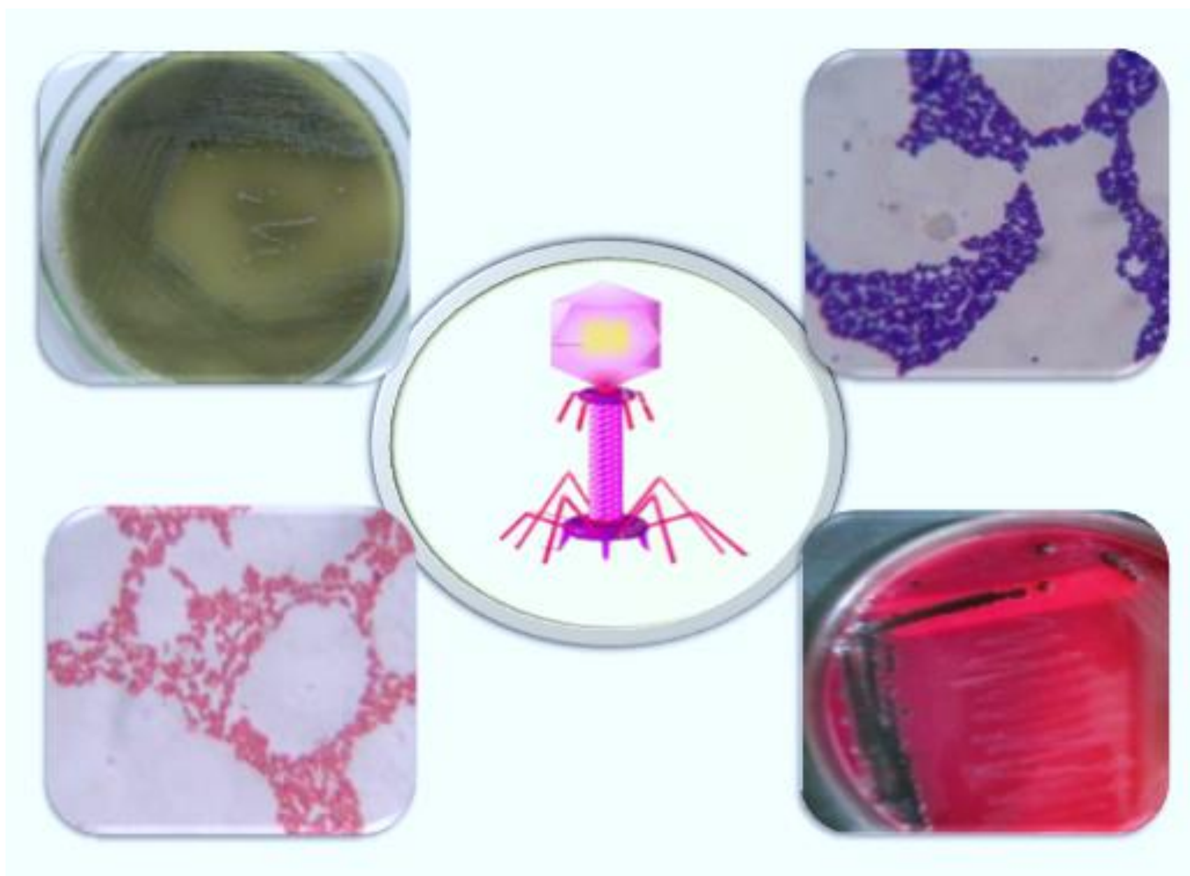
Laboratory Analytical Techniques Series (LATS)/…./2017-18

LAB SCALE FEASIBILITY STUDY OF USING BACTERIOPHAGE FOR REDUCTION OF ENTERIC PATHOGENS IN DOMESTIC SEWAGE & WASTEWATER EFFLUENT



CENTRAL POLLUTION CONTROL BOARD
BIOMONITORING, TOXICOLOGY & MICROBIOLOGY (BTM) LABORATORIES
(Ministry of Environment, Forest & Climate change, Govt. of India)
'Parivesh Bhawan', East Arjun Nagar, Delhi-110032

June 2017

एस.पी.एस. परिहार, भा.प्र.से.
अध्यक्ष
S.P.S. PARIHAR, I.A.S.
Chairman

केन्द्रीय प्रदूषण नियंत्रण बोर्ड
पर्यावरण, वन एवं जलवायु परिवर्तन मंत्रालय
(भारत सरकार)
CENTRAL POLLUTION CONTROL BOARD
Ministry of Environment, Forest & Climate Change
(Government of India)

FOREWORD

The occurrence of pathogenic microorganisms in environmental waters is a serious concern for public health officials, pollution control managers and those working in the water management area worldwide. During the early history of various countries, epidemics of water borne diseases such as Typhoid, Shigellosis, Cholera, Giardiasis, and Amoebiasis were common threats. It was subsequently determined that the primary source of these pathogens was sewage, which was generally untreated and easily polluting the environmental waters used for drinking and swimming. Thus, there is a need to determine the microbiological safety of these waters by analysing them for the presence of specific pathogenic organisms in environmental samples.

The Central Pollution Control Board (CPCB) has been assessing treated wastewater generated from different Wastewater Treatment Plants (WTP) across the country and consistent monitoring and microbiological analysis of the effluent from these Sewage Treatment Plants (STPs) has revealed the presence of enteric bacteria and pathogens in the inadequately treated sewage. Thus, to minimize the risk of environmental release of enteric bacteria and other pathogens, there is a need to improve and adopt more stringent methods for disinfection of effluent discharged from the Sewage Treatment Plants (STPs) using novel approaches.

In the present report, an attempt has been made to evaluate the potential of bacteriophage based techniques in wastewater treatment systems to improve the effluent and sludge quality before being discharged into the environment. The project was executed with the Department of Microbiology, Bhavan's College, Mumbai and coordinated by **Dr. Pratima Akolkar, Ex. Additional Director (Scientist 'E') and Dr. Z. P. Bhathena, Associate Professor, Department of Microbiology, Bhavan's College (Mumbai).** The contribution made by colleagues from CPCB, **Ms. Meenakshi Koul**, JRF and **Ms. Parul Baranwal**, JRF for reviewing and preparing this report, under the guidance of **Dr. Sanjeev Agrawal, Additional Director (Scientist 'E') & In-Charge Bioscience (BTM) Laboratory**, is noteworthy.

I hope this work would stimulate further research on the subject.

(S.P. Singh Parihar)

Date: 24/07/2017
Place: Delhi

'परिवेश भवन', सी.बी.डी.-कम-ऑफिस कॉम्पलैक्स, पूर्वी अर्जुन नगर, दिल्ली-110032
'PARIVESH BHAWAN', C.B.D.-CUM-OFFICE COMPLEX, EAST ARJUN NAGAR, DELHI-110 032
PHONE : 22307233, TEL. / FAX : +91-11-22304948, e-mail : ccb.cpcb@nic.in

Contributions by CPCB & Bhavan's Research Centre, Mumbai

Principal Coordinators	**Dr. A. B. Akolkar,** **Member Secretary, CPCB** **Dr. Pratima Akolkar** **Ex-Scientist 'E' & In-Charge,** **Bioscience (BTM) Laboratory, CPCB** **Dr. Sanjeev Agrawal,** **Scientist 'E' & In-Charge,** **Bioscience (BTM) Laboratory, CPCB**
Project Awarded To	**Bhartiya Vidya Bhavan's, Bhavan's College,** **Department of Microbiology,** **Bhavan's Research Centre, Mumbai**
Principal Investigator	**Dr. Zarine Bhathena, Professor,** **Head, Department of Microbiology,** **Bhavan's Research Center, Mumbai**
Project execution, Data Collection, Compilation and Report Preparation	***<u>Bhavan's Research Centre, Mumbai:</u>*** **Dr. Zarine Bhathena,** **Dr. Nishith Desai** **Ms. Sanjana Kuruwa**
Report Review and Editing	**Dr. Sanjeev Agrawal,** **Scientist 'E' & Divisional Head/Nodal Officer,** **Bioscience (BTM) Laboratories** **CPCB, Delhi** **Dr. D.K. Markandey** **Scientist 'D', CPCB, Bioscience (BTM) Laboratory** **Ms. Meenakshi Koul,** **JRF, CPCB, Bioscience (BTM) Laboratory** **Ms. Parul Baranwal,** **JRF, CPCB, Bioscience (BTM) Laboratory**
Technical Assistance & Laboratory Scale Tasks	***<u>Bhavan's Research Centre, Mumbai:</u>*** **Ms. Nishita D'souza** **Ms. Aparna Pandey** **Ms. Ronica Dsouza**
Sampling	**Dr. Gandhi and Dr. Shinde** **Excel Industries Limited**

CONTENTS

Chapter No.	Topic	Page No.
CHAPTER 1	**INTRODUCTION**	9-12
1.1	Scope of Project	12
CHAPTER 2	**MATERIALS AND METHODS**	13-19
2.1	Sample collection	13
2.2	Detection of enteric bacteria	13
2.3	Identification of pathogenic bacteria	14
2.4	Determination of baseline microbial load and specific pathogen load in sewage samples	15
2.5	Confirmation of the identity of the pathogenic bacteria using VITEK systems	15
2.6	Enrichment of bacteriophages against enteric bacteria	15
2.7	Detection and assay of bacteriophages	16
2.8	Purification of bacteriophages	16
2.9	Determination of host range of phages	16
2.10	One step growth curve	17
2.11	Determination of Multiplicity of Infection	18
2.12	Use of selected bacteriophages formulations for effective reduction of pathogens within domestic sewage water and effluents from Sewage Treatment Plant (STP)	18-19
CHAPTER 3	**RESULTS AND DISCUSSION**	20-47
3.1	Characterization and baseline identification of bacterial pathogens predominantly observed in domestic wastewater	20-24
3.2	Isolation of bacteriophages against enteric pathogens like *E. coli* and *E. faecalis* from domestic wastewater and wastewater effluent samples	24-26

Chapter No.	Topic	Page No.
3.3	Characterization of purified bacteriophages based on host range	27-32
3.4	Determination of infectivity of phage and optimization of feed concentration of phage to effectively reduce specific pathogen in wastewater/effluent	32-36
3.5	Preparation of bacteriophages formulation to reduce pathogen load within wastewaters at laboratory scale	36-40
3.6	Use of select bacteriophages formulations to effectively reduce pathogen load in secondary treated wastewater obtained from Sewage Treatment Plant (STP)	41-47
CHAPTER 4	**CONCLUSIONS & RECOMMENDATIONS**	48-49
	REFERENCES	50

LIST OF TABLES

Table No.	Title	Page No.
Table-1	Selective media used for the isolation of pathogens as per BIS standard reference	14
Table-2	Typical Colony characteristics of the pathogens on selective agar	14
Table-3	Biochemical reactions used for Identification of the selected pathogens	14
Table-4	Host cultures used for determination of phage host range	17
Table-5	Experimental setup for determination of optimal MOI	18
Table-6	Experimental setup for determination of effective reduction of enteric pathogens	19
Table-7	Target pathogens isolated from the 10 sewage samples	20
Table-8	Different strains identified from sewage samples	22
Table-9	API based identification of selected isolates obtained from sewage samples	22-23
Table-10	Microbial load present in the treated sewage samples	23
Table-11	Spot test for Detection of bacteriophage against reference host bacteria	25
Table-12	Plaque clones selected for further purification	26
Table-13	Determination of host range of bacteriophage using reference and environmental *E. faecalis* host	27
Table-14	Determination of host range of bacteriophage using reference and environmental *E. coli* host	27
Table-15	Determination of host range of bacteriophage using reference and environmental Salmonella host bacteria	28
Table-16	Determination of host range of *E. faecalis* bacteriophage against reference and environmental host bacteria	29
Table-17	Determination of host range of *E. coli* bacteriophage against reference and environmental host bacteria	30
Table-18	Determination of host range of *Salmonella* bacteriophage against reference and environmental host bacteria	31
Table-19	Final selection of phage lysates showing activity against specific hosts	32
Table-20	Turbidity and pH of sewage samples	41

LIST OF FIGURES

Figure No.	Title	Page No.
Figure-1	Structure of bacteriophage	10
Figure-2	Lytic and lysogenic cycle of phages	11
Figure-3	Typical colony characteristics on selective agar media and Gram staining of the presumptive positive isolates of *E. coli*, *E. faecalis* and *Salmonella*	21
Figure-4	Average Microbial load present in sewage samples	23
Figure-5	Lysates showing different activity when spotted on reference host	25
Figure-6	Plaques of varied morphology observed following enrichment which were selected for further purification	26
Figure-7	Isolated plaques following purification	26
Figure-8	Types of plaques showing clear and turbid clearance	28
Figure-9a	One step growth curve of phage T1- Lysate 4 against *E. coli*	33
Figure-9b	One step growth curve of phage T3- Lysate 4 against *E. coli*	34
Figure-10a	One step growth curve of phage T3- Lysate 2 against *Salmonella*	34
Figure-10b	One step growth curve of phage T3- Lysate 4 against *Salmonella*	35
Figure-11a	One step growth curve of phage R7- Lysate 1 against *E. faecalis*	35
Figure-11b	One step growth curve of phage T1- Lysate 2 against *E. faecalis*	36
Figure-12a	Growth of reference host cultures in sewage in absence of phage	37
Figure-12b	Reduction of *E. coli* reference host when infected with phage Sample T1 – Lysate 4	38
Figure-12c	Reduction of *E. coli* reference host when infected with phage Sample T3 – Lysate 4	38
Figure-12d	Reduction of *Salmonella* reference host when infected with phage Sample T3 – Lysate 2	39
Figure-12e	Reduction of *Salmonella* reference host when infected with phage Sample T3 – Lysate 4	39
Figure-12f	Reduction of *E. faecalis* reference host when infected with phage Sample R7 – Lysate 1	40
Figure-12g	Reduction of *E. faecalis* reference host when infected with phage Sample T1 – Lysate 2	40
Figure-13a	Reduction of *E. coli* host in presence of phage infected in 100ml sterile sewage	41
Figure-13b	Reduction of *Salmonella* host in presence of phage infected in 100ml sterile sewage	42
Figure-13c	Reduction of *E. faecalis* host in presence of phage infected in 100ml sterile sewage	42
Figure-13d	Reduction of *E. coli* host in presence of phage infected in 100ml sterile sewage in combination with *Salmonella* and *E. faecalis* host and phage consortia.	43
Figure-13e	Reduction of Salmonella host in presence of phage infected in 100ml sterile sewage in combination with *E.coli* and *E. faecalis* host and phage consortia.	43
Figure-13f	Reduction of *E, faecalis* host in presence of phage infected in 100ml sterile sewage in combination with *E.coli* and *Salmonella* host and phage consortia.	44
Figure-13g	Reduction of *E. coli* host in presence of phage infected in 1litre sterile sewage in combination with *Salmonella* and *E. faecalis* host and phage consortia.	44
Figure-13h	Reduction of *Salmonella* host in presence of phage infected in 1 litre sterile sewage in combination with *E.coli* and *E. faecalis* host and phage consortia.	45
Figure-13i	Reduction of *E, faecalis* host in presence of phage infected in 1 litre sterile sewage in combination with *E.coli* and *Salmonella* host and phage consortia.	45
Figure-13j	Reduction of *E, faecalis* host in presence of phage infected in 1 litre unsterile sewage in combination with *E.coli* and *Salmonella* host and phage consortia.	46

EXECUTIVE SUMMARY

The occurrence of pathogenic microorganisms in environmental waters is an ongoing concern for public health officials and those in the water management area worldwide. Enteric pathogens in environmental surface waters originate mainly from effluent discharged from Sewage Treatment Plants (STPs). Currently, the microbial quality of water is monitored by enumerating the levels of fecal indicator bacteria (e.g., thermotolerant *E. coli* and *Enterococci*), to determine levels of fecal input and the possible presence of pathogens. The present study reveals the identification of potential bacteriophage hosts and characterization of types of bacterial pathogens predominantly observed in domestic wastewater. Three target pathogens *viz. E. coli, Salmonella* sp. and *Enterococcus faecalis* were successfully isolated from 7 domestic sewage and 3 secondary effluent samples of which *E. coli* population formed about 6% while *E. faecalis* and *Salmonella* formed 0.1% of the total aerobic microbial load. Of 10 sewage samples, *Salmonella* was detected in 4, *E. coli* in 8 samples while *E. faecalis* was detected in all the 10 sewage samples.

Bacteriophages were isolated against enteric pathogens like *E. coli* and *Enterococcus faecalis* from wastewater samples. 90% and 80% of primary lysates were found to infect *E. coli* and *E. faecalis* respectively. In contrast only 50% of the lysates showed their ability to lyse *Salmonella* host. Clear plaques but with varied morphology were selected for further purification using repeated cycles of propagation of a single clone of plaque that was enriched and used for further rounds of propagation. The characterization of the isolated bacteriophages was done based on host range. Following purification, of the 34 *E. coli* lysates, 8 having a broad host range were identified, 4 out of the 18 lysates for *Salmonella* and all 4 lysates of *E. faecalis* had broad host ranges. Final phage lysates were shortlisted based on their characteristics of being lytic, having broad host range and ability to grow to high titers in the lab. Thus, 3 *E. coli* and *Salmonella* lysates each, and 2 lysates of *E. faecalis* were selected.

