Isolation and characterization of virulent bacteriophages infecting *Bacillus cereus*

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ABSTRACT

Bacillus cereus causes food borne illness with diarrheal and emetic syndromes and other non gastrointestinal infections. Bacteriophage is considered as an imperative biocontrol agent to eliminate the growth of deleterious microorganisms. In our study, B. cereus ATCC 33013 was used as a representative B. cereus for phage isolation. Four B. cereus phages (BCP_1, BCP_2, BCP_3 and BCP_4) were isolated from 62.5 % from tested clay soil samples. According to the phage morphology, three phages (BCP_1, BCP_2 and BCP_4) are belonging to Siphoviridae, but BCP_3 belongs to Myoviridae. The genome size of the four isolated phages ranged between 28.7 and 33.6 kbp. All phages had lytic activity against only Bacillus thuringiensis which was tested with other eight Bacillus spp. to determine the host range. SDS-PAGE analysis revealed that only one phage, BCP_3, had five protein bands with molecular weights ranging from 37.0 to 110.0 kDa. The effect of UV, temperature and pH on the lytic activity of BCP_1 was evaluated. BCP_1 retained 58.7% of activity after 75 min exposure to UV light. Conversely, it lost 72.5 and 18.1% of activity after LTLT and HTST pasteurization, respectively, and lost whole activity after boiling for 10, 20 and 30 min. BCP_1 was sensitive to extreme acidic and alkaline conditions as its activity was completely lost at pH 1.0, 3.0 and 13.0.

Keywords: *Bacillus cereus, Bacteriophage, Characterization, Phage stability*

INTRODUCTION

Bacillus cereus is a Gram-positive, facultative anaerobic and endospore-forming bacterial pathogen. Due to its ability to form endospores and to grow at a wide temperature range from 5.0 to 55.0°C, *B. cereus* is widely distributed in different environments (Shin *et al.*, 2011). This pathogen causes diarrheal and emetic syndrome. In 2011, the Centers for Disease and Control and Prevention (CDC) reported that *B. cereus* outbreaks represent 2.0 to 5.0% of food-borne diseases in the United States. *Bacillus cereus* is also associated with non-gastrointestinal tract infections as bacteremia, septicemia endocarditis, infection of the central nervous system and respiratory tract (Akesson *et al.*, 1991and Drobniewski, 1993).

B. cereus is generally resistant to beta-lactam antibiotics, and some strains are also resistant to erythromycin, tetracycline, and fluoroquinolones (Bottone, 2010 and Simm *et al.*, 2012). Organji *et al.* (2015) studied the incidence of toxigenic *B. cereus* in food in Egypt, especially in raw rice, raw milk, long life pasteurized milk, pasteurized milk, yoghurt and different infant milk powder formulas. They found that 31.8% of samples contained *Bacillus*-like growth. Of which

54.28% were positive for the presence of *B. cereus*, and all isolates were enterotoxin producers and resistant to penicillin G. All previous motives confirmed the importance of including *B. cereus* in disease control programs and search for alternative methods to control it. The harmless route to achieve the previous objectives is applying the bacteriophages as biocontrol agents against *B. cereus*. Also, the bacteriophages could be used in bacterial detection and serotyping (Petty *et al*., 2007). Bacteriophages are viruses that infect and lyse bacterial cells. They are defined as obligatory intracellular bacterial parasites and most of them are diverse in shape, size, host range, type of nucleic acid ……. etc.

The International Committee on the Taxonomy of Viruses (ICTV) used virus morphology and nucleic acid composition as a basis for the classification of phages into 13 families. Over 95.0% of all phages described in the literatures belong to the order *Caudovirales* as ds DNA phages (Ackermann, 2007). The aims of the present study were to isolate *B. cereus* phages from different sources under the Egyptian conditions, and to characterize them chemically and physically.

MATERIALS AND METHODS

Bacterial strain

Bacillus cereus ATCC 33013, obtained from Cairo University Research Park (CURP), Faculty of Agriculture, Cairo University, Giza, Egypt, was used for phage isolation from food and clay soil samples. Initially, the bacterium was tested for the presence of lysogenic phages (lysogeny state) through irradiation of exponentially growing cells with UV light with wavelength of 254.0 nm / 30.0 sec and 1 min. The irradiated cells were cultivated using tryptone soy agar (TSA) with incubation at 30.0°C / 24 h. The presence of lysogenic phages in the bacterial cells is evident by appearance of plaques. The culture was maintained on TSA at 4°C and transferred periodically.

Sampling

Twenty-seven food samples and eight clay soil samples collected from Giza governorate were screened for the presence of phages infecting *Bacillus cereus* ATCC 33013. The food samples comprised seventeen koshry (traditional Egyptian food), seven green salad and three mayonnaise samples.

