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Research Paper

Stability and infectivity of bacteriophages under different environmental conditions

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Abstract

Now a days, 80 % of the water resources have been polluted with various kinds of wastes, viz., domestic, industrial and agricultural wastes that includes salts, sugars, detergents, metals, agrochemicals especially pesticides etc. These pollutants may have an adverse impact on the microbial communities in the water bodies. Viruese of bacteria i.e. bacteriophages play a crucial role in maintaining ecological balance of the ecosystem. Pollutants present in the water may have direct effect on the stability and infectivity properties of such viruses present in the water environment. The main objective of the present work was to study the effect of chemical pollutants on stability and infectivity on phage particles in the natural environmental conditions. Stability of lytic9able to destroy its host bacterium) phages of *Salmonella Paratyphi B, Pseudomonas aeruginosa and Klebsiella pneumoniae* (viz., φSPB, BVPaP-3 and KPP respectively) was studied by exposing respective phage particle to various chemical factors for 1h and then determining remaining active phage particles by double agar layer plaque assay technique. Infectivity of φSPB, BVPaP-3 and KPP was studied at the sub-inhibitory concentration (SIC) values of chemical pollutants for the respective bacterial pathogen The infectivity of phage was determined in terms of number of plaque forming units after 24 h at 37 ˚C. They are found sensitive to SDS and Cetrimide but resistant to Tween 80 with 100% stability. Phages are found resistant to pesticide like Mancozeb but sensitive to Malathion. Zn^{2+}, Co^{2+}, Cd^{2+} exhibited stimulatory effect on stability of these phages. Phages viz., φSPB, BVPaP-3 and KPP could not able to infect their hosts in the presence of SDS and Cetrimide but in presence of Tween 80 infectivity was 100%. At lower concentrations of metals (Zn^{2+}, Co^{2+}, Cd^{2+}), infectivity was almost 100 %. However, this particular study could be helpful in the use of φSPB, BVPaP-3 and KPP phages as biocontrol agent in water bodies against life threatening pathogens in the future.

Keywords: detergents, *Klebsiella pneumonia*, pesticides, plaque forming units, *Pseudomonas aeruginosa, Salmonella Paratyphi* B.

Introduction

Microbial ecosystems consist of a variety of microorganisms that include algae, fungi, protozoa, bacteria, nematodes and viruses. These microorganisms interact with the abiotic factors in the system and amongst themselves. Therefore, such microbial ecosystems play an important role in maintaining ecological balance in the environment. Viruses are the obligate intracellular parasites that are present in the natural environment abundantly. These viruses are of three types viz., plant viruses, animal viruses and bacterial viruses (bacteriophages). There are very few fundamental differences among the viruses infecting plants, animals and bacteria [1]. The detailed study of bacterial viruses offers as easy introduction to basic virology.

Bacteriophages are the viruses of prokaryotes, which can either instantly kill a bacterial cell or integrate its DNA into the host genome [2]. Bacteriophages i.e. eaters of bacteria are found abundantly

in the biosphere. Amongst the predator community, they are the most prominent predators in the ecosystem. Bacterial viruses are the most numerous organisms on the earth surface therefore they can be easily isolated from a variety of the natural sources, including water, sewage, intestinal contents, vegetables, and some insects such as cockroaches and flies.

In the natural water systems bacterial viruses are likely to get affected by various physico-chemical and biological factors. The factors that are likely to affect phages are water, temperature, hydrostatic pressure, radiation, ionic environment, oxygen, pH, organic matter and host availability [3]. Dangerous chemicals, detergents, toxic metals, pesticides, organic matter are regularly discharged in the main water systems [4] .These chemicals can interact with the phages and may have adverse effects on the phage structures. These chemicals may interact with phages and can decrease their survivability and their ability to infect their hosts. Therefore, in-vitro study of effect of environmental factors on stability and infectivity of the phages is very essential. Viruses can survive in the aquatic systems for more than 500 days [5], but there could be many factors in the environment that can reduce the stability and the infectivity of many viruses. Second important fact is that multidrug resistant pathogenic bacterial strains are ever emerging in the nature, therefore in future, to overcome these problems, bacteriophages could be the alternative option in the near future.

In the present study, phages specific to *Salmonella entrrica* serovar Paratyphi B, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were isolated from the natural habitats and in-vitro study of effect of environmental factors such as detergents, pesticides, and metals on the stability and infectivity of phages was studied in detail.

Materials and Methods

River water samples were used for the isolation of bacterial hosts, viz., *Salmonella entrrica* serovar Paratyphi B, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and their specific viruses (bacteriophages). Each water sample was divided into two equal parts. One part was subjected to the isolation of above mentioned pathogens. The second part was subjected to the isolation, of phages against these bacterial pathogens.

Isolation of bacterial pathogens

Bacterial pathogens were isolated from the water sample by standard microbiological methods as described by Ahiwale et al [6].

Isolation of bacteriophages

Bacteriophages specific for *Salmonella entrrica* serovar Paratyphi B, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were isolated from the river water by standard double agar layer plaque assay technique as described earlier by Ahiwale et al [6].

Enrichment of phages

Well isolated single, clear plaques of the respective lytic phages obtained on the nutrient agar plate were transferred into sterile flasks (200 ml) containing phage broth separately along with the mid log phage culture (0.5 ml) of the for *Salmonella entrrica* serovar Paratyphi B, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* respectively.Flasks were incubated at 37˚C for 24 h. Then, contents of the flasks were treated with 1:50 diluted chloroform, shaked vigorously and then incubated at 8-10˚C for 16-18 h. Then the aqueous layer was centrifuged (10,000 rpm, 20 min, 4˚C).Then then the supernatant was filtered through membrane filter of pore size 0.20 µm (diameter 47 mm). Bacteriophage titre in the filtrate (lysate) was determined by double agar layer (DAL) plaque assay technique. Hereafter phages of *Salmonella entrrica* serovar Paratyphi B, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are designated as φSPB, BVPaP-3 and KPP respectively.

Electron micrograph of phage

Bacteriophage particles (φSPB, BVPaP-3 and KPP) were sedimented at 25,000 × g for 60 min using a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, CA). Phages were washed twice in 0.1 Molar ammonium acetate buffer (pH 7.0), stained with 2 % phosphotungstate (pH 7.2) solution,

deposited on carbon-coated Formvar films and examined under a Philips EM 300 electron microscope.

Stability of phages

The effect of varied concentrations of different environmental factors on the stability of phages was studied. An aliquot (1ml) of lysate (φSPB, 2.4 × 108 pfu/ml, BVPaP-3, 6.6 × 109 pfu/ml and KPP 1× 109 pfu/ml) was exposed to various environmental factors viz., pesticides, detergents and metals in tubes. Tubes were incubated at room temperature for 1 h. Contents of the tubes were serially diluted and pfu (plaque forming units) in each tube were determined by double agar layer plaque assay with the host bacteria viz., *Salmonella paratyphi* B, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in which, the mid –log phase culture (0.5 ml) of the respective host bacterium is mixed with the respective lysate (0.1 ml) in (4 ml) soft agar (0.6 % w/v) and then poured on the sterile nutrient agar medium. Plates were incubated at 37 ˚C for 24 h .After incubation, plaques were counted and recorded. A tube with (1 ml) SM buffer and the respective phage lysate (1 ml) was kept as a control in determining the effects of pesticides, detergents and metal effect.

Determination of stability of bacteriophages at varying concentrations of pesticides

Filter sterilized, varied concentrations of Malathion (1:10 – 1:500 v/v) and Mancozeb (2 -20 µg /ml) in sterile distilled water were taken (1 ml) in sterile screw capped tubes.

Determination of the stability of bacteriophages at varying concentrations of detergents (anionic, cationic and nonionic)

Filter sterilized (0.45 µm), varied concentrations of SDS (0.01 -1 % w/v), Cetrimide (0.01 -1 % w/v) and Tween 80 (2:0.5 -2:50000 v/v) were used for the study.