The infectivity of phage and optimization of feed concentration (infectivity) of phage was determined to effectively reduce the specific pathogen in wastewater/ effluent. The lysates as selected in the previous objective were further tested for their infectivity using one step growth curve that would determine the number of phages released per host cell on infection. This aided in the identification of the optimal growth stage of the host and amount of phage to be seeded. To apply a mixture of select bacteriophages effective in reducing pathogens in wastewater at laboratory scale. The effective Multiplicity of Infection (MOI) i.e. ratio of No. of phage to No. of bacterial host, was determined in order to obtain maximum reduction pathogens using minimal amount of phage in wastewater at laboratory scale. This was carried out in sterile sewage sample spiked with phage and host at an MOI of 1, 10 and 50. An MOI of 1 and 10 seemed more effective, in achieving a 4 log reduction of all 3 host pathogens within a short time period of 6 hrs.

To apply the mixture of select bacteriophages against domestic sewage water as well as wastewater from Sewage Treatment Plant (STP) to check for effective reduction in pathogens. The ability of the phage to infect and reduce target host bacteria varied in each sewage sample. It was particularly noted that the reduction was lower in certain sewage samples. *E. coli* showed a reduction ranging from 1-4 log, *Salmonella* showed a reduction of 0.5-4 log while *E. faecalis* showed a reduction ranging from 0.5-3 log.

CHAPTER-1

INTRODUCTION

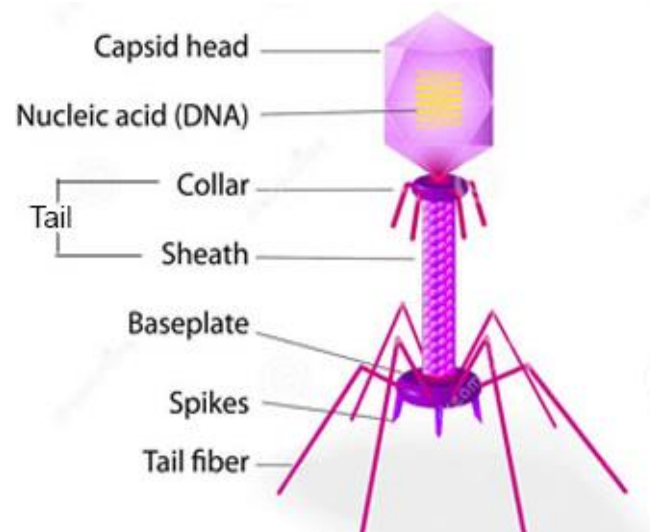
Freshwater is an essential resource for the survival of human and other life forms. India, being a developing country, is facing reduction in the per capita average freshwater availability due to ever increasing population and all round development works (R. Kaur *et al*. 2012). As a result, the wise management, prevention of pollution of available water resources and efficient recycling of wastewater by the wastewater treatment plants is the need of the hour to overcome the freshwater crisis prevailing in the country.

However, during random inspections of various sewage treatment facilities by the Central Pollution Control Board, it has been observed that a large number of sewage treatment plants are operating at sub optimal efficiency due to the ever widening gap between sewage generation and treatment capacity. Although, conventional wastewater treatment technologies reduce the number of enteric microbes, wastewater effluent from Sewage Treatment Plants (STPs) has still shown the presence of fecal coliform bacteria specially *Escherichia coli* and *Enterococcus faecalis*. The inefficient functioning of Sewage Treatment Plants (STPs) has further led to the contamination of water bodies and increased risk of multiple outbreaks of disease caused by these pathogenic organisms.

The persistence of these human pathogens in waters has thus led researchers to explore different approaches for pathogen reduction. This is especially true within the developing countries and waterborne bacterial pathogens in wastewater remains an important public health concern, not only because of the environmental damage, morbidity and mortality that they cause, but also due to the high cost of disinfecting wastewater within treatment plants. An additional hazard has been the concentration of multiple drug resistance strains found in sewage promoting several developed and developing countries to embark on programs that can reduce water-borne multidrug resistant(MDR) bugs that have gained entrance into the Sewage Treatment Plants (STPs) due to the indiscriminate release of hospital wastewater into public sewage (Summers 2001, Chitnis *et al*. 2000, Ekhaise and Omavwaya 2008).

In spite of the various treatment options available persistence of such human pathogens in waters has led researchers to explore different approaches for pathogen reduction. Based on the 2020 clean water mandate, many waste treatment systems are aiming for complete pathogen removal; there by necessitating search for novel approaches that do not harm the environment. Bacteriophage-mediated bacterial reduction is one such novel approach.

Bacteriophages are viruses that infect and replicate within bacterial cells. Bacteriophages are ubiquitous viruses, found wherever bacteria exist. They consist of a head and tail made up of nucleic acids and proteins. The head encapsulates a DNA or RNA genome, which is injected into the cytoplasm of the bacterial cell during infection followed by replication of the phage genome with in the bacterium.

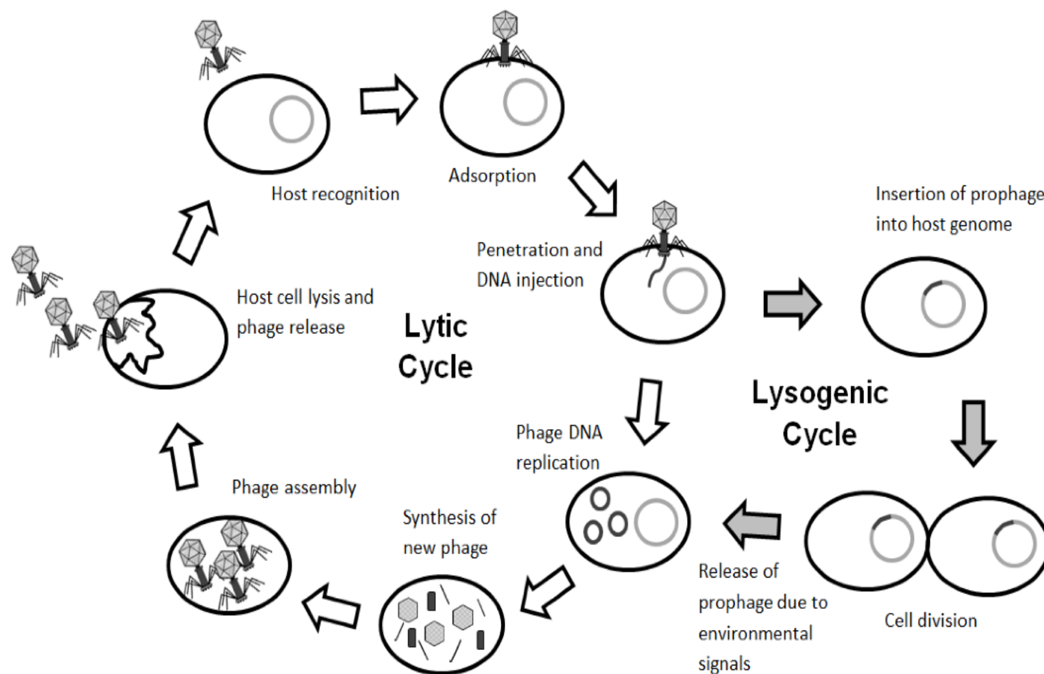


Source:www.dreamstime.com

Figure 1: Structure of bacteriophage

Bacteriophages may replicate within the bacteria through a lytic cycle or a lysogenic cycle. During lytic cycle, phages attach themselves to specific receptors on the bacterial cell with the help of tail fibres and inject their genetic material into the bacterium. The host bacterial cell's normal synthesis of proteins and nucleic acids is seized, and it is forced to manufacture viral products instead. Once the replication of the virion takes place, bacterial cells are lysed and destroyed immediately.

In contrast, the lysogenic cycle involves integration of the viral genome with the host bacterial genome after the injection of the viral genome into the host cell. The host cells are not immediately lysed and the viral genome replicates along with the host DNA relatively harmlessly till favourable conditions are available. When the conditions within the host become unfavourable, the endogenous phages become active and initiate the reproductive cycle, resulting in lysis of the host cell.



Source: Adapted from Doss J *et al.,* 2017 http://www.mdpi.com/1999-4915/9/3/50/htm

Figure 2: Lytic and lysogenic cycle of phages

A large number of bacteriophages have been isolated from water and wastewater and their properties studied (Withey *et al*., 2005). Bacteriophage-mediated bacterial reduction has the potential to influence wastewater treatment performance by controlling the abundance of key microbiological groups. Bacteriophage treatments have the potential to control environmental wastewater process problems such as, foaming in activated sludge plants; sludge dewater ability and digestibility; pathogenic bacteria; and to reduce competition between nuisance bacteria and functionally important microbial populations.

Despite this, very little work with respect to their potential to reduce contamination of waters caused by pathogenic bacteria has been reported (Snyder 2012; Choi *et al*., 2011; Periasamy *et al*., 2013), though they have found application in controlling pathogens in food and water (Petrovski *et al*., 2011, Zackek *et al*., 2015). While there are limitations in preparing effective bacteriophage mixtures that retain a species-wide host range numerous review articles have been published highlighting the benefits and disadvantages of applying bacteriophages in health, food and agricultural industries (Sulakvelidze *et al*., 2001).

Bacteriophage based approach may have the potential as an environmentally safe option for tackling worldwide sewage treatment problems. Nevertheless, phage based biocontrol approach has certain limitations:

1. High concentration of phages must be applied for the successful application;

2. Specific phage must be identified to counter specifically the pathogenic bacteria without affecting other bacteria;

3. The microbial analysis of the system is a prerequisite to phage application as the bacterial population may vary between wastewater treatment plants.

1.1 Scope of Project:

This project aims to provide a proof-of-concept evaluation for the potential application of bacteriophage based techniques in wastewater treatment systems to improve effluent and sludge emissions into the environment keeping the following objectives in mind:

1. To conduct a baseline to identify pathogenic bacteria that may serve as potential bacteriophage hosts and characterize types of bacterial pathogens predominantly observed in domestic wastewater;

2. To isolate bacteriophages against enteric pathogens like *E. coli* and *Enterococcus faecalis* from the domestic wastewater and wastewater effluent samples;

3. To characterize the isolated bacteriophages based on host range;

4. Determination of infectivity of phage and optimization of feed concentration (infectivity) of phage to effectively reduce the specific pathogen in wastewater/ effluent;

5. To apply a mixture of select bacteriophages effective in reducing pathogens in wastewater at laboratory scale;

6. To apply the mixture of select bacteriophages against domestic sewage water as well as wastewater from Sewage Treatment Plant (STP) to check for effective reduction in pathogens.

CHAPTER-2

MATERIALS AND METHODS

2.1 Sample collection:

Seven (07) raw domestic sewage samples were collected (1 litre each) from in and around Andheri, located in north-west Mumbai while three (03) sewage samples were collected (5 litre each) from secondary effluent treatment tanks/lagoons located in Andheri West, Mumbai, in sterile containers. The samples were collected from the following areas:

1. Sample R1- Domestic Waste- Andheri West location- 1
2. Sample R2- Domestic Waste- Andheri West location- 2
3. Sample R3- Domestic Waste- Andheri West location- 3
4. Sample R4- Domestic Waste- Andheri West location- 4
5. Sample R5- Domestic Waste- Andheri West location- 5
6. Sample R6- Mithi River
7. Sample R7- Dead well leached with sewage
8. Sample T1- Lagoon A- A1
9. Sample T2- Lagoon A- A2
10. Sample T3- Lagoon B- B1

For validation of phage reduction as required for the final objective; 2 sets of Sewage Treatment Plant (STP) samples of 5 Litre each were collected at different time lines as follows:

1. Batch 1 collected on 18th April, 2016: Sample from Lagoon A and Lagoon B
2. Batch 2 collected on 2nd May, 2016: Sample from Lagoon A and Lagoon B

2.2 Detection of enteric bacteria:

Ten (10) waste water samples (07 domestic waste and 03 secondary effluent samples) collected were assessed for their total aerobic microbial load as well as direct detection of specific pathogenic load within 24 hours of their receipt in the laboratory. The samples were also set up for enrichment and isolated on the specified selective media as per standard protocols (Table 1), so as to enable the recovery for low numbers of pathogens. For enrichment, 25 ml of sewage samples were individually dispensed in 225 ml of buffered peptone water and incubated at 37°C for 18-20 hours. Post incubation, the enriched broth was isolated on selective media as described in Table 1 and incubated at 37°C for 24 hours, while for *Salmonella*, the enriched broth was further selectively enriched in Rappaport Vassiliadis (RV) medium, incubated at 42°C for 20-24 hours and then isolated on Xylose Lysine Deoxycholate (XLD) agar plates. The plates were incubated at 37°C for 24 hours and observed for typical colony characteristic of the specific pathogen as described in Table 2.

Table 1: Selective Media Used for the Isolation of Pathogens as per Standard Reference

Target Bacterial Pathogen	Selective Media	Standard References
E. coli	Eosin Methylene Blue (EMB) Agar	APHA 22nd Edition, 2012, IS 5887- Part 1, 1976- Reaffirmed 2005
***Salmonella* sp.**	Xylose Lysine Deoxycholate (XLD) Agar	APHA 22nd Edition, 2012 IS 5887- Part 3, 1999- Reaffirmed 2005
E. fecalis	Bile Esculin Agar	APHA 22nd Edition, 2012 IS 15186: 2002

Table 2: Typical Colony Characteristics of the Pathogens on Selective Agar

Bacterial Pathogen	Selective Growth Media	Typical Colony Characteristics
E. coli	Eosin Methylene Blue (EMB) Agar	Metallic sheen colonies with blue black appearance
***Salmonella* sp.**	Xylose-Lysine Deoxycholate (XLD) Agar	Black centred medium sized red colonies.
E. faecalis	Bile Esculin Agar	Colourless colonies with black coloration of medium due to esculin hydrolysis

2.3 Identification of pathogenic bacteria:

Isolates showing typical colonies were further purified and subjected to identification based on biochemical reactions as described in Table 3.

Table 3: Biochemical Reactions used for Identification of the Selected Pathogens

Bacteria	Biochemical Reactions:
E. coli	Gram Negative rod shaped; Catalase positive; Acid/ Acid KIA reaction; Gas production; No H_2S Production; Positive Methyl Red Reaction; Negative Voges-Proskauer Reaction; Positive Indole Reaction; No Citrate Utilization; No Urease Hydrolysis, Positive Lysine Decarboxylase Reaction
Salmonella sp.	Gram Negative rods; Catalase positive; Alkaline/ Acid KIA reaction; Gas production(V); H_2S Production; Positive Methyl Red Reaction; Negative Voges-Proskauer Reaction; Negative Indole Reaction; Citrate Utilization; No Urease Hydrolysis, Positive Lysine Decarboxylase Reaction
E. faecalis	Gram positive cocci; Catalase negative Sugar fermentations: Glucose, Sucrose and Mannitol fermentation, No Lactose fermentation.

2.4 Determination of baseline microbial load and specific pathogen load in sewage samples:

Before setting up the samples for enrichment, sewage samples obtained from the sewage treatment plant were enumerated for its total microbial load and specific pathogen load. The sewage samples were serially diluted (10 fold) up to 10^{-4}. 0.1 ml of each dilution was surface spread on the selective media as described in Table 1 as well as on general purpose medium i.e. Plate Count (PC) Agar to obtain its aerobic total viable count after an incubation period of 72 hours at 37ºC. The total load of typical colonies obtained on selective agar media were used to estimate the counts of specific pathogens within the sewage sample.

2.5 Confirmation of the identity of the pathogenic bacteria using VITEK systems:

Analytical Profile Index (API) based identification as well as Antibiotic Susceptibility Testing (AST) of putative isolates of *E. coli*, *Salmonella*, and *E. faecalis,* as identified by biochemical analysis was undertaken. Testing was performed on a VITEK 2 system which automatically performs all of the steps required for identification and AST after a primary inoculum of 18-hour old culture at a cell density of 10 cfu/ml is used. This system allows kinetic analysis by reading each test every 15 minutes. The optical system combines multichannel fluorimeter and photometer readings to record fluorescence, turbidity and colorimetric signals that occur due to the metabolism of the substrate by the organisms into unique identification patterns that help to identify the pathogen.

