Biological control of *Pseudomonas aeruginosa* (ATCC-27853) by isolated lytic bacteriophage

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Background
Pseudomonas aeruginosa is a common bacterial species; it can grow in all fields, even in distilled water.
One of the obvious characteristics of *P. aeruginosa* species is its low antibiotic susceptibility (antibiotic-multi-resistant species); this reflects the urgent need for alternative strategies. Unconventionally, we try to use an alternative destructive antibacterial agent, phage. phage application in our environmental fields (engineering relevance) is a promising safe avenue for replacing chemical biocides.
Objective
To isolate a lytic phage as a bio-control agent for *Pseudomonas aeruginosa (ATCC*-27853), evaluate the ability of the isolated phage to inhibit or suppress the growth of the target bacteria. We study phage stability while performing biological and physical characterization, optimizing phage action against bacteria.
Materials and methods
We use an activated, purified young culture of *Pseudomonas aeruginosa (ATCC-27853)* as the target bacteria to isolate a specific lytic bacteriophage from a mixture of Enriched sewage and marine water through a sequence of experiments: a Broth clearing assay using nutrient broth and a spot assay test by using nutrient agar medium, both for the detection of phage presence and its lytic action. For the purification of phage, the plaque assay test was performed in many successive subcultures by using the serial dilution method on a semisolid medium. The high-titer lysate obtained from the confluent plates was investigated by TEM to determine its morphology and taxonomy. We performed biological characterization of the isolated phage: one-step growth curve, Phage adsorption rate, host range (using nutrient double layer agar), as well as a bacterial challenge test (using nutrient broth, based on the optical densities). Also, we performed physical characterization of the phage to determine phage Stability, pH, thermal stability, and the influence of UV and organic solvents on the lytic action of the phage.
Results and conclusion
Pyocyanin and pyoverdin pigments (both fluorescent under UV illumination) are secreted by *P. aeruginosa* (ATCC-27853), also their ability to thrive at 42°C. After spotting *P. aeruginosa* plates with enriched sewage filtrate, the results indicated lytic area (+ve). Plaques have a pin tip and are translucent. The lytic profile of bacteria after phage infection revealed that the bacteria was reduced to a MOI of 10 in 4 h. However, the multiplicity of infection(MOI) of 1 and 0.01, respectively, was longer (10, 22/h). The electron microscopy revealed that the phage is a member of the podoviridae family, with a noncontractile short tail (18 nm) and a polyhedral (heptagonal) head (45 nm) in diameter and a phage length of 63 nm. The adsorption rate of the phage was greatest at 20 min.
Our goal with the phage application is to replace harmful chemical biocides with lytic bacteriophages in various environmental systems (Phage for Biological Control). Phage bio-control treatment thus holds promise for eliminating the requirement for hazardous chemical biocides.

Keywords:
bacterial biocontrol, bacteriophage, pseudomonas aeruginosa

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Introduction

P. aeruginosa is a Gram-negative motile rod bacteria, largely distributed in our environment (water, soil, developed living organisms and industrial systems). It can grow in pure water, laboratory water baths

and other water containing containers. This explains why the organism is responsible for so many damaged systems. Any material, is extremely susceptible to microorganisms attachment [1] *P. aeruginosa* are acknowledged to be a biofilm former by forming an extracellular polymeric substances (EPSs). To protect themselves from external pressure and obtain nutrients for growth [2]. One of the most common characteristics of *P. aeruginosa* is its low antibiotic susceptibility [3]. Thus, prevention of such bacterial growth or even suppress its activity is a good target for protection of our environment. Such antibiotic-resistant isolates reflect the urgent need for alternative strategy such as biocontrol using phage. Unconventionally, we try to use a unique alternative antibacterial specific agent; phage [4]. The majority of marine bacteria may be infected by phages. They were used for a long time as antibiotics alternative, as a potent agent against multi-drug-resistant strains of many bacteria [5]. Viruses in seawater, primarily bacteriophage, play a major role in biogeochemical cycling [6].

The objective of this study is to isolate a specific lytic bacteriophage and to evaluate its inhibitory effect on *P. aeruginosa* growth, when used as a bio-control agent [7], where we use Phage as natural enemy to prevent the growth or eliminate the target bacteria in a safe manner without any hazard on human being [8]. Phage application in our environmental fields (engineering relevance) is a promising avenue for replacing the polluting chemical biocides [9].

Materials and methods

Bacterial strain

P. aeruginosa standard strain (ATCC-27853), Microorganism: Bacterial standard (ATCC) strain where, refresh of the strain occurred by incubation overnight at 37°C on nutrient agar.

Sewage samples used for isolation of phage

Samples were collected from sewage sewers and used for the isolation of a specific phage for *P. aeruginosa.* Raw sewage samples were collected from Al-Gable Al-Asffer station, Cairo.

Medium and reagents

Medium used for cultivation and maintenance of bacteria

Nutrient broth medium

Typical Composition/liter: Peptone 5.0 g; Meat extract 3.0 g, pH: 7.0–7.2 at 37°C, preparing solid medium (by adding 15 g/l agar) and semisolid medium (by adding 7.2 g/l agar) for culturing and propagation of bacteria. Also it was used for the characterization of isolated phage [10].