Bacteriophage enrichment and isolation

To isolate *Bacillus cereus* ATCC 33013 phages, the enrichment technique was conducted. Ten g of each food and soil sample were added to fifty mL of tryptone soy broth (TSB) inoculated with five mL of log phase *B. cereus* broth culture, and incubated at 30°C / 24 h. with shaking at 100.0 rpm. Thereafter, the supernatant was collected by centrifugation at 6000.0 rpm / 15.0 min, followed by filter sterilization through membrane filter with 0.45 µm pore size. The filtrate was assessed for the presence of phages applying the spot test in which five µl of the filtrate were spotted on TSA layer inoculated with *Bacillus cereus* ATCC 33013. Appearance of plaques on the lawn of *B. cereus* was an indicator for the presence of lytic phages infecting *B. cereus*.

Plaque assay

Phage quantification was carried out via titration for all food and soil samples which yielded positive phage isolation. The bacteriophage titer, in the filtrate (phage lysate) prepared from samples containing *B. cereus* phages, was determined by double agar layer technique according to Adams (1959). Serial decimal dilutions of phage lysate were prepared in phosphate buffer. Definite volume of each dilution (0.5 mL) was mixed with 0.5 mL of freshly prepared *B. cereus* broth culture.

This mixture was added to 3.0 mL of molten semi-solid TSA and poured on pre-solidified TSA base layer. The plates were then incubated at 30.0°C / 24 h. After the incubation period, phage assay plates were observed for the presence of viral plaques. The plaque counts were calculated according to ISO 7218:2007 applying the following equation.

$$\sum P / 1.1 \times d \times v$$

where:
$\sum$**P** is the sum of plaques counted from 2 successive dilutions.
(at least one of which contains a minimum of 10 plaques).
v is the volume of diluted phage lysate added to the plate, in ml.
d is the dilution corresponding to the first dilution counted.
For each dilution, the average number of two plates was applied. The phage count is expressed as pfu/mL.

Phage purification

Single plaques observed on the overlays were picked up, suspended in *B. cereus* broth culture, incubated at 30.0°C / 24 h. with shaking at 100.0 rpm to release the phage from agar and finally centrifuged at 6000.0 rpm/15.0 min. The obtained supernatant was filtrated through 0.45µm pore size membrane filter to remove any bacterial cells. Pure phage suspension (phage lysate) was stored at 4.0°C and it was amplified on freshly prepared *B. cereus* broth culture for further experiments.

Transmission electron microscopy (TEM)

The pure, fresh and high titer phage lysate (10^8 pfu / mL) was deposited on carbon-coated copper grids. The phage particles were negatively stained by 2% (w/v) phosphotungstate at pH 7.2. The stained sample was examined microscopically by transmission electron microscope JEOL (JEM-1400 TEM) in Electron Microscopy Unit, CURP, Faculty of Agriculture, Cairo University, Giza, Egypt. Images were captured by CCD camera model AMT, Optronics camera with 1632 x 1632 pixel format as side mount configuration.

Host range determination

The isolated phages were tested against eight *Bacillus* spp. strains using the spot test to determine the host range. These strains included one strain of either *B. cereus* (local identified isolate), *B. thuringiensis* HD-250, *B. megaterium* B-3254, *B. amyloliquenfaciens* B-14393, *Paenibacillus polymyxa* B-4317, *Paenibacillus lentimorbus* B-2522 and two strains of *B. subtilis* (ATCC 6633 and B-14472). All strains were obtained from ARS Culture Collection (NRRL), USA. The plates were checked for plaque formation after the incubation at 30.0°C / 24 h. The presence of a clear zone of lysis on the lawn of tested bacteria was considered as an evidence of phage susceptibility.

DNA and protein analysis

The phage particles were partially purified applying the method of Sambrook and Russell (2001). A definite volume of phage lysate of 1200.0 µL was mixed with 300.0 µL of polyethylene glycol (PEG) / NaCl mixture (5X) and incubated at 4.0ºC overnight. The phage was pelleted by centrifugation for 3.0 min at 13000.0 g. The supernatant was removed and the centrifugation was repeated. The pellet was suspended in 120.0 µL of TBS (1X) with incubation in ice for 1 h. The clear phage suspension was collected by the centrifugation as mentioned previously. This purified phage suspension was used for DNA extraction and SDS-PAGE protein analysis. The phage nucleic acid was extracted and

purified according to the protocol of Thermo Scientific GeneJET Genomic DNA Purification Kit. Determination of phage genome size was performed by pulse-field electrophoresis using agarose gel containing 1.0% agarose, TEA buffer and ethidium bromide. The DNA was migrated for 1 hour at 80.0 volte. The images were captured using a fluorescence imaging system (GelDoc). The molecular weight of each separated band was determined using 1D gel electrophoresis image analysis software GelAnalyzer (GelAnalyzer 2010a program) downloaded from www. Java.com. To determine the phage structural proteins, a definite volume of the concentrated phage was mixed with the sample buffer (10.0% w/v SDS, 10.0 Mm Dithiothreitol, 20.0% v/v glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% w/v Bromophenol blue) in a ratio of 4:1 and heated at 95.0°C / 5.0-10.0 min. The phage proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.0% acrylamide and visualized by Coomassie Blue G-250 staining.