Determination of stability of bacteriophages at varying concentrations of metals

Filter sterilized, varying concentrations (0.01 – 10 mM) of various metals solutions viz., $ZnCl_2$, $COCl_2$, $CdCl_2$ and $HgCl_2$ (0.01 – 10 mM) were dispensed separately into sterile screw capped tubes with an aliquot of the respective lysates (1 ml) were mixed separately. Stability of φSPB, BVPaP-3 and KPP was evaluated at shaking conditions (50 -500 rpm). Lysate of the respective bacteriophages (φSPB, 2.4 × 108 pfu/ml, BVPaP-3, 6.6 × 109 pfu/ml and KPP, 1× 109 pfu/ml) was dispensed (50 ml) into sterile flask. These flasks were incubated at 37 ˚C at different shaking conditions (50 – 500 rpm) in the incubator shaker for 1 h. Three flasks with the respective lysates (50 ml) were kept at stationary conditions in the incubator at 37 ˚C for 1 h. After incubation, the respective lysates from each flasks (both at shaking and stationary conditions) were serially diluted in sterile SM buffer and then plaque assay was performed as described above using the mid – log phage culture suspension of the respective host.

Effect of varied environmental factors on infectivity of phages

Environmental factors may have positive effect on the infection process at low concentration but at higher concentration may exert an adverse effect on the structure of bacteriophage that may lead to the loss of infectivity (ability to infect host cell).Factors viz., glucose, salt, metals may have stimulatory effects on the infection process, whereas detergents, pesticides may have inhibitory effects.

Determination of MIC (Minimum inhibitory concentration) and SIC (Sub-minimal Inhibitory Concentration) values of environmental factors for pathogens

To study, the ability of phages to infect its host, the Sub-Inhibitory Concentration (SIC) of each pollutant for *Salmonella paratyphi* B, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were determined. The Minimum-inhibitory Concentrations (MIC) of each pollutant for the above mentioned pathogens was determined in order to know the SIC value of each pollutant. Effect of various concentrations of solutes (sugar and salt) on the growth properties of the above mentioned pathogens were also evaluated.

Determination of MIC and SIC of detergents for pathogens

In this study, three different types of detergents were used. viz., Sodium Dodecyl Sulphate (anionic), Cetrimide (cationic) and Tween 80 (nonionic). Different concentrations of SDS (0.01 – 1 % w/v), Cetrimide (0.01 -0.5 % w/v) and Tween 80 (2:0.5 - 2:50,000 v/v) in sterile phage broth (9ml) tubes were taken and were inoculated with the respective pathogen. Tubes were incubated at 37 ˚C for 24 h and then observed for the growth in terms of turbidity. One loopful mixture from each concentration was streaked on sterile nutrient agar medium. Plates were then observed for growth after incubation and then results were recorded.

Determination of MIC and SIC of pesticides for pathogens

Pesticides viz., Melathion and Mancozeb were used in this study. Sterile phage broth in tubes (9 ml) containing varying concentrations of Melathion (1:10 -1:10000 v/v) and with varying concentrations of Mancozeb (1 -1000 µg/ml) separately and then inoculated with the mid – log phage culture suspension of the respective pathogen. After incubation at 37˚C, tubes were observed for the growth in terms of the turbidity. After that the mixture from each concentration of Melathion and Mancozeb was streaked on sterile nutrient agar medium. Then plates were observed for growth after 24 h at 37˚C, and then results were recorded.

Determination of MIC and SIC of Metals

Different metals in the form of salts viz., $ZnCl_2$, $COCl_2$, $CdCl_2$ and $HgCl_2$ were used. Varying concentrations of metals (0.01 -10 mM) were added to sterile phage broth tubes. The mid –log phase culture suspension of the respective pathogen was inoculated in phage broth medium containing different concentrations of metals. Tubes were then incubated at 37˚C for 24 h and observed for growth. A loopful of the mixture from each concentration was streaked on sterile nutrient agar medium. After incubation, results were recorded.

The infectivity of the phages was studied at SIC values of the respective environmental factors viz., sugar, salt, detergent, pesticides and metals for the respective host pathogen. To know the SIC values of the pollutants, it is very essential to determine the MIC values of the pollutants. Sterile nutrient agar medium with SIC and value less than that of the respective environmental factor was prepared. The mid–log phage culture (5 h) of the respective bacterial suspension (0.5 ml) was mixed with the respective lysates (0.1 ml) in a sterile tube (4 ml) containing soft agar (0.6 % w/v) and then plated onto sterile nutrient agar medium plates containing these factors. Plates were incubated at 37 ˚C for 24 h and then were observed for plaques. Plaques on sterile nutrient agar medium without the factor were considered as a positive control.

Effect of varied environmental factors on infectivity of phages

Environmental factors may have positive effect on the infection process at low concentration but at higher concentration may exert an adverse effect on the structure of bacteriophage that may lead to the loss of infectivity (ability to infect host cell).Factors like metals may have stimulatory effects on the infection process, whereas detergents, pesticides may have inhibitory effects.

Various environmental factors viz., detergents, pesticides, and metals were selected to study the infectivity of φSPB, BVPaP-3 and KPP.

Infectivity of phages

The infectivity of phages was studied at SIC values of the respective environmental factors viz., detergent, pesticides and metals for the respective host pathogen. To know the SIC values of the pollutants, it is very essential to determine the MIC values of the pollutants. Sterile nutrient agar medium with SIC and value less than that of the respective environmental factor was prepared. The mid–log phage culture (5 h) of the respective bacterial suspension (0.5 ml) was mixed with the respective lysates (0.1 ml) in a sterile tube (4 ml) containing soft agar (0.6 % w/v) and then plated onto sterile nutrient agar medium plates containing these factors. Plates were incubated at 37 ˚C for 24 h and then were observed for plaques. Plaques on sterile nutrient agar medium without the factor were considered as a positive control.

Effect of varying concentrations of detergents on the infectivity of phages

Filter sterilized, varied concentrations of SDS (0.01 -1 %), Cetrimide (0.01 -1 %) and Tween 80 (2:0.5 -2:50000 v/v) were mixed separately in sterile nutrient agar medium plates.

Effect of varied concentrations of pesticides on the infectivity of phages

The effect of varied pesticide concentrations on infectivity of φSPB, BVPaP-3 and KPP was studied. Sterile nutrient agar plates were prepared that were incorporated with filter sterilized, varied concentrations of Melathion (1:10 – 1:500 v/v) and Mancozeb (1 – 20 µg /ml).

Effect of varying concentrations of metals on infectivity of phages

Filter sterilized, varying concentrations (0.01 – 10 Mm) of different metals viz., $ZnCl_2$, $COCl_2$, $CdCl_2$ and $HgCl_2$ (metal stock solutions – 10 mM) were incorporated into sterile nutrient agar medium separately for the study.

Results and Discussion

Isolation and identification of bacterial pathogens and their specific phages

Bacterial Pathogens those isolated from river water were identified based on cultural, morphological and biochemical characteristics as Salmonella Paratyphi B, Pseudomonas aeruginosa and Klebsiella pneumoniae using Bergy's manual [7] of determinative bacteriology (data not shown). Bacteriophages specific for these pathogens were lytic nature as shown in Figure 1 (a).Figure 1a indicates lytic nature of phages as φSPB, BVPaP-3 and KPP gave clear plaques on the plates. Electron micrograph study Figure 1 (b) revealed phages as tailed phages. φSPB and BVPaP-3 phages belonged to Podoviridae family and KPP phage belonged to Myoviridae family.

