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ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES WITH LYTIC ACTIVITY ISOLATED FROM SILIGURI, WEST BENGAL, USING *ESCHERICHIA COLI* AS HOST ORGANISM

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ABSTRACT
Present research was performed to isolate and characterize bacteriophages with lytic activity against *Escherichia coli.* A total of 3 samples of house hold waste water (1), River and House hold waste water (1) and House hold and dumping waste water (1) were collected. Samples were preliminarily enriched with *E. coli* broth culture to facilitate the growth of the phages and then the phages were isolated and phage titre was determined for all the samples. Samples 1 showed the highest number of phages which also correlated with the maximum Electrical conductivity and total dissolved solids of the waste water. This study also correlated with the physiochemical properties of water with the phage titre. Multiplicity ratio of the sample 1 was found to be 29.67, which was significantly higher among all other samples investigated in the present study. Growth curve of the phages also revealed that the eclipse period of the sample 2 was lower among all other samples.

KEY WORDS: Bacteriophage, *Escherichia coli,* Electrical conductivity, Total dissolved solids, Multiplicity ratio, Eclipse period.

INTRODUCTION

Bacteriophages are found almost everywhere where bacteria exist as they replicate using bacteria as a host organism (Zhan *et al*., 2015). The environmental samples such as soil, water, sewage, animal excreta, factory wastes that contain the bacterial host are the suitable for isolation of bacteriophage and employing them as an alternative therapeutic approach against multidrug resistant bacteria (Naghavi *et al*., 2013). Presently antibiotic resistant bacteria creating a serious problem regarding the health and hygiene of human (WHO, 2014). Moreover, pharamaceutical industries are withdrawing from research and development of new antibiotics due to their unprofitability of the venture and the risk of emerging resistant bacteria day by day (Clarke, 2003) which trigger the urge to development of an alternative strategies which can combat against this current problem. Lytic phages are the possible replacement for antibiotics to treat bacterial infections not responding to conventional antibiotic therapy (O'Flynn *et al*., 2004). Therefore, the present investigation was conducted to isolate and characterize lytic bacteriophages from the waste water using *Escherichia coli* as host system and to assess their multiplicity ratio and growth curve in order to employ them as a suitable phage against bacterial pathogens.

MATERIALS AND METHODS

Waste water samples collection of and processing

The samples of house hold waste disposal were collected in sufficient amount from three different locations near University of North Bengal, West Bengal, India, in the month of September. 250ml water samples were collected in sterilized 500 ml flasks according to the method describe by Jothikumar *et al*., 2000, with slight modification. Samples were spun gently in a homogenizer for 3h continuously and then centrifuged at 2000 rpm for 10min. The supernatant was collected and again centrifuged at 8000 rpm for 10 min at 4^{o}C (Remi C-24). Then, supernatant was filtered through a 0.45 µm millipore syringe filters, and filtrate was tested for the presence of lytic activity against *B. subtilis* and *E. coli*.

Physicochemical Analysis of Waste water Samples

Temperature (^{o}C), pH, Dissolved oxygen (%), Total dissolved solids (mg/l), Electrical conductivity (µS/cm) of the collected waste water samples were determined using Eutech Instrument Cyber Scan series 600 probe.

Isolation and enumeration of bacteriophage from the waste water sample:

Bacteriophage was isolated from the sewage water samples according to the protocol described by Harrigan and McCance, 1993 with slight modifications. Initially 40 ml of raw sewage water samples were mixed with 5 ml of 10X nutrient broth and 5ml of 24h old *Escherichia coli* nutrient broth (1X) culture. The resulting mixture was incubated at 37^{o}C for 24h at 120 rpm. After incubation the 10ml of the mixture broth was centrifuged at 2000 rpm for 5min at 4^{o}C. Most of the bacterial cells and the suspended particulate large materials get pelleted and the resulting supernatant was taken as a crude source of bacteriophage (CB). To remove the remaining bacterial cells from the CB, 10 ml CB was pass through the sterilized 0.45 micron membrane filter fitted into a 10ml syringe barrel. The flow through was collected into the collection tube and kept at 4^{o}C for further use as a bacteriophage suspension. Bacteriophages were further serially diluted (figure 1) using 1.5 ml of sterilized microfuge tube each containing 0.9 ml of 1X Phosphate buffer saline (PBS, containing (g/l)-NaCl 8; KCl 0.2, Na_2HPO_4 1.44, KH_2PO_4 0.24) pH 7.4 up to 10^{-6}. Each of the serially diluted phage samples of 0.1ml was mixed with 0.5 ml of log phase (OD=0.5)

