). It was revealed that all 35 isolates were *Bacillus cereus* 80-100%.

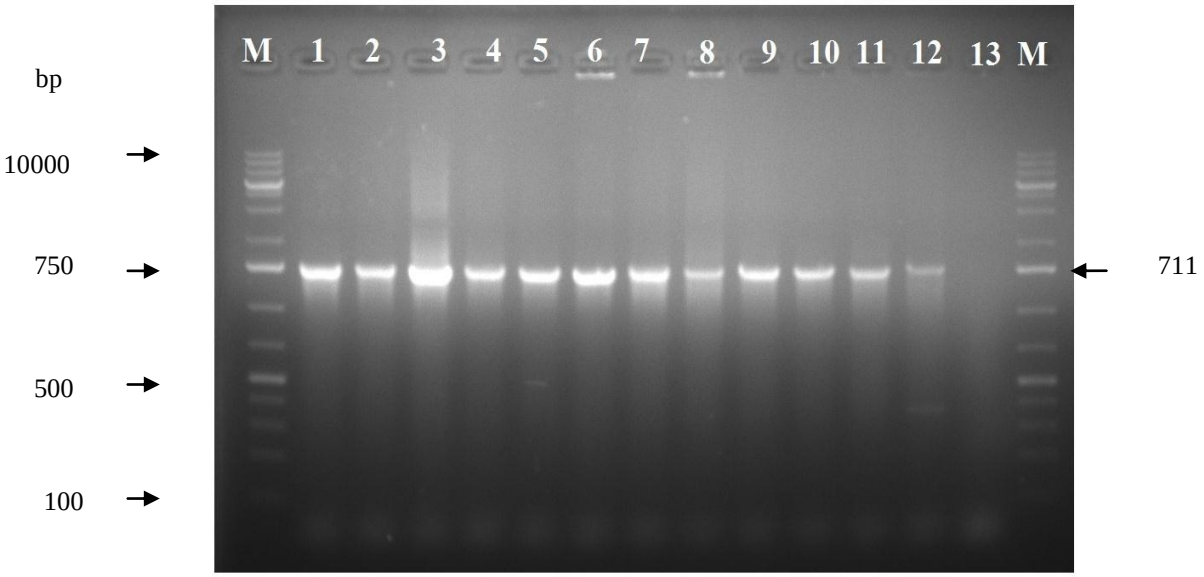

Figure 1: Amplicons of *Bacillus cereus* isolates using 16S rDNA universal primers on 1.5 % (w/v) agarose gel. Lane M: 1 kb DNA Lane 1-11: *B. cereus* isolates; Lane 12: Positive control

The 16SrDNAsequences of 35 *B. cereus* isolates were analysed phylogenetically. A Neighbour-Joining (NJ) tree comprising 15 16S rDNA*B. cereus* species was constructed (Figure 2). The 16S rDNA sequences from the plant (*Carduus* and *Syzygium*) were presented as the out-group. Phylogenetic analyses revealed that eukaryotic sequence are distinct from prokaryotic sequence and share low sequence identity, indicating that

these organism have undergone extensive evolutionary differentiation. Interestingly, BC26 did not clustered with other isolates that grouped in clade I which was supported by bootstrap values of 100. There are two isolate strains (BC29 and BC33) clustered together from the other isolates indicating the similarity in their sequences. Other isolates such as 16 were grouped together with the pathogenic bacteria species such as *Sterptomysis sp.*, *Closteridum acetobytlicum*, *Desulfovibrio vulgaris*, *Microbacterium oxydans* and *Enteriobacter cloacae.*