Bacteriophages are omnipresent and there are numerous types of phages appear in the environment. The concentration of viral particles in both fresh and sea water systems varies from 103 to 107 per ml [8]. Many viruses of humans and animals are released in faeces reach the water system and as a result of their known resistance to the natural inactivation or water treatment, they survive better [9,10]. The levels of enteric viruses that are excreted from the infected people are 1010 infectious particles per gram of faeces. Since the late 1980, bacteriophages have been considered as reliable indicators of viral pollution of drinking water by faeces or sewage [11] because bacteriophages closely resemble human viruses and are found to be highly resistant to environmental stresses. There could be many factors that affect the number and behavior of phages in the water environments. Viz., the density of host and phages, the association of phages and bacteria with the solids, organic matter content (especially, organic matter that influences the metabolic activity of the host bacteria), pH, temperature, concentration and type of ions, preys other than the host bacteria, prey-predator ratio, competition between microorganisms for nutrients [12,13] .

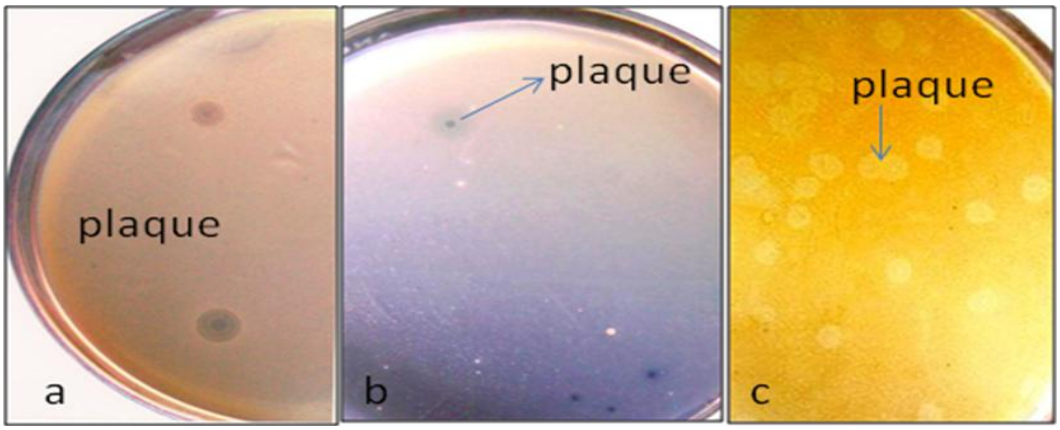

Figure 1a: Plaque nature of (a), φSPB, (b), BVPaP-3 and (c), KPP

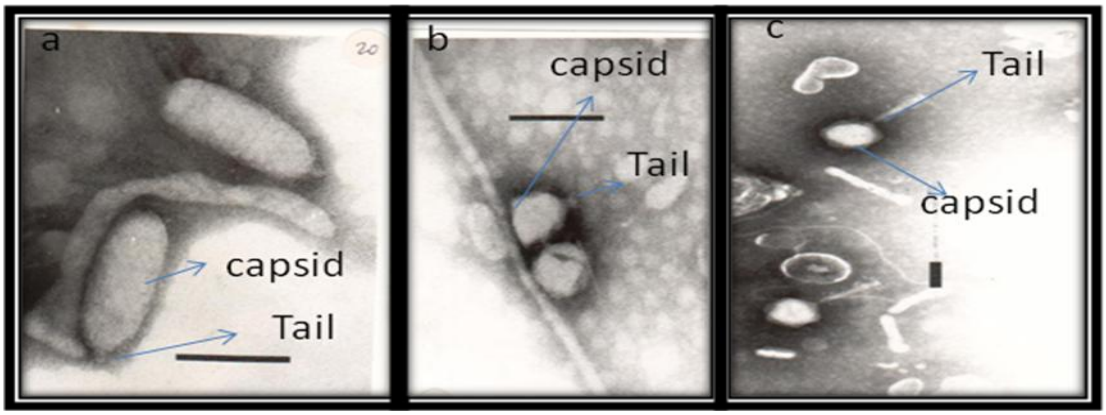

Figure 1b: Electron micrograph of (a), φSPB, (b), BVPaP-3 and (c), KPP

Effect of SDS on stability

In case of φSPB, only 25 % phages were stable at 0.01 to 0.05 % SDS concentrations, beyond that (1 to 6 %), stability was decreased to 17 % (Figure 2 a). BVPaP-3 was stable in the range of 10 to 20 % at all concentrations of SDS (Figure 2 b). Stability of KPP was also quite low, in the range of 10 – 25 % at varying concentrations (0.01 to 0.6 %) of SDS (Figure 2 c).

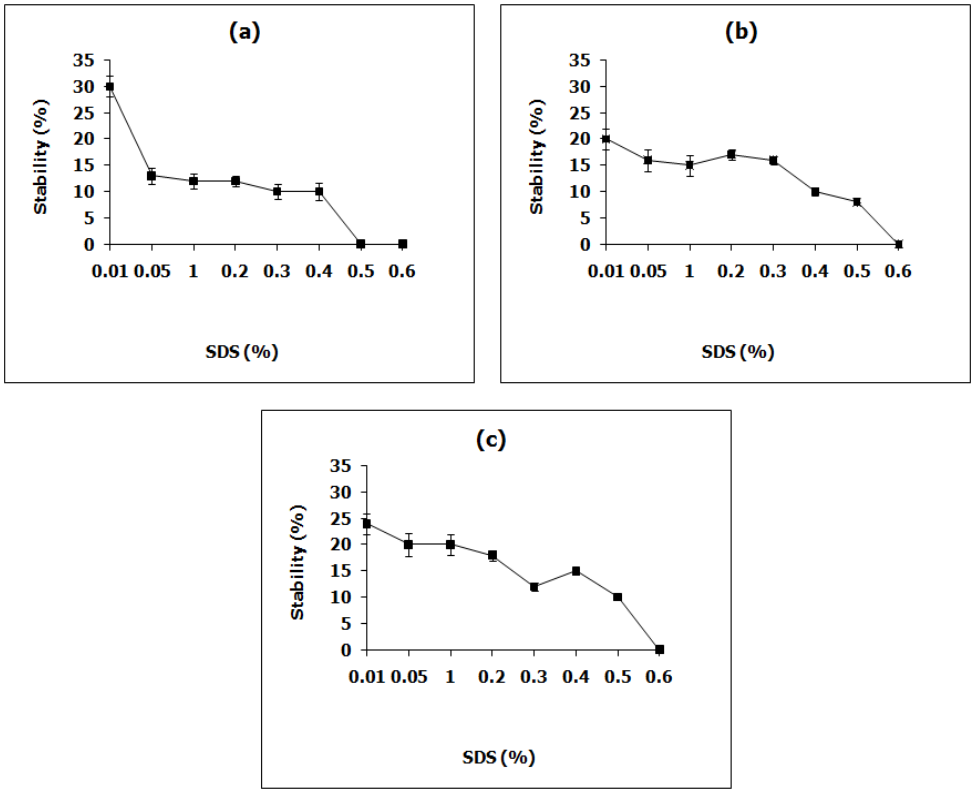

Figure 2: Effect of SDS on stability of (a), φSPB, (b), BVPaP-3 and (c), KPP % stability: N/N0 × 100, where N is the number of viable phages after 1 h of incubation at varying concentrations of SDS and N0 is the initial number of phages

Effect of Cetrimide on stability

In case of φSPB, only 25 % stability was recorded at 0.2 % Cetrimide. At rest of the concentrations, stability was only 17 %(3 a). BVPaP-3 was stable (35 %) at 0.01 % of Cetrimide. For rest of the concentrations stability was 15 % (3 b). KPP has optimal stability (35 %) at 0.01 % of Cetrimide, and then stability was decreased (15 %) as the concentrations were increased upto 0.6 % (3 c)