2.6 Enrichment of bacteriophages against enteric bacteria:

The ten samples (07 domestic waste and 03 secondary effluent samples) as collected in Section 2.1.1 were processed for recovery of bacteriophages within 24 hours of its receipt in the laboratory. 50 ml sample was filtered through a 0.45 uM filter to remove bacteria and retain phages in the filtrate. Bacteriophages were enriched in these sample lysates (filtrate) using various reference host strains viz; *Salmonella* spp. (ATCC 13311), *E. coli* (ATCC 8739) and *E. faecalis* (ATCC 29212). The enrichment process entailed the addition of 50 ml of the filtered sample to 50 ml 2X Nutrient Broth, to which 5 ml of 24 hour grown culture of the specific host against which phages are desired was added .The flask were incubated for 48 hours at 37°C during which the lytic cycle helped to increase the numbers of phage particles .The bacteriophages were then harvested by addition of 1:1 volume of chloroform, followed by vigorous shaking that helped to lyse the bacterial cells such that on settling the upper aqueous layer containing the inert phage particles was collected. Complete removal of bacterial debris was attained through 0.4 uM filtration (Petrovski *et al*.,2011; Tan *et al*., 2008; Beudoin *et al*., 2007).

2.7 Detection and assay of bacteriophages:

Following enrichment, the presence of bacteriophage was determined by Spot Test Technique wherein a 10-µl drop of purified bacteriophage was spotted onto the surface of a lawn of specific host bacterial strains grown. The presence of phage was identified by the presence of clearance of the bacterial host culture. In cases where clearance was observed, the titre of the bacteriophages were determined using the Agar overlay method. Briefly, the phage lysates were serially diluted (10 fold) up to 10^{-6} and 0.1 ml of the diluted lysate mixed with 0.5 ml of host culture. The mixture was incubated at 37°C for 10 minutes for adsorption to occur after which they were added to7 ml of 1% soft agar and overlaid on to LBT plates. The plates were incubated in upright position at 37°C for 24 hours and isolated plaques counted to obtain the pfu/ml counts.

2.8 Purification of bacteriophages:

Isolated clear plaques having varied morphology, with respect to their shape, size, clear/turbid plaques, indicates presence of different clones of bacteriophages within the lysates and thus such lysates need to be purified to attain bacteriophages that are able to give single morphology plaques against the specific host. Purification of the selected phage lysates showing clearance against reference host bacteria were performed through 3 consecutive isolation and propagation cycles wherein,

a. Specific reference host culture of *E. coli* (ATCC 11229), *E. faecalis* (ATCC29212) and *Salmonella* spp. (ATCC 13311) were prepared by growing overnight in Nutrient Broth;

b. 0.2 ml of this culture was inoculated in 20 ml Nutrient Broth and grown for 2 hours to obtain a log phase host culture that can be used to propagate the phages.

c. The phages showing well isolated clear plaques formed against each specific host were cut using a sterile borer and added to the above mentioned host culture and phage assay performed. The plates after incubation for 24 hours at 37°C showing single variant plaques were considered to be pure while those that showed varied plaque morphologies were picked up for the next cycle of propagation wherein again a single plaque was picked up and propagated. Such cycles of purification helped to attain high titer lysate wherein bacteriophages capable of showing single plaque morphology were obtained (Figure 3).

2.9 Determination of host range of phages:

Following the cycles of purification, the lysates obtained from propagated plaques were tested against varied strains of *E. coli*, *Salmonella* and *E. faecalis* with an aim to classify the lysates with broad host range. Host bacteria selected included the standard reference strains, environmental strains of *E. coli*, *Salmonella* and *E. faecalis* obtained from the samples collected in Objective 1 as well as other environmental cultures available in the lab (Table 4). The presence of activity of the phage was confirmed by spotting lysates on a lawn of host culture using the agar overlay method. Positive activity was determined by detection of clearance after incubation for 24 hours at37°C. The ability to target multiple strains was used to shortlist the lysates that can be used for creation of formulations for in-situ experiments.

Table 4: Host Cultures Used for Determination of Phage Host Range

Target Pathogen of Lysate	Host Cultures used		
	Reference strains	Environmental isolates	Total Host Culture
E. coli	*E. coli* ATCC 11229, *E. coli* ATCC 8739, *E. coli* ATCC 15597, *E. coli* ATCC 10536, *E. coli* ATCC 196.	5 isolates of *E. coli* obtained from Sewage sample T1 2 isolates of *E. coli* obtained from sample T2 2 isolates of *E. coli* obtained from Sample T3 Other *E. coli* : 1 isolate	15
Salmonella	*Salmonella* ATCC 13311, *Salmonella* ATCC 14028	2 isolates of *Salmonella* obtained from Sample R4 1 isolate of *Salmonella* obtained from Sample T2 Other *Salmonella* : 5 isolates	10
E. faecalis	*E. faecalis*: ATCC 29212	Sample R2 : 1 isolate Sample R4 : 1 isolate Sample R6 : 1 isolate Sample T1 : 1 isolate Sample T2 : 1 isolate Sample T3 : 1 isolate	7

2.10 One step growth curve:

In order to determine the infectivity of phage and optimize the feed concentration (infectivity) of phage required to effectively reduce the specific pathogen in wastewater/ effluent, one step growth curve was performed. The one step growth curve was performed for the selected phages to determine its burst pressure in accordance with the method described by Ellis and Delbruck (1939). Briefly, a suitable dilution of phage having a titre of 1×10^7 - 1×10^8pfu/ml was mixed with a suspension of bacteria containing 1×10^6 - 1×10^7 cfu/ml to obtain a Multiplicity of Infection (MOI) of 10 (i.e. 10 phage particles per CFU of bacteria). This mixture was allowed to stand at 37°C for 10 minutes which helped to obtain more than 90 percent adsorption of the phage. This mixture was then diluted 1: 10^4 in broth, and incubated under static conditions at 37°C. Samples of this diluted mixture were withdrawn at 30 minute intervals and assayed using the double layer agar plating method after performing serial dilutions. This was performed in duplicate for each phage lysate and the results of the same are plotted in Figure 6,7 and 8. The phage host mixture was again diluted 1:10 at the start of the first rise (60-120 minutes) to further decrease the rate of adsorption of the phage set free in the first step.

$$\text{Burst pressure was calculated as: } \frac{\text{maximum titre of the phage at } 1^{st} \text{ rise}}{\text{No of host bacterial cells in the adsorption tube at Time 0}}$$

The rise corresponds to the average number of phage produced per burst.

2.11 Determination of Multiplicity of Infection (MOI):

In order to determine the amount of phage required to effectively reduce pathogens in wastewater at laboratory scale, the Multiplicity of Infection (MOI) was determined. Multiplicity of Infection (MOI) is the ratio of phage to target host cells. In order to determine the optimal Multiplicity of Infection (MOI) required for maximum clearance of reference host bacteria, the bacteria were infected with phages as selected in Table 5. The reference host bacteria were seeded in sterile Sewage Treatment Plant (STP) sample at a concentration of 1 x 10^4 - 5 x 10^4 cfu/ml and phages selected based on their broad host range were added at a Multiplicity of Infection (MOI) of 1, 10 and 50 in order to determine the optimal ration of phages to target host cells required for effective clearance of the reference host culture (Table 5).

At time intervals of 0 hour, 6 hours, 24 hours and 48 hours the number of host bacteria in the Sewage Treatment Plant (STP) sample were determined through a viable count of the same using Nutrient Agar. At the mentioned point of time, 1ml sample was drawn after mixing and this was serially diluted (10 fold) up to 10^{-4}. One ml of each dilution was plated using pour plate technique for the aerobic total viable count. The plates were incubated at 37°C for 48 hours and then counted to determine the microbial load.

Table 5: Experimental setup for determination of optimal MOI

S. No.	Reference Host	Concentration of Host	Phage	Phage MOI	Time Interval
1	*E. coli* (ATCC 8739)	1x10^4 – 5x10^4 cfu/ml	Sample T1 - Lysate 4	1,10,50	0 hour 6 hours 24 hours 48 hours
2			Sample T3 – Lysate 4		
3			None (host control)		
4	*Salmonella* (ATCC 11331)		Sample T3 – Lysate 2		
5			Sample T3 – Lysate 4		
6			None (host control)		
7	*E. faecalis* (ATCC 29212)		Sample R7 – Lysate 1		
8			Sample T1 – Lysate 2		
9			None (host control)		
10	Sterile STP sample	Negative Control			

2.12 Use of selected bacteriophages formulations for effective reduction of pathogens within domestic sewage water and effluents from Sewage Treatment Plant:

Effluents from two oxidation ponds/lagoons A and B in sterile and non-sterile conditions were used as the base matrix to assess the ability of the phage formulation to reduce pathogen load of *E. coli*, *Salmonella* and *E. faecalis* added as reference host. Host cultures *E. coli* (ATCC 8739), *Salmonella* (ATCC 11331) and *E faecalis* (ATCC 29212) and phage lysates were seeded in sterilized effluent as single cultures in Set 1, 2 and 3 of this experiment, while host and phage were seeded in combination in 100 ml volumes in set 4 and a similar combination was seeded in set 5

but in a volume of 1 litre. The final set 6 was set up using unsterile sewage, in 1 litre volume, in which host bacteria and phages were seeded in combination.

Host bacteria without phage were seeded in sterile sewage as control (Table 6). The respective reference host bacteria were seeded in secondary effluent matrix at a concentration of $1x10^3 – 1x10^4$ cfu/ml while phage lysates were added at an MOI of 1-5 in order to determine their ability to clear the reference host culture (Table 6). At time intervals of 0 hour, 6hours, 24hours and 48 hours, the number of surviving host bacteria in the STP samples were determined through a viable count of the same using selective media as indicated in Table 1. At the mentioned time point, 1 ml sample was drawn, serially diluted (10 fold) up to 10^{-4}. 0.1 ml of each dilution was surface spread on the selective media as described in Table 1 to determine the total pathogen count after the plates were incubated at 37°C for 48 hours. Typical colonies on selective agar media as described in Table 2 were counted and the microbial load was estimated.

In addition to testing for reduction of reference host bacteria, turbidity and pH of the sewage samples was also determined in accordance with IS standard 3025 Part 10 and part 11 respectively, in order to determine if these physico-chemical parameters have any bearing on the activity of phage.

Table 6: Experimental setup for determination of effective reduction of enteric pathogens

Set No.	Reference Host	Phage Inoculated	STP Sample
1	*E. coli* (ATCC 8739)	Sample T3 – Lysate 4	Sterile STP (100 ml) – Sample A and B
2	*Salmonella* (ATCC 11331)	Sample T3 – Lysate 4	
3	*E. faecalis* (ATCC 29212)	Sample T1 – Lysate 2	
4	COMBINATION - *E. coli* (ATCC 8739), *Salmonella* (ATCC 11331), *E. faecalis* (ATCC 29212)	COMBINATION - *E. coli* (Sample T3 – Lysate 4), *Salmonella* (Sample T3 – Lysate 4), *E. faecalis* (Sample T1 – Lysate 2)	Sterile STP (100 ml) – Sample A and B
5			Sterile STP (1L) – Sample A and B
6			Neat STP (1L) – Sample A and B
7	*E. coli* (ATCC 8739)	None (host control)	Sterile STP (100 ml) – Sample A and B
8	*Salmonella* (ATCC 11331)	None (host control)	Sterile STP (100 ml) – Sample A and B
9	*E. faecalis* (ATCC 29212)	None (host control)	Sterile STP (100 ml) – Sample A and B
10	Sterile STP	Negative control	Sterile STP (100 ml) – Sample A and B

CHAPTER-3

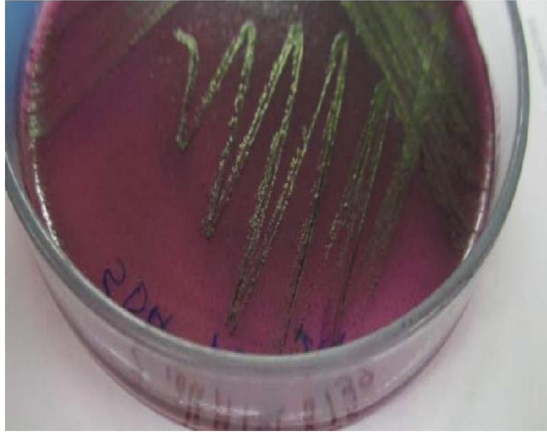
RESULTS AND DISCUSSIONS

3.1 Characterization and baseline identification of bacterial pathogens predominantly observed in domestic wastewater:

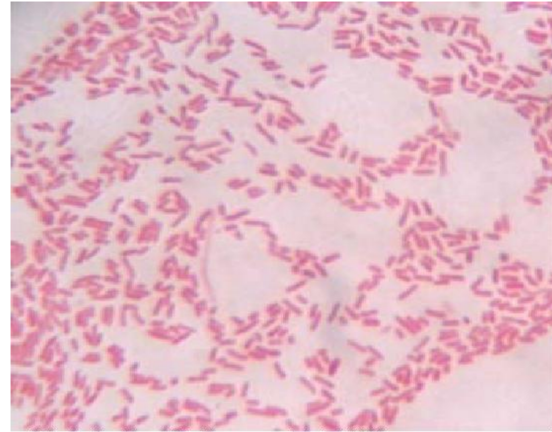
Ten (10) sewage samples collected from north-west suburban Mumbai (Andheri) were analysed microbiologically to ascertain the presence of target bacterial pathogens. These samples were enriched and isolated on specified selective media as prescribed in BIS standard protocols. Presumptive positive isolates showing typical colony characteristics on selective growth medium (Figure 1) were further identified based on their characteristic biochemical reactions. Based on these affirmations, presence of target pathogens in sewage was concluded and tabulated in Table 7.

Table 7: Target pathogens isolated from the 10 sewage samples

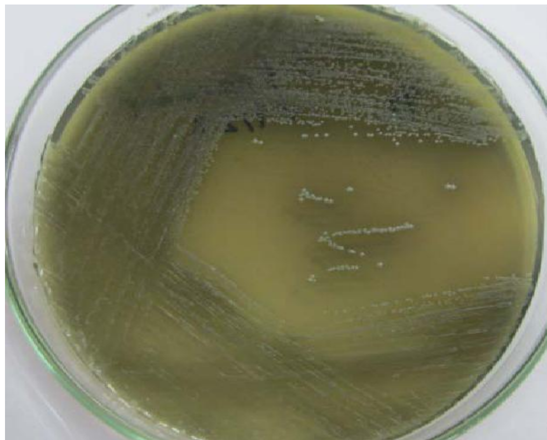
Samples	*E. coli*	*Salmonella* sp.	*E. faecalis*	Others
Sewage Samples (Total isolates = 105)				
No. of presumptive positive isolates	**73**	**22**	**10**	**-**
Sample R1	1	0	1	3
Sample R2	2	0	1	3
Sample R3	1	0	1	0
Sample R4	1	2	1	3
Sample R5	0	0	1	0
Sample R6	1	0	1	1
Sample R7	0	0	1	0
Sample T1	17	1	1	15
Sample T2	5	2	1	16
Sample T3	8	2	1	11
No. of isolates identified based on biochemical reactions	**36**	**7**	**10**	**52**



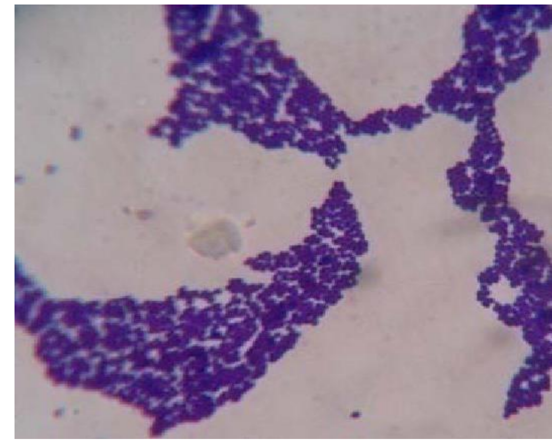
Presumptive *E. coli* on EMB Agar



Gram Stain (100X) of Presumptive *E. coli*

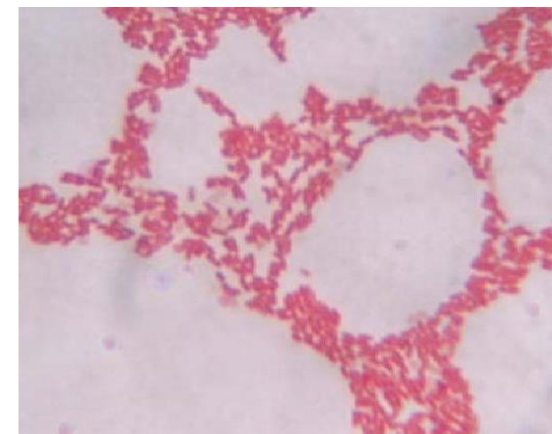


Presumptive *E. faecalis* on BE Agar



Gram Stain (100X) of Presumptive *E. faecalis*

Presumptive *Salmonella* on XLD Agar



Gram Stain (100X) of Presumptive *Salmonella*

Figure 3: Typical colony characteristics on selective agar media and Gram staining of the presumptive positive isolates of *E. coli*, *E. faecalis* and *Salmonella*

52 isolates classified as **"Others"** characterized based on the biochemical reactions and identified as different enteric bacterial strains are shown in Table 8.

