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Original Research Article

Bacteriophage Formulations for the Reduction of Multi-Drug Resistant *E.coli* in Water Sources

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Abstract

Production and supply of pathogen and toxic chemical free water is the preliminary aim of water treatment. The natural killing property of bacteriophages makes them the most prominent tool in eliminating unwanted bacteria, making them ideal candidate in water treatment research. In this study phage formulations against multidrug resistant *E. coli* KP005067 were fabricated by immobilizing them in broth, activated charcoal and alginate beads. The cross section of the phage formulation was analysed using Confocal Raman Microscope and the disinfection study was carried out in batch and packed bed reactor. The phage formulations remained stable for almost 4-8 and 3-5 months at 4^{o}C and 30^{o}C respectively. The batch reduction study using phage connoted the order of host reduction as phage-charcoal > phage-broth > phage-bead. Further more, the disinfection study using packed bed reactor showed 100 and 97% host reduction using phage-charcoal and phage-bead respectively. At the same time, there was concomitant increase in the phage population in the packed bed-reactor indicating that the phage formulations are highly potent in killing bacterial pathogen in water.

Keywords: Water treatment, bacteriophage formulations, multidrug resistance, E.coli KP005067.

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INTRODUCTION

Water resources are becoming depleted due to the contamination barrier caused by various pathogens, and disinfection is the last choice to counter seal this problem. One of the most exciting advances in the field is the use of eco-friendly disinfection techniques, which can protect ecosystem and human health [1]. Traditionally, several purification methods like chlorination, radiation, filtration etc. have been employed worldwide, which reduce the bacterial pathogens with certain disadvantages. Among which chlorination is the conventional one that can result in the formation of mutagenic/carcinogenic by-products [2]. Therefore, as an alternative solution, dechlorination followed by chlorination should be done to ensure safe drinking water. Also, some bacterial strains like *Legionella* and *Mycobacterium* have shown resistance to chlorine disinfection [3].

The disinfection resistance is further exacerbated by the overuse of antibiotics and emergence of antibiotic resistant bacteria. The third largest peril with regard to global public health in the 21st century is the spread of anti-microbial resistant (AMR) bacteria in water bodies. Presumably, the prevalence of antibiotic resistant bacteria in drinking water sources in developing countries is due to gross abuse in the use of antimicrobials [4]. Human and animal intestines can harbour commensal *E. coli* which is a good receptacle of AMR genes, and this can be further transferred to other bacteria in the environment [5]. Therefore, to control the spread of pathogens in environment, it is not only important to develop alternative treatment technologies, but also need to develop appropriate disinfectants that can target potentially life threatening bacteria.

The use of bacteriophage as bio-control agent is one of the possible options in controlling specific target bacteria. Due to their abundance in nature, they can outnumber bacteria as in 10:1 ratio [6]. Its ease in isolation, specificity and autocatalytic properties has made them a promising candidate in disinfection process [7]. As bacteriophages are natural antimicrobial agents against bacteria, their stability and infectivity is important while using as biocontrol agents. Encapsulation of phages in biodegradable polyester microcapsules and in freeze dried cakes of polyethylene glycol plus sucrose have been found to increase its shelf-life [8, 9]. Lyophilisation in liquid media and freezing of phages at low temperatures has also been

employed in storing active phages. Varying degree of success has been found in maintaining and stabilizing phage titer using these methods.

Therefore, an endeavour has been made to develop on-site low cost phage formulations by immobilizing the phages in different substrates and to analyse its stability during storage and compare the disinfection efficiency of these formulations in the management of waterborne bacterial pathogens.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition

E. coli KP005067 isolated from Canoli canal, Kozhikode district, Kerala, India was used as the host bacteria for phage treatment studies. For experiments, the strain was cultured on EMB agar, MacConkey agar and Nutrient agar and incubated at 37^{o}C for 24 hours. Further, the strain enrichment was done by transferring a single colony from EMB agar plate to alkaline petone water (5-10 mL) and incubated at 37^{o}C for 6-8 hours.

