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Original Research Paper

Bacteriophage Based Pathogen Reduction in Sewage Sludge

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ABSTRACT

Biological hazard in water resources in the form of pathogenic organisms are responsible for major outbreak in most of the developing countries. The goal which gains momentum is removal of pathogens. Every effort leading to reduction in sewage pollution and pathogenic microbes has to be promoted and implemented. This necessitates to search for novel approaches that does not harm the environment. One such novel approach is exploring the possibilities of bacteriophages for pathogen removal. Sewage sludge samples were collected from different locations of Tamil Nadu and analysed. The pH of the sludge samples varied from 6.26 to 8.23 and alkaline pH was observed in Coovum sample. Highest EC was recorded by Vellore sample (4.62 dSm^{-1}). The total heterotroph population ranged from 11×10^6 to 24×10^{14}/kg of dewatered sludge. Higher frequency of antibiotic resistant *E. coli, Pseudomonas* sp., *Streptococcus* sp. and *Bacillus* spp. were observed in all the places, which clearly indicated the extent of pollution. *E. coli* and *Salmonella typhi* showed resistance to almost all the antibiotics and intermediate resistance to 3 antibiotics. None of the sewage sludge samples had phages against MTCC culture. Phage treatment resulted in 100 % removal of *S. typhi* from sewage sludge.

INTRODUCTION

Sewage treatment systems were introduced in cities after Louis Pasteur and other scientists showed that sewage borne bacteria were responsible for many infectious diseases. From the early 1970 to 1990s, wastewater treatment objectives were based primarily on aesthetic and environmental concerns. The earlier objectives of reduction and removal of BOD, suspended solids and pathogenic microorganisms continued, but at higher levels.

In general during wastewater treatment process combinations of physical, chemical and biological methods were in practice. Many sewage waste treatment systems are aiming for complete pathogen removal. Several developed and developing countries embarked on programmes to reduce water-borne multidrug resistant bugs (MDR). The main cause for the emerging MDR is indiscriminate release of hospital wastewater into public sewage (Summers 2001, Chitnis et al. 2000, Ekhaise & Omavwaya 2008).

The purpose of disinfection in the wastewater treatment is to substantially reduce the number of living organisms in the water to be discharged back into the environment and that can be fulfilled with phage treatment (Ewert & Paynter 1980, Thiel 2004 and McDonald 2008). Interest in the ability of phages to control bacterial population has extended from medical application into the fields of agriculture, aquaculture, food industry and very recently for water treatment also. The reason is phages stop reproducing as long as the specific bacteria they target are dead, very specific, therefore dysbiosis and chances of developing secondary infections are avoided and can be targeted more specifically to bacterial surface receptors. So there is the potential application of phages in wastewater treatment system to improve effluent and sludge disposal into the environment. Most pathogens are associated with sludge flocs rather than liquid portions. Sludge biology should also be concentrated during the phage treatment. Hence, the following research work has been initiated to utilize the specific phages as biocontrol agents against the potential pathogens in sewage sludge. The research outcome of this project is directly applicable to Corporations and Panchayats, which face many difficulties in handling voluminous sewage water and sludge.

MATERIALS AND METHODS

Characterization of sewage sludge: Sewage sludge samples were collected from seven locations in different places of Tamil Nadu. The samples were collected in presterilised containers from the following places: 1. Ukkadam-1, Coimbatore; 2. Ukkadam-2, Coimbatore; 3. Kavundamplayam; 4. Coovum, Chennai, 5. Vellore, 6. Theni and 7. Perundurai, Erode. The physico-chemical and biological characterization of the samples was done as per the standard methods (APHA 1989).

Bacteriological analysis of sewage sludge: The bacteriological analysis was done to determine the sanitary condition of the water (Cappuccino & Sherma 1996). The