Table 8: Different Strains Identified from Sewage Samples

S. No.	Identification	No. of Isolates
1	*Proteus* sp.	11
2	*Citrobacter* sp.	10
3	*Serratia* sp.	9
4	*Morganella morganii*	5
5	*Edwardsiella tarda*	4
6	*Enterobacter* sp.	4
7	*Escherichia* sp.	4
8	*V. cholerae*	3
9	*Klebsiella oxytoca*	2
10	*Pantoea agglomerans*	2
11	Unidentified	1
Total Isolates		**52**

API based identification of a select putative isolates of *E. coli*, *Salmonella*, and *E. faecalis* was undertaken to confirm the identification of these isolates. These results have been tabulated in Table 9.

Table 9: API based Identification of Selected Isolates Obtained from Sewage Samples

<table>
<tr><th>S. No.</th><th>Isolates</th><th>Presumptive Identification</th><th>API based Identification</th></tr>
<tr><td>1</td><td>Sample T1 – Isolate 1</td><td rowspan="10">E. coli</td><td rowspan="10">E. coli</td></tr>
<tr><td>2</td><td>Sample T1 – Isolate 2</td></tr>
<tr><td>3</td><td>Sample T1 – Isolate 3</td></tr>
<tr><td>4</td><td>Sample T1 – Isolate 4</td></tr>
<tr><td>5</td><td>Sample T1 – Isolate 5</td></tr>
<tr><td>6</td><td>Sample T2 – Isolate 1</td></tr>
<tr><td>7</td><td>Sample T2 – Isolate 2</td></tr>
<tr><td>8</td><td>Sample T3 – Isolate 1</td></tr>
<tr><td>9</td><td>Sample T3 – Isolate 2</td></tr>
<tr><td>10</td><td>Other E. coli</td></tr>
<tr><td>11</td><td>Sample R4 – Isolate 1</td><td rowspan="10">Salmonella spp.</td><td>Citrobacter youngae</td></tr>
<tr><td>12</td><td>Sample R4 – Isolate 2</td><td>Salmonella spp.</td></tr>
<tr><td>13</td><td>Sample T2 – Isolate 1</td><td>Salmonella spp.</td></tr>
<tr><td>14</td><td>Sample T2 – Isolate 2</td><td>Pseudomonas putida</td></tr>
<tr><td>15</td><td>Sample T3 – Isolate 1</td><td>Citrobacter youngae</td></tr>
<tr><td>16</td><td>Other Salmonella – Isolate 1</td><td rowspan="5">Salmonella spp.</td></tr>
<tr><td>17</td><td>Other Salmonella – Isolate 2</td></tr>
<tr><td>18</td><td>Other Salmonella – Isolate 3</td></tr>
<tr><td>19</td><td>Other Salmonella – Isolate 4</td></tr>
<tr><td>20</td><td>Other Salmonella – Isolate 6</td></tr>
</table>

21	Sample R2 – Isolate 1	*E. faecalis*	*E. faecalis*
22	Sample R4 – Isolate 1		
23	Sample R6 – Isolate 1		
24	Sample T1 – Isolate 1		
25	Sample T2 – Isolate 1		
26	Sample T3 – Isolate 1		

Secondary treatment of effluent waters though able to reduce the bacterial load is not effective in causing complete removal of microbes. This was confirmed by determining the microbiological load within the Sewage Treatment Plant (STP) samples, the results of which are given in Table 10 and the average load has been graphically represented in Figure 2.

Table 10: Microbial load present in the treated sewage samples

Treated Sewage Samples	Total Microbial Load (cfu/ml)			
	Total Aerobic Viable Count	*E. coli*	*Salmonella* sp.	*E. faecalis*
Sample T1	5.40×10^5	2.30×10^4	1.95×10^2	1.05×10^2
Sample T2	4.45×10^5	4.00×10^4	6.10×10^2	1.10×10^2
Sample T3	2.00×10^5	1.00×10^4	2.50×10^2	4.35×10^2

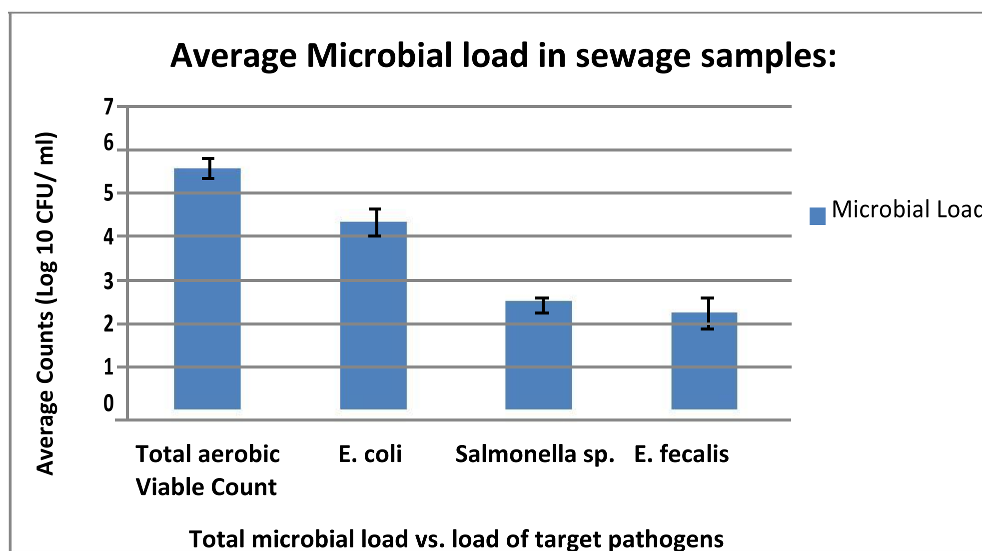


Figure 4: Average Microbial load present in sewage samples

Thus based on the above results it can be concluded that secondary treated water of treatment plant though able to reduce the microbial load are not effective in causing the complete removal as bacterial pathogens were isolated and identified from sewage samples. Three target pathogens viz. *E. coli*, *Salmonella* sp. and *E. faecalis* were successfully isolated from these samples. *Salmonella* was detected in four (04) sewage samples. Among *E. coli* and *E. faecalis*, *E. coli* was detected in eight (08) samples while *E. faecalis* was detected in all the ten (10) sewage samples substantiating the advocacy for targeting *E. coli* and *E. faecalis* as an indicator for fecal contamination.

50% of the total 105 presumptive isolates obtained across ten (10) different sewage samples, were identified to be belonging to genera that had been selected for attaining their reduction through phage intervention. 36 of 73 isolates (49%), 7 of 22 isolates (32%) and 10 of 10 isolates (100%) were biochemically identified as *E. coli*, *Salmonella* and *E. faecalis* respectively. The other 52 isolates were found to belong to *Proteus* sp. (21%), *Citrobacter* sp. (19%), *Serratia* sp. (17%), *Morganella morganii* (10%), *Edwardsiella tarda* (8%), *Enterobacter* sp. (8%), *Escherichia* sp. (8%), *V. cholerae* (6%), *Klebsiella oxytoca* (4%), *Pantoea agglomerans* (4%) while 1 isolate remained unidentified.

API tests confirmed the putative hosts to be *E. coli* (10/10 isolates), *Salmonella* spp (7/10 isolates) and E. faecalis (6/6 isolates). Antimicrobial sensitivity tests for *E. coli* isolates indicated its resistance to ampicillin (3/10), cephalosporin (7/10) ofloxacins (1/10); while *Salmonella* spp. were found to be sensitive to all three antibiotics and *E. faecalis* showed resistance to tetracycline (2/6). Additionally, not all environmental isolates that were identified to be target pathogens could be successfully sub-cultured in the laboratory, indicating the presence of viable but non-cultivable strains within the sewage samples. Based on the data obtained by enumeration studies it was found that *E. coli* population formed about 6% while *E. faecalis* and *Salmonella* formed 0.1% of the total microbial load (aerobic) in the representative sewage samples. Thus, a baseline identification of the pathogenic bacteria carried out showed a plethora of bacterial pathogens both target (*E. coli*, *Salmonella* and *E. faecalis*) as well as non-target in the representative sewage samples, which may serve as potential bacteriophage hosts.

3.2 Isolation of bacteriophages against enteric pathogens like *E. coli* and *Enterococcus faecalis* from the domestic wastewater and wastewater effluent samples:

Phage lysates obtained through enrichment were tested against the reference host cultures for its lytic activity, detected by the presence of clearance of reference host culture when phage lysates were spotted on lawn of host culture (Table 11 and Figure 3). 90% and 80% of all the lysates were found to infect *E. coli* & *E. faecalis* respectively. In contrast only 50% of the lysates showed their ability to lyse Salmonella culture. Additionally, all the lysates when plated to obtain isolated plaques showed variation in their morphology. Such variation normally indicates the presence of a mixture of phages within the lysate indicating a need to further purify the same.

Lysates that showed clear plaques but with varied morphology were selected for further purification (Table 12) using repeated cycles of propagation followed by pick of a single clone of plaque that was enriched and used for further rounds of propagation (Figure 4). Ideally, the final confirmation of the

purity of the lysates is undertaken using electron microscopy or molecular methods. Since this is out of the scope of this project, successive purifications steps were performed to ensure that the lysate gave plaques that were all of similar morphology and size (Figure 5).

Table 11: Spot test for Detection of bacteriophage against reference host bacteria

Sample No.	Detection of phage against reference host cultures		
	E. coli	***Salmonella***	***E. faecalis***
Sample R1	+	+	+
Sample R2	+	+	-
Sample R3	+	-	+
Sample R4	+	+	+
Sample R5	-	-	-
Sample R6	+	-	+
Sample R7	+	-	+
Sample T1	+	+	+
Sample T2	+	-	+
Sample T3	+	+	+

+: Clearance observed; -: No clearance/ turbid plaques observed

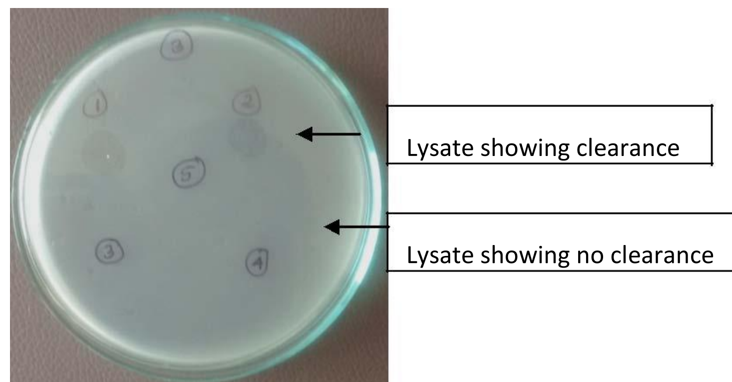


Figure 5: Lysates showing different activity when spotted on reference host

Table 12: Plaque clones selected for further purification

Lysates obtained from sewage	No. of plaques with varied morphology selected for purification with activity against		
	E. coli (ATCC 11229)	*Salmonella* (ATCC 11331)	*E. faecalis* (ATCC 29212)
Sample R1	6	1	0
Sample R2	0	5	0
Sample R3	0	0	0
Sample R4	0	4	0
Sample R5	0	0	0
Sample R6	4	0	0
Sample R7	2	0	1
Sample T1	4	4	1
Sample T2	10	0	1
Sample T3	8	4	1
Total lysates	**34**	**18**	**4**

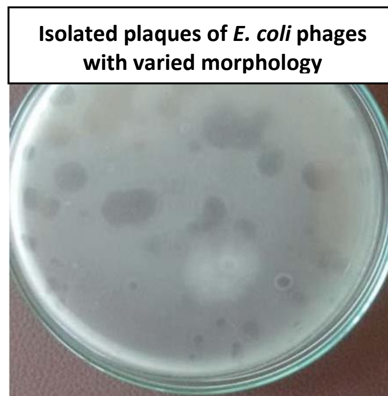


Figure 6: Plaques of varied morphology observed following enrichment which were selected for further purification

Figure 7: Isolated plaques following purification

3.3 Characterization of purified bacteriophages based on host range.

Use of a standard reference host that is well characterized is normally used to determine its purity and assess its lytic/lysogenic nature. But such purified lysates, if are to be used in natural environment to effectively reduce pathogen numbers, need to be checked for its activity against the routinely found flora/pathogen within the waste waters that are discharged after secondary treatment. With this aim in mind, purified Phage lysates were checked for their activity against selected standard reference pathogen host (ATCC cultures) and environmental pathogens that were earlier isolated and listed in Table 4. The results are summarized in Table 13, 14 and 15 and Figure 6.

Table 13: Determination of host range of bacteriophage using reference and environmental *E. faecalis* host bacteria

S. No	Host cultures	No. of lysates that showed plaques with			
		Complete clearance	Turbid clearance	No infectivity	Total lysates tested
1	*E. faecalis* (ATCC 29212)	4	0	0	4
2	Sample R2 – Isolate 1	4	0	0	
3	Sample R4 – Isolate 1	4	0	0	
4	Sample R6 – Isolate 1	4	0	0	
5	Sample T1 – Isolate 1	4	0	0	
6	Sample T2 – Isolate 1	4	0	0	
7	Sample T3 – Isolate 1	4	0	0	

Table 14: Determination of host range of bacteriophage using reference and environmental *E. coli* host bacteria

S. No	Host cultures	No. of lysates that showed plaques with			
		Complete clearance	Turbid clearance	No infectivity	Total lysates tested
1	*E. coli* ATCC 8739	32	2	0	34
2	*E. coli* ATCC 11229	34	0	0	
3	*E. coli* ATCC 15597	7	16	11	
4	*E. coli* ATCC 10536	22	6	8	
5	*E. coli* ATCC 196	13	10	11	
6	Sample T1 – Isolate 1	0	12	22	
7	Sample T1 – Isolate 2	2	0	32	
8	Sample T1 – Isolate 3	0	2	32	
9	Sample T1 – Isolate 4	0	9	25	
10	Sample T1 – Isolate 5	14	0	20	
11	Sample T2 – Isolate 1	0	4	30	
12	Sample T2 – Isolate 2	2	19	13	
13	Sample T3 – Isolate 1	3	5	26	
14	Sample T3 – Isolate 2	5	14	15	
15	Other *E. coli*	0	2	32	

Table 15: Determination of host range of bacteriophage using reference and environmental Salmonella host bacteria

S. No.	Host cultures	No. of lysates that showed plaques with			
		Complete clearance	Turbid clearance	No infectivity	Total lysates tested
1	*Salmonella* ATCC 11331	18	0	0	18
2	*Salmonella* ATCC 14028	2	0	16	
3	Sample R4 – Isolate 1	0	0	18	
4	Sample R4 – Isolate 2	16	2	0	
5	Sample T3 – Isolate 1	0	0	18	
6	Other *Salmonella* - Isolate	3	0	15	
7	Other *Salmonella* - Isolate	3	0	15	
8	Other *Salmonella* - Isolate	3	0	15	
9	Other *Salmonella* - Isolate	0	3	15	
10	Other *Salmonella* - Isolate	0	3	15	

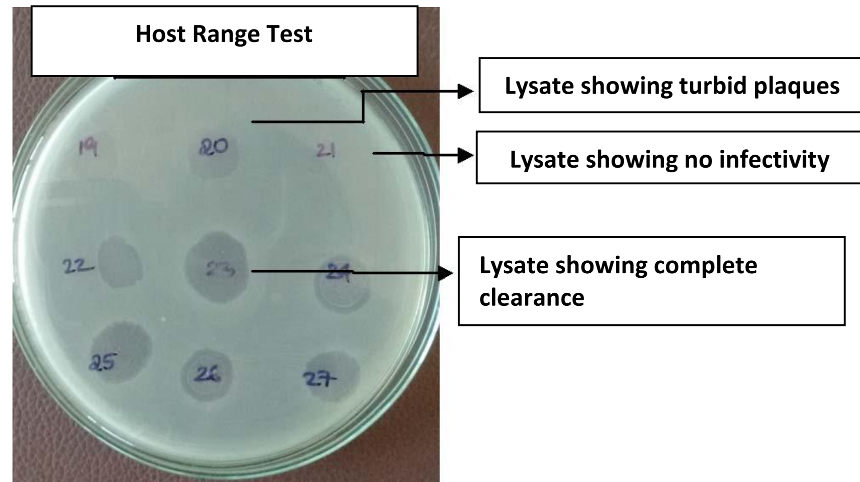


Figure 8: Types of plaques showing clear and turbid clearance

Since primary lysates are likely to have activity resulting from more than one phage being present, it was thus first purified and then tested for its host range, consistency in retaining the plaque morphology, along with being able to propagate to titers above 105 PFU/ ml helped to short list lysates such that of the 34 *E. coli* lysates, 8 having a broad host range were identified, while for *Salmonella* 4 out of the 18 lysates were found to have a broad host range. Surprisingly, all the 4 *E. faecalis* lysates could infect both reference and all 6 environmental strains isolated, the results of which are shown in Table 16, 17 and 18.

