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BACILLUS CEREUS IN FOOD: PARTIAL PURIFICATION AND CHARACTERIZATION OF HEMOLYSIN

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Abstract: *Bacillus cereus* is an aerobic spore former commonly found in soil, vegetables and in many raw, and processed foods. Consumption of foods that contain large numbers of *B*. *cereus* may result in food poisoning. Although, certain physiological and cultural characteristics are necessary for identifying *B*. *cereus*, its enterotoxigenicity indicates whether a suspect strain may be a public health hazard. Out of 75 food samples tested, we obtained 57 hemolytic strains of *B. cereus.* On further screening based on the antibiotic susceptibility pattern, three strains *viz., B. cereus* PCBC-06, *B. cereus* PCBC-52 and *B. cereus* PCBC-57 were selected and investigated for their growth pattern. Of all the three strains, *B. cereus* PCBC-06 exhibited good growth pattern. Test strains grew after the treatment at 40, 50, 60, 70, 80 and 90ºC and were killed only after treating at 121ºC for 20 min at 15 lb/sq. in. The optimum pH and glucose concentration for the growth of all strains were between 7.0 and 9.0, and 0.5%, respectively. *B. cereus* PCBC-06, *B. cereus* PCBC-52 and *B. cereus* PCBC-57 optimally grew at salt concentration of 0.75%, 0.5% and 0.25%, respectively. The heat resistance measurements exhibited that the test strains failed to grow at 2°C and the spore D_{90} value ranged from 5.40 to 5.50 min. *B. cereus* PCBC-06 was subjected to protein studies and its protein accumulation was directly proportional to its growth. In gel diffusion assay, discontinuous pattern of hemolysis by the crude toxin was observed and the zones measured 0.6 cm, 0.8 cm and 0.9 cm at 20 min, 65 min and 100 min, respectively. Hemolysin was produced by dialyzing membrane technique and partially purified hemolysin by ammonium sulphate precipitation had protein content of 44 µg/ml of sample. SDS-PAGE of the crude hemolysin showed four conspicuous bands of molecular weight 70 000, 68 000, and 57 000 and 43 000.

Keywords: Food, *B. cereus,* Growth, Hemolysin, Gel Diffusion, Partial Purification

INTRODUCTION

Foods that are nutritional value to humans are often ideal culture media for microbial growth. The foods that we eat are rarely sterile, carry microbial association whose composition depends upon which organism gain access and how they grow, survive and interact in the food overtime. Three gram-positive rods *viz., Clostridium botulinum, C. perfringens,* and *Bacillus cereus* are known to cause food intoxications. *B. cereus* is one of the 60 representatives of the widely varied genus *Bacillus* and found frequently as a saprophyte in soil, water, vegetation and air (Stanier *et al*. 1976). Two distinct types of illnesses are caused by *B. cereus* depending on the type of toxin produced: an emetic illness characterized by nausea and vomiting with an incubation time of 1 to 6 h, and a

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diarrheal type, with incubation time of 4 to 16 h. The emetic type is often associated with boiled or fried rice, while the diarrheal type is associated with a wider range of foods (Pelczar & Reid 1972; Davis & Walkinson 1973; Goepfert *et al*. 1973). Present routine detection methods for *B. cereus* rely on standard plate counting which require, approximately 4 days to be performed, including confirmatory testing. This is time-consuming when inspecting products with short shelf-live. The presence of *B. cereus* strains that cause food poisoning can also be indicated by detection of their toxins (Torkar & Mozina 2000). The purposes of the current study are to screen various food types for the presence of *B. cereus*, to isolate and to correlate various microenvironments for its growth, sporulation, detection, production and partial purification of hemolysin.

MATERIALS AND METHODS

Samples

Food samples were collected in and around the area of Peelamedu, Coimbatore, India from December 2003 to February 2004. Each sample unit consisted of at least 100 ml/g. Liquid food was subjected for continuous or periodic mixing and the samples were withdrawn into a sterile container, transported to lab and mixed thoroughly once again before pipetting the amounts required for investigation. Sampling of solids and semisolids was performed using sterile scalpels and spoons depending on the nature of material. Foods were examined by taking deep samples as well as surface sample. Deep sample was taken with care to minimize contamination from superficial levels. These large samples were treated separately, each being mixed thoroughly in the laboratory before processing. The samples were transported and stored under condition, which inhibited changes in microbial numbers. Frozen foods were kept frozen. Chilled/refrigerated foods were kept at 4°C and not frozen. Dried foods and canned foods were not cooled but stored and transported at a temperature less than 40°C. Insulated containers were used to hold and transport chilled or frozen samples. Samples were transported to the laboratory as soon as possible, preferably within 2 h. A total number of 75 samples were collected and designated from S1 to S75 (Table 1).

Table 1: Details of the food samples collected for isolation of *Bacillus* spp.