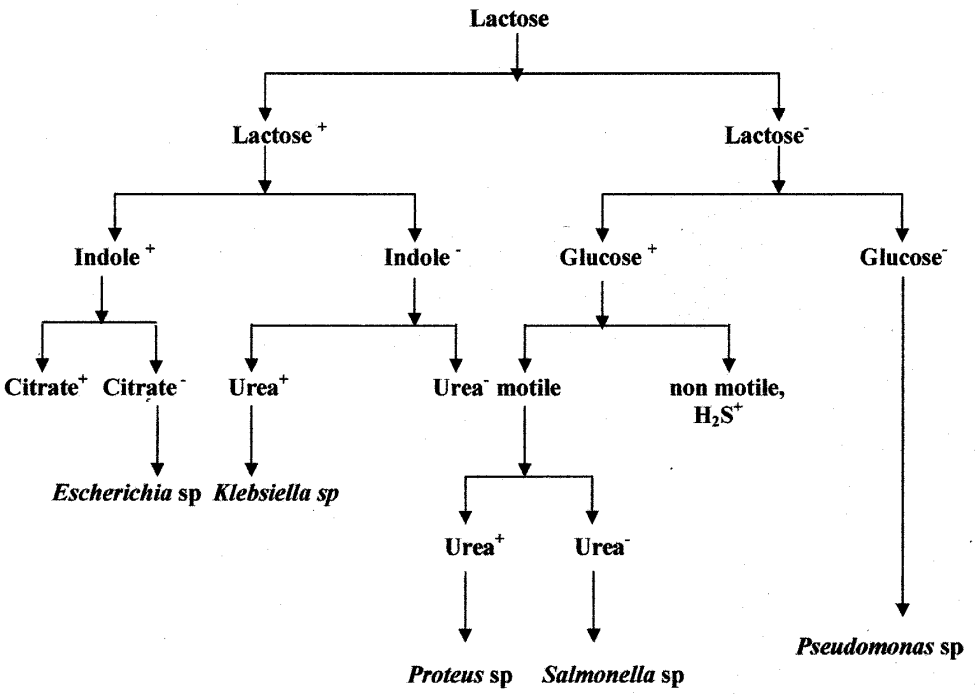

Fig. 1. Separation outline of target pathogens

samples were also plated in specific media to isolate the microorganisms. All the morphological, cultural and biochemical tests were performed based on the methods suggested by Holt et al. (1994) and Johnson & Case (1995). Potentially dreadful pathogens like *E. coli, Salmonella* sp., *Pseudomonas* sp., *Klebsiella* sp., *Staphylococcus* sp., *Streptococcus* sp., *Proteus* sp. and *Bacillus* spp. were isolated and characterized (Fig. 1).

Antibiogram of target pathogens: All the antibiotic tests were done based on the Kirby Bauer sensitivity disc method suggested by Bradshaw (1979) and Hiruta et al. (2001). For the estimation of the MDR bacteria, 100 µL diluted samples were spread over MacConkey agar plates supplemented with 30 µg/mL of chloramphenicol and 20 µg/mL of gentamycin (Saha et al. 1992).

Isolation of specific bacteriophages for target pathogens: Enrichment was done to increase the number of phage virions for the target pathogens isolated from the sludge sample, since host specificity is central to selection of suitable phages for particular wastewater treatment applications (Sulak Velidze et al. 2001). When confluent lysis has occurred, 5 mL of SM buffer was added to the plate and gently scrape the soft agarose into sterile centrifuge tube and tubes were spun at 4000 rpm for 10 min at 4°C, and the supernatant was recovered, to that one drop of chloroform was added to lyse the remaining cells. Thus, prepared bacteriophages were maintained as stock. Many bacteriophages require divalent cations such as Mg^{++} and Ca^{++} for attachment to bacterial host cells. Hence, it is essential to grow in bacterial growth medium with 10 mM $MgSO_4$ and 0.2% maltose. Magnesium and maltose facilitate the entry of phage particles into the cell (Marks & Sharp 2000).

Isolation of phages for MTCC cultures: Bacteriophages are highly specific and should be isolated from the same environment, where the host is isolated. To check the specificity of the phages, the following cultures were obtained from MTCC, Chandigarh and tested against the phages isolated from sewage.

MTCC code	Name of the organism
86	*Serratia marcescens*
98	*Salmonella typhimurium*
3917	*Salmonella typhi*
740	*Staphylococcus aureus*
1302	*Escherichia coli K-12*
1303	*Escherichia coli B*
1588	*Eschericha coli CSh 57.*
1650	*Escherichia coli KL 16*
1652	*Eschrichia coli DH5 a*
1748	*P. fluorescens*
310	*Sachharomyces cerevisiae*
7299	*Proteus vulgaris*
7664	*Enterobacter aerogenes*

Characterization of the identified bacteriophages: Characterization of phages viz., one step growth curve (Ellis &