Factors affecting the stability of isolated phage

UV radiation

Five mL of the phage lysate were exposed to UV lamp with a wavelength of 254.0 nm / 15.0, 30.0, 45.0, 60.0 and 75.0 minutes at the ambient temperature. The survival of the tested phage was determined by double layer agar technique.

Temperature

High temperature (boiling and pasteurization)

The effect of different heat treatments, employed in food processing, on the phage survival was studied. The phage lysate was heated at 100ºC / 10.0, 20.0 and 30.0 min, 63.0ºC / 30.0 min (low temperature long time pasteurization, LTLT) and 72.0ºC / 15.0 sec. (high temperature short time pasteurization, HTST). The survival of the tested phage was determined by double layer agar technique.

Low temperature (refrigeration)

The lysate of *B. cereus* phage was stored at 4.0°C and the titer value was recorded at ten-day intervals for seven months using the double layer agar method.

pH

The pH survivability of bacteriophage was estimated through exposure of phage to different pH values *e.g.* 1.0, 3.0, 5.0, 7.0, 9.0, 11.0 and 13.0 with incubation at the ambient temperature for 1 h. To achieve this experiment, TSB was pre-adjusted to tested pH values by 1.0 M HCL or NaOH, then 1.0 mL of the bacteriophage lysate was inoculated into 9.0 mL of pre-adjusted TSB. The titer of surviving phages was measured by the overlay technique.

Statistical analysis

All experiments were conducted in triplicates for the statistical analysis. Data were statistically analyzed using factorial ANOVA test MSTAT-C Version 2.10, 1989. The differences between means were analyzed using the least significant difference test (LSD) at 0.01.

RESULTS AND DISCUSSION

Bacillus cereus ATCC 33013, that was not lysogenic, was used as an indicator to isolate *B. cereus* phages. This was confirmed through exposure of the bacterial cells to UV radiation. It is known that the stress of phages using UV light or mitomycin C can release the phage DNA from bacterial genome, resulting in phage replication and bacterial cell lysis (Coffey and Coleman, 2001).