Sample No.	Type of sample	Name of sample	No. of collected sample
1		Milk	7
2		Apple juice	4
3	I Liquid	Coffee	6
4		Cooler water	8
5		Bottled drink	2

(continue on next page)

Table 1 *(continued)*

S. No.	Type of sample	Name of sample	No. of collected sample
6		Rose milk	5
7		Lassie	3
8		Drink in Carton	1
9		Paneer butter masala	2
10		Sambar	11
11	II Semi solid	Fish curry	6
12		Beef curry	3
13		Egg curry	7
14		Ghee rice	2
15	III Solid	Vada	5
16		Plain rice	3
	Total		**75**

Aerobic plate count (APC) and Enumeration of presumptive *Bacillus species.*

Serial dilutions from 10^{-2} to 10^{-6} were prepared from homogenized food samples (Andrews & Hammack 1998). Plate count agar was used for APC (Rhodehamel & Harmon 1998) while selective and differential media namely Polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PPEMBA) was used (Lancette & Harmon 1980) for the presumptive enumeration of *B. cereus*. Appropriate dilutions were spread on to the plates of plate count agar and PPEMBA. The plates were incubated in an inverted position for 48 h ± 4 h at 37°C. Colonies were counted immediately after the incubation period and observed for the presumptive *B. cereus* colonies (Rhodehamel & Harmon 1998).

Isolation and confirmation of B. cereus isolates

Five colonies with the typical morphology of *B. cereus* were isolated from PPEMBA plates from each sample and subcultured on Mannitol egg yolk agar (MYP). Colonies having the characteristic appearance of *B. cereus* were selected and subcultured on J agar (JA) plates (Choma *et al*. 2000). Identification and confirmation of the isolates to *B. cereus* group was done by Gram-staining, inoculation in *B. cereus* motility medium (BCMM), hanging drop method for motility identification, rhizoid growth, test for protein toxin crystals, growth in anaerobic medium, endospore staining and starch hydrolysis. Biochemical characterization of *Bacillus* species was performed by glucose fermentation, Voges-Proskauer (VP) test, citrate utilization test, catalase test and nitrate reduction test. Test for extracellular protein activity of the *Bacillus* spp. was performed by streaking the test strains on egg yolk agar, blood agar, starch agar and milk agar, followed by incubation at 37°C for 1 to 4 days and observation for lecithinase production, hemolysis, starch and casein hydrolysis, respectively (Claus & Berkeley 1986; Cappuccino & Sherman 1996; Atlas 1997). Based on

these analyses a total number of 57 hemolytic isolates were identified and strain numbers were designated as *B. cereus* PCBC-01 to 57.

Antibiogram pattern of the isolates of *Bacillus* species

B. cereus strains were further screened on the basis of antibiotic susceptibility as per the method of Bauer *et al*. (1966). Commonly used antibiotics for the treatment of *B. cereus* infection *viz:* ampicillin (A), cephalothin (Ch), cephotoximine (Ce), chloramphenicol (C), ciproflaxacin (Cf), clindamycin (Cd), erythromycin (E), gentamycin (G), lincomycin (L), neomycin (N), oxacillin (Ox), streptomycin (S) and tetracycline (T) each at the concentration of 30 µg/ml were used for the test.

Growth pattern of the selected isolates of *B. cereus*

Depending on antibiotic susceptibility, three stains of *B. cereus viz.,* PCBC-06, PCBC-52 and PCBC-57 were selected for further analysis. About 10 ml of the 24 h old culture was inoculated into 90 ml of sterile nutrient broth and incubated in a shaker at 37°C. Optical density (OD) of the cultures was measured periodically at 660 nm in ELICO, SL-159 UV-VIS spectrophotometer.

Effect of various growth conditions on *Bacillus* species.

Temperature

B. cereus suspension in nutrient broth was placed in the water bath for 10 min at different temperatures *viz.,* 40°C, 50°C, 60°C, 70°C, 80°C and 90°C and these cultures were inoculated on nutrient agar and incubated at 37°C for 48 h. The plate with no growth was considered as thermal death point.

pH

Sterile nutrient broth was prepared with varying pH of 5.0, 6.0, 7.0, 8.0, and 9.0, inoculated with 0.5 ml of the test culture and incubated at 37°C for 24 h. OD was measured at 660 nm in ELICO, SL-159 UV spectrophotometer.

Sodium chloride concentration

Sterile nutrient broth with different concentration sodium chloride (NaCl) *viz.,* 0.25%, 0.50%, 0.75%, and 1.00% was inoculated with 0.1 ml of the 24 h old culture, incubated at 37°C for 24 h and OD was measured at 660 nm in ELICO, SL-159 UV-VIS spectrophotometer.

Glucose concentration

Sterile nutrient broth with different glucose concentration (0%, 0.25%, 0.5%, 0.75%, and 1%) was inoculated with 0.1 ml of the 24 h old culture of test strains of *B. cereus*, incubated at 37°C for 24 h and OD was measured at 660 nm for each tube in ELICO, SL-159 UV VIS spectrophotometer.

Growth of *B. cereus* at various temperatures
J broth (JB), inoculated with the 24 h old culture of *B. cereus* was incubated at 2°C, 5°C, 30°C, 37°C and 42°C and observed for growth on 2, 3, 5, 7, 14 and 21 days. Positive cultures on JA were further analysed for spore production and spore morphology.

