Isolation and Characterization of Bacteriophages Against *Escherichia coli* And *Shigella dysenteriae* and Its Applications

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Abstract: ***Abstract:*** Environment populated by bacterial hosts such as soil, sewage and animal secretions are unique sources of all types of bacteriophages, offering the possibility to isolate them for purposes like therapy, typing, indicators etc. Lytic phages are the possible replacement for antibiotics to treat bacterial infections not responding to conventional antibiotic therapy. This study reports isolation and characterization of bacteriophages isolated from sewage water that effectively control growth of *Escherichia coli* And *Shigella dysenteriae*, Isolated phages demonstrated thermal stabilities and were able to survive in neutral and alkaline pH levels. The phage titre value obtained was 11 pfu/ml and 0.1 pfu/ml for 10^{-1} and 10^{-2} Dilutions respectively. Isolated phages were found to be effective in biocontrol of various food samples also the isolated phages were partially efficient in preventing biofilm formation. These findings indicate application of phages in medical and food industry.

Keywords: *Bacteriophage, Isolation, Therapeutic, Antibiotic.*

1. INTRODUCTION

Viruses are obligate intracellular parasites which require a specific host cell for its replication. Bacteriophage are viral parasites of bacteria and are some of the simplest self-replicating systems that contain an outer shell composed of proteins and genetic material in the form of DNA or RNA. They were discovered independently by Frederick Twort in 1915 and Félix d'Hérelle in 1917. The characteristics that make phage a good antimicrobial agent are:-1)bactericidal agents, 2)auto "dosing", 3)low inherent toxicity 4) minimal disruption of normal flora, 5)narrower potential for inducing resistance 6) lack of cross-resistance with antibiotics, 7) biofilm clearance (Catherine Loc-Carrillo 2011).
Viruses known as bacteriophages or phages selectively target and destroy specific bacteria without harming the host organism or other beneficial bacteria, such as normal gut flora, thereby minimizing the possibility of complications (Lara Marks 2019). The host specificity of bacteriophages offers an appealing technology for curing infections caused by bacteria or for biocontrol of contaminating pathogenic bacteria without disturbing the community of beneficial bacteria in our body. Also, unlike chemical therapeutics, phages are not susceptible to emergence of bacterial resistance as they evolve along with their hosts.
Due to the onset of antimicrobial resistance in recent years, phage associated treatment is now considered an important means to treat various bacterial infections resistant to antibiotics. Multi drug resistant bacteria are on the way to kill more people than cancer by the year 2050 (Aldo Tagliabue 2018). Thus, it's important that we develop ways to combat them.
Pathogenic enteric bacteria known as fecal coliforms.eg. *Escherichia coli* and *Shigella dysenteriae*, may produce severe disease symptoms when ingested by susceptible individuals (Rene N. Beaudoin 2007). Such bacteria are normal inhabitants of sewage sludge and thus act as the breeding site for bacteriophages. Other sources for isolating bacteriophages include sea water, soil, fresh water and sewage ecosystems (Mahadevan M. Sundar 2009).
The emergence of pathogens, such as *E. coli*, remains a threat for public health due to its low infective dose of <10 cells (Paul Schmid-Hempel 2007). Human infections occur through consumption of contaminated food products, drinking contaminated water and due to poor hygienic conditions (Abigail Clements 2012). *Shigella* can be isolated from numerous animal species and is the major cause of food poisoning. Severe infection may result in inflammatory bacillary dysentery or shigellosis. The infective dose is on the order of 10^3 organisms. *Shigella* is one of the leading cause of diarrheal morbidity accounting for about 13.2% of all diarrheal deaths (Ibrahim A Khalil 2018)
Currently much research is directed towards utilization of bacteriophages in phage based vaccines as an alternative to antibiotics, together with food biopreservation, biofilm prevention and surface disinfection. Thus, this study focuses on Isolating bacteriophages from sewage samples and characterizing its physical as well as biological parameters.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteriophages

Phage isolation was carried out from Sewage water samples collected from 23 different locations around Mumbai, Maharashtra. A slight modification in previously described phage enrichment method was used to enrich and isolate phages (Rohan Van Twest 2009). Sample was filtered through coarse filter paper to remove any particulate matter. 10% chloroform was added to the filtrate and vigorously shaken for 30 mins. It was the transferred to sterile centrifuge tube and centrifuged at 8000 rpm for 20 mins at 4°C. After centrifugation supernatant was removed and stored as lysate at 4°C.