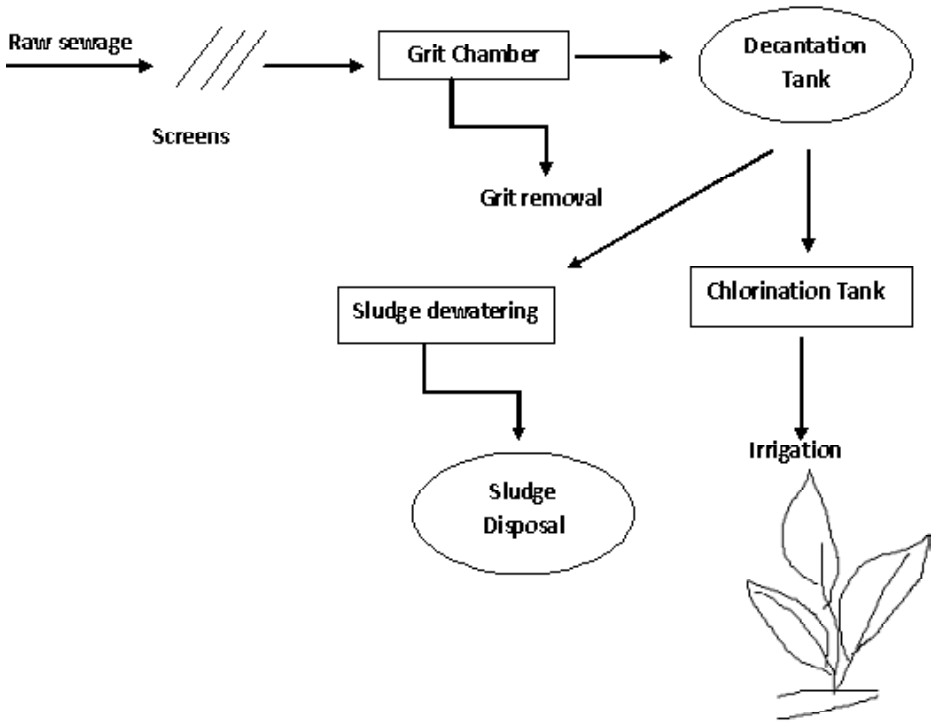

Fig. 2: Sewage treatment process at Coimbatore Corporation sewage farm.

Delbruck 1939) and multiplicity of infection are essential for fixing the time of treatment and dose of the phage dilutions to be used for wastewater purification (Sambrook & Russell 2001). The MOI of *E. coli* and *S. typhi* was assessed in one of our previous study (Sagkaguchi et al. 1989 and Dhevagi & Anusuya 2011) and used for the sludge treatment.

Developing an eco-friendly bioconsortium for augmenting the pathogen in sewage sludge: In the city of Coimbatore 650 km of drains were to be laid and linked to three sewage treatment plants coming up in Ukkadam, Nanjundapuram and Ondipudur, by the end of 2012. Coimbatore generates large quantity of sewage.

Ukkadam sewage treatment plant: The plant, built at Rs. 55 crore under the Jawaharlal Nehru National Urban Renewal Mission Scheme, has a capacity to treat 70 MLD (million litres a day) sewage. At present, the plant treats only about 20 MLD of wastewater which flows into it from the areas that already have underground drainage system. The sewage treatment plant has been constructed based on the 'Sequential Batch Reactor' (SBR) process, which was the most advanced method for sewage treatment (Fig. 2).

The *E. coli* and *Salmonella* sp. organisms were inoculated into sewage sludge. Sewage sludge collected from Ukkadam was used for the study. The following are the treatments.

T1: Sewage sludge inoculated with *E. coli* and *E.coli* specific bacteriophages.

T2: Sewage sludge inoculated with *Salmonella* sp. and *Salmonella* sp. specific bacteriophages.

T3: Sewage sludge inoculated with *E. coli* and *Salmonella* sp. specific bacteriophages.

T4: Control

Sewage sludge was collected and filtered and 100 mL of sewage sample (water and sludge) were taken in Din thread screw bottles and sterilized. After cooling it was inoculated with *E. coli* at @ 10^4/mL and *Salmonella* sp. at @ 10^3/mL. After inoculation of pathogens the cell count was assessed for checking the phage efficacy. Serial dilutions were carried up to 10 dilutions. From the serially diluted samples, 0.1 mL of pathogenic cultures were added to sterile plates containing LB (with sewage extract and without sewage extract) and incubated at 37°C for 24 hours. At every 1 hr the pathogen survival was assessed up to 14 hours.

RESULTS AND DISCUSSION

Characterization of sewage sludge: During the past decade, sewage water gets accumulated in the form of stagnant water and if there is drinking water pipes nearer, there is a chance for intrusion of sewage water into the drinking water. In

Table 1: Characterization of sewage sludge.