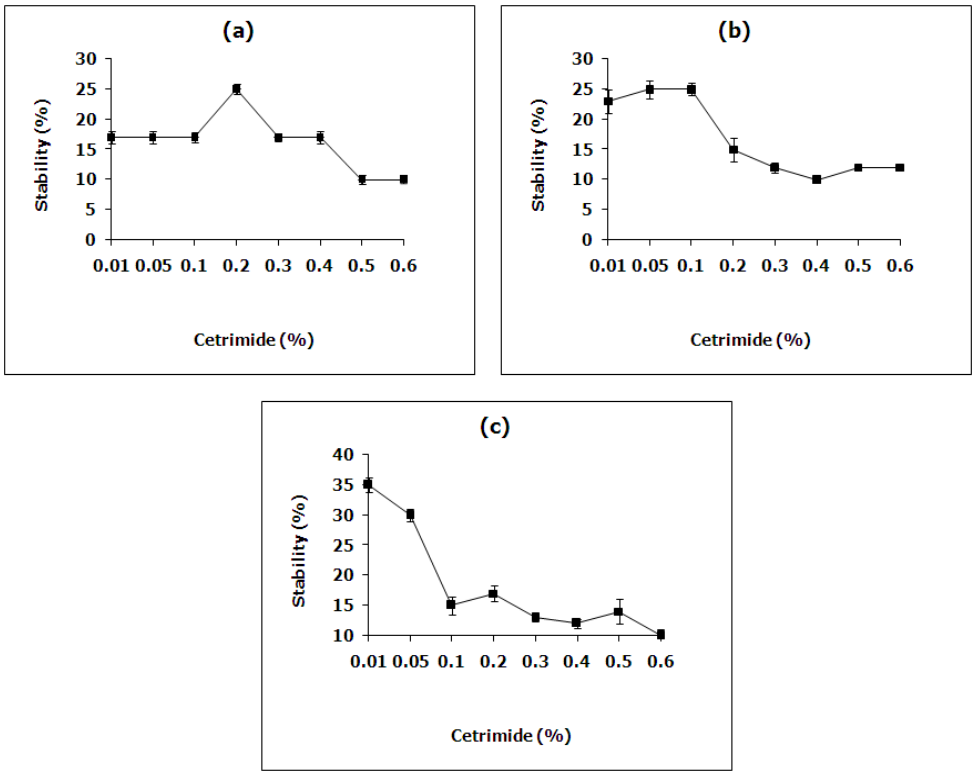

Figure 3: Effect of Cetrimide on stability of (a) φSPB, (b), BVPaP-3 and (c), KPP % stability: N/N0 × 100, where N is the number of viable phages after 1 h of incubation at varying concentrations of Cetrimide and N0 is the initial number of phages

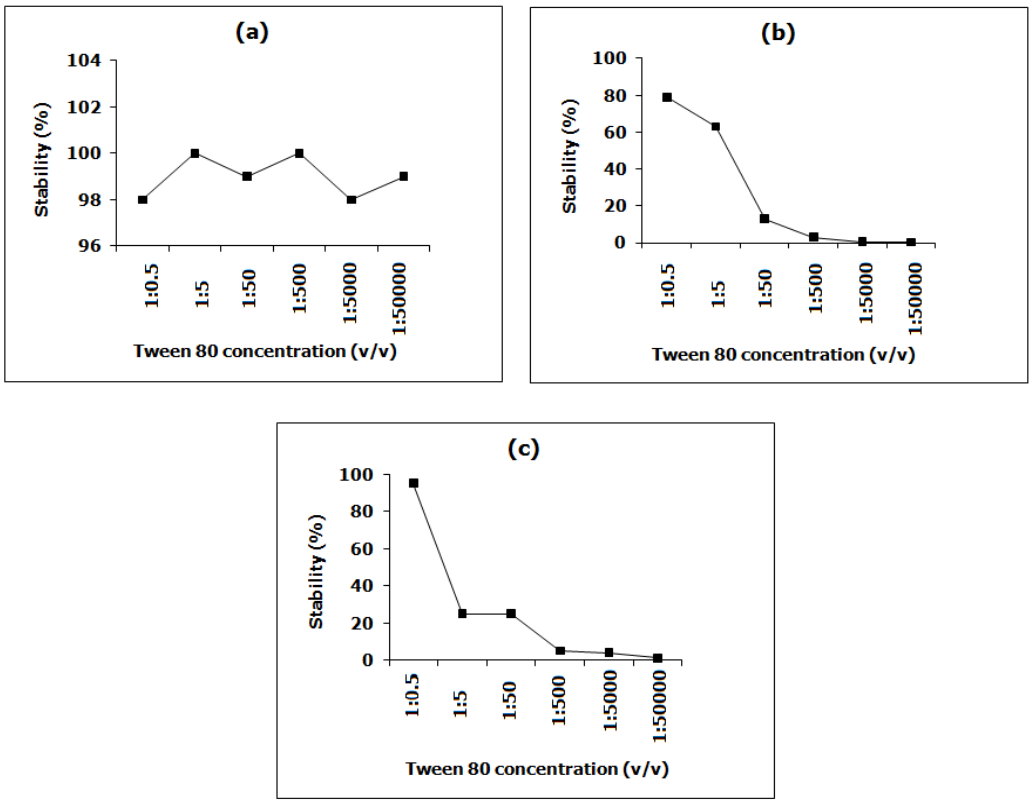

Figure 4: Effect of Tween 80 on stability of (a) φSPB, (b) BVPaP-3 and (c) KPP % stability: N/N0 × 100, where N is the number of viable phages after 1 h of incubation at varying concentrations of Tween 80 and N0 is the initial number of phages

Effect of Tween 80 on stability

Results indicated that φSPB, BVPaP-3 and KPP were quite stable to varying concentrations of Tween 80 with almost 100 % stability (Figure 4 a, b, c).

Effect of Mancozeb on stability

In the presence of Mancozeb, φSPB showed almost100% stability, at the concentrations of 2 to16 µg /ml, and then it was decreased to 70 % for rest of the concentrations of Mancozeb (Figure 5 a). BVPaP-3 showed optimal stability (100%) at the varying concentrations of Mancozeb, and then it was decreased gradually up to 60 % (Figure 5 b). KPP was quite stable (100 % stability) at varying concentrations (2 to 10 µg /ml) of Mancozeb. Then it was decreased to 90 % at rest of the concentrations (Figure 5 c).