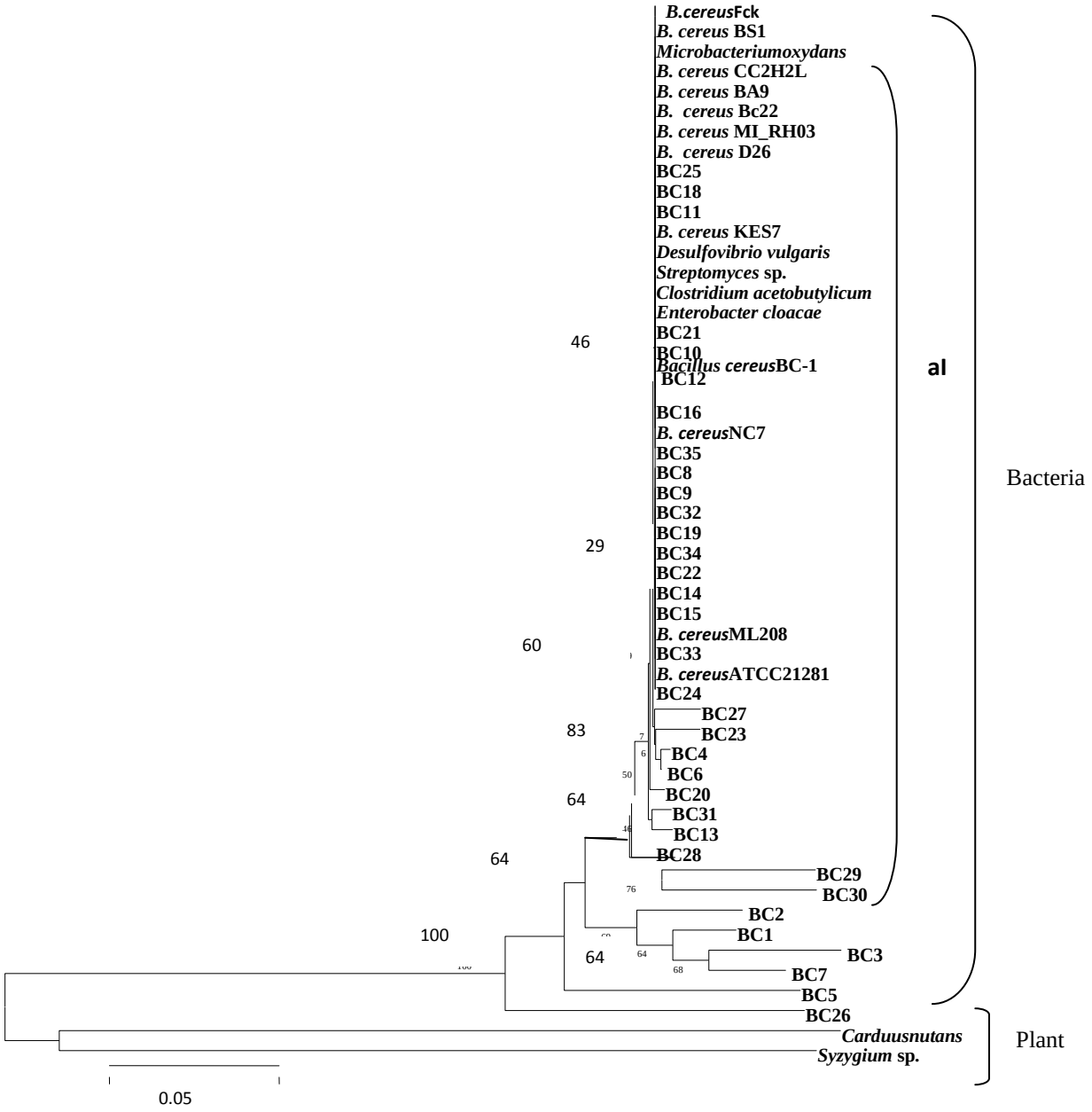

Figure 2:Phylogenetic tree displaying evolutionary relationships between *Bacillus cereus*isolatesin this study and other *Bacillus* species. The evolutionary history was inferred using the Neighbour-Joining method. Confidence values were assessed from 1,000 bootstrap replicates of the original sequence data and are shown next to the branches. The scale indicates the evolutionary distances computed using the p-distance method and are in the units of the number of nucleotide sequences differences per site. The analysis involved 53 nucleotide sequences. Evolutionary analyses were conducted using MEGA5.

Random Amplified Polymorphic-Dinucleic Acids (RAPD- PCR) Fingerprinting

RAPD method was applied in the present study to sub-type the *Bacillus cereus* isolates isolated from fried rice samples.Sub-typing would estimate their intraspecies diversity. One primer was used in the PCR analysis.RAPD-PCR with primer RAPD1 discriminated the *B. cereus* isolates into 35 RAPD fingerprinting. Using dendrogram it can be grouped into 5 clusters and 4 single isolates.For RAPD2 primers, they can discriminated the *B. cereus* isolates into 32 RAPD fingerprinting and can be group 3 clusters and 3 single isolates. at 70% similarity level examined. Results in the presence study demonstrated combination of phenotypically and genotypically methods show a wide heterogeneity among rice fried isolates of *B. cereus* (figure 3) and (figure 4).

The RAPD analysis help to elucidate the genetic diversity of the *B.cereus* isolates. In the present study, RAPD-PCR is used to differentiate theamong all 35 isolates of *B.cereus.*This findings was is agreement with[23] who reported that the RAPD-PCR analysis was effectively differentiated to isolates of *B.cereus.*The lost of specific site of bacteria is also reported by[24] but in *Ralstoniasolanacearum.*

