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Isolation and Characterisation of a Lytic Bacteriophage from Wastewater and its Application in Pathogen Reduction

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ABSTRACT

Present work was performed to isolate, enrich and characterise bacteriophage using basic laboratory set-up, and to demonstrate their lytic potential in pathogen reduction. Following standard microbiological and molecular biological procedures, we examined cultivable bacteria and bacteriophages in wastewater sample. Subsequently, from cultures of wastewater sample, we isolated and characterised bacterial isolate, which was then used to enrich lytic *bacteriophages,* using a combination of double layer plaque-assay, isolation and genetic techniques to isolate bacteriophage against *Pseudomonas aeruginosa*. Subsequently decontamination capability of this phage was assessed in form of spray and matrix adsorbed formulations. Storage stability of phage preparations at different temperatures was also studied by calculating reduction in phage titer. Present studies demonstrate lytic potential of phage as an eco-friendly alternative to conventional control approaches against bacterial contamination.

Keywords: Bacteriophage; Biocontrol; Water; Wastewater

1. INTRODUCTION

Bacteriophages (or phages) are ubiquitous in every ecosystem including human gut[1-3]. Bacteriophages against human pathogenic bacteria-*Salmonella*, *Escherichia coli*, and *Bacteroides fragilis* are abundant in feces, due to which wastewater receiving fecal matters harbor diverse bacteriophages[4]. Therefore, sewage is one of the rich sources of bacteriophages against pathogenic bacteria prevalent in a population, which could be harnessed for applications in a wide variety of settings, including clinics, industries and in treatment of wastes[3,5]. In particular, bacteriophages infecting various members of the bacterial family *Enterobacteriaceae* are predominantly present in wastewater[6]. Phages being natural and self-replicating enemies to bacteria, are increasingly being considered for bacterial control in a wide variety of settings ranging from wastewater treatment to therapeutic applications[3].

Pseudomonads, similar to other human pathogenic bacteria, are common in places like hospitals, animal farms, slaughterhouses, soil, aquatic environments and is associated with a number of serious health issues, including cystic fibrosis, urinary tract infections, dermatitis, soft tissue infections, complications in patients with severe burns and open wounds[7,8]. Recently, WHO has listed carbapenem-resistant *P. aeruginosa*, under priority 1 critical category of pathogens, against which new therapeutics are urgently required (WHO). *P. aeruginosa* has high adaptation rate, rapidly acquire resistance against potent antibiotics, and can survive in diverse adverse conditions, posing serious challenges in treatment[9,10]. It has recently been demonstrated that it can resist antibiotic therapy through synthesising resistive biofilms, causing failure of therapy[11]. *P. aeruginosa* contains flagella and type IV pili, which enable their motility on solid or semi-solid surfaces, a serious concern for contamination of surfaces, and tools, especially in clinical settings[12].

Therefore, as alternative to antibiotics and chemical based decontamination methods, biocontrol approaches are seriously being considered, of which phage therapy is the most potent. One of the biggest advantages of bacteriophages are their abundant presence in environment along with pathogenic bacteria, especially in sewage or wastewater sources that receive inputs from different contaminated settings[13,14]. Our present work was aimed at isolation, enrichment and characterisation of potentially lytic bacteriophages against *P. aeruginosa* from wastewater. Our results demonstrate that using simple microbiological and molecular biological approaches, bacteriophages with high lytic potentials could be easily isolated that can be used for pathogen reduction in different settings.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria and Biochemical Tests

Wastewater samples were collected from a human waste treatment facility in Tezpur, Assam (26° 39′ 4.3848″ N and 92° 47' 1.7268" E). An aliquot of sample was inoculated in tryptone soya broth (TSB) and incubated overnight for growth of bacteria along with multiplication of phages. Subsequently,

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total DNA was extracted (Qiagen GmbH, Hilden, Germany) to examine and confirm the presence of cultivable bacteria and bacteriophages. Extracted DNA was sent to a commercial facility for metagenomic analysis on Illumina Next generation sequencing (NGS) platform (Genotypic Technology Pvt. Ltd., Bengaluru, India). Post assembly (*de novo* assembly using MetaPlatanus), identification of contigs for genes and proteins was performed through alignment and homology search in different databases including viral RefSeq (BLASTx), NCBI-nr (BLASTn), MetaGeneMark, PHASTER (for prophage sequences).