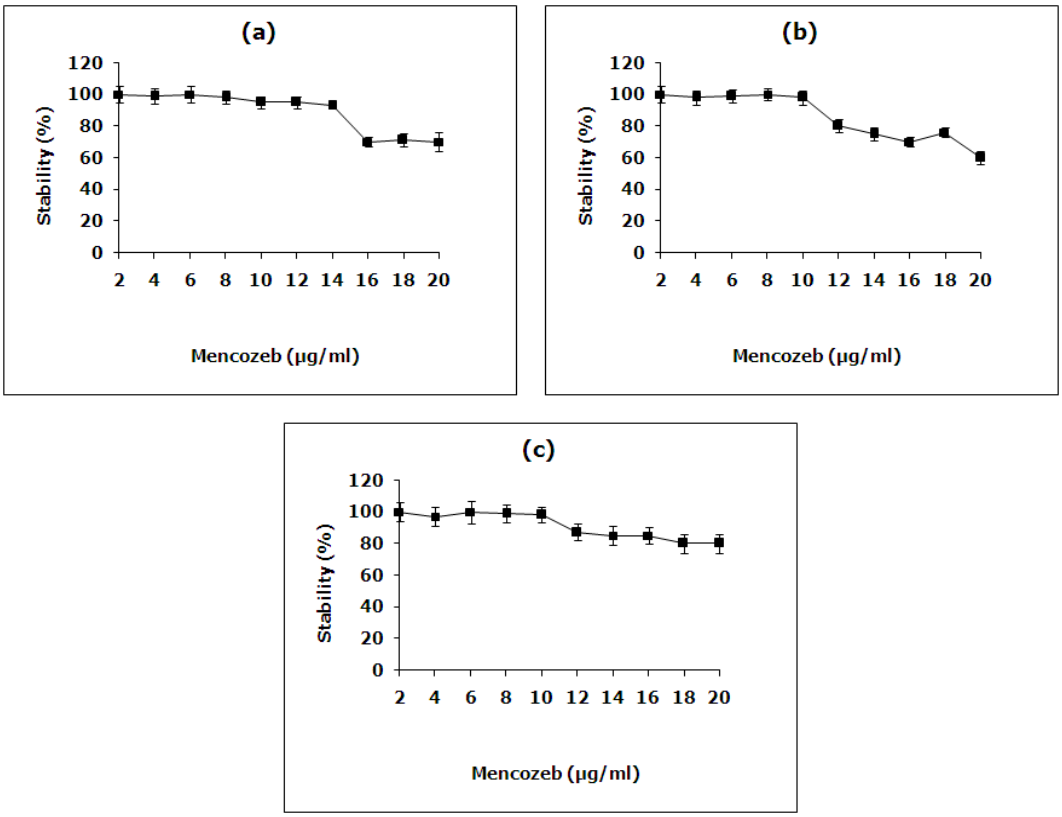

Figure 5: Effect of Mancozeb on stability of (a), φSPB, (b), BVPaP-3 and (c), KPP % stability: N/N0 × 100, where N is the number of viable phages after 1 h of incubation at different concentrations of Mancozeb and N0 is the initial number of phages.

Effect of Malathion on stability

φSPB, BVPaP-3 and KPP phages were not stable in the presence of Malathion.

Effect of Metals on stability

φSPB, found to be very stable at varying concentrations of metals, with almost 100 % stability upto 1mM concentrations of $ZnCl_2$, $CoCl_2$ and $CdCl_2$, except for $HgCl_2$, with only 10% stability at 0.05mM concentration of metal (Figure6 a).BVPaP-3 was also stable (100% stability) at varying concentrations of metals. At 1mM concentration of $ZnCl_2$, $CoCl_2$ and $CdCl_2$, stability was almost 80 % and at 5mM concentration, stability was 60 to 70 %. Stability was negligible at the lower concentrations of $HgCl_2$ (Figure 6 b). KPP was stable (100 %) at varying concentrations of (0.01 to 1Mm) of $ZnCl_2$, $CoCl_2$ and $CdCl_2$. In the presence of $ZnCl_2$ (5 mM) and $CoCl_2$ (5mM), the stability was 70 % and 35 %. At 5mM concentration of $CdCl_2$ KPP was not stable (Figure 6 c).

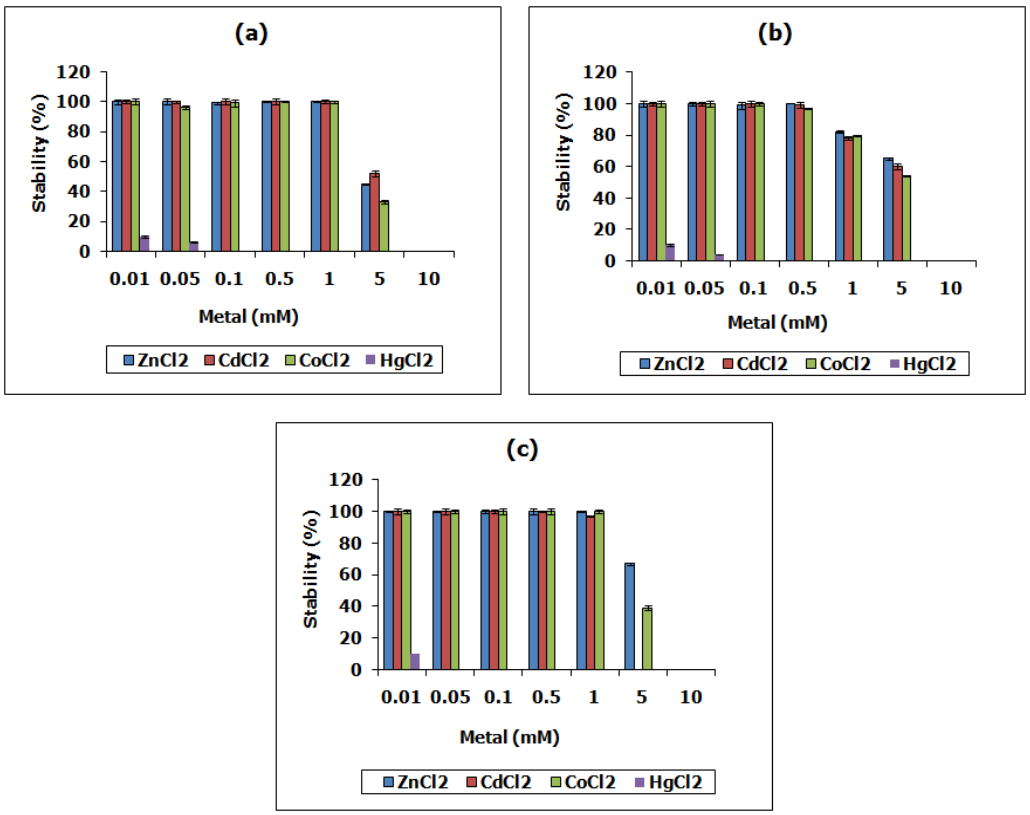

Figure 6: Effect of Metals on stability of (a), φSPB, (b), BVPaP-3 and (c), KPP % stability: N/N0 × 100, where N is the number of viable phages after 1 h of incubation at varying concentrations of metal and N0 is the initial number of phages, % stability with reference phage titer (pfu/ml) of the original lysate

Effect of varied environmental pollutants on infectivity of selected bacteriophages

The MIC and SIC values of the environmental factors for Salmonella Paratyphi B, Pseudomonas aeruginosa and Klebsiella pneumoniae were determined first and then infectivity was determined at the SIC values. The results of MIC and SIC values are listed in the tables (Table 3, 4, 5, 6, 7, 8) given below.

Table 1: MIC and SIC values of SDS

SDS (%)	*Salmonella Paratyphi B*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
0.01	+++	+++	+++
0.05	+++	+++	+++
0.1	+++	+++	+++
0.2	++	++	++
0.3	++	++	++
0.4	++	++	++
0.5	+	+	+
0.6	-	-	+
0.7	-	-	+
0.8	-	-	-
0.9	-	-	-
1.0	-	-	-

Negative Control*: Phage broth + respective concentration of SDS,
Positive control*: Phage broth + respective bacterium

Table 2: MIC and SIC values of Cetrimide

Cetrimide (%)	*Salmonella* Paratyphi *B*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
0.01	-	-	++
0.05	-	-	++
0.1	-	-	+
0.2	-	-	+
0.3	-	-	+
0.4	-	-	+
0.5	-	-	-

Negative Control*: Phage broth + respective concentration of Cetrimide only,
Positive control*: Phage broth + respective bacterium

Table 3: SIC values of Tween 80 for bacterial pathogens

Tween 80 (%)	*Salmonella* Paratyphi *B*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
1:0.5	+	+	+
1:5	++	++	++
1:50	+++	+++	+++
1:500	+++	+++	+++
1:5000	+++	+++	+++

Negative Control*: Phage broth + respective concentration of Tween 80 only,
Positive control*: Phage broth + respective bacterium