S.N	Parameters	Uk- 1	Uk- 2	Kvu	Cvum	Vellore	Theni	Per
1	pH	6.26	6.89	7.21	8.23	7.42	6.98	7.32
2	EC dSm^{-1}	2.23	1.98	2.88	4.23	4.62	1.89	1.87
3	Total N (%)	3.90	3.82	2.41	4.10	2.60	3.68	2.46
4	NH_4^+-N(%)	0.65	0.65	0.12	0.41	0.38	0.58	0.39
5	NO_3-N (%)	0.05	0.06	0.09	0.08	0.03	0.08	0.06
6	P (%)	2.50	2.40	1.80	2.30	1.20	1.78	1.42
7	K (%)	0.40	0.38	0.24	0.51	0.38	0.25	0.35
8	Na (%)	0.57	0.54	0.47	0.59	0.23	0.64	0.29
9	Ca (%)	4.9	4.3	3.9	2.7	3.2	2.8	4.3
11	Fe (%)	1.3	1.1	0.98	0.90	0.61	1.2	0.8
12	Total heterotrops (cfu/100 mL)	24×10^{14}	18×10^{14}	14×10^{10}	12×10^{14}	8×10^{10}	11×10^{6}	12×10^{6}
13	Arsenic (mg/kg DW)	9.9	9.8	8.9	7.8	8.1	7.4	8.6
14	Cadmium (mg/kg DW)	6.94	7.78	7.10	6.12	6.08	6.03	6.52
15	Chromium (mg/kg DW)	49	43	14	121	249	98	85
16	Copper (mg/kg DW)	741	698	623	597	621	710	705
17	Lead (mg/kg DW)	134.4	124.3	118.0	112.0	98.5	68.3	141.0
18	Mercury (mg/kg DW)	5.2	5.1	4.9	nil	3.8	1.2	0.9
19	Molybdenum (mg/kg DW)	9.2	8.6	8.3	7.1	6.5	4.9	6.7
20	Nickel (mg/kg DW)	42.7	40.8	39.8	nil	nil	21.3	14.9
21	Selenium (mg/kg DW)	5.2	5.1	4.9	3.9	1.8	3.2	4.6
22	Zinc (mg/kg DW)	1,202	1,104	1,009	987	1,023	Nil	Nil

Table 2: Microbiological analyses of sewage sludge collected from different locations.

S. No.	Location	Colony forming units								
		Total bacteria $\times 10^6$	*E. coli* $\times 10^2$	*Salmonella* sp $\times 10^2$	*Pseudomonas* sp $\times 10^2$	*Klebsiella* sp $\times 10^2$	*Azotobacter* sp	*Trichoderma* sp	*Aspergillus* sp	Yeast
1	Ukkadam-1, Coimbatore	24×10^8	76	80	2	-	28	25	32	14
2	Ukkadam-2, Coimbatore	18×10^8	35	78.56	-	-	20	12	16	8
3	Kavundampalayam	14×10^4	26	68.57	3	ND	15	32	21	9
4	Coovum, Chennai	12×10^8	46	101.24	4	ND	58	18	87	32
5	Vellore	8×10^4	38	45.62	-	-	12	15	11	18
6	Theni	11×10^1	24	57.89	-	-	11	8	14	17
7	Perundurai, Erode	12×10^1	12	5.46	-	-	12	7	22	61

developing countries 70% of the water is seriously polluted and 75% of illness and 80% of the child mortality is attributed to water pollution (Zoetman 1980, Sangu & Sharma 1987). Untreated wastewater contains numerous disease causing microorganisms and toxic compounds that dwell in the human intestinal tract may contaminate the land or water body where sewage is disposed. According to WHO estimate about 80% of water pollution in developing country, like India is carried by domestic waste (Moharir et al. 2002). Raw sewage disposal into the estuaries has been a common practice throughout the world (Yanggen & Born 1990, Tyagi et al. 2000, Das Gupta & Purohit 2000). It is therefore of interest to determine what levels of pollution indicator bacteria are owing to sewage disposal. This information would help to determine if careful waste treatment and disposal procedures are needed to safeguard the natural environment. The sludge samples collected from the seven locations were analysed and the results are given in Table 1.

The pH of the samples varied from 6.26 to 8.23 and alkaline pH was observed in Coovum sample. Highest EC was recorded in Vellore sample 4.62 dSm^{-1} and this may be due to high amount of salt discharge. The sludge had very high nutrient content; particularly phosphorus content was very high. Sodium content varied from 0.23 to 0.64 % (Ramalingam & Suniti 2010). The total heterotroph population was high and it ranged from 11×10^6 to 24×10^{14}/kg of dewatered sludge and this may be due to high organic content which facilitates the microbial growth. The heavy metal concentration was also high. Many of the samples had heavy metal concentrations above the acceptable limits prescribed by the Pollution Control Board. The presence of high concentration of nutrients, heavy metals and microbial population necessitates the sewage sludge treatment.

Bacteriological analysis of sewage sludge: The main objective behind the bacteriological analysis is to determine the faecal pollution, which is paramount in assessing the

Table 3: Resistance patterns of MDR bacteria isolated from sewage sludge.