Following metagenomic analyses, total culture was used to isolate different bacterial pathogens. Briefly, 100 µl of sample was serially diluted up to 10^{-12} folds in sterile normal saline, poured on Nutrient agar (NA) media and incubated overnight at 37 °C. Colonies were then visually inspected and based on morphological features, pure cultures were obtained using standard microbiological methods. Subsequently, isolates were tested on different selective media (HiMedia, India), such as MacConkey media (*Enterobacteriaceae*), EMB media (Fecal coliforms); King's A media & King's B media (*Pseudomonas*), Malachite green broth (*P. aeruginosa*). Additional biochemical tests were performed for identification of *P. aeruginosa*. All the microbiological manipulations were performed inside Class II biosafety cabinet (Esco, Singapore), strictly following good laboratory practices.

2.2 Bacterial Identification by 16S rRNA Gene Sequencing

Total bacterial genomic DNA was purified from overnight cultures of pure isolates (Sigma-Aldrich, St Louis, USA). 16S rRNA genetic region was amplified using universal PCR primers - 16S1 (gagtttgatcctggctca) and 16S2 (cggctaccttgttacgactt)[15,] and sent to a commercial facility for sequencing (Chromus Biotech, India). Strict precautions were followed to rule out cross contamination during amplification and appropriate controls were included during each step[16]. Sequences were examined, trimmed and edited using BioEdit program[17.] Homologous nucleotide sequences were searched in NCBI non-redundant database (nr database, excluding uncultured sequences) using blastn algorithm[18.]

2.3 Bacteriophage Enrichment, Purification and Protein Profiling

For isolation of bacteriophages, an aliquot of the collected wastewater was passed through sterile cotton bed filter, followed by centrifugation at 5,000 g for 20 mins for removal of bacterial cells and debris. Supernatant was then serially passed through 0.45-µm and 0.22-µm pore-size membranes (Whatman). Final filtrate was used as starting material for enrichment of bacteriophages. Briefly, filtrate was incubated with different bacterial isolates in their early log phase (approximately 10^6 CFU/ml). Phage particles were allowed to infect host cells overnight at 37 °C with shaking (180 rpm). Afterwards, enriched cultures were centrifuged at 5,000 g for 10 mins and supernatant was again serially passed through membranes of pore-sizes 0.45-µm and 0.22-µm. Lytic phages were identified through modified double layer agar method for plaque assays[19,20]. For plaque assay, soybean casein digest medium was used, to which divalent ions were added at final concentration of 10 mM $MgCl_2$ and 5 mM $CaCl_2$. After incubation at 37 °C for 16 h -18 h, plaques were visualised. Further bacteriophage purification was achieved by picking single plaques, following methods described elsewhere[21,22].

For phage protein profiling, purified phage preparation was concentrated by methanol precipitation method, reconstituted and quantified (Picodrop, Cambridge, UK). Subsequently, 10µg of total proteins were resolved in 12 % SDS-PAGE[23], and protein bands were visualised by silver staining (Fermentas, Villinius, Lithuania).

2.4 Decontamination Assay through Bacteriophage Spray

Decontamination assay through bacteriophage was performed using bacteriophage stock (10^9 PFU/ml) as described earlier[24]. Phage stock (10^9 PFU/ml), 10^3 fold dilution (10^6 PFU/ml) and 10^5 fold dilution (10^4 PFU/ml) of phage stock were used for assay. Sterile spray bottles were filled with phage dilutions. *P. aeruginosa* culture in log phage was poured on TSA agar and after 5 min, single spray (approximately 45 µl) of each dilution was applied on plates. Plates were incubated overnight at 37 °C. Results were interpreted according to visual clearance of bacterial lawn on the plates.