Antibiotic Susceptibility Testing

The antibiotic susceptibility of *E. coli* KP005067 was determined according to Clinical and Laboratory Standards Institute guidelines [10]. The inoculum was prepared by adjusting the turbidity to 0.5 McFarland's standard and spread on Muller-Hinton agar plates. Antibiotic discs containing piperacillin (10 µg), nalidixic acid (5 µg), erythromycin (15 µg), cefepime (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), imipenem (10 µg), and cefixime (5 µg) (Himedia Laboratories, Mumbai, India) were used as per the manufacturer's instructions. The discs were placed on the agar plates and incubated overnight at 37^{o}C for 24 hours. The zones of incubation were measured and the isolate was classified as sensitive, intermediate and resistant [10].

Preparation of Solid-Phage Formulation

Phage Immobilization on Charcoal

The immobilization of phage in charcoal was performed as per the method described by [11]. Activated charcoal powder (50 gram) was spread onto a sterilized glass dish and 10 mL of phage lysate was uniformly sprayed on to the matrix. The phage-charcoal powder was dried and mixed at 37^{o}C for two hours until it was completely dried. Further, it was ground to fine powder, and a particle size of 50-600 µm with an average particle size of 200 µm was maintained (Figure-1). One gram of this mixture was re-suspended in 10 mL of reverse-osmosis (RO) water and the recovery of phage was tested by spotting on host lawn in agar plates. Activated charcoal powder, without bacteriophage was kept as control.

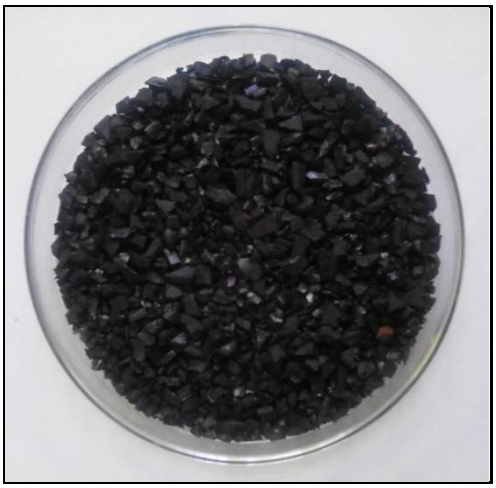

Fig-1: Phage immobilized on activated charcoal

Phage Immobilization on Alginate Beads

The immobilization procedure, a two-step approach was carried out as mentioned by [12]. Ca-alginate microspheres were prepared by extruding a mixture containing 10^{8} PFU mL^{-1} phage suspended in 4% (w/v) low viscosity sodium alginate solution into 50 mM $CaCl_2$ and gently stirred using a magnetic bar. The microspheres were prepared using a needleless 10 mL syringe, with a nozzle size of 300 µm from a height of 5 cm. Further, the microspheres were allowed to harden by placing them in 50 mM $CaCl_2$ solution for 30 minutes. Further, the beads were washed using a sterile saline solution (0.85 % w/v, pH 7.0) and stored at 4°C for further use (Figure-2). Calcium alginate beads, without bacteriophage served as control.

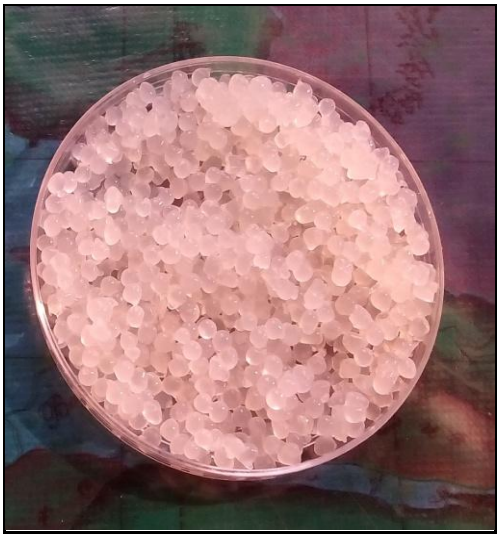

Fig-2: Phage immobilized on calcium alginate bead

Raman Confocal Microscopy

The images of immobilized phage in charcoal and Ca-alginate beads were analysed by using inverted Raman Confocal Microscope (WiTec, alpha 300). The substrate was prepared as per the procedure of [13] with certain modifications based on the particle size. The substrate was washed and immersed in petri dish with saline at 20^{o}C prior to Raman measurements. Here per cell, two spectra were acquired, one from nucleus and the other from cytoplasm and were recorded in 580–1750 cm^{-1} range. Different acquisition times were tested: two accumulations of 1, 2, 5, 10, 15, 20 and 30 seconds by adjusting the confocal pinhole at 150 mm. For Raman spectral imaging, isolated single cells were raster scanned with a 2 mm step and an acquisition time of 2 x 10 sec for each point. Labspec 4.18 software was used to carry out all spectral acquisitions.