Escherichia coli suspension. The resulting mixture was incubated at 37 ^{o}C for 10 min so that phage adsorption to the bacterial cell surface can take place. The bacteria and phage mixture was then mixed with 3 ml of 0.75%w/v molten sterilized agarose at 45^{o}C. The resulting mixture was then poured into 1X nutrient agar plates. The plates were then incubated at 37^{o}C for 24h and after incubation the phage titer were estimated in terms of plaque forming unit (pfu/ml) using the following equation 1.

Phage titer (pfu/ml) = Numbers of plaque X Dilution factor/ Volume plated (ml)……. (Eq1)

Plaque morphology determination

Plaque morphology was determined according to their size, edge and boundaries (Ellis *et al*., 1969) and was recorded as small (<2mm), medium (2mm) and large (>2mm) clear or diffused type plaques.

One step growth curve of bacteriophage

Growth curve of the isolated bacterio phage was prepared using the method described by Ellis and Delburck, 1938, with some modifications. Phage lysate of 0.1ml was mixed with 0.9ml of 24h old host bacterial culture *E. coli* and the resulting mixture was incubated for 10 min at 37^{o}C. After incubation the phage bacterial mixture were further serially diluted up to 10^{-4} using the phage broth media containing (g/l): casein enzymic hydrolysates 10, Yeast extract 5, Beef extract 5, Lactose 10, Dipotassium phosphate 5, pH 7. The resulting mixture was then incubated for different time interval (20, 25, 30, 40, 50, 60, 70 and 80 min) at 37 ^{o}C. After incubation 0.1 ml of the phage and bacterial mixture was mixed with 0.1 ml of host bacterial culture in a soft agar tube (0.75%w/v) and mix well. The resulting mixture was then poured into 1X nutrient agar plate and the plates were incubated at 37^{o}C for 24 h. Multiplicity ration of the phage in the adsorption tube was calculated using the following equation 2.

Multiplicity Ratio (MR) = Number of phage (pfu/ml) in adsorption tube/Bacteria (cfu/ml) in adsorption tube……..(Eq2).

RESULTS & DISCUSSION

Bacteriophage was isolated from the water sample collected from the sewage nearby University of North Bengal.

Physicochemical analysis of water

Physicochemical analysis of water (Table 1) showed that the sample 1 which mostly contain the house hold waste water and having the pungent and foul odour had maximum total dissolved solid of 3260 ±45 (mg/l) which was followed by sample 2 (2765 ±21 mg/l) and sample 3 (2100 ±11mg/l). With respect to the dissolved oxygen limit sample 2 had the lowest percentage of dissolved O_2 as compared to the other two waste water samples. It was also observed that the electrical conductivity was higher when the total dissolved solids were higher. Similar findings was observed in the research published by Uwidia and Ukulu, 2013, where it had been reported that a correlation coefficient of 0.95 was existed between the EC and TDS content of waste water, which suggested a strong positive correlation between this two parameters.

TABLE 1: Physicochemical analysis of the waste water

	Sample 1	Sample 2	Sample 3
pH	6.9 ± 1.2	6.9 ± 1.2	8.1± 1.9
Temperature (^{o}C)	30 ± 3.2	30 ± 3.7	28 ± 1.5
DO (%)	55 ± 8	63± 7	71± 5
TDS (mg/l)	3260 ± 45	2765 ± 21	2100 ± 11
EC (µS/cm)	367± 21	280 ± 15	206 ± 12
Sampling Conditions	House hold waste water	River and House hold waste water	House hold and dumping waste water
Colour	Black and Grey	Blue, Black and Grey	Black and Grey
Odour	Pungent and Foul	Pungent and Foul	Pungent and Foul

Isolation and enumeration of bacteriophage

Recovery of bacteriophage (Figure-1) was maximum in sample number 1 (92 X10^{5} Pfu/ml). Multiplicity ratio of the sample 1 was found to be 29.67. Higher number of phage was strongly correlated with the high TDS content and EC of the waste water. In case of the sample 2 which contain mainly the house hold and river waste water showed the phage titre of 73 X10^{5} Pfu/ml and multiplicity ratio of 23.54. Whereas in sample 3 phage titre and multiplicity ratio was recorded as 3 X 10^{5} Pfu/ml and 9.67, respectively.

