Novel application of bacteriophage for controlling foaming in wastewater treatment plant- an eco-friendly approach

Krishna Khairnar, Rajshree Chandekar, Aparna Nair, Preeti Pal, and Waman N. Paunikar

Environmental Virology Cell, Council for Scientific and Industrial Research - National Environmental Engineering Research Institute (CSIR-NEERI), Nehru Marg, Nagpur, Maharashtra, India

ABSTRACT
This addendum to “Novel application of bacteriophage for controlling foaming in wastewater treatment plant- an eco-friendly approach” includes characteristics of the phages NOC1, NOC2 and NOC3 not discussed in the previous paper. The phage adsorption and host interaction properties, their sensitivity to pH and temperature are inferred. NOC2 is seen to be more temperature resistant while others are not. All the phages show pH sensitivity. There is a variance observed in the behavior of these phages. Also, applicability of the phage based system to large scale reactors is studied and discussed here.

KEYWORDS
activated sludge; lytic bacteriophage; Nocardioforms

Introduction
We have reported previously, the use of bacteriophages in biocontrol of foaming by Nocardioforms in activated sludge process of wastewater treatment.1,2 In the previous study, we had isolated and tested specific bacteriophages against Nocardioforms – NOC1, NOC2 and NOC3 and showed that they can be used effectively to curb foaming. Their host range was determined and genome sequences studied. Submitted to GenBank under accession numbers – KF879861, KF879862, KF879863 for NOC1, NOC2 and NOC3 respectively. They were demonstrated to be potential inhibitors of nocardioform growth.3 In the present study, we have discussed the nature of these bacteriophages with respect to temperature and pH. Also, the applicability of this process was studied using lab scale reactors as a preliminary experiment.

The main objective of this work was to get a deeper understanding of phage characteristics and test the extendibility of this method from in vitro level to lab scale reactors. Study of specific properties and interaction of phage with bacteria would help fine tune the reaction conditions for maximum effect. Biocontrol of foaming bacteria by use of specific phages3,4,5 would be most ideal if efficiency of this technique can be maximized by detailed study of the system.

Characterization of lytic bacteriophages of nocardioforms

i) Phage adsorption and host interaction - single-step growth curve analysis

The method for this experiment has been followed as described in Petrovski et al, 2011.6,7 Phage stock and host cultures FB4, FB6, FB7 were infected with NOC1, NOC2, and NOC3 at a multiplicity of infection (MOI) of 1. After an adsorption period of 5 min at 30°C, these were used as a sample to calculate the single step growth curve using pour plate method. This culture was diluted 1 in 100 to minimize the possibility of non-adsorbed phages infecting cells. After 24 hours incubation at 30°C a graph was plotted on the basis of number of plaques observed (Fig. 1). The data is shown in Table 1 which is used for plotting the graph of time versus number of phages adsorbed.

ii) pH and thermal sensitivity

The method for this experiment has been followed as described in Yang et al, 2010.8 Phages NOC1, NOC2 and NOC3 were incubated under different pH values...
for one hour before determining the number of infectious phage particles. Results of pH stability tests are summarized in Table 2a and Fig. 2a. Experiments showed that all the 3 phages are stable at pH 6, 8 and only; they are non-tolerant to highly acidic or basic environment. At neutral or nearly neutral pH, phages are able to grow well and many plaques can be seen on the plates. Phage NOC1 is more stable than NOC2 and NOC3, as it can be clearly seen in table that NOC1 is giving more number of plaques at pH 8. A representative plate of plaque assay of pH stability is given in Fig. 2b where plaque count is about 200.