Shelf-Life of Phage Formulations

The plaque assay technique proposed by [14] was used to determine the phage titer in the formulations. Each formulation (phage-charcoal, phage-alginate bead and phage buffer) was equally dispensed into two sterile screw capped bottles and stored at 4 and 30^{o}C respectively. The density of phage (residual phage titer) in each formulation was monitored at an interval of one month for a year. The observations were analyzed, recorded and graphically presented.

Batch Reduction Study

Water sample (100 ml) was autoclaved separately in 500 ml screw capped containers in triplicates. Then it was spiked with saline suspension of the mid-log (5 hours old, 1 × 10^{6}colony forming unit ml^{-1}) cells of *E. coli* KP005067. Ten millilitre of phage formulations with multiplicity of infection (MOI) < 1 was added to each of the water samples except control flask (water sample + host bacterium). Further, the samples were incubated at 35 ± 2^{o}C for 24 hours in shaking conditions (60 rpm). Then 0.1 mL of the aliquots were withdrawn from each batch and plated on to agar media. After incubation at 37^{o}C for 24 hours, the numbers of colonies were recorded. The plaque forming units were also determined in each aliquot by double agar plaque assay technique. Percentage of reduction of colony forming units and plaque forming units in each aliquot was noted. In order to determine the most significant phage formulation on pathogen reduction, analysis of variance (ANOVA) was applied using the linear model. The parameters were evaluated using 5% (p = 0.05) significance level.

Packed Bed Reactor Study

A packed bed, with two cylindrical glass columns (450 x 30 mm) maintained under semi-continuous conditions were used as reactor for the biocontrol studies. The total reactor volume was 282.60 mL and the working volume was 176.63 mL (Figure-3). Non-adsorbent cotton was placed at the bottom to support the bed. One of the columns was filled with phage-immobilized charcoal and the other was filled with phage-immobilized Ca-alginate beads up to a height of 25 cm. A control was maintained for both reactors in which empty charcoal and Ca-alginate beads were also packed to the same height. Further, 100 mL of sterile water inoculated with 1 mL bacterial suspension (3x10^{8} CFU/mL) equivalent to 0.5 McFarland's standard were passed through the columns at the rate of 0.59 mL/min. The column was operated in this manner overnight and at this rate the hydraulic retention time was 300 minutes. The resulting samples were drawn from the effluent outlet periodically at every three hours and tested for the number of surviving bacteria, using standard procedures.

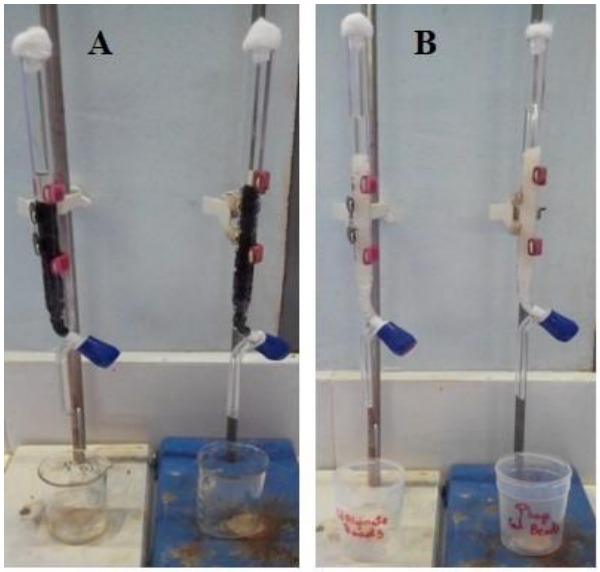

Fig-3: Packed bed column (A) phage-charcoal and (B) phage-beads

UV Action Spectra of Phage Particles

Samples were taken at each stage of the treatment process and the absorbance of phage was determined using UV-visible spectrophotometer (Thermo evolution 201) (optical density was measured at 280 nm). The samples after each hour of treatment was assayed to determine the concentration of phage by vortexing with 2% chloroform (disrupt host cells).

