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POTENTIAL THERAPEUTIC PH RESISTIVE SALMONELLA PHAGES FROM TROPICAL SEWAGE WATERS

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ABSTRACT
Bacteriophages are seen as the alterative for the antibiotics in the era of antibiotic resistant strains of pathogenic bacteria. Two lytic bacteriophage infecting *Salmonella typhi* (STP A and STP B) was isolated from sewage samples by enrichment method. Both the phage types were found to be chloroform resistant. The phage purification had been carried by serial sub culturing of the specific type of the plaques appeared in the crude lysate up to the 10^{th} passage. Phage concentration in the purified lysate was $2x10^8$ PFU/ml for Phage STP A and the concentration of the Phage STP B was $7x10^7$ PFU/ml. The phage was purified by repeated infecting process in *Salmonella typhi* and concentrated using Polyethylene glycol 8000. Both the phages were concentrated upto the order of 10^{12}. The phages STP A & STP B were able to infect *Salmonella typhi* and other *Salmonella* sp. occurred in the same habitat. But they were not of potential candidate to infect other species apart from their host species. They were unable to induce a lytic infection even against the *E.coli*, *Shigella* isolate 1 and *E.coli DH5α.* The biological parameters of phage infection were analyzed. Both the phages were highly stable in pH conditions ranging from pH 4 to 10 and stable only upto 45°C.

KEY WORDS: Salmonella serovar typhi, Bacteriophage, Phage growth curve, stabliity

INTRODUCTION

Enteric fever is prevalent world over and continues to be a major public health problem in developing countries. In India, *Salmonella enterica serotype typhi* remains the predominant Salmonella species causing enteric fever (Jesudason, John, & John, 1996; Pillai & Prakash, 1993) *Salmonella typhi* causes a protracted bateraemic illness referred to as typhoid fever. Since humans are the only reservoir for *S. typhi*, infection is most often acquired through ingestion of food or water contaminated by feces and urine of infected persons and chronic carriers. *Salmonella enterica* has 6 subspecies with more than 2,300 known serovars having different antigenic specificity (Porwollik et al., 2004). The resistance of *S. enterica* to oral antibiotics including ampicillin, chloramphenicol, co-trimoxazole (trimethoprim-sulfamethoxazole), ofloxacin, and ciprofloxacin was increasing across all endemic areas. Bacteriophage is a generic term for a large group of agents with the ability to lyse growing bacterial cultures and as the property of regenerating themselves during their contact with growing susceptible bacteria. They represent the largest of all virus groups and found in many diverse natural habitats in enormous numbers (Ackermann, 1987). They are widely distributed in nature and even found in the intestinal tracts of man and animals. These particles can exist independently outside the cells and must enter into a host bacterium to replicate. Phages are not only the most abundant biological entities but probably also the most diverse ones. The phages behave as a non living entity, outside the host cell and they always depend on the host cell for replication and other metabolic processes (Carlton, 1999). All phages consist of protein and nucleic acid, however some phages have lipid coat. The nucleic acid is either DNA or RNA but not both, either they may be single stranded or double stranded. As phages are the viruses specific for bacteria they can be considered as potential antibacterial agents. Unlike broad- spectrum antibiotics phages are highly specific. Hence they do not elicit resistance in untargeted bacterial strains (Sulakvelidze, 2005). Phages have thrown lights in the battle against bacterial infections, in particular multidrug resistant bacterial infections. They are considered as natural antimicrobial agents. Phage therapy has increased scope to control systemic and intracellular infections. Endolysins or virolysin are dsDNA bacteriophage encoded enzymes produced during the lytic cycle, late phase of gene expression to degrade the bacterial cell wall peptiodoglycan. Virolysins belong to a group of bacterial cell wall hydrolases. These virolysins have been demonstrated to control pathogens *E. faecalis* and *E. faecium*, *Staphylococci*, *B. anthracis*, Group A *Streptococci*, *S. pneumonia* and *Clostridium* bacteria.