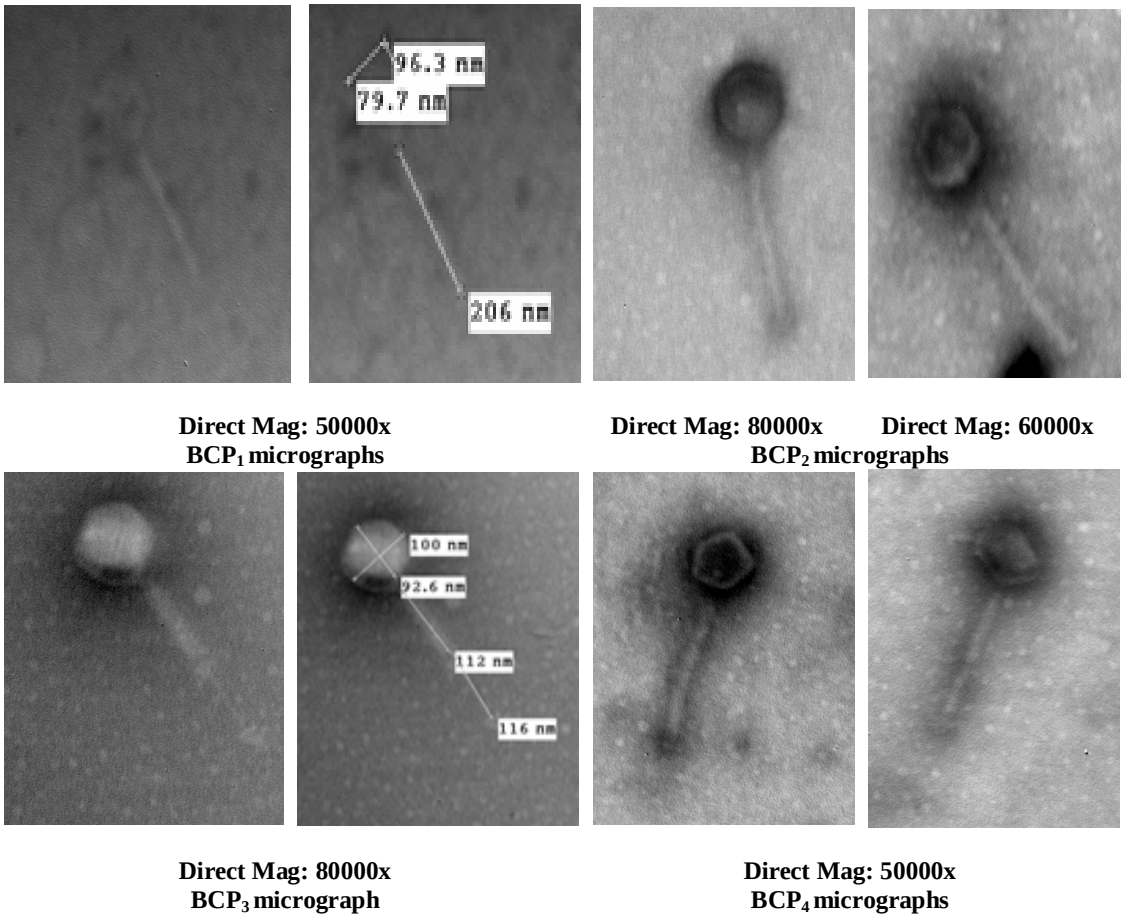
Isolation of *Bacillus cereus* phages

Eight clay soil samples and twenty-seven food samples including seventeen koshry, seven green salad and three mayonnaise samples were subjected to isolation of *B. cereus* phages. The results indicated the absence of *B. cereus* phages in all tested food samples. On the other hand, only five soil samples (62.5%) were revealed to contain bacteriophages. Some previous studies reported the presence of *B. cereus* phages in fermented foods. Shin *et al.* (2011), Kim *et al.* (2011) and Bandara *et al*. (2012) isolated the previous phage from Korean traditional fermented food samples as doenjang, gochujang and jangajji. Kong and Ryu (2015) isolated *Siphoviridae* virulent phage, PBC1, from sewage, and its lytic enzymes as antimicrobial agent against *B. cereus.* Also, Ji *et al*. (2015) could isolate a lytic cold-active bacteriophage against *Bacillus cereus* from Mingyong Glacier in China as an ecological system with low temperature and nutrient levels. Four bacteriophage isolates infecting *B. cereus* ATCC 33013 (BCP_1, BCP_2, BCP_3 and BCP_4) were selected based on the formation of single clear plaque, purified and propagated to obtain high titer phage lysate (10^8 pfu / mL).

Phage morphology

The morphological features of the isolated phages were identified using TEM (Fig. 1). Generally, the dark heads in TEM micrographs are considered as an evidence for losing the nucleic acid from the virions. The micrographs revealed that the isolated phages could be characterized by three morphological types which are:

a. Icosahedral capsid with hexagonal outlines with dimensions of 96.3 x 79.7 nm and 108.0 x 104.0 nm, and long non-contractile tail with a length of 206.0 and 271.0 nm for BCP_1 and BCP_2, respectively.
b. Icosahedral capsid with hexagonal outlines with dimensions of 100.0 x 92.6 nm, neck and contractile tail with a length of 116.0 nm for BCP_3.
c. Icosahedral capsid with pentagonal outlines with dimensions of 101.6 x 98.6 nm and long non-contractile tail with a length of 197.0 nm for BCP_4.



Direct Mag: 50000x
BCP_1 micrographs

Direct Mag: 80000x **Direct Mag: 60000x**
BCP_2 micrographs

Direct Mag: 80000x
BCP_3 micrograph

Direct Mag: 50000x
BCP_4 micrographs

Fig. (1): Transmission electron microscopy (TEM) micrographs of negatively stained B. cereus phage particles (BCP_1, BCP_2, BCP_3 and BCP_4).

The classification of bacteriophages depends principally on both morphological and molecular characterization. According to the morphology, 96% of bacteriophages belong to Caudovirales which is subdivided into three families. These families are *Myoviridae* (25%) characterized by icosahedral head and contractile tail with a neck and central tube, *Siphoviridae* (61%) with a noncontractile tail and flexible or rigid tubes, and *Podoviridae* (14.5%) with a short and noncontractile tail (Ackermann, 2007).

The morphological characterization suggests that the isolated phages could be considered as members of *Siphoviridae* family for BCP_1, BCP_2 and BCP_4 and *Myoviridae* family for BCP_3. Some previous studies reported the same morphological features for different *Bacillus* spp. phage isolates. Bandara *et al.* (2012) isolated two *B. cereus* phages with icosahedral head (95.0 nm in size) and long contractile tail (220.0 and 210.0 nm) from Korean traditional fermented food. El-Didamony (2014) isolated *B. thuringiensis* phages from clay soil in Yemen and classified these phages into two groups of tailed phages (*Siphoviridae* and *Podoviridae*). Krasowska *et al.* (2015) classified *B. subtilis* phages isolated

from soil to *Myoviridae* and *Siphoviridae*. Kong and Ryu (2015) isolated *B. cereus* phage characterized by isometric head with a diameter of 70 nm and a non-contractile tail with a length of 200 nm (*Siphoviridae*). The electron microscopy analysis performed by Ji *et al.* (2015) revealed that *B. cereus* phage isolated from glaciers has an icosahedral head (59.2 nm in length, 31.9 nm in width) and a tail (43.2 nm in length).