Table 16: Determination of host range of *E. faecalis* bacteriophage against reference and environmental host bacteria

S. No.	Host cultures	Lysates tested			
		Sample R7 – Lysate 1	Sample T1 – Lysate 1	Sample T2 – Lysate 1	Sample T1 – Lysate 2
1	*E. faecalis* ATCC29212	+	+	+	+
2	Sample R2 – Isolate 1	+	+	+	+
3	Sample R4 – Isolate 1	+	+	+	+
4	Sample R6 – Isolate 1	+	+	+	+
5	Sample T1 – Isolate 1	+	+	+	+
6	Sample T2 – Isolate 1	+	+	+	+
7	Sample T3 – Isolate 1	+	+	+	+

Table 17: Determination of host range of *E. coli* bacteriophage against reference and environmental host bacteria

S. No.	Host cultures	Lysates tested							
		Sample R1 - Lysate 3	Sample R6 - Lysate 1	Sample R6 - Lysate 2	Sample R6 - Lysate 3	Sample T1 - Lysate 4	Sample T2 - Lysate 1	Sample T3 - Lysate 4	Sample T3 - Lysate 5
1	*E. coli* ATCC 8739	+	+	+	+	+	0	+	0
2	*E. coli* ATCC 11229	+	+	+	+	+	+	+	+
3	*E. coli* ATCC 15597	0	0	0	0	0	0	0	0
4	*E. coli* ATCC 10536	+	+	+	+	+	0	+	0
5	*E. coli* ATCC 196	+	0	0	0	0	0	0	0
6	Sample T1 – Isolate 1	+	-	+	+	+	+	+	+
7	Sample T1 – Isolate 2	-	-	-	-	-	-	-	-
8	Sample T1 – Isolate 3	-	-	-	-	-	-	-	-
9	Sample T1 – Isolate 4	-	-	-	-	-	-	-	-
10	Sample T1 – Isolate 5	+	-	0	+	+	-	+	+
11	Sample T2 – Isolate 1	-	-	-	-	-	-	-	-
12	Sample T2 – Isolate 2	-	-	-	-	-	-	-	-
13	Sample T3 – Isolate 1	-	-	-	-	-	-	-	-
14	Sample T3 – Isolate 2	-	-	-	-	-	-	-	-
15	Other *E. coli*	-	-	0	`-	-	-	-	-

Table 18: Determination of host range of *Salmonella* bacteriophage against reference and environmental host bacteria

S. No.	Host cultures	Lysates tested				
		Sample R4 - Lysate 4	Sample T3 - Lysate 1	Sample T3 - Lysate 2	Sample T3 - Lysate 3	Sample T3 - Lysate 4
1	*Salmonella* ATCC 11331	+	+	+	+	+
2	*Salmonella* ATCC 14028	+	+	+	+	+
3	Sample R4 – Isolate 1	-	-	-	-	-
4	Sample R4 – Isolate 2	+	+	+	+	+
5	Sample T3 – Isolate 1	-	-	0	-	-
6	Other *Salmonella*-Isolate 1	-	0	-	-	-
7	Other *Salmonella*-Isolate 2	-	-	-	-	-
8	Other *Salmonella*-Isolate 3	-	0	-	-	-
9	Other *Salmonella*-Isolate 4	-	-	-	-	-
10	Other *Salmonella*-Isolate 5	-	-	-	-	-

Key:

(+) Complete clearance
(-) No clearance
(0) Turbid plaque

Thus following purification, phage lysates were shortlisted based on their characteristics of being lytic, having broad host range and ability to grow to high titers in the lab. On the basis of these criteria the final selection of phage lysates and their characteristics have been detailed in Table 19.

Table 19: Final selection of phage lysates showing activity against specific hosts

S. No.	Phage lysates	Target host	Host range (ref Table 4)	Titer (PFU/ml)
1	Sample R7 – Lysate 1	*Enterococcus faecalis*	7/7– complete clearance	3.6×10^{8}
2	Sample T1 – Lysate 2		7/7– complete clearance	4.8×10^{8}
3	Sample R6– Lysate 2	*E. coli*	4/15 – complete clearance 4/15 – turbid plaques 8/15 – no clearance	2×10^{14}
4	Sample T1 – Lysate 4		5/15 – complete clearance 2/15 – turbid plaques 8/15 – no clearance	4.1×10^{12}
5	Sample T3 – Lysate 4		5/15 – complete clearance 2/15 – turbid plaques 8/15 – no clearance	4.9×10^{9}
6	Sample R4 – Lysate 4	*Salmonella* spp.	3/15 – complete clearance 7/15 – no clearance	3.4×10^{6}
7	Sample T3 – Lysate 2		3/15 – complete clearance 1/15 – turbid plaques 6/15 – no clearance	5×10^{6}
8	Sample T3 – Lysate 4		3/15 – complete clearance 7/15 – no clearance	8×10^{6}

3.4 Determination of infectivity of phage and optimization of feed concentration (infectivity) of phage to effectively reduce the specific pathogen in wastewater/ effluent

Growth curves as per the method of Ellis and Delbruck (1939) were performed for selected phages against the reference host bacteria *E. coli* using lysates 4 obtained from waste water sample T1 and T3 respectively. For *Salmonella* host culture the lysates used in this assay were Lysate No. 2 and 4 obtained from waste waters sample T3 respectively while for *E. faecalis* host lysates 1 and lysates 2 obtained from sample R7 and T1 respectively were used. The results of the growth curves have been graphically represented as Log (P/Po) plotted versus time where Po is initial concentration of phage and P is concentration of phages obtained at time t.

Burst pressure was calculated as: $\frac{\text{Maximum titer of the phage at 1st rise}}{\text{No. of host bacterial cells in the adsorption tube at Time 0}}$

The rise corresponds to the average number of phage produced per burst.

One step growth curves for 2 phage lysates having activity against *E. coli* indicated that both lysates were able to burst the host culture in stationary phase with the first burst occurring around 150 minutes. In contrast, the pressure at which the host lysed due to the accumulation of formed phages for lysate 4 obtained from Sample T1 was 11 phages while for lysate 4 obtained from Sample T3 was 20 phages. This indicates that within the given host - phage system 11 and 20 phages respectively will be released every 150 minutes (Figure7). In case of *Salmonella,* the burst pressure for phages obtained from Sample T3 was found as 14 phages during the first burst occurring at 150 minutes while at the second burst occurring at 240 minutes, 24 phages per host cell were released. In contrast lysates obtained from Sample T3 burst the cell early i.e every 60 minutes to release 11 phage particles per host cell lysed (Figure 8). For *E. faecalis,* phage particles obtained from Sample R7 burst every 30 minutes to release 7 particles per host followed by a second burst that occurs every 120 minutes to release 13 particles per host cell lysed (Figure 9).

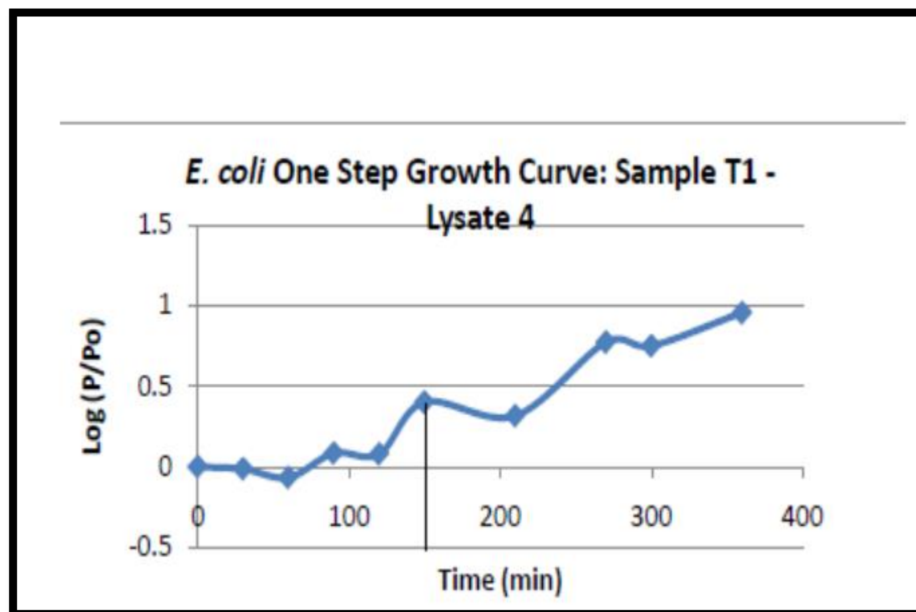


Figure 9a: One step growth curve of phage T1- Lysate 4 against *E. coli*

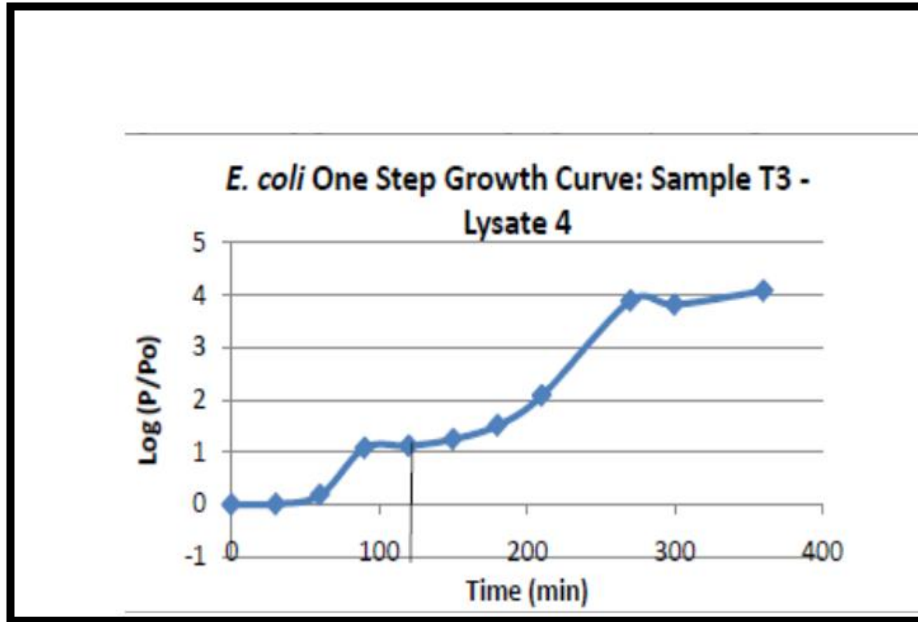


Figure 9b: One step growth curve of phage T3- Lysate 4 against *E. coli*

For Sample T1 – Lysate 4 – burst pressure observed at 150 minutes was calculated as 11phages/ host. For Sample T3 – Lysate 4– burst pressure observed at 150 minutes was calculated as 20phages/ host.

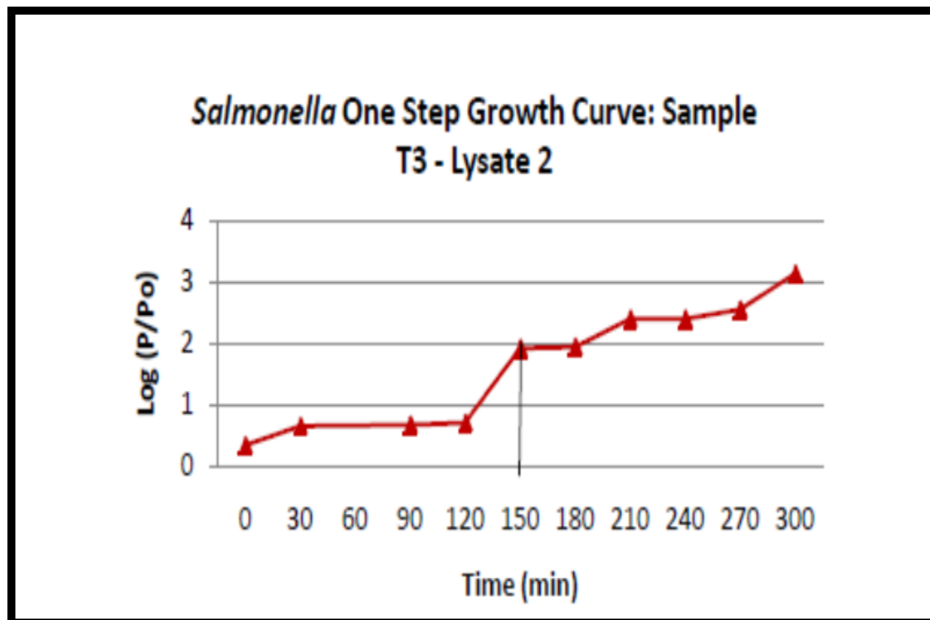


Figure 10a: One step growth curve of phage T3- Lysate 2 against *Salmonella*

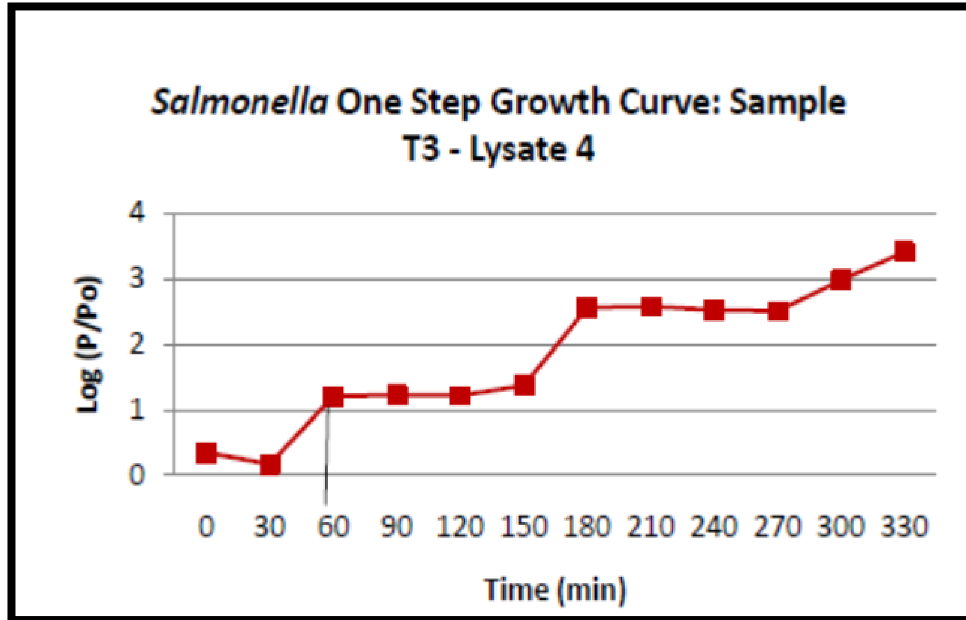


Fig 10b: One step growth curve of phage T3- Lysate 4 against *Salmonella*

For Sample T3 – Lysate 2– burst pressure observed at 150 minutes was calculated as 14phages/ host. For Sample T3 – Lysate 4– burst pressure observed at 60 minutes was calculated as 11phages/ host.