Detection of heat resistant spores of *B. cereus*
Sterile soil extract agar plates were inoculated with the test strains of *B. cereus* and incubated at room temperature of 28°C for five days. The spores were collected from the agar surface with a sterile cotton swab, suspended in 1.5 ml sterile distilled water and placed in ice for 45 min. The spore suspension was submerged in hot water at 90°C for 3, 5, 7, 10 and 15 min respectively, streaked onto JA plates and incubated at 30°C for 24 h.

Growth and protein accumulation of *B. cereus*
About 10 ml of the test strains of *B. cereus* PCBC-06 was inoculated into 90 ml of sterile nutrient broth and incubated at 37°C. Periodical estimation of the protein in the broth culture was carried out by Lowry *et al*. (1951).

Extraction and estimation of whole cell protein from *B. cereus*
Nutrient broth containing the 24 h old culture of *B. cereus* PCBC-06 was centrifuged and pellet was washed with phosphate buffer saline (pH 7.2). The pellet was incubated in boiling water bath with the sample buffer for 10 min and subjected to Lowry *et al*. (1951) method of protein estimation.

Hemolysin production by *B. cereus*
Aqueous suspension of *B. cereus* PCBC-06 was inoculated into brain heart infusion glucose (BHIG) broth and the turbidity was made equivalent to No. 1 on McFarland nephelometer scale (McFarland 1907). The broth was shaken at 3 ± 2^{o}C at 125 rev min^{-1} for 12 h in environmental shaker. The contents were centrifuged at 10 000 rpm for 10 min. The supernatant was examined for the presence of toxin.

Gel diffusion assay for hemolysin activity
Brain heart infusion broth supplemented with 0.1% glucose (BHIG) was inoculated with 1% (v/v) of an overnight stationary seed culture of *B. cereus* PCBC-06, grown in BHIG at 30°C and incubated on a rotary shaker at 200 rev min^{-1} for 5 h. Cells were removed by centrifugation at 10 000 rpm for 10 min and the supernatant was stored at –20°C. Using a gel cutter, 3.0 mm wells were made in the center of sterile blood agar plates (Beecher & Wong 1994), loaded with 10 µl of the supernatant and incubated at 37°C. Hemolysis was monitored at 15 min intervals.

Partial purification of extracellular hemolysin from *B. cereus*
Preparation of crude hemolysin was produced by dialysis membrane technique (Murphy & Haque 1967). Precipitation of hemolysin was accomplished with saturated solution of ammonium sulphate (pH 5.0) at 4°C overnight. The

precipitate was removed by centrifugation at 30 000 rpm for 20 min and then suspended in 10 ml of absolute alcohol. The precipitated protein was again removed by centrifuging at 5 000 rpm for 10 min. The supernatant was dialyzed against a 0.9% solution of NaCI. The partially purified hemolysin of *B. cereus* was analysed for protein content by Lowry *et al.* (1951) method and again tested for hemolysin activity on blood agar plates. The partially purified hemolysin was subjected to separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to analyse the hemolysin components. The crude samples were run at 30 mA per gel (constant current) and through the resolving gel at 60 mA per gel. After electrophoresis, the gels were stained with colloidal Coomassie brilliant blue G-250 (Beecher & Macmillan 1991).

RESULTS

APC and enumeration of presumptive *Bacillus* spp.

Among 75 food samples tested, higher APC of 290 × 10^2 bacterial cells/g or ml was obtained in S17 sample. Lower APC of 10 × 10^2 bacteria/g or ml was recorded in S20, S30, S66 and S74 samples (Fig. 1). On PPEMBA, *B. cereus* produced peacock blue or turquoise colonies surrounded by a precipitate, *B. subtilis* produced yellow colonies surrounded by a yellow zone and *B. megaterium* produced white colonies. With the exception of few samples, *B. cereus* was present in most of the samples. The samples *viz.*, S52, S54, S60 and S63 revealed more than 15 000 bacteria /g or ml whereas, the other samples *viz.,* S2, S4, S15, S18, S33, S46 etc., did not contain *B. cereus* (Fig. 2).