RESULTS

In this study, it is clearly evident that *E. coli* KP005067 exhibited high resistance pattern towards the antibiotics tested. Antibiogram results showed that *E. coli* was highly resistant to piperacillin, erythromycin, ceftazidime and imipenem, while being sensitive to nalidixic acid and cefepime (Table-1). An intermediate resistance was observed against cefixime and cefuroxime. The results indicated that majority of the antibiotics tested were ineffective against *E. coli* KP005067. The antibiogram profile of *E. coli* KP005067 denotes its selective resistance to four antibiotics viz., piperacillin, erythromycin, ceftadizime and imipenem. Even [15] reported the resistance of *E. coli* to more than three antibiotics. The prevailing antibiotic resistance in *E. coli* may be attributed to indiscriminate use of antibiotics, overcrowding of flock, poor sanitation etc [16].

Table-1: Antibiotic susceptibility pattern of *E. coli* KP005067

Bacteria/Antibiotic (µg)	CFM (30)	PT (10)	ERM (15)	CXM (5)	NA (30)	CPM (30)	CAZ (30)	IM (10)
E. coli KP005067	I	R	R	I	S	S	R	R
CFM-cefixime PT-Piperacillin, ERM-erythromycin, CXM-cefuroxime, NA- nalidixic acid,CPM-cefepime, CAZ-ceftazidime, IM- imipenemR-Resistant, S-Sensitive, I-Intermediate								

The developed phage formulations in broth, charcoal and Ca-alginate beads were analysed for its longevity in storage conditions both in room temperature and freezing temperature. Temperature is one of the important factors that determine phage activity [17]. Percentage of active phage particles was high during 4^{o}C rather than 30^{o}C in this study. One of the reasons is that, the phage used in this study was tailed one, and they are highly resistant to storage conditions that extended its shelf life [18]. Of the phages studied by various research groups, 96% are reported to be tailed and only 3% to be polyhedral, filamentous and pleomorphic [19]. Also, good phage stability was observed at 4^{o}C in buffer solution for more than six months, but not at 37^{o}C [20].

The nucleic acids of phages often contain unusual or modified bases and the size of the nucleic acid varies depending upon the type of phage. Both the optical image and the Raman image were considered for phage-charcoal and phage-bead combinations. The results showed peaks corresponding to nucleotides which were identified at 1578 cm^{-1} (guanine and adenine) and are represented in yellow color in both phage-charcoal and phage-bead. More number of nucleotides was visible in the phage-charcoal combination, indicating the high number of phages immobilized in charcoal than alginate bead.

The number and the amount of each kind of protein present in the phage particle will vary depending upon the type of phage, and these proteins function in the infection and also in the protection of

nucleic acids. The amide III region (1450 – 1660 cm^{-1}) was chosen to detect proteins. The two spectra generated (Figure 4) represent basic spectra of different regions of the phage inside the immobilized particle. The basic spectrum of protein and nucleotide regions is shown in false colors as red and yellow respectively. The cell components were distinguished from the two images generated by the fit procedure illustrated with different colors as seen in figure 5 and 6. The red features represent the protein and the yellow areas showed the nucleoli inside the nucleolus. Thus the presence of phage particles in the charcoal and alginate beads was confirmed by Confocal Raman images.