Medium used for routine purification of bacterium, enhancing the pigmentation (pyocyanin) of *P. aeruginosa* strain used in this study is standard strain (ATCC), overnight shaking cultures of this strain were cultured at 37°C in nutrient broth known to excrete pyocyanin [11].

Cetrimide agar medium: Cetrimide Agar Base is used to selectively isolate and purify *P. aeroginosa*. Magnesium chloride 1.4 g; peptone from gelatin 20.0 g; potassium sulfate 10.0 g; cetrimide 0.3 g; glycerol 10 g; agar 13.0 g; pH: 7.2–07.5 at 25°C when exposed to UV light, *P. aeruginosa* generates pigments and fluoresces [12].

Buffers

Phosphate-buffered saline (PBS) buffer, Typical composition (/liter): KCl 0.2 g; NaCl 8 g; $CaCl_2$ 0.1 g; $MgCl_2.6H_2O$ 0.1 g; KH_2PO_4 0.2 g; $Na_2HPO_4.2H_2O$ 1.44 g; pH 7.2–7.5 (PBS) was used for bacteriophage studies [13].

Preparation of bacterial inoculum

50 mL of nutrient broth is inoculated by a single cells from nutrient agar plate, then incubated with shaking (150 rpm) for 24 h at 37°C. The colonies were then obtained by centrifugation at 4000 rpm for 10 min at 4°C. The bacterial pellet was re-suspended in 10 mL saline to give the inoculums suspension 0.5 value at wave length 600 nm., The cultures were incubated at 37 C° for 2 days. Growth was evaluated by the optical density method (OD = 600 nm).

Isolation of the specific phage of p. aeruginosa

Two methods are used for isolation and cultivation of phage from natural sources:

(1) Cultures of *P. aeruginosa* (ATCC 27853) were exposed to 10, 20, 30 sec. of UV light exposure to induce any probable prophages .
(2) Raw sewage collected from Cairo sewers, one liter was filtered through a 0.4 μm pores membrane to allow phages only to pass through it, the produced filtrate was enriched by the bacterial host, then used as whole or diluted to test the phage presence. Experiments was performed in duplicates [14].

Enrichment and isolation of bacteriophage from sewage

These procedures were conducted based on vantwest [15]. Briefly, 20 mL of sewage-marine water sample was centrifuged at 3000 rpm for 30 min. to remove any

debris. 20 mL of The supernatant was transferred to a sterile flask containing 20 mL of young culture and an equal volume of double-concentration nutrient broth containing 2 mM $CaCl_2$. The mixture was incubated in shaker at 150 rpm for 48 h., at 37°C. the suspension was centrifuged at 3000 rpm for 40 min at 4°C, the supernatant was filtered with 0.4 um cellulose membrane to remove bacterial debris, stored at 4°C.

Broth clearing assay

It's a Fast phage quantitative detection method, based on optical density, a simple and inexpensive method, simply, one sample of the target bacteria only, another sample of the target bacteria mixed with phage sample (100 mL of bacterial Nutrient broth were infected with 100 µL of phage suspension), both was grown for 24 h. in Nutrient broth at 37 C° in a shaker incubator (150 rpm), then we determine the Optical density at 600 nm [16].

The spot assay for detection of phage

The spot assay used to determine the lytic activity of phages against the host Pereira [17]. Briefly, 100 uL of a young bacterial broth culture was mixed with 5 mL of semisolid agar held at 55°C in a water bath and then poured gently onto sterile nutrient agar plates to form a thin film of the tested bacteria. The lytic activity of the phage lysate was determined by spotting 10 uL of the crude extract (10^{11} PFU/mL) on the top of soft agar. Plates were incubated at 37°C for 24 h., plates were observed for the presence of the lytic zones at the pipetted lysate.

Plaque assay test

A clear lytic zones are evidence of the presence of virulent phages specific for *P. aeruginosa* in the filtrate. Select the confluent plates then add 2–3 ml of sterile PBS to each one, gently put these plates in refrigerator overnight, the materials from such selected plates was collected and centrifuged at 3000 rpm for 20 min., the supernatant was taken and filtered through 0.4 µm membrane, serially diluted to achieve a plaque assay on plates to determine PFU (plaque forming units) [14,18]. (3 ml of semisolid, melted nutrient agar, was kept in 55°C water bath, solid agar nutrient plates kept warm in 37 C° incubator [19].

(1) Add 0.3 ml of bacteria to the 3.0 ml semisolid agar, followed by 100 micron of diluted phage in one tube.
(2) Mix The bacterial-phage suspension gently for a few seconds then poured onto solid agar plate.
(3) Allow plates to harden. Incubate at 37 C°. Plaques should be visible within 24 h., prolonged incubation may result in confluent lysis. Calculate and determine the PFU/ml [20].

Purification of phages

As described by [21] The plates with plaques were selected, one plaque was picked up by a 1 mL pipette tip and placed in 2 mL Eppendorf tubes containing 500 uL (PBS) solution (pH 7.2). The suspension was kept overnight at 4°C. to obtain plaques with similar morphology, 2–3 successive experiments were performed. Lastly, a serial dilution was prepared, and the double layered technique was used to determine the PFU. Chloroform (1–2 drops) was added to The pure viral filtrate and stored at 4°C.