2.5 Bacteriophage Attachment over Sponge Matrix

Sponge matrix of 1.5 cm × 1 cm was dipped in phage stock (10^9 PFU/ml) for 10 min. A control sponge was dipped in sterile Tris magnesium buffer (TM). After 10 mins, soaked sponges were placed over lawn of *P. aeruginosa* culture and incubated as previously mentioned. The zone of clearance was observed and documented.

2.6 Storage Conditions for Bacteriophages

Aliquots of phage stocks were stored at three different temperatures, 4 °C, room temperature (25 °C - 30 °C and 37 °C for 3 months to determine stability of phage preparations. Post storage, PFU/ml of each of the test preparations was determined by plaque assay[25].

3. RESULTS

3.1 Diversity of Cultivable Bacteria and Bacteriophages in Wastewater

Diversity and abundance of cultivable pathogenic bacteria along with their respective phages in wastewater was analysed by NGS. Gram-negative bacteria *P. aeruginosa* (37 %) was most abundant in the sample. *Enterobacteriaceae* family was primarily represented by *Citrobacter* species (28 %), *Proteus* species (24 %), *Klebsiella* species (9 %) and other in very less abundance (data not shown). On the other hand, bacteriophages present in the sample have been shown in Fig. 1. Most abundant phage present in sample was Pseudomonas phage (36 %) followed by Enterobacteria phage (23 %), Salmonella phage (11 %), Shigella phage (5 %) and Erwinia phage (4 %). Other phages were also identified in sample, but in very less amount. Metagenomic data has been submitted to the Sequence Reads Archive (NCBI-SRA) and is available under the Project

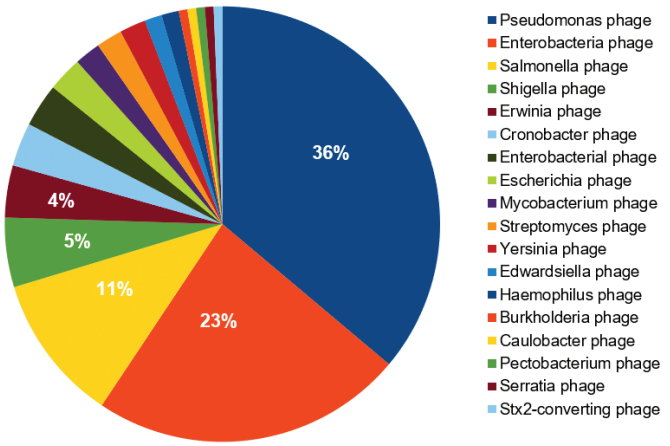

Figure 1. Pie chart showing abundance and diversity of bacteriophages (obtained from NGS data).

PRJNA369028. Detailed analyses of the NGS data have been submitted elsewhere for publication.

3.2 Morphological and Biochemical Analysis of Bacterial Isolates

For biochemical analyses, colonies were grown on appropriate media. Colonies showing both lactose fermenting (pink mucoid) and non-lactose fermenting (colourless, white in appearance) reaction on MacConkey agar were enumerated under *Enterobacteriaceae* family. Fluorescence and pyocyanin production were observed in *P. aeruginosa* colonies on Kings'B Media as shown in Fig 2. *P. aeruginosa* showed positive reaction for Oxidase and Catalase, while negative for Urease. Different pure isolates were subsequently identified from partial 16S rRNA gene sequences, which corroborated the results of metagenomic sequencing. 16S rRNA sequences are available in GenBank under accession numbers KX759177 through KX759188.