MATERIALS AND METHODS

Collection of sewage sample

Sewage water was sampled using sterile dark containers from the open sewage located at Alwarkurichi, Tirunelveli district during the month of January 2012. The sample collected was brought immediately to the laboratory and filtered with a filter paper and spinned at 1500 rpm for 5 mins to remove the debris. The filtrate was stored at 4°C.

Isolation of Salmonella phage

10 ml of sample and 5 ml of Logarithmic phase cells of Salmonella serovar typhi MTCC No 3917 (potential recipient) was mixed with 50 ml double strength

Trypticase Soy Broth broth (Himedia, Mumbai) and incubated at 37°C on rotary shaker to enrich the phages specific to the host. After 24 hours of incubation, 4ml of chloroform was added to the mixture and incubated at 4°C for 4hours. Later, 10 ml of the supernatant was centrifuged at 8000 g for 15 min and the supernatant was filtered through 0.22µm PVDF which is supposed to be the expected phage, free of live bacterial cells (cell lysate) was stored at 4°C.

Plaque assay

The cell lysate (0.1ml) and exponentially grown host cells (0.2 ml) were suspended in 3 ml aliquots of molten soft agar (0.7% agar) held at 45°C was mixed and uniformly dispensed over hard agar (1.5% agar), incubated at 37 ± 2˚C for 12 h. A positive control *S. typhi* specific bacteriophage was used. Negative control was maintained by using sterile phage free water as lysate.

Purification of bacteriophage

The phage purification was done by repeated sub culturing of the plaques. The plaques formed in the plaque assay were sub cultured by picking the plaques in a sterile inoculation loop and inoculated in a fresh overnight *Salmonella typhi* culture broth and the cell lysate were prepared as mentioned earlier and sub cultured repeatedly.

Enumeration of phage titre

The cell lysate was serially diluted in saline water (0.85%) and plated on the soft agar overlay with the host S*almonella* phage. From the plaque count the phage titre was enumerated and expressed in PFU.

Concentration of phages

The phage lysate solution was concentrated in two steps. By centrifugation, the phage lysate was centrifuged for 9000 rpm for 30 minutes discarding the pellet. This step was repeated two times. The supernatant obtained after the centrifugations were further concentrated using polyethylene glycol. PEG 8000 was added at a concentration of 8% w/v, stored for 12 hours at 4° C. The mixture was centrifuged for 8000 rpm for 15 minutes, the retained pellet was diluted in STE buffer (10 mM Tris (pH 8), 1mM EDTA (pH 8), 0.1 M NaCl) stored at 4°C.

Host range determination

The capability of the phages to replicate in different species of bacteria was analyzed using fifteen bacterial strains. Bacteriophage lysis assay was carried out using the strains of *Klebsiella pneumoniae* (MTCC – 109), *Salmonella typhimurium* (MTCC – 1251), *Staphylococcus epidermidis* (MTCC – 3615), *Salmonella typhimurium* B, *Staphylococcus aureus* (MTCC – 96), *Salmonella serovar typhi* (MTCC -3917), *Enterobacter aerogenes*, *E. coli*, Salmonella isolate 1, Salmonella isolate 2, Shigella isolate 1, *E. coli* DH5α by means of spot plaque technique. Early log phase bacterial culture was spread on trypticase soy soft agar with a drop of phage stock solution (10^8 PFU/ml) added on the bacterial lawn. The plates were incubated at 37 °C overnight and examined for the presence of clear zones of lysis.

Stability of the phages

The thermal stability of the phage was estimated. The phage lysate was incubated at different temperatures (35, 45, 55, 65, 75, 85, 95, 100° C) for 1 hour and subjected to spot assay technique. Similarly the stability of the phage in different pH conditions (2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) was attempted.