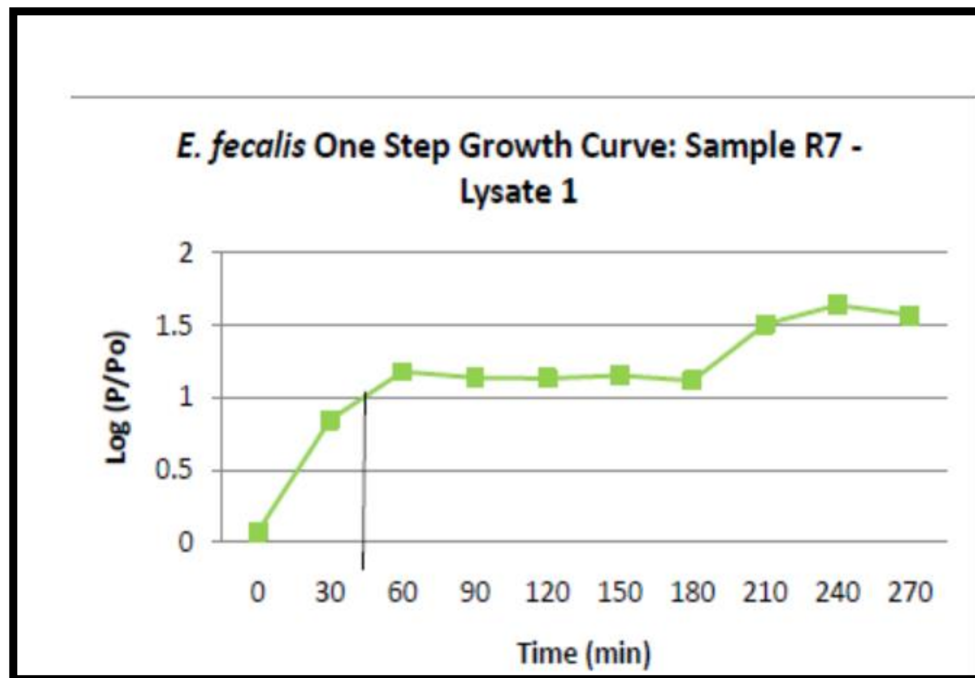


Figure 11a: One step growth curve of phage R7- Lysate 1 against *E. faecalis*

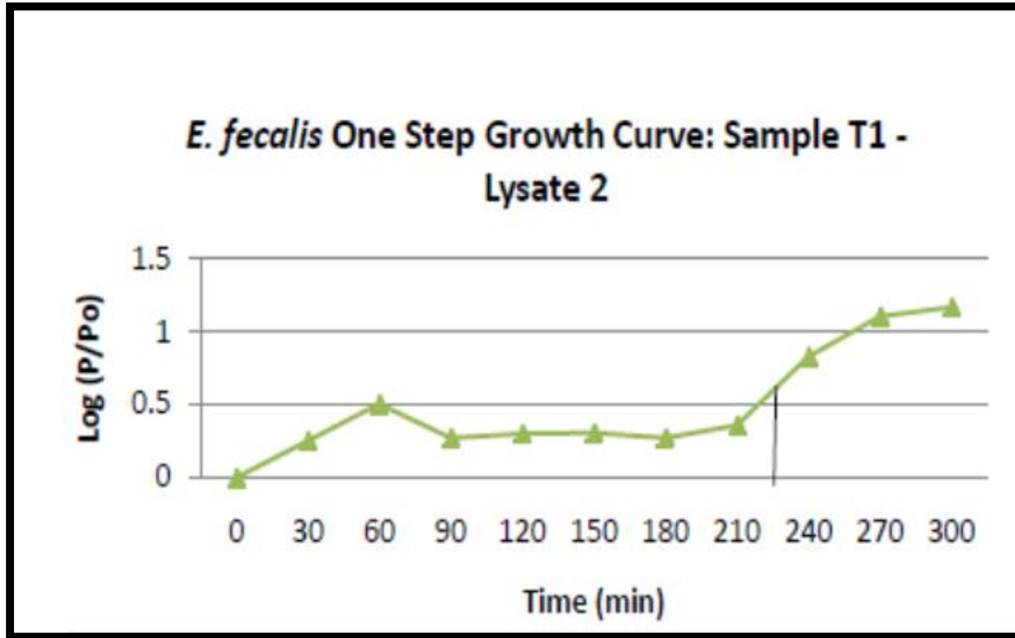


Figure 11b: One step growth curve of phage T1- Lysate 2 against *E. faecalis*

For Sample R7– Lysate 1 – burst pressure observed at 30 minutes was calculated as 13phages/ host. For Sample T1– Lysate 2 – burst pressure observed at 210 minutes was calculated as 34 phages/ host.

3.5 Preparation of bacteriophages formulation to reduce pathogen load within wastewaters at laboratory scale.

For an effective lysis of a bacterial cell the right level of phage particles need to be adsorbed on the cell surface. Too little phage particles will leave unabsorbed host cells while too high a phage titre will lead to super infection that will prevent cell lysis. Thus, in order to determine the amount of phage particles required to effectively reduce pathogens in wastewater at laboratory scale, effective Multiplicity of Infection (MOI)needs to be determined. Thus, a system of reference host (i.e. *E. coli* /*Salmonella*/*E. faecalis*) and its selected phage lysate were set up at concentrations that achieved a MOI ratio of 1, 10 and 50 respectively (Table 5) within the sterilized secondary treated effluent obtained from the STP plant.

At time intervals of 0 hour, 6 hours, 24 hours and 48 hours, the effect of phage induced lysis of the host cells was determined by performing total viable count of samples drawn from the same thereby knowing the number of host bacteria viable within the system at the specified time points. Within such a simulated system survival of the selected strains of *E. coli, Salmonella and E. faecalis* was observed indicating that secondary treated effluent have adequate nutrients to support their survival such that *E. coli* host culture survived up to 24 hours after which growth declined. In contrast, *Salmonella* grew over the 24-hour time period but *E. faecalis* naturally declined within that time span (Figure 10a). Even in cultures that naturally declined in sewage, the addition of phage was able to reduce their growth in a short time period of 6 hours as all the selected phage lysates were found to be capable of showing a reduction of reference host culture to less than 10 CFU/ml within 6 hours of infection. (Figure 10b-10g).

An MOI between 1-10 seemed more effective to use for further reduction experiments since it requires lower amount of phage and will also avoid superinfection which reduces the efficacy of phage action. Thus, based on their MOI, burst size and the ability to be propagated at high titres (> 1 x 10^8 PFU/ml). **Phage Lysates 4 obtained from Sample T3 were found to be ideal for reduction of *E. coli* and *Salmonella* spp. respectively, while for reduction studies of *E. faecalis*, Lysate 2 obtained from Sample T1 was selected for final experiments in STP set up at larger scale of 1 litre volume.**

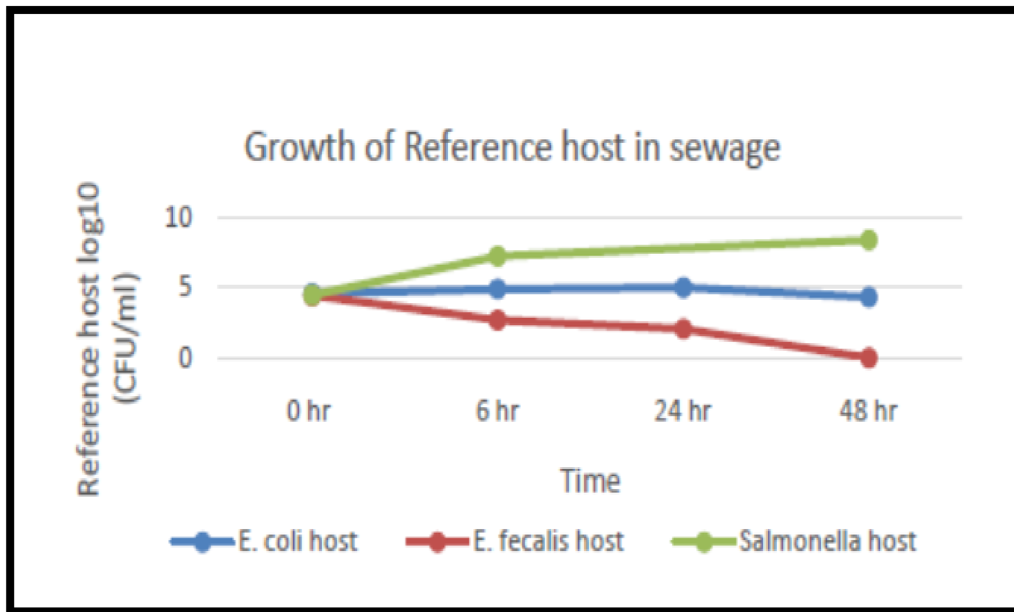


Figure 12a: Growth of reference host cultures in sewage in absence of phage

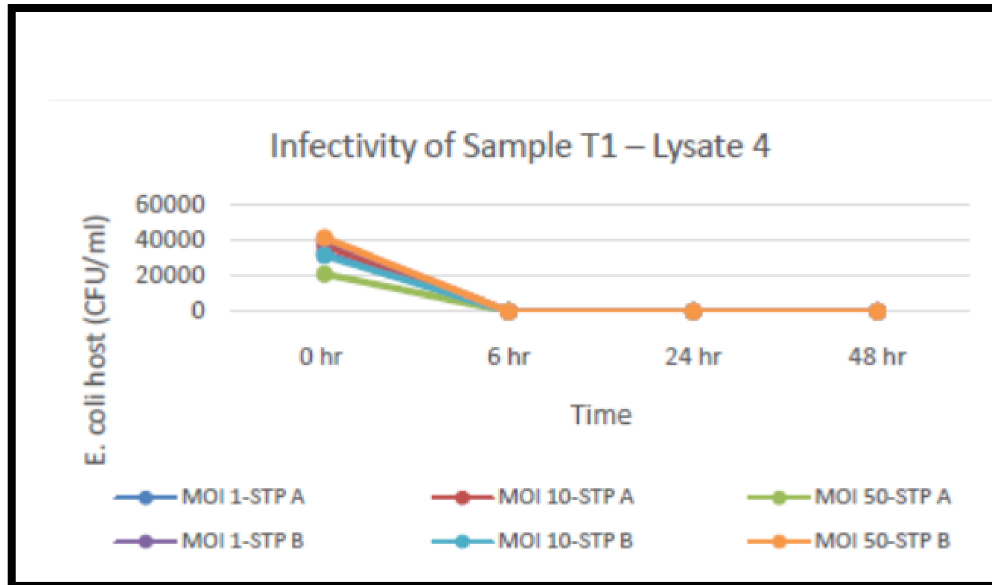


Figure 12b: Reduction of *E. coli* reference host when infected with phage Sample T1 – Lysate 4

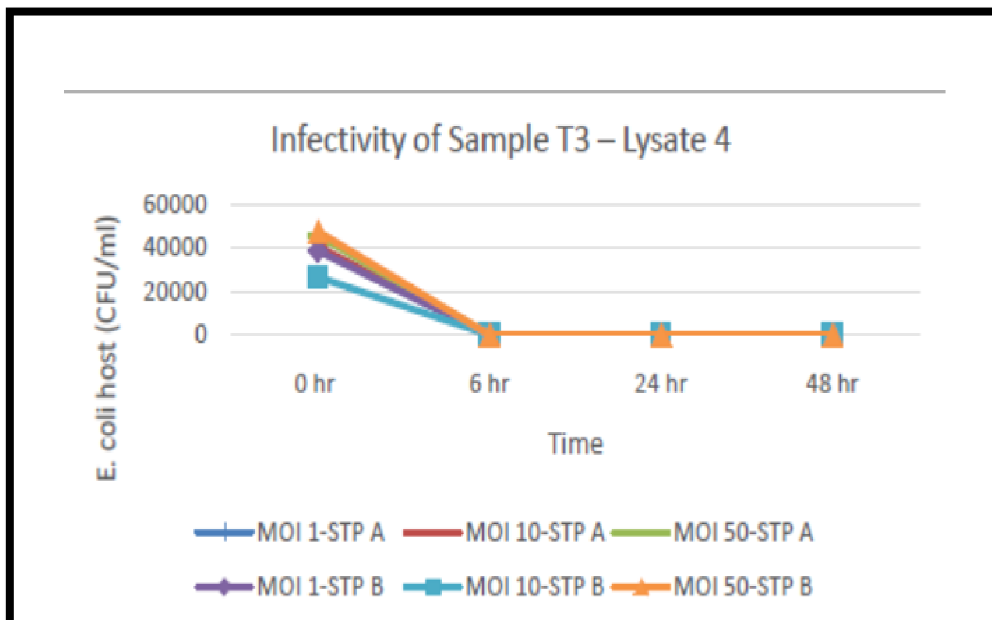


Figure 12c: Reduction of *E. coli* reference host when infected with phage Sample T3 – Lysate 4

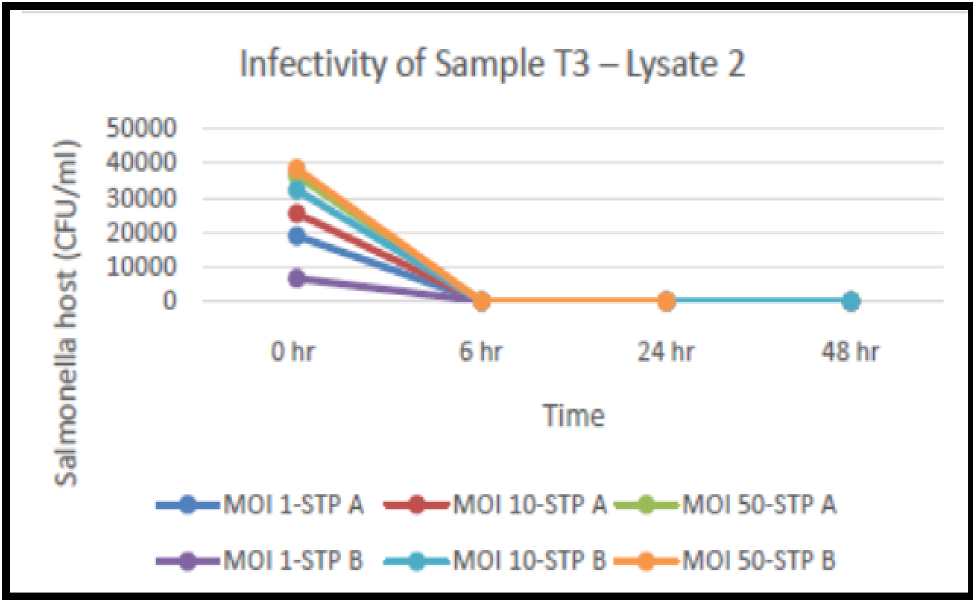


Figure 12d: Reduction of *Salmonella* reference host when infected with phage Sample T3 –Lysate 2

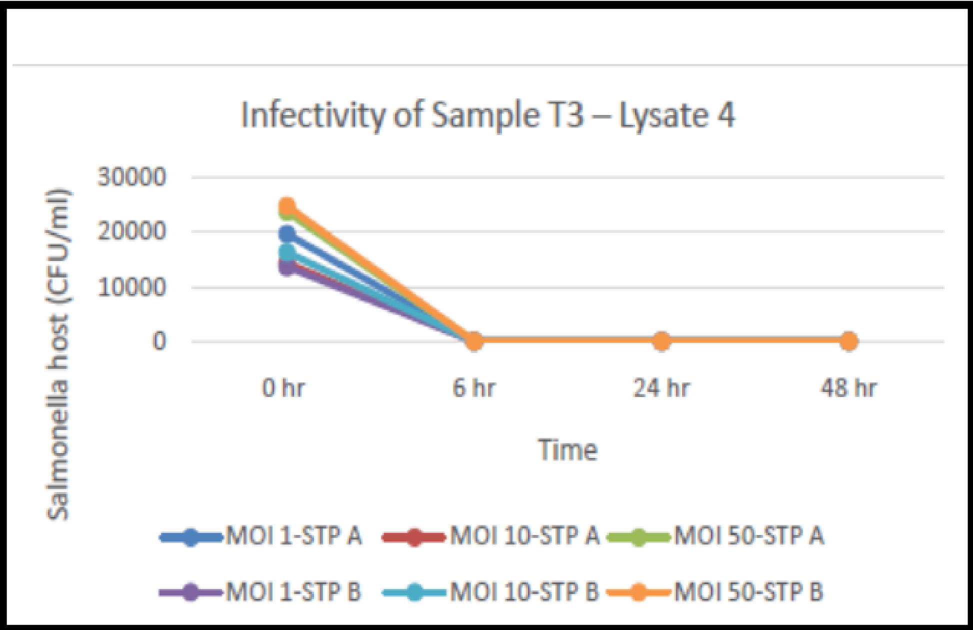


Figure 12e: Reduction of *Salmonella* reference host when infected with phage Sample T3 – Lysate 4

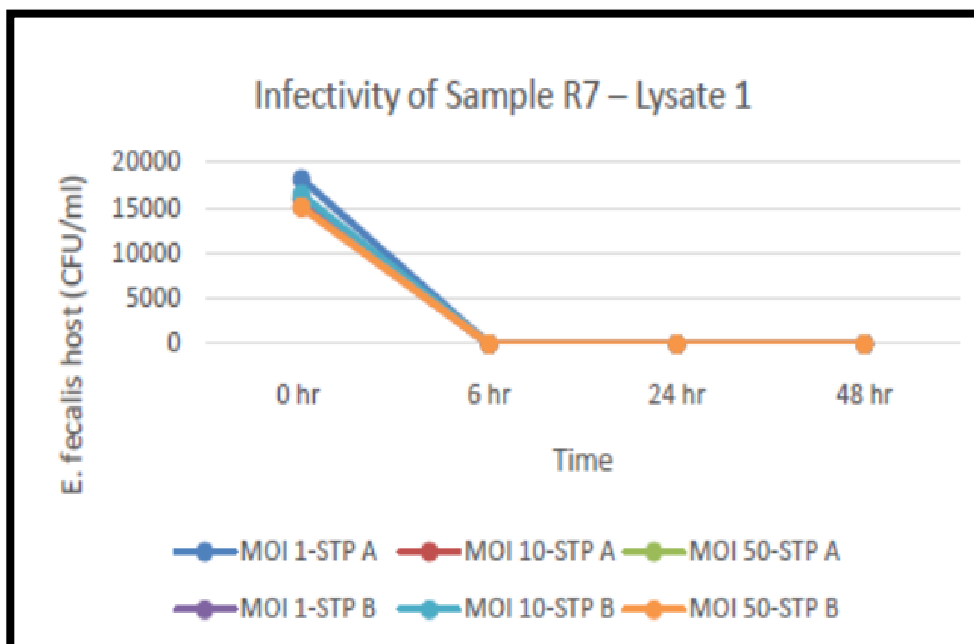


Figure 12f: Reduction of *E. faecalis* reference host when infected with phage Sample R7-Lysate 1