3.3 Bacteriophage Enrichment, Purification and Protein Profiling

Bacteriophage enriched cultures were purified as described in previous section. Viability of purified product was tested by spot tests and/or plaque-assays (Fig. 2(d)). In addition, phage protein profiling showed multiple visible bands corresponding to the phage structural proteins - internal core protein; head-tail connector protein; capsid protein; and tail fibre protein as shown in Fig. 3.

3.4 Decontamination Assay through Bacteriophage Spray

In this experiment, bacterial plates sprayed with phage stock (10^9 PFU/ml) have inhibited the growth of lawn, whereas the plates sprayed with 10^4 PFU/ml formulation did not show any clearance of the bacterial lawn and the plate that received 10^6 PFU/ml dilution showed intermediate clearance of lawns as shown in Fig. 4.

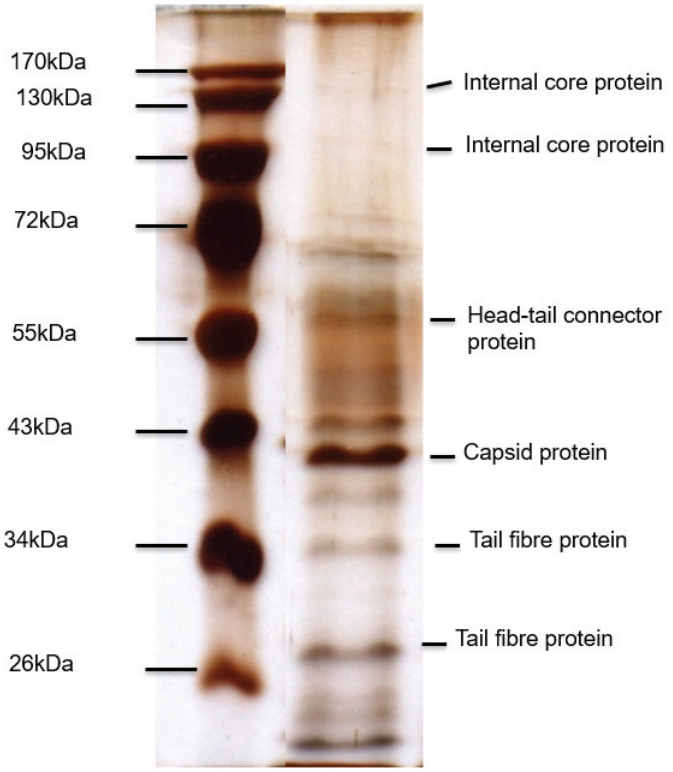

Figure 3. Protein profile of purified phages separated through 12 % SDS-PAGE. Bands were marked based on comparison with protein profile reported in Lavigne[37], *et al.*