Growth curve

One ml of bacterial (10^6CFU/ml) and viral suspension (10^5 CFU/ml) were mixed and incubated on a shaker at 120 rpm at 28±2˚C. Two 0.1ml of samples were retrieved from this incubated mixture at regular time intervals (5, 10, 15, 25, 35, 45 and 55 min) of which one sample was used as such for the plaque assay and the other was treated with chloroform to kill the remaining bacteria and used. The number of phage particles was determined. The plaques were counted and viral titre was estimated and expressed as plaque forming unit per millilitre (PFU/ml).

RESULTS

Isolation of *S. typhi* specific bacteriophage

Salmonella specific bacteriophages were isolated from the open sewage located at Alwarkurichi, Tirunelveli district in the month of October 2012 using enrichment method. The double layer agar method used for the isolation of phage yielded a mixed type of plaques (Figure – 1). The crude phage lysate produced a mixture of plaques, medium and small in size on the *Salmonella typhi* lawn grown on 0.6% soft agar. All the plaques were transparent with their edges clearly marked. The difference among the plaques were in their sizes with no evident other morphological characteristics. Two types of plaques were distinguished based on their sizes one with an average plaque size of 1.5mm in diameter (Phage STP A) and the other with 0.7mm in diameter (Phage STP B) on 0.7% soft agar. The total concentration of Salmonella specific phages of both types in the enriched crude lysate was $5x10^6$ PFU/ml and were chloroform resistant. They were stored along with 0.1% chloroform during the entire study period of 6 months. Cholroform did not elicit any significant reduction in the phage titre. The phage purification was carried by serial sub culturing of the specific type of the plaques in the crude lysate upto the 10^{th} passage. The plaque population produced by phage samples of the 10^{th} passage consisted of single type of plaques with smaller plaque size specifically. Phage concentration in the purified lysate was $2x10^8$ PFU/ml for phage STP A and $7x10^7$ PFU/ml for phage STP B respectively (Table – 1).

TABLE 1. Plaque assay of the crude enriched phage lysate

S.No	Dilution	Number of plaques
1	10^{-1}	TNTC
2	10^{-2}	TNTC
3	10^{-3}	TNTC
4	10^{-4}	56
PFU/ml – $5x10^6$		

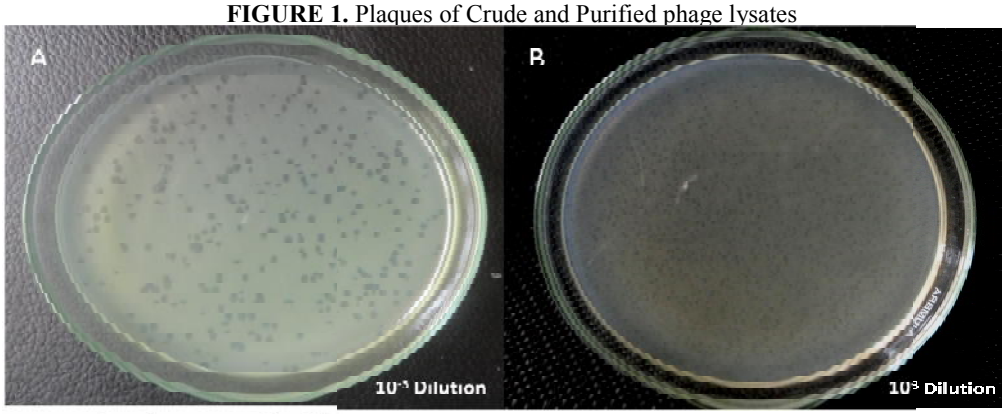
Both the phages were concentrated using PEG 6000 and assayed for their killer titres. The titre values of the phages were $8x10^{12}$ PFU/ml for the phage STP A and $2x10^{12}$ PFU/ml for phage STP B. For both the phages, the increase in the concentration of the titre was in the order of 10^{12}. On soft agar, STP A and STP B appeared to have a size of 0.6mm and 1.3mm respectively (Table – 2).