2.2 Spot Test

200µl of target bacteria suspension incubated overnight was spread on LA plates and incubated for 40 minutes at 30°C. Phages were spotted onto the surface of the plates by pipette holding 10µl of individual phage lysates. The plates were left to dry and were inspected for lysis zones after an overnight incubation at 30°C. The spot assay was used to confirm the bactericide ability of all of the isolated virulent phages to form clear zones on the bacterial strains

2.3 Characterization

2.3.1 Determination of Phage Host Range
A total of 18 different bacterial strains used in this study were obtained from Department of Biotechnology, Chikitsak Samuha's S.S & L.S. Patkar College of Arts & Science (Mumbai), India. Molten soft LB (0.5%) agar containing 0.2ml lysate and 0.4ml of bacterial suspension (OD 0.4 at 530nm) were spotted on sterile plate containing thin layer of LB agar. Plates were incubated at 37°C for 24 hrs Formation of clear zone on the bacterial lawn was recoded as a positive result (Ong Guang-Han 2016).

2.3.2 Determination of Phage Titer
Enriched lysate was subjected to 6-fold dilution. 0.3ml of culture suspension of *E.coli and S.dysenteriae* (OD 0.4 at 530nm) along with respective diluted lysate was added in molten soft LB agar tube. The following mixture was then poured to sterile plate containing a thin layer of LB agar (Soft agar overlay technique). Plate were incubated at 37°C for 24 hrs and checked for number of plaques in each dilution.
Phage Titre= Number of plaques/ ml of lysate

2.3.3 Effect of different incubation temperature on phage stability
Phage lysates were incubated at temperatures 4°C, 16°C, Room temperature, 37°C and 60°C. After 1 hr. incubation, 0.2ml of the following lysate and 0.4ml of culture suspension of *E.coli* and *S. dysenteriae* (OD 0.4 at 530nm) was added in molten soft LB agar and soft agar overlay technique was performed. The plates were incubated at 37°C for 24 hrs and after 24 hrs number of plaques were counted for each treatment (Collins Njie Ateba 2019).

2.3.4. Effect of different pH on phage stability
pH of LB broth containing phages was adjusted at following pH: 2, 4, 7, 9 and 10.5. After 18 hr incubation at room temperature, 0.2ml of the following lysate and 0.4ml of culture suspension of *E.coli* and *S. dysenteriae* (OD 0.4 at 530nm) was added in molten soft LB agar and soft agar overlay technique was performed. The plates were incubated at 37°C for 24 hrs and after 24 hrs number of plaques were counted (Collins Njie Ateba 2019).

2.3.4 Antimicrobial Susceptibility Test of Hosts
The selected hosts *E.coli* and *S.dysenteriae* were checked for its antibiotic sensitivity by disc diffusion method. Antibiotics chosen include Vancomycin, Kanamycin, Amoxicillin, Chloramphenicol, Streptomycin, Ampicillin, Bacitracin and Gentamycin. The plates were incubated at 37°C for 24 hrs. After incubation the diameter of zone of inhibition (mm) was measured and compared with Kirby Bauer chart.

2.3.5 Growth curve analysis of Hosts
Growth curve analysis of *E.coli* and *S.dysenteriae* was done in presence of lysate and antimicrobial drug respectively and the growth pattern was compared and growth rate & generation time was calculated. Control sample containing neither drug nor lysate was maintained.

2.4. Applications:

2.4.1 Bio preservation of Matki (*Vigna aconitifolia*) and Green peas (*Pisum sativum*)
Seeds of matki and peas were surface sterilized using tween 80 and washed with tap water, followed by sterile distilled water. Seeds were rinsed with 70% ethanol. Then further washed with distilled water. Then seeds were washed with Mercuric chloride and transferred to sterile petri plate and were maintained as Control(D/W), Standard (culture suspension of *E.coli* or *S.dysenteriae)* and Test (culture suspension and lysate) respectively.