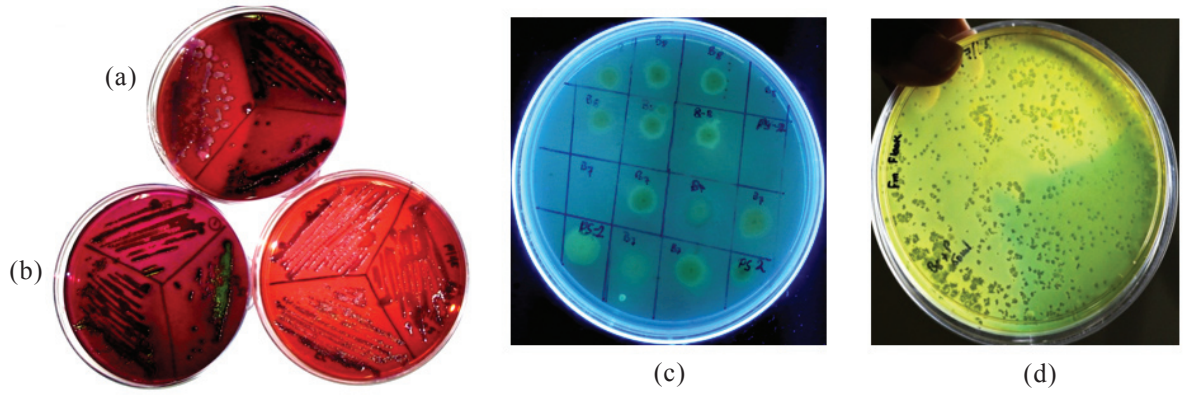

Figure 2. Growth of bacterial isolates on different media (a) MacConkey Agar, (b) EMB Agar, (c) Kings'B Media with florescent *P. aeruginosa* colonies under UV, and (d) Plaques on *P. aeruginosa* growing on agar plate.

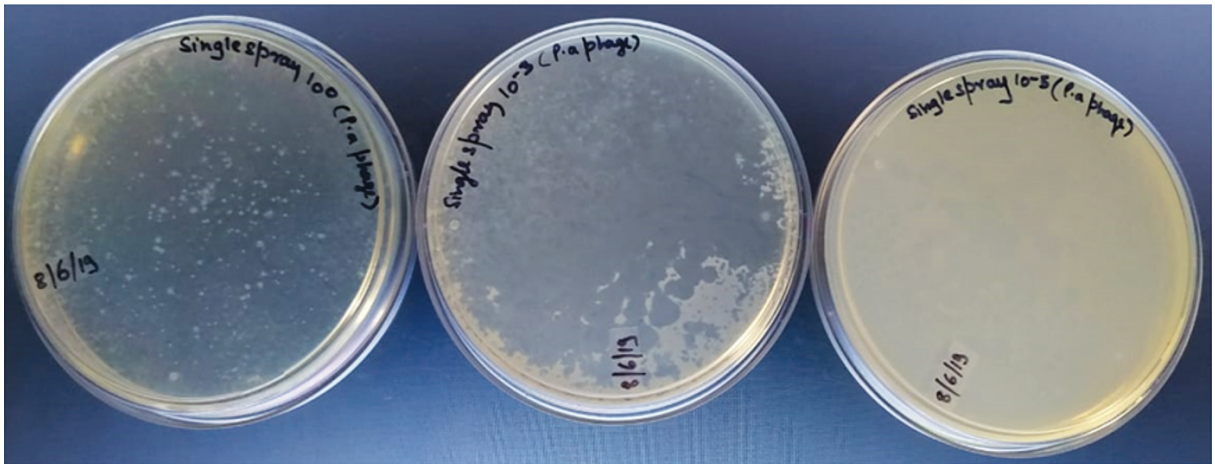

Figure 4. Decontamination Assay through bacteriophage spray (10^9 PFU/ml).

3.5 Bacteriophage Attachment over Sponge Matrix

A prominent zone of clearance was observed in plate with sponge matrix dipped in phage stock (10^9 PFU/ml) whereas, control plate receiving sponge dipped in sterile TM buffer did not showed any zone of clearance as shown in Fig. 5.

3.6 Storage Conditions for Bacteriophages

Phage preparations stored at 4 °C were stable for more than 12 months. No substantial changes in PFU/ml, phage viability and lytic efficiency was documented. However, in phage preparations stored at room temperature and at 37 °C, one log reduction in phage titer was noted after 3 months of storage.

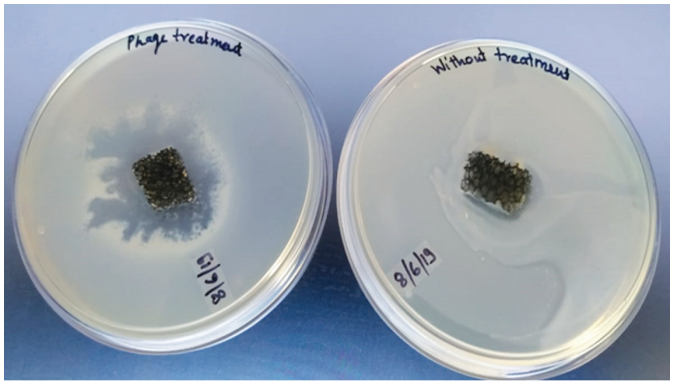

Figure 5. Activity of bacteriophage (stock 10^9 PFU/ml) adsorbed over sponge.