S. No	Antibiotics	Ukkadam-1 Coimbatore	Ukkadam-2 Coimbatore	Kavunda-mpalayam	Coovum Chennai	Vellore	Theni	Perundurai Erode
1	Ciproflaxin (10 mcg)	I	I	R	R	R	R	S
2	Tetracycline (30 mcg)	R	R	R	R	S	I	S
3	Streptomycin (10 mcg)	S	R	I	I	I	R	S
4	Kanamycin (10 mcg)	S	R	S	I	I	S	R
5	Ampicillin (10 mcg)	I	R	R	I	R	R	I
6	Erythromycin (15mcg)	R	R	S	R	R	I	S
7	Penicillin (10 mcg)	I	R	S	S	R	S	R
8	Cephalosporin (30 mcg)	R	R	R	R	R	R	R
9	Rifampicin (5mcg)	S	S	I	I	I	R	R

R = Resistant; S = Sensitive; I = Partially resistant. Drug concentration in µg/disc mentioned in parentheses.

Table 4: Morphological and biochemical characterization of specific pathogens.

S. No.	Tests performed	*E. coli*	*Salmonella typhi*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
1	Shape	Rods	Rods	Rods	Rods
2	Gram staining	Gram negative	Gram negative	Negative	Negative
3	Motility	Motile	Motile	Motile	Positive
4	Gelatin utilization test	Negative	Positive	Positive	Negative
5	Citrate utilization test	Positive	Positive	Positive	Positive
6	Methyl Red	Negative	Positive	Positive	Negative
7	Voges Proskeur	Positive	Negative	Negative	Negative
8	Acid from glucose	Positive	Positive	Positive	Positive
9	Gas from glucose	Negative	Positive	Positive	Negative
10	Triple Sugar Iron test	Acid was produced	Gas was produced	Acid was produced	Acid was produced
11	Urease test	Positive	Negative	Positive	Positive
12	Indole production	Negative	Positive	Positive	Negative

Table 5: Antibiogram of target pathogens.

S.No	Antibiotics	(Concentration 30 mcg)			
		E. coli	*Salmonella typhi*	*Pseudomonas aeruginosa*	*Klebsiella* sp
1	Gentamycin	R (1)	R (1)	R (1)	R (1)
2	Ciproflaxin	I (4)	R (1)	I (5)	I (4)
3	Chloramphenical	R (0)	R (1)	R (1)	R (2)
4	Tetracycline	R (2)	R (1)	R (1)	I (5)
5	Streptomycin	S (11)	I (3)	R (1)	R (2)
6	Kanamycin	S (10)	R (1)	R (2)	R (1)
7	Ampicillin	R (3)	R (1)	R (2)	R (2)
8	Erythromycin	R (1)	R (1)	R (1)	R (1)
9	Penicillin	I (4)	R (1)	R (1)	R (1)
10	Cephalosporin	R (1)	R (1)	R (0)	R (1)
11	Rifampicin	S (6)	R (4)	R (1)	R (2)

R = Resistant (R, < 3mm), I = Intermediate(I, 3-5mm), S = Susceptible (S, > 6mm)

associated health risks. The sewage sludge was analysed to determine the pollution load. Pathogenic population was high which suggests the essentiality of the treatment. Since *Salmonella* is a dreadful pathogen and has more aggregating property, more number of organisms was isolated from the sewage sludge. Sludge collected from Chennai had very high *Salmonella* population, lowest was recorded in Perundurai sample. Among the seven different locations, samples collected from Ukkadam recorded the maximum heterotroph population (172×10^6/mL of sample) and high *E. coli* population (24×10^8/mL of sample), followed by samples collected from Coovum, Chennai (142×10^6/mL of sample) (Table 2).

Antibiogram of target pathogens: The antibiotic resistance of the isolates was tested using disk diffusion test. For the estimation of the MDR bacteria, 100 µL diluted samples were

spread over MacConkey agar plates supplemented with 30 µg/mL of chloramphenicol and 20 µg/mL of gentamycin (Table 3) because they have greater *in vitro* stability. Simultaneous resistance to ciproflaxin, tetracycline, streptomycin, kanamycin, ampicillin, erythromycin, penicillin, cephalosporin and rifampicin formed the common MDR pattern. Sewage sludge of Ukkadam showed very high percentage of MDR bacteria, since Ukkadam is the prime area, where Coimbatore city wastes are disposed. The MDR pattern seen in the bacterial isolates from sewage sludge samples included most of the antibiotics used presently for treating human infections. The origin of such MDR bacterial strains appears to be the hospital environment and the selective pressure responsible for expanding such bacterial populations in hospitals must have been through the use of drugs in humans (Neema et al. 1997, Rangnekar 1981, Ogunseitan et al. 1990, Dhevagi & Anusuya 2011). The present observations suggest that indiscriminate release of sewage water can be a potential health hazard by adding MDR bacteria in to the environment.