Host range determination

The four isolated phages exhibited very limited host spectrum, infecting only 1 of 8 *Bacillus* spp. The isolated phages can lyse *B. thuringiensis* HD-250 only. The other tested *Bacillus* spp. including *B. cereus* (local isolate), *B. megaterium* B-3254, *B. amyloliquenfaciens* B-14393, *Paenibacillus polymyxa* B-4317, *Paenibacillus lentimorbus* B-2522 and two strains of *B. subtilis* (ATCC 6633 and B-14472) were resistant to the isolated bacteriophages infecting *B. cereus*. These results showed that the tested phages may be specific to *Bacillus cereus* and *Bacillus thuringiensis* strains. As reported by Low *et al*. (2011), Son *et al*. (2012) and Kong and Ryu (2015), phages infecting *B. cereus* show high specificity toward *B. cereus*, whereas their endolysins have broad antimicrobial spectrum.

Analysis of phage DNA

Determination of molecular size of DNA extracted from the four isolated *B. cereus* phages applying PFGE indicated that the genome size ranged approximately between 28.7 kbp in BCP_3 and 33.6 kbp in BCP_1 (Fig. 2). In comparison to DNA marker (GeneRuler DNA Ladder Plus, Fermentas), the DNA extracted from BCP_2 and BCP_4 has a molecular size of 31.8 and 30.2 kbp, respectively. All the isolated *Bacillus* phages, in previous studies, possess dsDNA with a size ranged between 18.0 kbp and 158.0 kbp (Klumpp *et al*., 2010, Lee *et al*., 2011, Bandara *et al*., 2012 and El-Arabi *et al*., 2013).

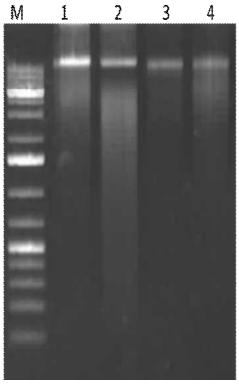


Fig. (2): Pulsed-field gel electrophoresis of phage DNAs. Lanes M-4: M, DNA marker; 1, BCP1 DNA; 2, BCP2 DNA; 3, BCP3 DNA; 4, BCP4 DNA.

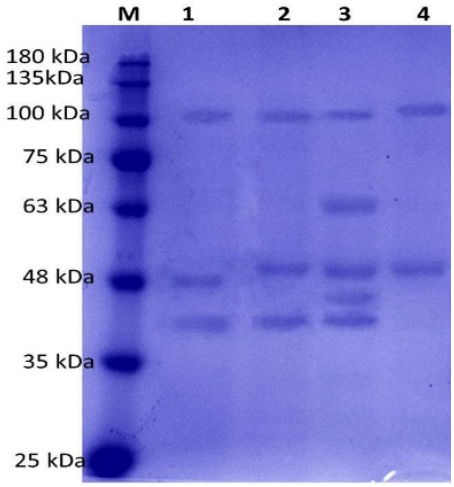


Fig. (3): SDS-PAGE analysis of phage structural proteins. Lanes M-4: M, protein marker; 1, BCP1 proteins; 2, BCP2 proteins; 3, BCP3 proteins; 4, BCP4 proteins.

Analysis of phage structural proteins

SDS-PAGE was applied to determine the structural proteins corresponding to the isolated phage particles (Fig. 3). A total of five proteins, with molecular weights ranging from 37.0 to 110.0 kDa, appeared in BCP_3 virions, followed by BCP_1 and BCP_2 which have three proteins ranged from 36.0 to 105.0 and from 37.0 to 121.0 kDa, respectively. Finally, the virions of BCP_4 have two proteins with molecular masses of 42.0 and 123.0 kDa.

The *B. cereus* phages isolated by Bandara *et al*.(2012) had three structural proteins with molecular weights of 50.0, 15.0 and 10.0 kDa. Also, Krasowska *et al*. (2015) found that the molecular weight of the structural proteins extracted from four isolated *B. subtilis* phages ranged between 31.0 and 52.0 kDa. One of these phages had fourteen structural proteins in three bands of 42.0, 37.0 and 31.0 kDa. The morphology and size of phage particles as well as the molecular weight of both DNA and structural proteins extracted from all purified *B. cereus* phages are summarized in Table (1).

Table (1): Morphology, size of phage particles and molecular weight of both DNA and structural proteins extracted from B. cereus phages.