2.4.2 Bio preservation of orange and tomato juice
Juice samples of tomato and orange were maintained as Control (juice sample), Standard (culture suspension of *E.coli* & *S. dysenteriae)* and Test (culture suspension and lysate). 1ml of sample was added in molten LB agar butts and poured onto respective plates. Plates were incubated at 37°C for 24 hrs.

2.4.3 Prevention of biofilm formation
A loopful of culture suspension of *E.coli* and *S.dysenteriae* was smeared on a clean sterile slide. Slide was immersed in sterile LB broth and in Lb containing phage lysate and incubated at room temperature for 1 week. After incubation the slide was stained with 15% crystal violet for 1 min and then rinsed with water (O'Toole 2011).

3. RESULTS AND DISCUSSION

3.1 Isolation of phage.
Spot test was successful for 1 out of 23 different sewage water samples Clear zones or plaques were observed on the bacterial lawn *E.coli* and *S.dysenteriae* after addition of isolated lysates sample.

Figure 1: Isolated plaques : a) *Escherichia coli* b) *Shigella dysenteriae*

3.2 Host Range Determination.

The isolated phage was able to form clear zones on 4 different test organisms out the 18 tested

Figure 2: Host that were affected are a) *E.coli* ampicillin sensitive b) *E.coli* ampicillin resistant c) *E.coli* 616 d) *Shigella dysenteriae*

3.3 Phage Titre.

The phage titre value obtained for 10^{-1} dilution is 11 pfu/ml and the phage titre value for 10^{-2} dilution is 0.1 pfu/ml for both organisms

3.4 Effect of different incubation temperature on phage stability.

The phages were thermally stable when incubated at all tested temperatures as proper plaques were observed on bacterial lawn of *E.coli* & *S.dysenteriae*

Figure 4: Visualization of plaques when incubated at different temperatures against *Escherichia coli*
a) 4°C b) 16°C c) Room Temperature d) 37°C e) 60°C

3.5 Effect of different pH levels on phage stability.

The phages were able to survive in neutral and alkaline pH conditions, but not able to survive in acidic pH. The phage stability was confirmed by visualization of plaques on bacterial lawn of *E.coli* and *S.dysenteriae*

Figure 6: Visualization of plaques when incubated at pH conditions against *Escherichia coli*
c) pH 7 d) pH 9 e) pH 10.5

Figure 7: Visualization of plaques when incubated at pH conditions against *Shigella dysenteriae*
c) pH 7 d) pH 9 e) pH 10.5

3.6 Antimicrobial Susceptibility Test of Hosts.

Zone of inhibition was observed around antibiotics disc like Kanamycin, Amoxicillin, Chloramphenicol, Streptomycin and Gentamycin against host *Escherichia coli* and Kanamycin, Amoxicillin, Chloramphenicol, Ampicillin and Gentamycin against *Shigella dysenteriae*

No zone of inhibition indicates bacterial resistance of host against antibiotics like Vancomycin, Ampicillin and bacitracin for *E.coli* and Vancomycin, Streptomycin and bacitracin for *S. dysenteriae*

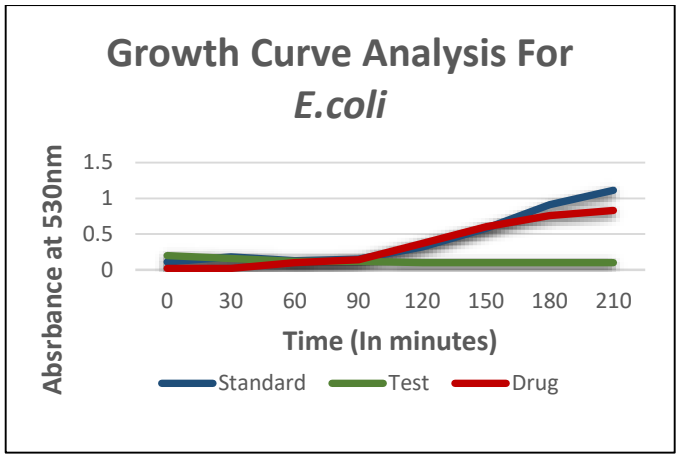
Figure 8: Zone of inhibition for a) *Escherichia coli* b) *Shigella dysenteriae*