Characterisation of target pathogens isolated from sewage sludge: Single colony, picked up from the culture plate was kept as stock. The picked colonies were streaked on MacConkey agar medium, Kings B medium and *Salmonella Shigella* agar medium. The streaked organisms were incubated at 37°C for 24 hours. Pink colonies on MacConkey agar plate resembling *E. coli* were further characterized. Pink colonies on *Salmonella Shigella* agar plates resembling *Salmonella* were evaluated by morphological and biochemical analysis to identify the organism. Fluorescent colonies from Kings B medium plates resembling *Pseudomonas aeruginosa* were evaluated by morphological and biochemical analysis to identify the organism (Table 4).

Bacterial strains *E. coli, Salmonella typhi, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were tested for the antibiotic resistance with different antibiotics in which they showed resistance to most of the antibiotics. By their zone of inhibition the organisms were chosen. *E. coli* showed resistance to 5 antibiotics, intermediate resistance to 3 antibiotics and susceptible to 3 antibiotics. *Salmonella typhi* showed resistance to almost all the antibiotics and intermediate resistance to 3 antibiotics. *Pseudomonas aeruginosa* showed resistance to almost all the antibiotics, and intermediate resistance to only one antibiotic. *Klebsiella* sp. showed resistance to 9 antibiotics and intermediate resistance to 2 antibiotics (Table 5). Compared to sewage water, more MDR pattern was observed in pathogens isolated from sludge samples (Poorani et al. 2006).

Isolation of specific bacteriophages for target pathogens: Interest in the ability of phages to control bacterial population has extended very recently for water treatment also. Antibiotic resistant pathogens are inevitable as survival is the key for existence. Phage therapy is an alternative to overcome these menacing organisms. This study highlights the potential to develop phage treatments for generalized control of bacterial populations. There is potential application of phages in wastewater treatment system to improve effluent and sludge disposal into the environment. Phage treatments have the ability to control environmental wastewater processes such as foaming in active sludge plants; sludge dewater ability and digestibility of pathogenic bacteria; and to reduce competition between nuisance bacteria and functionally important microbial population. When target bacteria have been identified, phage infective for those species must then be selected. Host specificity is central to selection of suitable phages for particular wastewater treatment applications (Sulak Velidze et al. 2001).

Enrichment was done to increase the number of phage virions in sewage sludge. It is essential for the success of any phage therapy; suitable phage should be isolated, enriched to produce sufficient numbers for the application. Phage enrichment normally involves the inoculation of mixed environmental samples and growth media with single host strain. Repeated phage purification using just one host strain may increase the specificity for that strain (Connon & Giovannoni 2002, Rappe et al. 2002, O'Sullivan et al 2004).

Plaque formation was observed due to the inhibition of growth and lyses of the phage infected cells. The clear plaque was used for purification of phages for further analysis

Table 6: Number of plaque forming units per mL of the *E. coli* lysate, *Salmonella typhi* lysate and *Pseudomonas aeruginosa* lysate.

S. No.	Dilution factor	*E. coli* lysate pfu/mL of sample	*Salmonella typhi* lysate pfu/mL of sample	*P. aeuroginosa* lysate pfu/mL of sample
1	10^{-2}	TNC	TNC	TNC
2	10^{-3}	175×10^5	214×10^5	237×10^5
3	10^{-4}	116×10^6	119×10^6	129×10^6
4	10^{-5}	83×10^7	86×10^7	94×10^7
5	10^{-6}	74×10^8	46×10^8	80×10^8

Values represent mean of three replications; TNC - Too Numerous to Count

Table 7: Cell count of *E. coli, Salmonella typhi* and *P. aeruginosa.*

S.No.	Dilution	*E. coli* (cfu/mL)	*Salmonella typhi* (cfu/mL)	*P. aeruginosa* (cfu/mL)
1	10^{-1}	uncountable	uncountable	ND
2	10^{-2}	uncountable	uncountable	ND
3	10^{-3}	uncountable	uncountable	ND
4	10^{-4}	uncountable	uncountable	uncountable
5	10^{-5}	uncountable	uncountable	uncountable
6	10^{-6}	uncountable	541	99×10^{-7}
7	10^{-7}	uncountable	398	30×10^{-8}
8	10^{-8}	uncountable	126	12×10^{-9}
9	10^{-9}	249	87	ND
10	10^{-10}	86	31	ND

Values represent mean of three replications.