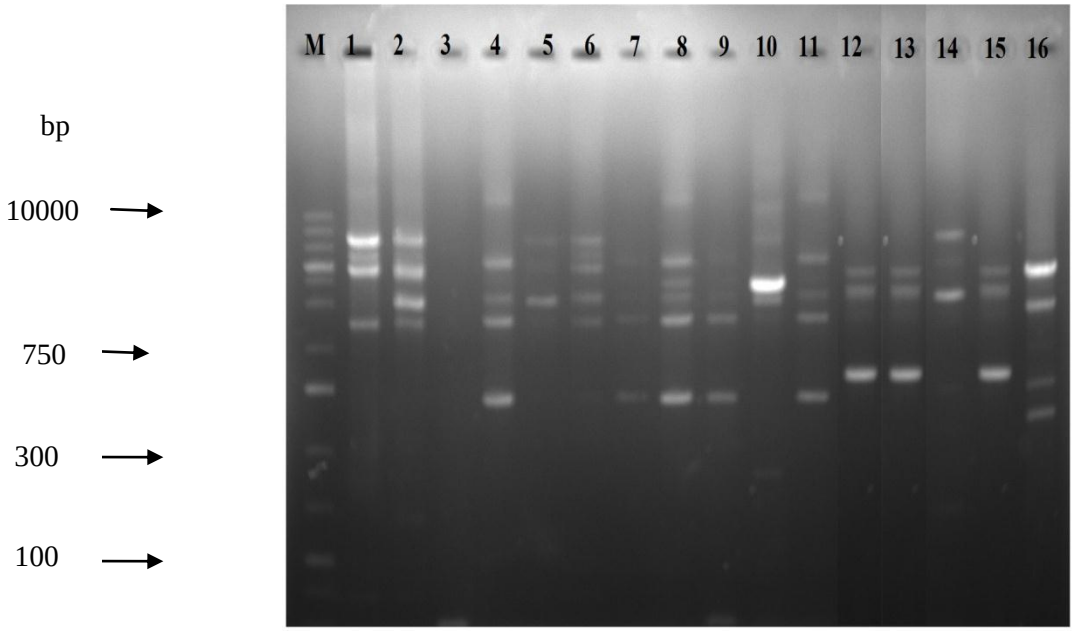

Figure3: RAPD1 fingerprinting generated from RAPD1 primer by*Bacillus cereus*isolates (isolates BC1-BC16). M: 1kb DNAladder; Lane1-16: *B. cereus* BC1- BC 16

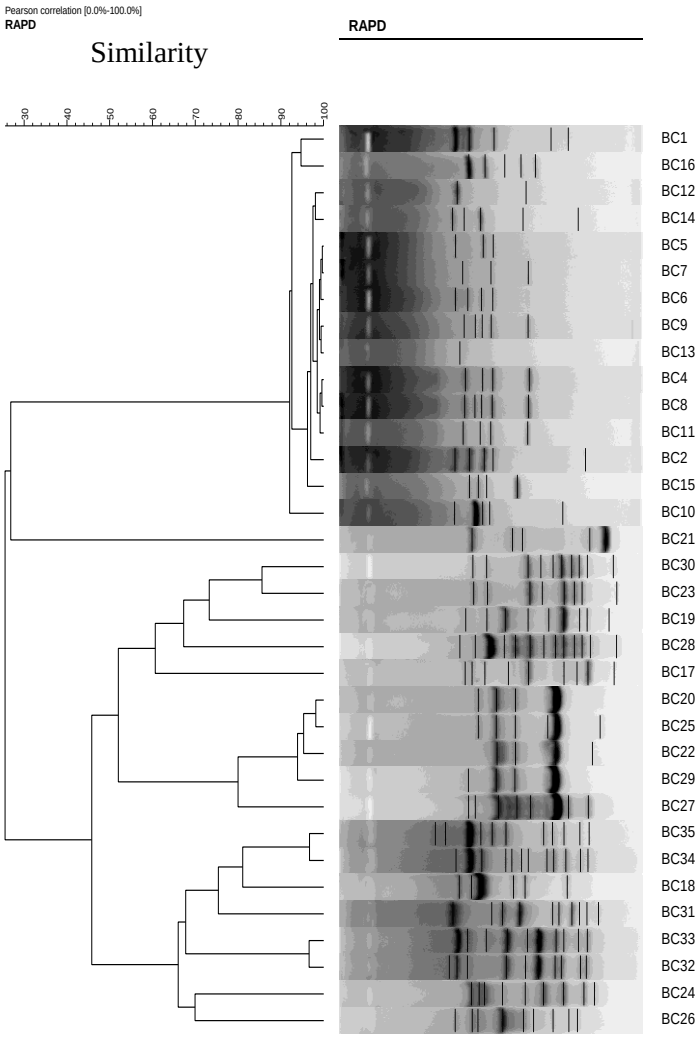

Figure4: Dendrogram of *Bacillus cereus* typable isolates produced from RAPD analysis (RAPD1) using average linkage unweighted group pair method with arithmetic averages (UPGMA)

Conclusion

Results of the presence study demonstrated the combination of phenotypically and genotypically methods show a wide heterogeneity among fried rice isolates of *B. cereus.* Hence, it is important to educate food handlers about their responsibilities for food safety and train them on personal hygiene policies and basic practices for safe food handlings.

Acknowledgments

The author thanks Associate. Prof. Sahilah AbdMutalib for their fruitful discussions and support, this research was supported by the School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), Malaysia.

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