Preparation of high-titer phage stock

According to the method of [22], Large quantities of phage suspension were prepared by inoculating of young *P. aeruginosa* broth with phage filtrate at a multiplicity of infection (MOI) of 0.1. The infected bacteria were incubated until clarification of the cultures occurred. Removing of Bacterial debris occurred by centrifugation at 3000 rpm for 30 min, add chloroform (10%) to the supernatant fluid, keep it at 4°C [23].

Transmission electron microscopy (TEM)

A sample of the prepared high titer stock was investigated under TEM (JEOL-JEM-2100) to determine its shape, its type, the phage was prepared by putting the virion onto the carbon film, using of phosphotangestic acid (2% (w/v) as a negative staining technique, pH 3.0–5.0) [24]. The concentrated phage lysate (10^{10}) was observed using a TEM. The phage morphology was determined using the International Committee of Taxonomy of Viruses (ICTV). Lastly, we classified the phage based on the criteria proposed by [25].

Biological characterization of the isolated phage

(1) Adsorption rate of phage

Bacteria in the stationary growth phase were diluted in nutrient broth to an optical density (O.D.600) of 0.1 nm, then 10 ml of the bacterial suspension and 10 ml of phage lysate were mixed to give MOI of 2. The mixture was left at room temperature with shaking (150 rpm), then100 µl were taken every 10 min during a total period of 60 min. Chloroform-treated samples were serially diluted and assayed quantitatively after overnight incubation at 37°C, plaques were counted [26].

(1) One-step growth curve

A single-round growth model was done to assess the latent period and burst size as reported by [17]. Briefly, 6 mL of the bacterial suspension in the exponential phase (OD600=0.5) was centrifuged at 6000 rpm for10 min. The pellet of bacterial mass was re-suspended in 300 mL of fresh nutrient broth, then 600 mL of purified phage suspension was added to get a 0.01 MOI. After a 5 min, the mixture was centrifuged at 6000 rpm for 20 min to remove the un-adsorbed phages. Then, re-suspend the pellet in 6,000 mL of fresh nutrient broth medium and incubated with shaking (150 rpm/min) overnight at 37°C at 10 min intervals for 1 h, the samples were collected. The phage titers were determined using the double layered technique [26]. These experiments were carried out in triplicate.

(1) Bacterial reduction assay

Bacterial challenge test; To determine the in vitro phage bacteriolytic activity after Phage Infection. The lysis challenge of the purified phage was assessed as, 100 mL of the high titer phage lysate (10^{10}PFU/mL) was mixed with an equal volume of broth culture at MOI values of 100, 10, 1, and 0.1 in a 96 well-microliter plate and incubated at 37°C. Measuring the OD at 600 nm at one-hour intervals for 15 h to evaluate the bacterial reduction rate. One well-containing PBS (equal proportions) act as a negative control. The results was plotted [20]. The experiments were undertaken with duplicate assay.

(1) Phage host range

The host range of this lytic phage was determined using the spot test, where we use another two gram –ve bacteria; *Pseudomonus aeruginosa* ATCC 10145, *Escherichia coli* ATCC 23282, Shortly, 5 uL of the purified phage lysate (10^{10} PFU/mL) was added carefully onto the selected young bacterial film, the lytic ability of the tested phage was estimated by the presence of clear zones, the results are two classes; (–) no plaques and (+) positive-clear plaques.

Physical characterization of phage (Phage stability tests)

(1) pH (acid–base) stability

To assess pH effect, 100 ml of the bacteriophage suspension, (10^{10} PFU/ml) were added to 900 ml of elution buffer, pH adjusted to 3.0, 4.0, .05, 0.6, 7.0, 8.0, 9.0, and 11.0, using 1 M HCl and NaOH that were added drop-wise into the nutrient broth to set at different values of pH. The samples were incubated for 2 h at 37°C, according to [27].

(1) The thermal stability

To determine the thermal stability, we add 100 ml of the phage suspension (10^{10} PFU/ml) to 900 ml of elution buffer and incubate them at different temperatures (20, 30, 40, 50, 60, 70, and 80°C) for 2 h. After the incubation, the phage titer was determined in triplicates, according to Cui, *et al.* [27].

(1) The influence of UV

We determined The influence of UV radiation In brief, the phage suspension of 10^{10} PFU/mL and then added to the open Petri dishes, incubated on ice and placed 20–30 cm away from the UV source. The phage supernatant was collected every 20 min for 1 h followed by titration. The experiments were undertaken independently in duplicate according to [28].

The effect of organic solvents

The stability of the isolated phage for organic solvents as indicated by Topka, G., *et al.*, [29]. Briefly, 1000 uL of phage suspension (10^{10} PFU/mL)was mixed with a 100uL of organic solvents in a ratio 10% (chloroform, ethanol, methanol, DMSO, DMFO, Ccl_4 and Acetone) separately in sterile tubes and incubated with shaking(50 rpm) at 37°C for 2 h, the mixtures were left statically, and the phage titer was determined by the double-layer agar technique.