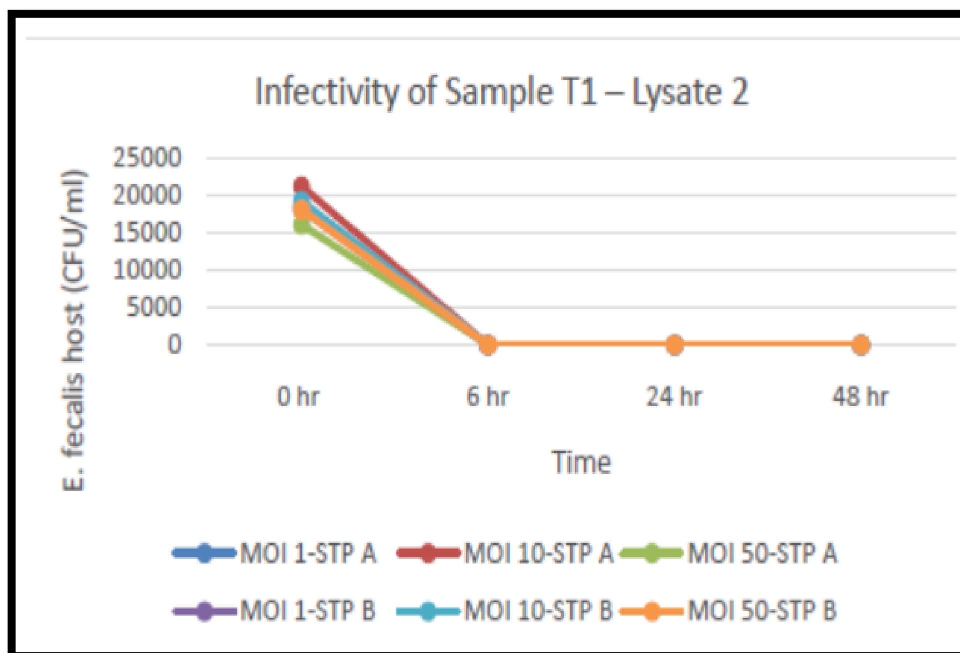


Figure 12g: Reduction of *E. faecalis* reference host when infected with phage Sample T1-Lysate 2

3.6 Use of select bacteriophages formulation to effectively reduce pathogen load in secondary treated wastewater obtained from Sewage Treatment Plant (STP):

Using the optimal MOI between 1-10, the selected phage and host system was set up in the STP waste water obtained from lagoon A and B. The system was set up in a volume size of 100 ml and 1 litre using both sterile and unsterile secondary treated effluents (Table 6). At time intervals of 0 hour, 6 hours, 24 hours and 48 hours, the reduction in the number of host bacteria within in the STP matrix was determined through a viable count of the same using selective media as indicated in Table 1. Sterile sewage maintained as negative control showed no growth at each time point. The values were plotted as Log (ln/lo) is plotted, where lo is the CFU/ml of reference host not infected with phage and ln is the CFU/ml of reference host after infection studies with phage at time t. In addition to the microbial load, the turbidity and pH of the sewage samples was also determined in order to estimate the effect these parameters may have on the activity of phage. (Figure 11)

Table 20: Turbidity and pH of sewage samples

S. No.	Sample	Turbidity (NTU)	pH
1	Batch 1- Lagoon A	27.4	6.33
2	Batch 1- Lagoon B	30.7	7.05
3	Batch 2- Lagoon A	79.0	6.48
4	Batch 2- Lagoon B	22.4	6.60

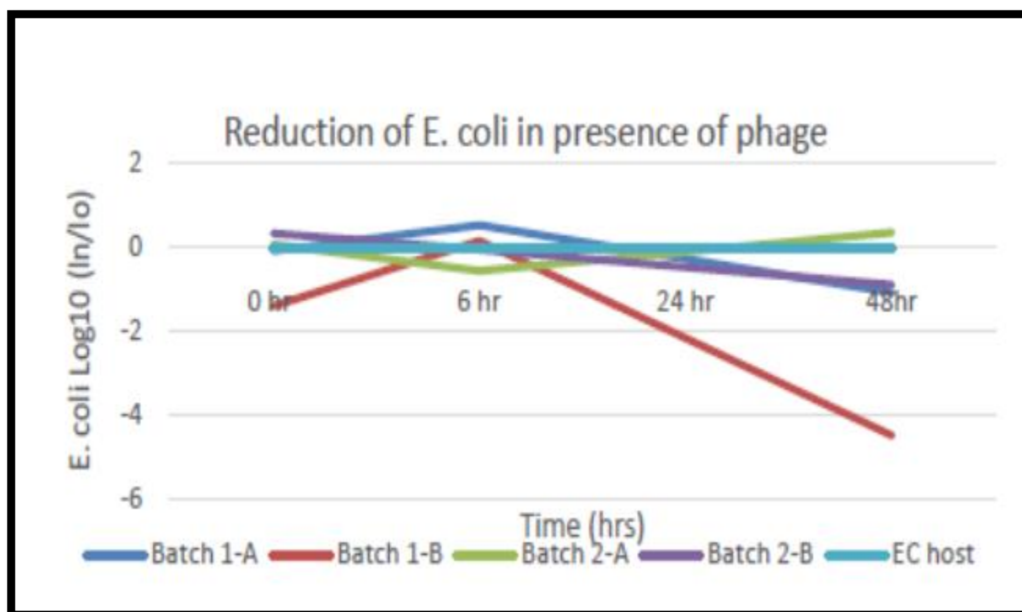


Figure 13a: Reduction of *E. coli* host in presence of phage infected in 100ml sterile sewage

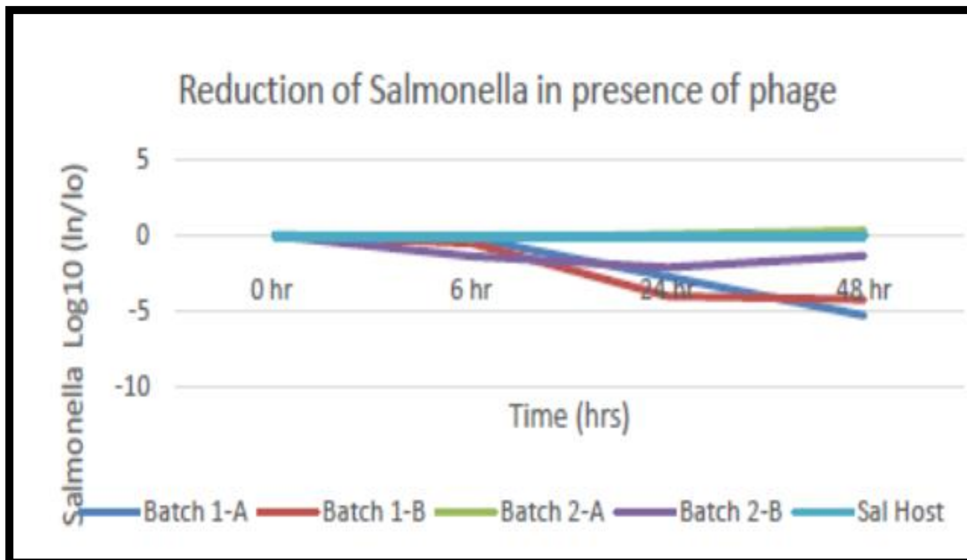


Figure 13b: Reduction of *Salmonella* host in presence of phage infected in 100ml sterile sewage

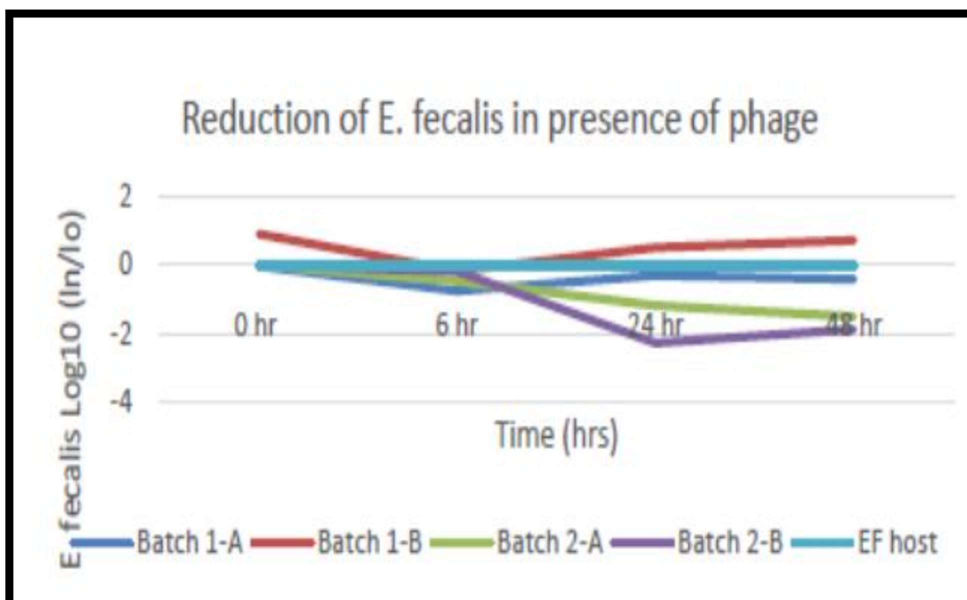


Figure 13c: Reduction of *E. faecalis* host in presence of phage infected in 100ml sterile sewage

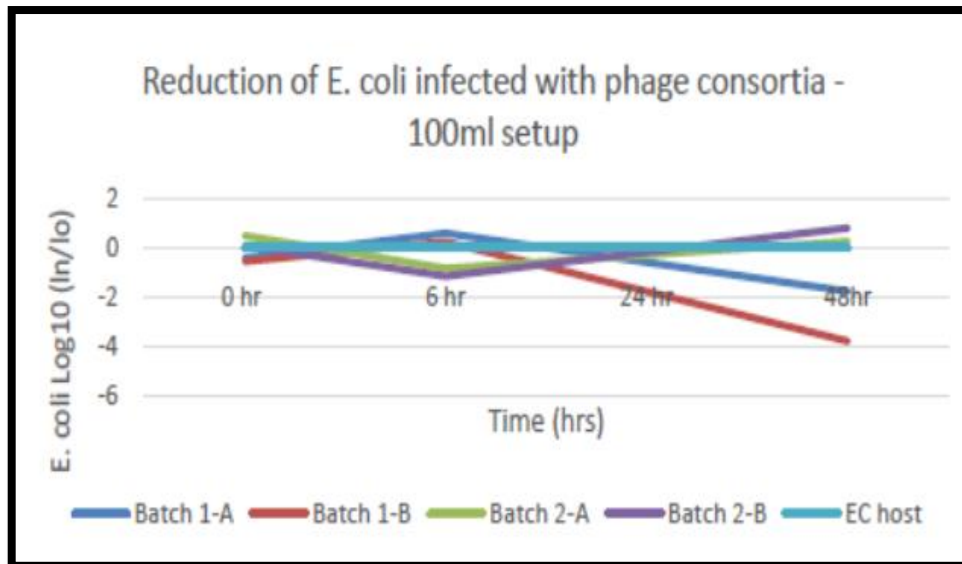


Figure 13d: Reduction of *E. coli* host in presence of phage infected in 100ml sterile sewage in combination with *Salmonella* and *E. faecalis* host and phage consortia

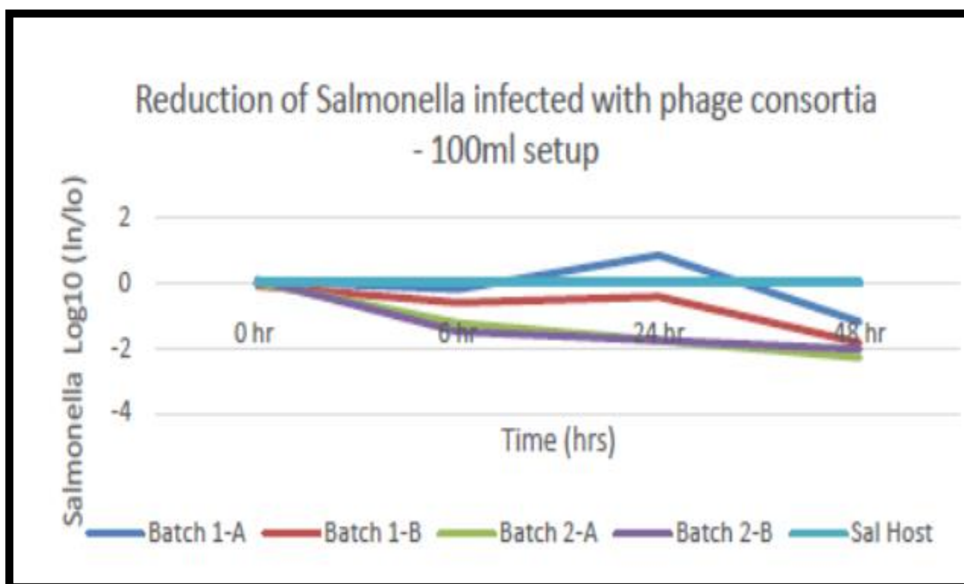


Figure 13e: Reduction of *Salmonella* host in presence of phage infected in 100ml sterile sewage in combination with *E. coli* and *E. faecalis* host and phage consortia

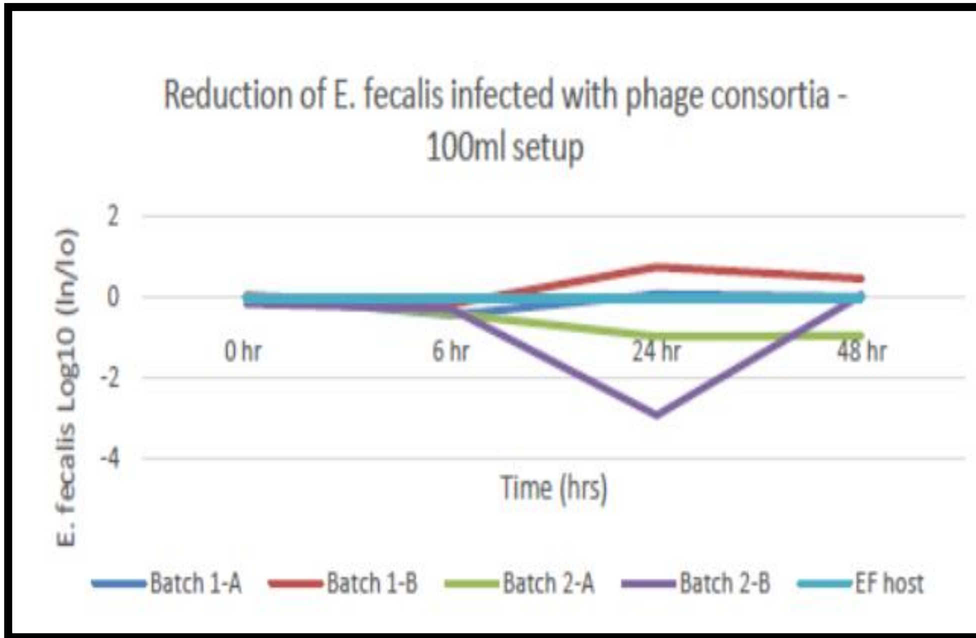


Figure 13f: Reduction of *E. faecalis* host in presence of phage infected in 100ml sterile sewage in combination with *E. coli* and *Salmonella* host and phage consortia

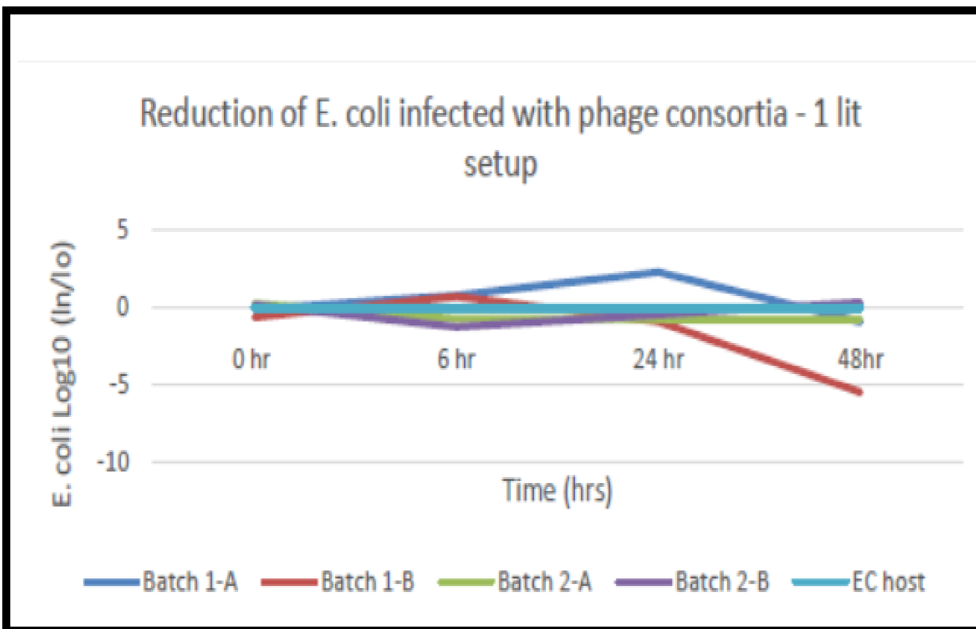


Figure 13g: Reduction of *E. coli* host in presence of phage infected in 1 litre sterile sewage in combination with *Salmonella* and *E. faecalis* host and phage consortia