Antibiotics	Zone of inhibition(mm)	Interpretation
Vancomycin	-	Resistant
Kanamycin	18.5	Sensitive
Amoxicillin	18	Sensitive
Chloramphenicol	32	Sensitive
Streptomycin	17	Sensitive
Ampicillin	-	Resistant
Bacitracin	-	Resistant
Gentamycin	22	Sensitive

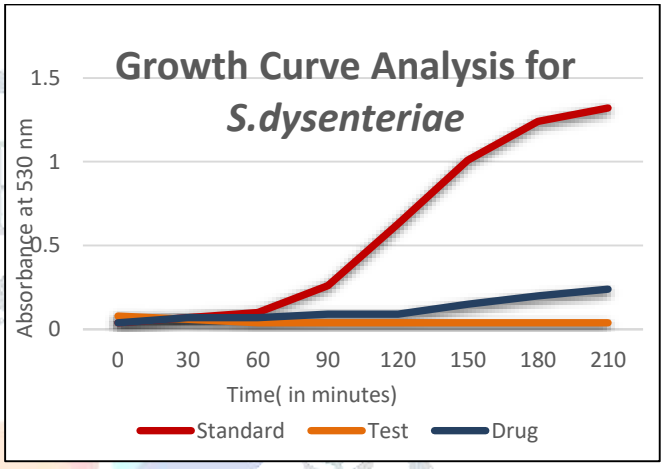
Antibiotics	Zone of Inhibition(mm)	Interpretation
Vancomycin	-	Resistant
Kanamycin	20	Sensitive
Amoxicillin	25.5	Sensitive
chloramphenicol	37	Sensitive
Streptomycin	-	Resistant
Ampicillin	30	Sensitive
Bacitracin	-	Resistant
Gentamycin	28.5	Sensitive

Table 1: Diameter of zone of inhibition for a) *Escherichia coli* b) *Shigella dysenteriae*

3.7 Growth curve Analysis.



Key: Std- E.coli
Test- *E.coli*+ Phage
Drug- *E.coli*+ Ampicillin (60mcg)



Key: Std- *S.dysenteriae*
Test – *S. dysenteriae* + Phage
Drug- *S.dysenteriae*+ Streptomycin (60mcg)

Growth curve analysis confirmed reduction in number of host cells in presence of bacteriophages, while in presence of standard conditions and drug conditions host cells showed normal growth pattern.

3.8 Bio preservation of Matki (*Vigna aconitifolia*) and Green peas (*Pisum sativum*)
Matki (*Vigna aconitifolia*) and Green peas (*Pisum sativum*) treated with lysate was found to be fresh as compared to the other controls.

3.9 Bio preservation orange and tomato juices.
The presence of bacterial load was checked with the help of different controls. The test control showed no growth, while standard showed growth of the respective organisms used.

Figure 13 Bio preservation of Orange juice against *E.coli*
a) Standard b) Test

c) Common control

Figure 14: Bio preservation of Orange juice *against S.dysenteriae*
a) Standard b) Test

Figure 15 Bio preservation of Tomato juice against *E. coli*
a) Standard b) Test

c) Common control

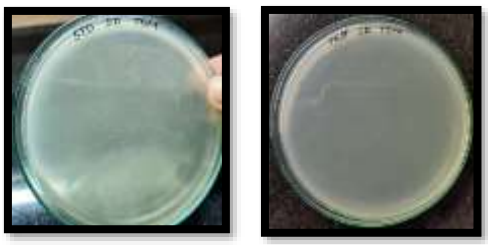


Figure 16: Bio preservation of Tomato juice against *S.dysenteriae*
a) Standard b) Test

3.10 Prevention of biofilm formation.

Crystal violet assay confirmed much lower biofilm mass in test as compared to standard this is because the host organisms has taken up the Crystal violet while in test as less amount of cells were present so the intensity of Crystal violet is less.

