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NATURAL WEAPONS AGAINST *PSEUDOMONAS*: APPLICATION OF BACTERIOPHAGES IN NITROGEN-RICH EFFLUENT TREATMENT PLANTS

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ABSTRACT

Bacteriophages are viruses used as biocontrol tools and are a vital alternative to antibiotics, currently being applied in several sectors. One of the practical applications of these organisms is in wastewater treatment plants. Biological processes for removing nitrogen, such as deammonification, stand out for removing high pollutant loads and minimizing operating costs in treatment plants. However, despite the benefits of deammonification, there are still some obstacles to the propagation of technology, one of which is the control of undesirable bacteria in this system through operational processes. Among these bacteria, pathogenic heterotrophs can grow in this environment, causing substrate competition and overgrowing the Anammox, which are the main responsible for the process. This study aimed to isolate a bacteriophage to control *P. aeruginosa,* a species that causes problems in these plants. The bacteriophage showed control over the growth of the bacterium, with inhibition being observed at infection multiplicities of 0.01 and 100. This promising biocontrol tool could optimize nitrogen removal processes such as deammonification.

Keywords: Phage. Biocontrol. Antimicrobial.

1 INTRODUCTION

Bacteriophages (phages) are viruses that specifically infect bacteria. The use of these organisms to prominence in the early 2000s, when they emerged as an alternative to antibiotics since bacteria were growing resistant to these drugs in the most diverse sectors of society.

Heterotrophic pathogenic bacteria in raw effluents that reach biological treatment processes are a point of attention and monitoring in wastewater treatment plants. Phage concentrations are estimated at approximately 10^8 - 10^9 mL^{-1} in wastewater treatment plants [1], and these viruses could be a viable alternative for combating unwanted bacteria [2]. Phages have shown potential to control problems in wastewater treatment plants [3], such as reducing foaming caused by microorganisms [4], dewatering and digestibility of sludge after the aerobic process [5], increasing substrate availability for further anaerobic treatment, combating pathogenic bacteria [6] and reducing competition between unwanted bacteria and functionally important microbial populations [7].

Deammonification consists of converting ammoniacal nitrogen using two microbial groups: ammonia-oxidizing bacteria (AOB), most of which belong to the *Nitrosomonas* genus, to generate nitrite, which, in combination with residual ammoniacal nitrogen, serves as a substrate for the subsequent Anammox process [8]. This process proposes the innovative removal of nitrogen, standing out for achieving greater efficiencies and reducing costs in treating concentrated effluents with a low carbon/nitrogen ratio. To successfully apply this technology on a large scale, it is necessary to have a balance between the bacterial communities. In the deammonification process, the appearance of aerobic heterotrophic denitrifying bacteria (AHDB), such as those of the genus *Pseudomonas*, is typical, and an increase in these populations can lead to system collapse, affecting the overall efficiency of nitrogen removal. AHDB can grow due to the effluent's remaining carbon, which can suppress the Anammox bacteria due to their sensitivity to high carbon concentrations.

AHBD control is carried out by manipulating process operating parameters such as airflow, temperature, and pH. The control of pathogenic bacteria is usually carried out at the end of the treatment process. It may also involve physical-chemical processes such as filtration, UV exposure, and chlorine or ozone disinfection. However, the diversity of bacteria involved in these processes is high, and modifying a parameter to disfavor a particular species can favor another, which is not desired. In this way, the control of the process using operating parameters has shown areas for improvement, which is one of the significant unresolved challenges in consolidating existing technologies. Therefore, this work aimed to isolate a phage-infecting bacterium from the genus *Pseudomonas* and demonstrate its viability as an antimicrobial agent against this microorganism through in vitro inhibition assays.

2 MATERIAL & METHODS

For phage isolation, samples were taken from an urban stream with effluent discharge in Dois Vizinhos, Paraná, Brazil. A *Pseudomonas aeruginosa* strain was used as a host. Portions of the environmental sample were mixed with the nutrient broth in a ratio of 1:1 (v/v), together with the host bacteria culture, which was enriched overnight (14 hours) at 37ºC and shaken at 160 rpm [9]. After this, the enriched solution underwent a physical-chemical process (addition of reagents to remove bacterial particles and centrifugation and sonication) [10]. The double-layer agar method [11] was used to plate the supernatant (heterogeneous stock) using soft agar in the upper layer. Aliquots of 800 µL of the heterogeneous phage stock were inoculated into 5mL of soft

agar medium, containing around 500µL of a culture of the host bacterium, and placed in a hot bath at 45ºC. The medium was then poured over the base agar, allowing it to solidify, and the plates were then incubated at 37°C [9]. The following day, the presence of lysis plates was observed. The observed lysis was collected using a tip and transferred to a microtube containing Salt-Magnesium Buffer (SMB). The solution was then vortexed and centrifuged. The supernatant was serially diluted in SMB (1:10) to concentrations of 10^{-4} and 10^{-5}, where new double-layer agar assays were carried out with these dilutions, followed by overnight (12-hour) incubation at 37°C.