Results and discussion

Activation of standard strain *P .aeruginosa* ATCC-27853

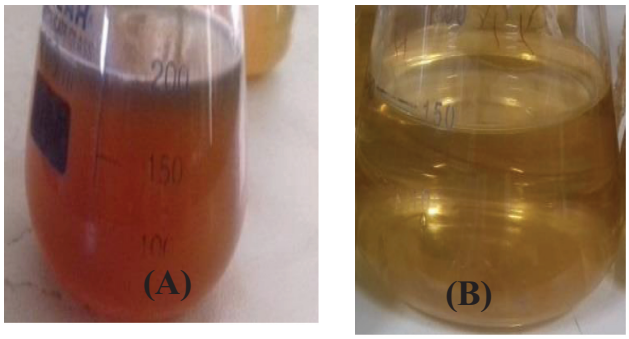
The bacterial Sample (host) were cultivated gradually (for activation), Fig. 1.

then using the selective medium, cetrimide (cetyl trimethyl ammonium bromide) agar medium, and the bacteria number was adjusted at (10^6); almost from 0.5–0.6 at O.D. $_{600}$, Table 1.

Isolation of specific bacteriophage active against *P . aeruginosa* ATCC-27853

In our case we isolated the lytic specific phage against *P .aeruginosa* ATCC-27853, from raw sewage collected from Cairo sewers; Algabal Alasffar Station; phage isolation passes throughout a series of subsequent steps, as follow;

Figure 1



Inoculated Nutrient broth medium with *p. aeruginosa* ATCC- **27853**, secretes pigments, (brown-green) . & activity at 42 C°

non- inoculated Nutrient (Sterile) broth medium . no growth, no pigments .

The pigmentation (brown-green ring) of *P. aeruginosa* ATCC- 27853 in broth medium (A), with –ve control (B).

Table 1 The great suppression of bacterial growth by phage action

The Culture O.D 600 nm	*P. aeruginosa* ATCC-27853	*Phage lysate*
	0.6	0.2

(1) Broth clearing assay

It's a fast phage detection and quantification: An optical density-based approach, simply, the decrease in optical density mean cell lysis by the effect of viral infection of bacterial culture, determines the percentage of growth inhibition .bacteria was grown overnight in Nutrient broth medium at 37°C in a shaker incubator (150 rpm). Optical density of bacterial broth was measured at 600 nm, similarly, The Phage lysate (1% phage suspension), also measured in spectrophotometer [16].

High sharp decreasing of the bacterial growth, where (O.D.) was normally 0.6 and declined to 0.2, this is a crucial proof of the presence of a lytic phage(s).

(1) Spot assay

Results presented in Fig. 2 showed that; lytic area(+) appeared on *P.aeruginosa* plates after spotting by enriched sewage filtrate.

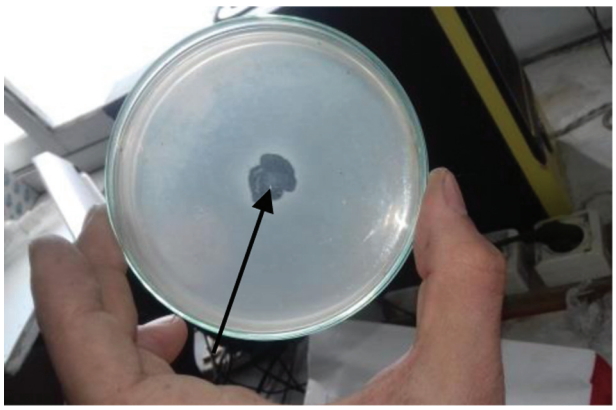
As *P. aeruginosa* is its low antibiotic susceptibility, (smith, *et al.*, [30], phage application is necessary and efficient, where, its involves the use of lytic phage (virulent phage) for inhibition or suppression of bacterial growth.

(1) Plaque assay

The positive samples for the presence of lytic phage was diluted and plaque assayed quantitatively to confirm the presence of phage. Results in Figs. 3 and 4, revealed that; the pin tip, transparent plaques appeared with *P.aeruginosa* plaque-forming units (PFU/ ml) is calculated by multiplying no. of plaques per plate X dilution factor.

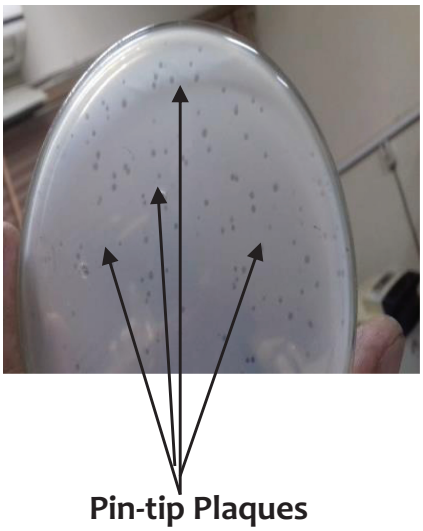
After enrichment, phage-containing sample was placed on semi solid nutrient medium with *P. aeruginosa*, the observed plaques of isolated phage was similar in all aspects as clear (transparent) pin tip, circular plaques, Table 2. This had also been observed in a study reported by [31].

Figure 2



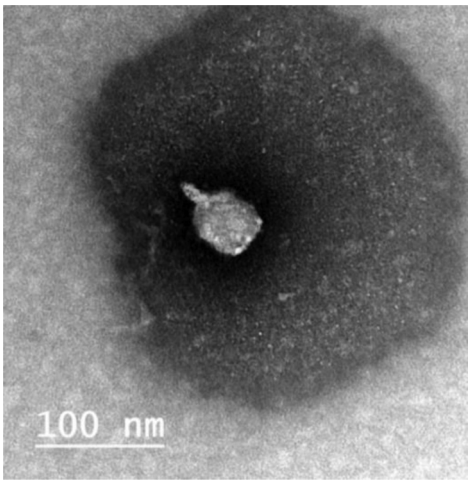
A spot assay on a young inoculated *Pseudomonas aeruginosa* (ATCC-27853) show positive spot assay (clear zone) of *P. aerogi-nosa* Phage.