Table 4: MIC of Mancozeb for bacterial pathogens

Mencozeb (µg/ml)	*Salmonella* Paratyphi *B*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
2	+++	+++	+++
4	+++	+++	+++
6	+++	+++	+++
8	+++	+++	+++
10	+++	+++	+++
12	+++	+++	+++
14	+++	+++	+++
16	+++	+++	+++
18	+++	+++	+++
20	+++	+++	+++
40	+++	+++	+++
60	+++	+++	+++
80	+++	+++	+++
100	+++	+++	+++
200	+++	+++	+++
300	+++	+++	+++
400	+++	+++	+++
500	+++	+++	+++

Negative Control*: Phage broth + respective concentration of Mencozeb only,
Positive control*: Phage broth + respective bacterium

Table 5: MIC of Malathion for bacterial pathogens

Malathion (v/v)	*Salmonella* Paratyphi *B*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
1:10	-	-	-
1:50	-	-	-
1:200	-	-	-
1:400	-	-	-
1:500	-	-	+
1:1000	-	-	+
1:10000	-	-	+

Negative Control*: Phage broth + respective concentration of Mencozeb
Positive control*: Phage broth + respective bacterium

Table 6: MIC and SIC values of Metals for pathogens

Metal		Pathogen		
Salt	Concentration (mM)	*Salmonella* Paratyphi *B*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
$ZnCl_2$	0.01	+++	+++	+++
	0.05	++	++	++
	0.1	++	++	++
	0.5	+	+	++
	1	+	+	+
	4	-	-	-
	5	-	-	-
	10	-	-	-
$CoCl_2$	0.01	+++	++	+++
	0.05	+++	++	++
	0.1	++	+	++
	0.5	+	+	++
	1	+	+	+
	4	+	+	+
	5	-	-	-
	10	-	-	-
$CdCl_2$	0.01	+++	++	++
	0.05	++	+	++
	0.1	+	+	+
	0.5	+	+	+
	1	-	-	+
	4	-	-	-
	5	-	-	-
	10	-	-	-
$HgCl_2$	0.01	-	-	-
	0.05	-	-	-
	0.1	-	-	-
	0.5	-	-	-
	1	-	-	-
	4	-	-	-
	5	-	-	-
	10	-	-	-

Effect of SDS on infectivity

In case of φSPB infectivity was in the range of 15 – 25 % (Figure 7 a) where as infectivity of BVPAP-3 was also less in the range of 5 to 20 % (Figure 7 b) and in case of KPP infectivity was in the range of 5 to 20 % at varying concentrations of SDS (Figure 7 c).

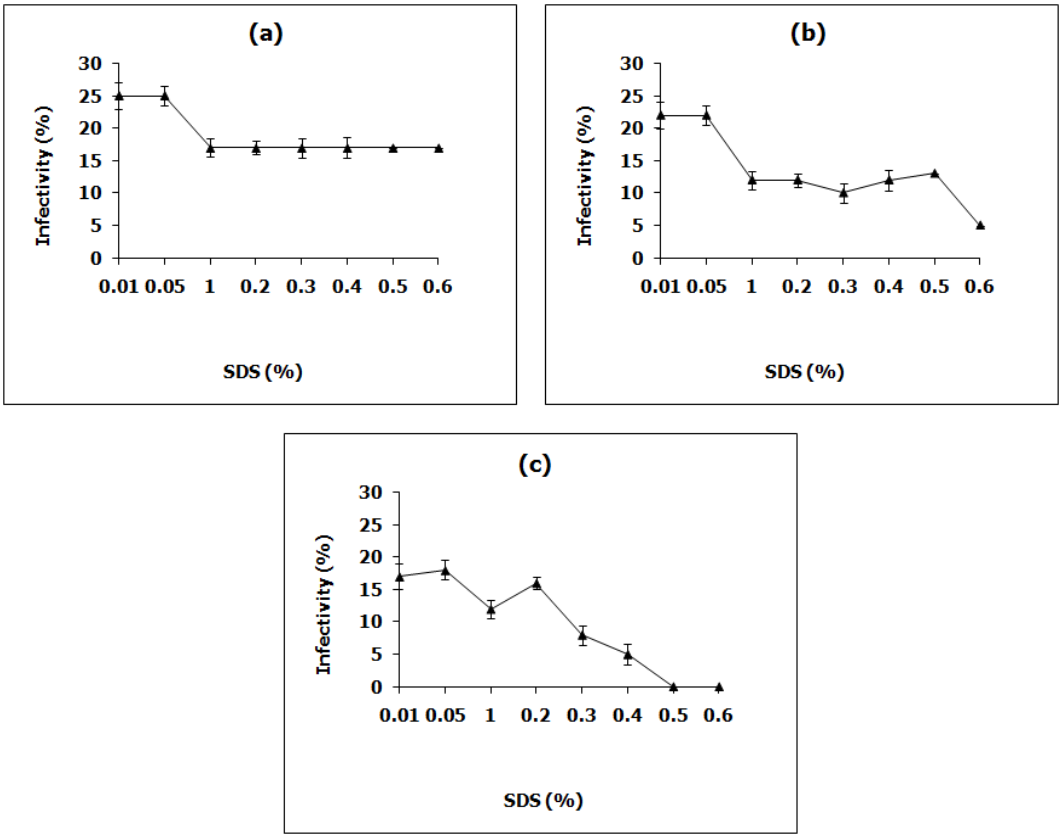

Figure 7: Effect of SDS on infectivity of (a), φSPB, (b), BVPaP-3 and (c), KPP % infectivity: N/N0 × 100 where N is the number of viable phages at different concentrations of SDS incorporated in the nutrient agar medium after incubation at 37˚C. N0 is the initial number of phages, % infectivity with reference phage titer (pfu/ml) of the original lysate

Effect of Cetrimide on infectivity

Infectivity is determined at the SIC value of Cetrimide for Salmonella Paratyphi B, and Klebsiella pneumoniae did not grow in the presence of Cetrimide, therefore, infectivity of φSPB and KPP can not be determined. Infectivity of BVPAP-3 was in the range of 10 to 35 % for all the concentrations (0.01 to 0.6 %) of Cetrimide (Figure 8).

Effect of Tween 80 on infectivity: Infectivity was almost 100 % at all concentrations of Tween 80, except at highest concentration, it was very less (Figure 9a, b, c).

Effect of Mancozeb on infectivity

Infectivity was optimal (90 %) at 2 µg /ml, of Mancozeb, then it decreased to 80 % at 4 to 8 µg /ml, of Mancozeb. At 16 to 20 µg /ml, infectivity was zero (Figure 10 a). Infectivity was also in the range of 85 to 95 % at 2 to 10 µg /ml of Mancozeb (Figure 10 b). KPP was infective (70 to 100 %) at varying concentrations of Mancozeb (Figure 10 c).

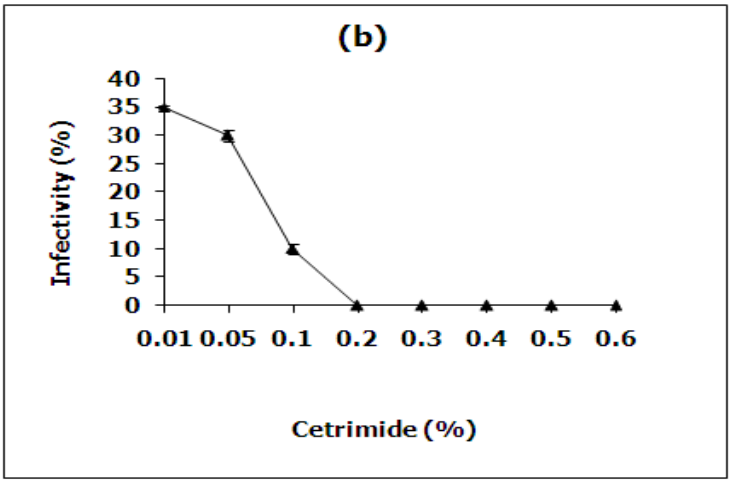

Figure 8: Effect of Cetrimide on infectivity of BVPaP-3 % infectivity: N/N0 × 100 where N is the number of viable phages at different concentrations of Cetrimide incorporated in the nutrient agar medium after incubation at 37˚C. N0 is the initial number of phages, % infectivity with reference phage titer (pfu/ml) of the original lysate