The preliminary experiments showed that phage NOC1, NOC2, NOC3 stock solution retained almost 100% infection activity after incubation at 30°C for

---

**Table 1.** Number of phage adsorbed within 30 minutes of phage and host interaction.

| S.No. | Time (min) | NOC1 | NOC2 | NOC3 |
|-------|------------|------|------|------|
| 1.    | 0          | 50   | 36   | 20   |
| 2.    | 5          | 95   | 50   | 30   |
| 3.    | 10         | 250  | 65   | 60   |
| 4.    | 15         | 275  | 90   | 100  |
| 5.    | 20         | 300  | 200  | 305  |
| 6.    | 25         | 405  | 350  | 300  |
| 7.    | 30         | 750  | 800  | 300  |

---

**Table 2(a).** pH sensitivity of isolated filamentous bacteria bacteriophages.

| Sr. No. | pH maintained | Plaque count (NOC1) | Plaque count (NOC2) | Plaque count (NOC3) |
|---------|---------------|---------------------|---------------------|---------------------|
| 1       | 2             | 0                   | 0                   | 0                   |
| 2       | 4             | 0                   | 0                   | 0                   |
| 3       | 6             | 98                  | 200                 | 35                  |
| 4       | 8             | 300                 | 250                 | 210                 |
| 5       | 10            | 60                  | 99                  | 52                  |
| 6       | 12            | 0                   | 0                   | 0                   |
| 7       | 14            | 0                   | 0                   | 0                   |

---

**Table 2(b).** Heat stability of bacteriophages NOC1, NOC2 and NOC3.

| Temp. | Incubation Time(min) | NOC1 | NOC2 | NOC3 |
|-------|----------------------|------|------|------|
| 20    | 10                   | 140  | 750  | 530  |
| 20    | 20                   | 200  | 800  | 210  |
| 30    | 30                   | 600  | 850  | 600  |
| 40    | 10                   | 580  | 700  | 150  |
| 20    | 20                   | 210  | 730  | 600  |
| 30    | 30                   | 300  | 450  | 350  |
| 60    | 10                   | 500  | 852  | 425  |
| 20    | 20                   | 305  | 650  | 320  |
| 30    | 30                   | 12   | 800  | 800  |
| 60    | 10                   | 65   | 600  | 55   |
| 20    | 20                   | 35   | 550  | 24   |
| 30    | 30                   | 0    | 520  | 9    |

---

**Figure 1.** Single step growth curve.

**Figure 2.** (a) pH sensitivity curve of NOC1, NOC2 and NOC3.

---

**Figure 2.** (b) Representative phage plate of pH stability test (pH = 6).

---

**Table 3.** Bacterial count reduction in the reactor by applying bacteriophages.

| Sample collected | Dilution in 1 ml | Time interval for sample collection (mins.) | Bacterial count after 24 hrs. incubation at 30°C (2 µl) | % reduction |
|------------------|------------------|--------------------------------------------|---------------------------------------------------------|-------------|
| 5 ml             | 10^-6            | 0                                          | 800                                                     | 0           |
| 5                | 750              | 6.25                                       | 500                                                     | 37.50       |
| 10               | 500              | 37.50                                      | 430                                                     | 46.25       |
| 15               | 380              | 52.50                                      | 200                                                     | 75.00       |
| 20               | 200              | 75.00                                      | 100                                                     | 87.50       |

Note: These data represent average values from three independent experiments.
one month (not shown), so temperatures chosen to test thermal stability of phage NOC1, NOC2 and NOC3 were 20°C, 40°C, 60°C, and 80°C, data is given in Table 2b. Phage count taken for incubation was 10³ phages. The results showed phage NOC2 was heat stable, 80% and 50% phages still remained alive after 30 minutes incubation at 60°C and 80°C, respectively; only 3% phages NOC1 were alive after 20 minutes incubation at 80°C; while more than 99% phages lost their infection ability in 30 minutes at 80°C. Similarly NOC3 phages also lost their infectivity after incubation at 80°C for 30 min while all the phages were growing well at incubation temperatures 40°C and 60°C.