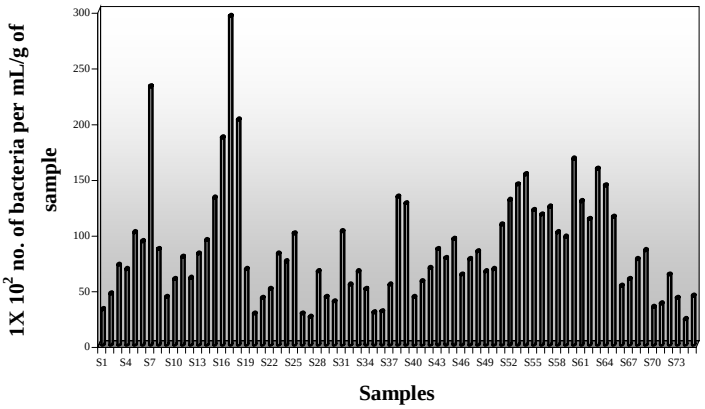

Figure 1: APC of food samples

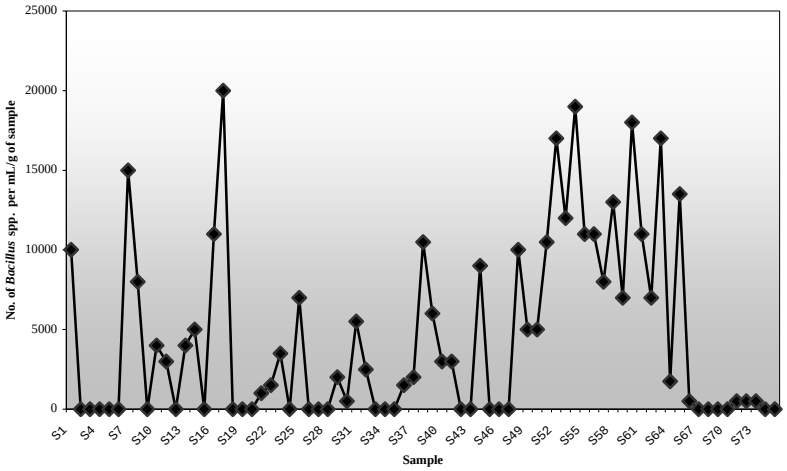

Figure 2: Presumptive enumeration of *B. cereus*

Isolation and confirmation of *B. cereus* isolates

The presumptive colonies of *B. cereus* were identified and differentiated from other species of *Bacillus* by morphological and biochemical characterization (Table 2). A total number of 57 hemolytic isolates of *B. cereus* were obtained which were designated from *B. cereus* PCBC-01 to 57.

Table 2: Morphological and biochemical characterization for the identification of *Bacillus* spp.

Sample No.	Test	*B. cereus*	*B. subtilis*	*B. megaterium*
1	Gram staining	Gram positive	Gram positive	Gram positive
2	Motility	+	–	–
3	Rhizoid growth	–	–	–
4	Protein toxin crystals	–	–	–
5	Spore staining	+	–	–
6	Glucose fermentation	A	A	A
7	VP test	+	+	–
8	Citrate utilization test	+	+	+
9	Catalase test	+	+	+
10	Nitrate test	+	+	+

+ – Positive reaction
- – Negative reaction
A – Acid reaction

Antibiogram pattern of the isolates of *Bacillus cereus*

Antibiogram pattern of the selected isolates of *B. cereus* demonstrated 89.47%, 91.22%, 7.00%, 77.20%, 15.79%, 89.47%, 17.54%, 85.90%, 15.79%, 94.70%, 8.77%, 10.53% and 82.46% of the strains were resistant to Ce, L, Cf, A, C, Ch, G, T, S, Cd, N, E and Ox, respectively. Based on the antibiotic susceptibility pattern the isolates *viz., B. cereus* PCBC-06, *B. cereus* PCBC-52 and *B. cereus* PCBC-57 were selected for further investigation as they were resistant to most of the antibiotic used when compared to other strains (Table 3).

Table 3: Antibiogram pattern of *Bacillus* species

Strains	Ce	L	Cf	A	C	Ch	G	T	S	Cd	N	E	Ox
B1	R	R	S	R	R	R	S	R	R	R	R	R	R
B2	R	R	S	R	S	R	R	R	R	R	S	S	R
B3	R	R	S	R	S	R	S	R	R	R	S	S	R
B4	R	R	S	R	S	R	S	R	R	R	S	S	R
B5	R	R	S	R	R	R	S	R	R	R	S	R	R
B6	R	R	R	R	S	R	R	R	R	R	R	R	R
B7	R	R	R	R	R	R	S	R	R	R	R	R	R
B8	R	R	S	R	R	R	R	R	R	R	S	S	R
B9	R	R	S	R	S	R	R	R	R	R	S	S	R
B10	R	R	S	R	S	R	S	R	R	R	S	S	R
B11	R	R	S	R	S	S	S	S	R	R	S	S	R
B12	R	R	S	R	S	R	S	R	R	R	S	S	R
B13	R	R	S	R	S	R	S	R	R	S	S	S	R
B14	R	R	S	R	S	R	S	R	R	R	S	S	R
B15	R	S	S	R	S	R	S	R	R	R	S	S	R
B16	R	R	S	R	S	R	S	R	R	R	S	S	R
B17	R	R	S	R	S	R	S	R	R	R	S	S	R
B18	R	R	S	R	S	R	S	R	R	R	S	S	R
B19	R	S	S	S	R	S	S	S	S	S	S	S	S

(continue on next page)