TABLE 2. Plaque assay of the purified and concentrated phage lysate

S.No	Dilution	Phage STP A lysate No of Plaques		Phage STP B lysate No of Plaques	
		Purified	Concentrated	Purified	Concentrated
1	10^{-1}	TNTC	TNTC	TNTC	TNTC
2	10^{-2}	TNTC	TNTC	TNTC	TNTC
3	10^{-3}	TNTC	TNTC	TNTC	TNTC
4	10^{-4}	TNTC	TNTC	TNTC	TNTC
5	10^{-5}	TNTC	TNTC	72	TNTC
6	10^{-6}	26	TNTC	-	TNTC
7	10^{-7}	-	TNTC	-	TNTC
8	10^{-8}	-	TNTC	-	TNTC
9	10^{-9}	-	TNTC	-	TNTC
10	10^{-10}	-	42	-	22
11	10^{-11}	-	8	-	-
	PFU/ml	$2x10^{8}$	$8x10^{12}$	$7x10^{7}$	$2x10^{12}$

TNTC – To Numerous To Count

FIGURE 1. Plaques of Crude and Purified phage lysates



A – Mixed types of wild type Plaques, B – Purified Phage STP B Plaques on the lawns of *Salmonella serovar typhi* MTCC No 391

Host range

Host range of the two phages was evaluated against wide range of bacterial species. Both the phages did not produce plaques on the spot assay experiment on the lawn of the tested bacterial strains viz., *Klebsiella pneumoneia* (MTCC – 109), *S. typhimurium* (MTCC – 1251), *Staphylococcus epidermidis* (MTCC – 3615), *S. typhimurium B*, *Staphylococcus aureus* (MTCC – 96), *E. aerogenes, E. coli, Shigella isolate 1* and *E. coli DH5α*

TABLE 3. Host range of the purified phage lysate

Culture	Infectivity	
	Phage STP A	Phage STP B
K. pneumoniae (MTCC – 109)	-	-
S. typhimurium (MTCC – 1251)	-	-
S.epidermis (MTCC – 3615)	-	-
S. typhimurium B	-	-
S. aureus (MTCC – 96)	-	-
S. serovar typhi (MTCC -3917)	+	+
E. aerogenes	-	-
E.coli	-	-
Salmonella isolate 1	+	+
Salmonella isolate 2	+	+
Shigella isolate 3	-	-
E.coli DH5α	-	-

\+ Denotes infectivity
\- Denotes non infective

Whereas visible plaques were produced on the bacterial lawns of the test bacterial cultures viz., *Salmonella* serovar *typhi* (MTCC -3917), Salmonella isolates 1 and 2 which indicates the induction of lytic infection on these bacterial strains (Table – 3). The bacterial strains isolated from the same sewage sample were susceptible for the phages with an exception of Shigella isolate.

Stability of the phages

STP A and B phages were incubated in different pH (2-11) for 1 hr at room temperature and their titres were calculated. The phage titre was highly stable in acidic condition up to pH 4 and started to decline at the pH 3. No visible plaques were seen at pH 2. Similarly in the basic conditions, the phage titre remained stable up to pH 10 after which if declined. Both the phages were highly stable in pH (4-11) expressing similar characteristics in extreme pH conditions (Table – 4).

Both the phages were unstable at higher temperatures. Both the phages were incubated on different temperature conditions for 2 hr and their titer was calculated. The titer of the phages were highly stable up to 45°C and the titer of the phages started to decline rapidly after the temperature reaching 55°C. Beyond this temperature both the phages have lost their characteristics of producing visible plaques on the bacterial lawns. No visible plaques were seen at and above the temperature of 55°C. The Phages STP A and STP B have behaved in the similar manner at higher temperatures (Table – 5).