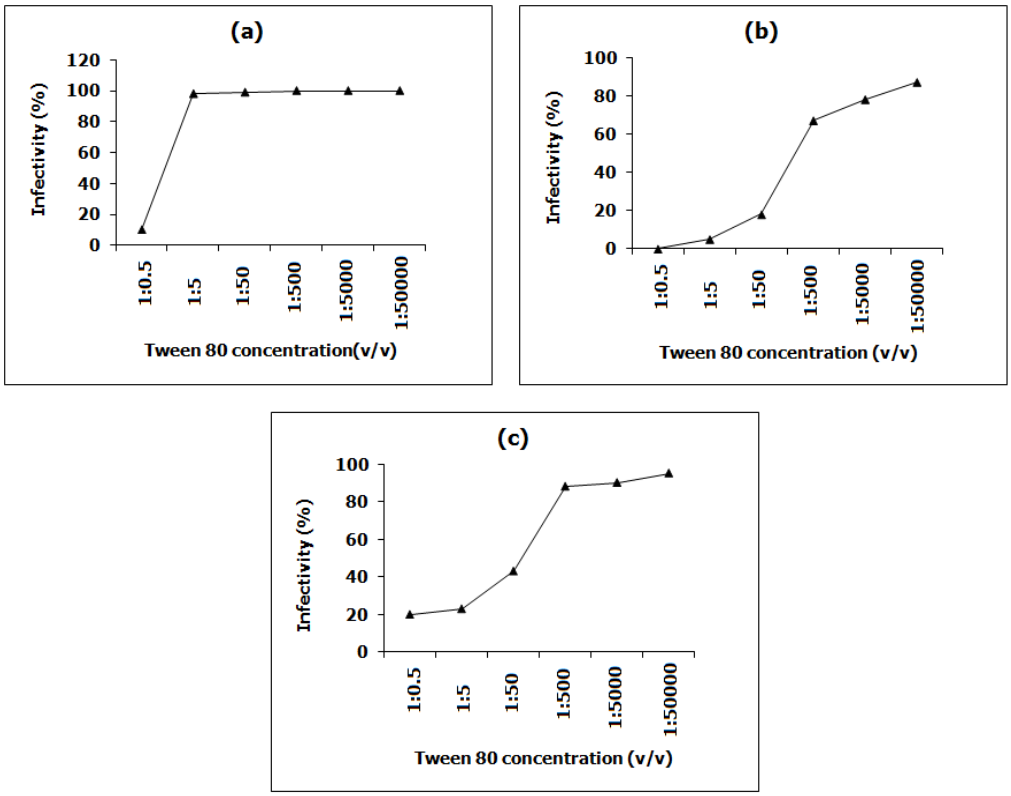

Figure 9: Effect of Tween 80 on infectivity of (a) φSPB, (b) BVPaP-3 and (c) KPP % infectivity: N/N0 × 100 where N is the number of viable phages at different concentrations of Tween 80 incorporated in the nutrient agar medium after incubation at 37˚C. N0 is the initial number of phages % infectivity with reference phage titer (pfu/ml) of the original lysate

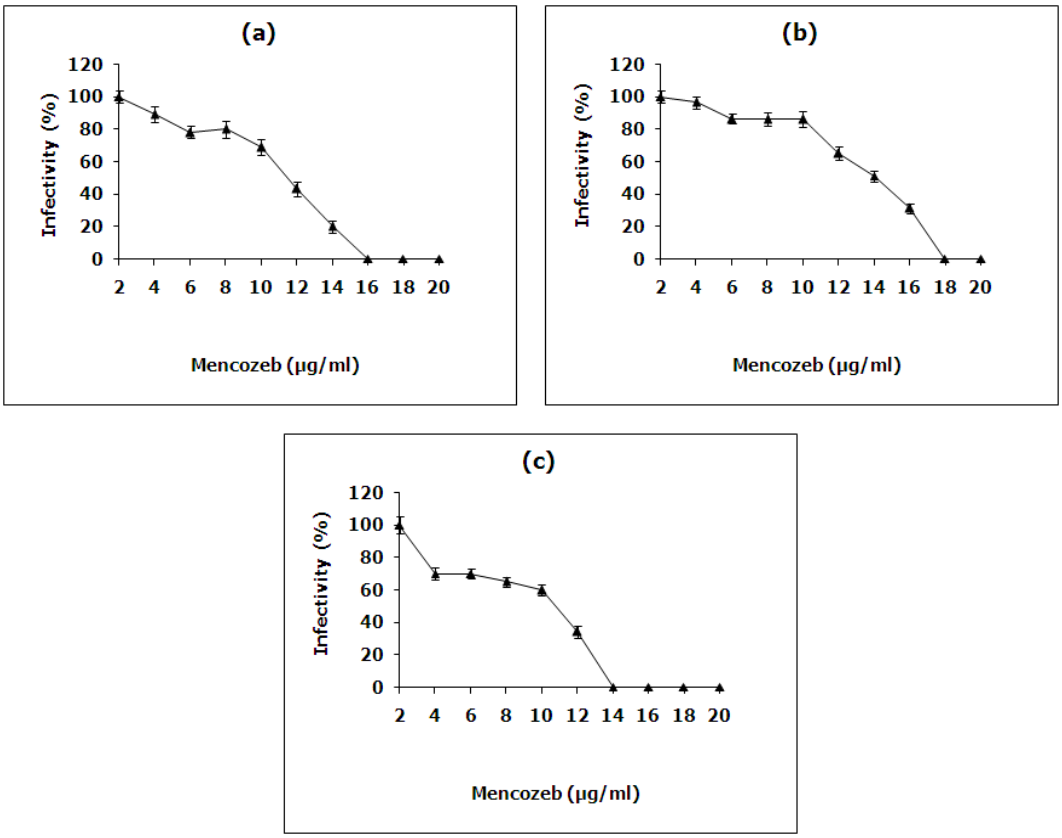

Figure 10: Effect of Mancozeb on stability of (a), φSPB, (b), BVPaP-3 and (c), KPP % infectivity: N/N0 × 100 where N is the number of viable phages at different concentrations of Mancozeb in the nutrient agar medium after incubation. % stability and % infectivity with reference phage titer (pfu /ml) of the original lysate

Effect of Metals on infectivity

φSPB showed 100 % infectivity at 0.01 mM concentration of all the metals. Then at 0.5 mM concentration ZnCl2, CoCl2 and CdCl2, infectivity was decreased to 80 to 85 % except for HgCl2, with no stability. At 1 mM concentration, infectivity was only 40 % (Figure 11 a). BVPaP-3 was infective at the concentrations (0.01 to 5 mM) with 100 % infectivity. In the presence of HgCl2, no infection was found (Figure 11 b). KPP was infective with almost 100 % infectivity at metal concentrations 0.01 to 1 mM, except for HgCl2, there was no infectivity (Figure 11 c).

The results obtained indicated that anionic (SDS) and cationic (Cetrimide) detergents have adverse effects on the stability and infectivity of φSPB, BVPaP-3 and KPP. Cetrimide had a toxic effect on Salmonella Paratyphi B and Klebsiella pneumoniae because detergents mainly act on the proteins and microbial membranes [14] by rupturing cytoplasmic membranes giving small empty envelopes. At high concentrations of Cetrimide granules appear in the cytoplasm, increasing the size with increasing concentration until the structure becomes transformed to a granular body [15] but *Pseudomonas aeruginosa* could be able to grow at lower concentrations of cetrimide, but not at higher concentrations, indicating that it is able to degrade Cetrimide. Phage isolates were stable to some extent in the presence of Cetrimide and infectivity of BVPaP-3 to Pseudomonas aeruginosa was also found to some extent, this might be because of the physical damage to the tail part of the phage by the Cetrimide.