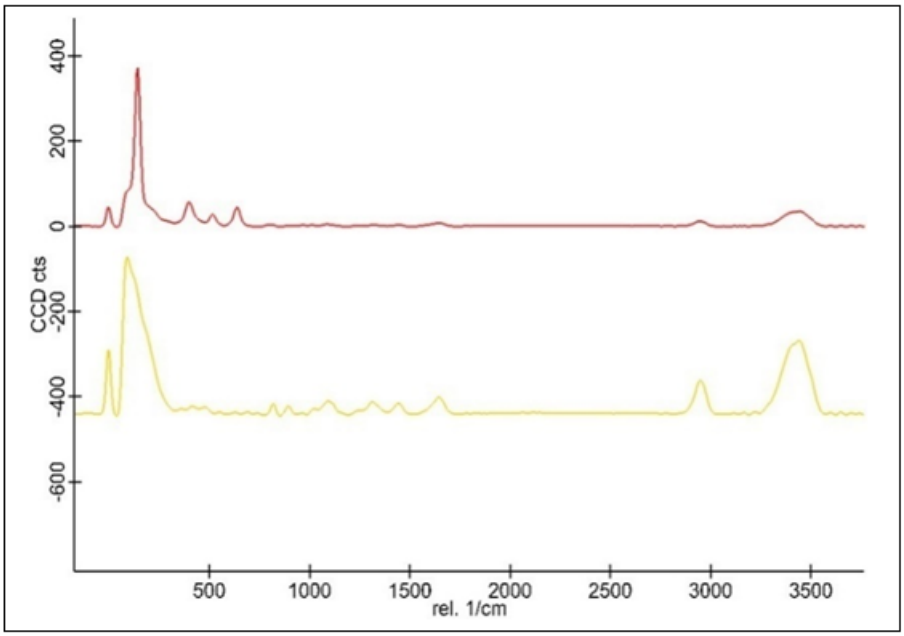

Fig-4: Raman spectra of nucleoli and protein of phage particles

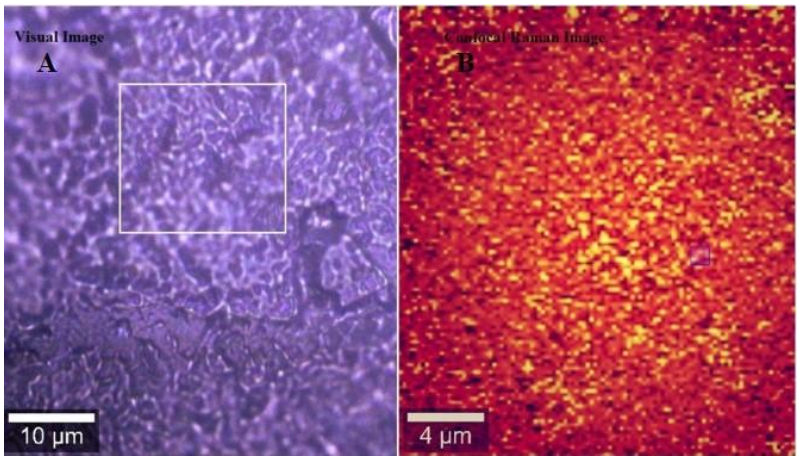

Fig-5: Microphotograph of the (A) visual image and (B) Raman image of phage immobilized in charcoal

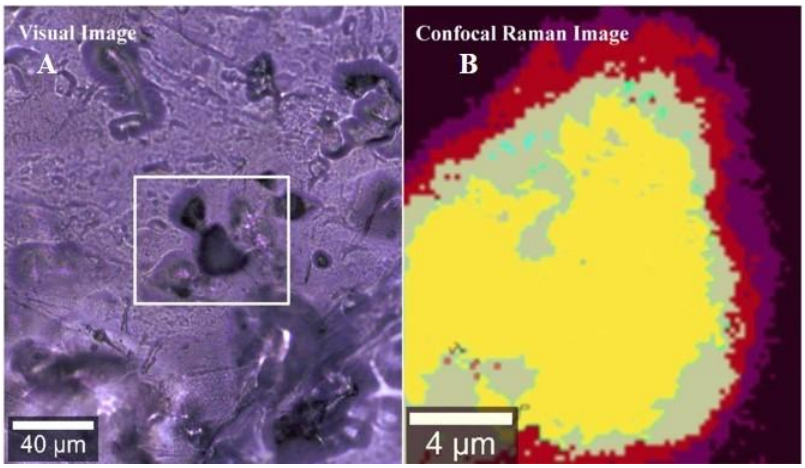

Fig-6: Microphotograph of the (A) visual image and (B) Raman image of phage immobilized in Ca-alginate bead

Phage formulations in broth, alginate bead and charcoal, stored at 30°C were less stable when compared to 4°C (Figure-7). Phage broth formulations stored at 4°C was stable for almost 6 months and reduced to almost 70% at the end of eighth month. Likewise, phage-charcoal was stable with 80% survival up to six months and then the phage titer was gradually reduced. The phage-beads were stable for first two months and then it was reduced to 50% by the end of fourth month. Overall, comparing the initial phage titer there was a decrease in phage titer stored at 30°C. Phage suspended in broth, phage charcoal and phage beads were stable for 50, 35 and 27 days respectively. The carriers used in this study have retained the active phages and did not damage the phage and its structural elements, which was clearly visible in the confocal Raman image. Powdered formulations of phages are applied worldwide for disease treatment, but phages get adsorbed on the matrix and may get inactivated due to various environmental conditions [21]. So it can be concluded that all the three matrices (phage broth, charcoal and alginate bead) played key a role in maintaining the activity of phages.