TABLE 4. Stability of the STP phages in different pH conditions

pH	Infectivity	
	Phage STP A	Phage STP B
2	-	-
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+

\+ Denotes infectivity
\- Denotes non infective

TABLE 5. Stability of the STS phages at different Temperatures

Temperature	Infectivity	
(°C)	Phage STP A	Phage STP B
35	+	+
45	+	+
55	-	-
65	-	-
75	-	-
85	-	-
95	-	-
100	-	-

\+ Denotes infectivity
\- Denotes non infective

Growth parameters of Phages

The growth parameters of the phages STP A & STP B were projected by one step growth analysis. The phage STP A & STP B lytic life cycle in *Salmonella typhi* host was characterized. Shortly after the initiation of infection, samples were collected and analysed for every 5 min, the dynamical change in the PFU/ml value of the total and free phages during the infection process was used to determine the different stages and properties of lytic life cycle of the phages. The eclipse period for the phage A was short period of 10 min and the latent period follows the eclipse quickly on 15 min from the initiation of the infection which also marks the initiation of the rise period. The rise period persisted for the next 10 min, (ie) 25 min. The end of the rise period specifies the completion of first round of lytic life cycle of the phage STP A. The burst size was of 101 PFU per infected cell after 25 min at RT. Similarly the Phage STP B exhibited an eclipse period of 15min, followed by a latent period of 20min and a rise period starting at 20min and lasting upto 30 min. the phage yielded a burst size of 112 PFU per infected cell (Table 6-7) (Figure – 2) .

FIGURE 2. Growth curve of the selected phages

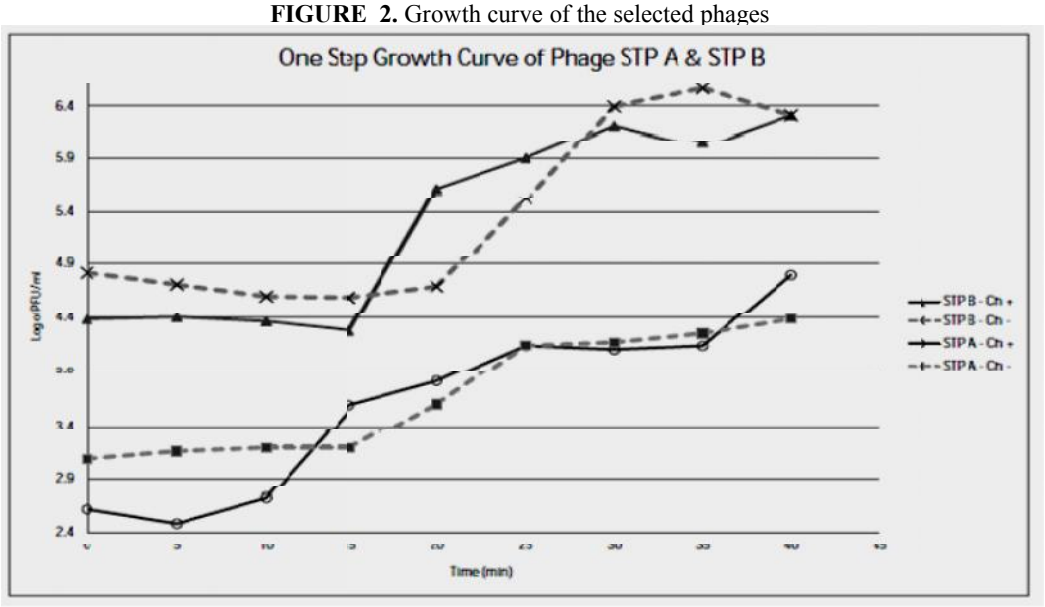


TABLE 6. One step growth curve of phage STP A

S.No	Phage sample	Time	Sample	Log of PFU
1	Phage STP A	0	Ch-	3.079
			Ch+	2.602
		5	Ch-	3.146
			Ch+	2.477
		10	Ch-	3.176
			Ch+	2.698
		15	Ch-	3.176
			Ch+	3.591
		20	Ch-	3.602
			Ch+	3.806
		25	Ch-	4.146
			Ch+	4.146
		30	Ch-	4.176
			Ch+	4.113
		35	Ch-	4.255
			Ch+	4.146
		40	Ch-	4.380
			Ch+	4.799