Phage particle	Phage morphology	Head dimensions (nm)	Tail length (nm)	M.W. of DNA (kbp)	M. W. of structural protein (kDa)	M. W. of protein bands (kDa)				
						band 1	band 2	Band 3	band 4	band 5
BCP_1	Hexagonal head with non-contractile tail	96.3 x 79.7[1]	206.0	33.6	180.0	105.0	39.0	36.0	-	-
BCP_2	Hexagonal head with non-contractile tail	108.0 x104.0[2]	271.0	31.8	201.0	121.0	43.0	37.0	-	-
BCP_3	Hexagonal head with contractile tail	100.0 x 92.6[3]	116.0	28.7	280.0	110.0	54.0	41.0	38.0	37.0
BCP_4	Pentagonal head with non-contractile tail	101.6 x 98.6[4]	197.0	30.2	165.0	123.0	42.0	-	-	-

1,4, magnification power is 50000x 2,3, magnification power is 80000x

Factors affecting the stability of isolated phages

The *B. cereus* phage with the highest DNA molecular weight (BCP_1) was selected to study the effect of some stress conditions as UV radiation, high and low temperatures, acidic and alkaline pH on the phage survivability.

UV radiation

The UV phage stability was assessed after phage exposure to the UV light with a wavelength of 254.0 nm / 15.0, 30.0, 45.0, 60.0 and 75.0 minutes. Table (2) and Figure (4) show that the phage survival is inversely proportional with exposure time to UV. After 75 minutes, 41.29% of the treated virions were inactivated. The UV rays are known to be capable of disrupting phage DNA which can result in phage inactivation, but Kokjohn *et al.* (2005) suggested that some phages have the ability to reverse the DNA damage due to UV radiation by repairing of DNA lesions.

Temperature

The results of thermal stability test indicate that the *B. cereus* phage was sensitive to heating where the significant decrease of phage count was observed after all heat treatments (Table 3). The phage retained only 27.5% lytic competence after LTLT pasteurization at 63°C / 30 min. On the other hand, the phage lost its infection activity entirely after boiling for 10, 20 and 30 minutes.

Table (2): UV stability of B. cereus phage.

Time (minutes)	Bacteriophage survival log average ±S.D.	Bacteriophage reduction (%)
Control	10.40 ± 0.047^{A}	0.0
15	8.43 ± 0.35^{B}	18.86
30	8.0 ± 0.5^{B}	23.0
45	7.61 ± 0.55^{BC}	26.76
60	6.69 ± 0.17^{CD}	35.61
75	6.1 ± 0.44^{D}	41.29

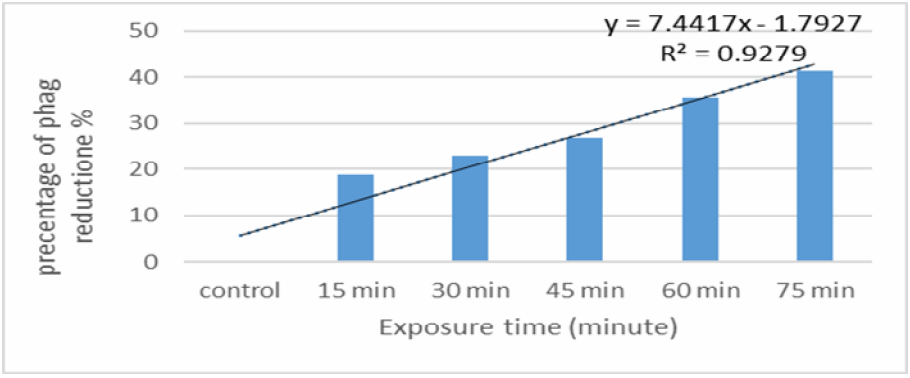


Fig. (4): Reduction percentage of Bacillus cereus bacteriophage irradiated with UV radiation at 254 nm.

Table (3): Thermal stability of B. cereus phage.

Time	Bacteriophage survival log average ±S.D.	Bacteriophage reduction (%)
Control	11.84 ± 0.13^{A}	0.0
72°C/15 sec	9.7 ± 0.15^{B}	18.1
63°C/30 min	3.26 ± 1.1^{C}	72.5
100ºC/10 min	0.0^{D}	100.0
100ºC/20 min	0.0^{D}	100.0
100ºC/30 min	0.0^{D}	100.0

As reported by Yates *et al*. (1985), Nasser and Oman (1999), Olson, *et al*. (2004) and Tey *et al*. (2009) the high temperatures play a critical role in bacteriophage survival, capacity for attachment, and the length of the latent period. Caldeira and Peabody (2007) proposed that the disulfide bonds between capsid proteins may be have a role in the protection of phages against thermal denaturation. They noticed the thermal loss of *Pseudomonas* phage PP7 activity after addition of 1,4-dithiothreitol as a reducing agent destroys the disulfide bonds. Cooney *et al*. (1975) reported that the *B. megaterium* ATCC 19213 phage has lost 99.8% of its activity after 5 minutes at 70°C. Ji *et al*. (2015) tested the thermolability of cold-active phage of *B. cereus* and they found that the phage could be tolerant with minimal activity loss at 20°C after 60 min. The four isolated *B. subtilis* phages by Krasowska *et al*. (2015) were rarely different in their thermal stability.