Host pathogens could able to grow in the presence of SDS at lower concentrations only, but not at higher concentrations, this might be because of the lethal effect of SDS. Literature survey indicates that SDS ions bind by means of the hydrocarbon chain to the specific binding sites on cell surfaces, thereby solubilising proteins [16]. Stability and infectivity of φSPB, BVPaP-3 and KPP was reduced in the presence of sodium dodecyl Sulphate (SDS) this might be because of binding of SDS ions by means of the hydrocarbon chain to the specific binding sites on the virion, so that the Sulphate groups

are near lysine- or arginine- phosphate interaction points, these interactions are neutralized and the phosphate groups are repulsed, resulting in virion dissociation [17,18].

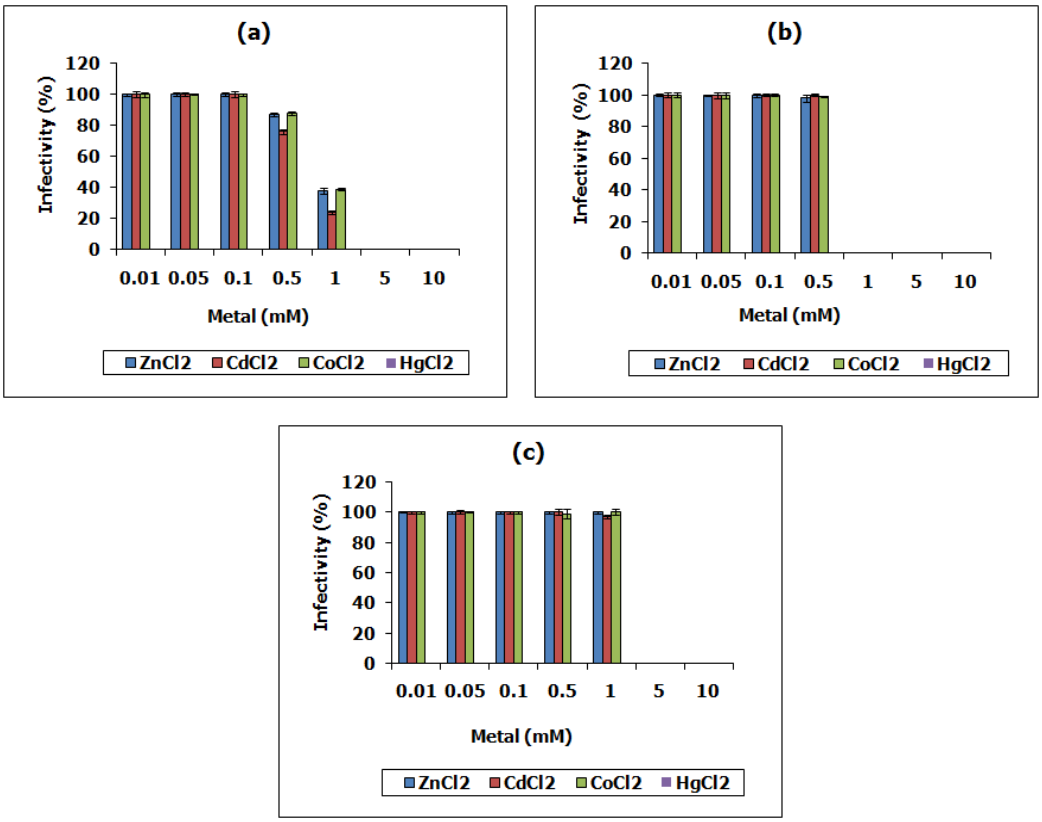

Figure 11: Effect of Metals on infectivity of (a)φSPB, (b), BVPaP-3 and (c), KPP % infectivity: N/N0 × 100 where N is the number of viable phages at different concentrations of metal incorporated in the nutrient agar medium after incubation at 37˚C. N0 is the initial number of phages, % infectivity with reference phage titer (pfu/ml) of the original lysate

Tween 80 is a non- ionic detergent, therefore, did not have adverse effects on the host pathogens as well as on the stability of φSPB, BVPaP-3 and KPP phage isolates. At the very high concentration the infectivity was reduced remarkably because Tween 80 may have adverse effects on the capsid proteins because of its amphiphilic nature it may bring about the disruption of hydrogen bonds and salt linkages [19]. Results indicated that phage isolates were quite stable at a high concentration of Tween 80, this might be due to the viscous nature (micelle form) of Tween 80, the polar side chains of ether and ester linkages may not got exposed to phages and therefore, did not inactivated phage isolates.

Toxic effects of metal ions on microbial cells have been known since a long back. Very low concentrations of heavy metals may display antimicrobial activity. This depends upon the ability of heavy metals to combine with the –SH group of a number of enzymes [19]. In this study, effect of metal ions (Zn, Co, Cd and Hg) on growth of Salmonella Paratyphi B, Pseudomonas aeruginosa and Klebsiella pneumoniae was studied. Metal ions (Zn, Co, Cd) at low concentration showed stimulatory effects on the growth because they are the essential parts of certain enzymes and cofactors, but at higher concentrations prevented the growth of pathogens. Mercury (Hg) ions could prevent the growth of the pathogens at all concentrations. Therefore it can be concluded that cells live in the environment of ions and need many ions, even though many are considered toxic [19].

Effect of the varied concentrations of Zn, Co, Cd and Hg ions was studied on the stability and the infectivity on φSPB, BVPaP-3 and KPP phage isolates. Zn, Co and Cd ions did not affect adversely on the protein conformation or the protein integrity of capsid of φSPB, BVPaP-3 and KPP phage isolates. At the same time, results indicated that, these metal ions had stimulatory effects on the infectivity property of these phage isolates at lower concentrations, whereas at higher concentrations

they exhibited inhibitory effects on the infectivity properties of phages. It was found that the stability of φSPB, BVPaP-3 and KPP phage isolates was negligible even at low concentrations of Hg .This indicates that, φSPB, BVPaP-3 and KPP phage isolates are very sensitive to the toxic effects (coagulation of capsid proteins) of Mercury. These findings are in consistence with the previous findings that heavy metals are responsible for virus inactivation in sea waters [20] whereas metal ions are involved in the phage multiplication and they have a stimulatory effect [21, 22].

Results indicated that, pesticides, viz., Melathion had inhibitory effects on the growth of Salmonella Paratyphi B and Klebsiella Pneumoniae at all the tested concentrations except for Pseudomonas aeruginosa, which was not inhibited in the presence of Melathion at lower concentrations. Mancozeb, which is a fungicide, did not inhibit the growth of these pathogens. Pesticides did not have much adverse effect on the stability of φSPB, BVPaP-3 and KPP, but the infectivity was lost at the higher concentrations of Mancozeb. These results are in consistent with the previous results in the literature, indicating that different pesticides alone or in combination do not inhibit the lytic activity of bacteriophages [23].

Infectivity property of φSPB, BVPaP-3 and KPP was evaluated at the SIC concentrations of the environmental factors. These SIC values reflect the viability and growth capacity of the host. Beyond the SIC values, there might be the lack of an active host, therefore, infectivity can not be determined. The phage might be infective, but the host is not in a suitable condition to allow phage amplification.

Conclusion

From the results, it can be concluded that φSPB, BVPaP-3 and KPP phages are quite resistant (able to survive) to unfavorable chemical factors. Thus they can settle in the natural water resources that constantly receive various types of pollutants. Therefore, these phages can be used as potential biological control agent in the natural water bodies to bring about targeted killing. Phages can be used as potential disinfectant in the natural water bodies alone or in combination with physical and chemical process.

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