Table 3 *(continued)*

Strains	Ce	L	Cf	A	C	Ch	G	T	S	Cd	N	E	Ox
B20	R	R	S	S	S	R	R	S	R	R	S	S	S
B21	R	R	S	S	R	R	S	R	R	R	S	S	S
B22	R	R	S	R	S	R	R	R	R	R	S	S	R
B23	R	R	S	R	S	R	S	R	R	R	S	S	R
B24	R	R	S	R	S	R	S	R	R	R	S	S	R
B25	R	R	S	R	S	R	S	R	R	R	S	S	R
B26	R	R	S	R	S	S	S	R	R	R	S	S	R
B27	R	R	S	R	S	R	S	S	R	R	S	S	R
B28	R	R	S	R	S	R	S	R	R	R	S	S	R
B29	R	R	S	R	S	R	S	R	R	R	S	S	R
B30	R	S	S	R	R	R	S	S	R	R	S	S	R
B31	R	R	S	R	R	R	R	R	R	R	S	S	R
B32	R	R	S	R	S	R	R	R	R	R	S	S	R
B33	R	R	S	R	S	R	S	S	R	R	S	S	R
B34	R	R	S	R	S	R	S	R	R	R	S	S	R
B35	R	R	S	R	S	R	S	R	R	R	S	S	R
B36	R	R	S	R	S	R	S	R	R	R	S	S	R
B37	R	R	S	R	S	R	S	R	R	R	S	S	R
B38	R	R	S	R	S	S	S	S	R	R	S	S	R
B39	R	R	S	R	S	R	S	R	R	R	S	S	R
B40	R	R	S	S	S	R	S	R	R	R	S	S	S
B41	R	R	S	R	S	R	S	R	R	R	S	S	R
B42	R	R	S	R	S	R	S	R	R	R	S	S	R
B43	R	R	S	R	S	R	S	R	R	R	S	S	R

(continue on next page)

Table 3 *(continued)*

Strains	Ce	L	Cf	A	C	Ch	G	T	S	Cd	N	E	Ox
B44	R	R	S	R	S	R	S	R	R	R	S	S	R
B45	R	R	S	R	S	R	S	R	R	R	S	S	R
B46	R	R	S	R	S	R	S	R	R	R	S	S	R
B47	S	R	S	R	S	R	S	R	R	R	S	S	R
B48	S	R	S	S	S	S	S	R	S	R	S	S	S
B49	S	S	S	S	S	R	S	R	S	S	S	S	S
B50	R	R	S	S	S	R	S	R	S	R	S	S	S
B51	R	R	S	S	S	R	S	R	S	R	S	S	S
B52	R	R	R	R	R	R	S	R	R	R	R	R	R
B53	S	R	S	S	S	R	R	R	S	R	S	S	S
B54	S	R	S	S	S	S	S	S	S	R	S	S	S
B55	S	S	S	S	S	R	S	R	S	R	S	S	S
B56	S	S	S	S	S	R	S	R	S	R	S	S	S
B57	R	R	R	S	R	R	R	R	R	R	R	R	R

R–Resistant; S–Sensitive
Ce – Cephalothin; L – Lincomycin; Cf – Ciprofloxacin; A`– Ampicillin,
C – Chloramphenicol; Ch – Cephalothin; G – Gentamycin; T – Tetracycline,
S – Streptomycin; Cd – Clindamycin; N – Neomycin; E – Erythromycin; Ox – Oxacillin

Growth experiments

B. cereus PCBC-06 exhibited good growth pattern compared to the other two isolates *viz*., *B. cereus* PCBC-52 and *B. cereus* PCBC-57 (Fig. 3). All the three test strains had good growth after the treatment at 40ºC, 50ºC, 60ºC, 70ºC, 80ºC and 90ºC. The culture of *B. cereus* PCBC-57 showed slight retardation in growth after the treatment at 100ºC whereas the other two strains grew normally. The test cultures exposed to 121ºC for 20 min at 15 lb/sq.in. did not show any growth (Table 4). The optimum pH for growth was between 7.0 and 9.0 for all the test strains. The optimum salt concentration for the strains *B. cereus* PCBC-06, *B. cereus* PCBC-56 and *B. cereus* PCBC-57 were 0.75%, 0.5% and 0.25%, respectively. All the three strains had optimum requirement of 0.5% of glucose (Table 5).

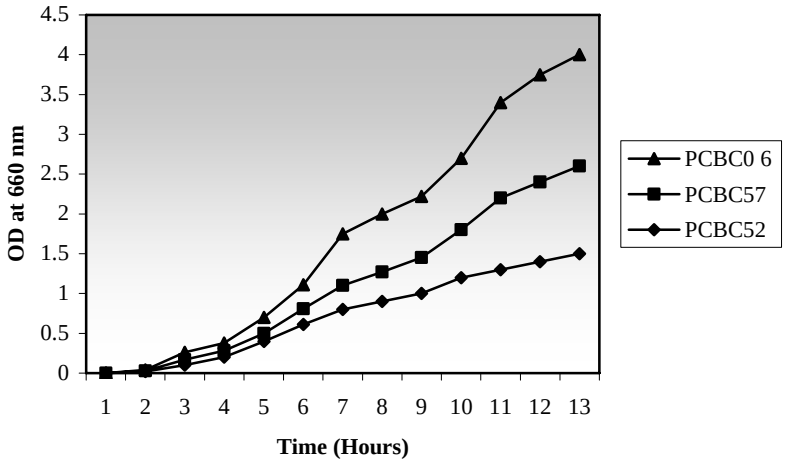

Figure 3: Growth pattern of the selected isolates of *B. cereus* at 37ºC

Table 4: Effect of temperature on the growth of *Bacillus* spp.

B. cereus	**Temperature in ºC**							
	40°C	50°C	60°C	70°C	80°C	90°C	100°C	Autoclaved at 121°C
PCBC - 06	+	+	+	+	+	+	+	–
PCBC - 52	+	+	+	+	+	+	+	–
PCBC - 57	+	+	+	+	+	+	R	–

+ good growth; – no growth; R- intermediate growth.