Ch- Denotes without chloroform
Ch+ Denotes with chloroform

TABLE - 7. One step growth curve of phage STP B

S.No	Phage sample	Time (min)	Sample	Log of PFU
1	Phage STP B	0	Ch+	4.380
			Ch-	4.819
		5	Ch+	4.397
			Ch-	4.716
		10	Ch+	4.361
			Ch-	4.612
		15	Ch+	4.278
			Ch-	4.602
		20	Ch+	5.633
			Ch-	4.699
		25	Ch+	5.903
			Ch-	5.518
		30	Ch+	6.230
			Ch-	6.397
		35	Ch+	6.041
			Ch-	6.556
		40	Ch+	6.322
			Ch-	6.322

Ch- denotes without chloroform
Ch+ denotes with chloroform

DISCUSSION

Phages can be isolated from diverse environment such as sea water, soil, sewage water/sludge etc. They have been found to be effective against almost all the pathogenic bacteria. The present study describes the isolation and partial characterization of bacteriophages against the human pathogen *Salmonella typhi*. Bacteriophages were isolated from sewage using the methods described by (Spencer, 1955). The sewage sample was enriched and assayed against *Salmonella typhi*. For almost all the bacteria that prevails there exists corresponding phage which represents them as a biological control for specific bacterial pathogens (Lederberg, 1996). In India, *Salmonella enterica serotype typhi* remains the predominant Salmonella species causing enteric fever (Jesudason et al., 1996). Hence sewage remains a better source for isolation of *S. typhi.* Likewise the probability of obtaining a phage specific for *S. typhi* was also very high as the sewage harbors Salmonella sp. Isolation of phages from the environment of suspected bacterium residing was a customary against various bacteria (Xie, Zhuang, Kong, Ma, & Zhang, 2005). The crude lystae of enriched sewage produced a mixed type of circular plaques. A single habitat can harbor many types of bacteriophages specific for single species of bacterium. Salmonella bacteriophages associated with swine effluent lagoons produced several bacteriophages with Podoviridae morphology (McLaughlin et al., 2003). It is usually assumed that a single virus particle kick off an single plaque but not all virus particles can initiate infections and form plaques in a given sample (Kleczkowski & Kleczkowski, 1951). The infection of a single particle of phage results in a circular plaque morphology because of repeated cycles of infection on the embedded host cells by the numerous viral progeny propagating in all directions like a wave raising for a single point (You & Yin, 1999). In a similar

environmental condition, bacterial strain and other factors, the size and morphology of a plaque of a particular phage remains the same. As the crude lysate produced two types of plaques of different sizes in a single experiment, it was believed to be two different types of phages. The phage STP A and STP B were resistant to chloroform which confirms that the phage coat was believed to be made up of protein with the absence of lipids.