Table 8: Measurement of bacteriophage growth with 4×10^9 cfu/mL of *E. coli* sp. and 2×10^7 pfu/mL *E. coli* specific phages and 4×10^9 cfu/mL of *Salmonella* sp. and 2×10^7 pfu/mL *Salmonella* specific phages.

S.No.	Time in hours	*E. coli* specific phages No. of pfu/mL	*Salmonella* specific phages No. of pfu/mL
1	1	8.4×10^6	9×10^5
2	2	6.8×10^6	9×10^5
3	3	8.9×10^7	1×10^6
4	4	9.5×10^7	1.5×10^6
5	5	7.2×10^8	2.2×10^6
6	6	8.6×10^8	3.6×10^6
7	7	9.6×10^8	5.6×10^6
8	8	8.1×10^9	2.12×10^7
9	9	8.2×10^9	2×10^7
10	10	Confluent lyses	Confluent lyses

(Maloy et al. 2008). The plaques appeared on the *E. coli* and *Salmonella typhi* lawn was individually isolated, and used for the sewage treatment. Since phages are very specific (Shuttle 2000 and Alonso et al. 2002) inoculation of the phage should coincide with bacterial population density sufficient to support phage replication (Payne & Jansen 2001).

Isolation of specific phages for MTCC cultures: The results indicated that none of the sewage sludge sample had bacteriophages against MTCC cultures. This clearly indicates the specificity of phages.

Characterization of the identified bacteriophages: When confluent lyses has occurred, 5 mL of SM buffer was added to the plate and gently scrape the soft agarose into sterile centrifuge tube. Tubes were spun at 4000 rpm for 10 min at 4°C, and the supernatant was recovered and to that one drop of chloroform was added to lyse the remaining cells. Thus, prepared bacteriophages were maintained as stock and used for further analysis. Bacteriophages were titrated with their respective dilutions to know the number of plaques formed for their respective host and results are given in Table 6.

After multiplication of specific pathogen, cell count in each mL of broth was assessed for the purpose of fixing the phage concentration. Serial dilutions were carried out up to 10 dilutions. From the serially diluted samples, 0.1 mL of pathogenic cultures were added to sterile plates containing LB and incubated at 37°C for 24 hours. In case of *E. coli* up to 10^{-8} dilutions, there are uncountable numbers. Countable numbers were observed only in 10^{-9} and 10^{-10} dilutions. In case of *Salmonella typhi*, up to 10^{-5} dilutions, there are uncountable numbers of colony forming units. Countable numbers was observed from 10^{-6} dilutions (Table 7).

Characterization of phages viz., one step growth curve, multiplicity of infection and burst size are essential for fixing the time of treatment and dose of the phage dilutions to be used for wastewater purification (Sambrook & Russell 2001). Even though phages specific to *E. coli, Salmonella* and *Pseudomonas* were isolated, characterization studies were restricted to *E. coli* and *Salmonella* sp.

One step growth studies: From one step growth curve of bacteriophage, multiplicity of infection was calculated to analyse the lytic activity of phage to host bacteria (Ellis & Delbruck 1939). The multiplicity of infection for the present isolate was observed as 0.3. Sagkaguchi et al. (1989) reported that the phage with MOI higher than 0.1 could effectively lyses the host bacteria after 5-7 hours of infection. Therefore *Salmonella* phage isolated is an effective lyric phage for *Salmonella typhus. Salmonella* started to form phage particles after 2 hours of infection and completed at 8 hours and 7 hours in case of *E. coli*. Therefore, one phage infected S*almonella typhi* can produce 68 phage particles (Table 8).

Developing an eco-friendly bioconsortium for augmenting the pathogen in sewage sludge: Most pathogens are associated with sludge flocs rather than liquid portions. Hence, sludge biology should also be concentrated during the phage treatment. Enumerated bacteriophages were tested for the biocontrol efficacy in controlling the target pathogens. The test organism selected for the study was *E. coli* and *Salmonella typhi*. The target pathogens with their specific bacteriophages were inoculated separately as well as in mixture. Then the survival rates of pathogens were studied. The number of bacteriophages should 3 to 10 times greater than bacteria (Hennes & Simon 2005). Reduction in population of viruses during activated sludge treatment occurs by viral adsorption to sludge flocs (Wellings et al. 1976 and Tanji et al. 2002, Ketratanakul & Ohgaki 1989). While mixing the host and phages the concentration should be sufficient. Payne & Jansen (2001) observed that insufficient host cell concentration may also contribute for phage decline. Inoculation of the phage should coincide with bacterial population density sufficient to support phage replication. Poor

Table 9: Quality of water treated at Ukkadam STP.