Table 5: Effect of various growth conditions on growth of B. cereus strains

Growth Conditions		OD at 660 nm		
		Test strains		
		B. cereus PCBC-06	*B. cereus* PCBC-52	*B. cereus* PCBC-57
pH	1.0	0.10	0.10	0.10
	2.0	0.20	0.22	0.20
	3.0	0.25	0.28	0.25
	4.0	0.29	0.35	0.28
	5.0	0.30	0.40	0.30
	6.0	0.35	0.45	0.34
	7.0	0.45	0.50	0.37
	8.0	0.44	0.48	0.36
	9.0	0.39	0.44	0.32
	10.0	0.37	0.39	0.30
Concentration of NaCl (%)	0.00	0.15	0.35	0.30
	0.25	0.20	0.25	0.28
	0.50	0.23	0.20	0.29
	0.75	0.45	0.15	0.18
	1.00	0.25	0.20	0.20
Concentration of glucose (%)	0.00	0.20	0.21	0.20
	0.25	0.30	0.40	0.23
	0.50	0.50	0.80	0.45
	0.75	0.45	0.60	0.40
	1.00	0.40	0.50	0.38

Heat resistance

Heat resistance experiments exhibited growth of all the strains at 5°C, 30°C, 37°C and 42°C whereas no growth at 2°C. Spore heat resistance analysis of *B. cereus* strains revealed the growth of spores exposed to 90°C for 3 min and 5 min. The spores that were exposed for 7 min and above lost their viability (Table 6). The spores that were treated for 5.40 min and above did not show any growth on JA plates that indicated the spore D_{90} value is from 5.40 to 5.50 min.

Table 6: Spore heat resistance analysis

B. cereus	Treatment of spores at 90ºC						
	Survival after time (min)						
	1.00	2.00	3.00	4.00	5.00	6.00	7.00
PCBC - 06	+	+	+	+	+	–	–
PCBC - 52	+	+	+	+	+	–	–
PCBC - 57	+	+	+	+	+	–	–

+ - survival of spores
– - death of spores

Protein studies

B. cereus PCBC-06 was selected for further analysis due to its profound hemolytic activity and growth pattern. Protein accumulation was directly proportional to the growth of *B. cereus* (Fig. 4). The protein from *B. cereus* PCBC-06 was extracted and was found to be 180 μg of per 100 μl of extracted sample.

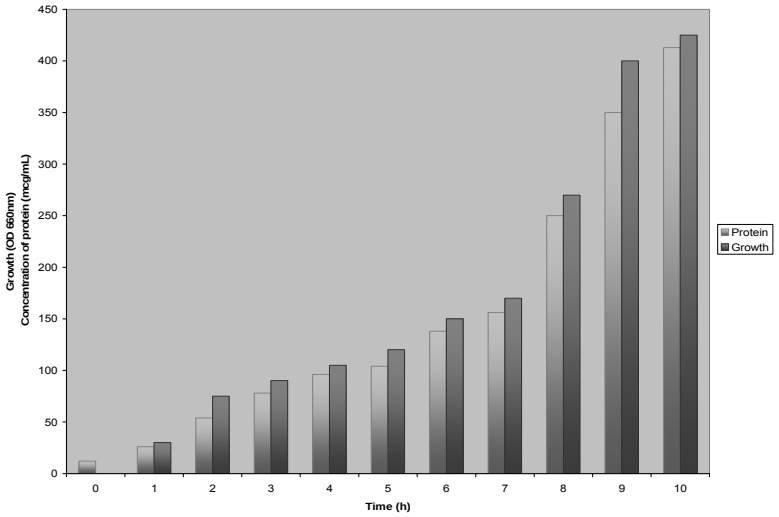

Figure 4: Growth and protein accumulation in *B. cereus* PCBC-06

Gel diffusion assay for hemolysin activity

The supernatant containing the hemolysin produced hemolysis on blood agar in 30 min, indicating the production of hemolysin by *B. cereus* PCBC-06. Discontinuous hemolytic pattern of hemolysin was observed (Fig. 5). The diameter of zone of hemolysis was 0.6 cm, 0.8 cm and 0.9 cm on 20 min, 65 min and 100 min respectively (Table 7).

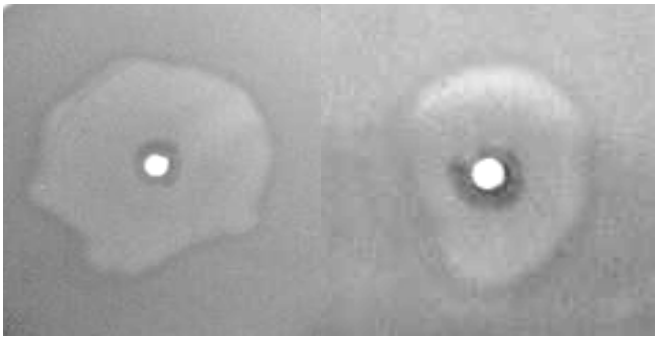

Figure 5: Hemolytic patterns produced by culture supernatants from cultures of *B. cereus* PCBC-06 in gel diffusion assay

Table7: Gel diffusion assay for activity of hemolysin from *B. cereus*

Time (min)	Diameter of zone of hemolysis
0	0.00
20	0.60
50	0.70
65	0.80
80	0.85
100	0.90

Partial purification and characterization of extracellular hemolysin from B. cereus

Partially purified hemolysin by ammonium sulphate precipitation produced clear zone of hemolysis around the point of application in 20 min on blood agar. The protein estimation revealed 44 µg/ml of sample. SDS-PAGE of the crude hemolysin showed four conspicuous bands with molecular weight of 70 000, 68 000, 57 000 and 43 000 (Fig. 6).