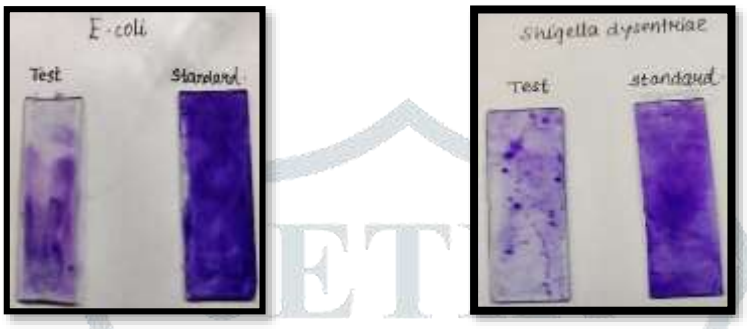


Figure 17: Prevention of biofilm formation against a) *Escherichia coli* b) *Shigella dysenteriae.*

4. CONCLUSION

In conclusion, stable and virulent lytic phages against *E.coli* and *S.dysenteriae* were isolated and characterized in this study. Isolated phages were found to be active against few bacteria belonging to *Enterobacteriaceae family* .They demonstrated thermal stabilities and were able to survive in neutral and alkaline pH levels. The phage titre value obtained was 11 pfu/ml and 0.1 pfu/ml for 10^{-1} and 10^{-2} Dilutions respectively. Growth curve analysis confirmed reduction in growth pattern of host in presence of phage.

Isolated phages were found to be effective in biocontrol of various food samples also the isolated phages were partially efficient in preventing biofilm formation. However, future research should be directed on detailed characterization of the phage its identification and sequencing the whole genome so as to ascertain their safety in biocontrol, biofilm inhibition and to optimize appropriate phage titre to ensure complete inhibition of *E.coli* and *S.dysenteriae* in both artificial media and real food systems.

5. REFERENCES

1. Abigail Clements, Joanna C. Young, Nicholas Constantinou, Gad Frankel. 2012. “Infection strategies of enteric pathogenic Escherichia coli.” *Gut microbes* 3: 71-87.

2. Aldo Tagliabue, Rino Rappuoli. 2018. “Changing Priorities in Vaccinology: Antibiotic Resistance Moving to the Top.” *Frontiers in Immunology* 9: 1068.

3. Catherine Loc-Carrillo, Stephen T Abedon. 2011. “Pros and cons of phage therapy.” *Bacteriophage* 111-114.

4. Collins Njie Ateba, Muyiwa Ajoke Akindolire. 2019. “Isolation and Characterisation of Bacteriophages with Lytic Activity Against Virulent Escherichia coli O157:H7: Potential Bio-Control Agents.” *Preprints.*

5. Ibrahim A Khalil, Christopher Troeger, Brigette F Blacker, Puja C Rao, Alexandria Brown, Deborah E Atherly, Thomas G Brewer, Cyril M Engmann,. 2018. “Morbidity and mortality due to shigella and enterotoxigenic Escherichia coli diarrhoea: the Global Burden of Disease Study 1990–2016.” *The Lancet Infectious Diseases* 1229-1240.

6. Lara Marks, Jakrin Bamrungthai. 2019. “Phage therapy.”

7. Mahadevan M. Sundar, Nagananda G.S., Arijit Das, Sourav Bhattacharya, Sandeep Suryan. 2009. “Isolation of Host-Specific Bacteriophages from Sewage Against Human Pathogens.” *Asian Journal Of Biotechnology* 1: 163-170.

8. Ong Guang-Han, Choh Leang-Chung,Kumutha Malar Vellasamy,Vanitha Mariappan,Chang Li-Yen,Jamuna Vadivelu. 2016. “Experimental Phage Therapy for Burkholderia pseudomallei Infection.” *PloS one.*

9. O'Toole, George A. 2011. "Microtiter Dish Biofilm Formation Assay." *Journal of visualized experiments.*

10. Paul Schmid-Hempel, Steven A Frank. 2007. "Pathogenesis, Virulence, and Infective Dose." *PLoS pathogens* 3: 1372-3.

11. Rene N. Beaudoin, Danielle R. DeCesaro, Debrah L. Durkee, Susan E. Barbaro. 2007. "ISOLATION OF A BACTERIOPHAGE FROM SEWAGE SLUDGE AND CHARACTERIZATION OF ITS BACTERIAL HOST CELL." *RIVIER ACADEMIC JOURNAL* 3.

12. Rohan Van Twest, Andrew M. Kropinski. 2009. "Bacteriophage Enrichment from Water and Soil." In *Bacteriophages. Methods in Molecular Biology*, by Andrew M. Kropinski Martha R.J. Clokie. Humana Press.