Figure 3



Plaque assay experiment.

Figure 4



Electron microscopy of the isolated lytic phage of *P. aeruginosa* (27853-ATCC), collected from confluent plates, after staining using uranile acetate.

Table 2 Morphological characteristics of phage

Isolated plaques	shape of plaque	Appearance of plaque	(PFU)/ ml
p. aeruginosa	Pin tip	transparent	10^9

Characterization of isolated phage

Morphological characterization of phage by transmission electron microscopy (TEM)

Investigation of phage by electron microscope is a very characterizing step for phage taxonomy, The purified particles of *P.aeruginosa*(27853-ATCC) – phage was negatively stained with Uranile acetate.

According to the TEM micrograph, the isolated phage belongs to *podoviridae* family, which is characterized by long contractile tail according to the classification of [32], having a polyhedral (heptagonal head 45 nm in diameter and non-enveloped, noncontractile tail (short), tail length (18) nm, the phage length = 63 nm, this coincided with the findings of [33]. 96% of all phages investigated in the last 45 years were members of the *Siphoviridae,* the *Myoviridae,* or the *Podoviridae* [25].

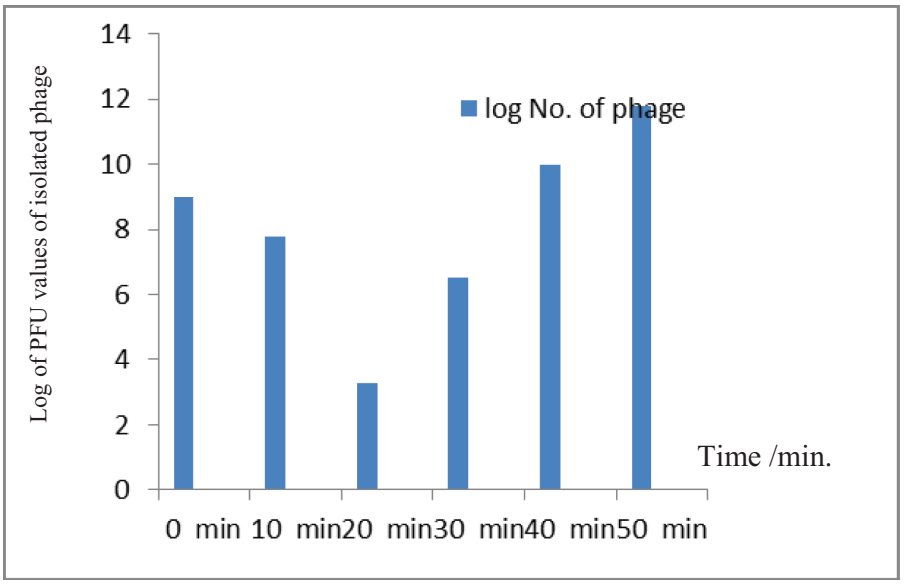
Biological characterization

(1) Adsorption rate of phage

Rate of adsorption depends on host physiological state and cultural condition [34]. The study showed that, phage particles are able to achieve a high level of adsorption at a period of time ~20 min and the increasing of time has significant effects of increasing the number of phage particles that adsorbed on the surface of bacterial cells. In this study, the highest reduction in number of free phages was observed after 20 min of incubation .The phage numbers was increased after this time. Figure 5.

This study showed that, phage particles are able to achieve a high level of adsorption (the highest reduction in number of free phages) of at a period of time ~20 min (60% of phage), and the increasing of time has no effects of phage particles – number that adsorbed on the surface of bacterial cells, but phage no. increases with increasing the incubation period.

Figure 5



Adsorption of phage at 10 min intervals.minutes.

(1) One-step growth curve analysis of phage

One-step growth curve analysis of phage used for determination of latent period and the Burst size of phage [35], the results illustrate A latent period of 30 min and a burst size of 125 PFU / host cell, were calculated for phage from the tri-phasic curve. (Fig. 6). Results agreed with those results being obtained by other studies [36], values of PFU of phage illustrate the latency and Rise phase and burst size: At MOI = 0.01.

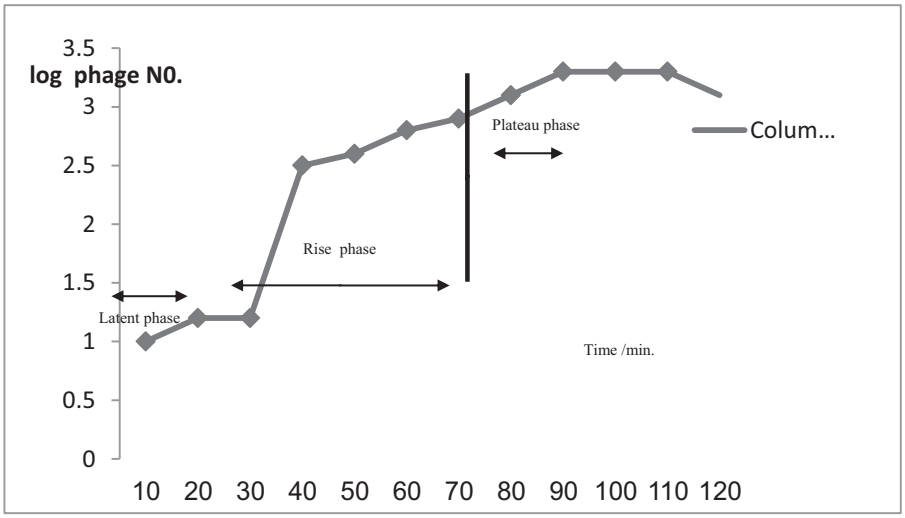
(1) Bacterial challenge test. To determine the in vitro phage bacteriolytic activity