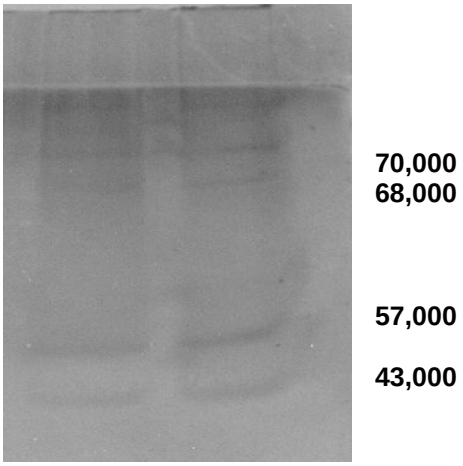

Figure 6: SDS-PAGE showing protein bands of hemolysin of *B. cereus*

DISCUSSION

Bacillus spp., including *B. cereus* are environmental microorganisms, present in a variety of foods. Among spore forming bacteria *B. cereus* has been responsible for food poisoning (Granum 1997) and frequently isolated from raw and processed food products such as rice, milk and dairy products, spices, vegetables (Roberts *et al*. 1982), meat products and farinaceous foods (Kramer & Gilbert 1989). A total number of 75 samples of food were collected and assessed for its microbial population with special reference to *Bacillus* spp. The aerobic plate count ranged from 1 000 to 29 000 bacteria per g/ml of the food sample. If those samples/foodstuff are held without proper storage or inadequate keeping temperature it may lead to severe consequences. The presumptive *Bacillus* count ranged from 2 500 to 16 000 bacteria per g/ml of sample. *B. cereus* was detected by Choma *et al*. (2000) in 20% of unstored vegetable purees pasteurized in their final package at less than 10 cfu/g. Hobbs and Gilbert (1974) have suggested that large numbers (≥ 10^5 cfu/g) of *B. cereus* are required to cause food poisoning. On the other hand, Dack *et al*. (1954) observed that the presence of large numbers of bacteria in food does not always result in illness.

On PPEMBA**,** peacock blue, yellow and white colonies were produced by *B. cereus, B. subtilis* and *B. megaterium* respectively. The population of *B. cereus* were determined after enumeration of the colonies having the characteristic appearance of *B. cereus* i.e., precipitation of hydrolyzed lecithin and failure to utilize mannitol (Lancette & Harmon 1980). After presumptive identification the isolates were characterized based on their morphological and biochemical properties. A provisional serotyping scheme was used by Gilbert and Parry (1977) to type cultures of *B. cereus* from 84 outbreaks of food poisoning in seven countries. Phylogenetic diversity of the genus *Bacillus* has been demonstrated by many researches on the basis of the 16S rRNA gene sequences (Rossler *et al.* 1991; Farrow *et al*. 1992; Ash *et al*. 1993; Nielsen *et al*.

1994; Suzuki & Yamasato 1994). By detailed morphological and biochemical characterization, 57 isolates of hemolytic *B. cereus* were obtained. Extracellular enzyme activity was performed and it was interesting to observe better hemolysis, lecithinase production, starch and casein hydrolysis by *B. cereus* PCBC-06 when compared to the other two strains. Kramer and Gilbert (1989) stated that emetic strains of *B. cereus* are usually unable to degrade starch. Identification of *B. cereus* was confirmed by catalase reaction and anaerobic growth (Claus & Berkeley 1986). To investigate bacteria resistant to antimicrobial drugs which penetrate into the human population with foods, antibiotic susceptibility test was conducted according to Bauer *et al*. (1966). This test was performed for two reasons. First, the population may represent a possible source of drug resistance for human pathogenic agents (Shryock 1999; Teuber *et al*. 1999) and second, to select the strains for further analysis. Based on the results of the susceptibility pattern strains PCBC-06, PCBC-52 and PCBC-57 were selected for further analyses.