Plaque morphology of bacteriophage lysates

Table 2 and the figure 2 represent the different morphology of the plaques produced due to the lytic activity of the phages on *E. coli* present in the waste water sample.

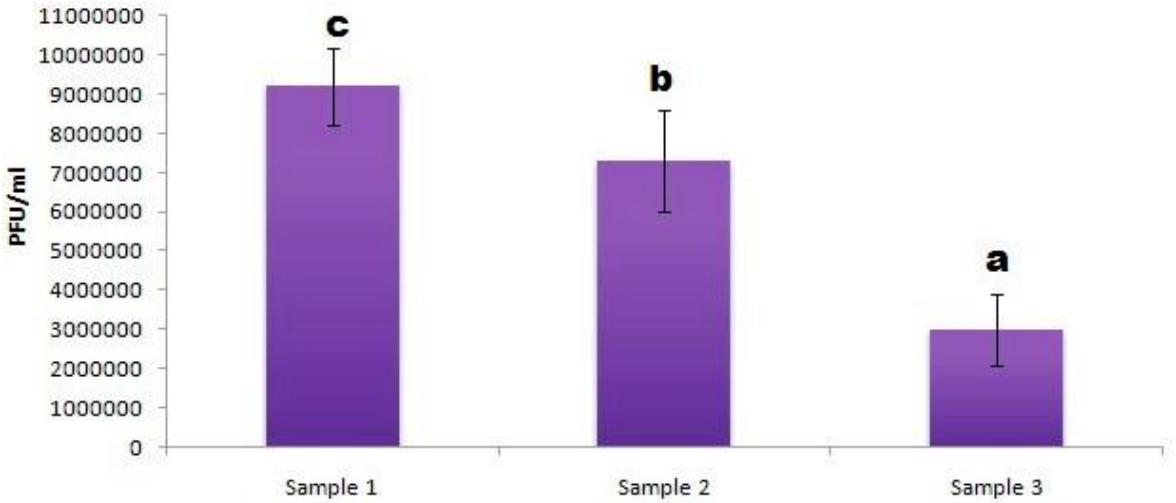

FIGURE 1: Enumeration of bacteriophage isolated from three different waste water samples. Data were represented as mean ±SD. Each bar marked with different alphabets were significantly ($p<0.005$) different as suggested by one way analysis of variance test.

TABLE 2: Morphology of the plaques produced due to the phages isolated from the waste water samples

Phage Identification No	No. of Isolates	Plaque Morphology
Pas_Eb1	14	Pin headed small size clear plaque
Pas_Eb22	2	Large size clear plaque
Pas_Eb32	8	Small size clear plaque
Pas_Eb45	1	Large sized diffused plaque
Pas_Eb48	1	Elevated type haloed plaque
Pas_Eb52	1	Small sized diffused plaque