Lytic Profile of Bacteria after Phage Infection; Kill curve. The host strain was decreased in the MOI of 10 in 4 h. But in the MOI of 1, and 0.01, the reduction time of bacteria was longer (10, 22/h), respectively, as in (Figs 7 and 8). the growth of *P.aeruginosa* was apparently inhibited after phage inoculation at MOI = 0.01, 1 and 10 this agree with [37].

(1) Phage host range

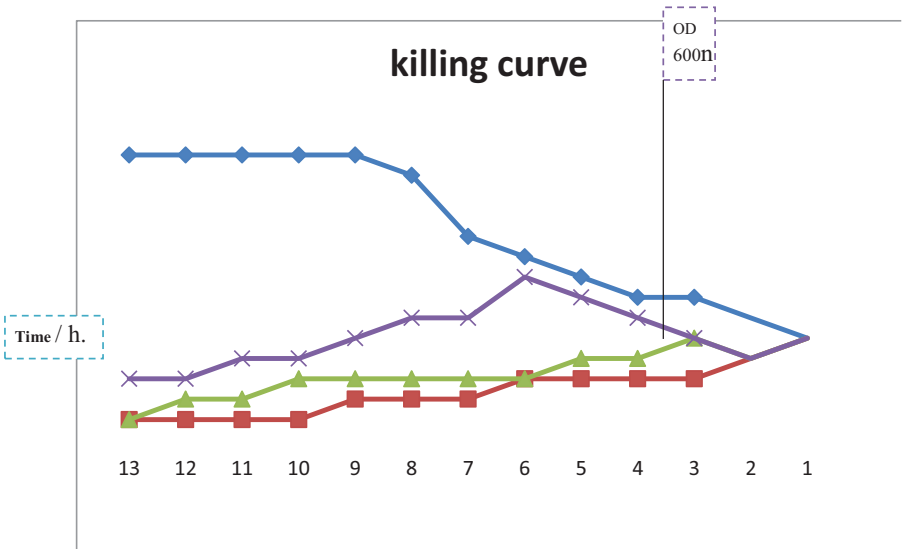
Data in Table 3 showed that, Isolated phage could infect only *P.aeruginosa* ATCC-27853 and does not infect any other tested gram –ve organisms

Figure 6



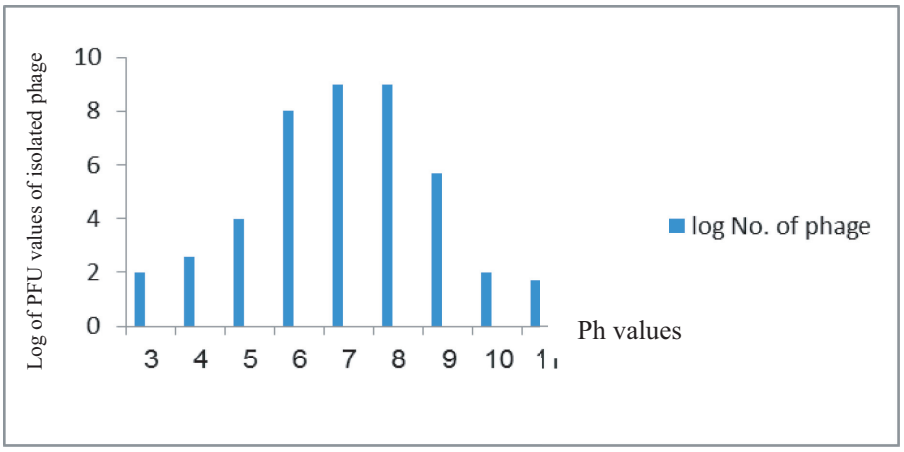
One-step growth curve analysis of $Øp_{a3}$ Phage, the growth curve of $Øp_{a3}$ phage illustrate the latency and rise phases and burst size : at MOI = 0.01.

Figure 7



The bacteriolytic challenge of phage at variety of MOI: at MOI = 0.01,1,10.

Figure 8



Effect of pH variation on phage viability.

(*Pseudomonus aeruginosa* ATCC 10145 and *Escherichia coli* ATCC 23282). Data reveals that, this phage is highly specific, have been found to be greatly specific for their receptors present on the host cell surface, this specificity enable it for phage typing, this results in agreement with finding of [38].

The narrow host range can be advantageous in phage application in a certain field affecting the target bacteria only, leaving other useful members, these results in agreement to the results of [39].

Physical characterization (Phage Stability Tests)

(1) Acid–base stability (pH effect)

The Neutral pH (7.0) and the slight alkaline one were the optimum pH value, where no any harmful effect on the phage viability (PFU still constant), but in case of pH 5.0 pH 5 (cause 45% reduction) and pH 9.0(reduce 35%), there is a small, nonsignificant changes in phage viability, whereas the values of pH 3.0, 4.0, 10, and pH 11.0, the phage viability largely affected, this isolated phage was one of the highly withstanding of pH variation. this results in agreement to the results of [40].