**Applicability of phage to lab scale reactor**

The method for this experiment has been followed as described in Petrovski 2011. Bacteriophages were grown in lab and a sufficient amount of bacteriophages were applied to their respective hosts. The data showed CFU/ml decreased markedly in the presence of NOC1, NOC2, and NOC3 when collectively applied to the lab scale reactor. Hence, it is considered that the foaming ability of bacteria is reduced if a specific formulation of bacteriophages is applied to the reactor (Table 3). Thus, the phage mediated cell number reduction eliminates foam under laboratory conditions (Fig. 3) and encourages its application in the control of foaming-activated sludge mixed liquor in full-scale plants.

**Conclusions**

In the present research bacteriophage NOC1, NOC2 and NOC3 were isolated from waste water of a lab scale reactor where foam was observed during aeration process. Significant reduction in the foam was observed when the isolated phages were applied to the reactor at lab scale. Various tests were performed on isolated bacteria and bacteriophages. Experiments showed that phage NOC2 was considerably stable after exposure to high temperature and pH, emphasizing that phage can withstand the seasonal/operational fluctuations. The isolated phage showed no cross infectivity with other bacteria most commonly found in activated sludge systems, thus validating its suitability for biocontrol of filamentous foaming caused by filamentous bacteria. The phage-based biocontrol, therefore, holds a great potentiality for large-scale applications as an economic agent in the mitigation of several water, wastewater and environmental problems.

The isolated and characterized phages in this study were showing similarity to those phages reported earlier as active against the stable foam forming bacteria under laboratory conditions. This observation implies that the isolated bacteriophages viz. NOC1, NOC2, NOC3 may be helpful in reducing the stable foaming problem in wastewater treatment plant. Bacterial count shows significant reduction in less time when exposed to these phages in lab scale reactors. Scale-up experiments and their standardization might prove to be beneficial to establish this technique in practicality in activated sludge process of waste water treatment.

**Disclosure of potential conflicts of interest**

The authors declare that they have no conflict of interest.

**Funding**

We are thankful to Department of Science and Technology, New Delhi, India for providing funding for completion of this study through project (Project No. GAP-1-2128).
References

[1] Khairnar K, Pal P, Chandekar RH, Paunikar WN. Isolation and Characterization of Bacteriophages Infecting Nocardioforms in Wastewater Treatment Plant. Biotechnology Research International. 2014;2014:151952. doi:10.1155/2014/151952.

[2] Petrovski S, Seviour RJ, Tillett D. Prevention of Gordonia and Nocardia stabilized foam formation by using bacteriophage GTE7. Appl Env Microbiol 2011; 77(21): 7864-7; PMID:21926218; http://dx.doi.org/10.1128/AEM.05692-11

[3] Pal P, Khairnar K, Paunikar W.N. Causes and remedies for filamentous foaming in activated sludge treatment plant. Global NEST Journal 2014; 16(4):762-772.

[4] Thomas JA, Soddell JA, Kurtbêoke D_I. Fighting foam with phages? Water Sci Technol 2002; 46(1-2):511-8; PMID:12216679

[5] Withey S, Cartmell E, Avery LM, Stephenson T. Bacteriophages –potential for application in wastewater treatment processes. Science of the Total Environment 2005; 339(1-3):1–18. View at Publisher; PMID:15740754; http://dx.doi.org/10.1016/j.scitotenv.2004.09.021

[6] Petrovski S, Seviour RJ, Tillett D. Genome sequence and characterization of the Tsukamurella bacteriophage TPA2. Appl Env Microbiol 2011; 77(4):1389–98; PMID:21183635; http://dx.doi.org/10.1128/AEM.01938-10

[7] Petrovski S, Seviour RJ, Tillett D. 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. Appl Env Microbiol 2011; 77(12):3923-9; PMID:21498753; http://dx.doi.org/10.1128/AEM.00025-11

[8] Yang, H., Liang, L., Lin, S., & Jia, S. (2010). Isolation and characterization of a virulent bacteriophage AB1 of Acinetobacter baumannii. BMC microbiology; 10(1):1.