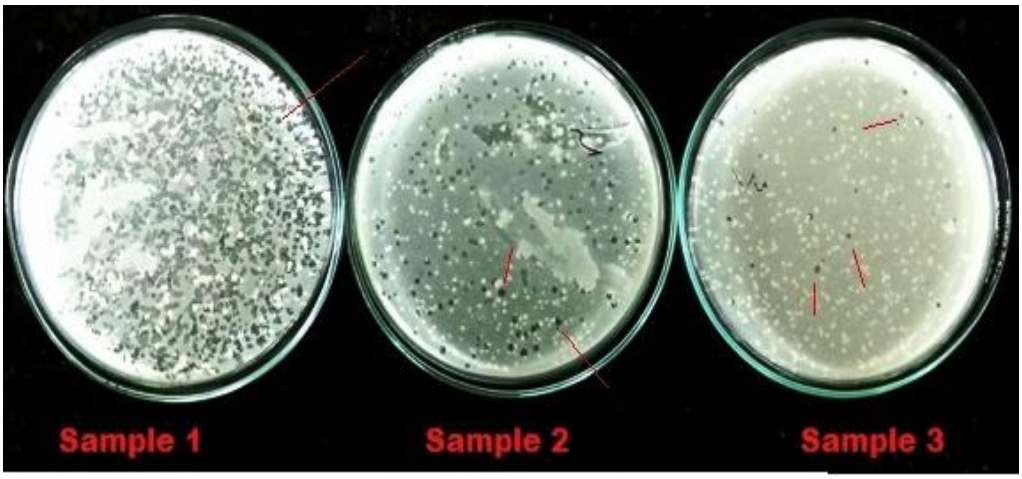

FIGURE 2. Different plaque morphologies observed due to the lytic activity of the phages isolated from three different waste water samples. Red line indicates the distinguish morphologies of plaques.

One step growth curve of bacteriophage

The mixture of different phages obtained from the 3 different waste water samples were further evaluated for their characteristic growth pattern using *E. coli* as the host bacteria. From the result was elucidated in the figure 3. it was evident that among the waste water samples phage present in the sample 2 had the smallest eclipse period of 10 min among all other the samples. Sample 2 had the exponential phase ranging from 10-70 min and then it gets declined, whereas sample sample 1 had its eclipse period upto 20 min and its exponential phase ranging from 20-70 min. Among the samples, phages present in the sample 3 takes a bit longer time of eclipse up to 25 min and its titre value were also low as compared to the other two samples. Similar finding was also reported by Xu *et al.*, 2018 where 18 min of eclipse period was recorded for the phage vB-EcoS-B2 infecting *E. coli* as the host organism.

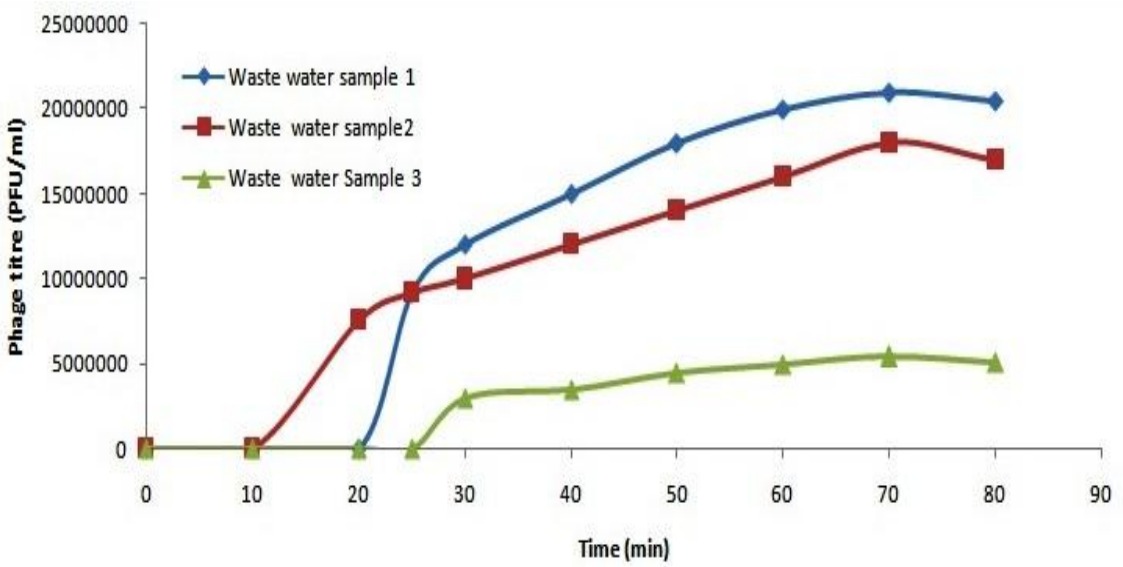

FIGURE 3. One step growth curve of the phages isolated from waste water samples using *E. coli* as host organism

CONCLUSION

In this study, isolation and characterization of coliphages from house hold and river waste water using *E. coli* as the host system was successfully conducted. The isolated phages have different degree of eclipse period and more importantly these phages can be used as an alternative approach to regulate the growth of multidrug resistant bacteria.

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