As a preliminary investigation, effect of various microenvironments *viz*., temperature, pH, NaCl and glucose on growth of *B. cereus* strains were observed. The observation of heat resistance revealed that*, B. cereu*s PCBC-06 and PCBC-52 were highly resistant as they could be killed only after autoclaved at 121°C. Temperature affects the kinetics of all reactions taking within a sporulating cell. *Bacillus* is usually isolated from unheated or heated samples. Some species produce heat resistant spores and determined after heating for 30 min at 100°C (Franklin *et al*. 1956). The effect of temperature on the maximal specific growth rate was studied by Choma *et al*. (2000) in *B. cere*us between 5°C and 40°C cultivated in courgette broth and growth was observed below 10°C. The optimum pH for growth was between 7.0 and 9.0 for all the test strains. Cultures of *B. cereus* strain T, grown in an unbuffered glucose-yeast extract-mineral salt medium and in the same medium buffered at pH 6.4, 7.0, or 7.4, were examined by Nakata (1963). Differences between microorganisms in respect of their pH tolerance can be screened qualitatively by using pH gradient plates (Sacks 1956). Goepfert *et al*. (1973) stated that the range of pH permitting the growth of *B. cereus* in laboratory media to be 4.9 to 9.3. The optimum salt concentration for the strains *B. cereus* PCBC-06, *B. cereus* PCBC-56 and *B. cereus* PCBC-57 were 0.75%, 0.5% and 0.25% respectively. All the three strains had optimum requirement of 0.5% of glucose. The effects of NaCl, pH, and water activity on the ability of vegetative cells of *B. cereus* to initiate aerobic growth in brain heart infusion broth at 30°C were studied by Raevuori and Genigeorgis (1975) and they observed decreased growth rate of *B. cereus* occurs when exposed to media with NaCl concentration increasing from 0 to 10%. Brain-heart infusion medium, modified by 1.0% w/v glucose supplement, 1.0% w/v soluble starch supplement, pH adjustment to 8.8 or to 5.0, was used by Garcia-Arribas and Kramer (1990) to investigate the influence of glucose, starch and pH on growth, enterotoxin and hemolysin production by *B. cereus*.

The isolates were grown at various temperatures and the growth was also observed at 5°C proving the ability of the strain to thrive even during refrigeration. A *Bacillus* food poisoning episode usually occurs because spores still survive during cooking or pasteurization and then germinate and multiply

when the food is inadequately refrigerated. Anon (1991) reported that *B. cereus* should not be a hazard whenever refrigeration is properly maintained throughout the shelf life of the product. Choma *et al*. (2000) stated that bacteria were not detected on selective media in products stored at 4°C, as 4°C is recommended storage temperature for seeds, cereals, flour etc. Jaaskelainen *et al*. (2003) found cereulide, an emetic toxin, in products stored at refrigerator temperature (4–8°C) and was not inactivated by heating. Spore heat resistance was measured and the D value ranged somewhere between 5.50 min to 6.0 min. Hence, proper cooking is essential to destroy the spores present. Sporulation generally occurs rapidly and with highest spore yields at or near the optimum growth temperature, the percentage of sporulation being reduced by unfavorable growth temperatures (Murrell 1961). The heat resistance of spores is also influenced by their growth temperature. *B. cereus* spores had the heat resistance when produced at 30°C (Murrell & Warth 1965). However, other work (Lechovich & Ordal 1960; Murrell 1961; Roberts & Hitchins 1969; Warth 1978; Khoury *et al*. 1987) has shown increased heat resistance with higher sporulation temperature. More analyses have to be performed so that the nature of the isolates can further be explored. From this point, the investigation was preferentially narrowed down to *B. cereus* PCBC-06 due to its hemolysis, better growth pattern and antibiotic resistance.

Hemolysin BL (HBL) is a diarrheal enterotoxin produced by *Bacillus cereus*. HBL, a membrane-lytic system composed of three antigenically distinct proteins thought to contribute to diarrheal food poisoning and necrotizing infections. Separately, the HBL components are nontoxic, but when combined they exhibit a variety of toxic activities including hemolysis, cytotoxicity, vascular permeability, dermonecrosis, enterotoxicity, and ocular toxicity. When HBL diffuses from a bacterial colony or a well in blood agar, it produces an unusual discontinuous hemolysis pattern (Beecher & Wong 1997). In the Gel Diffusion assay for hemolysin activity, there was a comprehensible revelation of hemolysis by HBL within 20 min of incubation and a similar pattern of hemolysis was noticed. Hemolysin was obtained by dialysis membrane technique suggested by Murphy and Haque (1967) and was partially purified by ammonium sulphate precipitation. Cereolysin, a hemolytic toxin was purified by Cowell *et al*. (1976) to apparent homogeneity by using ammonium sulphate fractionation, hydrophobic chromatography with AH-Sepharose, isoelectric focusing and gel filtration. In the present study SDS-PAGE of the partially purified hemolysin displayed four distinct components with molecular weight of 20 5000, 57 400, 68 000, and 43 000. Thompson *et al.* (1984) and, Bitsaev and Ezepchuk (1987) reported a multicomponent diarrheal enterotoxin from *B. cereus*. In the prototype strain F837/76, HBL is composed of three antigenically distinct proteins designated B, L1, and L2 that have molecular weight of 37.5, 38.2, and 43.5 kDa, respectively. All three proteins are required for biological activity (Beecher & Wong 1994).

To conclude, hemolytic enterotoxin seems to be broadly distributed among strains of *B. cereus* group and relates neither to a specific strain nor to a specific environment. The detection of toxin production is useful in determining the significance of isolates. The gel diffusion assay of hemolysin can be a good detection method for *B. cereus* in food and it is evaluated for many strains of *B.*

cereus in food and other samples by Beecher and Wong (1994). The advantage of this technique is that samples can be stored frozen and assayed when desired. The consequences of microbiological findings for food safety considerations are yet to be evaluated.

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