Table 3 Host-range of phage

Host	Host source	Presence of lytic area with isolated phage
P.aeruginosa ATCC10145	Biotech. Lab., processing Development Dep., (EPRI)	(–)
P.aeruginosa ATCC27853	MRCN, faculty of agriculture, In shams university .	(+)
E.coli ATCC 23282	Biotech. Lab., processing Development Dep., (EPRI)	(–)

(+) indicates that the strain is susceptible to the phage and produce plaques with titers, while. (–) indicates that no plaque were observed.

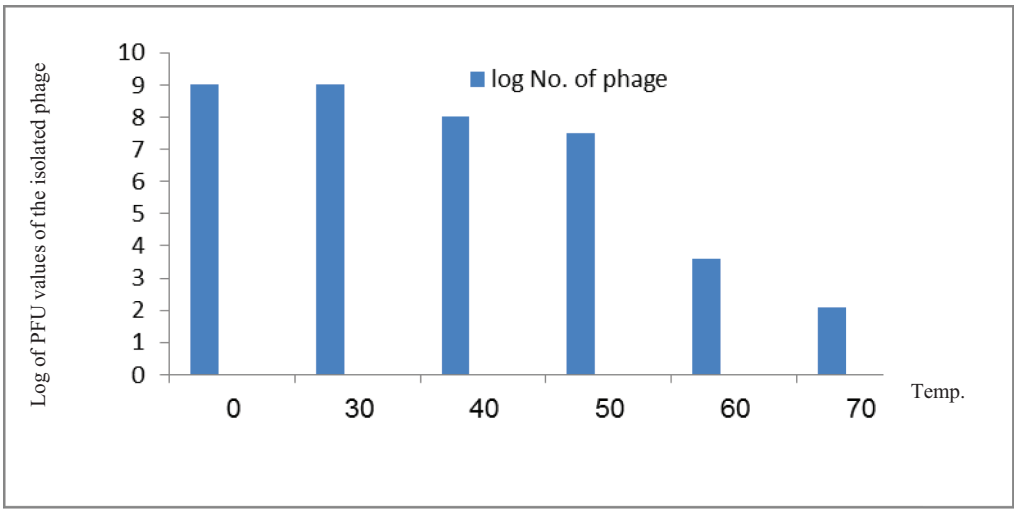
(1) Thermal stability tests (Effect of temperature on phage)

Figure 9, showed that exposure of phage (for 12 min) to temperature 30°C, do not affect phage activity. However, exposure of this phage to 40°C, causes a loss about 20% of their activity while, exposure to 50 and 60°C inhibit 30% and 60%, respectively of their infectivity and the phage disappeared after exposure to 80°C. Generally this phage is considered as thermostable. This study correlated with the results who showed that phages are heat resistant and even 23% phages remain viable up to 70 C° after 1 h incubation. On other study by alkhozai and alkabei [41], showed that the phage particles were effectively inhibited at 80°C after 10 min.

(1) Effect of UV irradiation on isolated phage

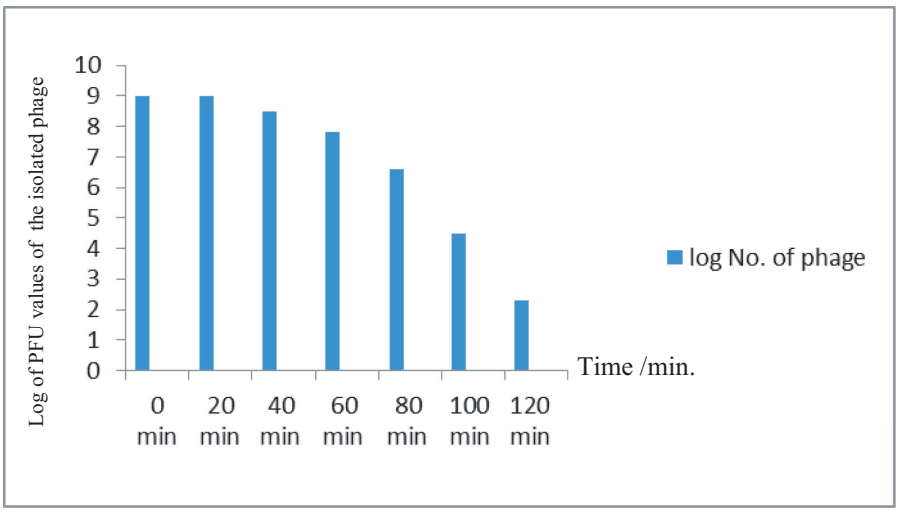
Results in Fig. 10 showed that the exposure of phage suspensions to UV irradiation for different period of times has affected the phage activity, with increasing the exposure time to UV the phage numbers (PFU/ml) obviously decreased. The lowest (PFU/ml) was observed after 120 min of exposure to UV. This indicated that the DNA of phage not completely damaged by exposure to UV for 120 min, [42] use UV to study phage reproduction. The exposure time to UV and the number of plaques illustrate the high survival rate of nonirradiated phage than irradiated one and showed that, lytic activity (PFU) decreased with increased exposure to UV. As shown in Fig. 10, the exposure of phage to UV for 100 min. reduce 50%

Figure 9



Effect of temperature on phage.