Parameter	Raw Sewage quality	Permissible Standard as per TNPCB	Treated sewage quality
BOD (Biochemical Oxygen Demand)	250 ppm	< 20 ppm	< 10 ppm
COD (Chemical Oxygen Demand)	580 ppm	No limit	< 100 ppm
Total nitrogen	15 ppm	No limit	< 10 ppm
Total phosphorus	5 ppm	No limit	< 2 ppm
Faecal coliforms	10^6 nos/100mL	No limit	< 200 nos/100 mL
pH	7.5	No limit	7.9

Table 10: Effect of phage consortium on pathogens.

S. No.	Treatment details	Initial population		After treatment (14 hours)	
		E. coli	*Salmonella* sp	*E. coli*	*Salmonella* sp
T1	Sewage water inoculated with *E. coli* and *E. coli* specific bacteriophages	2.48×10^3	35	Nil	22
T2	Sewage water inoculated with *Salmonella* sp. and *Salmonella* sp. specific bacteriophages	2.47×10^3	78	2.4×10^3	Nil
T3	Sewage water inoculated with *E. coli* and *Salmonella* sp. specific bacteriophages	2.46×10^3	65	Nil	Nil
T4	Control	2.58×10^3	89	2.6×10^3	102

penetration in the sludge flocs may reduce the efficacy of phage treatment. Kim & Unno (1996) showed ingestion of viral particles by bacteria, protozoa and metazoa, which may contribute to phage loss, should be addressed. In addition, radiation also reduces the numbers. Hantula et al. (1991) found that approximately 10% of phages isolated from activated sludge were polyvalent in nature.

In contrast, Jensen et al. (1998) and Wolf et al. (2003) found that multiple host isolation techniques may be more effective at isolating polyvalent phages. Thomas et al. (2002) found that 15 out of 17 phages isolated from activated sludge had broad host ranges. Despite the potential advantages of polyvalent phage, broader host range phage influencing not only the target strains, but also beneficial degradative bacteria. Phage enrichment was done to isolate a suitable host bacterium. Repeated phage purification using just one host strain may increase specificity for that strain potentially narrowing the host range of phage.

Reduction in population of viruses during activated sludge treatment occurs by viral adsorption to sludge flocs (Wellings et al. 1976 and Tanji et al. 2002). More than 97% coliphages are associated with suspended particles (Ketratanakul & Ohgaki 1989).

Pilot study: The initial characteristics of sewage were analysed and the results were given in Table 9. Raw sewage sample had very high pollution load as per the Pollution Control Board standards. After treatment, the treated sewage was analysed and all the parameters were below the Tami Nadu Pollution Control Board standards. As pilot study the developed bacteriophage preparations were tested in Coimbatore Corporation sewage treatment plant.

The sewage treatment facilities were installed only during the month of January 2011. As a preliminary study the developed bacteriophage preparations were tested in sample collected at Coimbatore Corporation Ukkadam sewage treatment plant, Ukkadam. The *E. coli* and *Salmonella* sp. organisms were inoculated into sewage sludge.

Hundred mL of sewage sample (water and sludge) was taken in Din thread screw bottles and sterile. After cooling it was inoculated with *E.coli* at @ 10^4/mL and *Salmonella* sp. at @ 10^3/mL. After inoculation, cell count of the inoculated pathogens was assessed for assessing the phage efficacy.

Serial dilutions were carried up to 10 dilutions. From the serially diluted samples, 0.1 mL of pathogenic cultures were added to sterile plates containing LB (with sewage extract and without sewage extract) and incubated at 37°C for 24 hours. Initial population and after 14 hours of incubation the survival was assessed (Table 10). In case of treatment T3 (Sewage water inoculated with *E. coli* and *Salmonella* sp. specific bacteriophages) after 14 hours of incubation the entire population was vanished, however individual inoculation also reduced the respective population. A detailed study on the interaction and survival is needed.

The reawakening of interest in the use of phages to control bacterial populations has spread from medical sector to

wastewater treatment process. The outcome of this project highlighted the aspects of wastewater treatment, where phage induced bacterial lysis might be harnessed.

Success would depend on accurate identification of problematic, effective isolation and unbiased enrichment of phage and ability of phage to penetrate flocs and remain infective in *in situ* condition. Density of non host cells may also be important in determining the success of phage treatment of wastewater. Thus, further substantial research is needed to explore the potential of phage treatment. Despite some of the potential hindrances to the phage treatment, the current awareness regarding phages indicates that phage application to wastewater treatment deserves attention. Growing levels of antibiotic resistance and the exit of major pharmaceutical industries from antibiotic development force to have no choice but to adopt phage therapy for growing number of otherwise untreatable infections.

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