Application of PEG to separate and concentrate viruses was been widely used. (Leberman, 1966). The broad spectrum of animal, plant, and bacterial viruses had been concentrated by PEG earlier. The phages STP A & STP B which were purified by repeated sub culturing process was concentrated by PEG. Both the phages were concentrated up to the order of 10^{12}. The host range of the bacteriophages was determined by Spot Test (Armon & Kott, 1993). The phages STP A & STP B were able to infect *S. typhi* and other Salmonella sp. occurred in the same habitat. But they were not potential candidate to infect other species apart from their host species. They were unable to induce a lytic infection even against the *E.coli*, *Shigella isolate 1* and *E.coli DH5α* which were closely related to bacterium species to *Salmonella*. The phages were unable to lyse other bacterium *K.pneumonia* (MTCC – 109), *S. typhimurium* (MTCC – 1251), *Staphylococcus epidermis* (MTCC – 3615), *S. typhimurium B*, *Staphylococcus aureus* (MTCC – 96) and *E. aerogenes*. Studies of bacteriophage infection have revealed that the process is initiated when the virion interacts with host cell surface receptor molecules. Many bacteriophages were known to be highly specific to their receptors which in turn make it highly specific for the host and show minimal or denial of interaction with receptors with a even slightly different structure within the same bacterium or from different species. Bacteriophage P1, a transducing phage can induce host cell lysis on several enteric species including *E. coli* (Yarmolinsky, 2004). Even though some bacteriophages do infect a range of bacterial species, the present research indicates that some bacteriophages were highly specific to their host. The occurrence of broad host- range phages depends on the origin of virus particles which comprises such a large percentage of the dissolved organic carbon in marine ecosystems (Torrella & Morita, 1979). The results of the host range analysis substantiate that the host specific phages can be frequently and readily isolated from complex natural microbial communities as judged by plaque-forming ability. It is convinced that many bacterial species existing in close proximity in many natural microbial communities which hold complex and rich assemblages of many bacterial species (Jensen et al., 1998). As of the results of Jensen et al. (1998) this study also convinced that sewage is a best source for the isolation of host specific bacteriophages against human pathogens. The phages were highly prevalent and can be readily isolated from sewage ecosystems against human pathogens as they were the reservoirs of human pathogen.

The phages STP A & STP B were assayed for their abiotic stress tolerance properties and characterized on the physical resistance parameters. The phages were highly stable in a wide range of alkaline and acidic conditions. Both the phages were highly stable in pH conditions ranging from pH 4 to pH 10. Below the pH of 4 and above the pH of 11, the titres of the phages starts to decrease which is inversely proportional to the increase in the acidic or basic conditions of its habitat. The phages were completely inactivated in pH 2 and 11. The phages were stable in a narrow spectrum of temperature higher than the room temperature. The phage STP A and STP B were stable only up to 45°C and the titre started to reduce rapidly beyond 45°C, and completely inactivated after 55°C. These results indicate that the phages were highly stable in extreme acidic and basic conditions but very unstable in higher temperatures. This contradictory abiotic stress resistant and sensitivity can be explained based upon the habitat of the phages where it originated/lived along with their host. Usually sewages in the temperate regions like Southern India which roughly lies in the 8.0°N, which is just 8.0° North of equator do not have high temperature variations during their entire year. As the phages in the sewage were exposed to minimal temperature variations, their adaptation to extreme temperatures may not be on their charts. For their stability in a wide range of pH can be explained by the following abiotic and biotic factors present in the sewage where the pH can be oscillated from acidic to basic. The factors responsible for acidic nature of the sewage may be due to the abundance of aerobic and anaerobic bacterial communities present in the sewage and their metabolic activities. Similarly the addition of new sewage from household and from industries may have influenced the pH bias towards basic. This oscillation in the pH might have induced the phages to adapt itself to survive in wide pH environments. There are few reports available stating the availability of thermal resistant phages in sewages. Moce-Llivina, Muniesa, Pimenta-Vale, Lucena, and Jofre (2003) isolated phages from sludge and sewage, even after a thermal treatment to the sample. Goodridge, Gallaccio, and Griffiths (2003) determined the growth kinetics of two bacteriophages with burst size 177 and 38 PFU/cell, latent periods of 52 and 40 min. The length of the latent period depends on the specific phage growth rate, physiological conditions, host, incubation conditions, medium, and temperature.

Phages represent a group of viruses that specifically infect and replicate in bacteria. Phages specific for *S. typhi* may be useful as biocontrol, therapeutic, or diagnostic agents to control and detect the prevalence of *S. typhi* in animals and food. Phage can be used as a biocontrol to reduce of Salmonella and Campylobacter contamination of chicken skin (Goode, Allen, & Barrow, 2003). The bacteriophage of *S. typhi* isolated and partially characterized can be easily propagated in the laboratory and used to reveal the interactions with *S. typhi*.

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