4. DISCUSSION

It is well established that sewage is a potent source of pathogens, which also reflects the pathogens ubiquitous in a community[2,13,14]. Sewage also provides habitat to a number of human pathogens including human gut microbes, such as *Escherichia*, *Citrobacter*, *Klebsiella* and *Enterobacter* and phages against them[26]. Therefore, these sources are good starting materials for isolating bacteriophages against epidemiologically important bacterial pathogens and previous studies have isolated phages from these sources[27,28]. In this work, through a systematic approach, we examined the diversity of cultivable microbial populations and isolated phages from wastewater. Previous studies based on metagenomics have demonstrated abundance and diversity of bacteria and viruses (phages in particularly) in diverse sources including feces and sewage treatment facilities[29,31-33]. Despite abundance of phages in these sources, their enrichment and isolation remain a challenge, since a large fraction of bacteria present in these environments are uncultivable *in vitro*. On the contrary, some of the potent pathogenic bacteria are cultivable and therefore, isolation of bacteriophages against them is possible.

In this study, among the cultivable bacterial species present in the wastewater, we aimed at isolating lytic phages, particularly against *P. aeruginosa*. Previously, it has been reported that *P. aeruginosa* can acquire resistance to multiple drugs through horizontal transfer of resistance genes or modified enzymes or by drug induced mutations and is also known to form drug resistant biofilms[34.] Moreover, *P. aeruginosa* has been shown to form biofilm in human infections, leading to antibiotics resistance and failure of therapeutic strategies[11,35,36].

We isolated phages against *P. aeruginosa,* purified, characterised and assessed their lytic potential. Our results of protein profile of purified *Pseudomonas* phage (as evident by the relative abundance of the capsid protein, gp32 and the tail fiber protein, gp 40) corroborated with protein profile reported previously for other *P. aeruginosa* phages[37,38]. Similarly, protein profiling through SDS PAGE has been widely utilised in characterisation of variety of phages against diverse bacterial hosts[39,40]. We also visualised purified particles under TEM, which showed phage belonging to *Caudovirales* order with a non-enveloped, icosahedral head-tail geometry and tail (manuscript submitted elsewhere).

Phage application for biocontrol of *P. aeruginosa* has been demonstrated *in vitro* by Hall[41], *et al.* and *in vivo* by Debarbieux[42], where efficiency of phage application was demonstrated in mice acute lung infection model. Interestingly, phage treatment was found to be as effective as therapeutic and prophylactic approaches in saving animals from lethal infection and also in preventing lung infection[43]. In contrast to other countries, bacteriophage mediated pathogen reduction or other applications have recently gained attention among Indian researchers. In a recent study, Periasamy and Sundaram[26], isolated phages from hospital wastewater along with host and

demonstrated significant *E. coli* controlling efficiency of these phages. Similarly, Nocardiophages were recently isolated from wastewater for control of *Nocardia* species that pose a foaming problem in activated sludge process (ASP), worldwide[28]. Likewise, other reports have also shown phage application in pathogen reduction[17,39,44-46]. Phage application as therapeutic agent to combat bacterial infection needs to be further explored along with *in vivo* models and clinical trials.

5. CONCLUSION

The results of this study suggest that phages against important bacterial pathogens prevalent in a given population can be easily isolated and used as a potential biocontrol agent in the form of spray formulation or matrix adsorbed formulation for pathogen reduction in different settings. In addition, these phages may further be considered as candidate for phage therapy against multidrug resistant *P. aeruginosa* in clinical settings. However, further studies are required to better understand the phage-host interaction for developing phage-based products and their trials.

Conflicts of Interest

The authors declare no conflict of interest.

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