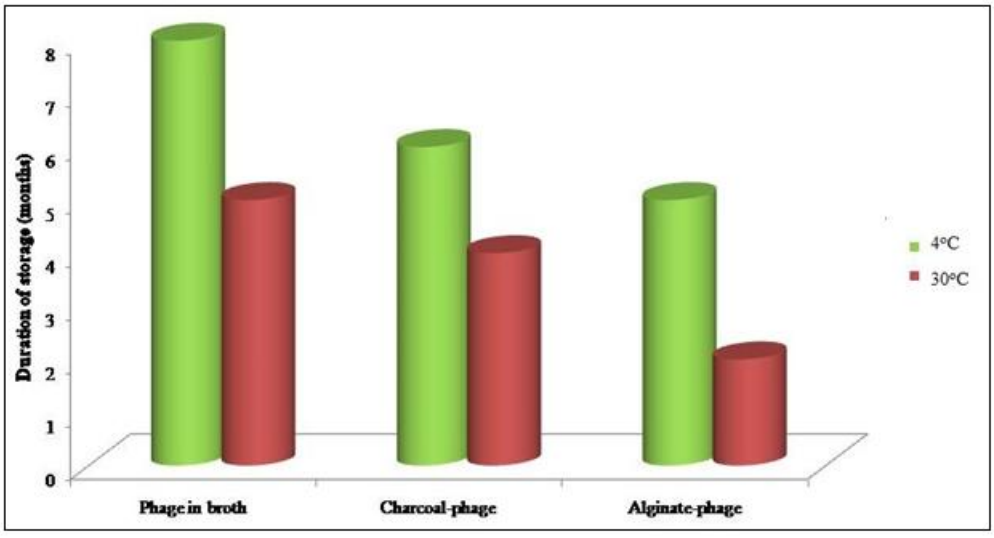

Fig-7: Phage survival at different temperature

As there are limited studies regarding the application of phages in water systems [22] therefore, free phages as well as immobilized phage cells were employed for treatment of water. Batch reduction experiments using free phage (broth), charcoal-phage and alginate-phage were maintained up to 12 hours. According to single step growth curve studies, the phage population reached the maximum level within 7 to 8 hours. So the incubation time in this experiment was maintained up to 12 hours. Maximum reduction (100%) was observed in the samples treated with phage-charcoal combination in 12 hours, where both the charcoal and phage had the property to remove bacteria from the water samples. In case of free phage and alginate-phage, the reduction of *E. coli* was found to be 90 and 84% respectively. The sequences of reduction, in batch treatment were as follows: phage-charcoal > free phage cells > phage-beads (Figure-8).

The phage formulations were found to show an active lysis pattern against host bacteria in the exponential phase of the growth cycle. Maximum reduction of 100% was observed in the samples treated with phage-charcoal combination making this formulation an effective biological disinfectant against antibiotic resistant *E. coli*. Phages adhere to different matrix and it is difficult to release them during various applications, due to their strong binding capacity. Also, the metal ions present in water do not have any adverse effect on the stability of phage formulation [23]. The phage broth carrier contains nutrients that helped the phage to remain in the active state, which can be the reason for its reduction efficiency (90%) in batch study. Application of phage broth formulation by [14] also showed similar results in the reduction of *S. enterica*

The analysis of data for significant variance was done by ANOVA. All the three groups showed significant variation between treatments with $F = 5.111$ ($P = 0.003$) as shown in table 2. Among the three groups tested, charcoal-phage formulation showed the maximum efficiency with a mean of 16.94, followed by free phage (14.29 MPa) and alginate-phage (10.63 MPa) in decreasing order. A 95% confidence level was used for the analysis, so that $P \leq 0.05$ were considered to be statistically significant.