All phages lost their infective activity at 80°C, 70°C and 60°C after an exposure time ranged between 1 – 2 min, 5 – 30 min and 1 – 3h, respectively. The thermostability of other phages of G^{+} bacteria were studied. Buzrul *et al*. (2007) found that the 20.0% and 50.0% of *Lactococcus* spp. phages were inactivated at 72.0°C / 15 min and 90.0°C / 5.0 min, respectively. Also, Atamer *et al*. (2008) showed that approximately 60.0% of the *Lactococcus lactis* phages were inactivated by heating at 80.0°C / 5.0 min.The survivability of *B. cereus* phage under refrigeration conditions was tested at 4.0ºC. Every ten- day intervals for seven months, the titer value was determined (Fig. 5). Interestingly, the phage is

not affected significantly during the storage period. It was noticed that, along the storage time, the phage count has fluctuated between 8.42 ± 0.37 and 9.79 ± 0.55 $\log_{10}$.

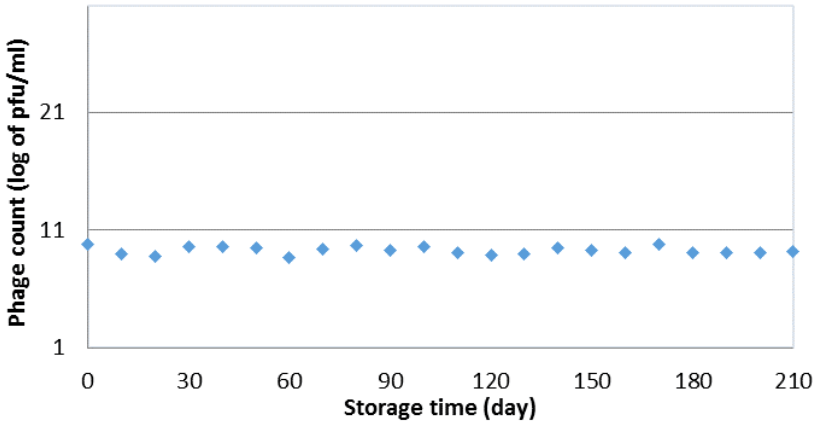


Fig. (5): Counts of Bacillus cereus phage during storage at 4º C for 7 months.

The temperature of bacteriophage storage is one of the most important factors to determine phage activity. Thorne and Holt (1974) showed that the *B. cereus* phage, CP-51, retained only 14.0 and 4.0% of its activity at 0.0°C after 7.0 and 24.0 hours, respectively. Also, they noticed that there was no activity loss after 2 weeks at 15.0°C suggesting that the optimum temperature for its stability was 15.0°C. Mullan (2001) recorded reduction in *Lactococcus* sp. phage titer by 5.0–10.0% after 6 months at 2.0–5.0°C. Some researchers suggested some recommendations for efficient phage storage. Warren and Hatch (1969) reported that the bacteriophage storage at −20.0°C is not recommended because the crystal structure of ice may cause their destruction, but addition of 5.0 – 10.0% glycerol to the phage suspension may maintain the viability and infectivity for 30 days at −20.0°C or −70.0°C (Olson *et al.* 2004). Ackermann *et al.* (2004) found that the lipid-containing phages were not stable after storage at 4.0°C but could be stored at −80°C or in liquid nitrogen.

pH

The phage ability to infect its bacterial host is also affected by the acidity and alkalinity of the environment. The pH stability of the *B. cereus* phage was evaluated at different pH values with incubation at the ambient temperature for 1 h (Table 4).

Table (4) : pH stability of B. cereus phage.

pH value	Bacteriophage survival log average ± S.D.	Bacteriophage reduction (%)
pH 1.0	0.0 ± 0.0^{D}	100.0
pH 3.0	0.0 ± 0.0^{D}	100.0
pH 5.0	8.82 ± 0.095^{B}	5.97
PH 7.0	9.38 ± 0.1^{A}	0.0
pH 9.0	8.72 ± 0.006^{B}	7.1
pH 11.0	5.0 ± 0.076^{C}	46.69
pH 13.0	0.0 ± 0.0^{D}	100.0

The effect of pH on the lytic capacity of the phage was statistically significant. The *B. cereus* phage appeared to be extremely unstable at very terrible acidic and alkaline environments as its infection ability was lost completely at pH 1.0, 3.0 and 13.0. Alternatively, the phage retained 94.03% lytic activity at pH 5.0. At alkaline pH, the pH 11.0 had influential effect compared to pH 9.0. The phage retained 53.31 and 92.2% activity at pH 11.0 and 9.0, respectively. These results propose that the tested phage is resistant comparatively to the effect of alkaline pH compared to the acidic pH.