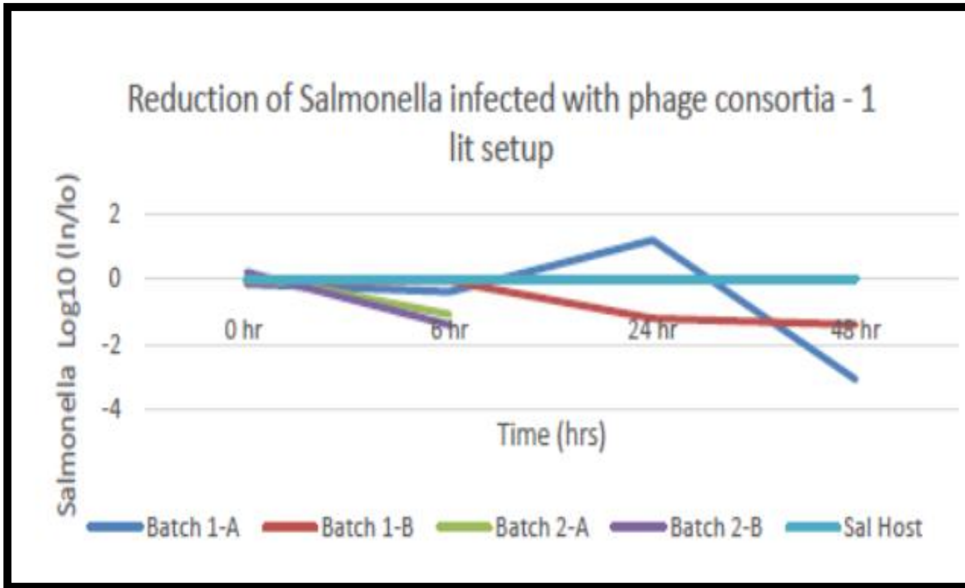


Figure 13h: Reduction of *Salmonella* host in presence of phage infected in 1 litre sterile sewage in combination with *E. coli* and *E. faecalis* host and phage consortia

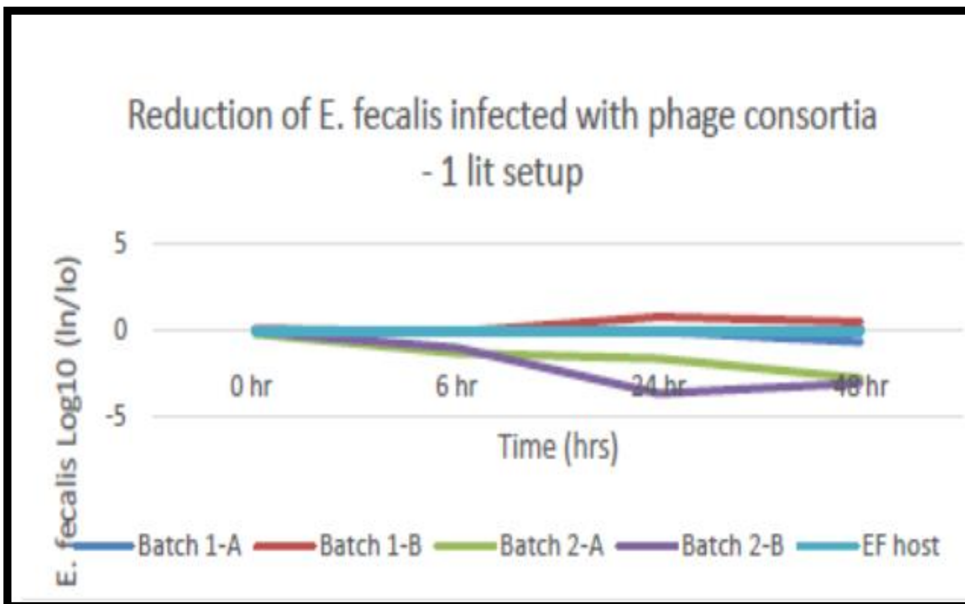


Figure 13i: Reduction of *E. faecalis* host in presence of phage infected in 1 litre sterile sewage in combination with *E. coli* and *Salmonella* host and phage consortia

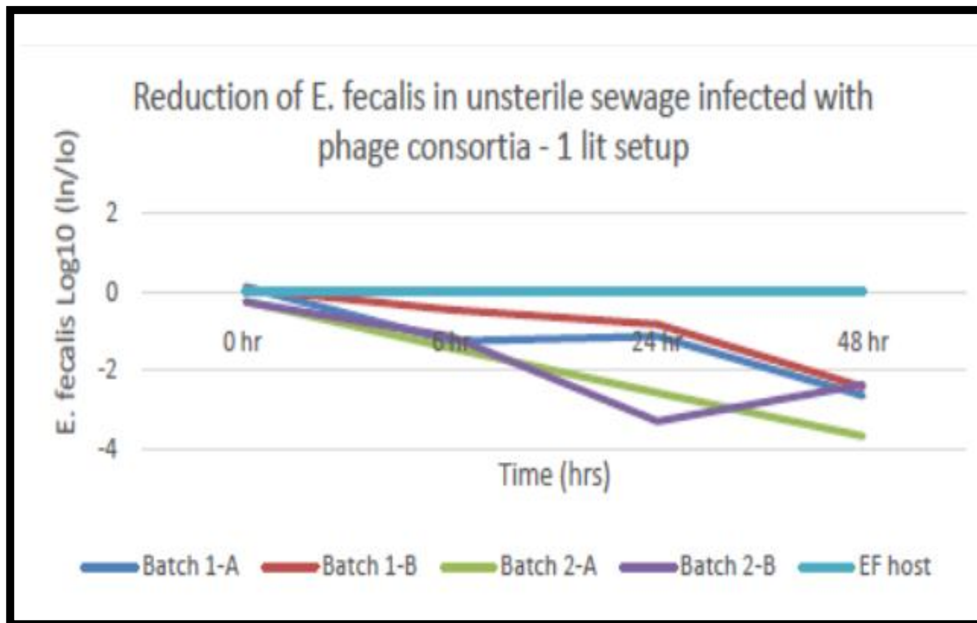


Figure 13j: Reduction of *E. faecalis* host in presence of phage infected in 1 litre unsterile sewage in combination with *E. coli* and *Salmonella* host and phage consortia

Note: In case of E. coli and Salmonella we were unable to estimate the reduction when spiked in unsterile sewage since a number of other bacteria overgrew on the XLD plate. In order to obtain such an estimation, molecular methods need to be employed which was beyond the scope of the current project. In contrast, E. faecalis load could be estimated without any background interference as the selective media of Bile Esculin Agar allows Gram positive organisms and did not allow the growth of gram negative organisms.

While an initial reduction of reference host cultures was observed the behaviour of host cultures varied in each sewage sample. It was particularly noted that the reduction was lower in certain sewage samples. Growth of host detected despite addition of phages could be due to cases where infection may not have been initiated either due to a resistant sub-population or due to improper conditions for infection. *E. coli* showed a reduction over 48 hours ranging from 1-4 log in case Batch 1 samples A and B as well as Batch 2 sample A. However, it showed an increase in growth in case of Batch 2-Sample B. In contrast *Salmonella* showed a reduction over 48 hours ranging from 0.5-4 log in case of Batch 1 as well as Batch 2 with the exception of 100 ml setup in Batch 2A of STP (Figure 11b) samples received. *E. faecalis* showed a reduction over 48 hours ranging from 0.5-3 log in case of Batch 1 as well as Batch 2 with the exception of Set 4 in Batch 1A and all sets of Batch 1B of STP samples received indicating that there may be underlying physico-chemical parameters that affect the infectivity of the phage.

In case of neat sewage inoculated in Batch 1 and 2 results were only obtained in case of *E. faecalis* since a large amount of natural flora overgrew on EMB and XLD selective medium (ref Table 1) which made it difficult to identify a reduction at lower than a 10^{-3} dilution. *E. faecalis* results could be deduced which was reduced with the addition of phage. The results indicate that while the selected phages are able to reduce the load of enteric pathogens, additional investigations are required to standard the effect of variation within the organic load and other physicochemical parameters of sewage like turbidity, levels of Calcium/Magnesium that are required to achieve phage adsorption. Due to the inherent limitations of using selective media to determine pathogen reduction, this study was unable to draw a conclusive result of the same in unsterile sewage. However, the use of molecular techniques due to their sensitivity and specificity can circumvent such issues.

The current study has shown a higher log reduction of up to 4-5 log for all bacterial pathogens under study in certain sewage samples. This is indicative of the potential of the bacteriophages to be used as a biocontrol agent following further studies to optimize parameters conducive to its infection as well as variations of physico-chemical parameters of sewage that may discourage infection. Thus, for bacteriophage based biocontrol approach to be successful, the following points have to be taken care of:

(1) Concentration of bacteriophages to be applied must be carefully optimized;

(2) Specific bacteriophage must be identified to counter specifically the pathogenic bacteria without affecting other bacteria;

(3) The microbial analysis of the system is a prerequisite to bacteriophage application as the bacterial population may vary between wastewater treatment plants;

(4) The bacteriophage should not carry any lysogenic genes that may endow it with the potential to integrate with the host nor should it contain any virulent gene that may endanger the environment by inducing virulence in an otherwise sensitive population of host pathogens.

CHAPTER-4

CONCLUSIONS & RECOMMENDATIONS

CONCLUSIONS:

1. Three target pathogens viz. *E. coli, Salmonella sp. and E. fecalis* were successfully isolated from 7 domestic sewage and 3 secondary effluent samples of which *E. coli* population formed about 6% while *E. fecalis* and *Salmonella* formed 0.1% of the total aerobic microbial load. Of 10 sewage samples, *Salmonella* was detected in 4, *E. coli* in 8 samples while *E. fecalis* was detected in all the 10 sewage samples;

2. 90% and 80% of primary lysates were found to infect *E. coli and E. fecalis* respectively. In contrast only 50% of the lysates showed their ability to lyse *Salmonella* host. Clear plaques but with varied morphology were selected for further purification using repeated cycles of propagation of a single clone of plaque that was enriched and used for further rounds of propagation;

3. Following purification, of the 34 *E. coli* lysates, 8 having a broad host range were identified, 4 out of the 18 lysates for *Salmonella* and all 4 lysates of *E. fecalis* had broad host ranges. Final phase lysates were short listed based on their characteristics of being lytic; titers in the lab. Thus, 3 *E. coli* and *Salmonella* lysates each, and 2 lysates of *E. fecalis* were selected;

4. The lysates as selected in the previous objective were further tested for their infectivity using one step growth curve that would determine the number of phages released per host cell on infection. This aided in the identification of the optimal growth stage of the host and amount of phage to be seeded;

5. The effective multiplicity of infection (MOI) i.e. ratio of No. of phage to No. of bacterial host, was determined in order to obtain maximum reduction pathogens using minimal amount of phage in wastewater at laboratory scale. This was carried out in sterile sewage samples spiked with phage and host at an MOI of 1,10 and 50. An MOI of 1and10 seemed more effective, in achieving a 4 log reduction of all 3 host pathogens within a short time period of 6 hrs.;

6. The ability of the phage to infect and reduce target host bacteria varied in each sewage sample. It was particularly noted that the reduction was lower in certain sewage samples. *E. coli* showed a reduction ranging from 1-4 log, *Salmonella* showed a reduction of 0.5-4 log while *E. fecalis* showed a reduction ranging from 0.5-3 log. However, in some cases growth of host was also observed despite addition of phages could be due to cases where infection may not have been initiated either due to a resistant sub-population. Alternatively, this could be due to variations in environmental and physic-chemical conditions of sewage that hamper phage infection. In case of unsterile sewage inoculated with host and phage results were only obtained in case of *E. fecalis* since a large amount of natural flora overgrew on EMB and XLD selective medium which prevented obtaining a count of *E. coli and Salmonella.*

RECOMMENDATIONS:

Although, the present study demonstrated the potential of bacteriophages for the improvement of effluent and sludge emissions from the wastewater treatment plants at the laboratory scale, further studies need to be carried out to apply the concept at large scale for minimizing the risk of environmental release of pathogens from sewage treatment plants. Future scope of the present study is as follows:

(1) While the selected phages were able to reduce pathogens, additional investigations are required in order to determine the optimal amount of phage required to reduce these pathogens with physico-chemical variations in sewage.

(2) In case of *E. coli* and *Salmonella,* we were unable to estimate the reduction when spiked in unsterile sewage since other bacteria overgrew on selective media. In order to estimate this parameter, molecular methods like real time PCR need to be employed which was beyond the scope of the current project.

(3) Phages have inherent capabilities to transduce host bacterial DNA to varying degrees. Although this is reduced in case of lytic phages, molecular studies are required to identify lysogenic characteristic or presence of virulent genes within the bacteriophage genome that may result in the transference of virulent genes in an otherwise sensitive host population.

(4) In the current study, the selected phages were purified only on the basis of plaque morphology. They require to be further characterized using electron microscopy and molecular methods in order to determine their taxonomy and purity over successive generations. This is particularly relevant in case of scaling up of the phage where minor variations will affect the ability of the phage to infect the target host pathogen.

REFERENCES

1. **Kaur R, Wani SP, Singh AK and Lal K.** 2012**.** Wastewater Production, Treatment and Use in India**.** National Report presented at the 2nd regional workshop on Safe Use of Wastewater in Agriculture, May 16-18, 2012, New Delhi, India (http://www.ais.unwater.org)**.**
2. **Anastasi EM, Matthews B, Stratton HM, Katouli M.** 2012. Pathogenic *Escherichia coli* found in **sewage** treatment plants and environmental waters. *Applied Environmental Microbiology,* 78(16):5536-41. doi: 10.1128/AEM.00657-12.
3. **Bain R, Cronk R, Hossain R, Bonjour S, Onda K, Wright J**. 2014. Global assessment of exposure to faecal contamination through drinking water based on a systematic review. *Tropical Medicine & International Health*, 19: 917–27. doi: 10.1111/tmi.12334.
3. **Beaudoin RN, DeCesaro DR, Durkee DL, Barbaro SE**. 2007. Isolation of a bacteriophage from sewage sludge and characterization of its bacterial host cell. *Rivier Academic Journal*, 3(1):00.
4. **Choi J, Kotay SM, Goel R.** 2011. Bacteriophage-based biocontrol of biological sludge bulking in wastewater. *Bioeng Bugs,* 2(4):214-7.
5. **Ellis EL, Delbrück M.** 1939. The growth of bacteriophage. *Journal of General Physiology*, 22(3):365-84.
6. **Hunter PR, Andersson Y, Von Bonsdorff CH, Chalmers RM, Cifuentes E, et al.** 2003. Surveillance and investigation of contamination incidents and waterborne outbreaks. Water quality series - Chapter 7. World Health Organization.
7. **Mulani MS, Azhar S, Azharuddin S, Tambe S.** 2015. Harnessing the power of bacteriophage for pathogen reduction in wastewater. *International Journal of Current Microbiology and Applied Sciences*, Special Issue -2:152-161.
8. **Periasamy D, Sundaram A.** 2013. A novel approach for pathogen reduction in wastewater treatment. *Journal of Environmental Health Science and Engineering*, 11(1):12.
9. **Petrovski S, Seviour RJ, Tillett D**. 2011. Characterization of the genome of the polyvalent lytic bacteriophage GTE2, which has potential for biocontrol of Gordonia-, Rhodococcus, and Nocardia-stabilized foams in activated sludge plants. *Applied Environmental Microbiology*, 77(12):3923-9.
10. **Snyder A.** 2012. Isolation and characterization of bacteriophage for the control of enterohemorrhagic *Escherichia coli* on fresh produce. Thesis. The Ohio State University.
11. **Tan GH, Nordin MS, Napsiah AB.** 2008. Isolation and characterization of lytic bacteriophages from sewage water. *Journal of Tropical Agriculture and Food Science*, 36(2):000-000.
12. **World Health Organization and Organisation** for Economic Co-operation. 2003. Assessing Microbial Safety of Drinking Water – Improving approaches and methods. Water Quality Series.

-------------*****----------