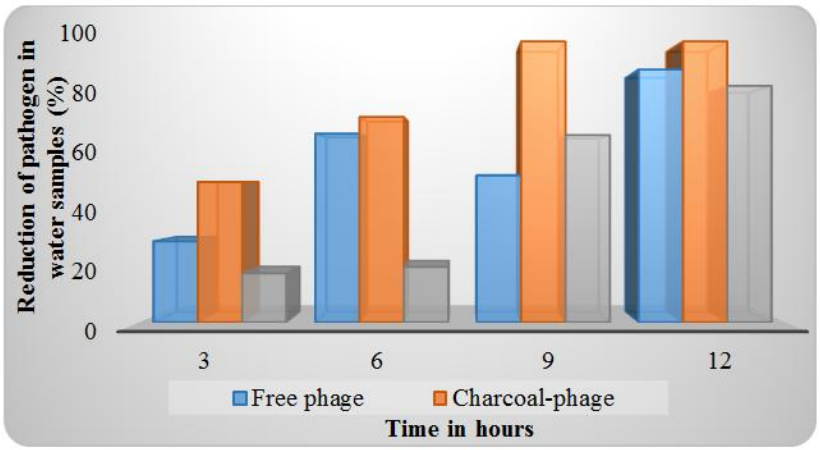

Fig-8: Effect of phage formulation on *E. coli* in batch reduction study

Table-2: Comparison of treatment efficiency among three groups using ANOVA

	n	**Mean**	**SD**	**SE**	**F**	***P* value**
Free phage	5	14.29	3.79	1.34	5.11	0.003
Charcoal-phage	5	16.94	5.01	1.77		
Alginate-phage	5	10.63	2.75	0.97		
Total	15	13.95	3.85	1.36		

(SD=Standard Deviation, SE=Standard error)

In packed column experiment, decrease in *E. coli* concentration was observed at regular intervals of time (hours). When the column was static (without feed), there was no significant change in *E. coli* concentration. Feasibility of using solid phage formulations for disinfection was found to be high in a packed bed reactor. There was a 100% removal of *E. coli* in the column packed with phage-immobilized charcoal during the 18th hour of experiment. On the other hand, only 35.35 ± 0.52% decrease in count was observed due to adsorption in the column with empty charcoal (Figure 9). In the column packed with phage-immobilized Ca-alginate beads, 97.24 ± 0.53% of reduction was observed within 18 hours (Figure-10). The column with empty phage showed very less removal of *E.coli* (12.75 ± 0.51%) and rapid decrease in bacterial population was observed after 9th hour of treatment.

In the packed bed reactor, charcoal-phage formulation established a rapid decrease in lysis after the 9th hour of treatment, which may be due to the growth cycle of phages, where maximum release occurs during 7-8 hours. It was also observed that 3 hours post addition there was a 10% increase in reduction pattern of host bacteria, using immobilized charcoal when compared to the immobilized Ca-alginate beads. This can be due to the entrapment in charcoal would have allowed the release of enough phage particles to cause considerable decrease of host population rather than the entrapment of phage in alginate beads. This explains the lesser reduction pattern observed while using phage-bead combination. Alginate beads released phage particles into the medium at a slower rate, thus causing a delayed decrease in host bacteria which is evident from 3 and 6 hours post addition of the beads. Similar results were reported in a study by [24] where the use of coliphage seeded beads indicated the release of efficient

amount of infective lytic phage that caused a significant decrease in host bacteria count of sewage water.

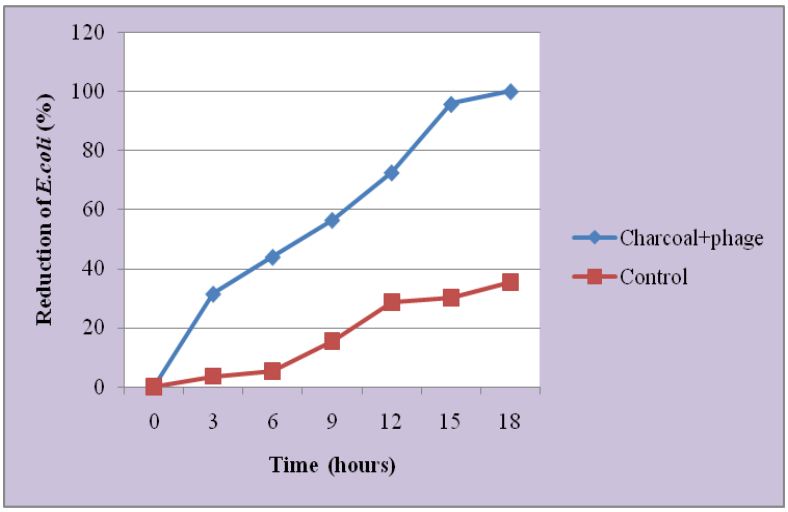

Fig-9: Percentage removal of *E. coli* treated with phage-charcoal in packed bed reactor