The bacteriophages may persist in an acidic or alkaline environment as described by some researchers. Thorne and Holt (1974) observed that a change in pH of the suspending medium for *B. cereus* CP-51 phage influenced its stability at 0°C. The optimal pH was 5.6, after 30 min of incubation, the phage titer decreased by only 11.0%, whereas at pH 7.0, it retained only 1.0% of the initial PFU (10^7). These observations indicate that the change in environmental pH may protect phage activity at a low temperature. The maximum stability of the cold-active *B. cereus* phage was observed at pH 8.0 and it was comparatively stable at pH 5.0–9.0 (Ji *et al*., 2015). Krasowska *et al*. (2015) assessed the pH stability of the four isolated *B. subtilis* phages after an incubation periods of 1 and 6 hours at the room temperature.

The optimum pH values for all phages were 7.0 and 8.0. Two phages are the most resistant to acidic pH of 4.0 and alkaline ones of 9.0 and 10.0. One phage was the most sensitive to acidic and alkaline conditions. One phage was extremely stable in the pH range of 6.0–8.0. Wunsche (1989) reported that the pH has an effect on the adsorption rate of phages on the host cell through the alteration of the charge of the protein capsid. Generally, the phage occurrence and lytic activity are affected by different physical and chemical factors, such as temperature, acidity, alkalinity, salinity, and light. The effects of these factors include damage of its structure, lipid loss, and DNA denaturation (Ackermann *et al*. 2004). Some authors stated that there is a relation between a phage morphology and its survival abilities. Phages with tail or large capsid with a diameter of 100.0 nm are more persistent to the unsuitable external conditions (Feng *et al*. 2003, Ackermann *et al*. 2004, Faquet *et al*. 2005, Prigent *et al*. 2005, Lin *et al*. 2010 and Jończyk *et al*., 2011).

Lasobras *et al*. (1997) suggested that phages belonged to the *Siphoviridae* family are the most resistant to adverse conditions. On the other hand, Prigent *et al*. (2005) suggested that the phages of *Myoviridae* family can protect themselves from unsuitable environment, as strong ultraviolet light, desiccation, and large temperature fluctuations, through intercellular location in pseudolysogens or confinement in the biofilm

created by bacterial hosts. Finally, there is no obvious systemic comparative studies to confirm this hypothesis.

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الملخص العربي

عزل وتوصيف الفيروسات البكتيرية التي تصيب *Bacillus cereus*

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تعد بكتيريا *Bacillus cereus* سببا للأمراض التي تنقلها الأغذية والتي من أعراضها الإسهال والقيء، كما أنها أيضا مسئولة عن بعض الأمراض غير المرتبطة بالجهاز الهضمي. يعتبر البكتريوفاج (فيروس البكتيريا) أحد عوامل المقاومة الحيوية الهامة للحد من نمو الكائنات الحية الدقيقة الضارة. في هذه الدراسة تم أستخدام *Bacillus cereus* ATCC 33013 كعائل لعزل الفيروسات البكتيرية المتخصصة التي تصيب *Bacillus cereus* وتم عزل أربع فيروسات بكتيرية (BCP_1-BCP_2-BCP_3-BCP_4) من ٦٢.٥% من عينات التربة الطينية المختبرة و بناءا على الشكل الخارجي للفيروسات وجد أن كلا من BCP_1-BCP_2-BCP_4 تنتمي إلى عائلة *Siphoviridae* بينما ينتمي BCP_3 لعائلة *Myoviridae*، كما يتراوح حجم الجينوم للفيروسات الأربع ما بين ٢٨٠٠٠.٧ و٣٣٠٠٠.٦ زوج قاعدة. كما أنها لديها القدرة على إصابة *Bacillus thuringiensis* والتي أستخدمت مع ثماني سلالات أخري تابعة لجنس *Bacillus* لأختبار المدي العوائلي. أوضح التفريد الكهربي SDS-PAGE أن BCP_3 يحتوي على خمس أنواع من البروتينات يتراوح الوزن الجزيئي لها ما بين ٣٧ و١١٠ كيلو دالتون. تم دراسة تأثير كلا من الحرارة والأشعة فوق البنفسجية والأس الهيدروجيني على نشاط فيروس BCP_1 ووجد أنه يحتفظ بـ ٥٨.٧% من حيويته بعد التعرض لمدة ٧٥ دقيقة للأشعة فوق البنفسجية وعلى الجانب الآخر فإنه يفقد ٧٢.٥ و١٨.١٪ من حيويته بعد تعرضه للبسترة البطيئة والسريعة على التوالي، كما يفقد نشاطه كاملا عند تعرضه لدرجة حرارة الغليان لمدة عشر أو عشرين أو ثلاثين دقيقة. كما وجد أنه يتأثر بدرجات الحموضة والقلوية المرتفعة حيث يفقد حيويته تماما عند درجات حموضة ١.٠، ٣.٠، ١٣.٠.