Tests in liquid culture were carried out to demonstrate the inhibitory potential of phages on the growth of host bacteria. These experiments are also a way of confirming the phage host range. The batch experiments were carried out in 96-well plates where the optical density (OD600) was monitored. Each well was inoculated with the bacterium in the exponential growth phase at 10^8 colony-forming units/mL. Different multiplicities of infection (MOIs), relative concentrations between phages and host, were tested (0.01 and 100). The Victor NivoTM PerkinElmer plate reader was set up with the following parameters: 600 nm, 37°C, 8 hours of processing, with a 2-second agitation before the readings, which will be taken every 30 min from time zero.

3 RESULTS & DISCUSSION

The isolation of the phage was successful, confirmed by the presence of plaques (transparent lysation halos), which is indicative of the release of the progeny of a phage. Figure 1 shows the lysis of phage from *P.aeruginosa.*

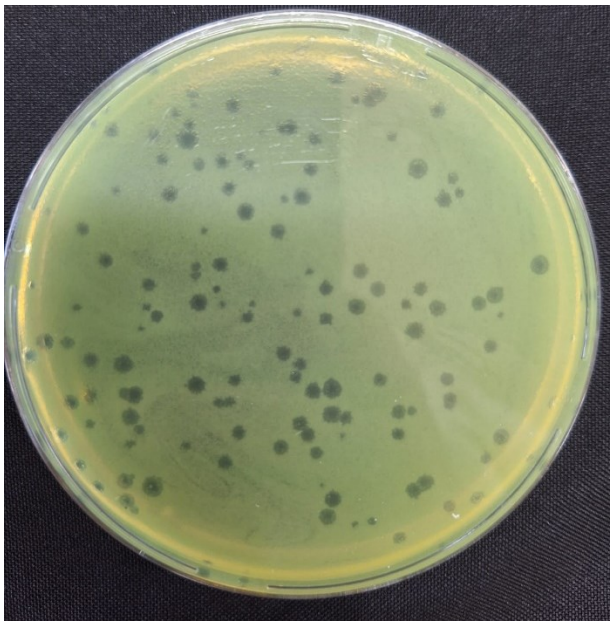

Figure 1 Lysis originated by the phage of *P.aeruginosa*

Phages survive wherever their bacterial hosts are found, so they are ubiquitous in the biosphere. Studies have already reported the isolation of *P. aeruginosa* phage in various wastewater-contaminated environments from different sectors [12, 13]

The results from liquid culture inhibition are shown by comparing the control (only the growth of bacteria is observed) with suspensions containing phages in different final titrations, thus corresponding to MOIs. Figure 2 shows the growth curves generated for the *P. aeruginosa*. The higher the number, the higher the ratio of phages to bacteria. At an MOI of 0.01, there are 100 times more bacteria than phages, while at an MOI of 100, there are 100 times more phages than bacteria.

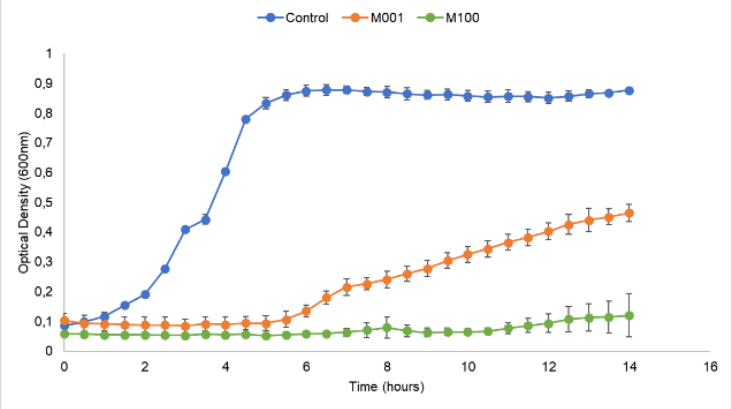

Figure 2 Growth curve comparing the control with different multiplicities of infection

At an MOI of 0.01, the phage-controlled growth for 6 hours, while at an MOI of 100, bacteria growth was inhibited for 14 hours. It is common knowledge that higher MOIs tend to show greater control over the host. Phages isolated from sewage samples showed an excellent ability to control the growth of the bacteria between the MOI of 0.1-10 over 6 hours [14]. In another study, at an MOI of 0.1, there was an insignificant reduction in bacterial growth, while from an MOI of 1, there was a significant bacterial reduction. The highest level of bacterial reduction was observed at MOIs of 10 and 100 [15]. When the antibiofilm activity of the phage against *P. aeruginosa* was tested, a dependence on MOI was observed, where maximum biofilm inhibition was observed at MOI of 10 compared to MOI of 1 and 0.1 [16]. These results corroborate those found in these studies.

4 CONCLUSION

Using phages as process control in deammonification plants is a promising area since they can potentially control bacteria of the *Pseudomonas* genus. It has been observed that the higher the proportion of phages about the host, the better the control efficiency. Using these viruses makes it possible to increase the efficiency of the process and make the effluent sanitary safe.

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