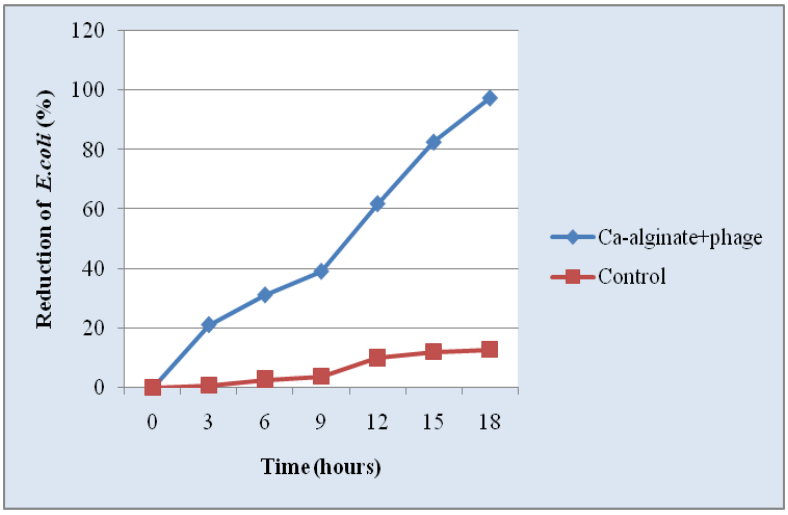

Fig-10: Percentage removal of *E. coli* treated with phage-alginate bead in packed bed reactor

The number of phage particles released during treatment in packed bed reactor was determined using UV spectrophotometry. During the time interval of 4, 8, 12 and 16 hours, a concomitant increase in the phage population in both charcoal-phage and alginate-phage treatment experiments was observed (Figure-11). The results indicated that the phage is highly potent in killing host bacteria.

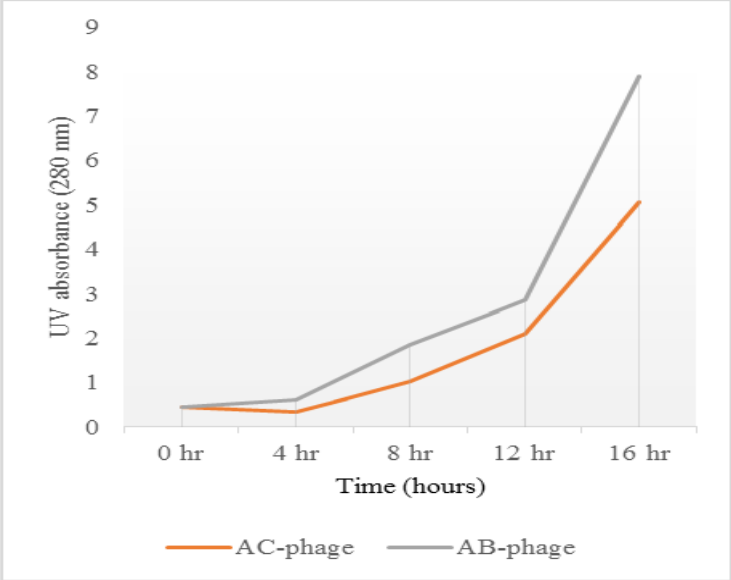

Fig-11: Quantification of phage particles in UV-Spectrophotometer (AC-activated charcoal, AB-alginate bead)

Furthermore, this work has elucidated an understanding of phage induced bacterial disinfection rates, and developed a foundation for the use of phages as bio-control agents in water systems, but additional challenges remain with regard to the control and growth of phage resistant bacteria. Thus, the emergence of phage resistant bacteria expresses the possible limitations for the use of phages for long-term use against bacterial proliferation.

CONCLUSION

The current research work focused on evaluating the potentiality of bacteriophage in reducing multi-drug resistant *E.coli* pathogens isolated from water sources. This study successfully fabricated eco-friendly, low-cost phage formulations which could be used as a bio-disinfectant by replacing chemical disinfection in water bodies. Within the time interval of 12-16 hours complete reduction (100%) of multi drug resistant host bacteria was observed. High efficiency was found in phage-charcoal combination, as both charcoal and phage had the property to remove bacteria from the water samples. However, it should be noted that only a single host pathogen was considered during this research, which covered a narrow geographical area. Nevertheless, the results obtained are important as it empowers us to consider the use of phage as an ideal tool in water research.

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