Figure 10



Effect of exposure to UV irradiation on phage.

of phage activity. However, UV exposure of this phage to 60 min. causes a loss about 20% of their activity. There is a high survival rate of the nonirradiated phage than irradiated one and the lytic activity (PFU) decreased with increased UV exposure, this results agree with that of [43].

Effect of a variety of chemicals on isolated phage

Results in Table 4 showed the exposure of phage suspensions to some chemicals used in different industrial fields for 2 h. at concentrations 50 and 100 ml/l. The effect on the phage activity at conc. 50 ml/l. for all chemicals was slight except for chloroform that has no effect(highly adapted with chloroform), Moderate effect of CCl_4, ethanol and methanol at 100 ml/l conc. The most effective chemicals against phage was acetone. Phage maintain its viability even when exposed for these chemicals.

Table 4 Effect of different organic chemicals (solvents) on phage

		5% conc.		10% conc.	
Chemical	Original PFU	No.	Log	No.	Log.
Chloroform	10×10^8	10×10^8	9	8×10^7	7.9
Ccl_4	10×10^8	5×10^7	7.6	9×10^8	9
Acetone	10×10^8	7×10^7	7.8	9×10^6	7
DMF	10×10^8	3×10^8	8.5	3×10^7	7.4
DMSO	10×10^8	10×10^8	9	8×10^7	8
Acetonitrile	10×10^8	2×10^7	7.3	8×10^7	7.9
Ethanol	10×10^8	6×10^6	6.7	9×10^5	6
Methanol	10×10^8	2×10^8	8.3	8×10^7	8

Preservation of isolated phage (phage longevity)

The high titer of the phage (11×10^9 PFU/ml) was held for 4 weeks at different temperatures: 0°C (freezing point), 4°C (refrigerator), 25°C, and 37°C. The phage titer was marginally reduced at 4°C, and it lost 3% of its infectivity after 4 weeks of incubation at this temperature. Storing the phage at 25°C or 37°C for four weeks lowered its infectivity by 15% and 25%, respectively. In general, these findings are comparable to those of [44]. The only published publications on long-term storage (5 years) and phage infectivity are from [43]. The results in Fig. 11 are generally consistent with the findings of [44].

Conclusions

As *Pseudomonas aeruginosa* is one of the most spread aerobic bacteria in our environment, including the industrial fields, so its responsible for so many damaged systems, beside *P. aeruginosa* is a multi-resistant antibiotic microbe, its a slime layer forming bacteria, so *P. aeruginosa* resist the common antimicrobial agents.

This study support our ideas with concept to apply phage as a unique anti-bacterial agent to prevent or even suppress the activity of *P. aeruginosa*, usage of the lytic specific phage against *P. aeruginosa* as a novel method is necessary.

Our target from the phage application is replacing the traditional chemical biocides with bacteriophages in different environmental engineered systems (Phage for Biological control) Thus, phage bio- control treatment holds promise for reducing the need for toxic, polluting chemical biocides.

The isolated lytic phage of *P. aeruginosa* (27853-ATCC) was purified and characterized by biological, chemical and physical methods. The electron microscopy illustrated that, phage belongs to podoviridae family, with non-contractile short tail (18 nm) and a polyhedral (heptagonal head (45) nm in diameter, and the phage length = 63 nm. The adsorption rate of $Øp_{a3}$ phage, was maximum at (20) min., also the one-step growth curve clear; burst size (125) and latent period (30 min.). We study the effect of different environmental factors on the phage lysate, it was generally, thermo-stable, with high preservative properties, with considerable stable genetic material withstand UV irradiation for (2) hours and withstand a large variety of pH values.

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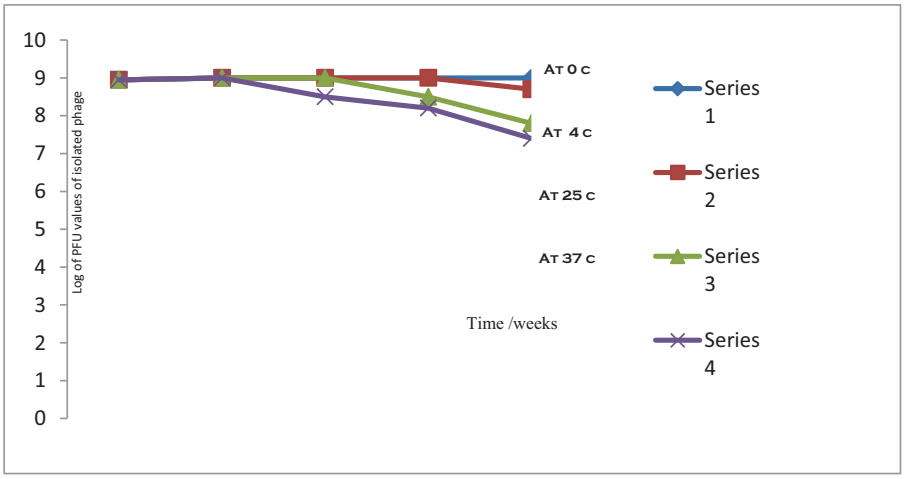
Figure 11



Effect of the storage time at different temperatures on the infectivity of phage.

Conflicts of interest

The authors declare there are no conflicts of interest.

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